Microwell perfusion array for high-throughput, long-term imaging of clonal growth

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Continuous cell tracking by time-lapse microscopy has led to detailed study of cell differentiation pathways using single cell fate maps. There are a multitude of cell fate outcomes, so hundreds of clonal division histories are required to measure these stochastic branching processes. This study examines the principle of condensing cell imaging information into a relatively small region to maximize live cell imaging throughput. High throughput clonal analysis of non-adherent cells by continuous live cell tracking was possible using a microwell perfusion array with an internal volume of $16\ \mu\text{l}$ and 600 microwells at the base. This study includes examination of biocompatibility of buffer systems, connecting tubing, cell culture substrates, and media degradation. An intermittent perfusion protocol was selected for long-term time-lapse imaging of KG1a cells in the microwell array; 1500 clones were simultaneously cultured and scanned every 3 min at $100\times$ magnifications for 6 days. The advantages of perfusion microwell culture are continuous long-term cell tracking, higher cell imaging throughput, and greater control over cell microenvironment. Microwell devices facilitate high throughput analysis of cell lineage development and measurement of the probability distribution for cell life events such as mitosis. © 2011 American Institute of Physics. [doi:10.1063/1.3669371]

I. INTRODUCTION

Time-lapse imaging of single cells provides detailed temporal information describing mitosis, gene expression, motility, as well as inheritance of these phenotypic traits.¹ Single cell lineage maps are generated from this data showing differentiation pathways.² High throughput live cell imaging has been combined with gene silencing by RNA interference to perform functional genome-wide screening for cell division, migration, and survival.³ These emerging technologies have a common requirement for long-term time-lapse imaging of the growth of large numbers of cells.

In comparison to traditional cell culture platforms, microbioreactors automate the control of microenvironment by supplying fresh media to cells and removing waste products for long term continuous imaging of cell development.⁴ Microgrooves or wells restrict cell migration and protect cells from shear stress.^{4,5} Constraining cell movement is necessary for atomic force microscopy⁶ or to study the formation of cell clusters.^{7,8} Lecault and colleagues⁹ employed $160\ \mu\text{m}$ cubic microchambers to study clonal culture of primitive mouse hematopoietic stem cells using live-cell imaging.⁹

Development of microdevices for long-term perfusion culture is a significant bioengineering challenge requiring in-depth analysis and optimisation of system properties. In Kim's review,

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relevant system properties are subdivided into (1) system design and fabrication, (2) perfusion culture operation, and (3) assessment of cellular phenotype and function.¹⁰ As discussed by Kim, biocompatibility of materials, their sterilisation, surface chemistry, and 3-D geometry are important design and fabrication considerations. Microfluidic devices have a high surface area to volume ratio (SAV), so surface adsorption of culture media components may limit growth. Regehr *et al.*¹¹ showed that particle desorption mass spectrometry (PDMS) oligomers leach into cells whilst a significant proportion of small hydrophobic molecules such as oestrogen from serum are absorbed by PDMS. At very low flow rates, evaporation across tubing and PDMS membranes increases the osmolality of culture media which is detrimental to cells.¹² Walker *et al.*¹³ introduced the concept of the effective culture volume, “an indicator of the cell’s ability to control its microenvironment during cell culture.” Cells in microscale culture generally have more control over their environment because the volume of media surrounding cells is much lower than tissue culture flasks. Cell seeding density will influence cell survival, so perfusion rates are to be scaled appropriately. Young and Beebe¹⁴ define the critical perfusion rate (CPR) as the media velocity at which substrate concentrations are depleted to zero at the bioreactor outlet. Thus, the rate of media exchange should exceed the nutrient requirement for the metabolising cell biomass. Furthermore, oxidative media degradation occurs rapidly at low cell density.¹⁵ These studies provide general guidelines for microdevice development.

