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Study Report and Preliminary Guidance on the Adaptation of the In Vitro micronucleus assay (OECD TG 487) for Testing of Manufactured Nanomaterials

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Foreword

This Study Report and Preliminary Guidance on the Adaptation of the *In Vitro* micronucleus assay (OECD TG 487) for Testing of Manufactured Nanomaterials has been prepared as part of the OECD Working Party of National Co-ordinators of the Test Guidelines Programme (hereafter WNT). The aim of this project was to develop guidance on the Adaption of *in Vitro* Mammalian Cell Based Genotoxicity TGs for Testing of MNs. This project was originally proposed and initiated by the EU Joint Research Centre in 2014. This resulted in two JRC Reports. The first one on *Physicochemical characterisation of gold, silica and silver nanoparticles in water and in serum-containing cell culture media*, published in 2018; and a second one published in 2020 addressing *In vitro cytotoxicity and cellular uptake evaluation of gold, silica and silver nanoparticles in five different cell lines: Caco-2, A549, CH0, V79 and TK6* .

Asa follow-up, the United Kingdom and Germany complemented the work by reporting on work carried out on an adapted protocol. This document compiles the information generated through their experimental work and aims to make the available the data, as well as the share lessons learned from their work with nanomaterials.

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Aim and scope of the project

1. The OECD Working Party on Manufactured Nanomaterials addresses all the components needed for thorough hazard assessment of engineered nanomaterials (ENM) for human health and the environment, including making available reliable test methods for the purpose of ENMs safety assessment. At an OECD expert meeting held in Paris in October 2014 it was concluded that the scientific information available was not sufficient to fully support development of a harmonised version of the *in vitro* micronucleus test protocol because the existing or published data were not generated in a harmonised way and did not provide answers to some key questions (e.g. the uptake on ENMs, dosing regimens, necessary length of exposure to ENMs or scheme of treatment to cytochalasin B). The OECD WPMN approved a JRC led project with the aim to identify needed adaptations of the *in vitro* micronucleus test (Test Guideline 487) for the assessment of manufactured ENMs and eventually provide appropriate guidance. The same project was subsequently included by the OECD-WNT in the work plan of the Test Guidelines Programme (Project 4.95). Therefore, work aiming to develop a Guidance Document (GD) addressing the necessary adaptations of current genotoxicity TGs for ENM testing was initiated in January 2019 under the coordination of JRC. In order to share the lessons learnt from work carried out so far under this project and guide the users of the Test Guideline 487 when testing ENM, it was agreed to publish this document, mindful that it will be refined in future with the testing of additional ENMs.

2. The whole study project was divided into 3 phases: 1-Clarification of main technical issues; 2-Interlaboratory comparison and 3- Guidance development. These are described as follows:

Phase 1: Clarification of main technical issues

- Physico-chemical characterization of gold, silver, and silica Nanoparticles as synthesised, in water and in serum-containing cell culture media.
- Cytotoxicity assessments of nanoparticles in A549, Caco-2, V79, TK6 cells
- Uptake of Nanoparticles into the various cell systems
- Define the doubling times

3. JRC coordinated this phase of work, which was completed and culminated in the following reports:

4. The Report on Physico-Chemical Characterisation of the nanomaterials chosen for the study was published in 2018 by JRC.

- Drewes, C., Ojea Jimenez, I., Mėhn, D., Colpo, P., Gioria, S., Bogni, A., Ponti, J., Kinsner-Ovaskainen, A., Gilliland, D. and Riego Sintes, J., Physicochemical characterisation of gold, silica and silver nanoparticles in water and in serum-containing cell culture media, EUR 29054 EN, Publications Office of the European Union, Luxembourg, 2018, ISBN 978-92-79-77705-9 (online),978-92-79-77704-2 (print), doi:10.2760/818663 (online),10.2760/58721 (print), JRC110379.

5. The Report on In vitro toxicity and uptake with the selected cell lines and selected nanomaterials was published on 2020 by JRC

- Bogni A., Ponti J., Drewes C., Kinsner-Ovaskainen A., Bremer-Hoffmann S., In vitro cytotoxicity and cellular uptake evaluation of gold, silica, and silver nanoparticles in five different cell lines: Caco-2, A549,

CHO, V79 and TK6, European Commission, Ispra, 2020, JRC120791. ISBN 978-92-76-44690-3, doi:10.2760/959826.

Phase 2: Interlaboratory comparison

- Selection, purchase and characterization of material
- Protocol development by lead laboratory
- Protocol transfer to other laboratories
- Interlaboratory study
- Study evaluation

6. The JRC coordinated this phase of work, while the experimental work was performed by BASF SE (Germany) and Swansea University (SU; UK). The outcomes of the experimental work conducted under this phase of the work programme are detailed within this document. Additionally, a publication detailing this phase of the study is in preparation.

7. The selection of cell lines to evaluate during this phase of study were agreed upon during an expert group meeting and included TK6 cells, HepG2, V79 and human lymphocytes. These cells were selected as they are cited as appropriate for use with the *in vitro* micronucleus assay in OECD TG487, while cells such as A549 and Caco-2 (which were considered in Phase 1) have not been evaluated for use with the assay.

8. The ENMs selected for exposure included all materials fabricated and characterized by JRC during Phase 1, CeO_2, $BaSO_4$ and Tungsten-Carbide/Cobalt (Wc-Co). Previous scientific literature has suggested that Wc-Co may be a suitable particle positive control for the *in vitro* micronucleus assay[1]; thus, to evaluate this further, Wc-Co was included as a trial positive particle control, together with all JRC materials, for exposure to the test cell lines. BASF have undertaken extensive *in vivo* genotoxicity testing with CeO_2 and $BaSO_4$; these materials were therefore included in selected cell exposures to determine their capacity for induction of micronuclei in the *in vitro* assay.

Phase 3: Guidance development

- Set up of expert drafting group
- Guidance development
- Presentation to OECD

9. The JRC was not able to coordinate this phase of work and so the leadership was transferred to the UK and Germany.

10. The OECD guideline 487 (adopted 29. July, 2016) is the current guideline under the Mutual Acceptance of Data (MAD) agreement that instructs how an *in vitro* micronucleus assay should be performed. The procedures described in the guideline are, however, directed at testing chemicals and not ENMs. In the guideline this deficiency is noted, yet a recommendation of a suitable adaptation of the protocol for the nanomaterial testing is not given. Thus, the current regulatory situation for the mutagenicity testing of ENMs is officially to follow the current procedure of the guideline, which is not necessarily the optimal procedure from a scientific point of view. Parameters such as the treatment interval or selection of the test concentration are a few examples

[1] Moche H, Chevalier D, Barois N, Lorge E, Claude N, Nesslany F. Tungsten carbide-cobalt as a nanoparticulate reference positive control in in vitro genotoxicity assays. Toxicol Sci. 2014 Jan;137(1):125-34.

of aspects, which need specific consideration when testing ENMs; a pulse treatment of cultures with ENMs do not provide sufficient time for the internalization process of a particular material and ENMs can per se only be tested at precipitating levels. Since there is no official document on how to adapt the OECD 487 protocol for ENM testing, there is a certain level of urgency in providing a guidance on the necessary adaptations, which would allow a more relevant assessment of the mutagenic potential of ENMs and avoid repetition of inadequately performed studies. This preliminary guidance document is therefore aimed at addressing this urgent deficiency in the guidance available.

Phase 2: Methods

Preparation of ENMs.

11. 5nm AuNPs (#OECD-RLS-03) & 30nm AuNPs (#OECD-RLS-10)), 22nm SiO_2 NPs (#OECD-RLS-0102) were supplied as a nano supension by JRC. JRC stock ENMs were sonicated in a 90W Ultrasonic Bath (Fisher Scientific #FB15046) for 20 minutes at 37°C to encourage destabilisation of agglomerate material. Following sonication, working concentrations of particles were prepared in cell culture media using a 1:1 dilution for the highest concentration and using serial dilutions to prepare the rest of the dose range (Supplementary Information Table 1). Given each JRC ENM stock were at different concentrations, this process was ENM-specific. CeO_2 (Lot#NM-212 Reference Nanomaterial), $BaSO_4$ (Lot#V106) were derived from the JRC repository and used as benchmark materials, since these two compounds have been tested in carcinogenicity studies. Physico-chemical characterization of both materials has been previously reported[2] Tungsten-Carbide/Cobalt, (8wt% WC/Co <200nm, 99.5% LOT#5561-072018, Nanostructured & Amorphous Materials Inc., USA), was used as a trial positive particle control and was weighed, suspended and sonicated according to the NANoREG protocol (Alstrup Jensen et al., 2014).

Cell culture (University of Swansea protocol).

12. All cell lines (irrespective of the laboratory) are maintained at the respective facility according to the OECD good *in vitro* methods practices guidance document (https://doi.org/10.1787/9789264304796-en). The human lymphoblastoid (TK6) cells were purchased from Public Health England (PHE). The cells were cultured in RPMI 1640 supplemented with 10% Horse serum and 1% L-glutamine. TK6 cells were routinely sub-cultured for 2 weeks prior to ENM testing, cells were regularly checked for potential changes to morphology and density by light microscopy. The human liver cancer (HepG2) cell line was purchased from American Type Culture Collection (ATCC). The cells were cultured in DMEM containing 10% FBS, 1% Penicillin/Streptomycin (P/S) and 1%L-glutamine. HepG2 cells were grown to 80% confluency before being routinely sub-cultured. The V79 cells (AbbVie GmbH) were cultured in MEM Eagle media (Pan-Biotech, UK) containing 1% L-glutamine, 1% Amphotericin, 10%FBS and 1% P/S. V79 cells could grow to 80% confluency before being routinely sub-cultured.

Cell culture (BASF SE protocol):

13. **V79 cell cultures:** V79 (adherent) cells were seeded at $5.0x10^5$ cells/ml in T25 flasks (attachment period 24-28 hours) and were treated with ENMs along with satellite flasks per concentration to be counted for cytotoxicity and cellular exposure assessment (see paragraph 21) for 1 cell-cycle (14 hours). Ethyl methanesulfonate (EMS) at 500µg/ml and 600µg/mL was used as the chemical positive control, and WC/Co at

[2] WOHLLEBEN W, HELLACK B, NICKEL C, HERRCHEN M, HUND-RINKE K, KETTLER K, RIEBELING C, HAASE A, FUNK B, KÜHNEL D, GÖHLER D, STINZ M, SCHUMACHER C, WIEMANN M, KELLER J, LANDSIEDEL R, BROßELL D, PITZKO S, KUHLBUSCH T. 2019. The nanoGRAVUR framework to group (nano) materials for their occupational, consumer, environmental risks based on a harmonized set of material properties, applied to 34 case studies. Nanoscale, 11, 17637-17654.

30 µg/mL & 100µg/ml was tested as a potential positive particle control. After 1 cell-cycle test substance treatment the cultures were rinsed twice with HBSS (Hanks Balanced Salt Solution). The cultures intended for mutagenicity assessment were incubated in MEM (incl. 10% [v/v] FCS) supplemented with CytB (final concentration: 3 µg/mL; stock: 0.6 mg/mL in DMSO; AppliChem, Cat.No. A7657) for 24 hours. Cultures used for LA ICP MS assessments were trypsinized, fixed twice in methanol : acetic acid (19:1; -20 °C) and spread on slides.

14. **Whole blood cultures:** After 48 hours the activated cell cultures were pooled and centrifugated in 10 mL aliquotes at 900 g for 5 minutes. After centrifugation the supernatant (culture medium) was removed, and the cells suspended in ENM dilutions in culture medium along with satellite tubes for each concentration for cellular exposure assessment (see paragraph 21). All tubes were transferred into cell culture flasks and incubated for 20 hours. Mitomycin C (MMC; Roche Diagnostics) at 0.04 µg/mL and Colchicine (Col; Roche Diagnostics) at 0.05 µg/mL were used as the chemical positive controls, and WC/Co at 10, 30, 60 and 100µg/mL was tested as potential positive particle control. At the end of the exposure period, the cells were transferred in tubes, centrifuged for 5 minutes at 900 g and resuspended in HBSS (Hanks Balanced Salt Solution). Washing of the cells was repeated at least once. Then the cells were centrifuged at (900 g, 5 min) and resuspended in DMEM/F12 medium with 10% [v/v] FCS and transferred into 25 cm² cell culture flasks. Cyt B (see 3.2.2) was added to the cultures intended for mutagenicity assessment and incubated at 37°C, 5% (v/v) CO2 and ≥ 90% relative humidity for 20 hours. To prepare the cells for the LA ICP MS assessments, they were separated from the nanoparticles using density centrifugation (Ficoll paque) and washed once at 900 g for 5 minutes. The obtained cells were fixed twice with methanol:acetic acid (19:1; -20°C) and spread on slides.

15. **Buffy coat cell cultures:** Buffy coat cells are nucleated cells that are remaining when the erythrocytes are removed from the whole blood. After 48 hours the activated cell cultures were pooled and centrifugated in 10 mL aliquotes at 900 g for 5 minutes. After centrifugation the supernatant (culture medium) was removed, and the cells suspended in ENMs dilution in culture medium along with satellite tube for each concentration for cellular exposure assessment (see paragraph 21). All tubes were transferred into cell culture flasks and incubated under agitation for 20 hours (corresponding to 1 cell cycle) . Mitomycin C (MMC; Roche Diagnostics) at 0.04 µg/mL and Colchicine (Col; Roche Diagnostics) at 0.05 µg/mL were used as the chemical positive controls, and WC/Co at 10, 25 30, 60 and 100µg/mL was tested as potential positive particle control. At the end of the exposure period, the cells were transferred in tubes, centrifuged for 5 minutes at 900 g and resuspended in HBSS (Hanks Balanced Salt Solution). Washing of the cells was repeated at least once. Then the cells were centrifuged at (900 g, 5 min) and resuspended in RPMI medium with 20% [v/v] FCS and transferred into 25 cm² cell culture flasks. Cyt B was added to the cultures intended for mutagenicity assessment and incubated at 37°C, 5% (v/v) CO2 and ≥ 90% relative humidity for 20 hours. In order to prepare the cells for the LA ICP MS assessments, they were separated from the nanoparticles using density centrifugation (Ficoll paque) and washed once at 900 g for 5 minutes. The obtained cells were fixed twice with methanol:acetic acid (19:1; -20 °C) and spread on slides.

Semi-automated in vitro cytokinesis-blocked micronucleus assay.

16. Two slightly different approaches were taken with the cytokinesis-blocked micronucleus (CBMN) assay depending on whether suspension or adherent cell lines were used. TK6 (suspension cells) and HepG2 (adherent) cells were seeded at $1.0x10^5$ cells/ml in T25 flasks along with satellite flasks per concentration to be counted for cytotoxicity and were then treated with ENMs for 1 cell-cycle (12 hours). Mitomycin-C (MMC) at 0.01µg/ml was used as the chemical positive control, and WC/Co at 20µg/ml & 100µg/ml was tested as a potential positive particle control. The *in vitro* cytokinesis-blocked micronucleus assay was performed as described previously by Evans *et al* & Burgum and colleagues (Evans et al., 2019, Burgum et al., 2020). On the day of exposures, cells are counted at least 2 hours before exposures (with suspension cells this can be done immediately prior to exposures). When using the HepG2 cells time is required for them to re-adhere to the flasks before exposures. Cells were dosed (both the satellite flasks and the CBMN-flasks) with ENMs prepared in cell-culture media and the negative control being media only. Exposures were performed as close to sonication

times as possible to avoid sedimentation. Following the exposure period, the satellite flask cells were counted for the calculation of relative population doubling (RPD), a measure of cytotoxicity (Equation 1). Cell counts were performed using a Beckman coulter counter by adding 100µl of cells to 10ml of diluent. Following ENM exposure for 1 cell cycle, TK6 cells were centrifuged and washed with PBS in triplicate to remove as much ENM as possible; while it cannot be guaranteed that all test ENM is removed, it should be noted that cytochalasin B blocks any further uptake of residual ENM[3]. Satellite flasks were counted to facilitate calculation of cytotoxicity data according to Relative Population Doubling (RPD; Equation 1). The cell viability (%) used in all subsequent figures for TK6 and HepG2 was then calculated by multiplying the RPD value by 100. V79 and HepG2 cells had exposure media aspirated and were then washed twice with PBS before being counted in the same manner. TK6 cells were re-seeded into clean T25 flasks in cell-culture media containing 3µg/ml of cytochalasin-B (cyto-B), the cells were returned to the incubator for a further 1.5 cell cycles. V79 and HepG2 cells remained in the same flasks and were supplemented with fresh media containing the same concentration of cyto-B.

Equation 1)

$$\text{RPD} = \frac{\text{No. of population doublings in treated cultures}}{\text{No. of population doublings in control cultures}} \times 100$$

Where population doubling = [log (post-treatment cell number/ Initial cell number)]/ log 2

Cell harvesting for semi-automated Metafer scoring (University of Swansea Protocol).

17. The TK6 and HepG2 cells were harvested by centrifugation (230g for 5 min), resuspended in 5ml of pre-warmed PBS and centrifuged at 230g for 10 minutes. The supernatant was discarded, this was repeated a second time. The cells were then resuspended in hypotonic solution (KCl 0.56%), before being centrifuged immediately at 230g for 10 minutes. The cells were resuspended in Fixative 1 (methanol: acetic acid: NaCl (0.09%) (5:1:6 parts)) and incubated at 4°C for 10 minutes before centrifugation at 230g for 10 minutes. Cells were resuspended in Fixative 2 (methanol: acetic acid (5:1 parts)) and incubated at 4°C for 10 minutes before centrifugation at 230g for 10 minutes, this was repeated 3 times. Cells can be maintained overnight in Fixative 2 at 4°C, tubes covered by foil. The day before making slides, freshly opened microscope slides were placed in a glass tank of Fixative 2 at 4°C, for 2 hours before slide preparation (ideally this is done overnight). On the day of preparing slides, the fixative was replaced with ddH_2O. On the day of slide preparation, the fixed cell suspensions were centrifuged at 230g for 10 minutes and thoroughly re-suspended in ~1ml of Fixative 2. Slides were removed from the ddH_2O and wiped dry with slide tissue. A total of 100µl of the cell suspension was evenly pipetted onto the slide held at an angle. The slides were then stood near-vertical on tissue paper to dry. The cell density was checked to ensure cells were evenly distributed, without clumping. Once dried, the slides were stained with 30µl of 1x Vectashield mounting medium with DAPI at 1.5ug/ml, coverslip applied and incubated in the dark for 15 minutes. Slides were scored using the Zeiss AxioCam HRc (Carl Zeiss Microscopy and Imaging, UK) semi-automated Metafer system. The details for the classifier used to support the analysis can be found in Table 1 of the protocol Supplementary Information. All experiments were performed in triplicate (*n*=3) and a minimum of 2000 binucleated (BN) cells per replicate were scored per concentration (6000 BN cells in total) on non-coded slides. It should be noted that evaluation of the accuracy of the Metafer system scoring has been previously demonstrated, with no difference between the semi-automated versus manual scoring of

[3] DOAK S, GRIFFITHS S, MANSHIAN B, SINGH N, WILLIAMS P, BROWN A, JENKINS G. 2009. Confounding experimental considerations in nanogenotoxicology. Mutagenesis, 24, 285 - 293

micronuclei[4]. Additionally, Appendix 2 (Figure 1) provides data that also illustrates the good comparability between both scoring systems following CeO_2 exposure in TK6 cells.

