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

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14

15 **SIGNIFICANCE STATEMENT: Nanoparticles are being produced for an ever-increasing range of applications and with such growth comes a need to efficiently assess any potential toxicity associated with these new materials. Here we describe in detail a step-by-step protocol that can be used to rapidly and effectively assess nanotoxicity, by combining mammalian cytotoxicity assessment with vertebrate abnormality scoring using *X. laevis* embryos. We have previously demonstrated that this approach is effective at determining low-toxicity nanomaterials in mice (Webster et al., 2016). This protocol can be used as a rapid screening approach for newly developed nanomaterials, with high predictive power for determining nanoparticle safety in vertebrate systems.**

ABSTRACT

The African clawed frog, *Xenopus laevis*, has been used as an efficient pre-clinical 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 a novel biomedical NP.

Keywords: Nanoparticles • nanotoxicity • physicochemical characterisation of nanoparticles • cytotoxicity • *Xenopus laevis* embryos

INTRODUCTION

The research and application of biomedical NPs is a rapidly evolving discipline (De Jong and Borm, 2008). For many, it is believed that biomedical nanomaterials can act as advantageous

47 tools in the treatment of several disease states. In particular, the unique physico-chemical
48 properties of NPs makes them an ideal therapeutic and diagnostic tool in oncology by
49 overcoming the limitations of conventional therapies, as we have previously discussed
50 (Bombelli et al., 2014). The main advantages of using biomedical NPs as drug delivery
51 systems include targeted drug delivery, increased biocompatibility and a decrease in drug
52 toxicity, whilst maintaining or improving the therapeutic effect. However, as a result of the
53 high surface area-to-ratio volume and complex composition of the nanomaterial, NPs can be
54 highly reactive, where combinations of NP size, shape, material, and functionalisation, can
55 result in toxicity within a biological systems (Lewinski et al., 2008; Nystrom and Fadeel,
56 2012).
57
58 Conflicting information regarding NP safety for a given material can impede the progression
59 of a NP from the early stages of formulation development through to the clinic.
60 Inconsistencies in NP toxicity data are largely attributable to a lack of a standardised protocol
61 for nanotoxicity assessment. Firstly, full characterisation of a NP system (including size,
62 surface charge, and stability in assay buffers) is required to understand the fate of the NP in a
63 biological system and its potential to cause toxicity. Different early developmental models,
64 such as *Xenopus* species (Bacchetta et al., 2014; Hu et al., 2016; Mouchet et al., 2008;
65 Tussellino et al., 2015; Webster et al., 2016) and zebrafish (George et al., 2011; Liu et al.,
66 2012; Rizzo et al., 2013), have been explored as systems that can provide rapid, accurate, cost
67 effective and abundant data for NP toxicology assessment. *X. laevis* (the African clawed frog)
68 is a species that produce large quantities of embryos allowing them to be used in a high-
69 throughput style assay to gain toxicology data relatively quickly. Furthermore, with an
70 individual embryo size at early developmental stages of ~1 mm, they are well suited for use
71 in a multi-well format. *X. laevis* has the advantage of being evolutionary closer to humans

72 than other early models such as *Caenorhabditis elegans*, *Drosophila*, and zebrafish (Wheeler
73 and Brandli, 2009). Although mouse models, as the gold standard, are evolutionary closer to
74 humans than *X. laevis*, they are expensive and not a viable option to test numerous NPs over
75 a wide range of concentrations, as far fewer embryos are produced compared to *X. laevis*.
76
77 Here we provide a detailed protocol for the use of *X. laevis* embryos in conjunction with
78 cytotoxicity analysis, for highlighting potential NP toxicity by observing phenotypic
79 abnormalities/lethality in response to NP exposure. *X. laevis* development is well documented
80 (Nieuwkoop and Faber, 1967), making it easy to detect when toxicity-induced deviation from
81 normal embryo development has occurred. The rationale for this approach has previously
82 been described (Webster et al., 2016) and involves a combined assessment of cytotoxicity
83 with *X. laevis* abnormality assessment in response to NP treatment, which offers a sensitive
84 nanotoxicity model to bridge standard *in vitro* assessment alone with further rodent testing
85 (Fig. i). Specifically, this methodology incorporates physicochemical characterization of
86 nanomaterials, followed by rapid cytotoxicity and phenotypic abnormality assessment as an
87 indicator of nanotoxicity prior to later testing in mammalian systems.
88

89 BASIC PROTOCOL 1 – PHYSICOCHEMICAL CHARACTERISATION OF NPs

90 This protocol describes the necessary steps to prepare nanoparticle (NP) dispersions suitable
91 for toxicological characterization by cytotoxicity and *X. laevis* phenotypic scoring assays.
92 This protocol is designed to be adaptable to different types of nanoformulations (thus it is not
93 addressed to a specific typology of NPs), but is to be used for NPs dispersed in aqueous
94 solutions. Physical-chemical characterization of NP dispersions is a critical step in a nano-
95 safety assessment protocol (Azhdarzadeh et al., 2015), in particular the experiments need to
96 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. 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 experiments (i.e. 10% v/v serum used in GM), but not in pure serum as in that case the protein signal overcomes that deriving 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 (Cedervall et al., 2007; Monopoli et al., 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 et al., 2010) or Fluorescence Correlation Spectroscopy (Rocker et al., 2009), which is beyond the interest of this protocol.

Materials

NP stock dispersions (concentrations and nanomaterials tested are to be pre-determined

122 by the experimenter)

123 Disposable DLS cuvettes

124 Dynamic Light Scattering apparatus

125 PBS (see recipe)

126 Mammalian cell culture growth media (GM; see basic protocol 2 for further details)

127 0.1X Marc's Modified Ringer's (MMR; see recipe)

128

129 1. Prepare the DLS cuvettes cleaning them with autoclaved Milli-Q-purified H_20 (d.H_20)
130 and then dry with particular care to protect them from dust.

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

134 *IMPORTANT NOTE: Never touch the middle-bottom part of the cuvettes with hands,*
135 *but always manage them touching them in their upper edge.*

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

141 *It is recommended to run a quick test for evaluating the averaged scattered intensity of*
142 *the chosen dilution that should be above 20 kcounts/s for be statically significant. If it is*
143 *lower than that value, a more concentrated sample should be prepared.*

144 3. Set the temperature to the desired value according to that at which the biological
145 experiments are performed.

146 *In this context these temperatures will be 37°C for mammalian and 12-23°C for X.*

147 *laevis work.*

148 4. The NP dispersion in the cuvette should be left to rest in the sample holder for
149 approximately 10 minutes before the measurement to reach the desired temperature and allow
150 the eventual dust to sediment.

151 5. Measure the scattered intensity at a set angle of detection. Generally, the most used
152 apparatus can measure the scattered intensity at a fixed angle (either 90° or 173°), but there
153 are also more advanced instruments that permit multi-angle detection, in that case it is better
154 to measure the scattered intensity at different angles (Fig ii). The detected signal will be
155 automatically sent to the correlator, which produces the auto-correlation function of the
156 scattered intensity $g_2(q,t)$ for each angle (equation 1):

157

158

$$g_2(q,t) = \frac{\langle I^*(q,0)\, I(q,t)\rangle}{\langle I(q,0)^2\rangle} \qquad (1)$$

159

160 where:

161

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

162

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

165 2 Analyze the auto-correlation functions to extract the NP hydrodynamic size by available
166 analysis softwares. The analysis of the auto-correlation functions at each angle gives a
167 decay rate $\Gamma(s^{-1})$ related to the NP dynamics and related to the translational diffusion
168 coefficient, D, through the following equation for Brownian systems (equation 2):

169

170 $$\Gamma(s^{-1}) = D \cdot q^2 \qquad (2)$$

171

172 Thus reporting the decay rates versus the scattering vectors the slope of the obtained curve is
173 the translation diffusion coefficient. The NP hydrodynamic radius, r_H, can be determined
174 through the Stokes-Einstein relationship (equation 3):

175

176 $D=k_BT/6\pi\rho r_H$ (3)

177

178 Where T is the experimental temperature and ρ the viscosity of the solvent.

179 *IMPORTANT NOTE: the fitting analysis of the auto-correlation functions for*
180 *determining the decay rates must be carefully chosen. If the auto-correlation*
181 *function is monomodal (the sample is mostly composed of a single population of NPs*
182 *of the same size), a Cumulant method (Koppel, 1972) can be used. This fitting*
183 *analysis gives an averaged $<\Gamma>$ together with a polydispersity index (PDI). If the*
184 *PDI is <0.2-0.25, it is reasonable to use this method. If the PDI is >0.25 the sample*
185 *is either very polydisperse or composed of two or more populations and an*
186 *alternative method must be used. The most common is the algorithm*
187 *CONTIN (Provencher, 1982)-based on the Laplace transform of the auto-*
188 *correlation function. This method gives a size-distribution of the NP dispersion*
189 *distinguishing different particle populations differing in scattered intensities of at*
190 *least $1:10^{-5}$. For monomodal polydisperse samples the two methods should give*
191 *comparable results.*

