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Preparation of Zygotes and Embryos of the Kelp *Saccharina latissima* for Cell Biology Approaches

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[Abstract] Embryogenesis and early development in kelps are poorly studied and understood. Cultivation protocols focus mostly either on the preparation of large volumes of vegetative gametophytes or on the production of conspicuous juvenile sporophytes as starting materials for aquaculture to grow large adult organisms for biomass. Hence, our protocol describes the optimal conditions for a) efficient gametogenesis, b) synchronization of egg release, c) control of zygote density, and d) optimal embryonic development of the kelp *Saccharina latissima*. This species is currently subject to aquaculture development in Europe, but the aim here is to provide tools for academic research aiming to identify the mechanisms underlying embryogenesis. These protocols, adaptable to different volume scales from Petri dishes to up to ten-liter containers, are the required first steps for robust downstream experiments scaled at the cytological level (immunochemistry, microdissection, laser ablation, and cell transcriptomics) and for physiological and phycopathogenesis studies at very early stages.

Graphic abstract:

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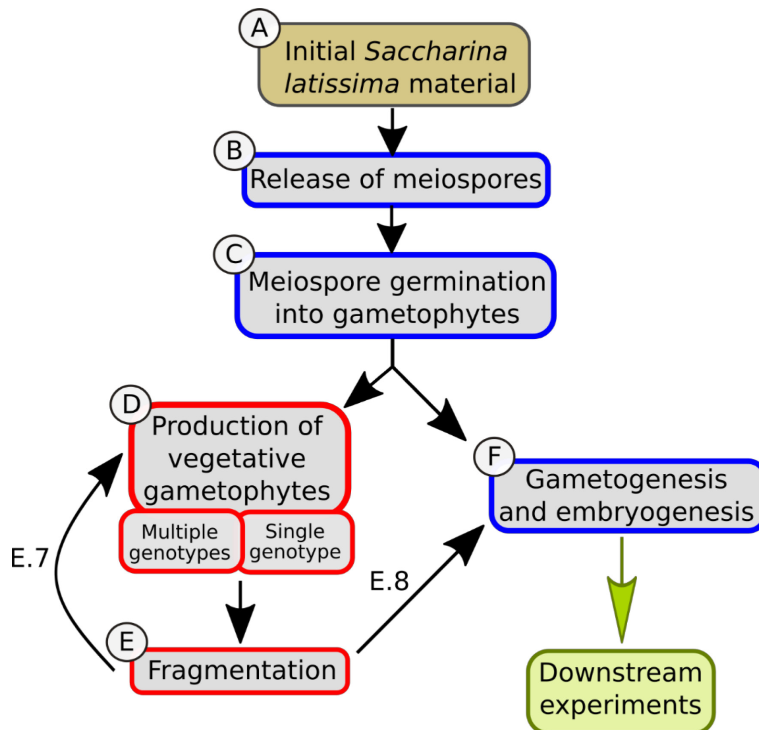

Boxes describing the different steps of the protocol. The color of the box contours (red or blue) indicates different light conditions, red light and white light, respectively.

Keywords: Brown alga, Gametogenesis, Embryogenesis, Kelp, Saccharina, Cultivation, Zygote

[Background] Brown algae are a group of mainly marine photosynthetic organisms with exclusively multicellular body plans (thallus). Their thallus ranges from small filaments like in *Ectocarpus* species to the large conspicuous parenchymatic kelps (Charrier *et al.*, 2012). Kelps in particular play a major ecological role as habitat for other organisms and are important primary producers in coastal ecosystems. Kelps also attract considerable economic interest, mainly as food sources (*e.g.*, kombu). In addition, extracts from brown algae have shown anti-tumor and anti-inflammatory effects (Cumashi *et al.*, 2007; Han *et al.*, 2019; Long *et al.*, 2019; Mohibbullah *et al.*, 2019), and their biomass is used as bioethanol (Adams *et al.*, 2009; Kraan, 2016). This altogether raises special interest in their metabolism and physiology. This is particularly the case for *Saccharina latissima,* as shown by an ~5-fold increase in publications over the past 10 years (from 40 in 2010 to more than 180 in 2020; Web of Knowledge), elevating it to currently being the most studied brown alga worldwide. However, this interest focuses mainly on its ecophysiology and less on its development, a balance that this protocol, in combination with expected publications, aims to improve.