Cell harvesting for manual scoring approach (University of Swansea Protocol).

18. After the Cyto B treatment the adherent V79 cells were rinsed with pre-warmed HBSS and trypsinized. The obtained cell suspensions from all different cultures were treated in the same manner, namely the cells were centrifuged, and the cell pellet treated with a hypotonic solution (10 minutes with 0.4% KCl for V79 and buffy coat cells; 20 minutes with 0.28% KCl for whole blood cultures). After the hypotonic treatment, the cells were fixed twice by adding fixative (19 parts methanol and 1-part acetic acid: -20°C). Slides were prepared by immersing in deionized water followed by pipetting the fixed cells on the slide. Three drops of DPX were placed on the slide and a 24mm x 60mm coverslip applied; the slides were then left to dry for 24 hours. Slides were viewed under a light microscope, 1000BN cells per replicate were scored per concentration (2000 BN total) on non-coded slides. Cells were scored to calculate Cytokinesis Block Proliferation Index (CBPI) as a measure of cytostasis (Equation 2). Relative cell viability (%) used in subsequent buffy coat-, whole blood- and V79-cell figures was then calculated by subtracting the (%) Cytotoxicity value (deduced using Equation 2) from 100.

Equation 2)

$$\% \text{ Relative cell viability} = 100 - 100\ (CBPI_T - 1 / CBPI_C - 1);\ \text{where T is treatment, C is control}$$

$$CBPI = \text{No mononucleated cells} + 2x\ \text{BN cells} + 3 \times \text{MNCs} / N;\ N \text{ is the total number of cells scored}$$

Cell harvesting for manual scoring approach (BASF SE Protocol).

19. After the CytB treatment the adherent cells were rinsed with pre-warmed HBSS and trypsinized. The obtained cell suspensions from all different cultures were treated in the same manner, namely the cells were centrifuged and the cell pellet treated with a hypotonic solution (10 minutes with 0.4% KCl for V79 and buffy coat cells; 20 minutes with 0.28% KCl for whole blood cultures). After the hypotonic treatment, the cells were fixed twice by adding fixative (19 parts methanol and 1-part acetic acid; -20°C). Slides were prepared by immersing in deionized water followed by pipetting the fixed cells on the slide. The cells were stained with May-Grünwald (3min) and 10%[v/v] Giemsa (in Titrisol, pH7.2, 20min), mounted and scored as described above.

Kinetochore staining of micronuclei.

20. To determine if the DNA damage was a consequence of an aneugenic or clastogenic response, kinetochore staining of micronuclei in BN cells was performed. Following the exposure period, the cells were washed three times with PBS before being cytocentrifuged (500g, 5 minutes) onto microscope slides and fixed in ice-cold 90% methanol at −20°C. Immunofluorescent staining of kinetochore proteins was performed as previously described (Singh et al., 2012, Burgum et al., 2020). Kinetochore scoring was performed on a Zeiss AxioCam HRc (Carl Zeiss Microscopy and Imaging, UK). Only the lowest and highest significant NP concentrations were used for each particle type, with WC/Co included at 100µg/ml, of which 50 micronuclei were scored for the presence or absence of FITC fluorescence in the micronucleus indicating the presence of a whole chromosome (K+) or chromosome fragment (K-) respectively (n=1 biological replicate).

[4] Seager AL, Shah U-K, Brüsehafer K, Wills J, Manshian B, Chapman KE, Thomas AD, Scott AD, Doherty AT, Doak SH, Johnson GE, Jenkins GJS. Recommendations, evaluation and validation of a semi-automated, fluorescent-based scoring protocol for micronucleus testing in human cells. Mutagenesis, 2014, 29, 155–164.

Cellular exposure assessment using the laser ablation inductively coupled plasma mass spectroscopy (LA-ICP-MS).

21. Common techniques to determine the uptake of NPs by cells are scanning electron microscopy (SEM) and transmission electron microscopy (TEM) with energy dispersive x-ray (EDX) detection. Although these methods allow for the visualization of NPs and the cell itself at a high lateral resolution, they demand for a complex sample preparation, expensive equipment, and high vacuums, hampering high throughput and the evaluation of a high number of cells. In contrast, laser ablation-inductively coupled plasma-mass spectrometry (LA-ICP-MS) has emerged to the method of choice for the evaluation of larger cell numbers[5]. It can be used either in scanning mode to generate maps of the elemental distribution or in single spot mode determining the elemental composition of each individual cell at once. Although images of the elemental distribution might provide subcellular resolution, single spot analysis are less time consuming and have a slightly better signal to noise ratio [Löhr et al 2020 & 2019]. Different quantification strategies were presented to (semi)-quantitatively determine the association of NPs with cells. External calibration based on microdroplets[6]or isotopic dilution[7] ensured reliable quantification of different elements in single cells. An alternative semi-quantitative approach is the use of an endogenous element as internal standard to compare same textured samples. Elements applied as internal standard require a homogenous distribution and moderate concentrations within the sample. In the literature, different elements are suggested, all providing limitations and advantages[8]. Phosphorus, which occurs in cellular DNA, proteins, and the cell membrane, was utilized as cell marker in several studies[9,10,11] and thus applied here as internal standard to compare the association within one cell type and applied test substance. An absolute quantification is not given so that the results of different elements cannot be compared with each other.

Experimental Procedure

22. For LA-ICP-MS analysis, a 193 nm ArF Excimer Laser (NWR193 Excimer Laser Ablation System, Elemental Scientific Lasers, Bozeman, MT USA) equipped with a two-volume cell (TwoVol^2 Ablation Cell, Elemental Scientific Lasers) was coupled to an ICP-MS Triple Quadrupole (8900 ICP-MS Triple Quad, Agilent Technologies, Santa Clara, CA, USA). The samples were transported in a carrier gas flow (He, 800 mL/min)

[5] WANG M, ZHENG L, WANG B, CHEN H, ZHAO Y, CHAI Z, REID H, SHARP B, FENG W. 2014. Quatitative analysis of Gold nanaoparticles in single cells by laser ablation inductively coupled plasma-mass spectrometry. Analytical Chemistry, 86, 10252-10256

[6] VAN MALDEREN SJM, EVERGUCHT E, DE RIJCKE M, JANSSEN C, VINCZE L, VANHAECKE F. Quantitative Determination and Subcellular Imaging of Cu in Single Cells via Laser Ablation-ICP-Mass Spectrometry Using High-Density Microarray Gelatin Standards. Anal. Chem. 2016, 88, 11, 5783–5789

[7] ZHENG L, SANG Y, LUO R, WANG B, YI F, WANG M, FENG W. 2019. Determination of silver nanoparticles in single cells by microwell trapping and laser ablation ICP-MS determination. Journal of Analytical Atomic Spectrometry, 34, 915-921.

[8] AUSTIN C, FRYER F, LEAR J, BISHOP J, HARE D, RAWLING T, KIRKUP L, MCDONAGH A, DOBLE P. 2011. Factos affecting internal standard selectionfor quantitative elemental bio-imaging of soft tissues by LA-ICP-MS. 2011.Journal of Analytical Atomic Spectrometry, 26, 1494-1501.

[9] GOMEZ-GOMEZ B, CORTE-RODRIGUEZ M, PEREZ-CORONA M, BETTMER J, MONTES-BAYON M, MADRID Y. 2020. Combined single cell and single cell particle ICP-TQ-MS analysis to quantiatively evaluate the uptake and biotransformation of tellurium nanoparticles in bacteria. Analytica Chimica Acta, 1128, 116-128.

[10] CORTE-RODRIGUEZ M, BLANCO-GONZALEZ E, BETMMER J, MONTES-BAYON M. 2019. Quantitative analysis of transferrin receptor 1 (TfR1) in individual breast cancer cells by means of labeled antibodies and elemental (ICP-MS) detection. Analytical Chemistry, 91, 15532-15538.

[11] MEYER S, LOPEZ-SERRANO A, MITZE H, JAKUBOWSKI N, SCHWERDTLE T. 2018. Metallomics, 24, 73-76.

and introduced *via* a Dual Concentric Injector (DCI, Elemental Scientific Lasers) to the ICP-MS. An additional gas flow (Ar, 1 L/min) was added, and the sample transferred *via* a quartz injector pipe (inner diameter: 3.5 mm) into the plasma. The ICP-MS was equipped with platinum sampler and skimmer. To resolve the issue of polyatomic interferences especially for low masses (*e.g.,* ^{31}P), it was operated in TQ modus with O_2 as reaction gas. The optimum RF was set to 1300 W and the set-up was tuned daily for maximum signal intensity and an oxide ratio (*m/z* 232/248) below 1.5 % with a NIST Glass standard (NIST SRM 612, National Institute of Standards and Technology, Gaithersburg, MD, USA). Each cell was ablated separately with 50 bursts and a spot size of 25 µm. A laser pulse frequency of 100 Hz and a laser energy of 0.5 J/cm^2 ensured a full ablation avoiding ablation of the glass slide. The isotope $^{31}P^{16}O^+$ was monitored with a dwell time of 50 ms for all experiments and chosen as internal standard. For Au NPs the isotope $^{197}Au^+$, for CeO_2 NPs the isotope $^{140}Ce^{16}O^+$ and for WC-Co NPs the isotopes $^{59}Co^+$ and $^{184}W^{16}O^+$were detected each with a dwell time of 50 ms. To distinguish a cell event from the continuous background, a threshold three times higher than the mean signal of the background was applied. For an identified cell, an association rate was calculated by dividing the summed signal intensities of each individual cell event. This results in an association rate describing to what extent the NPs are allocated to the cells.

Statistics

23. All data is presented as the mean +/- the standard deviation (SD). For data generated at the Swansea University lab: statistical analysis was performed in SPSS statistics software (v.20 IBM, UK) where all data sets were firstly analysed for normality (Shapiro-Wilk test, $p \leq 0.05$) and for equal variance $p \leq 0.05$). A one-way analysis of variance (ANOVA) was performed with post hoc Dunnett's multiple comparisons applied to evaluate pairwise statistical significance between control and concentrations; the alpha value was set to 0.05.

24. For data generated at the BASF lab: An appropriate statistical analysis was performed. The proportion of cells containing micronuclei was calculated for each test group. A comparison of the micronucleus rates of each test group with the concurrent vehicle control group was carried out for the hypothesis of equal proportions (i.e., one-sided Fisher's exact test, BASF SE). If the results of this test were statistically significant compared with the respective vehicle control ($p \leq 0.05$), labels (s) were printed in the tables. In addition, a statistical trend test (SAS procedure REG) was performed to assess a possible dose-related increase of micronucleated cells. The used model is one of the proposed models of the International Workshop on Genotoxicity Test procedures Workgroup Report. The dependent variable was the number of micronucleated cells, and the independent variable was the concentration. The trend was judged as statistically significant whenever the one-sided p value (probability value) was below 0.05.

Phase 2: Results

25. The data generated by SU with TK6 cells revealed no cytotoxic effects following 1 cell-cycle exposure to either of the AuNPs. Some significant (*) cytotoxicity was observed following the SiO_2 exposure but only at the highest test concentration of 100μg/ml. Chromosomal breakage, measured by the *in vitro* CBMN assay revealed some significant (*) genotoxicity following exposure to each JRC particle type, the significant responses were most apparent at the highest test concentration of each material where 2-fold increases over background levels (of %Mn/BN) were observed. The WC-Co particle control was observed to be positive following 1 cell-cycle exposures to TK6 cells, with 2-fold increases in micronuclei induced at the two test concentrations of 20 and 100μg/ml (Figure 1).

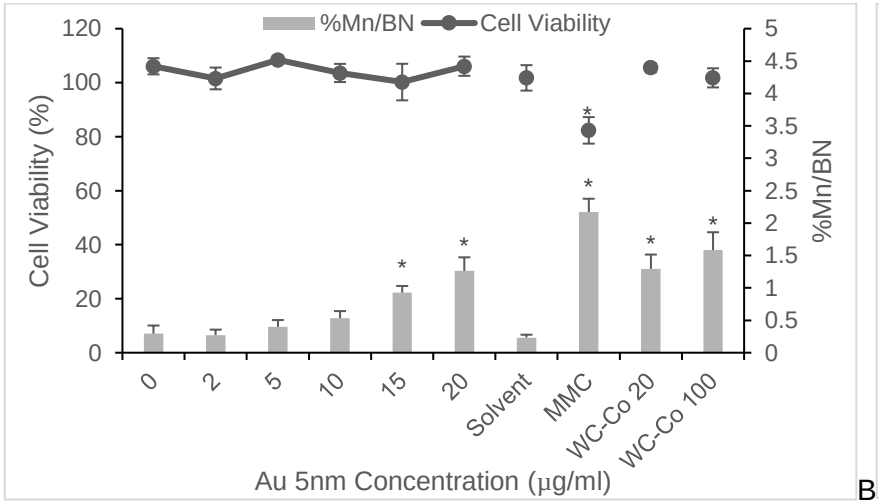
26. HepG2 cell line data generated by SU showed a more limited response to the JRC materials as compared to the TK6 suspension cells. Firstly, no significant cytotoxicity was observed across any test material. Secondly, the HepG2 cells showed no variation in %Mn/BN following 1 cell-cycle exposure to 30nm AuNPs. Whilst the 5nm AuNPs and the SiO_2 NPs did induce significant (*) %Mn/BN, in all cases, the increase in micronucleus frequency observed was less than 2-fold greater than the control. Unlike the TK6 response to WC-Co particles, the HepG2 cell line only showed significant (*) %Mn/BN at the highest test concentration of the material, 100μg/ml (Figure 2).

27. The BASF-generated data on buffy coat cells showed no significant cytotoxic response to the JRC materials. This limited response to the materials was mirrored in the genotoxicity data whereby the only significant (*) data was reported by the positive chemical controls and the WC-Co at 10, 60, and 100μg/ml (Figure 3). Whole blood exposures to JRC ENMs showed largely corresponding data sets with no significant (*) cytotoxic or genotoxic damage with exception of the positive chemical controls and the WC-Co particle control, however only at the highest test concentration of 100μg/ml (Figure 4). BASF-generated data on V79 cells using the JRC reference materials showed no significant (*) cytotoxicity or genotoxicity following 1 cell cycle exposures. The exception being the positive chemical control, MMC (Figure 5).

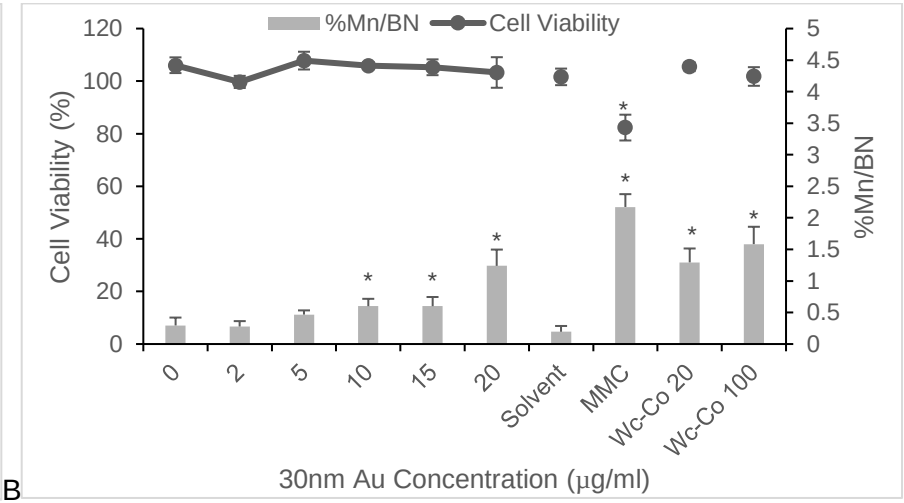
28. While in the above data sets, SU and BASF evaluated the response of differing cell lines to the test nanoparticles, one data set was generated using the same cell line, evaluated in both labs. This final data set involved exposure of the test nanoparticles to the V79 cells by SU, utilising the BASF protocol to determine the transferability of the protocol and ability to generate harmonised data sets. Following the 1 cell-cycle exposures to the JRC reference materials the V79 cells showed a limited response with no significant (*) cytotoxicity or genotoxicity with the only exception being the positive chemical control, EMS (Figure 6). Thus, there was no difference in the data generated by both the SU and BASF labs using this cell line, favouring the possibility of this SOP being transferable and reproducible.

Phase 2: TK6 human B lymphoblastoid cell line

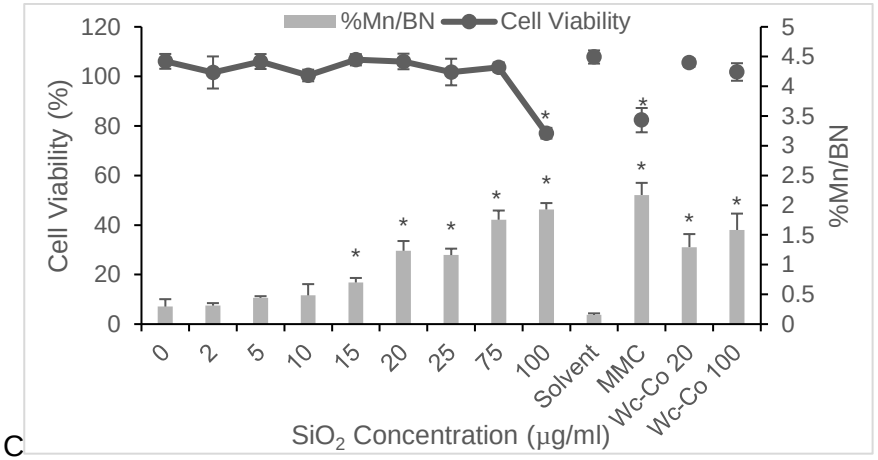
Figure 1. TK6 cytotoxicity & genotoxicity data following 1 cell-cycle exposure to 5nm AuNPs (A), 30nm AuNPs (B) and 22nm SiO2NPs (C). MMC (0.01µg/ml) was used as the positive chemical control, WC-Co was used as the particulate control at 20 and 100µg/ml. Results were considered statistically significant (*) when p<0.05, *n*=3.



