Flow Cytometry of Cultured Plant Cells for Characterization of Culture Heterogeneity and Cell Sorting Applications

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FLOW CYTOMETRY OF CULTURED PLANT CELLS FOR CHARACTERIZATION OF CULTURE HETEROGENEITY AND CELL SORTING APPLICATIONS

A Dissertation Presented

by

VISHAL GAURAV

Submitted to the Graduate School of the
University of Massachusetts Amherst in partial fulfillment
of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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Chemical Engineering
FLOW CYTOMETRY OF CULTURED PLANT CELLS FOR CHARACTERIZATION OF CULTURE HETEROGENEITY AND CELL SORTING APPLICATIONS

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Plant cells culture provides a production source for plant-derived pharmaceuticals in which environmental conditions can be more easily controlled, manipulated, and optimized to yield high quantities of these valuable natural products. The Roberts Laboratory focuses on development and optimization of bio-processes for production of the anti-cancer agent paclitaxel (Taxol®) in Taxus cell suspension cultures with an emphasis on understanding cellular metabolism at both the molecular and cellular level. Most studies concerning metabolite production via cell culture technology rely on culture-average parameters, which are often insufficient to describe culture heterogeneity. There are several limitations associated with this non-model system; and adapting optimization strategies similar to other cellular systems (e.g., yeast and bacterial) do not necessarily translate into productive and economical processes. The two primary challenges for plant cell culture processes are low yields and variability in productivity. One of the primary sources of variability is due to culture heterogeneity, which is characterized by metabolic differences amongst cells, induced by cell aggregation. To study this heterogeneity we optimized methods to isolate single cells from aggregated
Taxus cultures, thereby allowing analysis of single cell phenotypes in a culture population. In this work, flow cytometric characterization of Taxus cell subpopulations with respect to paclitaxel accumulation is presented. By analyzing cell populations with varying levels of paclitaxel accumulation, the inherent molecular and metabolic differences amongst cells in culture can be explored; and the phenomena that underlay culture heterogeneity and production variability can be more completely understood.

Following a statistically optimized enzymatic digestion procedure to yield intact single cells in high yields, a live cell-based indirect immunofluorescence assay for paclitaxel based on PE fluorescence was developed. Paclitaxel is primarily stored in the plant cell wall, and because our studies necessitated the sorting and selection of live cells both for metabolic analysis and reculturing, it was critical that cells remained intact and not permeabilized or damaged throughout the staining procedure. This immunoassay was sufficient to stain cell wall-associated paclitaxel in different subpopulations. A broad range of paclitaxel accumulation amongst Taxus cells was detected, which provided an excellent basis for fluorescence-activated cell sorting (FACS). FACS on plant cells is challenging due to their aggregated nature in suspension and relatively large size when compared to microbial and mammalian cells. A new technique for high-throughput plant cell sorting using BD flow cytometers was developed to accommodate large-sized Taxus cells. A BD FACSVantage® equipped with 200 μm nozzle with optimized optics and fluidics conditions was employed in analysis and sorting of Taxus cells based on paclitaxel accumulation. Additionally, a BD FACSaria®, with 100 μm nozzle was also used to sort Taxus cells. Following a successful (~80-90% purity) sort by size, cells were
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CHAPTER 1
INTRODUCTION AND OBJECTIVES

1.1 Introduction

Plant cell culture provides an important method for production of plant-derived pharmaceuticals in which environmental conditions can be controlled, manipulated and optimized in order to obtain high quantities of these valuable natural products. A prominent example of plant cell culture technology in both research and industrial applications is *Taxus* for the production of anti-cancer agent paclitaxel (Taxol®). Paclitaxel is a member of the taxane family of compounds and is derived from the yew tree. Paclitaxel has been approved by the FDA for the treatment of breast, ovarian and lung cancers as well as the AIDS-related Kaposi's sarcoma. Recently, paclitaxel has demonstrated efficacy in the prevention of Alzheimer’s disease (Doraiswamy and Xiong, 2006) and also in preventing re-stenosis following coronary angioplasty (Slavin et al., 2007). The NCI has predicted that the demand for paclitaxel is going to increase significantly in the coming years in view of the increasing number of cancer cases in the US and the novel emerging applications for this important pharmaceutically active agent. The original supply for paclitaxel was through harvestation of bark tissue from the yew tree. This method was cumbersome, environmentally harsh and low yielding due to the slow growth of the yew tree (it takes ~200 years for a mature tree to grow (Kolewe et al., 2008)). The amount of paclitaxel extracted is quite low (10,000 kg of dried bark weight yields only 1 kg of paclitaxel). Harvesting of two to four mature trees is necessary to provide paclitaxel to treat one patient (Stull and Jans, 1992). Due to these limitations
from natural sources, alternative production methods were sought, including semi-
synthesis from needle precursors, total synthesis (Paterson et al., 2010; Borman, 1994),
production from endophytic fungi (Gangadevi and Muthumary, 2009; Stierle et al., 1993)
and plant cell culture. Plant cell culture is considered an excellent alternative in meeting
supply needs for the drug, and plant cell culture technology has been adopted in the US to
partially supply paclitaxel for both patient treatments and clinical trials. It is expected that
the demand for paclitaxel is going to rise in future years, and plant cell culture technology
will be used to supply a more significant amount of the total clinical paclitaxel. Hence,
second generation plant cell culture processes are being established in a variety of
industries and benefit significantly from basic research concerning optimization of *Taxus*
growth and secondary metabolism for enhanced paclitaxel production.

While a majority of research on paclitaxel accumulation and other secondary
metabolites through plant cell cultures has been focused on system characterization -
reactor design, medium optimization and nutrient utilization, the Roberts laboratory
emphasizes the understanding of cellular metabolism and culture heterogeneity (Roberts
and Shuler, 1997; Naill and Roberts, 2004, 2005a, b, c). Development of an optimal plant
cell culture process for production of paclitaxel requires a successful combination of
bioprocess engineering and other directed biosynthesis approaches. Adapting these
optimization strategies, which are often developed using other, more commercially-
relevant cell types (e.g., mammalian, yeast and bacterial) do not necessarily translate into
productive and viable plant cell culture processes, because of the several inherent
challenges associated with plant cells that ultimately lead to low and variable product yields.

Cell-cell heterogeneity in culture results in unpredictable shifts in metabolite accumulation over time and within cultures cultivated at the same time. Cell aggregation creates distinct microenvironments, which induces cellular differentiation, resulting in alterations in gene expression and metabolic function and observable phenotypic changes in culture behavior. These changes include variations in metabolic pathway participation and metabolite accumulation as well as cell subpopulation variability where certain populations of cells do not participate in growth or secondary metabolite accumulation (Yanpaisan et al., 1999).

Most studies concerning metabolite production via cell culture technology rely on culture-average parameters. These measurements involve averages of culture properties over a group of cells, and are often insufficient to describe culture heterogeneity as they neglect variations at the single cell level. Analyzing cell populations with different properties (e.g., varying levels of paclitaxel accumulation) at the single cell level can significantly contribute to the understanding of the inherent molecular and metabolic differences amongst cells in culture which can in turn help to decipher the phenomena that underlay culture heterogeneity and production variability. Techniques for investigating single cell properties (e.g., flow cytometry) can provide insight into the nature of culture heterogeneity and when adapted with a sorting functionality, allow for the recovery, culture and establishment of new elite cells lines. In this thesis, we develop
flow cytometry methods to characterize cultural heterogeneity. An overview of the methodology for identifying and isolating distinct paclitaxel accumulating subpopulations of *Taxus* cells is presented in Figure 1-1.

### 1.2 Objectives

The basic objective of this work is to enable development and optimization of processes for production of pharmaceuticals in plant-based systems, with an emphasis on understanding cellular metabolic control and culture heterogeneity. The system employed in this study is *Taxus* plant cell culture for production of the secondary metabolite anti-cancer agent paclitaxel (Taxol®). Methodologies for investigating *Taxus* plant cells (e.g., statistical design of experiments (DOE), assay development, flow cytometry (FCM) and fluorescence-activated cell sorting (FACS)) to enable a fundamental study of culture dynamics and heterogeneity were developed.

The specific aims are as follows:

1. **Statistical Optimization of Single Cell Production** - Development of techniques for optimizing isolation of single cells from aggregated *Taxus* suspensions through statistical design of experiments (Chapter 3).

2. **Paclitaxel Detection Assay in Single Cells** – Development of a live cell-based assay to detect paclitaxel accumulation in isolated *Taxus* single cells using indirect immunofluorescence methods (Chapter 4).

3. **Fluorescence-Activated Cell Sorting (FACS) Technology** – Development of novel FACS technologies to enable sorting of intact *Taxus* single cells and protoplasts,
based on various parameters such as size, complexity, paclitaxel accumulation, etc. (Chapter 5).

4. **Culture of Sorted Cell Subpopulations** – Establishment of conditions for the culture and analysis of sorted single cells (Chapter 6).

5. **Nuclear DNA content and Genome Size Evaluation** – Development of a rapid and easy nuclei isolation and DNA staining procedure to enable genome size quantification (Chapter 7).
Figure 1-1: Overview of the protocol for identifying and isolating distinct paclitaxel-accumulating subpopulation of cells.

Note: Aggregated *Taxus* cell cultures are enzymatically digested to form intact single cells. Isolated single cells are then incubated with primary and secondary antibodies to stain for paclitaxel. Single cell viability is tested with fluorescein diacetate. Viable *Taxus* cells, stained for paclitaxel are sorted into subpopulations based on paclitaxel accumulation via FACS technology.
CHAPTER 2
MATERIALS AND METHODS

2.1 Materials

The enzyme pectolyase Y-23 was obtained from MP Biomedicals, Inc. (Aurora, OH). The anti-Taxol™ monoclonal antibody was obtained from Cardax Pharmaceuticals / Hawaii Biotechnology Group, Inc. (Aiea, HI) and the goat anti-mouse IgG-phycoerythrin secondary conjugate was from Southern Biotechnology Assoc., Inc. (Birmingham, AL). The Plant Preservative Mixture (PPM™) was attained from Plant Cell Technology (Washington, D.C.). Arabidopsis thaliana, Pisum sativum, and Nicotiana tabacum were obtained from various groups (Normanly Lab (Arabidopsis), Biochemistry and Molecular Biology; Omparkash Group, (Pisum and Nicotiana) Plant, Soil & Insect Sciences) at the University of Massachusetts at Amherst and used as reference standards for the DNA content analysis. All other chemicals were obtained from Sigma-Aldrich unless otherwise mentioned.

2.2 Cell Culture and Maintenance

The cell lines P991 (Taxus cuspidata), PO93X (T. cuspidata), P93AF (T. cuspidata), and CO93D (Taxus canadensis) were provided by US Plant Soil and Nutrition Laboratory (Ithaca, NY) and maintained in our laboratory. Every two weeks, cells were subcultured into fresh medium, which consisted of Gamborg’s B5 basal salts (Gamborg et al., 1968) with 20 g/L sucrose, supplemented with 2.7 μM naphthalene acetic acid (NAA) and 0.1 μM benzyladenine (BA). Glutamine, ascorbic acid, and citric
acid were filter sterilized and added after autoclaving at various concentrations for each cell line (Table 2-1). All cultures were maintained in glass shake flasks (125 ml) with Bellco (Vineland, NJ) foam closures. For subculture, 10 ml of 14 day-old culture was transferred into 40 ml of fresh medium with 2-3 ml of packed cell volume per flask. All cultures were incubated in gyratory shakers at 125 rpm and 23 °C in the dark.

2.3 Elicitation with Methyl Jasmonate (MJ)

The cell cultures were elicited with methyl jasmonate (MJ) seven days post-transfer (Ketchum et al., 1999), unless otherwise noted. For elicitation, 40 µl of MJ was added to 460 µl 95% (v/v) ethanol and 500 µl nanopure water. This solution was vortexed and then filtered through a 0.2 m PVDF filter into a sterile container and subsequently added to suspension cultures to yield the desired concentration. Nonelicited cell cultures were given a similar volume of ethanol/water (1:1) as a control.

2.4 Enzymatic Digestion for Single Cell Release

An osmoticum of 0.5 M mannitol was prepared in nanopure water with 0.3 % dextran sulfate and added to the cell cultures to enhance single cell release, and autoclaved (Takebe et al., 1968). The enzymes, cellulase at 0.04 % (w/v) and pectolyase Y-23 at 0.5 % (w/v), were dissolved in the osmoticum under sterile conditions. Cells were vacuum-filtered using Miracloth® (Calbiochem, San Diego, CA) and added to the enzyme mixture using the ratio of 1 g cell weight per 5 mL of enzyme mixture. Digestion was carried out for four hours at standard culture conditions (23 °C and 125 rpm in the dark). Subsequently, the resulting cell suspension was transferred to sterile centrifuge
tubes and centrifuged at 1000 x g for 5 minutes. The supernatant was removed and the digest was washed with one-half volume of enzyme-free osmoticum by centrifuging at 400 x g for 1 minute and removing the supernatant. Single cells were then suspended in equal volume of 10 mM phosphate-buffered saline (PBS) and filtered through an 80 µm nylon mesh (Sefar American, Depew, NY) to remove large debris and undigested aggregates.

2.5 Isolation of Protoplasts

For protoplast preparation, the enzymes cellulase (1.0% w/v) and pectolyase Y-23 (0.1% w/v) were dissolved in an osmoticum consisting of 0.5 M mannitol and 0.3% (w/v) dextran sulfate at a pH of 5.5. Suspension cells were isolated via filtration through Miracloth® and then added to the enzyme solution. The ratio of cell mass to enzyme solution was maintained at about 1 g:5 ml for consistency. Incubation was carried out at 23-24 °C and 125 rpm in the dark for four hours. Following incubation, the solution was characteristically light brown in color and contained very few or no large clumps. The protoplasts were purified by density gradient centrifugation. First, they were centrifuged at 100 x g for 4 minutes. The supernatant was decanted and the pellet was resuspended in 0.5 M sucrose. Centrifugation at 600 x g for 4 minutes resulted in a noticeable protoplast band near the top of the liquid level. This band was carefully removed via pipet in order to reduce protoplast breakage. Finally, the protoplasts were washed three times with 10 mM phosphate buffered saline (PBS).

2.6 Preparation of Nuclei Suspensions
Galbraith’s buffer was made from 45 mM MgCl$_2$, 20 mM MOPS, 30 mM sodium citrate, and 0.1% (v/v) Triton X-100. The pH was adjusted to 7.0 with 1 M NaOH and the solution was filtered through a 0.22 µm filter and stored at -20 ºC in 10 ml aliquots. 1 mg/ml stock solutions of both FITC and PI were made and filtered through 0.22 µm to remove small particulates and stored at -20 ºC in 1 ml aliquots. PI is a mutagen and care must be taken when handling it. RNase stock solution was made at a concentration of 1 mg/ml and was heated to 90 ºC for 15 minutes to inactivate any possible DNase constituents, filtered through a 0.22 µm filter and stored in 1 ml aliquots at -20 ºC. Plastic Petri dishes (~5.5 cm diameter) were used to place the tissues on while chopping. A double-edged razor blade and a GSM Mini-Glass scraper were used. Nylon mesh (80 µm pore size) was used to remove large particulates from the nuclei suspensions. Polystyrene BD Falcon tubes were used to store samples. An ice container was used to incubate the PI-stained samples.

Plant cells (50 g, ~ 2 scoops of spatula) were vacuum-filtered through Miracloth and placed in the center of a plastic Petri dish. 1 ml of ice-cold Galbraith’s buffer was added to the Petri dish and the tissue was immediately chopped with a new razor blade for five minutes. If necessary, additional quantities of the buffer were added to facilitate chopping. Chopping was done carefully and not vigorously, as too much of it may yield a large amount of debris. The nuclei were then transferred to a 15 ml conical tube and equal volume of PBS added to form a nuclei suspension, which was then filtered through 80 µm nylon mesh into a labeled sample tube. Sample loss, if any, could be reduced by presoaking the nylon mesh with the nuclear isolation buffer prior to filtration.
2.7 Hemacytometry

Measurement of cell aggregates was performed by counting cells using a hemacytometer. For each sample, three separate counts of at least 100 clusters, distributed over 10 grids each were taken. Because of the presence of large clusters, all aggregates containing 25 or more cells were combined into a single category. For the single cell count, any cluster having more than one cell was excluded. The SCY was defined as the percentage of cell clusters that contain a single cell. The SCY data are reported as the mean of the three sample replicates ± standard deviation.

2.8 Measurement of Viability

Viability was determined by staining cells with fluorescein diacetate (FDA) (Widholm, 1972). A stock solution of 5 mg FDA/ml in acetone was prepared and stored at -20 °C. A working dilution of 0.5 mg FDA/ml in acetone was thereafter prepared from the stock solution. For viability testing, 20 µl of this working dilution was added to 1 ml of cell suspension and incubated in the dark at room temperature for ten minutes. Analysis with flow cytometry was used to distinguish viable (fluorescent) from nonviable (nonfluorescent) cells. Unstained cells were used as a control, where autofluorescence was found to be negligible.

2.9 Flow Cytometry

A BD LSR II® flow cytometer (Becton Dickinson, San Jose, CA), configured with 488 nm and 633 nm fixed alignment, air-cooled lasers was used to analyze the single
cells, and study their viability and scatter properties. There were no instrument modifications. The isolated single cells were filtered through 80 µm nylon mesh before flow cytometric analysis. Noclogging was observed with the single cell preparations. Data were collected and analyzed with BD FACSDiva® software (Becton Dickinson, San Jose, CA). Data collected included dot plot profiles of scatter properties (forward scatter and side scatter) and one-parameter histograms of cellular fluorescent intensities.

**2.10 Statistical Experimental Design**

The critical parameters affecting the single cell formation from *T. cuspidata* P991 cell suspensions were optimized using response surface methodology (RSM) (Box and Hunter, 1957). These parameters were cellulase concentration (ce), pectolyase Y-23 concentration (pe), centrifugation speed (cs) and time of incubation (ti) (Table 3-1). RSM has been extensively used by researchers to determine the optimal values of the critical factors that produce a maximum or minimum value of the output response. Thus, RSM is used to design experiments in order to obtain consistent measurements for the output response, and is also helpful in development of suitable mathematical models through fitting of the experimental data.

Central composite design (Box and Wilson, 1951) was utilized to optimize the critical digestion parameters. Central composite design is a powerful statistical optimization tool which contains an imbedded factorial design to develop a second order model for the response variable and allows for estimation of all the related regression parameters. Experiments were designed using Minitab (v. 13, Minitab, Inc.). In this
design, the total number of combinations was \(2^k + 2k + n_o\) where ‘k’ is the number of independent variables and \(n_o\) the number of replicates at the center point. The variables \(X_i\) were coded as \(x_i\) according to the following equation, to simplify statistical design:

\[ x_i = \frac{(X_i - X_o)}{\Delta X}, \quad i = 1, 2, 3, \ldots, k \]

where, \(x_i\) is the dimensionless coded value of the independent variable \(X_i\), \(X_i\) is the real measurable value of the variable, \(X_o\) is the real value of the variable measured at the center point, and \(\Delta X\) is the step change. In this optimization, a \(2^3\) - factorial design with six axial points \((\alpha = 1.682)\) and six replicates at the center point \((n_o = 6)\) was employed, which resulted in a total of 20 experiments. The value of \(\alpha\) (1.682) was chosen so as to make the design rotatable, which improves the prediction quality and is often a desirable property in quadratic model response surface designs (Montgomery, 2004).

An average of three replicate values was obtained for the SCY, which was taken as the dependent variable, or the response \(Y_{\text{expt}}\) (%). Regression analysis on the data was performed using Minitab software. The following quadratic equation was used to explain the system behavior:

\[ Y_{\text{model}} = \beta_o + \sum (\beta_i x_i) + \sum (\beta_{ii} x_i^2) + \sum (\beta_{ij} x_i x_j); \quad i, j = 1, 2, 3, \ldots, k \]

where \(Y_{\text{model}}\) is the predicted response, \(\beta_o\) the offset term, \(\beta_i\) the linear effect, \(\beta_{ii}\) the squared effect, and \(\beta_{ij}\) the interaction effect. The polynomial coefficients of the above equation were calculated using Minitab. This equation was used to estimate the value of the dependent variable as a function of the independent variables. The optimum combinations of the independent variables were determined by maximizing the above polynomial using the ‘Solver Tool’ of Microsoft® Office Excel (2003 version).
surface 3-D wireframe plots using the polynomial model equation were drawn by Minitab.

