Project:

Determination of intracellular reactive oxygen species

Assessment of cellular reactive oxygen species induced by nanomaterial treatment by means of high-content imaged based screening

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Contents

1 Introduction

Reactive oxygen species (ROS) are chemically active substances that contain oxygen, including hydrogen peroxide and radicals such as hydroxyl radical (OH·) and superoxide (·O2−) [1]. ROS are generated endogenously both as a by-product of mitochondrial respiration, as well as a signalling molecule in physiological processes such as inflammation and cellular homeostasis. However, excessive generation of ROS through the perturbation of the mitochondrial membrane potential may cause macromolecule damage through oxidation of membranes, proteins and nucleic acids, that may cause cell injury, cell death or mutations [2]. Thus, to avoid such unwanted side-effects it is of great interest to evaluate if biomedical interventions cause ROS generation as an unwanted side effect.

The ability of nanomaterials (or other test substances) to generate ROS can be assessed following various exposure routes and exposure times using a High Content Screening (HCS) platform. Following exposure to the materials, the treated cells are stained with a dye that labels nuclei, and a dye that reports the presence of reactive oxygen species. Changes in the fluorescence intensity associated with CellRoxDeepRed can give indications of the cytotoxicity of the materials. Consideration should be taken to ensure that experimental design, cellular models, methods of exposure, and analytical metrics are in line with OECD [3, 4] and European Food Safety Authority (EFSA) guidelines [5].

As such, this protocol describes the approach for testing the cytotoxic responses of human cell lines to nanoparticles (NPs) using the HCS platform by automated imaging and subsequent multiparametric image analysis. The introduction of a “nano” specific SOP enables and expands the use of existing HCS instrumentations for the titration, assessment and safety screening of nanomaterials, or their engineered forms.

As it is an image-based technique for single-cell quantitative analysis, it allows for the avoidance of artefacts and ambiguities in the results produced. Furthermore, it will also circumvent any interference issues originated by the nanomaterials / nanoparticles. The multiparametric nature of the analysis also enables the collection of large volumes of data, providing opportunities for data-mining or bioinformatic analytical approaches.

2 Principle of the Method

Procedures followed are in accordance with REACH on the hazard assessment of chemical entities [6-8] and aspects of the ISO 10993-5 guidelines for in vitro cytotoxicity assessment on the use of liquid samples [9].

The principle of the method is the oxidative conversion of the CellRoxDeepRed probe from a non-fluorescent molecule to an entity that is fluorescent in the infrared region. Certain reactive oxygen species such as the superoxide radical. Thus, the intensity of cells imaged in the deep red channel will be a measure of intracellular reactive oxygen species. To locate the cells, the Nuclear Green LCS1-probe is used. This also provides secondary readouts such as an enumeration of nuclei in each well, and their area.

The HCS assays detailed in this SOP relate to the measurement of nuclear area and reactive oxygen species using the Nuclear Green LCS1 and CellRoxDeepRed probes, respectively. Cells are then imaged used an automated fluorescent microscope, imaging enough cells per well to obtain to be analysed to provide robust data. In the case of this SOP, cells are imaged using an ImageXpress confocal high content microscope (Molecular Devices, United States) Analysis is conducted using image analysis software, that first detects, counts and marks out cell nuclei in the green channel, and then quantitates the intensity of each cell in the deep red channel. This is then a measure of cellular ROS status, where higher intensities correspond to a more oxidative cellular environment. We have used the built-in software on the instrument (MetaXpress, Molecular Devices, United States). However, Other imaging and analysis instruments and software packages can also be used [10], provided the instrument has the appropriate detection filters and ability to read 96-well plates.

3 Applicability and Limitations (Scope)

This SOP describes the in vitro methods for the evaluation of ROS generation of test substances or nanoparticles using the HCS platform to measure nuclear area and ROS. The protocol is based on the assessment of the A549 and HepG2 cell lines, although other adherent cell lines may be used as well.

However, the assay, and this SOP, does not provide detailed mechanistic information of the toxic effect experienced by the cell.

