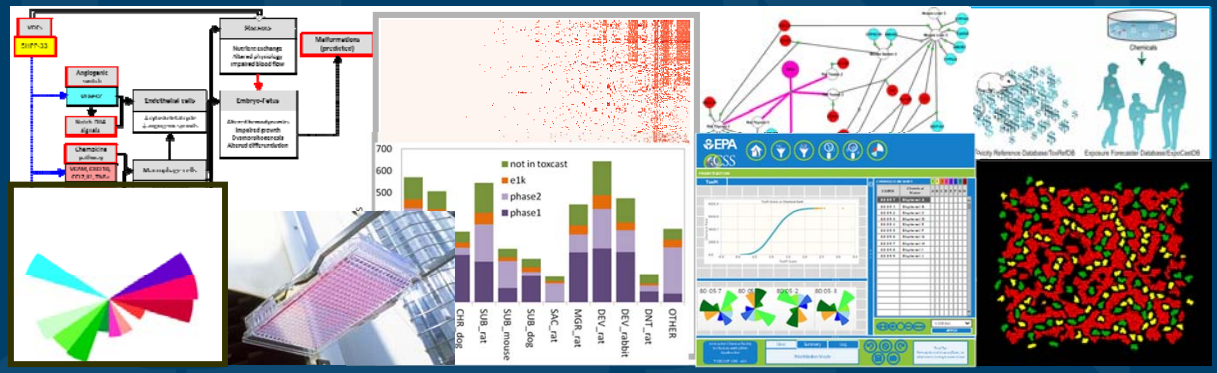
EPA
United States
Environmental Protection
Agency

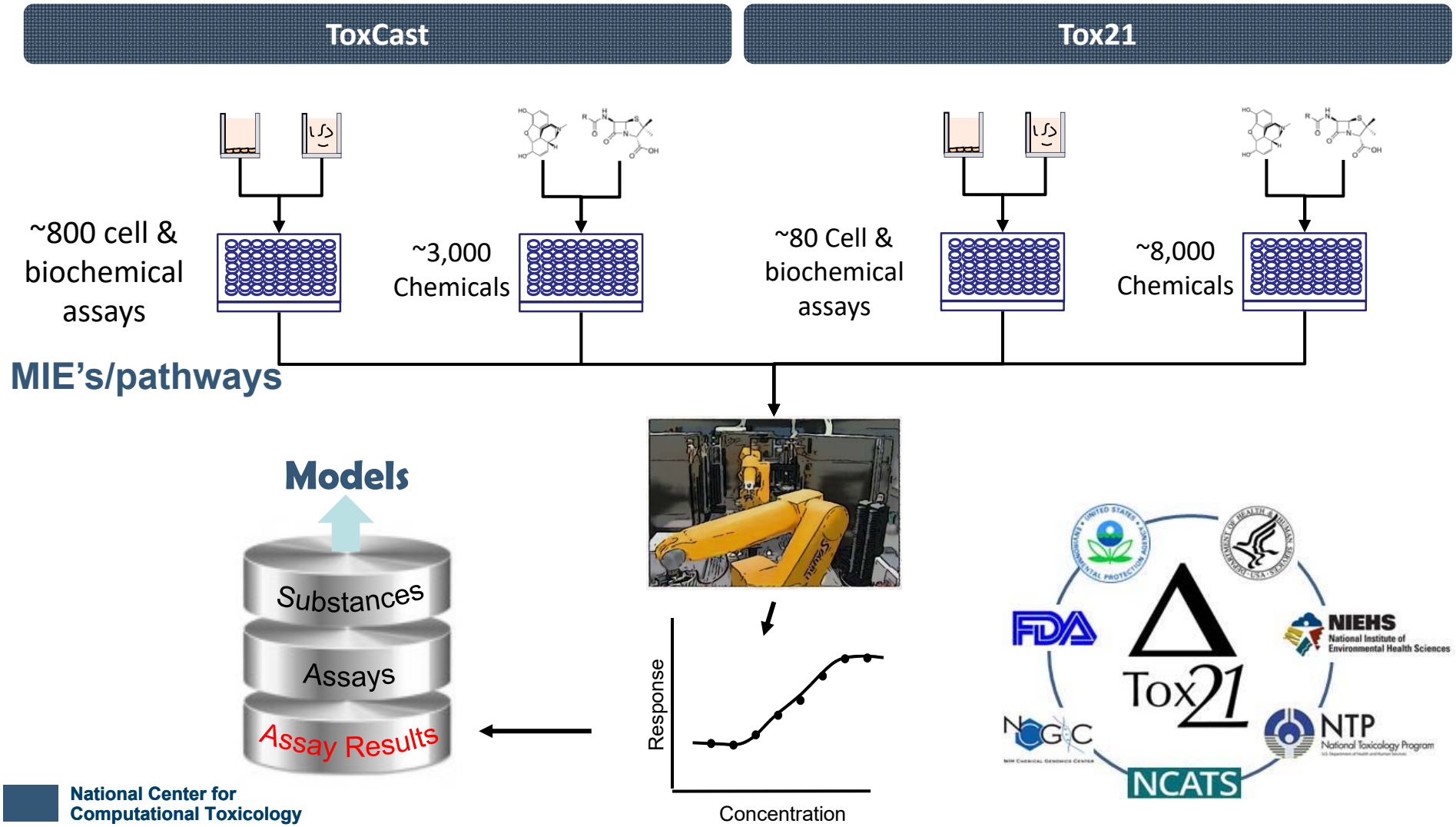
Update on the ToxCast Chemical Prioritization Project



CBCRP
Oakland, CA
February 12-13, 2018

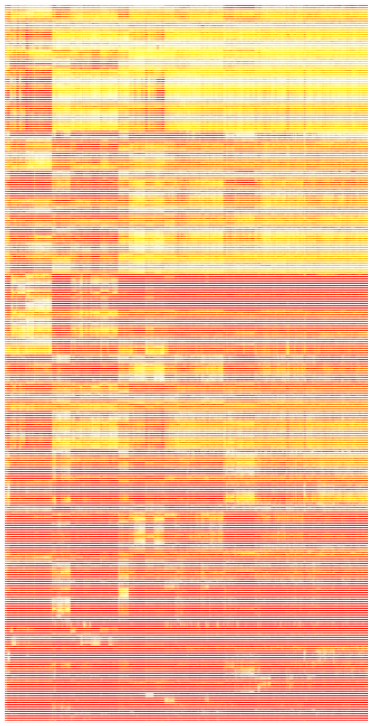
Keith Houck
National Center for Computational Toxicology

High-Throughput Hazard Screening

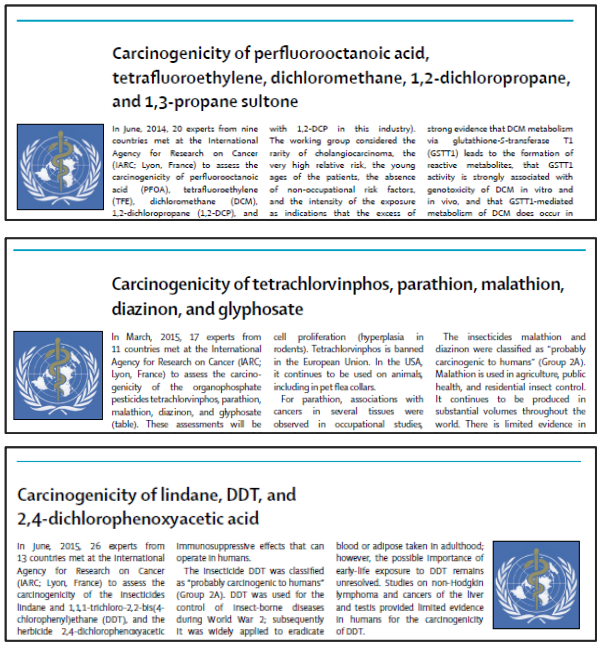


Broad Success Derived from High-Throughput Screening Approaches

Group Chemicals by Similar Bioactivity and Predictive Modeling



Provide Mechanistic Support for Hazard ID



Carcinogenicity of perfluorooctanoic acid, tetrafluoroethylene, dichloromethane, 1,2-dichloropropane, and 1,3-propane sultone

In June, 2014, 20 experts from nine countries met at the International Agency for Research on Cancer (IARC; Lyon, France) to assess the carcinogenicity of perfluorooctanoic acid (PFOA), tetrafluoroethylene (TFE), dichloromethane (DCM), 1,2-dichloropropane (1,2-DCP), and with 1,2-DCP in this industry). The working group considered the rarity of cholangiocarcinoma, the very high relative risk, the young ages of the patients, the absence of non-occupational risk factors, and the intensity of the exposure as indications that the excess of strong evidence that DCM metabolism via glutathione-S-transferase T1 (GSTT1) leads to the formation of reactive metabolites, that GSTT1 activity is strongly associated with genotoxicity of DCM in vitro and in vivo, and that GSTT1-mediated metabolism of DCM does occur in

Carcinogenicity of tetrachlorvinphos, parathion, malathion, diazinon, and glyphosate

In March, 2015, 17 experts from 11 countries met at the International Agency for Research on Cancer (IARC; Lyon, France) to assess the carcinogenicity of the organophosphate pesticides tetrachlorvinphos, parathion, malathion, diazinon, and glyphosate (table). These assessments will be cell proliferation (hyperplasia in rodents). Tetrachlorvinphos is banned in the European Union. In the USA, it continues to be used on animals, including in pet flea collars.

For parathion, associations with cancers in several tissues were observed in occupational studies, The insecticides malathion and diazinon were classified as "probably carcinogenic to humans" (Group 2A). Malathion is used in agriculture, public health, and residential insect control. It continues to be produced in substantial volumes throughout the world. There is limited evidence in

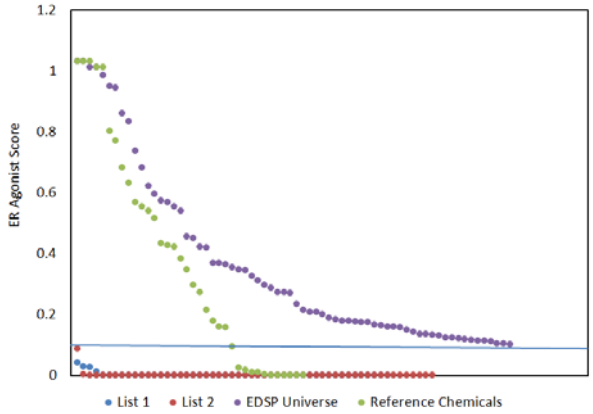
Carcinogenicity of lindane, DDT, and 2,4-dichlorophenoxyacetic acid

In June, 2015, 26 experts from 13 countries met at the International Agency for Research on Cancer (IARC; Lyon, France) to assess the carcinogenicity of the insecticides lindane and 1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane (DDT), and the herbicide 2,4-dichlorophenoxyacetic immunosuppressive effects that can operate in humans.

The insecticide DDT was classified as "probably carcinogenic to humans" (Group 2A). DDT was used for the control of insect-borne diseases during World War 2; subsequently it was widely applied to eradicate blood or adipose taken in adulthood; however, the possible importance of early-life exposure to DDT remains unresolved. Studies on non-Hodgkin lymphoma and cancers of the liver and testis provided limited evidence in humans for the carcinogenicity of DDT.

IARC Monographs 110, 112, 113

Prioritization of Chemicals for Further Testing



FIFRA SAP, Dec 2014

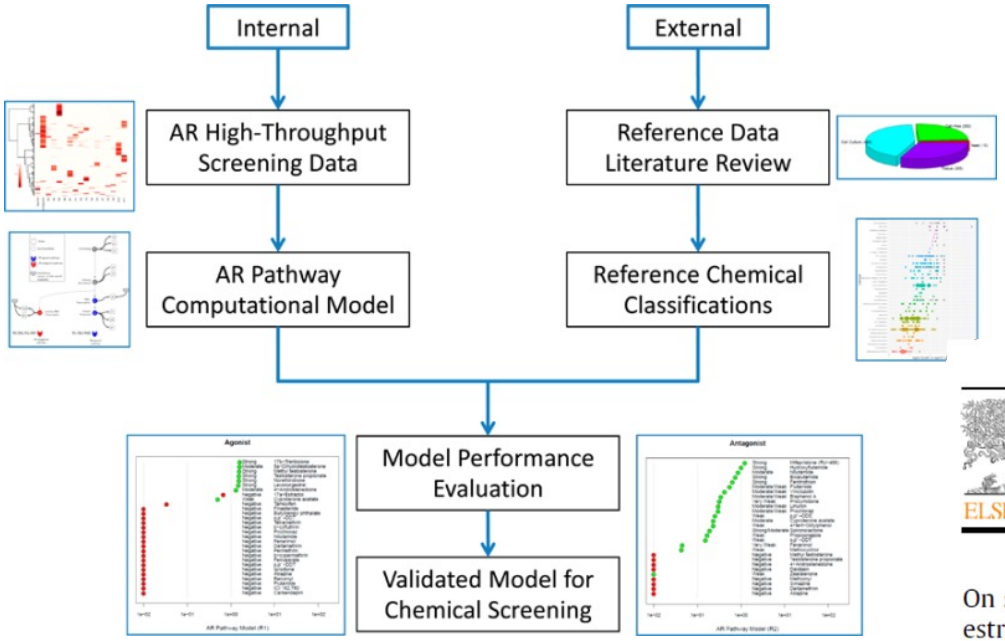
EPA
United States
Environmental Protection
Agency

What Are We Doing Now?

