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Nuclei isolation protocols for flow cytometry allowing nuclear DNA content estimation in problematic microalgal groups

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Abstract

Microalgae are fundamentally important organisms for global ecosystem functioning with high potential in biotechnology and its applications. The knowledge of their nuclear DNA content has become a prerequisite for many areas of microalgal research. Due to common presence of various pigments, secondary metabolites and complex cell walls, the nuclear DNA content estimation using flow cytometry (FCM) is, however, often laborious or even impossible with the currently used protocols. In this study the performance of six nuclei isolation protocols was compared on various problematic microalgae using FCM. The nuclei isolation methods involved osmotic bursting of cells, razor blade chopping of fresh biomass and two newly introduced protocols, razor blade chopping of desiccated biomass and bead beating. These techniques also involved the use of two different nuclei isolation solutions, Otto I + II solutions, and LB01 buffer. Performance of the particular protocols differed greatly, depending on the used nuclei isolation solution and microalgal group. The most successful method was a newly adopted chopping of desiccated biomass in LB01 buffer. This method seems more appropriate for nuclei isolation in filamentous microalgae; on the other hand, bead beating appears to be more suitable for nuclei isolation in solitarily living algae. Using the optimal protocol for a given species, their nuclear DNA content was estimated, resulting in first DNA content estimates for four investigated taxa (*Chlamydomonas noctigama*, *Gonyostomum semen*, *Microglena* sp. and *Stigeoclonium* sp.). The estimated DNA content spanned from 0.15 to 32.52 pg.

Keywords Nuclei isolation · Flow cytometry · Microalgae · Silica gel desiccation · Bead beating · Nuclear DNA content

Introduction

Microalgae are photosynthetic microorganisms that occur across a wide range of habitats from freshwater lakes to desert soils. Due to their polyphyletic origin across the tree of life, they are a remarkably diverse group of organisms. Moreover, microalgae play a key role in the global ecosystem as primary producers and major source of oxygen. Recently, considerable attention has been paid to microalgae as the potential source of next generation biofuels or usable metabolites (Brennan and Owende 2010; Hyka et al. 2013; Milano et al. 2016; Khan et al. 2018). This has led to a need for microalgal DNA content data due to a number of reasons. First, this knowledge enables us to select lineages with potentially higher secondary metabolite production given that an increase in DNA content is often coupled with an increase in gene dosage (e.g. due to aneuploidisation or polyploidisation; Mason 2016; Priyadarshan 2019; Qin et al. 2019). Second, the recent attention drawn to microalgae accelerated the whole-genome sequencing effort, and the DNA amount is the key to designing an optimal sequencing strategy. Further, the nuclear DNA content directly influences the cost of a sequencing project; hence, the low DNA content has become a major criterion in selection of appropriate algal strains (Waaland et al. 2004; Peters et al. 2004; Lin 2006). The combination of DNA content knowledge and high-level phylogeny also opens the ways to determine evolutionary trends in DNA content variation. Such innovative studies brought new insights into microalgal nutrition modes or cell-size changes (Poulíčková et al. 2014; Olefeld et al. 2018). Further, the nuclear DNA content, at least in relative units, is essential for cell cycle determination (Lemaire et al. 1999; Reinecke et al. 2018).

The most suitable method for precise and rapid nuclear DNA content estimation is flow cytometry (FCM). Using FCM, we are able to detect fluorescent-stained particles (e.g. cells, isolated nuclei) in a stream of fluid (Doležel et al. 2007).

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While FCM has found a broad spectrum of applications in genomic surveys on plants and animals (e.g. Dionisio Pires et al. 2004; Kron et al. 2007; Galbraith 2012; Chang et al. 2018; Sadílek et al. 2019), it has been only rarely applied in algal studies (but see Figueroa et al. 2010; Hyka et al. 2013).

There are several reasons causing the gap of nuclear DNA content estimates in microalgae. First, it is almost always necessary to cultivate microalgal strains from a single cell/filament to obtain sufficient amounts of biomass for FCM analysis. However, this is very time-consuming and for some species even hard to accomplish. Because of the great diversity of microalgae, there is also a wide range of pigments and metabolites that frequently interfere with fluorescent stain and/or create pronounced background noise, prominent especially when whole intact cells are analysed (Simon et al. 1994; Veldhuis et al. 1997; Mazalová et al. 2011). Although the pronounced cytoplasmic autofluorescence as well as nonspecific background fluorescence can be lowered by chemical fixation, such approach is far from optimal due to reduced quality of FCM analyses. Instead, protoplast extraction and preparation of nuclear suspension are much more suitable (Doležel and Bartoš 2005). To achieve this, several methods of cell wall disruption can be implemented in a sample preparation protocol for FCM analysis. However, currently used protocols for microalgae often do not work for FCM. Commonly used nuclei isolation method is chopping the biomass by a razor blade combined with various enzymatic treatments (Mazalová et al. 2011; Weiss et al. 2011; Poulíčková et al. 2014). In many cases the enzymatic treatment was applied to chemically dissolve the cell walls without the need for any further mechanical disruption (Mazalová et al. 2011; Poulíčková et al. 2014). The application of enzymatic treatment on algal samples was originally adopted from plant or fungal studies (Jazwinski 1990; Doležel et al. 2007) and the predominantly used enzymes for microalgal species are cellulase, macerozyme and lyticase (Mazalová et al. 2011; Weiss et al. 2011; Poulíčková et al. 2014). In Mazalová et al. (2011) the authors introduced an enzymatic treatment that was subsequently tested on a broad variety of microalgal species. The enzymatic mixture was primarily developed for streptophyte algae (e.g. the genus *Zygnema*), but also worked with some Chlorophyta (*Chloroidium ellipsoideum*, *Tetraselmis subcordiformis*) and Ochrophyta (*Tribonema vulgare*). Despite this, the introduced protocol did not work for nearly half of the tested microalgae, among others, for the green algae *Trentepohlia* sp. or *Chlamydomonas noctigama* (referred there as *C. geitleri*).

