Application Note

Cancer Biology

Agilent

Trusted Answers

Cell Viability Assessment by LUCS Assay

Using the Agilent BioTek Cytation 5 cell imaging multimode reader

Authors

Paul Held, PhD
Agilent Technologies, Inc.
Winooski, VT, USA

Damien Brechet
Agilent Technologies SAS
Les Ulis, France

Christophe Furger, Camille Gironde, Mylene Rigal, and Wenli Debord
Anti Oxidant Power (AOP)
Toulouse, France

Abstract

The Light-Up Cell System (LUCS) is a novel viability assay, which is based on the activation of an intracellular photosensitizer, in a simple protocol that only requires one light photoinduction and two fluorescence readings. As the cells remain alive during the entire LUCS process, the technology is also amenable to multiplexing. However, the photoinduction requires high energy, and the light flash is usually provided by an external light-source device. In this application note, chloroquine, an antimalarial drug known to be cytotoxic, was used to demonstrate that LUCS assay can be implemented in a "plug-and-play" mode using the Agilent BioTek Cytation 5 cell imaging multimode reader. A dose-response study carried out on human liver HepG2 cells led to a chloroquine 50% efficacy concentration (EC_{50}) of 79.43 µM/mL (R^2 = 0.97), in line with previously published data.

Introduction

The LUCS cytotoxicity, live cell assay resulted from the discovery of a photosensitizer, thiazole orange (TO), which presents an interesting property for cell biology: its fluorescence quantum yield (Φ) remains very low (2×10^{-4}) in the culture medium, due to free rotation of its two aromatic rings around the methine bridge that links them. Under these conditions, energy relaxation occurs in a nonradiative mode via internal conversion through an ultrafast intramolecular twisting (100 fs) at the excited state. This means that there is virtually no residual TO fluorescence before the photosensitizer has reached its intracellular target. TO is also known to interact with nucleic acids with an increase of its fluorescence quantum yield 500-fold; a property attributed to a restriction in its torsion capacity.[1] More interestingly, a recent electron paramagnetic resonance study conducted in HepG2 cells showed that excited TO also acts as a classical photosensitizer producing both $^1O_2$ (type II reaction) and OH• (type I reaction).[1] A model of the LUCS process is shown in Figure 1.

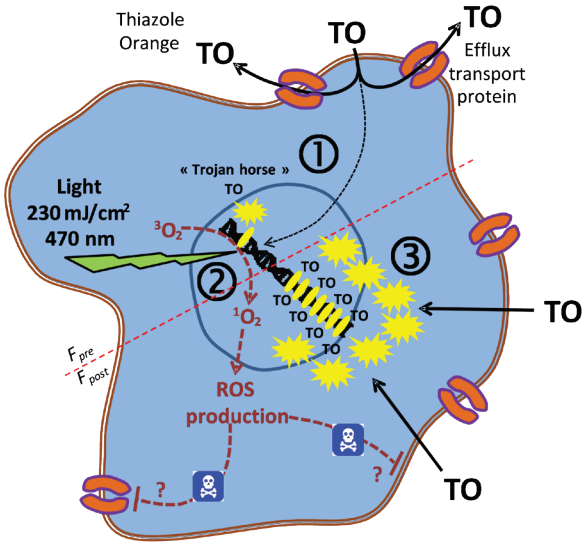

Figure 1. Model of LUCS process in homeostatic cells.[2] (1) TO is mainly removed from the cell by efflux transport, limiting its access to nucleic acid targets; low fluorescence (F_{pre}) is observed; (2) light is applied, inducing $^1O_2$ and °OH production; (3) Reactive oxygen species (ROS) deleterious effects alter efflux and/or other cell functions; (4) massive entry of TO triggers increase in fluorescence emission (F_{post}).

Lastly, TO presents another unique property in live cells: its fluorescence level increases during photoinduction in a process called LUCS. The intimate mechanisms underlying LUCS have been partially deciphered.[2] While TO passively enters the cells, it is mainly removed by efflux transport proteins (presumably of the MATE family), limiting its access to nucleic acids and resulting in a low fluorescence level. When light is applied, reactive oxygen species (ROS), induced by TO photoactivation, alter efflux and/or other cellular functions, perturbing cell homeostasis and triggering a massive entry of the biosensor. This progressively saturates nucleic acid binding sites, resulting in a relevant increase in fluorescence. According to the model shown in Figure 1, this increase in fluorescence is limited to viable cells in homeostasis, leading to a high "postlight" versus "prelight" fluorescence intensity ratio ($F_{post}/F_{pre} >> 1$). Inversely, in altered cytotoxic cells, efflux and other cellular functions do not work properly, and TO enters massively before the photoinduction process, leading to a $F_{post}/F_{pre} \approx 1$.

