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# APPLICATION OF SPECTROSCOPIC METHODS FOR THE AUTOMATION OF OIL PALM CULTURE

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**ABSTRACT**

*Oil palm tissue culture offers a potentially practical route to clonal propagation of high yielding palms. However, current tissue culture methods are laborious and costly, and the performance of the cultures can be difficult to describe quantitatively. Computer control of bioreactor processes increases reproducibility and permits quantitative description of the growth of oil palm cultures. Even so, there remain unmet needs in the areas of online metabolite measurement and of automation of the tissue culture process. In this work, we apply Raman spectroscopy for non-destructive off-line quantitation of sucrose, glucose, fructose, nitrate, potassium phosphate and magnesium sulphate metabolites in oil palm bioreactor culture supernatants. We also explore the feasibility of using fluorescence to discriminate between different morphotypes of oil palm calli. Finally, we report the use of flow cytometry to sort oil palm suspension cultures on the basis of size; selected samples were deposited into separate wells in a microplate with one callus particle per well. The technologies described in this article contribute to the development of automated methods for moving and positioning oil palm cells, and for online measurement of metabolites in oil palm bioreactor supernatant.*

**Keywords:** Raman, UV fluorescence, spectroscopy, cytometry, glucose.

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## INTRODUCTION

Malaysia is the world leader in palm oil production, and exports in 2006 exceeded USD 9.2 billion (Wahid, 2007). Palm oil is a major edible oil and industrial feedstock worldwide, as well as a key source of biodiesel (Wahid *et al*., 2005); thus, the cultivation of oil palm (*Elaeis guineensis*) is an important industry (Wahid *et al*., 2005). Currently, it takes at least five years to develop and mature oil palm, and there are no guarantees that progeny palms will be high yielding, even after careful selection and cultivation (Chin, 2004). Thus, clonal propagation of oil palm is needed to generate identical plants of superior productivity and improved yield (Jones, 1974; 1983; Rohani *et al*., 2003). The oil palm industry embarked on research to commercialize tissue culture, based on the prospect of increasing yield by cloning elite materials (James, 1984). Field trials have shown that up to a 30% increase in yield and a significant reduction in time to produce fruits is possible through the planting of tissue culture materials (Mutert and Fairhurst, 1999).

However, the unreliability and low efficiency of the tissue culture process has been a barrier to progress. A significant proportion (60%-80%) of ortets harvested from parent palms fail to differentiate into the pluripotent disorganized callus form when exposed to the necessary plant growth regulators (Mutert and Fairhurst, 1999). After propagation, the callus is manually classified as either embryogenic or non-embryogenic, based on

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morphological features. The rate of embryogenesis in oil palm is only about 6% (Wooi, 1995). In other plants, embryogenesis has been shown to be affected by growth conditions and stress (Ingram and Mavituna, 2000). Embryogenic callus is of value as it is amenable to growth as a suspension culture and can differentiate into embryos suitable for the formation of single plantlets (Tarmizi, 2002). Since plant cells are maintained in culture over the course of weeks or months, there is increased necessity for proper sterile techniques, as contamination from bacteria or fungi can overwhelm the plant cells. Excessive length of time in culture can also contribute to the undesired mantled phenotype, a problem brought about by changes in transcription due to DNA hyper- and hypo-methylation (Shah and Parveez, 1995; Cassells and Curry, 2001; Jaligot *et al*., 2002; Morcillo *et al*., 2006). It is therefore of critical importance to carefully monitor suspension cultures to get the maximum yield of cells that are both embryogenic and free of epigenetic alterations.

The traditional methods for tissue culture are staff intensive and time consuming. Automated processes offer the potential to reduce costs and time requirements, and sources of contamination and loss. The bioreactor cultivation of oil palm suspension cultures is accompanied by several advantages including reduced labour/operator handling and amenability to computerized control and monitoring of operating conditions. The computerized control and monitoring of conditions in the bioreactor, in turn, make it possible to carry out and develop statistically significant procedures to determine the optimal operating conditions. Our group developed conditions suitable for the growth of oil palm cells in suspension culture using a multiplexed bioreactor system and, with response surface experimental design, determined optimum concentrations of the nitrogen source in the medium, as well as the optimum inoculum size (Gorret *et al*., 2004). Tarmizi *et al*. multiplied oil palm suspension cultures in a 2-litre bioreactor system and achieved 10-14-fold biomass increase after 50-80 days of culture (Tarmizi *et al*., 2004).

Although many issues were addressed with the development of the oil palm bioreactor system, there remain many needs in the areas of online monitoring and automation. A great advantage of bioreactors over the traditional method of propagation in flask cultures is the ability to directly control and monitor culture conditions. The Sixfors™ bioreactor system (Infors AG, Bottmingen, Switzerland) permits simultaneous online monitoring and control of growth conditions, including temperature, agitation and dissolved oxygen, in six separate 0.5 litre reactor vessels equipped with marine impellers to reduce the shear stresses caused by aeration and impeller agitation (Sun and Linden, 1999; Abdullah *et al*., 2005). Currently, other culture conditions including the concentrations of sugars, ammonia, nitrate, amino acids and organic acids are monitored off-line; that is, samples are withdrawn from the bioreactor and processed, and quantities of the compounds of interest measured using analytical equipment. Standard off-line chemical separation techniques, such as High Performance Liquid Chromatography (HPLC), provide the flexibility of easily analysing a variety of chemical components. However, they require sampling and interfacing the measurement system with the sterile culture. Besides the contamination risk, the culture conditions can change significantly as the sample is being processed outside of the bioreactor, introducing a potentially large source of error in the measurements.

Online detection methods, that is, measurement of key culture parameters without the need for sample withdrawal, would result in further cost and time savings. By eliminating the need for sampling, non-invasive online systems also reduce the chances for contamination, resulting in decreased losses of materials and time. One possible approach to monitoring is based on *in situ* biosensors, where a chemically specific reagent is used to generate an electrical signal. For example, a glucose biosensor makes use of an immobilized enzyme which oxidizes glucose, producing hydrogen peroxide, which is subsequently oxidized at a platinum electrode, producing a measurable current. Sensors of this type can have a working measurement range up to 100 mM (Yacynych *et al*., 1994). Although their specificity and sensitivity are very good, integrated enzymatic assays degrade over time. Also, a different sensor is required for every chemical component whose analysis is desired.

One family of analysis tools that alleviate contamination and sterilization concerns and have the flexibility to analyse a variety of chemical components are optical techniques, *e.g*. Raman spectroscopy, that never require a physical interface with the sterile environment. We have demonstrated Raman spectroscopy as a technique for online monitoring of metabolites in bacterial bioreactors (Lee *et al*., 2004; Gil, 2005). In this work, we demonstrate the feasibility of Raman spectroscopy for measurement of metabolites in oil palm bioreactor supernatants.

