NANoREG

Grant Agreement Number 310584

Deliverable D 5.09
Summary report WP5

Author(s) and company:	NIA
Work package/task:	WP5
Document status:	Final
Confidentiality:	Public
Key words:	Categorisation, Read-across, Extrapolation, Intrapolation, QSAR

DOCUMENT HISTORY

Version	***Date***	***Reason of change***
1	20170421	First version (*in vitro* section)
2	20170424	Second version (HTS section added)
3	20170825	Final version (no changes from previous version)
4	20170830	Project Office harmonized lay-out
5	20170908	Final edits

This project has received funding from the European Union Seventh Framework Programme (FP7/2007-2013) under grant agreement no 310584

Lead beneficiary for this deliverable: NIA, partner 12 (main contact: David Carlander)

Owner(s) of this document	
Owner of the content	NIA, partner 12
Co-Owner 1	

Table of Content

1 Introduction

This report aims to provide a short overview of the main results and recommendations of the *in vitro* and *in vivo* correlation work in NANoREG as well as the main results and recommendations with High Throughput Screening (HTS) and High Content Analysis (HCA).

2 *In vitro* and *in vivo* work in NANoREG – The role of *in vitro* testing to support *in vivo* data, with reduced animal use

2.1 Summary

The benefits of *in vitro* over *in vivo* experimentation are well recognised. *In vitro* experimentation is easier to implement, more economically viable, free from ethical implications, able to use human cells directly and could be adapted into high-throughput methodologies and implemented into intelligent testing strategies. *In vivo* experiments remain, however, the gold standard in the field of toxicology.

The full potential of *in vitro* assays has yet to be realised in any sector, even where animal testing has been outlawed (cosmetics) but in sectors such as pharmaceuticals, *in vitro* testing is increasingly being recognised as a highly valuable tool to accelerate development, reduce costs and minimise animal use. Direct benefits include:

- Earlier stage identification and elimination of candidates with higher toxicity *in vitro* outcomes – the funnel effect.
- Ability to screen a broader range of candidates earlier, rather than proceeding on a narrow selection on cost basis, without any defining evidence for their selection over other candidates.
- Ability to refine and reduce the number of animals used and reduce likely adverse events, reducing cost, speeding process and ensuring higher quality of life (QoL) for animals within testing regimes.
- Ability of contract research organisations and other service providers to innovate and create new commercial opportunities, building additional sectors for European development.

In vitro testing for all sectors goes hand in hand with advances in other technologies, such as cell line, 3D tissue development and ‘organs on a chip’. It can be anticipated that as more validated tissue platforms emerge, the impact and value of *in vitro* testing will rapidly increase.

Within nanomaterial (NM) testing, *in vitro* technologies that have been assessed are able to rank NM by toxicity potential, although the following issues must be considered: cell lines should be chosen with care as they have different sensitivities towards NM exposure, more complex *in vitro* systems showed a better toxicity trend when compared with *in vivo* results (co-cultures ALI exposure) and further efforts should be taken into this direction, and care should be taken with potential interferences of NMs with detection methodologies. Even though *in vivo* studies were more sensitive, *in vitro* methodologies were able to rank NM by toxicological outcomes.

2.2 Suitable *in vitro* assays

NANoREG (D5.6) focused on the adaptation and harmonization of *in vitro* methodologies for genotoxicity and immunotoxicity (cytotoxicity, ROS and inflammation). The task used available *in vitro* assays and developed them further. The assays used included:

1) Adaptation of the current LAL assay to detect endotoxin in contaminated NMs. The proposed protocol worked well for all core materials but for nanotubes, which showed interference problems,
2) Modification of the human clonogenic assay to accommodate a more relevant cell line to represent the inhalation route,

3) Evaluation of standard cytotoxicity assays for the prediction of NM toxicity (MTS, Alamar blue, Neutral red, Colony Forming Efficiency (CFE),
4) Evaluation of standard genotoxicity assays for prediction of NM genotoxicity,
5) Relevance of dispersion protocols, selected cell lines and exposure times and
6) Relevance of ROS detection and evaluation of inflammation in a testing strategy.