Unfortunately, the utilization of enzymatic treatment is methodologically demanding as well as time-consuming. Moreover, due to the great algal diversity, enzymatic treatment often requires additional modifications for specific algal groups (Mazalová et al. 2011; Weiss et al. 2011; Potter et al. 2016). However, use of the enzymatic treatment predominates as a protoplast isolation technique in microalgal studies despite these disadvantages. To resolve the situation, new methods of nuclei isolation for FCM analysis need to be established for microalgae. For example, the most common way of nuclei isolation in plants or seaweeds is simple chopping tissue using a razor blade (Galbraith et al. 1983; Asensi et al. 2001; Doležel et al. 2007). Further, beat beating by zirconium or silica beads has been previously used to isolate nuclei of bacteria (Gryp et al. 2020), fungi (Griffin et al. 2002), plants (Roberts 2007) and animals (Harmon et al. 2006). Interestingly, despite its easy and rapid use, neither bead beating nor chopping by a razor blade alone was ever successfully applied to isolate microalgal nuclei for FCM.

The aim of this study is to develop new protocols of microalgal nuclei isolation and test them on a diverse set of species that were referred as problematic in the past (Mazalová et al. 2011; personal observation).

Materials and methods

Origin, cultivation and harvesting of investigated strains

Monoclonal cultures used in this study were obtained from Culture Collection of Algae of Charles University in Prague (CAUP), Culture Collection of Cryophilic Algae (CCCryo), Norwegian Culture Collection of Algae (NORCCA) and from collaborators (Table 1). The algal taxa chosen for this study were selected based on the previous difficulties with their nuclei extraction and/or FCM analysis (Mazalová et al. 2011; author's personal observation in pilot FCM analyses). A special focus is paid to *Zygnema* strains as this genus is the model organism in recent studies in our working group (e.g. Pichrtová et al. 2018; Trumhová et al. 2019).

The strains were cultivated either in 50 mm Petri dishes filled with Bold's Basal medium (BBM; Bischoff and Bold 1963) solidified with 1.5% agar or in 50-mL Erlenmeyer flasks filled with liquid BBM or modified WC medium (MWC; Guillard and Lorenzen 1972). The majority of cultures were maintained at 17 °C with constant light conditions under the illumination of 30–50 μmol photons m^{-2} s^{-1}. The *Chlamydomonas noctigama* and *Microglena* sp. strains were cultivated at 23 °C with 14 h light and 10 h dark conditions under the illumination of 100 μmol photons m^{-2} s^{-1}. Origin details and cultivation media for particular algal strains are listed in Table 1. The cultures were transferred into a fresh medium 2 to 5 weeks before the planned FCM analyses and their biomass growth regularly checked. Afterwards, the culture biomass was harvested in their exponential phase of growth. Approximately 15-30 mg bulk of biomass were collected from cultures growing on solidified medium (BBM-agar) using an inoculation needle with a bent tip. Similarly,

Table 1 Original collection site and cultivation media for the investigated algal strains

Class	Species	Strain	Original collection site	Cultivation medium
Zygnematophyceae	*Spirogyra* sp.	CAUP K902	Pond near Winterthur, Switzerland	BBM-agar
Zygnematophyceae	*Zygnema* sp.	13 179-4	Near Pyramiden, Svalbard	BBM-agar
Zygnematophyceae	*Zygnema* sp.	15 Osor 2	Puddle, near Osor, Croatia	BBM-agar
Zygnematophyceae	*Zygnema* sp.	CCCryo 171-04	Mountain creek, Poatina, Tasmania, Australia	BBM-agar
Chlorophyceae	*Chlamydomonas noctigama*	CAUP G224 (SAG 6.73/UTEX 2289)	Hvězda pond, Northern Moravia, Czech Republic	BBM
Chlorophyceae	*Microglena* sp.	Fio 17	Lake Fiolen, Småland, Sweden	BBM
Chlorophyceae	*Stigeoclonium* sp.	CAUP J603	Žebrákovský creek, river basin of Sázava, Czech-Moravian Highlands, Czech Republic	BBM-agar
Raphidophyceae	*Gonyostomum semen*	NIVA-2/10 (BO-182)	Lake Bökesjön, Scandia, Sweden	Modified WC
Ulvophyceae	*Trentepohlia* sp.	CAUP J1601	Bark, Singapore	BBM-agar
Xanthophyceae	*Tribonema vulgare*	CAUP D501	Palach Pond near Lednice, Czech Republic	BBM-agar

2 mL of strains cultivated in liquid medium (BBM or MWC) were transferred into an Eppendorf tube, centrifuged (5500 rpm for 5 min) and superfluous medium removed by pipetting.

