Research review paper

Flow cytometry for the development of biotechnological processes with microalgae

P. Hyka [a,b], S. Lickova [a,b], P. Přibyl [a,c], K. Melzoch [b], K. Kovar [a,*]

[a] Institute of Biotechnology, Zurich University of Applied Sciences (ZHAW), Campus Grüental, CH-8820 Wädenswil, Switzerland
[b] Institute of Chemical Technology (ICT), Technická 5, CZ-166 28 Prague, Czech Republic
[c] Algological Centre, Institute of Botany, v.v.i., Academy of Sciences of the Czech Republic, Dukelská 135, CZ-379 82 Třeboň, Czech Republic

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ABSTRACT

The current interest in microalgae as a sustainable source of next generation biofuels and other valuable substances is driving exploration of their use as unique biotechnological production systems. To design and optimise appropriate production strategies, the behaviour of particular microalgal species should be well characterised under different culture conditions. Thus, flow cytometric (FCM) methods, which are already well established in environmental and toxicological studies of microalgae, are also useful for analysing the physiological state of microalgae, and have the potential to contribute to the rapid development of feasible bioprocesses. These methods are commonly based on the examination of intrinsic features of individual cells within a population (such as autofluorescence or size). Cells possessing the desired physiological or morphological features, which are detectable with or without fluorescent staining, are counted or isolated (sorted) using an FCM device. The options for implementation of FCM in the development of biotechnological processes detailed in this review are (i) analysing the chemical composition of biomass, (ii) monitoring cellular enzyme activity and cell viability, and (iii) sorting cells to isolate those overproducing the target compound or for the preparation of axenic cultures.

Contents

1. Introduction

Microalgae cover a broad group of eukaryotic and typically photo-autotrophic microorganisms of very diverse phylogenetic positions.

* Corresponding author. Tel.: +41 796259589; fax: +41 589345001.
  E-mail address: koka@zhaw.ch (K. Kovar).

Accordingly, the ecology, morphology, physiology and biochemistry of microalgae are extremely diverse. The considerable metabolic versatility and flexibility of microalgae, also termed plasticity (Trainor, 2009), is awaiting exploitation in biotechnological manufacturing. To design and optimise appropriate production strategies, the behaviour of particular microalgal species should be well characterised under different culture conditions. These characteristics can be advantageously analysed using flow cytometric (FCM) methods, which are already well established in ecophysiological studies of microalgae (Collier, 2000; Sosik and Olson, 2007; Yentsch and Yentsch, 2008).

Products from microalgae are of emerging interest in a number of areas including supplements for human and animal nutrition, aquaculture, pharmaceutical and cosmetic products (Chisti, 2007; Rosenberg et al., 2008). Some most recent publications (Acien et al., in press; Heilmann et al., 2011) give the size of the microalgal biomass market worldwide as being about 5000 t year^{-1} of dry matter (Pulz and Gross, 2004) and generating a turnover of ca. $1.25 \cdot 10^9$ U.S. $ year^{-1} (Raja et al., 2008). The main commercial products are (in alphabetic order): β-carotene (*Dunaliella* sp.; Betatene or Western Biotechnology or Aqua-Carotene, Australia; Inner Mongolia Biological Engineering, China), astaxanthin (*Haematococcus* sp.; Cyanotech or Mera Pharmaceutcal, Hawaii, USA; Fuji Health Science, Japan), lipids and polyunsaturated fatty acids such as DHA (*Crypthecodinium* sp.; Martek or Omegatec, USA) or EPA (*Odontella* sp.; Innovalg, France), phycocyanine (*Spirulina platensis*), polysaccharides (*Chlorella* sp.; Ocean Nutrition, Canada; Solazyme, USA) and vitamins such as vitamin B_{12} (*Spirulina* sp.; Panmol or Madaus, Australia) (Pulz and Gross, 2004). Additionally, interest in microalgal biomass as a possible source for third generation biofuels has increased over the last decade (Montero et al., 2011). However, for economically sustainable production of biofuels from microalgal

biomass, additional enhancement of the productivity and cell density of microalgal cultures would be necessary (Parmar et al., 2011).

Improving the productivity and reproducibility of cultivation processes is also of great importance in order to establish microalgae as a unique biotechnological production system for high-value substances (Bumbak et al., 2011; Guedes et al., 2011). Therefore, it is essential to gain more detailed information on microalgal metabolism in general and on cultivation and production processes in particular. Some basic information is available from environmental and toxicological studies of phytoplankton (e.g. Adler et al., 2007; Franqueira et al., 2000). These studies mostly incorporate the use of flow cytometry (FCM), which enables single cells with different features or physiological states to be counted, sorted and/or examined on the basis of quantification of scattered and fluorescent light signals. The special feature of microalgae is the presence of photosynthetic pigments that exhibit strong autofluorescence, requiring specific FCM approaches (e.g. selection of an appropriate fluorescent dye). The application of FCM methods (both combined and not combined with fluorescent staining) to determine morphological features of microalgal cells, to identify species, and to examine the physiological state of individual cells in marine and freshwater microalgal communities was described in detail in several review papers (Collier, 2000; Dubelaar and Jonker, 2000; Sosik and Olson, 2007; Veldhuis and Kraay, 2000; Yentsch and Yentsch, 2008).

To date, FCM information has been successfully applied for the optimisation of growth conditions and for the production of a target compound only in yeast, bacterial and cell cultures (Davey, 2002; Davey and Kell, 1996; Weithoff, 2004; Yanpaisan et al., 1999). However, in principle, it can be also used with microalgae. In contrast to the routine methods used in bioprocess development and monitoring, FCM provides information on the intrinsic heterogeneity of a population in a bioreactor. The

Table 1
Applications of FCM analyses in bioprocesses with microalgae.

	Effect/product	FCM-method	Cultivation system	Microalga	Reference
Monitoring & optimisation of cultivation	Shear stress (>1 Pa) and rate (>10 s^{-1})	Viability (FDA)	Rotor-stator shear cylinders	*Chaetoceros muelleri*	Michels et al., 2010
	Shear stress (60 rpm)	Reactive oxygen species (DCFH, DHR 123)	Autotrophic, shaking flask	*Protoceratium reticulatum*	Rodriguez et al., 2009
	Ultrafiltration and cell density on growth	Cell cycle (PI)	Autotrophic, 1-L photobioreactor	*Chlorella vulgaris*	Javanmardian and Palsson, 1991
	Growth and DHA	Viability and membrane integrity (PI)	Heterotrophic, shaking flask	*Crypthecodinium cohnii*	Lopes da Silva and Reis, 2008
	Growth and oil	Viability and cell cycle (PI)	Heterotrophic, shaking flask	*Chlorella protothecoides*	Lopes da Silva et al., 2009b
	Cellulose and lipids, cell cycle phase and glucose concentration	Viability and cell cycle (PI), cellulose (CFW), neutral and polar lipids (NR)	Heterotrophic, shaking flask	*Crypthecodinium cohnii*	Kwok and Wong, 2005, 2010
	Growth and oil	Oil (NR)	Heterotrophic, shaking flask	*Chlorella protothecoides*	Lopes da Silva et al., 2009b
	Oil	Neutral and polar lipids (NR)	Autotrophic, outdoor photobioreactor	*Scenedesmus obliquus, Neochloris oleoabundans*	Lopes da Silva et al., 2009a
	Oil	Oil content (NR)	Autotrophic, 1-L air-lift reactor	*Neochloris oleabundans*	Gouveia et al., 2009
	Lipid and PUFA	Neutral and polar lipids, PUFAs (NR)	Autotrophic, shaking flask	*Tetraselmis suecica*	Guzman et al., 2010
	Lipid and DHA	Neutral and polar lipids (NR)	Heterotrophic, shaking flask	*Crypthecodinium cohnii*	de la Jara et al., 2003
	Astaxanthin	Autofluorescence	Autotrophic, shaking flask	*Haematococcus pluvialis*	Hu et al., 2008
Isolation	Neutral lipid overproducing strain	Cell sorting, neutral lipids (NR)	Autotrophic, air-lift photobioreactor	*Tetraselmis suecica*	Montero et al., 2011
	Lipid overproducing strain	Cell sorting, lipids (NR, autofluorescence)	Mixotrophic, shaking flask	*Nannochloropsis* sp.	Doan and Obbard, 2011a
	Carotenoid overproducing strain	Carotenoids (NR, autofluorescence), morphology (FSC, SSC)	Autotrophic, shaking flask	*Dunaliella salina*	Mendoza et al., 2008
	Carotenoid overproducing strain	Cell sorting (FSC, SSC, autofluorescence)	Autotrophic, outdoor reactor	*Dunaliella salina*	Nonomura and Coder, 1988
	Axenisation	Cell sorting (FSC, SSC autofluorescence)	Autotrophic, shaking flask	*Cyanophora paradoxa, Haematococcus* sp., *Monomastix* sp., *Scherffelia dubia, Spermatozopsis similis*	Sensen et al., 1993

All abbreviations of the designations of fluorescent dyes are explained in Table 2.

distribution of different cell features in the population of a single species is influenced by environmental conditions, the age of cells, cell cycle phases and mutations (Franqueira et al., 2000; Mendoza et al., 2008; Müller et al., 2010). The information gained on heterogeneity could therefore be exploited in combination with cell sorting to isolate cells overproducing a target compound (Montero et al., 2011; Müller and Nebe-von-Caron, 2010; Nonomura and Coder, 1988) as well as to sort cells for the preparation of axenic microalgal cultures (Sensen et al., 1993), which are required to successfully cultivate microalgae under heterotrophic conditions (Hammes and Egli, 2010; Mendoza et al., 2008; Müller and Nebe-von-Caron, 2010; Shapiro, 2003; Winson and Davey, 2000).