192

193 **SUPPORT PROTOCOL 1 – TEM FOR NP CHARACTERISATION**

194 As highlighted in Basic Protocol 1, a TEM study should be done on the NP stock dispersion
195 for evaluating NP morphology and better interpreting DLS results. TEM analysis allows the
196 determination of the size of single NPs that can be used for understanding the NP size

197 distribution obtained by DLS and highlight possible agglomeration effects. TEM equipment
198 comprises of complex instrumentation and usually a dedicated person(s) is/are responsible for
199 its maintenance and running experiments in a core facility within institutions. Thus, here we
200 only describe a protocol for preparing samples to be measured by TEM. It is necessary to
201 prepare a dispersion of the NPs in d.H_2O as the sample has to be dried (measurements are
202 performed in vacuum) and salt crystallization can occur if the NPs are dispersed in buffer
203 affecting the experiment. If the NP stock is dispersed in buffer, it is also possible to wash the
204 sample directly on the grid.

205

206 ***Materials***

207

208 TEM grid (the chosen material depends of the NP material and the specifics of the
209 apparatus and manufacturer)
210 TEM instrument with imaging modality
211 NP stock dispersions (concentrations and nanomaterials tested are to be pre-
212 determined by the experimenter)
213 1. Wash the grid with a suitable clean solvent as indicated by the supplier (it depends on
214 the material of the grid).
215 *IMPORTANT NOTE: Never touch the grid with hands but always use suitable*
216 *tweezers.*
217 2. Transfer the NP dispersion onto the grid by multiple depositions of 5-10 µl. After
218 each deposition let the solvent evaporate before adding the following drop.
219 *If it is necessary (i.e. if the NPs are dispersed in salt solutions) wash the grid with*
220 *d.H_2O to eliminate the salts as this operation should not remove the NPs, which are*
221 *adhered to the grid surface.*

222 *A rough calculation of the amount of NPs transferred to the grid should be done for*
223 *evaluating the number of depositions necessary to reach the minimum amount of*
224 *sample to perform a statistically significant measure.*

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

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

227 5. For each grid (sample) several images are taken and saved. The images are analyzed
228 with specific image software that allow extracting size information, thus a size-
229 distribution can be determined.

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

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

234

235 **BASIC PROTOCOL 2 – CYTOTOXICITY ASSESSMENT OF NP TREATMENT**

236 A crucial part of our nanotoxicity protocol is cytotoxicity assessment in mammalian cells, as
237 due to their unique material composition, some nanoformulations can have harmful toxic
238 effects in mammalian systems. Multiple factors can influence the extent of nanomaterial
239 toxicity such as NP size, morphology, chemical structure and surface chemistry (Caballero-
240 Diaz and Valcarcel Cases, 2016). A wide variety of conventional *in vitro* assays are available
241 to assess nano-cytotoxicity, for example; 3-(4,5 dimethylthiazol)-2,5 diphenyltetrazolium
242 bromide (MTT), which is a commonly used cytotoxicity assessment assay that has been
243 successfully used to detect nanotoxicity (Gulati et al., 2010; Hussain et al., 2005; Park et al.,
244 2010; Schubert et al., 2006; Webster et al., 2016; Yuan et al., 2010) and provides a simple,
245 reproducible and reliable test set-up. In addition to MTT, nanotoxicity in mammalian cells
246 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 luminiscence, 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, as some nanoformulations can affect cytotoxicity readings by certain experimental approaches (Belyanskaya et al., 2007; Davoren et al., 2007; Hillegass et al., 2010; Monteiro-Riviere et al., 2009; Wang et al., 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 i provides a list of conventional cytotoxicity assessment assays and which NPs are compatible with these methods. Here we describe a protocol that we have previously used for NP cytotoxicity assessment to analyse 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 (recommended a minimum of 3 should be used)

Liquid N_2 cryogenic cell storage Dewar flask (for long-term storage of cell stocks; Cole-Palmer)

272 Water bath (set to 37 ºC; Fisher Scientific; an anti-microbial agent should be added to
273 the water tray to limit contamination)
274 GM containing supplements as required (e.g. foetal bovine serum, amino acids,
275 antibiotics etc., as required depending on the chosen cell types. GM details
276 for specific lines are provided by the supplier or in the scientific literature.
277 All reagents must be cell culture grade)
278 70% ethanol (Sigma-Aldrich)
279 Class II biological safety cabinet (Monmouth Scientific)
280 Sterile, disposable cell culture plastic ware (including flasks, plates, tubes, tips etc.
281 For adherent cells, flasks and plates must be cell culture grade)
282 Humidified 37 ºC, 5% CO_2 cell culture incubator (New Brunswick; an anti-microbial
283 agent should be added to the water tray to limit contamination)
284 Inverted light microscope (Olympus)
285 Phosphate buffered saline (PBS; see recipe)
286 0.05% (w/v) Trypsin-EDTA solution (cell culture grade; Sigma-Aldrich)
287 Swing-out (bucket) centrifuge (Eppendorf)
288 Automated cell counter (e.g. Bio-Rad TC20™) or a Neubauer hemocytometer (Merck
289 Millipore)
290 Mycoplasma testing kit (we use the EZ-PCR mycoplasma test kit; Gene Flow)
291 Multichannel pipette (Fisher Scientific)
292 NP exposure solution (concentrations and nanomaterials tested are to be pre-
293 determined by the experimenter)
294 MTT solution (Sigma-Aldrich; prepared according to the manufacturer's instructions)
295 Dimethyl sulfoxide (DMSO; Sigma-Aldrich)
296 Sorensen's glycine buffer (see recipe)

Microplate spectrophotometer reader (SpectraMax)

1. Resuscitate mammalian cells from cryopreservation. Grow according to recommendations for the chosen cell lines, according to good lab practice (GLP). Correct handling and GLP for cell culturing involves the use of aseptic technique to avoid contamination of the cultures (Freshney, 2010). 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 in man, 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 NOTE: *GM is prepared in advance and can be used for several weeks if stored at 4 ^{o}C. It should be pre-warmed to 37 ^{o}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 NOTE: *Maintenance and preparation of mammalian cell lines should be conducted in a class II biological safety cabinet and 70% ethanol used to sanitise all reagents and plastic ware used in the hood. All reagents must be prepared under aseptic conditions.*

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

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

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

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

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

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

339 4. Following incubation with the NPs, remove the treatment media and wash the cells
340 twice with PBS. Prepare fresh media of 50 µl of MTT (2 mg/ml) in d.H_20, added to a total
341 volume of 250 µl/well and incubate the plate for a further 4 hrs.

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

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

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

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

6. Remove the plate cover and measure the absorbance in each well at 570 nm wavelength using a microtitre plate reader for optical absorbance.

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

*% cell viability = (*I*sample/*I*control)*100 [where* I = *absorbance intensity].*

SUPPORT PROTOCOL 2 – TRYPAN BLUE EXCLUSION ASSAY

As highlighted in Basic Protocol 2, >1 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 of the principle that intact plasma membranes exclude the dye, whereas damaged/dead cells do not (Avelar-Freitas et al., 2014). Mammalian cells stocks are maintained and prepared using GLP as described above (basic protocol 2, point 1).

