European Commission

High throughput screening in nanotoxicology

Dr. Kirsten Gerloff

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EURL ECVAM
European Union Reference Laboratory for Alternatives to Animal Testing

Serving society
Stimulating innovation
Supporting legislation

Overview

- Motivation
- HTS/HCA facility at EURL-ECVAM
- Translating data into a regulatory context
- HTS in nanotoxicology
- Summary

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Motivation

Reasons for assay automation

- Efficiency – generate data faster
- Coverage – test more materials
- Precision – minimise technical variance
- Application – make ready for industrial use
- Necessity – validation of HTS-specific assays

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Workflow assay automation

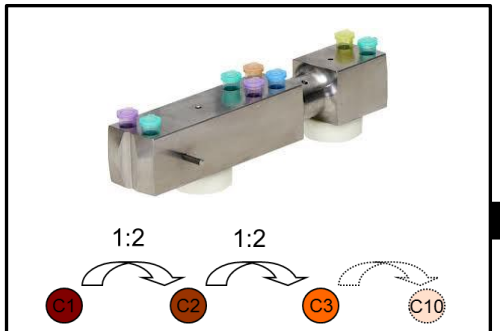
1. step

Cell culturing (manual)

2. step

Cell seeding (automated)

3. step



Particle preparation (semi-automated)

4. step

Cell treatment (automated)

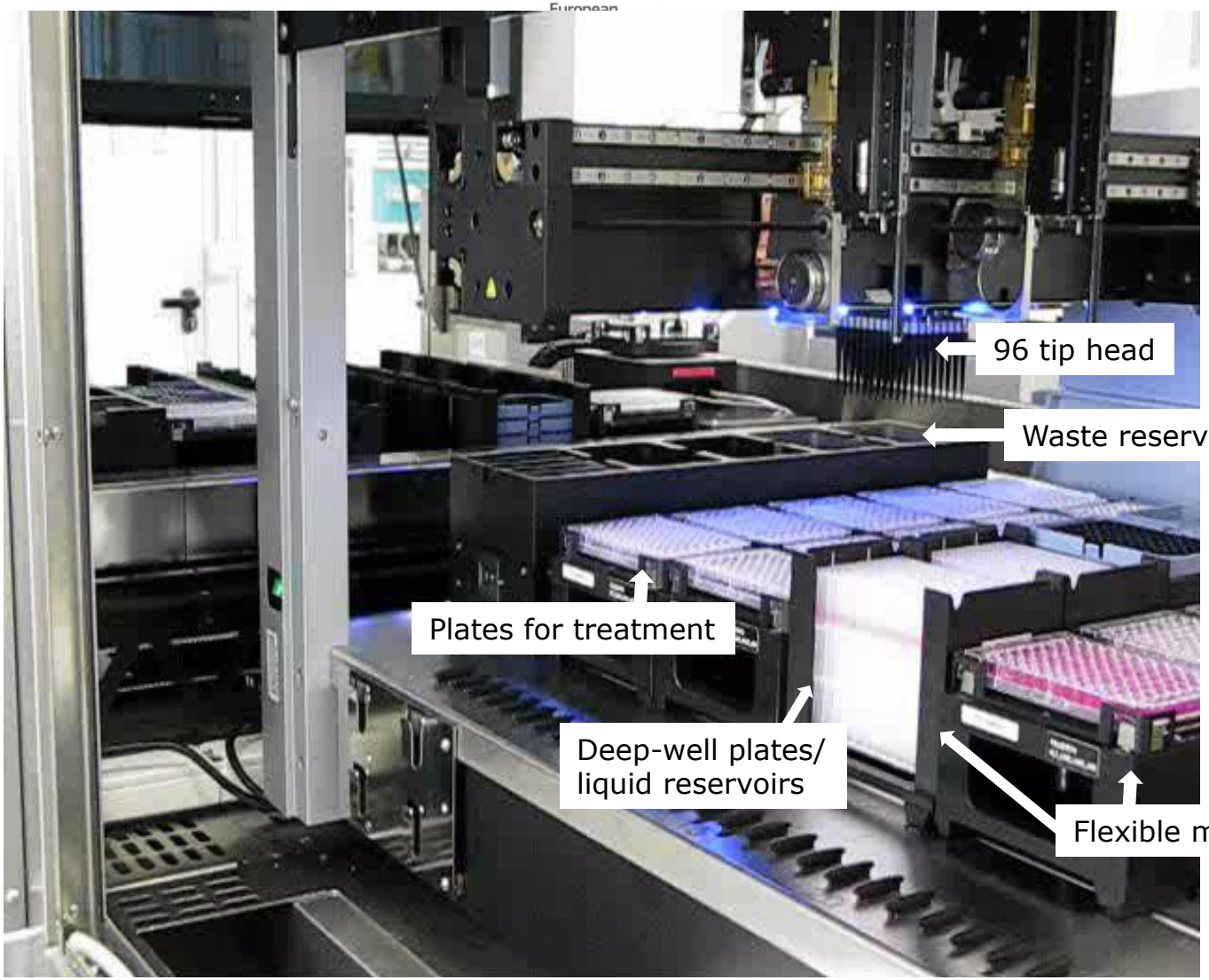
5. step

Staining (automated)

6. step

Automated detection (using "cellomics")

Automation platform



1 to 300µl volume handling

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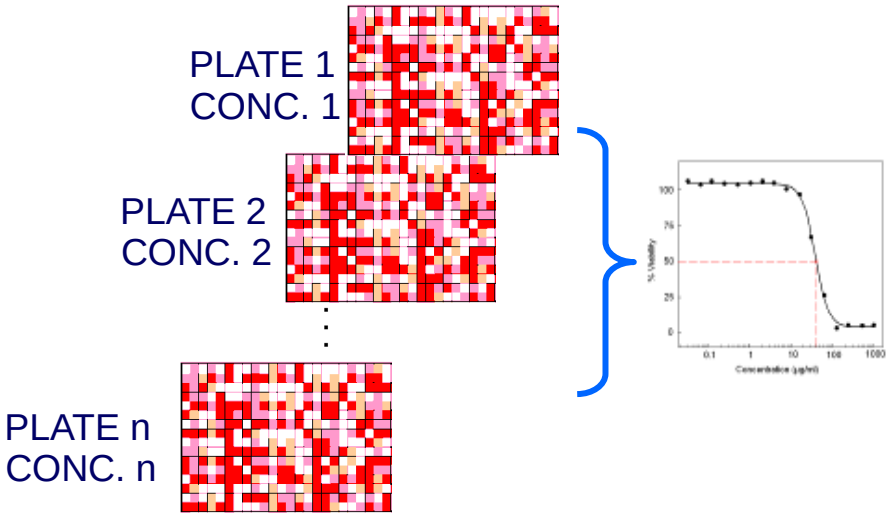
Quantative HTS*

Throughput



- Many compounds/plate
- Large (fixed) conc. range
- Conc. varies over plates

Quality of data

** Inglese J. et al. "Quantitative high-throughput screening…" PNAS 103,11473-11478, 2006*
Xia M. et al. "Compound cytotoxicity profiling…" Environmental Health Perspectives 116, 284-291, 2008.

