Supplementary material

Part 1: detailed protocol for the double alphazurine-crystal violet viability test

Safety warnings

Acetic acid is an irritant

Ethanol is toxic at high doses

Crystal Violet is a suspected carcinogen and is toxic for the environment

All solutions and wastes used in this protocol should be disposed of respecting applicable local regulations

Materials

Culture plates shall be with a transparent flat bottom and treated for mammalian cell adherence. We use 12 or 24 well culture plates from Falcon (#353043 and #353047, respectively)

Chemicals

Non-denatured ethanol should be used. 96% ethanol is acceptable without correction for titer

Acetic acid is 99% pure. We use reference A6283 from Sigma Aldrich

Alphazurine A was reference A2770 from Sigma Aldrich. This product is discontinued. Alternate references are #ICNA0215473025 from MP biomedicals or reference AC189370100 from ThermoFisher.

Crystal Violet is reference C0775 from Sigma Aldrich.

PBS, calcium and magnesium free, is reference D5652 from Sigma Aldrich

Equipment

A plate reader able to read absorbance is required. The required wavelengths to be read are 590 nm (Crystal Violet) and 637 nm (Alphazurine)

Test Setup

The rationale of the alphazurine stain (staining of dead cells in the plate) means that the absorbance reading at 637 nm will provide an index of the number of dead cells. This index must be scaled against wells that will contain only dead cells and thus represent a 100% dead cells level. This means that a plate designed for a complete test shall be arranged as follows

