Neutral red uptake assay for the estimation of cell viability/cytotoxicity

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Published online 12 June 2008; doi:10.1038/nprot.2008.75

The neutral red uptake assay provides a quantitative estimation of the number of viable cells in a culture. It is one of the most used cytotoxicity tests with many biomedical and environmental applications. It is based on the ability of viable cells to incorporate and bind the supravital dye neutral red in the lysosomes. Most primary cells and cell lines from diverse origin may be successfully used. Cells are seeded in 96-well tissue culture plates and are treated for the appropriate period. The plates are then incubated for 2 h with a medium containing neutral red. The cells are subsequently washed, the dye is extracted in each well and the absorbance is read using a spectrophotometer. The procedure is cheaper and more sensitive than other cytotoxicity tests (tetrazolium salts, enzyme leakage or protein content). Once the cells have been treated, the assay can be completed in <3 h.

INTRODUCTION

The neutral red uptake assay is one of the most used cytotoxicity tests with many biomedical and environmental applications[1–6], as shown in **Table 1**. It is used in basic and applied research and it is also included in the first nongenotoxicity *in vitro* assay accepted for the regulatory evaluation of chemicals, that is, the phototoxicity assay[7,8].

In vitro tissue culture studies using the neutral red dye were developed for assessment of viral cytopathogenicity, of immunotoxicity[9] and for the detection of toxic compounds. The neutral red uptake cytotoxicity assay was developed at the Rockefeller University as a cell viability chemosensitivity assay[1]. It is based on the ability of viable cells to incorporate and bind the supravital dye neutral red. This weakly cationic dye penetrates cell membranes by nonionic passive diffusion and concentrates in the lysosomes, where it binds by electrostatic hydrophobic bonds to anionic and/or phosphate groups of the lysosomal matrix[10,11]. The dye is then extracted from the viable cells using an acidified ethanol solution, and the absorbance of the solubilized dye is quantified using a spectrophotometer.

The uptake of neutral red depends on the cell's capacity to maintain pH gradients, through the production of ATP. At physiological pH, the dye presents a net charge close to zero, enabling it to penetrate the membranes of the cell. Inside the lysosomes, there is a proton gradient to maintain a pH lower than that of the cytoplasm. Thus, the dye becomes charged and is retained inside the lysosomes.

Figure 1 shows the morphology of different cells stained with neutral red, including the RTG-2 salmonid fish cell line, the PLHC-1 cells derived from the topminnow *Poeciliopsis lucida* and the SH-SY5Y human neuroblastoma cell line, with and without exposure to zinc chloride.

Figure 2 shows the increase in uptake of neutral red according to the period of incubation. The uptake is significantly increased up to 2 h of contact with the dye, with a maximum at 3.5 h. It is not recommended to use a more concentrated neutral red solution[4] because it causes cytotoxicity even after 1-h exposure, significantly increases the variability of the results, causes precipitates of the dye and obstructs the morphological examination of the cultures.

The relationship between the number of cells and the absorbance at 540 nm after the neutral red test is shown in **Figure 3**. Neuro-2a mouse neuroblastoma cells were seeded at different concentrations in the wells of a 96-well tissue culture plate. Neutral red medium was immediately added, and after 3-h incubation at 37 °C the dye was extracted and the uptake was quantified. The graph demonstrates the linear relationship between the number of cells and the absorbance.

When the cell dies or the pH gradient is reduced, the dye cannot be retained[12]. Consequently, the amount of retained dye is proportional to the number of viable cells. In addition, the uptake of neutral red by viable cells can be modified by alterations in cell surface or lysosomal membranes[13]. It is thus possible to distinguish between viable, damaged or dead cells[1] according to their specific lysosomal capacity for taking up the dye[6]. Lysosomal integrity, with the concomitant binding of the neutral red dye, is a highly sensitive indicator of cell viability. The assay quantitates cell viability and can be used to measure cell replication, cytostatic effects or cell death depending on the seeding density.

TABLE 1 | Some applications of the neutral red uptake assay.

