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Multi-parameter flow cytometry and cell sorting reveal extensive physiological heterogeneity in *Bacillus cereus* batch cultures

Andrew Want · Helen Hancocks ·
Colin R. Thomas · Stuart M. Stocks ·
Gerhard Nebe-von-Caron · Christopher J. Hewitt

Abstract Based on two staining protocols, DiOC$_6$(3)/propidium iodide (PI) and RedoxSensor Green (an indicator of bacterial reductase activity)/PI, multi-parameter flow cytometry and cell sorting has identified at least four distinguishable physiological states during batch cultures of *Bacillus cereus*. Furthermore, dependent on the position in the growth curve, single cells gave rise to varying numbers of colonies when sorted individually onto nutrient agar plates. These growing colonies derived from a single cell had widely different lag phases, inferred from differences in colony size. This further highlights the complex population dynamics of bacterial monocultures and further demonstrates that individual bacterial cells in a culture respond in markedly dissimilar ways to the environment, resulting in a physiologically heterogenous and dynamic population.

List of symbols
Ψ Electro potential
i Inside
o Outside
a Active (diffusible or unbound) concentration
R Universal gas constant
T Absolute temperature
Z Charge
F Faraday constant

A. Want · C. J. Hewitt (✉)
Centre for Biological Engineering, Department
of Chemical Engineering, Loughborough University,
Leicestershire LE11 3TU, UK
e-mail: c.j.hewitt@lboro.ac.uk

H. Hancocks · C. R. Thomas
Centre for Bioprocess Engineering, School of Chemical
Engineering, University of Birmingham,
Edgbaston B15 2TT, UK

S. M. Stocks
Novozymes A/S, Smørmosevej 25 (2JS.12),
2880 Bagsværd, Denmark

G. Nebe-von-Caron
Inverness Medical International, Priory Business Park,
Bedford MK44 3UP, UK

Introduction

Multi-parameter flow cytometry has many advantages over conventional microbiological analyses such as dilution plating (cfu per ml) and these have been extensively reviewed elsewhere (Davey and Kell 1996; Shapiro 2003; Hewitt and Nebe-von-Caron 2001, 2004) but, briefly, using various mixtures of fluorescent dyes, it is possible to resolve the

physiological state of individual microbial cells beyond culturabilty, in 'real-time' based on the presence or absence of an intact polarised cytoplasmic membrane and the transport mechanisms across it. The presence of both an intact polarised cytoplasmic membrane and active transport systems across it are essential for a fully-functional healthy cell. The cytoplasmic membrane potential (CMP) of prokaryotes is between 100 and 200 mV, the exact magnitude depending on the physiological state of the cell with the inside of the cell negative with respect to its exterior (Shapiro 2003). In general, Gram-negative cells have a higher maximum CMP than Gram-positive cells so anionic stains such as bis-(1,3-dibutylbarbituric acid) trimethine oxonol (bis-oxonol) are often used (Jepras et al. 1997; Hewitt et al. 1998) to reflect CMP in preference to fluorescent probes that are accumulated actively (e.g. Rhodamine 123). This is because Gram-negative bacteria, like *E. coli,* frequently do not take up cationic stains unless the outer membrane of the cell is permeablised (e.g. by repeatedly washing with an EDTA solution (Hewitt et al. 1998)). Therefore, it is not known if any subsequent staining reflects the true CMP at the time of sampling or whether it is an artefact due to the treatment regime employed (Davey and Kell 1996). This can be further complicated if active transport systems exist that pump the fluorescent stain out of the cell, since interference with or from such transport systems will obviously influence CMP (Nebe-von-Caron and Badley 1995).

In theory, distributional probes should partition themselves as described in the Nernst equation:

$$\psi_i - \psi_o = \frac{RT}{zF} \times \ln\left(\frac{a_o}{a_i}\right) \qquad (1)$$

However, theory and practise are often not in perfect alignment. In the case of cationic probes, such as rhodamine 123, potential-based staining is perceived to be diminished at high dye concentrations, leading to indistinguishable dye uptake in polarised and depolarised cells (Nebe-von-Caron and Mueller 2007). For anionic probes, an increase in target sites even with a low uptake of dye can lead to accumulation of dye intracellularly. Thus, if the CMP does not overcome the affinity of the probe to the intracellular target or a temporary 'leakage' across the cytoplasmic membrane exists, the dye accumulates inside the cell. The control applied to test for

CMP independent accumulation of a dye is to collapse the CMP using decouplers (Hewitt et al. 1999). However, this method fails if the cells do not stain because of their outer membrane or active dye export or because of uptake due to temporary 'leakiness'. In those cases the best control is to attempt staining with a CMP probe of the opposite charge.

In the case of a Gram-positive *Rhodococcus* sp., only $\sim$40% of the cell population showed exclusion of bis-oxonol during nutrient sufficient conditions (Hewitt and Nebe-von-Caron 2004). Since it is unlikely for the cells not to possess a CMP during exponential growth this indicates that the uptake of bis-oxonol in this case does not reflect the complete collapse of the CMP. Additionally, the cells still excluded propidium iodide (PI), indicating that a certain degree of cytoplasmic membrane integrity remained. Such counter intuitive phenomena are not unique, PI straining during exponential phase has been observed in actinomycetes (Sebastine et al. 1999; Stocks and Thomas 2001) and mycobacteria (Shi et al. 2007). While it may be possible to construct explanations for the reasons that "live" cells fail to stain negative with bis-oxanol, or can stain positive with PI, one must also consider that such populations contain cells which are in fact CMP negative, with intact membranes, or that there can be a minimum in "viability" during exponential growth.

