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Received: 25 January 2021 | Accepted: 16 February 2021

DOI: 10.1002/cyto.a.24328

ORIGINAL ARTICLE

MIFlowCyt

ISAC CYTOMETRY Journal of Quantitative Cell Science PART A

Best practices in the flow cytometry of microalgae

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Abstract

Microalgae are photosynthetic microorganisms with a major influence on global ecosystems. Further, owing to the production of various secondary metabolites, microalgae are also intensively studied for their enormous potential in biotechnology and its applications. While flow cytometry (FCM) is a fast and reliable method particularly suitable for genome size estimation in plant and animal studies, its application to microalgae often comes with many methodological challenges due to specific issues (e.g., cell wall composition, and presence of various secondary metabolites). Sample preparation requires considerable amounts of biomass, chemical fixation, and/or extraction of cellular components. In genome size estimation, appropriate methods for isolation of intact nuclei (using lysis buffers, razor-blade chopping, various enzymes, or bead-beating of cells) are essential for successful and high-quality analyses. Nuclear DNA amounts of microalgae diverge greatly, varying by almost 30,000-fold (0.01 to 286 pg). Even though new algal reference standards for genome size are now being introduced, animal red blood cells and nuclei from plant tissues are still predominantly used. Due to our limited knowledge of microalgal life cycles, particular caution should be taken during 1C/2C-value (or ploidy level) assignments.

KEYWORDS

best practices, microalgae, flow cytometry, nuclear isolation, genome size, algal FCM standards

1 | INTRODUCTION

Microalgae are an extremely diverse group of organisms, individual species of which are placed within different domains across the tree of life. These photosynthetic microorganisms occupying a wide range of habitats, from freshwater lakes to desert soils, also play key roles in the functioning of the global ecosystem. Because of their polyphyletic origin, microalgae differ greatly in their morphology, cell wall composition, protoplast content, and/or presence of specific organelles. Analyzing microalgae using flow cytometry (FCM) is, in general, methodologically more challenging and time-consuming as compared to the analysis of plant or animal tissues. This is particularly due to difficulties in obtaining sufficient amounts of biomass, and in protoplast extraction (corresponding to widespread cell wall heterogeneity and variation in the complexity of wall components), and due to the presence of a wide variety of pigments and secondary metabolites that can interfere with fluorescent staining [1–4]. As for other plant and animal species, FCM enables counting, sorting, and/or examination of different features or physiological states of microalgae on the basis of quantification of scattered light signals and emitted fluorescence. Here is provided a general FCM protocol and workflow for the analysis of microalgal samples. However, one should always keep in mind the enormous diversity among microalgae and the specific features of particular groups that may often require modifications of this protocol.

1.1 | Obtaining biomass

The first step in FCM analysis of microalgal samples is obtaining sufficient amounts of biomass. While this task is relatively straightforward with macroalgae consisting of multicellular thalli which can be sampled in the field, analysis of microalgae usually requires further

