



High throughput toxicity screening and intracellular detection of nanomaterials

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With the growing numbers of nanomaterials (NMs), there is a great demand for rapid and reliable ways of testing NM safety—preferably using *in vitro* approaches, to avoid the ethical dilemmas associated with animal research. Data are needed for developing intelligent testing strategies for risk assessment of NMs, based on grouping and read-across approaches. The adoption of high throughput screening (HTS) and high content analysis (HCA) for NM toxicity testing allows the testing of numerous materials at different concentrations and on different types of cells, reduces the effect of inter-experimental variation, and makes substantial savings in time and cost. HTS/HCA approaches facilitate the classification of key biological indicators of NM-cell interactions. Validation of *in vitro* HTS tests is required, taking account of relevance to *in vivo* results. HTS/HCA approaches are needed to assess dose- and time-dependent toxicity, allowing prediction of *in vivo* adverse effects. Several HTS/HCA methods are being validated and applied for NM testing in the FP7 project NANoREG, including

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Label-free cellular screening of NM uptake, HCA, High throughput flow cytometry, Impedance-based monitoring, Multiplex analysis of secreted products, and genotoxicity methods—namely High throughput comet assay, High throughput *in vitro* micronucleus assay, and γ H2AX assay. There are several technical challenges with HTS/HCA for NM testing, as toxicity screening needs to be coupled with characterization of NMs in exposure medium prior to the test; possible interference of NMs with HTS/HCA techniques is another concern. Advantages and challenges of HTS/HCA approaches in NM safety are discussed. © 2016 The Authors. *WIREs Nanomedicine and Nanobiotechnology* published by Wiley Periodicals, Inc.

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INTRODUCTION TO HIGH-THROUGHPUT SCREENING OF NANOMATERIALS

Manufactured nanomaterials (NMs—materials with at least one dimension <100 nm) and nanoparticles (NPs—NMs with all three dimensions <100 nm) are considered as distinct from normal chemical compounds on account of their size, chemical composition, shape, surface structure, surface charge, aggregation, and solubility.^{1,2} The extraordinary physicochemical properties of NMs have accelerated their incorporation into diverse industrial and domestic products. Although their presence in consumer products represents a major concern for public health safety agencies as well as for consumers, the potential impact of these products on human health has been poorly characterized. At present, the very limited, and often conflicting data derived from published literature—and the fact that different NMs are physicochemically so heterogeneous—make it difficult to generalize about health risks associated with exposure to NMs. There is therefore an urgent need to clarify the toxic effects of NPs and NMs and to elucidate the mechanisms involved in their toxicity. In view of the large number of NMs currently in use, high throughput screening (HTS) techniques aimed at accurately predicting and assessing toxicity are clearly needed; given the availability of reliable toxicity metrics, the HTS approach will generate large and valuable data sets.^{3,4}

Up to now, there has been no consensus regarding models and tests that should be used to analyze the *in vitro* toxicity of NPs/NMs and at present no clear regulatory guidelines on testing and evaluation are available.^{5–7} The heterogeneity of NMs severely limits the feasibility of producing general toxicity protocols to address NM risk assessment. However, reliable, robust and validated protocols for testing

NP/NM toxicity (Table 1) are essential for human and environmental risk assessment.^{5,8,9}

Compared with *in vivo* approaches, *in vitro* methods to address NM-induced toxicity have the advantages of simplicity, economy, and shorter time required for investigation; they can aid in revealing general mechanisms underlying the effects of NMs on cells, and can provide a basis for evaluating potential risks of exposure. However, obtaining toxicological data from *in vitro* assays alone has potential limitations since the behavior of cells with NMs in culture differs from their behavior in the complex biological systems of the whole organism.⁹ This is attributed to what is known as ‘coordinated tissue response,’ perhaps the most under-researched area in the field of toxicology.⁵ Ideally, when considering screening novel NMs for toxic effects we should use a combination of *in vitro* methods simulating as closely as possible *in vivo* conditions.

HTS is defined as the use of automated tools to facilitate rapid execution of a large number and variety of biological assays that may include several substances in each assay.⁴ HTS was introduced in the pharmaceutical and chemical industries as a rapid way of evaluating effects of many novel compounds. With the rapid growth of NM production, HTS methods are needed to allow toxicity testing of large numbers of materials in a timely manner and with savings in labor costs. HTS *in vitro* facilitates the hazard ranking of NMs, through the generation of a database with all reported effects on biological and environmental systems; thus novel NMs can be prioritized for *in vivo* testing. An effective HTS model for investigating the toxic effects of several metal-oxide NPs,¹⁰ based on a hazard ranking system using HTS, gave results that were mostly comparable to *in vivo* results in zebra fish embryos with the same NPs.

TABLE 1 | Advantages and Limitations of High Throughput Screening Methods to Study Toxicity of Nanomaterials

Technique	Advantages	Limitations
Label-free fast qualitative and quantitative assessment of uptake and cellular screening of NMs		
Flow cytometry	Fast, cost-effective and validated assay. Multiplexing capabilities. Interference compensation and gating accounted for by side scatter versus forward scatter. Uptake can be followed. Discriminates cells with and without NPs, to study heterogeneity. Recommended for fast cell assay in cases where NMs do not interfere.	Requires fluorescent labels which may interfere with surface properties of NMs. Optical properties of NM can interfere with measurement of absorbance or fluorescence. NPs may also adsorb reagents, markers. NPs situated on cell membranes may bind or mask surface receptors. So far only developed for use with 96 wells. Needs proper controls to identify interference due to NMs.
HTS flow cytometry	Side scatter signal can detect NMs. Imaging flow cytometry relates physicochemical characteristics of NMs to their uptake	
HTS flow cytometry for studying specific cellular uptake	Evaluates specific cellular uptake	If uptake is measured using side scatter, increased cellular density may also be due to apoptosis, so additional methods must be used for validation.
Confocal laser scanning spectroscopy	Cost-effective and validated method, with multiplexing capabilities, Z-stacks option and combined imaging options.	Assay is low throughput unless automated.
AES	Recommended for particle localization/uptake. Quantifies specific chemical elements of NMs in cultured cells and tissues with ppb accuracy.	Expensive. Technical knowledge needed.
ICP-MS	High accuracy and low limit of detection (ppb). Can selectively screen for battery of elements.	Only applicable to inorganic NMs. Not possible to distinguish between NMs that are internalized in cells, extracellularly associated and/or just located between cells or within extracellular fluid.
Single particle ICP-MS	Records pulses related to single NM and distinguishes between dissolved and particulate forms of NMs	Same as for ICP-MS.
IBM techniques (μ PIXE and/or μ RBS)	Powerful tools for spatially resolved elemental imaging. Possible to determine concentration of cellular elements, with sensitivity in ppm range. Distinguishes between internalized NMs and NMs attached on outside of plasma membrane. Can quantify total cellular NP concentration in cells and tissues.	In most cases, difficult to distinguish between dissolved and particulate forms of NMs.
EMPA	Quantifies and visualizes single elements in biological specimens.	Low signal/noise ratio and poor penetration of biological specimens compared to proton beam.
MRI, PET and SPECT	Clinical imaging applications, and detection of NPs in whole organism.	Only applicable with specifically designed and validated detection probes. Low resolution (about 1 mm).
TEM, ToF-SIMS	Simultaneous visualization of NMs and their biological environment at sub-cellular level.	TEM and ToF-SIMS methods are relatively costly and time-consuming, and require heavy equipment.
CRM	Noninvasive fast screening 3D method to visualize and quantify NMs at sub-cellular level as well as to study adverse effects of NMs such as apoptosis/necrosis, ROS, Cyt C redox status, DNA fragmentation based on spectroscopic marker of individual cells <i>in vitro</i> and <i>in vivo</i> . Economical and relatively fast technique highly recommended for HTS analysis.	CRM is not able to detect dissolved NMs.

TABLE 1 | Continued

Technique	Advantages	Limitations
High throughput screening and High Content Analysis for NM-induced cytotoxicity		
Flow cytometry	Fast, cost-effective and validated assay with multiplexing capabilities, Interference compensation and gating accounted for by side scatter versus forward scatter. Recommended for fast cell viability testing.	
HTS flow cytometry for studying cell death	Can investigate several parameters in one sample.	Further investigation needed to ensure there is no interference with NMs.
HTS flow cytometry for ROS	Detects several early intracellular indicators at much low NM concentrations. Flexible, allowing for study of several NPs in one single experiment.	Further investigation needed to ensure there is no interference with NMs.
Multiplex analysis of secreted products	Allows quantification of up to 500 analytes in same sample at same time. Reduction of reagents and sample volume, and number of experimented animals; Possible to perform repeated measures of multiplex panels in same experimental assay. Detection of analytes over broad range of concentrations.	Assay costs are moderately high.
Impedance-based monitoring of NM-induced cytotoxicity		
Impedance-based spectroscopy/ Impedance-based HTS	Enhanced sensitivity compared to traditional assays and label free (no interference with spectrophotometric readings).	Relatively high cost for plates with electrodes and for microfluidic impedance-based chips.
xCELLigence [®] , CellKey and ECIS systems	Not labor-intensive, label-free, noninvasive, biophysical assay, detecting dynamic cell responses. Measure real-time electrical impedance, up to 384-well plate format.	Observes cellular responses to effectors without giving any indication of underlying mechanisms. Adherent cells only. Relatively high cost for plates with electrodes. Effects of ions and particulate matter difficult to differentiate for soluble NMs.
IFC, Ampha Z30	Label-free. Measures single cells in suspension, giving information about size and number of cells, membrane capacitance and cytoplasmic conductivity, with capacity to differentiate between viable, apoptotic and necrotic cells.	Endpoint assay; does not identify underlying mechanisms. Recently introduced; more time needed for optimisation and validation.
HTS/HCA for imaging and quantitative screening of NMs		
HCA and High content Screening coupled with CLSM or EPI	Fast, reliable, real time, 384 wells. Quantitative and qualitative data. Allows multiparametric analysis, Easy to use.	Assay costs are moderately high compared to standard kit even allowing for multiparametric screening.
High-throughput omics	96–1536 wells. Label-free. Multiplexing of 50–1500 parallel gene expression measurements. High sensitivity with linear response: measurements possible without amplification of the target. Permits single-cell sensitivity and extremely high specificity. Results can be compared to/validated against existing repositories of gene expression profiles.	Assay costs are moderately expensive, but lower than traditional microarray or RNA-seq analyses. Not a substitute for phenotype assays.
Genotoxicity/mutagenicity		
HTS Comet Assay	Well-known and simple assay allowing testing of several NMs simultaneously. Automatic scoring systems have been developed.	Semi-automatic scoring is time-consuming. Fully automatic scoring is expensive and still might need validation.
'CometChip'	Uses microarray on an agarose-coated plate.	Long-term sample storage needs to be improved.

TABLE 1 | Continued

Technique	Advantages	Limitations
HCS approach to IVMN	Efficient method with high sensitivity and specificity.	Requires multiple assays as developed within FP7 (QualityNANO). Not fully automated. Tested on polystyrene and iron oxide NMs.
γ H2AX assay—foci	Several orders of magnitude more sensitive than method measuring overall protein level. Allows distinction between pan-nuclear staining and focus formation.	Automated scoring still needs improvements. Validation needed against other methods.

AES, Atomic Emission Spectroscopy; ICP-MS, Inductively Coupled Plasma Mass Spectrometry; IBM (μ PIXE and/or μ RBS), Ion Beam Microscopy; HTS, High Throughput Screening; HCA, High Content Analysis; CLSM, Confocal Laser Scanning Microscopy; EPI, EMPA-Electron microprobe analysis; MRI, magnetic resonance imaging; PET, positron emission tomography; SPECT, single-photon computed emission tomography; TEM, Transmission electron Microscopy; ToF-SIMS, time-of-flight secondary ion mass spectrometry; IVMN, *in vitro* micronucleus; H2AX, phosphorylated histone H2AX; γ H2AX, Foci of phospho-H2AX.

