ADVANCES IN GENOTOXICITY TESTING, PART 2:
The reconstructed human skin micronucleus assay

ASCCT webinar series

Stefan Pfuhler
Procter & Gamble
CE TG Genotoxicity

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Agenda

1. Background
2. Concept of the use of 3D skin tissue equivalents in genotoxicity testing
3. Reconstructed skin comet assay
4. Reconstructed skin micronucleus assay
5. Strategic fit of assays in testing strategies and examples
6. Summary/outlook

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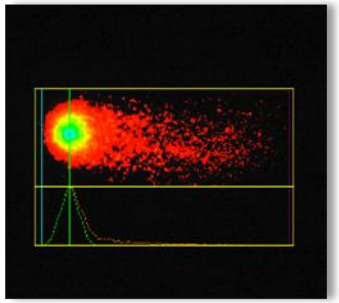
Dermal route

Dermal Route

3D Skin Comet

Reconstructed Skin Comet assay

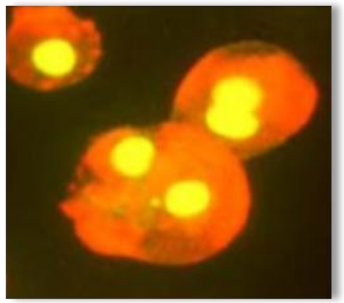


Phenion® Full-Thickness Skin Model

www.phenion.com

RSMN

Reconstructed Skin MicroNucleus test



EpiDerm™ (MatTek)

- Test systems combined with classical read-outs.
- Battery of two assays addresses all three endpoints.

Assay	Mutation	Structural	Numerical
		Chromosome damage	
RS Comet	x	x	
RSMN		x	x

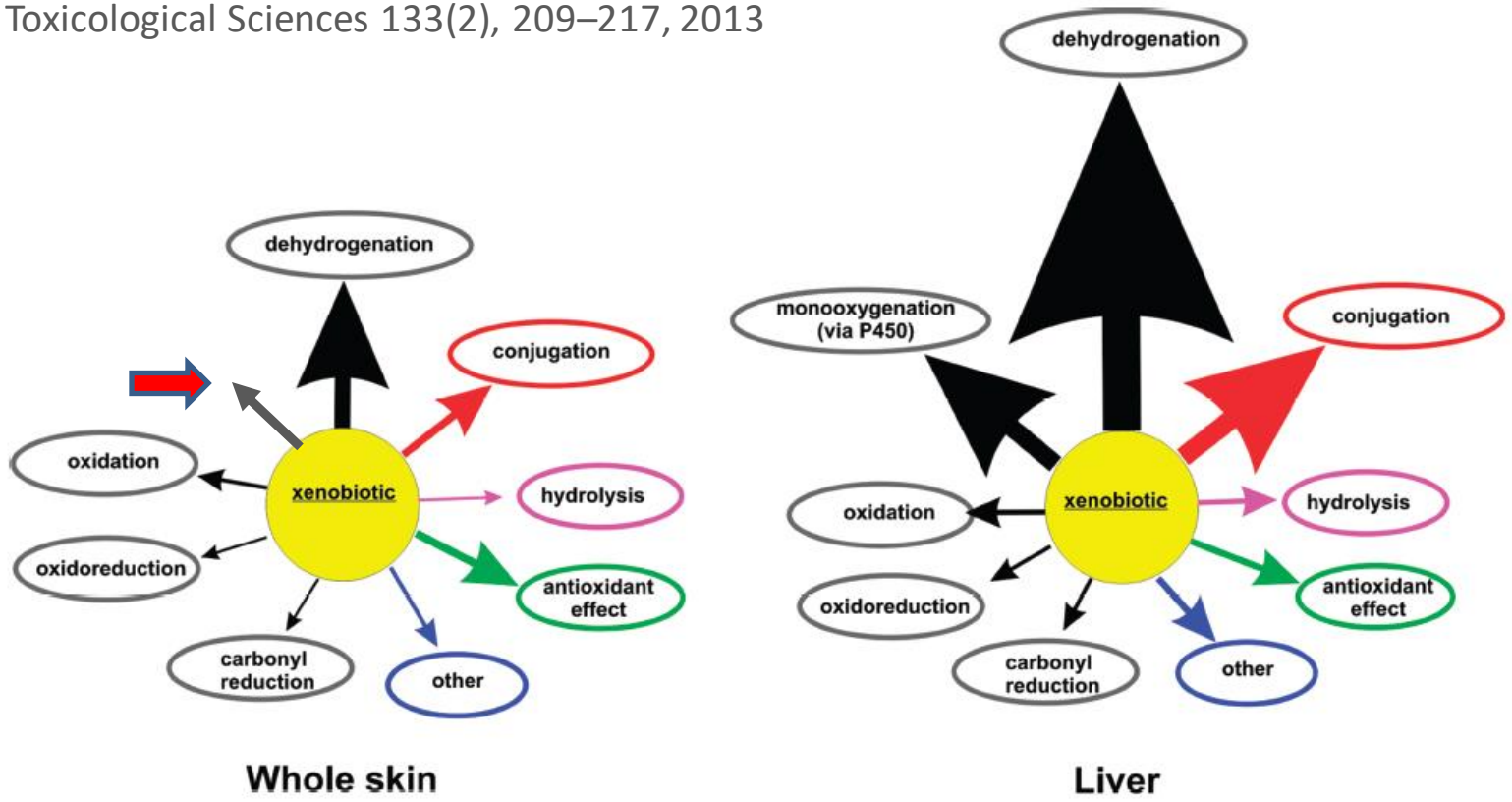
- Assays are intended to follow up on initial positive findings.

Understanding skin metabolism

Dermal application of test material, multiple application protocol (enables enzyme induction) ➡

Metabolic competency of 3D skin models similar to human skin

Hewitt et al, Toxicological Sciences 133(2), 209–217, 2013



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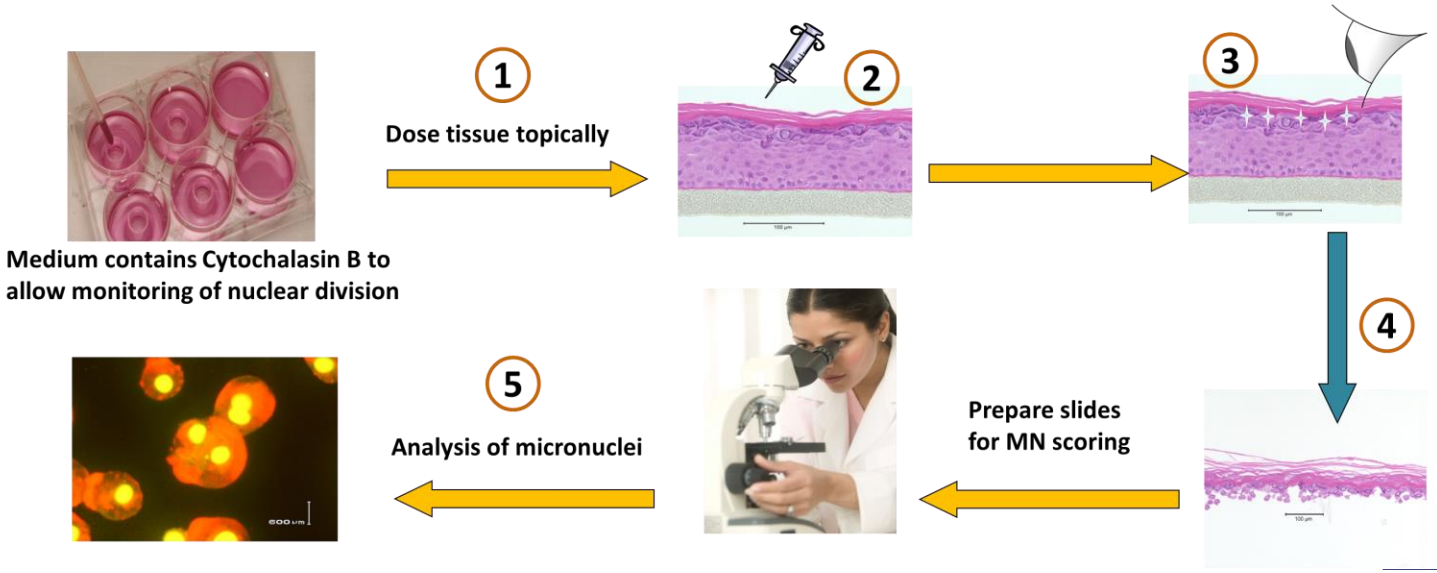
Test Principle: RSMN

- Assay is built on reconstructed human skin tissues using micronucleus OECD 487 technology
- Assay development: Collaboration between IIVS and P&G (Curren et al., 2006)
- Protocol refinement and start of an international validation effort in 2007



1. EpiDerm™ models are treated topically with test compound.
2. Dose at 24h intervals (48h or 72h total)
3. Precipitation at the beginning and the end of the treatment period is noted.
4. Keratinocytes are released by trypsinization
5. Micronuclei in binucleated cells are counted by visual scoring.

