

# A microfluidic 3D hepatocyte chip for drug toxicity testing†

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We have developed a microfluidic 3D hepatocyte chip (3D HepaTox Chip) for *in vitro* drug toxicity testing to predict *in vivo* drug hepatotoxicity. The 3D HepaTox Chip is based on multiplexed microfluidic channels where a 3D microenvironment is engineered in each channel to maintain the hepatocytes' synthetic and metabolic functions. The multiplexed channels allow for simultaneous administration of multiple drug doses to functional primary hepatocytes while an incorporated concentration gradient generator enables the *in vitro* dose-dependent drug responses to predict *in vivo* hepatotoxicity. The IC<sub>50</sub> values of 5 model drugs derived from the dose-dependent on-chip testing correlate well with the reported *in vivo* LD<sub>50</sub> values. The 3D HepaTox Chip can be integrated with on-chip sensors and actuators as the next generation cell-based on-chip drug testing platform.

## 1. Introduction

With the cost of bringing a drug into the market rising to US\$ 400 million,<sup>1</sup> there is a greater need to eliminate false lead candidates by performing pre-clinical drug screening using *in vitro* models.<sup>2</sup> Current *in vitro* models are based on high-density well plates, which require a diverse array of automated platforms for drug preparation, administration, and analyses.<sup>3,4</sup> To perform more efficient *in vitro* drug testing, microfluidic chips have emerged as a suitable platform to conduct *in vitro* drug testing (*i.e.*, drug preparation, administration, and analyses) in a continuous and integrated fashion.<sup>3–6</sup> Such microfluidic chips are already commercially available for non-cell based *in vitro* drug screening, involving molecular assays, such as affinity

binding assays.<sup>3,7,8</sup> However, it is important that such drug testing microfluidic chips incorporate cells,<sup>5,9</sup> in particular primary hepatocytes. Since primary hepatocytes contain a broad range of metabolizing enzyme activities, hepatic transporters, and other differentiated functions,<sup>10</sup> microfluidic hepatocyte chips will be able to achieve *in vitro* drug testing that is more predictive of *in vivo* responses.

Microfluidic hepatocyte chips will need to incorporate specific features to meet the requirements for drug testing. Firstly, the hepatic microenvironment must be recapitulated adequately in the microfluidic system so as to maintain the hepatocytes' differentiated functions. The maintenance of these functions ensures that drugs are metabolized in a similar manner to the *in vivo* situation *i.e.*, a better predictability is achieved for the *in vitro* model.<sup>11</sup> The microfluidic system must also support independent fluidic addressing to multiplexed cell culture chambers so that different drugs or drug concentrations can be administered simultaneously for increased throughput.<sup>12,13</sup> Current microfluidic hepatocyte chips have only been partially successful in fulfilling these requirements. Microfluidic systems incorporating 3D culture or hepatocyte-fibroblast co-culture have been developed to augment hepatocyte functions,<sup>11,13–15</sup> but these systems lack independent fluidic addressing for multiplexed drug administration. On the other hand, an independently addressed microfluidic chip capable of multiplexed drug administration has been designed.<sup>12,16</sup> However, the functional maintenance of the hepatocytes in such microfluidic chips remains to be validated. Hence, there is a need to overcome the challenges of culturing functional primary hepatocytes in multiplexed microfluidic systems, which can be used for *in vitro* drug testing, and thus for correlating to *in vivo* drug responses.

Here, we have developed a multiplexed microfluidic hepatocyte culture system (hereafter referred to as the 3D HepaTox Chip) tailoring to the specific requirements of *in vitro* drug testing. The 3D HepaTox Chip is based on a 3D microfluidic cell culture system (3D-μFCCS), where a 3D microenvironment is engineered within microfluidic channels.<sup>17</sup> We multiplex the 3D-μFCCS, yielding 8 parallel cell culture channels that are independently addressed by the outputs of a concentration

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† Electronic supplementary information (ESI) available: Schematic of one-pass perfusion system used during perfusion culture and drug testing with the 3D HepaTox Chip (Fig. S1); solubilizing agents can increase the solubility of hydrophobic drugs (ketoconazole and rifampin), which had a tendency to be adsorbed onto the tubing used to deliver the drug solutions into the 3D HepaTox Chip, resulting in a lower effective drug concentration (Fig. S2); confocal images of nuclei staining for necrotic and total cell population in the 3D HepaTox Chip after 24 hours of treatment with 14.29 mM acetaminophen (APAP) (Fig. S3); comparison of IC<sub>50</sub> values calculated from the 3D HepaTox Chip and reported IC<sub>50</sub> values from literature (Table S1). See DOI: 10.1039/b900912d

gradient generator. We then show that this 3D microenvironment is suitable for maintaining functional hepatocytes at a level that is higher than or comparable to multi-well plate controls. The utility of the 3D HepaTox Chip is demonstrated by assessing the hepatotoxicity of 5 model drugs in a dose-dependent manner. IC<sub>50</sub> values that are estimated from the dose-response curves are correlated to the reported *in vivo* LD<sub>50</sub> values. The 3D HepaTox Chip, when fully validated, can be readily integrated with other micro-devices, *e.g.*, analytical systems, as the next generation of *in vitro* drug testing platform.

## 2. Experimental

### 2.1 Materials

All chemicals and reagents were purchased from Sigma-Aldrich Pte Ltd, Singapore unless otherwise stated. Stock solutions of drugs used for hepatotoxicity testing were prepared in dimethylsulfoxide (DMSO). The stock concentrations for drugs used were as follows: acetaminophen (10 mM), diclofenac (1 mM), quinidine (1 mM), rifampin (1 mM) and ketoconazole (250 µM).

### 2.2 Microfluidic device design and fabrication

The 3D HepaTox Chip was designed as a modular system (Fig 1A). It consisted of a multiplexed cell culture chip coupled to a linear concentration gradient generator. The multiplexed cell culture chip consisted of 8 parallel single cell culture channels. The dimensions of each cell culture channel were 1 cm (length) × 600 µm (width) × 100 µm (height). An array of 30 µm × 50 µm elliptical micropillars further separated the cell culture channel into 3 compartments: a 200 µm wide central cell culture compartment flanked by 2 side perfusion compartments (Fig 1B). The central cell compartments of each of the 8 channels were fluidically connected to a single inlet for introducing cell suspension. The dimensions of the cell seeding distribution channels were specified to ensure a constant cell suspension flow rate from the reservoir to all the 8 cell culture channels. The side perfusion compartments for each of the 8 cell culture channels were individually addressed by the outputs of the gradient generator. Culture medium or drug solutions were introduced into the 2 fluidic inlets of the multiplexed cell culture chip, delivered into the gradient generator and discrete solution concentrations were fed back to the side perfusion compartments of each cell culture channel in the multiplexed chip.

