# Protocol for Cytophototoxicity Testing of Contrast-Enhanced Fluorescence Imaging Products

## Tool Reference

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**Protocol for cytophototoxicity testing of contrast-enhanced fluorescence imaging products**

**Overview:** This protocol covers cell culture, fluorophore selection and preparation, optical exposure, assaying and data analysis procedures.

1. Cell culture: BALB/c 3T3 clone A31 (ATCC, VA, USA) cells are cultured following recommendations from the aforementioned OECD 432 guidelines. Briefly, cells are cultured in T-75 flasks (Cell Treat, MA, USA) in a 37°C and 5% $CO_2$ incubator until they reached 80-90% confluency. Dulbecco's modified eagle's medium (DMEM) medium (ATCC, VA, USA) supplemented with 10% Fetal Bovine Serum (FBS) (Gibco, MD, USA), 1% penicillin-streptomycin (Corning, MA, USA) are used to maintain the cells. The medium is changed every 48 hours. BALB/c 3T3 cells are confirmed to be mycoplasma-free using the Mycoalert™ Plus mycoplasma detection kit (Lonza, Basel, Switzerland). To optimize the cell seeding for phototoxicity evaluation, BALB/c 3T3 cells are plated over a range of cell seeding numbers (from 1.0 $x10^3$ to 1.2 $x10^5$ cells per well) in 96-well tissue culture plates (Southern Labware, GA, USA). After 48 hours, an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Invitrogen, MA, USA) is performed following the vendor protocol. Briefly, 3T3 cells are incubated with 0.25 mg/ml of MTT for 1 hour. The formazan crystals formed are solubilized in 100% DMSO, and the absorbance is measured at 570 nm using a spectrophotometer

2. Fluorophore samples and illumination: Fluorophores are tested for concentrations ranging from 0 µM (negative control) to approximately twice the concentration used in the clinic for imaging. The 2X concentration range is based on parameters reported in the literature, like human blood plasma levels, pharmacokinetics data after intravenous injection, and clinical studies on using the test agents for FGS. Rose Bengal (RB) and BPD can serve as positive controls since they are known to generate RMS after photoactivation and are used as photodynamic agents [32, 33]. The concentration range of test agents is increased up to 100 µg/mL per OECD 432 guidelines to test the photocytotoxicity generated at higher concentrations to determine potential safety thresholds for the fluorophores. The $H_e$ and illumination wavelengths are determined from clinical studies.

3. Optical exposure system setup and validation: The laser light should be collimated and an aperture 1" x 1" used to restrict illumination to the sample(s). Power measurements should be taken to ensure uniform power distribution during laser exposure, for example, using an iris diaphragm attached to the power meter sensor. The location of each well during illumination should be marked to ensure that measurements were are from a fixed location.

4. Modified Neutral Red Uptake (NRU) assay: For testing, 1.0 $x10^4$ BALB/c 3T3 A31 clone cells are plated in 100 µL complete growth medium per well in two 96-well, black-walled, clear flat bottom microtiter plates. The cells are allowed overnight attachment at 37 °C in a humidified atmosphere containing 5% $CO_2$.

   On day 1, a fresh fluorophore solution is prepared in DMSO or HBSS based on the solubility of the agent. Eight different testing concentrations are prepared per fluorophore. Each testing concentration has six replicates per plate.

For testing, the wells are washed with 100 µL of HBSS, followed by adding 100 µL of the test fluorophore concentration, and incubated for 60 minutes in both plates. The control wells contain 100 µL of the solvent control (0.1% DMSO in HBSS or HBSS only). After 60 minutes, the IRR plate is illuminated to achieve the desired $H_e$ at set irradiance. If radiant exposure is sufficient, multiple wells can be irradiated simultaneously. Exposure time for the IRR group can be varied depending on the test $H_e$ for each agent. The NO IRR plate is stored in the dark at room temperature while illuminating the IRR plate.