Continued EDSP Support

United States Environmental Protection Agency

AR Model



Chemical Research in Toxicology

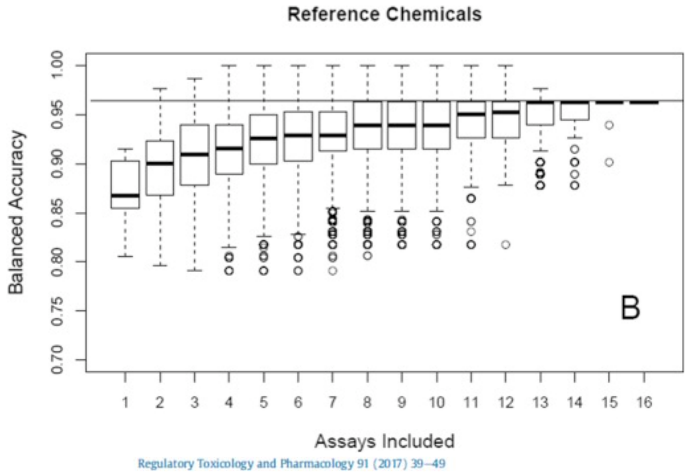
pu

Development and Validation of a Computational Model for Androgen Receptor Activity

Nicole C. Kleinstreuer,*,† Patricia Ceger,‡ Eric D. Watt,§ Matthew Martin,§ Keith Houck,§ Patience Browne,∥ Russell S. Thomas,§ Warren M. Casey,† David J. Dix,⊥ David Allen,‡ Srilatha Sakamuru,# Menghang Xia,# Ruili Huang,# and Richard Judson§

National Center for Computational Toxicology

ER Model



Regulatory Toxicology and Pharmacology 91 (2017) 39–49

Contents lists available at ScienceDirect

Regulatory Toxicology and Pharmacology

journal homepage: www.elsevier.com/locate/yrtph

On selecting a minimal set of *in vitro* assays to reliably determine estrogen agonist activity

Richard S. Judson*, Keith A. Houck, Eric D. Watt, Russell S. Thomas

US. Environmental Protection Agency, RTP, NC, United States

HPT Axis Targets

Assay Target	
OATP	TR
MCT8	Duox
Sulfation/Gluc	Deiodinases
Agonists	NIS
AhR	Pendrin
Antagonists	TBG
CAR	Thyroid Receptors
PXR	TPO
Phase I	TRH Receptor
Phase II	TSH Receptor
Hepatic Metabolism	TTR

Addressing Selected Criticisms of ToxCast Program

- You don't include metabolism in your *in vitro* assays
- You don't measure my favorite endpoint
- You don't cover all of biological space
- *In vitro* assays are not normal biology
- Assay (x) in your battery did not get the right answer for my chemical
- My assay disagrees with your assay (x), so your approach is flawed
- You can't test my favorite chemicals because of limitations in your methods (e.g., solvents, high LogP)
- Your assay descriptions to do not allow me to reproduce your results
- I get different answers when I analyze your data

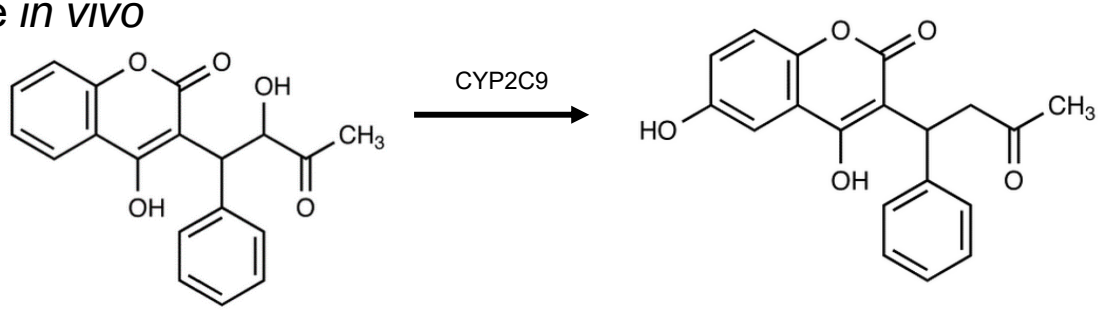
Updated from Bob K's original list

Why is Metabolic Competence Important for *in vitro* Assays?

Our existing *in vitro* assays have limited or no metabolic capacity. This leads to two problems:

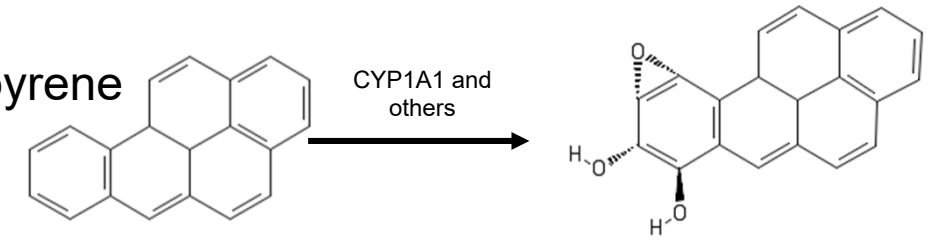
1. Overestimation of chemical hazard *in vitro* if the parent compound is detoxified to a less toxic or non-toxic metabolite *in vivo*

Example: Warfarin



2. Underestimation of chemical hazard *in vitro* if the parent compound is activated to a more toxic metabolite *in vivo*

Example: Benzo[a]pyrene



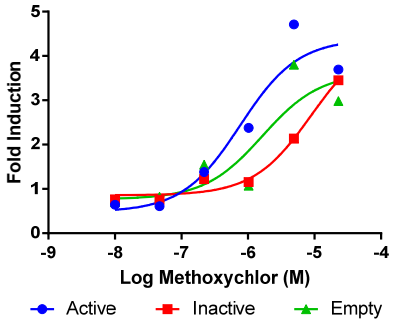
Steve Simmons/NCCT

Beginning to Address Metabolic Competence

United States Environmental Protection Agency

"Extracellular" Approach

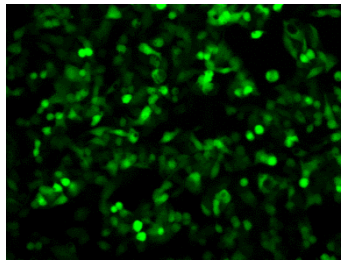
Chemicals metabolism in the media or buffer of cell-based and cell-free assays

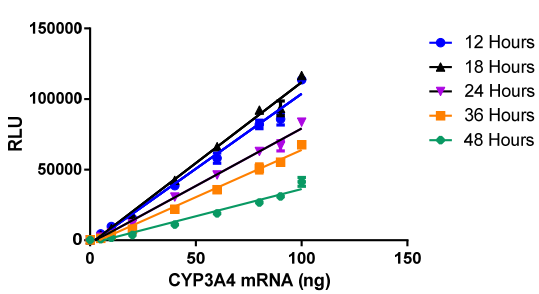


More closely models effects of hepatic metabolism and generation of circulating metabolites

"Intracellular" Approach

Capable of metabolizing chemicals inside the cell in cell-based assays





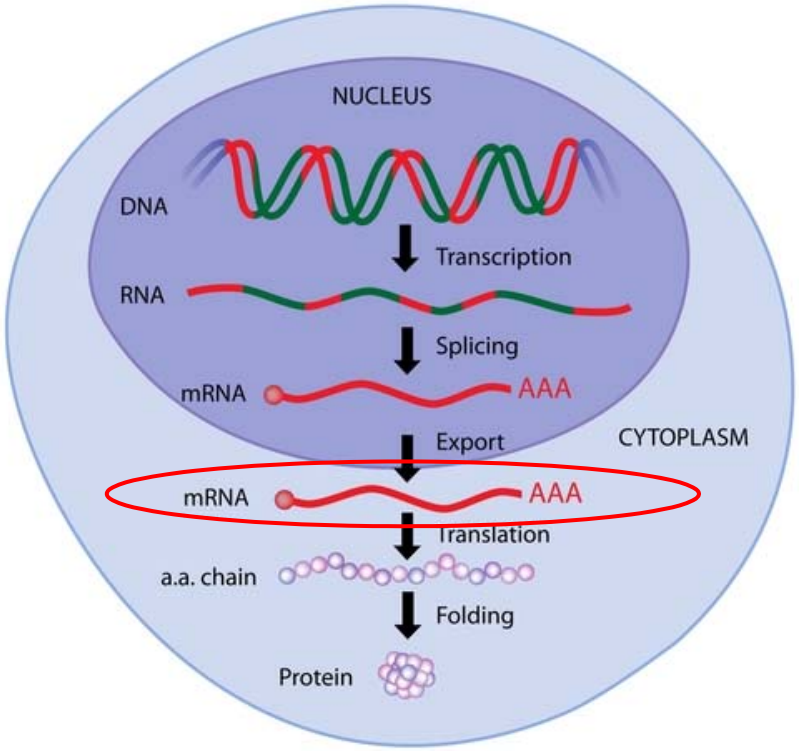
More closely models effects of target tissue metabolism

Integrated approach to model *in vivo* metabolic bioactivation and detoxification

Steve Simmons/NCCT

Collaboration with Unilever

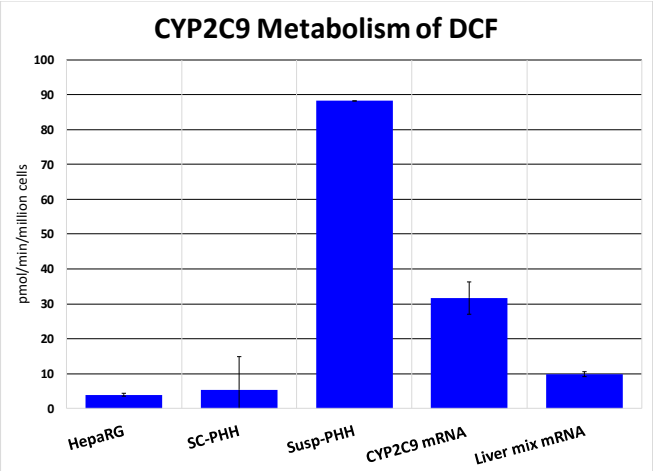
Intracellular Metabolism with mRNA Transfection

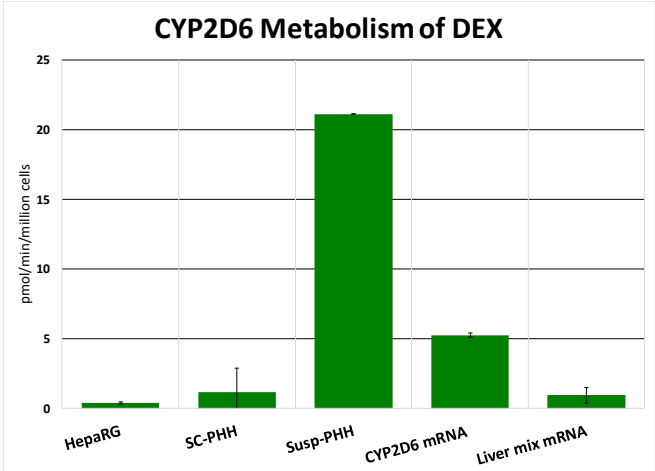


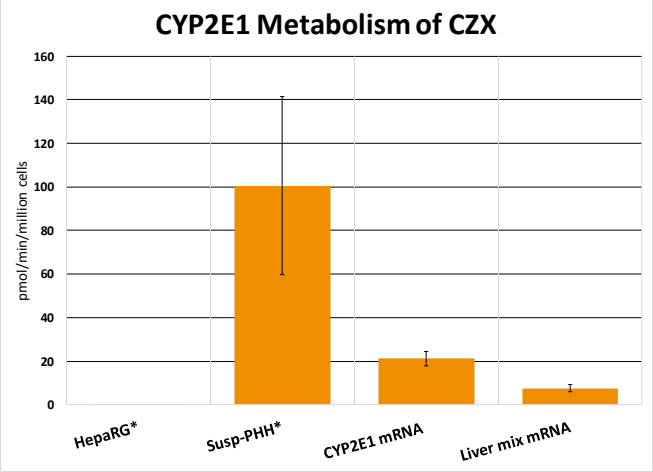
- Introducing xenobiotic-metabolizing enzyme (XME)-encoding genes back into cells with low/no expression is not a new idea
- Plasmid transfection, electroporation, and various viral vectors introduce XME-encoding genes (DNA) back into cells under control of gene promoters that drive strong expression (transcription)
- Transcription levels vary greatly between cell types and tightly controlled co-expression genes is difficult

