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New Insights into Cell Encapsulation and the Role of Proteins During Flow Cytometry

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1. Introduction

Modern approaches to science tend to follow divergent paths. On one hand, instruments and technologies are developed to capture as much information as possible with the need for complex data analysis to identify problematic issues. On the other hand, formulation focused, minimalistic approaches that gather only the most pertinent data for specific questions also represent a powerful methodology. This chapter will provide many examples of the latter by integrating Flow Cytometry (FACS - Fluorescence-Activated Cell Sorting) technology with high throughput screening (HTS) of encapsulation systems with extensive utility of one-dimensional (1-D) imaging for protein localisation. In this regard, less information is acquired from each cell, data files will be more manageable, easier to analyse and throughput screening will be significantly enhanced beyond traditional HTS analysis, irrespective of the protein concentration present in the background or delivery media.

1.1 Real-time on-line monitoring of bioprocesses

The production of heterologous therapeutic proteins is well established in today's biotechnology industry; however their presence during cytometric screening poses complex analytical obstacles for food and biotechnologists alike. The Process Analytical Technology (PAT) initiative, launched by the Food and Drug Administration (FDA) encourages extensive process understanding to achieve the desired quality of pharmaceutical and bioactive protein products rather than a 'quality-by-testing' approach. Thus, elucidating and monitoring variations within protein production, purification and encapsulation systems is fundamental for quality control, protein detection and localisation (Glassey et al., 2011). One key issue for PAT is the use of on-line process analysers; in this context, 'on-line' is used in the sense of 'fully automatic' or 'without any manual interaction'.

The foundation of early microscopy and its transition to the modern FACS nowadays, proved that cell growth within a population differs significantly from one cell to another. These heterogeneities are caused by either fluctuations in the micro-environment of individual cells (Dunlop and Ye, 1990); phenotypic changes during the cell cycle (Münch et al., 1992); or by mutations resulting in genotypic variations in the population (Hall, 1995) leading to different protein expression levels. Thus, the most important variable in a bioprocess - cell productivity of a heterologous protein product - is distributed over a wide range within the cell population. Protein analytical methods (i.e. SDS-PAGE, Western blot,

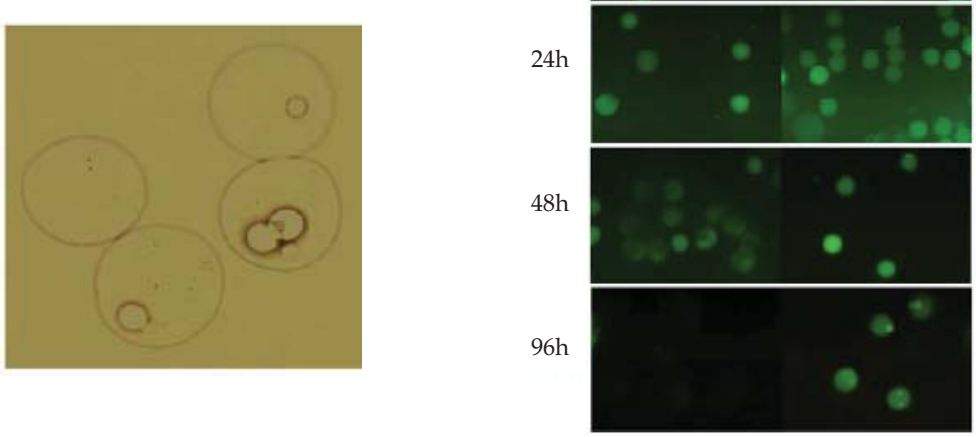
MALD-TOF-MS) are off-line, time and cost intensive, require human interaction and are not capable of exhibiting protein expression levels in single cell level. Earlier studies demonstrated that tagging the target protein with a fluorescent reporter molecule (green fluorescent protein GFP) permits detection of low protein concentrations by measuring the reporter molecule's specific fluorescence (Broger et al., 2011). Specific protein fluorescence signals can be measured through on-line, *in situ* fluorescence sensors (Jones et al., 2004, Reischer et al., 2004); but the main drawback of these measuring techniques is that they all result in population averaged data; hence analysis of single cell productivity was not possible. As mentioned FACS is one of the available methods for measurement of population distribution and has been exploited in biotechnology (Rieseberg et al., 2001). To overcome this disadvantage of FACS as an off-line analyser, a number of flow injection (FI) flow cytometer systems have been designed for the determination of proteins (Kelley, 1989). Ruzicka and Lindberg (1992) were the first to utilise FI as an interface between a bioreactor and FACS. Further progress in FI-FACS has been made by Srienc and co-workers (Zhao et al., 1999), examining population dynamics of *Saccharomyces cerevisiae* (Kacmar and Srienc, 2005) and growth dynamics of Chinese Hamster Ovary (CHO) cells and their associated proteins (Sitton and Srienc, 2008a, b). None of them, however, measure single-cell productivity using fluorescent reporter tag co-expressed with target protein, which restricts the purification of secreted protein products.

1.2 Efficacious phenotypic analyses of cell encapsulation systems

Cellular detection and characterization usually involves cell harvesting for more than 20 generations in order to achieve the formation of macro-colonies on solid media. What is more, encapsulation provides an innovative solution for the handling and use of live cells through the use of three-dimensional scaffolds. These scaffolds - commonly known as beads or capsules - can be used in a number of ways, such as for the isolation of individual cells to form clonal populations, for establishing partial barrier for cells from their environment, and for creating a matrix allowing the formation of 3D-cell cultures or clusters. These methods have important implications for the biomedical engineering field and the areas of biomanufacturing and bioprocessing. Nonetheless, a myriad of information remains to be discovered. For example, the chemical composition and engineered functionalities of these scaffolds affect the biology of the encapsulated cells. One particular system that has recently achieved significant recognition are the dairy proteins. A variety of cell types can be enclosed in casein or whey beads scaffolds in a range of sizes. While maintaining their morphology and function, these encapsulated cells can proliferate, form cell clusters, and even lay down extracellular matrix components. Furthermore, cells can be encapsulated in small particles that can then be handled, characterized and analysed with ease. These features make milk proteins an attractive encapsulation material for three dimensional scaffolds of live cells. Meanwhile, HTS of these encapsulated cells is restricted by several practical aspects including low sample throughput, protein matrix limitations and the absence of sorting capability for mixed cell cultures. Hence, a unique reconsideration of conventional cytometric approaches for accelerated food and/ or bioprocess intensification represents a timely research strategy for encapsulation technologists and cytometrists alike. It was recently discovered (Delgado et al., 2010) that microbial growth can be monitored in encapsulated cell systems by following the development of micro-colonies. Therefore, development and dissemination of a FACS technology capable of analysing encapsulated