A general overview of the state of the art of FCM applications to microorganisms has been presented in recent reviews (Diaz et al., 2010; Hammes and Egli, 2010; Müller and Nebe-von-Caron, 2010; Troussellier et al., 1993; Winson and Davey, 2000), which have focused on bacteria or yeast but paid no attention to microalgae. Only a limited number of specific applications of FCM to microalgal bioprocesses have been published to date (Table 1). A gap still remains between the FCM methods established for microalgae and their exploitation for the monitoring, development and optimisation of biotechnological production processes. In order to bridge this gap, we examine the suitability and limitations of the FCM-aided approach for microalgal-based processes. The main topics of this review paper relate to analysing the chemical composition of biomass, examining and monitoring cellular enzyme activity and cell viability, isolating cells overproducing a target compound, and the preparation of axenic cultures. Although the generic methods outlined are largely supported by experimental data for natural non-recombinant microalgae, in principle they are also applicable to emerging strains improved by genetic engineering (e.g. Hyka et al., 2010; Looser et al., 2005).

2. Utilisation of FCM methods

The combination of FCM and fluorescent dyes is an important approach to determining certain cell features. After selection of an appropriate dye targeting the cell feature to be studied, the staining protocol should be optimised. In fact, the optimum concentration of the fluorescent dye as well as the most appropriate incubation time and additional sampling pre-treatment should be ascertained (Strauber and Muller, 2010). Before being suitable for FCM analysis, the samples may require several preparation steps such as (i) proper dilution, (ii) disaggregation of cell clusters by agitation, ultrasound (5–10 min) or by resuspension in tetrasodium pyrophosphate (TSPP) buffer (Veal et al., 2000), (iii) pre-filtration through a 15–35 μm filter (e.g. using BD tubes with 35 μm nylon mesh) to prevent clogging of the flow system of the FCM device (Battye et al., 2000), (iv) cell centrifugation and washing in buffer with a defined pH and/or ionic strength, and/or (v) cell fixation or increasing the permeability of the fluorescent dye through the cell membrane or cell wall. Since some microalgae have a specific cell wall composition and structure, fixation and permeabilisation procedures require increased attention with respect to the staining method (Collier and Campbell, 1999; Toepel et al., 2004; Vives-Rego et al., 2000).

The specific advantage of using FCM to analyse microalgae is the autofluorescence of naturally occurring intracellular pigments, which can be employed to distinguish between different species or between microalgae and other microorganisms without applying toxic fluorescent probes (Sensen et al., 1993). On the other hand, the autofluorescence spectrum can overlap with the red spectral range of commonly used dyes. Therefore, FCM methods established with bacteria or yeasts which use, for instance, propidium iodide or Nile red are not optimal for microalgae (Sato et al., 2004; Veldhuis and Kraay, 2000). For this reason, careful attention should be paid to selection of an appropriate fluorescent dye with respect to the particular microalgal strain being studied and the lasers available with the FCM device being used. The specifically stained cells are analysed either online or offline by an FCM device tuned to appropriate settings.

2.1. Cell fixation and permeabilisation

Cell fixation and permeabilisation are preliminary steps that are required for many FCM methods, especially for the analysis of cell composition and cell cycles. Fixation of cells provides the following advantages: fixed samples may be stored and need not be analysed immediately, it is possible to analyse all samples in one run, and fixation improves the penetration of the fluorescent dye into the cell. On the other hand, fixation causes cell death, which rules out any use involving analysis of the vital functions of cells (such as enzyme activity measurements). Moreover, some fixation methods lead to lower scatter properties and fluorescence intensity, changes in cell volume, higher background, and conformational changes in cellular macromolecules (Collier and Campbell, 1999; Toepel et al., 2004; Vives-Rego et al., 2000).

A comparison of fixation methods suggests that paraformaldehyde or formaldehyde is the best choice of fixative for microalgae and phytoplankton (for both fixatives: 0.5 to 1% v/v final concentration, 15–20 min incubation time). These fixatives do not change cell morphology and alter autofluorescence intensity only slightly (Chao and Zhang, 2011; Vives-Rego et al., 2000). In contrast, glutaraldehyde can cause a higher green fluorescence of cells and reduce antigen binding sites (Peperzak et al., 2000; Tang and Dobbs, 2007; Vives-Rego et al., 2000). Ethanol, methanol or acetone fixation can lead to changes in cell surface ultrastructure or morphology, generation of debris, bleaching, and a decrease in the chlorophyll autofluorescence of cells (Chao and Zhang, 2011; Vives-Rego et al., 2000). The optimal concentration and incubation time has to be tested experimentally for each species. After fast freezing in liquid nitrogen, fixed samples can be stored at −20 °C for a few weeks or at −80 °C for longer periods (Collier and Campbell, 1999; Nicklisch and Steinberg, 2009; Tang and Dobbs, 2007).

Besides cell fixation, some fluorescent dyes require the enhancement of membrane permeability to optimise dye penetration. Two conventional permeates, ethylenediamine-tetraacetic acid (EDTA) or Triton X-100, are often applied (Strauber and Muller, 2010; Vives-Rego et al., 2000).

2.2. Fluorescent dyes and probes

The market offers a broad spectrum of fluorescent dyes/probes targeting specific cell compartments, molecules, or their biological functions without the need for expensive antibodies (Life-Technologies™, 2010; Table 2). As these fluorescent dyes/probes were mostly developed for use with mammalian cells, sample pre-treatment is typically required before their application to microalgae. Microalgae and other phytoplankton possess fluorescing endogenous pigments (chlorophylls, carotenoids, sometimes also phycobilins), which are detectable with FCM as strong red or orange autofluorescence (>600 nm). The strong pigment autofluorescence can cause interference or quenching of a dye if it fluoresces within the same spectrum. This affects the quantitative signal information from the fluorescent dye. Therefore, ideal fluorescent dyes/probes chosen for the investigation of microalgae should:

- have maximal fluorescence intensities in the range of 500–600 nm, at which there is no interference with the autofluorescence of the pigments (mainly chlorophyll a);
- be non-toxic and sensitive at low concentrations of the dye to achieve better recovery of the cells after cell sorting;
- show excitation at 488 nm, since affordable FCM devices used in industry are only equipped with an air cooled single-line argon blue laser tuned to this wavelength.

Dyes used for the determination of the physiological state of microalgae or phytoplankton are listed in Table 2. Unfortunately, not all of them meet the previously mentioned requirements (i.e. CFW, DAPI, Hoechst 33342 do not excite at 488 nm but in the UV range, and PI, NR, CTC have maximal fluorescence intensities above

Table 2
Dyes (probes) used for FCM analysis of phytoplankton.

	Probe[a]	Ex. (nm)	Em. (nm)	Target feature	Microalgae or phytoplankton species	Reference
Biomass composition	Autofluorescence	488	>600	Chlorophyll a phycoerythrin, pigments content	Phytoplankton	Olson et al., 1989
	BODIPY 505/515	505	515	Lipids and oil content	Haematococcus pluvialis Chrysochromulina sp. Mallomonas splendens Ophiocytium majus	Hu et al., 2008 Cooper et al., 2010
	CFW [Calcofluor White M2R]	347 UV	450	Cellulose content	Crypthecodinium cohnii	Kwok and Wong, 2003, 2010
	FITC [fluorescein-5-isothiocyanate]	494	518	Protein content	Agmenellum quadruplicatum[c] Chlorella kessleri	Paau et al., 1978 Hutter and Eipel, 1978
	Fluorescein-5-EX succinimidylester	494	518	Protein content	Chlamydomonas, Rhodomonas	Nicklisch and Steinberg, 2009
	NR [Nile red]	549	628[b]	Lipids (polar, neutral), oil	Chlorella sp., Navicula Chlorella protothecoides Crypthecodinium cohnii Dunaliella salina Nannochloropsis sp. Neochloris oleoabundans Scenedesmus obliquus Tetraselmis suecica	Cooksey et al., 1987 Lopes da Silva et al., 2009b de la Jara et al., 2003 Mendoza et al., 2008 Doan and Obbard, 2011b Gouveia et al., 2009 Lopes da Silva et al., 2009a Guzman et al., 2010
Nucleic acid, viability	DAPI [4′,6-diamidino-2-phenylindole]	358 UV	461	DNA content (A/T specific), cell cycle	Chlorophyta, Rhodophyta Acetabularia, Batophora,	Vitova et al., 2005 Coleman, 1979
	Hoechst 33342	352 UV	461	DNA content (A/T specific), cell cycle	Gonyaulax polyedra	Vicker et al., 1988
	PI [propidium iodide]	536	617[b]	Nucleic acid content, viability, cell cycle	Chlamydomonas eugametos Chlorella protothecoides Chlorella pyrenoidosa Chlorella vulgaris Crypthecodinium cohnii Phaeodactylum tricornutum Scenedesmus vacuolatus	Franqueira et al., 2000 Lopes da Silva et al., 2009b Liu et al., 2008a Gonzalez-Barreiro et al., 2006; Javanmardian and Palsson, 1991; Rioboo et al., 2009 Lopes da Silva and Reis, 2008 Cid et al., 1996 Adler et al., 2007
	PicoGreen	480	520	DNA content (A/T specific), viability, cell cycle	Chlamydomonas Phytoplankton Rhodomonas	Nicklisch and Steinberg, 2009 Marie et al., 1996; Veldhuis et al., 2001 Nicklisch and Steinberg, 2009
	SYBR Green I	497	520	Nucleic acid content, cell cycle	Phytoplankton Prochlorococcus[c] Chlorophyta, Rhodophyta	Marie et al., 1997 Zubkov et al., 2000 Vitova et al., 2005
	SYTO 9	483	503	Nucleic acid content, cell cycle	Phytoplankton	Leuko et al., 2004
	SYTO 13	488	509			Guindulain et al., 1997
	SYTO RNAselect	490	530	RNA content	Chlamydomonas, Rhodomonas	Nicklisch and Steinberg, 2009
	SYTOX Green I SYTOX Green II	504	523	Nucleic acid content, viability, cell cycle	Phytoplankton Chlamydomonas reinhardtii Micromonas pusilla Oscillatoria agardhii[c] Phaeocystis pouchetii Phormidium foveolarum[c]	Marie et al., 1997; Veldhuis et al., 2001 Sato et al., 2004 Brussaard et al., 2001 Sato et al., 2004 Brussaard et al., 2001 Sato et al., 2004
	TOTO-1	514	533	Nucleic acid content, viability, cell cycle	Phytoplankton	Guindulain et al., 1997; Marie et al., 1996
	TOPRO-1	515	531			
	YOYO-1	491	550			
	YOPRO-1	491	509			
Membrane potential	CTC [5-cyano-2,3-ditolyl tetrazolium chloride]	450	630[b]	Electron transport, membrane activity	Scenedesmus vacuolatus	Adler et al., 2007
	DHR 123 [dihydro-rhodamine 2-(3,6-diamino-9H-xanthene-9-yl)-, methyl ester]	505	534	Reactive oxygen species	Chlamydomonas reinhardtii Euglena gracilis Phaeodactylum tricornutum Scenedesmus obliquus	Jamers et al., 2009 Watanabe and Suzuki, 2001 Cid et al., 1996 Ronot et al., 1986
	DiOC₆(3) [3,3′-dihexyloxa-carbocyanine iodide]	484	501	Membrane potential, respiration rate	Dunaliella tertiolecta Phaeodactylum tricornutum	Gregori et al., 2002 Cid et al., 1996
	TMRM [tetramethyl-rhodamine-methyl ester]	555	580	Membrane potential and integrity	Chlamydomonas reinhardtii Dunaliella tertiolecta Phaeodactylum tricornutum	Jamers et al., 2009 Overnell, 1975 Cid et al., 1996
Cellular enzyme activity	Calcein-AM [calcein acetoxy-methyl ester]	494	517	Non-specific esterase activity, viability	Micromonas pusilla Phaeocystis pouchetii	Brussaard et al., 2001
	Carboxy-H₂DFFDA, 5(6)-carboxy-2′,7′-dihydrodifluoro-fluorescein diacetate	478	518	Reactive oxygen species	Chlamydomonas reinhardtii	Szivak et al., 2009
	CFDA-SE or CFSE [5(6)-carboxy-fluorescein diacetate N-succinimidyl ester]	492	517	Non-specific esterase activity, cell division	Chlorella vulgaris	Rioboo et al., 2009