There are also obvious differences between Gram-positive and-negative organisms, in their membrane architectures. The cationic stain, 3,3′-dihexylocarbo-cyanine iodide-DiOC$_6$(3), that accumulates actively (i.e. that enter and stain the cell when the cytoplasmic membrane is polarised) was preferential for reflecting changes in CMP for Gram-positive organisms (Müller et al. 1996; Lopes da Silva et al. 2005) especially since they do not have to traverse an outer cell membrane. Alternatively, intracellular reduction–oxidation activity can also be used as a measure of cell metabolic activity since such activity is also related to the function of the electron transport chain (hence CMP) in addition to the anabolic and catabolic activity of the cell (Haughland 2002).

In the work presented here we use multi-parameter flow cytometry and cell sorting to understand more of the staining phenomena for the Gram-positive bacterium *Bacillus cereus* during batch culture. Specifically, the fluorochromes used were DiOC$_6$(3) and the

proprietary RedoxSensor Green (an indicator of bacterial reductase activity, Molecular Probes, Invitrogen, UK) both counter-stained with propidium iodide to monitor the physiological state of *Bacillus cereus* throughout its growth curve.

Materials and methods

Organism and growth conditions

Bacillus cereus NCTC 11143, an asporulating mutant, was chosen as a model organism because it is a common rod-shaped, soil-dwelling bacterium that is easy to grow in the laboratory. It was grown in 50 ml nutrient broth (oxoid) without glucose, in 500 ml baffled shake-flasks at 37°C and 200 rpm in an orbital shaker for ∼14 h. This culture was then used to inoculate 24 similarly prepared flasks, with duplicate flasks taken periodically for analysis.

Flow cytometry protocols

Flow cytometric analysis and cell sorting were done by a coulter EPICS ELITE flow cytometer (High Wycombe, UK) with 488 nm excitation from an argon-ion LASER at 15 mW. Samples taken from the culture were immediately diluted, to a constant cell number, as measured by the flow cytometer data rate, (at least 1:2,000 v/v) with DBS (pH 7.2) and stained with one of two mixtures of fluorescent stains either PI/DiOC$_6$(3) or PI/RedoxSensor Green. Samples were kept in a sonication bath for 10 s prior to analysis, in order to reduce problems associated with cell aggregation. The sorting method was optimised using 660 nm diam. Fluorescent latex beads, with a 3-droplet sort, and visualised under a fluorescence microscope. Individual cells were sorted directly on nutrient agar plates (at least 100 cells on each of a minimum of 5 plates per sort) such that each cell could give rise to a discrete visible colony after 24 h at room temperature. For fluorescent microscopic examination, populations of like cells were sorted on to filter discs pre-wetted with Dulbecco's buffered saline (DBS, pH 7.2) and examined under the fluorescent microscope within 1 min. Stock solutions of each dye were prepared as follows: DiOC$_6$(3) was made at 10 μg ml^{-1} in dimethyl sulphoxide (DMSO), PI was made at 2 mg ml^{-1} in distilled water and RedoxSensor Green

was added at the concentration supplied by the manufacturer. The working concentrations of DiOC$_6$(3), RedoxSensor Green and PI were 0.3 μg ml^{-1}, 3 μM and 0.3 μg ml^{-1}, respectively. The DiOC6(3) or RedoxSensor were added to the diluted cell sample first, followed by PI, necessitating a 1 min incubation (at room temperature) for the former and 30 s for the latter, prior to analysis. All solutions were passed through a 0.2 μm filter immediately prior to use to remove particulate contamination. Signal discrimination was set on forward angle light scatter (FALS or FS) and right angle light scatter (RALS or SS) signal. The optical filters were set up so that PI fluorescence was measured using a 630 nm long pass while DiOC$_6$(3) and RedoxSensor Green fluorescence were measured using a 525 nm band pass. In all cases there was some spectral overlap between the emitted fluorescence of the stains in mixtures however, due to the large dynamic range of the signals compensation was kept to a minimum to aid cluster differentiation but to avoid overcompensation. Bacteria were identified by their light scattering cluster which was used as a gate for the fluorescence density plots.

Other analytical techniques

OD$_{580}$ values were measured with appropriate dilution of samples in DBS to be in the range 0–0.6 absorbance units. Colony forming units (cfu per ml) was determined by serial dilution spread plated onto nutrient agar. The dry cell weight (DCW g/l) was measured of samples dried at 100°C to constant weight. Microscopy was performed using a Leitz Ortholux II microscope with the I3 filter cube (513719) giving 470 ± 25 nm excitation at 50% transmission and emission 520 nm LP at 50% transmission. A 100× oil immersion objective was used and images were taken using a with a Nikon DS-2Mv camera.

Results and discussion

Duplicate batch fermentations of *Bacillus cereus* were carried out in which samples were taken periodically and stained with one of two mixtures of fluorescent stains, either DiOC$_6$(3)/PI or RedoxSensor Green/PI, over the course of a standard growth curve (Fig. 1). A 2 h lag phase was followed by 2 h

Fig. 1 $OD_{580\ nm}$, DCW (g/l), cfu/ml and pH profiles show mean values for four replicate 24 h batch fermentations with *Bacillus cereus*. *Error bars* show the standard deviation of the data used to calculate the mean values (minimum 14 measurements)
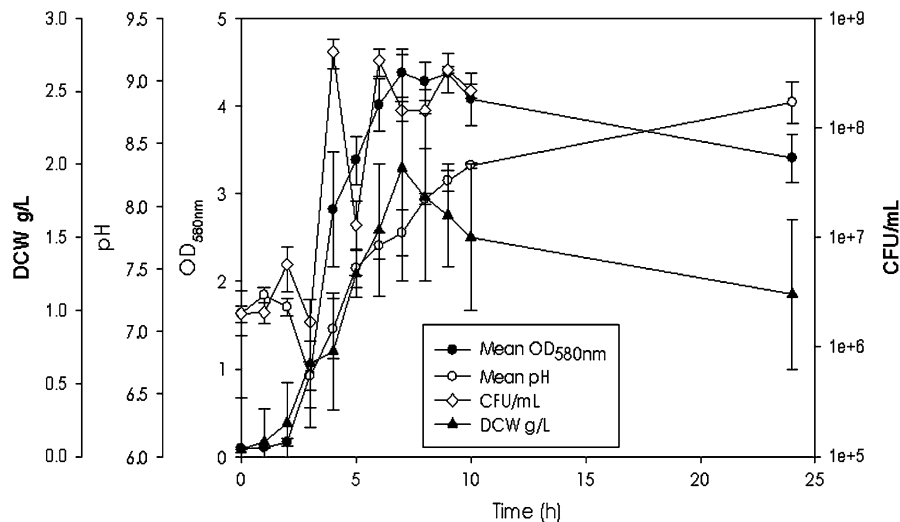