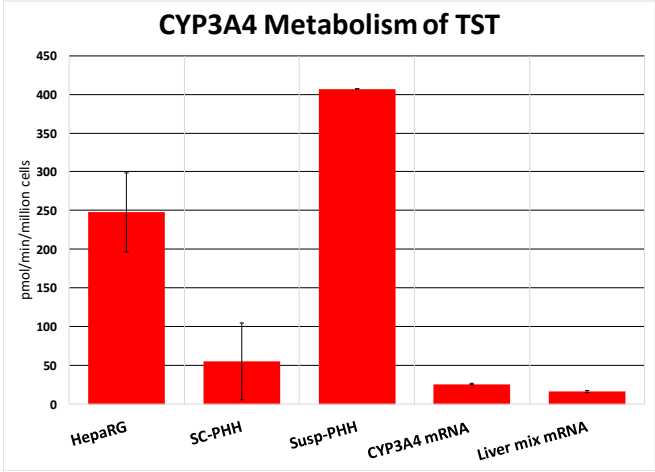
- Transfection of XME-encoding mRNAs is a novel approach that bypasses cellular transcription
- Chemically-modified nucleotides and cap eliminate the toxicity traditionally seen with RNA transfection
- Rapid XME expression and permits user to define composition and ratios of input mRNAs
- Method development focused on cytochrome P450 (CYP) enzymes, responsible for phase I metabolism

Comparison to "Gold-Standard" XM-Competent Cell Models

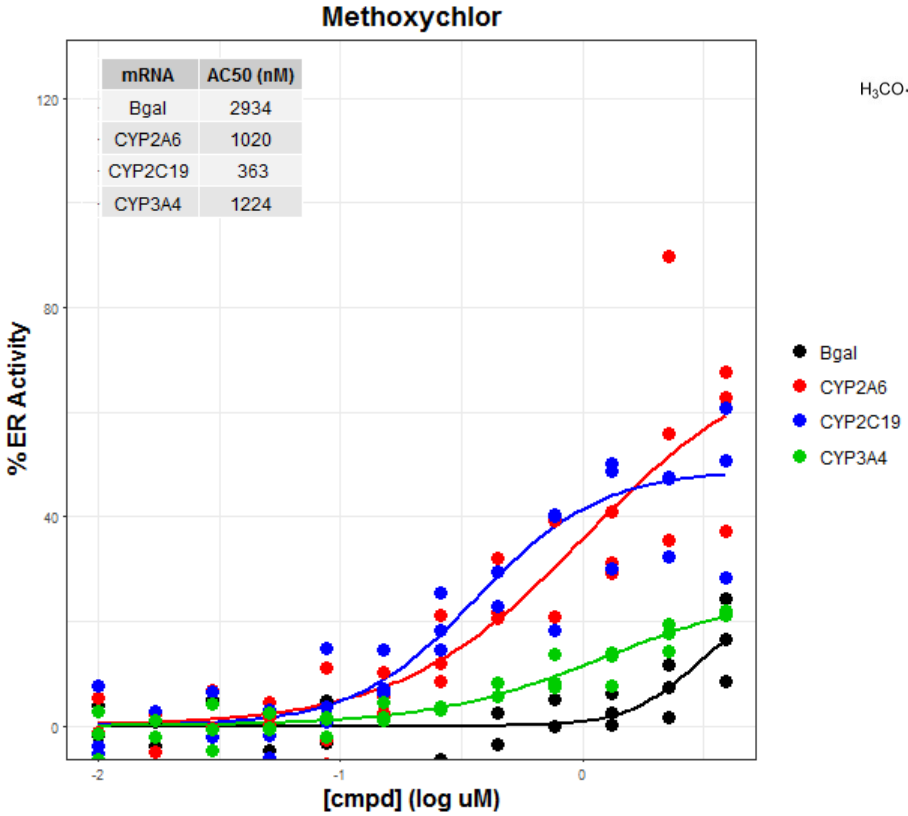


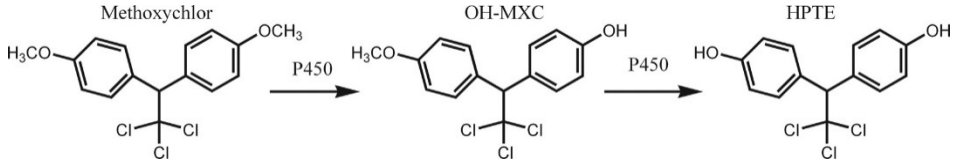






Deployment to ER Transactivation Assay





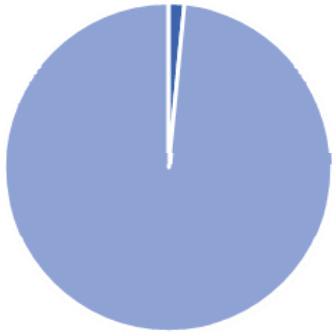
- Methoxychlor (MXC) has minimal ER agonist activity
- MXC is demethylated by certain human CYP450 enzymes to HPTE: 1A2, 2A6, 2C18, 2C19 > 2B6, 2C9
- HPTE is a more potent and efficacious agonist of ER

- VM7 cells (formerly BG1) transfected with CYP-encoding mRNA or B-gal control for 6 hours (384w)
- Exposed to MXC (10nM – 5μM) for 24 hours
- Activity normalized to maximal E2-induced activity (parallel wells on same plate)
- A minimal ER response was seen in cells transfected with B-gal or CYP3A4 mRNA
- A pronounced ER response was observed in cells transfected with CYP2A6 or CYP2C19

Beginning to Address Concerns for Increased Biological Coverage

United States Environmental Protection Agency

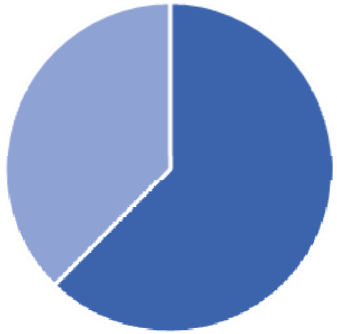
Gene Coverage



ToxCast

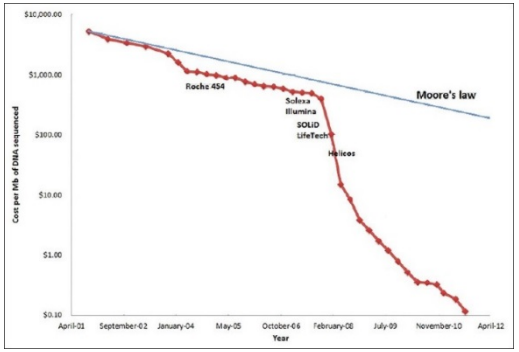
Not in ToxCast

Pathway Coverage*

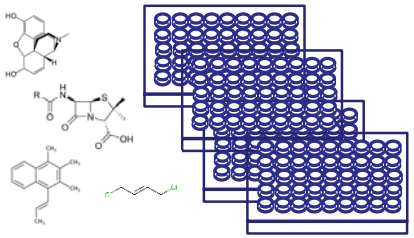


*At least one gene from pathway represented

High-throughput Genomics (HTTr)

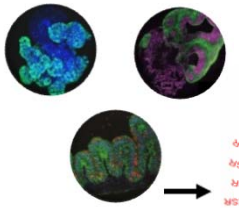


Thousands of chemicals

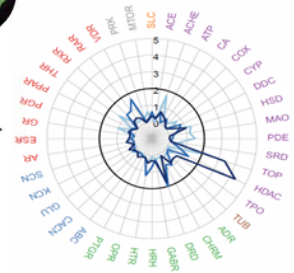


X

Multiple Cell Types



Inferred MOA/MIE



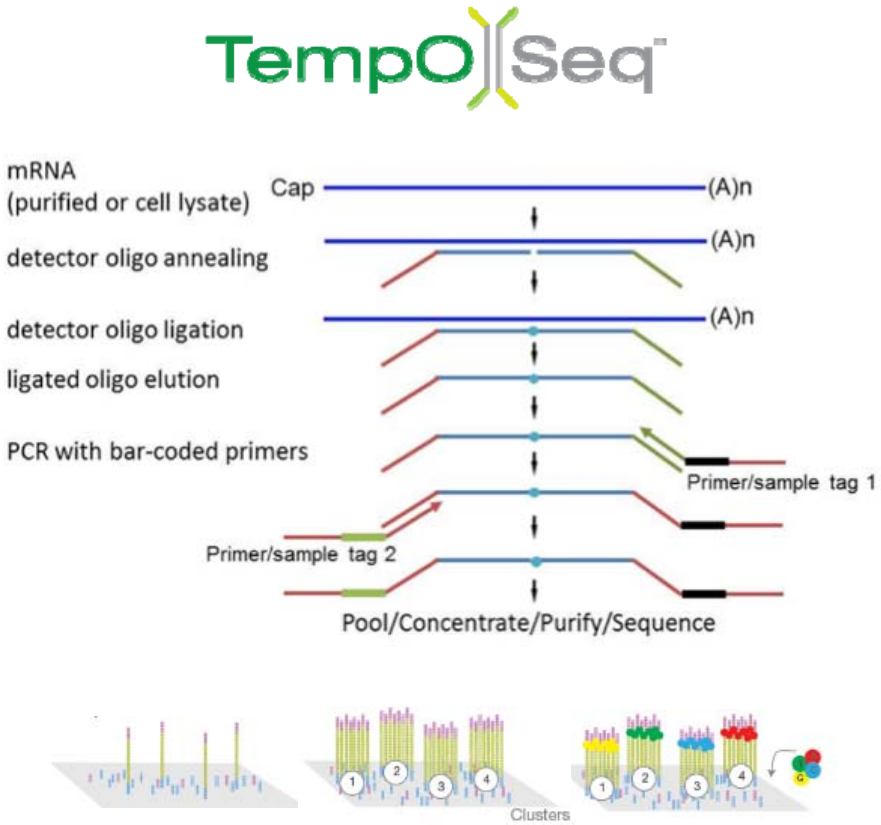
Requirements:

- Low cost
- Whole genome
- 384 well
- Automatable

National Center for Computational Toxicology

BioSpyder TempO-Seq

- Targeted RNA-Seq technology
- Whole transcriptome assay provides output on > 20,000 transcripts.
- Requires very low input (< 10 pg total RNA).
- Performed on “standard” PCR and Next Gen Sequencers.
- Compatible with purified RNA or cell lysates.



www.biospyder.com

HTTr Pilot: Workflow

Generate Cryopreserved Cell Stocks

Cell Expansion

Cell Plating

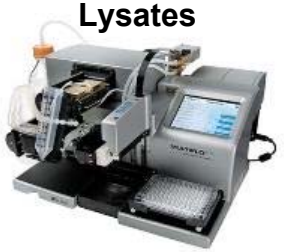
BioTek MultiFlo FX

Cell Dosing

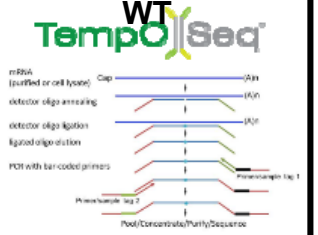
LabCyte Echo® 550 Liquid Handler

Track 1: Targeted RNA-Seq

Generating Cell Lysates



TempO-Seq WT



Track 2: Cytotoxicity / Apoptosis

Cell Labeling

High Content Imaging

Thermo Cellomics ArrayScan® VTI HCS Reader

Josh Harrill/NCCT

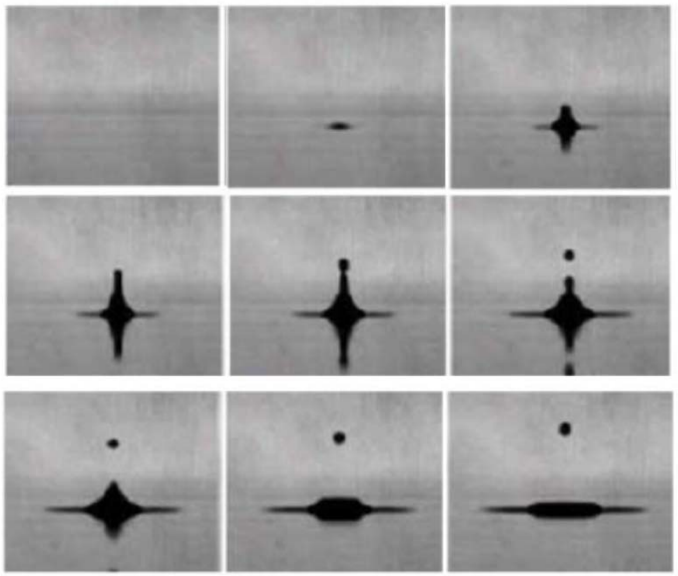
Dose Randomization using Echo 550

United States Environmental Protection Agency

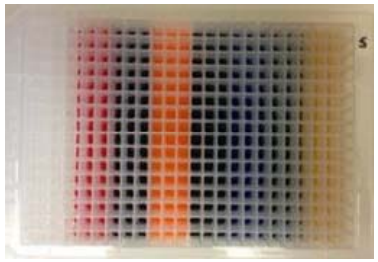
Acoustic dispensing technology:

- Uses soundwaves to precisely transfer small quantities of liquid (nL) from source plate to test plate.
- Allows for randomization of test wells → mitigate potential edge effects without "losing real estate."

