Chapter 5

Efficient Interpretation of Multiparametric Data Using Principal Component Analysis as an Example of Quality Assessment of Microalgae

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Additional information is available at the end of the chapter

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Abstract

Multiparametric flow cytometry (FCM) realizes high-throughput measurement, but multiparametric data make it difficult to interpret the complicated information. To present clear patterning graphs from FCM data, one must grasp the essence of the data. This study estimated the usefulness of principal component analysis (PCA), which reduces multidimensional information to arbitrary one-dimensional information. Recently, microalgae have attracted the attention of pharmaceutical, cosmetic, and food companies. Taking alga *Chlorella* as an example, this chapter presents the usefulness of PCA for the evaluation of algal quality using FCM. To evaluate the algal status effectively, *Chlorella* (control), heated algae, and metallic-treatment algae were prepared and quantified using FCM. FCM data were subjected to PCA analysis. To interpret correlativity among parameters, FCM data are generally expressed as histograms and scatter or contour plots. An operator using multiple parameters has difficulty finding high correlativity among parameters and presenting an effective graph. The PCA method produced new comprehensive axes with different inclination factors among parameters. Scatter plots using new axes showed patterns treatment dependently with different vectors. Results show that the PCA method can extract information of test objects from data and that it can contribute to effective interpretation of cell characteristics, even if data include multiparameters from FCM.

Keywords: flow cytometry, multivariable analysis, cell status, cell cycle, *Chlorella*, chlorophyll, trace metal elements, slag

1. Introduction

Flow cytometry (FCM) can provide cell optical information from microbes to model animal and plant cells. Over the last several decades, FCM with those fundamental characteristics has

served as a powerful and invaluable tool in fields such as cell biology, microbiology, protein engineering, and health care [1]. Actually, FCM has functions to conduct several procedures such as cell counting, biomarker detection, cell cycle analysis, and cell sorting. Clear patterning graphs from FCM data can elucidate correlation among several parameters. Recent FCM systems enable a user to analyze up to a dozen multiparameters including scattered light parameters in a single assay [2]. In fact, multiparametric detection realizes high-throughput measurement and cost-performance and is also time-saving of experiments in life science. For instance, ten combined experiments must be conducted when one examines five parameters of interest using several designed FCM experiments with three-color fluorophores (designated as three-color FCM). By contrast, when using a designed FCM experiment with five-color fluorophores (five-color FCM), correlation between the five parameters can be examined from only one experiment, in principle. Generally, the number of combined experiments is calculable using Pascal's triangle (**Figure 1**).

However, the number of available colors used in each experiment is restricted in conjunction with both numbers of excitation lasers and corresponding emission filters used in an instrument. **Figure 2** portrays excitation and emission spectra of representative fluorophores, as examined using online software (SpectraViewer; thermo Fisher Scientific Inc.). When only a single blue laser operating at 488 nm is used for multicolor FCM, the emission spectra of fluorophores shown in **Figure 2** resemble those in **Figure 3**. Several areas of overlapping of

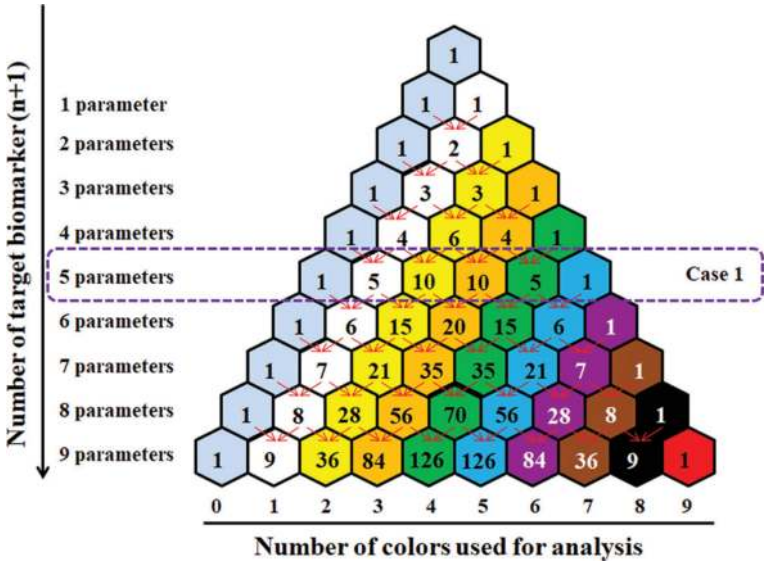

Figure 1. Correlation between the number of target parameters and that of colors used in an experiment, as shown by the Pascal's triangle. Ten combined experiments must be conducted to measure five parameters of interest using two-color FCMs for case 1 of **Figure 1**, although a system using four-color FCMs requires only five combined experiments to measure them. It is noteworthy that an experiment using just one-color FMC cannot examine any correlation between target parameters except for scattered light parameters. Multicolor FCM (more than two-color FCM) must be used to find correlation between parameters.

