In Situ Monitoring of 3D In Vitro Cell Aggregation Using An Optical Imaging System

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ABSTRACT: Bioreactor systems that maintain cells and tissues in suspension are increasingly popular for culturing 3D constructs to avoid the loss of in vivo cell function associated with traditional 2D culture methods. There is a need for the online monitoring of such systems to provide better understanding and control of the processes involved and to prevent the disruption of these processes caused by offline sampling and endpoint analysis. We describe a system for the imaging and analysis of cell aggregation, over long periods, within a high aspect rotating vessel (HARV). The system exploits side illumination, using an adjustable beam pattern, to restrict the detected light to that scattered by the cell aggregates, thus eliminating the need for the fluorescent labeling of the cells. The in situ aggregation of mammalian cells (MCF-7 breast carcinoma cells) was monitored over an 8 h period and image sequences showing the growth and motion of the aggregates within the bio-reactor were obtained. Detailed size and population data have been derived characterizing the development of the aggregates during this time. We show how the number of resolvable aggregates increases to reach a peak and then declines as these aggregates merge. Once formed, remaining aggregates are found to consolidate to form more tightly packed bodies, typically reducing in cross-sectional area by one third. These results provide the basis for the development of an automated feedback system to control the growth of 3D cell cultures for repeatable, reliable, and quality controlled experimentation.

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Introduction

Within the body, mammalian cells function in a complex 3D environment. However, conventional monolayer cell cultures, as used in biological and toxicological studies, are often constrained to two dimensions (Monks et al., 1997). As a result of losing their environmental architecture, cells in 2D culture can lose in vivo functions, when cultured over prolonged periods, as cellular perception and interpretation of biochemical signals from the surrounding microenvironment is lost (Anders et al., 2003). Pioneering research in the field of breast cancer has illustrated that 3D culture systems can maintain in vivo cell signaling events and subsequent tissue function (Abbott, 2003; Schmeichel and Bissell, 2003). This has also been illustrated in tissue engineering with, for example, primary hepatocyte:stellate cell co-cultures in which hepatocyte function is maintained for longer periods within the 3D culture environment (Riccalton-Banks et al., 2003; Thomas et al., 2005). There is now great interest in developing scalable methods of generating and monitoring these 3D cell cultures that can be applied to the growth of...
any new tissue for use as in vivo-like in vitro models for biological studies and drug testing, and in regenerative medicine.

Bioreactor systems that maintain cells and tissues in suspension within their culture media are increasingly popular for culturing 3D constructs. Additionally, these systems encourage efficient mass transport of nutrients and waste products to ensure quality tissue growth. Such bioreactors are varied in design, are often custom built to suit the needs of the tissue being cultured and include flow perfusion and rotary culture systems (Martin and Vermette, 2005). However, a major limitation of current bioreactor systems is that assessment of 3D tissue or aggregate formation and function currently often requires offline sampling and endpoint analysis, causing considerable disruption to the experimental process. To take these 3D cultures into an industrial arena, will also require repeatable, reliable and quality controlled experimentation.

Real time in situ monitoring of cell cultures within bioreactor systems has been reported using a number of imaging techniques and bioreactor systems. For example, partially submerged microscope systems (termed ‘in situ microscopy’, ISM), of cells cultured in static bioreactors has been reported for monitoring cell density and initial cell aggregation (Guez et al., 2004; Joeris et al., 2002). In-line monitoring of engineered cartilage constructs using magnetic resonance imaging (MRI) has been used to determine the permeability of the constructs and to measure the macroscopic flow of culture medium through and around the growing tissue (Neves et al., 2006). Although both methods illustrate that in situ monitoring of a growing tissue or cell aggregate is possible, ISM monitors cell culture at a microscopic level (rather than at a macroscale tissue level) and MRI is not easily accessible to all researchers in the fields of tissue engineering and in vitro modeling. These techniques are not easily adapted to all bioreactor systems, especially those that have moving components to encourage efficient mass transport, such as rotary culture systems. Another imaging technology used for in situ monitoring of tissue growth is optical coherence tomography (OCT), which is capable of providing high resolution, cross-sectional images and has been used to study engineered, tubular tissue structures (Mason et al., 2004). However, it too is more suited to imaging microstructure as well as being limited to a depth of 2–3 mm.

