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REVIEW

Applications of flow cytometry in environmental microbiology and biotechnology

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Abstract Flow cytometry (FCM) is a technique for counting, examining and sorting microscopic particles suspended in a stream of fluid. It uses the principles of light scattering, light excitation and the emission from fluorescent molecules to generate specific multiparameter data from particles and cells. The cells are hydrodynamically focussed in a sheath solution before being intercepted by a focused light source provided by a laser. FCM has been used primarily in medical applications but is being used increasingly for the examination of individual cells from environmental samples. It has found uses in the isolation of both culturable and hitherto non-culturable bacteria present infrequently in environmental samples using appropriate growth conditions. FCM lends itself to high-throughput applications in directed evolution for the analysis of single cells or cell populations carrying mutant genes. It is also suitable for encapsulation studies where individual bacteria are compartmentalised with substrate in water-in-oil-in-water emulsions or with individual genes in transcriptional/translational mixtures for the production of mutant enzymes. The sensitivity of the technique has allowed the examination of gene optimisation by a procedure known as random or neutral drift where screening and selection is based on the retention of some predetermined level of activity through multiple rounds of mutagenesis.

Keywords Fluorescence-activated cell sorting · Directed evolution · In vitro compartmentalisation · Non-culturable bacteria · Single cell analysis · Random (neutral) drift · Random mutagenesis · Protein optimisation

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Introduction

Flow cytometry (FCM) is a technique for counting, examining and sorting microscopic particles suspended in a stream of fluid. It allows simultaneous multiparametric analysis of the physical and/or chemical characteristics of single cells flowing through an optical and/or electronic detection apparatus. It is a powerful high-throughput technology that has been primarily used in the medical arena for the analysis of mammalian cells (Festin et al. 1987; Davis et al. 1998; Shapiro 2003). A greater understanding of the immune system was made possible through FCM when it was combined with the use of monoclonal antibodies. We now realise that even within a clonal population, cells are quite variable and populations are actually heterogeneous. In the past, the application of FCM to microbiology was limited, mainly due to the sensitivity required for the analysis of micron-sized microorganisms, but also due to a lack of available fluorescent stains for cell

differentiation (Vesey et al. 1994b; Davey and Kell 1996). In recent years, technical advances in the availability of fluorescent stains, including the development of green fluorescent protein (GFP) has opened the way for this technology to be recognised in the microbiological and biotechnological fields. Microbiologists now realise that FCM measurements targeting specific biomolecules and physiological processes can be used for the breakdown of cell heterogeneity within microbial populations (Veal et al. 2000; Czechowska et al. 2008). This diversity is often missed when using traditional measurements such as turbidity, which provide an average for a population only.

Cytometry is the measurement of an individual cell's size, shape and fluorescence, whereas FCM is the measurement of a cell's size, shape and fluorescence as individual cells pass in single file within a hydrodynamically focused fluid stream (Fig. 1). Single cells are focused so that they pass through an excitation source and information regarding each cell's light scattering and/or fluorescence properties can be captured and optically separated by the use of dichroic filters and detectors (usually one detector per fluorescent emission peak). This data is then converted into digital information on both the physical and chemical parameters of each individual cell, enabling population heterogeneity to be closely monitored.

Whilst the technology is complementary to fluorescence microscopy, it offers the opportunity for multiparameter analysis of several thousands of cells per second (Davey and Kell 1996; Shapiro 2003). Put simply, FCM can be compared to a fluorescence microscope that, in most cases does not capture a morphological image of a cell on a slide but instead offers the opportunity for real-time measurements of a cell's characteristics in the form of electronic signals as they pass by an excitation source. This data includes structural information on the size and granularity of a cell through forward scatter (FSC) and side scatter (SSC) measurements, as well as fluorescent information according to both the natural autofluorescence of a cell and the fluorescence attributable to the staining methodologies applied. The result is a measurement of the distribution of multiple parameters within a population and their resulting interrelationships (Mattanovich and Borth 2006). Whilst FCM carries out similar measurements to fluorescence microscopes, the majority of instruments do not provide a visual image but instead they provide quantitative, statistically significant data sets in a rapid timeframe.

Conventionally, the data generated by FCM can be plotted in a single dimension to produce a histogram or to give two-dimensional dot plots. The regions on these plots can be sequentially separated based on fluorescence intensity, by creating a series of subset extractions termed gates that can be employed for cell sorting. Different fluorescent dyes used for cell staining have overlapping emission spectra and consequently, signals received at the collection optics (detectors) have to be compensated electronically as well as computationally.

Cell sorting

Certain flow cytometers have the additional capability for allowing sophisticated fluorescence-activated cell-sorting technology or FACS (Fig. 1). That is, the ability to separate physically a user-defined sub-population of cells of interest away from a complicated mixture for further analysis such as downstream genomics or proteomics. Two major types of cell-sorting instrumentation are currently available, complicated droplet cell sorters which require highly trained operators and which are expensive but they can sort thousands of cells per second, or alternatively, simple analytical cell sorters which are easy to operate, are more cost-effective, yet are only capable of reliably sorting hundreds of cells per second (Davey 2002; Shapiro 2003; Ferrari et al. 2004). Depending on specific research requirements, each technology has advantages and disadvantages, each of which should be carefully considered for specific user-defined requirements.

