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Multiparametric High-Content Assays to Measure Cell Health and Oxidative Damage as a Model for Drug-Induced Liver Injury

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Abstract

Drug-induced liver injury is an important cause of non-approval in drug development and the withdrawal of already approved drugs from the market. Screening human hepatic cell lines for toxicity has been used extensively to predict drug-induced liver injury in preclinical drug development. Assessing hepatic-cell health with more diverse markers will increase the value of in vitro assays and help predict the mechanism of toxicity. We describe three live cell-based assays using HepG2 cells to measure cell health parameters indicative of hepatotoxicity. The first assay measures cellular ATP levels using luciferase. The second and third assays are multiparametric high-content screens covering a panel of cell health markers including cell count, mitochondrial membrane potential and structure, nuclear morphology, vacuolar density, and reactive oxygen species and glutathione levels.

Keywords

cell health; drug-induced liver injury; glutathione; HepG2; high-content analysis; high-throughput screening; mitochondrial dysfunction; multiparametric assays; reactive oxygen species

INTRODUCTION

Predicting and avoiding drug-induced liver injury (DILI) or drug-induced hepatotoxicity remains one of the major challenges in drug discovery (Kuna et al., 2018; Nouredin & Kaplowitz, 2018; Weaver et al., 2020). In a clinical setting, DILI presents with symptoms associated with acute hepatitis and/or cholestasis. However, the underlying etiopathogenesis of DILI is complex and involves a combination of genetic, immune, and metabolic factors

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Author Contributions

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(Ulrich, 2007). The pathogenesis of DILI usually involves the parent drug or its metabolite forming an unwanted interaction with hepatocytes. To avoid DILI in a clinical setting, it would be advantageous to determine which candidate molecules could induce hepatotoxicity in the early stages of drug discovery. Furthermore, it is important to predict the mechanisms implicated in hepatotoxicity. We have, therefore, developed multiparametric assays to be used together to measure damage to hepatocytes. These assays can use different types of cell lines or primary cells. Here, we describe the methods using the hepatocellular carcinoma HepG2 cell line.

This article provides three protocols that measure several toxicity endpoints including cellular ATP levels, cell count, nuclear morphology, mitochondrial membrane potential (MMP) and structure, vacuolar count, and reactive oxygen species (ROS) and glutathione (GSH) levels (Fig. 1 and Table 1). Basic Protocol 1 describes a standard, luminescence-based assay that measures cellular ATP count. Basic Protocols 2 and 3 are multiplexed, high-content assays. High-content analysis (also called high-content screening) uses automated microscopy for image acquisition and data analysis. Basic Protocol 2 measures cell count, MMP, mitochondrial structure, and ROS; Basic Protocol 3 measures vacuolar density and GSH levels. While the principles of these assays can be performed on any instrument and analyzed using various image-analysis software, our specific protocol uses an IN Cell Analyzer imager (Cytiva) and IN Cell Developer/IN Carta software (Cytiva). A typical workflow for a high-content screening assay, described in Basic Protocols 2 and 3, is shown in Figure 2.

BASIC PROTOCOL 1 MEASUREMENT OF CELLULAR ATP CONTENT

Metabolically active cultured cells maintain high levels of intracellular ATP and ATP is often used as a reporter of the number of viable cells present in culture. Here, we provide a protocol to measure ATP levels using a luciferase enzyme that converts luciferin to oxyluciferin in the presence of Mg^{2+} , O_2 , and ATP (Fig. 3A; Crouch, Kozlowski, Slater, & Fletcher, 1993; Current Protocols article: Tolliday, 2010). This enzymatic reaction produces luminescence, which is measured on a plate-based spectrophotometer such as the EnVision 2105 (PerkinElmer). This protocol uses the CellTiter-Glo 2.0 kit (Promega), which includes a cell lysis buffer and Ultra-Glo luciferase. The assay is performed in 384-well microplates with a “mix and read” format that is amenable to high-throughput screening.

HepG2 cells are grown in serum-supplemented medium. To capture variations in blood glucose levels, we use both high glucose (DMEM) and low glucose (Eagle’s minimum essential medium, EMEM) media. Cells are then exposed to a single concentration of potentially toxic compounds for either 6 or 24 hr. All assays are performed in triplicate.

Materials

CellTiter-Glo 2.0 Cell Viability Assay (Promega, cat. no. G9242)

Complete medium with antibiotics (either complete DMEM with antibiotics or complete EMEM with antibiotics; see recipes)

HepG2 cells (MilliporeSigma, cat. no. 85011430) subcultured and resuspended in complete medium containing antibiotics (see recipe)

Dimethyl sulfoxide (DMSO)

Corning 50-ml Centrifuge Tubes (Corning, cat. no. 430290)

Greiner Bio-One 384-well Polystyrene Cell Culture Microplates (Thermo Fisher Scientific, cat. no. 07-000-074)

EL406 Washer Dispenser (BioTek)

EnVision 2105 Multimode Plate Reader (PerkinElmer)

Eppendorf Easypet3 Serological Pipet (Thermo Fisher Scientific)

Forma Scientific CO₂ Water Jacketed Incubator (Thermo Fisher Scientific)

NOTE: Make sure that HepG2 cells used for every assay run are fewer than 20 passages. We consistently use cells between passage number 8 and 20.

NOTE: All incubations must be performed in a humidified 37°C, 5% CO₂ incubator unless otherwise specified.

Perform CellTiter-Glo assay

1. Thaw frozen CellTiter-Glo 2.0 reagent overnight at 4°C.
2. Plate cells (see Support Protocol 2) at 4.0×10^3 cells per 50 μ l of complete medium and incubate overnight in a humidified 37°C, 5% CO₂ incubator.

For two time points, two types of medium, and triplicate tests, each compound will require twelve wells. We recommend preparing twelve replicate plates so that each plate is processed through a single condition.

Positive control wells contain 50 μ l of complete medium with no cells (as recommended by Promega), while negative control wells contain 4.0×10^3 cells per 50 μ l of complete medium.
3. Pin transfer 300 nl of test compounds in 10 mM DMSO to experimental wells(see Support Protocol 3). Using another source plate containing only DMSO, pin transfer 0.6% DMSO to the remaining negative control and positive control wells.

Three hundred nanoliters of test compound dissolved in DMSO results in 60 μ M working compound concentration and 0.6% DMSO. These numbers were derived from the upper cellular tolerance limit for DMSO, which is 0.6% in culture medium; above this limit, HepG2 cell health will be compromised.
4. Incubate plates for 6 or 24 hr.
5. Thirty minutes before use, remove CellTiter-Glo 2.0 reagent from 4°C and leave the reagent bottle on the benchtop to equilibrate at room temperature.

Mix reagent carefully by inverting the contents, gently vortexing, or swirling the bottle. Set aside equilibrated reagent until ready to be administered to cells.

6. After 6 hr, remove plates from incubator and allow plates to sit at room temperature 30 min.
7. Add 25 μ l CellTiter-Glo 2.0 reagent to all wells in each 384-well plate using a peristaltic pump dispenser such as an EL406 washer dispenser. Multichannel pipets can also be used.

This step lyses cells and adds luciferase reagent.

8. Agitate plates for 10 s using the shake feature of the EL406 or place on an orbital shaker for 2 min.
9. Equilibrate cells 30 min at room temperature before measuring luminescence on the plate reader.

This step reduces thermal gradients that are greatest on the edges of the plates, which may result in an uneven luminescence signal.

10. Read plates using the luminescence protocol on a plate reader (such as PerkinElmer's EnVision 2105 Multimode Plate Reader).

Determine the background luminescence signal of the instrument by using the positive control wells containing only medium without cells. If significant, the background luminescence can be subtracted from test wells during data analysis, below.

11. Repeat steps 6–10 for plates incubated for 24 hr.

Data analysis

The goal of this section is to select which compounds reduce luminescence in the CellTiter-Glo assay, implying toxicity. Data were acquired in triplicate. Negative control wells contained cells, 0.6% DMSO, and CellTiter-Glo 2.0 reagent. Positive control wells contained no cells, 0.6% DMSO, and CellTiter-Glo 2.0 reagent. As noted above, a toxic compound could also be used as a positive control. Data were analyzed using Microsoft Excel.

12. Transfer data from the plate reader to a spreadsheet program, such as Excel. Data columns should include (1) well ID, (2) control or compound ID, and (3) luminescence in RLU for each assay plate.
13. Determine plate performance by calculating the mean (μ), standard deviation (σ), and percent coefficient of variation (%CV) for each plate using the negative and positive control wells. Also determine the signal-to-background ratio (S/B) using Equation 1.

$$S/B = \frac{\mu_{\text{negative control}}}{\mu_{\text{positive control}}} \quad \text{Equation 1}$$

14. Calculate the Z' value for each plate using Equation 2.

$$Z' = 1 - \frac{(3\sigma_{\text{negative control}} + 3\sigma_{\text{positive control}})}{|\mu_{\text{negative control}} - \mu_{\text{positive control}}|} \quad \text{Equation 2}$$

The Z' -factor (Z') is a widely accepted metric to evaluate assay quality. A Z' value between 0.5 and 1.0 denotes a statistically significant separation between the negative and positive controls (Zhang, Chung, & Oldenburg, 1999). If the Z' value is below 0.5, evaluate the source of error, e.g., a large standard deviation or a small luminescence signal in the untreated/negative controls. Address experimentally by varying cell density or plating procedures.

15. To allow comparisons across plates, normalize raw RLU values to percent inhibition values using Equation 3.

$$\% \text{ inhibition} = 100 \times \left(\frac{\text{RLU}_{\text{in the presence of compound}} - \text{RLU}_{\text{positive control}}}{\text{RLU}_{\text{negative control}} - \text{RLU}_{\text{positive control}}} \right) \quad \text{Equation 3}$$

Percent inhibition is also calculated for the control wells. The average of negative controls will be close to 0% and the average of the positive control wells will be 100%.

16. Calculate the standard deviation (σ) for positive and negative controls on each plate.
17. Calculate the average % inhibition value for replicate (triplicate) data.
18. Select active (potentially toxic) compounds based on percent inhibition relative to the mean and standard deviation of the negative control. A compound is active if:

$$\% \text{ inhibition} > \mu_{\% \text{inhib, negative control}} + 3\sigma_{\% \text{inhib, negative control}}$$

It is often helpful to plot percent inhibition data as a scatter plot, with compound number on the x axis and % inhibition on the y axis (Fig. 3B). The mean plus 3 standard deviations of the control is then added as a line across the scatter plot. All data points above the line are significantly active.

19. Active compounds may be analyzed in a dose-response manner to determine the IC_{50} value in the CellTiter Glo 2.0 assay (see Support Protocol 4).

For control compounds and hits that were run in a dose-response manner during the secondary screen, IC_{50} s were determined on GraphPad Prism (Fig. 3C). The pIC_{50} s (a positive-trending potency metric) are then determined using the equation below:

$$pIC_{50} = -\text{Log}_{10}(IC_{50})$$

Equation 4

The IC_{50} value should be in molar units in Equation 4.

BASIC PROTOCOL 2 HIGH-CONTENT ANALYSIS ASSAY TO ASSESS CELL COUNT, MITOCHONDRIAL MEMBRANE POTENTIAL AND STRUCTURE, AND REACTIVE OXYGEN SPECIES

Compounds can cause toxicity by multiple mechanisms. These mechanisms can be monitored at times and doses that do not yet induce cell death. For example, reaction with oxidative enzymes in hepatocytes can lead to the generation of reactive oxygen species (ROS) and induction of organelle stress, including mitochondrial stress. If adaptive responses cannot compensate, then organelle stress will either activate the intrinsic pathway of apoptosis or lead to necrosis. HepG2 cell health assessment should therefore include the detection of ROS and mitochondrial stress (Iorga, Dara, & Kaplowitz, 2017; Nouredin & Kaplowitz, 2018; Yuan & Kaplowitz, 2013). Basic Protocol 2 describes the development and use of a multiplex image-based assay to assess cell count, nuclear size, MMP, mitochondrial structure, and levels of ROS in HepG2 cells. As with the luciferase assay in Basic Protocol 1, we evaluate the effect of glucose and measure cell-health markers at 6 hr (early stress detection) and 24 hr.

