Automation of an Image-based Assay to Model Nonalcoholic Steatohepatitis (NASH)

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Abstract

Nonalcoholic steatohepatitis of NASH is a common liver disease whose major feature is fat in the liver along with inflammation, which can lead to liver damage and cirrhosis. While no definitive cause has been identified, the cellular toxicity of numerous fatty acid metabolites is suspected. The formation of fatty droplets has been proposed as a marker for lipotoxicity caused by fatty acid derived species. HepG2 liver cells exposed to fatty acid mixtures respond in a concentration dependent manner, with over 80% testing positive at high concentrations. While the liver is the organ primarily responsible for the *de novo* synthesis of lipids and the conversion of free fatty acids to neutral lipids, *in vitro* cell types other than liver cells can accumulate neutral lipids. Here, we examine the production of lipid droplets in a number of different cells types including fibroblasts, epithelial cells and primary hepatocytes when exposed to palmitic and oleic acids.

We show that liver cells, when exposed to various concentrations of a mixture of oleic and palmitic free fatty acids (FFA), accumulate intracellular neutral lipids in a dose-dependent fashion up to 1 mM. Dosages of FFA higher than 1 mM result in cell death and the loss of cells. Several other cell lines including HeLa, HT1080 and NIH3T3 cells show a response, albeit at different rates and fatty acid concentrations. Following treatment, cells are automatically fixed and stained with Nile red (lipid stain) and DAPI (nuclear stain). Fixed and stained cells were digitally imaged with a Cytation™ 5 Cell Imaging Multi-Mode Reader using a 10X objective.

In order to investigate the temporal relationship with lipid droplet formation and fatty acid exposure, we automated the assay process such that challenged cells are fixed and stained at defined intervals over a 24 hour period. Uptake of FFA and their conversion to neutral lipid was monitored over time using a BioSpa™ Automated Incubator to present plates to a liquid handler at periodic times after the addition of FFA in order to stop the reaction by adding fixative to the cells at various times. Automated image analysis was then used to determine the percentage of cells positive for neutral lipids. Recovery, after removal of the challenging fatty acids, as measured by lipid droplet depletion was also examined in a similar fashion.

Fatty Liver Tissue

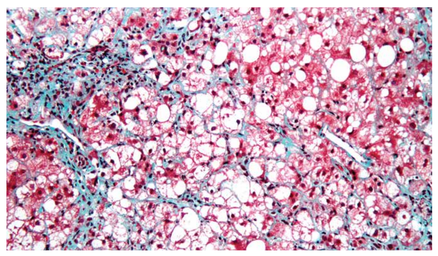

Figure 1. Trichrome stain of liver biopsy depicting nonalcoholic steatohepatitis (NASH). Image depicts balloon degeneration of hepatocytes (large cells with small centrally located nuclei) along with fine-chicken wire fibrosis, hepatocyte necrosis and inflammation. Image copyright © undated Michael Bonert; used per the CC-BY-SA 3.0 license. Accessed at https://commons.wikimedia.org/w/index.php?curid=7747223

Assay Process

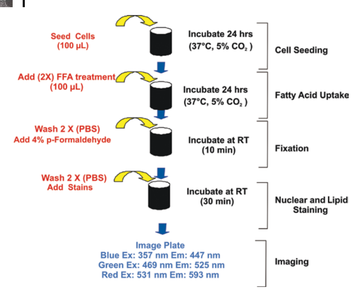

Figure 2. NASH model assay process. Cells were routinely seeded into 96-well microplates using the MultiFlo™ FX Multi-Mode Dispenser's peripump dispenser and allowed to attach overnight. The following day, cells were challenged with a mixture (3:1) of oleic and palmitic free fatty acids at various concentrations and for various exposure times. Cells were subsequently fixed for 10 minutes with 4% paraformaldehyde (PFA) and then stained with DAPI (nuclei), Texas Red-phalloidin (actin) and BODIPY 493/503 for 30 minutes. Cells were imaged using a Cytation 5 and images were analyzed with Gen5™ Microplate Reader and Imager Software.

Neutral Lipid Staining

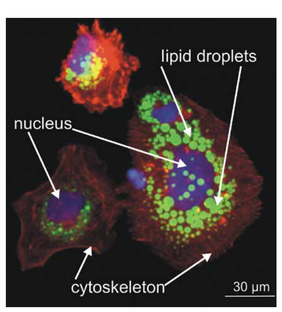

Figure 3. Neutral lipid staining of hepatocytes. Human hepatocytes were treated with a 3:1 mixture of oleic and palmitic fatty acids for 24 hours after which they were fixed and stained with BODIPY 493/503 (green), DAPI (blue); and Texas Red-phalloidin (red). 10X fluorescence images were captured using a Cytation 5 Cell Imaging Multi-Mode reader. Scale bar denotes 100 µm. The image has been digitally zoomed to provide greater structural detail.