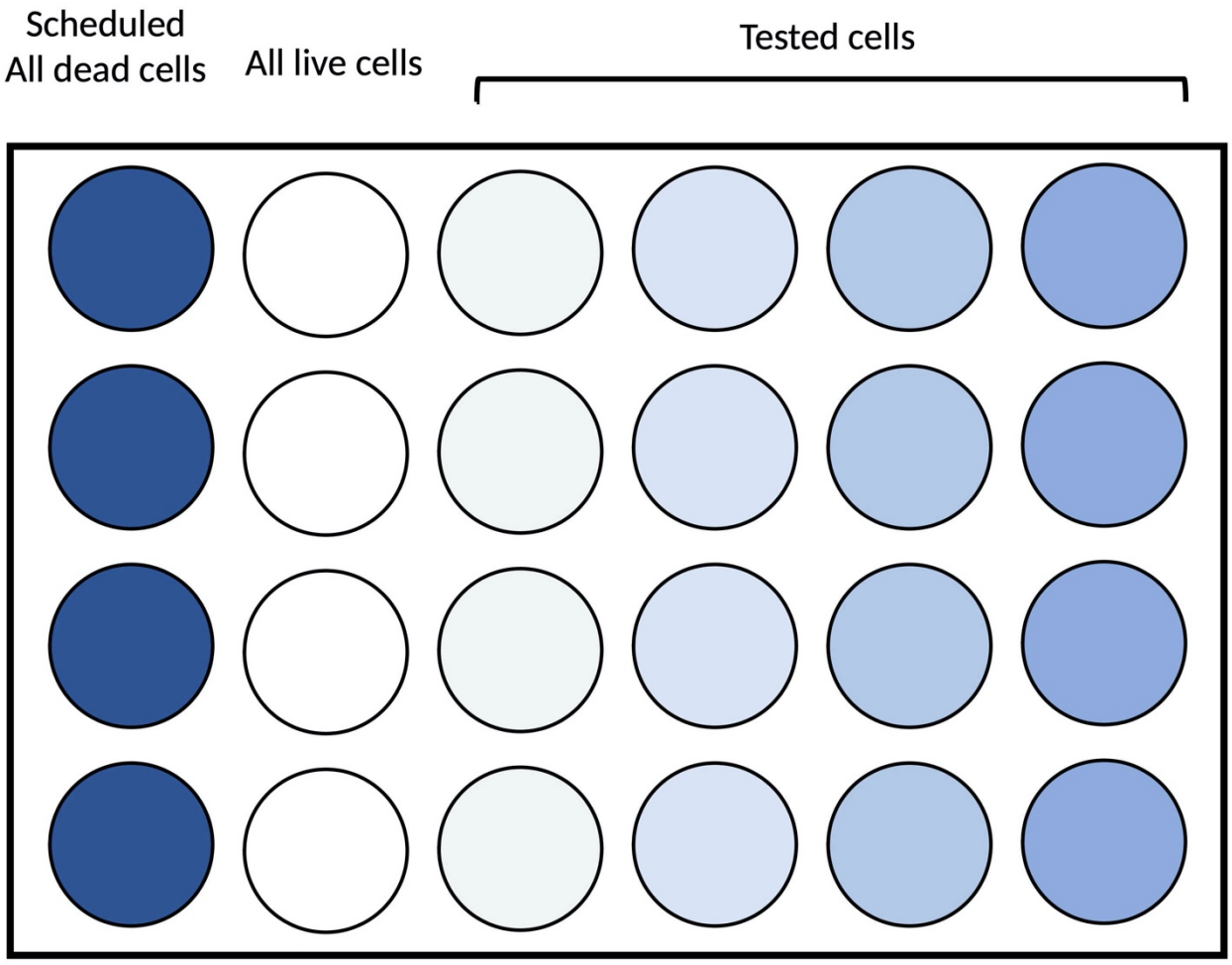

Fig. S1: Schematic rationale of the alphazurine staining

Note: we do not recommend to go below four replicates by condition, which is an absolute minimum.

Protocol

Table S1: step-by-step detailed protocol

1) Plate preparation	
Centrifuge cell culture plate for 2 min at 1200 rpm	Centrifugation prevents any loss of cells
Remove the medium from well scheduled for 100% dead cells control add 300µL of Ethanol (EtOH) 50% for 3-5 min at RT	The addition of ethanol induces cell death and mak their membranes permeable
Rinse with DMEM live cell wells	The medium may have been affected by the 48h culture period or the presence of particles, prompti its replacement with a fresh one
Remove EtOH 50% Rinse wells once with clean culture medium Add a volume of clean culture medium	The exposure to ethanol dehydrates the cells, necessitating hydration before dyeing them

2) Alphazurine dye staining	
Add AlphazurineA at a final concentration of 0,2 mg/mL in all wells (50 fold direct dilution from a 10 mg/ml in water)	Alphazurine is added to fresh medium to ma cells in a favourable environment while stai ensuring that viability remains uncomprom
Incubate 40 min at 37°C	Incubating cells under cultured conditions er that viability remains uncompromised
Centrifuge 3 min at 1200 rpm	Centrifugation prevents any loss of cells
Rinse 3x with 1X Phosphate-buffered saline (PBS) Centrifuge for 3 min at 1200 rpm after each rinse	Rinsing cells with PBS facilitates the removal that have not entered the cells Centrifugation prevents any loss of cells
Remove the supernatant	The last rinse with PBS has to be remove b adding the elution solution
Elute by adding a volume (equivalent to cell culture medium used) of 50% ethanol 1% acetic acid solution	Adding acetic acid to ethanol enhances the solubility
Incubate 30 min at RT on a shaker Centrifuge for 2 min at 1200 rpm	Centrifugation prevents any loss of cells
(Optional) Transfer 200µL into a 96-well plate	To eliminate the potential interference from absorbance on the original plate, it is advisa transfer a portion on supernatant into a new
Read absorbance at 637 nm	