Interference of the NP with the assay readouts should be considered, particularly if the NP under examination exhibits fluorescent properties (quantum dots for example), during experimental planning and interpretation of the results. Existing available literature should therefore be reviewed during design of experiments in order to evaluate the applicability of the assays. Alternatively, specific measurements should be carried out by incorporation of “no cells” controls which will allow for accounting of the additional contribution provided by the “nano” materials or particle. Moreover, the signal generated by reactive oxygen species should in principle be quenched by treatment with antioxidants such as N-acetyl cysteine (NAC). The proposed SOP, and technique adopted, can be used in a dual-measurement: single-cell quantitative fluorescence analysis and well-based fluorescence intensity averages. This is the main advantage of HCS techniques versus other cell-based approaches (e.g., MTT, WST-8, and others) for the cell viability assessment.

Test material dilutions are carried out in the presence of serum. This may cause aggregation of the test material, and so stability studies in such media should be carried out. Further details on experimental design and characterisation protocols can be found in scientific literature or by contacting EUNCL core expert teams. Methodology for the characterisation of test materials in complex media (such as cell culture medium) are available in EUNCL-PCC-21, EUNCL-PCC-22, EUNCL-PCC-23, and EUNCL-PCC-35

4 Equipment and Reagents

4.1 Equipment

1. Incubator, 37˚C with 5% CO2 and 95% humidity

2. Microscope compatible with 96-well plate format (ImageXpress Micro Confocal microscope (Molecular Devices) or equivalent.
3. P1000, P300 and P100 Multichannel pipettes or equivalent
4. Black walled, 96-well plates with optically clear bottom (Nunc 165305 or equivalent).
5. Image analysis software (MetaXpress 6, Softmax 6.5.1 or equivalent).

4.2 Reagents

1. Tert-butyl hydroperoxide 70% (SigmaAldrich 458139-100ML)
2. CellRoxDeepRed (2.5 mM in DMSO, 50 µl per tube, ThermoScientific C10422)
3. Nuclear Green LCS1 (Abcam ab138904)
4. HBSS (SigmaAldrich H6648)

4.3 Reagent Preparation

4.3.1 Cell culture medium

The complete medium for HepG2 and A549 is prepared by adding 5–10% Foetal bovine serum , 2 mM L-glutamine and 1% Penicillin-Streptomycin to RPMI medium (final concentrations). The medium is stable for up to 4 weeks at 4˚C.

4.3.2 Positive control

A 1 h incubation in the presence of 250 µM tert-butylhydroxide.

4.3.3 Wash buffer

Hanks' balanced salt solution (HBSS, SigmaAldrich # H9269)

4.3.4 10x Live cell staining solutions

A mix containing 5 µM Nuclear Green LCS1 is dissolved in HBSS supplemented or not with 50 µM CellRoxDeepRed.

5 Procedure

5.1 General remarks

5.2 Procedure

1. Seed cells (over night)
2. Change to fresh medium, then add test materials.
3. Incubate 4-24 h
4. Prepare concentrated positive control and cell staining solutions.
5. Add positive control (250 µM tert-butylhydroxidr final concentration) and cell staining solution. Incubate 1 h.
6. Remove test materials, positive controls and staining solution.
7. Wash cells thrice with 100 µl HBSS.
8. Image plate and analyze data.

5.3 Cell handling

The A549 and HepG2 cell lines used in this study were obtained from ATCC. Information relating to thawing, propagation, and creation of stocks is similar to that as described in EUNCL-GTA-001 and EUNCL-GTA-002. These stock ampoules serve as starting point for all experiments. After thawing of stock ampoules, cells are subcultured for 3 passages until enough cells are achieved, but no more than 25 passages (see Subcultivation). On the day of experiment, perform cell seeding as described in 5.4, continue with addition of samples the following day as described in 5.5.2.

5.3.1 Subcultivation

A549 and HepG2 cells are kept in a sub-confluent state by routinely passaging twice or three times a week to seeding densities between 6 x 10^4 – 1 x 10^5 cells /cm^2.

Given volumes are for 75 cm^2 flask – proportionally reduce or increase amount of dissociation medium for culture vessel of other size.

