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Targeted Laser Ablation in the Embryo of *Saccharina latissima*

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Citation

Boscq, S., Dutertre, S., Theodorou, I., Charrier, B. Targeted Laser Ablation in the Embryo of *Saccharina latissima*. *J. Vis. Exp.* (181), e63518, doi:10.3791/63518 (2022).

Date Published

March 11, 2022

DOI

10.3791/63518

URL

jove.com/video/63518

Abstract

In *Saccharina latissima*, the embryo develops as a monolayered cell sheet called the lamina or the blade. Each embryo cell is easy to observe, readily distinguishable from its neighbors, and can be individually targeted. For decades, laser ablation has been used to study embryo development. Here, a protocol for cell-specific laser ablation was developed for early embryos of the brown alga *S. latissima*. The presented work includes: (1) the preparation of *Saccharina* embryos, with a description of the critical parameters, including culture conditions, (2) the laser ablation settings, and (3) the monitoring of the subsequent growth of the irradiated embryo using time-lapse microscopy. In addition, details are provided on the optimal conditions for transporting the embryos from the imaging platform back to the lab, which can profoundly affect subsequent embryo development. Algae belonging to the order Laminariales display embryogenesis patterns similar to *Saccharina;* this protocol can thus be easily transferred to other species in this taxon.

Introduction

Laser ablation has been used for decades to study embryo development. Irradiating embryo cells with a laser beam makes it possible to monitor the regenerative potential and the modification of the cell lineage during embryogenesis and investigate the impact of targeted ablation on cell division and cell fate. The model organisms used in laser ablation methods are typically animals, such as insects[1,2], nematodes[3,4], vertebrates[5,6], and occasionally plants[7,8]. In addition, a laser micro-ablation approach was used on the brown alga *Fucus* in 1994 and 1998 to demonstrate the role of the cell wall in the photopolarization of the early embryo[9,10].

Brown algae belong to the group Stramenopiles, diverged at the root of the eukaryotic tree 1.6 billion years ago. As a result, they are phylogenetically independent of other multicellular organisms, such as animals and plants[11]. *Saccharina latissima* belongs to the order Laminariales, more commonly known as kelps, and they are among the largest organisms on earth, reaching sizes of over 30 m. *Saccharina*

sp. is a large seaweed used for many applications such as food and feed, and its polysaccharides are extracted for use in the agricultural, pharmacological and cosmetic industries worldwide[12,13]. Its cultivation, mainly in Asia and more recently in Europe, requires the preparation of embryos in hatcheries before releasing juveniles in the open sea. Like all kelps, it has a biphasic life cycle composed of a microscopic gametophytic phase, during which a haploid gametophyte grows and produces gametes for fertilization, and a diploid macroscopic sporophytic phase, where a large planar blade develops from its holdfast attached to the seafloor or rocks. The sporophyte releases haploid spores at maturity, thereby completing the life cycle[14,15,16].

S. latissima presents some interesting morphological features[17]. Its embryo develops as a monolayered planar sheet[15,18,19] before acquiring a multilayered structure coinciding with the emergence of different tissue types. In addition, Laminariales is one of the only taxa of brown algae whose embryos remain attached to their maternal gametophytic tissue (Desmarestiales and Sporochnales do too[15]). This feature offers the opportunity to study the role of maternal tissue in this developmental process and compare maternal control mechanisms in brown algae with those in animals and plants.

This article presents the first complete protocol for laser ablation in an early kelp embryo. This protocol involving UV ns-pulsed technique results in the specific destruction of individual embryo cells to study their respective roles during embryogenesis. The procedure offers a reliable approach for investigating cell interactions and cell fate during embryogenesis in Laminariales.

