

## Video Article

**Colonization of *Euprymna scolopes* Squid by *Vibrio fischeri***

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Specific bacteria are found in association with animal tissue<sup>1-5</sup>. Such host-bacterial associations (symbioses) can be detrimental (pathogenic), have no fitness consequence (commensal), or be beneficial (mutualistic). While much attention has been given to pathogenic interactions, little is known about the processes that dictate the reproducible acquisition of beneficial/commensal bacteria from the environment. The light-organ mutualism between the marine Gram-negative bacterium *V. fischeri* and the Hawaiian bobtail squid, *E. scolopes*, represents a highly specific interaction in which one host (*E. scolopes*) establishes a symbiotic relationship with only one bacterial species (*V. fischeri*) throughout the course of its lifetime<sup>6,7</sup>. Bioluminescence produced by *V. fischeri* during this interaction provides an anti-predatory benefit to *E. scolopes* during nocturnal activities<sup>8,9</sup>, while the nutrient-rich host tissue provides *V. fischeri* with a protected niche<sup>10</sup>. During each host generation, this relationship is recapitulated, thus representing a predictable process that can be assessed in detail at various stages of symbiotic development. In the laboratory, the juvenile squid hatch aposymbiotically (uncolonized), and, if collected within the first 30-60 minutes and transferred to symbiont-free water, cannot be colonized except by the experimental inoculum<sup>6</sup>. This interaction thus provides a useful model system in which to assess the individual steps that lead to specific acquisition of a symbiotic microbe from the environment<sup>11,12</sup>.

Here we describe a method to assess the degree of colonization that occurs when newly hatched aposymbiotic *E. scolopes* are exposed to (artificial) seawater containing *V. fischeri*. This simple assay describes inoculation, natural infection, and recovery of the bacterial symbiont from the nascent light organ of *E. scolopes*. Care is taken to provide a consistent environment for the animals during symbiotic development, especially with regard to water quality and light cues. Methods to characterize the symbiotic population described include (1) measurement of bacterially-derived bioluminescence, and (2) direct colony counting of recovered symbionts.

**Video Link**The video component of this article can be found at <http://www.jove.com/video/3758/>**Protocol****1. Preparation of Bacterial Inocula****1. Day 0**Two days prior to squid inoculation, plate the relevant bacterial strains on LBS<sup>13</sup> agar.

2. Incubate bacteria at 25-28°C overnight.

**3. Day 1**Inoculate 3 ml LBS medium in a glass culture tube with one colony of each *V. fischeri* strain for infection. Prepare duplicate tubes as backup.**4. Day 2****(Coordinate bacterial steps 1.4-1.6 with squid steps 3.7-3.10)**

1 h prior to inoculation, subculture bacteria 1:80 (37.5 µl) into 3 ml LBS in a glass culture tube and grow for 1 h with aeration.

5. Measure the OD<sub>600</sub> of the sample prior to inoculation. Typical measurements are 0.3-0.6 depending on the strain.6. For a target inoculum of 3-5 × 10<sup>3</sup> CFU/ml calculate the inoculum volume as follows: Inoculum volume (µl) = 1.25 / OD<sub>600</sub> (e.g., For OD<sub>600</sub> = 0.5, the calculated inoculum volume = 1.25/0.5 = 2.5 µl). This amount is added directly to the seawater containing squid in Step 4.1. This calculation may need to be adjusted for different strains of *V. fischeri* or for inoculation at lower or higher levels than those specified here.**2. Preparation of Agar Plates for Enumeration of the Inocula**

