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Combining Cytotoxicity Assessment and *Xenopus laevis* Phenotypic Abnormality Assay as a Predictor of Nanomaterial Safety

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The African clawed frog, *Xenopus laevis*, has been used as an efficient preclinical screening tool to predict drug safety during the early stages of the drug discovery process. *X. laevis* is a relatively inexpensive model that can be used in whole organism high-throughput assays whilst maintaining a high degree of homology to the higher vertebrate models often used in scientific research. Despite an ever-increasing volume of biomedical nanoparticles (NPs) in development, their unique physico-chemical properties challenge the use of standard toxicology assays. Here, we present a protocol that directly compares the sensitivity of *X. laevis* development as a tool to assess potential NP toxicity by observation of embryo phenotypic abnormalities/lethality after NP exposure, to in vitro cytotoxicity obtained using mammalian cell lines. In combination with conventional cytotoxicity assays, the *X. laevis* phenotypic assay provides accurate data to efficiently assess the safety of novel biomedical NPs. © 2017 by John Wiley & Sons, Inc.

Keywords: nanoparticles • nanotoxicity • physical-chemical characterization of nanoparticles • cytotoxicity • *Xenopus laevis* embryos

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INTRODUCTION

The research and application of biomedical nanoparticles (NPs) is a rapidly evolving discipline (De Jong & Borm, 2008). For many, it is believed that biomedical nanomaterials can act as advantageous tools in the treatment of several disease states. In particular, the unique physical-chemical properties of NPs make them an ideal therapeutic and diagnostic tool in oncology by overcoming the limitations of conventional therapies, as we have previously discussed (Bombelli, Webster, Moncrieff, & Sherwood, 2014). The main advantages of using biomedical NPs as drug delivery systems include targeted drug delivery, increased biocompatibility, and a decrease in drug toxicity, whilst maintaining or improving the therapeutic effect. However, as a result of the high

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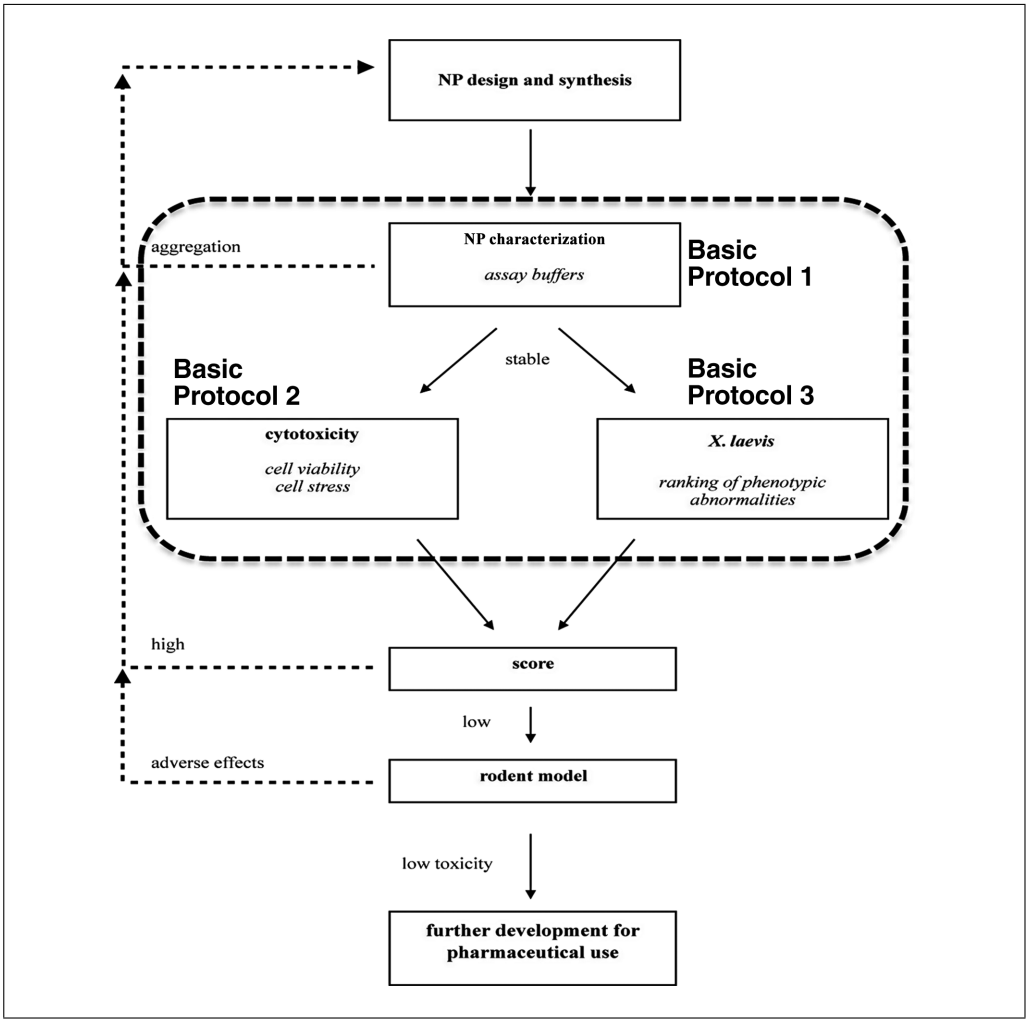


Figure 20.13.1 Flow diagram of proposed nanotoxicity screening protocol. This figure is adapted with permission from a previously published study from our group (Webster et al., 2016). Briefly, newly synthesized nanotherapeutics are firstly characterized in terms of their physical-chemical characteristics in biologically relevant media (Basic Protocol 1). Once identified as stable by this protocol, nanoparticles (NPs) are further assessed through an integrated approach of cytotoxicity analysis and phenotypic abnormality screening in *X. laevis* embryos (Basic Protocols 2 and 3, respectively). Here we provide detailed methodological descriptions of these three protocols as highlighted in the dotted box. Results from Basic Protocols 2 and 3 are then combined to provide a score that can indicate whether or not further in vivo nanotoxicity assessment should be made using mammalian models.

surface-area-to-volume ratio and complex composition of the nanomaterial, NPs can be highly reactive, where combinations of NP size, shape, material, and functionalization, can result in toxicity within biological systems (Lewinski, Colvin, & Drezek, 2008; Nystrom & Fadeel, 2012).

Conflicting information regarding NP safety for a given material can impede the progression of an NP from the early stages of formulation development through to the clinic. Inconsistencies in NP toxicity data are largely attributable to a lack of a standardized protocol for nanotoxicity assessment. Firstly, full characterization of an NP system (including size, surface charge, and stability in assay buffers) is required to understand the fate of the NP in a biological system and its potential to cause toxicity. Different early developmental models, such as *Xenopus* species (Bacchetta et al., 2014; Hu et al., 2016; Mouchet et al., 2008; Tussellino et al., 2015; Webster et al., 2016) and zebrafish (George et al., 2011; Liu et al., 2012; Rizzo et al., 2013), have been explored as systems that can

provide rapid, accurate, cost effective, and abundant data for NP toxicology assessment. *X. laevis* (the African clawed frog) is a species that produce large quantities of embryos allowing them to be used in a high-throughput style assay to gain toxicology data relatively quickly. Furthermore, with an individual embryo size at early developmental stages of ~1 mm, they are well suited for use in a multi-well format. *X. laevis* has the advantage of being evolutionarily closer to humans than other early models such as *Caenorhabditis elegans*, *Drosophila*, and zebrafish (Wheeler & Brandli, 2009). Although mouse models, the gold standard, are evolutionarily closer to humans than *X. laevis*, they are expensive and not a viable option to test numerous NPs over a wide range of concentrations, as far fewer embryos are produced compared to *X. laevis*.

Here we provide a detailed protocol for the use of *X. laevis* embryos in conjunction with cytotoxicity analysis, for highlighting potential NP toxicity by observing phenotypic abnormalities/lethality in response to NP exposure. *X. laevis* development is well documented (Nieuwkoop & Faber, 1967), making it easy to detect when toxicity-induced deviation from normal embryo development has occurred. The rationale for this approach has previously been described (Webster et al., 2016) and involves a combined assessment of cytotoxicity with *X. laevis* abnormality assessment in response to NP treatment, which offers a sensitive nanotoxicity model to bridge standard in vitro assessment alone with further rodent testing (Fig. 20.13.1). Specifically, this methodology incorporates physicochemical characterization of nanomaterials, followed by rapid cytotoxicity and *X. laevis* phenotypic abnormality assessment as an indicator of nanotoxicity prior to later testing in mammalian systems.

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) and must follow officially approved procedures for the care and use of laboratory animals.

PHYSICOCHEMICAL CHARACTERIZATION OF NANOPARTICLES (NPs)

BASIC PROTOCOL 1

This protocol describes the necessary steps to prepare nanoparticle (NP) dispersions suitable for toxicological characterization by cytotoxicity and *X. laevis* phenotypic scoring assays. This protocol is designed to be adaptable to different types of nanoformulations (thus it is not addressed to a specific typology of NPs), but is to be used for NPs dispersed in aqueous solutions. Physical-chemical characterization of NP dispersions is a critical step in a nano-safety assessment protocol (Azhdarzadeh et al., 2015). In particular, the experiments need to be performed not only in the NP dispersion medium, but also in the fluids in which the NPs will be dispersed during the biological assays. It is also important to monitor the colloidal stability of the NP dispersions over the duration of the nanotoxicity assessment period to detect any potential agglomeration effects over time (Cho et al., 2013). Generally, NP dispersions are commonly characterized in terms of hydrodynamic size of the particles through dynamic light scattering (DLS) measurements (Fig. 20.13.2). To better interpret DLS results it is also necessary to perform transmission electron microscopy (TEM) on the dried samples for evaluating the morphology and size of a single NP.

The presence of biomolecules (i.e., proteins) in the biological fluids affects the DLS results by producing a background signal, thus such experiments should be performed at a maximum protein concentration used in the nanotoxicity assessment protocols, e.g., 10% v/v serum used in cell culture growth medium (GM), but not in pure serum as the protein signal overcomes that derived from the NPs. Moreover, it has been shown that the presence of proteins or other biomolecules in the biological fluids affects the physical-chemical properties of the NPs through the formation of a protein corona around the NPs

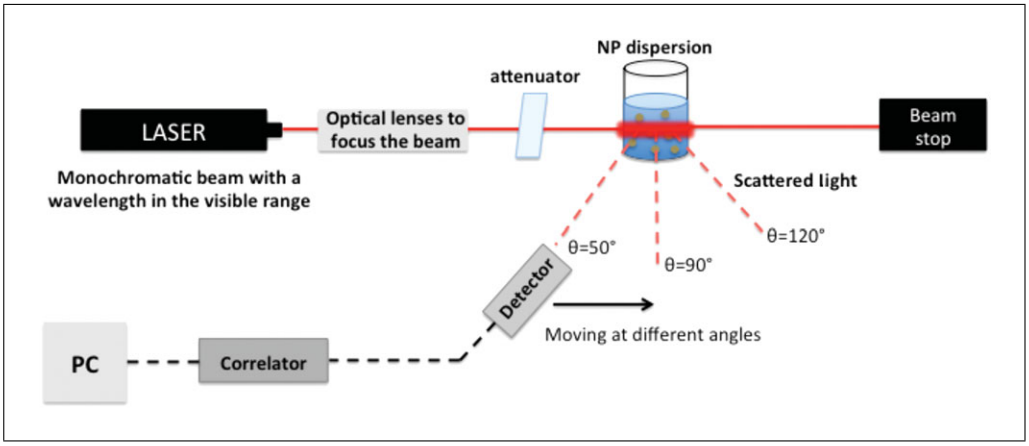


Figure 20.13.2 Schematic drawing of a dynamic light scattering (DLS) apparatus with a multi-angle detector. The equipment is composed of a monochromatic laser in the visible range, optical lenses to focus the beam on the sample, attenuator of the incident light, detector (equipped with a motor to move it at different angles with respect to the incident beam), correlator, and personal computer (PC) with specific software for the analysis of the raw data. The attenuator modulates the incident light to an optimal value that depends on the features of the detector. The detected scattered light reaches the correlator that builds an auto-correlation function of the scattered intensity for each angle. The auto-correlation functions and the raw signals (kcounts/sec) can be analyzed by the specific software provided by the supplier of the instrument.

