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# A Method for Photomicrography of Cytotoxicity in 96-Well Plates

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**Abstract:** Cell photomicrography is an indispensable part of many *in vitro* cytotoxicity experiments. Nevertheless, currently used methods for qualitative visual estimation have limitations including separate experimental set-up and economic feasibility. To surmount the restrictions of existing methods of photography that employ Propidium Iodide (PI) and trypan blue staining and to exploit the 96-well plate format, we showcase for the first time, a method to photograph Sulforhodamine B (SRB) stained cells. The conventional PI and trypan blue staining was compared with our method for photomicrography of A549 cells and other adherent cells (WI38, B16F1 and L929). It was found that our method achieved visualization results equivalent to those produced using PI staining and superior to the trypan blue staining for adherent cells. By doing away with the necessity for additional experiments, our method radically reduced the time needed for photomicrography, exactly reflected experimental conditions and circumnavigated the need for expensive dyes like PI which require a microscope with fluorescent attachment. In brief, our method is an improvement over the presently available methods especially for photography of adherent cells.

**Key words:** Photomicrography, SRB, cytotoxicity, propidium iodide, trypan blue

## INTRODUCTION

Cytotoxicity or toxicity to cells can follow many pathways, which include apoptosis, (Green and Kroemer, 2005), lysis of the cell membrane (Riss and Moravec, 2004) and alteration of topoisomerase activity (Leteurtre *et al*., 1994) among others. The study of cytotoxicity is an important aspect of biological inquiry in order to determine whether the compounds being used as pharmaceuticals or cosmetics are nontoxic, or whether they are designed as therapeutic agents for which cytotoxicity may be crucial to their action. New drugs, cosmetics, food additives, and many other substances go through extensive cytotoxicity testing before they are released for use by the public. Assessment of cell membrane integrity is commonly used to measure cell viability and cytotoxicity. Cytotoxic compounds frequently compromise membrane integrity. Healthy cells are normally impermeable to vital dyes such as trypan blue and PI. However, with loss of membrane

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integrity, these dyes can freely cross the membrane barrier and stain intracellular components due to which dead cells appear coloured blue in the case of trypan blue (Dougherty *et al*., 2006; Tolnai, 1975) and red in the case of PI staining (Düchler and Stepnik, 2008; Deitch *et al*., 1982). In the case of the latter, however, imaging techniques which employ fluorescence rely on the specific microscopy used as well as different bioanalytical techniques and chemicals used for its detection. The Sulforhodamine B (SRB) assay was developed by Skehan *et al*. (1990) to evaluate drug-based cytotoxicity for extensive drug-screening applications. The principle is based on the affinity of the fluorescent protein dye Sulforhodamine B to basic amino acid residues of Trichloroacetic Acid (TCA) fixed cells. Under acidic influence the dye binds to and under basic conditions it can be extracted from the cells and solubilized for colorimetric measurement. This method has worldwide application and is recommended by National Cancer Institute (NCI) for routine drug screening (Monks *et al*., 1991; Perez *et al*., 1993). Accordingly, the SRB assay for cytotoxicity can additionally be harnessed for quick and easy photomicrography of cytotoxicity and would benefit many biological researchers.

In this study, we have comparatively analysed the merits and demerits of this simple, precise, economical and user-friendly method with conventional methods for photomicrography. Some drawbacks of the routine staining methods include multiple steps and processing of cells which may damage the cell membranes and cause a shift in the percentage cell population of live and dead cells. For instance, the trypan blue dye exclusion assay requires detaching, staining and loading the cells on a hemocytometer. Alternately, PI staining is time consuming and requires expensive materials and an elaborate infrastructure not easily available to researchers in an environment where resources are scarce. PI and trypan blue staining methods only detect dead cells and can be used to quantify the percentage population of live and necrotic cells. However photomicrography using these staining techniques would entail separate experimental set-ups. Giemsa staining of cells (Freshney, 2000) is purely for visualization and cannot be used for quantification of cytotoxicity.

