Supplemental Materials for:

Using hybridization chain-reaction fluorescent *in situ* hybridization (HCR-FISH) to track gene expression by both partners during initiation of symbiosis

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Whole-Mount *In Situ* Hybridization of Juvenile Hawaiian Bobtail Squid using Hybridization Chain Reaction (HCR)

Modified from protocols by Lee, *et al*. (2009), and Choi, *et al*. (2014).

A. Solutions Needed

1) Filtered natural seawater or artificial Instant Ocean (Aquarium Systems, Mentor, OH)
2) Marine PBS (mPBS) – 50 mM phosphate buffer, pH 7.4; 0.45 M NaCl
3) Permeabilization Buffer – mPBS containing 1% (v/v) Tween-20
4) 50% Hyb Buffer – DNA Hybridization Buffer (Molecular Instruments; www.molecularinstruments.org) mixed 1:1 with mPBS
5) Probe Wash Buffer – Provided by Molecular Instruments
6) DNA Amplification Buffer – Provided by Molecular Instruments
7) 5X SSC (750 mM NaCl, 75 mM sodium citrate, pH 7)
8) 5X SSC_{Tw} – 5X SSC containing 0.05% Tween-20

B. Method

1. Collection and Fixation of *E. scolopes*

[1.1] Collect juvenile squid from hatching table (refer to colonization protocol as needed) and isolate into collection cups. Colonize with desired *V. fischeri* strain.

[1.2] Anesthetize squid for 2 min by placing into 2% ethanol in filtered seawater.

[1.3] Under dissecting scope, split and peel back mantle on anterior side. Then carefully pull back the funnel to expose the light organ.

[1.4] Place dissected squid into 4% paraformaldehyde (PFA) and incubate overnight at 4 °C on shaker to fix. The best vessels to use are 1.5mL screw-cap vials, to prevent leaking at later wash steps.

2. Permeabilization by Proteinase K

Use RNase-free equipment and solutions throughout the remainder of the protocol. Perform all treatments, hybridizations, and washes in a 500-µL volume, on a rotator/shaker, unless otherwise noted.

[2.1] Wash each sample five times (5 min per wash) with Permeabilization Buffer at room temperature (RT).

[2.2] Treat with 0.01 mg/mL Proteinase K Permeabilization Buffer at RT for 15-20 minutes. Do not place on shaker or rotator.

[2.3] Stop the proteinase K digestion with two washes of 2 mg/mL glycine in Permeabilization Buffer.

[2.4] Post-fix in Permeabilization Buffer with 4% PFA for 1 h at RT on the shaker.

[2.5] Wash with Permeabilization Buffer five times (5 min per wash).

Note: Permeabilized juveniles can be used immediately, or stored for no longer than 1 week at 4 °C in Permeabilization Buffer.

3. Pre-hybridization for Probes

[3.1] Remove as much of the Permeabilization Buffer as possible from the sample. Incubate sample in 500 µL of 50% Hyb Buffer at 65 °C for 30 min.

[3.2] Change sample into 500 µL of fresh 50% Hyb Buffer, and incubate at 65 °C for 2.5 h.

Note: Prevent drying during prolonged incubations. When using petri plates, place them in a humidified chamber or seal the cover of the plate with a strip of parafilm.

4. Probe Hybridization

To identify possible artifacts and confounding effects, the following alternate sample preparations should be performed:

a. Autofluorescence (AF) – Follow protocol but do not add probes (step 4) or hairpins (step 7)

b. Non-Specific Amplification of hairpins (NSA) – Sample incubated without probes (step 4) but with hairpins included.

c. Non-Specific Detection of targets (NSD) – This control is applicable only for (i) transgenic (non-endogenous) targets, where a wild-type sample missing the target transcript is treated using the same protocol, and with the test probes and hairpins; or (ii) non-ubiquitous endogenous target transcript, where the locus of expression

is known beforehand, and for which surrounding tissue can give an estimate of NSD in the same sample after treatment.

[4.1] Mix 1 pmol of each probe in 500 µL of 50% Hyb buffer at 45 °C for 30 min (*this step should be coordinated with step [3.2] so that they are completed at the same time*).

