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Expanding the *Symbiodinium* (Dinophyceae, Suessiales) Toolkit Through Protoplast Technology

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ABSTRACT

Dinoflagellates within the genus *Symbiodinium* are photosymbionts of many tropical reef invertebrates, including corals, making them central to the health of coral reefs. *Symbiodinium* have therefore gained significant research attention, though studies have been constrained by technical limitations. In particular, the generation of viable cells with their cell walls removed (termed protoplasts) has enabled a wide range of experimental techniques for bacteria, fungi, plants, and algae such as ultrastructure studies, virus infection studies, patch clamping, genetic transformation, and protoplast fusion. However, previous studies have struggled to remove the cell walls from armored dinoflagellates, potentially due to the internal placement of their cell walls. Here we produce the first *Symbiodinium* protoplasts from three genetically and physiologically distinct strains via incubation with cellulase and osmotic agents. Digestion of the cell walls was verified by a lack of Calcofluor White fluorescence signal and by cell swelling in hypotonic culture medium. Fused protoplasts were also observed, motivating future investigation into intra- and inter-specific somatic hybridization of *Symbiodinium*. Following digestion and transfer to regeneration medium, protoplasts remained photosynthetically active, regrew cell walls, regained motility, and entered exponential growth. Generation of *Symbiodinium* protoplasts opens exciting, new avenues for researching these crucial symbiotic dinoflagellates, including genetic modification.

Keywords

Cell wall, cellulase; cellulose; dinoflagellate; protoplast fusion; protoplast generation; somatic hybridization; genetic modification; zooxanthellae

PHOTOSYNTHETIC dinoflagellates are globally important primary producers that sustain unique ecological pathways and biogeochemical processes throughout Earth's aquatic biospheres (Murray et al. 2016). Species within the genus *Symbiodinium* are extensively studied due to their fundamental role as endosymbionts of reef-building corals and many other marine invertebrates (Fabina et al. 2013; LaJeunesse 2002). *Symbiodinium* not only drive coral productivity and reef growth (Muscatine 1990; Muscatine and Porter 1977), but their diverse genetic backgrounds and distinct physiologies can also determine the thermal bleaching thresholds of their coral host (Berkelmans and van Oppen 2006; Fitt et al. 2001; Howells et al. 2012; Levin et al. 2016a; Loram et al. 2007; Yuyama et al. 2012). With coral reefs currently under extreme threat from climate change (Ainsworth et al. 2016; Baker et al. 2008), there is a pressing need to enhance our understanding of *Symbiodinium* biology. Despite the extensive research focus on *Symbiodinium,* almost all the knowledge gained to date has relied on studies of wild-type (not genetically modified) cells (e.g., Howells et al. 2012; Levin et al. 2016a; Parkinson et al. 2016; Rosic et al. 2015; Suggett et al. 2008; Suggett et al. 2015; Xiang et al. 2015). Only two studies have validated successful genetic transformation of *Symbiodinium* (Ortiz-Matamoros et al. 2015, which did not produce transformants capable of cell division; ten Lohuis and Miller 1998), and only one study has reported artificial selection of *Symbiodinium* (Huertas et al. 2011). Our current inability to advance research in genetics and cell biology of *Symbiodinium* is due to a lack of tools that facilitate genetic modification of *Symbiodinium* (Davy et al. 2012). Consequently, understanding of this ecologically critical dinoflagellate still remains in its infancy.

One such missing tool is the ability to generate *Symbiodinium* protoplasts. The first protoplasts were produced from plant cells over a century ago through mechanical

disruption of the cell wall (Klercker 1892). In the mid 1900s, introduction of enzymes to digest cells walls (Bachmann and Bonner 1959; Cocking 1960; Eddy and Williamson 1957; Weibull and Bergström 1958) led to high protoplast viability and yields (Davey et al. 2005). Protoplasts have since unlocked major research areas of bacteria, fungi, plants, and algae – notably genetic modification methods (Carlson 1973; Davey et al. 2005; Gietz and Woods 2001; Hopwood 1981; Reddy et al. 2007) – as well as economically valuable agricultural improvements (Bajaj 2012; Bravo and Evans 2011; Wang et al. 2013). Absence of the cell wall enables alternative methods for intracellular delivery of foreign DNA into cells such as polyethylene glycol (PEG)- and liposome-mediated transformation that can improve genetic transformation efficiency in certain species (Caboche 1990; Hansen and Wright 1999; Mathur and Koncz 1998; Rakoczy-Trojanowska 2002) and intracellular delivery of RNA-protein complexes that can enhance CRISPR-Cas9 genome editing (Woo et al. 2015). Furthermore, removal of the cell wall has allowed for protoplast fusion (i.e., somatic hybridization), another method of genetic modification in which two protoplasts are joined to form one hybrid cell containing two genomes (one from each parent). Protoplast fusion can even be used to hybridize cells of sexually incompatible species such as potatoes and tomatoes (Grosser et al. 1990; Kito et al. 1998; Melchers et al. 1978; Sagadevan et al. 2009). When capable of cell division, the hybrid cell can be mitotically propagated to produce genetically enhanced plants expressing novel combinations of traits controlled by individual genes and/or entire gene networks inherited from each parent cell (Bravo and Evans 2011; Davey et al. 2005; Peberdy 1980).