of rapid growth, with a maximum specific growth rate (μ_{max}) of 1.02 h^{-1} recorded at 3 h, after which the culture entered the stationary phase before being terminated at 24 h. It can be seen from the pH curve that the initial fall, resulting from the depletion of the complex carbon-source present, was arrested after only 3 h. Following this point, the pH steadily and continuously rose, probably as a consequence of deamination as amino acids are consumed as a carbon-source, with the rising pH further influencing the metabolism of the cells, the culture eventually entering the conventionally termed stationary and decline phases.

For samples stained with $DiOC_6(3)$/PI when compared to the control the presence of up to three populations of cells could be expected. This was found to be true here (Fig. 2) although in the case of lag/stationary phase *Bacillus cereus* cells a fourth sub-population was also identified (C). This corresponded to cells stained with both PI and $DiOC_6(3)$; paradoxically this would mean that these cells had a permeablised but polarised cytoplasmic membrane. The presence of the fourth sub-population has been shown previously (Montfort and Baleux 1996) and it has been proposed that with lipophilic cationic carbo-cyanine dyes, such as $DiOC_6(3)$, non-specific energy-independent binding can occur when the hydrophobic regions of the cytoplasmic membranes are exposed to relatively harsh conditions such as exposure to dehydration in ethanol or high concentrations of the fluorochrome. However, this was not the case here.

Indeed when double $DiOC_6(3)$/PI stained cells were sorted onto a filter paper and examined under the microscope it could be seen (Fig. 5) that this population was made up of doublets i.e. two cells attached together, one stained positively for PI and the other stained positively for $DiOC_6(3)$. Due to the site of connection being along the longitudinal axis of the cells, it is likely that these doublets were the result of incomplete cell division (Haeusser and Levin 2008) rather than cells simply 'sticking together'. The same events sorted onto nutrient agar plates gave rise to a colony >65% of the time (Fig. 5) with each colony presumably being derived from the PI negative cell, approximately the same as that derived from the $DiOC_6(3)$ only stained population where a colony was produced ~70% of the time.

For cells stained with RedoxSensor Green/PI, the existence of three populations of cells could also be expected. This was the case here (Fig. 3) although in the case of lag/stationary phase *Bacillus cereus* cells a fourth sub-population could again be identified (C). This corresponds to cells stained with both PI and RedoxSensor Green; counter intuitively this would mean that cells had a permeablised cytoplasmic membrane but retained a reductase activity. Whilst this could be the case, as enzyme activity is independent of cell permeabilisation, the retention of the substrate would be less likely once the cell integrity is lost. Indeed when RedoxSensor Green and PI double stained cells were sorted onto a filter membrane and examined under the microscope it

Fig. 2 Flow cytometric analysis of cell samples taken **i** pre-inoculation then at **ii** 2 h, **iii** 4 h, **iv** 8 h, **v** 9 h and **vi** 24 h post-inoculation during the course of a 24 h batch fermentation with *Bacillus cereus* stained with DiOC$_6$(3)/PI. Up to four populations of cells could be identified. These correspond to cells with an intact depolarised cytoplasmic membrane, not stained (*a*), cells with an intact polarised cytoplasmic membrane, stained with DiOC$_6$(3) (*b*), cells with a permeablised depolarised cytoplasmic membrane stained with PI only (*d*), and cells stained with both PI and DiOC$_6$(3) (*c*)
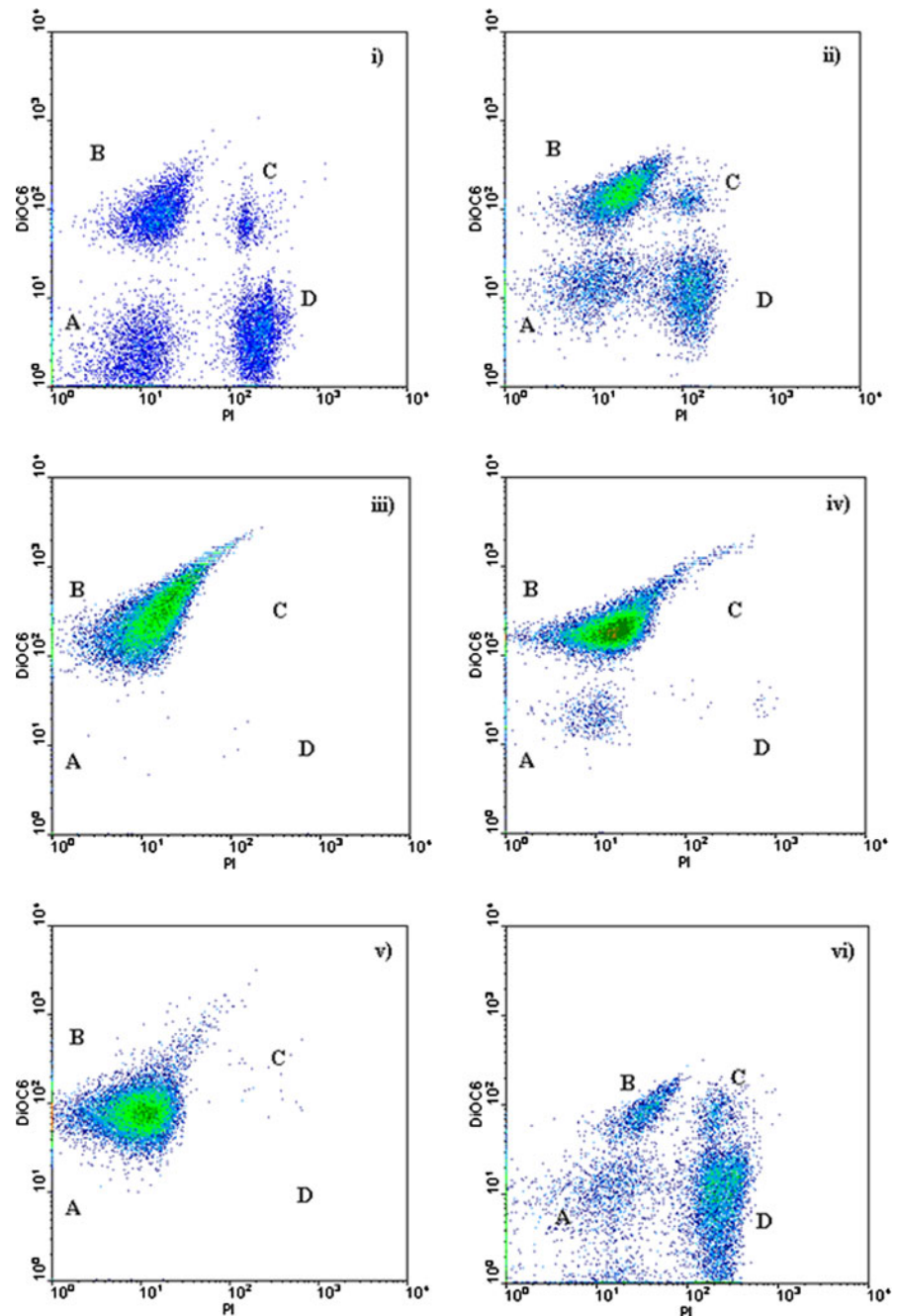
could indeed be seen (Fig. 6) that this population was, again, made up of doublets i.e. two cells attached together, one stained positively for PI and the other stained positively for RedoxSensor Green. As previously observed, this subpopulation, when sorted onto nutrient agar plates gave rise to a colony >70% of the time (Fig. 6) with each colony,

