Application Note

Cell Metabolism

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Comparison of Different Cell Types for Neutral Lipid Accumulation

Using image-based metrics to quantify lipid accumulation in cells

Authors

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Abstract

It has been postulated that liver cells accumulate neutral lipids as a response to stress from the exposure of free fatty acids. *In vivo*, the liver is the organ primarily responsible for the denovo synthesis of lipids, as well as the conversion of free fatty acids to neutral lipids by their esterification with glycerol. *In vitro* cell types other than liver cells can and do accumulate neutral lipids. This application note describes the use of the Agilent BioTek Cytation 5 cell imaging multimode reader to rapidly image and analyze several different lipid stained tissue culture cells.

Introduction

Nonalcoholic fatty liver disease (NAFLD) represents a spectrum of diseases ranging from hepatocellular steatosis, nonalcoholic steatohepatitis (NASH) to hepatocellular carcinoma.[1] NAFLD is associated with obesity and insulin resistance and is considered the hepatic manifestation of the metabolic syndrome. Both hepatic steatosis and NASH can develop through several molecular pathways, including: increased free fatty acid (FFA) uptake, denovo lipogenesis, and endoplasmic reticulum (ER) stress.[2,3] The ER is a membrane-bound organelle that provides a specialized environment for the production and post-translational modification of secretory and membrane proteins, lipid biosynthesis, and homeostasis of intracellular Ca^{2+}.

The first step in the development of NAFLD is hepatic steatosis, which is characterized by lipid droplet accumulation of triglycerides in the cytoplasm of hepatocytes (Figure 1). The formation of lipid has been used as an *in vitro* model to study NASH. This application note compares the response of several different cell lines to FFA known to cause intracellular lipid accumulation.

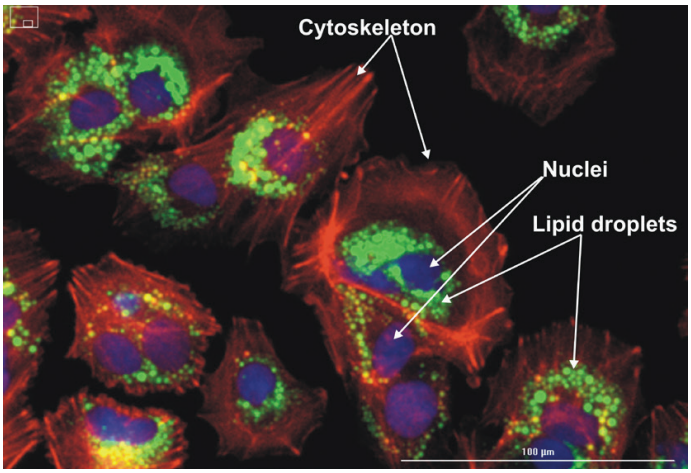

Figure 1. Neutral lipid staining of iCell hepatocytes 2.0. Human iPSC-derived hepatocytes (CDI) were treated with a 3:1 mixture of oleic and palmitic fatty acids for 24 hours. The CDIs were then fixed and stained with BODIPY 493/503 (green), DAPI (blue); and Texas Red phalloidin (red). 10x fluorescence images were captured using an Agilent BioTek Cytation 5 cell imaging multimode reader. Scale bar denotes 100 µm. The image has been digitally zoomed to provide greater structural detail.

Materials and methods

Cell culture

Hep G2, NIH3T3, HeLa, and HT1080 cells were cultured in Advanced DMEM supplemented with 10% fetal bovine serum and penicillin-streptomycin at 37 °C in 5% CO_2. Cultures were routinely trypsinized (0.05% trypsin-EDTA) at 80% confluency. For experiments, cells were plated into Corning 3904 black sided clear bottom 96-well microplates. Primary human hepatocytes from Corning (Corning, NY) and iCell hepatocytes 2.0 from Cellular Dynamics International (Madison, WI) were cultured according to the supplier's instructions.

These cell lines were seeded into 96-well collagen coated BioCoat plates (BD Biosciences) and allowed to normalize for three days before FFA challenge. Media were changed daily during this period. Unless indicated, cells were plated at 10,000 cells per well for experiments.

