Project: VIGO

Detection of reactive oxygen species in THP-1 cells

DCF assay inTHP-1 cells

AUTHORED BY:	DATE:
Cordula Hirsch	20.01.2014

REVIEWED BY:	DATE:
Harald Krug	09.04.2014

APPROVED BY:	DATE:

DOCUMENT HISTORY

Effective Date	Date Revision Required	Supersedes
15.02.2014	DD/MM/YYYY	DD/MM/YYYY

Version	Approval Date	Description of the Change		Author / Changed by
1.0	DD/MM/YYYY	All	Initial Document	Cordula Hirsch
1.1	19/04/2016	2	1. concentration L-glutamine (paragraph 5.3.1) 2. cell seeding density (paragraph 6.3.2)	Cordula Hirsch

Table of Content

1 Introduction

NMs can influence cellular systems in several ways. One very prominent and widely discussed cellular (as well as systemic) reaction towards NMs is the overproduction of reactive oxygen species (ROS) (see e.g. Donaldson et al. 2004; Johnston et al., 2010; Nel et al., 2006; Schins and Knaapen, 2007; Wiseman and Halliwell, 1996). In healthy cells ROS play an important role as messengers for both intra- and intercellular communication (e.g. Hancock, 2001; Held, 2010). Cells are generally used to handle such reactive species by anti-oxidant defense mechanisms that balance the absolute levels of ROS. Once these protective mechanisms fail, the increasing ROS levels lead to oxidative stress reactions. These include damage to DNA, proteins and lipids and can further lead to genotoxicity, inflammation and/or cell death. Thus, detecting elevated levels of ROS in cellular systems is an important tool to assess very early steps of potential cytotoxic effects.

2 Principle of the Method

The 2', 7'-dichlorodihydrofluorescein diacetate (H_2DCF-DA) assay is a widely used *in vitro* ROS-detection method. The non-fluorescent dye (H_2DCF-DA) is a chemically reduced form of fluorescein and cell-permeable. Intracellular esterases cleave off the diacetate (DA) moiety which renders the molecule (H_2DCF) sensitive to oxidation by ROS. In its oxidized form dichlorofluorescein (DCF) is highly fluorescent and easily detectable e.g. using a fluorescent plate reader.

3 Applicability and Limitations

H_2DCF is a non-specific ROS detector sensitive to a number of different reactive species. This can be an advantage for initial screening approaches where neither the general existence nor the type of reactive species is known. This easy and fast screening assay yields **qualitative** results that serve as an indication for further investigations and is as such valuable.

As the H_2DCF molecule is not completely retained inside the cell the measured fluorescence values are thus the sum of intra- as well as extracellular ROS formation.

Non-specific esterases not only exist intracellularly but also in serum which is usually added to cell culture medium. To avoid dye cleavage even before cellular uptake it is important to perform the assay under **serum free conditions**, preferentially in Hank's balanced salt solution (HBSS).

NM-related consideration: The large (most often reactive) surface area of NMs may be able to process the H_2DCF molecule to DCF without cellular contribution. This issue is addressed in this SOP in the cell free part of the 96-well plate. Furthermore NMs have been reported to interfere with fluorescence measurements by quenching an existing signal (for a review see Kroll et al., 2009). This

issue is addressed in the related SOP "NM interference in the DCF assay". Both cell free controls cannot be calculated against values from cellular measurements. They serve as qualitative estimations of NM only reactions that do not involve cellular contribution.

4 Related Documents

Table 1: Documents needed to proceed according to this SOP and additional NM-related interference control protocols.

Document ID	Document Title
O_DCF_interference	*NM interference in the DCF assay – Quenching effects – DCF*
cell culture_THP-1	*Culturing and differentiating THP-1 cells*
M_NM suspension_metal oxides	*Suspending and diluting Nanomaterials – Metal oxides and NM purchased as monodisperse suspensions*
M_NM suspension_ carbon based	*Suspending and diluting Nanomaterials – Carbon based nanomaterials*

5 Equipment and Reagents

5.1 Equipment

- Centrifuge (for cell pelleting; able to run 15 ml as well as 50 ml tubes at 200 x g)
- Conical tubes (15 ml and 50 ml; polypropylene or polystyrene; e.g. from Falcon)
- Flat bottom 96-well cell culture plates
- Fluorescence reader for multi-well plates (to measure excitation/emission at wavelength maxima of: λex=485 nm and λem=528 nm)
- Hemocytometer
- Laminar flow cabinet (biological hazard standard)
- Light microscope (for cell counting and cell observation)
- Microreaction tubes (1.5 ml; e.g. from Eppendorf)
- Multichannel pipette (with at least 8 positions; volume range per pipetting step at least from 50 µl to 200 µl)
- Vortex®

5.2 Reagents

For cell culturing and differentiation:

- Fetal Calf Serum (FCS)
- L-glutamine
- Neomycin[1)]
- Penicillin[1)]
- Phorbol 12-myristate 13-acetate (PMA) *[CAS number: 16561-29-8]*
  Note: Carcinogenic! Handle with special care! Special waste removal (see chapter 8)
- Phosphate buffered saline (PBS)
- Roswell Park Memorial Institute medium (RPMI-1640)

- Streptomycin[1)]
- Trypsin-EDTA (0.05%)

[1)] bought as a 100x concentrated mixture of Penicillin, Streptomycin and Neomycin (PSN) e.g. from Gibco.