(Cedervall et al., 2007; Monopoli, Aberg, Salvati, & Dawson, 2012). Thus, the analysis of DLS data in biological fluids can be more complex than in physiological buffer solutions. In fact, even if DLS is a good technique for testing the stability of NP dispersions in biological fluids, it does not give a quantitative estimation of the size of such complexes (as it cannot distinguish among dimer, trimer, or agglomerates of protein-NP complexes). For this purpose it would be necessary to implement the NP characterization with different analysis such as differential centrifugal sedimentation (Walczyk, Bombelli, Monopoli, Lynch, & Dawson, 2010) or fluorescence correlation spectroscopy (Rocker, Potzl, Zhang, Parak, & Nienhaus, 2009), which is beyond the scope of this protocol.

Materials

Nanoparticle (NP) stock dispersions (concentrations and nanomaterials tested are to be pre-determined by the experimenter)
PBS (see recipe)
Growth medium (GM) for mammalian cells, containing supplements (e.g., FBS, amino acids, antibiotics, as required depending on chosen cell types; GM details for specific lines are provided by the supplier or in the scientific literature; all reagents must be cell culture grade)
0.1× Marc's Modified Ringer's (MMR) solution (see recipe)

Disposable dynamic light scattering (DLS) cuvettes
Dynamic light scattering apparatus

1. Prepare DLS cuvettes: Clean them with autoclaved Milli-Q-purified H_2O (d.H_2O) and then dry with particular care to protect them from dust.

2. Transfer NP dispersions to the DLS cuvettes (necessary volume depends on the DLS apparatus) and dilute them if necessary; the solvent used to dilute the NP dispersions must be dust free as much as possible.

   For the purposes of the protocol described here, NP samples need to be dispersed in GM (for mammalian cell culture work) and also in MMR (for X. laevis work), and hydrodynamic size compared to NPs dispersed in PBS or dH_2O.

IMPORTANT: *Never touch the middle-bottom part of the cuvettes with hands, but rather manipulate them using their upper edge.*

The choice of the optimal concentration for DLS measurements should be based on both experimental and technical considerations. A concentration that is similar, as much as possible, to those used in the biological assays should be chosen (usually the most concentrated dose used in vitro is the safest choice to detect possible NP agglomeration).

We recommend a quick test for evaluating the averaged scattered intensity of the chosen dilution be run; the count should be above 20 kcounts/sec to be statistically significant. If it is lower than that value, a more concentrated sample should be prepared.

3. Set temperature to the desired value.

   In this context these temperatures will be 37°C for mammalian and 12° to 23°C for X. laevis experiments.

4. Allow cuvette to rest in the sample holder for ~10 min before measurement of the NP dispersion, to enable the sample to reach the desired temperature and allow the dust to settle.

5. Measure scattered intensity at a set angle of detection.

   Generally, the most commonly used apparatus can measure the scattered intensity at a fixed angle (either 90° or 173°), but there are also more advanced instruments that permit multi-angle detection, in that case it is better to measure the scattered intensity at different angles (Fig. 20.13.2). The detected signal will be automatically sent to the correlator, which produces the auto-correlation function of the scattered intensity $g_2(q,t)$ for each angle (Eqn. 20.13.1):

$$g_2(q,\ t) = \frac{\langle I^*(q,0)I(q,t)\rangle}{\left\langle I(q,0)^2\right\rangle}$$

Equation 20.13.1

where:

$$q = \frac{4\,\pi n}{\lambda}\sin(\theta/2)$$

is the scattering vector (with θ the detection angle, λ the wavelength of the incident light, and n the solvent refractive index).

6. Analyze auto-correlation functions to extract the NP hydrodynamic size using available analysis software.

   The analysis of the auto-correlation functions at each angle gives a decay rate $\Gamma(s^{-1})$ related to the NP dynamics and related to the translational diffusion coefficient, D, through the following equation for Brownian systems (Eqn. 20.13.2):

$$\Gamma(s^{-1}) = D \cdot q^2$$

Equation 20.13.2

Thus reporting the decay rates versus the scattering vectors, the slope of the obtained curve is the translation diffusion coefficient. The NP hydrodynamic radius, r_H, can be determined through the Stokes-Einstein relationship (Eqn. 20.13.3):

$$D = k_B T/6\pi\rho r_H$$

Equation 20.13.3

where T is the experimental temperature and ρ the viscosity of the solvent.

IMPORTANT: *The fitting analysis of the auto-correlation functions for determining the decay rates must be carefully chosen. If the auto-correlation function is monomodal (the sample is mostly composed of a single population of NPs of the same size), a Cumulant method (Koppel, 1993) can be used. This fitting analysis gives an averaged $<\Gamma>$ together with a polydispersity index (PDI). If the PDI is <0.2 to 0.25, it is reasonable to use this method. If the PDI is >0.25 the sample is either very polydispersed or composed of two or more populations and an alternative method must be used. The most common is the algorithm CONTIN, based on the Laplace transform of the auto-correlation function. This method gives a size distribution of the NP dispersion, distinguishing different NP populations differing in scattered intensities of at least 1:10^{-5}. For monomodal polydisperse samples, the two methods should provide comparable results.*

SUPPORT PROTOCOL 1

TRANSMISSION ELECTRON MICROSCOPY FOR NANOPARTICLE CHARACTERIZATION

As highlighted in Basic Protocol 1, a TEM study should be done on the NP stock dispersion for evaluating NP morphology and better interpreting DLS results. TEM analysis allows the determination of the size of single NPs that can be used for understanding the NP size distribution obtained by DLS and highlight possible agglomeration effects. TEM equipment is comprised of complex instrumentation and usually a dedicated person(s) is/are responsible for its maintenance and running experiments in a core facility within institutions. Thus, here we only describe a protocol for preparing samples to be measured by TEM. It is necessary to prepare a dispersion of the NPs in d.H_2O as the sample has to be dried (measurements are performed in vacuum) and salt crystallization can occur if the NPs are dispersed in buffer affecting the experiment. If the NP stock is dispersed in buffer, it is also possible to wash the sample directly on the grid.

Materials

Nanoparticle (NP) stock dispersions (concentrations and nanomaterials tested are to be pre-determined by the experimenter)

Transmission electron microscopy (TEM) grid (chosen material depends on NP material and specifics of apparatus and manufacturer)
Transmission electron microscopy (TEM) instrument with imaging modality

1. Wash grid with a suitable clean solvent as indicated by the supplier (the choice of solvent depends on the material of the grid).

   IMPORTANT: *Never touch the grid with hands, but rather use suitable tweezers.*

2. Transfer NP dispersion onto the grid by multiple depositions of 5 to 10 µl. After each deposition, let solvent evaporate before adding the following drop.

   If it is necessary (i.e., if the NPs are dispersed in salt solutions) wash the grid with d.H_2O to eliminate the salts as this operation should not remove the NPs, which are adhered to the grid surface.

   A rough calculation of the amount of NPs transferred to the grid should be done to evaluate the number of depositions necessary to reach the minimum amount of sample needed to obtain a statistically significant measurement.

3. Leave grid to dry overnight, ideally under a hood and protected from dust.

4. Perform measurement taking pictures of different areas on the grid.

5. Take and save several images for each grid (sample). To determine a size-distribution, analyze images with specific image software that allow the extraction of size information.

IMPORTANT: *To be statistically meaningful the size-distribution must be done on at least 100 NPs.*

TEM size is often 10% smaller than the hydrodynamic size that also includes the hydration layer.

CYTOTOXICITY ASSESSMENT OF NANOPARTICLE TREATMENT

BASIC PROTOCOL 2

A crucial part of our nanotoxicity protocol is cytotoxicity assessment in mammalian cells, as due to their unique material composition, some nanoformulations can have harmful toxic effects in mammalian systems. Multiple factors can influence the extent of nanomaterial toxicity such as NP size, morphology, chemical structure, and surface chemistry (Caballero-Diaz & Valcarcel Cases, 2016). A wide variety of conventional in vitro assays are available to assess nano-cytotoxicity, for example, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT), which is a commonly used cytotoxicity assessment assay that has been successfully used to detect nanotoxicity (Gulati, Rastogi, Dinda, Saxena, & Koul, 2010; Hussain, Hess, Gearhart, Geiss, & Schlager, 2005; Park, Yi, Kim, Choi, & Park, 2010; Schubert, Dargusch, Raitano, & Chan, 2006; Webster et al., 2016; Yuan, Liu, Qian, Wang, & Zhang, 2010) and provides a simple, reproducible, and reliable test setup. In addition to MTT, nanotoxicity in mammalian cells can be evaluated by a variety of other cytotoxicity assessment methods including 2′,7′-dichlorofluorescein (DFC) assay, proinflammatory cytokine ELISA, TUNEL, trypan blue exclusion assay, (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; MTS), CellTiter-Glo, adenosine triphosphate luminescence, alamar blue (resazurin assay), neutral red staining, lactate dehydrogenase content analysis, phosphatidylserine translocation monitored by Annexin V staining, mitochondrial membrane potential, and apoptotic protein level/activity, to name several.

Depending on their specific NPs and experimental conditions, users can select cytotoxicity methodologies to suit their needs, as some nanoformulations can affect cytotoxicity readings by certain experimental approaches (Belyanskaya, Manser, Spohn, Bruinink, & Wick, 2007; Davoren et al., 2007; Hillegass et al., 2010; Monteiro-Riviere, Inman, & Zhang, 2009; Wang, Yu, & Wickliffe, 2011). Two or more cytotoxicity protocols need to be employed to ensure that the nanotoxicity assessment is robust, which ideally should test more than one of the following cytotoxicity assessment parameters: Oxidative stress, cell death, cell viability, and inflammatory response. Table 20.13.1 provides a list of conventional cytotoxicity assessment assays and examples of NPs that are compatible with these methods. Here we describe a protocol that we have previously used for NP cytotoxicity assessment to analyze cell viability using two methods: MTT and trypan blue exclusion assay (Support Protocol 2), and cell death by assessing apoptotic markers (Support Protocol 3).

