

Validation of a 3-Dimensional Human Liver Microtissue Model for Long-term Hepatotoxicity Studies

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Promega



Abstract

Non-steroidal anti-inflammatory drugs (NSAIDs) are a class of drug commonly used as analgesics and antipyretics, as well as for management of rheumatological disorders. They are one of the most highly prescribed drug families around the world, and consequently, along with antimicrobial agents, are the most frequent causes of drug-induced liver injury (DILI) (Bjornsson *et al.*, 2010). Diclofenac, in particular, frequently used to treat chronic pain and inflammatory disorders, elicited an FDA warning in 2009 concerning potential hepatic effects.

Multiple *in vitro* animal models have been used to determine the varied mechanisms of action (MOA) of NSAID-related hepatotoxicity. Studies using rat liver mitochondria and freshly isolated rat hepatocytes demonstrated that diphenylamine, a common NSAID structure, uncoupled oxidative phosphorylation, and decreased hepatic ATP content (Masubuchi *et al.*, 2000). Mitochondrial permeability transition (MPT) has also been shown to be important in diclofenac-induced liver injury, as well as the role that oxidative stress plays in MPT induction (Gómez-Lechón *et al.*, 2003). Finally, according to work done by Schmitz *et al.*, 1992, cytochrome P450 (CYP)-related metabolic activation of the drug, and the formation of reactive metabolites is also related to diclofenac hepatotoxicity.

The combined hepatotoxic effects of these mechanisms usually occurs within weeks of therapy commencement. This emphasizes the fact that *in vitro* safety testing should incorporate dosing experiments that analyze the effects of a potential drug for periods reaching out to 7-14 days or longer. However, primary hepatocytes cultured in a two-dimensional (2D) manner on the bottom of a microplate, standard practice for *in vitro* liver toxicity testing, have been shown to undergo rapid loss of differentiated function and metabolic capacity (Cheng *et al.*, 2008), and have less complex inter-cellular and cell-matrix interactions compared to *in vivo*. What is required is a cell culture model where cells retain viability and function long-term, by creating a favorable environment for these interactions to reform.

Here we evaluate the suitability of 3D human liver microtissues for use in long-term toxicity studies. Primary human hepatocytes are reaggregated into functional microtissues by hanging drop technology. The microtissues demonstrate *in vivo* like cell-cell and cell-matrix interactions and retained viability over weeks. A panel of assays was run to assess cell health and the different MOA exhibited by diclofenac using the 3D liver microtissues, in addition to primary hepatocytes cultured in 2D. Quantification of the various luminescent and fluorescent emissions via microplate reading and imaging was carried out by a novel cell imaging multi-mode reader.

BioTek Instrumentation

Cytation™ 3 Cell Imaging Multi-Mode Microplate Reader combines automated digital widefield microscopy and conventional microplate detection. The instrument includes both high sensitivity filter-based detection and a flexible monochromator-based system for unmatched versatility and performance. The upgradable automated digital fluorescence microscopy module provides researchers rich cellular visualization analysis without the complexity and expense of standard microplate-based imagers. The instrument was used to perform all luminescent microplate reads and cellular imaging. Cell counting and quantification of the fluorescent signal from the images captured was done using the Gen5™ Data Analysis Software.

3D and 2D Culture Models

3D liver microtissues were obtained from InSphero, Inc. (Cambridge, MA). The microtissues were created using proprietary hanging drop technology with human hepatocytes from Bioreclamation IVT (Baltimore, MD) using lot IZT. Cryopreserved human hepatocytes were also provided to BioTek directly from Bioreclamation IVT from the same hepatocyte lot (IZT). The cells were cultured in BioCoat™ Collagen I 384-well black, clear bottom plates (Catalog No. 354667) from Corning Life Sciences (Corning, NY) for 2D cell culture comparison using media provided by Bioreclamation IVT.

Materials and Methods

Cell Viability and Activity Assays CellTiter-Glo (Catalog No. G7571) and CellTiter-Glo 3D from Promega Corporation (Madison, WI) were used to assess viability by means of cellular ATP measurement for cells cultures in 2D and 3D, respectively. The P450-Glo CYP3A4 assay with Luciferin-IPA (Catalog No. V9002), also from Promega Corporation, was used to measure cellular function via cytochrome P450 enzyme activity analysis.

