High-Content Imaging Detection of Cellular Lipids to Measure Lipid Toxicity and Cell Differentiation

Marie C. Nlend,[1] Nikki Jarrett,[1] Kay Opperman,[1] Suk J. Hong,[1] Surbhi Desai,[1] Matthias Wenzel,[2] Frank Lehman,[2] Peter Czerney[2] and Richik N. Ghosh[1]

[1]Thermo Fisher Scientific, Rockford, IL, USA. [2]Dyomics Gmbh, Jena, Germany

Thermo Fisher Scientific • 3747 N. Meridian Rd., PO Box 117, Rockford, Illinois U.S.A. 61105

Abstract

The detection and understanding of prelethal mechanisms in toxicological profiling and compound screening are important components of drug discovery. Cationic amphiphilic drugs can impact lipid metabolism and inhibit the normal phospholipid metabolism resulting in their intracellular accumulation (phospholipidosis). Other drug classes more adversely affect various aspects of fatty acids or neutral lipid metabolism leading to cytoplasmic accumulation of neutral lipids as lipid droplets (steatosis). We developed fluorescent reagents for performing multiplexed, cell-based high-content imaging cytotoxicity assays to automatically detect and quantify intracellular accumulation of phospho- and neutral lipids in mammalian cell lines. To label phospholipids, live or fixed HepG2 cells were treated with propranolol in a dose-dependent manner with new fluorescent lipid probes with green (ex/em: 498/532 nm) or orange (ex/em: 557/584 nm) fluorescence emission. Neutral lipid droplets are quantitatively detected either in a steatosis lipid toxicity model in HepG2 cells treated with cyclosporin A or in an adipogenesis model of 3T3L1 fibroblasts differentiated into adipocytes. Neutral lipids were detected with high affinity using a specific probe with red fluorescence emission (ex/em: 656/680 nm) on live or fixed cells without wash steps. The phospho- and neutral lipid probes were multiplexed with each other and other fluorescent markers to detect the lipid compartmentalization in the cell. We found that the cellular lipid detection reagents were specific for their respective targets by their correct cellular location, determined by colocalization analysis with a lysosome marker (for phospholipids) and anti-adipophilin immunofluorescence (for neutral lipids). In summary, quantitative imaging analysis was used to explore different lipid toxicity cellular models and lipid accumulation by cell differentiation, was found to be specific and sensitive, and was validated as an accurate screening assay method.

Introduction

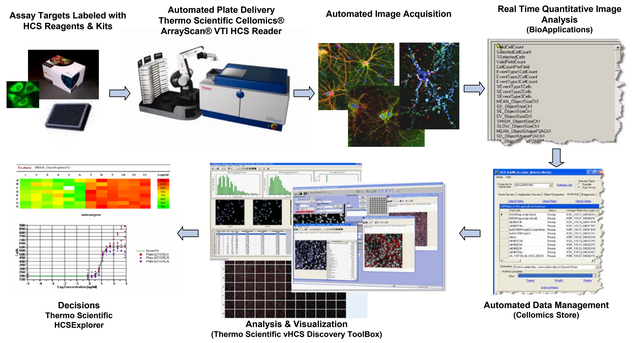
Early cytotoxicity detection of a substance and identification of its toxic mechanism are of great value during drug development. Automated, quantitative cellular imaging (i.e., high-content imaging) is a powerful assay method for predicting compound toxicity. Detection and determination of drug-induced cytotoxicity can be monitored by multiple markers that indicate cellular responses to a toxic insult. Some drug classes adversely affect various aspects of fatty acid or neutral lipid metabolism leading to the cytoplasmic accumulation of neutral lipids as lipid droplets or globules (steatosis), or of phospholipids in lysosomes (phospholipidosis). Abnormal lipid metabolism leading to phospholipidosis and/or steatosis is typically examined during the safety assessments of new drugs. The success in monitoring key cellular responses depends on fluorescent probes that enable specific and sensitive detection of the response by cellular imaging. For example, differentiation of preadipocytes to fat cells through adipogenesis involves the cytoplasmic accumulation of lipid droplets and a specific lipid probe is required to detect these lipid droplets. Therefore, researchers need validated tools for detecting and distinguishing the accumulation of phospholipids (phospholipidosis) and neutral lipids (steatosis) in the cytoplasm of mammalian cell lines after the incubation of test compounds.

We developed a set of fluorescent reagents for multiplexed, cell-based, high-content imaging cytotoxicity assays to automatically detect and quantify phospholipidosis. The two new detection reagents label the accumulation of phospholipids in live or fixed cells and have either green (ex/em = 498/532 nm) or orange (ex/em = 557/584 nm) fluorescence emission. We have also developed a novel fluorescent dye, Red Fat Dye (ex/em = 656/680 nm), to detect neutral lipid droplet formation and lipid overloading in cells in normal and pathological conditions; e.g., to study the toxic effects of compounds on lipid metabolism in mammalian cell lines and to monitor the formation and differentiation of adipocytes. Red Fat Dye shows a high affinity and specificity for neutral lipid droplets in live or fixed cells and it is compatible with our phospholipidosis detection reagents. These dyes can be detected by fluorescence microscopy and high-content screening instruments.

