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Microalgal cell division tracking using CFSE

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ABSTRACT

icroalgal cell division tracking could unlock new research means. It could help decipher cell response to an intentional stressor, aiming at increasing an added value molecule accumulation, or an accidental stressor, in the case of environmental pollution. It could also be used to monitor asynchrony in cultures exposed to photoperiod or determine the fate of dead cells. To date, because of the lack of guidelines specific to microalgae, microalgal ecotoxicology and biotechnology communities have not yet implemented such protocol in routine. Therefore, a systematic optimization methodology has been deployed to adapt the CarboxyFluorescein DiAcetate Succinimidyl Ester (*CF*-DA-SE, or, in short, CFSE) lymphocytes proliferation tracking technique to the microalga *Chlorella vulgaris*. The toxic effect of the CFSE solvent (DMSO) was delineated (stock solution at 10 mM). Then, incubation conditions (time, probe/cell ratio, illumination) were optimized (30 min, 4.50 nmol/MCell, in the dark). Finally, CFSE washing and cell recovery were robustified (low-acceleration - 100 *g* - centrifugation). Using a semi-synchronous culture as a test case, the method was successfully applied to count cell divisions. Up to four generations could be discriminated. The generation-to-generation signal ratio was exactly 1/4, corresponding to the natural division of *Chlorella vulgaris*. Furthermore, an advanced yet easy-to-implement signal processing technique was introduced to ease generation discrimination.

1. Introduction

Microalgae are one of the simplest and oldest forms of life. Throughout their evolution, they emancipated from their original aquatic environment to colonize deserts [1], volcanic water sources [2], or even glaciers [3]. Owing respect to their very long lifespan on Earth, they acquired a pivotal place in their environments by delivering key ecosystemic services. To name a few, they make up to half of the annual oxygen production on Earth [4] and are the foundation of aquatic food webs [5]. They draw human attention for three reasons. First, from the phycologist's point of view, studying their evolution allows garnering knowledge about past eras [6]. Second, from the biotechnologist perspective, they offer means to help society shift from its fossil-based, doomed, paradigm to a bio-based, sustainable, one [7]. Third, from the ecologist's standpoint, their pivotal place in the environment makes them valuable advanced bio-sensors to document the tremendous anthropic pressure humanity is exerting on the planet [8,9].

For their study on microalgae, these three communities have developed or implemented various top-tier techniques. For example, fluorometry is widely spread among ecologists and, to a lesser extent, biotechnologists. The most common tests are photosystem II quantum yield analysis (a.k.a. Fv/Fm measurements) and time-resolved photosystems' fluorescence induction monitoring (a.k.a. OJIP tests). Yet, their usages differ. The first community usually tries to pinpoint the origin of photosynthesis perturbations, classically associated with an environmental stressor (*e.g.*, microplastics [10], heavy metals such as zinc, copper, nickel, cadmium, or arsenic [11–13]). The second aims at better understanding energy management by the cells [1,14], with driving them to make the most out of impinging light as ultimate goal [15]. Another technique of note, imported from biomedical science, is flow cytometry. It revolves around two key technologies: hydrodynamic focusing and optoelectronic. Hydrodynamic focusing allows the individualization of cells from a suspension and carries those single cells into a succession of laser beams. At this point, cells, and their molecular content, interact sequentially with the lasers. Depending on their size, cytoplasmic complexity, and endogenous and exogenous molecules' fluorescence properties, emitted/scattered light is collected, sorted (by passband filters), amplified, and ultimately turned into a numerical signal. This technique allows access to individual cell properties and, by analyzing many cells, trait distribution among their population. Thanks

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Table 1
Reported CFSE staining conditions for microalgae. Examples with lymphocytes are provided as points of comparison.

Organism	Stock solution	Cell concentration/ number	Staining procedure	Incubation	Washing	Comment	Ref.
Planothidium frequentissimum	0.6 mM in 10 % vol/ vol DMSO	–	Staining concentration of 1.2 μM	10 min	–	–	[25]
Microcystis aeruginosa	10 mM in pure DMSO	9 MCell/mL	65 μL in 130 mL of cell suspension	1 h, 20 °C, in the dark	By dilution	Allowed 1 day for cells to recover	[26]
Chlamydomonas reinhardtii	–	150 Mcell	Staining concentration 7.2 μM	40 min	–	22 cm using a cell number and a CFSE concentration hinders reproductibility	[27]
Chlorella vulgaris	Unknown concentration, in pure DMSO	150 Mcell	Staining concentration 14.4 μM	30 min, 18 °C, in the dark	By dilution		[28]
Prorocentrum donghaiense	5 mM in pure DMSO	6.8 MCell/mL	6 μL in 994 μL of cell suspension	2 h, 20 °C, in the dark	By centrifugation	–	[29]
Lymphocytes	5 mM in pure DMSO	50 MCell/mL	Staining concentration 5 μM	10 min, 37 °C	By dilution	–	[23]
Lymphocytes	10 mM in pure DMSO	100 MCell/mL	Staining concentration 10 μM	5 min, 20 °C	By dilution	–	[30]

to its versatility, flow cytometry can be applied to a wide range of problems belonging to both ecotoxicology and biotechnology. Among them, one can cite morphology tracking [16], Reactive Oxygen Species level quantification [17], viability assessment [18], cell lipid content evolution when exposed to a stressor [19], marine populations composition analysis [20], pigment production induction monitoring [21], or high-lipid producing mutant selection [22]. Still, a very elegant and potentially ground breaking application is under-represented: cell division tracking.