Viral cytopathogenicity and treatment effectiveness
Estimation of cytotoxic T-lymphocyte populations
Ab-dependent complement cell damage (Filman)
Tumoral cell growth and chemotherapy effectiveness
Toxicity of physical agents either alone or in combination with chemicals, and, in particular, phototoxicity
Determination of toxins in biological and environmental samples and in biotechnological products
Ranking chemicals according to their toxic potencies or hazard
Identification of organ- and cell-specific toxicity
Comparison of species toxicity
Establishment of chemical structure–toxicity relationships
Elucidation of antagonistic/synergistic interactions between chemicals
Toxicity prevention and treatment effectiveness
The investigation of metabolism-mediated cytotoxicity (detoxication and toxification process)
The study of temperature-dependent toxicity

Most primary cells and cell lines from human, animal, fish and other origin may be successfully used with the same test protocol if appropriate culture media and incubation conditions, particularly of serum and temperature, are selected according to the specific needs of the cells. The assay has been used in many human cell lines, including melanoma cells, neuroblastoma cells, hepatocytes, melanocytes and fibroblasts and low passage cells, such as normal keratinocytes and endothelial cells, with other rodent and fish cell lines, including fibroblast, epithelioid and hepatoma cell types by numerous laboratories[2,14]. Non-adherent cells can also be used if a centrifugation step (400*g*) of the plate is included before each change of reagent[15]. For the assessment of the cytotoxicity of chemicals requiring metabolic activation to toxic metabolites, it is recommended to use cells with biotransformation capacity or to include a hepatic microsomal fraction.

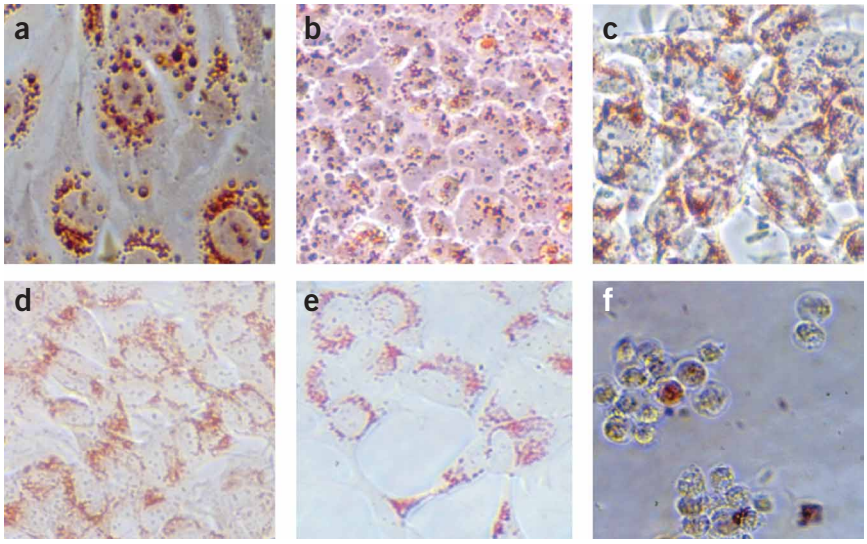

Figure 1 | Cultured cells stained with neutral red. (**a**) Morphology of different cells stained with neutral red (×1,600), including the RTG-2 salmonid fish cell line derived from the gonad of rainbow trout *Oncorhynchus mykiss*, (**b**) the PLHC-1 cells derived from a hepatocellular carcinoma of the topminnow *Poeciliopsis lucida*, (**c**) the SH-SY5Y human neuroblastoma cell line, and the same human cells after 24-h exposure to (**d**) 1, (**e**) 20 and (**f**) 50 μg ml^{-1} zinc chloride.

There are advantages and disadvantages of the neutral red uptake assay in comparison with other widely used viability tests. The procedure is very sensitive and readily quantifiable. It is at least two times cheaper (~0.2€/plate; Sigma-Aldrich Online Catalog), presents less interference, is more sensitive and does not use unstable reagents as required for the viability tests using tetrazolium salts (MTT, MTS, XTS, etc.) that quantify dehydrogenase activity by the chemical reduction of the salts to formazans[16].

The neutral red assay is more sensitive and requires less equipment than the estimation of cell death by enzyme leakage using lactate dehydrogenase. The neutral red assay also compares favorably to estimation of total cell number by assaying protein content. The neutral red uptake assay is cheaper and more simple, detecting only viable cells; however, once initiated it must be completed immediately, as it is not possible to freeze the cells, as is done for the determination of total protein assay. Nevertheless, the neutral red assay is compatible with the determination of total protein content, because it is possible to perform both the total protein content and the neutral red assays on the same culture, that is, neutral red estimates can be obtained and then the protein determination can be carried out[17,18].

