The reconstructed skin MN (RSMN) assay – next steps to improve use and aid implementation

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Overview

1. RSMN Assay – Development, Validation and Strategic Fit
2. Incorporation of Liver Metabolism
3. Automated RSMN Analysis

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RSMN Assay – Development, Validation and Strategic Fit

Regulatory change as driver for '2nd tier' in vitro assays

- 7th Amendment to the EU Cosmetic Directive a testing and marketing ban of cosmetic ingredients tested *in vivo* came into force 2009, many followed
- Catalyst for *in-vitro-only* testing concepts
- However, the 'test battery' approach leads to a reduced specificity – increase in fraction of 'misleading positives'
- Cosmetics Europe´s animal-free strategy for genotoxicity testing
  - "3D skin model" project, included MN and Comet
  - More "in-vivo-like" behavior and enable route of exposure specific assessments

RSMN Assay – Development, Validation and Strategic Fit

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Original Manuscript
Advance Access publication 5 February 2021

OXFORD

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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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.

- Formal validation peer review ongoing
- OECD guideline development to follow

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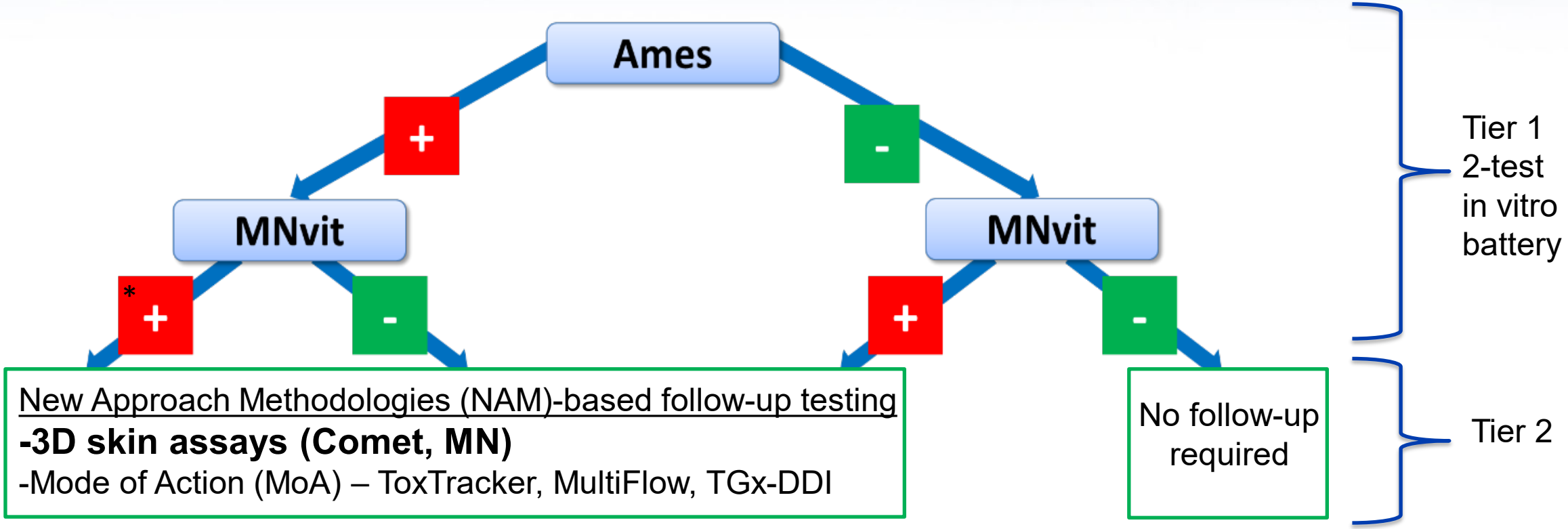
RSMN Assay – Development, Validation and Strategic Fit

Tiered approach



Overall Sensitivity of the skin assay battery (MN and comet)
increases from 75% to 89% when endpoint-specific strategy is applied!

*low priority for follow-up

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Incorporation of Liver Metabolism

- Reconstructed skin models known to reflect human skin specific metabolism
- RSMN assay demonstrated to detect compounds requiring metabolic activation
  - Improved with the 72-hour extended exposure (Aardema et al. 2013, Kidd et al. 2016)
  - However, in dermal exposure, there may be scenarios where substances penetrate the skin unchanged and undergo further metabolism in the liver
    - **Evaluate the ability of rat liver S9 to complement the standard RSMN assay**

*Aardema et al. Evaluation of chemicals requiring metabolic activation in the EpiDerm™ 3D human reconstructed skin micronucleus (RSMN) assay. Mut Res, 2013.
*Kidd et al. The 3D reconstructed skin micronucleus assay: considerations for optimal protocol design. Mutagenesis 2021

Incorporation of Liver Metabolism

- Standard RSMN procedures (48 and 72-hour protocols)
- Evaluated two S9 exposure scenarios
  - 4 hour + 20-hour recovery
  - Low concentration continuous
- Cyclophosphamide (CP) model compound

S9 Exposure	Day 1*	Day 2		Day 3		Day 4
No S9	Arrival	NMM + Cyto B		NMM + Cyto B		Harvest
Continuous S9		NMM + S9 + Cyto B		NMM + S9 + Cyto B		
4h S9 + Recovery		NMM + S9 (4hr)	NMM + Cyto B (20hr)	NMM + S9 (4hr)	NMM + Cyto B (20hr)	

