EPA
United States
Environmental Protection
Agency

Development and Use of High Content Tier 1 Screening Assays at the USEPA National Center for Computational Toxicology (NCCT)

Joshua A. Harrill, USEPA National Center for Computational Toxicology (NCCT)

Michigan State, Center for Research in Ingredient Safety
East Lansing, MI
November 14th, 2018

Disclaimer

The views expressed in this presentation are those of the author(s) and do not necessarily represent the views or policies of the U.S. Environmental Protection Agency, nor does mention of trade names or products represent endorsement for use.

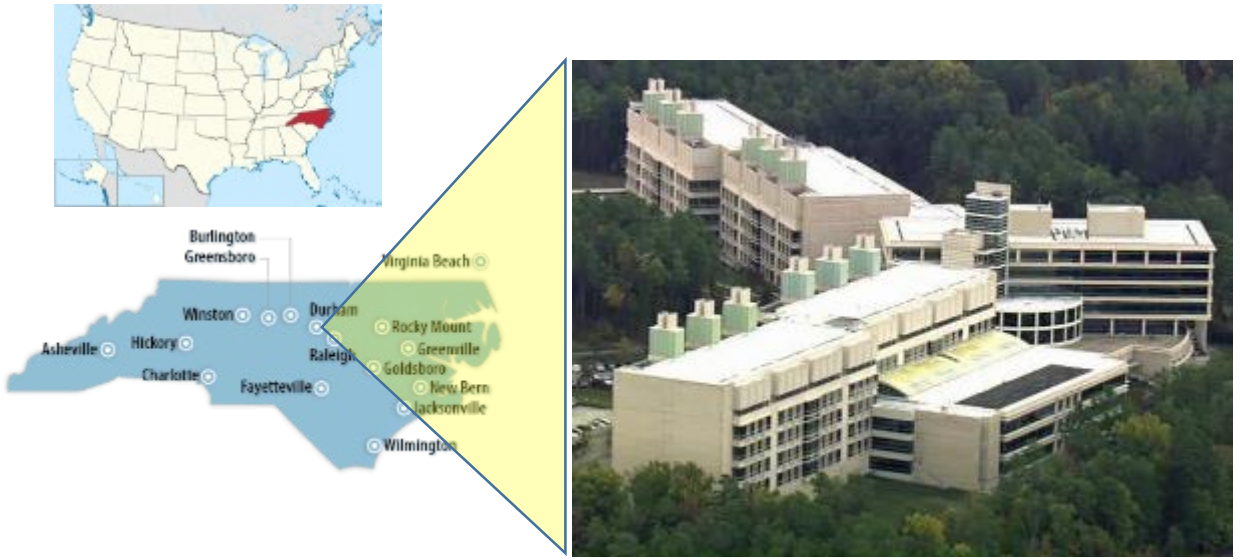
Outline

- **Background**
  - Who is NCCT?
  - What does NCCT Do?

- **High-Content Screening Assays**
  - High Throughput Transcriptomics (HTTr)
  - High Throughput Phenotypic Profiling (HTPP)
    - Technology Overview
    - Experimental & Computational Workflows
    - Concentration-Response Modeling

- **Potential Applications for Regulatory Decision Making**
  - Putative MIE/MOA Prediction with HTTR
  - Bioactivity Exposure Ration (BER) Analysis

Who is NCCT?



Research Triangle Park Campus

National Center for Computational Toxicology

Mission Statement:

A research organization tasked with advancing the science of toxicity testing through the **development and/or application of novel experimental and computational approaches** for rapidly characterizing the biological activity, exposure potential and potential human health risks associated with chemicals.

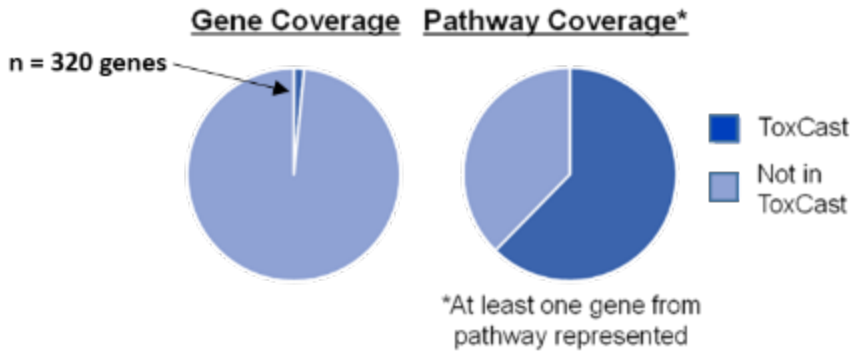
What Does NCCT Do?

NCCT has research programs focus on developing the **tools, approaches and data** needed to accelerate the pace of chemical risk assessment and foster incorporation of non-traditional toxicity testing data into regulatory decision-making processes.

- **ToxCast:** Use of high-throughput screening (HTS) assays to expose living cells or isolated proteins to chemicals and assess bioactivity and potential toxic effects.

	# of assays	# of chemicals	Types of chemicals
Phase 1 *(2007 – 2009)*	500	300	Mostly pesticides
Phase 2 *(2009 – 2013)*	700	2,000	Industrial, consumer product, food use, "green"

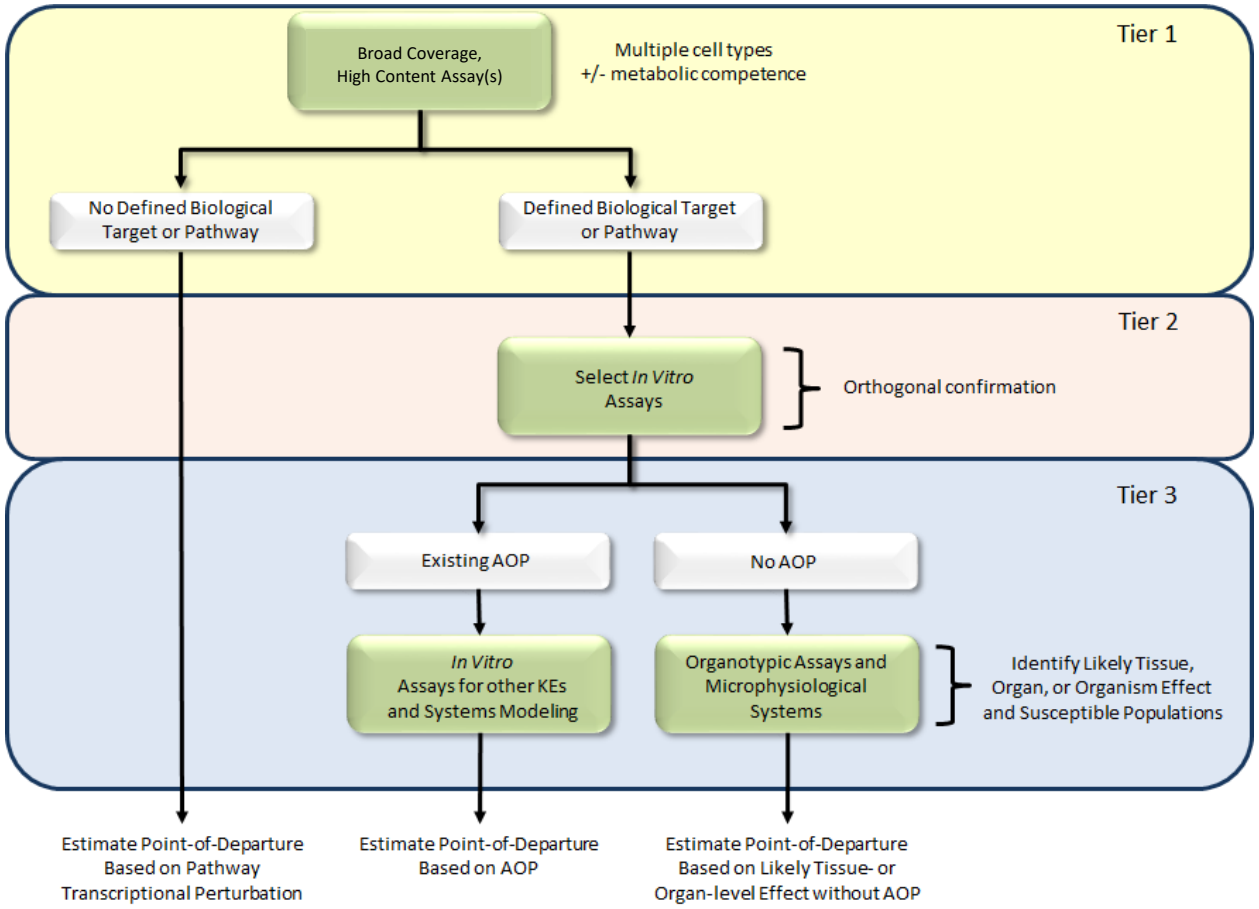
- Mostly targeted assays (*chemical X → protein Y*)
- Does not provide complete coverage of biological space



Broad Coverage, High Content Screening Assays

- Instead of targeted screening, NCCT proposes using screening strategies that cast the **broadest net possible** for capturing hazards associated with chemical exposure.
- Increasing efficiency and declining cost of generating whole transcriptome profiles has made **high-throughput transcriptomics (HTTr)** a practical option for broad coverage *in vitro* chemical screening.
- Activation or inhibition of protein targets by chemicals may manifest as changes in cellular morphology. Certain types of **high content imaging (HCI)** provide a cost effective means for broad coverage *in vitro* chemical screening.
- Both methods are **complementary** to each other and can be used in **human-derived** *in vitro* models.
- The resulting bioactivity profiles can potentially be used for **mechanistic prediction** and evaluation of **chemical similarity**.

A Strategic Vision and Operational Roadmap for Computational Toxicology at US EPA



EPA United States Environmental Protection Agency

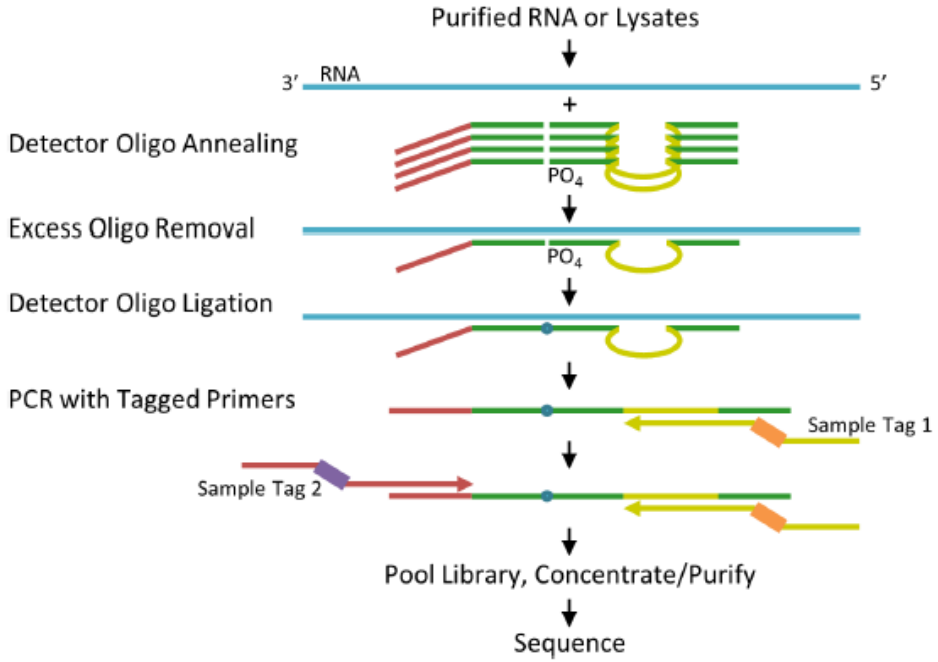
High Throughput Transcriptomics (HTTr)

Templated Oligo with Sequencing Readout (TempO-Seq)

Technology

- The **TempO-Seq** human whole transcriptome assay measures the expression of greater than 20,000 transcripts.
- Requires only picogram amounts of total RNA per sample.
- Compatible with purified RNA samples or **cell lysates**.
- Transcripts in cell lysates generated in 384-well format are barcoded according to well position and combined in a single library for sequencing using industry standard instrumentation.
- Scalable, targeted assay:
  - 1) specifically measures transcripts of interest
  - 2) ~50-bp reads for all genes
  - 3) requires less flow cell capacity than RNA-Seq
- Per sample fastq files are generated and aligned to BioSpyder sequence manifest to generate integer count tables.

TempO-Seq Assay Illustration



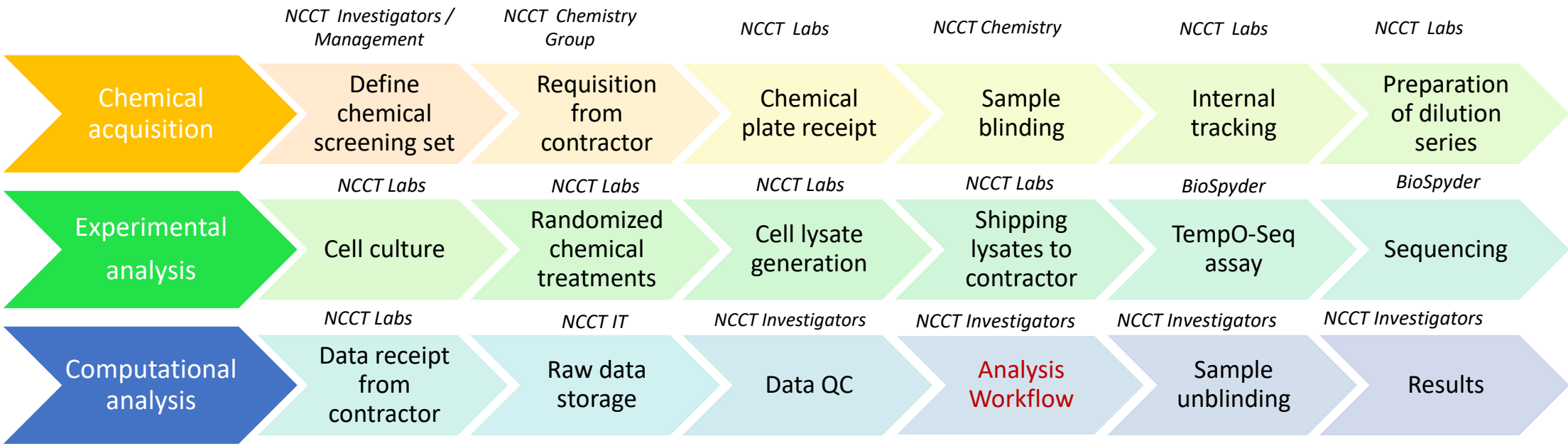
HTTr MCF-7 Screen: Experimental Design

Parameter	Multiplier	Notes
Cell Type(s)	1	MCF-7
Culture Condition	1	DMEM + 10% HI-FBS [a]
Chemicals	2,112	ToxCast ph1, ph2 Nominated chemicals from e1k / ph3
Time Points:	1	6 hours
Assay Formats:	2	TempO-Seq HCI Cell Viability & Apoptosis
Concentrations:	8	3.5 $\log_{10}$ units; semi $\log_{10}$ spacing
Biological Replicates:	3	--

- **Total number of samples:** 54,432
- **Total number of endpoint readouts:** 1.15×10^9
- **Total size of fastq files:** ~50 TB

[a] MCF7 cells cultured in DMEM + 10% HI-FBS was selected as the test system to facilitate comparability to the Broad Institute Connectivity Map (CMAP) database (http://portals.broadinstitute.org/cmap/).