1. Remove and discard culture medium.
2. Wash the cell layer twice by gently rinsing it with 10-15 mL preheated (37˚C) PBS.
3. Add 0.75-1 mL dissociation reagent (e.g TrypLE®), incubate at 37˚C for 5 minutes, and gently knock culture flasks to detach most of the cells.
4. Resuspend cells in 9 mL complete cell culture medium to stop trypsination.
5. Transfer and dilute in new culture vessels.
6. Incubate the culture at 37˚C in a humidified atmosphere with 5% CO2 in a suitable incubator.

5.4 Cell Seeding

1. Harvest cells from prepared flasks, the cells should be cultivated for minimum 3 passages before use for experiment.
2. Count cell number using a coulter counter or haemocytometer.
3. Dilute cells to a density of 1 x 10^5 cells/mL in cell culture media.
4. Plate 100 µL cells/well per plate per time point (4, 24 hr, etc).
5. Incubate plates over night at 5% CO_2, 37˚C and 95% humidity.

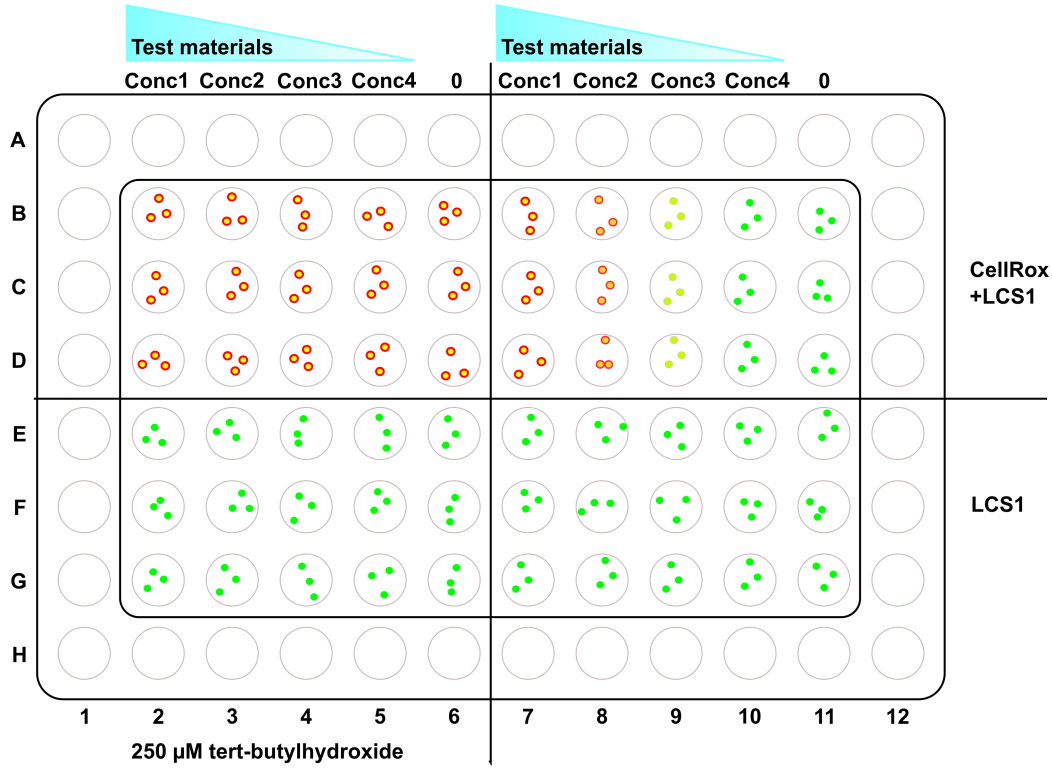

Figure 1: Representative plate layout for ROS assay. In this setup, cells are seeded in the inner wells of a 96-well plate (rows B–G, columns 2–11), and incubated with dilution series of one test substance added to columns 2–6 and columns 7–11. Following incubation for 3–23 h, the positive control tert-butylhydroxide is added to colums 2–6, the nuclear stain LCS1 is added to columns E, F, and G, and LC1 and CellRoxDeepRed are added to columns B,C, and D. The cells are further incubated for 1 h, washed and imaged. Detection of green fluorescence reveals the presence of individual cells. Exposure to ROS, or agents that induce ROS, chemically changes to CellRoxDeepRed reagent so it fluoresces in the infrared spectrum. By first using the fluorescence in the green channel to identify cells, one can quantify the red intensity associated with each cell. This is a measure for the oxidative environment in each cell. Final concentrations are typically 1000–37 ng/µl test substance, 250 µM tert-butylhydroxide, 0.5 µM LCS1, and 5 µM CellRoxDeepRed. This plate setup is designed to correct for agents that quench the fluorescence, as well as subtraction of background fluorescence from cells or test materials.

5.5 Assay procedure

5.5.1 Procedural Notes

1. Do not allow plate wells to become dry at any time during the protocol.
2. Perform all steps at room temperature unless otherwise indicated.
3. The protocol is optimized for A549 and HepG2 cells seeded in 96-well plates. Using conditions other than those indicated may necessitate optimization.
4. The DMSO concentration exposed to the cells are 1% or less.