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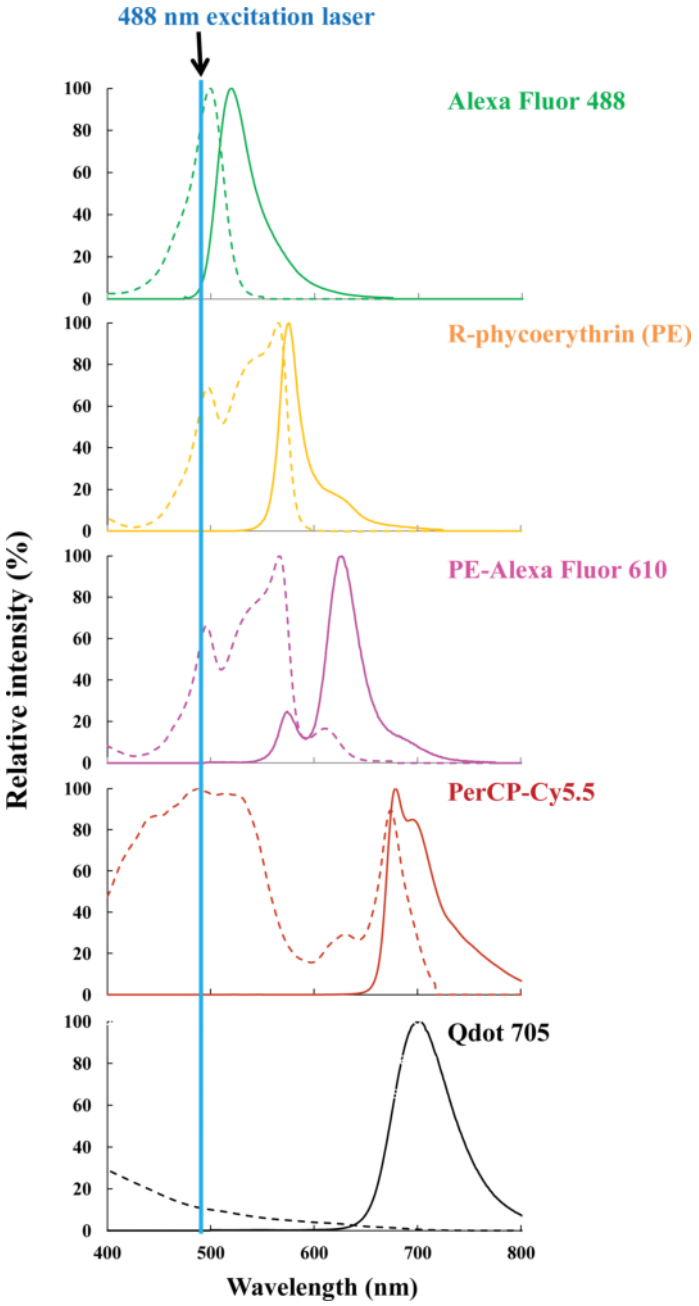

Figure 2. Fluorescence properties of representative fluorophores examined using online software (SpectraViewer; thermo Fisher Scientific Inc.). Dotted lines and solid lines respectively show the excitation spectrum and emission spectrum of each fluorophore. A vertical blue line signifying 488 nm as an example is included in each graph.

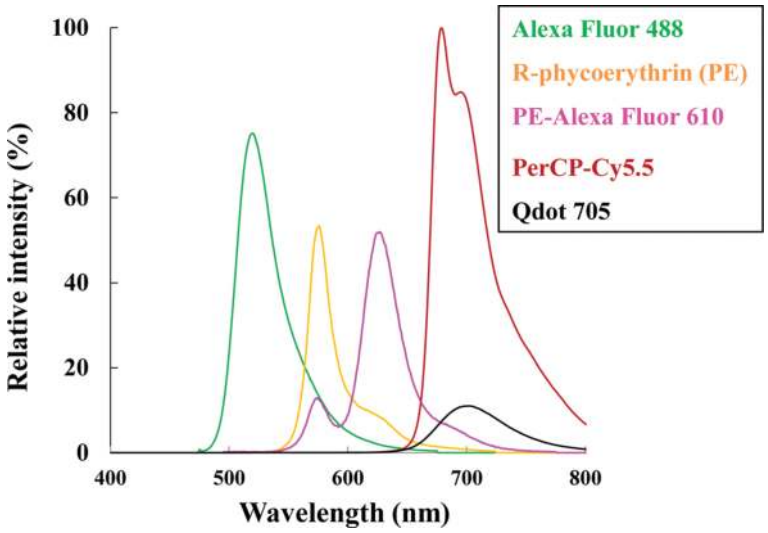

Figure 3. Fluorescence properties of representative fluorophores excited using a blue laser operating at 488 nm as an example. The graph reflects differences in the excitation intensity of each fluorophore excited using a blue laser. Here, the emission intensity of each fluorophore was calculated from each excitation spectrum in **Figure 2**.

emission spectra occur because the spectra of some fluorophores are flared at the bottom. Along with overlapping of emissions, differences of excitation efficiency might present simultaneous difficulties for multicolor FCM analysis (**Figure 3**). Using a flow cytometer detecting two colors to five colors per single laser, even when using a more high-end instrument than that described above, one must commonly discuss and interpret correlation between multiparameters based on several combined results. Just to be sure, all fluorophores excited by an arbitrary single laser does not necessarily work together because of differences in the emission efficiency of each fluorophore.

In contrast to the benefits of multiparametric FCM, multiparametric data make it difficult to get rid of extraneous data and reach an interpretation of the complicated information. Although one can make multi-dimensional graphs digitally, it is not easy to reach an accurate and clear conclusion from any multi-dimensional graph. To present clear patterning graphs from complicated FCM data, an analyst must be able to grasp the essence of the data.

