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2021-11

Nanotechnologies — High throughput screening method for nanoparticles toxicity using 3D model cells

Nanotechnologies — Méthode de criblage à haut débit de la toxicité des nanoparticules utilisant des systèmes cellulaires 3D

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Foreword

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This document was prepared by Technical Committee ISO/TC 229, *Nanotechnologies*.

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

Introduction

With an increasing number of nano-products including nanoparticles (NPs), potential exposure of consumers to NPs has increased. Therefore, the human and environmental impacts of NPs have recently emerged as an issue. High-throughput screening (HTS) approaches are often used for NPs toxicity screening. However, there are still limitations to provide the reproducible and reliable results based on a HTS method. To assess the potential toxicity of manufactured or engineered NPs, traditional in vitro toxicity studies have been performed using a surface attached two-dimensional (2D) culture system. 2D assays for cellular metabolic activity, cytotoxicity, or oxidative stress have been widely used in the first stage of hazard evaluation. However, several problems were encountered during assay validation, ranging from particle agglomeration in biological media to optical interference with the assay platform. There are ISO documents on the cytotoxic effects of NPs using cell viability assays and detection of reactive oxygen species (ROS) levels, but they can be applicable for a few classes of NPs that are well-dispersed in the media. Additionally, reagents used in the assays can interact with tested NPs or interfere with spectrophotometric reading.

This document describes a new assay platform, consisting of three-dimensional (3D) arrangement of cells on pillar inserts to evaluate cell viability and diminish artefacts arising from optical interferences and NP reactivity with assay components.

This document aims to overcome the optical interference of NPs and obtain reliable and reproducible cell viability results. The 3D-model cells are exposed to fresh cell viability reagent by simply transferring and immersing the pillar insert from one well to another well without optical interference from the NPs. In addition, 3D-model cell culture approaches facilitate cell-cell interactions and enhance cell-to-cell or cell-to-extracellular matrix (ECM) adhesion/signalling, ultimately leading to the expression of phenotypic proteins/genes and the formation of in vivo tissue-like morphology. It generates uniform cell-containing hydrogel droplets on the pillar insert and allows to easily change cell growth media or expose 3D-model cells to analytical reagents by immersing the tip of the pillar insert in different reaction plates.

Nanotechnologies — High throughput screening method for nanoparticles toxicity using 3D model cells

1 Scope

This document describes a method for high throughput evaluation of cytotoxic response of 3D model cells exposed to NPs without optical interference.

The method in this document is intended to be used in biological testing laboratories that are competent in the culture and growth of cells and the evaluation of cytotoxicity of NPs using 3D-model cells.

This method applies to materials that consist of nano-objects such as nanoparticles, nanopowders, nanofibres, nanotubes, and nanowires, as well as aggregates and agglomerates of these materials.

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO/TS 80004-2, *Nanotechnologies — Vocabulary — Part 2: Nano-objects*

3 Terms and definitions

For the purposes of this document, the terms and definitions given in ISO/TS 80004-2 and the following apply.

ISO and IEC maintain terminology databases for use in standardization at the following addresses:

— ISO Online browsing platform: available at https://www.iso.org/obp

— IEC Electropedia: available at https://www.electropedia.org/

3.1
agglomerate
collection of weakly bound particles or aggregates or mixtures of the two where the resulting external surface area is similar to the sum of the surface areas of the individual components

Note 1 to entry: The forces holding an agglomerate together are weak forces, for example, van der Waals forces, or simple physical entanglement.

Note 2 to entry: Agglomerates are also termed secondary particles, and the original source particles are termed primary particles.

[SOURCE: ISO/TS 80004-2:2015, 3.4, modified — "weakly or medium strongly bound particles" has been replaced with " weakly bound particles or aggregates or mixtures of the two".]

3.2
dispersion
microscopic multi-phase system in which discontinuities of any state (solid, liquid or gas: discontinuous phase) are dispersed in a continuous phase of a different composition or state

Note 1 to entry: If solid particles are dispersed in a liquid, the dispersion is referred to as a suspension. If the dispersion consists of two or more liquid phases, it is termed an emulsion. A super emulsion consists of both solid and liquid phases dispersed in a continuous liquid phase.

[SOURCE: ISO 19007:2018, 3.2]

3.3
nano-object
material with one, two or three external dimensions in the *nanoscale* (3.5)

Note 1 to entry: This is a generic term for all discrete nanoscale objects.

[SOURCE: ISO/TS 80004-2:2015, 2.2, modified — "discrete piece of" has been added to the definition and Note 1 to entry has been replaced.]

3.4
nanoparticle
NP
nano-object (3.3) with all three dimensions in the *nanoscale* (3.5)

Note 1 to entry: If the lengths of the longest to the shortest axes of the nano-object differ significantly (typically by more than three times), the terms nanorod or nanoplate are intended to be used instead of the term nanoparticle.

3.5
nanoscale
size range from approximately 1 nm to 100 nm

Note 1 to entry: Properties that are not extrapolations from a larger size will typically, but not exclusively, be exhibited in this size range. For such properties, the size limits are considered approximate.

Note 2 to entry: The lower limit in this definition (approximately 1 nm) is introduced to avoid single and small groups of atoms from being designated as *nano-objects* (3.3) or elements of nanostructures, which can be implied by the absence of a lower limit.

3.6
high-throughput screening
method that comprises the screening of a large number of chemicals via automation, miniaturized assays and large-scale data analysis

Note 1 to entry: This protocol can be applied to screen the toxicity of NPs based on 96-well plate or 532 microchip.

4 Background

4.1 General

With the increase in the number of consumer products containing NPs, potential exposure to NPs has increased, and potential human and environmental hazards of NPs have emerged. To assess the effects of NPs, a high-throughput screening method to evaluate cell viability following exposure to NPs is needed. High-throughput approaches have been used to screen for toxicity of manufactured NPs[1].

4.2 Effects of optical properties of NPs on in vitro cell viability assays

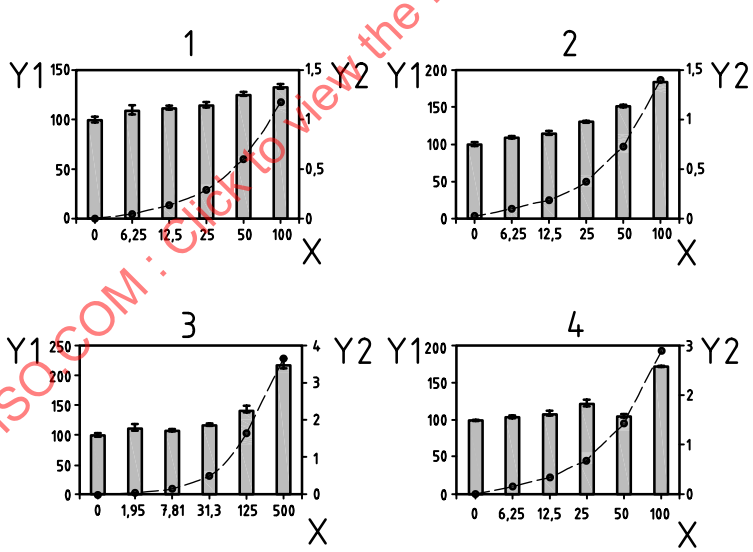
NPs possess linear/nonlinear optical absorbance and photoluminescence emission[2][3]. Because of their physicochemical and optical properties, NPs are used in various fields for disease diagnoses or as industrial products. Most NPs exhibit the optical properties in a wide range of absorbance wavelengths, where optical interference can be pronounced in cell viability assays based on absorbance read-outs.

To assess the potential toxicity of manufactured or engineered NPs, traditional in vitro toxicity studies have been performed using 2D model culture systems. During validation of 2D-model assays, several problems, for example, particle agglomeration in biological media and optical interference with the assay system, were encountered[4][5]. In traditional cell viability assays, colorimetric detection is generally used, and luminescent and fluorescent detection methods have been also applied to evaluate the cell viability assay. As shown in Table 1, some NPs such as the Ag NPs show an optical absorption at the wavelengths where the colorimetric assays are monitored.