LabCyte Echo® 550 Liquid Handler



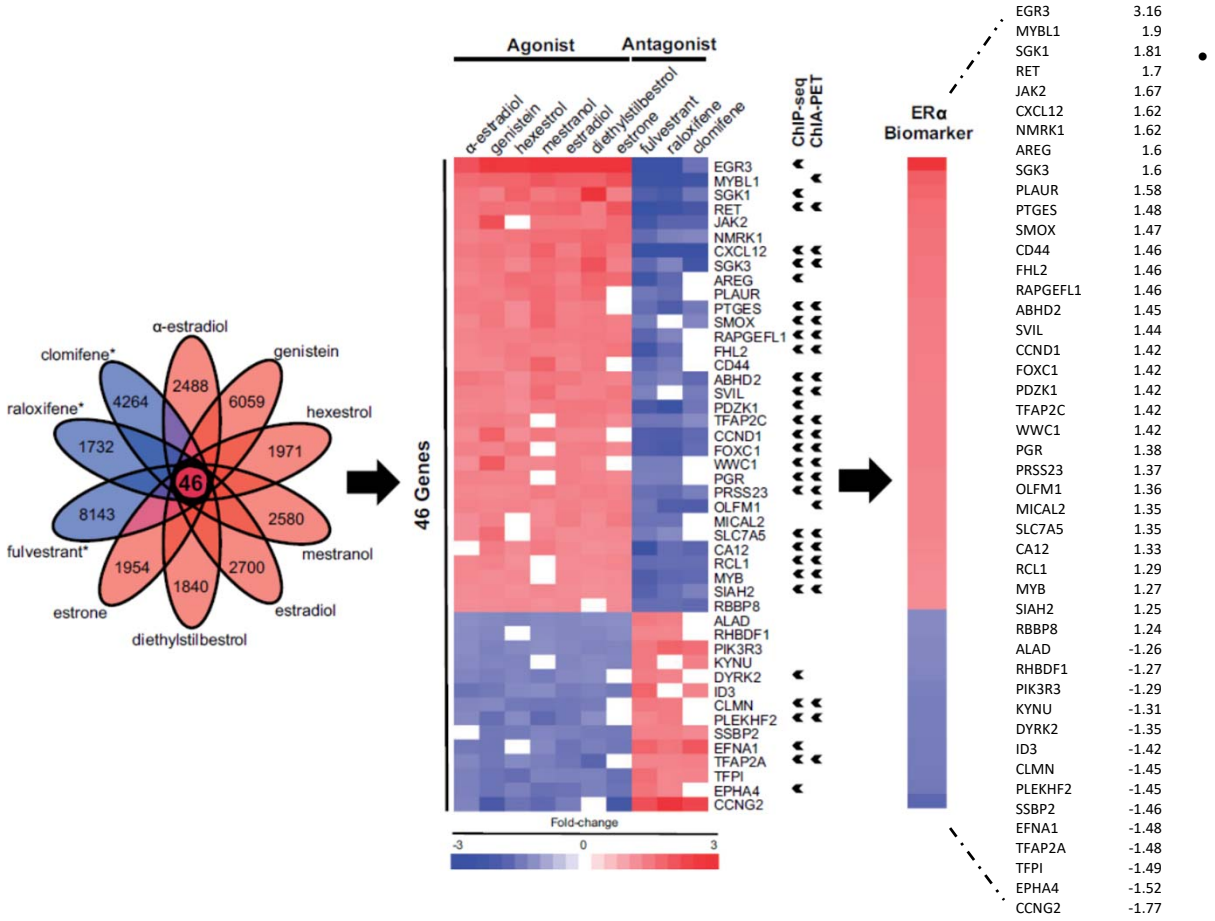
Source Plate



Test Plate

ERα Biomarker Signature

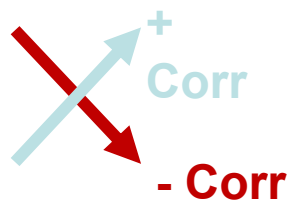
- Biomarker signature determined by consensus DEGs in MCF7 cells with various ERα agonists and antagonists.
- Can we use this to detect biologically meaningful signal in the BioSpyder data?

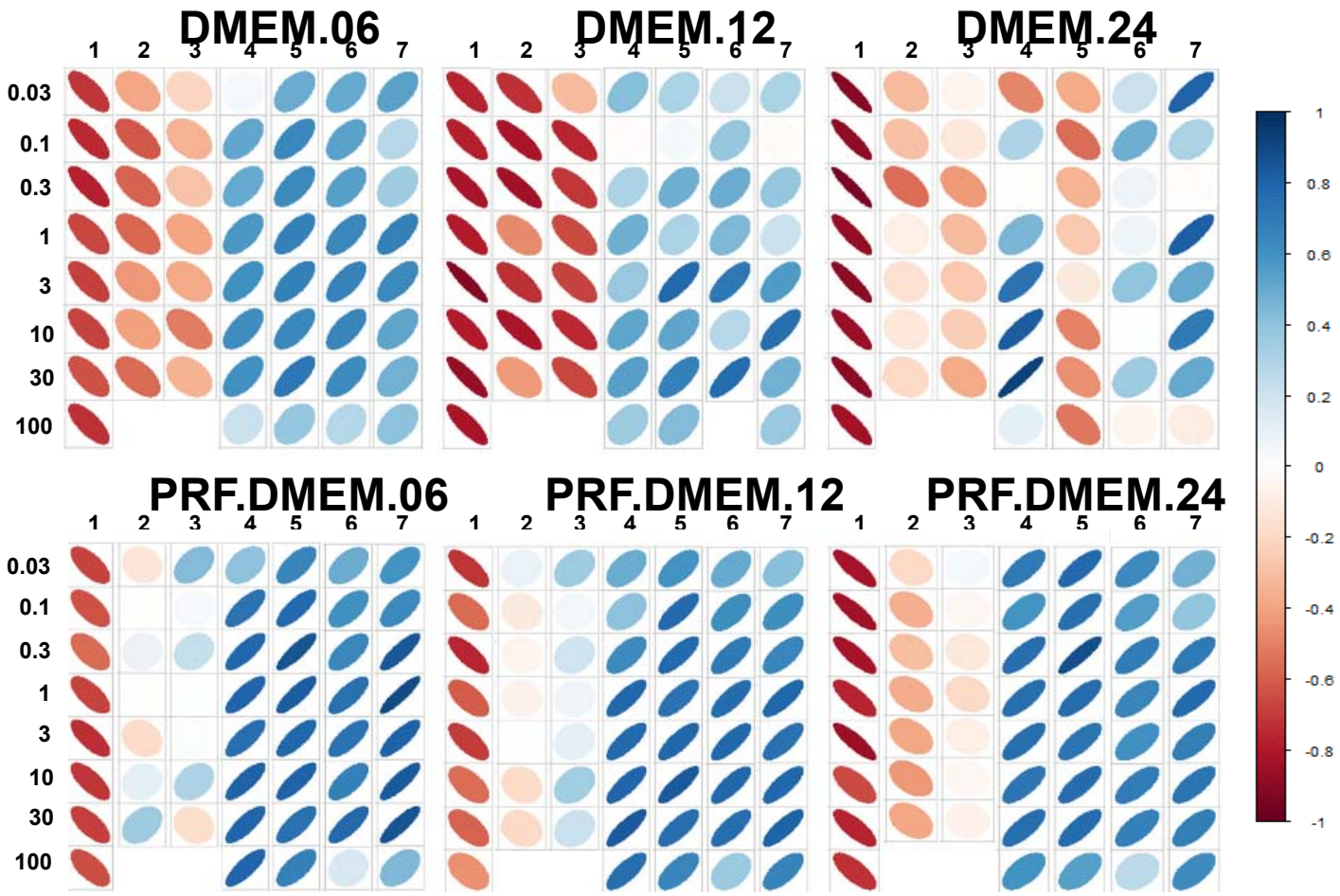


Ryan et al., 2016. Toxicol Sci. 2016 May;151(1):88-103.

Correlation with ERα Transcriptional Biomarker

	Chemical	MOA
1	Fulvestrant	Antiestrogen (SERD)
2	4-Hydroxytamoxifen	Antiestrogen (SERM)
3	Clomiphene Citrate	
4	Bisphenol A	Estrogenic
5	Bisphenol B	
6	4-Nonylphenol, branched	
7	4-Cumylphenol	





Josh Harrill, NCCT, unpublished

Pathway Enrichment

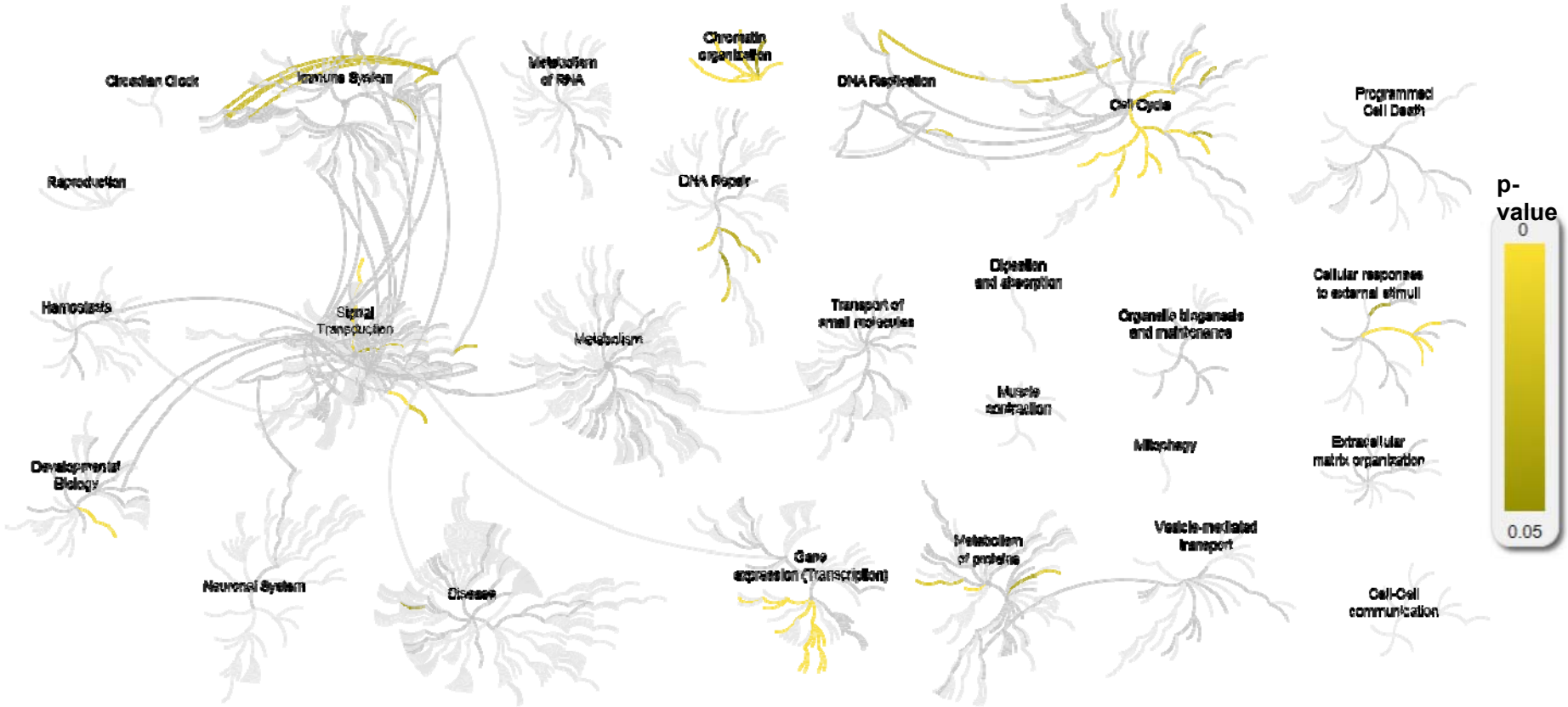
Numbers of Pathways Enriched

Chemical Name	MSigDB_C2	MSigDB_H	Reactome	Chemical Name	MSigDB_C2	MSigDB_H	Reactome
Ziram	1268	26	314	Propiconazole	20	1	2
4-Hydroxytamoxifen	1068	14	331	3,5,3'-Triiodothyronine	18	0	1
Cycloheximide	570	24	126	Fenofibrate	17	0	1
4-Nonylphenol, branched	533	7	127	Cyanazine	16	0	1
Amiodarone hydrochloride	524	12	136	Flutamide	10	0	1
Reserpine	523	11	80	Fulvestrant	9	1	0
Maneb	248	3	75	Cypermethrin	7	0	1
Rotenone	215	5	22	Lovastatin	6	0	0
Thiram	204	5	64	Simvastatin	5	0	0
4-Cumylphenol	198	4	27	Butafenacil	3	0	0
Bisphenol B	185	2	31	Vinclozolin	2	0	0
Fenpyroximate (Z,E)	183	5	14	Tetrac	2	0	1
Cyproterone acetate	166	5	4	Lactofen	2	0	0
Prochloraz	113	2	10	Cyproconazole	0	0	0
Clomiphene Citrate	68	3	0	Clofibrate	0	0	0
Nilutamide	56	0	29	PFOS	0	0	0
Trifloxystrobin	47	1	2	Simazine	0	0	0
Cladribine	47	0	71	Fomesafen	0	0	0
Bisphenol A	45	1	5	Troglitazone	0	0	0
Imazalil	41	0	4	PFOA	0	0	0
Pyraclostrobin	37	0	1	Atrazine	0	0	0
Farglitazar	22	1	0	Bifenthrin	0	0	0

- Heterogeneity in the amount and type of pathways enriched.
- Changing filtering stringency and BMD modeling strategy affects these results.