The multiplexed cell culture chip and concentration gradient generators were designed using AutoCAD (Autodesk Inc, USA). Silicon templates of the 3D HepaTox Chip were fabricated by the standard deep reactive ion etching (DRIE) process (Alcatel, France).<sup>17</sup> The microfluidic devices were then obtained by replica molding poly-dimethylsiloxane (PDMS) (Dow Corning, USA) on the silicon templates. Concentration gradient generators were fabricated by replica molding PDMS on a master template, which was obtained by patterning a 50 µm negative photoresist on a silicon wafer (SU8 2050, Microchem, USA). All PDMS structures were oxidized in oxygen plasma for 1 minute (125 W, 13.5 MHz, 50 sccm, and 40 millitorr) for irreversible chemical bonding to glass coverslips. Fluidic ports in the PDMS devices were made with a 23G punch (Technical Innovations Inc., USA). The 3D HepaTox Chip was connected to the concentration

gradient generator by 1 cm long 23G hypodermic tubings (New England Small Tubes Corp., USA). A cell reservoir, which comprised of a 4-way valve with 3 Luer connections (Cole-Palmer, USA) coupled to a 22G stainless steel hypodermic tubing with Luer fitting (Becton-Dickinson, USA), was then connected to the 3D HepaTox Chip to deliver cell suspension or methylated collagen to each of the microfluidic channels. The device was primed with ethanol, 0.2% bovine serum albumin (BSA) and 1X PBS consecutively using a pressure filling technique to remove all the bubbles.<sup>18</sup> The entire microfluidic system was sterilized by autoclaving at 105 °C for 30 minutes.

### 2.3 Methylated collagen and terpolymer preparation

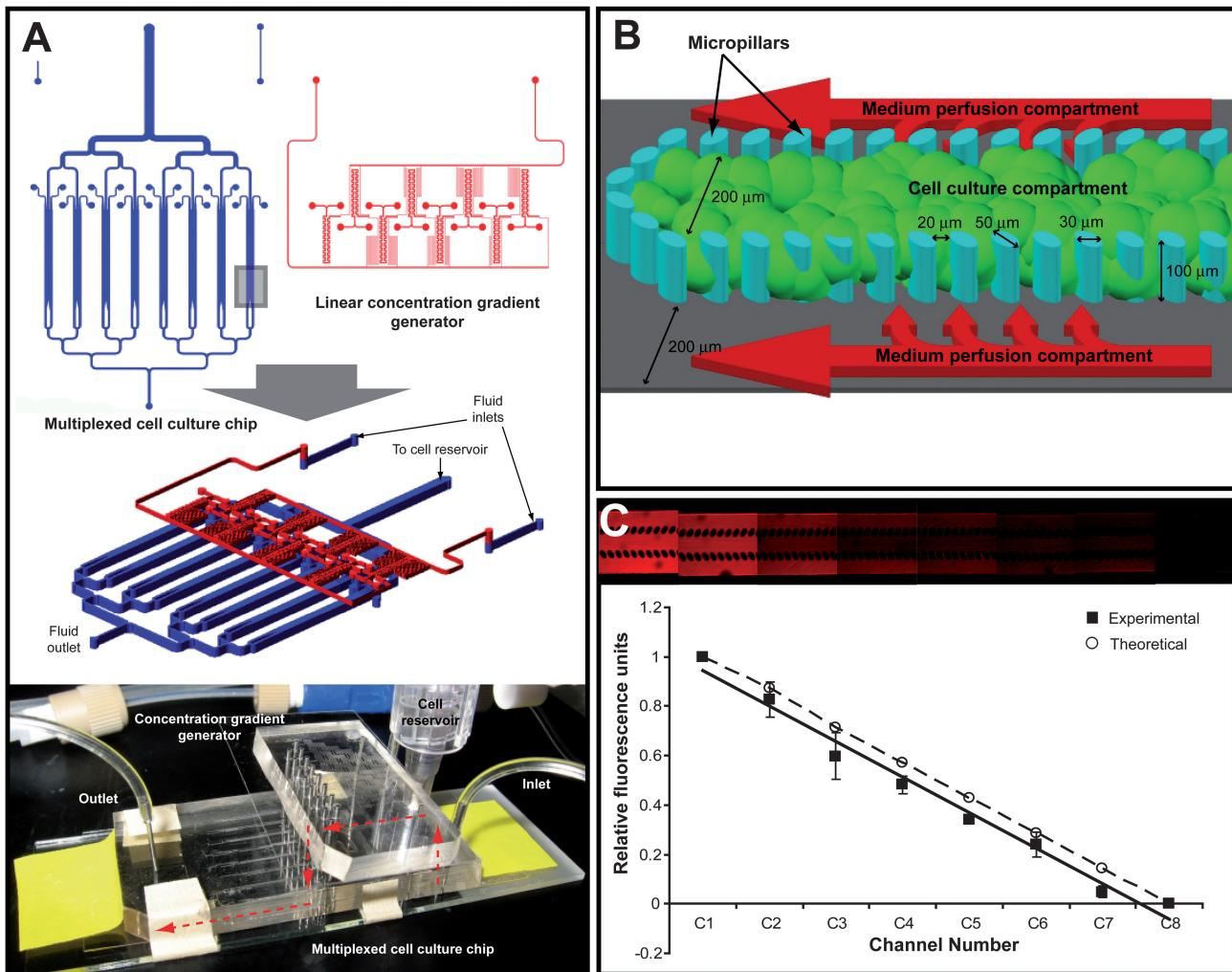
Positively charged methylated collagen and negatively charged HEMA-MMA-MAA terpolymer were chosen for laminar flow complex coacervation to form a localized 3D matrix because they have been shown to support hepatocyte functions.<sup>19</sup> Preparations of methylated collagen and HEMA-MMA-MAA terpolymer were as described previously.<sup>19</sup> 3% terpolymer solution (MW = 80.5 kDa), and 1.5 mg ml<sup>-1</sup> methylated collagen were used in this study.