Cytotoxicity Assays Cytotoxicity was determined with the CellTox Green Cytotoxicity Assay (Catalog No. G8731) from Promega Corporation. Mitochondrial superoxide concentration was used to monitor cellular oxidative stress with the MitoSOX Red Mitochondrial Superoxide Indicator (Catalog No. M36008), while apoptotic activity was assessed by measuring mitochondrial permeability transition pore opening using the Image-iT Live Assay (Catalog No. I35103). Both assays were purchased from Life Technologies (Carlsbad, CA).

Long-term Diclofenac Cytotoxicity Assessment

Liver microtissues were received in GravityTRAP plates, medium was exchanged, and the tissues were incubated overnight at 37 °C/5% CO₂. Cryopreserved hepatocytes were also thawed, plated using traditional 2D cell culture methods at concentrations of 2000, 5000, and 10,000 cells/well, and incubated overnight at 37 °C/5% CO₂. The following day cell viability and cytochrome P450 enzyme activity was determined using the assay chemistries mentioned above. Luminescent values generated were used as baseline “Day 1” results from which relative percent cellular viability and activity were calculated. Diclofenac, at final 1X concentrations of 300, 30, 3, and 0 μM was then added to separate, designated wells of the plates containing the liver microtissues or 2D plated hepatocytes. The cells and tissues were re-dosed with diclofenac on a daily basis over a two week timeframe by removing medium with compound and replacing with an equal volume of fresh compound in medium.

The microplate and imaging assays previously mentioned were performed on day 2, 4, 8, 11, and 16 of the diclofenac treatment.

Assay Procedures

3D Liver Microtissue Assay Procedures: Medium was removed from wells to be used for the particular time point assessment. 30 μL of medium containing either Luc-IPA substrate, CellTox Green and MitoSOX probes, or the components for the Image-iT assay was then added to designated wells and incubated at 37 °C/5% CO₂ for four hours. Following incubation, 50 μL of P450-Glo Luciferin Detection Reagent (LDR) was added to wells containing the Luc-IPA, and 35 μL of CellTiter-Glo 3D reagent was added to separate wells. A 5X tip mix was then completed, and the entire volume (including the tissue) was transferred to two individual white, 96-well plates. The P450-Glo plate was placed back at 37 °C/5% CO₂ for 20 minutes, while the CellTiter-Glo 3D plate was shaken for 5 minutes at room temperature (RT), followed by an additional 25 minute RT incubation. The luminescent signal was then quantified from both plates. In the microtissue plate, following transfer of the P450-Glo and CellTiter-Glo 3D volumes, the wells containing the fluorescent probes were washed 3X with PBS and imaged using 4x and 10x magnification.

2D Hepatocyte Assay Procedures: Medium removal and addition of Luc-IPA and fluorescent probes were as previously described, using a 25 μL volume for all reagents. A 60 minute incubation at 37°C/5% CO₂ then followed. 12.5 μL was then transferred from wells containing medium and Luc-IPA to a separate white, 384-well plate, an equal volume of LDR was added, the plate was shaken for 60 seconds, and incubated for 20 minutes at RT. In the cell plate, 12.5 μL of CellTiter-Glo was added to the same wells used for the P450-Glo assessment, and the plate shaken for 60 seconds. The wells containing the fluorescent probes were then washed 3X with PBS and imaged using 4x and 20x magnification. Following imaging, the luminescent signal from the wells containing the CellTiter-Glo reagent and the separate white, 384-well plate was then quantified.

2D/3D Long-Term Cell Health Analysis

A two week examination of hepatocytes cultured in 2D monolayers or 3D microtissues was conducted to assess whether cell health was maintained over the entire incubation period. This initial test was essential to validate whether cells cultured in these different manners could be used for long-term cytotoxicity studies.