cultivation steps. In the former case, collected thalli are cleaned from epiphytes or extraneous debris and rinsed in distilled water [5, 6]. However, in some macroalgal groups, where the FCM analysis of thalli may be problematic, the use of unicellular life stages (e.g., zoids or spores) provides an alternative [5, 7]. In that case, cells are processed in the same manner as microalgal samples. When working with microscopic algae, we first need to establish a unialgal, clonal (and, if possible, axenic) culture. For heterotrophic microalgae, prey must be often added to the culture (e.g., some dinoflagellates or cryptophytes), and later it may be difficult to differentiate prey nuclei from those of the microalgal taxa. This is probably the reason to why heterotrophic microalgae have been largely avoided as a subject of FCM (but see Reference [8]). Similarly challenging is the task of separating the nuclei of a studied sample from its symbionts. To avoid this problem, it is sometimes possible to switch to specific life-cycle stages, for instance to zoospores [9, 10]. In order to increase the proportion of biological materials in optimal condition, the culture is inoculated into fresh medium and placed under higher levels of illumination (e.g., 40 μmol m^{-2} s^{-1} or higher) a few weeks before the planned FCM analysis (usually 2 weeks, depending on the growth rate of studied algae). If possible, the use of material from different subcultures treated independently is preferable for repeated measurements. The optimal harvesting time is during the (mid-)exponential phase of culture growth. Furthermore, cells in young, exponentially-growing cultures may not have fully developed cell walls, which makes them more suitable for protoplast isolation based on enzymatic digestion (see below). This is especially crucial when working with desmids, where only young cells without fully developed cell walls are suitable for the use of enzymatic digestion [4, 11]. Although the culture should have a high cell density, even more, important is to aim to analyze cultures that are as young as possible, due to the potential accumulation of secondary metabolites (or even genomic changes in culture, see below) during long-term cultivation. A different approach is taken when the amount of storage compounds is the target of a study (e.g., oils or starch). In this case, microalgae are usually harvested during the stationary phase of culture growth [12, 13].

The microalgal biomass can be harvested either using a cell scraper from agar-based cultivation media or by centrifugation of liquid cultures (about 1–100 mL of culture, depending on cell density). After removing the supernatant, the pelleted cells should be visible to the naked eye, but the cell concentration should then be precisely determined by counting. The pellet should contain at least 10^5 cells for successful analysis [9, 14–16]. When working with mucilaginous species, ultrasonication for several minutes before sample preparation may help the subsequent release of individual cells from pellets.

1.2 | Chemical fixation

Since the first flow cytometric studies on microalgae, samples have been processed by analyzing the entire cells [1,2,15,17-19]. However, such analysis may well be affected by prominent cytoplasmic and cell wall autofluorescence due to the presence of high levels of pigments and other classes of molecules. Sometimes the autofluorescence spectrum of cells can overlap the spectra of the fluorochromes used for DNA content measurement. To help prevent unwanted autofluorescence, chemical fixation can be employed. Various fixation protocols have been employed by different authors, with none apparently prevailing [2, 15, 17, 18, 20–24]. Cell pellets are incubated in fixative, typically ethanol, methanol, methanol: acetic acid (3: 1) mixture, formaldehyde, or paraformaldehyde, with incubation times ranging from tens of minutes up to 48 h. Following centrifugation, this fixation step can be repeated up to three times and, if so, the sample is kept on ice between the washing steps. Optimal fixative concentration and incubation times need to be defined experimentally for each studied species. Glutaraldehyde fixation has also been tested as a pigment-removing fixative. However, it resulted in high background fluorescence and interfered with fluorochrome staining [2, 9, 25, 26]. Following fixation, the sample is washed in phosphate-buffered saline (PBS), methanol, or TE buffer [15, 18, 20, 23, 24, 27]. The PBS purification was also used to separate dinoflagellate *Oxyrrhis marina* from nuclei of its prey (although this approach was successful only for unfixed cells [8]). It is worth mentioning that in plant FCM studies, chemical fixation is not recommended for absolute DNA estimation [28] and the same might apply for microalgae.