putatively, being derived from the PI negative stained cell. This percentage was approx. the same as that derived from the RedoxSensor Green only stained population, where a colony was produced ∼72% of the time. For cells stained with RedoxSensor Green/PI, a fifth sub-population of cells could also be identified in the bottom corner of quadrant A.

Fig. 3 Flow cytometric analysis of cell samples taken **i** pre-inoculation then at **ii** 2 h, **iii** 4 h, **iv** 8 h, **v** 9 h and **vi** 24 h post-inoculation during the course of a 24 h batch fermentation with *Bacillus cereus* stained with RedoxSensor Green/PI. Up to four populations of cells could be identified. These correspond to cells with an intact cytoplasmic membrane with a low reductase activity, not stained *a* cells with an intact cytoplasmic membrane with a higher reductase activity stained with RedoxSensor Green *b*, cells with a permeablised cytoplasmic membrane with no reductase activity stained with PI only *d* and cells stained with both PI and RedoxSensor Green *c*
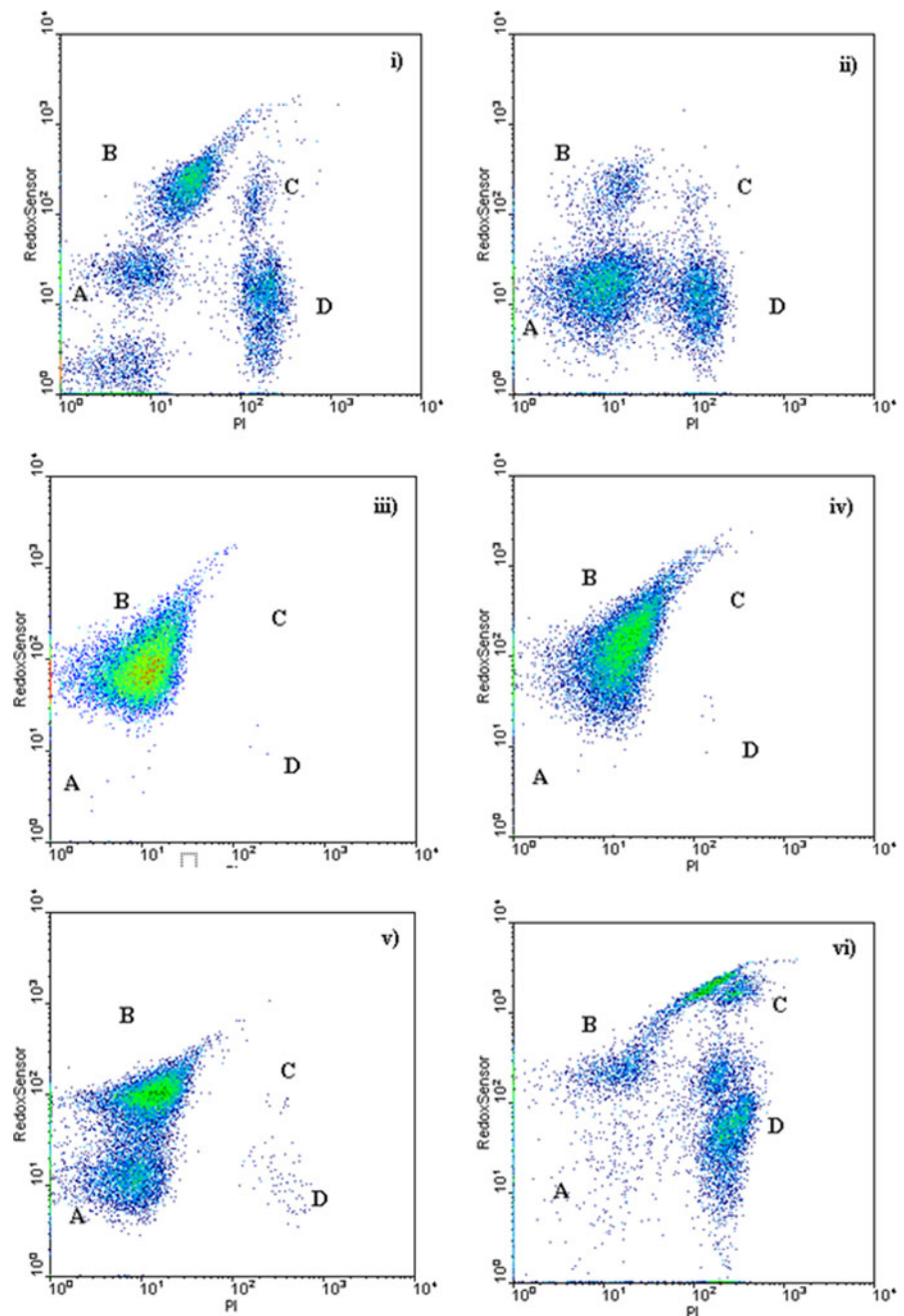
Following sorting, events did not give rise to any colonies on nutrient agar. Back gating identifies those events mainly to consist of debris by their typical meandering light scatter clustering (data not shown). Although, some events scatter like bacterial cells the conclusion here is that these events were related to cell ghosts, cell debris or other particulate 'noise' (Lewis et al. 2004).

