Can We Identify Tipping Points between Adaptive and Adverse Perturbations from In Vitro Data?

Applying New Approach Methodologies to Risk Assessment: Consideration of Exposure and Compensatory Mechanisms

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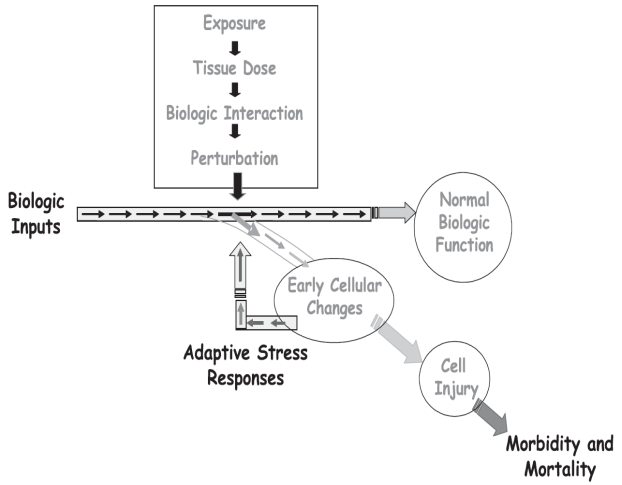
EPA Context

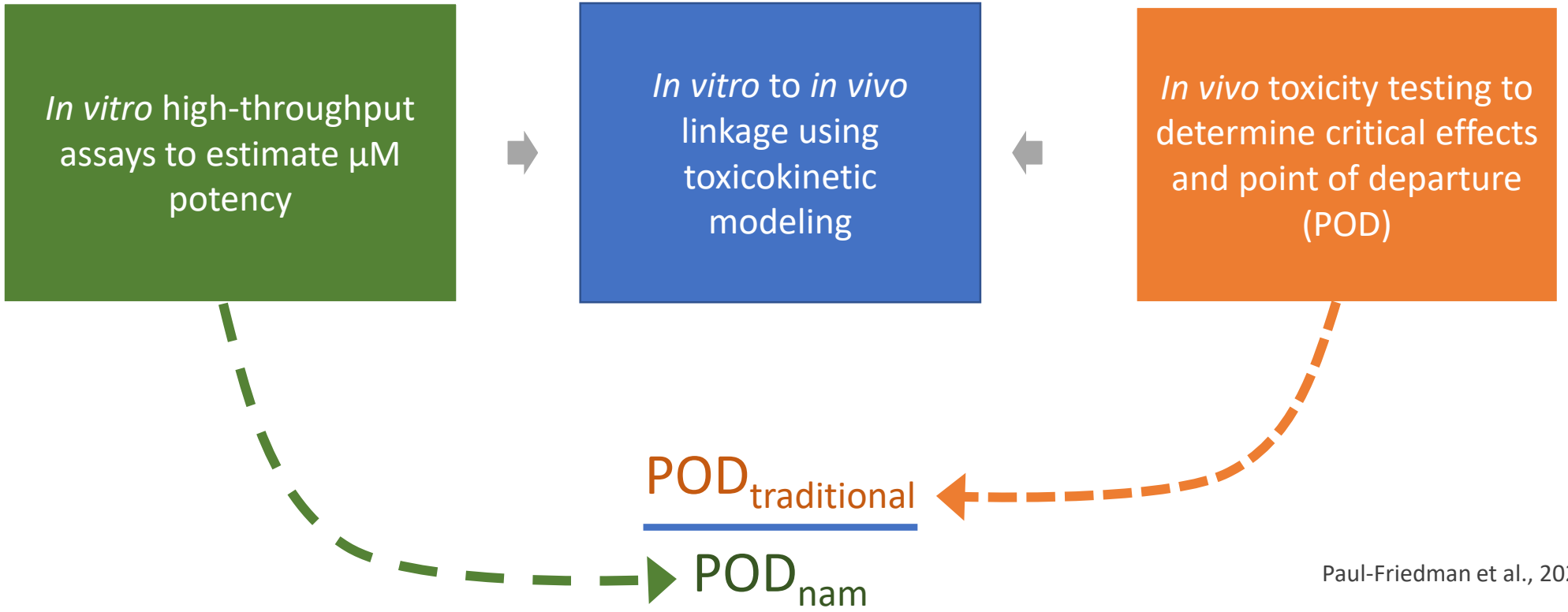
- Need new approach methodologies (NAMs) to evaluate thousands of untested chemicals effectively
- Chemicals cause toxicity via complex pathways that are poorly defined. Two main conceptual approaches to map adverse outcome pathways (AOPs):-
  - Specific receptor-mediated mechanisms (e.g. ER-mediated developmental or reproductive effects)
  - Non-specific adaptive stress response pathways (e.g. oxidative stress, unfolded protein response, etc.)
- We are interested in developing NAMs using *in vitro* and *in silico* models for systems-based analysis of toxicological pathways / AOPs
- Hypothesis: Increasing the level of chemical(s) beyond "tipping point" can overwhelm the adaptive stress responses and result in adverse outcomes
- Key questions:
  1. What *in vitro* approaches can serve as surrogates of tipping points?
  2. How do we estimate critical concentrations (c_{cr}) at tipping points?
  3. How do c_{cr} compare with AC_{50} and with doses that produce toxicity?



Krewski et al., 2010

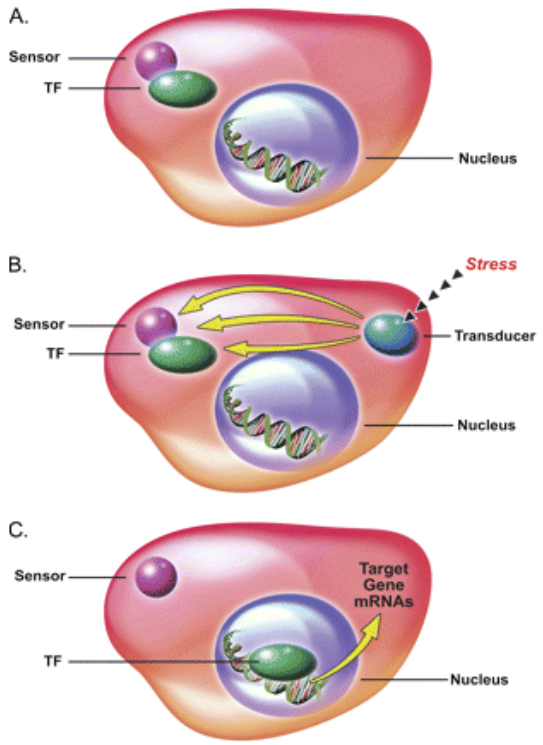
NAMs for Risk-based Prioritization

NAMs: Any technology, methodology, approach, or combination of methods that can provide information about chemical hazard and risk assessment without using whole animals.



Paul-Friedman et al., 2020

Adaptive Stress Response Pathways



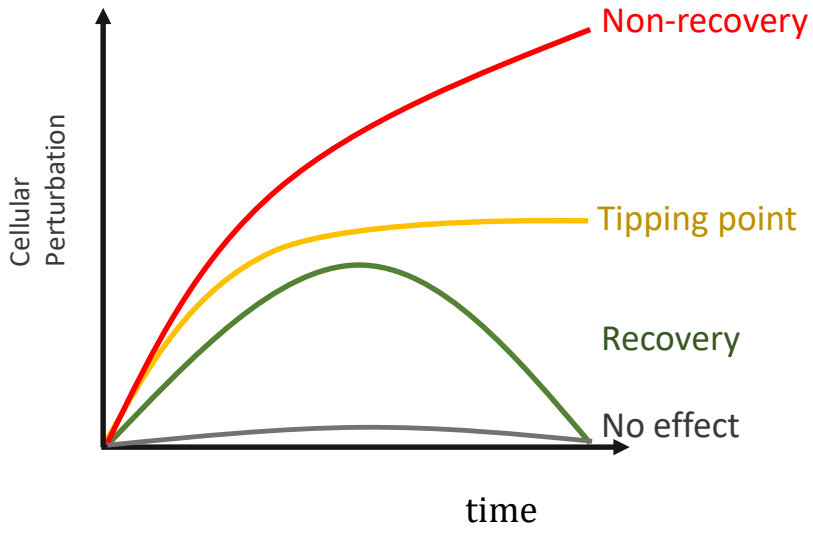
The Major Adaptive Stress response pathways

