Towards *in vitro* Subcellular Toxicology Screening, Implementing Novel Organelle Activity Assays for Environmental Contaminant Testing

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ABSTRACT

Certain xenobiotics are sequestered into subcellular organelles by different metabolically-driven mechanisms including mitochondrial membrane potential-driven concentration, nuclear concentration via DNA affinity and vacuolar-ATPase-driven trapping into lysosomes that subsequently swell by an osmotic mechanism. Concentration within these organelles may be initiated by the innate membrane permeability of the uncharged forms of these compounds or by means of specific transporters (organic cation transporters, choline transporters, etcetera). Such sequestration can contribute to overall cell toxicity, as well as prolong the duration of a compound's action within the body. Cell-based assays, wherein a physiological response is measured as an endpoint, have increasingly become important to high throughput compound screening (HTS) because these assays can often provide higher value data than conventional target-based, biochemical assays. However, most are geared towards simple measurement of cell proliferation, viability and death. A panel of novel fluorescence-based assays was devised to assess the impact of xenobiotics on overall subcellular organelle function, with particular emphasis on the lysosomal, mitochondrial and nuclear compartments. The assay workflow provides a rapid and quantitative high-throughput approach for determining drug- or toxic agent-induced live cell response, offering throughput advantages relative to methods based upon electron microscopy, fluorescence microscopy or flow cytometry. The described organelle-targeted approach offers the prospect of identifying subtle *in vitro* cellular responses that could serve as predictive surrogates for *in vivo* toxicity testing. The highlighted battery of assays should provide an early indication of xenobiotics that represent potential hazards towards human health and the environment.

Xenobiotics are Sequestered into Cells by a Variety of Mechanisms

- Membrane transporter-driven concentration
- Mitochondrial membrane potential-driven concentration
- Nuclear concentration via DNA affinity
- Vacuolar-ATPase-driven trapping into lysosomes

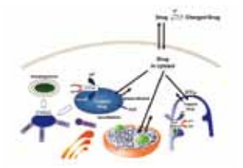


FIGURE 1: Xenobiotics are sequestered into cells by a variety of metabolically-driven, but often receptor-independent means including mitochondrial membrane potential-driven concentration, nuclear concentration via DNA affinity and vacuolar-ATPase-driven trapping into lysosomes. We describe a battery of cell-based assays for assessing the impact of xenobiotics on cellular physiology. (Model modified from Morissette et al., Toxicol Appl Pharmacol. 2008;228(3):364-77).

Proof of Concept Experiment: Selected Cell-Based Assays

Lyso-ID® Red Detection Kit (GFP-Certified™):	Measures lysosomal degradation response system (DRS).
Mito-ID™ Membrane Potential Cytotoxicity Kit:	Measures changes in the mitochondrial membrane potential.
Nuclear-ID™ Green Chromatin Condensation Kit:	Measures nuclear condensation arising from apoptosis. (Autophagy does not cause nuclear condensation)

Selected Test Compounds of Interest to the U.S. EPA:

2,4-Dichloro-phenoxyacetate (2,4-D):	A common systemic herbicide used in the control of broadleaf weeds.
Dibutyl phthalate	A commonly used plasticizer, that is also used as an additive to adhesives or printing inks.
Titanium Oxide (< 100 nm nanopowder):	Pigment for paint, sunscreen and food coloring.
Bisphenol A (BPA):	Building block of several important plastics and plastic additives.
Permethrin:	A common synthetic chemical, widely used as an insecticide, pesticide, and insect repellent
Rotenone:	A broad-spectrum insecticide, piscicide, and pesticide.

Cell-Based Lyso-ID® Red dye/Nuclear-ID™ Green Microplate Assays: Simple Protocol

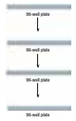


Plate cells. Allow to attach and grow with appropriate culture medium overnight.

Add test compound for desired time period at 37°C. (Generally 30 mins to 24 hours)

Remove the medium and add Lyso-ID® Red dye or Nuclear-ID™ Green dye for 15-30 min.

Wash the cells and analyze with a fluorescence microplate reader.

FIGURE 2: Generalized workflow for analyzing compounds that induce changes in lysosomal number and volume and nuclear-condensation using live cell fluorescence-based microplate assays for high-throughput screening.

Cell-Based Mito-ID™ Membrane Potential Cytotoxicity Assay for microplates: Simple Protocol

Plate cells. Allow to attach and grow with appropriate culture medium overnight.