### 2.4 Hepatocytes isolation and culture

Hepatocytes were harvested from male Wistar rats weighing between 250 and 300 g by a two-step *in situ* collagenase perfusion.<sup>20</sup> Animals were handled according to the IACUC protocol, approved by the IACUC committee of the National University of Singapore. Hepatocytes used in all experiments had cell viability of > 90%, as determined by a Trypan Blue exclusion assay, and a yield of 200–300 million cells. The hepatocytes were maintained in HepatoZYME SFM (Invitrogen, Singapore), supplemented with 0.1 µM dexamethasone, 100 units ml<sup>-1</sup> penicillin and 100 µg ml<sup>-1</sup> streptomycin. During the perfusion culture, a syringe pump (Cole-Parmer, USA) was used to drive culture medium in a one-pass perfusion system with an external bubble trap to remove bubbles generated during the course of the perfusion culture (ESI Fig. S1†). The perfusion culture medium was supplemented with 60 mM HEPES (Invitrogen, Singapore). The microfluidic system was placed onto a heating plate (MEDAX GmbH & Co. KG, Germany) maintained at 37 °C throughout the culture period. The medium perfusion flow rate was 480 µl hr<sup>-1</sup> (*i.e.*, 60 µl hr<sup>-1</sup> per cell culture channel). 2D multi-well plate hepatocyte cultures were established by plating hepatocytes on collagen type I-coated well-plates (Vitrogen collagen, Cohesion Technologies Inc., USA) at a density of 0.1 million cm<sup>-2</sup>, before culturing in a 5% CO<sub>2</sub> incubator.

### 2.5 Cell seeding in the 3D HepaTox Chip

Cell seeding in the 3D HepaTox Chip was adapted from cell seeding in the 3D-µFCCS with modifications.<sup>17</sup> Cell immobilization was initiated by withdrawing a hepatocyte suspension (3 × 10<sup>6</sup> cells ml<sup>-1</sup> of methylated collagen) from the cell reservoir *via* the device outlet at a flow rate of 130 µl hr<sup>-1</sup>. We used a low seeding flow rate to ensure high hepatocyte viability.<sup>17</sup> Therefore, the time taken for cells to travel from the inlet, through the flow distribution channels and finally fill up the 8 culture channels of the multiplexed microfluidic chip was approximately 1 hour. The culture medium was infused from the side perfusion channels at



**Fig. 1** 3D HepaTox Chip for the simultaneous administration of multiple drug concentrations. (A) Microfluidic design and assembly of the linear concentration gradient generator and multiplexed cell culture chip to construct the 3D HepaTox Chip. The bottom panel is a sample of the 3D HepaTox Chip. Dotted arrows depict the fluid flow path from the inlet to the outlet of the device. (B) Magnified view of a single cell culture channel of the multiplexed cell culture chip (grey box in Fig 1A). An array of  $30 \times 50 \mu\text{m}$  micropillars separated the channel into 3 compartments: a central cell culture compartment and 2 side media perfusion compartments. (C) Characterization of concentration gradient profile in the 3D HepaTox Chip coupled to a linear concentration gradient generator. The top panels are confocal micrographs showing gradients of Rhodamine G solution in the 3D HepaTox Chip. The bottom panels are the average fluorescence intensity in each microfluidic channel normalized against the fluorescence intensity of 100  $\mu\text{M}$  Rhodamine G. Solid lines are experimentally determined values, while dotted lines are theoretically predicted values. Data are represented as means  $\pm$  SD of 3 independent experiments.

a flow rate of  $90 \mu\text{l hr}^{-1}$  during the cell immobilization process to maintain cell viability. Laminar flow complex coacervation was implemented by simultaneously infusing methylated collagen (at  $10 \mu\text{l hr}^{-1}$ ) and terpolymer (at  $500 \mu\text{l hr}^{-1}$ ) via the cell reservoir and device inlets respectively with syringe pumps for 5 minutes. Upon the formation of the complex coacervated collagen-terpolymer matrix, excess polyelectrolytes were displaced by culture medium infused from the device inlets.

## 2.6 Functional assessment of hepatocytes

The culture conditions of different cell culture channels in the 3D HepaTox Chip are uniform, thus we used single-channel 3D HepaTox Chips to assess the differentiated functions of hepatocytes over a period of 72 hours.<sup>17</sup> The differentiated functions

of hepatocytes were assessed by measuring albumin production as well as basal and induced phase I and II metabolic activities. Hepatocytes cultured on collagen-coated multi-well plates were used as controls to benchmark the performance of the 3D HepaTox Chips. Phase I and II metabolic activities were indicated by cytochrome P450 (CYP) 1A1/2 isoenzymes and UDP-glucuronyltransferase (UGT) activities respectively. The basal and induced activities of the CYP isoenzymes, CYP1A1/2, were measured by the de-alkylation of the substrate, 3-cyano-7-ethoxycoumarin (CEC), to the highly fluorescent 3-cyano-7-hydroxycoumarin (CHC).<sup>21</sup> Basal and induced UGT activity was based on 4-methylumbelliferyl glucuronide (4-MUG) formation from 4-methylumbellifrone (4-MU).<sup>21</sup> All functional data were normalized to the DNA content in samples quantified using PicoGreen® assay (Molecular Probes, USA).

Albumin production was quantified with a rat albumin ELISA quantification kit (Bethyl Laboratories Inc., USA) in culture medium every 24 hours. Basal CYP 1A1/2 activities of hepatocytes in single-channel 3D HepaTox Chips were determined by infusing 50  $\mu\text{M}$  of 3-cyano-7-ethoxycoumarin (CEC) (Molecular Probes, USA) for 4 hours, while basal UGT activity was determined by infusing 100  $\mu\text{M}$  of 4-umbelliflferone (4-MU) (Merck, Singapore) for 4 hours. The perfusate was collected after which, 3-cyano-7-hydroxycoumarin (CHC) and 4-umbelliferyl glucuronide (4-MUG) were analyzed using capillary electrophoresis with laser induced fluorescence (CE-LIF) detection (Princ Technologies B.V., Netherlands).<sup>21</sup> Basal CYP 1A1/2 activities in multi-well plate controls were determined by incubating samples with 15  $\mu\text{M}$  of CEC for 4 hours before CE-LIF analysis. 15  $\mu\text{M}$  of CEC was used in the multi-well plate control since 15  $\mu\text{M}$  was found to be the effective concentration in the 3D HepaTox Chip after adsorption of the substrate onto tubing and connectors (data not shown). UGT activity in multi-well plate controls was determined by incubating the samples with 100  $\mu\text{M}$  of 4-MU for 4 hours before CE-LIF analysis. Induced phase I and II metabolic functions were assessed by dosing culture medium with inducers of CYP 1A1/2 activities (*i.e.*, 2  $\mu\text{M}$  3-methylcholanthrene) and UGT activity (*i.e.*, 50  $\mu\text{M}$  rifampin) for 20 hours prior to functional assessment at 24 and 72 hours of culture by CE-LIF as described above.