Buffers, solvents and detection dye itself:

- 2‘,7‘-Dichlorofluorescin-diacetate (H_2DCF-DA) *[CAS number: 4091-99-0]*
- 3-Morpholinosydnonimine hydrochloride (SIN-1)
- Calcium chloride dihydrate ($CaCl_2$*2 H_2O) *[CAS number: 10035-04-8]*
- D-Glucose *[CAS number: 50-99-7]*
- Dimethyl sulfoxide (DMSO) *[CAS number: 67-68-5]*
- Disodium phosphate (Na_2HPO_4*2H_2O) *[CAS number: 10028-24-7]*
- Magnesium chloride hydrate ($MgCl_2$*6H_2O) *[CAS number: 7791-18-9]*
- Magnesium sulfate heptahydrate ($MgSO_4$*7H_2O) *[CAS number: 7487-88-9]*
- Methanol *[CAS number: 67-56-1]*
- Monosodium phosphate (NaH_2PO_4) *[CAS number: 89140-32-9]*
- Pluronic F-127 *[CAS number: 9003-11-6]*
- Potassium chloride (KCl) *[CAS number: 7447-40-7]*
- Potassium hydrogen phosphate (KH_2PO_4) *[CAS number: 7778-77-0]*
- Sodium chloride (NaCl) *[CAS number: 8028-77-1]*
- Sodium hydrogen carbonate ($NaHCO_3$) *[CAS number: 7542-12-3]*
- Sodium hydroxide (NaOH) *[CAS number: 1310-73-2]*
  Note: Corrosive! Handle with special care! (see chapter 8)

For waste treatment:

- HCl (smoking) *[CAS number: 7647-01-0]*
  Note: Corrosive and Irritating! Handle with special care! (see chapter 8)
- NaOH *[CAS number: 1310-73-2]*
  Note: Corrosive! Handle with special care! (see chapter 8)

5.3 Reagent Preparation

5.3.1 Complete cell culture medium

Basic medium:

- RPMI-1640

supplemented with:

- 10% FCS
- 1x PSN, which results in final concentrations of:
  - 50 µg/ml Penicillin
  - 50 µg/ml Streptomycin
  - 100 µg/ml Neomycin
- 0.2 mM L-glutamine

5.3.2 PMA stock solution

Prepare a 1 mM stock of PMA in DMSO. Therefore resuspend the 1 mg (standard packaging size) PMA powder in 1.62 ml DMSO. Aliquote and freeze at -20°C. Can be stored for years.

Note: Carcinogenic! Handle with special care! Special waste removal. (see chapter 8)

5.3.3 NaOH

Prepare a 5 M solution NaOH for PMA waste treatment.

- Dissolve 200 g NaOH pellets in 1 l ddH_2O.

Note: Be careful, exothermic reaction, gets HOT. NaOH is corrosive, wear protective clothing (especially eye protection).

5.3.4 1x concentrated Hank's Balanced Salt Solution (HBSS)

1 g/l	D-glucose
185 mg/l	$CaCl_2 * 2\ H_2O$
400 mg/l	KCl
60 mg/l	KH_2PO_4
100 mg/l	$MgCl_2 * 6\ H_2O$
100 mg/l	$MgSO_4 * 7\ H_2O$
8 g/l	NaCl
350 mg/l	$NaHCO_3$
60 mg/l	$Na_2HPO_4 * 2\ H_2O$

Dissolve all reagents in ddH_2O and adjust the pH to 7.4. Store at 4°C.

5.3.5 2x concentrated Hank's Balanced Salt Solution (HBSS)

Double-concentrated HBSS will be necessary to dilute nanomaterials (NM) for final application.

2 g/l	D-glucose
370 mg/l	$CaCl_2 * 2 H_2O$
800 mg/l	KCl
120 mg/l	KH_2PO_4
200 mg/l	$MgCl_2 * 6 H_2O$
200 mg/l	$MgSO_4 * 7 H_2O$
16 g/l	NaCl
700 mg/l	$NaHCO_3$
120 mg/l	$Na_2HPO_4 * 2 H_2O$

Dissolve all reagents in ddH_2O and adjust the pH to 7.4. Store at 4°C.

5.3.6 Pluronic F-127

Stock:

- 160 ppm in ddH_2O: 160 µg/ml (=16 mg/100 ml)

5.3.7 H_2DCF-DA

Stock:

- 5 mM in DMSO: 2.44 mg/ml

Working concentration:

- 50 µM in HBSS: 70 µl [5 mM] in 7 ml HBSS

5.3.8 Deacetylation of H_2DCF-DA

Prepare the following solutions freshly:

NaOH (0.01 M):	0.4 mg/ml	NaOH	
NaH_2PO_4 (0.033 M):	5.2 mg/ml	$NaH_2PO_4 * 2 H_2O$	adjust to pH 7.4

Preparation of 50 ml 50 µM H_2DCF:

Add 0.5 ml 5 mM H_2DCF-DA, 2.5 ml Methanol and 10 ml 0.01 M NaOH in a beaker. Stir for 30 minutes at room temperature (RT) in the dark (beaker covered in aluminum foil). Stop the reaction by adding 37.5 ml 33 mM NaH_2PO_4.

This solution is stable for two weeks in the dark at 4°C.

5.3.9 Sin-1

Sin-1 is instable as soon as put in solution. Therefore work as fast as possible and prepare dilutions (compare 6.4.3) as freshly as possible.

Stock:

- 1 mM in HBSS: 0.21 mg/ml

If necessary this stock solution can be frozen in single use aliquots at -20°C.