Protocol

1. Production of *Saccharina latissima* gametophytes

1. Collect mature sporophytes of *S. latissima* from the wild as previously described[20,21]. Ensure that the selected sporophytes are devoid of epiphytes (small organisms visible on the blade's surface) or internal parasites (found in the bleached areas or spots on the blade).
2. Using a scalpel, cut the darkest part in the center of the blade (fertile spore-producing tissue[22]) into 1-5 square pieces (1 cm^2), avoiding any bleached spots, if present.
3. Remove any remaining epiphytes by gently cleaning the cut pieces with the back of a scalpel and some absorbent paper.
4. Place the cleaned pieces in a glass dish filled with sterile natural seawater (see **Table of Materials**) for 45 min to release spores following previously published report[22].
5. Remove the blade pieces and filter the seawater through a 40 µm cell strainer to remove debris or unwanted organisms.
6. Dilute the spores in the filtrate to a 20-40 spores/mL concentration in plastic Petri dishes[22].
7. Place the spore solution in a culture cabinet (see **Table of Materials**) configured with the optimal culture conditions (13 °C, 24 $\mu E.m^{-2}.s^{-1}$, photoperiod 16:8 L:D).
8. Allow the spores to germinate and develop into gametophytes.
   NOTE: Spore germination is visible after 2 days in the cabinet, and the first cell division of the gametophytic cells usually occurs within the following 48 h.

9. Replace the growth medium after 5 days with micro-filtered natural seawater enriched with a 0.5x Provasoli solution (NSW1/2) (see **Table of Materials**).

   NOTE: To avoid repeating these steps, specific male and female gametophytes can be selected and vegetatively propagated for several months. The gametophytes remain vegetative when grown under red light (4 $\mu E.m^{-2}.s^{-1}$ with a wavelength of at least 580 nm)[23] in the same culture conditions described above (step 1.7).

2. Fragmentation and induction of oogenesis

1. Harvest gametophytes with a cell scraper.
2. Using a small plastic pestle, crush the collected gametophytes in a 1.5 mL tube into 4-5-celled pieces.
3. Fill the tube with 1 mL NSW1/2 (step 1.9).
4. Add 2.5 µL of the crushed gametophyte solution into 3 mL of natural seawater enriched with 1x Provasoli solution (NSW) and place them in a Petri dish.

   NOTE: A 25 mm, glass-bottomed Petri dish is recommended for easier handling.
5. Place the prepared dishes in a culture cabinet and induce gametogenesis at 13 °C under white light with an intensity of 24 $\mu E.m^{-2}.s^{-1}$ (dim light, photoperiod 16:8 L:D).

   NOTE: The first gametangia (female oogonia and male archegonia) can be observed after 5 days. The male is hyper-branched with small cells, and the female is composed of larger cells forming long filaments[15,22]. The first eggs are observed ~10 days later, and the first division of zygotes usually occurs within the following 2 days.
6. Six days after observing the first eggs, transfer the dishes to brighter white light conditions: 50 $\mu E.m^{-2}.s^{-1}$, photoperiod 16:8 L:D, still at 13 °C.

3. Image acquisition for selecting embryos for ablation and monitoring subsequent growth

1. Image the entire Petri dish to (re)locate the embryos selected during the ablation step (no need to return the dish to the ocular microscope) and monitor the subsequent development of the selected embryos.

   NOTE: Use an inverted laser scanning confocal microscope (see **Table of Materials**) for imaging (**Figure 1**), and the laser ablation is described in step 5.
2. Place the Petri dish on the stage and orientate it with a visual mark (e.g., draw a line with a permanent marker).
3. Use the 10x/0.45 objective to focus on an embryo. Record the position of the four cardinal points of the Petri dish.
4. Start the tile scan. Acquire transmitted/fluorescent images of the whole Petri dish at low resolution: 256 x 256 pixels, a pixel dwell time of 1.54 µs with bidirectional scanning, and a digital zoom of 0.6x using a 561 nm laser at 1.2% transmission.

   NOTE: The scan time for a whole 2.5 cm Petri dish is ~6 min for 225 tiles (**Figure 2**). Here, the 561 nm laser was used for transmission and fluorescence imaging. The fluorescence signal was collected between 580–720 nm on the confocal photomultipliers (PMT) and the transmitted light was collected on the transmitted PMT. The 561 nm laser can also monitor chlorophyll at this step, but it is unnecessary because it only helps distinguish the signal noise and the organisms correctly.

5. Save the tile scan image and keep it open in the image acquisition software (see **Table of Materials**) window.

6. Change the objective, do not remove the Petri dish.
   NOTE: The 40x/1.2 water objective was moved to the side of the stage so that the immersion medium (water) could be added to the objective without moving the Petri dish from its initial position.

7. Navigate through the previously acquired tile scan image to select the appropriate embryo. Once an embryo has been identified on this image, move the stage to the exact position of the embryo and acquire transmitted/ fluorescent images of that embryo at high resolution.
   NOTE: High-resolution settings: 512 x 512 pixels, 0.130 µm/pixel, 0.208 µs pixel dwell time with mono-directional scanning and 2x digital zoom using a 561 nm laser at 0.9% transmission.