To extract the essence of FCM data, this study applied principal component analysis (PCA) for multivariate analysis to the complicated FCM data and estimated the usefulness of the PCA method. Recently, some microalgae have already generated a lot of attention from pharmaceutical developers, cosmetic manufacturers, and food companies. The industrial application of algae demands the assessment of their qualities in culture. Taking green alga *Chlorella* sp. as an example and as a convenient organism for FCM, this study presents the usability of PCA method for the assessment of algal quality using FCM.

2. FCM analysis of microalgae

In addition to the numerous but unappreciated roles of phytoplankton, including microalgae, in aquatic ecosystems to support yields of fish and shellfish, several microalgae have also attracted attention from several pharmaceutical and vitamin supplement developers, along with food companies [3, 4]. Biotechnologies are sometimes classified into colors based on their respective research areas: red biotechnologies are related to medicine and medical processes. White ones are associated with industrial processes including production of chemicals [3] and biofuels [5]. Gray ones are directly related to the environment. Green ones are connected to agricultural processes including environmentally friendly solutions as alternatives to traditional processes [3, 4, 6, 7]. Blue technologies are related to marine and aquatic processes. Finally, black ones are used to develop bioterrorism. Microalgal applications have the potential to be related to most of those biotechnologies. Autotrophic algal biorefineries, for instance, can present great advantages over conventional refineries that manufacture materials using fossil fuels and over conventional microbial biorefineries that use fermentation, which requires food nutrients for microbes.

The industrial application of algae demands the selection of useful algal species, the evaluation of algal features, and the assessment of their qualities in culture [4]. The algal quality demanded is particularly important because microalgal metabolisms are strongly affected by even trace levels in the concentration of various organic and inorganic pollutants such as heavy metals [1, 8]. When assessing algal quality in culture and using those algae in industrial application, analyzing their life (cell) cycle is a crucially important technique. Cell cycle analysis using FCM is a standard procedure in versatile application of FCM. Considering the cell size of microalgae, unicellular algae such as *Chlorella* sp. are convenient model organisms for microalgal studies using FCM [9].

Algae have chlorophyll as an endogenous fluorescent biomolecule (**Figure 4A** and **B**). FCM in analogy with spectrofluorometry can pick up the chlorophyll fluorescence of algae and can evaluate some properties including chlorophyll and scattered light signals of an individual alga [9–15]. **Figure 4A–C** portrays *Chlorella*-like alga and its fluorescence properties. The wavelength of the maximal fluorescence near 680 nm is from algal chlorophyll (solid curve in **Figure 4C**). Algae are sensitive to heat treatment (dotted curve in **Figure 4C**) [11–14] because the thermal stress damages the thylakoid membrane, which is related to structural and functional changes of the photosystem (PS) II and PS I, thereby interrupting the Calvin cycle [16, 17]. Inducing heat stress in algae reduces chlorophyll fluorescence (dotted curve in **Figure 4C**) and increases yellow fluorescence derived from chlorophyll degradation [11]. Consequently, red fluorescence can indicate vigorous algae, whereas yellow fluorescence indicates stressed and dying algae [11–14]. **Figure 4D** takes a dotted graph from FCM data using a *Chlorella*-like alga (SA-1 strain) to present an example. Both the cell size detected as forward scatter signals (FSS) and chlorophyll contents of algae as red fluorescence channel are correlated strongly with the algal cell cycle [9, 10, 15, 18]. Here, algae are categorized into three populations (Stages 1–3) as described in reports of previous studies [9, 10, 15, 18]: Stage (St.) 1, “growth” stage; St. 2, “maturation” stage; and St. 3, “division and autospore liberation” stage in **Figure 4D**.