Josh Harrill, NCCT, unpublished

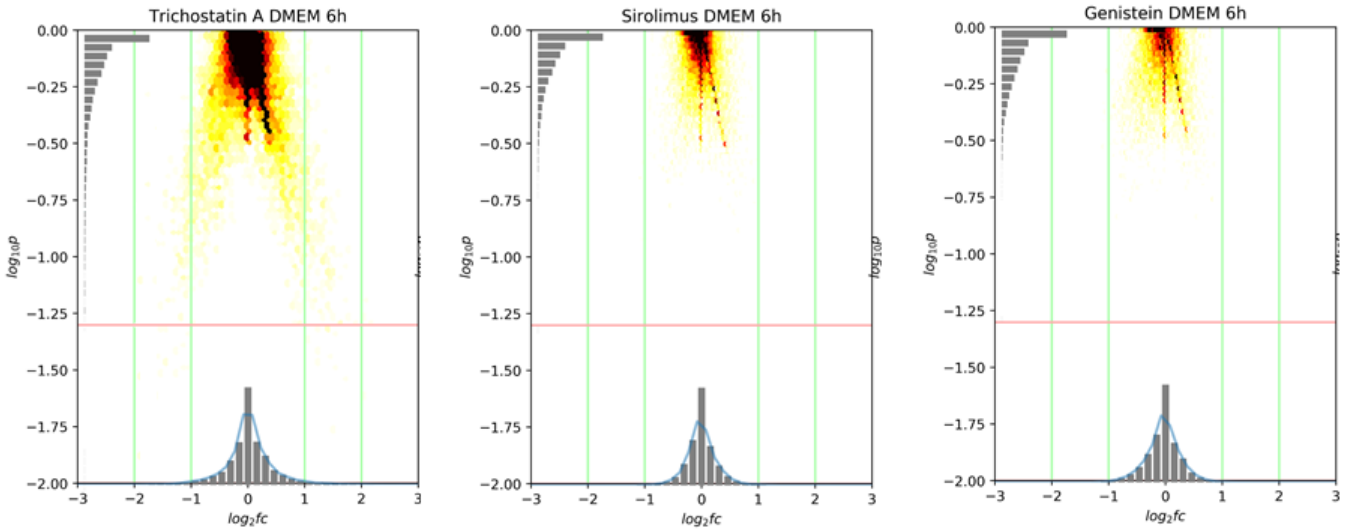
Network Mapping [Clomiphene Citrate]

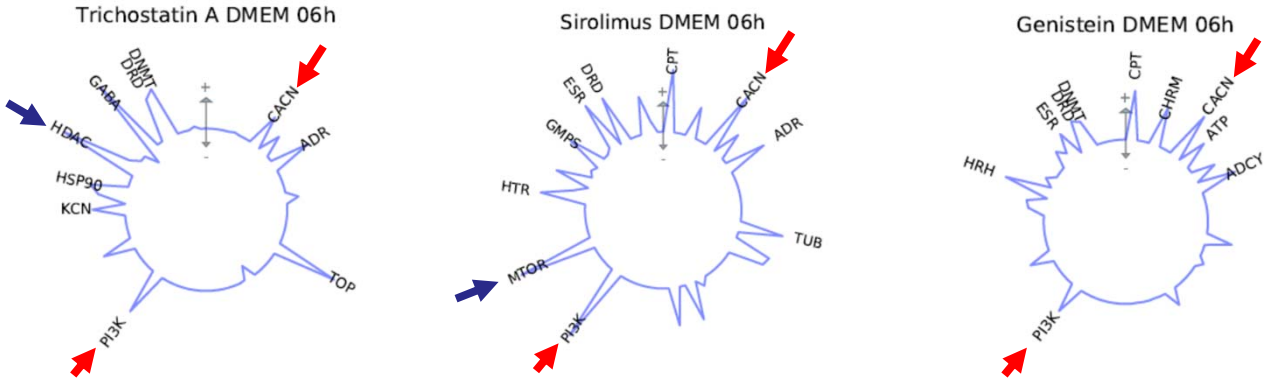


- Reactome (v60) Pathway Hierarchy → Overlaid with enrichment scores based on probes with acceptable BMD model fit
- Highlights different areas of biology affected by a chemical

Josh Harrill, NCCT, unpublished

Connectivity Mapping Demonstrates Multiple Pathway Matches





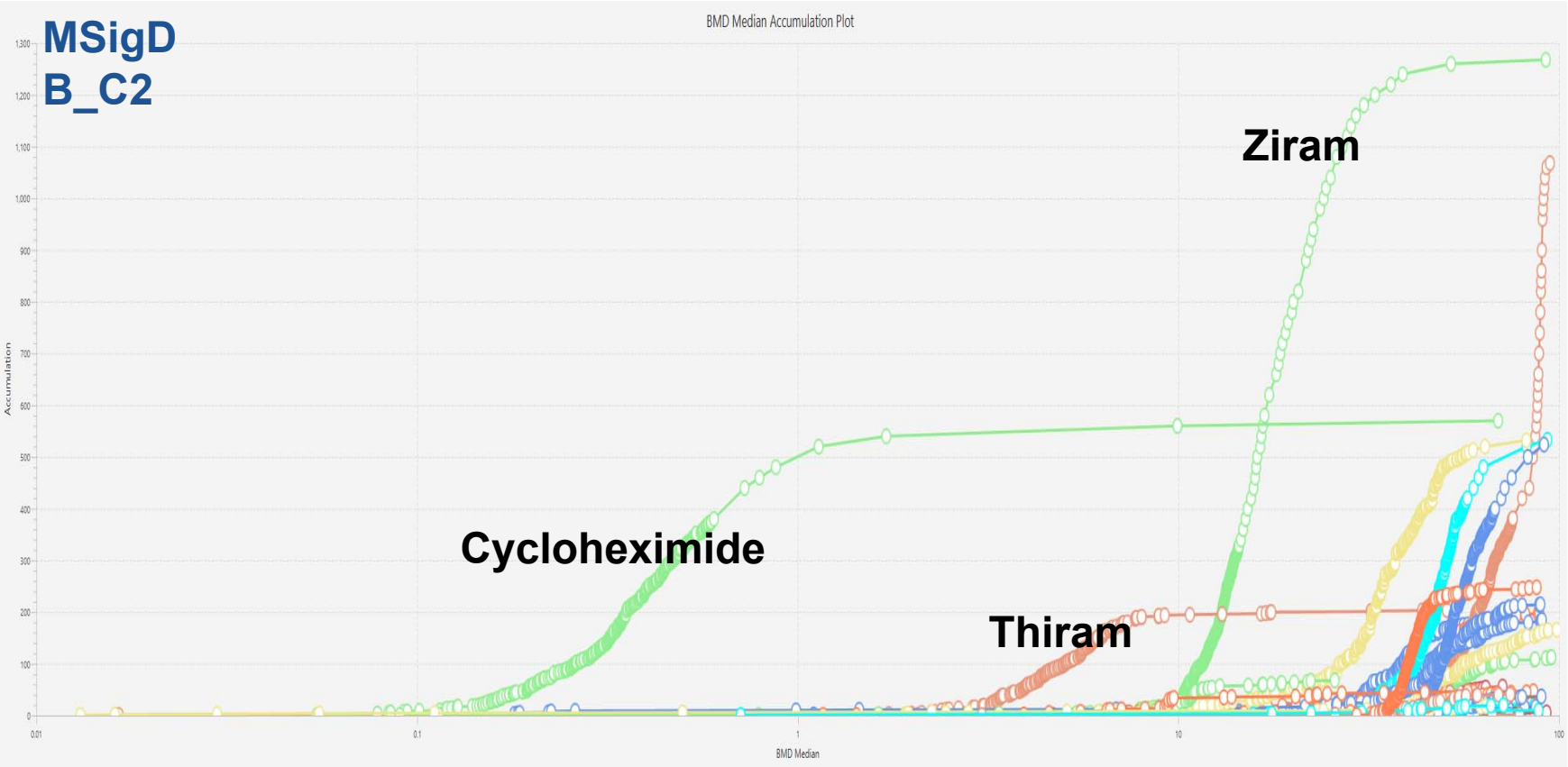
Putative target

Promiscuous Target Mapping

- Differential gene expression observed with reference chemicals
- Putative targets identified using Connectivity Mapping
- Large degree of promiscuity of predicted targets observed
- Currently evaluating additional methods for MIE prediction

Imran Shah, NCCT, unpublished

Pathway Potencies by BMD Analysis



- Broad range of pathway level potency estimates and number of pathways affected across chemicals.

Josh Harrill, NCCT, unpublished

Cell Painting Phenotypic Screen Background

- Cell Painting (Bray et al., 2016, *Nature Protocols*): A cell morphology-based phenotypic profiling assay multiplexing six fluorescent "non-antibody" labels, imaged in five channels, to evaluate multiple cellular compartments and organelles.
- Key Features:
  - Non-targeted screening (i.e. target agnostic)
  - Tractable across different adherent cell lines
  - High content 100s – 1000s of features measured at the cell level
  - Concentration-response analysis
  - Fingerprinting and clustering

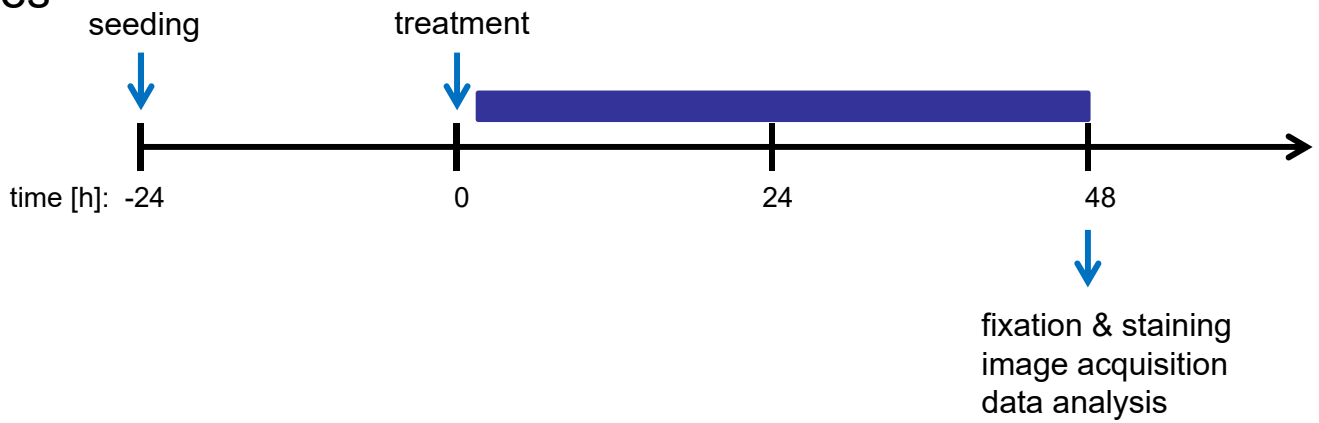
Marker	Cellular Component	Labeling Chemistry	Labeling Phase	Opera Phenix	
				Excitation	Emission
Hoechst 33342	Nucleus	Bisbenzamide probe that binds to dsDNA	Fixed	405	480
Concanavalin A – AlexaFluor 488	Endoplasmic reticulum	Lectin that selectively binds to α-mannopyranosyl and α-glucopyranosyl residues enriched in rough endoplasmic reticulum		435	550
SYTO 14 nucleic acid stain	Nucleoli	Cyanine probe that binds to ssRNA		435	550
Wheat germ agglutinin (WGA) – AlexaFluor 555	Golgi Apparatus and Plasma Membrane	Lectin that selectively binds to sialic acid and N-acetylglucosaminyl residues enriched in the trans-Golgi network and plasma membrane		570	630
Phalloidin – AlexaFluor 568	F-actin (cytoskeleton)	Phallotoxin (bicyclic heptapeptide) that binds filamentous actin			
MitoTracker Deep Red	Mitochondria	Accumulates in active mitochondria	Live	650	760

Experimental Objectives & Design

Objectives:

- Replicate phenotypes observed by BROAD group (Gustaffdottir, Bray)
- Compare sensitivity across cell models.
- Identify reference chemicals for use as assay controls in screening applications.