(continued on next page)

Table 2 (continued)

Probe[a]	Ex. (nm)	Em. (nm)	Target feature	Microalgae or phytoplankton species	Reference
CMFDA [5-(chlormethyl) fluorescein diacetate]	485	538	Non-specific esterase activity, viability	Euglenophyceae	Tang and Dobbs, 2007
ELF 97 – phosphate	345 UV	530	Alkaline phosphatase activity	Phytoplankton *Prochlorothrix hollandica*[c] *Scenedesmus dimorpha*	Duhamel et al., 2009; Strojsova and Vrba, 2009 Dignum et al., 2004 Fitzgerald and Nelson, 1975
FDA [fluorescein diacetate]	492	519	Non-specific esterase activity, viability	Phytoplankton *Chaetoceros muelleri* *Chlamydomonas reinhardtii* *Chlorella pyrenoidosa* *Chlorella vulgaris* *Euglena gracilis* *Microcystis aeruginosa*[c] *Nannochloropsis gaditana* *Rhodomonas salina* *Scenedesmus obliquus* *Selenastrum capricornutum* *Tetraselmis suecica*	Debenest et al., 2010; Jamers et al., 2009; Yoshida et al., 2004 Michels et al., 2010 Jamers et al., 2009 Liu et al., 2008a Hadjoudja et al., 2009; Stauber et al., 2002 Fleck et al., 2006 Hadjoudja et al., 2009; Shi et al., 2007 Hampel et al., 2001 Hampel et al., 2001 Shi et al., 2007 Stauber et al., 2002 Hampel et al., 2001
H$_2$DCFDA [2′,7′-dichlorodihydro-fluorescein diacetate]	505	535	Reactive oxygen species, respiratory activity	Green microalgae *Chlamydomonas reinhardtii* *Chlorella pyrenoidosa* *Protoceratium reticulatum*	Yoshida et al., 2004 Saison et al., 2010 Knauert and Knauer, 2008 Rodriguez et al., 2009
HE [hydroethidine, dihydroethidium]	510	595	Peroxidase activity	*Phaeodactylum tricornutum*	Cid et al., 1996
Rhodamine 123	507	529	Mitochondrial membrane potential, viability	*Phaeodactylum tricornutum* *Scenedesmus vacuolatus*	Cid et al., 1996 Adler et al., 2007

Only dyes suitable for use with an argon laser with an excitation at 488 nm are listed.

ex., em. wavelengths of maximal excitation and maximal fluorescence intensities.

[a] Only common or commercial brand names are given.

[b] Fluorescence intensities >600 nm (overlap with autofluorescence of native pigments).

[c] Categorised as cyanobacteria.

600 nm that overlap with autofluorescence of pigments). In these cases, electronic compensation can be used to correct the spectral overlap of fluorescent signals with cellular autofluorescence of unstained and stained cells (Marie et al., 1997). However, both features measured do not change proportionally, the result of which is an unequal shift in fluorescence intensity that can lead to different compensation settings for the same set of samples taken during one cultivation process. Moreover, such compensation should not be applied during data collection, but only computationally during data analysis (Collier, 2000).

2.3. Equipment specifications required

FCM analysis is a method that enables relative changes in cell physiological state (including biomass composition) to be detected during a cultivation process. The rapid analysis of cells at single cell level by FCM methods provides valuable feedback for efficient process development. Basically, the measurement principle of a flow cytometry device relies on counting individual particles with specific features, which is performed at a rate of thousands of events per second. Samples containing (stained or non-stained) cells are loaded onto the FCM device and are carried by a liquid phase through the light stream of a mercury lamp or laser of specific wavelength. This light excites the fluorescent molecules inside the cells, and the fluorescent intensity of the emitted and/or scattered light are determined individually for each cell by detectors collecting light at different wavelengths. The instrument settings of the FCM device (i.e. for its detectors, amplifiers and thresholds) are adjusted in accordance with the target cell features and population (Shapiro, 2003). Moreover, signal of the lasers alters with their increasing age. Therefore, controls during and calibration of each measurement series are required to obtain reliable data which are comparable to previously gained data.

Although today's market offers FCM devices which are equipped with more than one laser and several optical filters (e.g. BD FACS

Aria^{TM} III with 6 lasers and 18 detectors), most of the cheaper models are still equipped with one blue laser at 488 nm only and three optical filters (e.g. FACSCalibur^{TM} BD Bioscience with FL1 = 530/30 nm, FL2 = 585/42 nm and FL3 > 670 nm). Therefore, this review focuses on FCM applications to microalgae using simple instruments of this type. Further advanced on-line FCM devices, including flow injection analysis (FIA) modules, which enable process monitoring and control in real time mode are still specially assembled in research laboratories and are not available as an entire commercial system (Broger et al., 2011; Kacmar et al., 2006; Sitton and Srienc, 2008). Furthermore, the fractions of cells with specific features can be categorised and isolated using the FCM device together with a sorting module.

3. Current applications of FCM

Flow cytometry methods have the potential to contribute to the rapid development and optimisation of suitable production strategies using microalgae (Table 1). Possible applications of FCM are summarised in Fig. 1. Single microalgal cells can be counted, examined or sorted according to their features or physiological state, as metabolic activity, viability, composition or morphology. Monitoring physiological changes of the cells during cultivation process the information gained can be used for process control or determination of optimal conditions for growth or to reach the target biomass composition. Potentially, individual cells can be isolated to obtain axenic cultures or overproducers. Overproducer sub-populations isolated from a heterogeneous culture can be cultivated again and further analysed (Fig. 1).

3.1. Determination of cell features

For control of microalgal cultivation it is important not only monitoring the increase in biomass, but also changes in biomass composition.
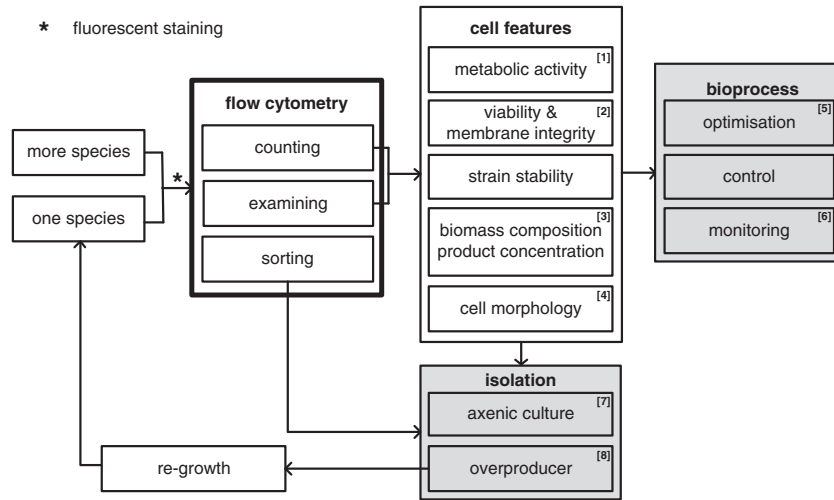
Fig. 1. Applications of FCM analyses for biotechnological processes with microalgae. The boxes with targets when using FCM are highlighted in grey. Single cells with different features or in different physiological states can be counted, sorted and examined on the basis of quantification of scattered and/or fluorescent light signals. Provided the changes in cell features can be monitored, the information gained can afterwards be used for process control or optimisation. In addition, individual cells with target features can be isolated, subsequently re-grown, and further analysed (regardless of whether a uniform or a heterogeneous population was obtained). The numbers in angled brackets refer to particular publications on microalgae.

- [1] (Rodriguez et al., 2009)
- [2] (Javanmardian and Palsson, 1991; Lopes da Silva and Reis, 2008; Lopes da Silva et al., 2009b; Michels et al., 2010)
- [3] (de la Jara et al., 2003; Gouveia et al., 2009; Guzman et al., 2010; Hu et al., 2008; Lopes da Silva et al., 2009b; Mendoza et al., 2008; Nonomura and Coder, 1988)
- [4] (Sensen et al., 1993; Surek and Melkonian, 2004)
- [5,6] (de la Jara et al., 2003; Guzman et al., 2010; Lopes da Silva and Reis, 2008; Lopes da Silva et al., 2009a; Lopes da Silva et al., 2009b; Michels et al., 2010; Rodriguez et al., 2009)
- [7] (Sensen et al., 1993; Surek and Melkonian, 2004)
- [8] (Mendoza et al., 2008; Nonomura and Coder, 1988).

