# A Continuously Perfused 3-D Tissue Culture Microdevice to Improve Hepatocyte Drug Tolerance Testing

**Dorian Perkins[1], Mimi Y. Zhang[2], Luke P. Lee[3]**

*[1]Department of Computer Science, University of California Riverside*
*[2]Bioengineering Graduate Program, [3]Department of Bioengineering*
*University of California Berkeley*

## ABSTRACT

Liver diseases such as liver cancer, cirrhosis, hepatitis, and acute or chronic liver failure affect many patients globally. Liver transplantation, although a feasible method of treatment, is limited by a short supply of donors and expensive post-operation treatments, which has led to the development of bio-artificial liver devices. In vitro drug tolerance testing of hepatocyte spheroids, or spherical multi-cellular aggregates, is essential to furthering the advancement of Bio-Artificial Liver (BAL) devices. Hepatocyte spheroids, because of their cell-cell interactions and in vivo-like physical formation, metabolize drugs in a more physiological relevant manner than sparse two-dimensional (monolayer) cultures. Our goal is to improve the functional maintainability of the primary source of cells used in these BAL devices by utilizing hepatic cell lines cultured in a spheroid configuration using a three-dimensional high-density hepatic spheroid trapping array microdevice with continuous perfusion to simulate physiological flow conditions.

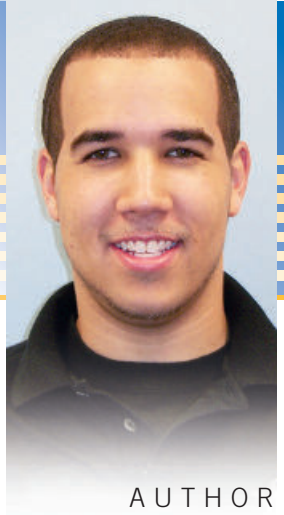
Our cytotoxicity assay uses the anti-inflammatory Diclofenac (sodium salt) with concentrations of 10μM, 100μM, and 1000μM in media, and a control with no drugs. HepG2/C3A (human hepatoma) cell proliferation will be characterized over a 4-day culture period using the spheroid array microdevice compared against static control cultures in a 12-well plate. Viability and proliferation is determined using Live/Dead and Hoescht stains in devices and Trypan Blue exclusion in the controls. Preliminary results have determined an exponential decrease in cell proliferation versus increasing drug concentrations while in 2D monolayer formation. Future results are predicted to show spheroid formation yields a stronger resistance amounting in increased proliferation versus the control.

KEYWORDS: hepatocyte, spheroid, drug metabolism, Diclofenac, HepG2/C3A, trapping array, microdevice, BioMEMS

## FACULTY MENTOR

**Luke P. Lee, Assistant Professor**
*Department of Bioengineering, UC Berkeley*

In addition to his teaching and mentoring responsibilities, Dr. Lee directs the Berkeley Sensor & Actuator Center and Biomolecular Nanotechnology Center. He also serves as a Core Member of the UCB/UCSF Graduate Group in Bioengineering. Dr. Lee's research specialty is biomedical microelectromechanical systems and he prides himself on mentoring students and encouraging them to explore this area of study. Before returning to graduate school and subsequently joining the UC Berkeley faculty, he worked in industry for ten years, first with TRW Space and Technology and later at Conductus, Inc.



## AUTHOR

**Dorian J. Perkins**

*Computer Science*

Dorian Perkins is a graduating senior majoring in Computer Science. He was recently admitted into the PhD program in Computer Science at UCR and selected to become an LSAMP Bridge-to-the-Doctorate Fellow, a prestigious program funded by the National Science Foundation. Dorian's research was completed in summer 2006 at UC Berkeley as part of the SUPERB research program under funding from NSF and Intel. He recently won a special merit award for his research poster presentation at the 2007 CAMP statewide symposium. In addition to research, Dorian is a scholar, mentor, and leader who currently serves as the president of both the National Society of Black Engineers and Alpha Phi Alpha Fraternity Inc., Pi Epsilon chapter.