EXPERIMENTAL DESIGN FOR EFFECTIVE HIGH-THROUGHPUT SCREENING: ACCELERATING TOXICITY ANALYSIS

Accurate design and planning of HTS for assessing the toxicity of NMs/NPs are essential; interlaboratory comparisons (before adopting a method for routine screening) help to reduce confidence variance and may identify possible sources of variability.¹¹ Adoption of automated and robotic liquid and sample handling is advisable since this will help to reduce systematic errors. In order to reduce such bias, the experimental design needs to be randomized.

HCA and HTS approaches should deliver information on key biological indicators of NM-cell interactions, such as cell proliferation, cellular morphology, membrane permeability, lysosomal mass/pH, DNA and chromosome damage, activation of transcription factors, mitochondrial membrane potential changes, oxidative stress monitoring and post-translational modification.¹²

Technical challenges can arise in HTS/HCA design, as toxicology screening needs to be coupled with characterization of NPs/NMs in the exposure medium. Characterization is of necessity time-consuming and cannot be automated. This limitation is partially overcome if NMs, once characterized, can then be tested (in an HTS/HCA mode) on a variety of cell lines, using different exposure times, a range of concentrations, etc.

To achieve statistical significance, experiments should be performed at least three times with replicate samples within each data point (three repeats).

Further basic requirements are: (1) clearly identified endpoints, (2) assay-related as well as NM-specific positive and negative controls, (3) toxicologically relevant (extracellular) concentrations of NMs,

(4) validated assays, (5) multiparametric statistical analysis of data, e.g., using ANOVA with post Bonferroni analysis, or general linear models, (6) well-designed graphical display of data (e.g., bar charts) and—in the case of multiparametric datasets—various graphical plots to visualize associations between NP/NM exposure and different endpoints. For example, a multilevel heatmap matrix has been used to illustrate effects of dose, concentration, and time for multiple NMs^{11–13} (Figure 1).

Various methods have been applied to the study of toxicity of NMs, employing diverse physical, chemical, and biological principles and endpoints; they will now be described in detail, with emphasis on high throughput adaptations.

LABEL-FREE DOSIMETRY AND IMAGING TECHNIQUES—TOWARD HIGH THROUGHPUT

While the intracellular distribution pattern of NMs is an important factor in investigating toxicological responses, it is generally difficult to predict and model, because it is the final result of several translocation events. Flow cytometry as well as confocal laser scanning spectroscopy have been frequently applied for studying NM translocation and semi-quantitative estimation of NM uptake. A potential problem with these methods is that they require fluorescently labeled NMs. The comparatively large dye molecules may significantly change the surface properties of NMs, and thus falsify the cellular distribution and uptake of NMs. Clearly, label-free dosimetry and imaging techniques are advantageous in that they allow the study of authentic NMs—a crucial requirement in the regulatory context. For this reason this section focuses on label-free techniques and on

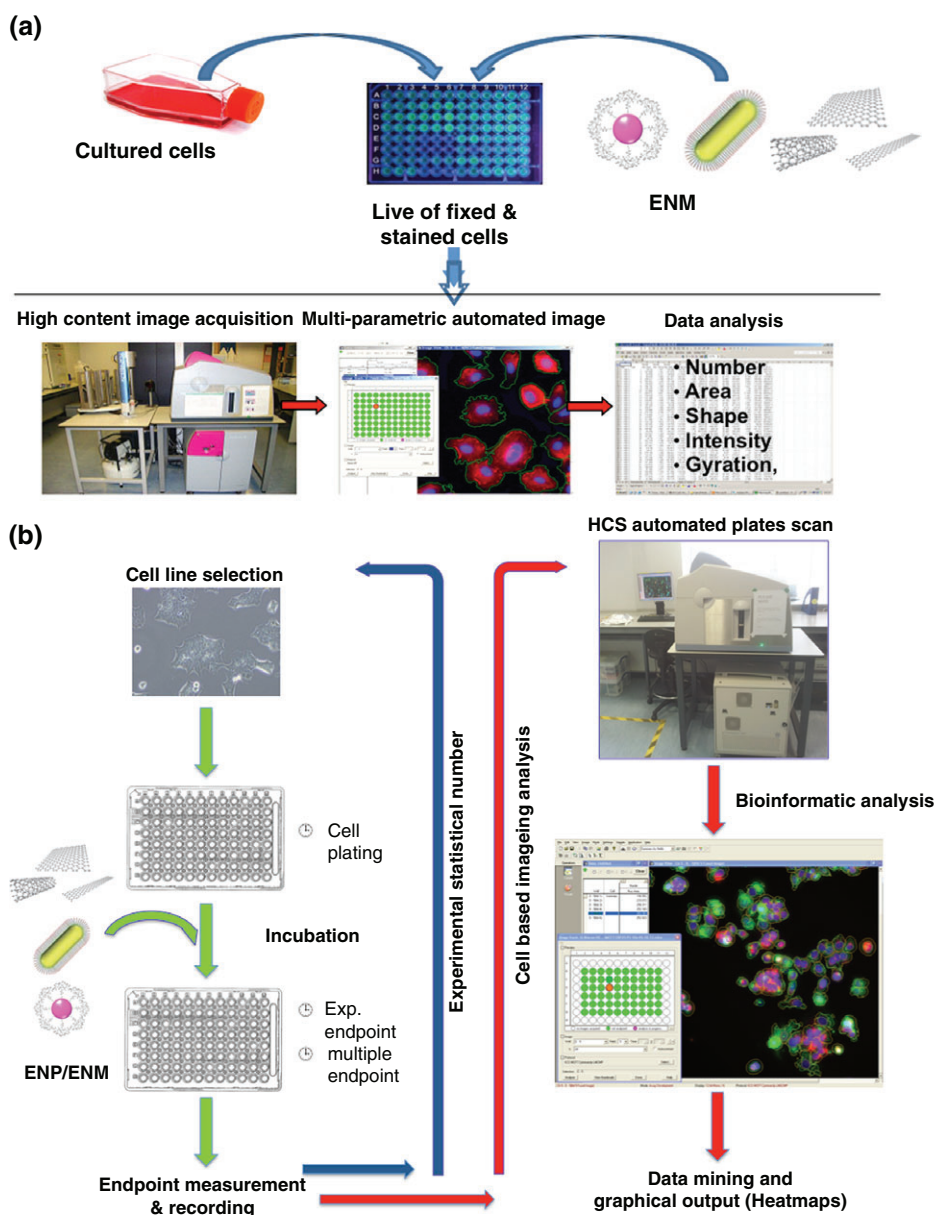


FIGURE 1 | (a) Experimental workflow and (b) experimental design for effective high-throughput screening

the potential and applicability of imaging techniques for high throughput requirements.

Atom emission spectroscopy (AES) has been shown to be a useful tool for the quantification of a large range of elements. AES is based on the emission of radiation, which is characteristic for every element. The method is capable of quantifying specific chemical elements of NMs in cultured cells and tissues with ppb accuracy.^{14,15}

Mass spectrometry (MS) is another rather sensitive technique for cellular NM quantitation. The elements of analyzed particles are ionized and separated by their mass-to-charge-ratio. Predominantly, mass

spectrometry with inductively coupled plasma (ICP-MS) is used for quantification of NM uptake in cultured cells.¹⁶ The specimen to be investigated is either a suspension of cultured cells or a tissue sample, which needs to be dissolved prior to ICP-MS. Studying the uptake of organic or inorganic NMs requires a comparatively large number of cells.¹⁷ ICP-MS was further developed as single particle ICP-MS (sp-ICP-MS). This technique is able to record pulses related to a single NM. sp-ICP-MS has the advantage of being able to distinguish between the dissolved and particulate form of NMs,¹⁸ because the dissolved analyte does not generate pulses.

A disadvantage of these techniques is that there is no possibility to distinguish between NMs that are internalized into the cells, extracellularly associated and/or just located between cells or within the extracellular fluid.¹⁹ It has been shown that there is a significant difference between uptake measured by means of ICP-MS and uptake based on the analysis of individual cells.¹⁷

Ion beam microscopy (IBM) techniques, such as micro-proton-induced X-ray emission (μ PIXE) and micro-Rutherford backscattering (μ RBS), are powerful tools for spatially resolved elemental imaging and quantitative analysis at the single-cell level. A proton beam is used for scanning the sample in the xy-plane at a resolution of about 1 μ m. With the combination of PIXE and RBS it becomes possible to determine the concentration of cellular elements (for example, P, S, Ca, K, Zn, and Fe), with a sensitivity in the ppm range.^{20,21} Recently, IBM was applied to quantify NM cellular uptake in cultured cells.^{17,22} The simultaneous application of μ RBS and μ PIXE methods delivers unique information on the genuine concentration and distribution of NMs down to the single-cell level. IBM allows visualization and quantification of a wide range of NMs in tissues and cells (Figure 2). Moreover, μ RBS can reveal the distribution of NMs in the z-direction with an accuracy of about 100 nm. The method distinguishes between NMs that are internalized and those that are attached on the outside of the plasma membrane, owing to the loss of energy of back-scattered protons from NMs located inside cells.

Electron microprobe analysis (EMPA) is a technique similar to μ PIXE. Here a scanning electron

beam is used instead of a proton beam. EMPA is able to quantify and visualize single elements in biological specimens^{23,24} in a spatially resolved manner.

The advantage of PIXE/RBS over electron-based X-ray emission is that proton beams are capable of analyzing a much higher probe thickness and so can quantify the total cellular NM concentration in cells and tissues. Moreover, the protons offer a better signal/noise ratio allowing the accurate detection of cellular trace elements at very low concentrations.

Magnetic resonance imaging (MRI), positron emission tomography (PET) and single-photon emission computer tomography (SPECT) are commonly used in clinical imaging applications as well as for the detection of NMs in the whole organism with a resolution of about 1 mm.^{25–27} The activation of oxygen by the $^{16}\text{O}(p,\alpha)^{13}\text{N}$ reaction within metal-oxide NMs avoids the undesired surface modification of NMs. Therefore it becomes possible to study the distribution of commercially available Al_2O_3 NMs in tissues and organs of rats by PET.²²

Transmission electron microscopy (TEM), time-of-flight secondary ion mass spectrometry (ToF-SIMS)²⁸ and confocal Raman microspectroscopy (CRM) are methods which allow the simultaneous visualization of NMs and their biological environment at a sub-cellular level. These techniques can be considered as semi-quantitative space-resolved imaging methods. TEM visualizes the intracellular localization of NMs in ultra-thin (50–100 nm) sections of tissue or cultured cells.^{29,30} Chemical identification of NMs can be done by