To date, in situ analysis of cell aggregation dynamics in rotating systems has largely been based on mathematical modeling (Begley and Kleis, 2000; Cummings and Waters, 2007; Lappa, 2003; Ramirez et al., 2003; Waters et al., 2006). Where imaging systems have been used to validate the modeling, the dynamics of solid microparticles, not cells, have generally been assessed hence the true potential of optical imaging has not yet been fully exploited (Pollack et al., 2000). The monitoring of cell aggregation kinetics within rotating bioreactors has recently been demonstrated but only for fluorescently tagged cells (Manley and Lelkes, 2006).

The work described here is intended to be the first step in the development of an automated feedback system to control 3D cell culture in a rotating bioreactor. Such a system would adjust the rotation speed and other culture conditions in response to the measured aggregate population, size and distribution etc. We expect that carefully controlling the aggregation process will ultimately enable us to target regimes leading to the formation of either multiple aggregates of a specified mean size or a single large aggregate, as presented in this study, to provide the repeatability and quality control mentioned above. We present an optical system for imaging and analyzing 3D cell aggregate formation inside a rotating bioreactor with a high aspect ratio, from a suspension of single cells to large aggregates (10 mm in diameter). We demonstrate that aggregation data, that is, detailed size and population information, can be obtained automatically, without interrupting the rotation of the vessel for sampling, without fluorescent labeling of the cells and at a rate that is high compared with that of the aggregation process, effectively achieving continuous monitoring. In the first instance, we have demonstrated our system in line with experiments ongoing within the Tissue Engineering Laboratories at Nottingham to establish an in vitro model of breast cancer using MCF-7 carcinoma cells (Worrall, 2006). However, the technique presented is equally applicable to a range of tissue and cell types, including stem cells and cells whose properties and behavior are affected by fluorescent labeling, and bioreactor systems.

Materials and Methods

MCF-7 Monolayer Culture

MCF-7 breast carcinoma cells (ATCC) were cultured as monolayers prior to seeding in the rotary culture vessel. Cells were cultured in complete MCF-7 media which consisted of RPMI 1640 media (Sigma, Poole, Dorset, UK) supplemented with 10% (v/v) fetal calf serum (FCS; Sigma), 1% (v/v) non-essential amino acids (NEAA; Sigma), 2 mM L-Glutamine (Sigma), 100 μg streptomycin, 250 ng amphotericin B, 1,000 units/mL penicillin B (Sigma; sold as a complete antibiotic and antymycotic stock solution) and 20 mM HEPES (Sigma; MCF-7 complete media). Cells were incubated at 37°C in a humidified atmosphere of 5% CO₂ in air and medium was replenished every 2–3 days. Experiments were performed between passage 28 and 38.

IHARV Culture

The Imaging High Aspect Ratio Vessel (IHARV; 43.5 mm internal diameter × 6.7 mm depth; Fig. 1) was manufactured in-house to a design with minimal obstruction to the illumination and imaging of its contents by vessel ports. Key components in the IHARV were ‘Alcast’ Clear Cast Acrylic UV stabilised Perspex (Rotormotion Midlands Ltd., Leicester, UK) and the gas permeable membrane (bioFOLIE 25,
In Vitro Systems and Services, Osterode, Germany). Prior to cell seeding, the IHARV components were washed with 70% (v/v) industrial grade methylated spirit (IMS) and then irradiated with UV light for 30 min, after which they were assembled to form a 10 mL vessel and treated as sterile (Maharbiz et al., 2004). Confluent (70–90%) cell monolayers were incubated with 0.25% (v/v) trypsin (Sigma) and 0.02% EDTA (w/v; Sigma) solution at 37°C for 4 min to achieve a single cell suspension. MCF-7 complete media was then added to the mixture to inhibit enzyme activity and the resultant suspension was passed 6 times through an 18-gauge needle to ensure a single cell population. The culture media was replaced with fresh and the IHARV seeded with a suspension of 2 × 10⁶ cells mL⁻¹ and rotated at a constant 15 rpm within the imaging incubator at 37°C. Care was taken to expel any air bubbles from the vessel.

Automated Aggregate Imaging and Subsequent Analysis

The imaging system was housed in a custom made black Perspex incubator that excluded background light and isolated the light source and camera from the heated compartment (Fig. 2). A HeNe laser (Power = 20 mW CW, wavelength λ = 633 nm; JDS Uniphase, Milpitas, CA) provided the illumination, with neutral density filters (Thorlabs, Ely, UK) being used to control the irradiance of the IHARV. The beam was expanded to a diameter of 10 mm using a lens pair: a plano-concave lens (f = −10 mm; Comar, Cambridge, UK) and a doublet (f = 80 mm; Comar) and passed through a rectangular aperture. This allowed both the height and width of the beam cross section to be adjusted and was used to control the volume of the bioreactor that was illuminated. The mirror directed the beam onto the side of the vessel, providing illumination

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**Figure 1.** Component parts of the imaging high aspect ratio vessel. The dashed circle outlines the gas permeable membrane.