Materials

Mammalian cell lines of choice (use a minimum of 3)
Growth medium (GM) for mammalian cells containing supplements (e.g., FBS, amino acids, antibiotics, as required depending on chosen cell types; GM details for specific lines are provided by the supplier or in the scientific literature; all reagents must be cell culture grade)
70% ethanol (Sigma-Aldrich)
0.05% (w/v) trypsin-EDTA solution (cell culture grade; Sigma-Aldrich)
Mycoplasma testing kit (we use EZ-PCR mycoplasma test kit; Gene Flow)
Nanoparticle (NP) exposure solution (concentrations and nanomaterials tested are to be pre-determined by the experimenter)
3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) solution (Sigma-Aldrich; prepared according to the manufacturer's instructions)

Dimethyl sulfoxide (DMSO; Sigma-Aldrich)
Sørensen's buffer (see recipe)
PBS (see recipe)

Liquid N_2 cryogenic cell storage Dewar flask (for long-term storage of cell stocks; Cole-Palmer)
Water bath (set to 37°C; Thermo Fisher Scientific; add an anti-microbial agent to the water tray to limit contamination)
Class II biological safety cabinet (Monmouth Scientific)
Sterile, disposable cell culture plastic ware (e.g., flasks, plates, tubes, tips; for adherent cells, flasks and plates must be cell culture grade)
Humidified 37°C, 5% CO_2 cell culture incubator (New Brunswick; add an anti-microbial agent to the water tray to limit contamination)
Inverted light microscope (Olympus)
Swing-out (bucket) centrifuge (Eppendorf)
Automated cell counter (e.g., Bio-Rad TC20) or Neubauer hemocytometer (Merck Millipore)
Multichannel pipet (Thermo Fisher Scientific)
Microplate spectrophotometer reader (SpectraMax)

1. Resuscitate mammalian cells from cryopreservation. Grow according to recommendations for the chosen cell lines, using good lab practice (GLP; see *APPENDIX 3B*, Phelan & May, 2016).

   Correct handling and GLP for cell culturing involves the use of aseptic technique to avoid contamination of the cultures (Freshney, 2011). Furthermore, cells should be used at low passage numbers (<25) to avoid genetic drift and lines should be validated, and checked for contaminants prior to experimental use.

   Three or more cell lines should be selected by the experimenter to assess nanotoxicity. The selection of these lines should be based upon the predicted exposure routes of the nanomaterial being assessed. For example, we have previously assessed iron oxide NP cytotoxicity in cell lines that represent possible exposure tissues or sites of NP accumulation in humans, i.e., lung epithelium (A549), skin (SK-MEL-28), and kidney epithelium (MDCK), and that are easy to grow (Webster et al., 2016).

   Supplementation of GM with antibiotics is optional. If it is used we recommend 100 μg/ml penicillin/streptomycin.

   IMPORTANT: *GM is prepared in advance and can be used for several weeks if stored at 4°C. It should be pre-warmed to 37°C using a water bath prior to use on the cells to avoid cold shock. Water baths are a source of contamination in cell culture facilities and therefore should be regularly checked and cleaned, and an anti-microbial agent added to the water.*

   IMPORTANT: *Maintenance and preparation of mammalian cell lines should be conducted in a class II biological safety cabinet and 70% ethanol used to sanitize all reagents, and plastic ware used in the hood. All reagents must be prepared under aseptic conditions.*

   IMPORTANT: *Like water baths, cell culture incubators represent another source of potential contamination. They too should be regularly checked, cleaned, and a non-toxic anti-microbial added to the water tray.*

2. Trypsinize and seed cells at 4500 cells/well in a 96-well, flat-bottomed plate in triplicate (as a minimum for experimental replicates). Incubate cells overnight in cell culture incubator.

   IMPORTANT: *Due to the edge effect on cell culture plates, conditions in the outer-most wells can lead to assay variability. We recommend not using the outer-most wells and rather only add GM or PBS to them.*

Table 20.13.1 Examples of Commonly Used Materials in Nanoformulations for Biomedical Applications and Their Compatibility with Different Cytotoxicity Assay Methods[a]

Nanoparticle (NP)-based materials	Compatible cytotoxicity assay	Reference
Aluminium	MTT	Hussain et al., 2005
	NRRT	Braydich-Stolle et al., 2005
	LDH	Hussain et al., 2005
Cadmium	LDH	Braydich-Stolle et al., 2005
	MTS	Braydich-Stolle et al., 2005
Cerium	ATP	Colon et al., 2009
	MTT	Schubert et al., 2006
	NRRT	Lanone et al., 2009
	GSH	Schubert et al., 2006
Cobalt	MTT	Lanone et al., 2009
Copper	NRRT	Hu et al., 2014; Lanone et al., 2009
Curcumin	Apoptotic protein detection by immunoblotting	Dhule et al., 2012
Gold	NRRT	Tedesco et al., 2008
	Apoptotic protein detection by immunoblotting	Patra et al., 2007
	PI	Patra et al., 2007
	MTT	Connor et al., 2005; Patra et al., 2007
	$\Delta\psi_m$	Wang, Liu, et al., 2011
Hydroxyapatite NP (HAPN)	MTT	Yuan et al., 2010
	Annexin V	Yuan et al., 2010
	Apoptotic protein detection by immunoblotting	Yuan et al., 2010
Iron	Annexin V	Berry et al., 2004
	MTT	Webster et al., 2016; Hussain et al., 2005
	Trypan blue	Webster et al., 2016; Rodríguez-Luccioni et al., 2011
	LDH	Hussain et al., 2005
Manganese	MTT	Hussain et al., 2005
	LDH	Hussain et al., 2005
Nickel	MTT	Lanone et al., 2009
Poly(*N*-isopropylacrylamide)-co-poly(ethylene glycol), PNIPAAM-PEG based NP	NBT	Gulati et al., 2010
Silica	SRB	Lin et al., 2006
	LDH	Lin et al., 2006

continued

Table 20.13.1 Examples of Commonly Used Materials in Nanoformulations for Biomedical Applications and Their Compatibility with Different Cytotoxicity Assay Methods[a], *continued*

Nanoparticle (NP)-based materials	Compatible cytotoxicity assay	Reference
	DCFH-DA	Lin et al., 2006
	GSH	Lin et al., 2006
	MDA	Lin et al., 2006
Silver	PI	Park et al., 2010; AshaRani et al. 2009
	Annexin V	AshaRani et al. 2009;
	LDH	Braydich-Stolle et al., 2005; Hussain et al., 2005
	DCFH-DA	AshaRani et al., 2009; Hussain et al., 2005
	GSH	Hussain et al., 2005; Park et al., 2010
	MTS	Braydich-Stolle et al., 2005
	NBT	Guo et al., 2008
	ATP content	AshaRani et al., 2009
	$\Delta\psi_m$	Hussain et al., 2005; Teodoro et al., 2011
Sodium	LDH	Braydich-Stolle et al., 2005
	MTS	Braydich-Stolle et al., 2005
Tungsten	LDH	Hussain et al., 2005
Yttrium	GSH	Schubert et al., 2006
Zinc	PI	Reddy et al., 2007

[a]Abbreviations: MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide; NRRT, neutral red retention time assay; LDH, lactate dehydrogenase; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium; ATP, adenosine triphosphate; GSH, glutathione; PI, propidium iodide; $\Delta\psi_m$, mitochondrial membrane potential; NBT, nitro blue tetrazolium chloride; SRB, sulforhodamine B assay; DCFH-DA, 2′,7′-dichlorofluorescin diacetate; MDA, malondialdehyde.

For non-adherent, suspension cells, treated samples should be collected, spun down, resuspended in a fresh medium and treated with MTT solution.

3. Wash cells with PBS (enough to cover the monolayer) and add NPs at the desired concentration in GM at a volume of 150 µl/well. For the control wells add 150 µl/well GM alone. Incubate cells 72 hr.

   IMPORTANT: *Careful pipetting technique must be used whilst washing, removing, and adding GM to the cells. For adherent cells, disturbance of the monolayer can dramatically affect the assay results.*

4. Following incubation with the NPs, remove treatment medium and wash cells twice with PBS. Prepare fresh medium: 50 µl MTT (2 mg/ml) in d.H_2O, added to a total volume of 250 µl/well and incubate plate a further 4 hr.

   During this time the cells can be checked for the development of formazan crystals (formed through the reduction of tetrazolium salts), which appear as an intracellular purple precipitate.

5. Carefully remove MTT solution to leave the insoluble formazan precipitate. Add 200 μl DMSO/well and 25 μl Sørensen's buffer/well. Mix gently to resuspend formazan crystals.

   From this point onwards the experiment does not need to be conducted using aseptic technique.

   IMPORTANT: *During mixing, avoid the production of air bubbles that could otherwise affect the optical absorbance readings.*

6. Remove plate cover and measure absorbance of each well at 570 nm wavelength using a microtiter plate reader for optical absorbance.

7. Calculate percentage cell viability as a ratio of mean absorbance from the replicates with respect to the control treatments, using the following formula (Eqn. 20.13.4):

$$\% \text{ cell viability} = (I_{sample}/I_{control}) \times 100$$

Equation 20.13.4

where I = absorbance intensity.

TRYPAN BLUE EXCLUSION ASSAY

SUPPORT PROTOCOL 2

As highlighted in Basic Protocol 2, more than one cytotoxicity assay should be employed to determine nanotoxicity in mammalian cells. Here we describe the use of trypan blue exclusion assay to support the findings from MTT analysis (see Basic Protocol 2). Trypan blue determines the number of live and dead cells depending on the principle that intact plasma membranes exclude the dye, whereas damaged or dead cells do not (Avelar-Freitas et al., 2014). Mammalian cell stocks for this assay are maintained and prepared using GLP as described above (Basic Protocol 2, step 1; see *APPENDIX 3B*, Phelan & May, 2016).

Additional Materials *(also see Basic Protocol 1)*

Mammalian cell lines of choice (use a minimum of 3; see Basic Protocol 1 for a detailed list of equipment and reagents required for growing mammalian cell lines)
Nanoparticle (NP) exposure solution (concentrations and nanomaterials tested are to be pre-determined by the experimenter)
0.05% (w/v) trypsin-EDTA solution (cell culture grade; Sigma-Aldrich)
0.4% trypan blue solution (Sigma-Aldrich)

1. Trypsinize and seed mammalian cells at 20,000 cells/well in a 24-well, flat-bottomed plate in triplicate (as a minimum number of replicates). Incubate cells overnight in a cell culture incubator.

2. Gently wash cells with PBS (enough to cover the monolayer) and add NPs at the desired concentration in GM at a volume of 500 μl/well. For the control wells, add 500 μl/well of GM alone. Incubate cells 72 hr.

3. Following incubation with NPs, gently wash cells twice with PBS and use 100 μl/well trypsin-EDTA to detach cells from the well. Mix 10 μl cell suspension 1:1 with 0.4% trypan blue solution. Incubate 2 min at room temperature.

   Trypan blue should be stored in a dark bottle at room temperature and filtered with a 0.2-μm filter if used after prolonged storage.