Nuclei isolation and staining

In total, six nuclei isolation protocols were subsequently tested on the studied algal strains. In each protocol, either LB01 buffer (15 mM Tris, 2 mM Na_2EDTA, 0.5 mM spermine tetrahydrochloride, 80 mM KCl, 20 mM NaCl, 0.1 % (v/v) Triton X-100; pH = 8.0; Doležel et al. 1989) or a two-step Otto protocol (Otto I solution consisting of 0.1 M citric acid, 0.5% Tween 20 with pH = 2.0–3.0 and Otto II solution consisting of 0.4 M $Na_2HPO_4{\cdot}12H_2O$ with pH = 8.0–9.0; Otto 1990) was used.

Protocol 1

Single-celled algal strains (*C. noctigama*, *Microglena* sp. and *Gonyostomum semen*) were prepared for the FCM analysis without any protoplast extraction, i.e. whole cells of each strain were mixed with 550 μL of ice-cold LB01 lysis buffer or Otto I solution, to attempt a release of nuclei by osmotic bursting of cells. The suspension was thoroughly mixed and filtered through a 42-μm nylon mesh into a special 3.5-mL cuvette for direct use with the flow cytometer. Following a 20 min incubation[1] at room temperature, staining solution consisting of either 1 mL Otto II solution or 550 μL LB01 lysis buffer, of 50 μg mL^{-1} propidium iodide, of 50 μg mL^{-1} RNase IIA and of 2 μ mL^{-1} β-mercaptoethanol was added to the sample.

[1] If visible sediment was present after 20 min incubation, an upper layer of nuclei suspension was transferred into a new cuvette and used as a material for analysis.

Protocol 2

Harvested biomass was transferred to a plastic Petri dish and chopped by a razor blade in 550 μL of ice-cold Otto I solution. The resulting suspension was thoroughly mixed and filtered through a 42 μm nylon mesh into a special 3.5-mL cuvette for direct use with the flow cytometer. Following a 20-min incubation[1] at room temperature, staining solution consisting of 1 mL of Otto II solution, of 50 μg mL^{-1} propidium iodide, of 50 μg mL^{-1} RNase IIA and of 2 μL mL^{-1} β-mercaptoethanol was added to the sample.

Protocol 3

Harvested biomass was transferred into a plastic Petri dish and chopped by a razor blade in 550 μL of ice-cold lysis buffer LB01. The resulting suspension was thoroughly mixed and filtered through a 42 μm nylon mesh into a special 3.5-mL cuvette for direct use with the flow cytometer. Following a 20 min incubation[1] at room temperature, staining solution consisting of 550 μL of LB01 lysis buffer, of 50 μg mL^{-1} propidium iodide, of 50 μg mL^{-1} RNase IIA and of 2 μL mL^{-1} β-mercaptoethanol was added to the sample.

Protocol 4

Harvested biomass was desiccated by transferring into 2-mL Eppendorf tube and placed with an open lid into a zip-lock bag filled with silica gel for 2 to 5 days. The dry algal biomass was then transferred in a plastic Petri dish and chopped by a razor blade in 550 μL of ice-cold lysis buffer LB01. The sample preparation was further completed according to Protocol no. 3.

Protocol 5

Approximately 10 glass beads of 1.5-mm diameter (Sigma-Aldrich) were added into 2-mL Eppendorf tube containing 550 μL of ice-cold Otto I solution and a biomass pellet. The cells were disrupted for 3 min at 25 Hz using Retsch MM200 mixer mill (Retsch, Inc., Germany). The nuclei suspension was then filtered through a 42 μm nylon mesh into a special 3.5-mL cuvette for direct use with the flow cytometer. Following a 20 min incubation[1] at room temperature, staining solution consisting of 1 mL Otto II solution, 50 μg mL^{-1} propidium iodide, 50 μg mL^{-1} RNase IIA and 2 μL mL^{-1} β-mercaptoethanol was added to the sample.

Protocol 6

Approximately 10 glass beads of 1.5 mm diameter (Sigma-Aldrich) were added into 2-mL Eppendorf tube containing 550 μL of ice-cold lysis buffer LB01 and the pellet of biomass. The cells were disrupted for 3 min at 25 Hz using Retsch MM200 mixer mill. The sample was filtered through a 42 μm nylon mesh into a special 3.5-mL cuvette for direct use with the flow cytometer. Following a 20 min incubation[1] at room temperature, staining solution consisting of 550 μL of LB01 lysis buffer, of 50 μg mL^{-1} propidium iodide, of 50 μg mL^{-1} RNase IIA and of 2 μL mL^{-1} β-mercaptoethanol was added to the sample.