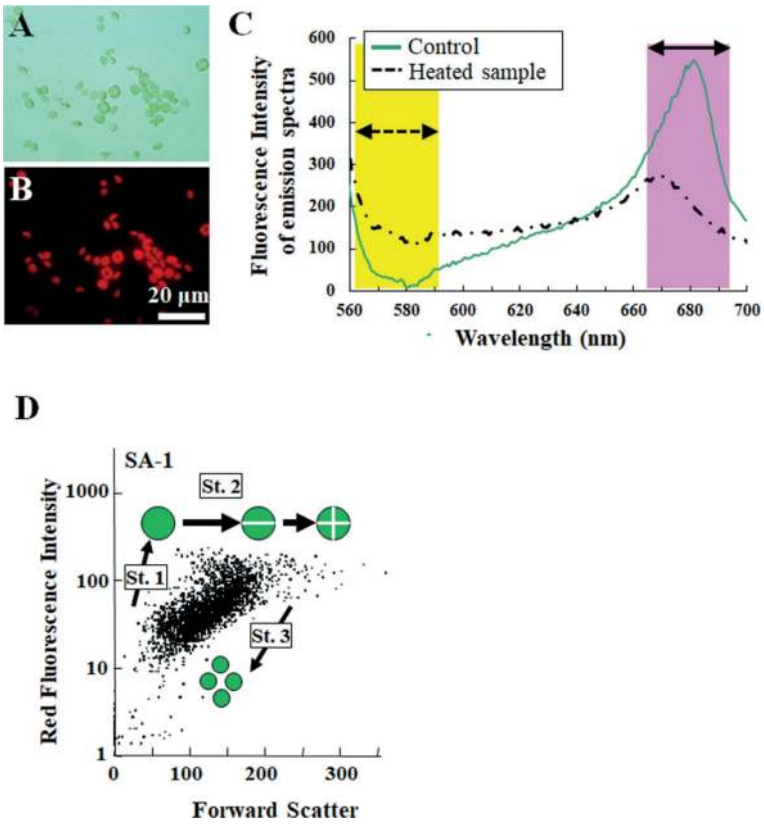

Figure 4. Fluorescence characteristics of algae and microphotographs of *Chlorella*-like alga experimentally isolated from ciliate *Paramecium bursaria*. A bright field image of *Chlorella*-like alga (A) and the corresponding fluorescence image derived from chlorophyll (B) are shown. Panel C presents fluorescence characteristics of *Chlorella*-like alga obtained using fluorescence spectroscopy. Emission spectra of algae are shown with (dotted line, heated algae) and without heat treatment (solid line, control algae). Yellow (dotted arrow) and pink (solid arrow) areas, respectively, represent detection ranges of yellow and red fluorescence channels for FCM used for this study (see *Research methods*). Panels A-D were referred and partly modified from the literature [1, 13, 15, 18].

3. Research methods

This study investigated the algal status such as viability using FCM after treatment of algae with the test condition. For this study, the author used *Parachlorella kessleri* (C-531 strain) (new nomenclature for *Chlorella kessleri*) as the model organism representing algae. The alga was obtained from the Institute of Applied Microbiology (IAM) culture collection at The University of Tokyo. Before experiments, algae were grown on CA agar plates at pH 7.2 [19] under an LD cycle (12 h light/12 h dark) at approximately 1100 lux of natural white fluorescent light and 23 ± 2°C. The algae were scratched with an inoculating needle and were suspended in fresh CA liquid medium at pH 7.2.

Chlorella (initial density of 1.0×10^4 cells/ml adjusted using hemocytometry) grown in CA medium as a control condition for 1 week under an LD cycle and algae treated with metal eluate for 1 week as a test condition were prepared, respectively, as described in earlier reports [1, 12, 14]. Moreover, algae treated with heat for 5 min at 100°C were prepared. Here, the test conditions were reference standards subjected, respectively, to metallic eluate from steelmaking by-products and heat stress. A detailed description of the metal eluate reveals that the metal eluate was made from stainless steel slag (**Table 1**) subjected to a leaching test based on JIS K0058-1: 2005 (method for chemicals in slags Part 1: Leaching test) [12, 14, 20–23]. **Table 1** presents compositions of stainless steel slag particles used for this study [12, 14, 20–22]. In brief, slag used for this study mainly contains SiO_2, CaO, Al_2O_3, MgO, MnO, and Cr_2O_3 [12, 14]. Here, all Fe and Cr compounds are described, respectively, as FeO or Cr_2O_3 because it is generally difficult to distinguish FeO and Cr_2O_3 formed from Fe and Cr in a suspended metal solution at the occasion of elemental analysis after alkali fusion of stainless steel slag [1, 12, 14].

After elution from slag at pH 6 adjusted with HCl, the solution was filtrated with a 0.45 μm pore filter to eliminate slag particles. Then the solution was used for bioassay with *Chlorella* as a test solution including trace metals. **Table 2**, which shows components of the metal eluate used for this study, includes environmental quality standards for soil pollution, marine pollution, and water pollution, along with other standards for eluent and drinking water for reference. In this study, CA medium containing eluates was first made from 25 vol% of the concentrated CA medium, which had four times that amounts of respective chemicals for making CA medium, and 75 vol% of mixture of arbitrary amounts of eluate, a definite number of algae (1.0×10^4 cells/ml), and ultrapure water. Therefore, nutrient amounts of CA medium containing eluates were the same as those of CA medium alone, but the concentrations of chemicals derived from eluate differed from those of CA medium without eluate as described in reports of earlier studies [1, 12, 14].

To characterize each algal sample using FCM, this study used a cell analyzer (Muse™; Merck Millipore Corp., Hayward, CA) with a green laser operating at 532 nm as an excitation light source, a photodiode for detection of FSS, and two fluorescence filters of a 680/30 nm band pass (BP) filter suitable for chlorophyll fluorescence (red fluorescence) and a 576/28 nm BP filter suitable for chlorophyll degradation (yellow fluorescence) (**Figure 4C**) [1, 11, 12, 14].

This study was undertaken to evaluate the correlativity between algal properties and the test condition. To evaluate the correlativity among multiple properties of algae and each stress factor, PCA of multivariate analysis was used for this study using software for multivariate analysis (Institute of Statistical Analyses, Inc.). A dimensional reduction technique, PCA, reduces multi-dimensional information to arbitrary one-dimensional information, which is a

	FeO	SiO_2	CaO	Al_2O_3	MgO	MnO	Cr_2O_3	ZnO	NiO	CuO
Slag A	0.74	44.1	33	5.39	7.68	4.09	3.29	0.01	0.06	0.024

Table 1. Chemical compositions of steel slag used for this study (mass%) referred from the literature [1, 12–14].