This technique was introduced in the field of lymphocytes study by Lyons and Parish over the 90's [23]. It relies upon a molecule, CarboxyFluorescein DiAcetate Succinimidyl Ester (*CF*-DA-SE, or, in short, CFSE). This molecule is made of three key components, each having a pivotal role in cell tagging and division tracking. The acetate groups ensure that the molecule can cross the cell membrane. Once within the cell, these acetate groups are cleaved by generic esterases. The removal of the acetate groups has two consequences: fluorescein shifts from a non-fluorescent to a fluorescent state, and the new polarity of the molecule prevents it from crossing back the cell membrane. Once trapped within the cell, the succinimidyl ester comes into play by binding to the free amine groups it encounters. One should note that this binding is not specific; hence, the molecule targets long-lasting cytoskeletal proteins, as well as other molecules presenting an amine group. After some time, the cells have degraded short turnover proteins, and only the fluorescein bonded to cytoskeletal proteins remains [24]. As these proteins are carried to daughter cells during cell division, they allow for individual cell division tracking, as daughters will exhibit a signal twice lower than their mother (in binary fission case). By applying this approach to lymphocytes study, Lyons and Parish have counted up to seven cell divisions. Returning to the world of microalgae, routine use of CFSE could unlock new research. For example, when applying stress to increase cell pigment content, CFSE could allow the identification of refractory (not dividing) and compliant (proliferating) populations and compute product expression kinetics accordingly. It could also be used to monitor asynchrony in cultures exposed to photoperiod or determine the fate of dead cells.

Still, the application of CFSE in the microalgal ecotoxicology and biotechnology communities remains limited (five to the authors' knowledge). Buhmann et al. used CFSE not for its cell proliferation monitoring capabilities but as low background noise viability dye to study diatom cryoinjury survival [25]. Cai et al. used it to track *Microcystis aeruginosa* cell regrowth and elucidate their overwintering strategy [26]. Anido-Varela et al. applied it to ascertain the dose-response effects of sunscreen molecules (benzophenone-3 and -4) on *Chlamydomonas reinhardtii* proliferation (among other indicators) [27]. Roiboo et al. were able to go further and used signal decay between generations to identify how terbutryn, a triazine herbicide, perturbates *Chlorella vulgaris* division (in four or in two) [28]. Zhou et al. used CFSE elegantly to document a predation pattern [29]. To do so, they tagged the prey but not the predators. Then, they used fluorescent microscopy to detect CFSE-positive cells, among which surviving prey but also predators that had fed (by absorbing prey's cytoplasmic content) returned positive.

All the above studies based their work on Lyons' one [23,24]. Yet, the cell staining conditions (summarized in Table 1) show large variations. Among them, the staining concentration and the incubation procedure are the most striking. Sadly, those two aspects of CFSE deployment are key. Indeed, they control the most sensible features of a successful CFSE staining, namely the intensity of the initial staining, its uniformity among the exposed cells (measured as the coefficient of variation, ideally below 15 %), and the low toxicity of the procedure [24]. As CFSE is admitted to be a low-toxicity molecule, increasing its staining concentration ensures most of those aspects. Yet, limits exist and have not been clearly identified for microalgae. Apart from the fact that a too-high concentration that is too high will damage the cells, it could also induce fluorescence signal leakage onto other detectors, or the amount of CFSE solvent itself (DiMethylSulfOxyde, in short, DMSO) will become toxic. Indeed, while it is sometimes used as a cryoprotectant, DMSO can induce cell damage (from osmotic or chemical origin) above 25 % vol/vol for green microalga *Nannochloropsis atomus* or above 20 % vol/vol for *Planothidium frequentissimum*.

This complexity may explain the relatively low number of work involving CFSE for cell proliferation tracking. This work aims to offer a systematic investigation of those parameters and provide clear guidelines for scholars and engineers willing to deploy cell division tracking procedures. Over its course, CFSE solvent (DMSO) toxicity was quantified, initial staining was optimized (duration, staining concentration, light), and cell washing procedures were analyzed. Once set, the protocol was applied to division tracking of cells in a partially synchronized culture, and advanced processing techniques were suggested to ease generation number and daughter cells quantity counting. As this work ambitions to be taken over by both microalgal ecotoxicology and biotechnology communities *Chlorella vulgaris* was chosen as the model strain. Indeed, *Chlorellae* make a fast-growing and ubiquitous genus often used for ecotoxicological studies [31,32]. Furthermore, from a biotechnological point of view, *Chlorella vulgaris* is commonly encountered in industrial and scientific communities, approved as food and feed by EFSA (Ares (2022) 1668627) and US FDA (GRN 00396), and features a sizable potential [33].

2. Materials and methods

2.1. Strain and culture medium

The strain used for this study was *Chlorella vulgaris* (CV 211-11b) obtained from SAG Culture Collection, Germany. Bold's Basal Medium with tripled nitrogen load was used throughout this study (sterilization by autoclaving) [34]. This medium was chosen as, from our group experience, it allows flourishing cultures of *Chlorella vulgaris*. Cultures were conducted in shake flasks (250 mL, 25 mL medium, 100 rpm) under continuous moderate light (50 μmolPhotonPAR/m^2/s, cool white spectrum LEDs). Cultures were not supplemented in carbon dioxide, and temperature was kept within a 20–22 °C range (all manipulations were led within this temperature range if not specified otherwise). Before entering the test phases, cells were subcultured under these conditions for more than five passagings to ensure acclimation to the subculturing conditions.

Chlorella vulgaris cells used for the study were collected in the exponential phase. Before being used for a test, the suspension concentration was adjusted to an optical density of 1.0 at 750 nm, corresponding to a cell concentration of 37 MCell/mL (hemocytometer counting, Zeiss Axioplan 2).

2.2. CFSE and stock solution

CFSE was bought as a mixture of 5-carboxyfluorescein diacetate succinimidyl ester and 6-carboxyfluorescein diacetate succinimidyl ester (unknown proportion), usually referred to as 5-(and 6)-carboxyfluorescein diacetate succinimidyl ester (AAT Bioquest). CFSE was dissolved in pure DMSO at different concentrations and placed in a dark vial before storage at −20 °C. The different concentrations were used to limit DMSO introduction in the culture (see Section 3.1). However, as it will be pointed out later, if the reader is not interested in a concentration screening, increasing this concentration to 10 mM is advised.

