Application Note

Cell Metabolism

Automation of Nonalcoholic Fatty Liver Disease Model Assay

Image-based analysis of neutral lipid in liver cells

Author

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Abstract

Nonalcoholic fatty liver disease is a common malady whose major feature is fat accumulation in the liver, which can lead to liver damage and cirrhosis. One phenotypic marker for this disease is the presence of intracellular neutral lipid droplets. An *in vitro* model for this phenotype uses the exposure of cells to free fatty acids to elicit the response. This application note describes the automation of the assay workflow using an Agilent BioTek BioSpa 8 automated incubator linked to an Agilent BioTek Cytation 5 cell imaging multimode reader and an Agilent BioTek MultiFlo FX multimode dispenser.

Introduction

Nonalcoholic fatty liver disease (NAFLD) is a common liver disease affecting millions worldwide, which is characterized by liver cell steatosis and lipidosis.[1] Nonalcoholic steatohepatitis, or NASH, is a variant of the disease where fat accumulation is accompanied by inflammation of the liver. The primary fatty acids in the human body are palmitic acid (PA) and oleic acid (OA) and are commonly used to induce steatosis *in vitro* and *in vivo*.[2] This phenotype presents with intracellular neutral lipid droplets (Figure 1).

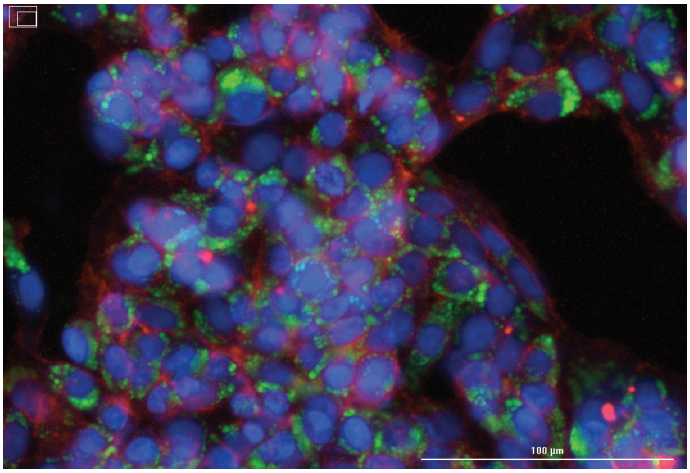

Figure 1. Neutral lipid staining of HepG2 cells. HepG2 cells were challenged with a 0.5 mM mixture of oleic and palmitic fatty acids for 24 hours after which they were fixed and stained with BODIPY 493/503 (green), DAPI (blue), and Texas Red phalloidin (red). 10x fluorescence images were captured using an Agilent BioTek Cytation 5 cell imaging multimode reader. Scale bar denotes 100 µm.

Materials and methods

Cell culture

HepG2 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. During experiments, cells were plated into Corning 3904 black-sided clear bottom 96-well microplates at 10,000 cells per well depending on the experiment.

Fixing and staining

All cell washes and reagent additions for cell fixation and fluorescence staining were accomplished using the MultiFlo FX multimode 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% paraformaldehyde (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 BODIPY 493/503 stain at room temperature followed by 2 washes with 200 µL of PBS. After aspiration 200 µL of PBS was added to all wells and imaged.

Imaging

Experiments were imaged using a Cytation 5 cell imaging multimode reader configured with DAPI, GFP, and Texas Red light cubes. LED light sources, in conjunction with band pass filters and dichroic mirrors, provide appropriate wavelength light. The DAPI light cube uses a 337/50 nm excitation filter and a 447/60 nm emission filter suitable for the nuclear stain; a GFP light cube uses a 469/35 nm excitation filter and a 525/39 nm emission filter suitable for BODIPY 493/503; and a light cube uses a 586/15 nm excitation and a 647/57 nm emission filter suitable for the cytoskeleton stain used in Figure 1.

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 stained nuclei. The secondary mask is used to quantify the neutral lipid build-up in the cells using the probe BODIPY 493/503 and is denoted as the region up to 30 µm surrounding each nuclei which represents the cell cytoplasm. Lipid positive cells were identified using a threshold of 1×10^6 in the total integrated BODIPY fluorescence in the secondary mask.

Automation

The assay workflow was automated using a BioSpa 8 automated incubator to interface a MultiFlo FX multimode dispenser with a Cytation 5 cell imaging multimode reader. The BioSpa 8 maintains temperature and humidity, as well as providing CO_2 and O_2 gas control for up to eight microplates. BioSpa control software manages the process and scheduling.