8. Annotate the tile scan image for each embryo candidate for laser ablation (**Figure 2B**) and proceed to the laser ablation step.

4. Laser calibration

1. Calibrate the laser and synchronize the image acquisition software with the laser-driver software in the "Click & Fire mode" and a pulsed 355 nm laser.
   NOTE: This step is crucial to ensure perfect synchronization between the mouse cursor's position in the laser-driver software (see **Table of Materials**) with the position in the live image of the acquisition software.

2. Open the laser-driver software package and click on **Live** in the image acquisition software package.

3. Synchronize both software packages by clicking on **Start acquisition** in the laser-driver software package. The live image is now also recorded in the UV laser-driver software.

4. Define an area of interest (AOI) by clicking on **Choose AOI** button and clicking on the edges of the image (right, left, top and bottom) in the UV laser-driver software package.
   NOTE: After this calibration step, the settings for pixel size, image format, and zoom in the acquisition software package must remain constant.

5. Select an empty area on the dish and lower the level of the stage to 20 µm below the sample focal plane to focus on the glass bottom.

6. Set the ablation laser and imaging laser trajectories by clicking on **Start calibration** and choose **Manual calibration**.

7. Select a laser power high enough to see a black dot in the center of the live image corresponding to the hole in the glass coverslip (all the shutters must be open).

8. Click on this central black dot with the mouse cursor and click on **18 additional dots** proposed by the software to complete the alignment procedure.

9. Check the calibration in the "Click & Fire mode" on the same coverslip.
   NOTE: Laser calibration depends on the imaging parameters. Once the laser has been calibrated, ensure that the imaging parameters (i.e., 512 x 512 pixels, 0.130 µm/pixel, 0.208 µs pixel dwell time with mono-directional scanning and 2x digital zoom) have not changed.

5. Laser ablation

1. Select an embryo of interest. Start a time-lapse recording in the image-acquisition software.

2. Acquire transmitted/fluorescence images with a 40x/1.2 W objective at high resolution (i.e., 512 x 512 pixels, 0.130 µm/pixel, 1.54 µs pixel dwell time with mono-directional scanning and 2x digital zoom using a 561 nm laser at 0.9% transmission). Acquire the time-lapse recording at maximum speed.
3. Zoom out of the area at the beginning of the time-lapse recording. Zoom in on the AOI.
4. Use the "Click & Fire" function of the laser-driver software to apply the damaging irradiation on the cell of interest in the embryo. Use the following parameters: 45% laser transmission (corresponding to a maximum of 40 µW) and 1 ms pulse time duration (step 4).
   NOTE: Video recording during the laser shooting is recommended.
5. Under 688 nm, monitor the ejection of autofluorescent chloroplasts from the cytoplasm.
6. If cell contents remain in the cell, use the "Click & Fire" function once more to increase the size of the breach in the cell. Repeat, keeping the number of shots to a minimum until most of the cell contents have been released.
7. Stop the time-lapse recording after the embryo has stabilized (i.e., no further intracellular movement can be detected (~1-5 min).
8. Update the annotation on the tile scan image, if necessary.

6. Monitoring the growth of irradiated embryos

NOTE: Monitoring is carried out over several days.

1. Determine the survival rate by monitoring the number of embryos that develop after laser ablation and compare them to those that die.
   NOTE: Some embryos die immediately after ablation for various reasons. A high and rapid mortality rate is usually a sign of inappropriate laser parameters or higher/longer exposure to stress during the experiment or subsequent transport.
2. Determine the growth delay by measuring the length of the laser-shot embryos every day and comparing it to intact embryos.
   NOTE: The growth rate of laser-shot organisms is generally slower than that of untreated organisms. However, some (inappropriate) laser settings can inhibit growth for more than a week, with growth resuming after that.
3. Find out the adjacent damage by monitoring the reaction of cells adjacent to the ablated cells. In some cases, post-burst depressurization may cause neighboring cells to burst.
4. Check for microbe contaminations. Monitor the growth of microalgae and bacteria in the medium. If an unusual level of microbes are present in the dish, then discard it and repeat the protocol from step 2 or step 3.
   NOTE: After laser ablation, damaged *S. latissima* embryos are already highly stressed, and additional external stress can cause increased mortality. Bacterial or viral outbreaks are possible because the treated embryos cannot grow in axenic conditions.
5. Check the global development of the shot organisms by studying the phenotype and understanding the role in the development of the targeted region.