In common to other cell culture procedures, there are certain limitations due to the character of the compounds to be tested: substances that are volatile, unstable or explosive in water, or with low solubility, present problems.

MATERIALS

REAGENTS

- Primary cells or established cell lines
- Neutral red (3-amino-7-dimethylamino-2-methyl-phenazine hydrochloride) (Sigma, cat. no. N4638) (see REAGENT SETUP)
- Dulbecco's formulation PBS tablets (Bio-Whittaker, cat. no. 17-512F) (see REAGENT SETUP)
- Trypsin–EDTA (0.05% wt/vol trypsin, 0.02% wt/vol EDTA) (BioWhittaker, cat. no. BE17-161E)
- Medium adequate for cell requirements (e.g., Eagle's minimum essential medium (EMEM)) (BioWhittaker, cat. no. BE12-125F)
- Medium additives adequate for cell requirements (e.g., 10% fetal or new born calf serum, heat inactivated) (GIBCO, cat. no. 10108-165); 200 mM L-GIn (BioWhittaker, cat. no. BE17-605E); 50 mg l^{-1} gentamycin sulfate (BioWhittaker, cat. no. 17-518Z); 2 mg l^{-1} Fungizone (BioWhittaker, cat. no. BE17-636E); nonessential aminoacids (BioWhittaker, cat. no. BE13-114E).
  ▲ **CRITICAL** Complete medium with additives and serum should be stored at 4 °C for no longer than 2 weeks.
- Glacial acetic acid (Sigma, cat. no. 537020)
- Ethanol 96% (Riedel-de Haën, cat. no. 32294)
- 0.4% (wt/vol) Trypan blue in 0.9% ClNa solution (Sigma, cat. no. T8154)
- Glutaraldehyde 5% by dilution of the commercial 25% (Sigma, cat. no. G6257) (optional)

EQUIPMENT

- Tissue culture incubator, humidified, 5% CO_2/95% air, with temperature adjustable to the requirements of the cells
- Inverted microscope

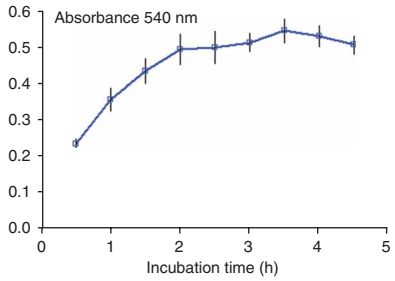

Figure 2 | Relationship between the time of incubation with neutral red and the absorbance at 540 nm after the assay of neutral red. SH-SY5Y human neuroblastoma cells were seeded in a 96-well tissue culture plate and incubated for 24 h. Neutral red medium was added to every column for an incubation period of up to 4.5 h, then the dye was extracted with acidified ethanol solution.

- Centrifuge
- Vertical spectrophotometer (540 nm) or spectrofluorimeter, ideally with excitation and emission wavelengths of 530 and 645 nm, respectively
- Flow hood (for hazardous chemicals)
- Shaker for microtiter plates
- Cell counter or hemocytometer
- Tissue culture flasks, 75 cm^2 (Falcon Becton Dickinson, cat. no. 353135)
- Flat-bottomed 96-well tissue culture plates (Falcon Becton Dickinson, cat. no. 353072)
- Centrifuge tubes, 50 ml polypropylene (Falcon Becton Dickinson, cat. no. 352070)
- 12-well multichannel pipette and tips (Gilson)

REAGENT SETUP

PBS, calcium and magnesium free Can be prepared from Dulbecco's formulation PBS tablets (Bio Whittaker, cat. no. 17-512F) and stored at 4 °C for no longer than 4 weeks.

Neutral red stock solution 4 mg ml^{-1} Dissolve 40 mg neutral red dye in 10 ml PBS. Store up to 2 months at room temperature (20–30 °C) protected from light by foil.

Neutral red medium 40 μg ml^{-1} In centrifuge tubes and under sterile conditions dilute 1:100 the neutral red stock solution with culture medium, that is, 12 ml of medium plus 0.12 ml of the stock solution per plate. Incubate overnight at the temperature of culture of the cells. ▲ CRITICAL Neutral red medium should by prepared the day before use.