Stress response pathway	Chemical inducers	TF	Activated gene promoters
Oxidative stress	Quinones, hydroperoxides, heavy metals, trivalent arsenicals	Nrf2	*HMOX1*, *NQO1*, *GST2A*
Heat shock response	Heat, Heavy Metals	HSF-1	*HSPA6*
DNA damage response	Etoposide, Methyl Methanesulfonate, N-Dimethylnitrosamine, Cyclophosphamide, UV radiation	p53	*CDKNIA*, *GADD45A*, *MDM2*, *BCL2*, *TP53I3*
Hypoxia	Hypoxia, Cobalt, Desferriozamine, Quercetin, Dimethyloxalylglycine	HIF-1	*VEGF*, *TF*, *EPO*
ER stress	Tunicamycin, Thapsigargin, Caplain, Brefeldin A	XBP-1, ATF6, ATF4	*HSP90B1*, *HSPA5*, *DNAJB9*
Metal stress	Heavy Metals	MTF-1	*MT1E*, *MT2A*
Inflammation	Metal, PCBs, Exhaust Particles, Smoke Particles	NF-κB	*IL1A*, *TNFA*
Osmotic stress	High salt, polyethylene glycol, mannitol	NFAT5	*AKR1B1*, *SLC6A12*, *SLC5A3*

Simmons et al., 2009

Adaptive Stress Responses & Tipping Points

- Adaptive stress response pathways are invoked to maintain homeostasis
- Dysregulation of stress responses can cause toxicity or lead to disease
- For chemical-induced toxicity three potential outcomes of stress response activation:
  - No perturbation of cellular endpoints
  - Perturbation of cellular endpoints followed by recovery
  - Perturbation of cellular endpoints without recovery
- Claim: if the perturbation exceeds a critical level – the "tipping point" – then recovery is not possible

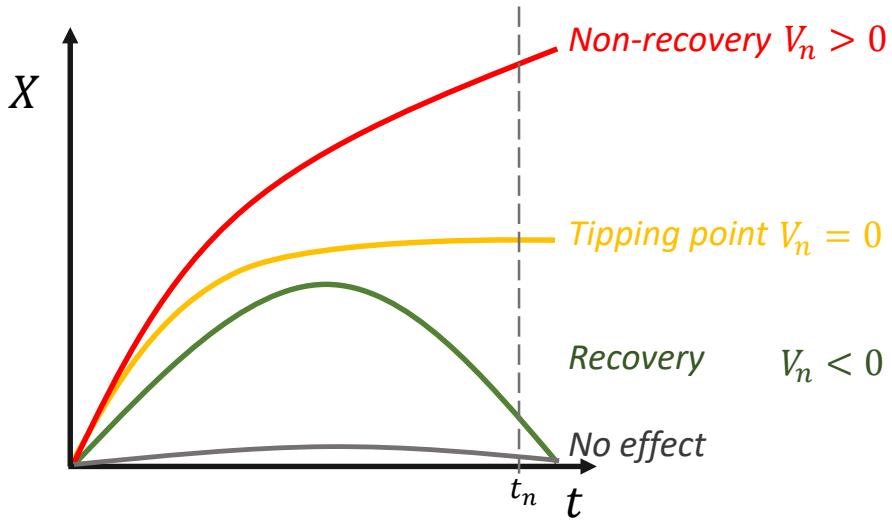


(Shah et al. 2016)

Identifying Tipping Points: from recovery to non-recovery

Measured using *in vitro* assays

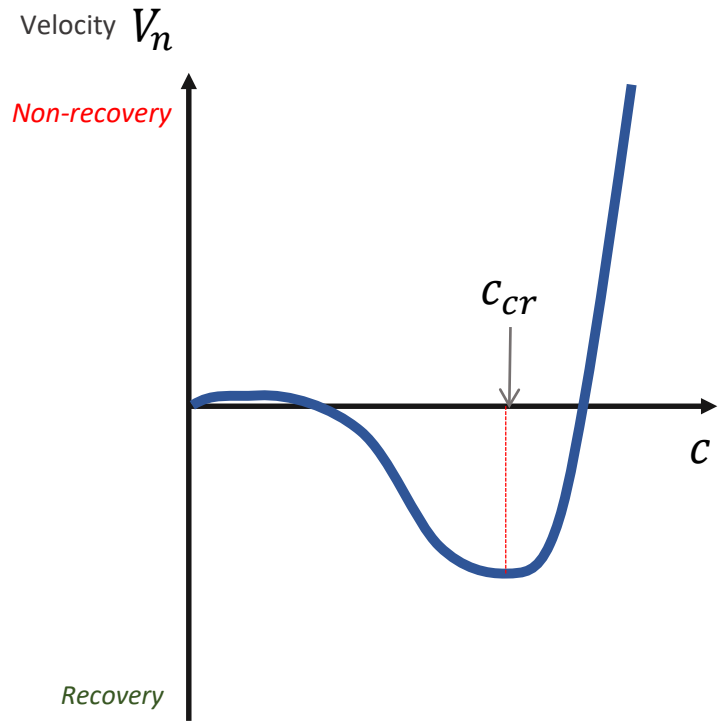
Cellular Perturbation



Measured using time-course *in vitro* data

Calculate velocity $V = \frac{dX}{dt}$

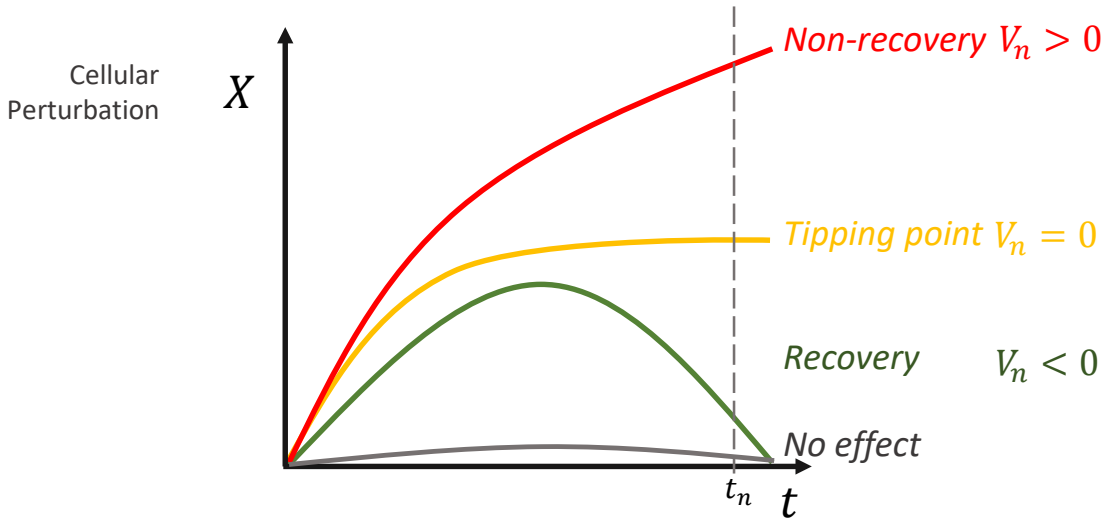
(Shah et al. 2016)



Tipping point concentration c_{cr} when $dV_c = \frac{dV}{dc} = 0$

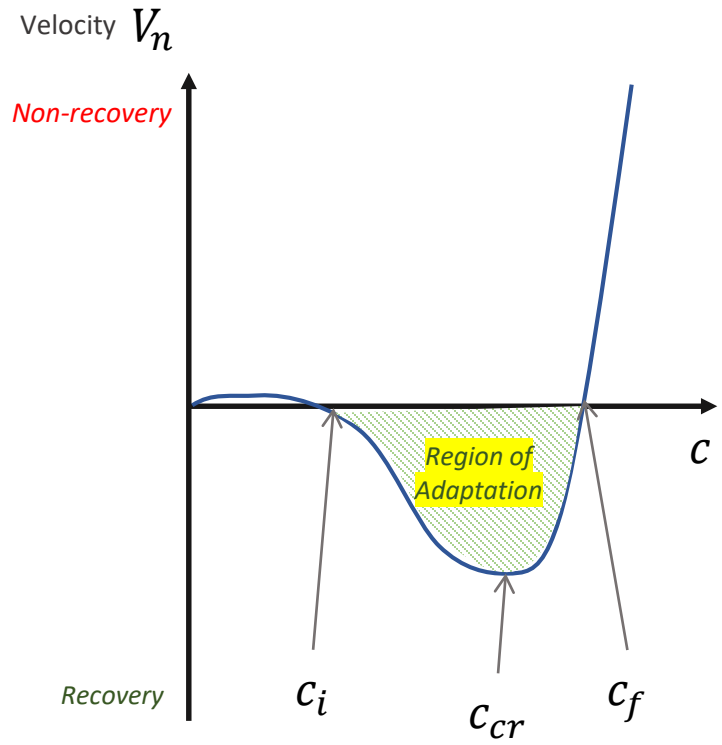
Shah et al. *in prep*

Tipping points and region of adaptation



Calculate velocity $V = \frac{dX}{dt}$

(Shah et al. 2016)