2.11 Taxane Quantification via High performance Liquid Chromatography (HPLC)

2.11.1 Measurement of Extracellular Paclitaxel

Medium samples (500 µl or 1000 µl) for the quantification of paclitaxel in cell culture were taken via micropipette and frozen at ~50 °C. Samples were later thawed and evaporated down to approximately 100 µbar dryness on a Savant SpeedVac® Plus (Holbrook, NY). The dried material was resuspended in 100 µl of acidified methanol (0.01% (v/v) glacial acetic acid in methanol) via sonication in a VWR Aquasonic® sonication bath for 30 minutes. The sample was then centrifuged for 25 minutes at 10,000 x g and the supernatant was filtered through a 0.2 µm PVDF filter and analyzed by HPLC (see Chapter 2.11.d).

2.11.2 Measurement of Cell-associated Paclitaxel

Suspension cultures were filtered through Miracloth® and washed with water in the same way as for the measurement of fresh weight. Known weights of fresh cells were placed into 1.5 ml centrifuge tubes and stored at ~50 °C. For sample preparation, the samples were allowed to thaw and then evaporated down to approximately 100 µbar dryness on a Savant SpeedVac® Plus. The dried material was resuspended in 1000 µl acidified methanol (0.01% (v/v) glacial acetic acid in methanol). After sonication for 30 minutes, the samples were vortexed and sonicated again for 30 minutes. Centrifugation for 25 minutes at 10,000 x g was followed by removal of the supernatant. The pellet was
resuspended in 1000 µl of acidified methanol and subjected to a second extraction. Next, the supernatants from the two extractions were combined in a new tube and dried completely under an air stream using an Evap-o-rac (Cole Parmer, Vernon Hills, IL). The dried material was resuspended in 100 µl of acidified methanol with 30 minutes of sonication. Following centrifugation (25 minutes, 10,000 x g), the sample was filtered through a 0.2 µm PVDF filter and analyzed by HPLC (see Chapter 2.11.d for details).

2.11.3 Measurement of Total Paclitaxel

Samples were taken via micropipetting with a cut pipet tip, where a 1000 µl capacity tip was trimmed approximately 1 inch from the end, to allow for the withdrawal of a 500 µl sample containing both cells and medium in the same proportion as in the original culture. The use of a cut pipet tip has been shown to not influence either the total volume or the dry weight of the pipetted sample (studied previously in our lab). The samples were frozen at –50 ºC and then extracted by the same method used for cell-associated paclitaxel measurements (see section 2.11.2).

2.11.4 Taxane Quantification via HPLC

All taxanes were identified and quantified using HPLC in combination with taxane standards provided by either Sigma Chemical Co., Inc. (St. Louis, MO) or Hauser Chemical Research, Inc. (Boulder, CO). Identification of unknown peaks was based upon the comparison with known standards of retention time and UV absorption spectra. Separation was accomplished on a Metachem (Torrance, CA) Taxsil®, 250 mm x 4.6 mm x 5 µm column equipped with guard cartridges. The mobile phase was acetonitrile and
water (48:52) at a flowrate of 1 ml/min, unless otherwise noted. The detector scanned from 210-400 nm, with peak detection at 228 nm. Paclitaxel, as with most taxanes, has a characteristic absorption peak at 228 nm. The HPLC system consisted of a Waters (Milford, MA) Alliance 2690 separation module HPLC with 996 photodiode array detector. Data acquisition, processing and equipment control was performed using Waters Millenium™ software version 3.05. Peak identification was based upon comparison of retention time and UV absorption spectra with known standards. For quantification, peak absorption at 228 nm was used.

2.12 Immunofluorescent Staining of Paclitaxel

Prior to staining, isolated single cells were washed three times with an equal volume of PBS. The cells were then divided into two equal volumes. The first volume served as a fluorescence control and was incubated with secondary antibody only. The second volume served as the stained sample and was incubated with both primary and secondary antibody. Both antibodies were prepared at a variety of dilution ratios in PBS. The cells were washed with PBS to remove any unbound antibodies a prescribed number of times (as noted), filtered through 80 µm nylon mesh and prepared for flow cytometric analysis.

2.13 Fluorescence Microscopy

Fluorescence images were acquired with an Olympus (Center Valley, PA) X71 inverted fluorescence microscope equipped with color-corrected 10X Plan-APO and 20X LCPlanFI fluorescence objectives and IPLab software (BD Bioscience, Rockville, MD).
Green and red fluorescence images were acquired using 470/40 nm excitation and 525/50 nm emission filters, and 535/50 nm excitation and 590 nm long pass emission filters, respectively (Chroma, Rockingham, VT). For paclitaxel staining, no fluorescence was observed in the control cells, which were only stained with secondary antibody (data not shown).

2.14 Quantification of Paclitaxel Content

The Quantum™ Simply Cellular® (QSC) kit (Bangs Laboratories, Inc., Fishers, IN) was used to quantify paclitaxel content in Taxus cells. The kit consists of five microsphere bead populations: one blank and four labeled with varying antibody concentrations, with capacities to bind mouse monoclonal antibodies in different proportions. The antibody binding capacity (ABC) values were provided with the QuickCal® analysis template v. 2.3 included with the kit. This method is described in more detail in Chapter 4.

2.15 Flow Cytometric Equipment Setup

2.15.1 BD LSR II® Flow Cytometer

This flow cytometer was configured with two lasers (fixed alignment) for excitation at 488 nm (primary blue) and 633 nm (red). Apart from measuring scatter properties, the cytometer should be able to excite the fluorophores/dyes used in the protocol to enable their fluorescence measurements. For instance, for FDA and PE fluorescence measurements, 530/30 and 575/26 band-pass filters were used respectively, under the optical configuration of 488 nm laser. We recommend using the BD
FACSDiva® (Becton Dickinson, CA) or FlowJo® (Tree Star, Inc., OR) software to record and analyze the flow cytometric data. Before running the samples, the LSR II should be calibrated with Sphero rainbow fluorescent particles (more details on the specific bead sizes can be found in Chapter 5). Refer to BD LSR II user’s guide for additional information.

2.15.2 BD Vantage and Aria Cell Sorter

The cell sorters were equipped with both 488 nm and 633 nm air-cooled lasers. To enable sorting of plant cells and other large particles, the FACSVantage sorter was functionalized with a MacroSort option, including a 200 µm flow nozzle. The FACSaria was equipped with 100 µm flow nozzle. Sorters were calibrated with AlignFlow fluorescence alignment beads, and with 25 µm Megabead NIST particles (Polysciences, Inc., Warrington, PA). Refer to the BD FACSVantage and FACSaria user guides for specific details. Also see Chapter 5 for further information.

2.16 Estimation of Nuclear DNA Content and Genome Size

The plant reference standards to determine Taxus DNA content were processed and stained for PI content. Their 2C nuclear DNA content was obtained from the literature (see Figure 6-1): Arabidopsis thaliana = 0.43 pg; Pisum sativum = 9.09 pg; Nicotiana tabacum = 10.04 pg.

With these references, nuclear DNA content of unknown samples was estimated using the following formula,
Sample 2C value [DNA in pg]

\[ = \text{Reference 2C value} \times \frac{\text{sample 2C mean peak position}}{\text{reference 2C mean peak position}} \]

Furthermore, genome size was estimated with the following formula (Dolezel and Bartos, 2005),

\[ \text{Genome size [in bp]} = \text{pg DNA} \times 0.978 \times 10^9 \text{ bp} \]
Table 2-1: Concentration of supplemental hormones and nutrients used for the maintenance of *Taxus* suspension cultures

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Ascorbic acid (mg/L)</th>
<th>Glutamine (mM)</th>
<th>Citric acid (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P991</td>
<td>150</td>
<td>6</td>
<td>150</td>
</tr>
<tr>
<td>P93AF</td>
<td>125</td>
<td>5</td>
<td>125</td>
</tr>
<tr>
<td>PO93X</td>
<td>125</td>
<td>5</td>
<td>125</td>
</tr>
<tr>
<td>CO93D</td>
<td>62.5</td>
<td>2.5</td>
<td>62.5</td>
</tr>
</tbody>
</table>
CHAPTER 3
STATISTICAL OPTIMIZATION OF SINGLE CELL PRODUCTION FROM
TAXUS CELL AGGREGATES

3.1 Introduction

Plant cell cultures can be used to produce and supply a variety of important compounds including medicinal (Kolewe et al., 2008), flavors (Mulder-Krieger et al., 1988), oils (Teixeira et al., 1995) and colors (Pepin et al., 1995). Plant cell suspensions are often a preferred culturing method as traditional microbial bioprocess optimization and scale-up strategies can be readily adapted (Roberts, 2007). However, plant cell suspensions are highly aggregated and have been shown to demonstrate high levels of heterogeneity, with significant variability amongst cells in culture, in terms of growth, morphology, cell cycle activity, and participation in secondary metabolism. This variability in secondary metabolite accumulation, coupled with low yields, limits their commercial utility. Traditional culture measurements used in designing optimization strategies for plant cell suspensions involve culture-average properties such as fresh/dry weight and overall product accumulation (Kim et al., 2005; Mirjalili and Linden, 1996). These measurements do not accurately describe the heterogeneous nature of plant suspension cultures and are not sufficient to characterize true culture behavior. The study of cell subpopulations through single cell analyses can establish relationships amongst important culture parameters such as growth and secondary metabolite accumulation (Yanpaisan, 1999). Characterization of cell cultures at the single cell level can provide insights into the underlying phenomena that govern intracultural subpopulation
variability. Single cell populations with specific genotypic and phenotypic traits (e.g., high metabolite-producing cells, cells with upregulated pathway genes, or any combination of different attributes) can be selectively stained and identified for various applications. Specific intact single cell populations can be sorted and propagated in culture for creation of superior cell lines. These populations can also be analyzed for cell-specific gene expression profiles to develop strategies for targeted metabolic engineering, with an ultimate aim to enable development of superior and efficient biological processes (Kolewe et al., 2008; Roberts, 2007).

Flow cytometry is a rapid and efficient technique to analyze single cells, and study subpopulation dynamics and behavior in a culture. It is considered as a superior method to investigate plant cell culture heterogeneity as it allows multiparameter analysis of cellular characteristics on a single cell basis (Davey and Kell, 1996; Naill and Roberts, 2004). It offers immense possibilities to identify, screen and isolate distinct populations of intact (with cell wall) and viable single cells, when coupled with a fluorescence-activated cell sorting (FACS) functionality. These isolated single cell subpopulations can be further studied for gene expression to design targeted metabolic engineering strategies or be aseptically propagated for the creation of specialized cell lines. However, one of the basic sample requirements while operating a flow cytometer is the need for single cell particles in liquid suspension. Plant cells tend to form aggregates in suspension resulting in technical difficulties with the operation of the equipment (Yanpaisan, 1999). Isolation of intact and viable single cells as opposed to protoplasts is, therefore, a crucial step in the flow cytometric study of plant cells, and reproducible methods for their formation
from aggregated suspension cultures must be established. Early studies have demonstrated the feasibility of isolating and culturing single cell particles derived from higher plants, albeit with limited success (Bergmann, 1960). Some of these methods were based on establishing long-term single cell cultures and were not suitable for rapid and high-throughput flow cytometric analyses. Other techniques to produce single cells employed chemical (Hayashi and Yoshida, 1988) and mechanical methods (Kurz, 1971), but were restricted to particular systems and, therefore, had very limited range of applicability. Also, most plant flow cytometric analyses utilize protoplasts or isolated plant organelles (e.g., nuclei (Lee and Lin, 2005)) due to the difficulty in rapidly achieving suspensions of intact and viable single cells from aggregated cultures in high yields.

_Taxus_ plant cell cultures are used commercially in the U.S. to supply the anti-cancer compound paclitaxel (Taxol®) for both patient treatments and clinical trials. Optimization of _Taxus_ cell cultures could provide superior cell lines for use in bioprocesses to supply this important agent for therapeutic use. In this work, statistical optimization of the critical parameters affecting generation of intact and viable single cells from aggregated _Taxus cuspidata_ cultures is presented. It was shown previously that the enzymatically-isolated single cells represented the aggregated cultures accurately, without adversely affecting cell physiology and metabolism (Naill and Roberts, 2004; Roberts et al., 2003). However, this method was not optimized for obtaining a high yield of intact single cells, which is particularly important for subsequent rapid and high-throughput flow cytometric analysis and cell sorting applications. In this work,
production of single cells is enhanced by maximizing the output variable, single cell yield (SCY). A general procedure to determine the optimum involves varying one variable while keeping others at constant levels. This single variable optimization (Fabregas et al., 2000; Pilat et al., 1976) is relatively less efficient compared to multi-variate statistical optimization (Francis et al., 2003; Sen and Swaminathan, 2004; Xua et al., 2006) owing to the fact that it excludes the interactive effects amongst variables and, therefore, does not always accurately predict the optimal output variable. In this work, multi-variate design of experiments (DOE) optimization is carried out using response surface methodology (RSM) (Box and Hunter, 1957) and central composite design (CCD) (Box and Wilson, 1951). The results presented here demonstrate a method to obtain high yields of live and intact single cells from aggregated plant cultures, thus enhancing their suitability for further research and analysis through flow cytometry. This is the first report of its kind to specifically employ statistical tools for the optimization of inter-dependent parameters affecting single cell formation in plant cell cultures. Although this technique was evaluated in the Taxus system here, this methodology can also be applied to optimize single cell/protoplast isolation methods, and investigate culture heterogeneity in other commercially relevant plant cell systems such as Catharanthus roseus (Runguphan and O'Connor, 2009; Gaines, 2004), Coptis japonica (Hara et al., 1989) and Artemisia annua (Baldi and Dixit, 2008), hence broadening the impact of this work.

3.2 Materials and Methods

A complete description of the materials and methods is located in Chapter 2. Of particular interest is section 2.10.
3.3 Results and Discussion

3.3.1 Dependent and Independent Variables Considered in the Design

SCY was the parameter of interest and hence the dependent variable considered in the design. Probable independent variables affecting single cell formation considered in the design are listed in Table 3-1. Initially, the effect of incubation time on the SCY was investigated at various times points of 2, 4, 6 and 8 hours. The data obtained (Figure 3-1a) suggested that incubation time be eliminated as a key independent variable. There was a significant 1.6-fold increase in SCY from 2-4 hours as opposed to relatively lower 1.1- and 1.0-fold increases from 4-6 and 6-8 hours, respectively. Additionally, longer incubation times may more significantly influence cellular metabolism and therefore single cells are less likely to be representative of aggregated suspensions (Roberts et al., 2003). Therefore, an incubation time of 4 hours was used in all further experiments.

3.3.2 Choosing a Design Region

To choose the design region, preliminary experiments were conducted to study the individual effects of the independent variables - ce, pe and cs on the SCY. Single cells were generated at various ce concentrations of 0% - 0.08% (w/v), while keeping pe and cs constant at 0.5% and at 1000 x g, respectively. The maximum SCY was obtained in the range of 0.02% - 0.06% ce (data not shown). Hence, this range was chosen for the experimental design. The center point was taken as $X_o = 0.04\%$ and the step change as $\Delta X = 0.02\%$. Next, the effect of pe on SCY was studied by varying its concentration from 0.1% to 0.9%, while keeping ce and cs constant at 0.04% and 1000 x g, respectively. The
maximum SCY was obtained between 0.3% and 0.7% pe (data not shown), which was chosen as the design range with center point as $X_o = 0.5\%$ and step change as $\Delta X = 0.2\%$. Finally, the effect of cs on SCY was examined. Speeds were varied from $600 \times g - 1400 \times g$, while keeping ce constant at 0.04% and pe fixed at 0.5%. A maximum SCY was observed between $800 \times g$ and $1200 \times g$ cs (data not shown). Therefore, this range was chosen for the design with center point as $X_o = 1000 \times g$ and the step change as $\Delta X = 200 \times g$.

### 3.3.3 Central Composite Design (CCD)

The experiments described above illustrated the individual effects of all the variables on the SCY. However, interactions and inter-dependence amongst the independent variables affecting the SCY may influence the optimum value. For example, it was unclear how the SCY changed when ce, pe and cs were allowed to vary in tandem. Therefore, the interaction effects amongst the variables were studied using statistical experimental design. The center points and difference levels (both coded and un-coded) to be considered in the central composite design are listed in Table 3-2. Based on these values, an experimental design was generated using Minitab (Table 3-3). In this design, 20 experiments were performed at various combinations of ce, pe and cs. The experimental results were fitted to a quadratic model, which enabled prediction of the output response ($Y_{model}$) under any given conditions of input variables within the design region.

### 3.3.4 Determination of Experimental SCY
From the experimental results (Table 3-3), it can be seen that a maximum SCY (54%) was obtained when the three independent variables $x_1$, $x_2$ and $x_3$ were at 0, 0 and 1.682 levels, respectively (run #18). When all the variables were kept at the +1 level, the SCY obtained was 43% (run #10). However, the SCY decreased significantly to 15% when all the variables were at the -1 level (run #1). Noticeably, the SCY dropped to 21% and 12% at extreme ce (-1.682 and 1.682; corresponding to runs #13 and #14, respectively), while other variables were kept at center points. Enzyme ce degrades the cell wall by hydrolyzing the cellulose. High values of ce may result in significant cell wall damage, lysis and debris formation, hence compromising cell integrity resulting in low SCY. This situation also results in loss of vital metabolic information, stored in the cell wall. On the other hand, at low ce, there may not be enough dissociation of aggregates to generate a significant number of single cells, again resulting in low SCY. Results show that optimization of pe is also critical to provide a high SCY. Pectin, another polysaccharide, which is present in the middle lamella of the cell wall and serves to cement adjacent cells in an aggregate, is degraded by pe. Hence, it is important that the enzyme concentration be sufficiently high (run #18) to enable the release of intact single cells.

3.3.5 Second Order Regression Equation

The second order regression equation used to calculate $Y_{model}$ is:

$$Y_{model} = 35.511 + 0.727x_1 + 4.913x_2 + 7.045x_3 - 6.505x_1^2 - 2.816x_2^2 + 0.059x_3^2 - 0.217x_1x_2$$
$$+ 0.683x_2x_3 + 1.770x_1x_3$$
$Y_{model}$ represents the SCY dependence on ce ($x_1$), pe ($x_2$) and cs ($x_3$). The coefficients of the equation were obtained by multiple regression analyses of the experimental data (Table 3-3). The values of the correlation coefficient, $R$ (0.918), and the squared correlation coefficient, $R^2$ (0.843), suggest an agreement between the experimental and model predicted values (Table 3-3). At the specified values of the parameters, the 95% confidence interval for $R^2$ was 0.725 to 0.96. These results show that the quadratic model adequately describes the experimentally determined SCY values. The coefficient of the term $x_3^2$ was found to be very low (0.059) and could be suitably omitted from the equation as it does not contribute to the overall $Y_{model}$ calculations (data not shown).

### 3.3.6 Analysis of Variance (ANOVA)

Statistical testing of the model was performed by the Fischer’s statistical test for ANOVA. The F value, which is defined as the ratio of the treatment (i.e., different experimental runs) mean square to the error mean square, was calculated to be 5.97 (for the data set in Table 3-3). At the 0.01 significance level, the tabulated F value ($F_{9, 10}; 9$ and $10$ being degrees of freedom of treatment and error, respectively) is 4.94 and the P value is 0.01. For a well-fitted model, the F value (calculated) should be greater than the F value (tabulated) (Dallal, 2004). Clearly, the calculated F value is greater than the tabulated value, which suggests that the null hypothesis (i.e., no statistical difference between individual experimental runs) is rejected at a P value of 0.01. This implies that the model is applicable in the defined design region.
3.3.7 Maximization of the Objective Function

The above quadratic equation was optimized by an iteration method. The SCY was chosen as the objective function to be maximized. \( x_1, x_2 \) and \( x_3 \) are the design variables within their respective domains in the design region. The optimal coded values of \( ce (x_1) \), \( pe (x_2) \) and \( cs (x_3) \) that result in the maximal \( Y_{model} (47.5) \) were determined to be 0.176, 0.987 and 1, respectively. The corresponding un-coded optimal values are 0.045\%, 0.7\% and 1200 \( x \) \( g \), respectively. From these results, it is clear that the optimal \( cs \) lies on the +1 level, while the optimal \( ce \) and \( pe \) concentrations lie within the design region. In order to verify the optimal values, experiments were performed at the suggested optimal \( ce \) and \( pe \) values, with varying \( cs \). The SCY was found to increase with increasing \( cs \) values and reached a plateau at 1200 \( x \) \( g \) (Figure 301b). This experimental result further validates the optimal values obtained from the model. Therefore, the optimal conditions for maximum SCY are 0.045\% \( ce \), 0.7\% \( pe \) and 1200 \( x \) \( g \) \( cs \).

Additionally, experiments were performed at enzyme concentrations outside the design region. The SCY was found to be lower than that obtained under the optimal conditions (data not shown), again validating the model outputs. It was, however, found that the model was not able to predict the SCY with accuracy for experimental points outside the design region (data not shown).

