

High Content Imaging and Analysis of Mitotoxicity and Cytotoxicity in Fixed Cells

Robert J. Aggeler, Bhaskar Mandavilli, Sangeeta Rojanala, and Michael S. Janes
 Invitrogen Corporation - Molecular Probes® Labeling and Detection Technologies
 29851 Willow Creek Road • Eugene, OR 97402 • USA

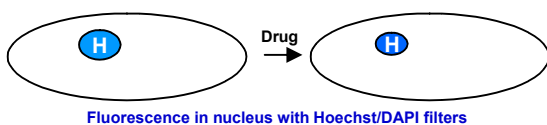
Abstract

The mitochondrial membrane potential is a central feature of healthy mitochondria. It is essential in Ca^{2+} uptake and storage, generation and detoxification of reactive oxygen species (ROS) and, most importantly, the synthesis of ATP by oxidative phosphorylation (1). The latter involves the oxidation of nicotinamide adenine dinucleotide (NADH) and controlled reduction of molecular oxygen to water generating a proton gradient which is then used by ATP synthase. Therefore, depolarization is a good indicator of mitochondrial dysfunction, which is increasingly implicated in drug toxicity (2-6).

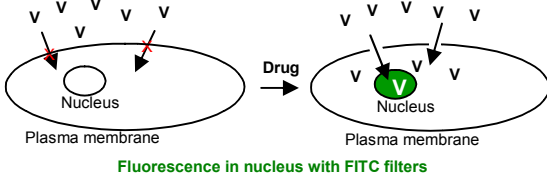
We developed a mitochondrial health assay to enable the simultaneous quantitation of two cell health parameters by high content analysis in the same cell: mitotoxicity and cytotoxicity. This new assay is based on two probes from Invitrogen which are used to label cells with signal retention after formaldehyde fixation and detergent permeabilization. While the mitochondrial stain accumulates in live cells proportional to the mitochondrial membrane potential, the cell viability stain distinguishes between live and dead cells by labeling only those with compromised plasma membrane integrity. Valinomycin, CCCP and troglitazone, known mitotoxic compounds, were used to treat HeLa or A549 cells as examples to validate the assay and demonstrate its robustness. The data demonstrated that this multi-parametric approach revealed cells which underwent only pre-lethal perturbation of mitochondrial function while lethal damage was also induced in other cells within the same wells, underscoring the value of high content imaging-based assays in cytotoxicity. The mitochondrial health assay presented here is a robust tool for high content imaging and analysis that allows for the simultaneous assessment of serious cell injury leading to cytotoxicity as well as less dramatic (pre-lethal) events related to mitotoxicity.

Figure 1. Mitochondrial Health Assay Concept

Segmentation - Nuclear Morphology - Hoechst 33342



Plasma Membrane Integrity - Image-iT® DEAD Green™ viability stain (V)



Mitochondrial Membrane Potential - MitoHealth stain



Figure 1. Multi-parametric mitochondrial health assay. Hoechst 33342 was used as segmentation tool. Plasma membrane integrity was assessed with Image-iT® DEAD Green™ viability stain and the mitochondrial membrane potential with MitoHealth stain.

Figure 2. Oxidative Phosphorylation in Mitochondria

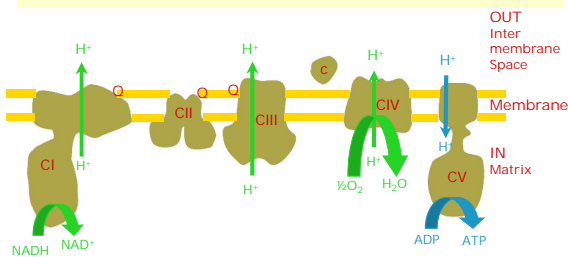


Figure 2. Mitochondrial membrane potential - generation and partial dissipation of proton gradient by respiratory chain complexes and ATP synthase, respectively.