We also report on the use of fluorescence spectroscopy and cytometry for oil palm culture. We evaluated oil palm calli of different morphologies and determined that these cell types may be distinguished based on their fluorescence signatures. In addition, we tested the feasibility of using a fluidic device to assist with sorting and selection of cells. We demonstrated that using a fluidic technique we could place one callus sample per well in a microplate with ~97% accuracy, at a rate of 36 samples per minute. These activities tie into the goal

of developing an automated, non-destructive technique for moving and sorting oil palm cells.

## EXPERIMENTAL

### Cultivation of Oil Palm Cells

The oil palm cell line E90L1 was provided by the Malaysian Palm Oil Board (MPOB) (Bangi, Malaysia). Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) was prepared using Sigma-Aldrich products (St Louis, MO, USA) and used for all the experiments. MS medium was supplemented with 1 mg $ml^{-1}$ 2, 4-dichlorophenoacetic acid (2,4-D), and 1 mg $ml^{-1}$ alpha-naphthalene acetic acid. Cells in Erlenmeyer flasks were incubated at 26°C on a rotary shaker and agitated at 125 rpm. The medium in the flask cultures was changed every 10 days, conditioned medium added as described and the cells passed through sterile brass 600 micron mesh during subculture as described (Gorret *et al*., 2004). Oil palm cell suspension cultures were propagated via sequential subculture in flasks to generate sufficient cell mass to inoculate the bioreactor. Cultures were carried out in Sixfors bioreactors (Infors, AG CH-4103 Bottmingen, Switzerland). Each reactor was equipped with two 2.5 cm diameter marine-type impellers, dissolved oxygen probes and pH probes. The temperature was regulated at 26°C.

### Raman Spectroscopy

***Raman data acquisition.*** Online Raman spectra were acquired from samples of oil palm cell supernatant using an InPhotonics Raman probe (Norwood, MA) and the experimental set-up outlined in *Figure 1*. The excitation was chosen to be 785 nm to reduce the background from fluorescence while staying within the sensitivity of commercial CCDs. Laser excitation at 785 nm was provided by an external cavity laser similar in design to that described by Arnold *et al*. (1998) with additional filtering by a Kaiser(TM) volumetric holographic grating. Coupling into the 105 μm excitation fibre of the fibre Raman probe resulted in 50-60 mW power at the sample. Optics internal to the probe provided shortpass filtering of the excitation and longpass filtering of the Raman scattering which was collected into a 200 μm collection fibre in a 180° backscattering geometry. The Raman scattering was analysed by coupling the 200 μm collection fibre into an Acton SpectraPro 300i

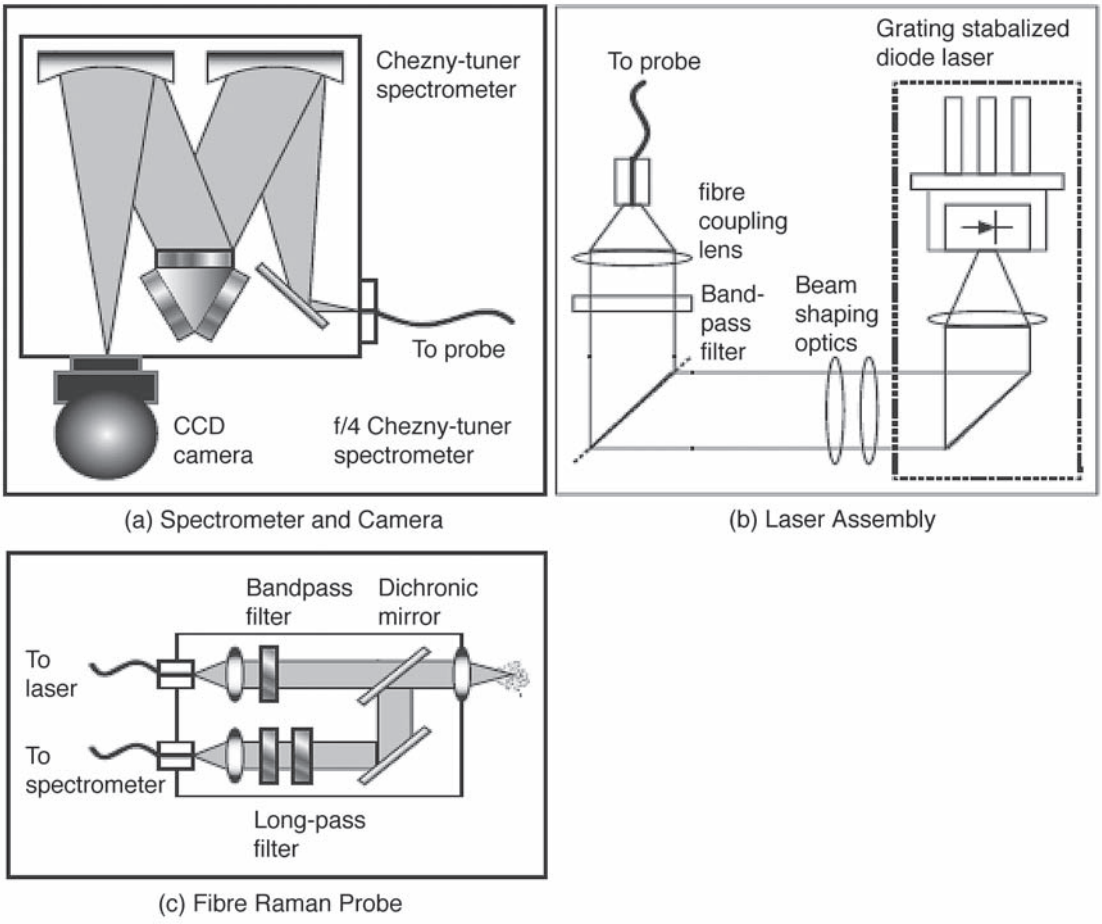



*Figure 1. Schematics of the dispersive Raman spectroscopy set-up used in these experiments.*

spectrograph (Acton, MA) equipped with a Princeton Instruments SPEC10: 400BR back-illuminated deep depletion CCD camera. Liquid nitrogen cooling of the CCD sensor to -85°C reduced the dark counts to an average of 2 count/pixel $hr^{-1}$, or essentially zero. The spectrograph was run in low-noise mode, with a gain of approximately 5.19e"/ count. All the spectra were acquired with WinSpec(TM) software (Princeton Instruments) and signal processing done in MATLAB.