## 2.7 Characterization of concentration gradient profile in 3D HepaTox Chip

The concentration profile generated by a linear concentration gradient generator was evaluated experimentally in the 3D HepaTox Chip. The reagent concentration in each channel of the 3D HepaTox Chip was determined by measuring the steady state fluorescence intensity of a tracer dye, Rhodamine G. 100  $\mu\text{M}$  Rhodamine G solution and 1X PBS were introduced into each of the 2 inlets of the device at a combined flow rate of 300  $\mu\text{l hr}^{-1}$ . Fluorescence images of the microfluidic channels after 24 hours of perfusion were captured with a confocal microscope (Zeiss LSM 510 Meta, Carl Zeiss, Germany), and the average fluorescence intensity of 100 random points in each image was determined (Zeiss LSM Image Browser, Carl Zeiss, Germany). The average fluorescence intensity in each microfluidic channel was normalized against the fluorescence intensity of 100  $\mu\text{M}$  Rhodamine G solution. Theoretical values of Rhodamine G concentrations were calculated based on the design of the linear concentration gradient generator,<sup>22</sup> where 2 input solutions were divided into 8 different volumetric proportions and then combined in varying proportions to create discrete concentration ratios. Thus, the theoretical concentrations were calculated from a linear equation,  $y = (a/7)x$ , where  $a$  is the highest specified concentration administered and  $x \in [0\dots7]$ .

## 2.8 Hepatotoxicity testing with the 3D HepaTox Chip

Hepatotoxicity testing was performed with 5 model drugs: acetaminophen, diclofenac, quinidine, rifampin and ketoconazole. Drug dosing commences 24 hours after seeding primary hepatocytes into the 3D HepaTox Chip. Stock solutions of the drugs were diluted in culture medium, where the final

concentration of DMSO was kept below 1% to prevent detrimental effects on cells. Some of the drugs are hydrophobic and were adsorbed onto the fluidic tubing resulting in a lower effective concentration. Thus, 5% BSA was added to the culture medium to aid in the solubilization of rifampin and ketoconazole (ESI Fig. S2†). One of the fluidic inputs of the 3D HepaTox Chip was replaced with either 20 mM acetaminophen, 1000  $\mu\text{M}$  diclofenac, 1000  $\mu\text{M}$  quinidine, 2000  $\mu\text{M}$  rifampin, or 420  $\mu\text{M}$  ketoconazole. A linear concentration gradient generator was used to feed 8 different drug concentrations into the 8 channels of the 3D HepaTox Chip. For multi-well plate cultures, the hepatocytes were incubated with drugs at concentrations corresponding to that generated by the linear concentration gradient generator. DMSO vehicle controls were established by diluting corresponding amounts of DMSO in the culture medium without drugs. Hepatotoxicity was examined after 24 hours of drug dosing by quantifying cell viability as a cytotoxic endpoint. The cell viabilities after treatment with different drug concentrations were normalized against that of untreated cells. To calculate IC<sub>50</sub> values (*i.e.*, the concentration that produces 50% inhibitory effect on cellular activity), the dose-response data were fitted by a sigmoidal curve (SigmaPlot 8.0, Systat Software Inc., USA), and the concentration yielding a 50% decrease in normalized percentage cell viability was determined.

## 2.9 Quantification of cell viability

The viability of hepatocytes was quantitatively determined by a dual-nuclear staining method. 2 fluorescent nuclear dyes (*i.e.*, Propidium iodide (PI) (Molecular Probes, USA) and SYTOX Green (Molecular Probes, USA) were used to selectively stain the necrotic and total cell population. The *in situ* staining was effected by perfusing reagents at a flow rate of 2.0  $\text{ml hr}^{-1}$  through the 3D HepaTox Chip. Necrotic cells were stained by perfusing 25  $\mu\text{g ml}^{-1}$  PI in culture medium for 30 minutes, followed by fresh culture medium (15 minutes) to remove the excess dye. The cells were then fixed by perfusing 3.7% paraformaldehyde (PFA) for 30 minutes, and washed in 1X PBS for 15 minutes. The entire cell population was labeled with 250 nM SG and 200  $\mu\text{g ml}^{-1}$  RNAase for 30 minutes before washing in 1X PBS for 15 minutes. Hepatocytes in multi-well plate cultures were stained by incubating with 25  $\mu\text{g ml}^{-1}$  PI (30 minutes), 3.7% PFA (30 minutes), and 250 nM SG and 200  $\mu\text{g ml}^{-1}$  RNAase (30 minutes); the samples were washed 3 times with 1X PBS between reagent changes.

All stained samples were imaged with a confocal microscope at 488 nm and 543 nm excitation. For each microfluidic channel, a 100  $\mu\text{m}$  optical section (with 1  $\mu\text{m}$  step size) was acquired at 3 points along the length of the channel. A 3D reconstruction of each optical section was obtained (Zeiss LSM Image Browser, Carl Zeiss, Germany), and the number of objects in the 3D volume for both red (indicative of dead cells) and green (indicative of all cells) channels were quantified (ESI Fig. S3†). For multi-well plate cultures, images from a 0.85  $\text{mm}^2$  area-of-interest (AOI) were acquired at 400X magnification using the tile scan function of the confocal microscope. Cell viability was quantified by counting the number of objects in the green (indicative of all cells), and red channels (indicative of dead cells) with an image processing software (Image-Pro® Plus 4.5.1,

Media Cybernetics Inc., MD, USA). 3 AOIs were acquired for each drug concentration tested. The average percentage cell viability of each microfluidic channel was calculated as:

#### Viability

$$= \frac{\sum_{\text{optical sections}} \left[ \frac{\text{No. of green objects} - \text{No. of red objects}}{\text{No. of green objects}} \right]}{\text{No. of optical sections}} \times 100\%$$

### 3. Results and discussion

Our approach for realizing a microfluidic hepatocyte model, which has utility in *in vitro* drug testing, involves engineering a microenvironment that is conducive for the maintenance of hepatocyte functions, and extending the design of the basic hepatocyte culture system to enable multiplexed drug testing. The 3D HepaTox Chip consists of a multiplexed microfluidic system with 8 parallel cell culture channels independently addressed by the outputs of a linear concentration gradient generator. A 3D cellular microenvironment is constructed in each cell culture channel,<sup>17</sup> which can maintain the differentiated functions of hepatocytes (*i.e.*, albumin synthesis, basal/induced CYP 1A1/2 and UDP-glucuronyltransferase activities) at a level that is comparable or higher than the multi-well plate controls. The 3D HepaTox chip enables *in vitro* toxicity testing by allowing for the simultaneous, dose-dependent administration of drugs to functional primary hepatocytes. We used the 3D HepaTox Chip to assess the hepatotoxicity of 5 model drugs. IC<sub>50</sub> values that are derived from the dose-response curves are correlated to the reported *in vivo* LD<sub>50</sub> values, illustrating the potential predictive value of the 3D HepaTox Chip for acute hepatotoxicity.