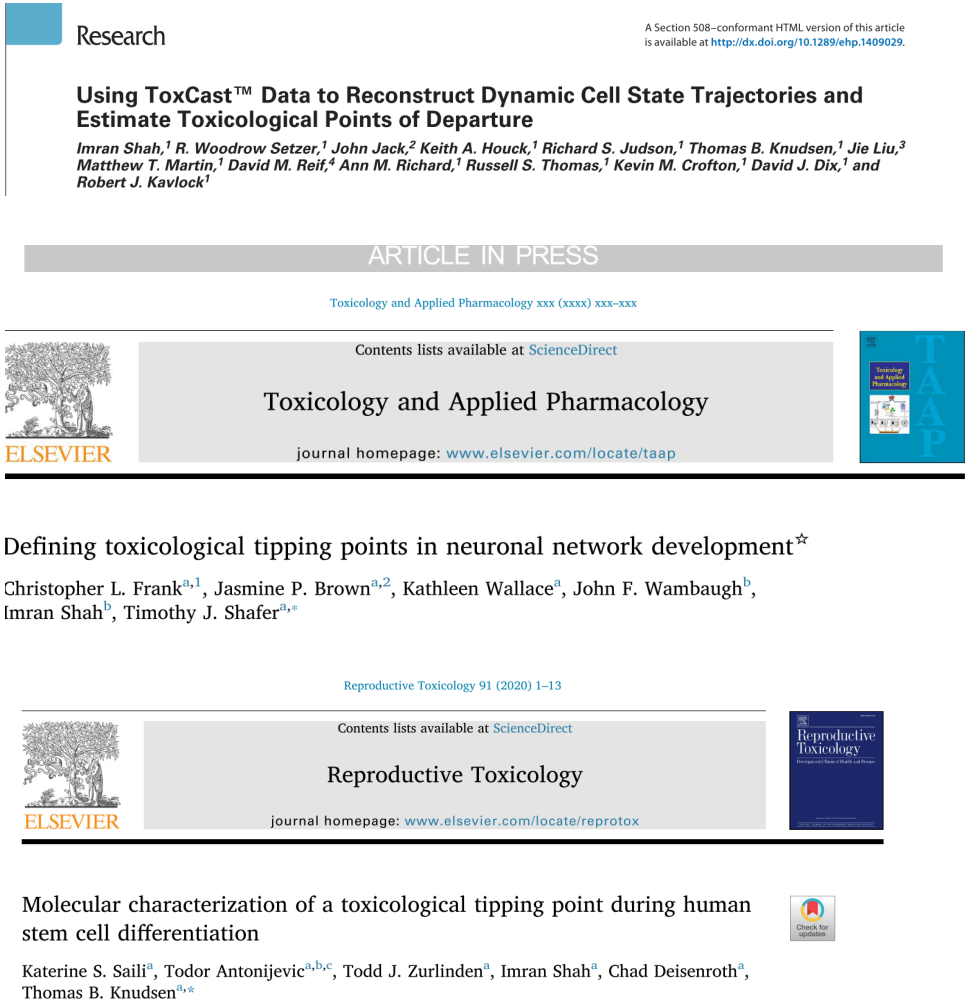
"Region of adaptation" defined by $c_i < c_{cr} < c_f$

Shah et al. *in prep*

Key Questions #1 & #2

In vitro surrogates of tipping points
Critical concentrations (c_{cr}) at tipping points

- Tipping points have been analyzed in three different *in vitro* models to demonstrate feasibility
- HepG2 cells using high-content imaging (HCI) to measure time-course cell phenotypic data (Shah et al. 2016)
- Developing rat neuronal networks and time-course microelectrode array data on electrophysiological activity (Franks et al. 2018)
- Induced pluripotent stem cells and time-course transcriptomic data during endodermal differentiation linked to ATRA signaling and toxicity (Saili et al. 2020)



Research

A Section 508–conformant HTML version of this article is available at http://dx.doi.org/10.1289/ehp.1409029.

Using ToxCast™ Data to Reconstruct Dynamic Cell State Trajectories and Estimate Toxicological Points of Departure

Imran Shah,[1] R. Woodrow Setzer,[1] John Jack,[2] Keith A. Houck,[1] Richard S. Judson,[1] Thomas B. Knudsen,[1] Jie Liu,[3] Matthew T. Martin,[1] David M. Reif,[4] Ann M. Richard,[1] Russell S. Thomas,[1] Kevin M. Crofton,[1] David J. Dix,[1] and Robert J. Kavlock[1]

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Defining toxicological tipping points in neuronal network development☆

Christopher L. Frank[a,1], Jasmine P. Brown[a,2], Kathleen Wallace[a], John F. Wambaugh[b], Imran Shah[b], Timothy J. Shafer[a,*]

Reproductive Toxicology 91 (2020) 1–13

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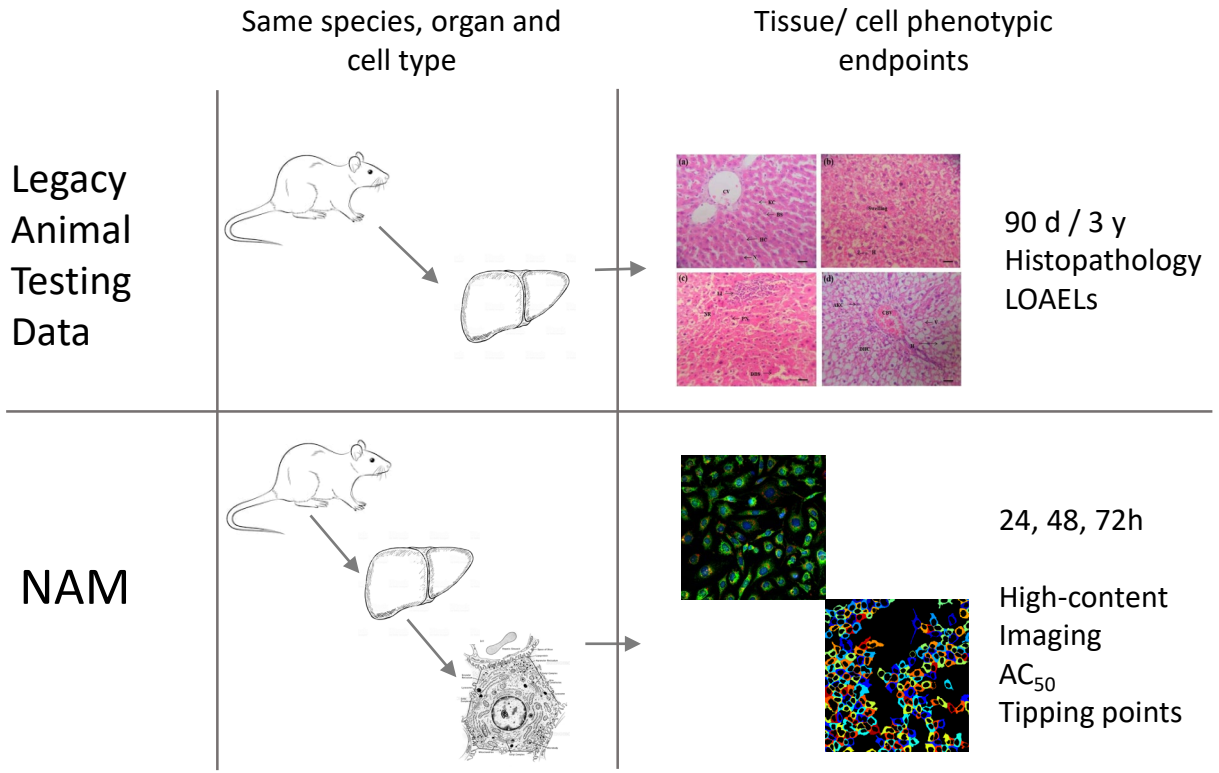
Molecular characterization of a toxicological tipping point during human stem cell differentiation