Calibration spectra were acquired by suspending the Raman probe vertically 0.318 cm above an aluminum cartridge containing 220 µl wells 0.476 cm in diameter. Liquid samples were deposited in the wells at the defined volume to ensure a flat meniscus. This method eliminated the interference from the Raman spectrum of a cuvette or other receptacle wall. Furthermore, the vertical geometry and defined volume wells controlled systematic distortions to the spectra to less than 1% of the collected signal. This geometry was sensitive to the exact shape of the meniscus protruding from the side of the well, causing µl changes in volume to cause systematic distortions of as much as 5%. In all cases, data were acquired for a total of 300 s in ten 30 s exposures. Cosmic ray events were removed from all spectra by comparing data for all the 10 exposures in each acquisition and removing points more than three standard deviations from the mean and averaging the resultant frames.

**Concentration and Error Estimation**

Assuming that the Raman spectrum of an aggregate is the linear combination of the components, then the concentration of the components can be determined by least squares fitting of a set of calibration spectra to the measured spectrum. To enable this, we assumed that background fluorescence results in additive error, that scattering from biomass and bubbles only caused a scalar multiplicative effect, and that the wavelength dependence of scattering is negligible. Under these conditions, shot-noise from the calibration spectra and the measured spectra should be the dominant source of error.

Calibration spectra are normalized to 1 mM concentration. Included with these calibration, Raman spectra is a fourth order polynomial to model the additive background fluorescence; together they formed the pure component spectrum matrix, *K*. For a given measured spectrum vector, *s*, the vector concentrations, *c*, can be acquired by solving s = Kc by means of pseudo-inverse methods (Trefethen and Bau, 1997). The scalar amplitude correction for scattering and laser drift is done by normalizing all the component concentrations to the concentration of water, as described (van den Brink *et al*., 2002). In this way, water is used as an internal standard.

**Chemical Analysis (HPLC and enzymatic methods)**

In order to establish reference measurements for comparison with the Raman concentration estimates, reagents and primary metabolites, sugars and organic acids were quantified by HPLC analysis as described (Gorret *et al*., 2004). Ten millilitre samples were collected regularly during the run and centrifuged at 3500 xg for 10 min. The supernatants were filtered through 0.2 µm Acrodisc filters. Sugar and organic acid concentrations were determined by HPLC (Agilent model 1050, Waldbronn, Germany) using an Aminex® HPX-87H column (Bio-Rad, Hercules, CA, USA). Sample analysis was performed at 40°C using 5 mM sulphuric acid as the mobile phase at a flow rate of 0.6 ml $min^{-1}$. Sugars were determined by a refractive index detector (Agilent model 1047A) and organic acids with the UV detector at 210 nm (Agilent model 1050).Amino acids were analysed as ortho-phthaldialdehyde (OPA) derivatives by reverse-phase chromatography using a C18 AminoQuant column with an Agilent series 1050 high-pressure liquid chromatography (HPLC) system. Nitrate and nitrite were analysed using a colorimetric method (Cat No. 11746081001, Roche Applied Science, Mannheim, Germany). Ammonia was analysed using an enzymatic method (Sigma-Aldrich, St Louis, MO, USA). Total phenolics were determined by a modified Folin-Ciocalteu colorimetric method (Gao *et al*., 2000).

**Fluorescence Analysis**

Oil palm cells were withdrawn from the suspension culture and applied to glass microsope slides. The callus particles in each sample were sorted based on size and morphology, then dried and granulated in order to prevent differences in callus geometry from affecting the signal amplitudes. Excitation from a 380 nm LED was filtered with a shortpass filter with cutoff wavelength of 480 nm (Omega Optical, Brattleboro VT) butt-coupled to a 20-fibre bundle of 500 µm core optical fibres. The excitation was then delivered at normal incidence 1 cm above the samples. The fluorescence was collected by a 20-fibre bundle-to-slit coupled directly into the spectrometer slit. Fluorescence spectra were acquired using the same spectrograph and CCD camera used for the Raman spectroscopy except with a 600 lines $in^{-1}$ grating, blazed at 500 nm.

**Cytometry**

Fluorescence-activated cell sorting (FACS) was employed to sort the oil palm suspension cultures in solution. Oil palm suspension cultures were filtered through a brass 600 micron mesh before samples were applied to a COPAS Device (Union

Biometrica, Somerville, MA), a flow cytometer designed to accommodate objects ranging in size from single plant cells to calli. Oil palm callus particles passed through a laser beam focused to the centre of the flow cell. This beam was narrower than the calli so that multiple measurements were made per particle and the calli optically scanned along their long axis as they flowed past the beam. Real-time light scattering and fluorescence at different wavelengths were monitored along the axis of the particle. Rapid puffs of air were employed by the COPAS device to gently divert calli from the flow stream. Calli were sorted by size into 'selected' and 'non-selected categories'. The 'selected' particles were deposited, one oil palm callus particle per well, into microplates.

## RESULTS AND DISCUSSION

### Offline Raman Spectroscopy for Plant Cell Bioreactions

In this work, we investigated the spectroscopic analysis of oil palm culture. We evaluated the utility of Raman spectroscopy, a non-invasive method, for the analysis of oil palm bioreactor culture supernatants. Raman spectroscopy takes advantage of Raman scattering (Raman, 1928; Raman and Krishnan, 1928). Raman scattering, also referred to as inelastic light scattering, is caused by the interaction between the optical oscillations of light with the vibrational motion of molecules.

Before sample analysis could be performed, it was necessary to investigate the Raman activity of the constituent components of the oil palm medium to generate the calibration spectra used to estimate component concentrations. The tests were conducted using the rigid universal calibration set-up described in the Experimental section. All spectra were the average of ten 30s acquisitions and underwent amplitude and wavelength corrections. Amplitude corrections were performed by normalizing the amplitude of the measured Raman spectra to the amplitude of the residual Rayleigh scattered light that passed through the optical filters. Wavelength corrections were similarly accomplished by shifting the measured spectra, typically by less than 1 nm, between frames such that the Rayleigh lines were congruent as described (Gil, 2005). The need for such calibrations would be minimized using more sophisticated commercial laser systems.

Medium constituents that are monitored in a typical bioreaction include sugars such as fructose, sucrose, and glucose. The concentrations of these constituents vary in the range of approximately 0 - 30 g litre$^{-1}$ (Gorret *et al*., 2004). The sensitivity of glucose has already been demonstrated at 0.11 g litre$^{-1}$, indicating the promise for sucrose and fructose concentrations (Lee *et al*., 2004). Other constituents of interest include ammonia and glutamine, which have been demonstrated to be Raman active in the near-infrared in the literature (Xu *et al*., 1997), although at much higher concentrations than those used in this study.

