

# Modeling Nonalcoholic Steatohepatitis (NASH): Automated Assay for Lipid Accumulation in Liver Cells



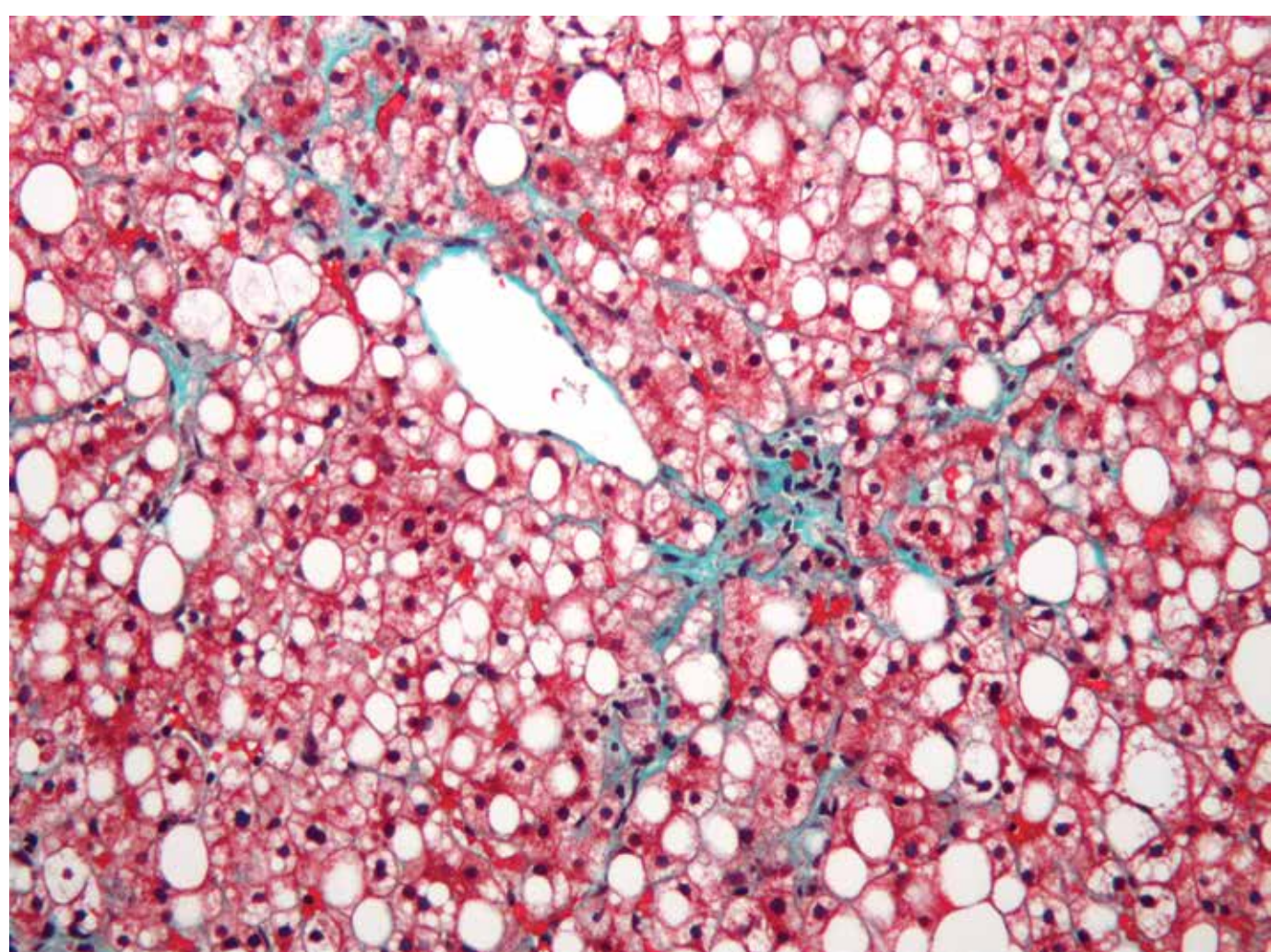
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## Abstract

Nonalcoholic steatohepatitis, or NASH, is a common, often “silent” liver disease that resembles alcoholic liver disease, but occurs in people who drink little or no alcohol. The etiology of the disease is the accumulation of neutral lipid droplets within the liver cell, which can be tracked with Nile red staining (Figure 1). To date, research in this area has suffered from the lack of a suitable human cell model as well as the means to automate the assay process and subsequent microscopic image analysis.

