

Unclassified**English - Or. English**

24 September 2020

**ENVIRONMENT DIRECTORATE
JOINT MEETING OF THE CHEMICALS COMMITTEE AND THE WORKING
PARTY ON CHEMICALS, PESTICIDES AND BIOTECHNOLOGY****ANNEX I: DETAILED ASSAY DESCRIPTIONS WITHIN CASE STUDY ON
THE USE OF AN INTEGRATED APPROACH TO TESTING AND
ASSESSMENT FOR IDENTIFICATION AND CHARACTERISATION OF
PARKINSONIAN HAZARD LIABILITY OF DEGUELIN BY AN AOP-BASED
TESTING AND READ ACROSS APPROACH****Series on Testing and Assessment
No. 326**

The corresponding monograph to this annex is available under the following cotes:
ENV/JM/MONO(2020)22

JT03465712

Methods

Seahorse

- Complexes
- Whole cells

Mitochondrial Membrane Potential

Mitochondrial viability

- lactate
- resazurin
- ATP levels

Proteasome assays

- Proteasome activity
- CHOP-GFP reporters

Neurite outgrowth/degeneration

- glucose
- galactose

Bioavailability

- model
- samples
- mass spectrometry

PBPK

Repeat dosing

Receptor docking

Some methods are still in the process of being described, see the next table.

Type	sub	Lab	Document	Method	Partner	Type
Seahorse	complex	UKN	Annex 1.1 Assessment of individual mitochondrial respiratory chain complex activity with permeabilised LUHMES cells	seahorse_complex_UKN	Johannes Delp (UKN)	SOP
		Cyprotex	Method will be soon available		Richard Maclennan (Cyprotex)	
	Whole	UKN	Annex 1.2 Mitochondrial stress test using LUHMES cells	seahorse_mitostresstest_UKN	Johannes Delp (UKN)	SOP
		VU	Annex 1.3 CS4 read across HepG2 Seahorse method description	seahorse_mitostresstest_VU	Giada Carta (VU)	description
MMP	JC1	VU	Annex 1.4. CS4 read across RPTEC/TERT1 methods description	Lactate_resazurin_JC1_VU	Giada Carta (VU)	description
	RHO	LU	Annex 1.5 DB-ALM Protocol HepG2 based Rhodamine123 assay - Last Update: Sept 2018 (v.9.4)	MMP_LU	Wanda van der Stel (LU)	DB-ALM type
	RHO	Swetox	Annex 1.6 Measurements mitochondrial membrane potential in differentiated human neuroblastoma SH-SY5Y cells after exposure to chemicals	MMP_Swetox	Anna Forsby (Swetox)	SOP
Mitochondrial viability	lactate	all partners	Annex 1.7 Lactate Assay (provided by Paul Jennings)	Lactate assay	Giada Carta (VU)	SOP
		VU	Annex 1.4. CS4 read across RPTEC/TERT1 methods description	Lactate_resazurin_JC1_VU	Giada Carta (VU)	description
		Swetox	Annex 1.8 Lactate measurement in supernatant after acute exposure (24h) in differentiated SH-SY5Y cells	Lactate assay_swetox	Anna Forsby (Swetox)	SOP
	resazurin	all partners	Annex 1.9 Resazurin Assay (provided by Paul Jennings)	Resazurin assay	Giada Carta (VU)	SOP
		VU	Annex 1.4. CS4 read across RPTEC/TERT1 methods description	Lactate_resazurin_JC1_VU	Giada Carta (VU)	description
		Swetox	Annex 1.10 Cell viability measurements with Resazurin after acute exposure (24h) in differentiated SH-SY5Y cells	Resazurin assay_Swetox	Anna Forsby (Swetox)	SOP
Proteosome	Assay	UKN	Annex 1.11 Assessment of proteasome activity in live cells	proteasomal_activity_UKN	Johannes Delp (UKN)	SOP
	Reporters	LU	Annex 1.12 DB-ALM Protocol HepG2 BAC-GFP Cell Stress Reporter Cell Line - Last Update: May 2018 (v.9.4)	GFP-reporters_LU	Wanda van der Stel (LU)	DB-ALM type
Neuron outgrowth	glucose	UKN	Method will be soon available		Johannes Delp (UKN)	
		Swetox	Annex 1.13 Neurite degeneration measurements in differentiated SH-Sy5Y cells after acute exposure (24 hours) to chemicals	neurite_degeneration_Swetox	Anna Forsby (Swetox)	SOP
	galactose	UKN	Annex 1.14 NeuriTox/UKN4 assay in galactose conditions	neuron_outgrowth_UKN	Johannes Delp (UKN)	SOP
Bioavailability	model	Cyprotex	Method will be soon available		Iain Gardner (Certara)	
	collection samples	Cyprotex	Method will be soon available		Richard Maclennan (Cyprotex)	
	mass spec	Cyprotex	Method will be soon available		Richard Maclennan (Cyprotex)	
PBPK	Model	Certara	Annex 1.15 Biokinetic Modelling Description	Biokinetic Modelling	Iain Gardner (Certara)	description
Repeat dosing	RDT	Swetox	Annex 1.16 Multiplex measurement of neurite degeneration, PI staining, ATP content.	Repeat dosing	Anna Forsby (Swetox)	SOP

	UKN	Method will be soon available		Johannes Delp (UKN)	
	LU	Method will be soon available		Wanda van der Stel (LU)	
	VU	Method will be soon available		Giada Carta (VU)	
Docking	UNIVIE	Method will be soon available		Florentina/Barbara	
Summary file	Swetox	Annex 1.17 CS4 read across SH-SY5Y method description (Swetox)	Summary	Anna Forsby (Swetox)	description
	VU	Annex 1.4. CS4 read across RPTEC/TERT1 methods description	Summary	Giada Carta (VU)	description
	LU	Annex 1.18 CS4 read across HepG2 method description (LU)	Summary	Wanda van der Stel (LU)	description
	UKN	Method will be soon available	Summary	Johannes Delp (UKN)	

Annex 1.1. Assessment of individual mitochondrial respiratory chain complex activity with permeabilised LUHMES cells

Method	LUHMES: Individual mitochondrial complex activity assessment
Contact	Johannes Delp (UKN) -> johannes.delp@uni-konstanz.de

Introduction

Aim

This protocol describes the analysis of how to assess the activity of the mitochondrial respiratory chain complex I-IV activities by using selectively permeabilised LUHMES cells (human neuronal).

Purpose

Many toxic substances inhibit mitochondrial function. Also, many compounds in research for pharmaceutical use inherit mitochondrial off-target effects. This assays aims to identify direct inhibition of the MRC complexes I-IV.

Limitations

Since the analysis of each MRC complex activity is based on the oxygen consumption at cIV, sometimes only indirect conclusions can be drawn. However, control experiments can confirm the suggested results. Additionally, adverse effects on cytochrome c or ubiquinone are not assessed.

Method outline

Cells get selectively, i.e. only their plasma membrane, permeabilised before the experiment starts. Then, their MRC complexes are sequentially fed with specific substrates, i.e. first cI, then cII, then cIII, finally cIV. At the same time, inhibitors for upstream complexes are added with the fuel substance for the next downstream complex to enable the analysis. Finally, complex activity is analysed simultaneously in treated samples relative to solvent control samples, based on the cIV-mediated oxygen consumption using the Seahorse device.

Method description

Chemicals and buffers

Table 1. Chemicals used in experiments

Product	Supplier	Cat. No.	Lot. No.	Cas. No.
ADP	Sigma	A5285	-	72696-48-1
Digitonin	Sigma	D141	0001432565	11024-24-1
D-Mannitol	Sigma	M4125	007K0166	69-65-8
DMSO	Merck	1.09678	K48040378 727	67-68-5
Duroquinol	TCI	T0822	5QCSN	527-18-4
EGTA	Sigma	E0396	SLBP2807V	67-42-5
Fatty-Acid free BSA	Roth	8076.5	197254757	90604-29-8
HEPES	Roth	HN77.4	468101696	-
KH ₂ PO ₄	Ridel-deHaen	30407	51530	7778-77-0
L-Ascorbic Acid	Sigma	A-4544	21H01855	50-81-7
L-Glutamine	Sigma	G3126	046K0009	56-85-9
Malic acid	Sigma	M6413	BCBJ3883V	97-67-6
Malonic acid	Sigma	M1296	BCBV3859	141-82-2
MgCl ₂ -Hexahydrate	Roth	2189.1	387253790	7791-18-6
Potassium Hydroxide	Sigma	30603	SZBE1180V	1310-58-3
Pyruvic acid	Sigma	107360	MKCC8726	127-17-3
Succinic acid	Sigma	S9512	057K01281	110-15-6
Sucrose	Merck	1076511000	K47396351613	57-50-1
TMPD	Sigma	T7394	STBC1186V	100-22-1
XF calibrant solution	Agilent/Seahorse	100840-000	090	-

Table 2. Reagent preparation

Reagent	Stock	Final (on cells)	10 x port solution	Solvent
ADP	100 mM	1 mM	10 mM	MAS
Ascorbate	200 mM	2.5 mM	20 mM	MAS
Digitonin	25 mg/ml	25 µg/µl	250 µg/ml	MAS
Duroquinol	500 mM	0.250 mM	2.5 mM	DMSO
Glutamate	200 mM	2 mM	20 mM	MAS
Glutamine	200 mM	2 mM	20 mM	MAS
Hydroquinone	500 mM	5 mM	50 mM	Water
Idebenone	250 mM	0.25 mM	2.5 mM	DMSO
Malate	250 mM	2.5 mM	25 mM	MAS
Malonate	250 mM	5 mM	50 mM	MAS
Pyruvate	500 mM	5 mM	50 mM	MAS
Succinate	250 mM	10 mM	100 mM	MAS
TMPD	500 mM	0.125 mM	1.25 mM	Ethanol

Table 3. Formulation of 1x MAS buffer

Reagent	1x MAS	Amount for 1.0 liter of 1x MAS
Sucrose	70 mM	23.96 g
Mannitol	220 mM	40.08 g
KH ₂ PO ₄	10 mM	1.36 g
MgCl ₂	5 mM	1.02 g
HEPES	2 mM	0.48 g
EGTA	1 mM	0.38 g
Fatty acid free BSA	4 mg/ml	4 g

Reagents were dissolved in MiliQ-water and pH was adjusted to 7.2 by using KOH. After 0.22 µm filter sterilisation, MAS buffer was stored at 4°C.

Preparation upfront

- Hydrate seahorse cartridge
- Have cells at 95% confluency in coated (normal PLO/fibronectin LUHMES coating, optionally with additional laminin) Seahorse plates ready, cultured at least 18 h in these plates for equilibration
- 1x MAS buffer
- Substrates and tool compounds

Experimental procedure

1. Cells were grown in T175 cell culture flasks until they reached a confluency of 95%
2. For assays, conducted one day after cell seeding in Seahorse cell culture plates, proliferating cells were seeded at a density of 60.000 cpw in 100 µl proliferation medium (PM). For assays, conducted two days after cell seeding in Seahorse cell culture plates, proliferating cells were seeded at a density of 40.000 cpw in 100 µl PM.
3. After one hour of attachment, 150 µl of PM was added to proliferating cells.
4. The Seahorse cell culture plates were incubated at 37°C in a humidified 5%-CO₂ incubator until the day of assay.
5. At the day of assay, the reagents for the respective assay were prepared. Therefore, 1x-MAS buffer was warmed to 37°C in the water bath.
6. The prepared stock solutions of mitochondrial effectors and oxidisable substrates had to be thawed if they were frozen.
7. MAS-buffer is used to prepare the 10x port solutions, which are loaded into the ports of the cartridges. The desired final concentrations for oxidisable substrates and mitochondrial inhibitors are listed in above tables.
8. After preparing the desired concentrated port solutions, they are loaded into the appropriate ports of the cartridge, by adding 56 µl/62 µl/69 µl/77 µl into ports A/B/C/D, respectively.
9. Until the start of the assay, the cartridge was incubated at 37°C without CO₂.

10. Create an XF assay template with Mix/Wait/Measure times of 2 min/1 min/2 min, respectively. The equilibration step is excluded from the assay template. Each step is repeated two cycles.
11. After the calibration step of the XF assay template is finished, the cell culture plate is removed from the incubator.
12. The medium was replaced with pre-warmed 1x MAS-buffer (37°C). Therefore, the medium was carefully aspirated, and 500 µl of MAS-buffer was added to each well of the cell culture plate.
13. The utility plate was removed from the analyser and was discarded, while the cartridge remained in the instrument.
14. Finally, the cell culture plate was inserted into the XF analyser and the assay was started.

Data analysis

- Each experiment has to contain control treated (solvent) samples
- Oxygen levels have to be checked before deeper data analysis (mmO₂, not OCR!)
- If oxygen doesn't get depleted, the data can be analysed.
- First, basal respiration of all wells gets normalised to the last measurement value before compound injection via port A
- Then the oxygen consumption rate of the first measurement cycle after each tool substrate/tool inhibitor mix injection undergoes comparison. Inhibition results in OCR values smaller in treated samples than in control samples, uncoupling vice versa.
- Compound effects are expressed as percent inhibition relative to control
- Over several experiments (4 weeks, 30 compounds, 4 complexes assessed), the SD of the measurement was 12.5%. Thus compound effects of >25% (=2xSD) were considered as "biologically significant"

Annex 1.2. Mitochondrial stress test using LUHMES cells

Method	LUHMES: Mitochondrial stress test
Contact	Johannes Delp (UKN) -> johannes.delp@uni-konstanz.de

Introduction

Aim

This protocol describes the analysis of how to assess the utilised and spare mitochondrial activity of LUHMES cells (human neuronal).

Purpose

Many toxic substances impair mitochondrial function. Also many compounds in research for pharmaceutical use inherit mitochondrial off-target effects. This assays aims to identify direct or indirect impairment of mitochondrial function.

Limitations

This assay assesses only the mitochondrial and non-mitochondrial oxygen consumption, but gives not directly an explanation why these parameters might be impaired. E.g., mitochondrial pyruvate uptake inhibitors might result in the same reduction in oxygen consumption as complex I inhibitors. Additionally, redox cyclers might result in the same increased oxygen consumption as mitochondrial uncouplers. Therefore, follow-up experiments might be needed.

Method outline

Cells are cultured in Seahorse assay plates (100,000 cells per well in 24 well plates) and allowed to equilibrate to their environment. At the day of the assay, their normal cell culture medium gets replaced by Seahorse assay medium, supplemented with pyruvate (1 mM), glucose (18 mM), glutamine (2 mM), N₂ supplement (1x) and tetracycline (2.25 µM) 1 h prior to the assay.

Then the Agilent Seahorse Mitostress test is performed according to the manufacturer's recommendation. Port A is used for the injection of the compound of interest or the solvent control, while ports B-D are used for oligomycin, FCCP and rotenone/antimycin a, respectively.

Finally, mitochondrial oxygen consumption is analysed simultaneously in treated samples relative to solvent control samples, based on the oxygen consumption using the Seahorse device, as the manufacturer recommends.

Method description

Chemicals and buffers

- Agilent Seahorse Mitostress test:
- Oligomycin: final concentration on cells is 1 µM

- FCCP: final concentration on cells is 1.5 μM

Rotenone/antimycin A: final concentration on cells is 0.5/0.5 μM

Agilent Seahorse basal DMEM

Glucose, pyruvate, glutamine, N_2 supplement and tetracycline

Preparation upfront

- Hydrate seahorse cartridge
- Have cells at in coated (normal PLO/fibronectin LUHMES coating, optionally with additional laminin) Seahorse plates ready, cultured at least 18 h in these plates for equilibration
- Substrates and tool compounds

Experimental procedure

1. Change the normal cell culture medium to the assay medium, place the plate in a 37°C non- CO_2 incubator for at least 1 h before the start of the experiment
2. Prepare the cartridge with the compounds solutions (10x solution in ports A/B/C/D, 56 μl /62 μl /69 μl /77 μl , respectively), incubate in a 37°C non- CO_2 incubator for at least 1 h before the start of the experiment
3. Set up the Seahorse analyser and the measurement program; use mix/wait/measure times of 3/2/3 minutes, respectively. Enable “calibrate” and “equilibrate”
4. Calibrate the cartridge and when the instrument is ready, the utility plate is removed from the analyser and, while the cartridge remained in the instrument.
5. Finally, the cell culture plate was inserted into the XF analyser and the assay was started.

Data analysis

- Each experiment has to contain control treated (solvent) samples
- Oxygen levels have to be checked before deeper data analysis (mmO_2 , not OCR!)
- If oxygen doesn't get depleted, the data can be analysed.
- First, basal respiration of all wells gets normalised to the last measurement value before compound injection via port A
- Then the oxygen consumption rate of the first measurement cycle after each tool inhibitor injection undergoes comparison. Inhibition results in OCR values smaller in treated samples than in control samples, uncoupling vice versa.
- Compound effects are expressed as percent inhibition relative to control
- The manufacture's Excel sheets were used for data analysis.

Annex 1.3. CS4 read across HepG2 Seahorse method description

Method	HepG2: Resazurin Assay
Contact	Giada Carta (VU) -> g.cart@vu.nl

Cell Model: Human Hepatocellular Carcinoma cells. It is an immortal non-tumorigenic cell line with high proliferation rates and an epithelial-like morphology (Donato, Tolosa, & Gómez-Lechón, 2015). HepG2 don't need differentiation and use glycolysis as primary source of energy.

Cell splitting and routine culture: For routine purposes cells are cultured on Sarstedt 10 cm dishes, seeded at the density approximately 1.5×10^5 cells/mL and fed every 2nd / 3rd day with 10 mL of cell culture medium Lonza DMEM (25 mM glucose) containing: Pen/Strep (100 U/ml and 100 µg/ml) and 10 % FBS.

Cell splitting for experiments: for CS4 experiments, cells are seeded as described above in a 96 well plastic seahorse cell culture microplate pre-coated with 5 µg/cm² collagen IV, at density of approximately 3×10^5 cells/mL one day prior to experiment.

Chemical stock maintenance and use: Stock solution of chemicals are dissolved in 100% DMSO at the concentration of 50 mM and stored at -20 °C until use. Exposure dilutions are prepared fresh for each experiment in cell culture medium at DMSO concentration of 0.1%. Controls cells are treated with cell culture medium at DMSO concentration of 0.1%

Mitostress assay with Seahorse analyser

The Seahorse mitostress test measures key parameters of mitochondrial function by directly measuring the oxygen consumption rates (OCR) of cells. OCR measured after sequential injections of modulators of respiration that target components of the electron transport chain permits derivation of ATP production, maximal respiration, proton leak, spare respiratory capacity and non-mitochondrial respiration (Agilent Seahorse, 2017).

For this system, cells are injected with test compounds prior to Mitostress modulators.

Cells plate preparation: Cells are plated on seahorse cell culture microplate one day before experiment. On the day of experiment medium is exchanged one hour prior to chemical exposure with the Seahorse XF Base Medium (DMEM based with no glucose, bicarbonate and phenol red) supplemented with 1 mM pyruvate, 2 mM glutamine, 10 mM glucose and 5 mM HEPES, pH 7.4.

Assay workflow:

- Baseline OCR of cells is measured for 20 minutes
- Test compound is injected, and the OCR is measured for 30 minutes
- After compound injection mitostress modulators are injected in the following order and OCR is measured for three times after each injection; oligomycin 2 µM (complex V inhibitor, allows ATP production calculations), FCCP 2 µM (uncoupler, allows maximal respiration capacity calculations), mixture of Antimycin A and Rotenone 0.5 µM each (complex III and complex I inhibitors, allows calculation of non-mitochondrial respiration).

CS4 read across reported data: reported data consist of a dose response curve given by OCR measurements in intact cells after test compound injection for 30 minutes and expressed as percentage of their own OCR baseline measurements prior to chemical injection.

Re-normalisation of datasets for BMR calculations:

Values of at least two no effect concentrations were forced to upper asymptote exactly at 100 %, data sets were re-normalised as percentage of no effect concentrations. Curve fitting and BMRs values are generated using the *in vitro* toxicology on-line tool provided by AG. Leist, University of Konstanz (<http://invitrotox.uni-konstanz.de:3838/BMC/>).

Agilent Seahorse. (2017). Mito Stress Test. Agilent.

Donato, M. T., Tolosa, L., & Gómez-Lechón, M. J. (2015). Culture and Functional Characterization of Human Hepatoma HepG2 Cells (pp. 77–93). Humana Press, New York, NY.
https://doi.org/10.1007/978-1-4939-2074-7_5

Annex 1.4. CS4 read across RPTEC/TERT1 methods description

Method	RPTEC/TERT1: Lactate Assay, Resazurin Assay, MMP assay (JC-1)
Contact	Giada Carta (VU) -> g.cart@vu.nl

Cell Model: Human Renal Proximal Tubular Cells. Telomerase immortalised, non-cancerous RPTEC/TERT1 (Wieser *et al.*, 2008). Prior to differentiation, the cells are bundle-shaped and highly glycolytic. Upon contact inhibition, RPTEC/TERT1 cells stop proliferating and start differentiating in polarised epithelial cells. Differentiated cells slow down glycolytic rates to increase oxidative phosphorylation as source of energy, furthermore acquire the characteristics of proximal tubule cells, including the expression of numerous transporters. The transport of water and solutes across the monolayer causes the formation of the characteristic “dome” structures on plastic. Microscopic assessment of domes is used as differentiation criteria.

Cell splitting and routine culture: For routine purposes cells are cultured on Sarstedt 10 cm dishes, seeded at the density approximately 1.5×10^5 cells/mL and fed every 2nd / 3rd day with 10 mL of hormonally defined cell culture medium DMEM/F12 (5 mM glucose) containing: Pen/Strep (100 U/ml and 100 µg/ml), Glutamax (2 mM), ITS (~5 µg/mL, 5 ng/mL), EGF (10 ng/mL), Hydrocortisone (36 ng/mL).

Cell splitting and differentiation for experiments: for CS4 experiments, cells are seeded as described above in a plastic 96 well plate format at density of approximately 1.5×10^5 cells/mL and fed every 2nd / 3rd day with 100 µL cell culture medium. After confluence is reached, 7 days of spontaneous differentiation are counted, time in which formation of domes is assessed.

Experimental exposure to mitotoxins: differentiated cells on 96 well plates are fed 24 h prior to chemical exposure. Stock solution of chemicals are dissolved in 100% DMSO at the concentration of 50 mM and stored at -20 °C until use. Exposure dilutions are prepared fresh for each experiment in cell culture medium at DMSO concentration of 0.1%. Controls cells are treated with cell culture medium at DMSO concentration of 0.1%. For this system, cells are treated for 24 h before cell viability and cellular stress assays are performed.

Endpoints:

1. Supernatant lactate quantification:

Most cells use glucose as a primary energy source. The RPTEC/TERT1 medium contains 5 mM glucose that can be used by the cells in glycolysis (leading to the final product lactate) and/or oxidative phosphorylation for the production of ATP. When lactate is produced, it is quickly extruded from the cells and can be measured in cell culture supernatants. We have previously shown that exposure of RPTEC/TERT1 and other cell lines to chemicals cause an increase in supernatant lactate, likely due to injury or dysfunction of mitochondria leading to or resulting from various types of cellular stress (Limonciel *et al.*, 2011).

24 h after chemical exposure supernatant medium is collected. Produced lactate is quantified using a colourimetric assay. The lactate assay is based on the conversion of lactate to pyruvate by the enzyme lactate dehydrogenase (LDH), reducing the co-factor NAD to NADH (Babson & Phillips, 1965). In the assay, NADH reduces PMS to PMSH, which reduces INT to INT^H. INT^H is the reagent measured by

colourimetry. Optical density is read at 490 nm. Unknown lactate values in mM are extrapolated from lactate standard curve using a spline fit function. Values are expressed as percentage of 0.1% DMSO controls.

2. Resazurin reduction quantification:

This method assesses cell viability based on the irreversible reduction of the blue dye resazurin to the pink product resorufin, which is highly fluorescent in red. Although the exact enzyme(s) involved in the reaction have not been identified, the assay is considered to be based on the global REDOX capacity of the cells in culture.

24 h after chemical exposure supernatant medium is collected for lactate quantification, cells are washed with PBS and a fresh cell culture medium containing 44 μ M resazurin is applied to the cells for 1.5 h. Fluorescence of the redox product resorufin is read at excitation 540 nm / emission 590 nm. Values are expressed as percentage of 0.1% DMSO controls.

3. Mitochondrial membrane potential assessment with JC-1:

JC-1(tetraethylbenzimidazolylcarbocyanine iodide) is a cationic dye that accumulates in energised mitochondria. At low concentrations (due to low mitochondrial membrane potential) JC-1 is predominantly a monomer that yields green fluorescence with emission of 530 ± 15 nm. At high concentrations (due to high mitochondrial membrane potential), the dye aggregates yielding a red to orange coloured emission (590 ± 17.5 nm). The emission spectra of JC-1 shifts from green to red with increasing concentration (i.e., aggregation) in the mitochondria, thus allowing for a dual-colour (green/red) and radiometric semiquantitative assessment of mitochondrial polarisation states (Perry, Norman, Barbieri, Brown, & Harris, 2011).

24 h after chemical exposure, cells are treated with cell culture medium containing 2 μ L/mL JC-1 2 mg/mL and 10 μ L/mL Pluronic. After approximately 1.5 h the JC-1 solution was replaced with fresh medium. In a plate reader, cytoplasmatic JC-1 (green monomers) is read at 492 nm excitation and 535 nm emission and mitochondrial JC-1 (red dimers) at 492 nm excitation and 590 nm emission. The polarisation of the membrane is calculated as ratio of mitochondrial over cytoplasmatic JC-1, the higher the ratio the higher the polarisation of the membrane. Values are expressed as percentage of 0.1% DMSO controls.

Re-normalisation of datasets for BMR calculations:

4. **Supernatant lactate:** values of at least two no effect concentrations were forced to upper asymptote exactly at 100 %, data sets were re-normalised as percentage of no effect concentrations. Curve fitting and ECs values couldn't be generated using the *in vitro* toxicology on-line tool provided by AG. Leist, University of Konstanz (<http://invitrotox.uni-konstanz.de:3838/BMC/>) as the algorithm is suitable only for inhibition curves. Lactate production increase with increase of the insult leading to activation curve, therefore curve fitting and ECs values are generated using point-to-point extrapolation in GraphPad Prism.
5. **Resazurin reduction and MMP with JC-1:** values of at least two no effect concentrations were forced to upper asymptote exactly at 100 %, data sets were re-normalised as percentage of no effect concentrations. Curve fitting and BMRs

values are generated using the *in vitro* toxicology on-line tool provided by AG. Leist, University of Konstanz (<http://invitrotox.uni-konstanz.de:3838/BMC/>).

- Babson, A. L., & Phillips, G. E. (1965). A rapid colorimetric assay for serum lactic dehydrogenase. *Clinica Chimica Acta*, 12, 210–215.
- Limonciel, A., Aschauer, L., Wilmes, A., Prajczner, S., Leonard, M. O., Pfaller, W., & Jennings, P. (2011). Lactate is an ideal non-invasive marker for evaluating temporal alterations in cell stress and toxicity in repeat dose testing regimes. *Toxicology in vitro*, 25(8), 1855–1862. <https://doi.org/10.1016/j.tiv.2011.05.018>
- Perry, S. W., Norman, J. P., Barbieri, J., Brown, E. B., & Harris, A. (2011). Mitochondrial membrane potential probes and the proton gradient: a practical usage guide. *Biotechniques*, 50(2), 98–115. <https://doi.org/10.2144/000113610.Mitochondrial>
- Wieser, M., Stadler, G., Jennings, P., Streubel, B., Pfaller, W., Ambros, P., Grillari-Voglauer, R. (2008). hTERT alone immortalizes epithelial cells of renal proximal tubules without changing their functional characteristics. *Am J Physiol Renal Physiol*, 295, 1365–1375. <https://doi.org/10.1152/ajprenal.90405.2008.-Telomere-dependent>

Annex 1.5. DB-ALM Protocol HepG2 based Rhodamine123 assay - Last Update: Sept 2018 (v.9.4)

The present template is based on the Content Criteria for Protocols, which are designed for the provision of technical details that enable the documented alternative method to be transferred to other laboratories without the need of additional information.

The Content Criteria, on which the presented template is based, are generic and based on the analysis of common descriptors from hundreds of different non-animal experimental methods and techniques. However, not all parameters or sections indicated are applicable to all protocols. It is the responsibility of the author to provide content where relevant and as appropriate related to a precise protocol. The compilation is to be performed by completing the individual sections with the information related to your method. A review for consistency, completeness in relation to the technique described and compliance with the Content Criteria in place is always performed by the JRC staff. Furthermore, before any protocol is published *via* the DB-ALM, the final draft is reviewed and approved by designated contact person(s) (method's owner and/or experienced user).

A summary description of the main method features bringing it in a context regarding its intended purpose(s) and application(s), as well as the scientific rationale is always to be provided with each protocol. The content criteria for the method summary will be sent in due course.

The DB-ALM is operated by the European Reference Laboratory for Alternatives to Animal Testing of the Joint Research Centre.

NOTE: All statements shall be supported with references within the text and full details listed under "Bibliographic References" data sector.

Part A. Protocol Introduction

Protocol Name: LU HepG2 Rhodamine123 assay

Abstract:

This protocol describes how to culture HepG2 cells, how to perform compound exposures, how to perform imaging of the reporters, how to perform image analysis and how to perform additional functional assays.

Résumé

With the Rhodamine123 mitochondrial membrane potential sensitive dye the presence of the membrane potential can be monitored over time using confocal imaging systems. This approach provides the option to determine dye intensity and localisation. Furthermore, the dye is not invasive meaning that the cells can subsequently be used in assays including cell death assays using propidium iodide, galactose capacity determinations by measuring lactate levels in the supernatant and viability assays by using the resazurin reduction read-out. See references.

Experimental Description

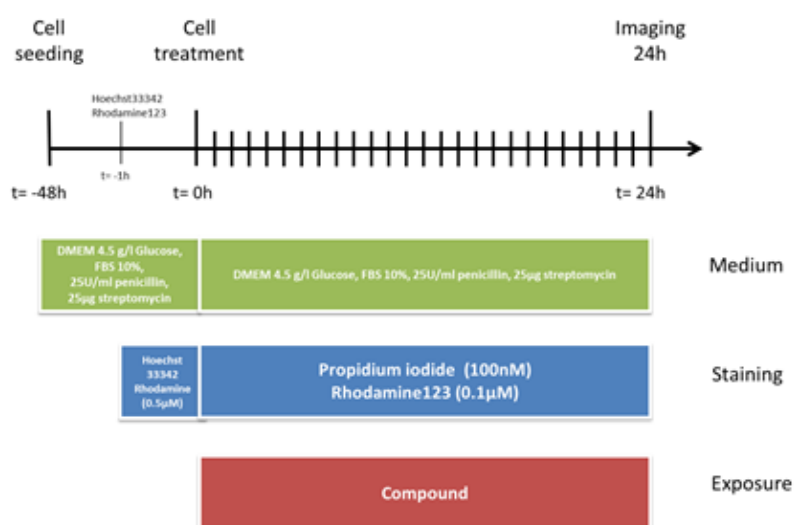
Biological Endpoint and Endpoint Measurement:

The endpoints of our toxicity test system include:

1. Mitochondrial integrity based on membrane potential sensitive dye (rhodamine123)
2. Cytotoxicity based on Propidium Iodide (PI)
3. Glycolytic capacity based on lactate concentrations
4. Viability based on resazurin reduction

The acquisition of microscopic pictures is done at specific timepoints, i.e. 24h/48h/72h or live (every 1h).

Figure 1. Exposure scheme read out every hour live



Endpoint Value:

1. The number of cells is quantified using the Hoechst 3342 staining.
2. The mitochondrial membrane potential is determined based on the EGFP signal intensity of the Rhodamine123 in the cytoplasmic compartment. The intensity is quantified based on the integrated intensity of all pixels in the cytoplasm (circle of 10 pixels around identified nuclei).
3. The cytotoxicity measurement is based on the percentage of PI positive nuclei.
4. The glycolytic potential of the cell based on lactate concentration
5. The viability measurement is based on the reduction of resazurin to resorufin.
6. Dose response modelling, four-parametric log logistic curve fitting or point of departure calculation might be used to compare responses triggered by different compounds.

Experimental System:

The assay is performed in HepG2 (ATCC® HB8065™) using Rhodamine123, and PI plus lactate concentration measurements and resazuring reduction measurements.

Basic procedure

The HepG2 reporter cell lines are seeded in multi-well imaging plates, stained with Hoechst 33342, propidium iodide, and treated with different concentration of testing compounds. The cells are then imaged live on a confocal laser scanning microscope. The imaging procedure is followed by the collection of supernatant for lactate concentrations. The cells are submerged in the resazurin staining for 2 hours after with the intensity of the reduction product (resafurin) is measured.

Discussion

This bioassay is based live monitoring of the various dyes; therefore, an image acquisition a confocal laser scanning microscope including a cell culture incubator is required. Depending on the test compounds, further safety measures might be necessary.

The person conducting the experiment needs to be properly trained for culturing HepG2 cells, confocal microscopy and high content data analysis; therefore, a Master of Science level education is recommended.

Depending on the desired time point, the experiment itself can be conducted in 3 to 5 days. The amount of time of the data analysis is depending on the computing capacity.

The critical point of this experiment is the seeding of the HepG2 cells as a single cell suspension. HepG2 cells tend to form clumps, which negatively affect the image acquisition with a confocal laser scanning microscope and the single nuclei segmentation during the high content image analysis.

Status

In Development:

Data analysis pipeline: Point of departure, EC values

Known Laboratory Use:

The usage of the Rhodamine 123 dye for MMP measurements is widely accepted. However, the use of the usage in a live confocal setting is limited to the group of Bob van de Water at the University of Leiden.

The usage of the lactate and resazurin assay in the bellowed described setting are based on the SOP provided by the group of Paul Jennigs at the Vrije University of Amsterdam.

Participation in Evaluation Study:

The SOP was developed within the frame of the EU-ToxRisk project:

<http://www.eu-toxrisk.eu/>

Grant agreement No 681002

Participation in Validation Study:

The test method did not undergo any validation as defined in OECD Guidance Document No. 34 so far.

Regulatory Accepted:

There are no relevant guidelines available.

Proprietary and/or Confidentiality Issues

The use of rhodamine123 technology is not restricted by patents. The protocols for the lactate concentration assay and the resazurin assay are published (see references). The protocol regarding the experimental and microscopy procedures is open. However, the protocol regarding the automated data-analysis is owned by the Van de Water lab and should not be shared among others.

Health and Safety Issues**General precautions:**

Follow general safety precautions of a mammalian cell culture lab. Wear lab coat, gloves and safety goggles. For the laser scanning fluorescence microscope, safe work with the involved lasers has to be guaranteed. The involved cell lines are safe to work with in a biosafety level 1 according to council directive 90/679/EEC.

MSDS Information:

For safety data sheets, please contact the compounds' manufacturer.

Abbreviations and Definitions

ATCC...American Type Culture Collection
 BAC...Bacterial artificial chromosome
 BEVC ...best-fit ellipse of Voronoi cell
 DMEM... Dulbecco modified eagle medium
 DMSO... Dimethyl sulfoxide
 EDTA... Sodium ethylenediaminetetraacetate
 EGFP...Enhanced green fluorescent protein
 FBS...foetal bovine serum
 MMP...Mitochondrial membrane potential
 PoD...Point of Departure
 PI...propidium iodide
 P/S...Penicillin/Streptomycin solution
 RT...room temperature
 T25/T75/T175...Tissue flask 25 cm² /75cm²/175cm²
 WMC...watershed masked clustering

Part B. Technical Description

Procedure Details, Latest Version: 1.

Protocol Name: LU HepG2 Rhodamine123 assay

Contact person

Bob van de Water

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Materials and Preparations*Cell OR Experimental system*

The original HepG2 wildtype cells were obtained from ATCC (ATCC® HB-8065™). The obtained cell line has been biobanked and stored at -80°C for short term storage and -150°C for long term storage.