It is clear that the quality of the inoculum for the growth experiments would be sub-optimal for an industrial fermentation, with only ∼28% of cells being positively stained with only either $DiOC_6(3)$ or RedoxSensor Green at the start. However, as a tool for development of novel assays for detecting cellular stress, this feature is invaluable. In both cases within 4 h of inoculation almost all cells were either only

Fig. 4 Functional assessment of cells: **i** unstained, **ii** stained with DiOC6(3)/PI, **iii** RedoxSensor Green/PI and cell sorting. Rapid growth phase cells of *Bacillus cereus* i.e. 4 h after inoculation sorted directly onto nutrient agar plates
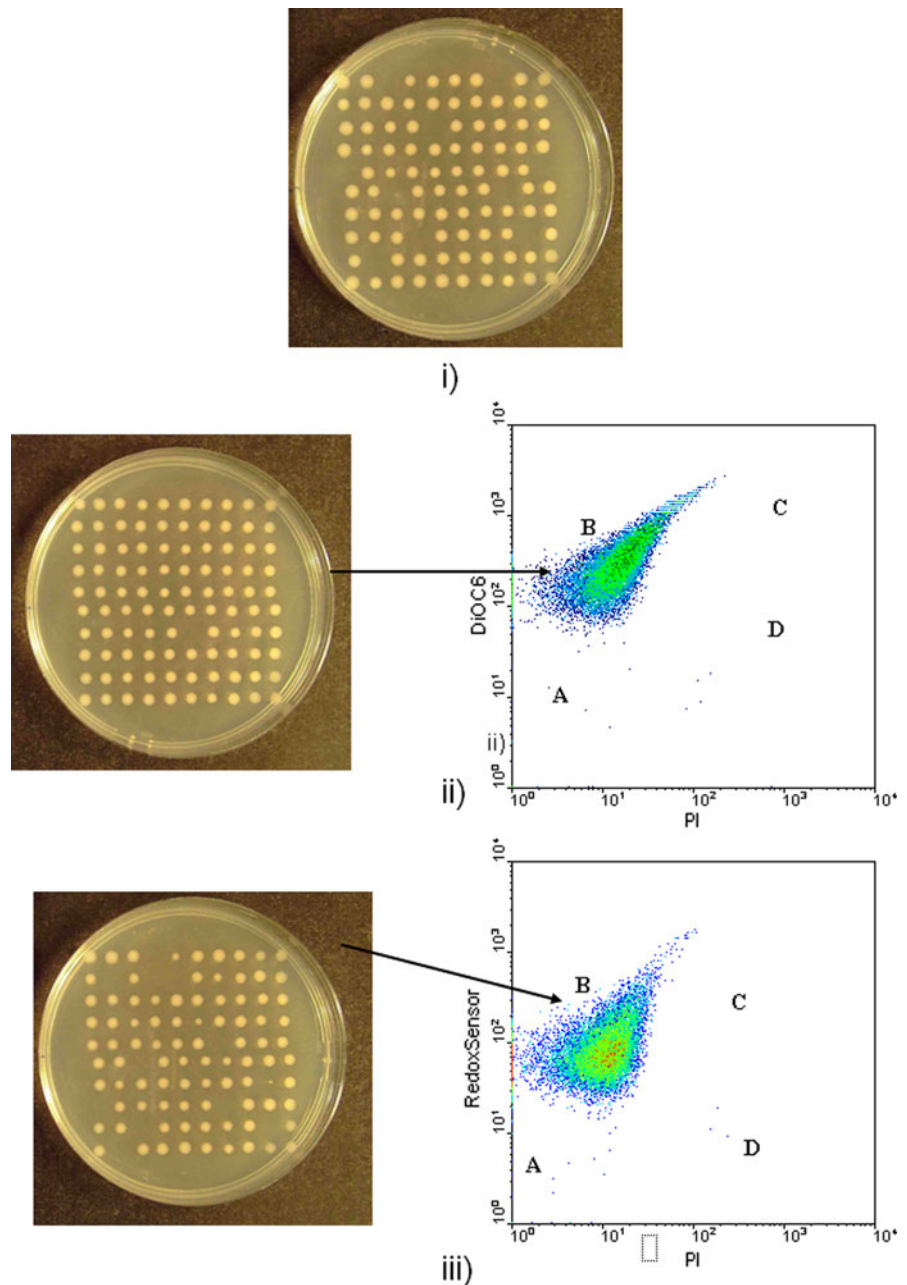
DiOC6(3) or RedoxSensor Green positive, as was to be expected, because the culture had, by then, entered the period of most rapid growth. This remained unchanged through 8 h until the culture began to enter the stationary phase of growth whereupon there was a progressively detrimental change in cell physiological state until the experiment was terminated at 24 h.