Standardization

Initially, nuclei suspensions for FCM analysis were prepared without a standard. When a suitable nuclei extraction protocol was found for a given species, an internal standard was included into following analyses. Four different plants were used as standards in this study—wild clone of *Carex acutiformis* (2C = 0.82 pg; Veselý et al. 2012), commercial clone of *Solanum pseudocapsicum* (2C = 2.59 pg; Temsch et al. 2010), wild clone of *Bellis perennis* (2C = 3.38 pg; Schönswetter et al. 2007) and *Vicia faba* cv. Inovec (2C = 26.90 pg; Doležel et al. 1992). To release the standard nuclei, ca. 20 mg piece of fresh leaf tissue was chopped with a razor blade either together with an algal sample (Protocols 2, 3 and 4) or separately, in a fraction of used nuclei isolation solution and later mixed with the protoplast suspension containing the remaining solution (Protocols 5 and 6). When razor chopping was used to isolate nuclei of both algal sample and plant standard, the algal biomass was chopped slightly less than the plant standard. The resulting nuclei suspension was filtered and stained as described in Protocols 1–6.

DNA content estimation

The stained samples were immediately analysed using a Partec CyFlow SL cytometer (Partec GmbH, Germany) equipped with a green solid-state laser (Cobolt Samba, 532 nm, 100 mW), and aside from PI fluorescence intensity, optical parameters forward scatter (FSC) and side scatter (SSC) were recorded. Each sample measurement was taken for up to 5000 particles. The success rate of particular protocol was evaluated as follows: (1) no peak, sample peak undistinguishable from the background noise or not detected; (2) poor analysis, sample peak visible but its position hardly recognizable from the background noise (yet apparent on a relative fluorescence vs. side scatter plots); and (3) good result, sample peak clearly visible with reduced background noise.

To properly analyse DNA content of the studied algal strains, at least three measurements were done on separate days to obtain precise value and to minimize the effect of random instrumental shift. The resulting FCM histograms were analysed using FloMax ver. 2.4d (Partec). The lowest fluorescence intensity sample peaks were identified as G_1 (vegetative cells) and additional peaks with double fluorescence intensity (if observed) as G_2. Gating of sample nuclei in fluorescence vs. side scatter plots was necessary to remove the background noise connected to the populations of interest in order to obtain more accurate results (with an exception of *G. semen*). The absolute nuclear DNA content was calculated as the sample G_1 peak mean fluorescence/standard G_1 peak mean fluorescence × standard 2C DNA content (according to Doležel and Bartoš 2005). Since the ploidy level or life cycle stage of studied organisms is generally unknown, the DNA content results are given in pg $cell^{-1}$, i.e. the absolute nuclear DNA content measured per cell (1 pg ≈ 978 Mbp; Doležel et al. 2003). The quality and accuracy of resulting DNA content estimates was expressed by averaged coefficient of variation (CV) for individual sample peaks and standard deviation (SD) for measurements error averaged from the three independent measurements.

Results

Comparison of isolation protocols

Altogether, six nuclei isolation protocols were tested and compared on a set of ten problematic algal taxa (Table 1). Protocol success rate was evaluated using a three quantitative scale (see Materials and methods). The results differed greatly according to the used protocol and algal sample tested (Table 2).

Except analysing cells only mixed with nuclei isolation solution (Protocol 1), the broadly used technique of razor blade sample chopping (Protocols 2 and 3) was the least

Table 2 Comparison of six different nuclei isolation protocols applied across the studied algal strains, including the type of biomass, used nuclei isolation solution and cell disruption technique

	Biomass	Nuclei isolation solution	Cell disruption	*Spirogyra* sp.	*Zygnema* spp.	*Chlamydomonas noctigama*	*Microglena* sp.	*Stigeoclonium* sp.	*Gonyostomum semen*	*Trentepohlia* sp.	*Tribonema vulgare*
Protocol 1	Fresh	LB01/Otto I + II	osmotic	Not tested	Not tested	No peak	No peak	Not tested	No peak	Not tested	Not tested
Protocol 2	Fresh	Otto I + II	razor chopping	No peak	No peak	No peak	No peak	No peak	No peak	No peak	No peak
Protocol 3	Fresh	LB01	razor chopping	No peak	Poor analysis	No peak	No peak	Good result	No peak	No peak	Good result
Protocol 4	Desiccated	LB01	razor chopping	Good result	Good result	No peak	No peak	Poor analysis	No peak	Good result[2]	Poor analysis
Protocol 5	Fresh	Otto I + II	bead beating	Not tested	Not tested	No peak	No peak	Not tested	No peak	Not tested	Not tested
Protocol 6	Fresh	LB01	bead beating	Not tested	No peak	Poor analysis	Poor analysis	No peak	Good result	No peak	No peak

The outcomes are categorized as no peak (sample peak undistinguishable from the background noise or not detected), poor analysis (sample peak visible but its position hardly recognizable from background noise; yet apparent on a side scatter), good result (sample peak clearly visible with minimum background noise) and not tested

[2] The same quality of FCM analysis in *Trentepohlia* strain was observed when Otto I + II solutions instead of LB01 buffer were used in Protocol 3

successful method in this study. When Otto I + II solutions were used (Protocol 2), none of the tested strains resulted in a visible sample peak. The razor blade chopping technique was successful only in combination with LB01 isolation buffer (Protocol 3), resulting in clearly visible and well separated peaks for *Stigeoclonium* sp. and *Tribonema vulgare*. A partial success of Protocol 3 was also achieved for all *Zygnema* strains, however, still leading to a high background noise and often hardly distinguishable sample peak (Fig. 1a,d).