FCM, often and advantageously combined with specific fluorescent probes, enables monitoring of these changes. Biomass composition can be determined as either cell granularity or the intensity of a specific fluorescent probe signal, and is related to the amount of target compound in the cell. Since the amount of target compound does not need to be proportional to cell volume, measurement of cell size is necessary for determination of relative amount of target compound in individual cells of the population. According to the maximal relative concentration of target compound the overproducers can be isolated (Kwok and Wong, 2003;

Fig. 2. Morphological and physiological characteristics of cells in high-cell-density fedbatch cultures of *Chlorella vulgaris*. The exponential growth of the culture in a mineral medium with glucose in the dark was accompanied by a linear decrease in the autofluorescence of cells, while the viability did not change. A remarkable decrease in cell volume after 12 h of fedbatch cultivation was recorded. Viable cells were determined by FCM as the percentage of cells stained by fluorescein diacetate (FDA, i.e. esterase active cells relative to total cells). Biomass concentration was determined as cell dry weight (CDW). Cell volume was measured by the correlation of the intensity of the forward scatter signal (FSC) of standard beads (Invitrogen F-13838) with microalgal cells. The chlorophyll concentration was determined as the median autofluorescence intensity (MFI) detected by the fluorescent filter (FL3) of the FCM and normalised to cell volume.

Resina et al., 2009). Specific components of microalgal biomass are photosynthetic pigments having distinct autofluorescence that can be measured. Another important application of FCM is determination of cell viability. However, there is no universal method for its determination. It is recommended to use a combined approach: both the identification of damaged cells with reduced cell membrane integrity and the more sensitive detection of intracellular enzymatic activity of intact cells (Brussaard et al., 2001).

3.1.1. Cell size and granularity

Morphology, including cell size and granularity, is characteristic for certain microalgal species and is influenced by cultivation conditions as well as culture density (Umorin and Lind, 2005). These cell features correlate with two scattering signals measured by FCM, namely forward scatter (FSC) and side scatter (SSC) signals.

FSC is normally assumed to be proportional to cell size or cell volume, because the signal intensity increases linearly with the square of the cell diameter or cross sectional area (Cunningham and Buonnacorsi, 1992; Jochem, 2000; Koch et al., 1996; Shapiro, 2003; Toepel et al., 2004). Cell volume can be quantified using a calibration curve obtained from the measurement of spherical dextran gel beads of a known diameter. Due to the morphological variability of microalgae, the more exact relationship between FSC and cell volume was previously determined using image analysis and geometric models, which depend on cell shape and cell size (Becker et al., 2002; Koch et al., 1996; Sun and Liu, 2003; Toepel et al., 2004). For example, Eq. (1) was used for the estimation of cell volume of the microalga *Crypthecodinium cohnii* (Kwok and Wong, 2003) and Eq. (2) to estimate the cell volume of ovoid (yeast) cells (Resina et al., 2009), both after previous calibration using beads of a known diameter in μm. However, an exact universal correlation between cell size and scatter signal does not exist due to varying optical properties of different instruments or the refractive index of particles, and each calibration requires mathematical adjustment (Mieplot, 2010; Müller and Nebe-von-Caron, 2010; Vives-Rego et al., 2000). The decrease in cell volume of *Chlorella vulgaris* cultivated under heterotrophic conditions in
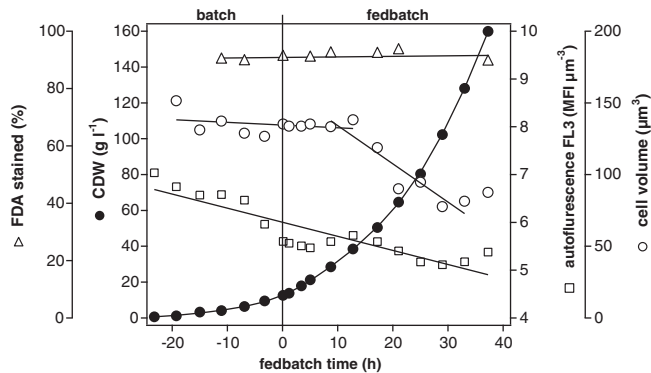
high-cell density cultures (>160 g l^{-1}) in carbon limited fedbatch was determined using calibration of the FSC signal with dextran gel beads (Invitrogen F-13838). The cell volume after 36 h of cultivation was 53% compared to the initial cell volume (Fig. 2). Similarly, a decrease in cell size during exponential growth was shown most recently in autotrophically cultured C. vulgaris (Pribyl et al., 2012). Probably, decrease in cell volume was caused by the intensive division of cells and shorter cell cycle, which limited the synthesis of storage compounds.

$$cell\ volume\left[\mu m^3\right] \sim FSC^3 \qquad (1)$$

$$cell\ volume\left[\mu m^3\right] \sim FSC^{3/2} \qquad (2)$$

SSC has been shown to be affected by cell morphology, and especially by intracellular structures that are determined by chemical composition (e.g. starch content). A higher SSC intensity is usually obtained from cells with a higher level of cytoplasmic granularity (Davey and Kell, 1996). Besides granularity, SSC intensity was also demonstrated to be related to cell volume of very small cells such as picoplankton (Gutierrez-Rodriguez et al., 2010; Jochem, 2000), to a change in cell granularity caused by exposure to metals (Debelius et al., 2009), or to different stages in the life cycle of Haematococcus pluvialis (Wang and Miao, 2004).

3.1.2. Autofluorescence of native pigments

Photosynthetic pigments present in microalgae are chlorophylls, carotenoids, or sometimes phycobilins. Both pigment content and composition is taxon specific and depends on cultivation conditions (illumination, nutrient status, temperature, pH, exposure to photosynthetic inhibitors) (Demers et al., 1991; Leya et al., 2009; Liu and Lee, 2000; Toepel et al., 2004). Pigment fluorescence, the major component of endogenous fluorescence (autofluorescence), has been proven to be linearly related to cellular pigment content (Collier, 2000; Veldhuis and Kraay, 2000; Yentsch and Yentsch, 2008). Therefore, autofluorescence intensity was used for the quantification of photosynthetic capacity and the identification of cellular pigments (Phinney and Cucci, 1989). In environmental studies, autofluorescence is often mentioned in relation to cell biomass of phytoplankton in oceanography (Burkill and Mantoura, 1990; Phinney and Cucci, 1989), but it is also influenced by the light history of cells prior to sampling. Since pigment composition is specific for microalgal species, FCM measurement of autofluorescence of individual pigment is used for the classification of phytoplankton from mixed environmental samples (Davey and Kell, 1996; Olson et al., 1989; Veldhuis and Kraay, 2000; Veldhuis et al., 2001). Moreover, chlorophyll autofluorescence is a unique biomarker for photosynthetic organisms and enables setting an FCM gate for the separation of microalgae from other particles and microorganisms (Burkill and Mantoura, 1990).

The primary photosynthetic pigment, chlorophyll a, is the only one pigment present in all photoautotrophic microalgae. It emits light in the spectral range of UV-blue (<450 nm) and in the red-far red spectral range (>650 nm). The other chlorophylls, b, c, and d, together with carotenoids, have accessorial functions. They capture photons and pass them to chlorophyll a. Other microalgal pigments, however present only in some species, include phycoerythrin with maximal fluorescent intensity at 575/10 nm, phycocyanin, and allophycocyanin that both have maximal fluorescence intensities at 660/20 nm (Phinney and Cucci, 1989; Reckermann, 2000; Veldhuis and Kraay, 2000). Since the fluorescence intensities of individual pigments overlaps, FCM device with appropriate combination of lasers and filters is needed for their signal separation. For example, employing a red laser line (633 nm) phycocyanin fluorescence was used for FCM detection of phycocyanin-rich species in phytoplankton samples (Reckermann, 2000). To quantify chlorophyll content, autofluorescence intensity must be calibrated against an internal standard (e.g. chlorophyll beads; Phinney and Cucci, 1989) after

their inter-calibration using another quantitative method of pigment analysis such as spectrophotometry (Jeffrey and Humphrey, 1975), fluorimetry or reverse phase HPLC (Mackey et al., 1998; Mantoura and Llewellyn, 1983). Cell size limits the amount of chlorophyll that chloroplasts are able to accommodate. Therefore, to compare chlorophyll content, the autofluorescence of pigments (FL) per cell should be expressed relative to cell volume (Eq. (3)). Furthermore, it is necessary to establish a threshold for 'positive' chlorophyll fluorescence which separates true chlorophyll fluorescence from spurious signals other autofluorescence molecules such as reduced pyridine nucleotides, flavins, tryptophan, and elastin (Shapiro, 2003). This threshold can be determined by measurement of cells, from which chlorophyll was extracted using 90% acetone, followed by bleaching the cells under high intensity light for 20–30 min until no pigment was detectable via epifluorescence microscopy (Phinney and Cucci, 1989; Yentsch and Campbell, 1991).

$$relative\ fluorescence = \frac{FL}{cell\ volume} \qquad (3)$$

Changes in photosynthetic activity during dark and light phases of the cultivation process were investigated by FCM. During the dark phase of cultivation (without a carbon source), a considerable decrease in autofluorescence was observed in Chlorella pyrenoidosa while only a slight increase in autofluorescence occurred in the cyanobacterium Microcystis aeruginosa compared with the previous state at light (Zhang et al., 2007a). The decrease in autofluorescence was also determined during the cultivation of C. vulgaris under heterotrophic conditions (without access to light) in high-cell density cultures (>160 g l^{-1}) in carbon limited fedbatch. After 36 h of cultivation cellular autofluorescence was 74% of the initial value (Fig. 2). The decrease in autofluorescent pigments during the dark phase was expected in Chlorella sp., because the cells divided without synthesising new chlorophyll (Zhang et al., 2007a). The increase in autofluorescence in M. aeruginosa was explained as a delayed effect of photosynthetic products produced during a previous light incubation (Zhang et al., 2007a) or as recovery after photoinhibition in response to previous high illumination (Collier, 2000; Vaulot and Marie, 1999). Moreover, M. aeruginosa is the dominant species in eutrophic lakes where there is lack of light due to an algal bloom and therefore it is better adapted to suboptimal light conditions than C. pyrenoidosa (Zhang et al., 2007b).