Detailed methodology info:
See Dahl et al, 2011

Contents lists available at ScienceDirect
Mutation Research/Genetic Toxicology and Environmental Mutagenesis
journal homepage: www.elsevier.com/locate/gentox
Community address: www.elsevier.com/locate/mutres

The reconstructed skin micronucleus assay (RSMN) in EpiDerm™: Detailed protocol and harmonized scoring atlas

Erica L. Dahl[a,*], Rodger Curren[a], Brenda C. Barnett[b,g], Zubin Khambatta[b], Kerstin Reisinger[c], Gladys Ouedraogo[d], Brigitte Faquet[d], Anne-Claire Ginestet[d], Greg Mun[a], Nicola J. Hewitt[e], Greg Carr[b], Stefan Pfuhler[b], Marilyn J. Aardema[f]

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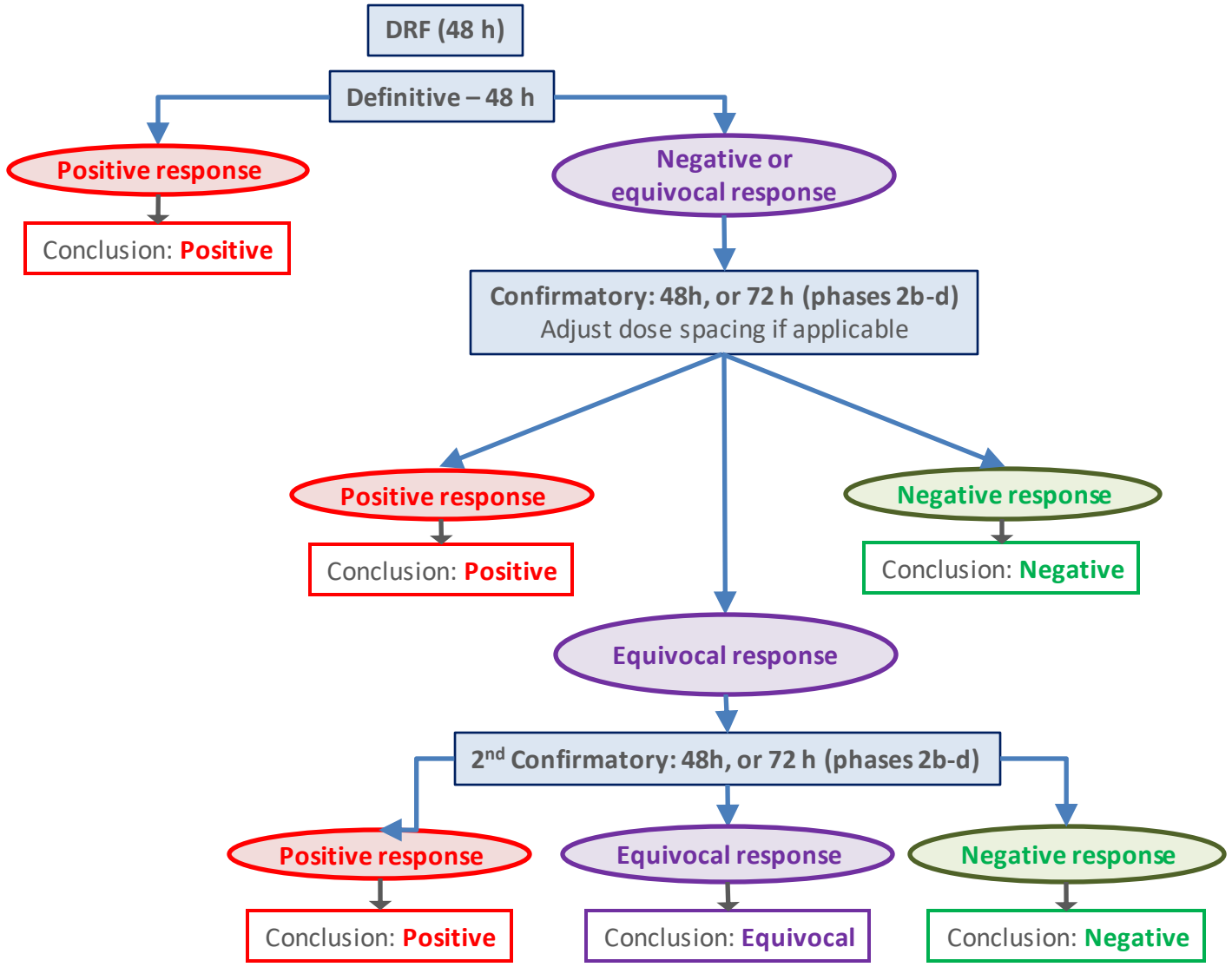
Experimental design

- OECD 487 cytoB method, modified
- 2 or 3 treatments at (-72), -48 and -24h
- Minimum of 3 doses
- 3 tissues/dose (2 acceptable)
- 500 binucleated cells evaluated/tissue
- Maximum dose: 1600 ug/cm^2
- If cytotoxic, aiming at:
  - 50 ± 10% (high cytotoxicity)
  - 30 ± 10% (intermediate cytotoxicity)
  - 10 ± 10% (low cytotoxicity)
- Toxicity measures:
  - % binucleation (>40% control)
  - Cell count (>40% control)
  - More sensitive defines cutoff

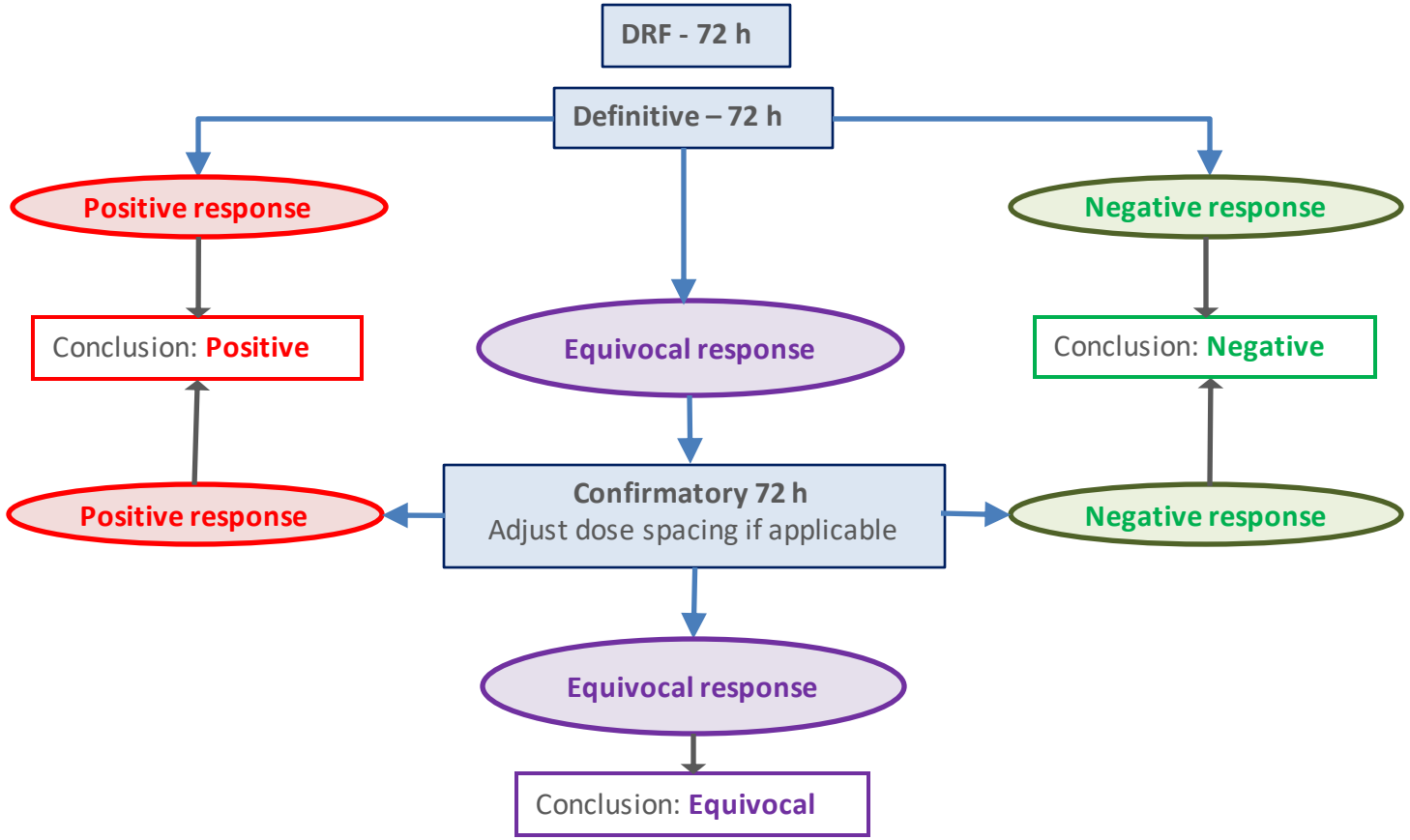
Decision tree for **validation** exercise



Experimental design – new (as per IWGT recommendation)

Pfuhler et al., 2020

Recommended decision tree, using the 72h protocol only. Decsion tree is in line with OECD 487 where clear positive or clear negative results do not need to be reproduced.



