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Coral sperm collection for genome sequencing protocol, prepared by Shini Sunagawa

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Materials needed:

Centrifuge (e.g., Sorvall Biofuge Primo R, Rotor: Heraeus #7588)

50 mL Falcon tubes

Filtered (0.22 um) sea water (FSW)

Pipettors and wide-bored pipet tips (P200 and P1000)

Hemocytometer

Microscope

For agarose plugs only:

Sterile ddH_2O

Water bath

CHEF (mammalian) Genomic DNA Plug Kit (Cat. no: 170-3591)

Phenylmethylsulfonyl fluoride (PMSF) and isopropanol

1. Collection of bundles from a single colony.

The collection of bundles basically follows the spawning protocol and there is no standard protocol on how to do it. The most important thing is the outcome, i.e. obtaining approximately 150 mL of bundles (in the future, 50 mL will be sufficient) with as little contamination (eggs, broken bundle fragments, contaminating organisms) as possible. Using small cups (e.g. 350 mL) will ease the concentration of sperm cells. In our example (see below), we used a 350 mL cup, which yielded about 100 mL of sperm cell suspension after filtration (see below). We then distributed the sperm cells to 50 mL Falcon tubes that fit into a centrifuge, so that in one run all sperm cells could be spun down.

After the separation of coral sperm cells from predators, eggs, debris, or Symbiodinium!!! (see below), the sperm cells were pelleted, washed and:

a) finally flash frozen, and

b) used to produce plugs of agarose-embedded sperm DNA.

Example Mexico 2009:

Custom made nets were deployed with floating 350 mL collection cups (Figure 1A) on five healthy looking *Montastraea faveolata* colonies at “La Bocana” reef near Puerto Morelos (20°52’28.77”N and 86°51’04.53”W) on 10 September 2009 at around 5:30 pm. At around 10:30 pm, about 80% of sampled coral colonies started to spawn. Care was taken not to shine lights directly at cups to avoid the attraction of predators.

One colony was selected for sperm collection and tagged with cattle tag number 452. The collection cup, carefully closed with the labeled lid, contained about 150 mL of unbroken bundles at the time of collection. The samples were left at ambient temperature and brought to the laboratories of the UNAM - Instituto de Ciencias del Mar y Limnología field station within 1.5 hours.

NOTE: We kept another collection cup from a different colony on ice to preserve the integrity of bundles. Result: Aggregations formed and it seemed impossible to make use of this sample, thus do not place samples on ice before the centrifugation steps.

photo by Chris Voolstra

Photo by Shini Sunagawa

photo by Chris Voolstra

Top, left: collection net deployed on *M. faveloata* colony. Top, right: *A. palmata* bundles floating into collection cups (from field trip Florida 2006). Bottom, left: collection cups with filtered coral sperm suspension. Bottom, right:

2. Concentrate sperm and wash pellet

Once the bundles broke up completely, the content of the cup was passed 3 times through a 100 mm mesh (eggs are >300 um in diameter; (Szmant et al., 1997)), then filtered through a 20 mm cell strainer into four 50 mL Falcon tubes. The volume was adjusted to 50 mL with filtered seawater (FSW; 0.22 mm) and the suspension centrifuged at 1,000 x g for 5 min at 4°C.

After centrifugation, the pellet contained many pigmented cells (putatively, *Symbiodinium*) as identified by microscopy. Thus, the SUPERNATANT was taken, transferred to a new set of 50 mL Falcon tubes, and used for the concentration of sperm.

After centrifugation (same settings as above**),** the supernatant was discarded and the pellet resuspended in 50 mL FSW. Repeated microscopy indicated no presence of contaminating cells. The pellet was washed two more times by resuspension and centrifugation in 0.22um FSW. After the last centrifugation, as much seawater as possible was removed and the pellets from three Falcon tubes were combined to yield approximately 1 mL of highly concentrated sperm suspension. Aliquots of 100 uL were transferred to 2.0 mL cryovials using a wide-bore pipet tip and frozen at -80°C.

The last tube was used to make agarose plugs.

3. Making HMW DNA agarose plugs

Calculate amount of DNA per cell, given that 1Gigabase=1pg DNA:

If 15 mg DNA/plug are needed, calculate the amount of cells needed for ~50 plugs. *Acropora palmata* has an estimated #genome size of 0.75 gigabases (haploid), which translates to 1.5pg/cell.

Montastraea faveolata has an estimated genome size of 0.25 gigabases (haploid), which translates to 0.5pg/cell.

For *M. faveolata:*

Given an estimated haploid genome size of 500 Mbp, and given that the mass of 1 Gbp = 1 pg, there are 0.5 pg of DNA per sperm cell (haploid).

$$0.5 \text{ pg} \times (x \text{ cells}) = 15 \text{ ug}$$

$$x \text{ cells} = 15 \times 10^6 \text{ pg} : 0.5 \text{ pg} = 30 \times 10^6 \text{ cells}$$

Volume of disposable plug in CHEF kit is 100 uL. Thus, bring cells to concentration of **30×10^7 cells / mL**.

1. Microwave (in small time intervals) 2% CleanCut Agarose solution until liquid, then equilibrate at 50 degrees in water bath.

2. Remove approx. 15×10^8 cells and fill to 3.15 mL* with Cell Suspension Buffer (CHEF kit) in a 15 mL Falcon tube. Equilibrate tube in a water bath at 50°C.

*Note: Later, 1.85 mL of 2% agarose will be added, so that the final concentration will be 15 x 10^8 cells / 5 mL, which equals 30 * 10^7 cells / mL (i.e., the desired final concentration).

3. Add 5 x 0.37 mL (=1.85 mL) of equilibrated 2% CleanCut Agarose to equilibrated sperm cell suspension (3.15 mL).

4. Mix gently (but thoroughly). Keeping the mixture at 50 degrees, transfer mixture (100 uL per plug) to plug molds using sterile, wide-bore transfer pipets.

5. Place plug molds at 4 degrees for 10 – 15 min.

6. Add 5 x 100 uL (=500 uL) of Proteinase K stock solution to 5 x 2.5 mL (=12.5 mL) Proteinase K Reaction Buffer in a 50 mL Falcon tube.

7. Push solidified plugs into the solution prepared in step 5.

8. Incubate plugs overnight (up to 4 days) at 50 degrees without agitation.

9. Prepare 200 mL Wash Buffer (using the 10 x solution and sterile ddH_2O).

10. Wash plugs four times in 1 x Wash Buffer for 1 hour at room temperature with gentle agitation. In the second wash, add PMSF to a final concentration of 1 mM. Perform the third and fourth wash without PMSF.

11. Store plugs in 1 x Wash Buffer at 4 degrees.

Szmant, A.M., Weil, E., Miller, M.W. and Colon, D.E. (1997) Hybridization within the species complex of the scleractinan coral Montastraea annularis. ***Marine Biology*****, 129, 561-572.**