#### 3.1 3D HepaTox Chip for administration of multiple drug concentrations to 3D hepatocyte cultures

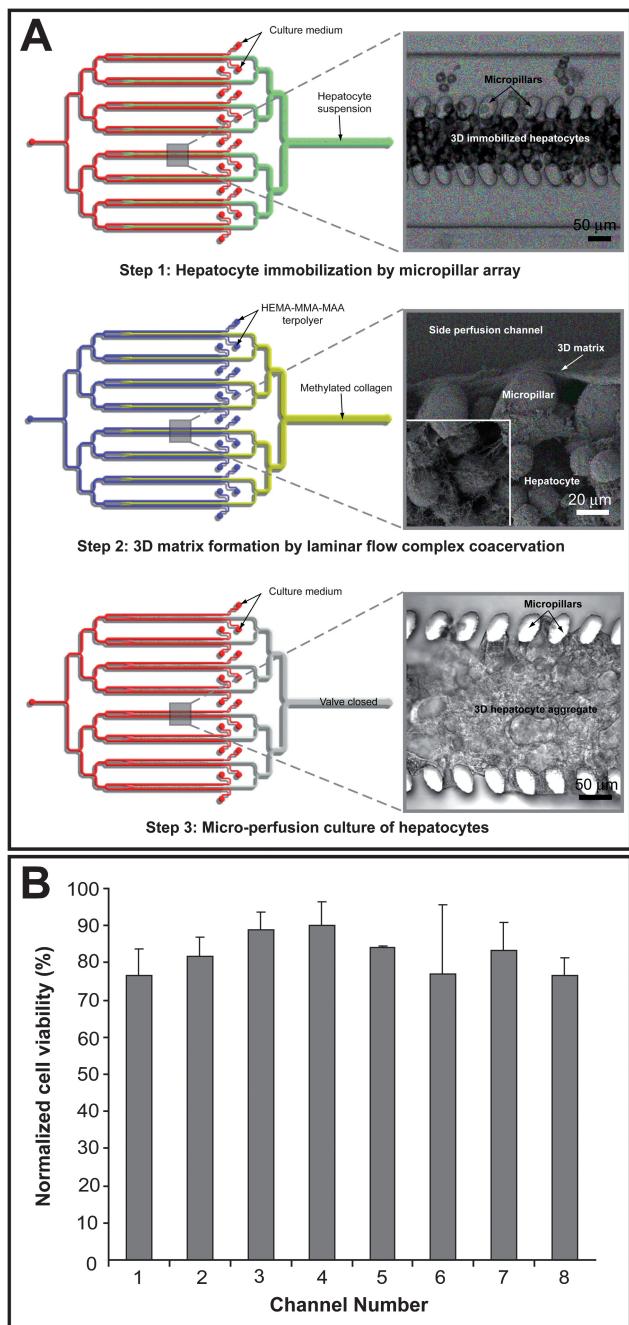
An important criterion to facilitate drug testing pertains to the multiplexing capability of the *in vitro* model. We designed the 3D HepaTox Chip to be a modular multiplexed microfluidic system. A multiplexed cell culture chip and a linear concentration gradient generator were independently fabricated and connected to each other *via* stainless steel hypodermic tubings (Fig. 1A). The multiplexed cell culture chip consists of 8 parallel microfluidic cell culture channels that are further divided into a central cell culture compartment and 2 side perfusion compartments (Fig. 1B). The side perfusion compartments of each channel in the multiplexed cell culture chip are independently addressed by outputs from the linear concentration gradient generator. Independent fluidic addressing allows for higher throughput drug testing, where multiple drug concentrations can be administered simultaneously. Drug solutions are introduced *via* the fluidic inlets in the multiplexed cell culture chip and delivered into the linear concentration gradient generator, where the fluid streams are split and recombined at varying volumetric proportions to create 8 different concentrations (Fig 1A).<sup>22</sup> The fluid streams with discrete drug concentrations are then delivered to the side

perfusion compartments of each cell culture channel in the multiplexed chip. We experimentally determined the linear concentration profile of a tracer dye, Rhodamine G, in the 3D HepaTox Chip, which concurred with the calculated theoretical values (Fig. 1C).

Besides facilitating multiplexed drug administration, it is important to establish a microenvironment that is conducive for the maintenance of hepatocyte functions. A 3D culture environment has been shown to be one of the important factors for hepatocytes to remain functional.<sup>11,23</sup> We have previously implemented a 3D microenvironment in single microfluidic channels by presenting cells with 3D cell-cell and cell-matrix interactions.<sup>17</sup> Here, we demonstrate that the process of constructing the 3D microenvironment can be adapted to a multiplexed microfluidic system. Firstly, hepatocytes must be immobilized at high density by the micropillar array in each of the cell culture channels to form 3D cell-cell interactions (Fig. 2A). Hepatocyte suspension was introduced from a single inlet and distributed into the central compartments of each cell culture channel. The cells were immobilized by the micropillars as they flowed through the central cell compartment since the gap size of the micropillar array (*i.e.*, 20 µm) was smaller than hepatocytes. After the hepatocytes were seeded, a localized 3D matrix was formed to present the cells with 3D cell-matrix interactions without obstructing the side perfusion channels (Fig. 2A). This is achieved by the laminar flow complex coacervation of a positively-charged modified collagen and negatively-charged acrylate based terpolymer.<sup>24</sup> Thus, hepatocytes cultured in our 3D HepaTox Chip are maintained in a 3D microenvironment experiencing both 3D cell-cell and cell-matrix interactions. We observed that after 72 hours of perfusion in the 3D HepaTox Chip, hepatocytes formed multi-cellular aggregates with 3D cyto-architecture, reminiscent of highly-functional 3D hepatocyte spheroids<sup>25</sup> (Fig. 2A). We further validated the uniformity of the culture conditions within the 3D HepaTox Chip by determining the viability of hepatocytes after 72 hours of perfusion culture; and observed that there were not significant differences across different cell culture channels (single factor ANOVA, p < 0.05) (Fig. 2B).

#### 3.2 Functional validation of hepatocytes cultured in 3D HepaTox Chip

Maintenance of hepatocyte differentiated functions in the 3D HepaTox Chip is pivotal to its application in *in vitro* drug testing, particularly when evaluating hepatotoxicity and drug-drug interactions, which are major causes for withdrawal of drugs from the market.<sup>26,27</sup> To ascertain whether the microenvironment in the 3D HepaTox Chip sustains functional hepatocytes, we perfusion-cultured primary hepatocytes for 72 hours and assessed their differentiated functions. The differentiated functions of hepatocytes were indicated by albumin production, cytochrome P450 (CYP) 1A1/2 (a phase I metabolic enzyme) activity and UDP-glucuronyltransferase (UGT) (a phase II metabolic enzyme) activity.<sup>11,19,28</sup> We measured both basal and induced levels of metabolic enzymatic activities because some drug toxicity may be caused by drug-drug interactions where one of the co-administered drug is non-toxic but induces metabolic enzymatic activity.<sup>29</sup> Hepatocytes in the 3D HepaTox Chip could



**Fig. 2** Engineering a 3D microenvironment for hepatocyte culture in the 3D HepaTox Chip. (A) Schematics for seeding and culturing hepatocytes three-dimensionally in the multiplexed cell culture chip. An array of micropillars within a microfluidic channel immobilized cells at high density resulting in 3D cell-cell interactions (step 1). 3D cell-matrix interactions were presented by forming a localized 3D matrix *via* laminar flow complex coacervation of methylated collagen and HEMA-MMA-MAA terpolymer (step 2). Hepatocytes cultured by perfusing culture media from the side perfusion channels formed 3D aggregates (step 3). The images in the right panel are magnified views of a single channel after each step. (B) Cell viability in each channel of the 3D HepaTox Chip after 72 hours of perfusion culture. There was no significant difference in cell viability between the different channels (single factor ANOVA,  $p < 0.05$ ).