Representative Results

Gametophytes of *S. latissima* were grown, and gametogenesis was induced to produce zygotes and embryos. Twelve days after the induction of gametogenesis, the embryos underwent laser ablation. Here, the experiment aimed to assess the role of specific cells in the overall development of *S. latissima* embryos. The most apical cell, the most basal cell, and the median cells were targeted. After tile scanning, the entire Petri dish (**Figure 2A**), an embryo of interest, was identified as a suitable candidate for laser shooting (**Figure 2B**). A specific cell of this embryo was chosen, targeted, and shot with a pulsed UV laser beam with a maximum of 40 µW of laser power (corresponding to 45% of maximum power for the equipment used here) for 1 ms (**Movie 1**). The cell released its contents (chloroplasts and cytoplasm). Interestingly, adjacent cells responded to the bursting of the irradiated cell by expanding into the intercellular space. The position of the irradiated embryos was recorded for subsequent monitoring over 10 days (**Figure 3**). Most of the irradiated embryos continued to develop but showed growth alteration (embryo shape). A detailed analysis of the morphological changes needs to be undertaken before a specific function can be attributed to each irradiated cell in controlling the overall developmental mechanism.

In contrast, embryos tested with other laser parameters (e.g., 33 ms for the shooting duration of 60%-80% laser power; **Movie 2**) quickly showed signs of severe stress such as cell bleaching, fading, or shape changes (rounding). Almost all embryos shot in this way died within five days after the experiment (**Figure 4**).

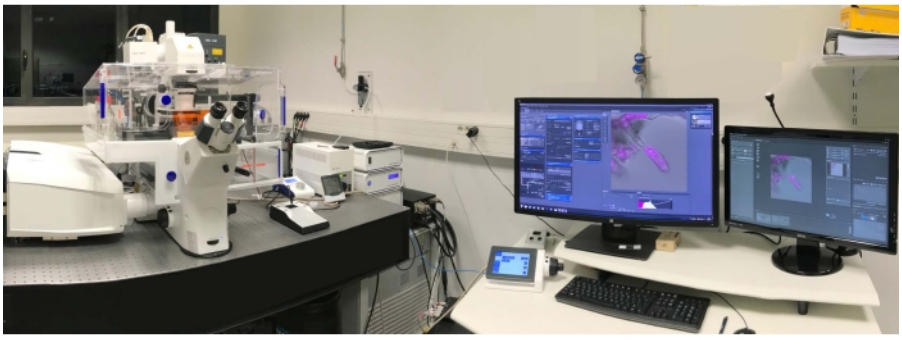

Figure 1: Photograph of the laser ablation microscope set-up. Please click here to view a larger version of this figure.

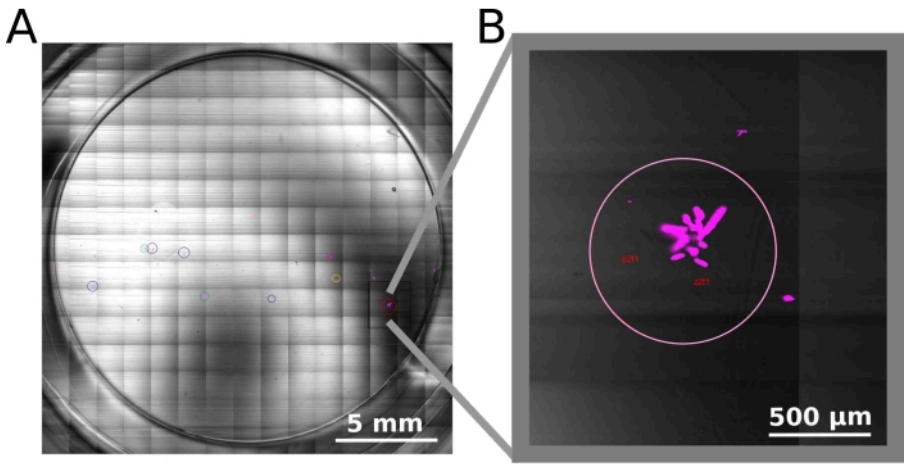

Figure 2: Annotated tile scan of a whole Petri dish. (**A**) Tile scan of the entire Petri dish used for laser ablation. The transmission PMT was used to obtain a brightfield scan. Once an embryo of interest has been located, the user clicks on the embryo's position in the tile scan, and the software positions the stage directly over the embryo. (**B**) The tile scan can then be annotated specifically to locate the embryo in the subsequent steps, to track and monitor the embryo. Here, the image shows an embryo attached to the bottom of the Petri dish, which can be easily located using the 561 nm laser. Please click here to view a larger version of this figure.