Neutral red destain solution 50% ethanol 96% (Riedel-de-Häen), 49% deionized water, 1% glacial acetic acid (Sigma): prepare the required volume, that is, 10 ml water, 10 ml ethanol 96% and 0.2 ml glacial acetic acid. ! CAUTION Glacial acetic acid is corrosive (see material safety data sheet available at http://www.sigmaaldrich.com/catalog/search/ProductDetail/SIAL/537020/) and ethanol is highly flammable (see material safety data sheet available at http://www.sigmaaldrich.com/catalog/search/ProductDetail/RIEDEL/32294/). Therefore, prepare in a flow hood wearing safety glasses and gloves.

Treatment medium As the presence of serum may cause interference and, in particular, reduction in sensitivity of chemicals with high protein affinity, the treatment step can be carried out using 5 or 1% serum or even chemically defined medium in all the columns. However, a decrease in sensitivity could be produced in the assay if the growth of control cells is reduced under these conditions. For this reason, 5% serum is a good option for most cases. Treatment medium with additives and serum should be stored at 4 °C for no longer than 2 weeks.

Treatment solutions Test solutions must be freshly prepared in treatment medium immediately before use, unless stability data demonstrate the acceptability of storage.

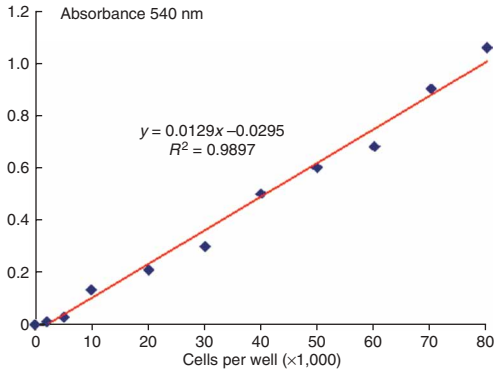

Figure 3 | Relationship bewteen the number of cells and the absorbance at 540 nm after the assay of neutral red. Neuro-2a mouse neuroblastoma cells were seeded at different concentrations in a 96-well tissue culture plate. Neutral red medium was immediately added, and after 3-h incubation at 37 °C the uptake was quantified at 540 nm, extracting the dye with acidified ethanol solution.

PROCEDURE

Cell seeding ● TIMING Day 1, 35 min

1| Decant the medium the cells are growing in and rinse the cells by gentle agitation in PBS-without Ca^{2+} and Mg^{2+} at the culture temperature; this removes any remaining serum, which might inhibit the action of the trypsin, for example, 20 ml is used for a 75-cm^2 flask. Discard the PBS. It is best if primary cells are used as soon as possible after they are obtained and that cell lines are in an exponential growth phase before use.

▲ CRITICAL STEP Although most cultured cells grow at 37 °C, this assay is applicable to other cell types with different optimal growth temperatures, if the reagents and incubation conditions are considered.

▲ CRITICAL STEP The procedures on the first 2 d (Steps 1–17) should be carried out under aseptic conditions and in the sterile environment of a laminar flow cabinet.

2| Add ~2 ml trypsin–EDTA solution at the culture temperature to the monolayer, agitate gently, remove excess trypsin–EDTA solution and incubate the flask at the culture temperature for 2–5 min.

3| Lightly tap the flask to detach the cells and add complete culture medium, that is, 4 ml for a 75-cm^2 flask. Gently triturate to ensure that a single-cell suspension is obtained.

▲ CRITICAL STEP To avoid adhesion of the cells, it is recommended to transfer the suspension to a cell culture-treated tube and proceed quickly.

4| Count a sample of the cell suspension obtained from the cell line or the primary culture from the donor using a cell counter or a hemocytometer. It is also recommended to check that cell viability is ≥95%, for example, using trypan blue exclusion.

5| Dilute the cells with complete medium, preparing at least 15 ml per plate of a uniform cell suspension of an adequate cell density according to the cell type and the design of the study. Approximately 5×10^4 cells ml^{-1} is adequate for most cells and applications. Some recommendations for several commonly used cells are given in **Table 2**. Note that nonadherent cells can be used, preferably in round-bottomed 96-well plates. It is important to include a 5-min centrifugation step (400*g*) before each change of reagents.

TABLE 2 | Recommended cell concentration of the suspension to be applied in 0.2 ml to the wells of the 96-well plates.