Table 1 — Range of the wavelengths of conventional cell viability assays

Assay	Wavelength used for measurement nm
WST-1	420 to 480
XTT	450 to 500
WST-8	450 (450 to 490)
MTS	490 (450 to 540)
NRU	540
MTT	570 (500 to 600)

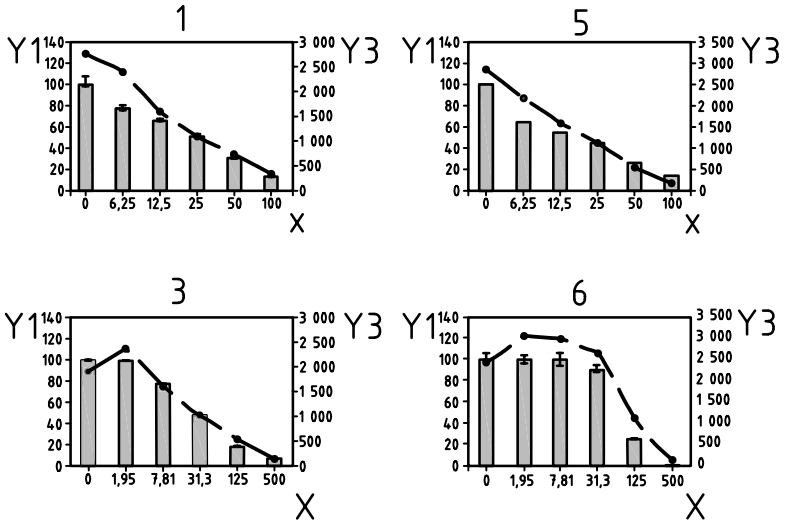
Representative cell viability assays include water-soluble tetrazolium (WST), 2,3-Bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide salt (XTT), 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS), neutral red uptake (NRU), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assays as well as ATP detection based on luminescence, but the optical properties of NPs can influence all of these detection methods. The effects of the optical absorbance of NPs (Ag, silver-shelled gold (Au@Ag), and iron (II, III) oxide (Fe_3O_4) NPs and single-wall carbon nanotube (SWCNT) on cell viability were evaluated as shown in Figure 1, a). The absorbance increased according to the concentration of NPs. Although some remedies, such as multiple washing steps, NPs attached to the cultured cells can still influence the optical absorption reading. These optical properties can lead to false positive or false negative results[6]. The luminescence detection method is also vulnerable to the optical interference of NPs, as shown in Figure 1, b). As the number of NPs increased, the intensity of luminescence remarkably decreased, showing false positive cytotoxicity.



a) Colorimetric detection using CCK-8 (Cell Counting Kit-8) reagent (Dojindo)[1)]

CCK-8 (Cell Counting Kit-8) reagent (Dojindo) is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.

1) CCK-8 (Cell Counting Kit-8) reagent (Dojindo) is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.



b) Luminescence detection using CellTiter-Glo® reagent (Promega)[2)]

CellTiter-Glo® reagent (Promega) is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.

Key

X	concentration (μg/ml)	1	Ag
Y1	cell viability (%)	2	Ag-shelled Au
Y2	absorbance	3	Fe_3O_4
Y3	luminescence	4	SWCNT
	cell viability	5	Au
----•----	background absorption	6	QD
— — —	background values		

Figure 1 — Effects of optical properties of NPs on cell viability

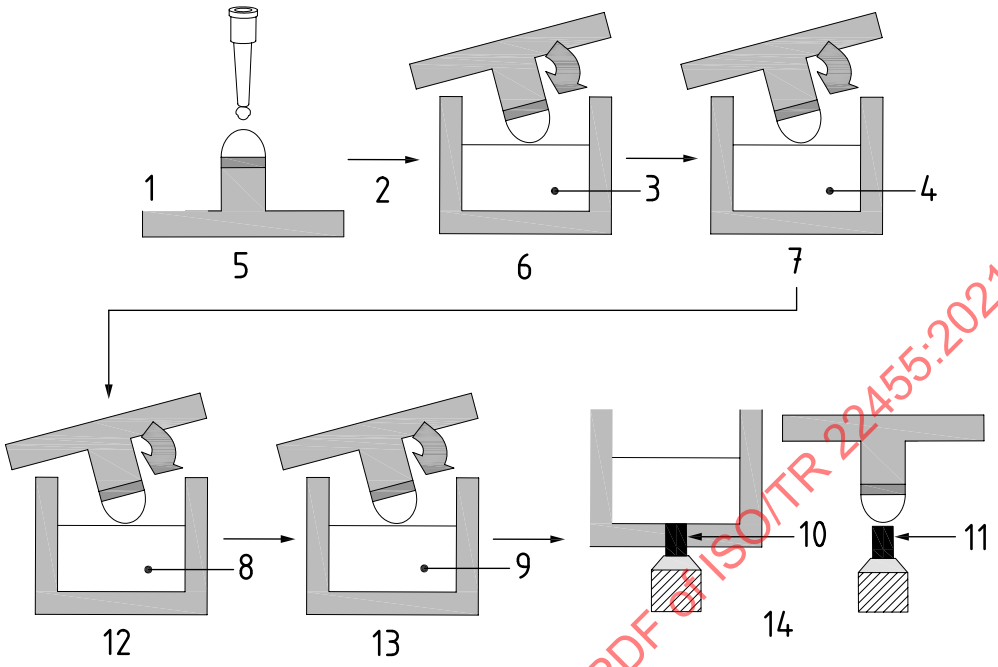
A549 cells were treated with NPs under the pre-dose finding ranges for 24 h, and cell viability was measured using the colorimetric and luminescence detection method. The absorbance of NPs without cells was measured as the background. The left axis represents the relative cell viability, and the right axis represents the absorption values at 450 nm (A) and luminescence (B). For colorimetric detection using CCK-8 (Cell Counting Kit-8) reagent (Dojindo) in panel (A), the cell viability and background absorption are represented with bars and dashed lines respectively. For luminescence detection using CellTiter-Glo® reagent (Promega) in panel (B), the cell viability and background values are represented with bars and dashed lines respectively. SWCNT, single-walled carbon nanotube; QD, quantum dot.

4.3 New assay platform for in vitro toxicity screening of NPs diminishing optical interference

3D-model cells on a pillar insert are used to evaluate the cell viability while minimizing artefacts such as those associated with optical absorption and undesirable reactions with an assay reagent. In the use of these platforms, the 3D-model cells are exposed to fresh cell viability reagent by simply transferring and immersing the pillar insert from a column of a well to another column of that well without the optical interference of the NPs, and the schematic flow is shown in Figure 2. This platform allows to easily exchange cell growth media and to expose the 3D-model cells to analytical reagents by immersing

2) CellTiter-Glo® reagent (Promega) is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.

the tip of the pillar insert in different reaction plates[7][10]. Thus, this method allows high-throughput screening of NPs cytotoxicity by reducing optical interference and reactivity with assay reagents.