The life cycle of *S. latissima* and kelps in general, is divided in a haploid and a morphologically distinct diploid phases (Kanda, 1936; Fritsch, 1945). During the haploid phase, the organism is prostrate and filamentous, and two sexes can be distinguished (dioicous condition). Given the appropriate conditions for gametogenesis (see next paragraph), the female gametophyte releases a spherical egg that remains fixed to the empty gametangium (also named oogonium). When the male gamete released from the

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male gametophyte is chemically attracted by the egg (Maier and Müller, 1986; Kinoshita *et al*., 2017), their fusion results in the onset of the diploid phase starting with an elongated zygote. The developing embryo will eventually grow into an impressive (>3 m) mature sporophyte after only a few months (Andersen *et al.*, 2011). Sporiangia on the surface of the mature fertile sporophyte contain the haploid meiospores. After being released, these meiospores settle and germinate into gametophytes. While the ecophysiology of both phases is well studied (an old review: Bartsch *et al.*, 2008), little is known about the embryonic development of the sporophytes, and historic studies only provide descriptive observations (reviewed in Fritsch, 1945). Given both the importance of this resource to the environment and as feed and food, as well as advances in science, this is expected to change. Therefore, to boost the developmental and cellular approaches aiming to decipher the mechanisms underlying embryogenesis in this alga, we propose a protocol focusing on the production and cultivation of zygotes and early embryos.

Gametogenesis is an important step for promoting normal embryogenesis in artificial culture conditions. Considerable work focused on environmental factors like light quality, temperature, and nutrients that can disrupt or inhibit gametogenesis. Lüning and Dring (1972) demonstrated that blue light induces the process while red light acts repressively. Normal white light acts like blue light, given low light intensity (Hsiao and Druehl, 1971; Lüning, 1980; Lee and Brinkhuis, 1988). Temperature is also crucial: temperatures above 15°C inhibit gametogenesis, while temperatures between 10 and 15°C are optimal (Lüning, 1980). A parameter often missed in these earlier works is density. For *Saccharina japonica*, high density of gametophytes inhibits gametophytic growth in *in vitro* cultures (Petri dishes; Yabu, 1965) and fertility at larger scales (20 L tanks, Zhang *et al.*, 2008). Recent work from Ebbing *et al.* (2020) further clarifies the density parameter and the effect of its combination with light quality, a correlation not considered in previous works. Specifically, gametogenesis-induction by blue light occurs only in low-density cultures combined with low light intensity. Red light has the opposite effect. In high light intensity, red light promotes higher fertility than blue light or white light. Regarding nutrients, high concentrations of chelated iron into the culture medium seem to promote gametogenesis in other kelp species (Motomura and Sakai, 1984; Lewis *et al.*, 2013). Considering the above parameters, the present protocol relies on the biological properties of different light conditions in combination with the concentration of nutrients in the medium to either arrest or promote gametogenesis.

In addition, this protocol allows the production of zygotes and embryos amenable to downstream experiments, like immunolocalization protocols, in which the steps of chemical fixation, cell wall digestion, antibody incubations, and staining can occur inside glass bottom Petri dishes. Alternatively, and depending on the size and the developmental stages of embryos, embryos can be transferred onto manufactured or homemade poly-L-Lysine (1 mg ml^{-1}) coated slides or coverslips using tweezers with thin ends. In transmission electron microscopy (TEM) protocols, while fixation and post fixation steps can occur in common plastic Petri dishes, dehydration requires incubation in acetone, which would dissolve most plastic Petri dishes. Therefore, material transfer into, *e.g.*, Falcon or Eppendorf tubes or glass Petri dishes before dehydration and infiltration will be necessary in this case.