2.3. Maximal admissible DMSO amount and viability staining

As DMSO is known to induce cell damage, various cell suspension/pure DMSO ratios were tested. The tested ratio ranged from 1/60^{th} to 1/4^{th}, with a constant volume of initial *Chlorella vulgaris* suspension of 1 mL. As pointed out by Canavate et al., contact time matters [35]. In our case, it was set to 30 min as preliminary tests showed no difference on viability between a 30-min and a 90-min DMSO exposure. Incubation was carried out in the dark. Viability was assessed using propidium iodine. This molecule enters dead cells and binds to their DNA, incidentally inducing its fluorescence upon excitation. To lead this assay, 10 μL of 1 g/L fresh propidium iodide (Sigma Chemicals) were added to the test tube. Afterward, cells were washed by centrifugation (15,000 rpm, 4 °C, 5 min), and the supernatant was discarded. The pellet was resuspended before immediate analysis. The dye signal was recovered using the yellow-green laser (561 nm, 610/20 nm detection). Heat-treated (90 °C, 10 min) cells were used as positive control (*i.e.*, dead cells), and pristine cells were used as negative control (*i.e.*, alive cells). Each of the tested conditions was duplicated.

2.4. CFSE staining optimization

A successful CFSE staining would exhibit three traits, namely, uniform staining of the cell (no subpopulations), a narrow spread of the stained population, and a high contrast between stained cells and control (to count as many generations as possible). The two first quality indicators can be measured as the coefficient of variation, ideally below 15 % [24]. The last one can be accessed by computing the mean fluorescence signal of the tagged population. Several factors can influence the quality of the staining. We selected three of them to undergo a systematic optimization by a design of experiment approach: the incubation time (15 and 45 min), the illumination (presence or absence), and the probe/cell ratio (1.8 to 7.2 nmol/MCell, ±50 % with respect to a previous work we led on *Chlorella vulgaris* [18]). After incubation, the remaining CFSE was washed by centrifugation. This design yielded eight conditions to test. Two additional center points (30 min, 4.50 nmol/MCell, no light) were added to assess for lack of fit. Finally, as results pointed toward a sole effect of probe/cell ratio, two additional points were tested (2.7 and 3.6 nmol/MCell, 30 min, no light). The trials were realized with 1 mL of exponential phase culture with an optical density adjusted to 1.0 (at 750 nm). Manipulations were performed on the same day by the same operator, with cells coming from the same culture, all in duplicate.

2.5. Cell recovery and culture conditions

Once cells have been stained, one last critical step remains to be investigated: their recovery (and the incident removal of remaining CFSE). Three protocols were tested. First, a classical washing by high-acceleration centrifugation was applied (16,600*g*, 20 °C, 5 min) as it yields a solid pellet that is easy to handle. Second, a gentle washing by low-acceleration centrifugation was employed (100*g*, 20 °C, 30 min). The characteristics of this protocol were determined iteratively through preliminary tests to allow for cell pelleting at a minimal acceleration. Third, a filter-rinsing method was implemented. Cells were spread onto a cellulose filter (0.22 μm) and washed using culture medium; no vacuum nor pressure was applied. After about half an hour (time for the gravity filtration to be completed), the filter was rinsed with a culture medium to recover the cells. This last method was tested in case the reader would like to work with very fragile cells. Still, it should be noted that in this case, axeny and liquid volumes are more challenging to control.

Cells were then resuspended in fresh culture medium (25 mL to ensure unlimited access to light) and placed back in the incubator under a 10 h:14 h (light:dark) photoperiod. The photoperiod was set, in accordance with the literature for *Chlorella* strains [36], to obtain a semi-synchronized culture. By doing so, one avoids mistaking a refractory subpopulation for daughter or mother cells populations. As cells would need time to divide, those tests were carried out on Friday, and cells were analyzed on Monday (76 h after inoculation).

2.6. Test case

The optimized protocol was tested by monitoring the proliferation of semi-synchronized cells (10 h:14 h photoperiod). Here, we chose this test case to ensure that at least two generations of cells would be present simultaneously. Hence, we could assess the efficiency of CFSE staining in discriminating cell generations. The cultures (1 mL, optical density at 750 nm of 1.0) were tagged and inoculated on Monday (25 mL of fresh culture medium). Then, 1-mL samples were withdrawn twice daily over a week. With them, optical densities at 680 and 750 nm were measured (not reported). Part of the sample was then used for cell counting using a hemocytometer (Zeiss Axioplan 2). The remaining part was analyzed by flow cytometry. Cultures were carried out in triplicate with an unstained culture as control.

2.7. Flow cytometry, data handling and statistical testing

Flow cytometry analyses were carried out using a BD Fortessa x20 (with BD FACS Diva software). Four parameters were recorded: forward scatter (or FSC, blue laser at 488 nm) as a proxy of cell size, side scatter (or SSC, blue laser at 488 nm, 488/10 nm detection) as a proxy of cell complexity, chlorophyll fluorescence (red laser at 620 nm, 780/60 nm detection), and CFSE fluorescence (blue laser at 488 nm, 530/30 nm detection). At least 100,000 events (FSC above 5000) were acquired for each run. Post-treatment was realized with the *FlowCal* python library. Cells were identified based on the FSC and SSC signals. Chlorophyll