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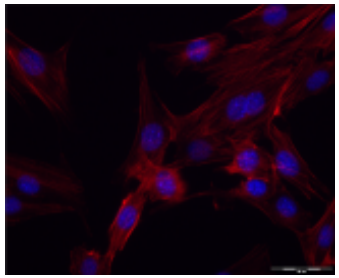
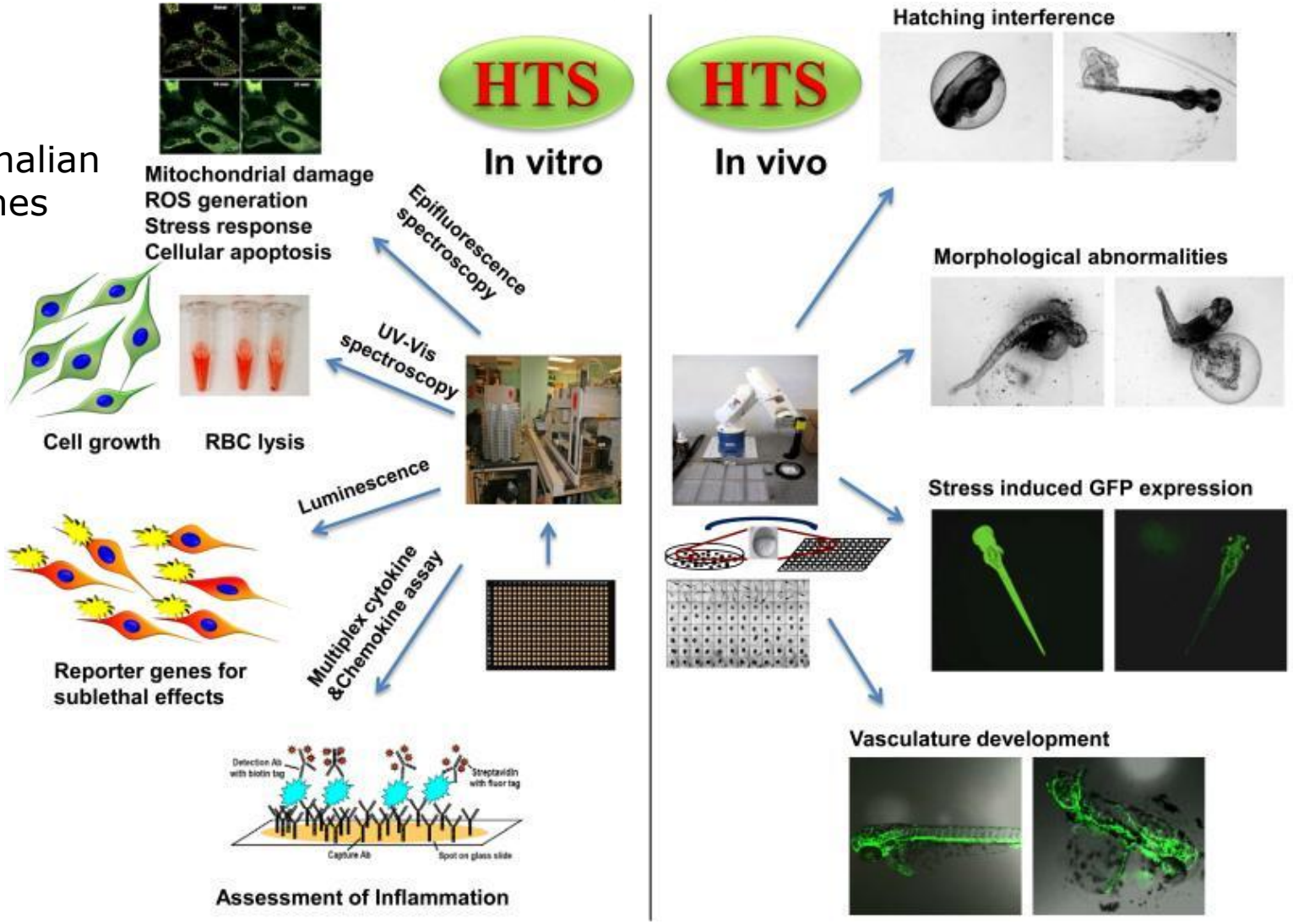
Challenges

- High setup and maintenance costs
- Handling of nanomaterials (sonication prior to treatment, sedimentation,…)
- Availability and quality of large material libraries
- Scale and reproducibility of cell culturing
- Operational complexity
- Heavy price for small mistakes



Mammalian cell lines

Zebrafish embryos



Nel et al., Acc Chem Res, 2013

Automated image acquisition

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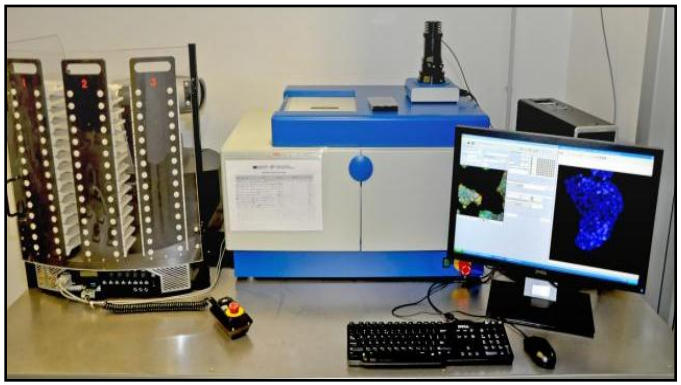
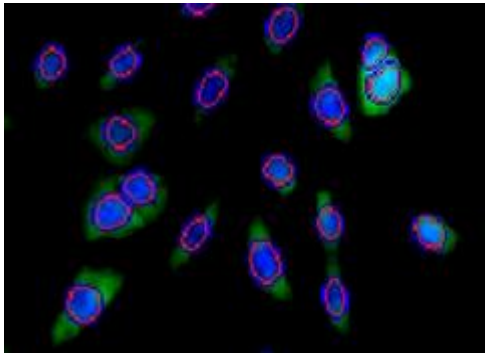
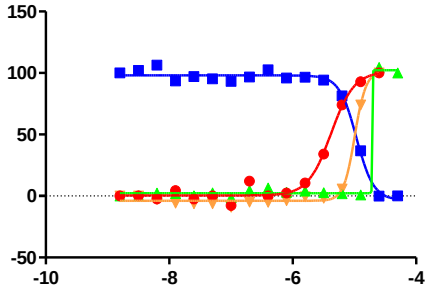
Cellomics