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Finally, the protocol describes how to control the density of growing embryos, which impacts the production of i) healthy embryos, ii) embryos sufficiently spread to allow subsequent monitoring of, *e.g.*, growth dynamics in time-lapse observation, and iii) embryos amenable to isolation and experimentation, *e.g.*, through microdissection and laser ablation.

Clean (close to sterile) conditions are essential through the entire protocol, especially during gametogenesis induction and embryogenesis.

Altogether, this protocol will promote further studies on the microscopic stages of kelp development.

Materials and Reagents

1. 1.5 ml sterile Eppendorf tubes
2. Sterile scalpel
3. Filter tips 1,000 µl, 100 µl, and 20 µl (Starlab, TipOne)
4. Plastic Petri dishes Ø35 mm (Sarstedt, catalog number: 82.1135.500)
5. Petri dishes with glass bottom Ø28.2 mm (NEST, catalog number: 801001)
6. Pellet pestles, blue polypropylene (autoclavable) (Sigma-Aldrich, catalog number: Z359947-100EA)
7. Cell scraper (Sarstedt, catalog number: 83.1830)
8. Cell strainer 40 µm (Falcon, catalog number: 352340)
9. Autoclaved, 0.2-5 µm filtered seawater (SW), stored at 14°C
10. Counting chamber slides (Kova glasstic slide 10 with counting grids, catalog number: 87144)
11. Pasteur pipettes, long
12. Nalgene bottles, 2 and 10 L
13. H_3BO_3
14. $FeCl_3$
15. $MnSO_4$
16. $ZnSO_4$
17. $CoSO_4$
18. EDTA
19. $(NH_4)_2Fe(SO_4)_2 \cdot 6H_2O$
20. $NaNO_3$
21. $C_3H_7Na_2O_6P$
22. Vitamin B12 (cyanocobalamin)
23. Thiamin (vitamin B1)
24. Biotin
25. Tris
26. Provasoli solution (see Recipes)

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Equipment

1. Inverted Microscope Leica DMi8, light source: CTR compact, camera: RGB Leica DMC4500
2. Laminar flow hood
3. Light source: Philips, Master TL-D 18W/865, commercial sheet of red filter (LEE filters, 026 Bright Red)
4. Climate chambers with controllable temperature and light source
5. Automatic pipettes: 1,000, 100 or 50, and 10 µl
6. Lighter

Procedure

A. Collecting fertile sporophytic material from the wild

1. Depending on the geographical region, mature sporophytes are fertile from early winter until middle to late spring. They might be collected from the beach during low tide or directly underwater while diving. Fertile sporophytes have dark areas, called sori (singular: sorus), where meiosporiangia reside (Figure 1).

Figure 1. Comparison of fertile and non-fertile areas. A. Non-fertile area of a sporophytic blade held upside down. B. Fertile areas (sori) on the same blade a couple of meters below the non-fertile area shown in A.

Note: Look for large blades with normal morphologies: twisted stipe or other symptoms of disease (like white spots at the surface of the blade) reveal the presence of pathogens and endosymbionts. In addition, avoid collecting material covered with epiphytes. It is common for other filamentous brown algae or red algae to grow on the thallus of kelps.

2. In the Western part of France, on the Atlantic coast (Brittany), fertile months are October to April,

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and our material was collected from thalli stranded on the beach of Perharidy (48°43'33.5"N 4°00'16.7"W).

3. Alternatively, large fragments (>1 m) of sporophytes can be collected, and sporogenesis can be induced. Parts of the thallus should be cut away from the intercalary meristem and the stipe. Then, they are kept in short day conditions (8 h light: 16 h dark) and 100-120 photons m^{-2} s^{-1} for at least two weeks to induce sporogenesis (Pang and Lüning, 2004; Forbord *et al.*, 2012). Depending on the geographical location where the strain has been collected and on the season, the incubation time needed to induce sporogenesis under short day conditions can vary up to 12 weeks.

