2.3. Transgenic constructs for injections, general protocol for 'cut and paste' cloning

- Restriction enzymes and buffer
- Gelelution kit (e.g. Invitrogen Gelelute Pure Link)
- rAPid kit
- T4 ligase & buffer
- Miniprep solutions (Merlin I-III)
- Isopropanol
- 70% EthOH
- 10x loading buffer
- DNA ladder
- LB medium, LB Agar plates
- Midi prep kit (e.g. Nucleo Bond Xtra Midi from Machery-Nagel)
- RNAse free H_2O

Day 1: Preparation of fragments for Ligation:

- ❑ Digest 3-5 µg DNA in 40µl volume (see 2.8.)
- ❑ Run 1ul on gel to check whether digest complete
- ❑ If completely digested, run complete digest
- ❑ Carefully cut out bands that match expected sizes
- ❑ Elute DNA from gel with Invitrogen Gelelute Pure Link
- ❑ Dephosphorylation of 5'ends with rAPid Alkaline Phosphatase (Roche)
- ❑ Ligation with T4 Ligase (over night)

Day 2: Transformation of Ligation into bacteria

- ❑ Deactivate T4 Ligase (10min at 65°C)
- ❑ Transform ligation (see 2.8.) and plate cells on Amp-plates (37°C over night)

Day 3: Grow bacteria for minipreps

- ❑ Compare numbers of colonies of ligation & control to get an idea about
- ❑ Select positive colonies and inoculate 4-5 ml LB medium (37°C on shaker for ~16 hours)

Day 4: Minipreps and plasmid analysis

❑ if the sizes of the two ligated fragments differ more than 1kb and many colonies are to analyze, there is a quick (and 'dirty') method to screen for positive clones to see whether the fragment is inserted:

Mix 20 µl overnight culture with 30 µl H_2O and add 50 µl 1:1 Phenol:Cloroform, vortex briefly, take 10µl of supernatant, add 10x Loading buffer that contains RNAse., and run on gel.

The positives from this quick screen will still have to be miniprepped and analyzed with suitable restriction enzymes to verify insertion and to see in which direction the insert is clones into the vector – but this saves many minipreps and restriction enzymes

❑ miniprep 3-4ml bacteria culture with Merlin solution I-III (see 2.8.)

❑ analytical digest (see 2.8.) with appropriate enzymes (to see whether ligation worked, and if yes, in which direction the insert was placed)

❑ Run restriction on gel and determine the right clone for further cloning steps. Or, if this is the end product, inoculate 100ml LB medium with the respective clone and incubate on shaker at 37°C over night for midi prep the next day.

2.3. Transgenic constructs for injections, general protocol for 'cut and paste' cloning

Day 5: if produced plasmid is end product to inject, Midi Preps:

- ❑ Follow protocol of midi prep kit (e.g. Plasmid DNA Purification Nucleo Bond Xtra Midi, Machery-Nagel)
- ❑ Perform wash steps in RNAse free 70%EthOH
- ❑ Resolve DNA in 50 µl RNAse free H_2O
- ❑ Nanodrop and use recommended concentration for injection (see 2.5.2.), store at -20°C.

- PCR purification kit (e.g. invitrogen PureLink)
- Mmessage Machine T7 kit (Ambion)
- Isopropanol
- RNAse free 70% EthOH
- RNAse free water
- RNAse free 1x loading buffer
- DNA ladder

2.4. Transposase mRNA preparation

Work as clean and RNAse free as possible:

- ❑ Linearize pBluescript {Transposase mRNA} with NotI in 100µl volume
- ❑ Purify digest with PCR Purification Kit, elute in 20µl RNAse free ddH2O
- ❑ set up Transcription reaction of Transposase (=MiTR) mRNA with mMessage Machine T7 kit and follow protocol until precipitation with isopropanol
- ❑ Store at -20°C/-80°C in isopropanol

Preferable on the same day as injections are plann ed:

- ❑ Whenever needed for injections, vortex the tube with MiTR mRNA in isopropanol tube quickly
- ❑ Take 10-15µl from this tube, put back original tube and centrifuge the aliquot for at least one hour at 13000rpm at 4°C
- ❑ Remove isopropanol carefully
- ❑ Add 50µl RNAse free 70%EthOH and centrifuge for 15-30 min at 13000rpm at 4°C
- ❑ Remove EthOH as much as possible (use dry tip to remove rests)
- ❑ Dry pellet at RT for ca. 5 min
- ❑ Resolve pellet in 6.5 µl RNAse free H2O
- ❑ Nanodrop 1µl (usually >300ng/µl)
- ❑ Run 1µl on gel to check quality (run with 4µl 1x RNAse free Loading buffer) (see Fig. 4).

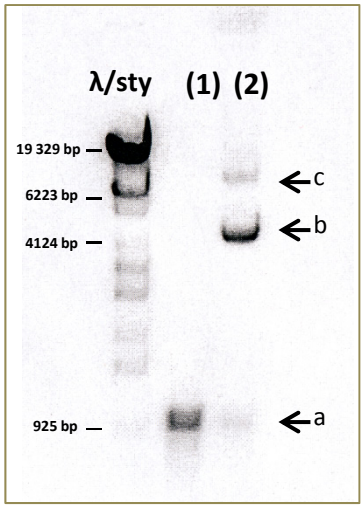


Fig.4 Example of gel of (1) fresh capped Transposase-mRNA and of (2) used injection mix (2 days old), in which the mRNA is degraded.
The first lane contains 5µl of λ/sty digest. Lane 1 contains 1 µl of capped Transposase mRNA and 4 µl 1x RNAse free loading buffer and lane 2 contains 1 µl injection mix with DextranRed and pMi construct and 4 µl 1x RNAse free loading buffer.

0.8% agar gel, ethidiumbromide, 100V

2.5. *Parhyale* cell injection

2.5.1. Collecting embryos for injections

Couples are separated from the *Parhyale* culture the evening before embryos are needed. The next day females with eggs in their ventral brood pouch can be collected over the day. Anaesthetize the females using either clove oil (20µl clove oil in 50 µl FASW, mixewell before use) or CO2 enriched FASW. As the animals stop moving, they should then be transferred to FASW.

To collect the eggs, grip the female on one side (at the coxal plates) with fine forceps and hold it in FASW under a dissection scope. This allows to open carefully the brood pouch with a sharply bent tungsten needle (Fig. 6B in 6.2.6.) and to subsequently flush out the embryos without damaging the adult female. Collect the embryos in dishes according to stages and collect all females in a FASW dish to let them wake up and to return them to the main *Parhyale* culture as soon as they are fully mobile.

2.5.2. Injection mix

The concentration of the MiTR mRNA in the injection mix should be around 100-200 ng/µl, the concentration of the pMinos vector should be around 200-400 ng/µl. In order to be able to see the injection mix during the injections, it is useful to add the vital dye PhenolRed into the mix at a dilution of 1:10.
As a fluorescent tracer, Dextran-Red of Dextran-Green can be added to the mix (careful with Dextran-Green, see photoablation 2.6.1.). Adjust the concentrations with RNAse free H2O to a volume of 6-10 µl. The injection mix should be fresh due to the degradation of the RNA, therefore 6-10 µl are recommended to prepare for one or two days of injections. To clean the injection mix additionally, microfilter can be used (be aware that here additional 1-2µl can be lost in the filter). You can check the quality of the injection mix before injections, if needed (see also Fig. 4).

Work as RNAse free as possible.

Also keep in mind for the injections: Keep the injection mix on ice after loading the needle. Freeze the mix if you anticipate to not having to reload for the next few hours. Check quality of injection mix again on a gel towards the end of injections to estimate the quality of the mix (Fig. 4).