The MultiFlo FX is a modular upgradable reagent dispenser that can have one or two peripump (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-wells. As many as five different reagents can be dispensed without user intervention. The peristatic pump cassettes are autoclavable making them useful for dispensing sterile solutions.

The Cytation 5 is a modular, upgradable multimode 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 turret holds six 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 four cubes.

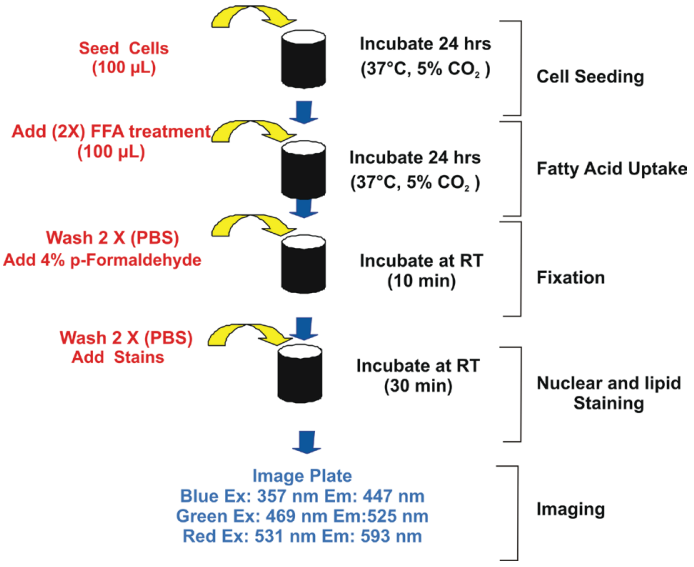

Figure 2. NAFLD model assay process. Cells were routinely seeded into 96-well microplates using the Agilent BioTek MultiFlo FX multimode dispenser and allowed to attach overnight. The following day cells were challenged with a mixture (3:1) of oleic and palmitic free fatty acids at various concentrations and for various exposure times. Cells were subsequently fixed for 10 minutes with 4% paraformaldehyde and then stained with DAPI (nuclei), Texas Red-phalloidin (actin) and BODIPY 493/503 for 30 minutes. Cells were imaged using an Agilent BioTek Cytation 5 cell imaging multimode reader and images analyzed with the Agilent BioTek Gen5 multimode reader and imager software.

Results and discussion

A number of different experimental formats revolving around investigating NAFLD have been automated using a BioSpa automated incubator in conjunction with a MultiFlo FX multimode dispenser and a Cytation 5 cell imaging multimode reader. The BioSpa software provides Gantt charts depicting the proposed assay processing steps prior to running the assay. Session results showing process times and traces for temperature, CO_2/O_2 and humidity are provided during the run and saved with the session information (Figure 3).

Using the Agilent BioTek BioSpa live cell imaging system to perform the assay, HepG2 cells were seeded into two 96-well plates and allowed to attach for 24 hours. Following attachment, media containing four different concentrations of FFA were added to the wells of the microplate using the peripump dispenser. Reagents were added such that two rows each received one of the three different concentrations of FFA to initiate the assay (Figure 4). Subsequent to the addition of FFA, individual strips were fixed with 4% paraformaldehyde for 10 minutes at timed intervals over 24 hours. The two plates are staggered such that one strip was processed every hour, but each plate was only removed every two hours. Once all of the strips had been fixed, wells were stained with DAPI and BODIPY 493/508 and imaged using the Cytation 5.

Exposure of HepG2 cells to FFA shows a time and concentration dependency. Image data analysis shows that the percentage of cells registering positive for lipid increases with time. In addition, the rate and extent is also dependent on FFA concentration (Figure 5).