Materials

369 Mammalian cell lines of choice (recommended a minimum of 3 should be used)
370 See basic protocol 1 for a detailed list of equipment and reagents required for growing
371 mammalian cell lines.
372 NP exposure solution (concentrations and nanomaterials tested are to be pre-
373 determined by the experimenter)
374 0.4% trypan blue solution (Sigma-Aldrich)
375

376 1. Trypsinise and seed mammalian cells at 20000 cells/well in a 24-well, flat-bottomed
377 plate in triplicate (as a minimum). Incubate cells overnight in a cell culture incubator.
378 2. Gently wash cells with PBS (enough to cover the monolayer) and add NPs at the
379 desired concentration in GM at a volume of 500 µl/well. For the control wells add 500 µl/well
380 of GM alone. Incubate cells for 72 hrs.
381 3. Following incubation with NPs, gently wash cells twice with PBS and use 100 µl/well
382 trypsin/EDTA to detach cells from the well. Mix 10 µl of the cell suspension 1:1 with 0.4%
383 trypan blue solution. Incubate for 2 min at room temperature.
384 *Trypan blue should be stored in a dark bottle at room temperature and filtered with a*
385 *0.2 µM filter if used after prolonged storage.*
386 4. Count the unstained (viable) and stained (non-viable) cells. Calculate cell viability
387 using the following equations:
388 *% cell viability = (*unstained cells/total cells*)*100*
389 *% non-viable cells = (stained cells/total cells)*100*
390

391 **SUPPORT PROTOCOL 3 – IMMUNOBLOTTING FOR APOPTOTIC MARKERS**
392 Immunoblotting (or Western blotting) is a molecular technique used to detect proteins in a
393 complex milieu. Following extraction from cells, proteins are separated (usually by sodium

394 dodecyl sulphate-polyacrylamide gel electrophoresis; SDS-PAGE) and then immunoblotted
395 by transferring the proteins to a solid substrate and proteins of interest detected using
396 antibodies targeted against them. Here we describe a protocol using immunoblotting to assess
397 apoptotic cell death in response to NP treatment. A variety of markers can be used to assay
398 apoptosis, should reduced cell numbers be detected in Basic Protocol 2/Support Protocol V
399 (e.g. cleaved Caspase [3,8 and 9], Puma, Noxa and p7056K). Here we describe the use of
400 cleaved Poly (ADP-ribose) polymerase-1 (PARP1) as a read-out of apoptosis. During this
401 type of cell death, Caspase/protease-mediated cleavage of PARP1 in fragments of 89/24kDa
402 is a useful and easily detectable apoptotic hallmark (Kaufmann et al., 1993). The basic
403 protocol is adapted from immunoblot protocols used in our previous work (Jenei et al., 2009;
404 Webster et al., 2016).

405

406 ***Materials***

407

408 Mammalian cell lines of choice (recommended a minimum of 3 should be used)
409 See basic protocol 1 for a detailed list of equipment and reagents required for growing
410 mammalian cell lines.
411 NP exposure solution (concentrations and nanomaterials tested are to be pre-
412 determined by the experimenter)
413 A cytotoxic agent that can be used as a positive control in the cell lines of choice (e.g.
414 cisplatin; this agent and dose should be pre-determined for each cell line selected).
415 PBS (see recipe); 0.1-0.5 L needs to be cooled to 4°C
416 Protein extraction buffer (containing protease inhibitors; see recipe; cooled to 4°C)
417 Plastic cell scrapers (Thermo Fisher Scientific)
418 1.5 ml Eppendorf microcentrifuge tubes (Thermo Fisher Scientific)

419 Sonicator (Diagenode™ Bioruptor® Pico Ultrasonicator; Thermo Fisher Scientific)

420 -20ºC freezer

421 Pierce™ BCA Protein Assay kit (Thermo Fisher Scientific)

422 UV-Vis Spectrophotometer (Orion™ AquaMate 8000; Thermo Scientific)

423 Dithiothreitol (DTT; Sigma-Aldrich)

424 SDS (Sigma-Aldrich)

425 Loading buffer (see recipe)

426 Dry block heating system

427 Tris-HCl buffer (see recipe; Sigma-Aldrich)

428 40% acrylamide/bisacrylamide (Sigma-Aldrich)

429 Ammonium persulfate (APS; Sigma-Aldrich)

430 >99.5% tetramethylethylenediamine (TEMED; Sigma-Aldrich)

431 Mini gel tank and associated casting plates, combs etc. (Mini-PROTEAN® Tetra

432 Vertical Electrophoresis Cell ; Bio-Rad)

433 Running buffer (10X; see recipe)

434 Protein Molecular Weight Standards (range = 6500-205,000 Daltons; Thermo Fisher
435 Scientific)

436 Gel-loading tips (range 0.5–200 µL; Thermo Fisher Scientific)

437 Universal Power Supply (PowerPac™ ; Bio-Rad)

438 Polyvinylidene difluoride (PVDF) membrane (Thermo Fisher Scientific) or

439 nitrocellulose membrane (Thermo Fisher Scientific)

440 Bent-tip stainless-steel forceps (Thermo Fisher Scientific)

441 10X transfer buffer (see recipe)

442 Sponge pad for blotting (Invitrogen)

443 Tris-buffered saline/Tween20 (TBST; see recipe)

444 Shaker plate/roller

445 Ponceau S solution (Sigma-Aldrich)

446 Blocking solution (see recipe)

447 Rabbit anti-PARP-1 antibody (sc-7150; Santa Cruz Biotechnology)

448 Anti-rabbit Horseradish-peroxidase (HRP)-conjugated secondary antibody (#7074;

449 Cell Signalling Technology)

450 ECL^{TM} Western blotting detection reagent (GE Healthcare)

451 ChemiDoc^{TM} XRS+ system (Bio-Rad)

452 Image analysis software (ChemiDoc Touch, Bio-Rad)

453 Mouse anti-α-tubulin (DM1A; Cell Signalling Technology)

454 Anti-mouse Horseradish-peroxidase (HRP)-conjugated secondary antibody (#7076,

455 Cell Signalling Technology)

456

457 ***Protein preparation from mammalian cells***

458 1. Trypsinise and seed mammalian cells at $1x10^6$ cells/10 cm diameter petri dish (cell

459 culture grade) and incubate cells overnight in a cell culture incubator.

460 2. Gently wash cells with PBS (enough to cover the monolayer) and add NP/control

461 treatments at the desired concentration in GM, at a volume of 5-10 ml/plate. Incubate cells

462 for 72 hrs.

463 *A positive control (pro-apoptotic drug) treatment should be used to ensure the*

464 *detection of apoptosis in the cell type of choice.*

465 3. Remove the GM and wash cells twice in ice-cold PBS (enough to cover the

466 monolayer). Remove PBS and add 300 µl/plate ice-cold lysis buffer. Using a cell scraper

467 (chilled to 4°C), scrape the cells off the dish then gently transfer the resulting lysate in a pre-

468 cooled microfuge tube.

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

471 4. Sonicate sample for 15-30 secs, typically 20 to 50 kHz.

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

474 5. Centrifuge lysate at 4ºC for 20 min at 16000*g*. Gently aspirate the supernatant
475 containing the protein extract and store in fresh cold tubes.

476 *At this point samples can be stored as aliquots at -20 ºC. Avoid repeated freeze-*
477 *thawing as this can reduce sample integrity.*

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

481 ***Perform SDS-PAGE***

482 7. Prepare 10-25 µg of total protein by adding DTT at a final concentration of 0.1 M, 1%
483 SDS in loading buffer (4X stock volume) to a total volume of 10-25 µl/sample. Denature
484 samples at 90 ºC for 10 min.

485 *DTT functions as a reducing agent to reduce disulphide bridges, whilst SDS functions*
486 *as an anionic denaturing detergent.*

487 IMPORTANT NOTE: *Wear gloves at all times when handling SDS-PAGE gels, as*
488 *acrylamide is a potent, cumulative neurotoxin.*

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

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

494 *Prepare the gel the same day or the day before (storing overnight in running buffer at*
495 *4 °C). Alternatively pre-cast gels can be purchased.*

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

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

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

504 ***Perform immunoblotting***

505 11. Pre-soak a nitrocellulose transfer membrane in 1 X transfer buffer for 5 min.

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

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

509 12. Prepare the transfer sandwich as previously described (Gallagher et al., 2008).
510 Briefly, sandwich the gel and membrane between layers of pre-soaked filter paper/blotting
511 sponges (in 1 X transfer buffer) in a transfer cassette, ensuring tight contact between the gel
512 and membrane. For tank blotting, assemble the transfer sandwich in the gel tank and perform
513 protein transfer in 1 X transfer buffer at 4 °C, ensuring the membrane faces the anode.