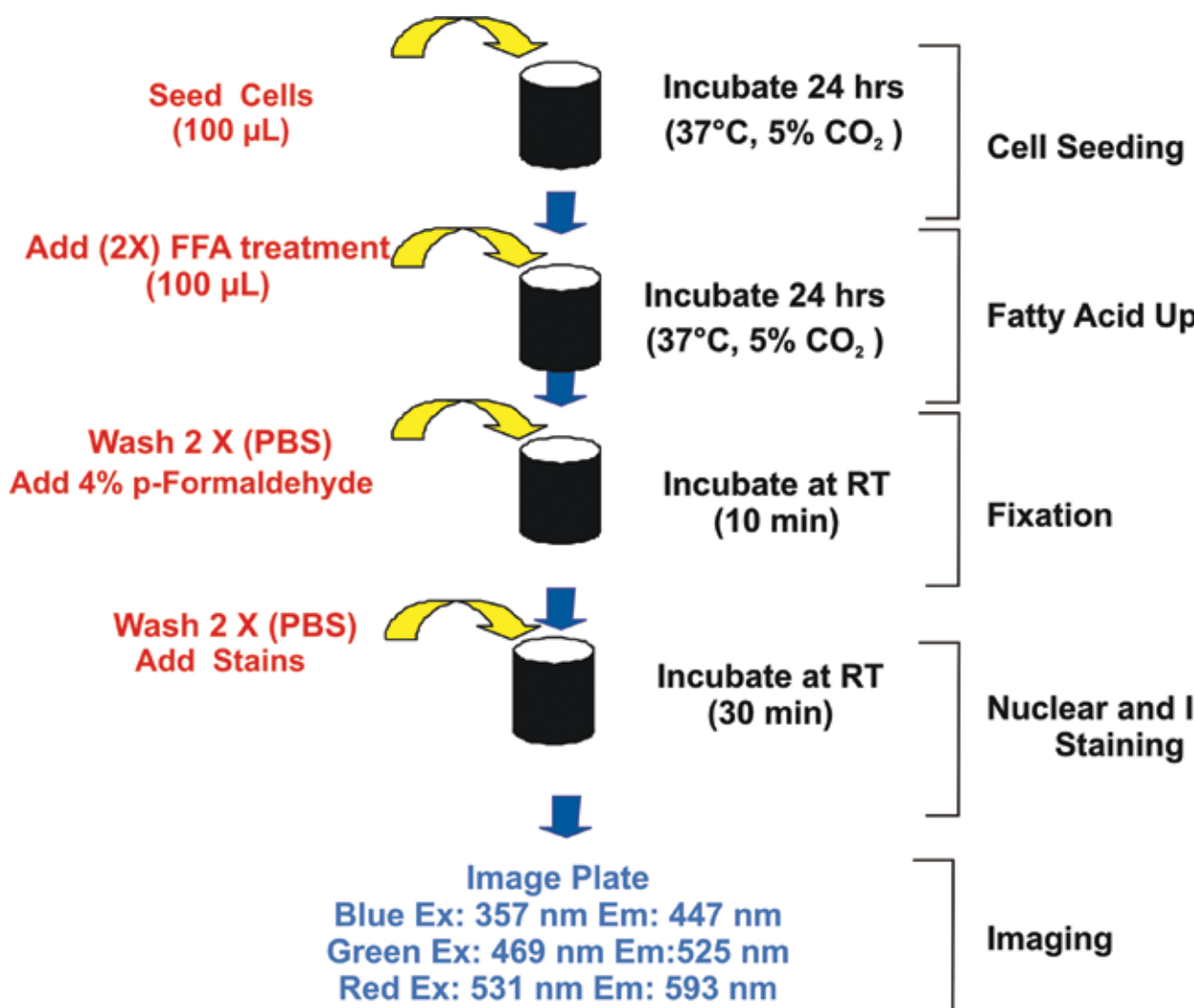
We have shown here that liver cells, when exposed to various concentrations of a mixture of oleic and palmitic free fatty acids (FFA), accumulated intracellular neutral lipids in a dose dependent fashion up to 1 mM FFA treatment. Doses of FFA higher than 1 mM resulted in cell death and the loss of cells. Following treatment, cells were fixed and stained with Nile red (lipid stain) and DAPI (nuclear stain) using automation. Fixed and stained cells were digitally imaged with a Cytation™ 5 Imaging Multi-Mode Reader using a 10x or 20x objective. Uptake of FFA and conversion to neutral lipid droplets was monitored over time using a BioSpa™ 8 Automated Incubator to present plates to a MultiFlo™ FX Multi-Mode Dispenser at periodic times after the addition of FFA in order to stop the reaction by adding fixative to the cells. Automated Image analysis was then used to determine the percentage of cells positive for neutral lipids.

## Fatty Liver Tissue



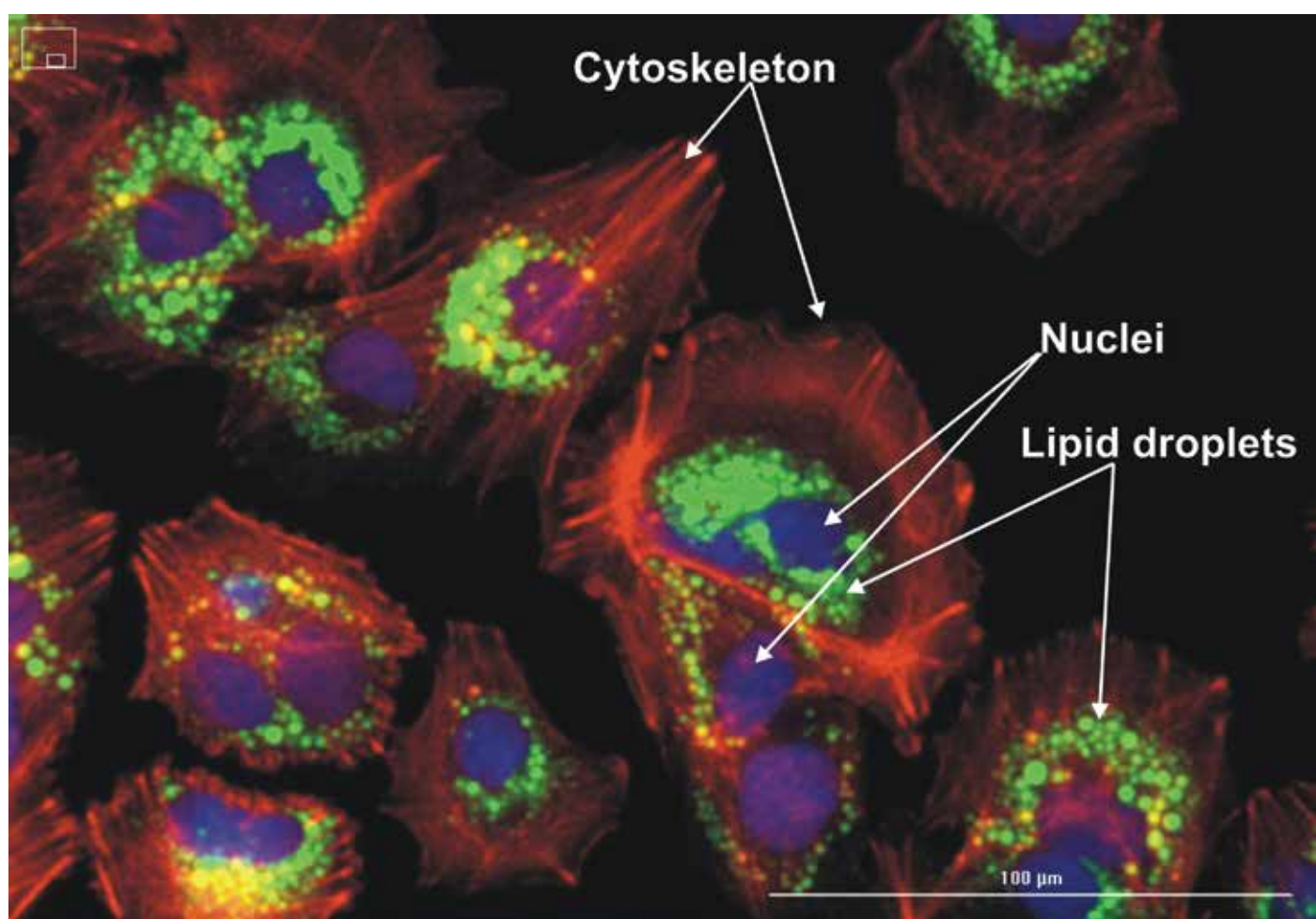
**Figure 1. Trichrome stain of liver biopsy depicting nonalcoholic steatohepatitis (NASH).** Image depicts balloon degeneration of hepatocytes (large cells with small centrally located nuclei) along with fine-chicken wire fibrosis, hepatocyte necrosis and inflammation. Image courtesy of Nephron, [1] CC BY-SA 3.0, <https://commons.wikimedia.org/w/index.php?curid=7747223>