Screening Overview



Experimental Workflow

Cryopreserved Cell Stocks

Cell Expansion

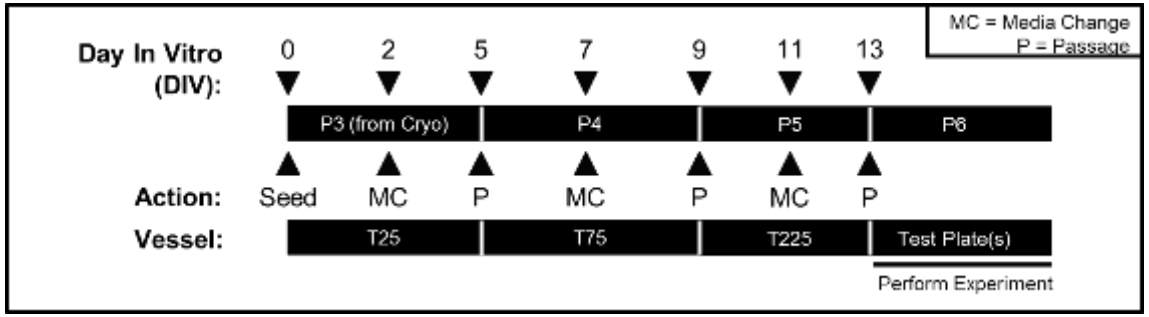
Cell Plating

BioTek MultiFlo ™ FX

Dispensing Test Chemicals

LabCyte Echo® 550 Liquid Handler

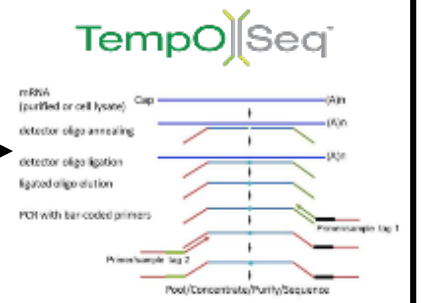
Standardized Expansion Protocol



Track 1: Targeted RNA-Seq

Generating Cell Lysates

TempO-Seq WT



Track 2: Apoptosis / Cell Viability

Reagent Dispensing

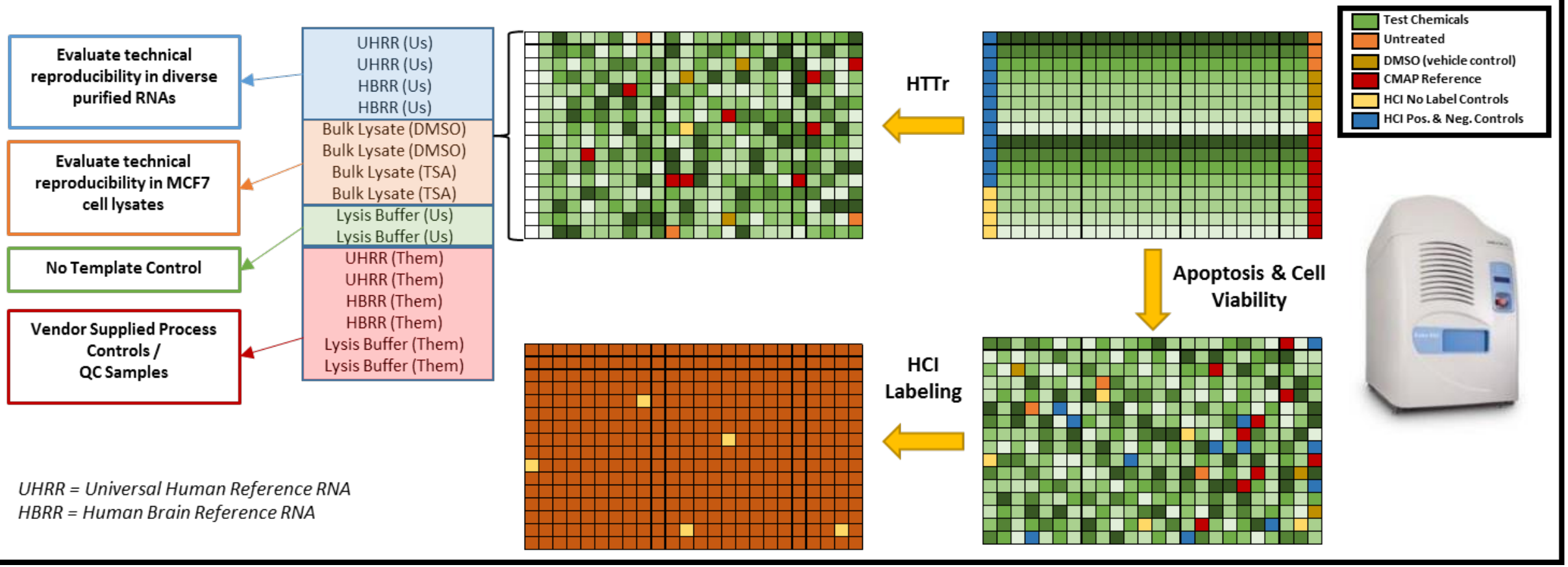
High Content Imaging

Perkin Elmer Opera Phenix™ High Content Screening System

Treatment Randomization & Quality Control Samples

Treatment Randomization: *Each test plate uniquely randomized with respect to treatment.*

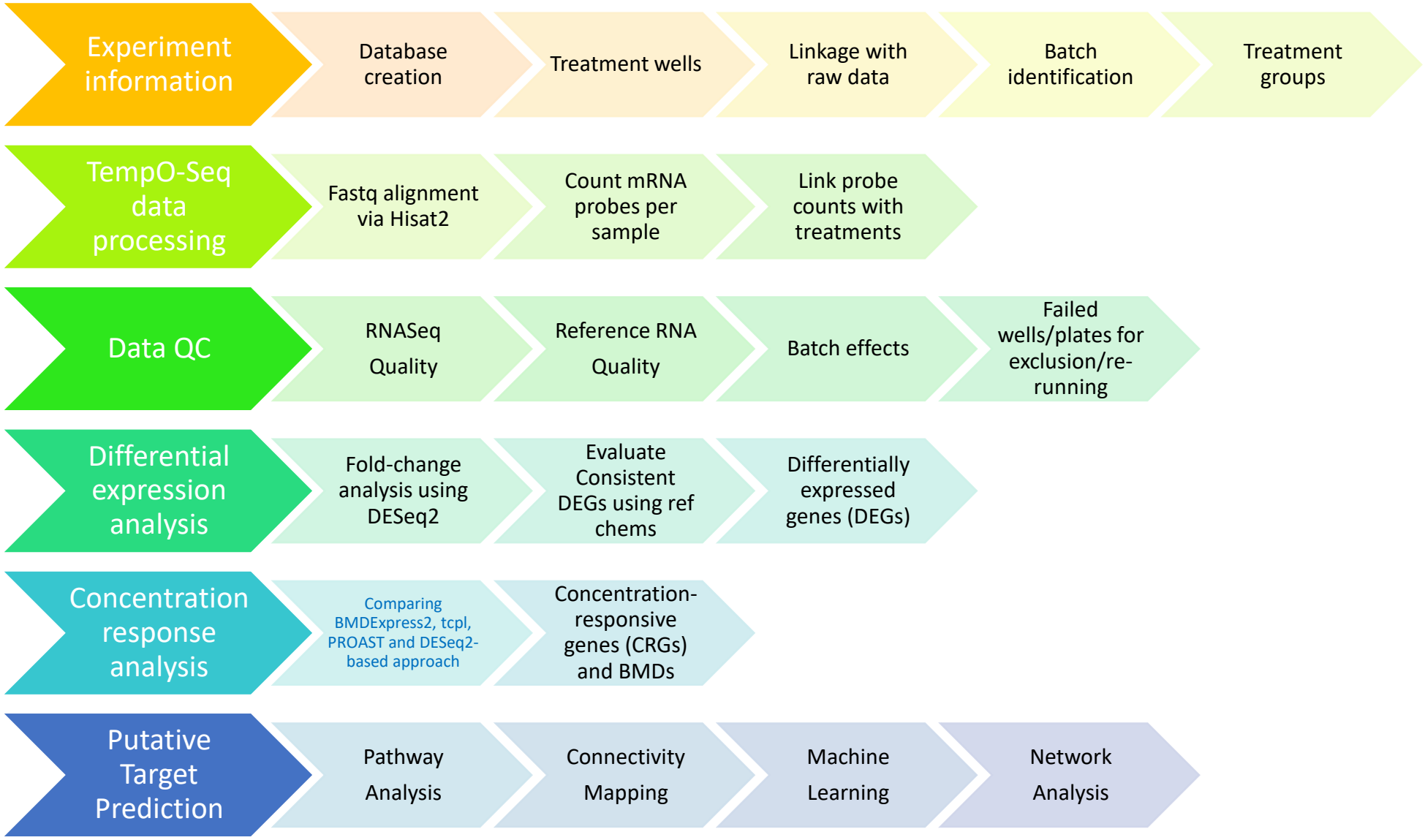
QC Samples: *Quality Control samples included on each plate*



UHRR = Universal Human Reference RNA
HBRR = Human Brain Reference RNA

EPA United States Environmental Protection Agency

HTTr Analysis Pipeline (Nov 2018)



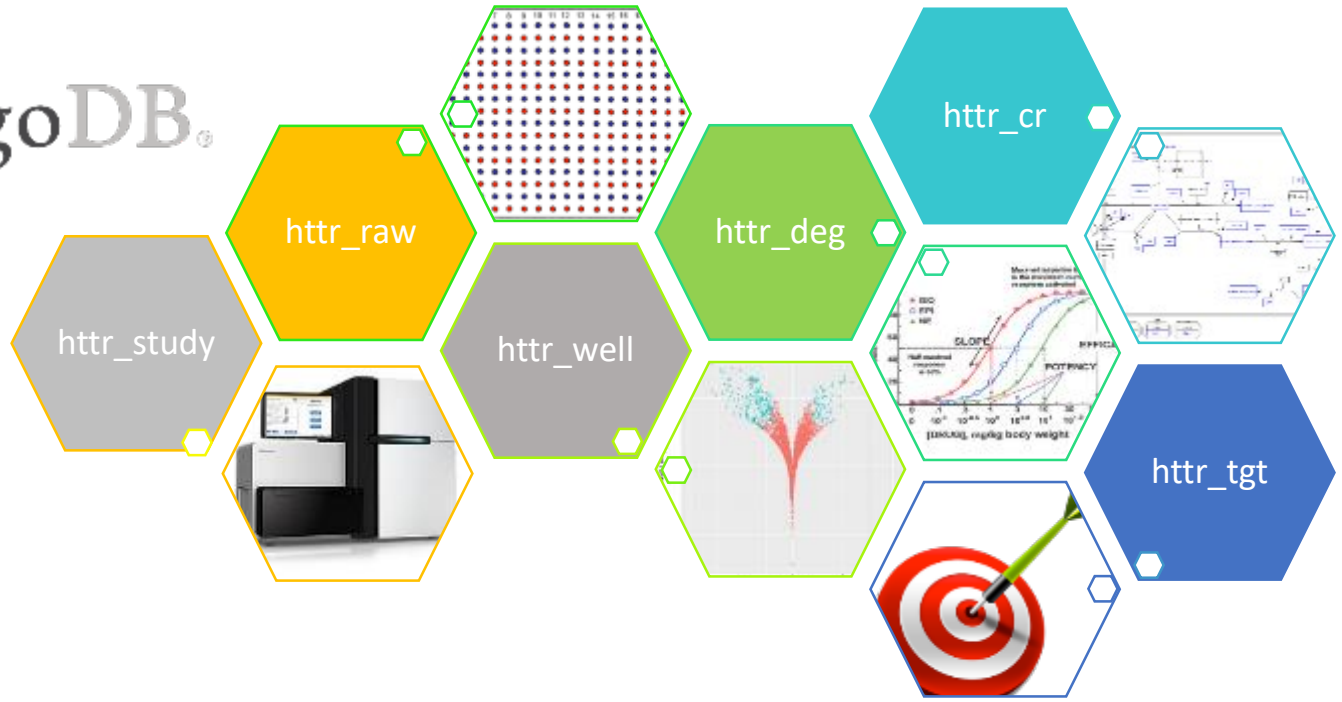
HTTr Computational Framework and Infrastructure

Python & R analysis pipeline

jupyter

TempO-Seq data processing → Differential expression analysis → Concentration response analysis → Target/ Pathway Prediction

mongoDB.



REST API

Flask

```
http://httr-dev.epa.gov/api/httr/v1/

searchChem
getChemPlates
getPlateInfo
getPlateGroups
getChemProbeCounts
getChemDEG

getChemCRG
getChemTargets
```

http://bitbucket.zn.epa.gov/projects/HTTR

Concentration Response Modeling

Parameter	Criteria [a]
Pre-filter:	William's Trend Test (p_{raw} < 0.05 & |FC| ≥ 1.5)
Models	Hill, Exponential 3, *poly2, power, linear*
BMR Factor:	1.349 (10 %)
Best Model Selection:	Lowest AIC
Hill Model Flagging [b]:	'k' < 1/3 Lowest Positive Dose Retain Flagged Models
Pathway Analysis:	Genes with BMD <= Highest Dose ≥ 3 ≥ 5% Gene Set Coverage
Gene Set Collections [c]:	GO-BP MSigDB_H Reactome

[a] *Exploratory analysis – modeling criteria not finalized*

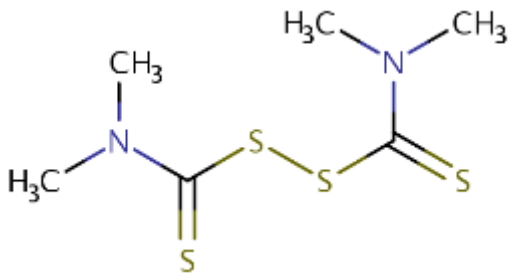
[c] Gene Set Collections:

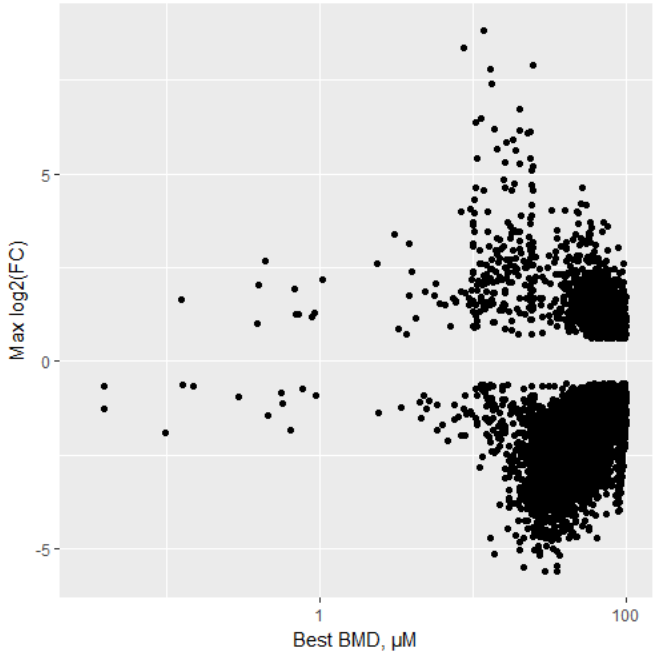
- **GO-BP:** Framework for annotation of gene functions and how these functions relate to each other in terms of a particular set of molecular functions carried out by specific gene products in an often highly regulated manner or particular temporal sequence (n = 4206).
- **MSigDB_H:** Coherently expressed signatures derived by aggregating many MSigDB gene sets to represent well-defined biological states or processes (n = 50).
- **Reactome:** Open-source, curated and peer reviewed pathway database with hierarchical pathway relationships in specific domains of biology. (n = 1764).