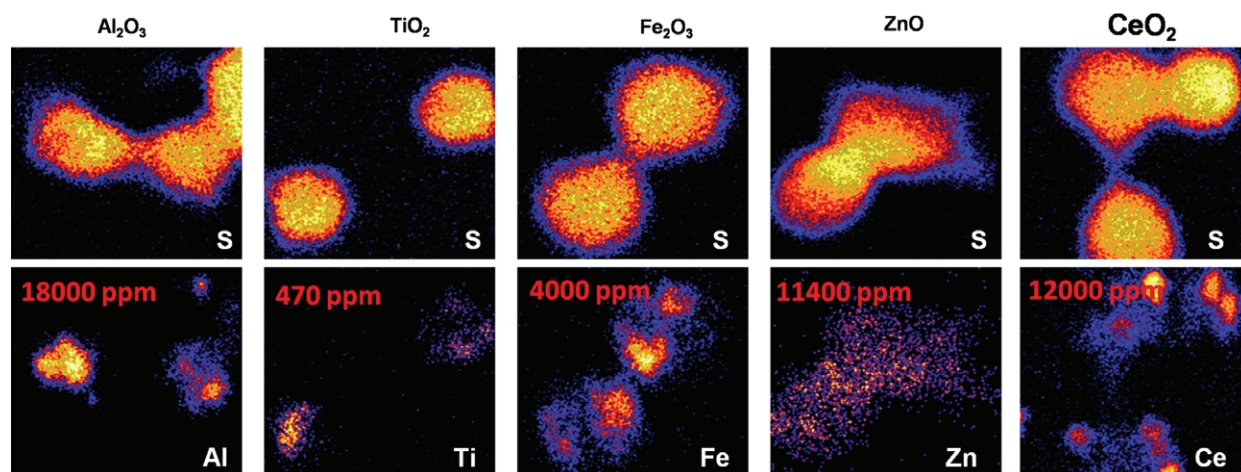


FIGURE 2 | Micro-proton-induced X-ray emission (μ PIXE) elemental mapping of A549 cells exposed to different metal-oxide NMs at a concentration of 30 $\mu\text{g}/\text{mL}$ for 48 h. Top and bottom images demonstrate S (Sulfur) and NM related element distributions, respectively. The color code is as follows: yellow is the maximum, black represents the minimum. The size of all images is 50 \times 50 μm . (Reprinted with permission from Ref 22. Copyright 2014 Wiley)

X-ray energy-dispersive spectrometry (EDS) and electron energy-loss spectrometry (EELS) in TEM. The 3D reconstruction of NMs internalized by tissues and cells with suitable contrast and high depth resolution has been accomplished by tomography ('slice and view') using Focused Ion Beam-Scanning Electron Microscopy (FIB-SEM).³¹ With ToF-SIMS it is possible to measure the 3D distribution of NMs in cells and tissues by means of a layer-wise high energy beam raster technique. The lateral resolution is about 300 nm. Pathological changes in the pattern of cellular lipids following NM exposure have been detected.^{32,33} TEM and ToF-SIMS methods are relatively cost-intensive and time-consuming, and so might not be useful as high throughput techniques for NM screening. CRM, in contrast, is an economical and relatively fast technique, which could well be adopted for regulatory purposes in the future. CRM provides 3D chemical composition images with a resolution of about 200 nm at tissue and cellular levels. CRM reveals not only the 3D NM distribution but also their co-localization within cell compartments. CRM is especially suitable for the detection of manufactured NMs, as the nanostructures show a specific Raman signal, which distinguishes the NMs from the signals of their chemical constituents. Biochemical changes in cells that have been exposed to toxic chemical agents, drugs or NMs can be detected, identified and quantified.^{34,35} CRM imaging was used to follow the different stages of the cell cycle,³⁶ DNA condensation in late stages of apoptosis and also for the assessment of cell viability.^{37,38} DNA damage, lipid changes, and protein denaturation were analyzed as a response to drugs and chemicals.^{39,40} However, the spatial resolution and sensitivity of CRM are limited compared with TEM which typically has a threshold of ~60 nm.⁴¹

The power of CRM regarding cell imaging, subsequent analysis of biochemical and cell physiological processes as well as diagnostic power has been demonstrated in a number of studies with cells and tissues. Label-free imaging of cell organelles,^{42,43} uptake and intracellular fate of drug carriers^{44–46} and NMs inside individual cells^{32,47,48} have been studied.

CRM has a strong potential as a label-free, nondestructive technique for time-course imaging of individual cells and tracking of cell metabolism. It may become a useful tool for *in vitro* toxicological studies for estimation and prediction of cell response to agents at the cellular level. This qualifies CRM as an innovative high throughput technique for *in vitro* toxicity/uptake studies.

HIGH-THROUGHPUT SCREENING FOR NM-INDUCED CYTOTOXICITY

Assays for cellular metabolic activity, oxidative stress evaluation, apoptosis detection, and cell membrane integrity^{49–55} have been developed for analyzing cytotoxicity of chemicals and these are widely used also in NM-induced cytotoxicity screening. The methods are time-consuming, labor-intensive, complex, and in some instances unreliable owing to NM interferences.^{53,56,57} The reagents employed in such methods may interact with some of the tested NMs or may interfere with spectrophotometric readings, leading to unreliable results.^{49,50,55} Conventional assays focus on specific endpoints, without providing information about the dynamic biological processes leading to those endpoints, or the specific time points and concentrations at which different toxic effects are induced by NMs.^{54,58} Walker and Bucher⁵⁹ suggested that, owing to the unpredictable behavior of many NMs, HTS approaches based on conventional methods may only be applicable for a few classes of NMs that are compatible with the available test systems. However, these issues do not preclude the use of HTS approaches to screen for NM-induced cytotoxicity.⁶⁰ Assay interferences can be avoided by using label-free methods.^{61,62} Impedance-based spectroscopy is suggested as a method that does not need markers or dyes, and that has enhanced sensitivity compared to traditional assays.^{63,64} In addition, live cytotoxicity screening provides information at any given point throughout the progress of an experiment—based on which, relevant time points and concentrations can be identified for further mechanistic studies.

HIGH CONTENT ANALYSIS

Combining automated image acquisition and powerful algorithms designed to quantify and extract a maximum of information from a population of cells, HCA generates great quantities of data for a large number of cellular characteristics, including changes in fluorescence intensity and distribution of intracellular targets, as well as detailed information on cellular and nuclear morphology. From its debut in the mid-1990s, developments in cellular imaging have rendered HCA an important tool for understanding biological processes induced by diverse xenobiotic molecules. Originally an approach used almost exclusively in the pharmaceutical industry to screen potential drug candidates aimed at specific targets, this technology is now widely used by researchers in many disciplines to study a wide range of cellular

responses. Although a relatively new approach, the development of HCA technology has been accelerated by advances in optics, automated imaging, and great improvements in fluorescent molecular probes and reagents. In addition, rapid advances in information technology, including image analysis software, and increases in computing power and memory for storage of vast amounts of images and results have been a driving force in the success of HCA.

Quantitation of fluorescence corresponding to relevant molecular targets in cellular compartments allows researchers to characterize and quantify biological responses at the level of the individual cell as well as for whole cell populations. Moreover, since high content imaging allows multiparametric analyses of several markers at the same time, correlations between cellular markers can be readily analyzed on the same cell populations. The speed of analysis, the multiparametric nature of the analysis, combined with the quantity and quality of data, make HCA an efficient and powerful approach to study a wide range of cellular processes and responses.

A number of commercial benchtop HCA instruments are currently available, each offering specific advantages for imaging and analysis. These systems are equipped with powerful image analysis software based on the automatic identification of cells, and—depending on the instrument—they offer considerable flexibility for analysis. Currently, most HCA microscopes have optional environmental control systems which can regulate temperature, atmosphere, and humidity, thereby allowing live cell imaging in real time. Although with most instruments HCA can be performed on histological samples, the approach is best adapted to studies using cultured cell lines in multiwell plates (6–1536 wells) where multiple conditions (various compounds, range of concentrations, etc.) can be tested in a single experiment.

The wide selection of high quality primary antibodies and specific fluorescent molecular probes has given rise to limitless possibilities for analysis of biological responses. In addition, a broad range of fluorescent secondary antibodies with relatively narrow excitation and emission characteristics allows the multiplexing of several markers simultaneously. Many cell-permeable fluorescent probes are currently available which allow the visualization of changes in membrane permeability, reactive oxygen species (ROS), mitochondrial and lysosomal functions, among many other cellular processes, in fixed or living cells. Indeed, the vast array of possible analyses of specific cellular endpoints has made HCA a key approach in various domains including toxicology,

genotoxicology, oncology, neurobiology, and research on metabolic disorders.⁶⁵

HCA approaches offer a number of advantages regarding assay cost and data output. Miniaturization of HCA runs on 96-, 384- or 1536-well plates increases the number of compounds analyzed, and reduces the volumes of reagent used. The incorporation of robotic handling systems into the workflow, combined with automated image and data analysis, permit a significant reduction in hands-on time in the laboratory. Furthermore, the throughput, speed and the number of cells analyzed using HCA approaches generate great quantities of statistically robust quantitative data in considerably less time when compared to manual image acquisition and analysis.

HCA approaches have been widely used for many years by the biotech and pharmaceutical industries in drug discovery and toxicity testing⁶⁶ of extensive libraries of chemical compounds,⁶⁷ and have accurately predicted the toxicity of novel compounds. Indeed, a multiparametric assay quantifying oxidative stress, mitochondrial membrane potential and intracellular glutathione levels was capable of accurately predicting hepatotoxicity with a low false positive rate.⁶⁸

There is an urgent need to develop HCA assays to evaluate the toxicity of manufactured NMs. The sheer number of NMs currently being used in consumer products means that HCA-based approaches will undoubtedly be key tools for safety testing, as suggested by the growing number of published HCA studies in the field of nanotoxicology. HCA can provide detailed information concerning the pathways of cellular responses to treatment with NMs.^{69–71} Recently, several studies have applied high content imaging to hazard characterization of NMs.^{10,72,73}

High content imaging represents a promising approach in the prediction and evaluation of toxicity of NMs. Combined with complementary HTS methods as described in this article, HCA will provide valuable information on the mechanistic pathways involved in toxicity and cellular responses.

HIGH-THROUGHPUT FLOW CYTOMETRY

From simple cell-based fluorescent, colorimetric, luminescent, and radiologic plate reader assays, to high-content fluorescent imaging systems, the ability to screen NMs in the context of living cells is essential in toxicology-screening programs. The emerging field of high throughput flow cytometry extends the capabilities of cell-based screening technologies.⁷⁴

The process involves the rapid introduction of multiple samples through a single length of tubing, and it is even possible to process samples from microplates through a flow cytometer. This allows the implementation of large screening campaigns using assays with multiple readouts per well. High throughput flow cytometry is an ideal tool for cell-based applications involving screening of cells in suspension, where multiple readouts are desired.⁷⁵ Potential applications include cell viability, intracellular incorporation of NMs, and ROS detection. However, careful consideration should be given to the selection of appropriate flow cytometry assays in the light of possible NM interference with fluorescent markers.

Cell Death (Apoptosis/Necrosis Determination)

Cellular death may be divided into programmed cell death, commonly referred to as apoptosis, and necrosis, which indicates accidental cell death. Cellular apoptosis occurs in a sequential manner, starting from cell shrinkage, increased cellular permeability, membrane asymmetry and chromatin condensation. Flow cytometry analysis coupled to a high throughput system allows investigation of several parameters in one sample, producing a complete picture of the cell death profile. Collapse of the mitochondrial membrane, an early stage in apoptosis, can easily be assessed by positively charged fluorescent probes that are able to locate inside the negatively charged mitochondria. As mitochondrial potential is lost, the emission of the fluorescent probe changes, indicating the onset of apoptosis in affected cells. Intermediate apoptotic events include the activation of caspases, which can be easily detected in permeable cells upon incubation with nonfluorogenic substrates.

Loss of membrane asymmetry can be studied by incubating cells with Annexin-V conjugated with green-fluorescent dye—apoptotic cells giving a positive fluorescent signal. Subsequent incubation with propidium iodide detects necrotic cells. Proportions of apoptotic and necrotic cells are estimated by flow cytometry.⁷⁶ However, unpublished experiments⁵⁸ indicate that Annexin V may not be a reliable assay, as false negatives were obtained; the more TiO₂ NMs were added, the lower was the apparent percentage of apoptotic (Annexin V-positive) cells. Obviously, some type of interference occurred: cell membrane attachment masking NMs, or adsorption of Annexin V to the NMs (possibly both).