3.3.8 Model Validation through Response Surface Methodology (RSM)

Response surface 3-D wireframe plots were drawn using the second order regression equation, when one of the variables was fixed at the optimum value and the other two were varied (Figure 3-2). The response surface plot showed a SCY peak
(Figure 3-2a) near the model-derived optimal points \((x_1 = 0.176, x_2 = 0.987)\) when cs was kept at optimal value \((x_3 = 1)\) and the enzyme concentrations varied within the design region. Peaks for maximum SCY were also observed when the enzyme concentrations were kept at their respective optimal values and other variables were varied within the design region (Figure 3-2b, 3-2c). For instance, in Figure 3-2b, ce was kept at the optimal value \((x_1 = 0.176)\) and the maximum SCY peak was observed near the optimal points as obtained earlier \((x_2 = 0.987, x_3 = 1)\). In order to further validate the RSM analyses, experiments were conducted at both the optimal condition and center point, and experimental SCY data were directly compared to model predictions (Figure 3-3). Results demonstrate that under optimal conditions, the SCY was improved by nearly 1.5-fold when compared to the center point and that the model accurately predicted this increase.

### 3.3.9 Viability Measurements by Flow Cytometry

Maintaining viability of isolated single cells during the preparation is vital, since this will have direct consequences on the ability to analyze cells through flow cytometry. Single cells were isolated from \(T. cuspidata\) P991 at the obtained optimal values of the independent parameters. Isolated single cells were then stained for viability using fluorescein diacetate (FDA). Living cells have the ability to hydrolyze FDA retained within them by intracellular esterases and convert it to a fluorescent product, fluorescein, which can be used to distinguish live cells from dead (non-fluorescent). Population gating analysis using the 1\% relative fluorescence cut-off method (Shapiro, 2003) between the control (unstained) and sample (stained with FDA) histograms indicated that ~99.8 \%
cells were viable after the single cell isolation procedure (Figure 3-4). The mean fluorescence value increased from 44 units for the control (unstained cells) to 10,707 units when the cells were stained with FDA. This result provides further validation of this method to obtain live and high yields of single cells from *T. cuspidata* aggregated cell cultures.

3.3.10 Microscopic Analysis of Isolated Single Cells

Single cells were isolated from *T. cuspidata* P991 at optimum conditions, and brightfield images were acquired with an Olympus X71 inverted microscope (Center Valley, PA) using the 40X objective (Figure 3-5). Images clearly reveal that the isolated single cells were undamaged and remained morphologically intact after the procedure.

3.4 Conclusions

Flow cytometric characterization of plant cell culture growth and metabolism at the single cell level is a superior method over traditional culture average measurements to collect population information. Investigation of culture heterogeneity and production variability by obtaining information about different culture subpopulations is crucial for optimizing bio-processes for enhanced productivity. Obtaining high yields of intact and viable single cells from aggregated plant cell cultures is an enabling criterion for their analysis and isolation using high-throughput flow cytometric methods. Cellulase, pectolyase Y-23 and centrifugation speed were the critical parameters affecting generation of single cells from *Taxus* plant cell aggregates. RSM and central composite design were successfully utilized to optimize these independent parameters in order to
obtain a maximum SCY. The optimum values obtained were 0.045% cellulase concentration, 0.7% pectolyase Y-23 concentration and 1200 x g centrifugation speed. At these conditions, SCY was determined to be 55%, which is 72% higher than that previously reported. A second order regression model equation was developed to predict the SCY. This model was shown to hold well in the design region through ANOVA. The optimization procedure was validated through microscopy and flow cytometric characterization. The isolated single cells were undamaged and morphologically intact, as observed by microscopy. Flow cytometry was used to analyze the isolated single cells after staining with fluorescein diacetate, which measures the activity of intracellular esterases in living cells and, therefore, is an indicator of cell viability. The optimum procedure obtained in this work generated more than 99% viable cells, thereby facilitating the subpopulation analysis at the single cell level through rapid and high-throughput flow cytometry. In addition, RSM was useful in understanding the interaction effects amongst the variables. For example, the coefficient of interaction term $x_1x_3$ was higher than that of the linear term $x_1$, suggesting that the interaction effects amongst the variables cannot be neglected during the optimization. This multivariate statistical technique can be broadly applied in the development of models to predict maximal SCY for other cell systems as well as for the optimization of other output variables in biochemical processes, in general. This procedure will be particularly useful for isolation of single cell populations from other commercially relevant plant systems used to synthesize medicinal secondary metabolites such as *Catharanthus*, *Coptis* and *Artemisia*. The ability to rapidly prepare high yields of viable single cells will facilitate high-throughput flow cytometric investigation of cell culture heterogeneity at the single cell
level, which will enable a better understanding of metabolite production variability so that superior engineering strategies can be applied to optimize productivity.
Table 3-1: Probable independent variables\(^a\) affecting the SCY, and their corresponding suggested values (Naill and Roberts, 2004)

<table>
<thead>
<tr>
<th>Independent Variable</th>
<th>Unit</th>
<th>Suggested value</th>
</tr>
</thead>
<tbody>
<tr>
<td>( X_1 )</td>
<td>% (w/v)</td>
<td>0.04</td>
</tr>
<tr>
<td>( X_2 )</td>
<td>% (w/v)</td>
<td>0.5</td>
</tr>
<tr>
<td>( X_3 )</td>
<td>x g</td>
<td>1000</td>
</tr>
<tr>
<td>Time of incubation</td>
<td>hours</td>
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</tr>
</tbody>
</table>

\(^{a}\)\( X_1 \) = cellulase; \( X_2 \) = pectolyase Y-23; and \( X_3 \) = centrifugation speed
Table 3-2: Coded and un-coded values of the independent variables - cellulase, pectolyase Y-23 and centrifugation speed considered for the central composite design\textsuperscript{b}

<table>
<thead>
<tr>
<th>Variable</th>
<th>Coded</th>
<th>Un-coded</th>
<th>Range and levels</th>
<th>( -\alpha = -1.682 )</th>
<th>-1</th>
<th>0</th>
<th>+1</th>
<th>+( \alpha = +1.682 )</th>
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</thead>
<tbody>
<tr>
<td>Cellulase</td>
<td>( x_1 )</td>
<td>( X_1 )</td>
<td>0.006</td>
<td>0.020</td>
<td>0.040</td>
<td>0.060</td>
<td>0.073</td>
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<tr>
<td>Pectolyase Y-23</td>
<td>( x_2 )</td>
<td>( X_2 )</td>
<td>0.164</td>
<td>0.300</td>
<td>0.500</td>
<td>0.700</td>
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<td>Centrifugation speed</td>
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<td>( X_3 )</td>
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<td>800</td>
<td>1000</td>
<td>1200</td>
<td>1336.400</td>
<td></td>
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</table>

\[ \alpha = F^{1/4} = (2^k)^{1/4} = 8^{1/4} = 1.682 \] (for rotatability), where \( k = \) number of independent variables (3) used in the design (Montgomery, 2004). The units of the un-coded variables \( X_1, X_2, \) and \( X_3 \) are % (w/v), % (w/v) and g respectively. The un-coded values of the variables at different levels were calculated according to the formula: \( x_i = (X_i - X_o) / \Delta X, i = 1, 2, 3, \ldots, k \), as described in the ‘Materials and Methods’ section.
Table 3-3: An experimental design of 20 runs at various combinations of cellulase concentrations ($X_1$ and $x_1$), pectolyase Y-23 concentrations ($X_2$ and $x_2$) and centrifugation speeds ($X_3$ and $x_3$); with results in the form of experimental SCY ($Y_{expt}$) and the model predicted SCY ($Y_{model}$).```

<table>
<thead>
<tr>
<th>Run #</th>
<th>$X_1$ (%)</th>
<th>$x_1$</th>
<th>$X_2$ (%)</th>
<th>$x_2$</th>
<th>$X_3$ (x g)</th>
<th>$x_3$</th>
<th>$Y_{expt}$ (%)</th>
<th>$Y_{model}$ (%)</th>
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$R^2 = 0.843; R = 0.918$. $Y_{model}$ was calculated using the second order regression equation. $X_i =$ un-coded values of variables; $x_i =$ coded values of variables. The units of the un-coded variables $X_1, X_2, \text{ and } X_3$ are % (w/v), % (w/v) and x g respectively. Tabulated $Y_{expt}$ values are the average of three samples with standard deviations of less than 5% (data not shown).
Figure 3-1: SCY of *T. cuspidata* P991: (a) effect of incubation time: single cells were isolated at conditions listed in Table 3-1, incubated with enzyme solution for 2, 4, 6 and 8 hours and centrifuged. (b) effect of centrifugation speed: single cells were isolated at optimum cellulase (0.045 %) and pectolyase Y-23 (0.7 %) concentrations, but with varying centrifugation speeds.

Note: SCYs were calculated using hemacytometry. Reported values are the average and standard deviation of three samples.
Figure 3-2: Model-predicted response surface 3-D wireframe plots: (a) effect of cellulase ($x_1$) and pectolyase Y-23 ($x_2$) on SCY at optimal centrifugation speed ($x_3$); (b) effect of pectolyase Y-23 ($x_2$) and centrifugation speed ($x_3$) on SCY at optimal cellulase concentration ($x_1$); (c) effect of cellulase ($x_1$) and centrifugation speed ($x_3$) on SCY at optimal pectolyase Y-23 concentration ($x_2$).

Note: $x_i$ represents the coded value of the variable.
Figure 3-3: Comparison of SCY of *T. cuspidata* P991 at center point and optimal conditions of the independent variables.

Note: Reported values are the average and standard deviation of three samples.
Figure 3-4: Flow cytometric histograms: (a) control (no staining) and (b) sample, stained with fluorescein diacetate (FDA).

Note: Single cells were isolated from *T. cuspidata* P991 at the optimized conditions. A total of 10,000 events were recorded at a rate of 200-300 events/sec using a 488 nm (FITC) laser in the BD LSR II flow cytometer. The 1% fluorescence cut-off method was applied by gating the high-fluorescent 1% population in the control histogram and applying the same gate in the stained sample histogram. The percentage of cells in the stained sample histogram lying within this gate represents the positively-stained (viable) population.
Figure 3-5: Brightfield image of single cells of *T. cuspidata* P991: single cells were isolated at the optimum values of the input parameters obtained in this work.

Note: The figure clearly shows that intact single cells, with minimal debris, were obtained after the enzymatic digestion procedure under the optimum conditions. Scale bar represents 40 µm.
CHAPTER 4

PACLITAXEL DETECTION ASSAY IN LIVE TAXUS SINGLE CELLS:
DEVELOPMENT, OPTIMIZATION AND QUANTIFICATION

4.1 Introduction

Plants are a renewable resource for the production of a variety of important products, including pharmaceutics, oils, colors, flavors and specialty compounds. Plant cell culture is used throughout the world to supply these products (e.g., paclitaxel, anthocyanin, vanilla, thiopene); however, there are several challenges in the commercial adaptation of this technology (Kolewe et al., 2008). Importantly, plant cell cultures display significant variability in product accumulation across cell lines, culture replicates and over time. In addition, plant cell cultures show disparity in cell sizes, cell-specific functions, cell cycle progression, and secondary metabolite accumulation (Naill and Roberts, 2004; 2005a). This variability is related to culture heterogeneity which results from the aggregated nature of cells in culture, possibly because of incomplete separation of cell walls during cell division. Large aggregates, such as those observed in Taxus cell cultures, can lead to oxygen and nutrient gradients amongst the cells, resulting in the formation of subpopulations. Despite the problems which arise from aggregation, Taxus plant cell culture is currently used to supply paclitaxel, a secondary metabolite used in the treatment of cancer, with opportunities for bioprocess optimization through genetic and metabolic engineering. Elucidating the underlying phenomena behind culture
heterogeneity and subpopulation variability is critical to increase secondary metabolite yields and manage variability in cell culture.

Subpopulations can exist in plant cell cultures that are capable of accumulating higher levels of secondary metabolites as compared with the overall culture. For example, the production of solasonine, an alkaloid found in *Solanum laciniatum* cells (Zenk, 1978), varied over a wide range of concentrations, with some cells producing as high as six times more solasonine than others. Here, colonies of *S. laciniatum* cells were grown on agar plates and varying numbers of colonies were randomly selected and their solasonine content measured to obtain a frequency distribution. Similarly, for *Catharanthus roseus* suspension cells (Hall and Yeoman, 1987), only 10% of the cell population was found to be pigmented and shown to accumulate anthocyanin, which was measured using microscopic and microdensitometric techniques. This was attributed to an intrinsic ability of some cells to accumulate higher amounts of secondary metabolites as compared to the rest of the population.

Traditional population average measurements (e.g., cell growth/biomass, metabolite concentration) are often employed by researchers to study plant cell culture performance, but these methods do not accurately characterize diverse subpopulations of cells within a culture. Investigating plant cell culture traits at a single cell level will help illuminate the underlying causes of variability in cellular metabolism and secondary metabolite biosynthesis. Flow cytometry is an efficient and reliable method for analysis of single cell particles, but cannot be used directly for aggregated plant cells. Because
plant cells can be isolated as single cell particles, using cell wall digesting enzymes, and stained for various secondary metabolites, their rapid and high-throughput flow cytometric analysis can be performed. Several secondary metabolites such as thiophenes (Adamse, 1990), berberine (Hara et al., 1989), and serpentine (Brown et al., 1984) that accumulate in plant cells are autofluorescent and can be identified directly by measuring either the fluorescence or fluorescence quenching in the cytometer, while others do not autofluoresce and, therefore, require staining by a suitable dye or fluorochrome. Most secondary metabolites will demonstrate some cell-associated accumulation, in the cell wall or vacuole, thus their analysis using flow cytometry is possible. However, procedures must produce intact and pure single cell suspensions to minimize product loss and allow for accurate representation. Vacuoles are fragile and sensitive to shear, which can result in product diffusion through the vacuolar membrane. As a result, these products can diffuse out of the cell during flow cytometric analysis (Yanpaisan et al., 1999). Isolation and staining of protoplasts and other organelles (e.g., nuclei) have similar limitations resulting in the loss of metabolites, and has been observed with the loss of serpentine and berberine from protoplasts of *C. roseus* (Brown et al., 1984) and *C. japonica* (Hara et al., 1989), respectively.

*Taxus* cells accumulate a significant proportion of the secondary metabolite paclitaxel in the cell wall (Roberts et al., 2003; Aoyagi et al., 2002). In order to stain cells for paclitaxel, it is required that the cell wall not be compromised and that intact single cell suspensions are prepared. Additional limitations may exist depending on the intended assay applications (e.g., mRNA expression analysis, re-culture for propagation of elite
cell lines, etc.). For instance, it is imperative when identifying and sorting specific subpopulations of cells for further culture and propagation, the cells remain unfixed and are not irreparably damaged. Thus, maintenance of cell health and viability of isolated single *Taxus* cells is crucial while staining for subpopulations which accumulate variable amounts of paclitaxel. Cellular staining for paclitaxel has been performed on cell aggregates to demonstrate accumulation variability (Kawamura et al., 1998). However, this method was based on the manual selection of aggregates and neglected cell-cell variability within a single aggregate. A method to identify paclitaxel accumulating populations was previously developed in our laboratory for fixed *Taxus* cells; however, for analyzing cellular metabolism in single cells and to isolate cells for the propagation of superior cell lines, it is necessary that cells remain viable and membrane and wall integrity is not compromised by the assay.

In this work, a novel live cell-based indirect immunoassay was developed to detect paclitaxel accumulation in *Taxus* plant cell suspensions. Flow cytometry was used to analyze paclitaxel accumulation in stained single cells. Fluorescence microscopy was performed on the stained cells to visualize paclitaxel-related fluorescence and qualitatively assess the degree of variability in *Taxus* subpopulations. Cell viability, in terms of esterase activity, was also evaluated using flow cytometry after costaining with fluorescein diacetate (FDA). Further experiments were performed with variations in antibody concentrations, volume and number of washes, cell densities, and incubation times to establish an optimal immunoassay procedure. Dissociation constants were determined to assess binding capacities. This new assay was applied to evaluate the effect
of sampling day and elicitor (i.e., methyl jasmonate (MJ)) concentration on the percentage of paclitaxel-accumulating cells in culture. Finally the assay was applied to quantify paclitaxel accumulation per cell by using a series of beads with different calibrated antibody binding capacities (ABC) for mouse monoclonal antibodies. This work, to our knowledge, is the first report on staining of live and intact plant cells according to secondary metabolite accumulation, thus facilitating their high-throughput analysis and recovery through flow cytometry.

4.2 Materials and Methods

A complete description of the materials and methods is located in Chapter 2. Of particular interest is section 2.12.

4.3 Results and Discussion

4.3.1 Immunofluorescent Assay for Detection of Paclitaxel in Live Cells

A novel vital staining procedure was designed and evaluated to detect accumulation of paclitaxel in live Taxus cells, which involved development of an indirect immunofluorescent assay using a commercially available monoclonal anti-paclitaxel primary antibody. A PE-conjugated secondary antibody was subsequently used for detection and amplification of paclitaxel-related fluorescence. To enable subsequent sorting, isolation and propagation of distinct cell populations as well as gene expression analysis, cells must remain viable throughout the procedure. Additionally, the assay must accurately reflect the level of paclitaxel accumulation in viable cells. Previously, various immunocytochemical methods have been applied primarily to investigate cellular
localization of paclitaxel using solvent fixation and cross-linking strategies (Fonseca and Brown, 1997; Straubinger, 1995; Choi et al., 2001; Russin et al., 1995). These techniques are not applicable here as the cells need to remain viable after the procedure is completed. The use of detergents or other reagents known to increase cell permeability and affect membrane integrity were avoided in the assay.

Aggregated *T. cuspidata* P991 cell cultures were elicited with MJ on day 7 of the culture period. Five days post-elicitation, single cells were isolated for paclitaxel staining. Sample cells were incubated with both primary anti-paclitaxel antibody and secondary PE-conjugated antibody, while control cells were only incubated with secondary antibody. The monoclonal anti-paclitaxel antibody used in this study exhibits a high degree of specificity (minimally 50-fold) for paclitaxel over other structurally similar taxanes (Grothaus et al., 1995). Therefore, this antibody can be specifically employed to investigate paclitaxel accumulation and variability in *Taxus* cultures. Brightfield (Figure 4-1a) and fluorescent (Figure 4-1b) images of the stained cells were acquired following the vital staining procedure. Figure 4-1 shows that the cell wall remained intact and that cellular integrity was not compromised after the staining procedure. Since no fluorescence was detected in the control (data not shown), it became evident that the visible cellular fluorescence was due to the binding of the monoclonal anti-paclitaxel antibody. It can be ascertained that the antibody specifically detected paclitaxel in the immunostaining procedure and approximately 80-90% (through visual observation) of the cells accumulated paclitaxel in the MJ-elicited *Taxus* cultures (Figure 4-1b).
To study the subpopulation variability in paclitaxel accumulation and to validate the immunoassay developed here, the stained cells were analyzed in a flow cytometer. The cells were stained for paclitaxel and approximately 10,000 cells were run at (300-1000) events/s through the 488 nm laser in the BD LSR II to detect the PE fluorochrome. The cells were categorized by PE-related fluorescence to approximate the level of paclitaxel accumulation. Typical sets of cytometric data collected for analysis included forward scatter (FSC), side scatter (SSC), and PE-fluorescence (Figure 4-2). The control cells were run first. The voltage settings for the FSC and SSC photomultiplier tubes (PMT) were adjusted to include the cells (events in the dot plot) in the window of analysis. A manual gate was drawn in the scatter dot plot to exclude noise, debris and doublets from the population of interest (Figure 4-2a). The voltage corresponding to the PE-detector was then adjusted so that the control histogram fell at the lower end of the fluorescence axis (Figure 4-2c). The stained sample was subsequently run through the cytometer at the same voltage settings. The scatter dot profiles of both the control and the stained sample closely resembled each other in terms of size (FSC) and complexity (SSC) (Figs. 4-2a and 4-2b), which indicated that there were no adverse effects of the vital staining procedure on the cell size distribution, cellular ultra-structure and overall morphology of the cells. Figure 4-2d, the histogram for the stained sample, can be used to evaluate the relative amount of paclitaxel accumulation with respect to the control. This is usually obtained by calculating the difference in mean fluorescent intensities between the sample and the control. The mean PE-fluorescence in the stained sample was recorded to be 802 units, while that in the control was 161 units. This difference in fluorescence (641 units) can be used to compare the degree of paclitaxel accumulation
across various samples when measured under different conditions (e.g., MJ-elicited vs. nonelicited). Additionally, other histogram analysis techniques (described in the next section) can be employed to compare populations and determine the percentage of paclitaxel-accumulating cells in a given cell sample. These results indicate that cellular heterogeneity and paclitaxel accumulation variability can be assessed through this method, which can be used to investigate cell populations with varying amounts of paclitaxel levels. It is anticipated that following the development of this live cell-based assay to detect paclitaxel in plant cells, specific subpopulations can be aseptically sorted and propagated to obtain new cell lines with potentially higher stability and reduced variability in paclitaxel accumulation.