Fixing and staining

All cell washes and reagent additions for cell fixation and fluorescent staining were carried out using the EL406 Washer Dispenser. Cells were washed two times with 200 µL of PBS (NaCl 137 mM, KCl 2.7 mM, Na_2HPO_4 10 mM, KH_2PO_4 7.4 mM) after which 100 µL of 4% para-formaldehyde (PFA) solution was added. Cells were fixed for 10 minutes at room temperature followed by two washes of 200 µL using PBS. Cells were stained for 30 minutes with 50 µL of working solution of DAPI and either Nile Red or BODIPY 493/503 stain at room temperature followed by two washes with 200 µL of PBS. After aspiration, 100 µL of PBS was added to all wells and the plates sealed using an optically clear TopSeal-A adhesive plate sealer (PerkinElmer, Waltham, MA). Prepared plates were kept at 5 °C and protected from light before imaging.

Imaging

Experiments were imaged using an Agilent Cytation 5 cell imaging multimode reader configured with DAPI, GFP, RFP, and Texas Red light cubes. The Cytation 5 uses a combination of LED light sources in conjunction with bandpass filters and dichroic mirrors to provide appropriate wavelength light. The DAPI light cube uses a 337/50 excitation filter and a 447/60 emission filter suitable for the nuclear stain; a GFP light cube consisting of a 469/35 excitation filter and a 525/39 emission filter for imaging BODIPY 493/503; an RFP light cube with a 585/29 excitation and 624/40 emission filters for Nile Red; and the Texas Red light cube with a 586/15 excitation and a 647/57 emission filter for imaging phalloidin labeled F-actin.

Image analysis

Primary and secondary mask analyses of the captured digital images were used to determine the percentage of lipid positive cells. Primary mask analysis of the DAPI channel identifies individual cells by their nuclei. The secondary mask is designated as the region up to 30 µm surrounding each nuclei and represents the cell cytoplasmic region containing lipid droplets. Threshold analysis was used to identify lipid positive cells by the total integrated fluorescence of the fluorescent probe used in the secondary mask.

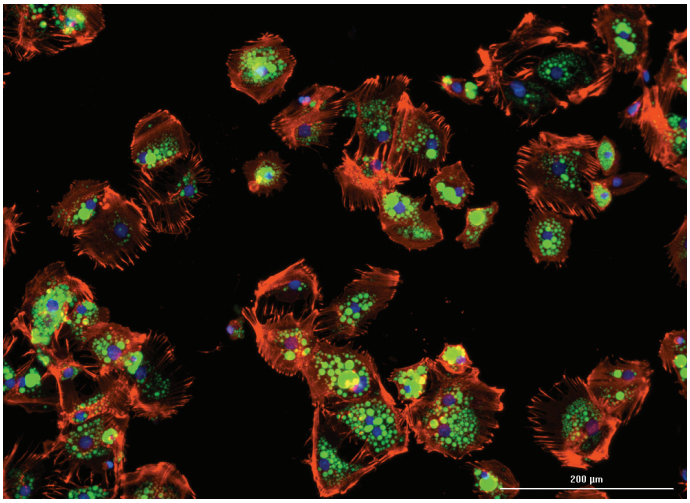

Figure 2. Neutral lipid staining of primary hepatocytes. Primary hepatocytes were challenged with a 0.5 mM mixture of oleic and palmitic fatty acids for 24 hours. Next, they were fixed with 4% PFA and fluorescently stained. Blue fluorescence illustrates DAPI-stained nuclei; green fluorescence depicts BODIPY 493/503 stained neutral lipids; and red fluorescence shows Texas Red phalloidin stained F-actin. 10x fluorescence images were captured using an Agilent BioTek Cytation 5 cell imaging multimode reader. Scale bar denotes 200 µm.

Results and discussion

Figure 3 demonstrates positive (0.5 mM FFA) and negative (vehicle) control images of hepatocytes stained with DAPI and BODIPY 493/503. It is evident in the negative control that few hepatocytes in the field of view show lipid accumulation (green fluorescence); yet the positive control demonstrates almost all cells with lipid accumulation. The negative control illustrates the power of microscopy to determine individual cellular responses and differentiate the response from just a background measure that would be available with conventional microplate readers that use PMTs to detect collective optical responses.

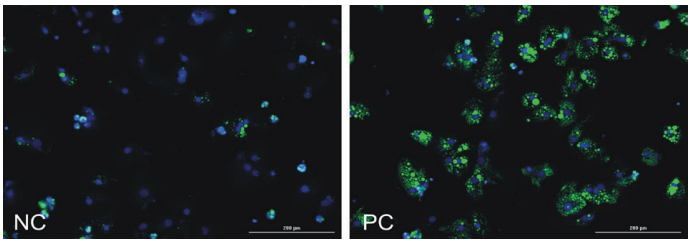

Figure 3. Negative and positive control images of hepatocytes stained with BODIPY 493/503. Primary hepatocytes were treated with 0.5 mM FFA mixture (positive control) or vehicle (negative control) for 24 hours, then fixed with 4% PFA and stained with DAPI (nuclei) and BODIPY 493/503 (lipid). Images were captured using an Agilent BioTek Cytation 5 cell imaging multimode reader with a 20x objective. Scale bar represents 100 µm.

