Exploring Human Fibrosis Models Using a Real-time Cellular Biosensor- Discovery-Q

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Technology

We have developed a multi-well cell-based sensor that can monitor real-time biological changes in living cells, such as mass redistribution, and viscoelasticity. Our platform is designed to maintain physiologically appropriate temperature, humidity and CO_2 (Fig1).

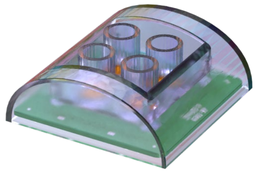

Figure 1. Discovery-Q Device, in incubator, with humidity, temperature and CO_2 control

This system provides unique kinetic information regarding phenotypic cellular changes post treatment, using the cell as a sensor (Fig 2A). The system measures change in frequency and resistance. (Fig 2B,C).

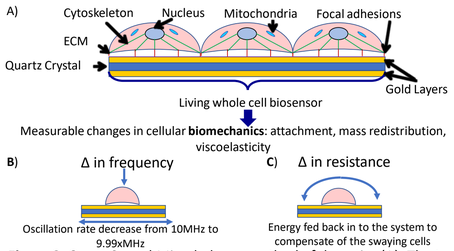

Figure 2. Quart Crystal Microbalance method of detection(A). The two unique measurements are change in frequency (B) that relates to the physiological state of the cytoskeleton and cell to cell connections, and change in resistance (C) relates to the viscoelasticity and sway of cells.

When cells bind a matrix there is a initial negative change in frequency (Δf), and then an increases as cells become internally organized and polarized, leading to ECM attachment and intercellular connectivity (fig 3)**.** For HepG2 cells, all extrinsic and intrinsic forces reach homeostasis at 24 hours.

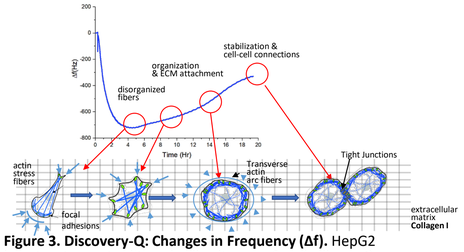

Figure 3. Discovery-Q: Changes in Frequency (Δf). HepG2 hepatocytes on traditional cell culture surface (collagen I)

When the cells bind the matrix there is an initial increase in resistance (ΔR), followed by a decrease as cells become connected to the ECM and form cell-cell connection (fig 4). This results in polarization of the monolayer causing the cells to oscillate on the quartz crystal in unison.

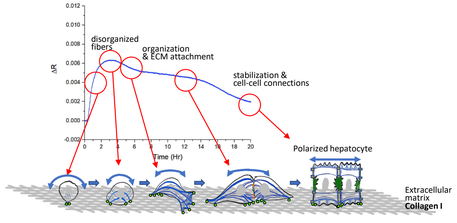

Figure 4. Discovery-Q: Changes in Resistance (ΔR). HepG2 Hepatocytes on traditional cell culture surface (collagen I)

Biomechanics of Liver Disease

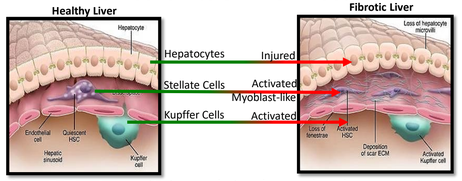

Figure 5. The changes that occur in the liver during disease onset, and the cellular components that drives progression.

The liver is the organ responsible for multiple vital functions in the human body. Compromised liver function a leading reason for death in the US(1). To be able to study liver disease progression, the ability to form and acces primary liver co-cultures is necessary. (1) In addition it is necessary to study how physiologically relevant extracellular matrices (ECMs) affects the co-culture (2-5). However, there is no method currently available to provide real time tracking of primary liver cell de-differentiation, or the cell-to-cell and cell-to-ECM effects in co-cultures. Using the In-Vitro-Q with primary liver hepatocytes and stellate cells provided by Thermo Fisher Scientific, we have developed a novel method to track both cell-to-cell interactions in co-cultures, and cell-to-ECM interactions using primary liver cells, thereby simulating physiological conditions.

Methods

Thermo Fisher Scientific Life Technologies Primary Hepatocytes (HMCPIS) were used in the first experiment to test the In-Vitro-Q's ability to measure in real-time across multiple days, the binding, monolayer formation and hepatocyte de-differentiation. An ECM of poly lysine (P6407 Sigma-Aldrich) was added to each Discovery-Q well according to manufacturer's instructions. Warmed plating media was added to the Discovery-Q before cells were added, and data acquisition was initiated. Cells were thawed in plating media and added to the Discovery-Q according to manufacturer's instructions. All subsequent cell maintenance steps were followed, and the cells were trypsinized 8 days later (Fig 6.)

For the co-culture experiment either Collagen I from rat tail (C7661, Sigma-Aldrich) or Collagen IV from human placenta (C5533, Sigma-Aldrich) was used as the ECM and applied according to manufacturer's instructions. Co-cultures were created by mixing hepatocytes (HMCPTS) and stellated cells (HMFHSC) at ratios provided by Dr. Witek. All culture methods were followed as above (Fig 7-10).