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Validation setup

- International validation team, with involvement of EURL ECVAM from the start
- Substance selection via external subject matter experts
- Steering Team of experts, extended team as needed (e.g., decision making for next steps)
- International laboratories (6 total) experienced in genotoxicity testing and with working with 3D skin models
- Constant discussion/calibration with scientific community (over 100 presentations and publications)

RS assay project – validation outline

Phase 1
Optimization and transferability with 2 model genotoxins

Phase 2
Intra- and inter-lab reproducibility with 5-10 coded compounds

Phase 3
Validation with 30+ coded Compounds per assay

Selection of compounds:

Initial selection by international subject matter experts (assay experts, skin metabolism and skin cancer experts): final selection of validation subset by Raffaella Corvi (EURL-ECVAM), David Kirkland (Kirkland consulting)

Coding & shipment of chemicals:

EURL-ECVAM, Italy; ZEBET, Germany; Covance, UK; VitroScreen, Italy; Integrated Laboratory Systems, Inc. USA, BioTeSys, Italy

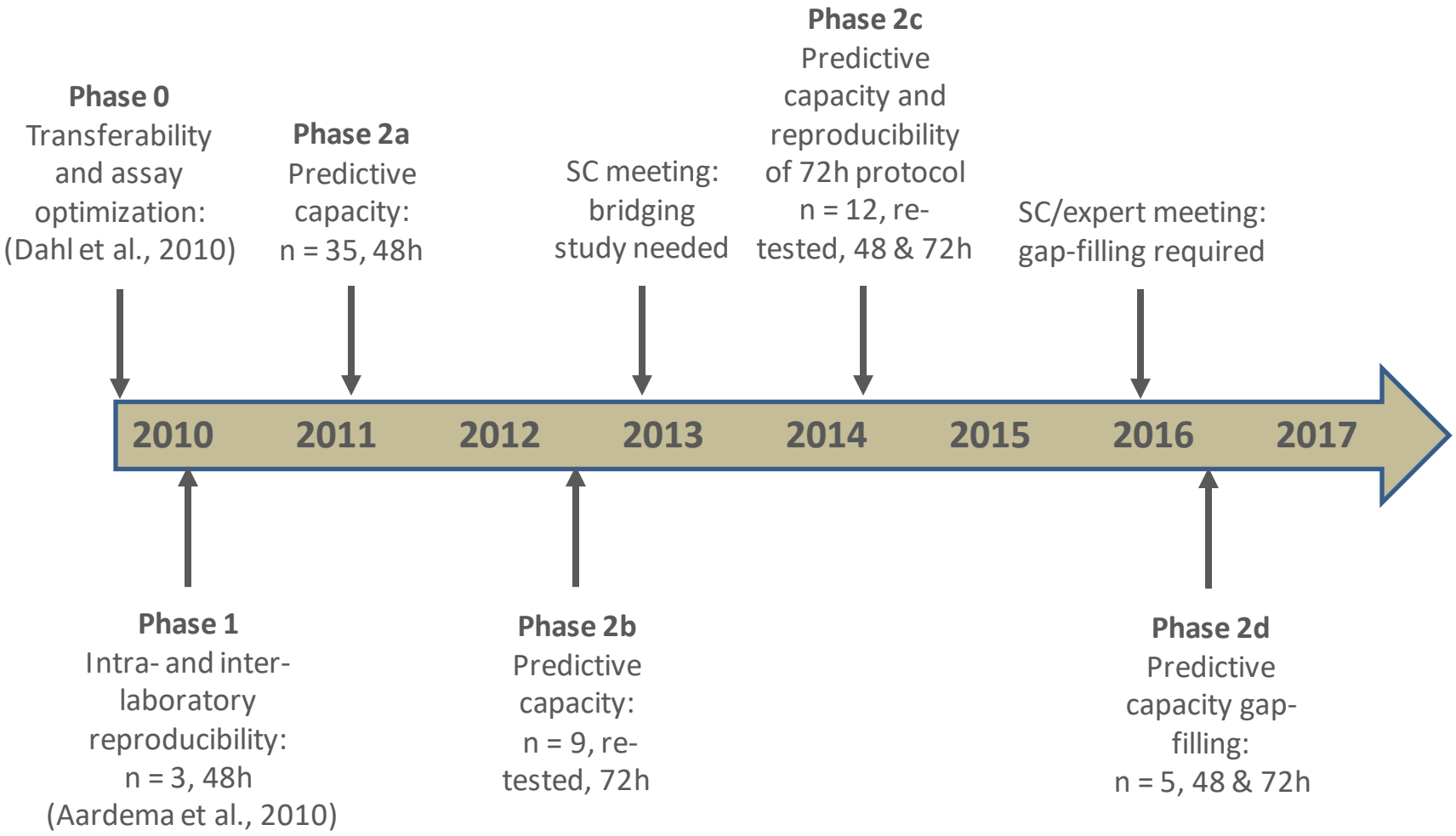
Decoding:

Raffaella Corvi (EURL-ECVAM)

Independent analysis of data:

Sebastian Hoffmann (seh consulting & services); Ralph Pirow, BfR, Germany

Validation timeline



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Validation outcome - Mutagenesis Special Topic “3D Skin”

- Edited by Shareen Doak; Guest Editors: Rafaella Corvi & Stefan Pfuhler
- April 2021
- 5 manuscripts, including the RS Comet and RSMN validation papers
- [Volume 36 Issue 1 | Mutagenesis | Oxford Academic (oup.com)]

Mutagenesis, 2021, 36, 1–17
doi:10.1093/mutage/geaa035
Original Manuscript
Advance Access publication 5 February 2021

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Editor's choice

Original Manuscript

Validation of the 3D reconstructed human skin micronucleus (RSMN) assay: an animal-free alternative for following-up positive results from standard *in vitro* genotoxicity assays

Stefan Pfuhler[1,*], Thomas R. Downs[1], Nicola J. Hewitt[2], Sebastian Hoffmann[3], Greg C. Mun[4], Gladys Ouedraogo[5], Shambhu Roy[6], Rodger D. Curren[4] and Marilyn J. Aardema[7]

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Mutagenesis, 2021, 36, 19–35
doi:10.1093/mutage/geaa009
Original Manuscript
Advance Access publication 10 March 2020

OXFORD

Original Manuscript

Validation of the 3D reconstructed human skin Comet assay, an animal-free alternative for following-up positive results from standard *in vitro* genotoxicity assays

Stefan Pfuhler[1,*], Ralph Pirow[2], Thomas R. Downs[1], Andrea Haase[2], Nicola Hewitt[3], Andreas Luch[2], Marion Merkel[4], Claudia Petrick[4], André Said[2,5], Monika Schäfer-Korting[5] and Kerstin Reisinger[4]

