Characterization of Cryopreserved HepaRG Cells for Multiparameter High Content Analysis

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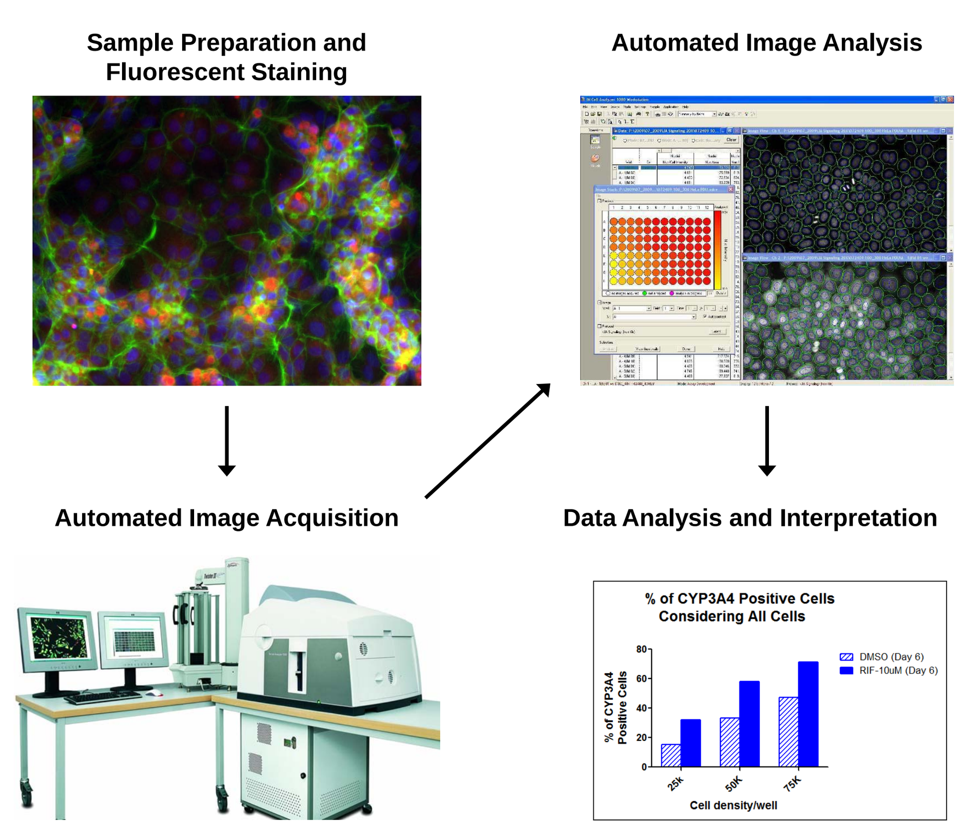
Introduction

The promise of High Content Analysis (HCA) and systems biology techniques for human hepatotoxicity has been limited by a. scarcity, variability and short lifespan of primary human hepatocytes, b. lack of metabolic activity in widely used cell lines such as HepG2, and c. complex long-term protocols required to differentiate progenitor cells. Thus, alternative model cell systems are needed.

We evaluated newly available cryopreserved HepaRG cells, a metabolically competent immortal hepatoma cell, for use in multiparameter HCA assays. HepaRG is a human bipotent cell line which differentiates toward two distinct cell phenotypes (hepatocyte and biliary) with characteristic dense organization. HepaRG cells, unlike many other immortalized hepatic cell lines, maintain characteristics of primary human hepatocytes including cytochrome P450 mediated metabolism, transporter functions and also expresses key nuclear receptors involved in signal transduction pathways which are known to play important role in liver injury following drug exposure.

Our key objective was to determine if these cells are amenable to multiparameter HCA under conditions where a hepatocyte phenotype is retained. Furthermore, we aimed to determine if these cells could be used for imaging-based CYP activation studies and multiparametric HCA toxicity studies.

Workflow for High Content Analysis



Methods

1. Cell Culture
Cryopreserved HepaRG cells (EMD Millipore) were thawed and cultured at 25K, 50K and 75K cells/well in collagen coated 96-well plates. Basal and induced CYP3A4, 2C9 and 1A2 activities were measured at the beginning and end of induction period using P450-Glo Assays (Promega).

2. Compound Treatment
On day 3 the cells were treated with CYP inducers Rifampicin (10uM) or Omeprazole (50uM) for 72 hrs with DMSO (<0.1%) used as a control.

