

THE APPLICATION OF FLOW CYTOMETRY FOR KELP MEIOSPORE ISOLATION

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Abstract

The goal of this study was to evaluate flow-cytometric techniques for isolating individual kelp meiospores into 96-welled plates. Previously reported low success rates for sorting tens of thousands of meiospores at a time have been improved by technological developments, specifically, the low nozzle pressure of the commercially available, JSAN

(Bay Bioscience Co. Ltd) instrument. We monitored growth and gametophyte development post-sorting for 10 months. Our data demonstrate that successful kelp meiospore isolations of up to 76% viability can be achieved with flow-cytometry. This method can save time as compared to traditional, manual isolations using pipettes and improves confidence that self-fertilized individuals will not contaminate specific crosses of resulting gametophytes. Our results highlight a new application for the flow cytometer to produce clonal kelp gametophytes with direct applications for germplasm and culture collection development.

Keywords: chlorophyll a, cytometry, meiospores, *Saccharina*, single cell sorting

1. Introduction

Kelp aquaculture products are gaining popularity beyond Asia in South America, North America, and Europe [1–8]. The current, global seaweed production is valued at US\$11.7 billion [9] with predictions for growth and expansion of the industry. Several kelp species, including *Undaria pinnatifida*, *Saccharina japonica*, *Saccharina latissima* and *Laminaria digitata* are incorporated into human foods and animal feed, bioenergy development, and are used for bioremediation of coastal waters [10,11]. Other species are used in the phycocolloid industry as additives for thickening. With high consumer demand and growing population, cultivated seaweed can contribute to the predicted gap in global food security [12]. As the seaweed industry continues to grow, it is now of the utmost importance to develop techniques to lower the reliance on the fragile wild populations and invest in propagating kelp from germplasm “seed” collections. Most

existing commercial kelp nurseries rely on wild populations for collection of reproductive thalli; this may place a burden on some populations that are already stressed from habitat loss, overharvesting, and climate change. Seed banking for microscopic stages of macroalgae as storage of genetic diversity of species and storage of strains for cultivation purposes has been gaining interest both for economic development and for conservation in a changing environment [13-14].

Kelps have a biphasic life cycle with a macroscopic, diploid, blade phase that becomes reproductive to produce biflagellate meiospores. When meiospores are released from the sporangia, they become part of the plankton and rely upon endogenous lipid reserves for survival before settling onto a suitable substrate, germinating, and growing into microscopic, filamentous male and female gametophytes[15-17]. In the wild, these haploid male and female gametophytes will undergo cross fertilization and produce a new generation of sporophytes.

For decades, kelp meiospore selection for propagation has been done by micropipetting meiospores and/or developing gametophytes under a compound microscope. Because of how time-consuming this is, the technique is a bottleneck for industrial-scale propagation. High-throughput cell sorting by flow-cytometry is a contemporary technique for isolation of live, single cells of various origins for cultivation or experimentation. Technological advances have been made to increase the accuracy of sorting based upon fluorescence and light-scattering of cells [18,19]. Flow cytometry (FC) has been used to sort single microalgal cells to obtain clonal cultures, and reports show taxon-specific variability in cell recovery after cytometric sorting [20]. Algal cells have optical characteristics that can be used in sorting, including natural properties such

as cell size, structure, and endogenous pigmentation as well as chlorophyll autofluorescence [21]. While FC has been proposed for kelp when coupled with stable isotope analysis to investigate the detritus or particulate organic matter in the water column [22], most of the applications for algal sorting have been done on counting, analysis and identification of phytoplankton [23-24].

Druehl, et al. (1989) used flow cytometry to characterize meiospores of three kelp species (*Alaria marginata*, *Saccharina latissima*, and *Cymathaere triplicata*), specifically to assess size, DNA, and chlorophyll contents, as well as to investigate sex ratios of developing microscopic gametophyte stages [25]. In that descriptive study, approximately 10,000 meiospores were sorted at a time with the application of several stains (Hoechst and DAPI), and very low success rates (0.9-1.5%) were obtained [25], making this method inefficient for sex determination [26] and uniclonal isolation.

Our kelp-breeding project, funded by the US Department of Energy ARPAC MARINER program, is an ongoing effort to cultivate superior strains of sugar kelp (*Saccharina* spp.) for aquaculture. This project demands isolation of hundreds of kelp cultivars for genetic screening and production of hybrids to plant out at near-shore, open-water farm sites. To screen hundreds of samples from over 20 populations throughout the Northeast Atlantic, we needed a way to improve isolation techniques to optimize viability and make the process faster than the traditional pipetting methods that are very labor intensive and prone to error and contamination. Accordingly, the overall goal of this study was to evaluate flow-cytometric sorting for isolating individual meiospores into 96-well plates for propagation of clonal gametophytes. Here we report the efficiency and

effectiveness of using a commercially available sorting cytometer (JSAN, Bay Bioscience Co., Ltd) to isolate kelp meiospores for clonal propagation.

