Received: 2 July 2021 | Revised: 10 July 2021 | Accepted: 13 July 2021

DOI: 10.1002/cyto.a.24485

COMMENTARY

MIFlowCyt

ISAC INTERNATIONAL SOCIETY FOR ADVANCEMENT OF CYTOMETRY

CYTOMETRY Journal of Quantitative Cell Science PART A

# Meet the challenges of analyzing small genomes using flow cytometry

**Dora Čertnerová**

Faculty of Science, Department of Botany, Charles University, Prague, Czech Republic

**Correspondence**
Dora Čertnerová, Faculty of Science, Department of Botany, Charles University, Prague, Czech Republic.
Email: dora.certnerova@gmail.com

In many fields of biodiversity research, nuclear DNA content is a crucial parameter of the study organism (individual, cellular type), allowing, for example, ploidy determination, cell-cycle analysis or selecting suitable organisms and optimal strategy for whole genome sequencing (WGS). Due to lower sequencing costs, small genome size represents a major advantage for WGS projects. Not surprisingly, most DNA content estimates available for small genomes have been derived from WGS data. On the other hand, the routine use of WGS as a method for genome size estimation has been discouraged due to its poor quantification of genomic content of repetitive elements (e.g., present in centromeres or telomeres) that may significantly underestimate the true DNA amount. Currently, the most suitable method for the task is flow cytometry (FCM), a rapid and easy to perform technique, using which the DNA content is estimated from the mean fluorescence intensity of nucleic acid binding dye (e.g., propidium iodide, ethidium bromide). The FCM is routinely used in immunology, cancer research or plant and animal studies, however, its application on organisms with small genomes can be highly challenging.

Even though, the complexity of organisms is not directly linked with the amount of their nuclear DNA, the small genomes are very often found among microorganisms, specifically in nano/picoplankton, unicellular parasites and most fungi, as a consequence of the positive genome size—cell size correlation [1]. However, even microorganisms in assumed clonal populations commonly differ in morphology, physiology or biochemistry. In fungi, the smallest measured nuclear DNA content (2.2 Mbp in *Encephalitozoon romaleae*; [2]) also reaches the lowest end of known DNA content among all eukaryotes. Moreover, the DNA content of other fungal species is generally not much higher (with a median value <40 Mbp; [3]). In the study by Talhinhas et al. [4], the authors nicely summarized the currently used methods for fungal genome size estimation using FCM and addressed the potential pitfalls. Interestingly, these pitfalls are widely shared with many other groups of microorganisms with small genomes.

Until the modern sequencing techniques have been introduced, the microorganisms were largely understudied and their diversity, phylogenetic relationships, life cycles, and so forth widely unexplored. Despite their major importance for the global ecosystem and common applications in biotechnology, the microorganisms' research has lagged behind plant and animal studies up to the present. However, limited research of microorganisms had consequences in low number of DNA content data, especially pronounced in contrast to their estimated diversity.

Because of the small size of their bodies, microorganisms usually need to be cultivated to obtain sufficient amounts of biomass for the FCM, which is not only time-consuming but also sometimes unrealistic. For the uncultivated microorganisms in trophic interactions, another approach could be taken in simultaneous analysis of studied microorganism and its symbiont/host/prey and then to analyze these partners separately to correctly distinguish peaks of each organisms. Such approach seems especially suitable for parasites as is nicely illustrated by Talhinhas and colleagues [4] for pathogenic fungi and its host plant. However, simultaneous analysis might not be suitable for organisms substantially differing in their genome size. Moreover, microorganisms commonly live in microbial communities and this makes them harder to isolate or preserve in cultivation. Nonetheless, when possible, it is best to conduct the analysis on unistrain culture, ideally young and actively growing, as was also pointed out by Talhinhas and colleagues [4]. Unfortunately, residual of culture media may increase background fluorescence. In fact, the background noise is one of the major challenges when analyzing small genomes. For FCM analysis of plant or animals, even low sensitive flow cytometers such as CyFlow (Sysmex/Partec) are adequate, however, for FCM of microorganisms, instruments like CytoFLEX (Beckman Coulter) or FACS/LSR II (BD Biosciences), high-sensitive to small particles are more appropriate (see Figure 1). Further, there are several ways how to reduce the background noise. Nuclei should be isolated from cells, either chemically (using enzymes) or mechanically (razor-blade chopping, bead-beating). Although razor-blade chopping is routinely used