*Table 1* shows the results of the tests along with their initial concentrations. Fructose and glucose were not added initially; they are the natural by-products of sucrose cleavage and thus, appear in large quantities only later in the cell culture lifecycle. All other components can be assumed to be either consumed in the bioreactor or remain constant. *Table 1* also shows the results of tests for the Raman activity of the components at the nominal starting concentrations. Fructose and glucose were tested at the nominal beginning concentration of sucrose. The carbon sources (sugars) and the nitrogen sources (nitrates) exhibited comparatively strong Raman spectra, and the nitrate salts had identical Raman spectra. Since nitric acid ($HNO_3$) is a strong acid, it disassociates completely into the nitrate ion, ($H_3NO^+$) and hydronium ion ($H_3O^-$) (Spencer *et al*., 2003). Likewise, a nitrate salt will disassociate in solution into the nitrate ion and cation. It is therefore logical that the nitrate solutions had identical Raman spectra, since the ammonium ion has a very weak Raman cross-section and a single atom has none at all. The medium buffer components were also Raman active, and must be included in any calibration set. Significant Raman spectra are shown in *Figure 2*.

The comparative strengths of the Raman spectra of the sugars, nitrates and buffer components, as shown in *Table 1*, indicate that they will be detected by Raman spectroscopy. The other components, however, are likely to be in quantities too low to be detectable. Phenolics and other components, added or created by the oil palm cells, are only produced to levels up to the 1 mM range, and are therefore below the sensitivity of the instrument. *Figure 3* shows the concentrations of fructose, glucose and sucrose from one of the bioreactions as assessed by Raman spectroscopy and HPLC. As had been previously shown, the sucrose carbon source is converted to fructose and glucose within 10 days of the bioreaction (Gorret *et al*., 2004); this is likely due to the action of sucrose synthase or invertase enzymes although we have not tested this directly.

The theoretical values for the error are derived using an algorithm employing a first order error analysis of the pseudoinverse as described (Gil, 2005). These data show similar error performance in previous offline measurements for *Escherichia coli* bioreactions (Lee *et al*., 2004). The values we obtained by Raman spectroscopy are in agreement with those assessed by HPLC. The beginning concentration of sucrose was overestimated by HPLC, which accounted for some of the error between the HPLC

**TABLE 1. RAMAN ACTIVITY TOWARD OIL PALM MEDIUM COMPONENTS**

| | Composition of oil palm medium | | |
|---|---|---|---|
| **Component** | **Stock conc. (mg litre$^{-1}$)** | **Starting conc. (mg litre$^{-1}$)** | **Raman activity** |
| Ammonium nitrate | 16 500.0 | 1 650.0 | Degenerate |
| Calcium chloride anhydrous | 3 322.0 | 332.2 | Undetectable |
| Magnesium sulphate | 1 807.0 | 180.7 | Strong |
| Potassium nitrate | 19 000.0 | 1 900.0 | Degenerate |
| Potassium phosphate monobasic | 1 700.0 | 170.0 | Strong |
| Boric acid | 6.2 | 0.62 | Undetectable |
| Cobalt chloride ($6H_2O$) | 0.025 | 0.0025 | Undetectable |
| Cupric sulphate ($5H_2O$) | 0.025 | 0.0025 | Undetectable |
| $Na_2$ EDTA | 37.3 | 3.73 | Undetectable |
| Ferrous sulphate ($7H_2O$) | 27.8 | 2.78 | Undetectable |
| Manganese sulphate $H_2O$ | 16.9 | 1.69 | Undetectable |
| Molybdic acid ($2H_2O$) | 0.25 | 0.025 | Undetectable |
| Potassium iodide | 0.83 | 0.083 | Undetectable |
| Zinc sulphate ($7H_2O$) | 8.6 | 0.86 | Undetectable |
| Glycine | 2.0 | 0.002 | Undetectable |
| Myo-inositol | 100.0 | 0.1 | Undetectable |
| Nicotinic acid | 0.5 | 0.0005 | Undetectable |
| Pyroxidine hydrochloride | 0.5 | 0.0005 | Undetectable |
| Thiamine hydrochloride | 0.1 | 0.0001 | Undetectable |
| Sodium 2,4-D monohydrate | 1.0 | 0.001 | Undetectable |
| Alpha-naphthalene acetic acid | 1.0 | 0.001 | Undetectable |
| Glutamine | 100.0 | 0.0 | Undetectable |
| Sucrose | 30 000.0 | 30 000.0 | Strong |
| Fructose | 0.0 | 0.0 | Strong |
| Glucose | 0.0 | 0.0 | Strong |

and Raman measurements. The error between the HPLC concentration estimate and nominal beginning concentration of sucrose was 7.0 mM. Finally, the lack of pH control in this bioreaction is a potential source of error in the concentration estimates due to protonation of high pKa acids. A previous bioreaction of the same cell line showed that the pH in the bioreactor dropped dramatically in the first 10 days and then gradually rose over time (Gorret *et al*., 2004).

These results show the potential of Raman spectroscopy as an online bioprocess monitoring system for plant cell cultures. Accurate online measurements of sugar concentrations is desirable in plant micropropagation since the concentration and type of sugar in the supernatant can have an effect on rate of growth of cells in suspension (Yu *et al*., 2000), as well as on processes such as somatic embryogenesis (Levi and Sink, 1990) and shoot germination (Hilae and Te-chato, 2005). In order to bring the results online, current systematic errors should first be addressed. Changes in the pH currently invalidate some calibration spectra, adversely affecting the concentration estimates. Controlling pH in the plant cell cultures would eliminate this source of error. Alternatively, if pH control is not available, acquiring calibration spectra at multiple pH levels and adapting the calibration spectra used in the pure component matrix as a function of pH would reduce this error. In addition, since the plant cell bioreactions grow at a slow rate, online measurements can use longer acquisition times to increase the signal to noise ratio. An additional consideration for online Raman analysis are the effects of scatterers on the measured spectra. These include air bubbles and the calli particles themselves. Because light scattering from particles has a wavelength dependence, it effectively distorts the measured Raman spectra thereby introducing an error in the pure component calibration spectra which were measured without scattering. For the slow impeller speed, large particle size and relaxed