Figure 3. Mitochondrial Health Assay Protocol

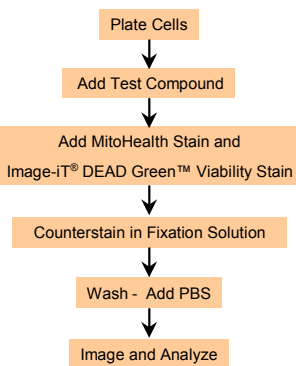


Figure 4. Imaging of Mitotoxicity and Cytotoxicity - Effect of Valinomycin on HeLa Cells

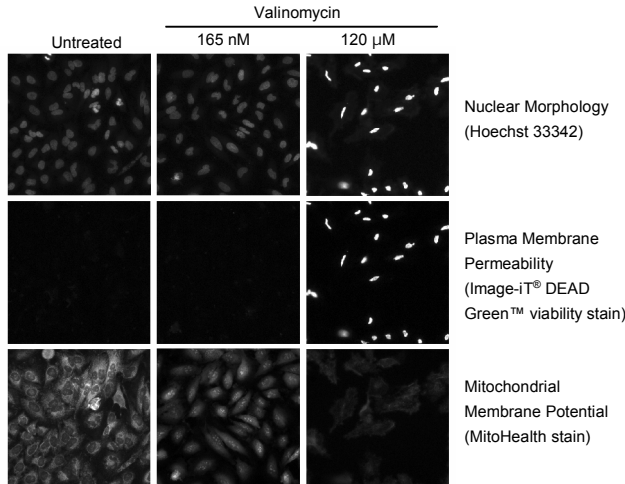


Figure 4. HeLa cells treated with 165 nM and 120 μM valinomycin (a K^+ ionophore) or with an equal volume of DMSO (untreated) and incubated for 24 hours at $37^\circ\text{C}/5\% \text{CO}_2$ and assayed using the HCS Mitochondrial Health Kit. Images of fixed cells were obtained at 20x. Mitochondrial membrane potential was impaired at low nanomolar concentrations of valinomycin while the plasma membrane integrity was compromised in the micromolar range.

Figure 5. Mitochondrial Health Assay to Determine EC_{50} Values

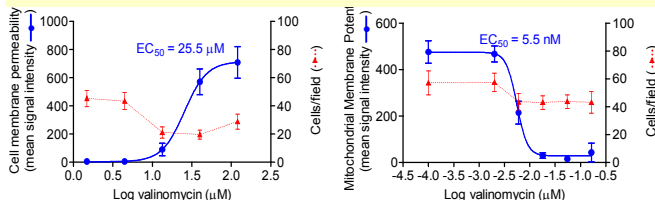


Figure 5. HeLa cells treated with 2 nM to 120 μM valinomycin, incubated for 24 hours at $37^\circ\text{C}/5\% \text{CO}_2$ and assayed using the HCS Mitochondrial Health Kit.

Figure 6. Effect of CCCP on HeLa Cells Determined with the Mitochondrial Health Assay

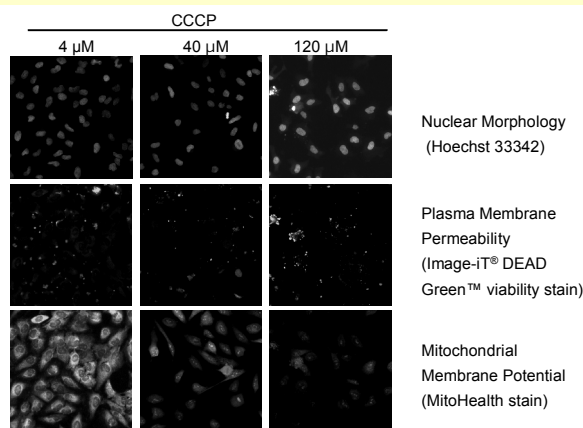


Figure 6. HeLa cells treated with 2 nM to 120 μM of the protonophore carbonyl cyanide 3-chlorophenylhydrazone (CCCP) or with an equal volume of DMSO (untreated), incubated for 24 hours at $37^\circ\text{C}/5\% \text{CO}_2$ and assayed using the HCS Mitochondrial Health Kit. Only the mitochondrial membrane potential was affected by up to 120 μM CCCP ($\text{EC}_{50} = 20 \mu\text{M}$). Plasma membrane integrity was not affected.