The evaluation of genotoxicity, ROS and inflammation by the used assays, were in general reproducible among partners and unravelled cellular outcomes not observed by standard cytotoxicity methodologies (MTS, Alamar blue, Neutral red and CFE). *In vitro* methodologies were also useful to rank NMs by toxicity outcomes and could therefore be used as a first line of tools in a testing strategy.

An SOP for a long term Human Lung Cell Transformation Assay (hLCTA) was evaluated where cells were exposed for a maximum of 26 weeks to three different NMs.

2.3 Conclusions related to *in vitro* assays

Conclusions related to cytotoxicity assays:

- In general, there was good correlation between the different techniques for all NMs under study.
- Cells lines derived from different organs show different sensitivity towards NM exposure. It is therefore important, while designing experiments, to select the cell line which best represent the intended exposure route.
- The experimental procedures were properly harmonized and are robust enough to be widely used.
- Cell culture media composition directly affects physico-chemical state of the NM. It is therefore recommended that toxicity results are directly compared to physico-chemical state of NM. These finding may also be directly related to the cell media used.
- A limited effect of the dispersion procedure was observed for some of the tested NMs suggesting that dispersion procedure could modify the biological effect of some NMs. A detailed experimental dispersion procedure should be reported alongside toxicity evaluation since dispersion methodologies may be responsible for toxicological outcomes.
- Data suggest that the toxic effect of NMs could be cell type-dependent.
- Cytotoxicity experiments allow to rank NM by toxicity outcomes.

Conclusions related to genotoxicity assays:

- In general, most NMs were not genotoxic under the conditions of the study.
- In spite that Comet and micronucleus assays are able to detect different types of genetic lesions, a good correlation between results obtained using these two methods was found (with one exception).
- The Comet assay may represent a complementary test to the micronucleus assay. The Comet assay allows for high-throughput adaptations and could potentially be included in the battery of *in vitro* tests for genotoxicity assessment of NMs.
- Results from the Comet assay indicate that time points should be carefully taken into consideration when designing genotoxicity experiments. In particular, short time points such as 3 hours are important in the Comet assay, to understand initial steps provoking a genotoxic response and a potential recovery capacity from cells additionally from 24 hours treatment.

- Genotoxicity results indicate that 1) Two different times (3 h and 24 h) should be used in the Comet assay to assess early DNA damage followed by a potential recovery, 2) The use of Formamido Pyrimidine Glycosylase (FPG) in the course of the Comet assay allows the detection of oxidative damage to DNA which would otherwise be missed and 3) A small increase in concentration can have profound effects in terms of genotoxicity.

Conclusions related to inflammatory effects and immunotoxicity:

- It is mandatory to evaluate the potential interference of NMs with the assay, to exclude false negative or positive results. Interference may be evaluated by incubation of the NM with experimental reagents in the absence of cells.
- Inflammatory effects where usually observed at concentration levels lower than those observed for cytotoxic effects, highlighting effects at non-cytotoxic conditions.

2.4 Conclusions related to *in vitro* and *in vivo* correlation

From the experiments performed in NANoREG WP4 and WP5, several conclusions can be drawn regarding *in vivo* and *in vitro* correlations.

- *In vivo* approach appears to be the most sensitive and exhaustive one to assess absolute pulmonary toxicity of poorly soluble NM. *In vivo* approach remains at that time the reference strategy to characterise NMs immunotoxicity and genotoxicity.
- *In vitro* approach may provide valuable information regarding relative ranking of NM, provided that the cell model is sensitive enough (THP-1 cells were able to provide significant signals in response to NM exposure, both in monoculture or when co-cultivated with epithelial lung cells whereas monocultures of lung epithelial cells (A549 or BEAS- 2B) are poorly sensitive models).
- The mode of exposure must be considered. When cultivated and exposed to aerosols of poorly soluble NM at the ALI (Air-Liquid interface), cells show responses at lower doses compared to submerged exposure.
- *In vitro* experiments may contribute to prediction of toxic effects once knowledge on the intracellular effective dose is available for both cultures and tissues.