6 Procedure

6.1 General remarks

This SOP includes an optimized plate setup and dilution scheme to assess the oxidative potential of nanomaterials in differentiated THP-1 cells and in parallel in a cell free environment. Therefore the following plate layout is used:

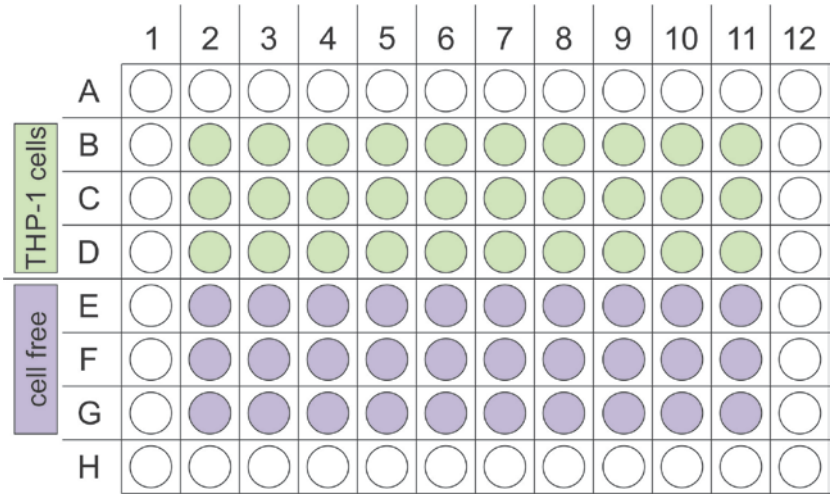

Figure 1: General plate layout.
Cells are only seeded into wells B2-D11. Outermost wells A1-A12; A1-D1 and A12-D12 receive complete cell culture medium only. Wells E1 to H12 remain empty for the first 72 h and are then treated as described in the text below.

6.2 Flow chart

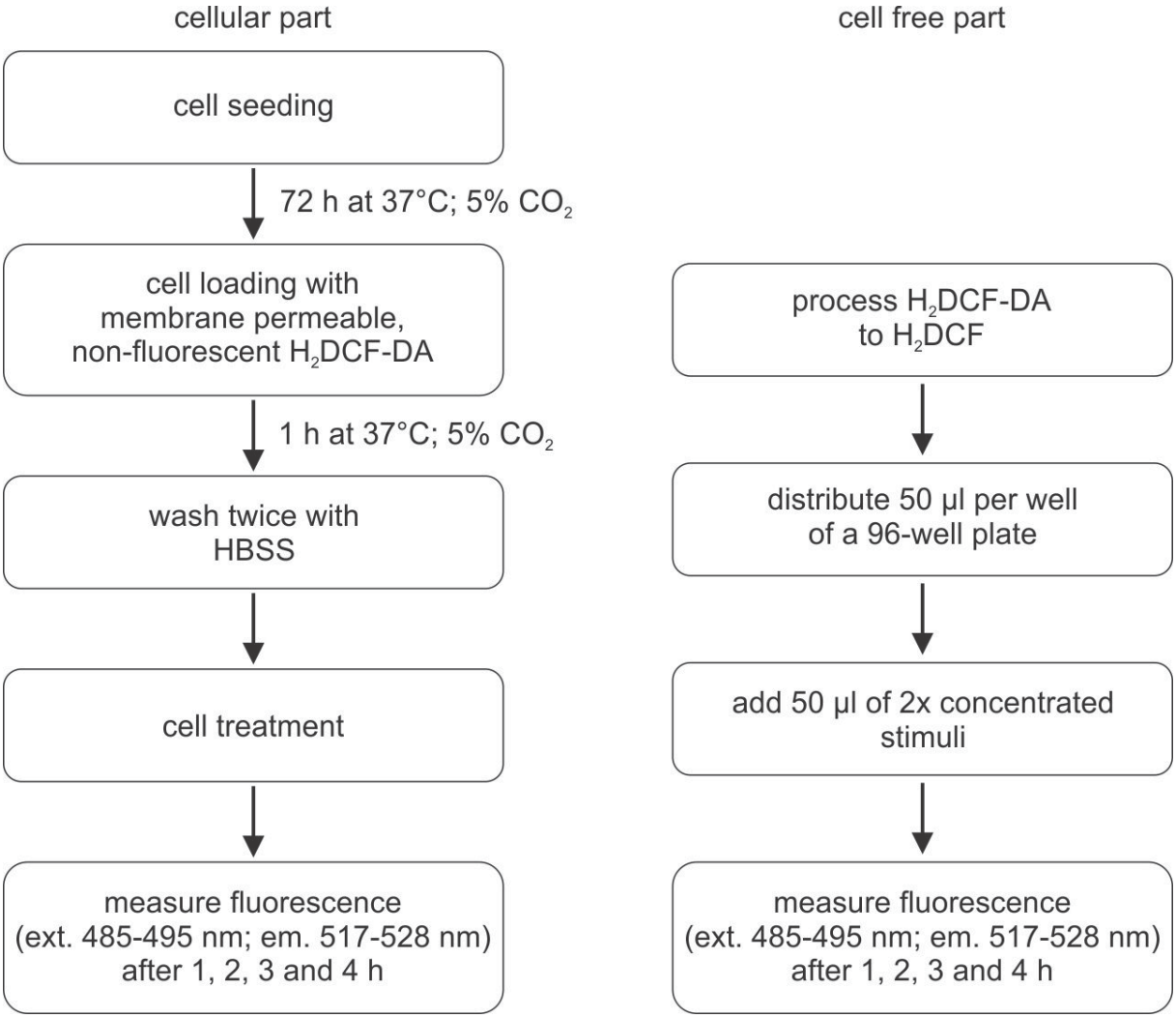

Figure 2: Brief outline of the workflow.

6.3 Cell seeding

6.3.1 Cell culture

THP-1 cells are grown in T75 cell culture flasks in a total volume of 20 ml of complete cell culture medium. They are kept at 37°C, 5% CO_2 in humidified air in an incubator (standard growth conditions according to SOP "Culturing and differentiating THP-1 cells").