4. Count unstained (viable) and stained (non-viable) cells. Calculate cell viability using the following two equations (Eqns. 20.13.5 and 20.13.6):

$$\% \text{ cell viability} = (\text{unstained cells/total cells}) \times 100$$

Equation 20.13.5

$$\% \text{ non-viable cells} = (\text{stained cells/total cells}) \times 100$$

Equation 20.13.6

SUPPORT PROTOCOL 3

IMMUNOBLOTTING FOR APOPTOTIC MARKERS

Immunoblotting (or Western blotting) is a molecular technique used to detect proteins in a complex milieu. Following extraction from cells, proteins are separated (usually by sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SDS-PAGE) and then immunoblotted by transferring the proteins to a solid substrate and proteins of interest detected using antibodies targeted against them. Here we describe a protocol using immunoblotting to assess apoptotic cell death in response to NP treatment. A variety of markers can be used to assay apoptosis, should reduced cell numbers be detected in Basic Protocol 2 or Support Protocol 2 (e.g., cleaved caspase 3, 8, and 9; Puma; Noxa; and p7056K). Here we describe the use of cleaved poly (ADP-ribose) polymerase-1 (PARP1) as a read-out of apoptosis. During this type of cell death, caspase/protease-mediated cleavage of PARP1 in fragments of 89 and/or 24 kDa is a useful and easily detectable apoptotic hallmark (Kaufmann, Desnoyers, Ottaviano, Davidson, & Poirier, 1993). This protocol is adapted from immunoblot protocols used in our previous work (Jenei et al., 2009; Webster et al., 2016).

Additional Materials *(also see Basic Protocol 1)*

Mammalian cell lines of choice (use a minimum of 3; see Basic Protocol 1 for a detailed list of equipment and reagents required for growing mammalian cell lines)
Nanoparticle (NP) exposure solution (concentrations and nanomaterials tested are to be pre-determined by the experimenter)
Cisplatin or other cytotoxic agent (used as a positive control in cell lines of choice; agent and dose should be pre-determined for each cell line selected)
PBS (0.1 to 0.5 liter, cooled to 4°C; see recipe)
Lysis buffer (containing protease inhibitors, cooled to 4°C; see recipe)
Pierce BCA Protein Assay kit (Thermo Fisher Scientific)
Dithiothreitol (DTT; Sigma-Aldrich)
SDS (Sigma-Aldrich)
4× loading buffer (see recipe)
Tris·Cl buffers (pH 8.8 and pH 6.8; see recipes)
40% acrylamide/bisacrylamide (Sigma-Aldrich)
Ammonium persulfate (APS; Sigma-Aldrich)
Tetramethylethylenediamine (TEMED), >99.5% (Sigma-Aldrich)
10× running buffer (see recipe)
10× transfer buffer (see recipe)
Tris-buffered saline/Tween 20 (TBST; see recipe)
Ponceau S solution (see recipe)
ECL Western blotting detection reagent (GE Healthcare)
Blocking solution (see recipe)
Methanol
Mouse anti-PARP1 antibody (F-2; Santa Cruz Biotechnology, cat. no. SC-8007)

Mouse anti-α-tubulin (DM1A; Cell Signalling Technology, cat. no. 3873)
Anti-mouse Horseradish-peroxidase (HRP)-conjugated secondary antibody (Cell Signaling Technology, cat. no. 7076)

Plastic cell scrapers (Thermo Fisher Scientific)
1.5-ml Eppendorf microcentrifuge tubes (Thermo Fisher Scientific)
Sonicator (e.g., Diagenode Bioruptor Pico Ultrasonicator; Thermo Fisher Scientific)
−20°C freezer
UV-Vis Spectrophotometer (Orion AquaMate 8000; Thermo Fisher Scientific)
Dry block heating system
Mini-gel tank and associated casting plates, combs, and related items (Mini-PROTEAN Tetra Vertical Electrophoresis Cell; Bio-Rad)
Protein molecular weight standards (range: 6500 to 205,000 Da; Thermo Fisher Scientific)
Gel-loading tips (range: 0.5 to 200 µl; Thermo Fisher Scientific)
Universal power supply (PowerPac; Bio-Rad)
Polyvinylidene difluoride (PVDF) membrane (Thermo Fisher Scientific) or nitrocellulose membrane (Thermo Fisher Scientific)
Bent-tip stainless-steel forceps (Thermo Fisher Scientific)
Sponge pads and filter paper (for blotting; Invitrogen)
Shaker plate/roller
ChemiDoc XRS+ system (Bio-Rad)
Image analysis software (ChemiDoc Touch, Bio-Rad)

Protein preparation from mammalian cells

1. Trypsinize and seed mammalian cells at 1×10^6 cells/10 cm diameter Petri dish (cell culture grade) and incubate cells overnight in a cell culture incubator.

2. Gently wash cells with PBS (enough to cover the monolayer) and add NP or control treatments at the desired concentration in GM, at a volume of 5 to 10 ml/plate. Incubate cells 72 hr.

   A positive control (pro-apoptotic drug) treatment should be used to ensure the detection of apoptosis in the cell type of choice.

3. Remove GM and wash cells twice in ice-cold PBS (enough to cover the monolayer). Remove PBS and add 300 µl/plate ice-cold lysis buffer. Using a cell scraper (chilled to 4°C), scrape cells off the dish then gently transfer the resulting lysate in a pre-cooled microcentrifuge tube.

   IMPORTANT: *This step should be carried out on ice. From this step onwards keep all fractions and reagents used on ice throughout.*

4. Sonicate sample 15 to 30 sec, typically 20 to 50 kHz.

   At this frequency, sonication ensures complete cell lysis and shears the DNA to reduce sample viscosity.

5. Centrifuge lysate at 4°C, 20 min at 16,000 × *g*. Gently aspirate supernatant containing the protein extract and store in fresh cold tubes.

   At this point samples can be stored as aliquots at −20°C. Avoid repeated freeze-thawing as this can reduce sample integrity.

6. Determine protein concentration using the Pierce BCA Protein Assay kit (Thermo Fisher Scientific) according to the manufacturer's protocol, or using a similar technique (e.g., the Bradford assay; Bradford, 1976).

Table 20.13.2 12% SDS-PAGE Mini-Gel Recipe[a]

12% resolving (lower) gel	5% stacking (upper) gel
d.H_2O: 3.45 ml	d.H_2O: 2.9 ml
40% acrylamide/bisacrylamide: 2.4 ml	40% acrylamide/bisacrylamide: 0.75 ml
1.5 M Tris·Cl, pH 8.8: 2 ml[b]	0.5 M Tris·Cl, pH 6.8: 1.25 ml[b]
10% SDS: 80 µl	10% SDS: 50 µl
10% APS: 80 µl	10% APS: 50 µl
TEMED: 8 µl	TEMED: 5 µl

[a]Abbreviations: APS, ammonium persulfate; d.H_2O, Milli-Q-purified H_2O; SDS, sodium dodecyl sulfate; TEMED, tetramethylethylenediamine.
[b]Adjust pH of Tris·Cl buffer accordingly.

Perform SDS-PAGE

7. Prepare 10 to 25 µg total protein by adding DTT at a final concentration of 0.1 M 1% SDS in loading buffer (four times stock volume) to a total volume of 10 to 25 µl/sample. Denature samples at 90°C, 10 min.

   DTT functions as a reducing agent to reduce disulfide bonds, whilst SDS functions as an anionic denaturing detergent.

   IMPORTANT: *Wear gloves at all times when handling SDS-PAGE gels, as acrylamide is a potent, cumulative neurotoxin and probable human carcinogen.*

8. Assemble SDS-PAGE gel tank system and add 1× running buffer to the top. Carefully load protein in the desired sequence and load protein markers according to the manufacturer's instructions.

   Alternative gel tank systems are available from different manufacturers, so follow the assembly instructions for each different apparatus accordingly.

   Prepare the gel (Table 20.13.2) the same day or the day before (storing overnight in running buffer at 4°C). Alternatively pre-cast gels can be purchased.

   Careful loading is critical to avoid sample spill over between adjacent gel lanes. We recommend using gel-loading tips to prevent spill over.

9. Using gel electrophoresis, separate proteins in a 12% SDS-PAGE resolving gel, overlaid with a 5% stacking gel (Table 20.13.2). Run protein separation at 90 V through the stacking gel and 120 V through the resolving gel.

10. Once the proteins are fully resolved, dismantle SDS-PAGE apparatus. Carefully remove gels from the casting plates, remove stacking gel, and discard. Keep resolving gel moist in transfer buffer, whilst preparing for immunoblotting.

Perform immunoblotting

11. Pre-soak nitrocellulose transfer membrane in 1× transfer buffer 5 min.

    If using a PVDF membrane, pre-soak in 100% methanol.

    Membrane handling should be kept to a minimum and only use membrane forceps when manipulating to reduce background staining.

12. Prepare transfer sandwich as previously described (Gallagher, Winston, Fuller, & Hurrell, 2008). Briefly, sandwich gel and membrane between layers of pre-soaked filter paper/blotting sponges (in 1× transfer buffer) in a transfer cassette, ensuring tight contact between the gel and membrane. For tank blotting, assemble transfer

sandwich in the gel tank and perform protein transfer in 1× transfer buffer at 4°C, ensuring the membrane faces the anode.

IMPORTANT: *Avoid air bubbles between the gel and membrane as this can lead to poor protein transfer. Using a clean pipet to roll over the membrane when assembling the transfer sandwich can easily remove bubbles.*

Alternatively protein transfer can be done using semi-dry blotting apparatus. These systems should be used according to the manufacturer's instructions.

13. Once protein transfer is complete, carefully dismantle transfer sandwich. Wash membrane twice in 1× TBST (enough to cover the membrane) 5 min on a shaker plate/roller.

14. Stain membrane with Ponceau S solution (enough to cover the membrane) 1 min, to visualize proteins and ensure complete transfer (protein bands will stain red). Then wash stain away with d.H_2O several times with agitation until all the Ponceau S solution is removed from the membrane.

15. Block membrane 1 to 2 hr at room temperature with agitation in blocking solution (containing 5% milk; enough to cover the membrane).

    5% BSA can also be used as a blocking reagent and, for alternative antibodies to the ones suggested here, should be used as recommended for individual antibody clones.

16. Dilute anti-PARP-1 antibody in enough blocking solution to cover the membrane and incubate with the membrane overnight at 4°C with constant gentle agitation.

    We standardly use a 1/200 dilution, but this will require optimization for individual cell types to determine the optimal antibody/protein ratio.

17. Wash membrane three times in 1× TBST 10 min each at room temperature with constant agitation.

    This step is required to remove any unbound antibody.

18. Add cognate secondary antibody diluted in blocking solution 1 hr at room temperature with gentle agitation.

    Use the secondary antibody at a minimal dilution of 1/2500, although this will require optimization for the cell types used.

19. Repeat step 17. Incubate membrane with ECL reagent (according to the manufacturer's instructions) and detect chemiluminescent signal using the desired imaging system (e.g., the ChemiDoc XRS+ system; Bio-Rad). Use image analysis software to analyze protein band intensity.

    X-ray film (with or without automated developing) is also a commonly used method for signal detection.

20. Rinse membrane in methanol and then repeat step 17. Dilute anti-α-tubulin antibody in enough blocking solution to cover the membrane and incubate with the membrane overnight at 4°C with constant gentle agitation.

    Detection of α-tubulin in the cells is used as a loading control. The choice of a loading control can be modified depending on the cell type used and the size of the protein(s) of interest being detected by immunoblotting.

21. Repeat steps 17 through 19.

    Determining the ratio between the cleaved PARP-1 (89 kDa) and full-length PARP-1 (116 kDa) bands relative to the gel loading control can be used as a readout for caspase-mediated apoptosis.