In contrast, Protocol 4, combining sample desiccation with razor blade chopping in LB01 isolation buffer, was the most successful of all the tested methods. This protocol resulted in clearly visible and well separated peaks for *Spirogyra* sp., *Trentepohlia* sp. (Fig. 1h,k) and all three analysed strains of *Zygnema* sp. (Fig. 1g,j). Interestingly, the same quality of analysis with *Trentepohlia* was observed when Otto I + II solutions were used instead of LB01 buffer; however, this was not examined for any other microalgal strain. Protocol 4 was further partially successful for the species *Stigeoclonium* sp. and *T. vulgare*, however, leading to a more pronounced background noise compared to the same method without the desiccation step (Protocol 3). On the other hand, this method failed to result in any sample peaks for *Chlamydomonas noctigama*, *Microglena* sp. and *Gonyostomum semen* (Fig. 1c,f). The only successful method for analysing these microalgal species was Protocol 6. In this protocol, the nuclei were extracted by bead beating cells in LB01 isolation buffer. This method was particularly suitable for *G. semen*, where it led to a high-quality analysis with nearly no visible background noise (Fig. 1i,l). Contrarily, the analyses of *C. noctigama* and *Microglena* sp. were of very low quality (pronounced background noise and poor peak delimitation). However, Protocol 6 was the only protocol leading to any sample peak for these species. Interestingly, the same method of nuclei isolation by bead beating successful for *C. noctigama*, *Microglena* sp. and *G. semen* did not work when Otto I + II solutions were used (Protocol 5) instead of LB01 isolation buffer (Protocol 6). Therefore, Protocol 5 was not further examined for the remaining strains.

Nuclear DNA content estimation

When the most suitable protocol for particular species was found, their absolute nuclear DNA content per cell was thoroughly investigated (Table 3). The nuclear DNA content of studied microalgal strains is given in pg of absolute nuclear DNA per cell with equivalent values in Gbp (1 pg ≈ 0.978 Gbp; Doležel et al. 2003). The DNA content differed greatly, spanning from 0.15 (0.14) to 32.52 pg (31.81 Gbp). The smallest DNA content belonged to the representatives of the class Chlorophyceae with 0.15 pg (0.14 Gbp) for *Stigeoclonium* sp., 0.33 pg (0.33 Gbp) for *C. noctigama* and 0.44 pg (0.43 Gbp) for *Microglena* sp. and to the

representative of the class Xanthophyceae with 0.34 pg (0.34 Gbp) for *T. vulgare*. In contrast, the largest measured DNA content of 32.52 pg (31.81 Gbp) belonged to *G. semen* from the class Raphidophyceae. The three analysed strains of the genus *Zygnema* varied in their DNA content (1.11–2.86 pg ≈ 1.09–2.73 Gbp). The highest quality of DNA content estimates was observed within *G. semen* and one of *Zygnema* strains, with coefficients of variation (CVs) < 2% (1.14 and 1.75%, respectively). On the other hand, the lowest quality of DNA content estimates was documented in *Stigeoclonium* sp., *Microglena* sp. and *Spirogyra*, exceeding 13% (13.51, 13.54 and 13.65%, respectively).

Fig. 1 Flow cytometric fluorescence histograms (**a-c, g-i**) and fluorescence vs. side scatter plots (**d-f, j-l**) summarizing the results of poor quality (**a-f**) and suitable (**g-l**) nuclei isolation protocols of *Zygnema* sp. OS2, *Trentepohlia* sp. and *G. semen* strains with internal reference standards. The nuclei of *Zygnema* sp. OS2 isolated by a razor blade chopping of fresh biomass in LB01 buffer (Protocol 3) resulted in visible sample and standard peaks with pronounced background noise (**a**, **d**), while using razor blade chopping of desiccated biomass (Protocol 4) led to prominent sample and standard peaks (**g**, **j**). Isolation of *Trentepohlia* sp. nuclei using Protocol 3 did not result in visible sample peak (**b**, **e**), contrary to nuclei isolation with the Protocol 4, where the sample peak is clearly visible and well separated (**h**, **k**). Note that in fluorescence vs. side scatter plot, the peak of presumed haploid zoospores can be identified (**k**, indicated by the arrow). Protocol 4 did not result in any sample peak for *G. semen* (**c**, **f**); however, when nuclei were isolated by cell bead beating in LB01 buffer (Protocol 6), it led to a clear sample peak with nearly no background noise (**i**, **l**)

Discussion

Nuclei isolation protocols In this study, two new nuclei isolation protocols for FCM are proposed and applied on various samples of microalgae. The newly introduced methods involve either sample desiccation before razor blade chopping or bead beating of the sample biomass. Both methods are easy to use and bring satisfactory results of DNA content estimation in microalgae, even for problematic taxa. These new methods were compared with more broadly used techniques for microalgae, i.e. analysis of osmotic bursting of cells (applied on unicellular algae) and razor blade chopping of fresh biomass (all tested algae).