Final steps in apoptosis usually include chromatin condensation and fragmentation. At this stage,

cells are smaller and can be detected with the traditional UV-excited Hoechst 33342.

Reactive Oxygen Production

ROS occur as by-products of mitochondrial respiration and inflammation processes. In addition, xenobiotics can induce ROS production, either directly or via inflammation. The physicochemical characteristics of NMs enable them, in many cases, to catalyze ROS production and oxidative damage to biomolecules, with potential pathological consequences. Most of the commercially available probes to monitor ROS production by flow cytometry in living cells are cell-permeating chemicals that undergo changes in their fluorescence spectral properties once oxidized by ROS. Two such probes are represented by dihydroethidium (DHE, also called hydroethidine) and CM-H₂DCFDA (chloromethylchlorodihydrofluorescein diacetate). DHE emits blue fluorescence in the cytoplasm until oxidized by superoxide to 2-hydroxyethidium, which intercalates within the DNA staining the cell nucleus a bright fluorescent red. On the other hand, the nonfluorescent CM-H₂DCFDA is first hydrolysed to DCFH by intracellular esterases and DCFH is then oxidized to form the highly fluorescent DCF in the presence of ROS⁷⁷ (Figure 3). Detection of ROS species by flow cytometry coupled to a high-throughput system allows the detection of several early intracellular indicators at much lower NM concentrations than those needed for standard cytotoxicity assays. This system is also very flexible, allowing for the study of several NMs in one single experiment, or alternatively, a few NMs in cell lines

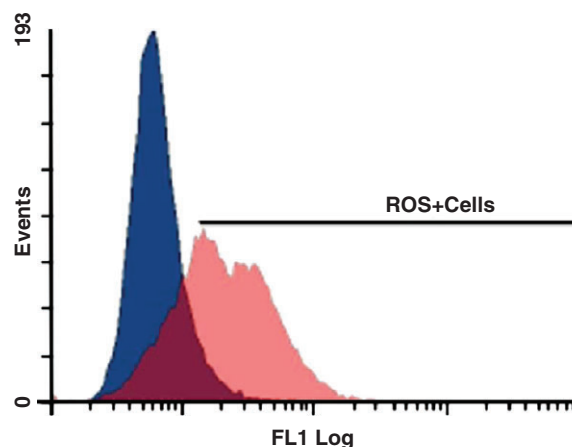


FIGURE 3 | Flow cytometric detection of reactive oxygen species produced in 3T3 cells after 24 h CeO₂ NP exposure. FL1 represents fluorescence from oxidation of chloromethylchlorodihydrofluorescein diacetate (CM-H₂DCFDA). Blue area, control group without CeO₂ NP exposure; Red area, 0.1 mg/mL CeO₂ NP exposure.

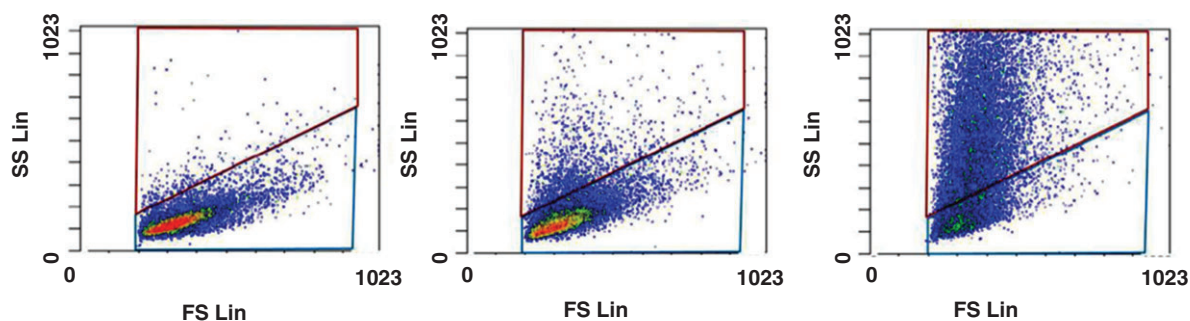


FIGURE 4 | Forward scatter (FS)/side scatter (SS) plots to determine NP uptake in 3T3 cell line after 24 h CeO₂ NP exposure. Blue box, cells with no NP incorporation; Red box, uptake of NPs by 3T3 cells. (a) Control group with no CeO₂ NPs. (b) CeO₂ NP exposure at 0.01 mg/mL. (c) CeO₂ NP exposure at 0.1 mg/mL.

representing different tissues susceptible to NM exposure.

Specific Cellular Uptake

Uptake of NMs labeled with rhodamine fluorescent probe is measured by flow cytometric detection of rhodamine-positive cells. In the case of nonlabeled NMs, it is possible to analyze the specific cellular uptake through forward scatter (FS) (size) and side scatter (SS) (Figure 4). Higher side scatter can also be indicative of apoptosis, and so additional methods such as microscopy should be used for validation.

While particular properties of NMs can interfere with conventional cytotoxicity assays and induce bias, this can be turned to advantage in the case of flow cytometry. It was demonstrated that TiO₂^{78–80} and Ag⁸¹ NMs induce an increase of SS signal in cell populations after exposure to both NMs. SS indicates the granularity of the cells and is strongly linked to their content of NMs. As few as 5–10 NMs per cell can be detected.⁷⁹ Recently, SS signal analysis was successfully applied for detecting carboxylated nanodiamonds and tungsten carbide-cobalt (WC-Co) NMs in cells, results being validated by Raman and confocal microscopy associated with 3D reconstruction.^{82,83} Finally, imaging flow cytometry (an integrative approach combining flow cytometry analyses with confocal microscopy) relates the physicochemical characteristics of NMs to their uptake, with a view to designing safe NMs.⁸⁴ Flow cytometry appears to be a good alternative method to detect NM internalization compared to TEM which is very time-consuming and requires heavy equipment.

IMPEDANCE-BASED MONITORING

Real-time cell monitoring using electrical properties (electric cell-substrate impedance sensing, or ECIS)

was introduced by Giaever and Keese over 20 years ago.⁸⁵ As a label-free, noninvasive biophysical assay detecting dynamic cell responses, it provides a valuable tool for the investigation of NM toxicity and early stage efficacy testing of NP-linked drugs. Impedance-based methods have been used in applications ranging from food processing to clinical research^{86–90} and recently have been adapted to HTS techniques in order to examine the impedance characteristics of cell monolayers after exposure to certain bio-reactive agents, for instance in the pharmaceutical industry.⁸⁶

Impedance-based devices used for adherent cells consist essentially of electrodes attached to cell culture vessels. When cells are grown in such vessels, their growth, attachment and proliferation result in changes in the measured impedance output, as growing cells—with their insulating bilipid membranes—act as dielectric objects.^{55,85,91} An alternating electrical current (AC) is applied through the electrodes and the extent to which the cells impede that current is measured.^{58,89} When the cells grow and attach to the electrodes at the bottom of the culture vessels the impedance increases; this provides information about the cell count, cell morphology, attachment to the substrate and viability. When cells die, they detach from the electrode surface, causing a drop in the recorded impedance, which indicates a reduction in the number of viable cells.^{53,58}

Commercially available impedance-based instruments for *in vitro* analysis on cell monolayers monitor the changes in impedance properties of cells after exposure to bioactive agents. The xCELLigence®, CellKey, and ECIS systems, probably the most used such instruments, work on essentially the same principle. They utilize cell culture well plates with gold-plated electrodes attached to the bottom of the wells and measure the real-time opposition of the seeded cells to the applied electric

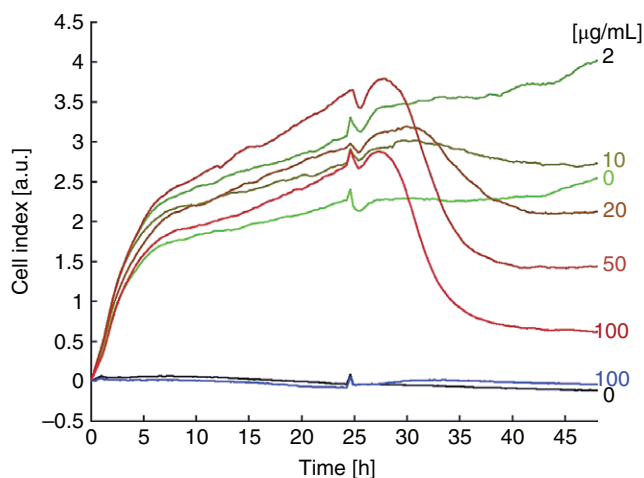


FIGURE 5 | Impedance-based measurements of A549 cells exposed to NM-100 titanium dioxide particles (110 nm diameter, anatase): Representative data collected with the xCELLigence instrument (ACEA Biosciences, USA) (See Figure 8 for illustration). The figure shows the plot of the cell index (CI) which reflects real-time cellular proliferation. Exposure of cells started at 24 h. The conditions are color coded from green to red in concentrations 0, 2, 10, 20, 50, and 100 $\mu\text{g}/\text{mL}$. Medium only (black) or with 100 $\mu\text{g}/\text{mL}$ NM-100 (blue) are included for reference.

current, providing information about the cells' attachment to the well, their proliferation and their reaction to the bioactive agent in question.^{86,92} The format of cell culture plates that these instruments employ varies from 16-well to 96-well and 384-well plates, allowing the live screening of a large number of materials.^{86,93,94} A limitation of such impedance-based devices is that they observe cellular responses to effectors without giving any indication of how the effects took place. However, they enable real-time observations to be made of cell changes throughout an experiment without the need for destructive cell sampling. They collect data about both short-term and long-term responses of cells to NMs,⁹⁵ facilitating the identification of key time points (Figures 5 and 6). Toxicity assays with other endpoints can then be applied to investigate the underlying mechanisms. Impedance-based methods, being easy to implement, can also be used in HTS fashion to test several NMs at different concentrations simultaneously.^{54,96}

Real-time measuring of cellular impedance is a useful and sensitive method for screening effects of NMs, at different concentrations, on a range of cell lines simultaneously,⁹⁷ without variation due to artifacts affecting the measured signal.^{82,98–100} It can also be used to assess changes in cellular motility and adhesion in physiological conditions.¹⁰¹

In contrast to the real-time impedance methods, impedance-based flow cytometry (IFC) is an endpoint

assay for cells in suspension that examines the impedance characteristics directly for each single cell. A microfluidic chip-based IFC developed by Amphasys AG (Switzerland) can analyze single cells without any specific sample preparation prior to measurement.^{97,101,102} Compared to other impedance-based cytometers, e.g., Z series Coulter Counters or the CASY from Roche, the microfluidic chip-based IFC can cover impedance measurements at a broader frequency range, and thus yield information regarding the size and number of cells and, in addition, their membrane capacitance and cytoplasmic conductivity.^{54,103} IFC gives a snapshot of the cellular state of single cells based on the changed resistance within the chip-channel caused by the passing cells.¹⁰² The advantages of microfluidic chip-based IFC are that it measures the dielectric properties of cells directly; it can analyze the state of each single cell (Figure 7); and it is suitable for cells in suspension.¹⁰² Additionally, it can apply multifrequency impedance measurements to obtain diverse information regarding the state of the cells and cellular identity.