Materials

HepG2 cells (MilliporeSigma, cat. no. 85011430) subcultured and resuspended in complete medium containing antibiotics (see recipes)

Complete medium with antibiotics (either complete DMEM with antibiotics or complete EMEM with antibiotics; see recipes)

DMEM, high glucose, GlutaMAX supplement, pyruvate (Thermo Fisher Scientific, cat. no. 10569010)

BioWhittaker Medium EMEM Cell Culture Media (Lonza, cat. no. 12001–570)

BenchMark FBS (Gemini Bio-Products, cat. no. 100–106)

GlutaMAX Supplement (Thermo Fisher Scientific, cat. no. 35050061)

MEM Non-Essential Amino Acids Solution, 100× (Thermo Fisher Scientific, cat. no. 11140050)

Penicillin-Streptomycin, 10,000 U/ml (Thermo Fisher Scientific, cat. no. 15140122)

Dimethyl Sulfoxide, Fisher BioReagents (Thermo Fisher Scientific, cat. no. BP231100)

Menadione, 25 g (MilliporeSigma, cat. no. M9429–25G)

Raloxifene Hydrochloride, 500 mg (MilliporeSigma, cat. no. R1402–500MG)

PBS, no phenol red, no sodium (Gibco, Molecular Probes, cat. no. 20–012-050)

Invitrogen Hoechst 33342, Trihydrochloride, Trihydrate, 100 mg (Thermo Fisher Scientific, cat. no. H3570)

CellROX Green Reagent (Thermo Fisher Scientific, cat. no. C10444)

MitoTracker Deep Red FM (Thermo Fisher Scientific, cat. no. M22426)

MitoTracker Red CMXRos (Thermo Fisher Scientific, cat. no. M7512)

Multiplex high-content analysis (HCA) staining solution (see recipe)

Falcon Cell Strainers, 40 μ m Pore (Corning, cat. no. 087711)

Microplate, 384-well, deep well small volume polypropylene (Greiner Bio-One, cat. no. 784201)

Microplate, 384-well, V-shape (Greiner Bio-One, cat. no. 781280)

CELLCOAT poly-D-lysine coated 384-well plate (Greiner Bio-One, cat. no. 781946)

Matrix WellMate (Thermo Fisher Scientific)

Biomek FX^P Laboratory Automation Workstation (Beckman Coulter)

EL406 Washer Dispenser (BioTek)

IN Cell Analyzer 2500 HS (GE Healthcare Life Sciences)

IN Cell Developer Toolbox (GE Healthcare Life Sciences)

Forma Scientific CO₂ Water Jacketed Incubator (Thermo Fisher Scientific)

NOTE: To capture variations in blood glucose level, we used two types of assay media: high glucose (DMEM) and low glucose (EMEM) complete media. Typically, 1% of penicillin-streptomycin antibiotics were added into each medium to prevent potential microbial contamination.

NOTE: All incubations must be performed in a humidified 37°C, 5% CO₂ incubator unless otherwise specified.

NOTE: The following protocol uses live-cell imaging. Depending on the speed of the high-content imager and the size of the experiment, plates read at the beginning of the experiment may be treated for a significantly shorter time than plates read at the end of the experiment. The following protocol takes up to 35 min for one 384-well plate to be scanned on an IN Cell Analyzer 2500 HS instrument. Timing can be handled in two ways. First, one could stagger the addition of test compounds and then reagents so that each plate is treated for the same length of time. Second, all plates can be treated at the same time and left in PBS until imaged; we have found that four plates can be included in a single assay run without altering the results.

1. Plate cells (see Support Protocol 2) in poly-D-lysine coated 384-well plates at a seeding density of 4.0×10^3 cells per well then incubate overnight. We typically use 50 μ l complete medium in each well.

Experiments are usually run in duplicate. For two time points, two types of medium, and duplicate tests, each compound will require eight wells. We recommend preparing replicate plates so that each plate is processed through a single condition.

2. Pin transfer (see Support Protocol 3) 300 nl test compounds (10 mM stocks in DMSO), positive controls, and DMSO (negative control wells).

Three hundred nanoliters of test compound results in 60 μ M concentrations and 0.6% DMSO. These numbers were derived from the upper acceptable concentration for DMSO which is 0.6% in culture medium; above this limit, HepG2 cell health will be compromised.

Organize 384-well plates so that the first two and last two columns are reserved for negative and positive controls, respectively, while the rest of the wells are reserved for test compounds. Negative control wells are treated with 0.6% DMSO. Positive control wells are treated with 100 μ M menadione (Fig. 4).

3. Incubate plates for 6 or 24 hr.
4. Aspirate medium from cells and add 50 μ l of live multiplex HCA staining solution to the cells using a bulk dispenser such as an EL406 washer dispenser or multichannel pipettor, then incubate 30 min.
5. Aspirate staining solution and wash plates twice with 100 μ l PBS, aspirating after each wash; add a final 50 μ l PBS.

The culture is now ready for live imaging. We use an IN Cell Analyzer 2500 HS high-content analysis imaging system (Cytiva). Similar instruments are manufactured by PerkinElmer, Molecular Devices, Thermo Fisher Scientific, Vala Sciences, and others.

6. Images are obtained with a 20 \times magnification objective, four wavelength channels (excitation/emission of Blue channel: 390/435 nm, Green channel: 473/525 nm, Red channel: 575/623 nm, and Far Red channel: 631/684 nm), and four field-of-views per well (Fig. 4).

Prior to starting batch image collection, perform autofocus and exposure time adjustments in all four channels.

Data Analysis 1: Image segmentation and quantification

The following analysis protocol uses the terminology of IN Cell Developer Toolbox (Cytiva). Other commercial instruments have analogous software but terminology and the details of data processing might be different.

7. Open the IN Cell Developer Toolbox software and load image files containing an XCDE file extension.
8. Perform a two-step object segmentation on cell nuclei (Nucleus 1 and Nucleus 2) captured in the Blue channel. When segmenting for Nucleus 1 and Nucleus 2, set the kernel size and sensitivity to 17 and 1, respectively (Fig. 5A).

Nuclear segmentation is performed in two steps because HepG2 cells tend to aggregate as they grow. Hence, performing the two-step object segmentation ensures an optimal clump-breaking process at the end of nuclear segmentation.

- a. Postprocessing of “Nucleus 1”:
 - i. Perform an erosion function of kernel size to 21 as the first postprocessing step for Nucleus 1 to sufficiently reduce the object size and to leave approximately one object mark per nucleus.

At the end of this stage, there will still be some nuclei in close proximity that are identified as one object.
 - ii. Perform a watershed clump-breaking function to separate clumps based on loss of signal intensity at the cell-to-cell contact points.
 - b. Postprocessing of “Nucleus 2”:
 - i. Set an acceptance criterion of [Form Factor] >0.4.
 - ii. Add a sieves (binary) function for Nucleus 2 to keep targets with an area >50 μm^2 .

This step eliminates unspecific objects identified in the background.
 - iii. Perform a clump-breaking function with Nucleus 1 as a second segmentation to obtain the final nuclear segmentation outcome.

The objective of this setting is to accurately segment nuclei with the input of object segmentation from Nucleus 1.
9. Quantify nuclear count, nuclear area, and ROS from Nucleus 2:
 - a. Measure nuclear count (statistical function: sum) and nuclear area (statistical function: mean) from the Blue channel.
 - b. Measure nuclear density-level (statistical function: mean) from the Green channel as a measure of ROS levels.

“Nuclear count,” “nuclear area,” and “ROS levels” (nuclear density-level in the Green channel) are endpoints of this

protocol. These values will be used for the identification of active compounds.

10. Segment cells using the mitochondrial signal captured in the Far Red channel with the Mitotracker Deep Red FM dye (Fig. 5B).
 - a. Use the Far Red channel for intensity segmentation and set the minimum and maximum intensity thresholds to 1,000 and 65,535, respectively. These settings eliminate background intensity.
 - b. Using Nucleus 2 as a second segmentation, perform a clump-breaking function to separate cells.
11. Measure mitochondrial density-level (statistical function: mean) from the Far Red channel. This value is “mitochondrial structure.”
12. Measure mitochondrial membrane potential density level (statistical function: mean) from the Red channel. This value is “mitochondrial membrane potential” (MMP).

“Mitochondrial structure” and “MMP” are endpoints of this protocol. These values will be used for identification of active compounds (Fig. 5C–D).

Data Analysis 2: Compound hit selection and pEC₅₀ value calculation

Assay performance is based on the signal-to-background ratio, percent coefficient of variation, and the Z-factor (*Z*) for each plate. When determining compound hits, the measured segmentation data is normalized to the average of the negative controls, generating a “fold change” value. A change in signal that differs by more than three standard deviations from the mean of the negative controls is considered significant. Depending on the parameter and active compound’s mechanism of toxicity, signals may increase or decrease relative to control (Table 1).

3. Transfer data from the IN Cell Developer Toolbox to a spreadsheet program, such as Excel. Data columns should include (1) well ID, (2) control or compound ID, (3) nuclear count, (4) nuclear area, (5) ROS levels, (6) mitochondrial structure, and (7) MMP for each assay plate.

We performed data rearrangement and analysis using a script written in Python using the Pandas software library via the Jupyter notebook platform.

The Python script that supports the data analysis of this protocol is available in: <https://github.com/gracepohan/HCA-Data-Analysis-Current-Protocols>.

4. Determine plate performance by calculating the mean (μ), standard deviation (σ), and percent coefficient of variation (%CV) for each parameter using the negative and positive control wells. Also determine the signal-to-background ratio (S/B) using Equation 5.

$$S/B = \frac{\mu_{\text{negative control}}}{\mu_{\text{positive control}}} \quad \text{Equation 5}$$

5. Calculate the Z' value for each plate using Equation 6.

$$Z' = 1 \frac{(3\sigma_{\text{negative control}} + 3\sigma_{\text{positive control}})}{|\mu_{\text{negative control}} - \mu_{\text{positive control}}|} \quad \text{Equation 6}$$

6. To allow comparisons across plates, normalize each measured parameter (nuclear count) into fold change values using Equation 7.

$$\text{fold change} = \frac{\text{raw value}}{\mu_{\text{negative control}}} \quad \text{Equation 7}$$

Fold change is also calculated for the control wells. The average of negative controls will be close to 1 and the average of the positive control wells will vary with the assay window and whether signals increase (ROS) or decrease (nuclear count).

7. Calculate the standard deviation (σ) for the positive and negative controls on each plate.
8. Calculate the average fold change value for replicate data.
9. Select active compounds based on fold change, relative to the mean and standard deviation of the negative control. Depending on the parameter measured and/or an active compound's mechanism of action, a compound is active if:

$$\text{fold change value} > \mu_{\text{fold change, negative control}} + 3 \sigma_{\text{fold change, negative control}}$$

or:

$$\text{fold change value} < \mu_{\text{fold change, negative control}} - 3 \sigma_{\text{fold change, negative control}}$$

It is often helpful to plot fold change data as a scatter plot, with compound number on the x axis and fold change on the y axis (Fig. 6A). If plotting a parameter whose signal can either increase or decrease, then the mean \pm 3 standard deviations of the control are added as two lines across the scatter plot. All data points above or below these lines are significantly active.

10. Active compounds may be analyzed in a dose-response manner to determine the EC_{50} value for a given assay parameter (see Support Protocol 4).

For control compounds and hits that were run in dose-response manner during the secondary screen, EC_{50} s were determined on GraphPad Prism. The pEC_{50} s are then determined using the equation below:

$$pEC_{50} = -\text{Log}_{10}(EC_{50})$$
 Equation 8

The EC_{50} value should be in molar units in Equation 8. Examples of dose-response curves of raloxifene are shown in Figure 6B.

