Project: VIGO

NM interference in the DCF assay

Quenching effects – DCF

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DOCUMENT HISTORY

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

Version	Date	Description of the Change		Author / Changed by
1.0	15/02/2014	All	Initial Document	Cordula Hirsch
1.1	20/10/2016	1	NM "solvent" for pre-dilutions (old: ddH_2O; new: HBSS; paragraph 6.2)	Cordula Hirsch

Table of Content

1 Introduction

NMs have been shown to interfere with several colorimetric as well as fluorimetric read outs (e.g. Belyanskaya, 2007; Casey, 2007; Guo, 2008; Monteiro-Riviere, 2006; Pulskamp, 2007; Wörle-Knirsch, 2006). Especially carbon-based NMs may quench existing fluorescence signals (for a review see Kroll et al., 2009). The detection of potential quenching effects of NMs in the DCF-assay is addressed in this SOP.

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.

The fluorescent DCF molecule is commercially available. It is used in a cell free 96-well plate setup to uncover potential quenching effects of NMs.

3 Applicability and Limitations

The results from these 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_A549	*Detection of reactive oxygen species in A549 cell – DCF assay in A549 cells*
O_DCF_THP-1	*Detection of reactive oxygen species in THP-1 cell – DCF assay in 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

- Flat bottom 96-well cell culture plates
- 15 ml conical tubes (polypropylene or polystyrene; e.g. from Falcon)
- Vortex®
- Multichannel pipette (with 12 positions; volume range per pipetting step at least from 50 µl to 200 µl)
- Fluorescence reader for multi-well plates (to measure excitation/emission at wavelength maxima of: λex=485 nm and λem=528 nm)

5.2 Reagents

- 2',7'-Dichlorofluorescin (DCF) *[CAS number: 76-54-0]*
- Calcium chloride dihydrate ($CaCl_2$*2 H_2O) *[CAS number: 10035-04-8]*
- D-Glucose *[CAS number: 50-99-7]*
- Disodium phosphate (Na_2HPO_4*2H_2O) *[CAS number: 10028-24-7]*
- Ethanol *[CAS number: 64-17-5]*
- Magnesium chloride hydrate ($MgCl_2$*6H_2O) *[CAS number: 7791-18-9]*
- Magnesium sulfate heptahydrate ($MgSO_4$*7H_2O) *[CAS number: 7487-88-9]*
- Pluronic F-127 *[CAS number: 9003-11-6]*
- 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]*

5.3 Reagent Preparation

5.3.1 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.

5.3.2 2x concentrated HBSS

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.

5.3.3 DCF

Stock:

- 500 µM in Ethanol: 0.2 mg/ml
- Prepare aliquots and store at -20°C. **Note:** can be stored for several month.

Working concentration:

- 2.5 µM in HBSS: dilute 15 µl of the 500 µM stock solution in 3 ml HBSS

5.3.4 Pluronic F-127

Stock:

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

Can be stored for up to 4 weeks at 4°C.

6 Procedure

6.1 Flow chart

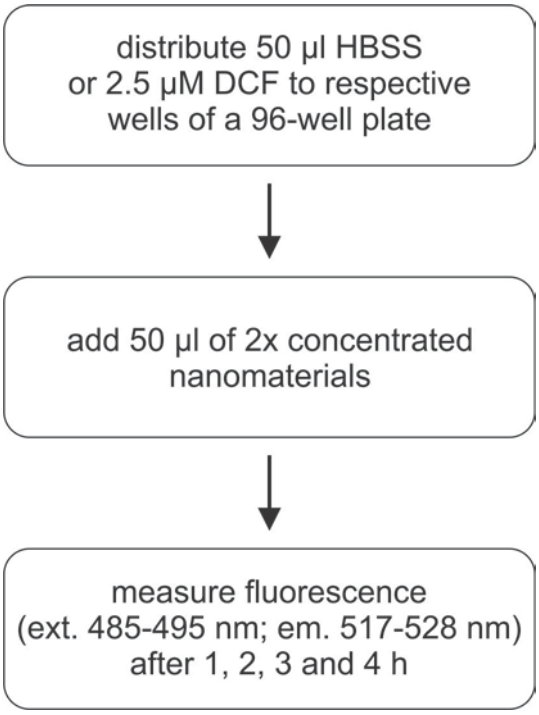

Figure 1: Brief outline of the workflow.

6.2 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.

Note: A series of experiments showed that quenching reactions of certain NM (i.e. gold nanoparticles) can be stronger in ddH_2O compared to HBSS as a solvent. As cell based experiments are run in HBSS, this interference protocol was adapted accordingly to mimic the cellular situation as good as possible. Thus all NM pre-dilutions described below use HBSS as a solvent.

(1) Metal oxide NM:

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

- Label nine conical tubes (15 ml total volume) with 1 to 9 (relates to steps 1-9 below).
- Mix 1.5 ml 2x concentrated HBSS and 300 µl ddH_2O in tube no. 1 (mixture A).
- Add 1.5 ml (1x concentrated) HBSS to tubes 2 to 9.