1. For each treatment, label LBS plates (2 per treatment) to plate samples of the inoculum in Step 4.1.

2. Add 5 sterile plating beads per plate.

### 3. Collection of Squid Juveniles

1. Measure the salinity of Instant Ocean using the refractometer and adjust to 35‰.
2. Filter 1 L of Instant Ocean using the filtration unit and an attached vacuum line or vacuum pump, to generate filter-sterilized Instant Ocean (FSIO). Oxygenate the water by swirling vigorously prior to each dispensing. The filter unit can be reused for 2 days.
3. Aliquot 40-50 ml of FSIO into each of two (2) disposable sample bowls. Label one as *earlies* and one as *timelies*.
4. Prepare an excess of plastic transfer pipettes for acquiring juvenile squid by cutting the pipette approximately 1 cm from the tip, above the lowest ridges (see Figure 3). This facilitates a wider area through which the squid can pass upon collection. Discard any transfer pipettes in which there is a rough exposed surface.
5. Using prepared transfer pipettes, collect *E. scolopes* that hatched overnight and transfer to the *earlies* bowl of FSIO. Early hatchlings have been in the egg system for over 1 h and are susceptible to colonization by contaminating *V. fischeri* in the egg system. Do not use *earlies* for sensitive colonization experiments.
6. Check egg tanks every 30-45 min for new hatchlings. Ensure that all hatchlings are cleared during each check. Remove hatchlings with a transfer pipette, and deposit into the *timelies* bowl of FSIO. Animals collected in a timely fashion are available for colonization experiments.
7. When the collection has finished (~ 45 min after dusk), transfer the squid to the main laboratory. Empirically it is advantageous to colonize the animals under uninterrupted laboratory light conditions for 3 h inoculations.
8. For each treatment, prepare a bowl with 40 ml FSIO. Add squid to the bowls for the assay (maximum n=40 per bowl).
9. Prepare an additional bowl as an aposymbiotic (negative) control.
10. Prepare a dedicated transfer pipette for each treatment.
11. Euthanize extra squid in 2% ethanol.

### 4. Squid Colonization

1. **Day 2** - Using a P10 Pipetman, dispense the calculated aliquot of bacteria (Step 1.5) into each squid bowl (Step 3.8) for each treatment. Start a 3 h timer immediately after the first inoculation.
2. For each treatment, create a "vortex" in the bowl with the dedicated transfer pipette by placing the pipette near the edge of the bowl and pipetting up and down repeatedly to mix the water and squid for approximately 10 sec. Thorough mixing is critical.
3. Plate 50  $\mu$ l from each bowl onto an LBS agar plate from Step 2.2 (for technical replicates, plate two 50  $\mu$ l plates per treatment). Incubate at 25-28°C overnight.
4. Prepare wash bowls (100 ml FSIO/ea) for each treatment.
5. Prepare *Drosophila* vials (4 ml FSIO/ea) for each squid.
6. After exactly 3 h, transfer the squid to their respective wash bowls (complete for all treatment). This stops the inoculation.
7. Proceed to transfer each individual squid to its own *Drosophila* vial with FSIO. Use a designated transfer pipette for each treatment.
8. Move trays of *Drosophila* vials to the squid facility to return to the day/night light cycle the animals experienced during embryogenesis.
9. **Day 3** - Prepare *Drosophila* vials (4 ml FSIO/ea) for each squid.
10. Prior to dusk at 22-24 h post-inoculation, transfer each squid to a new *Drosophila* vial. Use a designated transfer pipette for each treatment.
11. **Day 4** - Prepare labelled 1.5 ml microcentrifuge tubes (1/squid).
12. Prior to dusk at 46-48 h post-inoculation, measure and record the luminescence of each squid in the *Drosophila* vial (luminometer set for 6 s integration and auto-read on lid closure).
13. As a negative control for background luminescence, measure a vial with FSIO that does not contain any squid.
14. Transfer each squid in a volume of approximately 700  $\mu$ l to a 1.5 ml microcentrifuge tube from Step 4.12. Move to a cardboard freezer box. Once the lid is placed on the box, do not remove it as the light cues for bacteria expulsion are not well-understood.
15. Freeze microcentrifuge tubes at -80°C overnight.

### 5. Determination of Colonization Levels

1. For each squid, prepare two (2) microcentrifuge tubes, each with 475  $\mu$ l FSIO (or autoclaved 70% Instant Ocean).
2. Prepare pestles by first using a Kimwipe to clean the pestle and remove gross debris and/or tissue.
3. Place pestles tip-down in a 50 ml beaker containing 95% ethanol. Ethanol should be added to a height of approximately 3 cm.
4. For each pestle, remove from the beaker and wipe the tip with a Kimwipe.
5. Dip pestle back into the ethanol bath, remove and insert (tip up) into a microcentrifuge tube rack and allow to air dry completely for approximately 15 minutes.
6. Thaw squid in a microcentrifuge tube rack (maximum n=8).
7. If necessary, adjust the volume to 700  $\mu$ l.
8. Using a pestle from Step 5.5, disrupt the animal tissue until the ink sac ruptures (the water will turn a murky grey color).
9. Remove the pestle and ensure all tissue remains in the tube.
10. Vortex the tissue briefly for exactly 10 seconds (use a timer).
11. Allow the tissue to rest for 10 min. The tissue will settle and the bacteria and ink remain in solution. For the calculations that follow, the bacteria/ink solution is the [A] dilution (i.e. the *E. scolopes* light organ homogenate in 700  $\mu$ l). Serial 1:20 dilutions ([B], [C]) are described below.
12. For the [B] dilution, add 25  $\mu$ l [A] to one of the microcentrifuge tubes prepared in Step 5.1. Vortex.
13. For the [C] dilution, add 25  $\mu$ l [B] to one of the microcentrifuge tubes prepared in Step 5.1. Vortex.
14. Plate 50  $\mu$ l of each dilution onto LBS agar, 2 replicates per treatment.
15. Incubate the plates at 25-28°C overnight.