High content imaging (Cellomics)



Bioapplication mask



Dose - Response

Cell viability Endpoints (Channel 1)

- Cell count (nuclear identification)
- Nuclear morphology (size, shape)
- Nuclear brightness (apoptosis vs. necrosis)

Specific Endpoint – (up to 5 channels)

- Additional markers of cell viability (e.g. cell membrane integrity)
- Specific apoptosis markers
- Mitochondrial health
- DNA damage
- ROS induction
- Specific target organ toxicity (e.g. Steatosis development in the liver)

……

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Automated image acquisition

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Cellomics

Determining the best assay conditions

Suitable cell model
→ More complex if 3D cell models shall be used

Time lapse to find the best experimental time point

Assay needs to be suitable for automation
→ Fixation of cells

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Our cell system: HepaRG cells

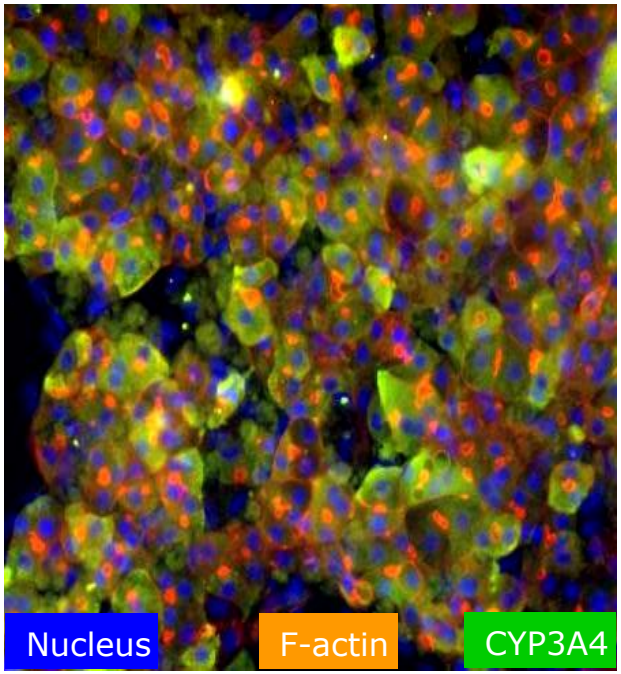
- **Human liver** cell line

- bipotent undifferentiated progenitor cells
→ Differentiation to hepatocytes

- expression of most of the liver specific genes:

  - **phase I and II enzymes**
  - **nuclear receptors**
  - **liver specific proteins**

→ Closely resemble **human primary hepatocytes**



Determining the best assay conditions

Suitable cell model
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Time lapse to find the best experimental time point

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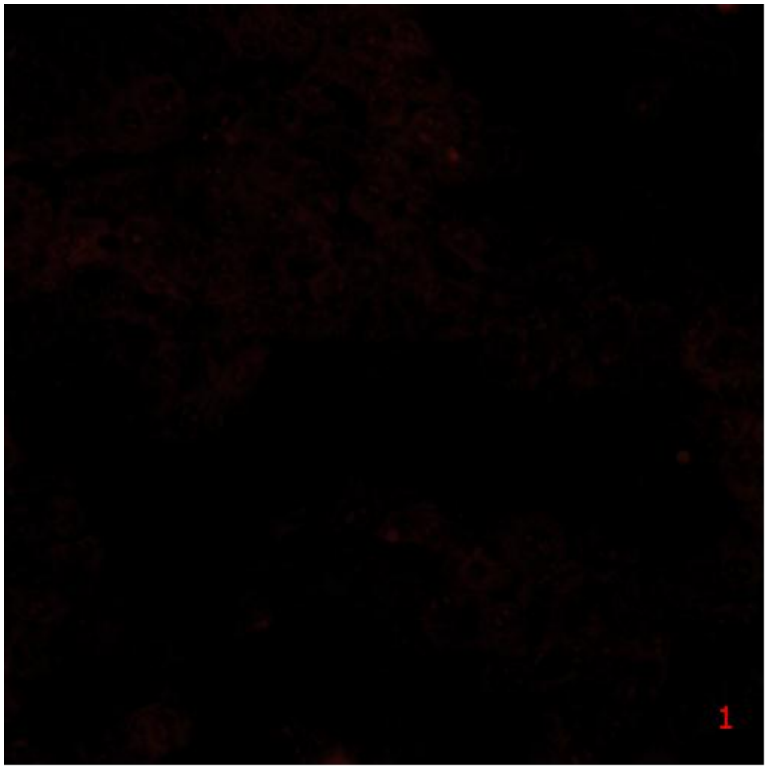
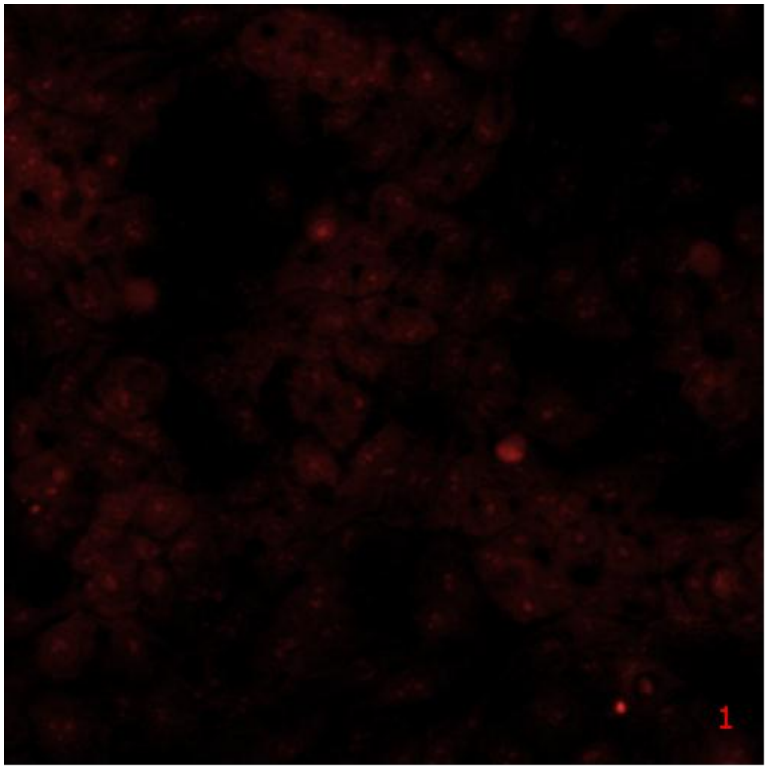
Formation of ROS (reactive oxygen species)
using DHE stain