*Equipment***Fixed Equipment**

Material/Equipment
Centrifuge
TC20™ Automated Cell Counter
Fridge (4°C)
Freezer (-20°C, -80°C, -150°C)
Humidified incubator (37°C, 5% CO ₂)
Ice machine
Laminar flow hood for sterile atmosphere
Laminar flow hood for sterile atmosphere
Water Bath
Stereoscopic microscope
Nikon Eclipse Ti or Nikon Eclipse Ti2, equipped with: four lasers (408, 488, 561 and 644 nm lasers), a A1, C1 or C2 box, live cell incubator, automated stage, perfect focus system or equivalent.
Milli-Q water dispenser
FluoStar plate reader

Material/Equipment	Supplier	Catalogue No
Micropipettes Pipet-Lite XLS LTS		
Pipette L-1000XLS+	Rainin	17014382
Pipette L-200XLS+	Rainin	17014391
Pipette L-20XLS+	Rainin	17014392
Pipette L-2XLS+	Rainin	17014393
Multichannel pipets		
Eppendorf Research® plus, 8-channel, variable, incl. epT.I.P.S.® Box, 0.5 – 10 µL, medium gray	Eppendorf	312500010
Eppendorf Research® plus, 8-channel, variable, incl. epT.I.P.S.® Box, 10 – 100 µL, yellow	Eppendorf	312500036
Eppendorf Research® plus, 8-channel, variable, incl. epT.I.P.S.® Box, 30 – 300 µL, orange	Eppendorf	312500052
Software		
NIS viewer	Nikon	
CellProfiler® (version 2.1.1)	Carpenter Lab at the Broad Institute of Harvard and MIT.	
R Studio (version R version 3.3.2 or newer)	RStudio,	
Plastics		
Sterile 15 plastic tubes	Sarstedt	62.554.502
Sterile 50 plastic tubes	Greiner Bio-One	210270
Sterile cell culture flasks T25	Corning	430639
Sterile cell culture flasks T75	Corning	430641U
Sterile cell culture flasks T175	Corning	431080
Sterile pipettes 2 ml	Greiner Bio-One	710180
Sterile pipettes 5 ml	Greiner Bio-One	606180
Sterile pipettes 10 ml	Greiner Bio-One	607180
Sterile pipettes 25 ml	Greiner Bio-One	760180
Cryovials		
Cryo.s U-starand-2ml	Greiner Bio-One	122263
Cryo.s U-starand-2ml	Greiner Bio-One	122277
Cryo.s U-starand-2ml	Greiner Bio-One	122278
Cryo.s U-starand-2ml	Greiner Bio-One	122279
Cryo.s U-starand-2ml	Greiner Bio-One	122280
Micro tube 1.5ml	Sarstedt	72.690.001
Micro tube 2ml	Sarstedt	76.695.500
Sterile Pipette tips		
LTS tips 20ul	Mettler Toledo	17005091
LTS tips 200ul	Mettler Toledo	17005093
LTS tips 1000ul	Mettler Toledo	17005089
Biopur 2-200 ul refills	VWR	613-3569
Biopur 20-300 ul refills	VWR	613-3570
Gloves EcoSHIELD wit econitrl PF 250	Boom BV	38055162; 38055163; 38055164
Imaging plates		
96 µclear wells plate	Greiner Bio-One	655090
96 ScreenStar plate	Greiner Bio-One	655 866
384 µclear wells plate	Greiner Bio-One	781 091
Cell Counting Kit, 30 dual-chambered slides, 60 counts, with trypan blue	BIO RAD	1450003
50µm non-sterile CellTrics® filters	SysmexNederland	04-0042-2317

Testing compounds

Compound	abbreviation	Sigma Aldrich Cat. No.	Labelling accord. Reg. (EC) 1272/2008
Rotenone	ROT	R8875	
Deguelin	DEG	D0817	
Azoxystrobin	AZO	31697	
Kresoxim-methyl	KRE	37899	
pyraclostrobin	PYR	33696	
Picoxystrobin	PIC	33658	
trifloxystrobin	TRI	46477	

Media, Reagents, sera, others

Material	Supplier	Catalogue Number
DMEM, high glucose, pyruvate	Fisher Scientific	11504496
Foetal Bovine Serum	South American, Fisher Scientific	S181L-500
Penicillin Streptomycin solution	Fisher Scientific	15070-063
DMSO	Biosolve	04470501/1
Trypan blue	Biorad	1450021
DULBECCO'S PHOSPHATE BUFFERED SALINE MODIFIED	Sigma Aldrich	D8537
Trypsin EDTA	In house	NA
Hoechst 33342, Trihydrochloride, Trihydrate	Life technologies	H1399
Propidium iodide =94.0% (HPLC)	Sigma Aldrich	P4170
Rhodamine123	Sigma Aldrich	R8004-5MG
Distilled Water	Gibco/Thermo Fisher Scientific	15230147

Lactate concentration assay

Material	Supplier	Catalogue Number
Triethanolamine HCl	Sigma Aldrich	T9534
EDTA.Na ₂	Sigma Aldrich	E4884
MgCl ₂ anhydrous	Sigma Aldrich	M8266
PMS (N-Methylphenazonium methyl sulphate)	Sigma Aldrich	P9625
INT (p-iodonitrotetrazolium violet)	Sigma Aldrich	I8377
Triton X-100	Sigma Aldrich	X100
?NAD	Sigma Aldrich	N7004
Lactate dehydrogenase (LDH)	Sigma Aldrich	L2500-25KU
L-lactic acid sodium salt (standard)	Fluka Chemika	71718

Resazurin assay

Material	Supplier	Catalogue Number
Resazurin	Sigma Aldrich	R7017

PREPARATIONS**Media and Endpoint Assay Solutions**

Medium/Solution	Preparation
Foetal Bovine Serum aliquots	Thawed, aliquoted into Sterile 50 plastic tubes, frozen again and stored at -20°C.
PenicillinStreptomycin aliquots	Thaw out Penicillin Streptomycin bottle, prepare aliquots of 2 ml in sterile 15 plastic tubes, frozen again and stored at -20°C.
Complete DMEM	Mix 450 ml of DMEM, 50 ml of FBS and 2 ml PenicillinStreptomycin solution (sterile). Store at 4°C. Medium does not need pre-warming before use.
Trypsin EDTA aliquots	Dissolved in a total volume of 1l in Milli-Q® Type 1 Ultrapure Water : 0.05 % (m/V) trypsin; 8 gr NaCl; 0.25 gr KCl; 1.43 gr Na ₂ HPO ₄ *2 H ₂ O; 0.25 gr KHP ₂ PO ₄ . pH set to 7.2 with 1M HCl Filtered with a 0.2 µm filter (sterile)
Hoechst 33342 aliquots	1mg/ml Hoechst 33342 in sterile distilled water, Filtered with a 0.2 µm filter (sterile)
Rhodamine123 aliquots	2500uM in DMSO
Propidium iodide aliquots	Stock solution 100M in sterile distilled water, sterile filtered
Hoechst + rhodamine123 staining solution	Create 200ng/ml Hoechst 33342 (1:5000) plus 0.5uM rhodamine (1:5000) in complete DMEM
Propidium iodide + rhodamine123 staining solution	Create 200 nM Propidium iodide aliquot (1:500) plus 0.1uM rhodamine (1:25.000) in complete DMEM
Freezing medium	20% DMSO in FBS
Test compound stock solution	
DMSO stock	DMSO stocks are created by dissolving the provided test compound in in DMSO by vortexing or resuspending it with a pipette. The stock concentration has to be at least 1000 x higher than the highest testing concentration (=0.1% v/v of DMSO). (The weight provided by JRC was used for concentration calculations)
Lactate and resazurin assay solutions	
Assay solutions	The solutions for the lactate and resazurin assay were created based in the SOPs provided by Paul Jennings lab at the Vrije Universiteit Amsterdam.

Compound stock solutions

Test compound stock solutions were prepared as described in the following table. All stock compounds solutions were stored in brown glass bottles at -80°C under light exclusion. Furthermore, stocks for usage were stored at -20°C under light exclusion.

Freeze thaw cycles were kept minimal. For replicates, different aliquots of the same stock solution were used. The stock solutions were diluted out with full DMEM. All treatments, excluding DMEM vehicle, contained 0.1% (V/V).

Compound	Solvent stock solution	Concentration stock solution [mM]	Concentration stock solution [g/l]	Highest testing concentration [µM]
Rotenone	DMSO	10	394,4	10
Deguelin	DMSO	10	394,4	10
Azoxystrobin	DMSO	10	403,39	10
Kresoxim-methyl	DMSO	10	313,4	10
pyraclostrobin	DMSO	10	387,8	10
Picoxystrobin	DMSO	10	367,3	10
trifloxystrobin	DMSO	10	408,4	10

Compound	Testing concentration [μM]							
	A	B	C	D	E	F	G	H
ROTENONE	10	2	0.4	0.08	0.016	0.0032	0.00064	0.000128
DEGUELIN	10	2	0.4	0.08	0.016	0.0032	0.00064	0.000128
AZOXYSTROBIN	10	2	0.4	0.08	0.016	0.0032	0.00064	0.000128
KRESOXIM-METHYL	10	2	0.4	0.08	0.016	0.0032	0.00064	0.000128
PYRACLOSTROBIN	10	2	0.4	0.08	0.016	0.0032	0.00064	0.000128
PICOXYSTROBIN	10	2	0.4	0.08	0.016	0.0032	0.00064	0.000128
TRIFLOXYSTROBIN	10	2	0.4	0.08	0.016	0.0032	0.00064	0.000128

Positive Control(s)

Positive controls decrease the membrane potential. Therefore, besides Rotenone we also included FCCP.

Negative Control(s)

Complete DMEM medium, with and without 0.1% DMSO.

Method

EXperimental System Procurement

Routine culture procedures

Cultureware	Cell culture maintenance vessels		Seeding densities *1000	
	Growth Area (cm ²)	Media Volume (ml)	Trypsin-EDTA Volume (mL)	PBS Volume (mL)
T25	25	5	1	5
T75	75	15	2	10
T175	175	30	4	20

Imaging plates			
Plate format	Seeded cells per well for 24h of exposure	Seeded cells per well for 48h of exposure	Seeded cells per well for 72h of exposure
96 well plate	20000 in 100 μl	15000 in 100 μl	12000 in 100 μl
384 well plate	8000 in 30 μl	6000 in 30 μl	5000 in 30 μl

Thawing of Cryopreserved HepG2 cell lines

1. HepG2 reporter cell lines are stored at -80°C for short term storage and long storage at -150°C .
2. Cells are thawed in 37°C water bath and poured into 4 ml of complete DMEM.
3. After centrifugation (1000 rpm, 5 min, 4°C), the supernatant is replaced by fresh medium (total volume 5 ml).
4. Then the cells were carefully resuspended and transferred into a T25 tissue flask and moved in an incubator (37°C , 5% CO_2 and 95% humidity).
5. After one further passaging step, the cells can be used in an assay.

Splitting of HepG2 cell lines

1. After removing the medium, cells are washed with PBS.
2. Then the cells are incubated with trypsin-EDTA for 5 minutes in an incubator (37 °C, 5% CO₂ and 95% humidity).
3. Before neutralising the trypsin- EDTA with complete DMEM, the cell suspension is forcefully resuspended 20 times with a 10 ml pipette (smallest opening) in order to achieve a single cell suspension.
4. Passage according to demands in according to table 'Cell culture maintenance vessels'.
5. HepG2 BAC-GFP reporter should not be passaged more than once per 48h.

Cell counting with TC20™ Automated Cell Counter

1. Under sterile condition remove 10µl of the cell suspension with a microliter pipette, (resuspend well before taking the sample).
2. Pipet the 10µl into a dual-chambered slide, avoid air bubbles.
3. Insert dual-chambered slide into TC20™ Automated Cell Counter.
4. Inspect bright field image and histogram of counted particles in order to assess the single cell suspension. Clumps can be removed with Celltrix® 50µm filter.

Cryopreservation HepG2 cell lines

1. Trypsinise cells as described earlier.
2. Add 800 µl cell suspension containing 2×10^6 Cells into cryovials containing precooled (4°C) 800 µl freezing medium.
3. Transfer cells into precooled freezing rack.
4. Freeze at -80°C overnight.
5. Transfer for long term storage to -150°C or maintain on -80°C.

Test Material Exposure Procedures

Seeding HepG2 cell lines

1. Remove medium.
2. Wash with PBS (RT).
3. Add Trypsin EDTA and incubate at 37°C for 5 min.
4. Reduce cell clumps to single cell suspension by pipetting up and down, 20 times, while pressing the pipet's tip to the tissue flasks wall.
5. Determine cell number with TC20™ Automated Cell Counter (see above)
6. If necessary, remove clumps with Celltrix® 50µm filter.
7. Dilute and seed out cells with multichannel pipet in multi-well imaging plates.

Consult table 'Imaging plates' for plating densities.

Added volumes	96 well plate	384 well plate
Hoechst+rho staining solution	100µl	30µl
PI+rho staining solution	50µl	25µl
Compound solution	50µl	25µl

1. Add Hoechst + Rhodamine123 staining solution with multi-channel pipette.
2. Incubate 45 minutes
3. In the meantime: Prepare compound solutions (as mentioned under “test compounds”).
4. Aspirate medium containing Hoechst staining solution (3 to 5 rows at a time).
5. Add PI + Rhodamine123 staining solution to each well with multi-channel pipette.
6. Add compounds in the desired concentration with multi-channel pipette.
7. If live imaging: Proceed to the microscope (incubator preheated)
8. If time point imaging: Place imaging plate in incubator until imaging time point arrives.

FLUORESCENT Endpoint Measurement

For the laser and filter setup, consult Table 1 Fluorescence Microscope Channels. For live imaging, it is recommended to pre-expose one well with positive control in order to setup the EGFP imaging.

- Preheating incubator of the microscope to 37°C under saturated humidity in time before imaging.
- Hoechst 33342
 - Check in the Hoechst 33342 channel whether the whole plate’s cells are in focus.
 - Set intensity to a low enough level to still identify all nuclei, but as low as reasonably possible in order reduce oversaturation as far as possible.
- eGFP
 - Orientate setting of GFP reporter readout at the negative control. Since this measurement ought to be quantified, do not oversaturate.
- Propidium iodide
 - Use a compound concentration, which induces cytotoxicity and one negative control to set up microscopic settings to differentiate between background and signal. For PI is an object identification foreseen to classify a cell as positive, therefore a clear visualisation of PI staining of cells is required.

Fluorescence microscopy:

Table 1. Fluorescence Microscope Channels

Fluorophore	Laser	Emission filter [nm]	Filter Range [nm]
Hoechst 33342	408 nm	450/50	425 - 475
eGFP	488 nm	525/50	500 -550
Propidium iodide	561 nm	595/50	570 -620
Annexin V Alexa 633	633 nm	700/75	663 - 738

ACCEPTANCE CRITERIA

The quality of the test is based on the cell death marker (PI) and the GFP intensity

A test is discarded when:

1. More than 15% of the cells are PI-positive in the solvent control (DMSO)
2. To determine if the control provides the expected result, we determine the robust Z score per plate.
3. To be able to do so you need at least 3 wells of your positive control (σ_p and μ_p) and of your negative control (σ_n and μ_n). (σ = standard deviation and μ = mean) The estimated Z-factor should be above 0.25.

$$\text{Estimated Z-factor} = 1 - \frac{3(\hat{\sigma}_p + \hat{\sigma}_n)}{|\hat{\mu}_p - \hat{\mu}_n|}$$
4. At least 4 negative controls (complete DMEM), solvent control (complete DMEM + 0.1% (V/V) DMSO), and 4 positive controls should be taken along.

LACTATE AND RESAZURIN ENDPOINT MEASUREMENT

The lactate and resazurin assay were performed following the lactate and resazurin assay protocol provided by Paul Jennings lab at the Vrije Universiteit Amsterdam.

Lactate assay

The supernatant was collected after live confocal imaging and stored upon ice until further usages. The provide protocol was followed:

1. 10uL culture medium was combined with 90uL working lactate assay reagent mix
2. The mixture was stored for 7min in the dark
3. Subsequently the absorbance was measured at 490nm using the FluoStar plate reader.

Resazurin assay

The residual medium was removed from the imaging plates. The plates were not washed with PBS (because of the cell loss it would induce).

1. 30uL of pre-warmed 20x diluted resazurin mixture was added to all wells.
2. After 2h of incubation at 37degrees the resorufin fluorescence was measured: excitation at 540nm, emission at 590nm.

Data Analysis

Disclaimer

The nuclei segmentation in cellprofiler relies on the watershed masked clustering developed by Kuan Yan (see Di *et al* 2012). The WMC might not be available to the public. It can be done alternatively via segmentation in imageJ. The segmented pictures can then be read into cellprofiler.

The R-scripts for the data readout out of the HDF5 file is not available publicly.

Image processing

The image format retrieved from the microscopy session is a ND2 file. This .ND2 file contains the images (well /channel/timepoint). Furthermore, it includes the technical information about the microscope composition and settings (laser settings, excitation/emission, detector settings, plate position, etc...). Since the ND2 files can reach a significant size, a fast data connection is recommended. At the Division of Drug Discovery and Safety, the data is retrieved by USB 3 compatible hard drives and an in-house server.

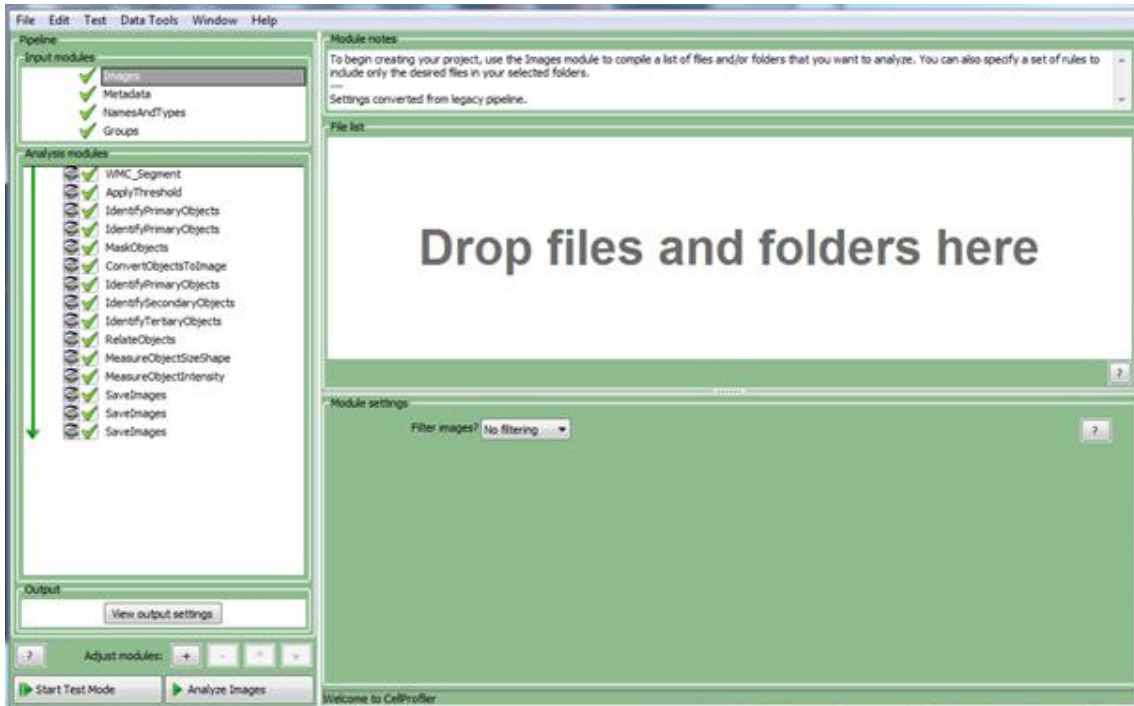
A program freely provided by Nikon (NIS-Elements Viewer) was used to export the .ND2 file to single .tiff images per well /channel/timepoint (xy_c_t).

The exported tiff files were then sorted into image location specific folders (i.e. B02_1, meaning well B02 first imaging location). These were used while reading in the images in cell profiler to associate the images location_ID.

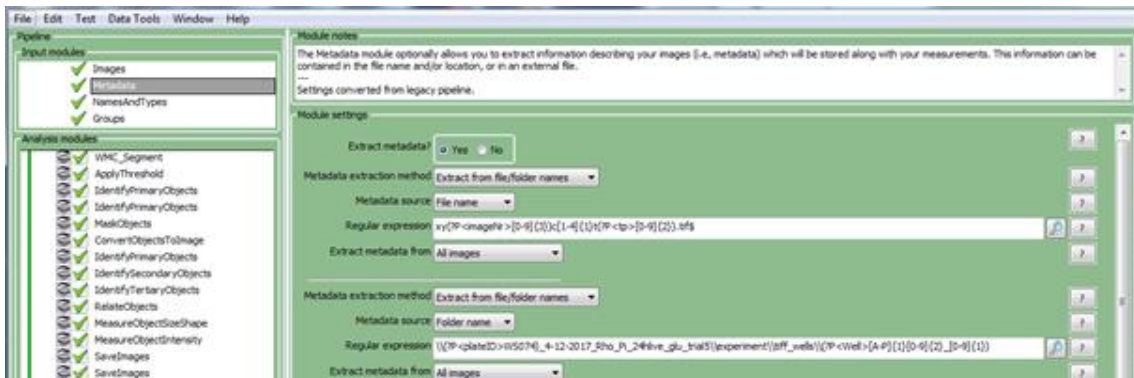
Image analysis

The structured folders with .tiff files are subsequently used for analysis in the free and open-source software program CellProfiler® (version 2.1.1). To be able to study the intensity and shape of objects the following pipeline was used for object identification:

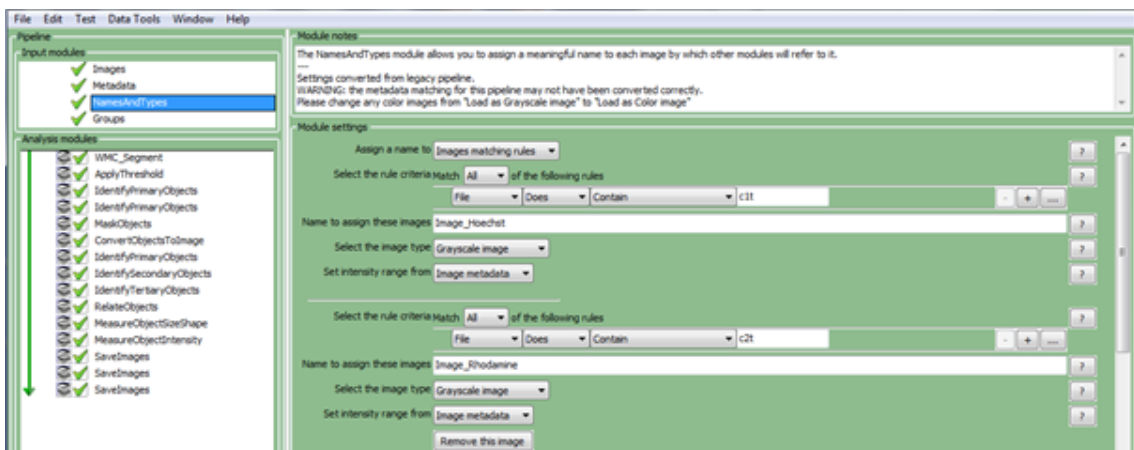
1. Identification of the nucleus based on the Hoechst image
 2. After segmentation of the nuclei, a circle of 10 pixels around the nuclei is created.
 3. Identification of PI object based on the PI image.
If there is an overlap between the nucleus and the PI the cell is considered PI positive.
1. **Cell Profiler analysis > set Input modules**
 - Open CellProfiler (see what features need to be analysed in the figure above)
 - Drag and drop images in CellProfiler:



- Set regular expressions in “metadata”.. Add four variables: 1. plateID 2. imageNr 3. Timepoint 4. Well location. Click on update



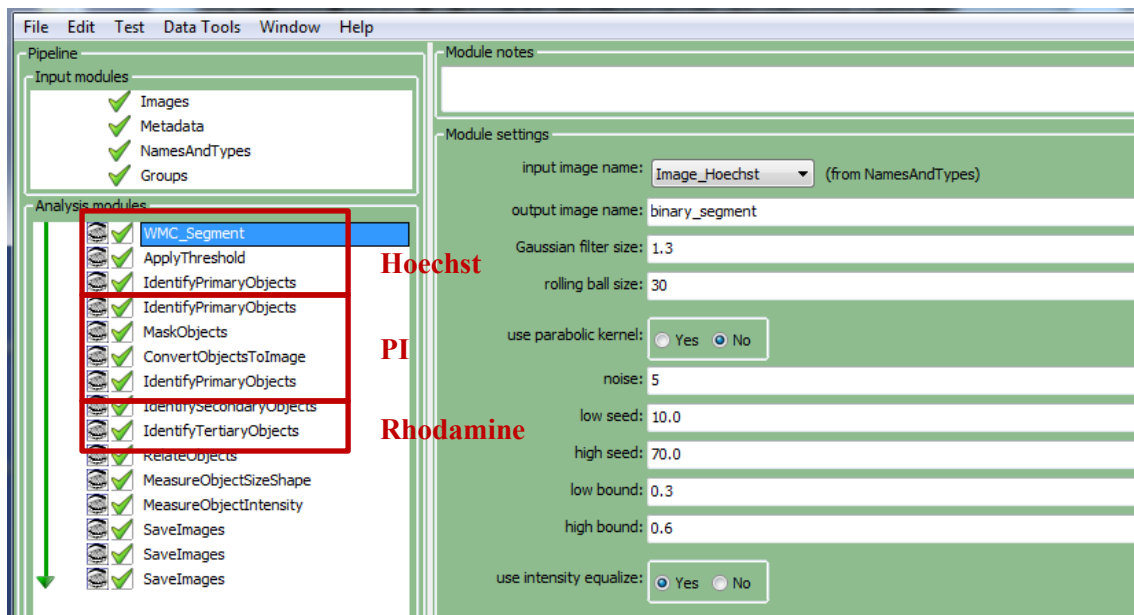
- Set the different channels (Hoechst, Rho, and PI) in the “names and types” module. Click on update



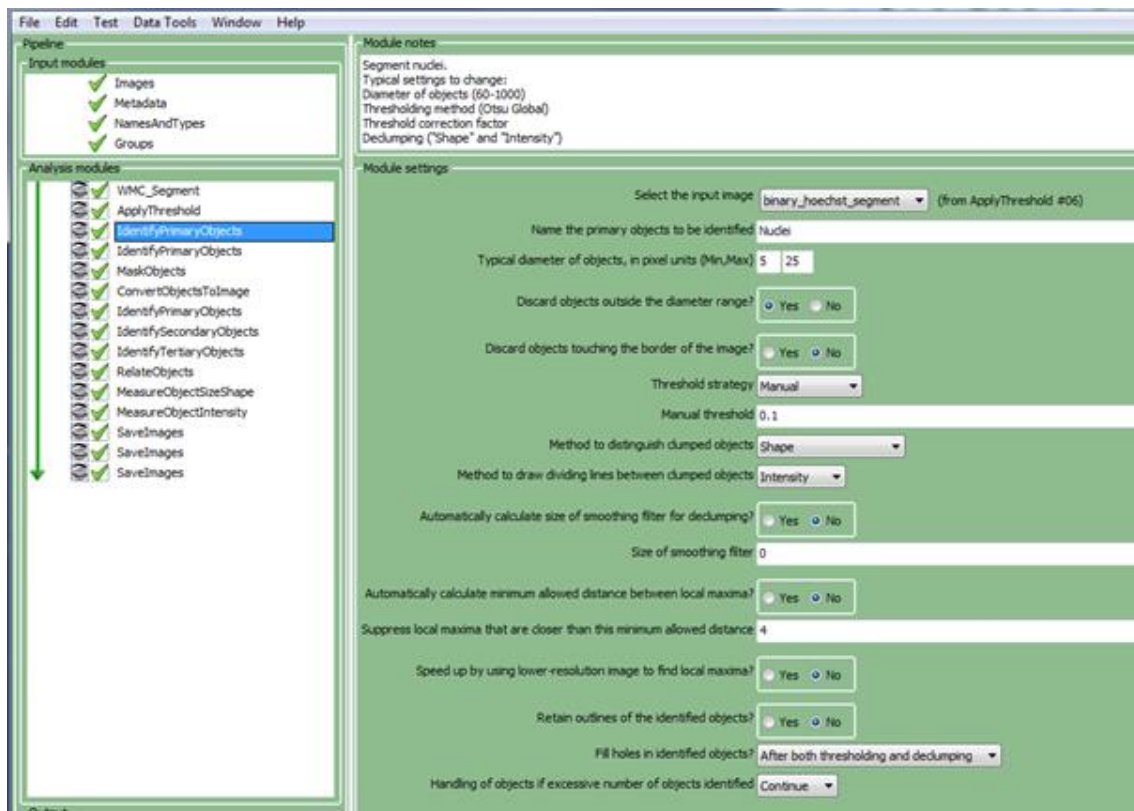
- OPTIONAL: set groups
- 2. **Cell Profiler analysis > set Analysis modules**
- Start test mode (bottom left corner)

Nucleus identification

- Input = Hoechst Channel
- Check the separation between foreground and background pixel in the Hoechst channel using the watershed masked clustering (WMC) ImageJ plugin.
- The WMC ImageJ plugin was developed by Kuan Yan (see Di *et al* 2012).

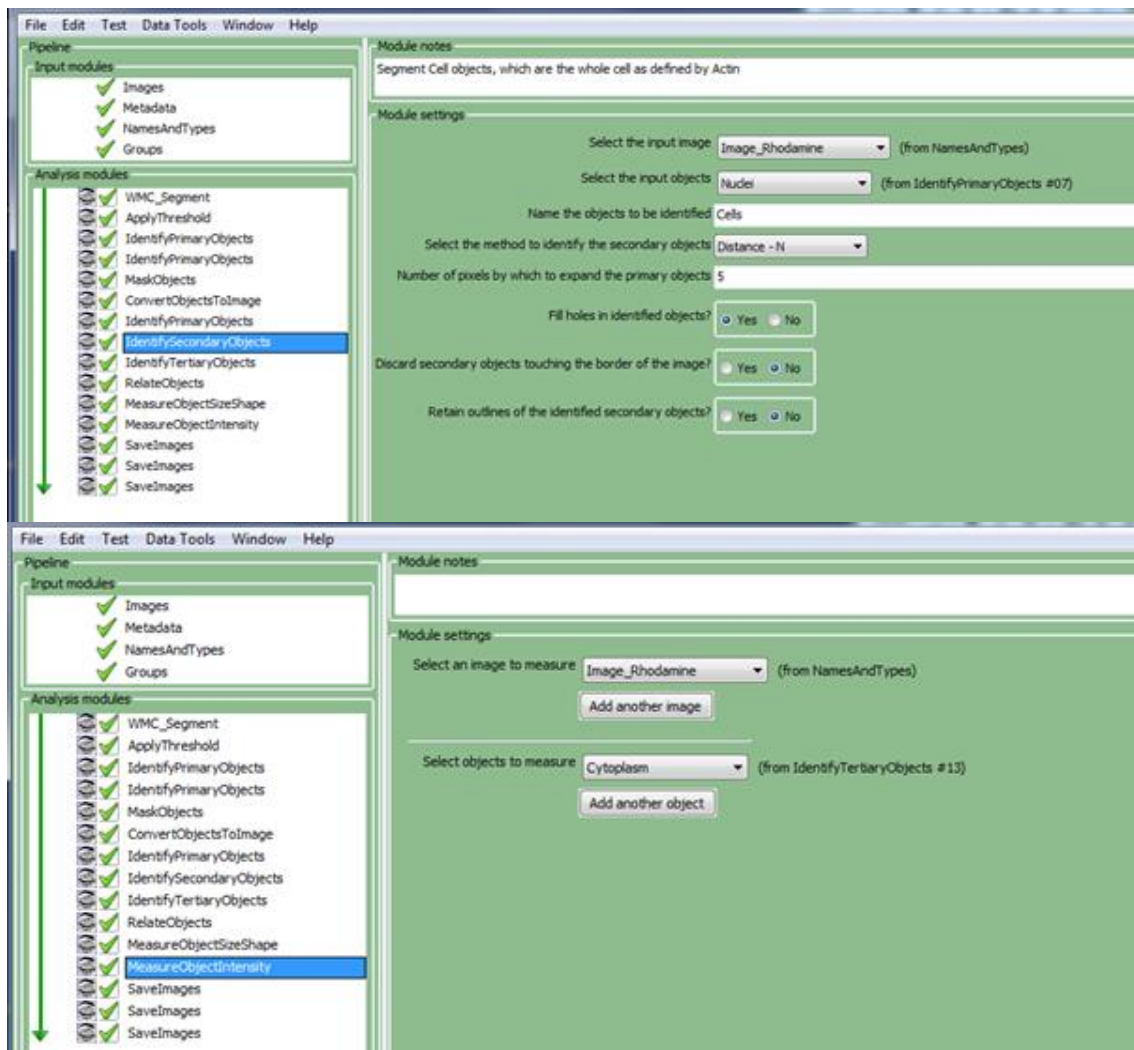


- Check the identification of the different objects in “Identify Primary Objects”



Rhodamine123 signal identification > in the cytoplasm

- Input = eGFP channel
- When identification of the cytoplasm is desired, check the identification of the cytoplasm using the “Identify Secondary Objects” module
- The “Identify Tertiary Objects” is an objected created by subtracting the “Identify Primary Objects” from the “Identify Secondary Objects”.
- MeasureObjectIntensity => Measure GFP intensity in the identified Tertiary Object (Cytoplasm)

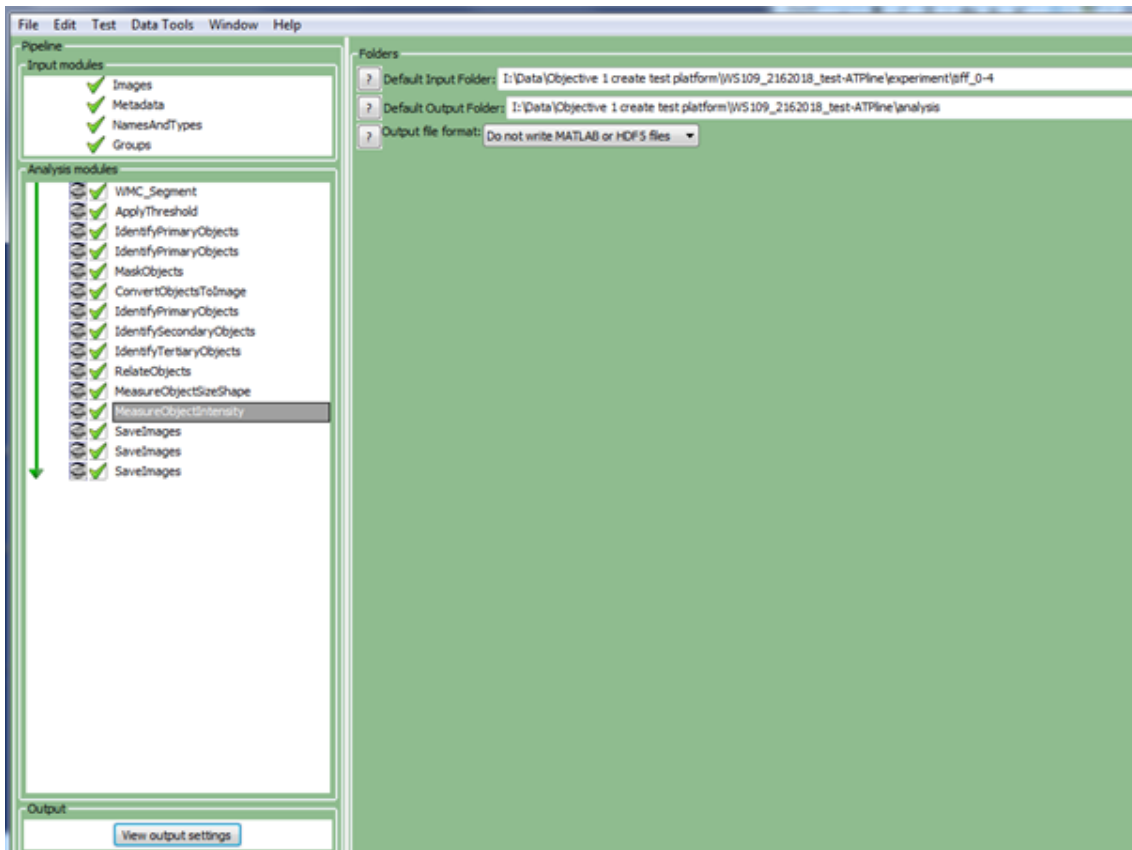


PI staining

- Input = PI/AnV image
- Identify the PI staining in the primary picture (IdentifyPrimaryObject)
- Overlay these PI objects with the nuclei objects and save the overlapping areas (MaskObjects)
- Create new image (ConvertObjectsToImage)
- Identify the PI objects that are still present after the overlay (IdentifyPrimaryObject)

3. **Cell Profiler analysis > set Output**

- Update “view output settings” by updating the input and output folders



- Start Cellprofiler analysis.
The output of the CellProfiler run is a HDF5file with all measured parameters and when asked for folders with segmentation images per created .tiff.

Reading out data from an HDF5 can be challenging. The summary data per image can be read out as an .csv file as well. This won't allow single cell data read outs.

Data collection

The output files of the CellProfiler® analysis are the segmentation images (.png) to make a manual take of the segmentation possible. Furthermore the measured objects (nuclei, pi and AnV objects), and intensities are saved in an .HDF5 file.

An R-script assembled in house is used to extract the features of interest for the researcher. Based on the packages: rhdf5, stringr, plyr, data.table, doParallel, ggplot2, reshape2, grid, shiny and ggvis.

Within a graphical user interface, the desired readouts for further analysis were picked:

- Nuclei number (imageCountparentObj)
- Integrated GFP intensity for reporter with GFP signal in the cytoplasm. (Cytoplasm_Intensity_IntegratedIntensity_image_GFP)
- Fraction GFP positive cells (countGFPpos, amount of cells with a GFP intensity more than two times DMSO control).

- Fraction PI positive cells
(count_PI_masked_primaryID_AreaShape_Area.DIV.Nuclei_AreaShape_Area_larger_0.1_)

DOSE RESPONSE MODELING

The dose response modelling and calculation of the EC/IC values was based on a Konstanz developed R script.

Step 1 = An in-house script was used to determine the maximal value of the sigmoidal curve. This maximal point was set to hundred per cent to be able to compare all inter lab data.

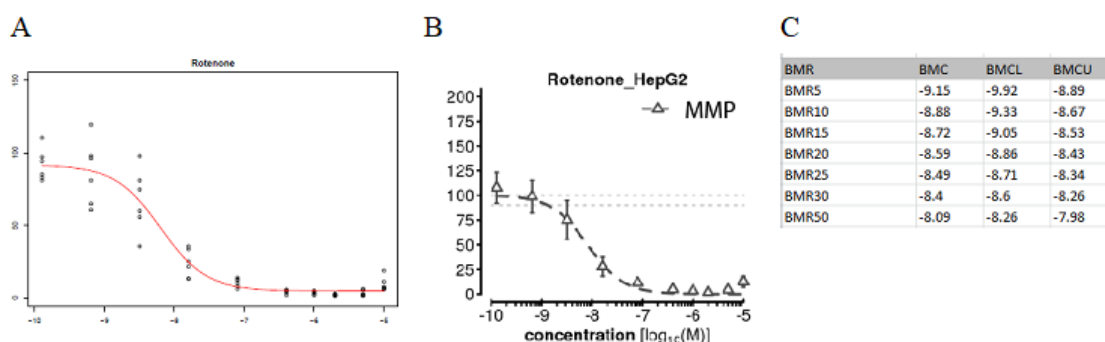
Step 2 = The created dose responses curves were used to perform the calculation of the EC/IC values using the Konstanz R script.