*if using 72hr RSMN, 1st treatment occurs after at least 1hr equilibration

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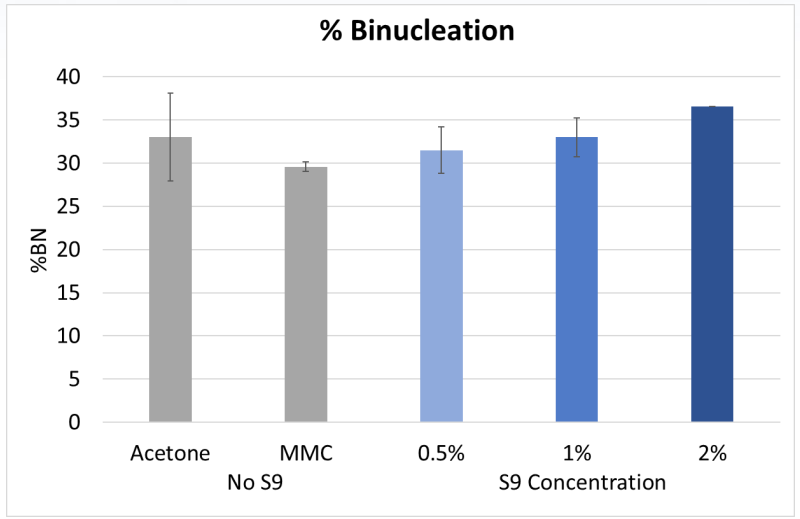
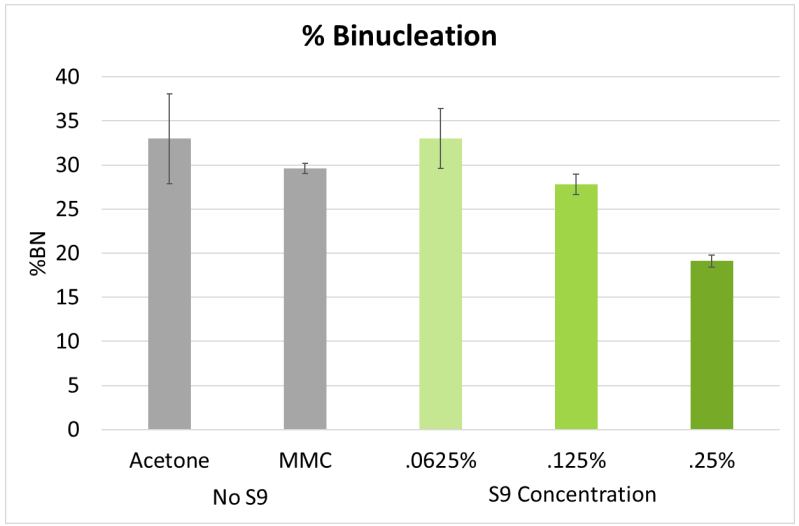
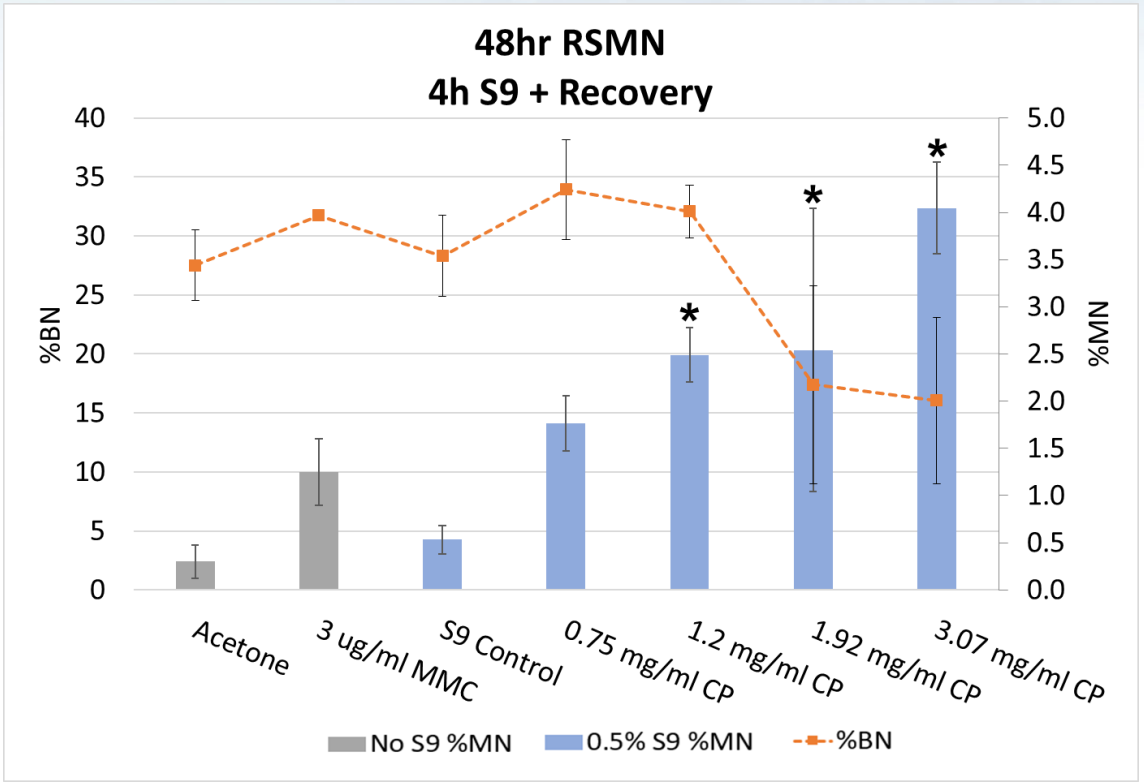
Incorporation of Liver Metabolism