In this study, we apply these principles to develop a microwell perfusion array (MPA) for continuous long-term imaging of non-adherent cells at high density. This design maximises the number of cells that are imaged for each field of view. Additionally, proliferating cells are retained at the bottom of the same microwell during media exchange overcoming the inherent limitation of flat bottomed culture vessels (Petri dishes or flasks) where media replacement can displace cells from the microscopic field of view. The aim of this study was to optimise MPA parameters for high throughput clonal analysis of motile cells. This included an assessment of the biocompatibility of the perfusion system (buffers, tubing, cell culture substrates, and media degradation), selection of a perfusion protocol, and analysis of the relationship between microwell seeding density and growth rate. Furthermore, this study benchmarks the cell imaging system with respect to the number of clones that can be continuously imaged in parallel.

II. MATERIALS AND METHODS

A. Static cell culture experiments

KG1a cells, an acute myeloid leukemic cell line,¹⁶ were used as a model system to study growth of non-adherent cell types. KG1a cells were cultured in T25 flasks (CELL STAR, Greiner bio-one, Germany) with Iscove’s Modified Dulbecco’s Medium (IMDM) (Sigma–Aldrich, Castle Hill, Australia) supplemented with 10% fetal bovine serum (FBS, JRH BIOSCIENCES, Kansas, USA), 1% penicillin/streptomycin (PS, Sigma–Aldrich, Castle Hill, Australia), and sodium bicarbonate buffer (3.5 g/L).

The effect of buffer system ($\pm$ sodium bicarbonate), polymer materials (polystyrene versus PDMS), and geometry (flat versus microwell) were studied in static culture using 35-mm Petri dishes. Triplicate 2 ml Petri dish cultures inoculated with 5×10^4 KG1a cells were incubated in a humidified incubator at 37 °C with 5% CO₂. A humidified incubator without added CO₂ was used to assess 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid (HEPES) buffer systems. Cells were counted daily for 6 days (18 parallel cultures in each experimental group) using an automated cell counter (Vi-cell, BECKMAN COULTER, California, USA). A growth curve was plotted for each experimental condition. The buffer systems examined were sodium bicarbonate with a 5% CO₂ in air gas mixture or 25 mM HEPES buffer in air.

PDMS culture surfaces were compared to tissue grade polystyrene. Petri dishes were modified by casting a 1 mm thick layer of PDMS at their base. Sylgard 184 elastomer base and curing agent (Dow Corning Corporation, Midland, USA) were mixed thoroughly at a weight ratio of 10:1 and cured overnight at 60 °C.

The PDMS microwell array was manufactured by soft lithography.¹⁷ Wells had a diameter of 200 μ m and depth of 35 μ m. They were arranged as a square lattice with 50 μ m interval

among wells. The PDMS microwell array was glued to the floor of Petri dishes by uncured PDMS. PDMS modified dishes were sterilized by 80% ethanol for 30 min, followed by rinsing by 0.9% sodium chloride (Baxter Healthcare, NSW, Australia) and IMDM.

B. Media degradation study

The rationale for this study was to quantify the effect of container, temperature, FBS, and light on the rate of media degradation. A simple bioassay was developed to measure media degradation by determining the growth rate of KG1a in test media diluted with fresh media.¹⁵ For the study of media degradation in different containers, sodium bicarbonate buffered IMDM (with 10% FBS and 1% PS) was stored in sealed sterilized glass bottles or polyethylene copolymer (PPC) syringes (TERUMO, Japan) at 4 °C and 37 °C for 72 h. The percentage (V/V) of test media in fresh media was 0%, 25%, 50%, 75%, and 90%. Half millilitre cultures inoculated with 4×10^4 cells in 24-well tissue grade polystyrene culture plates (Becton Dickinson Labware, NJ, USA) were incubated for 48 h in a humidified incubator at 37 °C with 5% CO₂ in air. Cell numbers were counted by Vi-cell.

The interaction of light, storage temperature, and serum on degradation of sodium bicarbonate buffered IMDM was studied in more detail by a three-factor, two-level full factorial experiment. In replicate experiments, media was stored in sterile glass bottles with or without 10% FBS, at temperature 23 °C or 37 °C and in the light or dark for 72 h (Table I). Treated media was assessed by the media degradation assay described above. The final concentration of treated media in cultures was 80%. It was necessary to add fresh FBS to non-serum containing groups to adjust the final FBS concentration to 10% for the cell culture assay (S5-S8, Table I). One milliliter cultures were inoculated with 1×10^5 cells and counted after 48 h of incubation as described above.