Figure 2. Example of dose response modelling

A) Curve used for normalisation to 100%

B) Normalised curve used for BMC modelling in Konstanz tool

C) The results from the IC/EC value calculations



Prediction Model

Rationale of your test method (= scientific purpose)

The combination of the MMP dye, glycolytic, viability and cell death measurements enables us to quantify and monitor mitochondrial functioning over time in the HepG2 cell line. The usage of automated confocal imaging creates a high throughput manner to study time and dose response relationships. This screening platform provides the opportunity to correlate the various mitochondrial functional read outs, to be able to establish the point of departure in dose and time of mitochondrial perturbation.

Prediction model

This cellular system can be used to predict to what extent, in which timeframe and at which concentration a specific compound induces mitochondrial perturbation and or will be cytotoxic.

Mitochondrial perturbation

The mitochondrial membrane potential can be used as a measure for the integrity of the mitochondria. Furthermore, the glycolytic capacity demonstrates the switch in ATP source which is correlated with malfunctioning of mitochondria. The processes can be described with the following read outs.

The dye intensity per cell demonstrates the status of the potential compared to a non-treated condition. The glycolytic capacity of the cell can be based on the lactate concentration which is a by-product of the glycolysis.

Cytotoxicity

The threshold of cytotoxicity is determined by dose response modelling of the PI and resazurin readout.

Prediction model setup

The options for the use of this set of assays as a prediction model are not jet investigation.

Dose response modelling can be done via BMDExpress.

IVIVE In vitro – in vivo extrapolation

The specific measurements are not jet used for *in vivo* extrapolation.

Applicability domain

The applicability of this testing system will be a first tier high throughput testing screen for the identification for the test compounds ability to induce mitochondrial perturbation.

Incorporation in test battery

The mitochondrial functions assays in HepG2 can be used in combination with the other assays for comprehensive testing of a broad range of mitochondrial stress responses.

Strengths	Weaknesses
1. High-content and –throughput	1. Correct handling is crucial for HepG2 cells
2. Live time dynamics	2. Lack of metabolic capacity in HepG2 cell system
3. Identification of AOP while cell death measures can be observed	3. The need of a powerful confocal microscopy system including heater and CO ₂ for live cell imaging
4. Stand-alone test system	
5. Single cell measurements	
6. Live monitoring of mitochondrial functioning	

Annexes

Bibliography

- Rhodamine123 assay
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Annex 1.6. Measurements mitochondrial membrane potential in differentiated human neuroblastoma SH-SY5Y cells after exposure to chemicals

Method	SH-SY5Y: MMP using Rho123
Contact	Anna Forsby (Swetox) -> anna.forsby@swetox.se

Standard Operating Procedure (SOP)

1. PURPOSE

The SOP describes how mitochondrial membrane potential in SH-SY5Y cells can be determined with Rhodamine 123 staining.

2. SCOPE

Mitochondrial membrane potential (MMP) is correlated with the cells' ability to produce ATP through oxidative phosphorylation. Acute changes in MMP are monitored by measuring the changes in fluorescence intensity after loading the cells with Rhodamine 123 and exposing them to a selection of chemicals for 24 hours.

3. INTRODUCTION

The mitochondria in all cells produce ATP by means of the proton electrochemical gradient potential (ΔP) which takes place at the inner mitochondrial membrane. The two key factors generating the force need for the movement of electrons across the mitochondrial membrane are the mitochondrial membrane potential (MMP) and to a lesser degree the mitochondrial pH gradient. A variety of dyes designed for measurement of MMP have been design over the years, these dyes have the ability to equilibrate across the mitochondrial membrane displaying a relationship between the accumulation of dye and the MMP of the cells. Thus, factors affecting the polarisation state of the mitochondrial membrane can be assessed by measuring the fluorescence intensity of the mitochondria after incubation with the dye.

4. RESPONSIBILITY

All personnel performing this assay should follow this SOP.

5. PROCEDURES

METHOD OUTLINE

Rhodamine 123 is for staining mitochondria, Hoechst for nuclear staining and Propidium Iodide (PI) for viability measurements.

For experiments, SH-SY5Y cells are plated in 96-well plates (10 000 cells/well) in EMEM with 10% foetal bovine serum, 2 mM glutamine, 1% non-essential amino acids, 100g streptomycin/mL and 100 U penicillin/mL, which is exchanged to differentiation medium

with 1 μM RA and without serum (DMEM:F12 [1:1], N2 supplements [diluted 1:100], 1 mM glutamine, 100g streptomycin/mL and 100 U penicillin/mL) after 24 h.

Six (6) days-differentiated SH-SY5Y cells are stained with 1 μM Rhodamine123 for 45 minutes before the exposure with test chemicals. The effect of the test chemicals on the mitochondrial membrane potential is reflected as an increased (hyperpolarisation) or decreased (depolarisation) Rhodamine 123 fluorescence intensity.

DEFINITIONS/ABBREVIATIONS

The following abbreviations are used:

ΔP	Proton electrochemical gradient potential
DMEM/F12	Dulbecco's modified medium and Ham's F12 medium (1:1)
DMSO	Dimethyl sulfoxide
EMEM	Minimum essential medium with Earle's salts
RA	All-trans retinoic acid
MMP	Mitochondrial membrane potential
FBS	Foetal bovine serum
PEST	Penicillin Streptomycin
NEAA	Non-essential amino acids
PI	Propidium Iodide

MATERIALS

A. Cell Culture Type

Human neuroblastoma SH-SY5Y cell line is cultured and differentiated as described in SOP_SH-SY5Y_16_v01.

B. Controls

- a Positive controls: FCCP 2 μM and 400 nM, Rotenone 10 μM .
- b Negative controls: DMSO 0,1%.

C. Technical Equipment

1. Cell cultures

- a Laminar Air Flow cabinet, class 2, for protection of laboratory personnel and material
- b Cell culture incubator with humidified CO₂ atmosphere (5% in air), 37°C
- c Multichannel pipette (100-200 μl)

2. Mitochondrial membrane potential measurements

- a Fume hood for work with test chemicals

- b. Multichannel pipette (50, 100 μ l)
- c. Incubator, 37°C, 5% CO₂
- d. ImageXpress® Micro (Molecular Devices, UK)
- e. MetaXpress® Software (Molecular Devices, UK)

D. Reagents, Chemicals, Media, Sera, Plastic ware

1. Reagents and chemicals

- a. Rhodamine 123 (ThermoFisher, cat no R302).
- b. Hoechst 10 mL (ThermoFisher, cat no H3570), 10mg/mL.
- c. Propidium iodide solution (Sigma, cat no P4170).
- d. DMSO (Sigma, cat no D4540).
- e. Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) (Sigma, cat no C2920).
- f. All-trans RA (Sigma, cat no R2625), 10 mM in 99% ethanol.

2. Media, sera and other cell culture reagents

Cell culture medium is prepared as described in SOP_SH-SY5Y_16_v01. Briefly;

- a. Cell culture medium, denoted Complete EMEM: EMEM with 10% foetal bovine serum, 2 mM glutamine, 1% non-essential amino acids, 100 μ g streptomycin/mL and 100 U penicillin/mL.
- b. Differentiation medium, denoted **N2-RA medium**: DMEM:F12 [1:1], N2 supplements [diluted 1:100], 1 mM glutamine, 100 μ g streptomycin/mL and 100 U penicillin/mL. On the day of start of differentiation, RA [1mM] (dissolved in ethanol) is added to a final concentration of 1 μ M (0.1% ethanol).
- c. N2 supplements must be aliquoted in 1 mL volumes for 100 mL complete N2-RA medium. The aliquots must be stored at -20°C.

All-trans retinoic acid

[Note: RA must be handled and stored in dark to avoid degradation.]

1. Prepare a 10 mM stock solution of RA in 99% ethanol. Store the RA stock solution at -20°C. Moderate heating may be needed to dissolve all RA.
2. Prepare a working solution of 1 mM in 99% ethanol. Store the solution at -20°C.
3. When applied to cells, dilute the working solution [1mM] 1:1000 in cell (N2) medium. The final concentration of RA will be 1 μ M (in 0.1% ethanol).

3. Plastic ware and other materials

- a. Black cell culture microplate, 96-well plate with microclear bottom (Greiner, No 655090)
- b. 1.5 mL eppendorf tubes, polypropylene (VWR, cat no 700-5239)

- c. 2 mL eppendorf tubes, polypropylene (VWR, cat no 211-2120)
- d. 50 mL Reagent reservoir, polystyrene (Sigma, cat no CLS4870-200EA)
- e. 15 mm syringe filter with regenerated cellulose membrane, pore size 0.2 μM (Sigma, cat no CLS431215-50EA)

METHODS

A. Preparations of Media and Solutions

Cell culture media

Cell culture media are prepared as described in SOP_SH-SY5Y_16_v01. Briefly;

- a. Cell culture medium: EMEM with 10% foetal bovine serum, 2 mM glutamine, 1% non-essential amino acids, 100 μg streptomycin/mL and 100 U penicillin/mL. To prepare one bottle add:
 - 500 mL EMEM
 - 50 mL FBS
 - 5 mL PEST
 - 5 mL L-Glutamine
 - 5 mL NEAA
- b. Differentiation medium: DMEM:F12 [1:1], N2 supplements [diluted 1:100], 1 mM glutamine, 100 μg streptomycin/mL and 100 U penicillin/mL. On the day of start of differentiation, RA [1mM] (dissolved in ethanol) is added to a final concentration of 1 μM (0.1% ethanol).



B. Preparation of Cells for Assays

[Note: work with cell cultures is performed under sterile conditions.]

1. Detach confluent SH-SY5Y cell cultures by trypsination.
2. Prepare single cell suspension: Count the cells, and dilute the cell suspension to 100 000 cells/mL in EMEM with supplements.
3. Add 200 μl cell culture medium (EMEM without supplements) to the wells in columns 1 and 12 and rows A and H.
4. Plate 100 μl of the cell suspension in each well of rows B-G in columns 2-11 to obtain a final cell density of 10 000 cells per well. Use a multi-channel pipette

with 100 µL aliquot dispenser function. Flush at the lowest possible speed in the centre of each well to get an even cell distribution over the surface.

5. Wrap the plates in plastic foil and place them immediately in the incubator.

Figure 1. Plate setup when seeding. Grey box- 200 µL EMEM and blue box- 100 µL cell suspension

	1	2	3	4	5	6	7	8	9	10	11	12					
A	200 µL	200 µL	200 µL	200 µL	200 µL	200 µL	200 µL	200 µL	200 µL	200 µL	200 µL	200 µL					
B	200 µL											200 µL					
C	200 µL			100 µL/well 100 000 cell/mL in EMEM									200 µL				
D	200 µL																200 µL
E	200 µL																200 µL
F	200 µL																200 µL
G	200 µL											200 µL					
H	200 µL	200 µL	200 µL	200 µL	200 µL	200 µL	200 µL	200 µL	200 µL	200 µL	200 µL	200 µL					

C. Differentiation of cells

[Note: RA must be handled and stored in dark to avoid degradation.]

1. On the day after plating, the cells are differentiated by changing the EMEM + supplements medium to N2-RA medium.
2. Prepare the estimated volume of N2-RA medium in a 50 mL reagent reservoir (Corning Costar 4870).

Table 1. Differentiation medium (N2-RA) preparation

Number of plates	N2 medium (mL)	RA [1mM] µL
1	7	7
2	14	14
3	20	20
4	26	26

3. Remove the EMEM + supplements row by row with a 12 channel pipette and immediately replace with 100 µL N2 –RA added gently to avoid flushing away the cells.
4. Wrap the plates in plastic foil and place them immediately in the incubator.
5. After 72 hours remove 50 µL of the N2RA medium inside the wells and replace with 50 µL of freshly prepared N2RA medium. The exposure with test chemicals will be performed after the cells have been differentiated for 6 days.

D. Exposure with chemicals

Preparation of Test Chemicals

General

- a. All work with test chemicals must be performed in a fume hood.
- b. Test chemicals are stored as recommended by the suppliers.

- c. Before use, risk evaluation must be performed for each test chemical. The risk assessment must be approved by the SHE group.
- d. Solubility properties are checked in Material Technical Sheets provided by suppliers or at the Santa Cruz website and indicated in the cross systems chemicals table.
- e. The test chemicals are dissolved by vortexing or heating. No precipitates must be present in the stock solutions. Careful notes are taken about the solubility of the test chemicals.

Test chemicals

- a. Weigh an amount that is close to the indicated amount of the test chemical in a 2 mL Eppendorf tube.
- b. Dissolve the test chemical in 100% DMSO yielding a concentration of 10 mM or to the solubility limit.
- c. Aliquot the stock solutions and store them at -20 °C.

pH of test chemical solutions

1. Check for any pH changes in the medium for the highest concentration by visual analysis of the colour of phenol red.

[Note: The work has to be performed under sterile conditions.]

General

1. Prepare 1-5 mL of a 20 µM solution of the test chemicals dissolved in DMSO in exposure medium, N2-RA.
2. Sterile filter the 20 µM solution into a sterile 1.5 mL eppendorf tube.
3. For each experiment, 5 dilutions of the test chemical are prepared. All dilution steps must contain the same concentration of vehicle, this is usually 0.2% for DMSO. This will be diluted to 0.1% DMSO in the cell plate.
4. The dilutions series are prepared in sterile polypropylene 1.5 mL eppendorf tubes. See Table 3.

Concentrations for main experiment

All compounds are tested at fixed concentrations of 10 µM, 2µM, 400 nM, 80 nM and 16 nM. The cells in the controls are exposed to DMSO 0.2% which will be diluted to DMSO 0.1% when added to the control wells:

Table 2. Preparation of DMSO 0.2% for exposure of controls

Number of plates	N2-RA medium (mL)	DMSO 100% (µL)
1	1	2
2	2	4
3	3	6
4	4	8

Test chemical dilutions

The dilution series are prepared in a stepwise manner to the final well concentrations (table 3). The highest concentration (D1) is prepared by diluting the stock solution 500x in N2RA medium, yielding 0.2% DMSO. Thereafter, the dilution series are prepared in N2-RA medium + 0.2% DMSO. Every dilution step needs to be vortexed before the next dilution step.

Table 3. A typical pipetting scheme for preparation of the dilution series for compounds dissolved in DMSO

Sample name	Conc. In ep-tube (M)	Test chemical (µL)	N2-RA Medium (µL)	Conc. In wells (M)
D1	2,00E-05	1000	0	1,00E-05
D2	4,00E-06	100 of D1	400	2,00E-06
D3	8,00E-07	100 of D2	400	4,00E-07
D4	1,6E-07	100 of D3	400	8,00E-08
D5	3,2E-08	100 of D4	400	1,60E-08

The dilution series and the assay controls are transferred to rows B-G (50 µl per well) according to the cell plate configuration Figure 2.

Figure 2. Plate configuration for MMP measurements.

Four compounds are measured in the same plate.

	1	2	3	4	5	6	7	8	9	10	11	12
A	EMEM	EMEM	EMEM	EMEM	EMEM	EMEM	EMEM	EMEM	EMEM	EMEM	EMEM	EMEM
B	EMEM	DMSO	DMSO	DMSO	DMSO	DMSO	Co3 D1	Co4 D1	Co4 D1	FCCP 400 nM	FCCP 400 nM	EMEM
C	EMEM	Co1 D1	Co1 D1	Co2 D1	Co2 D1	Co3 D1	Co3 D2	Co4 D2	Co4 D2	FCCP 2 µM	FCCP 2 µM	EMEM
D	EMEM	Co1 D2	Co1 D2	Co2 D2	Co2 D2	Co3 D2	Co3 D3	Co4 D3	Co4 D3	FCCP 2 µM	FCCP 2 µM	EMEM
E	EMEM	Co1 D3	Co1 D3	Co2 D3	Co2 D3	Co3 D3	Co3 D4	Co4 D4	Co4 D4	Rotenone 10 µM	Rotenone 10 µM	EMEM
F	EMEM	Co1 D4	Co1 D4	Co2 D4	Co2 D4	Co3 D4	Co3 D5	Co4 D5	Co4 D5	Rotenone 10 µM	Rotenone 10 µM	EMEM
G	EMEM	Co1 D5	Co1 D5	Co2 D5	Co2 D5	Co3 D5	DMSO	DMSO	DMSO	DMSO	DMSO	EMEM
H	EMEM	EMEM	EMEM	EMEM	EMEM	EMEM	EMEM	EMEM	EMEM	EMEM	EMEM	EMEM

Preparation of positive control and staining stocks

Rhodamine 123:

Prepare a 1 mM stock solution of Rhodamine 123 as follows:

- Weigh in 25 mg Rhodamine 123 (MW 380.83) and dilute them in 2525 µL 100% DMSO under sterile conditions. The final concentration of the Rhodamine 123 solution will be 26 mM.
- Dilute 39 µL of the previously made 26 mM Rhodamine 123 solution 961 µL 100% DMSO. The final concentration of the Rhodamine 123 solution will be 1 mM.
- Store the solutions at -20°C.

FCCP:

- Prepare a 50mM stock solution by dissolving 10 mg FCCP in 787 μ L 100% DMSO.
- Prepare a 5mM FCCP working solution by diluting 100 μ L of the 50mM FCCP stock solution in 900 μ L 100% DMSO.
- Store at -20°C.

Propidium iodide (PI)

- Prepare a 5 mg/mL (7.48 mM) stock solution by diluting 10 mg PI in 2 mL PBS. Store at 4°C protected from light.
- Prepare a 20 μ M PI working solution by diluting 27 μ L of the 5 mg/mL PI stock solution in 9973 μ L PBS. Store at 4°C protected from light until further use.

E. Rhodamine staining and exposure to chemicals

1. Approximately 72 hours after start of differentiation remove 50 μ L cell medium from the 60 innermost wells and replace with 50 μ L fresh N2-RA containing 2 μ M RA.
2. The exposure starts about 6 days after start of differentiation in N2-RA medium.
3. Prepare a staining solution containing 2 μ M Rhodamine 123. For two plates, dilute 140 μ L of 1 mM Rhodamine 123 in 6860 μ L N2-RA medium.
4. Remove 50 μ L N2-RA medium from all wells one row at the time with a 12 channel pipette and immediately replace with 50 μ L of staining solution containing 2 μ M Rhodamine 123 (the final concentration of Rhodamine in the wells is 1 μ M).
5. Incubate for 45 minutes at 37°C with 5% CO₂ in darkness.
6. Meanwhile prepare the dilutions of the test compounds and controls (10 μ M rotenone, 2 μ M FCCP and 0.4 μ M FCCP).
 - a. FCCP: FCCP in two concentrations is used as a positive control. Dilute 4 μ L of the 5mM FCCP stock in 996 μ L N2-RA to prepare a 20 μ M FCCP working solution. Dilute 100 μ L of the 20 μ M FCCP working solution in 400 μ L N2RA to obtain the 4 μ M FCCP positive control. Repeat the same procedure but this time diluting 100 μ L of the 4 μ M FCCP in 400 μ L N2RA to obtain a 0.8 μ M FCCP positive control. These solutions will be diluted to 2 and 0.4 μ M respectively once added to the cell plate.
 - b. Rotenone: Rotenone 10 μ M is used as an internal standard, a decrease in the MMP of about 60% is expected after 24 hours of exposure. To prepare the working solution dilute 2 μ L of the stock (10mM) in 998 μ L N2-RA medium, the final concentration will be 20 μ M which will be diluted to 10 μ M in the cell plate.

7. After incubation with Rhodamine 123, remove completely the N2-RA + staining solution one row at the time with a 12 channel pipette and wash once with 200 μ L pre-warmed DMEM:F12 medium without additives.
8. Add 50 μ L N2-RA medium to all wells and 50 μ L of each concentration according to the plate layout (Figure 2). Start by adding N2-RA + DMSO 0.2% to the controls.
9. Make a note on the plate lid stating which chemicals were added to the cells, date and time.
10. Place the plate immediately in the incubator (37°C, 5% CO₂) for 24 hours.
11. On the day of measurements prepare a staining solution containing PI and Hoechst as follows:

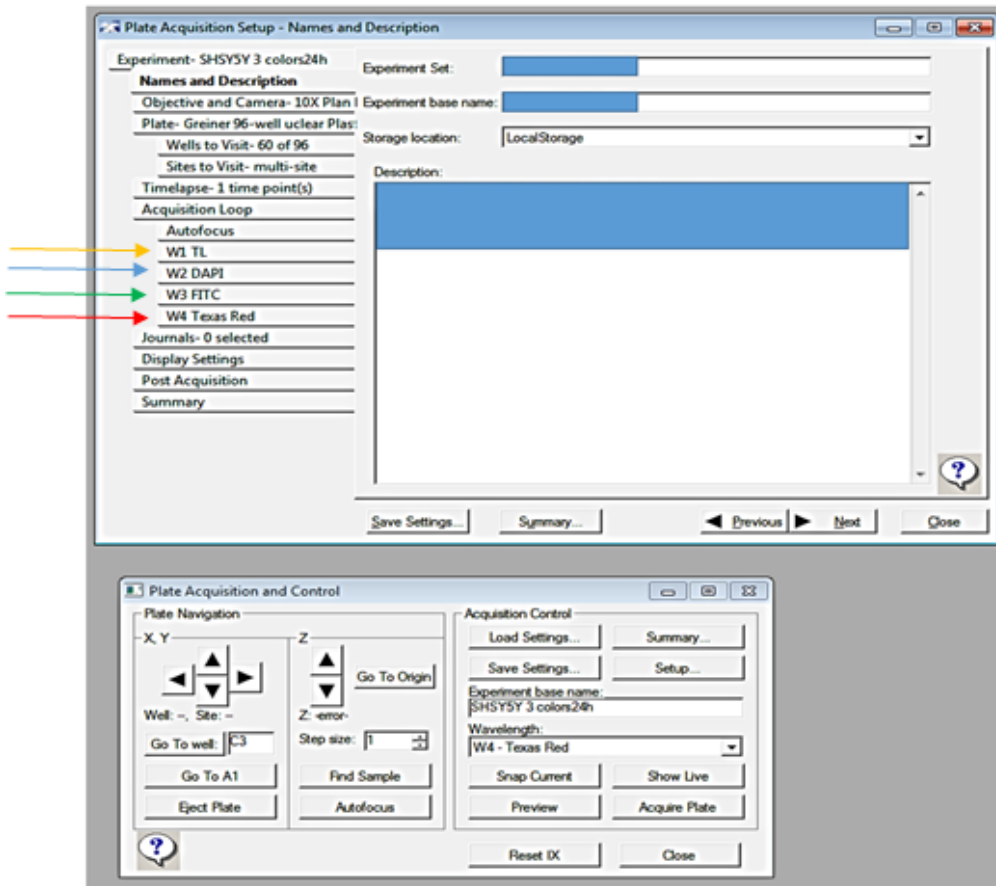
PI + Hoechst: Sonicate the Hoechst stock for 15 minutes before use. Add 500 μ L of 20 μ M PI and 2.5 μ L of 10 mg/mL Hoechst to 1500 μ L N2-RA medium. The final concentration of PI in the staining solution will be 5 μ M which is diluted to 833 nM in the cell plate. The final concentration of Hoechst in the staining solution will be 20 μ M, which is diluted to 3.3 μ M in the cell plate.
12. Add 20 μ L of the staining solution containing PI + Hoechst to all wells 30 minutes before measuring (after 23 hours 30 minutes incubation with compounds). Place the plate inside the incubator until it is time to start the measurements.

F. Measurement of MMP

ImageXpress® Micro (Molecular Devices, UK) is used to obtain images of the exposed cells. Nine images per well are taken with 4 channels: DAPI (blue arrow), TL (yellow arrow), FITC (green arrow) and Texas red (red arrow).

1. Turn on the machine 30 minutes before starting the measurements.
2. Open MetaXpress®
3. On the top menu of the MetaXpress® software, select screening → plate acquisition set up.
4. On the top menu of the MetaXpress® software, select screening → plate acquisition and control.
5. Once the plate acquisition set up and plate acquisition and control windows are opened, start by writing the name of the experiment and a description under the “Names and description” tab (see below, Figure 3).

Figure 3. “Names and description” tab.

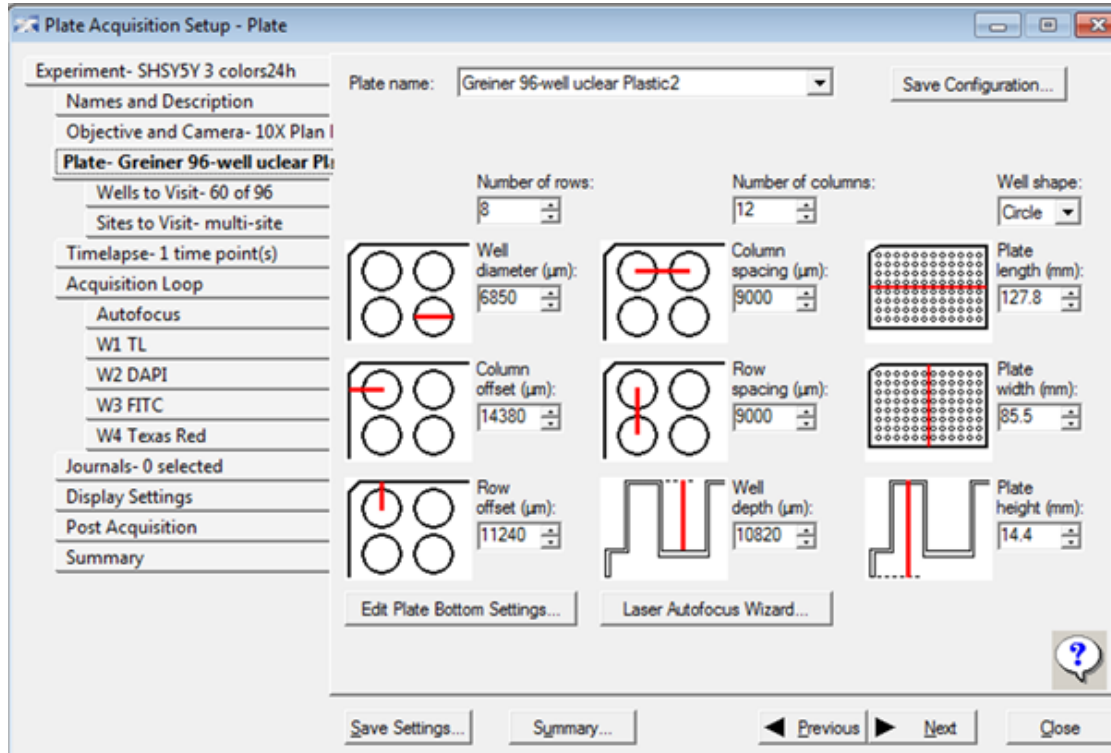


6. Under the “objective and camera” tab, select a 10X magnification, camera binning 1 and gain 1. These settings should only be modified by the instrument’s responsible person or technician.
7. Under the “Plate” tab, use the settings optimised for 96 well black microclear bottom cell culture plates (Greiner, No 655090). Figure 4 contains a description of the settings used for 96 well plates in this instrument, these settings should only be modified by the instrument’s responsible person or technician.

[Note: different batches of plates can have differences in the well depth within the same plate, this will affect the sharpness of the image taken during the testing of the settings but it will not affect the final images]

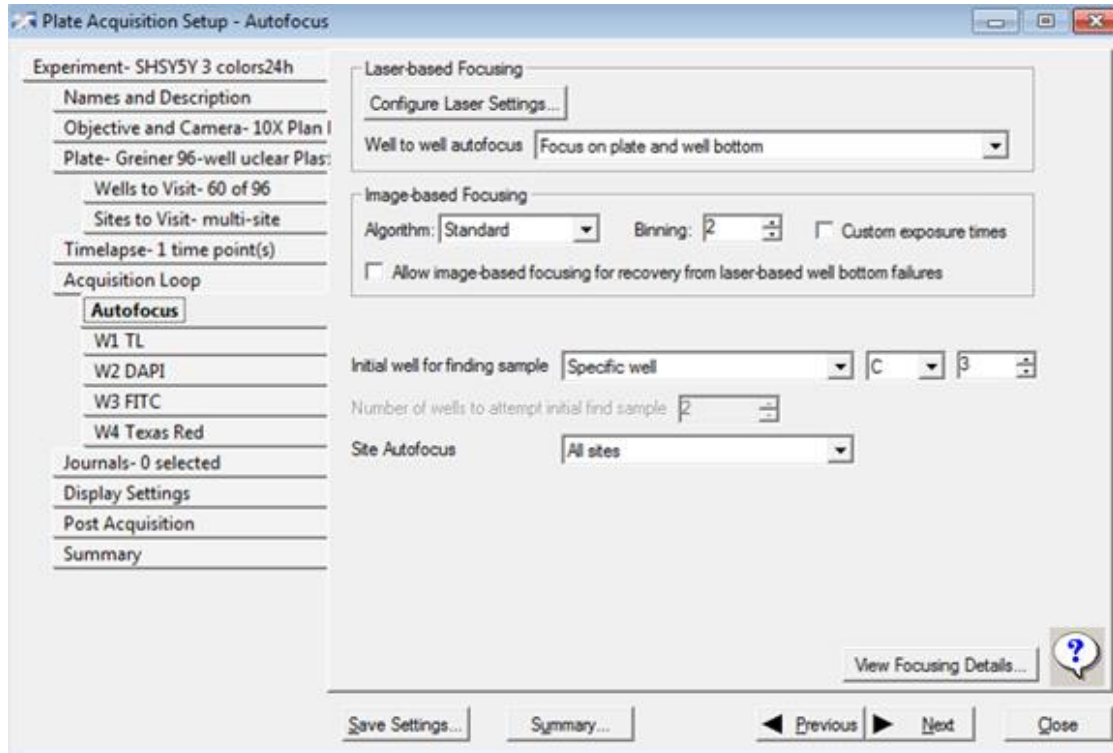
Figure 4. "Plate" tab including the setting that have been standardised for measurements in 96 well plates.

These settings should only be modified by the person responsible for the instrument.



8. Under the “wells to visit” tab, select the 60 innermost wells for analysis. Make sure that the selected wells are showed in a green colour.
9. Under the “sites to visit” tab select 3 columns and 3 rows to obtain 9 images/well.
10. Under the “timelapse” tab select number of endpoints = 1.
11. Under the “autofocus” tab (see Figure 5) select:
 - a. Well to well autofocus: focus on plate and well bottom.
 - b. Imaged based focusing:
 - i. Algorithm: standard
 - ii. Binning: 2
 - c. Initial well for finding sample: specific well
 - d. Site autofocus: all sites.

Figure 5. “Autofocus” tab with the settings used for measurements in 96 well plates.



12. Under the tabs W1 to W4 the setting for image acquisition in these four wavelengths is specified:

Table 4. Settings used for the acquisition of images with MetaXpress®.

The specific exposure time, target maximum intensity and Z- offset set for each of the four wavelengths used to measure MMP in SH-SY5Y cells are described.

Illumination setting	TL	DAPI	FITC	Texas Red	
Exposure time (ms)		5	150	400	400
Target max intensity		16960	34464	33000	33000
Z-offset		8.94	4.48 μm	11	11.96
Calculate Offset (use Z stack and Custom range)	Range: 50 um Step: 1.5 um	Range: 50 um Step: 1.5 um	Range: 50 um Step: 1.5 um	Range: 50 um Step: 1.5 um	Range: 50 um Step: 1.5 um

13. Insert the plate in the imager
14. With the mouse, right click button select one of the 9 sites and one control well, select W1 TL or W2 DAPI to evaluate cell distribution and morphology within the well. To be able to see the cells click on “find sample” in the plate acquisition and control window.
15. With the mouse right click button select one of the 9 sites and one control well, select W3 FITC to evaluate Rhodamine 123 staining.
16. With the mouse, right click button select one of the 9 sites and one FCCP exposed well. Select W4 Texas Red to evaluate PI staining.
17. Click under the “summary” tab → acquire plate.

18. All obtained images are automatically stored in MetaXpress® in the D disk of the computer. The SOP “procedure for backing up old experiments from the imager” describes the steps to follow in order to create back up files.

Data analysis

MetaXpress® Software (Molecular Devices, UK) is used for data acquisition, the fluorescence intensity of the different dyes is measured using the following settings:

Table 5. Settings used for analysis of the images obtained with MetaXpress® after MMP measurements in SH-SY5Y cells.

Dye	Rhodamine123	PI	Hoechst
Filter	FITC	Texas red	DAPI
Approximate min width	1 µm = 2 pixels	5 µm = 8 pixels	5 µm
Approximate max width	20 µm = 31 pixels	8 µm = 12 pixels	8 µm
Intensity above local background	100 graylevels	50 graylevels	150 graylevels
Measurement	Mean fluorescence intensity per well	Number of cells stained	Number of cells stained

To analyse the images:

1. Open MetaXpress® software.
2. On the top menu, select screening → Review plate data.
3. Select the plate for analysis.
4. Select all the wells to be analysed.
5. On the “Run analysis” tab select:
 - a. Analysis: <transfluor HT>
 - b. Settings: FITC, Texas red or DAPI. Add the settings described in Table 5.

To export the values to Microsoft Excel:

1. Open MetaXpress® software.
2. On the top menu, select screening → Review plate data.
3. Select the plate for analysis.
4. On the “Measurements” tab select:
 - a. Analysis: Hoechst/Rhodamine or PI
 - b. Measurement: nuclei (transfluor HT) or Integrated granule intensity (transfluor HT).
 - c. Configure log:
 - i. Excel file
 - ii. Open log
 - iii. Log data

In Microsoft Excel, the mean Rhodamine123 fluorescence intensity is normalised against the number of cells stained with Hoechst in the same well. The results are expressed as the

% of change in the Rhodamine123 fluorescence intensity in comparison with the control (DMSO 0.1%).

$$MMP (\%) = \frac{\text{Rhodamine123 mean FI exposed cells}}{\text{Rhodamine123 mean FI controls}} * 100\%$$

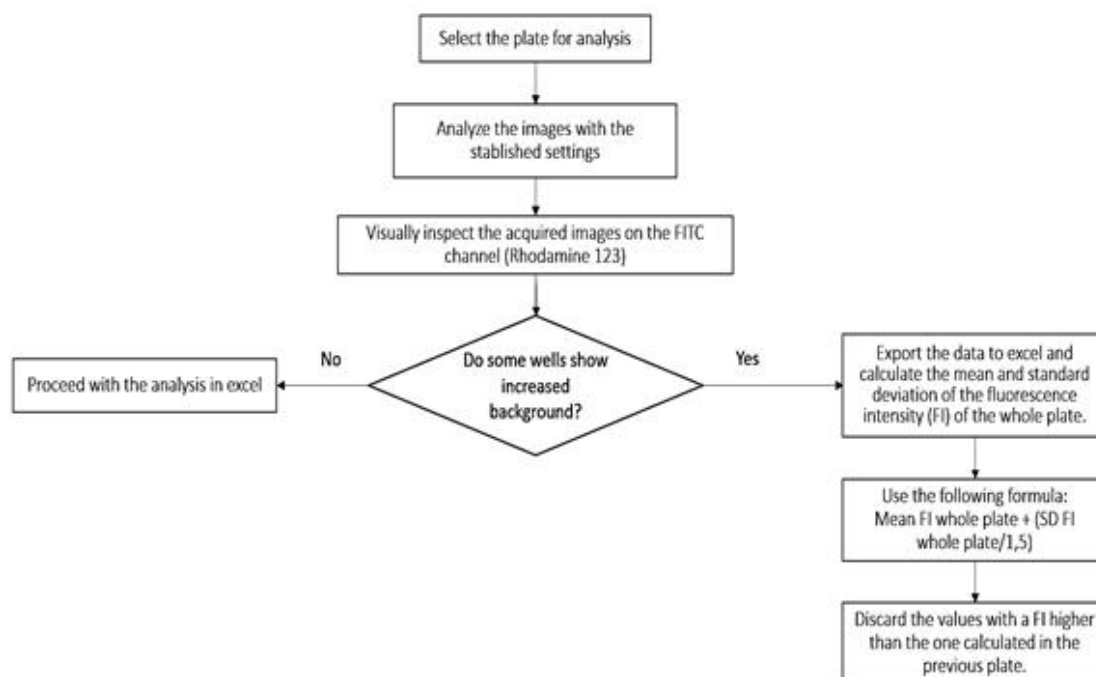
Cell viability is calculated by subtracting the number of PI stained cells from the Hoechst stained cells and normalising against the number of Hoechst stained cells in the same well. The results are expressed as the % of viable cells in comparison with the control (DMSO 0.1%).

$$\text{Viable cells per well } (\%) = \frac{(\text{Hoechst stained cells} - \text{PI stained cells})}{\text{Hoechst stained cells}} * 100\%$$

$$\text{Cell viability } (\%) = \frac{\text{Mean viable cells exposed}}{\text{Mean viable cells control}} * 100\%$$

Outliers:

Uneven background can be produced due to difficulties during the washing steps after the first incubation with Rhodamine 123. Wells with higher background can incorrectly report increased fluorescence intensity, to avoid this problem follow these steps:



6. HEALTH SAFETY AND ENVIRONMENT

Cell culture procedures take place in a laminar air flow cabinet, class 2. All work with test chemicals is performed in a fume hood. Experiments are planned in order to reduce the exposure time of the laboratory personnel to the test chemical. Wastes of environmentally hazardous compounds are handled according to local regulations.