  

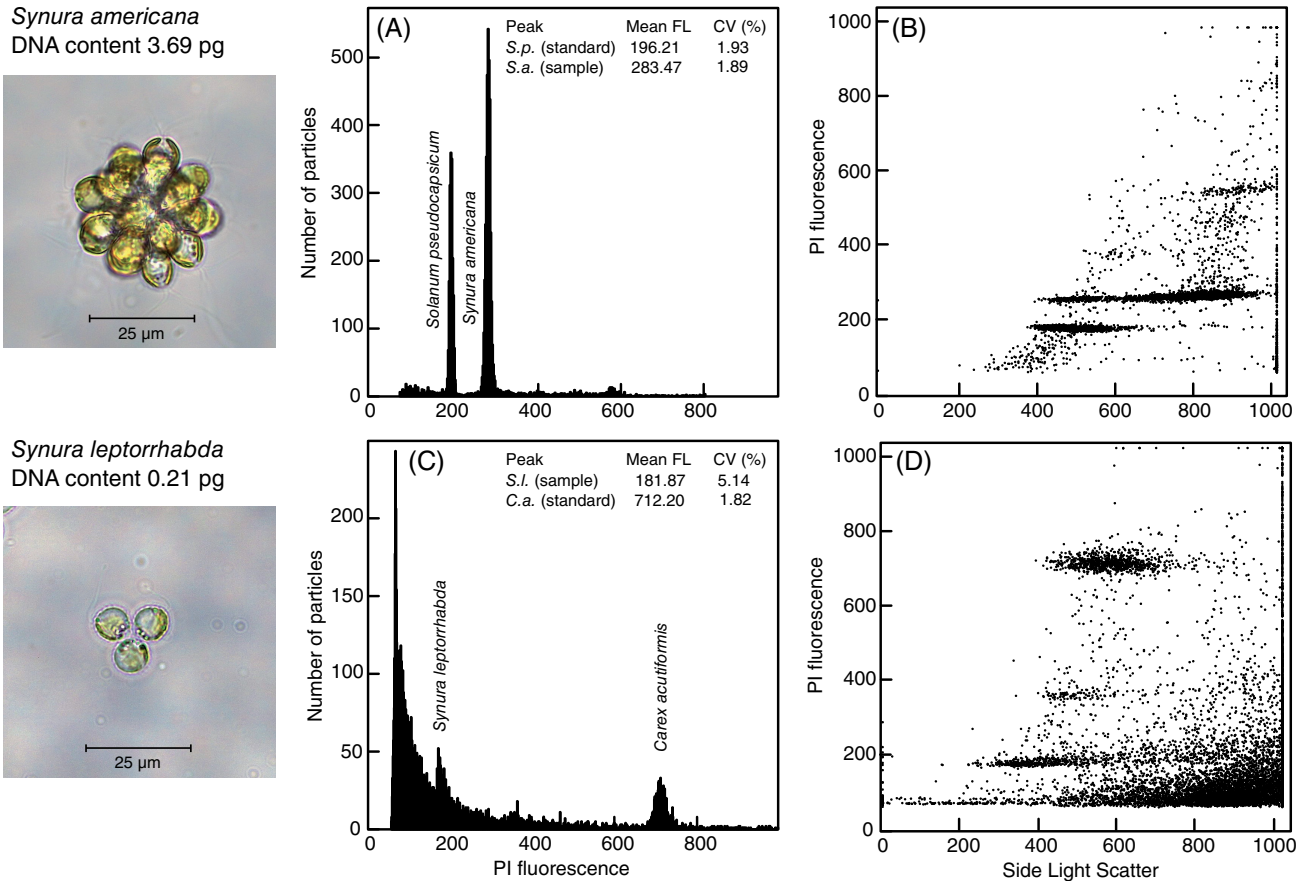


**FIGURE 1** CyFlow flow cytometry (FCM) outputs of two chrysophyte algae of the genus *Synura*—*S. americana* with higher DNA content (3.69 pg) and *S. leptorhabda* with lower DNA content (0.21 pg) and its plant standards. Note clearly visible peaks with only minor background noise on both fluorescence histogram (A) and fluorescence versus side scatter plot (B) in case of the first sample analysis. Conversely, higher amount of debris is present in the second analyzed sample (C) with the sample DNA content approaching the limits of resolution for CyFlow instrument, yet with peaks still sufficiently separated on fluorescence versus side scatter plot (D); unpublished data

in plant FCM, it seems unsuitable for protists (i.e., single-celled eukaryotes) but useful for filamentous microorganisms as was shown by Talhinhas and colleagues [4] or Čertnerová [5]. To reduce autofluorescence or adverse effect of secondary metabolites, sample can be fixed with various fixatives (ethanol, methanol, methanol: acetic acid mixture, formaldehyde, paraformaldehyde, or acetone), although the chemical fixation may not be suitable for precise genome size estimation [6]. Another possibility is to test different isolation buffers. For example, the Woody Plant Buffer or Tris-$MgCl_2$ buffer seems to work with fungal samples and LB01 buffer found wide application in FCM of microalgae (Talhinhas et al. [4]; Čertnerová [5]. However, new lysis buffers reflecting the specifics of particular groups of microorganisms still need to be developed. The lysis buffer may be further supplemented with PVP (polyvinylpyrrolidone) and/or with mercaptoethanol [7]. In addition, Talhinhas and colleagues [4] suggested using a lower concentration of propidium iodide, however, still adequate enough to properly stain the sample nuclei. It is also convenient to visualize measurements on a side-scatter versus fluorescence plot and apply gating to distinguish population of nuclei from a background noise if needed, as was also highlighted by the authors. In case of problematic plant or animal sample, alternative tissue/organ might help, though, this is not a possibility for most microorganisms (except few rare cases). However, despite a great effort, analyzing organisms with small genomes usually leads to higher CVs and, therefore, the criteria on acceptable precision of FCM analysis should not be generally as stringent.