3. Immunofluorescent Staining
Following the culture period, the cells were fixed using HCS fixation solution at room temperature. Cells were washed with immunofluorescence buffer and stained using CYP3A4 primary antibody (Millipore AB1254), CK19 (Millipore MAB3238) followed by Cy3- and FITC- labeled secondary antibodies for CYP3A4 and CK19, respectively (EMD Millipore). The nuclei were stained using Hoechst dye (Sigma 14533). Wells were rinsed and sealed prior to HCA imaging. MitoTracker, CellTracker and Phalloidin dyes were from Life Technologies.

4. HCA Imaging and Analysis
Plates were imaged on a GE IN Cell Analyzer 1000 at 20X objective magnification with 20 fields of view/well. Images were analyzed with GE IN Cell Analyzer 1000 Workstation (3.7) software, utilizing the Multi Target Analysis algorithm.

Results

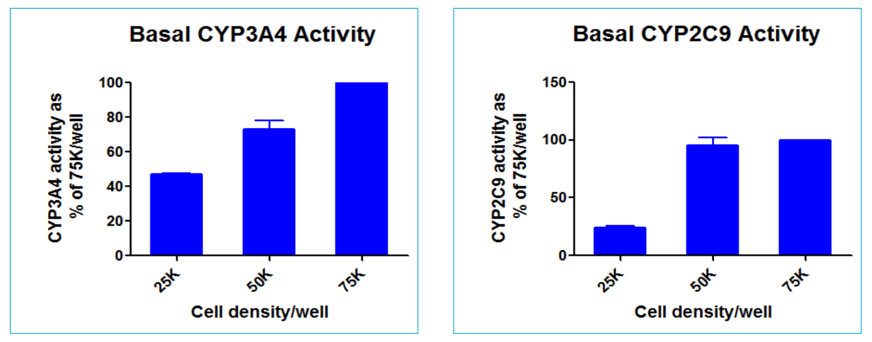


Figure 1. Basal CYP activity in HepaRG cells. HepaRG cells were plated on 96-well collagen coated plates in growth media at 25K, 50K and 75K cells/well and cultured for 72 hours. Basal CYP3A4 (Fig.1. left panel) and 2C9 (Fig.1. right panel) activities were determined. Data presented are % enzyme activity compared to 75K/well seeding density, n = 3. 25K and 50K seeding densities showed 50% and 25% lower CYP3A4 while CYP2C9 basal activity at 50K is comparable to 75K but is 70-75% lower at 25K.

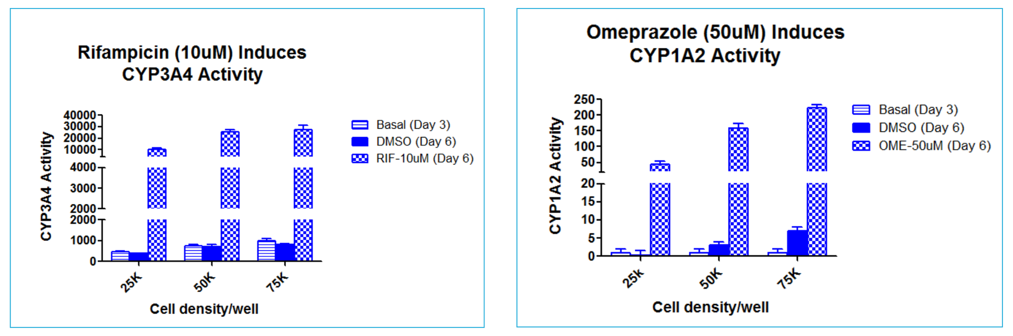


Figure 2. CYP induction in HepaRG cells. HepaRG cells were plated on 96-well collagen coated plates in growth media at 25K, 50K and 75K cells/well and treated with DMSO (0.1%), Rifampicin (RIF,10uM) or Omeprazole (OME,50uM) for 72hrs starting at Day 3 in culture. RIF induced 30-50 fold increase in CYP3A4 activity at all seeding densities (Fig.2.left panel). The basal CYP1A2 activity was undetectable but was induced 40-200 fold in presence of OME (Fig.2. Right panel). Data presented as CYP activity after normalization to protein content in a representative experiment.

Results

We began by assessing CYP activity in HepaRG cells. For CYP activity vs. seeding density studies we chose to perform 72 hr inductions beginning at Day 3 in culture. Basal CYP activities, but not fold-inductions, were found to be closely related to seeding density. 25K and 50K seeding densities showed lower basal CYP3A4 activity (50% and 20% respectively) than 75K, while rifampicin (10uM, 72h) evoked 30-50 fold CYP3A4 induction at each seeding density. CYP2C9 basal activity at 75K and 50K were equivalent, but 70-75% lower at 25K. The CYP1A2 basal activity was very low or undetectable but was highly induced by omeprazole (40-200 fold) at all seeding densities (50uM, 72h). From these experiments we concluded that seeding densities under 50K per well are unsuitable for experiments requiring CYP activity.