The applications of FCM and cell sorting in microbiology and biotechnology are expanding and include the isolation of rare pathogens from the environment, the recovery of rare uncultivated microorganisms from marine or terrestrial environments, differentiation of physiological

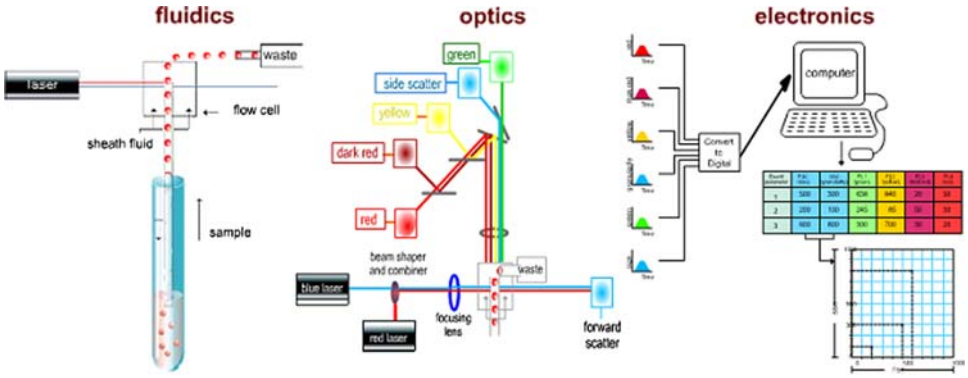

Fig. 1 Three major components of flow cytometers include fluidics, optics and electronics. (From http://www.bdbiosciences.com/immunocytometry_systems/support/training/online/ITF/index.html)

states of cells for the characterisation of viable and non-viable cells, screening approaches for selection of viable mutants—mutant libraries, or strain selection in industrial microbiology (Bell et al. 1998). One recent application of FCM has been in the high-throughput identification and analysis of recombinant cells expressing mutant proteins, usually enzymes, as part of the process of in vitro or directed evolution.

Directed evolution

Proteins have evolved for the survival and benefit of an organism and so may not exhibit features essential for in vitro applications. Therefore, there is considerable room for improvement of properties for which natural selective pressure was never applied. Protein engineering has the potential to dramatically enhance protein performance in a wide variety of non-natural, but technologically interesting, environments (see recent reviews, Bessler et al. 2003; Turner 2003; Chopra and Rananathan 2003; Bershtein and Tawfik 2008; Jäckel et al. 2008). We have developed two related technologies that can be used to improve the performance and characteristics of proteins such as enzymes, as described below, one of which is dependent on FCM for high-throughput screening of mutated populations.

Shuffling techniques can be used on a collection of mutants of the same gene, or related members of a gene family can be shuffled to produce mutants encoding chimeric gene products. One difficulty with current shuffling procedures is the predominance of unshuffled ('parental') molecules regenerated. We have developed a procedure for gene shuffling using degenerate primers that allows control of the relative levels of recombination between the genes that are shuffled and reduces the regeneration of unshuffled parental genes [degenerate oligonucleotide gene shuffling (DOGS), Gibbs et al. 2001; Bergquist et al. 2005; Bergquist and Gibbs 2006]. This procedure has the advantage of avoiding the use of endonucleases for gene fragmentation prior to shuffling and allows the use of random mutagenesis of selected segments of the gene as part of the procedure. This procedure can be combined with random drift mutagenesis (RNDM) for wider exploration of the sequence space of shuffled genes.

The DOGS procedure demonstrated that it is possible to shuffle members of a gene family that are not particularly closely related and still generate chimeric molecules at a high enough frequency so that comprehensive and time-consuming screens are not necessary.

RNDM has been developed to determine whether a phenotype that is derived from the interaction of multiple amino acids might require the accumulation and interaction of neutral mutations (neutral in isolation) and adaptive mutations (Bergquist et al. 2005). The procedure uses iterative misincorporation mutagenesis but no screening for adaptive mutations occurs. Instead, screening is only done for retained ability (whether unchanged, improved or reduced) to catalyse the hydrolysis of a substrate. This procedure is intended to provide high speed screening of mutants for retained activity without tedious assay procedures and to allow a comprehensive examination of sequence space for superior mutants. It is a platform technology applicable to any protein for which there is a colorimetric or fluorescent assay, for example, the precipitation of indigo as the result of hydrolysis of an indoxyl group as the fluorescent signal when combined with FCM analysis. All positive recombinants are combined and used as template for a further round of mutagenesis and so on for as many rounds as are necessary. In this manner, it is assumed that accumulation of multiple adaptive, neutral and harmful (but not inactivating) mutations occurs. Once generated, this library is then screened for recombinants with modified biochemical activity.

Similar directed enzyme evolution procedures have been explored by others, particularly from Tawfik's group who also use FCM for high-throughput screening (Gupta and Tawfik 2008; Bershtein et al. 2008; Bershtein and Tawfik 2008). They term the process 'neutral drift' with the same emphasis on the gradual accumulation of mutations to maintain the protein's original function. Gupta and Tawfik (2008) have shown that relatively small libraries can be used for this purpose as demonstrated using directed evolution of serum paraoxenase to show improved kinetic activity with toxic organophosphates. These experiments were accomplished by gene fusion to a superfolder mutant of GFP (Pédelacq et al. 2006) for flow cytometric detection of beneficial mutants. Similar considerations were proposed by Bloom et al. (2006; 2007) but their methodology did not involve FCM.

Flow cytometry as an aid to high-throughput screening for directed evolution

Cell sorting has great potential for high-throughput enzyme screening since such instruments are capable of rapid multiparametric analysis of individual cells at rates of more than 10^7 cells screened per hour (Aharoni et al. 2005). However, enzyme screening via FACS poses several challenges—the biggest obstacle being the lack of suitable enzyme substrates that remain associated with individual cells. FACS can be used to screen libraries where the natural product of the enzyme reaction remains inside the cells and there is a detectable change in fluorescence when the substrate is converted to product. Whilst many substrates can cross a cell membrane readily to act as a

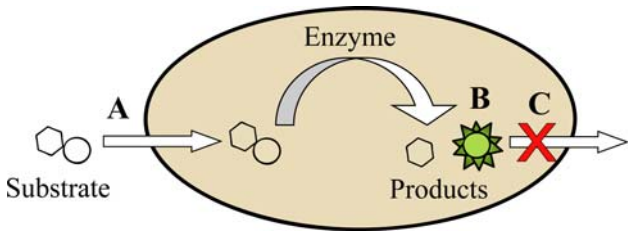

Fig. 2 A cell expressing an enzyme encapsulated with substrate in water-in-oil-in-water emulsion. **a** The substrate readily crosses the cell membrane; **b** a detectable fluorescent product is released upon hydrolysis; **c** the product remains associated with the cell. Cells and substrates are encapsulated in FACS-compatible water-in-oil-in-water emulsions

substrate for an expressed enzyme, the product can also diffuse out of the cell just as rapidly. FCM and cell-sorting analyses individual cells so it is essential that the detectable product remains associated with the cell that produced it. Ideally, the enzyme reaction would produce an insoluble or non-diffusable fluorescent product (Fig. 2).