4 Hour S9 Exposure + Recovery



Continuous S9 Exposure





- 4 hour 0.5% S9 had no impact on binucleation and dose dependent increases in MN detected after CP treatment (48 and 72-hour protocols)
- Continuous S9 exposure resulted in excessive toxicity upon treatment

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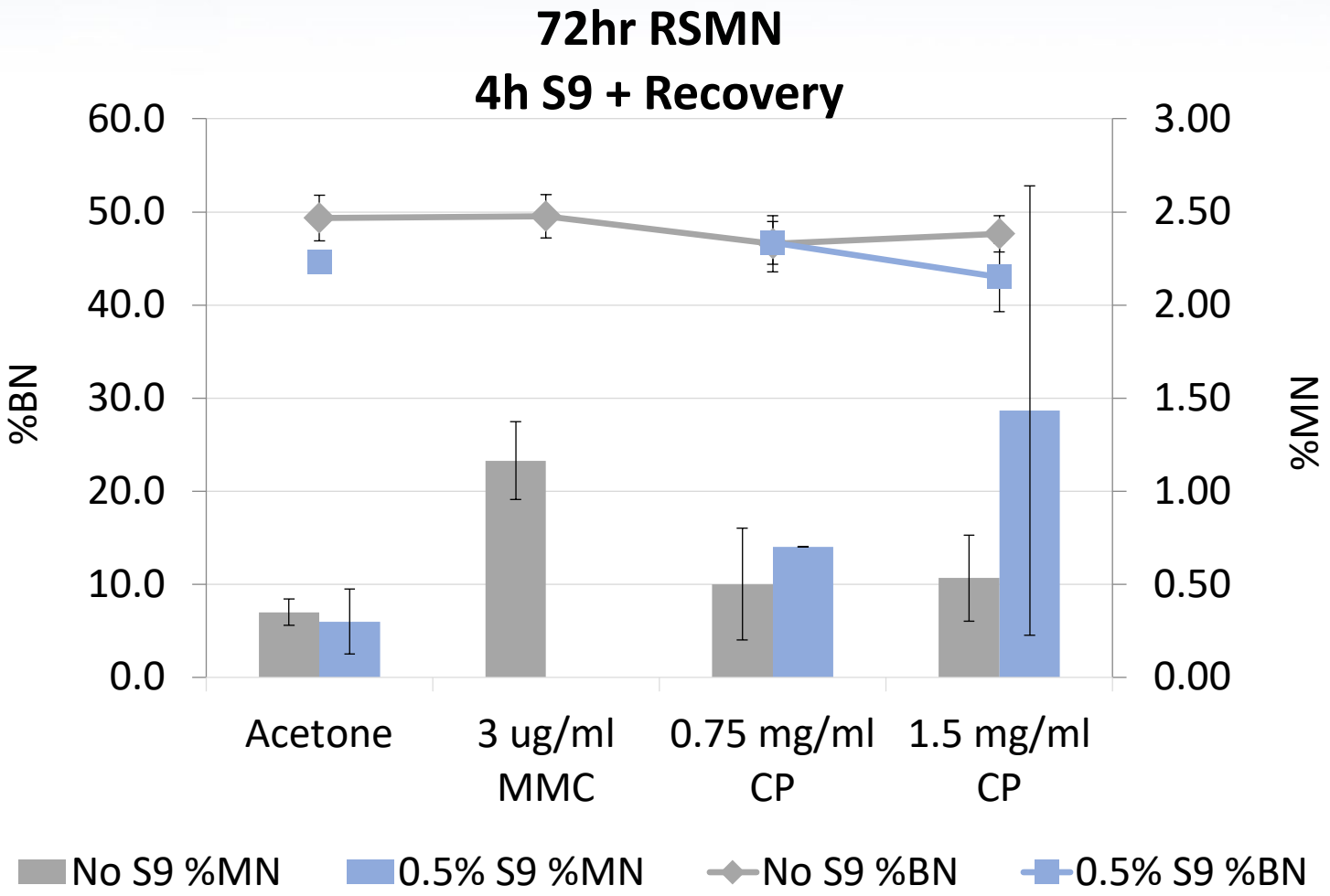
Incorporation of Liver Metabolism



Next Steps

- Continue to optimize the S9 concentration for the 4h S9 + recovery approach to maximize effect
- Validate the method with additional compounds requiring metabolic activation