## Assay Process



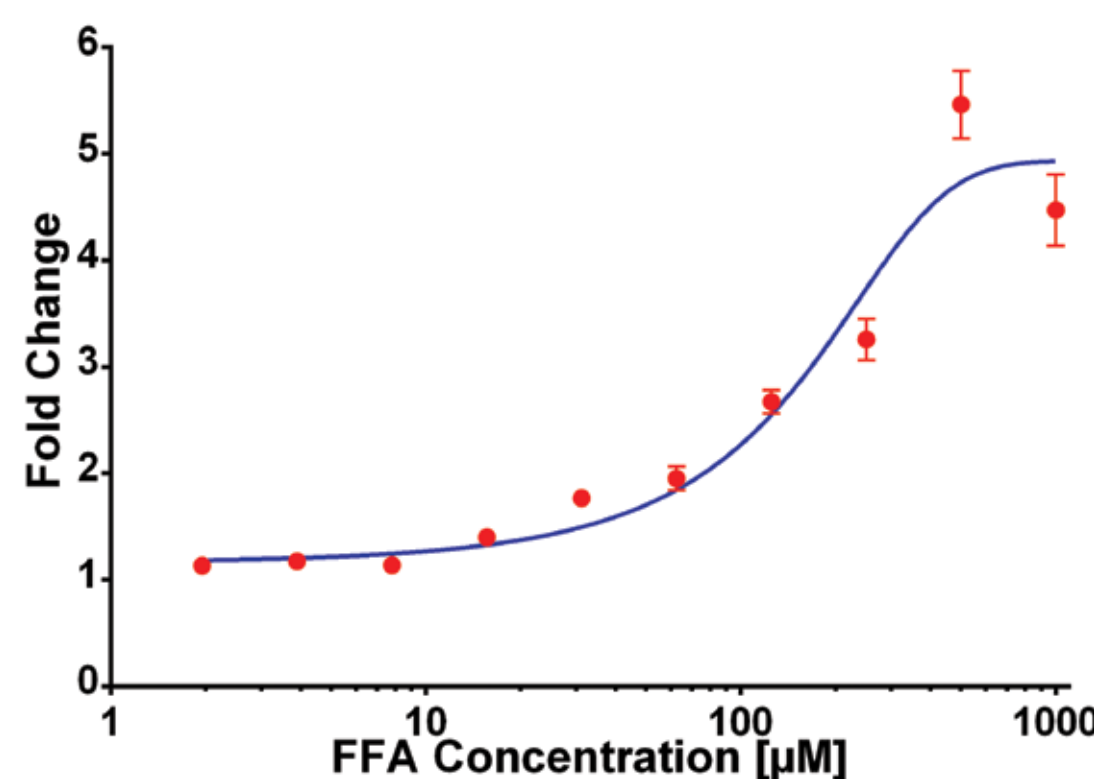
**Figure 2. Lipid Accumulation and Staining Process.** Cells were routinely seeded into 96-well microplates using the MultiFlo FX peripump dispenser and allowed to attach overnight. The following day, cells were challenged with a mixture (3:1) of oleic and palmitic free fatty acids at various concentrations and for various exposure times. Cells were subsequently fixed for 10 minutes with 4% paraformaldehyde. Cells were then stained with DAPI (nuclei), Texas Red-phalloidin (actin) and either BODIPY 493/503 or Nile red (neutral lipid) for 30 minutes. Cells were then imaged using a Cytation 5, and images analyzed with Gen5™ Microplate Reader and Imager Software.