1.3 | Extraction of cellular components

The most common application of FCM is to detect fluorescence from stained nuclei. Despite the fixation effort, FCM analysis of entire cells may result in prominent background fluorescence and/or incompletely-stained nuclei, causing high CVs of the studied samples, or even preventing successful analysis. Hence, for total genome size estimation of microalgal samples, only the analysis performed on properly extracted nuclei is accurate enough to allow high precision of measurement. In samples without a cell wall (e.g. zoids of Ectocarpales or chrysophytes), nuclei can be extracted simply by adding lysis buffer, sometimes combined with incubation under higher temperature (e.g., 50°C for 5–10 min; [7, 16, 29]). Alternatively, bead-beating of cells in a mixer mill can be employed to isolate nuclei. This method was particularly successful in the raphydophyte *Gonyostomum semen* [30]. In many cases, however, enzymatic treatment to disrupt cell walls needs to be implemented before protoplast content extraction [4, 11, 31]. It should be noted that enzymatic treatments are time-consuming and often require optimization specific to the studied group of algae. Enzymatic cell-wall disruption in microalgae is based on protocols adopted from plant or fungal studies [32, 33]. The predominantly used enzymes are cellulase, macerozyme or lyticase, sometimes dissolved in a rinsing solution of PGly (composition: 27.2 mg·l $^{-1}$ KH_2PO_4, 101 mg l^{-1} KNO_3, 1117.6 mg·l^{-1} $CaCl_2$, 246 mg·l^{-1} $MgSO_4 \cdot 7H_2O$, 11.5 g·l^{-1} glycine, 18.016 g·l^{-1} glucose, 0.58572 g·l^{-1} MES and 65.58 g·l^{-1} mannitol; [4, 11, 31]). The enzymatic mixture dissolved in rinsing solution was primarily developed for streptophyte algae, but also worked for some Chlorophyta (*Chloroidium ellipsoideum*, *Tetraselmis subcordiformis*) and Ochrophyta (*Tribonema vulgare*). Nonetheless, the enzymatic mixture may not

digest the cell wall completely, and, for example, only young cells of desmids with partially dissolved cell walls were suitable for FCM analysis. Sometimes the enzymatic treatment needs to be complemented by chopping algal biomass using a razor blade (e.g., in case of *Zygnema* spp.; [4]). It is also a common practice to check the successful enzymatic cell-wall degradation under a microscope. Grinding algal biomass in a mortar for nuclear isolation has also been tested, however without success [4].

1.4 | Isolation buffers

In studies on microalgae, commonly-used buffers are LB01 (with streptophytes, Chlorophyta, Ochrophyta, raphidophytes; [4, 11, 30]), a MOPS-based buffer (3-(N-morpholino) propanesulfonic acid (used with dinoflagellates; [21, 24]), or Otto buffers (with chrysophytes; [16, 29]). Triton X-100 (to a final concentration of 0.05%–1%) may be added to improve the sample staining, though its effect varies across different groups of algae [2, 7, 14, 19, 34]. Phenols, tannins, and other secondary metabolites are commonly present in microalgae and may act as staining inhibitors or lower the quality of the FCM analysis. Their adverse effect can be lowered, to some extent, by adding PVP (polyvinylpyrrolidone) and/or mercaptoethanol to the lysis buffer [35].

1.5 | Standardization

For precise total DNA content estimation, it is essential to include a FCM standard. The use of internal DNA standards is highly recommended for microalgal samples, considering the frequent presence of secondary metabolites with the potential to interfere with FCM analysis. An appropriate internal standard is closely related to the studied organism with similar but not overlapping genome size. Unfortunately, due to the lack of a broad range of algal DNA standards, animal red blood cells and plant tissues are still predominantly used for this purpose [2, 4, 9, 15–17, 21]. However, the nuclear DNA within the most commonly used standard, chicken red blood cells, has a high packing density, and red blood cells from male and female chickens differ in genome size by 2.7% due to the contributions of sex chromosomes; these factors likely contribute to non-systematic errors in DNA content estimates [36, 37]. Although the number of available algal standards is now rising, it is still a negligible number in contrast to algal diversity. Examples of standards include the green microalga *Chlamydomonas reinhardtii* CC-400 cw15 mt+, which, being a cell-wall deficient mutant, is therefore easy to use (2C = 0.24 pg; available at Chlamydomonas Resource Center, University of Minnesota; [14,34]). The chrysophyte *Synura sphagnicola* CCAC 2959 B (2C = 0.4 pg; otherwise designated LO234K-E, and available at The Central Collection of Algal Cultures [CCAC]) was recently introduced as an internal standard by Olefeld et al. [16]. The desmid *Micrasterias pinnatifida* SVCK 411 (2C = 3.4 pg; available at the Microalgae and Zygnematophyceae Collection Hamburg) was established as a streptophyte standard by Mazalová et al. [4]. It should be noted that this standard requires enzymatic treatment that degrades cell wall structure and thus enables the release of nuclei (see above; [4, 11]). While the cultivated microalgal lineages can serve as reliable references for specific genome size classes, their limited numbers can be compensated by field-collected standards. Examples include the use of the red alga *Chondrus crispus* (2C = 0.33 pg) as an internal standard [6, 7]. Also, the number of microalgae with available complete genome sequences is increasing and these offer promise as new FCM standards. In von Dassow et al. [18], the authors employed the diatom *Thalassiosira pseudonana* strain CCMP1335 as an internal standard (2C = 0.07 pg; available at Provasoli-Guillard National Center for Marine Algae and Microbiota). However, the use of diatoms as genome size standards may be problematic due to considerable genome flexibility during cultivation, as documented in the referenced study (see below). Even though the internal standard should be optimally treated in an identical way to the experimental microalgal sample, this is nearly impossible in most studies. As a common practice, the standard nuclei are extracted separately and are later mixed with the microalgal sample (i.e., pseudo-internal standardization [9, 16, 21, 24]).