A



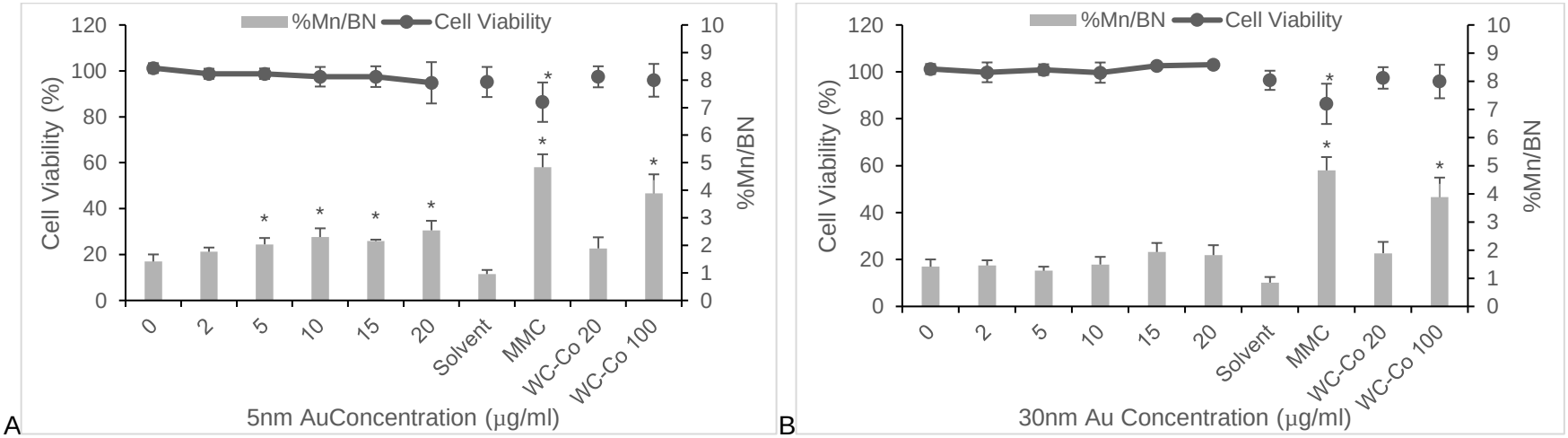
B


%Mn/BN
Cell Viability
Cell Viability (%)
%Mn/BN
120
100
80
60
40
20
0
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
0
2
5
10
15
20
25
75
100
Solvent
MMC
Wc-Co 20
Wc-Co 100
SiO2 Concentration (µg/ml)

C

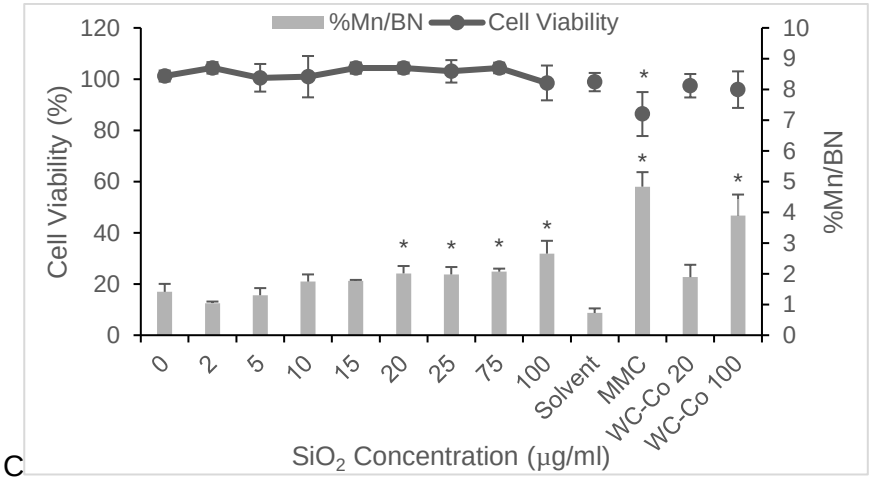
HepG2 human hepatocyte carcinoma cell line

Figure 2. HepG2 cytotoxicity & genotoxicity data following 1 cell-cycle exposure to 5nm AuNPs (A), 30nm AuNPs (B) and 22nm SiO2NPs (C). MMC (0.01µg/ml) was used as the positive chemical control, WC-Co was used as the particulate control at 20 and 100µg/ml. Results were considered statistically significant (*) when p<0.05



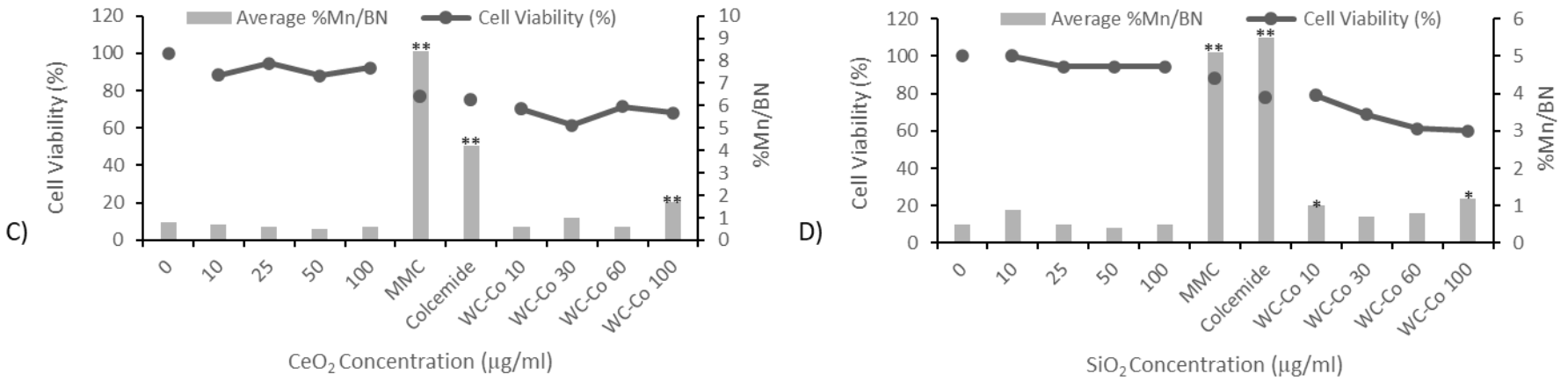
A B

C

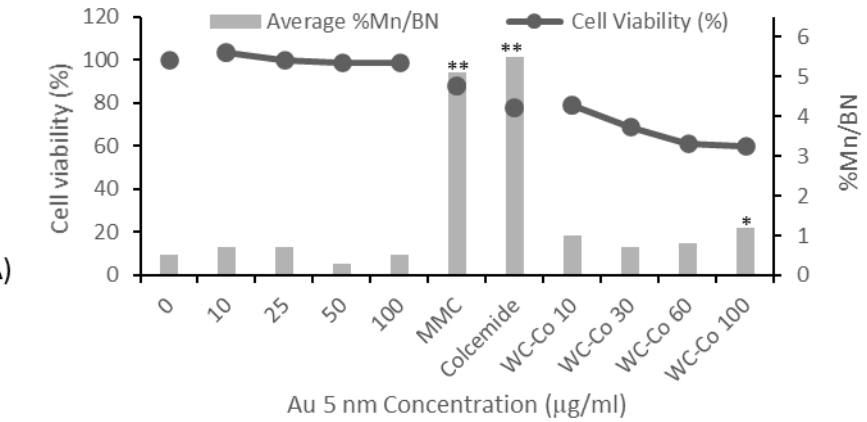

%Mn/BN
Cell Viability
Cell Viability (%)
%Mn/BN
120
100
80
60
40
20
0
10
9
8
7
6
5
4
3
2
1
0
0
2
5
10
15
20
25
75
100
Solvent
MMC
WC-Co 20
WC-Co 100
SiO2 Concentration (µg/ml)

Buffy coat fraction of blood

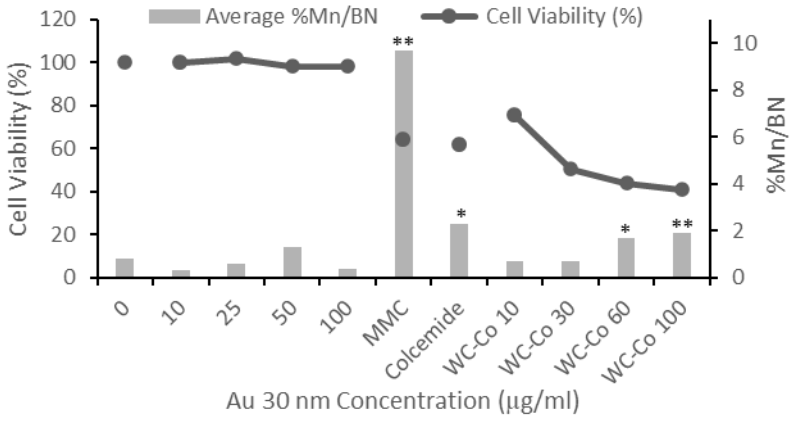
Figure 3. **Buffy coat CBPI and frequency of micronuclei determined by the *in vitro* CBMN assay following 1 cell-cycle exposure to 5nm AuNPs (A), 30nm AuNPs (B), CeO_2 NPs (C), 22nm SiO_2 NPs (D). Positive controls used were MMC at 0.04µg/ml, Colcemide at 0.05µg/ml and WC/Co at 10, 30, 60 & 100µg/ml. Results were considered statistically significant (*) when $p \leq 0.05$ or (**) when $p \leq 0.01$.**



A)

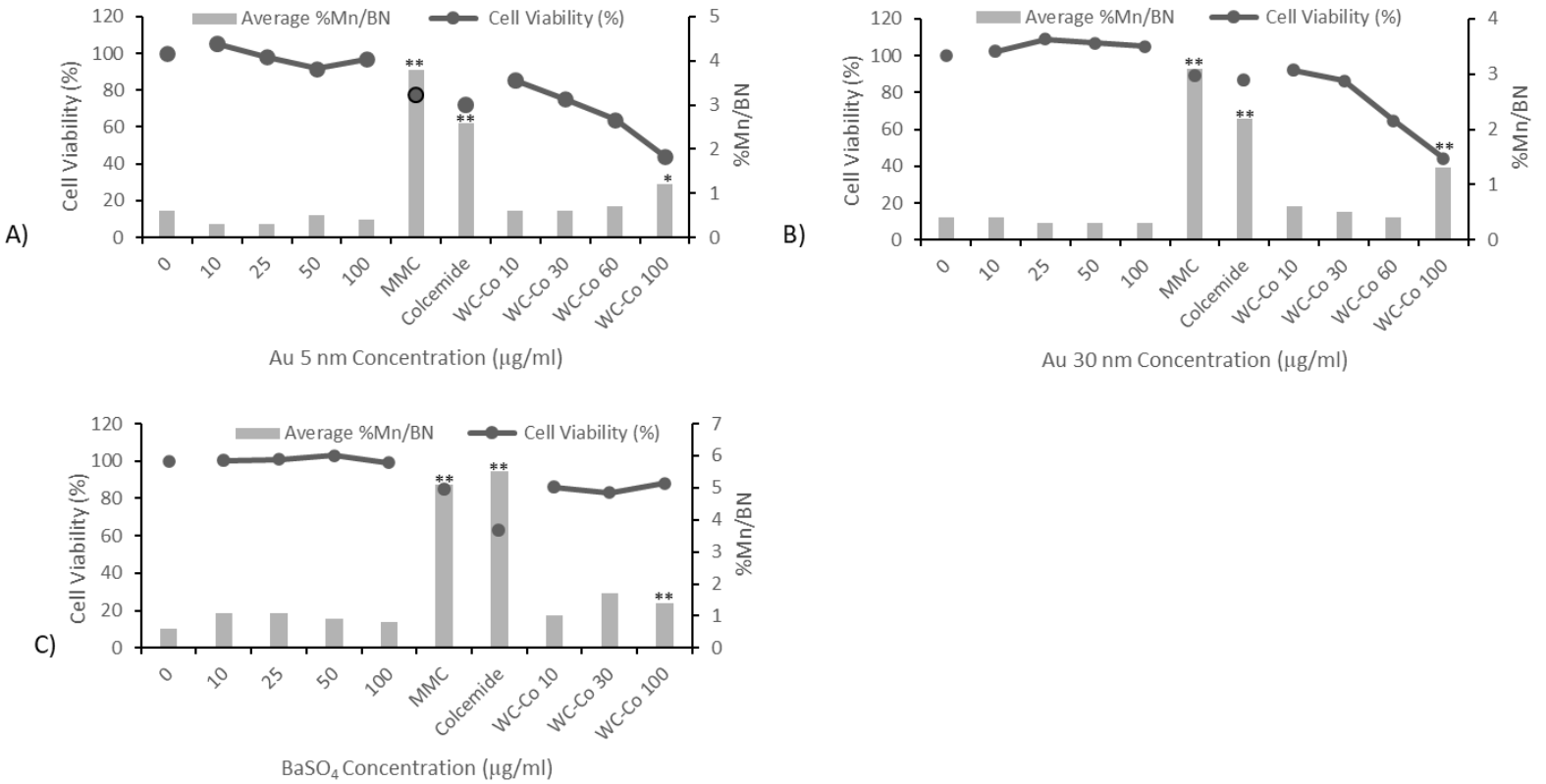

Average %Mn/BN
Cell Viability (%)
Cell viability (%)
%Mn/BN
0
10
25
50
100
MMC
Colcemide
WC-Co 10
WC-Co 30
WC-Co 60
WC-Co 100
Au 5 nm Concentration (µg/ml)

B)


Average %Mn/BN
Cell Viability (%)
Cell Viability (%)
%Mn/BN
0
10
25
50
100
MMC
Colcemide
WC-Co 10
WC-Co 30
WC-Co 60
WC-Co 100
Au 30 nm Concentration (µg/ml)

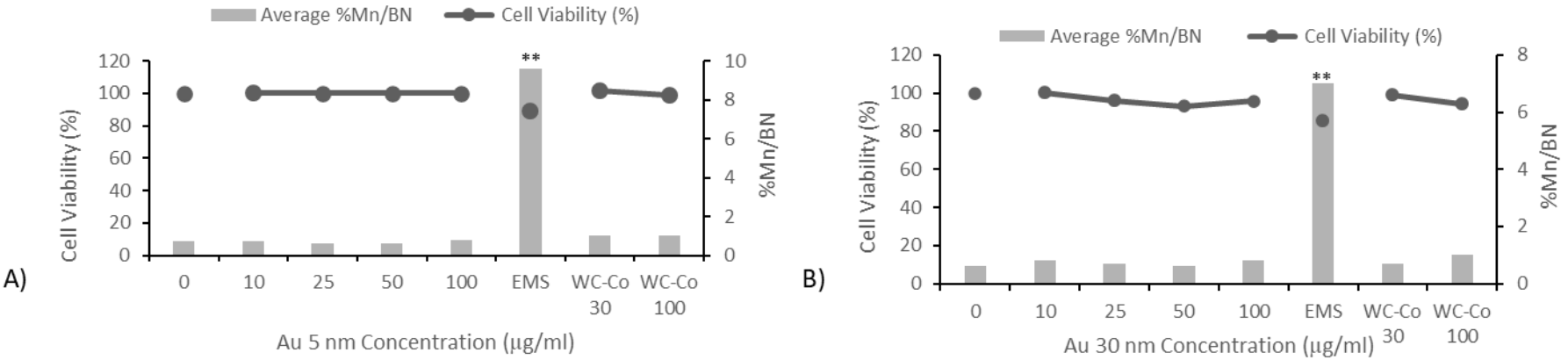
Whole blood

Figure 4. Whole blood CBPI and frequency of micronuclei determined by the in vitro CBMN assay following 1 cell-cycle exposure to 5nm AuNPs (A), 30nm AuNPs (B), BaSO4 NPs (C), 22nm SiO2 NPs (D). Positive controls used were MMC at 0.04 µg/ml, Colcemide at 0.05 µg/mL and WC/Co at 10, 30, 60 & 100µg/ml. Results were considered statistically significant (*) when p≤0.05 or (**) when p≤0.01.

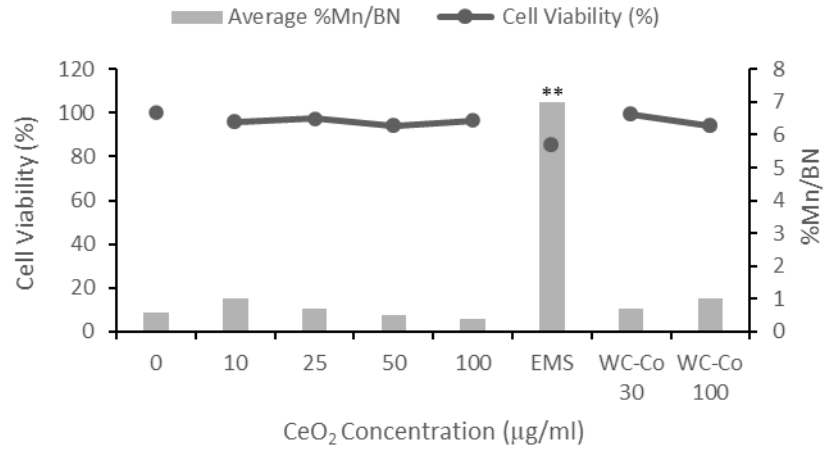


V79 Chinese hamster lung fibroblast cells

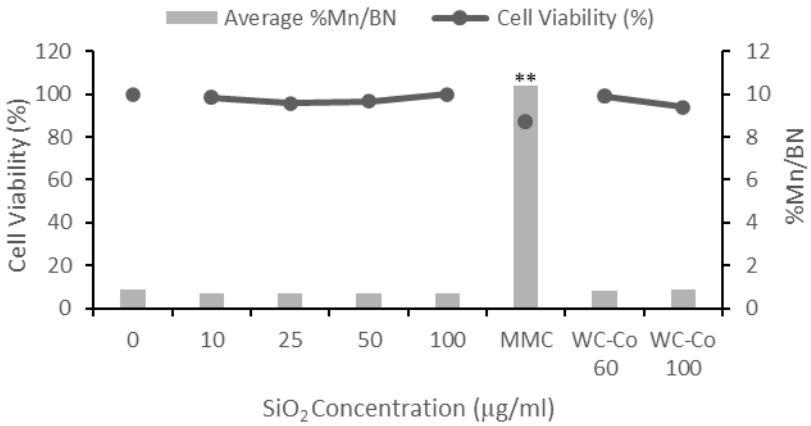
Figure 5. V79 CBPI and frequency of micronuclei determined by the in vitro CBMN assay following 1 cell-cycle exposure to 5nm AuNPs (A), 30nm AuNPs (B), CeO2 NPs (C), 22nm SiO2 NPs (D), BaSO4 NPs (E). Positive controls used were EMS 600 µg/ml and WC/Co at 60 & 100µg/ml. Results were considered statistically significant (*) when p≤0.05 or (**) when p≤0.01.