## Neutral Lipid Staining



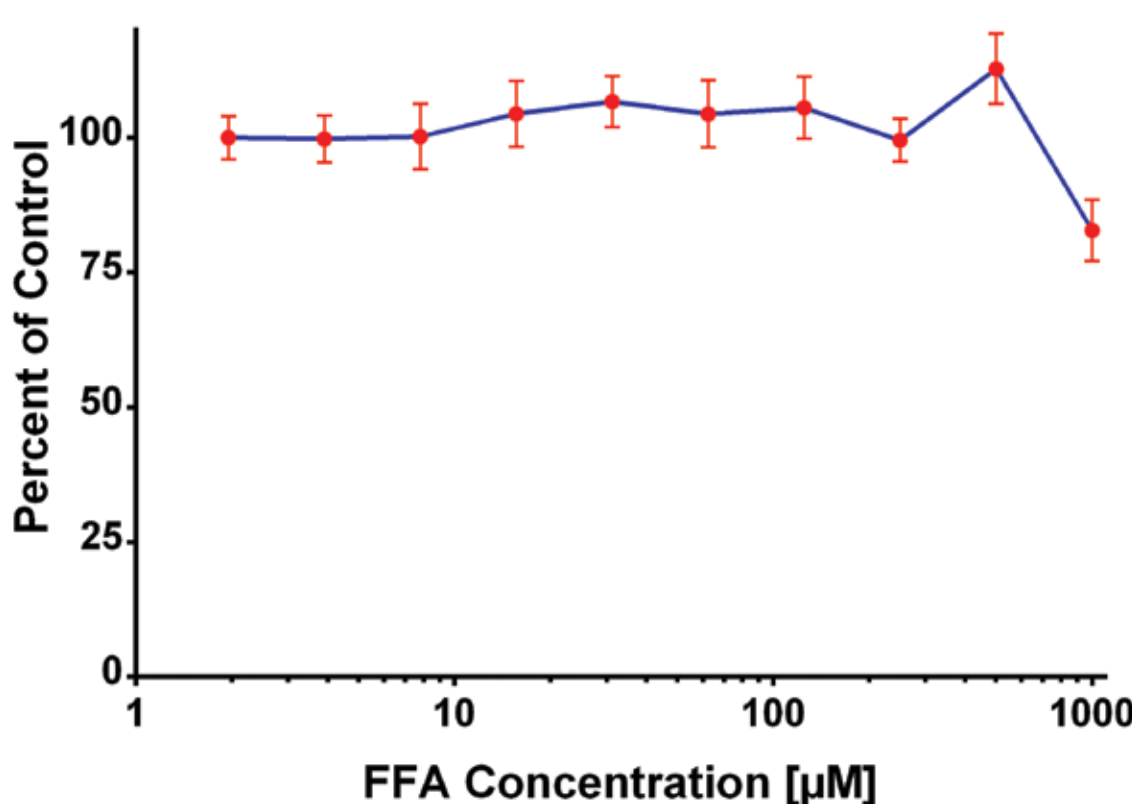
**Figure 3. Neutral Lipid Staining of iCell Hepatocytes 2.0.** Human iPSC-derived hepatocytes (Cellular Dynamics International, Madison, WI) were treated with a 0.5 mM mixture of oleic and palmitic fatty acids for 24 hours. Next, they were fixed with 4% PFA and stained with BODIPY 493/503 (green), which stains neutral lipids; DAPI (blue), which stains nuclei; and Texas Red Phalloidin (red), which stains actin. Three color 10x images were captured using Cytation 5. Scale bar denotes 100 µm.

## PMT Detection of Lipid Dyes



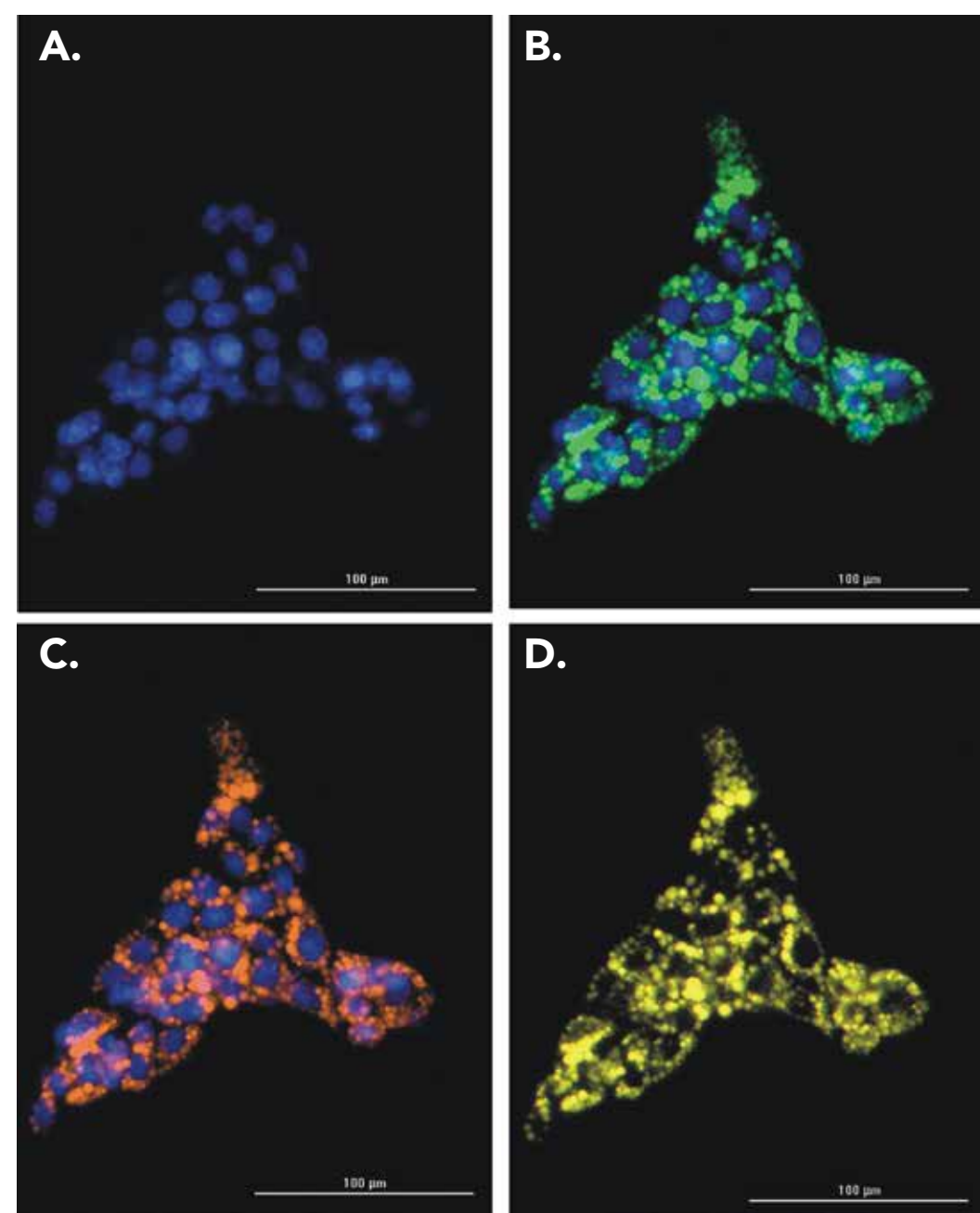
**Figure 4. Fluorescent Detection of Neutral Lipids.** The epifluorescence of DAPI and Nile Red stained HepG2 cells was determined using Cytation 5. DAPI fluorescence was determined at excitation and emission wavelengths of 360 nm and 460 nm, respectively, while Nile red was measured at 530 nm and 570 nm. Data was captured from the bottom using automatic gain selection. Nile red stained lipid fluorescence of each well was normalized by dividing by the DAPI fluorescence of that well after blank subtraction. The fold change was calculated from the results of the untreated controls. Data represents the mean and standard deviation of eight determinations.

