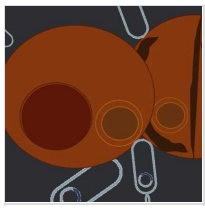

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Isolating non-axenic monoclonal Symbiodinium cultures from Aiptasia pallida

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Symbiodiniaceae-bacterial protocols at CEE Lab

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ABSTRACT

To isolate non-axenic, monoclonal *Symbiodinium* from the anemone *Aiptasia pallida* (CC7). Although there is no guarantee that all bacterial associates will be conserved using this method, 16S rDNA sequencing (unpublished data) suggested that previously documented bacteria[1] in the Symbiodiniaceae phycosphere (in a study not affiliated with our group, done on 5 clades of Symbiodiniaceae) were also prevalent in our monocultures even after three years of consistent subculturing with L1 (no Si) media.

[1] Lawson, C. A., Raina, J. B., Kahlke, T., Seymour, J. R., & Suggett, D. J. (2018). Defining the core microbiome of the symbiotic dinoflagellate, Symbiodinium. *Environmental Microbiology Reports*, *10* (1), 7-11.

NOTE: This protocol requires an inverted microscope. Please assure you have access to one for the entirety of the process.

DOI

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PROTOCOL CITATION

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GUIDELINES

This protocol requires an inverted microscope. Please assure you have access to one for the entirety of this protocol.

MATERIALS TEXT

- 0.2 µm filtered seawater (FSW) (~ 2 L)
- 15 mL Eppendorf tubes
- 2 mL microcentrifuge tubes
- L1 media prepared with 0.2 µm FSW (no Silica added), we used Bigelow's L1 Media Kit (SKU: MKL150L, https://ncma.bigelow.org/MKL150L), as making this media from scratch is quite costly
- Inverted microscope
- VWR Homogenizer
- Centrifuge
- Sterile, filter-tip pipettes/ pipettors (1000 µL, 200 µL and 20 µL)
- Protoslo® Quieting Solution, Laboratory Grade, 15 m (Item #: 885141, Carolina Biological), or alternatively, methyl cellulose
- Parafilm
- General purpose bacteriological agar (no extra additions, just a plain agar)
- 6-well plates
- Microscope slides w concave wells (Multitest Slide, 10 Well Capacity, MP Biomedical)
- Cell culture flasks (VWR flask tissue culture, 50ML plug seal or 0.2 µm filter seal)
- DNeasy Plant Mini Kit (Cat. No. / ID:69104)
- Qubit Fluorometer
- ITS2 Primers (ITS2-F: GTG AAT TGC AGA ACT CCG TG, ITS2-R: CCT CCG CTT ACT TAT ATG CTT)
- 4.5 mm plating glass beads, sterilized
- Incubator 25- 27 ºC, lights PAR ~10-50 µmol with a timer of 14:10 (light: dark)

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BEFORE STARTING

This protocol will take a minimum of 12 weeks. *Symbiodinium* growth takes time but it is mostly a background process, and patience is key. This project requires minimal expenditure on the human side after the initial isolations.

INITIAL ISOLATION FROM ANEMONE

1 

Rinse an anemone (preferably ~ 1cm +) with 0.2 µm FSW at least 3x, prior to disruption with a homogenizer. Place the anemone in a 15 mL Eppendorf tube with ~ 5mL of 0.2 µm FSW and homogenize at "medium" speed for >30 seconds or until all visible anemone tissue has been completely disrupted.

2 

Remove any chunky, clear floating tissue. Transfer the slurry to three, 2 mL microcentrifuge tubes. Aliquot evenly and centrifuge at 8000 rpm for 2 minutes. This will bring the algal cells to the bottom.

3 Remove the supernatant and add 1 mL of 0.2 µm FSW to each tube. Centrifuge at 8000 rpm for 1 minute. Repeat 3x.

4 Prepare a 6-well plate with 3mL of 1:1 L1 media (no Si added) and 0.2 µm FSW.

5 

Transfer the pellets of one tube to a well in the 6-well plate. Allow to incubate for up to 5 days (14:10, light: dark cycle, at 25-27ºC) or use an inverted microscope to check whether some *Symbiodinium* have expressed flagella and returned to a motile state.