### 4.3.2 Histogram Analysis for Population Comparison

Comparing histogram distributions obtained from flow cytometric data is an important step in many biotechnological applications. The primary purpose of this comparison is to determine the statistically significant difference between two samples (e.g., control and stained sample), which can be used to detect the positive response and, therefore, identify the positively stained population in a given sample. Another application is in the field of instrumentation and operation, where similar comparisons can be made using the data generated over time to provide feedback on the machine performance and reliability.

The basic approach used to evaluate and compare multiple histograms (e.g., control and stained sample) is based on a relative fluorescence cut-off method. The
primary hypothesis in this method is that the cells in the stained sample having fluorescence that exceed 99% of those in the control sample are defined as positively stained cells (Shapiro, 2003). This step is performed by defining a specific fluorescence channel (i.e., by gating the high fluorescent 1% of the control population), and then calculating the percentage of cells in the stained sample having fluorescence equal to or above this threshold. This percentage of cells in the test sample is then considered positively stained.

*T. cuspidata* P991 single cells were isolated on day 12 from MJ-elicited cultures and stained for paclitaxel using the assay developed in this work. PE fluorescence histograms of 10,000 cells each were obtained from flow cytometric analyses of both control (secondary antibody only) (Figure 4-3a) and stained samples (Figure 4-3b). The top (high fluorescent) 1% of the control population was gated using FlowJo software (Tree Star, Inc., Ashland, OR) and the same gate was applied to the corresponding stained sample for determination of percent positives using the method described above. It was found that 84.7% of the stained *Taxus* cells were accumulating paclitaxel (Figure 4-3b). This number is an implicit indicator of the relative fluorescence shift of the stained sample with respect to the control and results suggest that a specific subpopulation of *Taxus* cells in culture accumulate paclitaxel. The specific subpopulations (accumulating and nonaccumulating) can be sorted to for metabolic analyses to understand the underlying phenomena behind this observed heterogeneity.

4.3.3 Cell Viability Assay
In order to successfully sort, recover and propagate specific *Taxus* subpopulations following paclitaxel identification, it is crucial that the viability of cells is maintained during the assay. The immunostaining procedure was carefully designed to avoid any potential detrimental steps causing cell damage or membrane rupture. To evaluate the effect of the paclitaxel detection immunoassay on cell health and viability, we chose fluorescein diacetate (FDA), a dye that measures metabolic activity correlating to cell viability. The FDA assay has been used to reliably assay cell viability and cytoplasmic integrity of many plant cell systems (Widholm, 1972; Castro-Concha et al., 2006). Esterases present inside living cells convert the FDA to fluorescein, and its fluorescence represents the degree of cell viability in a given sample. Following staining for paclitaxel, cells were incubated with FDA for ten minutes. Multiparameter fluorescence microscopy was performed on co-stained *Taxus* cells (for both paclitaxel, PE, and viability, FDA) and allows for simultaneous determination of two or more physical or immunological parameters. This method helps to establish informative correlations between various parameters of interest in a cell, which is crucial for obtaining a fundamental understanding of cellular function and metabolism. Unstained cells were used as a control to determine the autofluorescence, which was found to be negligible in all instances. Fluorescent images of the stained cells were acquired (Figure 4-1c) and suggest high levels of cell viability. Here, both PE and FDA-related fluorescence images were acquired following the multiparameter staining, and merged using Image J software. Figure 4-1 shows that the cells which were stained for FDA were fluorescent, indicating that they were viable after completion of the assay; furthermore, only a proportion of cells accumulated paclitaxel and demonstrated PE-related fluorescence.
The cells were stained for PE and subsequently by FDA and tested for fluorescence using the FITC detector in the BD LSR II flow cytometer. PE-stained cells and unstained (for FDA) were used as a control. The stained sample was then analyzed, which exhibited a distinct histogram shift, indicating that approximately 99% of the cell population was viable after paclitaxel staining (data not shown). The quantitative results from flow cytometry were in agreement with the qualitative results from fluorescence microscopy, establishing the efficacy of this procedure.

### 4.3.4 Establishing a Superior Immunoassay Procedure

There are several important parameters associated with any immunoassay procedure which should be optimized to obtain consistent and reliable staining data. Optimal antibody concentrations should be determined by staining cells and determining saturation concentrations. This is typically the concentration at which the increase in fluorescence is less than 10% of that corresponding to the previous lower concentration evaluated (Chabner and Longo, 2010). This concentration should then be employed in the immunostaining procedure as it allows for optimal antibody usage, and is a necessary criterion in using flow cytometry as a quantitative tool. Washing steps are also a critical component of indirect immunoassays. The primary purpose of washing is to remove excess unbound antibody from the sample, which may interfere with subsequent staining steps or lead to false positive results. Time of incubation is another parameter that should be minimized to prevent changes in cellular metabolism. Moreover, the effect of cell
density on staining should also be considered, as it is directly related to the amount of antibody used.

Both the primary and secondary antibodies employed in this assay were used at increasing volumetric dilution ratios of 1/1600, 1/800, 1/400, 1/200 and 1/100 to stain *T. cuspidata* P991 cells (Figure 4-4a). The sample cell density was 1 x 10⁶ cells/ml and the time of incubation was 60 minutes. The experiment was performed with the following three conditions for washing: (number of washes) and (volume of wash buffer) = 2 and 2 ml, 3 and 3 ml, and 4 and 4 ml. Figure 4-4a shows the antibody titration curves under the different wash conditions evaluated. Saturation in antibody staining, as measured by flow cytometric PE fluorescence, was observed at a dilution ratio of 1/200 for both 3 and 3 ml and 4 and 4 ml wash conditions. At this concentration, there was no saturation with the 2 and 2 ml wash condition. The absence of a plateau-like profile in the antibody titration curve for the 2 and 2 ml wash condition can be attributed to insufficient washing, which resulted in a reduced degree of staining. This suggests the presence of excess unbound primary antibody, which can bind with the secondary antibody in solution, reducing interaction of the secondary antibody with the bound primary antibody. The secondary antibody conjugated to the PE moiety (only PE has a molecular weight of 240 kDa), is prone to steric hindrance, and therefore fails to access and saturate the bound primary antibody.

Figure 4-4b shows the effect of incubation time and sample cell density on immunostaining. *T. cuspidata* P991 cells were stained with antibodies at a dilution ratio
of 1/200 and a wash condition of 3 and 3 ml. Cells were incubated for 30, 60 and 90 minutes under two sets of cell densities per time period. For the sample with 1 x 10^6 cells/ml, increasing the incubation time from 60 minutes to 90 minutes had no effect on the fluorescent staining; but incubation time of less than 60 minutes showed a decrease in the degree of staining, thereby suggesting a minimum incubation time of 60 minutes. Furthermore, the sample with a lower cell density of 1 x 10^6 cells/ml exhibited more than six-fold higher fluorescence after 60 minutes as compared to the sample with a higher cell density of 2 x 10^6 cells/ml, which showed a significant increase only after 90 minutes. The antibody concentration used with the higher cell density sample would need to be increased to account for the increased number of cells in the sample. The higher cell density sample likely exhibited a slower binding rate as higher fluorescence levels were observed at 90 minutes. The recommended conditions are: incubation time of 60 minutes, cell density of 1 x 10^6 cells/ml, and wash steps of 3 and 3 ml.

4.3.5 Binding Affinity Measurements

Determination of dissociation constant (K_d) and analysis of binding kinetics is an essential component in the development of an immunoassay procedure. These measurements can be used to characterize antibody systems, and also provide a predictive approach for antibody binding performance in biological assays (Neri et al., 1996). A smaller value of K_d suggests stronger binding and stable assay conditions. Typical K_d values for antibodies and adhesion molecules lie in the range of nM-µM (Mattes, 1995). Antibody staining data (at 1 x 10^6 cells/ml and 60 minutes) for T. cuspidata P991 cells at 3 and 3 ml wash conditions were collected using various antibody dilution ratios. The
value of $K_d$ can be estimated from the concentration of the antibody which corresponds to half of the maximum observed binding. The concentration of the antibody at half of the maximum binding (41.5 %) was determined by fitting the staining data via a non-linear regression analysis using Prism software (GraphPad Software, Inc., La Jolla, CA), which was estimated to be 0.1 µM (Figure 4-5a). The dilution ratio obtained at half of the maximum binding was 1/1000. Note that $K_d$ is the actual concentration of the antibody used at the dilution ratio of 1/1000. Since the stock antibody concentration was 1 mg/ml, it was used for calculating the $K_d$ value of 0.1µM. This value of $K_d$ for the paclitaxel immunoassay indicated strong binding and stable assay conditions.

### 4.3.6 Effect of Methyl Jasmonate on Paclitaxel Accumulation

Paclitaxel accumulation in *Taxus* cell cultures is significantly enhanced upon the addition of methyl jasmonate (MJ) (Yukimune et al., 1996; Wang et al., 2004; Wu and Lin 2003). MJ is considered a fundamental component of the signal transduction process that regulates plant inducible defense systems. It is helpful in upregulating secondary metabolite accumulation in a variety of plant culture systems. Durrant et al. (Durrant et al., 2000) have shown that MJ is capable of upregulating genes within hours after elicitation, yet it is only after several days that paclitaxel accumulation is stimulated and observed in *Taxus* cultures (Ketchum et al., 1999).

It was observed through flow cytometric population analysis that on day 12 of the culture the percentage of cells accumulating paclitaxel increased up to 84 % with an increase in the MJ concentration (Figure 4-5b). The percentage of paclitaxel
accumulating cells was found to be 45% when the culture was sampled on day 8 and elicited with 100 µM MJ, and 20% when not elicited. Even though a higher percentage of the MJ-elicited cells accumulated paclitaxel, there was still a large subpopulation of nonaccumulating cells in the elicited cultures. Interestingly, there was an identifiable 20% of the cell population that accumulated paclitaxel even in the nonelicited cultures. This is likely due to the increased sensitivity of flow cytometry as opposed to HPLC (Naill and Roberts, 2005a). Typically, HPLC measurements of paclitaxel accumulation indicate that nonelicited cultures do not accumulate paclitaxel. A definitive increase in the accumulation patterns was found from day 8 to day 12. Increasing MJ concentration correlated with an increased percentage of paclitaxel accumulating cells. These results demonstrate the applicability of the vital staining assay. Various subpopulations (accumulators and nonaccumulators) from both MJ-elicited and nonelicited Taxus cultures can be sorted for further population characterization, which is described in Chapter 5.

4.3.7 Bead-based Immunofluorescent Quantification of Paclitaxel Content

The use of an indirect immunoassay to quantify small molecules and other metabolites has become a preferred route over other methods, primarily because of its ease of use, shorter analysis times, and high level of accuracy (Vogt et al., 2000). Quantifying bound cellular molecules via staining by antibodies at saturating concentrations has several advantages over traditional methods like ELISA, radioactive binding and chromatography, since there are no harsh chemical extraction steps involved. Unlike radioactive substance usage, no major regulatory requirements need to be
addressed. Moreover, analysis via flow cytometry enables both high sensitivity and rapid analysis. Use of flow cytometry also bypasses another common problem encountered with immunocytochemical techniques like ELISA, which involves setting up a staining cut-off limit above which a sample can be considered positive. Employing an indirect immunoassay for quantification significantly helps in overcoming the above issue through the use of a control (i.e., cell stained with secondary antibody only).

To quantify paclitaxel content per cell using the vital immunostaining procedure, we used a series of beads with varying antibody binding capacities (ABCs) (i.e., 1991, 15,943, 58,330, 506,749) to bind mouse monoclonal antibodies. ABC represents the number of monoclonal antibodies a sample (bead, cells, etc.) will bind to, and therefore, can be correlated to the number of paclitaxel molecules bound to the cells at saturating conditions. Beads were stained in the same manner as cells, with primary antibodies bound first followed by the PE-conjugated secondary antibody. PE-fluorescence data for the bead staining experiment was obtained using the BD LSR II and plotted against their respective ABC values (Figure 4-6a). A linear regression of the data yielded an equation correlating ABC with fluorescence intensity: \[ ABC = 211.68 \times (\text{fluorescence intensity}) - 3853.7 \] with an \( R^2 \) of 0.99 (Figure 4-6a). This equation was then used to determine the number of bound paclitaxel molecules, corresponding to the ABC of the cell sample. \( T. cuspidata \) P991 cells were stained at varying antibody concentrations and run under the same PMT voltage settings in the BD LSR II as the calibrating beads (under same conditions as mentioned in section 4.3.4). A saturating antibody concentration of 1/200 was obtained (Figure 4-6b) and the corresponding value of the mean fluorescence
intensity (2207.43; CV = 3.2 %) was used to determine the ABC value, which was obtained as 463,415.08. Thus, every cell in the sample can be assigned an ABC, which can be directly related to its paclitaxel content. The ABC value is independent of the machine settings, sampling day/time, and variations in sample preparation, etc. This number can provide a universal measure of paclitaxel, which can be compared across all cell samples. Furthermore, ABC can be employed to calculate the amount (in gram units) of bound molecules on a per cell basis, using the molecular weight and Avogadro number, and on a per sample (volumetric) basis, using the cell density value. A sample calculation, with appropriate formula is shown below:

\[
\text{Since ABC} = \# \text{paclitaxel molecules / cell; Avogadro Number (} N_A) = 6.023 \times 10^{23} \text{ molecules / mole;}
\]

\[
\text{Molecular Weight (MW)} = \text{MW g / mole; Cell Density (d)} = \text{d cells / ml.}
\]

Rearranging the terms and balancing the units to obtain the paclitaxel amount in mg / L, we get

\[
\text{Paclitaxel (mg / L)} = \frac{\text{MW (g / mole)} \times \text{ABC (molecules / cell)} \times \text{d (cells / ml)} \times 10^3 (\text{ml / L}) \times 10^3 (\text{mg / g})}{N_A \text{ (molecules / mole)}}
\]

4.4 Conclusions

Regular use of plant cell culture technology in industry is currently limited due, in large part, to production variability across cell lines and culture periods. Methods have been developed to characterize plant cell cultures using whole culture averages; however, characterization at the single cell level is necessary to study cell-cell heterogeneity, which we have shown influences culture growth and secondary metabolite production. Here, the
subpopulation variability in *Taxus* plant suspension cultures was investigated through the development of a live cell-based assay, which detects cellular paclitaxel accumulation while maintaining cell viability and integrity. Fluorescence microscopy was used to analyze paclitaxel-related fluorescence and assess the degree of variability in *Taxus* subpopulations. To further validate the assay and obtain a more robust, quantitative assessment, flow cytometry was performed to analyze paclitaxel accumulation in stained cells. Histogram comparisons for population analysis were performed on the control (i.e., secondary antibody only) and stained (i.e., primary and secondary antibodies) samples to quantify positively stained populations. A relative fluorescence cut-off method was employed to determine the percentage of paclitaxel-accumulating cells, which can be used in conjunction with cell-sorting to investigate accumulation patterns amongst subpopulations. In order to identify, sort and recover *Taxus* subpopulations, it is essential that cells remain viable and intact. Cell viability was assayed using flow cytometry after co-staining with FDA, which detects esterase activity. Multifluorescent images of cells stained for both paclitaxel and viability were acquired to demonstrate that the cells remained viable and a proportion of them accumulated paclitaxel. To establish a superior immunoassay procedure to obtain consistent and reliable staining data, the experimental variables in the process were optimized. These included antibody concentrations, volume and number of washes, cell density, and incubation times. Binding affinity measurements, to estimate the dissociation constant ($K_d$), were carried out using a non-linear regression analysis, which suggested strong binding and stable immunoassay conditions. MJ elicitation of *Taxus* cultures results in increased paclitaxel accumulation, which was assessed using the immunostaining procedure and flow cytometry. The effect
of sampling day and MJ concentration on the percentage of paclitaxel accumulating cells was evaluated, indicating an increase with both sampling day and MJ concentration. Presence of paclitaxel accumulating subpopulations (20%) was also shown in the nonelicited cultures. Subsequently, this assay was employed to quantify paclitaxel accumulation per cell through the use of a series of precalibrated beads with varying antibody binding capacities. This quantification approach can be routinely applied to quantify paclitaxel on a single cell basis across samples independent of instrument settings, sampling day or sample preparation. This assay can be applied to identify and sort high- and low-secondary metabolite accumulating subpopulations from commercially-relevant plant cell culture systems, enabling their analysis and propagation for creation of superior cell lines for use in bioprocesses. These subpopulations can also be collected for cell-specific metabolic analyses, which can be used to design targeted metabolic engineering strategies for enhanced product biosynthesis.
Figure 4-1: Microscopic images of stained *T. cuspidata* P991 single cells: (a) brightfield; (b) fluorescent (red) images of cells after staining for paclitaxel using the indirect immunofluorescence assay; (c) fluorescent (green) image of cell stained for viability using fluorescein diacetate (FDA) after the immunoassay; and (d) multi-fluorescent merged image of cells stained for both paclitaxel (red) and viability (green).

Note: Single cells were isolated on day 12 of the culture, elicited with MJ on day 7. The immunofluorescent assay was then employed to stain for paclitaxel accumulation. Co-staining with FDA was performed to evaluate the viability post-assay. The scale bar represents 40 µm.
Figure 4-2: Flow cytometric (a) scatter dot plot of control (stained with secondary antibody only); (b) scatter dot plot of sample stained for paclitaxel; (c) PE-histogram of control; and (d) PE-histogram of stained sample.

Note: The scatter dot plots were gated to exclude debris and aggregates, and the resulting population was recorded in the histogram.
Figure 4-3: Flow cytometric histograms of (a) control (stained with secondary antibody only) for paclitaxel staining, with top fluorescent 1% region gated; (b) stained sample for paclitaxel staining, with the control gate applied to determine the percentage of paclitaxel-positive cells.
Figure 4-4: (a) Effect of dilution ratios of antibodies and wash conditions (number and volume of washes) on the degree of staining. Cells were stained at varying antibody dilution ratios with different sets of wash conditions and their corresponding PE-fluorescence values were analyzed using BD LSR II. (b) Effect of sample cell density and staining time on the degree of staining.

Note: Relative fluorescence intensity was calculated as the ratio of the intensity difference between sample and control to sample. Reported values are mean of three replicates (from one flask) and error bars represent one standard deviation. The coefficient of variation (CV) across all the sample peaks was found to be less than 5%.
Figure 4-5: (a) Binding affinity determination using the antibody staining data at the wash condition of 3 x 3 ml. The value of $K_d$ (0.1 µM) was obtained as the concentration of the antibody at half of the maximum binding (41.5%) from a non-linear regression analysis using the Prism software. (b) Effect of MJ concentration and sampling day on the percentage of paclitaxel accumulating cells, determined using the relative fluorescence cut-off method between the stained sample and control (secondary antibody only).

Note: The error bars represent standard deviation across three replicates from one flask.
Figure 4-6: (a) Linear regression relationship between the antibody binding capacities and PE-fluorescence intensities of the calibration beads stained using the immunoassay. The beads were stained in a similar manner as cells using the same antibodies, and analyzed in the BD LSR II. (b) Flow cytometric PE-histograms of *T. cuspidata* P991 cells stained for paclitaxel (at conditions mentioned in section 4.3.4) at varying antibody dilution ratios of (a) 1/800, (b) 1/400, (c) 1/200, and (d) 1/100, for determining saturating antibody concentrations to be employed for quantifying cellular paclitaxel content.
5.1 Introduction

Plants produce secondary metabolites as part of their defense mechanism. While these molecules may not be considered essential from a primary metabolism standpoint, they are useful in the treatment of several human diseases, including cancer, primarily because of their relevant biological activity against these disorders (Kolewe et al., 2008). Traditional harvesting and chemical methods to synthesize these secondary metabolites are often limited by factors such as low yields, slow plant growth rates, and high cost. These limitations can be overcome through the use of in vitro plant cell cultures (Roberts and Shuler, 1997). Apart from providing a more efficient production route, they are also advantageous from regulatory and environmental perspectives. However, plant cell culture systems exhibit significant variability and unpredictability in metabolite production, which limits their use commercially. The reasons behind this variability and the concomitant culture heterogeneity are not fully understood, but can be considered either genetic, phenotypic (e.g., variations in gene expression and hence metabolic functions) or morphological (e.g., presence of cellular aggregates which induces different microenvironments), or a combination. Previous data in our laboratory suggest the presence of distinct intracultural subpopulations of cells with specific traits. In order to understand and control variability in product accumulation, it is important that these subpopulations are characterized at the single cell level using tools like flow cytometry.
and fluorescence-activated cell sorting (FACS) (Naill and Roberts, 2004; 2005a; 2005b; 2005c). Strategies employing bioprocess and metabolic engineering tools can then be suggested to optimize and stabilize secondary metabolite production in plant cell cultures (Roberts, 2007; Yanpaisan et al., 1999).