Although additional investigations would be useful to strengthen these conclusions, several key points should be considered before performing *in vitro* experiments, to improve the *in vitro* predictivity after acute exposure to poorly soluble NMs:

- Assessing the real mass of NM deposited on the cell surface *in vitro* is fundamental.
- Using compatible and relevant dose metrics between the *in vivo* and the *in vitro* is critical.
- It appears that more realistic cell models and exposure methods are important.
- It seems important to use similar timing of the dose delivery and exposure duration *in vitro* and *in vivo* to assess the acute toxicity of NMs using *in vitro* methods.

2.5 *In vitro* testing strategy

Currently, *in vitro* experimentation plays a minor role in regulatory safety assessment of chemicals. However, given the unlimited number of NMs which may be generated by one chemical entity, *in vitro* experimentation represent a feasible approach to generate relevant information for either 1) decision-making at early stages of product development, 2) waive animal experimentation and/or 3) provide further information for read-across and grouping.

The NANoREG WP5 strategy has focused on inhalation and the oral routes as the main exposure routes of entry into the body. The scheme below (Figure 1) indicates recommended cell lines per route of exposure, relevant end points and their corresponding assays. As a summary, cytotoxicity

is recommended as a first step to assess overall cellular damage and to obtain sub-toxic concentrations for further testing. Secondly, genotoxicity *in vitro* is a well described end point for drugs and chemicals, and, as such, it is also recommended in the scheme. The battery of recommended tests should cover all potential mechanisms of action, such as DNA damage, gene mutations and chromosomal damage. It is therefore proposed to perform the Comet assay, micronucleus assay, the mammalian gene mutation test (HPRT or TK mutations) and the cell transformation assay on Bhas42 (OECD no. 231).

Detection of ROS is also recommended under the proposed scheme given the higher reactivity of NMs compared to their larger-sized counterparts. Detection of ROS may also turn into a key parameter since ROS induction may be responsible for indirect genotoxicity (genotoxicity produced via ROS induction rather than by NM direct interaction with DNA). If genotoxicity is induced by ROS, it may indicate that it is NM dose dependent. Further investigations may then set a threshold value and so, under certain conditions, product development may continue. This may not be the case for direct genotoxicity, which may not be dependent on a threshold value and, under these conditions; product development may be stopped at early developmental stages.

From here, and depending on the results obtained and intended usage of final product, two further pathways may apply: 1) to investigate potential inflammatory outcomes and 2) to screen for potential physiological barrier penetration. Inflammation is a well reported endpoint for fibre-like and other materials, as such, a set of recommendations on assays is provided. On the other hand, physiological barrier models *in vitro* are still under development and further investigations apply to detection methodologies. Information collected from *in vitro* assays will result in preliminary hazard information which may allow to 1) flag a NM at early stages of product development, and/or 2) guide future *in vivo* experimentation based on mechanistic understanding/mode of action information collected by fast and economically feasible *in vitro* approaches.