6.3.2 Cell seeding into 96-well plate

- Three days (72 h) prior to experimental start harvest and count cells as described in SOP "Culturing and differentiating THP-1 cells".
- Seed $4x10^4$ cells in 200 µl complete cell culture medium containing 200 nM PMA per well into a 96-well cell culture plate.
- For one 96-well plate (see Figure 3) $2x10^6$ cells are suspended in 10 ml complete PMA containing cell culture medium ($2x10^5$ cells/ml).
  Note: PMA is diluted 1:5000 from the 1 mM stock (2 µl/10 ml medium).

- Using a multichannel pipette (10 channels) 200 µl of this cell suspension are distributed into each of the green wells (B2 to D11, Figure 3).

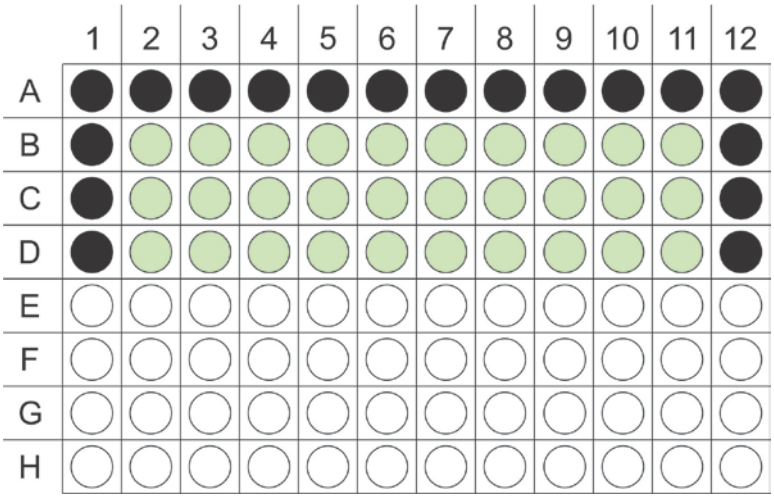

Figure 3: Cell seeding into a 96-well plate.
Cells are seeded at a density of $4x10^4$ cells per well in 200 µl complete cell culture medium containing 200 nM PMA into each of the green wells. Black wells receive 200 µl complete cell culture medium each.

- Outermost wells (labeled in black in Figure 3) receive 200 µl complete cell culture medium only. These wells will serve as blank values later on and will be treated exactly the same as the cell containing wells (apart from not containing cells).
- Differentiate cells for three days (72 hours) in a humidified incubator at standard growth conditions.

6.4 Prearrangements

6.4.1 Deacetylation of H_2DCF-DA

Perform deacetylation as described in 5.3.8. This can be done in advance as the H_2DCF is stable for two weeks at 4°C in the dark.

6.4.2 Dilution of nanomaterials

For this SOP we distinguish two types of nanomaterials (NM) according to their solvent, suspension properties and highest concentrations used in the assay. See also respective related documents (3).

(1) Metal oxide NM, Polystyrene beads and all NM delivered as monodisperse suspensions by the supplier: solvent either determined by the supplier or ddH_2O; sub-diluted in ddH_2O; highest concentration in assay 100 µg/ml
(2) Carbon based NM: suspended and sub-diluted in 160 ppm Pluronic F-127; highest concentration in assay 80 µg/ml

Volumes given in the following dilution schemes are enough for one 96-well plate.

Note: "Mixing" in the context of diluting NMs means, the solvent containing tube is put on a continuously shaking Vortex® and the previous sub-dilution (or stock suspension, respectively) is put dropwise into the shaking solvent. The resulting suspension stays on the Vortex® for additional 3 seconds before proceeding with the next sub-dilution.

(1) Metal oxide NM:

Prepare serial sub-dilutions of the stock suspension (1 mg/ml) in ddH_2O:

- Label seven microreaction tubes (1.5 ml total volume) with 1 to 7 (relates to steps 1-7 below).
- Add 400 µl of the 1 mg/ml stock suspension to tube 1.
- Add 200 µl ddH_2O to tubes no. 2, 3, 5 and 7.
- Add 240 µl ddH_2O to tube 4.

1. 400 µl NM stock suspension in ddH_2O → 1 mg/ml (1)
2. 200 µl of 1 mg/ml stock suspension (1) are mixed with 200 µl of ddH_2O → 500 µg/ml (2)
3. 200 µl of 500 µg/ml (2) are mixed with 200 µl ddH_2O → 250 µg/ml (3)
4. 160 µl of 250 µg/ml (3) are mixed with 240 µl ddH_2O → 100 µg/ml (4)
5. 200 µl of 100 µg/ml (4) are mixed with 200 µl ddH_2O → 50 µg/ml (5)
6. 200 µl of 50 µg/ml (5) are mixed with 200 µl ddH_2O → 25 µg/ml (6)
7. 200 µl ddH_2O → solvent control (7)

Preparation of final dilutions:

- Label seven microreaction tubes (1.5 ml) as follows:
    1. 200 µg/ml
    2. 100 µg/ml
    3. 50 µg/ml
    4. 20 µg/ml
    5. 10 µg/ml
    6. 5 µg/ml
    7. Solvent control: ddH_2O
- Mix 3200 µl 2x HBSS with 1920 µl ddH_2O. This mixture (A) is used in all following steps for the preparation of the final NM concentrations. Mixing NM sub-dilutions with (A) will result in 1x HBSS containing the respective NM concentrations.
- Add 640 µl (A) to each of the seven tubes.
- Mix on the Vortex® with 160 µl of the respective NM sub-dilutions or the solvent (ddH_2O):
    1. 160 µl of the stock suspension (1 mg/ml) are mixed with 640 µl (A) → 200 µg/ml (1)
    2. 160 µl of 500 µg/ml sub-dilution are mixed with 640 µl (A) → 100 µg/ml (2)
    3. 160 µl of 250 µg/ml sub-dilution are mixed with 640 µl (A) → 50 µg/ml (3)
    4. 160 µl of 100 µg/ml sub-dilution are mixed with 640 µl (A) → 20 µg/ml (4)
    5. 160 µl of 50 µg/ml sub-dilution are mixed with 640 µl (A) → 10 µg/ml (5)
    6. 160 µl of 25 µg/ml sub-dilution are mixed with 640 µl (A) → 5 µg/ml (6)
    7. 160 µl ddH_2O are mixed with 640 µl (A) → solvent control (7)