Flow cytometry is a powerful tool to count, analyze and sort single particles (e.g., cells, protoplasts, nuclei, chromosomes, beads, etc.) in suspension. Flow cytometry allows for simultaneous multiparameter analysis of cell and/or cellular molecules, based on their light scatter and fluorescence properties, within heterogeneous populations (Shapiro, 2003). The quantitative evaluation of cellular characteristics on a per cell basis across the entire population set facilitates rapid, accurate, and sensitive analyses of culture heterogeneity. A variety of measurement techniques, including fluorescent probes, are used to identify certain cellular components to enable analysis and/or sorting. Some examples of cell and/or cell-associated properties with their respective probe/staining methods which can be measured using flow cytometry are provided in Table 5-1.

Flow cytometry has been used extensively by scientists for the study of mammalian and microbial cell populations for biotechnological applications (Sklar, 2005). Plant cell flow cytometry is, however, still an emerging field where many researchers adapt existing techniques developed for mammalian and microbial cells. Sorting and analysis of various plant particles/organelles (e.g., nuclei, protoplasts, chromosomes, and pollen grains) have been performed, with applications ranging from
production of pharmaceuticals and specialty chemicals to crop breeding. A genomic chromosome library has been constructed through flow cytometric sorting of banana (Musa balbisiana) nuclei (Safar et al., 2004). Specific green fluorescent protein (GFP)-positive protoplasts of Arabidopsis thaliana have been isolated using FACS and analyzed for gene expression (Birnbaum et al., 2005). Chromosomes differing in DNA content have been sorted and isolated from suspension cells of Haplopappus gracilis using flow cytometry (de Laat and Blaas, 1984). Transcriptional profiling of Arabidopsis tissues was performed by obtaining highly purified pollen grains through flow cytometric sorting (Becker et al., 2003). However, because of the large size of some plant protoplasts and nonuniformity of intact single cells (i.e., those cells that contain an intact cell wall), it is challenging to use conventional methods and flow cytometers for analysis and sorting (Dolezel et al., 2007). Plant cells are relatively large in size (ca. 20-100 µm), limiting the use of a typical nozzle in the cytometer. The dimensions of the particles to be sorted, including intact single cells, should be compatible with the flow nozzle. It is generally assumed that for a smooth and clog-free run, the flow nozzle/orifice should be at least four times the particle size, therefore demanding special size instrument nozzles when sorting larger plant cells (Harkins and Galbraith, 1987).

Intact single cells, as opposed to protoplasts, are relatively nonisotropic, which adds complexity in the application of flow cytometric methods. Moreover, large sized particles create instabilities in the flow stream, which mandates that the system flow rate and sheath pressure be maintained at low values, necessitating longer runs. Researchers have studied the physics of the cytometric sorting process to establish correlations which
explain and can ultimately predict flow and droplet formation in a sorter. The flow velocities are empirically regulated to avoid potential adverse effects on cellular integrity as a consequence of sudden flow stream acceleration and deceleration. For instance, there have been reports of interdependence between parameters such as sorting efficiency, particle diameter, flow cell nozzle diameter, sheath pressure, and drive frequency (Harkins and Galbraith, 1987). An extensive optimization of these parameters can lead to stable hydrodynamic flow conditions, resulting in efficient droplet formation and successful sorting of plant cells. Vacuoles, which are largely comprised of water and may constitute up to 90% of a plant cell, render plant cells fragile and shear-sensitive, thus affecting cell health and viability during sterile operations such as live cell staining and sorting. Special care (e.g., low centrifugation speeds and reduced agitation rates) must be taken during sample preparation and instrument operation to avoid any potential detrimental cellular effects. Plant cells, unlike animal and microbial cells, are nonuniform in shape, creating problems with signal detection that lead to incorrect optical measurements. To overcome this issue, a significant number of cells (~10,000 or more) should be analyzed in the cytometer, to ensure an accurate representation of the entire population set.

Another crucial difference between plant cells and other systems that limits the application of flow cytometry is the tendency of plant cells to aggregate in suspension. A first step in isolating a single particle suspension from aggregated plant cell suspensions is to induce single cell generation using enzymatic digestion to weaken the middle lamella that cements adjoining cells in an aggregate (Naill and Roberts, 2004; see
Chapter 3 for more detailed information). Following single particle isolation, cells tend to sediment in suspension, which can complicate cytometer operation. Xanthan gum, a relatively inert material, has been used to keep large biological particles suspended during flow cytometric analysis and sorting (Freyer et al., 1989). The sorting is performed aseptically under controlled conditions (e.g., temperature, mixing, exposure to light, etc.). In order to accurately represent plant cells from the size and scatter data, the cytometer is calibrated with large sized beads. Using these conditions, the feasibility of sorting plant cells on the basis of scatter properties can be established. Subsequently, plant cells can be sorted based on metabolite-related fluorescence. Distinct subpopulations with high level of sort purities (greater than 85 %) can be isolated. These sorted populations with distinct phenotypes can be directed for analysis, propagation and other applications, thereafter.

Here, a rapid, efficient and high-throughput method for sorting of *Taxus* cells based both on size and secondary metabolite accumulation is presented. These techniques can be used for both development of higher-accumulating cell lines and characterization of production variability through gene expression analyses, to ultimately suggest rational strategies to enhance metabolite accumulation.

### 5.2 Materials and Methods

A complete description of the materials and methods is located in Chapter 2. Of particular interest is section 2.15.
The methodology applied is presented here in a protocol format that is easy to adapt in a lab setting, and is also augmented with critical steps and troubleshooting tips and solutions. A complete methodology is presented that involves all steps prior to sorting (e.g., formation of single cells, staining for paclitaxel, flow cytometric analysis for both paclitaxel level and viability). Although some of this information is presented in other chapters, this presentation allows for future researchers to follow the complete procedure in an organized format.

5.2.1 Preparation of Single Cell Suspensions from Aggregated Cultures

(see Chapter 3 for more details)

Timing: 4-4.5 h

1) Prepare an enzyme mixture by dissolving 0.04 % (w/v) cellulase and 0.5 % (w/v) pectolyase Y-23 in 10 ml osmoticum solution in a 25 ml shake flask. Filter the enzyme solution through a 0.22-µm filter into a sterile 25 ml shake flask.

Critical Step: The choice of enzymes which efficiently dissociate aggregates into single cells is empirical and should be determined for given plant species and cell line.

2) Set up a vacuum filter unit using sterilized vacuum flask, funnel, and Miracloth in a laminar flow hood. Filter the MJ-elicited Taxus cultures (day 12) using a vacuum pump and collect the cells over the Miracloth. Using a clean and sterile spatula, gently transfer 2 g of cells to the 10 ml enzyme solution in the 25 ml shake flask. The sterile filtrate obtained in the vacuum flask is the conditioned medium (Naill and Roberts, 2005d); store in a sterile container at 4 °C for subsequent usage.
Critical Step: The amount of cells added to the enzyme mixture depends on the ability of the species to release single cells. This ratio should be optimized in order to maximize the single cell yield upon enzymatic digestion.

3) Incubate the aggregated cells in the enzyme mixture at standard culture conditions (23 °C and 125 rpm in the dark). Carry out the enzymatic digestion for 4 h.

Critical Step: Optimize the digestion time according to the species and the cell lines used. This can be crucial as longer digestion times may affect cellular integrity and viability.

4) Transfer the digest to a sterile 15 ml centrifuge tube and centrifuge at 1000g for 5 minutes at 23 °C.

5) Remove the supernatant without disturbing the cells and gently resuspend the cells in 10 ml of enzyme-free osmoticum.

6) Filter the cells through an 80 µm Nylon mesh into a sterile 50 ml centrifuge tube. Use a wide-bore pipette tip to transfer the cell suspension on the nylon mesh,

Pause Point: The isolated single cells can be stored overnight under normal culture conditions in a 50-50 medium (50 % fresh medium + 50 % conditioned medium). A suitable storage medium to prevent viability loss should be designed for the species of interest. The cells should be removed from the osmoticum and resuspended in the storage medium.

5.2.2 Live Cell-based Assay for Secondary Metabolite Accumulation

(see Chapter 4 for more details)

Timing: 2-2.25 h
7) Carefully remove the osmoticum or 50-50 medium (if the cells are stored overnight) by centrifuging at 1000 x g for 5 minutes at 23 °C.

8) Re-suspend the single cells in PBS. Measure the density of the single cell suspension using a hemacytometer. If necessary, dilute them with PBS to achieve a density of about \(1 \times 10^6\) cells per ml.

9) Split the cell suspension into two parts for staining. Transfer 5 ml each into two 15 ml shake flasks. Mark one flask as the fluorescence control (to be stained with secondary antibody only) and the other as the stained sample.

**Critical Step:** It is preferable to carry out the staining in glass containers as opposed to tubes, six-well plates, etc., which can affect the staining process. Cells and dyes/stains absorb to the walls of the plastic container.

10) Stain to detect paclitaxel accumulation using the primary anti-paclitaxel antibody first. Add the primary antibody at a dilution ratio of 1:200 to the cells in one of the 15 ml flasks (marked as stained) only. Do not add the primary antibody to the flask marked as control. Incubate under standard culture conditions for 45-60 minutes.

**Critical Step:** Staining variables, including antibody dilution ratios, time of incubation, etc., should be adjusted according to the cell type in order to obtain a superior staining procedure (see Chapter 4).

10) Wash the cells three times with PBS to remove excess antibody. Perform washing by centrifuging the cells in 15 ml tubes for 5 min at 200 x g, decanting the supernatant and adding an equal volume of PBS.
11) Stain both flasks (control and stained sample) using the PE-conjugated secondary antibody. Add the secondary antibody at the dilution ratio of 1:200 to both the samples. Incubate the flasks at standard culture conditions for 45-60 minutes. 

_Critical Step:_ Staining with secondary antibody should be done in the dark to avoid fluorescence loss. Wrap the flask in aluminum foil or use a sterile opaque container while performing incubation.

12) Wash the cells three times with PBS (as mentioned in step 10) to remove any unbound stain, and resuspend the cells in an equal volume of PBS.

13) Filter both the control and stained samples through the nylon mesh. Divide both the control and stained samples into 3 parts each for (i) flow cytometric analysis of paclitaxel accumulation (about 1 ml), (ii) multiparameter staining for paclitaxel accumulation and viability (about 1 ml), and (iii) fluorescence-activated cell sorting (FACS) and recovery (about 3 ml). The number of cells to be sorted can be altered based on the intended application.

_Critical Step:_ Care should be taken to minimize loss of cell number and viability during washing and filtering steps.

5.2.3 **Flow Cytometric Analysis of Paclitaxel Accumulation**

_Timing:_ 15-20 min

14) Transfer 1 ml of both control and stained samples to 5 ml polystyrene round-bottom tubes for flow cytometric analysis of paclitaxel accumulation.

15) Perform the set up for operation of the BD LSR II flow cytometer by following the user’s guide for recommended instructions. Perform a quality control (QC) before
analyzing the samples. Calibrate the flow cytometer. Select the settings for the scatter properties and the fluorophore of interest, phycoerythrin (PE).

16) Run the control sample through the cytometer first. Acquire data for forward and side scatter. Adjust the photo multiplier tube (PMT) voltages so that the data are included within the dot plot region, and do not fall out of range. Since the stained cells will have a higher degree of PE-fluorescence than the control cells, adjust the PE voltage accordingly to bring the control histogram at the lower end of the fluorescence axis. Do not change these voltage settings for subsequent analysis.

17) Collect data for at least 10,000 cells from the control sample. Draw a polygon gate to de-select the debris and doublets from the analysis. Record the PE histogram data next. Draw an interval gate in the top 1% fluorescent region in the histogram, using the relative cut-off method for determination of percent positive populations (Shapiro, 2003).

18) Subsequently run the stained sample and record the data for identical number (10,000) of cells. Apply the same histogram interval gate from the control sample, to estimate the paclitaxel-positive populations in the stained sample. Use the BD FACSDiva and FlowJo software packages to analyze the recorded data. Other histogram subtraction methods can be employed to further validate the results obtained from this analysis.

5.2.4 Multiparameter Staining for Paclitaxel and Viability

*Timing:* 15 min

19) Transfer 1 ml of both control and stained samples to sterile 5 ml flasks for subsequent viability staining via FDA.
20) From the FDA working solution, add FDA at a ratio of 20 µl per 1 ml cell suspension to the stained (for paclitaxel) sample only. Incubate in dark for 8-10 min.

21) Transfer to 5 ml polystyrene round-bottom tubes for multiparameter flow cytometric analysis.

22) Run the control sample in the BD LSR II and acquire data, as described previously. On a biparametric scatter dot plot of PE vs. FITC fluorescence, draw an appropriate quadrant gate to delineate top 1 % fluorescent regions in both the channels along both axes, respectively.

23) Run the costained (paclitaxel and viability) sample and record the data for identical number of cells. Apply the same quadrant gate in the biparametric dot plot to correlate paclitaxel accumulation and viability, using the 1 % relative cut-off method. Analyze the data using the recommended software.

5.2.5 Fluorescence-activated Cell Sorting (FACS) and Recovery

*Timing*: 1-1.5 h

24) Transfer the remaining control and stained samples (3 ml each) to sterile 5 ml polystyrene round-bottom tubes for cell sorting and recovery. Use a BD FACSVantage cell sorter, with a 200 µm flow tip and configured with a MacroSort option. Alternatively, for a faster sort operation, perform the sort with a BD FACSARia. In either case, perform the instrument set up according to the user’s manual. (see troubleshooting Table 5-2). Users may compare and contrast the two sorters to evaluate their performances based on the specific needs – sort rate, time, sort purities, etc. For our system, the BD FACSARia was found to consume about 24-fold less time than
BDFACSVantage, and is therefore recommended. The following paragraph shows a comparison between the FACSVantage and FACSaria, with some of the readings as observed on our system:

FACSVantage -

- Better and manual control but less automated
- Adaptability to using larger (200 µm) nozzles => slower sorting conditions
- Cell analysis rates: (300-1000) s⁻¹
- Cell sorting rates: (30-50) s⁻¹
- Estimated time to isolate 1 million cells: ~7 hours [= 1000000 / (40 x 60)]

FACSaria -

- More automated settings
- Largest nozzle available: 100 µm
- Cell analysis rates: (3000-4000) s⁻¹
- Cell sorting rates: (800-1200) s⁻¹
- Estimated time to isolate 1 million cells: ~17 minutes [= 1000000 / (1000 x 60)]

Minimum number of cells required for mRNA analysis is (3-4) million per subpopulation set, while those necessary for a 3 ml culture initiation for scale-up is 1.5 million per subpopulation set.

25) In order to ensure that all the lines are clean and decontaminated, sterilize the sorter with the sterilization mixture according to the manufacturer recommended guidelines. Use prefiltered and sterile sheath fluid. Clean all external surfaces with 70 % ethanol.

26) Use the Alignflow QC beads (AlignFlow™ flow cytometry alignment beads, 2.5 µm for 488 nm excitation; and AlignFlow™ flow cytometry alignment beads, 2.5 µm for 633
nm excitation (Molecular Probes, Inc., Eugene, OR)) to align the lasers and calibrate the sorter. Additionally, sort the large sized Megabead NIST particles (Megabead NIST Traceable Particle Size Standard, 25.0 µm (Polysciences, Inc., Warrington, PA)) and attain optimum instrument settings (FSC and SSC voltages, FSC threshold, fluorescent voltages, etc) corresponding to high sort performance and purity.

27) At these settings, achieve a stable hydrodynamic flow condition in the sorter. Use the camera view to monitor the streams, and check for any unwanted deflections or loss of stream. (see troubleshooting Table 5-2).

28) Run the control sample in the cell sorter first and record the data. Adjust the PMT voltage to be able to later accommodate the stained histogram within the scale.

29) Subsequently, run the stained sample through the sorter. Acquire and record data for identical number of cells.

30) Select subpopulations to be sorted by defining regions as high- and low-paclitaxel accumulating cells in the histogram using appropriate interval gating.

31) Aseptically, sort the two subpopulations and collect them simultaneously via a two-way sort into two 5 ml polypropylene round-bottom tubes, each containing 200 µl of the suitable storage media. Regulate the sheath pressure, flow rate and sample volume differential, etc. of the FACSVantage to maintain a sorting rate of (1000-2000) events/s.

**Critical Step:** Carefully increase sheath pressure and other variables to maximize the sorting rate so that the stream is not disrupted. Avoid high pressures which may be detrimental on cell health and integrity

**Critical Step:** Polypropylene tubes are recommended for collecting sorted cells. The cells are charged as they pass through the electric field in the sorter, which may lead to poor
collection and recovery owing to the high electrostatic charge build-up on the polystyrene tube walls.

32) Sort 10,000 cells per tube and analyze the sort purity by analyzing each sorted subpopulation through the sorter again and maintaining an analysis rate of (3000-5000) events/s. Acquire the histogram data and apply the respective pre-sorting gates to determine the sort purities, as the percentage of sorted events lying in a particular gate.

33) Following a successful run with high sort purity collect about (3-4) x 10⁶ cells per subpopulation.

*Critical Step:* Determine and sort the required number of cells for respective applications.

34) Initiate new cell cultures from the isolated cell populations by removing the sheath fluid and transferring to suitable growth media. Monitor their growth, metabolite accumulation, etc., over time for subsequent applications. Additionally, RNA can be isolated from the sorted cells for gene expression analysis. To achieve this, sort the cells in tubes over dry ice or in commercially available RNA stabilizing buffers.

5.3 Results and Discussion

5.3.1 Flow Cytometric Histograms from the Immunoassay

In this complete protocol, flow cytometry was extensively used to both validate the staining assay and for cell sorting. Flow cytometric scatter dot plots and fluorescence histograms (Figure 5-1) were collected to demonstrate subpopulation variability in paclitaxel accumulation in *Taxus* cells. The similarity in scatter dot plot profiles (FSC and SSC) between the control (Figure 5-1a) and stained (Figure 5-1b) samples indicates that the staining procedure does not affect the overall population distribution with respect to size, morphology and structural complexity of the cells. The polygon gates, used to
exclude debris and doublets, comprise similar percentage of cells in both the control and stained samples (83.79 and 84.21, respectively). This is a quantitative justification that identical sets of cell populations (control and stained) in terms of scatter properties are obtained. PE-fluorescence histograms were used to compare the relative amounts of paclitaxel accumulation between the control and stained samples (Figure 5-1c, 5-1d). Statistical data (e.g., mean PE fluorescence) obtained directly from the LSR II can be used to analyze paclitaxel accumulation in samples under different conditions to estimate the percentage of a positive population in an individual sample.

### 5.3.2 Histogram Population Comparison

The commonly employed 1% relative cut-off method was used to analyze the histogram data (see Chapter 4 for more complete description of this method). The top 1% fluorescent population of the control histogram was gated to differentiate positively stained populations from the control sample (Figure 5-1c). The percentage of cells in the stained sample lying within this gate was defined as the positively stained population (84.84% in Figure 5-1d) (Shapiro, 2003).

A second method of population comparison applied to these data was absolute and involved subtraction of cell counts in every channel of the control histogram from the corresponding channels of the stained sample histogram (Overton, 1988). This method to determine the positive population yields a difference histogram, and is particularly advantageous in cases where the histograms to be compared have overlapping regions. Statistically, this method is more robust as it provides a computationally adaptive
platform to employ different algorithms (Overton cumulative histogram subtraction; and Super-enhanced $D_{\text{max}}$ Subtraction) (Overton, 1988) for percent positive determination. At the same time, several statistical tests (Kolmogorov-Smirnov (K-S)) (Lampariello, 2000); Probability binning (Roederer et al., 2001; 2001a); and Cox chi-square (Cox et al., 1988)) can be easily performed with this method to determine the confidence interval within which the two compared populations are statistically different. This method of channel-by-channel subtraction was applied on the same set of histograms using FlowJo Mac v.8 (Figure 5-1e). A third plot (green) was superimposed as a difference histogram between the stained and the control samples. This plot was above the midline for all the channels where the control has higher cell counts than the stained sample and vice versa. The Overton cumulative histogram subtraction algorithm was run on this data set, which showed a presence of 86.8 % positively stained cells in the test sample. The super-enhanced $D_{\text{max}}$ subtraction algorithm gave similar results; furthermore, it was found that the maximum positive difference in the integrated histogram values between the stained sample and the control occurs at a PE-fluorescence intensity value of 3677 (Figure 5-1e). Statistical testing of these comparison algorithms were performed by K-S test and Cox chi-squared test using FlowJo. K-S test shows that the confidence in difference between both populations was greater than 99.9 %, thus justifying the fact that the two populations were not drawn from a common distribution. Chi-squared testing resulted in a $T(X)$ value of 878 and a maximum $T$ value of 1097. For two distributions to be statistically different with $p<0.01$ (99 % confidence), it is required that the value $T(X)>4$. Thus, these results suggested that only a certain population of *Taxus* cells in the culture accumulate
paclitaxel, which can be determined within statistical confidence levels using the methods discussed here.