Further analysis of the cell sorting data revealed some interesting points. Unstained cells from the most rapid phase of growth when sorted directly onto nutrient agar produced a colony >90% of the time (Figs. 4, 5, 6). Therefore, it can be concluded that passage through the flow cell and laser at 15 mW had little detrimental effect on the reproductive viability of the cells. Whilst in the absence of staining it is not

Fig. 5 Functional assessment by *multicolour staining* with $DiOC_6(3)$/PI and cell sorting. Lag phase cells of *Bacillus cereus* i.e. 2 h after inoculation stained and sorted directly onto nutrient agar plates
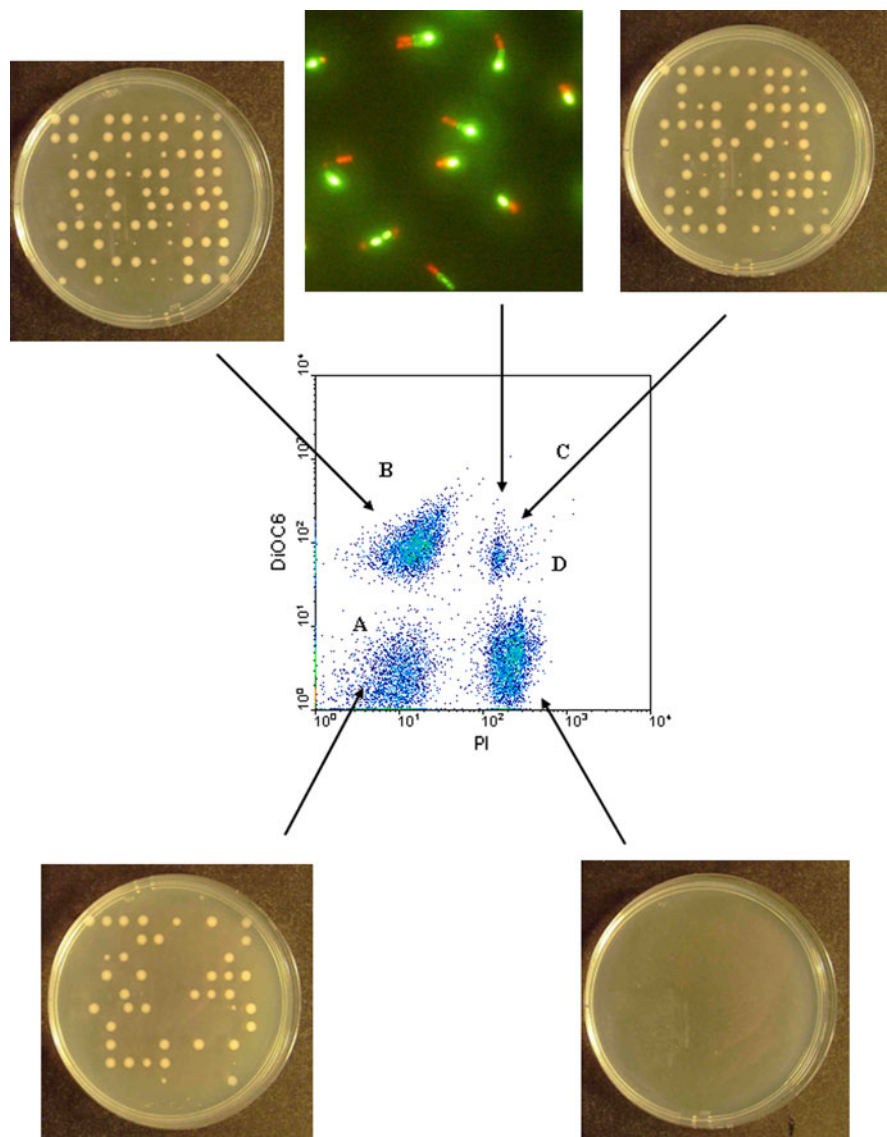
possible to eliminate interfering particles by the absence of dye uptake, it is to be noted that a 100% recovery of cells on the nutrient agar plate should not be expected since cells only divide when conditions are absolutely perfect such that sub-lethally injured and other so called 'viable but nonculturable' cells are often missed (Oliver 2010) unless the right artificial growth medium is employed. Indeed a 3 log difference in the number of cells recovered from the same *Salmonella* spp. sample has been observed dependent on the type of solid agar medium used (Nebe-von-Caron and Badley 1995). When cells from

the most rapid phase of growth, stained only with $DiOC_6(3)$, were sorted based on light scatter only, a colony was produced >99% of the time and when stained only with RedoxSensor Green >90% of the time (Fig. 4) implying that positive staining with $DiOC_6(3)$ somehow increased the ability of cell to produce a colony on nutrient agar. Why this is so is not clear and will need further investigation.