**Figure 2.** A schematic diagram showing the arrangement of the imaging system in the custom incubator.
parallel to the front face. The cylindrical lens \((f = -10 \text{ mm}, 16 \text{ mm} \times 6.3 \text{ mm}; \text{Comar})\) caused the beam to diverge in the vertical plane and ensured that the full height of the bioreactor cavity was illuminated. The volume of the vessel illuminated was typically a 2 mm thick slice close to the front face. With this arrangement, absorption of light by the cells was found to be negligible at the seeding density used. Also, illumination of the vessel faces through light scattered by the cellaggregate suspension was kept to acceptable levels to achieve good quality images. Although we only used one bioreactor design, the optics provide the flexibility to tailor the illumination pattern to suit different bioreactor geometries.

The IHARV was screwed onto the shaft of a gearbox driven by an electric motor (12 V DC motor, 200:1 ratio gearhead spur; Maxon Motors, Finchampstead, UK), the speed of which was controlled by adjusting the voltage applied by the motor power supply (EL302T Triple power supply; TTi, Huntingdon, UK). Images of the aggregate formation within the illuminated slice were acquired using a CCD camera (Jai CV-A11, maximum exposure time 1/30 s; Firstsight Vision, Tongham, UK) fitted with a zoom lens (Navitar Zoom 7000, f5.5–f28, 18–108 mm focal length; Edmund Optics, York, UK). At the highest resolution, 1 pixel corresponds to a 22 μm square in the object plane. The iris (Thorlabs) masked unwanted light scattered from the edges of the IHARV. A frame grabber card (Imagenation PXR800) installed in a PC was used to capture the desired images at 30 frames per second (fps). Software running on the PC controlled the exposure time, number of images taken, the intervals between them and their storage during processing. As the change in the subject brightness during the aggregation process, and therefore the correct camera exposure time, could not be determined in advance, exposure bracketing (i.e., using a range of exposure times) allowed the set of images taken at the optimum exposure to be selected before subsequent processing.

The stored images were processed using a program written in Matlab (The MathWorks, Natick, MA). This identified the aggregates, counted the number present and determined their cross sectional areas (CSAs). Scattering resulted in a residual, uneven background, varying with time, against which the aggregates had to be detected. Larger aggregates appeared as bright objects and were readily identified against the background by setting a high amplitude threshold. However, this missed smaller aggregates which appeared as fainter features in the images. A second stage of detection, that involved removing the background and large aggregates from the images, was necessary to enhance performance of the smaller signals. An estimate of the background in each image was obtained using Matlab’s morphological opening function (Bashtanov et al., 2004). This function removes features from an image that cannot completely enclose a user specified, structuring element. In this case a disc a little larger than the faint aggregates to be detected was used to filter out all such aggregates from each image, leaving the background. Removing contributions due to the detected bright aggregates and the estimated backgrounds yielded images in which the smaller aggregates were the predominant features. These, being less bright, were detected by means of a second, lower amplitude threshold (the thresholds were set empirically). Next, contiguous small and large aggregates, fragmented by the thresholding process, were recomposed. The final step was to automatically remove artifacts incorrectly identified as aggregates, based on measurements of their distribution within the vessel, their shape and their brightness. The processed images were used to obtain aggregate population and size data, that is, CSA. Values of each of these parameters were calculated for every image in the subset, giving fifteen values per parameter at each time interval. Although parts of the processing algorithm are currently manual, in principle, the entire process can be automated.

Particle size standards (polystyrene DVB microspheres; Duke Scientific Corporation, Fremont, CA) with a certified mean diameter of 300 μm ± 6 μm were used to verify the accuracy of the method. A suspension of these particles was imaged at with an exposure time of 1/125 s at maximum resolution (22 μm pixel\(^{-1}\)).

**Results**

**Measurements on Particle Size Standards**

The processed, output images (Fig. 3b) of the microsphere suspension were manually compared with the raw, input images (Fig. 3a) to confirm the correct detection of all the microspheres present. Note that there is no scattering from the center of monodispersed microspheres as, unlike similarly sized aggregates, they are isotropic. Features in the processed images due to monodispersed microspheres were identified and their diameters were measured at 331 μm ± 30 μm. This is consistent with the certified mean diameter: the slight overestimation of the size results from quantization errors due to pixelation of the image and from blurring due the motion of the particles. The quantization error is less at higher resolutions. Similarly, blurring is less at shorter exposure times.