Katerine S. Saili[a], Todor Antonijevic[a,b,c], Todd J. Zurlinden[a], Imran Shah[a], Chad Deisenroth[a], Thomas B. Knudsen[a,*]

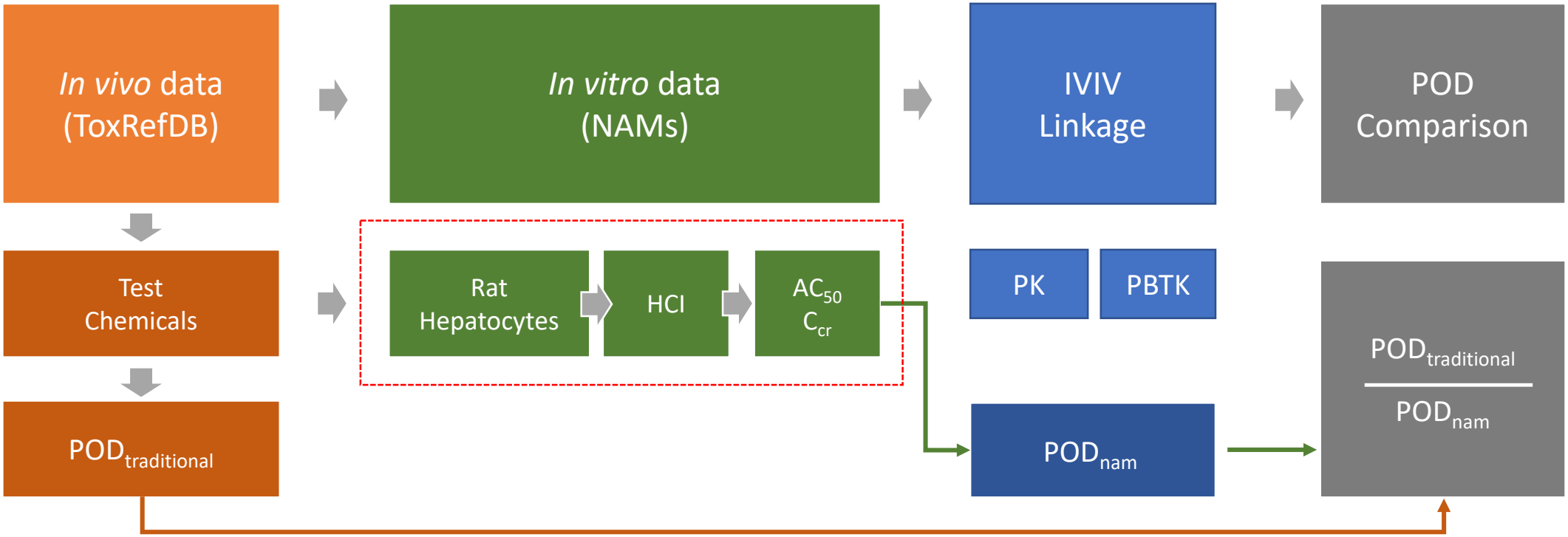
Key Question #3

Comparing critical concentrations with AC_{50} and LOAELs

- Case-study with 51 rat hepatotoxicants
  - *In vivo r*epeat oral dose toxicity in rats:
    - 37 chemicals: Subchronic (90d)
    - 45 chemicals: Chronic (2 y)
    - Hepatic lowest observed adverse effect levels (LOAELs)
  - *In vitro* assay:
    - Rat primary hepatocytes
    - 51 chemicals: 10 concentrations for 1, 2, 3 d
    - High-content Imaging (HCI) of cell phenotypes
    - ToxCast assay data (for comparison)
  - *In silico*:
    - Physiologically based toxicokinetic modeling (PBTK)
    - Estimate *in vitro* doses corresponding to POD values
- Compare $POD_{traditional}$ and POD_{nam}
  - $POD_{nam} = AC_{50}$ (using concentration-response analysis)
  - $POD_{nam} = \{c_{cr}, c_i, c_f\}$ (from tipping point analysis)



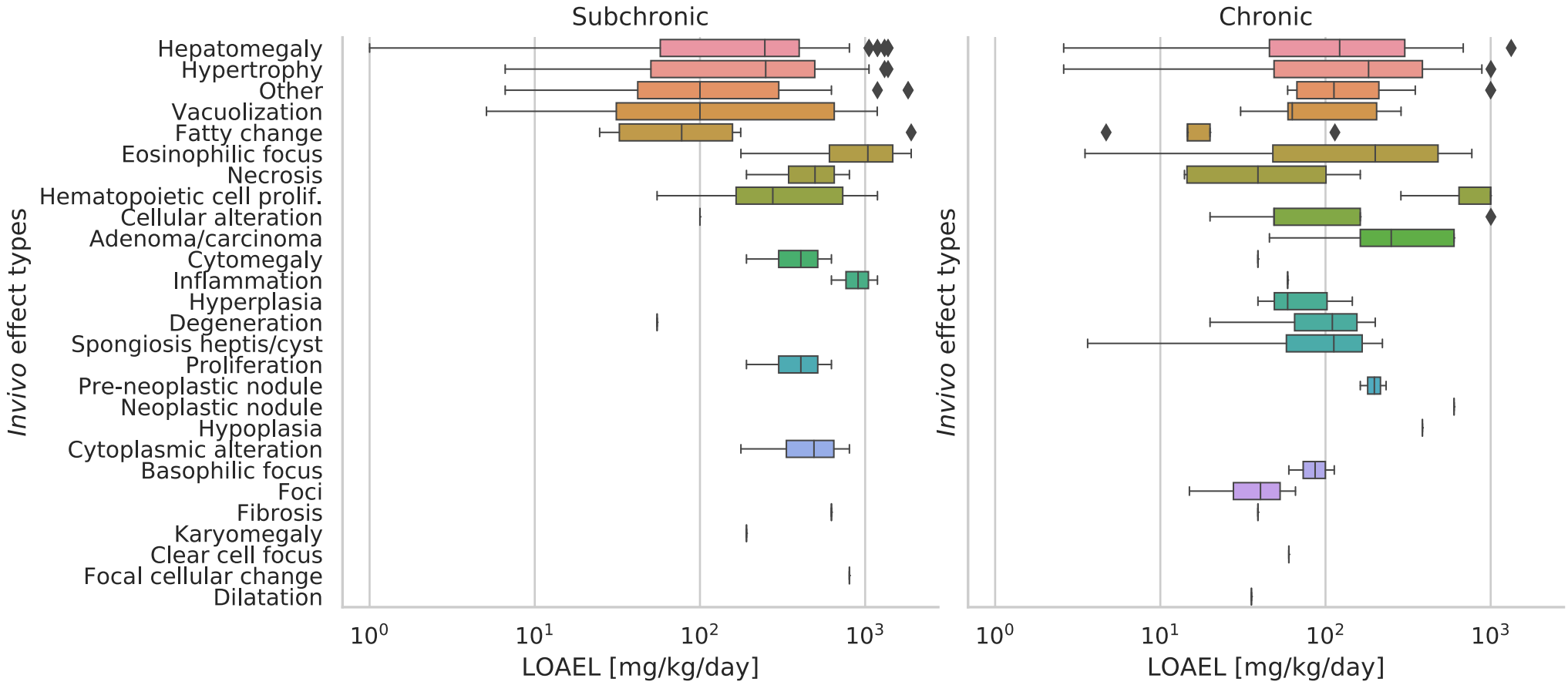
Workflow



(Shah et al. *in prep*)

Sub-chronic & Chronic Effects & LOAELs

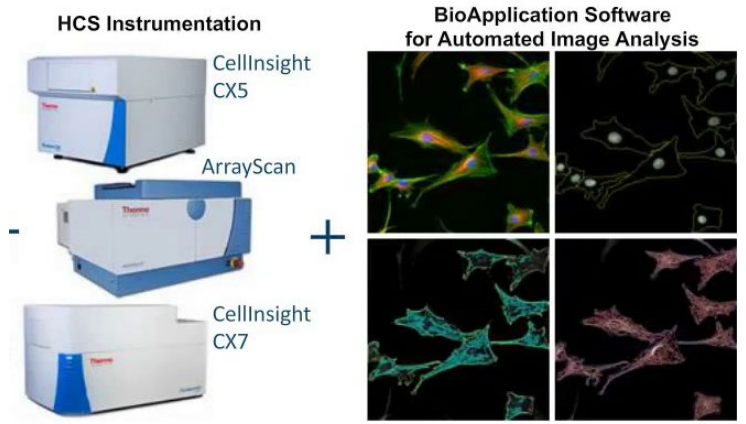
Results from repeat-dose guideline toxicity testing studies