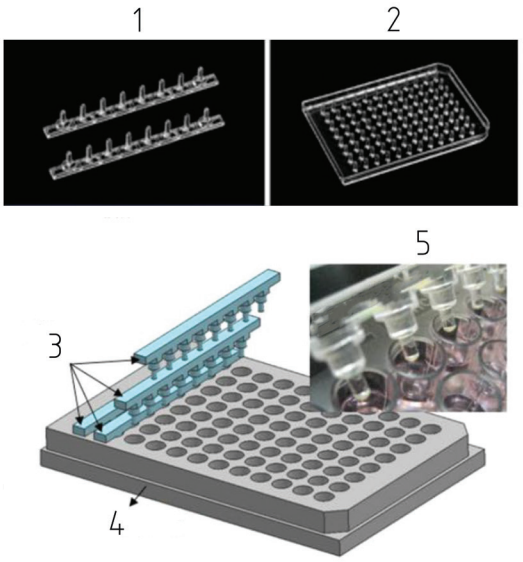
Key

1	pillar insert	8	PBS
2	96-well	9	reagent
3	growth medium	10	absorbance
4	nanoparticles	11	fluorescent intensity
5	cell seeding	12	washing
6	cell culture	13	assay
7	treatment	14	measurement of cell viability (10 or 11)

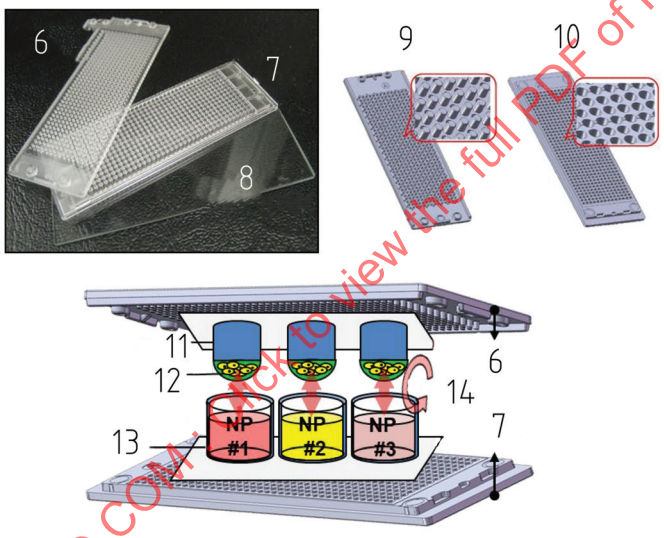
Figure 2 — Schematic flow of the pillar insert system for evaluating the cell viability of NPs

The cell-alginate mixture was dispensed onto a pillar insert. The encapsulated 3D cells on the pillar insert were cultured in the medium and then exposed into NPs. The following exposed 3D cells on pillar insert is easily transferred into an independent well with WST-8 or calcein AM reagent and the cell viability can be measured by absorbance or fluorescence.

The method is applicable to 96-well plates and can be extended to 532-well plates microchips (see Figure 3).



a) 96-well system



b) 532-well microchip system

Key

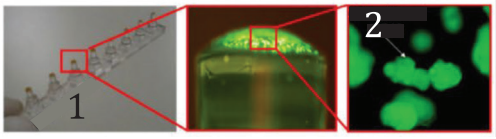
1	8-pillar strip	8	slide glass (75 mm × 25 mm)
2	96-pillar plate	9	top micropillar
3	pillar insert	10	bottom microwell
4	96-well plate	11	pillar
5	pillar insert	12	cells in alginate
6	micropillar chip	13	well
7	microwell chip	14	stamping

NOTE Each pillar insert was immersed into 96 wells or 532 microwells.

Figure 3 — Schematics of the assay platform to screen the cell viability of NPs based on a 96-well or 532-well microchip system

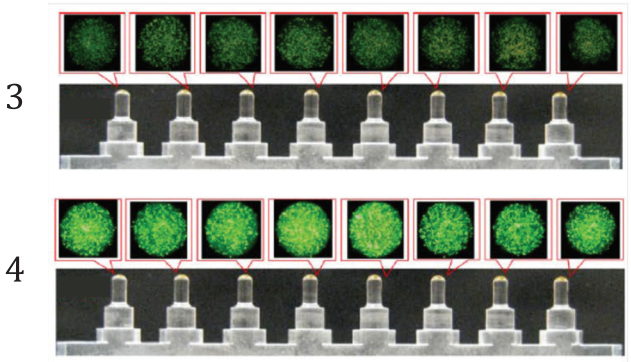
4.4 Characteristics of 3D model cells

Owing to the lack of physiological functions in vivo in traditional 2D culture models, 3D cells have been described as more predictive in hazard evaluation[11]. Advantages and challenges for evaluating the toxicity of NPs have been discussed between 2D and 3D culture models[12]. A plastic pillar insert is used to facilitate miniaturized 3D cell culture in a 96-well plate and 532-well microchip by forming 3D hydrogel droplets containing cells on the tip of the pillar insert. 2D cell cultures have been generally used previously in toxicity studies, but many cells of normal and malignant origin lose some of their phenotypic properties when grown in vitro as a 2D monolayer[13][14]. The formation of tissue-like structures is highly inhibited in a 2D monolayer culture due to the strong affinity of cells for most artificial surfaces and the restriction to a 2D space, which severely limits intercellular contacts and interactions. In addition, 3D-model cell culture approaches facilitate cell-cell interactions and cell-to-cell or cell-to-ECM adhesion/signalling, ultimately leading to the expression of phenotypic proteins/genes and the formation of in vivo tissue-like morphology[15]-[18]. It is evident that a 3D model restores the morphological and functional characteristics of tissues. For instance, cytoskeletal structure or ECM adhesion molecules are more similar to in vivo features in 3D fibroblasts[18]. 3D A549 also showed the increased expression of structural and functional markers including tight junction, epithelial proteins and mucin-specific proteins[15]. The previous investigations demonstrated that the toxic effects of NPs in 3D cell model correlated well with the animal study data[16]. To demonstrate the feasibility of pillar insert for 3D cell culture, A549 and PC9 cell lines from human non-small cell lung cancer (NSCLC) were grown on the pillar insert, and their 3D morphology, growth rate and cytotoxicity (with Erlotinib) were tested, as shown in Figure 4. The dispensed single cells in alginate matrix grew on the pillar insert, forming a unique 3D structure by cell-cell and cell-ECM interaction during proliferation [see Figure 4, a)]. The increase of green fluorescence and morphological observation over time also evidenced the growth of 3D A549 cells [see Figure 4, b)]. The number of cells in alginate droplets was linearly proportional to the cell seeding density, indicating the uniformity of encapsulated 3D cells on the pillar insert [see Figure 4, c)]. As a proof of concept showing the functionality of 3D cells, the cytotoxic effects of Erlotinib, an inhibitor of epidermal growth factor receptor (EGFR) on PC9 cells (EGFR mutant type) and A549 (EGFR wild type) were compared between 2D and 3D culture system. PC9 cells with EGFR mutation are known to show a great response to Erlotinib (i.e. extremely low half-maximal inhibitory concentration, $C_{i,50,}$ values were obtained)[19]. Interestingly, the $C_{i,50}$ value of 3D PC9 cells grown on the pillar insert was six times lower than those obtained from 2D PC9 cells, whereas the $C_{i,50}$ value of 3D A549 cells were five times higher than those from 2D A549 cells [see Figure 4, d) and Figure 4, e)]. This kind of disparity on $C_{i,50}$ values between 2D and 3D cell culture systems has been reported in the literature and is considered as a distinctive characteristic of cells cultured in 3D system[20][21]. The result can represent that A549 and PC9 cells grown on the pillar insert better mimic tissue environment.