## Free Fatty Acid Cytotoxicity

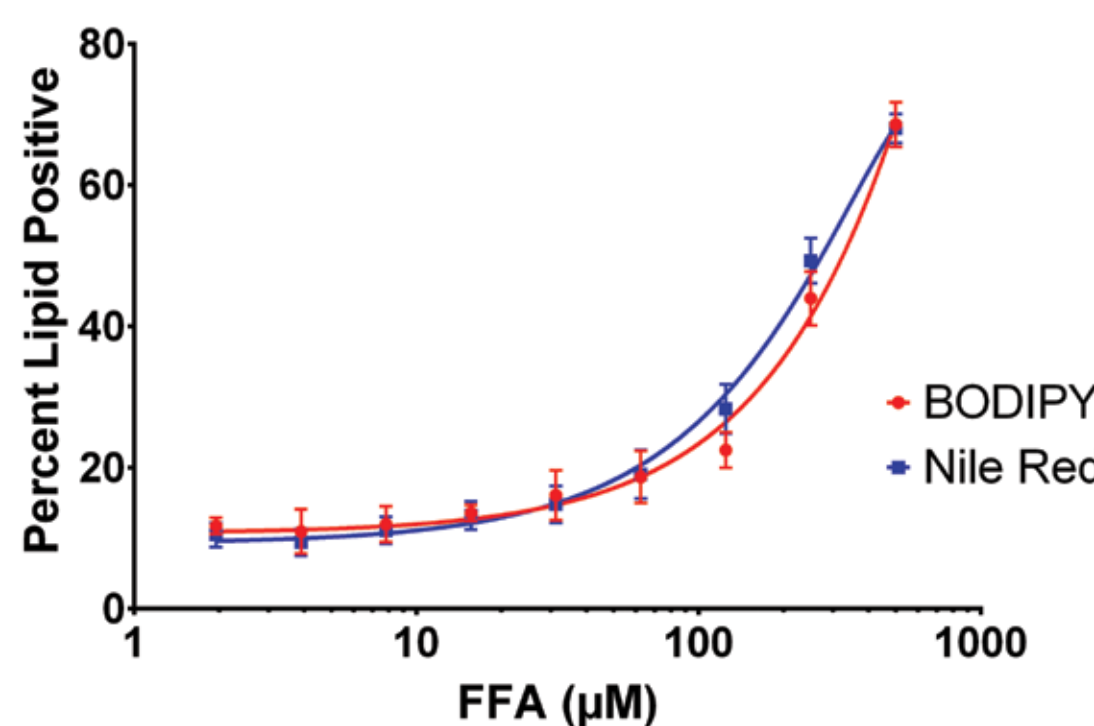


**Figure 5. Cell Counts of HepG2 Cells Treated with Various Concentrations of Free Fatty Acids.** Using a 4x objective, montage images (3 x 4) of fixed and DAPI stained HepG2 cells were captured and stitched together. Cell counts were made using a single object mask with a threshold of 10,000 and expressed as a percentage of the untreated control. Data represents the mean of eight determinations.