Production of viable protoplasts has not been accomplished for many microalgae strains, due to the complexity of microalgal cell walls and diversity of cell wall types across different species (Coll 2006). Particularly, armored dinoflagellates, unlike plants, have an intricate cell covering composed of an internal cell wall (a cellulose-enforced pellicle and cellulosic thecal plates) positioned between membranous layers (Markell et al. 1992; Morrill and Loeblich 1981; Wakefield et al. 2000). The membranous outer layer and thecal vesicle membrane serve as potential obstacles in the generation of

dinoflagellate protoplasts by reducing access to the internal cell wall. Several prior attempts have been made to produce protoplasts of armored dinoflagellates (Adamich and Sweeney 1976; Kwok et al. 2007; Pozdnyakov et al. 2014; Trench and Blank 1987), but success has been limited. Adamich and Sweeney (1976) reported the first protoplast generation of *Gonyaulax polyedra* using the detergent Liquinox, but trials of the Liquinox method failed to produce *Symbiodinium* protoplasts (Jit Ern Chen, pers. comm.). Cellulase was also considered to be unsuccessful at digesting cell walls in live *Symbiodinium* cells (Trench and Blank 1987), despite cellulase fully digesting isolated cell walls extracted from *Symbiodinium* cells (Markell et al. 1992). However, cellulase has been indicated to digest the cell walls of the dinoflagellate *Lingulodinium* (Wang et al. 2005). Cell walls of *Crypthecodinium cohnii* have been reduced, but not removed, from growing cells on agar plates with polyethylene glycol (PEG) (Kwok et al. 2007), and finally, treatment of *Prorocentrum minimum* with a cellulose synthesis inhibitor had low efficacy in preventing cellulose production (Pozdnyakov et al. 2014).

Here we produce protoplasts of *Symbiodinium* that are subsequently capable of regenerating their cell walls and returning to a healthy physiological status. Additionally, we explore the potential for fusion of *Symbiodinium* protoplasts, which may unlock the gateway for investigating intra- and inter-specific somatic hybridization of genetically distinct *Symbiodinium* isolates.

MATERIALS AND METHODS

Culture maintenance and genotyping

ITS2-type A3 *Symbiodinium* (University of Technology, Sydney, Climate Change Cluster culture collection: monoclonal strain CS73; host: *Tridacna maxima*; geographic origin: Heron Island, Great Barrier Reef) (Suggett et al. 2015), ITS2-type C1 *Symbiodinium* (Australian Institute of Marine Science culture collection: monoclonal strain SCF055; host: *Acropora tenuis*; geographic origin: Magnetic Island, Great Barrier Reef), and a mixed population of *Symbiodinium* (University of Technology, Sydney, Climate Change

Cluster culture collection: undefined combination of ITS2-types from strains described in Suggett et al. 2015) were maintained at 25 °C in culture medium composed of filtered seawater supplemented with Diago's IMK (Wako Pure Chemical Industries Ltd., Osaka, Japan) at pH 7.9. Different *Symbiodinium* ITS2-types are considered different species. Light was provided at an irradiance of 50 µmol photons m^{-2} s^{-1} (Philips TLD 18W/54 fluorescent tubes, 10,000 K) on a 12 h light : 12 h dark cycle starting at 9:00 am each day. Genomic DNA was extracted from each strain with the PowerPlant Pro DNA Isolation Kit (MoBio). The ITS2 region from genomic DNA of each strain confirmed by amplification with ITS2 primers from Stat et al. (2011) and subsequent Sanger sequencing by the Australian Genome Research Facility.

Enzymatic digestion and regeneration of the cell wall

For digestion, aliquots (~10^7 cells) of each *Symbiodinium* culture in exponential growth phase were pelleted at 2,000 × *g* for 5 min. Culture medium was decanted, and the cell pellet was re-suspended in 10 ml of digestion solution composed of 0.5 M D-sorbitol (Sigma-Aldrich, St. Louis, MO) dissolved in culture medium along with either 1.5 kilounits (KU) or 3 KU of cellulase from *Trichoderma sp.* (catalog number C1794, Sigma-Aldrich, St. Louis, MO). The cells in digestion solution were then incubated at 29-30 °C (to increase cellulase activity) in the dark on a shaking platform at 100 rpm for 36-48 h.

After digestion, protoplasts were pelleted at 200 × *g* for 5 min. Digestion solution was replaced with 10 ml of protoplast wash solution containing of 0.5 M D-sorbitol, 0.5 M sucrose, and 25 mM $CaCl_2$ (Sigma-Aldrich, St. Louis, MO) dissolved in culture medium supplemented with 100 µg/ml kanamycin. The protoplasts were then incubated at 25 °C in the dark on a shaking platform at 100 rpm for 3 h, after which they were pelleted and the wash step was repeated. Kanamycin was included in the protoplast wash solution to prevent overgrowth of *Symbiodinium*-associated bacteria due to the high concentration of sucrose that serves as a metabolically active osmoticum and carbon source. D-sorbitol is also important as an inert osmoticum, and $CaCl_2$ promotes protoplast

membrane stability (Gamborg 1977). After washing, protoplasts were pelleted, suspended in 10 ml of regeneration medium (0.5 M D-sorbitol and 25 mM $CaCl_2$ dissolved in culture medium, pH 7.0), and returned to *Symbiodinium* maintenance conditions (25 °C, 50 µmol photons m^{-2} s^{-1}, 12 h light : 12 h dark cycle).