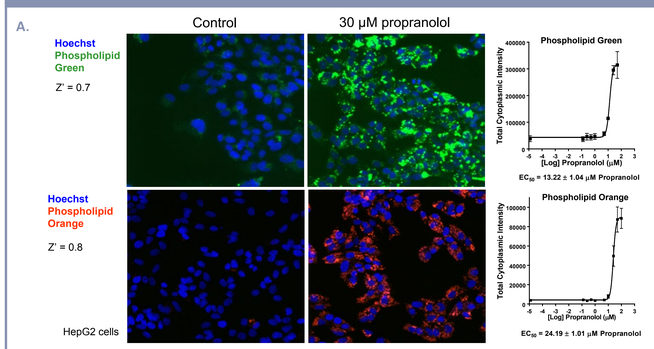
New Thermo Scientific Cellomics Dyes for Lipid Toxicity:

Dye:	Phospholipid Green	Phospholipid Orange	Red Fat Dye
Use:	phospholipidosis	phospholipidosis	steatosis & adipogenesis
Excitation λ:	500 nm	550 nm	656 nm
Emission λ:	522 nm	566 nm	680 nm
Molecular Weight:	1,633 g/mol	1,326 g/mol	698 g/mol
ε:	> 62,000 mol^{-1} cm^{-1}	> 90,000 mol^{-1} cm^{-1}	> 93,000 mol^{-1} cm^{-1}

Thermo Scientific Cellomics High Content Imaging: Seamless Integration of all Steps in Cellular Analysis



High Content Imaging of Phospholipidosis



Specificity: Phospholipid Orange is perfectly colocalized with LysoTracker Yellow in lysosomes

Figure 2: A. Images: HepG2 cells were treated with 1 µM Thermo Scientific Cellomics Phospholipid Green or Phospholipid Orange and 30 µM propranolol for 48 hours, and nuclei were counterstained with Hoechst 33342. Half of each plate was treated, while the other half was not treated with propranolol. Images were automatically acquired and quantitatively analyzed by the Thermo Scientific Cellomics® ArrayScan® VTi HCS Reader. The performance of this assay is robust (Z' factor = 0.7 and 0.8, respectively). Dose Response Curves: HepG2 cells were treated with 1 µM of Phospholipid Green or Orange and various propranolol concentrations (0.125-100 µM) for 48 hours (y-axis is total fluorescence intensity measured in the cytoplasm). B. Phospholipid Green and Orange are specific for phospholipids. HepG2 cells were treated with 1 µM Thermo Scientific Cellomics Phospholipid Orange, 75 nM LysoTracker Yellow, and 30 µM propranolol for 48 hours. Colocalization analysis yielded a Pearson's correlation coefficient of 0.97 ± 0.02 and a Z' factor of 0.74. Thus the Phospholipid Orange is perfectly colocalized with LysoTracker Yellow in lysosomes, showing its specificity.

Comparison with Other Phospholipid Probes

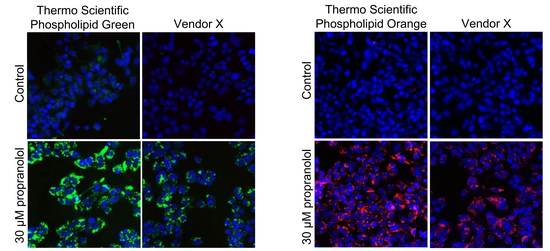


Figure 3: HepG2 cells were treated with either 1 µM of our Phospholipid Green or the green phospholipid detection reagent from Vendor X, or with 1 µM of our Phospholipid Orange or the orange phospholipid detection reagent from Vendor X. Vendor X's probes were used according to the manufacturer's instructions. The cells were either treated with 30 µM propranolol for 48 hours, or left as controls. Nuclei were counterstained with Hoechst 33342. Dye performance was assessed after image acquisition using a Cellomics ArrayScan VTi HCS Reader with identical fixed exposure times; images represent raw data. The Thermo Scientific Cellomics Phospholipid Green and Orange were much brighter than the competitor's products.