## Comparison of Lipid Dyes

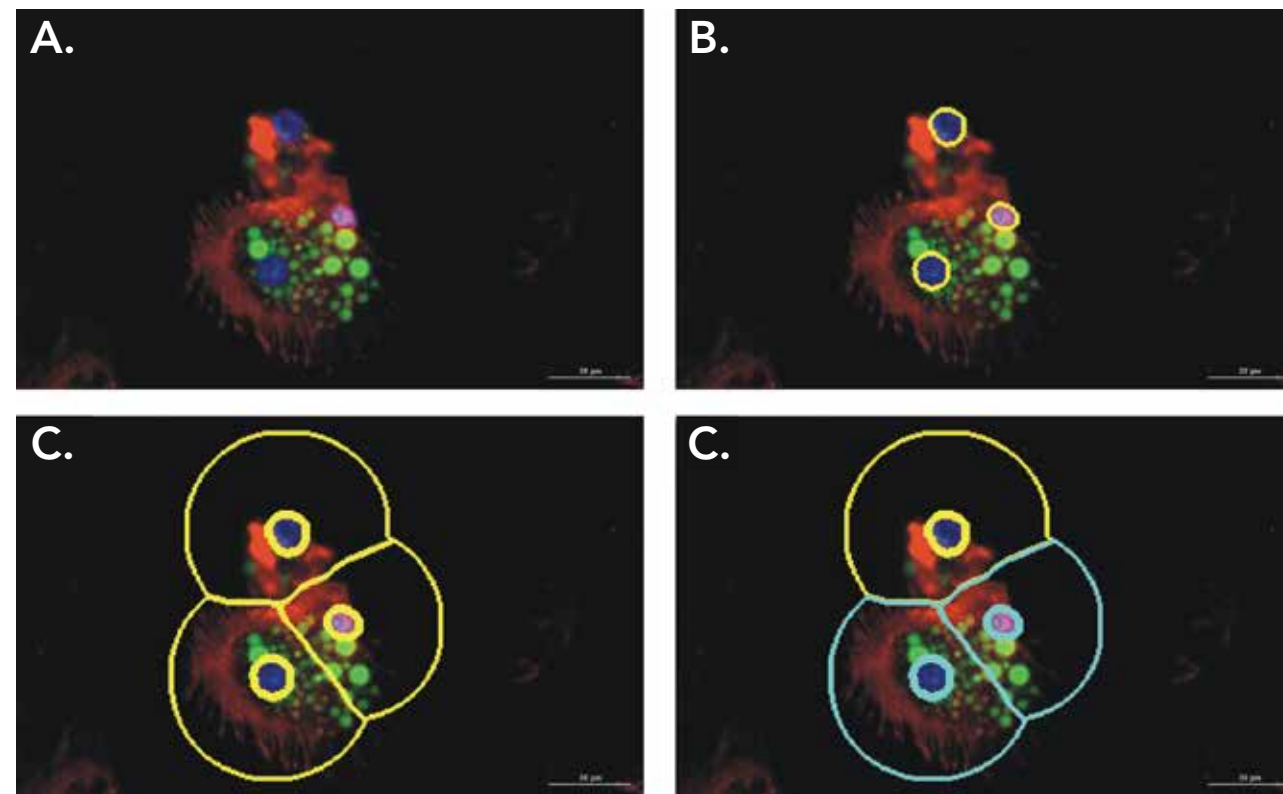


**Figure 6. Co-localization of Lipid Staining Dyes.** HepG2 cells were challenged with 0.25 mM FFA for 24 hours. Cells were then fixed with 4% PFA and stained with DAPI (nuclei) and both Nile red and BODIPY 493/503 lipid stain. (A) DAPI image only; (B) DAPI and BODIPY; (C) DAPI and Nile red; (D) Nile red, and BODIPY. Scale bar denotes 100 µm.

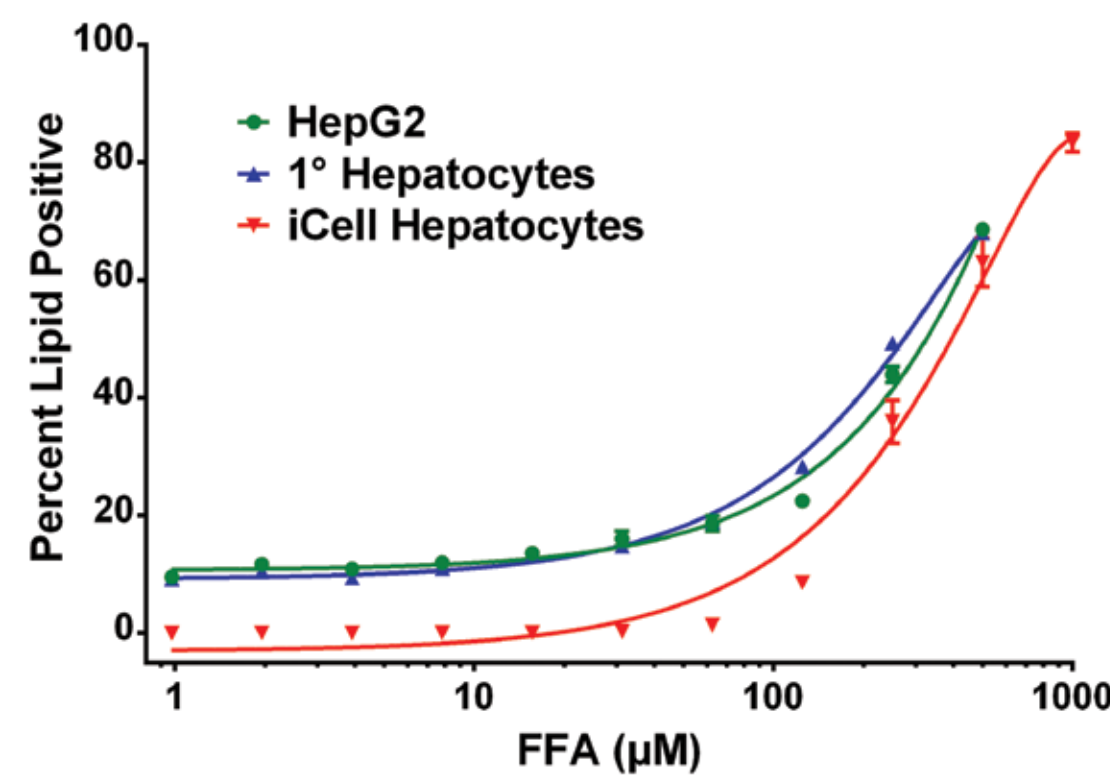


**Figure 7. Comparison of Lipid Dyes to Detect Increases in Lipid Content.** Primary hepatocytes were challenged with increasing concentrations of free fatty acids (oleic and palmitic) for 24 hours. Cells were then fixed and stained with DAPI to identify nuclei and either BODIPY 493/503 or Nile red to identify neutral lipid. The percentage of lipid positive cells was calculated using a subpopulation dual mask object analysis. The primary mask identified individual nuclei, the secondary mask was spatially linked to the primary mask. The lipid positive subpopulation was identified with a threshold of  $6 \times 10^6$  and  $2 \times 10^7$  for BODIPY and Nile red stains, respectively.