2.5.3. Preparing embryos for injection – Alternatives of mounting embryos

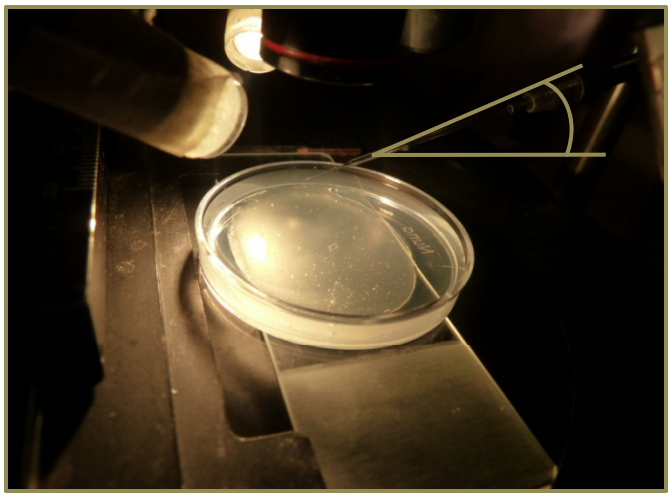
For *Parhyale* injections there at least three alternatives of mounting embryos for injections. Depending on the specific aim of the injection (e.g. which cell stage needs to be injected, whether a large quantity is needed or quality of injection is more important, or whether specific blastomeres are targeted) one of these methods may be more efficient and reasonable than the other. One technique that has been established for injections of the amphipod *Orchestia cavimana* (Scholtz & Wolff 2002, Wolff & Scholtz 2002) using cover slips has not been successful in my hands with *Parhyale* embryos.

2.5.3. 1. Agar embedding

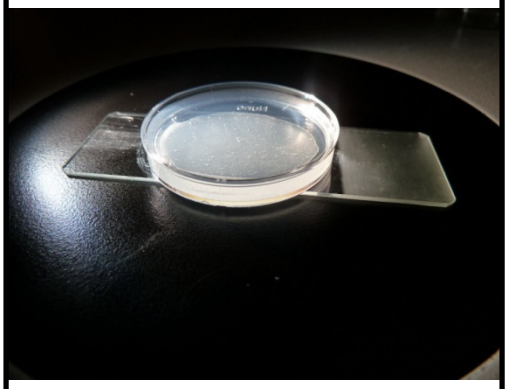
Embedding embryos in Agar is very helpful for observation, documentation or taking pictures when the embryos need to be oriented in a certain way. Also this embedding can be helpful to orient 8-cell stages so that a specific micromere can be targeted for injection.

The down side of this embedding, however, is that it is difficult to position more than few embryos at a time, since the embryos tend to change their positions in the agar or they get squeezed too much between the agar.
While fixing the embryos in the right position one has to keep in mind that the needle will be having access, therefore the embryos should be fixed superficially to the agar surface (also make sure that no agar residues block the area from where needle is coming).

The micromanipulator should be set up so that the needle points at an angle of around 30° towards the surface of the agar.



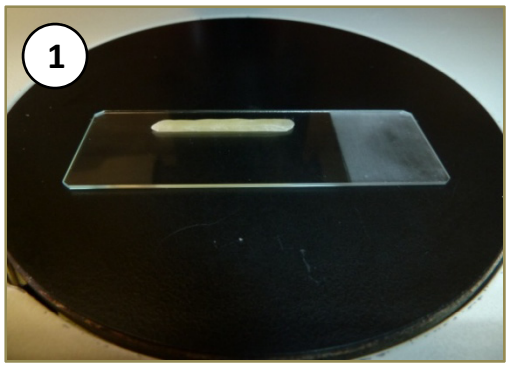
Preparation:

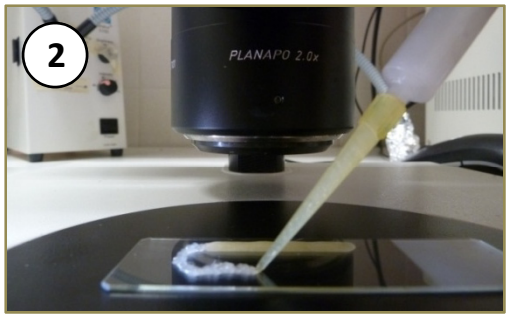


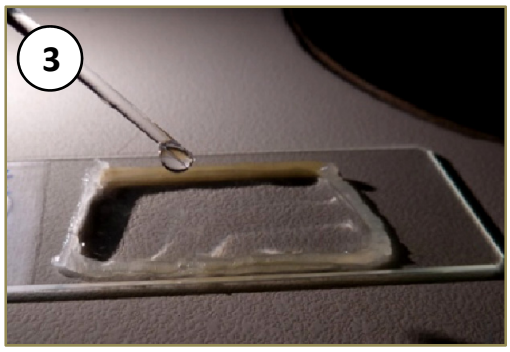
- ❑ Fill small petri-dish lids (ø 3cm) with 1% Agar-FASW (leave 1-2mm from the rim for FASW later) and let cool off
- ❑ stick lid with vaseline onto micsoscopic slide
- ❑ add FASW and embryos
- ❑ prick little holes in agar and orient embryos so that embryos are fixed in their orientation, but not squeezed too much (also try to position them close to the surface so that they are accessible for the needle)
- ❑ position under injection microscope (orient needle so that it comes from above in an angle of around 30°

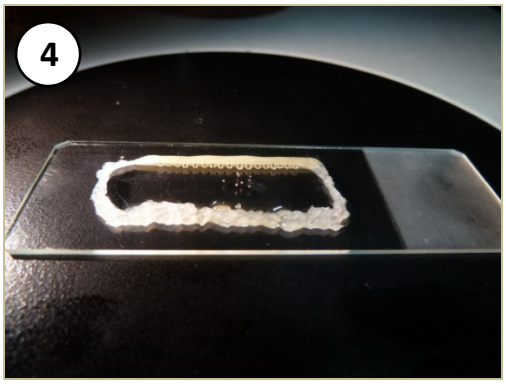
2.5.3. 2. Bee wax embedding

This embedding also allows for embryo orientation and therefore for injections targeting specific blastomeres. The set up is a little more elaborate, however, once routinely performed, the quantity of successfully injected embryos is much higher than embryos oriented in agar and results in a higher percentage of correctly targeted blastomeres.









Preparation:

1 Form a long role of beewax (~1inch/2.5 cm and ca. 3-4mm in diameter) and fix it onto one side (about one quarter) of a microscopic slide.

2 Form a basin around the other side of the bee was with vaseline using a syringe (using a pipette tip regulates better the amount of vaseline coming out of the syringe – cut off the tip, if opening it too small).

3 Fill basin with FASW (use salinity on the lower side around 1.018).
The basin side of the wax should be covered with FASW. Press little holes into the wax with an insect needle (cut the tip of a regular insect needle so that the cutting plane is flat).