B. Release of meiospores

1. Using a scalpel, remove the fertile tissue from the rest of the blade or fragments. The fertile tissue where meiosporiangia reside is darker and easily distinguishable from the rest of the blade (Figure 1). Try to dissect undamaged dark parts showing no interruption of their color and morphology.
2. Clean the surface with a paper towel dampened with distilled (osmosis) water, remove any excess water, and dry with a new paper towel.
3. Remove epiphytes by softly scratching the surface of the blade with the scalpel and wipe with a dry paper towel.
4. You can choose to release meiospores a) at this point or b) one of the next two days.
5. If a), cut the tissue in small fragments of up to 1-3 cm^2 and place them in sterile autoclaved and filtered 0.2 µm SW under ambient room light (in our case, it was 8-15 µmol photons s^{-1} m^{-2} on a cloudy day or 9-16 µmol photons s^{-1} m^{-2} in a well-lit room).
6. If b), keep large fertile fragments in a humid chamber at 4°C. This humid chamber is made of a damp (but not wet) piece of paper towel or cotton placed in a Petri dish sealed with parafilm. Under these conditions, the tissue can be preserved for up to 48 h. For the release, proceed at step 5.
7. After ~40 min, an adequate amount of meiospores swimming in the medium is visible (Figure 2A; Video 1). They color the medium amber.

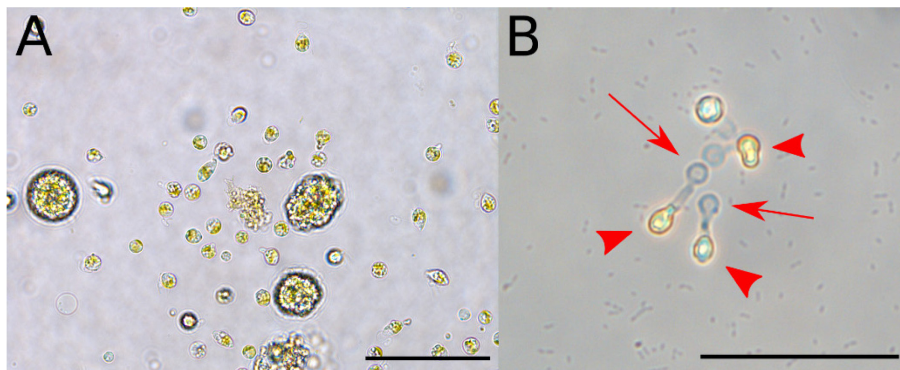

Figure 2. Meiospores. A. Freshly released meiospores. B. Meiospores germinating three days

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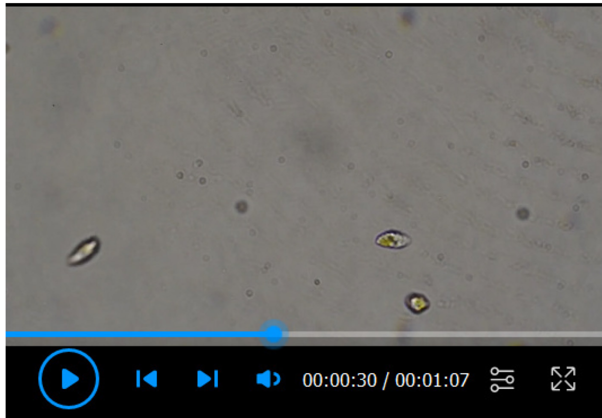
after release; arrows: empty spore and germinating tube, arrowheads: first gametophytic cell. Scale bars = 50 µm.



Video 1. Swimming spores with visible flagella (different magnifications: 10×, 20×, 40×, and 63×). Credit: David Wahnoun.

Note: It is essential to not let the fertile tissue into the medium for more than 1 h because, epiphytes and endophytes will be released into the medium together with the spores. This can tamper with the germination and normal vegetative development of future gametophytes.