C. Biocompatibility of tubing

Tubing biocompatibility was measured by testing the growth of KG1a cells in media extracted by flow through four tubing types. Media was extracted at 4 °C with flow adjusted so that the extraction time was set to 6.6 h (Table II). SilasticTM (Dow Corning Corporation, Midland, USA), PharmedTM (Cole-Parmer Instruments, Illinois, USA), and polyethylene (Dural Plastics & Engineering, NSW, Australia) were sterilized by autoclave at 121 °C for 20 min. TygonTM (Cole-Parmer Instruments, Illinois, USA) was sterilized with 80% ethanol by perfusion at a flow rate of 20 μ l/min for 1 h. The internal diameter (I.D), flow rate, and lengths for each type of tubing are shown in Table II. The sterile perfusion system consisted of a syringe pump, and 1 ml PPC syringe containing IMDM with sodium bicarbonate connected to tubing using a polypropylene connector (Cole-Parmer Instruments, Illinois, USA). Media was collected into sterile glass vials and tested using the media degradation assay described above.

TABLE I. Media treatment method in the full factorial design.

Sample No.	Treatment		
	10% FBS	Temperature (°C)	Light
S1	Present	23	Present
S2	Present	37	Absent
S3	Present	23	Absent
S4	Present	37	Present
S5	Absent	23	Present
S6	Absent	37	Absent
S7	Absent	23	Absent
S8	Absent	37	Present

TABLE II. Tubing specification and media flow rates for biocompatibility studies. The residence time of media inside tubing was 6.6 h.

Tubing	I.D (mm)	Length (m)	Surface area/Volume (SAV) (mm^{-1})	Flow rate ($\mu\text{L}/\text{min}$)
Polyethylene tubing	0.8	3	5	3.79
Silastic TM tubing	0.64	3	6.25	2.43
Tygon TM tubing	0.25	5	16	0.62
Pharmed TM tubing	0.225	5	17.8	0.5

D. Microscope incubator

Figure 1(a) shows a schematic of the microscope incubator system. An acrylic incubator housing an Olympus inverted fluorescence microscope was maintained at 37.0 ± 0.1 °C by a heater fan enclosure inside the incubator controlled by a custom-built proportional-integral-derivative (PID) microcontroller. A smaller transparent gas-tight environmental chamber mounted on the microscope stage maintained a 5% CO₂ in air gas mixture around the MPA. An infrared CO₂ detector was connected to the output of the culture pod to check that the 5% CO₂ concentration was maintained within the environmental chamber. The air/CO₂ mix was humidified by recirculation through an underwater sealed sparging system.

The Olympus IX70 microscope was equipped with a $10\times$ phase contrast objective and automated x, y, z-stage. A 12-bit Peltier-cooled camera (1376×1032 pixels) with FireWireTM interface (F-View II, SIS XM10 Trigger supplied by Olympus) captured images. Cell/M software (Olympus) set the protocol. The microscope stage scanning and imaging protocol were controlled by Cell/M software (Olympus) and a custom-designed microprocessor for advancing stage positions.

E. Microwell perfusion system

Manufacture of the MPA was described in detail by Chen *et al.*¹⁷ Briefly, a PDMS flow-cell (with a $280\text{-}\mu\text{m}$ plate separation) directing flow over a microwell array (as described in Sec. II A) and immobilizing cells was fabricated by standard soft lithography (Figures 1(b) and 1(c)). Inlet and outlet ports on the flow-cell layer were punched out using a 23-gauge hole punch (Technical Innovations, Inc. Brazoria, Texas). The microwell array was irreversibly bonded to the flow-cell header by oxygen plasma etching. TygonTM tubing (Table II) was connected to 23 gauge inlet and outlet ports. As shown in Figure 1(a), the microwell array was placed inside the transparent gastight environmental chamber (Compartment C). Tubing from the MPA communicated with the outside through the wall of the environmental chamber via gastight collars sealed with PDMS. Inlet and outlet tubing was connected to a syringe and a waste container, respectively. The syringe pump (Harvard Apparatus, cat no 70-2212) was housed inside a fridge at 4 °C. The formation of gas bubbles inside the microdevice was prevented by a 20 cm hydraulic pressure head generated by the raised waste container (Figure 1(a)).