In spite of the many hallmarks of cytotoxicity estimated by current methods, like cell membrane lysis or release of cytoplasmic enzymes (Mitchell *et al*., 1980) a decrease in the viable cell population remains the trademark of a cytotoxicity assay. Renvoize *et al*. (1998) have suggested that as a rule, classification of cell death in a given model should always include morphological examination coupled with at least one of the other assays. Differential uptake of fluorescent protein binding dyes (such as SRB staining) is a preferred method for its speed, simplicity and precision. This assay involves a cell fixation step, due to which the 96-well plates can be conveniently stored and processed later. SRB permeates all cells and stains them pink (Vichai and Kirtikara, 2006). Adherent cells after fixation are stained by the SRB dye and indirectly infer cell viability. A comparison of the treated cells with the untreated cells would give an estimate of cytotoxicity as the color intensity is directly proportional to the number of cells. Consequently, the absorbance values are indirectly proportional to the cytotoxicity i.e., more the color, less the cytotoxicity and vice versa.

The 96-well plate format is well suited for investigation of multiple cell types using a broad concentration range of test compounds. It is also appropriate for high throughput drug screening. To surmount the limitations of current methods that visualize cytotoxicity *in vitro*, and to exploit the microplate format, we submit a technique for photomicrography of SRB stained cells performed exclusively in a 96-well plate.

## MATERIALS AND METHODS

The SRB and trypan blue dye exclusion experiments were carried out during the period January 2005-June 2007 at the Animal Biotechnology Department, The Kelkar Education Trust's Scientific Research Centre, Mumbai. The PI staining and photomicrography were carried out at Advanced Centre for Treatment, Research and Education in Cancer (ACTREC), Mumbai between April-May 2006.

**Cell Lines and Normal Culture Conditions**

All cell lines A549 (a human lung carcinoma cell line), WI38 (a human normal lung fibroblast cell line), B16F1 (a murine melanoma cell line) and L929 (a murine normal fibroblast cell line) were obtained from NCCS (National Centre for Cell Sciences, India). Cells were cultured in either RPMI 1640 medium, MEM or DMEM procured from Sigma St. Louis, MO, USA and supplemented with 10% fetal bovine serum from HiMedia and 20 μg $mL^{-1}$ Genticyn procured from Nicholas Piramal, India Ltd. L929 was supplemented with 10% horse serum from HiMedia. The cells were grown under humidified conditions in an incubator at 37°C with 5% $CO_2$. All tissue culture plates and other plastic-ware were from Nunc (USA).

**Reagents**

Sulforhodamine B (SRB), trypan blue and PI were procured from Sigma St. Louis, MO, USA. All reagents were of AR grade from Qualigens.

**Induction of Cytotoxicity in A549 Cells with PP Treatment**

After 24 h of pre-incubation, cells were treated with the alcoholic extract of an orthopteran insect, designated as PP, at a final concentration of 1 and 8 μg $mL^{-1}$ [prepared in Calcium Magnesium Free Phosphate Buffered Saline (CMFPBS)] for 48 h before they were subjected to SRB, PI or trypan blue staining.

**Microplate Based SRB Staining**

Procedures followed were as described previously by Skehan *et al.* (1990). Briefly, 10 mL of 1-5×$10^5$ cells $mL^{-1}$ was seeded in a 96-well plate. Following treatment, cells were fixed in 30% TCA and placed for 1 h at 8°C, followed by tap water wash. After drying the fixed cells were stained with 0.4% SRB (prepared in 1% acetic acid), washed with 1% acetic acid and dried. At this point the cells were photographed and later quantified by reading on a plate reader at 540 nm (reference filter 630 nm), after solubilization with 10 mM Tris buffer. The inverted microscope (Zeiss Axiovert S100) was set at brightfield (H) position for condenser, under 10X objective, using blue filter with the aperture diaphragm towards the centre. The cells were photographed using a manual Pentax camera K1000.

The calculation was as follows:

$$\text{Percentage cell viability} = \frac{\text{OD of test}}{\text{OD of control}} \times 100$$

$$\text{Percentage inhibition} = 100\text{-Percentage viability}$$

**Propidium Iodide Staining**

Petridishes (35 mm) containing 1-5×$10^5$ cells $mL^{-1}$ were treated with varying concentrations of PP, stained with 20 μL of 1 mg $mL^{-1}$ PI in CMFPBS and immediately photographed using a Zeiss Axiovert 200 M Inverted fluorescent microscope at Cy 3 (excitation 514 nm and emission 566 nm).

**Trypan Blue Staining and Dye Exclusion**

Petridishes (35 mm) containing 1-5×$10^5$ cells $mL^{-1}$ were treated with varying concentrations of PP. On termination the media was decanted to avoid dilution of the dye and 0.5 mL of 0.4% trypan blue: deionised water (1:1) was added to the monolayer of adherent cell lines. Photomicrography was carried out using a manual Pentax camera K1000. The cell viability was estimated using a hemocytometer in separate experiments. Cell mortality was expressed as the percentage of trypan blue positive cells compared to the total number of cells.