Depending on the experimental situation it is often necessary to have an assortment of fluorescent dyes with different spectral characteristics available to detect neutral lipids. BODIPY 493/503 is an exceptionally bright fluorescent stain for the detection of lipids, but cannot be used with cells that express genetically encoded GFP, due to signal overlap. Nile Red is an alternative lipid staining probe with red-shifted emission properties. Figure 4 demonstrates the equivalence of these two different probes to quantify lipid accumulation in primary hepatocytes. Each demonstrate that approximately 10% of hepatocytes test positive for lipid accumulation without FFA challenge, but then this percentage begins to increase after a dose of 30 to 50 nM FFA after which the percentage of cells positive for lipid increases in a concentration-dependent fashion.

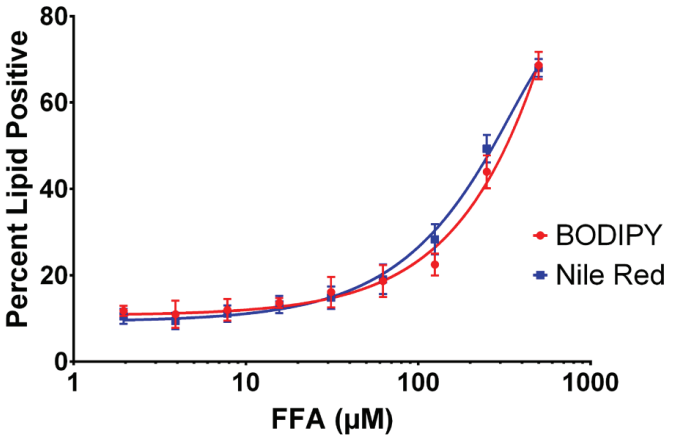

Figure 4. Comparison of lipid dyes to detect increases in lipid content. Primary hepatocytes were challenged with increasing concentrations of free fatty acids (oleic and palmitic) for 24 hours. Cells fixed and stained with DAPI to identify nuclei and either BODIPY 493/503 or Nile Red to identify neutral lipid. The percentage of positive cells was calculated using a subpopulation dual mask object analysis. The primary mask identified individual nuclei; with the secondary mask spatially linked to the primary mask. The lipid positive subpopulation identified with a threshold of 6×10^6 and 2×10^7 for BODIPY 493/503 and Nile Red stains respectively.

Several different hepatocyte-derived cell lines were stained with BODIPY 493/503 after free fatty acid treatment and all responded with lipid formation (Figure 5). HepG2 cells, an immortal cell line derived from a well-differentiated hepatocellular carcinoma, primary hepatocytes isolated from liver tissue, and iCell Hepatocytes 2.0, a pluripotent derived hepatocyte, all produce lipid in response to free fatty acid challenge.

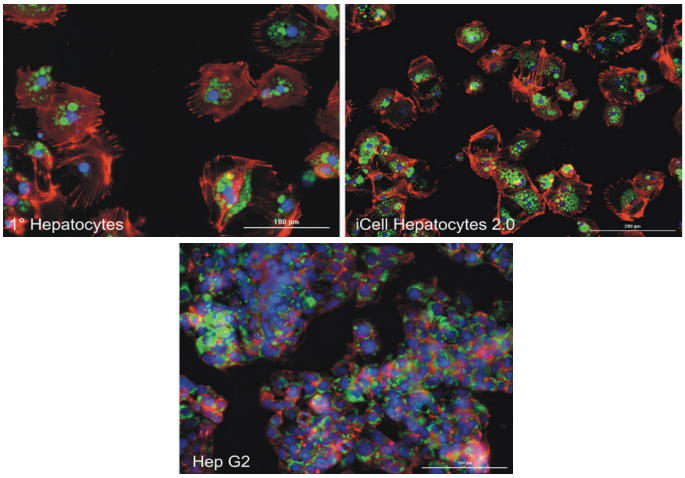

Figure 5. BODIPY staining of lipids in different hepatic cell types treated with free fatty acids. HepG2, primary hepatocytes, and iCell hepatocytes were treated with 0.5 mM FFA mixture for 24 hours, then fixed with 4% PFA and stained with DAPI (nuclei), BODIPY 493/503 (lipid) and Texas Red phalloidin (actin). Images were captured using an Agilent BioTek Cytation 5 cell imaging multimode reader with a 20x objective. Scale bar represents 100 µm. The image has been digitally zoomed to provide greater structural detail.