1. 1.2 ml of 1 mg/ml stock suspension are mixed with 1.8 ml of mixture A → 400 µg/ml (1)
2. 1.5 ml of 400 µg/ml (1) are mixed with 1.5 ml HBSS → 200 µg/ml (2)
3. 1.5 ml of 200 µg/ml (2) are mixed with 1.5 ml HBSS → 100 µg/ml (3)
4. 1.5 ml of 100 µg/ml (3) are mixed with 1.5 ml HBSS → 50 µg/ml (4)
5. 1.5 ml of 50 µg/ml (4) are mixed with 1.5 ml HBSS → 25 µg/ml (5)
6. 1.5 ml of 25 µg/ml (5) are mixed with 1.5 ml HBSS → 12.5 µg/ml (6)
7. 1.5 ml of 12.5 µg/ml (6) are mixed with 1.5 ml HBSS → 6.25 µg/ml (7)
8. 1.5 ml of 6.25 µg/ml (7) are mixed with 1.5 ml HBSS → 3.13 µg/ml (8)
9. 1.5 ml of 3.13 µg/ml (8) are mixed with 1.5 ml HBSS → 1.56 µg/ml (9)

As these sub-dilutions will be mixed 1:2 with the DCF dye (or ddH_2O) in the 96-well plate (see Figure 2 and Figure 3), the final concentrations will be halved to the following final concentrations:

1. 200 µg/ml
2. 100 µg/ml
3. 50 µg/ml
4. 25 µg/ml
5. 12.5 µg/ml
6. 6.25 µg/ml
7. 3.13 µg/ml
8. 1.56 µg/ml
9. 0.78 µg/ml
10. Solvent control: HBSS

(2) Carbon based NM:

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

- Label nine conical tubes (15 ml total volume) with 1 to 9 (relates to steps 1-9 below).
- Add 1.5 ml 2x concentrated HBSS to tube no. 1.
- Mix 10 ml 2x HBSS with 10 ml 160 ppm Pluronic F-127.
  Note: Using this mixture (A) for NM sub-dilutions will result in 1x HBSS containing the appropriate amount of NM as well as Pluronic F-127.
- Add 1.08 ml mixture (A) to tube no. 2.
- Add 1.5 ml mixture (A) to tubes 3 to 9.

1. 1.5 ml of 500 µg/ml stock suspension are mixed with 1.5 ml 2x HBSS → 250 µg/ml (1)
2. 1.92 ml of 250 µg/ml (1) are mixed with 1.08 ml (A) → 160 µg/ml (2)
3. 1.5 ml of 160 µg/ml (2) are mixed with 1.5 ml (A)→ 80 µg/ml (3)
4. 1.5 ml of 80 µg/ml (3) are mixed with 1.5 ml (A)→ 40 µg/ml (4)
5. 1.5 ml of 40 µg/ml (4) are mixed with 1.5 ml (A)→ 20 µg/ml (5)
6. 1.5 ml of 20 µg/ml (5) are mixed with 1.5 ml (A)→ 10 µg/ml (6)
7. 1.5 ml of 10 µg/ml (6) are mixed with 1.5 ml (A)→ 5 µg/ml (7)
8. 1.5 ml of 5 µg/ml (7) are mixed with 1.5 ml (A)→ 2.5 µg/ml (8)
9. 1.5 ml of 2.5 µg/ml (8) are mixed with 1.5 ml (A)→ 1.25 µg/ml (9)

As these sub-dilutions will be mixed 1:2 with the DCF dye (or ddH_2O) in the 96-well plate (see Figure 2 and Figure 3), the final concentrations will be halved to the following final concentrations:

1. 125 µg/ml
2. 80 µg/ml
3. 40 µg/ml
4. 20 µg/ml
5. 10 µg/ml
6. 5 µg/ml
7. 2.5 µg/ml
8. 1.25 µg/ml
9. 0.625 µg/ml
10. Solvent control: mixture (A) = 80 ppm Pluronic F-127

6.3 Preparation of DCF working solution:

Dilute the 500 µM stock solution 1:200 in ddH_2O resulting in a 2.5 µM working solution. For one 96-well plate a final volume of 3 ml is needed.

- Mix 2985 µl ddH_2O with 15 µl of the 500 µM stock solution.

6.4 Distribution into 96-well plate

- Add 50 µl ddH_2O into each of the outermost wells (black wells in Figure 2, A1-A12; H1-H12; B1-G1; B12-G12).
- Add 50 µl ddH_2O also into wells B2-D11 (also black in Figure 2).
- Add 50 µl 2.5 µM DCF working solution into each blue well (Figure 2, E2 to G11).