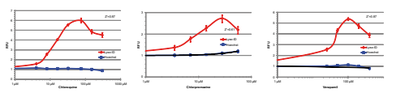
Incubate the cells with Mito-ID™ MP dye for 30 minutes @ RT
(No serum or medium removal required)

Using the BioTek two-syringe pump dispenser, the cells are treated with test compounds. Kinetic readings are collected on the Synergy™ Mx microplate reader for up to 2 hours, at 5 minute intervals (Ex/Em = 490/570 nm).

FIGURE 3: Generalized workflow for analyzing compounds that induce changes in mitochondrial membrane potential using a live cell fluorescence-based microplate assay for high-throughput screening.

Lysosome-mediated Degradation Response System (DRS): Phospholipidosis and Aberrant Autophagosome Accumulation

Changes in the Lysosomal Compartment Arising from Phospholipidosis



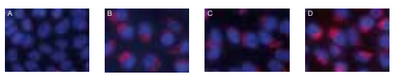


FIGURE 5: Using a BioTek Synergy™ Mx fluorescence microplate reader, the half maximal effective concentration (EC_{50}) of Chloroquine, Chlorpromazine, and Verapamil in U2OS cells was estimated. The high Z-factor (≥ 0.6) obtained using the assay demonstrates excellent signal-to-noise and signal-to-background ratios. The error bars denote the standard deviation of at least six determinations. The red line denotes the Lyso-ID® dye signal, and the blue line highlights the Hoechst nuclear signal. Corresponding fluorescent microscopy images of untreated U2OS cells (A), Chloroquine-treated cells (B) Chlorpromazine-treated cells (C) Verapamil-treated cells (D).

EPA Test Compounds and Lyso-ID® Red Assay

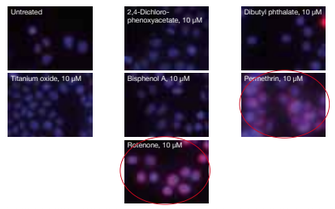


FIGURE 6: Fluorescent microscopy images of cells treated with the given concentrations of the various test agents. Control (HeLa cells), cells pre-treated for 18 hours with 10 µM 2,4-Dichloro-phenoxyacetate, 10 µM Dibutyl phthalate, 10 µM Titanium oxide (< 100 nm nanopowder), 10 µM Bisphenol A, 10 µM Permethrin and 10 µM Rotenone. Cells were subsequently stained with Lyso-ID® Red dye for 15 minutes. Nuclei were counterstained with Hoechst 33342 dye. Permethrin and Rotenone increased general staining with Lyso-ID® Red.

Mito-ID™ Membrane Potential Assays

Subcellular Staining Patterns of Mito-ID™ Membrane Potential Dye

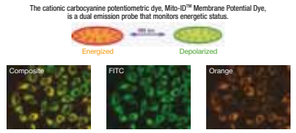


FIGURE 7: The mitochondria of HeLa cells were stained with Mito-ID™ Membrane Potential dye, and visualized by fluorescence microscopy. Orange fluorescent aggregates are localized in the mitochondria, while green fluorescent monomers mainly stain the cytosol.

Comparison of JC-1 and Mito-ID™ Membrane Potential Dye

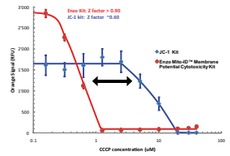


FIGURE 8: Using a BioTek Synergy™ Mx fluorescence microplate reader, Mito-ID™ Membrane Potential dye was shown to decrease as a function of CCCP concentration (decrease in orange signal). Mito-ID™ MP dye is at least 10-fold more sensitive to mitochondrial membrane potential loss than the commonly used dye, JC-1. The high Z-factor (≥ 0.9) obtained using the Mito-ID™ MP dye arises from the no-wash protocol.

Real Time Monitoring of Mitochondrial Membrane Potential Changes with Permethrin

FIGURE 9: Time course study of mitochondrial membrane potential change with BioTek Synergy™ Mx fluorescence microplate reader: HeLa cells were seeded overnight in 25,000 cells per 100 µl per well in a 96-well black wall/clear bottom plate. Next day, 100 µl of Mito-ID™ Membrane Potential dye was added directly into each well and incubated for 30 minutes at room temperature (No serum or media removal). Permethrin (10 µl/well) was added using the BioTek two-syringe pump dispenser to achieve concentrations of 4 µM , 10 µM and 40 µM, respectively. For each sample, 8 replicates were used. Mito-ID™ MP dye was shown to be responsive to permethrin, as demonstrated by a decrease in Mito-ID™ MP dye orange signal.