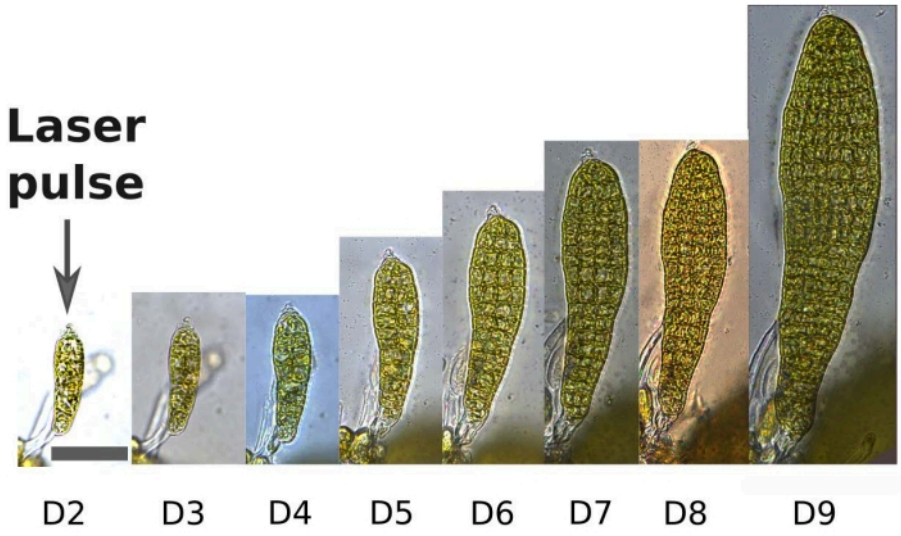

Figure 3: Time series of the growing *S. latissima* embryo after irradiation. This is shown in Movie 1. Laser ablation of the most apical cell of the embryo did not bleach the adjacent cells of the embryo. They continued to grow and divide, forming a normal embryo after a few days. Images were taken every 24 h, 2-9 days after ablation. The scale bar is 50 µm and is the same for all photos. D2-D9 corresponds to day-2 to day-9. Please click here to view a larger version of this figure.