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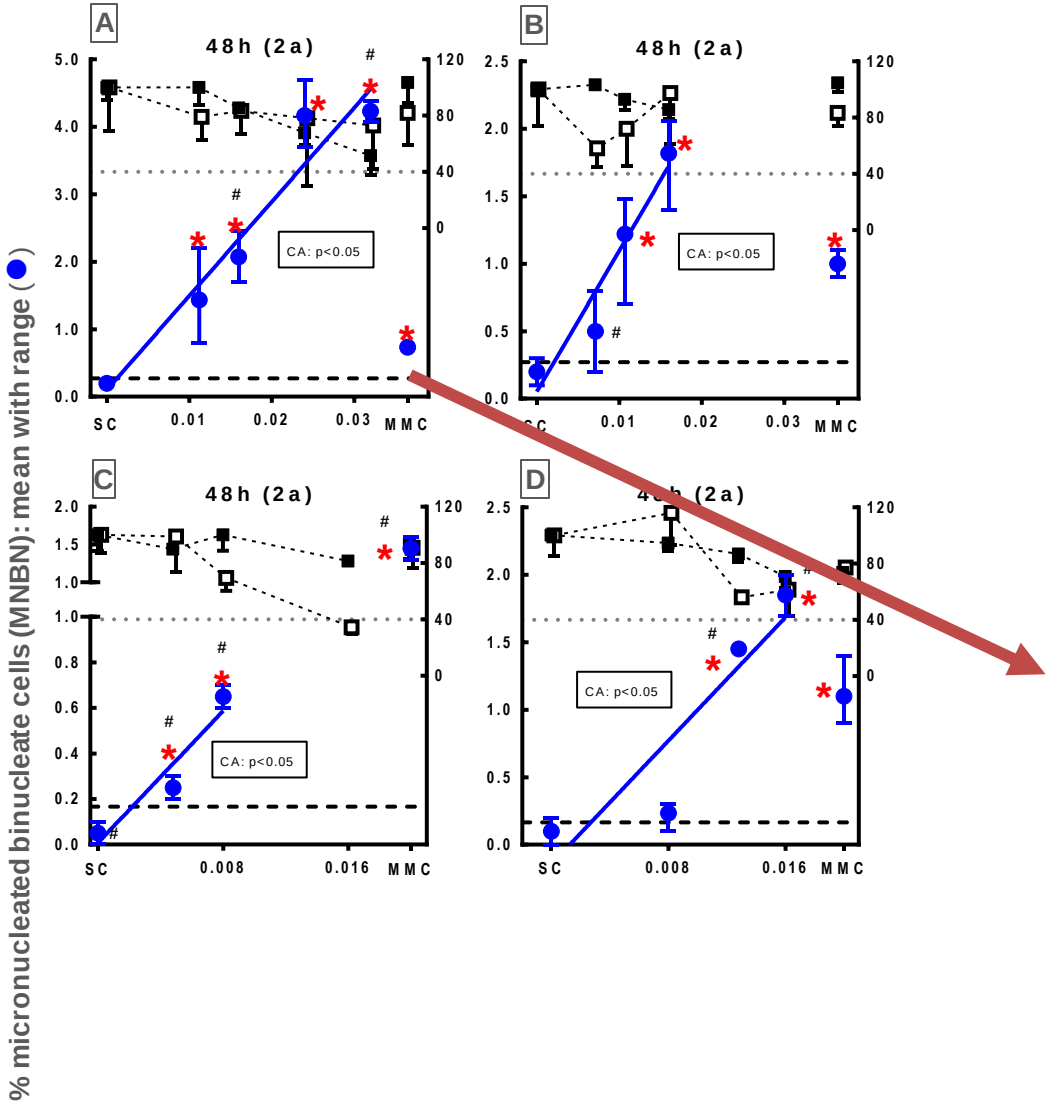
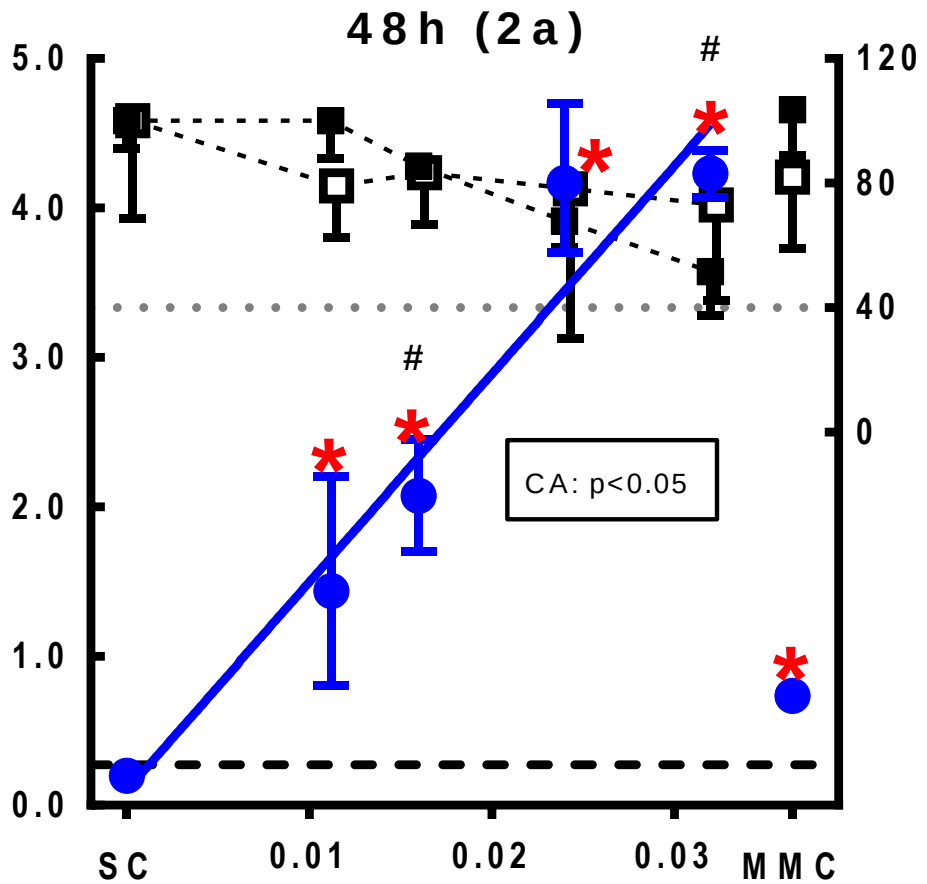
Examples from Validation dataset

a) Figure S5: Colchicine
b) Figure S14: 5-fluorouracil

(Data from: Pfuhler et al, Mutagenesis, 2021, 36, 1–17 – Supplemental figures)

Figure S5: colchicine





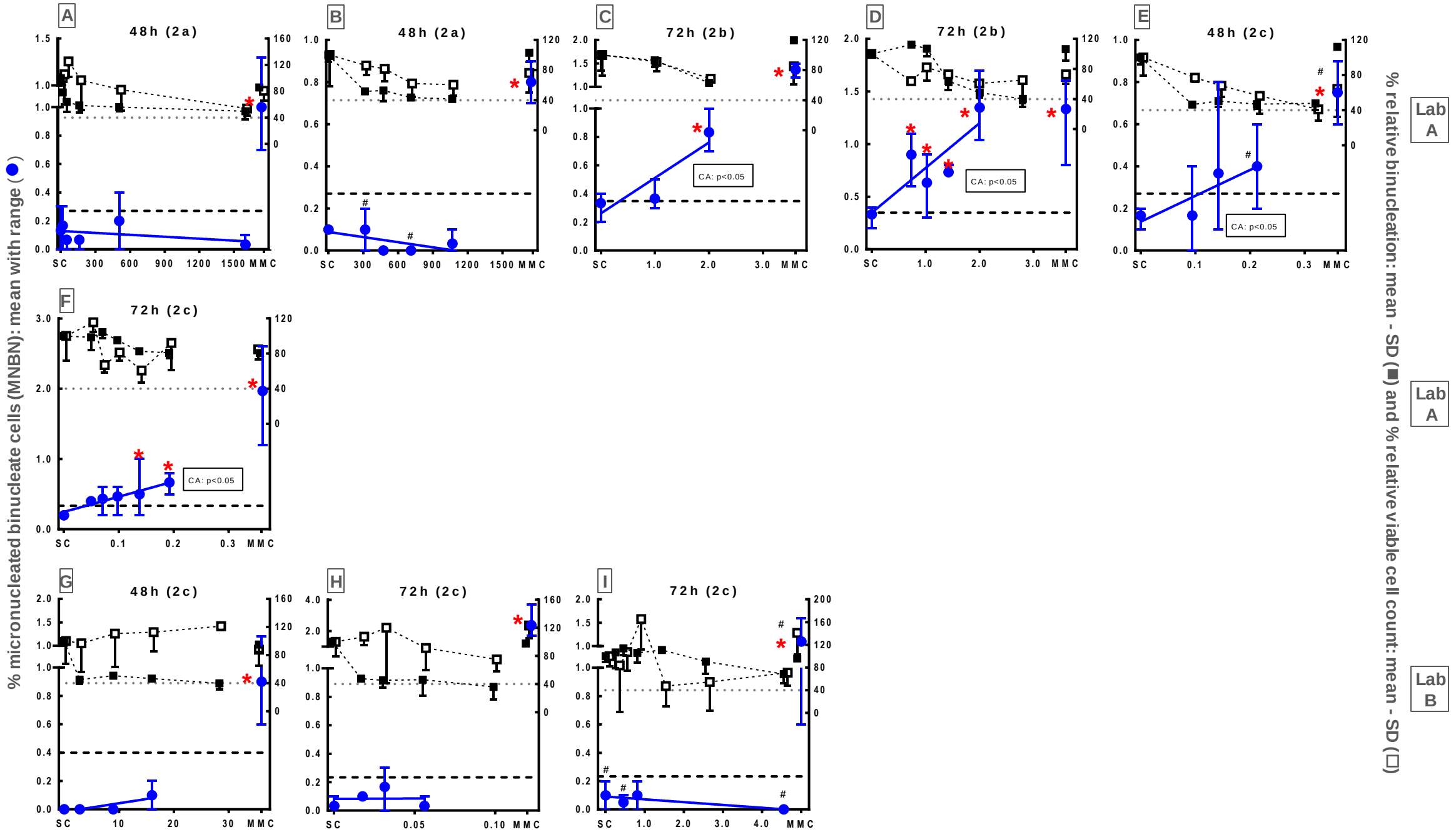
Lab A

Lab C

% relative binucleation: mean - SD (■) and % relative viable cell count: mean - SD (□)

Dose (µg/cm^2)

Figure S14: 5-fluorouracil



Validation outcome

See Pfuhler et al, 2021

Table 1. Overview of validation outcome of the RSMN experiments conducted within the coded validation effort in all phases

