The cellular response to cytotoxic exposure is controlled by a series of complex biochemical pathways such as necrosis or apoptosis which results in cell death. In apoptosis, typical morphological changes include pseudopodia retraction, reduction of cellular volume (pyknosis), nuclear fragmentation (karyorrhexis) and eventually loss of plasma membrane integrity. Morphological changes that characterize necrosis include cytoplasmic swelling and early rupture of the plasma membrane. Compounds that have cytotoxic effects often compromise cell membrane integrity regardless of the pathway, a phenomenon which can occur early in the exposure (< 2 hours). Measurement of membrane integrity in vitro therefore serves as a valuable tool for determining cellular viability and cytotoxicity.

Assays designed to measure cytotoxicity in vitro have long been used to predict tissue-specific toxicity or to identify and classify leads for anti-cancer therapies. Multiplexed, high-throughput screening (HTS) in vitro cytotoxicity assays measuring a variety of different readouts are being employed to assess the cytotoxicity of compounds in early drug development. Commonly used cytotoxicity assays evaluate a range of endpoint parameters, such as the release of lactate dehydrogenase (LDH) and glutathione (GSH) following membrane rupture, generation of reactive oxygen species (ROS), cell proliferation, and disruption of mitochondrial transmembrane potential. Although these multiplexed assays are able to simultaneously measure multiple indicators of in vitro cytotoxicity, they typically assess a single time point and are unable to assess biological activity over time.

The introduction of the highly sensitive cyanine nucleic acid dyes, IncuCyte™ Cytotox Reagents have been validated long term real time assessment of in vitro cytotoxicity testing. These exhibit an increase in fluorescence of 100-1000-fold upon binding to genomic deoxyribonucleic acid (DNA) therefore allowing cell membrane integrity to be measured kinetically without the need for washing or complicated protocols. The use of these reagents gives the user the ability to monitor morphological changes in parallel with quantification, the combination of which is a powerful and unique tool for detecting pharmacological or genetic manipulations that alter cell viability.

We have developed a new image-based methodology that combines IncuCyte™ Cytotox Reagents and image-based fluorescent measurements, enabling simple mix and read protocols suitable for screening.

1. Real-time detection of cytotoxicity in living cultures
2. Simple mix-and-read 96/384-well protocols - no washing, no fixing, no lifting
3. Validate cytotoxicity measurements with images and movies
4. Automated analysis and quantification
5. Multiplex with other cell health measurements

IncuCyte™ Cytotoxicity Assay General Protocol

This protocol provides an overview of the IncuCyte™ Cytotoxicity Assay methodology which uses the mix-and-read IncuCyte™ Cytotox Green Reagent or IncuCyte™ Cytotox Red Reagent to detect cell death in real time. The protocol is compatible with the IncuCyte® ZOOM instrument and your choice of cells and treatments. Furthermore, this protocol can be used with cells labelled using the IncuCyte™ NucLight™ nuclear labeling reagents to provide multiplexed measurements of proliferation alongside apoptosis in the same well.
Dead cell quantification using the Cytotoxicity Application

The cellular response to cytotoxic exposure is controlled by a IncuCyte™ Cytotoxicity Assay concept. Cells are combined with IncuCyte™ Cytotox Reagent in the presence or absence of modulating treatments while being imaged within your incubator using the IncuCyte™ live-cell imaging system. As cell membrane permeability increased, the IncuCyte™ Cytotox Reagent enters the nucleus and binds to DNA, thus emitting a fluorescent signal and enabling cytotoxicity to be quantified directly and in real-time.

IncuCyte™ Cytotoxicity Assay General Protocol

Day 0

1) Seed cells (100 µL per well) at an appropriate density into a 96-well plate such that by day 1 the cell confluence is approximately 20 - 50%. The seeding density will need to be optimized for each cell type used; however we have found that 1 x10³ to 5 x10³ cells per well are reasonable starting points.

   • After seeding, allow the cells to settle at ambient temperature for 30 minutes before placing in to the IncuCyte ZOOM® system. This will ensure cells are evenly distributed across the surface of each well before imaging.

   • Remove bubbles at the liquid surface by gently squeezing a wash bottle (containing 100% ethanol with the inner straw removed) to blow vapor over the surface of each well.

   • Monitor cell growth using the IncuCyte ZOOM® system to capture phase contrast images every 2 hours and analyze using the integrated confluence algorithm.

Day 1

2) Once the cells have reached an appropriate confluence, remove the cell plate from the incubator and add desired treatments prepared in media containing the IncuCyte™ Cytotox Reagent. We recommend adding 50 µL/well, prepared at 3x final assay concentration.

   • Add 45 uL of full media, or PBS, to a single vial of IncuCyte™ Cytotox Reagent to provide a stock concentration of 100 µM

   • Transfer the contents of the vial (50 µL) to 6.6 mL full media to yield a Cytotox Reagent working solution (750 nM)

   • Prepare treatments at 3x final assay concentration in the Cytotox Reagent working solution

   • Add 50 µL of Cytotox Reagent solution containing 3x treatment to each well of the 96-well plate for a final 1:3 dilution to give a final assay volume of 150 µL (final assay concentration of Cytotox Reagent 250 nM)

   • Remove bubbles at the liquid surface by gently squeezing a wash bottle (containing 100% ethanol with the inner straw removed) to blow vapor over the surface of each well.

3) Image the plate in the IncuCyte ZOOM® instrument with a 20x or 10x objective using the Standard Scan Type. We recommend 2 images per well, and scanning of phase and fluorescence every 2 hours until the assay is complete (2 to > 120 hours).