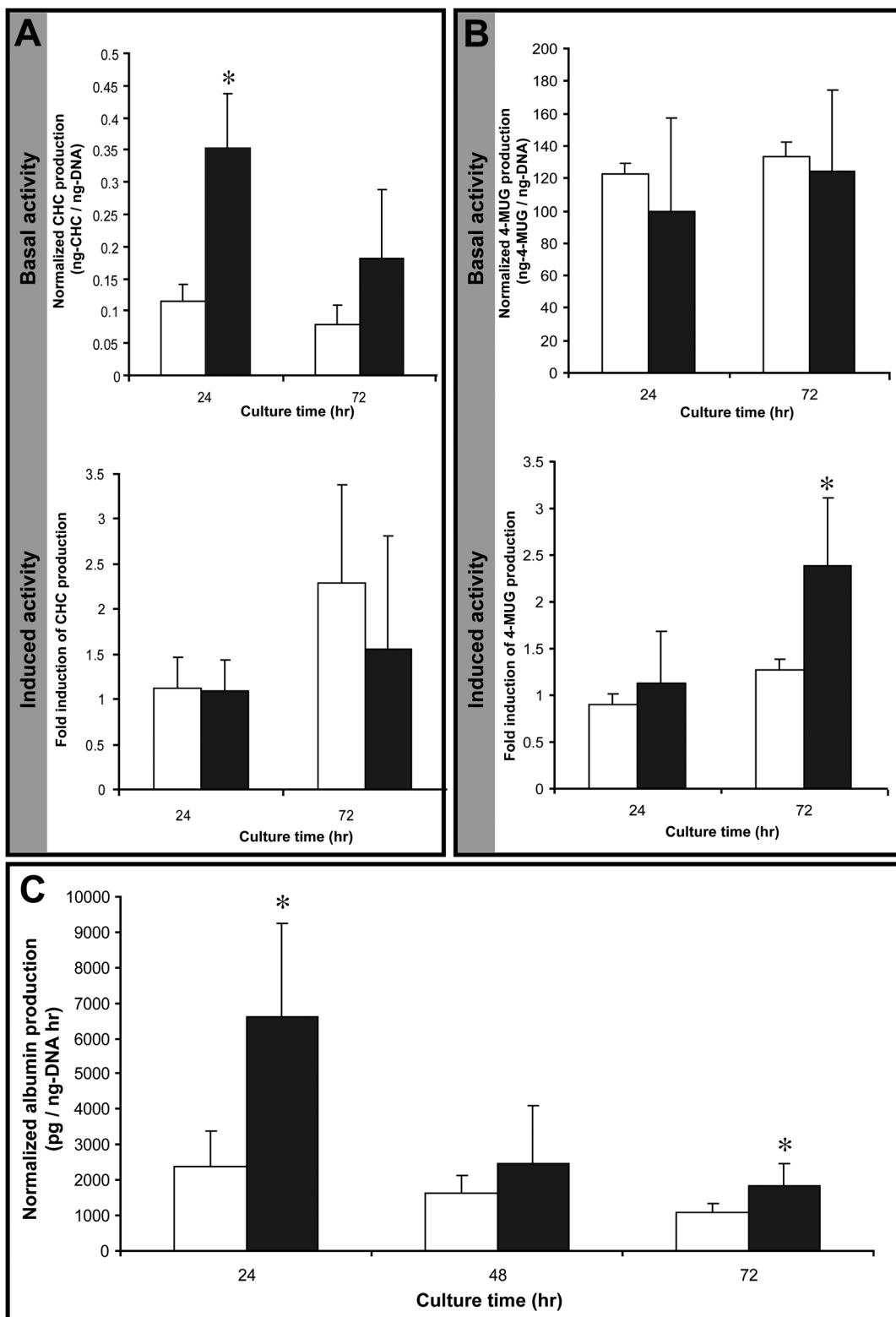
maintain a comparable, if not, higher level of phase I and II metabolic functions than monolayer cultures in multi-well plates. After 24 hours of culture in the 3D HepaTox Chip, basal CYP 1A1/2 activities were approximately 3-fold higher than that of the monolayer culture in multi-well plate, as indicated by CHC production (Fig. 3A). Induced CYP 1A1/2 activities in the 3D HepaTox Chip were similar to that in multi-well plate cultures (Fig. 3A). UGT activity in the 3D HepaTox Chip was comparable to the monolayer culture in multi-well plate over the 72 hour culture period (Fig. 3B). However, this enzyme can be induced to a greater extent by rifampin in the 3D HepaTox Chip than their multi-well plate counterpart (Fig. 3B). The synthetic functions of the hepatocytes were assessed by albumin production. Albumin production in the 3D HepaTox Chip ranged from 1833–6619 pg/(ng-DNA hr) over the culture period and was significantly higher (Student's t-test,  $p < 0.05$ ) than that of the multi-well plate controls on day 1 and 3 of culture (Fig. 3C).

Thus, we have demonstrated that hepatocytes cultured in the 3D HepaTox Chip maintained some basal or induced metabolic functions better than the current multi-well plate systems. The 3D HepaTox Chip may be more sensitive than the current multi-well plate-based hepatocyte models for detecting drug-mediated hepatotoxicity and drug-drug interactions. We also ascertained that hepatocytes cultured in the 3D HepaTox Chip remained functional for at least 72 hours, which is sufficient to conduct most established drug testing assays. For instance, most acute hepatotoxicity studies do not exceed 24 hours,<sup>30–32</sup> while drug inducibility studies typically do not exceed 72 hours.<sup>33</sup> Drug testing with the 3D HepaTox Chip can be extended to include chronic toxicity and pharmacokinetics studies as the inherent perfusion culture feature allows for longer term hepatocyte culture.<sup>9</sup>

### 3.3 Hepatotoxicity testing with the 3D HepaTox Chip

Having successfully established the 3D HepaTox Chip, we utilize it for hepatotoxicity testing. Hepatotoxicity mediated by drugs is dependent on their metabolism by hepatocytes, which determines whether the drug or its metabolites are present at a toxic concentration. Since hepatocytes cultured in our 3D HepaTox Chip can preserve their metabolic functions, it can serve as a useful *in vitro* model for predicting *in vivo* drug hepatotoxicity. We demonstrate this by using the 3D HepaTox Chip to simultaneously assess the toxicity at 8 different concentrations for 5 model hepatotoxic drugs (acetaminophen, diclofenac, quinidine, rifampin, and ketoconazole). The drugs were selected based on the criteria that (i) they are well-characterized hepatotoxic drugs, (ii) they span over a range of toxic concentrations, and (iii) their *in vivo* lethal dose 50 (LD<sub>50</sub>) in rats are available.<sup>34,35</sup> We then correlated the *in vitro* hepatotoxicity data to the published *in vivo* acute lethal toxicity to assess the predictive value of the 3D HepaTox Chip.

