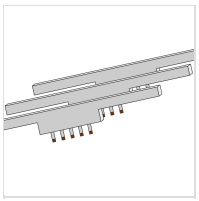

Jun 12, 2022

Preparation of Symbiodiniaceae for cryopreservation and laser-warming

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This protocol is used to prepare Symbiodiniaceae samples for cryopreservation, specifically for vitrification and laser-warming. It uses Symbiodiniaceae freshly extracted from scleractinian corals as described here. The materials listed here are for 45 blades of encapsulated Symbiodiniaceae.

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cryopreservation, alginate encapsulation, algal symbiont, zooxanthellae, Scleractinia, Cladocopium, vitrification, laser-warming

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Abbreviations
FSW: Filtered seawater (0.22 µm)

Biological Materials
·Symbiodiniaceae freshly isolated as detailed in Bouwmeester et al. (2022)
(Reference: Bouwmeester J, Daly J, Quinn M, Hagedorn M (2022) Isolation of Symbiodiniaceae from scleractinian coral hosts suitable for cryopreservation and laser-warming. Protocols.io,

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Solutions

·Filtered seawater (FSW), freshly collected and filtered each day
·Deionised water (DI)

Chemicals and Nanoparticles

·Trehalose (Pfanstiehl Inc. Waukehan, IL, USA, CAS # 6138-23-4)
Note that this chemical cannot be replaced by a cheaper version as it needs to be of high grade to be tolerated by the algal symbionts
·Alginic acid sodium salt (Sigma-Aldrich, St. Louis, MO, USA, CAS # 9005-38-3)
·Calcium chloride (Sigma-Aldrich, St. Louis, MO, USA, CAS # 10043-52-4)
·Gold nanorod nanoparticles with PEG surface and peak absorbance at 1064 nm, custom-made (Nanocomposix, San Diego, CA, USA)

Glassware and Consumables

·1000 mL glass beaker
·250 mL glass beaker (e.g., Pyrex No 1000)
·Extra fine tea strainer to fit into 250 mL beaker (e.g., Yoassi, ASIN # B01LQ7NQTW)
·20-µL cell strainer basket (Pluriselect, Leipzig, Germany, SKU 43-50020-03)
·1-µL pipette tips
·20-µL pipette tips
·200-µL pipette tips
·1.5-mL Eppendorf tubes
·15-mL conical Falcon tubes
·Polyresin foam, 6 mm thick (Ben Franklin Craft Store, Hawaii)
·Acetate transparency film (Avatar AT1000 Transparency Film)
·Parafilm
·Kimwipes

Equipment and Tools

·1-µL pipette
·20-µL pipette
·200-µL pipette
·Magnetic stir bar (n=3)
·Magnetic stir plate (n=3)
·Centrifuge for 1.5-mL Eppendorf tubes
·Block heater with aluminium blocks for 1.5 mL and 15 mL tubes (e.g., Isotemp 125D, Fisher Scientific)
·Microbalance for weighing chemicals
·Hemacytometer (Hausser Scientific, Horsham, PA, USA, #02-671-51B)
·Cell counter
·15-mL Dounce tissue grinder (Wheaton, DWK Life Sciences, Millville, NJ, USA) – use the loose pestle
·Microscope
·Ultrasonic cleaner for sonicating gold nanoparticles (e.g., Co-Z 2L Professional Ultrasonic Cleaner, ASIN # B075FTFD3R)
·Sand paper (P150)
·Paper cutter
·Cutter knife
·Metal ruler or similar object to be used as guideline for cutting through the foam craft sheets with a cutter knife

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·Optional: Chemical-friendly microwave

Prepare Materials

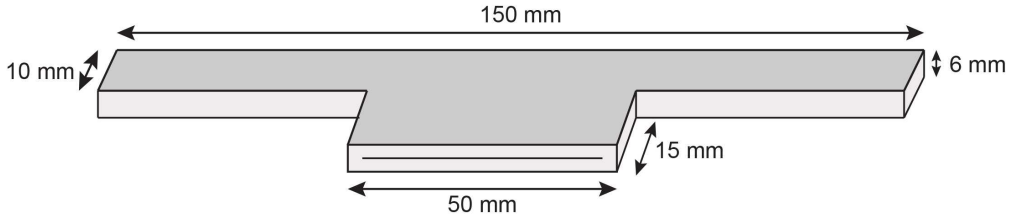
1. Making the cryo-blades

- One sheet of acetate transparency film can produce one thousand cryo-blades
- Using P150 sand paper, sand down one side of the acetate sheet until a liquid droplet (e.g., 10 µL) becomes flat when deposited on the sanded side of the sheet instead of forming a round droplet. Note: if you are only making a hundred cryo-blades, you only need to sand down a 30 mm strip across the width of your acetate transparency film
- Cut the sheet into small blades of exactly 3 x 15 mm using a sharp paper cutter, starting with cutting a 15 mm strip across the width of the sheet (a consistent length is very important for the laser to warm the sample at the right spot during laser-warming).
- With the sanded side facing up, add a mark at the bottom right end of the blade with a sharpie