(2) Carbon based NM:

Prepare serial sub-dilutions of the stock suspension (500 µg/ml) in 160 ppm Pluronic F-127:

- Label seven microreaction tubes (1.5 ml total volume) with 1 to 7 (relates to steps 1-7 below).
- Add 600 µl of the NM stock suspension in 160 ppm Pluronic F-127 to tube 1.
- Add 300 µl 160 ppm Pluronic F-127 totubes 2 to 7.

1. 600 µl NM stock suspension in Pluronic F-127 → 500 µg/ml (1)
2. 300 µl of the 500 µg/ml stock suspension (1) are mixed with 300 µl of Pluronic F-127 → 250 µg/ml (2)
3. 300 µl of 250 µg/ml (2) are mixed with 300 µl Pluronic F-127 → 125 µg/ml (3)
4. 300 µl of 125 µg/ml (3) are mixed with 300 µl Pluronic F-127 → 62.5 µg/ml (4)
5. 300 µl of 62.5 µg/ml (4) are mixed with 300 µl Pluronic F-127 → 31.25 µg/ml (5)
6. 300 µl of 31.25 µg/ml (5) are mixed with 300 µl Pluronic F-127 → 15.63 µg/ml (6)
7. 300 µl 160 ppm Pluronic F-127 → solvent control (7)

Preparation of final dilutions:

- Label seven microreaction tubes (1.5 ml) as follows:
    1. 160 µg/ml
    2. 80 µg/ml
    3. 40 µg/ml
    4. 20 µg/ml
    5. 10 µg/ml
    6. 5 µg/ml
    7. Solvent control: Pluronic F-127
- Mix 3200 µl 2x HBSS with 1152 µl ddH_2O. This mixture (B) is used in all following steps for the preparation of the final NM concentrations. Mixing NM sub-dilutions with (B) will result in 1x HBSS containing the respective NM concentrations.
- Add 544 µl (B) to each of the seven tubes.
- Mix on the Vortex® with 256 µl of the respective NM sub-dilutions or the solvent (160 ppm Pluronic F-127):
    1. 256 µl of the stock suspension (500 µg/ml) are mixed with 544 µl (B) → 160 µg/ml (1)
    2. 256 µl of the 250 µg/ml sub-dilution are mixed with 544 µl (B) → 80 µg/ml (2)
    3. 256 µl of the 125 µg/ml sub-dilution are mixed with 544 µl (B) → 40 µg/ml (3)
    4. 256 µl of the 62.5 µg/ml sub-dilution are mixed with 544 µl (B) → 20 µg/ml (4)
    5. 256 µl of the 31.25 µg/ml sub-dilution are mixed with 544 µl (B) → 10 µg/ml (5)
    6. 256 µl of the 15.63 µg/ml sub-dilution are mixed with 544 µl (B) → 5 µg/ml (6)
    7. 256 µl 160 ppm Pluronic F-127 are mixed with 544 µl (B) → solvent control (7)

6.4.3 Dilution of Sin-1 (chemical positive control)

Sin-1 is not stable in solution. Assure that the Sin-1 stock is thawed right before usage. Prepare the following dilutions as shortly before application (see chapter 6.7) as possible. Tube labeling and HBSS distribution should be done in advance to speed up the final process of Sin-1 dilution and distribution into the 96-well plate.

Prepare serial dilutions of the stock solution (1 mM) in HBSS. For one plate at least 800 µl of each dilution and 1.5 ml of the stock solution are needed:

- Label five microreaction tubes (1.5 ml total volume) with 1 to 5 (relates to steps 1-5 below).
- Add 600 µl HBSS to tubes 1, 3 and 5.
- Add 1200 µl HBSS to tubes 2 and 4.

Shortly before usage finalize the dilution series as follows:

1. 600 µl of 1 mM Sin-1 (stock solution) are mixed with 600 µl HBSS → 500 µM (1)
2. 300 µl of 500 µM Sin-1 (1) are mixed with 1200 µl HBSS → 100 µM (2)
3. 600 µl of 100 µM Sin-1 (2) are mixed with 600 µl HBSS → 50 µM (3)
4. 300 µl of 50 µM Sin-1 (3) are mixed with 1200 µl HBSS → 10 µM (4)
5. 600 µl of 10 µM Sin-1 (4) are mixed with 600 µl HBSS → 5 µM (5)

Application of NM as well as Sin-1 dilutions are shown in Figure 5 and described in 6.7 “Application of stimuli and measurement”.

6.5 Loading of cells with H_2DCF-DA

- Prepare the 50 µM working concentration of H_2DCF-DA in HBSS. For one 96-well plate a final volume of 7 ml are needed:

  70 µl 5 mM H_2DCF-DA stock + 7 ml HBSS

- Remove medium from wells A1 to D12 using a vacuum pump and a multichannel adapter. Be careful not to remove cells from the bottom of wells B2 to D11.

Note: Assure special waste removal for PMA containing medium (see chapter 8).