Glycosyltransferases (GT) are enzymes that have been assayed using FCM by detection of a non-diffusable product. These enzymes transfer a monosaccharide, usually from a nucleotide-sugar donor, to an acceptor molecule such as a protein or an oligosaccharide (Breton et al. 2001). The sugar donor, sialic acid (Neu5Ac) and a fluorescently labelled sugar-acceptor molecule, Bodipy-lactoside, were both taken up actively by cells expressing the glycosyltransferase, CstII. Addition of the donor sugar to the fluorescent-acceptor molecule resulted in a fluorescent product that was retained in the cell due to its size and charge. A mutant CstII population was screened in this manner using FACS. Whilst a variant with 400-fold improved activity to the fluorescently labelled acceptor was isolated, its activity against the natural substrates and substrates with different fluorophores attached was unchanged (Aharoni et al. 2006). This study provides an example of how cell sorting can be utilised for screening for enzyme catalysis.

Many enzymes of industrial interest are hydrolases, which break large biomolecules down into smaller subunits. A substrate that can be hydrolysed to an insoluble fluorescent product would be ideal for these enzymes. ELF-97 (enzyme-linked fluorescence) forms a highly fluorescent, water insoluble precipitate (Baty et al. 2000) that has an excitation wavelength of 365 nm and emission of 525 nm (Zhou et al. 1996) which is detectable by FCM. Phosphatase activity in phytoplankton has been screened using this system and ELF-97 phosphate (Dignum et al. 2004). However, ELF substrates have poor permeability (Baty et al. 2000) and this fact maybe one of the reasons that they have not been widely used in enzyme screening.

Cloned genes for directed evolution generally are expressed in *Escherichia coli*, which has a complex cell membrane (Beveridge 1999; Roodveldt et al. 2005). Initially, we investigated a lipophilic derivative of fluorescein digalactoside (FDG) that was non-fluorescent until cleaved but the hydrolysis product was prone to leaching out of the cells. A derivative of FDG with a lipophilic tail has been added to the fluorescein portion of the FDG molecule to help retain it within the cells has been used successfully. However, the hydrophobic tail hinders passage across the bacterial cell wall which must be permeabilised by heating the cells to 65°C for 5 min in the presence of the substrate. However, reducing the integrity of the host *E. coli* cell wall increases permeability both ways and the contents of the cells can leak out. Over time, the amount of fluorescence observed in the positive cells dropped and the cells were probably losing product. A substrate that could be used directly to screen enzyme libraries via FCM would have allowed a straightforward means to separate functional mutants and non-functional variants. In its absence, we utilised a method that compartmentalises the cells in water-in-oil-in-water emulsions (Tawfik and Griffiths 1998; Aharoni et al. 2005). This modification allowed the incubation of cells at high temperatures, the use of a more cell permeable substrate and overcame the problem of substrate leakage.

In vitro and in vivo compartmentalisation

One of the key requirements of directed evolution experiments is maintaining a linkage between a protein, the gene that encodes it and the activity of that protein. In vitro compartmentalisation (IVC) is a technique that has been developed to maintain this association by creating cell-like structures where single genes are encapsulated in an artificial membrane along with the components required to transcribe and translate them, plus any additional substances such as enzyme substrates (Tawfik and Griffiths 1998). A major development that has expanded the potential of IVC for application in the screening of enzymes for directed evolution has been the conversion of water-in-oil emulsions to water-in-oil-in-water emulsions, thus making the particles themselves amenable for FCM.

IVC has been used successfully to evolve a number of enzymes. Variations of the original concept have been developed to broaden its applicability, especially with regards to screening for multiple turnover reactions and it has been extended to allow in vitro transcription–translation to occur after a PCR of individual mutant genes in the compartments (reviewed in Taly et al. 2007).

A compartmentalised in vitro system offers several advantages over expressing proteins in vivo as well as preserving the link between the genotype and phenotype. In directed evolution experiments, library size is often

restricted by the need for cloning and low transformation efficiencies (Matsuuraa and Yomo 2006). However, IVC allows for the selection or screening of libraries in the range of 10^8–10^{11} mutants, because the proteins can be expressed directly from a PCR product (Miller et al. 2006). In addition, an in vitro system is not limited by the fitness of the organism expressing the protein of interest, thus permitting the expression of toxic proteins and screening conditions that would be detrimental to most living organisms (Martemyanov et al. 2001; Mastrobattista et al. 2005). Another problem that can be overcome by expressing enzymes in vitro is the reduction of background activity from native enzymes of the host organism (Aharoni et al. 2005). By packaging everything in the one compartment, enzyme activity assays are not limited by substrates having to penetrate cellular membranes (Mastrobattista et al. 2005).