### 6. Data Analysis

1. To calculate the CFU/light organ (LO), count colonies on the plate for each treatment in which 10-400 colonies are present, and use the appropriate formula:

$$\text{CFU/LO} = (\text{colonies on [A] plate}) \times 14; \text{ or}$$

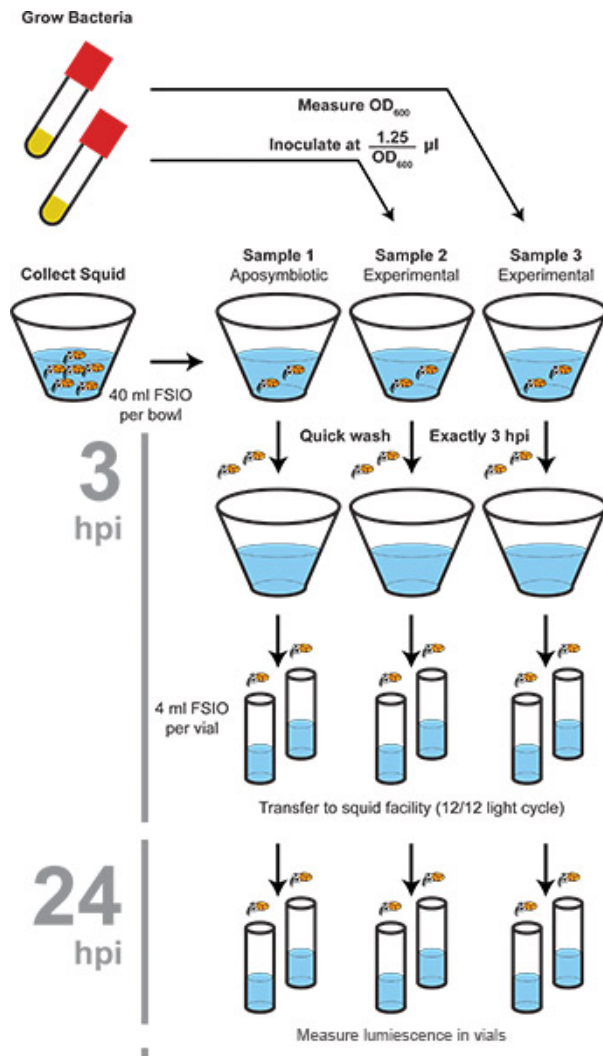
CFU/LO = (colonies on [B] plate) x 280; or

CFU/LO = (colonies on [C] plate) x 5600.