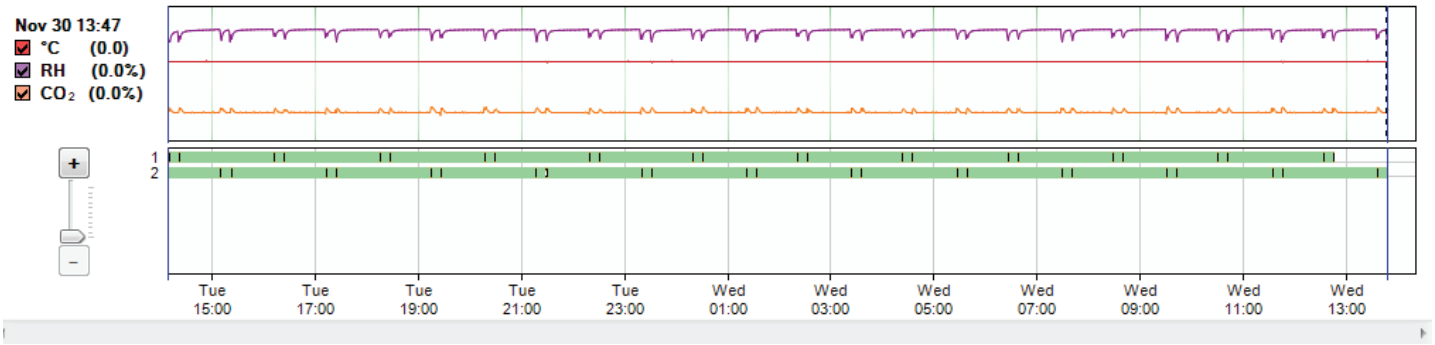

Figure 3. Gantt chart of an Agilent BioTek BioSpa 8 FFA Uptake session. A series of wash and dispense routines are carried out with the Agilent BioTek MultiFlo FX multimode dispenser to remove media and wash columns with PBS, then add 4% paraformaldehyde fixative. After 10 minutes the fixative is removed 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 every two hours. Plates 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.

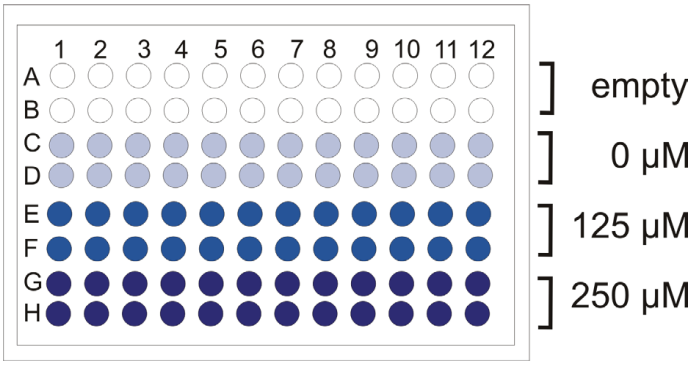

Figure 4. Plate map configuration of FFA treatment. FFA mixture is added to the plate using the Agilent BioTek MultiFlo FX multimode dispenser such that three different concentrations (0, 125, and 250 mM) of FFA are added to two rows each with pairs of the eight separate dispense tubes in rows A to F. The concentration for each is indicated.

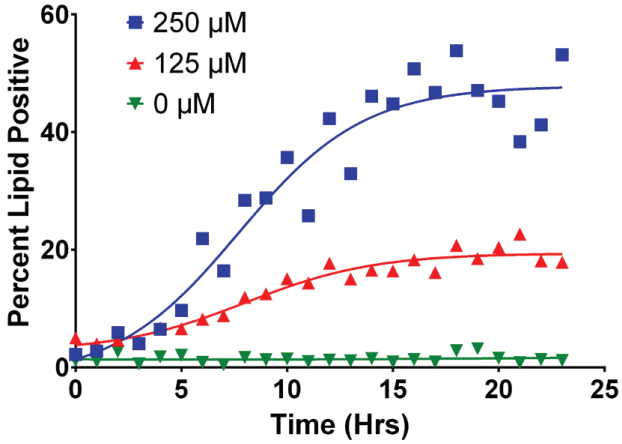

Figure 5. Comparison of neutral lipid accumulation. HepG2 cells were treated with different concentrations of FFA, then fixed and stained for neutral lipid at various times. Using an Agilent BioTek BioSpa to control timing and maintain the necessary environmental control, individual columns of a plate 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 represent the mean of two determinations.

Longer-term exposure of HepG2 cells to FFA over a period of 4 days reveals that the lipid content reaches a steady state after 20 and 36 hours of exposure for 0.25 mM and 0.125 mM FFA, respectively (Figure 6). The steady state level of fluorescence lipid staining is FFA concentration-dependent. Long-term exposure to FFA was also tested in a similar fashion as described above with eight plates. After initiation, pairs of plates, representing the 24 hourly time points of each of four days were manually removed from the system after fixative was added and refrigerated. At the completion of the BioSpa session, all eight plates were stained as a group and imaged.