3.1.3. Biomass composition

The biomass composition of microalgae is extremely variable depending on the species and cultivation conditions such as light, nutrient status, temperature, pH or inhibitors. For example, variations in the basic biomass composition of Chlorella protothecoides, depending on nitrogen availability in heterotrophic cultures are, in % of dry biomass; lipids, 25–45; carbohydrates, 17–24; proteins, 11–26; and for nucleic acids, 10–12 (Xiong et al., 2010). Therefore, the main efforts in the optimisation of cultivation conditions are related to producing biomass with the required ratio of lipid, protein, carbohydrate, and nucleic acid.

3.1.3.1. Lipids. Oils and other lipids, as important storage compounds of microalgae, are mostly synthesised during the stationary phase of growth, and their content was enhanced in nitrogen limited cultures (Chisti, 2007; Cooksey et al., 1987; Elsey et al., 2007; Li et al., 2010; Pribyl et al., 2012; Shen et al., 2010). In biotechnological processes, lipid content is controlled for the production of nutraceutical compounds such as omega-3 and omega-6 polyunsaturated fatty acids (PUFAs) used in human and animals diets, and neutral lipids, which have potential for the production of biodiesel (Brennan and Owende, 2010; de la Jara et al., 2003; Doan et al., 2011; Gouveia et al., 2009; Lopes da Silva et al., 2009b; Tokusoglu and Unal, 2003). For FCM determination of oil content, two lipophilic fluorescent dyes have been employed, Nile red (NR), or BODIPY 505/515 (4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-s-indacene).
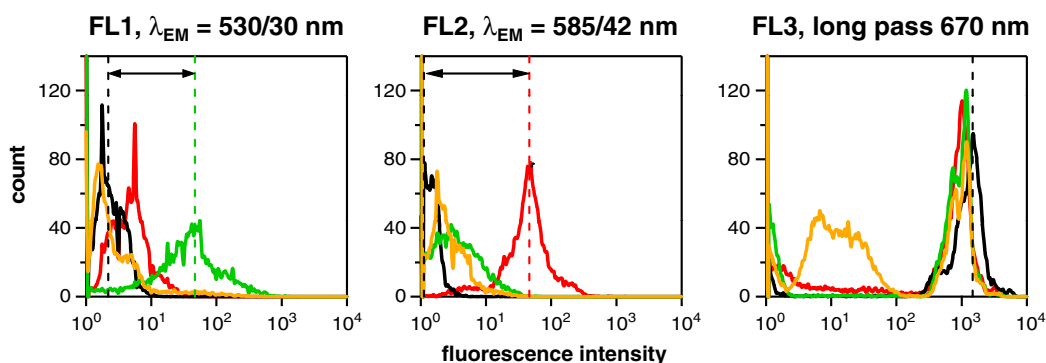
Fig. 3. Fluorescence intensity of lipid-staining dyes in *Chlorella vulgaris* cells. Four histograms determined by analysis of one sample using three different optical filters (FL1, FL2, FL3) and autofluorescence are displayed in one overlay graph. Measurements were processed using a FACSCalibur™ device (BD Bioscience) with blue laser and excitation at 488 nm. They demonstrate interferences in the autofluorescence intensity of microalgal pigments when using fluorescence dyes for staining intracellular lipids (Nile red or BODIPY 505/515). However, BODIPY 505/515 diluted in DSMO and Nile red diluted in acetone showed a useful signal when using an FL1 filter and FL2 filter sets respectively. Black line: unstained cells, autofluorescence originating solely from chlorophyll; red line: cells stained with Nile red diluted in acetone; orange line: cells stained with Nile red diluted in DMSO (comparable histograms were obtained when no solvent was used, data not shown); green line: cells stained with BODIPY 505/515 diluted in DMSO.

NR has been used over the last 20 years, mostly in a lipid-directed research. A comparison between gravimetric and gas chromatographic (GC) analytical methods has shown a correlation between fluorescence intensity of NR, measured as yellow-gold fluorescence (585/40 nm) and as red fluorescence (635/20 nm), and neutral and polar lipid content (e.g. PUFAs) respectively (Cooksey et al., 1987; de la Jara et al., 2003; Doan and Obbard, 2011a; Elsey et al., 2007). However, this correlation should be interpreted carefully for the determination of lipid content, for example in *Nannochloropsis* (Doan and Obbard, 2011b). NR staining has been used several times for the optimisation of neutral and polar lipids in a variety of microalgal taxa such as *Chlorella*, *Scenedesmus*, *Crypthecodinium*, or *Neochloris*. In addition, the NR-method was applied to determine the ratio of polar/neutral lipids, which is believed to be associated with the relative fatty acid content in the microalgae *Tetraselmis suecicca* and *C. cohnii*. In general, polar lipids have a higher PUFA content than other lipids, which means that the polar/neutral ratio could be used in screening studies as an effective indicator of the relative PUFA content in microalgae (de la Jara et al., 2003; Guzman et al., 2011). The disadvantage of this dye is interference of the NR fluorescence intensity in the red spectral range with the autofluorescence signal derived from photosynthetic pigments. This problem can be partly overcome by enhancement of NR fluorescence using an optimal concentration of DMSO as a stain carrier. The concentration of DMSO used was, however, very high (20–25%) (Chen et al., 2009; Doan and Obbard, 2011b; Elsey et al., 2007) potentially impairing cell viability and hampering the possible successful revitalisation of stained cells subsequent to their isolation by a sorter. Dilution of Nile red in acetone (1% v/v) led to a good separation of autofluorescence signal from the fluorescence of lipids in *C. vulgaris* cells using fluorescence filter FL2 (585/42 nm). On the other hand, if NR was diluted in DMSO (2% v/v), both signals interfered with all three fluorescence filters. Moreover, it seems that NR in 2% DMSO was not able to stain all cells in the population, as indicated by two separate peaks when using the long pass filter FL3 (>670 nm, Fig. 3). Flow cytometry using NR staining was applied to the monitoring and screening of *in situ* lipid production in *Neochloris oleabundans* during testing of different cultivation conditions. This method facilitated the determination of lipid overproducing conditions, yielding the maximal lipid content (56% of cell dry weight) after 6 days under nitrogen limitation and without CO_2 supplementation (Gouveia et al., 2009; Table 1).

The recently introduced BODIPY 505/515 dye has a narrow emission spectrum at 515 nm, which prevents interference with autofluorescence in the green spectral range (Chen et al., 2009; Cooper et al., 2010; Lopes da Silva et al., 2009a). BODIPY 505/515 selectively stains lipids including fatty acids, cholesteryl esters, phospholipids and sphingolipids and their analogues (Cooper et al., 2010). In contrast to NR, BODIPY 505/515 (in 2% v/v DMSO) showed a well separated fluorescence signal from autofluorescence when using the green fluorescent filter FL1 (530/30 nm, Fig. 3). Unlike NR, BODIPY 505/515 was shown to efficiently penetrate cells with the application of only 2% v/v DMSO. In spite of its advantageous characteristics, exploitation of this dye in microalgae is still poorly established (Cooper et al., 2010) compared to NR.

3.1.3.2. Carbohydrates. Carbohydrates in microalgae include mainly starch, cellulose, hemicellulose and soluble sugars (Branyikova et al., 2011; Graham et al., 2009). Analogous to lipids, starch is an important microalgal storage compound. An increase in the production of starch in stationary phase of sulphur limited cultures was observed in *C. vulgaris* (Branyikova et al., 2011) and in *Chlamydomonas reinhardtii* (Deng et al., 2011). Unfortunately, a starch-specific fluorescent dye is not available. Starch content can be roughly estimated by the change in intensity of the flow cytometric SSC, which increases with increasing cell granularity (Yang et al., 2002). However, the SSC signal determining cell granularity is influenced not only by starch accumulation (see above, Chapter 3.1.1), so for example quantitative changes in starch content can be measured in cells where only starch accumulation is expected.

Cellulose synthesis and content were studied in the dinoflagellate microalga *C. cohnii* using cellulose and chitin specific dye Calcofluor White M2R (CFW). A higher rate of cellulose synthesis was determined in the G1 phase of the cell cycle, and the cellulose content was proportional to cell size (Kwok and Wong, 2003, 2010). However, CFW is excited in the UV spectrum, which unfortunately prevents the use of the argon blue laser (Veldhuis and Kraay, 2000).

3.1.3.3. Proteins. Total microalgal protein is of high nutritional quality and it is comparable to conventional vegetable proteins. In the 1950s, this was one of the alternatives for single cell protein (SCP) production and currently 30% of the world's algal production is sold for animal or aquaculture feeding (Becker, 2007).

The standard FCM method for determination of proteins *in vivo* is based on fluorescein isothiocyanate (FITC) staining (Hutter and Eipel, 1978; Paau et al., 1978). Alternatively, staining with fluorescein-5-EX succinimidyl ester, after washing the cells with fresh 0.1 M $NaHCO_3$ solution at pH 8.5, was applied for protein staining in *Chlamydomonas* and *Rhodomonas*. Using this method, in combination with fluorescent staining of RNA with SYTO RNAselect, a relationship between the RNA/protein ratio and growth was determined in continuous cultures at various specific growth rates. Based on these data the relationship between specific growth rate and RNA/protein ratio was estimated for *Chlamydomonas* and *Rhodomonas* (Nicklisch and Steinberg, 2009). Unfortunately, FCM detection of proteins in microalgae has been described or used only rarely.