cells within intact micro-capsules encapsulation systems would represent a novel cytometric strategy for academic and industrial applications. This approach would require knowledge management of cytometric control parameters, which would generate reduced capital cost, human error and environmental deterrents associated with conventionally high-throughput screening strategies. However, there is a need for addressing physico-chemical properties of micro-capsules for efficient cytometric screening without i) occlusion of capsular flow in the fluidics system during hydrodynamic focusing or ii) adverse interactions between protein (originating from the micro-capsule or cell metabolic activity) and cytometric buffer media during the screening process.

Delgado et al., (2010) demonstrated that cell proliferation within encapsulation systems can be detected via FACS, provided that the population of capsules exhibit appropriate optical and mechanical properties and are mono-dispersed in size and shape. Encapsulated cells can be further utilized for a variety of applications: from characterizing secreted enzymes to detection of thermo-sensitive mutants. Delgado et al., (2010) successfully revealed the application of Flow Focusing® technology for microencapsulated cells of different types in mono-disperse hydrogel microspheres. Using a CellENA® Flow Focusing® microencapsulator, monodisperse capsules were fabricated containing one single cell with sizes ranging from



Source: (Delgado et al., 2011). http://www.cellena.net/en/documents/CellenaPoster.pdf

Fig. 1. Data detecting glutenases in micro-encapsulated bacteria. Colonies of bacteria were grown in gliadin-containing capsules. Images on the right illustrate the change in fluorescent intensity as a function of incubation time in culture media after micro-encapsulation. Gliadin content was detected by incubating the particles with the monoclonal antibody G12, conjugated to FITC. The micrograph on the left illustrated colonies of bacteria growing gliadin-containing micorparticles (Delgado et al., 2011)

100 µm to over 600 µm diameter. This offers a plethora of applications including the characterization of secreted therapeutic proteins to detect encapsulated cells or thermo-sensitive mutants. More importantly, cell proliferation inside the micro-capsules was detected by FACS without the need for fluorescent labelling, which represents a significant development (Delgado et al., 2011). Furthermore, bacteria expressing glutenase activity, isolated from agrochemical samples were detected by their ability to degrade gliadin when growing inside capsules as shown in Figure 1. Gliadin content was detected by incubating the particles with the monoclonal antibody G12, conjugated to FITC (Delgado et al., 2010). Further information relating to COPAS analysis can be obtained from Union Biometrica (http://www.unionbio.com/).

Figure 2 illustrates capsules containing yeast colonies incubated for different times. Data revealed that capsules had a similar size (Time of Flight; TOF) but differed in optical complexity (Extinction; EXT). Hence, different yeast concentrations were predicted as a function of time. This Flow Focusing® Technology from Union Biometrica, Belgium, will inevitably bring additional utility and commercial recognition to FACS as an economically viable biochemical application for improved analytical monitoring of downstream processing during fermentation and drug/ protein generation. Figure 3 illustrates the successful analysis of protein capsules based on size (TOF) and extinction (EXT). The best resolution was obtained with the 561nm solid state laser due to higher penetration into the protein capsule, which will depend upon membrane thickness, coating or presence of a

Source: (Delgado et al., 2011). http://www.cellena.net/en/documents/CellenaPoster.pdf

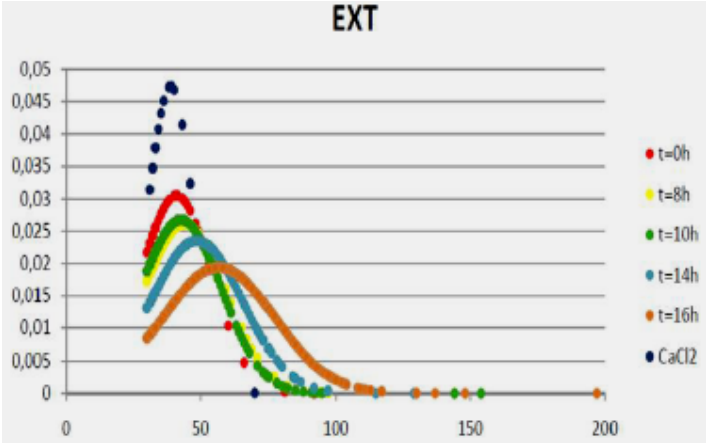


Fig. 2. Optical density (Extinction; EXT; x-axis) measurements of encapsulated yeast following the time points indicated (above, the images of the encapsulated yeast)

surface cross-linker. It was also demonstrated that maximum peak height can be used as a sorting parameter for cytometric sorting of encapsulated cells in protein capsules. Further research is currently being conducted in this promising area of cytometric analysis.