F. Microwell perfusion culture

The MPA was sterilized by 80% ethanol for 30 min at a flow rate of $20\text{ }\mu\text{L}/\text{min}$. The sterilization was followed by 30-min rinsing with 0.9% NaCl at the same flow rate. Finally, IMDM with bicarbonate supplemented with 30% FBS and 1% PS was injected at $10\text{ }\mu\text{L}/\text{min}$ for 30 min.

KG1a cell suspension with 1.5×10^5 cells/ml was injected into the flow-cell manually. Cells deposited by gravity into microwells without flow over 10 min. Residual cells from tubing and microwells were washed out of the device at a flow rate of $15\text{ }\mu\text{L}/\text{min}$ for 1 h using culture media. Prior studies have characterized the relationship between the number of cells retained in

FIG. 1. Schematic drawings of the perfusion cell culture system (a), the top view (b) and cross-sectional view in the flow direction (c) of the MPA. Plate separation $h = 280 \mu\text{m}$ and well radius is $100 \mu\text{m}$. The syringe pump and a syringe containing fresh media were placed inside a fridge (compartment A) at 4°C . The PDMS MPA was placed inside a gastight environmental chamber (compartment C). Inlet and outlet ports of the flow-cell were connected to a syringe and a waste container, respectively. Air with 5% CO_2 was recirculated through a humidifier with a sealed sparging system. The gastight environmental chamber, microscope, and humidifier were surrounded by a purpose-built incubator (compartment B).

microwells for various well geometries and shear stress. At this flow rate, an average of 3 cells was retained per microwell.¹⁷ The syringe pump and a 3-ml syringe containing media were placed inside a fridge at 4°C as shown in Figure 1(a). The total length of the inlet tubing was 1 m; the first 20 cm was inside the fridge, the middle 20 cm was at room temperature, and the remainder (60 cm) was inside the purpose-built incubator at 37°C . For continuous perfusion culture, media was injected at a flow rate of $0.3 \mu\text{l}/\text{min}$, while for intermittent perfusion culture, media was injected at the same flow rate for 1 h every 3 h with an average flow rate of $0.1 \mu\text{l}/\text{min}$.

min. Cells were continuously cultured for 6 days. During this time, 600 microwells were imaged every 3 min (100 microscopic fields of view).

G. Image and statistical analysis

Images acquired from long-term culture were processed by MATLAB software utilizing the image processing and statistics toolboxes (MATLAB R2010a). The software automatically identifies 6 microwells for each image and counts the number of cells in each well. The purpose designed segmentation algorithm identifies pixel patterns with circular geometry. An editing software package was used to inspect segmented cells and correct errors for each frame. Two-way analysis of variance (ANOVA) (Minitab™) was used to determine the effect of system properties on KG1a growth rates. Errors are standard deviations unless stated otherwise. The lag and exponential phases of KG1a cell cycle were estimated by the empirical cumulative distribution function (MATLAB statistics toolbox).

III. RESULTS AND DISCUSSION

A. Biocompatibility of the perfusion system

HEPES buffering of culture media has the advantage that there is no need for the gas tight environmental chamber for maintaining a 5% CO₂ atmosphere, a significant design simplification. However, it was shown that the growth of KG1a was greater with a sodium bicarbonate/CO₂ buffer system (Figure 2(a)). The biocompatibility of unmodified PDMS was confirmed; Figure 2(b) shows that the growth of KG1a on PDMS was similar to tissue grade polystyrene. The SAV for Petri dish culture was 10-fold lower than the microwell perfusion culture device (Table III). The absorption of small molecules and leaching of PDMS oligomers^{11,18} did not significantly affect the growth of KG1a cells.

It has been shown that HEPES buffer, riboflavin, and light generate cytotoxic reactive oxygen species (ROS) in cell culture media.^{19–21} Furthermore, serum contains essential components such as albumin to prevent oxidative damage to media.¹⁵ Most cell types can eliminate ROS via their glutathione reductase pathway, however excessive oxidative stress will occur if there are fewer cells per unit volume to metabolize ROS (i.e., high effective culture volumes). Thus, ROS generated by media in tubing and containment vessels may limit cell growth in microdevices.