Chemical	CAS No.	Cat	Phase	Lab A	Lab B	Lab C	Lab D	BLR
2-Acetylaminofluorene (2-AAF)	53-96-3	TP	2a,c		Neg	Neg	Neg	1
2-Amino-3-methylimidazo[4,5-*f*]quinolone (IQ)	76180-96-6	TP	2d	Pos				–
Azidothymidine (AZT)	30516-87-1	TP	2d	Pos				–
Cadmium chloride ($CdCl_2$)	10108-64-2	TP	2a,b,c	Pos	Neg	Pos		0
Colchicine	64-86-8	TP	2a	Pos		Pos		1
Cyclopenta[*c,d*]pyrene (CPPE)	27208-37-3	TP	2a,b		Pos	Neg[a]		–
Cytosine arabinoside	147-94-4	TP	2a,b		Neg			–
2,4-Diaminotoluene (2,4-DAT)	95-80-7	TP	2a,b	Pos	Neg		Neg	0
2,3-Dibromo-1-propanol	96-13-9	TP	2a	Pos				–
Diethylstilbestrol	56-53-1	TP	2a,b		Pos			–
7,12-Dimethylbenz[*a*]thracene (DMBA)	57-97-6	TP	2d	Neg				–
Ethyl methanesulfonate (EMS)	62-50-0	TP	2a,c	Pos		Pos		1
N-Ethyl-*N*-nitrosourea (ENU)	759-73-9	TP	1	Pos	Pos		Pos	1
Etoposide	33419-42-0	TP	2a	Pos	Pos			1
5-Fluorouracil	51-21-8	TP	2a,b,c	Pos	Neg			0
Methyl methanesulfonate (MMS)	66-27-3	TP	2a		Pos			–
N-Methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG)	70-25-7	TP	2d	Pos				–
Mitomycin C	50-07-7	TP	1	Pos	Pos		Pos	1
Potassium bromate	7758-01-2	TP	2a,b,c	Pos	Pos	Pos		1
Taxol	33069-62-4	TP	2a			Pos		–
4-Vinyl-1-cyclohexene diepoxide	106-87-6	TP	2a,b,c	Pos[b]	Pos	Pos		1
Ampicillin sodium salt	69-52-3	TN	2a	Neg				–
Beclomethasone dipropionate	5534-09-8	TN	2a		Neg			–
N-Butyl chloride	109-69-3	TN	2a,c	Neg	Neg	Neg	Neg	1
Curcumin	458-37-7	MP	2a		Pos			–
Cyclohexanone	108-94-1	TN	1	Neg	Neg		Neg	1
2,6-Diaminotoluene (2,6-DAT)	823-40-5	MP	2a				Neg	–
2,4-Dichlorophenol	120-83-2	MP	2a	Neg			Neg	1
Diclofenac	15307-79-6	TN	2a,c	Pos	Pos		Pos	1
Ethionamide	536-33-4	MP	2a,c	Neg	Neg	Neg		1
Eugenol	97-53-0	MP	2d	Pos				–
8-Hydroxyquinoline	148-24-3	MP	2a				Neg	–
d-Limonene	5989-27-5	TN	2a,c	Neg	Neg	Neg		1
d-Mannitol	69-65-8	TN	2a		Neg		Neg	1
Nifedipine	21829-25-4	TN	2a	Neg				–
Nitrofurantoin	67-20-9	MP	2a		Neg			–
1-Nitronaphthalene	86-57-7	MP	2a	Neg				–
4-Nitrophenol	100-02-7	MP	2a,c	Neg	Neg	Neg	Neg	1
Phenanthrene	85-01-8	TN	2a,b	Pos	Neg	Neg	Neg	0
Phenol	108-95-2	MP	2a				Neg	–
Propyl gallate	121-79-9	MP	2a		Neg			–
Resorcinol	108-46-3	MP	2a,c	Equiv	Neg			0.5
Tolbutamide	64-77-7	TN	2a	Equiv	Neg	Neg		0.5

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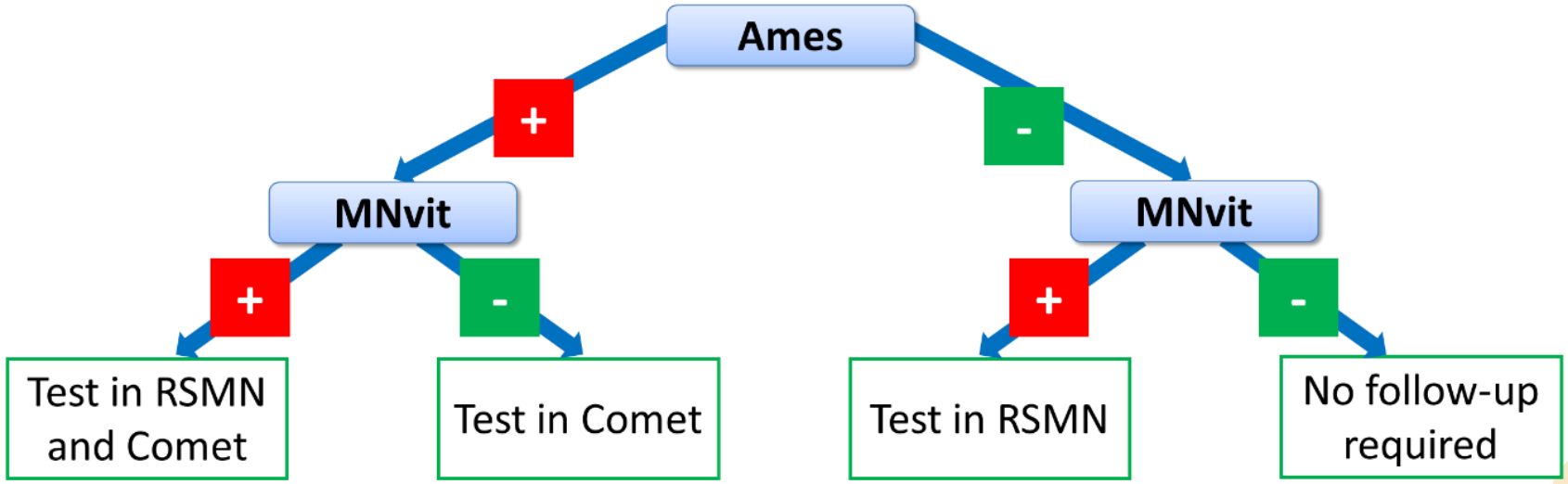
Strategic fit of RS assays

Follow-up options for dermally exposed substances, as a function of the outcome of the 2-test *in vitro* battery



*low priority for follow-up

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Validation outcome - RSMN

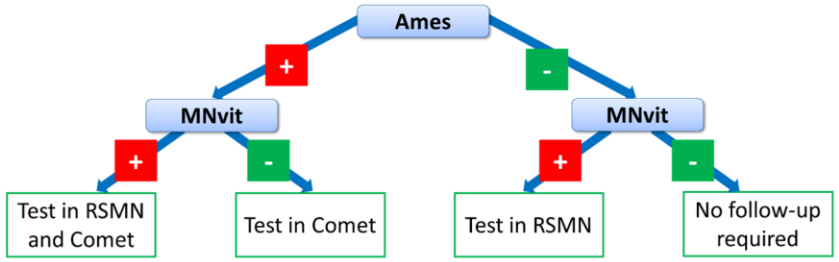
Table 3. Overall reproducibility within and between laboratories over time [within-laboratory reproducibility (WLR) and between-laboratory reproducibility (BLR)] in Phases 1 and 2a–2d

		Discordant	Concordant	Total	%
WLR	Lab A	6	17	23	73.9
	Lab B	3	21	24	87.5
	Lab C	1	6	7	85.7
	Lab D	1	14	15	93.3
	All labs	11	58	69	84.1
BLR		5	17	22	77.3

Table 4. Predictive capacity of the RSMN calculated based on the evaluation criteria agreed on by the Steering Committee and other external experts

Parameter	Lab A	Lab B	Lab C	Lab D	Overall
Sensitivity (%)	93.3	61.5	75.0	50.0	75.0
Specificity (%)	71.4	85.7	100	90.0	84.1
Accuracy (%)	82.8	74.1	85.7	78.6	79.8

For a per lab view, also see Supplementary Table S1.



Overall Sensitivity of the skin assay battery **increases to 89%** when endpoint-specific strategy is applied!