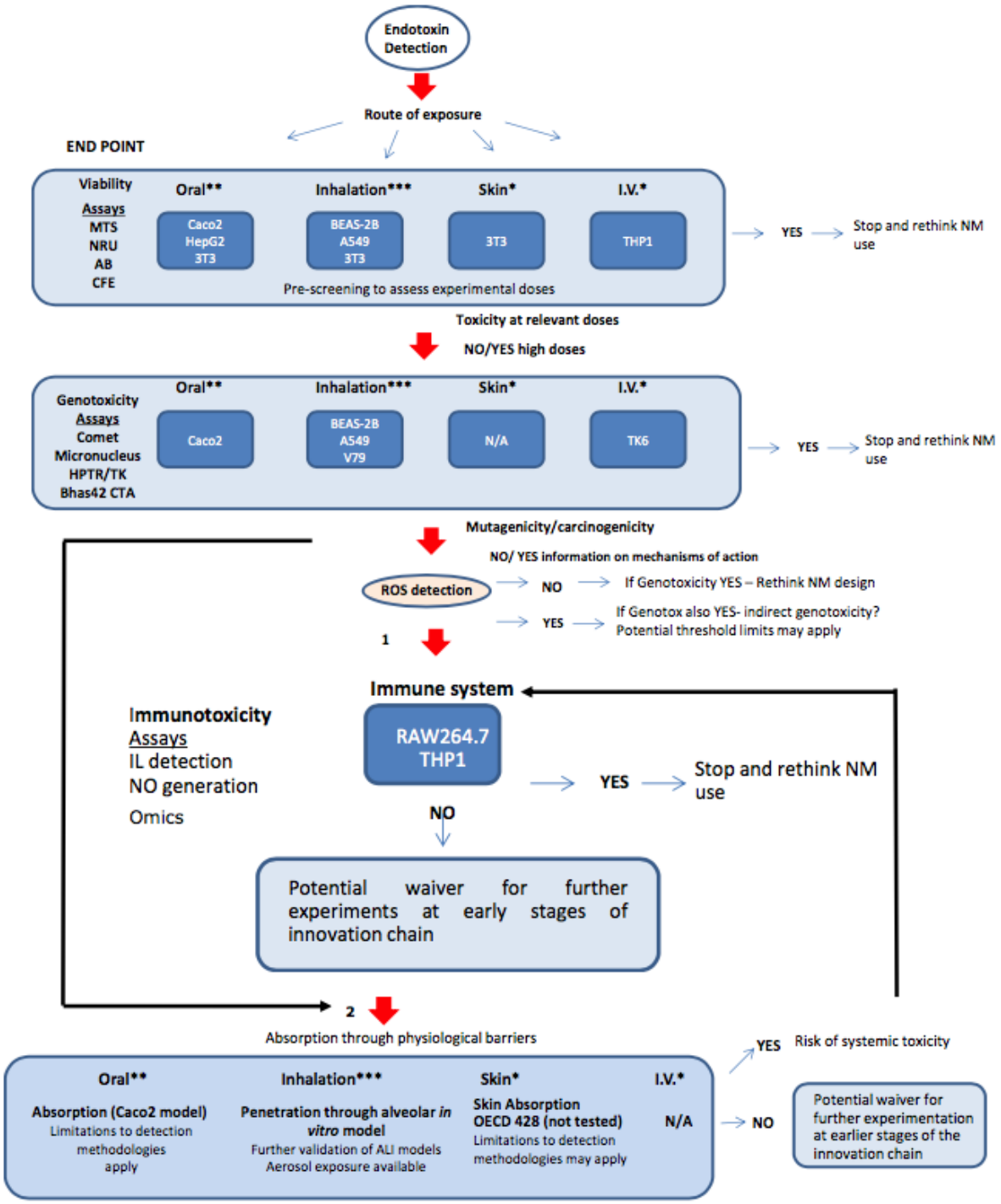

Figure 1: *In vitro* experimental scheme proposed under WP5 Task 5.5 at early stages of product development to collect information to guide further testing. *** High priority exposure route, ** medium priority exposure route, *low priority exposure route. I.V. indicates intravenous route. Dark blue squares indicate cell lines. N/A; not implemented under NANoREG (for further information, see NANoREG D.5.6).