X. LAEVIS PHENOTYPIC ABNORMALITY ASSAY FOR NANOTOXICITY ASSESSMENT

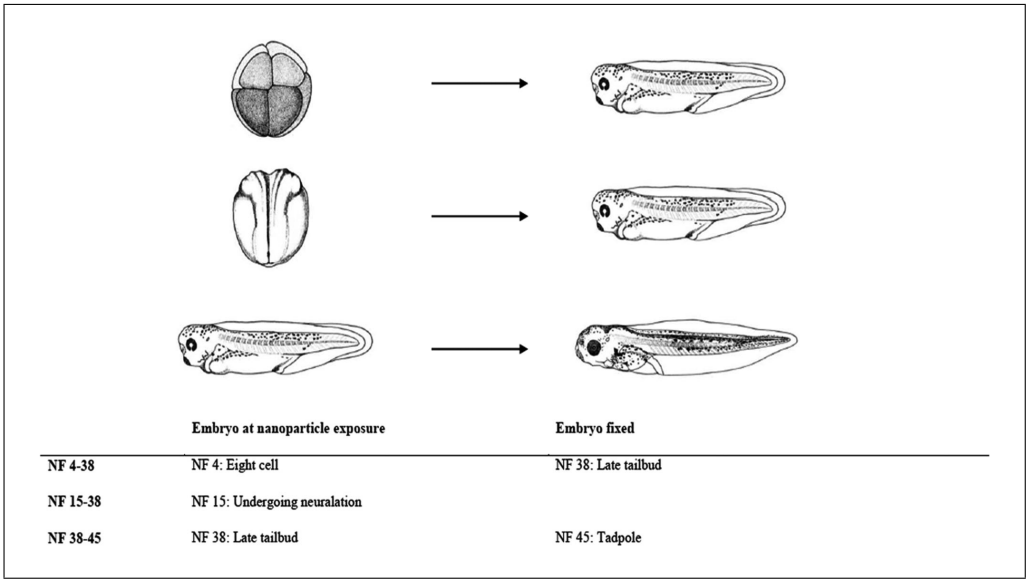
This protocol is designed to be used in parallel with cell-based cytotoxicity assays as part of an integrated toxicity assessment in order to obtain a complete safety profile of a novel NP (Fig. 20.13.1). *X. laevis* is an ideal model organism to be used for comparatively high-throughput screening (Tomlinson, Rejzek, Fidock, Field, & Wheeler, 2009) and has been used as a toxicity model in the frog teratogenesis assay-*Xenopus* (or FETAX assay) for drugs in their early stages of drug safety evaluation (Leconte & Mouche, 2013). This is largely due to *X. laevis* being a relatively inexpensive and rapid model that can be easily scaled up as a large number of embryos can be produced. *X. laevis* embryos develop externally, making them an easily accessible system for exposure to NPs. Previous work has shown that this methodology allows both external NP exposure and internal exposure to key internal organs for assessing potential toxicity (Webster et al., 2016). Briefly, *X. laevis* embryos are exposed to an NP-containing incubation solution over a desired developmental period that can be adapted depending on the specific aims of the nanotoxicity assessment protocol.

Materials

Nieuwkoop and Faber (NF) stage 1 *X. laevis* embryos (see Support Protocol 4)
0.1× Marc's Modified Ringer's (MMR) solution (see recipe)
Nanoparticle (NP) exposure solution (concentrations and nanomaterials tested are to be pre-determined by the experimenter)
Ethyl 3-aminobenzoate methanesulfonate (0.6 mg/ml; see recipe)
MEMFA fixative (see recipe)
PBS (see recipe)
PBS/Tween 20 (PBST; see recipe)
2% (w/v) agarose gel (see recipe)
Methanol (analytical grade; Sigma-Aldrich)
25%, 50%, and 75% (v/w) methanol (analytical grade; Sigma-Aldrich) in PBS
Gentamycin (25 µl/ml; optional)

Pasteur pipet (we recommend glass; whole embryos are too large to fit into a standard pipet, therefore mark the end with a diamond pen, break off cleanly and fire the end briefly to melt any sharp edges; alternatively, plastic Pasteur pipets can be used with the end removed)
10-cm^2 Petri dish (Thermo Fisher Scientific)
Culture incubator (set to desired temperature; see below for details)
Stereomicroscope with two-armed fiber optic illuminator (to allow the angle of illumination to be easily adjusted)
Dumont #5 forceps (stainless steel; ultrafine and can be used for carefully manipulating embryos throughout the protocol; Sigma-Aldrich).
24-well culture plate (non-cell culture grade; Thermo Fisher Scientific)
3-cm^2 Petri dish (Thermo Fisher Scientific)
Long-handled scalpel (10 A blades)
Light microscope with charge coupled-device (CCD) digital camera (for whole-mount imaging of embryos)
Glass vials with screw caps (3.5 ml; SGL)
Parafilm M wrapping film (Thermo Fisher Scientific)
−20°C freezer

1. Harvest NF stage 1 *X. laevis* embryos (see Support Protocol 4) and incubate 12° to 23°C until required developmental stage is reached (Fig. 20.13.3).



	Embryo at nanoparticle exposure	Embryo fixed
NF 4-38	NF 4: Eight cell	NF 38: Late tailbud
NF 15-38	NF 15: Undergoing neuralation	
NF 38-45	NF 38: Late tailbud	NF 45: Tadpole

Figure 20.13.3 Suggested *X. laevis* Nieuwkoop and Faber (NF) stages for nanoparticle (NP) exposure. Schematic depicts *X. laevis* embryos at different developmental NF stages that have been selected for treatment to assess nanotoxicity (Webster et al., 2016). Embryo physiology images (Nieuwkoop & Faber, 1967) depicted above the line, with their associated NF staging description provided below the line. Images not to scale. The selected NF stages for NP exposure provide analysis of two critical teratogenic assessment stages: Gastrulation through to neuralation (NF 4-NF 38) and neuralation alone (NF 15-NF 38), and at stages that can more accurately represent an adult system during organogenesis (NF 38-45).

During the incubation times it is important to regularly observe the embryos (at least twice daily or more at early stages) to remove any dead embryos and ensure the correct NF stage has been reached.

Developmental times of embryos are dependent on incubation temperature and culturing them at differing temperatures can speed or slow development. Typically, after incubation at 23°C, embryos are NF stage 4 after ~2 hr, NF stage 15 after ~17 hr, and NF stage 38 after incubation for ~ 2 days 5 hr.

IMPORTANT: *Bacteria grow well at the higher incubation temperatures so embryos cultured at 18° to 25°C should be regularly monitored and washed twice daily. To avoid this problem, the 0.1× MMR culture medium of later stage embryos (NF stage 23 onwards) can be supplemented with 25 μg/ml of gentamicin.*

2. In a 24-well plate, add 200 µl NPs in 0.1× MMR solution to each well at a concentration that is ten times higher than that of the desired final concentration. For the control wells, add 200 µl 0.1× MMR alone.

3. At the required NF stage, select five healthy embryos and transfer into 1800 µl 0.1× MMR using a Pasteur pipet, in one well of the prepared 24-well plate (as described in step 2). Repeat until wells for each of the desired NP concentrations (along with the control wells) contain five embryos to a final volume of 2 ml. Incubate at the same temperature that the embryos were initially developed.

4. Continue to incubate *X. laevis* embryos until they have reached the desired end stage (Fig. 20.13.3).

Again it is important that the embryos are checked several times a day to identify any dead ones and to assess developmental progress. Dead embryos should be removed from the well during this incubation period and the number of dead recorded.

Phenotypic description	Anatomical location	Example image	Comments
Normal (control treated embryo)	Whole mount		No abnormalities scored
Bent axis	Dorsal observation		Curvature of the spine; can be restricted to the tail or observed along the entire posterior-anterior axis
Eye deformities	Eyes		Absence of eye and eye deformities, including loss/reduction of pigmentation
Stunted growth	Whole mount		Overall shortened length of embryo
Developmental delay	Whole mount		Embryos appear to be developing without gross abnormalities, but at a reduced rate relative to control embryos
Edema/blistering	Pericardium region/skin		Abnormal accumulation of fluid often observed in the pericardium region; swelling or blistering can sometimes also be observed under the skin
Loss of pigmentation	Skin and eyes		Reduction/loss of observable melanocytes and/or their pigment
Tissue loss	Whole mount		Extensive loss of tissue throughout embryo
Gross malformations (other)	Whole mount		Easily identifiable, non-discrete gross abnormalities

Figure 20.13.4 Examples of phenotypic toxic response abnormalities commonly observed in *X. laevis* larvae at NF 45. More than one phenotype can be observed/animal, but one or more abnormality is sufficient for a positive score for nanotoxicity. Examples provided in these images have been treated with either $10^{15.8}$ or $10^{14.2}$ CdSe Quantum dots (QDs), with the exception of the control treated embryo (gray section). Adapted from Webster et al. (2016).

5. Make a note of any dead *X. laevis* embryos at the end of the incubation time. Wash embryos with 0.1× MMR and using a Pasteur pipet, gently transfer to a new 24-well plate containing 1 ml 0.6 mg/ml ethyl 3-aminobenzoate methanesulfonate salt to anesthetize the embryos. Incubate 20 min at room temperature to ensure embryos are fully anesthetized prior to fixing (Sherwood, Manbodh, Sheppard, & Chalmers, 2008; Webster et al., 2016).

   A variety of nanomaterials are synthesized for use as fluorescent bioimaging tools (Wolfbeis, 2015). If such fluorescent NPs are being tested using this protocol (e.g., metal chalcogenide quantum dots [QDs]) they can be detected in the embryos using live whole-mount fluorescent imaging at this stage in the protocol (Webster et al., 2016; see Support Protocol 5).

6. Wash away anesthetic solution with several rinses 0.1× MMR before fixing the embryos with MEMFA 1 hr at room temperature or overnight at 4°C.

   IMPORTANT: *Fresh MEMFA should be prepared for each experiment.*

If the embryos are going to be used for transmission electron microscopy (TEM; which can be used to determine exposure to non-fluorescent NPs) then MEMFA should not be used for embryo fixation. Rather an alternative fixing protocol provides improved ultrastructural analysis of X. laevis embryos by TEM (see Alternate Protocol).

7. Following fixation, aspirate off as much MEMFA as possible and wash embryos twice with excess PBST.

8. Take whole-mount images of the embryos to assist with phenotypic scoring: Prepare 2% (w/v) agarose gel by heating 100 mg agarose in 5 ml PBS until all agarose has dissolved. Then pour the 2% agarose into the bottom of a 10-cm^3 culture dish and leave to cool and set (~30 min).

   Agarose gel-containing imaging wells (as described above) can be prepared in advance of the experiment and stored at 4°C prior to use.

   Once set, a small indentation or notch can be made in the agarose gel using a scalpel to help position the embryos for imaging.

9. Pour a small layer of PBS over the agarose gel. Gently transfer *X. laevis* embryos into the agarose gel-containing dish using a Pasteur pipet and use this as a platform for imaging.

   The PBS should cover the embryos so that they remain hydrated, but not be in excess such that it is difficult to retain the embryos in the desired position for imaging.

10. Observe each embryo using a light microscope and rank for phenotypic abnormalities (Fig. 20.13.4). Calculate phenotypic abnormality; the number of malformed larvae as a percentage of the total number at the beginning of the experiment. Likewise, calculate percentage mortality in the same way.