7. REFERENCES

ftp://ftp.meta.moleculardevices.com/support/mx/MX51CDR3/Manuals/MetaXpress_Analysis_Guide.pdf

Annex 1.7. Lactate Assay (provided by Paul Jennings)

Method	Lactate Assay
Contact	Giada Carta (VU) -> g.cart@vu.nl

Aim: This SOP describes the enzymatic analysis of supernatant lactate, a marker of glycolysis, using a colourimetric assay.

Purpose: Since cells increase glycolysis rates when in stress conditions (for many reasons, including mitochondrial injury) and also in tissue repair due to increased cell proliferation, lactate is a useful indicator of sub-lethal injury (Limonciel et. al.). This is especially so in renal epithelial cells, which have low levels of glycolysis once differentiated and quiescent.

Limitations: Supernatant lactate generally increases when cells are sub-lethally injured (Limonciel et. al. and Jennings et. al.). However, when there is a significant loss of viable cells supernatant lactate will decrease.

Method Outline: The lactate assay is based on the conversion of lactate to pyruvate by the enzyme lactate dehydrogenase (LDH), reducing the co-factor NAD to NADH (Babson *et al*). In the assay, NADH reduces PMS to PMSH which reduces INT to INT_H. INT_H is the reagent measured by colourimetry.

Required

TRAM buffer:

- Triethanolamine HCl – Sigma T9534, FW=185.65
- EDTA.Na₂ – Sigma E4884, FW=372.24
- MgCl₂ anhydrous - Sigma M8266, FW=95.21

Colour reagent:

- PMS (N-Methylphenazonium methyl sulphate) – Sigma P9625, FW=306.3
- INT (p-iodonitrotetrazolium violet) – Sigma I8377) FW=505.7
- 100% Ethanol
- Triton X-100 – Sigma X100

β-NAD - Sigma N7004 / N0632 - FW=663.4 (- 20°C)

Lactate dehydrogenase (LDH) - Sigma L2500-25KU, approx. 12KU/mL

L-lactic acid sodium salt (standard) –Fluka Chemika 71718, FW=112.06

1. Stock TRAM solution

Concentrations: 108 mM Triethanolamine HCl, 10.7 mM EDTA.Na₂, 42 mM MgCl₂

Weigh out:

- 20.0 g Triethanolamine HCl
- 4.0 g EDTA.Na₂
- g MgCl₂ anhydrous

Bring to 1 L with ddH₂O

pH to 7.5

Store at 4°C for up to 12 months

2. Stock Colour reagent

Concentrations: 1.63 mM PMS, 3.95 mM INT, 35% ethanol, 2% Triton-X-100

This solution is light sensitive. Do not leave in direct light.

- weigh out 0.050 g PMS and 0.200 g INT
- dissolve in 35 mL 100% ethanol
- add 63 mL ddH₂O
- add 2 mL Triton X-100 for stabilisation (2%)
- sonicate for 5 to 10 min (until powders are fully dissolved)
- store at 4°C for up to 1 year in an amber glass vial
- discard if light yellow colour darkens towards red

3. Lactate standard

Dissolve 56 mg lactate powder into 20 mL ddH₂O to obtain a 25 mM stock.

Store at 4°C for several months.

4. Lactate assay reagent – prepare immediately before incubation

Sample/mix ratio

For 10 µL supernatant sample, use 90 µL reagent mix (Limonciel *et al*, 2011)

Reagent mix

Concentrations: 326 µM PMS, 790 µM INT, 3.3 mM β-NAD, 4 U/mL LDH

The volume of mix depends on the number of samples to be measured. Where few biological replicates are available, it is recommended to measure each sample with 2 technical replicates.

Typically, the volume of mix in µL is: $(X*90) + (16*90) + 1000$

where X is the number of samples where lactate levels must be determined, “16” corresponds to the wells for standard curves, “1000” corresponds to an extra volume of 1 mL.

Calculate the volume needed of each mix component for the volume needed for your experiment, relative to the composition below.

For 10 mL reagent:

- 8 mL TRAM
- 1.98 mL colour reagent
- 22.4 mg β-NAD
- 3.3 µL LDH

Mix TRAM and colour reagent, dissolve β -NAD in, add LDH just before starting the incubation.

Mix by inversion and protect from direct light (aluminium)

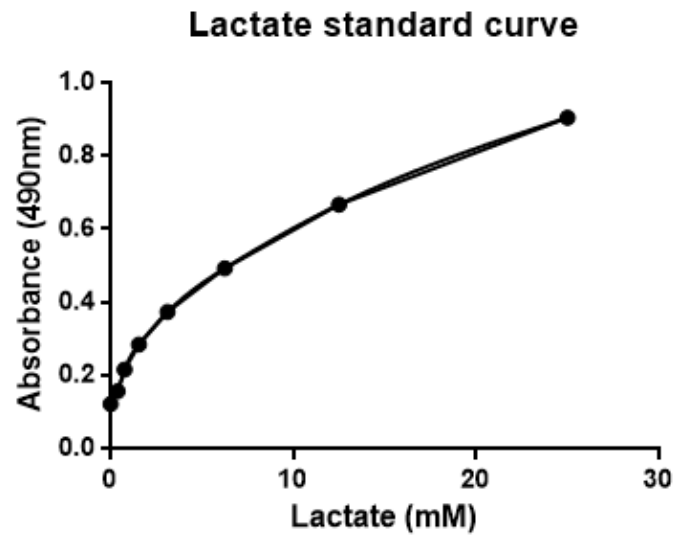
Note: do not vortex the mix, as 1) it damages the enzyme, 2) it produces a lot of foam and considerably reduces the final volume.

Assay

- prepare the lactate assay reagent mix fresh as described
- generate 2 **standard curves** as follows:

Add 40 μ L of 25 mM lactate standard to well A1. Add 20 μ L of ddH₂O (or medium) to wells B1 through H1. Take 20 μ L of standard from A1 to B1 and then C1, D1, E1, F1 and G1 (not H1). Discard volume left from G1. Using a fresh pipette tip transfer 10 μ L H1 to H2. Using the same tip transfer G1 to G2 working upwards and finish with A1 to A2.
- add 10 μ L of cell culture supernatant in the blank wells (starting with A3)
- assay all samples in duplicate
- once all samples and standards are added to the 96 well plate, add 90 μ L of working lactate assay reagent mix using a multichannel pipette
- leave in dark for about 7 min at room temperature
- the mix goes from yellow to orange, to red. Measure before the samples develop to dark brown.
- if necessary (e.g. there is no close access to the plate reader), the assay can be stopped with 50 μ L 1N HCl. Stopped assay should be light protected and read within 1 h
- read optical density (absorbance) at 490 nm (do not use a reference wavelength)
- using a **spline fit function**, e.g. Prism, extrapolate unknowns from standard curve values. Do not use a linear fit as there is a slight saturation over 8 mM lactate.

Typical standard curve: Curve was generated as described using a Tecan Rainbow plate reader with a spline fit / LOWESS (cubic spline) in GraphPad Prism.



References

- Babson, A.L. and G.E. Phillips, *A rapid colorimetric assay for serum lactic dehydrogenase*. Clin Chim Acta, 1965. **12**(2): p. 210-5.
- Limonciel A, Aschauer L, Wilmes A, Prajczek S, Leonard MO, Pfaller W, Jennings P. Lactate is an ideal non-invasive marker for evaluating temporal alterations in cell stress and toxicity in repeat dose testing regimes. *Toxicol In vitro*. 2011 May 24. [Epub ahead of print] PubMed PMID: 21635945.

Annex 1.8. Lactate measurement in supernatant after acute exposure (24h) in differentiated SH-SY5Y cells

Method	SH-SY5Y: Lactate Assay
Contact	Anna Forsby (Swetox) -> anna.forsby@swetox.se

Standard Operating Procedure (SOP)

1. PURPOSE

The SOP describes how cell viability can be analysed by fluorescence in differentiated SH-SY5Y cells.

2. SCOPE

The assay is a modified version of the protocol provided by Paul Jennings, adapted for differentiated SH-SY5Y cells.

This SOP describes the enzymatic analysis of supernatant lactate, a marker of glycolysis, using a colourimetric assay. Since cells increase glycolysis rates when in stress conditions (for many reasons, including mitochondrial injury) and also in tissue repair due to increased cell proliferation, lactate is a useful indicator of sub-lethal injury (Limonciel et. al.). This is especially so in renal epithelial cells, which have low levels of glycolysis once differentiated and quiescent.

3. INTRODUCTION

The lactate assay is based on the conversion of lactate to pyruvate by the enzyme lactate dehydrogenase (LDH), reducing the co-factor NAD to NADH (Babson *et al*). In the assay, NADH reduces PMS to PMSH which reduces INT to INTH. INTH is the reagent measured by colourimetry.

4. RESPONSIBILITY

All personnel performing this assay should follow this SOP.

5. PROCEDURES

LIMITATIONS

- a) Supernatant lactate generally increases when cells are sub-lethally injured (Limonciel et. al. and Jennings et. al.). However, when there is a significant loss of viable cells supernatant lactate will decrease.

METHOD OUTLINE

For experiments, supernatant collected before the resazurin viability measurements in 24 h exposed, 6 days differentiated SH-SY5Y cells was used for lactate analysis.

DEFINITIONS/ABBREVIATIONS

The following abbreviations are used:

DMEM/F12	Dulbecco's modified medium and Ham's F12 medium (1:1)
DMSO	Dimethyl sulfoxide
EMEM	Minimum essential medium with Earle's salts
RA	All-trans retinoic acid
FBS	Foetal bovine serum
PEST	Penicillin Streptomycin
NEAA	Non-essential amino acids
PMS	N-methylphenazonium methyl sulphate
INT	p-iodonitrotetrazolium violet
LDH	Lactate dehydrogenase

IDENTIFICATION OF TEST AND CONTROL SUBSTANCES***A. Test Chemicals***

The cells were pre-exposed to test chemicals before the viability measurements, simulating acute exposure.

B. Controls

- a Negative control:** Cells exposed to N2-RA medium with 0.1% DMSO.
- b Positive control:** Lactate standard curve (25 to 0.78 mM).

MATERIALS***A. Cell Culture Type***

Human neuroblastoma SH-SY5Y cell line is cultured and differentiated as described in **SOP plating and differentiation**.

B. Technical Equipment

- a. 96-well standard plate with flat bottom, solvent resistant polypropylene (Corning, cat no 3364); used as compound plate for storage of lactate samples.
- b. 96-well plate with clear bottom (Corning, cat no CLS3595-50EA).
- c. Triethanolamine HCl (Sigma, cat no T9534)
- d. EDTA.Na₂ (Sigma, cat no E4884)
- e. MgCl₂ anhydrous (Sigma, cat no M8266)
- f. PMS (N-methylphenazonium methyl sulphate) (Sigma, cat no P9625)
- g. INT (p-iodonitrotetrazolium violet) (Sigma, cat no I8377)
- h. Ethanol 99.8% (VWR chemicals, cat no 20821.296)

- i. Triton X-100 (Sigma, cat no X100)
- j. B-NAD (Sigma, cat no N7004)
- k. Lactate dehydrogenase (LDH) (Sigma, cat no L2500)
- l. L-lactic acid sodium salt (standard) (Fluka Chemika, cat no 71718)
- m. Tecan® Infinite M200 Pro
- n. Tecan® i-control 3.7.3.0 software

METHOD:

A. Preparation of stock solutions

Stock TRAM solution

1. Weigh out:
 - 20 g Triethanolamine HCl
 - 4 g EDTA.Na₂
 - 3,97 g MgCl₂
2. Bring to 1 L with ddH₂O. The final concentrations are: 108 mM Triethanolamine HCl, 10.7 mM EDTA.Na₂ and 42 mM MgCl₂
3. Adjust pH to 7,5
4. Store at 4 °C for up to 12 months

Stock colour reagent

This solution is light sensitive, do not leave in direct light.

1. Weigh out 0.050 g PMS and 0.2 g INT
2. Dissolve in 35 mL 100% ethanol
3. Add 63 mL ddH₂O
4. Add 2 mL Triton X-100 for stabilisation
5. Sonicate for 5 to 10 min (until powders are fully dissolved)
6. Store at 4 °C for up to 1 year in an amber glass vial

The stock colour reagent should have a light yellow colour. Discard if the colour darkens towards red. The final concentrations of all reagents are: 1.63 mM PMS, 3.95 mM INT, 35% ethanol, 2% Triton X-100

Lactate standard

Dissolve 56 mg lactate powder into 20 mL ddH₂O to obtain a 25 mM stock. Store at 4 °C for several months.

Lactate assay reagent

Prepare **immediately before** incubation

Sample/mix ratio: For 10 µL supernatant sample, use 90 µL reagent mix.

Reagent mix

The final concentrations of all reagents are: 326 µM PMS, 790 µM INT, 3.3 mM β-NAD, 4 U/mL LDH.

The volume of mix depends on the number of samples to be measured.

1. Calculate the volume of mix needed:

$$\text{Needed volume of mix in } \mu\text{L} = (X \cdot 90) + (16 \cdot 90) + 1000$$

Where X is the number of samples where lactate levels must be determined, “16” corresponds to the wells for standard curves, and “1000” corresponds to an extra volume of 1 mL. For the experiment, 12 mL of reagent mix are needed to measure one plate.

2. Calculate the volume needed of each mix component for the volume needed for the experiment, relative to the composition below:

Mix component	To prepare 10 mL reagent	To prepare 12 mL reagent (one plate)
TRAM	8 mL	9,6 mL
Colour reagent	1,98 mL	2,38 mL
B-NAD	22,4 mg	26,88 mg
LDH	3,3 µL	3,96 µL

3. Mix TRAM and colour reagent
4. Dissolve β-NAD in
5. Add LDH just before starting the incubation, mix by inversion (vortexing the mix damages the enzyme and produces foam).
6. Protect from direct light (aluminium)

B. Exposure

SH-SY5Y cells were plated at a density of 10 000 cells per well and exposed for 24 hours to a selection of chemicals for subsequent measurements of cell viability using the resazurin assay. The day the cell viability was performed 150 µL of supernatant were collected and stored at -20 °C for lactate analysis.

C. Lactate assay

Once all the chemicals have been tested, the collected supernatant from all the plates is analysed.

1. Bring all the plates with supernatant to room temperature
2. Prepare the reagent mix fresh as described in the previous section
3. In a clear 96 well plate generate 2 standard curves as follows:
 - a. Add 40 µL of 25 mM lactate standard to well A1. Add 20 µL of ddH₂O (or medium) to wells B1 through H1. Take 20 µL of standard from A1 to B1 and then C1, D1, E1, F1 and G1 (not H1). Discard volume left from G1. Using a

fresh pipette tip transfer 10 μ L H1 to H2. Using the same tip transfer G1 to G2 working upwards and finish with A1 to A2.

4. Add 10 μ L of cell culture supernatant in the blank wells (See plate configuration, Figure 1)
5. Once all samples and standards are added to the plate, add 90 μ L of reagent mix using a multichannel pipette
6. Cover the plate with aluminium foil
7. Shake the plate for 2 minutes
8. Incubate at room temperature for 5 minutes (the mix goes from yellow to orange, to red. Measure before the samples develop to dark brown)
9. If needed, the assay can be stopped with 50 μ L 1M HCl. The stopped assay should be light protected and read within 1 hour
10. Open the Tecan Infinite M200 Pro reader
11. Read the plate:
 - a. Mode: absorbance
 - b. Wavelength: 490 nm (Do not use reference wavelength)
 - c. Bandwidth: 9 nm
 - d. Number of flashes: 25
 - e. Settle time: 0 ms
 - f. Temperature: off
12. Store the data in the S-disk: S:\Projects\PHC33_EUToxRisk\Case study 4_Mitotox\Lactate.
13. Microsoft Excel is used to analyse the data.
14. The average absorbance of each concentration (duplicates) and standard deviation (SD) are calculated. The average absorbance value of each concentration is normalised against the mean absorbance value of control wells (12 wells) and subsequently multiplied by 100 in order to obtain the % of lactate production in comparison to the negative controls.

Figure 1. Plate configuration used for the lactate assay

	1	2	3	4	5	6	7	8	9	10	11	12
A	Standard curve	DMSO control	EMPTY									DMSO control
B			Co1 D1	Co1 D2	Co1 D3	Co1 D4	Co4 D1	Co4 D2	Co4 D3	Co4 D4		
C			Co2 D1	Co2 D2	Co2 D3	Co2 D4	Co5 D1	Co5 D2	Co5 D3	Co5 D4		
D			Co3 D1	Co3 D2	Co3 D3	Co3 D4	Co6 D1	Co6 D2	Co6 D3	Co6 D4		
E			EMPTY									
F			EMPTY									
G			EMPTY									
H			EMPTY									

IX. HEALTH SAFETY AND ENVIRONMENT

Cell culture procedures take place in a laminar air flow cabinet, class 2. All work with test chemicals is performed in a fume hood. Experiments are planned in order to reduce the exposure time of the laboratory personnel to the test chemical. Wastes of environmentally hazardous compounds are handled according to local regulations.

X. REFERENCES

1. Babson, A.L. and G.E. Phillips, *A rapid colorimetric assay for serum lactic dehydrogenase*. Clin Chim Acta, 1965. **12**(2): p. 210-5.
2. Limonciel A, Aschauer L, Wilmes A, Prajczek S, Leonard MO, Pfaller W, Jennings P. Lactate is an ideal non-invasive marker for evaluating temporal alterations in cell stress and toxicity in repeat dose testing regimes. *Toxicol In vitro*. 2011 May 24. [Epub ahead of print] PubMed PMID: 21635945.

Annex 1.9. Resazurin Assay (provided by Paul Jennings)

Method	Resazurin Assay
Contact	Giada Carta (VU) -> g.cart@vu.nl

This method assesses cell viability based on the irreversible reduction of the blue dye resazurin to the pink product resorufin, which is highly fluorescent in red. Although the exact enzyme(s) involved in the reaction have not been identified, the assay is considered to be based on the global REDOX capacity of the cells in culture.

Resazurin stock

Resazurin [Sigma R7017], formula weight = 251.2 g/mol.

A 20X resazurin stock (880 µM) can be prepared and stored at 4°C for several months if kept sterile.

To prepare 20X stock:

- weigh out 0.011g resazurin
- add 1mL 0.1 N NaOH
- stir gently to dissolve
- bring to just under 50 mL with PBS
- mix
- adjust pH from about 11 to 7.8
- bring to exactly 50 mL with PBS
- filter sterilise (0.2 µm) under laminar flow
- store cold (4°C) and protected from light (aluminium) for up to 3 years

Assay

- warm up the 20X resazurin stock to 37°C to dissolve resazurin crystals
- **dilute 1 in 20** into warm complete culture medium (final conc: 44 µM)
- remove cell culture supernatant
- wash with sterile PBS (room temperature)
- add xx µL of diluted resazurin to the cells for 1 to 3 h

Volume depends on the format and should be the same as that used for cell seeding and feeding, in order to cover the whole cell population in the well/dish.

Duration depends on the cells and their REDOX capacities. For HK-2 and RPTEC/TERT1 cells, 2 h is sufficient. For HepaRG, 1h is enough. Incubation was too long if all the wells have turned from blue to pink.

- include a **blank with no cells** to determine background resazurin fluorescence. This should be incubated in similar conditions as the cells.

- measure resorufin fluorescence: **excitation at 540 nm, emission at 590 nm**. Either measure in the plate from the top or collect supernatant to a 96-well plate, including the blank with no cells.

References

1. Jennings P, Koppelstaetter C, Aydin S, Abberger T, Wolf AM, Mayer G, and Pfaller W. Cyclosporine A induces senescence in renal tubular epithelial cells. *Am J Physiol Renal Physiol*, 2007.
2. Jennings P, Koppelstaetter C, Pfaller W, Morin JP, Hartung T, and Ryan MP. Assessment of a new cell culture perfusion apparatus for *in vitro* chronic toxicity testing. Part 2: toxicological evaluation. *Altex* 21: 61-66, 2004.

Annex 1.10. Cell viability measurements with Resazurin after acute exposure (24h) in differentiated SH-SY5Y cells

Method	SH-SY5Y: Resazurin Assay
Contact	Anna Forsby (Swetox) -> anna.forsby@swetox.se

Standard Operating Procedure (SOP)

1. PURPOSE

The SOP describes how cell viability can be analysed by fluorescence in differentiated SH-SY5Y cells.

2. SCOPE

The cell viability is determined by the number of viable cells in culture. The assay is a modified version of the protocol provided by Paul Jennings, adapted for differentiated SH-SY5Y cells.

3. INTRODUCTION

This method assesses cell viability based on the irreversible reduction of the blue dye resazurin to the pink product resorufin, which is highly fluorescent in red. Although the exact enzyme(s) involved in the reaction have not been identified, the assay is considered to be based on the global REDOX capacity of the cells in culture.

4. RESPONSIBILITY

All personnel performing this assay should follow this SOP.

5. PROCEDURES

METHOD OUTLINE

We used resazurin which is irreversibly reduced into resorufin by live cells producing thereof a dye fluorescent in red. The obtained fluorescence intensity correlates to the number of live cells in culture.

For experiments, SH-SY5Y cells are plated in black 96-well plates with clear bottom in EMEM with 10% foetal bovine serum, 2 mM glutamine, 1% non-essential amino acids, 100g streptomycin/mL and 100 U penicillin/mL, which is exchanged to differentiation medium with 1 µM RA and without serum (DMEM:F12 [1:1], N2 supplements [diluted 1:100], 1 mM glutamine, 100g streptomycin/mL and 100 U penicillin/mL) after 24 h. Six (6) days differentiated SH-SY5Y cells are exposed to test chemicals and incubated for 24 hours after which the effect on cell viability is measured with resazurin.

DEFINITIONS/ABBREVIATIONS

The following abbreviations are used:

DMEM/F12	Dulbecco's modified medium and Ham's F12 medium (1:1)
DMSO	Dimethyl sulfoxide
EMEM	Minimum essential medium with Earle's salts
RA	All-trans retinoic acid
FBS	Foetal bovine serum
PEST	Penicillin Streptomycin
NEAA	Non-essential amino acids

IDENTIFICATION OF TEST AND CONTROL SUBSTANCES

A. Test Chemicals

The cells were pre-exposed to test chemicals before the viability measurements, simulating acute exposure.

B. Controls

- a. **Negative control:** Cells exposed to N2-RA medium with 0.1% DMSO.
- b. **Blank:** DMEM:F12 cell medium.

MATERIALS

A. Cell Culture Type

Human neuroblastoma SH-SY5Y cell line is cultured and differentiated as described in **SOP plating and differentiation**.

B. Technical Equipment

- a. Tecan® Infinite M200 Pro
- b. Tecan® i-control 3.7.3.0 software
- c. Resazurin (Sigma, Cat no R7017)
- d. PBS, pH 7.4 (Gibco, Cat no 10010056)
- e. Syringe filtration unit Filtropur S 0.2 (Sarstedt, Cat no 83.1826.001)
- f. Black cell culture 96-well plate with clear bottom (Corning, cat no 3603).
- g. 96-well standard plate with flat bottom, solvent resistant polypropylene (Corning, cat no 3364); used as compound plate for storage of lactate samples.

METHOD:

A. Preparation of the resazurin stock

The 880 µM resazurin stock is prepared as follows:

1. Weigh out 0,011g resazurin
2. Add 1000 μ L 0,1 M NaOH
3. Stir gently to dissolve
4. Bring to just under 50 mL with PBS
5. Mix
6. Adjust pH from about 11 to 7,8
7. Bring to exactly 50 mL with PBS
8. Sterile filter (0,2 μ m) under laminar flow
9. Store at 4 °C and protected from light (aluminium) for up to 3 years

B. Exposure

For experiments, 10 000 SH-SY5Y cells are plated in black 96-well plates with clear bottom in complete EMEM with 10% foetal bovine serum, which is exchanged to N2-RA differentiation medium after 24 h. After 3 days of differentiation 100 μ L of N2-RA differentiation medium are added on top of all wells, the plate is then placed in the incubator and the cells are allowed to differentiate for 3 days more (total differentiation time of 6 days). On the 6 day of differentiation 100 μ L of cell medium are removed from the wells containing cells and replaced with 100 μ L of the assigned treatment.

C. Resazurin cell viability assay

The measurement of resazurin reduction is performed 24 hours after exposure to test chemicals

1. Warm up the 880 μ M resazurin stock
2. For one plate, dilute 1000 μ L of the resazurin stock in 9000 μ L DMEM:F12 to prepare a 2X working solution
3. Remove 150 μ L of cell medium from the wells and collect it in a compound plate (supernatant can be used for lactate measurements)
4. Seal the compound plate and store it at -20 °C
5. Add 50 μ L from the resazurin working solution to all wells including blanks
6. Incubate the plate for 2 hours at 37 °C
7. Open the Tecan Infinite M200 Pro reader.
8. Measure the resorufin fluorescence:
 - a. Excitation wavelength: 540 nM
 - b. Emission wavelength: 590 nM
 - c. Mode: Fluorescence top reading
 - d. Excitation bandwidth: 9 nm
 - e. Emission bandwidth: 20 nm
 - f. Gain: Calculated from B2

- g. Number of flashes: 25
- h. Integration time: 20 μ s
- i. Lag time: 0 μ s
- j. Settle time: 0 ms
- k. Z-position: Calculated from B2
- l. Temperature: 37 °C
9. Store the data in the S-disk: S:\Projects\PHC33_EUToxRisk\Case study 4_Mitotox\Resazurin results\24h exposure.
10. Microsoft Excel is used to analyse the data.
11. The average fluorescence intensity of each concentration (triplicates) and standard deviation (SD) are calculated. The average fluorescence intensity value of each concentration is normalised against the mean fluorescence intensity value of control wells (12 wells) and subsequently multiplied by 100 in order to obtain the % of viable cells in each concentration.

IX. HEALTH SAFETY AND ENVIRONMENT

Cell culture procedures take place in a laminar air flow cabinet, class 2. All work with test chemicals is performed in a fume hood. Experiments are planned in order to reduce the exposure time of the laboratory personnel to the test chemical. Wastes of environmentally hazardous compounds are handled according to local regulations.

X. REFERENCES

1. Jennings P, Koppelstaetter C, Aydin S, Abberger T, Wolf AM, Mayer G, and Pfaller W. Cyclosporine A induces senescence in renal tubular epithelial cells. *Am J Physiol Renal Physiol*, 2007.
2. Jennings P, Koppelstaetter C, Pfaller W, Morin JP, Hartung T, and Ryan MP. Assessment of a new cell culture perfusion apparatus for *in vitro* chronic toxicity testing. Part 2: toxicological evaluation. *Altex* 21: 61-66, 2004.

XI. APPENDIX

Summary of the procedure for measurement of cell viability with resazurin in differentiated SH-SY5Y cells:

Day 1

Plating of SH-SY5Y cells, 10 000 cells/well.

Day 2

Start differentiation.
Change complete EMEM to N2-RA medium.

Day 3

Addition of fresh N2-RA differentiation medium

Day 6

Exposure to test chemicals.
Remove 100 µL N2-RA medium.
Add 100 µL of the corresponding chemical dilution.

DMSO Control	Co1 D1	Co1 D2	Co1 D3	Co1 D4	Co4 D1	Co4 D2	Co4 D3	Co4 D4	DMSO Control
	Co2 D1	Co2 D2	Co2 D3	Co2 D4	Co5 D1	Co5 D2	Co5 D3	Co5 D4	
	Co3 D1	Co3 D2	Co3 D3	Co3 D4	Co6 D1	Co6 D2	Co6 D3	Co6 D4	

Day 7

Resazurin viability assay (Protocol Cell viability)
Remove 150 µL of supernatant and collect it for lactate measurements.
Add 50 µL of 2X resazurin working solution.
Incubate the plate for 2 hours at 37 °C.
Record the resorufin fluorescence at 540 nM excitation and 590 nM emission with the Tecan Infinite M200 Pro reader.

Annex 1.11. Assessment of proteasome activity in live cells

Method	LUHMES: Proteasome activity assay
Contact	Johannes Delp (UKN) -> johannes.delp@uni-konstanz.de

Introduction

Aim

This protocol describes the analysis of how to assess the proteasome activity of live LUHMES cells (human neuronal).

Purpose

Proteasomal activity is an essential process of cellular homeostasis. It can be directly inhibited, e.g. by proteasomal inhibitors, or decreased as a consequence of e.g. energy depletion, since the proteasome is ATP-dependent.

Limitations

The proteasome has three major catalytic activities: the $\beta 1$ subunit has a caspase-like activity (i.e. cleaves after acidic amino acids), the $\beta 2$ subunit has a trypsin-like activity (i.e. cleaves after basic amino acids) and the $\beta 5$ subunit has a chymotrypsin-like activity (i.e. cleaves after hydrophobic amino acids). The used substrate (MeOSuc-Gly-Leu-Phe-AMC) is thus most likely mainly cleaved by the $\beta 5$ subunit, while the activity of the other subunits might not be assessed to a similar extent. The positive control (e.g. MG-132) is an inhibitor which impairs all catalytic subunits.

Additionally, for proper cellular proteasomal protein degradation, also the ubiquitin system has to work functionally, which is not assessed by this assay.

During the assay (while the proteasomal substrate is incubated), no treatment substances are on the cells, thus treatment substances might be diluted out of the cells during assessment of proteasomal activity.

On top, other intracellular proteases could cleave the substrate. This background/ non-blockable cleavage should be assessed by controls, e.g. broad-spectrum proteasome inhibitors.

Method outline

Cells are cultured normally according to established lab protocols in 96 well plates. Around the time of endpoint assessment, the medium is removed from the wells and the assay mix with the proteasomal substrate is added to replace the medium. The proteasomal substrate is cell-permeable and diffuses into the proteasome, as well as the cleavage products diffuse out of the proteasome.

The proteasome activity dye consists of a fluorophore which is linked to a small peptide chain which quenches the fluorescence activity. Upon proteasomal cleavage, the fluorescence increases. Thus, the increase in fluorescence activity between directly after addition of the substrate and 2 h later is correlated with the proteasomal activity.

Method description

Chemicals and buffers

- Proteasome substrate: MeOSuc-Gly-Leu-Phe-AMC from Bachem (I1430). Prepare 10 mM Stock in DMSO, store at -20°C.
- HBSS buffer
- Cells in 96 well plates (otherwise adapt the volumes)
- Fluorescence plate reader

Preparation upfront

- Prepare working mix of proteasome substrate: dilute 1:400 in HBSS, final concentration 25 μ M

Experimental procedure

- Remove medium from the wells (2 h before the endpoint should be determined)
- Add 100 μ l/well working mix (25 μ M proteasome substrate in HBSS)
 - Measure fluorescence directly after addition (=baseline measurement; ex. 360 nm, em: 465, fixed gain)
- Incubate the cells in their standard incubation conditions, thereby wait for 1-3 h, depending on the cellular activity
 - Measure fluorescence again (=end measurement; ex. 360 nm, em: 465, fixed gain – same as for baseline)
 - Time course measurements can be performed to find the best waiting time
- Use assay positive controls: cells treated with a sufficient concentration of a proteasome inhibitor, e.g. MG-132 (alternatively: lactacystin, bortezomib, epoxomicin or a combination of them)

Data analysis

- Subtract from each value of the end measurement its baseline value
- Calculate fluorescence intensity relative to control
 - Positive controls can be used to determine the amount of not proteasome inhibitor blockable substrate cleavage

Annex 1.12. DB-ALM Protocol HepG2 BAC-GFP Cell Stress Reporter Cell Line - Last Update: May 2018 (v.9.4)

The present template is based on the Content Criteria for Protocols, which are designed for the provision of technical details that enable the documented alternative method to be transferred to other laboratories without the need of additional information.

The Content Criteria, on which the presented template is based, are generic and based on the analysis of common descriptors from hundreds of different non-animal experimental methods and techniques. However, not all parameters or sections indicated are applicable to all protocols. It is the responsibility of the author to provide content where relevant and as appropriate related to a precise protocol. The compilation is to be performed by completing the individual sections with the information related to your method. A review for consistency, completeness in relation to the technique described and compliance with the Content Criteria in place is always performed by the JRC staff. Furthermore, before any protocol is published *via* the DB-ALM, the final draft is reviewed and approved by designated contact person(s) (method's owner and/or experienced user).

A summary description of the main method features bringing it in a context regarding its intended purpose(s) and application(s), as well as the scientific rationale is always to be provided with each protocol. The content criteria for the method summary will be sent in due course.

The DB-ALM is operated by the European Reference Laboratory for Alternatives to Animal Testing of the Joint Research Centre.

NOTE: All statements shall be supported with references within the text and full details listed under “**Bibliographic References**” data sector.

Part A. Protocol Introduction

Protocol Name: LU HepG2 BAC-GFP reporter cell line assay

Abstract:

This protocol describes how to culture HepG2 BAC-GFP reporter cells, how to perform compound exposures, how to perform imaging of the reporters and how to perform image analysis.

Résumé

With the HepG2 BAC GFP reporter platform the activation of cellular stress pathways can be accurately measured over time on a single cell level. The platform includes HepG2 reporters for oxidative stress (KEAP1, NRF2, SRXN1, HMOX, NQO1), ER stress (ATF4, XBP1, CHOP, BIP), inflammation (IkBa, RelA, ICAM, A20), DNA damage (53BP1, P53, P21, BTG2), Heat shock (HSF-1/2, HSP70), Heavy metal (MTF1) and hypoxia (VHL, HIF-1a, HUMMR). The combination of a protein based reporter system with confocal imaging has proven to be a powerful tool in drug safety testing as well as mechanistic research in cellular stress responses. See references.

Experimental Description

Biological Endpoint and Endpoint Measurement:

The endpoints of our toxicity test system include:

1. Activation of responsive part of the cellular stress response pathway of interest by GFP signal intensity in the nucleus or cytoplasm
2. Cytotoxicity based on Propidium Iodide (PI) and Annexin V Alexa 633 staining

The acquisition of microscopic pictures can be done at specific timepoints, i.e. 24h/48h/72h.

Figure 1. Exposure scheme read out after 24h

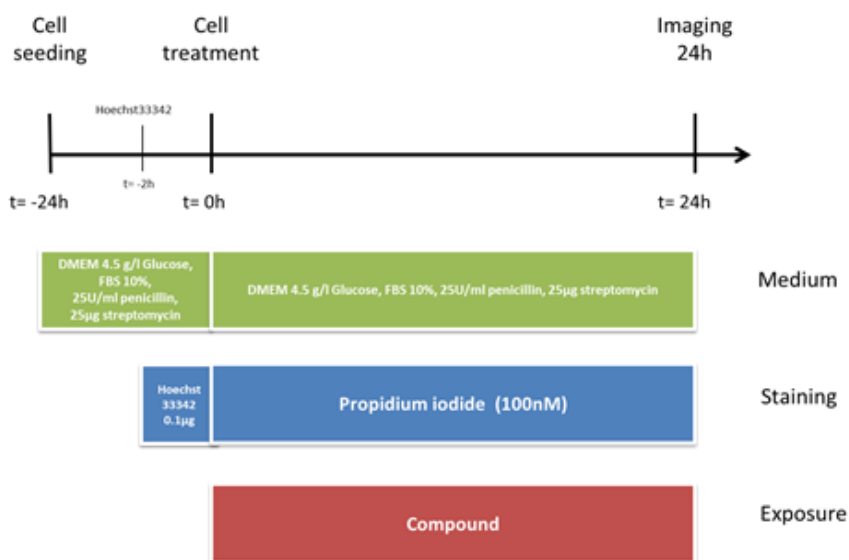


Figure 2. Exposure scheme read out after 48h

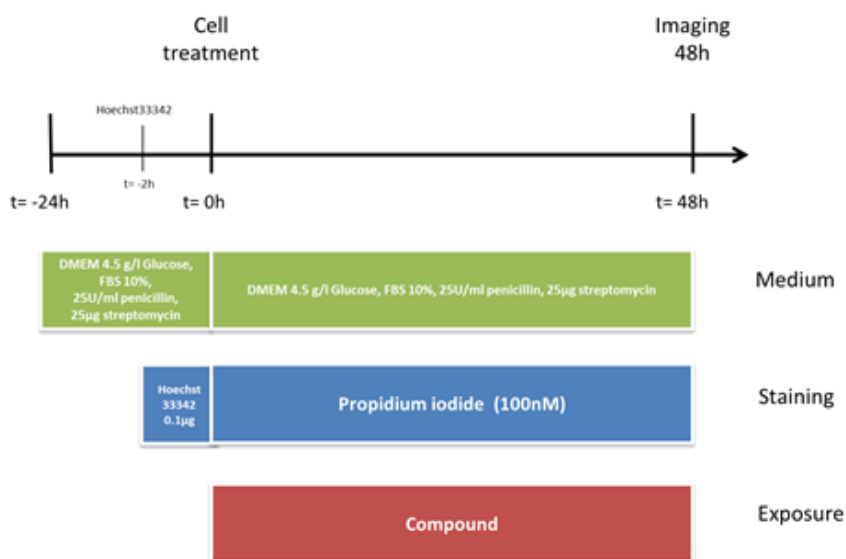
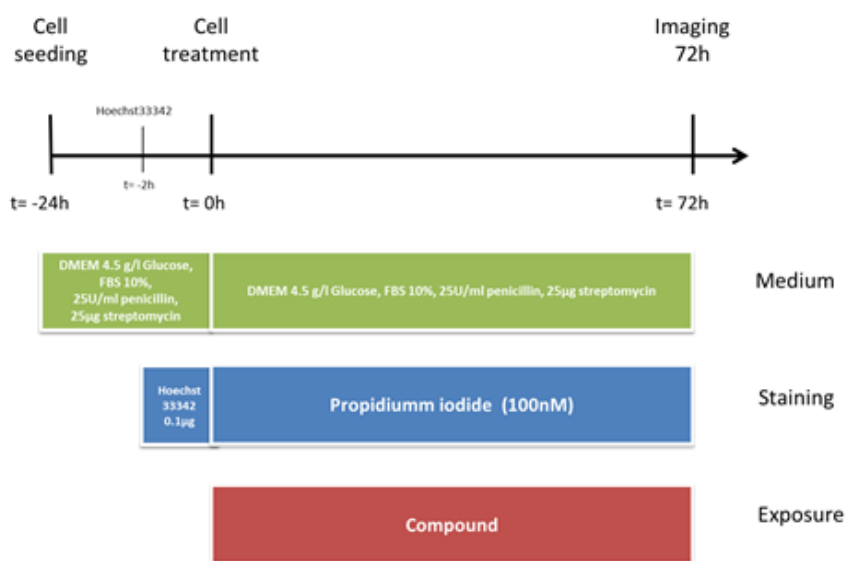


Figure 3. Exposure scheme read out after 72h

**Endpoint Value:**

1. The number of cells is quantified using Hoechst 33342 staining.
2. Upregulation of the EGFP-tagged protein of interest is determined based on the fluorescence signal in the EGFP-channel. Proteins which are located in the nucleus is quantified by the integration of pixels in the
3. Single cell location is based on the nuclear Hoechst 33342 staining and automated high content image analysis.
4. The cytotoxicity measurement is based on the percentage of PI positive nuclei.
5. Dose response modelling, four-parametric log logistic curve fitting or point of departure calculation might be used to compare responses triggered by different compounds.