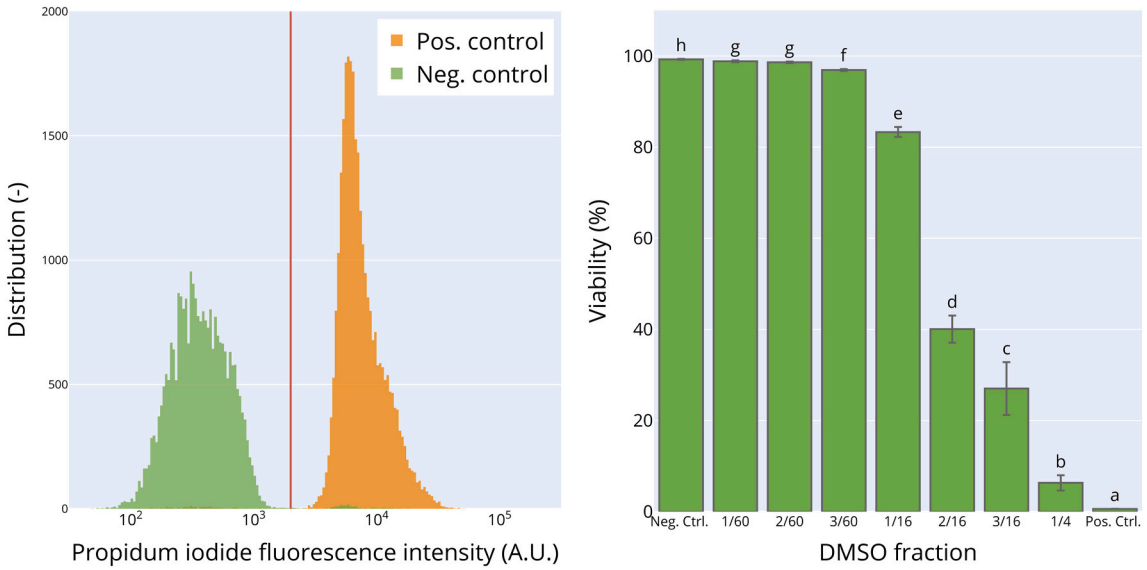

Fig. 1. Left - Cell fluorescence intensity on the propidium iodide channel (yellow-green laser, 610/20 nm detection). Red bar - threshold value to differentiate alive and dead cells. Right - Culture viability for different fractions of DMSO, presented as mean and standard deviation (error bars). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

fluorescence was not needed to identify *Chlorella vulgaris* cells as the samples were almost noise-free. Hypothetical outliers were removed by two-side 95 % Windsorisation [37] and data were log-transformed before being used for statistical analysis. Statistical testing was performed using *statsmodels* package's ANOVA model with pairwise *t*-test as a post-hoc test when a significant difference ($p < 0.05$) was reported [38].

3. Results and discussion

3.1. DMSO-induced mortality

Before assessing the effect of DMSO on cell viability, the first step was to ensure that alive and dead cells could be differentiated. Fig. 1 (left) presents the fluorescence signals on the propidium iodide channel for the positive and negative controls. As one can see, the staining procedure allows for a clear discrimination between the two cell conditions. The threshold was chosen at a fluorescence value of 2000 (red bar). Interestingly, the careful observer could note that the negative control exhibits a very small, yet non-zero, amount of dead cells (0.73 ± 0.17 %). The signal intensity of those dead cells is located at the same place as the positive control, a token of the quality of the procedure. Confident in the ability to discriminate between alive and dead cells, the influence of DMSO on the culture viability could be analyzed (Fig. 1 (right)). As one can see, even a minimal introduction of DMSO (22 µL to 1 mL, condition 1/60) induces a statistically significant decrease in cell viability (99.27 ± 0.17 % for negative control, 98.90 ± 0.30 % for 1/60 condition). Yet, the effect can be deemed marginal until 3/60 (66 µL to 1 mL), for which viability drops to 89.48 ± 0.27 %. Unsurprisingly, further increasing the DMSO fraction leads to a sizable loss in culture viability. In our case, it was deemed acceptable to work with up to 44 µL of DMSO in 1 mL of microalgal suspension (97.76 ± 0.26 % for 2/60 condition) to limit the number of stock solutions to prepare for the concentration screening. Yet, given the toxicity of DMSO, if the reader is not interested in a concentration screening, it is advised to work with a high concentration (10 mM) stock CFSE solution to limit the amount of DMSO introduced into the culture.

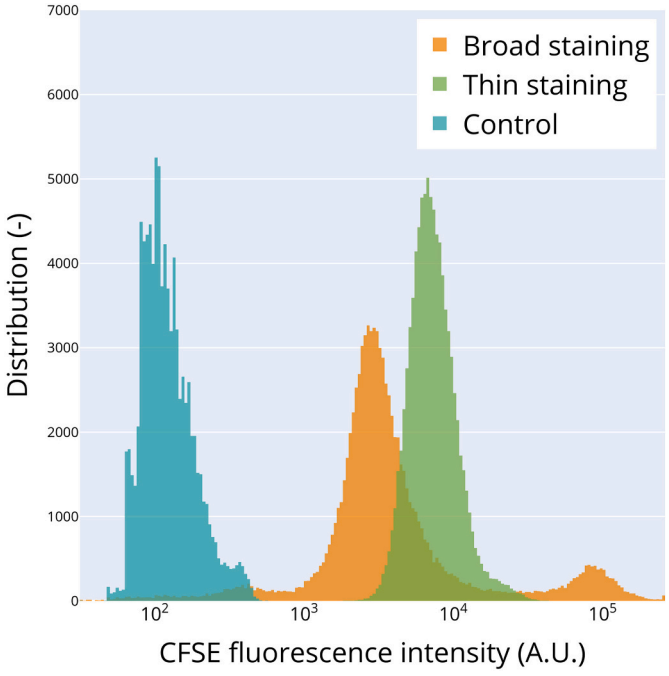

Fig. 2. Cell fluorescence intensity on the CFSE channel (blue laser, 530/30 nm detection). Control - unstained cells. Orange - 15 min, 1.80 nmol/MCell, dark. Green - 45 min, 7.20 nmol/MCell, dark. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.2. CFSE staining optimization

First of all, the diversity of staining quality is to be commented on. Fig. 2 presents three configurations: the control in blue, a successful thin staining in green, and suboptimal staining in orange. Two differences can be noted between the two stainings. First of all, the average intensity of the fluorescence peak is higher for successful staining. But more importantly, the run pictured in orange features a main population (around 2000) and two small subpopulations (around 500 and 10^5). This

Table 2
Results of the ANOVA treatment of the design on experiments. * factor deemed significant (p-value <0.05).