Impedance-based methods have been compared with conventional cytotoxicity assays, examining effects on various types of cells induced by NMs as well as other chemicals. Toxicological information obtained by impedance-based methods were found to be consistent with results obtained via conventional methods, and impedance-based methods have been recommended as a fast and reliable alternative to conventional methods for cytotoxicity testing.^{53,54,58,104} Recently, impedance-based monitoring was used to screen TiO_2 NM-induced cytotoxicity on fibroblasts⁵⁸; the authors suggested that the method has merit when addressing NM-induced cytotoxicity. In another study, Moe et al.¹⁰³ investigated the cytotoxic effects of nano- TiO_2 and nano-Ag on three cell lines simultaneously. The authors recommended such techniques for the initial assessment of the potential cytotoxic effects of NMs and to direct further toxicological testing.

A chip-based system measuring electrochemical impedance was used to monitor cytotoxicity in human hepatocellular carcinoma cells (HepG2) and immortalized mouse fibroblasts (BALB/3T3). The results were consistent with findings from the traditional MTT assay.¹⁰⁵

In summary, impedance-based methods for cytotoxicity testing are simple, cost-effective, label-free, lend themselves to HTS, and importantly allow in situ monitoring of not just cytotoxicity but also other aspects of cell physiology such as proliferation, morphology, attachment, and intercellular adhesion. These methods are widely used in pharmaceutical

research, oncology, cardiology, and other fields of biomedicine and clinical research, to investigate responses of cells to exposure to bioactive chemical agents and toxicants. However, further validation of HTS applications of impedance-based instruments/techniques is advisable. To date, several studies using cell impedance have demonstrated its ability to discriminate cytostatic from cytotoxic effects¹⁰⁶ and also to predict different outcomes in cell-based functional assays.⁹² Various cell types have already been tested: astrocytes,¹⁰⁷ cardiomyocytes,^{94,108,109,110} cervix cells,^{111,112} eye cells,⁹⁴ intestinal cells,^{99,113} lung cells,^{82,94,99,100,103} hepatocytes,^{82,93,94,99} kidney cells,^{82,99} macrophages,¹⁰⁰ fibroblasts,⁵⁸ breast,⁸³ and neuronal cells.¹¹⁴

In conclusion, besides eliminating interferences and allowing continuous follow-up measurement, cellular impedance provides qualitative and quantitative cytotoxicity data, and also directs further studies into the mode of action (toxicity) of a NM.¹⁰³

MULTIPLEX ANALYSIS OF SECRETED PRODUCTS

Cytotoxic effects induced by NMs may be detected by conventional methods. Besides the possibility of interference effects of certain NMs that impact on interpretations of cytotoxicity, these methods only focus on specific endpoints and do not consider relevant intracellular biological events. Secreted proteins such as cytokines, chemokines, and growth factors are the largest class of soluble factors, and are generally determined by enzyme-linked immunosorbent assays (ELISA). ELISA allows the quantification of a single protein in serum, supernatants of cultured cells, and tissue lysates. ELISA depends on an enzymic reaction, for instance between hydrogen peroxide and horseradish peroxidase (HRP), which means that possible interference by NMs in the reaction could affect the estimation of protein levels.^{61,115,116}

Multiplex analysis of soluble factors has emerged as the 'next generation' of ELISA and can be applied in different systems such as flow cytometry and chemiluminescence and fluorescence measurements. The purpose of this HTS technology is to quantify several analytes in the same sample at the same time, avoiding enzymic reactions and minimizing eventual biochemical interferences. Instruments are available that analyze up to 500 analytes in the same assay kit. Multiplex panels comprise polystyrene or magnetic beads developed by biotechnology companies, where each capture antibody is conjugated to a specific bead. In general, a common

biotinylated detection antibody is added to the samples (beads plus analytes) followed by incubation with phycoerythrin-labeled streptavidin. Beads and binding events are recognized and quantified by red and green lasers or LEDs, respectively. Advantages of this technology include the reduced use of reagents and sample volume, the possibility to perform repeated measures of the multiplex panels in the same experimental assay condition, detection of analytes in a broad range of concentrations, and customization of analytes in the assay plates. In addition, there is a considerable positive impact in reduced time and cost for the assay development.^{117,118} Proteins related to intracellular signaling pathways are also covered by multiplex assay kits, and it is possible to study the effect of an exogenous effector on multiple pathways in the same cell population.

The multiplex analysis of secreted products is important for identification of alterations that conventional methods would not detect efficiently. For instance, gold NMs are considered nontoxic, nonimmunogenic and have biocompatibility relevant to applications in nanomedicine.^{119–121} A recent study demonstrated that PEGylated gold nanoparticles did not affect viability in C2C12 muscle cells determined by conventional methods (MTT conversion into formazan or altered intracellular calcein activity). However, multiplex analysis based on magnetic beads for several soluble factors contained in the cell supernatant showed a sharp increase of IFN- γ , a pleiotropic cytokine related to induction of pro-inflammatory mediators as well as differentiation of T and B cells, macrophages, granulocytes, endothelial cells, fibroblasts, and NK cells. Also, levels of TGF- β 1 increased, suggesting an involvement in fibrosis induction in muscle cells. These data suggest that this NM has the potential to induce inflammation and fibrosis, promoting cell vulnerability, and susceptibility to death stimuli.¹²² It is unlikely that these results would have been obtained if analytes had been chosen at random for conventional analysis.

HIGH-THROUGHPUT COMET ASSAY

The mismatch between the speed with which new NMs appear on the market and the current low-throughput, time-consuming, and laborious approaches for evaluating their genotoxicity⁷³ has led to the development of rapid, efficient, and high-throughput genotoxicity testing strategies for safety/risk assessment of NMs. It is important to identify and distinguish the existing genotoxicity testing

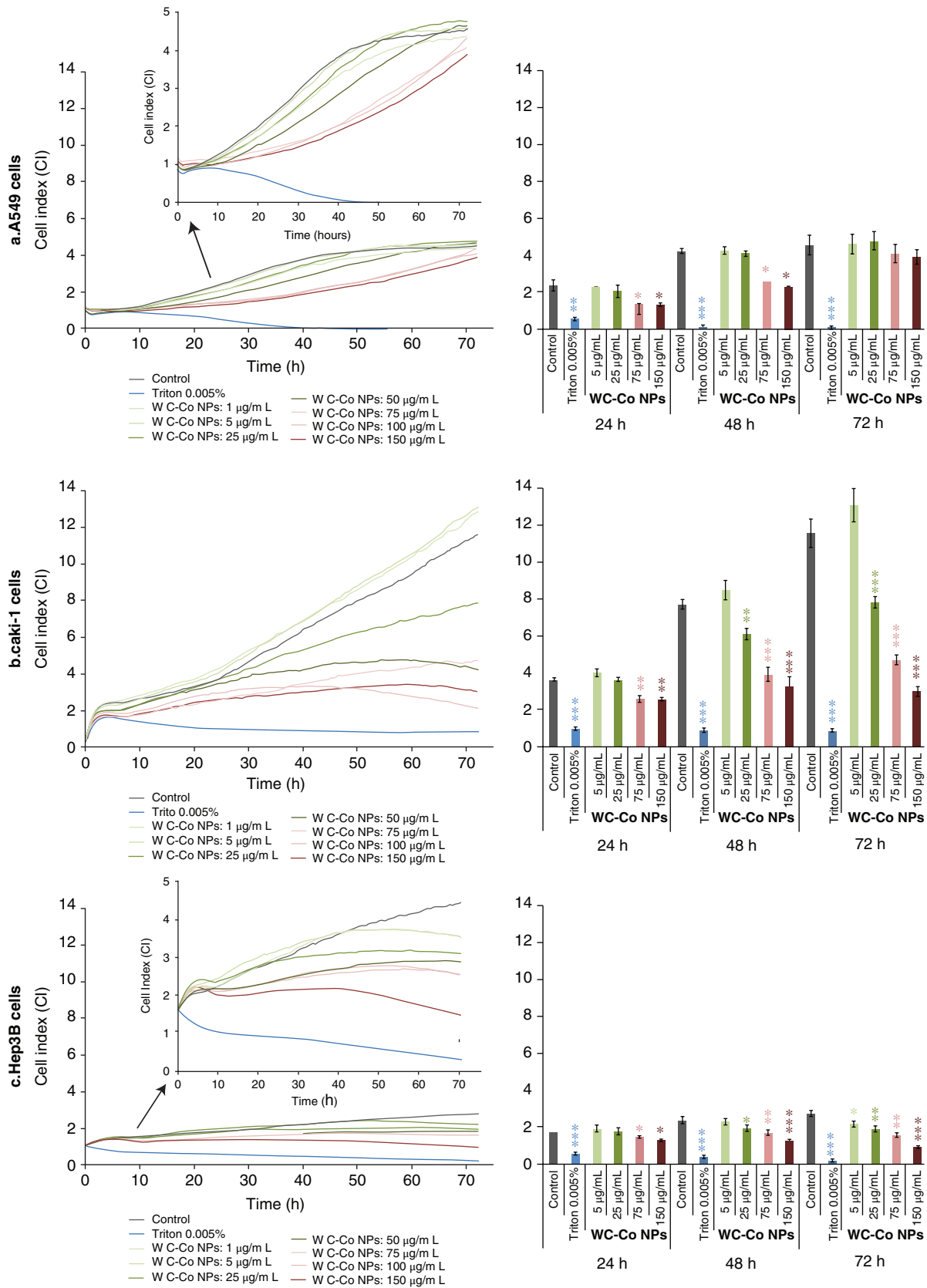


FIGURE 6 | Legend on next page.

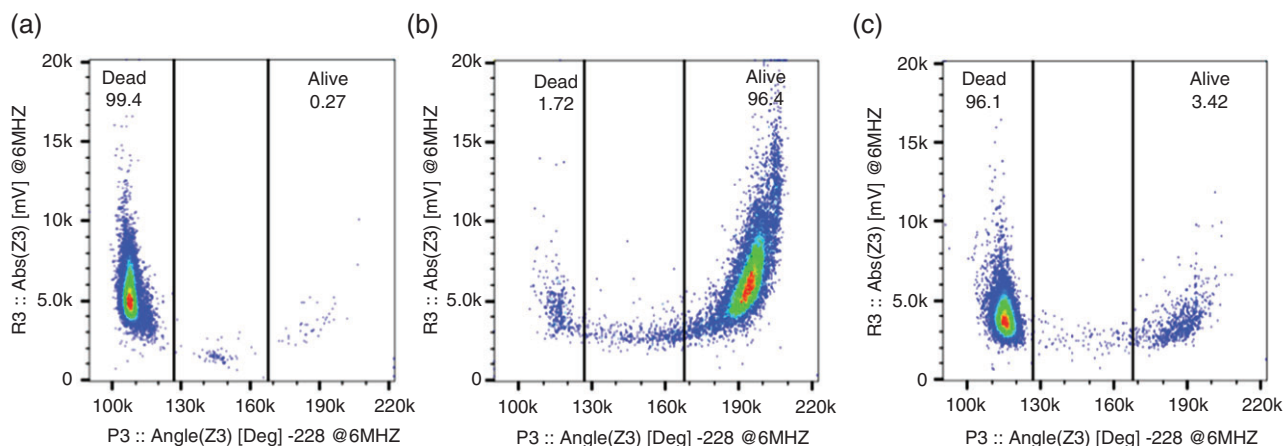


FIGURE 7 | Impedance-based measurements of U937 monoblastoid cells exposed to NM 300 K silver particles (15 nm, spherical): Representative data collected with the Ampha Z30 microchip-based flow cytometer (Amphasys AG, Switzerland) (See Figure 8 for illustration). The figure shows the dotplots of (a) necrotic cells (heated at 70°C), (b) unexposed cells, and (c) cells exposed for 24 h to 100 µg/mL NM 300 k.

methods that are amenable to HTS/HCA approaches.