51 chemicals. ToxRefDB v2.0 production. LOAELs & effects filtered by oral admin. studies in rats only

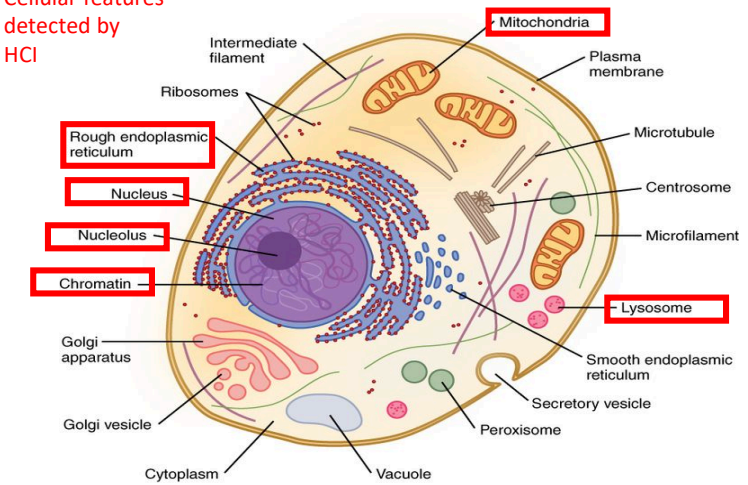
Rat Primary Hepatocyte Assay

- Chemical treatments
  - Controls: (-) DMSO; (+) CCCP, Bupivacaine, Tamoxifen, Nimesulide
  - Conc: 0.2, 0.39, 0.78, 1.56, 3.12, 6.24, 12.5, 25, 50 and 100 µM
  - Duration: 24, 48 and 72 h.
- Assay: 384 Well High-content imaging (HCI) (conducted by Cyprotex)
  - St/Steatosis: LipidTox®
  - ES/ER Stress: GADD153 (CHOP)
  - MF/Mitochondrial function/mass: MitoTracker Red
  - LM/Lysosomal Mass: LysoTracker Red
  - Ap/Apoptosis: Cytochrome C
  - DT/DNA texture: Hoechst 33342
  - NS/Nuclear size: Hoechst 33342
  - CN/Cell number: Hoechst 33342



Thermofisher.com

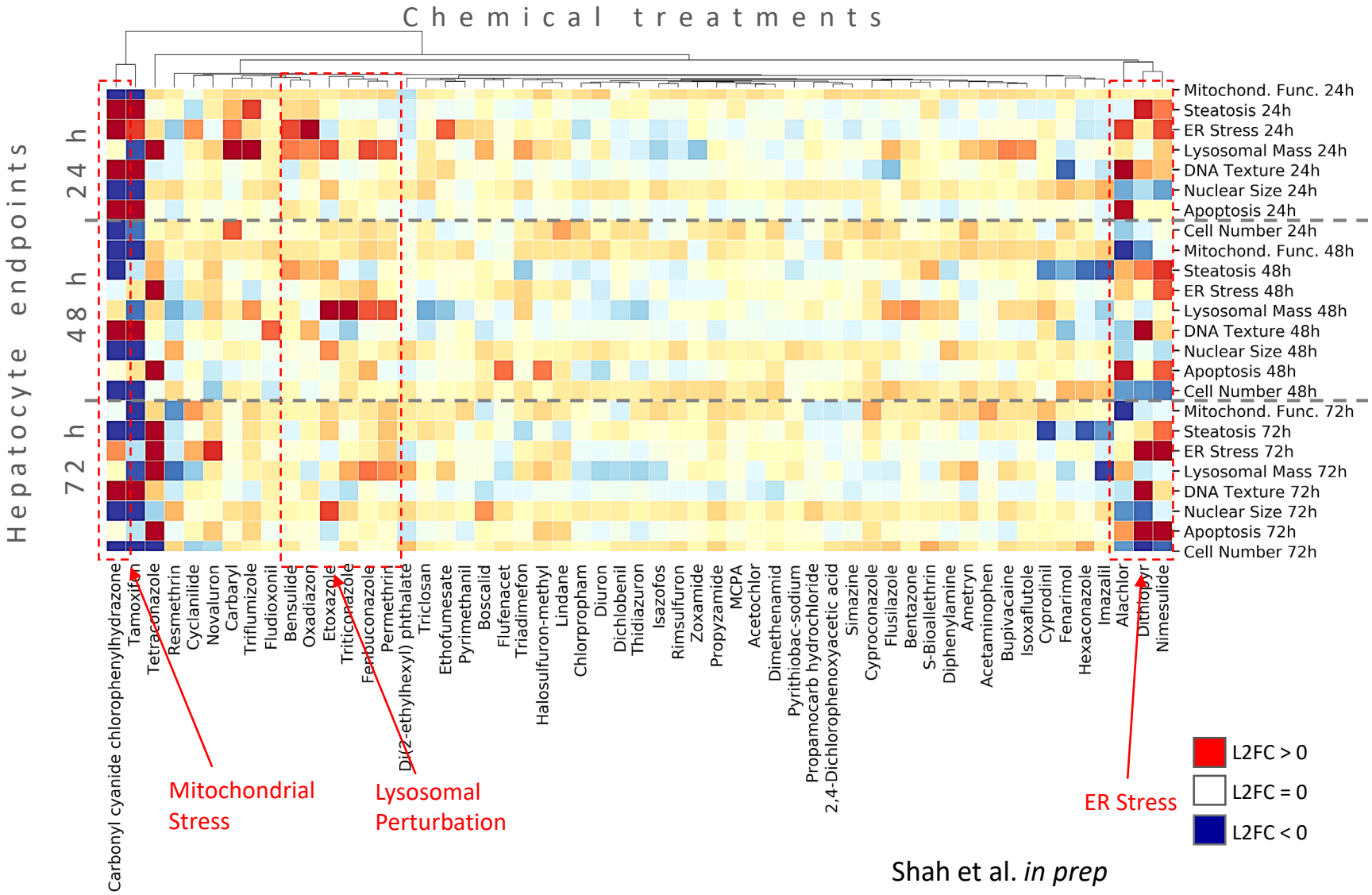
Cellular features detected by HCI



Wikimedia.org

Rat Hepatocyte HCI Effects – 50µM

- Log2 Fold Change (L2FC) by comparison with DMSO controls
- Summarize L2FC of all chemicals at 50µM
- Heatmap shows chemicals (columns) vs HCI features at 24, 48 and 72h and L2FC values (blue=decrease and red=increase)
- Phenotypic response categories
  - No significant effects
  - Mitochondrial stress ± cell death
  - Lysosomal mass ± cell death
  - ER Stress ± cell death