The compartments are made by creating an emulsion, where an aqueous phase (containing the genes and other components) is dispersed in an oil phase and stabilised by surfactant molecules (Tawfik and Griffiths 1998). Modifications have been made to this formulation due to problems arising with the surfactants interfering with protein expression in some in vitro systems (Ghadessy and Holliger 2004) and the rapid exchange of small molecules between compartments (Mastrobattista et al. 2005). Other modifications include re-emulsifying the water-in-oil compartments in a continuous aqueous phase compatible with cell sorters (Bernath et al. 2003). Several methods that have been used to create emulsions including stirring (Tawfik and Griffiths 1998), homogenising (Miller et al. 2006) and extruding (Mastrobattista et al. 2005). The method used will dictate the degree of polydispersity of the droplets (Rothe et al. 2006). Miller et al. (2006) have shown that mean size and distribution of the droplets can be controlled by modifying the homogeniser speeds. Droplets can be as small as bacteria ($\sim$1 μm diameter; Taly et al. 2007) and contain femtolitre (10^{-15} l) volumes (Aharoni et al. 2005). Typically, there are 10^{10} aqueous compartments per ml of an emulsion (Miller et al. 2006). If these individual compartments are thought of as single wells in a microtitre plate, it is easy to see how reducing the volume of reagents required to a single well will dramatically decrease the costs and liquid handling required when performing such experiments (Kelly et al. 2007).

IVC has proved to be very useful as a strategy for allowing the alteration of the properties of DNA modifying enzymes such as polymerases (Ghadessy et al. 2001), methyltransferases (Cohen et al. 2004) and restriction endonucleases (Doi et al. 2004). In these reactions, the DNA plays a dual role of encoding for a protein and acting as the substrate for that enzyme. Methods have been developed that use this aspect as a selection strategy. The genes that encode functional proteins survive to the next round of selection, whilst genes encoding non-functional enzymes are eliminated (Griffiths and Tawfik 2006). Only one example each of cell compartmentalisation and IVC for gene enrichment will be given for screening by cell sorting in this type of directed evolution strategy. Other examples can be found in the references provided.

IVC for gene enrichment has been used to improve the activity of EbG, a protein of unknown function that previously has been evolved to confer on *lacZ E. coli* the ability to grow on lactose (Mastrobattista et al. 2005). They found several improved variants after two rounds of mutagenesis and screening by FACS that were characterised further. Of these mutants, all had k_{cat}/K_m ratios at least 300-fold higher than wild type. Many of the mutations that conferred improved activity were actually stop codons or frameshifts in the smaller subunit of the protein. The difference in mutations may be a reflection of the different substrates used in this study compared to the in vivo studies, e.g. lactose, more natural substrates compared to fluorescein digalactoside used for FACS screening (Mastrobattista et al. 2005). Whilst high-throughput screening was successful in finding mutants with improved enzyme activity, it is limited by the quality of library.

Cell compartmentalisation with IVC

Although the general technique has been termed IVC, an equivalent procedure is to compartmentalise the individual bacteria. We term this procedure in vivo compartmentalisation. This type of screening for enzyme activity has been applied also to whole cells by Aharoni et al. 2005. The use of cells compared to in vitro enzyme production may be desirable if the enzyme activity is low. IVC produces micelles containing approximately 10–10^2 enzyme molecules per droplet compared to 10^4–10^5 per cell (Aharoni et al. 2005). One of the problems encountered with FCM analysis of droplets is that the majority will be empty (Bernath et al. 2003) and others will have multiple cells, reducing the efficiency of the screen and leading to the inclusion of false positives. In order to make the screen more efficient, Aharoni et al. 2005 set up a situation where the cells also expressed GFP, so only droplets containing single cells were analysed for activity. This was not an option in our experiments because the GFP version suitable for FCM has almost identical excitation and emission spectra to fluorescein, the molecule released when FDGlu is hydrolysed by β-glucosidase. This substrate was the only suitable one that released a fluorescent product that can be detected by commonly available lasers/filters on most flow cytometers.

IVC experiments where the gene, the machinery required to express the enzyme and the appropriate substrates are

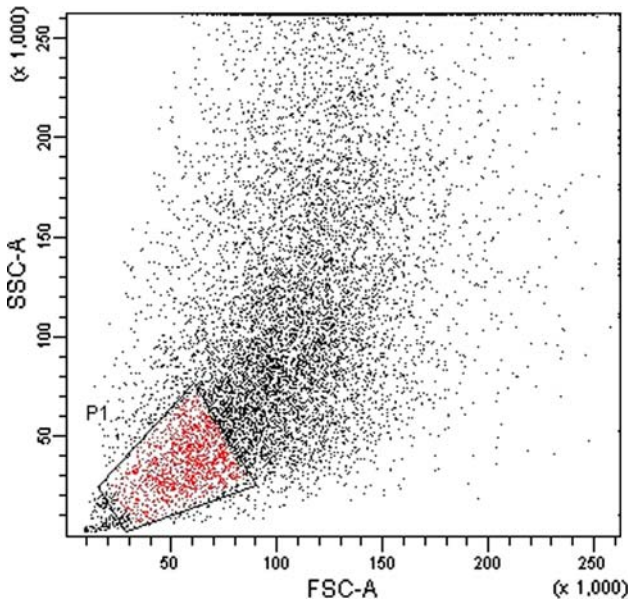

Fig. 3 Plot of forward scatter (FSC) against side scatter (SSC). The compartments defined by the P1 gate were collected separately and their status evaluated by microscopy

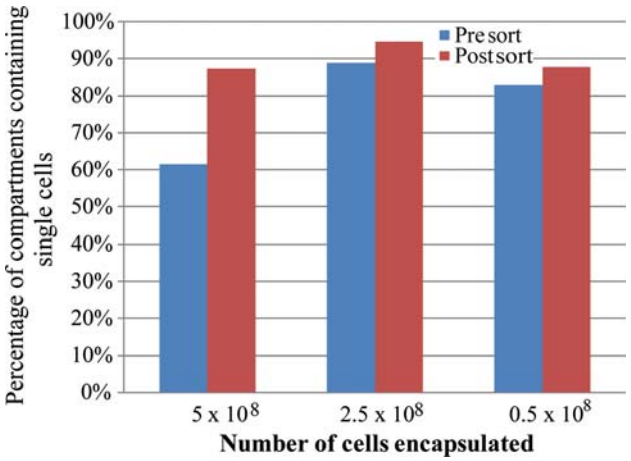

Fig. 4 Number of compartments containing single cells as a function of cell concentration. The *left hand bars* show the percentage of occupied compartments before sorting in the flow cytometer, the *right hand bars* show the percentage after sorting

encapsulated together does not use a fluorescent marker to highlight droplets that contain genes. This process relies on the fact that on average, each droplet will contain one gene. Therefore, we looked also at how many compartments in an emulsion contain single cells and if this number could be enriched by a selection based on the smaller size and complexity (FSC vs. SSC) of individual cells. We determined also whether or not this number could be improved by reducing the concentration of cells emulsified.