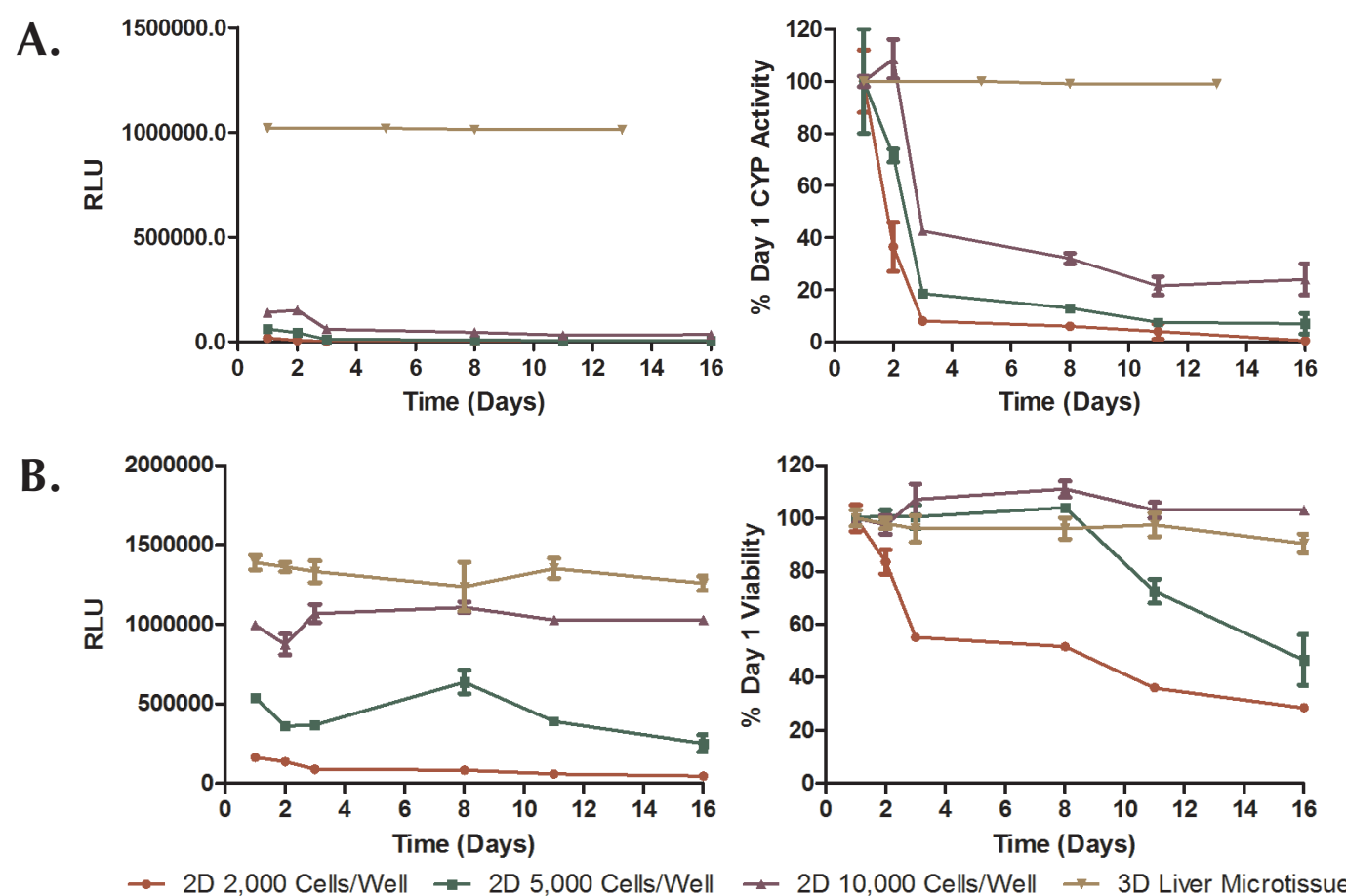


Figure 1 – Cellular health findings for long-term 2D/3D hepatocyte culture. (A) CYP3A4 enzyme activity, and (B) cell viability results for liver microtissues and 2D hepatocyte cell culture using three separate cell concentrations. Left side graphs display raw luminescent values for each cell culture and concentration tested, while right side graphs exhibit normalized comparisons to Day 1 values. % CYP Activity and Viability calculated by the following formula: $RLU_{(Day X)} / RLU_{(Day 1)} * 100$.