*these *Symbiodinium* cells can range in size from 6-10 µm. The magnification you use will be limited to your microscope but please keep in mind their size so you can adjust your own settings.

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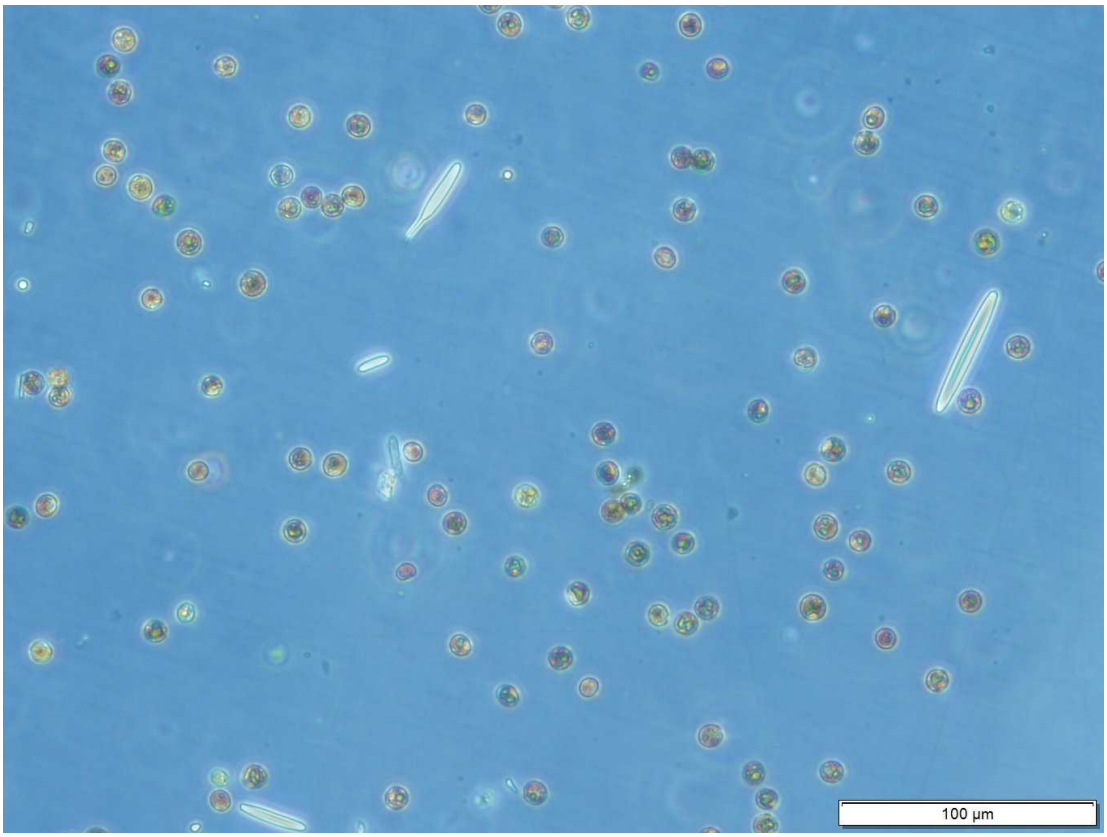

Symbiodinium, freshly expelled from the anemone tissue (steps 1-5, before incubation) and still in their symbiosomes (thin layer surrounding the algal coccoid cells). The clear, oblong-shaped cells are anemone stinging cells that were released during homogenization, as a defense mechanism of *Aiptasia*. Micrograph taken by Emily Aguirre.

6 While waiting for Step 5, prepare 1% agar according to the manufacturer's instructions, but modify with 0.2 µm FSW, instead of DI water. After the agar has cooled down and it is at a temperature of ~60-65ºC, add the components for L1 media, including the F/2 vitamins, no Si. Deposit plates in incubator at 27ºC for 24 hours. Store at 4ºC in a plastic sleeve.

7

After ~ 5 days, check for motile *Symbiodinium* in the 6-well plates using an inverted microscope, and if any are spotted swimming around, add a couple drops (or as needed) of *Protoslo* (or methyl cellulose) a protozoa slowing agent, to "catch them".