[4.2] Remove the 50% Hyb buffer from [3.2], and add this probe solution to samples for overnight (16 h) incubation at 45 °C.

5. Probe Washes

All solutions used here must be pre-warmed to 45 °C. Probe solution is not reused, and hence discarded at the start of the washes.

[5.1] Wash samples in 500 µL Probe Wash Buffer for 15 min at 45 °C.

[5.2] Wash samples in 500 µL (75% of Probe Wash Buffer + 25% of 5X SSC) for 15 min at 45 °C.

[5.3] Wash samples in 500 µL (50% of Probe Wash Buffer + 50% of 5X SSC) for 15 min at 45 °C.

[5.4] Wash samples in 500 µL (25% of Probe Wash Buffer + 75% of 5X SSC) for 15 min at 45 °C.

[5.5] Wash samples 2 times, in 500 µL of 5X SSC for 15 min at 45 °C.

[5.6] Wash samples 2 times, in 500 µL of 5X SSC for 30 min at 45 °C.

6. Pre-hybridization for Hairpins

[6.1] Incubate samples in 500 µL of DNA Amplification Buffer at RT for 30 min.

[6.2] Incubate samples in fresh 500 µL of DNA Amplification Buffer at RT for 30 min.

[6.3] Aliquot 6 pmol (for every 100 µL of DNA Amplification Buffer) of each hairpin in a separate PCR tube.

[6.4] Heat the hairpins to 95 °C for 90 sec (*e.g.*, using a PCR machine/thermal cycler).

[6.5] Store the heated hairpins in the dark for 30 min at RT (keep hairpins unmixed).

[6.6] Prepare 100 µL of fresh DNA Amplification Buffer equilibrated at RT.

(*Steps [6.2] through [6.6] should be coordinated so that they are completed at the same time for all samples)*

7. Hairpin Amplification

[7.1] Mix all hairpins in 100 µL of pre-equilibrated DNA Amplification Buffer at RT. (Final concentration of each hairpin is 60 nM.)

Note: The volume of this incubation can be scaled up if needed (*i.e.*, for high-abundance squid transcripts); however, the hairpin concentration must be kept constant.

[7.2] Remove final wash solution from [6.2] and add the hairpin solution to the samples for an overnight (16 h) incubation at RT.

[7.3] Wrap the sample tubes (or incubation oven) in aluminum foil to keep light out.

8. Hairpin Washes

Note: All solutions used here must be pre-equilibrated to RT.

[8.1] Wash samples 4 times, in 500 µL of 5X SSC_{Tw} for 5 min each at RT

[8.2] Wash samples 2 times, in 500 µL of 5X SSC_{Tw} for 30 min each at RT

9. Imaging

[9.1] Samples can be imaged directly in 5X SSC_{Tw}, or stored in 5X SSC_{Tw} at 4 °C.

Note: The processed samples can be counterstained with phalloidin or wheat germ agglutinin following standard protocols.