### 5.3.3 Multiparameter Staining of Paclitaxel and Viability

The efficacy of the immunostaining assay in maintaining cell viability after identifying paclitaxel accumulation was critical with respect to subsequent applications. This was demonstrated by staining cells with a viability stain (FDA), and analyzing in a flow cytometer (Figure 5-2a, 5-2b). The control sample, used to distinguish viability-related fluorescence, was not stained with FDA. The FDA-stained histogram (Figure 5-2b) exhibited a distinct shift from the control, which upon applying the 1 % relative cut-off method for determination of percent positive population yielded a value of 99.8 %. This is the percentage of cells that remain viable after the immunoassay, and thus validates the live cell-based assay. Bi-parametric flow cytometric scatter dot plots of both FITC and PE-associated fluorescence were simultaneously captured along the horizontal and vertical axis respectively (Figure 5-2c, 5-2d). The control plot (Figure 5-2c) is gated to define the four regions lying within the following quadrants: Q1 (low FITC, high PE); Q2 (high FITC, high PE); Q3 (high FITC, low PE); and Q4 (low FITC, low PE). The gates were placed according to the 1 % relative cut-off method, such that (Q1 + Q2) = (Q2 + Q3) = 1 % in the control plot. A majority of the cells (c.a. 99 %) lied in Q4, thus validating the gating for an appropriate control for co-stained cells. The sample plot was obtained and the same gates were applied (Figure 5-2d). Approximately, 82 % of cells lied in Q2, suggesting that as many viable cells accumulated paclitaxel. On the other hand, Q3 consisted of about 18 % of cells, which implies that these cells were viable but
did not accumulate detectable amounts of paclitaxel. Additionally, 0.16 % of the cells lied in Q4, representing the less viable and less paclitaxel accumulating subpopulation. The vital staining procedure mandates that the cells expressing paclitaxel are kept alive and cellular damage is minimized in the process, which was validated through this analysis.

5.3.4 Cell Sorting based on Size and Paclitaxel Accumulation

First, the feasibility to sort intact *Taxus* single cells based on size and complexity was tested. Numerous experiments were performed to empirically optimize sorting of single cells. We first evaluated sorting of protoplasts, which was successful (data not shown). We also evaluated effect of changing operating parameters (i.e., flowrate and sheath pressure) and sample processing parameters (i.e., addition of Xantham gum for dispersion and cell density) (data not shown). The data presented in this thesis represent the final reproducible conditions for sorting of *Taxus* single cells.

The sorter was calibrated with larger beads that are better representative of plant cells (see section 5.2.). Cells were sorted on the basis of scatter properties. Figure 5-3a shows the presort dot plot, with a gated area (named P1) for high FSC and SSC, encompassing 36 % of the entire cell population. The cells within this gate were sorted and analyzed for purity by running through the sorter again. Figure 5-3b denotes the post-sort scatter dot plot of the sorted population. The sort purity was found to be 84 %, which is the percentage of cells in the dot plot of Figure 5-3b that fall within P1. Following a successful sort by size, cells were subsequently sorted based on paclitaxel accumulation.
Representative histograms in Figure 5-4 indicate that distinct subpopulations of Taxus cells, designated as Low- and High-paclitaxel accumulators, were gated within low 44 % and high 40 % fluorescent regions in the histogram of the stained sample, respectively. This gating strategy served as a criterion for cytoselection and subsequent sorting. The populations were sorted and collected separately under sterile conditions. To analyze the sort purity, an indicator of sorting accuracy, the sorted populations were evaluated for key parameters by reanalyzing under the same conditions of gating, voltage, etc. For example, both Low and High subpopulations were expected to display similar values of mean PE-fluorescence before and after the sorting. Additionally, both sorted subpopulations were expected to contain significantly higher (more than 85 %) percentage of cells in respective gates. Figure 5-4a, 5-4b showed that approximately 97 % and 87 % of the Low- and High-accumulators fell within their respective gates after sorting. The results presented here demonstrate a proof-of-concept of this methodology, as exhibited by the efficient sorting of intact plant cells according to secondary metabolite accumulation, which ultimately serves as an enabling technology for subsequent applications described above.

The feasibility of sorting based on paclitaxel accumulation was verified on two different instruments: BD FACVantage (Figure 5-5) and BD FACSaria (Figure 5-6). Section 5.2.5 lists the basic conditions and instrument settings for the two sorters. The minimum time required to sort a certain amount of cells for both culture and RNA extraction was also calculated. Based on this information, it is recommended that BD
FACSAria be used to sort *Taxus* cells. It has been proposed in the literature that the sorter nozzle should be at least 3-4 times to that of the cell size (Dolezel et al., 2007; Harkins and Galbraith, 1987). Successful use of the 100 µm nozzle to sort *Taxus* cells was surprising but adds flexibility to sorting and analysis options.

### 5.3.5 Size Distribution Profiles for Sorted Subpopulations

Figure 5-7 shows condensed FSC histograms of presort (or unsorted), sorted high accumulators and sorted low accumulators of *T. cuspidata* P991 cells stained for paclitaxel and subsequently sorted in the FACSAria using a 100 µm nozzle. In this analysis, FSC is used as an indicator of cell size (Yanpaisan et al., 1999; Shapiro, 2003). The mean FSC values of the three subpopulations were recorded. It was found that the size distribution profiles of sorted high accumulators closely resembled that of the unsorted single cells. On the other hand, the sorted high accumulators showed a 1.32-fold higher FSC mean value over the sorted low accumulators. Additionally, the sorted low accumulators exhibited a size distribution profile with a mean value equal to 0.7 times that of the unsorted cells, implying that the sorted low accumulators were smaller in size. While the low accumulating subpopulation predominantly consisted of smaller cells, the high accumulators included both small and large sized cells. This observation is interesting and calls for further analysis to study the interdependence between cell size and paclitaxel accumulation in distinct *Taxus* subpopulations. Further study should be aimed at investigating whether the cells are high accumulators due to an increase in cell wall size (resulting in an overall increase in cell size), since it is known that a majority of paclitaxel accumulates in the cell wall (Roberts et al., 2003).
If this relationship between cell size and paclitaxel accumulation is confirmed, size may be able to be used as a sort criteria to isolate high accumulators. This strategy may be used to bypass the vital staining step, which is time consuming and can alter cellular metabolism. For example, the histogram of the unsorted cells can be obtained. Subsequently, all cells with FSC values less than a critical size may be sorted as low accumulators, while cells with higher FSC values may be sorted as high accumulators.

5.4 Conclusions

Investigating plant cell culture heterogeneity at the subpopulation level through enabling flow cytometric technologies is a new strategy for development of bioprocesses to enhance metabolite accumulation. A rapid and high-throughput method to isolate, stain and sort distinct paclitaxel accumulating cell subpopulations using FACS technology was developed, thus enabling the subsequent recovery of cells and propagation for improved culture productivity. This methodology represents a new approach to optimization of plant cell culture processes and it is anticipated that these techniques will facilitate propagation of potentially superior cell lines.
Table 5-1: Examples of some important flow cytometric measurable parameters and measurement techniques and/or fluorescent probes.

<table>
<thead>
<tr>
<th>Measurable variables</th>
<th>Measurement techniques and/or associated probe(s)</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell count and size</td>
<td>Scatter dot plots</td>
<td><em>Nicotiana tabacum</em></td>
</tr>
<tr>
<td>DNA, ploidy, cell cycle</td>
<td>DAPI, mithramycin, propidium iodide</td>
<td><em>Dioscorea</em></td>
</tr>
<tr>
<td>Chlorophyll content</td>
<td>Autofluorescence</td>
<td><em>Nicotiana tabacum</em></td>
</tr>
<tr>
<td>Cell viability</td>
<td>PI and fluorescein diacetate (FDA)</td>
<td><em>Brassica napus</em></td>
</tr>
<tr>
<td>Cell-wall components</td>
<td>Specific antibodies, Calcofluor white</td>
<td><em>Brassica napus</em></td>
</tr>
<tr>
<td>Apoptosis and related studies</td>
<td>Annexin-V</td>
<td><em>Nicotiana plumbaginifolia</em></td>
</tr>
<tr>
<td>Membrane fluidity</td>
<td>Diphenylhexatriene (DPH)</td>
<td><em>Lupinus albus L.</em></td>
</tr>
<tr>
<td>Membrane potential</td>
<td>Rhodamine 123</td>
<td><em>Solanum tuberosum L.</em></td>
</tr>
<tr>
<td>Intracellular Ca(^{2+})</td>
<td>Indo-1, fura-2</td>
<td><em>Barley aleurone</em></td>
</tr>
<tr>
<td>Gene expression</td>
<td>GFP or other reporter genes</td>
<td><em>Arabidopsis</em></td>
</tr>
<tr>
<td>Intracellular molecules</td>
<td>Specific dyes, antibodies tagged with fluorescence after cell fixation and permeabilization</td>
<td><em>Coptis japonica</em></td>
</tr>
</tbody>
</table>
Table 5-2: Troubleshooting information.

<table>
<thead>
<tr>
<th>Problem</th>
<th>Possible reason</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poor single cell yield</td>
<td>Aggregated cultures too old</td>
<td>Use a healthy and actively-growing culture</td>
</tr>
<tr>
<td></td>
<td>Inefficient enzymatic action</td>
<td>Optimize enzyme concentrations and digestion time</td>
</tr>
<tr>
<td></td>
<td>Degradation of enzymes</td>
<td>Avoid heating or vortexing the enzyme solution</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cells do not remain viable</td>
<td>Harsh and careless handling of single cells</td>
<td>Perform the steps carefully and aseptically</td>
</tr>
<tr>
<td></td>
<td>Poor condition of aggregated cultures</td>
<td>Do not use senescent and old initial aggregated cultures</td>
</tr>
<tr>
<td></td>
<td>Membrane permeabilization during staining</td>
<td>Avoid fixatives, detergent and harsh steps during staining</td>
</tr>
<tr>
<td></td>
<td>Breakage and loss of cellular integrity while sorting</td>
<td>Adapt a suitable sized nozzle and amenable sorting conditions</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weak fluorescent signal</td>
<td>Secondary metabolite not expressed</td>
<td>Elicit the cultures to induce production</td>
</tr>
<tr>
<td></td>
<td>Fluorescence bleaching</td>
<td>Avoid exposure to light; wrap the container</td>
</tr>
<tr>
<td></td>
<td>Antibodies concentrations are too dilute</td>
<td>Use optimized antibody concentrations</td>
</tr>
<tr>
<td></td>
<td>Suboptimal staining</td>
<td>Optimize the wash steps during staining</td>
</tr>
<tr>
<td></td>
<td>Incorrect filter and voltage settings in the cytometer</td>
<td>Make sure the correct filter is installed for the fluorochrome of interest; adjust the voltage setting to bring the data within the window of analysis</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clogging of flow cytometer</td>
<td>Presence of aggregates or clumps</td>
<td>Filter the cells through an 80 µm mesh to remove clumps</td>
</tr>
<tr>
<td>Problem</td>
<td>Possible reason</td>
<td>Solution</td>
</tr>
<tr>
<td>--------------------------------------------------</td>
<td>--------------------------------------------------------------------------------</td>
<td>---------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Flow is not stable</td>
<td>Obscured observation point; irregular and out of phase side streams</td>
<td>Monitor the stream and check for unwanted deflections and instabilities. Adjust the sheath pressure, vibration frequency and other flow conditions to control the side streams. Adjust the laser alignment, if needed.</td>
</tr>
<tr>
<td>Fewer cells sorted than expected</td>
<td>Debris causes too many sorting events</td>
<td>Choose a higher forward scatter threshold to eliminate debris</td>
</tr>
<tr>
<td>Unexpected and erratic sort rate</td>
<td>Event rate too high</td>
<td>Decrease the event rate by sample differential control</td>
</tr>
<tr>
<td></td>
<td>Laser and instrument noise</td>
<td>Decrease the drop drive amplitude</td>
</tr>
</tbody>
</table>
Figure 5-1: Flow cytometric scatter dot plots of (a) control (secondary antibody only) and (b) stained samples of *T. cuspidata* P991 cells stained for paclitaxel via the immunoassay procedure. The plot was gated to exclude debris and aggregates, and PE-fluorescence histograms were subsequently obtained for both (c) control and (d) stained samples. The 1% relative cut-off method was applied on the histograms to determine the percentage of paclitaxel accumulating cells. Additionally, an absolute channel by channel histogram subtraction method was also applied on the (e) control and stained samples, to obtain a difference histogram (shown in green).
Figure 5-2: Flow cytometric histograms of (a) control (unstained) and (b) stained sample of *T. cuspidata* P991 cells stained for viability using FDA, after the paclitaxel immunostaining procedure. 10,000 cells were analyzed at (300-1000) events/s using the 488 nm laser in the BD LSR II. The 1 % relative cut-off method was applied on the histograms to determine the percentage of viable cells. Subsequently, biparametric PE vs. FITC flow cytometric scatter dot plots were acquired on the cells co-stained for paclitaxel and viability. The (c) control was gated according to the 1 % relative cut-off method along both axes. The same gate was applied to the (d) stained sample to correlate paclitaxel accumulation and viability amongst the cells.
Figure 5-3: Flow cytometric scatter dot plots for *T. cuspidata* P991 cells. Side scatter (SSC) and forward scatter (FSC) indicate complexity and size respectively. (a) Unsorted cells (36% of cells fall within region P1). (b) Sorted cells (84% of cells fall within region P1, indicating the sort purity).

Note: This operation was performed using a 200 µm sized nozzle at (1000-2000) events/s per tube in the BD FACS Vantage cell sorter.
Figure 5-4: Flow cytometric histograms of *T. cuspidata* P991 cells stained for paclitaxel using the immunostaining procedure. (a) Cells before sorting, with designated sorting regions as ‘Low’ (low-paclitaxel accumulators) and ‘High’ (high-paclitaxel accumulators) consisting of 44.35 % and 40.32 % of the entire cell population, respectively. These subpopulations were sorted using a 200 µm sized nozzle at (1000-2000) events/s per tube in the BD FACSVantage cell sorter. Re-analysis of the sorted subpopulations showed that the (b) sorted ‘Low’ subpopulation consisted of 96.50 % of the original events; and (c) sorted “High” subpopulation consisted of 87.28 % of the original events, indicating high sort purities.
Figure 5-5: Flow cytometric histograms for T. cuspidata P991 cells stained for paclitaxel using the vital staining procedure. Higher PE fluorescence values indicate higher paclitaxel content. These subpopulations were sorted using a 200 µm sized nozzle in the BD FACSVantage cell sorter. (a) Unsorted cells, with designated sorting regions P1 and P2. (b) Sorted cells: Low paclitaxel accumulators (91 % of the events fall within P1). (c) Sorted cells: High paclitaxel accumulators (88 % of the events fall within P2).
Figure 5-6: Flow cytometric PE histograms for *T. cuspidata* P991 cells stained for paclitaxel using the vital staining procedure. Higher PE fluorescence values indicate higher paclitaxel content. These subpopulations were sorted using a 100 µm sized nozzle in the BD FACSARia cell sorter. (a) Unsorted cells, with designated sorting regions Low and High. (b) Sorted cells: Low paclitaxel accumulators (97.9 % of the events fall within Low). (c) Sorted cells: High paclitaxel accumulators (88.3 % of the events fall within High).
Figure 5-7: Flow cytometric FSC histograms of sorted *T. cuspidata* P991 subpopulations.

Note: Single cells were stained for paclitaxel using the vital staining procedure. The two subpopulations were sorted using a 100 µm sized nozzle in the BD FACSArria cell sorter. The presort histogram corresponds to the unsorted population. The arrow represents the FSC value/size below which all the cells are low accumulators.
6.1 Introduction

It has been shown in the previous chapters that *Taxus* cell aggregates can be enzymatically digested to yield intact single cells in high yields (Chapter 3). These single cells can be successfully stained for paclitaxel using a live cell-based assay (Chapter 4). A method to sort and isolate distinct subpopulations has been established (Chapter 5). This new sorting technology can be used to both establish superior and stable cell lines and to isolate distinct populations for further metabolic analyses. In order to establish new and potentially improved cell lines, there needs to be a reliable method in place for the reculture of single cell populations. A method to culture freshly prepared *Taxus* single cells was developed previously in the Roberts Laboratory (Naill and Roberts, 2005d). This method served as the basis for the establishment of reculture conditions for sorted single cells described herein.

Culture and regeneration of plant systems have been primarily performed using protoplast platforms. Protoplasts have been used because of their ease of processing with techniques such as cell fusion, transformation and hybridization (Ma et al., 2003; Hou and Jia 2004). After isolation and manipulation, protoplasts are cultured to fuse together, regenerate cell walls and finally grow into plants (Blackhall et al., 1994). Common types of protoplast cultures employ semi-solid medium such as alginate, agar, etc., to facilitate growth with minimum breakage and cell density losses, since protoplasts are relatively
more shear sensitive in nature due to the absence of cell walls (Damm et al., 1989). Culturing in semi-solid medium also allows the use of lower seeding densities when compared to a liquid suspension culture. These protoplast culture systems, however, lack the vital metabolic information stored in the cell wall, and may not be suitable for our studies since paclitaxel is primarily stored in the cell wall. Moreover, protoplasts in culture often form aggregates quickly, which results in a rapid reversion to the original heterogeneous aggregated state (Blackhall et al., 1994). Although there have been few reports of culturing single cell plant suspensions (Street et al., 1965; Kurz, 1971), most of these methods are system-specific and do not necessarily translate to other systems (Kubek and Shuler, 1978). For the Taxus system, protoplast cultures have not been evaluated, however a method to isolate protoplasts upon enzymatic digestion has been established (Roberts et al., 2003). A method to culture single cells was developed in our laboratory (Naill and Roberts, 2005d).

In this work, stained and sorted intact single cells of Taxus were cultured and analyzed over time. Sorted cells were propagated in the appropriate medium under suitable conditions (see section 6.2). Cultures can be scaled up over time and analyzed for growth and paclitaxel accumulation to determine the efficacy of sorting for establishing higher, more stable paclitaxel accumulating cultures. In this chapter, preliminary data are presented with regards to reculture and analysis that can be used as a basis for future comprehensive studies.

6.2 Materials and Methods
Both sorted and unsorted single cells of *T. cuspidata* P991 were propagated in conditioned medium (obtained from day 7-10 of the P991 aggregated cultures and filter sterilized using 0.22 µm Nalgene cellulose nitrate membranes), supplemented with 20 g/l sucrose and 0.2 % (v/v) Plant Preservative Mixture (PPM). The cultures were kept under normal incubation conditions (see section 2.2). The cell seeding density was kept at 0.5 million cells/ml for each population. Medium was supplemented with plant antioxidants at final concentrations of 6 mM glutamine, 150 g/l ascorbic acid and 150 g/l citric acid. Medium was exchanged completely at each subsequent subculturing after centrifuging the cultures in sterile centrifuge tubes (15 or 50 ml) at 600 x g for 3 minutes and removing the supernatant. Cell growth was monitored by measuring the cell density using a hemacytometer over time. Upon doubling of cell density, cultures were scaled-up to twice the volume. It is recommended that any possible evaporation in the single cell cultures is also accounted for while measuring their cell densities. In this phase, the culture volumes were scaled from 3 ml (6-well plates) to 6 ml (6-well plates), 12 ml (25 ml flasks), 24 ml (50 ml flasks) and 40 ml (125 ml flasks) after 2.5 months.

During each scale-up, cultures were also analyzed for paclitaxel accumulation via the vital staining procedure (Chapter 4) in conjunction with flow cytometric analysis. Cells were stained using a primary anti-paclitaxel antibody and PE-conjugated secondary antibody, following which they were analyzed for paclitaxel-related fluorescence using the BD LSR II flow cytometer. All data were tabulated using BD FACSDiva software. The reported values represent relative paclitaxel fluorescence of sorted cultures, normalized with that of the fluorescence obtained from the unsorted culture. The
difference between mean PE fluorescence values of the stained sample and the control sample (secondary antibody only) was used to obtain the relative fluorescence for each culture. Furthermore, the 1 % relative cut-off method (as described in Chapter 4) was used on the PE histograms (stained and control) to determine the percentage of paclitaxel-positive cells.