- U-2 OS / MCF7
- 384-well plate
- 16 chemicals, 7 concentrations
- 3 technical replicates / plate
- 3 biological replicates

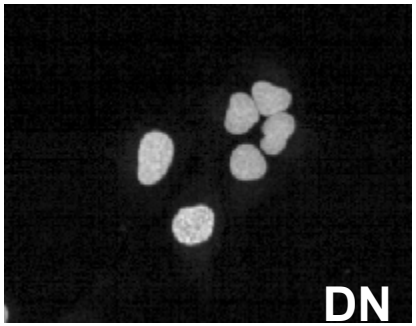


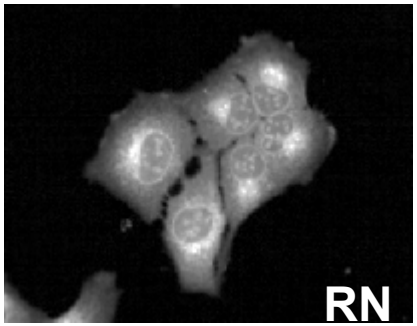
EPA United States Environmental Protection Agency

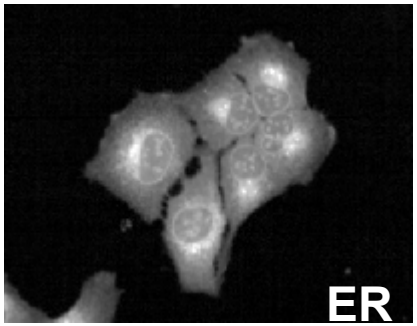
Image Acquisition

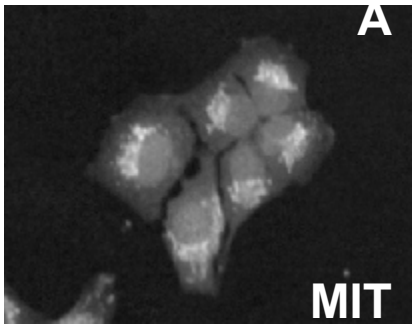
Image Acquisition

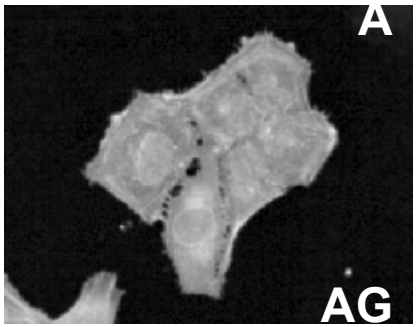
- Perkin Elmer Opera Phenix
- 20x Water Immersion Objective
- Confocal Mode, Single Z
- CellCarrier-384 Ultra Microplates

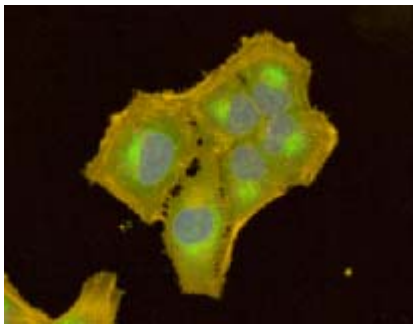








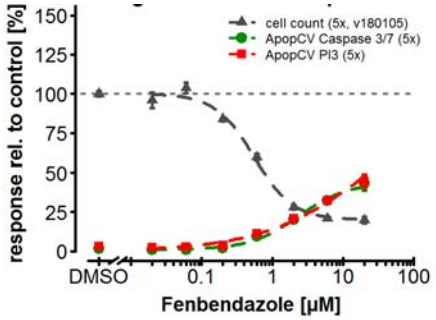


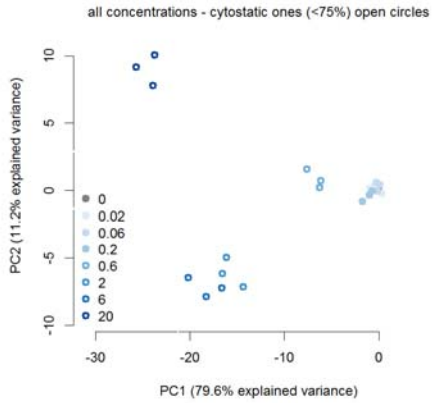


United States Environmental Protection Agency

Fenbendazole

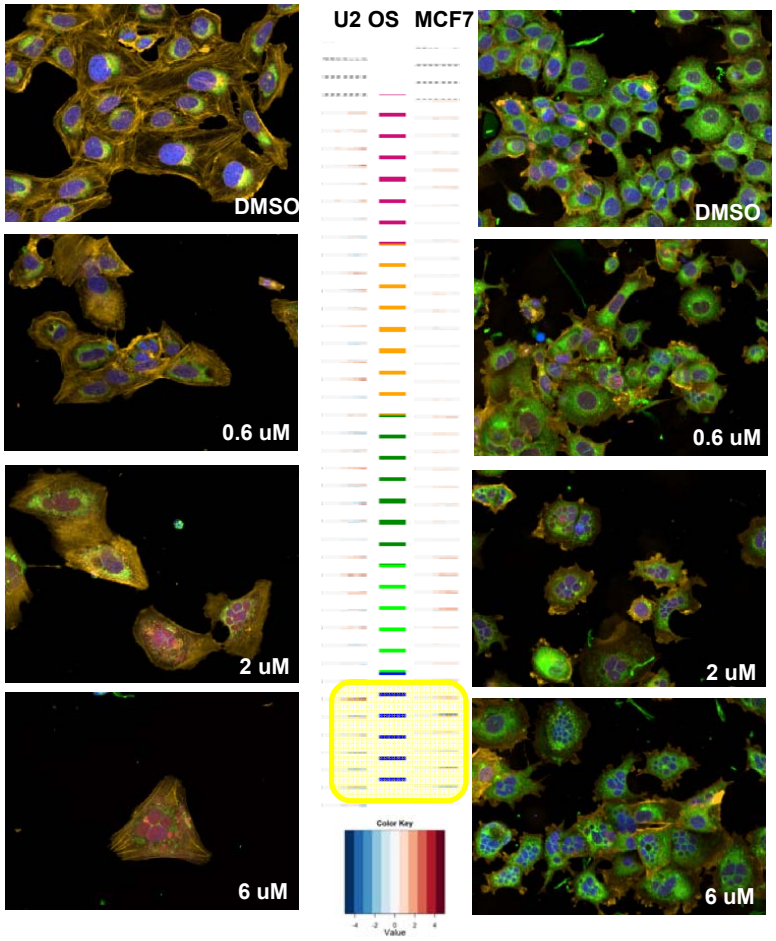
U-2 OS (-SYTO)



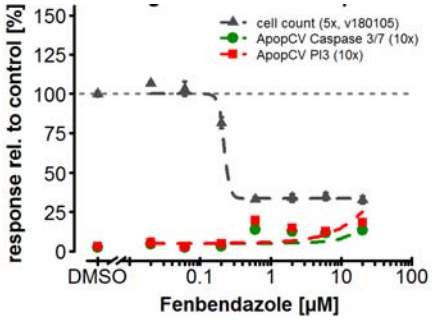


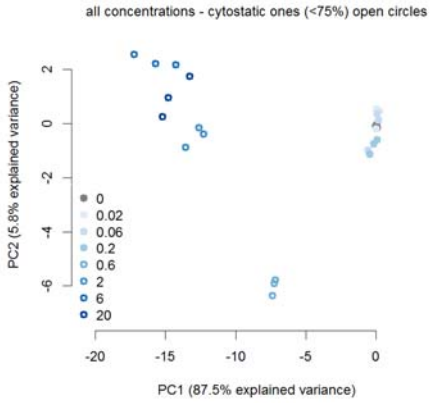
- multinucleated cells!

Expected phenotype: Giant, multi-nucleated cells



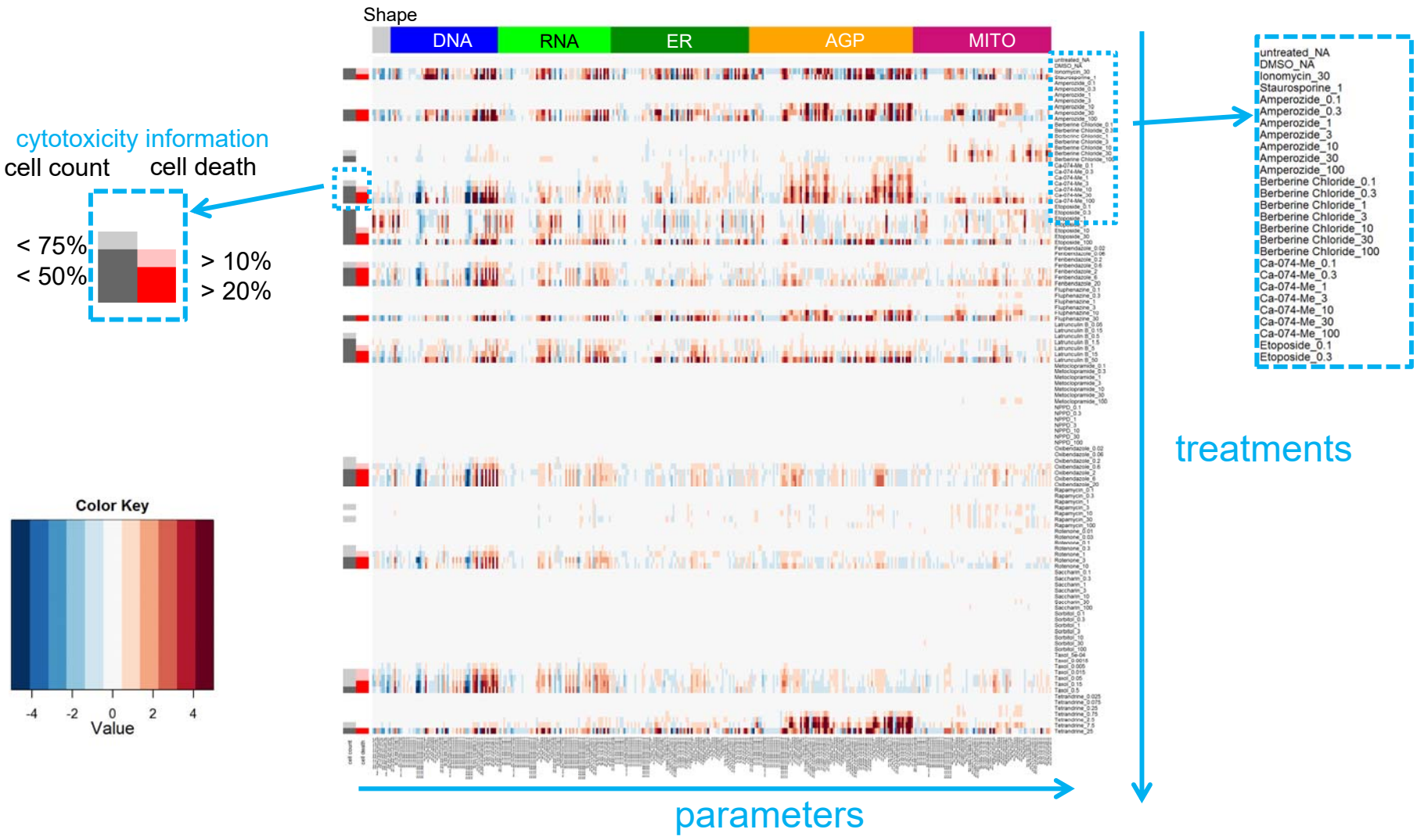
MCF7 (-SYTO)