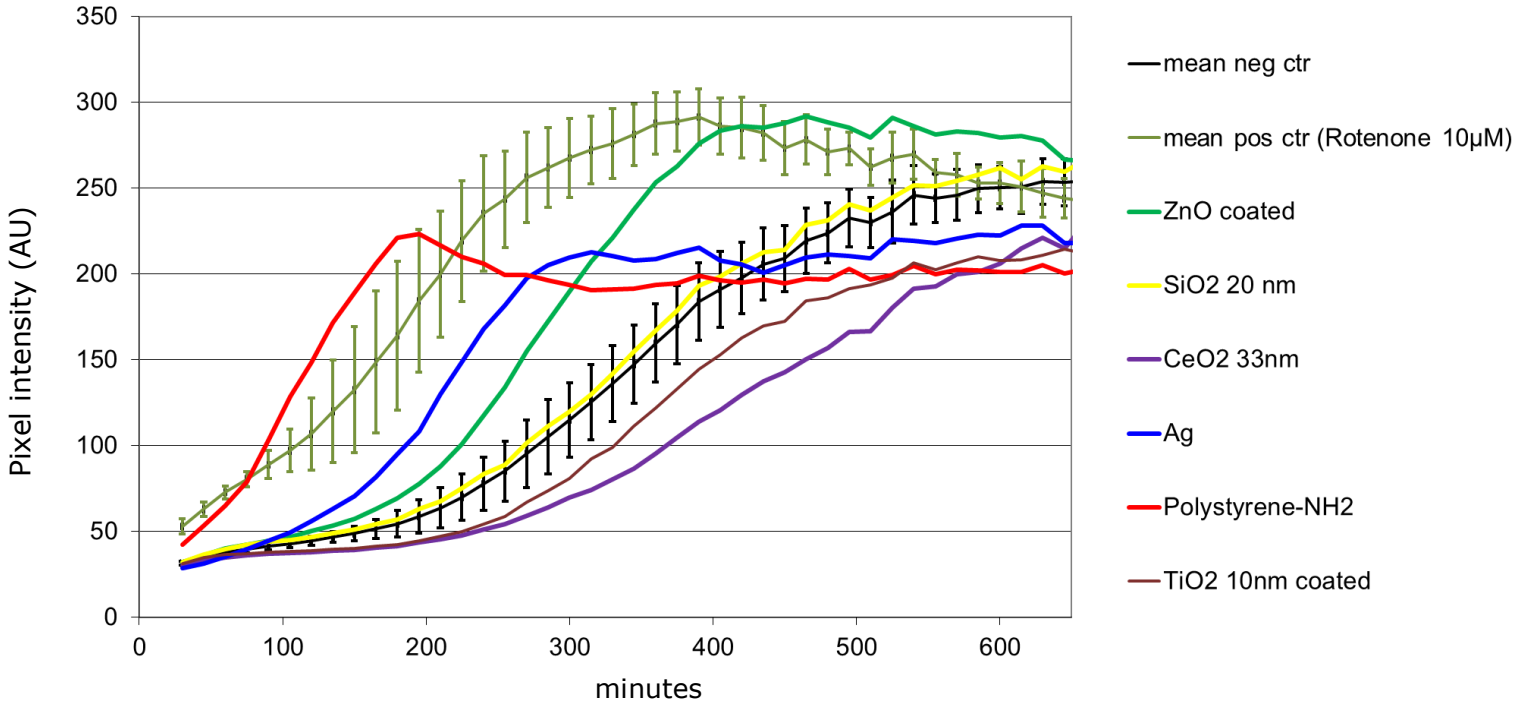
negative control (untreated cells)



positive control (cells treated with Rotenone)

1 time unit = 15 min

Formation of ROS in HepaRG cells



Determining the best assay conditions

Suitable cell model
→ More complex if 3D cell models shall be used

Time lapse to find the best experimental time point

Assay needs to be suitable for automation
→ Fixation of cells
to account for time delays that occur during image acquisition

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To integrate and make use of HTS data:

Adverse Outcome Pathways (AOPs)

→ Framework to allow usage of data

"a **conceptual framework** that portrays existing knowledge on the links between a **Molecular Initiating Event (MIE)** and an **Adverse Outcome (AO)**"

→ adverse health or ecotoxicological effect of regulatory concern

Launched by OECD in 2012: Guidance document available on OECD webpage

→ http://www.oecd.org/chemicalsafety/testing/adverse-outcome-pathways-molecular-screening-and-toxicogenomics.htm

→ http://ihcp.jrc.ec.europa.eu/our_activities/alt-animal-testing-safety-assessment-chemicals/improved_safety_assessment_chemicals/adverse-outcome-pathways-aop

Adverse Outcome Pathway (AOP)