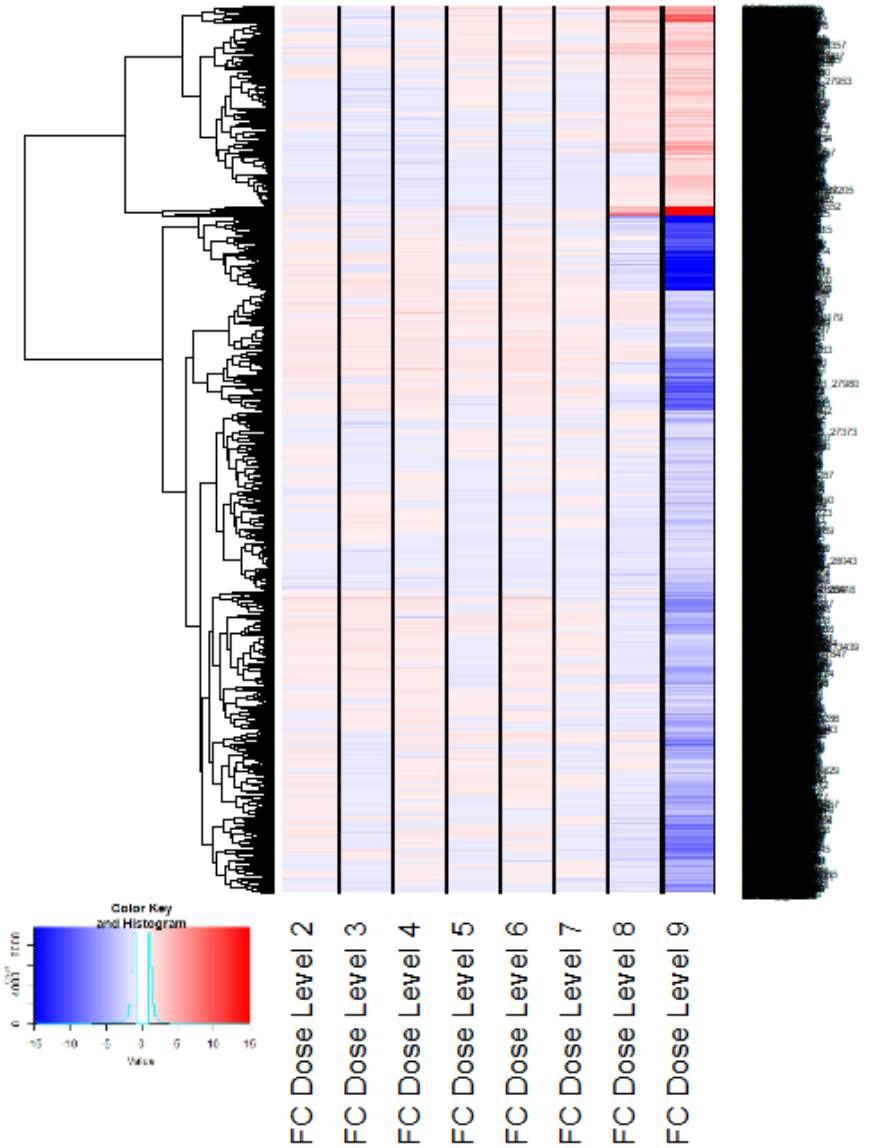
Concentration Response Modeling Example

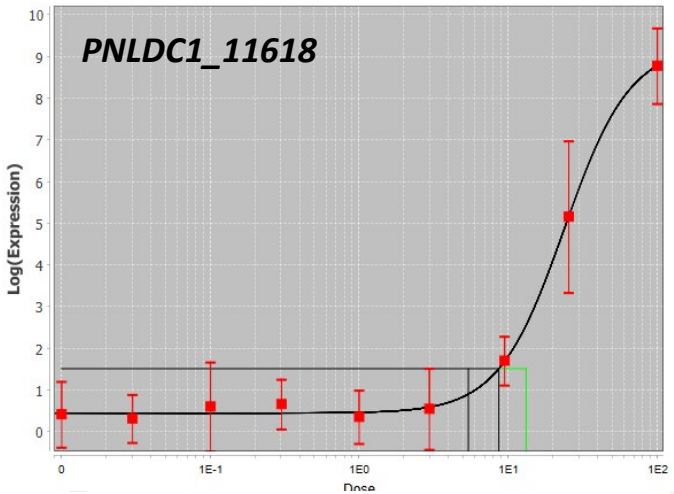
Thiram

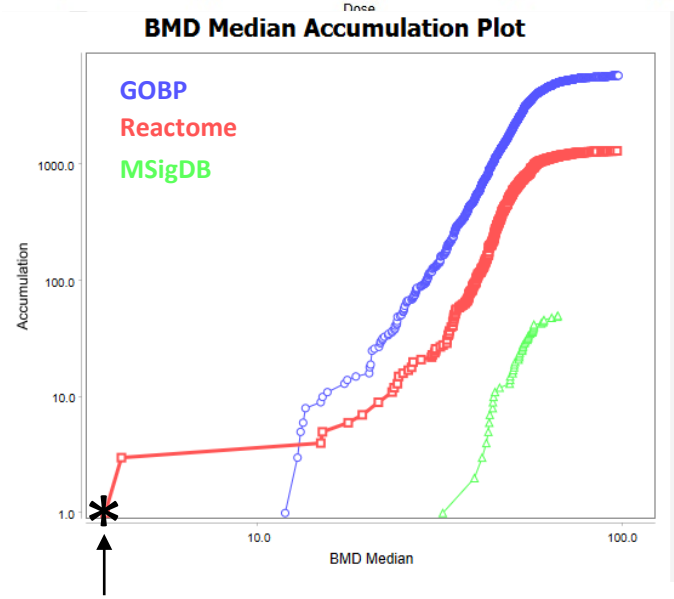
137-26-8 | DTXSID5021332





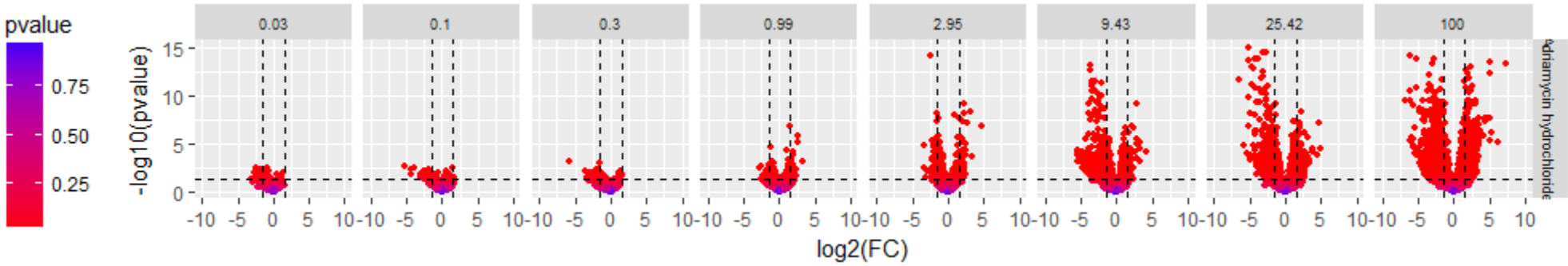


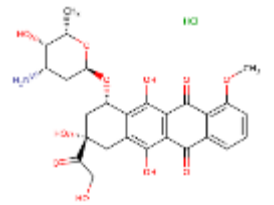




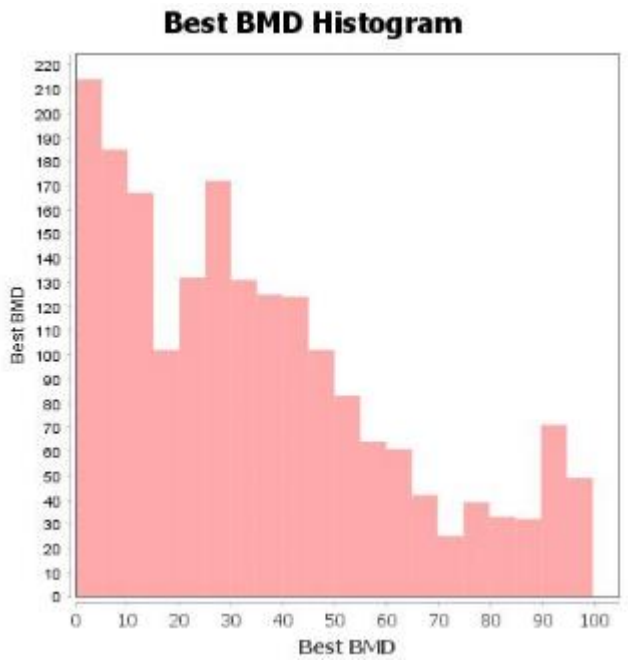
Biological Pathway Altering Concentration (BPAC)

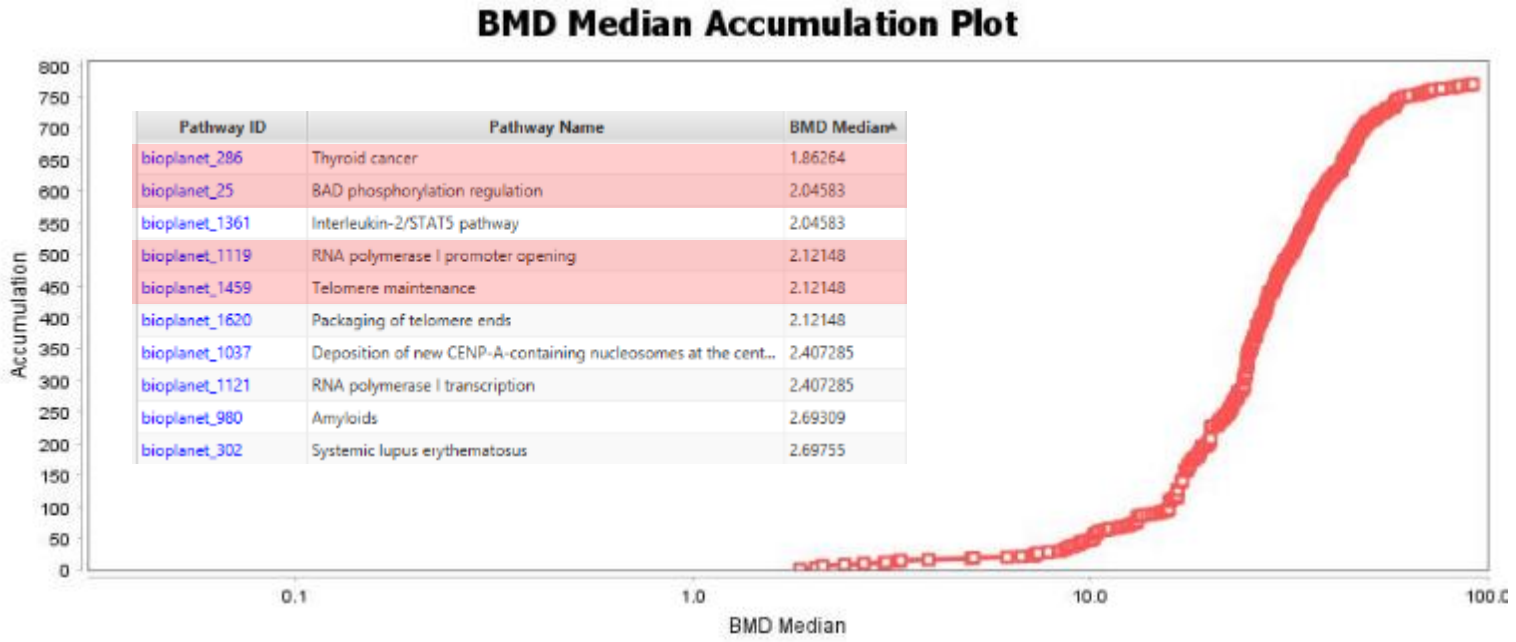
Doxorubicin (aka Adriamycin hydrochloride)





Adriamycin hydrochloride | DTXSID3030636





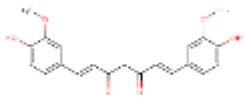
Pathway ID	Pathway Name	BMD Median
bioplanet_286	Thyroid cancer	1.86264
bioplanet_25	BAD phosphorylation regulation	2.04583
bioplanet_1361	Interleukin-2/STAT5 pathway	2.04583
bioplanet_1119	RNA polymerase I promoter opening	2.12148
bioplanet_1459	Telomere maintenance	2.12148
bioplanet_1620	Packaging of telomere ends	2.12148
bioplanet_1037	Deposition of new CENP-A-containing nucleosomes at the cent...	2.407285
bioplanet_1121	RNA polymerase I transcription	2.407285
bioplanet_980	Amyloids	2.69309
bioplanet_302	Systemic lupus erythematosus	2.69755

Int J Mol Cell Med. 2015 Spring;4(2):94-102.

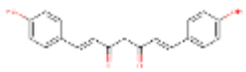
Elevation of cAMP Levels Inhibits Doxorubicin-Induced Apoptosis in Pre- B ALL NALM- 6 Cells Through Induction of BAD Phosphorylation and Inhibition of P53 Accumulation.

Fatemi A[1], Kazemi A[1], Kashiri M[1], Safa M[2].

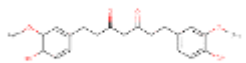
Gene Set Analysis Using BMD Modeling Results



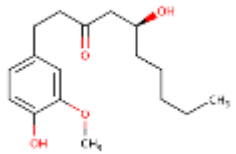
Curcumin | DTXSID8031077



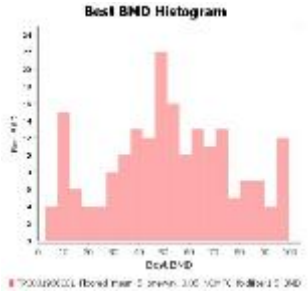
Bisdemethoxycurcumin | DTXSID00872663

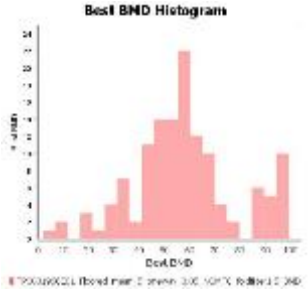


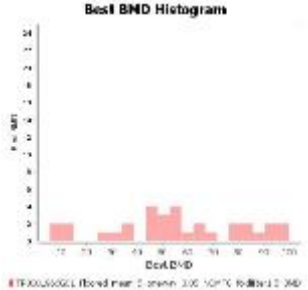
Tetrahydrocurcumin | DTXSID30865801

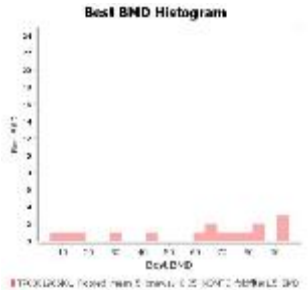


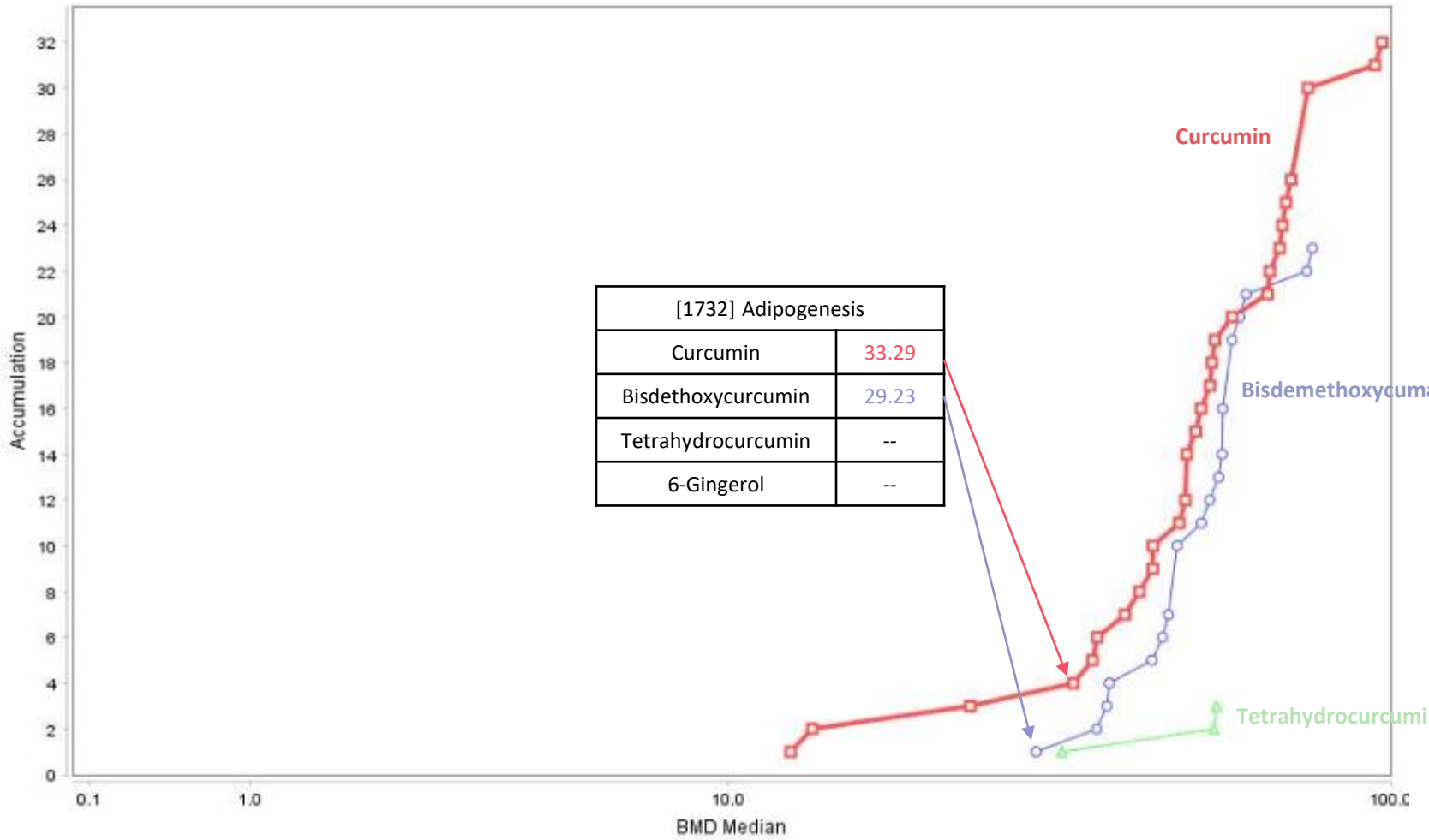
(6)-Gingerol | DTXSID3041035









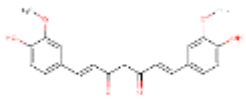


[1732] Adipogenesis	
Curcumin	33.29
Bisdethoxycurcumin	29.23
Tetrahydrocurcumin	--
6-Gingerol	--

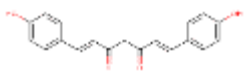
Toxicol Appl Pharmacol. 2017 Aug 15;329:158-164. doi: 10.1016/j.taap.2017.05.036.
Curcumin inhibits adipogenesis induced by benzyl butyl phthalate in 3T3-L1 cells.
Sakuma S[1], Sumida M[2], Endoh Y[2], Kurita A[2], Yamaguchi A[2], Watanabe T[2], Kohda T[2], Tsukiyama Y[2], Fujimoto Y[3].

J Agric Food Chem. 2016 Feb 3;64(4):821-30. doi: 10.1021/acs.jafc.5b05577. Epub 2016 Jan 25.
Bisdemethoxycurcumin Inhibits Adipogenesis in 3T3-L1 Preadipocytes and Suppresses Obesity in High-Fat Diet-Fed C57BL/6 Mice.
Lai CS[1,2], Chen YY[1], Lee PS[1], Kalyanam N[3], Ho CT[4], Liou WS[5], Yu RC[1], Pan MH[1,6,7,8].