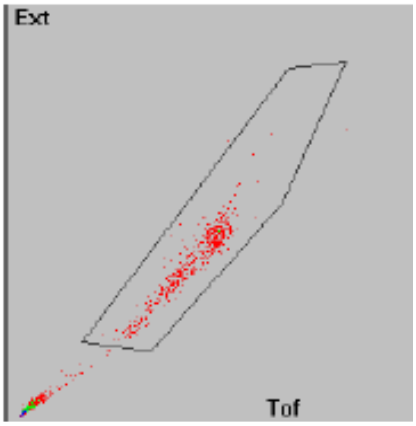


Fig. 3. Displays the dot plots TOF vs. EXT for empty protein capsules. TOF is an indicator of size, while EXT represents the density of the detected object

These developments may aid bioprocessing technology, improve process development and analytical control by sorting cells based on i) micro-capsule size; ii) cell type; iii) cell density; iv) monoclonality and v) protein expression. In this way, fluorescent tagging is not an essential requirement for the characterisation of encapsulated cell cultures and their respective recombinant proteins, which demonstrates a significant development for cost- and time-efficient cytometric screening. Moreover, encapsulation evaluation using Flow Focusing® Technology, can be used for phenotypic analysis of encapsulated cells, such as the expression of specific characteristics of microbial colonies (glutenase expression by bacteria) or growth-related phenotypes (antibiotic resistance and proliferation in thermo-sensitive yeast). Mammalian cells are delicate, difficult to handle and manipulate individually. However, these cells can be encapsulated within a polymer material such as protein or alginate, and these encapsulated scaffolds remain intact throughout all steps of cytometric analysis and dispensing. Furthermore, fragile cells like adipocyte stem cells remain viable for extended periods of time (2 weeks) (Delgado et al., 2010) and can be released and recovered to permit further analysis and use, which endorses an exclusive cytometric development.

1.3 High throughput processing of encapsulated bacterial libraries

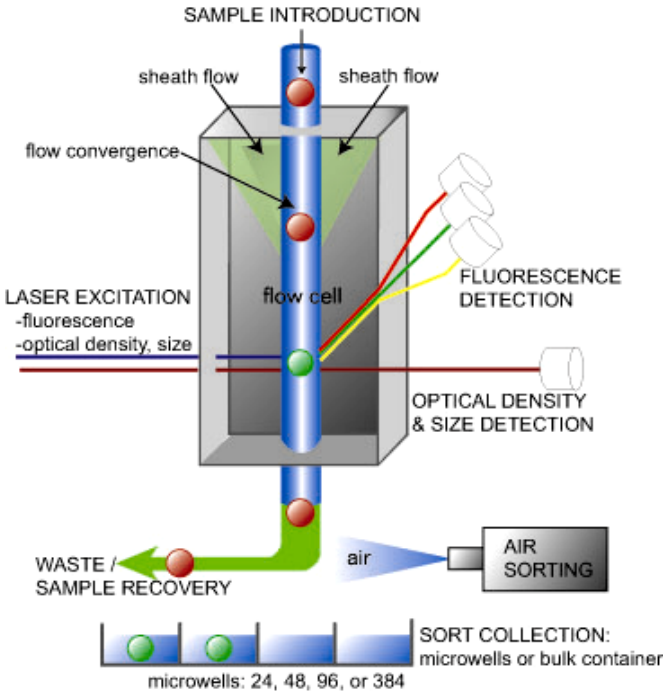
Industrial interest in encapsulation has grown exponentially in the fields of bioprocessing, fermentation and elucidation of bacterial libraries, especially in high-throughput environments exceeding 10^6 samples per day. Fundamental pre-requisites reveal the necessity for a one-to-one relationship between individual cells and analytical algorithms. Essentially, each micro-carrier (i.e. capsule) would therefore contain exactly one cell or colony. However, synthesis of larger numbers of capsules containing exactly one cell is not feasible as cells are randomly distributed during capsule production. The problem is clear -

high dilution conditions will yield an adequate degree of monoclonality; however, this will be coupled with the generation of a significant fraction of empty micro-capsules. Conversely, distribution under low dilution conditions will generate unacceptable numbers of polyclonal capsules for whose removal no satisfactory technologies exists to date. Recent finding demonstrated (Walser et al., 2008, 2009) that hydrogel micro-carriers can be applied as growth milieu for individual cell colonies. *Escherichia coli* cells expressing green fluorescent protein (GFP) were encapsulated at low dilution thereby intentionally producing a considerable amount of polyclonal micro-carriers. Empty and polyclonal micro-carriers were then removed from the desired monoclonal fraction using a particle analyzer. Data was compared to model predictions in order to investigate possible limitations in the analysis and sorting of monoclonal micro-carriers. Fluorescent *E. coli* cells (GFP) were randomly distributed throughout the micro-carrier population and cells successfully propagated to colonies in the micro-carrier with enrichment to 95% monoclonality. Interestingly, colony diameter represents a limiting factor for enrichment-efficiency in encapsulation systems. With increasing colony size, two antagonistic effects are associated with the cytometric approach: First, improved sorting efficiency due to increased fluorescence intensity and thus higher detection efficiency, and second, deterioration of sorting efficiency due to occlusion occurring in polyclonal micro-capsules. Hence, encapsulation under low dilution conditions with high-throughput sorting via FACS represents a practical economically viable initiative for isolating large quantities of monoclonal micro-capsules from bacterial libraries and at the same time, keeping the amounts of empty micro-capsules at a moderate level.

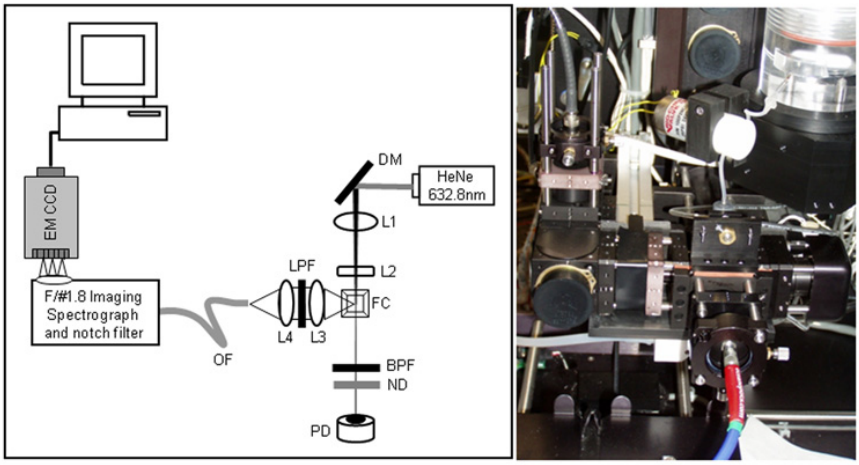
1.4 Utilization of micro-capsules for next generation cytometric assays