- Test a few blades to make sure that they break down surface droplet tension by dipping them in filtered seawater (FSW)
- Make at least 50 blades (but we recommend making more for later use)
- The cryo-blades can be re-used as long as they are not bent or visibly damaged (e.g., laser damage from overheating)

2. Making the cryo-blade holders

- One Letter-size polyresin foam sheet can make 11 cryo-blade holders
- Each cryo-blade holder can sustain 5 cryo-blades



- Cut a 25 x 150 mm rectangle out of the foam sheet using a cutter knife with a sharp blade, along a metal ruler or other straight metal guide
- Draw the shape below on the cut-out rectangle using a fine sharpie and cut the foam holder along the lines using the sharp cutter knife along a straight metal guide
- Cut a central line through the side of the short end, where the blades will be inserted
- Make at least 10 foam holders
- Write “Sample” on one side, and “blank” on the other side, to make sure the blades and the samples are always mounted on the same way on the foam holder
- The foam holders can be re-used

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Prepare solutions

1. Prepare 5 mL of 1M trehalose in DI water

- Prepare 1M trehalose (1890 mg) in a 15 mL Falcon tube
- Add DI water until the 5 mL mark is reached
- Vortex the tube and dissolve the trehalose into the DI water by heating the tube in a block heater (60-100°C). Vortex the tube every few minutes
- This trehalose in DI solution can be prepared up to 48 hrs in advance but must be kept in fridge and brought to room temperature just before use

2. Prepare 2.5 mL of 4% alginate solution in 1M trehalose in DI

- The alginic acid powder can be hard to dissolve so we recommend preparing the solution as follows:
- Transfer 1 mL of 1M trehalose in DI (prepared above) into a 15 mL Falcon tube
- Add 100 mg alginic acid into the tube
- Gently add 1M trehalose in DI solution until the 2.5 mL mark is reached
- Thoroughly vortex the tube
- Place the alginate solution in a block heater (100-120°C) to help dissolve the alginic acid and regularly vortex the solution. This step might take a while. The process can also be accelerated by short bursts of rapid heating in a chemical-friendly microwave (~3-4 seconds) and subsequent off-gassing (by very carefully unscrewing the lid) and vortexing, but care must be taken to not overheat the tube (the tube will burst if it contains too much pressure from a too high increase in heat) and to not burn oneself (the tube will be hot – use protective gloves)
- The alginate solution is ready when it looks clear and homogenous
- Double check that the 2.5 mL mark has been reached and if not, add some 1M trehalose in DI
- The solution can be prepared up to 48 hrs in advance but must be kept in fridge and brought to room temperature just before use

3. Prepare 2% calcium chloride solution in FSW

- For three 250-mL beakers: 15 g $CaCl_2$ in 750 mL FSW
- Prepare in the 1000 mL beaker and transfer to the smaller beakers just before use
- Add a stir bar in the beaker, set the beaker on the stir plate at higher speed until the calcium chloride has fully dissolved and then bring down to half power

Preparing the Symbiodiniaceae solution for encapsulation

1 Transfer freshly isolated Symbiodiniaceae (as in Bouwmeester et al. 2022, doi.org/10.17504/protocols.io.b4e3qtgn) into a 50-mL Falcon tube. Add FSW if needed to reach a total volume of 50 mL.

2 Spin the 50-mL tube in a centrifuge for 10 min at RCF = 2300 x g.

3 Discard the supernatant, add 5 mL FSW, and resuspend the pellet using a 1-mL pipette.

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4 Add FSW to reach a total volume of ~10 mL. At this volume and concentration, the Symbiodiniaceae can be maintained for several hours.