Moreover, these new techniques allowed for the first time DNA content estimation in *C. noctigama*, *G. semen*, *Microglena* sp. and *Stigeoclonium* sp. Further, identical strains of *C. noctigama* (strain CAUP G224) and *Stigeoclonium* sp. (strain CAUP J603) were already examined in the study Mazalová et al. (2011) using enzymatic mixture for protoplast extraction, however, without any success. In this work, *C. noctigama* was successfully analysed by applying bead beating of the biomass in LB01 isolation buffer (Protocol 6). Interestingly, a suitable method for analysing *Stigeoclonium* sp. was simple razor blade chopping of the biomass in LB01 isolation buffer (Protocol 3), not a protoplast extraction using enzymatic mixture (Mazalová et al. 2011) or any other method used in this study (except Protocol 4, see later). Moreover, Protocol 3 was also the best method to analyse *T. vulgare*. Although this taxon was already successfully analysed with the use of enzymatic mixture in the study Mazalová et al. (2011), the enzymatic treatment is methodologically demanding as well as time-consuming. In contrast, razor blade chopping of a fresh sample is very simple and rapid method and sometimes, as seen on the example of *Stigeoclonium* sp. and *T. vulgare*, also the optimal method for FCM without the need for further optimization. Therefore, this simple method is still worth a try when conducting pilot FCM measurements on other microalgal species. Both *Stigeoclonium* sp. and *T. vulgare* were also successfully analysed using desiccation step followed by razor blade chopping (Protocol 4), however, resulting in a reduced quality of the FCM analysis.

In general, razor blade chopping of biomass in LB01 isolation buffer either preceded by the desiccation step (Protocol 4) or without it (Protocol 3) appears to be a more efficient way of nuclei isolation in filamentous microalgae. The success of the desiccation using silica gel is especially interesting since this led to a decrease of quality in FCM analysis of vascular plants (Kolář et al. 2012). However, desiccating the biomass of microalgae might have reduced the negative effect of secondary metabolites interfering with DNA staining. For example, high amounts of secondary metabolites such as phenols have been documented in *Trentepohlia* sp., *Spirogyra* sp. and *Zygnema* spp. (Simić et al. 2012; Pichrtová et al. 2013; Mridha et al. 2017). Phenolic compounds can significantly decrease the quality of FCM analyses (Loureiro et al. 2006a), and the desiccation might reduce their negative effect (along with possibly other metabolites) on FCM analysis. On the other hand, optimal algal material for FCM analysis are young cultures approximately 3 to 5 weeks after their inoculation into a fresh medium, yet young *Zygnema* cells are known to contain high amounts of phenolic compounds (Holzinger et al. 2018), contradicting the benefits of using young cultures. However, analysis of *Zygnema* spp. cultures older than 5 weeks resulted only in a background noise (data not shown). Another explanation could be the putative role of desiccation in disturbing layers of polysaccharide present on *Zygnema* and *Spirogyra* filaments (Palacio-López et al. 2019), facilitating the release of their nuclei.

Bead beating of biomass in LB01 buffer (Protocol 6) seems to be more suitable for solitarily living algae. Even though the cell disruption by bead beating was previously used to isolate DNA of algae (e.g. Countway and Caron 2006), to my knowledge, it has never been used as a method for nuclei isolation in algal FCM. This technique was particularly suitable for *G. semen*, where it resulted in clear FCM histograms with very limited background noise (Fig. 1l,i). Bead beating of cells in