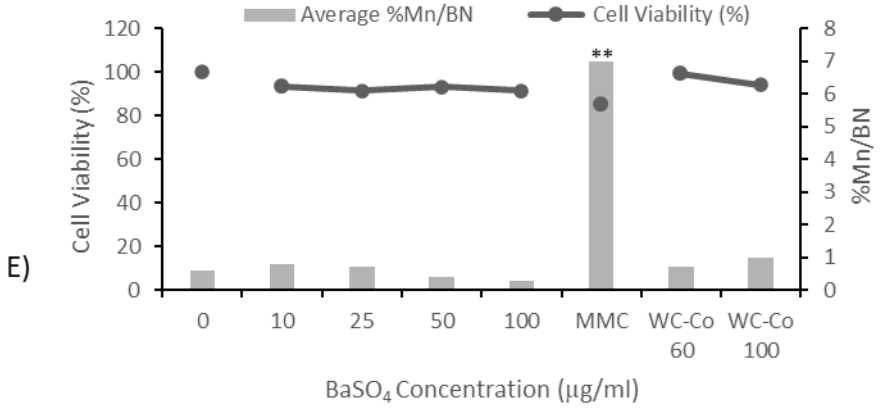
C)


Average %Mn/BN
Cell Viability (%)
Cell Viability (%)
%Mn/BN
**
0
10
25
50
100
EMS
WC-Co 30
WC-Co 100
CeO2 Concentration (µg/ml)

D)

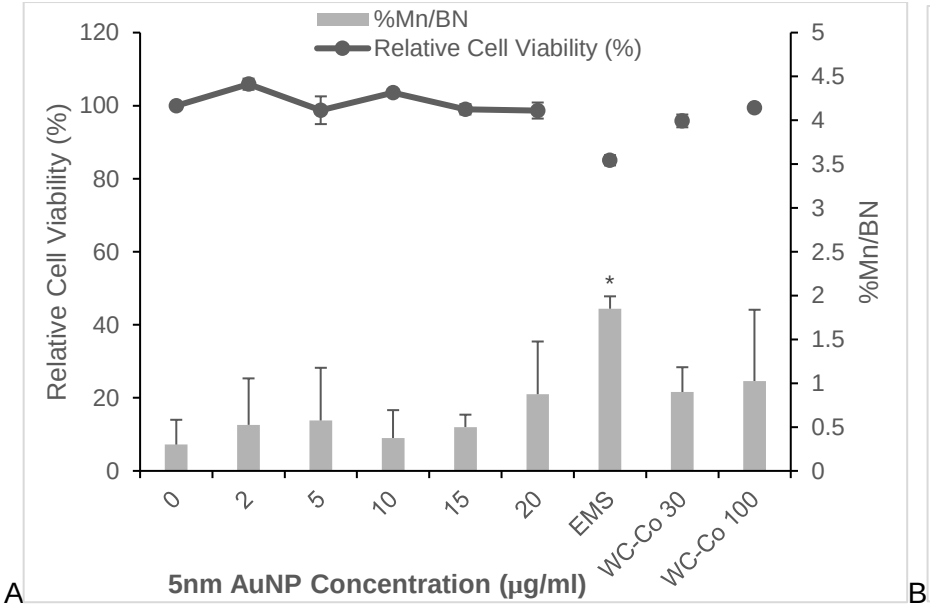

Average %Mn/BN
Cell Viability (%)
Cell Viability (%)
%Mn/BN
**
0
10
25
50
100
MMC
WC-Co 60
WC-Co 100
SiO2 Concentration (µg/ml)

E)

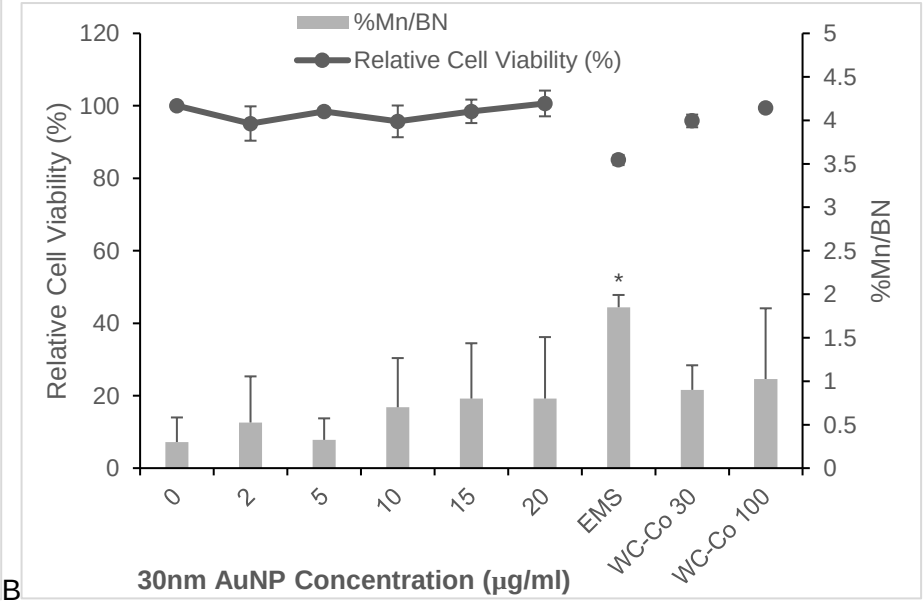

Average %Mn/BN
Cell Viability (%)
Cell Viability (%)
%Mn/BN
**
0
10
25
50
100
MMC
WC-Co 60
WC-Co 100
BaSO4 Concentration (µg/ml)

V79 cells, using BASF SOP

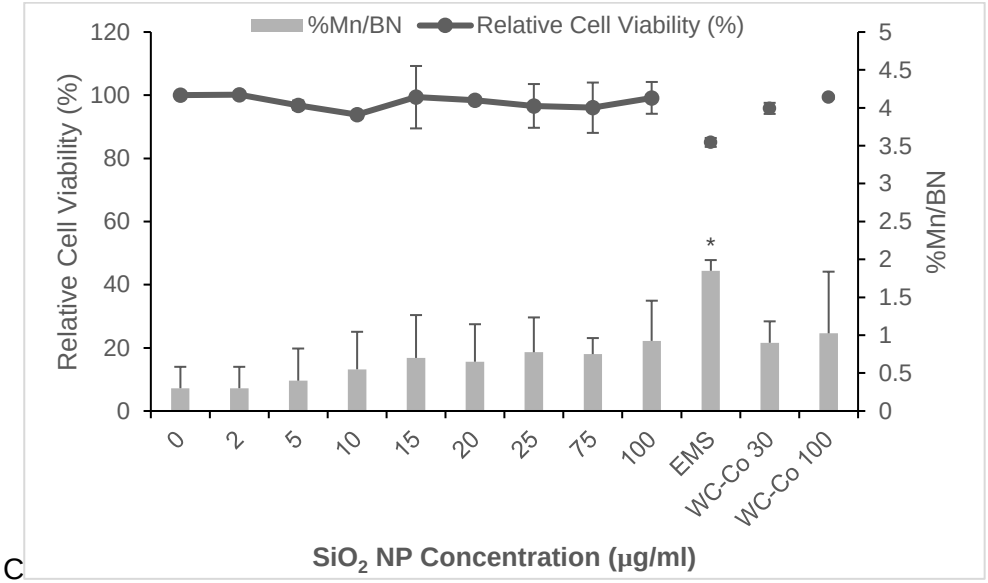
Figure 6. V79 cytostasis & genotoxicity data following 1 cell-cycle exposure to 5nm AuNPs (A), 30nm AuNPs (B) and 22nm SiO2NPs (C). EMS (600µg/ml) was used as the positive chemical control, WC-Co was used as the particulate control at 30 and 100µg/ml. Results were considered statistically significant (*) when p<0.05, (*n*=2).



A



B

C 

Cellular exposure assessment

29. In addition to the cytotoxicity and genotoxicity analysis, BASF evaluated the association of the test nanoparticles with the buffy coat cells, whole blood cultures and V79 cells by LA-ICP-MS analysis. The ratio of the respective elements Au, Ce, Ba and Co to Phosphorus is given as a semi-quantitative measure for the association of the tested nanoparticles with the cells. The data given in **Table 1** show that ratio of the individual element to the intracellular phosphorus increased concentration relatedly in each individual experiment. A comparison of the ratios between the elements is not possible. However, the ratios between the cell culture type used can be compared. Thus, it can be observed that V79 cells in all cases have a higher ratio of the element to their intracellular phosphorus levels as compared to buffy coat or whole blood cells. Consequently, despite the lack of genotoxicity in V79 cells, this data demonstrates that cell association did occur following exposure to the test nanoparticles.

Table 1. The ratio of the indicated elements to Phosphorus in buffy coat, whole blood and V79 cells after treatment with the indicated concentrations of the given nanoparticles.

µg/mL	Buffy coat cells				Whole blood cultures				V79				
	Au_{5nm}	Au_{30nm}	CeO_2	WC/Co	Au_{5nm}	Au_{30nm}	$BaSO_4$	WC/Co	Au_{5nm}	Au_{30nm}	CeO_2	$BaSO_4$	WC/Co
	Au/P	Au/P	Ce/P	Co/P	Au/P	Au/P	Ba/P	Co/P	Au/P	Au/P	Ce/P	Ba/P	Co/P
0	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0.02	<LOD	<LOD	<LOD	<LOD	0.03	<LOD
10	0.01	0.003	0.04	--	0.008	0.01	--	--	0.23	0.46	57.53	--	--
25	--	--	--	--	--	--	--	--	--	--	--	--	--
30	--	--	--	0.08	--	--	1.24	0.019	--	--	--	27.32	0.45
50	0.02	0.010	0.36	--	0.035	0.03	--	--	0.48	2.01	219.86	--	--
60	--	--	--	0.1	--	--	1.61	0.045	--	--	--	32.53	0.95
100	0.04	0.058	3.81	0.16	0.089	0.16	1.97	0.072	0.76	4.56	334.19	35.71	1.26

--: not tested

Recommendations

30. From the Phase 2 experimental work presented here, the following recommendations can be made to answer the original questions posed during the expert meeting held in Paris in October 2014:

1. *Appropriate cell lines to use in the in vitro micronucleus assay when testing* ENMs*:*

31. Within the Phase 2 work, it was evident that suspension cells (TK6 and Buffy coat cells) were more sensitive as compared to adherent V79 and HepG2 cells. However, considering the scope of the work performed, a recommendation on the most adequate test system cannot be made.

32. It is important to note that the most sensitive cell system, preferably of human origin (regardless the expected route of exposure to the nanomaterial in the final consumer product) and p53 competent should be used. Intracellular uptake of the ENMs should be demonstrated, and only cells that can efficiently internalise ENMs should be used.

33. As this Phase 2 study only included a small number of ENMs, it is recommended that expanding the sensitivity comparison of TK6 versus V79 cells is continued through further inter-laboratory trials using a wider range of ENMs.

2. *Positive controls:*

34. Currently, there are no suitable particle positive controls that have been identified for the *in vitro* micronucleus assay. Thus, in the absence of particle positive controls, it is recommended that chemical positive controls continue to be used (e.g. MMC, EMS).

35. Previously, there has been a suggestion in the scientific literature that tungsten carbide nanoparticles could be considered as a particle positive control for the *in vitro* micronucleus assay with human lymphocytes[12]. Thus, within the Phase 2 study, the nanoparticle tungsten carbide was included for further evaluation. This study demonstrated that tungsten carbide nanoparticles have the potential to be a suitable particle positive control, as observed for TK6 or buffy coat cells (however, the *in vivo* relevance and the biological significance, i.e. germ cell mutagenicity or somatic cell carcinogenicity have not yet been demonstrated).

3. *Top dose:*

36. In the case of cytotoxic materials, the limits of allowed cytotoxicity should be those stated in OECD 487.

37. In cases where there is lack of induced cytotoxicity, it is recommended that the top dose should be restricted to 100 µg/mL or 100 µg/cm^2, whichever is highest. Doses higher than this are not physiologically relevant, but more importantly, can result in interference with scoring due to high deposition on cells. Lower concentrations can be considered if they are justified by *in vivo* organ burdens. Furthermore, agglomeration dynamics need to be carefully considered. Typically, at high doses, agglomeration is more predominant with lower dispersion stability than at lower doses when a stable

[12] Moche H, Chevalier D, Barois N, Lorge E, Claude N, Nesslany F. Tungsten carbide-cobalt as a nanoparticulate reference positive control in in vitro genotoxicity assays. Toxicol Sci. 2014 Jan;137(1):125-34.

dispersion of the particles can be more readily achieved, which will result in cellular uptake variation across a dose range. Thus, a dose-dependent genotoxicity response will not be likely for all ENMs.

4. Treatment time:

38. One cell cycle treatment time, followed by 1.5 cell cycle exposure to cytochalasin B is recommended for the cytokinesis blocked micronucleus assay, since the uptake of ENM is different from the uptake of soluble substances and needs longer periods. Pulse treatments as stated in the OECD 487 are considered as redundant[13].

39. It should be noted that this experimental time can be challenging to conduct within normal working hours for the TK6 cells, which have a cell cycle time of 12-14h. Swansea University have therefore repeated the Phase 2 *in vitro* micronucleus assay experiments using 1.5 cell cycle exposures. The data demonstrated that there was no significant differences in the data generated when using either 1 or 1.5 cell cycle exposure with the TK6 cells. Thus, to enable the assay to be conducted during standard working hours, for TK6 cells, a 1.5 cell cycle exposure is recommended and is detailed within the SOP in Annex 1.

5. Dispersion:

40. Several methods can be utilised to disperse ENMs, however within the Phase 2 trial conducted, the NANoREG protocol (Alstrup Jensen et al., 2014) was implemented as this is a well-established method.

*6. BASF and Swansea University have generated a harmonised SOP for the in vitro micronucleus assay for testing of manufactured ENMs (**Annex A**).*

[13] Rosalie Elespuru, Stefan Pfuhler, Marilyn J Aardema, Tao Chen, Shareen H Doak, Ann Doherty, Christopher S Farabaugh, Julia Kenny, Mugimane Manjanatha, Brinda Mahadevan, Martha M Moore, Gladys Ouédraogo, Leon F Stankowski, Jr, Jennifer Y Tanir, Genotoxicity Assessment of Nanomaterials: Recommendations on Best Practices, Assays, and Methods, Toxicological Sciences, Volume 164, Issue 2, August 2018, Pages 391–416

Limitations

41. The described project has addressed several key issues regarding the testing of ENMs in the *in vitro* micronucleus assay, nevertheless there are some issues which have either not been addressed in this study or not resolved.

42. The recommendations in this guidance document are derived from data obtained from a small number of compounds performed in two laboratories using different cellular test systems. A cross validation of the protocols was only partly performed by Swansea University using V79 cells. A larger scale inter-laboratory validation comprising of several independent laboratories would definitely add value to the obtained results. It should be noted that if future ring-trials are to be conducted, it is critical to ensure that all laboratories use an identical approach to dispersing their materials; if harmonisation in dispersion approach across the participating laboratories cannot be achieved then the data may not align. For example, if sonication is being applied to disperse ENMs, then delivery of specific energy levels is required.

43. The number and type of ENMs used for this project were more or less predefined by the previous work carryout out by JRC[14]. A follow up trial should focus on a broader range of ENMs representing a larger portion of the existing ENMs. In this context, the assessment of the *in vivo* relevance of the obtained results with the tested ENMs is very limited. In this study CeO_2 was the only compound with relevant *in vivo* follow up data[15]. Thus, a selection of further ENMs with *in vivo* genotoxicity data would provide a better opportunity for interpretation of *in vitro* data.

44. During the meeting where the structure of the protocol was decided upon by the expert team the subject of the highest concentration to be tested was thoroughly discussed. Thus, 100 µg/mL was selected as the optimal highest concentration to be used in the experiments. Despite the unanimous decision by the expert team, it is recommended to discuss this topic in a broader community to establish a range finding workflow for the selection of the top concentration to be used for mutagenicity assessment.

45. Inclusion of S9 mix in the study protocol was not considered as relevant, since all selected ENMs. were inorganic and not likely subject to metabolization for induction of mutagenicity. However, ECHA recommends the use of S9 mix for organic ENMs. The issues not addressed in this document relate to firstly identify the plausibility of use of S9 for organic ENMs, the protocol on how to incorporate an S9 Treatment within a test substance treatment period corresponding to one cell cycle as well as the identification of a corresponding positive nanomaterial requiring metabolization. Future metabolism considerations with respect to the replacement of animal derived S9 mix could include in vitro metabolism assays such as TGP project 4.150.

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[4] Drewes, C., Ojea Jimenez, I., Mėhn, D., Colpo, P., Gioria, S., Bogni, A., Ponti, J., Kinsner-Ovaskainen, A., Gilliland, D. and Riego Sintes, J., Physicochemical characterisation of gold, silica and silver nanoparticles in water and in serum-containing cell culture media, EUR 29054 EN, Publications Office of the European Union, Luxembourg, 2018, ISBN 978-92-79-77705-9 (online),978-92-79-77704-2 (print), doi:10.2760/818663 (online),10.2760/58721 (print), JRC110379.

[15] Ma-Hock, L., Gröters, S., Strauss, V., Keller, J., Wiench, K., van Ravenzwaay B. and Landsiedel, R., Long-term inhalation study with CeO_2 and $BaSO_4$ nanomaterials. Manuscript in preparation.

Additional Supporting Interlaboratory Trial

46. Since completion of the Phase 2 experimental work, the Horizon 2020 project RiskGONE has agreed to undertake an interlaboratory trial using the harmonised SOP generated through this Phase 2 project. This interlaboratory trial will include three laboratories: Swansea University (UK), Norwegian Institute for Air Research (NILU; Norway) and the French Agency for Food, Environmental and Occupational Health & Safety (ANSES; France). The interlaboratory trial will involve each partner exposing the TK6 cell line to the ENMs:

- Zinc Oxide (ZnO) – Sigma #MKCJ4155
- Titanium dioxide (TiO_2) – JRC #JRCNM01005a990582
- Tungsten carbide-cobalt (WC/Co) – NanoAmor #5561HW
- Chemical positive control: Mitomycin C (MMC) – Sigma, #M4287-2MG. Suspend in double distilled water at 1mg/ml, keep at 4°C.