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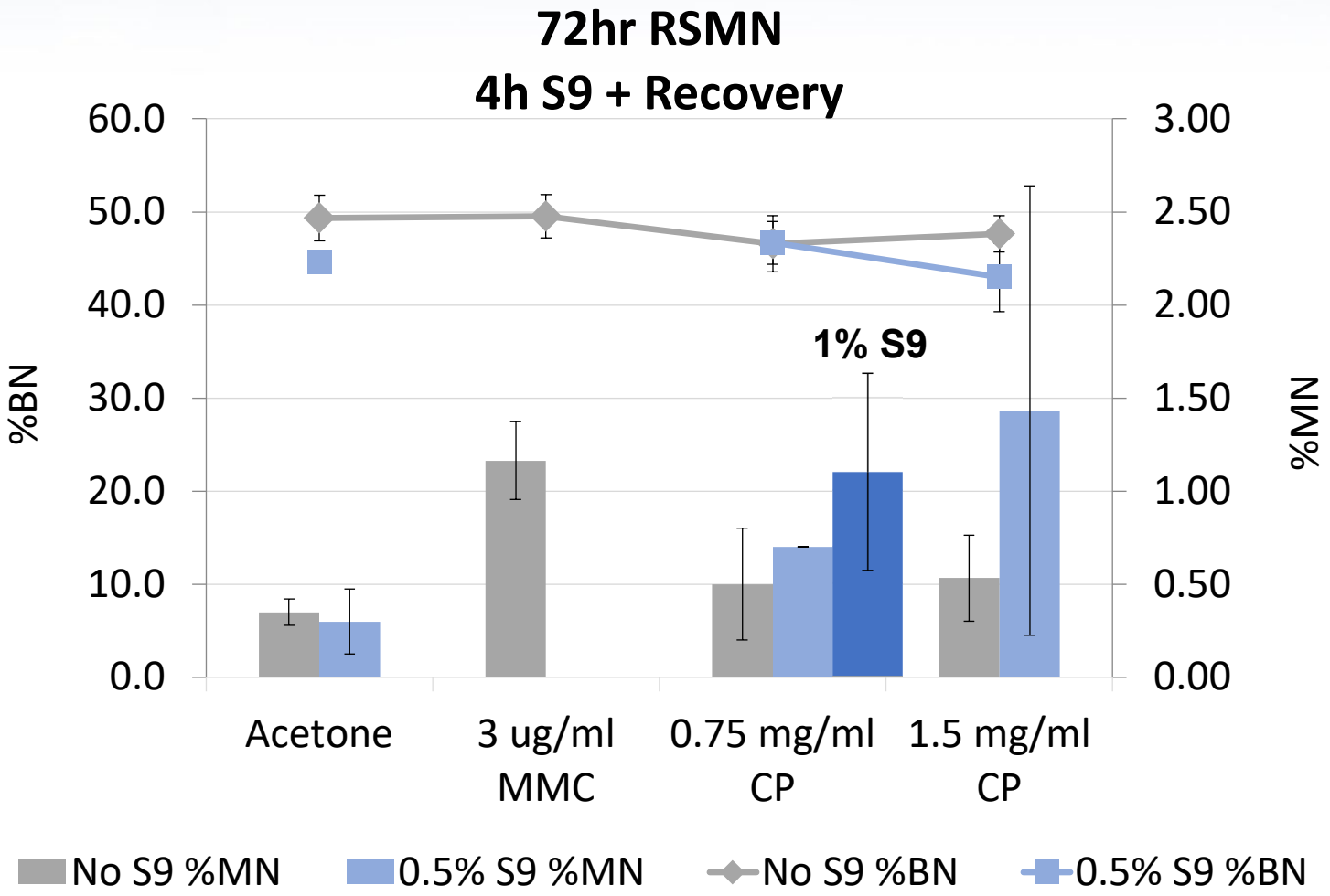
Incorporation of Liver Metabolism



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Automated RSMN Analysis

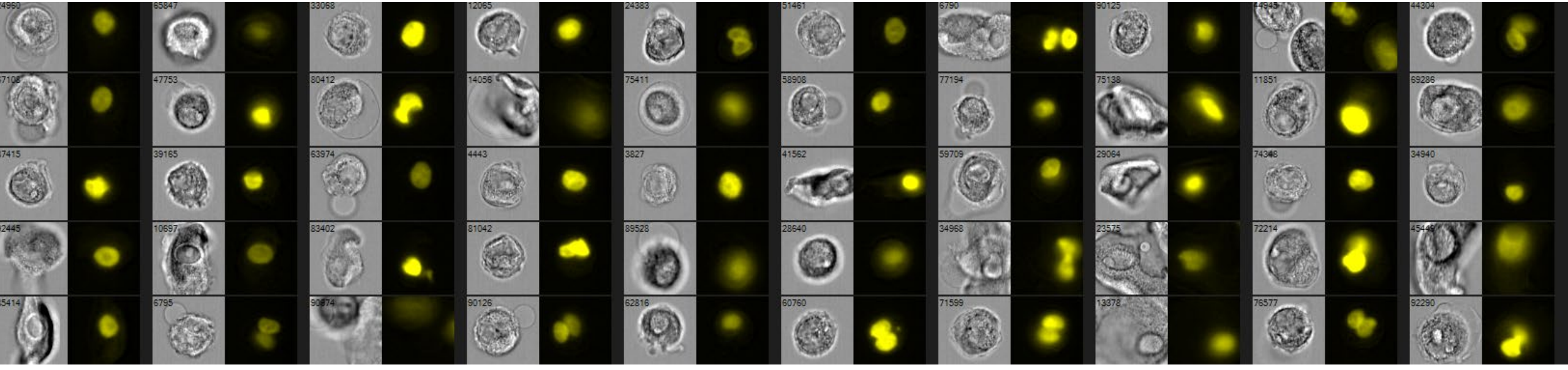
- Challenges of the RSMN assay
  - Technical expertise
    - Laborious sample and slide preparation
    - Scoring – time intensive and subjective
  - Limited statistical power
- Some success with automated slide scanning methods (Chapman et al. 2014)
- 3D skin not compatible with flow cytometric MN analysis (lysis based)

Compromise → **Imaging flow cytometry**

*Chapman et al. Automation and validation of micronucleus detection in the 3D EpiDerm™ human reconstructed skin assay and correlation with 2D dose responses. Mutagenesis, 2014.