Table S1

Probe Sequences		
Probe	Hairpin (2)/Fluorophore	Probe Sequence
V. fischeri 16S probe #1	B3 / Alexa488	5′-TGTGCGGGCCCCCGTCAATTCATTTGAGTTTTAATCTTGCGACCGTACTC
V. fischeri 16S probe #2	B3 / Alexa488	5′-GTAGGTAAGGTTCTTCGCGTTGCATCGAATTAAACCACATGCTCCACCGC
V. fischeri luxA probe #1	B4 / Alexa546	5′-GAAAGTTGATCTAACAGCAATACATCCTCTGCTTGCCGTACGGGGTGAGC
V. fischeri luxA probe #2	B4 / Alexa546	5′-CCAACTAAGTACCATTGGCATCCCTTGCCTTGCTAGCCACTCAGTAGTAC
V. fischeri luxA probe #3	B4 / Alexa546	5′-CTGATCAAGAACTTCCGACATACCTCACGAGCTCTATCCCCATCTTCGTG
V. fischeri luxA probe #4	B4 / Alexa546	5′-CGCTGAATAATCTCAAGGCACTCTGCAGGAGTTCCAACAGGATTAAGTCC
V. fischeri luxA probe #5	B4 / Alexa546	5′-TCAGTGCCATTAGCCTCAAAACCACAGGTGACGTTAGTAATACCAGTCGC
E. scolopes Actin probe #1	B1 / Alexa488	5′-CCGTGTTCAATGGGGTATTTCAAGGTAAGGATACCTCTCTTGCTCTGGGC
E. scolopes Actin probe #2	B1 / Alexa488	5′-CGACACGGAGCTCGTTGTAGAAGGTGTGATGCCAGATTTTCTCCATGTCA
E. scolopes Actin probe #3	B1 / Alexa488	5′-CTGGATGGCAACATACATGGCGGGAGCGTTGAAGGTCTCGAACATGATTT
E. scolopes Actin probe #4	B1 / Alexa488	5′-TTCATAGATGGGTACAGTGTGGGTGACACCATCGCCGGAGTCAAGAACAA
E. scolopes Actin probe #5	B1 / Alexa488	5′-ACGTTCGGTCAAGATCTTCATGAGGTAGTCAGTAAGATCACGTCCGGCCA
E. scolopes HSP90 #1	B5 / Alexa628	5′-TCCAGATGTTTCTTGGCTGCCATATATCCCATCGTTGAGGTGTCTCGCAG
E. scolopes HSP90 #2	B5 / Alexa628	5′-TATCAGCATCCACTTTCTCCTTCAGGGATTTGATGATAGGGTGGTCGGGG
E. scolopes HSP90 #3	B5 / Alexa628	5′-TCATCATCTTCCAAAGGTGGGAGTTCAGCATCAACAGAGTCTCCTGCTCC

E. scolopes HSP90 #4	B5 / Alexa628	5′-GCTGGATCAGTTTTTGGAACCTACACCAATTCCGCAATGATACGCAGCGC
E. scolopes HSP90 #5	B5 / Alexa628	5′-TTTTTTTTCCGTTAGCAGTCACCTCTTTCAGACCCAAGCTCTAGCCGCGC
E. scolopes PGRP1/2 #1	B2 / Alexa546	5′-CGTATTTTTACCGCAGTCGAGATGGCGCTCTTTACGAATGATGGGAGGTC
E. scolopes PGRP1/2 #2	B2 / Alexa546	5′-CCGTTTCTTCTTTCGTTGCTGTTCTGCCTACTAGACGAGCGATTTGCTGG
E. scolopes PGRP1/2 #3	B2 / Alexa546	5′-AGGGTAACGCCTTTACAGGTGCCATTTCCGAAACTGCAAGCTGAGGACAT
E. scolopes PGRP1/2 #4	B2 / Alexa546	5′-AACCATTTTGACGGGCATCGGTATAGAAACGACTTCTTTGGGTGGACGCG
E. scolopes PGRP1/2 #5	B2 / Alexa546	5′-GCCGATATCGAACCATCCTCGGTCGTCCATATGAAAGTTCTGGATTTTTC
E. scolopes EsCadDP1 #1	B2 / Alexa546	5′-AGGATCCATGGCTGTGCATGGTTCCTCTGCTGCATACCCTGATGTAATCA
E. scolopes EsCadDP1 #2	B2 / Alexa546	5′-TAGGTCGAATTCCGGCATCTGGTCATCTCCATCGATCACTGTCAGGTTCA
E. scolopes EsCadDP1 #3	B2 / Alexa546	5′-TAGCAGCCATCGAGTCTCCATCTTTCGCTTTGATTTGCATTGCTGGACCC
E. scolopes EsCadDP1 #4	B2 / Alexa546	5′-GTAGACAACTGTTCCGACCAGGTCCGACTTATCAACGGGTTTCACCATAC
E. scolopes EsCadDP1 #5	B2 / Alexa546	5′-GGGGAAAACACTCATAATCGGATCACAAGCTCATCCCCTCATTGACATGC