Cell name	Cell concentration (cells ml^{1})	Adaptation*/treatment periods (h)	Temperature (°C)
B9 Mouse hybridoma (suspension)	2.5×10^4	24 + 24	37
Balb/c mouse 3T3 fibroblasts	5×10^4	24 + 24	37
CTLL-2 mouse cytotoxic T cells (suspension)	2.5×10^4	24 + 24	37
HepG2 human hepatoma	1×10^5	24 + 24	37
Macrophages	5×10^5	24 + 24	37
Neuro-2a mouse neuroblastoma	5×10^4	24 + 24	37
PLHC-1 topminnow *Poeciliopsis lucida* hepatocellular carcinoma	4.5×10^5	24 + 24	30
RTG-2 rainbow trout gonad fibroblasts	4.5×10^4	48 + 24	20
SH-SY5Y human neuroblastoma	9×10^4	48 + 24	37
Vero monkey kidney fibroblasts	6×10^4	24 + 24	37

This is appropriate for most applications and should use the cells under exponential growth conditions with 60–70% confluence.*Adaptation period, time necessary for the adhesion of the cells to the surface of the plate and to proliferate adequately.

6| Using a multichannel pipette, dispense 200 µl of PBS only into the peripheral wells of a 96-well tissue culture microtiter plate (blanks). As different authors have detected a decrease in cell growth in the wells on the outer perimeter of the plates, they recommend the use of cells only in the inner wells (see **Fig. 4**).

7| Agitate gently the cell suspension and place it in a sterile reservoir.
▲ **CRITICAL STEP** Ensure a uniform cell suspension by filling and ejecting contents back several times with the multichannel pipette. Repeat periodically for even distribution.

8| Dispense 200 µl of the cell suspension into the 60 remaining wells of the plate.

9| Cover the plate and incubate the cells at adequate conditions (temperature, CO_2, humidified atmosphere) until they form a half-confluent monolayer, generally overnight. This incubation period allows for cell recovery and adherence and for exponential growth.

Cell culture treatment ● TIMING Day 2, 30 min

10| After incubating, check the correct growth of the cells under a phase-contrast inverted microscope.

11| Prepare all test solutions immediately before use to avoid problems of stability and precipitation of the compound or medium proteins. Use sterile conditions and, if possible, sterilize the solutions through a 0.22-µm filter. There are many possible designs according to the aims of the study, but a very common format may be the following distribution of the columns (C) (see Steps 12–15): C1 and C12: blanks, with medium, C2: control culture with treatment medium, C3: solvent control, with treatment medium containing solvent in the maximum used (if necessary, DMSO, methanol, ethanol) and C4–C11: the test chemical, virus, etc. in treatment

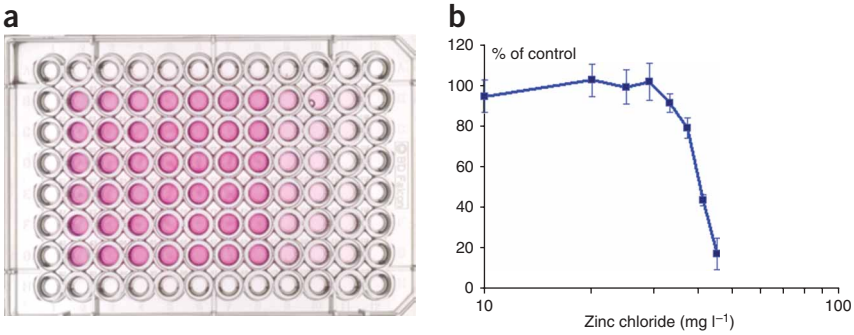

Figure 4 | Neutral red cytotoxicity test. (**a**) A typical image of a 96-well culture plate with extracted neutral red at the end of the assay. SH-SY5Y human neuroblastoma cells were exposed to a range of concentrations of zinc chloride for 24 h. Neutral red medium was added, and after 3 h incubation at 37 °C the plate was washed and the dye was extracted with acidified ethanol solution. A decrease in color is evident in the columns on the right. (**b**) The same plate was quantified at 540 nm. The mean value from the wells without cells was subtracted from the rest of wells, and the values of treated cultures were referred to control nonexposed cultures, to obtain a typical dose–response inhibition curve.

medium in increasing concentrations, e.g., 0.0001, 0.001, 0.01, 0.1, 1.0, 10, 100, 1,000 μg ml^{-1} in a range-finding experiment. Rows A and G can be used to check possible interference of the test solutions with neutral red.