Removal and regeneration of cell walls from incubation in digestion solution was assessed by cellulose staining with Calcofluor White (Sigma-Aldrich, St. Louis, MO). Culture aliquots (200 µl) were pelleted, resuspended in 20 µl of regeneration medium, and gently pipetted onto the 10 mm well of a FluoroDish (World Precision Instruments, Sarasota, FL). In the dish, cells were pre-treated with 20% dimethyl sulfoxide (DMSO) and a drop of 10% potassium hydroxide (BD Biosciences, Franklin Lakes, NJ) to ensure rapid permeablization of the cell membranes and wall then stained with 2 µl of Calcofluor White. After 5 min, cells were imaged on an Olympus FV1000 inverted confocal microscope (Tokyo, Japan) using the oil-immersion PL SAPO 100×/1.4 objective in a sequential scan with a 405 nm laser (dichroic mirror 490 nm, emission filter 425/50 nm) to detect stained cellulose and a 543 nm laser (dichroic mirror 560 nm, emission filter LP560) to detect chlorophyll fluorescence of cells.

To further verify protoplast generation, control cells and cellulase-treated cells were exposed to a range of osmotic pressures: culture medium alone, culture medium + 0.5 M D-sorbitol, culture medium + 1 M D-sorbitol. After 4 h of incubation, bright-field images of cells were captured with a Nikon Eclipse N*i* upright microscope on the 20× objective. The cross-sectional area of imaged cells was calculated with the equation Area = π(length/2)(width/2), generalizing cell shape as an ellipsoid.

To evaluate protoplast fusion, culture aliquots (750 µl) were fixed with 1% paraformaldehyde for 24 h and stained with 1 µM DAPI (4',6-diamidino-2-phenylindole) for 20 min. The 100× (oil-immersion) objective of a Nikon Eclipse N*i* upright microscope was used to capture bright-field, autofluorecence (excitation: 460-490 nm, emission:

500-∞ nm), and DAPI fluorescence (excitation: 340-380 nm, emission: 435-485 nm) images of cells.

Protoplast physiology

Physiological assessment occurred throughout four phases: Pre-digestion (day -2), digestion (4:00 pm on day -2 to 8:00 am on day 0), washing (8:00 am to 2:00 pm on day 0) and cell wall regeneration (2:00 pm on day 0 to day 28). *Symbiodinium* cultures (n = 3) were sampled on day -2 (pre-digestion) and on days 1, 3, 7, 14, and 28 (post-digestion, cell wall regeneration phase) to track cell health and morphology. Each physiological measurement was recorded at the same time on each sampling day. On days 2 and 6, cells were pelleted, and 15 ml of fresh regeneration medium was added. On days 13 and 27, cells were pelleted, and regeneration medium was replaced with 40 ml of culture medium.

Two active chlorophyll *a* fluorescence approaches were used to assay the photophysiological status of cells after 10-15 min of low-light acclimation in order to fully oxidise the photosynthetic electron transport chain (Suggett et al. 2015). First, samples were assessed using pulse-amplitude modulated imaging (PAM) microfluorometry (Imaging-PAM M-series Chlorophyll Fluorometer, Heinz Walz GmbH, Effeltrich, Germany) to yield a relative description of photosystem II (PSII) physiological heterogeneity amongst each culture at the single cell level. Additionally, samples were subjected to Fast Repetition Rate fluorescence analysis (FRRf; Soliense Inc.) to determine absolute quantification of PSII photosynthetic performance.

For the Imaging-PAM, the following configuration was used to deliver a multiple turnover (MT) excitation protocol, following the manufactures guidelines (Imaging-PAM M-series Chlorophyll Fluorometer, Instrument Description and Information for Users, 2.152 / 07.06, 5. Revised Edition: March 2014): An IMAG-RGB LED Lamp Module (Heinz Walz GmbH, Effeltrich, Germany) mounted on a Zeis Axio A1 microscope (Zeiss,

Göttingen, Germany) with a Zeiss Fluar 20× objective, RG665 detector filter, and a 420-640 nm dichroic mirror, allowed for computer-assisted measuring of maximum quantum yield of PSII (F_v/F_m, dimensionless). The Special SP-routine of the Imaging-WIN software (Heinz Walz GmbH, Effeltrich, Germany, version 2.41a) was used for increased sensitivity at low levels of excitation intensity. After a non-biological fluorescence standard was used to normalize measurements of the maximum fluorescence yield (F_m), 10 µl of low-light acclimated cells were then mounted onto a glass slide on the microscope. Once the steady state fluorescence yield (F_0) stabilized, a saturating pulse of blue (460 nm) light was applied and F_V/F_M ($[F_m-F_0]/F_m$) was imaged (measuring light intensity = 2, gain = 5, frequency = 8, damping = 2, F_0 averaging n = 3).