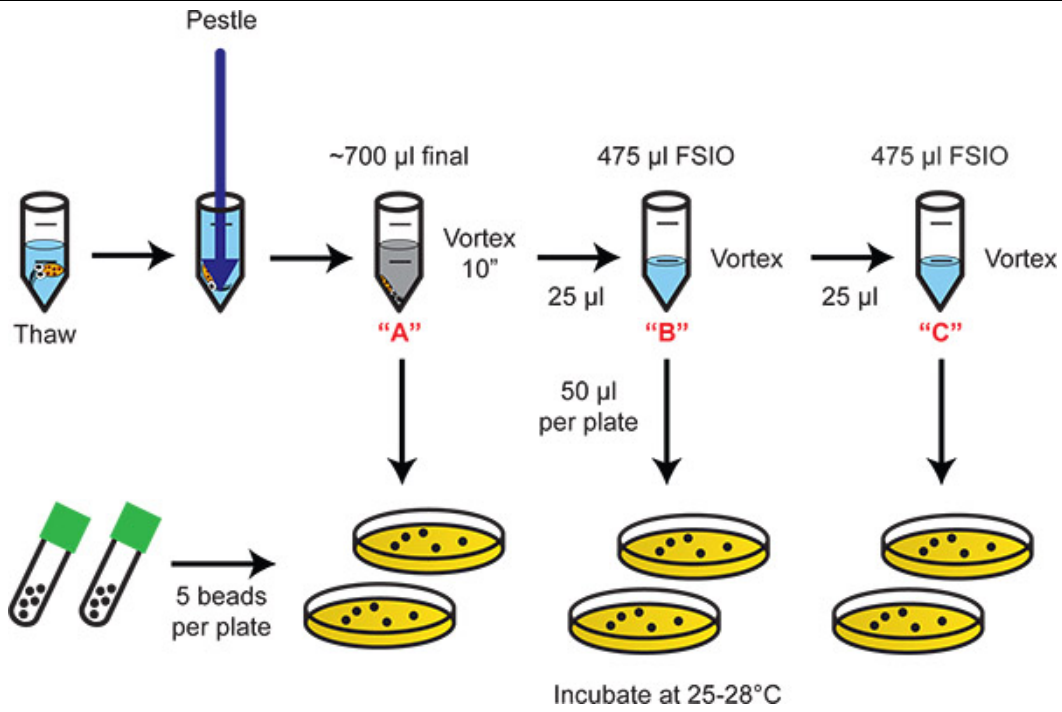
2. Plot individual data points and medians on a logarithmic scale.
3. The data are often not normally distributed, with different variances, and the outliers may contain biologically meaningful information. Therefore, non-parametric tests provide a useful method to determine whether the treatments differ significantly.
4. Use GraphPad Prism software for statistical analysis. For two treatments, use the Wilcoxon Rank Sum test. For comparisons among greater than two treatments, use the Kruskal-Wallis test with appropriate post-tests.

## 7. Representative Results

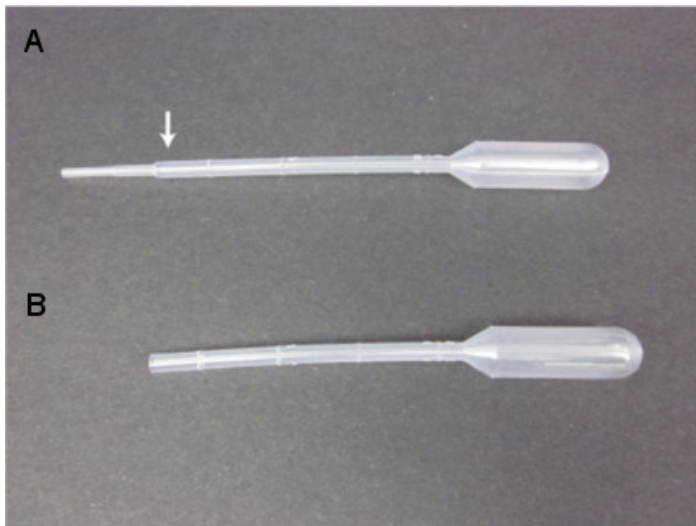
Results from a sample colonization assay are shown in Figure 4. Two strains of *V. fischeri* that exhibit different relative levels of luminescence were each inoculated into six squid, along with six squid that served as aposymbiotic (uncolonized) controls. The *E. scolopes* symbiont, ES114<sup>14</sup>, and the brighter *Sepiolo robusta* symbiont, SR5<sup>15,16</sup>. Similar inoculum levels (Fig. 4A) lead to 100% colonization within 3 h. At 48 h, the luminescence levels (Fig. 4B) and CFU counts (Fig. 4C) were determined to assess the colonization proficiency of the strain. Determination of the specific luminescence (Fig. 4D; per bacterium) allows for determination of the brightness of each bacterial strain during the symbiosis.