It is evident that cytometric screening is a pragmatic approach for integration of cell encapsulation within continuous bioprocesses. Subsequent segregation and sorting of microcapsules containing high cell densities of animal cells and associated proteins has recently catalysed interest in cytometric screening. Research performed by Union Biometrica Inc. (www.unionbiometrica.com) applied this technology and found COPAS™ (Complex Object Parametric Analyzer and Sorter) suitable for automated analysis, sorting, and dispensing of 'large' objects such as capsules. The COPAS™ PLUS is capable of analysing particles with diameters of 30 - 800 µm in a continuously flowing stream at a rate of 25-50 objects/second. Physical properties such as size, optical density and intensity of fluorescent markers are taken into consideration. To avoid damaging or changing the fragile biological samples, a gentle pneumatic device located after the flow cell is used for sorting encapsulated cells and therefore makes the instrument suitable for handling live biological materials or sensitive proteins (Figure 4). It is interesting to note that the fluid pressures of the instrument are also significantly lower than those of traditional flow cytometers. The COPAS™ XL instrument has a 2,000 µm flow cell, which allows the analysis of larger beads (30-1,500 µm) compared to the COPAS™ PLUS. If an encapsulated sample contains certain fluorophores that can be excited by light of 488 nm, the emission levels can be detected for each of the objects in the encapsulated system.

Figure 4 details the analysis of intact encapsulation capsules inside the flow cells. Objects are carried through the flow cell by a liquid stream while their physical properties are being measured. Convergence of the sheath and sample fluid allows "hydrodynamic focusing" of the micro-capsules, forcing them to go through the centre of the flow cell along their



(a)



(b)

Fig. 4. Schematic of Flow Focus Technology™ shown in (a). Figure (b) illustrates a schematic (left) and an image (right) of the spectral detection leg constructed on the open face of the COPAS™ flow cell (Watson et al., 2009). DM: dichroic mirror; L1: 80 mm spherical lens; L2: 6 mm cylindrical lens; FC: flow cell; L3, L4: 21 mm aspherical lenses; OF: optical fiber; ND: 0.8 OD neutral density filter

longitudinal axes. Inside the flow cell objects are illuminated by two low energy lasers that measure the microcapsule optical properties inclusive of size, optical density and fluorescence. Micro-capsules that meet the sort criteria are permitted to drop into sort collection, while those that do not are diverted to waste recovery using a pneumatic sorting device. Cells may be classified according to their fluorescent intensity or optical density, which will accommodate a greater efficiency for detection of recombinant proteins during encapsulation procedures. Preliminary studies performed using COPAS™ instrumentation has demonstrated the discrimination of hydrogel microcapsules containing mixed cell cultures. During initial sampling, micro-capsules were selected based on their size (TOF), monoclonality and optical density (EXT). Preparation of a standard curve using micro-capsules of known size and TOF measurements provided a practical correlation between TOF measurements generated by the flow cytometer and the actual size. Hence, it is plausible to select micro-capsules that meet specific size and optical density criteria. Furthermore, encapsulated cells can also be chosen to meet criteria of size, optical density and levels of green and red fluorescence. Hence, it is also possible to sort micro-capsules with various magnitudes of cell growth, cell density and recombinant protein content.

Watson et al., (2009) successfully adapted Union Biometrica COPAS™ Plus instrument to allow red excitation and optical fiber-based light collection and spectral analysis using a spectrograph and CCD array detector (Figure 4B). These modifications did not compromise the ability of the instrument to resolve different sized capsules based on their extinction (EXT) and time of flight (TOF) signals. The modified instrument has the sensitivity and spectral resolution to measure the fluorescence and Raman signals from individual particles with signal integration times of 10 usecond. The high speed spectral analysis of individual particles in flow will enable new applications in biological encapsulation systems

2. High content screening – getting more for less

High content screening (HCS) can identify proteins via automated image analysis and, in general, is designed to capture image information regarding cells and associated protein products. The availability of commercial imaging systems has made HCS increasingly practical for protein determination and has placed HCS into a standard tool for protein and drug discovery (Zanella et al., 2010). The value of this protein determination approach is apparent since a vast number of cellular characteristics can be captured in large data files. Over the past decade advances in information technology and biological probes have led to practically automated image analysis with throughput far above that possible by manual cell analysis in protein-rich environments. Several commercial instruments are now available for HCS of proteins, for example the *BD Pathway* (BD Biosciences), *In cell Analyzer* (GE healthcare), *ImageXpress Ultra* (Molecular Devices) and *Scan^R* (Olympus), to name a few. The *Imagestream* (Amnis) is a relatively new instrument that combines aspects of FACS with image analysis, resulting in a device capable of imaging cells at multiple fluorescent wavelengths with a throughput of hundreds of cells per second (Reardon et al., 2009). In HCS devices, the presence of environmental protein presents trade-offs between the quantity (and quality) of data acquired and throughput. Hence, environmental protein is a hindrance to the efficacy of cytometric screening. The *Imagestream*, for example, trades ultrahigh image resolution for speed, and many cellular images are captured in one data file, the size of which can easily exceed 3 gigabytes.