Symbiodinium, freed from the cnidarian symbiosome, ~ five days after separation from the host and after incubation (step 6). An accumulation of debris from anemone tissue degradation has occurred, which is why the many rinsing steps are crucial in this protocol. Video by Emily Aguirre.

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8

Pipet motile *Symbiodinium* and coccoid *Symbiodinium* into a sterile, small petri dish containing 1 mL of 0.2 µm FSW.

9 Meanwhile, place L1 agar plates in a 27ºC incubator for 30 minutes or until they are at room temperature.

FIRST TRANSFER/INCUBATION STEP (AGAR) 6w

10

Using an inverted microscope, transfer 1 or up to 10 individual cells onto a microscope slide with concave wells, each containing 20-50 µL of 0.2 µm FSW. Transfer the cells, GENTLY, through 3 wells to further rinse them. When the last well is reached, pipet the liquid (including the cell/s) and aliquot onto a warmed L1 agar plate (from step 9).

*This is a tedious process and will take a couple hours (depending on your pipetting skills) to go through all cells. But please try to be gentle and go as fast as you safely can, since the harsh microscope light (even in dimmer settings) can exacerbate their photosynthetic systems and stress them out, if exposed too long.

11

Add as many "cleaned" cells per agar plate (you can mix and match, motile vs coccoid shape) as you'd like. The total aliquot should be no more than 200 µL. I recommend starting off with 20-60 cells per plate.

The more cells you rinse and transfer to an agar plate, the faster your colonies will visibly show up on the plate.

However, transferring too many cells at once may introduce other eukaryotic microbes if not adequately rinsed. These can outcompete the dinoflagellates in the media immediately, leading to no *Symbiodinium* growth. Rinse plenty.

12 Add 3-4 plating beads, shake until all the liquid has been adsorbed on the surface. Discard beads and wrap in parafilm.

13

Incubate as before (step 5), but allow cells to grow for 2 weeks to 6 weeks after plating, or until

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visible, brown clusters appear on the surface of the plates.

SECOND TRANSFER/INCUBATION STEP (AGAR) 4w

14 Pick a colony "cluster" out, using a sterile filter-tip/ pipettor and transfer to a sterile, small petri dish containing 1mL of 0.2 µm FSW. Pipet the cluster up and down, gently, to disperse the cells and make it easier to visualize them.

15 

Visibly verify (by ASEPTICALLY taking a 10 µL aliquot from Step 14) it is a *Symbiodinium* cluster (and not brown diatoms) on an inverted or compound microscope.

16 Take 200 µL of the aliquot from step 14 and plate/incubate (as seen in steps 12 and 13) for 2-4 weeks.

THIRD TRANSFER/INCUBATION STEP (AGAR) 4w

17 

The resulting plate should clearly be all brown clusters, evenly distributed throughout the surface. From this plate, pick a colony and streak a new L1 agar plate with this colony. Incubate for 2-4 weeks, as before.

FOURTH TRANSFER/INCUBATION STEP (LIQUID, L1 MEDIA) 4w

18 After incubation, inspect the plate, aseptically pick out the biggest colony and add it to a sterile, 50 mL cell culture flask containing 25 mL of L1 media (no Si). Using a sterile 1000 µL pipet tip, pipet the liquid up and down to disperse the *Symbiodinium* and associated bacterial cells. Allow oxygen to pass through by loosely capping the cultures. A culture flask with a 0.22 µm filter cap can be tightly capped.

19 

Incubate for ~2-6 weeks or until there is sufficient visible, brown biomass.

20 I usually subculture once every 1.5 months when the flasks display a distinguishable brown film.

To subculture: Disturb the cultures gently with a 1000 µL pipet, and then pipet 500 µL of culture into a new cell culture flask containing 25 mL of L1 media (no Si). Repeat as needed.

VERIFYING IDENTITY OF SYMBIODINIUM

21 

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To verify and identify *Symbiodinium*, you can use any DNA extraction protocol for dinoflagellates, but I used DNeasy Plant Mini Kit. Quantify your DNA concentrations (I used Qubit fluorometer).

The ITS2 gene was sequenced and sent in for Sanger sequencing.

21.1

ITS2 Primers

- ITS2-F: 5'-GTG AAT TGC AGA ACT CCG TG-3'
- ITS2-R: 5'-CCT CCG CTT ACT TAT ATG CTT-3'

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