At each time point where a media change occurred, cell counts were conducted. Supernatant was preserved to run an LDH cytotoxicity assay using a kit (88953, Pierce), and Albumin assay using a kit (MAK124, Sigma-Aldrich), both according to manufacturer's specifications (Fig 11).

Results

Analysis of Life Science Primary Hepatocytes using Discovery-Q

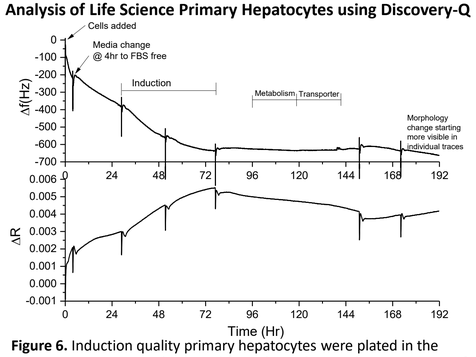

Figure 6. Induction quality primary hepatocytes were plated in the Discovery-Q system and cultured for 8 days. This graph is an average of many 24 well traces, and the Thermo Fisher Scientific experimental window times are superimposed.

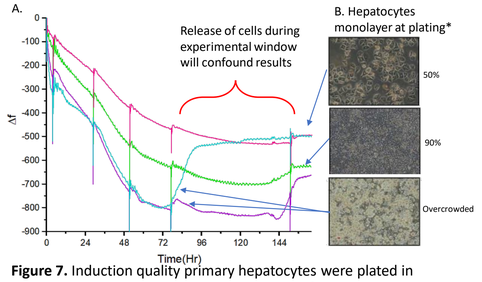

Figure 7. Induction quality primary hepatocytes were plated in Discovery-Q. The variability in plating is quantified by the system, and indicates potential for confounding data due to lifting if the optimal density is not obtained. B) Images from Thermo Fisher Hepatocyte workshop

The Discovery-Q can follow the culture dynamics of the primary hepatocytes, and demonstrate that due to variability some wells at 72 hours exhibited loss of cells (Fig 7A). Cell loss due to overcrowded cells is suspected. Microscopic images were provided by Thermo Fisher Scientific (Fig. 7B)

Simulation of primary liver cells in co-culture using the Discovery-Q

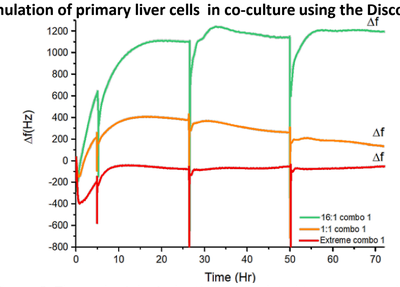

Figure 8. Three physiological ratios were plated to compare culture biomechanics as disease progression is modeled. Green - 16 Hep: 1 Stellate- Simulation of a normal liver. Orange - 1 Hep : 1 Stellate- Simulation of a fibrotic liver. Red - 1 Hep: 4 Stellate- Simulation of extreme inflamed fibrotic liver. All cells were plated on a Collagen I ECM.

Figure 8 shows an experiment conducted to determine if the Discovery-Q could detect the differences in cell-cell interaction created by changing hepatocyte to stellate ratios to simulate disease states. Importantly, total cell counts are the same within each co-culture, regardless of ratio. The Discovery-Q can readily identify altered co-culture ratios, and how each alters the degree of intercellular interaction (Fig 8). The Δf decreases as the establishment of cytoskeletal structures and cell-cell interactions declines with a more diseased state.

In Figure 9, Discovery-Q data shows that the addition of only 6% stellates into a 94% percent hepatocyte co-culture (16:1 ratio) changes cytoskeletal structures to a degree which is significantly greater than the sum of the individual cell-type frequency changes. This shows that there is a synergistic effect of the cellular cytoskeletal interactions. The resistance shows that the monolayer moves like a hepatocyte-only monolayer.

QCM can detect changes in cell-ECM

Collagen I vs Collagen IV

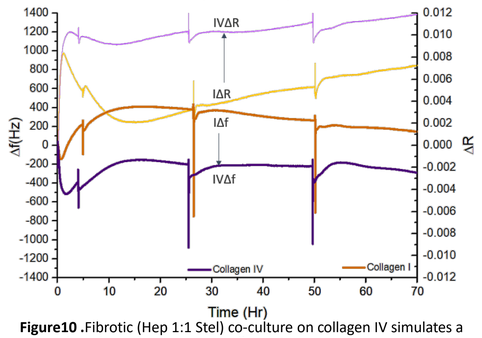

Figure10 . Fibrotic (Hep 1:1 Stel) co-culture on collagen IV simulates a more physiological response vs collagen I

We examined the impact of different ECM by changing the collagen I matrix to a collagen IV matrix, more typical of fibrosis. As seen in Figure 10, changing the ECM from a elastic (I) to a rigid (IV) ECM causes the cells to adapt to interaction with a more rigid matrix. This was seen in frequency (Δf) decreases as cells attempt to bind to stiff crosslinked collagen IV fibers (Fig 10). This results in a stressed cytoskeletal conformation and decreased cell-to-cell interactions. The stellates are involved in active remodeling of the ECM. The resistance (ΔR) continues to rise as the cells cannot bind well to the ECM and create a polarized monolayer (Fig 10). Eventually a stressed, stable confirmation is reached.