(many true pos are double-positive)

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Practical use of the RSMN – Case examples

- RSMN (and Comet) assays are offered by CRO's, under GLP
- Several examples exist of how these assays have been used for (regulatory) decision making:
  - RS Comet examples, as already presented by K. Reisinger
  - Example 1: Use of the RSMN as '2nd Tier' tool in an *in-vitro-only* testing strategy for fragrance materials (concordance with in vivo)
  - Example 2: Use in the context of a hair dye precursor (skin-specific metabolism)
  - Example 3: Use for a nanomaterial (barrier)
  - Example 4: Use for an aneugenic dermal drug (hazard/risk, limitations)

Not discussed today:

Cosmetics Europe project with IIVS to establish a **photo-RSMN** that enables detection of genotoxins that are activated by UV irradiation

Example 1: Research Institute for Fragrance Materials (RIFM) genotoxicity program

- Part of RIFM screening for genotoxicity potential of >2500 fragrance components
- Bluescreen® used to prioritize for further testing, then a 2-test *in vitro strategy* (Ames plus *in vitro* MN)
- Many fragrance materials are also used as flavor -> EFSA* requires *in vivo*-follow-up testing
- Aspiration to avoid *in vivo* testing in the future also in the context of oral exposure! (HET-MN)
- Manuscript in press

https://doi.org/10.1093/mutage/geab040

*EFSA: European Food Safety Authority

Mutagenesis, 2021, XX, 1–23
https://doi.org/10.1093/mutage/geab040
Advance access publication 30 November 2021
Original Manuscript

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Original Manuscript

Use of the EpiDerm™ 3D reconstructed skin micronucleus assay for fragrance materials

Yax Thakkar[1,*], Holger Moustakas[1], Marilyn Aardema[2], Shambhu Roy[3], Stefan Pfuhler[4], Anne Marie Api[1]

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RIFM dataset

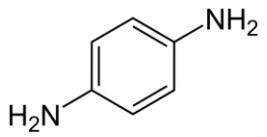
- 19 RSMN/*in vivo* MNT pairs
- 100% concordance
- RSMN = GLP compliant
- 18/19 *in vivo* MNT are state-of-art, GLP and OECD compliant studies

Table 1. Summary table describing all genotoxicity data for materials

Material	CAS #	*In vitro* MNT	3D Skin MNT	*In vivo* MNT
sec-Butyl ethyl ether	2679-87-0	+	-	-
Cadinene	29350-73-0	+[a]	-	
2,3-Dihydro-1,1-dimethyl-1H-indene-ar-propanal	300371-33-9	Equivocal	-	
1,5-Dimethylbicyclo[3.2.1]octan-8-one-oxime	75147-23-8	+	-	-
2,2′-(Dithiodimethylene)difuran	4437-20-1	+	-	-
Ethyl formate	109-94-4	+	-	-
2-Ethyl-1,3,3-trimethyl-2-norbornanol	18368-91-7	-	-	-
Furfuryl thioacetate	13678-68-7	+	-	-
Isobornyl methyl ether	5331-32-8	Equivocal	-	-for read-across[b]
Lauric Aldehyde	112-54-9	+	-	-
p-Methoxy cinnamaldehyde	1963-36-6	+	-	-
6-Methoxy-2,6-dimethylheptan-1-al	62439-41-2	+	-	-
2-Methyl-2-pentenal	623-36-9	+	-	-
Methyl beta-phenylglycidate	37161-74-3	+*	-	-
Nona-2 trans- 6-cis-dienal	557-48-2	+	-	-
2-Octenoic acid, 4-ethyl-, (2Z)	60308-75-0	+	-	-
2-Octen-4-one	4643-27-0	+	-	-
4-Phenyl-3-buten-2-ol	17488-65-2	+	-	-
5-Phenylhex-3-en-2-one	60405-50-7	Equivocal	-	-for read-across[c]
4-Thujanol	546-79-2	+	-	-
3,3,5-Trimethylcyclohexaneacetic acid	3213-73-8	+	-	-
Veratraldehyde	120-14-9	+	-	-

aResults did not meet all criteria for a positive.
bRead-across analogue is 1-ethyl-3-methoxytricyclo[2.2.1.02,6]heptane (CAS # 31996-78-8).
cRead-across analogue is 4-Phenyl-3-buten-2-one (CAS#122-57-6).

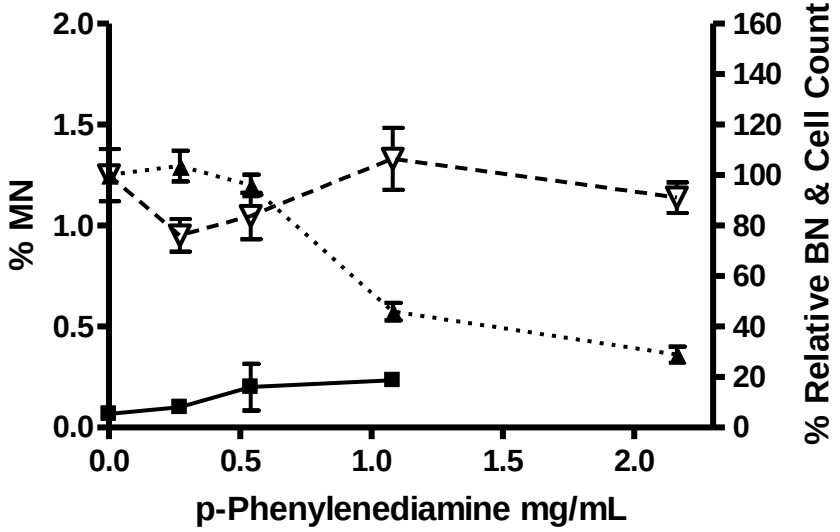
Example 2: Hair dye precursor paraphenylene diamine (PPD)

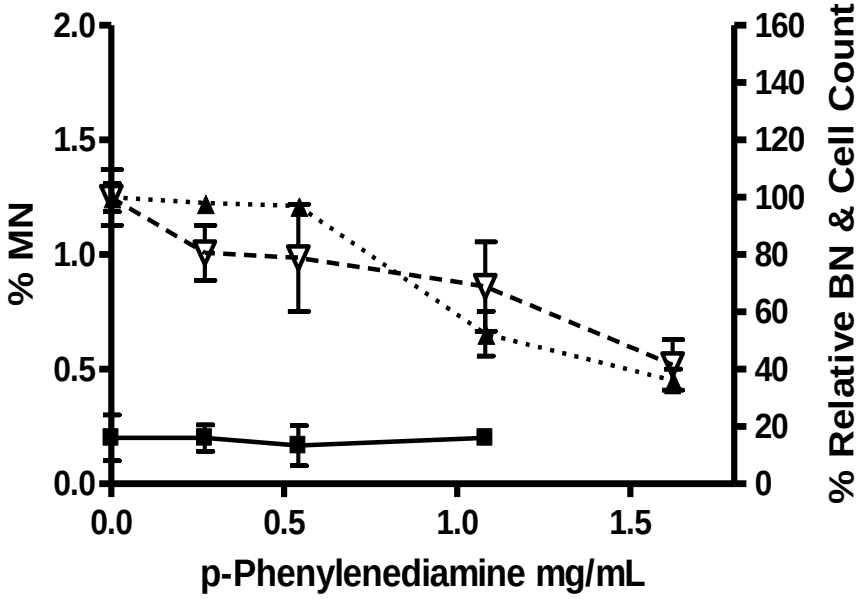


- Data situation: (from dossier, SCCS/1443/11)
  - pos in *in vitro* standard battery: Ames, CA, MLA *tk* (new criteria: negative)
  - neg in HPRT assay
- Was assessed non-genotoxic by SCCS since it was:
  - neg *in vivo*: MN (bone marrow), UDS (liver), Comet (8 organs; Sasaki 2000))
- Shown to be N-acetylated when applied to human volunteers in hair dye formulation (Nohynek et al, *Food Chem Toxicol*, **42**, 1885-1891)

Case study: PPD

Evaluation of PPD in the 3D Human Reconstructed Skin Micronucleus Assay, 2 independent studies





Legend:
closed squares: % micronucleated cells
open triangles: relative cell counts
closed triangle: % relative binucleation