Gene Set Analysis Using BMD Modeling Results

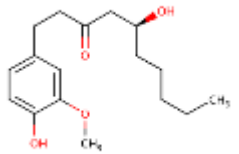


Curcumin | DTXSID8031077

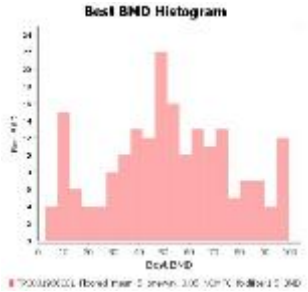


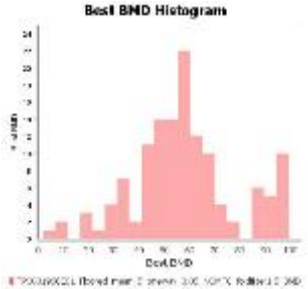
Bisdemethoxycurcumin | DTXSID00872663

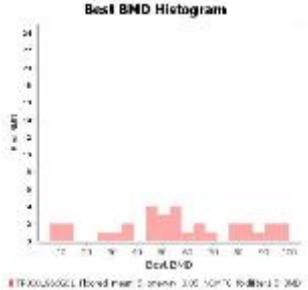
Tetrahydrocurcumin | DTXSID30865801

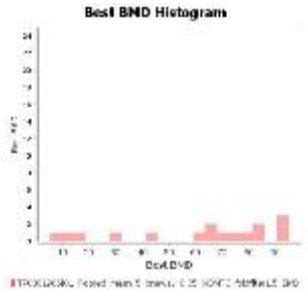


(6)-Gingerol | DTXSID3041035



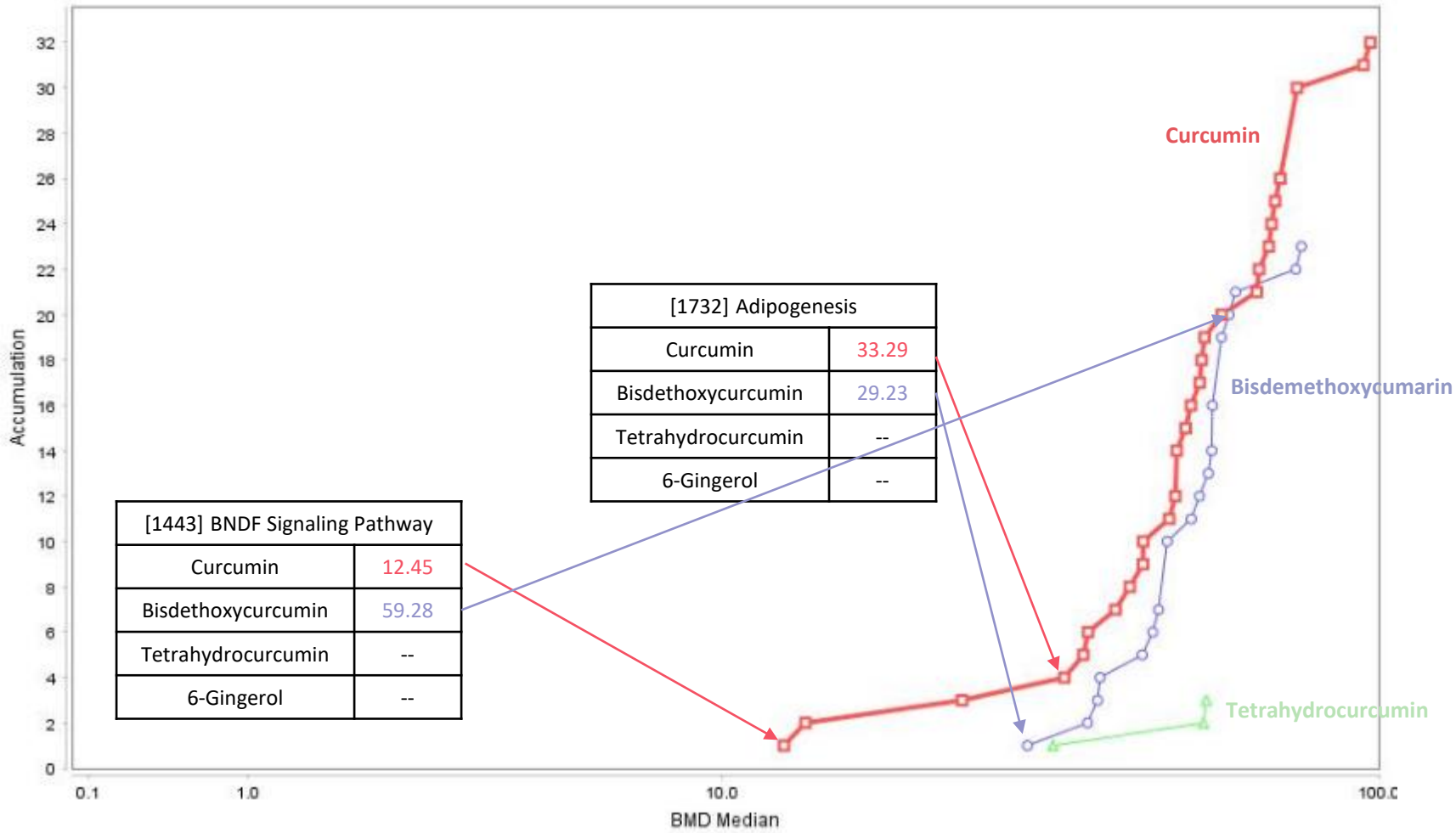






[1443] BNDF Signaling Pathway	
Curcumin	12.45
Bisdethoxycurcumin	59.28
Tetrahydrocurcumin	--
6-Gingerol	--

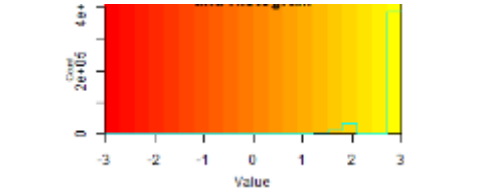
[1732] Adipogenesis	
Curcumin	33.29
Bisdethoxycurcumin	29.23
Tetrahydrocurcumin	--
6-Gingerol	--



Neuropeptides. 2016 Apr;56:25-31. doi: 10.1016/j.npep.2015.11.003. Epub 2015 Nov 11.
Effect of curcumin on serum brain-derived neurotrophic factor levels in women with premenstrual syndrome: A randomized, double-blind, placebo-controlled trial. Fanaei H[1], Khayat S[2], Kasaeian A[3], Javadimehr M[4].

Biomed Pharmacother. 2017 Mar;87:721-740. doi: 10.1016/j.biopha.2016.12.020. Epub 2017 Jan 14.
Curcumin confers neuroprotection against alcohol-induced hippocampal neurodegeneration via CREB-BDNF pathway in rats.
Motaghinejad M[1], Motevalian M[2], Fatima S[3], Hashemi H[1], Gholami M[4].

Gene Set Analysis Summary (2)



Observe both promiscuous chemicals and profile similarities across chemicals

Predicting Putative Chemical Targets with HTTr

Connectivity mapping

Compare entire transcriptomic profile to reference database to find target using kNN

Pros:
- Need just one profile / target

Cons:
- Sensitive but not specific within platform
- Low cross-platform accuracy
- Requires chemical annot.

Pathway analysis

Compare entire transcriptomic profile to pathways (bags of genes) to find pathway 'hits' using different scoring schemes

Pros:
- More accurate (specificity)
- Derive conc-response

Cons:
- Needs curated pathways
- No ideal pathway collection
- Multiple scoring schemes
- Pathways ≠ Targets
- Requires chemical annot.

Signatures/classifiers

Using profiles for target to create classifiers / signatures ("biomarkers"); search using entire profiles of test chemicals

Pros:
- More accurate
- Derive conc-response
- Target/mode-specific

Cons:
- Requires chemical annot.
- Require LARGE profile db

Network analysis

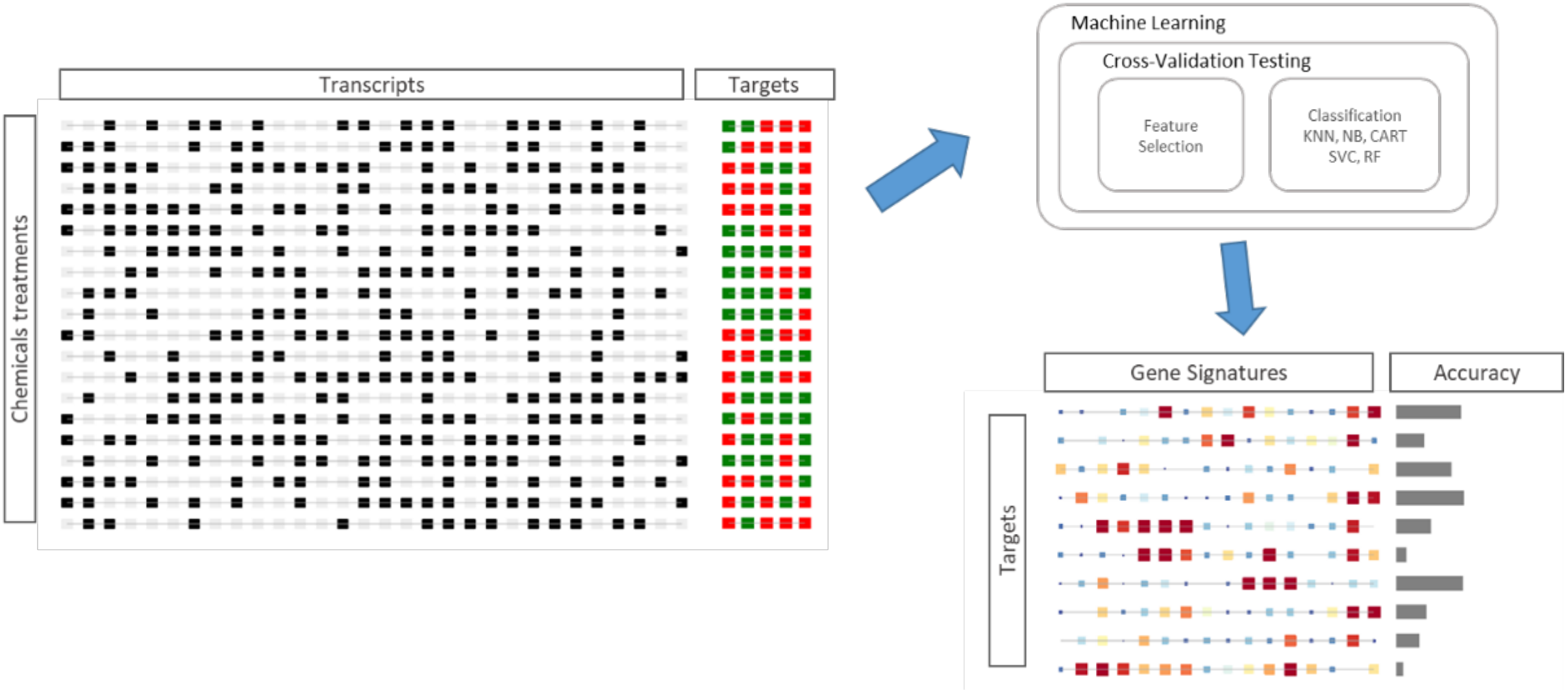
Use transcriptomic profile alone with genetic-regulatory and signaling data to infer putative targets

Pros:
- TBD

Cons:
- TBD

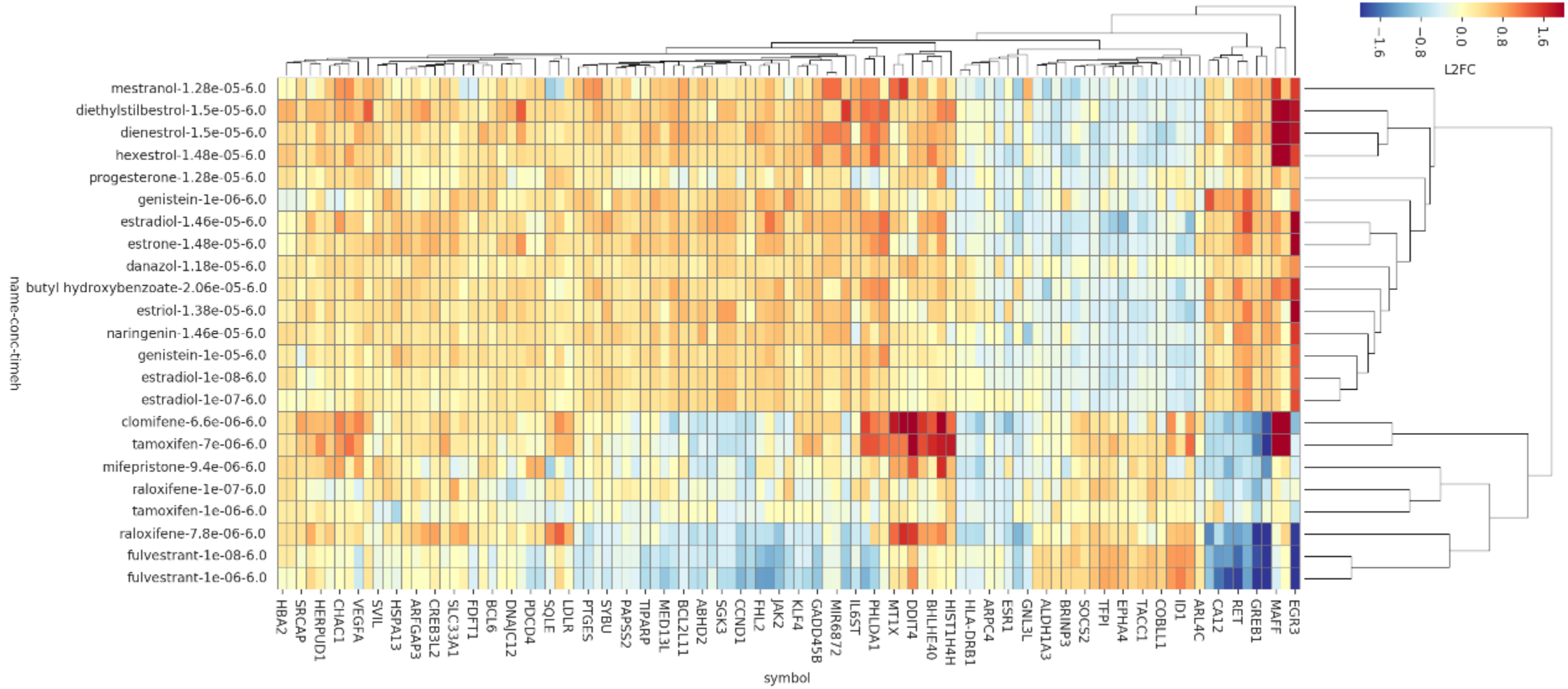
Slide by Imran Shah

Signatures via Machine Learning



Slide by Imran Shah

ER Model (any Mode) Derived from CMAP



Searching CMAP v2 Using ER Signature

dsstox_sid	name	target	cell	conc	jaccard	olap	timeh
Other	equilin	Other	MCF7	0.000015	0.176	110	6
Other	equilin	Other	MCF7	0.000015	0.166929	106	6
Other	lynestrenol	Other	MCF7	0.000014	0.163303	89	6
Other	prasterone	Other	MCF7	1.22E-05	0.162055	82	6
DTXSID3020465	diethylstilbestrol	ESRRG|ES	MCF7	0.000015	0.153846	82	6
DTXSID3020465	diethylstilbestrol	ESRRG|ES	MCF7	0.000015	0.144531	74	6
Other	trifluoperazine	Other	MCF7	0.00001	0.140777	87	6
Other	epitiostanol	Other	MCF7	0.000013	0.137876	87	6
Other	equilin	Other	MCF7	0.000015	0.1376	86	6
Other	etynodiol	Other	MCF7	1.04E-05	0.135314	82	6
DTXSID0020814	mestranol	ESR1	MCF7	1.28E-05	0.134052	87	6
DTXSID8022371	testosterone	AR|ESR2	MCF7	1.16E-05	0.131068	81	6
DTXSID6023656	thioridazine	DRD2|KCN	MCF7	0.00001	0.127303	76	6
DTXSID6023656	thioridazine	DRD2|KCN	MCF7	0.00001	0.126761	72	6
DTXSID4022369	fulvestrant	ESR2|ESR1	MCF7	1E-08	0.12623	77	6
Other	suloctidil	Other	MCF7	1.18E-05	0.124756	64	6
Other	prochlorperazine	Other	MCF7	0.00001	0.124214	79	6
DTXSID5022308	genistein	ESR2|ESR1	MCF7	0.00001	0.123664	81	6
Other	suloctidil	Other	MCF7	1.18E-05	0.120553	61	6
Other	mometasone	Other	MCF7	7.6E-06	0.119869	73	6
DTXSID2022880	danazol	AR|ESR1	MCF7	1.18E-05	0.119449	78	6
Other	trifluoperazine	Other	MCF7	0.00001	0.11936	82	6
Other	fluphenazine	Other	MCF7	0.00001	0.119163	74	6
DTXSID9020110	astemizole	KCNH2|HI	MCF7	8.8E-06	0.119005	67	6
Other	butyl hydroxyben	Other	MCF7	2.06E-05	0.118902	78	6
Other	ciclosporin	Other	MCF7	3.4E-06	0.118699	73	6
Other	ivermectin	Other	MCF7	4.6E-06	0.118098	77	6