From the luminescent (RLU) values generated with the P450-Glo assay (Figure 1A left graph), it can be seen that CYP3A4 activity is approximately 100X greater in liver microtissues than with the equivalent concentration of hepatocytes cultured in 2D, and remains 10X greater than that from a 2D concentration which is 5X higher (10,000 cells/well). Enzyme activity in the microtissues is also maintained at a consistent level during the entire incubation period, while activity in 2D cell culture continues to decrease rapidly over the first week of culture (Figure 1A right graph). Loss of activity is generally confirmed to be at least partially due to loss of cell viability over the same timeframe (Figure 1B left graph). Cellular ATP levels for 2000 and 5000 cell/well 2D concentrations fall to 50% or lower by the end of the incubation period (Figure 1B right graph). Levels remain closer to 100% with the highest 2D cell concentration and liver microtissues. However, only when using liver microtissues do both CYP activity and cell viability remain consistent over a two week period, confirming that 3D cell culture provides the most relevant method to generate reliable results from long-term studies.

Diclofenac Cytotoxicity Study – Liver Microtissue Model

The cytotoxic effect of diclofenac on hepatocytes in the liver microtissue model was then assessed using compound concentrations spanning 3 logs. Cell culture conditions and concentrations were the same as previously mentioned.

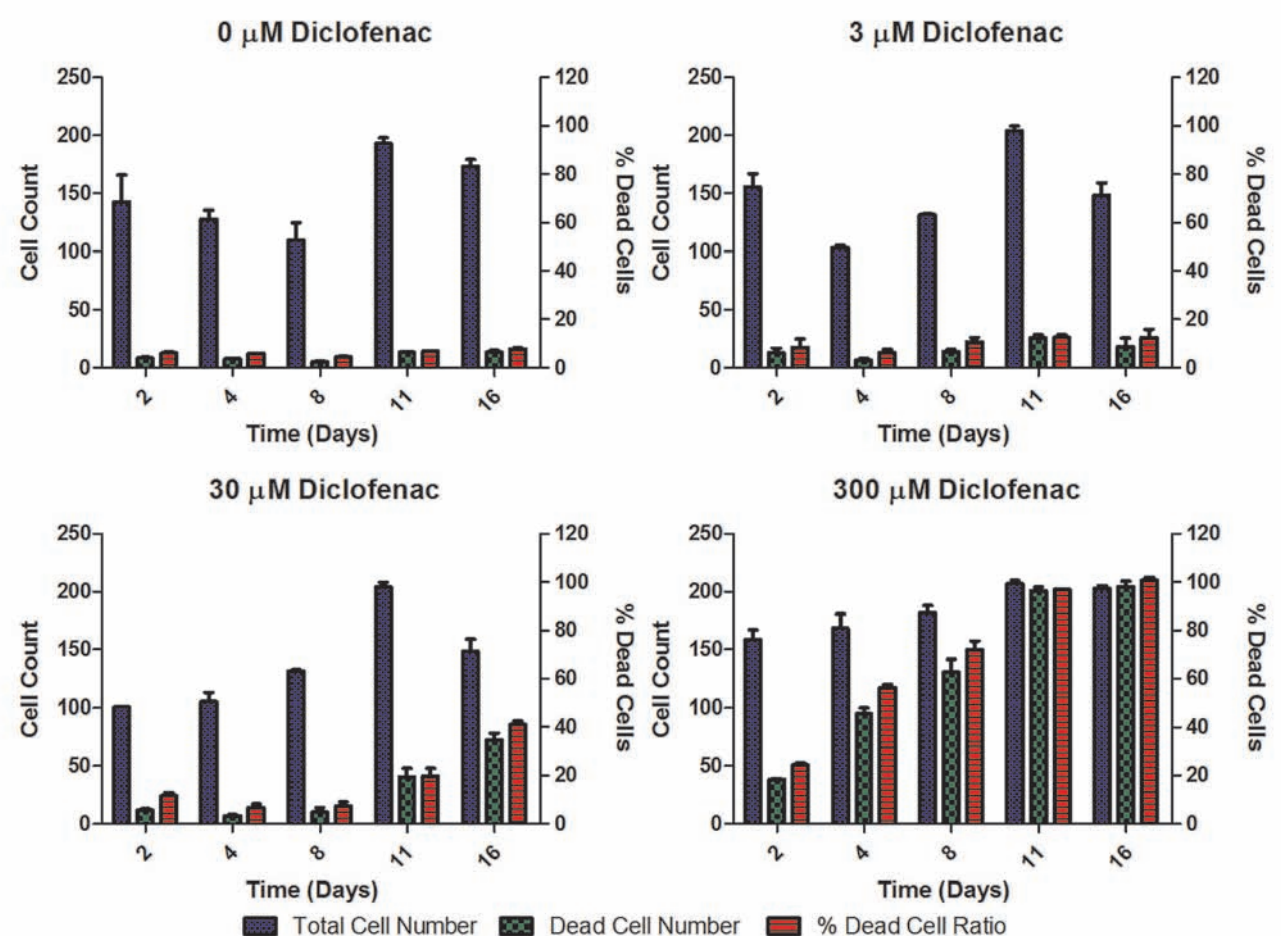