3.1.3.4. Nucleic acids. The nucleic acid content depends on the cell cycle phase and is of interest in the investigations of circadian rhythms in cells, cell composition where most storage compounds are formed in the late G2/M phase (Kwok and Wong, 2010); product synthesis (Müller et al., 2010; Vicker et al., 1988; Yanpaisan et al., 1999); or the application of mutagenic agents, which should be applied in late S-phase of the cell cycle (Harper et al., 1995). Although there is a large number of nucleic acid dyes (Table 2) emitting blue fluorescence (4′-6-diamidino-2-phenylindole (DAPI), Hoechst 33342), green fluorescence (PICO green, SYBR Green I or II, SYTO 9, SYTO 13, SYTOX-green, TOTO-1, TO-PRO-1, YOYO-1, YO-PRO-1) or red fluorescence (propidium iodide), no universal staining procedure exists over the wide taxonomic diversity of microalgae (Life-Technologies™, 2010). Among the dyes tested, SYBR Green I and II are the most favoured and are used as nucleic acid dyes in oceanography and water ecology (Duhamel and Jacquet, 2006; Evans et al., 2006). The application of classic dyes such as DAPI, Hoechst 33342 and propidium iodide (PI) is limited. DAPI and Hoechst 33342 need additional excitation in the UV range. PI emits fluorescence in the same spectral range as the microalgal pigments (Marie et al., 1996). To overcome this problem, the cells were fixed with ethanol (Kwok and Wong, 2005, 2010) or methanol (Javanmardian and Palsson, 1991) prior to determination of the cell cycle using PI, which lead to a favourable suppression of autofluorescence due to extraction of cell pigments. Since most nucleic acid dyes are G/C but not A/T base pair-specific, they bind both DNA and RNA. Dyes used to study the cell cycle should have a strong affinity for double-stranded DNA (T-A base pairs) without interference from RNA (e.g. SYTO RNAselect) (Nicklisch and Steinberg, 2009), unless it is necessary to treat the cells with RNase (0.1 g l^{-1} final concentration, for 30 min at 37 °C). Moreover some of the nucleic dyes such as DAPI, SYTOX Green, PicoGreen, PI, TOTO-1, TO-PRO-1, YOYO-1 and YO-PRO-1 require cell fixation before staining.

The quantification of microalgal RNA content and the determination of cell cycle phase by FCM were used to identify physiological factors influencing cell growth and production processes. An increased lipid and cellulose content during the cell cycle, with a maximum in late G2/M, was detected in *C. cohnii* using the dyes PI, Nile red and calcofluor white for the determination of cell cycle phases, lipid and cellulose contents respectively (Kwok and Wong, 2005, 2010). Using PI it was determined that the accumulation of autoinhibitory compounds in autotrophic HCD cultures (30 g$_{CDW}$ l^{-1}) of *C. vulgaris* in a photobioreactor was responsible for alteration of the cell cycle, where cell growth is halted both at the commitment and division stages (Javanmardian and Palsson, 1991).

3.1.4. Cellular enzyme activity

Enzymatic activities are a basic feature of vital cells and so provide information on their physiological state. Some enzymatic activities (esterase, respiratory or phosphatase) can be detected by FCM. The activities of intracellular enzymes are proportional to the fluorescence intensity of the dye and depends on the concentration of the respective enzymes inside the cell, the amount of the dye penetrating and simultaneously being excreted from the cell, the incubation time of the dye in the culture, and conditions during incubation (i.e. temperature, pH, ionic strength). Therefore, it is important to measure enzymatic activity immediately after sampling and to use standardised staining conditions. In contrast to cell composition, the normalisation of fluorescence intensity to the cell volume is not necessary to be performed, because the cell metabolic activity does not need to be proportional to cell volume (i.e. smaller cells can possess higher relative metabolic activity).

Esterase activity is the most common way how to measure cellular enzyme activity. For its detection, uncharged non-fluorescent probes from the family of fluorescein diacetates (FDA, cFDA, CMFDA) or calcein acetomethyl ester (Calcein-AM) are used (Jochem, 1999). They diffuse passively into the cells, where the non-fluorescent substrates are hydrolysed by cellular enzymes becoming polar fluorescent

products that are retained by cells with an intact cytoplasmic membrane. Since penetration of fluorescein diacetates into the cells is species dependent, the application of these dyes need to be optimised for individual species (Strauber and Muller, 2010).

The fluorescent dyes cFDA and calcein-AM are preferred due to low rates of dye extrusion and higher stability in comparison to FDA. However, the penetration of cFDA into the cell is very low, therefore cell treatment with EDTA is usually necessary to enhance it (Diaz et al., 2010; Joux and Lebaron, 2000; Müller and Nebe-von-Caron, 2010). Moreover, the detection of esterase activity can be influenced by intracellular pH, because fluorescence intensity of fluorescein dyes is pH dependent (Comas-Riu and Rius, 2009). Cellular enzyme activity was determined by staining with FDA in many applications, such as in cultivation processes with *Chaetoceros muelleri*, *Brachiomonas submarina*, *Pavlova lutheri*, and *Chrysochromulina hirta* under dark conditions (Jochem, 1999; Michels et al., 2010); in cryopreservation studies with *Euglena gracilis* (Fleck et al., 2006); in environmental studies with *M. aeruginosa*, *Scenedesmus obliquus*, *C. pyrenoidosa* (Jochem, 1999; Shi et al., 2007; Zhang et al., 2007a); in toxicity of pollutants such as to copper in *C. vulgaris*, *M. aeruginosa*, *Pseudokirchneriella subcapitata*, *Entomoneis cf punctulata*, *Selenastrum capricornutum*, *Dunaliella tertiolecta*, *Phaeodactylum tricornutum*, *Tetraselmis* sp. (Franklin et al., 2001; Hadjoudja et al., 2009; Stauber et al., 2002); cadmium to *C. reinhardtii* (Jamers et al., 2009); tetrabromobisphenol A (TBBPA) treated *C. reinhardtii*, *C. pyrenoidosa*, *Nitzschia palea*, *P. subcapitata* (Debenest et al., 2010; Liu et al., 2008a); perfluorosulphonic or perfluorocarboxylic acids treated *S. obliquus* (Liu et al., 2008b); and linear alkylbenzene sulphonate homologues were evaluated in four marine microalgae, i.e. *Nannochloropsis gaditana*, *T. suecica*, *Rhodomonas salina*, and *Isochrysis galbana* (Hampel et al., 2001).

Specifically in biotechnological processes, FDA was applied as an indicator of cell vitality during cultivation of *C. vulgaris* under heterotrophic conditions in high-cell density fedbatch culture, where maximal biomass over 160 g l^{-1} was achieved with more than 85% vital cells. The percentage of esterase active cells did not change during the whole cultivation indicating no negative effects on cell vitality (Fig. 2). In contrast, a decrease in esterase activity was observed during the dark phases of *C. pyrenoidosa*, *M. aeruginosa*, or phytoplankton growth during phototrophic cultivation (Dorsey et al., 1989; Wu and Shi, 2008; Zhang et al., 2007a), as well as in *S. capricornutum* after exposure to high concentrations of metals (e.g. copper, 450 ppb; Jochem, 2000). In addition to these studies, FDA was applied to determine the effect of shear stress on cellular enzyme activity of *C. muelleri* where a pressure higher than 1 Pa and shear rate over 10 s^{-1} decreased the percentage of enzyme active cells to 66% (Michels et al., 2010; Table 1).

Respiratory activity in mitochondria is characterised by the electron transport system and can be measured with dyes sensitive to changes in the proton electrochemical potential, such as the green fluorescent dyes Rhodamine 123 or DiOC$_6$(3) (Gregori et al., 2002; Ronot et al., 1986). Alternatively, cellular respiratory activity can be detected with non-fluorescent 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) that is absorbed via electron transport mechanisms and reduced with dehydrogenase into an insoluble, red-fluorescent formazan product (Life-Technologies™, 2010).

In order to investigate the respiratory rate of *D. tertiolecta* under different cultivation conditions, carbonyl cyanide m-chlorophenylhydrazone (CCCP, 3 mM in ethanol, 10 min) was used to uncouple mitochondrial electron transport from proton translocation. A linear relationship between the green fluorescence of DiOC$_6$(3) and respiratory rate of *D. tertiolecta* was observed (Gregori et al., 2002). Damage to respiratory activity was related to an increase in reactive oxygen species (Jamers et al., 2009).

Intracellular generation of reactive oxygen species (ROS) and lipid peroxidation (peroxidase activity) are induced by oxidative stress that can be caused by exposure of cells to shear stress, heavy metals or metal detoxification. The generation of ROS can be

monitored using dihydrorhodamine 123 (DHR123) or dihydroethidium (EH) that are oxidised by free radicals, H_2O_2 or NO_3^-, to the fluorescent dyes rhodamine 123 or hydroethidine, respectively. Alternatively the non-polar probe, 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) is transformed by an intracellular esterase to its non-fluorescent form, 2′,7′-dichlorodihydrofluorescein (H_2DCF). When ROS formation is induced within the cell, H_2DCF is converted into fluorescent 2′,7′-dichlorofluorescein (DCF) (Saison et al., 2010; Yoshida et al., 2004). Another probe for the detection of ROS is a fluorinated carboxylated variant of H_2DCFDA, 5(6)-carboxy-2′,7′-dihydrodifluorofluorescein diacetate (carboxy-H_2DFFDA) that is transformed upon oxidation to fluorescent carboxy-DFF (Life-Technologies™, 2010; Szivak et al., 2009). As a positive control (i.e. presence of ROS) the stained cells can be incubated with H_2O_2 (5%, v/v) for 20 min (Jamers et al., 2009).

Production of ROS was observed in *C. reinhardtii* during osmotic (>0.4 M sorbitol) and salt stresses (>0.2 M NaCl) (Yoshida et al., 2004), or shear stresses of *Protoceratium reticulatum*. The simulation of photo-oxidative stress in *P. reticulatum* cells led to a higher production of pigments (e.g. chlorophyll a, peridinin and dinoxanthin) that have antioxidant properties (Rodriguez et al., 2009; Table 1). This finding can be used to enhance the production of secondary carotenoids such as astaxanthin or lutein that are of interest for biotechnological production (Hu et al., 2008). However, any FCM application for this purpose has not yet been investigated.

Alkaline phosphatase (AP) activity provides information about the nutrient status of cells with respect to the surplus/lack of inorganic phosphate. Since the production of different storage compounds is coupled to limitations in various elements, it is of relevance to control the cellular reserve of such elements (Branyikova et al., 2011). A surplus of phosphate can be measured using the phosphatase substrate 2-(50-chloro-20-phosphoryloxyphenyl)-6-chloro-4-(3H)-quinazolinone (ELF-97) phosphate, which yields intensely green fluorescent precipitates of ELF-97 alcohol (ELFA) upon enzymatic dephosphorylation (Dignum et al., 2004; Fitzgerald and Nelson, 1975; Novotna et al., 2010; Rengefors et al., 2003). Although the manufacturer describes detection of ELF after excitation with a blue laser at 488 nm (Pecorino et al., 1996), other authors were successful only with UV excitation at 363 nm (Dyhrman and Palenik, 1999).