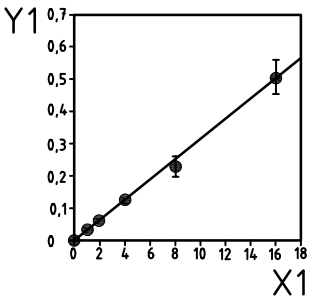


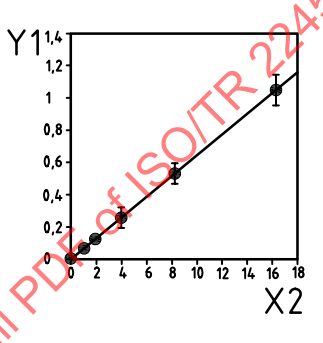
NOTE A549 cells were stained with calcein AM.

a) Alginate droplet containing A549 cells on the tip of the pillar inserts and A549 cells forming 3D structures in the droplet



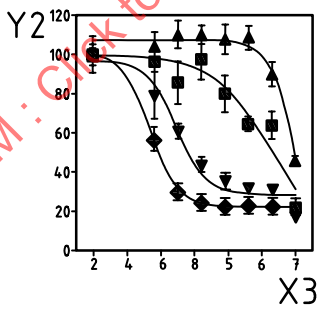
b) Pictures of stained A549 cells in alginate on the tip of the pillar insert after 1-day and 4-day incubations





NOTE The linear increase in absorbance indicates that a uniform number of cells are encapsulated in alginate droplets.

c) Absorbance of A549 cells encapsulated in alginate on the tip of the pillar inserts at different seeding densities measured by the CCK-8 reagent



d) Dose-response curves of Erlotinib with A549 and PC9 cells cultured in 3D alginate droplets on the pillar inserts and 2D monolayers

	2D	3D
Doubling time (h)		
A549	15,3	15,9
PC9	14,3	16,1
$C_{i,50}$ values		
A549	3,0 µM	15,2 µM
PC9	9,7 nM	1,7 nM

e) Comparison of the doubling times of A549 and PC9 cells cultured in 2D monolayers and 3D alginate droplets and their $C_{i,50}$ values obtained with Erlotinib

Key

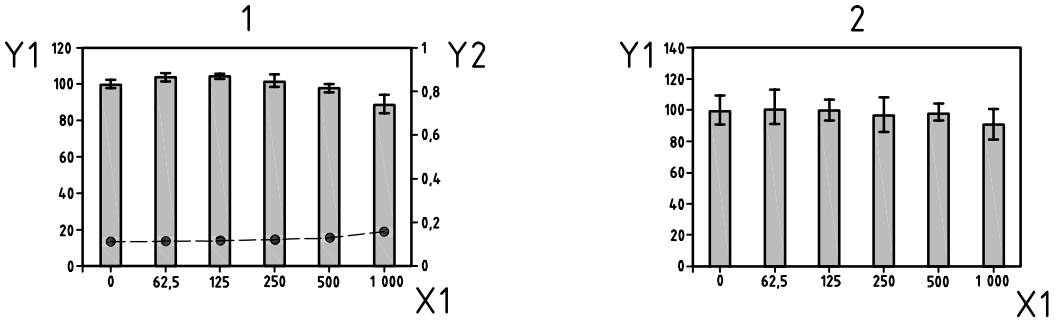
X1	# of A549 cells (1 000 cells)	3	day 1
X2	# of PC9 cells (1 000 cells)	4	day 4
X3	log [erlotinib (pM)]	■	A549_2D
Y1	absorbance	▼	PC 9_2D
Y2	live cell (%)	▲	A549_3D
1	pillar insert	◆	PC9_3D
2	3D cultured A549		

SOURCE: Reference [7], reproduced with the permission of the authors.

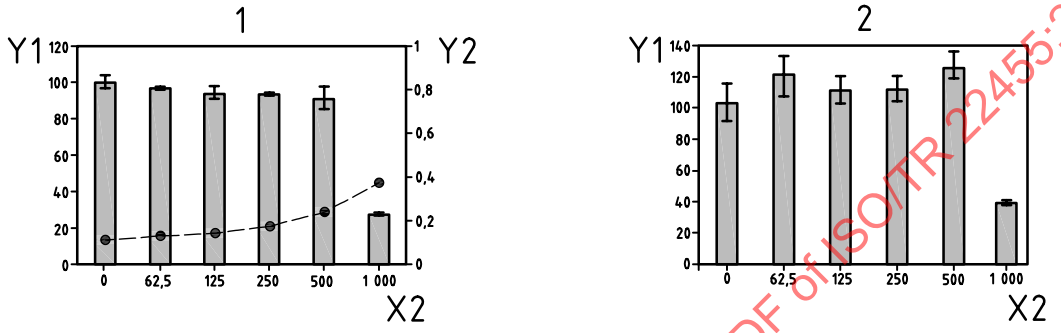
Figure 4 — Characteristics of 3D-model cells on pillar insert[7]

4.5 Cell viability in response to NPs assessed using 3D model cells on a pillar insert

The cell viability of NPs with or without optical interference was evaluated in both 2D monolayer and 3D-model cells on pillar insert. At first, the applicability of the pillar insert system for cell viability assay was tested with NPs showing no significant optical interference. The 70-nm silica (SiO_2) NPs and QD selected as negative and positive reference NPs for cytotoxicity and the cell viability was compared between the 2D monolayer and 3D-model cells on pillar insert (see Figure 5). The cell viability of negative SiO_2 NPs and positive QD were similar between both culture systems, indicating that cell viability assay is applicable in the pillar insert system.



a) Measurement of cell viability of 70 nm SiO_2 in 2D monolayer and 3D model A549 cells



b) Measurement of cell viability of QD in 2D monolayer and 3D model A549 cells

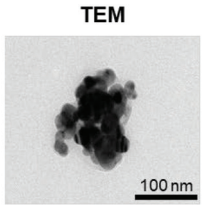
Key

X1	70 nm SiO_2 (µg/ml)	1	2D monolayer
Y1	cell viability (%)	2	3D culture on pillar insert
X2	QD (µg/ml)		CCK-8
Y2	absorbance		NPs only

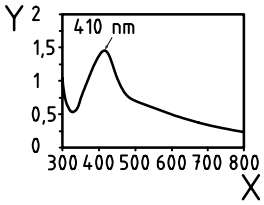
Figure 5 — Cell viability of NPs without optical interference in 2D and 3D culture systems

A549 cells were cultured in a conventional 2D monolayer and as 3D-model cells on a pillar insert. Well-dispersed 70 nm SiO_2 NP and QD were selected as negative and positive reference NP to test the cytotoxicity, respectively. The cell viability was compared between the two systems after NPs treatment for 24 h. The pre-range dose finding test was performed to determine the concentration at which cell viability is reduced for both negative and positive NPs. Alternative concentration can be used depending on dispersion or precipitation. Cell viability was measured using a WST-8 (CCK-8) assay.

On the other hand, the cell viability of Ag NPs showing optical interference was compared in both culture system using a WST-8 assay (Figure 6). The size of the Ag NPs was certified by the manufacturer to be 7,9 nm ± 0,95 nm based on transmission electron microscopy (TEM) and 11,5 nm ± 10,5 nm based on dynamic light scattering (DLS). The Ag NPs showed a unique absorbance with a maximum at 410 nm. Light microscopy showed that many Ag NPs were attached to HepG2 cells. In the 2D culture platform, cell viability differs depending on whether the background is subtracted. The background subtraction in the 2D culture system has limitations to provide reproducible and reliable data depending on the degree of optical absorbance of NPs. When the pillar insert system was used, the cell viability showed patterns similar to that of the background-subtracted group for the 2D culture system. This finding demonstrated that the pillar insert system can be a useful tool to evaluate the cell viability of NPs for 3D-model cells without optical interference effects.