Figure 2 – Diclofenac Hepatotoxicity Results using Liver Microtissues. (A) Total cell counts using Hoechst 33342 stained cells, and dead cells using CellTox Green stained cells calculated for liver microtissues treated with diclofenac for dosing times listed, using Gen5 data analysis software (left y-axes). Hoechst stained cells quantified using an RFU threshold of 5000, and a minimum object size of 5 μm and a maximum object size of 30 μm. CellTox Green stained cells quantified using an RFU threshold of 9000 and equal minimum and maximum object sizes as previously stated. % dead cells (right y-axes) also calculated by Gen5 using the following formula: $(Dead Cell Number / Total Cell Number) * 100$.

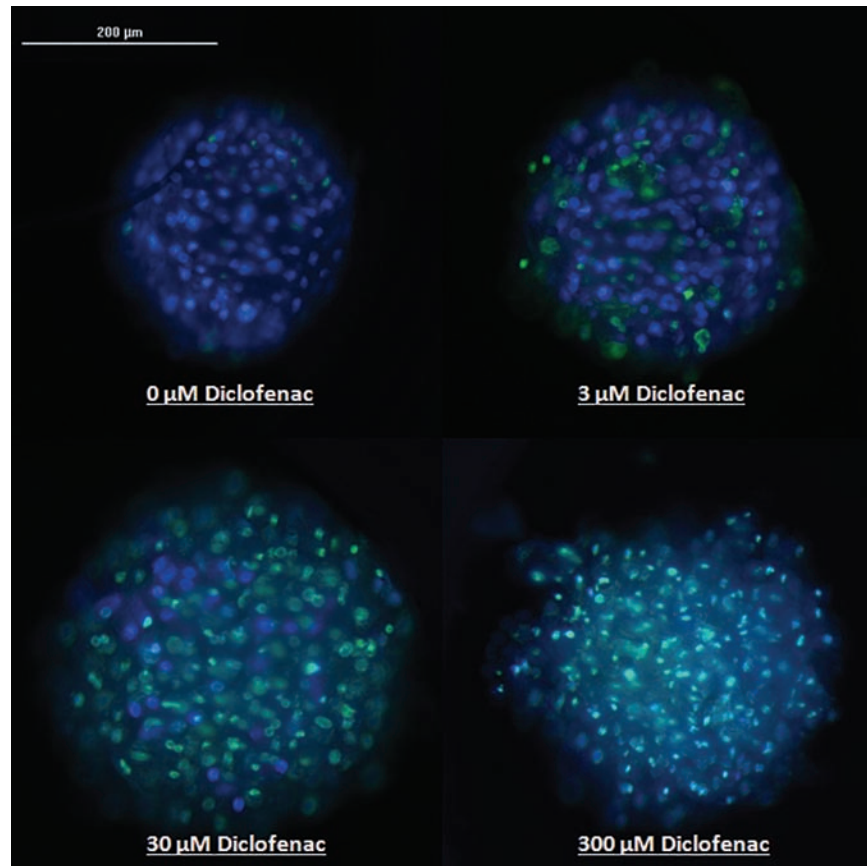


Figure 3 – Cytotoxicity Imaging. Representative overlaid images of liver microtissues after a 10 day diclofenac treatment, stained with Hoechst 33342 (blue) and CellTox Green (green), and captured using the DAPI or GFP Cytation 3 imaging channels, respectively. Autofocus performed on CellTox Green stained cells.

The results illustrated in the graphs from Figure 2 and images from Figure 3 demonstrate that total cell numbers counted in the liver microtissue remain relatively constant, while the number of dead cells increases during the 16 day dosing period in a manner dependent on diclofenac concentration. This demonstrates that the microtissues represent a viable option to monitor potential cytotoxic effects of test compounds, particularly when chronic dosing evaluations are desired.

