# Cell viability assessment by LUCS assay using EnSight™ multimode plate reader

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## Abstract

LUCS (Light Up cell System) is a new viability assay based on the activation of an intracellular photosensitizer in a simple protocol that only requires one light flash and two fluorescence readings. As the cells remain alive during the entire LUCS process, the technology is also open to multiplexing. However, the photoinduction requires high energy and the light flash is usually provided by an external light source device. Here, we used the chloroquine, an antimalarial drug known to be cytotoxic, to show that LUCS assay can be implemented in a "plug-and-play" mode using the EnSight™ multimode plate reader from Perkin Elmer. A dose-response study carried out on human liver HepG2 cells led to a chloroquine $EC_{50}$ of 49.85 µM/ml ($R^2$ = 0.98), in line with previous published data. The present study demonstrates that the EnSight™ multimode plate reader provides robust LUCS data using a drastically simplified protocol compatible with HTS campaigns in robotic environment.

## Introduction

The idea of a new cytotoxicity live cell assay came from the discovery of a photosensitizer, namely thiazole orange (TO), which presents a very interesting property for cell biology: its fluorescence quantum yield remains very low ($2 \times 10^{-4}$) in the culture medium due to free rotation of its two aromatic rings around the methine bridge that links them. In this situation, energy relaxation occurs on a non-radiative 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 to 0.1, denoting a 500-fold gain, a property attributed to a restriction in its torsion capacity[1]. More interesting, a recent electron paramagnetic resonance (EPR) study conducted in HepG2 cells showed that excited TO also acts as a classical photosensitizer producing both $^1O_2$ (type II reaction) and $OH^{\cdot}$ (type I reaction)[2].

Lastly, TO presents another quite unique property in live cells: its fluorescence level increases during photoinduction in a process called light-up cell system (LUCS). The intimate mechanisms underlying LUCS have been partially deciphered[2]. TO passively enters the cells but is mainly removed out 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, ROS induced by TO photoactivation alter efflux and/or other cellular functions, perturbing cell homeostasis and triggering a massive entry of the biosensor, which progressively saturates nucleic acid binding sites, resulting in a relevant increase of fluorescence level. According to the model depicted below (Fig. 1), this increase of fluorescent is limited to cells in homeostasis, leading to a high "post light" *versus* "pre light" fluorescence intensity ratio ($F_{post}/F_{pre}$>>1). Inversely, in altered cells, efflux and other cellular functions do not work properly and TO enters massively before the photoinduction process, leading to a $F_{post}/F_{pre} \approx 1$.

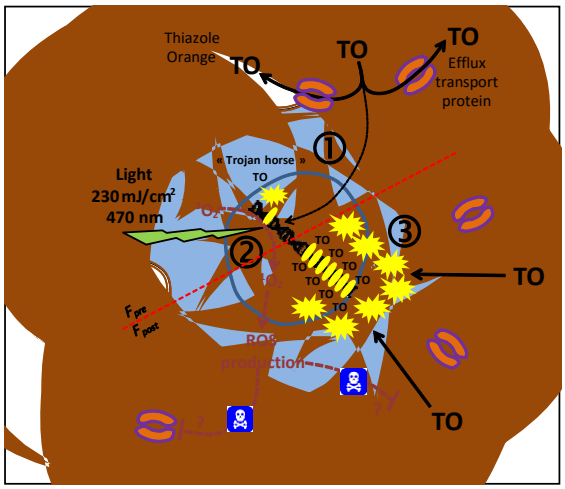


*Fig. 1 Model of LUCS process in homeostatic cells. (1) TO is mainly removed out of the cell by efflux transport limiting its access to nucleic acid targets; low fluorescence (Fpre) is observed; (2) light is applied inducing 1O2 and °OH production; (3) ROS deleterious effects alter efflux and/or other cell functions; (4) massive entry of TO triggers increase in fluorescence emission (Fpost). (diagram taken from ref. 2).*

Apart from industrial applications, LUCS assay was also recently applied as a live cell method to predict human acute oral toxicity as an alternative to animal testing[3].