Compartments containing single cells were enriched from 60% of droplets containing cells to almost 90% using the original number of cells suggested by Miller et al. (2006). Lower dilutions of cells had more droplets containing single cells prior to FACS. Starting with a population of 2.5×10^8 cells resulted in a final population after sorting with 95% of droplets containing one cell (Figs. 3, 4).

A population of $BglA^-$ and $BglA^+$ cells were grown separately, counted and mixed in equal proportions prior to encapsulation. The mixed cells were plated on XGal and the encapsulated cells were sorted based on fluorescence (FITC released by positive cells from FDGlu) and size (Fig. 4). FITC-positive cells were collected and plated onto XGal and IPTG to assess the proportion of positive cells. The population was found to have been enriched so that almost 90% expressed the wild type gene.

The random drift method (RNDM) of directed evolutions is summarised in Fig. 5, where FACS can be used to collect all functional mutants for further rounds of mutagenesis and screening. The idea behind RNDM is to evolve proteins by the accumulation and combination of neutral as well as beneficial mutations. A traditional directed evolution approach would discard neutral mutations at each round of mutagenesis. By mutating whole populations of functional mutants at each round, we hoped to isolate mutants with unforeseen and unique combinations of mutations that would not have been discovered using a traditional mutagenesis/screening approaches.

Four rounds of RNDM were performed on the *Cs. saccharolyticus* β-glucosidase enzyme. A sample of mutants was sequenced to monitor the accumulation of mutations at each round of mutagenesis and screening. In addition, the most active mutants identified by FACS were collected and screened in 96-well plate format to identify any variants showing enhanced β-glucosidase activity compared to the wild type enzyme.

Several directed evolution studies have shown how neutral drift can act as a stepping stone for the evolution of promiscuous enzyme functions and changes in substrate specificity (Amitai et al. 2007; Bloom et al. 2007). Furthermore, it has been suggested that mutations accumulated during a neutral drift could act as a means of increasing protein stability to allow higher mutational loads and the incorporation of mutations that can significantly alter enzyme function (Bershtein et al. 2008).

Compartments were then selected based on size and complexity (FSC vs. SSC) to enrich for compartments containing single cells that were analysed for enzyme activity based on the release of fluorescein by active enzymes and detected as an increase in fluorescence intensity. All fluorescent compartments (10^6 compartments) and the most active 5% of variants (1×10^4–5×10^4) were collected and assayed using *p*-nitrophenol glucopyranoside (pNPGlu) substrate. The activity of mutants was normalised

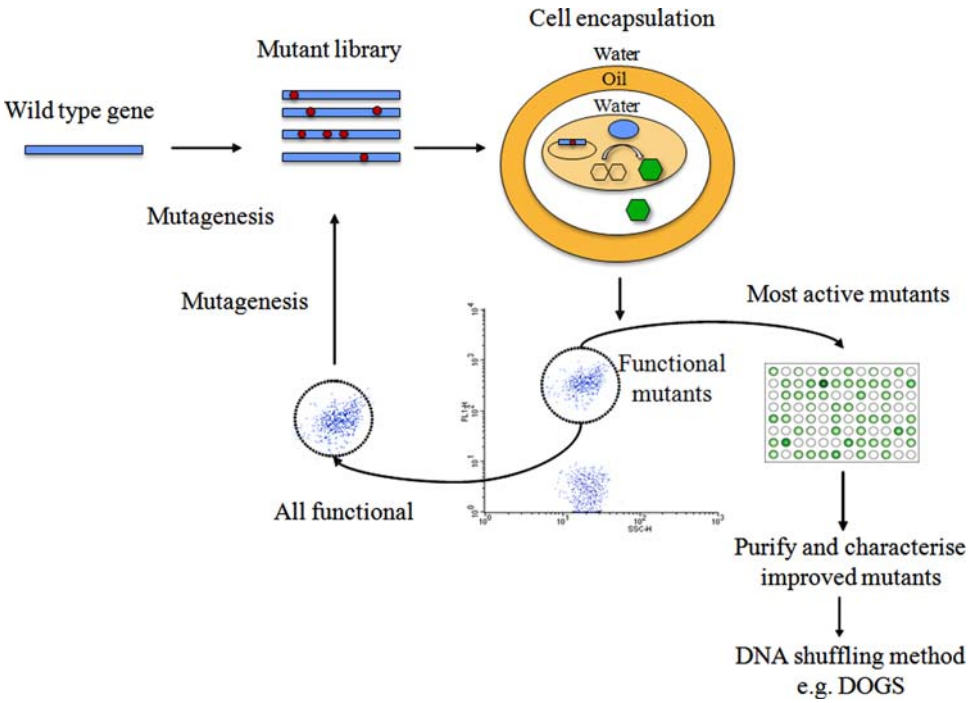

Fig. 5 Overview of the random drift mutagenesis (RNDM) process. A mutant library of the gene encoding the protein of interest is created. The library contains variants that have random point mutations throughout the gene. Each round of mutagenesis introduces on average 1–2 amino acid changes per molecule. *E. coli* cells expressing the mutant enzymes are encapsulated with substrate and enzyme activity is measured using FCM. All functional mutants are collected by FACS and used as the template for the next round of mutagenesis. The most active mutants at each round are also collected and screened in 96 well plates. Any mutants displaying enhanced activity are purified and characterised. Further increases in enzyme activity also can be achieved by recombining improved mutations using a DNA shuffling method such as DOGS (Gibbs et al. 2001)

to cell density and wild type activity and the most active mutants compared to the wild type enzyme were rescreened in triplicate to remove false positives. Mutant enzymes that displayed better β-glucosidase activity compared to the wild type were purified for further characterisation. One mutant, E1, with three mutations, showed an improved kinetic performance compared to the parental enzyme (Table 1).