Talhinhas and colleagues [4] further discussed the lack of appropriate FCM standards, which is yet another important issue accompanying analysis of small genomes. In recent years, the number of newly introduced FCM standards is slowly rising up, with, for example, *Saccharomyces cerevisiae*, *Aspergillus fumigatus* or *Chlamydomonas reinhardtii* possessing very small genome sizes (1C values of 24.1, 29.2, and 0.12 pg, respectively; [8, 9]). In the previous work, Talhinhas et al. [10] introduced additional fungal FCM standards with various genome sizes. Even so, there is still a dearth of FCM standards suitable for microorganisms, with those already introduced not easily accessible, leading to a frequent use of suboptimal standards such as chicken red blood cells or plant standards. However, these are

ISAC CYTOMETRY Journal of Quantitative Cell Science PART A



biologically different and could be therefore influenced differently from analyzed sample resulting in change of sample and standard peaks proportion.

When evaluating FCM outputs, we might have to deal with some additional challenges. The DNA content data are available for only a fraction of microorganisms and thus the range of genome size variation is widely unknown but often more diverse than expected. Fungi particularly are known for their high degree of genome size plasticity. Additionally, dearth of knowledge on life cycles of the studied organisms may lead to misinterpretation of detected fluorescence peaks in FCM histograms. Some fungal species are even heterokaryotic, that is, possessing multiple different-sized nuclei, and hence generating several $G_1$ peaks [11]. Further, variations in chromosome number and chromosome size seem to be the rule rather than the exception [12]. Unfortunately, chromosome counts are generally problematic in microorganisms due to the small size of their cells and asynchronous cell division. This also had an impact on missing ploidy level data.