BASIC PROTOCOL 3 HIGH-CONTENT ANALYSIS ASSAY TO ASSESS NUCLEAR MORPHOLOGY, VACUOLES, AND GLUTATHIONE CONTENT

In vitro detection of oxidative stress requires not only the measurement of ROS production but also the measurement of antioxidant defenses. GSH is an intracellular antioxidant containing a thiol group that balances out both intracellular and intra-organelle redox states and reduces ROS (Aquilano, Baldelli, & Ciriolo, 2014; Circu & Yee Aw, 2008). The balancing of these redox states is considered a compensatory mechanism that makes GSH a sensitive parameter for early cell health detection of DILI (Zheng et al., 2015). Nuclear morphology is also a sensitive measure of cell damage, e.g., by oxidative stress (Kreuz & Fischle, 2016) or apoptosis, which induces chromatin condensation (Oberhammer, Hohegger, Froschl, Tiefenbacher, & Pavelka, 1994; Redza-Dutordoir & Averill-Bates, 2016). Furthermore, changes in intracellular osmotic pressure are often reflected as an increase in vacuolar density as cells try to maintain homeostasis after exposure to toxic agents (Shubin, Demidyuk, Komissarov, Rafieva, & Kostrov, 2016); vacuolization may also be a measure of necrotic cell death. Therefore, changes in GSH, nuclear morphology, and vacuolar density are indicative of potentially early stages of DILI (Fig. 1). In this protocol, we assess these three additional cell health readouts: GSH, nuclear morphology, and vacuolar content.

Additional Materials (see also Basic Protocol 2)

HepG2 cells (MilliporeSigma, cat. no. 85011430) subcultured and resuspended in complete medium containing antibiotics (see recipes)

Complete medium with antibiotics (either complete DMEM with antibiotics or complete EMEM with antibiotics; see recipes)

DMEM, high glucose, GlutaMAX supplement, pyruvate (Thermo Fisher Scientific, cat. no. 10569010)

BioWhittaker Medium EMEM Cell Culture Media (Lonza, cat. no. 12001-570)

BenchMark FBS (Gemini Bio-Products, cat. no. 100-106)

GlutaMAX Supplement (Thermo Fisher Scientific, cat. no. 35050061)

MEM Non-Essential Amino Acids Solution, 100× (Thermo Fisher Scientific, cat. no. 11-140-050)

Penicillin-Streptomycin, 10,000 U/ml (Thermo Fisher Scientific, cat. no. 15140122)

Dimethyl Sulfoxide (DMSO), Fisher BioReagents (Thermo Fisher Scientific, cat. no. BP231100)

Raloxifene Hydrochloride, 500 mg (MilliporeSigma, cat. no. R1402–500MG)

PBS, no phenol red, no sodium (Gibco, Molecular Probes, cat. no. 20–012-050)

PBS with Ca²⁺ and Mg²⁺ (Thermo Fisher Scientific, cat. no. 14040133)

HCS NuclearMask Deep Red Stain, 250× Concentrate in DMSO (Thermo Fisher Scientific, cat. no. H10294)

ThiolTracker Violet (Thermo Fisher Scientific, cat. no. T10096)

Duplex high-content analysis (HCA) staining solution (see recipe)

Falcon Cell Strainers, 40 µm Pore (Corning, cat. no. 087711)

Microplate, 384 Well, Deep Well Small Volume Polypropylene (Greiner Bio-One, cat. no. 784201)

Microplate, 384 Well, V-shape (Greiner Bio-One, cat. no. 781280)

CELLCOAT Collagen Type I coated 384-Well Plate (Greiner Bio-One, cat. no. 781956)

NOTE: To capture variations in blood glucose level, we used two types of assay media: high glucose (DMEM) and low glucose (EMEM) complete media. Typically, 1% of penicillin-streptomycin antibiotics were added into each medium to prevent potential microbial contamination (see recipe).

NOTE: All incubations must be performed in a humidified 37°C, 5% CO₂ incubator unless otherwise specified.

NOTE: The following protocol uses live-cell imaging. Depending on the speed of the high-content imager, plates read at the beginning of the experiment will be treated for a significantly shorter time than plates read at the end of the experiment. The following protocol takes up to 30 min for one 384-well plate to be scanned on an IN Cell Analyzer 2500 HS instrument. Timing can be handled in two ways. First, one could stagger the addition of test compounds and then reagents so that each plate is treated for the same length of time. Second, we have found that two plates can be included in a single assay run without altering the results. Compared to the assay in Basic Protocol 2, in this assay, cells are more prone to detaching after staining with ThiolTracker Violet dye when left in PBS for an extended amount of time.

1. Plate cells (see Support Protocol 2) in collagen type I-coated 384-well plates at a seeding density of 4.0×10^3 cells per well and incubate overnight. We typically use 50 µl complete medium in each well.

Experiments are usually run in duplicates or triplicates.

2. Treatment: Pin transfer (see Support Protocol 3) 300 nl of test compounds (10 mM stocks in DMSO) on experimental wells and 0.6% DMSO on remaining negative control wells.

Three hundred nanoliters of test compound results in 60 μM concentrations and 0.6% DMSO. These numbers were derived from the upper cellular tolerance limit for DMSO which is 0.6% in culture medium; above this limit, HepG2 cell health will be negatively affected.

Organize compound addition in such a way that the first two columns are reserved for negative controls, while the rest of the wells are reserved for test compounds.

3. Incubate plates for 6 and 24 hr.
4. Aspirate medium from cells and wash plate twice with PBS containing Ca^{2+} and Mg^{2+} .
5. Begin the staining procedure using the EL406 washer dispenser by priming the lines with a 1-ml dispense from each of the dispensing tubes.

This priming step is critical for an automated washer dispenser to prevent strong ThiolTracker dye absorption on the dispensing tubes, which could lead to irregular dye distribution across the plate. However, users of multichannel pipets can skip this step.

6. Dispense 25 μl of live duplex HCA staining solution to the cells and incubate 30 min.
7. Wash plates twice with 100 μl PBS containing Ca^{2+} and Mg^{2+} with a low dispensing speed to prevent cell detachment.
8. Aspirate the last wash and add 50 μl PBS into each well; the plate is now ready for live imaging.
9. Image plates using IN Cell Analyzer 2500 HS high-content analysis imaging system.
10. Obtain images with a 20 \times magnification objective, two wavelength channels (excitation/emission of Blue channel: 390/435 nm and Far Red channel: 631/684 nm), and four field-of-views per well (Fig. 7A).

Prior to starting the batch imaging, perform autofocus and exposure time adjustments in both channels.

Data Analysis 1: Image segmentation and quantification

Nuclear segmentation (Fig. 5A)

11. Open the IN Cell Developer Toolbox software and load the image files containing an XCDE file extension.
12. Perform a two-step object segmentation on cell nuclei (Nucleus 1 and Nucleus 2) captured in the Far Red channel. When segmenting for Nucleus 1 and Nucleus 2, set the kernel size and sensitivity to 17 and 1, respectively.

Nuclear segmentation is performed in two steps because HepG2 cells tend to aggregate as they grow. Hence, performing the two-step object

segmentation ensures an optimal clump-breaking process at the end of nuclear segmentation.

- a. Postprocessing of “Nucleus 1”:
 - i. Perform an erosion function of kernel size to 21 as the first postprocessing step for Nucleus 1 to sufficiently reduce the object size and to leave approximately one object mark per nucleus.

At the end of this step, there will still be some nuclei in close proximity that are identified as one object.
 - ii. Perform a watershed clump-breaking function to separate clumps based on loss in signal intensity at the cell-to-cell contact.
- b. Postprocessing of “Nucleus 2”:
 - i. Set an acceptance criterion of [Form Factor] >0.4.
 - ii. Add a sieves (binary) function for Nucleus 2 to keep targets with an area >50 μm^2 .

This eliminates unspecific objects identified at the background.
 - iii. Perform a clump-breaking function with Nucleus 1 as the second segmentation to obtain the final nuclear segmentation outcome.

The objective of this setting is to accurately segment the area of nuclei with the input of object segmentation from Nucleus 1.

13. Quantifying “Nucleus 2”: Measure nuclear count (statistical function: sum), nuclear area (statistical function: mean), and nuclear density-level (statistical function: mean) from the Far Red channel.

The generated data will be used for subsequent data processing and hit selection.

Cellular segmentation (Fig. 8A–C)

14. Segment cells using the GSH signal, captured in the Blue channel. The segmentation type in this case is done with an intensity segmentation because the shape and distribution of GSH is not well-defined.
 - a. Under GSH intensity segmentation, select the automatic threshold function to compensate for the variability in ThiolTracker Violet staining.

10. Perform a sieve function on the images by keeping any bright targets with an area >1 pixel and <75 pixels.
The images are now converted into a format that is easier to postprocess and quantify into a vacuolar count.
11. To further exclude cellular contour from the vacuolar segmentation, a postprocessing step is done through the sieve function to keep targets with an area <25 μm^2 and >1 μm^2 .
12. To acquire the cell area number, perform an object segmentation with kernel size 99 and sensitivity 50. Perform a sieve function as the final postprocessing step to keep targets with an area >25 μm^2 .
13. Vacuolar density is calculated by taking the ratio of the measured vacuole over the measured cell area. The generated data will be used for subsequent data processing and hit selection.

Data Analysis 2: Compound hit selection and pEC₅₀ value calculation

Assay performance is based on the signal-to-background ratio, percent coefficient of variation, and the Z-factor (Z') for each plate. When determining compound hits, the measured segmentation data is normalized to the average of the negative controls, generating a “fold change” value. A change in signal that differs by more than three standard deviations from the mean of the negative controls is considered significant. Depending on the parameter and active compound’s mechanism of toxicity, signals may increase or decrease relative to control (Table 1).

14. Transfer data from the IN Cell Developer Toolbox to a spreadsheet program, such as Excel. Data columns should include (1) well ID, (2) control or compound ID, (3) nuclear count, (4) nuclear area, (5) GSH density level, (6) GSH area, and (7) vacuolar density for each assay plate.

We performed data rearrangement and analysis using a script written in Python using the Pandas software library via the Jupyter notebook platform.

The Python script that supports the data analysis of this protocol is available in: <https://github.com/gracepohan/HCA-Data-Analysis-Current-Protocols>.

15. Determine plate performance by calculating the mean (μ), standard deviation (σ), and percent coefficient of variation (%CV) for each parameter using the negative and positive control wells. Also determine the signal-to-background ratio using Equation 9.

$$S/B = \frac{\mu_{\text{negative control}}}{\mu_{\text{positive control}}} \quad \text{Equation 9}$$

16. Calculate the Z' value for each plate using Equation 10.

$$Z' = 1 - \frac{(3\sigma_{\text{negative control}} + 3\sigma_{\text{positive control}})}{|\mu_{\text{negative control}} - \mu_{\text{positive control}}|} \quad \text{Equation 10}$$

17. To allow comparisons across plates, normalize each measured parameter (e.g., nuclear count) into fold change values using Equation 11.

$$\text{fold change} = \frac{\text{raw value}}{\mu_{\text{negative control}}} \quad \text{Equation 11}$$

Fold change is also calculated for the control wells. The average of negative controls will be close to 1 and the average of the positive control wells will vary with the assay window and whether signals increase or decrease.

18. Calculate the standard deviation (σ) for the positive and negative controls on each plate.
19. Calculate the average fold change value for replicate data.
20. Select active compounds based on fold change, relative to the mean and standard deviation of the negative control. Depending on the parameter measured and/or an active compound's mechanism of action, a compound is active if:

$$\text{fold change value} > \mu_{\text{fold change, negative control}} + 3\sigma_{\text{fold change, negative control}}$$

or:

$$\text{fold change value} < \mu_{\text{fold change, negative control}} - 3\sigma_{\text{fold change, negative control}}$$

It is often helpful to plot fold change data as a scatter plot, with compound number on the x axis and fold change on the y axis. If plotting a parameter whose signal can either increase or decrease, then the mean \pm 3 standard deviations of the control are added as two lines across the scatter plot. All data points above or below these lines are significantly active.

21. Active compounds may be analyzed in a dose-response manner to determine the EC₅₀ value for a given assay parameter (see Support Protocol 4).

For control compounds and hits that were run in a dose-response manner during the secondary screen, EC₅₀s were determined on GraphPad Prism. The pEC₅₀s are then determined using the equation below:

$$\text{pEC}_{50} = -\text{Log}_{10}(\text{ES}_{50}) \quad \text{Equation 12}$$

The EC₅₀ value should be in molar units in Equation 12. Examples of dose-response curves of raloxifene are shown in Figure 7B.

SUPPORT PROTOCOL 1 SUBCULTURING AND MAINTAINING HEPG2 CELLS

HepG2 cells have the tendency to clump with each other because of their natural tumor-like growth properties as a hepatocarcinoma cell line. Clumps are problematic when performing segmentation during high-content analysis and may affect the normalization and quantification of many structural biomarkers like nuclear count and size. It is advisable that when subculturing these cells, each flask should not reach a confluency of >70% to avoid cell cycle arrest and changes in mitochondrial morphology due to crowding (Current Protocols article: Fu & Lippincott-Schwartz, 2018). In addition, when HepG2 cells reach full confluency, de-clumping becomes a difficult process in succeeding passages.