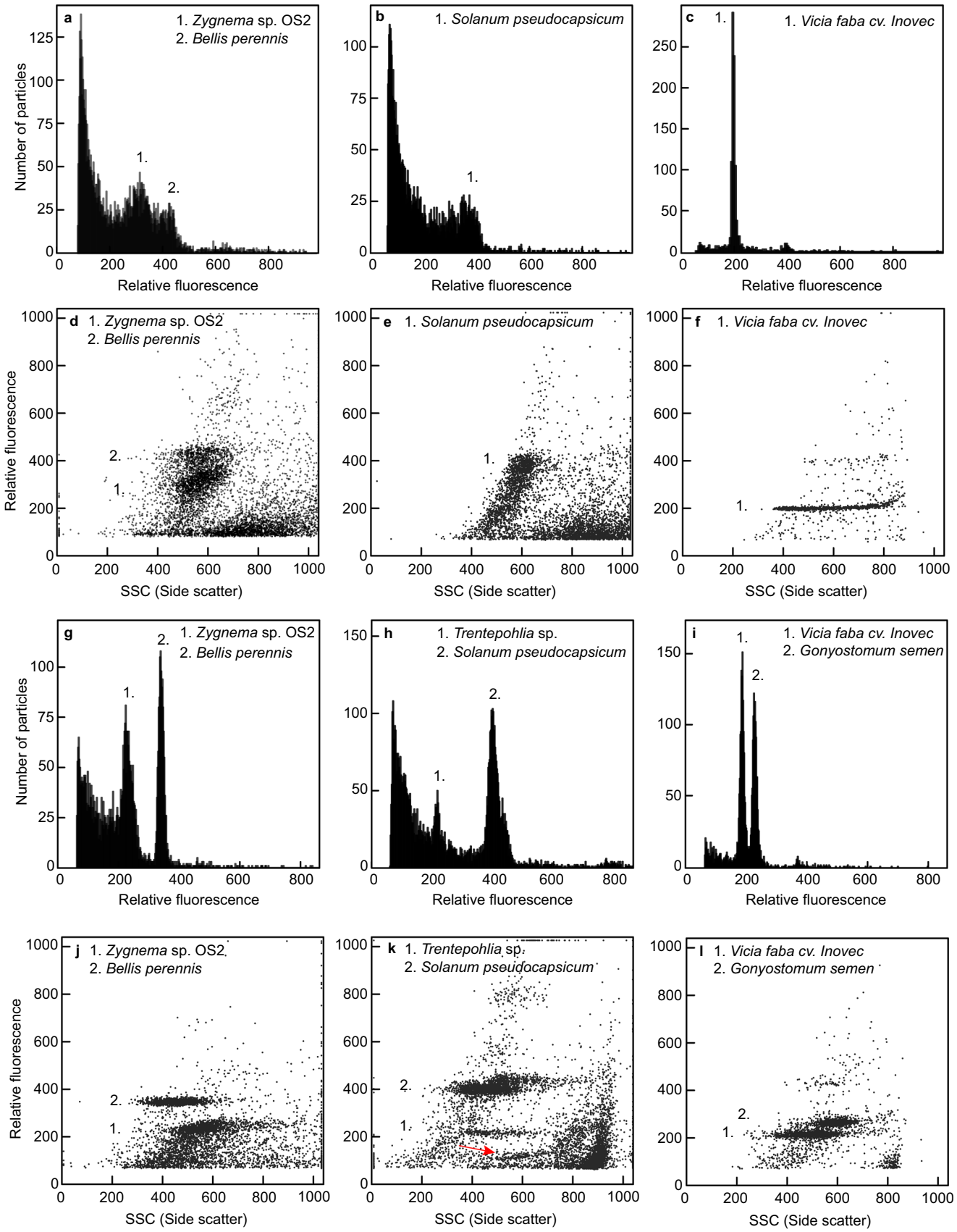
a
1. Zygnema sp. OS2
2. Bellis perennis
Number of particles
Relative fluorescence
b
1. Solanum pseudocapsicum
Relative fluorescence
c
1. Vicia faba cv. Inovec
Relative fluorescence
d
1. Zygnema sp. OS2
2. Bellis perennis
Relative fluorescence
SSC (Side scatter)
e
1. Solanum pseudocapsicum
SSC (Side scatter)
f
1. Vicia faba cv. Inovec
SSC (Side scatter)
g
1. Zygnema sp. OS2
2. Bellis perennis
Number of particles
Relative fluorescence
h
1. Trentepohlia sp.
2. Solanum pseudocapsicum
Relative fluorescence
i
1. Vicia faba cv. Inovec
2. Gonyostomum semen
Relative fluorescence
j
1. Zygnema sp. OS2
2. Bellis perennis
Relative fluorescence
SSC (Side scatter)
k
1. Trentepohlia sp.
2. Solanum pseudocapsicum
SSC (Side scatter)
l
1. Vicia faba cv. Inovec
2. Gonyostomum semen
SSC (Side scatter)

Table 3 Absolute nuclear DNA content per cell estimated for the studied algal strains

Species	Strain	Average DNA content		Mean CV (%)	Internal reference standard
		[pg] ± SD	[≈ Gbp]		
Zygnema sp.	13 179-4	1.112 ± 0.05	1.087	4.50	*Bellis perennis* (2C = 3.38 pg)
Zygnema sp.	15 Osor 2	2.394 ± 0.10	2.342	4.18	*Bellis perennis* (2C = 3.38 pg)
Zygnema sp.	CCCryo 171-04	2.856 ± 0.05	2.793	1.75	*Bellis perennis* (2C = 3.38 pg)
Stigeoclonium sp.	CAUP J603	0.148 ± 0.02	0.144	13.51	*Carex acutiformis* (2C = 0.82 pg)
Tribonema vulgare	CAUP D501	0.342 ± 0.01	0.335	2.92	*Carex acutiformis* (2C = 0.82 pg)
Trentepohlia sp.	CAUP J1601	1.167 ± 0.02	1.141	1.56	*Solanum pseudocapsicum* (2C = 2.59 pg)
Gonyostomum semen	NIVA-2/10 (BO-182)	32.523 ± 0.37	31.807	1.14	*Vicia faba* cv. Inovec (2C = 26.9 pg)
Microglena sp.	Fio19	0.443 ± 0.06	0.434	13.54	*Carex acutiformis* (2C = 0.82 pg)
Chlamydomonas noctigama	CAUP G224 (SAG 6.73/UTEX 2289)	0.333 ± 0.01	0.326	3.00	*Carex acutiformis* (2C = 0.82 pg)
Spirogyra sp.	CAUP K902	1.026 ± 0.14	1.003	13.65	*Solanum pseudocapsicum* (2C = 2.59 pg)

The average DNA content estimates based on three independent measurements are provided in pg of DNA (with equivalent values in Gbp), along with average coefficient of variation for analyses (CV) and details on the used internal reference standard

LB01 buffer (Protocol 6) is also the only method that gained any DNA content estimates for *C. noctigama* and *Microglena* sp. not only in this study but also including unsuccessful attempts in Mazalová et al. (2011). However, the outcomes were of very poor quality, and further optimization is needed to obtain more precise results.