Image Analysis

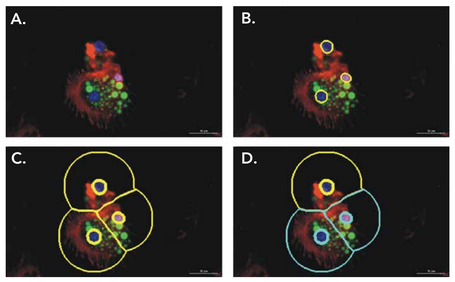

Figure 4. Quantitative analysis of image data. Primary mask analysis of the DAPI channel identifies individual cells by their nuclei. Secondary mask is identified by the space up to 30 µm surrounding the primary mask. **(A)** Raw image; **(B)** Primary mask identified by yellow trace; **(C)** Primary and secondary masks identified by yellow trace; **(D)** Primary and secondary masks with lipid positive cells identified with blue trace. Scale bar indicates 30 µm.

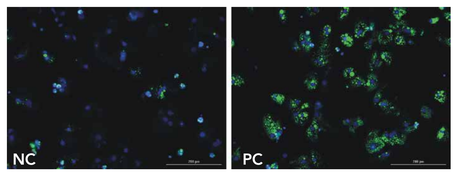

Figure 5. Negative and positive control images of hepatocytes stained with BODIPY 493/503. Primary hepatocytes were treated with 0.5 mM FFA mixture (positive control) or vehicle (negative control) for 24 hours, then fixed with 4% PFA and stained with DAPI (nuclei) and BODIPY 493/503 (lipid). Images were captured using a Cytation 5 with a 20x objective. Scale bar represents 100 µm.

Image vs. PMT Detection

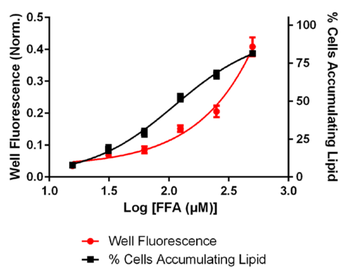

Figure 6. Lipid accumulation in HepG2 cells treated with various concentrations of free fatty acids. Lipid droplet formation after 24-hour exposure was assessed using Nile Red fluorescence using either PMT-based microplate reader optics (well fluorescence) or image-based subpopulation analysis (% cells accumulating lipid).

BioSpa Automation

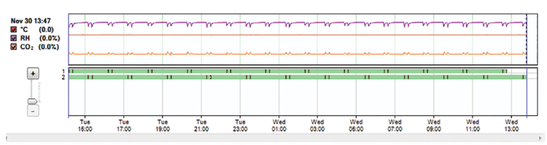

Figure 7. Gantt chart of a BioSpa 8 FFA uptake session. A series of wash and dispense routines were carried out with the MultiFlo FX to remove media and wash columns with PBS, then added 4% PFA fixative. After 10 minutes the fixative was removed and replaced with PBS. Two plates were treated in parallel such that a single column from one of the plates was treated every hour and each plate was only removed from the BioSpa every 2 hours. Plate were incubated in the BioSpa 8 at 37 °C, with a humidified 5% CO_2 atmosphere between reagent additions. After 24 hours, the plates were stained and imaged.

Cell Lineage Uptake Comparison

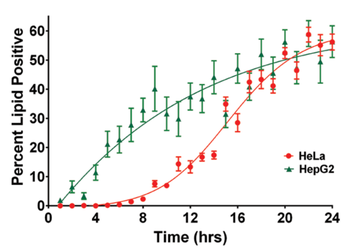

Figure 8. Comparison of lipid formation of HepG2 and HeLa cell lines. HeLa and HepG2 cell lines were seeded into 96-well microplates and challenged with 500 µM FFA mixture various amounts of time. At the indicated time after challenge, cells were fixed with 4% PFA. Total cell counts were determined using a primary mask to identify nuclei. Lipid positive cells were determined using a secondary mask surrounding the nuclei with a cutoff threshold of 1x10^6 on the integral of the GFP channel. Data points represent the mean and standard deviation of 8 data points.

Non-Hepatic Cell Response

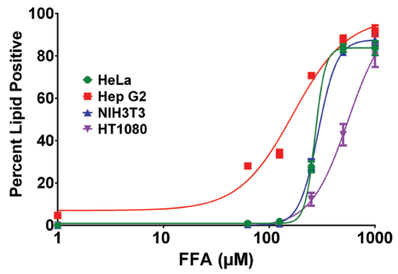

Figure 9. Comparison of non-hepatic derived cell lines. Cell lines were treated with various concentrations of free fatty acid mixtures for 24 hours and then fixed with 4% PFA and stained with DAPI (nuclei) and BODIPY 493/503 (lipid). Analysis reflects the percentage of lipid positive cells as a function of the cell number total. Total cell counts were determined using a primary mask to identify nuclei. Lipid positive cells were determined using a secondary mask surrounding the nuclei with a cutoff threshold of 1x10^6 on the integral of the GFP channel. Data points represent the mean and standard deviation of 2 data points.