The comet assay is the method of choice for measuring DNA damage in cellular DNA. Briefly, cells in suspension are embedded in a thin layer of agarose on a microscope slide, lysed, and electrophoresed. Lysis removes membranes, releases soluble cell components, strips histones from DNA, and leaves compact structures known as nucleoids in which the DNA is attached at intervals to the nuclear matrix. The DNA is in effect a series of supercoiled loops. Under electrophoresis (normally at alkaline pH), the DNA is attracted to the anode, but only those loops that contain breaks, relaxing supercoiling, are able to extend significantly. They form comet-like structures when viewed by fluorescence microscopy, and the relative intensity of the tail ('% tail DNA') reflects the frequency of DNA breaks. While the basic comet assay detects strand breaks, a common modification—incorporating digestion with a lesion-specific endonuclease after the lysis step—allows detection of damaged bases. Formamidopyrimidine DNA glycosylase (FPG) has been particularly useful; its primary substrate is the oxidized base 8-oxoGua, and it has therefore been employed to measure the effects of oxidative stress on DNA.

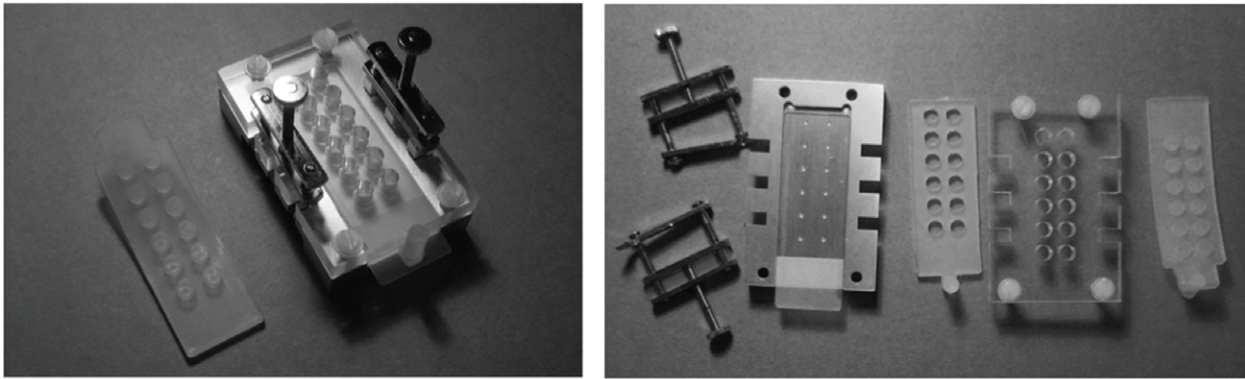
The comet assay is popular on grounds of sensitivity, accuracy, simplicity, and economy. However, it has limitations. The number of samples that can be analyzed in one experiment is limited by the size of the electrophoresis tank, typically accommodating 20 slides with one or two gels each. Also, it is relatively labor-intensive, especially at the stage of scoring; normally 50 or 100 comets per gel are selected by the operator for analysis of % tail DNA using dedicated comet image analysis software. Various approaches have been used to increase the number of samples per experiment; the higher throughput entails a commensurate increase in scoring time, and fully automated scoring becomes a necessity.

Increasing Throughput

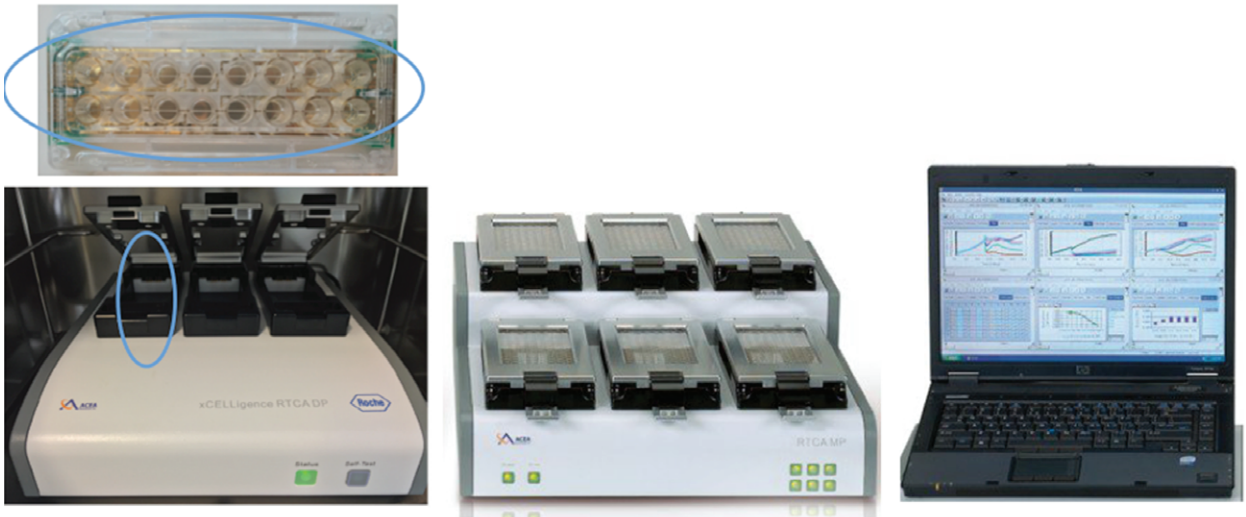
Options for increasing throughput include increasing the size of tank, for instance incorporating a stack of platforms for slides, and reducing the size of gels. The latter approach has been more popular to date. The standard gel of 70–100 µL, covering an area of about 4 cm², contains thousands of cells, of which only a tiny fraction are actually scored. Reducing the gel to a volume of 4 or 5 µL, with just a few hundred cells, allows twelve gels to be set on a standard slide,

FIGURE 6 | Impedance-based measurements: cell index (CI) real-time monitoring and viability of cells exposed to WC-Co NPs. Index real-time monitoring and viability of A549 (a), Caki-1 (b), and Hep3B (c) cells exposed to tungsten carbide-cobalt (WC-Co) NPs. Impedance measurements (one representative experiment among three independent experiments) were carried out for 72 h and cell indices were normalized at time 0 to ensure no inter-well variability prior to the addition of NPs. Control cells were not exposed to WC-Co NPs. Positive control cells were exposed for 72 h to 0.005% Triton in the case of Caki-1 and Hep3B cells and to 0.01% Triton for A549 cells. The histograms correspond to CI values at three endpoints (24, 48, and 72 h) for control cells, to a positive control (Triton), and to cells exposed to 1, 5, 25, 50, 75, 100, and 150 µg/mL of WC-Co NPs. Statistical analysis was performed for each exposure condition compared to nonexposed cells (Student's *t*-test, **p* < 0.01; ***p* < 0.001; ****p* < 0.0001).

(a)



(b)



(c)



(d)

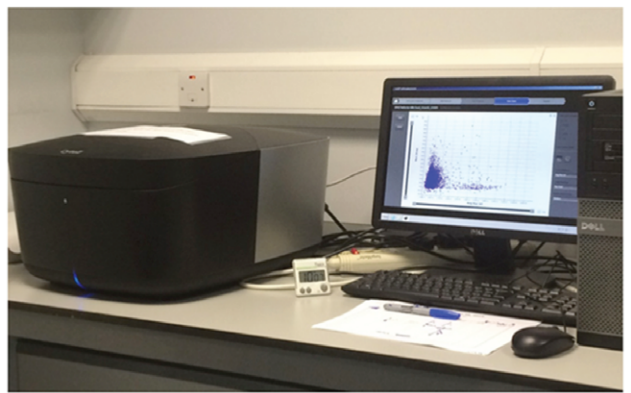


FIGURE 8 | Legend on next page.

or 96 as a standard 8×12 array. Such formats are available commercially (Figure 8(a)). For the 12-gel format, standard slides precoated with agarose are placed on a metal template and gels are set on the positions marked on the template. If gels are to be

treated with different reagents or enzymes, the slides are clamped in a chamber device creating individual wells above the gels. The throughput is further increased with the 96 gel arrays, four of which can be fitted into one tank. The gels can be set on films

of GelBond, which are then held under slight tension in special frames.

These HTS approaches were developed in the EC-FP6 project COMICS, and for nanogenotoxicity in the FP7 NanoTEST.^{71,123} The performance of the 12-gel and 96-gel formats was compared with that of the standard comet assay¹²⁴; the damage induced in cultured cells by X-rays or methylmethanesulphonate (MMS) was detected equally well by the three formats—and with very similar coefficients of variation between replicate experiments. Further characterization of the 96-gel format has been published.¹²⁵

The HTS comet assay, together with FPG, has recently been applied to study the potential induction of ROS by NMs. Cos-1 fibroblast-like kidney cells were treated with different concentrations of iron oxide NMs, and cells embedded in minigels (12 per slide) Subsequent incubation with FPG revealed damage not seen with the basic assay for strand breaks (without FPG).⁷¹ Further, Huk et al. used the 12 gel system to study genotoxicity of nine well-defined nanosilvers in relation to their size and surface properties.^{126,127} Reservations have been expressed about the use of the comet assay, because of potential interference of NMs with the assay. However, recent studies^{128,129} showed that this is unlikely for most NMs; the comet assay can therefore be considered reliable and useful for testing NM genotoxicity, especially in the HTS version.

Automated Scoring

Automated scoring systems are dependent on accurate positioning of gels on slide or GelBond film, optimal intensity of comets and low background fluorescence, and a density of embedded cells such that few comets overlap. The gels are located and comet images focused automatically; images are captured, and later the comets are analyzed one by one to obtain % tail DNA. Noncomet fluorescence, from cell debris, fibers, etc. must be recognized and

eliminated from the analysis. Automated scoring is fraught with difficulties, but has been successfully developed by a few companies, notably Imstar with the Pathfinder system, and MetaSystems with Metafer. (High content imaging systems have also been adapted for automated comet scoring.) In the COMICS project the Pathfinder system of Imstar was compared with semi-automated image analysis (comets selected by the operator for analysis), and also with manual scoring (comets categorized by visual examination into one of 5 classes). While there were differences between the three methods, all were capable of accurate damage measurement, with comparable sensitivities.¹³⁰

The ‘CometChip’ integrates a HTS comet assay with automated scoring in a novel way; cells are deposited at predefined positions stamped in a microarray on an agarose-coated plate, so that it is possible to locate comets precisely for image capture and analysis.^{131,132}

The comet assay is currently the most used method for genotoxicity testing of NMs/NPs. The need for NM-specific positive and negative controls (reference standards) should be met with the candidate materials identified in projects such as FP7 NanoTEST and Nanogenotox.^{123,133}

HIGH-THROUGHPUT *IN VITRO* MICRONUCLEUS ASSAY

The *in vitro* micronucleus assay is a likely choice among a battery of genotoxic assays for rapid and effective screening of NMs using HTS/HCA platforms. Different approaches have been proposed to increase the speed of this assay. Classically, the long and tedious visual scoring of slides has been relieved by using automated platforms scoring many slides in each run.^{134–136} Different commercial automatic scoring devices are now on the market and the time saving, based on person-hours, due to the automation is approximately 70%.