Automated RSMN Analysis

- Evaluating micronuclei with imaging flow cytometry has been previously established for isolated blood and standard cell lines
- Translating the method to 3D skin is not straightforward…
  - Complex sample preparation (optimize existing methods)
  - Heterogenous cell population (retrain existing artificial intelligence (AI) analysis methods vs. create new)



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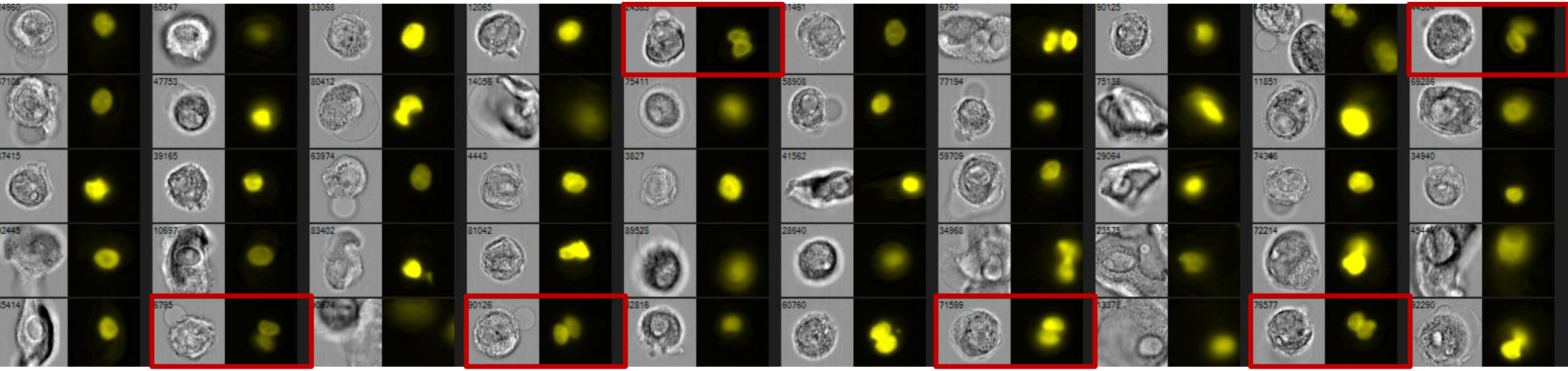

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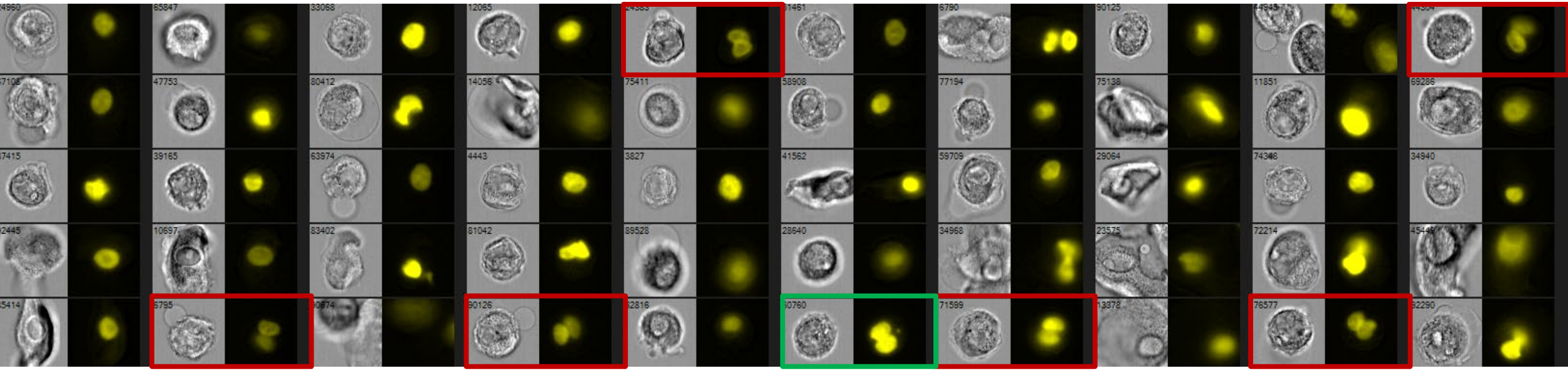