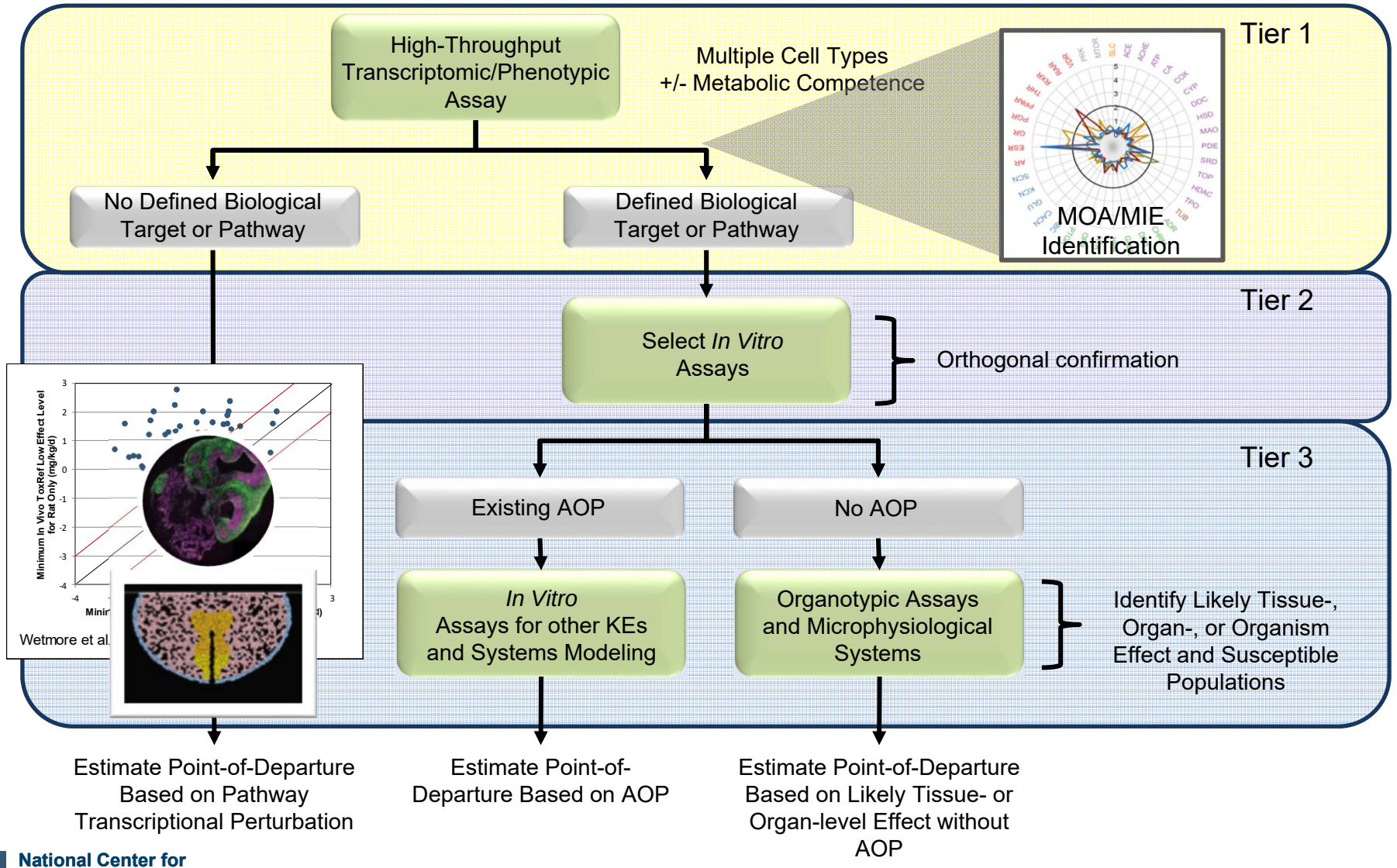
Josh Harrill, NCCT, unpublished

Results

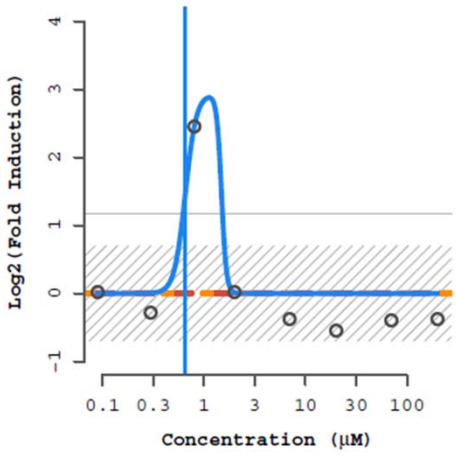


Josh Harrill, NCCT, unpublished

Framework for Integrating Hazard Components…



Regulatory Applications Require More Focus on Quality and Transparency

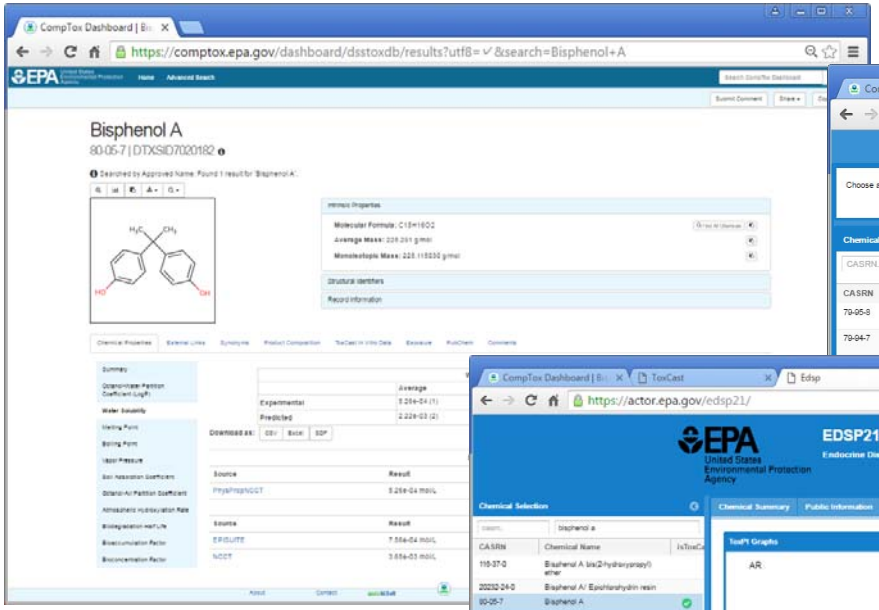



```
ASSAY:   AEID117 (ATG_ERa_TRANS)

NAME:    Thioglycolic acid
CHID:    26141   CASRN: 68-11-1
SPID(S): TX007664
L4ID:    420385

HILL MODEL (in red):
      tp       ga       gw
val:  3.1e-11  -2.15    0.416
sd:   NaN      NaN      NaN

GAIN-LOSS MODEL (in blue):
      tp       ga       gw       la       lw
val:  2.93     -0.184   8        0.173    18
sd:   3.56     0.334    9.48     5.82     814

      CNST       HILL       GNLS
AIC:  20.14      26.14      17.79
PROB: 0.23       0.01       0.76
RMSE: 0.92       0.92       0.32

MAX_MEAN: 2.45      MAX_MED: 2.45      BMAD: 0.233

COFF: 1.17   HIT-CALL: 1    FITC: 50   ACTP: 0.77
```

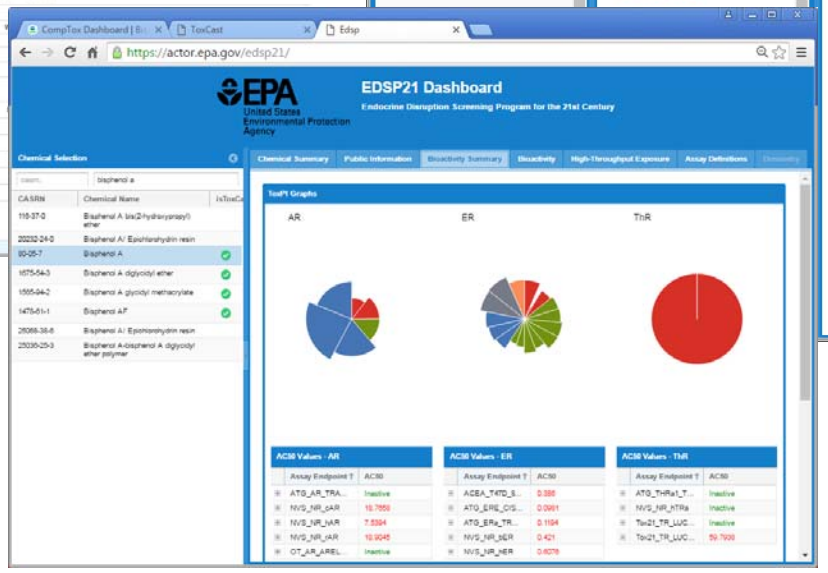
```
FLAGS:
Only one conc above baseline, active
Borderline active
```

- Public release of Tox21 and ToxCast data on PubChem and EPA web site (raw and processed data)
- Publicly available ToxCast data analysis pipeline
  - Data quality flags to indicate concerns with chemical purity and identity, noisy data, and systematic assay errors
- Tox21 and ToxCast chemical libraries have undergone analytical QC and results publicly available
- Public posting of ToxCast procedures
  - Chemical Procurement and QC
  - Data Analysis
  - Assay Characteristics and Performance
- External audit on ToxCast data and data analysis pipeline
- Migrating ToxCast assay annotations to OECD 211 compliant format

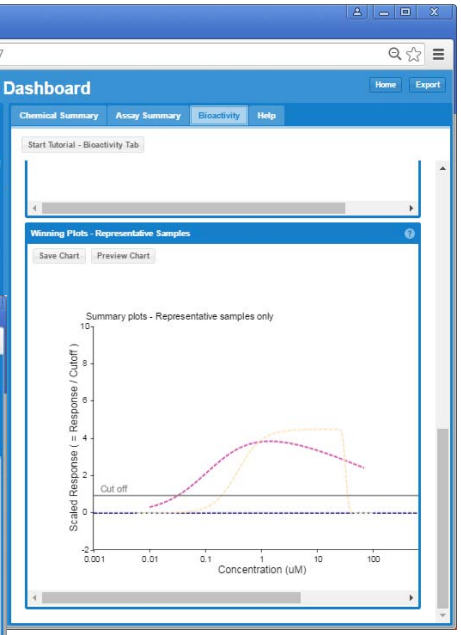
Effort to Provide Data Through Display and Decision Support Dashboards



Enhanced Chemistry Dashboard (https://comptox.epa.gov/dashboard)



EDSP21 Dashboard (https://actor.epa.gov/edsp1)

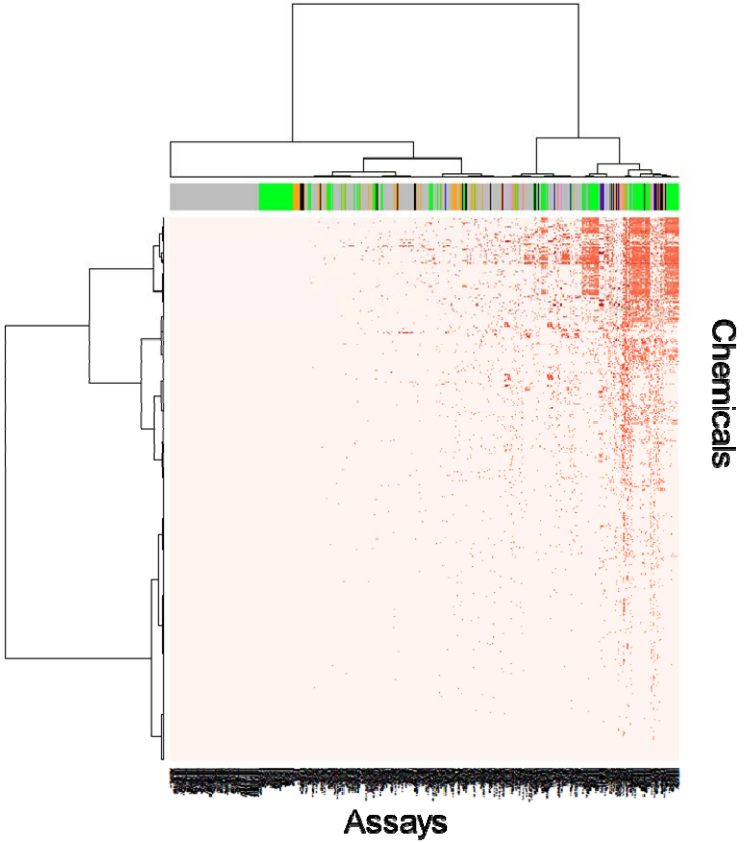


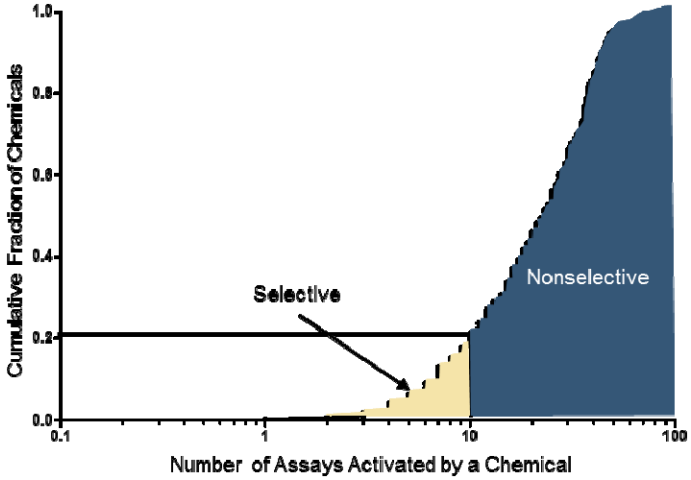
ToxCast Dashboard (https://actor.epa.gov/dashboard)