Origin of slag		Eluate of stainless steel slag	Environmental quality standards				
			Soil pollution	Marine pollution	Water pollutant	Effluent standard	Drinking water standard
Regulated substances	Total As	ND[1] (RDL[2]: 0.001)	0.01	0.1	0.01	0.1	0.01
	Total B	0.16	1		1[4]	10[4], 230[5]	1
	Total Be	ND (RDL: 0.0005)		2.5			
	Total Cd	ND (RDL: 0.0001)	0.01	0.1	0.01	0.03[6]	0.003
	Chromium (VI)	ND (RDL: 0.005)	0.05	0.5	0.05	0.5	0.05
	Total Cu	0.003	0.001	3		3	1
	Total Pb	ND (RDL: 0.0005)	0.01	0.1	0.01	0.1	0.01
	Hg	ND (RDL: 0.0001)	0.0005	0.005	0.0005	0.005	0.0005
	Total Ni	0.001	0.001	1.2			0.02
	Total Se	0.012	0.01	0.1	0.01	0.1	0.01
	Total V	ND (RDL: 0.001)		1.5			
	Total Zn	0.099		2	0.03[7], 0.02[8], 0.01[9]	2	1
	F^-	ND (RDL: 0.1)	0.8	15	0.8[4]	8[4], 15[5]	0.8
Substances out of regulation	Total Al	ND					
	Total Ca	9.3					300[10]
	Total Fe	ND				10	0.3
	Total Mg	0.9					300[10]
	Total Mn	0.028				10	0.05
	Total Si	1.8					
	Total N	0.4			0.1–1[11] 0.2–1[8]	100	0.04[12], 10[13]
	Total P	ND (RDL: 0.1)			0.005–1[11] 0.02–0.09[8]	16	

[1]Not detected.

[2]Reportable detection limit.

[3]These data from a previous study reported by Takahashi et al. [17].

[4]Standard value is not applied to coastal waters.

[5]Standard value is applied to coastal waters.

[6]The Cd value has changed from 0.1 to 0.03 mg/L since December 2014.

[7]Habitable river or lake for aquatic life.

[8]Habitable coastal water for aquatic life

[9]Habitable coastal water that requires conservation in particular for nidus and nursery ground.

[10]Total concentrations of both calcium and magnesium are limited for water hardness.

[11]Habitable lake for aquatic life.

[12]Total N contents derived from nitrite nitrogen.

[13]Total N contents derived from both nitrite nitrogen and nitrate nitrogen.

Table 2. Environmental quality standards regarding pollutions and others for effluent and drinking water, and concentrations of elements of eluate (mg/L) referred from the literature [1, 12–14].

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dataset from a new axis produced by PCA [15]. According to results of the correlation matrix analysis for the data, the author calculated the contribution rate of each component, the factor loading of each parameter, and the score plot of each component. Here, each factor loading (PC1-3) generally indicates correlation factors between each parameter and each component (**Figure 5**). The statistical results obtained using PCA were interpreted to evaluate the algal status between control and test conditions.

After treatment of algae with and without eluate, the algae were quantified using hemocytometry. Here, CA medium containing several concentrations (0–70 vol%) of the metallic eluate was used for the experiment using hemocytometry. The algal proliferation ratio (average ± standard error) was expressed as a proportion of the number of algae treated with eluate to that of control without eluate [1, 12, 14].

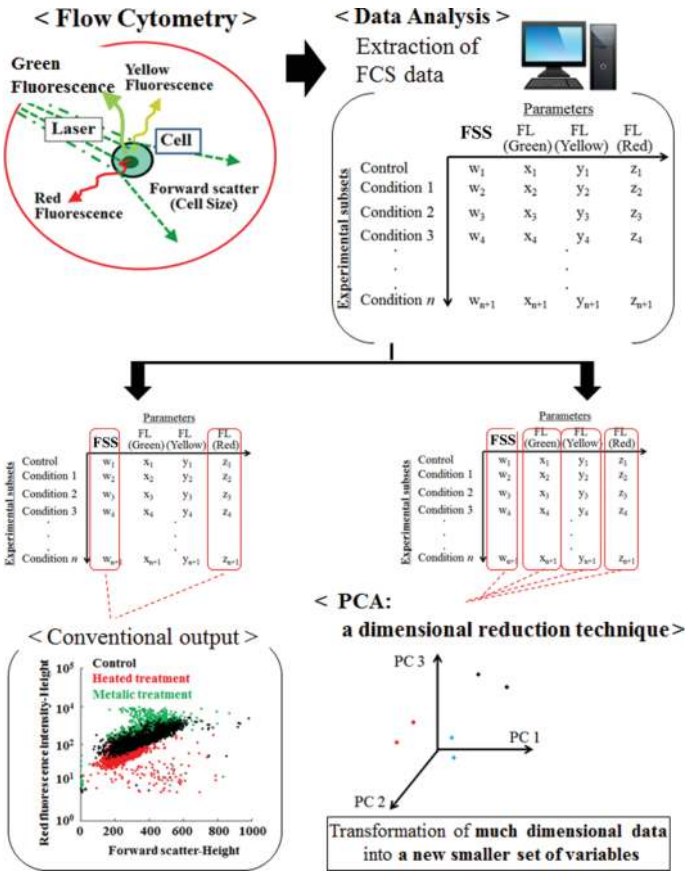

Figure 5. Outline from FCM analysis to PCA analysis of multivariate analysis. All data from FCM were extracted and subjected to PCA. Only one or two parameters are used to produce a one-dimensional (1D) histogram and 2D scatter or contour plots in conventional FCM analysis. These graphs, however, ignore some parameters of data (left below in **Figure 5**). To incorporate all information of the data, PCA transforms multi-dimensional data into a new smaller set of variables (right below in **Figure 5**).

