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Notes & Tips

# Interference in MTT cell viability assay in activated macrophage cell line

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The MTT[1] (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay is a simple colorimetric method to measure cytotoxicity, proliferation, or cell viability first developed by Mosmann in 1983 [1]. MTT is a yellow, water-soluble, tetrazolium salt. Metabolically active cells are able to convert this dye into a water-insoluble dark blue formazan by reductive cleavage of the tetrazolium ring [1]. Formazan crystals, then, can be dissolved and quantified by measuring the absorbance of the solution at 570 nm, and the resultant value is related to the number of living cells. By using 96-well microtiter plates and a multiwell spectrophotometer this assay can be semiautomated to process a large number of samples and provide a rapid measurement of cell number. Therefore this method has been mainly used in the past decade for anticancer drug screening assays on human and mammalian cell lines [2–4], further validated by comparative assays in several cellular systems [3].

The cellular reactions involved in MTT reduction are not completely understood, but the mitochondrial succinate dehydrogenase system seems to be primarily involved [1,5]. Nevertheless, there is currently no convincing evidence that the mitochondria are the only site of MTT reduction in the intact cell and in addition some authors have observed the possible appearance of artifacts when the assay is used on a lung cancer cell line treated with interferon [6].

The aim of this work was to study the reliability of the MTT assay on activated macrophage cell line, to verify the possible interference of metabolism of activated macrophages on MTT reduction. Experiments were performed using the murine macrophage cell line RAW 264.7 and the ovarian carcinoma cell line HeLa as control cells. MTT cell viability tests were compared to the SYTOX Green nucleic acid stain method. This method is based on a cationic nonsymmetrical membrane-impermeant cyanine dye that is normally used to stain dead cells with membrane damage [7]. The dye develops a fluorescent signal after binding to cellular nucleic acids [8]. Here we employed this method to quantitate living adherent cells on 96-well microtiter plates, previous removal of dead cells and after the opportune permeabilization. The SYTOX Green method was chosen as the control assay because it is based on a completely different quantification approach. In fact, while the MTT process depends on enzymatic cellular activities that could be subjected to alterations during different cellular phases, the nucleic acid stain is simply proportionally related to the cell nuclear content, thus avoiding the interference by metabolic artifacts.

## Materials and methods

### *Cells and media*

The RAW 264.7 murine tumor macrophage cell line and the HeLa cell line were grown in DMEM with Glutamax I (GIBCO, Invitrogen, Carlsbad, CA, USA) supplemented with 10% defined fetal bovine serum (HyClone, Logan, UT, USA). They were maintained at 37 °C in a humidified atmosphere containing 5% $CO_2$.

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[1] *Abbreviations used:* MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DMEM, Dulbecco's minimum essential medium; LPS, lipopolysaccharide; NP-40, Nonidet P-40; NBT, nitroblue tetrazolium; PBS, phosphate-buffered saline; iNOS, inducible nitric oxide synthase; DMSO, dimethyl sulfoxide.

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Cells were plated at $10^5$/ml, $2 \times 10^5$/ml, or steady-state confluence in 96-well plates, and after 2 h of incubation, required for cell adhesion, they were stimulated with 10 or 100 ng/ml bacterial LPS [9] or killed with 50 mM $NaN_3$. Cell viability assays were performed after 18 h of incubation with or without stimuli. The iNOS inhibitor *S*-(2-aminoethyl)isothiourea (Calbiochem, Darmstadt, Germany) [10] was added, when necessary, at a final concentration of 100 μM 1 h before the assays.

Standard curves of both cell lines were also performed for each method, to verify the linearity of colorimetric or fluorescence reactions.

*Cell viability assays*

MTT and SYTOX Green assays were carried out simultaneously, in quadruplicate, in the same 96-well plates. The MTT assay was performed following the well-described procedure with minor modifications [3]. Briefly, the wells were washed three times with complete medium, then 180-μl aliquots of medium and 20-μl aliquots of MTT solution (5 mg/ml of PBS) were added to each well at the established time. After 2 h of incubation at 37 °C and 5% $CO_2$ for exponentially growing cells and 15 min for steady-state confluent cells, the media were removed and formazan crystals were solubilized with 175 μl DMSO. The plates were then read on a Microplate reader Model 450 (Bio-Rad Laboratories, Hercules, CA, USA) at 570-nm wavelength.

The SYTOX assay was performed as follows: medium was removed and the wells were washed three times with PBS at 37 °C to completely remove dead cells. Living cells were then permeabilized with lysis buffer (100 mM Tris–HCl, pH 7.4, 154 mM NaCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.1% NP-40). Cell DNA was then stained with 2 μM SYTOX Green nucleic acid stain (Molecular Probes Europe BV, Leiden, The Netherlands).

After dye incubation at 37 °C for 15 min, the plates were immediately read on a Fluostar OPTIMA (BMG Labtechnologies GmbH, Offemburg, Germany) using a 485-nm excitation and a 520-nm emission wavelength.