3)Crystal violet dye	
Remove the remaining alphazurine eluate Rinse once with elution solution, then once with PBS	The elution solution dehydrates the cells, necessita hydration before dyeing them with another dye
Add one volume (equivalent to cell culture medium used) of CV solution at 4 µg/ml in PBS	As the cells are dead, exposure to water poses no ri them, so this solution can be prepared using either w PBS
Incubate 30 min at RT	It is not necessary for cells to be under culture cond for this staining, as all cells are dead
Centrifuge 3 min at 1200 rpm	Centrifugation prevents any loss of cells
Rinse 3x with PBS Centrifuge for 3 min at 1200 rpm after each rinse	Rinsing cells with PBS facilitates the removal of dy have not stained the cells Centrifugation prevents any loss of cells
Remove the supernatant	The last rinse with PBS has to be remove before add elution solution
Elute by adding a volume (equivalent to cell culture medium used) of 50% EtOH 1% acetic acid solution	Adding acetic acid to ethanol enhances the dye solu
Incubate 45 min at RT on a shaker Centrifuge for 2 min at 1200 rpm	Centrifugation prevents any loss of cells
(Optional) Transfer 200µL into a 96-well plate	To eliminate the potential interference from cell abso on the original plate, it is advisable to transfer a port supernatant into a new plate
Read absorbance at 590 nm	

Readout

The alphazurine stain provides an index of the number of dead cells. Thus, in addition to the desired measurements, the plate(s) must contain wells with all live cells (or the best approximation thereof) and wells with only dead cells. There are therefore three sets of measurements in the plate(s).

$A637_{\text{all live cells}}$

$A637_{\text{all dead cells}}$

$A637_{\text{tested cells}}$

The $A_{\text{all live cells}}$ values can be used as a background value, as it integrates the absorbance due to stain adsorbed onto plastic, absorbance of the plate plastic itself and the absorbance due to the few dead cells that may exist in a healthy cell culture.

Then the 100% scale can be calculated with the formula $A637_{\text{all dead cells}} - A637_{\text{all live cells}}$

Then, for each tested condition, the fraction of dead cells in the well can be calculated by the formula

$\text{Fraction}_{\text{dead}} = (A637_{\text{tested cells}} - A637_{\text{all live cells}}) / (A637_{\text{all dead cells}} - A637_{\text{all live cells}})$

However, these fractions also depend of the total number of cells that are present at the end of the alphazurine reading. Depending on how the tested chemicals act on cells, dead cells may still be adherent to the plate (which is the case for e.g. ethanol) or dead cells can detach or disintegrate and thus be no longer present in the plate when the alphazurine absorbance is read. To compensate for this phenomenon, the crystal violet staining will provide an index of the total number of cells (either initially dead or alive) that are still present at the bottom of the well.

Thus, the fraction of cells present in the well is given by the formula

$\text{Fraction}_{\text{present}} = (A590_{\text{tested cells}} / A590_{\text{all live cells}})$

This allows to calculate the fraction of live cells compared to the initial cell input, which is the required value

$\text{Fraction}_{\text{live}} = \text{Fraction}_{\text{present}} - \text{Fraction}_{\text{dead}}$

Part 2: Evaluation of interference between zinc oxide or silver nanoparticles and the dyes used in the VVBlue assay

Methodology:

J774A.1 cells were seeded at 500,000 cells/ml in 24 well plates. After adhesion for 24 hours, the cells were treated with 5 or 15 µg/ml of zinc oxide nanoparticles or with either 25, 50 or 100µg/ml of silver nanoparticles. The cells were left in contact with the nanoparticles for 24 hours, after which the culture medium was removed and the cells killed with 50% ethanol. The cells were then submitted to the VVBlue assay, and microscopy images were taken at the end of the alphazurine staining step (i.e. just before elution) and at the end of the crystal violet staining step. The absorbance of the eluates was then compared to the one of untreated, control cells. The experiment was carried out on four independent wells per conditions.

Results:

1) Zinc oxide nanoparticles

Treatment of the cells with zinc oxide resulted in massive cell death accompanied with massive cell detachment (Fig S2a and S2b).