- Wash cell twice with pre-warmed (37°C) PBS. Be again careful not to remove cells from the bottom of wells B2 to D11.
- Using a multichannel pipette (12 channels) add 100 µl 50 µM H_2DCF-DA in HBSS per well (A1 to D12).
- Incubate plate in a humidified incubator at standard growth conditions for 60 minutes.
- Wash cells (wells A1 to D12) twice with pre-warmed (37°C) HBSS. Do not remove second HBSS solution before cell-free wells are prepared.

6.6 Preparation of cell-free control wells

- Add 50 µl ddH_2O into each of the outermost wells (black wells in Figure 4, E1-H1; E12-H12; H2-H11).
- Add 50 µl deacetylated H_2DCF (prepared according to 5.3.8) into each purple well (Figure 4, E2 to G11).

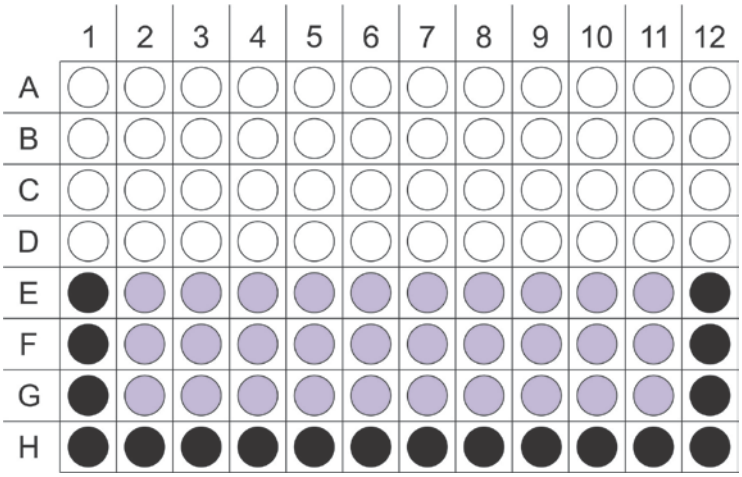

Figure 4: Cell free controls. Outermost wells (E1-H1; E12-H12; H1-H12) receive 50 µl ddH_2O each. Inner wells (E2-G11) receive 50 µl deacetylated H_2DCF.

6.7 Application of stimuli and measurement

Stick to the following chronological order.

Note: All NM dilutions have to be vortexed directly before application to the cells.

1. Make sure to have NM dilutions ready (see 6.4.2).
2. Prepare Sin-1 dilutions (see 6.4.3).
3. Remove HBSS from wells A2 to D11. Leave HBSS in outer wells A1 to D1 and A12 to D12.
4. Add **100 µl** of the respective NM dilution per well to wells A6 to D11 (shown in Figure 5 a).

wells	metal oxide NM concentration	carbon based NM concentration
A11-D11	100 µg/ml	80 µg/ml
A10-D10	50 µg/ml	40 µg/ml
A9-D9	20 µg/ml	20 µg/ml
A8-D8	10 µg/ml	10 µg/ml
A7-D7	5 µg/ml	5 µg/ml
A6-D6	solvent (ddH_2O)	solvent (160 ppm Pluronic F-127)

5. Add **50 µl** of the respective NM dilution per well to **cell free control** wells E6 to H11 (shown in Figure 5 b).

wells	metal oxide NM concentration	carbon based NM concentration
E11-H11	200 µg/ml	160 µg/ml
E10-H10	100 µg/ml	80 µg/ml
E9-H9	50 µg/ml	40 µg/ml
E8-H8	20 µg/ml	20 µg/ml
E7-H7	10 µg/ml	10 µg/ml
E6-H6	solvent (ddH_2O)	solvent (160 ppm Pluronic F-127)

Note: Applied concentrations are different for cellular and cell free wells! However, due to the 1:2 dilution of the NMs in the cell free wells (50 µl H_2DCF + 50 µl NM dilution) final concentrations are the same.

6. Add **100 µl** of respective Sin-1 dilution per well to wells A2 to D5 (shown in Figure 5 c).

wells	**Sin-1 concentration**
A5-D5	500 µM
A4-D4	50 µM
A3-D3	5 µM
A2-D2	solvent (HBSS)

7. Add **50 µl** of the respective Sin-1 dilution per well to **cell free control** wells E2 to H11 (shown in Figure 5 d).

wells	**Sin-1 concentration**
E5-H5	1000 µM
E4-H4	100 µM
E3-H3	10 µM
E2-H2	solvent (HBSS)

Note: Applied concentrations are different for cellular and cell free wells! However, due to the 1:2 dilution of Sin-1 in the cell free wells (50 µl H_2DCF + 50 µl Sin-1 dilutions) final concentrations are the same.

8. Incubate plate in a humidified incubator at standard growth conditions.
9. Measure fluorescence in a multi-well plate reader after 1, 2, 3 and 4 hours. After each measurement place plate back into incubator.
   Fluorescence settings: excitation at λ=485-495 nm, emission at λ=517-528 nm.