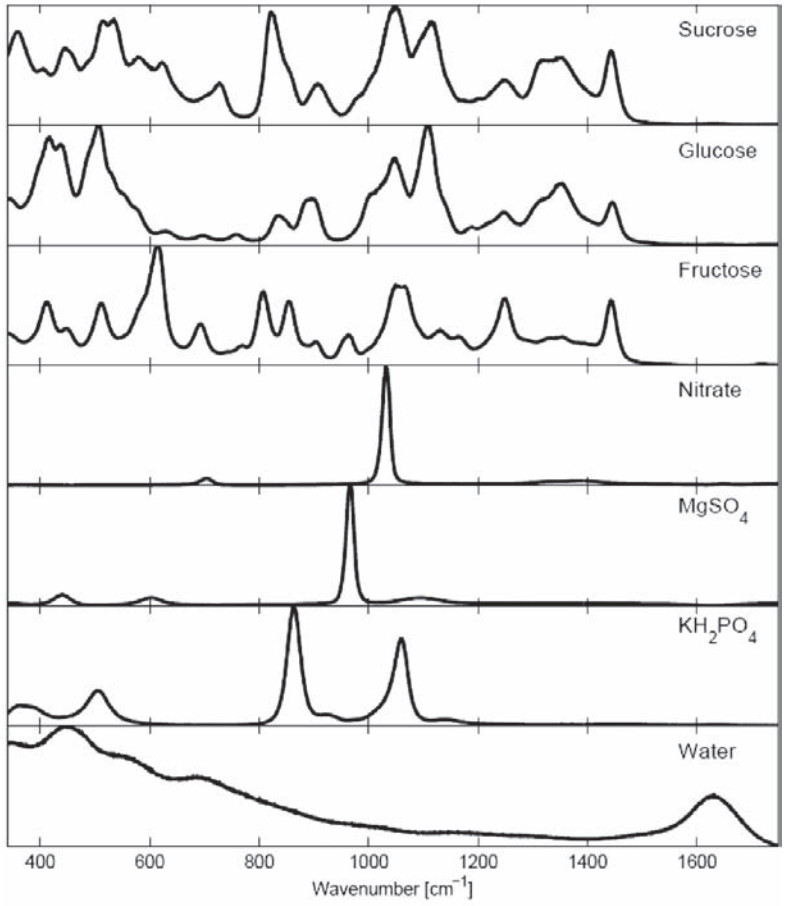



*Figure 2. Raman spectra of oil palm medium components.*

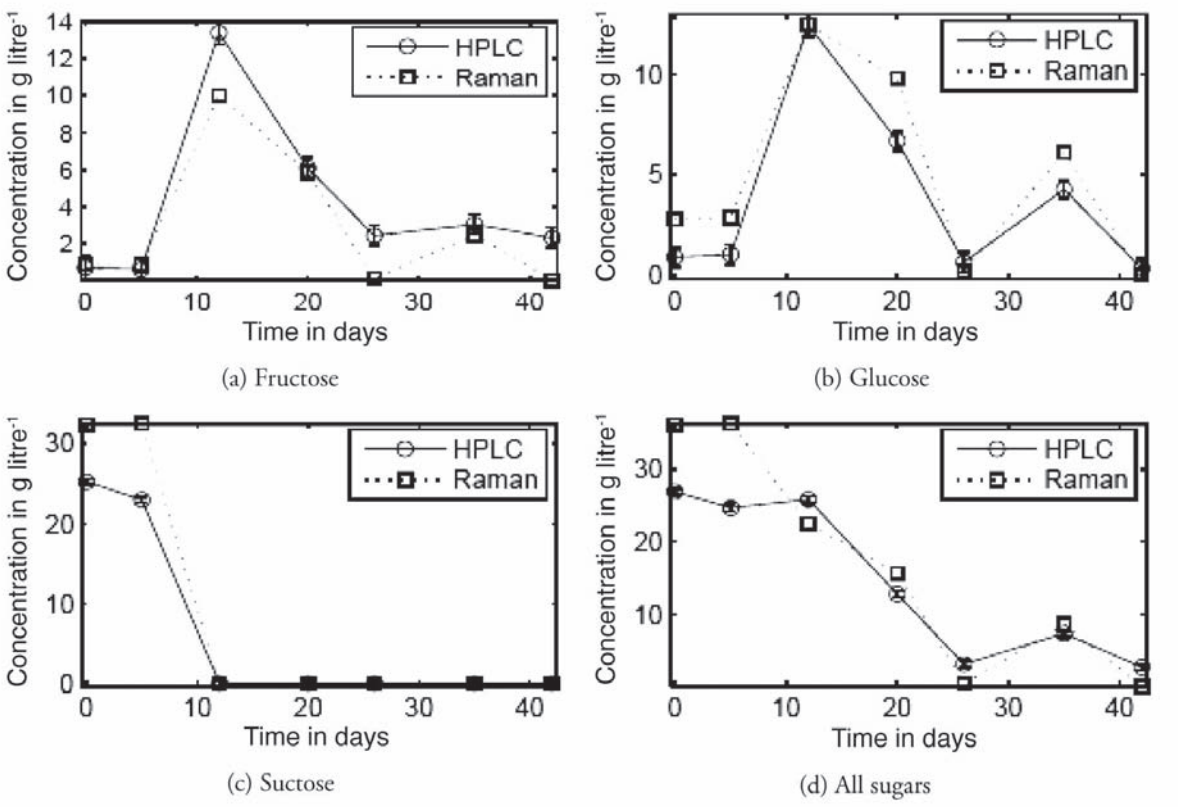



*Figure 3. Raman measurements are consistent with HPLC measurements. Concentrations of (a) fructose, (b) glucose, (c) sucrose and (d) all sugars (combined fructose, glucose and sucrose) measured by HPLC (O) and Raman spectroscopy (□). All measurements were performed on cell-free culture supernatant.*

time requirements of plant cell cultures, removing scatterers by filtering particles through a sterile filter or dialysis membrane into a chamber surrounding the Raman probe is a possibility, and has already been performed for a shake-flask culture of baker's yeast (Shaw *et al.*, 1999). With these steps implemented, Raman spectroscopy can provide a solution for online concentration estimation of oil palm cell culture.

**Fluorescence Spectroscopy for Calli Differentiation**

A valuable application of plant cell culture is the propagation of many clones of a plant with a desired phenotype. Even in the most controlled conditions, somaclonal variation and methylation of DNA caused by the unnatural environment can trigger mechanisms that prevent the calli from growing into plants. These non-embryogenic calli are essentially waste. It is therefore useful to determine as early as possible in a growth process whether a callus is embryogenic, *i.e.* whether it can develop into a plant. Current methods for differentiating embryogenic calli from non-embryogenic calli involve trained observations of callus morphology that involve the laborious, time consuming and qualitative visual inspection of each sample. These morphological differences can also correlate with differences in chemical composition. For *Medicago arborea* L. calli, for example, non-embryogenic calli were found to contain less starch (Martin *et al.*, 2000), implicating that a non-invasive test for starch could be used as a possible differentiation mechanism.

Raman spectroscopy was initially investigated as a means to differentiate calli samples; however, even at 785 nm excitation, any Raman scattering was overwhelmed by fluorescence from the sample. Fluorescence spectroscopy was therefore investigated as a method to determine the chemical contrast between calli with a white and friable phenotype and calli with a yellow and non-friable, or hard, phenotype. *Figure 4* displays photographs of the calli before and after sample preparation.