The Cytation 5 cell imaging multimode reader combines automated microscopy and conventional microplate detection in a configurable, upgradable platform. The microscopy module offers up to 60x magnification in fluorescence, brightfield, high-contrast brightfield, color brightfield, and phase contrast to address many applications and workflows. The multimode detection modules include filter- and monochromator-based fluorescence detection, luminescence, and UV-Vis absorbance detection.

Experimental

Materials

HepG2 cell line (part number HB8065) was purchased from the American Type Culture Collection (ATCC) (LGC Standards, Molsheim, France). DMEM high glucose (part number 11965092), penicillin-streptomycin 10,000 U/mL solution (part number 15140122), GlutaMAX Supplement (part number 35050061), and sodium pyruvate (part number 11360070) were obtained from Gibco/Life Technologies (Carlsbad, CA). HyClone fetal bovine serum (FBS) and HyClone 0.05% trypsin EDTA were from Thermo Fisher Scientific SAS (Illkirch-Graffenstaden, France). Chloroquine was obtained from Sigma-Aldrich (Fallavier, France). The LUCS kits (part number K-2001) were obtained from Anti Oxidant Power (Toulouse, France).

Cell culture

HepG2 cells were cultured at 37 °C/5% CO_2 in GlutaMAX DMEM medium, complemented with 10% FBS and 1x penicillin-streptomycin solution. Cells were grown up to 70 to 80% confluence, then transferred in clear-bottom 96-well microplates for 24 hours, at a density of 75,000 cells/well in 75 µL.

Experimental protocol

Nine different chloroquine concentrations (1.95 to 500 µM) were obtained by serial two-fold dilutions. Experiments were carried out in 96-well microplates. All cell treatments were performed in serum-free medium to avoid potential interaction with serum components. Each experimental condition was assayed in triplicates, including the solvent control without sample. Cells were incubated for 24 hours at 37 °C in 5% CO_2 with each experimental condition. Solution A (Anti Oxidant Power LUCS kit, part number K-2001) was added to the cells for 30 minutes at 37 °C, in 5% CO_2. After incubation, the fluorescence was measured using 505 nm excitation and 535 nm emission wavelengths before and after each well was illuminated with the Cytation 5 imaging lamp: intensity 10 with GFP filter set for 1 second, repeated 3 times.

Instrumentation

Data was collected on an Agilent BioTek Cytation 5 cell imaging multimode reader configured with a GFP imaging filter cube and a 4x objective, controlled by the Agilent BioTek Gen5 microplate reader and imager software.

Analysis

Raw data were analyzed by Prism 8 software (GraphPad, San Diego, CA, USA) to generate dose-response curves. F_{post}/F_{pre} fluorescence ratios were calculated for each experimental condition. Ratio values were then used to evaluate EC_{50} values from a mathematical nonlinear regression model (sigmoid fit) given by Prism 8, following Equation 1, where Hill slope is equal to the slope coefficient of the tangent at the inflection point. EC_{50} and determination coefficient R^2 values were deduced from this regression model.

Equation 1.
$$Y = \frac{Bottom + (Top - Bottom)}{1 + 10^{((LogEC50 - X) \cdot Hill\ slope))}}$$

Results and discussion

These data indicate that the Cytation 5 cell imaging multimode reader can be used to automate both the light exposure and fluorescence detection necessary for the LUCS assay technology. Initial experiments optimized the amount of photoinduction light necessary to produce an adequate assay window. As demonstrated in Figure 2, a minimum of three separate one-second exposures with maximal LED intensity is required to photoactivate TO in HepG2 cells.