Simplification of a toxic process

Systematic AOP development in the "AOP-wiki" → formation of an AOP network

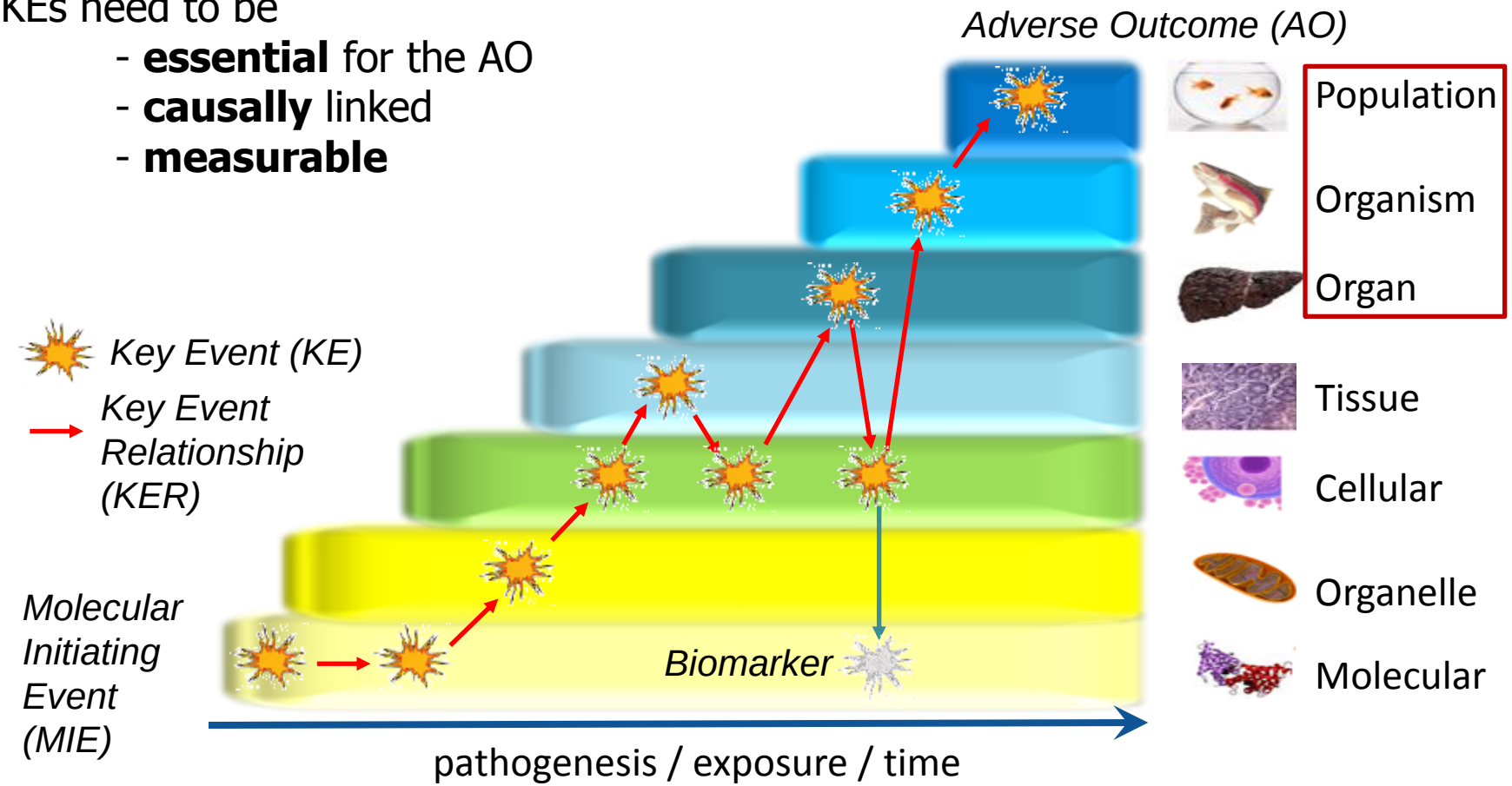
Adverse Outcome Pathway Wiki https://aopkb.org/

Integration of data from many different sources

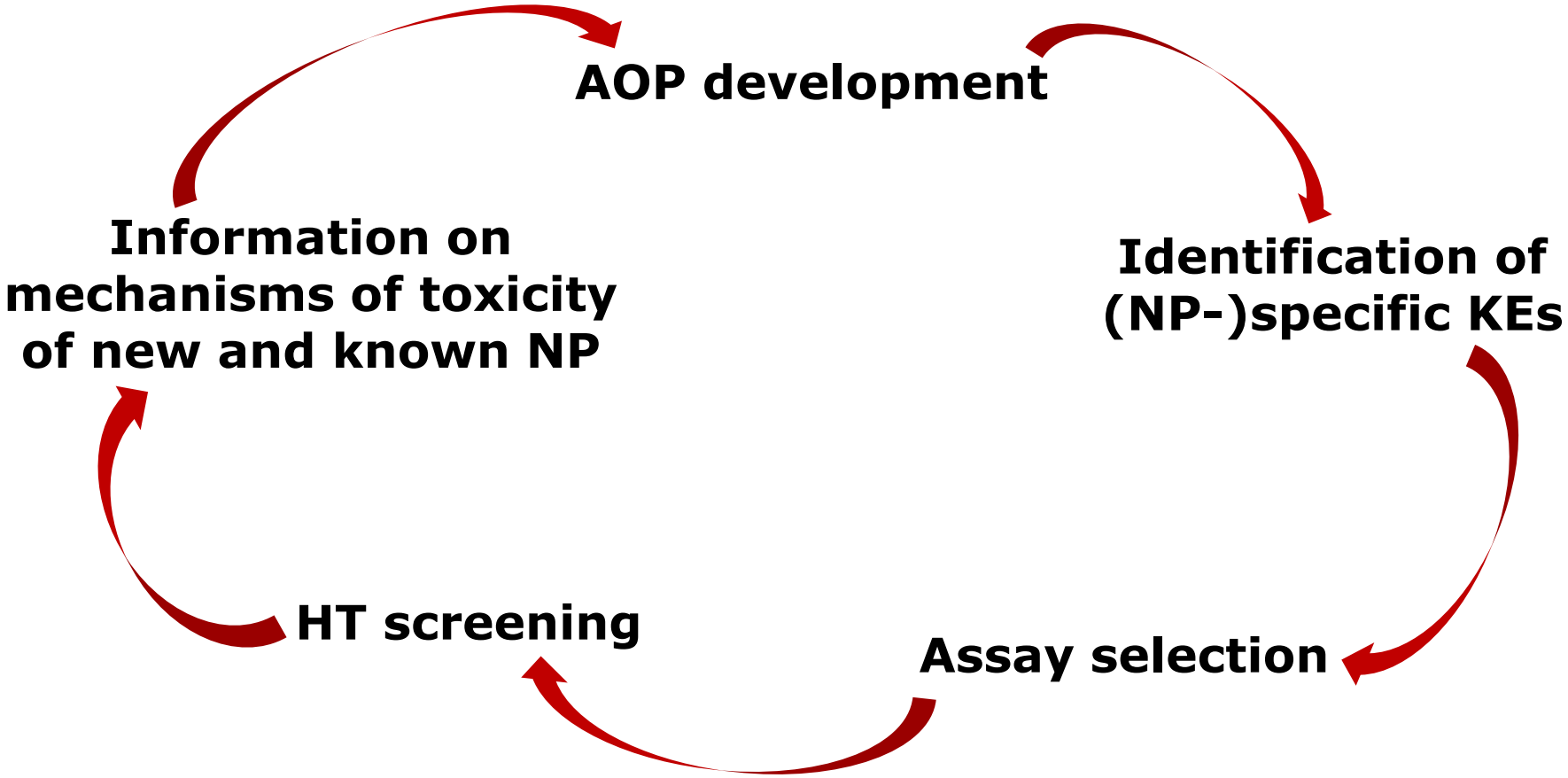
HTS-assay selection based on key events relevant for regulatory endpoints

Background – Adverse Outcome Pathway (AOP)

→ KEs need to be
- **essential** for the AO
- **causally** linked
- **measurable**