Real Time Monitoring of Mitochondrial Membrane Potential Changes with Rotenone

FIGURE 10: Time course study of mitochondrial membrane potential changes using a BioTek Synergy™ Mx fluorescence microplate reader: HeLa cells were seeded overnight in 25,000 cells per 100 µl per well in a 96-well black wall/clear bottom plate. Next day, 100 µl of Mito-ID™ Membrane Potential dye was added directly into each well and incubated for 30 minutes at room temperature (No serum or media removal). Rotenone (10 µl/well) was added using a BioTek two-syringe pump dispenser to achieve concentrations of 1 µM, 5 µM and 9µM, respectively. For each sample, 8 replicates were used. Mito-ID™ MP dye was shown to was shown to be responsive to rotenone, as demonstrated by a decrease in Mito-ID™ MP dye orange signal.

Nuclear-ID™ Green Chromatin Condensation Assay

Stages of Nuclear Condensation During Cell-Free Apoptosis

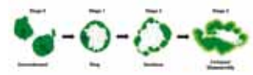


FIGURE 11: During the apoptotic process, chromatin undergoes a phase change from a heterogeneous, genetically active network to an inert highly condensed form that is subsequently fragmented and packaged into apoptotic bodies. Recently, three stages of apoptotic chromatin condensation have been defined based upon morphological and biochemical criteria using a cell-free system: stage 1 ring condensation; stage 2 necklace condensation; and stage 3 nuclear collapse/disassembly. Cells appear to possess an apoptosis-specific system for the induction of chromatin condensation, with phase 2 of the process requiring DNase(s) and phase 3 requiring hydrolysable ATP. (Model based upon Toné et al Exp Cell Res. 2007;313(16):3635–44.) Nuclear-ID™ Green dye signal progressively increases as cells undergo nuclear condensation.

Chromatin Condensation Assay

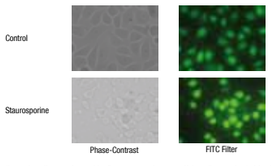


FIGURE 12: Chromatin condensation, as observed by fluorescence microscopy. HeLa cells were treated for 4 hours with DMSO (Control) or 2 µM Staurosporine and stained with 5 µM Nuclear-ID™ Green dye.

Monitoring Chromatin Condensation by Flow Cytometry

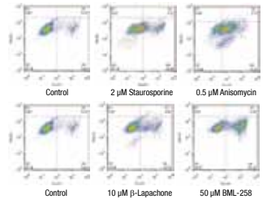


FIGURE 13: Jurkat cells (1x 10^6 cells/ml) were treated with the indicated drugs for 4 hours and analyzed by flow cytometry.

EPA Compounds and Nuclear-ID™ Green Chromatin Condensation Assay

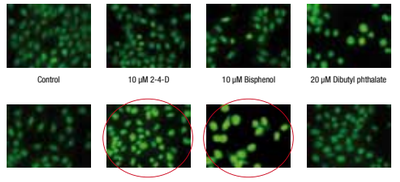


FIGURE 14: Chromatin condensation, as observed by fluorescence microscopy. Control HeLa cells, cells pre-treated for 4 hours with 10 µM Dibutyl phthalate, 20 µM Titanium oxide 10 µM Bisphenol A, 10 µM Permethrin or 10 µM Rotenone. Cells were subsequently stained with with 5 µM Nuclear-ID™ Green dye for 15 minutes. Permethrin and Rotenone induced nuclear condensation.

EPA Compounds and Nuclear-ID™ Green Chromatin Condensation Assay (24 hour exposure)

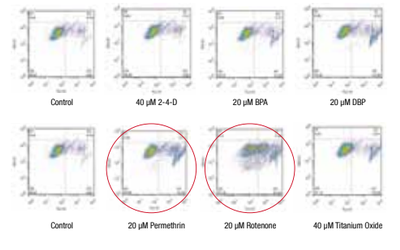


FIGURE 15: Jurkat cells (1x 10^6 cells/ml) were treated with the indicated EPA compound for 4 hours and analyzed by flow cytometry. Permethrin and Rotenone induced nuclear condensation.

CONCLUSIONS

- The effect of drugs or other xenobiotics (e.g. toxins, environmental contaminants) upon living organisms depends very much upon their concentration at the site(s) of their action.
- We present a panel of fluorescence-based live cell assays designed to assess the impact of xenobiotics on overall cell function, with particular emphasis on the lysosomal, mitochondrial and nuclear compartments.
- The assays have been optimized for analysis by microplate-based cytometry, providing a rapid and quantitative high-throughput approach for determining drug- or toxic agent-induced live cell response.
- The assays provide significant throughput advantages relative to methods based upon electron microscopy, fluorescence microscopy or flow cytometry.
- The cited assays could aid in selecting the best candidate compounds for further drug development efforts, as well as provide preliminary benchmarking of dosing limits in preclinical toxicity studies.