The data also shows that significant cytotoxicity is not seen until the third day of dosing with diclofenac, using a 300 μM concentration. This finding contrasts with previously published data stating that cytotoxicity from diclofenac concentrations ranging between 200-450 μM can be seen in as little as 24 hours with hepatocytes plated in 2D (Gómez-Lechón *et al.*, 2003), and in as little as 2 hours with suspension hepatocytes (Pourahmad *et al.*, 2011), and underlies the need to use the most relevant cell model for cytotoxicity studies.

Diclofenac Cytotoxicity Study – 2D Hepatocyte Model

Cytotoxicity assessments were also performed using the CellTox Green and Hoechst 33342 assays with hepatocytes cultured in 2D. This was done to further assess whether differences in induced toxicity levels could be seen between the two cell models. A single cell concentration of 10,000 cells/well was used due to the fact that cellular ATP levels remained the most consistent over the two week dosing period, compared to lower cell concentrations.

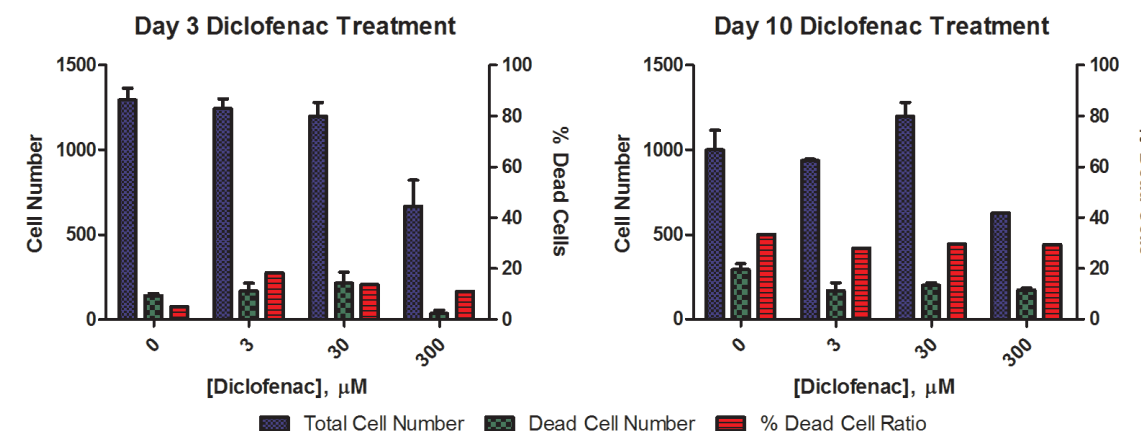


Figure 4 – Diclofenac Hepatotoxicity Results using 2D Cell Culture. Total cell counts using Hoechst 33342 stained cells, and dead cells using CellTox Green stained cells calculated for 10,000 cell/well concentration cultured in 2D, treated with diclofenac for 3 or 10 days. Hoechst and CellTox Green stained cells quantified using an RFU threshold of 10,000, and a minimum object size of 5 μm and a maximum object size of 35 μm.

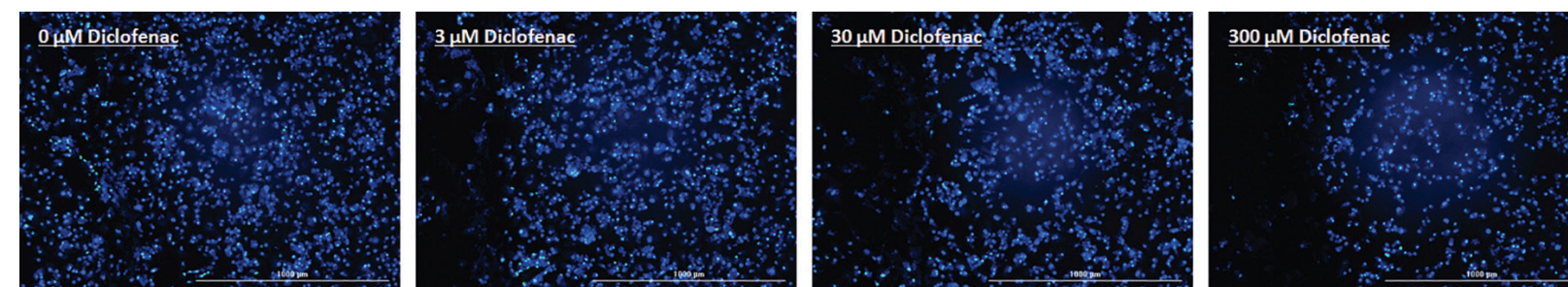


Figure 5 – Representative Overlaid Images of 2D Cultured Hepatocytes. Images captured of hepatocytes cultured in 2D following a 10 day diclofenac treatment with the listed concentrations. Cells were stained and images captured using the same procedures as previously described. Autofocus performed on Hoechst 33342 stained cells.