Practical implementations into HTS strategy

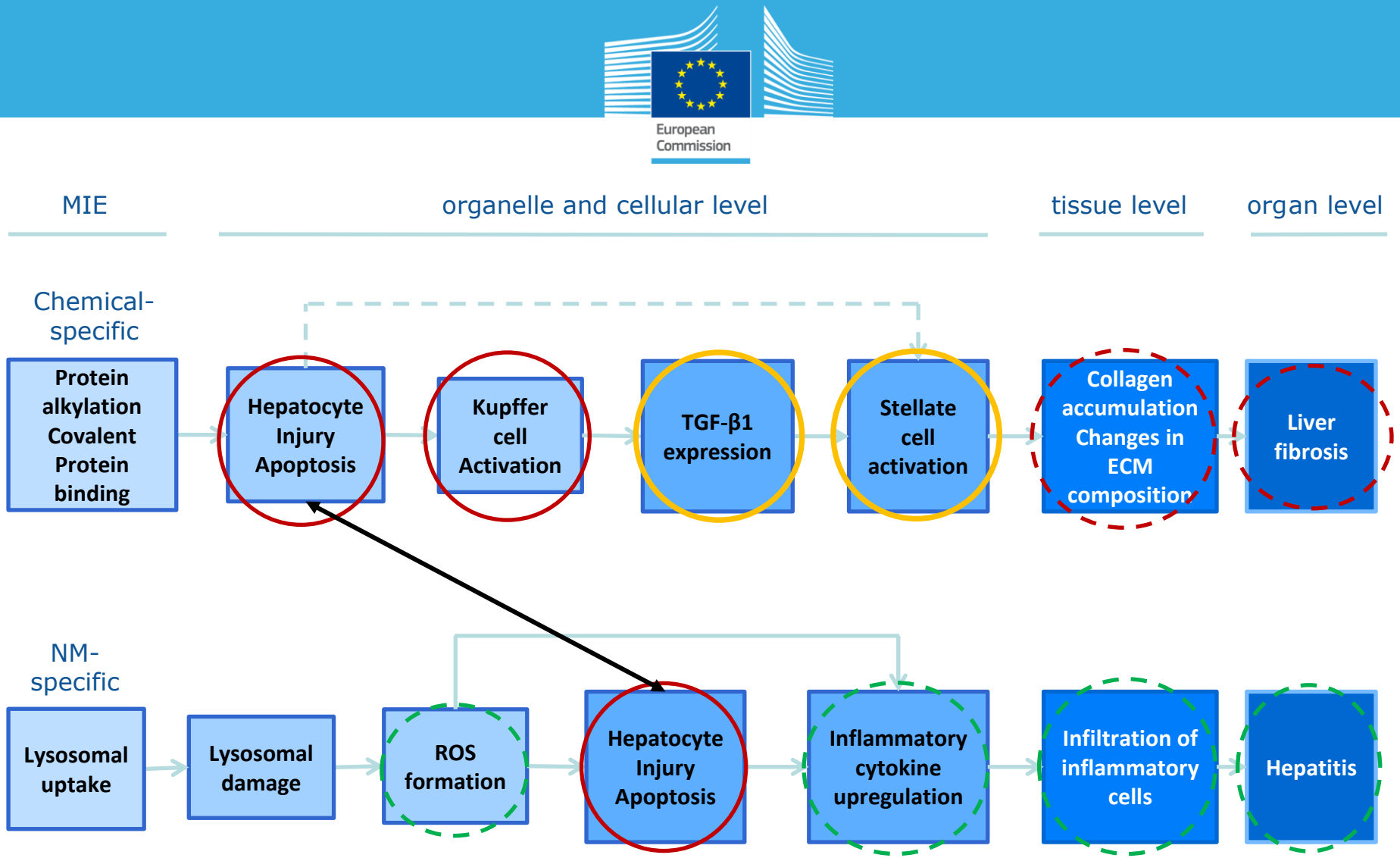


Opportunities in nano-AOP development

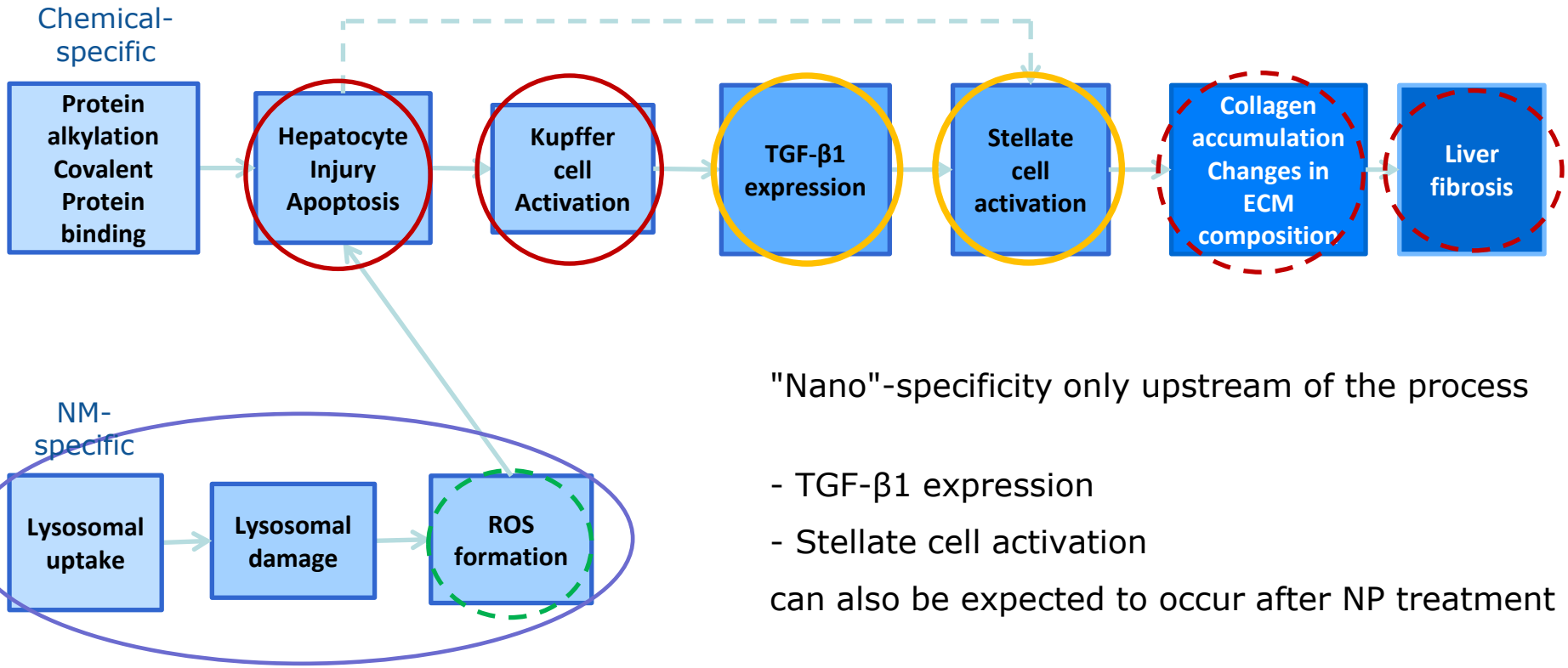
- Available AOPs based on chemical-induced AOs can inform nano-AOPs