Factor	Avg. CFSE fluo. Intensity (log_{10})		Coefficient of variation (%)	
	Coefficient	p-Value	Coefficient	p-Value
Intercept	3.586	0.000	10.183	0.000
Time	0.047	0.082	0.138	0.661
Probe/cell	0.056	0.045*	−5.040	0.000*
Illumination	0.003	0.912	0.275	0.391
Time × probe/cell	0.097	0.003*	−0.783	0.032*
Time × illumination	−0.059	0.037*	1.083	0.007*
Probe/cell × illumination	0.017	0.489	−0.339	0.296
All combined	−0.019	0.443	−0.785	0.032*

observation raises the question of the origin of such different results. The run leading to thin staining was incubated for a long time (45 min) in the dark at a high probe/cell ratio (7.20 nmol/MCell), while the orange run leading to broad staining was incubated for a short time (15 min) in the dark at a low probe/cell ratio (1.80 nmol/MCell). This example underlines the need for a systematic approach to disambiguating the effect of the different factors (time and probe/cell ratio in this example).

Table 2 reports the results of the design of experiment analysis for the two outputs of interest, which are the cell's average fluorescence intensity on the CFSE channel and its coefficient of variation. The magnitude of the effect and statistical significance are reported for both parameters. Indeed, taken individually, these indicators bear only limited meaning [39].

Starting with the average cell fluorescence intensity on the CFSE channel, one can clearly see a difference of about two orders of magnitude between the intercept and the coefficients. Indeed, the largest one is the Time × Probe/Cell interaction, which represents 2.71 % of the intercept. Therefore, even though statistically significant, the effect is marginal. This conclusion is even more relevant for the other factors. Thus, the average cell fluorescence intensity on the CFSE channel can be deemed relatively independent from the tested factors

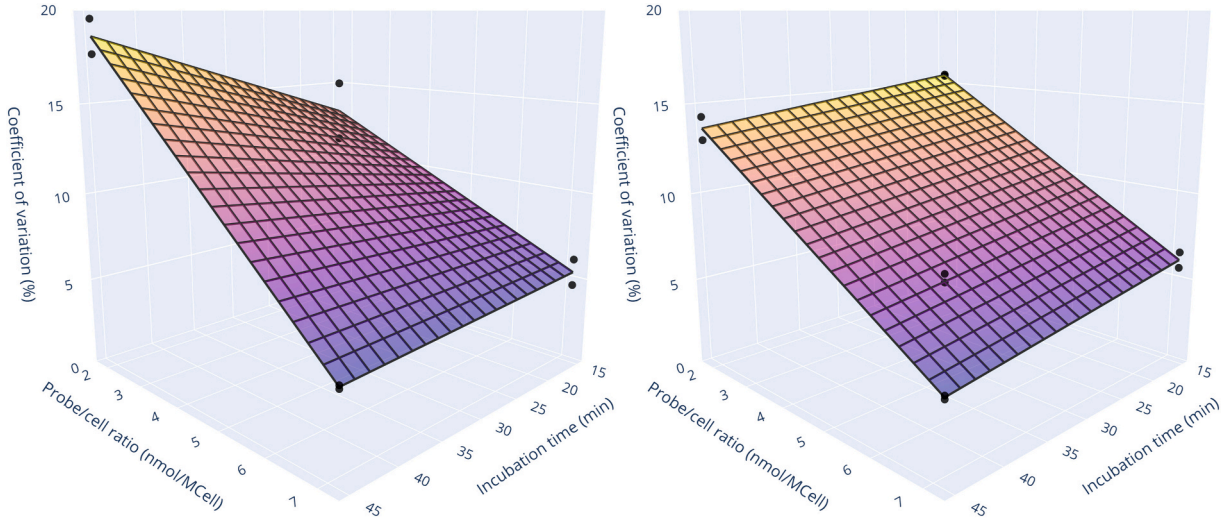

Fig. 3. Left - Response surface for the coefficient of variation built using samples processed under ambient illumination. Right - Response surface for the coefficient of variation built using samples processed in the dark. Note that the center points only appear in the case of incubation in the dark.