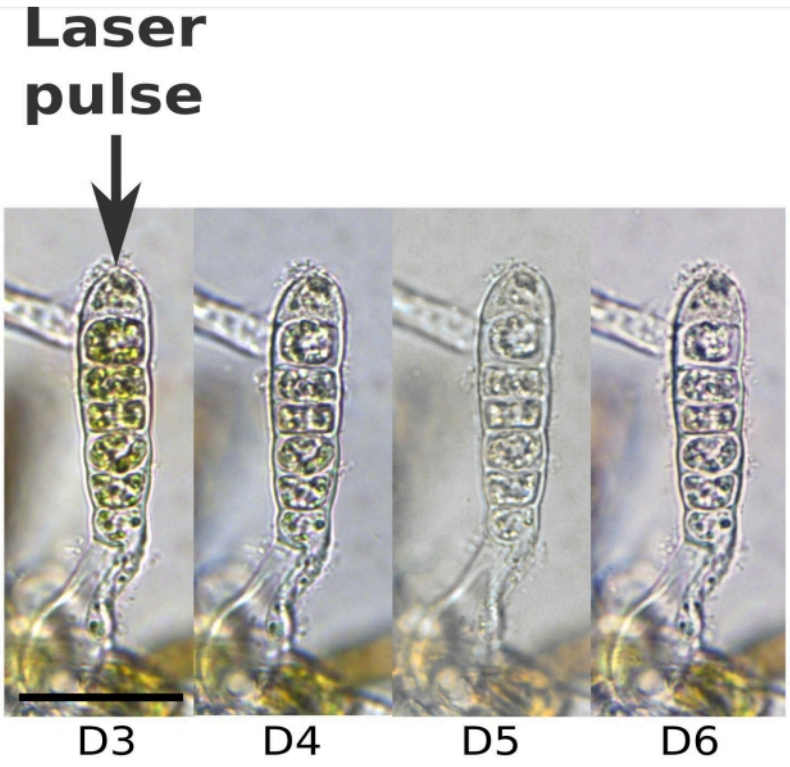

Figure 4: Time series of *S. latissima* embryo death after laser ablation using suboptimal parameters. Overexposure to laser irradiation can easily lead to higher death rates of the target embryo and also the neighboring ones. A time series of the embryo shot in Movie 2 is shown, with the time after ablation indicated above each photo (3-6 days after ablation). Scale bar is 30 µm and applies to each photo. Please click here to view a larger version of this figure.

Movie 1: Laser ablation of the most apical cell of an 8-celled embryo of *S. latissima*. The focus was first adjusted on the embryo selected among a bouquet of *Saccharina* sporophytes (at different fields, first wide, then narrow). The most apical cell of the embryo was then shot using 45% laser power for 1 ms. The apical cell rapidly burst and released its contents. The adjacent cells, freed from the turgor of the burst cell, filled the empty space. After 5 min, the cell and its contents have stopped moving and have reached a state of equilibrium. The movie has been accelerated 4 times (from 1 image per 632 ms to 6 fps). The follow-up of embryo development is shown in **Figure 3**. Please click here to download this Movie.

Movie 2: Significant bleaching of cells adjacent to a burst cell caused by suboptimal laser settings. Using a laser power of 60%-80% for 1 ms or a laser power of 45% for 10 ms resulted in significant bleaching of adjacent cells and a much lower survival rate. The settings used for the laser should not cause bleaching or partial damage to adjacent cells. Best results were obtained by reducing the power and/or duration of the laser pulse, and targeting the point furthestfrom the adjacent cells. Here, the most apical cell of a 4-celled embryo

of *S. latissima* is*shot (*80% laser power, 1 ms) (lateral/top view). Overbleaching of the lower and lateral cells is readily observable. The subsequent development of the embryo is shown in **Figure 4**. Please click here to download this Movie.

Supplementary Figure 1: Schematic flowchart of the entire protocol. Please click here to download this File.

Discussion

Local cellular laser ablation allows for temporal and spatial ablation with a high level of precision. However, its efficiency can be hampered by the non-accessibility of target cells; for example, all the cells are of a three-dimensional embryo. This protocol was developed on the embryo of the alga *Saccharina latissima*, which develops a monolayered lamina in which all cells can be easily distinguished and destroyed individually with a laser beam.

Laser power and wavelength

NIR fs-pulsed laser is commonly used in developmental studies on animals[24,25]. However, in the case of brown algae such as *Saccharina,* the laser could not burst the cell without burning the whole embryo. This reaction may be related to the high activity of the chloroplast light harvesters.

Using the ns-pulsed UV laser for ablation, two approaches were tested: (1) using a high power pulse over a short period of time or (2) using a lower laser exposure over a longer period of time. The energy delivered by the laser, a combination of laser power and pulse duration, impacts the energy delivered to the irradiated cell. The delivered energy can also affect the surrounding cells by reflection and diffraction. Therefore, the lowest settings of the two parameters that gave reproducible results were selected to reduce any unwanted collateral effects. Laser power between 25-40 $\mu W.cm^{-2}$ with a pulse of 1 ms appeared to be optimal. Using these parameters, the effect of the UV laser was contained at a very local level with no visible bleaching in the surrounding cells but efficient enough to cause the target cell to burst. These parameters were repeatedly applied without any direct lethal effect on the embryos. Longer pulse times or higher laser power caused bleaching of the surrounding cells or even death of the embryo, whereas lower values were insufficient to break the cell wall.

Algal material and culture conditions

Although allowing for precise shooting at the subcellular level, this technique requires the experimenter to address four critical factors to allow reliable interpretation of the results. These points must be considered both before and after the laser ablation experiment.