Materials

- Complete DMEM without antibiotics (see recipe)
- Complete EMEM without antibiotics (see recipe)
- Gibco Trypan Blue Solution, 0.4% (Thermo Fisher Scientific, cat. no. 15–250-061)
- HepG2 cells (MilliporeSigma, cat. no. 85011430) growing at a low passage number in 75-cm² flasks
- Hyclone PBS solution (GE Life Sciences, cat. no. SH30256.01)
- Trypsin-EDTA, 0.05%, phenol red (Thermo Fisher Scientific, cat. no. 25300054)
- Avanti J-15R Benchtop Centrifuge (Beckman Coulter)
- Cell Counting Slides for TC20 Cell Counter, Dual-Chamber (Bio-Rad, cat. no. 1450015)
- Corning 50-ml Centrifuge Tubes (Corning, cat. no. 430290)
- Corning T-75 Cell Culture Flask with Vent Cap (Corning, cat. no. 430641U)
- Eppendorf Easypet3 Serological Pipet with Tips (Thermo Fisher Scientific, cat. no. 12–654-105)
- Falcon 40- μ m Cell Strainer (Corning, cat. no. 352340)
- Fisherbrand Isotemp Digital Control Water Baths Model 225 (Thermo Fisher Scientific)
- Forma Scientific CO₂ Water Jacketed Incubator (Thermo Fisher Scientific)
- Motic AE2000 Inverted Compound Microscope (VWR)
- P1000 and P10 Micropipets with Tips (Eppendorf)
- TC20 Automated Cell Counter (Bio-Rad)

NOTE: All incubations must be performed in a humidified 37°C, 5% CO₂ incubator.

NOTE: Confluency should not exceed 70% using a 75-cm² flask when maintaining and subculturing cells.

NOTE: Medium is replaced in flasks when cells have been seeded at a lower than usual cell density of 6.5×10^6 cells/ml after two days of incubation.

NOTE: Make sure that HepG2 cells used for every assay run are lower than 20 passages. We consistently used cells between passage number 8 and 20.

1. Warm complete medium without antibiotics, PBS without Ca^{2+} and Mg^{2+} , and 0.05% trypsin-EDTA in a 37°C water bath.

We prefer to maintain our cells without antibiotics to avoid adverse effects of antibiotics on cells. Cells can be grown with antibiotics as an added precautionary measure to prevent contamination.

2. Examine cells under an inverted phase-contrast microscope to assess cell morphology, viability, degree of confluency, and the absence of bacterial and fungal contamination.

Regular examination of cells ensures that the cells are in optimal condition before plating for any experimental procedure. If any problems are detected, then cells are discarded and fresh batches of cells are thawed. This ensures quality and reproducibility of data.

3. Aspirate medium from cells grown in a 75-cm² flask and wash with 20 ml warmed PBS. Swirl flask very gently to detach dead cells before aspirating PBS completely.

4. Trypsinize cells using 4 ml of 0.05% trypsin-EDTA and incubate 10 min.

Before setting flask with trypsin in incubator, ensure that enough trypsin has coated the entire surface of the cells.

5. Remove flask from the incubator, gently tap flask, and swirl to help detach cells.

Verify that cells have completely detached using a phase contrast microscope.

6. Neutralize trypsin by adding 20 ml complete medium to the flask.

Gently pipet up and down to resuspend or homogenize cell suspension.

7. Transfer the entire volume of liquid containing neutralized trypsin, cells, and complete medium to a 50-ml centrifuge tube.

8. Centrifuge tube at $300 \times g$ for 5 min.

9. After centrifuging, verify that a cell pellet has collected on the bottom of the centrifuge tube then aspirate supernatant.

10. Resuspend cell pellet using 4 ml medium.

- a. Use a 5-ml serological pipet and carefully pipet up and down 20–40 times to de-clump cells, making sure that bubbles do not form on the surface.

- b.** Resuspend a second time using a 1-ml tip of a P1000 micropipet up and down 20–40 times to prevent further clumping.
- c.** Save 10 μ l of medium/cell suspension for counting (step 13).
- 11.** Sieve the resuspended cells through a 40- μ m cell strainer to exclude larger clumps of cells from your cell suspension.
- 12.** Seed 1 ml of resuspended cells in a new 75-cm² flask containing 20 ml medium.
- Swirl newly seeded flask several times to ensure a homogenous monolayer of cells attach before returning flask to the incubator.
- Multiple 75-cm² flasks can be grown simultaneously according to experimental needs.
- 13.** Count cells, e.g., using the TC20 automated cell counter, to verify the seeding density.
- a.** Mix 10 μ l medium and cells from step 10 and 10 μ l trypan blue.
- CAUTION: Trypan blue is toxic and a potential carcinogen. Relevant personal protective equipment (PPE) must be worn.
- b.** Use a P20 pipet to fill both chambers of a cell counting slide, each with 10 μ l trypan blue-cell suspension mixture.
- Carefully touch the small well on one end of the slide with the pipet tip and allow the chamber to fill via capillary action.
- c.** Insert slide for automated cell counting in hemocytometer and record total cell count, live cell count, and percentage of live cells.
- Seeding density to maintain at least 70% confluency should be $\sim 6.5 \times 10^6$ cells/ml.

SUPPORT PROTOCOL 2 PLATING HEPG2 CELL LINE

When plating cells in 384-well plates, cells must be passaged (see Support Protocol 1), treated with trypsin to allow cell detachment from culture flasks, and reattached to 384-well assay plates. Medium with antibiotics is used as a standard precautionary measure to prevent bacterial or fungal contamination and to maintain high assay accuracy throughout. To automate the process, a liquid dispenser such as a Matrix WellMate (Thermo Fisher Scientific) can be used. The operation of this liquid dispenser is explained in this support protocol.

Additional Materials (see also Support Protocol 1)

70% ethanol, 200 Proof (VWR, cat. no. TX89125–170SFU)

Penicillin-Streptomycin, 10,000 U/ml (Thermo Fisher Scientific, cat. no. 15140122)

HepG2 cells (MilliporeSigma, cat. no. 85011430) growing at a low passage number in 75-cm² flasks

Complete medium with antibiotics (either complete DMEM with antibiotics or complete EMEM with antibiotics; see recipes) warmed to 37°C

Clorox Bleach (Office Depot, cat. no. CLO30966CT)

Complete medium without antibiotics (either complete DMEM without antibiotics or complete EMEM without antibiotics; see recipes)

384-well plate (type of plate depends on assay readout, e.g., luminescence- or fluorescence-based screening)

Accuflo Reagent Reservoir, 25 ml (Thomas Scientific, cat. no. 1148U24)

Cell Counting Slides for TC20 Cell Counter, Dual-Chamber (Bio-Rad, cat. no. 1450015)

Color Squid Magnetic Stirrer (IKA)

Corning 35-mm TC-Treated Culture Dish (Corning, cat. no. 430165)

Corning 50-ml Centrifuge Tubes (Corning, cat. no. 430290)

Costar Polystyrene Storage Bottles, 250 ml (Corning, cat. no. 8390)

Fisherbrand Octagon Stir Bars (Thermo Fisher Scientific, cat. no. 145121481)

Matrix WellMate Microplate Dispenser (Thermo Fisher Scientific)

Matrix WellMate Disposable Tubing Cartridges Small Bore (Thermo Fisher Scientific, cat. no. 201–30002)

Nunc Square Bioassay Dish (Thermo Fisher Scientific, cat. no. 12–565-224) TC20 Automated Cell Counter (Bio-Rad)

NOTE: All solutions and equipment that have contact with cells must be sterile. Proper sterile technique should be used at all times when working in the laminar flow hood.

NOTE: All incubations must be performed in a humidified 37°C, 5% CO₂ incubator unless otherwise specified.

1. Passage cells (see Support Protocol 1) from 75 cm² flasks, spin down at 300 × *g* for 5 min, and resuspend cell pellet in 5 ml medium without antibiotics.

Ensure the cells are passed through a 40-µm cell strainer to avoid cell aggregates in assay plates.

2. Determine cell density (see Support Protocol 1) in the 5-ml resuspension using a cell counter, such as the TC20 automated cell counter.

The hemocytometer calculates cell density in cells/ml units and differentiates between total cell count and live cell count.

3. Calculate the corresponding volume of live cell suspension needed to be aliquoted into a predetermined volume of assay medium with antibiotics. Total

volume of medium needed depends on the number of 384-well plates used and amount of medium in each well. We typically use a cell density of 4.0×10^3 cells per 50 μ l of complete medium in each well.

When using six 384-well plates—three plates for 6-hr incubation and three plates for 24-hr incubation—50 μ l of medium with cells in each well will require roughly 150 ml of complete medium with antibiotics.

Add an additional 100 ml of medium to account for the volume of tubing in the liquid dispenser and also volume needed to prime the tubes with medium before plating. Total medium needed for six plates will be 150 ml plus 100 ml for tubing and priming, 250 ml total.

We plate cells in assay medium with antibiotics to avoid contamination during the compound pinning process.

4. Pipet resuspended cells from step 3 into 250 ml complete medium with antibiotics.
5. Add a magnetic stir bar into the bottle containing the 250-ml live cell suspension then set it on a magnetic stir plate. Set stir plate to 350 rpm.

Stirring the suspension ensures that cells remain homogenous during the plating procedure.

6. Attach disposable tubing cartridge to the liquid dispenser (Matrix WellMate) using the instrument user manual as a guide.

The open end of the tubes where cells enter the tubing should be completely submerged in the cell suspension to prevent air bubbles during priming and dispensing.

7. Attach the waste-fluid vessel to waste-fluid tubing and ensure this tubing is connected to a vacuum aspirator trap within a laminar flow hood.
8. Turn on the liquid dispenser and vacuum aspirator.
9. Lower the nozzle tips so that they are at the correct dispensing height above the waste-fluid vessel.
10. Press and hold the “PRIME” button. Observe that all tubing fills with medium, which then properly flows through the tubes without blockage.

Prime for up to 1 min using the cell suspension while making sure that the waste-fluid vessel is being properly aspirated.

11. Select plate type by moving the plate type switch to the 384-well position.
12. Set the dispense volume to 50 μ l by pressing “MODE” and observe the “VOL” (μ l) LED illuminate.

Press the “UP” and “DOWN” arrows to either increase or decrease the volume in 1- μ l increments until the screen displays 50 μ l.

13. Select the dispense pattern to choose which plate columns to be filled.

Press “MODE” and observe the “PATTERN” LED illuminate. Note that the two left-most digits on the display show the column number and the right-most digit is either a 0 (indicating an unselected column) or a 1 (indicating a selected column). Press the “UP” and “DOWN” arrows to switch between columns and the “SELECT” button to toggle between on or off dispensing for each column.

14. Place a test plate in the plate carriage and observe that the selected wells are receiving the same volume of cell suspension.
15. Press “START” to run the program on the test plate and once dispensing is determined to be accurate, run the program on the experimental plates.
16. When the cell plating procedure is finished, press the “EMPTY” button to return any remaining medium in the tubes to the 250-ml bottle.

Rinse the tubing first by pressing “PRIME” with 50 ml bleach, followed by 50 ml double-distilled water (ddH₂O), and finally 70% EtOH.

17. After plating, set plates out to equilibrate at room temperature for 1 hr.

This step minimizes uneven distribution of cells and reduces edge effects (Lundholt, Scudder, & Pagliaro, 2003).

18. To avoid further edge effects due to evaporation at the edge of 384-well plates, place up to four plates inside a Nunc Square bioassay dish containing a 35-mm TC-treated culture dish filled with 5 ml ddH₂O in the center.

The Nunc Square bioassay dish can fit four standard 384-well plates with corresponding lids side by side. When arranged accordingly, a small space in the middle of the dish is present to fit the 35-mm dish containing ddH₂O for hydration.

19. Transfer the Nunc Square bioassay dish containing the 384-well plates into a humidified 37°C, 5% CO₂ incubator.

SUPPORT PROTOCOL 3 TRANSFERRING COMPOUNDS BY PIN TOOL

When using 384-well plates, typically 10 to 500 nl volumes of compound are transferred into each well. Acoustic dispensing (Echo, Beckman Coulter) and pins (VP Scientific) are two common methods for nanoliter volume transfers. This protocol describes how to use a pin tool containing stainless steel pins, attached to a Biomek FXp liquid handler. Manual pin tool instruments are also available and pin tools can be added to other liquid handling workstations.