    Common abnormalities induced by NP exposure include loss of melanocytes, blistering, edema, tail loss, bent spine, degradation of tissue, developmental delay, eye deformities, and stunted growth (Webster et al., 2016; Fig. 20.13.4).

    Exposure should be confirmed of NPs that do not produce notable nanotoxicity as scored in this phenotypic abnormality assay. If the NP is fluorescent this can be done as described in step 5 of this protocol (see Support Protocol 5 for detailed instructions on whole-mount imaging), but if not we propose that TEM imaging of X. laevis tissue will facilitate confirmation of NP uptake in the embryos (see Alternate Protocol).

11. Following scoring, dehydrate embryos for long-term storage: Transfer embryos into glass vials using a Pasteur pipet. Gently aspirate PBST and replace with 25% methanol in PBS 5 min, completely immersing all embryos in the glass vial.

12. Aspirate the 25% methanol and immerse embryos in 50% methanol. Repeat this step with 75% methanol and finally 100% (with 5 min between each concentration).

    If required, embryos can be rehydrated for further analysis by reversing steps 12 and 11.

13. After dehydration, *X. laevis* embryos can be stored long-term in 100% methanol at −20°C. Finally, seal the glass vial cap with Parafilm for long-term storage at −20°C.

HARVESTING *X. LAEVIS* EMBRYOS

SUPPORT PROTOCOL 4

X. laevis have been used as model organisms for biological research for decades, particularly as developmental vertebrate systems. As a result, detailed methodologies have been devised to obtain and work with *X. laevis* embryos (Sive, Grainger, & Harland, 2000). Ethical legislation and considerations must be in place when working with adult *X. laevis* frogs, the specific requirements of which will be dependent upon geographical and institutional location. This is not only a legal requirement in many countries, but such ethical considerations will also assist with maintaining a well-cared for population of

adult frogs for generating healthy embryos. You will need access to an aquarium facility for holding *X. laevis* colonies, where males and females should be housed in separate tanks. The following protocol describes the steps required to collect eggs and conduct fertilizations in order to obtain *X. laevis* embryos for nanotoxicity assessment (see Basic Protocol 3).

Materials

Female *X. laevis* adults (two or more)
Pregnant mare serum gonadotrophin (PMSG; Intervet)
Human chorionic gonadotrophin (hCG; Intervet)
0.1× Marc's Modified Ringer's (MMR) solution (see recipe)
One male *X. laevis* adult
Ethyl 3-aminobenzoate methanesulfonate (0.6 mg/ml; see recipe)
Testes buffer (see recipe)
2% cysteine (w/v) de-jellying solution (see recipe)

25-G needle (BD Biosciences) and 1-ml syringe (Thermo Fisher Scientific)
Non-textured, powder-free gloves (Thermo Fisher Scientific)
Culture incubator (set to 17°C)
10-cm^2 Petri dish (Thermo Fisher Scientific)
Surgical equipment including scalpels, forceps, and curved scissors
−20°C freezer

1. Prime female(s) with an injection of 100 units PMSG into the dorsal lymph sac 5 to 7 days before requiring embryos.

   We recommend priming and inducing ovulation in more than one female, in case egg yield and quality is not good, as this can vary greatly between individual animals.

2. Isolate testes from an adult male *X. laevis* by first anesthetizing by submersion in 0.6 mg/ml ethyl 3-aminobenzoate methanesulfonate for a minimum of 3 hr. Remove testes by exposing the abdominal cavity and drawing out the fat body with forceps.

   The testes lie at the base of the fat body and can be identified as white, oval shaped organs covered in a fine network of capillaries.

   Remove both testes and store in testes buffer at 4°C up to 14 days post-isolation. Store the testes at 4° to 17°C until step 5.

   IMPORTANT: *The male should be dead due to the overdose of anesthetic. Confirm no reaction by pinching the toes before starting the harvesting. Snip the heart prior to harvesting and freeze the sacrificed male, post-isolation of the testes.*

3. Induce ovulation in females by injection of 250 units hCG into each of the dorsal lymph sacs (500 units total) using a 25-G needle. Incubate induced females at 17°C.

   The dorsal lymph sac is located directly rostral to the hind limbs. It can be located between the lateral line (that appears as "stitch marks" on the adult's skin) and the spine.

   IMPORTANT: *The skin covering the dorsal lymph sac is loose and therefore it is straightforward to insert the needle subcutaneously and inject the hCG, however it is crucial not to penetrate too deeply into the muscle.*

4. After 12 to 14 hr the females should be ready to lay, which can be seen as the cloaca will appear red and swollen (due to the oocytes collecting in a sac close to this region). Gently squeeze abdomen of female *X. laevis* to encourage egg release into a 10-cm^2 Petri dish containing 0.1× MMR (enough to cover the eggs); this is done by very gently applying lateral and/or vertical pressure to the lower abdomen.

IMPORTANT: *Eggs should be fertilized immediately when collected in this manner. From this point onwards in the protocol it is critical to progress as rapidly as possible through the remaining steps; this helps ensure quality of the resulting embryos.*

As an alternative to squeezing, eggs can be collected passively by allowing females to lay in 1× MMR, where eggs will be viable for fertilization for up to 8 hr post-laying.

5. Fertilize harvested eggs by cutting off a small piece of one testis (<25%) and homogenize testis section using a scalpel blade and forceps. Add 1 ml 1× MMR to the mashed up testis piece. Mix testis slurry well with the eggs across the entire dish to promote fertilization. Leave 5 min then flood dish with 0.1× MMR and leave 20 to 30 min.

6. Incubate eggs at 17°C and monitor regularly for successful fertilization.

   The first sign is a cortical contraction of the animal pole ∼5 min post-fertilization. However, by 15 to 30 min fertilized eggs will reorient such that the animal pole faces up, which is the most reliable sign that fertilization has been successful.

   The release of cortical granules into the space between the fertilized egg and the vitelline membrane blocks polyspermy and causes the eggs to turn with their membranes according to gravity, with their pigmented animal poles facing up. At this point fertilized eggs will be much firmer than unfertilized ones, so it is easy to tell if the fertilization has been successful or not by 30 min post-fertilization.

7. Continue to incubate fertilized eggs at 17°C another 1 to 2 hr.

   Upon entering the first cell cycle, cortical rotation occurs, which is required for formation of dorsal tissues and usually occurs within 2 hr of fertilization at 17°C.

   IMPORTANT: *Do not disturb the embryos during this incubation period too much, as it can interfere with correct dorso-ventral patterning. For example, shaking the embryos during this time is known to produce spontaneous secondary axis formation through microtubule reorientation.*

8. In a glass beaker, gently swirl embryos in 2% cysteine (w/v) de-jellying solution until they pack closely together.

   X. laevis embryos are surrounded by a thick layer of protective jelly that must be removed prior to further experimentation. Ideally this should be done after cortical rotation to reduce the likelihood of developmental defects (see step 7).

   The time required for this step can vary depending on differences between embryo batches, however it should normally take ∼5 min and no longer than 10 min.

   IMPORTANT: *The de-jellying solution needs to be made fresh on the day of use and used at room temperature.*

   IMPORTANT: *Do not over treat as this can lead to developmental defects and can contribute to poor embryo quality.*

9. Remove cysteine solution and wash eggs several times with distilled water (more than five washes) followed by several washes with 0.1× MMR. Grow embryos in 0.1× MMR, until ready for further experimental procedures.

WHOLE-MOUNT IMAGING OF *X. LAEVIS* EMBRYOS FOR FLUORESCENT NANOPARTICLE UPTAKE

SUPPORT PROTOCOL 5

This protocol can be used to investigate internalization of fluorescent NPs in *X. laevis* embryos. We have previously demonstrated that this protocol works well using 20 nm fluorescent carboxylate-modified NPs (PS-COOH; Molecular Probes FluoSphere beads; Thermo Fisher Scientific; catalog number F8887), thus we propose that these NPs offer

a useful positive control for NF stage 45 embryos, exposed to 10^{15} NP/ml from NF stage 38 (Webster et al., 2016).

Materials

2% (w/v) agarose gel (Sigma-Aldrich; see recipe)
Tadpole stage *X. laevis* embryos (from NF stage 38 onwards; see Fig. 20.13.3; anesthetized [in 0.06% ethyl 3-aminobenzoate methanesulfonate: Dilute 0.6 mg/ml solution 1/10 in dH_2O, until movement is inhibited] and pre-exposed to florescent NPs, see step 5, Basic Protocol 3 for details; use 20 nm PS-COOH NPs as a positive control)
PBS (see recipe)

3-cm^2 Petri dish (Thermo Fisher Scientific)
Long-handled scalpel (10 A blades)
Glass Pasteur pipet (prepared as described in Basic Protocol 3)
Dumont #5 forceps (stainless steel; ultrafine and can be used for carefully manipulating embryos throughout the protocol; Sigma-Aldrich)
Fluorescent microscope with CCD digital camera

1. Prepare agarose imaging plates for whole-mount *X. laevis* embryos as described in step 8, Basic Protocol 3.
2. Pour a small layer of PBS over the agarose gel and gently transfer *X. laevis* embryos into the agarose gel-containing imaging plate (see step 9, Basic Protocol 3 for details).
3. Image embryos using a fluorescent microscope according to the emission filter required to excite the NPs being tested.

   For the PS-COOH NPs, an emission filter of 509 to 547 nm should be used. The fluorescence from these NPs will appear bright throughout the embryo (Webster et al., 2016).

4. Monitor fluorescent NPs: Use time-lapse images (with time-frame stills of 0.7 sec) to monitor fluorescent NPs traveling through the vasculature of the *X. leavis* embryos, which are particularly clear in the embryonic intersomitic blood vessels (Webster et al., 2016).

ALTERNATE PROTOCOL

TEM IMAGING OF *X. LAEVIS* EMBRYO SECTIONS FOR NANOPARTICLE UPTAKE

Support Protocol 5 cannot be used to confirm uptake of non-fluorescent NPs in *X. laevis* embryos and for this reason such NPs require an alternative procedure to ensure embryo exposure to these nanomaterials. Electron microscopic techniques facilitate high-resolution visualization of NPs in tissues and in particular TEM has been used for a long time in NP research. Due to the complexity of sample preparation, imaging, and interpretation of ultrastructural NP localization within tissues, the infrastructure required for TEM analysis is often housed in centralized facilities, where it is possible to seek pertinent advice about TEM experimental design with expert staff within such core facilities. This will assist with optimization of advanced TEM imaging for specific nanomaterials, but here we describe a protocol that is convenient for preparing high-quality *X. laevis* embryo sections and that is suitable at least for imaging iron oxide core NPs (Webster et al., 2016). The processes of fixing, embedding, and sectioning *X. laevis* embryos for TEM is based on a previously described method developed for imaging carbon NPs in vivo (Bacchetta et al., 2012).