1.6 | Fluorescent staining

The use of different fluorescent stains allows the detection of a number of different content amounts and enzymatic activities of microalgal cells. Even without the addition of any stain, it is possible to determine chlorophyll content in the sample due to its autofluorescence [38]. To assess cellulose content, Calcofluor White can be used due to its ability to bind cellulose and emit blue fluorescence following UV excitation [39]. Staining lipid globules with Nile Red fluorescent stain (9-diethylamino-5-benzo[a]phenoxazinone; [5]) allows FCM estimation of the cell lipid content. Intracellular peroxidase and reactive oxygen species (ROS) can be detected using hydroethidine or DCFH-DA (2′,7′-dichlorodihydrofluorescein diacetate acetyl ester). The presence of intracellular peroxidase and ROS leads to the conversion of hydroethidine to ethidium, which is accompanied by a change of emitted fluorescence (40). Similarly, non-fluorescent DCFH-DA is oxidized by either intracellular peroxidase or ROS to form fluorescent DCF (7′-dichlorofluorescein; 40). FCM can also be used to detect changes in cellular (or mitochondrial) membrane potential. Positively charged lipophilic stains, such as $DiOC_6$ (3,3′-dihexyloxacarbocyanineine) and rhodamine 123, can penetrate through organelle membranes to reach their negatively charged interiors. When an equilibrium concentration is reached, membrane depolarization or hyperpolarization causes release or uptake of the fluorescent stain, respectively [19,41]. However, by far the widest application of FCM in microalgal studies is the detection of fluorescence-stained nuclei. Analysis of protoplasts following staining with a membrane-impermeable fluorochrome (e.g., propidium iodide [PI]) allows determination of cell viability in terms of plasma membrane integrity [42]. Other applications are directed at the analysis of DNA base composition (GC content) and genome size characteristics, allowing, for example, detection of different life-cycle stages within

populations, and intraspecific ploidy level diversity or cell-cycle stages in microalgal cultures [14,17,19,27,38,43-46]. It is also possible to distinguish taxa and identify cryptic species on the basis of differences in genome size [22,47]. When estimating genome size in microalgae using PI, caution should be taken as the emission spectrum of PI fluorescence can overlap with the autofluorescence of photosynthetic pigments, PI can bind polysaccharides from the remaining cell walls and thus contribute to background noise and increase of CV [34], and its ability to fluoresce in the presence of double-stranded RNA must be considered and, if necessary, eliminated by including RNAse in the staining protocol.