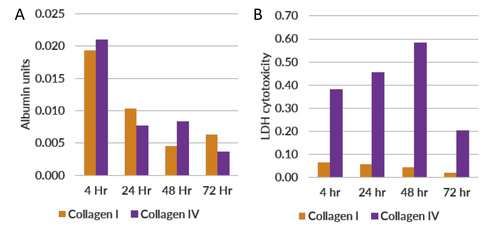

Figure 11. Albumin (A) and LDH (B) levels were checked at every feeding, as indicators of co-culture health.

Synergistic response of co-culture

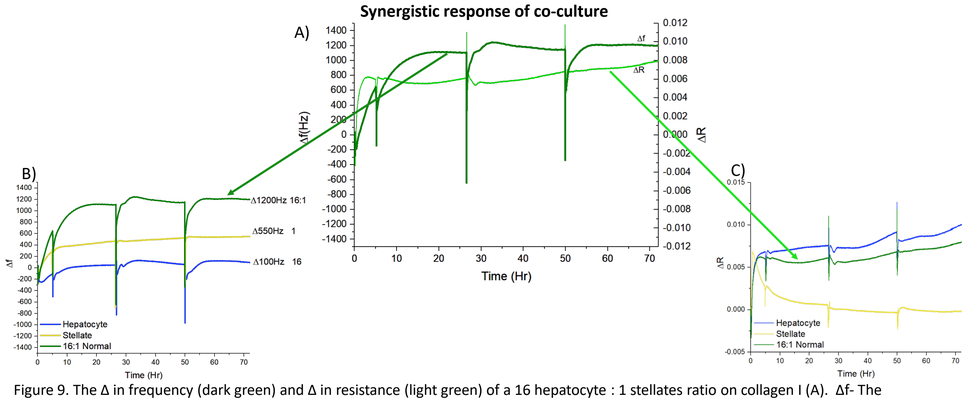

Figure 9. The Δ in frequency (dark green) and Δ in resistance (light green) of a 16 hepatocyte : 1 stellates ratio on collagen I (A). Δf- The introduction of 6% stellate cells significantly increases the health of the co-culture (B).The addition of the individual cell components creates a synergistic response. ΔR- monolayer is mostly influenced by 94% polarized hepatocytes (C).

In addition to the real-time phenotypic measurements made by the Discovery-Q assessing cell-to-cell and cell-to-ECM interactions, standard molecular assays were performed on the same cultures to quantify cell health. This was the second test that was conducted at each media change. Albumin serum level were tracked at every media change and are indicative of liver health. A decrease being indicative of a compromised liver (1), (Fig 11A). Lactate dehydrogenase (LDH) serum level were similarly tracked, are indicative of fibrosis, and can be a prognostic factor for treatment (6) (Fig 11B). The benefit of being able to obtain supernatant during Discovery-Q use is that recordings can continue, while additional quantitative readings are taken.

The albumin and LDH levels showed that in addition to the 1:1 hepatocyte: stellate ratio, ECM alteration to collagen IV significantly altered culture characteristics and may better resemble physiologically fibrotic tissue.

Conclusion

Discovery-Q:

I. Revealed otherwise obscure effects of initial cell density on culture health during experimental use, which may confound pharmacology evaluation.
II. Demonstrated significant physiological effect of the changing ratios of primary liver cells in co-cultures
III. Demonstrated powerful synergies in primary liver cell co-cultures due to cell-cell and cell-ECM interactions
IV. Readily distinguished between different ECMs and their effects on primary cells and co-cultures
V. Demonstrated that a fibrotic ECM with a fibrotic co-culture ratio yielded physiologically relevant albumin secretion and LDH levels

Discovery-Q & modeling liver fibrosis

The extracellular matrix is a very complex and integral part of overall tissue health. To mimic disease, a physiological tissue extracellular matrix environment for cells to grow on must be simulated. A physiologically "normal" extracellular matrix slows de-differentiation of human primary hepatocytes, whereas a "fibrotic" matrix will drive disease progression. The Discovery-Q is a biomechanical sensing platform that can detect and quantify these cell-ECM effects, as demonstrated with the measurements of the co-culture cellular response to a varying collagen matrix.

Discovery-Q & Liver Models

Discovery-Q can rapidly assess the characteristics of primary liver cell donors and determine optimal windows for in vitro experimental use. Our ability to detect and characterize the biomechanical interplay between cells and extracellular matrix enables quantitative measurements of disease models: NASH, Fibrosis, Pharmacology, Toxicology, and Tumorigenicity, and the impact of pharmacology on each.

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