47. The ring trial will consist of each partner testing the TK6 cell line with the selected ENMs, following the SOP in **Annex A**. Experiments are to be performed in biological duplicate for an *n*=2 (Two independent experiments). The ENM exposure period will be for 1.5 cell cycles of TK6 cells and a cyto B time of 1.5 cell cycles.

48. This trial started in September 2021 and the data collation is planned for completion by January 2022.

Annex A. Harmonised SOP generated by BASF & SU

49. This annex includes the finalised SOPs for testing ENMs with the *in vitro* micronucleus assay using TK6 and buffy coat cells, as these were the most sensitive cell lines identified.

Experiments for characterization of nanoparticles, recommended by both BASF & Swansea University

50. The interpretation of *in vitro* genotoxicity requires a minimum physical-chemical characterisation of the test item, including both intrinsic properties (size, shape, specific surface area) and extrinsic properties (agglomeration and solubility in the genotoxicity test medium). The following (non-GLP) technologies can be applied to generate this information:

1. Transmission Electron Microscopy (TEM) to determine the particle size distribution and shape distribution. These are ECHA requirements to identify the Nanoform (or non-nano-form) of the test item. (European_Chemicals_Agency_(ECHA), 2019) The method is selected according to the NanoDefine Method Manual, published by JRC in 2020 (Mech et al., 2020). Specifically, a tip of a spatula of material is dispersed in 5ml of ethanol. The dispersion is treated for 5min in an ultrasonic bath. 5 drops of the dispersion are applied between two glass slides to create a thin liquid film. A carbon coated TEM grid is dipped on the film to transfer the particles to the TEM substrate. The TEM samples are analysed using a Tecnai Osiris machine (Thermo-Fisher) operated at 200 keV under bright-field (BF) conditions (Spotsize; 100µm condenser aperture; 20µm objective aperture). About 10 Images per sample are acquired using a Gatan 1000XP 2K CCD camera with an acquisition time of 2s at magnifications of 13500x and 26500x. The particle size distribution and aspect ratio distribution are evaluated using the free ParticleSizer plugin for ImageJ developed and validated within the EC FP7 project NanoDefine. Automated evaluation is faster, reproducible and limits operator bias to a minimum compared to manual image analysis. The report includes distributions, median D50 and statistics on particle size and aspect ratio.
2. Brunauer-Emmett-Teller (BET) method to determine the specific surface area. This is an ECHA requirement to identify the Nanoform (or non-nano-form) of the test item (European_Chemicals_Agency_(ECHA), 2019). The method is selected according to the NanoDefine Method Manual, published by JRC in 2020 (Mech et al., 2020). Specifically, the method conforms with ISO_9277:2014 “Determination of the specific surface area of solids by gas adsorption – BET method. If the test item is organic, then the degassing temperature is limited to 80°C, or at least 50°C below melt temperature.
3. Analytical Ultracentrifugation (AUC) method to determine the agglomeration in genotoxicity test medium. Characterise two concentrations (0.03 mg/mL and 0.1 mg/mL) and repeat one concentration at 20h (conforming with the duration of the genotoxicity testing) to assess the stability throughout the incubation time. AUC implements an optical system that is

synchronized with the centrifugal frequency to monitor the radial concentration profile during centrifugal separation. The samples are prepared by the identical protocol as for the genotoxicity testing in the identical medium. Suspensions are then directly loaded in AUC cells without further preparation. All measurements are done on a Beckman Coulter XLI Proteome Lab (Beckman Coulter Inc., Brea, USA). The cells are mounted on a 4-sample or 8-sample titanium rotor, and data is acquired during a gravitational sweep from 73g to 116000g over 2h. The samples are measured using either interference or absorbance optics to detect sedimentation as described by the NanoDefine project (Mehn et al., 2018). Both types of sedimentation data are evaluated with sedfit v15.0 software and are represented in particle size distributions in mass metrics and in number metrics.

4. Incubation and filtration with analysis by Inductively-Coupled-Plasma Mass Spectrometry (ICPMS) or by UV Vis-Spectrometry, to determine the static solubility in genotoxicity test medium. Characterise two concentrations (0.03 mg/mL and 0.1 mg/mL). The samples are prepared by the identical protocol as for the genotoxicity testing in the identical medium. Suspensions are then incubated at 37°C for 20h, conforming with the conditions of the genotoxicity testing. After 20 hours, the suspensions are filtered through a 1 µm glass filter directly followed by a 0.02 µm (= 20 nm) alumina membrane. Thereafter, the filtrates are analysed by UV-Vis (for organic pigments) or ICP-MS (for metal-based particles). If the test item consists of Si or Al, polymeric filters are used instead.

    a) Calibration of UV-Vis at peak pigment wavelength for quantification of the dissolved material is performed by dissolving the pigment in concentrated sulfuric acid to get the mass attenuation coefficient. The UV-Vis instrument Agilent Cary is used with 50mm quartz cells to optimize detection down to 0.001 mg/L. Blank controls (no test item in the dissolution setup) and medium controls (pure medium) are conducted. The general procedure is following “UV-VIS Absorption Spectra (spectrophotometric method)”, OECD guideline for Testing of Chemicals, guideline 101, adopted 12th May 1981. For pigments containing metals, the dissolved ions are quantified by ICP-MS (Perkin Elmer Nexion 2000b). The samples are diluted by a factor of 10 to 100 in 1% HNO_3, then measured with 50ms integration time on each mass. External calibration uses concentrations of 0 / 0.01 / 0.1 / 1 / 10 µg/L with matrix-matched standards. The ultra-high purity quartz nebulizer has an argon flow of 0.94 mL/min and a sample flow of 0.29 mL/min at a pump rate of 35rpm.

In vitro micronucleus test on primary human lymphocytes; Cytochalasin B method with nanoparticles BASF Standard Operating Procedure 2020

Preparation of the cells

51. The blood used for testing comes from volunteer test persons, the administration of personal data as well as the sampling procedure are described in the SOP "Organization of blood sampling for genotoxicological tests". The selection of possible test persons is based on the criteria specified in OECD guideline 487.

- Included are; Men and women aged 18 to 35, non-smokers, do not depend on regular medication, and do not use drugs.
- Excluded are; pregnant women, people with known diseases or exposure to genotoxic substances (chemicals / ionizing radiation).
- The cells will be incubated at 37°C, 5% (v/v) CO_2 and ≥ 90% relative humidity in the incubator. The culture medium RPMI contains stable glutamine and is supplemented with 20% [v/v] FCS and 1% (v/v) Penicillin / Streptomycin. The culture medium is preheated to 37°C.

Stimulation of the cells with phytohemagglutinin

52. Erythrocytes are first separated from the whole blood. For this purpose, the blood is first diluted 1:1 with culture medium. The erythrocytes are then separated from the remaining nucleated cells (buffy coat) by means of a Ficoll separation.

53. For the Ficoll separation, 25 mL Ficoll-PaqueTM (GE Healthcare Bio-Sciences AB) are placed in 50 mL centrifuge tubes and carefully covered with 20 mL of the diluted blood. The centrifuge tubes are centrifuged at 1055g for 20 minutes. The centrifuge's braking function should be switched off. The "buffy coat" layer (the layer between the medium and Ficoll) is carefully collected and centrifuged again at 491g (15 minutes with braking function). The pellet is then washed twice with medium and centrifuged (5 minutes at 900g). The pellet is taken up in a volume of cell culture medium (RPMI; 20% FCS, 0.6 mg / mL PHA) that corresponds to 5 times the blood volume (e.g. for 20 mL whole blood, the cells are taken up in 100 mL).

54. 50 mL of each batch are placed in a 175 cm^2 cell culture flask and cultivated in the incubator for 48 hours.

Test substance preparation and preparation of the particulate positive control

55. Tungsten carbide-Cobalt will be used as particulate positive control. The nanoparticle test substance and particulate positive control will be prepared as described by the NANoREG protocol (Alstrup Jensen et al., 2014).

56. In summary, a certain amount of the respective nanoparticle is weighed and mixed with ethanol (pre-wetting) so that the final ethanol concentration is 0.5 vol%. Then the dispersion medium (0.05% w / v BSA water) is added so that a particle concentration of 2.56 mg / mL is reached. The dispersion is treated with a probe ultrasonicator (Branson Sonifier 550 W) for 16 minutes at 10% amplitude (approx. 7 W). This stock dispersion is used to create the different testing concentrations.

The test substance is tested with the following concentrations:

- 1, 3, 10, 30, 60, 100 µg/mL

Tungsten-Carbide/Cobalt (8wt% WC/Co <200nm, 99.5% LOT#5561-072018, Nanostructured & Amorphous Materials Inc., USA) particulate positive control is tested with the following concentrations:

- 30, 60, 100 µg/mL

Preparation of the chemical positive control

57. The positive controls are prepared fresh on the day of the test shortly before the substance is administered or prepared in larger quantities at an earlier point in time, stored in ready-to-use portions at 80°C, and thawed and used shortly before use.

58. Mitomycin C (0.04µg/ml) and colchicine (0.05µg/ml) are used as chemical positive controls.

59. Several concentrations of a positive control substance can be used, it being enough if only one concentration used is evaluated if it shows a sufficiently strong genotoxic effect in the experiment.

Preparation and treatment of test cultures

60. The substance preparations and the preparations of the particulate positive control are diluted to the final concentration in culture with culture medium, the total volume for two cultures (duplicate determination) is 30mL. The pipetting scheme for dilution is shown in Table 1.

Table 1. Pipetting scheme of test substance preparations

			dilution in BSA	dilution in culture medium RPMI incl. 20% FCS	
Dose [µg/mL]	[mL]	from Dosis	+ vehicle [mL]	[mL] aus Ansatz	+ vehiclel [mL]
100*	5	2560	7.8	3	27
60*	6	100	4	3	27
30*	5	60	5	3	27
10	0.5	100	4.5	3	27
3	0.5	30	4.5	3	27
1	0.5	10	4.5	3	27

*: applies to both test substance and particulate positive control

61. The blood cultures are removed from the incubator, suspended and pooled. For each culture (two cultures per test group = double determination), 10 mL are pipetted into a prepared 15 mL centrifuge tube. The cells are centrifuged at 900g for 5 min and the supernatant is removed.

62. For each culture (double determination) 10 mL of the prepared substance dispersion are added to the cells. The cells are suspended with a 10 mL serological pipette and transferred into prepared 25 cm² cell culture flasks. The cells are incubated on a shaker (approx. 150 rpm) in the incubator for 20 hours (1-cell cycle).

Checking or determining further parameters

63. Changes in the pH value can be recorded by the colour change of the indicator dye of the culture medium (phenol red: no colour change from pH 6.7 - 8.3).

64. The pH value is measured at the start of treatment at least for the highest dose and for the untreated control or the vehicle control (pH 6.7-8.3).

Test substance removal

- The exposure phase is ended by washing the cells several times with HBSS
- Transfer the cultures into prepared 15 mL centrifuge tubes
- Centrifugation (5 min at 900g)
- Remove the supernatant
- Suspend the cells in 5 mL HBSS
- Centrifugation (5 min at 900g)
- Remove the supernatant
- Suspend the cells in 5 mL HBSS
- Centrifugation (5 min at 900g)
- Remove the supernatant
- Suspend the cells in 10 mL fresh medium
- Transfer of the cultures into prepared 25 cm^2 cell culture flasks

Cytochalasin B treatment

- Following a 1-cell cycle exposure to the test nanoparticles, each culture is treated with 30 µL initial solution (final concentration 6 µg/mL culture medium). The cultures are incubated for an additional 20h (1-cell cycle) in the incubator.

Cell harvest and preparation of slides

- The preparation takes place under a fume hood under non-sterile conditions.
- The fixative (19 parts methanol: 1 part acetic acid) is freshly prepared every working day, during the incubation steps the fixative is stored at -20°C.
- The hypotonic solution (0.65% KCl) is pre-warmed to 37°C in a water bath, it can be prepared the day before and stored in the refrigerator until use.

Hypotonic treatment and fixation of the cells:

65. The cells will be transferred into 15mL tubes, centrifugated at 900g for 5 min (room temperature) and washed with HBSS. Then the cells will be treated with a hypotonic solution and fixative:

- Centrifugation (5 min, 900g, at room temperature)
- Suspend in 5 mL KCl, cover with Parafilm

- Incubate for 10 min at 37°C
- Fix the cells by adding 1 mL of cold fixative and suspending them in the solution
- Centrifugation (5 min at 900g and 4°C)
- Remove the supernatant
- Suspend in 5 mL cold fixative
- Incubate for 20 min at 4°C
- Repeat the fixation step with 5 mL fixative (incubation for 20 min at 4°C)
- Centrifugation (5 min at 900g and 4°C)
- Suspend in 3 mL fixative
- After the last fixation step, the cells can be stored for up to six hours at 4°C or applied directly to the slide

Preparation of the cells on microscope slides

- Three preparations are made per culture as follows:
- Centrifuge the cells (5 min at 900g and 4°C) and suspend in 1-2 mL cold fixative. Depending on the pellet size / cloudiness of the cell suspension, additional fixatives can be added.
- Moisten the slide by immersing it in deionized water, allow it to drain briefly
- Apply 250 µL of the cell suspension evenly to the microscope slides
- Pull the slide through the flame of the Bunsen burner
- Extinguish the flame after approx. 1 second
- The cell density can already be assessed microscopically on the native preparation and, if necessary, adjusted (by varying the amount of fixative). The cells should be isolated and not overlapping on the slide.
- Let the preparations dry at least overnight.

Staining of the slides

66. The cells were stained with May-Grünwald (3 min) and 10% [v/v] Giemsa (in Titrisol, pH 7.2, 10 min) and mounted.

67. Note: whilst this protocol describes Giemsa staining and evaluation with a light microscope, it is also possible to use DAPI (fluorescent) staining as an alternative option for manual scoring. However, for DAPI staining a fluorescence microscope is needed to enable scoring.

Cytotoxicity – proliferation index (CBPI)

68. Micronuclei can only arise in proliferating cells. The cytokinesis block proliferation index (CBPI) is a direct measure for determining the cell division activity. The number of mononuclear, binuclear and multinucleated cells is determined in 500 cells per culture, in two cultures per test group = 1000 cells.

69. If only isolated mononucleated cells are found, no analysis is carried out. This is noted on the evaluation form (n.s. = not scorable).

70. The CBPI calculated from this distribution indicates the average number of nuclei per cell and can be used as a measure to determine the proliferation activity of a culture in the presence of the actin polymerization inhibitor Cyto B. The CBPI is given as an absolute value without a unit.

$$CBPI = \frac{((\text{No. mononucleate cells}) + (2 \times \text{No. binucleate cells}) + (3 \times \text{No. multinucleate cells}))}{(\text{Total number of cells})}$$

71. The CBPI was used to calculate the % cytostasis (relative inhibition of cell growth compared to the respective vehicle control group) - a CBPI of 1 (all cells are mononucleate) is equivalent to 100% cytostasis.

% Cytostasis = 100 - 100 {(CBPIT - 1) / (CBPIC - 1)}

T = test substance treated culture C = vehicle control culture

Micronucleus analysis

72. The selection of the dose groups that are used to record the micronucleus frequency depends on the quality of the preparations or cells, the cytotoxicity and possible other physiological parameters (e.g. pH value). This dose selection is made considering the applicable international test regulations. The evaluation of the cells for micronucleus frequency is carried out manually with a transmitted light microscope (e.g. Axio Scope.A1, Zeiss) on coded preparations. A magnification of 400 times is used. The findings are recorded manually on an evaluation form.

73. At least 1000 binuclear cells are analysed per culture and the number of micronucleated cells is recorded. At least 2000 binuclear cells per test group are evaluated. The criteria for evaluating a cell are:

- The cell membrane must be intact
- The cytoplasm should not be overstained
- The main nuclei should not differ in size
- The cell must be clearly distinguishable from the neighbouring cell
- Apoptotic cells with a fragmented nucleus are not assessed

The following criteria must be fulfilled for the assessment as a micronucleus

- The micronucleus should be round (no angular / crystalline structures)
- The diameter of the micronuclei should be less than 1/3 of the main nucleus
- The micronucleus should not be linked to the main nucleus
- Only micronuclei in binucleated cells should be scored
- The micronucleus/micronuclei should be completely within the cytoplasm of the binucleated cell

Appendix 1 for BASF SOP

Materials and chemicals

Materials	Manufacture	Order-no.
Cell culture flasks, 25 cm^2	TPP	P 90025
Cell culture flasks, 75 cm^2	Greiner	661160
Centrifuge tubes 15 mL	TPP	TPPA 91015
Centrifuge tubes 50 mL	TPP	TPPA 91050

microscope slide, free of grease	Superfrost	631-1320

Reagents und medium	Hersteller	Best.-Nr.
RPMI 1640, w: Stab. Glutamine, w: NaHCO3, ohne Phenolrot	PAN biotech	P04-16520
Hanks' Balanced Salt Solution ohne Ca^{2+}, Mg^{2+} (HBSS)	PAN biotech	P04-34500
Fetal Calf Serum (FCS)	Biowest	-
Penicillin/Streptomycin (10 000 E/10 000 µg/mL)	PAN biotech	P06-07100
Mitomycin C (MMC)	Roche	10107409001
Colchicin	Roche	10295892001
KCl	Bernd Kraft	4016056
methanol	Bernd Kraft	34940
glacial acetic acid	Bernd Kraft	4146307
Mounting Pertex®	Medite	MEDT 41-4011-00

74. A product or supplier of comparable quality can be used for all articles at any time. Reagents not listed here are described in more detail in the test plan if required. Other chemicals used are customary in the laboratory and are obtained from common manufacturers or are available in the laboratory.

Appendix 2 for BASF SOP

Recipe

Culture medium

75. RPMI 1640, w: Stab. Glutamine, w: NaHCO3, ohne Phenolrot unter Zusatz von:

- fetal calf serum (FCS), ca. 20% (v/v)
- Penicillin/Streptomycin (10 000 E/10 000 µg/mL), ca. 1% (v/v)

76. All components are mixed under sterile conditions in the medium bottle and marked with the ingredients, date and stored for a maximum of 6 weeks at approx. +4°C.