*iNOS enzymatic assay*

Aliquots of 100 μl containing 0.1 U recombinant murine iNOS (Sigma), 2.5 mM NADPH, 5 mM EDTA, 1 mM $MgCl_2$, different concentrations of MTT (10, 50, 100, 200, and 500 μM), and 100 μM *S*-(2-aminoethyl)isothiourea [10] when necessary were incubated for 20 s, then the reaction was stopped by adding 6 vol *n*-2-propanol with vigorous mixing. The mixture was then centrifuged in a microfuge for 3 min at 14,000 rpm at room temperature. The amount of solubilized MTT formazan salt was then read at 570 nm on a DU 640 spectrophotometer (Bekman, Milan, Italy).

## Results and discussion

Data plotted in Fig. 1 show that the absorbance was directly proportional to the number of cells for both methods and the linearity extended from $7 \times 10^3$ to $112 \times 10^3$ cells. Only data from RAW 264.7 cells are shown. The same experiments performed with HeLa cells gave similar values and patterns.

In our experiments we used SYTOX Green after lysis of adherent living cells with NP-40. This rapid fluorescence staining method is normally used to evaluate dead cells, the dye being impermeable to plasma membrane. The same dye is also suitable for quantifying living cells (after plasma membrane permeabilization) following the previous removal of dead cells. This specific variation of the method can be easily performed when adherent cells are used. In addition, to further validate the method, a group of control cells was treated with 50 mM $NaN_3$, which causes a complete and rapid cell death and the fluorescence signal of which, after accurate PBS washing and dye addition, remained similar to blank values, as expected. So, the reduced relative values observed with the SYTOX stain in cells treated with LPS (Fig. 2, dark bars) are really indicative of partial cell death that occurred after LPS activation.

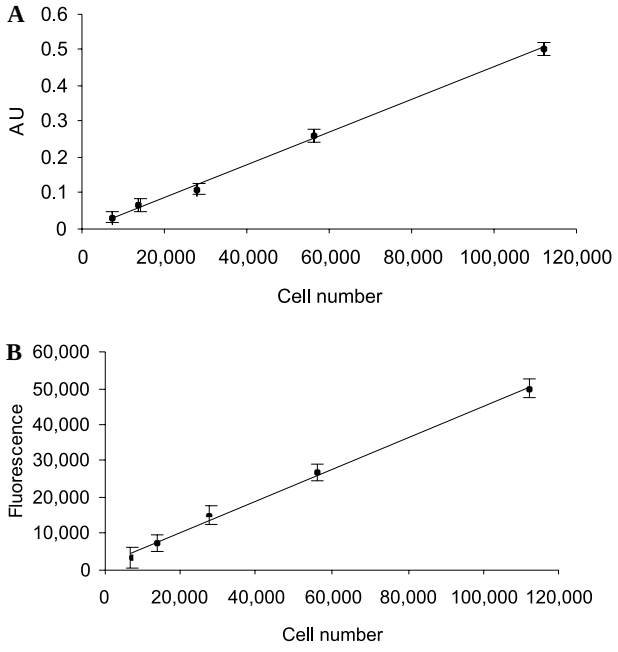


Fig. 1. Linearity of the MTT (A; $r = 0.9975$) and SYTOX Green (B; $r = 0.9989$) assays. RAW 264.7 cells were plated on 96-well microtiter plates starting from $7 \times 10^3$ to $112 \times 10^3$ cells. All data represent the means of four experiments, each of them performed in quadruplicate ($p < 0.05$).

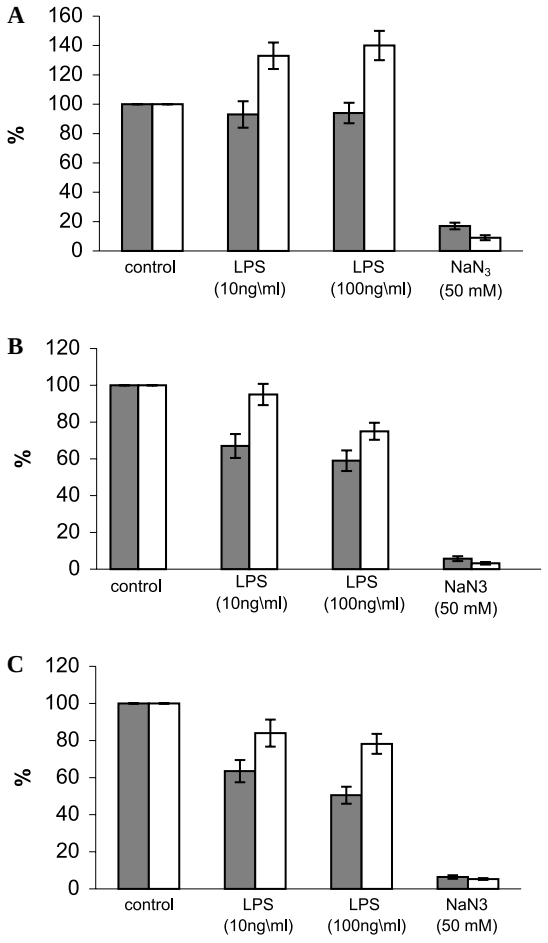


Fig. 2. Comparison of SYTOX Green (gray bars) and MTT (white bars) assays. Values are expressed as percentages of absorbance (MTT) or fluorescence (SYTOX Green) with respect to controls. Plots represent experiments performed on exponentially growing cells at $10^5$ cells/ml (A) and $2 \times 10^5$ cells/ml (B) or at steady-state confluence (C). All data represent the means of four experiments, each of them performed in quadruplicate ($p < 0.05$).