Steatosis Model: New Dye for Neutral Lipid Staining

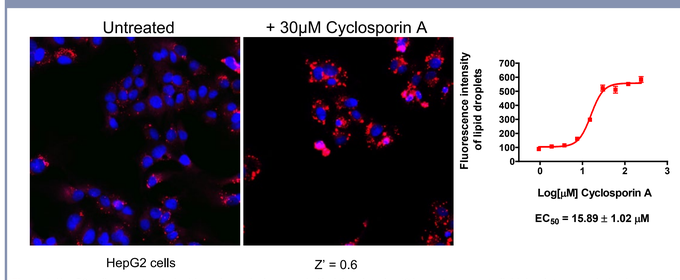


Figure 4: HepG2 cells were treated with cyclosporin A on one half of the plate for 48 hours to generate lipid droplet accumulation by steatosis, while the other half was maintained in fresh DMEM complete media. Cellomics Red Fat Dye (1 µM) was loaded to either live or fixed cells and incubated for 30 minutes at 37°C. Nuclei were stained with Hoechst 33342. Dye performances were assessed after image acquisition on the ArrayScan VTi HCS Reader using the same exposure time; images represent raw data. The assay produces robust performance (Z' factor = 0.6 ± 0.09 and COV = 7.4%).

Adipogenesis Detection with Thermo Scientific Cellomics Red Fat Dye

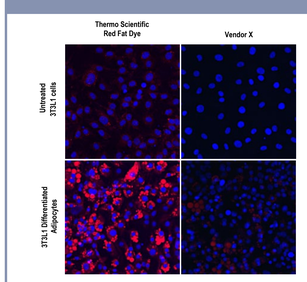


Figure 5: 3T3 L1 cells were differentiated into adipocytes for 7 days using MDI induction media and insulin media. One half of each plate was differentiated, while the other side was replaced with fresh media. Cellomics Red Fat Dye (1 µM) or a neutral lipid dye (1 µM) with similar spectra from Vendor X was added after fixation. Nuclei were stained with Hoechst 33342. Dye performance was assessed after image acquisition on the ArrayScan VTi HCS Reader using the same exposure time; images represent raw data. The total fluorescence intensity of neutral lipid droplets was used to calculate the Z' scores. The robustness of the assay using the Cellomics Red Fat Dye was measured in duplicate plates (Z' factor = 0.51 ± 0.13 and COV = 7.2%). The images using Vendor X's dye were not analyzed as neutral lipid staining was hardly seen. The Cellomics Red Fat Dye produces stronger lipid droplet staining.

Thermo Scientific Red Fat Dye is Specific for Neutral Lipid Droplets

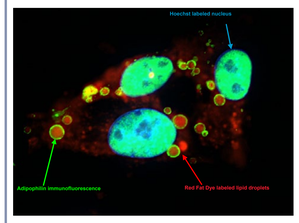
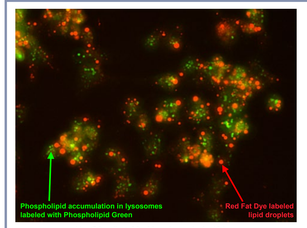


Figure 6: Cellomics Red Fat Dye specifically stains neutral lipid droplets. Neutral lipid droplets in differentiated 3T3L1 adipocytes were stained with Red Fat Dye, and then permeabilized and labeled with an anti-adipophilin antibody by immunofluorescence. Adipophilin is an adipose differentiation-related protein (ADRP) intrinsically associated with the surface of lipid droplets. Images were acquired using a higher resolution microscope objective (40X oil, 1.3 NA). The Red Fat Dye is specific for neutral lipid droplets, as it co-localizes with adipophilin. Furthermore, this experiment shows that the Red Fat Dye can be used with immunofluorescence enabling multiplexing of intracellular targets.

Neutral Lipid and Phospholipid Co-Detection



HepG2 cells + 30 µM cyclosporin A, 48 hrs.

Figure 7: HepG2 cells were treated with 30 µM cyclosporin A and 1 µM of Thermo Scientific Cellomics Phospholipid Green for 48 hours. The cells were then treated with 3 µM Thermo Scientific Cellomics Red Fat Dye for 30 minutes, fixed, and analyzed. Pearson's correlation coefficients indicate the two dyes label different cellular structures and are able to be multiplexed (control wells Pearson's = 0.43 ± 0.07; treated wells Pearson's = 0.52 ± 0.03).

Conclusions

Thermo Scientific Cellomics Phospholipid Green and Orange reagents:

- **Enable specific detection of phospholipidosis in cells**
- **Can be multiplexed with other fluorescent markers, including the Thermo Scientific Red Fat Dye for simultaneous detection of steatosis**
- **Are brighter than competitor's products and give robust assay performance with high Z' factors (>0.7)**
- **Are well-suited to automated, cell-based imaging analysis.**

Thermo Scientific Cellomics Red Fat Dye:

- **Labels neutral lipid droplets that occur in steatosis and adipogenesis**
- **Gives robust assay performance with high Z' factors (>0.5) and with brighter signal than dyes with similar spectra from competitors**
- **Labels cells both pre- and post fixation, and can be used with immunofluorescence for the multiplexed detection of other cellular targets**
- **Is specific, sensitive, and validated for quantitative measurements in high-content imaging assays for adipogenesis and steatosis.**