5. For best results, use low velocity fluid aspiration and dispensing (≤ 4 ml/minute) with an automatic pipetting device. Toxic compounds may weaken cell adherence to the substrate, thus aspiration with moderate and high velocity may cause significant cell loss. To minimize cell loss, add and remove solutions at the edge of the well while tilting the plate without ever touching the bottom of the plate.
6. Please refer to the guide and instructions provided with the software and the HCS instrument for optimal implementation of the assay.

5.5.2 Procedure for 4, 24 hr exposure

1. Dilute test compounds to appropriate concentration in complete culture medium.
2. Remove growth medium from cells using an aspirator or a multichannel pipette. Add 100 µL of the test compound to the desired wells using a multichannel pipette and incubate for the desired amount of time.
3. Incubate plates at 37°C in 5% CO_2 for the desired time (3–23 hours).
4. Add 10 µl 3.0 mM tert-butylhydroxide to the positive control wells (columns 2-6), and 11 µl medium to the rest of the wells (columns 7-11).
5. Add 10 µl 6 µM Nuclear Green LCS1 stain dissolved in medium to all wells in rows F, G, and H.
6. Add 10 µl 6 µM Nuclear Green LCS1 and 600 µM CellRoxDeepRed stains to all wells in rows B, C, and D.
7. Incubate for one hour at 37°C in 5% CO_2.
8. Gently aspirate liquid from all wells and add 100 µl HBSS, using a multichannel pipette. Repeat this twice.
9. Acquire images using ImageXpress confocal microscope using the following settings:
   * Objective and camera. Magnification:10x Plan Apo Lambda. Binning: 1. Use Widefield mode.
   * Plate: Use setup optimized for 96-well Nunc 165305 at room temperature.
   * Acquisition: Enable laser-based focusing. 3 Wavelengths: W1 FITC, W2 Cy5, W3 TL50.
   * W1 FITC: Bright sample. First calculate offset in one of the positive control wells B6, C6, or D6 to find optimal focus. The use AutoExpose to calculate Target max intensity at 30,000. The optimal exposure time is usually between 3 to 10 ms.
   * W2 Cy5: Bright sample. Calulate the offset to identify the optimal focus in one of the positive control wells B6, C6, or D6. Repeat AutoExpose to calculate Target max intensity, using an upper threshold of 20,000. Validate that all the three positive control wells are far from saturated, and that there is a healthy difference between cells and background of at least 1,000 in pixel intensity values.
   * W3 TL50: Bright sample. Calculate offset to identify optimal focus. Use AutoExpose as above with a Target Max Intensity value of 30,000.
10. Image the plate. The same settings can be re-used for subsequent plates treated in parallel.
11. Analyze data in MetaMorph 6, using Cell Scoring Analysis. The following setting is recommended, using the Fast Algoritm.
    * W1 source image: FITC.
    Approximate min width 7 µM,
    Approximate max width: 30 µM.
    Intensity over background: 200 gray levels.