8. Monitor the release of spores every 10 to 20 min by counting them using the counting chamber. Calculate the concentration of spores. An optimal concentration is 300,000-400,000 cells ml^{-1}.
   *Note: For counting meiospores using a counting chamber (like Neubauer), the following equation can be used. Concentration = Number of cells per volume unit (ml). Alternatively, use: Concentration (cell ml^{-1}) = Average number of cells per square × 10^4 * × dilution factor.*
   **: 0.1 µl is the volume loaded in the square. Therefore, for a final volume of 1 ml, the total number of spores = calculated number × 10^4.*
9. Transfer your material and work under the laminar flow hood for the rest of the protocol.
10. Filter 5-7 ml of meiospore-containing medium with the cell strainer.
    Note: Filtration and dilution steps additionally help to remove part of the mucus released together with the spores, which may carry contaminants or be harmful to spores.
11. Dilute adequately with fresh ½ PES SW (see Recipes) and filter once or twice more. We used 30-40 spores ml^{-1} as the final concentration. At that meiospores concentration, the gametophytes will be spread of ~300 µm, which will facilitate their isolation in the downstream steps if required (Figure 2A).

C. Meiospore germination and production of gametophytes

For the production of vegetative gametophytes as stock material, proceed to step 1.

For immediate production of zygotes, proceed to step 2.

1. Keep meiospores under red light conditions: 3 µmol photons m^{-2} s^{-1} light intensity and 14:10 light:dark photoperiod at 13°C (Figure 3A). After 12 h, the spores settle to the bottom of the Petri

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dish and germinate after 2-3 days. Proceed with **D**.

2. Put the meiospores in 16 µmol photons m^{-2} s^{-1} white light intensity (low light) and 14:10 light:dark photoperiod at 13°C (Figure 3B). After approximately 10 days, polarized gametophytes are developing female gametangia on top of one to two cells (Figure 2B). Use an inverted microscope to observe them. For more details, proceed with **E**.

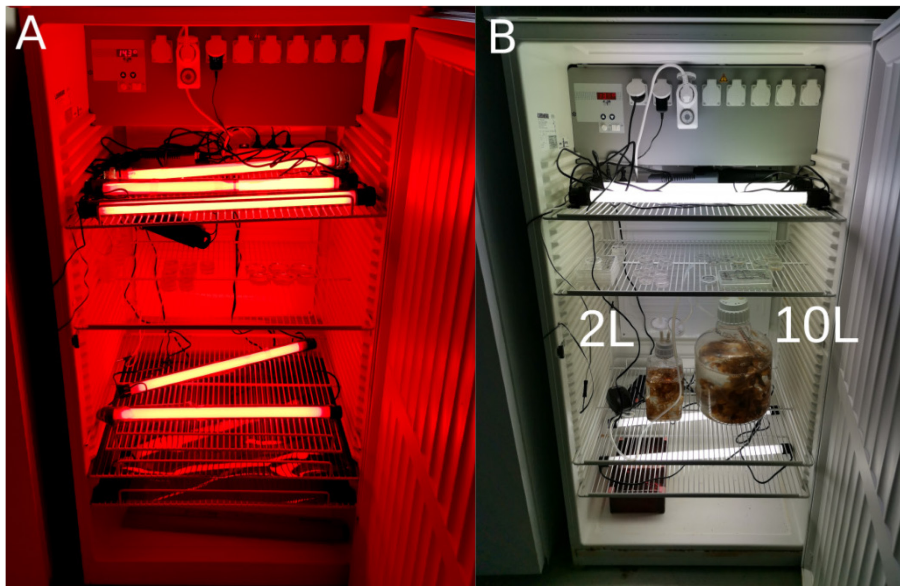

Figure 3. Culture cabinets. A. Red light conditions cabinet. B. White or normal light conditions cabinet, with 2-L and 10-L Nalgene bottles.

D. Working with stock material

1. Cultivation of mixed vegetative gametophytes

   When released from one parental sporophyte, germinating meiospores will produce a mix of gametophytes with different genotypes. At each release, the population of produced gametophytes has a different genotypic composition because the parental sporophyte is freshly collected from the wild.

   a. Avoid any exposure to white light (red light: 3 µmol photons m^{-2} s^{-1} light intensity and 14:10 light:dark photoperiod at 13°C). At the stage, the gametophytes are growing (Figure 4).