The first factor to address is embryo population density. Negative crowding impacts the development rate of *S. latissima*[26]. Low densities provide for (1) better pulse repeatability because the presence of other embryos in the laser path can decrease its power, and (2) easier monitoring of the embryos shot by the laser, which grow slightly slower than intact embryos; the latter quickly covering the shot embryos. The second critical factor of this protocol is the temperature at which the experiment is carried out. The laser beam itself heats the sample, but the temperature of the room and the local environment of the microscope add to the overall temperature conditions experienced by the sample, close to 18-22 °C. Here, the microscope's room was not refrigerated because this equipment is mainly used for animal cells that tolerate ambient temperatures. Fortunately, *Saccharina* embryos were able to withstand temperatures of 22 °C (9 °C higher than the optimal culture temperature of 13 °C) for up to 4 h. However, devices to help maintain a low ambient temperature, such as adequate ventilation or a cooling plate

holder, can help mitigate the risk of adverse effects on the survival and development of the algal embryos. Furthermore, slow pre-acclimation of gametophytes to 16 °C under red light can help the embryos withstand temperature spikes during the ablation process. Third, in addition to the increase in temperature, the embryos are deprived of a suitable light source for the duration of the experiment. Moreover, the chloroplasts may be partially bleached by the 561 nm laser. Using the lowest possible power on the 561 nm laser helps reduce this effect. Fourth, transport is also a critical factor to take into account because laser ablation equipment is not available in all laboratories. Temperature spikes and external movements or shocks should be avoided to prevent the algal material's dislodgement, loss, or death. Whenever possible, the transport boxes need to be refrigerated, vibration-resistant (e.g., shock-absorbing foam), and equipped with lights. Several transport boxes that meet these requirements are available commercially.

Even if all these factors are controlled as much as possible, weak but potentially significant environmental stress may still occur. This possibility must be considered in analyzing and interpreting the embryo response to laser ablation.

In addition to the environmental conditions that must be kept stable throughout the experiment, keeping track of the irradiated organisms throughout the monitoring step is also a challenge. The growth of the different embryos changes the original landscape over time. Unless an automated microscope can be dedicated to monitoring the experiment, following the embryo after laser ablation can easily become time-consuming and generate large amounts of data. Video recordings during the initial laser ablation pulse are highly recommended to track the embryo's immediate reaction to the pulse. This rapid monitoring of the pulse impact is of significant importance because in some instances, the pulse, when targeted to the middle of the cell, caused the target cell to burst rapidly, most likely due to the difference of osmolarity between the interior of the cell and the medium (seawater). When the leakage was too fast, the neighboring cells also burst. Shooting at the most distal region of the targeted cell proved to be an efficient way of buffering the burst effect on any adjacent cells. Another efficient way to avoid violent bursts is to increase the osmolarity of the medium with sucrose[27]. However, the difference in osmolarity is necessary to eliminate the targeted cell's contents. Thus, maintaining sufficient osmolarity potential is required. In a few cases, chloroplasts and other compounds obstructed the opening made by the laser ablation, which resulted in cells recovering from the laser shot, with growth resuming after a few days. Additional pulses directed at unclogging the opening proved sufficient to prevent obstruction.

Limitations

A trade-off must be struck between laser power, which should be high enough to make the cells empty their contents, and the survival of the neighboring cells, which in algal embryos are particularly sensitive to heat stress and photobleaching. Therefore, close monitoring of the cells after the laser shot is the key to controlling this step and ensuring that the target cells are dead. Otherwise, the interpretation of the targeted cells on the fate of the neighboring cells, and thus the subsequent development of the embryo, can be misleading.

Puncturing cells with a needle may seem to be an alternative in which the algal cells would not experience any heat or light stress. However, this approach would be challenging because brown algal embryo cells grow immersed in seawater and have no contact with any solid surface. The thick and elastic cell wall resists needle penetration even

when maintaining the embryo in contact with a solid, glass surface using a micromanipulator in a standard microinjection system.

In summary, this protocol describes the complete experimental procedure and settings for the laser ablation and monitoring of brown algal embryos and the critical steps for a successful experiment (**Supplementary Figure 1**). This unique approach is a promising method for studying cell interactions and cell fate in the early embryos of brown algae.

Disclosures

The authors have nothing to disclose.

Acknowledgments

S.B.'s PhD grant is funded by Region Bretagne (ARED grant Number COH20020) and Sorbonne Université. I.T.is PhD grant is funded by Region Bretagne (ARED grant Number COH18020) and the Norvegian NMBU University. This project has received financial support from the CNRS through the MITI interdisciplinary programs. MRic is member of the national infrastructure France-BioImaging supported by the French National Research Agency (ANR-10-INBS-04).

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