**Monitoring Cell Aggregation**

Images of the cell aggregation process within the IHARV were captured at hourly intervals, starting with a newly seeded vessel. The full data set contained sequences of 15 images, taken at each of three different exposures (1/30, 1/60, and 1/125 s) every 10 min. Multiple images were captured at each interval to ensure that sufficient reliable image data could be obtained.
Example images are shown in Figure 4. Images taken following seeding of the vessel depicted the cell suspension as a cloudy haze (Fig. 4a). As the magnification of the imaging system was configured to view the whole vessel, it is not possible to resolve single cells and very small aggregates that were present at the start of the experiment. Light scattered by these aggregates gave rise to diffuse illumination of the bioreactor walls so that its structure, for example, the cut outs in the rear face (Fig. 1), is visible in the images taken at early times; this effect decreased as the aggregates grew. These structures are above the detection thresholds and are identified as possible aggregates during the initial stages of the image processing, however they are mostly rejected at later stages as they are linear features. Two hours after seeding, one large aggregate and a large number of smaller aggregates had started to form and rotated, clockwise, with the vessel (i.e., performed solid body wall rotation). Both the large and smaller aggregates continued to increase in size, with the greatest number of aggregates seen after 3 h (Fig. 4b). Further observations revealed that once the large aggregate had started to form, it merged with smaller ones with which it came into contact. Between the third and fourth hours there was a transition period during which the large aggregate ceased to be part of the solid body rotation and started to fall through the medium on the rising
side of the vessel. After 4 h, the large aggregate remained stationary relative to the observer at a position in the vessel corresponding to approximately 8 o’clock (Fig. 4c). Once the large aggregate started to fall through the medium, the capture of smaller aggregates was accelerated such that by the 5th hour, only one large aggregate remained.

Image Processing

The identification of aggregates within an image is illustrated in Figure 5. The first image shows the raw data (Fig. 5a). The final threshold image, showing both large and small aggregates, was used for subsequent quantitative data analysis (Fig. 5b).

Aggregate Population Data Derived From the Automated Image Collection

Aggregate Number

The total number of aggregates detected as a function of time is plotted in Figure 6. Initially, a low background count of false alarms was reported due to the diffuse illumination of the vessel by light scattered by the cell suspension. As the cells aggregated this effect reduced and discrete aggregates were detected in increasing numbers as they approached the system resolution of ~100 μm. The numbers reached a peak and then fell as aggregates merged until they were again fewer in number than the false detections. In contrast to the start of the experiment, the false detections were limited to parts of the vessel closest to the remaining large aggregate that scattered light onto these areas. Hence the number of false alarms was fewer at the end of the experiment than at the start. Some of the variation in the results is caused by shadowing of parts of the image by vessel ports at certain vessel orientations. Although this was taken into account during the vessel design it could not be eliminated completely. The effect is also seen in other results that follow. Taking a sequence of images at each datum point ensures complete illumination of the vessel.

Aggregate Size

The size of the largest detected aggregate throughout the experiment is shown in Figure 7. After 1.5 h, one large aggregate starts to dominate and continues to increase in size to attain a maximum measured CSA of 66 mm², reached 3 h and 40 min after the seeding of the IHARV. The aggregate subsequently compacts and its measured area decays to approximately two-thirds of its peak value.

Figure 8 illustrates the changing distribution of measured aggregate sizes. It shows the distribution of aggregates with areas of 25 pixels² or less at two different time points, 2 h and 40 min and 3 h and 20 min following seeding. Figure 8a was obtained with the number of aggregates at its peak and shows an increasing proportion of larger aggregate sizes within the population. Figure 8b shows the distribution of aggregate sizes, when the mean aggregate size was at its peak and illustrates the reduction in number of smaller aggregates.

The mean CSA of the small aggregates as a function of time is plotted in Figure 9. The area of the single large aggregate is excluded as it would dominate and conceal the evolution of the small aggregates that are far greater in number. The plot shows that the period during which a large number of detectable aggregates were present is from 2 to 4.5 h after seeding. Before this period the aggregates were not
large enough to be resolved. Afterwards, the vast majority of the smaller aggregates had been captured by the large aggregate. The peak in this plot shows that larger aggregates merged before all the smaller ones were captured.