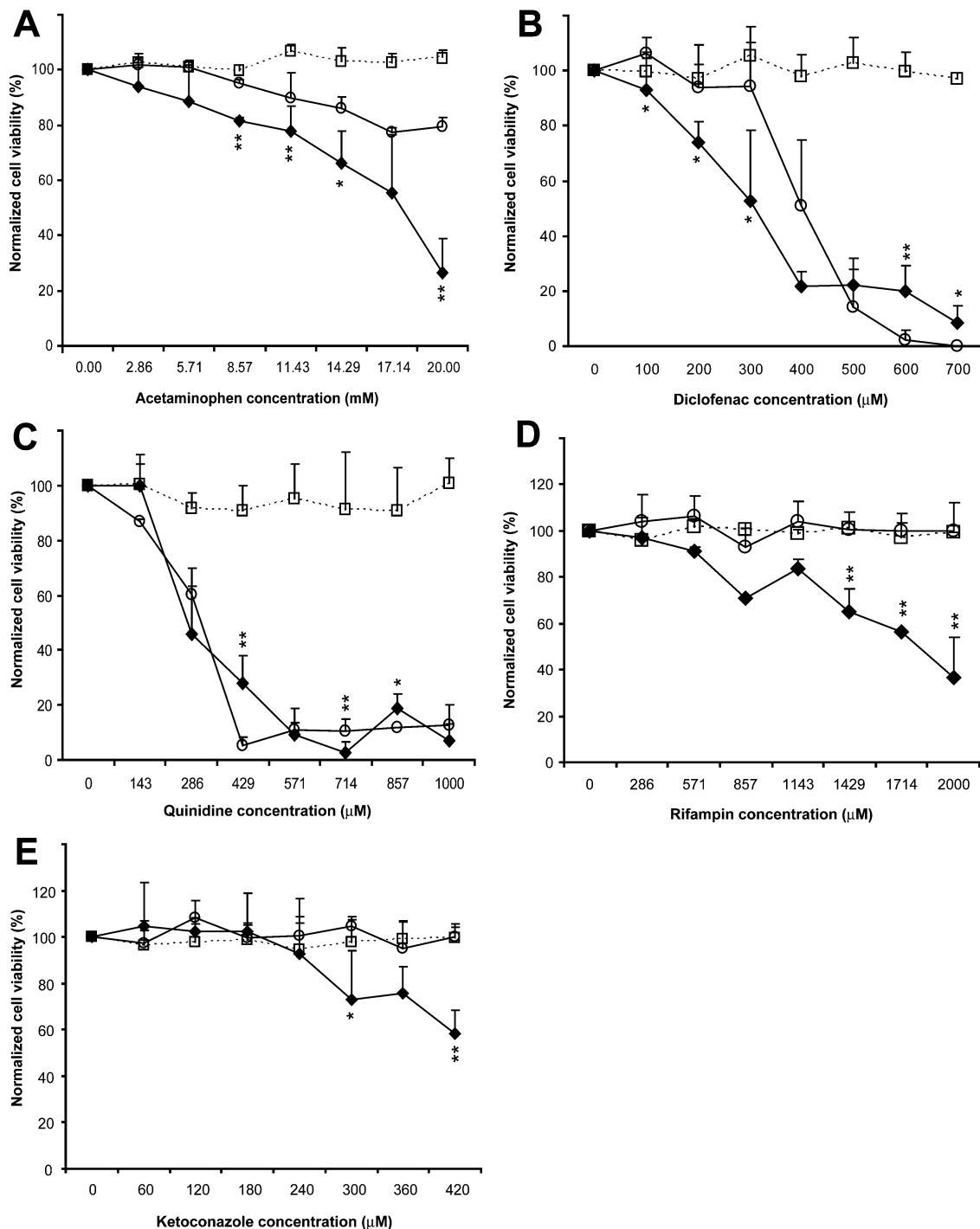
Hepatocytes were perfusion-cultured in the 3D HepaTox Chip for 24 hours prior to treatment at different concentrations of drugs. The drug solutions were perfused for 24 hours before the hepatotoxic effect was evaluated by quantifying cell viability (Fig. 4). To benchmark the performance of the 3D HepaTox Chip, we compared the hepatotoxicity profiles generated with that of the conventional multi-well plate cultures that are commonly used in hepatotoxicity studies.<sup>30,32,36</sup> The 3D HepaTox



**Fig. 3** Functional maintenance of hepatocytes in the 3D HepaTox Chip. Hepatocytes were perfusion-cultured in 3D HepaTox Chips (■) for 72 hours and assessed for their differentiated functions *i.e.*, cytochrome P450 (CYP) 1A1/2 activity, UDP-glucuronyltransferase (UGT) activity and albumin production. Conventional collagen-coated multi-well plate cultures (□) were used to benchmark hepatocyte functions. (A) Basal (top panel) and induced (bottom panel) CYP 1A1/2 activities. (B) Basal (top panel) and induced (bottom panel) UGT activities. (C) albumin production. All functional data were normalized to the DNA content in samples. Data are represented as means  $\pm$  SD of 3 devices (3D HepaTox Chip) or 3 culture wells (collagen-coated multi-well plate). \* indicates statistical significance compared with multi-well plate culture (Student's t-test,  $p < 0.05$ ).