The compartmentalisation process results in a large portion of empty droplets so during the FACS screening process it can be likened to analysing a microtitre plate where only one well contains a cell and all the other wells are empty. Although FCM allows rapid screening (10,000 particles per second) and the event threshold level was set so these "empty wells" can effectively be ignored, the process is still relatively inefficient and the screening and sort rates are much lower than the expected capability of the machine. In addition, the cells are encapsulated in droplets of variable sizes which is equivalent to screening enzymes in a microtitre plate where all the wells hold different volumes so big wells will have more substrate and give off a higher signal. The emerging technology of microfluidics offers promise to overcome some of these challenges as it has been used to encapsulate single eukaryotic cells in uniformly sized water-in-oil droplets. These types of droplets have the potential to be passed through a second microfluidics device that detected changes in fluorescence and incorporated a sorting mechanism such as dielectrophoresis (Clausell-Tormos et al. 2008).

Table 1 Kinetic parameters of mutant E1 isolated from a random drift experiment and the parental wild type β-glucosidase

Substrate	Enzyme	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M
pNPGlu	WT	0.40 ± 0.03	154 ± 2	384
	E1	0.25 ± 0.02	177 ± 3	710
pNPGal	WT	1.92 ± 0.12	181 ± 4	94
	E1	1.76 ± 0.07	203 ± 3	115
pNPFuc	WT	0.44 ± 0.03	209 ± 3	473
	E1	0.39 ± 0.02	243 ± 3	624
Cellobiose	WT	7.2 ± 0.8	275 ± 12	39
	E1	7.3 ± 0.8	311 ± 13	44
Lactose	WT	101 ± 12	742 ± 52	7
	E1	83 ± 6	985 ± 38	12

The wild type enzyme has a broad substrate range and hydrolyses a variety of aryl glycosides. Results are shown for the *p*-nitrophenyl derivatives of the glucoside, the galactoside, the fucoside and cellobiose and lactose. Mutant E1 has three mutations, one of which is located adjacent to the active site

However, it would be necessary to move into an in vitro system in order to increase the number of mutants that could be screened. The main advantage of this system is that transformation is unnecessary and production and screening of a much greater array of mutant enzymes is permitted. In addition, since this approach does not require a living host, enzyme reactions can be carried out at temperatures appropriate for thermophilic enzymes. Nevertheless, the in vitro system is not without its challenges. First, droplet size affects transcription/translation and consequently, the amount of enzyme produced per droplet (Miller et al. 2006). Secondly, there is only one copy of the gene and 10–100 enzyme molecules in a compartment, so a highly sensitive assay may be required to produce a detectable signal from the action of the enzyme on the substrate (Bernath et al. 2003). Finally, the enzyme is still in the presence of the transcription/translation mixture and this fact may affect its activity. Ideally, once the enzyme was produced, it should be linked to the DNA that encoded it, the emulsions broken and the linked DNA enzyme re-encapsulated and assayed under the desired conditions in the absence of any cellular components. This procedure has been done to evolve the activity of phophotriesterases, using a complicated procedure involving microbeads (Griffiths and Tawfik 2003).

Flow cytometry as a tool for accessing microbial diversity of the 'uncultivated' majority

Whilst phylogenetics approaches targeting rRNA have revolutionised our understanding of microbial ecology, studies are still dominated by highly abundant species (Woese 1987; Amann et al. 1990, 1995). Despite the emergence of metagenomics, methods to isolate total microorganisms from within environmental samples such as soil, which are predicted to contain between 100 and 7,000 species per gram, are still affected by selection biases towards fast-growing abundant organisms (Hugenholtz and Tyson 2008). In light of these problems, both culture-independent and culture-dependant approaches are required to reduce the complexity of mixed microbial communities so that the isolation and characterisation of the uncultivated majority is possible.

Culture-independent approaches towards the 'uncultivated' majority

Culture-independent approaches that combine the use of fluorescent probes with fractionation by cell sorting are becoming more popular for the characterisation of less abundant microbial species. Since 1996, the isolation and identification of rare microorganisms from the marine and terrestrial environments by combining FACS and molecular analysis through fluorescence in situ hybridisation (FISH) and/or DNA staining has been reported (Wallner et al. 1996, 1997). Low abundance microorganisms can be enriched for by cell sorting, enabling more comprehensive phyogenetic data to be obtained. A growing number of studies now combine FISH and cell sorting for cell enrichment followed by taxonomic identification of selected populations through PCR amplification of 16S rDNA genes, library construction and sequencing of differentiating gene fragments.

FISH combined with cell sorting has enabled enrichment for target communities from within industrial processes in order to gain a better understanding of the microbial interactions involved. In 2007, the diversity of nitrite reductase genes in rare species present in wastewater treatment plants provided insight into a difficult-to-isolate organism 'Candidatus *Accumulibacter phosphatis*' (Miyauchi et al. 2007). Similarly, in order to gain a better understanding of microbial interactions within an activated sludge community, cell sorting and FISH targeting Alphaproteobacterial tetrad-forming organisms was applied to enrich samples so that the full extent of the phylogenetic diversity of this group could be determined. This approach resulted in diverse taxonomic identifications which would have been missed had FACS enrichment not been carried out.

Fractionation of microbial populations using fluorescent staining. FACS and molecular tools also have been used to characterise the efficacy of water treatment plant processes. Hoefel et al. (2005) characterised bacterial populations that were resistant to disinfection in drinking water systems. By combining live/dead fluorescent staining with esterase activity dyes, cell sorting of active populations and subsequent DGGE and sequencing revealed that *Nitrospira*-related species were still present within the system. This approach was useful in highlighting the effectiveness of the current treatment process and identified where improvements were to be made to reduce viable microbial loads.