It is worth emphasizing the importance of selecting optimal nuclei isolation solution when employing FCM on algal samples. In this study, only two nuclei isolation solutions were used (LB01 buffer and Otto I + II solutions); however, their performance was completely different. When using Otto I + II solutions (Protocol 1, Protocol 2 and Protocol 5), the analyses led to no visible sample peaks (with the exception of *Trentepohlia* sp. with equally good results under the use of both buffers). Vast majority of the successful analyses were done using LB01 isolation buffer. The differences between LB01 buffer and Otto I + II solutions are in their different chemical composition but also in strikingly distinct pH level (2–3 and 8, respectively; Loureiro et al. 2006b). This stresses the importance of selecting an optimal isolation solution and comparing to others might be a next step in further optimization.

DNA content estimates of the studied algae The nuclear DNA content of four algal taxa was successfully estimated with the smallest measured DNA content represented by *Stigeoclonium* sp. with 0.15 pg (0.14 Gbp). To my knowledge this also represents the first DNA content estimate for the whole order Chaetophorales. On the other hand, the largest DNA content measured in this study belongs to the raphidophyte *G. semen* with 32.52 pg (31.81 Gbp). The only representative of the class Raphidophyceae that has been analysed for DNA content so far was marine *Heterosigma carterae* possessing a genome five times smaller (5.43–6.12 pg/5.31–5.98 Gbp; Veldhuis et al. 1997). In contrast, more DNA content data are available for the genus *Chlamydomonas* with estimates ranging from 0.08 to 0.40 pg (0.08–0.39 Gbp; Chiang and Sueoka 1967; Kates et al. 1968; Cattolico and Gibbs 1975; Spring et al. 1978; Veldhuis et al. 1997; Merchant et al. 2007; Reinecke et al. 2018; Nelson et al. 2019). However, only a few of these estimates were acquired using FCM. The DNA content of *C. noctigama* estimated in this study (0.33 pg/Gbp) is rather large but still falling within the previously published range.

The identical strain of *T. vulgare* (CAUP D 501) was previously analysed by Mazalová et al. (2011), leading to a slightly different result of 0.41 pg (0.40 Gbp) compared to 0.34 pg/Gbp estimated in this study. This variance might be induced by use of a different FCM standard. In this study, the plant *Carex acutiformis* was used in opposite to *Raphanus sativus* cv. Saxa used in the study by Mazalová et al. (2011). However, the latter FCM standard displays many difficulties like high CVs, polyploidy, higher presence of secondary metabolites and reported genome size of different values; therefore, its use was repeatedly discouraged (Doležel et al. 1992; Praça-Fontes et al. 2011; Park et al. 2016; Šmarda et al. 2019). The only available DNA content data for the genera *Trentepohlia* and *Spirogyra* originate from DAPI microdensitometry (Kapraun 2005, 2007; López-Bautista et al. 2006). However, DAPI fluorescent stain binds to adenine-thymine-rich regions and therefore may bring the erroneous estimates of AT:GC ratio of the sample and the reference standard

(Doležel et al. 1992). For the both genera, the DNA content estimates in this study were the first ones acquired using FCM. The previously published estimates for *Trentepohlia* sp. span from 1.08 to 4.01 pg (1.10–4.10 Gbp; López-Bautista et al. 2006; Kapraun 2007), and thus the estimate measured in this study (1.17 pg/1.14 Gbp) falls within the published range. Interestingly, in relative fluorescence vs. side scatter plots of some *Trentepohlia* sp. FCM analyses, it is possible to identify three sample peaks that differ in their ploidy level (Fig. 1 k). An abundant population of nuclei belonging to the intermediate ploidy was identified as nuclei of vegetative filament (G_1). The peak of highest *Trentepohlia* sp. ploidy was determined as dividing nuclei of the vegetative filament (G_2), unfortunately partially overlapping with the standard nuclei. The peak of the lowest ploidy with smallest population of nuclei may represent haploid zoospores. The presence of sporangia containing zoospores was subsequently confirmed by observation using light microscopy. The previous DNA content estimates of *Spirogyra* sp. (3.91–4.01 pg/4.00–4.10 Gbp; Kapraun 2005) were four times higher than in this study (1.03 pg/1.00 Gbp). Despite the fact that the measurements were conducted on different *Spirogyra* strains and by different techniques, these results probably reflect high DNA content variability within the *Spirogyra* genus. Similarly, DNA content variability within the genus *Zygnema* will likely be much higher than documented to date. The known DNA content range is from 0.49 to 1.5 pg (0.50–1.54 Gbp; Kapraun 2005; Mazalová et al. 2011). However, analyses of three *Zygnema* strains displayed DNA content between 1.11 and 2.86 pg (1.09–2.80 Gbp), and thus the previous DNA content range for the genus was nearly doubled.

I believe that the presented new nuclei isolation protocols will provide alternative ways of microalgal FCM and apply to a broad range of various species of microalgae. Hopefully, the newly introduced protocols will help to extend yet very limited DNA content data of microalgae, and these data will subsequently serve to various microalgae applications.

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Author's contributions The author confirms sole responsibility for the following: study conception and design, data collection, analysis and interpretation of results and manuscript preparation.

Data Availability All data are provided within the manuscript.

Declarations

Conflict of interest The author declares no competing interests.

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