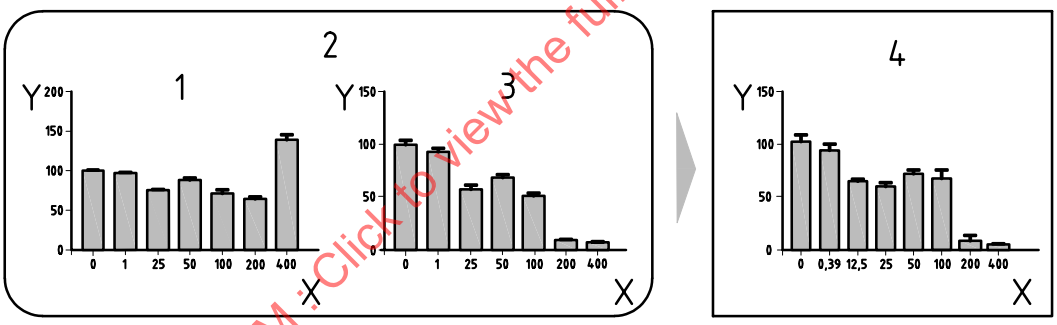


a) TEM image of Ag NPs



b) UV-visible spectrum of Ag NPs

c) Cell image after treated with Ag NPs for 24 h



NOTE Left hand box: 2D monolayer culture; right hand box: 3D culture.

d) Cell viability measured using the WST-8 (CCK-8) assay

Key

X	Ag NPs (µg/ml)	2	2D monolayer
Y	cell viability (%)	3	after background subtraction
1	before background subtraction	4	3D culture on pillar insert

Figure 6 — Cell viability of NPs showing optical interference in 2D and 3D culture systems

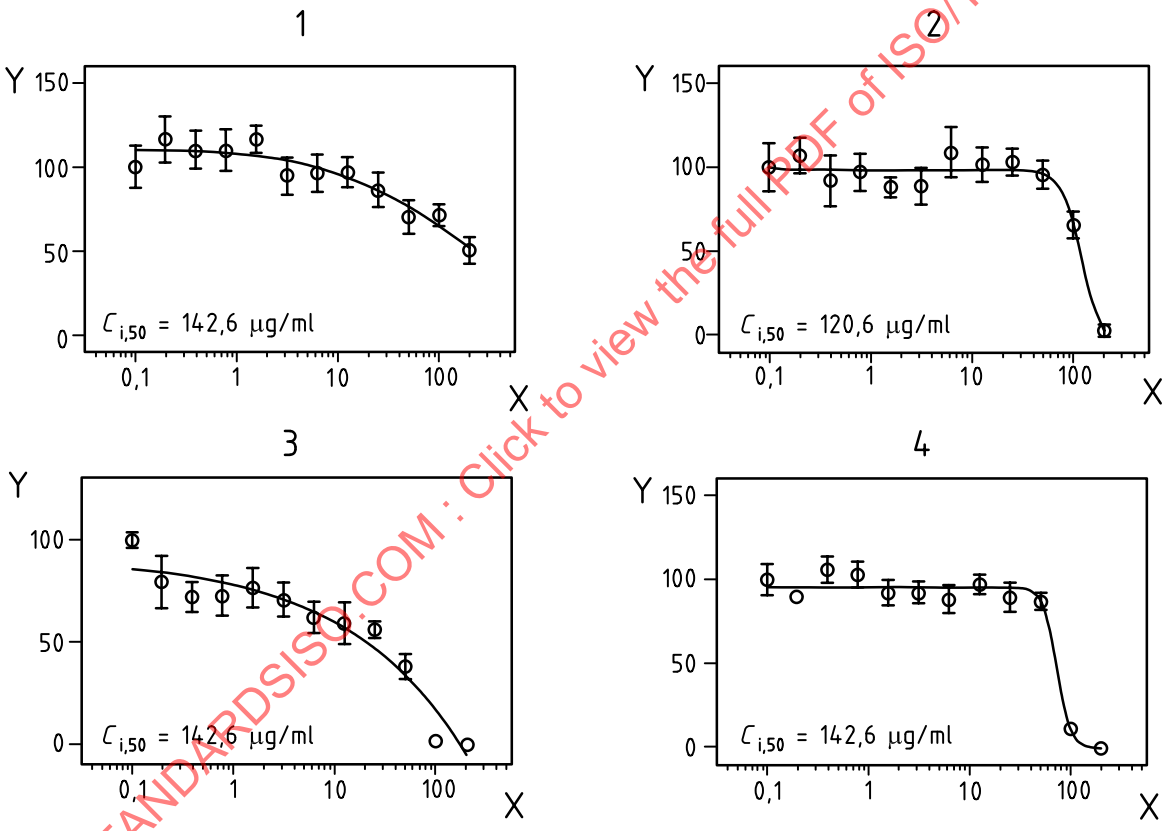
HepG2 cells were cultured in a conventional monolayer and as 3D-model cells on a pillar insert, as described in 5.3. 10-nm Ag NPs were incubated with HepG2 cells for 24 h.

The cell viability of different types of NPs showing the cytotoxicity was also evaluated based on a 532-well microchip system to test the applicability of pillar insert system in automated high-throughput screening. Information about the test NPs is listed in Table 2.

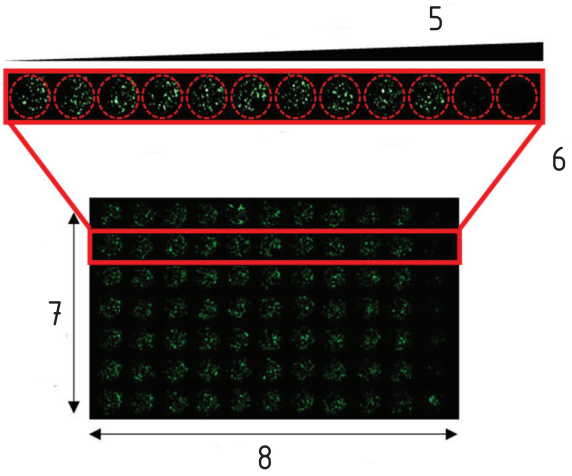
Table 2 — List of test NPs

Class	Product name	Size	Company
Ag	10 nm citrated BioPure silver	10 nm	Nanocomposix
	10 nm PVP BioPure silver	10 nm	Nanocomposix
Cu_2O	50 nm copper oxide NPs	50 nm	Nanocomposix
SWCNT	SWCNT mass fraction of 0,1 % (>95 %)	$D < 2$ nm 5 µm ≤ L ≤ 30 µm	Nanostructured and amorphous materials
Key D: diameter L: length			

The cell viability was calculated by the intensity of calcein AM staining (see Figure 7). The cytotoxic effects of different types of NPs, including Ag NPs, Cu_2O NPs, and SWCNT were observed in 3D A549 cells on a pillar insert system. The results showed that the suggested pillar insert system can be applied to evaluate various type of NPs.



a) Measurement of cell viability of nanoparticles in 3D A549 cells on a microchip



b) Representative image of measuring cell viability by staining 3D cells with calcein AM on a microchip

Key

X	concentration (μg/ml)	4	SWCNT
Y	cell viability (%)	5	concentration of SWCNT
1	Ag-Citrate	6	cell viability by calcein AM staining
2	Ag-PVP	7	6 replicates
3	Cu_2O	8	12 serial dose points

Figure 7 — Dose-response curves for cell viability in 3D-model A549 cells treated with NPs

The NPs were incubated with 3D A549 cells on a microchip for 24 h, and the cell viability was measured by calcein AM staining. The percentage of live cells was calculated according to NPs concentrations using the fluorescence intensity and area values as described previously[9]. The cell viabilities were normalized to the untreated sample, which was assigned a value of 100 %. The sigmoidal dose-response curves (variable slope) and $C_{i,50}$ values were obtained based on sigmoidal response fitting routines.

4.6 Cellular uptake of NPs using 3D model cells on a pillar insert

In the pillar insert system, the cells were encapsulated in alginate droplets, and the cells were cultured as 3D-model cells on the pillar insert. The encapsulated cells can be exposed to fresh growth medium, NPs, or staining dyes by simply transferring the tip of the pillar insert from one plate to another plate, where it is immersed, without damaging the 3D structure. To evaluate the cellular uptake of the test NPs through the alginate gel matrix, the pore size of the alginate was measured using scanning electron microscopy (SEM) analysis. As shown in Figure 8, the average pore diameter of the alginate was approximately 20 μm when dehydrated.