Ideally, media not in contact with cells should be stored at 4 °C out of the light. However on its way to the perfusion bioreactor, media passes through containment vessels and tubing at room temperature or 37 °C. Careful attention to media degradation processes is necessary for design of microfluidic perfusion bioreactors.

Media degradation was assessed using a biological assay where KG1a cells were grown in media extracts. At the end of the culture period, the cell growth inhibition was calculated by

FIG. 2. KG1a growth curves for (a) cells cultured in IMDM with sodium bicarbonate buffer and 5% CO₂ in air or IMDM with HEPES in air, and (b) cells cultured on PDMS or polystyrene substrates.

TABLE III. Calculation of critical perfusion rate for KG1a cells.

Parameter	Value
Device cell number (N)	1500-30 000 cells
Flow cell area (A)	56 mm ²
Flow cell length (L)	20 mm
Flow cell volume (V)	16 μ L
Glucose uptake by KG1a (U)	6×10^{-17} mol/cell/s
Glucose concentration in IMDM (C)	25 mM
Device glucose consumption (NU)	$9-180 \times 10^{-14}$ mol/s
Critical media flow rate (NU/C)	$2.2-43 \times 10^{-4}$ μ L/min
Critical perfusion time (NU/VC)	2.5-51 days
Critical perfusion rate (LNU/VC)	$2.7-55 \times 10^{-5}$ cm/min

dividing the difference between the control cell number (cultured in fresh media) and the experimental cell number (cultured in extracted media) by the control cell number, and expressing this ratio as a percentage. Therefore, a positive percentage indicates growth inhibition, and the level of growth inhibition increases with the concentration of testing media (Figure 3(c), $p=0.0025$). We found that media stored in glass bottles and PPC syringes at both 4 °C and 37 °C did not inhibit cell growth.

A factorial design experiment was used to determine the interaction of light, serum, and temperature on media degradation. Figure 3(a) shows the responses for the experimental groups shown in Table I, and Figure 3(b) is normalised probability plot showing the main factor effects or their interactions.²² Exposure to light (effect C) increased media degradation, whilst FBS (effect A) reduced media degradation. FBS can reverse light-mediated media degradation (effect AC). The storage of media at the higher temperature (37 °C versus 23 °C, effect B) resulted in a modest reduction in media degradation compared to 23 °C. Detailed biochemical studies are required to determine the media degradation reactions and their temperature dependence. Therefore, for microfluidic perfusion culture, media was stored at 4 °C with FBS and protected from light.

Media residence time in tubing may be significant when flow rates are very low (0.1 μ L/min). Therefore, it was necessary to relate tubing residence time to the level of media degradation and tubing cytotoxicity. Of the studied tubing, only polyethylene tubing and TygonTM tubing were suitable for perfusion culture; Figure 3(c) shows that PharmedTM and SilasticTM tubing had unacceptable levels of media degradation. PharmedTM and TygonTM had similar surface area to volume ratios (Table II) but TygonTM had much lower growth inhibition ($p=0.0025$). TygonTM has a higher level of growth inhibition compared to polyethylene tubing. This result is consistent with the observation that TygonTM causes cell death or detachment in long-term perfusion culture.²³ Polyethylene tubing with a small internal diameter (I.D) (0.25 mm) would be the best choice for long-term perfusion culture. However, the only available polyethylene tubing for this study had an internal diameter of 0.8 mm with an unacceptably long residence time of 28 h at a flow rate of 0.3 μ L/min.