2.6 Next steps to improve impact, commercial suitability and role of *in vitro* assays within nanomaterial testing

Europe has already invested significant funds in assays that could potentially reduce the use of animals within toxicity and other testing. This has resulted in a broad portfolio of assays, some of which are finding their way into industrial processes but for many, either have not been validated sufficiently to industry standards or stay within small scale use within the originating organisation. For *in vitro* assays to have a meaningful impact on industrial process and production of regulatory dossiers, the following steps are recommended:

Within assays specifically utilised within the NANoREG studies

- The development of more complex *in vitro* models, mimicking closer lung physiology and the reality of environmental exposure to assess pulmonary toxicity of low soluble NM should be a priority in the short term. The assays should take account of latest developments in 3D tissue modelling available from all sources, public and commercial.
- The long term Human Lung Cell Transformation Assay (hLCTA) should be further evaluated with additional NMs.

Within broader in vitro assay development

- *In vitro* assays and tissue models should be assessed from across sectors, specifically through those commercially provided through Clinical Research Organisations and currently used within the pharmaceutical sector to establish minimum criteria for assay delivery at commercial standards.
- Funding should be focussed towards validating an industry-selected set of *in vitro* assays in order to establish such assays within commercial use within the short term and where the assays are widely available for commercial development post-validation (pre-competitive research for public domain availability).

3 High Throughput Screening (HTS) for nanomaterials

3.1 Where is HTS/HCA used today

High Throughput Screening (HTS) and High Content Analysis (HCA) approaches can 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.

HCA approaches have been widely used for many years by the biotech and pharmaceutical industries in drug discovery and toxicity testing of extensive libraries of chemical compounds, and have accurately predicted the toxicity of novel compounds.

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.

3.2 NANoREG experience with HTS/HCA

Accurate design and planning of HTS for assessing the toxicity of NMs/NPs are essential (Figure 2). Adoption of automated and robotic liquid and sample handling is advisable since this will help to reduce systematic errors.

Technical challenges arise in HTS/HCA design, as toxicology screening needs to be coupled with characterization of NPs/NMs in the exposure medium. Characterisation is of necessity time-consuming and cannot be automated. This limitation is partially overcome if NMs, once characterised, 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 3 times with replicate samples within each data point (three repeats).

Further basic requirements are: a) clearly identified endpoints, b) assay-related as well as NM-specific positive and negative controls, c) toxicologically relevant (extracellular) concentrations of NMs, d) validated assays, e) multiparametric statistical analysis of data, e.g. using ANOVA with post Bonferroni analysis, or general linear models, f) well-designed graphical display of data (e.g. bar charts) and – in the case of multi-parametric datasets – various graphical plots to visualise associations between NP/NM exposure and different endpoints.