Culture-dependent approaches towards the 'uncultivated' majority

Capturing the uncultivated majority through culture-dependant routes are also coming to the forefront in microbiology. One major pitfall of combining cell sorting with FISH is that cells must be fixed or killed prior to analysis hence downstream cultivation is not possible. Alternative FCM strategies for the elucidation of heterogeneity within complex microbial populations are through monitoring the physiological or functional aspects of target

populations. Recently, real-time redox sensing of active methylotrophs using a novel fluorogenic redox indicator dye and cell sorting resulted in the ability to fractionate actively respiring methylotrophs in situ (Kalyuzhnaya et al. 2008). This approach resulted in enriched cultures of a previously recalcitrant *Methylobacter* species and further attempts to isolate this organism into pure culture are continuing. High-throughput screening of metagenomic libraries by combining cell sorting with the reporter molecule GFP have been explored also (Green and Keller 2006).

Substrate-induced gene expression (SIGEX) screening incorporates the use of FACS with selective enrichment for specific enzyme activities through the isolation of recombinant cells that are substrate-induced to express the reporter molecule GFP (Uchiyama et al. 2005). Positive cells containing novel catabolic genes can be detected through the expression of GFP and physically separated for sequence analysis. In the future, this approach promises to fast track the isolation of novel biocatalysts from the environment. DNA staining, cell sorting, FISH and DGGE were used to define the community composition of marine bacterioplankton over short-term incubation periods following limiting dilution (Fuchs et al. 2000). The results obtained highlighted major difficulties experienced in the cultivation of fastidious bacteria, as dominant slow-growing *Cytophaga–Flexibacter–Bacteroides* (CFB) members identified in an undiluted, nutrient-poor marine system were rapidly outcompeted by fast-growing Alpha- and Gamma-proteobacteria when cells were diluted into a nutrient-rich marine system. In this case, enrichment of the slow-growing species through physiological staining and cell sorting may improve the recovery of hard-to-culture species.

Novel cultivation-dependant strategies that rely on FACS have now emerged as high-throughput solutions to cultivating large numbers of undescribed species (Leadbetter 2003; Green and Keller 2006, Kramer et al. 2007). Microdroplet encapsulation of single bacterial cells combined with micro cultivation under natural environmental conditions promotes microcolony formation and subsequent cultivation of undescribed bacterial species (Zengler et al. 2002). Flow cytometric differentiation of microcapsules containing microcolonies (20–100 cells) combined with sorting of individual microcapsules into microtitre plates containing a nutrient-rich medium resulted in the potential growth of up to 10,000 bacterial and fungal isolates for every environmental sample analysed (Keller and Zengler 2004; Zengler et al. 2005). The major limitation is now screening for unique microbial signatures within the resulting micro cultures.

Simpler cell-sorting strategies are also useful in the cultivation of fastidious microorganisms. The soil substrate membrane system (SSMS), developed for the cultivation of mixed communities of recalcitrant microcolony-forming soil bacteria (Ferrari et al. 2009), combined with cell sorting for the isolation of individual microcolonies or filamentous bacterial morphotypes, has been used to enrich for novel filamentous bacterial species (Ferrari and Winsley 2008). In this example, the expected regions containing live filamentous cells (and/or microcolonies), after removal from the SSMS, were determined following total bacterial staining of

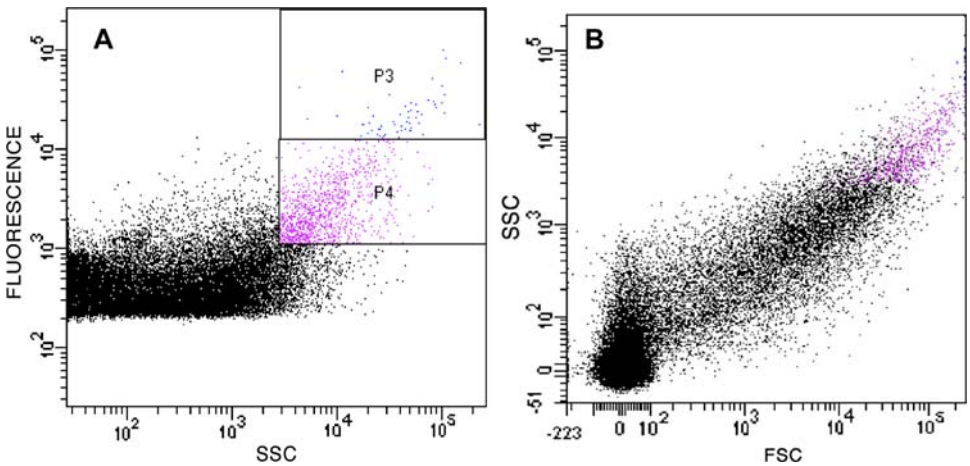

Fig. 6 **a** A bivariate dot plot of fluorescence versus side scatter (SSC) in arbitrary units, one dot represents a single cell or particle. Gated regions were defined after total bacterial staining with SYBR Green II to distinguish large filamentous cells from the dominant smaller rods and cocci. Previous analysis of a control sample of *Escherichia coli* and *Micrococcus luteus* enabled small rods and cocci to be defined into the left hand quadrant of the dot plots. By binomial elimination, gate P4 was found to contain the most filamentous cells, whilst P3 also contained some filamentous cells but also contained a high proportion of intact microcolonies. **b** A bivariate dot plot of side scatter (SSC) versus forward scatter (FSC) in arbitrary units, one dot represents a single cell or particle. *Purple dots* correspond to gate P4 from Fig. 6a. This region was then sorted to isolate unstained live filamentous cells for downstream cultivation into nutrient-poor media

SSMS bacteria and analysis by FCM (Fig. 6). Sorting of cells from within defined gates on a bivariate dotplot enables identification of a FCM region where a high proportion of fluorescent (A) and unstained or live filamentous cells (B) predominate. Subsequently, live filamentous cells can be removed from the SSMS for physical separation into nutrient-poor media (Fig. 7). In a pilot study, novel filamentous species from within three divisions were recovered using this approach, including a potentially new genus from within the *Cytophaga-Flexibacter-Bacteroides* phylum and novel stalked *Methylobacterium* and *Caulobacter* species from within the Alpha-proteobacteria group.