B. Microwell array perfusion culture

Potential media degradation by inlet tubing was minimized by selecting TygonTM tubing because of low internal diameter and lower rates of media degradation compared to SilasticTM and PharmedTM tubing. Polyethylene tubing would have been superior; however this study did not have access to low internal diameter tubing. A shorter length of tubing (1 m) was required to connect the refrigerated media supply to the microscope incubator compared to that used in the media degradation study (5 m, see Table II). The total residence time for continuous perfusion and intermittent perfusion culture was 2.7 h and 4.7 h, respectively, which was less than

FIG. 3. Media degradation studies. (a) Factorial design experiment to assess the effect of serum, light, and temperature on media degradation (Table I). (b) Normalised plot showing main effects FBS (A), 23 °C versus 37 °C (B), and light (C) and factor interactions (ABC and AC were significant). (c) Degradation of media by tubing. Error bars show standard deviations.

the tubing biocompatibility study (6.6 h, see Table II). The length of tubing at 37 °C inside the microscope incubator was 60 cm, so media was heated towards 37 °C for at least 90 min before entering the device. Additionally, the humidifier system (Figure 1(a)) minimized media evaporation. The pH was buffered using a bicarbonate/CO₂ buffer because of its superior performance (Figure 2(a)). The concentration of FBS was increased to 30% to counteract the absorption of FBS to tubing and PDMS.

The fold expansion (output cell number/input cell number) for both perfusion protocols is shown in Figure 4(a). The growth rate of KG1a in tissue culture dishes was higher than micro-well culture. Intermittent perfusion (1 h at 0.3 μ l/min followed by 2 h without flow) resulted in better growth of KG1a compared to continuous perfusion at 0.3 μ l/min. Static microwell culture had a similar growth rate compared to intermittent perfusion.

The effect of culture time and well inoculums on growth rate was determined by two-way ANOVA. For intermittent flow, there was a relatively minor positive effect of inoculum cell number on mean fold expansion (averaged over time) whereas for continuous flow, there was a modest negative effect (Figure 4(b)). The correlation coefficients for 0.1 μ l/min and 0.3 μ l/min were $r^2 = 0.1$ ($p = 0.002$) and $r^2 = 0.64$ ($p < 0.001$), respectively.

The observed opposing trend of cell density on growth rate may be related to local secretion of autocrine or paracrine factors which can influence the rate of cell division or apoptosis. Locally secreted inhibitory or stimulatory factors may explain either of these cell density dependent effects.²⁴ The much lower rate of growth for continuous perfusion culture may be related to increased exposure to media degradation products. Media cytotoxicity may also be reduced by recirculation, whilst maintaining the concentration of oxygen and nutrients.²⁵

To calculate the CPR for this device using the definition proposed by Young and Beebe,¹⁴ one selects a substrate that will limit cell growth if significantly depleted. As a first approximation, this calculation was based on glucose metabolism since its consumption would scale with

FIG. 4. Microwell perfusion culture: (a) Fold expansion versus culture time; Comparison among growth rates in tissue culture dish (solid line), microwell perfusion culture (0.1 $\mu\text{L}/\text{min}$ —dashed line and 0.3 $\mu\text{L}/\text{min}$ —dotted line) and microwell static culture (dash-dotted line). Error bars show standard error of the mean. (b) Effect of perfusion rates and inoculum on the fold expansion. Cell growth was inhibited at high flow rate. Note, “0.1 $\mu\text{L}/\text{min}$ perfusion” indicates the intermittent perfusion for 1 h at 0.3 $\mu\text{L}/\text{min}$ followed by 2 h without flow, while “0.3 $\mu\text{L}/\text{min}$ perfusion” represents the continuous perfusion at 0.3 $\mu\text{L}/\text{min}$.

other metabolites such as lactate production, amino acid consumption, and so on. One assumes that oxygen is in excess because of its relatively high solubility in PDMS. The critical perfusion rate for KG1a culture was calculated using the parameters shown in Table III.

In microscale culture, the absorption of growth factors becomes significant due to a large SAV compared to flask culture, which should be considered when determining the CPR.¹³ The SAV (4875 m^{-1}) is around 10 fold larger than a T25 flask (500 m^{-1}). The perfusion rates tested in this study (0.1–0.3 $\mu\text{L}/\text{min}$) are at least 1000 fold greater than the critical media flow rate ($<0.0043 \mu\text{L}/\text{min}$, Table III). One would anticipate that glucose will be depleted by less than 0.1% at the device outlet. For standard tissue culture of KG1a cells, media is replaced at a rate of at least $1 \text{ ml}/\text{day}/10^6$ cells. The equivalent media replacement rate to support 30 000 cells inside a microdevice would be $0.02 \mu\text{L}/\text{min}$. Thus, the microdevice flow rate (0.1 $\mu\text{L}/\text{min}$) is at least 5-fold higher than the flask tissue culture media exchange rate.