### Features of LUCS cell viability assay

- Fluorescent technology compatible with fluorescent plate readers,
- Based on the controlled cytosolic production of $^1O_2$ and free radicals by a photoinduction process,
- **Keeps cells alive during the entire process**,
- **Open to multiplexing** as the signal is measured on live cells after a moderate (non-destructive) production of free radicals,
- **Results not affected by cell number** as measures are made on a ratio mode in the same well,
- **Very easy to implement** using "one-step" kits,
- Easy to standardize on 96 and 384 well-plates,
- Open to HTS campaigns (interplate Z' > 0.8),
- Works on adherent and suspension cells,
- Compatible with organotypic models.

## Material & Methods

### Instruments

- PerkinElmer EnSight™ Multimode Reader
  - Excitation light source is a LED with center wavelength of 465 nm

*Fig. 2 view of the EnSight™ multimode plate reader PerkinElmer*

### Reagents

- Antioxidant-power LUCS kit #K-2001, Toulouse France
- Sigma-Aldrich Chloroquine, Saint-Quentin Fallavier, France
- Gibco DMEM high glucose, GlutaMAX supplement and pyruvate,

- HyClone fetal bovin serum (FBS)
- Gibco pen-strep solution (100X)
- HyClone 0.05 % Trypsin-EDTA Thermo Fisher Scientific (Illkirch-Graffenstaden, France)

### Cell culture

HepG2 (#HB8065) cell line was purchased from the American Type Cell Collection (ATCC) (LGC Standards, Molsheim, France).

HepG2 cells were cultured at 37 °C/5% $CO_2$ in Glutamax DMEM medium complemented with 10% FBS and 1X pen-strep solution. Cells were grown up to 70–80% confluence then transferred in clear bottom 96-well microplates for 24h at a density of $10^6$ cells/ml (75µL, 75000 cells/well).

## Experimental protocol

Nine different chloroquine concentrations (1000 µM - 3.9 µM) were obtained by serial factor 2 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 h at 37°C in 5% $CO_2$ with each experimental condition. Solution A (Antioxidant-power LUCS kit, #K-2001) was added to the cells for 30 min at 37°C in 5% $CO_2$.

The fluorescence level was measured (time 30 min in figure 3) using a EnSight™ multimode plate reader set up at 505/535 nm (excitation/emission wavelength). Each well was then illuminated with EnSight™ imaging LED: excitation at 465 nm, 100% intensity for 1500 milliseconds, repeated 4 times. Fluorescence was read again just after this lighting sequence.

## Results and discussion

Raw data were further analyzed by Prism8 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 50% Efficacy Concentration ($EC_{50}$) value from a mathematical non-linear regression model (sigmoid fit) given by Prism8, following the equation (1):

(1) $Y = Bottom + (Top-Bottom)/(1 + 10^{((LogEC_{50}-X)*HillSlope)})$

where HillSlope = slope coefficient of the tangent at the inflection point. $EC_{50}$ and determination coefficient $R^2$ values were deduced from this regression model (Fig. 3).

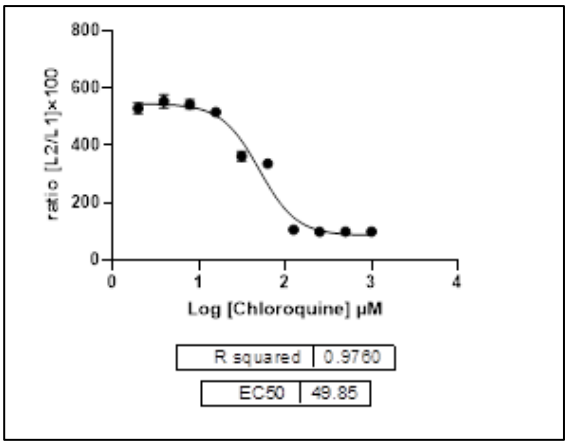


*Fig. 3 dose-response curved obtained after sigmoid fitting. Chloroquine HepG2 cytotoxicity EC50 was evaluated at 30.03 µM with an R2 = 0.98*

***Fig. 3.*** With a chloroquine $EC_{50}$ evaluated at 49.85 µM ($R^2$ = 0.98), results are in line with previously published data (chloroquine $EC_{50}$ = 39.7 µM)[2].

## Conclusion

**By its ability to connect both illumination and fluorescence reading in a unique protocol, the EnSight™ multimode plate reader was able to fully integrate LUCS assay in a "plug-and-play" configuration, adapted to routine and HTS applications.**

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