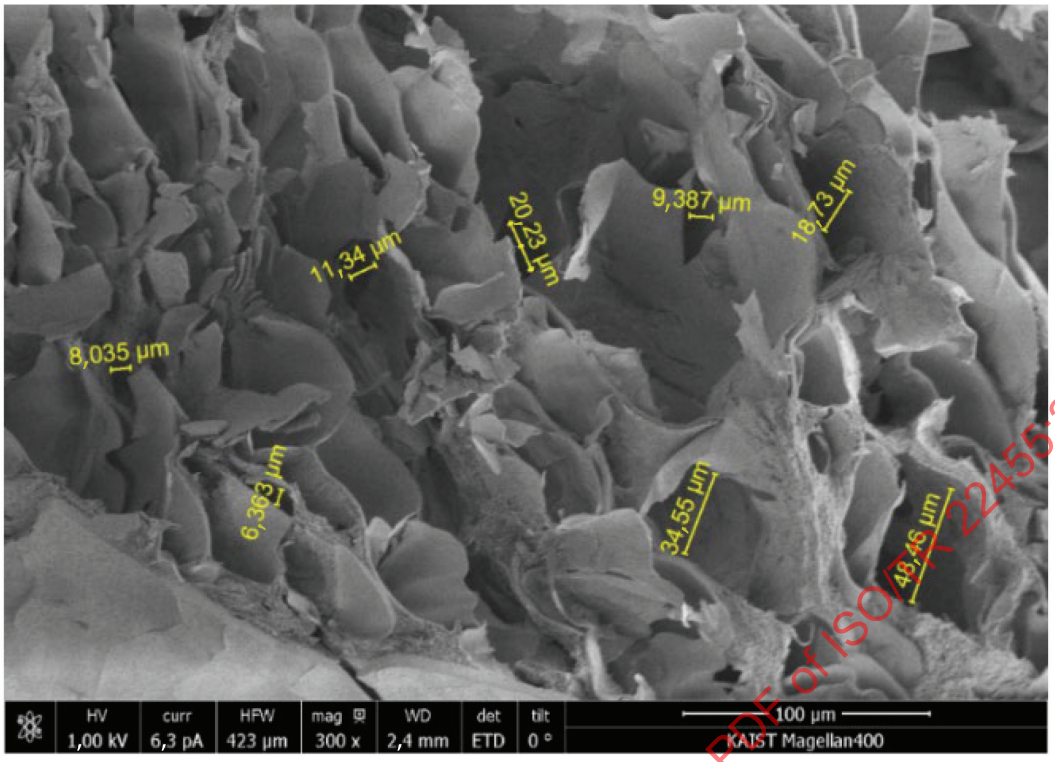
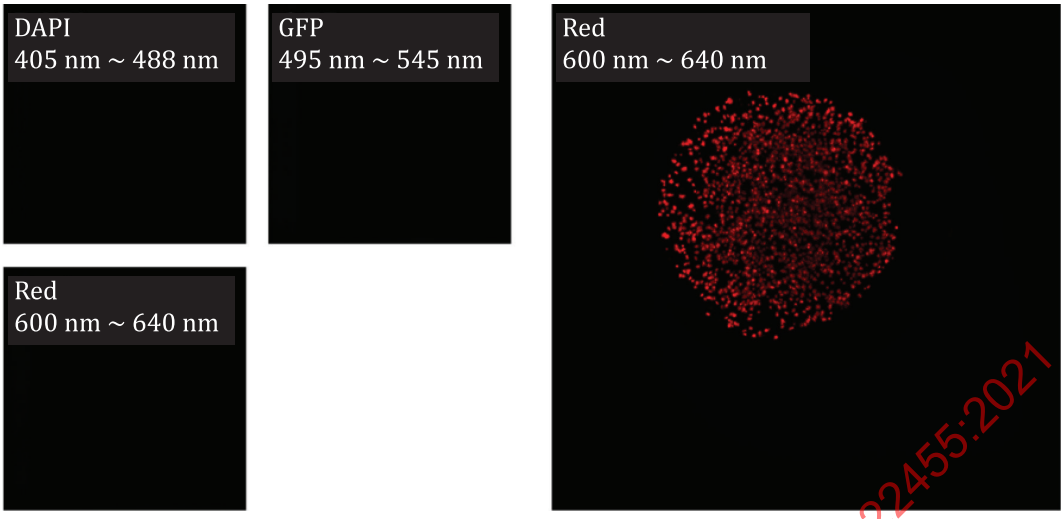


Figure 8 — SEM image of an alginate matrix

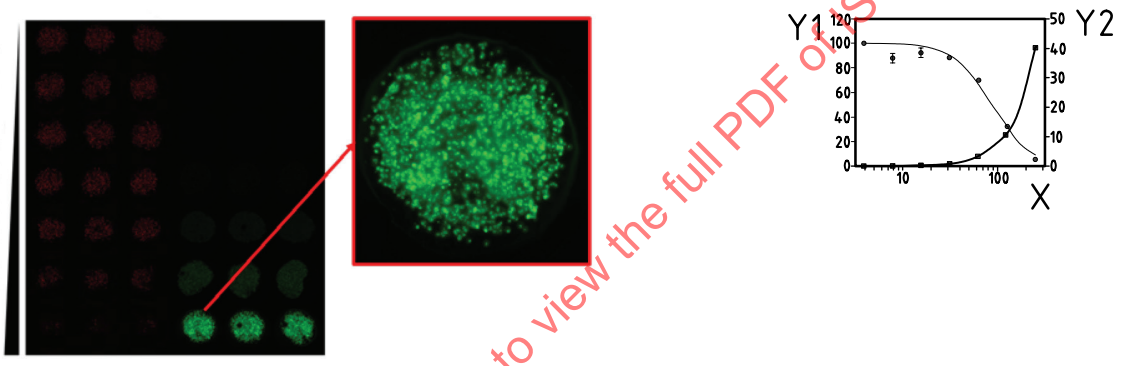
The morphologies and sizes of the porous hydrogel scaffolds were observed using a SEM instrument with a working voltage of 10 kV. The average pore diameter of alginate was measured to be approximately 20 µm, which is sufficient for NP access to immobilized cells.

Alginate is a polysaccharide that is widely used in industrial applications, including 3D-model cell culture[22]. At neutral pH values, alginate is polyanionic, and ionic alginate hydrogels can be formed after alginate chelates divalent cations. Alginate hydrogels enable cellular access to nutrients and removal of waste products[23]. The 20-nm SiO_2 was used to monitor the cellular uptake in the 3D BEAS-2B cells through the alginate hydrogel (see Figure 9). It was confirmed that there is no interaction between NPs and calcein AM. In the 3D culture on pillar insert, BEAS-2B cells encapsulated with alginate was treated with 20-nm SiO_2 and the fluorescence and TEM image was analysed. The fluorescence image showed the intracellular uptake of 20-nm SiO_2 through alginate hydrogel in 3D BEAS-2B cells. Intra-/inter- cellular uptakes of NPs can also influence the fluorescence detection depending on the types of NPs, and optical interferences of the remaining NPs can be considered to interpret the cell viability. As shown in Figure 9, a) cell viability was measured using calcein AM Red, the live cell staining dye, and there was no background fluorescence in the detection filter before calcein AM staining. If background fluorescence is detected by NPs treatment, further background subtraction can be considered to calculate the fluorescent intensity. TEM image showed that 20-nm SiO_2 were localized in cell organelles, and that SiO_2 seem to penetrate inside the cells. Overall, the results demonstrated that 3D BEAS-2B cells encapsulated with alginate hydrogel were able to uptake the NPs.