FIGURE 8 | Examples of high throughput equipment. (a) Apparatus for performing comet assay on 12 minigels on one slide. Right: Component parts of 12-gel chamber unit (Severn Biotech, Kidderminster, UK), including metal base with marks for positioning gels on slide, silicone rubber gasket, plastic top-plate with wells, and silicone rubber seal. Left: chamber unit assembled. (b) The xCELLigence[®] instruments (ACEA Biosciences Inc, San Diego, CA, USA) employ impedance-based label-free real-time monitoring of cells. Cell number, proliferation, viability, morphology, and adhesion are quantified. Electronic microtiter plates: 16-well (left), 96-well (middle), and 384-well (not shown), can be used for high throughput nanotoxicity screening. The instrument is placed in a standard CO₂ incubator and is cable-connected with analysis and control units outside the incubator (right). The data and the performance of the instrument are displayed real-time (right). (c) The Ampha[™] Z30 impedance flow cytometer (left) (Amphasys AG, Lucerne, Switzerland) uses microfluidic chips with microelectrodes (right) to measure changes in the electrical resistance of the fluid, in which cells are suspended, when cells pass through the applied AC electric field. Cell viability and mode of cell death (apoptosis vs. necrosis) can be detected and quantified. (d) GE Healthcare Cytell Cell Imaging System captures cellular and sub-cellular images in a benchtop unit equipped with on-board data analysis and visualization tools. It streamlines and simplifies routine assays, such as cell cycle and cell viability assays, to save time and help research progress more rapidly.

Another high-throughput change in the micronucleus assay includes the use of flow cytometry, based on the pioneering work of Nüsse and Kramer.¹³⁷ The standard protocol involves (1) the lysis of membranes by a nonionic detergent; (2) the use of one or more nucleic acid dyes that can permit discrimination between the liberated nuclei and micronuclei, according to their DNA-dye associated fluorescence intensities; (3) the separation of micronuclei and nuclei by flow cytometry. Further modifications use a 96-well format in conjunction with a robotic auto-sampling device. This adaptation requires less test material than conventional test methods, and has a greater compatibility with HTS instrumentation.^{138–141}

The use of Chinese Hamster Ovary K1 (CHO-K1) and human hepatocarcinoma (HepG2) cells in a HCA approach has demonstrated its potential as an alternative to labor-intensive manual scoring of micronuclei.^{142–145} The micronucleus assay on this HTS platform has proved to be an efficient methodology with high sensitivity and specificity to detect genotoxic compounds. Cells are cultured in 96-well plates pre-loaded with a dye that stains the cytoplasm. After incubation with the test compounds the cells are fixed and their DNA is stained with a Hoechst dye. The visualization and scoring of the cells are done using an automated fluorescent microscope coupled with proprietary automated image analysis software.

Until now, these approaches have not been applied to evaluating the genotoxic potential of NMs. However, a recent collaboration between Flinders University, University of South Australia, CSIRO and Safe Work Australia has developed an automated HTS procedure for assessing the genotoxic potential of NMs.¹⁴⁶ As a proof of concept, the development and validation of the method was carried out in several steps. A mixture of HR1K (Burkitt lymphoma) cells stained with Vybrant™ DiO Cell-labeling, and Jurkat cells (an immortalised line of T lymphocytes) stained with Hoechst-33342, was separated on an antibody microarray slide printed with different antibodies. Three monoclonal antibodies that were capable of differentiating between human leukocytes were used; anti-CD2 (specific for T lymphocytes), anti-CD20 (specific for B lymphocytes), and anti-CD 45 (common for T and B lymphocytes). Silver NPs (citrate and PVP-capped), and H₂O₂ as a positive control were used in the genotoxicity assay. Results showed the usefulness of the method; 10 nm citrate-capped AgNPs induced a strong genotoxic effect after 24 h exposure, double that induced by 70 nm citrate-capped AgNPs, and

greater than the effect of treatment for 1 h with 20 μM H₂O₂. In addition, 10 nm citrate-capped AgNPs induced much greater genotoxicity than did 10 nm PVP-capped AgNPs.

A crucial aspect of the HTS micronucleus assay—as with other assays—is the choice of suitable NM-based positive and negative controls. This has been a focus of many European Union funded projects including NANOGENOTOX and FP7 Quality-NANO or FP7 NanoTEST.

Overall, we can conclude that the micronucleus assay can be the subject of HTS approaches and that this can be applied to the testing of the genotoxic potential of NMs. Nevertheless these different approaches need to be validated with a wide range of NMs.

THE γ H2AX ASSAY

A number of studies have proposed C-terminal phosphorylated histone protein, γ H2AX, as a potential biomarker of DNA double-strand breaks (DSB) caused by genotoxicants.^{147–150} After DSB formation large numbers of γ H2AX molecules accumulate around the break site, making possible their detection. The importance of this biomarker arises from the fact that DSB are considered the most critical kind of DNA damage, initiating genomic instability and, potentially, leading to cancer.^{151,152} Paradoxically, DSB can also help to kill cancer cells.¹⁵³ Thus, this method is used in different fields ranging from cancer chemo- and radiotherapy¹⁵⁴ or drug discovery¹⁵⁵ to *in vitro* toxicology testing of environmental pollutants.¹⁵⁰

Two types of methods are often used for γ H2AX detection; those counting foci or other γ H2AX-containing structures in images of cells and tissues (by immunofluorescence microscopy), and those measuring overall γ H2AX protein levels (by immunoblotting or flow cytometry). Although both methods are currently used, counting γ H2AX foci is several orders of magnitude more sensitive, and allows the distinction between pan-nuclear staining and focus formation, and it is this approach that is being employed in efforts to develop high throughput techniques.¹⁴⁷

Although initially this biomarker was used to identify ionizing radiation effects, several studies have already used the γ H2AX phosphorylation technique to measure DSB caused by different NMs, including carbon nanotubes,^{156,157} zinc oxide,¹⁴⁹ gold,¹⁵⁸ silica,¹⁵⁹ nanodiamonds,^{83,99} tungsten cobalt,⁸² polystyrene,¹⁰⁰ TiO₂ NPs,^{160,161} and WC-

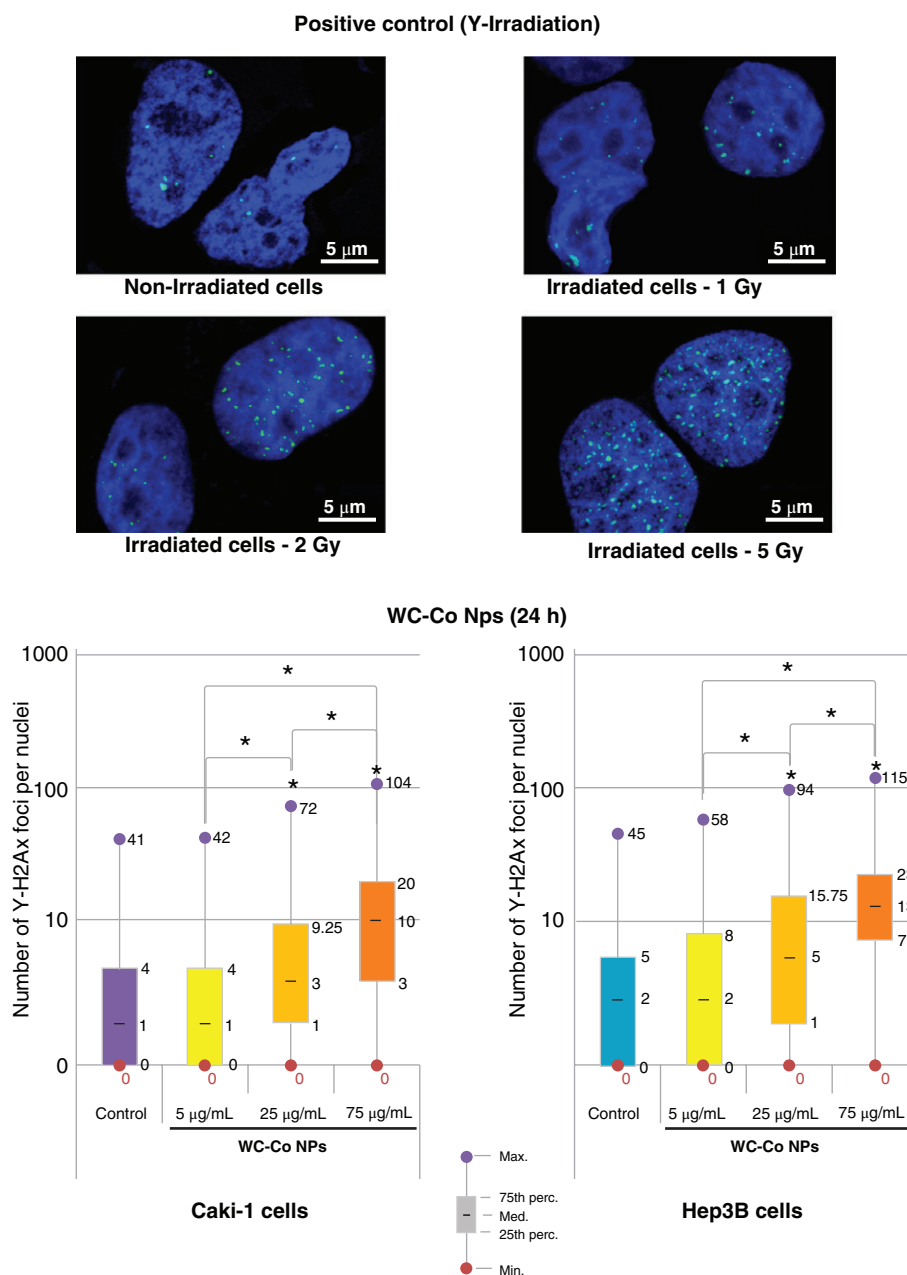


FIGURE 9 | Tungsten carbide-cobalt (WC-Co) NP genotoxicity determined by measuring foci of γ -H2Ax (phosphorylated H2Ax histones), which are directly proportional to the number of DNA double-strand breaks. Counts of γ -H2Ax foci were performed on at least 200 cells per condition and the results are depicted as box plot distribution values [minimum (min), maximum (max), median, 25th and 75th percentiles] of the number of foci obtained for each tested condition. A Wilcoxon rank test was performed for statistical comparisons (i.e., vs. control cells not exposed to NPs; $*p < 0.01$). For both cell lines Caki-1 and Hep3B, WC-Co NPs were found to be genotoxic in a dose-dependent manner (b). For γ -H2Ax positive control, Caki-1 cells were exposed to γ irradiation (a).

Co NPs (Figure 9) showing that this technique can be a useful tool to assess the genotoxic potential of NMs. However, counting γ H2AX foci is usually done manually by microscopy, which is time-consuming and cumbersome. γ H2AX dots are sometimes very small and need to be detected by sophisticated confocal microscopy. A highly advanced

confocal based high content imaging system may be needed. In addition, a 100 \times objective is required for imaging, which reduces the speed of the instrument, rendering high throughput as low throughput. Therefore, efforts have been made to improve this method, based on microscopy techniques such as imaging modalities in cell culture and in tissues,¹⁶² and

computer-assisted approaches.¹⁶³ The computational approaches are supported by image analysis software such as NIH Image and its Windows counterpart ScionImage, ImageJ, HistoLab, AutoQuantX, or Image Pro, among others, which rely on different computational algorithms for foci identification or the calculation of quantitative foci parameters.^{164–166} High content imaging systems are also capable of γ H2AX quantitation. Specialized software for counting foci (FociCounter) has been developed.¹⁶⁷ The latest development is the incorporation of focus counting into an automated high throughput image acquisition and processing platform.¹⁶⁸ Recently, Harris et al.⁷¹ have analyzed the H2AX phosphorylation induced by iron oxide NPs using a high content platform, demonstrating the possibility of using this technique with exposures such as NPs. These different computational approaches render conventional γ H2AX assay as a highly efficient HTS technique. At the same time they allow the analysis of other parameters in a cell population, while avoiding possible operator manipulation error.

These are some of the several options that could contribute to utilizing the full potential of the γ H2AX assay for assessing the DSB produced by NMs.