→Fill in knowledge gaps

→Allows to focus research needs



Landesmann, aop-wiki
Gerloff et al., The AOP approach in nanotoxicology, in preparation



"Nano"-specificity only upstream of the process

- TGF-β1 expression
- Stellate cell activation

can also be expected to occur after NP treatment

Choice of assays in HTS based on KEs

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Landesmann, aop-wiki
Gerloff et al., The AOP approach in nanotoxicology, in preparation

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HTS workpackages/theme contents in FP7 project clusters

- NanoMILE
- NanoTest
- NanoSolutions
- NanoReg
- Marina
- Sun

- UC-CEIN (USA, under A. Nel)

NanoMILE WP4 HTS data gathering

Snapshot of preliminary results

	JRC	partner 1		partner 2	partner 3		effect
	HepaRG liver cells	HepG2 liver cells	RAW264.7 macrophages	A549 lung cells	A549 lung cells	zebrafish embryos	
incubation time	24 h	24h	24h	24h	24h	120 h	
TiO2 10nm, uncoated		n.d.	n.d.		n.d.		membrane damage/cell count
	n.d.	n.d.	n.d.				mitochondrial health
					n.d.		apoptosis
			n.d.				lysosomal acidification
	n.d.						steatosis
						n.d.	mortality
						n.d.	hatching
						n.d.	morphology
TiO2 10nm, coating 1		n.d.	n.d.		n.d.		membrane damage/cell count
	n.d.	n.d.	n.d.				mitochondrial health
					n.d.		apoptosis
			n.d.				lysosomal acidification
	n.d.						steatosis
						n.d.	mortality
						n.d.	hatching
						n.d.	morphology
TiO2 10nm, coating 2		n.d.	n.d.		n.d.		membrane damage/cell count
		n.d.	n.d.				mitochondrial health
					n.d.		apoptosis
			n.d.				lysosomal acidification
	n.d.						steatosis
						n.d.	mortality
						n.d.	hatching
						n.d.	morphology
TiO2 10nm, coating 3		n.d.	n.d.		n.d.		membrane damage/cell count
	n.d.	n.d.	n.d.				mitochondrial health
					n.d.		apoptosis
			n.d.				lysosomal acidification
	n.d.						steatosis
						n.d.	mortality
						n.d.	hatching
						n.d.	morphology
TiO2 20nm, hydrophobic	n.d.				n.d.		membrane damage/cell count
	n.d.						mitochondrial health
					n.d.		apoptosis
							lysosomal acidification
	n.d.						steatosis
						n.d.	mortality
						n.d.	hatching
						n.d.	morphology
TiO2 20nm, hydrophilic	n.d.				n.d.		membrane damage/cell count
	n.d.						mitochondrial health
					n.d.		apoptosis
							lysosomal acidification
	n.d.						steatosis
						n.d.	mortality
						n.d.	hatching
						n.d.	morphology

Adverse Effect (AE) intensity

empty	Not analysed
n.d.	AE not detectable
	AE at high concentrations
	AE at intermediate concentrations
	AE at low concentrations

Earlier time point?

Later time point?

24 h treatment of HepaRG cells

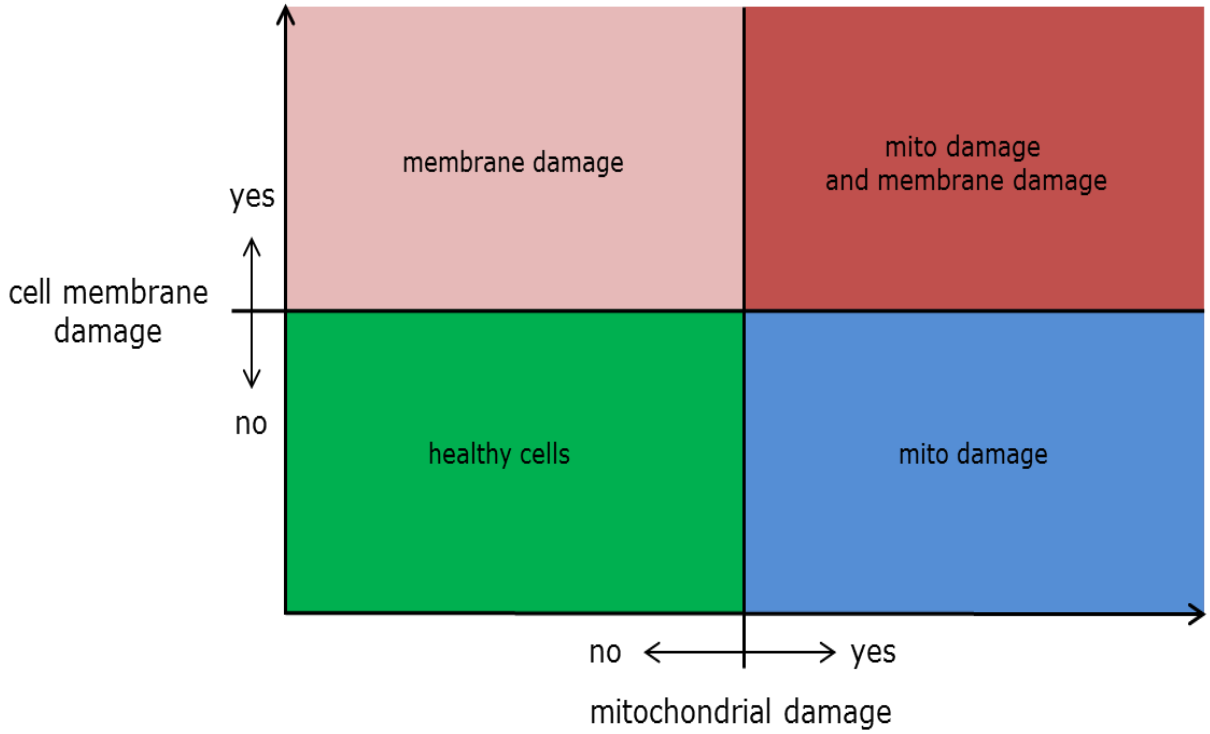
particle	mitochondrial membrane potential	cytoplasmic caspase3	nuclear caspase3	nuclear size	nuclear intensity	lipid droplet size	cell membrane damage	cell count
CeO2 20 nm	31.3	15.6	62.5	125	31.3	>250	31.3	>250
CeO2 33 nm	>250	>250	>250	>250	125	>250	>250	>250
Ag	15.6	>250	3.9	1.95	7.8	3.9	1.95	1.95
TiO2 10nm, uncoated	31.3	7.81	62.5	7.81	7.81	>250	7.8	31.3
TiO2 10nm, coating 1	31.3	7.81	15.625	7.81	7.81	>250	31.3	31.3
TiO2 10nm, coating 2	31.3	7.81	7.8125	7.81	7.81	>250	7.8	15.6
TiO2 10nm, coating 3	31.3	7.81	31.25	7.81	7.81	>250	15.6	31.3
TiO2 20nm, hydrophobic	>250	15.63	125	>250	>250	>250	125	125
TiO2 20nm, hydrophilic	>250	7.81	62.5	>250	>250	>250	62.5	>250
ZnO 150nm, uncoated	62.5	125	15.6	125	31.3	31.3	15.6	62.5
ZnO 140nm, coated	31.3	31.3	15.6	31.3	15.6	15.6	31.3	31.3
SiO2 < 20 nm	>250	>250	>250	>250	>250	>250	>250	>250
SiO2 25-30 nm	>250	>250	>250	>250	>250	>250	>250	>250
SiO2 50-60 nm	>250	>250	>250	>250	>250	>250	>250	>250
SiO2 100 nm	>250	>250	62.5	>250	>250	>250	>250	>250
SiO2-NH2 < 20 nm	31.3	31.25	62.5	>250	31.3	15.6	2.0	>250
SiO2-NH2 25-30 nm	62.5	31.3	62.5	>250	62.5	125	2.0	>250
SiO2-NH2 50-60 nm	>250	125	62.5	1.95	31.3	125	3.9	>250
SiO2-NH2 100 nm	>250	>250	125	>250	125	125	7.8	>250
SiO2-COOH < 20 nm	>250	>250	62.5	125	125	125	62.5	125
SiO2-COOH 25-30 nm	>250	>250	>250	>250	>250	>250	>250	>250
SiO2-COOH 50-60 nm	>250	>250	>250	>250	>250	>250	125	>250
SiO2-COOH 100 nm	>250	>250	>250	>250	>250	>250	>250	>250