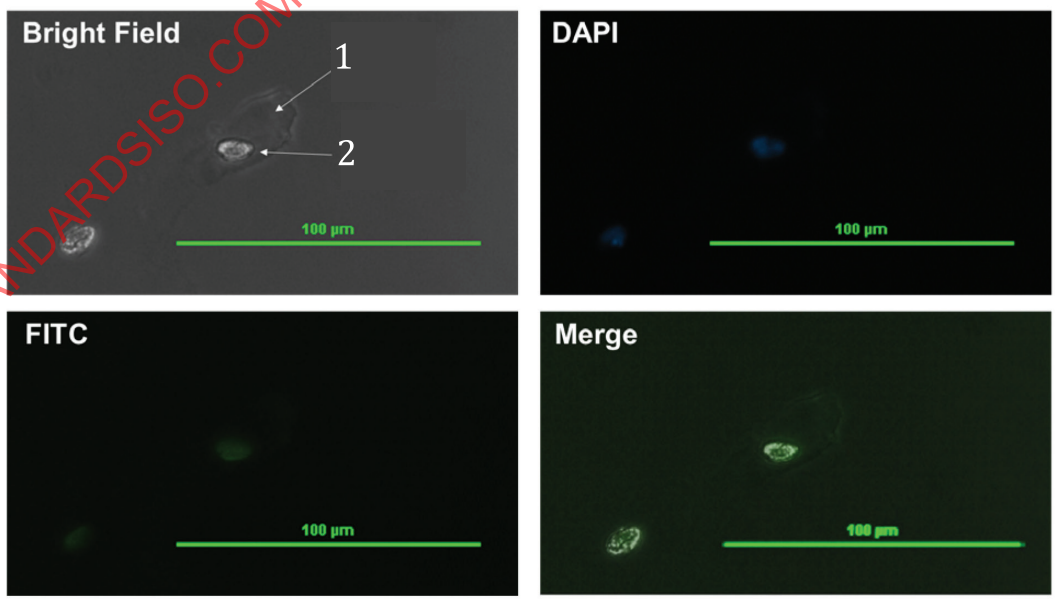
Before staining After staining (Calcein AM-Red)



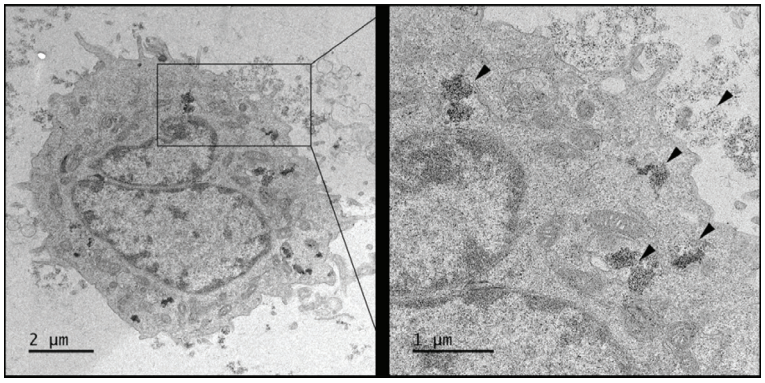
a) 3D BEAS-2B cells treatment with 20 nm SiO_2, followed by fluorescence measurement before and after calcein AM staining



b) Cell viability measurement by calcein AM staining and cellular uptake of NPs in 3D BEAS-2B cells encapsulated with alginate, measured by fluorescence image



c) Cellular uptake of 20 nm SiO2-FITC into microsectioned 3D BEAS-2B cells, detected by the bright field and fluorescence microscopy



d) Uptake and distribution of NPs in 3D BEAS-2B cells encapsulate with alginate, detected using TEM

Key

X	20 nm SiO_2-FITC (µg/ml)	2	BEAS-2B
Y1	cell viability (%)	●	cell viability
Y2	green intensity (a.u.)	■	20 nm SiO_2
1	alginate debris		

Figure 9 — Cellular uptake and intracellular localization of NPs by fluorescence image and TEM analysis (JEOL JEM1400 plus, at 120 kV)

20 nm SiO_2 was treated to 3D BEAS-2B embedded in alginate for 24 h. To confirm the interaction between NPs and calcein AM, 3D BEAS-2B cells were treated with 20 nm SiO_2 and then fluorescence was measured before and after calcein AM staining [see Figure 9, a)]. The 20 nm SiO_2-FITC was treated in 3D BEAS-2B cells on a microchip for 24 h. The cell viability was measured by calcein AM staining and cellular uptake of NPs in 3D BEAS-2B cells encapsulated with alginate measured by fluorescence image [see Figure 9, b)]. The 3D BEAS-2B cells were microsectioned, and cellular uptake of 20 nm SiO_2-FITC into the cells was detected by the bright field and fluorescence microscopy (see Figure 9, c)]. The uptake and distribution of NPs in 3D BEAS-2B cells encapsulated with alginate were detected using TEM [see Figure 9, d)].

4.7 Discussion of alternative strategies to evaluate in vitro toxicity testing of NPs

Conventional in vitro assays have limitations in evaluating the in vitro toxicity of NPs because of their physicochemical properties. It is important to assess the physicochemical properties of NPs based on reliable tests as described previously in ISO/TS 19337[24]. Assessing the physicochemical properties is also useful for interpreting the in vitro toxicity of NPs. If the NPs are well dispersed without optical interference, the conventional MTS assay, which has been described in ISO 19007[25] can be applied to evaluate the cell viability of NPs. However, it is necessary to establish an alternative testing strategy applicable to a wide range of NPs with various physicochemical characteristics. Here, alternative strategies are suggested to reliably test cell viability of NPs depending on their physicochemical properties (see Figure 10). For example, if there is optical interference from NPs, alternative methods such as impedance and pillar insert systems can be applied to measure cell viability. However, the impedance method developed in ISO/TS 21633[26] has limitations when sedimentation of NPs occurs. In the case of sedimentation, the effective dose for in vitro toxicity can be estimated using computational modelling such as in vitro sedimentation, diffusion and dosimetry (ISDD) model[27]. On the other hand, the insert pillar system allows changing the exposure direction in both of up-right and inverted exposure. This approach can give information about sedimentation of NPs effects on cell viability by comparing the cell viability from a different exposure direction, however, further experimental evidence is needed in the future. Collectively, this kind of strategic approaches can give a more reliable interpretation of the in vitro toxicity of NPs, and the standardization of the whole process can support the screening of potentially toxic NPs during the development of NPs in industrial fields.