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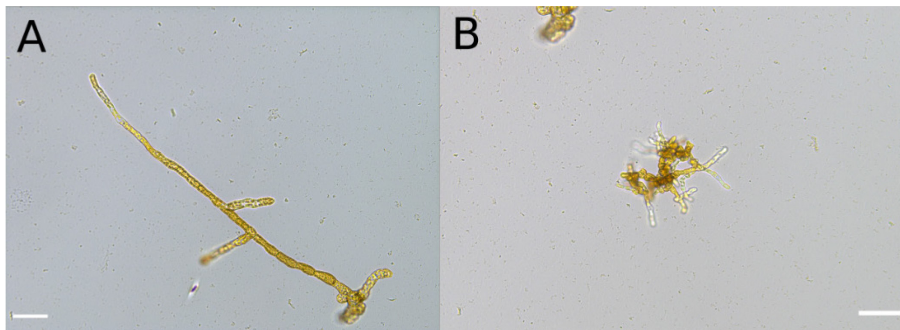

Figure 4. Two-month-old gametophytes. A. Female gametophyte. B. Male gametophyte. Scale bars: 50 μm.

b. Renew ½ PES medium once every month; decant the old medium and refill the Petri dish with freshly prepared medium.
   Note: Fragment and test gametogenesis and embryogenesis once every six months or once a year. Follow the same protocol as in ***E*** *and* ***F****.*
c. Keep the gametophytic cultures dense enough to prevent gametogenesis (see Notes).
d. Fragment the cultures every 3-6 months as indicated in Procedure E and place again in same culture conditions as above.
   Note: Fragment the gametophytes in the middle of the day of a 14:8 light:dark photoperiod. That way, the fragments stay under light for several hours to mitigate stresses caused by fragmentation.
e. Keep ambient to very low light conditions (maximum of 3 μmol m^{-2} s^{-1}) while handling the stock.
f. Be mindful of sterile conditions.

2. Isolating gametophytes for unialgal cultures
   To control the genotype of the produced embryos at each experiment, monogenic cultures of both female and male gametophytes are prepared and maintained in stock in vegetative conditions.
   a. Make a glass tool using a sterile Pasteur pipette and a lighter under the hood (Video 2).

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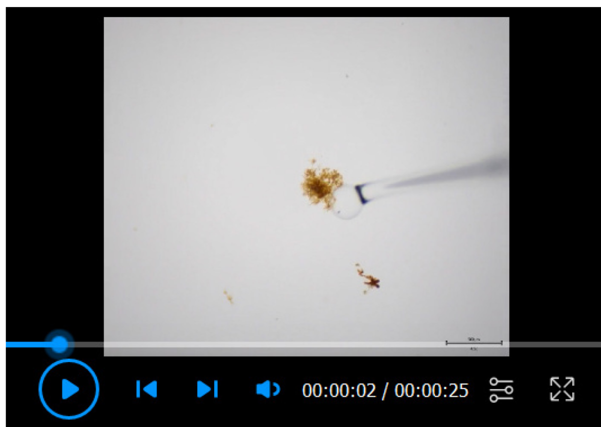
Video 2. Glass tool-making process using a glass Pasteur pipette and a lighter. Hold one side of the Pasteur pipette with your index and middle finger, specifically where the thinnest part ends. Hold the other end with your ring finger and thumb. With your other hand, place the lighter below the middle of your fingers, aiming the flame at the middle of the thinnest part of the Pasteur pipette. After 1 s, start pulling slowly without turning off the flame. When the glass becomes easy to pull, pull both ends in one go, as straight as possible.

b. Choose a gametophyte and scrape it with the glass tool. Another tool with a different diameter or a commercial tip used with an automatic or Pasteur pipette may help if the gametophyte is too small or not sticky enough (Video 3).



Video 3. Using a glass tool to detach and then transfer the gametophyte to another Petri dish using a sterile Pasteur pipette under the hood

c. Transfer the gametophyte carefully into new ½ PES.
d. Keep it under red light conditions until it is large enough for fragmentation. This requires about 6 months (see Notes).
e. Then, fragment the overgrown gametophyte as in E, and keep it in same culture conditions as stock.

E. Fragmentation

The aim is to induce gametogenesis and embryogenesis from 3-months-old or older material.