Following the fluorescence staining but before FCM analysis, the microalgal samples are filtered (the mesh size 5–150 μm, depending on the particular application) to prevent clogging of the fluidic system of a flow cytometer.

1.7 | Special considerations for estimating genome size in microalgae

Based on the currently limited knowledge, the DNA content of microalgae varies 28,600-fold, from 0.01 detected in *Nannochloropsis* sp. (Eustigmatophyceae) to 286 pg in *Valonia* sp. (Ulvophyceae; [2,48]). When evaluating the outcomes of FCM analysis, authors should be very cautious in their interpretations, in light of the general lack of data on genome size variation in many groups of microalgae, a dearth of information about life cycles, as well as the possibility of rapid genome size evolution across species and within genera.

In case of two peaks being observed in a FCM uniparametric histogram of a microalgal sample, the first peak (1C) is usually considered to represent G_1-phase cells and the second (2C) belonging to G_2 cells (Figure 1; [4, 11, 16]). Sometimes, however, only a single sample peak is observed. In this case, it is critical to determine whether the sample is in the G_1 phase with no dividing cells (e.g., extremely slowly growing cultures), or whether it represents extremely synchronized cells in the G_2 phase before mitosis. In phytoplankton species, the timing of cell division may strongly depend on the time at which cell biomass is harvested and processed [45,46]. For instance, in diatoms and dinoflagellates, a peak representing the G_2 nuclei can be either prominent or completely missing [9,15,47]. On the other hand, in a highly synchronized culture of the genus *Chlamydomonas*, the G_1 peak was always present and never represented less than 29% of analyzed nuclei [14].

The G_1 sample peak is commonly referred as either *n* or 2*n* stage, usually without the knowledge of a particular life-cycle stage of the analyzed sample. Unfortunately, our understanding of ploidy levels and reproduction strategies in the majority of microalgae is extremely limited. Thus, vegetative cells (G_1 phase) may be dominantly haploid (e.g., in the majority of dinoflagellates, some desmids, and presumably in chrysophytes) or diploid (e.g., in diatoms or raphidophytes; [16,18,45,47,48,50-53]). Many species of microalgae are capable of vegetative growth in both sexual and asexual stages (i.e., the biphasic

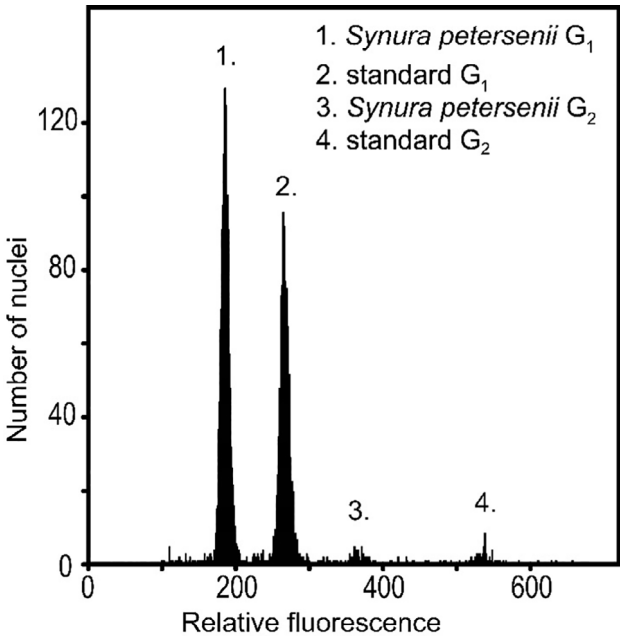

FIGURE 1 Flow cytometric histogram showing relative fluorescence of propidium iodide-stained nuclei of chrysophyte *Synura petersenii* and *Solanum pseudocapsicum* (reference standard; [49]) with G_1 and G_2 phase nuclei apparent for both analyzed sample and standard. In this case, the CV values are 2.97% and 2.31%

life cycle) and the sexual and asexual phases can be morphologically indistinguishable (as in the case of sea lettuce *Ulva* spp.; 54). In addition, numerous species or strains across microalgae are putative polyploids, which further complicates ploidy level assignments. Therefore, in case of any ambiguity, the DNA content of microalgal samples should be referred in pg·cell^{-1} rather than attempting to assign it to a specific 1C/2C-value (or ploidy level).