3. Cytometric limitations associated with environmental protein

Contemporary approaches to food fermentations and drug discoveries frequently use HTS to measure the fluorescence of cellular features tagged with fluorochrome-conjugated antibodies or other fluorescent labels in a very rapid manner. This is quite often performed by FACS, a technique well-suited to this purpose. However, tagged cells are often entrapped within a dense protein matrix or lattice, which represents an obstacle against true microbial counts. Hence, applications can be significantly hindered by the presence of proteins since environmental protein particles, matching the size range of cells, are often recognised as cellular bodies during hydrodynamic focusing. An appropriate methodology is therefore required to eliminate interfering environmental protein - non-cellular in origin - from cytometric analysis for successful application of cytometric screening. Traditional FACS can easily capture emissions from ten or more different fluorochromes on a cell at rates exceeding 25,000 cells per second. Although this is appropriate for many assays, FACS cannot provide accurate details on cell morphology or subcellular localisation of fluorescence in the presence of obtrusive environment protein. In the interim, manual microscope examination of fluorescent staining can provide information for these characteristics. However, this approach can be too subjective and lowers throughput substantially. Hence, environmental protein originating from e.g. encapsulation matrices or carrier media may exhibit adverse effects for accurate cytometric analysis.

4. Flow Cytometry for cell viability assessment in complex protein matrices

Proof of principle that product quality can be assessed within complex systems using FACS may raise awareness and further develop cytometric technologies within the industrial domains of quality management and product /process optimization. Rapid and efficient viability assessment is essential for regulation and legislation on therapeutic bioactives or drug product quality. Hence, there is a clear need for real-time cell enumeration techniques. Data procurement in minutes rather than days may identify a problem faster than usual. Within the food industry, FACS represents a major development for high throughput screening (HTS) of high cell density cultures in addition to providing real-time quality control. Numerous technologies, inclusive of FACS have been developed to accelerate data acquisition compared to traditional culture methods. In the food industry, techniques utilizing antibodies for cell labelling prior to FACS analysis have been developed for *Salmonella enterica* serovar Typhimurium and *Listeria monocytogenes* in milk and other dairy products (Patchett et al., 1991). Moreover, Doherty et al., (2010) demonstrated that FACS, coupled with fluorescent techniques, can be successfully applied for the assessment of cell viability in seven different protein matrices ranging in structural complexity. Food containing complex protein matrices can frequently generate unpredictable results regarding cell viability. Cell entrapment within protein networks can severely affect accurate cell enumeration, an issue which requires special attention as it has an impact on both quality and safety of the product. Cell viability can be accurately determined by FACS, specialised for cell encapsulation and protein systems. The distinctive features of this strategy can be summarised as follows: while Delgado et al., (2010) performed cytometric screening on encapsulated cells within intact micro-carriers, Doherty et al., (2010) enumerated cell viability following complete protein matrix digestion. Cell extraction and digestive pre-treatments were designed to liberate cells from the scaffold in order to minimise

the protein background, the predominant compositional obstacles for efficient FACS analysis. It is interesting to compare the success of these reverse strategies for use in encapsulation systems. Cell extraction by Doherty et al., (2010) required 40-minute sample preparation and distinct functional cell populations were discriminated based on fluorescent labelling by of Thiazole Orange (TO) and Propidium Iodide (PI). This assay yielded 45–50 samples/hour, a detection range of 10^2–10^{10} cfu mL^{-1} of homogenate and generated correlation coefficients (r) of 0.95, 0.92 and 0.93 in relation to standard plate counts during heat, acid and storage trials, respectively. This cytometric approach could also alleviate problems relating to environmental compatibility during the production of nutraceutical products; a formulation problem generating a strong current of industrial activity. However, uptake of this technology is dependent on cost-efficiency and the scope for extension of product applications. Both of these pre-requisites are satisfactory for food and pharmaceutical manufacture environments due to the i) multi-disciplinary function of the assay for cell viability assessment; ii) minimal personnel training required for instrument commission and iii) rapid, reproducible cytometric signature responses in a variety of encapsulation matrices in the presence of protein. The timely availability of cytometric results also provides manufacturers with the necessary skills to promote problem-solving investigations in bioactive and/ drug development for enhanced performance of therapeutic cultures with subsequent detection and utilisation of recombinant proteins.

4.1 Significance of a protein clearing procedure

Flow Cytometry is commonly associated with inaccuracies due to its basic operating principle involving 'hydrodynamic focusing' whereby each particle, either cell aggregate or single bacterium, is counted as one cell (Maukonen et al., 2006). Hence, fluorescent techniques like FACS are not universal and successful application necessitates detailed tailoring of pre-treatments and buffer compositions for cell lines and product types. Previous research (Bunthof and Abee, 2002, Gunasekera et al., 2000) failed to generate a procedure capable of consistent cellular discrimination within a diverse range of protein environments inclusive of dairy scaffolds, clinical protein supplements and encapsulation polymers. This challenge is associated with the fluctuating proteolysis response of various protein environments due to differing structural orientation and protein complexity. Doherty et al., (2010) optimised sample pre-treatments (Figure 5) buffer composition and probe concentrations in order accurately detect live, injured and dead cells within encapsulation systems. Moreover, this strategy liberated cells from encapsulation and protein networks without any adverse cellular injury as visualised in Figure 6.

The assay advocates rapid, reproducible cell liberation compared to lengthy extraction procedures previously reported for immobilized systems (Sun and Griffiths, 2000). Since cells are in the micron range, the signal-to-noise ratio in FACS analysis is of paramount importance especially in clinical protein supplements, which are normally viscous in nature. More importantly, encapsulation polymers and protein matrices will inevitably generate increased particle scatter due to high concentrations of colloidal particles in the cell size range. It is evident that protein clearing strategy is fundamental for the achievement of reproducible reliable cytometric viability screening in dense environmental milieu commonly encountered in drug and cell delivery models.