Next, low-light acclimated culture aliquots (50 µl) were diluted (1 : 30) in regeneration or culture medium and placed inside the Soliense FRRf optical head. The sample was subjected to 10 consecutive single turnover (ST) induction protocols (each separated by 150 ms) with an excitation sequence of 1.0 µs × 100 flashlets (2.5 µs interval) followed by a relaxation sequence of 50 × 1.0 µs flashlets (20 µs interval) as previously described (Schuback et al. 2015; Suggett et al. 2015). The final induction transient acquired for each sample was the average of the 10 × ST inductions, which was then fit against the KPF model (Kolber et al. 1998) to yield values for minimum and maximum fluorescence (F_0, F_m; and hence F_v/F_m, dimensionless), PSII effective absorption cross section (σ_{PSII}, $Å^2$ $quanta^{-1}$) and the electron turnover time of the primary PSII quinone acceptor, QA (τ_{QA}, µs), using custom software (Zbigniew Kolber, pers. comm.). It should be noted that all induction protocols were generated using only the LED with a 478 nm peak excitation, hence all σ_{PSII} values reported here are specific to this wavelength only. Values for τ_{QA} are represented by the initial of three decay constants (t) from the relaxation phase of the induction. All induction curve fits accounted for any formation of P680 triplet fluorescence quenching. Finally, all fits were performed relative to a sample blank consisting of an aliquot of regeneration or culture medium.

To measure motility, culture aliquots (100 µl) were added to wells of a 96 well plate, and cells were allowed to settle for 15 min. The total number of motile cells out of 100 counted cells was recorded for each aliquot through bright-field observations with a Nikon Eclipse T*i* inverted microscope (Tokyo, Japan) using the 20× objective. The total number of cells in each culture was quantified using a hemocytometer. Morphology of cells was assed from bright-field images captured with a Nikon Eclipse N*i* upright microscope (Tokyo, Japan) using the 100× (oil-immersion) objective.

RESULTS AND DISCUSSION

Three KU of cellulase per digestion of 10^7 cells was necessary for reliable protoplast generation within 36-48 h at 29-30 °C, as 1.5 KU only resulted in partial digestion of the cell walls (Fig. 1a). Exposure to hypotonic culture medium caused protoplasts to exhibit swelling compared to control cells (Fig. 1b) since digestion of the cell wall (lack of cell wall pressure) leads to increased turgor pressure. Addition of D-sorbitol to culture medium improved protoplast isotonicity (Fig. 1b). Complete regeneration of cell walls, indistinguishable from cells pre-digestion, occurred by day 14 post-digestion (Fig. 1a). Our results are consistent with previous findings that cellulose is the major structural component of *Symbiodinium* cell walls (Markell et al. 1992) and with previous evidence that internal cellulosic dinoflagellate cell walls can be digested with cellulase (Wang et al. 2005). Furthermore, our study overcomes a previous shortcoming where the use of cellulase failed to digest cell walls of live *Symbiodinium* (Trench and Blank 1987). Unfortunately, Trench and Blank (1987) did not detail their cellulase protocol, but an explanation for their contrasting results may be that their cellulase concentrations had been too low and/or their digestion times had been too short. In our study, cellulase was able to partially or completely digest *Symbiodinium* cell walls despite their internal placement, possibly because the *Symbiodinium* outer "membrane" is a thin layer composed of polysaccharides or glycoproteins (Blank 1987; Trench and Blank 1987; Wakefield et al. 2000), substances through which enzymes and other proteins can diffuse (Kingshott and Griesser 1999; McArthur et al. 2000; Olmsted et al. 2001), and the

thecal vesicle membrane is delicate causing it to easily rupture (Wakefield et al. 2000), which would allow cellulase access to the thecal plates. Moreover, *Symbiodinium* undergo ecdysis of their cell covering layers during the cell cycle and under stress (Bricheux et al. 1992; Pozdnyakov and Skarlato 2012; Wakefield et al. 2000), which might increase contact of the digestion solution and the underlying cellulose. Turgor pressure and osmotic stress due to disruption of the cell wall can also reduce membrane integrity (Fellows and Boyer 1976), potentially granting the digestion solution increased internal access. However, the mode through which cellulase contacted the internal cellulose was not directly investigated here.

Pearl chain assemblies (Fig. 2a), which create fusion interfaces between protoplasts (Zimmermann 1982), formed without exposure to a chemical fusogen or electric current. Protoplast fusion (Fig. 2b) occurred across all strains in < 1% of cells, which is still notable considering that no separate protocol to induce fusion (e.g., chemical fusion or electrofusion) was applied (Anne and Peberdy 1976; Zimmermann and Scheurich 1981). Yet, fusion was not necessarily spontaneous as the presence of salts and calcium can induce fusion (Boss et al. 1984; Kameya and Takahashi 1972). Furthermore, some protoplasts still exhibited swelling when exposed to 0.5 M D-sorbitol (Fig. 1b), which enhances efficiency of protoplast fusion through disruption of the membrane skeleton (Bhojwani 2013, Ahkong and Lucy, 1986). Massive size, irregular shape, and unsegregated choloroplasts clearly differentiated fused protoplasts from protoplasts of resting phase, interphase, or mitotic phase cells (Fig. 2b, S1). Fusion was further confirmed by detection of two nuclei in hybrid cells (Fig. 2b) and by scanning multiple focal planes to ensure that a continuous membrane encased the hybrid cell as opposed to two separate protoplasts atop one another (S1). No cells with morphology equivalent to hybrid cells were observed in control *Symbiodinium* cultures. While hybrid cells were not isolated from cultures for long-term assessment, they were viable on day 1 post-digestion since uniform cytoplasmic streaming across the hybrid cells was apparent, which also showed that the cytoplasms from each parent protoplast had joined (S1). Protoplast fusion in other organisms has been applied to combine desired