"Horse" estrogen

Synthetic progesterone

Synthetic progesterone

Pro-androgen/estrogen

Dopamine antagonist / Antipsychotic
gynecomastia in males

Most of the top hits are ER-related

United States Environmental Protection Agency

Performance of CMap v2 Affymetrx-derived signatures for predicting targets using BioSpyder HTTr data

Curation of hits necessary to determine specificity

Putative Target	CMap v2 / Affymetrix	BioSpyder HTTr-Phase I			
	Signature size	PPV	Positives	Positive Chemicals found	Top 5 Prediction (Uncurated)
CYP2C9	131	1	1	Fluconazole	Emodin, Phenazopyridine hydrochloride, Lactofen, Hexachlorophene, 2-Amino-5-azotoluene
ESR1	257	1	11	o,p'-DDT, Genistein, 4-Nonylphenol, 4-Hydroxytamoxifen, Diethylstilbestrol, Raloxifene hydrochloride, Bisphenol A, 17beta-Estradiol, 5alpha-Dihydrotestosterone, Mifepristone, 4-(1,1,3,3-Tetramethylbutyl)phenol	dl-Norgestrel, SSR504734, Haloperidol, Cyclosporin A, Astemizole
HDAC1	124	1	2	Trichostatin A, Valproic acid	2-(Thiocyanomethylthio)benzothiazole, Azinphos-methyl, Sodium (2-pyridylthio)-N-oxide, 3,3'-Dichlorobenzidine dihydrochloride
DHFR	215	1	2	Pyrimethamine, Methotrexate	Adriamycin hydrochloride, PharmaGSID_48505, Etoposide, Resveratrol, Nisoldipine
NR1I2	139	1	2	17beta-Estradiol, Bisphenol A	dl-Norgestrel, Endosulfan, Isodrin, Genistein, 17alpha-Estradiol
PGR	115	1	1	Mifepristone	Flurandrenolide, Fluorometholone, Dexamethasone, Melengestrol acetate, Betamethasone
HMGCR	236	1	1	Lovastatin	Resveratrol, dl-Norgestrel, o,p'-DDT, Tamoxifen, Chlorhexidine
ABCC2	357	1	1	Methotrexate	4-Nitrosodiphenylamine, Resveratrol, Adriamycin hydrochloride, Nisoldipine, 8-Hydroxyquinoline sulfate
TYMS	329	1	1	Methotrexate	Etoposide, Resveratrol, 4-Nitrosodiphenylamine, Cytarabine hydrochloride, PharmaGSID_48505
ESR2	281	0.86	7	Genistein, Diethylstilbestrol, 4-Nonylphenol, Bisphenol A, 4-Hydroxytamoxifen, 17beta-Estradiol	dl-Norgestrel, 17alpha-Estradiol, Haloperidol, Cyclosporin A, Isodrin
AR	261	0.78	9	o,p'-DDT, 17beta-Estradiol, 5alpha-Dihydrotestosterone, Flutamide, Bisphenol A, Mifepristone, 17-Methyltestosterone	dl-Norgestrel, Melengestrol acetate, Dehydroepiandrosterone, 8-Hydroxyquinoline, Genistein
NR3C2	352	0.5	2	Mifepristone	Fluocinolone acetonide, Bexarotene, 1-Naphthol, Dexamethasone, dl-Norgestrel
ABCB1	117	0.5	2	Reserpine	Fabesetron hydrochloride, Abamectin, SAR115740, SSR69071, Chlorobenzilate
NR3C1	148	0.5	4	Triamcinolone, Mifepristone	Medroxyprogesterone acetate, Fluorometholone, Melengestrol acetate, Dexamethasone, Prednisolone
CA1	176	0.5	4	Phenol, Sodium nitrite	Triclopyr, Triclopyr butotyl, p-Bromodiphenyl ether, 2-Fluoroacetamide, 1-Ethyl-2-methylbenzene
CA2	341	0.5	4	Celecoxib, Phenol	PharmaGSID_48509, Acenaphthylene, CP-105696, Aloe-emodin, 2-Fluoroacetamide
PTGS1	307	0.25	4	Indomethacin	SSR69071, 17alpha-Estradiol, Chlordane, Cetylpyridinium bromide, Zoxamide

EPA United States Environmental Protection Agency

HTTr Summary Slide

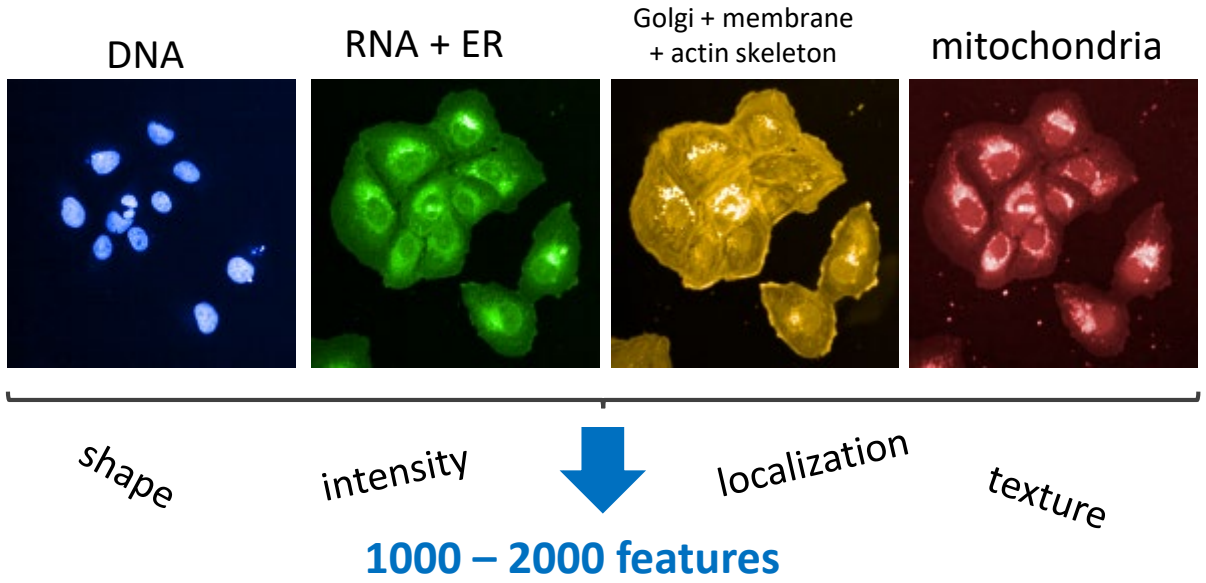
- **Technology:** Targeted RNA-Seq based HTTr is a promising platform for comprehensive and cost-effective evaluation of chemically-induced disruption of biological processes/pathways.
- **Workflow:** We have developed a standardized, scalable and portable workflow to generate large-scale HTTr data for thousands of chemicals.
- **Performance Standards:** The use of reference materials / QC standards on each plate enable development of performance standards for comparison within and across laboratories.
- **Concentration-Response Analysis:** Incorporation of concentration-response modeling into the analysis pipeline enables identification of transcriptional BPACs at the biological pathway/process level.
- **MIE/MOA Identification:** Multiple analysis approaches are being investigated for identification of MIE/MOA. Current methods are generally sensitive, but not specific and may be confounded by non-specific secondary/tertiary transcriptional cascades. Cross-platform connectivity mapping currently shows limited accuracy, but alternative analysis approaches are being explored that may improve this.

EPA
United States
Environmental Protection
Agency

High Throughput Phenotypic Profiling via High Content Image Analysis

Phenotypic Profiling

- Image-based phenotypic profiling is a chemical screening method that measures a large variety of morphological features of individual cells in *in vitro* cultures.
- Successfully used for functional genomic studies and in the pharmaceutical industry for compound efficacy and toxicity screening.
- No requirement for *a priori* knowledge of molecular targets.
- **May be used as an efficient and cost-effective method for evaluating the chemical bioactivity.**



- **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.

Experimental Workflow

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

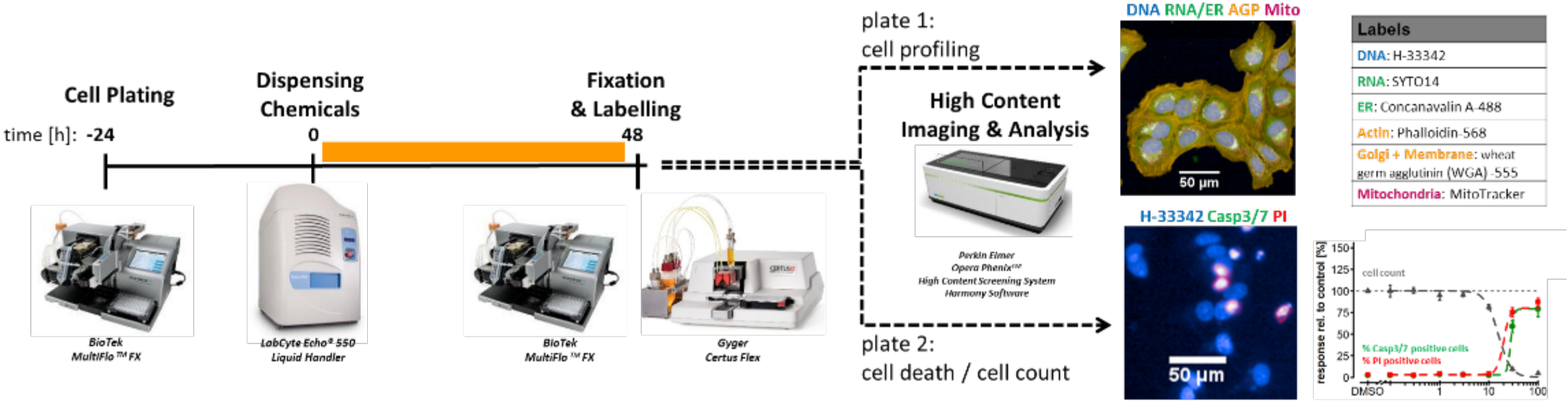
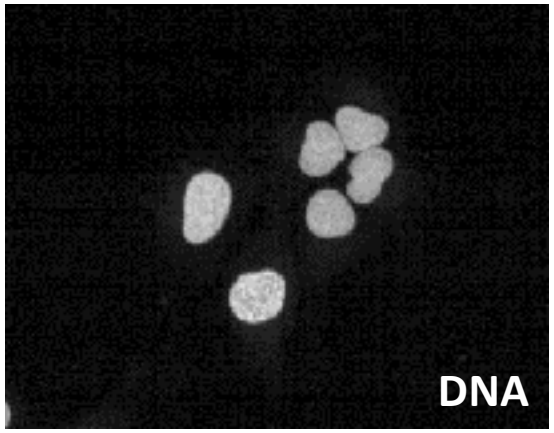
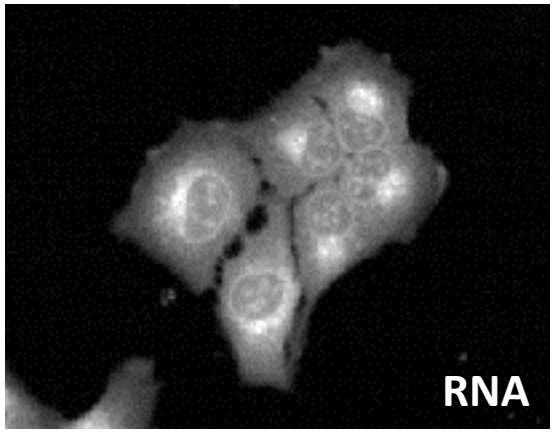


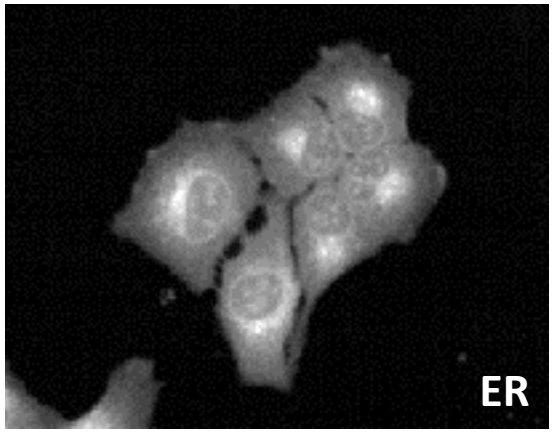
Image Analysis Workflow → Image Acquisition

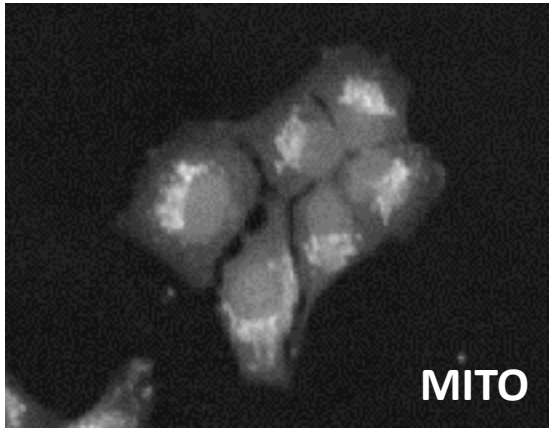
Image Acquisition

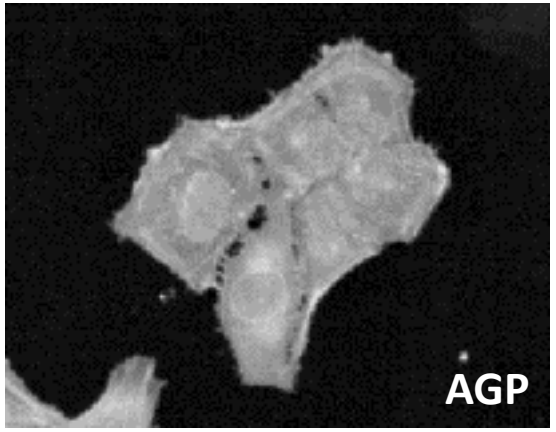
- Perkin Elmer Opera Phenix
- 20x Water Immersion Objective
- Confocal Mode, Single Z
- CellCarrier-384 Ultra Microplates