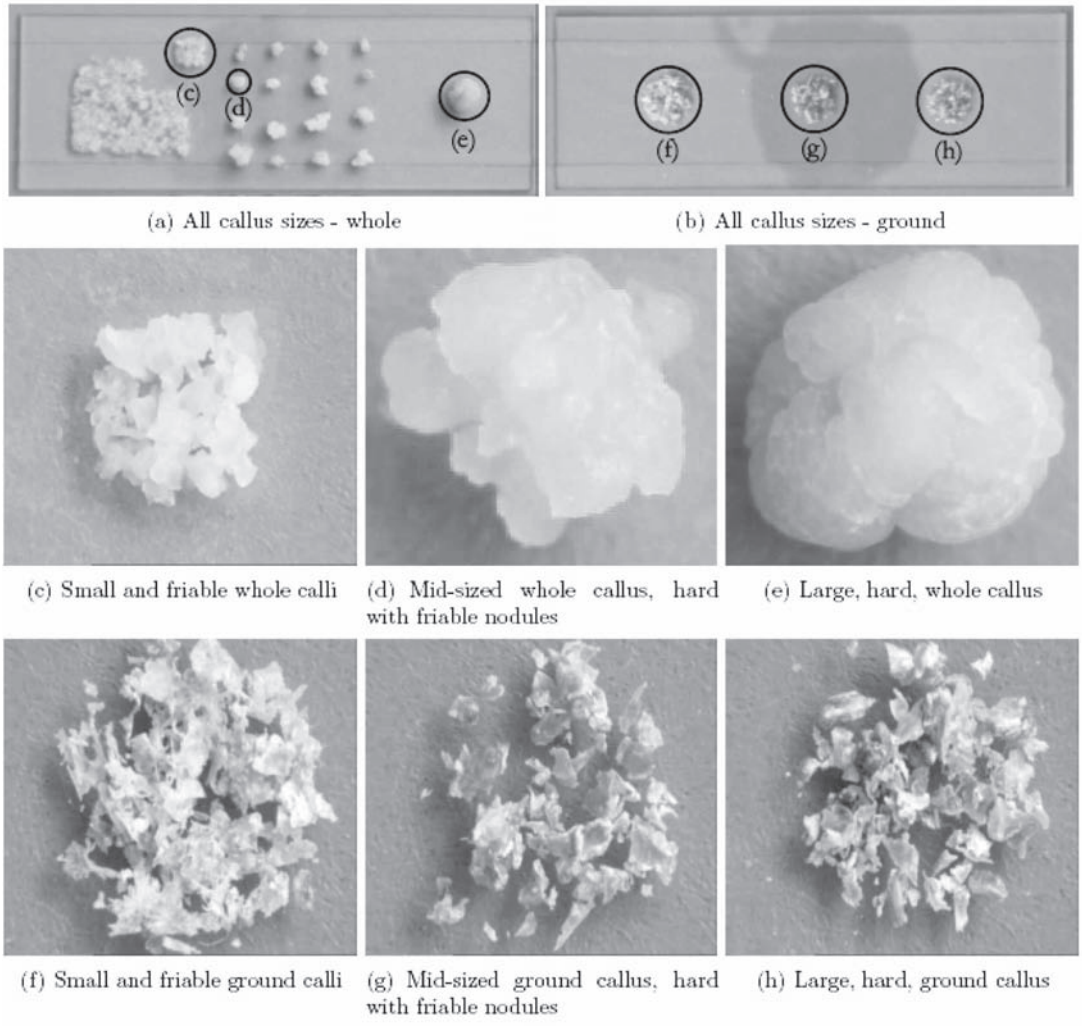



*Figure 4. Oil palm callus samples used for spectroscopic analysis. Panel (a): samples of oil palm suspension culture sorted based on morphology on a microscope slide; circled insets are expanded in panels (c),(d) and (e). Panel (b): dried and ground callus samples on a microscope slide; circled insets are expanded in panels (f), (g) and (h).*

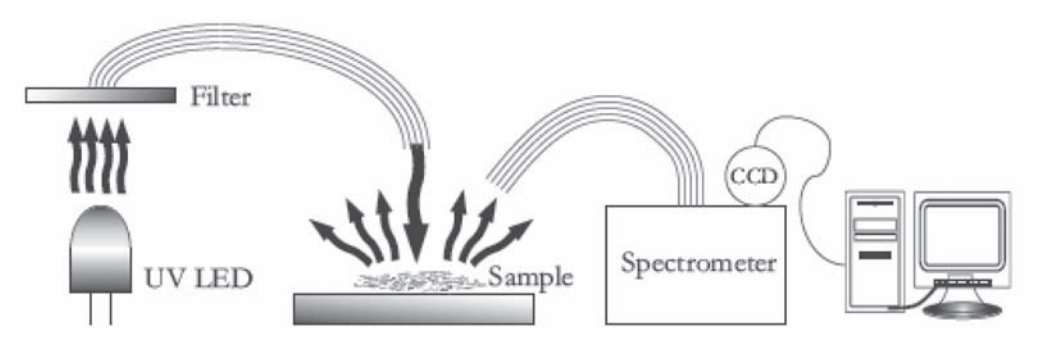



*Figure 5. Schematic of fluorescence spectroscopy set-up used in these experiments.*

Fluorescence spectra were acquired using the experimental setup shown in *Figure 5*.

The fluorescence spectra results shown in *Figure 6* are the averages of 10 frames taken with 100 s integration times. Contrast between the friable and non-friable phenotypes was discovered in the range of 430 nm to 560 nm and is shown in *Figure 6a*, normalized to the signal at 560 nm. The contrast region extended into shorter wavelengths, but the large signal from the tail of the excitation light dominates the total signal at these wavelengths, masking any fluorescence. A high intensity narrow band excitation source with proper shortpass excitation filtering and long-pass collection filtering would help resolve any fluorescence contrast in this wavelength region. To highlight the contrast, the ratio of the relative fluorescence spectra is shown in *Figure 6b*. The white phenotype had 20 % more signal in the blue-green region and more than 5% contrast up to 510 nm. Due to the limitations of the experimental set-up, the short wavelength contrast could also be due to a difference in absorption or scattering, and more work is required to confirm the source of the contrast.