Additional Materials *(also see Basic Protocol 3)*

Tadpole stage *X. laevis* embryos (from NF stage 38 onwards; see Fig. 20.13.3; anesthetized and pre-exposed to NPs, see step 5, Basic Protocol 3 for details)

TEM fixing buffer (see recipe)
1% (v/w) osmium tetroxide (OsO_4; Sigma-Aldrich) in PBS
Propylene oxide resin (TAAB Laboratories Equipment)

Incubator (set to 60°C)
Microtome (Reichert Ultracut E)
Carbon-coated 300-μm mesh copper grids (Agar Scientific)
TEM instrument with imaging modality

1. Immerse *X. laevis* embryos in 0.6 mg/ml ethyl 3-aminobenzoate methanesulfonate salt 20 min at room temperature to anesthetize.
2. Wash away anesthetic solution with several rinses of 0.1× MMR and fix embryos in TEM fixing buffer (enough to immerse the embryos) 1 hr at room temperature. During this time, replace TEM fixing buffer twice with fresh buffer.
3. Post-fix embryos in 1% OsO_4 1.5 hr at 4°C.

   This step is needed to increase the electron density in lipids and proteins.
4. Dehydrate fixed embryos in a decreasing concentration of methanol, as described for Basic Protocol 3, step 12.
5. Once dehydrated, wash embryos in 75% propylene oxide resin and leave in 100% pure resin overnight.
6. Submerse embryos in fresh resin and then polymerize at 60°C, 48 hr.
7. Using a microtome, cut semi-thin 1-μm sections of embryos.

   Cut in an anterior-to-posterior direction to produce transverse sections along the entire embryo. Analyze all tissues across the anterior-posterior axis as the location of the NPs will depend upon the biodistribution of specific nanomaterials within X. laevis embryos.

   Ultrathin sections (~50 nm) can also be used if required for NP detection.
8. Mount sections onto carbon-coated 300-μm mesh copper grids.
9. Image sections using TEM according to the settings required for the instrument.

   As an example, we have successfully used a Tecnai 20 TEM (FEI; Thermo Fisher Scientific) with AMT cameras, operating at an acceleration voltage of 200 kV to image iron oxide core NPs (Webster et al., 2016). Likewise carbon NPs have been successfully imaged in X. laevis embryos using a Zeiss LEO 912ab Energy Filtering TEM at 80 kV (Bacchetta et al., 2012).

REAGENTS AND SOLUTIONS

Use Milli-Q-purified water (d.H_2O) or equivalent in all recipes and protocol steps. General laboratory reagents are supplied by Sigma-Aldrich, unless otherwise stated. For common stock solutions, see APPENDIX 2A.

Agarose gel, 2% (w/v)

100 mg agarose
5 ml PBS
Prepare fresh before use.

Ammonium persulfate (APS), 10% (w/v)

0.1 g ammonium persulfate (APS)
Distilled water (dH_2O)
Final volume to 1 ml.
Store at −20°C. Prepare fresh stocks every 2 weeks.

Blocking solution

7.5 g nonfat dry milk
15 ml 10× TBS
0.15 ml Tween 20 (100%)
Final volume to 150 ml. Prepare fresh before use.

Cysteine de-jellying solution, 2% (w/v)

3 g cysteine
100 ml 0.1× Marc's Modified Ringer's (MMR) solution (see recipe)
Adjust to pH 7.8 with 10 M NaOH. Prepare fresh before use.

Ethyl 3-aminobenzoate methanesulfonate solution, 0.6%

6 g ethyl 3-aminobenzoate methanesulfonate (Fluka)
Distilled water (dH_2O)
Final volume to 1 liter. Adjust to pH 7.2.
Store protected from light at 4°C for up to 1 month. If the solution turns brown during storage, discard and prepare fresh solution.

Loading buffer, 4×

3 ml 1 M dithiothreitol (DTT)
1.5 ml 1 M Tris·HCl, pH 6.8 (*APPENDIX 2A*)
0.6 g SDS
2.4 ml glycerol
0.03 g bromophenol blue
Final volume to 7.5 ml. Store at −20°C for up to 1 year in aliquots

Lysis buffer

50 mM Tris·HCl, pH 7.4 (*APPENDIX 2A*)
1% Triton X-100
150 mM NaCl
Add 4 complete, EDTA-free protease inhibitor tablets.
Final volume to 200 ml in PBS. Adjust pH to 7.4. Store at 4°C and use within 3 months.

Marc's Modified Ringer's (MMR) solution, 0.1×

100 mM NaCl
2 mM KCl
1 mM $MgCl_2$
2 mM $CaCl_2$
5 mM *N*-(2-hydroxyethyl)piperazine-*N*-2-ethanesulfonic acid (HEPES), pH 7.6
Adjust to pH 7.4. Store at room temperature and use within 3 months.

MEM salts, 1×

For 10× MEM salts:
1 M 3-(*N*-morpholino)propanesulfonic acid (MOPS)
20 mM ethylene glycol-bis(2-aminoethylether)-*N,N,N′,N′*-tetraacetic acid (EGTA)
10 mM $MgSO_4$
5 mM *N*-(2-hydroxyethyl)piperazine-*N*-2-ethanesulfonic acid (HEPES), pH 7.6
Adjust to pH 7.4 with NaOH pellets. Autoclave and store in the dark at room temperature for no longer than 3 months provided the solution remains clear and not yellow in color.
Dilute in $d.H_2O$ for a 1× working solution. Prepare working solution fresh before use.

MEMFA fixative, 1×

3.7% formaldehyde
1× MEM salts (see recipe)
Prepare fresh before use.

PBS, 10×

1.4 M NaCl
26.8 mM KCl
100 mM Na_2HPO_4
17.6 mM KH_2PO_4
Adjust to pH 7.4 with HCl. Dilute in d.H_2O for a 1× working solution. Store at room temperature and use within 3 months.

PBS/Tween 20 (PBST), 1×

0.1% Tween 20
1× PBS (see recipe)
Store at room temperature and use within 1 month.

Ponceau S solution

0.2 g Ponceau S
5 ml glacial acetic acid
Final volume to 100 ml. Store at room temperature and use within 5 years.

Running buffer, 10×

30.2 g Tris base (25 mM)
144 g glycine (190 mM)
0.1% SDS
Final volume to 1 liter. Adjust to pH 8.3. Store at 4°C for up to 2 weeks.

SDS, 10% (w/v)

10 g SDS
Distilled water (dH_2O)
Final volume to 100 ml. Store at room temperature and use within 1 month.

Sørensen's buffer

39 ml NaH_2PO_4 (0.2 M)
Final volume to 100 ml. Adjust to pH 7.0. Store at 4°C for up to 1 month.

TEM fixing buffer

4% paraformaldehyde
2% glutaraldehyde
0.1 M sodium cacodylate buffer (4.28 g sodium cacodylate in 200 ml d.H_2O)
Adjust to pH 7.4. Prepare fresh before use.

Testes buffer

80% fetal calf serum
50 μg/ml gentamycin sulfate
Final volume to 50 ml in 1× MMR (see recipe). Store at 4°C and use within 2 weeks.

Transfer buffer, 10×

30.2 g Tris base (25 mM),
144 g glycine (190 mM)
0.1% SDS
Volume to 1 liter. Adjust to pH 8.3. Store at 4°C and use within 1 month.

Tris-buffered saline, 10×, and Tween 20 (TBST)

24.23 g Tris base
80.6 g NaCl
0.1% Tween 20
Final volume to 1 liter. Adjust to pH 7.6. Dilute in d.H_2O for a 1× working solution. Add 1 ml Tween 20. Store at room temperature and use within 1 month.

Tris·HCl buffer (pH 6.8), 0.5 M

61 g Tris base
Distilled water (dH_2O)
Final volume to 1 liter. Adjust to pH 6.8 with HCl. Store at room temperature for up to 6 months.

Tris·HCl buffer (pH 8.8), 1.5 M

181.65 g Tris base
Distilled water (dH_2O)
Final volume to 1 liter. Adjust to pH 8.8 with HCl. Store at room temperature for up to 6 months.

COMMENTARY

Background Information

Here we have described the use of non-specialist cytotoxicity testing protocols in combination with an *X. laevis* embryonic phenotypic abnormality screening assay for nanotoxicity assessment. Specifically, testing well-characterized nanomaterials at the physical-chemical level (Basic Protocol 1) with standard cytotoxicity assessment (Basic Protocol 2) and using this in combination with the *X. laevis* embryonic phenotypic assay (Basic Protocol 3), can bridge the gap between conventional in vitro (cell culture models) and in vivo (mammalian systems) nanotoxicity assessment (Webster et al., 2016).

We have shown that direct comparison of the cytotoxicity and *X. laevis* data can provide a logical ranking system to generate an overall hazard score for NPs (Webster et al., 2016). Briefly, a simple scoring system ranging from 0 to 2 can distinguish a hazard score, where NPs score 0 when the percentage of cell viability and healthy *X. laevis* embryos is >76%, 1 when this percentage ranges from 50% to 75% and 2 when it is <50%. From these criteria only NPs that score 0 in all nanotoxicity assessment protocols should progress to further toxicity assessment in mammalian models (Fig. 20.13.1). This approach can reduce false negatives that could otherwise be generated from cell-based assays used in isolation. Thus, only NPs that produce no-to-low toxicity assessment in the described protocol progress to further evaluation in mammalian systems, thereby reducing investment in time and money spent on more costly rodent models, which is important given the year-on-year increase in the development costs of nanotherapeutics. Overall, this protocol provides biomedical researchers with nanotoxicity assessment at an early stage in nanotherapeutic design to quickly and easily identify nanomaterials that require additional modifications for improved safety prior to mammalian testing (Fig. 20.13.1).

Critical Parameters and Troubleshooting

There are several critical parameters that will affect the successful outcome of the described protocols and therefore must be considered by users. These parameters include the following:

Dosing and storage of NPs

The most suitable conditions of NP storage depend on the type of material from which the NPs are composed. It is not possible to state general conditions. The chosen medium should guarantee stability of the NPs over time. If the material is not sensitive to low temperature it is suggested that stock solutions are stored in the fridge, mostly if they contain organic and/or biological moieties to avoid degradation. Before making any measurements it is also necessary to check the stock solution in terms of homogeneity in order to guarantee the right evaluation of the dose. Often, NP dispersions can be affected by flocculation over time. If flocculation is

reversible then this process does not represent a problem. It is necessary to re-disperse the sediment in the dispersion through simple shaking and/or 5 to 10 kHz sonication of the NP dispersion before measurement or preparation of the samples.

Cell culture considerations

There are several important considerations when conducting cytotoxicity analysis for NP testing. The first is to select cell types (three or more) that best model the exposure route(s) and target organ(s) of the nanomaterial of interest. Next, an appropriate methodology must be selected that can accurately assess cytotoxicity of the NP of interest without the development of false negatives and/or false positives, which is important to carefully consider because not all nanomaterials are compatible with commonly employed methods. For example, the MTT assay (the method described here; Basic Protocol 2)—although being easy, quick, and readily affordable—is not compatible with several types of NPs. Wang, Yu, and Wickliffe (2011) indicated that titanium oxide nanoparticle (nano-TiO_2) induces superoxide formation in mammalian cells that reduces tetrazolium salts and produces the absorbant formazan end products. Monteiro-Riviere et al. (2009) showed that single-walled carbon nanotubes (SWCNT) and carbon black alone (absence of cells) interact with the MTT to cleave the tetrazolium ring and lead to a false-positive reaction, whilst Belyanskaya et al. (2007) found that SDS-suspended SWCNTs interfere more with the MTT assay than polyoxyethylene sorbitan monooleate-suspended SWCNTs. Table 20.13.1 lists types of NP-based materials that have previously been demonstrated to be compatible with commonly employed cytotoxicity assays. Finally, it is essential to use GLP when conducting in vitro cell work; cell line validation, equipment validation and/or maintenance, mycoplasma contamination testing, employment of strict aseptic technique, and usage of low-passage cell culture are all critical in obtaining high-quality, reproducible cytotoxicity data.