    * W2 source image: Cy5.
    Stained area: Nucleus and Cytoplasm
    Approximate min width: 7 µM
    Approximate max width: 30 µM
    Intensity over local background: 1000 gray levels.

    * Use Preview in positive and negative control wells to validate that cells are scored correctly, and adjust cell size or intensity accordingly.

5.6 Calculations

All samples, positive, negative, and CellRoxDeepRed staining controls are run in triplicate. To calculate the final results, each well is subtracted from its respective CellRoxDeepRed-free control well to account for any fluorescence that may be due to the test material, or fluorescence due to the experimental treatment. The average of these three values should be used in the equations below.

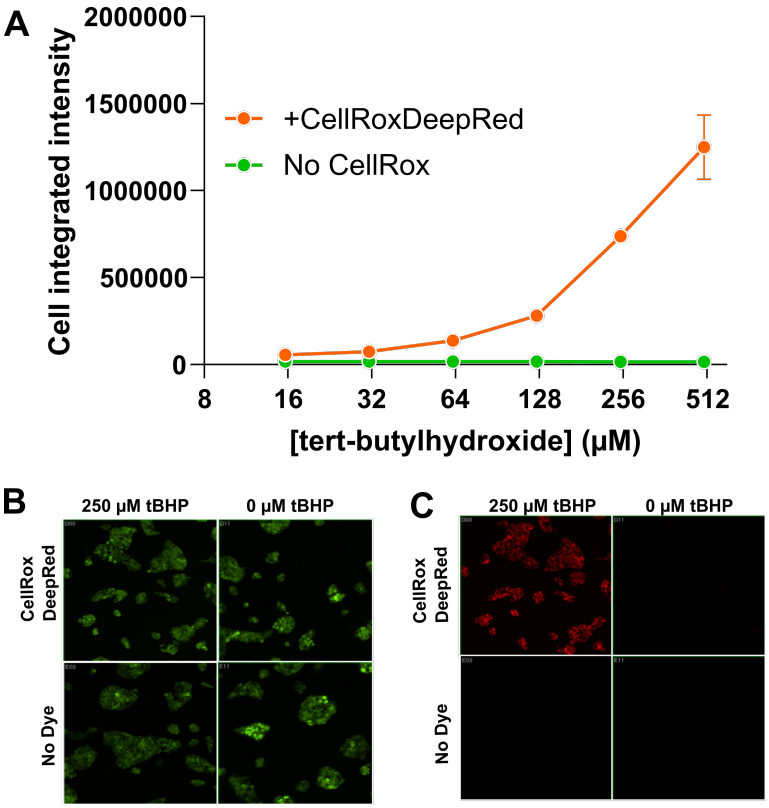

Figure 2: A. Quantitation of images of HepG2 cells exposed or not to tert-butylhydroxide. Cells were treated or not with the indicated doses tert-butylhydroxide for 1 h in the presence or absence of 50 µM CellRoxDeepRed, and 0.5 µM Nuclear Green LCS1. Cellular objects were identified based on signals from LCS1 in the green channel, and the intensity in the red channel associated with each cell was quantitated. The data show the integrated cell intensity per condition from >600 cells per well (n=3 wells per data point). B. Example images of HepG2 cells stained with LCS1 and exposed or not to 250 µM tBHP. No dye indicates the absence of CellRoxDeepRed dye. C. Example images of the same HepG2 cells as in B, imaged in the red channel.