4 Add the embryos and brush them into the wax holes, each hole one embryo.

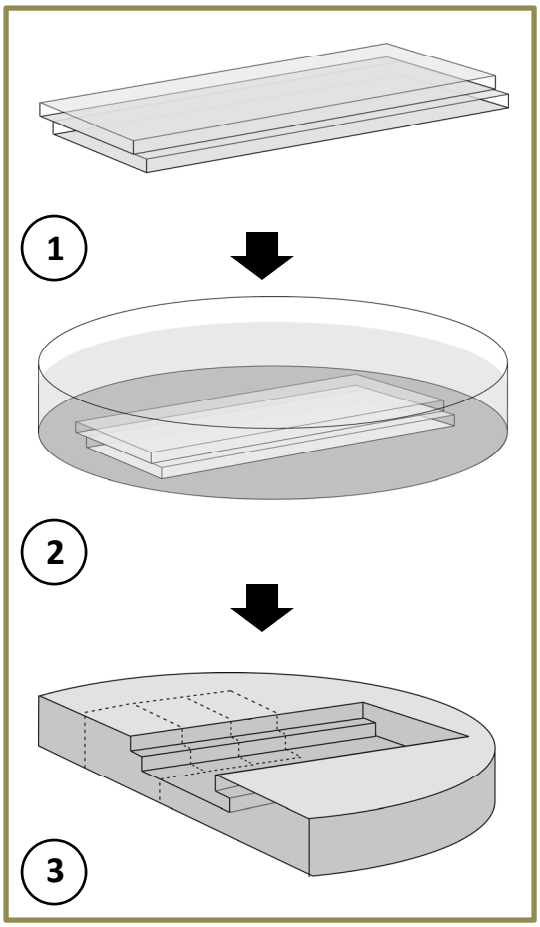
Orient the embryos so that the blastomere that is planned to be injected is facing to the out side, accessible for the needle. To fix the position of the egg carefully by pressing the wax sides of the hole from both sides towards each other (works best with fine forceps).

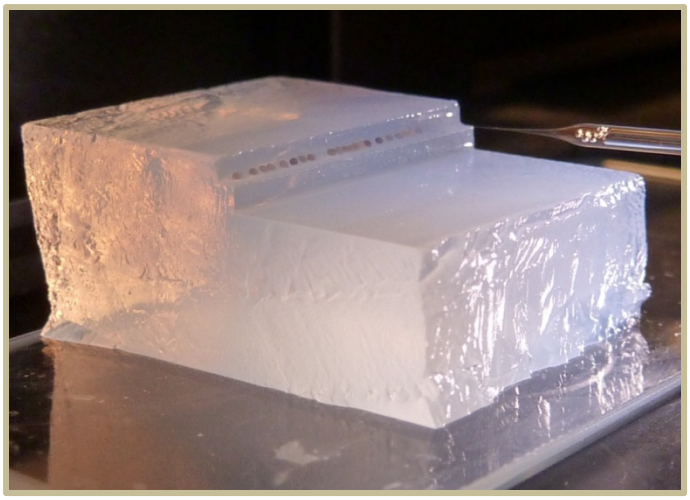
If this process takes too long in the beginning, make sure to start with embryos at a little earlier stage than needed for injection. If evaporation and therefore increase of salinity (changes in osmotic pressure in embryos) appear to be an issue, adjust with H_20.

5 After injection the eggs can be flushed out of the wax openings with a Pasteur pipette easily and then collected for further experiments/culturing. (If eggs cannot be removed easily from pouches, increase salinity, however, make sure to transfer them quickly to the normal salinity between 1.018-1.020).

2.5.3. 3. Agar stages

Agar stages are very suitable if large quantities of injected embryos are needed and the targeting of specific blastomeres is secondary. If transgenic lines are the aim, especially one of two cell stages are aimed to be injected so that the integration of the respective construct can occur as early as possible to obtain unilateral or bilateral mosaics (i.e. to increase the chance that the transgenic cell clones comprise the germ line).





Add embryos with water droplet and align with an eyelash tool.
Make sure that the embryos are always covered with FASW (adding water with brush is very convenient. To prevent the increase of salinity in this small amount of water, ddH2O can be added instead of FASW).
After injection, the embryos can be carefully removed with a brush and transferred back to a Petri dish with FASW.

Making of Agar stages:

1 Tape/glue two microscopic slides onto each other, shifted to each other by ca. 2mm.

2 Place (tape) them into a Petridish, fill with 2% Agar-FASW and let cool down.

3 Cut the side that is forming the 'step' into ca. 1x2 cm pieces (as seemed convenient).

Store in FASW @ 4°C (if stored for longer, add fungizides and antibiotica).

Before placing stages onto microscopic slides, dry them so that they attach to the surface and don't slip.