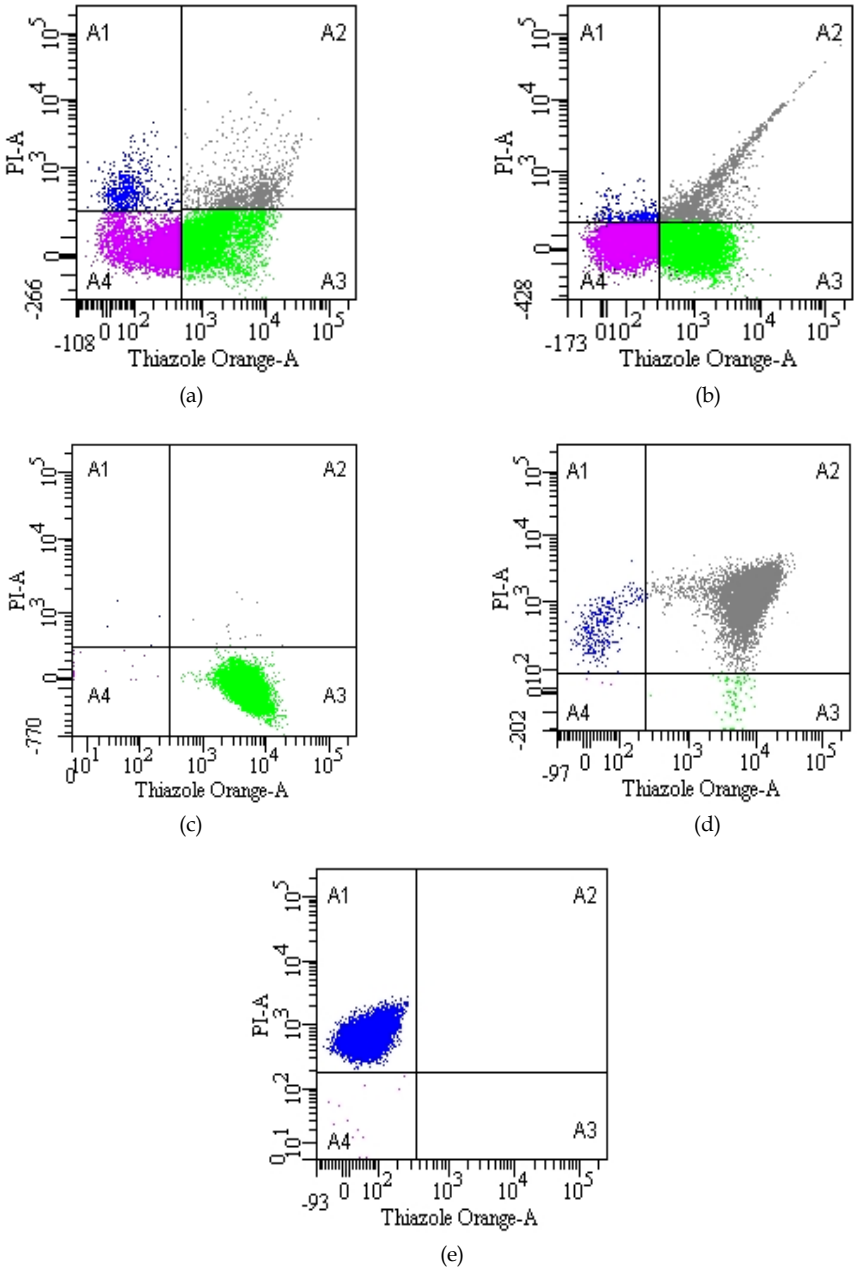


Fig. 5. Flow cytometric dot plots illustrating probiotic bacteria Lb. rhamnosus GG encapsulated in protein gel matrix and pre-sample extraction (a), post-homogenization (b) and after proteolysis digestion of the protein capsule (c). Results also demonstrated the clear distinction between live (c), injured (d) and dead probiotic cells (e)

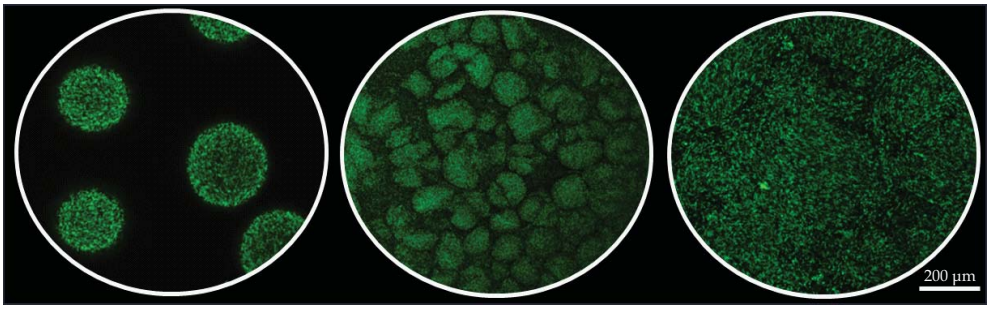


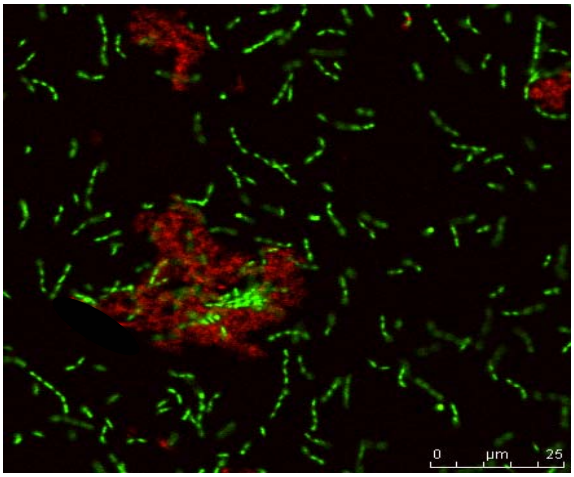
Fig. 6. Confocal image visualising the release of live probiotic bacteria from intact encapsulation scaffolds (left) and their progressive digestion during the protein clearing procedure (left- right)