To minimize the exposure of cells to room temperature for prolonged time intervals (<35 minutes) during illumination, (i) Two instead of eight fluorophores concentrations, with dark and solvent controls, were tested per plate; (ii) Four replicates per condition were tested at higher $H_e$. For $H_e$ of 6 J/cm$^2$, 12 out of 24 wells (IRR group only) per plate were irradiated in 12/4 irradiations.

After illumination, the solution is removed and the cells are washed twice with 200 µL of HBSS and incubated with the cell growth medium for 24 hours. The NRU assay can be performed on day 2 using the Neutral Red Assay kit (Abcam, Waltham, MA). Briefly, treated cells are incubated with 150 µL of the 1x neutral red solution for 3 hours. The cells are washed with 200 µL of the wash solution (1x PBS).

Subsequently, cells are incubated with 200 µL of solubilization solution to release the incorporated neutral red dye under acidified-extracted conditions. The absorbance of NR dye in the solubilization solution is measured at 540 nm (e.g., using a spectrophotometer). Results for surviving cells are normalized to the no-treatment (solvent controls) on each plate.

To determine if the absorbance spectrum of the test fluorophore affects the NRU absorbance spectrum during phototoxicity evaluation, cells are incubated with the fluorophore on day 1 for about 1.5 hours, considering the time for fluorophore incubation and illumination. Control wells are treated with 100 µL of HBSS. After incubation, 3T3 cells are washed with HBSS and incubated with media overnight. For the fluorophore--alone group, cells are incubated with fluorophores overnight. As described previously, the NRU assay can be performed on day 2, and the absorbance spectrum should be recorded using a spectrophotometer or similar instrument.

**5.** Data analysis: The concentration-dependent cytotoxic responses in the presence of light (IRR) and in the absence of light (NO IRR) are determined in the NRU assay. The metric recommended in OECD 432, the Mean Photo Effect (MPE), can be calculated using Phototox version 2.0 software. MPE is calculated based on a comparison of the IRR and NO IRR response curves on a set of commonly tested concentration ranges (i =1, …,n) of IRR and NO IRR curves (Equation 1).

$$MPE = \frac{\sum_{i=1}^{n} w_i PE_{c_i}}{\sum_{i=1}^{n} w_i}$$

Photo-effect ($PE_c$) at any concentration C is a product of dose-effect ($DE_c$) and the response effect ($RE_c$).

$$PE_c = DE_c * RE_c$$

The dose effect ($DE_c$) is calculated as:

$$DE_C = \left| \frac{C \,/\, C^* - 1}{C \,/\, C^* + 1} \right|$$

where C* represents the concentration at which the IRR response equals the NO IRR response at concentration C. If C* cannot be determined because the response of the IRR curve is higher or lower than the NO IRR, DE is set to 1 for that concentration. The response effect ($RE_c$) at concentration C is the difference between responses observed in the absence and presence of light:

$$RE_c = \frac{R_c\ (NO\ IRR) - R_c\ (IRR)}{100}$$

where $R_c$ is determined from measurements of absorbance (OD = log (1/T)) by the spectrophotometer:

$$R_c = \frac{OD[test\ concentration] - OD[blank]}{OD[concentration\ =\ 0] - OD[blank]} * 100$$

and the *blank* represents measurement of a cuvette with only solvent.

Weighting factors ($w_i$) are determined by the highest response value, i.e., $w_i$ = MAX [Ri (IRR), Ri(NO IRR)]. The MPE is subsequently obtained by averaging across all $PE_c$ values. For calculating MPE using Phototox software, concentration-response values are input into the software. Fitting the curve to the data by non-linear regression is performed by the software. A bootstrap resampling (i.e., selecting data points randomly with replacements) is performed to assess the influence of data variability on the fitted curve. Based on the MPE values, the fluorophore product at each tested $H_e$ level is determined to be non-phototoxic, equivocal-phototoxic, or phototoxic, using the standard in OECD 432.

Cell viability response is calculated using the normalized mean absorbance values obtained using the NRU assay. Cell viability is presented as mean ± standard error of the mean (SEM). Data can be analyzed using a paired Student's t-test, where a value of $p \leq 0.05$ is considered statistically significant. All experiments should be repeated at least three times to assess repeatability ($n \geq 3$).