Dorian J. Perkins

## Introduction

*In vitro* drug-concentration dependency testing of hepatocyte spheroids, or spherical multi-cellular aggregate [3], is essential to furthering the advancement of Bio-Artificial Livers (BALs). Hepatocytes are cells that synthesize proteins, produce bile, and regulate metabolism of carbohydrates, fats, and protein [4]. Spheroid culture has advantages over monolayer culture because it has been shown that spheroids are much better *in vitro* models than monolayer arrangements [4]. This claim is supported by the fact that hepatocyte spheroids metabolize drugs in a more physiological relevant manner than monolayer hepatocytes cultures. In a monolayer configuration, cells spread out creating a large surface-area to volume ratio, facilitating fast drug interaction, while in a spheroid formation, they are fortified and in turn, harder to penetrate. It has also been said that cell-cell interactions are presumed to be a reason why spheroids are better models than dispersed culture [3]. Ultimately, hepatocyte spheroids have characteristics similar to native liver function, sometimes referred to as being a 'mini liver', and have been found to be effective in maintaining long-term hepatocellular functions *in vitro* [2][3][4][6].

Despite these findings, some studies have questioned the usefulness of the spheroid formation over monolayer hepatocyte cultures for *in vitro* toxicity applications. However, drug metabolism using hepatocyte spheroids is important to study because it is one of the main reasons why drugs often fail in Phase II clinical tests. Drugs that fail the *in vitro* liver metabolism test are thought to be harmful to hepatocytes [2][5]. With the FDA's recent adoption of the "drug metabolism by the liver" toxicity test as part of the New Drug Application (NDA) it is hard to challenge the legitimacy of this methodology in high-throughput screening (HTS) [2] and bio-micro-electro-mechanical systems (BioMEMS) technology.

In order to model drug-concentration dependence, we need to know what factors indicate the toxicity of a drug to the hepatocyte spheroids. Because of aforementioned traits of hepatocyte cells to stray from normal functioning if not cultured under *in vivo*-like conditions, hepatocyte cell lines have been created. Cell lines are genetically-engineered, immortalized cells normally cultured in the lab for scientific purposes. These cells are used because of their high tolerance and cancer-like ability to reproduce indefinitely. Use of hepatocyte cell lines, such as HepG2/C3A, allows for comparison and validation of cell behavior and characteristics within the scientific community. Many functional assays can be run to see if hepatocytes are behaving as they would in native tissue, such as the ability of hepatocytes to maintain production of glucose, albumin, and other molecule [1][4][6]. For our purposes, we will measure hepatocyte viability and proliferation in our drug-dependent experiment over a timed trial. This will give us further insight in the dose-dependent drug tolerance properties of the HepG2/C3A cell line.

An assortment of drugs has been used to study hepatotoxicity, characterized by fatty infiltration, inflammation, cellular necrosis and fibrosis [5]. One of the most common of these is Diclofenac. Diclofenac is a non-steroidal, anti-inflammatory drug used clinically to treat several rheumatic diseases [1][6]. It has also been shown to cause liver injury in some individuals and has been reported to cause hepatic cytotoxicity *in vitro* [1][6]. This drug also can increase swelling of mitochondrial permeability and cause a mitochondrial bio-energetic dysfunction or swelling and a decrease of cellular adenosine tri-phosphate (ATP), a high energy phosphate molecule required to provide energy for cellular function [1][6].

In our experiment, we will culture cells using a BioMEMS device that will allow continuous perfusion of media through a 3-D high-density trapping array microdevice (Figure 1). This method is superior to a stagnant 2-D monolayer culture, and even when used with a rotary device or gyrotatory shaker the cells do not mimic *in vivo*-like function as similarly as constant perfusion with media in a microdevice.

## Materials and Methods

**Materials.** Diclofenac sodium salt ($C_{14}H_{10}Cl_2NNaO_2$) and Trypan Blue (0.4%) stain were obtained from Sigma Chemical Co. (St. Louis, MO). Culture media (MEM), fetal bovine serum (FBS), Trypsin, phosphate-buffered saline (PBS), Live (Calcein-AM) and Dead (Ethidium Homodimer-1) stain, and Hoescht stain were obtained from Invitrogen (Auckland, NZ). Polydimethylsiloxane (PDMS) was obtained from Dow Corning Corp. (Midland, MI). A hemacytometer was obtained from Reichert (Buffalo, NY), and ImageJ software was obtained from the National Institutes of Health (http://rsb.info.nih.gov/ij/).