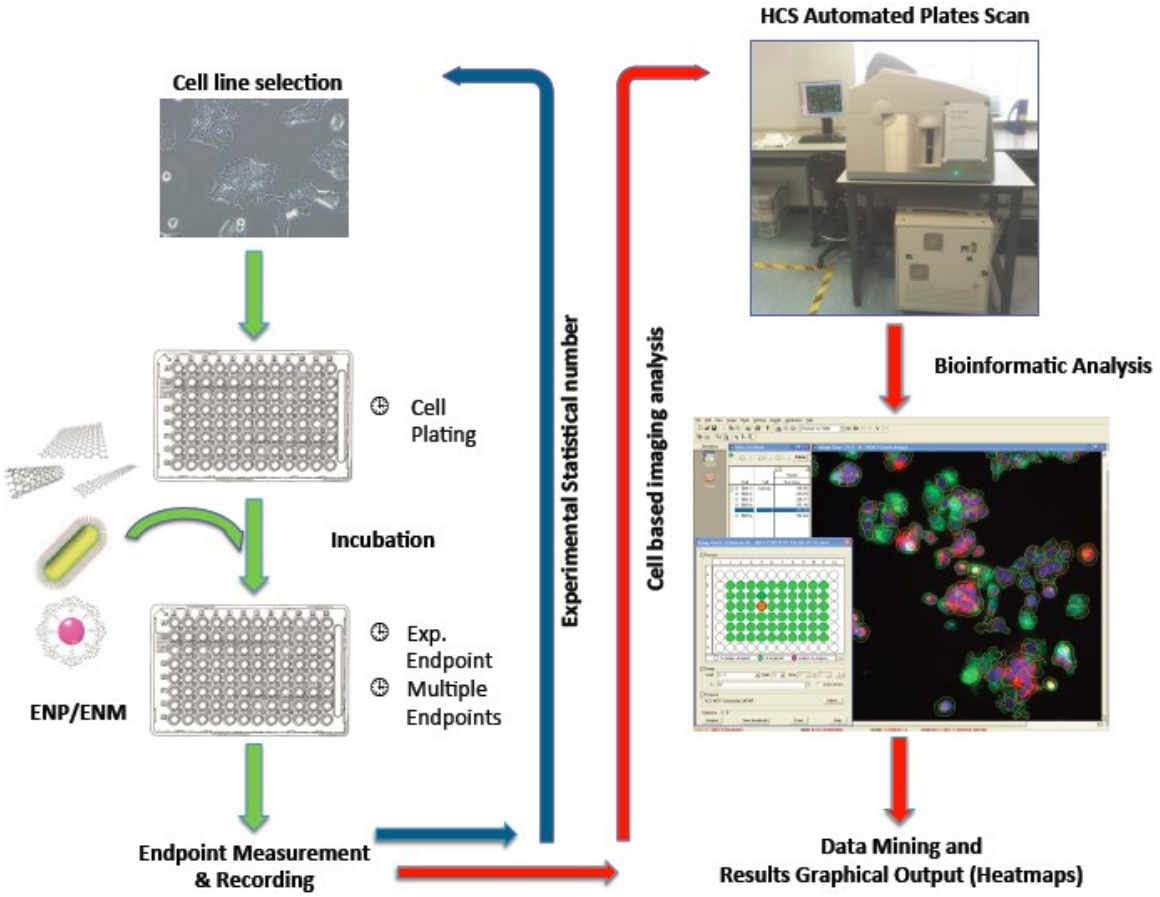

Figure 2. Experimental design for effective High-Throughput Screening

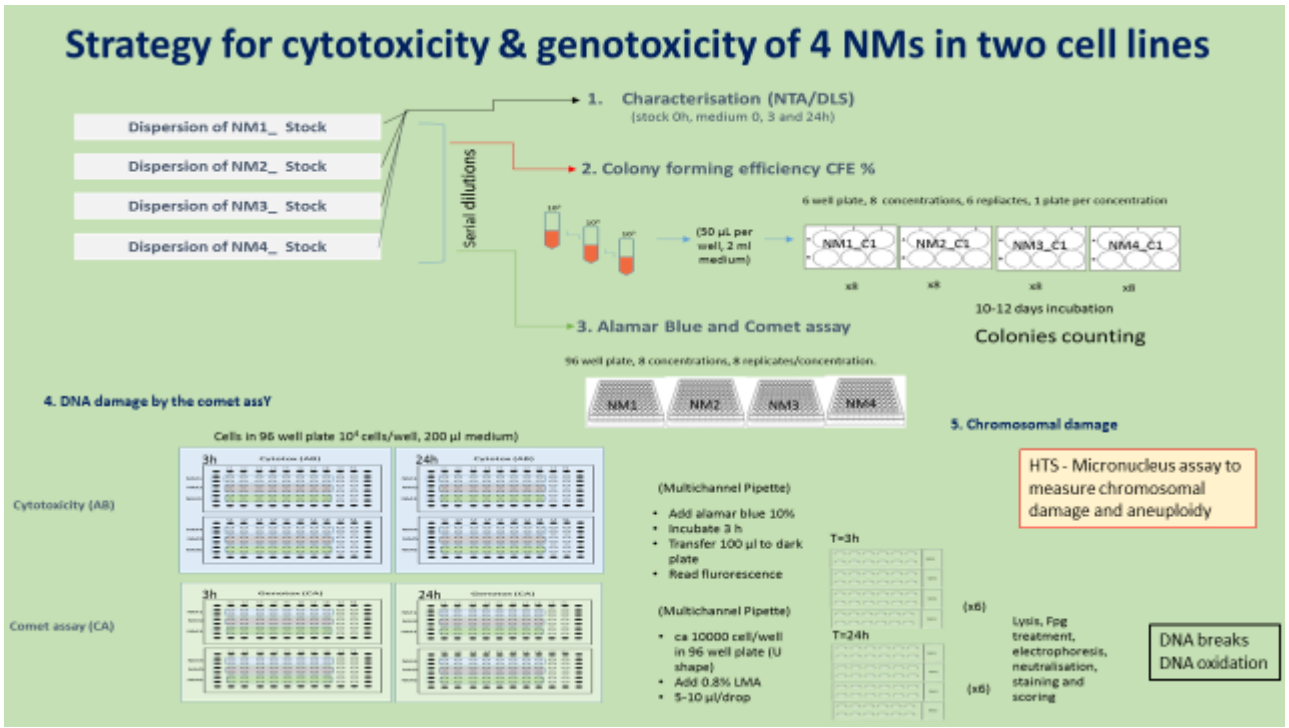

Figure 3: Suggested strategy with increased throughput for cytotoxicity (AlamarBlue and Colony Forming Efficiency – CFE) and genotoxicity (DNA damage by the Comet assay and Micronucleus assay by flow cytometry) for testing four NMs. Limitation is based on number of NMs that are possible to be tested thus throughput is increased with number of cell lines and time endpoints.

The experiments performed with reference NMs showed great potential for HTS/HCA methods with high capacity to test several dozen NMs per day (depending on type of HTS/HCA method) (Figure 3). However, this appeared not be practically possible due to the low throughput NM characterisation by the NANoREG guideline to always characterise NMs before, during and after the treatment of cells.

3.3 NANoREG HTS results

Preliminary evaluation of data shows that HTS/HCA approach has great potential and methods are reliable to test toxicity of NMs. However, methods for characterization are not yet developed to be coupled with HTS approaches.

The partners in task 5.6 tested 20 NMs with 10 NMs in common. All data are in ISA-TAB and uploaded in the NANoREG database. The data is still under evaluation and several publications are in progress to show comparisons between HTS methods versus standard method, comparison of several endpoints with same NMs, ranking of NMs and overall evaluation.

The HTS Comet assay can be considered reliable and useful for testing NM genotoxicity.

Preliminary comparison with standard assays show that both standard as well as HTS/HCA approaches are giving similar results.