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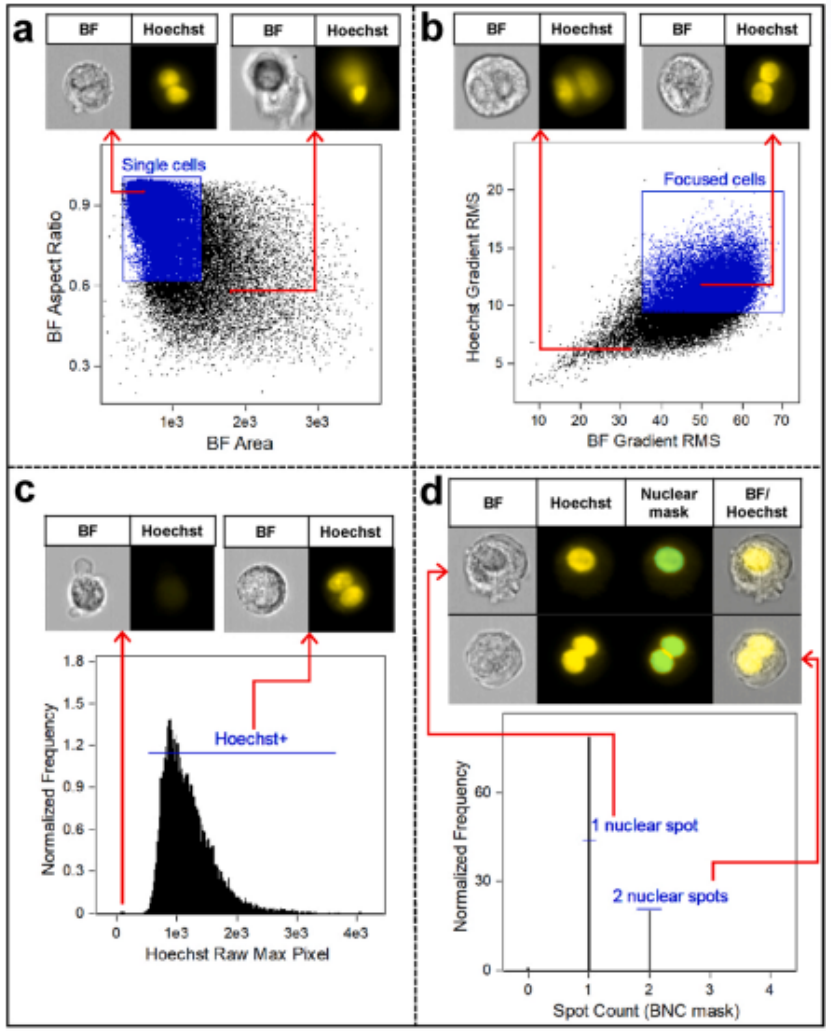
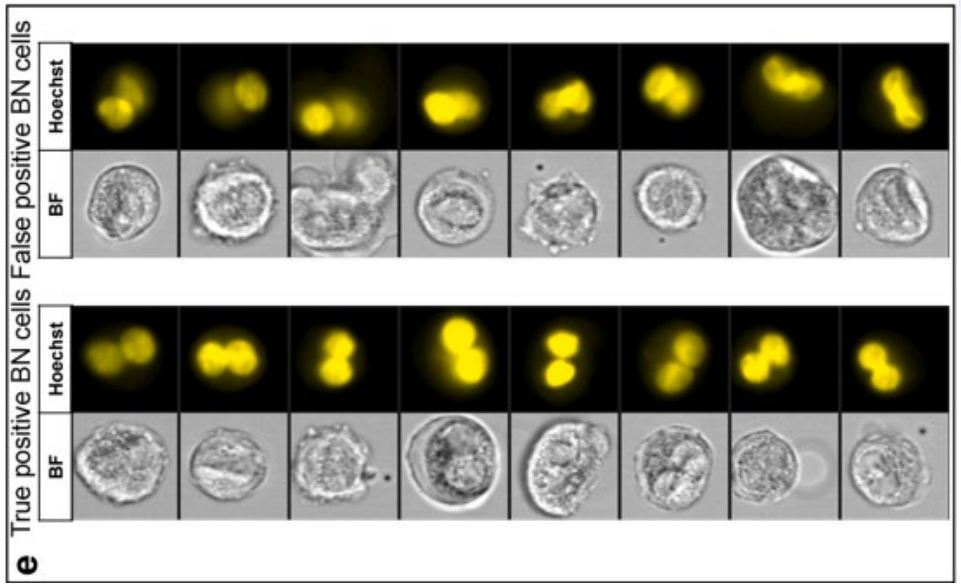




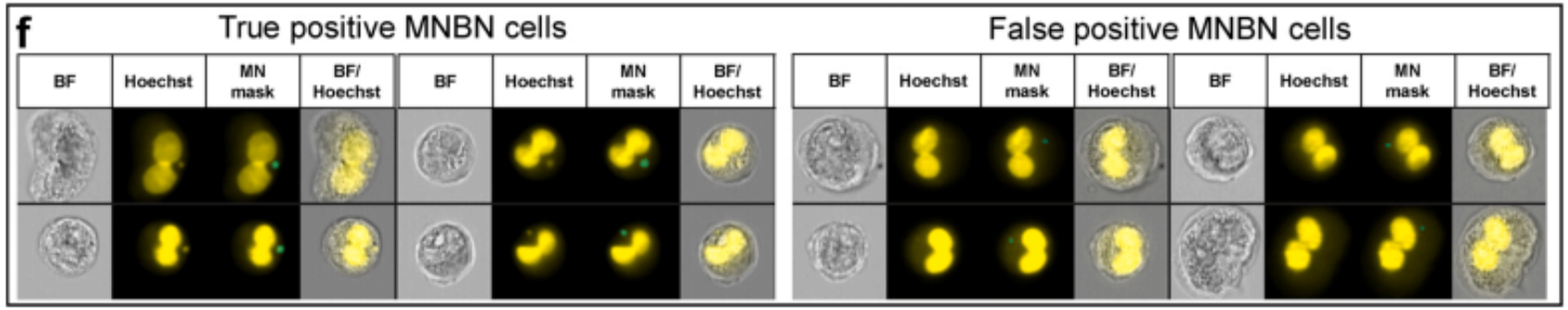
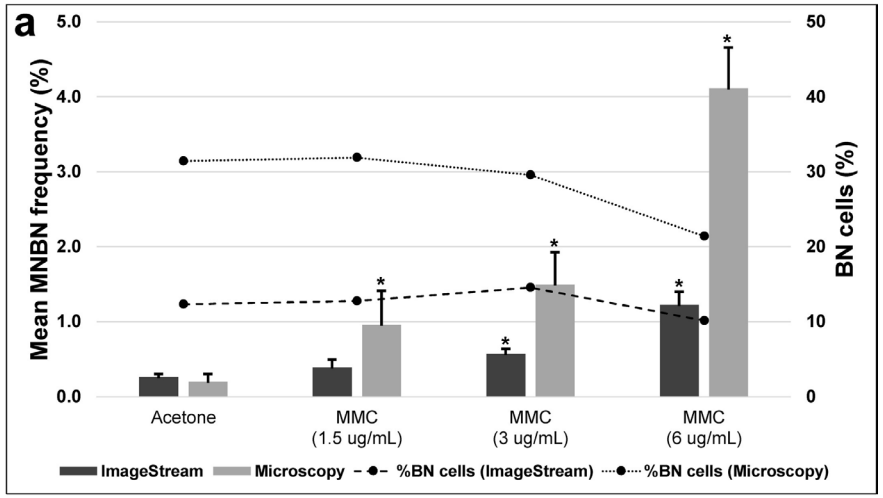