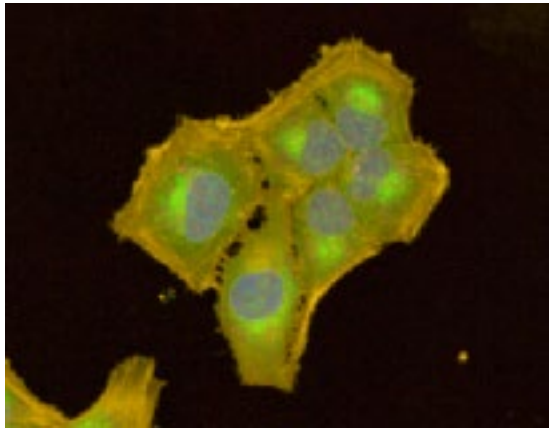
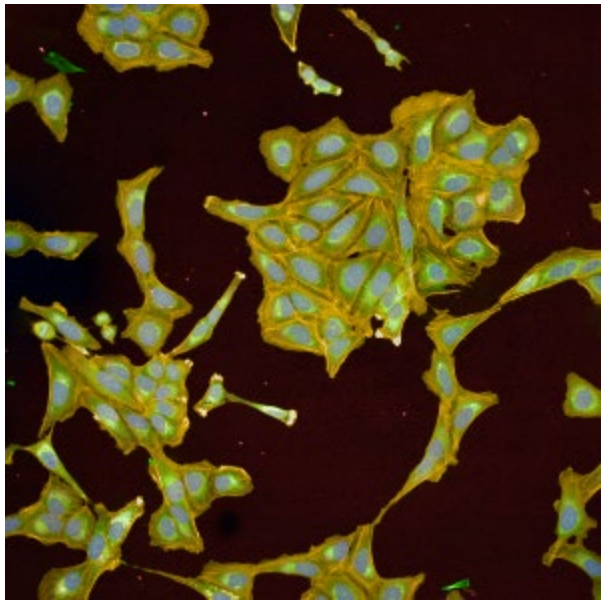
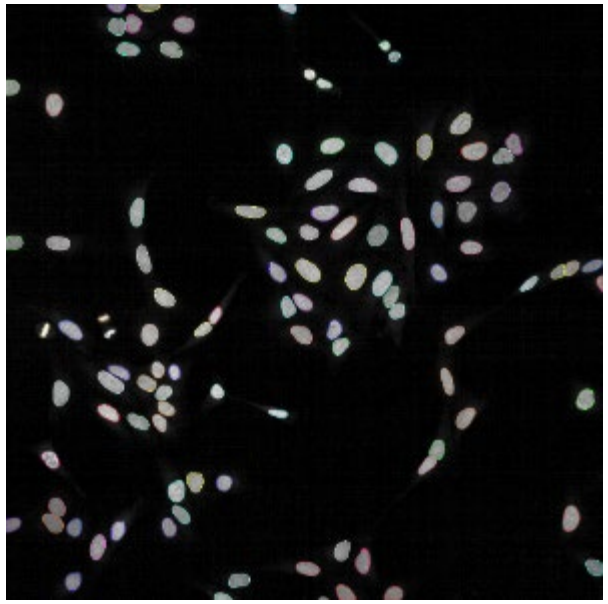
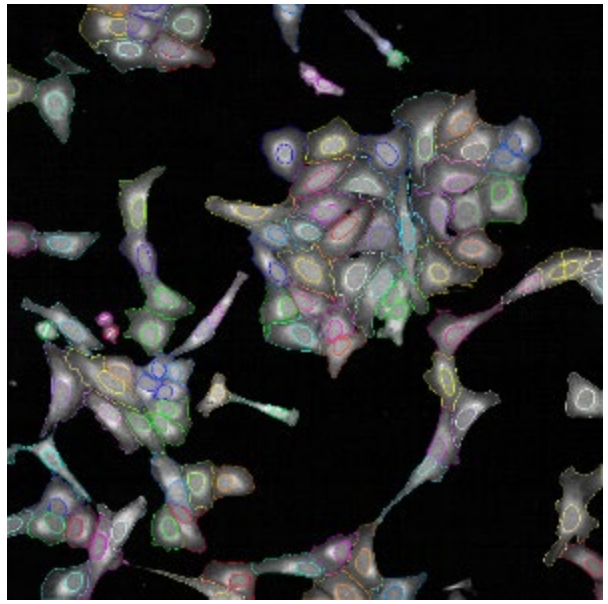
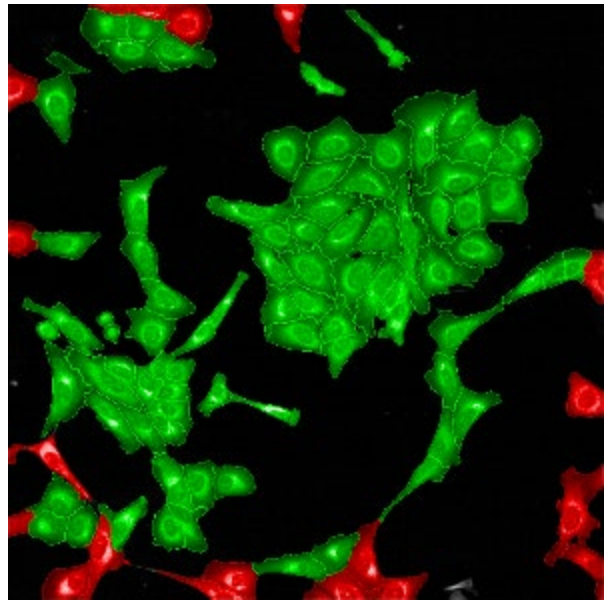
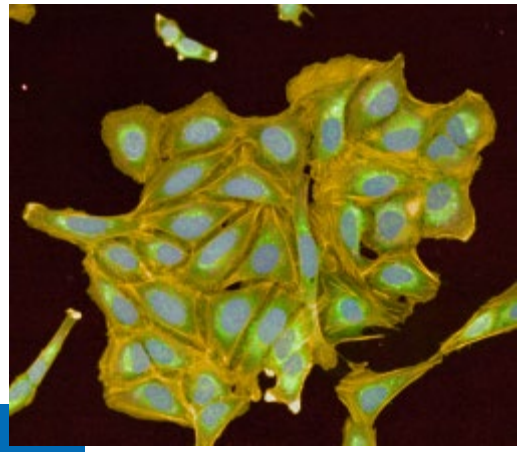
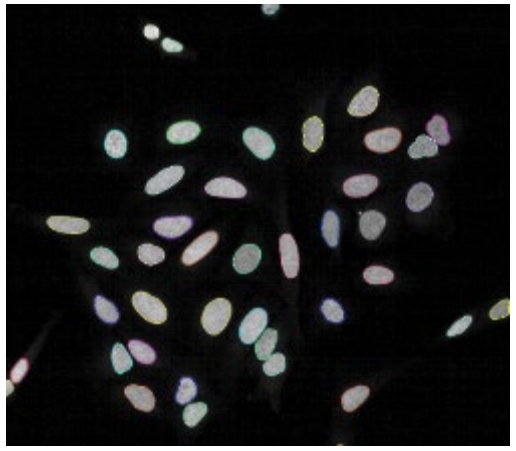
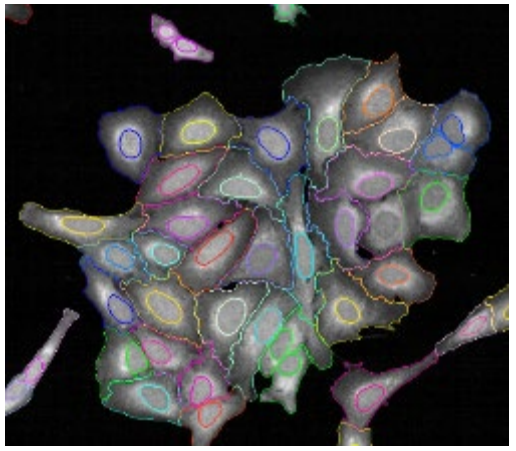


Image Analysis Workflow → Image Segmentation

1. find nuclei

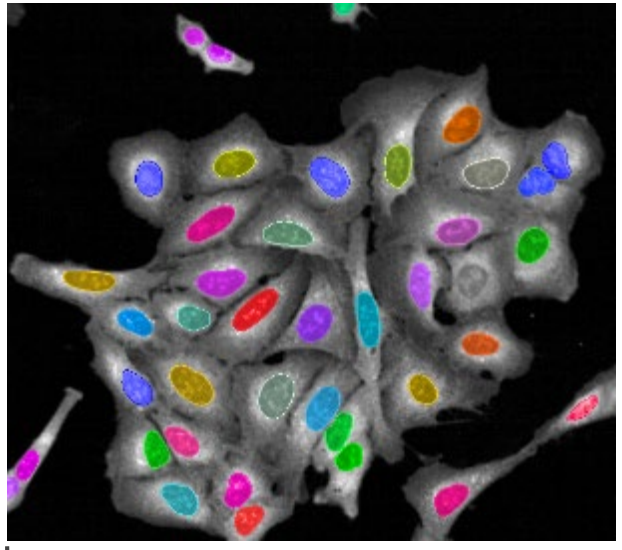
2. find cell outline

3. reject border objects

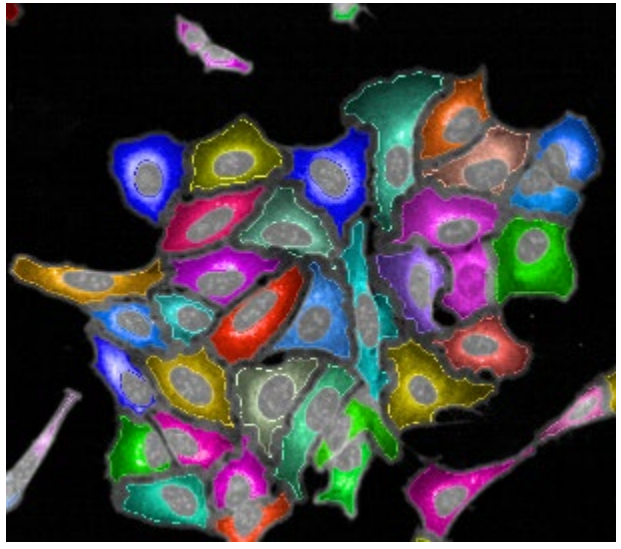








Define Cellular Compartments

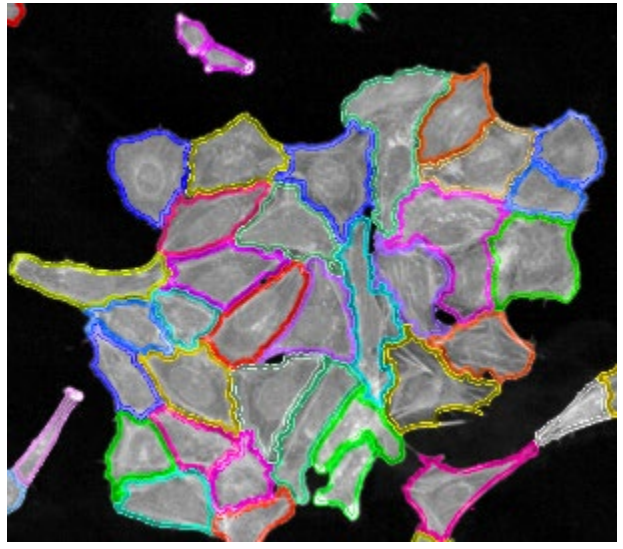
nuclei



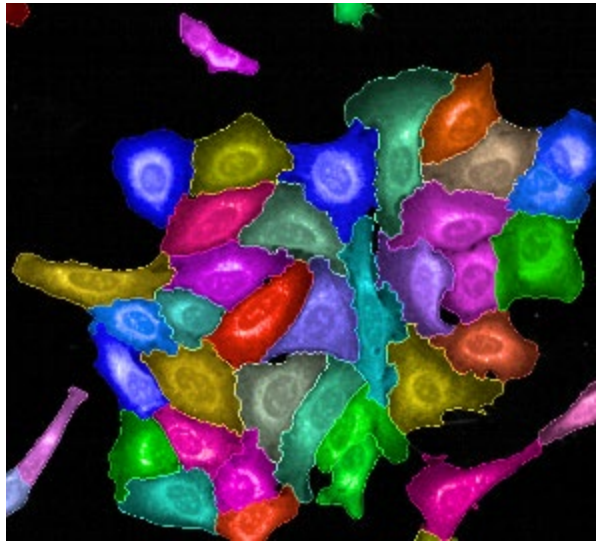
cytoplasm



membrane



cell



ring

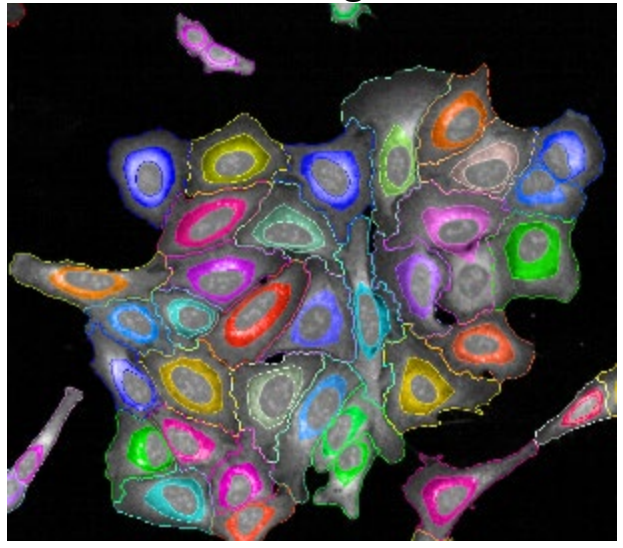


Image Analysis Workflow

Endpoints

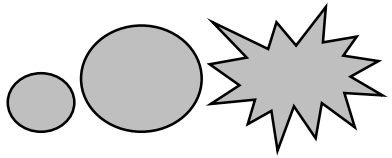
Profiling
with Harmony Software

	NUCLEUS	RING	CYTOPLASM	MEMBRANE	CELL
DNA	S,C,A,R, P,I,T,M	--	--	--	S,C,A,R, P,M
RNA	S,C,A,R, P,I,T	--	--	--	S,C,A,R, P
ER	S,C,A,R, P,I,T	I,T	I,T	I	S,C,A,R, P
AGP	S,C,A,R, P,I,T	I,T	I,T	I,T	S,C,A,R, P
MITO	S,C,A,R, P,I,T	I,T	I,T	I	S,C,A,R, P

~ 1300 endpoints

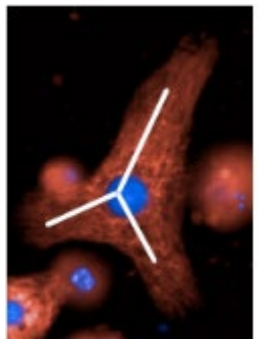
Ontologies:

- AGP_Texture _Cytoplasm
- Mito_Compactness _Ring
- DNA_Intensity _Nuclei

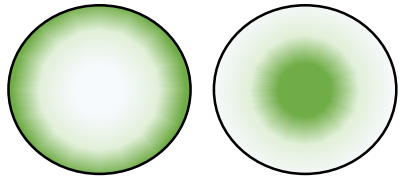


Shape (M)

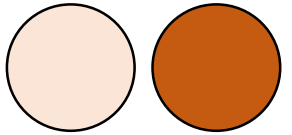
Threshold Compactness (C)



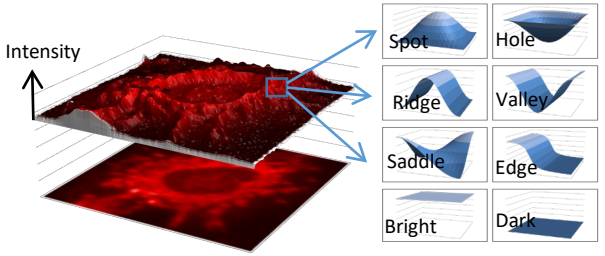
Symmetry (S)



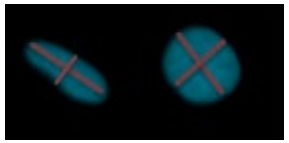
Radial distribution (R)



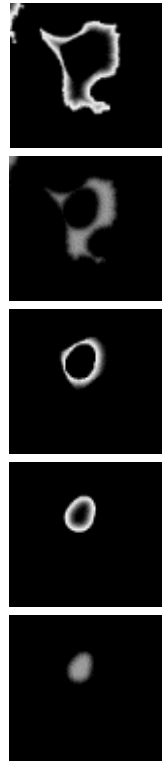
Intensity (I)



Texture (T)



Axial (A)



Profile (P)

Data Processing

Data Reduction
in R

cell-level data (~500 cells/well)

$$\frac{\text{cell value} - \text{median}_{DMSO}}{1.4826\ \text{MAD}_{DMSO}}$$

normalized cell-level data

median

well-level data

Caspase/PI BMD
BMD_3SD
Conc. above LEC removed

clipped well-level data

Benchmark dose (BMD) modelling
using BMDExpress 2.2

1293 endpoints

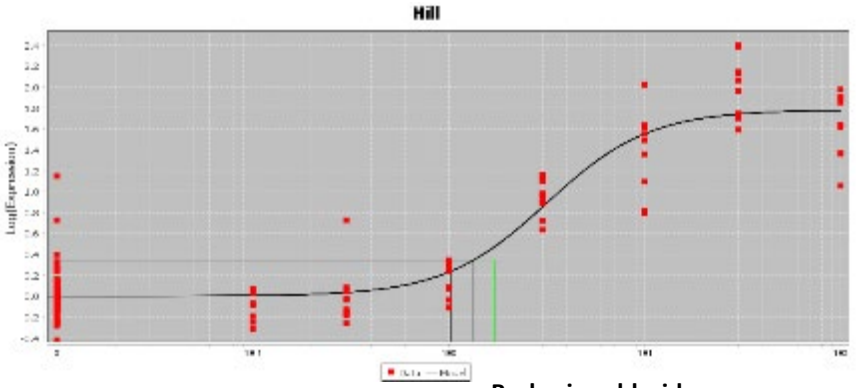
Pre-filtering

BMD modelling

4 models: Hill, Linear, Poly2, Power

Model selection

Model with best AIC



Berberine chloride
Mito_Cells_Morph_STAR

BMD_10%

Phase 1: Identification of Reference Chemicals

Experimental Design

Parameter	Multiplier	Notes	
Cell Type(s)	1	U-2 OS [a]	Bone
Culture Condition	1	DMEM + 10% HI-FBS	
Chemicals	16	14 phenotypic reference chemicals 2 negative control chemicals	
Time Points:	1	48 hours	
Assay Formats:	2	Cell Painting HCI Cell Viability & Apoptosis	
Concentrations:	8	3.5 $\log_{10}$ units; semi $\log_{10}$ spacing	
Biological Replicates:	3	--	

[a] Reference cell line (Bray et al. 2016).