2. Materials and methods

Reproductive sugar kelp, *Saccharina latissima*, blades were collected using SCUBA at 19 locations throughout coastal New England, USA (n=10-20 blades per site) over the course of 3 months (April-June of 2018). After collection, blades were kept in coolers during transport to maintain integrity. Sorus tissue was excised, cleaned, and desiccated overnight, and meiospores were released into a beaker with 100mL sterilized seawater the following morning as per standard protocols [27]. Only 13 of the sampled populations (Supplement Table) released meiospores and were used in the subsequent analyses. Meiospore density was quantified using a hemocytometer and compound, light microscope. After meiospores were observed in the seawater, they were immediately sorted.

Isolations were completed at the NOAA NMFS Lab in Milford, CT, using a commercially available sorting cytometer (JSAN, Bay Bioscience Co., Ltd.). We tested different settings of the flow cytometer to increase viability of isolated meiospores and quantified the success of this isolation technique. The concentration of spore suspension used with the cytometer was variable and was diluted only when the sample differential was too low to keep the event rate below the ideal 200 s^{-1} . Settings used for spore isolations were as follows: nozzle tip size 70 μm , sheath fluid 0.2 μm filtered sea-water, and sheath pressure at the lowest setting with mean sample differential of 0.76 kPa (+/- 0.59 kPa). Meiospores were sorted based upon size (forward scatter, FSC), internal complexity (side scatter, SSC), and chlorophyll *a* fluorescence (FL3), with the following

sort mode: three drops, high purity. Samples were sorted into 96-well plates, with each well pre-filled with 250 μ L of autoclaved seawater enriched with $\frac{1}{2}$ strength PES (Provasoli Enrichment Seawater, [28]) and 2 mL/L germanium dioxide (GeO_2) [29]. Immediately after sorting, wells were checked for meiospores, but they were too small to observe. As a control, thousands of meiospores were sorted and placed under red light to observe fluorescence and presence of meiospores.

After isolations, plates were incubated at 10°C in the dark for 24 hours to allow for settlement and germ tube formation. Afterwards, plates were brought into red light with a 12:12 light: dark cycle for over 2-3 weeks before being checked under a dissecting microscope (SZH Olympus, TYO, Japan) for the presence of filaments to indicate viability. Red light was used to suppress reproduction and promote vegetative growth of the gametophytes [30]. Gametophytes were then allowed to grow for another 1-2 months and checked periodically for presence/absence of growth in each of the wells, and sexed based upon size and morphology. The final count was completed 10 months after the initial isolation and yielded the highest results. All statistical analyses were done on these final counts.

We ran descriptive statistics comparing the sampled kelp populations to assess the success of flow cytometry to isolate and maintain the viability of meiospores for germination. Results of isolations were ranked from low to high density of gametophyte success in each plate. We then quantified correlations of the optical characteristics (size, internal complexity, and chlorophyll *a* fluorescence) as a function of successful gametophyte growth.

We tested to see if there was a relationship between successful germination and the quality of meiospores released as a function of two metrics - time after isolation and motility. First, we evaluated the relationship between the count of wells containing gametophytes and time after parental blade collection as well as collection location (two-way ANOVA). Time differences resulted from inability to process all blades immediately after collection. Occasionally, some of the blades were kept suspended in flow-through outdoor tanks (each with 18,500 L capacity and a flow rate of about 100 L/min) at Woods Hole Oceanographic Institution at ambient light and temperature at 10 °C. Time, in days, varied from 1-27 days after collection to FC sorting. Second, as the FC does not discriminate motile from non-motile meiospores, we ran a correlation to see if motility had an influence on germination post sorting and used the ratio of motile to non-motile spores as a possible predictor of success.

3. Results

Viability

Meiospores were identified by an initial analysis trial of 5,000 spores, as there were very few other particles in suspension. The optical characteristics used to identify meiospores in cytograms were size, internal complexity, and chlorophyll *a* fluorescence (Figure 1). The sort gates were applied to include some variation in the three variables and also adjusted to account for observable variations between populations and samples.

Viable kelp meiospores were isolated successfully using the JSAN sorting flow cytometer. The mean rate of sorting was estimated at 1150 meiospores/hr. At least two to three months were needed to allow for gametophyte growth before an accurate

assessment of gametophyte development could be made. Populations with low meiospore release were not included in the analyses, yielding a final count of 13 populations (n=1-12 blades/ population, Supplement Figure). The highest counts recorded were >76% success at 73 gametophytes in one 96-welled plate (sample from Casco Bay, Maine). The mean percentage for successful isolations was 37.5 (± 24.6 SD) gametophytes in two 96-well plates. Overall, the distribution of viable gametophytes was ranked into 6 categories from low success to high (Figure 2). The counts of successful gametophytes varied from 0-144 summed from two 96-welled plates. Highest frequency counts at 30% were found for the category of 24-48 meiospores sorted. Lowest frequency counts at 1% or only one sample, were in the category of 120-144 meiospores counted.