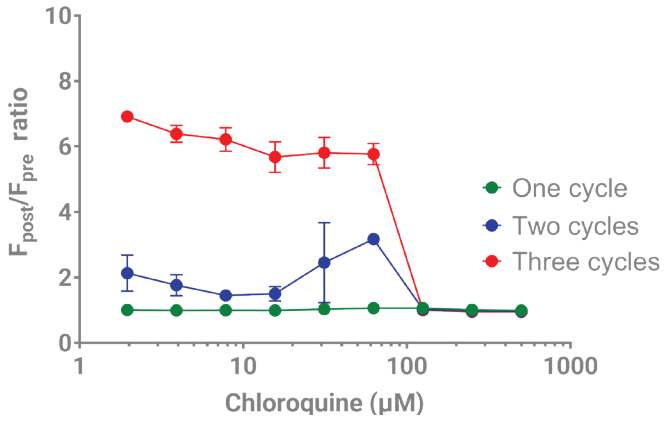

Figure 2. Optimization of imaging cube light exposure. Solution A was added to HepG2 cells, treated with various concentrations of chloroquine for 24 hours, for 30 minutes. The fluorescence was determined before and after the indicated number of one-second exposures of light generated from the GFP imaging light cube channelled through a 4x objective. The ratio of those two measurements was plotted as a chloroquine concentration function.

When HEPG2 cells were exposed to various concentrations of chloroquine, a linear relationship between drug concentration and cell viability is observed, as seen in Figure 3, where a chloroquine with an EC_{50} of 79.43 µM (R^2 = 0.97) was determined. These results are in line with previously published data (chloroquine EC_{50} = 39.7 µM).[2] Separate duplicate experiments using the Cytation 5 have produced very similar results, with differences in EC_{50} of less than 2.5% (data not shown).

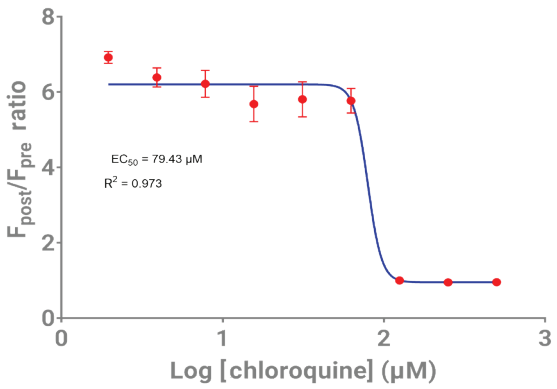

Figure 3. Chloroquine dose–response curve. Cytotoxicity was measured using a LUCS kit for HepG2 cells after 24-hour exposure to various concentrations of chloroquine. The F_{post}/F_{pre} ratio represents the ratio of fluorescence before and after three rounds of light exposure. Data were fitted with a four-parameter logistic fit, with each data point representing the mean and standard deviation of three replicates. After sigmoid fitting, the chloroquine HepG2 cytotoxicity EC_{50} was evaluated at 79.43 µM with an R^2 = 0.97.

The LUCS cell viability assay is a robust assay as evidenced by a high Z'. Figure 4 depicts a Z' determination with 15 replicates that were either untreated or treated with 500 µM chloroquine for 24 hours. Untreated cells had a F_{post}/F_{pre} fluorescence ratio that was 7.7 times greater than the chloroquine-treated replicates, and a Z' of 0.714. The Z' value considers the difference in signal between a positive and a negative control, as well as the signal variation amongst replicates. A scale of 0 to 1 is used, with values greater than or equal to 0.5, indicating an excellent assay.[4]

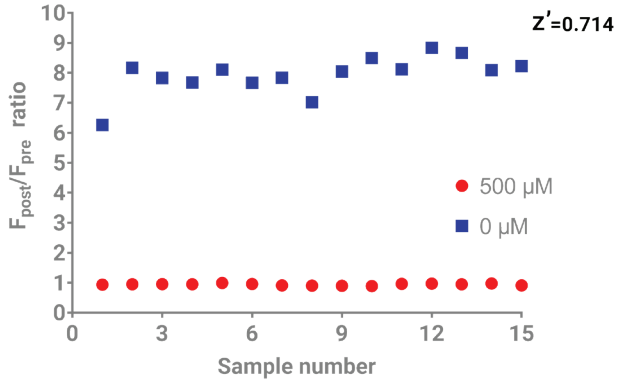

Figure 4. Z' analysis of chloroquine cytotoxicity using a LUCS assay kit. Fifteen replicates of HepG2 cells were either left untreated or treated with 500 µM chloroquine for 24 hours. The cytotoxicity was determined using a LUCS assay kit and the assay Z' was also determined.