Figure 2.
Allemang, et al. 2021. The 3D reconstructed skin micronucleus assay using imaging flow cytometry and deep learning: A proof-of-principle investigation, Mutat Res Genet Toxicol Environ Mutagen

Automated RSMN Analysis

- Initial efforts with Cytek (formerly Luminex) have demonstrated feasibility of the approach using ImageStream
  - ↑ analysis speed – 20 mins/sample
  - ↑ statistical power – on average, 2.5X more BN cells evaluated per sample
- Amnis® Artificial Intelligence (AAI) software



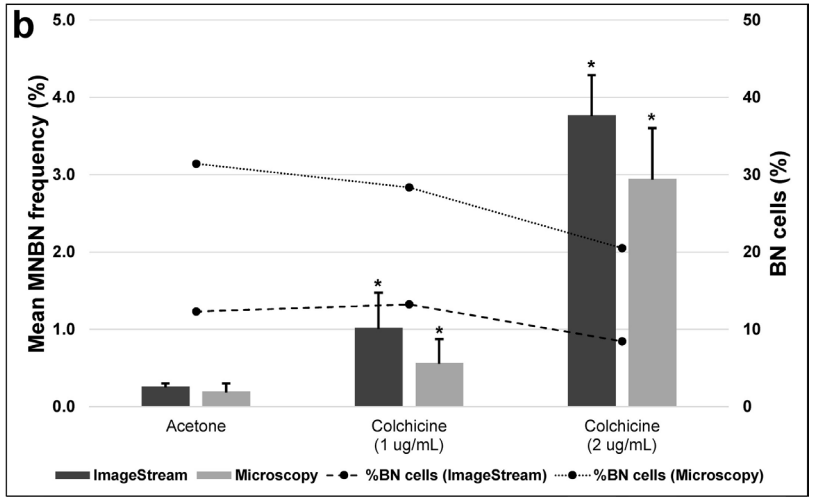
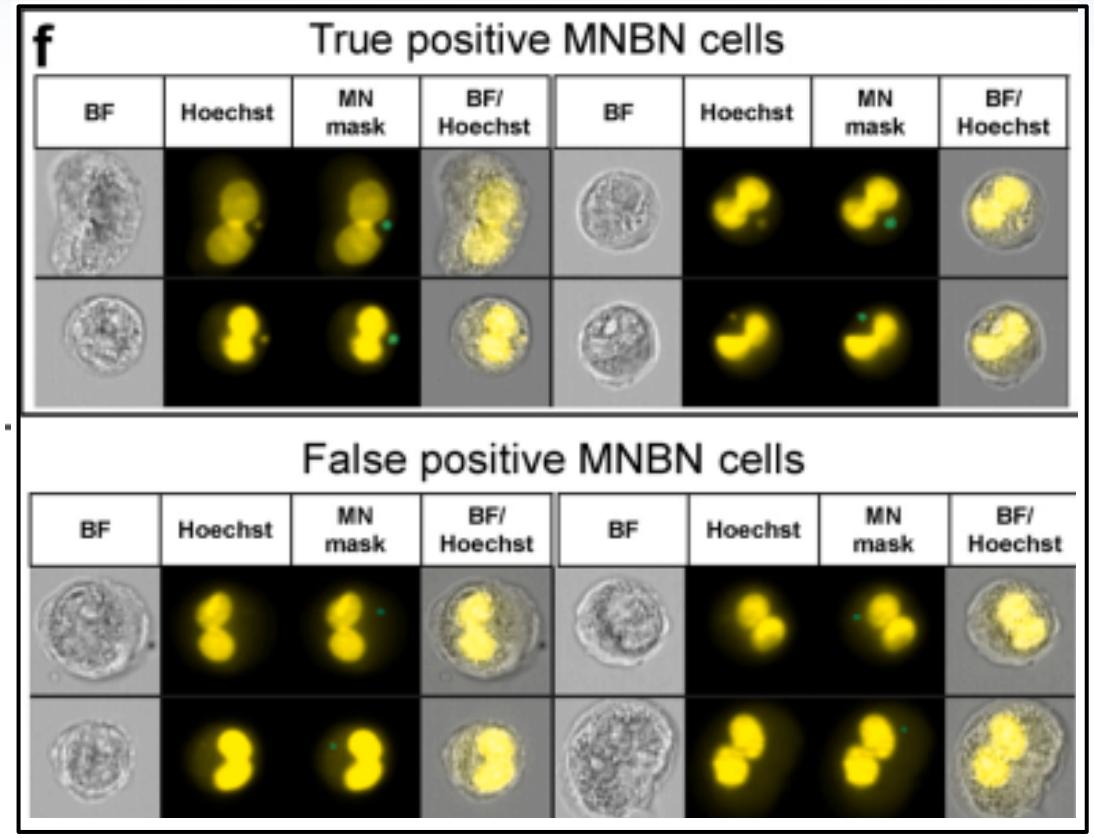


Figure 4.
Allemang, et al. 2021. The 3D reconstructed skin micronucleus assay using imaging flow cytometry and deep learning: A proof-of-principle investigation, Mutat Res Genet Toxicol Environ Mutagen

