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Cell size selection in *Chlamydomonas reinhardtii* gametes using fluorescence activated cell sorting

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ABSTRACT

Existing approaches to measuring the size of single cells or, alternatively, disrupting the size distributions of populations, do not often allow the high throughput collection and separation of live cells post-measurement. An emerging method for the measurement and collection of single cells based on cellular properties is flow fluorescence activated cell sorting (FACS). Here, we use cell sorting to demonstrate the utility of selecting on size in live gametes of the single celled algae *Chlamydomonas reinhardtii*; an important model in cell cycle research, cell physiology and biofuel modelling. Using three methods, we demonstrate the capacity to sort live cells without the need for extrinsic fluorophores. By selecting on size related characteristics in gametes, we are able to show the capacity for sensitive selection of small variations in cell size. Furthermore, we demonstrate the use of autofluorescence properties of algae, and further support existing literature showing effective disruptive selection on size when incident light scatter and fluorescence parameters are combined as selection criteria. Estimates demonstrate significant heritability of cell size in both gametes and vegetative cells, while a strong correlation between gametic and vegetative size suggests that selection on gamete size could be used to evolve vegetative size under experimental evolution.

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1. Introduction

The extent and dynamics of cell growth and division represent a complex interplay of processes. For example, the size that a cell grows to, as well as the dynamics of cell growth, exists as a response to intrinsic factors such as the molecular processes governing growth and division, as well as extrinsic factors mediated by the relationship between that cell and its environment that shape the evolution of cell growth. Elucidating such processes further requires the ability to control a cell's environment, the use of transgenic approaches or chemical methods to control the cell cycle stage, in addition to species demonstrating short generation times and the ability to exert phenotypic selection. All three of these approaches rest on the capacity to control for cell size in a high throughput and accurate way. Here we develop a method using flow cytometry in a model alga, which not only enables the separation of unstained cells based on cell size, but is capable of sorting individual cells into different media. *Chlamydomonas* is both highly autofluorescent and amenable to transgenic approaches or the application of extrinsic fluorophores. As a result, flow cytometric based methods for cell size selection would be compatible with a range of other methodologies. Such approaches have the potential to radically change the resolution of our understanding of the cell cycle and the relationship to and modulation of cell size.

Chlamydomonas reinhardtii is a single celled algae and an established model in biofuel production (biohydrogen and triacylglycerol (TAG) accumulation) [1,2], evolution [3,4] and biochemistry [5,6]. Often reproducing asexually, under situations of synchrony (where division is separated from growth and restricted to the dark phase of a light:dark cycle [7]). *Chlamydomonas* shows division by binary fission, where growth 2^n fold is followed by n divisions. *Chlamydomonas* is also amenable to transgenic approaches [8–10] and the genome of this alga is now readily available and annotated [11]. Finally, the single celled nature and capacity for asexual reproduction of *Chlamydomonas* enable mutagenesis screening or experimental evolution, and cells can be isolated to enrich or further characterise populations with specific light scatter or autofluorescent profiles.

Under conditions of nitrogen limitation, cycling haploid vegetative cells of *C. reinhardtii* undergo a program of gametogenesis, producing sexually competent haploid gametes that are smaller in size than their vegetative counterparts [12], and which have high TAG synthesis, useful in biofuel production [13]. Gametes possess a single autofluorescent chloroplast, and are capable of mating with a cell of the opposite mating type, to produce meiotic offspring [14]. As a model for biofuel production, the evolution of reproduction and the evolution of anisogamy (sperm and eggs), a method capable of disrupting *Chlamydomonas* populations based on cell size would have wide ranging applications.

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Flow cytometry is a high throughput, quantitative method for measuring the various structural and functional characteristics of cells. Such parameters may indicate size and complexity as well as the presence and quantity of intrinsic or extrinsically applied fluorescence tags, all with single cell resolution. The cells pass through a laser path in single file [15,16]. Detectors placed both in line with, and orthogonally to the lasers, detect refracted incident light and excited fluorescence which is scattered as cells pass through the light beam [16]. Light collected in the orthogonally placed detectors is divided into wavelength bands by a series of dichroic mirrors and filters, in front of photomultiplier tubes, to identify specific ranges of fluorescence emitted by the cell [16]. Thus the measurement of scattered light and fluorescence provides relative indications of cell size (measured as forward scatter), cell complexity (measured as side scatter), and the intensity and range of cellular fluorescence at different wavelengths for each cell [17]. Flow cytometry based cell sorting, which is an extension of the flow cytometry technology, allows the sorting of cells with predetermined characteristics into independent culture for analysis, whether as enriched populations or single cells. As such, flow cytometry cell sorting offers the potential for both the high resolution and control required for investigating cell size.

As a tool of size discrimination, Fluorescence Activated Cell Sorting (FACS) is an appealing method. The main alternative method is the Coulter counter; a common and established method for measuring size, which measures changes in electrical current (impedance) as cells pass through an opening which carries electrical charge [18,19] and where impedance is proportional to cell volume [19]. While this method is common and high-throughput, modified cell sorting coulter counters, which allow the enrichment of populations based on coulter counter measures are less commonly found. Similarly, methods using serial dilution and manual measurements of fixed *Chlamydomonas* cells to select on cell size are time consuming, are based on population averages rather than individual variation, and increase the risk of unmonitored genetic variation which may be problematic depending on the protocol. Thus the high throughput nature, manipulation of live cells, and amenability to other molecular techniques make FACS a useful candidate for routine cell size selection.