Experimental System:

HepG2 BAC-GFP reporter cell lines are based on HepG2 (ATCC® HB8065™) cells which were transfected with a bacterial artificial chromosome containing an EGFP-tagged-gene including the flanking regulatory domains. After G418 selection, a monoclonal cell lines were generated and after experimental confirmation (western blot, siRNA and time lapse microscopy), functional clones were selected (see also Wink *et al.* 2014).

Basic procedure

The HepG2 reporter cell lines are seeded in multi-well imaging plates, stained with Hoechst 33342, Annexin V alexa 633 and propidium iodide, and treated with different concentration of testing compounds. The cells are then imaged live on a confocal laser scanning microscope.

Discussion

This bioassay is based on the HepG2 cell line and the genetic modification is done with at biosafety level 1, therefore no further management of ethical issues nor higher biosafety

standards are required. Depending on the test compounds, further safety measures might be necessary. For image acquisition, a confocal laser scanning microscope including a cell culture incubator is required.

The person conducting the experiment needs to be properly trained for culturing HepG2 cells, confocal microscopy and high content data analysis, therefore a Master of Science level education is recommended.

Depending on the desired time point, the experiment itself can be conducted in 3 to 5 days. The amount of time of the data analysis is depending on the computing capacity.

The critical point of this experiment is the seeding of the HepG2 cells as a single cell suspension. HepG2 cells tend to form clumps, which negatively affects the image acquisition with a confocal laser scanning microscope and the single nuclei segmentation during the high content image analysis.

Status

In Development:

Data analysis pipeline: Point of departure, EC values

Known Laboratory Use:

The laboratory history of HepG2 BAC-GFP reporter so far is limited to the group of Bob van de Water at the University of Leiden.

Participation in Evaluation Study:

The SOP was developed within the frame of the EU-ToxRisk project:

<http://www.eu-toxrisk.eu/>

Grant agreement No 681002

Participation in Validation Study:

The test method did not undergo any validation as defined in OECD Guidance Document No. 34 so far.

Regulatory Accepted:

There are no relevant guidelines available.

Proprietary and/or Confidentiality Issues

The use of HepG2 BAC-GFP reporter technology is not restricted by patents. The protocol regarding the experimental and microscopy procedures is open. However, the protocol regarding the automated data-analysis is owned by the Van de Water lab and should not be shared among others.

Health and Safety Issues

General precautions:

Follow general safety precautions of a mammalian cell culture lab. Wear lab coat, gloves and safety goggles. For the laser scanning fluorescence microscope, safe work with the involved lasers has to be guaranteed. The involved cell lines are safe to work with in a biosafety level 1 according to council directive 90/679/EEC.

MSDS Information:

For safety data sheets, please contact the compounds' manufacturer.

Abbreviations and Definitions

AnV...Annexin V Alexa 633

ATCC...American Type Culture Collection

BAC...Bacterial artificial chromosome

BEVC ...best-fit ellipse of Voronoi cell

CDDO-me... 2-Cyano-3,12-dioxo-oleana-1,9(11)-dien-28-oic acid methyl ester

DEM...Diethyl maleate

DMEM... Dulbecco modified eagle medium

DMSO... Dimethyl sulfoxide

EDTA... Sodium ethylenediaminetetraacetate

EGFP...Enhanced green fluorescent protein

FBS...foetal bovine serum

PoD...Point of Departure

PI...propidium iodide

P/S...Penicillin/Streptomycin solution

RT...room temperature

T25/T75/T175...Tissue flask 25 cm² /75cm²/175cm²

WMC...watershed masked clustering

Part B. Technical Description***Procedure Details, Latest Version: 1.******Protocol Name: LU HepG2 reporter cell line assay******Contact person***

Bob van de Water

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Materials and Preparations

Cell OR Experimental system

The original HepG2 wildtype cells were obtained from ATCC (ATCC® HB-8065™). Using bacterial artificial chromosome (BAC) recombination a copy of the biomarker protein with at least 10kb upstream of the promoter region and at least 10kb downstream of the gene was inserted together with GFP was transfected in HepG2, resulting in a C-terminally tagged stable cell line. Multiple clones per biomarker protein were picked and the clone was selected that showed endogenous regulation of the reporter-GFP fusion protein. The created cell lines have been biobanked and stored at -80°C for short term storage and -150°C for long term storage.








Equipment

Fixed Equipment

Material/Equipment
Centrifuge
TC20™ Automated Cell Counter
Fridge (4°C)
Freezer (-20°C, -80°C, -150°C)
Humidified incubator (37°C, 5% CO2)
Ice machine
Laminar flow hood for sterile atmosphere
Laminar flow hood for sterile atmosphere
Water Bath
Stereoscopic microscope
Nikon Eclipse Ti or Nikon Eclipse Ti2 , equipped with: four lasers (408, 488, 561 and 644 nm lasers), a A1, C1 or C2 box, live cell incubator, automated stage, perfect focus system or equivalent.
Milli-Q water dispenser

Material/Equipment	Supplier	Catalogue No
Micropipettes Pipet-Lite XLS LTS		
Pipette L-1000XLS+	Rainin	17014382
Pipette L-200XLS+	Rainin	17014391
Pipette L-20XLS+	Rainin	17014392
Pipette L-2XLS+	Rainin	17014393
Multichannel pipets		
Eppendorf Research® plus, 8-channel, variable, incl. epT.I.P.S.® Box, 0.5 – 10 µL, medium gray	Eppendorf	312500010
Eppendorf Research® plus, 8-channel, variable, incl. epT.I.P.S.® Box, 10 – 100 µL, yellow	Eppendorf	312500036
Eppendorf Research® plus, 8-channel, variable, incl. epT.I.P.S.® Box, 30 – 300 µL, orange	Eppendorf	312500052
Software		
NIS viewer	Nikon	
CellProfiler® (version 2.1.1)	Carpenter Lab at the Broad Institute of Harvard and MIT.	
R Studio (version R version 3.3.2 or newer)	RStudio,	
Sterile 15 plastic tubes	Sarstedt	62.554.502
Sterile 50 plastic tubes	Greiner Bio-One	210270
Sterile cell culture flasks T25	Corning	430639
Sterile cell culture flasks T75	Corning	430641U
Sterile cell culture flasks T175	Corning	431080
Sterile pipettes 2 ml	Greiner Bio-One	710180
Sterile pipettes 5 ml	Greiner Bio-One	606180
Sterile pipettes 10 ml	Greiner Bio-One	607180
Sterile pipettes 25 ml	Greiner Bio-One	760180
Cryovials		
Cryo.s U-starand-2ml	Greiner Bio-One	122263
Cryo.s U-starand-2ml	Greiner Bio-One	122277
Cryo.s U-starand-2ml	Greiner Bio-One	122278
Cryo.s U-starand-2ml	Greiner Bio-One	122279
Cryo.s U-starand-2ml	Greiner Bio-One	122280
Micro tube 1.5ml	Sarstedt	72.690.001
Micro tube 2ml	Sarstedt	76.695.500
Sterile Pipette tips		
LTS tips 20ul	Mettler Toledo	17005091
LTS tips 200ul	Mettler Toledo	17005093
LTS tips 1000ul	Mettler Toledo	17005089
Biopur 2-200 ul refills	VWR	613-3569
Biopur 20-300 ul refills	VWR	613-3570
Gloves EcoSHIELD wit econitriil PF 250	Boom BV	38055162; 38055163; 38055164
Imaging plates		
96 µclear wells plate	Greiner Bio-One	655090
96 ScreenStar plate	Greiner Bio-One	655 866
384 µclear wells plate	Greiner Bio-One	781 091
Cell Counting Kit, 30 dual-chambered slides, 60 counts, with trypan blue	BIO RAD	1450003
50µm non-sterile CellTrics® filters	SysmexNederland	04-0042-2317

Testing compounds

Compound	abbreviation	Sigma Aldrich Cat. No.	Labelling accord. Reg. (EC) 1272/2008
Rotenone	ROT		
Deguelin	DEG		
Azoxystrobin	AZO		
Kresoxim-methyl	KRE		
paraclostrobin			
Picoxystrobin	PIC		
trifloxystrobin	TRI		

Media, Reagents, sera, others

Material	Supplier	Catalogue Number
DMEM, high glucose, pyruvate	Fisher Scientific	11504496
Foetal Bovine Serum	South American, Fisher Scientific	S181L-500
Penicillin Streptomycin solution	Fisher Scientific	15070-063
DMSO	Biosolve	04470501/1
Trypan blue	Biorad	1450021
DULBECCO'S PHOSPHATE BUFFERED SALINE MODIFIED	Sigma Aldrich	D8537
Trypsin EDTA	In house	NA
Hoechst 33342, Trihydrochloride, Trihydrate	Life technologies	H1399
Propidium iodide =94.0% (HPLC)	Sigma Aldrich	P4170
Annexin V Alexa 633	In house	NA
Alternative to the one above:	Annexin V, Alexa Fluor™ 647 conjugate	Invitrogen™ A23204
CDDO-Me	Cayman Chem/Bio-Connect	Cay-11883-5
Tunicamycin	Sigma Aldrich	T7765
Etoposide	Sigma Aldrich	E1383-25MG
Distilled Water	Gibco/Thermo Fisher Scientific	15230147

PREPARATIONS

Media and Endpoint Assay Solutions

Medium/Solution	Preparation
Foetal Bovine Serum aliquots	Thawed, aliquoted into Sterile 50 plastic tubes, frozen again and stored at -20°C.
PenicillinStreptomycin aliquots	Thaw out Penicillin Streptomycin bottle, prepare aliquots of 2 ml in sterile 15 plastic tubes, frozen again and stored at -20°C.
Trypsin EDTA aliquots	Dissolved in a total volume of 1l in Milli-Q® Type 1 Ultrapure Water : 0.05 % (m/V) trypsin; 8 gr NaCl; 0.25 gr KCl; 1.43 gr Na ₂ HPO ₄ *2 H ₂ O; 0.25 gr KHP ₂ PO ₄ . pH set to 7.2 with 1M HCl Filtered with a 0.2 µm filter (sterile)
Hoechst 33342 aliquots	1mg/ml Hoechst 33342 in sterile distilled water, Filtered with a 0.2 µm filter (sterile)
Hoechst staining solution	200ng/ml in complete DMEM by diluting from Hoechst 33342 aliquot (1:5000).
Propidium iodide aliquots	Stock solution 100M in sterile distilled water, sterile filtered
Alternative to the one above:	Propidium iodide solution (1.0 mg/ml in water)
Propidium iodide staining solution	Sigma-Aldrich, P4864 Adapt the dilution factor in order to result in the same end concentration of 100 nM.
Annexin V Alexa 633 solution	200 nM in complete DMEM by diluting from Propidium iodide aliquot (1:500). Produced in house by E. Coli strain BL21-pLys pET-ASH and labelled with Alexa Fluor™ 633 Protein Labelling Kit (cat. A20170, Fisher Scientific). See also Puigvert <i>et al.</i> , 2010.
Annexin V Alexa 633 staining solution	Staining solution 0.1 % (V/V) stock solution in complete DMEM (1:2000).
Complete DMEM	Mix 450 ml of DMEM, 50 ml of FBS and 2 ml PenicillinStreptomycin solution (sterile). Store at 4°C. Medium does not need pre-warming before use.
Freezing medium	20% DMSO in FBS
Test compound stock solution	
DMSO stock	DMSO stocks are created by dissolving the provided test compound in in DMSO by vortexing or resuspending it with a pipette. The stock concentration has to be at least 1000 x higher than the highest testing concentration (=0.1% v/v of DMSO). (The weight provided by JRC was used for concentration calculations)

Compound stock solutions

Test compound stock solutions were prepared as described in the following table. All stock compounds solutions were stored in brown glass bottles at -80°C under light exclusion. Furthermore, stocks for usage were stored at -20°C under light exclusion.

Freeze thaw cycles were kept minimal. For replicates, different aliquots of the same stock solution were used. The stock solutions were diluted out with full DMEM. All treatments, excluding DMEM vehicle, contained 0.1% (V/V).

Compound	Solvent stock solution	Concentration stock solution [mM]	Concentration stock solution [g/l]	Highest testing concentration [µM]
Rotenone	DMSO	10		10
Deguelin	DMSO	10		10
Azoxystrobin	DMSO	10		10
Kresoxim-methyl	DMSO	10		10
paraclostrobin	DMSO	10		10
Picoxystrobin	DMSO	10		10
trifloxystrobin	DMSO	10		10

Compound	Testing concentration [μM]							
	A	B	C	D	E	F	G	H
ROTENONE								
DEGUELIN								
AZOXYSTROBIN								
KRESOXIM-METHYL								
PARACLOSTROBIN								
PICOXYSTROBIN								
TRIFLOXYSTROBIN								

Positive Control(s)

Positive controls should induce GFP expression. Therefore, positive controls differ per BAC-GFP reporter cell line.

HepG2 BAC-GFP reporter cell lines	Positive control compound	Concentration
SRXN1, HMOX1	CDDO-me	0.32 μM
BIP, CHOP	Tunicamycin	6 μM
BTG2, P21	Etoposide	25 μM

Negative Control(s)

Complete DMEM medium, with and without 0.1% DMSO.

Method

EXperimental System Procurement

Routine culture procedures

Cultureware	Cell culture maintenance vessels				Seeding densities *1000	
	Growth Area (cm ²)	Media Volume (ml)	Trypsin-EDTA Volume (mL)	PBS Volume (mL)	2 nights	3 nights
T25	25	5	1	5	2000	1300
T75	75	15	2	10	6000	4000
T175	175	30	4	20	12000	8000

Plate format	Imaging plates		
	Seeded cells per well for 24h of exposure	Seeded cells per well for 48h of exposure	Seeded cells per well for 72h of exposure
96 well plate	20000 in 100 μl	15000 in 100 μl	12000 in 100 μl
384 well plate	8000 in 30 μl	6000 in 30 μl	5000 in 30 μl

Thawing of Cryopreserved HepG2 BAC-GFP reporter cell lines

1. HepG2 reporter cell lines are stored at -80°C for short term storage and long storage at -150°C .
2. Cells are thawed in 37°C water bath and poured into 4 ml of complete DMEM.
3. After centrifugation (1000 rpm, 5 min, 4°C), the supernatant is replaced by fresh medium (total volume 5 ml).

4. Then the cells were carefully resuspended and transferred into a T25 tissue flask and moved in an incubator (37 °C, 5% CO₂ and 95% humidity).
5. After one further passaging step, the cells can be used in an assay.

Splitting of HepG2 BAC-GFP reporter cell lines

1. After removing the medium, cells are washed with PBS.
2. Then the cells are incubated with trypsin-EDTA for 5 minutes in an incubator (37 °C, 5% CO₂ and 95% humidity).
3. Before neutralising the trypsin- EDTA with complete DMEM, the cell suspension is forcefully resuspended 20 times with a 10 ml pipette (smallest opening) in order to achieve a single cell suspension.
4. Passage according to demands in according to table 'Cell culture maintenance vessels'.
5. HepG2 BAC-GFP reporter should not be passaged more than once per 48h.

Cell counting with TC20™ Automated Cell Counter

1. Under sterile condition remove 10µl of the cell suspension with a microliter pipette, (resuspend well before taking the sample).
2. Pipet the 10µl into a dual-chambered slide, avoid air bubbles.
3. Insert dual-chambered slide into TC20™ Automated Cell Counter.
4. Inspect bright field image and histogram of counted particles in order to assess the single cell suspension. Clumps can be removed with Celltrix® 50µm filter.

Cryopreservation HepG2 BAC-GFP reporter cell lines

1. Trypsinise cells as described earlier.
2. Add 800 µl cell suspension containing 2*10⁶ Cells into cryovials containing precooled (4°C) 800 µl freezing medium.
3. Transfer cells into precooled freezing rack.
4. Freeze at -80°C overnight.
5. Transfer for long term storage to -150°C or maintain on -80°C.

Test Material Exposure Procedures

Seeding HepG2 BAC-GFP reporter cell lines

1. Remove medium.
2. Wash with PBS (RT).
3. Add Trypsin EDTA and incubate at 37°C for 5 min.
4. Reduce cell clumps to single cell suspension by pipetting up and down, 20 times, while pressing the pipet's tip to the tissue flasks wall.
5. Determine cell number with TC20™ Automated Cell Counter (see above)
6. Dilute and seed out cells with multichannel pipet in multi-well imaging plates.

Consult table 'Imaging plates' for plating densities.

Added volumes	96 well plate	384 well plate
Hoechst staining solution	100µl	30µl
PI staining solution	50µl	25µl
Compound solution	50µl	25µl

1. Add Hoechst staining solution with multi-channel pipette.
2. Incubate 45 minutes
3. In the meantime: Prepare compound solutions (as mentioned under “test compounds”).
4. Aspirate medium containing Hoechst staining solution (3 to 5 rows at a time).
5. Add PI staining solution to each well with multi-channel pipette.
6. Add compounds in the desired concentration with multi-channel pipette.
7. If live imaging: Proceed to the microscope (incubator preheated)
8. If time point imaging: Place imaging plate in incubator until imaging time point arrives.

Endpoint Measurement

For the laser and filter setup consult Table 1 Fluorescence Microscope Channels. For live imaging, it is recommended to pre-expose one well with positive control in order to setup the EGFP imaging.

- Preheating incubator of the microscope to 37°C under saturated humidity in time before imaging.
- Hoechst 33342
 - Check in the Hoechst 33342 channel whether the whole plate’s cells are in focus.
 - Set intensity to a low enough level to still identify all nuclei, but as low as reasonably possible in order reduce oversaturation as far as possible.
- eGFP
 - Orientate setting of GFP reporter readout at the reporters respective positive control compound. Since this measurement ought to be quantified, do not oversaturate.
- Propidium iodide
 - Use a compound concentration, which induces cytotoxicity and one negative control to set up microscopic settings to differentiate between background and signal. For PI is an object identification foreseen to classify a cell as positive, therefore a clear visualisation of PI staining of cells is required.
- Annexin V Alexa 633
 - AnV staining has to be done with a drastically increased pinhole. Therefore, it is recommended to image it with Hoechst 33342 on a setting with increased pinhole. For example, after a plate-wide imagine run for Hoechst, GFP and PI, run another for Hoechst and AnV with increased (up to maximum) pinhole.

- Use a compound concentration, which induces cytotoxicity and one negative control to set up microscopic settings to differentiate between background and signal. For AnV is an object identification foreseen to classify a cell as positive, therefore a clear visualisation of AnV staining of cells is required.

Fluorescence microscopy:

Table 1. Fluorescence Microscope Channels

Fluorophore	Laser	Emission filter [nm]	Filter Range [nm]
Hoechst 33342	408 nm	450/50	425 - 475
eGFP	488 nm	525/50	500 -550
Propidium iodide	561 nm	595/50	570 -620
Annexin V Alexa 633	633 nm	700/75	663 - 738

ACCEPTANCE CRITERIA

The quality of the test is based on the cell death marker (PI) and the GFP intensity

A test is discarded when:

1. More than 15% of the cells are PI-positive in the solvent control (DMSO)
2. To determine if the control provides the expected result, we determine the robust Z score per plate.
3. To be able to do so you need at least 3 wells of your positive control (σ_p and μ_p) and of your negative control (σ_n and μ_n).
(σ = standard deviation and μ = mean)
The estimated Z-factor should be above 0.25.
4. At least 4 negative controls (complete DMEM), solvent control (complete DMEM + 0.1% (V/V) DMSO), and 4 positive controls should be taken along.

$$\text{Estimated Z-factor} = 1 - \frac{3(\hat{\sigma}_p + \hat{\sigma}_n)}{|\hat{\mu}_p - \hat{\mu}_n|}$$

Data Analysis

Disclaimer

The nuclei segmentation in cellprofiler relies on the watershed masked clustering developed by Kuan Yan (see Di *et al* 2012). The WMC might not be available to the public. It can be done alternatively via segmentation in imageJ. The segmented pictures can then be read into cellprofiler.

The R-scripts for the data readout out of the HDF5 file is not available publicly.

Image processing

The image format retrieved from the microscopy session is a ND2 file. This .ND2 file contains the images (well /channel/timepoint). Furthermore, it includes the technical information about the microscope composition and settings (laser settings, excitation/emission, detector settings, plate position, etc...). Since the ND2 files can reach a significant size, a fast data connection is recommended. At the Division of Drug Discovery and Safety, the data is retrieved by USB 3 compatible hard drives and an in-house server.

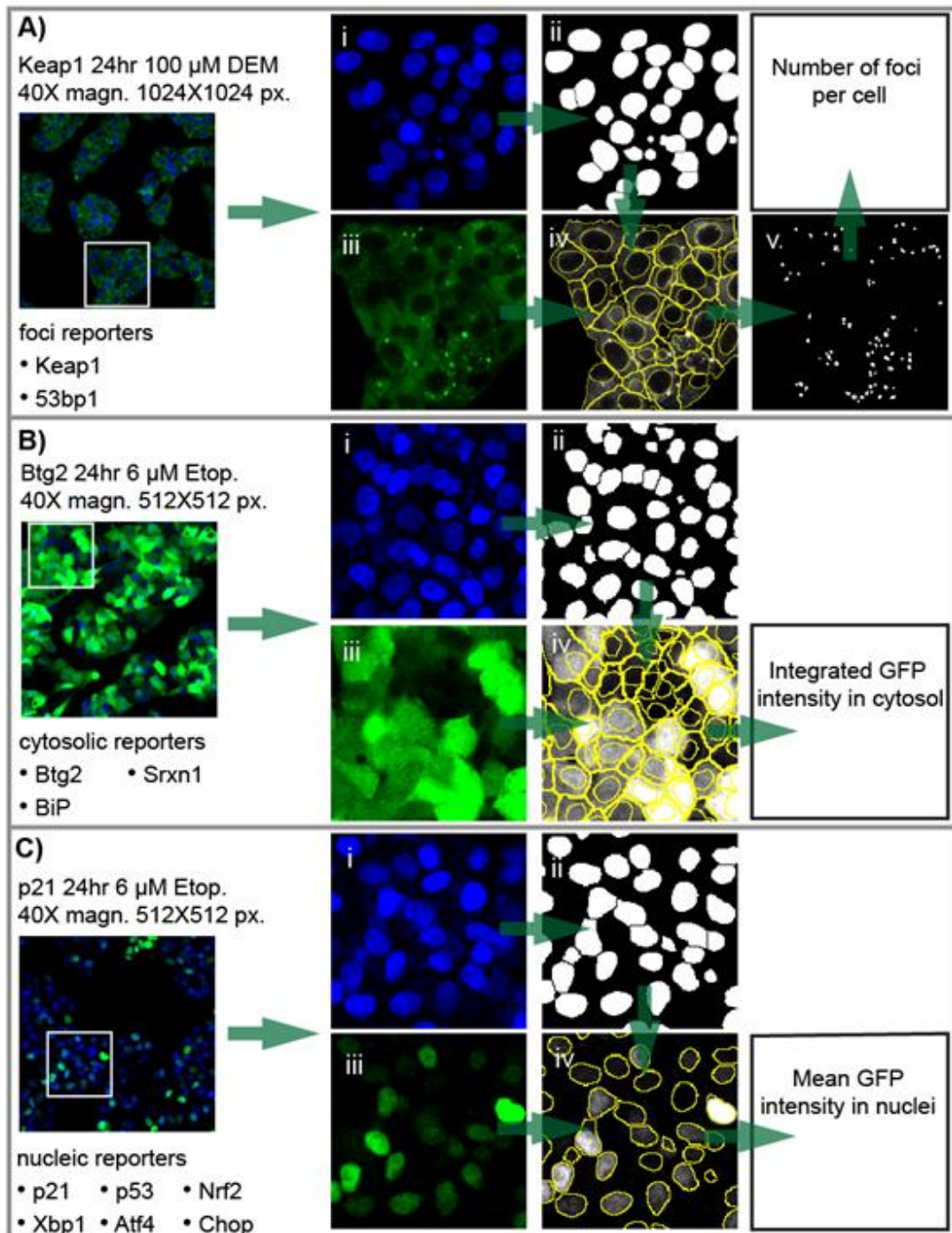
A program freely provided by Nikon (NIS-Elements Viewer) was used to export the .ND2 file to single .tiff images per well /channel/timepoint (xy_c_t).

The exported tiff files were then sorted into image location specific folders (i.e. B02_1, meaning well B02 first imaging location). These were used while reading in the images in cell profiler to associate the images location_ID.

Image analysis

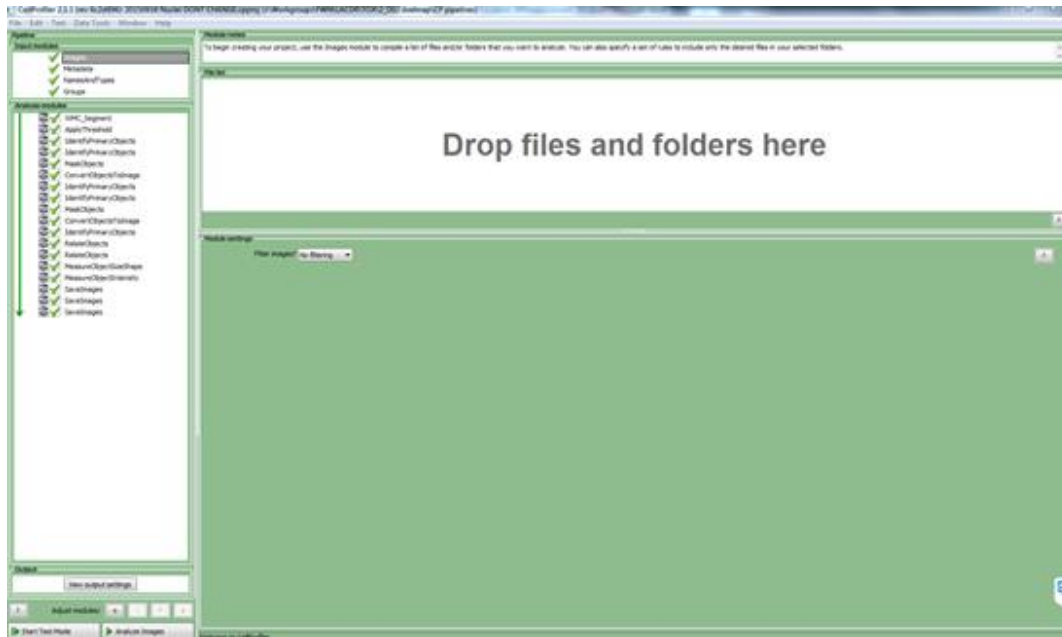
The structured folders with .tiff files are subsequently used for analysis in the free and open-source software program CellProfiler® (version 2.1.1). To be able to study the intensity and shape of objects the following pipeline was used for object identification:

1. Identification of the nucleus based on the Hoechst image
2. After segmentation of the nuclei, GFP intensity is determined in the nuclei. If the reporter protein is located in the cytoplasm, the cytoplasm is identified as a tertiary object based on the cell's nucleus and the intensity of the GFP channel picture.
3. Identification of PI object based on the PI image
If there is an overlap between the nucleus and the PI the cell is considered PI positive.

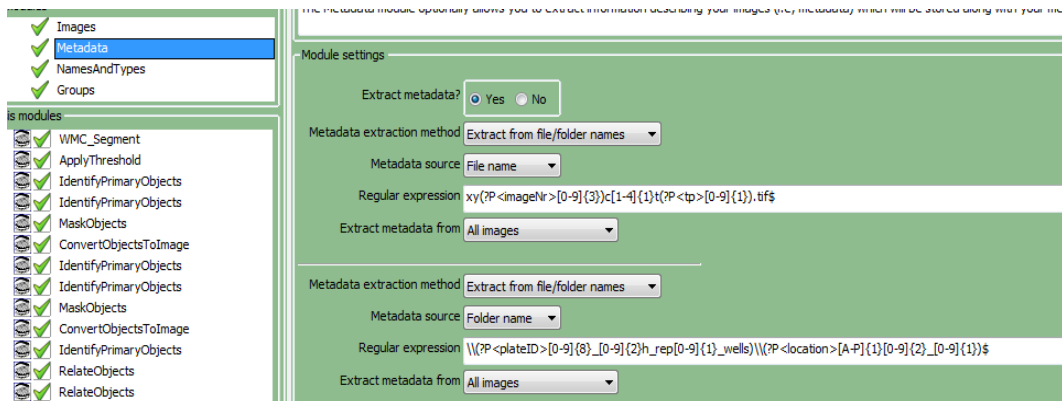
Figure 4. See also Wik *et al* 2014

1. Cell Profiler analysis > set Input modules

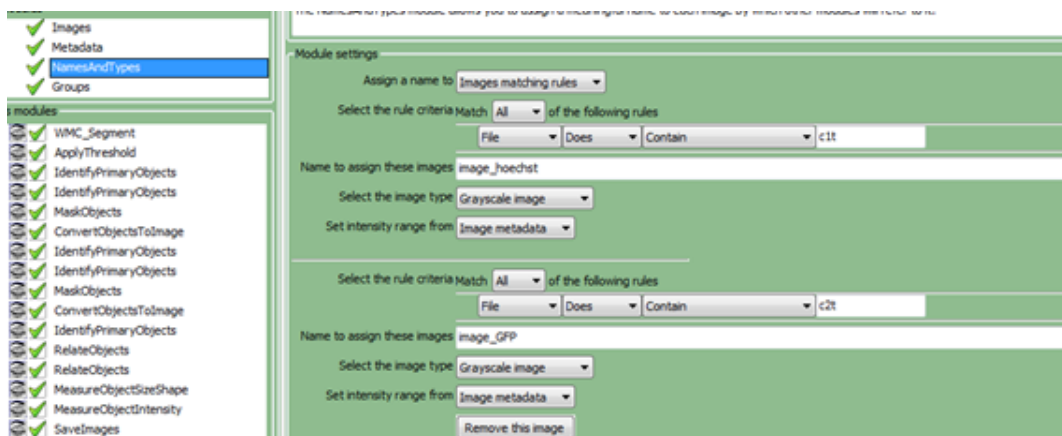
- Open CellProfiler (see what features need to be analysed in the figure above)
- Drag and drop images in CellProfiler:



- Set regular expressions in “metadata”. Add four variables: 1. plateID 2. imageNr 3. Timepoint 4. Well location. Click on update



- Set the different channels (which channel is Hoechst or GFP) in the “names and types” module. Click on update



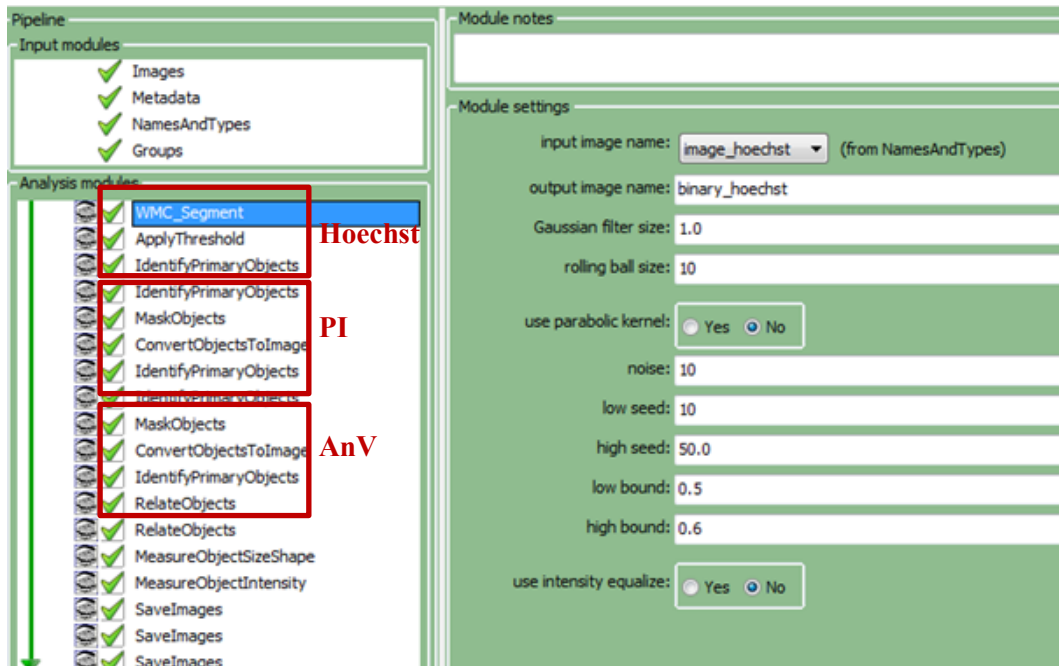
- OPTIONAL: set groups

2. Cell Profiler analysis > set Analysis modules

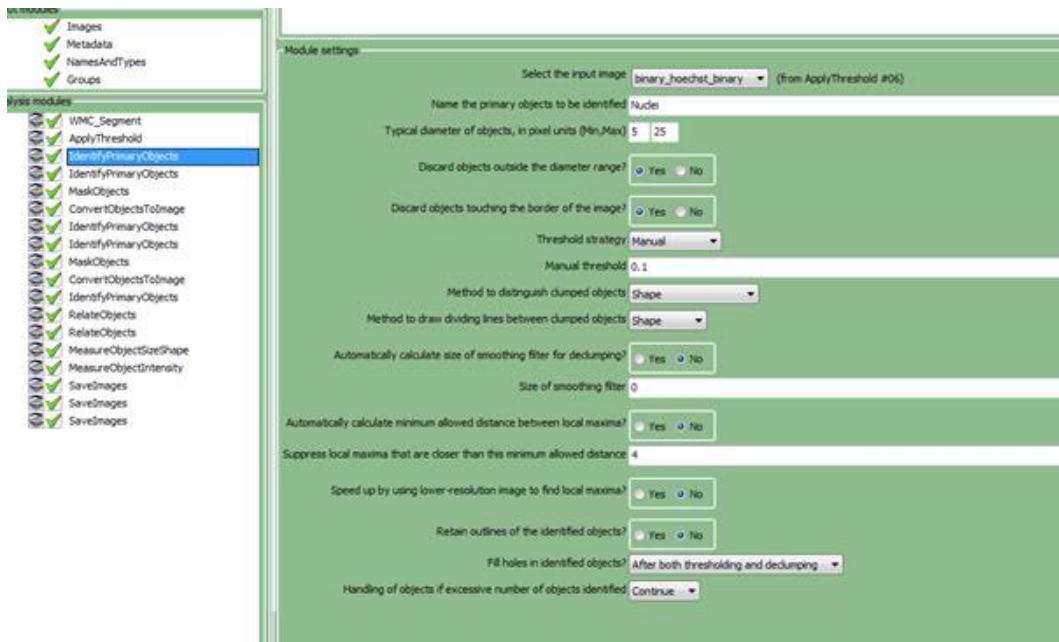
- Start test mode (bottom left corner)

Nucleus identification

- Input = Hoechst Channel
- Check the separation between foreground and background pixel in the Hoechst channel using the watershed masked clustering (WMC) ImageJ plugin.
- The WMC ImageJ plugin was developed by Kuan Yan (see Di *et al* 2012).