We then examined the suitability of HepaRG cells for quantitative imaging experiments. Triple labeling of the cells with Hoechst dye, CYP3A4 and CK19 antibodies enabled clear distinction of the hepatocyte and biliary cell populations, which could then be segmented and quantified using image analysis software. Based on quantitative image analysis, we determined that approximately of 60% cells were hepatocytes and 40% biliary cells in Day 3 cultures of HepaRG cells. Creating image segmentation masks for each cell type enabled downstream analysis of each cell type in isolation, rather than as a mixed population. This offers a significant advantage over analysis techniques which cannot distinguish between each cell type. Subsequent analysis correlating CYP3A4 and CK19 expression with nuclear measurements indicated that nuclear size and intensity measurements can serve as classifiers for distinguishing between hepatocyte and biliary cell populations, suggesting that antibody labeling of each cell type may not be necessary to distinguish between the two cell types.

We went on to use the CYP3A4 staining procedure to examine if image analysis could be used to quantify rifampicin-induced CYP3A4 induction. This experiment clearly showed that CYP3A4 was detectable and quantifiable using HCA algorithms. A final set of HCA experiments were performed using simultaneous four color staining with Hoechst, Mitotracker, Lysotracker and Celltracker dyes; these indicated that multiparametric image based measurements of hepatotoxic endpoints is readily achievable using HepaRG cells.

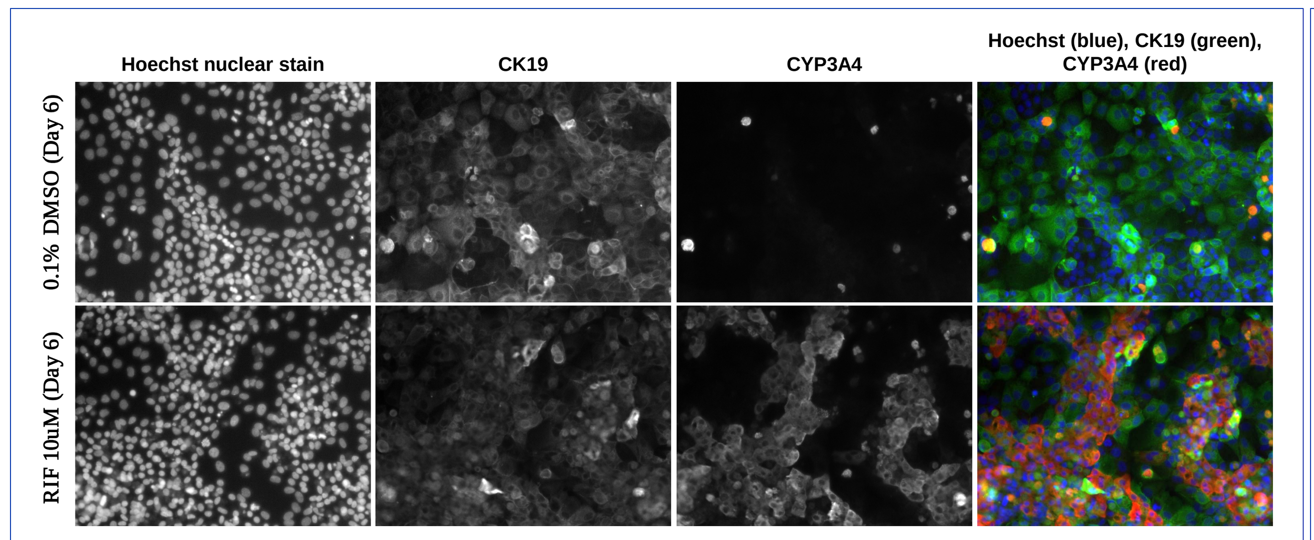


Figure 3. Multiplex immunolabeling of HepaRG hepatocyte and biliary populations. HepaRG cells were plated on 96-well collagen coated plates in growth media at 50K cells/well and treated with DMSO (0.1%) or Rifampicin (RIF,10uM) for 72hrs starting at Day 3 in culture. At the end of treatment period, cells were fixed and triple stained using antibodies against CYP3A4 (Cy3-Red), CK19 (FITC-Green) and Hoechst nuclear dye (Blue). Fig.3 shows increased CYP3A4 staining in presence of RIF (bottom panel) over DMSO ctrl (top panel) indicating RIF mediated induction of CYP3A4.