This protocol begins with a 384-well plate of compounds dissolved in DMSO to a concentration of 10 mM and yields a final concentration of 60 μM in the assay plate. Thus, we pin 300 nl of each compound/DMSO or DMSO alone, yielding 0.6% DMSO. HepG2 cells can tolerate up to 0.6% DMSO without any adverse cell health effects. For other cell lines, a DMSO tolerance test should be run as part of assay development.

Materials

384-well deep well small volume microplate containing DMSO in 25 μ l volume

384-well deep well small volume microplate containing test compounds in 25 μ l volume

Dimethyl Sulfoxide (DMSO; Fisher BioReagents) diluted with ddH₂O to 50% concentration (Thermo Fisher Scientific, cat. no. BP231–100)

Ethanol, 200 Proof (VWR, cat. no. TX89125–170SFU)

384-Well Deep Well Small Volume Microplate, Polypropylene, Greiner Bio-One (VWR, cat. no. 784201)

Avanti J-15R Benchtop Centrifuge (Beckman Coulter)

Biomek FX^P Automated Workstation with 50 nl Pin Tool (Beckman Coulter)

PlateLoc Thermal Microplate Sealer (Agilent Technologies)

Lint Free Blotting Paper, cut to fit into VP 413 Omni Tray (V&P Scientific, cat. no. 540D-100)

NOTE: All solutions and equipment that have contact with cells must be sterile. Proper sterile technique should be used at all times when working in the laminar flow hood.

1. Prepare source plates, one or more containing the test compounds and one containing DMSO and the positive control compound when available, by setting them at room temperature to thaw overnight.

The negative and positive controls are usually not included within the source compound plates to ensure that the source compound plates can be utilized for different assays that use different positive controls.

2. Once completely thawed, spin source plates in a plate-compatible centrifuge for 15 s at $\sim 100 \times g$. Carefully remove plate seal.
3. Prepare reservoirs containing 50% DMSO and 70% EtOH for washing the pin tool. Place blotting sheets to dry tips.
4. Turn on the pump switches connected to both 50% DMSO and 70% EtOH reservoirs and observe that both are continuously being cycled through a vacuum system.
5. Using the Biomek software user manual, create a new method that will allow for 300 nl of each compound or DMSO to be pinned to their respective wells. Include wash steps as needed by placing pins in the 50% DMSO reservoir, the 70% EtOH reservoir, then touching to blotting sheets.

Note that this assay uses a 50-nl pin tool and will therefore need six successive pins from both the compound and DMSO or positive control source plates to achieve 300 nl each.

Organize compound addition in such a way that the first and the last two columns are reserved for negative and positive controls, respectively, while the rest of the wells are reserved for test compounds. The negative control wells are treated with DMSO, while the positive control wells are treated with a positive control compound dissolved in DMSO when available.

6. On the software click on “INSTRUMENT” and then select “home all axes” before beginning.
7. Start the cleanup protocol to wash tips through 50% DMSO, 70% EtOH, and 100%EtOH. This process ensures tips are free of any contaminants.
8. Open the method made for this run (step 5) by clicking “OPEN” and selecting the method.

Verify that under “set plate no” that the number of plates selected for this run is correct.

9. Begin the process by clicking “START”. Ensure that each destination plate is properly placed and that plate covers are removed before pinning.

Note that at least two source plates are used. Replace the source plate(s) containing the test compounds with the source plate containing DMSO and positive control after the round for all plates have finished. Then restart the procedure to allow for DMSO and positive control compounds to be pin transferred onto the assay plates.

10. After pinning, remove source plates and seal them. Replace soiled blotting sheets.
11. Run the cleanup protocol and then select “home all axes”.
12. Transfer assay plates to a humidified 37°C, 5% CO₂ incubator and incubate either 6 or 24 hr.

SUPPORT PROTOCOL 4 GENERATING DOSE-RESPONSE CURVES

Dose-response curves are used to measure the effect of potentially toxic compounds on HepG2 cells. The biomarker involved can either be excitatory or inhibitory and EC₅₀s and/or IC₅₀s can be determined, respectively. Here, a 384-well source plate containing test compounds at a 10 mM concentration dissolved in DMSO undergoes a 2× serial dilution resulting in ten stock concentrations between 0.02 and 10 mM. The source plate is then pin transferred (see Support Protocol 3) to a 384-well assay plate containing cells in 50 µl of complete medium resulting in a ten-point serial dilution between 0.117 and 60 µM. Dose-response assays are usually conducted in triplicate.

Additional Materials (see also Basic Protocol 1 and Support Protocol 3)

Microplate, 384 Well, Deep Well Small Volume Polypropylene (Greiner Bio-One, cat. no. 784201)

P100 12-Channel Pipet (Eppendorf, cat. no. 312500044)

1. Obtain a fresh 384-well microplate and distribute 20 μ l of each selected compound (at 10 mM in DMSO) to columns 3 and/or 13. This plate will be subsequently referred to as the source plate.

The 384-well plates are organized so that the first two and last two columns are reserved for negative and positive controls, respectively, while the rest of the wells are reserved for test compound serial dilutions.

2. Distribute 10 μ l DMSO into columns 4 to 12 and 14 to 22.
3. Using a multichannel pipet, perform a 2 \times serial dilution of the test compounds in column 3. Transfer and mix 10 μ l of compound from column 3 into column 4, reducing its concentration by half.

Repeat this step until ten concentrations are obtained for each compound with equal volumes of 10 μ l on all wells. Pipet at least six times up and down between each dilution. Perform the same serial dilution on corresponding wells present in columns 13 to 22.

Final compound concentrations on the source plate are 10, 5, 2.5, 1.25, 0.625, 0.313, 0.156, 0.078, 0.039, and 0.02 mM.

4. Centrifuge plate for 15 s at $\sim 100 \times g$.
5. Pin transfer (see Support Protocol 3) 300 nl of each compound concentration from the source plate to a 384-well assay plate containing cells and 50 μ l complete medium.

Final working compound concentrations on the assay plate are 60, 30, 15, 7.5, 3.75, 1.88, 0.938, 0.469, 0.234, and 0.117 μ M.

Each experimental well should contain 300 nl of test compound dissolved in DMSO resulting in ten working compound concentrations and 0.6% DMSO. These numbers were derived from the upper cellular tolerance limit for DMSO, which is 0.6% in culture medium; above this limit, HepG2 cell health will be compromised. Hence, a maximum working compound concentration of 60 μ M is ideal.

6. Run the assay under its specific protocol and use GraphPad Prism to process the dose-response data to determine EC₅₀ and/or IC₅₀ for each biomarker (Figs. 3C, 6B, and 7B).

REAGENTS AND SOLUTIONS

Complete DMEM with antibiotics

DMEM containing high glucose (4.5 g/L), GlutaMAX supplement, pyruvate, and phenol red supplemented with:

10% FBS

1% GlutaMAX
1% penicillin-streptomycin
Store up to 1 month at 4°C.
This is a high glucose medium.

Complete DMEM without antibiotics

DMEM containing high glucose (4.5 g/L), GlutaMAX supplement, pyruvate, and phenol red supplemented with:
10% FBS
1% GlutaMAX
Store up to 1 month at 4°C.
This is a high glucose medium.

Complete Eagle's minimum essential medium (EMEM) with antibiotics

EMEM containing low glucose (1.0 g/L) and phenol red supplemented with:
10% FBS
1% GlutaMAX
1% MEM non-essential amino acid solution (NEAA)
1% penicillin-streptomycin
Store up to 1 month at 4°C. *This is a low glucose medium.*

Complete EMEM without antibiotics

EMEM containing low glucose (1.0 g/L) and phenol red supplemented with:
10% FBS
1% GlutaMAX
1% MEM non-essential amino acid solution (NEAA) Store up to 1 month at 4°C.
This is a low glucose medium.

Duplex high-content analysis (HCA) staining solution

Dissolve all ThiolTracker Violet from vial in 75 µl DMSO to form a 20 mM solution.
The ThiolTracker Violet dye must be made fresh for each experiment.
Then, to 100 ml PBS containing Ca²⁺ and Mg²⁺ add:
100 µl 20 mM ThiolTracker Violet (Thermo Fisher Scientific, cat. no. T10096) 400 µl 250× HCS NuclearMask Deep Red Stain (Thermo Fisher Scientific, cat. no. H10294)

Use within 15 min of preparation.

This solution is used for staining nuclei and GSH on live HepG2s.

Multiplex high-content analysis (HCA) staining solution

Dissolve 50 µg MitoTracker Deep Red FM (Thermo Fisher Scientific, cat. no. M22426) in 92 µl DMSO to form a 1 mM stock.

Dissolve 50 µg MitoTracker Red CMXRos (Thermo Fisher Scientific, cat. no. M7512) in 94 µl DMSO to a form 1 mM stock.

1 mM MitoTracker stocks can be stored at –20°C or below for at least 6 months protected from light.

To 60 ml of warmed complete medium (EMEM or DMEM depending on the assay conditions) with antibiotics add:

6 µl 1 mM MitoTracker Deep Red FM

6 µl 1 mM MitoTracker Red CMXRos

120 µl 2.5 mM CellROX Green Reagent (Thermo Fisher Scientific, cat. no. C10444)

60 µl 10 mg/ml Hoechst 33342 (Thermo Fisher Scientific, cat. no. H3570)

Use within 15 min of preparation.

This solution is used for staining nuclei, ROS, and mitochondria on live HepG2s.

COMMENTARY

Background Information

In the past two decades, in vitro cell-based toxicology studies using multiparametric high-content assays have been extensively used for predicting hepatotoxicity (Tolosa, Gómez-Lechón, & Donato, 2015). Nevertheless, most assays lack a comprehensive list of endpoints to detect drug-induced liver injury (DILI) but have focused only on ATP levels, nuclear count and morphology, and mitochondrial membrane potential (MMP) detection (Anguissola, Garry, Salvati, O'Brien, & Dawson, 2014; Garside et al., 2014; O'Brien et al., 2006; Persson, Løye, Mow, & Hornberg, 2013; Xu, Diaz, & O'Brien, 2004). Furthermore, there has been a lack in standardization of in vitro liver toxicology assay conditions (e.g., concentrations and cutoffs, end points, cell types), which needs to be urgently addressed as the use of in vitro DILI assays is increasing rapidly (Atienzar & Nicolas, 2018). Here, we describe a comprehensive multiparametric suite of high-content assays as a model for DILI and we detail the assay conditions used with the goal of opening a dialogue with the DILI community to achieve assay condition standardization. In addition to the usual endpoints of nuclear and mitochondrial health, our assays include physiologically relevant measures of mitochondrial structure, ROS levels, and GSH levels. We also measure vacuolar density, based on our observations that some compounds induced these changes. This observation highlights a benefit of high-content analysis: Visualizing cells allows for discovery of new phenotypes that might shed light on new mechanisms of action for active compounds.

The quantification of cell health readouts is the first step towards building a comprehensive *in silico* model for drug-induced liver injury. Oftentimes, image processing and segmentation are rate-limiting factors in assay development. We therefore provide a detailed guide to performing image processing and segmentation via the IN Cell Developer Toolbox software. As noted, these protocols can be adapted to other high-content instruments and image-analysis software.

Critical Parameters and Troubleshooting

Because HepG2 cells have a tendency to aggregate with each other, we recommend a cell seeding density of 4.0×10^3 cells per well in a 384-well plate. This low density keeps cells in a monolayer and increases accuracy during image segmentation. Cells should also be passed through a 40- μm cell strainer to remove clumps. Moreover, we recommend using poly-D-lysine-coated 384-well plates and leaving the plated cells at room temperature for at least 30 min prior to incubation at 37°C to facilitate better cell spreading and to minimize cell aggregation. At times, cells remain highly clustered, making image feature extraction problematic. We resolve this issue by performing a two-step nuclear segmentation process as described in Image Segmentation and Quantification (Figs. 5 and 8).