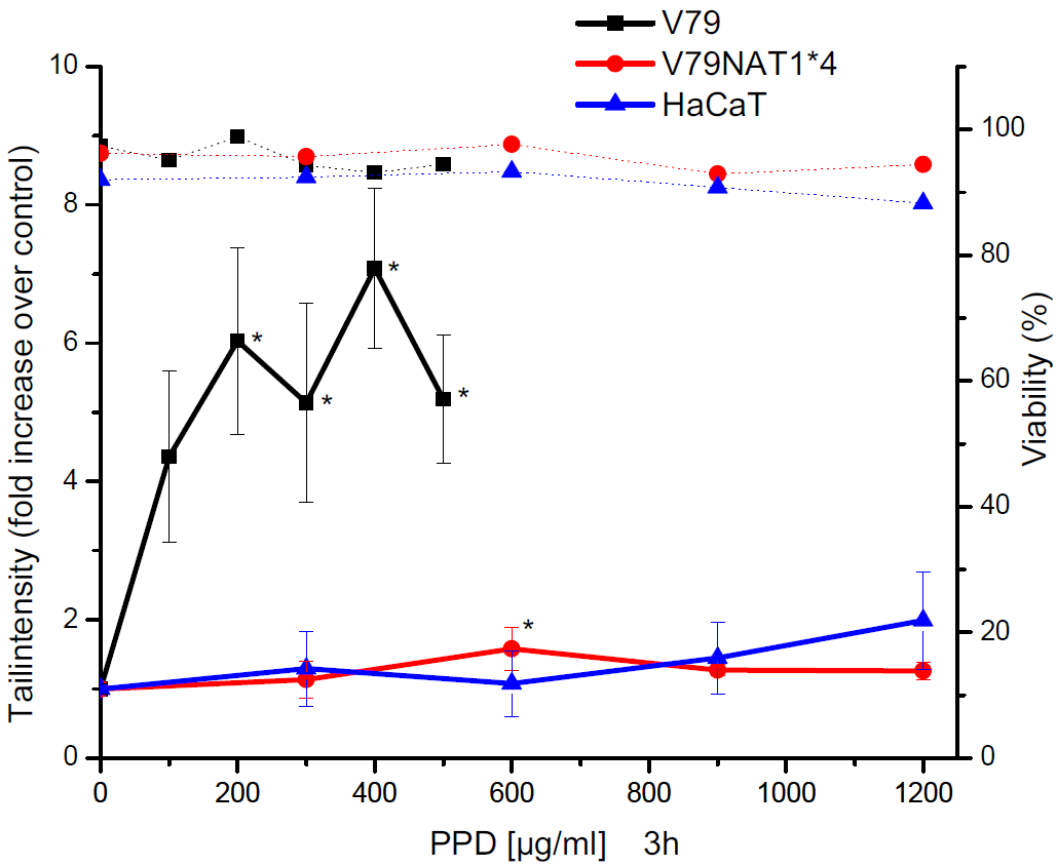
PPD tested negative in the 3D skin MN test – skin "first pass" effect?

skin = N-Acetyltransferase (NAT) proficient

Case study: PPD

Comet assay with PPD in three different cell lines:

- NAT1 deficient (**V79**) and NAT1 proficient (**V79NAT1*4**, **HaCaT**)



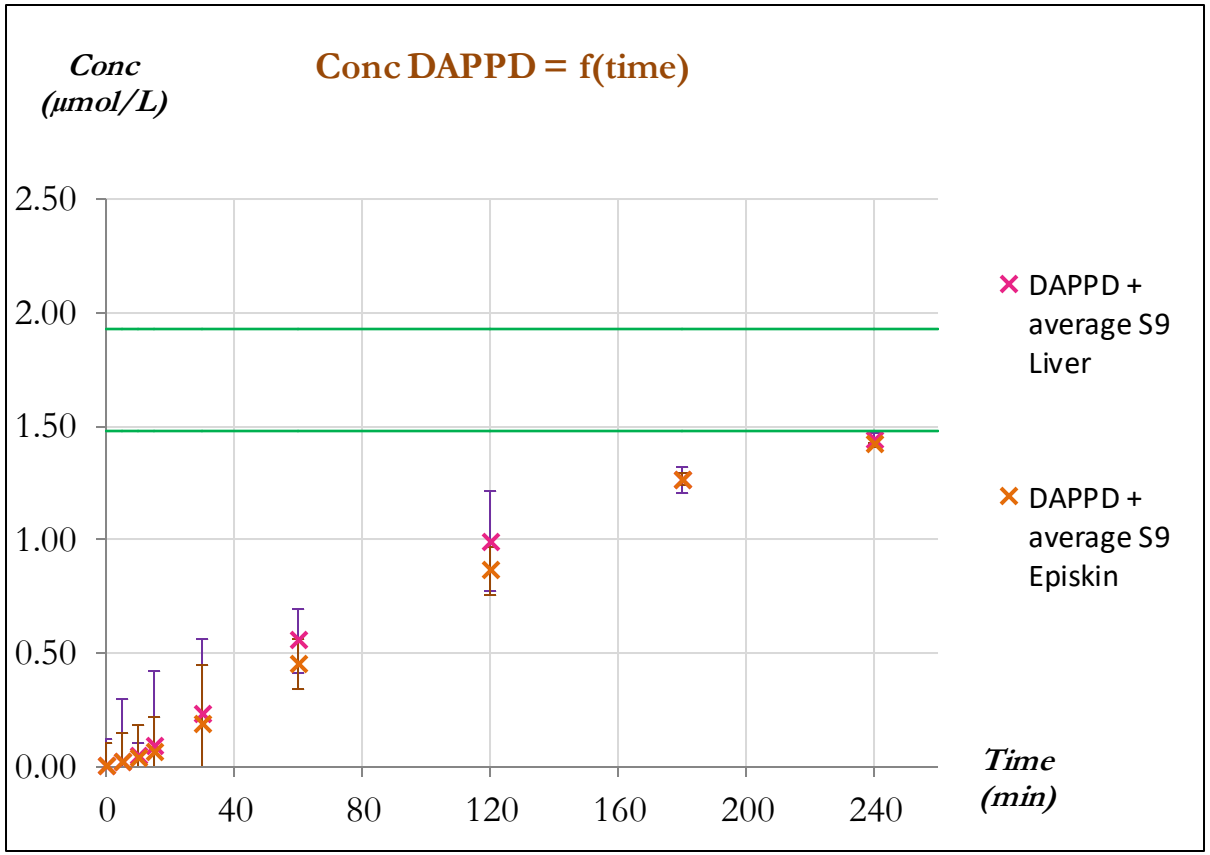
From Zeller and Pfuhler, Mutagenesis 29(1):37-48, 2014

- Genotoxic effect abolished in NAT competent cell lines

Metabolism data support negative result in Skin assay - "first pass" effect!

Case study: PPD

Formation of Diacetyl-PPD : Comparison between liver S9 and skin S9



→ speed of NAT conversion in skin similar to liver S9!

- PPD disappears at the same rate Diacetyl-PPD (DAPPD) is formed

Data from Cosmetic Europe Metabolism Project; Eilstein et al., 2019

Example 3: Skin models as a penetration barrier

Slides courtesy of Shareen Doak, Swansea University

Wills et al. Particle and Fibre Toxicology (2016) 13:50
DOI 10.1186/s12989-016-0161-5