Phytohemagglutinin

77. The PHA (1.2 mg) is dissolved in 2 mL of ultrapure water in a container and can be stored in the refrigerator for up to 6 weeks. The documentation is carried out in the raw data across studies.

Cytochalasin B – solution

78. The cytochalasin B (10 mg) is dissolved in the container in 5 mL DMSO (final concentration 2 mg/mL) and can be stored in the refrigerator for up to 6 weeks.

Hypotonic treatment

79. The hypotonic solution (0.65% KCl) can be prepared on the day before and stored in the refrigerator.

80. Approach: Weigh of 0.65g KCl/ 100 mL with ultrapure water

Fixative

81. The fixative is freshly applied on a working day and stored at -20°C during the fixation steps.

82. Approach: 19 parts methanol (cooled overnight at -20°C) + 1-part glacial acetic acid

In vitro cytokinesis-blocked micronucleus (CBMN) test protocol for use with TK6 cells and engineered nanomaterials (ENMs) Swansea University Standard Operating Procedure 2020

Biological Setting & Test System

83. This SOP should be carried out under strict laboratory conditions, with all work performed under sterile conditions and in a Class II Laminar Tissue Culture Hood.

Chemicals & Reagents

Table 2. List of chemicals and reagents required for *in vitro* CBMN assay.

Reagent	Supplier
PBS	ThermoFisher; 10010023
Cell culture medium	ThermoFisher; 21870076
Cytochalasin B	Sigma; C6762
Methanol	Sigma; 34860
Beckman Coulter Diluent	Beckman; 628017
Giemsa Stain	VWR; 350864
Gurr pH 6.8 Buffer tablets	VWR; 331542
Glutamine	ThermoFisher; 25030081
Horse Serum	ThermoFisher; 16050122
Trypsin-EDTA (0.25%)	ThermoFisher; 25200056
Potassium Chloride (KCl)	Sigma; P9333
Sodium Chloride (NaCl)	Sigma; S7653
Acetic acid	Sigma; 1005706
VECTASHEILD Mounting Medium with DAPI	VECTOR Laboratories; H-1200
DPX Mountant	VWR; 13510
Xylene	VWR; 214736

Cell culture preparation

84. In preparation for this SOP, it is advised that cell culture medium is prepared and pre-warmed at 37°C for 30 minutes prior to use. TK6 complete cell culture medium is prepared by adding 50ml of horse serum to 500ml of media (RPMI 1640), 5ml of L-glutamine is also added to complete the media. The full cell culture medium should then be mixed prior to use by inverting the bottle.

Procedure

85. To thaw cells from Liquid Nitrogen:

- Place a bottle of complete medium in the water bath at 37°C (25-30 minutes before use). Remove your vial of cells from the Liquid N_2 and place in a foam float in the water bath. Do not allow the vial to be immersed. Cells will thaw in ~1 minute, transfer them to a flask of the warm medium slowly and carefully using a Pasteur pipette. (The amount of medium depends on cell type and on rate of cell division; 40-50ml for suspension cell lines and 10-15ml for adherent).

- Label the flask with the name of the cells, the passage number, your name, the date of resuscitation and the date that the cells were previously frozen. (This is helpful because if there is a problem with them, the other vials from the batch can be identified.)
- Place the flask in the CO_2 incubator.
- Check them after 24 hours for growth and contamination. They can be counted to determine when they will need splitting. Cells should not be allowed to become too confluent (split once 70-80% confluent). If required change media every 2 days.
- Monitor passage number in your experiments; do not let this rise beyond a reasonable range. TK6 cells should not be grown continuously for more than 4 weeks.
- At day 4 the SOP can be performed 2 different ways, such that scoring can be conducted using a manual approach, or alternatively, a semi-automated scoring approach using the Metafer microscope.

DAY 1 – seed cells

- Count cells using a Beckmann Coulter Counter and seed at $1x10^5$ cells/ml in 10ml of media in $25cm^3$ flasks per treatment. Two sets of flasks are required per treatment dose: one for genotoxicity (micronucleus frequency analysis) and a second additional satellite flask per treatment for cytotoxicity calculations.
- Incubate overnight at 37°C/ 5% CO_2

DAY 2 – cytotoxicity count 1 & dosing of cells with test agent

Concurrent positive and solvent/vehicle controls should be included in each replicate. For TK6 cells, the recommended chemical positive control is:

MMC CAS 50-07-7 (Fisher Scientific; BP2531-2), dose for 1 – 1.5 cell cycles at 0.01µg/ml

This procedure must be conducted in a Class II Laminar Tissue Culture Toxic Hood, with the user wearing double gloves to ensure safety when dosing with chemicals and / or ENMs.

Preparation of ENMs

ENMs will require preparation, suspension and sonication prior to use in toxicology testing, allow time to prepare ENMs fresh on the day of dosing. User's should refer to a recognised dispersion procedure which generates a stable ENM suspension and where the final quantity of ENM suspension added to the cells does not exceed 1:100 as to avoid disturbing cell culture conditions (OECD TG487). For this protocol we use the NANoREG guidance document for ENM suspension and sonication (Alstrup Jensen et al., 2014).

- Count satellite flasks for cytotoxicity (initial cell number) 1h before dosing. 100µl of cell suspension is added to a cuvette containing 10ml of diluent for cell concentration determination using a Beckmann Coulter Counter.
- Dose cells in both sets of flasks (for genotoxicity and cytotoxicity evaluation) for 1 – 1.5 cell cycles (for TK6 cells, 1 cell cycle time is typically 12-15 hours) with the ENM at the desired final concentration; this is to be performed in triplicate per concentration, including triplicate negative control (media only, or vehicle used to suspend ENM) and positive controls.

DAY 3 – cytotoxicity count 2 & addition of cytochalasin B

- Count satellite flasks (post-treatment cell number) to calculate cytotoxicity. Add 100µl of cell suspension directly from satellite flasks to a cuvette containing 10ml of diluent for cell concentration determination using a Beckmann Coulter Counter.
- Transfer the content of the genotoxicity flasks to a 15 ml centrifuge tube, centrifuge at 230g for 5 min and discard the supernatant. Re-suspend the pellet in pre-warmed PBS and re-centrifuge at 230g for 5 min, repeat this wash step a second time and discard the supernatant.
- Resuspend cell pellets in fresh media containing 3-6 µg/ml cytochalasin B and place into new T25 flasks.
- Incubate for 1.5 cell cycles (18 hours)

DAY 4 – harvesting cells for automated scoring protocol using the Metafer system

- Harvest cell pellets by centrifugation (230g for 5 min), resuspend in 5 ml pre-warmed PBS and centrifuge cells at 230g for 10 min. Discard the supernatant and repeat this wash step a second time.
- Re-suspend cell pellets with hypotonic solution (KCl 0.56%), then centrifuge immediately at 230g for 10min
- Re-suspend pellets in Fixative 1 (methanol: acetic acid: 0.9% NaCl (5:1:6 parts)) and incubate at 4°C for 10min before centrifugation at 230g for 10min.
- Re-suspend pellets in Fixative 2 (methanol: acetic acid (5:1 parts)) and incubate at 4°C for 10 min before centrifugation at 230g for 10 min. Repeat this wash step a further 3 times. Maintain cells in the last fix wash overnight at 4°C.
- Place freshly opened microscope slides in a glass tank of Fixative 2 at 4°C, at least a 2 hours before slide preparation (ideally overnight). On the day of preparing slides, replace the fix with ddH_2O.
- Centrifuge (230g for 10 minutes) the fixed cell suspensions and thoroughly re-suspend in ~1ml Fixative 2
- Take a slide out of the ddH_2O and wipe the water off the upper side with slide tissue, ideally with one movement (the surface should be dry or with only a faint film of water remaining). Pipette 100µl of the cell suspension evenly onto the slide
- Wait a few seconds until the suspension is evenly spread over the slide, and then put it in a vertical position for drying
- Check cell density of binuclear cells and if required adjust the final re-suspension volume by either lowering or increasing the volume of Fixative 2 added. Cells should not be overlapping, densely packed or too sparse.
- Stain slides with 30µl (3 dots of 10µl) of 1x Vectashield mounting medium with DAPI (1.5µg/ml), apply coverslip and incubate in the dark for 15 min
- Score slides on a TK6 cell classifier using the automated Metafer microscope (Axio-imager Z2 fluorescent microscope, Carl Zeiss UK) Metafer 4 software version 3.5 (MetaSystems, Germany).

Not all cell lines will be suited to this classifier – it is specific to TK6 cells. Score 2000 BN cells per ENM concentration/per replicate, (6000 BN cells in total per ENM concentration). Classifier information can be found in the work by Seager and colleagues (Seager et al., 2014). We have also provided nuclei and micronucleus classifier settings in the Appendix.

DAY 4 – harvesting cells for manual scoring

- Harvest cell pellets by centrifugation (230g for 5 min), resuspend in 5 ml pre-warmed PBS and centrifuge cells at 230g for 5 min. Discard the supernatant and repeat this wash step a second time.
- Resuspend cells in 10ml of PBS (if the cell pellet looks small adjust the volume as necessary)
- Place labelled slides (90% methanol-cleaned) into cyto-clips, place a filter card (with 5 mm cyto-dot hole) on top of the side and clip in place a cyto-funnel. Load 100μl of cell suspension into each funnel and centrifuge at 200g for 10 min in a Shandon Cytospin.
- Examine slides for correct cell density and adjust the volume of cell suspension as required. If the cells are too sparse, centrifuge the cell suspension at 230g for 5 min and resuspend in a smaller volume of PBS.
- Fix slides for 10min in ice cold 90% methanol and leave to air dry (at this point slides can be stored at -20°C)
- Stain slides in 20% Giemsa solution (5ml of Giemsa + 20ml pH 6.8 Gurr buffer, filtered) for 10 minutes
- Rinse slides in pH 6.8 Gurr buffer, then soak in pH 6.8 phosphatase buffer for 1-2min. Note: two slide tanks can be set up simultaneously to improve efficiency
- Leave slides to air dry standing vertically
- Dip slides in xylene for 10 seconds using tweezers and drain off the excess
- Drop DPX over the area of cells
- Place 22x22 mm coverslip over the DPX, ensuring there are no air bubbles by pressing lightly on the coverslip
- Leave slides to dry for 24h in the fume hood
- View slides under a light microscope, evaluate 1000 BN cells per replicate per concentration of ENM for the presence of micronuclei (3000 BN cells in total).
- Note: whilst this protocol describes Giemsa staining and evaluation with a light microscope, it is also possible to use DAPI (fluorescent) staining as an alternative option for manual scoring. However, for DAPI staining a fluorescence microscope is needed to enable scoring.

Cytotoxicity Calculations

As described in OECD TG487, CBPI, RPD or RICC are the most suitable for quantifying cytostasis and cytotoxicity (paused growth and cell death respectively).

Relative Population Doubling (RPD):

$$\text{RPD} = \frac{\text{No. of population doublings in treated cultures}}{\text{No. of population doublings in control cultures}} \times 100$$

$$\frac{\text{No. of population doublings in control cultures}}{}$$

Where population doubling = [log (Post-treatment cell number/ Initial cell number)]/ log 2

Relative Increase in Cell Counts (RICC):

$$\textbf{RICC} = \frac{\text{Increase in number of cells in treated cultures (final – starting)}}{\text{Increase in number of cells in control cultures (final – starting)}} \times 100$$

Cytokinesis Block Proliferation Index (CBPI):

% Cytotoxicity = 100 – 100 ($CBPI_T$ – 1 / $CBPI_C$ – 1); where T is treatment, C is control

CBPI = No mononucleated cells + 2x BN cells + 3 x MNCs / N; N is the total number of cells scored

Appendices for Swansea University SOP

TK6 classifier settings on the Metafer microscope (Axio-imager Z2 fluorescent microscope, Carl Zeiss UK) Metafer 4 software version 3.5 (MetaSystems, Germany)

Nuclei

- Image Processing Operations: Sharpen (3,2) Median V (3) Median H (3)
- Object Threshold: 30%
- Minimum Area: 20.0µm^2
- Maximum Area: 400.0µm^2
- Maximum Relative Concavity Depth: 0.900
- Maximum Aspect Ratio: 1.500
- Maximum Distance: 30.0µm^2
- Maximum Area Asymmetry: 70%
- Region of Interest Radius: 40.0µm^2
- Maximum Object Area in ROI: 50.0µm^2

Micronuclei

- Image Processing Operations: Median V (3) Median H (3) Sharpen (5,3) SB Histomax
- Object Threshold: 8%
- Minimum Area: 1.50µm^2
- Maximum Area: 55.0µm^2
- Maximum Relative Concavity Depth: 0.900
- Maximum Aspect Ratio: 4.000
- Maximum Distance: 25.0µm^2

References

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Annex B. Raw & additional data

Annex Table 1. Raw data sets for the 24-hour exposure of 5nm AuNPs, 30nm AuNPs, SiO_2, positive particle control WC-Co and positive chemical control, mitomycin (MMC) to human lymphoblast (TK6) cells. Data has been displayed for the individual replicates of cell viability (%) and the %Mn/BN, with the supporting average and standard deviation values to generate Figure 1.

TK6		Replicate 1	Replicate 2	Replicate 3	Average	StDev	TK6		Replicate 1	Replicate 2	Replicate 3	Average	StDev
Material	Dose (ug/ml)	% Viability					Material	Dose (ug/ml)	%Mn/BN				
Au 5nm	0	100	100	100	100	0	Au 5nm	0	0.4166667	0.16666667	0.3	0.294444	0.125093
	2	105.39235	101.96608	102.697951	98.72659	4.033629		2	0.2833333	0.35	0.1833333	0.272222	0.083887
	5	107.26709	107.90773	97.3541894	107.2029	1.536302		5	0.3666667	0.31666667	0.5166667	0.4	0.104083
	10	103.75565	106.88128	110.189878	97.90478	3.36687		10	0.4166667	0.55	0.6333333	0.533333	0.109291
	15	97.629756	95.129758	100.153171	104.0845	6.77204		15	0.95	1.01666667	0.8166667	0.927778	0.101835
	20	109.62284	102.49873	107.907727	108.5454	3.562626		20	1.1	1.18333333	1.5	1.261111	0.211038
		Replicate 1	Replicate 2	106.171175	Average	StDev			Replicate 1	Replicate 2	Replicate 3	Average	StDev
Material	Dose (ug/ml)	% Viability					Material	Dose (ug/ml)	%Mn/BN				
Au 30nm	0	100	100	100	100	0	Au 30nm	0	0.4166667	0.16666667	0.3	0.294444	0.125093
	2	97.216201	100.15317	101.765814	108.9901	2.306707		2	0.35	0.18333333	0.3	0.277778	0.085527
	5	108.92665	110.5039	104.018821	108.2269	3.382078		5	0.4	0.46666667	0.5333333	0.466667	0.066667
	10	105.91205	107.26709	104.609141	102.5652	1.329058		10	0.5166667	0.55	0.7333333	0.6	0.116667
	15	102.49873	104.8707	108.481788	107.9077	3.012837		15	0.7666667	0.5	0.5333333	0.6	0.145297
	20	103.42598	97.423132	109.053488	102.4987	5.816187		20	0.9833333	1.5	1.2333333	1.238889	0.258378
		Replicate 1	Replicate 2	Replicate 3	Average	StDev			Replicate 1	Replicate 2	Replicate 3	Average	StDev
Material	Dose (ug/ml)	% Viability					Material	Dose (ug/ml)	%Mn/BN				
SiO2	0	100	100	100	100	0	SiO2	0	0.4166667	0.16666667	0.3	0.294444	0.125093
	2	98.726591	108.99008	97.0089606	97.00896	6.478641		2	0.35	0.26666667	0.3166667	0.311111	0.041944
	5	107.20286	108.22694	102.565171	102.5652	3.016968		5	0.4666667	0.45	0.4166667	0.444444	0.025459
	10	97.904778	102.56517	100.625462	100.6255	2.341077		10	0.7	0.4	0.35	0.483333	0.189297
	15	104.08454	107.90773	108.099344	108.0993	2.264663		15	0.6333333	0.78333333	0.6833333	0.7	0.076376
	20	108.54543	102.49873	107.010002	107.01	3.143024		20	1.4166667	1.1	1.1833333	1.233333	0.164148
	25	107.71585	100.28828	97.2852122	97.28521	5.36944		25	1.1666667	1.05	1.2666667	1.161111	0.10844
	75	101.8326	103.49198	105.717393	105.7174	1.949256		75	1.8	1.58333333	1.8833333	1.755556	0.15486
	100	75.034874	79.340177	76.8050188	76.80502	2.16395		100	1.8166667	1.93333333	2.0333333	1.927778	0.10844
		Replicate 1	Replicate 2	Replicate 3	Average	StDev			Replicate 1	Replicate 2	Replicate 3	Average	StDev
Material	Dose (ug/ml)	% Viability					Material	Dose (ug/ml)	%Mn/BN				
MMC	0.01	77.204199	82.828758	86.9769612	82.33664	4.904932	MMC	0.01	1.95	2.21666667	2.35	2.172222	0.20367
WC-Co	20	103.8873	105.32725	105.262124	105.4725	1.662545	WC-Co	20	1.25	1.53333333	1.1	1.294444	0.220059
	100	98.179256	101.89936	107.202861	101.7802	3.542936		100	1.45	1.9	1.4	1.583333	0.275379

Annex Table 2. Raw data sets for the 24-hour exposure of 5nm AuNPs, 30nm AuNPs, SiO_2, positive particle control WC-Co and positive chemical control, mitomycin (MMC) to human lymphoblast (HepG2) cells. Data has been displayed for the individual replicates of cell viability (%) and the %Mn/BN, with the supporting average and standard deviation values to generate Figure 2.