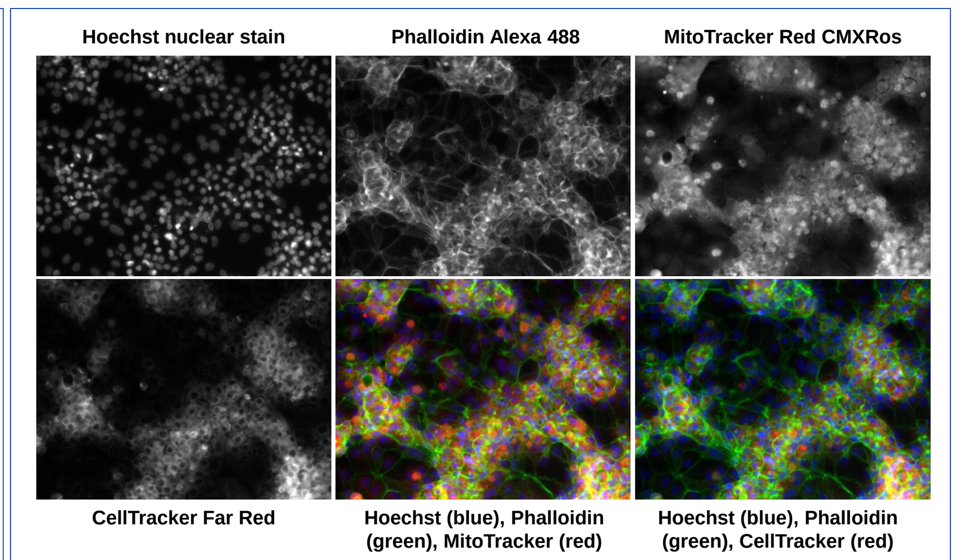


Figure 5. Multiplex labeling of HepaRG with cell function dyes. HepaRG cells were plated on 96-well collagen coated plates in growth media at 50K cells/well and cultured for 3 days. Cells were stained with MitoTracker Red, Phalloidin Alexa 488, CellTracker and Hoechst nuclear dye. Fig.5 shows that HepaRG cells may be readily stained with multiple cell function dyes for High Content Analysis, indicating these cell's utility for multiparametric image based hepatotoxicity screening.

Fig.4A.

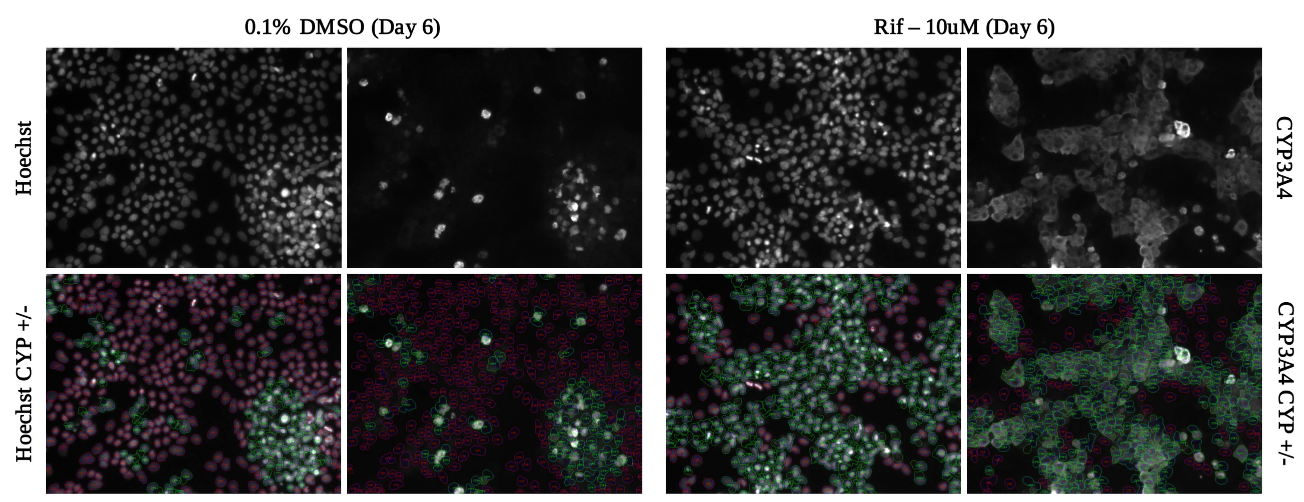


Fig.4B.