514 IMPORTANT POINT: *Avoid air bubbles between the gel and membrane as this can*
515 *lead to poor protein transfer. Using a clean pipette to roll over the membrane when*
516 *assembling the transfer sandwich can easily remove bubbles.*
517 *Alternatively protein transfer can be done using semi-dry blotting apparatus. These*
518 *systems should be used according to the manufacturer's instructions.*
519 13. Once protein transfer is complete, carefully dismantle the transfer sandwich. Wash the
520 membrane twice in 1 X TBST (enough to cover the membrane) for 5 min on a shaker
521 plate/roller.
522 14. Stain the membrane with Ponceau S solution (enough to cover the membrane) for 1
523 min, to visualise proteins and ensure complete transfer (protein bands will stain red). Then
524 wash the stain away with d.H_20 several times with agitation until all the Ponceau S solution is
525 removed from the membrane.
526 15. Block the membrane for 1-2 h at room temperature with agitation in blocking solution
527 (containing 5% milk; enough to cover the membrane).
528 *5% BSA can also be used as a blocking reagent and for alternative antibodies to the*
529 *ones suggested here, should be used as recommended for individual antibody clones.*
530 16. Dilute the anti-PARP-1 antibody in enough blocking solution to cover the membrane
531 and incubate with the membrane overnight at 4 ^oC with constant gentle agitation.
532 *We standardly use a 1:200 dilution, but this will require optimisation for individual*
533 *cell types to determine the optimal antibody/protein ratio.*
534 17. Wash the membrane three times in 1 X TBST for 10 min each at room temperature
535 with constant agitation.
536 *This step is important to remove any unbound antibody.*
537 18. Add the cognate secondary antibody diluted in blocking solution for 1 h at room
538 temperature with gentle agitation.

539 *Use the secondary antibody at a minimal dilution of 1:2500, although this will*
540 *require optimisation for the cell types used.*
541 19. Repeat step 17. Incubate the membrane with ECL reagent (according to the
542 manufacturer's instructions) and detect the chemiluminescent signal using the desired
543 imaging system (e.g. the ChemiDoc^{TM} XRS+ system; Bio-Rad). Use image analysis software
544 to analyse protein band intensity.
545 *X-ray film (with/without automated developing) is also a commonly used method for*
546 *signal detection.*
547 20. Rinse membrane in methanol and then repeat step 17. Dilute the anti-α-tubulin
548 antibody in enough blocking solution to cover the membrane and incubate with the
549 membrane overnight at 4 ^{o}C with constant gentle agitation.
550 *Detection of α-tubulin in the cells is used as a loading control. The choice of a*
551 *loading control can be modified depending on the cell type used and the size of the*
552 *protein(s) of interest being detected by immunoblotting.*
553 21. Repeat steps 17-19.
554 *Determining the ratio between the cleaved PARP-1 (89 kDa) and full-length PARP-1*
555 *(116 kDa) bands relative to the gel loading control, can be used as a readout for*
556 *caspase-mediated apoptosis.*
557
558 **BASIC PROTOCOL 3 – *X. LAEVIS* PHENOTYPIC ABNORMALITY ASSAY FOR**
559 **NANOTOXICITY ASSESSMENT**
560 This protocol is designed to be used in parallel with cell-based cytotoxicity assays as part of
561 an integrated toxicity assessment in order to obtain a complete safety profile of a novel NP
562 (Fig. i). *X. laevis* is an ideal model organism to be used for comparatively high-throughput
563 screening (Tomlinson et al., 2009) and has been used as a toxicity model in the frog

564 teratogenesis assay-*Xenopus* (or FETAX assay) for drugs in their early stages of drug safety
565 evaluation (Leconte and Mouche, 2013). This is largely due to *X. laevis* being a relatively
566 inexpensive and rapid model that that can be easily scaled-up as a large number of embryos
567 can be produced. *X. laevis* embryos develop externally, making them an easily accessible
568 system for exposure to NPs. Previous work has shown that this methodology allows both
569 external NP exposure and internal exposure to key internal organs for assessing potential
570 toxicity (Webster et al., 2016). Briefly, *X. laevis* embryos are exposed to a NP-containing
571 incubation solution over a desired developmental period that can be adapted depending on the
572 specific aims of the nanotoxicity assessment protocol.
573
574 ***Materials***
575
576 Nieuwkoop and Faber (NF) stage 1 *X. laevis* embryos (see Support Protocol 4)
577 MMR solution (see recipe)
578 Pasteur pipette (we recommend glass. Whole embryos are too large to fit into a
579 standard pipette, therefore mark the end with a diamond pen, break off
580 cleanly and fire the end briefly to melt any sharp edges. Alternatively, if
581 desired, plastic Pasteur pipettes can be used with the end removed)
582 10 cm^2 Petri dish (Fisher Scientific)
583 Culture incubator (set to desired temperature; see below for details)
584 Stereomicroscope with two-armed fibre optic illuminator to allow the angle of
585 illumination to be easily adjusted
586 Dumont #5 forceps (stainless steel; Sigma-Aldrich). These are ultrafine and can be
587 used for carefully manipulating embryos throughout the described protocol.
588 24-well culture plate (non-cell culture grade; Fisher Scientific)

589 NP exposure solution (concentrations and nanomaterials tested are to be pre-
590 determined by the experimenter)
591 Ethyl 3-aminobenzoate methanesulfonate (0.6 mg/ml; Fluka)
592 MEMFA fixative (see recipe)
593 Phosphate-buffered Saline (PBS) and PBST (see recipes)
594 3 cm^2 Petri dish (Fisher Scientific)
595 Agarose gel (2% [w/v]; Sigma-Aldrich; see recipe)
596 Long-handled scalpel (10A blades)
597 Light microscope with charge coupled-device (CCD) digital camera for whole-mount
598 imaging of embryos
599 Methanol (analytical grade; Sigma-Aldrich), 25%, 50%, 75%, 100% [v/w] in PBS
600 Glass vials with screw caps (3.5 ml; SGL)
601 Parafilm M™ wrapping film (Fisher Scientific)
602 -20 °C freezer
603
604 1. Harvest NF stage 1 *X. laevis* embryos (see Support Protocol 4) and incubate between
605 12–23°C until at required developmental stage (Fig. iii). During the incubation times it is
606 important to regularly observe the embryos (at least twice daily or more at early stages)
607 to remove any dead embryos and ensure the correct NF stage has been reached.
608 *Developmental times of embryos are dependent on incubation temperature and*
609 *culturing them at differing temperatures can speed or slow development. Typically,*
610 *after incubation at 23°C, embryos are NF stage 4 after ~ 2 h, NF stage 15 after ~ 17*
611 *h, and NF stage 38 after incubation for ~ 2 days 5 h.*
612 IMPORTANT NOTE: *Bacteria grow well at the higher incubation temperatures so*
613 *embryos cultured between 18-25 °C should be regularly monitored and washed twice*

614 *daily. To avoid this problem, the 0.1X MMR culture media of later stage embryos*
615 *(NF stage 23 onwards) can be supplemented with 25 µg/ml of gentamicin.*
616 2. In a 24-well plate, add 200 µl of NPs in 0.1 X MMR solution to each well at a
617 concentration that is 10 X higher than that of the desired final concentration. For the
618 control wells, add 200 µl 0.1 X MMR alone.
619 3. At the required NF stage, select 5 healthy embryos and transfer using a volume of
620 1800 µl 0.1 X MMR using a glass Pasteur pipette into one well of a 24-well plate. Repeat
621 until the wells for each of the desired NP concentrations (along with the control wells)
622 contain 5 embryos to a final volume of 2 ml. Incubate at the same temperature that the
623 embryos were initially developed at.
624 4. Continue to incubate *X. laevis* embryos until they have reached the desired end stage
625 (Fig. iii).
626 *Again it is important that the embryos are checked several times a day to identify*
627 *any dead ones and to assess developmental progress. Dead embryos should be*
628 *removed from the well during this incubation period and the number of dead*
629 *recorded.*
630 5. Make a note of any dead *X. laevis* embryos at the end of the incubation time. Wash
631 embryos with 0.1 X MMR and using a Pasteur pipette, gently transfer to a new 24-well
632 plate containing 1 ml of 0.6 mg/ml ethyl 3-aminobenzoate methanesulfonate salt to
633 anesthetise the embryos. Incubate for 20 min at room temperature to ensure embryos are
634 fully anesthetised prior to fixing (Sherwood et al., 2008; Webster et al., 2016).
635 *A variety of nanomaterials are synthesised for use as fluorescent bioimaging tools*
636 *(Wolfbeis, 2015). If such fluorescent NPs are being tested using this protocol (e.g.*
637 *metal chalcogenide quantum dots [QDs]) they can be detected in the embryos*

638 *using live whole-mount fluorescent imaging at this stage in the protocol (Webster*
639 *et al., 2016); see support protocol 5).*

640 6. Wash away the anaesthetic solution with several rinses of 0.1 X MMR before fixing
641 the embryos with MEMFA for 1 h at room temperature or overnight at 4°C.

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

643 *If the embryos are going to be used for transmission electron microscopy (TEM;*
644 *which can be used to determine exposure to non-fluorescent NPs) then MEMFA*
645 *should not be used for embryo fixation. Rather an alternative fixing protocol*
646 *provides improved ultrastructural analysis of* X. laevis *embryos by TEM (see*
647 *alternate protocol 1).*

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

650 8. Whole-mount images of the embryos should now be taken to assist with phenotypic
651 scoring. To do this, prepare a 2% (w/v) agarose gel by heating 100 mg agarose in 5 ml
652 PBS until all the agarose has dissolved. Then pour the 2% agarose into the bottom of a
653 10 cm^3 culture dish and leave to cool and set (should take approximately 30 minutes).