HepG2		Replicate 1	Replicate 2	Replicate 3	Average	StDev	HepG2		Replicate 1	Replicate 2	Replicate 3	Average	StDev
Material	Dose (ug/ml)	% Viability					Material	Dose (ug/ml)	%Mn/BN				
Au 5nm	0	100	100	100	100	0	Au 5nm	0	1.2	1.7	1.3333333	1.411111	0.258915
	2	96.207628	99.961368	100.1	98.74017	2.193709		2	1.6333333	1.9333333	1.7333333	1.766667	0.152753
	5	96.091269	100.0713	100	98.73803	2.292181		5	1.7666667	2.2	2.1333333	2.033333	0.233333
	10	92.518215	99.761101	100.052	97.44361	4.267988		10	2.2666667	2	2.6333333	2.3	0.31798
	15	92.284054	100.12409	100.05152	97.48655	4.505643		15	2.2	2.1	2.1666667	2.155556	0.050918
	20	84.412843	99.96343	100.05	94.80926	9.003671		20	2.2666667	2.9333333	2.4333333	2.544444	0.346944
		Replicate 1	Replicate 2	Replicate 3	Average	StDev			Replicate 1	Replicate 2	Replicate 3	Average	StDev
Material	Dose (ug/ml)	% Viability					Material	Dose (ug/ml)	%Mn/BN				
Au 30nm	0	100	100	100	100	0	Au 30nm	0	1.2	1.7	1.3333333	1.411111	0.258915
	2	94.923001	102.34633	102.14183	99.80372	4.228062		2	1.6666667	1.3333333	1.3666667	1.455556	0.183586
	5	98.304254	102.33372	102.17757	100.9385	2.282674		5	1.1666667	1.2	1.4333333	1.266667	0.145297
	10	94.69034	102.14936	102.19423	99.67798	4.319478		10	1.8	1.3666667	1.3	1.488889	0.271484
	15	102.14936	102.03228	103.54096	102.5742	0.839285		15	1.5666667	2.0666667	2.1666667	1.933333	0.321455
	20	102.36963	102.11499	104.72525	103.07	1.439166		20	2.2	1.5	1.7666667	1.822222	0.353291
		Replicate 1	Replicate 2	Replicate 3	Average	StDev			Replicate 1	Replicate 2	Replicate 3	Average	StDev
Material	Dose (ug/ml)	% Viability					Material	Dose (ug/ml)	%Mn/BN				
SiO2	0	100	100	100	100	0	SiO2	0	1.2	1.7	1.3333333	1.411111	0.258915
	2	105.59959	101.82657	105.77117	104.3991	2.229536		2	1.1	1	1.0333333	1.044444	0.050918
	5	101.11347	94.850647	105.58924	100.5178	5.394021		5	1.4	1.0333333	1.4666667	1.3	0.233333
	10	105.55789	91.63692	105.66827	100.9544	8.069328		10	1.5	1.7666667	1.9666667	1.744444	0.234126
	15	105.56821	101.81969	105.56173	104.3165	2.162343		15	1.8	1.7666667	1.7333333	1.766667	0.033333
	20	105.56459	101.85733	105.57846	104.3335	2.144402		20	1.7333333	2.1333333	2.1666667	2.011111	0.241139
	25	105.5541	97.974881	105.57508	103.0347	4.381932		25	2.1	2.1333333	1.7	1.977778	0.241139
	75	105.54855	101.98226	105.57486	104.3686	2.066638		75	2.1666667	2.0666667	1.9666667	2.066667	0.1
	100	92.065299	97.851974	105.60696	98.50808	6.794632		100	2.2333333	3.0666667	2.6666667	2.655556	0.416778
		Replicate 1	Replicate 2	Replicate 3	Average	StDev			Replicate 1	Replicate 2	Replicate 3	Average	StDev
Material	Dose (ug/ml)	% Viability					Material	Dose (ug/ml)	%Mn/BN				
MMC	0.01	96.145824	80.094454	82.839029	86.35977	8.585355	MMC	0.01	4.4666667	4.6666667	5.3666667	4.833333	0.472582
WC-Co	20	100.05152	92.130155	100.07395	97.41854	4.57989	WC-Co	20	2.2	1.4333333	2.0333333	1.888889	0.403228
	100	100.04995	87.647766	99.965499	95.88774	7.136151		100	4.3666667	3.1	4.2	3.888889	0.688261

Annex Table 3. Raw data sets for the 24-hour exposure of 5nm AuNPs, 30nm AuNPs, SiO_2, CeO_2, positive particle control WC-Co and positive chemical controls, mitomycin (MMC) and colcemide to Buffy coat cells. Data has been displayed for the individual replicates of cell viability (%) and the %Mn/BN, with the supporting average and standard deviation values to generate Figure 3.

BC	Replicate 1						Replicate 2						CBPI cytostasis [%]		%Mn/BN	
Material	Dose (ug/ml)	Mononucleate	Binucleate	Multinucleate	CBPI cyto-stasis [%]	%Mn/BN	Dose (ug/ml)	Mononucleate	Binucleate	Multinucleate	CBPI cyto-stasis [%]	%Mn/BN	Average	StDev	Average	StDev
Au 5nm	0	62	399	39		0.4	0	68	402	30		0.7	0.0		0.6	
	10	77	403	20		0.8	10	76	406	18		0.3	5.8		0.6	
	25	99	369	32		0.5	25	103	371	26		0.8	8.8		0.7	
	50	121	342	37		0.2	50	121	358	21		0.4	13.1		0.3	
	100	118	368	14		0.0	100	143	338	19		1.0	17.8		0.5	
BC	Replicate 1						Replicate 2						CBPI cytostasis [%]		%Mn/BN	
Material	Dose (ug/ml)	Mononucleate	Binucleate	Multinucleate	CBPI cyto-stasis [%]	%Mn/BN	Dose (ug/ml)	Mononucleate	Binucleate	Multinucleate	CBPI cyto-stasis [%]	%Mn/BN	Average	StDev	Average	StDev
Au 5nm	0	78	399	23		0.4	0	85	376	39		0.6	0.0		0.5	
	10	79	381	40		0.7	10	61	407	32		0.7	-3.7		0.7	
	25	81	389	30		0.5	25	83	381	36		0.8	-0.3		0.7	
	50	80	397	23		0.4	50	89	379	32		0.2	1.4		0.3	
	100	102	371	27		0.4	100	75	382	43		0.5	0.7		0.5	
BC	Replicate 1						Replicate 2						CBPI cytostasis [%]		%Mn/BN	
Material	Dose (ug/ml)	Mononucleate	Binucleate	Multinucleate	CBPI cyto-stasis [%]	%Mn/BN	Dose (ug/ml)	Mononucleate	Binucleate	Multinucleate	CBPI cyto-stasis [%]	%Mn/BN	Average	StDev	Average	StDev
Au 30nm	0	56	430	14		0.8	0	72	405	23		0.3	0.0		0.6	
	10	63	413	24		0.4	10	65	401	34		0.2	-2.3		0.3	
	25	57	427	16		0.4	25	56	429	15		0.3	-1.0		0.4	
	50	94	396	10		0.2	50	67	406	27		1.2	3.6		0.7	
	100	49	433	18		0.3	100	60	419	21		0.6	-2.3		0.5	
BC	Replicate 1						Replicate 2						CBPI cytostasis [%]		%Mn/BN	
Material	Dose (ug/ml)	Mononucleate	Binucleate	Multinucleate	CBPI cyto-stasis [%]	%Mn/BN	Dose (ug/ml)	Mononucleate	Binucleate	Multinucleate	CBPI cyto-stasis [%]	%Mn/BN	Average	StDev	Average	StDev
Au 30nm	0	50	374	76		0.4	0	47	378	75		1.2	0.0		0.8	
	10	53	355	92		0.2	10	63	360	77		0.3	0.1		0.3	
	25	56	374	70		0.4	25	37	373	90		0.8	-1.2		0.6	
	50	47	382	71		1.1	50	56	379	65		1.5	2.0		1.3	
	100	64	381	55		0.3	100	28	405	67		0.4	2.3		0.4	
BC	Replicate 1						Replicate 2						CBPI cytostasis [%]		%Mn/BN	
Material	Dose (ug/ml)	Mononucleate	Binucleate	Multinucleate	CBPI cyto-stasis [%]	%Mn/BN	Dose (ug/ml)	Mononucleate	Binucleate	Multinucleate	CBPI cyto-stasis [%]	%Mn/BN	Average	StDev	Average	StDev
SiO2	0	78	399	23		0.4	0	85	376	39		0.6	0.0			
	10	77	393	30		1.1	10	77	398	25		0.6	-0.2			
	25	120	356	24		0.4	25	84	388	28		0.5	5.7			
	50	102	375	23		0.3	50	88	396	16		0.4	5.6			
	100	96	383	21		0.5	100	96	382	22		0.4	5.3			
BC	Replicate 1						Replicate 2						CBPI cytostasis [%]		%Mn/BN	
Material	Dose (ug/ml)	Mononucleate	Binucleate	Multinucleate	CBPI cyto-stasis [%]	%Mn/BN	Dose (ug/ml)	Mononucleate	Binucleate	Multinucleate	CBPI cyto-stasis [%]	%Mn/BN	Average	StDev	Average	StDev
CeO2	0	68	412	20		1	0	53	433	14		0.5	0.0		0.8	
	10	111	375	14		1	10	125	352	23		0.4	12.3		0.7	
	25	122	364	14		0.5	25	38	453	9		0.7	5.5		0.6	
	50	120	364	16		0.4	50	104	384	12		0.6	11.9		0.5	
	100	73	401	26		0.5	100	136	346	18		0.6	8.5		0.6	
BC	Replicate 1						Replicate 2						CBPI cytostasis [%]		%Mn/BN	
Material	Dose (ug/ml)	Mononucleate	Binucleate	Multinucleate	CBPI cyto-stasis [%]	%Mn/BN	Dose (ug/ml)	Mononucleate	Binucleate	Multinucleate	CBPI cyto-stasis [%]	%Mn/BN	Average	StDev	Average	StDev
MMC	0.04	191	304	5		8.7	0.04	165	326	9		5.3	29.9		7.0	
	0.04	121	358	21		4.8	0.04	125	358	17		5.4	11.9		5.1	
	0.04	119	376	5		5.1	0.04	167	330	3		6.7	20.6		5.9	
	0.04	186	302	12		8.3	0.04	179	297	24		11.0	36.3		9.7	
	0.04	121	358	21		4.8	0.04	125	358	17		5.4	11.9		5.1	
	0.04	156	333	11		8.0	0.04	171	317	12		8.7	23.8		8.4	
CoI	0.05	220	265	15		2.9	0.05	186	300	14		2.3	33.7		2.6	
	0.05	168	308	24		5.9	0.05	186	288	26		5.0	22.6		5.5	
	0.05	138	343	19		4.9	0.05	111	381	8		2.6	14.4		3.8	
	0.05	192	280	28		3.2	0.05	221	241	38		1.4	38.0		2.3	
	0.05	168	308	24		5.9	0.05	186	288	26		5.0	22.6		5.5	
	0.05	179	311	10		3.7	0.05	160	330	10		4.6	25.4		4.2	
WC-Co	10	126	360	14		0.7	10	173	322	5		0.9	23.3		0.8	
	10	208	286	6		0.7	10	156	340	4		0.4	28.9		0.6	
	10	122	363	15		0.7	10	118	366	16		0.6	25.0		0.7	
	10	152	336	12		1.4	10	149	349	2		0.6	20.7		1.00	
	10	111	375	14		0.3	10	125	352	23		0.8	12.3		0.60	
	30	261	235	4		0.4	30	280	220	0		0.6	50.7		0.5	
	30	320	178	2		0.2	30	263	232	5		0.1	53.4		0.2	
	30	223	274	3		0.5	30	259	235	6		0.9	50.0		0.7	
	30	197	297	6		0.9	30	193	302	5		0.5	30.9		0.7	
	30	227	271	2		0.9	30	213	286	1		1.1	38.3		1.0	
	60	290	210	0		n.s.	60	315	185	0		n.s.	57.9		n.s.	
	60	341	157	2		1.2	60	385	115	0		1.2	69.6		1.2	
	60	278	218	4		1.6	60	265	233	2		1.7	56.1		1.7	
	60	230	265	5		1.1	60	234	261	5		0.4	39.3		0.8	
	60	160	337	3		0	60	190	309	1		1.1	28.4		0.6	
	100	305	195	0		2.2	100	282	205	13		1.6	59.6		1.9	
	100	226	271	3		1.2	100	244	245	11		1.1	39.5		1.2	
	100	204	294	2		1.5	100	176	322	2		1.8	31.7		1.7	

Annex Table 4. Raw data sets for the 24-hour exposure of 5nm AuNPs, 30nm AuNPs, SiO_2, $BaSO_4$, positive particle control WC-Co and positive chemical controls mitomycin (MMC) and colcemide to whole blood cultures. Data has been displayed for the individual replicates of cell viability (%) and the %Mn/BN, with the supporting average and standard deviation values to generate Figure 4.

WB	Replicate 1						Replicate 2						CBPI cytostasis [%]		%Mn/BN	
Material	Dose (ug/ml)	Mononucleate	Binucleate	Multinucleate	CBPI cyto-stasis [%]	%Mn/BN	Dose (ug/ml)	Mononucleate	Binucleate	Multinucleate	CBPI cyto-stasis [%]	%Mn/BN	Average	StDev	Average	StDev
Au 5nm	0	67	362	71		0.5	0	105	324	71		0.7	0.0		0.6	
	10	78	353	69		0.4	10	55	360	85		0.2	-5.3		0.3	
	25	99	328	73		0.2	25	90	340	70		0.3	1.6		0.3	
	50	109	325	66		0.4	50	127	318	55		0.6	8.8		0.5	
	100	100	336	64		0.2	100	92	344	64		0.5	3.5		0.4	
WB	Replicate 1						Replicate 2						CBPI cytostasis [%]		%Mn/BN	
Material	Dose (ug/ml)	Mononucleate	Binucleate	Multinucleate	CBPI cyto-stasis [%]	%Mn/BN	Dose (ug/ml)	Mononucleate	Binucleate	Multinucleate	CBPI cyto-stasis [%]	%Mn/BN	Average	StDev	Average	StDev
Au 30nm	0	29	420	51		0.4	0	49	409	42		0.4	0.0		0.4	
	10	36	403	61		0.4	10	40	407	55		0.4	-2.5		0.4	
	25	35	387	78		0.6	25	33	372	95		0.0	-8.9		0.3	
	50	44	373	83		0.2	50	40	369	91		0.3	-7.4		0.3	
	100	42	375	83		0.3	100	39	390	71		0.3	-5.7		0.3	
WB	Replicate 1						Replicate 2						CBPI cytostasis [%]		%Mn/BN	
Material	Dose (ug/ml)	Mononucleate	Binucleate	Multinucleate	CBPI cyto-stasis [%]	%Mn/BN	Dose (ug/ml)	Mononucleate	Binucleate	Multinucleate	CBPI cyto-stasis [%]	%Mn/BN	Average	StDev	Average	StDev
Au 30nm	0	50	374	76		0.4	0	47	378	75		1.2	0.0		0.8	
	10	53	355	92		0.2	10	63	360	77		0.3	0.1		0.3	
	25	56	374	70		0.4	25	37	373	90		0.8	-1.2		0.6	
	50	47	382	71		1.1	50	56	379	65		0.4	2.0		0.8	
	100	64	381	55		0.3	100	28	405	67		0.4	2.3		0.4	
WB	Replicate 1						Replicate 2						CBPI cytostasis [%]		%Mn/BN	
Material	Dose (ug/ml)	Mononucleate	Binucleate	Multinucleate	CBPI cyto-stasis [%]	%Mn/BN	Dose (ug/ml)	Mononucleate	Binucleate	Multinucleate	CBPI cyto-stasis [%]	%Mn/BN	Average	StDev	Average	StDev
BaSO4	0	71	374	55		0.5	0	55	379	66		0.6	0.0		0.6	
	10	47	396	57		0.9	10	48	419	33		1.2	0.0		1.1	
	25	54	398	48		0.9	25	36	408	56		1.3	-1.9		1.1	
	50	53	388	59		1.2	50	37	407	56		0.6	-3.0		0.9	
	100	55	392	53		0.4	100	59	395	46		1.2	1.0		0.8	
WB	Replicate 1						Replicate 2						CBPI cytostasis [%]		%Mn/BN	
Material	Dose (ug/ml)	Mononucleate	Binucleate	Multinucleate	CBPI cyto-stasis [%]	%Mn/BN	Dose (ug/ml)	Mononucleate	Binucleate	Multinucleate	CBPI cyto-stasis [%]	%Mn/BN	Average	StDev	Average	StDev
MMC		148	308	44		3.5	0.04	189	269	42		4.0	22.8		3.8	
	0.04	97	354	49		3.3	0.04	79	386	35		2.8	10.5		3.1	
	0.04	186	302	12		8.3	0.04	179	297	24		11.0	36.3		9.7	
	0.04	112	342	46		5.9	0.04	126	332	42		4.2	14.6		5.1	
Col	0.05	234	224	42		2.8	0.05	154	299	47		2.3	27.7		2.6	
	0.05	82	316	102		2.5	0.05	162	314	24		1.9	13.1		2.2	
	0.05	192	280	28		3.2	0.05	221	241	38		1.4	38.0		2.3	
	0.05	214	249	37		6.4	0.05	223	252	25		4.5	37.2		5.5	
WC-Co	10	112	346	42		0.7	10	121	356	23		0.5			0.6	
	10	42	428	30		0.7	10	65	420	15		0.5	7.6		0.6	
	10	122	363	15		0.7	10	118	366	16		0.6	25.0		0.7	
	10	97	370	33		1	10	104	368	28		0.9	13.6		1.00	
	30	161	329	10		0.2	30	125	370	5		1.0	24.8		0.6	
	30	83	398	19		0.3	30	68	418	14		0.7	13.1		0.5	
	30	223	274	3		0.5	30	259	235	6		0.9	50.0		0.7	
	30	103	368	29		1	30	116	363	21		2.3	16.5		1.7	
	60	203	296	1		0.7	60	181	318	1		0.7	36.3		0.7	
	60	186	310	4		0.7	60	167	327	6		0.1	35.3		0.4	
	60	278	218	4		1.6	60	265	233	2		1.7	56.1		1.7	
	100	290	208	2		0.9		281	218	1		1.4	55.5		1.2	
	100	301	197	2		1.3	100	257	241	2		1.2	56.1		1.3	
	100	305	195	0		2.2	100	282	205	13		1.6	59.6		1.9	
	100	98	364	38		1.4	100	n.s.	n.s.	n.s.		n.s.	11.6		1.4	

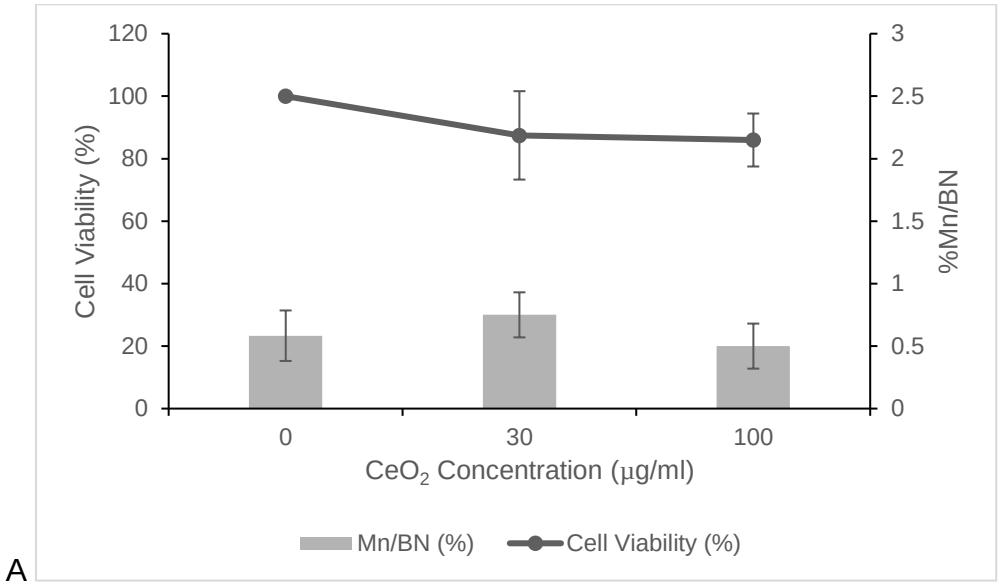
Annex Table 5. Raw data sets for the 24-hour exposure of 5nm AuNPs, 30nm AuNPs, SiO_2, CeO_2, $BaSO_4$, positive particle control WC-Co and positive chemical control, ethyl methanesulfonate (EMS) to mouse fibroblast (V79) cells. Data has been displayed for the individual replicates of cell viability (%) and the %Mn/BN, with the supporting average and standard deviation values to generate Figure 5.