strains based on phenotypic traits such as increased thermal tolerance, increased viral tolerance, and increased antioxidant activity (Jbir-Koubaa et al. 2015; Pereira de Carvalho Costa et al. 2003; Zhang et al. 2012) – all of which are traits in *Symbiodinium* that have been implicated to reduce coral bleaching (Correa et al. 2016; Howells et al. 2012; Levin et al. 2016a; Levin et al. 2016b; Ragni et al. 2010; Suggett et al. 2008). Moving forward, protocols for increasing fusion frequency (reviewed by Bajaj 2012 and Sowers 2013) should be trialled to promote somatic hybridization, and the ability of hybrid cells to produce daughter cells should be evaluated to establish the feasibility of *Symbiodinium* breeding/genome shuffling.

Photosynthetic assessment of individually imaged protoplasts (Imaging-PAM, Fig. 3) and culture aliquots (FRRf, Fig. 4a-c) revealed that protoplasts remained photosynthetically active following digestion of cell walls. However, on day 1 post-digestion, F_v/F_m decreased by 4.9-32.1%, σ_{PSII} increased by 9.5-22.5%, and τ_{QA} decreased by 15.8-38.1% across all cultures compared to on day -2 pre-digestion (Fig. 4a-c). Initially, photophysiology of type C1 was the least affected, correlating with its chloroplast structure being the least altered: bright-field imaging of entire type C1 protoplasts detected slight pigmentation loss (Fig. 5) while confocal imaging of only autofluorescence in a single plane of type C1 protoplasts did not detect noticeable change (Fig. 1a). The photophysiology trends observed across the cultures were not due to cell death, but rather the transient decline in health status of live protoplasts because parameters of active photophysiology cannot be recorded from dead cells. Parallel decreased F_v/F_m and increased σ_{PSII} are often observed when PSII reaction centres (RCIIs) become increasingly deactivated via cell stress (Suggett et al. 2009), most likely induced from a combination of removal of the cell wall, increased temperature during cell wall digestion (Takahashi et al. 2008), and osmotic and pH differences (Berkowitz and Gibbs 1982) in regeneration medium verses culture medium. Transient reduction of τ_{QA} on day 1 post-digestion relative to day -2 pre-digestion suggests enhanced electron turnover by RCIIs despite net RCII inactivation, for example via compensatory photoprotection (Behrenfeld et al. 1998) or transient thylakoid membrane (and/or

trans-thylakoid membrane pH) instability (Tchernov et al. 2004). Nonetheless, the exact mechanism at play cannot be confirmed from these data alone.

Along with PSII inactivation, protoplasts were not motile on day 1 post-digestion (Fig. 4d), potentially due to cell cycle arrest in the coccoid (flagella-lacking, dark-cycle) stage from 36-48 h of incubation in the dark (Pozdnyakov and Skarlato 2012; Wang et al. 2008) or incidental mechanical loss of flagella (Bricheux et al. 1992) during the cell wall digestion procedure. Total cell abundance on day 1 post-digestion was reduced to 51-85% of that on day -2 pre-digestion from lysis during cellulase treatment (Fig. 4e). The decrease continued on day 3 post-digestion when abundance of cells ranged from 32.2-79.0% relative to day -2 pre-digestion from an inability of some cells to recover from stress following cellulase treatment.

By day 7 post-digestion, protoplast cultures substantially improved photophysiological performance (Fig. 3, 4a-c) and morphology (Fig. 5), demonstrating that the stress experienced from exposure to cellulase and increased temperature during digestion was not sustained. Likewise, the potential effects of turgor pressure and osmotic stress on membrane ultrastructure, which can reduce PSII activity (Fellows and Boyer 1976), may have gradually decreased from regenerating wall pressure in conjunction with cells undergoing intracellular osmotic adjustments to prevent photosynthetic damage (Downton 1983). Uniquely, σ_{PSII} of only type C1 post-digestion continuously increased over time in regeneration medium, indicating that type C1 may be less capable of osmotic acclimation than type A3 and the mixed population. The differential photophysiological responses amongst types clearly highlight the broad phenotypic diversity apparent for the genus *Symbiodinium* (e.g., Suggett et al. 2015).

Complete morphological improvement, indistinguishable from cells pre-digestion, occurred by day 14 post-digestion (Fig. 5). However, while F_v/F_m returned to a healthy state in all cultures, τ_{QA} of type A3 and type C1 decreased. Again, it is not possible to fully confirm the cause of these trends, but they coincide with the switch

from regeneration medium to culture medium that occurred on day 13 post-digestion (Fig. 4c). Type A3 and type C1 may therefore require more than one day to acclimate to the absence of osmotic agents and increased pH of the culture medium compared to the regeneration medium. Gradual dilution of regeneration medium with culture medium over several days could be trialled to alleviate this cell stress response (Davey et al. 2005). Regardless, by day 28 post-digestion, all cultures were in exponential growth phase and exhibited F_v/F_m, σ_{PSII}, τ_{QA}, and motility equivalent to cells on day -2 pre-digestion (Fig. 4a-e).