The experiments were performed in 96-well microtiter plates; cells in exponential growth at different concentrations ($10^5$/ml, $2 \times 10^5$/ml; Figs. 2A and B, respectively) or at steady-state confluence (Fig. 2C) were used. Values are expressed as percentages of untreated control cells. In all three cases, cell treatment with LPS at 10 or at 100 ng/ml showed higher relative MTT values compared to those obtained with the SYTOX Green method. In contrast, parallel experiments performed with equally LPS-treated HeLa cells did not show appreciable differences between MTT and SYTOX Green methods (data not shown), as should be expected.

Although Mosmann did not detect any further MTT formazan product in LPS-activated macrophage-like cell line P338D1 [1], our experiments, performed on RAW 264.7 cells, show remarkable differences in the detection of activated cells between the MTT and the SYTOX assays. In particular the MTT method overestimates the number of stimulated cells (varying from 18 to 34% for cells stimulated with 10 ng/ml LPS and from 11 to 45% for cells stimulated with 100 ng/ml LPS) with respect to the SYTOX quantitation, which reveals a partial cell death after LPS treatment (Fig. 2).

As a possible explanation of these results, an earlier study described an increased production of formazan blue from nitroblue tetrazolium (NBT) salts by activated macrophages compared to unactivated cells [11]. Moreover, years ago, the neuronal isoform of nitric oxide synthase was reported to feature a NADPH diaphorase activity that leads to reduction of NBT to blue formazan [12,13]. Furthermore it is well known that activated macrophages deeply modify their metabolism, compared with the corresponding quiescent cells, and express various enzymes responsible for the production of inflammatory cytokines and cellular mediators, among which inducible nitric oxide synthase is one of the best characterized [14]. All this evidence, together with the comparable chemical reactivity of NBT and MTT, seems to suggest a possible involvement of macrophage iNOS in the overestimation of the MTT assay. To validate this hypothesis some preliminary "in vitro"

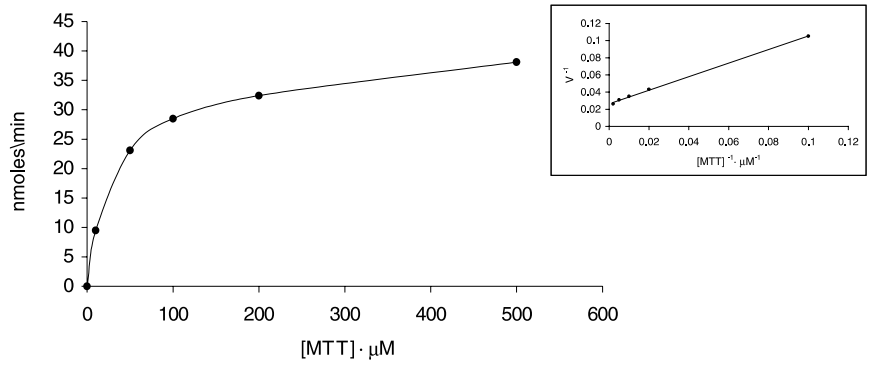


Fig. 3. Activity of recombinant murine iNOS incubated with increasing concentrations of MTT. The plot shows the nanomoles of reduced MTT produced in 1 min by 0.1 U of recombinant murine iNOS at different MTT concentrations. The Lineweaver–Burk plot is also shown, and the resulting $K_m$ for this enzymatic reaction is 28.6 μM.

experiments were performed on recombinant murine iNOS commercially available from Sigma (Milan, Italy). This enzyme was able to reduce the MTT to the blue formazan product in the presence of NADPH, and the kinetic characterization of iNOS activity on MTT, shown in Fig. 3, evidenced a $K_m$ of 28.6 μM. A co-incubation of the enzyme with the iNOS-specific inhibitor *S*-(2-aminoethyl)isothiourea [10] did not modify the kinetic plot (data not shown), and similarly cell pretreatment with this iNOS inhibitor did not modify the MTT overestimation. The high affinity of iNOS for MTT suggests an active interaction of the enzyme with the dye also in living cells, while the ineffectiveness of the inhibitor used suggests the existence of a site for MTT interaction with iNOS, related to the NADPH-oxidizing domain and not to the arginine-binding site where in contrast *S*-(2-aminoethyl)isothiourea and the majority of iNOS inhibitors normally bind [10].

## Conclusions

The MTT assay is widely used to test cellular cytotoxicity of many drugs and other biological agents on various cell types, including macrophages. Here we demonstrate that macrophage activation causes some interference in this assay, leading to overestimation of cell number. Moreover, we provide evidence that iNOS could be primarily involved in the reduction of MTT, suggesting that this could create artifacts also in other iNOS-expressing cell lines.

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