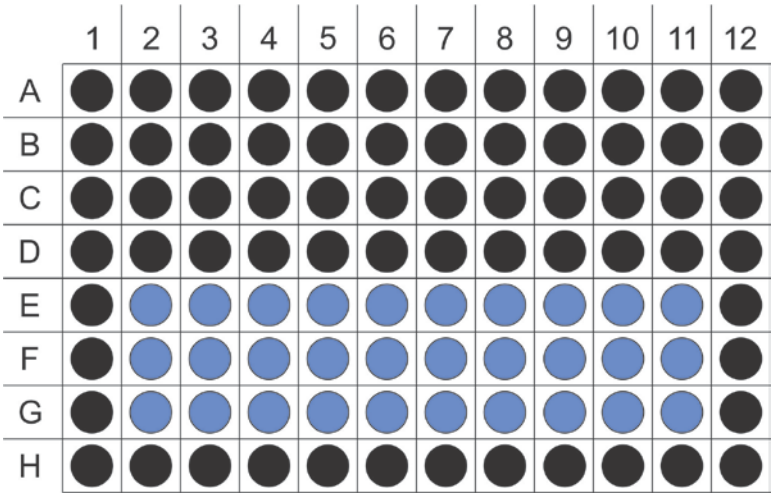

Figure 2: Plate layout. Rows A to D as well as outermost wells (E1-H1; E12-H12; H2-H11) receive 50 µl ddH_2O each. Inner wells (E2-G11) receive 50 µl 2.5 µM DCF.

6.5 Application of NMs and measurement

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

- Add NM sub-dilutions (see 6.2 "Dilution of nanomaterials") to appropriate wells according to Figure 3 and the table below:

wells	metal oxide NM concentration	carbon based NM concentration
B11-G11	400 µg/ml	250 µg/ml
B10-G10	200 µg/ml	160 µg/ml
B9-G9	100 µg/ml	80 µg/ml
B8-G8	50 µg/ml	40 µg/ml
B7-G7	25 µg/ml	20 µg/ml
B6-G6	12.5 µg/ml	10 µg/ml
B5-G5	6.25 µg/ml	5 µg/ml
B4-G4	1.13 µg/ml	2.5 µg/ml
B3-G3	1.56 µg/ml	1.25 µg/ml
B2-D2	solvent (HBSS)	solvent (80 ppm Pluronic F-127 in HBSS)

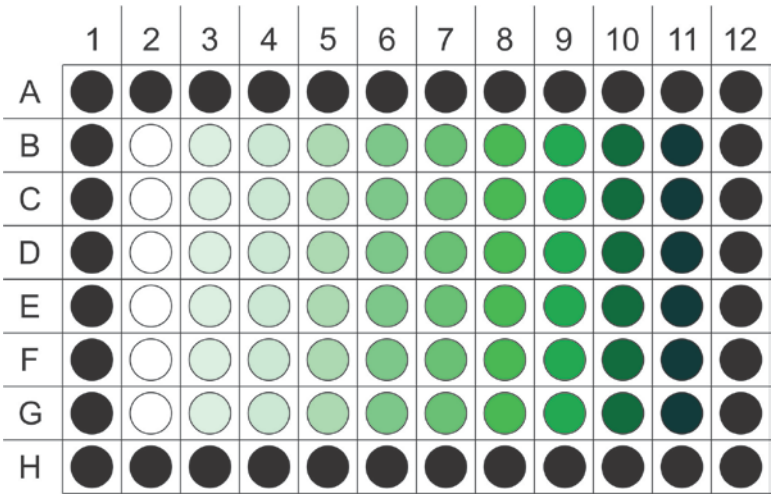

Figure 3: Distribution of NM dilutions. 50 µl of the NM sub-dilutions are added to the green wells. Declining green intensity indicates declining NM concentrations (from row 11 to row 2).

Note: Due to the 1:2 dilution of the NMs final concentrations are halved.

- Incubate plate in a humidified incubator at 37°C and 5% CO_2.
- 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.

6.6 Data evaluation

Data are presented as fluorescence values and represent the mean of three technical replicates and their standard deviation.

Wells B2-D11 (which do not receive DCF dye) serve as control wells to determine NM intrinsic fluorescence. If these values show any concentration dependent trend, they should be subtracted from DCF-containing corresponding values (blank correction as described in SOP "Detection of reactive oxygen species in A549 or THP-1 cells").

To better illustrate the quenching effect of a given NM, solvent control values (E2-G2) can be normalized to 100%. All other values are then expressed in % of the solvent control and can be interpreted as "quenching efficiency in %".

7 Quality Control, Quality Assurance, Acceptance Criteria

8 Health and Safety Warnings, Cautions and Waste Treatment

9 Abbreviations

DA	diacetate
DCF	2', 7'-Dichlorofluorescein
ddH_2O	double-distilled water
H_2DCF	2', 7'-Dichlorodihydrofluorescein
H_2DCF-DA	2', 7'-Dichlorodihydrofluorescein-diacetate
HBSS	Hank's balanced salt solution
NM	nanomaterial
ppm	parts per million
RT	room temperature

10 References

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