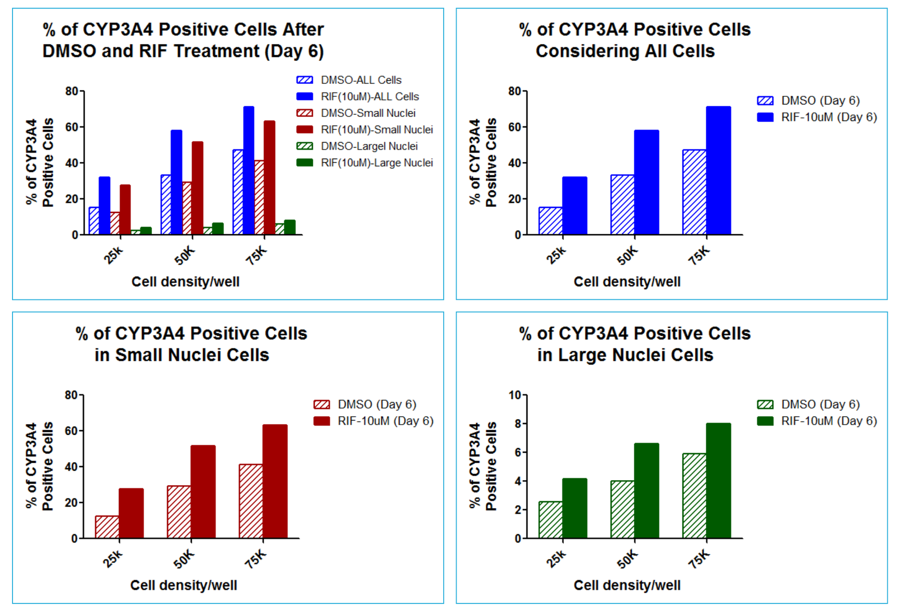


Figure 4. Quantitation of CYP induction via High Content Analysis. HepaRG cells were plated on 96-well collagen coated plates in growth media at 50K cells/well and treated with DMSO (0.1%) or Rifampicin (RIF,10uM) for 72hrs starting at Day 3 in culture. At the end of treatment period, cells were fixed and triple stained using antibodies against CYP3A4, CK19 and Hoechst nuclear dye. Following automated imaging, cells underwent sequential segmentation and analysis based on CYP3A4 expression (red, CYP3A4 –ve and green, CYP3A4 +ve) followed by nuclear size (small vs. large) as shown in Fig.4A. The CYP3A4 staining and segmentation analysis depicts that CYP3A4 is inducible after RIF treatment and is quantifiable using HCA algorithms. The % of CYP3A4 positive cells is increased 1.5- 2 times with RIF treatment at all seeding densities, in entire cell population and cells with small nuclei (Fig. 4B). We also determined that in 3 day culture approximately 60% cells were hepatocytes while 40% cells were biliary cells.
Segmenting HepaRG cell images on the basis of nuclear size enables specific analysis of the hepatocyte population within HepaRG cells, and excludes the biliary subpopulation, without the need for additional cell labeling steps.

Conclusions

- From the CYP activity experiments we concluded that the high levels of basal and induced CYP activity observed in HepaRG cells require seeding densities of at least 50K per well (96 well plate). Thus, seeding densities under 50K per well are unsuitable for experiments requiring CYP activity as it significantly affects basal as well as induced CYP activities. We recommend a seeding density of 50K as this is optimal for both CYP studies and cellular segmentation for High Content Analysis.
- Immunostaining followed by High Content Analysis correlating CYP3A4 and CK19 expression with nuclear measurements indicated that nuclear size and intensity measurements can serve as classifiers for distinguishing between hepatocyte and biliary cell populations, suggesting that antibody labeling of each cell type may not be necessary to distinguish between the two cell types.
- CYP3A4 staining and downstream image analysis can be applied to quantify CYP3A4 induction, our data clearly showed that rifampicin-induced CYP3A4 induction was detectable and quantifiable using HCA algorithms.
- Despite the dense organization of metabolically competent HepaRG cells, these cells are extremely amenable to multiparameter imaging-based applications and may readily be analyzed by image analysis algorithms.

Summary

Hepatotoxicity remains a major factor in the high fail rate of drug development and withdrawal of drugs from the market; and an active area of current research. With this study we have demonstrated that cryopreserved HepaRG cells may be an enabling cellular system for hepatotoxicity profiling, offering fast, highly reproducible results from metabolically competent cells. By developing optimal assay conditions and proof of concept studies for multiparameter HCA, we have demonstrated that comprehensive assessment and analysis of the cellular systemic response to toxin challenge in a metabolically competent cellular model is possible, creating the opportunity for novel mechanistic systems level studies of hepatotoxicity and greatly improved productivity in drug discovery and development.

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