A slight, positive trend indicating that samples with meiospores having higher chlorophyll *a* fluorescence (R-squared= 0.179), size (R-squared= 0.066), and internal complexity (R-squared= 0.217) yielded higher isolation success was noted. Overall, higher side scatter seemed to be the best indicator of viability rather than size or chlorophyll concentration.

Both time after collection (two-way ANOVA, p-value < 0.001) and collection location (two-way ANOVA, p-value < 0.01) were found to be statistically significant and as having an impact on eventual germination success rates. Overall, samples kept in a flowing seawater-holding tank for a long period yielded fewer gametophytes. Finally, a clear trend was not observed correlating meiospore motility with success (R-squared= 0.0629, not significant, Figure 4).

4. Discussion

To our knowledge, this study reports the first time a sorting flow cytometer was used to isolate single, individual kelp meiospores successfully for germinating viable gametophytes. The work by Druehl et al. (1989) used cytometry to isolate thousands of meiospores at a time with very low survival rates. Our protocol using the JSAN flow-cytometer proved to be an effective technique for high-throughput isolations of individual kelp meiospores that developed into single gametophytes that can then be vegetatively propagated.

In the marine environment, timing of meiospore release and settlement is critical. If suitable substrate for attachment is not found within 0.5 - 24 hours, the spores exhaust their resources and die. Several species of kelp produce spores capable of net photosynthesis – an advantage for prolonged dispersal with recorded transport of over several kilometers [13, 15, 31- 33]. Here we did not find any clear correlation between motile vs. non-motile meiospores and successful germination rates of gametophytes. Neither did we observe a strong correlation between motility and success, suggesting that even non-motile spores have the potential to settle and germinate into gametophytes post-sorting. It has been shown that settlement of kelp meiospores for a closely related species, *Saccharina japonica*, can occur after the settlement of both the motile stage as well as the free-floating stage after the spores have exhausted their reserves [34]. What did have a significant effect on gametophyte development success was the time kelp blades were stored post collection. Presumably, meiospores may have been released in the holding tank before arrival at the sorting facility. Additionally, collection location is also an important factor to consider as some locations presumably had blades that were ripe at collecting time.

Our flow cytometry technique enabled high-throughput, clonal gametophyte isolation and the development of a germplasm library for the emerging North Atlantic kelp aquaculture industry. Furthermore, this technique can be transferred to industry on a global scale and may even be tested for isolations of other macroalgal species. In contrast with manual isolation techniques [35] such as serial dilutions, single-cell FC establishes in hours thousands of clonal gametophyte isolates at the pre-development stage, improving confidence in the genetic identity of the germplasm subsequently used in breeding. Although the cost of owning the cytometer is prohibitive for this application alone, buying time at a core facility likely can be cost-effective compared to labor needed for manually isolating.

In conclusion, our results highlight a new application for the flow cytometer to produce vegetative kelp gametophytes with direct applications for germplasm and culture collection development. Isolations rates were as high as 76 % for some samples. Overall, the application of this method can save valuable time as compared to traditional manual isolations using glass pipettes. Using the flow cytometer can improve confidence that self-fertilized individuals will not contaminate specific crosses of resulting gametophytes.

Acknowledgements

We would like to thank J. Li, M. Dixon and the staff at NOAA NMFS Milford Lab; and M. Stekoll for sending samples. Funding was provided by the US Dept. of Energy, ARP Ae MARINER project contract number DE-AR0000915.

Statement of Informed Consent, Human/Animal Rights

No conflicts, informed consent, or human or animal rights are applicable to this study.

Author contribution

Author Simona Augyte was involved in the design and execution of research and writing of the manuscript; author Gary Wikfors was involved in experiment set up, data analysis and critical revisions, author Steve Pitchford was in charge of running the flow cytometer; author Michael Marty-Rivera was involved in data acquisition; Schery Umanzor was involved in data analysis and providing critical feedback during manuscript edition, author Scott Lindell provided the sporophyte holding facilities at WHOI, prepared blades for shipping and manuscript revisions, author David Bailey collected and prepared sporophytes and manuscript revisions, and Charles Yarish was involved in conception and design of the study and manuscript revisions.

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Figure captions

Fig. 1. Cytograms showing optical characteristics of meiospores and the gates. SSC-H is the side scatter, FSC-H is the internal complexity and Chl *a* – refers to the chlorophyll *a* pigmentation of the meiospores.

Fig. 2. Frequency distribution of viable gametophyte counts within several different categories in two 96 well plates.

Fig. 3. Success of isolated gametophyte growth as a function of days after parental sporophyte collection.

Fig. 4. Flow cytometry gametophyte isolation success in relation to ratio of motile to non-motile spores at the time of sorting. The data are for one blade sample (two 96-welled plates). (R-squared= 0.0629, not significant).

Fig. Supplement. Successful isolations (mean, \pm standard deviation) of samples in two 96-welled plates based on wild kelp populations.

Graphical abstract. Flow cytometry cytogram based on chlorophyll *a* concentration and internal complexity of meiospores, a close up of *Saccharina* meiospore with visible flagella and a 96-welled plate for sorting.