## Image Analysis

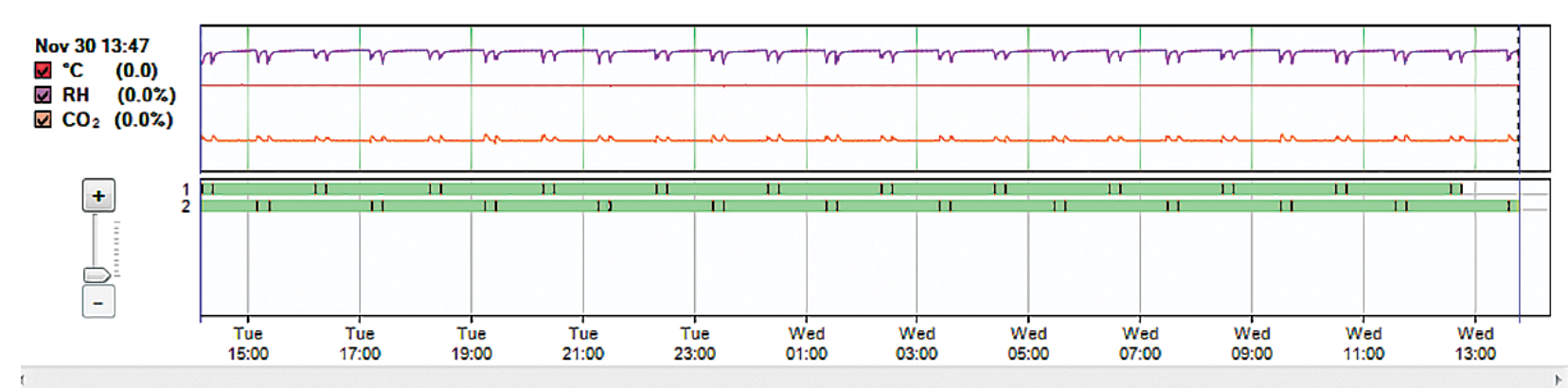


**Figure 8. Quantitative Analysis of Image Data.** Primary and secondary mask analysis was used to determine the percentage of lipid positive cells. Primary mask analysis of the DAPI channel identifies individual cells by their nuclei. Secondary mask is identified by the space up to 30 µm surrounding the primary mask. (A) Raw image; (B) Primary mask with nuclei identified by yellow traces; (C) Primary and secondary masks identified by yellow traces; (D) Primary and secondary masks with lipid positive cells identified with blue traces.



**Figure 9. Effect of Free Fatty Acid Concentration on Neutral Lipid Formation.** Three liver cell lines were challenged with various concentrations of free fatty acid mixture for 24 hours. Cells were then fixed and stained with DAPI and BODIPY 493/503. Montage (2x2) images were taken with a 10x objective and then stitched together and preprocessed to remove background fluorescence. Quantitative image dual mask subpopulation analysis was used to determine total number of cells as well as lipid positive cells.

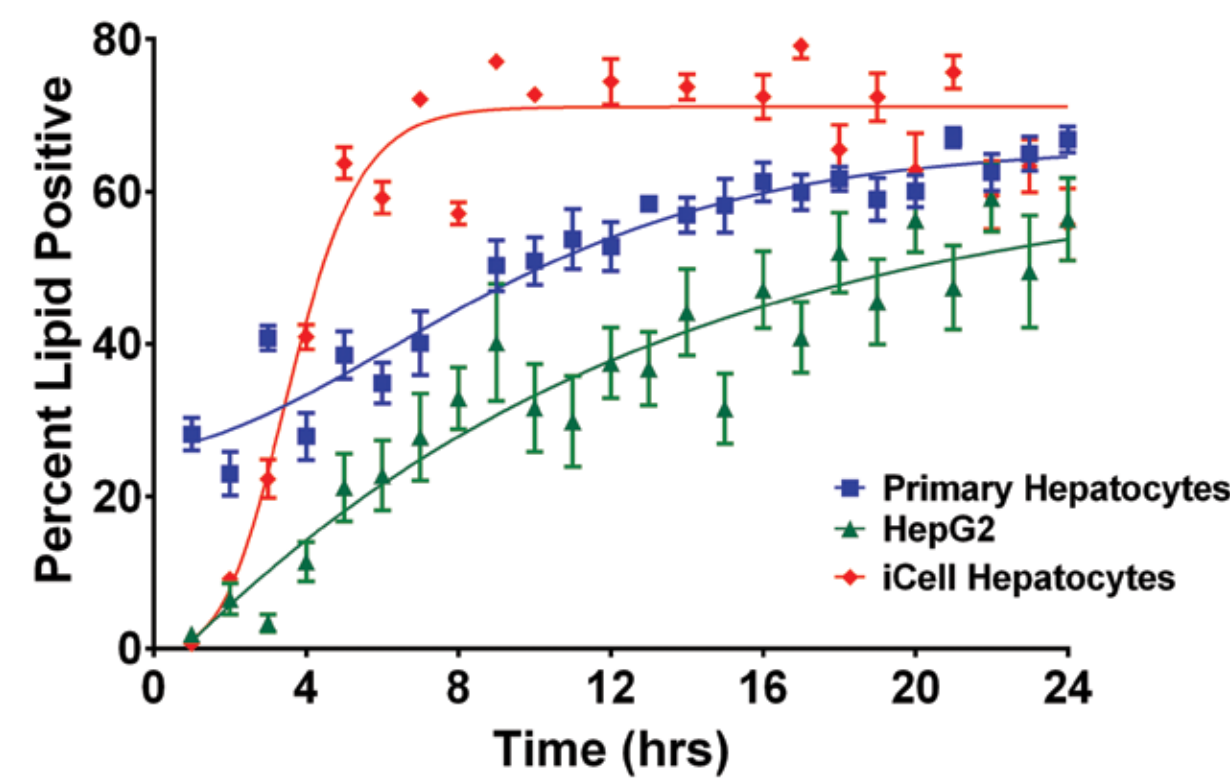
## BioSpa Automation



**Figure 10. Gantt Chart of a BioSpa 8 FFA Uptake Session.** A series of wash and dispense routines are carried out with the MultiFlo FX to remove media and wash columns with PBS, then add 4% paraformaldehyde fixative. After ten minutes, the fixative is removed and replaced with PBS. Two plates are treated in parallel such that a single column from one of the plates is treated every hour and each plate is only removed from the BioSpa 8 every two hours. Plates are incubated in the BioSpa 8 at 37 °C, with a humidified 5% CO<sub>2</sub> atmosphere between reagent additions. After 24 hours, the plates are stained and imaged.