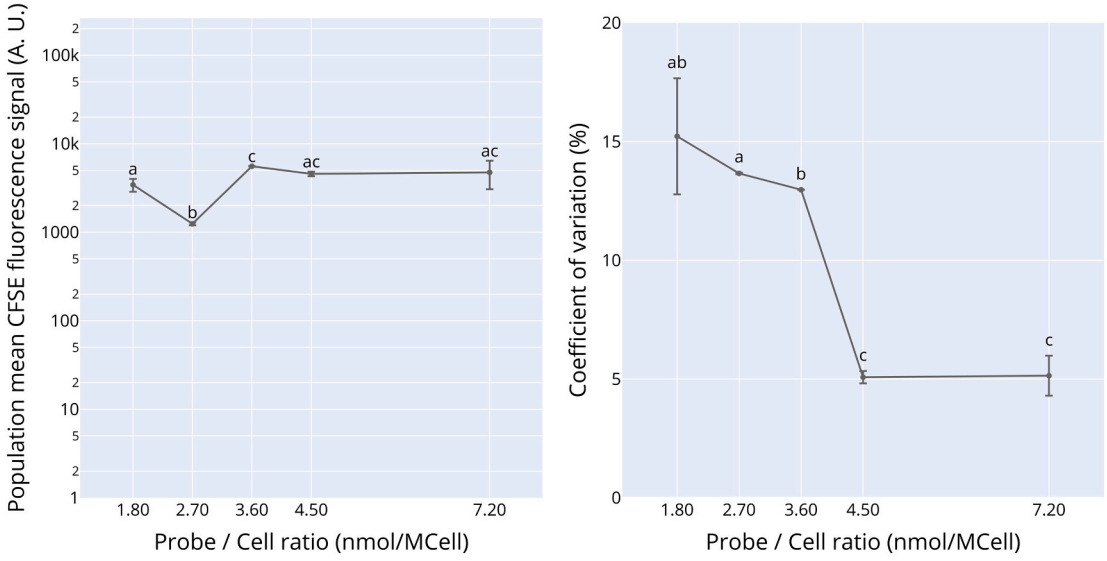

Fig. 4. Left - Average cell fluorescence intensity on the CFSE channel (blue laser, 530/30 nm detection). Right - Coefficient of variation cell fluorescence intensity on the CFSE channel (blue laser, 530/30 nm detection). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

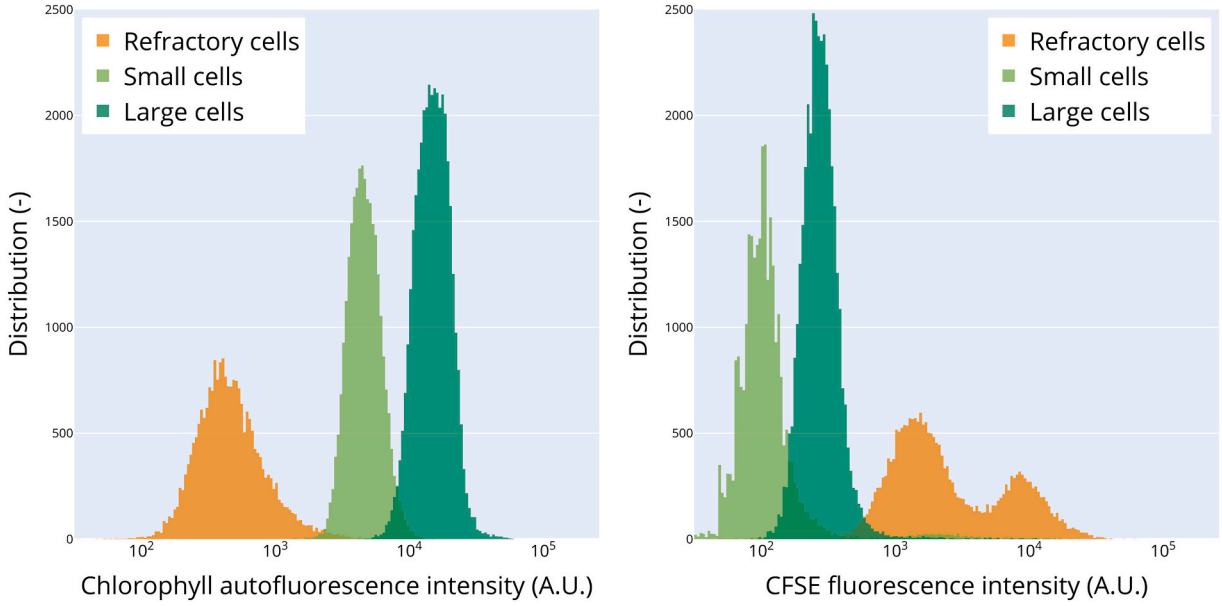

Fig. 5. Left - Cell fluorescence intensity on the chlorophyll channel (red laser, 780/60 nm detection). Right - Cell fluorescence intensity on the CFSE channel (blue laser, 530/30 nm detection). Medium inoculated with high-acceleration washed cell. Sample withdrawn 76 h after inoculation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

under the explored conditions. This assertion is reinforced by the high *p*-value for the lack of fit ($p = 0.583$).

Moving onto the coefficient of variation of cell fluorescence intensity distribution on the CFSE channel, a different analysis can be drawn. The most striking observation is the high magnitude of the effect of the probe/cell ratio. Taken alone, it can drive the dispersion from 15 % to 5 %. On the contrary, the time factor is only significant through its interactions, and its cumulated effects are nearing zero. Finally, illumination also has an effect through its interactions but not as a main factor. While Table 2 allows to dissect interactions and ascertain conclusions with figures, it is often easier to get a hold of this type of complex behavior through a graphical representation. Consequently, Fig. 3 presents two response surfaces, one for samples processed under ambient illumination (left) and the other for those processed in the dark (right). As one can see, the main effect indeed comes from the probe/cell ratio. The Time × Illumination interaction explains the difference in points placed the farthest from the viewpoint. Overall, it can be concluded that the response surface for samples incubated in the dark is consistently lower than its lit counterpart. It is, therefore, advisable to incubate samples in the dark. Regarding the incubation, under dark conditions and maximal probe/cell ratio, a duration of 15 min led to a coefficient of variation of 6.15 %, 30 min to 5.20 %, and 45 min to 4.27 %. Given the fact that the estimate of pure error lies at 1.22 %, it is difficult to advise with certainty. In our case, we continued the work by incubating our samples for 30 min, as 45 min was deemed too long. Yet, the reader willing to save time would only lose a marginal amount of precision by cutting this time to 15 min.

Focusing on the dark-incubated samples' response surface, the lack of fit ($p = 0.002$) indicates high non-linearity. Unreported tests showed that this effect was due to the probe/cell ratio and not the time. The probe/cell ratio was therefore refined with additional points (2.70 and 3.60 nmol/MCell). Fig. 4 presents the two quality indicators for CFSE staining. As one can see, average cell fluorescence intensity on the CFSE channel shows only minimal variations around a stable value (about 5000). This is in good agreement with the previous analysis of the response surface. On the contrary, the coefficient of variation exhibits a non-linear trend with a clear optimum at a probe/cell ratio of 4.50 nmol/MCell yielding a coefficient of variation of 5 %. Therefore, this ratio was chosen for the rest of the study.