We observed that staining HepG2 live cultures with ThiolTracker Violet can adversely affect cellular attachment. This effect is minimized by seeding HepG2 cells on collagen type I-coated plates instead of poly-D-lysine-coated plates. Additionally, washing and staining steps are performed using PBS (1 \times) containing Ca^{2+} and Mg^{2+} whenever possible to avoid any modulation of cell-adhesion molecules (Riikonen, Vihinen, Potila, Rettig, & Heino, 1995; Shimizu & Mobley, 1993). Finally, both the aspirating and dispensing steps on the EL406 washer dispenser are set to a lower speed to prevent cell monolayer detachment.

In all experiments involving liquid dispensers with tubing, e.g., Matrix WellMate and EL406 washer dispenser, adding at least 20 to 25 ml extra liquid is recommended to allow for dead volume and adequate priming. Particularly for solutions containing ThiolTracker Violet, the absorption of this dye to the tubing causes uneven staining when each tube is not primed with at least 1 ml of dye solution prior to dispensing into a culture plate.

Live cell imaging is ideal for ROS and GSH detection. However, it may be a challenge to maintain the consistency of each resulting live cell image because it takes ~30 min to image a single plate. To mitigate this problem, experiments can be staggered to maintain consistent incubation times for each plate. Two different time points were chosen in all the experiments to investigate the effect of short-term(6hr) and long-term (24 hr) compound exposure on cells leading to acute and chronic cytotoxicity, respectively. In addition, short-term compound exposure can identify compounds responsible for early organelle stress induction. This information will help build potentially more accurate *in silico* models for DILI prediction.

Understanding Results

Nine cell-health readouts are obtained from running Basic Protocols 1 through 3. The details and rationale of each of these readouts can be found in Table 1. We define a compound as “active” or “toxic to hepatocytes” when at least one readout changes by more than three

standard deviations from the negative control mean. The change in signal could be greater or less than the negative control, depending on the readout, exposure time, compound concentration, and the mechanism by which the compound is damaging cells. For example, at a 60 μM concentration and 24 hr exposure, raloxifene causes an increase in both MMP and mitochondrial structural intensity (Fig. 6), while at a 100 μM concentration and the same exposure duration, menadione causes a decrease in both MMP and mitochondrial structural intensity (Fig. 4). Despite the difference in the directionality of both MMP and mitochondrial structural intensity, ROS levels increase in either case. Menadione is known to cause overproduction of ROS and mitochondrial membrane potential disruption that leads to cell death, which is also in agreement with the experimental data (Loor et al., 2010). Raloxifene's mechanism of toxicity is unclear. There are reports of cholestatic hepatitis cases found in patients after taking raloxifene but the probability of its occurrence is deemed rare and idiosyncratic (Selective Estrogen Receptor Modulators, 2012). Nevertheless, the cell-health assays described here are able to capture raloxifene as a potentially hepatotoxic compound.

We developed a vacuolar segmentation protocol to capture observed increases in vacuolization. This protocol generated a few false positives caused by mis-assigning gaps in between cells as vacuoles. Errors might be minimized by defining the area of an individual cell. Alternatively, vacuolar segmentation could be accomplished using phase-contrast images and a previously reported method (Chiang et al., 2018).

Relevant activities in preclinical liver toxicity screens can be as weak as 100 μM . It can be challenging to select a dose of compound that balances detecting this low-affinity activity with solubility. We use concentrations between 50 and 200 μM depending on the compound solubility and cellular tolerance to DMSO; because we find a tolerance of 0.6% DMSO and are working with 10 mM stock concentrations, our highest available dose is 60 μM . On the other hand, with multiple readouts and a high compound concentration, it is possible to overestimate a compound's hepatotoxic potential, risking inaccurate DILI predictions. Further information, such as the in vivo exposure or C_{max} that is achievable for a given compound, can minimize overinterpretation of an in vitro toxicity result.

The high-content, multiparametric assays described herein provide a comprehensive set of readouts for DILI prediction. Moving forward, the data gathered from running a collective set of DILI-positive and DILI-negative compounds using these assays can become inputs to various in silico models. The models can then be validated with the corresponding clinical and/or preclinical DILI data to minimize false DILI prediction.

Time Considerations

The cumulative time taken to run Basic Protocol 1 is ~5 hr and to run Basic Protocols 2 and 3 is ~8 hr; these are spread over the span of three consecutive days. These numbers do not include cell incubation times or automated image segmentation process on Basic Protocols 2 and 3; the latter could take up to 5 hr depending on the image complexity. Using a 384-well plate format, one can run a single screening experiment with up to 336 different compounds, and 24 negative and positive control wells, respectively.

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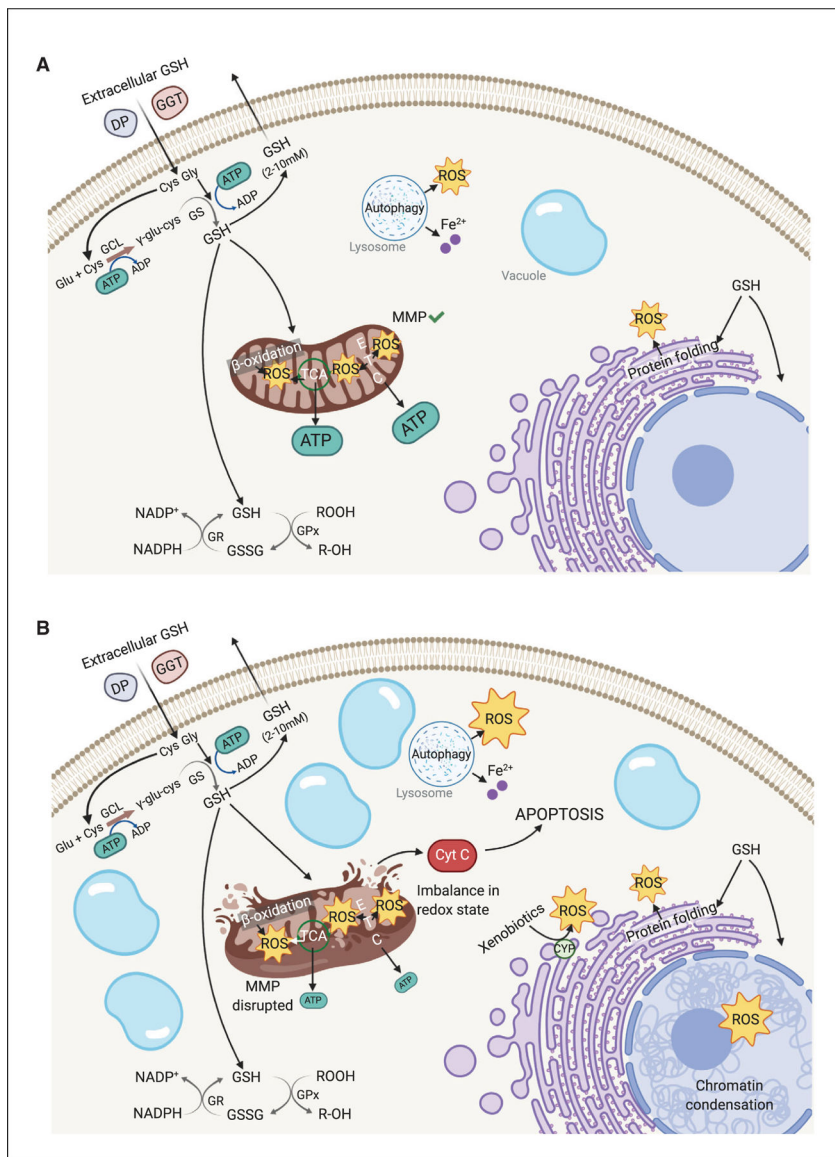


Figure 1. Overview of liver cell health parameters used to detect an imbalanced redox state and other cell damage. **(A)** Schematic depicting a healthy liver cell with a balanced intracellular redox state. **(B)** Schematic depicting a liver cell with an imbalanced intracellular redox state upon exposure to toxic compounds. Overproduction of reactive oxygen species (ROS) leads to an imbalanced intracellular redox state, mitochondrial membrane potential disruption, and apoptosis. Cell health parameters described in this paper are mitochondrial membrane potential (MMP), intracellular levels of ROS and glutathione (GSH), chromatin condensation, and cellular ATP levels. Created with [BioRender.com](https://www.biorender.com). DP, dipeptidase; GGT, gamma-glutamyl transferase; GCL, glutamate-cysteine ligase; GR, glutathione reductase; GPx, glutathione peroxidase; ETC, electron transport chain; CYP, cytochrome P450.

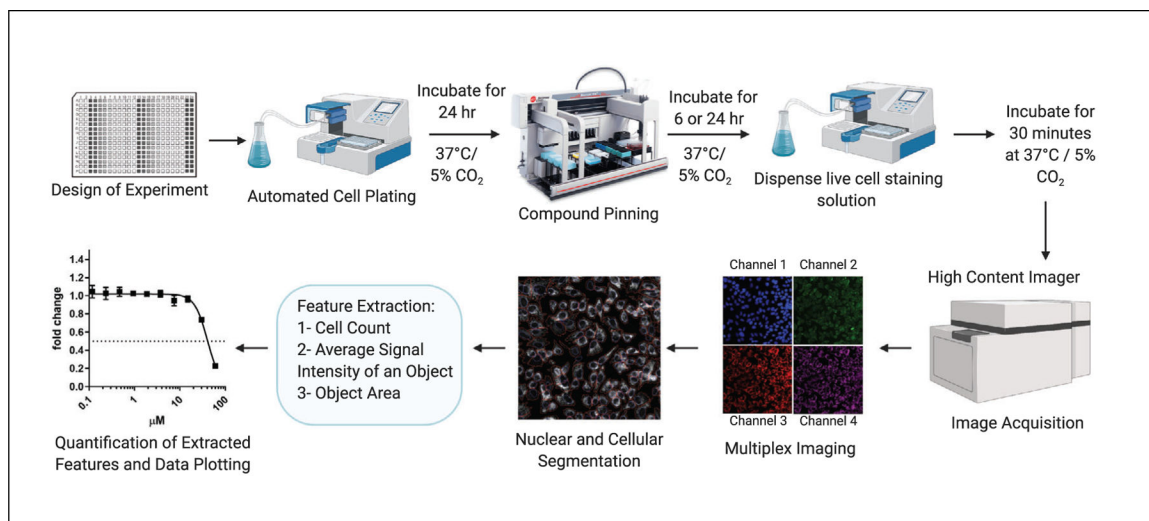


Figure 2.

High-content imaging workflow. Cells are plated using liquid dispensers into 384-well plates and incubated for 24 hr at 37°C with 5% CO₂. Compounds are then added using a pin tool and the plates incubated for either 6 or 24 hr. Cells are subsequently stained and images are acquired using a high-content imager (IN Cell Analyzer 2500 HS; Cytiva). Acquired multiplex images then undergo nuclear or cellular segmentation to define the areas of interest and allow normalization of data per cell. Features are then extracted according to the rationale of each particular readout. Features include, but are not limited to, cell count, signal intensity, and area of a particular object. Numerical data from extracted features are then plotted in graphs. Created with [BioRender.com](https://www.biorender.com).