12| In sterile conditions, aspirate or decant the culture medium from the plates.

13| Add 200 μl of treatment medium without chemicals to the wells of columns 1, 2 and 12.
▲ **CRITICAL STEP** The treatment medium can be serum-free medium or with 1 or 5% serum. It is important to both minimize the binding of the molecule being evaluated to proteins and also assure the good health of the cells within the exposure period (see ANTICIPATED RESULTS).

14| Add 200 μl of treatment medium containing an equivalent amount of solvent to that applied to the treatment columns (C4–C11) to column 3. Complete medium should be used if solvent is not used.

15| Add 200 μl of the treatment test solutions in increasing concentrations to the wells of columns C4–C11.

16| Incubate the plate at appropriate conditions, generally for 24 h.

17| Prepare the 40 μg ml^{-1} neutral red working solution and incubate overnight at the same temperature as the cells.

Microscopic evaluation (optional) ● **TIMING Day 3, 10 min**
18| Examine the cultures under a phase-contrast inverted microscope, recording changes in morphology of the cells due to cytotoxic effects of the test chemical. The highest tolerated dose (HTD) can be determined, that is, the concentration causing only minimal morphological changes compared with control cultures. Score severe alterations such as growth inhibition, vacuolization, rounding, detachment and lysis by a + to ++++ rating system for evaluation of the slope of effective concentrations.

Neutral red uptake assay ● **TIMING Day 3, 3 h**
19| Centrifuge the neutral red medium for ~10 min at 600*g* (~1,800 r.p.m) to remove any precipitated dye crystals.

20| Aspirate off or decant medium from cells.
! **CAUTION** When discarding medium from the cells, the properties and concentrations of the test agents applied should be taken into consideration; discard all chemicals and biologicals in accordance with appropriate regulations.
▲ **CRITICAL STEP** The final part of the procedure (Steps 20–33) does not need to be carried out under sterile conditions or with sterilized materials.
▲ **CRITICAL STEP** A washing step has been omitted in this version of the assay, as it is not necessary in most cases. However, if precipitation of neutral red is induced by the chemical, it is recommended to wash the cells with 150 μl PBS per well or covering or immersing the plate in PBS and to remove the washing solution by gentle tapping.

21| Place the neutral red medium into a reservoir. Do this gently to avoid disturbing the crystals at the bottom of the tube.

22| Add 100 μl of neutral red medium to each well of the plate.

23| Incubate the plate for 2 h at the appropriate culture conditions. Note that 2 h is generally adequate and 1 h can be used with a small loss of sensitivity. If there are low cell densities or cells with low metabolic activity, this can be increased up to 4 h.

24| Inspect the plates with an inverted microscope to check the possible precipitation of neutral red. The differences in neutral red uptake can also be evaluated morphologically (optional).

25| Remove the neutral red medium.

26| Wash the cells with 150 μl PBS per well by covering or immersing the plate in PBS and removing the washing solution by gentle tapping.
▲ **CRITICAL STEP** A fixation step before the addition of the destain solution has been omitted in this version of the assay. This is less hazardous than the original method. However, for low adherent cultures it is recommended to include a centrifugation step or to perform a quick washing–fixation, for example, no more than 2 min with 5% glutaraldehyde in a flow hood.
Note: Glutaraldehyde is toxic (see material safety data sheet available at http://www.sigmaaldrich.com/catalog/search/ProductDetail/SIAL/G6257/).

27| Add 150 μl neutral red destain solution per well.
! **CAUTION** The solution is flammable.

28| Shake the plate rapidly on a microtiter plate shaker for at least 10 min, or until the neutral red has been extracted from the cells and has formed a homogeneous solution.

29| Measure the OD of neutral red extract at 540 nm in a microtiter plate reader spectrophotometer, using blanks which contain no cells as a reference.
▲ **CRITICAL STEP** Higher sensitivity with less interference can be obtained using a spectrofluorimeter with excitation and emission wavelengths of 530 and 645 nm, respectively.
? TROUBLESHOOTING

30| Save the data in an appropriate file format for subsequent analysis.