In summary, we report the first generation of *Symbiodinium* protoplasts and the first observations of protoplast fusion in any dinoflagellate. Protoplasts have led to major breakthroughs in the biological understanding and genetic enhancement of a vast range of organisms (Bravo and Evans 2011; Carlson 1973; Davey et al. 2005; Gietz and Woods 2001; Hopwood 1981; Reddy et al. 2007), thus we anticipate our study may also significantly expand the experimental scope of *Symbiodinium* studies. Considering the recent calls for heightened focus on genetic modification of dinoflagellates (Murray et al. 2016) and genetic enhancement of *Symbiodinium* to sustain the health of coral reefs (van Oppen et al. 2015), *Symbiodinium* protoplast generation and fusion are valuable and timely technologies ready to be utilized.

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FIGURE LEGENDS

Figure 1. Cell wall digestion confirmed by cellulose staining and by swelling of protoplasts in hypotonic culture medium. **A.** Cellulose staining of type C1 *Symbiodinium* cells with Calcofluor White on day -2 pre-digestion (control cell wall), on day 1 after exposure to 1.5 KU of cellulase (partially digested cell wall), on day 1 after exposure to 3 KU of cellulase (digested cell wall), and on day 14 after exposure to 3 KU of cellulase (regenerated cell wall). Scale bar (white, bottom left) = 10 µm. **B.** Box-and-whisker plot

showing the size (cross-sectional cell area) distribution (10^{th}-90^{th} percentile) of type C1 *Symbiodinium* cells (n = 25) exposed to varying concentrations of D-sorbitol in culture medium.

Figure 2. Somatic hybridization (fusion) of *Symbiodinium* protoplasts. **A**. Pearl chain assembly of type C1 *Symbiodinium* protoplasts preceding somatic hybridization. **B**. Bright-field, autofluorescence, and DAPI fluorescence images of a hybrid cell compared to a dividing (mitotic phase) cell from the type C1 *Symbiodinium* strain after cellulase treatment. Scale bar (black, bottom left) = 10 µm. Focal plane scanning and cytoplasmic streaming of a live hybrid cell from the mixed *Symbiodinium* population after cellulase treatment are shown in S1 (video).

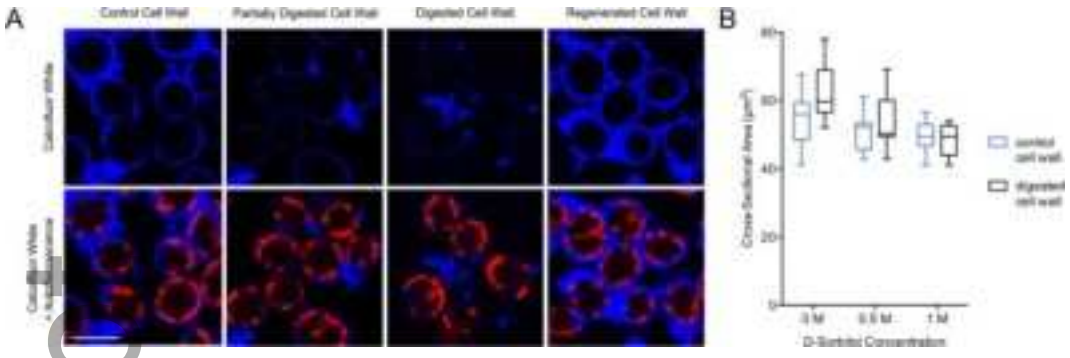
Figure 3. Single-cell photobiology throughout protoplast generation and cell wall regeneration. Individual *Symbiodinium* cells are shown according to a false-color spectrum where red indicates a relative maximum quantum yield of PSII approaching 0 and purple indicates a relative maximum quantum yield of PSII approaching 1. Panels from left to right indicate the pre-digestion sampling day -2 and post-digestion sampling days 1, 3, 7, 14, and 28 for type A3 (top row), type C1 (middle row), and the mixed population (bottom row). Scale bar (white, bottom left) = 20 µm.

Figure 4. Physiological status of cultures throughout protoplast generation and cell wall regeneration. **A**. Maximum quantum yield of PSII (mean ± standard error, n = 3). **B**. PSII effective absorption cross section (mean ± standard error, n = 3). **C**. Electron turnover time of the primary PSII quinone acceptor (mean ± standard error, n = 3). **D**. Cell motility (mean ± standard error, n = 3). **E**. Growth rate (mean ± standard error, n = 3). The blue solid line on day 0 marks when fresh protoplasts were resuspended in regeneration medium. The blue dotted line on day 13 marks when cells were returned to culture medium.

Figure 5. Bright-field imaging of cell morphology throughout protoplast generation and cell wall regeneration. Microscopy revealed morphological differences between *Symbiodinium* with cell walls and *Symbiodinium* protoplasts. Panels from left to right indicate the pre-digestion sampling day -2 and post-digestion sampling days 1, 3, 7, 14, and 28 for type A3 (top row), type C1 (middle row), and the mixed population (bottom row). Scale bar (black, bottom left) = 10 µm.

SUPPORTING INFORMATION

Supporting Video 1. Cytoplasmic streaming across fused cytoplasms of a live, hybrid cell from the mixed *Symbiodinium* population after cellulase treatment. Bright-field video was recorded with a Nikon Eclipse N*i* upright microscope using the 100× (oil-immersion) objective.