**Cell Cultures.** HepG2/C3A cells were cultured in minimal essential medium supplemented with 10% of fetal bovine serum (FBS) and 1% each of non-essential amino acids (NEAA), sodium pyruvate (NaPyr), Glutomax, and Penicillin/Streptomycin. For 2-D monolayer cultures, cells were plated in standard 12-well plates at a density of $1x10^5$ cells in 1mL medium per well and used 24 hours later. For 3-D microdevice cultures, about 1.5mL of cell solution was injected into a previously described cell trapping array microdevice bonded to a glass slide pretreated with 70% Ethanol and phosphate-buffered saline (PBS). A continuous flow of 1uL/minute was administered to each device using a syringe pump. (Table 1)

**Evaluation of Toxicity of Diclofenac to Hepatocytes in 2-D monolayer culture.** A single high-concentration solution of Diclofenac in PBS was mixed into media at increasing concentrations of 10µM, 100µM and 1000µM. Drugged media was added to each well after first media renewal at 24-hours of incubation. Three 12-well plates were used, each using one of the aforementioned concentrations throughout the entire plate. Four day trials were run on each of the 12-well plates analyzing three wells per day per plate.

The effect of diclofenac on the proliferation of hepatocytes was measured using a cytotoxicity assay that consisted of counting the number of the cells per well over regular time intervals. At each 24-hour time interval, three wells per each of the individual plates were analyzed in order to get a real distribution with minimum, maximum and median points. During analysis, the wells were trypsinized with 500µL of Trypsin 1X EDTA and incubated for 5 minutes. After all cells were broken loose from the culture well, 500µL of culture media was added to the well to neutralize the Trypsin. The cells in this solution were then homogenized to break them apart and reduce the amount of clumping. After homogenizing each well, 100µL of each well of solution was then transferred into a microfuge tube and 100µL of Trypan Blue stain was added to the solution. After re-homogenization, a small amount of cell solution was analyzed using a hemacytometer to count the approximate number cells/well using the standard Trypan Blue exclusion procedure and counting daily images by hand.

**Evaluation of control in 2-D monolayer culture.** The control experiment used to compare proliferation over time for non-drugged cells against the effects of Diclofenac on Hepatocytes was conducted by using a non-drugged media. Instead of having three separate plates for each drug concentration, we use on only one 12-well plate and only one type of non-drugged media. The cells were analyzed using the same techniques as aforementioned. The incubation period, media changing technique, trypsinizing method, photography, and cell counting are identical to the methods used for the drugged cytotoxicity assay. Cell proliferation was determined using Trypan Blue exclusion.

**Evaluation of control in 3-D microdevice culture.** In the microdevice, the control data was determined using a non-drugged media flowing through a high-density trapping array microdevice pre-loaded with cells as previously described. After cells have been trapped, media is flowed through the device for a short period of time to clear any non-trapped cells from the channel pathways. At this point and at each 24-hour point thereafter, the cells were photographed in the

Dorian J. Perkins

device. At the final 24-hour marker, media flow through the device ceases and is replaced by 1mL of a dye mixture containing 4µL Live stain, 2µL Dead stain, and 1µL Hoescht stain in 2mL PBS. After this solution has been flowed through the device for ten minutes, the device is then incubated for an additional ten minutes so the stains can react better with the cells. After incubation, the cells are photographed using a laser-equipped microscope with ultraviolet laser illuminating the Hoescht stained cells, blue the live (green) stained cells, and green for the dead (red) stained cells. Cells are counted by hand and using the ImageJ analysis tool.

**Evaluation of Toxicity of Diclofenac to Hepatocytes in 3-D microdevice culture.** The cytotoxicity assay for the 3-D microdevice is similar to the control experiment for the microdevice, but rather using the different concentrations of drugged media as used in the 2-D toxicity assay. Assays are run for each concentration for 1-, 2-, 3-, and 4-day trials. The proliferation of the cells in the microdevice is counted exactly as in the control using the same stain solution and technique. Photographs are also taken at regular intervals.