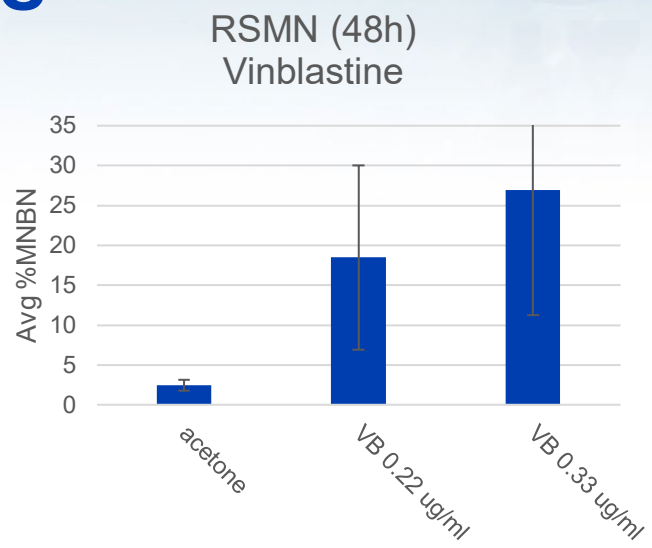
Automated RSMN Analysis

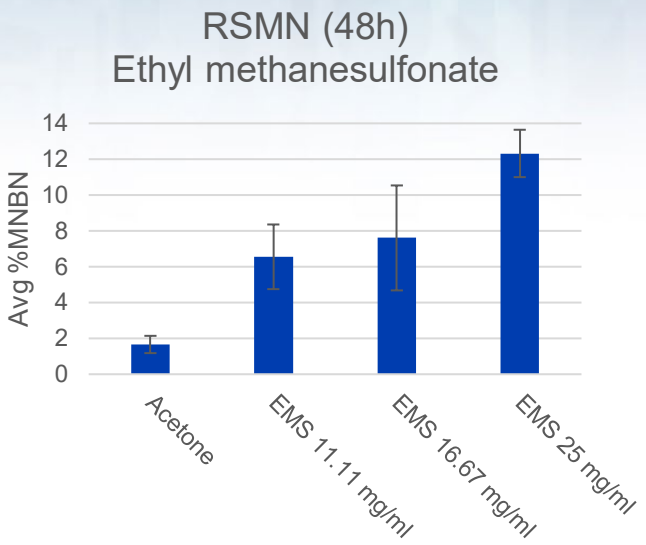
- Challenges
  - Currently, not fully automated
    - AI correctly identified BN cells 90%
    - MN needed visual verification
  - Artificial intelligence methods dependent upon large number of training images (“truth” populations)
    - Cell number limited by tissue size
    - Low MN rates, even in positive controls

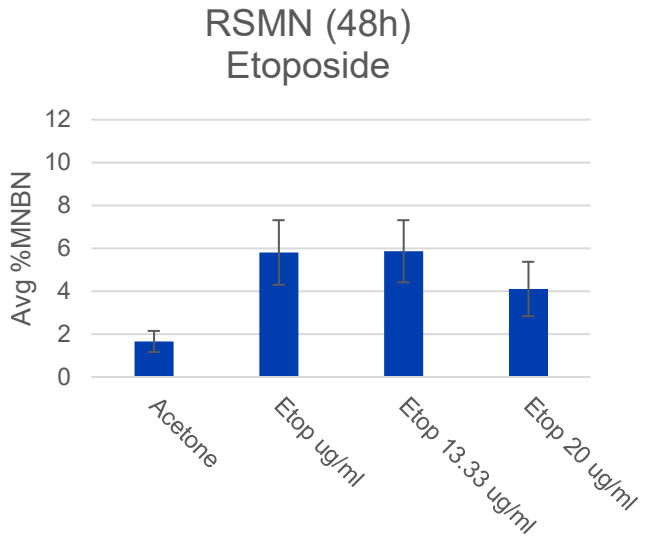


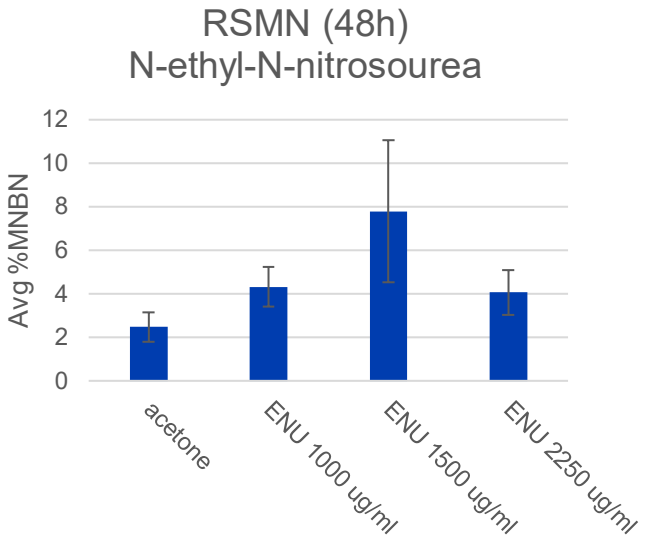
Automated RSMN Analysis

- New dataset generated with 4 additional compounds – analysis in progress!
  - Initial results using model based on TK6 cell images
  - Skin cell data will be added soon for further training of the AI model









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Summary

- The 3D reconstructed skin micronucleus assay is a valuable “3R” friendly tool for follow-up of in vitro positive results
- Proof of concept studies demonstrate feasibility of incorporating S9 to evaluate systemic metabolism in the RSMN assay
  - Work ongoing to optimize concentration and further validate
- Automated analysis of the RSMN assay using imaging flow cytometry can improve throughput and statistical power
  - Additional data has been generated, refining AI model currently in progress

➢ **Increase the utility and support implementation of the RSMN assay into regulatory schemes**

Acknowledgements

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- Rob Thacker
- Richard DeMarco

Thank you for your attention!

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