To assess the influence of autocrine factors on cell growth, the cell doubling time in wells with 1 cell/well and 8 cells/well was compared. Assuming cells in the microwell array grew with an exponential growth rate, the number of cells (N) at time t is given by

$$N = N_0 e^{kt}, \quad (1)$$

where N_0 is the initial cell number and k is a constant.

Since the doubling time $DT = \ln 2/k$, the ratio of doubling time DT_1 and DT_8 for initial cell density of 1 cell/well and 8 cells/well, respectively, was given below,

$$\frac{DT_1}{DT_8} = \frac{k_8}{k_1} = \frac{\ln(E_8)}{\ln(E_1)} = 1.057, \quad (2)$$

where $E_1 = 3.6$ and $E_8 = 3.87$ (see Figure 4(b)) were fold expansions during the 6-day culture when cell seeding density was 1 cell/well and 8 cells/well, respectively.

The cell doubling time with seeding density of 1 cell/well is only 5.7% larger than that with seeding density of 8 cells/well. So, the autocrine effect from the neighboring cells has a minor effect on cell growth.

C. Clonal throughput and analysis of cell cycle time heterogeneity

On average 3 cells were deposited per well for both perfusion cultures and around 500 microwells were tracked in parallel for 6 days at an imaging sampling interval of 3 min. Thus, the

throughput of this live cell clonal imaging system was around 1500 colonies per device. Individual cell trajectories could be tracked because cells migrate by less than a single cell diameter between two frames. Figure 5(a) (enhance online video) shows the division and movement of cells in a microwell in a 6-day culture period.

The live cell imaging data from microwell arrays can be analyzed in a number of ways. First, individual cell pedigrees are represented by binary branching trees as shown in Figure 5(b). Four clones were manually tracked over the first 100 h of culture. The y axis indicates the time from the beginning of culture, and the time of mitosis is recorded at each bifurcation. The division trees are used to observe cell cycle synchronization and the variation in cycle times for related progeny.

Second, the statistics of cell division (or other events) are performed by cell lifetime analysis. The empiric survival function (no division) was determined by the Kaplan–Meier (KM) statistic. The survival ($\pm 95\%$ confidence interval) is shown in Figure 5(c). The Smith–Martin model of cell cycle was used to approximate the KG1a cell generation time distribution.²⁶ The probability of survival (non-division) at a specified cell age T is

$$Pr[T > t] = \exp[-\lambda(t - \tau_0)], \quad t > \tau_0. \quad (3)$$

The Smith–Martin cell cycle parameters were estimated by linear regression with $\lambda = -0.1439 \text{ h}^{-1}$ and $\tau_0 = 25.4 \text{ h}$ ($R^2 = 0.924$). The time lag τ_0 is the obligate period required for cells to replicate DNA and divide, whereas the rate parameter gives an indication of the variability of cell cycle. The average cell cycle time calculated using this model was

FIG. 5. (a) Still image from a video showing cell division and movement over 6 days in a microwell. The image stack was thinned and time interval between 2 frames was 6 min. (b) Cell lineages of four individual clones in four wells created by manual processing of image stacks for 100-h cell culture (8000 images). Dots represent cells. (c) Empirical survivor function for cell generation time. LCB and UCB are the 95% lower and upper confidence interval for the Kaplan–Meier estimate. Dash-dotted line is a linear regression model. (a), (b), and (c) are generated from cells cultured with the average perfusion flow rate of $0.1 \mu\text{l}/\text{min}$. (enhanced online) [URL: <http://dx.doi.org/10.1063/1.3669371.1>].