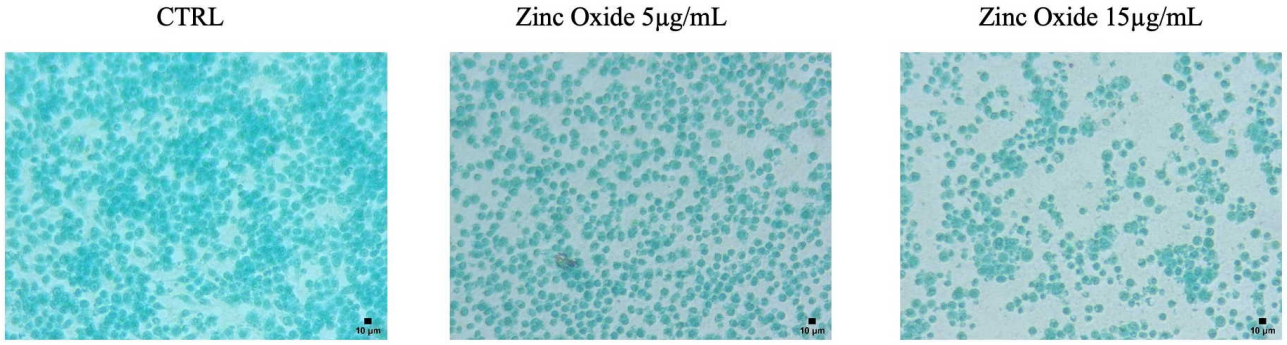

Figure S2a: microscopy imaging of the zinc oxide-treated cells after alphazurine staining

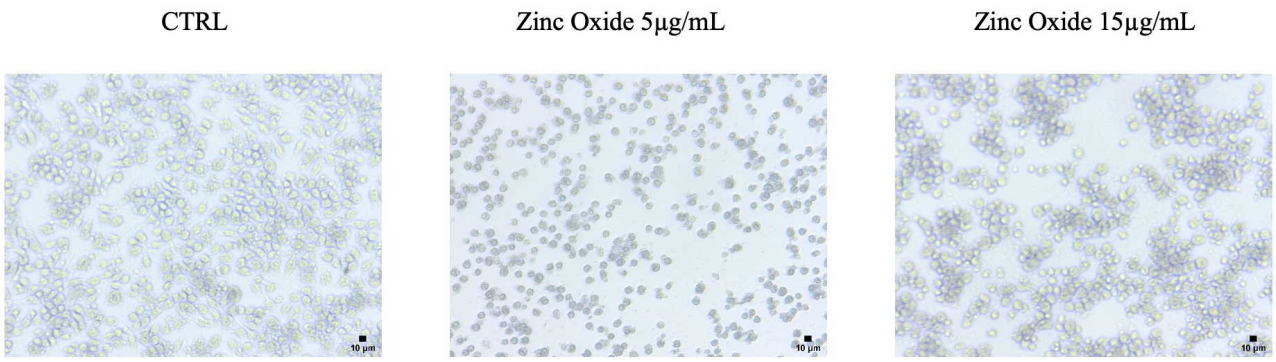

Figure S2b: microscopy imaging of the zinc oxide-treated cells after crystal violet staining

This translated into much lower signals compared to the control wells, as can be seen in Table S2a

Table S2a: absorbance obtained for both dyes for ZnO-treated J774A.1 cells

Condition	Control ((mean±Std dev)	ZnO 5 µg/ml	ZnO 15 µg/ml
Alphazurine absorbance (637 nm)	1.22±0.1	0.62±0.04	0.67±0.04
Crystal violet absorbance (590 nm)	0.2±0.01	0.16±0.004	0.16±0.007

2) Silver nanoparticles

Treatment of the cells with silver nanoparticles did not result in a massive cell detachment (Fig S2c and S2d), as opposed to zinc oxide.