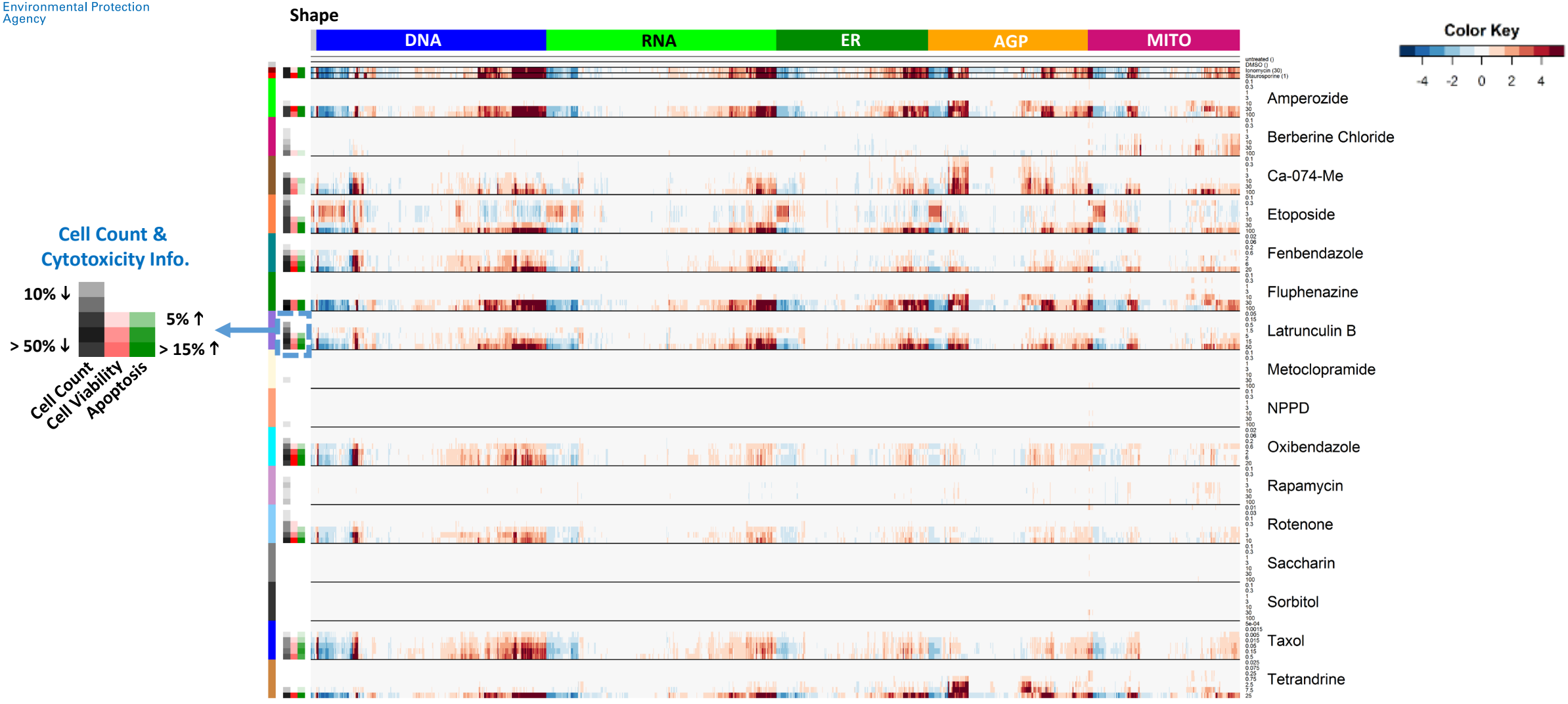
Reference Chemical Set

- Reference chemicals (n=14) with narrative descriptions of observed phenotypes were identified from Gustafdottit et al. 2013.
- Candidate negative control chemicals (n=2) with no anticipated affect on cell phenotype were included in the reference set.

Compound Name	Chemical Use	Expected Phenotype
Amperozide	Atypical antipsychotic	Toroid nuclei
Berberine Chloride	Mitochondria complex I inhibitor	Redistribution of mitochondria
Ca-074-Me	Cathepsin B inhibitor	Bright, abundant golgi staining
Etoposide	Chemotherapeutic	Large, flat nucleoli
Fenbendazole	Anthelmintic	Giant, multi-nucleated cells
Fluphenazine	Typical antipsychotic	Enhanced golgi staining and some cells with fused nucleoli
Latrunculin B	Actin cytoskeleton disruptor	Actin breaks
Metoclopramide	D_2 dompaine receptor antagonist	Enhanced golgi staining and some cells with fused nucleoli
NPPD	Chloride channel blocker	Redistribution of ER to one side of the nucleus
Oxibendazole	Anthelmintic	Large, multi-nucleated cells with fused nucleoli
Rapamycin	Macrolide antibiotic / antifungal	Reduced nucleolar size
Rotenone	Mitochondria complex I inhibitor	Mitochondrial stressor
Saccharin	Artificial Sweetener	Negative Control
Sorbitol	Artificial Sweetener	Negative Control
Taxol	Microtubule Stabilizer	Large, multi-nucleated cells with fused nucleoli
Tetrandrine	Calcium channel blocker	Abundant ER

Gustafsdottir, et al. 2013

Phenotypic Profiles for Reference Chemicals [U-2 OS]



- Effects on morphology observed at sub-cytotoxic concentrations.
- Unique phenotypic profiles observed across the reference chemical set.
- Some chemicals did not produce any effects.

Phenotypic Profiles Are Consistent with Previous Literature Studies

Parameters with marked effects:

Channel	Compartment	Domain
Mito	Cytoplasm	Texture
Mito	Cytoplasm + Ring	Intensity Maximum
Mito	Entire Cell	Morphology: Compactness

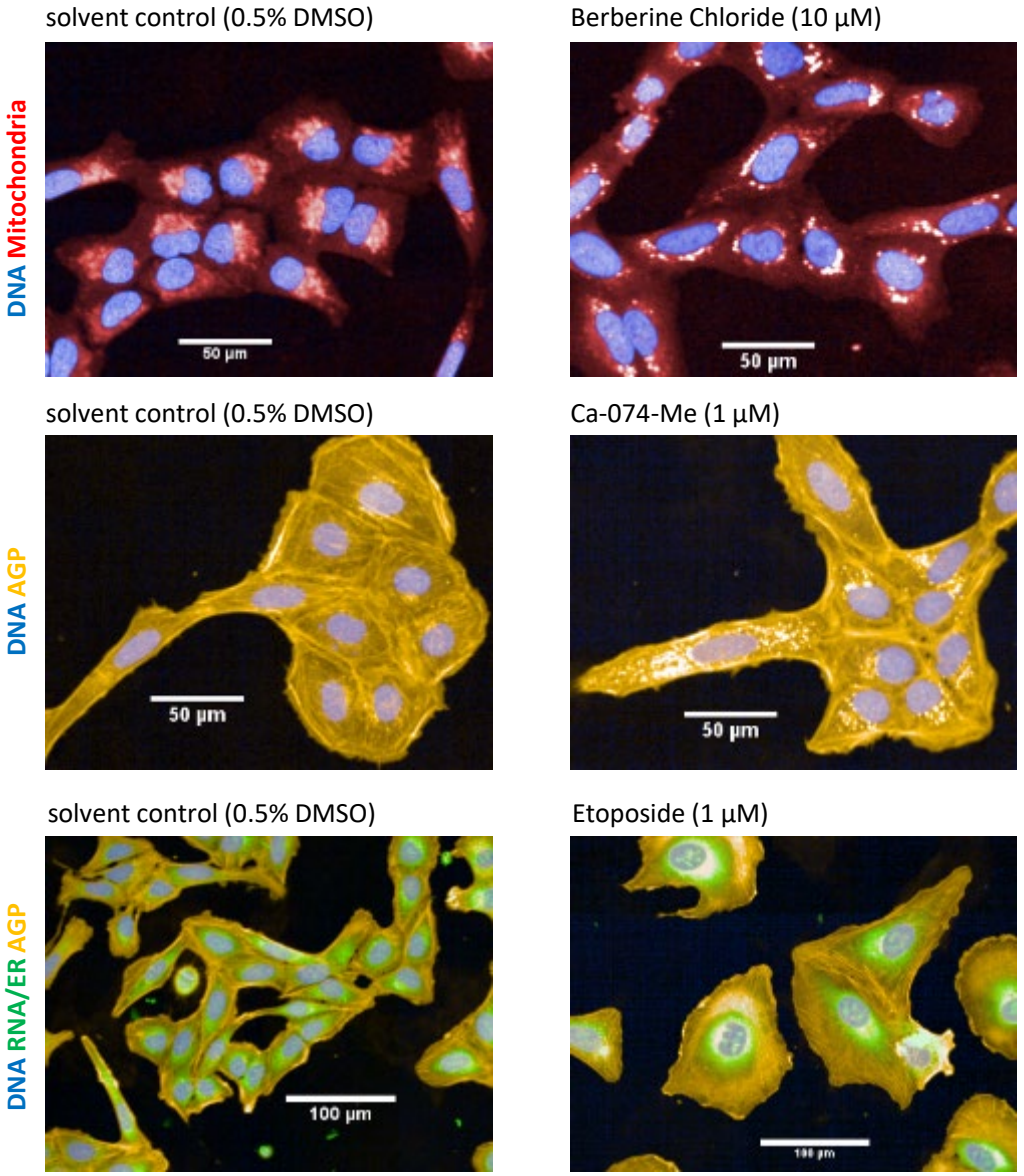
Literature: redistribution of mitochondria

Channel	Compartment	Domain
AGP	Cytoplasm + Ring	Texture
AGP	Cytoplasm + Ring	Intensity Maximum
AGP	Entire Cell	Morphology/Texture

Literature: bright, abundant Golgi stain

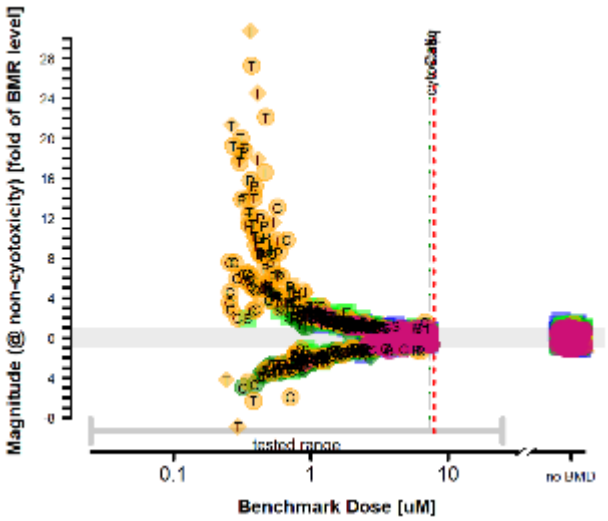
Channel	Compartment	Domain
"Shape"	Entire Cell	Morphology: Area
DNA + RNA	Nuclei	Morphology: Compactness Texture
ER + AGP	Cytoplasm + Ring	Intensity: Sum
all	Entire Cell	Morphology