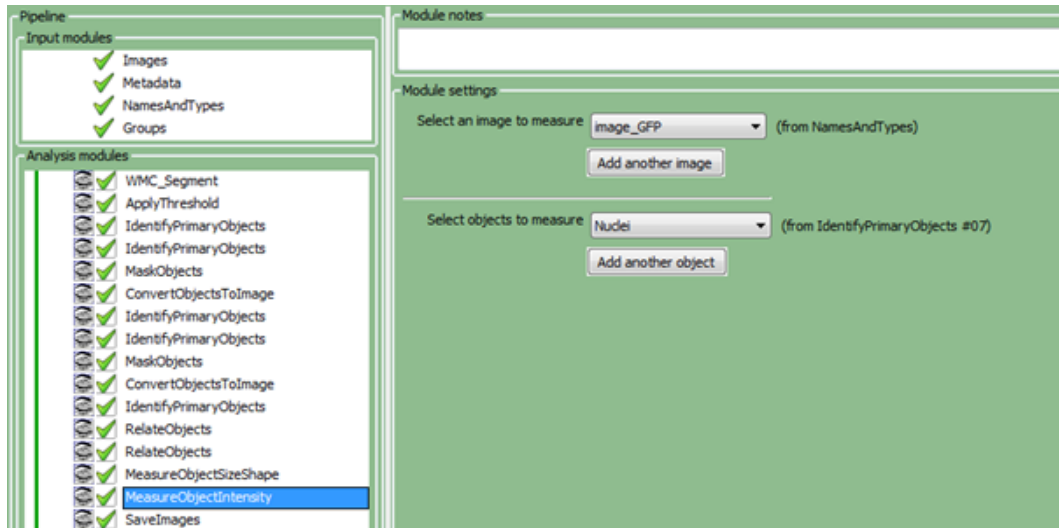


- Check the identification of the different objects in “Identify Primary Objects”

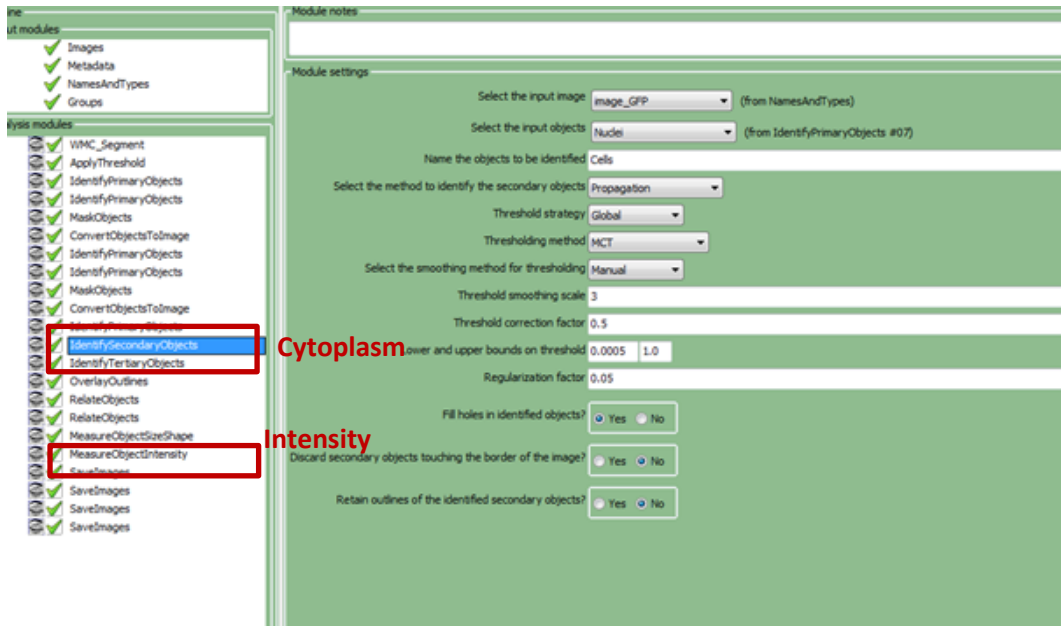


GFP signal identification > in the nucleus (p21, CHOP)

- Input = GFP channel
- For monitoring of GFP intensity in the nucleus, you do not need to create extra objects
- MeasureObjectIntensity => Measure GFP intensity in the earlier identified Hoechst-based nuclear objects

*GFP signal identification > in the cytoplasm (Srxn1, BiP, Btg2, HMOX1)*

- Input = GFP channel
- When identification of the cytoplasm is desired (for: Srxn1, BiP, Btg2, HMOX1), check the identification of the cytoplasm using the “Identify Secondary Objects” module
- The “Identify Tertiary Objects” is an object created by subtracting the “Identify Primary Objects” from the “Identify Secondary Objects”.
- MeasureObjectIntensity => Measure GFP intensity in the identified Tertiary Object (Cytoplasm)

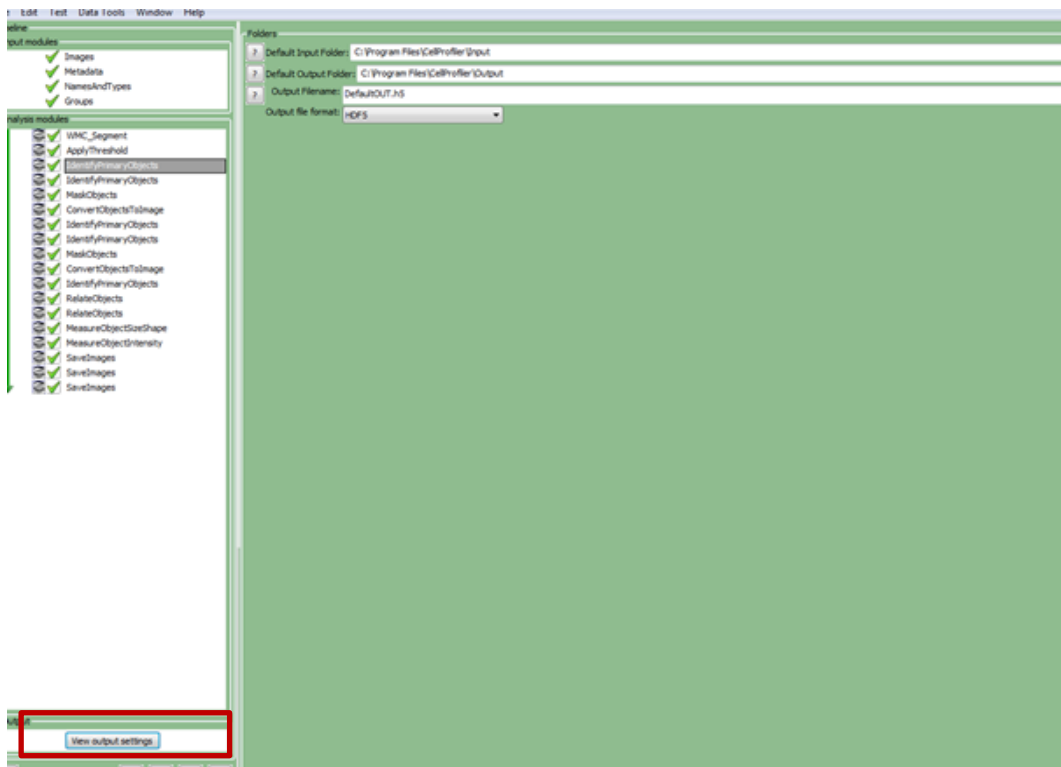


PI and AnV staining

- Input = PI/AnV image
- See SOP: PI-AnV staining for the analysis

3. Cell Profiler analysis > set Output

- Update “view output settings” by updating the input and output folders



- Start CellProfiler analysis. The output of the CellProfiler run is a HDF5 file with all measured parameters and when asked for folders with segmentation images per created .tiff.
- Reading out data from an HDF5 can be challenging. The summary data per image can be read out as an .csv file as well. This won't allow single cell data read outs.

Data collection

The output files of the CellProfiler® analysis are the segmentation images (.png) to make a manual take of the segmentation possible. Furthermore the measured objects (nuclei, pi and AnV objects), and intensities are saved in an .HDF5 file.

An R-script assembled in house is used to extract the features of interest for the researcher. Based on the packages: rhdf5, stringr, plyr, data.table, doParallel, ggplot2, reshape2, grid, shiny and ggvis.

Within a graphical user interface, the desired readouts for further analysis were picked:

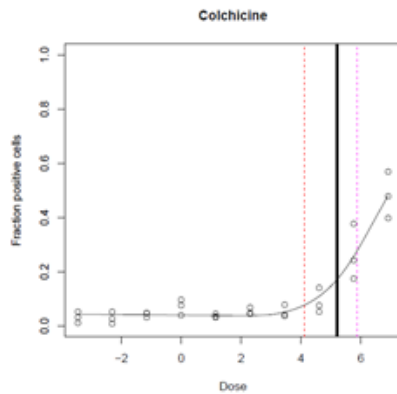
- Nuclei number (imageCountparentObj)
- Mean GFP intensity for reporter with GFP signal in the nucleus (Nuclei_Intensity_MeanIntensity_image_GFP)
- Integrated GFP intensity for reporter with GFP signal in the cytoplasm. (Nuclei_Intensity_IntegratedIntensity_image_GFP)
- Fraction GFP positive cells (countGFPpos, amount of cells with an GFP intensity more than two times DMSO control).
- Fraction PI positive cells (count_PI_masked_primaryID_AreaShape_Area.DIV.Nuclei_AreaShape_Area_larger_0.1_)
- Annexin V positive cells

Dose response analysis

The dose response modelling is based on a four parametric log logistic function. A house internal R script created by Steven Wink (s.wink@lacdr.leidenuniv.nl) was used to derive the effect concentration values. The script includes the R packages DRC, reshape2, magic and ggplot2.

Point of Departure (PoD) modelling

The PoD modelling is based on a house internal R script created by Steven Wink (s.wink@lacdr.leidenuniv.nl) was used to derive the effect concentration values. The script includes the R packages splines and ggplot2. The PoD is calculated based on a non-mechanistic regression analysis. A line is fitted through the data and based on the variance of the data points a curve is fitted and upper and lower intervals are calculated.



	SRXN1			
	24h			
treatment	Count GFP positive			
	PoD_est	PoD_L	PoD_U	
Colchicine	181.84	60.63577	353.5558	Concentration uM
Colchicine	3.74	4.217271	3.451542	$-\text{LOG}(M)$
line in graph	black	red	magenta	

Prediction Model

Rationale of your test method (= scientific purpose)

This HepG2 GFP reporter panel test system enables us to visualise and quantify the induction of various adaptive cellular stress responses in a systematic approach. This panel includes specific GFP reporters for the oxidative stress, ER stress, inflammation, DNA damage or heat shock response; heavy metal stress and hypoxia. For every stress response, an upstream regulator, transcription factor and target gene has been tagged in order to follow the activation of the whole signalling cascade. By combining these reporters with high content confocal imaging, perturbations of specific stress response signalling cascades by chemicals can be observed in a high-throughput manner including time dynamics of each key event. On single cell level, the induction and localisation of GFP can be observed also revealing the heterogeneity between cells in stress response induction. In this way, this test system can reveal the molecular mechanisms of toxicity. By combining these reporter with PI staining, the activation of these stress pathways can be related to induction of cell death to reveal the tipping point at which the activation of adaptive pathways are insufficient to overcome stress.

Prediction model

This cellular system can be used to predict to what extent, in which timeframe and at which concentration a specific compound induces a stress response and or will be cytotoxic.

Stress response activation

Protein expression and/or translocation of stress response pathway's proteins display the onset, extent and dynamic of pathway activation. This can be described with the following readouts

The fraction of cells which are higher than two times the average GFP-level of cells exposed to DMSO are considered GFP positive. When a compound induces the number of GFP positive cells with 20% it is being considered as a stress response activator.

Cytotoxicity

The threshold of cytotoxicity is determined by dose response modelling or point of departure modelling of the PI and AnV readout.

Prediction model setup

The options for the use of this cell system as a prediction model are under investigation.

Dose response modelling can be done via BMDEExpress.

IVIVE In vitro – in vivo extrapolation

The BAC GFP-reporter cell system can be linked to *in vivo* data by comparing the data with rat liver and hepatocytes gene expression microarray data from TG-GATEs (Igarashi Y *et al*, Nucleic Acids Res. 2015). Up or downregulation of similar networks can be verified and support the translation to the *in vivo* situation. Models for the *in vitro* kinetics will be developed soon but don't exist so far. Up until now, the test system has not been used for accurate IVIVE determination.

Applicability domain

The applicability of this testing system will be a first tier high throughput testing screen for the identification for the test compounds ability to induce the respective cellular stress response.

Incorporation in test battery

The HepG2 GFP reporter cell line can be used in combination with the other reporter cell line for comprehensive testing of a broad range of stress response pathway activation.

Strengths	Weaknesses
1. High-content and –throughput	1. Correct handling is crucial for HepG2 cells
2. Live time dynamics	2. Lack of metabolic capacity in HepG2 cell system
3. Identification of AOP while cell death measures can be observed	3. The need of a powerful confocal microscopy system including heater and CO ₂ for live cell imaging
4. Stand-alone test system	
5. Single cell measurements	
6. Protein based reporter with endogenous regulation	

Annexes

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Annex 1.13. Neurite degeneration measurements in differentiated SH-Sy5Y cells after acute exposure (24 hours) to chemicals

Method	SH-SY5Y: neurite degeneration
Contact	Anna Forsby (Swetox) -> anna.forsby@swetox.se

Standard Operating Procedure (SOP)

1. PURPOSE

The SOP describes how neurite degeneration can be determined by high content fluorescence microscope imaging using calcein AM in combination with a cell viability assay, using propidium iodide exclusion as marker.

2. SCOPE

The cell permeant dye Calcein-AM was used to stain the cytoplasm of differentiated cells allowing the visualisation and quantification of the neurite length. Once inside the cells, calcein-AM is hydrolysed by intracellular esterases producing a green fluorescent dye. Viable cells are visualised under the fluorescence microscope by exclusion of propidium iodide (PI).

3. INTRODUCTION

Neurite degeneration initiated at non-cytotoxic concentrations *in vitro* is an alert for neurotoxicity directed to pathologies in axons and dendrites *in vivo*. Herein, we examine neurite degeneration and cell viability in 6 days differentiated human neuroblastoma SH-SY5Y cells after 24h exposure with chemicals.

4. RESPONSIBILITY

All personnel performing this assay should follow this SOP.

5. PROCEDURES

LIMITATIONS

To be added

METHOD OUTLINE

We used the cell permeant dye calcein AM to stain the cells' cytoplasm and the dye Hoechst for nuclear staining.

For experiments, SH-SY5Y cells are plated in 96-well plates with micro-clear bottom (8 000 cells/well) in EMEM with 10% foetal bovine serum, 2 mM glutamine, 1% non-essential amino acids, 100g streptomycin/mL and 100 U penicillin/mL, which is exchanged to differentiation medium with 1 µM RA and without serum (DMEM:F12 [1:1], N2 supplements [diluted 1:100], 1 mM glutamine, 100g streptomycin/mL and 100 U

penicillin/mL) after 24 h. Six (6) days differentiated SH-SY5Y cells are exposed to test chemicals and incubated for 24 hours after which the effect on neurite degeneration is measured by fluorescent microscopy.

DEFINITIONS/ABBREVIATIONS

The following abbreviations are used:

DMEM/F12	Dulbecco's modified medium and Ham's F12 medium (1:1)
DMSO	Dimethyl sulfoxide
EMEM	Minimum essential medium with Earle's salts
RA	All-trans retinoic acid
FBS	Foetal bovine serum
PEST	Penicillin Streptomycin
NEAA	Non-essential amino acids

IDENTIFICATION OF TEST AND CONTROL SUBSTANCES

A. Test Chemicals

The cells were pre-exposed to test chemicals before the neurite degeneration measurements, simulating acute exposure.

Dilutions:

An initial screening was performed by testing all chemicals at 10 μ M, 2 μ M and 0.4 μ M. For those chemicals showing effect at the lowest tested concentration during the screening, extra 5X dilution steps were performed until a no effect concentration was reached. If no effect was observed during the screening, the experiment was repeated only a second time.

Table 1. A typical pipetting scheme for preparation of the dilution series for test chemicals dissolved in DMSO.

Dilution step	Conc. In ep-tube (μ M)	Test chemical (μ l)	Medium + 0.2% DMSO (μ l)	Conc. In wells (mM)
D1	2,00E+01	2	998	1,00E+01
D2	4,00E+00	200 of D1	800	2,00E+00
D3	8,00E-01	200 of D2	800	4,00E-01
D4	1,60E-01	200 of D3	800	8,00E-02
D5	3,20E-02	200 of D4	800	1,60E-02
D6	6,40E-03	200 of D5	800	3,20E-03
D7	1,28E-03	200 of D6	800	6,40E-04
D8	2,56E-04	200 of D7	800	1,28E-04
Controls	0	0	1000	0

B. Controls

- a. **Negative control:** Cells exposed to N2-RA with DMSO 0.1%.
- b. **Positive control:** Rotenone 10 μ M.

MATERIALS

A. Cell Culture Type

Human neuroblastoma SH-SY5Y cell line is cultured and differentiated as described in **SOP plating and differentiation**.

B. Technical Equipment

- a. ImageXpress® Micro (Molecular Devices, UK)
- b. MetaXpress® Software (Molecular Devices, UK)
- c. Calcein (Life technologies, Cat no C1430)
- d. Hoechst 10 mL (ThermoFisher, cat no H3570), 10mg/mL.
- e. Black cell culture microplate, 96-well plate with microclear bottom (Greiner, No 655090)

METHOD:

A. Preparation of Calcein stocks

1. Dilute the calcein powder (1mg) in 1 mL 100% DMSO
2. Warm up the diluted calcein to ensure proper dilution
3. Prepare 20 µL Aliquots
4. Store at 4 °C

B. Exposure

For experiments, 8 000 SH-SY5Y cells are plated in black microclear bottom 96-well plates in complete EMEM with 10% foetal bovine serum, which is exchanged to N2-RA differentiation medium after 24 h. After 3 days of differentiation 100 µL of N2-RA differentiation medium are added on top of all wells, the plate is then placed in the incubator and the cells are allowed to differentiate for 3 days more (total differentiation time of 6 days). On the 6 day of differentiation 100 µL of cell medium are removed from the wells containing cells and replaced with 100µL of the assigned treatment.

Figure 1. Plate lay out for screening of CS4 test chemicals. Six days differentiated SH-SY5Y are exposed to 10 μ M (D1), 2 μ M (D2) and 0.4 μ M (D3) in an initial screening.

Plate 1:												
	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		DMSO 0,1%				Co6 D1			Co11 D1		Rotenone 10 μ M	
C		Co1 D1				Co7 D1			Co12 D1		Rotenone 10 μ M	
D		Co2 D1				Co8 D1		DMSO 0,1%		Rotenone 10 μ M		
E		Co3 D1				Co9 D1			Co13 D1		Rotenone 10 μ M	
F		Co4 D1				Co10 D1			Co14 D1		Rotenone 10 μ M	
G		Co5 D1			DMSO 0,1%			DMSO 0,1%			Rotenone 10 μ M	
H												

Plate 2:												
	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		DMSO 0,1%				Co6 D2			Co11 D2		Rotenone 10 μ M	
C		Co1 D2				Co7 D2			Co12 D2		Rotenone 10 μ M	
D		Co2 D2				Co8 D2		DMSO 0,1%		Rotenone 10 μ M		
E		Co3 D2				Co9 D2			Co13 D2		Rotenone 10 μ M	
F		Co4 D2				Co10 D2			Co14 D2		Rotenone 10 μ M	
G		Co5 D2			DMSO 0,1%			DMSO 0,1%			Rotenone 10 μ M	
H												

Plate 3:												
	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		DMSO 0,1%				Co6 D3			Co11 D3		Rotenone 10 μ M	
C		Co1 D3				Co7 D3			Co12 D3		Rotenone 10 μ M	
D		Co2 D3				Co8 D3		DMSO 0,1%		Rotenone 10 μ M		
E		Co3 D3				Co9 D3			Co13 D3		Rotenone 10 μ M	
F		Co4 D3				Co10 D3			Co14 D3		Rotenone 10 μ M	
G		Co5 D3			DMSO 0,1%			DMSO 0,1%			Rotenone 10 μ M	
H												

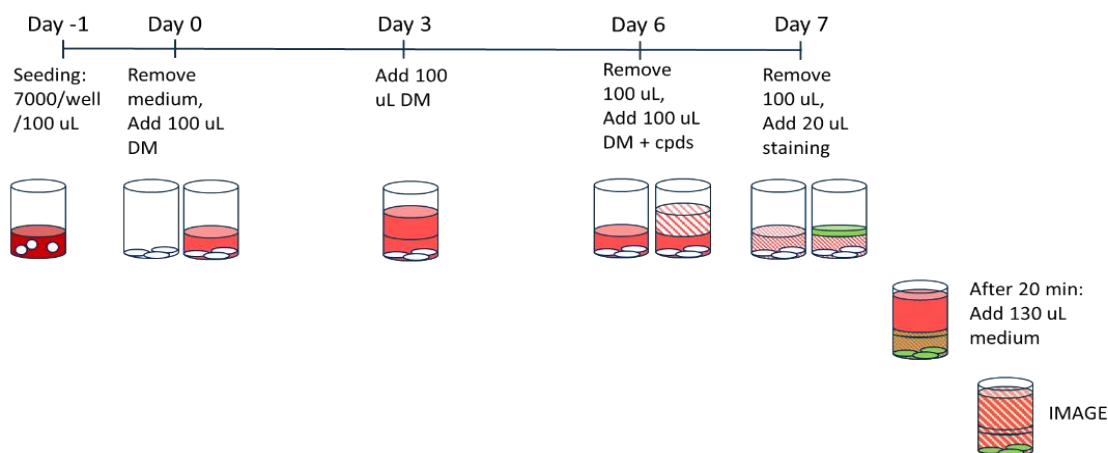
C. Neurite staining

The measurement of neurite degeneration is performed 24 hours after exposure to test chemicals

1. Be very careful not to keep plates out of incubator for longer time than necessary, and be extremely gentle with pipetting
2. Take one plate at a time out, carefully remove 100 μ L of medium from the wells
3. Put the plate back in the incubator as fast as possible, do not let it cool down
4. Prepare and warm up 22 mL of DMEM:F12 medium for later dilution of the staining
5. Ultrasonicate Hoechst-H33342 stock solution in ultrasonic water bath for 15 min
6. Prepare dye solution (enough for two plates):
 - a. 4 mL of DMEM/F-12
 - b. 12 μ L Hoechst 33342 stock 10 mg/mL (16,2 mM) [final conc: 5 μ g/mL]
 - c. 24 μ L Calcein-acetoxymethylester (CAM) stock of 1 mg/mL (1 mM) [final conc: 1 μ M]
 - d. Vortex

- e. Protect from direct light (switch off light in the sterile hood)
7. Put dye solution into a reservoir
8. Add 20 μL of dye solution to all wells in one plate, quickly but gently pipet up and down twice in each row (without changing tips for the same treatment)
9. Incubate the plate for 20 min at 37°C, 5% CO₂
10. Put 11 mL of warm DMEM/F-12 into a reservoir
11. Take the plate out
12. With a 12-channel pipette, quickly but gently, add 130 μL of DMEM:F12 in each well
13. Immediately image the plate (calcein in FITC channel, H33342 in DAPI channel, 4 sites/well)

Figure 2. Overview of the experimental set up used for imaging of neurite degeneration on six days differentiated SH-SY5Y cells.

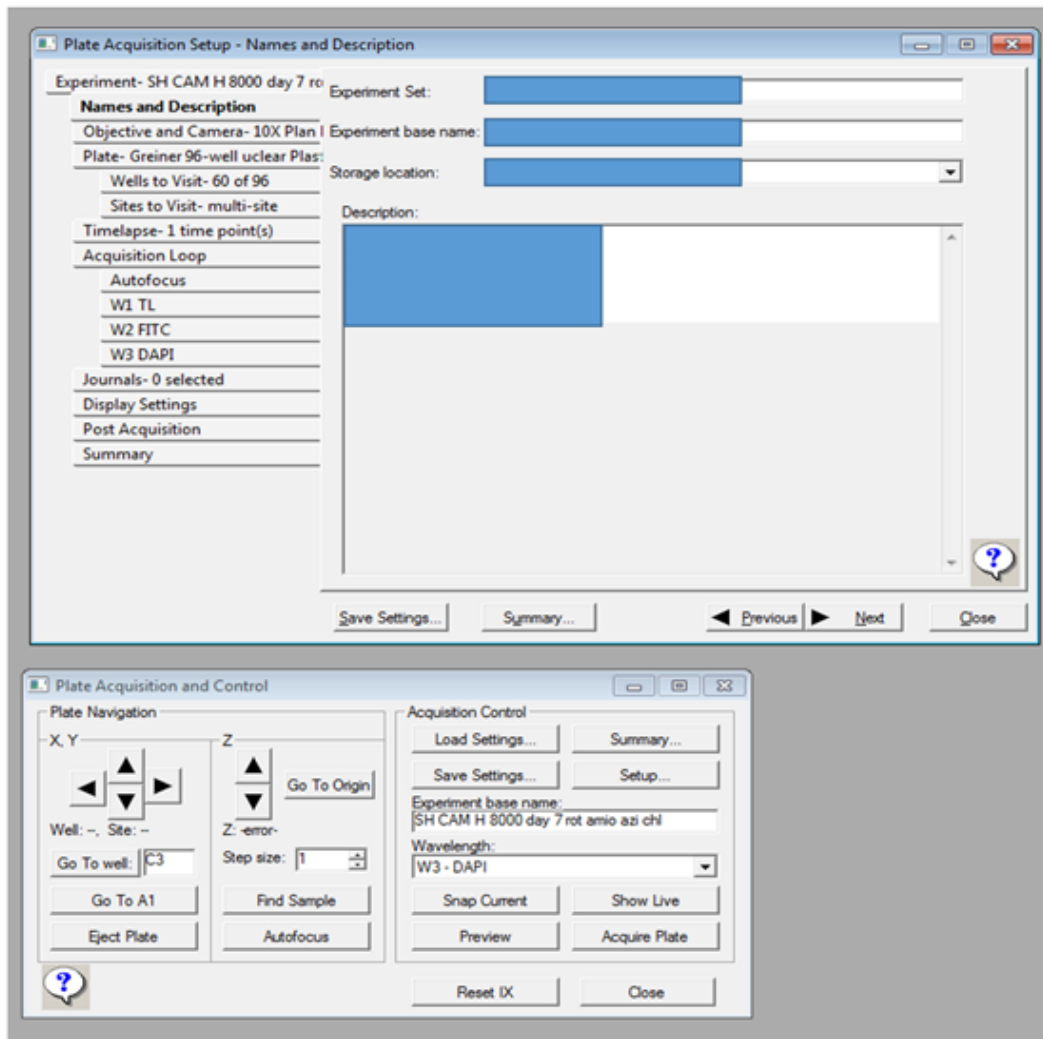


D. Neurite degeneration imaging

ImageXpress® Micro (Molecular Devices, UK) is used to obtain images of the exposed cells. Four images per well are taken with three channels: Transmitted light (TL, yellow arrow), Hoechst in DAPI channel (nuclear staining, blue arrow), and calcein in FITC Channel (cytoplasm/neurite staining, green arrow).

1. Turn on the machine 30 minutes before starting the measurements.
2. Open MetaXpress®
3. On the top menu of the MetaXpress® software, select screening → plate acquisition set up.
4. On the top menu of the MetaXpress® software, select screening → plate acquisition and control.
5. Once the plate acquisition set up and plate acquisition and control windows are opened, start by writing the name of the experiment and a description under the “Names and description” tab (see below, Figure 3).

Figure 3. "Names and description tab"

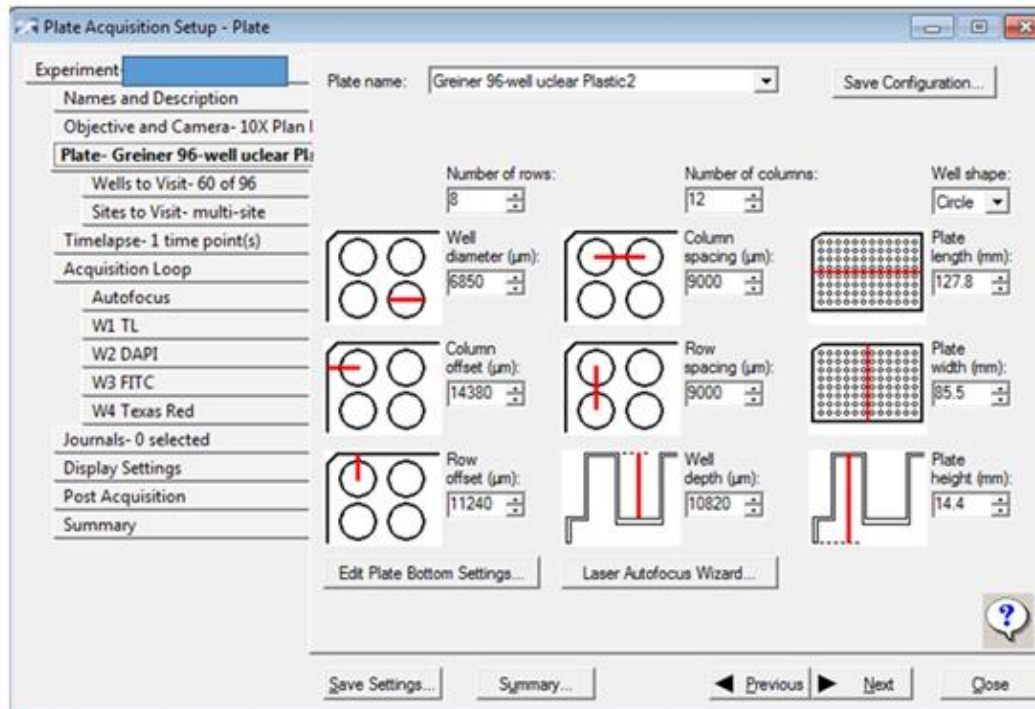


6. Under the “objective and camera” tab, select a 10X magnification, camera binning 1 and gain 1. These settings should only be modified by the instrument’s responsible person or technician.
7. Under the “Plate” tab, use the settings optimised for 96 well black microclear bottom cell culture plates (Greiner, No 655090). Figure 4 contains a description of the settings used for 96 well plates in this instrument, these settings should only be modified by the instrument’s responsible person or technician.

[Note: different batches of plates can have differences in the well depth within the same plate, this will affect the sharpness of the image taken during the testing of the settings but it will not affect the final images]

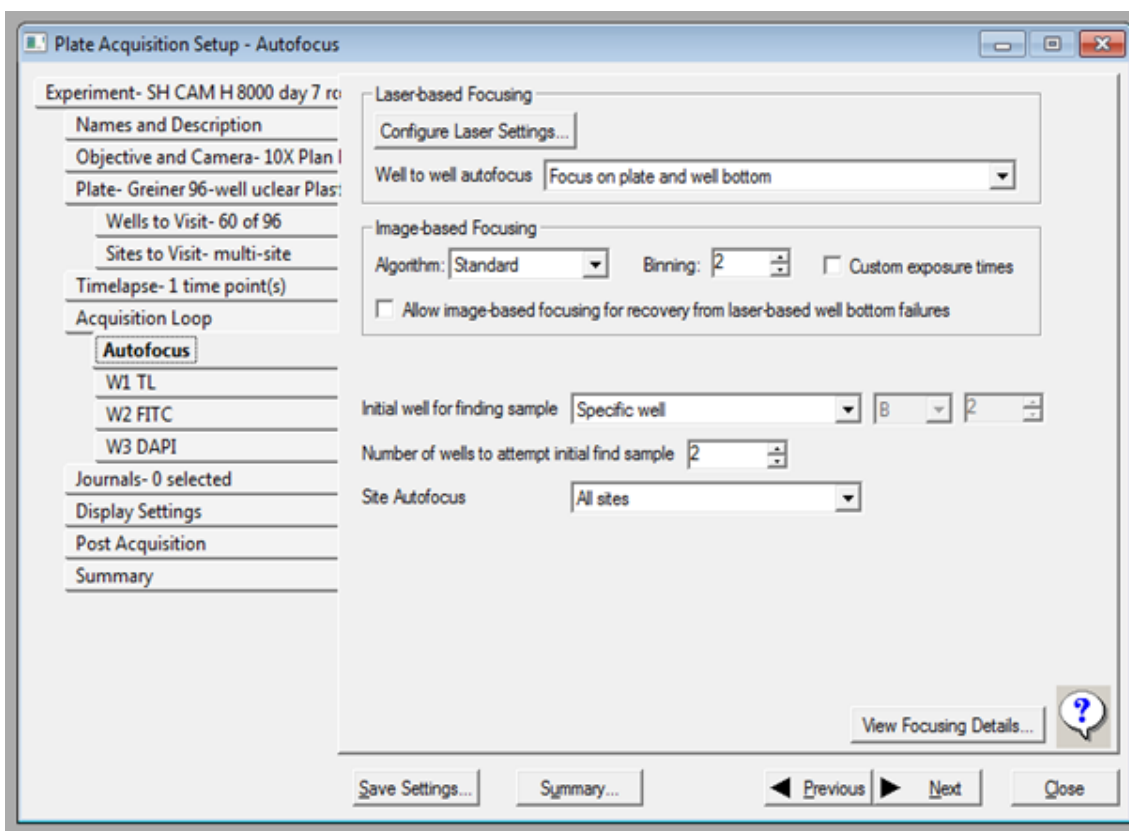
Figure 4. "Plate" tab including the setting that have been standardised for measurements in 96 well plates.

These settings should only be modified by the person responsible for the instrument.



8. Under the "wells to visit" tab select the 60 innermost wells for analysis. Make sure that the selected wells are showed in a green colour.
9. Under the "sites to visit" tab select 2 columns and 2 rows to obtain 4 images/well.
10. Under the "timelapse" tab select number of endpoints = 1.
11. Under the "autofocus" tab (see Figure 5) select:
 - a. Well to well autofocus: focus on plate and well bottom.
 - b. Imaged based focusing:
 - i. Algorithm: standard
 - ii. Binning: 2
 - c. Initial well for finding sample: specific well
 - d. Site autofocus: all sites.

Figure 5. “Autofocus” tab with the settings used for measurements in 96 well plates.



12. Under the tabs W1 to W3 the setting for image acquisition are specified:

Table 2. Settings used for the acquisition of images with MetaXpress®.

The specific exposure time, target maximum intensity and Z- offset set for each of the three wavelengths used to measure neurite degeneration in differentiated SH-SY5Y cells are described.

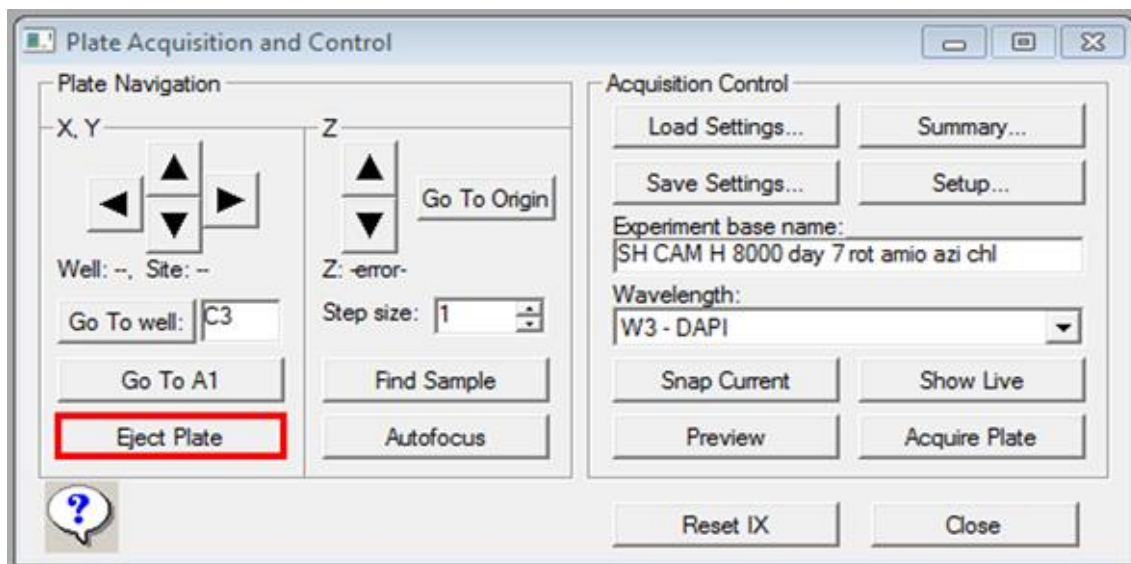
illumination setting	TL	DAPI	FITC
Exposure time (ms)	8	100	120
Target max intensity	16960	33000	34464
Z-offset (µm)	8.96	2.92	10.44
Calculate Offset (use Z stack and Custom range)	Range: 50 µm Step: 1.5 µm	Range: 50 µm Step: 1.5 µm	Range: 50 µm Step: 1.5 µm

13. Insert the plate in the imager (see Figure 6)

- a. In the plate acquisition and control menu choose eject plate
- b. Place the plate inside the imager
- c. In the plate acquisition and control menu choose load plate

Figure 6. Plate acquisition and control window.

To insert the plate in the imager press “eject plate”.



14. With the mouse, right click button select one of the 4 sites and one control well, select the DAPI channel to evaluate the nuclear staining with Hoechst. To be able to see the cells click on “find sample” in the plate acquisition and control window.
15. With the mouse right click button select one of the 4 sites and one control well, select the FITC channel to evaluate the calcein staining. To be able to see the cells click on “find sample” in the plate acquisition and control window.
16. Click under the “summary” tab → acquire plate.
17. All obtained images are automatically stored in MetaXpress® in the D disk of the computer. The SOP “procedure for backing up old experiments from the imager” describes the steps to follow in order to create back up files.

E. Image analysis

1. Open MetaXpress®
2. On the top menu of the MetaXpress® software, select screening → Review plate data [DB].
3. Select the plate for analysis.
4. Select all the wells to be analysed.
5. On the “Run analysis” tab select:
 - a. Analysis: <Neurite Outgrowth>
6. Settings: Press configure settings, and select:
 - a. Neurite image: FITC
 - b. Illumination: Fluorescence
 - c. Cell bodies:
 - i. Approximate max width: 75 μm = 115 pixels

- ii. Intensity above local background: 100 graylevels
 - iii. Minimum area: $90 \mu\text{m}^2 = 213$ pixels
 - d. Nuclear stain:
 - i. Nuclear image: DAPI
 - ii. Approximate min width: $5 \mu\text{m} = 8$ pixels
 - iii. Approximate max width: $13 \mu\text{m} = 20$ pixels
 - iv. Intensity above local background: 80 graylevels
 - e. Outgrowths:
 - i. Maximum width: $2 \mu\text{m} = 3$ pixels
 - ii. Intensity above local background: 15 graylevels
 - iii. Minimum cell growth to log as significant: $15 \mu\text{m} = 23$ pixels
7. In the configure settings window press “test run” to visualise the correct identification of cell bodies and outgrowths in the software (Figure 7 and 8)

Figure 7. ”configure settings tab”.