Steps can be reused as often as they are intact.

2.5.3. Injections

In order to inject, needles have to be pulled, beveled and loaded with the injection mix. Necessary equipment for the production of needles and techniques described in detailed by Rehm et al. (2009). The specific procedure will depend on the available equipment. The general set-up of the microscope, the manipulator and the agar stage with the embryos used here are shown in the Fig. 5.
Needles can be pulled in larger numbers and stored, if kept in a sealed container (e.g. in large petridishes: stick plastiline onto the bottom of the dish, press the needles in the plastiline so that the tip of the needles are not contact to anything and close with lid). The beveling of the needles (at an angle of 30-25° for 15-30min e.g. while setting up the embryo stage) is preferably performed on the day of the injection or few days ahead.

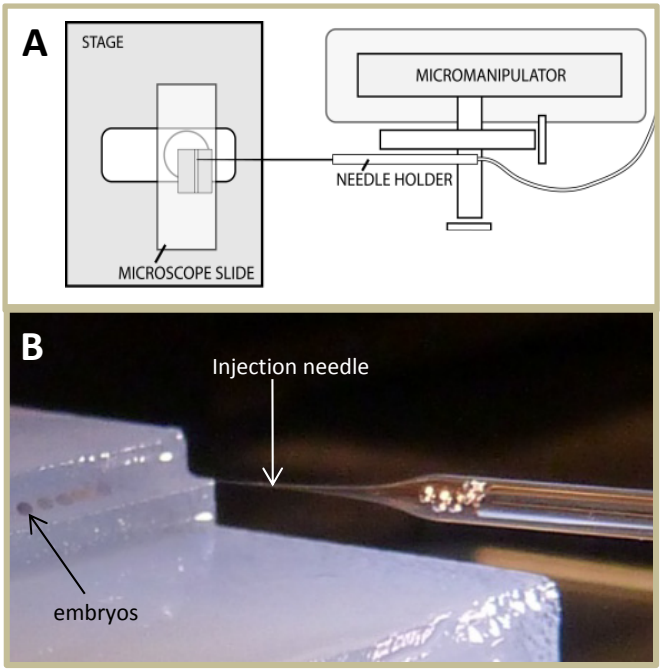


Fig. 5 A Schematic of the general set up of the microscope, micro manipulator, and the position of the needle holder.

2.8. Standard methods for cloning

Minipreps with Merlin

- ❑ Pellet 3-4ml cells
- ❑ Resuspend cells in 100 µl solution I by vortexting
- ❑ Add 100µl solution II, mix carefully and incubate at RT for max. 5 min
- ❑ Add solution III, mix gently and incubate on ice for 10 min
- ❑ Centrifuge for 15 min at 13000rpm at 4°C
- ❑ Transfer supernatant to a clean tube
- ❑ (extract with phenol/chloroform if cleaner DNA is required)
- ❑ Add 300µl isopropanol and vortex
- ❑ Leave at RT for 2 min and the centrifuge for 15 min at 13000rpm at 4°C
- ❑ Wash pellet with 500µl 70% EthOH , centrifuge for 5 min at 13000rpm at 4°C
- ❑ Dry pellet and resuspend in 20-30 µl $_{dd}H_2O$

Solution I (store at 4°C)**:**

50mM Tris-HCl, pH 7.5
10mM EDTA
100 µg/ml RNAseA

Solution II (should be fresh)**:**
0.2 M NaOH
1% SDS

Solution III:
Dissolve 6.13 mg solid potassium acetate and 3.57 mg glacial acetic acid in $_{dd}H_2O$ to get 50ml solution

Transformation

- ❑ Thaw chemically competent cells (DH1α *E. coli*) on ice (ca. 20 min)
- ❑ Add 20-30 ng plasmid DNA (dilute so that volume added is around 1 µl)
- ❑ Leave cells on ice for 20-30min
- ❑ Heat shock at 42°C for 1min30
- ❑ Put back on ice immediately and plate on Amp plates
- ❑ Colonies grow at 37°C within 14-16 hours (over night)

Restriction digest

Ratios are generally the same. For analytical digest usually a volume of 10µl is set up, for fragments that will be used in further cloning a volume of 40µl is useful. Check specific optimal conditions for each restriction enzyme and recommendation for double digests etc.