Figure 7. Effect of Troglitazone on HeLa Cells Determined with the Mitochondrial Health Assay

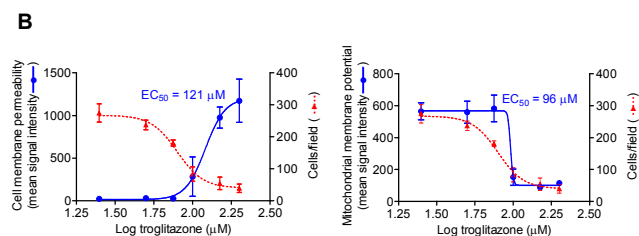
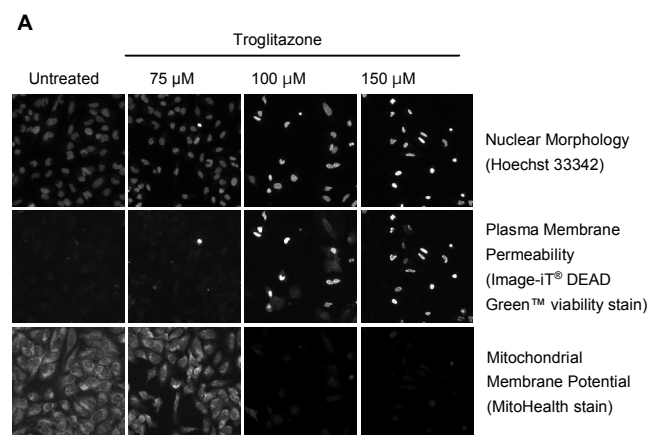


Figure 7. A) HeLa cells treated with 75 μM, 100 μM and 150 μM troglitazone or with an equal volume of DMSO (untreated) showing loss of both plasma membrane integrity and mitochondrial membrane potential at higher concentrations of troglitazone. B) HeLa cells treated with 25 μM to 200 μM troglitazone, incubated for 24 hours at $37^\circ\text{C}/5\% \text{CO}_2$ and assayed using the HCS Mitochondrial Health Kit. EC_{50} values were calculated from the dose response curves with respect to plasma membrane integrity and mitochondrial membrane potential.

Table 1. Robustness and Consistency of the Mitochondrial Health Assay

Measured Parameter	Coefficient of Variance	Z factor	Fold change
Cell Membrane Permeability (Image-iT® DEAD Green™ viability stain)	16.0% ± 2.1%	0.51 ± 0.06	190 ± 41
Mitochondrial Membrane Potential (MitoHealth stain)	14.6% ± 2.8%	0.43 ± 0.05	3.2 ± 0.3

Table 1. HeLa cells treated with 120 μM valinomycin or with an equal volume of DMSO for 24 hours at $37^\circ\text{C}/5\% \text{CO}_2$. Cells were labeled and fixed according to the protocol in Figure 3. The data above represent the consistency, screenability, and robustness achieved in measuring mitotoxicity and cytotoxicity between 3 plates using the HCS Mitochondrial Health Kit.

Conclusions

- The mitochondrial health assay (HCS Mitochondrial Health Kit, H10295) allows for the simultaneous detection of mitotoxic and cytotoxic effects of drugs on cells by high content imaging and analysis.
- The mitochondrial health assay is robust and consistent as indicated by coefficients of variance (< 20%), Z' factors (> 0.4), and by valinomycin-induced changes in MitoHealth stain and Image-iT® DEAD Green™ viability stain (Invitrogen I10291) signals of >3-fold and >100-fold, respectively.
- Because the fluorescent probes in the HCS Mitochondrial Health Kit survive formaldehyde fixation and detergent permeabilization, the assay is amenable with antibody labeling for multiplex detection of on-target proteins with mitotoxicity and cytotoxicity.

References

- Nicholls, D. G. (2004) Aging Cell 3, 35-40
- Tirmenstein, M. A. et al (2002) Toxicol. Sci. 69, 131-138 (2002)
- Amacher, D. E. (2005) Curr. Med. Chem. 12, 1829-1839
- O'Brien, P. J., et al (2006) Arch. Toxicol. 80, 580-604
- Dykens, J. A and Will, Y. (2007) Drug Discovery Today 12, 777-785
- Dykens, J. A. et al (2008) Toxicol. Sci. 103, 335-345