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

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

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

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

663 10. Observe each embryo using a light microscope and rank for phenotypic abnormalities
664 (Table iii). Calculate phenotypic abnormality; the number of malformed larvae as a
665 percentage of the total number at the beginning of the experiment. Likewise percentage
666 mortality should be calculated in the same way.

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

670 *Exposure should be confirmed of NPs that do not produce notable nanotoxicity as*
671 *scored in this phenotypic abnormality assay. If the NP is fluorescent this can be done*
672 *as described in point 5 (see support protocol 5), but if not we propose that*
673 *transmission electron microscopy (TEM) imaging of X. laevis tissue will facilitate*
674 *confirmation of NP uptake in the embryos (see alternate protocol 1).*

675 11. Following scoring, dehydrate the embryos for long-term storage. To do this, transfer
676 the embryos into glass vials using a Pasteur pipette. Gently aspirate the PBST and
677 replace with 25% methanol in PBS for 5 min, completely immersing all embryos in the
678 glass vial.

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

681 *If required, embryos can be rehydrated for further analysis by reversing steps 12 and*
682 *11.*

683 13. After dehydration, *X. laevis* embryos can be stored long term in 100% methanol at -
684 20°C. Finally, seal the glass vial cap with Parafilm for long-term storage at -20°C.

685

686 **SUPPORT PROTOCOL 4 – HARVESTING *X. LAEVIS* EMBRYOS**

687 *X. laevis* have been used as model organisms for biological research for decades, particularly
688 as developmental vertebrate systems. As a result, detailed methodologies have been devised
689 to obtain and work with *X. laevis* embryos (Sive et al., 2000). Ethical legislation and
690 considerations must be in place when working with adult *X. laevis* frogs, the specific
691 requirements of which will be dependent upon geographical and institutional location. This is
692 not only a legal requirement in many countries, but such ethical considerations will also assist
693 with maintaining a well cared for population of adult frogs for generating healthy embryos.
694 You will need access to an aquarium facility for holding *X. laevis* colonies, where males and
695 females should be housed in separate tanks. The following protocol describes the steps
696 required to collect eggs and conduct fertilisations in order to obtain *X. laevis* embryos for
697 nanotoxicity assessment (see Basic Protocol 3).
698
699 ***Materials***
700
701 Female *X. laevis* adults (2 or more)
702 Pregnant mare serum gonadotrophin (PMSG; Intervet)
703 25-gauge (25G; BD Biosciences) needle and 1 ml syringe (Fisher Scientific)
704 Non-textured, powder-free gloves (Fisher Scientific)
705 Human chorionic gonadotrophin (hCG; Intervet)
706 Culture incubator (set to 17°C)
707 10 cm^2 Petri dish (Fisher Scientific)
708 MMR (see recipe)
709 1 male *X. laevis* adult
710 0.05% Ethyl 3-aminobenzoate methane sulfonate (Fluka)
711 Surgical equipment including scalpels, forceps and curved scissors

712 Testis buffer (see recipe)

713 -20°C freezer

714 d.H_2O

715 Cysteine de-jellying solution (see recipe)

716 Glass beaker

717

718 1. Prime female *X. laevis* with an injection of 100 units of PMSG into the dorsal lymph
719 sac 5–7 days before requiring embryos.

720 *We recommend priming and inducing ovulation in >1 female, in case egg yield and*
721 *quality is not good, as this can vary greatly between individual animals.*

722 2. Isolate testes from an adult male *X. laevis* by first anesthetising him by submersion in
723 0.05% Ethyl 3-aminobenzoate methanesulfonate for a minimum of two hours. Remove
724 the testes by exposing the abdominal cavity and drawing out the fat body with forceps.
725 The testes lie at the base of the fat body and can be identified as white, oval shaped
726 organs covered in a fine network of capillaries. Remove both testes and store in testes
727 buffer at 4 ^{o}C for up to 14 days post-isolation.

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

731 3. Induce ovulation by injection of 250 units hCG into each of the dorsal lymph sacs
732 (500 units total) using a 25G needle. Incubate induced females at 17°C.

733 *The dorsal lymph sac is located directly rostral to the hind limbs. It can be located*
734 *between the lateral line that appears as 'stich' marks on the adult's skin and the*
735 *spine.*

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

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

744 IMPORTANT NOTE: *Eggs should be fertilised immediately when collected in this*
745 *manner. From this point onwards in the protocol it is critical to progress as rapidly*
746 *as possible through the remaining steps, this helps ensure quality of the resulting*
747 *embryos.*

748 *As an alternative to 'squeezing', eggs can be collected passively by allowing females*
749 *to lay in 1X MMR, where eggs will be viable for fertilisation for up to 8 h post-laying.*

750 5. Fertilise the harvested eggs by cutting off a small piece of one testis (<25%) and
751 homogenise the testis section using a scalpel blade and forceps. Add 1ml 1X MMR to the
752 mashed up testis piece. Mix the testis slurry well with the eggs across the entire dish to
753 promote fertilisation. Leave for 5 minutes then flood the dish with 0.1X MMR and leave
754 for 20-30 minutes.

755 6. Incubate the eggs at 17 ^{o}C and monitor regularly for successful fertilisation. The first
756 sign is a cortical contraction of the animal pole approximately 5 min post-fertilisation.
757 However, by 15-30 min fertilised eggs will reorient such that the animal pole faces up,
758 which is the most reliable sign that fertilisation has been successful.

759 *The release of cortical granules into the space between the fertilised egg and the*
760 *vitelline membrane blocks polyspermy and causes the eggs to turn with their*

761 *membranes according to gravity, with their pigmented animal poles facing up. At this*
762 *point fertilised eggs will be much firmer than unfertilised ones, so it is easy to tell if*
763 *the fertilisation has been successful or not by 30 min post-fertilisation.*
764 7. Continue to incubate the fertilised eggs at 17 ºC for another 1-2 h. Upon entering the
765 first cell cycle, cortical rotation occurs, which is required for formation of dorsal tissues
766 and usually occurs within 2 h of fertilisation at 17 ºC.
767 IMPORTANT NOTE: *Do not disturb the embryos during this incubation period too*
768 *much as it can interfere with correct dorso-ventral patterning. For example, shaking*
769 *the embryos during this time is known to produce spontaneous secondary axis*
770 *formation through microtubule reorientation.*
771 8. *X. laevis* embryos are surrounded by a thick layer of protective jelly that must be
772 removed prior to further experimentation. Ideally this should be done after cortical
773 rotation to reduce the likelihood of developmental defects (see point 7). In a glass beaker,
774 gently swirl the embryos in 2% cysteine (w/v) de-jellying solution until they pack closely
775 together.
776 *The time required for this step can vary depending on differences between embryo*
777 *batches, however it should normally take around 5 min and no longer than 10 min.*
778 IMPORTANT NOTE: *The de-jellying solution needs to be made fresh on the day of*
779 *use and used at room temperature.*
780 IMPORTANT NOTE: *Do not over-treat as this can lead to developmental defects*
781 *and can contribute to poor embryo quality.*
782 9. Remove cysteine solution and wash the eggs several times with distilled water (>5
783 washes) followed by several washes with 0.1 x MMR. Embryos are then reared in 0.1 X
784 MMR, ready for further experimental procedures.
785

786 **SUPPORT PROTOCOL 5 – WHOLE-MOUNT IMAGING OF *X. LAEVIS* EMBRYOS**
787 **FOR FLOUORESCENT NP UPTAKE**

788 This protocol can be used to investigate internalisation of fluorescent NPs in *X. laevis*
789 embryos. We have previously demonstrated that this protocol works well using 20 nm
790 fluorescent carboxylate-modified NPs (PS-COOH; Molecular Probes FluoSphere beads®;
791 Thermo Fisher Scientific®; catalog #F8887), thus we propose that these NPs offer a useful
792 positive control for NF stage 45 embryos, exposed to 10^{15} NP/ml from NF stage 38 (Webster
793 et al., 2016).