Hepatocyte-derived cells all produce neutral lipid in response to free fatty acid exposure, but respond slightly differently. Both HepG2 cells and primary hepatocytes demonstrate approximately 10% of cells positive for lipids without FFA challenge (Figure 6). The pluripotent-derived iCell hepatocytes 2.0 however exhibit little to no lipid present without FFA illustrative of a cell population expressing a more homogeneous phenotype under no stress. Yet all three cell lines demonstrate increasing lipid accumulation after approximately 30 µM FFA (Figure 5).

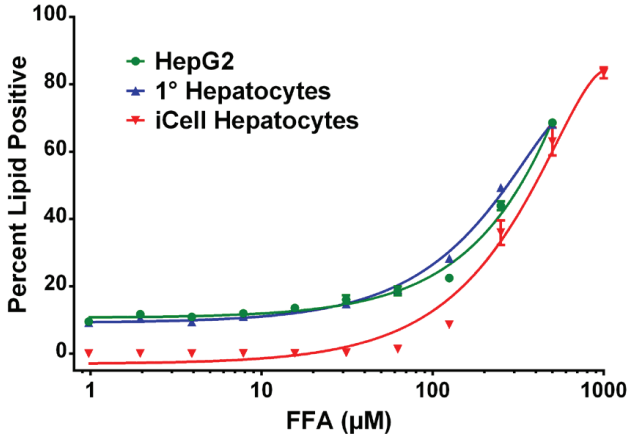

Figure 6. Comparison of hepatically derived cell lines. Cell lines were treated with various concentrations of free fatty acid mixtures for 24 hours and then fixed with 4% PFA and stained with DAPI (nuclei) and BODIPY 493/503 (lipid). Analysis reflects the percentage of lipid positive cells as a function 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 1×10^6 on the integral of the GFP channel. Data represent the mean and standard deviation of eight data points.

The temporal response to FFA exposure is also different with the three hepatic cell lines (Figure 7). While HepG2 and primary hepatocytes behave similarly in that they gradually increase the lipid content to a maximal amount over 24 hours, primary hepatocytes appear to possess a significant subpopulation of cells demonstrating lipid accumulation at the earliest time point, while HepG2 cells do not. This suggests that the HepG2 response shown in Figure 6 of 10% lipid positive cells under low FFA dose is brought about the additional time in the microplate (i.e. 23 hours), rather than reflective of the original population before the experiment. iCell hepatocytes 2.0 behave dissimilarly and uptake FFA more rapidly than either of the other two hepatic cell types, but similar to HepG2 possess a more homogeneous lipid-free cell population at the outset.

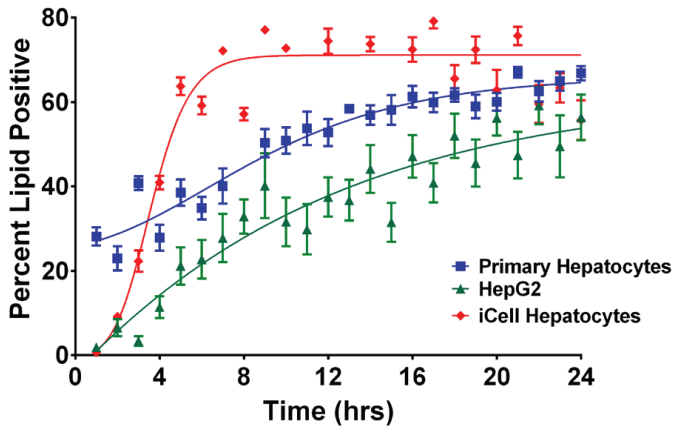

Figure 7. Comparison of lipid formation from different hepatic cell lines. Different hepatic cell lines were seeded into 96-well microplates and challenged with 0.5 mM FFA mixture various amounts of time. At the indicated time after challenge, cells were fixed with 4% PFA. Total cell counts were determined using a primary mask to identify nuclei. Lipid positive cells were determined using a secondary mask surrounding the nuclei with a cutoff threshold of 1×10^6 on the integral of the GFP channel. Data represent the mean and standard deviation of eight data points.