**Discussion**

We have successfully imaged the in situ aggregation of mammalian cells within a rotary culture vessel over an 8 h period without the need for fluorescent labeling of the cells. The experiments were terminated at this point as the aggregation process was complete. The overall duration of the imaging is only limited by data storage needs and therefore, to all intents and purposes, can be performed indefinitely. Prior to this, manual sampling of the vessel at set time points was our only method of quantitatively assessing the aggregation process. Not only did this interfere with the experiment, as the rotation had to be stopped and the cell suspension agitated whilst sampling the vessel, but only data from a small sample of the cell suspension (1 mL from a 10 mL vessel) could be analyzed. This image analysis system allows data collection from any desired sampling volume, from a thin slice up to entire volume of the vessel. We have eliminated the complications of using fluorescence, such as photo-bleaching, and are therefore able to

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**Figure 6.** Plot of the number of aggregates within the bioreactor as a function of time since seeding (data set indicates variation).

**Figure 7.** Plot of the area of the largest detected aggregate as a function of time since seeding (data set indicates variation).

**Figure 8.** Bar charts showing the distribution of aggregate areas up to 25 pixels at (a) 2 h and 40 min and (b) 3 h and 20 min after seeding (error bars indicate SD).
continuously monitor the experiment over long periods of time. In addition, using the automated system allowed us to gather detailed information about the aggregation process as images were taken every 10 min; to collect and process this amount of data manually is impracticable. From automated image collection, we observed that small aggregates appeared first and adopted a ‘solid body rotation regime,’ that is, they have the same angular velocity as the vessel. These smaller aggregates continued to collide and combine to form a single large aggregate which entered the ‘settling regime’ where it falls through the medium so as to appear stationary relative to the observer (Freed and Vunjak-Novakovic, 1995). Again, this is data that we could not gather when performing these experiments manually.

Image processing enabled the quantification of parameters describing the aggregates throughout the aggregation process: measurements of the number of aggregates (Fig. 6), the largest aggregate CSA (Fig. 7) and small aggregate CSA (Figs. 8 and 9) were monitored. Care must be taken in using CSA to estimate the volume of aggregates. Small aggregates are free to tumble in the vessel and grow in all directions, however larger aggregates are constrained by the vessel dimensions to grow parallel to its faces so different relationships between CSA and volume, depending on aggregate size, will apply.

Aggregate number data (Fig. 6) was corroborated by aggregate size data which illustrated that the large aggregate increased in area rapidly between 3 h and 3 h and 40 min after seeding as merging of small aggregates occurred (Fig. 7). From this point onwards, fewer and smaller aggregates remained for the large aggregate to combine with and, although it continued to sweep them up, it started to consolidate to form a more tightly packed body as illustrated in the decrease in large aggregate area. This was confirmed by TEM (Worrall, 2006) which indicated that a loose association between cells occurred at first followed by enhanced cell–cell adhesion and compaction of the aggregate.

Although most of the image acquisition and processing has been automated, some areas, such as the setting of exposure times and detection thresholds and the selection of images without shadowing, still require manual intervention. Future developments of the system will see these operations being automated to take into account changes in the image characteristics during the aggregation process to give improved performance.

Applying imaging techniques to monitor 3D cell growth is rapidly becoming an essential tool in tissue engineering. In bioreactor applications, other groups have reported the use of optical methods (Ulber et al., 2003; Wolfbeis, 2004) to monitor glucose levels (Hantelmann et al., 2006; Jung et al., 2002), pH (Ge et al., 2006; Jeevarajan et al., 2002), cell viability (Gloeckner et al., 2001), and oxygen (Roy et al., 2001) during in situ culture and we envisage being able to incorporate these technologies into the system described here by adding the necessary sensors. We intend to use the data obtained using such techniques, combined with detailed aggregate measurements, to develop a fully automated bioreactor system for cell aggregation. In particular, we will investigate how the feedback of size and population statistics and other data can be used to control the reactor conditions and optimize aggregate formation. Where measured data are not available, for example, accurate measurements of the spatial distribution of nutrients within a HARV, we will use mathematical models to identify the optimum experimental conditions.

**Conclusions**

A fluorescence-free computerized imaging system has been demonstrated which provides aggregate formation data, including a count of the number of aggregates formed and measurements of their CSAs as a function of time. The system provides aggregation statistics much more frequently than can be achieved manually and does not require the rotation of the vessel to be stopped for sampling. In addition, it is limited by neither the problems associated with obtaining a representative sample of the aggregates, especially when their number is low, nor those of fluorescent labeling. In principle, the system is capable of providing data for feedback to control the reactor conditions and optimize the formation of the aggregates depending on the state of the process.

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Sawyer et al.: Monitoring Cell Aggregation In Situ 167

Sawyer et al.: Monitoring Cell Aggregation In Situ

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