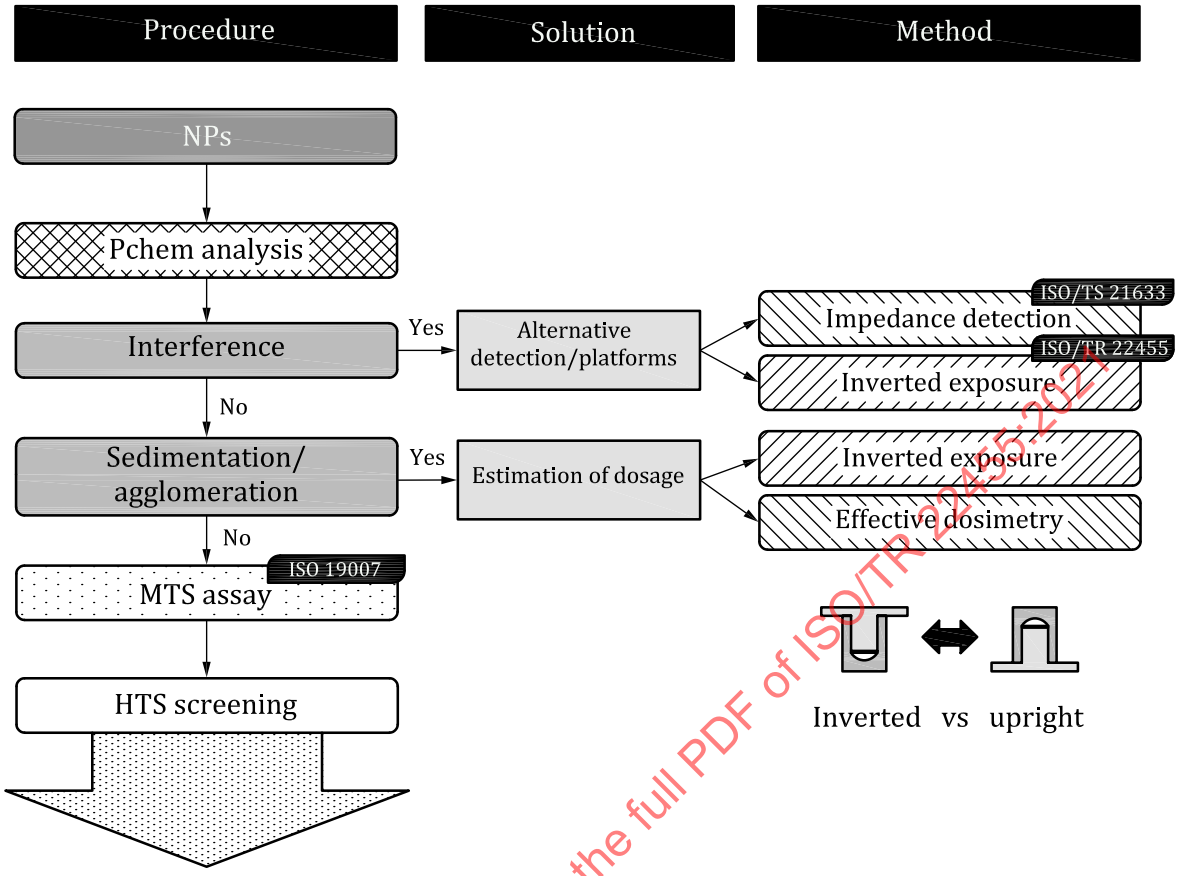


Figure 10 — Schematic flow of alternative strategies for evaluating the in vitro toxicity of NPs

5 Methods for cell viability screening of NPs using 3D-model cells

5.1 General

The information in Clause 6 illustrates through examples of actually performed testing, how 3D testing can be performed, and issues to consider.

5.2 Cell culture

For in vitro toxicity screening of NPs, the lung cells are preferred because the lung tissues are a main target airborne organ for NPs. Other cells such as hepatocytes and macrophages can be used, but the culture conditions are optimized according to the cell type. Initially, A549 and BEAS-2B cells were confirmed to generate the 3D-model cells in this approach and the cell preparation proceeds as follows. A549 and BEAS-2B cells were maintained in T-75 flask cell culture dishes using RPMI1640 (10 % fetal bovine serum and 1 % penicillin-streptomycin) and BEGM medium, respectively. Cells were maintained in a 37 °C, 5 % CO_2 incubator, and cells were subcultured every 2 d to 3 d. A suspension of cells was prepared by trypsinizing a confluent layer of cells with 1 ml of 0,05 % trypsin-EDTA from the culture flask and resuspending the cells in the culture medium. After centrifugation at 125 g for 5 min, the supernatant was removed, and the cell pellets were resuspended with culture medium to a final concentration of 6×10^6 cells/ml. For the different type of cells, culture duration and cell numbers can require different optimization to form the 3D characteristics[9]-[11].

5.3 Preparation of the pillar insert for in vitro screening

The pillar insert is made by plastic injection moulding and is a robust and flexible system for mammalian cell cultures, enzymatic reactions, viral infection, and compound screening. Polystyrene (PS), a widely

used biocompatible plastic, is used for the micropillars. For the 96-well-based system, a single pillar insert consisted of eight pillars, each pillar being 2 mm in diameter and 9 mm in height, and there was a 9-mm pillar-to-pillar distance, which rendered them compatible with the conventional 96-well plates. The cell encapsulation apparatus was a pair of grooves to help guide the pillar insert and a long and narrow trench (5 mm per width, 70 mm per length, and 0,8 mm per height) to accommodate reagents for encapsulating cells in a hydrogel. For the 532-well microchip system, the micropillar chip contained 532 micropillars (0,75-mm pillar diameter and 1,5-mm pillar-to pillar distance). The microwell chip also contained 532 microwells (with a 1,2-mm well diameter and 1,5-mm well-to-well distance). Both the micropillar chip and the microwell chip were similar to conventional microscopic glass slides in terms of size (75 mm × 25 mm). Before the pillar inserts and the cell-encapsulation apparatus were used, the pillar inserts and cell-encapsulation apparatus were immersed in 70 % ethanol for 30 min for sterilization, followed by complete drying at room temperature. A 2-µl poly-L-lysine (PLL)-$BaCl_2$ mixture was dispensed for surface coating the PS of the pillar. A PLL-$BaCl_2$ mixture was prepared by adding 0,1 mg of $BaCl_2$ to 10 ml of 0,01 % PLL. The PLL mixture was completely dried at room temperature before use. A plastic pillar insert is also commercially available, and it is readily applied to cell culture without the procedure mentioned above.

5.4 Encapsulation of cells on a micropillar chip to generate 3D-model cells

One millilitre of alginate (3 %) in distilled water was mixed with 1 ml of cell culture medium to make a 1,5 % alginate solution (cell culture or bioreagent grade). To culture cells on the pillar chip, 1 ml of cell suspension (6 × 10^6 cells/ml) was mixed with 1 ml of 1,5 % alginate solution. The alginate-cell mixture contained 3 × 10^6 cells/ml in 0,75 % alginate. For the 96-well system, 1,5 µl of the cell-alginate mixture was dispensed onto a pillar. Approximately 4 500 cells were encapsulated in a single spot of 0,75 % alginate per pillar. For the 532-well microchip system, 40 nl of the cell-alginate mixture was dispensed onto a microwell chip with 60 nl of dried 0,01 % PLL and 0,1 M BaCl2 mixture (2:1 volume ratio) using an automated chip spotter. Approximately 120 cells were encapsulated in a single spot of 0,75 % alginate per micropillar. After 1 min to 2 min of alginate gelation on a chilling deck at 4 °C, the pillar insert containing cell spots was laid on top of the microwell plate containing culture medium overnight before exposure to NPs.

5.5 NPs sample preparation

All solutions (except culture media), glassware, and relevant materials are sterile, and all procedures carried out under aseptic conditions and in the sterile environment of a laminar flow cabinet. Following the basic principle of sample preparation, NPs are dispersed in a biologically compatible fluid with a reproducible procedure. These procedures can include sonication and mixing by vortexing. Alternatively, NPs can be dispersed with biologically compatible chemical stabilizers, coatings such as albumin, or directly in the culture medium using the appropriate serum. Specific dispersion techniques were not discussed in this document. Details for dispersion can be found in the references cited in the notes and ISO/TS 19337. All NPs solutions were prepared fresh from stock solutions (e.g. 10 mg/ml). For cell culture experiments, this stock was agitated in a vortex mixer immediately before collecting a 20-µl aliquot in the middle of the stock tube and diluting it into 1,98 ml of complete cell culture medium. Approximately 2 ml (including excess) was required for each plate. A suggested method for preparing the NPs-cell culture medium preparation was to place 1,98 ml of complete cell culture medium in the bottom of a 5-ml sterile microtube. This procedure was performed in a hood or in a cleaned area that minimizes contamination of the cell culture medium with bacteria or fungus. Different concentrations of NPs were prepared with twofold serial dilutions to cover at least 6 to 12 dosing ranges.

5.6 Exposing 3D-model cells to NPs

Different concentrations of NPs were prepared with a twofold serial dilution containing at least six dosing ranges in 1,5-ml microtubes. After the prepared microtubes were gently vortexed, each dosing sample was dispensed into 96-well plates or 532-well microchip systems in at least triplicate, as shown in Figure 11. To introduce NPs to the microchip, NPs were dispensed onto the plate. After the prepared microtubes were strongly vortexed, each dosing sample was dispensed into 96-well plates or 532-well microchips in at least triplicate. The sandwiched chips were incubated in a 5 % CO_2 incubator at 37 °C