794

795 ***Materials***

796

797 3 cm^2 Petri dish (Fisher Scientific)
798 Agarose gel (2% [w/v]; Sigma-Aldrich; see recipe)
799 Long-handled scalpel (10A blades)
800 Tadpole stage *X. laevis* embryos (from NF stage 38 onwards; see Fig. iii),
801 anesthetised and pre-exposed to florescent NPs (see point 5, Basic Protocol 3
802 for details). As described above, 20 nm PS-COOH NPs should be used as a
803 positive control.
804 Glass Pasteur pipette (prepared as described in Basic Protocol 3)
805 Dumont #5 forceps (stainless steel; Sigma-Aldrich). These are ultrafine and can be
806 used for carefully manipulating embryos throughout the described protocol.
807 PBS (see recipe)
808 Fluorescent microscope with CCD digital camera
809

810 1. Prepare agarose imaging plates for whole-mount *X. laevis* embryos as described in
811 point 8, Basic Protocol 3.
812 *2.* Pour a small layer of PBS over the agarose gel and gently transfer the *X. laevis*
813 embryos into the agarose gel-containing imaging plate (see point 9, Basic Protocol 3 for
814 details).
815 3. Image the embryos using a fluorescent microscope according to the emission filter
816 required to excite the NPs being tested.
817 *For the PS-COOH NPs, an emission filter of 509-547 nm should be used. The*
818 *fluorescence from these NPs will appear bright throughout the embryo (Webster et*
819 *al., 2016).*
820 4. Time-lapse images (with time-frame stills of 0.7 seconds) can be used to monitor
821 fluorescent NPs traveling through the vasculature of the *X. leavis* embryos, which is
822 particularly clear in the embryonic intersomitic blood vessels (Webster et al., 2016).
823
824 **ALTERNATE PROTOCOL 1 – TEM IMAGING OF *X. LAEVIS* EMBRYO**
825 **SECTIONS FOR NP UPTAKE**
826 Support protocol 5 cannot be used to confirm uptake of non-fluorescent NPs in *X. laevis*
827 embryos and for this reason such NPs require an alternative procedure to ensure embryo
828 exposure to these nanomaterials. Electron microscopic techniques facilitate high-resolution
829 visualization of NPs in tissues and in particular TEM has been used for a long-time in NP
830 research. Due to the complexity of sample preparation, imaging and interpretation of
831 ultrastructual NP localisation within tissues, and that the infrastructure required for TEM
832 analysis is often housed in centralised facilities, where possible it is pertinent to seek advice
833 about TEM experimental design with expert staff within such core facilities. This will assist
834 with optimisation of advanced TEM imaging for specific nanomaterials, but here we describe

835 a protocol that is suitable for preparing high-quality *X. laevis* embryo sections that is suitable
836 at least for imaging iron oxide core NPs (Webster et al., 2016). The processes of fixing,
837 embedding and sectioning *X. laevis* embryos for TEM is based on a previously described
838 method developed for imaging carbon NPs *in vivo* (Bacchetta et al., 2012).
839

840 ***Materials***

841

842 Tadpole stage *X. laevis* embryos (from NF stage 38 onwards; see Fig. iii),
843 anesthetised and pre-exposed to NPs (see point 5, Basic Protocol 3 for details).
844 Glass Pasteur pipette (prepared as described in Basic Protocol 3)
845 MMR (see recipe)
846 TEM fixing buffer (see recipe)
847 Osmium tetroxide (OsO_4; Sigma-Aldrich), 1% [v/w] in PBS
848 Methanol (analytical grade; Sigma-Aldrich), 25%, 50%, 75%, 100% [v/w] in PBS
849 Propylene oxide resin (TAAB Laboratories Equipment Ltd.)
850 Incubator (set to 60 °C)
851 Microtome (Reichert Ultracut E)
852 Carbon-coated 300 µM mesh copper grids (Agar Scientific)
853 TEM instrument with imaging modality
854

855 1. Immerse *X. laevis* embryos in 0.6 mg/ml ethyl 3-aminobenzoate methanesulfonate salt
856 for 20 min at room temperature to anesthetise.
857 2. Wash away the anaesthetic solution with several rinses of 0.1 X MMR and fix the
858 embryos in TEM fixing buffer (enough to immerse the embryos) for 1 h at room temperature.
859 During this time replace the TEM fix twice with fresh buffer.

860 3. Post-fix the embryos in 1% OsO_4 for 1.5 h at 4 °C.

861 *This step is needed to increase the electron density in lipids and proteins.*

862 4. Dehydrate the fixed embryos in a decreasing concentration of methanol, as described

863 for point 12 of basic protocol 3.

864 5. Once dehydrated, wash the embryos in 75% propylene oxide resin and leave in 100%

865 pure resin overnight.

866 6. Submerse embryos in fresh resin and then polymerise at 60 °C for 48 h.

867 7. Using a microtome, cut semi-thin 1 µm sections of the embryos.

868 *Cut in an anterior to posterior direction to produce transverse sections along the*

869 *entire embryo. Analyse all tissues across the anterior-posterior axis as the location of*

870 *the NPs will depend upon the biodistribution of specific nanomaterials within X.*

871 *laevis embryos.*

872 *Ultrathin sections (~50 nm) can also be used if required for NP detection.*

873 8. Mount the sections in onto carbon-coated 300 µm mesh copper grids.

874 9. Image sections using a TEM according to the settings required for the instrument.

875 *As an example, we have successfully used a Tecnai™ 20 TEM (FEI; Thermo Fisher*

876 *Scientific) with AMT cameras, operating at an acceleration voltage of 200 kV to*

877 *image iron oxide core NPs (Webster et al., 2016). Likewise carbon NPs have been*

878 *successfully imaged in* X. laevis *embryos using a Zeiss LEO 912ab Energy Filtering*

879 *TEM at 80 kV (Bacchetta et al., 2012).*

880

881 **REAGENTS AND SOLUTIONS**

882 General laboratory reagents are supplied by Sigma-Aldrich. Use d.H_20 in the following

883 recipes (unless otherwise stated):

884

885 ***Agarose gel***

886 100 mg agarose

887 5 ml PBS

888

889 ***Blocking solution***

890 5 g BSA

891 100 ml 1 TBST

892

893 ***Blocking Buffer:***

894 7.5 g nonfat dry milk

895 15 ml 10X TBS

896 0.15 ml Tween-20 (100%)

897 Final volume 150 ml

898

899 ***Cysteine solution***

900 3 g cysteine

901 100 ml 0.1 x MMR

902 Adjust to pH 7.8 with 10 M NaOH

903

904 ***4X Loading buffer***

905 3 mL of 1 M DTT

906 1.5 mL of 1 M pH 6.8 Tris-HCl

907 0.6 g of SDS

908 2.4 mL of glycerol

909 0.03 g of bromophenol blue

910 Final volume to 7.5 ml. Store at -20°C

911

912 ***Lysis buffer***

913 50 mM Tris-HCl

914 1% Triton X-100

915 150 mM NaCl

916 Final volume to 200 ml in PBS. Adjust pH to 7.4.

917

918 ***MMR***

919 100 mM NaCl

920 2 mM KCl

921 1 mM $MgCl_2$

922 2 mM $CaCl_2$

923 5 mM HEPES (pH 7.6)

924 Adjust to pH 7.4.

925

926 ***MEMFA***

927 ***i) 10 X MEM salts*** *(autoclave and store in the dark)*

928 1 M MOPS

929 20 mM EGTA

930 10 mM $MgSO_4$

931 5 mM HEPES (pH 7.6)

932 Adjust to pH 7.4 with NaOH pellets. Dilute in d.H_2O for a 1 X working

933 solution.

934 ***i) 1 X MEMFA***

935 3.7% formaldehyde

936 1 X MEM salts

937

938 ***PBS/PBST***

939 ***i) 10 X PBS***

940 1.4 M NaCl

941 26.8 mM KCl

942 100 mM Na_2HPO_4

943 17.6 mM KH_2PO_4

944 Adjust to pH 7.4 with HCl. Dilute in d.H_2O for a 1 X working solution

945 ***ii) 1 X PBST***

946 0.1% Tween-20

947 1 X PBS

948

949 ***Ponceau stain***

950 0.2 g Ponceau S

951 5 ml glacial acetic acid

952 Final volume to 100 ml

953

954 ***10 X Running buffer***

955 30.2 g Tris-base (25 mM)

956 144 g Glycine (190 mM)

957 0.1% SDS

958 Final volume to 1 L. Adjust to pH 8.3

959

960 ***Sorensen's glycine buffer***

961 121 g Tris Base

962 28.55 ml Acetic Acid

963 50 ml, 0.5 M EDTA

964 Final volume to 500 ml. Adjust to pH 8.0

965

966 ***TBST***

967 24.23 g Tris-HCl

968 80.6 g NaCl

969 0.1% Tween-20

970 Final volume to 1 L. Adjust to pH 7.6. Dilute in d.H_2O for a 1 X working

971 solution. Add 0.1% Tween-20.