Immunoblotting considerations

Immunoblotting is a simple molecular procedure for the quantitative detection of proteins in cells and tissues. Here we describe a protocol to detect apoptotic markers in response to NP-induced cytotoxicity (Support Protocol 3). Despite its simplicity, an array of problems can be encountered that require troubleshooting to prevent unexpected results and a comprehensive description of effective immunoblotting troubleshooting has previously been provided (Mahmood & Yang, 2012). Briefly, use fresh protein samples using lysis buffer containing protease inhibitors to prevent sample degradation and ensure the transfer sandwich is effectively prepared by avoiding air bubbles between the gel and membrane. A final crucial consideration for immunoblotting is effective optimization of antibody concentration for specific samples, as too low and the signal will not be visible, and too high could result in over-exposed (negative) bands on the blot, including a high background signal. Altering membrane-washing times, the blocking reagent used, and membrane exposure times can also dramatically affect the signal-to-noise ratio and therefore also require optimization.

X. laevis egg quality

A major critical parameter for nanotoxicity assessment in *X. laevis* embryos is the quantity and quality of egg production (and thus the zygotes generated from these), which has a major influence on the collection of reliable data. The *Xenopus* research community is aware that egg quality and production levels are variable, which is often attributed to differences between individual females. Therefore, experimental replication can be improved by acquiring eggs from consistently good producers. Acceptable methods for identification of individuals include tagging (with beads or microchips), tattooing, branding, monitoring of dorsal markings in pigmented frogs, and perhaps more simply (if space is available), housing individuals in designated tanks. Implementing the following basic policies will increase the chances of quality egg harvests:

(1) Comprehensive training of personnel preforming the procedures.

(2) Introducing a robust system for identifying individual animals.

(3) Ensuring a compulsory rest period of at least 4 months between ovulations (Green, Parker, Davis, & Bouley, 2007). This will allow females to be reused for several years provided they remain healthy.

(4) Daily monitoring of post-procedure females for up to 2 weeks in a separate recovery tank, to ensure there are no complications caused by ovarian hyper-stimulation (Green et al., 2007).

(5) Detailed record keeping of all procedures conducted.

(6) Strict quarantine procedures for incoming animals into the aquarium.

There is also awareness in the community that *X. laevis* husbandry can also greatly influence egg quality. Seasonal changes, food, temperature, water quality, and environmental enrichment are all factors that have been suggested to affect the quality and quantity of *X. leavis* eggs (Delpire, Gagnon, Ledford, & Wallace, 2011; Godfrey & Sanders, 2004; Green, 2002; Hilken, Dimigen, & Iglauer, 1995; Sigel, 1990; Wu & Gerhart, 1991). Although some of these effectors are difficult to control, they can be minimized by maintaining a 12 hr light/12 hr dark cycle, a constant temperature (21° to 23°C), feeding once every 2 to 3 days, enriching the environment with functional items for the frogs (e.g., plastic plants, logs, dishes), and careful monitoring of water quality.

NP exposure in X. laevis embryos

NP exposure time in *X. laevis* embryos is an important consideration for this protocol. Embryos must be exposed to NPs for a sufficient length of time in order for the key internal organs to be exposed to the nanomaterial being tested. It is also important to consider at what developmental stage the embryos are exposed to these nanomaterials. The described protocol can be adapted depending on the aims of the toxicity screen. For example, embryos can be exposed to NPs very early on during the developmental process, such as at NF stage 4 and fixed at NF stage 38. Over this time, the embryos are exposed to NPs during key developmental processes such as gastrulation (NF stage 10) and neurulation (NF stage 15). The NF stage at which the NPs are applied will greatly affect exposure, too. For example, between NF stages 38 to 45, the gills and mouth of the embryos are open, providing additional routes of exposure for NPs aside from the porous skin, as we have previously discussed (Webster et al., 2016). As highlighted in the protocol description, it is essential to confirm that the embryos have been exposed to the NPs being tested by the experimental end point, which we propose can be done using microscopy (see Support Protocol 5 and Alternate Protocol). This is of particular importance for nanomaterials that do not produce visible toxicity in the embryos.

Anticipated Results

NP physical characterization is crucial step in a toxicity evaluation of NP dispersions for both in vitro and in vivo experiments. Importantly, it is necessary to evaluate stability and size distribution of the NP dispersions in experimental conditions that mimic, as much as possible, the conditions similar to those used in the biological nanotoxicity assays (that is, for example, temperature, dispersion medium, NP dose). Stability of the NP dispersion in its dispersing medium does not guarantee that such NPs are equally stable in the media used in the biological study. Biological media are complex fluids containing biomolecules and salts that can strongly affect NP self-assembly in solution, and in some cases also cause agglomeration and precipitation.

It is known that NP cellular interaction and uptake are affected by NP physical properties and size, thus to interpret NP biological response(s) it is necessary to know the features of NPs in the biological environment. DLS is the best technique to investigate the stability of the NP dispersions in different media over time at biologically relevant temperatures. It is important to note that this technique provides the hydrodynamic size distribution of the NPs in the solution (highlighting possible aggregation effects), but it does not provide the exact size of the single NP. For this reason TEM experiments should be done to complement the DLS investigation.

TEM is an imaging technique that gives information on the morphology and size of the NPs, providing exactly the size of the NP units in the dispersion. This knowledge permits better interpretation of the DLS results. It is also important to underline that TEM sizes are not representative of the NP distribution in solution. In fact, the drying process necessary to measure the NPs, could promote agglomeration. Nevertheless, qualitative information can be extracted that can be related to NP dispersibility. In fact, if the images show single well-separated NPs on the grid, it is reasonable to assume that they are also well dispersed in the dispersion. In the same way, if big NP agglomerates are visible in the grid, it suggests that NPs are also aggregated when dispersed in aqueous solutions.

Cytotoxicity assessment is an essential step in the described process of NP hazard assessment (Fig. 20.13.1). As detailed above in Basic Protocol 2, the researcher should select cytotoxicity assessment methodologies that are compatible with their nanomaterials of choice (see Critical Parameters, Cell culture considerations section for discussion). Ideally the selected methodologies should cover more than one cytotoxic assessment parameter (oxidative stress, cell death, cell viability, and

inflammatory response). Here we detail three protocols (Basic Protocol 2, Support Protocol 2, and Support Protocol 3) that combine to robustly assess cell viability (MTT and trypan blue exclusion assays) and cell death in response to NP treatment, providing percentage cell viability readings and an indication of apoptosis by immunoblotting. As detailed above, this data is then combined with results from the *X. laevis* phenotypic abnormality assay (Basic Protocol 3) to provide a hazard ranking score for NP safety assessment.

The *X. laevis* phenotypic abnormality assay (Basic Protocol 3) results in the percentage of embryos that did not survive NP exposure and the percentage that display phenotypic abnormalities relative to the total number of embryos tested, and therefore represents the percentage lethality and percentage abnormality, respectively. Expected abnormalities commonly include eye malformations, bent anterior-posterior axis, edema, blistering, stunted growth, and pigmentation loss (Fig. 20.13.4). We have previously described example results for a range of high-to-low toxicity-inducing nanomaterials (Webster et al., 2016). As discussed above, comparison between the *X. laevis* phenotypic abnormality data and the cytotoxicity results provides a hazard ranking score for NP safety, which can be used to determine whether or not further nanotoxicity assessment in mammalian systems is permissible or if further optimization of NP design and/or synthesis is first needed to reduce toxicity of the developed nanoformulation (Fig. 20.13.1).

Time Considerations

Basic Protocol 1: Preparation of the samples for DLS measurements is a quick procedure that generally involves the dilution of the NP stock dispersions in the different biological media. A DLS experiment is quite fast, it will take between 5 to 15 min depending on whether the measurement is performed at fixed angle or at different angles (in the latter case it will be longer). The measurements should be repeated over the experimental time of the biological assay with closer repetitions in the first day. Overall, the experimental time depends on the sample numbers and duration of the biological experiments. Moreover, additional time should be considered for the analysis of DLS data for multi-angle measurements for which the operator needs to make some more analysis work after the experiments.

Support Protocol 1: Preparation of TEM samples on suitable grids requires at least overnight incubation to guarantee complete evaporation of the solvent. Generally, the grids will be analyzed by a specialized technician, thus the experimental time is not predictable. The actual measurement takes approximately half an hour for each sample (as different areas of the grids need to be imaged). After that the operator will need to analyze the images with specific imaging software for extracting a size distribution of the NPs. The duration of this analysis depends on the quality of the images and the properties of the sample; if the NPs are well separated usually it is possible with most imaging software to automatically measure the size of all the NPs, while if the NPs formed agglomerates on the grid, size measurement of each single NP has to be done manually and this will take a longer time.

Basic Protocol 2: Preparation of mammalian cell line stocks, validation and preparation of cells for experiments will take 2 to 3 weeks depending on how well the specific cells grow in culture. Seeding and growing cells will take 1 day and NP treatment takes 3 days. The MTT assay takes a further 5 to 6 hr (depending on sample numbers) and the reading and generation of results ~1 to 2 hr: ~3 to 4 weeks in total, depending on how well the cell lines grow.

Support Protocol 2: As stated above for Basic Protocol 2, cell line preparation, seeding, and treating with NP will take ~2 to 3 weeks plus an additional 4 days. The trypan blue exclusion assay will take a further 30 min to 2 hr depending on how many samples are to be analyzed. Likewise, cell counting will take 10 min to 2 hr depending on sample numbers and count methodology: ~3 to 4 weeks in total, depending on how well the cell lines grow.

Support Protocol 3: Sample preparation, including treatment times and protein preparation, will take ~4 to 5 days. SDS-PAGE and completion of immunoblotting will then take a further 0.5 and 3 days, respectively: ~7 to 8 days in total, depending on optimized conditions.

Basic Protocol 3: Depending on the requirements of the NF stage needed for specific experiments, *X. laevis* embryo exposure and incubation times can vary from a few hours to several days. This is also influenced by the incubation temperature used (see steps 1 through 4 of Basic Protocol 3 for discussion of time estimates). At the end of the incubation period, fixing the embryos can take 2 to 24 hr depending on the temperature used. Washing, mounting, imaging, and scoring the embryos will take a few hours depending on how many

embryos need to be analyzed. Finally, dehydration of embryos for long-term storage takes ~30 to 40 min: ~1 week in total.

Support Protocol 4: Priming of females can take up to 1 week and induction of ovulation, up to 14 hr. Fertilizations and de-jellying will take 2.5 hr: ~6 to 8 days in total.

Support Protocol 5: Preparation of imaging plates (1 hr) and live, whole-mount fluorescent imaging of embryos will take ~1 to 3 hr (depending on the number of embryos to analyze): 2 to 4 hr in total.

Alternate Protocol: Anesthetizing, fixing, and dehydrating embryos takes 3.5 hr in total. Embedding the embryos in resin takes 3 days, whilst sectioning, mounting, and imaging could take up to 2 to 3 days (depending upon the number of samples to process): ~5.5 to 6.5 days in total.

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