1. Mean, SD and %CV should be calculated for each set of samples tested.
2. Results should be plotted and presented as average ±standard deviation for the three technical replcates.
3. Two values should be reported: Total nuclei count, and the average cellular integrated intensity from the Cy5 channel. The first is a measure of cellular proliferation and viability, and cell death. The last measurement is the intensity of the ROS reactive dye CellRoxDeepRed, corrected for background levels from wells with identical treatment of test materials, but not stained with CellRoxDeepRed reagent.
4. The sample EC50 values can be calculated by monitoring the increase in CellRoxDeepRed intensity as the test material concentration increases. It is recommended to generate a nonlinear fit calculating the EC50 as the concentration of the test material that induces half the maximum response for the given parameter.
5. Example images and quantitations is shown in figure 2.
6. The Z'-factor of the assay should be calculated and reported, where Z' =1 is an ideal assay, Z'=0 is suitable for binary yes/no responses, and Z' < 0 in unacceptable. The Z-factor is calculated as shown below:

$$Z'factor = 1 - \frac{3(\sigma_p + \sigma_n)}{|\mu_p - \mu_n|}$$

Where μ_p and σ_p are the mean and standard deviation values of the positive control (or treated samples) and μ_n and σ_n are those of the negative control [11]. A Z' Factor > 0.5 is an determined as being an "excellent assay for screening" [12].

6 Quality Control, Quality Assurance, Acceptance Criteria

1. Assay wells exposed to 250 µM tert-butylhydroxide or an equivalent dose solvent should be used as internal quality controls.
2. Z' for positive and solvent negative control should be > 0.5.
3. Caution is advised upon marked decreases in nuclear count and area, indicative of a proliferation defect and/or toxicity.
4. For each experimental run, a separate plate including an 8-concentration dilution series of tert-butylhydroxide should be included, with 1 h incubation time (Figure 2). The resulting EC50-value should be calculated and recorded. Highly deviant EC50-values, i.e. a more than a 2-fold variation from the historical average, will prompt caution when evaluating data from the relevant experiment. It is also advised to monitor the EC50 value for trends indicating shifts in the assay performance.
5. Good practice in multiparametric analysis or titration study is to ensure consistency between the data generated and the recorded images where the data is processed.
    a) If excessive cell fragmentation is present in the well, please revise your cell seeding density or check for exceeding nanomaterial aggregation (not quantified during physico-chemical characterisation).
    b) Nanoparticle aggregates could be counted as cells or organelles depending on the size of aggregates and sensitivity of the analytical thresholds.
    c) If fluorescent data generated does not align with the images recorded that there may be possible fluorescent interference or saturation points within the selected fields of view where to process the data. This could be either an outlier or systematically present across all wells. The common procedure here is to repeat the assay by revising the cell seeding and or nanomaterial test concentrations.

7 Health and Safety Warnings, Cautions and Waste Treatment

Please refer to available H.S.E information for any nano formulations evaluated in the assays. Note that some of the listed reagents are hazardous and must be handled with precaution. Please refer to safety data sheets for each reagent, wear protective equipment, and dispose waste according to local regulations.

8 Abbreviations

NP: Nanoparticle

RT: Room temperature

HBSS: Hanks' balanced salt solution

SD: standard deviation

% CV: Percentage coefficient of variation

FBS: fetal bovine serum

ROS: Reactive oxygen species

NAC: N-acetyl cysteine

tBHP: tert-butylhydroxide.

9 Related Documents

Table 1:

Document ID	Document Title	URL
EUNCL-GTA-001	LLC-PK1 Kidney Cytotoxicity Assay	http://www.euncl.eu/about-us/assay-cascade/PDFs/Toxicology/EUNCL_GTA_001.pdf?m=1468937871&
EUNCL-GTA-002	HepG2 hepatocarcinoma cytotoxicity	http://www.euncl.eu/about-us/assay-cascade/PDFs/Toxicology/EUNCL_GTA_002.pdf?m=1468937871&
EUNCL-PCC-21	Measuring NP aggregation propensities with DLS	http://www.euncl.eu/about-us/assay-cascade/PDFs/PCC/EUNCL-PCC-021.pdf?m=1468937870&
EUNCL-PCC-22	Size, Size Distribution	http://www.euncl.eu/about-us/assay-cascade/PDFs/PCC/EUNCL-PCC-022.pdf?m=1468937868&
EUNCL-PCC-23	Particle Tracking Analysis	http://www.euncl.eu/about-us/assay-cascade/PDFs/PCC/EUNCL_PCC_023.pdf?m=1526712237&
EUNCL-PCC-35	Drug release in complex media	

10 References

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