972

973 ***TEM fixing buffer***

974 4% paraformaldehyde

975 2% glutaraldehyde

976 0.1M sodium cacodylate buffer (4.28 g sodium cacodylate in 200 ml d.H_2O)

977 Adjust to pH 7.4

978

979 ***Testis buffer*** *(in 1 X MMR)*

980 80% Foetal Calf Serum

981 50 µg/ml gentamycin-sulfate

982

983 ***10 X Transfer buffer***

984 30.2 g Tris-base (25 mM),

985 144 g Glycine (190 mM)

986 0.1% SDS

987 Volume to 1 L. Adjust to pH 8.3

988

989 ***10 X Tris-HCl buffer***

990 61 g Trizma Base

991 Volume to 1 L. Adjust to pH 7.6 using HCl

992

993 **COMMENTARY**

994

995 **Background Information**

996 Here we have described the use of non-specialist cytotoxicity testing protocols in
997 combination with a *X. laevis* embryonic phenotypic assay for nanotoxicity assessment.
998 Specifically, testing well characterised nanomaterials at the physico-chemical level (Basic
999 protocol 1) with standard cytotoxicity assessment (Basic protocol 2) and using this in
1000 combination with the *X. laevis* embryonic phenotypic assay (Basic protocol 3), can bridge the
1001 gap between conventional *in vitro* (cell culture models) and *in vivo* (mammalian systems)
1002 nanotoxicity assessment (Webster et al., 2016). We have shown that direct comparison of the
1003 cytotoxicity and *X. laevis* data can provided a logical ranking system to generate an overall
1004 hazard score for NPs (Webster et al., 2016). Briefly, a simple scoring system ranging from 0-
1005 2 can distinguish hazard score, where NPs score 0 when the percentage of cell viability and
1006 healthy *X. laevis* embryos is >76%, 1 when this percentage ranges from 50-75% and 2 when
1007 it is <50%. From these criteria only NPs that score 0 in all nanotoxicity assessment protocols
1008 should progress to further toxicity assessment in mammalian models (Fig. i). This approach
1009 can reduce false negatives that could otherwise be generated from cell-based assays used in

1010 isolation. Thus, only NPs that produce no-to-low toxicity assessment in the described
1011 protocol progress to further evaluation in mammalian systems, thereby reducing investment
1012 in time and money spent on more costly rodent models, which is important given the year on
1013 year increase in development of nanotherapeutics. Overall, this protocol provides biomedical
1014 researchers with nanotoxicity assessment at early stage in nanotherapeutic design to quickly
1015 and easily identify nanomaterials that require additional modifications for improved safety,
1016 prior to mammalian testing (Fig. i).

1017

1018 **Critical Parameters and Troubleshooting**

1019 There are several critical parameters that will affect successful outcome of the described
1020 protocol and therefore must be considered by users. These parameters include the following:

1021

1022 ***Dosing and storage of NPs.***

1023 The most suitable conditions of NP storage depend on the type of material from which
1024 the NPs are composed of. It is not possible to state general conditions. The chosen medium
1025 should guarantee stability of the NPs over time. If the material is not sensitive to low
1026 temperature it is suggested to store stock solutions in the fridge, mostly if they contain
1027 organic/biological moieties to avoid degradation. Before making any measurements it is also
1028 necessary to check the stock solution in terms of homogeneity in order to guarantee the right
1029 evaluation of the dose. Often, NP dispersions can be affected by flocculation over time, if
1030 flocculation is reversible, this process does not represent a problem. It is only necessary to re-
1031 disperse the sediment in the dispersion through simple shaking and/or 5-10' sonication of the
1032 NP dispersion before the measurement or preparation of the samples.

1033

1034 ***Cell culture considerations***

1035 There are several important considerations when conducting cytotoxicity analysis for
1036 NP testing. The first is to select cell types (3 or more) that best model the exposure route(s)
1037 and target organ(s) of the nanomaterial of interest. Next, the appropriate methodology must
1038 be selected that can accurately assess cytotoxicity of the NP of interest without the
1039 development of false-negatives/-positives, which is important to carefully consider because
1040 not all nanomaterials are compatible with commonly employed methods. For example, MTT
1041 (the method described here; Basic protocol 2) although being easy, quick and readily
1042 affordable, is not compatible with several types of NPs. Wang Yu and Wickliffe, 2011
1043 indicated that titanium oxide nanoparticle (nano-TiO2) induces superoxide formation in
1044 mammalian cells that reduces tetrazolium salts and produces the absorbant formazan end
1045 products (Wang et al., 2011). Monteiro-Riviere, Inman and Zhang, 2009 showed that single-
1046 walled carbon nanotubes SWCNT and carbon black CB alone (absence of cells) interact with
1047 the MTT to cleave the tetrazolium ring and lead to a false positive reaction (Monteiro-Riviere
1048 et al., 2009). Whilst Belyanskaya et al., 2007 found that sodium dodecyl sulfate-suspended
1049 SWCNTs interfere more with MTT assay than polyoxyethylene sorbitan monooleate-
1050 suspended SWCNTs (Belyanskaya et al., 2007). Table i lists which types of NP-based
1051 materials have previously been demonstrated to be compatible with commonly employed
1052 cytotoxicity assays. Finally, it is essential to use GLP when conducting *in vitro* cell work;
1053 including cell line validation, equipment validation/maintenance, mycoplasma contamination
1054 testing, employment of strict aseptic technique and using low-passage cell culture are all
1055 critical in obtaining high-quality, reproducible cytotoxicity data.

1056

1057 ***Immunoblotting considerations***

1058 Immunoblotting is a simple molecular procedure for the quantitative detection of
1059 proteins in cells/tissues. Here we describe a protocol to detect apoptotic markers in response

1060 to NP-induced cytotoxicity (Support protocol 3). Despite its simplicity, an array of problems
1061 can be encountered that require troubleshooting to prevent unexpected results and a
1062 comprehensive description of effective immunoblotting troubleshooting has previously been
1063 provided (Mahmood and Yang, 2012). Briefly, use fresh protein sample using lysis buffer
1064 containing phosphatase inhibitors to prevent sample degradation and ensure the transfer
1065 sandwich is effectively prepared by avoiding air bubbles between the gel, and membrane. A
1066 final crucial consideration for immunoblotting is effective optimisation of antibody
1067 concentration for specific samples, as too low and the signal will not be visible and too high
1068 could result in over-exposed (negative) bands on the blot, and high background signal.
1069 Altering membrane-washing times, the blocking reagent used and membrane exposure times
1070 can also dramatically affect signal-to-noise ratio, and therefore can also require optimisation.
1071

1072 **X. laevis *egg quality***

1073 A major critical parameter for nanotoxicity assessment in *X. laevis* embryos is the
1074 quantity and quality of egg production (and thus the zygotes generated from these), which has
1075 a major influence on the collection of reliable data. The *Xenopus* research community are
1076 aware that egg quality and production levels are variable, which is often attributed to
1077 differences between individual females. Therefore, experimental replication can be improved
1078 by acquiring eggs from consistently good producers. Acceptable methods for identification of
1079 individuals include tagging (with beads or microchips), tattooing, branding, monitoring of
1080 dorsal markings in pigmented frogs and perhaps more simply (if space is available), housing
1081 individuals in designated tanks. Implementing the following basic policies will increase the
1082 chances of quality egg harvests:

1083 i) Comprehensive training of personnel preforming the procedures.
1084 ii) Introducing a robust system for identifying individual animals.