- Several HTS/HCA methods have been established and adjusted for NM testing including Label-free cellular screening of NM uptake, HCA, HTS flow cytometry, Impedance-based monitoring, Multiplex analysis of secreted products, and genotoxicity methods – namely HTS Comet assay, HTS in vitro micronucleus assay, and γH2AX assay.
- Several methods have been improved and can be applied in hazard assessment of NMs (uptake, quantification, bioimpedance test, colony forming efficiency, micronucleus assay, HCA).
- The use of a microchip-based bioimpedance flow cytometry method (Ampha Z30) for nanotoxicity testing was successfully established. A microfluidic prototype for HTS impedance-based cell analysis of NM-toxicity was designed.
- It was shown that HTS/HCA methods are faster, robust, more economical. HTS/HCA methods are generally of higher quality and show lower variation.
- Preliminary cytotoxicity and genotoxicity ranking shows good concordance with standard approaches.

3.4 Next steps for HTS/HCA

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.

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.

Future perspective

- Main technical challenge and limitation is still low throughput of characterization methods. Progress in this field is urgently needed.

- The HTS/HCA methods can be further developed as ISO or OECD guidance documents
- Research towards new approaches in *in vitro* toxicity testing to develop *in vitro* methods that mimic *in vivo* situation is needed. The focus should be on miniaturization and on increasing the throughput.
- Chronic exposure models need further development also towards HTS.

4 References

This report is based on NANoREG Deliverables D5.05, D5.06 and D5.07.