## RESULTS AND DISCUSSION

**Microplate Based SRB Staining**

The SRB method was extrapolated for photomicrography in order to visualize adherent cells in a 96-well plate, greatly reducing experimentation time for photography of cells treated with a broad range of concentrations. Using this method, we obtained concurrent photomicrography and quantification of cytotoxicity of the A549 cells (Fig.1a-c). This procedure also aided the study of external morphology of the cells. The same SRB dyed plates were quantified in a plate reader. The untreated cells were assumed to have 100% viability. In comparison to the untreated cells, the A549 cells after exposure to 1 and 8 μg $mL^{-1}$ for 48 h had cell viabilities of 51.42 and 26.9%, respectively.

Figure 2a-c represent SRB stained WI38, B16F1 and L929 adherent cells (untreated) using our 96-well based technique which enhances visual evaluation of cytotoxicity by photomicrography in addition to quantification

**Propidium Iodide Staining**

In Fig. 3a-c, PI staining of A549 cells reveals dead cells stained red while live cells remain unstained. In contrast to SRB based photomicrography and quantification of cytotoxicity, the PI method could not be quantified in the same experiment.

**Trypan Blue Staining and Dye Exclusion**

A549 cells stained with trypan blue indiscriminately appear blue as seen in Fig. 4a and b as this method does not appear suitable for staining cells preceding the disaggregation

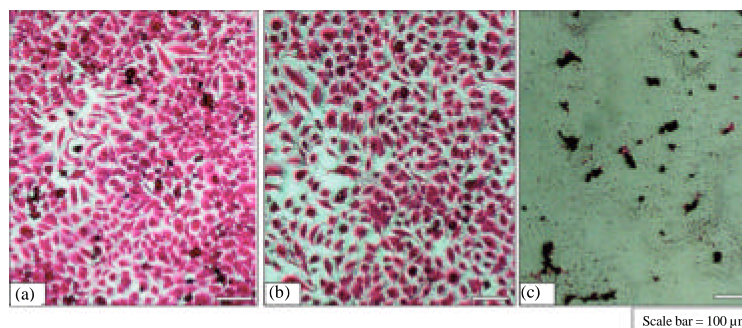


Fig. 1: 96-well plate based SRB staining: (a) A549 untreated cells. (b, c ) A549 cells treated with 1 and 8 μg $mL^{-1}$ of a natural product extract, PP, having cell viabilities of 51.42 and 26.9%, respectively. Treated cells show increasing dose dependent cytotoxicity

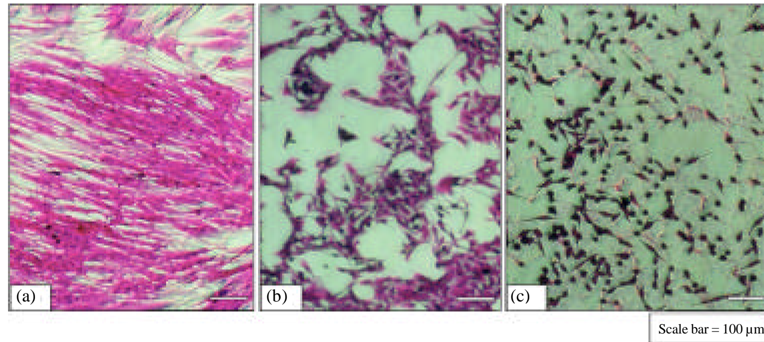


Fig. 2: 96-well plate based SRB staining of untreated cells (a) WI38, (b) B16F1 and (c) L929 cells

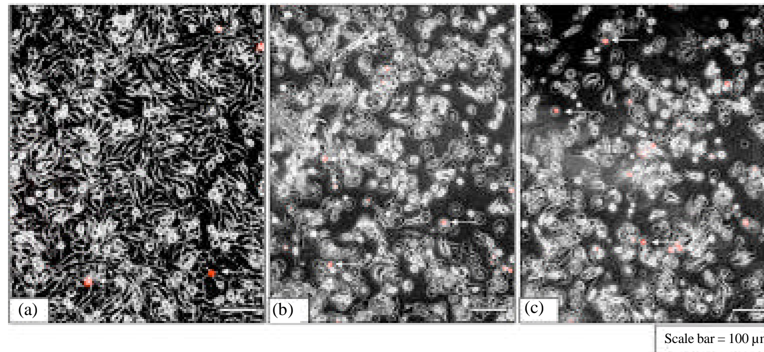


Fig. 3: Conventional PI staining: (a) shows A549 untreated cells which are unstained. (b) and (c) show A549 cells treated with 1 and 8 μg $mL^{-1}$ PP, respectively. Live cells are unstained and dead cells (⟶) appear red in color