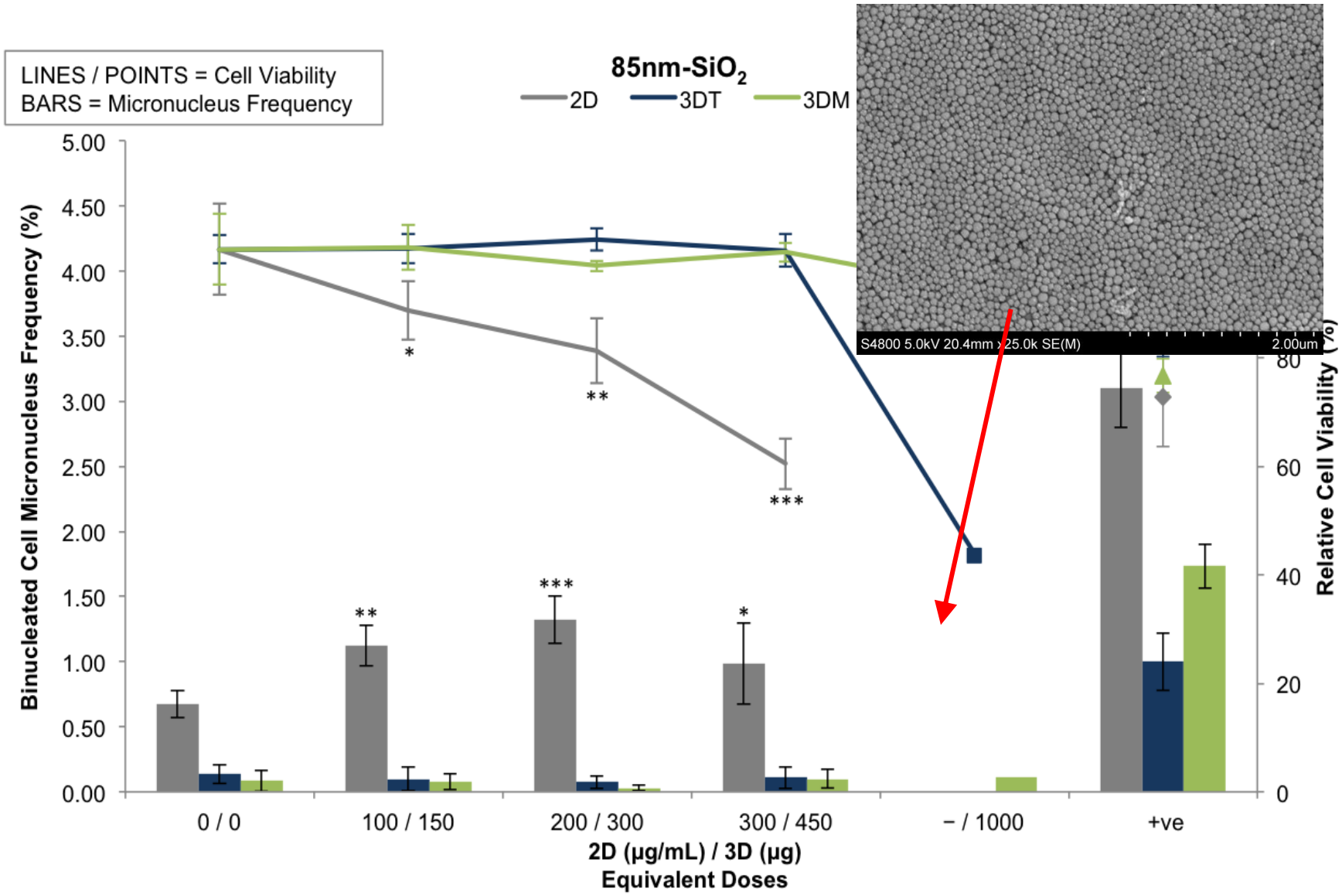
Particle and Fibre Toxicology

RESEARCH Open Access

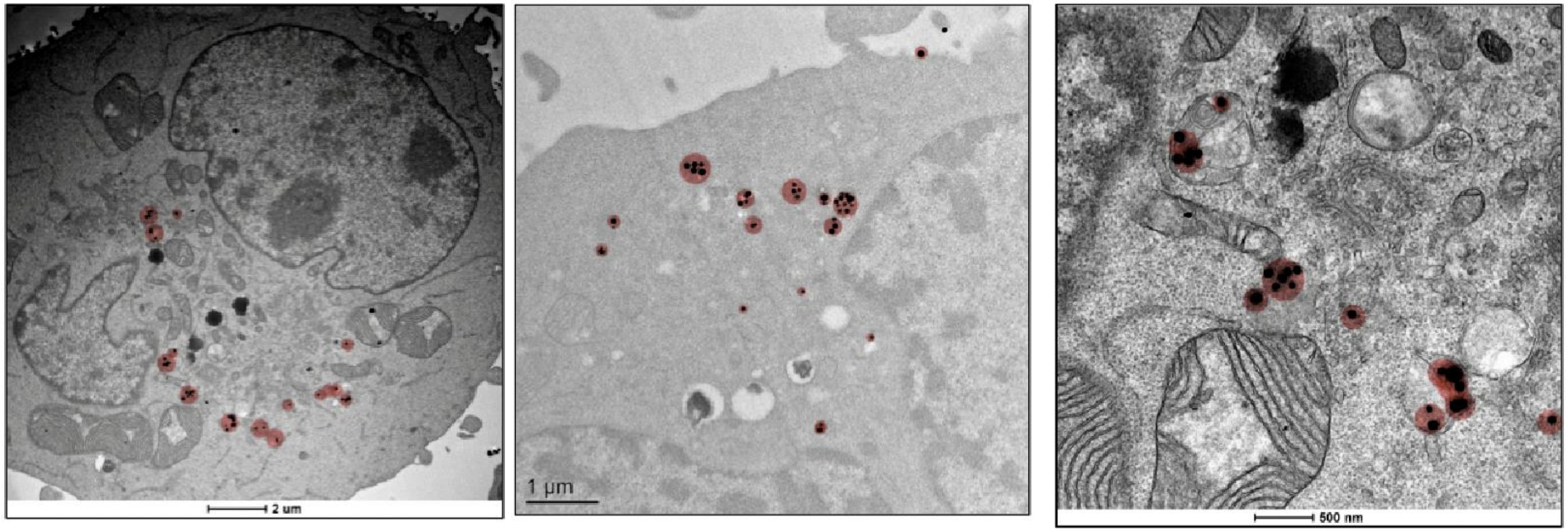
Genetic toxicity assessment of engineered nanoparticles using a 3D in vitro skin model (EpiDerm™)

John W. Wills[1*], Nicole Hondow[2], Adam D. Thomas[1], Katherine E. Chapman[1], David Fish[1], Thierry G. Maffeis[3], Mark W. Penny[3], Richard A. Brown[3], Gareth J. S. Jenkins[1], Andy P. Brown[2], Paul A. White[4] and Shareen H. Doak[1*]

2D vs 3D micronucleus assay

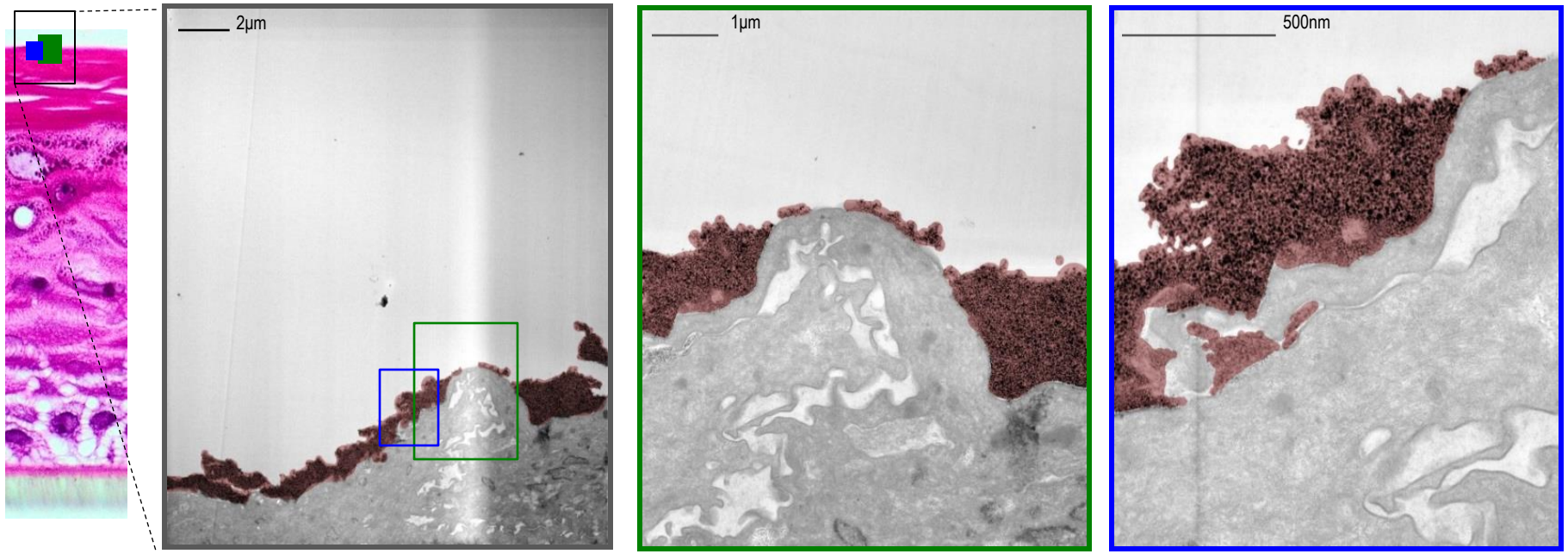


TK6 Cell Uptake (16nm Amorphous Silica)



Clear particle uptake into cells

Uptake into RS (16nm Amorphous Silica)



No particle uptake into the cells – more realistic exposure conditions for dermal route

Example 4: Dermally applied aneugenic drugs (Schuler et al, 2021)

OXFORD SOT Society of Toxicology academic.oup.com/toxsci

TOXICOLOGICAL SCIENCES, 180(1), 2021, 103–121

doi: 10.1093/toxsci/kfaa189
Advance Access Publication Date: 22 January 2021
Research Article

Experiments in the EpiDerm 3D Skin In Vitro Model and Minipigs In Vivo Indicate Comparatively Lower In Vivo Skin Sensitivity of Topically Applied Aneugenic Compounds

Maik Schuler,[1] Lindsay Tomlinson, Michael Homiski, Jennifer Cheung, Yutian Zhan, Stephanie Coffing, Maria Engel, Elizabeth Rubitski, Gary Seitis, Katherine Hales, Andrew Robertson, Saurabh Vispute, Jon Cook, Zaher Radi, and Brett Hollingshead

- Attempt to use RSMN assay for risk assessment
- Authors could rank-order results according to potency of aneugens

"….demonstrate that the EpiDerm RSMN is sensitive for the hazard identification of aneugens"

- BUT: substance in question was negative in minipig assay *in vivo*
- Also promotes use of flow-based alternative biomarkers

Highlights assay limitations:

- Limited selection of qualified solvents available to date
- Aqueous solvents are problematic – solvents like acetone and ethanol force penetration
- Evaluation is time consuming, automation desired!

Summary

- Use of RS models considers main route of exposure of cosmetics as well as skin-specific metabolic fate
- The 3D skin comet and micronucleus assays have been successfully validated
- If used as intended: Overall sensitivity = 89%, overall specificity = 79%
- Assays are offered commercially under GLP at several CROs
- 19 fragrance ingredients with positive results in standard *in vitro* genotoxicity assays tested negative in RS assays and *in vivo* (100% concordance)
- Case studies show the relevance as an exposure-route specific tool
- OECD approved the development of 2 separate guidelines
- Currently undergoing formal validation peer-review by ECVAM
- If successful, OECD guideline development will start

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Cosmetics Europe TF Genotoxicity

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Cosmetics Europe
the personal care association

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