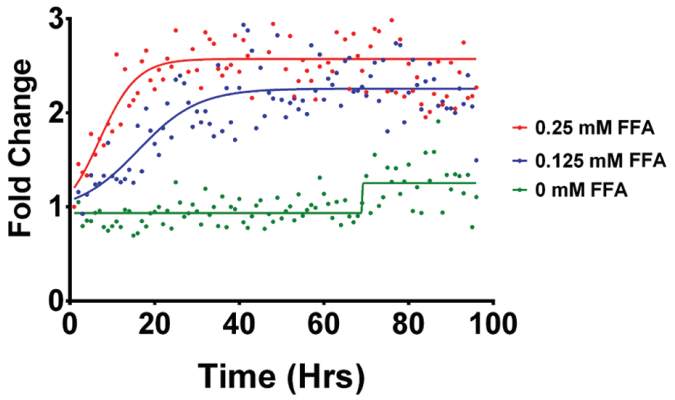

Figure 6. Long-term lipid accumulation. HepG2 cells were treated with a single dose of FFA mix, and then fixed with 4% paraformadehyde at intervals over four days. At the completion of the experiment, all plates were stained for nuclei and neutral lipid. Using an Agilent BioTek BioSpa 8 automated incubator to control timing and maintain the necessary environmental control, individual columns of a plate were fixed with 4% PFA at 1-hour intervals using an Agilent BioTek MultiFlo FX multimode dispenser. Eight plates were staggered such that any one plate was removed from the BioSpa at most every two hours. Data represent the fold change from time zero. Each data point represents the mean of two separate determinations.

Lipid degradation after the removal of stimuli takes place at much slower rates, if at all. Cells that have been exposed to FFA mixtures and accumulated intracellular lipids do not show any significant decrease in lipid content after removal of FFA. As demonstrated in Figure 7, treated Hep G2 cells maintain lipid content for at least 24 hours after removal of the stimuli. This is markedly different from the increase in lipid content with exposure.

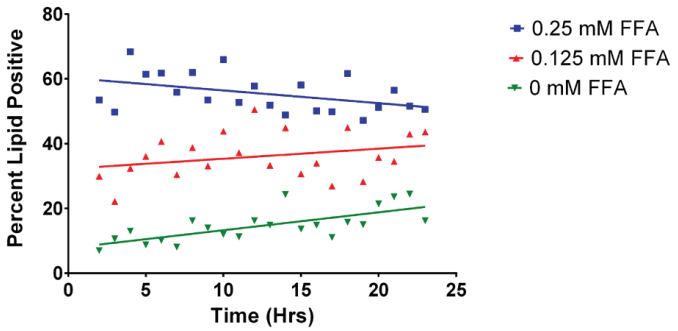

Figure 7. Lipid containment after removal of FFA stimuli. HepG2 cells were treated with various concentrations of FFA mix for 24 hours. Media was then replaced without FFA and cells were then fixed with 4% paraformadehyde hourly for 24 hours. At the completion of the experiment, all plates were stained for neutral lipid. Using an Agilent BioTek BioSpa 8 automated incubator to control timing and maintain the necessary environmental control, individual columns of a plate were fixed with 4% PFA at 1-hour intervals using an Agilent BioTek MultiFlo FX multimode dispenser. Plates were staggered such that any one plate was removed from the BioSpa at most every two hours. Each data point represents the mean of two separate determinations.

Conclusion

Cellular changes that take place over hours or days are often difficult to monitor precisely and in a timely fashion with manual methods. Round-the-clock experimentation requires effort over multiple work shifts by separate individuals or sleep deprivation by a single researcher, either of which can lead to experimental error due to different technique by separate individuals or poor performance by the tired researcher. These experiments demonstrate the utility of a low-cost high-value system involving the Agilent BioTek BioSpa 8 automated incubator to store and shuttle microplates while maintaining suitable conditions for cell health over long kinetic experiments. This system has the capability to seed cells, add reagents, fixatives and stains, provide environmental control, as well as image and analyze the results. The use of automation provides reproducible reagent addition for every reagent, as well as exact timing for each process step. Constant monitoring of process steps and environmental conditions in conjunction with the session log provides after the fact prove that the necessary assay conditions were followed. Because this system is small enough to be placed into a HEPA-filtered biosafety cabinet, sterile conditions can be maintained throughout the experiment.

References

1. Mehta, K. *et al.* Nonalcoholic Fatty Liver Disease: Pathogenesis and The Role of Antioxidants, *Nutritional Reviews* **2002**, *60*, 289–293.
2. Chui, H. C. *et al.* A Novel Mouse Model of Lipotoxic Cardiomyopathy. *J. Clin. Invest.* **2001**, *107*, 813–822.

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