By examining the images in Figure 5, as well as the total cell count numbers from the two graphs in Figure 4, it is evident that the cytotoxic effects of diclofenac on hepatocytes cultured in 2D causes the cells to detach from the bottom of the well. This phenomenon causes the total cell and dead cell counts to be artificially lowered, with the end effect being that the % dead cell ratio does not change, as was seen with the liver microtissues in Figure 2. Therefore it is impossible to accurately assess long-term diclofenac cytotoxicity using this method of cell culture, and further supports the notion that liver microtissues provides the most relevant cell model for long-term cytotoxicity assessments.

Diclofenac Cytotoxicity Mechanism of Action Confirmation

Confirmation studies were then conducted to determine whether the different mechanism of action (MOA) purported to be involved in diclofenac cytotoxicity, specifically effects on the mitochondria, using rat hepatocytes could also be seen using human hepatocytes cultured into 3D liver microtissues. Oxidative stress at the mitochondrial level and MPT pore opening were monitored on the same days in which cytotoxicity was assessed.

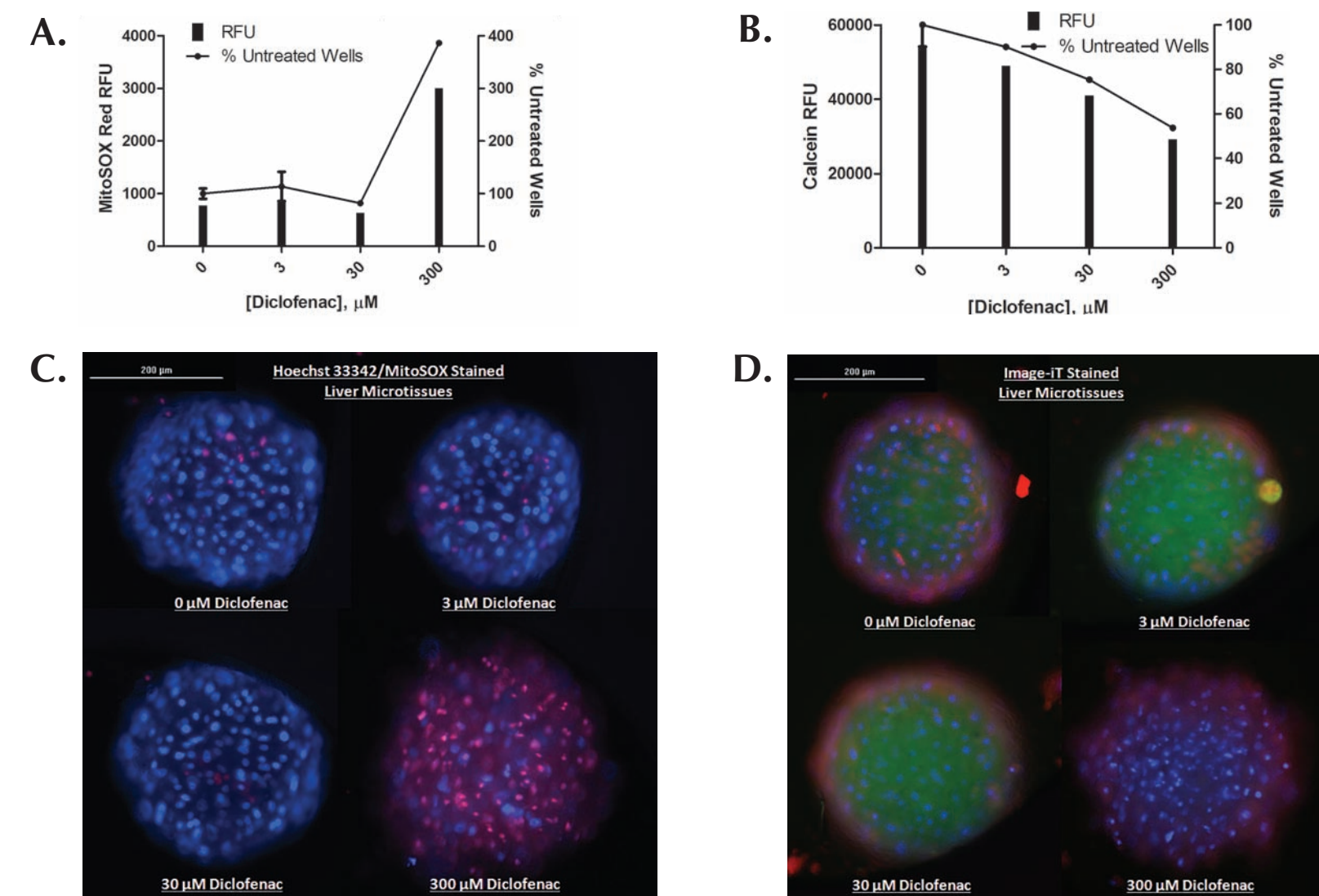


Figure 6 – Mitochondrial Oxidative Stress and MPT Induction Assessment. Mean pixel RFU values from the field of view (left y-axes) measured from MitoSOX assay signal following a three day dosing with diclofenac (A), or from Image-iT calcein signal following a seven day dosing with diclofenac (B). % signal for diclofenac treated wells (right y-axes) calculated by comparing to untreated well signal using the following formula: $(Diclofenac Treated Well Signal / Untreated Well Signal) * 100$. Representative overlaid images shown of liver microtissues after a (C) 3 day diclofenac treatment, stained with Hoechst 33342 (blue) and MitoSOX Red (red), and captured using the DAPI or RFP Cytation 3 imaging channels, respectively; and (D) after a 7 day diclofenac treatment, stained with Hoechst 33342 (blue), MitoTracker Red (red), and calcein (green), and captured using the DAPI, Texas Red, or GFP Cytation 3 imaging channels, respectively. Autofocus performed on DAPI stained cells for all imaging performed with the two assays.