4. Results and discussion

This study compared the effects of metallic eluate from stainless steel slag and heat treatment as an experimental stress factor on algal status, specifically that of *Chlorella* sp. [1, 12–14]. Here, CA medium containing 50 vol% of the metallic eluate was used for FCM analysis. To interpret correlativity among several parameters, FCM data are generally expressed as a 1D histogram and 2D scatter or contour plots. The more parameters an operator uses, the more difficult it becomes for the user to find high correlativity among parameters and to present an effective graph. The PCA method produced new comprehensive axes including several parameters, which have different inclination factors among parameters. The primary (PC1) and secondary (PC2) and tertiary (PC3) components, respectively, reflect 53.4, 34, and 12.6% of information for the data examined in this study (data not shown). **Figure 6A** and **B** presents the principal component loading of PC1 and PC2. Each loading shows that all parameters, including the algal size (FSS-H), red fluorescence intensity (Red-H), and yellow fluorescence (Yellow-H), are positively correlated with PC1 (**Figure 6A**). Particularly, correlation factors for both the algal size and the red fluorescence intensity were more strongly positive with PC1 than the yellow fluorescence intensity was. By contrast, the red fluorescence intensity and the yellow fluorescence intensity, respectively, show inverse and positive correlation with PC2 (**Figure 6B**). The 2D scatter plots using new axes show patterns with individually different vectors treatment dependently, as expressed by the score plot of PC1 versus PC2 (**Figure 6C**). The graph using new axes from PCA helps us to infer strong correlation between a particular parameter and the corresponding one. Consequently, the characteristics of both algal size and red fluorescence intensity are mainly reflected as the variation of algae on the positive PC1 axis (**Figure 6A** and **C**), whereas only yellow fluorescence mainly affected the variation of algae on the positive PC2 axis (**Figure 6B** and **C**). Results show that both the cell size (or red fluorescence intensity) and yellow fluorescence intensity of algae can be indicators that facilitate assessment of the variation for comparison of algae between control and heat treatment (**Figure 6C**), whereas both the cell size and red fluorescence can be indicators for comparison of algae between control and the metallic treatment (**Figure 6D**).

The results (**Figure 6**) from PCA analysis prompted us to produce plots of FSS or the red fluorescence for algae versus the yellow fluorescence intensity for algae (**Figure 7**). The 2D-dotted graph of the red versus yellow fluorescence intensity for control algae, for instance, showed 10^2–10^3 on the red channel and 10^1–10^2 on the yellow, whereas that for the heated algae showed 10^1–10^2 on the red channel and 10^1–10^3 on the yellow. By contrast to the heat treatment, the dot distribution of algae treated with metallic eluate closely resembled that of control, although that with the eluate shifted slightly upward relative to that of control algae [1, 12–14]. In analogy with the result (**Figure 6C**) from PCA analysis, the difference of algae between the control condition and metallic treatment is slight compared to the difference of algae between control and heat stress (**Figure 7**).

To conduct a precise comparison of algae of control and metallic treatments, the plot of FSS versus red fluorescence for algae was produced (**Figure 8**). Although the dot distribution

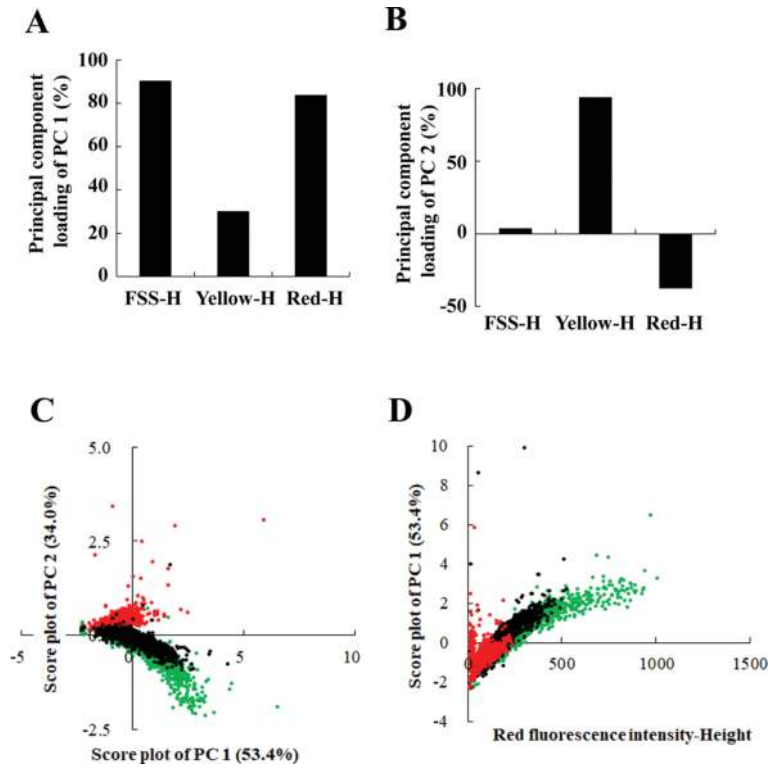

Figure 6. Condition-dependent distribution of *Chlorella* obtained using PCA method. The PCA reduces multi-dimensional information to arbitrary one-dimensional information and produces new components such as PC1–PC3. Here, factor loading plots of each parameter for PC1 (A) and PC2 (B) are shown. A score plot of PC1 vs. PC2 (C) and that of PC1 vs. red fluorescence intensity of algae (D) were produced using data from different test conditions.