5 Measure the cell concentration C_1 using a hemacytometer. Dilute the sample if necessary for counting, so that cells don't overlap on top of each other, but remember to adjust the final number to reflect the initial concentration

6 You will now need to concentrate a subsample of Symbiodiniaceae to obtain 0.1 mL Symbiodiniaceae at a final concentration of 4 (±1) x 10^7. Calculate the volume V_1 to concentrate (in mL), using the following formula:

$$V_1 = (C_2 \times V_2)/C_1 = ((4 \times 10^7) \times 0.1)/(C_1) = (4 \times 10^6)/(C_1)$$

7 Transfer the determined volume V_1 of Symbiodiniaceae solution into one or more 1.5 mL Eppendorf tubes

8 Spin the 1.5-mL tube(s) in a centrifuge for 3 min at RCF = 6100 x g.

9 For each tube, gently remove most of the supernatant with a 1-mL pipette, and gently remove any remaining supernatant with a 200-µl pipette.

10 Resuspend the Symbiodiniaceae pellet of the first tube in 100 µl of 1M trehalose in DI solution (prepared in advance). If there is more than one tube, use the resuspended Symbiodiniaceae solution of the first tube to resuspend the pellets of the other tubes.

11 Make sure the Symbiodiniaceae solution is homogenous by thoroughly mixing the solution with the pipette, and transfer 98 µl into a new Eppendorf tube.

12 Sonicate the gold nanoparticles in the ultrasonic cleaner for at least 30 seconds and immediately add 2 µl gold nanoparticles to the 98 µl Symbiodiniaceae solution. Mix the solution well, using a pipette or by flicking the tube.

13 Your Symbiodiniaceae are now ready for encapsulation. They should ideally be processed within 60 minutes to avoid stress from being highly concentrated in the Eppendorf tube.

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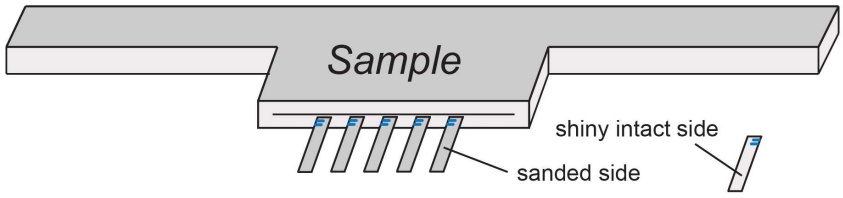
Encapsulation in alginate and polymerisation of the alginate

14 Setting the cryo-blades into the cryo-blade holders:
For each cryo-blade holder, position the blade holder with the “Sample” side facing up, and insert 5 acetate blades with the marked end of the blade towards the cryo-blade holder and the sanded side of the blade facing up. The marks on the acetate blades should all be facing the same side. Prepare three cryo-blade holders per cryopreservation round to reach a total of 15 blades.



15 Preparing the glass beakers for the calcium chloride solution.
Place each 250-mL glass beaker on a stir plate, add a stir bar in the bottom, and add a Yoassi tea strainer into the beaker. The stir bar mixing will prevent the calcium chloride from precipitating once poured into the beakers, and the tea strainer is needed to prevent the mixing at the bottom of the beaker from causing disturbance at the solution surface during the polymerization phase, during which the samples could fall off from the blades if too heavily disturbed. Set the stirring at ~270 rpm.

16 Pouring the calcium chloride solution into the glass beakers.
Sit a foam holder with its acetate blades on the top of the beaker (with the acetate blades facing downwards) for visual reference. Pour the calcium chloride solution into the 250-mL beakers, so that 50-75 % of the blades are immersed in calcium chloride but making sure that the calcium chloride solution does not reach the foam holders. Having the solution level too high will cause the blades to rotate and/or fall out. Having the solution too low will prevent polymerisation of the alginate.

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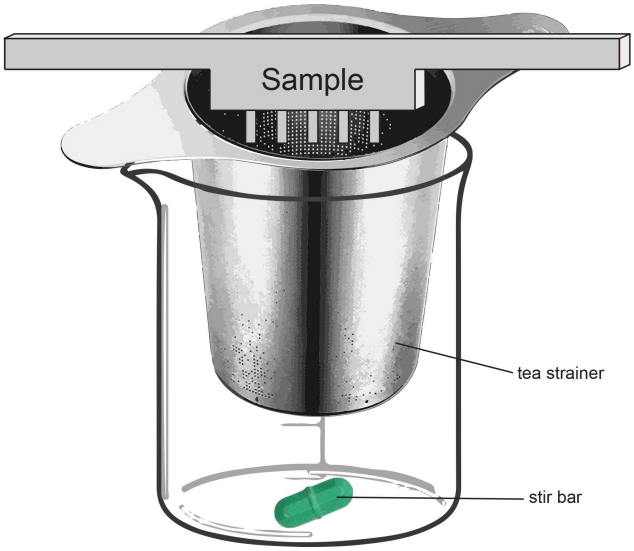