**Cytometry**

Methods for the automated positioning of oil palm plants are valuable tools for the plantation worker and the tissue culturist. Others have developed robotic methods for planting oil palm seedlings (Pebrian and Yahya, 2003), and for picking micropropagated plantlets (Wang *et al*., 1999). We focused on an earlier step in the micropropagation pipeline and tested a fluidic method of positioning oil palm suspension cells with the ultimate goal of sorting on the basis of embryogenicity. Fluorescence-activated cell sorting (FACS) is a method wherein objects in solution pass through specially designed flow-cells, where they are optically profiled and the specimens of interest diverted and collected. The COPAS (Complex Object Parametric Analyser and Sorter) Device (Union Biometrica, Somerville, MA) is a flow cytometer designed to accommodate objects

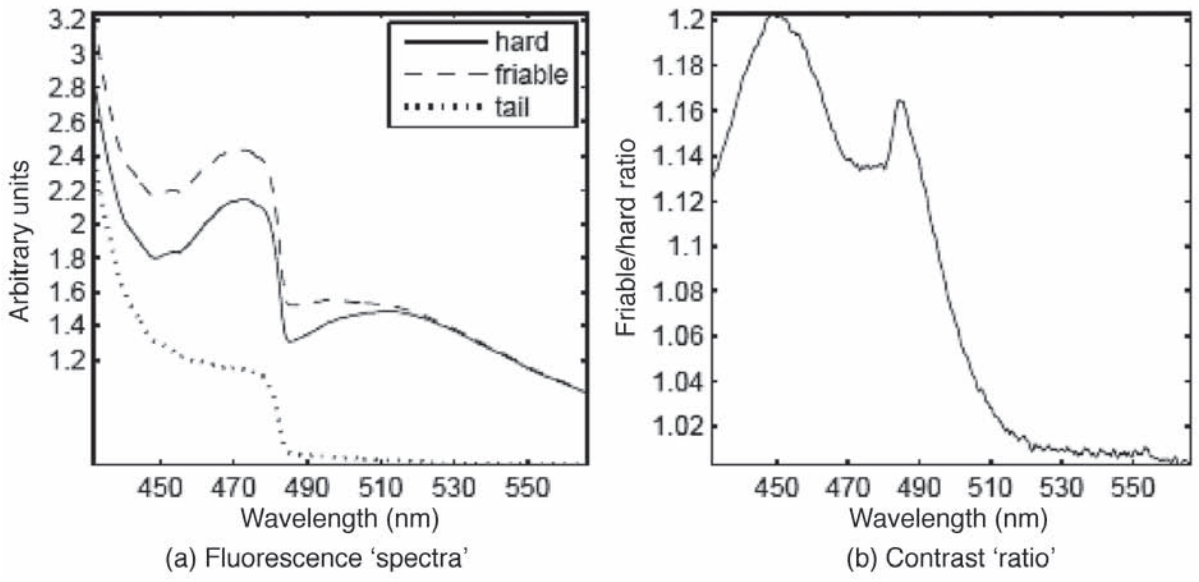
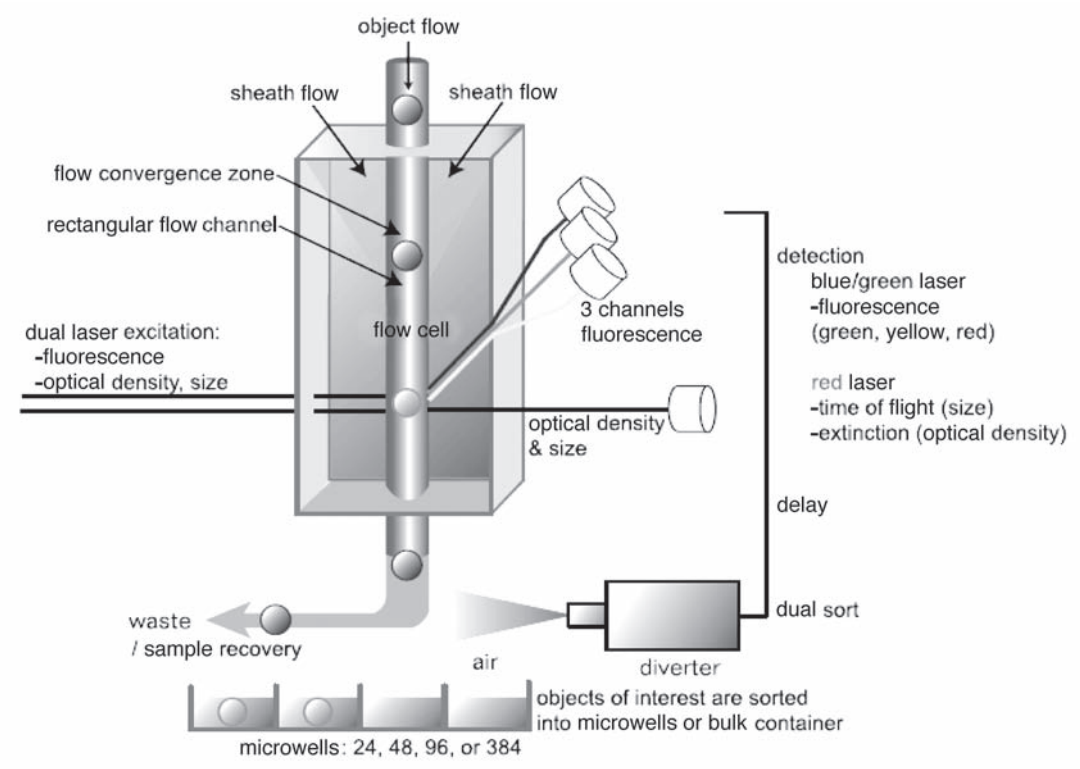



*Figure 6. Fluorescence signature of calli. (a) Samples of hard and friable oil palm calli were analysed via fluorescence spectroscopy. Tail refers to the signal from the excitation light, as described in the text. (b) The ratio of fluorescence signals from friable* vs. *hard calli.*




Source: Union Biometrica.

*Figure 7. Schematic of the Complex Object Parametric Anyalser and Sorter (COPAS) device.*

ranging in size from 10 to 1500 microns (diagrammed in *Figure 7*).

We harvested a sample of oil palm suspension cells, subjected the sample to sieving through 600 micron mesh, as in our standard subculture procedure, and then processed the cells through the fluidic device. As each oil palm particle, in an aqueous solution, passed in front of a sensor it was analysed for its size and optical spectrum. Particles were sorted on the basis of size. Based on the results of the optical analysis each callus particle was sorted into the 'selected' or 'non-selected' category. The sorting is non-destructive. The 'selected' particles were dispensed into the wells of microplates. During the run, the machinery processed oil palm samples at a rate of 1.67 s per well. We achieved ~97% accuracy of placing exactly one particle in each well. Data for one particle are shown in *Figure 8*. For this demonstration, yellow and green fluorescence data were collected as the particles were sorted using the standard optics on the equipment; for optimal sorting of oil palm samples based on fluorescence, the optics would need to be modified for measurement of shorter wavelengths, blue (430 nm) to blue-green (510 nm), where the constrast between friable and non-friable phenotypes is expected to be maximal. This methodology holds promise for sorting embryogenic from non-embryogenic calli.