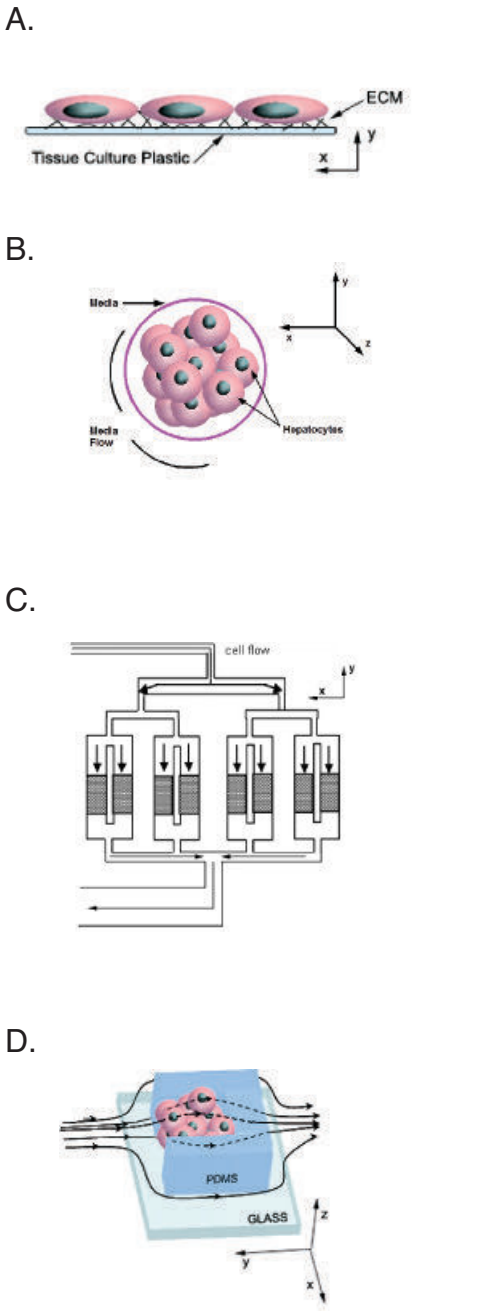



***Figure 1.*** *(**a**) Traditional 2-D culture method which forms a monolayer cell formation (**b**) An in-vivo-like 3-D spheroid formation that promotes cell-cell interactions (**c**) Schematic of spheroid trapping array device. (**d**) Individual traps present physical barriers to multiple cells and spheroid formation occurs by hydrodynamic flow in PDMS trapping chamber.*

Dorian J. Perkins

| | 2D monolayer | 3D microdevice |
|---|---|---|
| Control (+) | 12-Well Plate w/ 105 cells/well | Cell Trapping Device (500cells/channel) |
| Media Delivery | 1mL / well / day | Continuous (1.44mL/day) Flow rate: 1 L/min |
| Drug Concentrations | 10 M/100 M/1000 M 1,6 | 10 M / 1000 M |
| Viability | Trypan Blue exclusion | Live/Dead Stain |
| Growth | Trypan Blue exclusion By Hand / ImageJ | Hoescht (Nuclei) Stain ImageJ |

*Table 1.*

## Results

**Control culture for 2-d monolayer arrangement.** We use the 2-D monolayer culture as our control in this experiment to see whether 3-D spheroid formation shows increased proliferation and a higher resistance to Diclofenac over a timed trial. Over this 4-day trial, we observed the increase in number of non-drugged hepatocytes in normal conditions. At the end of the trial, we noticed a 4-fold increase in total cells per well. This trend is shown in figure 2.

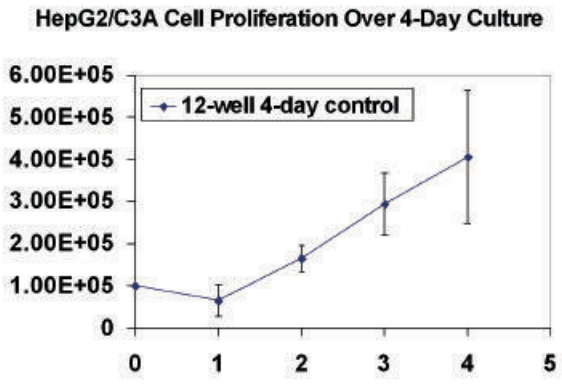



***Figure 2.*** *Proliferation for HepG2/C3A control after 4-days. To be later compared to 3-D configuration control data.*

**Cytotoxicity of Diclofenac on Hepatocytes in 2-D monolayer culture.** Diclofenac showed a concentration-dependent cytotoxicity when added to cultured human hepatocytes (HepG2/C3A). We observed hepatocytes reaction to Diclofenac over a 4-day trials, gathering data at 24-hour time-points. As expected, there was a notable decline in the number of cells per well with each of the three concentrations of Diclofenac in media. The concentration of Diclofenac in media has an inverse relationship with the cell proliferation. In other words, as the concentration of Diclofenac in the media exponentially increased, the decline in number of cells became more drastic, with the 1000µM concentration actually ending with fewer cells than plated at Day 0 as seen in figure 3. A comparison between cell density of the 10µM concentration and 1000µM concentration can be seen in figure 4.