V79	Replicate 1						Replicate 2						Relative Cell Viability (%)		%Mn/BN	
Material	Dose (ug/ml)	Mononucleate	Binucleate	Multinucleate	Relative Cell Viability (%)	%Mn/BN	Dose (ug/ml)	Mononucleate	Binucleate	Multinucleate	Relative Cell Viability (%)	%Mn/BN	Average	StDev	Average	StDev
Au 5nm	0	19	432	49		0.5	0	33	425	42		0.9	100.0		0.7	
	10	28	430	42		0.2	10	14	448	38		1.1	100.0		0.7	
	25	14	446	40		0.1	25	23	437	40		1.0	100.0		0.6	
	50	20	439	41		0.6	50	26	429	45		0.6	100.0		0.6	
	100	17	435	48		0.5	100	24	433	43		1.0	100.0		0.8	
V79	Replicate 1						Replicate 2						Relative Cell Viability (%)		%Mn/BN	
Material	Dose (ug/ml)	Mononucleate	Binucleate	Multinucleate	Relative Cell Viability (%)	%Mn/BN	Dose (ug/ml)	Mononucleate	Binucleate	Multinucleate	Relative Cell Viability (%)	%Mn/BN	Average	StDev	Average	StDev
Au 30nm	0	26	375	99		0.8	0	24	376	100		1.0	100		0.9	
	10	18	386	96		0.7	10	22	374	104		0.9	101		0.8	
	25	21	420	59		4.5	25	15	396	89		1.0	96.8		2.8	
	50	18	379	103		1.1	50	31	382	87		1.4	99.3		1.3	
	100	19	373	108		1.4	100	13	392	95		0.8	101.9		1.1	
V79	Replicate 1						Replicate 2						Relative Cell Viability (%)		%Mn/BN	
Material	Dose (ug/ml)	Mononucleate	Binucleate	Multinucleate	Relative Cell Viability (%)	%Mn/BN	Dose (ug/ml)	Mononucleate	Binucleate	Multinucleate	Relative Cell Viability (%)	%Mn/BN	Average	StDev	Average	StDev
Au 30nm	0	27	377	96		0.8	0	14	393	93		0.3	100.0		0.6	
	10	34	367	99		0.85	10	21	376	103		1.1	100.0		0.8	
	25	37	384	79		0.6	25	30	385	85		0.7	95.7		0.7	
	50	36	376	88		0.5	50	41	398	61		0.7	93.0		0.6	
	100	13	418	69		0.9	100	22	411	67		0.6	95.7		0.8	
V79	Replicate 1						Replicate 2						Relative Cell Viability (%)		%Mn/BN	
Material	Dose (ug/ml)	Mononucleate	Binucleate	Multinucleate	Relative Cell Viability (%)	%Mn/BN	Dose (ug/ml)	Mononucleate	Binucleate	Multinucleate	Relative Cell Viability (%)	%Mn/BN	Average	StDev	Average	StDev
SiO2	0	26	375	99		0.8	0	24	376	100		1.0	100.0		0.9	
	10	24	385	91		0.7	10	25	386	89		0.6	98.26		0.7	
	25	29	396	75		0.6	25	16	410	74		0.7	95.65		0.7	
	50	30	391	79		0.8	50	21	394	85		0.6	96.52		0.7	
	100	16	373	111		0.5	100	22	401	77		0.9	100.0		0.7	
V79	Replicate 1						Replicate 2						Relative Cell Viability (%)		%Mn/BN	
Material	Dose (ug/ml)	Mononucleate	Binucleate	Multinucleate	Relative Cell Viability (%)	%Mn/BN	Dose (ug/ml)	Mononucleate	Binucleate	Multinucleate	Relative Cell Viability (%)	%Mn/BN	Average	StDev	Average	StDev
CeO2	0	27	377	96		0.8	0	14	393	93		0.3	100.0		0.6	
	10	16	435	49		1.1	10	6	419	75		0.8	95.7		1	
	25	19	385	96		0.6	25	21	412	67		0.7	97.4		0.7	
	50	35	395	70		0.5	50	26	404	70		0.4	93.9		0.5	
	100	6	427	67		0.2	100	20	415	65		0.6	96.5		0.4	
V79	Replicate 1						Replicate 2						Relative Cell Viability (%)		%Mn/BN	
Material	Dose (ug/ml)	Mononucleate	Binucleate	Multinucleate	Relative Cell Viability (%)	%Mn/BN	Dose (ug/ml)	Mononucleate	Binucleate	Multinucleate	Relative Cell Viability (%)	%Mn/BN	Average	StDev	Average	StDev
BaSO4	0	33	372	95		1.1	0	29	356	115		1.2	100.0		1.2	
	10	15	377	108		1.1	10	38	364	98		1.2	100.4		1.2	
	25	25	371	104		0.9	25	32	361	107		0.3	100.5		0.6	
	50	33	353	114		0.5	50	50	408	42		2.5	93.5		1.5	
	100	32	401	67		0.7	100	30	375	95		1.0	95.8		0.9	
V79	Replicate 1						Replicate 2						Relative Cell Viability (%)		%Mn/BN	
Material	Dose (ug/ml)	Mononucleate	Binucleate	Multinucleate	Relative Cell Viability (%)	%Mn/BN	Dose (ug/ml)	Mononucleate	Binucleate	Multinucleate	Relative Cell Viability (%)	%Mn/BN	Average	StDev	Average	StDev
BaSO4	0	27	377	96	100	0.8	0	14	393	93		0.3	100.0		0.6	
	10	24	410	66		0.7	10	29	417	54		0.8	93.0		0.8	
	25	30	408	62		0.9	25	37	409	54		0.4	91.3		0.7	
	50	31	396	73		0.3	50	28	414	58		0.4	93.0		0.4	
	100	38	394	68		0.1	100	26	424	50		0.5	91.3		0.3	
V79	Replicate 1						Replicate 2						Relative Cell Viability (%)		%Mn/BN	
Material	Dose (ug/ml)	Mononucleate	Binucleate	Multinucleate	Relative Cell Viability (%)	%Mn/BN	Dose (ug/ml)	Mononucleate	Binucleate	Multinucleate	Relative Cell Viability (%)	%Mn/BN	Average	StDev	Average	StDev
EMS	500	38	449	13		9.6	500	53	437	10		9.5	89.4		9.6	
	500	29	405	30		10.5	500	34	436	30		10.3	86.8		10.4	
	500	25	460	15		7.6	500	27	456	17		6.4	85.2		7.0	
	500	29	405	30		10.5	500	34	436	30		10.3	87.0		10.4	
	500	42	426	32		9.2	500	63	417	20		10.2	82.5		9.7	
	500	25	460	15		7.6	500	27	456	17		6.4	85.2		7.0	
WC-Co	10	39	363	98		1.1	500	29	400	71		1.0	82.5		1.1	
	30	20	435	45		1.1	30	23	420	57		0.9	101.9		1.0	
	30	18	374	108		0.6	30	31	387	82		1.0	99.3		0.8	
	30	17	393	90		0.6	30	11	411	78		0.7	99.1		0.7	
	30	18	374	108		0.6	30	31	387	82		1.0	99.1		0.8	
	30	20	339	141		0.8	30	34	350	116		1.1	104.8		1.0	
	30	17	393	90		0.6	30	11	411	78		0.7	99.1		0.7	
	100	22	431	47		1.6	100	36	428	36		0.4	99.0		1.0	
	100	36	393	71		1.0	100	22	412	66		0.8	93.9		0.9	
	100	18	411	71		0.7	100	25	422	53		1.2	93.9		1.0	
	100	36	393	71		1	100	22	412	66		0.8	93.9		0.9	
	100	33	350	117		1.3	100	27	383	90		0.9	99.9		1.1	
	100	18	411	71		0.7	100	25	422	53		1.2	93.9		0.7	

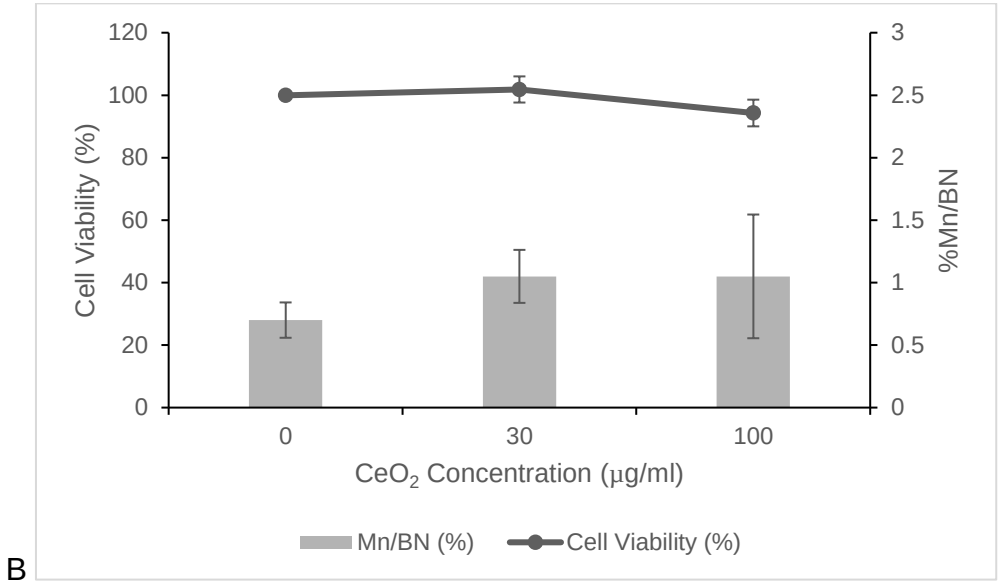
Annex Table 6. Raw data sets for the 24-hour exposure of 5nm AuNPs, 30nm AuNPs, SiO_2, positive particle control WC-Co and positive chemical control, ethyl methanesulfonate (EMS) to mouse fibroblast (V79) cells. Data has been displayed for the individual replicates of cell viability (%) and the %Mn/BN, with the supporting average and standard deviation values to generate Figure 6.

V79	Replicate 1						Replicate 2						Relative Cell Viability (%)		%Mn/BN	
Material	Dose (ug/ml)	Mononucleate	Binucleate	Multinucleate	Relative Cell Viability (%)	%Mn/BN	Dose (ug/ml)	Mononucleate	Binucleate	Multinucleate	Relative Cell Viability (%)	%Mn/BN	Average	StDev	Average	StDev
Au 5nm	0	1148	1050	37	100	0.2	0	1182	1040	29	100	0	100	0	0.1	0.141421
	2	1156	1005	21	104.8085827	0.1	2	1240	1000	14	106.9516877	0.2	105.8801	1.515404	0.15	0.070711
	5	1117	1000	33	101.4308439	0	5	1130	1002	54	96.06253077	0.3	98.74669	3.795971	0.15	0.212132
	10	1158	1004	33	103.166698	0.1	10	1265	1000	41	103.9582326	0.2	103.5625	0.559699	0.15	0.070711
	15	1072	1000	28	100.0101688	0.4	15	1150	1020	40	98.00008077	0.4	99.00512	1.421347	0.4	0
	20	1119	998	43	100.2105055	0.4	20	1265	1000	92	97.10346453	0.5	98.65698	2.19701	0.45	0.070711
V79	Replicate 1						Replicate 2						Relative Cell Viability (%)		%Mn/BN	
Material	Dose (ug/ml)	Mononucleate	Binucleate	Multinucleate	Relative Cell Viability (%)	%Mn/BN	Dose (ug/ml)	Mononucleate	Binucleate	Multinucleate	Relative Cell Viability (%)	%Mn/BN	Average	StDev	Average	StDev
Au 30nm	0	1148	1050	37	100	0.2	0	1182	1040	29	100	0	100	0	0.1	0.141421
	2	1023	1001	22	98.46414694	0.3	2	937	1010	17	91.76304784	0	95.1136	4.738393	0.15	0.212132
	5	1133	1000	55	99.13182982	0.2	5	1020	990	9	97.70181507	0.1	98.41682	1.011173	0.15	0.070711
	10	1034	1005	22	98.80781313	0.3	10	921	1000	8	92.6115952	0.3	95.7097	4.381388	0.3	0
	15	1112	1006	34	100.7689584	0.2	15	1019	1009	13	96.18990594	0.5	98.47943	3.237879	0.35	0.212132
	20	1222	1000	55	103.1641574	0.4	20	1142	1000	43	98.14054404	0.2	100.6524	3.552231	0.3	0.141421
V79	Replicate 1						Replicate 2						Relative Cell Viability (%)		%Mn/BN	
Material	Dose (ug/ml)	Mononucleate	Binucleate	Multinucleate	Relative Cell Viability (%)	%Mn/BN	Dose (ug/ml)	Mononucleate	Binucleate	Multinucleate	Relative Cell Viability (%)	%Mn/BN	Average	StDev	Average	StDev
SiO2	0	1148	1050	37	100	0.2	0	1182	1040	29	100	0	100	0	0.1	0.141421
	2	1033	1000	12	100.4343191	0.2	2	1153	1000	34	99.88594333	0	100.1601	0.38776	0.1	0.141421
	5	1025	1000	43	95.76559095	0.1	5	1057	999	17	97.8871819	0.1	96.82639	1.500191	0.1	0
	10	952	1000	25	94.69044423	0.3	10	1024	1000	42	92.9667995	0.1	93.82862	1.218801	0.2	0.141421
	15	1144	1003	8	106.3559703	0.2	15	931	1001	13	92.37958506	0.4	99.36778	9.882797	0.3	0.141421
	20	1050	997	29	98.96090843	0.4	20	1066	1002	20	97.74389032	0.2	98.3524	0.860562	0.3	0.141421
	25	932	1000	41	91.70406944	0.3	25	1122	1000	13	101.502646	0.6	96.60336	6.92864	0.45	0.212132
	75	935	1000	53	90.39617138	0.6	75	1154	1000	22	101.6682241	0.6	96.0322	7.970545	0.6	0
	100	1140	1002	31	102.7086172	0.4	100	1047	1012	26	95.58533718	0.7	99.14698	5.03692	0.55	0.212132
V79	Replicate 1						Replicate 2						Relative Cell Viability (%)		%Mn/BN	
Material	Dose (ug/ml)	Mononucleate	Binucleate	Multinucleate	Relative Cell Viability (%)	%Mn/BN	Dose (ug/ml)	Mononucleate	Binucleate	Multinucleate	Relative Cell Viability (%)	%Mn/BN	Average	StDev	Average	StDev
EMS	600	745	1019	26	84.05283068	1.9	0.01	855	1020	30	86.03953798	1.6	85.04618	1.404814	1.75	0.212132
WC-Co	30	1042	1019	33	97.05897999	0.8	20	1059	1002	41	94.58674374	0.6	95.82286	1.748135	0.7	0.141421
	100	1042	998	26	98.95319058	0.5	100	1138	1000	30	99.76547111	0.4	99.35933	0.574369	0.45	0.070711

Annex Figure 1. A comparison of semi-automated Metafer scoring (A) against manual scoring (B) of TK6 cells exposed to cerium dioxide (CeO_2) for one cell cycle. Data displayed represents the average +/- the standard deviation (*n*=3).



A



B

Annex C. List of Abbreviations

ANOVA	Analysis Of Variance
ANSES	French Agency For Food, Environmental And Occupational Health & Safety
ATCC	American Type Culture Collection
AUC	Analytical Ultracentrifugation
AuNP	Gold Nanoparticle
$BaSO_4$ NP	Barium Sulfate Nanoparticle
BET Method	Brunauer-Emmett-Teller Method
BN	Binucleated
CBMN	Cytokinesis-Blocked Micronucleus
CBPI	Cytochalasin B Blocked Proliferaiton Index
CeO_2 NP	Cerium Dioxide Nanoparticle
CO_2	Carbon Dioxide
Col	Colcemide
CytB	Cytochalasin B
ddH_2O	Double Distilled Water
DMSO	Dimethyl Sulfoxide
ECHA	European Chemical Agency
EDTA	Ethylenediaminetetraacetic Acid
EDX	Energy Dispersive X-Ray
EMS	Ethylmethane Sulfonate
ENM	Engineered Nanomaterial

FBS	Fetal Bovine Serum
FCS	Fetal Calf Serum
GD	Guidance Document
HBSS	Hanks Buffered Salt Solution
HS	Horse Serum
JRC	Joint Research Center
KCl	Potassium Chloride
LA ICP MS	Laser Ablation Inductively Coupled Plasma Mass Spectrometry
MAD	Mutal Acceptance Of Data
MMC	Mitomycin C
MMS	Methyl Methanesulphate
Mn/BN	Micronucleated Binucleate Cell
MNU	N-Nitro-N-Methylurea
NaCl	Sodium Chloride
NILU	Norwegian Institute For Air Research
OECD	Organization For Economic Co-Operation And Development
P/S	Penicillin/Streptomycin
PBS	Phosphate Buffered Saline
PHA	Phytohemagglutinin
PHE	Public Health England
RPD	Relative Population Doubling
SD	Standard Deviation
SEM	Scanning Electron Microscopy
SiO_2NP	Silicium Dioxide Nanoprticle
SU	Swansea University
TEM	Transmission Electron Microscopy

TG	Test Guideline
WC-Co	Tungsten Carbide Cobalt