Neutral Lipid Accumulation

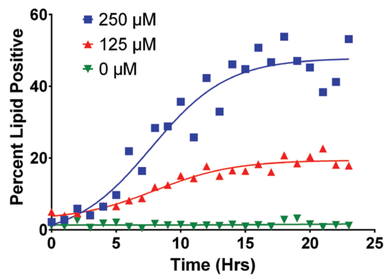

Figure 10. Comparison of neutral lipid accumulation. HepG2 cells were treated with different concentrations of FFA, then fixed and stained for neutral lipid at various times. Using a BioSpa to control timing and maintain the necessary environmental control, individual columns of a plate were fixed with 4% PFA at 1 hour intervals using a MultiFlo FX. Two plates were staggered such that each plate was removed from the BioSpa every two hours. Data represents the mean of 2 determinations.

Long-term Lipid Production

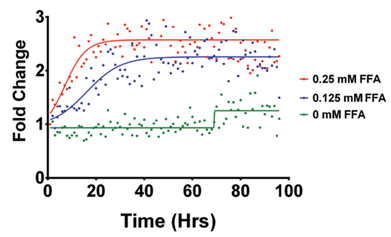

Figure 11. Long-term lipid accumulation. HepG2 cells were treated with a single dose of FFA mix and then fixed with 4% PFA at intervals over 4 days using a MultiFlo FX and a BioSpa to control timing and maintain the necessary environmental control. At the completion of the experiment, all plates were stained for nuclei and neutral lipid. Data represents the fold change from time zero. Each data point represents the mean of 2 separate determinations.

Lipid Containment

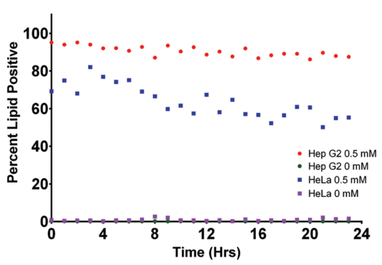

Figure 12. Comparison of lipid depletion of HepG2 and HeLa cell lines. HeLa and HepG2 cell lines were seeded into 96-well microplates and challenged with 0.5 mM or 0 mM, FFA for 24 hours, after which the media was replaced with fresh media without FFA. At the indicated time after FFA removal, cells were fixed with 4% PFA. Total cell counts were determined using a primary mask to identify nuclei. Lipid positive cells were determined using a secondary mask surrounding the nuclei with a cutoff threshold of 1x10^6 on the integral of the GFP channel. Data points represent the mean and standard deviation of 2 data points.

BioTek Instrumentation

Figure 13. BioSpa 8 Automated Incubator. The BioSpa 8 is a microplate incubator that can interface a BioTek microplate liquid handing device with a BioTek microplate reader/imager. The BioSpa 8 maintains temperature and humidity, as well as provides CO_2 and O_2 gas control for up to 8 microplates.

Figure 14. MultiFlo FX Multi-Mode Dispenser. The MultiFlo FX is a modular upgradable reagent dispenser that can have as many as two peri-pump (8 tube dispensers), two syringe pump dispensers and a strip washer. The syringe and washer manifolds can be configured for plate densities from 6- to 384-well.

Figure 15. Cytation 5 Cell Imaging Multi-Mode Reader. The modular, upgradable Cytation 5 combines automated digital microscopy and conventional microplate detection. Cytation 5 includes both filter- and monochromator-based detection; the microscopy module provides up to 60x magnification in fluorescence, brightfield, color brightfield and phase contrast. Incubation to 65 °C and plate shaking are standard features. The imaging module uses a turret to hold up to 6 objectives. Excitation and emission wavelengths for fluorescence microscopy are provided using LED light cubes in combination with specific band pass filters and dichroic mirrors. The imaging module holds up to 4 LED cubes. In conjunction with the multi-mode reader, Gen5 Microplate Reader and Imager Software controls reader function, and also provides image analysis and data reduction.

Conclusions

1. Long-term assays can be automated using the BioSpa 8 System.
   - Incubation (temp, gas and humidity)
   - Liquid handling (reagent addition)
   - Image analysis
2. Exposure of liver cells to free fatty acids results in intracellular neutral lipid accumulation.
3. Image-based and PMT-based analysis can track lipid accumulation.
   - Image analysis is more responsive
4. Both hepatic and non-hepatic derived cell lines are capable of lipid accumulation.
5. Production of neutral lipid is FFA dose- and time-dependent.
6. Different cell types have different neutral lipid production kinetics.
7. Once accumulated lipids are retained for lengthy periods of time.