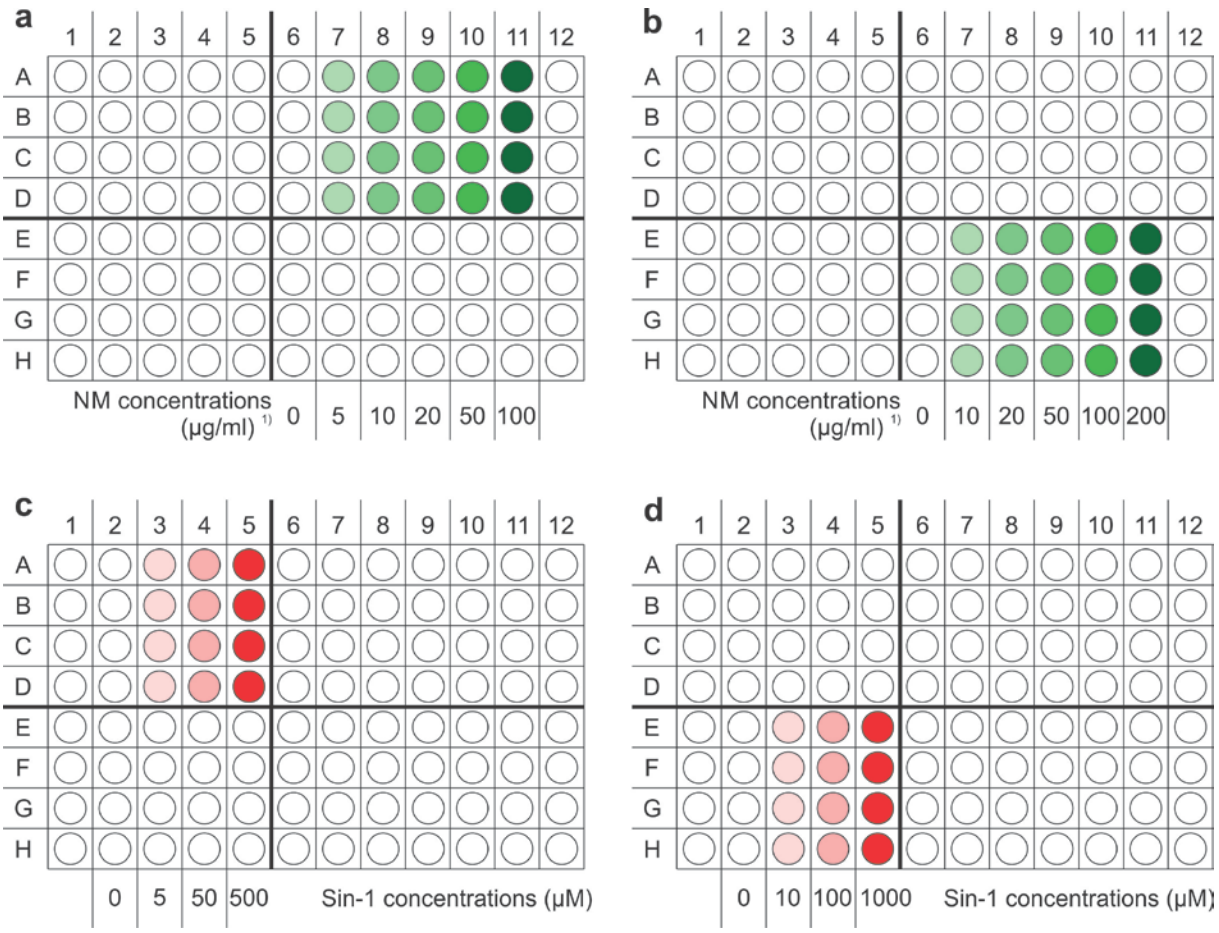

Figure 5: Application of stimuli.
a) Application of NM to cells first (wells A6-D11). **b)** Secondly, apply NM to cell free wells (E6-H11). **c)** Subsequently add Sin-1 to cells (A2-D5) and **d)** finally also to cell free wells (E2-H5).
1) NM concentrations given here refer to metal oxide NM. Carbon based NM concentrations are detailed in the text.

6.8 Data evaluation

Data are presented as blank corrected fluorescence values and represent the mean of three technical replicates. To calculate this, blank values (wells A2 to A11 for the cellular part and wells H2 to H11 for the cell free part) are subtracted from the respective sample values. For example: value in well A11 is subtracted from value in well B11, C11 and D11. B11*=B11-A11; C11*=C11-A11; D11*=D11-A11. The mean and standard deviation is then calculated from the resulting blank corrected values B11*, C11* and D11*.

7 Quality Control, Quality Assurance, Acceptance Criteria

8 Health and Safety Warnings, Cautions and Waste Treatment

Cell seeding has to be carried out under sterile conditions in a laminar flow cabinet (biological hazard standard). For this only sterile equipment must be used and operators should wear laboratory coat and gloves (according to laboratory internal standards). Special care has to be taken during PMA handling (carcinogenic potential of the substance!).

PMA waste treatment: use a separate exhaust extraction system with a collecting flask containing already 20 ml 5 M NaOH to neutralize PMA. The resulting non-toxic solution is very alkaline and has to be neutralized using HCl before final disposal in the sink.

NaOH is **corrosive**. It causes severe burns. Wear especially eye/face protection. Dissolution of NaOH is an exothermic reaction, the solution will get fairly hot – be careful! It is strongly recommended to wear eye protection when handling 5 M NaOH.

HCl is **corrosive and irritant**. It is very hazardous in case of skin contact, of eye contact and of ingestion. It is slightly hazardous in case of inhalation. Therefore avoid inhalation as well as contact with skin and eyes and avoid exposure in general.

Discard all materials used to handle cells (including remaining cells themselves) according to the appropriate procedure for special biological waste (i.e. by autoclaving).