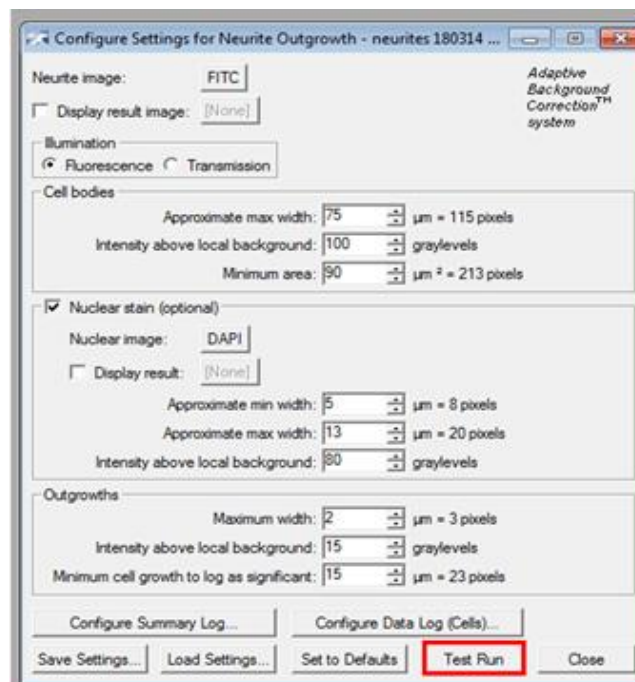
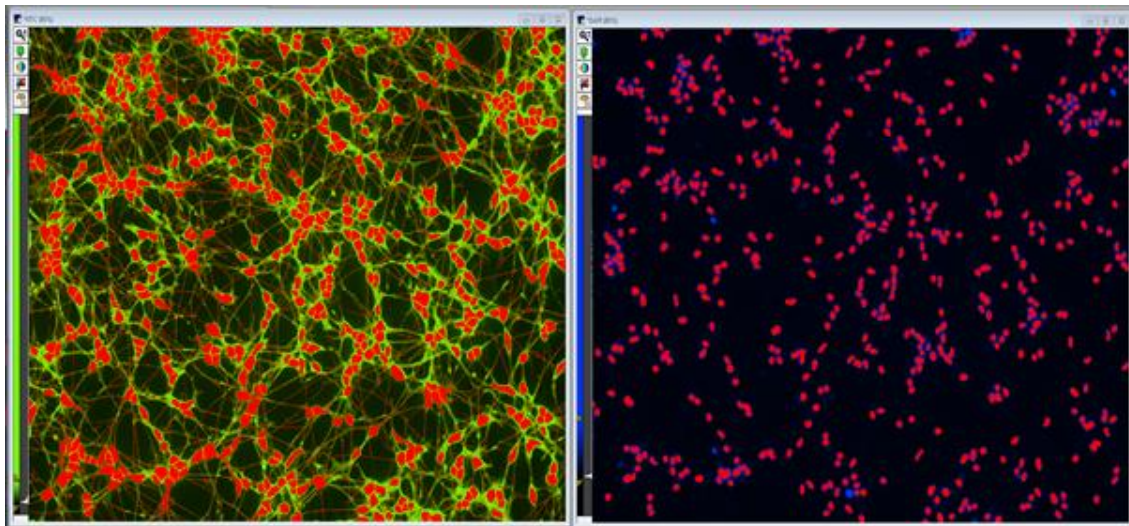


Figure 8. Analysis of neurite length in differentiated SH-SY5Y cells.

Left: staining with calcein in FITC channel, Right: nuclear staining with Hoechst in DAPI channel. Live cells are recognised as structures with both calcein and Hoechst staining.



Note: variations in the intensity of the staining can lead to inappropriate identification of cells bodies and/or neurites. If needed, the settings in the “configure settings” tab should be modified to assure proper identification of most of the imaged structures.

8. Analyse the plate by pressing “run analysis” on the “Review plate data” window.
9. After analysis, the data can be exported to an excel file.
10. Store the data in the S-disk: S:\Projects\PHC33_EUToxRisk\Case study 4_Mitotox\Neurite degeneration\Compilation of results 24h.
11. Microsoft Excel is used to analyse the data.

F. Data analysis

1. The differences in the mean process length (cell: mean process length) used for establishing the effect of the test compounds on the neurites (neurite degeneration).
2. Normalise the mean process length value of each well against the mean of all negative controls:

$$ND (\%) = \frac{\text{Mean process length exposed cells}}{\text{Mean process length negative controls}} * 100\%$$

3. Take the mean of the effect in the three technical replicates of each concentration.
4. Plot the values in GraphPad Prism.

HEALTH SAFETY AND ENVIRONMENT

Cell culture procedures take place in a laminar air flow cabinet, class 2. All work with test chemicals is performed in a fume hood. Experiments are planned in order to reduce the exposure time of the laboratory personnel to the test chemical. Wastes of environmentally hazardous compounds are handled according to local regulations.

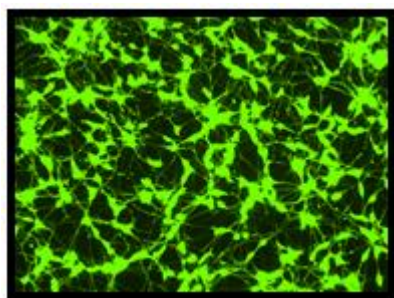
X. REFERENCES

To be added

XI. APPENDIX

Summary of the procedure for measurement of neurite **degeneration** in differentiated SH-SY5Y cells:

Day 1	Plating of SH-SY5Y cells, 8 000 cells/well.
Day 2	Start differentiation. Change complete EMEM to N2-RA medium.
Day 3	Addition of fresh N2-RA differentiation medium Add 100 μ L N2-RA to all wells
Day 6	Exposure to test chemicals. Remove 100 μ L N2-RA medium. Add 100 μ L of the corresponding chemical dilution.
Day 7	Neurite degeneration imaging Remove 100 μ L of supernatant. Add 20 μ L/well of Calcein + Hoechst staining solution. Incubate the plate for 20 minutes at 37 °C. Add 130 μ L DMEM:F12. Image the plate (Calcein in FITC channel, H33342 in DAPI channel, 4 sites/well)



Annex 1.14. NeuriTox/UKN4 assay in galactose conditions

Method	LUHMES: Neuron outgrowth in galactose medium
Contact	Johannes Delp (UKN) -> johannes.delp@uni-konstanz.de

Introduction

Aim

This protocol describes the analysis of how to assess the impairment of neurite outgrowth of live LUHMES cells (human neuronal) in galactose conditions, which make the cells more dependent on mitochondrial metabolism.

Purpose

In standard cell culture conditions, cells can use only glycolysis to meet their metabolic needs since glucose is highly abundant (e.g. 18 mM). Additionally, many cells don't have energetic requirements except from proliferation. Our biological model system are non-proliferating neurons, so they have low energy needs and don't need to be electrically active, thus they can afford to use mainly glycolysis. Galactose metabolism yields the same energy per molecule as glucose does, but has much slower kinetics, thus cells become more dependent on mitochondrial metabolism, which resembles the *in vivo* situation of neurons much better. Since mitochondria are then stronger involved in homeostasis, the hazard potential of mitochondrial inhibitors can be assess better.

Limitations

Galactose is a non-normal substrate for neuronal cells, therefore general cell viability and functionality parameters have to be controlled to ensure healthy, but metabolically tuned cells.

Method outline

Cells are cultured normally according to established DM-ALM protocol for UKN4/NeuriTox. The only difference is that as differentiation and centrifugation medium, medium with 18 mM galactose instead of 18 mM glucose is used.

Method description

Chemicals and buffers

- Medium: Thermo A2494301 (Silac AdvDMEM/F12 no glucose) to be supplemented with galactose, lysine and arginine to have the same composition as the medium mentioned in the DB-ALM protocol
- No other changes to the DB-ALM protocol

Preparation upfront

- Prepare the medium

- No other changes to the DB-ALM protocol

Experimental procedure

- Cells are cultured normally according to established DM-ALM protocol for UKN4/NeuriTox. The only difference is that as differentiation and centrifugation medium, medium with 18 mM galactose instead of 18 mM glucose is used
- No other changes to the DB-ALM protocol

Data analysis

- No changes to the DB-ALM protocol

Annex 1.15. Biokinetic Modelling Description

Method	Biokinetic Modelling
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Introduction to Biokinetic Modelling

Effective concentrations determined in *in vitro* toxicological assays are routinely based on a range of nominal treatment concentrations. However, the use of the nominal treatment concentration as the driving concentration for observed toxicity *in vitro* does not account for factors that reduce the free concentration within the assay and determine the true effective concentration. For cell based assay systems, this will dictate the concentration available for distribution into the cell, and subcellular compartments, and so determine the driving concentration at the target site mediating toxicity. In order to more accurately translate concentration driven toxicity from *in vitro* to *in vivo*, it is necessary to correct the nominal effect concentration, accounting for the distribution of the compound within the assay system. Factors to be considered in modelling this *in vitro* distribution include binding to the plastics used in assays, exchange at the interface between culture media and the air in the culture vessel, and binding to components that may be included in the culture media (e.g. lipids and proteins originating from foetal bovine serum, FBS); the modelling of these processes is termed biokinetics (1).

A number of biokinetic models have been published that account for some or all of the factors listed above in cell based assays. Armitage and colleagues (2) published a model framework to predict intracellular concentrations, correcting for some of the distribution factors in monolayer cell culture. This steady-state framework assumes instantaneous partitioning between media, headspace, serum-lipids, serum-proteins, dissolved organic material, and the cultured cell volume. However, a critical assumption of the Armitage model is that the test compounds are neutral or not significantly ionised under the conditions of the *in vitro* assay. This assumption of neutrality was, to some degree, addressed in the model developed by Fischer *et al* (3) where the authors adopted the same steady-state assumption, but excluded the partitioning of compound into the headspace. The proposed model incorporated separate partition constants for both the ionised and unionised fraction of test compound, determining the fraction ionised assuming a uniform pH=7.4 throughout the test system. This neglects the differential ionisation potential between the culture media and intracellular water resulting from their differing pH. Furthermore, the interior of the cell itself is not a uniform environment, the microenvironment of specific organelles being maintained at pH specific to their function (i.e. lysosomes (pH≈4.5), mitochondria (pH≈8), cytosol (pH≈7)). Indeed, the differential ionisation of compounds between organelles and intracellular water can result in the preferential sequestration of compounds within organelles; a phenomena commonly known by the misnomer ‘ion-trapping’(4). It is also critical to note that differences in the intrinsic permeability of the unionised/ionised form are not the only factors determining the distribution of ionised compound into cells. The potential difference maintained across the cell membrane, membrane potential (mV) can actively promote the uptake or exclusion of ionised compounds from the cell interior and can vary significantly between cell types.

Steady-state Biokinetic Model 2D Monolayer Cell Culture

Given that compounds in this read-across are monoprotic acids, significantly ionised at physiological pH, the assumption of neutrality or uniform ionisation is not applicable. As such, an alternative model revising the relevant assumptions of the published approaches was used to predict the intracellular concentrations in 2D monolayer test systems. Based on partition coefficients between different mediums, and physical volumes, we can predict an apparent volume of distribution in the *in vitro* system. Given this volume, we can calculate the unbound medium concentrations, $C_{medium,u}$.

$$C_{medium,u} = \frac{C_{nominal} \cdot f_{u_{FBS,dilu}} \cdot V_{medium}}{V_{medium} + k_{air} f_{ui} V_{air} + k_{cell} V_{totalcell} + k_{plastic} SA_{medium} \cdot 10^3}$$

Where $C_{nominal}$ is the nominal concentration, V_{medium} (L) is the volume of culture medium, V_{air} (L) the volume of air in the headspace above the media, and $V_{totalCell}$ (L) is the total volume of cultured cells at the time of the assay, SA_{medium} (m²) is the surface area of plastic in direct contact with culture medium, $f_{u_{FBS,dilu}}$ is the fraction unbound in foetal bovine serum accounting for the dilution of FBS in culture (where FBS is not included in the culture medium $f_{u_{FBS,dilu}}=1$), the partition coefficients between culture medium and air, cells and plastic are k_{air} , k_{cell} , and $k_{plastic}$, respectively, and are defined below. The fraction unionised, f_{ui} , is calculated based on the Henderson Hasselbalch equation using the compound specific pKa and the compartment relevant pH.

$$f_{ui} = \frac{1}{1 + Y}$$

$$Y_{neutral} = 0$$

$$Y_{acid} = 10^{(pH-pKa)}$$

Binding to Serum Components

The predominant binding protein present in untreated foetal bovine serum (FBS) is albumin. FBS also contains lipids and free fatty acids. The lipids within FBS are diverse and not individually characterised or quantified routinely. However, the neutral lipid triacylglyceride (TAG) is routinely quantified and reported in the certificate of analysis, as is the concentration of albumin. Assuming albumin and TAG to be the most significant binding components in FBS and complete cell culture medium, we can predict the fraction unbound in FBS, $f_{u_{FBS}}$.

$$f_{u_{FBS}} = \frac{1}{1 + K_{protein} f_{protein} + \frac{P_{nl} f_{nl,FBS}}{1 + Y_{FBS}}}$$

Where $f_{protein}$ (v/v) is the fraction of FBS comprised of protein, $K_{protein}$ is the albumin:water partition coefficient, P_{nl} is the neutral lipid partition coefficient (defined below), and f_{nl} is the fraction of FBS comprised of neutral lipid. Assuming that the fraction of albumin in the FBS is representative of the total protein fraction responsible for protein binding in the FBS, this can be calculated from the mass of albumin reported in the certificate of analysis for a batch of FBS.

$$f_{protein} \approx f_{alb,FBS} = \frac{mass\ albumin \cdot PSV_{albumin}}{1000}$$

Where $PSV_{albumin}$ is the partial specific volume of albumin (0.73 mL/g (5)). The albumin to water partition coefficient, $K_{protein}$, can be determined experimentally or can be calculated based on a previously described relationship with the octanol to water partition coefficient (6).

if $\log P_{ow} < 4.5$

$$\log k_{albumin} = 1.08 \cdot \log P_{ow} - 0.7$$

if $\log P_{ow} \geq 4.5$

$$\log k_{albumin} = 0.37 \cdot \log P_{ow} + 2.56$$

In much the same way the volumetric fraction of neutral lipid in FBS can be calculated, using TAG as a surrogate for neutral lipid content. TAG concentration is routinely determined using an enzymatic assay and so reported as a molar concentration.

$$f_{nl,FBS} \approx f_{TAG} = \frac{[TAG] \cdot 10^{-3} \cdot MW_{TAG} \cdot PSV_{TAG}}{1000}$$

Where PSV_{TAG} is the partial specific volume of TAG (1.09 mL/g (7)) and the molecular weight of TAG is taken to be 885.453 g/mol; specifically, this corresponds to the molecular weight of trioleate, a TAG molecule comprising a glycerol backbone and three oleic acid residues.

A dilution factor, D , can then be calculated to correct f_{uFBS} for the volumetric fraction of media comprising serum, f_{serum} , and so $f_{uFBS,dilu}$ can be calculated.

$$D = \frac{1}{f_{serum}}$$

$$f_{uFBS,dilu} = \frac{f_{uFBS}}{\frac{1}{D} \cdot (1 - f_{uFBS}) + f_{uFBS}}$$

Calculation of Partition Coefficients

The partition coefficient between the culture medium and air is derived from the test compounds Henry's Law constant, which may be determined experimentally or predicted using a variety of *in silico* tools. The dimensionless air medium partition coefficient is determined:

$$k_{air,u} = \frac{k_H}{RT}$$

Where K_H is the Henry's Law constant expressed in SI units ($\text{Pa m}^3 \text{ mol}^{-1}$), R is the universal gas constant ($8.314 \text{ Pa m}^3 \text{ K mol}^{-1}$), and T is the reference temperature at which Henry's Law constant as been defined (K).

The plastic to medium partition coefficient is predicted based on the octanol to water partition coefficient using a linear relationship established by Kramer (8) and used in the

model published by Comenges *et al* (9). Note that this partition-coefficient is not dimensionless and has units (m).

The partition coefficient between cells and culture medium is based on an adaption of the published approach of Rodgers and Rowland for predicting the partitioning of different compound classes between plasma and tissues, based on composition (10, 11). We derive $K_{cell\ uu,uu}$ and $K_{iw\ uu,uu, organelle}$ from the steady-state Fick-Nernst-Planck equation to describe the passive permeation of electrolytes across the cell and organelle membranes, respectively, as well as the passive permeation of neutral molecules,

$$k_{cell\ uu,uu} = \frac{1 + \frac{P_{unbound,ionised}}{P_{unbound,unionised}} \frac{N}{e^{N_1} - 1} Y_{ew}}{1 + \frac{P_{unbound,ionised}}{P_{unbound,unionised}} \frac{N}{e^{N_1} - 1} e^{N_1} Y_{iw}}$$

$$k_{IW_{organelle}^{uu,uu}} = \frac{1 + \frac{P_{unbound,ionised}}{P_{unbound,unionised}} \frac{N_1}{e^{N_1} - 1} Y_{iw}}{1 + \frac{P_{unbound,ionised}}{P_{unbound,unionised}} \frac{N_1}{e^{N_1} - 1} e^{N_1} Y_{organelle}}$$

$$N_{neutral} = 0$$

$$N_{monoacid} = -\frac{\phi F}{RT}$$

Where P is the permeability coefficient of either the ionised or unionised moiety. Using a ratio of the permeability coefficient between the unionised and ionised species, we can describe the differential permeability of the two molecular forms. It has previously been assumed that the permeability coefficient of the ionised species is 3-4 log units lower than that of the neutral form (12). Here we assume ionised, unionised permeability coefficient ratio of 3.3 log units for the monoprotic anion. F is the Faraday constant (96484.56 C mol⁻¹) and ϕ is the cell membrane potential (V).

$$k_{cell,u} = \frac{C_{cell}}{C_{media_{unbound}}} = \left(\begin{array}{l} (1 - f_{lyso} + f_{mito})(f_{iw}(1 + Y_{iw}) + P_{nl}f_{nl} + P_{np}f_{np}) \\ + f_{lyso}(f_{iw}(1 + Y_{lyso}) + P_{nl}f_{nl} + P_{np}f_{np})K_{iw\ lyso}^{uu,uu} \\ + f_{mito}(f_{iw}(1 + Y_{mito}) + P_{nl}f_{nl} + P_{np}f_{np})K_{iw\ mito}^{uu,uu} \end{array} \right) \frac{1}{1 + Y_{ew}} k_{cell\ uu,uu}$$

Incorporating $K_{cell\ uu,uu}$ and $K_{iw\ uu,uu, organelle}$ we adapt the original Rodgers and Rowland approach where f_{iw} , f_{lyso} , f_{mito} , f_{nl} , f_{np} denote the fractional cellular volumes of the intracellular water, lysosomes, mitochondria, neutral lipids, and neutral phospholipids, respectively. P_{nl} and P_{np} describe the partitioning of the compound between intracellular water and neutral lipids and neutral phospholipids, respectively. Where the olive oil to water ($P_{vo:w}$) and octanol to water ($P_{o:w}$) partition coefficients are used as surrogates, respectively.

This revises a fundamental assumption of the published Rodgers and Rowland approach, and previously published biokinetic models that only unionised molecular species can passively traverse biological membranes (2, 9-11). The approach also expands on previously published biokinetic models by describing distribution into two subcellular organelle compartments (lysosome and mitochondria). Based on the approach described above, total intracellular concentrations corresponding to nominal effect concentrations determined experimentally can then be calculated.

$$C_{cell} = k_{cell,u} \cdot C_{media,dissolved,u}$$

It should be noted that while this approach can be used to model ampholytes, monoprotic and diprotic acids and bases, the equations above are described in forms with specific to neutral compounds and monoprotic acids, relevant to the compounds investigated in this read across. A description of this model has been presented previously (13), and is currently submitted for peer-review.

Summary of Model Assumptions

The model described above, like those previously published (2, 3), is a steady-state approximation of multiple dynamic processes. Critically, it assumes a closed system such that the loss of test compounds within the *in vitro* system is assumed to be negligible with no metabolic clearance or instability. When that is not the case, the steady-state assumption may lead to overestimation of intracellular concentrations of the parent molecule, particularly for highly metabolised chemicals. In line with the assumption of a closed system, the culture system is assumed to be hermetically sealed, such that the air above the culture medium is a defined volume. For volatile compounds, tested in unsealed systems, this could also result in an overprediction of intracellular compounds, since distribution into the air will act as a clearance mechanism. As part of the steady-state assumption, the model assumes that the volume of cultured cells is constant with no significant increase or decrease over the course of the test assay. Finally, the model assumes that all binding and partitioning processes are non-saturable, with the distribution of test compound into the cell mediated through passive diffusion. The model assumes that there are no active uptake or efflux transport processes relevant to the partitioning of the test compound within the test system.

Alternative Biokinetic Predictions

An assumption of the biokinetic model used to predict intracellular concentrations is that cells are cultured as a 2D monolayer. Permeability into cells cultured in multi-layer, three dimensional systems may not be well described based on the approach detailed above; particularly permeability into cells that may not be in direct contact with the culture medium (i.e sandwiched between adjacent cells). Thus, we adopt a simplified approach to predict the corresponding concentration of free drug in test medium, as if the determined effective concentration was made up in complete culture medium prior to cell treatment. This predicted unbound effective concentration can then translated to an unbound plasma concentration *in vivo*; such approaches have been described previously (14).

As described above, the predominant binding components present in untreated FBS are albumin and neutral lipids. In order to predict the free concentration of test compounds in treatment medium it is necessary to account for binding to these media components. Here we assume that the binding to albumin and lipid (TAG) in complete culture (treatment) medium, are the only significant processes limiting the availability of test compound for distribution into subsequently treated cells. Thus, loss of compound due to volatility, or the binding to the plastics used in cell culture are not accounted for in this approach.

If we consider the threshold for significant volatility to be an air-water partition coefficient ($K_{air} < 0.03$), as previously assumed by Fischer *et al*¹, then the assumption that volatility has no significant impact on the freely dissolved media concentration holds for all of the compounds investigated here. Polymer-water partition coefficients have been shown to be

significantly lower than octanol-water partition coefficients (P_{ow}) (2), here used to determine the partitioning to TAG and albumin, as described below. As such, we assume here that binding to plastics used in the handling and preparation of culture medium have no significant impact on the free concentration of test compound. Finally, assuming that the maximal tested concentration does not exceed the solubility of the compound in complete culture medium, and taking the binding to protein and lipid in culture media to be linear across the tested concentration range, we can calculate an unbound fraction of test compound given the composition of the medium and the P_{ow} of the test compound.

$$f_{u_{media}} = \frac{1}{1 + K_{albumin}f_{albumin} + \frac{P_{vow}f_{nl}}{1 + Y}}$$

$$Y_{neutral} = 0$$

$$Y_{monoprotic\ acid} = 10^{(pH-pKa)}$$

$$\log K_{vow} = 1.115 \cdot \log K_{ow} - 1.35^4$$

Where $f_{albumin}$ is the volumetric fraction of medium comprised of protein, $K_{albumin}$ is the albumin-water partition coefficient, K_{vow} is the olive oil-water partition coefficient (derived from P_{ow}), f_{nl} is the volumetric fraction of medium comprised of neutral lipid (TAG), and Y is the ratio of the ionised to unionised concentrations in the culture medium calculated using the Henderson-Hasselbalch equation. As above, the binding of test compound to neutral lipids is assumed to be limited to the unionised fraction of the solubilised compound in medium. The volumetric fractions of albumin and TAG in the culture medium can be calculated using an analogous approach to that described above using the partial specific volumes of albumin and TAG.

$$f_{albumin} = \frac{[albumin] \cdot PSV_{albumin}}{1000}$$

$$f_{nl} \approx f_{TAG} = \frac{[TAG] \cdot PSV_{TAG}}{1000}$$

It should be noted that this relationship assumes that the neutral and the ionised fractions of compound partition equally into the hydrophobic phase and so $\log K_{albumin}$ is not influenced by the ionisation state for ionisable compounds. Here, both the concentration of albumin and TAG are taken to be in units of mg/mL.

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Annex 1.16. Multiplex measurement of neurite degeneration, PI staining, ATP content.

Method	SH-SY5Y: repeat dosing
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Standard Operating Procedure (SOP)

1. PURPOSE

The SOP describes the procedures for multiplexing measurements of neurite degeneration and cell viability determined by high content fluorescence microscope imaging together with quantification of ATP content determined by luminescence in differentiated SH-SY5Y cells after repeated exposure.

2. SCOPE

A multiplex assay for neurite degeneration, cell viability and ATP measurement has been developed to be able to estimate all three endpoints in the same plate of exposed human neuroblastoma SH-SY5Y cells. The cell permeant dye Calcein-AM was used to stain the cytoplasm of differentiated cells allowing the visualisation and quantification of the neurite length. Once inside the cells, calcein-AM is hydrolysed by intracellular esterases producing a green fluorescent dye. Viable cells are visualised under the fluorescence microscope by exclusion of propidium iodide (PI). After monitoring calcein and PI staining in a fluorescence microscope imaging system, the ATP content in each well was analysed by the ATP luminescence assay, using the CellTiter Glo kit.

3. INTRODUCTION

Neurite degeneration initiated at non-cytotoxic concentrations *in vitro* is an alert for neurotoxicity directed to pathologies in axons and dendrites *in vivo*. Furthermore, imbalances in the energy metabolism and ATP deficiency is a key event for neurotoxicity. Herein, we have combined assays for neurite degeneration, cell viability and ATP measurements in the same plates with SH-SY5Y cells after repeated exposure with chemicals.

4. RESPONSIBILITY

All personnel performing this assay should follow this SOP.

5. PROCEDURES

LIMITATIONS

To be added

METHOD OUTLINE

Neurite degeneration and cytotoxicity were evaluated by fluorescent microscopy with the dyes calcein-AM (for neurite staining) and propidium iodide (PI) (for early apoptotic/necrotic cell staining). In addition, ATP content was quantified after imaging by luminescence with the CellTiter-Glo® assay (Promega).

For experiments, SH-SY5Y cells were plated in 96-well plates with micro-clear bottom (8 000 cells/well) in EMEM with 10% foetal bovine serum, 2 mM glutamine, 1% non-essential amino acids, 100g streptomycin/mL and 100 U penicillin/mL, which is exchanged to differentiation medium with 1 µM RA and without serum (DMEM:F12 [1:1], N2 supplements [diluted 1:100], 1 mM glutamine, 100g streptomycin/mL and 100 U penicillin/mL) after 24 h. The cells are exposed to test chemicals after three and six days from the start of differentiation. The total exposure time is 120 hours. Neurite degeneration and cytotoxicity are measured by fluorescent microscopy with the dyes calcein-AM (for neurite staining) and propidium iodide (for staining of late apoptotic/necrotic cells). In addition, ATP content is quantified after imaging by luminescence using the CellTiter-Glo® 2.0 assay (Promega).

The Promega's CellTiter-Glo® assay system which employs the properties of a thermostable luciferase to enable reaction conditions that generate a stable "glow-type" luminescent signal while simultaneously inhibiting endogenous enzymes released during cell lysis (e.g., ATPases). The luminescence signal is recorded by luminescence reader (Tecan Infinite M200 Pro) for 300ms per well.

DEFINITIONS/ABBREVIATIONS

The following abbreviations are used:

DMEM:F12	Dulbecco's modified medium and Ham's F12 medium (1:1)
DMSO	Dimethyl sulfoxide
EMEM	Minimum essential medium with Earle's salts
RA	All-trans retinoic acid
FBS	Foetal bovine serum
PEST	Penicillin Streptomycin
PI	Propidium iodide
NEAA	Non-essential amino acids

IDENTIFICATION OF TEST AND CONTROL SUBSTANCES

A. Test Chemicals

The cells were pre-exposed to test chemicals for 120 h before the measurements, simulating repeated exposure.

Dilutions:

All chemicals were tested at 10 µM, 2 µM, 400 nM, 80 nM and 16 nM. For those chemicals showing effect at the lowest tested concentration during the screening, extra 5X dilution steps were performed until a no effect concentration was reached.

Table 1. A typical pipetting scheme for preparation of the dilution series for test chemicals dissolved in DMSO.

Dilution step	Conc. In ep-tube (μM)	Test chemical (μl)	Medium + 0.2% DMSO (μl)	Conc. In wells (mM)
D1	2,00E+01	2	998	1,00E+01
D2	4,00E+00	200 of D1	800	2,00E+00
D3	8,00E-01	200 of D2	800	4,00E-01
D4	1,60E-01	200 of D3	800	8,00E-02
D5	3,20E-02	200 of D4	800	1,60E-02
D6	6,40E-03	200 of D5	800	3,20E-03
D7	1,28E-03	200 of D6	800	6,40E-04
D8	2,56E-04	200 of D7	800	1,28E-04
Controls	0	0	1000	0

B. Controls

- a. **Negative control:** Cells exposed to N2-RA with DMSO 0.1%.
- b. **Positive control:** Rotenone 400 nM.

MATERIALS*A. Cell Culture Type*

Human neuroblastoma SH-SY5Y cell line is cultured and differentiated as described in [SOP plating and differentiation](#).

B. Technical Equipment

- a. ImageXpress® Micro (Molecular Devices, UK)
- b. MetaXpress® Software (Molecular Devices, UK)
- c. Tecan® Infinite M200 Pro
- d. Tecan® i-control 3.7.3.0 software
- e. Calcein (Life technologies, cat no C1430)
- f. Hoechst 10 mL (ThermoFisher, cat no H3570), 10mg/mL.
- g. Propidium iodide solution (Sigma, cat no P4170)
- h. CellTiter-Glo® Assay (Promega, cat no G7571 or G9242)
- i. Black cell culture microplate, 96-well plate with microclear bottom (Greiner, No 655090)

METHOD:*A. Preparation of Calcein stocks*

- a. Dilute the calcein powder (1mg) in 1 mL 100% DMSO
- b. Warm up the diluted calcein to ensure proper dilution
- c. Prepare 20 μL Aliquots

- d. Store at 4 °C

B. Preparation of propidium iodide (PI) stock

1. Prepare a 5 mg/mL (7.48 mM) stock solution by diluting 10 mg PI in 2 mL PBS. Store at 4 °C protected from light.
2. Prepare a 1 mM PI working solution by diluting 134 μ L of the 5 mg/mL PI stock solution in 866 μ L PBS. Store at 4 °C protected from light.

C. Exposure

For experiments, 8 000 SH-SY5Y cells are plated in black microclear bottom 96-well plates in complete EMEM with 10% foetal bovine serum, which is exchanged to N2-RA differentiation medium after 24 h. After 3 days of differentiation 100 μ L of the assigned treatment (test chemical dilution) are added on top of the wells. The plates are incubated with the test chemicals for 3 days. On the 6 day of differentiation 100 μ L of cell medium are removed from the exposed cells and 100 μ L of the same treatment are added on top of each well. The cells are placed in the incubator for 2 days more, the total time of exposure is 120 hours.

Figure 1. Plate lay out for multiplex assays (Neurite degeneration, propidium iodide and ATP content) of CS4 test chemicals.

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		DMSO 0,1%			Co2 D1			Co3 D1			Rotenone 400 nM	
C		Co1 D1			Co2 D2			Co3 D2			Rotenone 400 nM	
D		Co1 D2			Co2 D3			DMSO 0,1%			Rotenone 400 nM	
E		Co1 D3			Co2 D4			Co3 D3			Rotenone 80 nM	
F		Co1 D4			Co2 D5			Co3 D4			Rotenone 80 nM	
G		Co1 D5			DMSO 0,1%			Co3 D5			Rotenone 80 nM	
H												

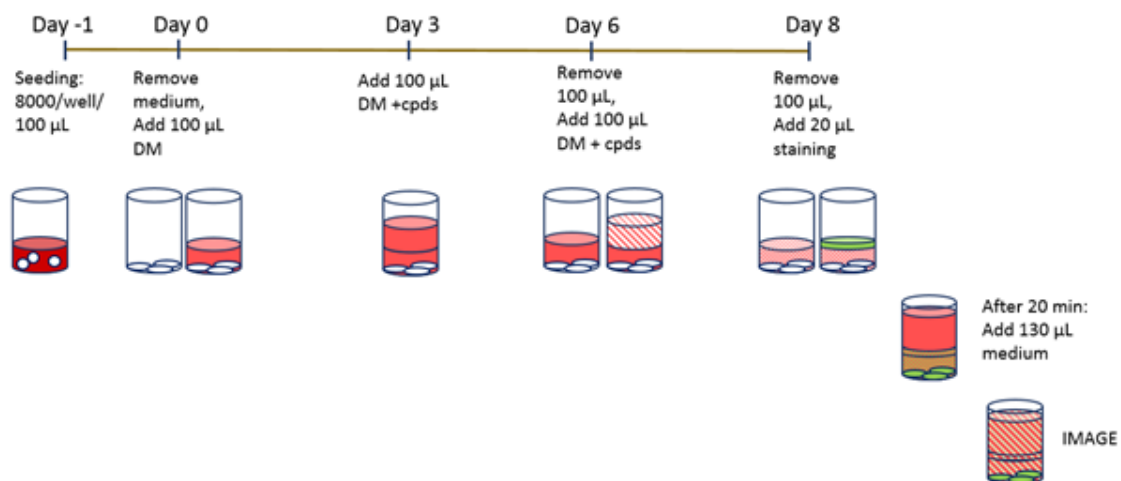
D. Neurite and propidium iodide staining

The measurement of neurite degeneration is performed 120 hours after exposure to test chemicals

1. Be very careful not to keep plates out of incubator for longer time than necessary, and be extremely gentle with pipetting
2. Take one plate at a time out, carefully remove 100 μ L of medium from the wells
3. Put the plate back in the incubator as fast as possible, do not let it cool down
4. Prepare and warm up 22 mL of DMEM:F12 medium for later dilution of the staining
5. Ultrasonicate Hoechst-H33342 stock solution in ultrasonic water bath for 15 min
6. Prepare dye solution (enough for two plates):
 - a. 4 mL of DMEM/F-12
 - b. 24 μ L Hoechst 33342 stock 10 mg/mL (16,2 mM) [final conc: 5 μ g/mL]

- c. 24 μL Calcein-acetoxymethylester (CAM) stock of 1 mg/mL (1 mM) [final conc: 1 μM]
 - d. 24 μL PI 1 mM [final conc: 5 μM]
 - e. Vortex
 - f. Protect from direct light (switch off light in the sterile hood)
7. Put dye solution into a reservoir
 8. Add 20 μL of dye solution to all wells in one plate, quickly but gently pipet up and down twice in each row (without changing tips for the same treatment)
 9. Incubate the plate for 20 min at 37°C, 5% CO₂
 10. Put 11 mL of warm DMEM/F-12 into a reservoir
 11. Take the plate out
 12. With a 12-channel pipette, quickly but gently, add 130 μL of DMEM:F12 in each well
 13. Immediately image the plate (calcein in FITC channel, H33342 in DAPI channel, PI in Texas red channel, 4 sites/well)

Figure 2. Overview of the experimental set up used for imaging of neurite degeneration and cytotoxicity on differentiated SH-SY5Y cells after repeated exposure to test chemicals.



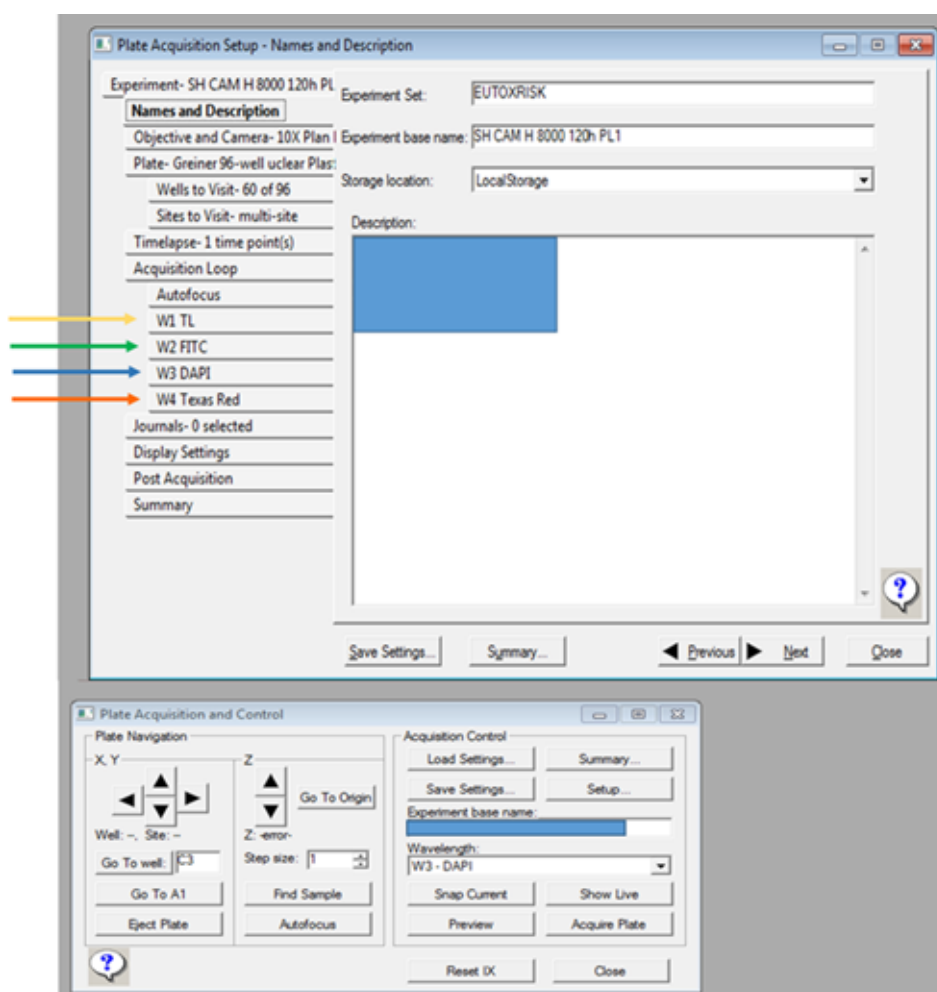
E. Neurite degeneration imaging

ImageXpress® Micro (Molecular Devices, UK) is used to obtain images of the exposed cells. Four images per well are taken with three channels: Transmitted light (TL, yellow arrow), Hoechst in DAPI channel (nuclear staining, blue arrow), calcein in FITC Channel (cytoplasm/neurite staining, green arrow) and PI in Texas red channel (cytotoxicity, orange arrow).