Diclofenac induction of mitochondrial oxidative stress was confirmed using the MitoSOX assay (Figure 6A and 6C). Following a 3 day dosing with a 300 μM concentration, a significant increase in signal from the red fluorescent probe was seen, indicating an elevation in superoxide levels. This is in keeping with previously published data stating that diclofenac cytotoxicity is a result of ROS formation (Pourahmad *et al.*, 2011). Increases in mitochondrial oxidative stress also led to opening of the MPT pore, as witnessed by the decrease in calcium signal from increasing diclofenac concentrations (Figure 6B and 6D). Cobalt chloride, a component of the Image-iT assay, quenches cytosolic calcein, but not mitochondrial calcein as it cannot freely pass through the mitochondrial membrane. Upon opening of the MPT pore, cobalt chloride can then enter the mitochondria and quench the calcein signal, which was observed after a 7 day dosing with the compound. This again agrees with literature findings (Gómez-Lechón *et al.*, 2003), and confirms that these important MOA are also seen using human hepatocytes as 3D microtissues.

Conclusions

- 3D Human Liver microtissues retain viability and normal function over extended culturing periods, and represent a viable option for performing long-term dosing studies
- The CellTiter-Glo 3D and P450-Glo luminescent assays, in addition to the CellTox Green, MitoSOX, and Image-iT imaging assays deliver accurate, reliable results when used with the incorporated 3D cell model
- The dedicated luminescence detection system on the Cytation 3 is able to easily quantify the signal from the CellTiter-Glo, CellTiter-Glo 3D, and P450-Glo luminescent assays
- Fluorescence microscopy of the liver microtissues, and cellular analysis, is also accomplished using the imaging module and Gen5 data analysis software
- Diclofenac mitochondrial injury and eventual cytotoxicity seen previously with other hepatocyte models are also seen in 3D liver microtissues using human hepatocytes
- The differences seen in the observance of diclofenac induced cellular events using liver microtissues, when compared to published results using other hepatocyte models, underscores the need for incorporation of the most relevant cell model when performing long-term cytotoxicity studies