The formation of lipid droplets resulting from free fatty acid challenge is not a phenomenon exclusive to hepatocytes or liver-derived cells. As demonstrated in Figure 8, HeLa cells, HT1080, and NIH3T3 cells also produce neutral lipid when challenged with FFA. Neutral lipid formation can be tracked using Nile Red. All cell lines exhibit significant Nile Red fluorescence after FFA treatment.

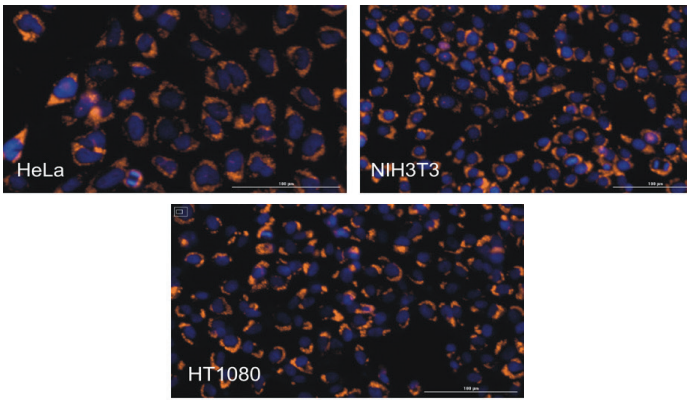

Figure 8. Nile Red staining of lipids in different cell types treated with free fatty acids. HeLa, NIH3T3, and HT1080 cells were treated with 0.25 mM FFA mixture for 24 hours, then fixed with 4% PFA and stained with DAPI (nuclei) and Nile Red (lipid). Images were captured using an Agilent BioTek Cytation 5 cell imaging multimode reader with a 10x objective. Scale bar represents 200 µm. The image has been digitally zoomed to provide greater structural detail.

FFA titrations with the different cell lines reproduce the residual lipids in HepG2 cells observed with BODIPY stain. The nonliver-derived cell lines do not show any lipid unless challenged with FFA more than 100 µM. Interestingly, human derived HeLa and murine-derived NI-H3T3 cells respond similarly (Figure 9). The HT1080 cell line, which was created from a biopsy from a fibrosarcoma, shows the most resistance to lipid formation.[5]

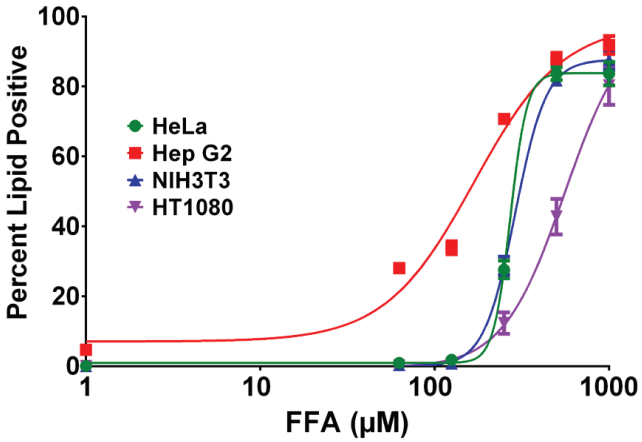

Figure 9. Comparison of nonhepatically-derived cell lines. Cell lines were treated with various concentrations of free fatty acid mixtures for 24 hours and then fixed with 4% PFA and stained with DAPI (nuclei) and BODIPY 493/503 (lipid). Analysis reflects the percentage of lipid positive cells as a function 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 1×10^6 on the integral of the GFP channel. Data represent the mean and standard deviation of two data points.

The dynamics of neutral lipid formation between liver-derived and nonliver-derived cell lines was examined. HeLa and HepG2 cells were treated with 500 µM free fatty acids and the rate of lipid formation was examined using image-based analysis. The number of lipid positive HepG2 cells immediately increased with exposure; reaching a steady state in approximately 24 hours. The epithelial derived HeLa cells did not demonstrate significant changes in lipid positive cells for nearly eight hours (Figure 10). In addition, HeLa cells produced significantly less lipid on a per cell basis. This difference is reflected in threshold value that is 50% less than that used for HepG2 cells.