Closer examination of the data reveals that cells positively stained with either $DiOC_6(3)$ or Redox-Sensor Green taken from the phase of most rapid growth gave rise to a colony ∼95% of the time,

Fig. 6 Functional assessment by multicolour staining with RedoxSensor Green/PI and cell sorting. Lag phase cells of *Bacillus cereus* i.e. 2 h after inoculation stained and sorted directly onto nutrient agar plates
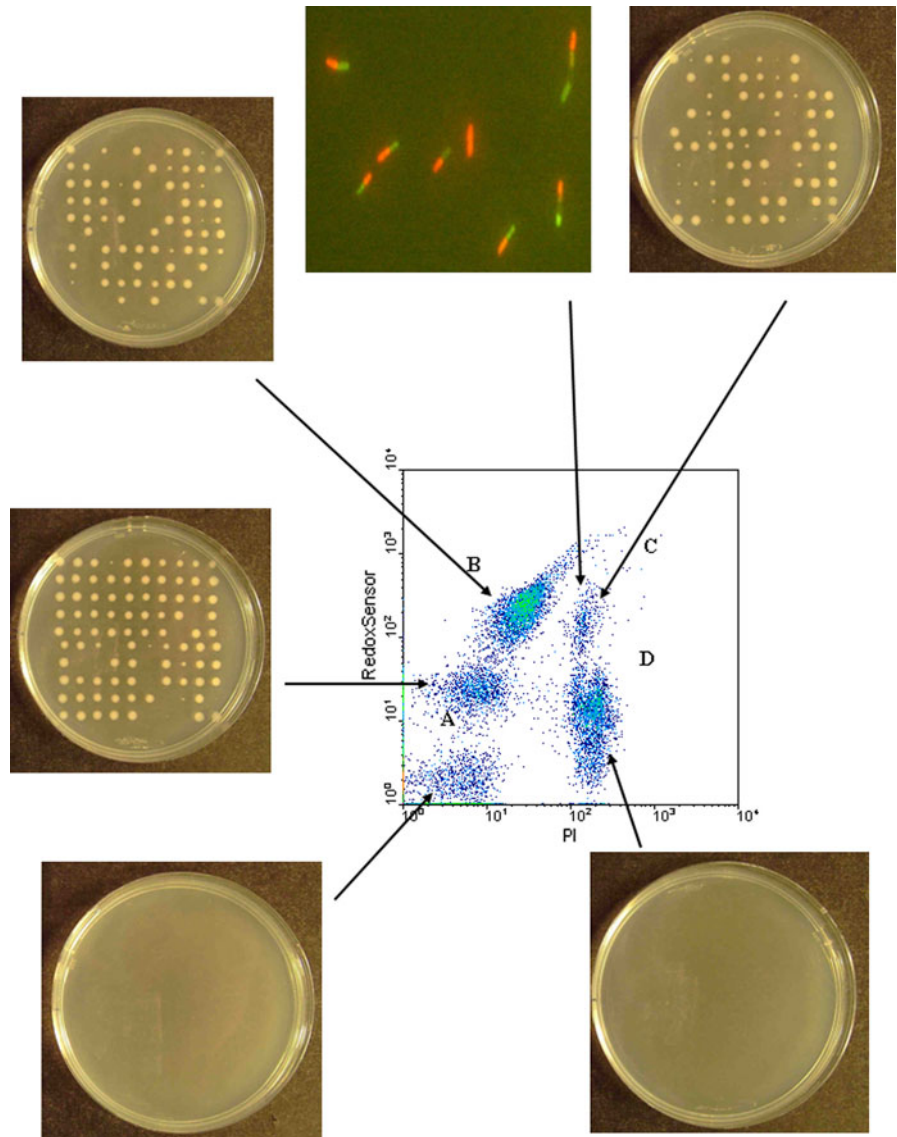
almost 3× as often as cells (∼30%) stained positively with either fluorochrome during the lag phase of growth. This demonstrates that the future ability of a cell to grow and divide is not contingent on having a higher intracellular reductase activity or a polarised cytoplasmic membrane. Indeed, the fact that >40% of cells unstained with $DiOC_6(3)$/PI and >85% unstained with RedoxSensor Green/PI gave rise to a colony when sorted onto nutrient agar shows that an absence of a detectable cytoplasmic membrane potential or reductase activity is not sufficient to render cells incapable of future growth and division.

Further, since it is impossible for a cell to replicate without a cytoplasmic membrane potential or reductase capability, then their absence must be reversible: a staining artefact to be challenged by further staining methods or, for the reductase deficiency, the condition might not be associated with the ability of the cell to propagate (Rezaeinejad and Ivanov 2010).

Closer examination of the colonies derived from sorted cells highlights another level of heterogeneity. Colonies of varying size appear on all of the sorts, implying variations in lag time, as it is unlikely that the cells have altered their growth rate. In all cases

cells stained only with PI when sorted onto nutrient agar did not produce a colony implying that under the conditions investigated here the cells staining with PI only are dead.

Conclusion

Recently, we have sought a universal dual staining technique, suitable for all bacterial cell types, to be used (by multi-parameter flow cytometry) in the assessment of bacterial cell physiological state during the course of various types of commercially important bioprocesses. This has proved difficult because of the difference in magnitude of membrane potential between Gram-positive and Gram-negative cells and the hindrance to entry of fluorochromes presented by the additional, outer membrane in the latter. However, the conclusion from this study (and informed by our previous work) is that the dual staining combination with PI/DiOC$_6$(3) is likely to be suitable for use with the Gram-positive cells e.g. *Bacillis cereus, Bacillus licheniformis, Corynebacter glutamicum, Rhodococcus* spp, (Lopes da silva et al. 2005; Chamsartra et al. 2005; Amanullah et al. 2002) and the dual staining combination with PI/bisoxanol is suitable for Gram-negative cells e.g. *E. coli, Salmonella* spp., *Acinetobacter johnsonii* (Boswell et al. 1998; Want et al. 2009.; Hewitt and Nebe-von-Caron 2001; Nebe-von-Caron et al. 2000; Boswell et al. 1998). This is likely to be species and process specific whilst the interpretation of such data can only be reliably made when the necessary control experiments have been carried out properly.

It is recognised that the relatively complex and high cost of quantitative fluorescent microscopy or flow cytometry equipment, when compared with other more traditional microbiological techniques, has prohibited it from becoming a routine analytical tool for use in most microbiology laboratories. Nevertheless, the data presented here has identified at least four distinguishable physiological states with the two staining protocols alone, highlighting further the complex population dynamics of bacterial cultures.