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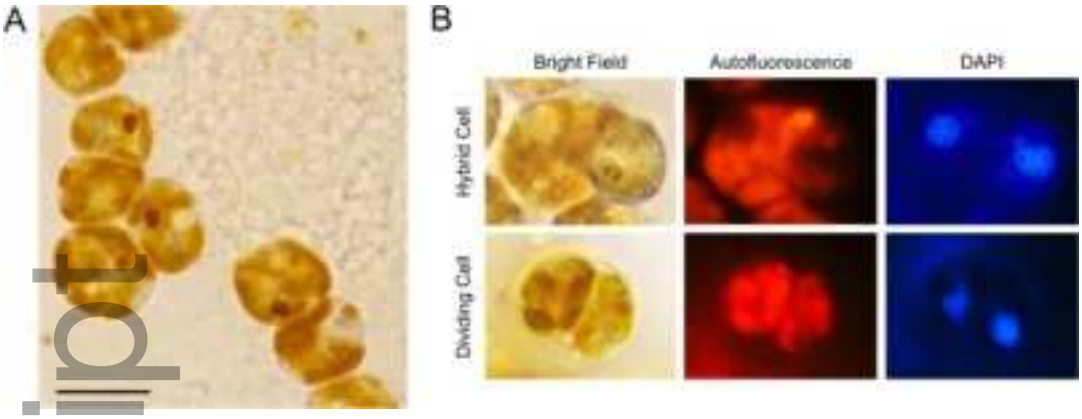

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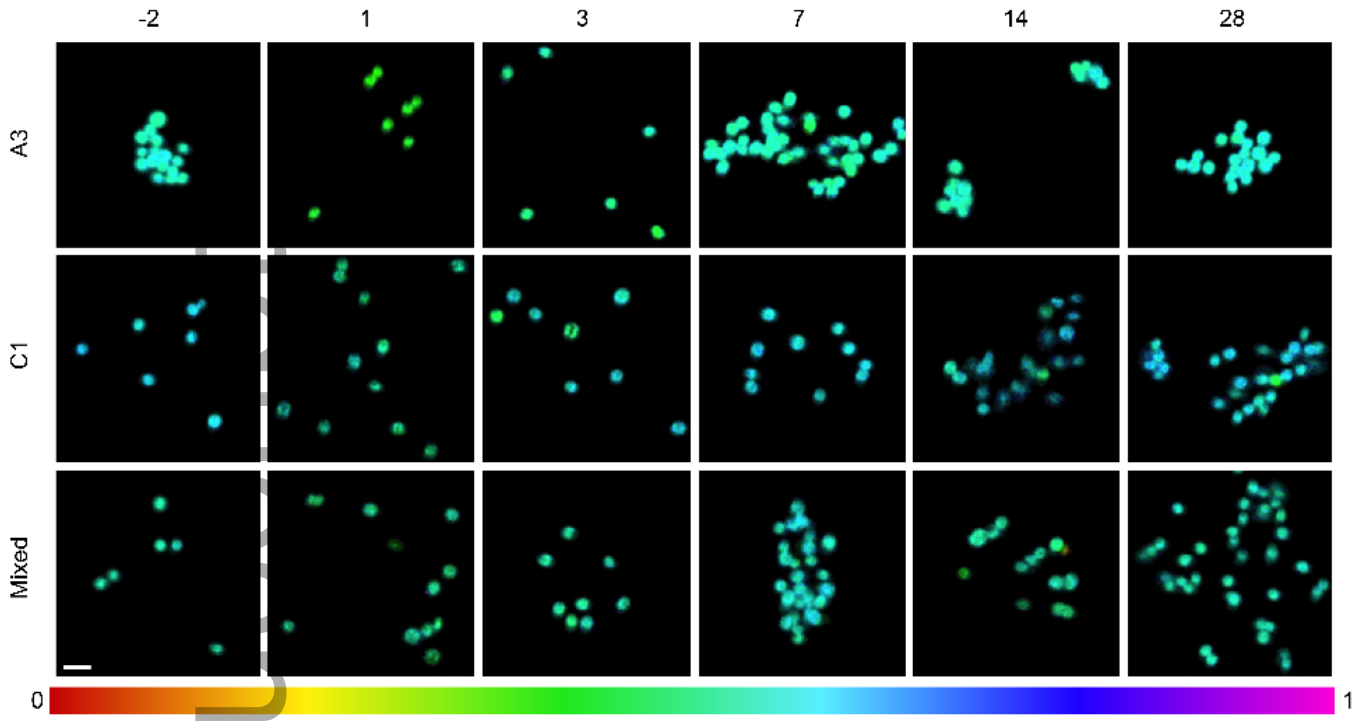