In contrast to other groups of microorganisms, fungal genome size data are listed in their own database [3]. Talhinhas and colleagues [4] analyzed these data from many different angles. They highlighted that the majority of genomes size data were obtained using WGS or static microscope-based cytometry methods, and only less than 5% were obtained with FCM. More frequent employment of FCM might thus allow researching high resolution estimates. The authors further pointed out several interesting correlations. Among others that fungal evolution toward plant mutualism or parasitism seems to be accompanied by genome size expansion and fungi interacting with plants thus possess bigger genomes when compare to saprotrophs or those interacting with animals. Similarly interesting associations with genome size were found also in different groups of microorganisms, for example, correlation of genome size with growth rate and nutritional modes in chrysophytes [13, 14]. However, much more is still waiting to be discovered with more DNA content data available for microorganisms. This could be achieved with more routine use of FCM in microorganism research so I fully support the authors' call for more frequent applications of FCM in fungal research (as well as in other microorganism studies). I also believe many of these tips might find their use in other FCM applications on microorganisms, such as detecting autofluorescence or testing cell viability.

## PEER REVIEW

The peer review history for this article is available at https://publons.com/publon/10.1002/cyto.a.24485.

## ORCID

*Dora Čertnerová* https://orcid.org/0000-0001-8219-554X

## REFERENCES

1. Cavalier-Smith T. Cell volume and the evolution of genome size. The evolution of genome size. Chichester: Wiley; 1985. p. 105–84.
2. Pombert J-F, Selman M, Burki F, Bardell FT, Farinelli L, Solter LF, et al. Gain and loss of multiple functionally related, horizontally transferred genes in the reduced genomes of two microsporidian parasites. Proc Natl Acad Sci USA. 2012;109:12638–43.
3. Kullman B, Tamm H, Kullman K. Fungal genome size database. 2005. http://www.zbi.ee/fungal-genomesize/.
4. Talhinhas P, Carvalho R, Loureiro J. The use of flow cytometry for fungal nuclear DNA quantification. Cytom Part A. 2021;99(4):343–7.
5. Čertnerová D. Nuclei isolation protocols for flow cytometry allowing nuclear DNA content estimation in problematic microalgal groups. J Appl Phycol. 2021;XX:XX–X. https://doi.org/10.1007/s10811-021-02433-z
6. Doležel J, Greilhuber J, Suda J. Estimation of nuclear DNA content in plants using flow cytometry. Nat Protoc. 2007;2:2233–44.
7. Loureiro J, Kron P, Temsch EM, Koutecký P, Lopes S, Castro M, et al. Isolation of plant nuclei for estimation of nuclear DNA content—overview and best practices. Cytom Part A. 2021;99:318–27.
8. Potter EE, Thornber CS, Swanson JD, McFarland M. Ploidy distribution of the harmful bloom forming macroalgae *Ulva* spp. in Narragansett Bay, Rhode Island, USA, using flow cytometry methods. PLoS One. 2016;11:1–15.
9. Veselská T, Svoboda J, Růžičková Ž, Kolařík M. Application of flow cytometry for genome size determination in *Geosmithia* fungi: a comparison of methods. Cytom Part A. 2014;85:854–61.
10. Talhinhas P, Tavares D, Ramos AP, Gonçalves S, Loureiro J. Validation of standards suitable for genome size estimation of fungi. J Microbiol Methods. 2017;142:76–8.
11. Catal M, King L, Tumbalam P, Wiriyajitsomboon P, Kirk WW, Adams GC. Heterokaryotic nuclear conditions and a heterogeneous nuclear population are observed by flow cytometry in *Phytophthora infestans*. Cytom Part A. 2010;77:769–75.
12. Kullman B. Application of flow cytometry for measurement of nuclear DNA content in fungi. Folia Cryptog Est. 2000;36:31–46.
13. Čertnerová D, Škaloud P. Substantial intraspecific genome size variation in golden-brown algae and its phenotypic consequences. Ann Bot. 2020;126:1077–87.
14. Olefeld JL, Majda S, Albach DC, Marks S, Boenigk J. Genome size of chrysophytes varies with cell size and nutritional mode. Org Divers Evol. 2018;18:163–73.