4.2 Cell release mechanism for enhanced cytometric screening

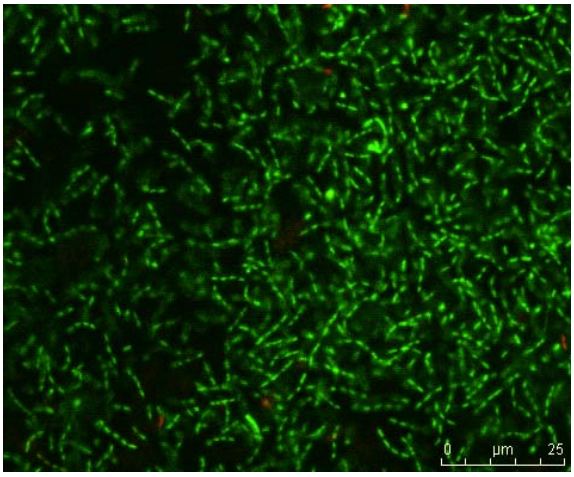
Current research illustrates a poor correlation between standard plate counts and cytometric screening due to non-specific binding of fluorescent dyes to protein particles (Gunasekera et al., 2000), which failed to be adequately removed by enzymatic treatments or commercial protein-clearing agents (McClelland and Pinder, 1994a, b). Meanwhile, Doherty et al., (2010) further resolved the negative effect of environmental proteins by introducing a mild homogenisation step in order to break-down cellular chains for the provision of true cytometric cell counts. Essentially, this two-stage pre-treatment substantially reduced particle counts or cytometric 'events' that were similar in size to, or larger than typical cellular dimensions i.e. 1 to 5 µm (Figure 7). Interestingly, further physico-chemical analysis e.g. zeta potential and hydrophobicity, provided details of charge interactions within protein-cell systems. This procedure allows to release of clean cell populations from complex or encapsulated protein matrices (Doherty et al., 2010).

4.3 Optimum compensation and fluorescent staining

Spectral overlap of the different fluorochromes used during FACS, if uncorrected, will lead to misinterpretation of data from false positives or artifactual populations. Compensation values in excess of 90% have been reported (Gunasekera et al., 2000, Gunasekera et al., 2003), which can be substantially reduced by pre-treatment to values as low as 28%, which may alleviate distortion of true viable counts. Pre-treatment of the preparations can influence fluorescence compensation values via optimization of instrument settings and equilibration of overlapping spectral channels. Despite dense protein environments in some products, the regression between FACS and plate counts can closely match the guidelines of Feldsine et al., (2002) for validation of qualitative and quantitative microbiological methods. Since all matrix material in the size range of cells can be adequately digested, cell overestimation as a result of matrix staining during FACS analysis was reduced. However, these findings may be interpreted as evidence of a dormant or an active but non-culturable cell condition, since Lahtinen et al., (2005) discovered a subpopulation of non-culturable cells with a functional cell membrane typical of viable cells. Interestingly, non-viable probiotic cells can also illustrate adherence to intestinal mucus for subsequent conveyance of immunomodulatory effects (Ouwehand et al., 2000, Vinderola et al., 2005). Therefore, the detection of compromised



(a)



(b)

Fig. 7. Fluorescent Confocal Scanning Laser Microscope (CSLM) images showing the incomplete protein digestion (a) generated after single protein digestion and the homogenous cell suspension generated (b) for FACS analysis following cell extraction and double proteolysis

cells or possibly dead cells may be screened by FACS for the provision of therapeutic cell cultures. Furthermore, Maukonen et al., (2006) highlighted the choice of fluorescent stain, sample pre-treatment and product matrix as additional cytometric factors influencing cell stainability, contrary to general credence based solely on strain characteristics. Retention of fluorescent dyes is synonymous with hydrophilic cell surface properties, both of which were illustrated for probiotic bacteria *Lactobacillus rhamnosus* GG by Doherty et al., (2010). Interestingly, surfaces of lactic acid bacteria studied in literature are also hydrophilic

(Boonaert and Rouxhet, 2000, Pelletier et al., 1997). This acquired knowledge relating to cytometric screening of protein-cell systems will diversify the choice of cell lines and the product range applicable for efficacious cell delivery via encapsulation.

4.4 Next generation flow cytometry for encapsulation applications

As conventional flow cytometers with advanced capabilities mature as large powerful laboratory systems for obtaining highly complex information, a new generation of 'personal flow cytometers' have evolved. These bench-top user-friendly cytometers focus on specific functions (Vignali, 2000) and include less flexible laser selection systems (Shapiro, 1995), less sensitive detectors (www.accuricytometers.com) and/or elimination of sheath fluid (www.millipore.com). These smaller systems can accomplish most customary tasks, including cell counting, measurement of cell viability, antibody quantification and detection of cell death. The Luminex® family of cytometers, in particular, have advanced the use of micro-particle-based assays to provide multiplexed analytical capability while keeping the optics relatively simple. With recent developments in micro-fabrication and microfluidics, such as small, optimised chip design, developers are miniaturising current cytometers even further to create systems for point-of-use applications and some of these systems will continue to employ particle-based assays (Kim et al., 2010).

Micro-particle assays often rely on antibodies as capture molecules on the surface of the capsule. Although, other types of capture molecules can be used, the high specificity and affinity of antibodies in the presence of complex sample matrices has made them a reagent of choice. In 1977, Horan and Wheeless published a manuscript in *Science* detailing the first microsphere-based immunoassay (Horan and Wheeless, 1977). Since then, others have followed with technology of increasing sophistication (Vignali, 2000). Capture antibodies are generally immobilized onto the micro particles using available reactive groups, for example amines, hydroxyls or thiols but carboxyls are the most frequently used. Functionalisation of carboxyl is followed by exposure to ethydimethylaminopropylcarbodiimide and *N*-hydroxysuc-cinimide, which provides a mild procedure for antibody attachment to the surface of micro-particles. The attached antibody can captures antigen from a sample on to the surface for the microsphere. The signal can then be generated as an aggregation event measured using light scattering or electrical or magnetic properties. More frequently, a fluorescent tracer is included in the particle-capture antibody-antigen complex, and fluorescence is measured.

Light scattering in flow cytometers is a staple phenomenon for detecting and characterising particles in FACS and more recently micro-capsules. A light beam directed at a particle can interact through reflective, refractive and diffractive effects. Then, information about the particle, aggregate or micro-capsule can be derived from the change in direction and intensity of a scattered light beam. Collecting scattered light at various angles from the incident beam has been reported to provide different types of information about the particle, e.g. size and density (Zharinov et al., 2001). The diameter of the particle/ capsule should be within the range of 1-50 wavelengths of the incident light beam.