Flow cytometry parameters may be used to determine relative size distributions of samples based on the properties of the cells being measured. However, which parameters are suitable may vary depending on the cell type under study. According to Mie theory [17], forward scatter (FSC) is relative to the size of the cell passing through the laser beam, with relative particle size being the ratio of the circumference of the particle to the wavelength of incident light [20,21]. This measure of size is shown to be dependent on pulse width (duration to pass through the laser beam) as demonstrated with dextran beads [22], and is expected to be less affected by cell shape and refractive index than other measures [23]. The amount to which light is scattered at greater (90°) angel (side scatter SSC), may also be related to the size of the cell as it correlates with total protein content and dry weight [18] in some species [24].

However, the assumption of a tight correlation between scatter and cell size may not be simple, as most models are based on idealised cells [22,25] and scatter profiles result from complex interplay between-and-within cell refractive index variations as well as light absorbing cell components [26], which are expected to show species differences and cell-type variations. It is also possible that cell shape and appendages may affect the cell's signal. It has been argued that relative scatter parameters may therefore be combined with quantitative fluorescent signals to increase the accuracy of selection [27]. Indeed, parameters related to cell size are assumed to provide such information based on different cellular characteristics that scale with size [17]. Combining multiple parameters may minimise non-specific selection, increase the stringency of selections by using multiple measures which correlate with size, and may minimise collection of false measures on a single parameter.

Flow cytometry is already widely applied in algal biology [15]. For example in identifying [28] as well as quantifying the relative abundance of taxonomic groups of algae and mixed species samples using scatter and autofluorescence [29–32], describing community dynamics [33], and determining lipid content for biofuel production [34–38]. Previous approaches have demonstrated the suitability of *Chlamydomonas* for flow cytometric study, and document the presence of autofluorescent compounds such as Chlorophylls A and B and nicotinamide adenine dinucleotide (NAD) which fluoresce highly in the red, far-red and infrared spectra [39]. Furthermore flow cytometry is becoming a key method for investigating biofuel production in *Chlamydomonas* [40], and there is a developing pool of literature demonstrating the usefulness of cell sorting for *Chlamydomonas* research, especially in biofuel enrichment [38,41]. Literature also shows the utility of cell-size based selection to synchronise cell cycles in mammalian cell lines [42], while a further emerging application of flow cytometry is in monitoring the progress of experimental evolution [36]. The method is gaining increasing attention as a method for experimental evolution, establishing selection criteria and methods [43], in contrast to the role of monitoring selection experiments which has been used in the past.

In this paper we applied flow cytometric methods to the cell sorting on gamete populations of *C. reinhardtii*. Such a method would have applications for studying the process of gametogenesis, the relationship to the environment during gametogenesis, as well as applying experimental evolution methods to the evolution of gamete size in this as well as other species.

2. Materials and methods

2.1. Strains and culture conditions

C. reinhardtii wild type strain (CC1690) was obtained from the *Chlamydomonas* Resource Centre (University of Minnesota). Cells were maintained for long term storage on Tris-Acetate-Phosphate (TAP) [44] agar plates supplemented with yeast extract. TAP Media was inoculated with cells taken directly from agar plates. Liquid and agar cultures were maintained at 22 ± 1 °C [45] with a 12:12 light/dark diurnal photoregime under 'cool white' fluorescent bulbs. Liquid cultures were agitated using an orbital shaker at 120 rpm. Three replicate pre-growth cultures of strain CC-1690 were grown vegetatively for 3 light/dark cycles. To produce populations of gametes, cells were diluted to 250,000 cells/ml and grown vegetatively to a concentration of ~1×10^6 cells/ml, then cultures were centrifuged at 3000*g* for 2 min and resuspended in dd$H_2$0. Gametes will form 24 h after suspension in N free water [46], hence after maintaining the resuspended cells in constant light for 24 h. This population was measured to be at a concentration of 2.5–3.5×10^6 cells/ml, and was used in subsequent experiments. Samples were passed through a 45 μm filter. All cell concentration measures were calculated using a haemocytometer counting chamber using Lugols solution to fix the sample as described previously [47].

2.2. Flow cytometer and cell sorter

Cultures of *C. reinhardtii* gametes were analysed and sorted using a BDInflux cell sorter (BD, USA). Spectral properties of gamete autofluorescence were examined by exciting cells with 355 nm, 405 nm, 488 nm, 561 nm, and 642 nm lasers and emission was measured in all 18 available parameters, ranging in wavelength spectrum from 435 nm to >750 nm. Cells were sorted at a sheath pressure of 30 psi, using an 86 μm nozzle, at a sample pressure of 30.2 psi, with a 1 drop purity mask. Forward scatter (FSC) and side scatter (SSC) measures of polystyrene beads (SpheroTech, USA) showed that modal intensities of the peaks of all distributions increased monotonically with bead diameter, as in [17]. Following single cell discrimination (based on FCS-A and FCS-H signal), ~ 15% low (small cells), and 15% high distribution

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