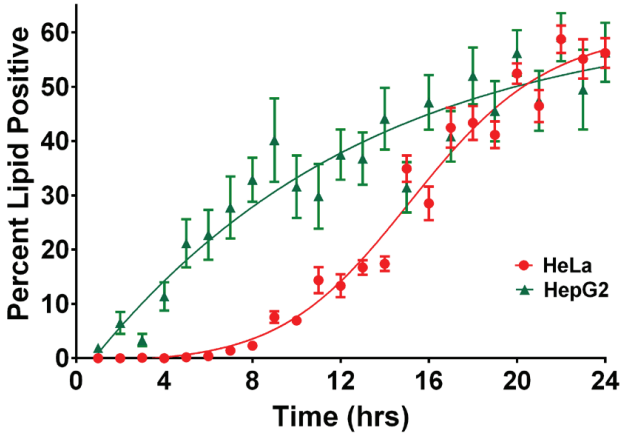

Figure 10. Comparison of lipid formation of Hep G2 and HeLa cell lines. HeLa and HepG2 cell lines were seeded into 96-well microplates and challenged with 500 µM FFA mixture various amounts of time. At the indicated time after challenge, cells were fixed with 4% PFA. Total cell counts were determined using a primary mask to identify nuclei. Lipid positive cells were determined using a secondary mask surrounding the nuclei with a cutoff threshold of 1×10^6 on the integral of the GFP channel. Data represent the mean and standard deviation of eight data points.

This application note has demonstrated that various cell lines produce significant amounts of neutral lipid in response to free fatty acid treatment. In fact, liver-derived cell lines respond quickly. We then examined whether these cells dissipated the neutral lipid upon removal of the stimuli. As shown in Figure 11, lipid positive cells (HepG2 or HeLa) remain positive for at least 24 hours after removal of the free fatty acid.

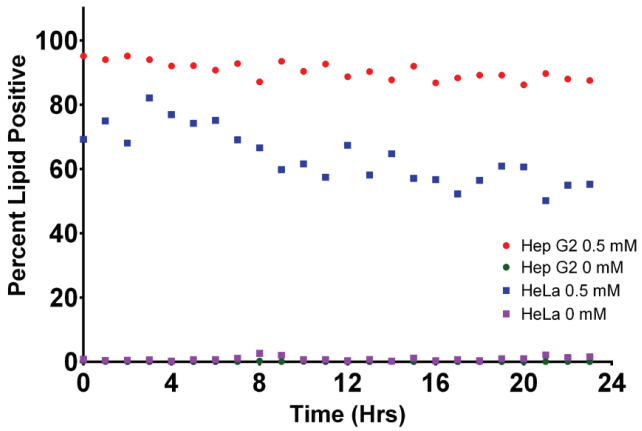

Figure 11. Comparison of lipid depletion of Hep G2 and HeLa cell lines. HeLa and HepG2 cell lines were seeded into 96-well microplates and challenged with 0.5 or 0 mM. FFA for 24 hours, After which the media was replaced with fresh without FFA. At the indicated time after FFA removal, cells were fixed 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 1×10^6 on the integral of the GFP channel. Data represent the mean and standard deviation of two data points.

Conclusion

As previously shown, hepatocarcinoma-derived cell line HepG2 cells will produce neutral lipid in response to free fatty acid treatment. This application note shows that other cell lines, both liver- and nonliver-derived cells, produce neutral lipids. However, depending on the cell type they respond differently temporally and in magnitude. Nonliver-derived cell lines generally respond slower to the stimuli and require greater amounts of free fatty acid when compared to hepatic cells.

The ability to use fluorescent dyes with different spectral properties to detect lipids allows the researcher to coordinate multicolor fluorescence imaging much easier. While BODIPY 493/503 is a bright fluorescent dye, its fluorescence precludes it use with GFP expressing cell lines. Nile Red signal will interfere with RFP expressers. Multiple dyes allow more flexibility regarding counterstains.

It has been suggested that the production of neutral lipids is an adaptive stress response to the deleterious nature of free fatty acids. Recent data suggest that disruption of endoplasmic reticulum (ER) homeostasis (i.e. ER stress) is a key element in the progression of NASH.[6] Because hepatocytes, like other secretory cells, are rich in ER, they would be more prone to this response. Other cell types produce lipids in response to environmental conditions. For example, single cell algae produce neutral lipid is response to poor environmental nutrient levels.[6]

Nonalcoholic fatty liver disease (NAFLD) is generally a silent disease that does not have a specific research model. The detection and quantification of neutral lipid formation by image-based analysis of cells grown *in vitro* is a first step in elucidating the etiology of this disease.

References

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RA44412.3980208333

Printed in the USA, May 17, 2022
5994-3381EN

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