## CONCLUSION

Plant cell cultures can be used to produce biochemicals metabolically in the suspension culture directly (Kieran *et al.*, 1997), and they can also be used for somatic clonal propagation of certain plant species (Ibaraki and Kurata, 2001). In this work, we explore the use of spectroscopic methods for oil palm cell culture processes. Raman spectroscopy has been examined in two separate clonal propagation bioprocess applications. Firstly, Raman analysis of the individual components of oil palm culture medium indicates that glucose, fructose and sucrose can be differentiated, and that overall nitrate content can be detected. We then carried out offline concentration estimation of filtered samples from a batch liquid oil palm bioreactor culture and confirmed these findings with estimation errors in the 10 mM range for fructose, glucose and sucrose. Incomplete HPLC data prevented comparison of nitrate concentration estimates with HPLC analysis. Error estimation indicated that 98.7% (3σ) of all sugar concentration estimates should fall within 3.4 mM of the actual value for shot-noise limited detection. Peak analysis of the residuals shows that interference of the phosphate buffer could be a major source of error. Research into oil palm cell culture shows that these errors correspond in time with changes in the

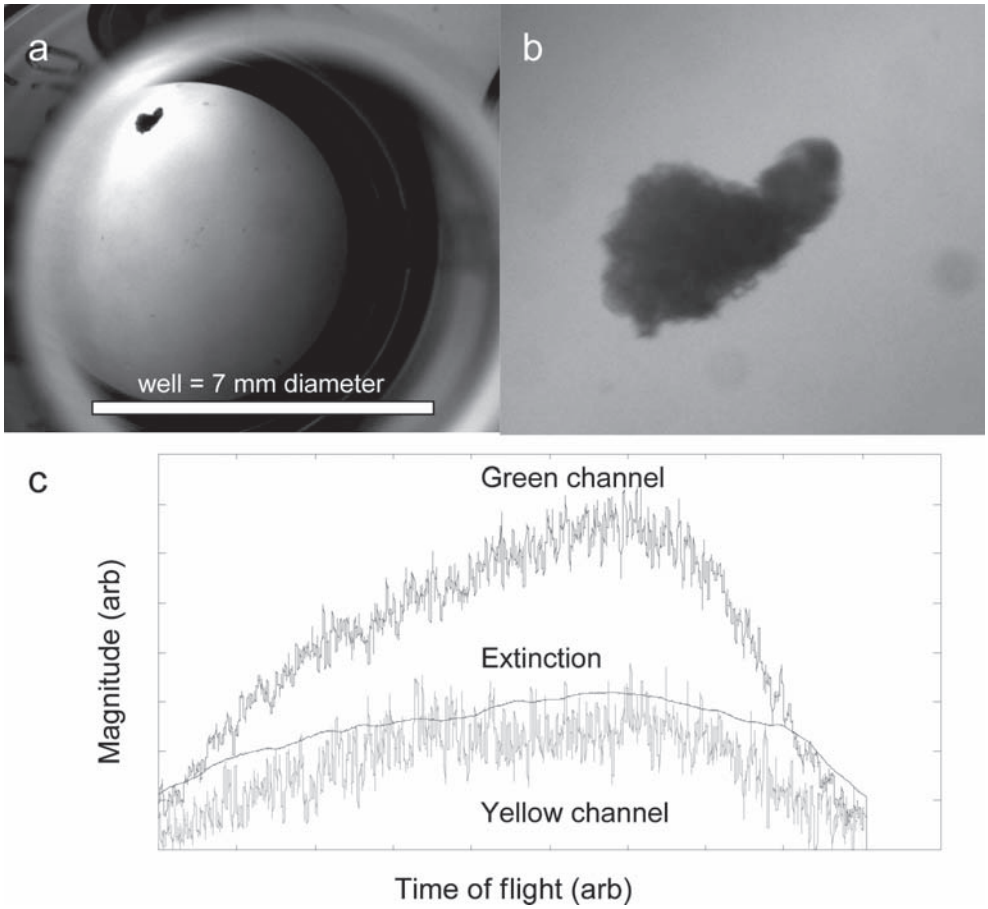



*Figure 8. Automated sorting of oil palm callus particles. Oil palm suspension cultures were sorted using a COPAS device and deposited, one particle per well, in a 96 well microplate. (a) Close-up of one particle in one well of the microplate (diameter of well=7 mm). (b) Magnified image of particle in panel (a). (c) Fluorescence data for oil palm particle shown in panels (a) and (b). Fluorescence in the green and yellow channels, as well as extinction, are plotted during the time of flight of the particle past the detectors. Magnitude of fluorescence and time of flight are shown in arbitrary units.*

pH of the medium (Gorret *et al*., 2004), suggesting that this source of error could be eliminated by controlling the pH in the bioreactor.

Secondly, we probed oil palm calli via Raman spectroscopy, revealing too much interference from fluorescence to resolve the Raman spectra for 785 nm excitation. However, this led to investigation of the fluorescence spectra of oil palm calli of a hard and yellow phenotype compared with calli of friable and white phenotype. These phenotypes were tested due to a correlation between a callus's morphology and its embryogenic properties (Teixeira *et al*., 1993). Fluorescence contrast was observed in wavelengths shorter than 510 nm. White phenotype calli had a 20% contrast compared to the yellow phenotype for these wavelengths. Additional experiments are required to determine whether the callus morphology, as differentiated by fluorescence spectroscopy, correlates with embryogenicity. If the criteria used in the experiment conducted are correct, the next step is to conduct fluorescence experiments using a narrower band of UV excitation with improved optical filtering. The fluorescence spectroscopy experiments reported in this work were carried out with dried and ground cells, in order to prevent differences in callus geometry from affecting the signal. Further experiments could be conducted on living calli to establish the difference in fluorescent contrast that can be observed with live cells in growth medium and to integrate the appropriate optics into a device to sort and separate calli for bioprocess integration.

We also demonstrated that oil palm suspension samples can be separated using the non-destructive microfluidic COPAS Device. Separation and sorting of pollen from other particles using flow cytometry with fluorescence as a differentiation mechanism has already been demonstrated (Byrne *et al*., 2003). A similar technique has also been applied to mouse embryoids (Bohlen *et al*., 2004) and human islets (Hanson *et al*., 2005). It is therefore clear that understanding the effect that embryogenesis has on fluorescence in oil palm calli can lead to high throughput automation with adaptation of available commercial equipment.

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