Fig S1

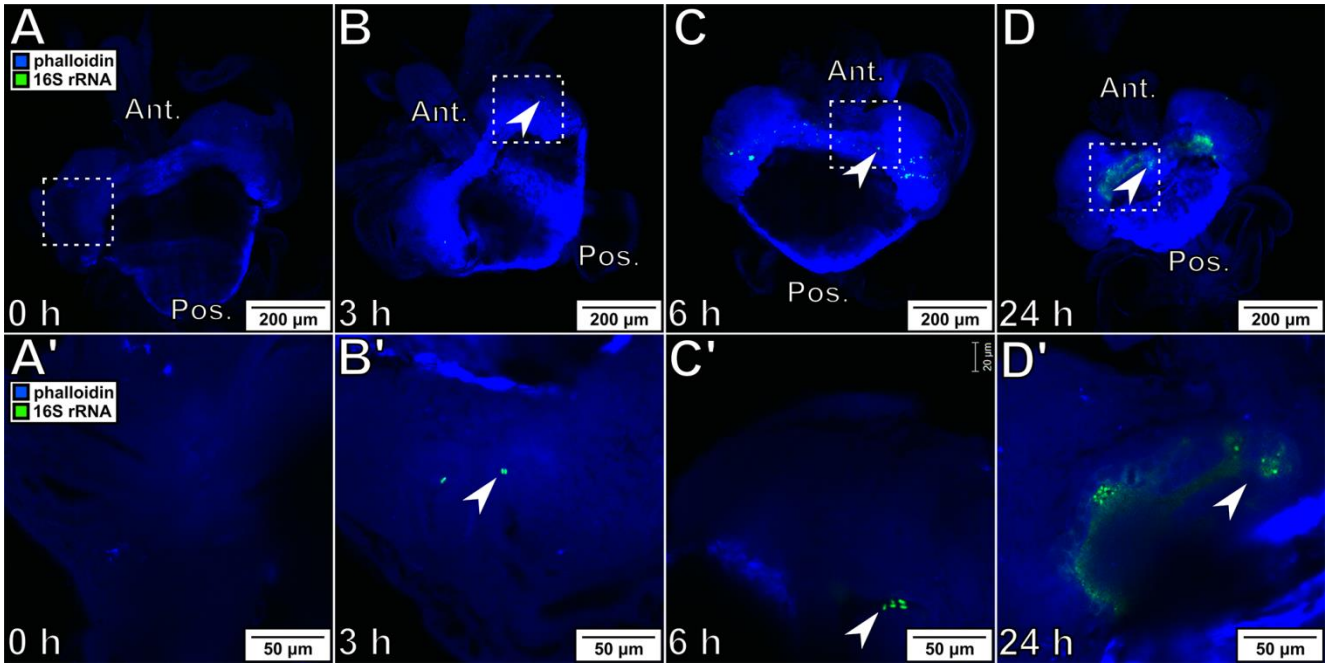

Figure S1. Tracking the position of bacteria during colonization. For all images, colonizing bacteria were labeled by probes to the 16S ribosome (green), and squid tissue was stained with 633-phalloidin (blue); panel X' represents a higher magnification (40X) image of the area in the dashed box for the light organ in X (10X). Prior to exposure (**A, A'**) no bacteria are visible within the light organ. By 3 h after exposure (**B, B'**), bacteria (arrows) have associated with the host tissue, and are visible within the light-organ ducts, located immediately interior of the pores. After 6 h of exposure (**C, C'**), bacteria have migrated into the light organ and begun to colonize the crypt space. By 24 h (**D, D'**), the host is bioluminescent, and bacteria are visible throughout the crypts.