LUCS technology requires high-intensity light to photoactivate detection fluorescence. Previously, this was accomplished using a light source external to the fluorescence reader. However, the Cytation 5 enables both microscopic fluorescence imaging and conventional fluorescence detection. The microscopic imaging module provides a focused, wavelength-specific, high-energy light to excite fluorescent molecules, which can be used as a substitute for the external light source necessary for this technology. The region of the well illuminated with the 4x objective for photoinduction encompasses approximately one-third of the area of the well of a 96-well plate, as shown in Figure 5.

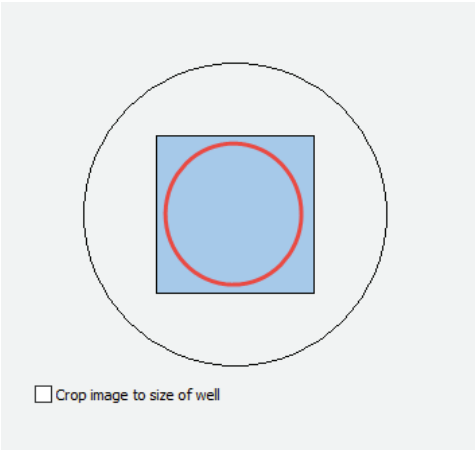

Figure 5. Exposure area of photoinduction light source. The blue rectangle represents the area of light exposure with a 4x objective, while the red circle indicates the region of fluorescence detection within the well of a 96-well microplate.

The viewing area of the 4x objective with the Cytation 5 wide field of view (shaded region) has an area of 12 mm^2, while the typical 96-well has a bottom area of 34 mm^2. When both the image area and fluorescence detection regions are aligned in the center of the well, the photoactivation area encompasses the detection region for fluorescence detection. The Cytation 5 is unique in that both photoactivation and fluorescence detection can be accomplished without any manual intervention.

The LUCS cell viability assay is a fluorescence technology based on the controlled cytosolic production of $^1O_2$ and free radicals by a photoinduction process. Because this live cell technology measures the signal after the generation of a nondestructive level of free radicals, it is open to multiplexing with other assays. The assay technology works well with both adherent and suspension cells, and is amenable to high-throughput screening (HTS) campaigns. The assay works in both 96- and 38-well microplates with high signal-to-noise ratios (interplate Z' > 0.7).

Conclusion

The LUCS technology is a novel way to assess cell viability using relatively inexpensive reagents. By using the imager lamp as an internal photoinduction light source, the Agilent BioTek Cytation 5 cell imaging multimode reader has become the first microplate reader able to fully integrate LUCS assay in a "plug-and-play" configuration. This allows a simple and robust assay protocol, compatible with HTS robotic environment.

References

1. Nygren, J; Svanvik, N; Kubista, M. The Interactions Between the Fluorescent Dye Thiazole Orange and DNA. *Biopolymers.* **1998** Jul;*46*(1):39-51. doi: 10.1002/(SICI)1097-0282(199807)46:1<39::AID-BIP4>3.0.CO;2-Z.
2. Derick, S; Gironde, C; Perio, P; Reybier, K; Nepveu, F; Jauneau, A; Furger, C. LUCS (Light-Up Cell System), A Universal High Throughput Assay for Homeostasis Evaluation in Live Cells. *Sci Rep.* **2017** Dec 22;*7*(1):18069. doi: 10.1038/s41598-017-18211-2.
3. Gironde, C; Dufour, C; Furger, C. Use of LUCS (Light-Up Cell System) as an Alternative Live Cell Method to Predict Human Acute Oral Toxicity. *Toxicol Rep.* **2020** Feb 19;*7*:403-412. doi: 10.1016/j.toxrep.2020.02.010. PMID: 32140424; PMCID: PMC7047139.
4. Zhang, JH; Chung, TDY; Oldenburg, KR. "A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays". *Journal of Biomolecular Screening.* **1999**;*4*: 67–73. doi: 10.1177/108705719900400206. PMID 10838414.

www.agilent.com/lifesciences/biotek

RA44616.4048726852

Published in the USA, February 28, 2022
5994-4598EN

Agilent
Trusted Answers