9 Abbreviations

DA	diacetate
DCF	2‘, 7‘-Dichlorofluorescein
ddH_2O	double-distilled water
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
FCS	fetal calf serum
g	constant of gravitation
H_2DCF	2‘, 7‘-Dichlorodihydrofluorescein
H_2DCF-DA	2‘, 7‘-Dichlorodihydrofluorescein-diacetate
HBSS	Hank’s balanced salt solution
NM	nanomaterial
PBS	phosphate buffered saline
PMA	phorbol 12-myristate 13-acetate
ppm	parts per million
PSN	Penicillin, Streptomycin, Neomycin
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute medium
RT	room temperature
Sin-1	3-Morpholinosydnonimine

10 References

Donaldson K, Stone V, Tran CL, Kreyling W, Borm PJ; 2004: Nanotoxicology. *Occup. Environ. Med.* 61(9): 727-728

Hancock JT, Desikan R, Neill SJ; 2001: Role of reactive oxygen species in cell signaling pathways. *Biochemical and Biomedical Aspects of Oxidative Modification* 29(2): 345-350

Held P; 2010: An Introduction to reactive oxygen species. BioTek White Paper: Winooski, VT, USA.

Johnston HJ, Hutchison GR, Christensen FM, Peters S, Hankin S, Aschberger K, Stone V; 2010: A critical review of the biological mechanisms underlying the in vivo and in vitro toxicity of carbon nanotubes: The contribution of physic-chemical characteristics. *Nanotoxicology* 4(2): 207-246

Kroll A, Pillukat MH, Hahn D, Schnekenburger J; 2009: Current *in vitro* methods in nanoparticle risk assessment: Limitations and challenges. *Eur. J. Pharm. Biopharm.* 72(2): 370-377

Nel A, Xia T, Mädler L, Li N; 2006: Toxic potential of materials at the nanolevel. *Science* 311(5761): 622-627

Schins RP, Knaapen AM; 2007: Genotoxicity of poorly soluble particles. *Inhal. Toxicol.* 19: 189-198

Wiseman H, Halliwell B; 1996: Damage to DNA by reactive oxygen and nitrogen species: role in inflammatory disease and progression to cancer. *Biochem. J.* 313: 17-29

11 Annex A:

Additional background information:

As H_2DCF should be retained inside the cell the H_2DCF-DA method has been reported to specifically detect intracellular ROS production (see e.g. Kohno, 2010; Wardman, 2008). However, some leakage of the dye to the extracellular space is possible (Tarpey, et al., 2004). Reactive species outside the cell can then process the H_2DCF-molecule. The resulting fluorescence values are thus the sum of intra- as well as extracellular ROS formation.

Measurements cannot be performed **quantitatively**: ROS – as implicated in the name – are highly reactive and short lived species. Therefore it is important to measure as shortly after ROS induction as possible. This is achieved by loading the H_2DCF-DA dye prior to cell treatment. With the processed dye (H_2DCF) in place any ROS pulse can be directly measured. However finding a “stable” positive reaction control for this assay is difficult. Any chemical used has to be as reactive as naturally occurring ROS and as a consequence cannot be stable. 3-Morpholinosydnonimine (Sin-1) is frequently used as the chemical positive control for the H_2DCF-DA assay (see e.g.: Buerki-Thurnherr et al., 2012; Limbach et al., 2007; Lipton et al., 1993; Piret et al., 2013; Wang et al., 1999). It works well to qualitatively see, if the assay per se worked or not. However, absolute values vary strongly due to the reactive and thus instable nature of the molecule. This is the case not only for Sin-1 but for any ROS-inducing compound. Prolonged handling steps during e.g. Sin-1 dilution or repeated freeze-thaw cycles (which have to be avoided!) reduce the activity of the molecule and with it also fluorescence values. Quantitatively comparing results from one experiment to another – or from one lab to another – is therefore difficult to impossible (Roesslein et al., 2013).

References in Annex A:

Buerki-Thurnherr T, Xiao L, Diener L, Arslan O, Hirsch C, Maeder-Althaus M, Grieder K, Wampfler B, Mathur S, Wick P, Krug HF; 2012: *In vitro* mechanistic study towards a better understanding of ZnO nanoparticle toxicity. *Nanotoxicology* 7(4): 402-416

Kohno M; 2010: Applications of electron spin resonance spectrometry for reactive oxygen species and reactive nitrogen species research. *J. Clin. Biochem. Nutr.* 47: 1-11

Limbach LK, Wick P, Manser P, Grass RN, Bruinink A, Stark WJ; 2007: Exposure of engineered nanoparticles to human lung epithelial cells: Influence of chemical composition and catalytic activity on oxidative stress. *Environ. Sci. Technol.* 41: 4158-4163

Lipton SA, Choi YB, Pan ZH, Lei SZ, Chen HS, Sucher NJ, Loscalzo J, Singel DJ, Stamler JS; 1993: A redox-based mechanism for the neuroprotective and neurodestructive effects of nitric oxide and related nitroso-compounds. *Nature* 364: 626-632

Piret JP, Jacques D, Audinot JN, Mejia J, Boilan E, Noel F, Fransolet M, Demazy C, Lucas S, Saout C, et al.; 2012: Copper(II) oxide nanoparticles penetrate into HepG2 cells, exert cytotoxicity via oxidative stress and induce pro-inflammatory response. *Nanoscale* 4: 7168-7184

Roesslein M, Hirsch C, Kaiser JP, Krug HF, Wick P; 2013: Comparability of *in vitro* tests for bioactive nanoparticles: A common assay to detect reactive oxygen species as an example. *Int. J. Mol.Sci.* 14: 24320-24337

Tarpey MM, Wink DA, Grisham MB; 2004: Methods for detection of reactive metabolites of oxygen and nitrogen: *in vitro* and *in vivo* considerations. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 286: 431-44

Wang H, Joseph JA; 1999: Quantifying cellular oxidative stress by dichlorofluorescein assay using microplate reader. *Free Radic. Biol. Med.* 27: 612-616

Wardman P; 2008: Methods to measure the reactivity of peroxynitrite-derived oxidants toward reduced fluoresceins and rhodamines. *Methods. Enzymol.* 411: 261-282