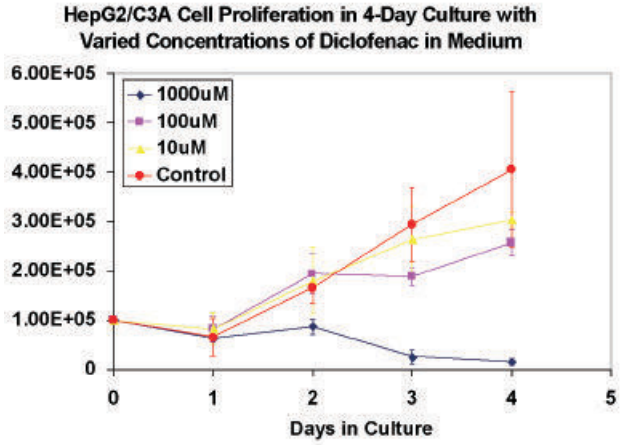
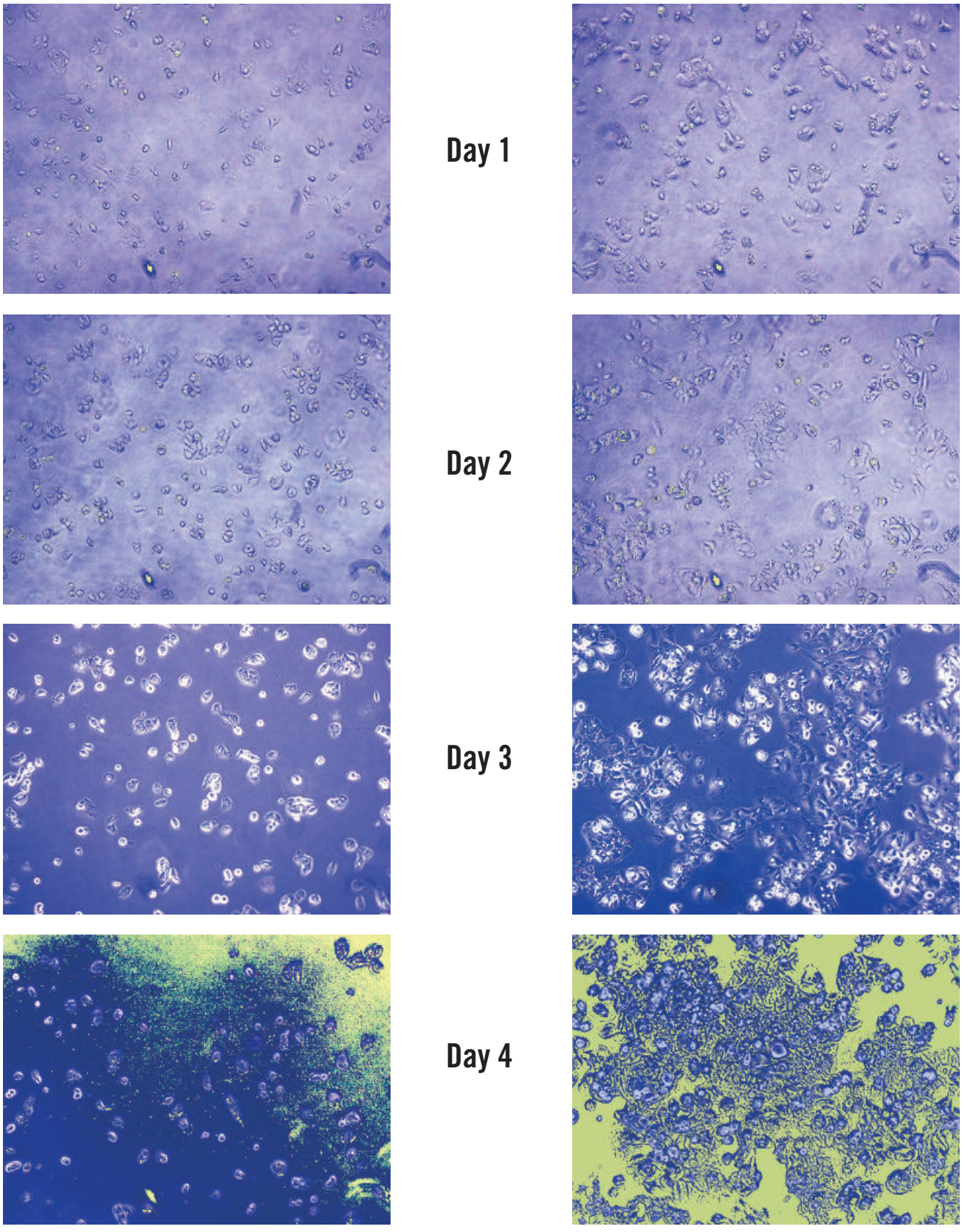