3.3. Cell recovery

Once cells have been tagged, their separation from the remaining CFSE is to be addressed. From our experiments [16,18], high-acceleration centrifugation is a convenient method that is well-suited for microalgal flow cytometry arrays. Indeed, a pellet can be obtained in 5 min, and the temperature lowered to 4 °C to block cell metabolism. Still, this treatment can be considered as harsh and may increase the lag/recovery phase of culture washed with this method. Over the course of this work, we noticed an intriguing behavior. While this washing technique does not influence the flow cytometry reading conducted right after cell staining, it induces the appearance of a refractory population when cells are transferred to a fresh culture medium. As one can see in Fig. 5, even 76 h after inoculation, this refractory population stands out by its low chlorophyll autofluorescence intensity and high CFSE fluorescence signal, even exhibiting two subpopulations. These two observations drive the conclusion that those cells might have been internally damaged by the centrifugation (potentially ruptured chloroplast leading to a decrease in chlorophyll autofluorescence intensity). Ultimately, those damages would have put their division cycle to a halt. Furthermore, even after 76 h, these cells represent 25.09 % of the total population. As the analysis of the by-standing cells suggests that they are healthy (classical chlorophyll autofluorescence intensity) and dividing (CFSE fluorescence intensity nearing background level), the original amount of refractory cells must have been much higher than 25.09 %.

In contrast to high-acceleration centrifugation, low-acceleration centrifugation (100 *g*, 30 min) and filter-rinsing washing and recovery methods allowed to conduct cultures free of refractory population. Therefore, low-acceleration centrifugation is advised, as it is more convenient, if the cells can handle it. Otherwise, the filter-rinsing procedure can be employed, yet it requires more material and takes more time.

3.4. Test case - cell proliferation tracking

As a test case, three semi-synchronous *Chlorella vulgaris* cultures were tagged and monitored over one week. The three replicates yielded similar behavior with small cells (divided overnight) and large cells (not divided overnight) present at the same time. Fig. 6 (left) presents the

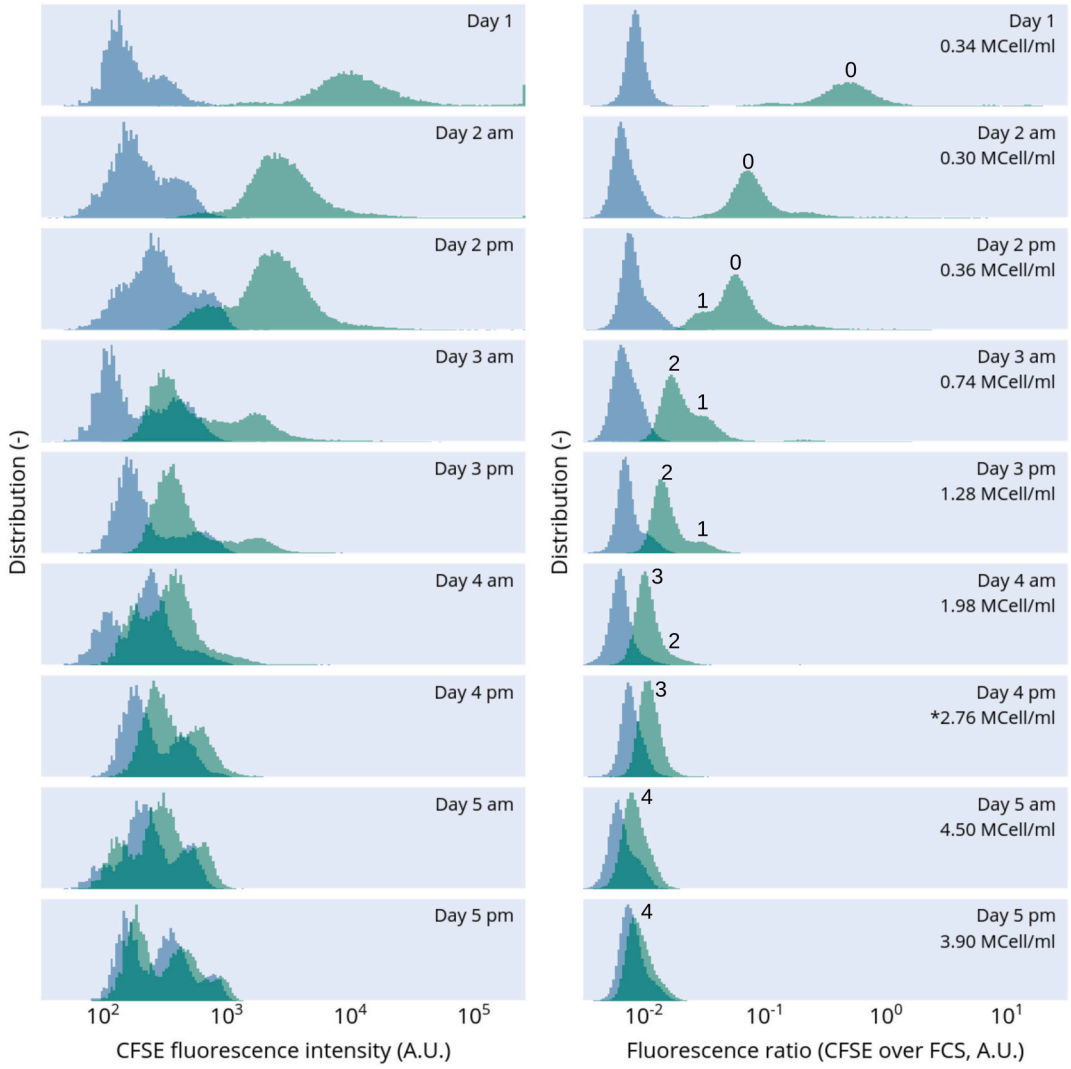

Fig. 6. Left - Cell fluorescence intensity on the CFSE channel (blue laser, 530/30 nm detection). Right - Ratio between the cell fluorescence intensity on the CFSE channel (blue laser, 530/30 nm detection) and Forward SCatter channel (blue laser). Blue - unstained cells used as control. Green - CFSE-stained cells. * on day 4 afternoon the cell counting is believed to be an artifact (corroborated by optical density measurements). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

evolution of one of the triplicates. In addition to cell fluorescence intensity on the CFSE channel, cell density was also obtained by hemocytometer counting. Several observations are to be commented on.