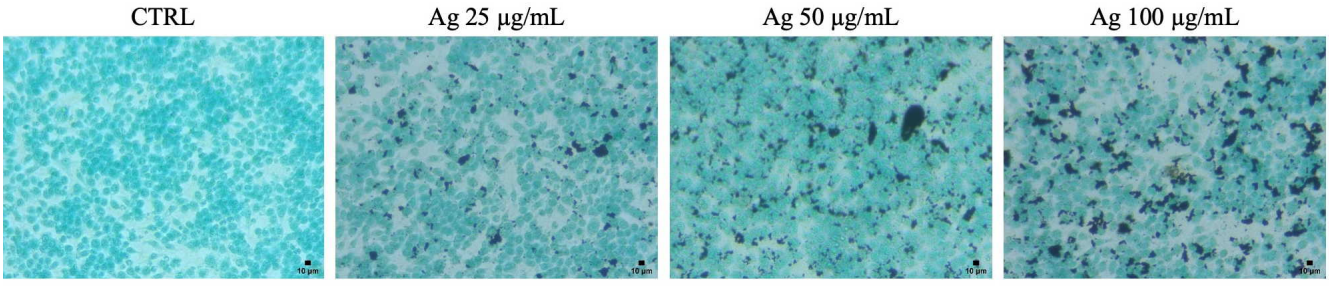

Figure S2c: microscopy imaging of the silver-treated cells after alphazurine staining

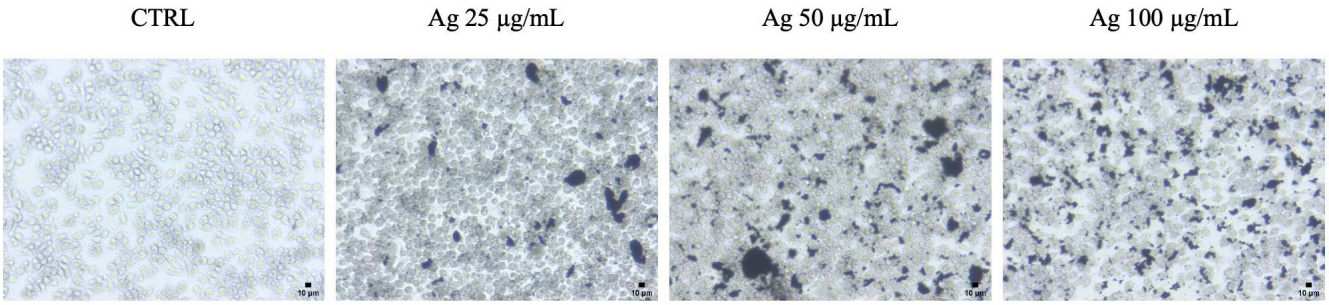

Figure S2d: microscopy imaging of the silver-treated cells after crystal violet staining

Therefore, the signals associated with both dyes were similar to those observed in the control cells, as can be seen in Table S2b

Table S2b: absorbance obtained for both dyes for silver nanoparticles treated J774A.1 cells

Condition	Control (mean±Std dev)	Ag 25 µg/ml	Ag 50µg/ml	Ag 100µg/ml
Alphazurine absorbance (637 nm)	1.22±0.1	1.22±0.15	1.15±0.14	1.07±0.1
Crystal violet absorbance (590 nm)	0.2±0.01	0.21±0.01	0.2±0.01	0.195±0.009

Conclusions:

The data obtained with the silver nanoparticles were the easiest to interpret. As no massive cell detachment occurred, the signal was linked to i) the number of cells and ii) the possible adsorption of dyes onto the nanoparticles. The latter part of the absorbance should vary with the concentration of the nanoparticles, which was not the case. The slight decrease in both alphazurine and crystal violet signals that occurred at 100µg/ml correlate with the slight decrease in cell density that can be observed at this concentration.

As in these experiments all cells are killed and fixed with ethanol, then rehydrated in PBS to completely expose the cell cytosol and the nanoparticles to the dyes, the data cannot be compared to the classical viability data in which the cells are exposed to alphazurine while still alive.

Regarding the data obtained with zinc oxide, the massive cell detachment renders them more difficult to interpret. However, the observed absorbances did not change between the two concentrations used,

which argues against an adsorption onto the nanoparticles, as this phenomenon should be concentration-dependent.