3.1.5. Cell viability and membrane integrity

One of the basic tasks in physiological analysis is the determination of cell viability. It is relevant for a wide variety of research applications and optimisation of production processes, since only viable cells participate in cell growth and production (Davey, 2011). Thus, a decrease in the number of actively producing cells reduces process efficiency and lysed cells may also release undesirable host cell proteins and/or proteases, accelerating product degradation (Cregg et al., 2000; Hyka et al., 2010; Jahic et al., 2003). Despite its frequent use, cell death, viability, or viable-but-not-culturable (VBNC) states are not easily defined terms. Therefore, different approaches for their identification were developed (Berney et al., 2007; Mattila et al., 1997; Peperzak and Brussaard, 2011; Troussellier et al., 1993; Umeno et al., 2005). Cell death is accompanied by a series of processes, among which are the loss or reduction of enzymatic activity or membrane integrity. Each of the methods enables discrimination between stained and unstained cells as two separate sub-populations (Fig. 4), damaged (inactive, dead) and intact (vital, viable). The methods should be tested and optimised for each strain with both living cells and cells that have been killed by incubation at 100 °C for 10–20 min, by microwave heating for 10–60 s or by fixatives (formaldehyde or paraformaldehyde) as negative control.

3.1.5.1. Permeabilised cell membrane.
The detection of damaged or permeabilised cell membranes is the most common and widely used technique for detection of cell viability employing nucleic acid dyes emitting green fluorescence (e.g. SYTOX-green, PICO green, YOYO-1, TOTO-1, YO-PRO-1, TO-PRO-1). These dyes are unable to penetrate intact cells, but they strongly stain cells with permeabilised membranes (Table 2). Application of the most commonly used nucleic acid dye, propidium iodide (PI), is limited for microalgae, because its red fluorescence interferes with autofluorescence of the photosynthetic pigments (Brussaard et al., 2001; Veldhuis et al., 2001). Nevertheless, PI was used to monitor cell viability in several studies (Table 2), where authors do not describe any problem with interference or quenching of autofluorescence during PI staining. In these works several control experiments were carried out with intact exponential growing cells, starved cells, and heat-killed cells (e.g. in a water bath at 100 °C for 10 min), and the authors achieved good
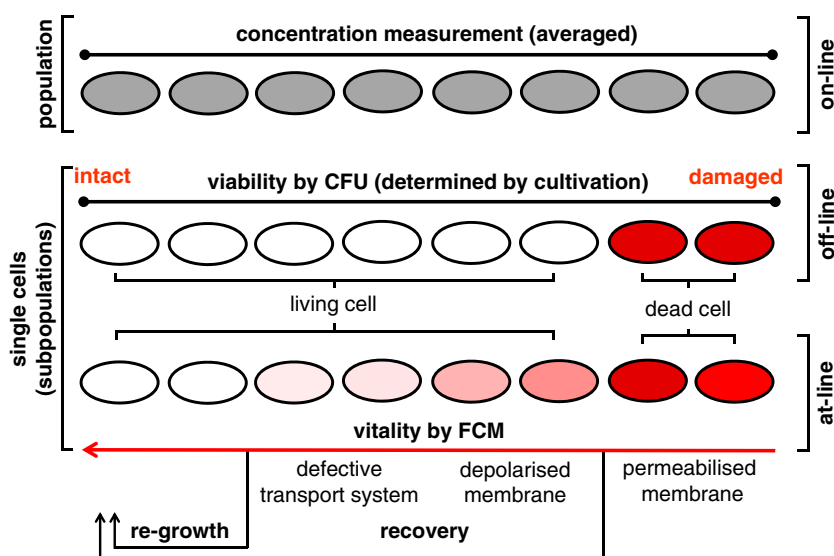

Fig. 4. Approaches for determination of cell viability and vitality. Concentration measurements (e.g. determination of on-line of exhaust gas, metabolites) detect features, which suggest that all cells are in the same (average) physiological state. Single cell analysis methods such as at-line application of FCM or off-line methods such as colony forming units (CFU) as well as epifluorescence microscopy enable live and dead cells to be distinguished. In addition, multi-parametric analysis with FCM enables more specific characterisation of cell viability, based on detection of enzymatic activity and membrane integrity. Live cells in different vitality states can be (easily) re-grown and recovered. However, dead cells are irreversibly damaged.

separation of stained (damaged) and unstained (intact) cells (Adler et al., 2007; Cid et al., 1996; Gonzalez-Barreiro et al., 2006; Lopes da Silva et al., 2009b; Rioboo et al., 2009). The cell membrane integrity of *C. cohnii* determined by PI was not affected by n-dodecane (0.5% v/v), a stimulant for the production of docosahexaenoic acid (DHA) (Lopes da Silva and Reis, 2008), which is important with respect to improved production process of this commercially valuable compound (Table 1).

3.1.5.2. Depolarised cell membrane. The depolarisation of cell membranes can be detected by charged and slightly lipophilic dyes such as the anionic bis-oxonols (DiBAC$_4$(3)), and cationic tetramethylrhodamine methyl ester TMRM, carbocyanines (DiOC$_6$(3)) or Rhodamine 123. The penetration of both anionic and cationic dyes is dependent on the electrochemical membrane potential. Therefore, cationic dyes accumulate in cells with polarised membranes (intact cells) and anionic dyes accumulate in cells with depolarised membranes (damaged cells or resting stages). The fluorescence intensity is influenced by the penetration and extrusion of the fluorescent dye across the cell membrane and with the number of lipophilic binding sites within the cell.

Dyes showing charge-dependent distribution are sensitive markers in ecotoxic assays, because many toxic substances are membrane active and energetically burden the cell (Jochem, 2000; Shapiro, 2003). To validate this method, cells with depolarised membranes were prepared by treatment with uncouplers such as CCCP or dinitrophenol, or inhibitors antimycin or dinitro-o-cresol, which dissipate the proton gradient and depolarise membranes, and afterwards stained with an appropriate fluorescent dye (Adler et al., 2007; Jamers et al., 2009).

3.2. Cell sorting

Flow cytometry (FCM) equipped with a sorting module enables the separation of cells that differ in cell size, morphology, or fluorescence being derived from photosynthetic pigments (autofluorescence) or from applied fluorescent probes. This technique is used in biotechnological processes for the isolation of cells with target features (e.g. isolation of overproducers, strain improvement) or for the preparation of axenic cultures (Table 1). For the latter, staining with undesirably toxic fluorescent dyes should be avoided so that microalgal cells can be separated from other microorganisms due to their strong autofluorescence of native pigments and different morphologies.

Most FCM sorters are based on droplet sorting, where the flow stream of cells is broken into individual droplets containing single cells. Afterwards, the single cells with target features can be gated and isolated (Reckermann, 2000; Shapiro, 2003). Isolated cells with specific properties are deposited onto an agar plate, into 96-multiwell plates or into glass tubes containing an appropriate sterile medium. The cells are recovered for the next cultivation or for further investigation using different analytical methods such as genomics and/or transcriptomics (Andersen, 2005).

3.2.1. Preparation of axenic cultures

Axenic cultures are required for physiological studies and further exploitation, where contamination with other microorganisms is undesirable, or in cultivation under heterotrophic conditions with a higher risk of overgrowth of the microalgal culture by contaminants (Sensen et al., 1993). Nevertheless, microalgal cultures from natural samples and also from culture collections are mostly contaminated, at least by bacteria. The traditional, most widely used methods for the isolation of axenic cultures employs microscopy and micropipetting, together with streaking cultures on a selective medium in agar plates, or using a serial dilution technique. However, due to the limitation of visual methods and the fast reproduction of bacterial cells, these methods are time consuming and mostly successful only for the separation of microalgae from co-occurring contaminating cells larger than 5 μm (Li, 2002; Sinigalliano et al., 2009). These obstacles can be overcome by cell sorting (Fig. 5), which permits the
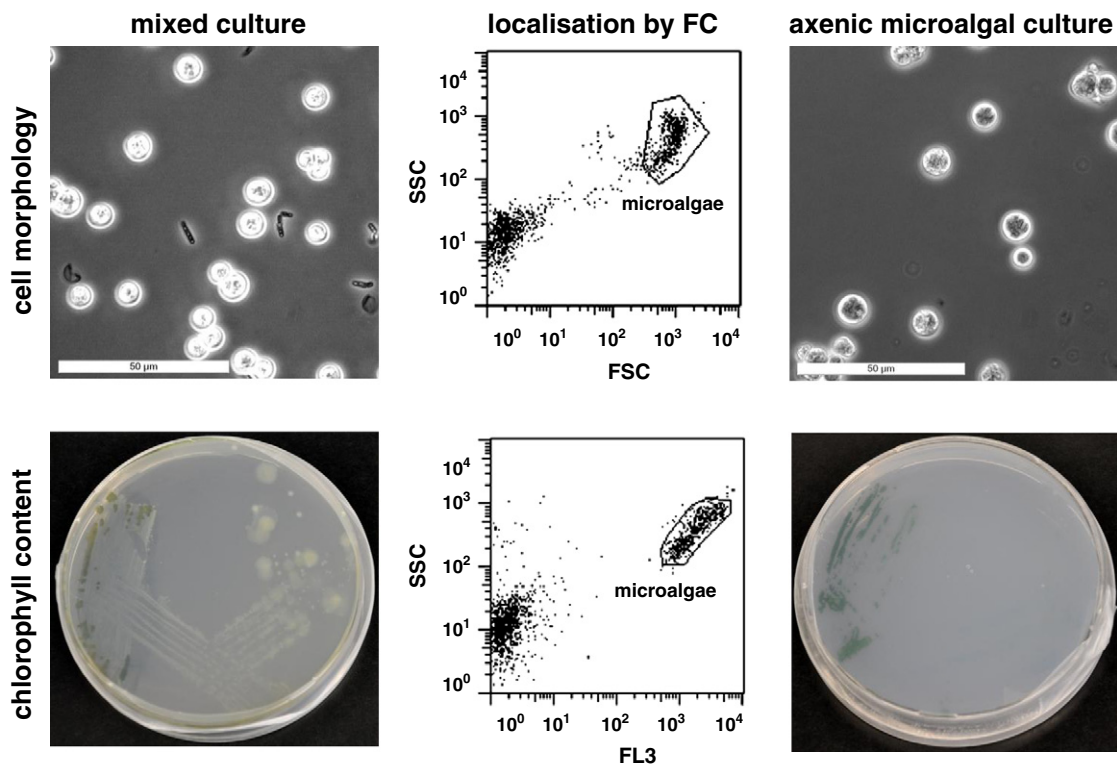