Chip was more sensitive to drug-mediated hepatotoxicity than multi-well plate cultures for most drugs tested (*i.e.*, acetaminophen, diclofenac, rifampin, and ketoconazole) (Fig. 4A–B, D–E). However, the 3D HepaTox Chip did not show increased

sensitivity to quinidine-mediated toxicity (Fig. 4C). Based on the hepatotoxicity profiles generated by the 3D HepaTox Chip, we estimated the drug concentration that produced a 50% inhibitory effect ( $IC_{50}$  value) for each drug (Table 1). The  $IC_{50}$  values



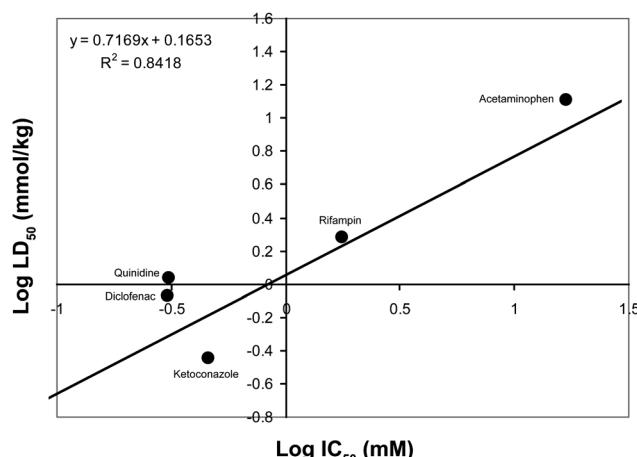
**Fig. 4** Hepatotoxicity profiles of 5 model hepatotoxicants obtained from the 3D HepaTox Chip and conventional multi-well plates: (A) acetaminophen, (B) diclofenac, (C) quinidine, (D) rifampin, and (E) ketoconazole. A linear concentration gradient generator was used to feed 8 different drug concentrations simultaneously into the microfluidic chip for 24 hours before assessing for cell viability. Cell viabilities after treatment with different drug concentrations were normalized against that of untreated cells. Data are represented as means  $\pm$  SD of 3 experiments. Asterisks indicate statistical significance compared to conventional multi-well plate culture; Student's t-test, \*  $p < 0.1$  and \*\*  $p < 0.05$ . 3D HepaTox Chip (◆); Multi-well plate (○); DMSO control (□).

calculated from the 3D HepaTox Chip in this study were of the same order of magnitude as the previously reported  $IC_{50}$  values using the same cell type and drug exposure time (ESI Table S1†).<sup>30–32</sup> Here, we chose to quantify cell necrosis because it is one of the accepted indicators for drug toxicity<sup>34</sup> and it can be assessed by imaging-based assays that are compatible with the transparent microfluidic system. This may explain why the  $IC_{50}$  values obtained in this study, using cell necrosis as the cytotoxic marker, were slightly higher than the  $IC_{50}$  values reported in some studies, which employed metabolic assays as cytotoxic markers, such as MTT reduction, LDH leakage, and protein synthesis assays.<sup>30,32,36</sup>  $IC_{50}$  values that are based on metabolic endpoints may be lower than those predicted from actual cell necrosis since a decrease in metabolic activity precedes cell necrosis during drug intoxication. Ideally, a robust drug  $IC_{50}$  prediction should be performed by using multiple cytotoxic endpoints.<sup>34</sup> However, the majority of the existing cytotoxicity assays, such as MTT and LDH assays require minimum sample volumes that exceed the typical operating volumes in microfluidic systems and are not readily implemented in the 3D HepaTox Chip. Thus, drug  $IC_{50}$  estimated with multiple cytotoxic endpoints using the 3D HepaTox Chip will require integration with other highly sensitive micro-biosensors that are currently in development.<sup>37,38</sup>

*In vitro* toxicity data, as indicated by  $IC_{50}$  values, have been shown to be positively correlated to *in vivo* toxicity data, indicated by  $LD_{50}$  values.<sup>34</sup> The  $LD_{50}$  value of a drug is the concentration that results in 50% mortality in animals. A linear regression between  $IC_{50}$  values and  $LD_{50}$  values is often used to predict the *in vivo* acute toxic potential of a drug from its *in vitro* hepatotoxicity data, and is particularly useful for estimating initial doses for acute oral lethality assays during preclinical *in vivo* testing.<sup>34</sup> Although the  $IC_{50}$  values from the 3D HepaTox Chip concurred with the reported  $IC_{50}$  values from literature, we wanted to determine whether the  $IC_{50}$  values obtained from our 3D HepaTox Chip can be used to estimate *in vivo*  $LD_{50}$  values. Thus, we correlated the  $IC_{50}$  values derived from the 3D HepaTox Chip with the published *in vivo*  $LD_{50}$  values (listed in Table 1). We could obtain a linear correlation between the  $IC_{50}$  and  $LD_{50}$  values ( $R^2 = 0.84$ ) (Fig. 5). The correlation between  $IC_{50}$  values derived using our 3D HepaTox Chip and *in vivo*  $LD_{50}$  values was similar to that obtained with the freshly isolated rat hepatocyte (< 6 hours culture) on collagen-coated multi-well plates ( $R^2 > 0.8$ ),<sup>36,39</sup> and was better than that obtained with cell lines ( $R^2 < 0.7$ ).<sup>32,34</sup> While the routine use of the 3D HepaTox Chip as a fully validated *in vitro* model for prediction of *in vivo* hepatotoxicity requires a larger dataset of drugs,<sup>34</sup> we have demonstrated the feasibility of the Chip for *in vitro* drug testing that can be correlated to *in vivo* toxicity.

**Table 1**  $IC_{50}$  values calculated from the 3D HepaTox Chip and published  $LD_{50}$  values for 5 model hepatotoxic drugs

Drugs	$IC_{50}$ (mM) (3D HepaTox Chip)	$LD_{50}$ (mmol/kg) <sup>34,35,40</sup> (acute oral toxicity in rats)
Acetaminophen	16.90	12.86
Diclofenac	0.31	0.84
Quinidine	0.31	1.08
Rifampin	1.77	1.91
Ketoconazole	0.46	0.31



**Fig. 5** Correlation of  $IC_{50}$  values calculated from the 3D HepaTox Chip to reported  $LD_{50}$  values in rats.

## 4. Conclusion

Microfluidic hepatocyte chips are promising for performing *in vitro* drug testing in a biologically relevant and efficient manner, which can reduce the number of animals used in *in vivo* testing. The use of microfluidics allows efficient handling and analyses of minute volumes of drugs and cells, while primary hepatocyte cultures allow better prediction of *in vivo* drug responses than cell-free or cell-line based models. Successful drug testing using microfluidic hepatocyte chips that will be predictive of *in vivo* drug responses requires the maintenance of functional hepatocyte in a microfluidic system that supports multiplexed drug administration. Here, we demonstrated *in vitro* hepatotoxicity testing with the 3D HepaTox Chip that has potential utility in predicting *in vivo* toxicity. The 3D HepaTox Chip is the first demonstration of the use of a microfluidic hepatocyte model for obtaining *in vitro* toxicity data that can be correlated to *in vivo* toxicity data, where we overcame challenges to seed and culture functional primary hepatocytes in a multiplexed microfluidic system. The development of such a microfluidic hepatocyte model is important as it enables the realization of on-chip drug testing.

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