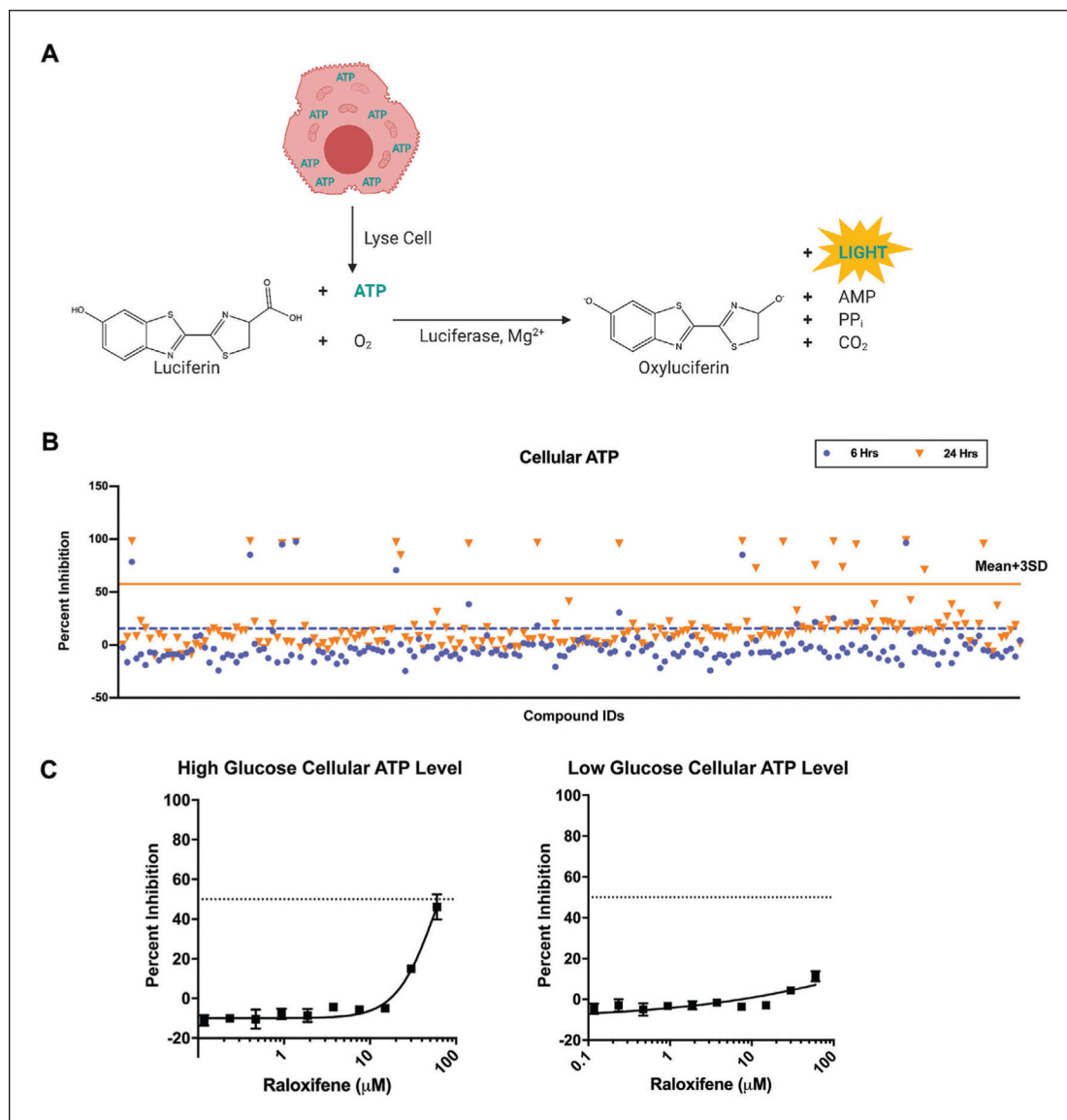


Figure 3. Quantification of ATP from viable hepatocytes. **(A)** A schematic of the reaction involved in the CellTiter-Glo 2.0 luminescent assay. In the presence of ATP liberated via cell lysis, molecular oxygen, and Mg²⁺, luciferase catalyzes the mono-oxygenation of luciferin to produce oxyluciferin and light. Created with [BioRender.com](https://www.biorender.com). **(B)** A scatterplot depicting percent inhibition of cellular ATP levels when HepG2 cells are exposed to 60 μM compounds for 6 and 24 hr in high glucose medium. **(C)** Dose-response curves depicting percent inhibition of cellular ATP levels when HepG2 cells are exposed to varying concentrations of raloxifene for 24 hr in low and high glucose medium. N = 3.

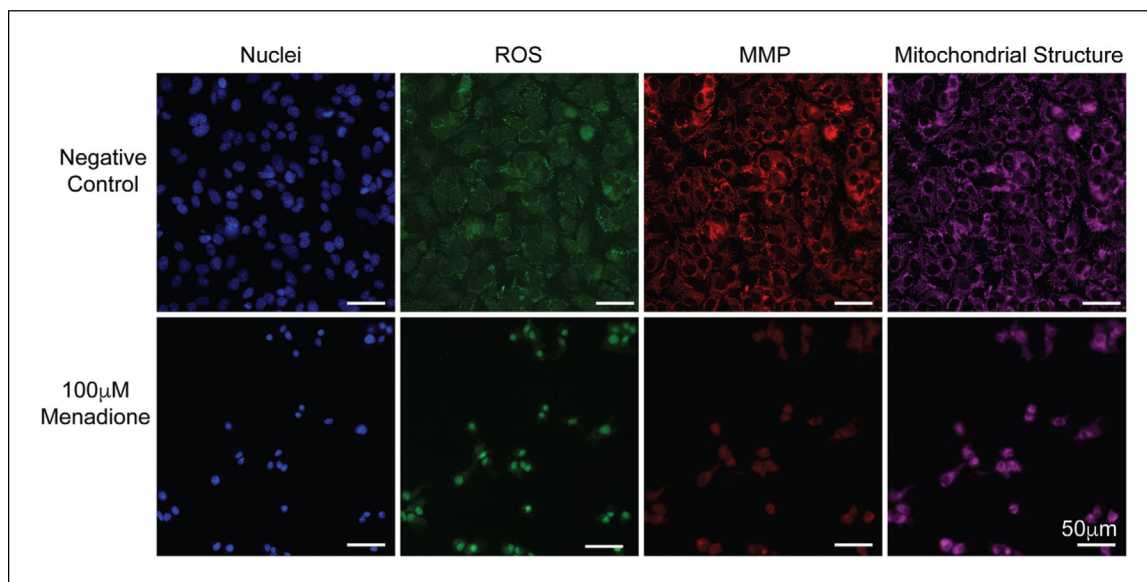


Figure 4. Example image data obtained using the multiplexed high-content analysis (HCA) assay described in Basic Protocol 2. Fluorescent images of negative (0.6% DMSO) and positive controls (100 µM menadione) used to calculate Z value. Scale bar = 50 µm. ROS, reactive oxygen species; MMP, mitochondrial membrane potential.

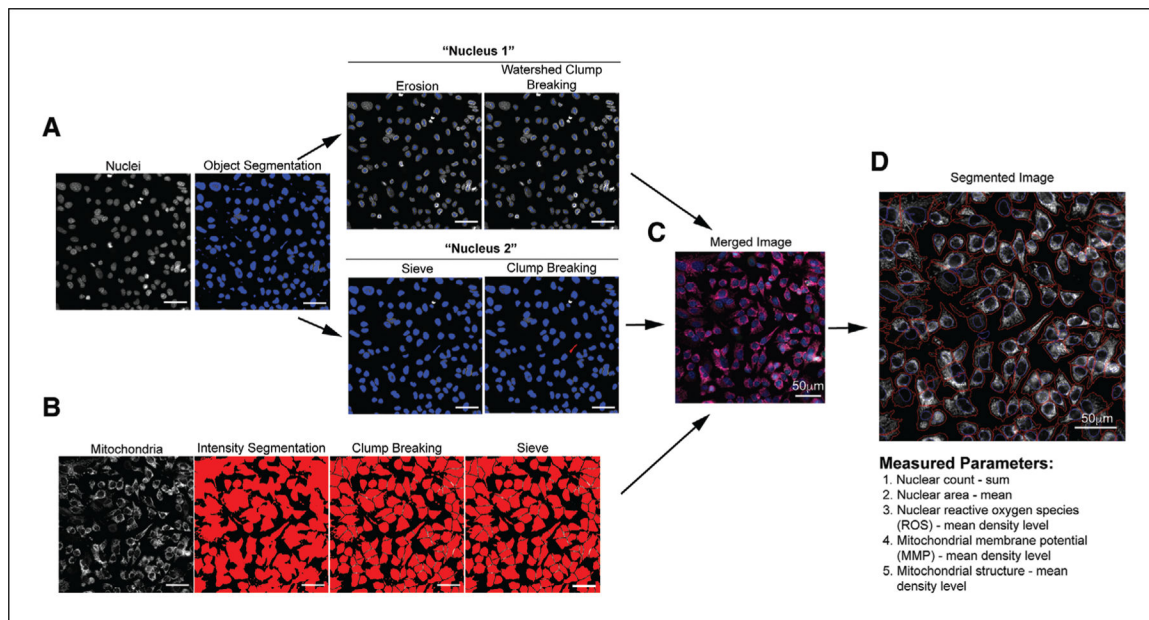
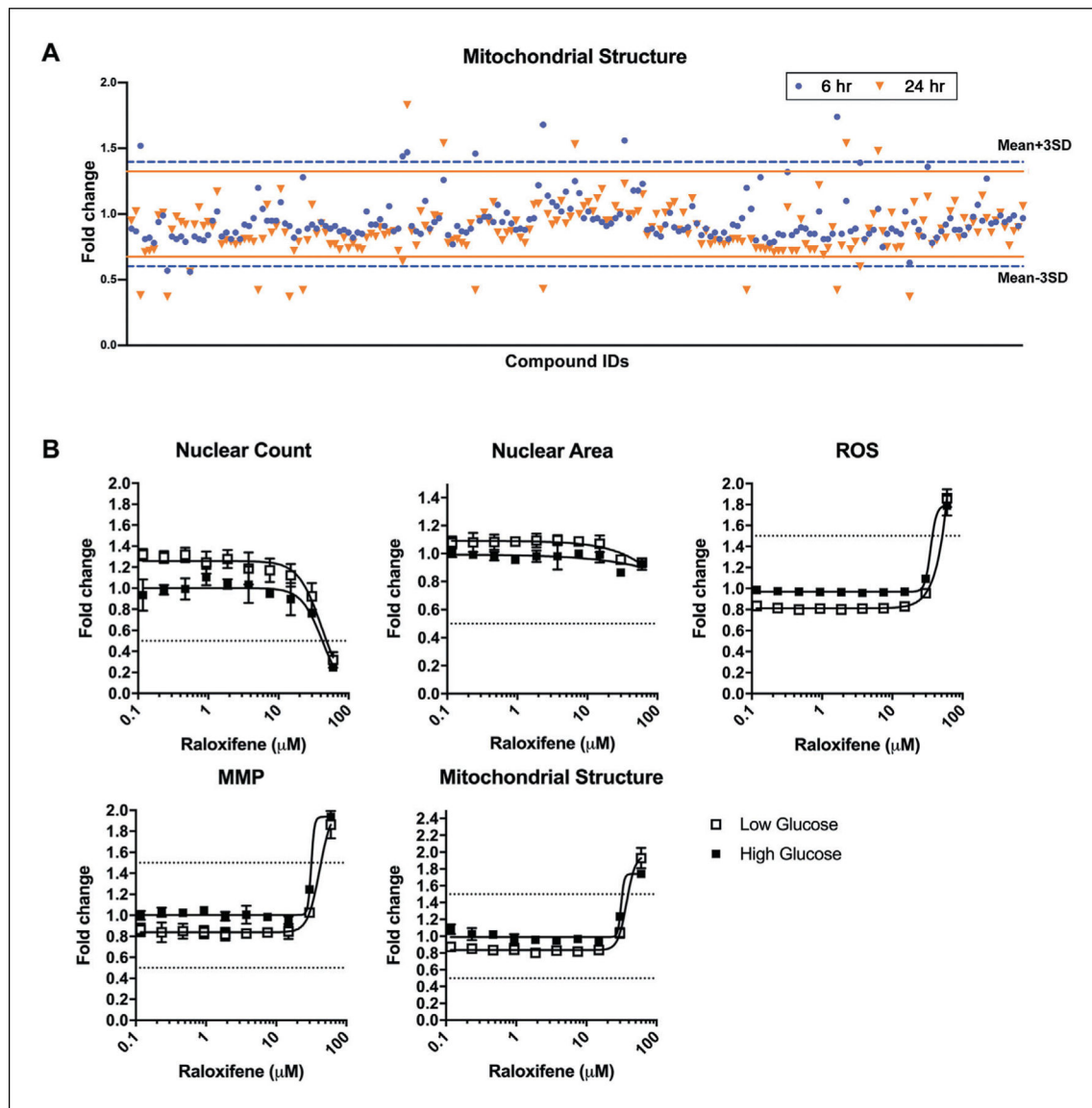


Figure 5. Segmentation of HepG2 cells for multiplexed high-content analysis (HCA) using Basic Protocol 2. **(A)** Images depicting the steps involved in nuclear segmentation. Nuclear segmentation is performed in two steps (“Nucleus 1” and “Nucleus 2”) to ensure an optimal clump-breaking process. **(B)** Images depicting the steps involved in mitochondrial intensity-based segmentation of individual cells. **(C)** Combined nuclear and cellular segmentation results: blue indicated nuclear stain and red indicates mitochondrial stain. **(D)** Cellular segmentation: blue lines indicate nuclear boundaries and red lines indicate cellular boundaries. List of parameters measured based on segmented nuclear area [nuclear count, nuclear area, and nuclear reactive oxygen species (ROS)] and segmented cellular area [mitochondrial membrane potential (MMP) and changes in mitochondrial structure using MitoTracker deep red]. All scale bars = 50 μm .