1. Turn on the machine 30 minutes before starting the measurements.
2. Open MetaXpress®

3. On the top menu of the MetaXpress® software, select screening → plate acquisition set up.
4. On the top menu of the MetaXpress® software, select screening → plate acquisition and control.
5. Once the plate acquisition set up and plate acquisition and control windows are opened, start by writing the name of the experiment and a description under the “Names and description” tab (see below, Figure 3).

Figure 3. "Names and description tab"

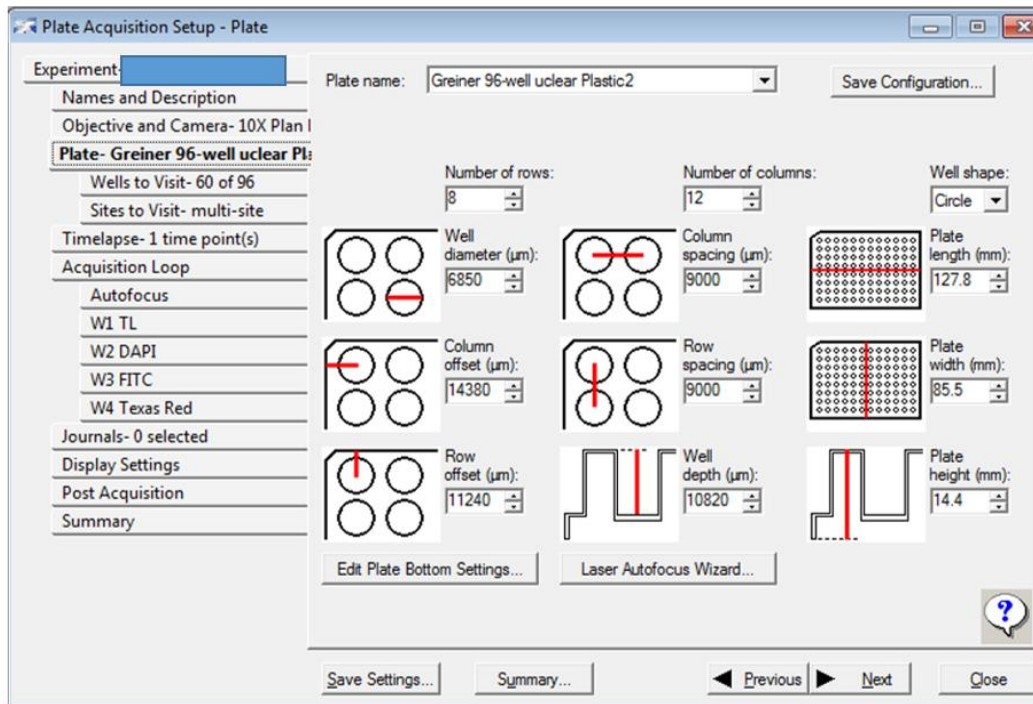


6. Under the “objective and camera” tab select a 10X magnification, camera binning 1 and gain 1. These settings should only be modified by the instrument’s responsible person or technician.
7. Under the “Plate” tab use the settings optimised for 96 well black microclear bottom cell culture plates (Greiner, No 655090). Figure 4 contains a description of the settings used for 96 well plates in this instrument, these settings should only be modified by the instrument’s responsible person or technician.

[Note: different batches of plates can have differences in the well depth within the same plate, this will affect the sharpness of the image taken during the testing of the settings but it will not affect the final images]

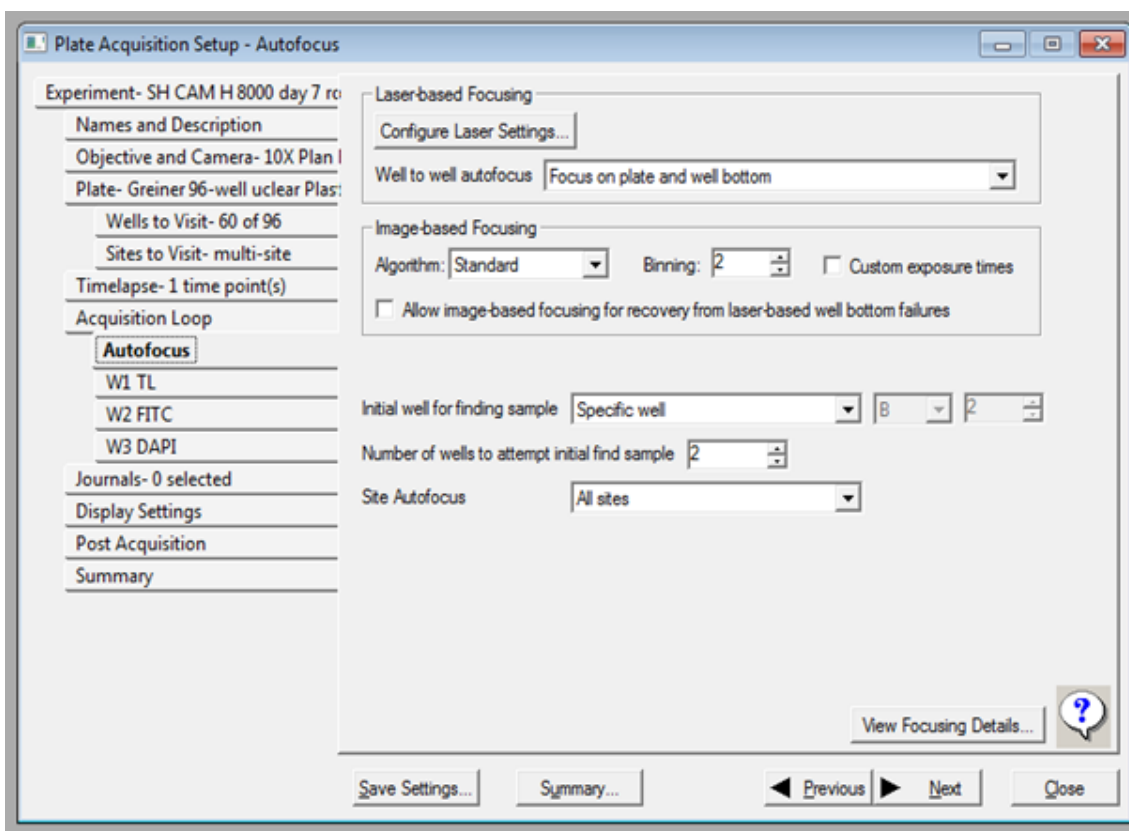
Figure 4. "Plate" tab including the setting that have been standardised for measurements in 96 well plates.

These settings should only be modified by the person responsible for the instrument.



8. Under the "wells to visit" tab select the 60 innermost wells for analysis. Make sure that the selected wells are showed in a green colour.
9. Under the "sites to visit" tab select 2 columns and 2 rows to obtain 4 images/well.
10. Under the "timelapse" tab select number of endpoints = 1.
11. Under the "autofocus" tab (see Figure 5) select:
 - a. Well to well autofocus: focus on plate and well bottom.
 - b. Imaged based focusing:
 - i. Algorithm: standard
 - ii. Binning: 2
 - c. Initial well for finding sample: specific well
 - d. Site autofocus: all sites.

Figure 5. “Autofocus” tab with the settings used for measurements in 96 well plates.



12. Under the tabs W1 to W3 the setting for image acquisition are specified:

Table 2. Settings used for the acquisition of images with MetaXpress®.

The specific exposure time, target maximum intensity and Z- offset set for each of the three wavelengths used to measure neurite degeneration in differentiated SH-SY5Y cells are described.

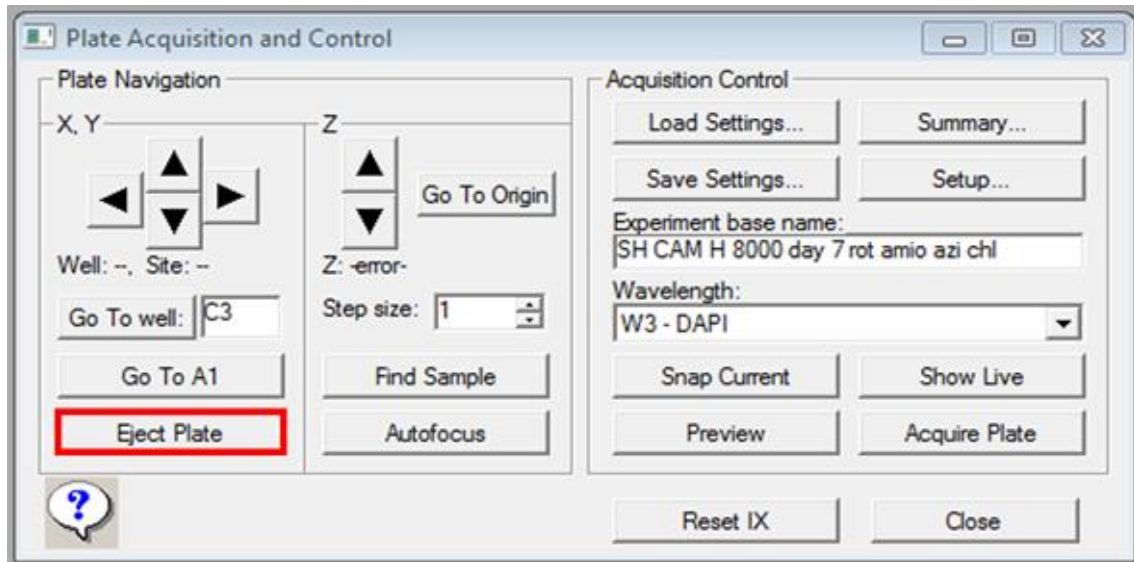
Illumination setting	TL	DAPI	FITC	Texas Red
Exposure time (ms)	8	100	120	400
Target max intensity	16960	33000	34464	33000
Z-offset (µm)	8.96	2.92	10.44	4.2
Calculate Offset (use Z stack and Custom range)	Range: 50 µm Step: 1.5 µm	Range: 50 µm Step: 1.5 µm	Range: 50 µm Step: 1.5 µm	Range: 50 µm Step: 1.5 µm

13. Insert the plate in the imager (see Figure 6)

- a. In the plate acquisition and control menu choose eject plate
- b. Place the plate inside the imager
- c. In the plate acquisition and control menu choose load plate

Figure 6. Plate acquisition and control window.

To insert the plate in the imager press “eject plate”



14. With the mouse, right click button select one of the 4 sites and one control well, select the DAPI channel to evaluate the nuclear staining with Hoechst. To be able to see the cells click on “find sample” in the plate acquisition and control window.
15. With the mouse right click button select one of the 4 sites and one control well, select the FITC channel to evaluate the calcein staining. To be able to see the cells click on “find sample” in the plate acquisition and control window.
16. With the mouse right click button select one of the 4 sites and one control well, select the Texas Red channel to evaluate the PI staining. To be able to see the cells click on “find sample” in the plate acquisition and control window.
17. Click under the “summary” tab → acquire plate.
18. All obtained images are automatically stored in MetaXpress® in the D disk of the computer. The SOP “procedure for backing up old experiments from the imager” describes the steps to follow in order to create back up files.

F. ATP content (CellTiter-Glo cell viability assay)

The measurement of ATP content is performed after imaging the cells:

1. Thaw the CellTiter-Glo® 2.0 (Promega, cat no G9242) and make 10 ml aliquots (for 2 plates). Store aliquots at -20°C until use.
2. Thaw the CellTiter-Glo® 2.0 and equilibrate to room temperature prior to use. For convenience the CellTiter-Glo® 2.0 (Promega, cat no G9242) may be thawed and stored at room temperature for up to 48 hours prior to use.
3. Alternatively, thaw the CellTiter-Glo® buffer (Promega, cat no G7571) and transfer 10 mL into the bottle containing CellTiter-Glo® substrate. Mix by gently vortexing.
4. Remove 200 µl from each well and add 50 µl of Reagent.

5. Put the plates on an orbital shaker for 2 minutes to induce cell lysis.
6. Allow the plate to incubate at room temperature for 15 minutes to stabilise luminescent signal.
7. Record the luminescence for 300 ms per well by using the Tecan Infinite M200 Pro reader.
8. Store the data in the S:\Projects\PHC33_EUToxRisk\Case study 4_Mitotox\Repeated exposure folder.

G. Image analysis

Neurite degeneration

1. Open MetaXpress®
2. On the top menu of the MetaXpress® software, select screening → Review plate data [DB].
3. Select the plate for analysis.
4. Select all the wells to be analysed.
5. On the “Run analysis” tab select:
 - a. Analysis: <Neurite Outgrowth>
6. Settings: Press configure settings, and select:
 - a. Neurite image: FITC
 - b. Illumination: Fluorescence
 - c. Cell bodies:
 - i. Approximate max width: 40 μm = 62 pixels
 - ii. Intensity above local background: 100 graylevels
 - iii. Minimum area: 30 μm^2 = 71 pixels
 - d. Nuclear stain:
 - i. Nuclear image: DAPI
 - ii. Approximate min width: 5 μm = 8 pixels
 - iii. Approximate max width: 13 μm = 20 pixels
 - iv. Intensity above local background: 10 graylevels
 - e. Outgrowths:
 - i. Maximum width: 2 μm = 3 pixels
 - ii. Intensity above local background: 10 graylevels
 - iii. Minimum cell growth to log as significant: 15 μm = 23 pixels
7. In the configure settings window press “test run” to visualise the correct identification of cell bodies and outgrowths in the software (Figure 7 and 8)

Figure 7. "configure settings tab".

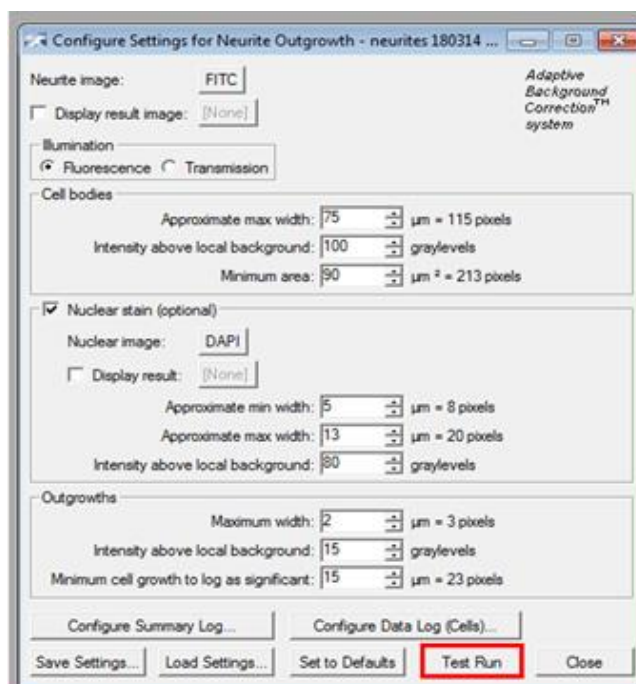
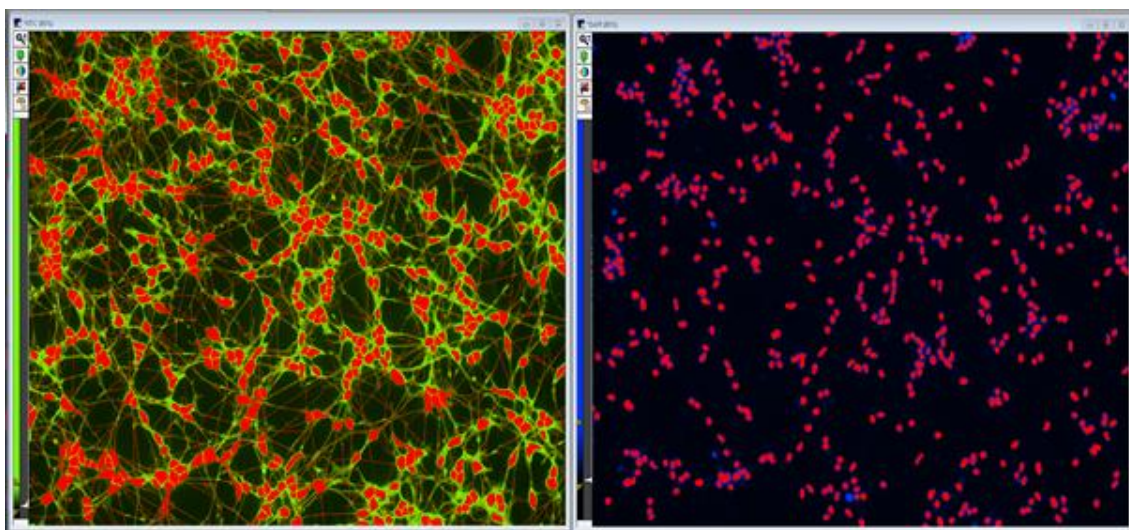


Figure 8. Analysis of neurite length in differentiated SH-SY5Y cells.

Left: staining with calcein in FITC channel, Right: nuclear staining with Hoechst in DAPI channel. Live cells are recognised as structures with both calcein and Hoechst staining.



Note: variations in the intensity of the staining can lead to inappropriate identification of cells bodies and/or neurites. If needed, the settings in the "configure settings" tab should be modified to assure proper identification of most of the imaged structures.

8. Analyse the plate by pressing "run analysis" on the "Review plate data" window.
9. After analysis, the data can be exported to an excel file.
10. Store the data in the S-disk: S:\Projects\PHC33_EUToxRisk\Case study 4_Mitotox\Neurite degeneration\Compilation of results 24h.

11. Microsoft Excel is used to analyse the data.

Cytotoxicity (PI staining)

1. Open MetaXpress®
2. On the top menu of the MetaXpress® software, select screening → Review plate data [DB].
3. Select the plate for analysis.
4. Select all the wells to be analysed.
5. On the “Run analysis” tab select:
 - a. Analysis: <transfluor HT>
6. Settings: Press configure settings, and select:
 - a. Check the box “Nuclear stain”
 - b. Nuclear image: Texas Red
 - i. Approximate min width: 2 µm = 3 pixels
 - ii. Approximate max width: 8 µm = 12 pixels
 - iii. Intensity above local background: 10 graylevels
7. In the configure settings window press “test run” to visualise the correct identification of PI stained cells. **Note:** variations in the intensity of the staining can lead to inappropriate identification of cells bodies. If needed, the settings in the “configure settings” tab should be modified to assure proper identification of most of the imaged structures.
8. Analyse the plate by pressing “run analysis” on the “Review plate data” window.
9. After analysis, the data can be exported to an excel file.
10. Store the data in the S-disk: S:\Projects\PHC33_EUToxRisk\Case study 4_Mitotox\Neurite degeneration\Compilation of results 24h.

H. Data analysis

Neurite degeneration

1. The differences in the mean process length (cell: mean process length) used for establishing the effect of the test compounds on the neurites (neurite degeneration).
2. Normalise the mean process length value of each well against the mean of all negative controls:

$$ND (\%) = \frac{\text{Mean process length exposed cells}}{\text{Mean process length negative controls}} * 100\%$$

3. Take the mean of the effect in the three technical replicates of each concentration.
4. Plot the values in GraphPad Prism.

Cytotoxicity (PI staining)

1. Add the total number of imaged cells per well to the number of PI stained cells in the same well to obtain the total number of imaged cells.
2. Calculate the % of viable cells per well as follows:

$$\text{Cell viability (\%)} = 100 - \left[\frac{\text{Number of PI stained cells}}{\text{Total number of imaged cells}} \right] * 100\%$$

3. Take the mean of the effect in the three technical replicates of each concentration.
4. Plot the values in GraphPad Prism.

ATP content

1. Microsoft Excel is used to analyse the data.
2. The average of luminescence of each concentration (triplicates) and standard deviation (SD) are calculated. The average luminescence value of each concentration is normalised against the mean luminescence value of control wells (6 wells) and subsequently multiplied by 100 in order to obtain the % of viable cells in each concentration.
3. Plot the values in GraphPad Prism.

IX. HEALTH SAFETY AND ENVIRONMENT

Cell culture procedures take place in a laminar air flow cabinet, class 2. All work with test chemicals is performed in a fume hood. Experiments are planned in order to reduce the exposure time of the laboratory personnel to the test chemical. Wastes of environmentally hazardous compounds are handled according to local regulations.

X. REFERENCES

To be added

XI. APPENDIX

Summary of the procedure for multiplex analysis of neurite degeneration, cytotoxicity and ATP content in differentiated SH-SY5Y cells:

Day 1

Plating of SH-SY5Y cells, 8 000 cells/well.

Day 2

Start differentiation.

Change complete EMEM to N2-RA medium.

Day 3

First exposure to test chemicals.

Add 100 µL of the corresponding chemical dilution.

	1	2	3	4	5	6	7	8	9	10	11	12
A	DMSO 0,1%		Co2 D1			Co3 D1		Rotenone 400 nM				
B	Co1 D1		Co2 D2			Co3 D2		Rotenone 400 nM				
C	Co1 D2		Co2 D3			DMSO 0,1%		Rotenone 400 nM				
D	Co1 D3		Co2 D4			Co3 D3		Rotenone 80 nM				
E	Co1 D4		Co2 D5			Co3 D4		Rotenone 80 nM				
F	Co1 D5		DMSO 0,1%			Co3 D5		Rotenone 80 nM				
G												
H												

Day 6

Second exposure to test chemicals.

Remove 100 µL from all wells.

Add 100 µL of the corresponding chemical dilution.

	1	2	3	4	5	6	7	8	9	10	11	12
A	DMSO 0,1%		Co2 D1			Co3 D1		Rotenone 400 nM				
B	Co1 D1		Co2 D2			Co3 D2		Rotenone 400 nM				
C	Co1 D2		Co2 D3			DMSO 0,1%		Rotenone 400 nM				
D	Co1 D3		Co2 D4			Co3 D3		Rotenone 80 nM				
E	Co1 D4		Co2 D5			Co3 D4		Rotenone 80 nM				
F	Co1 D5		DMSO 0,1%			Co3 D5		Rotenone 80 nM				
G												
H												

Day 8

Multiplex

Remove 100 µL of supernatant.

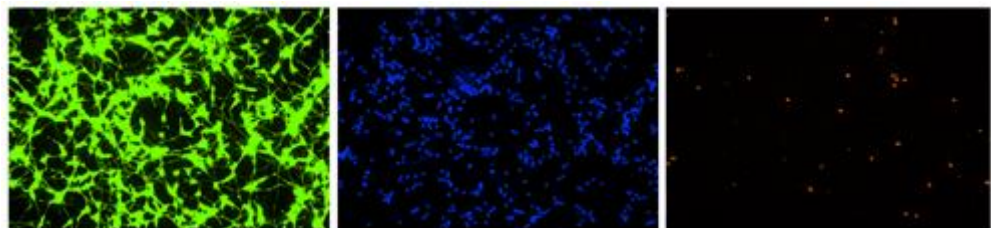
Add 20 µL/well of Calcein + Hoechst + PI staining solution.

Incubate the plate for 20 minutes at 37 °C.

Add 130 µL DMEM:F12.

Image the plate (Calcein in FITC channel, H333342 in DAPI channel, PI in Texas red channel 4 sites/well).

After imaging, measure ATP content with CellTiter-Glo® 2.0 (Promega)



Annex 1.17. CS4 read across SH-SY5Y method description (Swetox)

Method	SH-SY5Y: Lactate, Resazurin, Rho, Calcein, ATP, neuron degeneration
Contact	Anna Forsby (Swetox) -> anna.forsby@swetox.se

Cell Model:

SH-SY5Y is a thrice-cloned sub-line of the bone marrow biopsy-derived line SK-N-SH (Biedler *et al.*, 1973). The cells are characterised as sympathetic cells with high expression of muscarinic acetylcholine receptors and voltage operated Ca²⁺ channels, among other receptors and ion channels. The cells grow in monolayer cultures and consist of a neuronal phenotype (90-100%) and sometimes an epithelial-like phenotype (0-10%). The cultures shall not be used as models for neurons when the epithelial cells exceed 10% of the cell population, since these cells do not display any neuronal characteristics (Ciccarone *et al.*, 1989). The cells can be differentiated by all-trans retinoic acid (RA), thereby the cells develop into a phenotype with neuronal structural and functional properties and transcriptional profiles (Cheung *et al.*, 2009; Korecka *et al.*, 2013; Krishna *et al.*, 2014; Xicoy *et al.*, 2017). Herein, viability is studied after exposure of differentiated cells with test chemicals.

The cells can be purchased from ATCC (cat# CRL-2266), ECACC (cat# 94030304) or from other labs, e.g. Uppsala University or Stockholm University (Sweden). The native Stockholm cells display a more developed neuronal phenotype as compared to the cells obtained from ECACC.

Routine cell culture procedures:

The cells are maintained in culture in 75 cm² flasks and fed with MEM supplemented with 10% foetal bovine serum, 2 mM glutamine, 1% non-essential amino acids, 100g streptomycin/mL and 100 U penicillin/mL (named complete EMEM). The cells are subcultured every 7th day and the medium is replaced on the 3rd to 4th day after subcultivation.

Cell splitting and differentiation for experiments:

For experiments, SH-SY5Y cells are plated in white 96-well plates complete, which is exchanged to differentiation medium with 1 µM RA and without serum (DMEM:F12 [1:1], N2 supplements [diluted 1:100], 1 mM glutamine, 100g streptomycin/mL and 100 U penicillin/mL) after 24 h. The cells are allowed to differentiate for 6 days before the exposure to the test chemicals. On the third day of differentiation, half of the cell medium in the wells is removed and replaced by fresh differentiation medium. The cell density used varies between assays, 10 000 cells/well were used for viability (resazurin), lactate, mitochondrial membrane potential and propidium iodide staining. For measurements of neurite degeneration, a cell density of 8 000 cells/well was used.

Experimental exposure to test chemicals:

After arrival, all complex inhibitors were dissolved in Dimethyl Sulfoxide (DMSO) in a concentration of 10, 50 or 100 mM. All test chemicals were then diluted to obtain a 10mM stock which is subsequently aliquoted and stored at minus 20°C.

Six days differentiated SH-SY5Y cells were exposed to the test chemicals at fixed concentrations of 10 µM, 2µM, 400nM, 80nM and 16nM. Further testing at lower concentrations was performed for chemicals yielding an effect at 16 nM. For the exposure, the DMSO concentration in cell medium was 0.1 % (v/v) in all samples.

Endpoints:

A separate SOP is provided for each assay, a general description of the procedures is found below.

1. Cell viability (resazurin reduction) and lactate quantification:

For experiments, SH-SY5Y cells were plated in black 96-well plates with clear bottom (10 000 cells/well) in complete EMEM, which was exchanged to differentiation medium after 24 h. Six (6) days differentiated SH-SY5Y cells were exposed to the test chemicals and incubated for 24 hours after which the effect on cell viability was measured with resazurin.

Procedure: After the 24 hours incubation, the supernatant was collected and stored at -20 °C for lactate measurements. An 88µM resazurin working solution was then added on top of the cell medium in the wells yielding final concentration of 44µM resazurin. The plates were incubated for two hours, afterwards the resorufin fluorescence was measured at 540 nm excitation and 590 nm emission.

The amount of lactate in the collected supernatant was quantified by a colourimetric assay. The lactate assay is based on the conversion of lactate to pyruvate by the enzyme lactate dehydrogenase (LDH), reducing the co-factor NAD to NADH (Babson *et al*). In the assay, NADH reduces PMS to PMSH which reduces INT to INTH. INTH is the reagent measured by colourimetry. The samples are incubated with a reagent mix for 7 minutes, the absorbance is then measured at 490 nm.

2. Mitochondrial membrane potential with Rhodamine 123 and cytotoxicity assessment with propidium iodide:

Mitochondrial membrane potential (MMP) is correlated with the cells' ability to produce ATP through oxidative phosphorylation. Acute changes in MMP are monitored by measuring the changes in fluorescence intensity after loading the cells with Rhodamine 123 and exposing them to a selection of chemicals for 24 hours.

For experiments, SH-SY5Y cells were plated in 96-well plates with micro-clear bottom (10 000 cells/well) in complete EMEM, which was exchanged to differentiation medium after 24 h. Six (6) days differentiated SH-SY5Y cells were stained with 1 µM Rhodamine123 for 45 minutes before the exposure with test chemicals. After 24h, incubation with the test chemicals Hoechst and propidium iodide were added to the wells in order to stain the nuclei for cytotoxicity measurements. The effect of the test chemicals on the mitochondrial membrane potential was reflected as an increased (hyperpolarisation) or decreased (depolarisation) Rhodamine 123 fluorescence intensity.

3. *Neurite degeneration assessed by calcein staining:*

The cell permeant dye Calcein-AM was used to stain the cytoplasm of differentiated SH-SY5Y cells allowing the visualisation and quantification of the neurite length. Once inside the cells, calcein-AM is hydrolysed by intracellular esterases producing a green fluorescent dye. For experiments, SH-SY5Y cells were plated in 96-well plates with micro-clear bottom (8 000 cells/well) in complete EMEM, which was exchanged to differentiation medium after 24 h. Six (6) days differentiated SH-SY5Y cells were exposed and incubated with the test chemicals for 24 hours. After incubation, the cells were stained with calcein and imaged. All test chemicals were initially screened at 10 μ M, 2 μ M and 0.4 μ M. For those chemicals showing effect at the lowest tested concentration during the screening, extra 5X dilution steps were performed until a no effect concentration was reached. If no effect was observed during the screening, the experiment was repeated only a second time.

4. *Neurite degeneration, cytotoxicity and ATP content after repeated exposure:*

Neurite degeneration and cytotoxicity were evaluated by fluorescent microscopy with the dyes calcein-AM (for neurite staining) and propidium iodide (PI) (for early apoptotic/necrotic cell staining). In addition, ATP content was quantified after imaging by luminescence with the CellTiter-Glo® assay (Promega). For experiments, SH-SY5Y cells were plated in 96-well plates with micro-clear bottom (8 000 cells/well) in complete EMEM, which was exchanged to differentiation medium after 24 h. Three days after the start of differentiation the cells were exposed to the test chemicals, the exposure was repeated on the day six of differentiation. On the eighth day of differentiation (120h after the first exposure to test chemicals), the cells were stained with calcein-AM, PI and Hoechst and imaged. After imaging, the ATP content is measured with the Promega's CellTiter-Glo® assay system which employs the properties of a thermostable luciferase to enable reaction conditions that generate a stable "glow-type" luminescent signal while simultaneously inhibiting endogenous enzymes released during cell lysis (e.g., ATPases). The luminescence signal is recorded by luminescence reader (Tecan Infinite M200 Pro) for 300ms per well.

Normalisation of asymptotes for BMR calculations:

The values obtained from all assays are expressed as percentage of 0.1% DMSO controls. The data is transfer to GraphPad prism where the concentrations (expressed in M) are transformed to the logarithm (log 10). The data is then analysed by fitting a nonlinear regression with least squares fitting method and without constrains to obtain the best fit values for Top. If the lowest tested concentration is not exactly 100% of control (no effect), the asymptotes are normalised by calculating a factor as follows:

Factor = (100 / best fit values for Top as a factor)

Each obtained data point is then multiplied by the previously calculated factor.

If after the normalisation the values in the lowest tested concentration still don't fall in 100% of control, the mean of the two lowest concentrations is calculated and used to replace the best fit values for Top for calculation of the factor.

After normalisation of asymptotes, the BMR values were calculating using the online tool "BMC, Bench mark concentration" provided by AG Leist, University of Konstanz (<http://invitrotox.uni-konstanz.de:3838/BMC/>). If the reported BMR value was higher than the highest tested concentration, the BMR were reported as >highest tested concentration.

Exceptions:

Mitochondrial membrane potential: Azoxystrobin produced hyperpolarisation (values over 100%) at some of the lower tested concentrations. To provide a proper fit for these values, the data points were not normalised, instead the BMR for both effects (depolarisation and hyperpolarisation) were calculated.

Calculation of depolarisation: the values were uploaded in the online tool “BMC, Bench mark concentration” and the BMR were calculated as usual.

Calculation for hyperpolarisation: the section of the dose-response curve corresponding to the hyperpolarisation effect was inverted as follows:

Hyperpolarisation effect= $100 - (\text{data point} - 100)$

The obtained values correspond to an inhibition curve, these values were then uploaded in the online tool “BMC, Bench mark concentration” and the BMR were calculated as usual.

References:

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System genomics evaluation of the SH-SY5Y neuroblastoma cell line as a model for Parkinson's disease. *BMC Genomics* 15:1154.

10. Xicoy H., Wieringa B. and Martens J.M. (2017). The SH-SY5Y cell line in Parkinson's disease research: a systematic review. *Molecular Neurodegeneration* 12:10.

Annex 1.18. CS4 read across HepG2 method description (LU)

Method	HepG2: Lactate, Resazurin, Rho123/PI
Contact	Wanda van der Stel (LU) -> w.van.der.stel@lacdr.leidenuniv.nl

Cell Model

The assay is performed in HepG2 (ATCC® HB8065™).

Routine cell culture procedures

The cell are maintained in 75 cm² flasks using DMEM supplemented with 10% foetal bovine serum, 25g streptomycin/mL and 25U penicillin/mL.

Cell splitting

For routine culture the cells are splitted every 3 à 4 days. The medium is replaced during the procedure of cell splitting. For the CS4 experiments, cells are seeded in 384wells black unclear plates at a density of 10.000 cells per well. The seeded cells were used for the various assay 2 days after seeding.

Experimental exposure to mitotoxicants

After arrival, all complex inhibitors were dissolved in Dimethyl Sulfoxide (DMSO) in a concentration of 100 mM. All test chemicals were then diluted to obtain a 10mM stock which is subsequently aliquoted and stored at minus 80°C. Aliquots used for experiments were stored at minus 20°C.

Endpoints

A separate SOP is provided for each assay, a general description of the procedures is found below.

All assays are performed using the same cells in the following order:

1. Mitochondrial functioning based on mitochondrial membrane potential (rho123) and Cell death based on PI staining

With the Rhodamine123 mitochondrial membrane potential sensitive dye the presence of the membrane potential can be monitored over time using confocal imaging systems. 75min before compound exposure the cells are treated with 0,5uM Rho123 and 0.1ug/ml Hoechst (by adding 2x concentration solution on top of the cells). Subsequently the medium is replaced by a mixture of Rho123 (final conc = 0.1uM) and PI (final conc = 100nM), followed by the various chemicals. The Hoechst, rho123 intensity and PI intensity were monitored using a Nikon eclipse system with an automated stage, incubator, A1 confocal system and either equipped with photon multiplier (PMT) or Gasp detector (Hoechst = 408nM, Rho123 = 488nM and PI = 561nM). Using an in house pipeline (including CellProfiler and R scripts) the intensity and the localisation of the various signals were quantified. For the mitochondrial membrane, potential assessment the intensity of the dye

is used and for the assessment of viability, the fraction of PI positive nuclei is represented. All values are percentages of 0.1% DMSO controls.

2. Glycolysis based on lactate concentrations

The concentration of excreted lactate can be used to assess the glycolytic status of a cell system. To do so, the medium was collected after 24h of exposures (when the imaging was completed). The concentration of lactate is assessed using a colourimetric assay. The change in 490nm absorption upon the reduction of INT to INT_H (see SOP for complete description) can be assessed as a measure for the lactate concentration. All values are percentages of 0.1% DMSO controls.

3. Viability based on resazurin reduction

Cellular viability was finally quantified by assessing the reduction of resazurin in to resorufin, which is considered to be a measure for global REDOX capacity of the cell. Upon collection of the medium for the lactate assay, a mixture of 44µM resazurin in medium was applied to the cells for 2h. Subsequently, the Fluorescence of the resorufin is read at excitation 540nm/emission 590nm. All values are percentages of 0.1% DMSO controls.

Re-normalisation of datasets for BMR calculations:

1. Supernatant lactate

Values of at least two no effect concentrations were forced to upper asymptote exactly at 100 %, data sets were re-normalised as percentage of no effect concentrations. Curve fitting and ECs values couldn't be generated using the *in vitro* toxicology on-line tool provided by AG. Leist, University of Konstanz (<http://invitrotox.uni-konstanz.de:3838/BMC/>) as the algorithm is suitable only for inhibition curves. Lactate production increase with increase of the insult leading to activation curve, therefore curve fitting and ECs values are generated using point-to-point extrapolation in GraphPad Prism.

2. Resazurin reduction

Values of at least two no effect concentrations were forced to upper asymptote exactly at 100 %, data sets were re-normalised as percentage of no effect concentrations. Curve fitting and BMRs values are generated using the *in vitro* toxicology on-line tool provided by AG. Leist, University of Konstanz

3. MMP with Rho123

An in house script was used to determine the maximal value of the concentration response curve. This value was forced to upper asymptote exactly at 100. Curve fitting and BMRs values are generated using the *in vitro* toxicology on-line tool provided by AG. Leist, University of Konstanz

- Lactate assay
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- Resazurin assay
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