***Figure 3.*** *The control is equivalent to the data shown in figure 2. This graph compares the total cells per well over a 4-day trial with varied concentrations of Diclofenac in the culture media.*

Dorian J. Perkins

Media + 1000 M Diclofenac

Media + 10 M Diclofenac



**3-D microdevice control culture.** The control culture for the 3-D microdevice consisted of a previously described 3-D microdevice loaded with hepatocyte cells. For the control, we run 4 separate devices for a separate timed trial to get a 1-, 2-, 3-, and 4-day control experiment. At loading, the trapping chambers in the microdevice are filled will cells and any excess cells are washed away by the constant media perfusion that takes place. Over the timed trial, these cells begin to form together to create spheroids, and often tend to grow out of the limited space inside the trapping chamber. Because we are using a device that was not specifically designed for our experiment, the optimum spheroid formation is not formed because the trapping chamber is not square or circular. Instead, the rectangular chamber forms a clump of cells that tends to grow out of the chamber into a mushroom shape. It is undetermined whether this non-spherical shape causes any undesired effects. Only preliminary results using the microdevice are available.

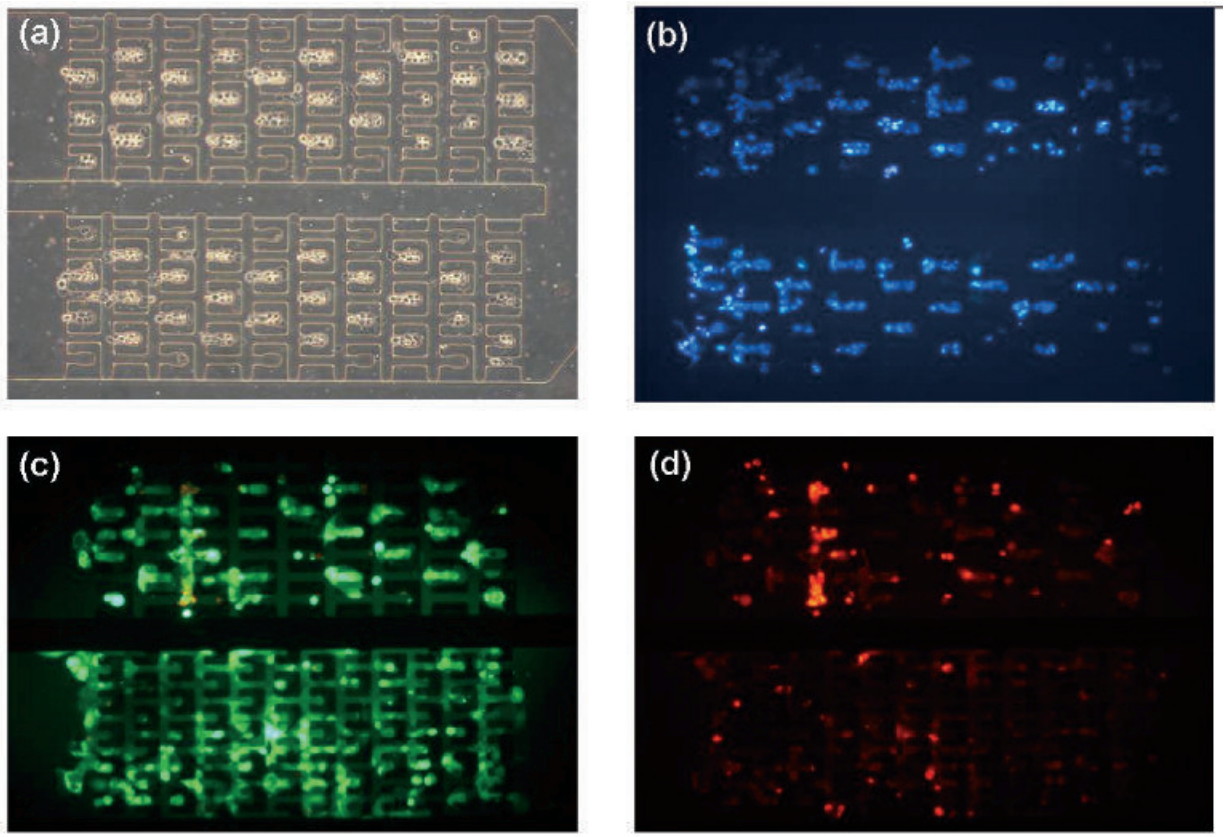



***Figure 5.*** *(a) Microdevice channels on Day 0 after loading cells. (b) All cells in channels on day 3 using Hoescht fluorescence nuclei stain. (c) Live cells in channels on day 3 using Live (Calcein-AM) fluorescence stain. (d) Dead cells in channels on day 3 using Dead (Ethidium Homodimer-1) fluorescence stain.*

## Discussion

Diclofenac is an anti-inflammatory drug known to cause severe adverse reactions with liver and kidney cells [1]. We use this drug in our toxicity examination to ensure that cells would show a noticeable reaction to the drug in the media. Using this information we seek to find the best method to culture hepatocyte spheroids in a Bio-Artificial Liver bioreactor.

The current device, because of uneven flow between channels, is not the optimum design for the current task. Because of these flow differences, the density of trapped spheroids in each channel is unequal. This wastes time because we are not utilizing the full capacity of the device, and also may cause disproportionate flow of drugged media in each channel, causing slightly skewed data.