Fig. 5. Separation of microalgae from bacteria. The outer left and right columns with microphotographs from liquid cultures and photographs of agar plates represent the situations before and after sorting the suspended *Chlorella vulgaris* cultures respectively. The middle column shows a dot-plot as result of a FCM analysis with the gated region of microalgal populations, which are distinguishable from contamination due to their size (FSC), granularity (SSC) and chlorophyll content (FL3, detected as autofluorescence at >650 nm).

efficient differentiation and subsequent isolation of single cells based upon their morphology (size) and variations in autofluorescence (Cellamare et al., 2010; Sensen et al., 1993; Surek and Melkonian, 2004). The rate of success for the isolation of axenic microalgal cultures collected from natural habitats was reported to be considerably higher than with a classical approach (Sensen et al., 1993). Using this method with a flow cytometer FACSAria™ (BD Bioscience) and without application any fluorescent dye, several species such as *Chlamydomonas* sp., *Chlorella* sp., *Coelastrum* sp., *Scenedesmus* sp. contaminated with unidentified bacteria were axenised in our laboratory. An example of a contaminated *C. vulgaris* axenisation is given; the initial microalgal culture, heavily contaminated by bacteria, was successfully cleared of this contaminant by cell sorting, producing an axenic culture (Fig. 5). In special cases, isolated cells can be highlighted by applying surface antibodies with fluorescent labels (Peperzak et al., 2000).

Due to the post-sorting decrease in cell viability, it is recommended to collect more than 100 cells from the population (Crosbie et al., 2003). Cells might be affected or destroyed by fluid acceleration, electrical or mechanical shock, as well as by optical stress (Cellamare et al., 2010; Sinigalliano et al., 2009). However, mostly these effects tend to be temporary or have minimal impacts on the long-term growth potential (Haugen et al., 1987). After sorting, the isolated cells are recovered, mostly in an appropriate sterile medium with organic carbon as an energy source, simultaneously controlling the axenicity of the culture. During recovery, the medium composition (salinity, nutrient concentration) and light conditions for growth of the sorted cells as well as for the detection of possible contaminants should be optimised. When isolating unknown microalgae, it is appropriate to use a sterile natural medium such as filtered lake or sea water.

The axenicity of the microalgal sample can be in turn checked by FCM. The total number of potentially contaminating microbial cells is enumerated after staining the sample with a nucleic acid dye (e.g. SYTO 9, SYTO 13, SYBR Green; with trigger at FL; see Table 2), while the concentration of microalgal cells is determined by their autofluorescence. The relationship between total cell number and the number of autofluorescent microalgal cells then shows the degree of contamination of the sample (Mendoza et al., 2008).

3.2.2. Isolation of overproducers

The goal of optimising production processes is the enhancement of productivity of a target compound. Besides the optimisation of cultivation conditions, there is the possibility to select strains with improved features or overproducers (e.g. of lipids, carotenoids, see Table 1). The ability of microalgae to adapt their metabolism to various culture conditions provides opportunities to modify and maximise the formation of target compounds using non-recombinant cells. For instance, adaptive responses, which help microalgae to survive environmental stress, lead to maximising the content of lipids including polyunsaturated fatty acids (PUFAs) or antioxidants (Jiang and Chen, 1999). Thus, achieving a better understanding of the underlying regulatory mechanisms is mandatory to implementing such 'production triggers' (Rosenberg et al., 2008).

Overproducing strains can be isolated after exposing the cells to stress conditions or after treating them with a chemical mutagen such as the alkylating agent ethyl methane sulphonate (EMS) (Mendoza et al., 2008). The desired overproducing strains are identified on the basis of their higher autofluorescence intensity or the higher fluorescence intensity of a specific probe, and then sorted and analysed. In this way mutant strains of *Dunaliella salina* with higher production of ß-carotene (20–40 pg carotene per cell) than in the wild type (5–15 pg carotene per cell) were selected on the basis of their higher autofluorescence of pigments (Nonomura and Coder, 1988). After addition of the mutagen EMS, the overproducing mutant with carotenoid productivity of 1.7 µg ml^{-1} day^{-1} was isolated based on higher fluorescence after NR staining (Mendoza et al., 2008). Using NR staining of lipids in combination with FCM cell sorting, a *Nannochloropsis* sp.

overproducing strain with lipid content of up to 45% CDW (Doan and Obbard, 2011a) and a *T. suecica* strain with a stable and four times higher fluorescence intensity than the original population, were isolated (Montero et al., 2011). In these studies, over 50% cell recovery of sorted cells was achieved. Recently, Jehmlich et al. (2010) introduced a combination of FCM sorting with liquid chromatography (LC) and mass spectrometry (MS) to identify proteins from sorted cells. FCM and gel-free proteomic analysis enabled the identification of more than 900 proteins from sorted microbial cultures (Jehmlich et al., 2010).

Alternatively to cell sorting, magnetic cell sorting (MACS), dielectrophoresis-activated cell sorting (DACS) or panning can be used for cell selection according to expression of surface molecules that can be detected with specific ligands. However, in comparison with cell sorting, these methods have lower precision or throughput and require binding with specific ligands. For details and a description see reviews (Füchslin et al., 2010; Ishii et al., 2010; Jamers et al., 2009; Keserue et al., 2011).

4. Opportunities and unfulfilled needs

Increasing interest in microalgal biotechnology calls for an adaptation of methods for studying culture heterogeneity, which have so far only been applied in the development of bioprocesses using bacteria or yeasts. Demand for such studies at single cell level has driven the development of many new analytical methods (Brehm-Stecher and Johnson, 2004; Moore et al., 2009). Among them, FCM in combination with fluorescent staining stands out for its high accuracy and rapid speed when used to examine multi-parametric targets (Wang et al., 2010) as well as for the opportunity to select (sort) cells of interest. FCM analysis is a method that allows relative changes in cell physiological state (including biomass composition) to be detected during a cultivation process. Various FCM methods for the determination of the physiological state of microorganisms have been established on the basis of know-how from medical and environmental applications of FCM (Diaz et al., 2010).

The most limiting disadvantage of FCM applications seems to be the necessary standardisation of methods and measurements. Comparison between data from various laboratories using different FCM devices is generally complex and occasionally not possible. Method standardisation, calibration, control and validation are therefore mandatory to provide consistent and reliable FCM analyses. Standardisation is applied in three ways: (i) standardisation of methods including sample manipulation and processing, (ii) instrument standardisation (i.e. calibration of fluidics, optics and electronics), and (iii) standardisation of data analysis and interpretation (Nebe-von-Caron, 2009). A guide for fluorescent instruments by ASTM International (the American Society for Testing and Materials, www.astm.org) provides criteria for the choice of suitable standards, design of fit-for-purpose calibration tools, and general instructions for use (ASTM_E_2719, 2010; Hoffman, 1997). Moreover, FCM requires sophisticated data analysis and is restricted to liquid samples (Wang et al., 2010). Other downsides of FCM are the high price of the instruments and high demands on operator skills. However, high-resolution, easy-to-handle and affordable FCM devices (e.g. BD Accuri® C6 and Partec CyFlow SL, which cost less than €40,000) have been developed. They allow FCM-based methods to be used as routine techniques in the monitoring and control of bioprocesses.

The specific physiological state of a microalgal culture can be monitored using FCM methods during the cultivation process (Fig. 2). Various cell features are determined at every stage of the process, which can be controlled by settings to achieve the desired performance. Although several FCM methods have been used for the optimisation of production processes with microalgae (Table 1), the current literature reveals further opportunities for implementation of innovative FCM methods that focus on the development of new process strategies, strain design and process control. For instance, the control of ROS by FCM would enable optimisation of carotenoid production after addition

of H_2O_2 or Fe^{2+} as a production trigger (Ip and Chen, 2005; Ma and Chen, 2001). Moreover, an increase in salinity is known to be the trigger for enhanced DHA production (Jiang and Chen, 1999). An as yet unrealised potential for rapid strain development is presented by the combination of mutagenesis (for instance, by UV irradiation or N-methyl-N-nitro-N-nitrosoguanidine; Hu et al., 2008) and selection of the desired mutant using an FCM. An application for selection of overproducing microalgal cells, combined with their in-depth analysis by omics-methods, would also accelerate the molecular design of strains with desired features (Ishii et al., 2010; Jamers et al., 2009; Müller and Nebe-von-Caron, 2010). As new fluorescent dyes/probes are being introduced, the list of possible targets (and measurable parameters) is also expanding (Hammes and Egli, 2010). Moreover, the development of FCM techniques applicable in an automated on-line mode (Kacmar et al., 2006) promises the incorporation of FCM into process analytical technology (PAT) as well as quality by design (QbD) concepts (Broger et al., 2011; Sitton and Srienc, 2008).

5. Conclusions

A boom in microalgal biotechnology has been evident in the last decade. In this review flow cytometry (FCM) exploiting the natural autofluorescence of photosynthetic pigments or in combination with fluorescent dyes is described as a rapid, accurate and sensitive method with the potential to contribute to efficient development of biotechnological processes with microalgae.

FCM offers opportunities for the following applications:

- control of biomass composition and cell viability,
- identification and counteracting of stress factors,
- selection of cells with targeted (improved) features, and
- isolation of axenic cultures.

As FCM has found its place in the monitoring, control and optimisation of bioprocesses with bacteria, yeasts and mammalian cells, it is expected to become established with microalgae. Its implementation will be further facilitated by information already available from environmental and toxicological studies of microalgal populations.

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