	Volume of 10µl	**Volume of 40µl**
dd H2O	Add to 10µl	Add to 40µl
10x enzyme buffer	1µl	4µl
10x BSA	1µl	4µl
DNA (plasmid)	Between 500ng and 1µg in max. 7.5µl volume	3-5 µg in max. 32µl
Restriction enzyme	0.5µl	2µl
Incubate for most restriction enzymes at 37°C, in general for analytical digest 1-2 hours is fine, 3-4 hours for larger digests (check on gel to make sure that digest is complete)		

2.9. References and helpful literature/links

Parhyale embryology:

• Gerberding, M., Browne, W.E., Patel, N.H., 2002. Cell lineage analysis of the amphipod crustacean *Parhyale hawaiensis* reveals an early restriction of cell fates. Development 129, 5789-5801.

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• Zeng, V., Ewen-Campen, B., Alwes, F., Browne, W.E., Extavour, C.G., 2011. *De novo* assembly and characterization of a maternal and developmental transcriptome for the emerging model crustacean *Parhyale hawaiensis*. BMC Genomics 12(1): 581.

Parhyale transgenesis:

• Pavlopoulos, A., Averof, M., 2005. Establishing genetic transformation for comparative developmental studies in the crustacean *Parhyale hawaiensis*. Proc. Natl. Acad. Sci. USA 102, 7888-7893.

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Parhyale established protocols:

• Rehm, E. J., Hannibal, R.L. , Chaw, R.C. , Vargas-Vila, M.A. , and Patel, N.H., 2009. The Crustacean *Parhyale hawaiensis*: A New Model for Arthropod Development *Cold Spring Harb Protoc; doi:10.1101/pdb.emo114*

• Rehm, E. J., Hannibal, R.L. , Chaw, R.C. , Vargas-Vila, M.A. , and Patel, N.H., 2009. Protocol Injection of *Parhyale hawaiensis* Blastomeres with Fluorescently Labeled Tracers *Cold Spring Harb Protoc; doi:10.1101/pdb.prot5128*

• Rehm, E. J., Hannibal, R.L. , Chaw, R.C. , Vargas-Vila, M.A. , and Patel, N.H., 2009. Antibody Staining of *Parhyale hawaiensis* Embryos. *Cold Spring Harb Protoc; doi:10.1101/pdb.prot5129*

• Rehm, E. J., Hannibal, R.L. , Chaw, R.C. , Vargas-Vila, M.A. , and Patel, N.H., 2009. Fixation and Dissection of *Parhyale hawaiensis* Embryos. *Cold Spring Harb Protoc; doi:10.1101/pdb.prot5127*

• Rehm, E. J., Hannibal, R.L. , Chaw, R.C. , Vargas-Vila, M.A. , and Patel, N.H., 2009. In Situ Hybridization of Labeled RNA Probes to Fixed *Parhyale hawaiensis* Embryos. *Cold Spring Harb Protoc; doi:10.1101/pdb.prot5130*

More protocols, images and other information:

• http://patelweb.berkeley.edu/

• http://extavourlab.com/protocols/index.html

Amphipod development:

• Scholtz, G., Wolff, C., 2002. Cleavage, gastrulation, and germ disc formation of the amphipod *Orchestia cavimana* (Crustacea, Malacostraca, Peracarida). Contributions to Zoology 71, 9-28.

• Wolff, C., Scholtz, G., 2002. Cell lineage, axis formation, and the origin of germ layers in the amphipod crustacean *Orchestia cavimana*. Dev Biol 250, 44-58