Size effect?

first concentration (in µg/ml) at which a significant difference with respect to the negative ctr is found

Information from a single assay:

"mitochondrial damage" assay provides multiple parameters



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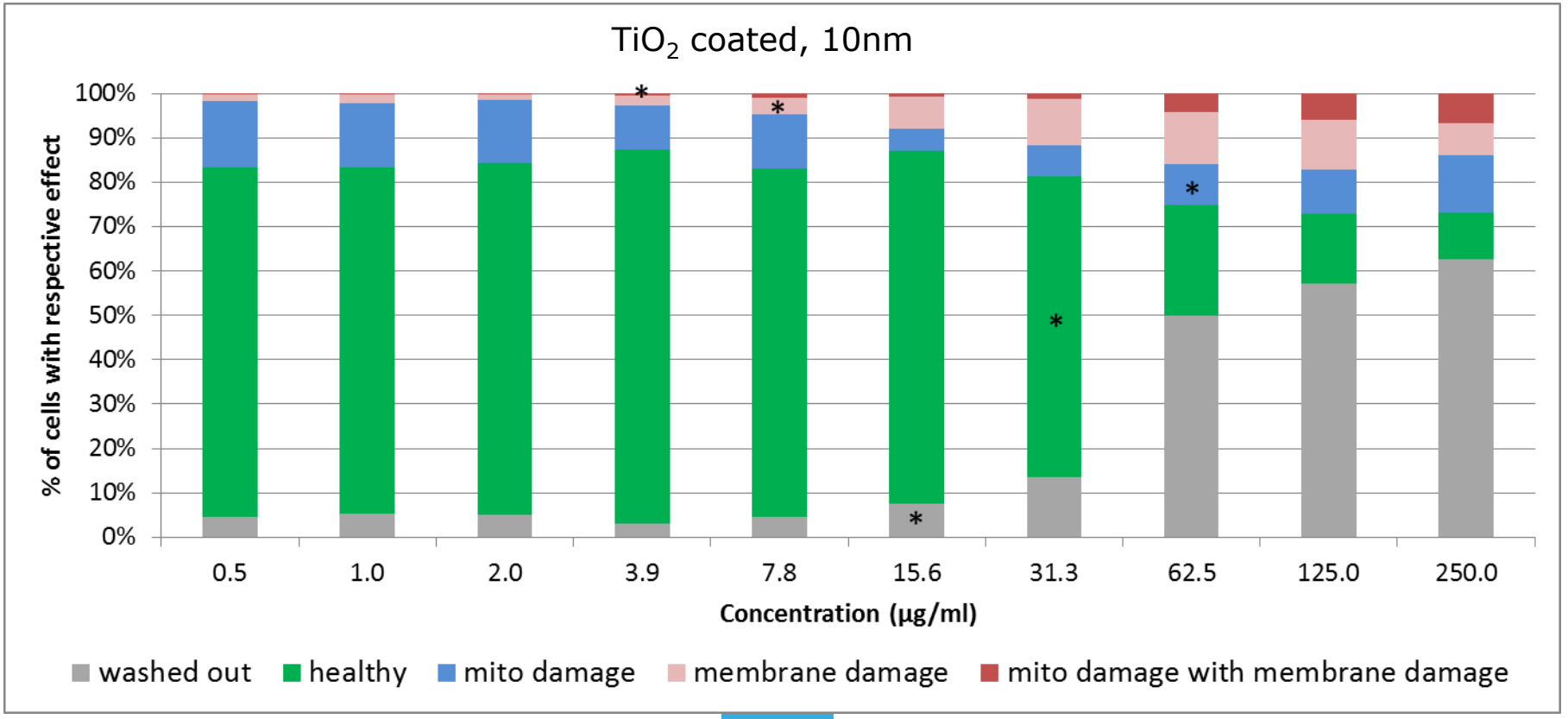

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Summary

- HTS of NMs is a useful tool for predictive nanotoxicology and safe NM design
- Shortcomings as for any *in vitro* assay
- opportunities: ultimate goal is the reduction of *in vivo* experiments and rapid acquisition of toxicity data
- Useful implementation of large amount of data by applying AOPs

Joint Research Centre (JRC)

The European Commission's in-house science service

Maurice Whelan
Brigitte Landesmann
Sharon Munn
Andrew Worth

Taina Palosaari
Elisabeth Joossens
Peter Macko
Jean-Michel Gineste

Douglas Gilliland
Isaac Ojea Jimenez

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WP4
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(KIT Germany)

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