1. Fragment dense gametophytic cultures (3 months or older) into a 1.5-ml Eppendorf tube with 50 µl ½ PES using a sterile scraper and autoclaved pestles.
   a. Decant the culture.
   b. Scrape gametophytes from the bottom of the Petri dish and place them into the Eppendorf tube with 50 µl of ½ PES.
   c. Place the gametophytes at the bottom of the Eppendorf tube.
   d. With the pestle, press onto the gametophytes while rotating.
   e. Lift up and down to check on your progress. You can additionally press the gametophytes

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on the sidewalls of the Eppendorf tube while observing the process.

f. The gametophytes can cope with high pressure on them; however, you should stop grinding when you observe that the gametophyte biomass is homogenized into the 50 µl.

g. Before committing a large number of your cultures into gametogenesis or for stock renewal, try part of them to find the right pressure and speed of rotation to be applied.

Note: Fragment gametophytes in the middle of the day of a 14:8 light:dark photoperiod. That way, the fragments stay under light for several hours to mitigate stresses caused by fragmentation.

2. Dilute fragments into 1 ml ½ PES.
3. Pipette up and down several times with a steady, slow rhythm.
4. Depending on the size of the donor Petri dish and the recipient dish, choose an adequate dilution. The aim is to have fragments distanced approximately 200 µm. A dilution series can help choose the necessary volume.
5. If female and male gametophytes were kept separated, proceed as described above.
   Note: In any case (mixed gametophyte cultures), try to use an almost equal number of male and female fragments.
6. While dispensing fragmented gametophytes into new Petri dishes, avoid material clotting by pipetting up and down at least twice and stir the medium in the Eppy before inoculation of each Petri dish.
7. For stock renewal (Procedure E), part (~20%) of the vegetative fragments can be kept in Petri dishes with ½ PES.
8. For zygote production, proceed with Procedure F.

F. Production of *S. latissima* zygotes and embryos

1. Inoculate the rest of the fragmented gametophytes in full PES SW.
2. Transfer the freshly inoculated Petri dishes into low light conditions (16 µmol photons m^{-2} s^{-1} light intensity and 14:10 light:dark photoperiod, 13°C). The fragments stick slightly to the bottom (mind motion during transportation).
3. Check gametogenesis progression every day. After 5 days, several eggs should be released, and a few polarized zygotes can be potentially observed (Figure 5). The major release of eggs and male gametes should take place during the 6th and 7th days.

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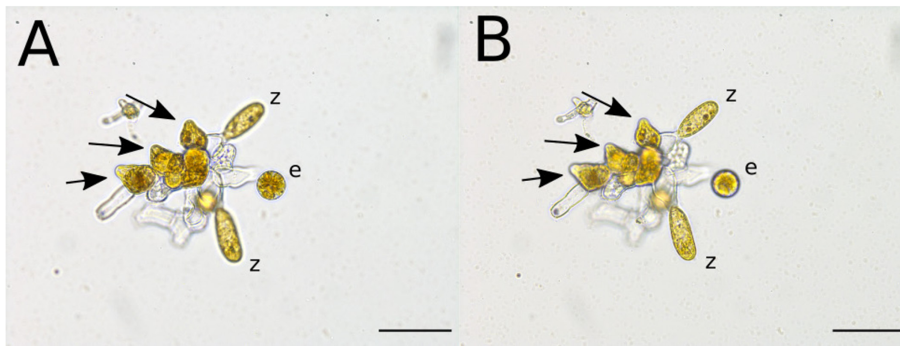

Figure 5. Gametangia, egg, and zygotes. A. Ripe gametangia (arrows), ready to release an egg (see e). B. Different focus plane showing zygotes (z) more clearly. Scale bars: 50 μm

4. To keep the release synchronized, transfer the material from low light to normal light conditions (50 μmol photons m^{-2} s^{-1}) at the beginning of the sixth day. This represses any further gametogenesis. Nevertheless, few male gametes and eggs will occasionally emerge. Most of the developing embryos will have approximately the same developmental stage (Figure 6).