of algal signals between the control and the metallic treatment was almost identical to that of the graph of the red versus the yellow fluorescence (**Figure 7**), both distributions differed on the graph of FSS versus the red fluorescence (**Figure 8**). A distinctive population (arrow in **Figure 8**) was found from algae treated only with metal eluate but not control. Drawing on the result from algal life (cell) cycle (**Figure 4D**), detection of the distinctive population in algae treated with metal eluate indicates that the algal cell cycle proceeds smoothly under the condition with metal eluate. By contrast to algae treated with metal eluate, the cell cycle of control algae seems to reach a stable stage such as a stationary phase, resulting in the near cessation of algal proliferation or extremely low proliferation activity.

In addition to estimation of algal population dynamics using FCM coupled with PCA analysis, direct quantification of algae using hemocytometry was conducted as described in earlier reports [1, 12–14]. The quantification specifically examined whether algal growth dynamics implied from the result of PCA analysis (**Figure 8**) was confirmed on algae

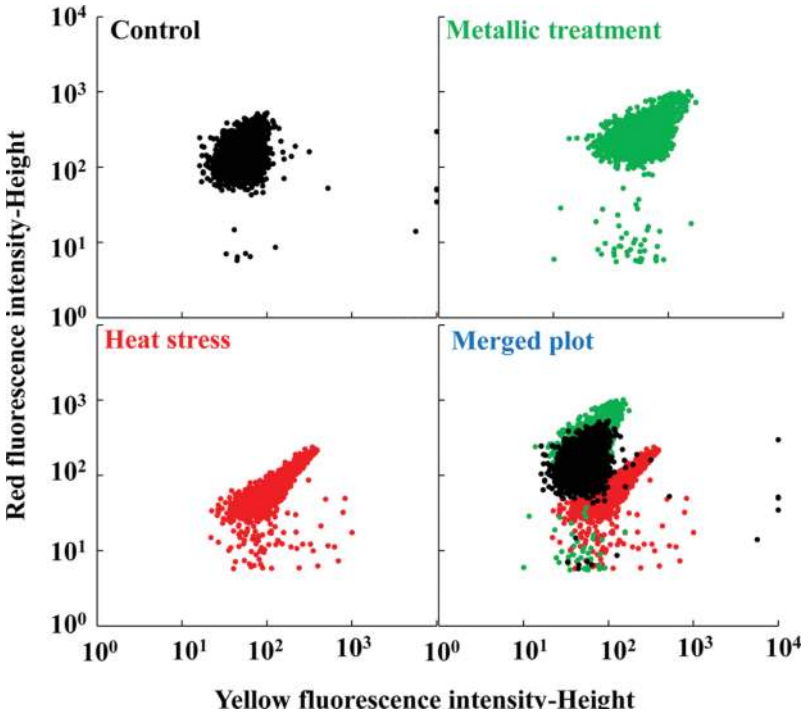

Figure 7. Distribution of *Chlorella* obtained using FCM on a graph of the red fluorescence intensity vs. the yellow fluorescence intensity, modified from the literature [1, 12–14]. The heat stress and the metallic treatment samples, respectively, derive from algae treated with heat and algae treated with a metallic solution containing concentrations of eluate of 50 vol%.

treated with metallic eluate. **Figure 9** shows the relation between the *Chlorella* proliferation ratio and the concentrations of the metallic eluate from steel slag in the test solution. As described in the explanation of research methods, all nutrient amounts derived from the CA medium, other than elements derived from slag eluate, were constant with each experiment condition. Results show that the number of algae increased according to the concentration of eluate up to 30 vol% (**Figure 9**). The algal numbers under more than 30 vol% of eluate (up to 70 vol%) were almost constant [12–14]. Reportedly, the addition of metallic eluate used for this study increases the concentration of aquatic CO_2 related to photosynthesis of algae [12–14]. The increased aquatic CO_2, which is found to be related to the presence of Ca^{2+} in eluate, might improve the rates of photosynthesis and algal proliferation [12–14].

It is noteworthy that approaches using PCA method (mainly **Figure 8**) have already exposed the effects of metallic eluate on algal growth without the proliferation test of algae treated with metallic eluate. Actually, 2–4 cells of autospore (St. 2) and algae after division (St. 3) other than algae at the growth stage (St. 1) were detected from control, whereas all types of algae at each stage (Sts. 1–3) were done from algae treated with metallic eluate (**Figure 10**).