**Figure 6.**

Example of scatterplot and dose-response graphs obtained using the multiplexed high-content analysis (HCA) assay described in Basic Protocol 2. **(A)** Scatterplot depicting the fold change in mitochondrial structure (measured with MitoTracker Deep Red FM) when HepG2 cells are exposed to 60 μM compounds for 6 and 24 hr in high glucose medium. **(B)** Dose-response curves of nuclear count, nuclear area, nuclear reactive oxygen species (ROS), mitochondrial membrane potential (MMP), and mitochondrial structure when HepG2 cells are exposed to varying concentrations of raloxifene for 24 hr in both low and high glucose media. $N = 2$.

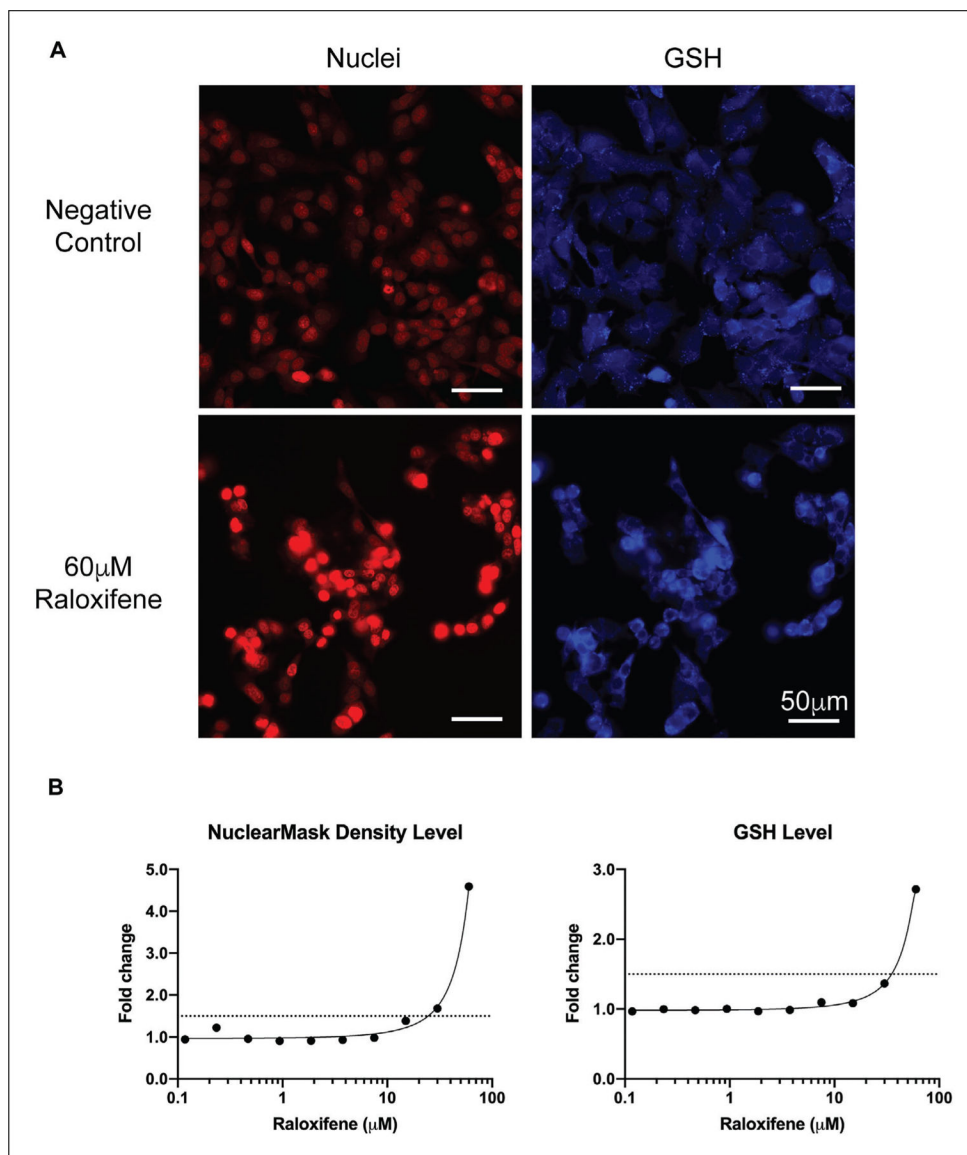


Figure 7. Example data obtained with the duplex high-content analysis (HCA) assay depicted in Basic Protocol 3. (A) Fluorescent images of negative (0.6% DMSO) and positive controls (60 μ M raloxifene). Scale bar = 50 μ m. (B) Dose-response curves of NuclearMask density level and glutathione (GSH) level upon exposure of HepG2 cells to varying concentrations of raloxifene for 24 hr in low glucose medium. N = 1.

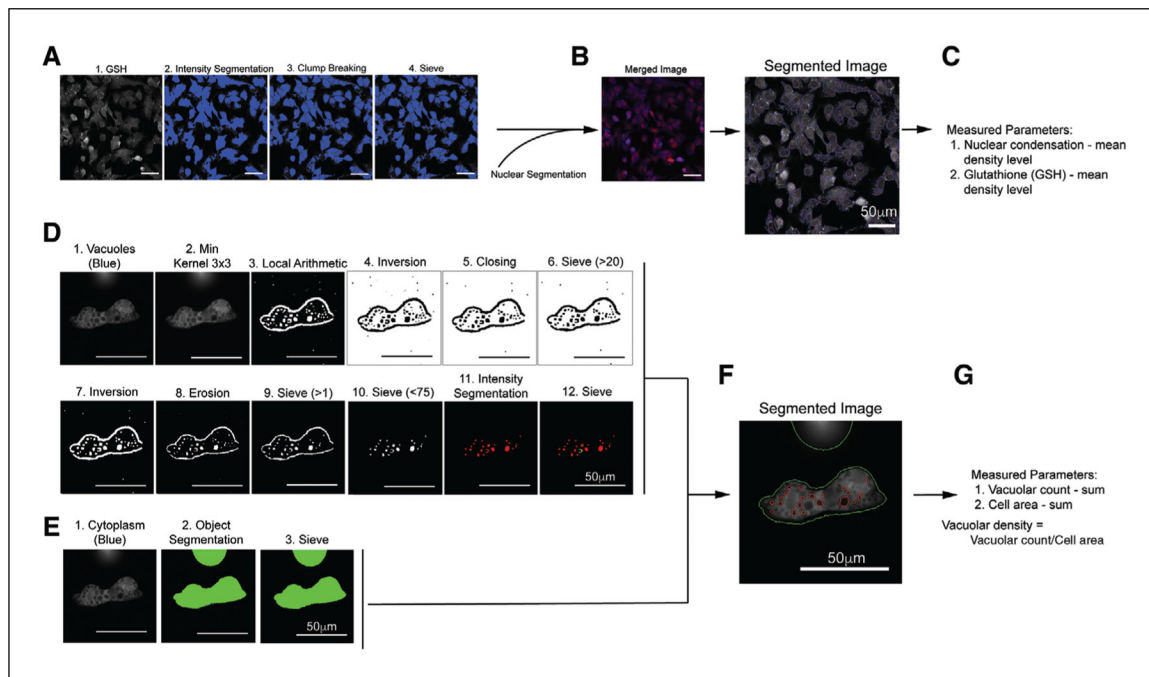


Figure 8.

Segmentation of HepG2 cells stained with duplex high-content analysis (HCA) assay in Basic Protocol 3. **(A)** Images depicting glutathione (GSH) intensity-based cellular segmentation. Nuclear segmentation was performed similar to Figure 5A. **(B)** Combined nuclear and GSH segmentation: pink lines indicate nuclear boundaries and blue lines indicate GSH or cellular boundaries. **(C)** List of parameters measured based on respective segmented areas. **(D)** Images depicting the steps involved in the segmentation of vacuoles using macro preprocessing, intensity segmentation, and postprocessing. Vacuoles were observed in the GSH/blue channel as dark spots within cells. **(E)** Cell area object-based segmentation using the GSH/blue channel. **(F)** Combined vacuolar and cellular area segmentation: red lines indicate vacuolar boundaries and green lines indicate cellular boundaries. **(G)** List of parameters measured based on segmented vacuoles and cell area. These readouts were then used to calculate the vacuolar density (vacuolar count/cell area). All scale bars = 50 μm .

Table 1

Summary of Assay Readouts Covered in the Multiparametric High-Content Assays

Readouts	Dyes	Rationale	Reference
A. Luminescence-based high-throughput screening (HTS)			
ATP Expected activity: decrease in ATP	Luciferin ^a	Cellular ATP levels are an indicator of metabolic activity in cells. ATP levels in cell lysates are directly correlated with cell number. Cellular ATP levels decrease as cell metabolism is reduced, e.g., due to changes in mitochondrial membrane potential or cell death.	Crouch et al., 1993; Current Protocols article: Tolliday, 2010
B. Fluorescence image-based high-content screening (HCS)			
Nuclear count Expected activity: increase or decrease in nuclear count	Hoechst 33342	Nuclear count is a terminal cell health parameter and is critical for detecting acute cell health effects due to toxic compounds.	Gilbert et al., 2011; Gomes, Harman, Centuori, Wolgemuth, and Martinez, 2018
Nuclear size Expected activity: increase or decrease in nuclear size	Hoechst 33342	Changes in nuclear size is a subtle marker of cell health. Unhealthy cells can show either a decrease or an increase in nuclear size depending on a compound's mechanism of action.	O'Brien, 2014; Tham et al., 2019
Reactive oxygen species (ROS) Expected activity: increase in ROS	CellROX Green	ROS, along with glutathione (GSH), are the main determinants for intracellular redox state. Lower levels of ROS are normally produced during metabolic activities like the citric acid cycle and electron shuttling in the electron transport chain (ETC). An increase in ROS intracellular production can activate cell death signaling pathways.	Nouredin and Kaplowitz, 2018; Redza-Dutordoir and Averill-Bates, 2016; Yuan and Kaplowitz, 2013
Mitochondrial membrane potential (MMP) Expected activity: increase or decrease in MMP	MitoTracker Red CMXRos	MMP is directly associated with mitochondrial health. MMP can decrease or increase depending on a toxic compound's mechanism of action. Changes in MMP could potentially lead to cytochrome <i>c</i> release from the mitochondria which triggers an apoptotic cascade.	Cottet-Rousselle, Ronot, Lerverve, and Mayol, 2011
Mitochondrial structure Expected activity: increase or decrease in mitochondrial structure	MitoTracker Deep Red FM	In healthy cells, mitochondria form tubular networks. However, a change in mitochondrial morphology can be detected due to toxic compound exposure.	Bray et al., 2016; Current Protocols article: Fu and Lippincott-Schwartz, 2018
Chromatin condensation/nuclear morphology Expected activity: increase or decrease in stain intensity	HCS NuclearMask Deep Red	Nuclear chromatin can undergo condensation in the early stages of the apoptotic process. Chromatin condensation increases NuclearMask intensity.	Current Protocols article: Mandavilli and Janes, 2018
GSH Expected activity: increase or decrease in GSH	ThiolTracker Violet	GSH is a cellular antioxidant that stabilizes the intracellular redox state. In the presence of excessive ROS, GSH concentration can either increase or decrease depending on the cell state.	Aquilano et al., 2014; Current Protocols article: Mandavilli and Janes, 2018; Tossige-Gomes et al., 2016
Vacuolar density Expected activity: increase in vacuolization	ThiolTracker Violet	Cells form vacuoles as a way to compensate changes in cellular osmotic pressure due to toxic compound exposure. Vacuolization is more prominent when cells are cultured in high glucose medium.	Hunter, Bianchi, Cheng, and Muldrew, 2007; Shubin et al., 2016

^aLuciferin from CellTiter-Glo 2.0 kit (Promega).