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

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

v) Detailed record keeping of all procedures conducted.

vi) 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 et al., 2011; Godfrey and Sanders, 2004; Green, 2002; Hilken et al., 1995; Sigel, 1990; Wu and Gerhart, 1991). Although some of these effectors are difficult to control, they can be minimised by maintaining a 12 h light/12 h dark cycle, a constant temperature (21-23°C), feeding once every 2-3 days, enriching the environment with functional items for the frogs (e.g. plastic plants, logs, dishes etc.) 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

1110 fixed at NF stage 38. Over this time, the embryos are exposed to NPs during key
1111 developmental processes such as gastrulation (NF stage 10) and neurulation (NF stage 15).
1112 The NF stage at which the NPs are applied will greatly affect exposure too. For example,
1113 between NF stages 38-45 the gills and mouth of the embryos are open, providing additional
1114 routes of exposure for NPs aside from the porous skin, as we have previously discussed
1115 (Webster et al., 2016). As highlighted in the protocol description, it is essential to confirm
1116 that the embryos have been exposed to the NPs being tested by the experimental end point,
1117 which we propose can be done using microscopy (see Support Protocol 5 and Alternate
1118 Protocol 1). This is of particular importance for nanomaterials that do not produce visible
1119 toxicity in the embryos.
1120

1121 **Anticipated Results**

1122 NP physical characterization is crucial step in a toxicity evaluation of NP dispersions
1123 for both *in vitro* and *in vivo* experiments. Importantly, it is necessary to evaluate stability and
1124 size distribution of the NP dispersions in experimental conditions that mimic, as much as
1125 possible, the conditions similar to those used in the biological nanotoxicity assays (i.e.
1126 temperature, dispersion medium, NP dose, etc.). Stability of the NP dispersion in its
1127 dispersing medium does not guarantee that such NPs are equally stable in the media used in
1128 the biological study. Biological media are complex fluids containing biomolecules and salts
1129 that can strongly affect NP self-assembly in solution, in some cases also causing
1130 agglomeration and precipitation. It is known that NP cellular interaction and uptake are
1131 affected by NP physical properties and size, thus to interpret NP biological response it is
1132 necessary to know their features in the biological environment. DLS is the best technique to
1133 investigate the stability of the NP dispersions in different media over time at biologically
1134 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 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.

Cytototoxicity assessment is an essential step in the described process of NP hazard assessment (Fig. i). 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 section; cell culture considerations for discussion). Ideally the selected methodologies should cover >1 cytotoxic assessment parameters (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 combined, 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.

1159 The *X. laevis* phenotypic abnormality assay (Basic Protocol 3) results in the
1160 percentage of embryos that did not survive NP exposure and the percentage that display
1161 phenotypic abnormalities relative to the total number of embryos tested, and therefore
1162 represents the percentage lethality and percentage abnormality, respectively. Expected
1163 abnormalities commonly include eye malformations, bent anterior-posterior axis, oedema,
1164 blistering, stunted growth and pigmentation loss (Table iii). We have previously described
1165 example results for a range of high-to-low toxicity-inducing nanomaterials (Webster et al.,
1166 2016). As discussed above, comparison between the *X. laevis* phenotypic abnormality data
1167 and the cytotoxicity results provides a hazard ranking score for NP safety, which can be used
1168 to determine whether or not further nanotoxicity assessment in mammalian systems is
1169 permissible or if further optimisation of NP design/synthesis is first needed to reduce toxicity
1170 of the developed nanoformulation (Fig. i).
1171
1172 **Time Considerations**
1173 *Basic Protocol 1:* Preparation of the samples for DLS measurements is a quick
1174 procedure that generally involves the dilution of the NP stock dispersions in the different
1175 biological media. A DLS experiment is quite fast, it will take between 5-15 min depending on
1176 if the measurement is performed at fixed angle or at different angles (in the latter case it will
1177 be longer). The measurements should be repeated over the experimental time of the
1178 biological assay with closer repetitions in the first day. Overall, the experimental time
1179 depends on the sample numbers and duration of the biological experiments. Moreover,
1180 additional time should be considered for the analysis of DLS data for multi-angle
1181 measurements for which the operator needs to make some more analysis work after the
1182 experiments.

1183 Support Protocol 1: Preparation of TEM samples on suitable grids requires at least
1184 overnight incubation to guarantee complete evaporation of the solvent. Generally, the grids
1185 will be analysed by a specialized technician, thus the experimental time is not predictable.
1186 The actual measurement takes approximately half an hour for sample (different areas of the
1187 grids need to be imaged). After that the operator will need to analyse the images with specific
1188 imaging softwares for extracting a size distribution of the NPs. The duration of this analysis
1189 depends on the quality of the images and the properties of the sample, if the NPs are well
1190 separated usually it is possible with most imaging softwares to automatically measure the size
1191 of all the NPs. While if the NPs formed agglomerates on the grid, size measurement of each
1192 single NP has to be done manually and this will take longer time.

1193 *Basic Protocol 2:* Preparation of mammalian cell line stocks, validation and
1194 preparation of cells for experiments will take 2-3 weeks depending on how well the specific
1195 cells grow in culture. Seeding/growing cells will take 1 day and NP treatment takes 3 days.
1196 The MTT assay takes a further 5-6 h (depending on sample numbers) and the
1197 reading/generation of results ~1-2 h: ~3-4 weeks in total, depending on how well the cell
1198 lines grow.

1199 *Support Protocol 2:* As stated above for basic protocol 2, cell line preparation,
1200 seeding and treating with NP will take ~2-3 weeks plus an additional 4 days. The trypan blue
1201 exclusion assay will take a further 30 min-2 h depending on how many samples are to be
1202 analysed. Likewise, cell counting will take 10 min-2 h depending on sample numbers and
1203 count methodology: ~3-4 weeks in total, depending on how well the cell lines grow.

1204 *Support Protocol 3:* Sample preparation including treatment times and protein
1205 preparation will take ~4-5 days. SDS-PAGE and completion of immunoblotting will then
1206 take a further 0.5 and 3 days, respectively: ~7-8 days in total, depending on optimised
1207 conditions.

1208 *Basic Protocol 3:* Depending on the requirements of the NF stage needed for specific
1209 experiments, *X. laevis* embryo exposure and incubation times can vary from a few h to
1210 several days. This is also influenced by the incubation temperature used (see points 1-4 of
1211 Basic Protocol 3 for discussion of time estimates). At the end of the incubation period, fixing
1212 the embryos can take 2-24 h depending on the temperature used. Washing, mounting,
1213 imaging and scoring the embryos will take a few hours depending on how many embryos
1214 need to be analysed. Finally, dehydration of embryos for long-term storage takes ~30-40 min:
1215 ~1 week in total.

1216 *Support Protocol 4:* Priming of females can take up to 1 week and induction of
1217 ovulation, up to 14 h. Fertilisations and de-jellying will take 2.5 h: ~6-8 days in total.

1218 *Support Protocol 5:* Preparation of imaging plates (1 h) and live, whole-mount
1219 fluorescent imaging of embryos ~1-3 h (depending on the number of embryos to analyse): 2-
1220 4 h in total.

1221 *Alternate Protocol 1:* Anesthetising, fixing and dehydrating embryos takes 3.5 h in
1222 total. Embedding the embryos in resin takes 3 days, whilst sectioning, mounting and imaging
1223 could take up to 2-3 days (depending upon the number of samples to process): ~5.5-6.5 days
1224 in total.

1225

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1230

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1361

1362 Figure Legends

1363

1364 **Figure i: Flow diagram of proposed nanotoxicity screening protocol.** This figure is
1365 adapted from a previously published study from our group (Webster et al., 2016). Briefly,
1366 newly synthesised nanotherapeutics are firstly characterised in terms of their physicochemical
1367 characteristics in biologically relevant media (basic protocol 1). Once identified as stable by
1368 this protocol, NPs are further assessed through an integrated approach of cytotoxicity analysis
1369 and phenotypic abnormality screening in *X. laevis* embryos (basic protocol 2 and basic
1370 protocol 3, respectively). Here we provide detailed methodological descriptions of these three
1371 protocols as highlighted in the dotted box. Results from basic protocols 2 and 3 are then

1372 combined to provide a score that can indicate whether or not further *in vivo* nanotoxicity
1373 assessment should be made using mammalian models.
1374
1375 **Figure ii:** Schematic drawing of a DLS apparatus with a multi-angle detector. The equipment
1376 is composed of a monochromatic laser in the visible range, optical lenses to focus the beam
1377 on the sample, attenuator of the incident light, detector (equipped with a motor to move it at
1378 different angles with respect to the incident beam), correlator and PC with a specific software
1379 for the analysis of the raw data. The attenuator modulates the incident light to an optimal
1380 value that depends on the features of the detector. The detected scattered light reaches the
1381 correlator that builds an auto-correlation function of the scattered intensity for each angle.
1382 The auto-correlation functions and the raw signals (kcounts/s) can be analyzed by the specific
1383 software provided by the supplier of the Instrument.
1384
1385 **Figure iii: Suggested *X. laevis* NF stages for NP exposure.** Schematic depicts *X. laevis*
1386 embryos at different developmental NF stages that have been selected for treatment to assess
1387 nanotoxicity (Webster et al., 2016). Embryo physiology images (Nieuwkoop and Faber,
1388 1967) depicted above the line, with their associated NF staging description provided below
1389 the line. Images not to scale. The selected NF stages for NP exposure provide analysis of two
1390 critical teratogenic assessment stages; gastrulation (NF 4-NF 38) and neuralation (NF 15-NF
1391 38), and at stages that can more accurately represent an adult system during organogenesis
1392 (NF 38-45).