HIGH-THROUGHPUT OMICS ASSAYS

Omics-based methods have the potential broadly to indicate toxicity of NMs on a systems biology level. Recent studies have shown that data from such methods, in combination with phenotypical HTS results, can be integrated to reveal complex physiological and toxicological effects of NMs as well as of chemicals, drug molecules, or the complex mixtures in consumer products (reviewed in Refs 169,170). Omics analysis is normally classified as a form of HCA, but is relatively costly and slower than typical robotics-assisted HTS methods. Typically, omics testing involves the use of chip-based or sequencing technologies for genome-wide profiling of gene activities, e.g., measuring mRNA levels following a toxic insult. However, reduced sets of toxicity-associated genes can be assayed at higher throughput and lower cost, e.g., with Luminex[®] technology.¹⁷¹ Future high-throughput transcriptomics platforms, e.g., in the LINCS and the Tox21 Phase III projects, enable rapid gene profiling experiments with both several doses and biological replicates using multiple model of 800–1500 genes.^{172,173} NM effects analyzed using traditional microarrays, such as Affymetrix

GeneChips[®], form the basis for most existing gene profiling analyses of NMs,^{174,175} providing reference values for recent next-generation sequencing (e.g., Ref 176 and future generation of HTS data from selected toxicity-reflective gene sets. Open source or commercial bioinformatics tools, such as—respectively—InCroMap or Ingenuity Pathway Analysis (Ingenuity[®] Systems, www.ingenuity.com), rapidly sort omics-derived data into mechanistically meaningful results, enabling grouping of NMs into clusters by gene or pathway activation levels.¹⁷⁷ Connectivity mapping, i.e., grouping for similarities in gene expression profiles, can be viewed as a form of biological read-across.¹⁷⁸ Modeling efforts have indicated a need for investigating more than 200 and even thousands of agents, whether NMs or chemicals, to effectively characterize toxicity mechanisms through omics analysis.¹⁷⁹ The well-known Connectivity Map project,¹⁸⁰ and its successor LINCS, have addressed this issue with over 1.5 million gene expression profiles covering over 10^5 variables to date.¹⁷³ Overall, data integration across high-throughput, high-content, pathway-based cellular assays and omics profiling will enable a diversified view of the potential toxicological activity of a NM. Tiered HTS approaches including toxicity assessment and immunochemical assays followed by omics analysis, lead to gradually broader characterization of intoxicating concentrations of selected, potentially class-representative NMs.^{169,170} Such efforts promise efficiently to define relevant toxic modes-of-action of NMs, via comprehensive evaluations of existing omics data collections aimed at systems toxicology.

COST-EFFECTIVENESS OF HIGH-THROUGHPUT SCREENING OF NANOMATERIALS

Even though only safe NPs/NMs should reach the market place, the conventional methodologies for conducting hazard assessment have not been able to keep pace with innovation, leading to the urgent need for innovative high throughput and cost-effective technologies. It has recently been estimated that the time taken to complete evaluation of existing NMs would be more than 30 years and the costs for testing them on an individual basis would be prohibitive.¹⁸¹ The HTS approaches for hazard assessment of NMs described above clearly allow a reduction of the time required for toxicity testing while increasing data outcomes, but the cost-effectiveness of those approaches needs also to be considered. Analyses of

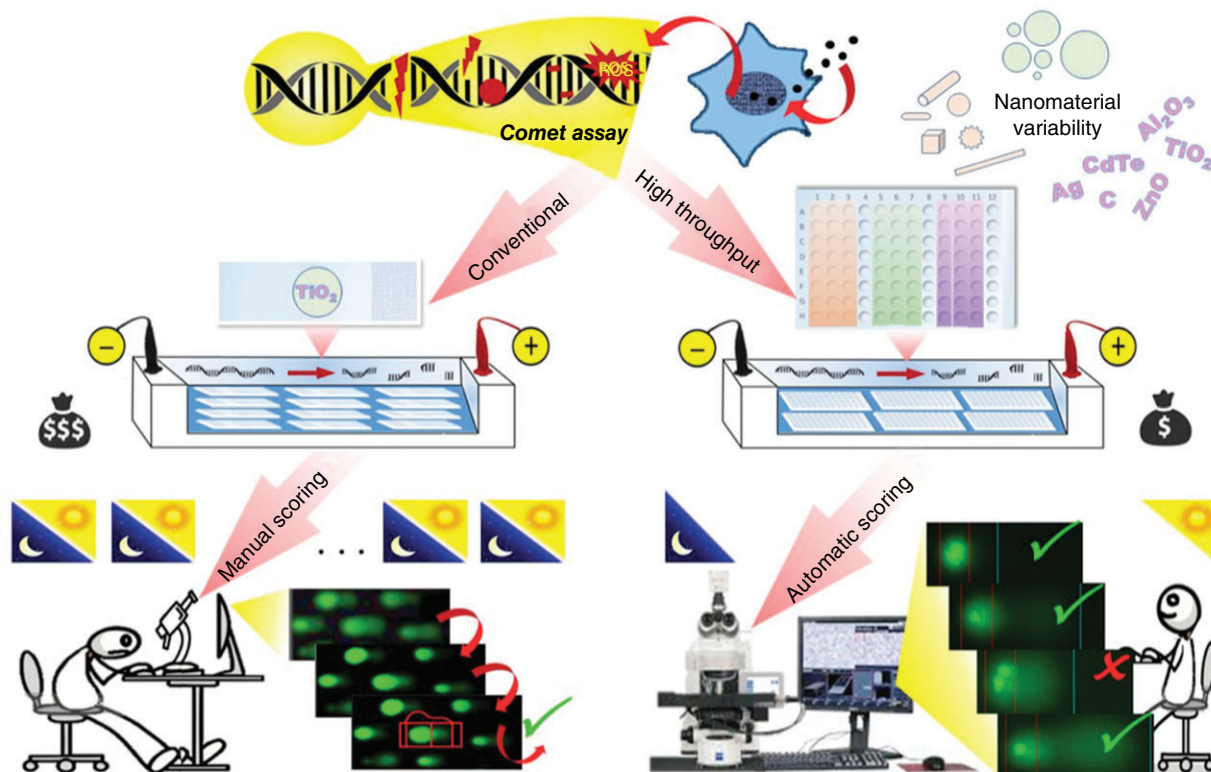


FIGURE 10 | Savings in time and cost with HTS comet assay.

cost-effectiveness involve complex economic indicators and have been used to measure the relative value of a new or modified technology in terms of the cost per benefit gained. This type of analysis takes into account short-term costs, e.g., the cost per new endpoint identified or per time saved, and long-term costs, e.g., the cost per hazardous NM identified versus the gains in terms of human disease prevention and environment protection. Thus, while short-term costs comprise mainly the direct costs associated with laboratory expenditure, the long-term costs are related to the societal costs and are much more complex to measure. Focusing merely on direct costs, HTS for toxicity is expected to reduce the costs of NM development, as has happened in drug discovery,¹⁸² as a result of the greater number of NPs/NMs and experimental conditions simultaneously assayed, and the lower amounts of test samples and consumables required, provided that the adequate equipment or accessories are available in the laboratory. There should also be savings related to direct labor (e.g., decreased time required to complete each task and lower degree of expertise or training necessary) (Figures 1 and 10). Pictures of some of the most recent equipment used in high throughput assays are shown in Figure 8.

These advantages have been realized in an integrated study of iron oxide NP toxicity in which high-content-imaging endpoints for cell viability, oxidative stress and DNA damage (double-strand breaks) were employed, as well as the HTS comet assay.⁷¹ Three laboratories using the comet assay have estimated the time taken to process one sample in the form of a mini-gel (excluding scoring) as between 4 and 9 times less than the time taken to process a sample in the conventional large gel format. Scoring time per gel using nonautomated image analysis was roughly halved with minigels compared with standard gels, as time was saved on changing slides and refocusing. However, scoring is still the main bottleneck, and it needs to be made fully automatic.

Another example is the replacement of traditional microscopy by automated imaging in the micronucleus assay, also contributing to time saving and consequent decrease in labor costs. Moreover, the faster scanning of micronucleus slides using an automated platform has given the possibility of increasing the statistical power of results, through maximizing the number of cells scored, while still saving time compared with visual scoring.¹⁸³ Furthermore, the flow cytometric micronucleus assay allows the possibility of scoring tenfold more cells in

the same period of time compared with the conventional microscopic evaluation, also increasing the statistical power of the assay.

Despite the unequivocal direct gains from applying HTS to nanotoxicology, the costs incurred by the laboratory can be prohibitive if the acquisition of expensive laboratory equipment (e.g., fully automated equipment for image analysis), and sophisticated tools for data analysis or on-line data storage capacity are needed for its implementation.¹⁸⁴ The investment can, however, be justified depending on the number of tests and samples to be analyzed, among other factors related to laboratory management.

Finally, regarding long-term costs versus societal benefits, the promotion of more robust, diverse and adaptable HTS techniques for the safety assessment of NMs, providing information early in the process of NM development, will further minimize the costs resulting from a delayed finding of potential harm to human health and/or the environment, thus maximizing the benefits of innovation.

CONCLUSIONS AND FUTURE PERSPECTIVES

With the growing numbers of engineered NMs, there is a huge demand from the scientific community as well as the legislative institutions to come up with ways of accurate and rapid testing of NM safety *in vitro*. The adoption of HTS techniques for this task not only allows the examination of large numbers of different materials at different concentrations and on different types of cells, but also makes substantial savings in time and cost, as well as reducing the effect of experimental variation.

Validation of *in vitro* HTS tests is essential, with regard to their relevance to *in vivo* conditions. Also, validated HTS approaches to assess dose- and time-dependent toxicity that are predictive of *in vivo* adverse effects are required. HTS/HCA methods for studying cellular uptake and intercellular transfer, with automated imaging and image analysis, and reduced-feature gene sets and biomarkers predictive of toxicity effects should be developed. The crucial

toxicity endpoints include cytotoxicity, oxidative stress, genotoxicity and markers indicative of cell transformation and carcinogenicity.

Automation should further streamline testing procedures, and—linked with appropriate standard operating procedures—this should contribute substantially to reducing variability and operator bias. HTS is bound to generate large data collections, and to encourage research groups to establish databases on relevant toxicological determinants of NMs. The availability of a bank of reliable information about NM toxicity will facilitate grouping approaches and the selection of class-representative materials that require animal testing. Finally, future efforts of HTS and HCA should also consider means of potentially automating the preparation and dispersion of NMs, as this task is so far mostly manually performed, leading to a less than desirable output of data from the increasingly growing array of HTS approaches reviewed here.

NMs display singular physicochemical properties that can bias the results of conventional toxicity assays^{61,185,186} depending both on the assay and on the NM. Positive and negative controls should be systematically included in experiments, in order to confirm the sensitivity of the techniques used, to assess potential NM interferences with assays or detection systems, and to benchmark the cytotoxic/genotoxic effects of tested NMs. Recently, several suitable candidate control NMs have been described. Iron oxide was suggested as a positive control for cytotoxicity, oxidative stress and genotoxicity endpoints and PLGA-PEO as a negative control.^{123,133} Also aminated polystyrene nanobeads were suggested as a positive control for acute toxicity,^{99,110} including cytotoxicity and membrane damage¹⁸⁷ but also activation of the inflammasome pathway,^{188,189} while carboxylated nanodiamonds (as negative control) were found to be neither cytotoxic nor genotoxic on several human cell lines.⁹⁹ These last two kinds of NMs have already been assessed on the xCELLigence® system, and results were very close to (1) those obtained by cell mortality detection using flow cytometry,⁹⁹ and (2) results using conventional toxicity evaluation methods^{110,190–192}

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