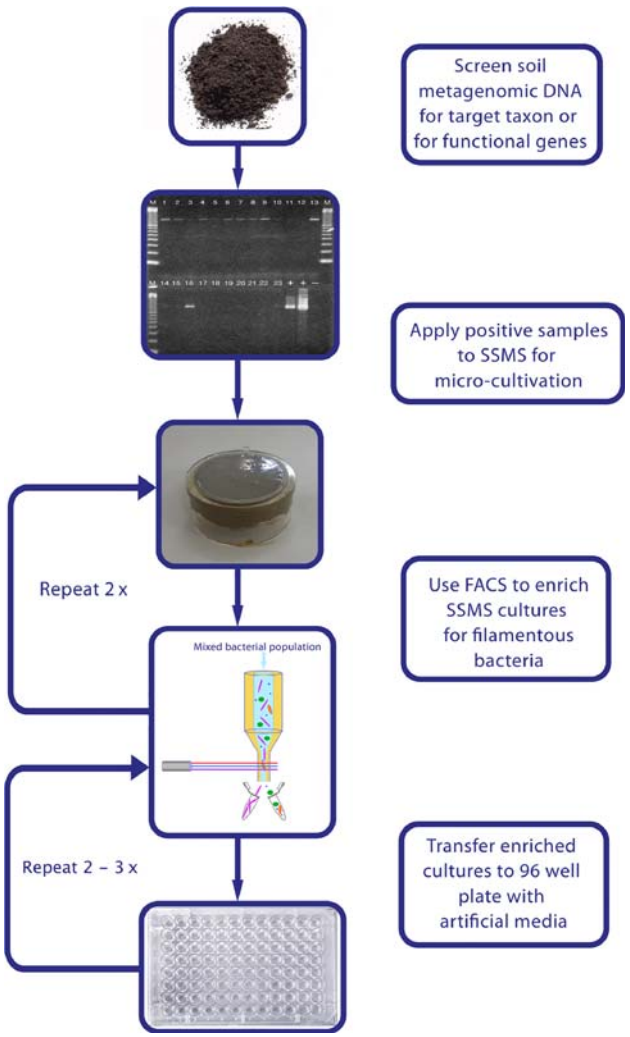

Fig. 7 A schematic representation of the strategy utilised for the successful cultivation and isolation of filamentous bacterial species targeted in soil environments. The strategy combines the screening of environmental samples for presence of target organisms, followed by SSMS micro cultivation and subsequent FACS enrichment of filamentous populations. This process allows for the transfer of oligophilic species from the SSMS into artificial media following sufficient enrichment of the filamentous bacterial populations and can be applied to any target taxa that can be cultivated with the SSMS

Flow cytometry in environmental biotechnology

Novel approaches combining the construction of whole-cell bacterial GFP-labelled biosensors have been developed for in situ detection of bioavailable compounds by FCM to improve our understanding of microbial ecology and microbial processes in complex environmental systems, (Hansen et al. 2001). A bacterial biosensor was constructed to express GFP following exposure to tetracycline in this study. When placed into sterile soil containing *Streptomyces rimosus*, a known producer of oxytetracyline, these bacterial biosensors switched on GFP and were detected by FCM after 2 days incubation, confirming antibiotic production was occurring in soil. This powerful approach has now been extended to include detection of the quorum sensing signal, N-acyl homoserine, by indigenous bacterial communities in litter (Burmolle et al. 2005) and for monitoring tetracycline production in the intestines of rats (Bahl et al. 2004). Moreover, this molecular reporter strategy has been used to monitor horizontal gene transfer within indigenous bacterial species from various environments (Sorensen et al. 2003; Musovic et al. 2006).

FCM has been applied successfully to the detection of rare cells in the environment. A decade of research has been carried out on its application for rapid detection of the infamous waterborne pathogens *Cryptosporidium* and *Giardia* from the within water samples (Vesey et al. 1993, 1994a). The detection limits required for these organisms are in the order of one cell within 10–100 l of water or one in 10^{11} particles. Therefore, innovative methods are required for their detection and most recently, fluorescence-based approaches in this area include the development of FISH probes for the differentiation of the major species of concern to public health and the application of quantum dots and biosensors towards field-based tests for real-time detection (Ferrari and Bergquist 2007; Kramer et al. 2007; Alagappan et al. 2008; Ibáñez-Peral et al. 2008).

Conclusions

FCM is a powerful technique that has been used extensively in health bioscience-related applications, particularly with antibodies. But the technique is versatile and has applications in other areas such as environmental microbiology and in molecular biology and genetics. It is exquisitely sensitive in being able to allow cell sorting at high speed of defined population subsets or individual cells. It has applications in taxonomy and bioprospecting when coupled to appropriate culturing techniques for rare or novel microorganisms from all environments, whether mesophilic or extremophilic. The instrumentation is able to

be modified for anaerobic uses and accordingly, should allow the isolation, identification and cultivation of an under-represented area of microbiological diversity, particularly when combined with whole genome amplification for total sequencing or 16S taxonomic identification (Ishoey et al. 2008). FCM has considerable potential in the high-throughput analysis of mutants of proteins for improved characteristics by in vitro evolution techniques. It is probably best suited to compartmentalisation techniques for whole cells or genes until more suitable fluorescent substrates are synthesised. This area appears to be a fruitful one for collaboration between synthetic chemists and molecular and microbiologists for the future.

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