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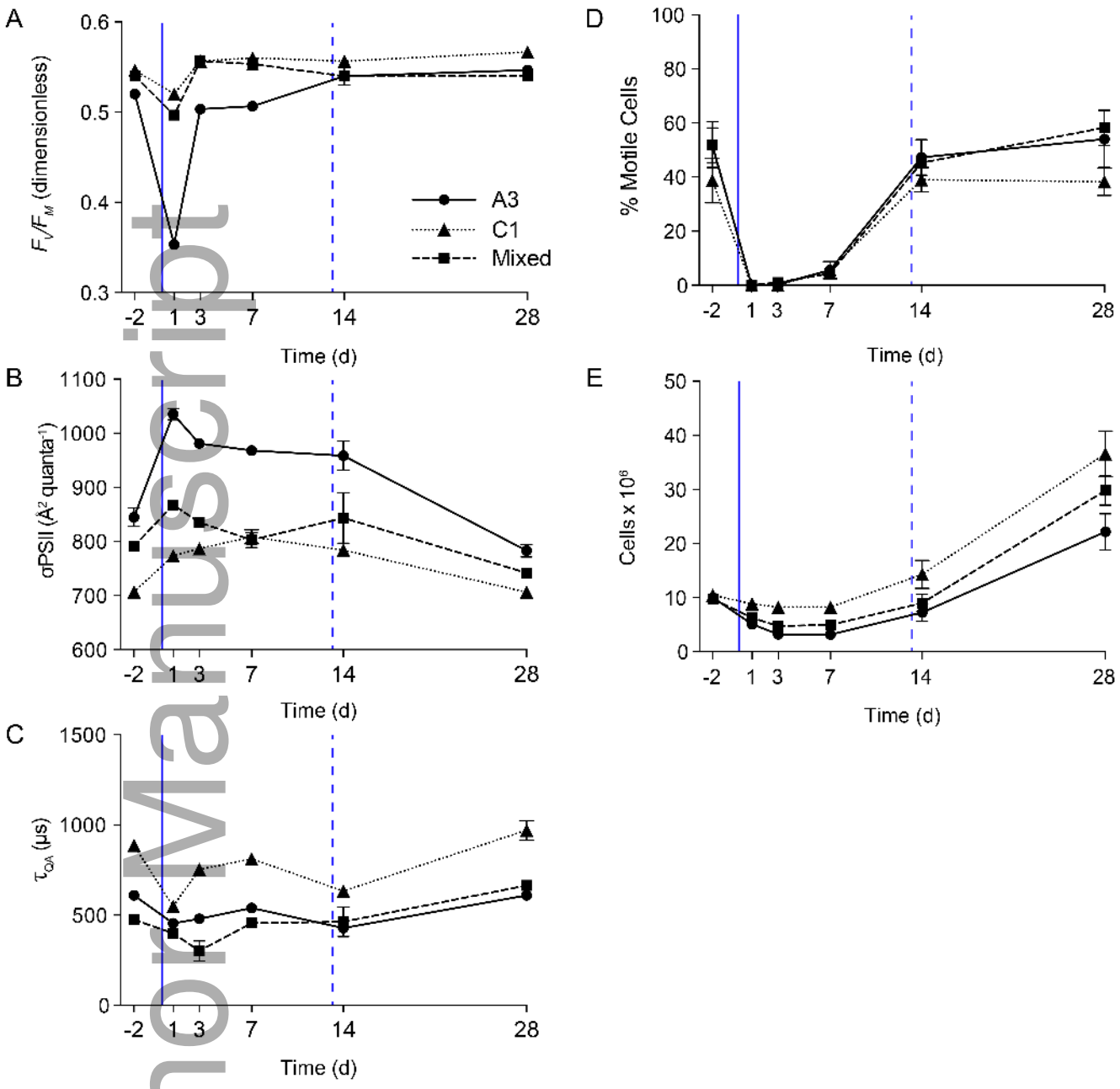

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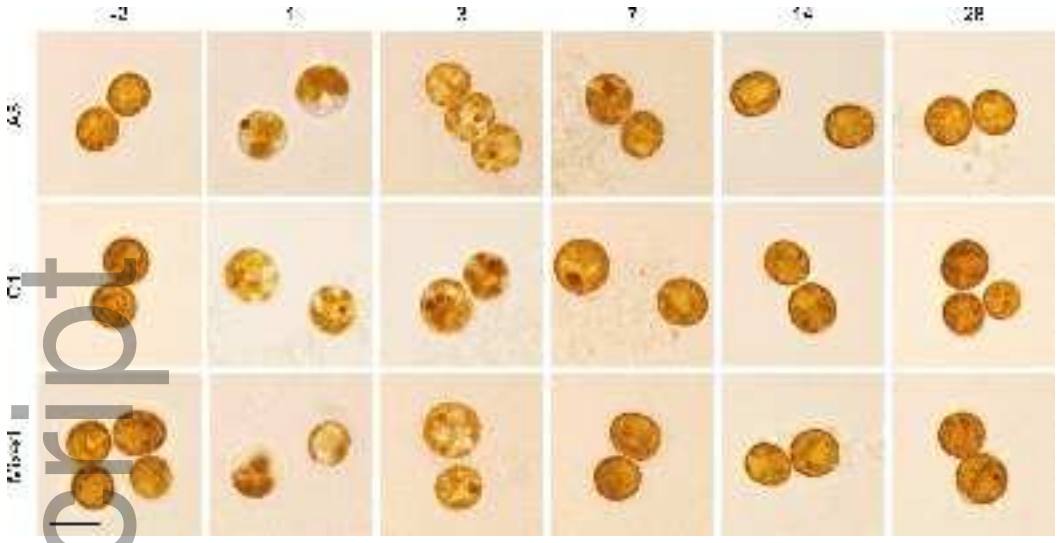

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