Additionally, cluster size distribution was evaluated by counting 100 cell clusters using a hemacytometer. For further details on hemacytometry see section 2.7. However, viability of the single cells was not measured at various time points during the reculture process, which is recommended in order to accurately characterize the culture state.

6.3 Results and Discussion

Post-sorting, the entire reculture experiment can be divided into the following four phases (a, b, c, and d), as described below.

6.3.1 Phase (a)

This phase spans from initiation of sorted cultures to the final scale-up. At month/time 0, T. cuspidata P991 cells were sorted, and three cultures (3 ml volume per culture) of (i) unsorted (and unstained), (ii) (stained and) sorted low, and (iii) (stained and) sorted high accumulating cells were started. Additionally, a fourth culture was maintained (unsorted and stained). This culture performed similarly to the unsorted and unstained culture (except where noted below) and therefore the data are not presented
here. It was observed that the growth rate gradually decreased through the scale-up procedure (Figure 6-1).

The percentage of clusters containing single cells remained in the range of (95-100) % in this phase (Figure 6-2). The sorted cultures exhibited specific accumulation patterns upon propagation. The sorted high accumulators showed 3-4-fold higher relative fluorescence level when compared to unsorted cultures, while the sorted low accumulators were consistently lower at approximately 0.5-fold relative fluorescence level (Figure 6-3). Results showed that the high accumulators remain high and the low accumulators remain low during the reculture period (Figure 6-4). Coefficients of variation (CV) as a measure of peak distribution were also calculated for all cultures. Consistently the sorted cultures (either high or low accumulators) exhibited lower CV values, which is indicative of decreased cell-cell variability as compared to the unsorted culture. During this phase we also compared unsorted and unstained cells to unsorted and stained cells to determine if staining for paclitaxel had an effect on either growth or paclitaxel accumulation. There was no significant effect on growth throughout this scale-up period (data not shown) between the unstained and stained samples. The unsorted stained cells exhibited an increase in paclitaxel content of 1.4-fold over the unsorted unstained cells throughout this scale-up period, potentially suggesting stability of the bound primary antibody used to detect paclitaxel.

6.3.2 Phase (b)
This phase extends from 2.5 months to 3.1 months. At 3.1 months, cells from one 40 ml culture were scaled up to 80 ml and divided into two separate cultures containing a volume of 40 ml each. During this phase, there was more than 3-fold increase in cell growth rate (Figure 6-1), which may be attributed to the importance of culture conditions and size (i.e., cultures were maintained at 40 ml volume, which is the typical culture maintenance volume for the aggregated cultures). The percentage of clusters containing a single cell remained high in the range of (92-97) % (Figure 6-2). Paclitaxel accumulation results and cell-cell variability in accumulation were consistent with phase (a) (Figures 6-3, 6-4 and 6-5).

6.3.3 Phase (c)

This phase spans from 3.1 months to 4.5 months, and marks the onset of aggregation in the previous predominantly single cell cultures. The growth rate, which was evaluated using fresh weight (g/ml) during this phase (measured by the weight of cell cake obtained upon centrifuging 1 ml culture in 0.22 µm nylon polypropylene centrifuge filter tubes at 800 x g for 5 mins), did not show any significant increase (Figure 6-1), prompting media modification (see Phase (d) description below). However, the percentage of clusters containing single cells dropped to as low as 85 % (Figure 6-2). Paclitaxel accumulation results and cell-cell variability in accumulation were consistent with phases (a) and (b) (Figures 6-3, 6-4 and 6-5).

6.3.4 Phase (d)
The final phase in the reculture extends from 4.5 months until 8 months. 10 %
(v/v) concentrated fresh medium (10X) was added to the cultures at the beginning of this
phase, which immediately resulted in an increase in the growth rate (Figure 6-1), which
was accompanied by an increase in cell aggregation, as the percentage of clusters
containing single cells dropped to as low as 48 % (Figure 6-2). In this phase, paclitaxel
content could not be routinely measured as before, owing to the increased aggregation
levels. Having a single cell suspension is a necessary requirement for flow cytometric
analysis. At 6.4 months, paclitaxel content was measured via flow cytometry after
filtering the cells through 80 μm nylon mesh, collecting the flow through which contains
single cells, and staining for paclitaxel content (Figure 6-3). Note that at this time point,
the flow cytometric data corresponds to (50-61) % of the culture population, which is the
percentage of clusters that contain single cells, and may not accurately represent the
overall paclitaxel content of the cultures.

6.4 Conclusions

Single cell cultures were propagated from sorted subpopulations of Taxus. Cultures were scaled up in volume over time, and their growth, aggregation and accumulation profiles analyzed. Earlier, cells were stained for paclitaxel accumulation and sorted into high- and low- accumulating subpopulations. Single cell cultures were initiated for unsorted, high- and low-accumulating cells. Approximately similar growth rate profiles were observed for all the three subpopulations in all the phases. There was a significant increase in aggregation of the cultures when concentrated fresh medium was introduced. Cultures also exhibited specific paclitaxel accumulation patterns, which may
be the first step towards creation of superior cells for use in bioprocesses. Furthermore, lower (50-75 %) CV values of paclitaxel accumulation for sorted populations were obtained, suggesting a decrease in accumulation variability. The percentage of paclitaxel-positive cells was high in the sorted high accumulators over time. At the same time, we noticed high values in the overall paclitaxel content in the high accumulators as well. The presence of large number of positive cells may be the primary reason for high paclitaxel content in the high-accumulating cultures, as opposed to a uniform increase in the paclitaxel content of all cells. The methodologies presented in this chapter will facilitate the process towards obtaining productive and stable cell lines from aggregated plant cell cultures.
Figure 6-1: Fractional cell growth rate of *T. cuspidata* P991 cultures (unsorted/unstained, sorted/stained low paclitaxel accumulators and sorted/stained high paclitaxel accumulators) over a period of 8 months.

Note: Fractional cell growth, over a period of ‘t’ days was calculated using the following formula: \((x_{\text{final}} - x_{\text{initial}})/(x_{\text{initial}} \times t)\); where \(x\) is the measured value of cell density or cell weight (here cell density). Note the four phases (a, b, c and d) in the plot are distinguished by the dotted lines. For example, fractional cell growth at the second data point for unsorted cultures can be calculated as: \(\frac{(1.21 \times 10^6 \text{ cells/ml} - 0.59 \times 10^6 \text{ cells/ml})}{(0.59 \times 10^6 \text{ cells/ml} \times 19)} = 0.055 \text{ day}^{-1}\)
Figure 6-2: Percentage of clusters containing single cells in *T. cuspidata* P991 cultures (unsorted/unstained, sorted/stained low paclitaxel accumulators and sorted/stained high paclitaxel accumulators) over a period of 8 months.

Note: Cultures were analyzed using hemacytometry and the percentage of clusters containing single cells were determined by counting 100 clusters. Note the four phases – a, b, c and d. Also note that the cultures started to aggregate during phase (c) and this phenomenon continued further in phase (d). Note the four phases (a, b, c and d) in the plot are distinguished by the dotted lines.
Figure 6-3: Relative paclitaxel content of *T. cuspidata* P991 cultures (unsorted/unstained, sorted/stained low paclitaxel accumulators and sorted/stained high paclitaxel accumulators) over a period of 5 months.

Note: Single cell cultures were stained for paclitaxel via the vital immunostaining procedure and analyzed using the BD LSR II flow cytometer. Mean paclitaxel-associated phycoerythrin (PE) fluorescence values were recorded as a difference between stained and control (secondary antibody only) samples, which were normalized with that of the unsorted cultures. Note the specific accumulation patterns amongst distinct populations. Note the four phases (a, b, c and d) in the plot are distinguished by the dotted lines.
Figure 6-4: Percentage of paclitaxel-positive cells in *T. cuspidata* P991 cultures (sorted low paclitaxel accumulators and sorted high paclitaxel accumulators) over a period of 5 months.

Note: Single cell cultures were stained for paclitaxel via the vital immunostaining procedure and analyzed using the BD LSR II flow cytometer. The relative 1% cut-off method (described in the text) was used to determine the percentage of paclitaxel positive cells. Note the four phases (a, b, c and d) in the plot are distinguished by the dotted lines.
Figure 6-5: Paclitaxel accumulation variability in *T. cuspidata* P991 cultures (unsorted/unstained, sorted/stained low paclitaxel-accumulators and sorted/stained high paclitaxel-accumulators) over a period of 5 months.

Note: Single cell cultures were stained for paclitaxel via the vital immunostaining procedure and analyzed using the BD LSR II flow cytometer. Coefficients of variation (\% CV) of the phycoerythrin (PE) histograms were used to analyze the accumulation variability across the entire population.
CHAPTER 7
NUCLEAR DNA CONTENT AND GENOME SIZE EVALUATION IN *TAXUS* CELLS

7.1 Introduction

In a cell, the DNA content can be estimated through isolation and staining of nuclei (Dolezel et al., 2007). Staining for nuclear protein content enables the understanding of cell cycle progression and its control in greater detail (Sgorbati et al., 1988). The ability to simultaneously quantify nuclear DNA content and genome size with protein content can be important in assessing the metabolic stability of any plant species. These analyses can also help in establishing relationships between primary and secondary metabolism. In addition, the correlation of nuclear DNA content with protein content allows for determination of the position of the cell within the cell cycle to predict its future development trajectories. These measurements can also help elucidate how and under what conditions (e.g., DNA content, protein content, cell size and metabolic state) do the mechanisms governing genome and metabolic stability get activated. Understanding these mechanisms in *Taxus* to establish the relationship to paclitaxel accumulation may suggest new strategies to maximize paclitaxel production in plant cell cultures. Quantifying and comparing nuclear DNA content across several species of a certain plant (e.g., *Taxus*) will also provide information regarding cell line stability and ploidy levels (Baebler et al., 2005).
It is important to choose appropriate stains for detection of nuclear DNA and protein. Analysis through multiparameter flow cytometry requires at least two fluorescent dyes, one for each parameter; hence care must be taken when selecting the dyes to avoid any spectral overlap (excitation and emission).

Only a few fluorochromes bind selectively and stoichiometrically to plant DNA, a condition required for estimation of DNA content. Sgobarti used propidium iodide (PI) as a fluorochrome for estimation of nuclear DNA content (Sgobarti et al., 1988). PI works by intercalating between the bases of the double-stranded DNA (as well as double stranded RNA (dsRNA)) without base-dependent bias. Consequently, treatment with RNase is required when using PI as a fluorochrome for estimation of nuclear DNA content. To achieve maximal fluorescence and highest resolution, PI is used at saturating concentrations (Dolezel and Bartos, 2005). With information from nuclear DNA content studies on PI-stained suspension cell cultures, Creemers-Molenaar (Creemers-Molenaar et al., 1992) demonstrated a change in ploidy level, going from euploidy to aneuploidy, with suspension culture age in ryegrass. In addition, Kreuger (Kreuger et al., 2008) demonstrated through DNA content analysis that cucumber pro-embryogenic masses remained genetically stable over several years. Greisbach and Kamo (Greisbach and Kamo, 1996) reported that the ploidy level can, in fact, affect plant secondary metabolism as well. They found that in the flavonol biosynthetic pathway of *Petunia 'Mitchell',* polyploidy results in a decrease in relative amounts of secondary metabolite (quercetin-3,7-diglucoside) concentration. In another report, it was found that there was a 40% increase in the accumulation levels of the secondary metabolite carotenoid in seed of
colchicine-induced tetraploid *Zea mays* L. as compared with seed from the diploid progenitor (Randolph and Hand, 1940).

In tandem with performing nuclear DNA content analysis, Blair and Roti (Blair and Roti, 1980) showed that fluorescein isothiocyanate (FITC) can be used to quantify protein content in cells. When bound with FITC, proteins typically turn fluorescent. Roti (Roti et al., 2005) demonstrated the ability to quantify protein content by staining HeLa nuclei with FITC. In relation to multiparameter flow cytometry, Sgorbati used PI and FITC to stain for nuclear DNA content and protein content, respectively (Sgobarti et al., 1988). In order to be able to specifically stain the nuclear protein, the isolated sample should be purified using density-based centrifugation to increase the yield of nuclei. A major challenge with the protein staining dye, FITC, is the longer reaction and incubation time that may limit rapid and high-throughput flow cytometric analysis. It is also advised to investigate possible effects of interferences during co-staining between FITC and other dyes, when used on the same sample. Longer (8 h) incubation times may also result in auto-bleaching and a decrease in staining efficiency.

In this chapter, a rapid and efficient method to prepare nuclei from aggregated cell suspensions of *Taxus* is presented. Staining protocols for nuclear DNA and protein content using PI and FITC, respectively, are detailed. Subsequently, nuclear DNA content and genome size of various *Taxus* cell lines were determined through comparison with standard plant references.
7.2 Materials and Methods

A complete description of the materials and methods is located in Chapter 2. Of particular interest are sections 2.6 and 2.16.

Additionally, the following section details the step by step protocol for isolation, staining and flow cytometric analysis of nuclei so that experiments can be effectively repeated.

7.2.1 Preparation of Nuclei Suspensions from Aggregated Cultures

1. For preparation of nuclei suspensions, cells from day 12 of the culture (note: here cells were day 12, but cells could be isolated from any culture time) are vacuum- filtered through Miracloth® and approximately 0.5 g of cells are transferred to the center of a plastic Petri dish.

2. The frozen nuclei isolation buffer is thawed at room temperature (approximately 30 minutes) and allowed to liquefy completely. Approximately 1-3 mL of the cold nuclei isolation buffer is added to the Petri dish (Galbraith et al., 1983).

3. Cells are immersed in the buffer and chopped immediately with the mini-glass scraper for five minutes (Galbraith et al., 1983). The amount of cells and buffer as well as chopping time should be determined according to the species to enable effective nuclei isolation. Note that some cells may be more resistant to breakage. The razor should be sharp and used only once. Do not chop vigorously or significant nuclei damage will occur.
4. The resultant sample is mixed properly with the buffer several times by tilting the Petri dish to ensure uniform chopping of the entire sample. If necessary, additional buffer can be added to facilitate chopping.

5. The chopped sample is then transferred to a 15 mL centrifuge tube and PBS is added to increase the volume to 10 mL.

6. The nuclei suspension is then filtered through 80 µm mesh and transferred into sterile 15 mL centrifuge tubes for staining of DNA.

### 7.2.2 Staining of Nuclear DNA and Protein

1. For staining of DNA, both PI and Ribonuclease A are added to the nuclei suspension (here isolated from both reference standards and nonelicited cultures as described above) at final concentrations of 50 µg/mL each. As PI also binds to dsRNA, add RNase simultaneously with PI to the nuclei suspension. Also, heat the RNase solution at 90 °C for 15 minutes to deactivate any DNase activity. The sample is mixed by inverting the tubes several times. The sample is then incubated on ice for 15 minutes.

2. The PI stained sample is filtered through 80 µm mesh and transferred to a 5 mL round-bottom tube for flow cytometric analysis.

3. Following the PI staining, fluoresceinisothiocyanate (FITC) is added to the nuclei suspension at a final concentration of 50 µg/mL to stain for protein. The tubes were covered with aluminum foil and incubated at 4 °C for 8 h. Subsequently, the samples were washed twice with PBS to remove any unbound dye.

### 7.2.3 Flow Cytometric Analysis of Stained Nuclei
1. The BD LSR II analytical flow cytometer is used to measure the fluorescence of the stained nuclei suspensions. The fluorochrome used is PI, which can be detected using the 488 nm laser under the PI detector.

2. The PI stained sample is run in the flow cytometer and data for 5,000 events are acquired in a histogram chart containing PI fluorescence intensity as a measure of relative nuclear DNA content. The scatter dot plot is gated manually to eliminate background noise arising from debris or aggregates. In order to obtain PI peaks without undesirable low channel signals, choose an appropriate PMT voltage threshold to minimize off signals coming from cell debris and autofluorescent compounds. The scatter dot plots may be gated to exclude any debris and aggregates, as described previously. In the case of significant debris and aggregates in the isolated nuclei suspension, the concentration of the nonionic detergent (i.e., Triton X-100) can be increased in the isolation buffer. Increasing concentrations aid in the release and cleaning of nuclei solutions, by decreasing the aggregation affinity of nuclei and debris without affecting the fluorescence properties of the dye molecule (Kapuscinski, 1995). There are several possible reasons (e.g., improper staining protocol, vigorous chopping, blunt razor blade, incorrect instrument operation and data acquisition, negative cytosolic effects, recalcitrant tissues, etc.) for broad peaks and a large amount of debris background that should be appropriately addressed (Dolezel et al., 2007).

7.3 Results and Discussion

7.3.1 Evaluation of DNA Content and Genome Size
Three commonly used plant reference standards (*Arabidopsis thaliana*, *Pisum sativum*, and *Nicotiana tabacum*) were analyzed using the procedure mentioned above and their PI fluorescence intensities plotted as shown in Figure 7-1. The nuclear DNA content of the standards was taken from the literature (Dolezel et al., 1998; Johnston et al., 1999) and related to the peak area of the 2C peak. This relationship can be used to determine the genome size of *Taxus* cell suspension lines in our laboratory. It is to be, however, noted that the DNA content values of the reference standards may differ with respect to the type of cultivar (e.g., genetically modified, clones, wild-type, etc.). Consequently, the values for DNA content of the reference species reported here (obtained from labs on campus as opposed to the labs that published the DNA content) may vary from what is published. Three plant reference standards were independently tested to avoid this problem.

The nuclear DNA content, and hence genome size, was determined for four *Taxus* cell lines in our laboratory (Figure 7-2). The amount of nuclear DNA can be calculated using the data collected (i.e., 2C peak area vs. known DNA content) from the reference plant tissues. For example, for the *Taxus* P991 cell line DNA content was quantified using all three standards. Using the values shown in Figure 7-1, the *Taxus* P991 cell line contained 31.6 pg of DNA using *Arabidopsis thaliana* as a reference standard, 36.0 pg of DNA using *Pisum sativum* as a reference standard, and 36.3 pg of DNA using *Nicotiana tabacum* as a reference standard. Furthermore, the genome size of *Taxus* P991 was calculated using the correlation in section 2.16 to be $3.09 \times 10^{10}$ bp using *Arabidopsis thaliana* as a reference standard, $3.52 \times 10^{10}$ bp using *Pisum sativum* as a reference standard.
standard, and \( 3.55 \times 10^{10} \) bp using *Nicotiana tabacum* as a reference standard. The coefficient of variance, CV, was calculated for each cell line, and did not exceed 7.6 %. Table 7-1 lists the DNA content (pg) and genome size (bp) values for four cell lines used in our laboratory. DNA content of *Taxus* suspension cultures has only been reported once. Baebler (Baebler et al., 2005) has shown that there exists major genomic instability in a majority of *Taxus* x media cell suspension lines. They reported a wide range of DNA content values for different *Taxus* cell lines, ranging from a low 21.06 pg to a high 50.72 pg. They also found subpopulation variability in terms of DNA content in those cell lines. Use of jasmonic acid did not affect the DNA content of any of the cell lines – a similar result observed in our studies using methyl jasmonate (MJ) (Figure 7-4; the location of the 2C and 4C peaks are the same). In another report, it was found that different subpopulations of cells existed with different ploidy levels in *Doritaenopsis* (Mishiba et al., 2001). It cannot be ruled out that these variations in DNA content across distinct subpopulations in a culture may lead to different growth rates among these subpopulations. This may, in turn, affect the metabolic activity of the cells, including protein and secondary metabolite synthesis. Therefore, the methods employed in this chapter could be utilized to monitor the amount, activity and subpopulation distribution of nuclear DNA content of *Taxus* suspension cultures over a period of time, which can further help to develop strategies to control changes and optimize culture performance.

### 7.3.2 Co-staining for Protein in Isolated Nuclei

In addition to determining nuclear DNA content and genome size, the nuclear protein content of nonelicited cells was compared with cells elicited with 200 \( \mu \)M MJ;
results are shown in Figure 7-3. According to the mean distribution of both histograms shown in Figure 7-3, MJ-elicited cell cultures contained two-fold more nuclear protein than nonelicited cell cultures. MJ is known to induce secondary metabolism and upregulate gene expression. Use of MJ has also been associated with a decrease in growth rate (Kim et al., 2005). The reason behind the two-fold protein increase in MJ-elicited cultures may be attributed to its role in upregulating secondary metabolism. In other words, the increase in secondary metabolic activity may be more substantial than the decrease in primary metabolism (Hermsmeier et al. 2001).