References

Amanullah A, Hewitt CJ, Nienow AW, Lee C, Chartrain M, Buckland B, Drew S, Woodley J (2002) Measurement of strain dependent toxicity in the indene bioconversion using multi-parameter flow cytometry. Biotechnol Bioeng 80:239–249

Boswell CD, Hewitt CJ, Mackaskie LE (1998) An application of bacterial flow cytometry: evaluation of the toxic effects of four heavy metals on *Acinetobacter* sp. with potential for bioremediation of contaminated wastewaters. Biotechnol Lett 20:857–863

Chamsartra S, Hewitt CJ, Nienow AW (2005) The impact of fluid mechanical stress on *Corynebacterium glutamicum* during continuous cultivation in an agitated bioreactor. Biotechnol Lett 27:693–700

Davey HM, Kell DB (1996) Flow cytometry and cell sorting of heterogeneous microbial populations: the importance of single-cell analyses. Microbiol Rev 60:641–696

Haeusser DP, Levin PA (2008) The great divide: coordinating cell cycle events during bacterial growth and division. Curr Opin Microbiol 11:94–99

Haugland RP (2002) Handbook of fluorescent probes and research chemicals. Molecular Probes, Inc., Eugene, OR

Hewitt CJ, Nebe-von-Caron G (2001) An industrial application of multi-parameter flow cytometry: assessment of cell physiological state and its application to the study of microbial fermentations. Cytometry 44:179–187

Hewitt CJ, Nebe-von-Caron G (2004) The application of multi-parameter flow cytometry to monitor individual microbial cell physiological state. Adv Biochem Eng Biotechnol 89:197–223

Hewitt CJ, Nienow AW (2007) The scale-up of microbial batch and fed-batch fermentation processes. Adv Appl Microbiol 62:105–135

Hewitt CJ, Boon LA, McFarlane CM, Nienow AW (1998) The use of flow cytometry to study the impact of fluid mechanical stress on *E. coli* during continuous cultivation in an agitated bioreactor. Biotechnol Bioeng 59:612–620

Hewitt CJ, Nebe-von-Caron G, Nienow AW, McFarlane CM (1999) The use of multi-parameter flow cytometry to compare the physiological response of *Escherichia coli* W3110 to glucose limitation during batch, fed-batch and continuous culture cultivation. J Biotechnol 75:251–254

Jepras RI, Paul FE, Pearson SC, Wilkinson MJ (1997) Rapid assessment of antibiotic effects on *E. coli* by bis-(1,3-dibutylbarbituric acid) trimethine oxonol and flow cytometry. Antimicrob Ag Chemother 41:2001–2005

Lewis G, Taylor IW, Nienow AW, Hewitt CJ (2004) The application of multi-parameter flow cytometry to the study of recombinant *Escherichia coli* batch fermentation processes. J Ind Microbiol Biotechnol 31:311–322

Lopes da Silva T, Reis A, Kent CA, Kosseva M, Roseiro JC, Hewitt CJ (2005) Stress-induced physiological responses to glucose and lactose pulses in *Bacillus licheniformis* continuous culture fermentation processes as measured by multi-parameter flow cytometry. Biochem Eng J 24:31–41

Monfort P, Baleux B (1996) Cell cycle characteristics and changes in membrane potential during growth of *Escherichia coli* as determined by cyanine fluorescent dye and flow cytometry. J Microbiol Methods 25:79–86

Müller S, Loffhagen N, Bley T, Babel W (1996) Membrane-potential-related fluorescence intensity indicates membrane injury. Microbiol Res 151:127–131

Nebe-von-Caron G, Badley RA (1995) Viability assessment of bacteria in mixed populations using flow-cytometry. J Microsc 179:55–66

Nebe-von-Caron G, Mueller S (2007) Bugs in the Beam. In: (ed) JP Robinson, MMK Holdings, Cytometry vol 10, ISBN 978-1-890473-10-5

Nebe-von-Caron G, Stephens PJ, Hewitt CJ, Powell JR, Badley RA (2000) Analysis of bacterial function by multi-colour and single cell sorting. J Microbiol Methods 42:97–114

Oliver JD (2010) Recent findings on the viable but non-culturable state in pathogenic bacteria. FEMS Microbiol Rev 34:414–425

Reis A, Lopes da Silva T, Kent CA, Kosseva M, Roseiro JC, Hewitt CJ (2005) The use of multi-parameter flow cytometry to study the impact of limiting substrate, agitation intensity and dilution rate on *Bacillus licheniformis* CCMI 1034 aerobic continuous culture fermentations. Biotechnol Bioeng 92:568–578

Rezaeinejada S, Ivanov V (2010) Heterogeneity of *Escherichia coli* population by respiratory activity and membrane potential of cells during growth and long-term starvation. J Micres doi:10.1016/j.micres.2010.01.007

Sebastine IM, Stocks SM, Cox PW, Thomas CR (1999) Characterisation of percentage viability of *Streptomyces clavuligerus* using image analysis. Biotechnol Technol 13:419–423

Shapiro HM (2003) Practical flow cytometry. Alan R. Liss Inc, New York

Shi L, Günther S, Hübschmann T, Wick LY, Harms H, Müller S (2007) Limits of propidium iodide as a cell viability indicator for environmental bacteria. Cytometry 71:592–598

Stocks SM, Thomas CR (2001) Viability, strength, and fragmentation of *Accharopolyspora erythraea* in submerged fermentation. Biotechnol Bioeng 75:702–709

Want A, Thomas ORT, Kara B, Liddell J, Hewitt CJ (2009) Studies related to the production of antibody fragments (Fabs) in *Escherichia coli* W3110 fed-batch fermentation processes using multi-parameter flow cytometry. Cytometry 75:148–154