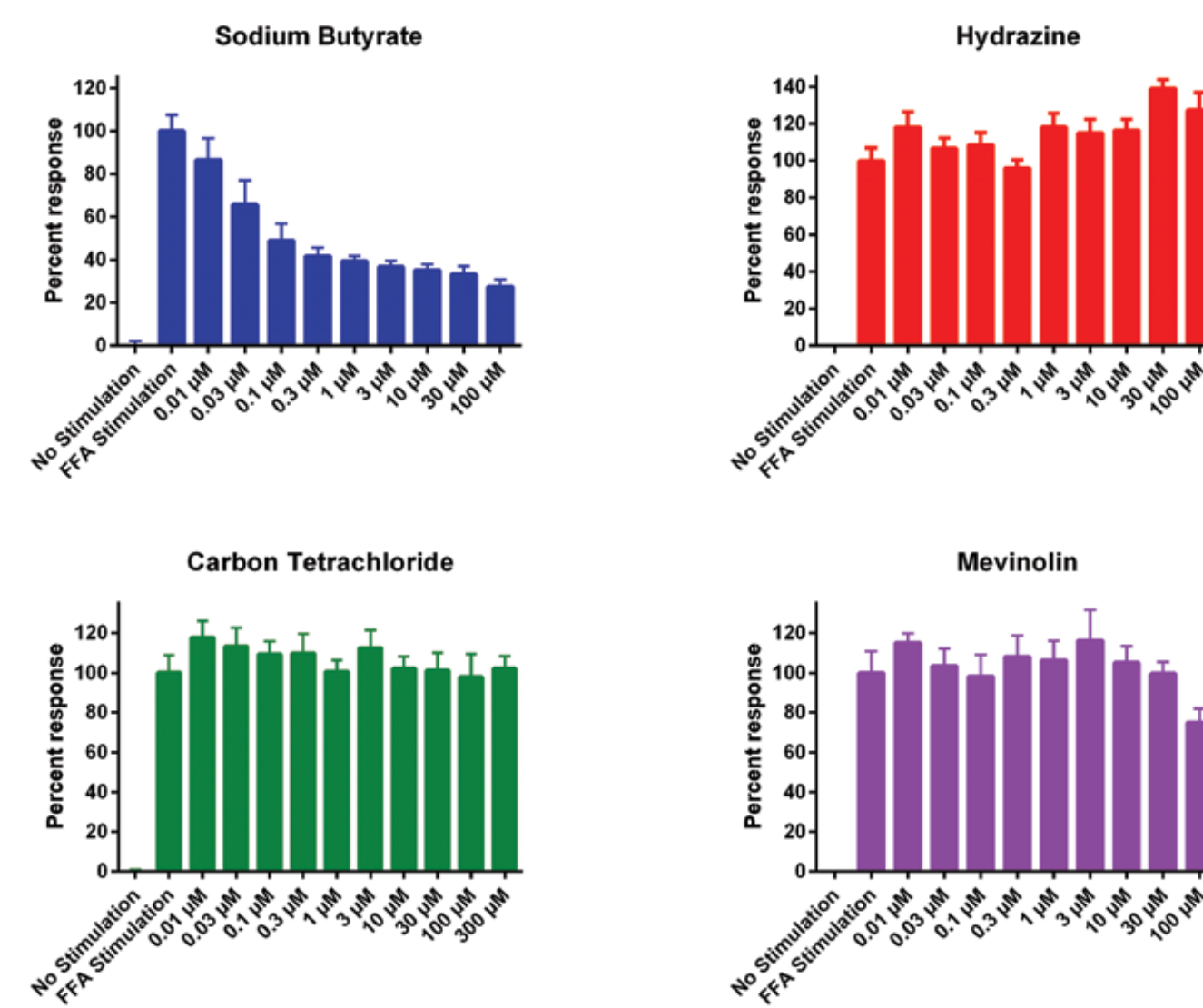
## Neutral Lipid Accumulation

### Cell Line Comparison



**Figure 11. Comparison of Neutral Lipid Accumulation.** Three different cell lines were treated with 0.25 mM FFA, then fixed and stained for neutral lipid at various time intervals. Using a BioSpa 8 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 8 every two hours. Data represents the mean of eight determinations.

## Effect of Compounds on Lipid Accumulation



**Figure 12. Effect of Compounds on Neutral Lipid Formation.** Various concentrations of known effectors of fatty liver formation were added simultaneously with 0.25 mM FFA mixture to HepG2 cells. After 24 hours, cells were fixed and stained for lipid. Percent response reflects the ratio of total lipid staining normalized for cell number for each concentration to the FFA stimulated control after the no stimulation control has been subtracted. Data represents the mean of seven determinations.

## Instrumentation



**Figure 13. BioSpa 8 Automated Incubator.** The BioSpa 8 is an microplate incubator that can interface a BioTek microplate liquid handling device with a BioTek microplate reader/imager. The BioSpa 8 maintains temperature and humidity, and provides CO<sub>2</sub> and O<sub>2</sub> gas control for up to eight microplates or other labware.



**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.** Cytation 5 is a modular, upgradable multi-mode reader that 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 dichro mirrors. The imaging module holds up to 4 LED cubes. In conjunction with the multi-mode reader, Gen5 software, which controls reader function, also provides image analysis and data reduction.

## Conclusions

1. Nile red and BODIPY 493/503 are capable of staining intracellular neutral lipids:
  - Dyes co-localize intracellularly
  - Offer different colors for fluorescent imaging
  - BODIPY 493/503 is brighter
2. Exposure of liver cells to free fatty acids results in intracellular neutral lipid accumulation.
3. Production of neutral lipid is FFA dose and time dependent.
4. Different cell types have different neutral lipid production kinetics.
5. Not all known effectors of fatty liver formation influence neutral lipid accumulation during *in vitro* FFA challenge.