7.3.3 Multiparameter Analysis of DNA, Protein and Scatter Properties

Multiparameter analysis can be used to quantify the dependence of a given parameter (e.g., DNA content) on a secondary parameter (e.g., protein content). Taxus nuclei were analyzed and their distributions are shown in Figure 7-4.

To determine the extent of emission overlap and other factors as part of staining with both FITC and PI, aliquots of nuclei suspension were prepared and stained with FITC only. Analysis of nuclei stained with both FITC and PI showed the same FITC mean intensity fluorescence as those nuclei that were stained with FITC only (data not shown). In addition, coefficients of variation (CVs) between co-stained and single-stained nuclei remained the same. These results agree with previously attained results (Sgorbati et al., 1988; Roti et al., 2005).
The scatter plots shown in Figure 7-4 can be characterized by three identifiable regions: starting from the left, 1) a 2C region defined by nuclei with increasing FITC fluorescence and constant PI fluorescence. This region represents nuclei with increasing protein content and constant nuclear DNA content; 2) an S region (between 2C and 4C) characterized by nuclei with relatively narrower FITC fluorescence values (as compared to the 2C and 4C regions) and increasing PI fluorescence, representing a narrower range of protein content and increasing DNA content; 3) a 4C region characterized by nuclei with increasing FITC fluorescence and constant PI fluorescence. These regions are similar to those found in previous studies with mammalian cells and plant tissues (Roti et al., 2005; Sgorbati et al., 1988, respectively).

Figure 7-4a shows a two parameter DNA-protein comparison between nonelicited cells (Figure 7-4a i) and MJ-elicited cells (Figure 7-4a ii). The dashed lines represent the mean FITC fluorescence in the 2C and 4C regions. As shown, the average protein content increases as the nuclei progress through S phase from 2C to 4C regions in the nonelicited cells. Notably, the trend is reversed in the MJ-elicited cells. In addition, the mean protein content in the 2C region for MJ-elicited cells is higher than that for nonelicited cells. Figure 7-4a also shows a critical value (indicated by the arrow located at the initial FITC fluorescence intensity of the S phase nuclei in Figure 7-4a i) of the protein/DNA ratio that was reached by 2C cells before entering S phase. Roti (Roti et al., 2005) and Sgorbati (Sgorbati et al., 1988) agree that cells having protein content lower than that needed to enter S phase, which can be indicated by the cells below the arrow in Figure 7-4a i, could be considered noneycling cells.
Figure 7-4b shows a two-parameter DNA-forward scatter (FSC) comparison between nonelicited cells (Figure 7-4b i) and MJ-elicited cells (Figure 7-4b ii). As the cells progress from 2C to 4C, the mean intensity of FSC increases in both nonelicited and MJ-elicited cells. The increase in FSC directly correlates with an increase in cell volume, associated with DNA synthesis (S phase). An aspect of this distribution should be noted. There are more nuclei below the mean in the 2C region, while there are relatively less nuclei below the mean in the 4C region. One study (Yen and Pardee, 1979) claims that larger nuclei are more prone to enter S phase. While our results are inconsistent with that study, they agree with those obtained by Roti (Roti et al., 2005), suggesting that this may either be a system-specific property or could be explained by subpopulation variability in culture. The light scatter results, presented here, are inconsistent with those obtained by the study (Roti et al., 2005) which demonstrates that the light scatter (both FSC and SSC) is increased uniformly across the cell cycle. In Figure 7-4c, there is a noticeable increase in the SSC only until the 2C stage, which diminishes in the S phase. Additionally, as illustrated in Figure 7-4b ii, it was found that the mean of 2C nuclei of MJ-elicited cells was lower than that of nonelicited cells.

Lastly, Figure 7-4c shows a two parameter DNA-side scatter (SSC) comparison between nonelicited cells (Figure 7-4c i) and MJ-elicited cells (Figure 7-4c ii). Figure 7-4c displays an overall decrease in SSC as the cells progress from the 2C to the 4C region in both nonelicited and MJ-elicited cells. Notably, the decrease in SSC going from 2C to 4C is relatively uniform in both nonelicited cells and MJ-elicited cells. This distribution
is different from those shown in Figure 7-4a and Figure 7-4b in that the debris (nuclei in M-stage lacking a nuclear membrane) shown in this distribution (before the 2C phase) contains significantly more SSC than the manually gated 2C and 4C nuclei. Finally, by careful comparison of Figure 7-4c i and Figure 7-4c ii, it can be seen that MJ affects cells in the later stage (4C) by increasing their SSC when compared with nonelicited cells. The mean SSC of nuclei in the 2C region is the same for both nonelicited and MJ-elicited cells.

7.4 Conclusions

The nuclear DNA content and estimated genome size were quantified for four Taxus cell lines currently used in our laboratory using three reference standards with known genome sizes. The calculation of nuclear DNA content and genome size depends on the reference standard used. Using three standards, a mean and a standard deviation were obtained, and the ratio of the standard deviation to the mean (CV) did not exceed 7.6 % in any of the four cell lines. In a single parameter study using FITC to stain for protein content, our results suggested that cells elicited with MJ contained more than two-fold more protein than nonelicited cells. The methods proposed in this chapter may be used to monitor and optimize plant cell cultures over a period of time in terms of amount, activity and subpopulation distribution of nuclear DNA content. Based on these methods, further DNA-protein studies can be proposed a) to further investigate normal and perturbed cells within different phases of the cell cycle; b) to enhance the fundamental understanding of cell cycle progression and regulation; and c) to engineer cell cultures by controlling cell cycle events such as cell cycle arrest, synchronization and transition.
under different physiological conditions through the addition of external agents such as *aphidicolin* (S-phase blocker) (Sorrell et al., 2001), *okadaic acid* (premature mitosis inducer) (Polit and Kaz’mierczak, 2003), *endothal* (G2/M phase activator) (Ayaydin et al., 2000), etc.
Table 7-1: Tabulated DNA content and genome size values of four cell lines used in our laboratory - *T. cuspidata* P991, P93AF and PO93X; and *T. canadensis* CO93D.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Standard used</th>
<th>DNA content (pg)</th>
<th>Genome size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P991</td>
<td><em>Arabidopsis thaliana</em></td>
<td>31.6</td>
<td>3.09E+10</td>
</tr>
<tr>
<td></td>
<td><em>Pisum sativum</em></td>
<td>36.0</td>
<td>3.52E+10</td>
</tr>
<tr>
<td></td>
<td><em>Nicotiana tabacum</em></td>
<td>36.3</td>
<td>3.55E+10</td>
</tr>
<tr>
<td></td>
<td>average</td>
<td><strong>34.6</strong></td>
<td><strong>3.39E+10</strong></td>
</tr>
<tr>
<td>P93AF</td>
<td><em>Arabidopsis thaliana</em></td>
<td>49.0</td>
<td>4.79E+10</td>
</tr>
<tr>
<td></td>
<td><em>Pisum sativum</em></td>
<td>55.8</td>
<td>5.46E+10</td>
</tr>
<tr>
<td></td>
<td><em>Nicotiana tabacum</em></td>
<td>56.2</td>
<td>5.50E+10</td>
</tr>
<tr>
<td></td>
<td>average</td>
<td><strong>53.7</strong></td>
<td><strong>5.25E+10</strong></td>
</tr>
<tr>
<td>PO93X</td>
<td><em>Arabidopsis thaliana</em></td>
<td>56.8</td>
<td>5.56E+10</td>
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<tr>
<td></td>
<td><em>Pisum sativum</em></td>
<td>64.8</td>
<td>6.34E+10</td>
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<td></td>
<td><em>Nicotiana tabacum</em></td>
<td>65.3</td>
<td>6.38E+10</td>
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<tr>
<td></td>
<td>average</td>
<td><strong>62.3</strong></td>
<td><strong>6.09E+10</strong></td>
</tr>
<tr>
<td>CO93D</td>
<td><em>Arabidopsis thaliana</em></td>
<td>31.8</td>
<td>3.11E+10</td>
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<td></td>
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<td>3.54E+10</td>
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<tr>
<td></td>
<td><em>Nicotiana tabacum</em></td>
<td>36.5</td>
<td>3.57E+10</td>
</tr>
<tr>
<td></td>
<td>average</td>
<td><strong>34.8</strong></td>
<td><strong>3.41E+10</strong></td>
</tr>
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</table>

Note: Three reference standards were used in these studies, and the calculations were performed on the basis of the correlations described in section 2.16.
Figure 7-1: PI staining for the plant reference standards used in the study.

Note: Nuclei suspensions were isolated from various plant tissues in the same way as *Taxus* and stained for DNA using PI. The nuclei were analyzed using the BD LSR II flow cytometer equipped with a 488 nm argon laser. The scatter dot plots were gated to eliminate any debris or aggregates. Peak intensities (i.e., areas) and percentage of events within each interval were determined. The 2C DNA content for the three species was taken from the literature as indicated in the figure, and was used to determine the DNA content for *Taxus*. 

<table>
<thead>
<tr>
<th>Plant Name</th>
<th>DNA Content (2C)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Arabidopsis thaliana</em></td>
<td>0.43 pg DNA</td>
<td><em>Dolezel et al., 1998</em></td>
</tr>
<tr>
<td><em>Pisum sativum</em></td>
<td>9.09 pg DNA</td>
<td><em>Dolezel et al., 1998</em></td>
</tr>
<tr>
<td><em>Nicotiana tabacum</em></td>
<td>10.04 pg DNA</td>
<td><em>Johnston et al., 1999</em></td>
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</table>

<table>
<thead>
<tr>
<th>Peaks: Mean Intensity (% of events)</th>
<th>Peaks: Mean Intensity (% of events)</th>
<th>Peaks: Mean Intensity (% of events)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2C: 301 (38.9%)</td>
<td>2C: 5582 (79.4%)</td>
<td>2C: 6120 (44.3%)</td>
</tr>
<tr>
<td>4C: 586 (29.1%)</td>
<td>4C: 9890 (11.4%)</td>
<td>4C: 13008 (39.5%)</td>
</tr>
<tr>
<td>8C: 1038 (17.3%)</td>
<td>8C: 19660 (3.2%)</td>
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</tr>
<tr>
<td>16C: 2009 (6.7%)</td>
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</table>
Figure 7-2: Flow cytometric histograms depicting nuclear DNA content of *Taxus* cell lines in our laboratory – *T. cuspidata* P991, P93AF and PO93X; and *T. canadensis* CO93D.

Note: All the four cell lines exhibit a typical diploid pattern. The cell lines P991 and CO93D have overlapping 2C peaks with mean intensities of 22,110 and 22,236 respectively; while the lines P93AF and PO93X have 2C mean intensities of 34,285 and 39,787 respectively.
Figure 7-3: Two histograms of *T. cuspidata* P991 cell suspension cultures showing FITC-stained nonelicited (left) and MJ-elicited (right) nuclei.

Note: The mean FITC fluorescence for nonelicited sample was 391 units, while that for MJ-elicited sample was 804 units.
Figure 7-4: Multiparameter flow cytometric analysis of *T. cuspidata* P991 cell suspension cultures. Figure 7-4a compares the nuclear DNA and protein content of nonelicited (Figure 7-4a i) and MJ-elicited (Figure 7-4a ii) cells. Figure 7-4b compares the cell size (FSC) and nuclear DNA content of nonelicited (Figure 7-4b i) and MJ-elicited (Figure 7-4b ii) cells. Figure 7-4c compares the cell complexity (SSC) with nuclear DNA content of nonelicited (Figure 7-4c i) and MJ-elicited (Figure 7-4c ii) cells.

Note: The dotted lines represent approximate mean values of the respective variables on the y-axes. The arrow in Figure 7-4a.i represents the minimum protein content at which the cells pass on to the S phase from 2C phase.
CHAPTER 8
CONCLUSIONS AND RECOMMENDATIONS

In this thesis, use of flow cytometry for both characterization of Taxus cell culture heterogeneity and sorting of distinct populations is presented. There are several limitations associated with Taxus cell culture systems which have been addressed in this work. The main challenge is the low and variable production, owing to culture aggregation and inherent heterogeneity induced through varying cellular microenvironments. Other limitations include inconsistency in production over time and the culture reversion to a biologically unproductive state, thereby making the process uneconomical and inefficient. By isolating intact single cells, staining, sorting and analyzing various cell subpopulations with differing levels of paclitaxel accumulation, the inherent molecular and metabolic differences amongst cells in culture can be explored and the phenomena that underlay culture heterogeneity and production variability understood.

8.1 Statistical Optimization of Single Cell Production

In Chapter 3, statistical optimization of the critical variables affecting the single cell formation of Taxus suspension cultures was performed. This optimization was the basic and enabling criterion for the analysis and isolation of cell subpopulations using high-throughput flow cytometric methods. Cellulase, pectolyase Y-23 and centrifugation speed were the critical parameters shown to affect generation of single cells from Taxus plant cell aggregates. Response Surface Methodology (RSM) and central composite design were successfully utilized to optimize these independent parameters in order to
obtain a maximum single cell yield (SCY). The optimum values obtained were 0.045% cellulase concentration, 0.7% pectolyase Y-23 concentration and 1200 x g centrifugation speed. At these conditions, SCY was determined to be 55%, which is 72% higher than that previously reported. A second order regression model equation was developed to predict the SCY. This model was shown to hold well in the design region through ANOVA. The optimization procedure was validated through microscopy and flow cytometric characterization. The isolated single cells were undamaged and morphologically intact, as observed by microscopy. Flow cytometry was used to analyze the isolated single cells after staining with fluorescein diacetate, which measures the activity of intracellular esterases in living cells and, therefore, is an indicator of cell viability. The optimum procedure obtained in this work generated more than 99% viable cells, thereby facilitating the subpopulation analysis at the single cell level through rapid and high-throughput flow cytometry. In addition, RSM was useful in understanding the interaction effects amongst the variables.

Although the SCY was statistically maximized, there is a need to minimize the total time of the isolation procedure. It was kept at 4 h in this work. Decreasing the time for isolation would minimize cellular metabolic or physiological changes. This is particularly useful in further analyses (i.e., mRNA isolation and gene expression), where an accurate cellular state is important for data interpretation. Certain other variables could also be tested to evaluate the effect on SCY and preparation time. For example, incubation speed (RPM) at which the digestion occurs can be optimized to minimize cellular stress while promoting disaggregation. This procedure can be applied to isolate
single cell populations from other commercially relevant plant systems used to synthesize medicinal secondary metabolites such as *Catharanthus*, *Coptis* and *Artemisia*. The multivariate statistical design of experiments (DOE) technique can be broadly applied in the development of models to predict maximal SCY for other cell systems as well as for the optimization of output variables in other biochemical processes. Additional optimization can be done in choosing the digestion enzymes. For example, xylanase and hemicellulase target the lamellar structure specifically (as opposed to cellulase) and may be more suitable for the preparation of single cells from aggregated suspension. Previous work in our laboratory explored the use of additional enzymes (e.g., pectinase and macerozyme) but a comprehensive evaluation may yield better options.

**8.2 Paclitaxel Detection Assay in Live Cells**

In Chapter 4, the subpopulation variability in *Taxus* cell cultures was investigated through the development of a live cell-based assay which detects cellular paclitaxel accumulation while maintaining cell viability and integrity. Fluorescence microscopy was used to analyze paclitaxel-related fluorescence and assess the degree of variability in *Taxus* subpopulations. To further validate the assay and obtain a more robust, quantitative and representative data set, flow cytometry was performed on stained cells. Histogram comparisons for population analysis were performed on the control and stained samples to determine the paclitaxel-positive population. Cell viability was assayed using flow cytometry after costaining with FDA, which detects esterase activity. To establish a superior immunoassay procedure to obtain consistent and reliable staining data, the experimental variables in the process were empirically regulated. The effect of sampling
day and methyl jasmonate (MJ) concentration on the percentage of paclitaxel accumulating cells was evaluated, indicating an increase with both sampling day and MJ concentration. Subsequently, this assay was employed to quantify paclitaxel accumulation through the use of a series of precalibrated beads with varying antibody binding capacities.

The staining time in the procedure could be decreased, which is crucial for experiments where minimizing time is critical, including sorting and RNA extraction for gene expression analysis. Antibody concentrations, temperature and incubation speed are some of the variables to be considered. The paclitaxel detection assay can be utilized in conjunction with another biomarker staining procedure to establish correlative profiles. For example, time-based paclitaxel accumulation profiles could be studied by staining for cell growth and cell division markers to establish integrative metabolic relationships in Taxus cell cultures. This can be of great value from an industrial perspective, as two different culture properties like cell growth and metabolite production could be cooptimized. A logical next step after the vital staining procedure is to utilize novel cell sorting platforms to isolate distinct subpopulations of Taxus cells. Sorting, not just based on one property (paclitaxel accumulation), but a combination of two or more properties that can be examined through multiparameter flow cytometry would provide a tool for comprehensive analysis of different subpopulations that exist in a single culture system.

8.3 Development of Fluorescence-Activated Cell Sorting (FACS) Technology
Investigating plant cell culture heterogeneity at the subpopulation level, through enabling flow cytometric sorting technologies is a new approach for development of plant bioprocesses to enhance metabolite accumulation. This methodology development was outlined in Chapter 5, which described sorting of *Taxus* plant cells based on both size and paclitaxel accumulation. A rapid and high-throughput method to isolate, stain and sort distinct paclitaxel accumulating cell subpopulations through the FACS technology was described, thus enabling their subsequent recovery and propagation for improved culture productivity. This methodology is the first to sort intact single cells (i.e., with intact cell walls). Because many hydrophobic secondary metabolites are stored in the cell wall (including paclitaxel), retaining this cellular compartment is important and necessary when aiming to isolate specific populations based on metabolite accumulation.

While *Taxus* cells were stained for paclitaxel accumulation and sorted according to low- and high-accumulating subpopulations, this technology provides tremendous opportunities to sort subpopulations exhibiting different combinations of properties (e.g., paclitaxel content and cell cycle stage/rapidly dividing cells). Since the isolated cells are subsequently utilized for reculture, it may be important to identify cells which are both actively dividing and have a specific level of paclitaxel accumulation. Multiparameter flow cytometry may be utilized to stain for protein content and other biomolecules. Instrument parameters can be further optimized to enable collection of a larger amount of cells in a shorter period of time, which will facilitate the reculture process. There exist physics-based correlations which govern the operation of a sorter, and the working conditions could be optimized using this information. To enable a controlled and
completely sterile sorting and recovery, a mini laminar flow chamber set up could be installed at the collection port in the sorter, with controlled humidity, temperature and air flow. Additionally, observations can be made by analyzing other sort parameters (e.g., FSC, SSC, time, etc.) in conjunction with paclitaxel-associated fluorescence to draw key inferences about the basic behavior of Taxus subpopulations.

8.4 Culture of Sorted Taxus Cell Subpopulations

In Chapter 6, methods were developed to propagate sorted single cell populations and analyze them over time for different biochemical parameters. More specifically, cultures were scaled up in volume over time, and their growth, aggregation and accumulation profiles analyzed. Specific paclitaxel accumulation profiles were observed during the culture period. There were some trends in the growth rate and the rate of aggregation, which indicated some relationship between the two parameters which could be induced by the action of concentrated medium. Moreover, the accumulation variability in the sorted populations was shown to decrease by as much as 50%. The percentage of paclitaxel-positive populations in the high-accumulators increased to approximately 90%.

While a method was developed to facilitate the growth and analysis of sorted populations, there exist several possibilities to improve the culture performance of these populations. Several key process variables could be optimized (statistically, if required) to maximize the output variables, mainly growth and accumulation. In order to be able to translate this process to an industrially viable one, it may be important to enhance the
culture throughput and ramp up the growth rate of these cultures. Other variables that could be optimized in this process include medium composition, scale-up strategy, feed medium, incubation conditions, etc.

8.5 Nuclear DNA content and Genome Size Evaluation

In Chapter 7, a multiparameter study of nuclear DNA content and estimated genome size were conducted for four *Taxus* cell lines currently used in our laboratory using three reference standards with known genome sizes. Additionally, in a single-parameter study using FITC to stain for protein content, our results suggested that cells elicited with MJ contained more than two-fold more protein than nonelicited cells. These methods can further help our understanding of the cellular metabolism and protein content, which could be used to suggest optimization strategies to significantly affect secondary metabolite accumulation in these cultures. These results are preliminary and further confirmation is necessary.

Although a rapid and easy method to isolate and stain *Taxus* nuclei was employed, the FITC staining assay for proteins did not provide information regarding protein localization and specificity. Since the cells were chopped indiscriminately, the protein stained represents the average content from the entire aggregate, and therefore may not be suitably used for multiparameter studies. Methods like density-based centrifugation could be developed to separate nuclei from larger debris. That might help better understand the distribution of FITC fluorescence, and correlate it with the DNA content.
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