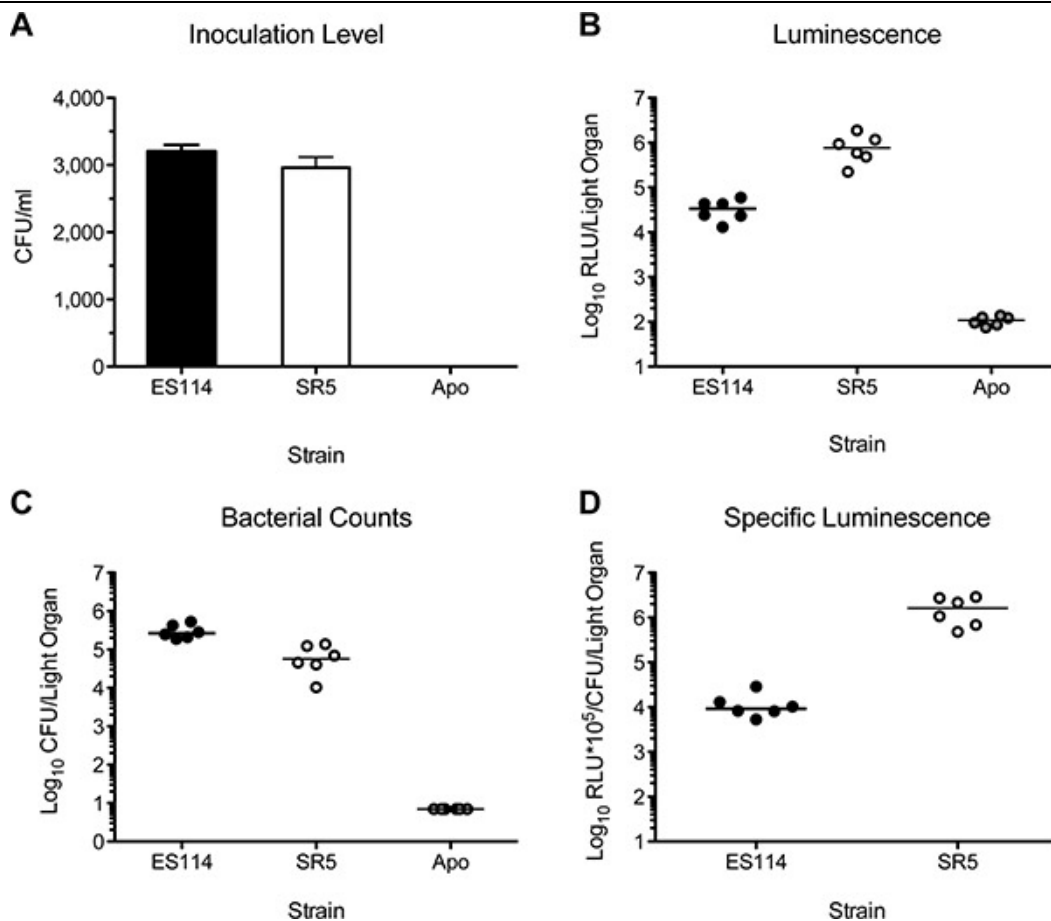
**Figure 1.** Flow chart of the colonization procedure. Bacteria and squid are harvested separately, then mixed at the specified inoculum. Squid are washed, then transferred to new water at 3 h, 24 h, and 48 h post inoculation. At 48 h the luminescence is measured and the animals are frozen, which serves to surface-sterilize the animals. Light-organ colonized bacteria remain viable at -80°C through one thaw (no additional freeze-thaw cycles).



**Figure 2.** Flow chart illustrating homogenization and dilution plating of the bacteria. Serial 20-fold dilutions provide the appropriate dynamic range for enumeration of colonized bacteria.



**Figure 3.** Transfer pipettes with an appropriately narrow shaft taper to a narrow bore (A) that would damage juvenile squid. Pretreatment by cutting off the narrowest section with scissors or a razor blade yields an appropriate tool (B) for transferring juvenile squid.



**Figure 4.** Sample data for a colonization assay. (A) Levels of the bacteria in the inoculum bowls. (B) Luminescence of individual squid. (C) Colony counts of individual squid. (D) Specific luminescence of individual squid. Apo, Aposymbiotic (uncolonized negative control).

## Discussion

The colonization assay described allows for analysis of a natural symbiotic process in a controlled laboratory environment. As such, it can be used to assess colonization by mutant strains, by different natural isolates, and under different chemical regimes. Variations on the experiments described are commonly used to assess different aspects of the symbiosis. The kinetics of colonization can be measured by examining luminescence during the first 24 h, which can be detected automatically in a scintillation counter in which the coincidence detector has been removed. Furthermore, the relative colonization ability of one strain relative to another can be measured by a competitive colonization assay, in which the output ratio of the two strains in a set of animals are normalized to the input ratio (differential detection by distinct antibiotic resistance, fluorescence, or chromogenic [LacZ/Xgal] markers). Finally, colonization can be imaged directly by confocal microscopy.

It is critical to use healthy juvenile squid for the experiments. Behavioral indicators of poor squid health include swimming in circles (euthanize the animal), or animals that remain white and do not alter their chromatophores to turn brown on a dark surface (use with care). As there exists variation in the host populations, a greater number of replicate experiments with smaller numbers of animals is often more valuable than a smaller number of replicate experiments with large sample sizes.

## Disclosures

The authors have nothing to disclose.

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