Figure 6. *Saccharina* embryos. The embryos are all at similar developmental stages. Scale bar = 100 μm.

5. Check the progression of gametogenesis and embryogenesis. Fertilization and embryogenesis may be delayed by a few days and may not be efficient if the number of female and male gametophytes is unequal. Differences may exist among species or strains.
6. After one month, transfer the embryos to aerated 2-L Nalgene bottles (Figure 3B). Full PES should be changed every 4-7 days.
7. After 2-3 months, the young sporophytes should be transferred to a 10-L container (Figure 3B), depending on the needs of your experiments.
   Note: Sterile conditions are not required to maintain larger embryos and healthy sporophytes if seawater medium is changed regularly. However, gametophytes show better vegetative growth

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in sterile culture medium and handling conditions (note that the algal material is not axenic as spores were originally collected from the wild and were not treated to make them axenic before germination into gametophytes).

The main conditions for the cultivation of vegetative gametophytes, gametogenesis induction, embryo formation, and production of juvenile sporophytes are summarized in Table 1.

Table 1. Summary of different culture conditions. Different light and medium conditions are necessary for induction of gametogenesis, optimization of embryo growth, and maintenance of stock cultures. Temperature is always at 13°C, and the photoperiod is 14:10 (light:dark).

Ontogenic stage	Light quality and intensity	Medium composition (SW completed with)	Change medium
Vegetative gametophytic growth	Red, 3 μmol photons m^{-2} s^{-1}	½ PES	Once every month
Gametogenesis	White, 16 μmol photons m^{-2} s^{-1}	Full PES	-
Embryogenesis	White, 50 μmol photons m^{-2} s^{-1}	Full PES	After 20-30 days (together with glass and plasticware)
Juveniles and Young sporophytes	White, 50 μmol photons m^{-2} s^{-1}	Full PES	Once every four to seven days

Notes

The gametophytes grow slowly and need to be maintained in red light conditions always. If gametophytes are the main interest, high culture density must be maintained as this will prevent any spontaneous embryogenesis (Ebbing *et al.*, 2020).

Recipes

1. Provasoli enriched seawater (PES) solution (Table 2)

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Table 2. Provasoli solution recipe. A comprehensive recipe with chemical composition and concentration on how to prepare vitamin and trace solution for natural or artificial seawater.

Components	Final concentrations
Solution 1 (10×)	
H_3BO_3	30.0 mM
$FeCl_3$	0.3 mM
$MnSO_4$	1.6 mM
$ZnSO_4$	0.13 mM
$CoSO_4$	0.028 mM
EDTA	5.7 mM
Solution 2 (10×)	
$(NH_4)2Fe(SO_4)2{\cdot}6H_2O$	3.0 mM
EDTA	3.4 mM
Solution 3 (10×)	
$NaNO_3$	270 mM
Solution 4 (10×)	
$C_3H_7Na_2O_6P$	15.4 mM
Vitamin mix (100×)	
Vitamin B12 (cyanocobalamin)	0.0067 g L^{-1}
Thiamin (vitamin B1)	0.33 g L^{-1}
Biotin	0.0033 g L^{-1}
Tris	333 g L^{-1}

These are stock solutions in 1 L volume. Adjust the volume according to your needs and resources. The vitamin mix should be kept in the dark after filtering at 0.2 µm. Maintain all solutions at 4°C. When making Provasoli medium, mix all solutions in numbered order (*i.e.*, from solution 1 to solution 4) and dilute to 1×; then, adjust the pH to 7.8 with HCl, and then add the vitamin mix (it should be slightly diluted before adding it to the other solution). Aliquot in falcons or small bottles and autoclave. Provasoli solution is kept at 4°C and used at 20 ml L^{-1} in artificial seawater or natural seawater for full Provasoli enriched seawater (PES) medium or 10 ml L^{-1} for ½ PES. In our case, we autoclaved and filtered (at 0.2 µm) natural seawater.
Our Provasoli recipe is based on Le Bail and Charrier (2013).

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Competing interests

The corresponding author certifies no financial and non-financial competing interests.

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