The results from our cytotoxicity assay have determined a notable decrease in cell proliferation versus increase in drug concentrations while in 2-D monolayer formation. We are seeking results using the 3-D microdevice to show that spheroid formation yields a stronger resistance amounting in increased proliferation versus the control. Utilizing this information, we can move forward in developing a better microdevice for conducting cytotoxicity assays that will lead to the development of an improved hepatocyte spheroid microchip system to be implemented in Bio-Artificial Liver devices.

In the future studies we will be examining new 3-D microdevices that will be more optimally designed for cytotoxicity testing on hepatocyte spheroids. The next steps for this project will be completing the control testing for the current microdevice and then running the cytotoxicity assay using the microdevice.

## ACKNOWLEDGEMENTS

I would like to thank the following people for their generous support through funding, resources, and technical guidance: SUPERB, UC Berkeley, Intel, BioPOETS Lab, Dr. Luke P. Lee, and Mimi Y. Zhang. Also, I would like to give special thanks to UC Riverside and the CAMP-UCR program for their continued support.

## REFERENCES

[1] Bort R, Ponsoda X, Jover R, Gomez Lechon M, and Castell J. Diclofenac toxicity to hepatocytes: A role for drug metabolism in cell toxicity. The Journal of Pharmacology and Experimental Therapeutics **288**, 1, 1998.

[2] Elkayam T, Amitay-Shaprut S, Dvir-Ginzberg M, Harel T, and Cohen S. Enhancing the drug metabolism activities of C3A—a human hepatocyte cell line—by tissue engineering within alginate scaffolds. Tissue Engineering **12**, 2006.

[3] Nakazawa K, Mizumoto H, Kaneko M, Ijima H, Gion T, Shimada M, Shirabe K, Takenaka K, Sugimachi K, and Funatsu K. Formation of porcine hepatocyte spherical multicelluar aggregates (spheroids) and analysis of drug metabolic functions. Cytotechnology **31**, 61-68, 1999.

[4] Strain A, and Neuberger J. A Bioartificial Liver—State of the Art. Science **295**, 1005-1009.

[5] Walker T, Rhodes P, and Westmoreland C. The differential cytotoxicity of methotrexate in rat hepatocyte monolayer and spheroid cultures. Toxicology in Vitro **14**, 475-485, 2000.

[6] Xu J, Ma M, and Purcell W. Characterisation of some cytotoxic endpoints using rat liver and HepG2 spheroids as in vitro models and their application in hepatoxicity studies. I. Glucose metabolism and enzyme release as cytotoxic markers. Toxicology and Applied Pharmacology **189**, 100-111, 2003.