17 Encapsulation: mixing the Symbiodiniaceae with the alginate.
On the plastic side of a square of parafilm (no need to peel it), mix a 20-µl droplet of alginate solution with a 20-µl droplet of Symbiodiniaceae in solution with gold nanoparticles, as prepared in step (13), using a 20-µL pipette. Make sure that you mix the Symbiodiniaceae really well before sampling the droplet, by flicking the tube, or by pipetting. Also make sure to first lay the alginate droplet on the parafilm and to then add the Symbiodiniaceae droplet on top of the alginate droplet. The two droplets will mix better in this way. Homogenise the droplet really well by pipetting the mixed droplet for 30 seconds.

18 Pre-wetting the cryo-blades in the calcium chloride solution.
Dip the acetate blades of the first foam holder into the calcium chloride.

19 Partially drying the cryo-blades.
Dry the non-sanded side of the blades by laying a Kimwipe on the laboratory bench and gently laying the non-sanded side of the blades on to the Kimwipe. Then turn the cryo foam holder around so that the sanded-side of the blades are facing down and just dry the last 3 mm of the blades. Some calcium chloride should remain visible between that 3-mm zone at the end of the blade and the foam holder.

20 Depositing the encapsulated Symbiodiniaceae at the end of the cryo-blades.
Using a 2-µL pipette, for each blade, transfer a 2-µL droplet of mixed alginate/Symbiodiniaceae onto the end of the blade and gently flatten the droplet to let it cover a 3 x 3 mm area at the end of the blade by swiping over the top of the droplet with the pipette tip. The swiping may remove part of the droplet, which is fine. Do not swipe more than once as the droplet will start polymerising once it has been in contact with the wet portion of the blades (which still carries some calcium chloride), and will break apart if you disturb it after that. Make sure to use a clean tip for each droplet.

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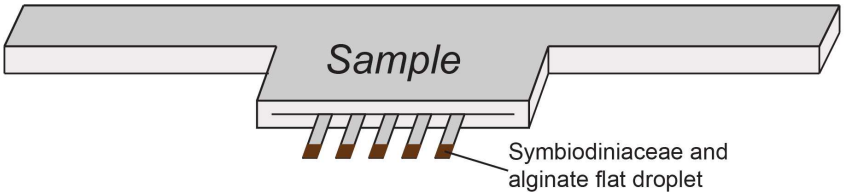
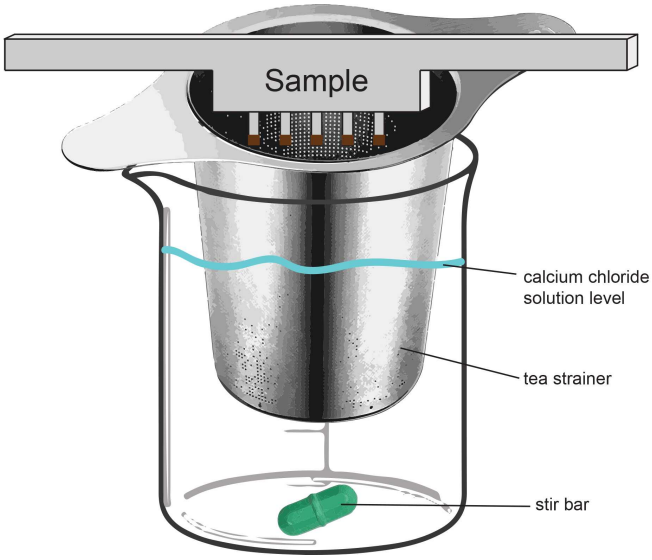


21 Polymerisation of the alginate.
Sit the foam holder on the top of the beaker, making sure that the encapsulated samples on the blades are all fully submerged in the calcium chloride solution.



22 Repeat steps 17-20 for the 2nd and 3rd cryo foam holders.

23 Start a timer as soon as the samples from the 3rd foam holder are submerged in the calcium chloride solution.

24 After 30 minutes of polymerisation, the samples are ready to be processed for cryopreservation. They should be used immediately.

25 To prepare a 2nd and 3rd round of cryopreservation, return to step (14). We recommend leaving a 15-30 minute gap between each round to allow for enough time to process the samples.

26 If you plan more than three rounds of cryopreservation, we recommend starting the 4th round from step (7).

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