Fig S2

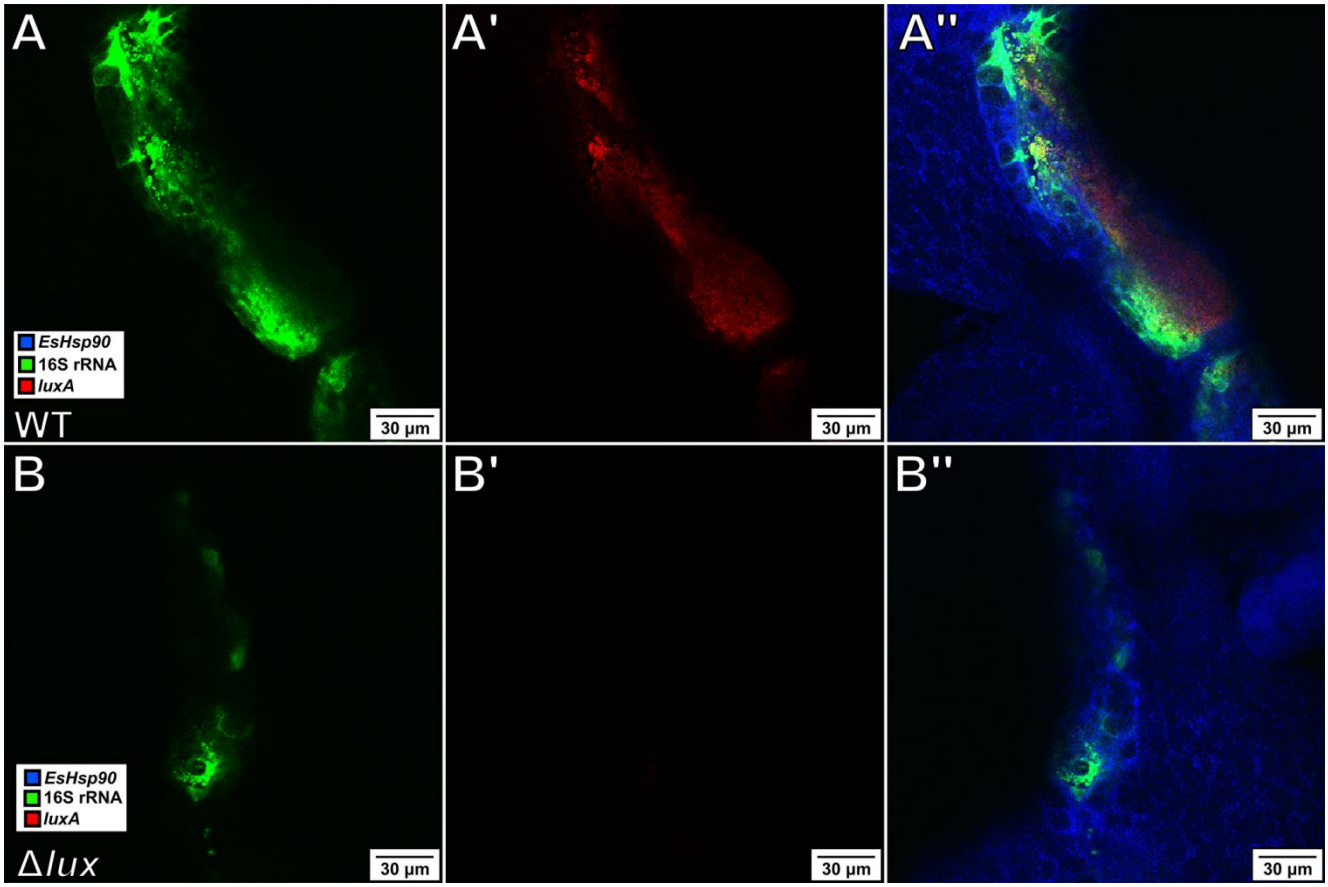

Figure S2. Validation of the specificity of the *luxA* probe. For all images, panel X shows labeling of the transcript for the bacterial 16S ribosomal subunit (green), panel X' shows labeling of the *luxA* transcript, and panel X'' shows host tissue labeled for HSP90 transcript (blue) overlaid with the two bacterial labels. (**A**) Wild-type *V. fischeri* were used to colonize squid, and samples were collected at 24 h post-inoculation, at which time the symbionts were fully bioluminescence. (**B**) Alternatively, Δ*lux V. fischeri* were used to colonize squid, and sampled at the same time point.

References

1. **Lee PN, McFall-Ngai MJ, Callaerts P, de Couet HG**. 2009. Whole-mount *in situ* hybridization of Hawaiian bobtail squid (*Euprymna scolopes*) embryos with DIG-labeled riboprobes: II. Embryo preparation, hybridization, washes, and immunohistochemistry. Cold Spring Harb Protoc 2009:pdb prot5322.
2. **Choi HM, Beck VA, Pierce NA.** 2014. Next-generation *in situ* hybridization chain reaction: higher gain, lower cost, greater durability. ACS Nano **8:**4284-4294.