31| Discard the destain solution, preferably into a waste container for nonhalogenated solvents, and discard the plate. If it is convenient, before discarding the plate, a subsequent quantification of the total content of protein can be performed. Wash the plate once with PBS, twice with deionized water, remove the washing solution by gentle tapping and store the plate at 20 °C until analysis[17,18].
■ **PAUSE POINT** Quantification of the total content of the protein and data analysis can be performed at a later date.

32| Draw dose–response curves and, where possible, calculate the concentration of a test chemical reflecting a 50% inhibition of the uptake (IC_{50}) and the confidence interval using a mathematical model, for example, a Hill function or logistic regression. Alternatively, graphical fitting methods can be employed. In this case, the use of probability paper is recommended (*x* scale: log, *y* scale: probit), as in many cases the concentration–response function will become almost linear after this transformation.

33| It is recommended to repeat the experiment at least three different times, if possible using a range of concentrations covering 0–100% effects.

? TROUBLESHOOTING
Troubleshooting advice can be found in **Table 3**.

TABLE 3 | Troubleshooting table.

Problem	Possible reasons	Solution
Low signals in nontreated cultures	Low cell density	Increase cell density
	Cells probably have low metabolic rate	Increase cell density
	Cell detachment	Use fixation
	Cell culture aging or microbial degeneration	Use another batch of cells
	Dye precipitation	Check preparation and temperature
Low signals in treated cultures	Dye precipitation induced by the test agent	Wash with PBS after decanting the test medium
		Increase buffering capacity of NR medium
High signals	Interference of chemical	Wash with PBS after decanting the test medium
	Reading interferences	Use fluorescence reading

● **TIMING**
Steps 1–9, cell seeding: day 1, 35 min
Steps 10–17, cell culture treatment: day 2, 30 min
Step 18, microscopic evaluation (optional): day 3, 10 min
Steps 19–32, neutral red uptake assay: day 3, 3 h

ANTICIPATED RESULTS
The results of the standard neutral red cytotoxicity assay are dependant on two main factors. First, they are dependent on the number of viable cells in the culture. There is a linear relationship between the neutral red extractable from a culture and the

number of viable cells in that culture[1]. **Figure 3** shows the relationship between the number of Neuro-2a mouse neuroblastoma cells and the absorbance at 540 nm after the uptake of neutral red. Second, the results are dependent on the precise lysosomal viability or function of these cells. The term 'viability of a culture' represents, in the classical sense, the percentage of cells which express certain characteristics reserved to live cells, for example, the exclusion of a dye such as trypan blue.

Figure 4a shows a 96-well tissue culture plate with extracted neutral red at the end of the assay. SH-SY5Y human neuroblastoma cells were exposed to a range of concentrations of zinc chloride for 24 h and the assay was then carried out. A decrease in the color is evident in the columns of the right, exposed to higher concentrations of the chemical. An estimation of the number of viable cells according to the neutral red uptake method is made on each group of treated wells. The results obtained under test conditions are compared with the appropriate control and habitually converted to a percentage value. The concentration–response cytotoxicity curves obtained from neutral red uptake assay are usually sigmoidal or exponential in shape. **Figure 4b** shows the graph obtained with the neutral red uptake assay in SH-SY5Y human neuroblastoma cells exposed to zinc chloride for 24 h. The IC_{50} value obtained was 40.07 mg l^{-1}.

The system is likely to underestimate the toxicity of chemicals which require metabolic activation to a toxic product and of substances which bind to serum proteins. However, in some cases it is possible to use metabolically competent cells and serum-free medium. Occasionally, some chemicals may induce the irreversible precipitation of the neutral red dye into fine, needle-like crystals, resulting in an overestimation of the toxic effects.

Any chemical having a localized effect on the lysosomes will result in low or high uptake. However, this factor makes the system useful in mechanistic studies to detect those chemicals which selectively affect the lysosomes, for example, chloroquine[19]. To have an estimation of lysosomal function avoiding the interference produced by changes in cell proliferation when the procedure is performed in the standard manner in nonstatic cell cultures, the assay can be used in conjunction with other tests capable of determining cell number[6]. It is interesting to note that the sequential use of the neutral red assay followed by total protein may lead to a reduction in the amount of protein estimated to be present in the cultures[18].

ACKNOWLEDGMENTS This work was partially supported by a grant from the Spanish Ministry of Education and Science and FEDER, project CTM 2006-06618.

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