Promiscuous Chemical Response is the Rule

United States Environmental Protection Agency

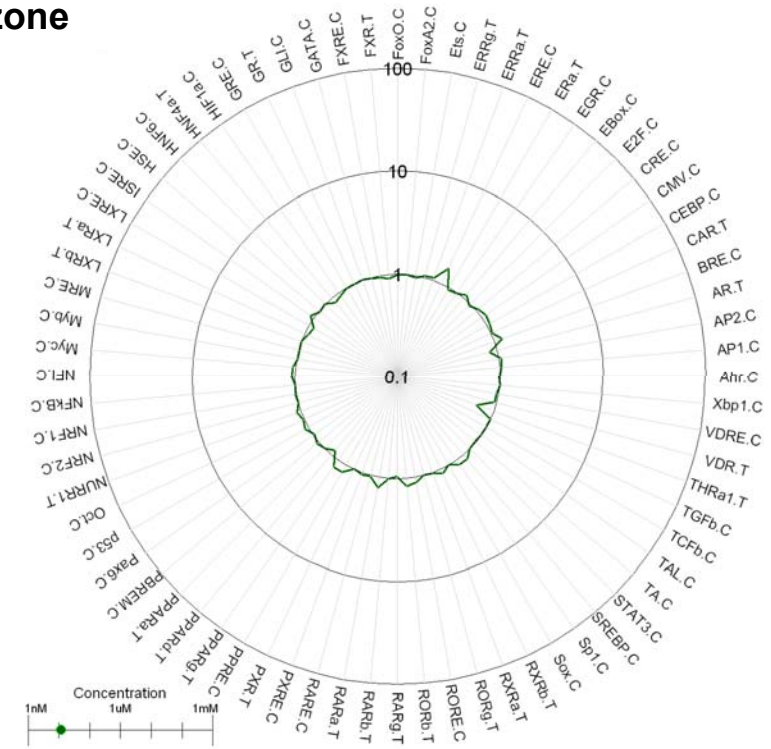
1000 chemicals/
800 assay endpoints





Thomas et al., 2013

Troglitazone



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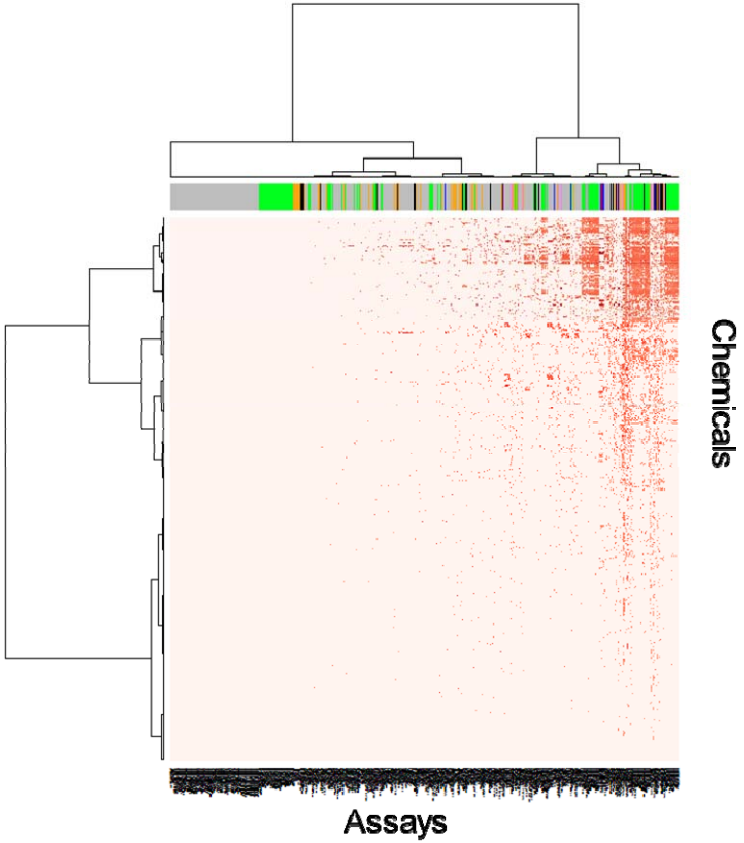
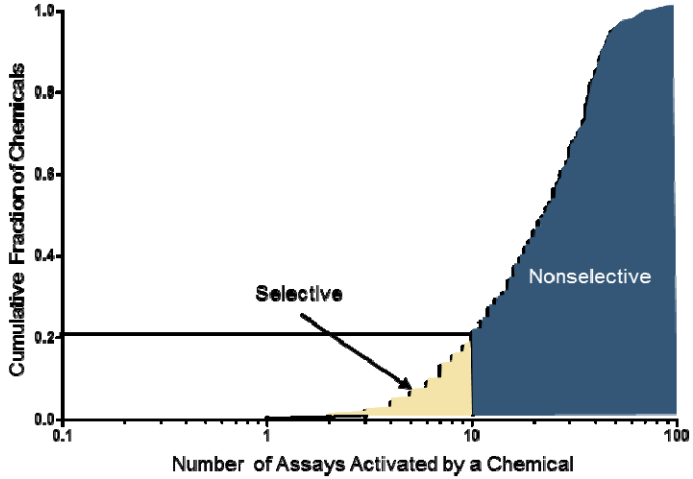
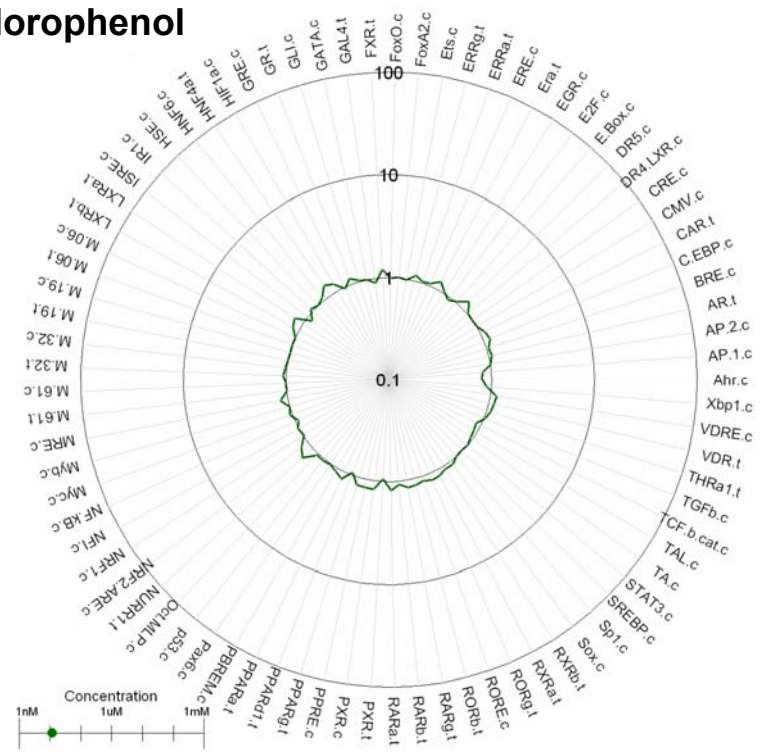




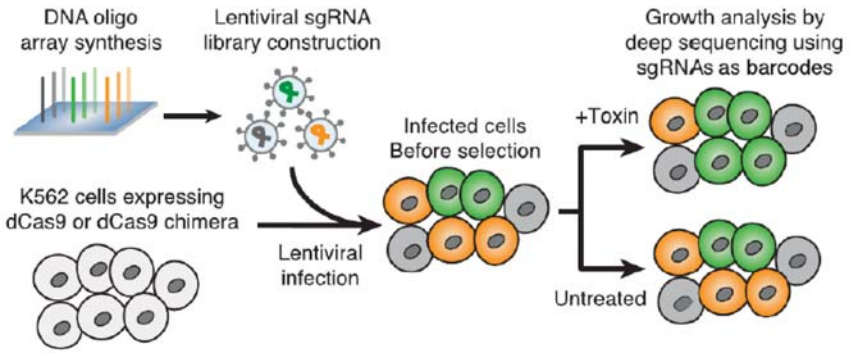
Pentachlorophenol



Functional genomics: Defining Relevancy

- Most chemicals have apparent polypharmacology—what is the critical/relevant MOA?
  - Could use potency to define but this may not be linked to adversity
  - Transcriptomics is high content but function is generally inferred

- Functional genomics allows for bridging between genotype and phenotype
- Previously mostly used in prokaryotic systems such as *S. cerevisiae*
- Advent of CRISPR-Cas9 opens door for higher throughput applications in mammalian cells



Gilbert et al., Cell, 2014

EPA United States Environmental Protection Agency

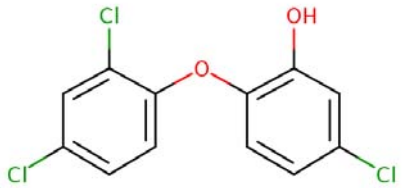
Pilot Project

- Collaboration between University of Florida (Chris Vulpe) and USEPA (NCCT, Keith Houck)
- Funded by USEPA SMARTi award to Keith Houck and Audrey Bone
- **Goal of the project is to test the feasibility of using CRISPR-Cas9 genome editing in human cells for screening environmental chemicals in a functional genomics toxicology format**

Chemical Selection

- Criteria
  - Mix of uses (pharmaceutical, pesticide, consumer, industrial)
  - Well-characterized mechanisms of cytotoxicity
    - Mitochondrial toxicity
    - DNA damage
    - Oxidative stress
    - Microtubule disruption
    - Proteosome inhibition
  - Known cytotoxic in Tox21/ToxCast assays without metabolic activation

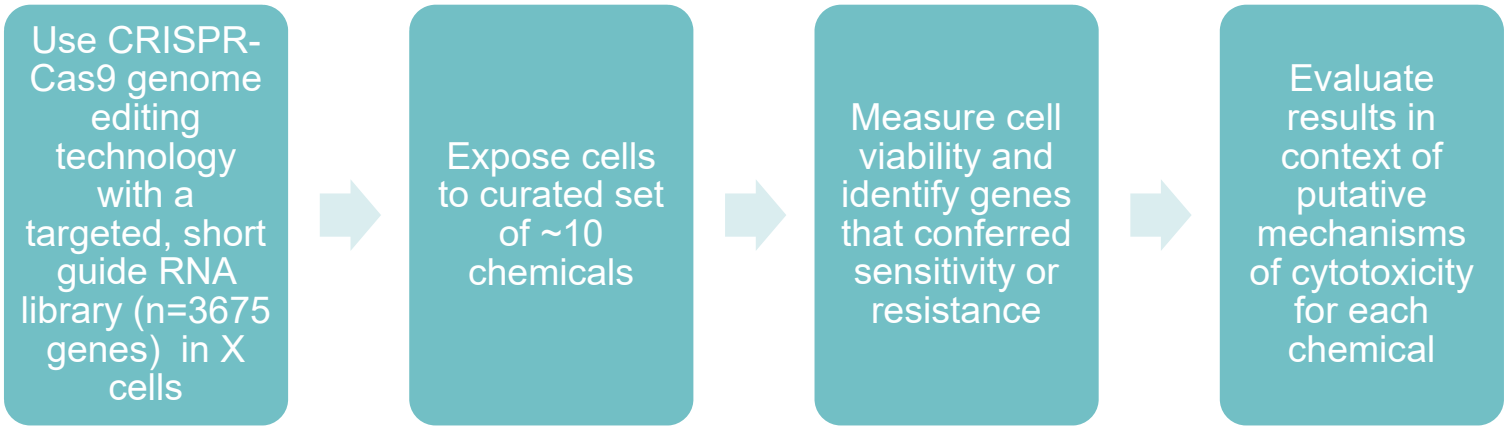
- 11 chemicals
  - Colchicine
  - Triphenyltin chloride
  - Triglycidyl isocyanurate
  - Cytembena
  - Propargite
  - Octhilinone
  - Triclosan
  - Tralopyril
  - Dibutyltin dichloride
  - Malachite green
  - Bisphenol A glycidyl methacrylate



Triclosan

EPA United States Environmental Protection Agency

Experimental Design



Ideas for ToxCast Assays for Prioritization of Carcinogens

- **Current**
  - Assays selected by commercial availability
  - Broad bioactivity to cover all types of toxicity
  - Challenges
    - Chronic exposures
    - Many diseases
    - Epigenetic events
    - Evolutionary development/stochastic genetic effects key

- **CarciCast**
  - Focus on key characteristics
  - Best-in-class existing assays
  - Development may benefit from:
    - genome editing tools
    - complex/organotypic cell models
    - phenotypic screening


EPA
United States
Environmental Protection
Agency
Thank You for Your Attention!
• EPA:
• Imran Shah
• Joshua Harrill
• Woody Setzer
• Richard Judson
• Rusty Thomas
• EPA:
• Steve Simmons
• Danica DeGroot
• Johanna Nyffeler
• Stacie Flood (ORAU)
• U of Florida:
• Chris Vulpe
• Abderrahmane Tagmount