Interestingly, genome size may differ greatly between closely related species or even between strains of the same species. Major intraspecific variation, reaching up to sevenfold differences, has been described in desmids (*Micrasterias rotata*, *Triploceras gracile*), haptophytes (*Emiliania huxleyi*), chrysophytes (*Synura* petersenii), and diatom species (*Thalassiosira punctigera*, *T. weissflogii*; [2,4,11,29,55,56]). Astonishingly, genome size changes within the same strain kept in cultivation were also documented. The DNA content estimates for diatom *Thalassiosira weissflogii* CCMP 1049 differed tremendously in comparisons of three independently-conducted studies (from 0.95 to 17.25 pg; [2, 15, 18]). Von Dassow et al. ([18]) have even reported genome size diversification of three *T. weissflogii* sub-cultures over a few years of cultivation (strains BILB2001, CCMP 1336, and CCMP 1587). A series of whole-genome duplications (polyploidy events) are the likely drivers of these genome size shifts, as previously proposed in studies on streptophytes [57,58] and dinoflagellates [59,60]. Further, the cultured microalgae may delete or amplify specific genomic regions depending on their current environmental conditions [18]. Other possible sources of genome size variation in culture include aneuploidy or meiosis introducing DNA amount variation [61]. Therefore, it is highly recommended to avoid long-term cultivation, and analyze the samples as soon as possible after isolation. On the

other hand, strains of the dinoflagellate genus *Symbiodinium* analyzed multiple times after a varying length of cultivation, exhibited a highly similar genome size suggesting its stability during the cultivation [21].

Due to the often challenging preparation of microalgal FCM samples and the frequently high contents of secondary metabolites interfering with the analysis, it is unusual to obtain measurements having CV values at or below 3%, although CVs are typically below 10% [9, 21, 34].

2 | GENERAL RECOMMENDATIONS

Aside from following the general best practices in FCM as indicated in other chapters of this series, recommendations specific for microalgal studies are worth highlighting:

- Ensure a sufficient amount of input biomass by optimized cultivation (high cell abundance, absence of contamination by other organisms).
- Try to avoid analyses of strains subjected to long-term cultivation that might result in genomic or other changes.
- For DNA amount measurements, use as young cultures as possible to avoid the accumulation of secondary metabolites and pigments. Also, isolate nuclei rather than attempt to analyze whole intact cells.
- In case of unsuccessful analysis, test different isolation buffers and protoplast extraction techniques (varying the intensity of razor-blade chopping or bead-beating of cells, or enzymatic treatments, as appropriate).
- In all cases, to the extent possible, and before FCM analyses, validate input cell populations in terms of number, purity, viability, cellular (or subcellular i.e., nuclear) integrity, and homogeneity of fluorescence staining, using light and fluorescence microscopy. Ensure the results of FCM analyses are consistent with these observations.
- When estimating genome size, use internal or pseudo-internal standardization, as high cellular contents of secondary metabolites or pigments may lead to potential shifts in relative fluorescence.
- In case of any uncertainty regarding the life cycle stage of the analyzed microalgal samples, the report in publications genome size estimates in absolute units (e.g., pg·cell^{-1}) rather than attempt to assign it to 1C/2C-value.

CONFLICT OF INTERESTS

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

Dora Čertnerová: Conceptualization; writing-original draft. **David Galbraith:** Supervision; writing-review & editing.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

How to cite this article: Čertnerová D, Galbraith DW. Best practices in the flow cytometry of microalgae. *Cytometry*. 2021;99:359–364. https://doi.org/10.1002/cyto.a.24328