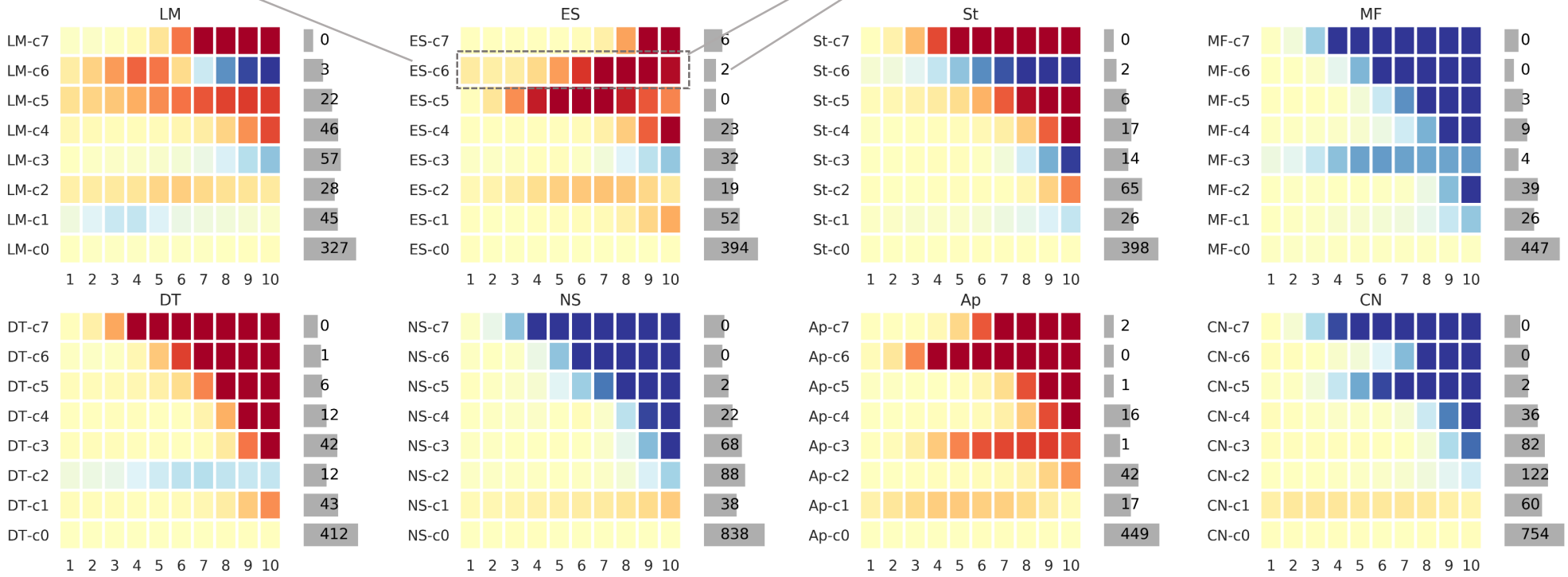
Shah et al. *in prep*

Conce r onse trends

Group of chemicals producing similar conc-response trends

Conc-response shape

Number of "test" chemicals in group



L2FC > 0
L2FC = 0
L2FC < 0

Conduct curve-fitting using tcplFit (Filer et al, 2014) to estimate AC_{50}
Summarize all concentration-response data by endpoint using clustering

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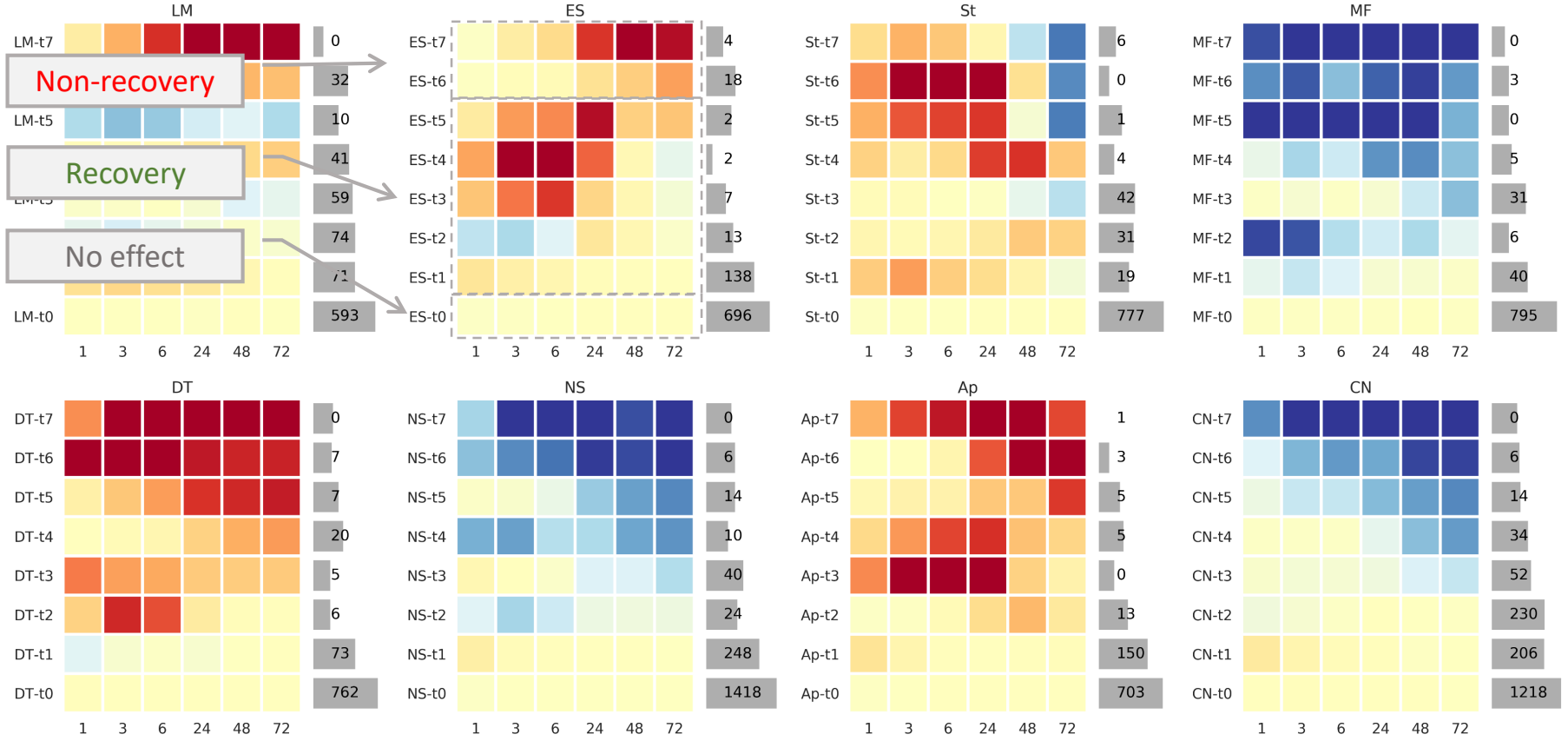
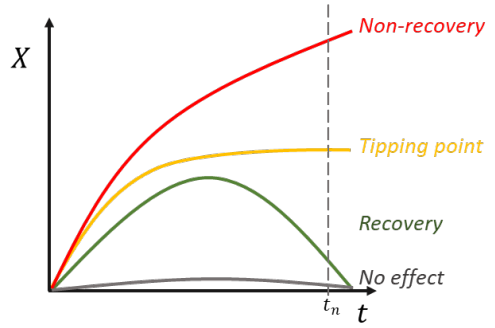
Concentration-Time-Response Trends





L2FC > 0
L2FC = 0
L2FC < 0

Summarize the concentration-time-response data by clustering
Mostly no effect, some recovery (transient) and some non-recovery trends

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Calculating Tipping Points for each chemical