Typically, forward scatter can provide approximate information about size of a protein gel micro-bead or capsule (Shapiro, 2003). It should however, be noted that an increase in the intensity of forward-scattered light does not always correlate with increasing particle size.

Side scattered light is often collected at 90^{o} and provides information about smaller particles and structures within protein particles, scaffold, capsule or gel aggregates. Proportionally more light is scattered by micro-capsules or encapsulated structures at a wide angle than at a small angle, and thus side-scattered light can provide information about the relative roughness or shape of micro-particles and capsules in addition to the granularity of their internal structures. Measuring side-scattered light and forward scattered light has become a standard FACS for biomedical research because this behaviour enables cells to be distinguished by size and granularity, providing insight into mixed populations, viability, or change in internal structure of encapsulation entities.

Using information derived from scatter light at different angles, particles can be classified and studied. Zharinov, et al., (2001) used light scattering data from a scanning FACS to distinguish lymphocytes, erthyrocytes, encapsulation capsules and milk-fat particles of various size and refractive index (Zharinov et al., 2001). These cells and particles generated different scattered light profiles dependent on scattering angle. Steen et al., (1990) custom-built a flow cytometer to characterise viruses of different size using light scattering (Steen et al., 1990).This device could easily distinguish particles with diameters in the range of 30-700 (Steen et al., 1990). In the 1980 Masson and co-workers presented a strong body of work describing particle-counting agglutination immunoassays (PACIA) (Sindic et al., 1981). Initially, PACIA was publicised as a replacement for expensive assay utilizing radioactive labels for characterisation of antigen and antibody interactions. In these assays, polystyrene particles coated with antigens were incubated with antibodies to cause agglutination or aggregation of the particles. Key aspects of this assay were:

- Use of an antibody with multivalent binding sites to enable particle-particle interaction
- Determination of particle concentrations that would allow aggregation; and
- Prevention of non-specific interactions between particles

The samples before and after agglutination were measured in an optical particle counter based on light scattering. The aggregated particles were larger in size than unaggregated particles and resulted in more side-scattered light. In 2003, Pamme, Koyama and Manz described a microfluidic device that used light scattering to analyse agglutination immuno assays (Pamme et al., 2003). The device used a design that focused particles in two dimensions into an optical interrogation region. Particles ranging from 2-9 μm in diameter were distinguished, which is significantly larger than the 70-300 nm range reported previously (Steen et al., 1990). Light scattering is extensively studied with many advantages to developers of microflow cytometry assays for encapsulation purposes. Using just a beam of light of suitable wavelength (relative to the capsule) and detectors at various angles, information regarding size, shape and granularity of a capsule are easily derived. Additionally, scattered light signals tend to be strong and do not need the most advanced or expensive detectors. Yet, distinguishing differing in diameter of a few microns can be challenging and represents the fundamental pre-requisite for encapsulation screening assays.

4.5 High content encapsulation screening

Literature also shows that high resolution two-dimensional (2-D) images consume limited detector bandwidth, introduce a data-acquisition delay that is a barrier for real-time

decisions needed for sorting capsules and introduce noise via inaccuracies in image segmentation. This impediment is further addressed to provide the foundation knowledge for a parallel microfluidic cytometer (PMC) using a high-speed scanning photomultiplier-based detector. Development of parallel flow channels within this model PMC would inevitably decouple count rates from signal-to-noise ratio for cytometric analysis of encapsulation systems. Essentially, this approach would demonstrate the feasibility for high throughput visual screening of encapsulated animal cells and stem cell clusters with concomitant determination of recombinant protein generation and localisation.

Imaging flow cytometers based on wide-field-charge-coupled device (CCD) imagers have demonstrated throughput limitations similar to those of microscopes (George et al., 2006). To address these restrictions, industry has developed a multi-channel parallel microfluidic cytometer (PMC) based on analog detection with parallel microfluidics. This design may potentially reduce i) data-buffering and storage requirements and ii) simplify the classification algorithm required to differentiate encapsulated animal or stem cells. Hence, parallel microfluidics will bypass sample-changeover restrictions commonly encountered with single channel flow cytometers. This novel strategy will enhance process efficiency of the PMC detector by increasing the diameter of a laser spot from 1 μm and 4 μm. Furthermore, sample flowing into the focal volume will potentially eliminate the focusing and stage volumes that limit high content screening on a microscope to approx. 2-6 wells min^{-1}. This new cytometric design circumvents many throughput limitations for both high cell density encapsulation and flow cytometer analysis by combining the best features of each technology to achieve efficient cell encapsulation screening.

Although much can be borrowed from the methods of 2-D high content screening (de Vos et al., 2010), 1-D algorithms are fundamentally different. Notably, microscope or flow cytometers draw boundaries around 'primary and secondary objects' (known as segmentation). However, this user-defined aspect of segmentation is a source of assay variability and is often considered the most challenging and time-consuming step. However, the recent application of 1-D cytometric imaging strategy for encapsulation systems integrated resolution issues experience within the standard hardware systems in order to eliminate potential segmentation problems. This was achieved by accepting any resolution element as an 'object'. Hence, this demonstrates the feasibility to develop efficient high content screening algorithms for micro-capsules using relatively low input.

5. Conclusion

Incorporation of bioactive compounds such as probiotics, animal cells and stem cell clusters into encapsulation matrices for food and pharmaceutical purposes can provide a simple way to develop novel nutraceuticals and drug treatment delivery systems with physiological benefits. Exact elucidation of cell proliferation and stability during encapsulation is of utmost importance for industrial or academic application. Hence, this chapter outlines the importance of cytometric analysis of encapsulation systems, in addition to the role of protein during this screening process. Proteins present various challenges for cytometric analysis; however, several techniques have been developed to overcome these limitations for commercial development of this novel technology.

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