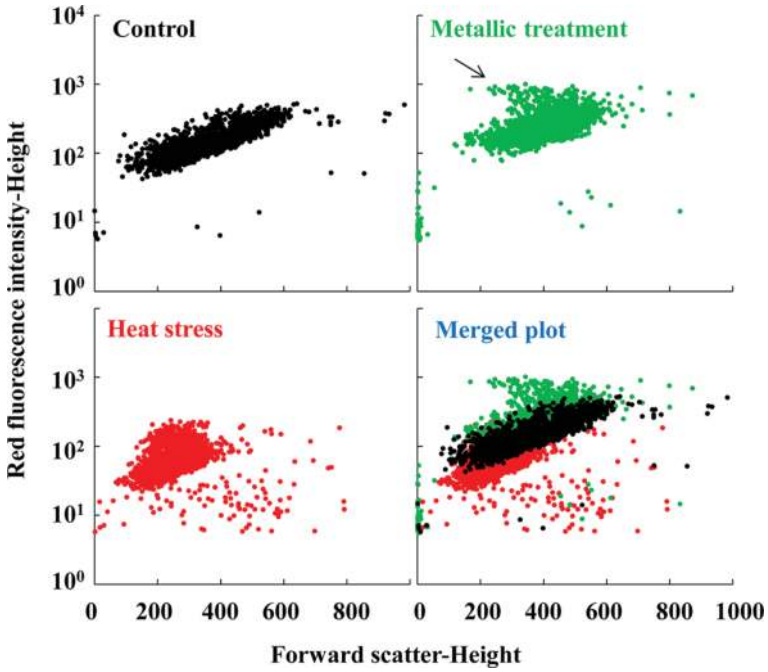

Figure 8. Distribution of *Chlorella* obtained using FCM on a graph of algal size vs. red fluorescence intensity. The heat stress and the metallic treatment samples, respectively, derive from algae treated with heat and those treated with a metallic solution containing concentrations of eluate of 50 vol%. The arrow indicates the distinctive population detected only from those treated with metallic eluate.

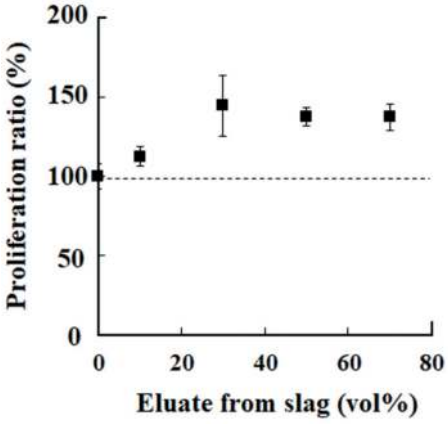

Figure 9. Effect of metallic eluate used for this study on algal growth modified from the literature [1, 12, 14]. The dotted line shows the proliferation ratio of control algae.

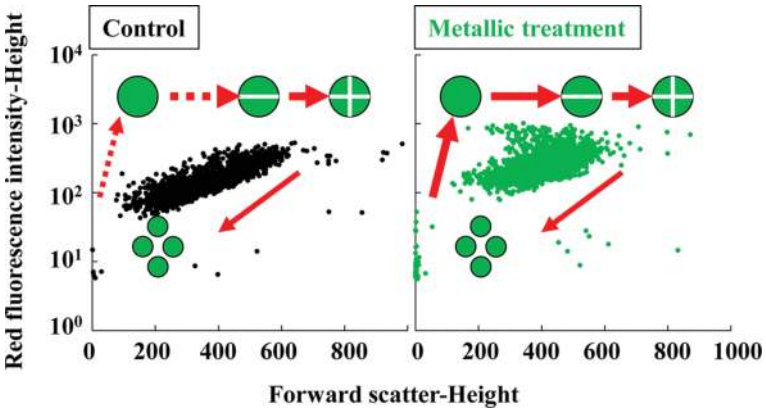

Figure 10. Effects of metallic eluate used for this study on algal growth. Here, each dotted graph in this figure is made from **Figure 8**.

Consequently, the cell cycle of algae treated with metallic eluate could continue to proceed smoothly even for algae after 7-day incubation when the control algae proliferation activity occurred at a low rate.

5. Conclusion

Multicolor FCM systems enable us to analyze up to a dozen multiparameters in a single assay and realize high-throughput measurement in life science. Countervailing the advantages of multiparametric FCM, multiparametric data make it difficult to interpret the resultant complicated information. Although multiparametric FCM is attractive relative to single or little parametric FCM in terms of cost performance and saving time of experiments, those benefits are meaningless unless the method leads to accurate and clear conclusions from multiparametric data. To elicit clear patterning graphs from FCM data and to grasp the essence of the data, this study examined the usefulness of PCA method of multivariate analysis. Comparison of control algae with several algae treated with test conditions such as heat and metallic eluate was conducted using FCM. To ascertain differences between control and test conditions about algal properties, FCM data were subjected to PCA analysis. Consequently, results from PCA analysis imply that both the red fluorescence intensity and the yellow one of algae can be an indicator for assessment of the variation for comparison of algae between control and heat treatment (**Figure 6C**), whereas both the cell size and the red fluorescence of algae can be an indicator for comparison of algae between control and metallic treatment (**Figure 6D**). It is striking that approaches coupled with PCA analysis have already exposed the effects of metallic eluate on algal growth with no proliferation test of algae. The result reveals that the low concentrations of metallic eluate used for this study induce algae to increase for a more prolonged period than in the control condition. Results show that PCA

method can extract information of test objects from data and that it can contribute to effective interpretation of cell characteristics, even if the data include several optical parameters from multiparametric FCM.

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