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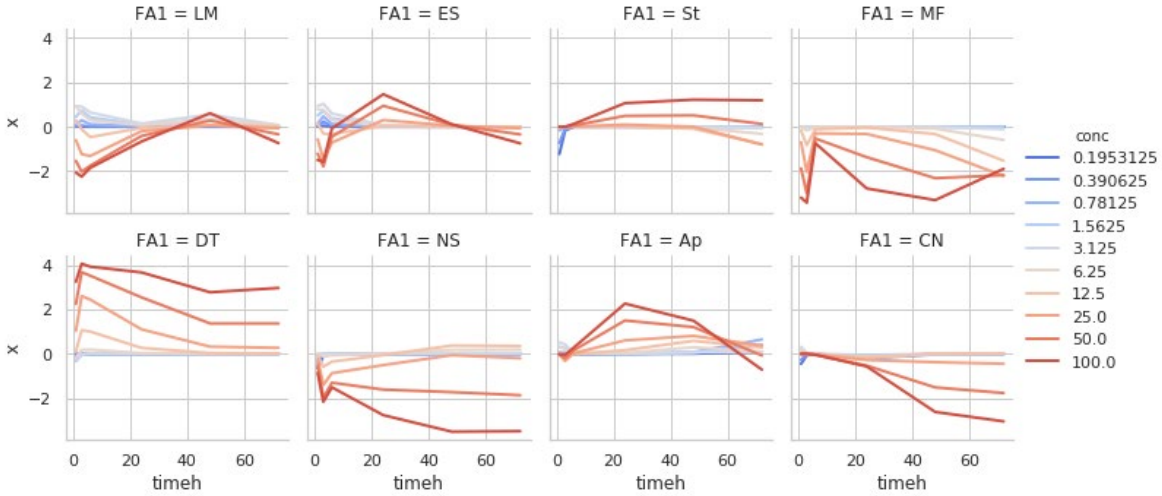
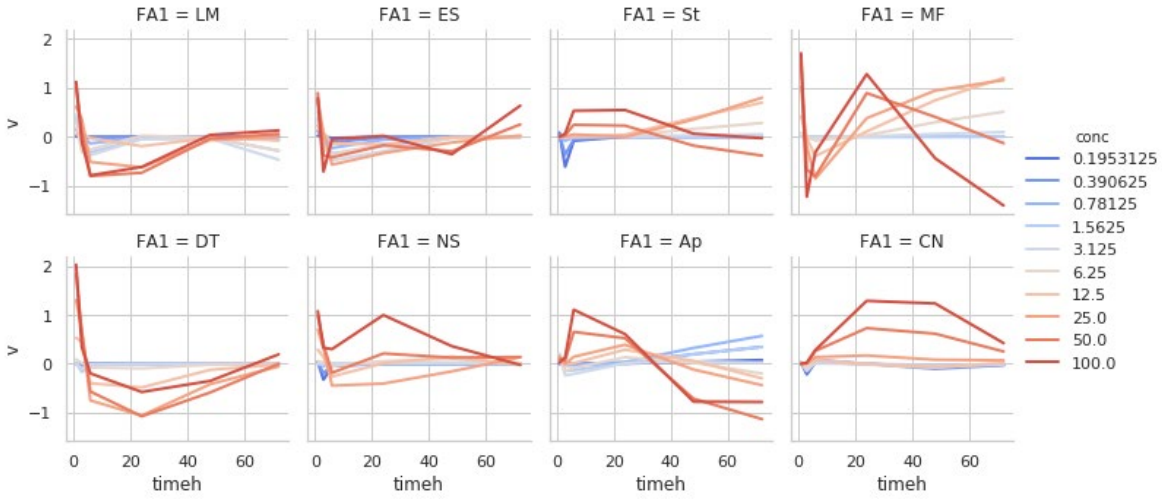
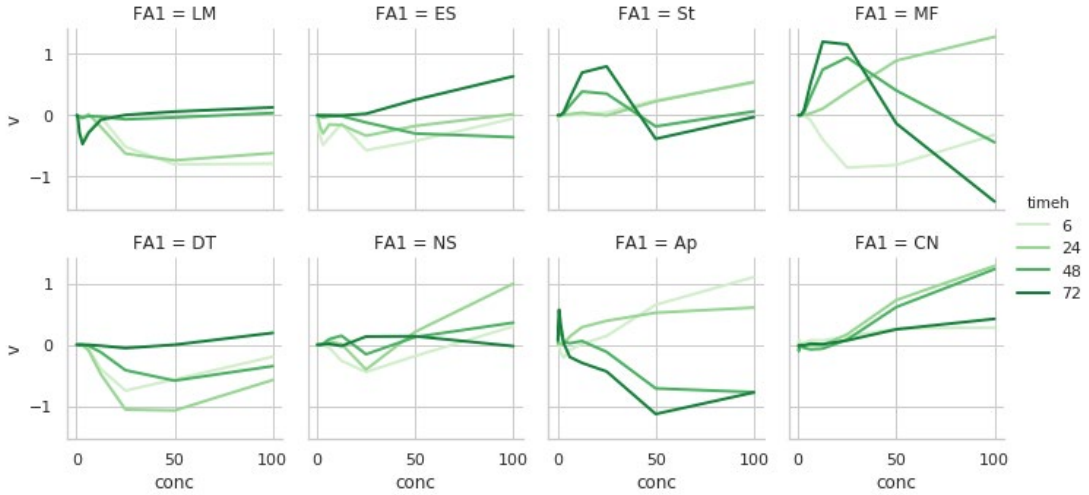
1. Perturbation (X) vs time (t) for all concs (c)



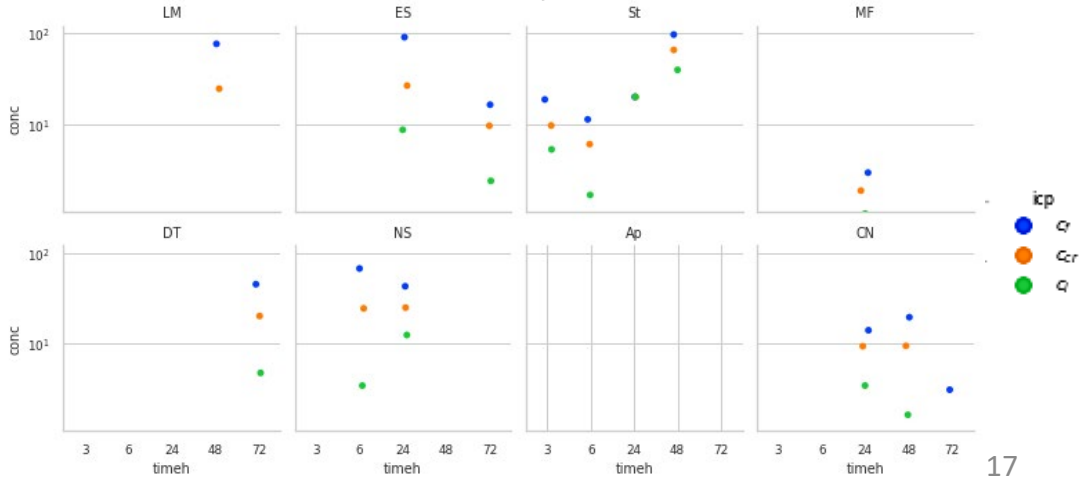
2. Velocity (V) vs time (t) for all concs (c)



3. Velocity (V) vs conc (c)



4. Critical concentrations: c_f, c_{cr}, c_i



Analyzed 51 Chemicals

In vitro high-throughput assays to estimate µM potency (POD_{nam})

In vitro to *in vivo* linkage using toxicokinetic modeling

In vivo toxicity testing to determine critical effects and point of departure ($POD_{traditional}$)

Potency metrics:
Conc-response: AC_{50}
Tipping points: c_i, c_{cr}, c_f

Endpoints: 8
Exposure durations: 1, 2, 3 d

IVIV approaches:
Steady-state (SS)
PBTK: AUC, Cmax, Cave (90, 730d)

Study types: Subchronic and Chronic repeat-dose testing
PODs: LOAEL

Comparing POD_{nam} to POD_{trad}

In vitro (NAM)

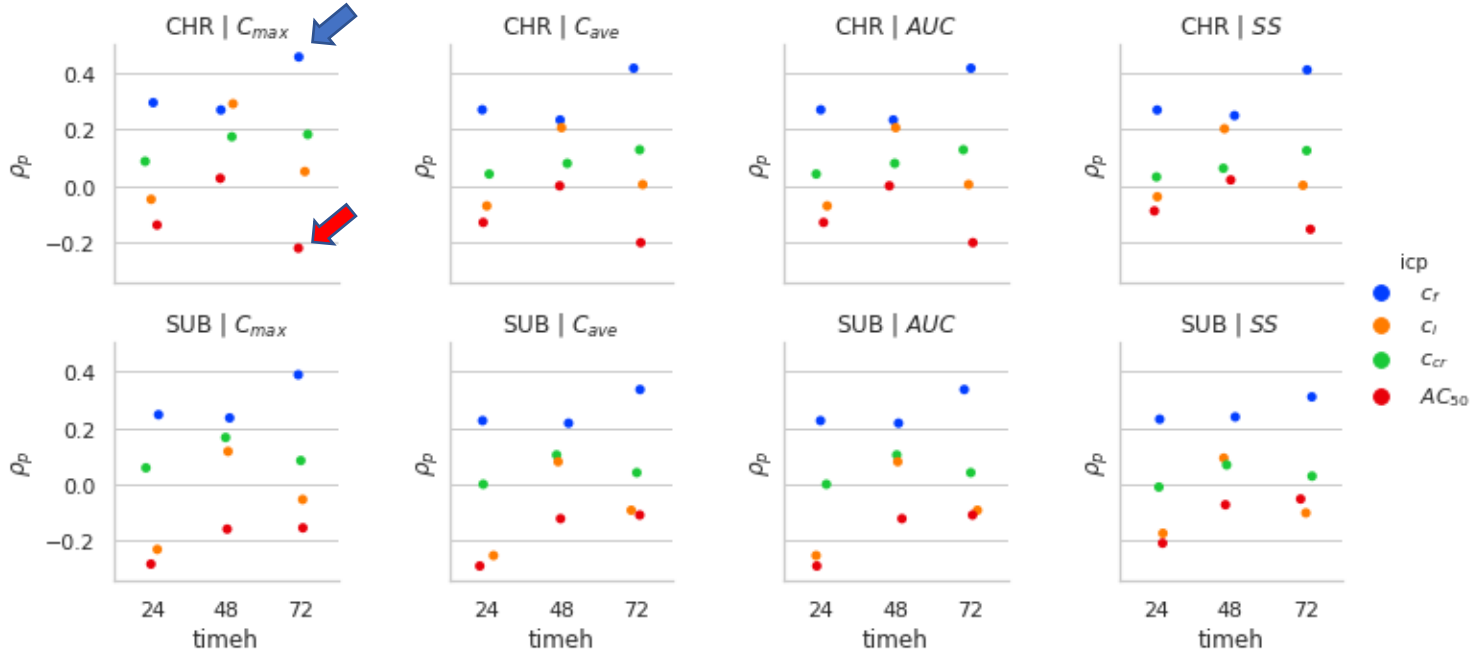
- 24 h exposure
- 50th percentiles of c_f, c_{cr}, c_i and AC_{50} across all endpoints