First, the cell fluorescence intensity on the CFSE channel decreased between days 1 and 2 without a significant change in cell count. It can be explained by the fact that CFSE binds to free amine groups. As this binding is not specific, CFSE targets both long-lasting cytoskeletal proteins and other short-turnover molecules presenting an amine group. The degradation of these molecules induces a natural decrease in CFSE fluorescence intensity. This behavior was originally observed and commented on by Lyons et al. [24], but not reported explicitly for microalgae (either not observed [28], or clouded by a 1-day recovery stage [26]). Hence, the observed decrease in the fluorescence signal is not to be mistaken for a cell division. This comment also underlines the need for a cell counting procedure parallel to the CFSE staining one.

Second, when associated with cell division (day 2 and on), CFSE fluorescence intensity decreased sequentially. Yet, the raw cell fluorescence intensity on the CFSE channel quickly overlaps the background signal. By processing the raw signal, it is possible to identify two generations with certainty, maybe three. The possibility to count numerous generations of *Chlorella* cells is hindered by two factors: the fact that microalgae feature a non-negligible carotenoid pigment autofluorescence on the CFSE detection channel [40], and the fact that *Chlorella* cells divide in four, *versus* two for lymphocytes. Nevertheless, it is possible to go further and use this raw signal to analyze the decrease in CFSE fluorescence intensity between the two generations. Taking the reading for the second day afternoon as an example, the ratio between the two populations' averages is 0.2518, 1/4 in short. This is a perfect agreement with the expected value, as a *Chlorella vulgaris* mother cell naturally divides into four daughter cells.

Still, discriminating generation is made even more difficult by the simultaneous presence of small and large cells. Indeed, as flow cytometry is based on a cell-laser interaction, change in cell shape (small or large) modulates the way fluorescence is emitted by the cells and harnessed by the detector. To overcome this Lyons et al. [24] suggested to sort cells (with a cell sorter) prior to their analysis. While doable, it is impractical and extremely expensive. Here, we suggest the use of the ratio between the CFSE and the FSC (proxy of size) signals intensities. As one can see in Fig. 6 (right), this technique dramatically improves the signal quality and allows for an easier generation counting. With this approach, up to four generations can be discriminated.

3.5. Applicability

Before parting, the proposed protocol is to be summarized and discussed. From this work, it is advised to dissolve CFSE in pure DMSO at a concentration of 10 mM and store this stock solution in the dark at −20 °C. Staining is to be carried out at a probe/cell ratio of 4.50 nmol/MCell while minimizing the amount of DMSO introduced in the culture. If resistant enough, cells can then be washed by low-acceleration centrifugation (100 *g*). Otherwise, a gentle filter-rinsing method is advised. Over the course of a culture, the obtained signal should be analyzed in conjunction with cell counting. Finally, when processing the results, it is advised to work with the ratio between the CFSE and the FSC signals intensities, as it nullifies cell morphology contribution to the signal distribution and ease generations discrimination.

To be deployed, this workflow only relies on the most common laser in flow cytometry (blue 488 nm). Microalgal chlorophyll autofluorescence has been shown not to be a problem. Yet, carotenoid pigments autofluorescent create a background noise on the CFSE fluorescence detection channel. To overcome this problem, one is advised to take advantage of the low quantum yield of carotenoids and increase the blue laser power to further set apart CFSE and pigment signals. In this regard, increasing the probe/cell ratio above 4.50 nmol/MCell has not brought any benefit in our case.

Finally, if one uncompromisably needs the detection window of CFSE for another probe, Quah et al. reviewed two alternative cell division tracking dyes detectable on other fluorescence channels [30]. Cell Trace Violet was deemed an acceptable alternative to CFSE (coefficient of variation below 20 %, and up to 5 lymphocyte divisions counted), while Cell Proliferation Dye eFluor 670 was judged inferior on all dimensions, and even transfers between stained and not pristine cells.

4. Conclusion

A systematic optimization methodology has been deployed to adapt the CFSE cell proliferation tracking technique to the microalga *Chlorella vulgaris*. The toxic effect of the CFSE solvent (DMSO) was delineated. Then, incubation conditions (time, probe/cell ratio, illumination) were optimized. Finally, CFSE washing and cell recovery were robustified. Using a semi-synchronous culture as a test case, the method was successfully applied to count cell divisions. Up to 4 generations could be discriminated. The generation-to-generation signal ratio was exactly 1/4, corresponding to the natural division of *Chlorella vulgaris*. Furthermore, an advanced yet easy-to-implement signal processing technique was introduced to ease generation discrimination.

CRediT authorship contribution statement

Victor Pozzobon: Methodology, Formal analysis, Conceptualization, Project administration, Software, Supervision, Validation, Writing – original draft. **Jules Lagirarde:** Formal analysis, Validation, Writing – review & editing. **Clarisse Arnoudts:** Formal analysis, Investigation, Methodology, Validation, Writing – review & editing. **Wendie Levasseur:** Formal analysis, Methodology, Validation, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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