$\tau_0 + \lambda^{-1} = 32.4$ h which was close to the observed mean cycle time (32.2 h, $N = 60$). KG1a generation time has a variability that is approximated by an exponential probability distribution with standard deviation $\lambda^{-1} = 6.95$ h. The coefficient of variation for cell cycle time was $6.95/32.4 \times 100\% = 21\%$. Only a small proportion of the variation could be explained by the effect of inoculation number on average doubling time (5.7%, Eq. (2)). The intrinsic variability of the observed cycle time highlights the stochastic nature of cell division and the importance of analyzing hundreds or thousands of clones to acquire statistically relevant observations.²⁶

The automated cell segmentation algorithm could identify wells (green circles, refer to color version online) and cells (green circles, refer to color version online) from phase contrast images (Figure (6)). An interactive software platform was developed to correct cell segmentation using mouse clicks. The number of cell segmentation errors increases at higher cell density, or if there was a large fraction of dead cells in the microwell. There were 288 000 images recording cell movement and divisions at 100 positions in a 6-day experiment. This software fell well short of automated trajectory mapping and is unable to perform detailed analysis of trajectory paths and division times for all 1500 clones. Semi-automated software for tracking division pedigrees as shown in Figure 5(b) is still under development.

Figure 7 shows a “heat” map which illustrates the distribution of cells with respect to individual wells on consecutive days of culture. The color map key on the right shows the number of cells per well. Rows and columns of the heat map index well identifications (IDs) and the sampling day, respectively. The wells were ordered from the lowest to highest cell number, so that cell seeding numbers increase down the page. Growth results in an increase in cell number from left to right, though cell number does not increase at a uniform rate; there are a range of cell expansion outcomes. This is particularly evident for the continuous flow experiment (Figure 7(b))

FIG. 6. Semi-automated cell counting. KG1a cells were randomly distributed between microwells using a flow protocol which deposited 3 cells per well on average. The images were taken on consecutive days from the same position on the microwell array. Green and red circles show automatically segmented wells and cells, respectively. This automated segmentation results were then inspected and corrected manually using an interactive software platform.

FIG. 7. Heat map showing the cell number in each well. (a) 0.1 $\mu\text{l}/\text{min}$ and (b) 0.3 $\mu\text{l}/\text{min}$.

where there was an increased rate of cell death. The heat map representation confirms that the growth rate at 0.3 $\mu\text{l}/\text{min}$ (Figure 7(b)) was lower than 0.1 $\mu\text{l}/\text{min}$ (Figure 7(a)).

IV. CONCLUSION

This study demonstrates the principle of condensing cell imaging information into a relatively small region to maximize live cell imaging throughput. The examination of biocompatibility was important to minimise media degradation during transport from a refrigerated supply to the microdevice. An intermittent perfusion protocol was selected for long-term, time-lapse imaging of KG1a cells in the microwell array with growth rates lower than standard tissue culture. The lower growth rate may be related to the large SAV with adsorption of media components.

For standard culture devices (Petri dishes, 96 well plates), it is not practical to scan the entire surface area at this sampling interval (3 min), so current live cell imaging systems rely on user selected regions of interest. Often cells pass outside of these fields of view, resulting in a high rate of “lost” cells. Microwell arrays constrain cell motion so that many clones are scanned in a single image, greatly enhancing the efficiency and throughput of live cell imaging microscopy. The high density microwell array ensures that each field of view at $100\times$ magnification has 6 microwells with at least 1 cell per microwell. High throughput is achieved by imaging 100×6 wells every 3 min. In these studies, we were able to track 1500 clones per experiment. Even higher throughput would be achieved at lower magnifications. The microwell array lends itself to clonal analysis of very motile, non-adherent cell types such as haematopoietic or immune cells (T and B lymphocytes). This study clearly illustrates the application of microwell devices to cell analysis; namely for high throughput analysis of cell lineage development and to measure the probability distribution of cell life events such as mitosis.

There are off-the-shelf microwell plates for static culture. Static systems require manual media replacement by hemidilution during which cell tracking trajectories may be lost because of media flow. The advantages of perfusion are greater control over cell microenvironment and continuous long-term tracking. In this study, there was little difference between the growth rate of cells in static microwell culture compared to perfusion microwell culture, though continuous perfusion at 0.3 $\mu\text{l}/\text{min}$ inhibited cell growth.

Future studies should focus on development of semi-automated software for segmenting clonal division trees,²⁷ vital fluorescent staining methods to facilitate cell tracking and identification of specific cellular states, and selection of more appropriate manufacturing materials to minimise physical adsorption of media components.

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