In vivo (Subchronic)

- 50th percentile of LOAEL values
- PBTK modeling to estimate venous C_{ave}

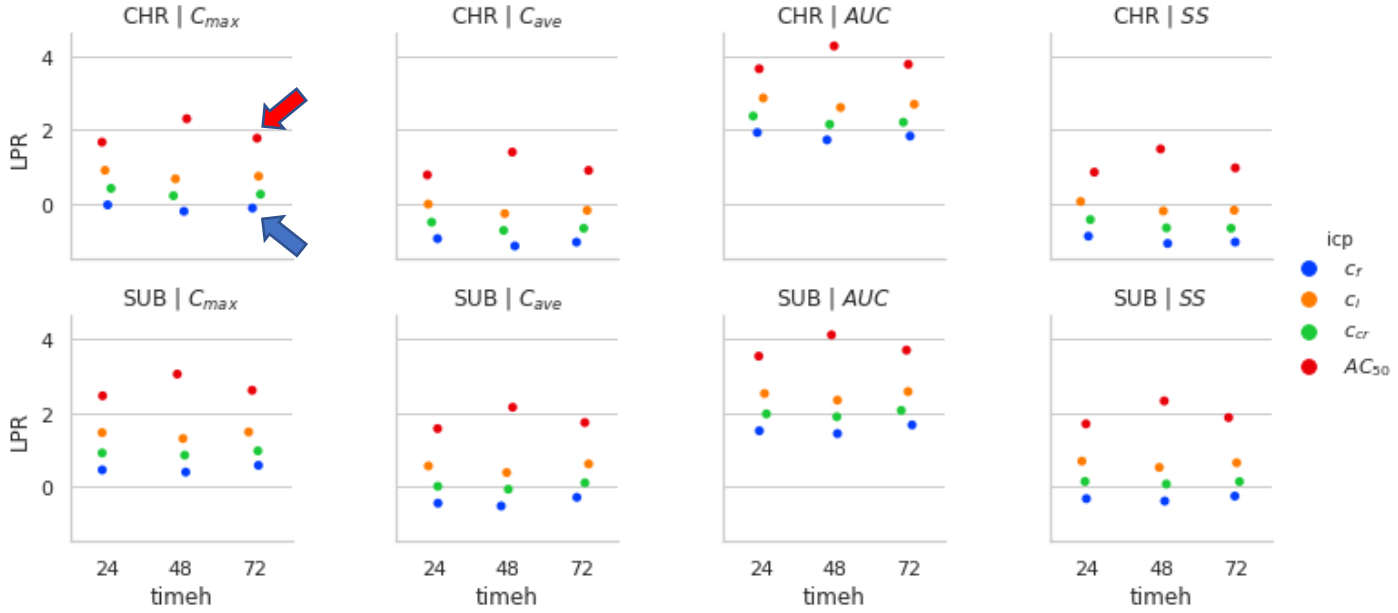
How close are POD_{nam} to $POD_{trad.}$?

- Use Pearson correlation (ρ_p)
- ρ_p : $c_f > c_{cr} > c_i >> AC_{50}$
- ρ_p best for 72 h *in vitro* exposure
- C_{max} PBTK dose metric has maximum ρ_p



How health-protective are POD_{nam} ?

- Use LPR = log10($POD_{traditional}$/POD_{nam})
- LPR~2 AC_{50} is the most conservative
- AUC is the most health-protective LPR>2
- Similar LPR for chronic and subchronic POD_{trad}



Shah et al. *in prep*

Summary

1. *In vitro* surrogates of tipping points

   Using time-course *in vitro* data it may be feasible to identify a region of adaptation and critical points related to cellular non-recovery. This may not capture higher tissue-level adaptative responses but is a useful starting point for consider cellular resilience.

2. Estimating chemical critical concentrations at tipping points

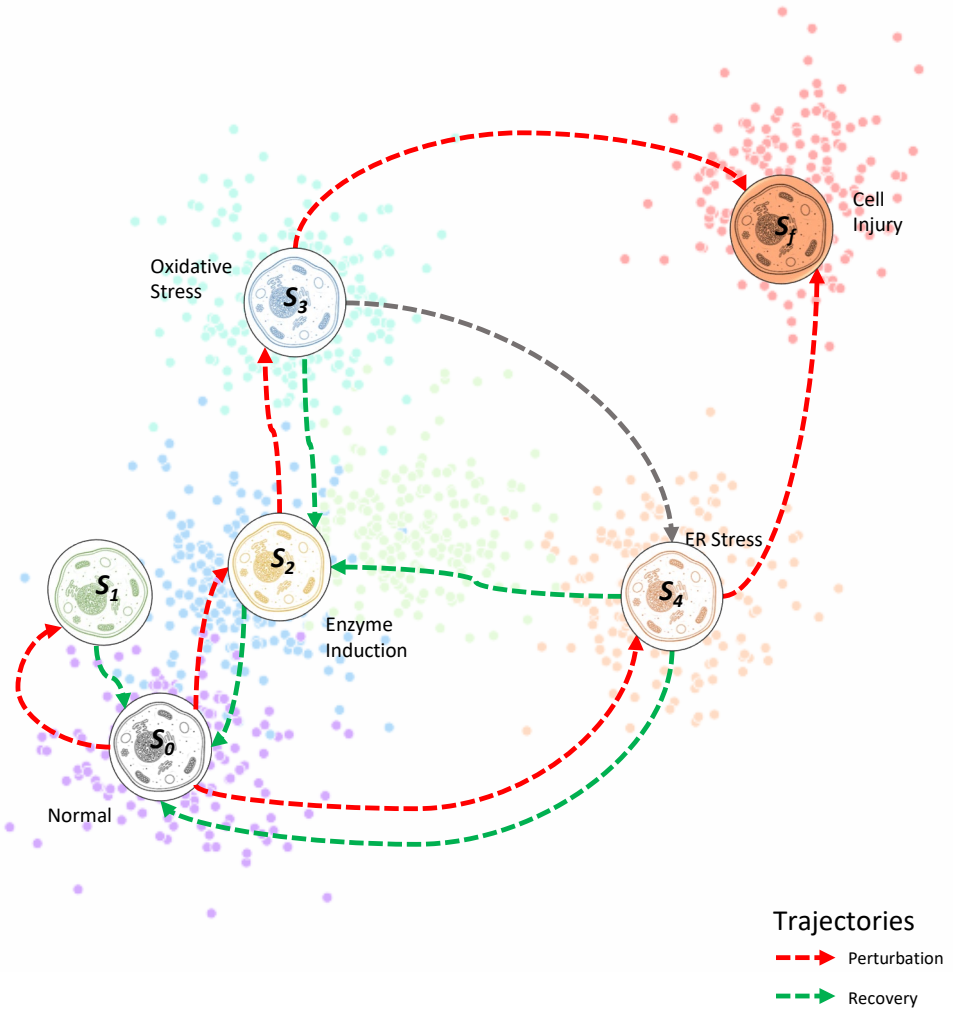
   We hypothesize an adaptative region defined by c_i, c_{cr}, c_f that can be identified from time-course multi-parameter data. We estimate these critical concentrations using HCI.

3. Comparing tipping point concentrations with *in vitro* AC_{50} and LOAELs

   We estimated c_i, c_{cr}, c_f and AC_{50} for rat primary hepatocytes endpoints and compared them with rat subchronic and chronic hepatic LOAELs using PBTK. While AC_{50} are highly-health protective (20x lower than LOAELs) the c_f are highly correlated with LOAELS ($\rho_p \sim 0.4$).

4. Future directions

   Analyzing the systems biology of adaptive stress response pathways in order to further investigate the molecular basis of cellular resilience and tipping points, to streamline the development of NAMs for evaluating untested chemicals based adaptive stress responses and overcome barriers to acceptance.



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