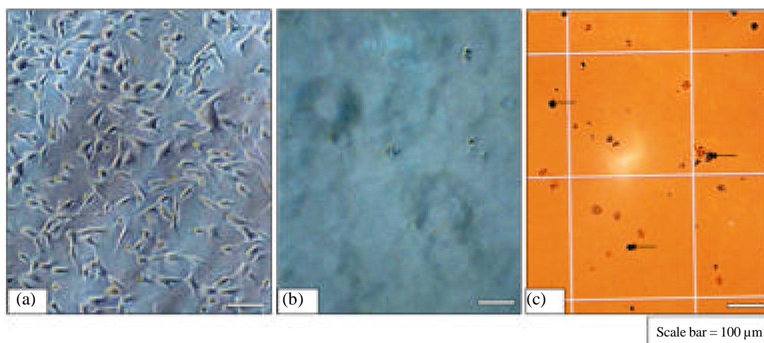


Fig. 4: Conventional Trypan Blue staining: (a) shows A549 untreated cells and (b) shows cells treated with 8 μg $mL^{-1}$ PP. The stained cells do not show distinct differentiation in stain except for reduction in number of cells as seen in (b). (c) depicts A549 cells treated with 8 μg $mL^{-1}$ PP, disaggregated, stained with trypan blue and loaded on a hemocytometer. Dead cells (⟶) are stained blue while live cells are unstained

step. However, if disaggregation is followed by staining and mounting the cell suspension on the hemocytometer, dead cells appear stained blue, while live cells are unstained as observed in Fig. 4c. Nevertheless separate experiments were required for quantification of cytotoxicity by the trypan blue dye exclusion assay.

In summation, visual determination of the live and dead A549 cells was good with PI staining while the SRB method clearly depicted a decrease in cell population with increasing cytotoxicity. We observe that the clarity and definition is sharper in the photographs of the SRB and PI stained cells compared to the trypan blue stained cells, despite the comparatively poor optical quality of the plastic bottomed 96-well plates. Additionally, for high optical clarity, glass-bottomed 96-well plates may be used in order to eliminate this possible limitation, or a microscope with Hoffman modulation contrast may be preferable for optimized quality of the plastic (Davidson *et al*., 1998).

Kashii *et al.* (1994) quantified toxicity on retinal cultures of fetal rats by the trypan blue dye exclusion assay. They were able to concomitantly visualize cell toxicity due to a cell fixation step after staining with trypan blue and photographed the cells under a Hoffman modulation microscope. Similarly, Takeuchi *et al.* (1993) studied cytotoxicity on lymphocytes by the trypan blue method and also employed a cell fixation step which enabled visualization. Since we did not fix the cells for the trypan blue dye exclusion assay, the photomicrography was not optimal. Due to cytotoxicity most cells had lost their adherent properties, which was a deterrent to acceptable focal clarity. Furthermore separate cytotoxicity experiments were required which necessitated disaggregation and counting the cells on a hemocytometer.

Wu *et al.* (1997) achieved visualization of neonatal rat cardiac myocytes using fluorescent DNA-binding dyes H33342 and PI aided by fluorescent microscopy. Nevertheless this method requires costly dyes and sophisticated infrastructure which may not be easily affordable. The present proposed application of the SRB method for analyzing the same experiment for quantification of cytotoxicity as well as photomicrography has the advantages of semi-automation, economic feasibility and accuracy as the cells are fixed *in situ.* Besides, numerous experiments can be carried out in a single microplate as opposed to the conventional methods discussed earlier.

## CONCLUSION

Here, we recommend an added value of photomicrography using the SRB method for cytotoxicity estimation in 96-well plates. Since this method lends itself well to the microplate format, it could be very helpful for cultures difficult to grow in large volumes (e.g., primary explants from patient biopsies or animal samples). Our technique is a significant improvement over conventional cell photography techniques as it is simple, time-saving and can be well adapted for any adherent cell line being analysed for cytotoxicity by the SRB assay. We concluded that by eliminating unnecessary processing, the loss of cells is minimized and this facilitates photomicrography and quantification of cytotoxicity in a single experiment.

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