Literature: large, flat nucleoli

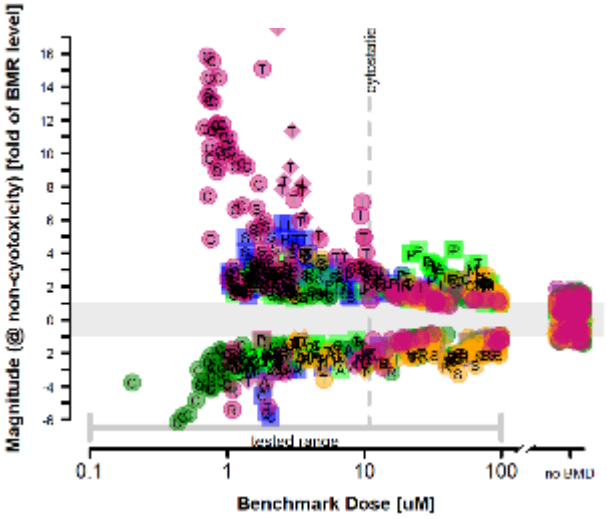


Visualizing Phenotypic Profiles: Potency vs. Efficacy Plots

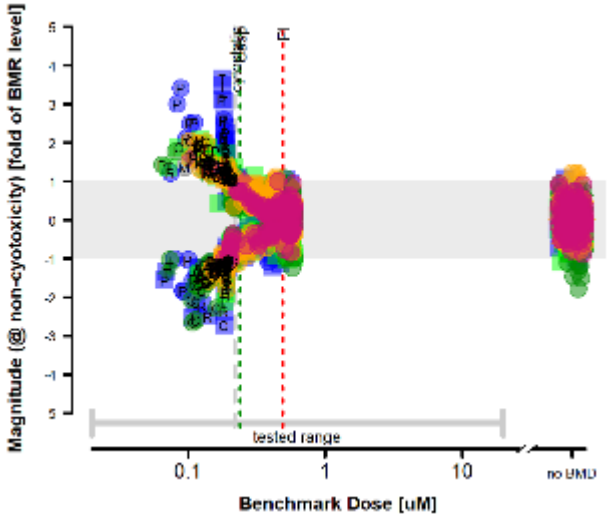
Tetrandrine



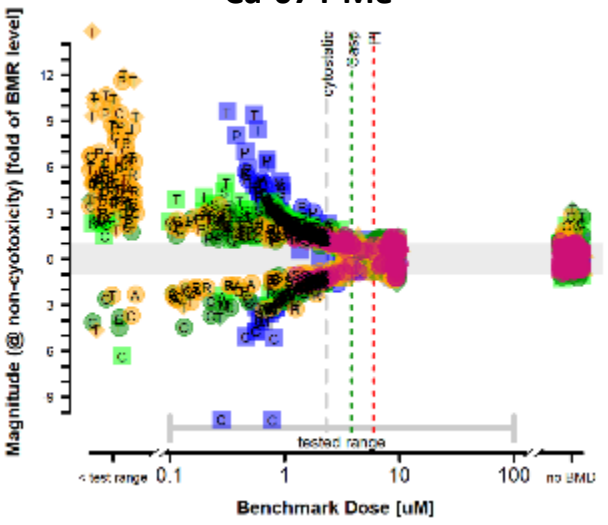
Berberine Chloride



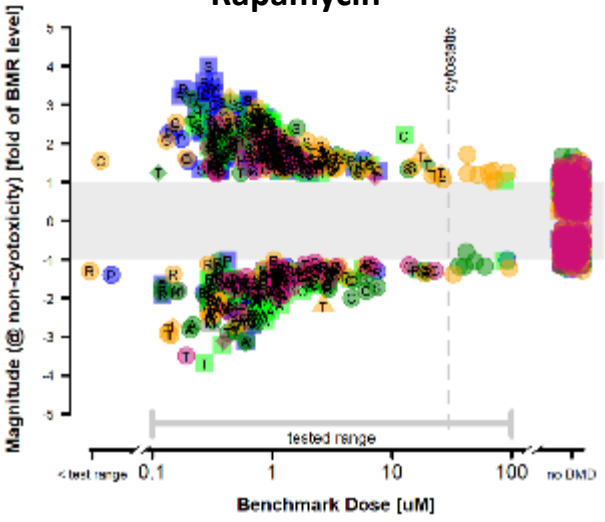
Oxibendazole



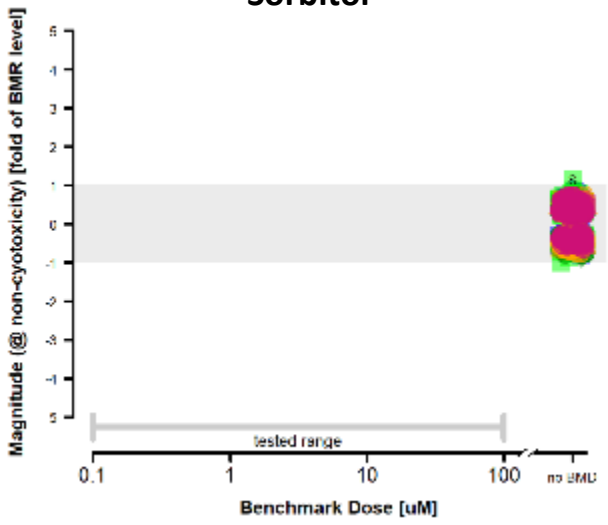
Ca-074-Me



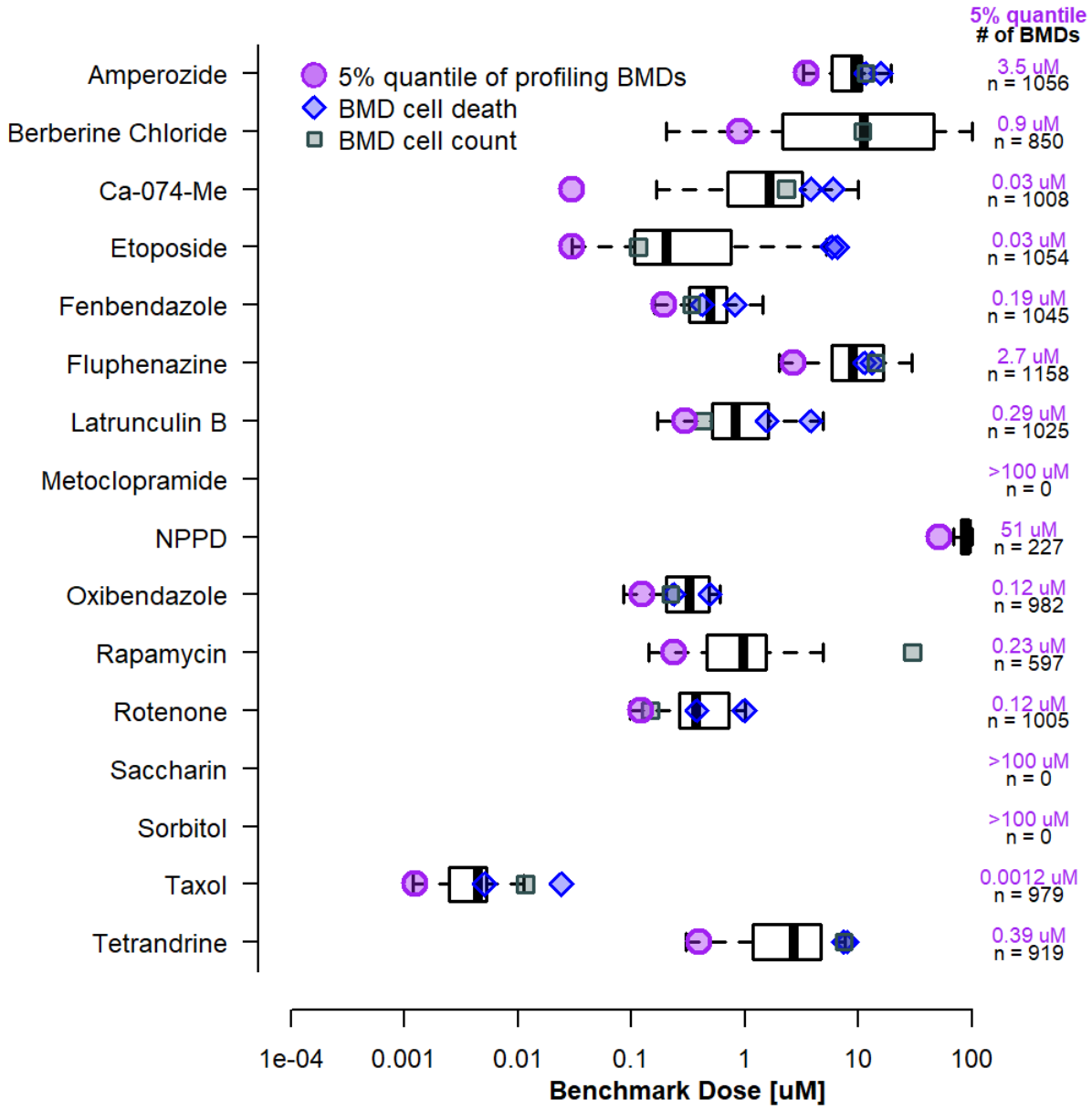
Rapamycin



Sorbitol



In Vitro BPAC Determination



- *In vitro* BPACs calculates as lower 5th percentile of affected endpoints
- Effects on cell morphology observed at concentrations well below cytotoxicity.
- Potency varies across reference chemical set

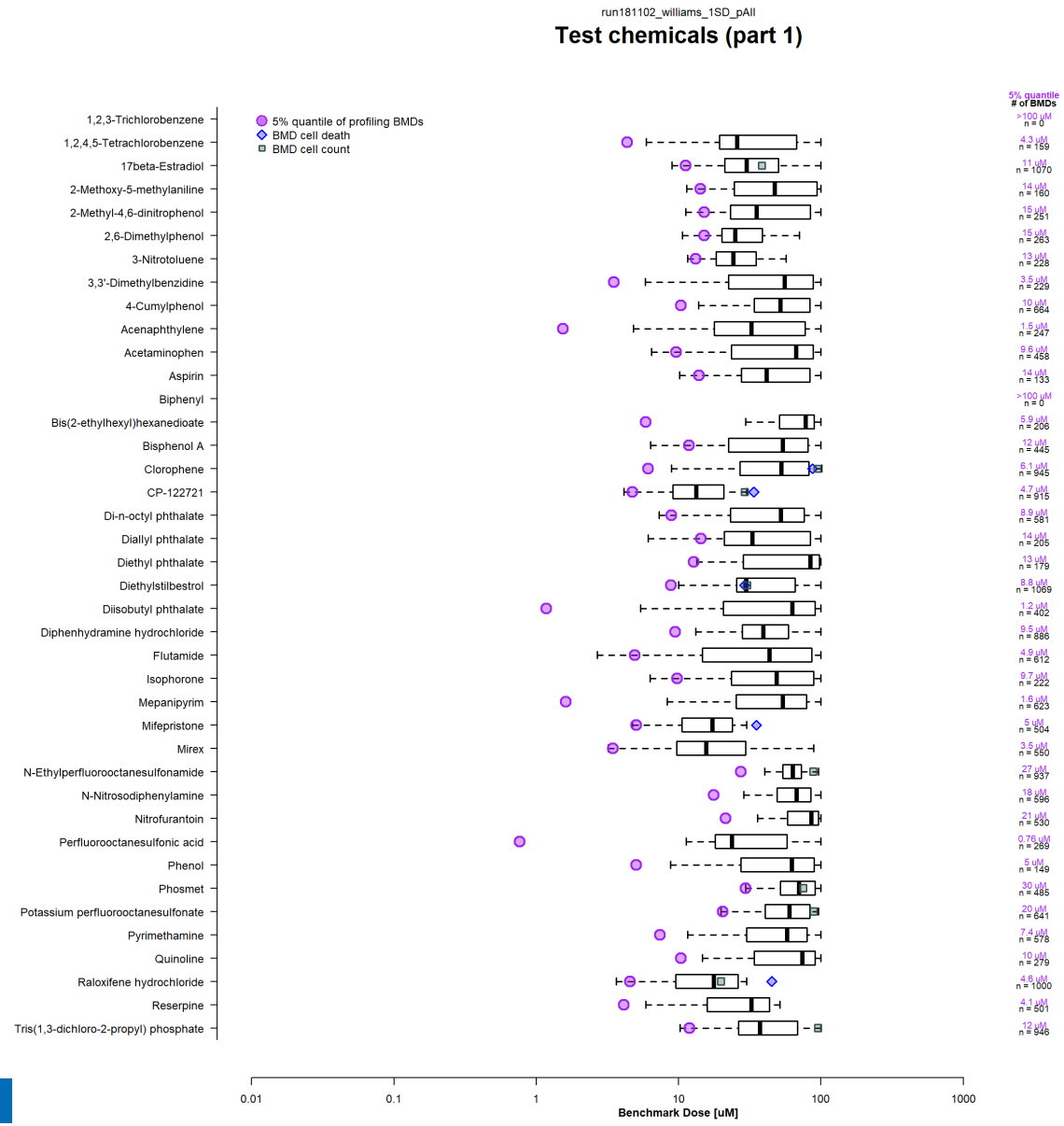
EPA United States Environmental Protection Agency

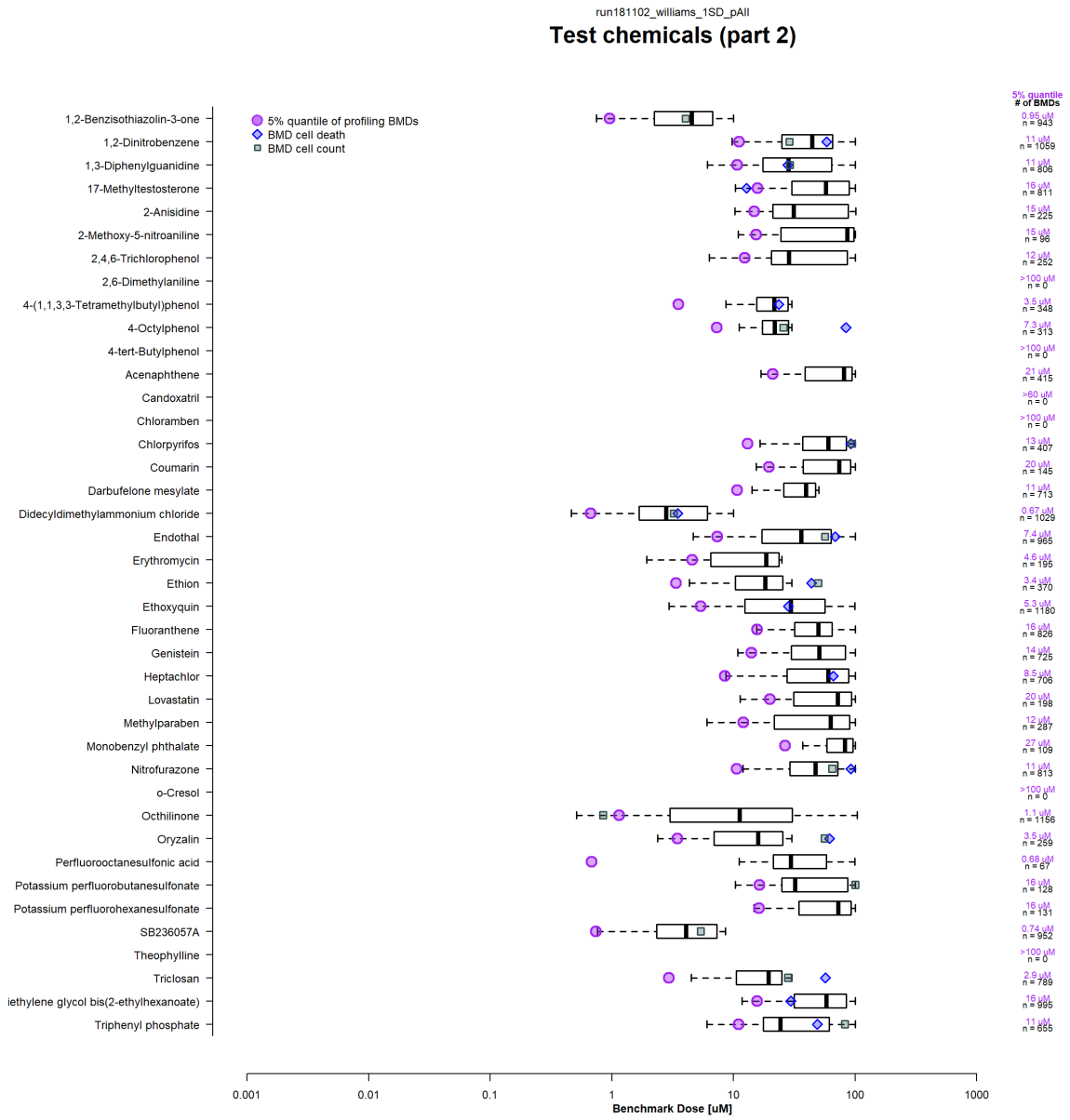
Phase 2: Pilot Screen
Experimental Design

Parameter	Multiplier	Notes	
Cell Type(s)	1	U-2 OS [a]	Bone
Culture Condition	1	DMEM + 10% HI-FBS	
Chemicals	80	Selected from ToxCast HTTK parameters	
Time Points:	1	48 hours	
Assay Formats:	2	Cell Painting HCI Cell Viability & Apoptosis	
Concentrations:	8	3.5 $\log_{10}$ units; semi $\log_{10}$ spacing	
Biological Replicates:	3	--	

[a] Reference cell line (Bray et al. 2016).

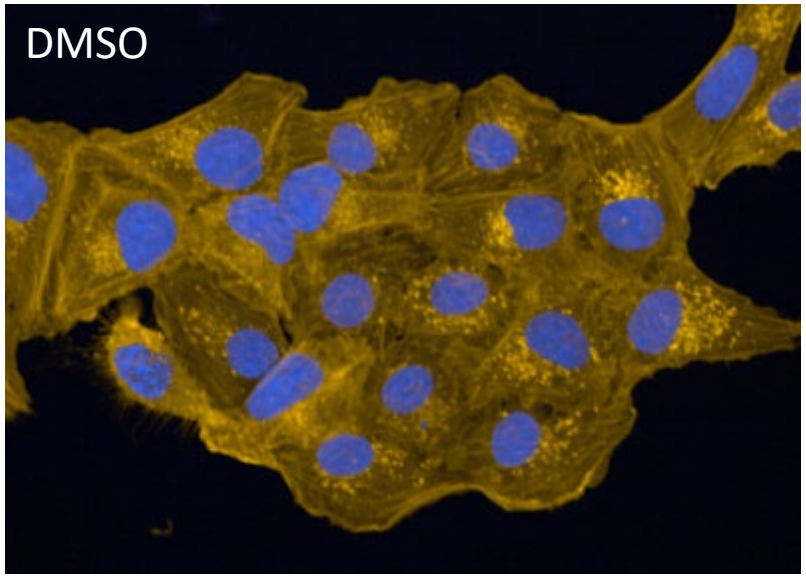
HTPP-Derived BPACs, ToxCast Chemicals

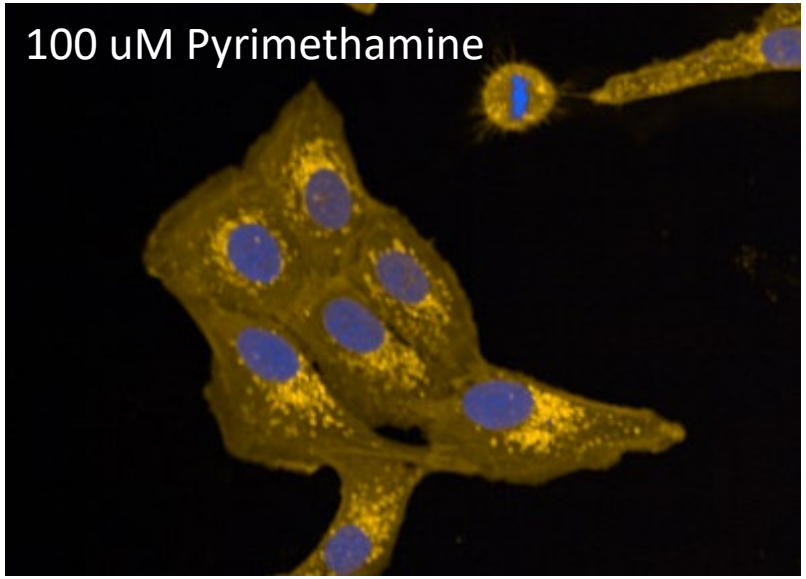




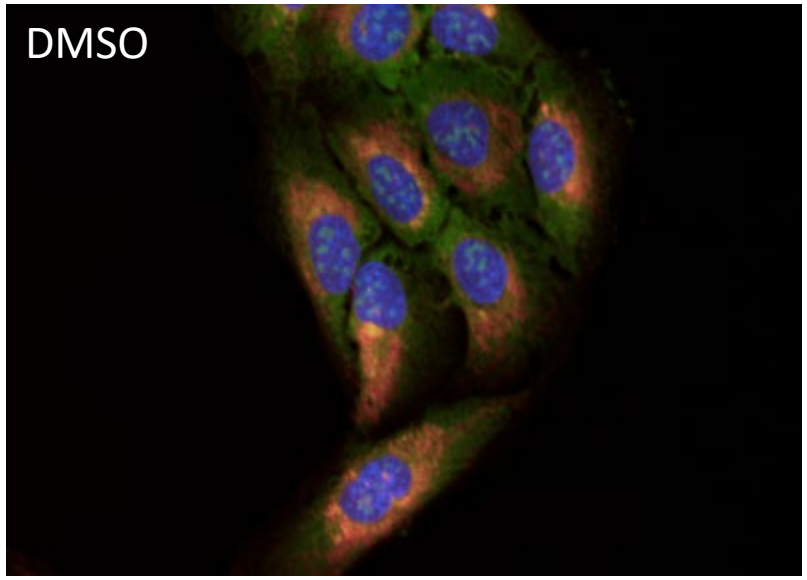
Examples of Morphological Phenotypes

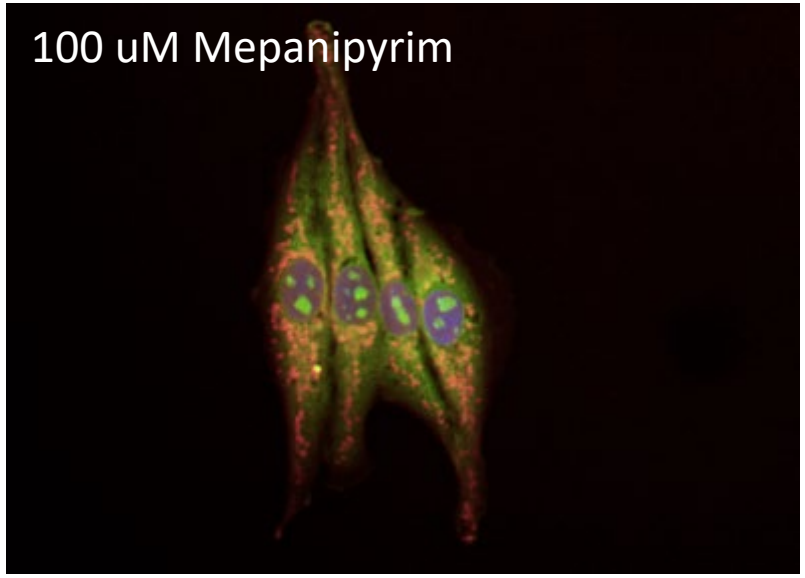
Hoechst / AGP



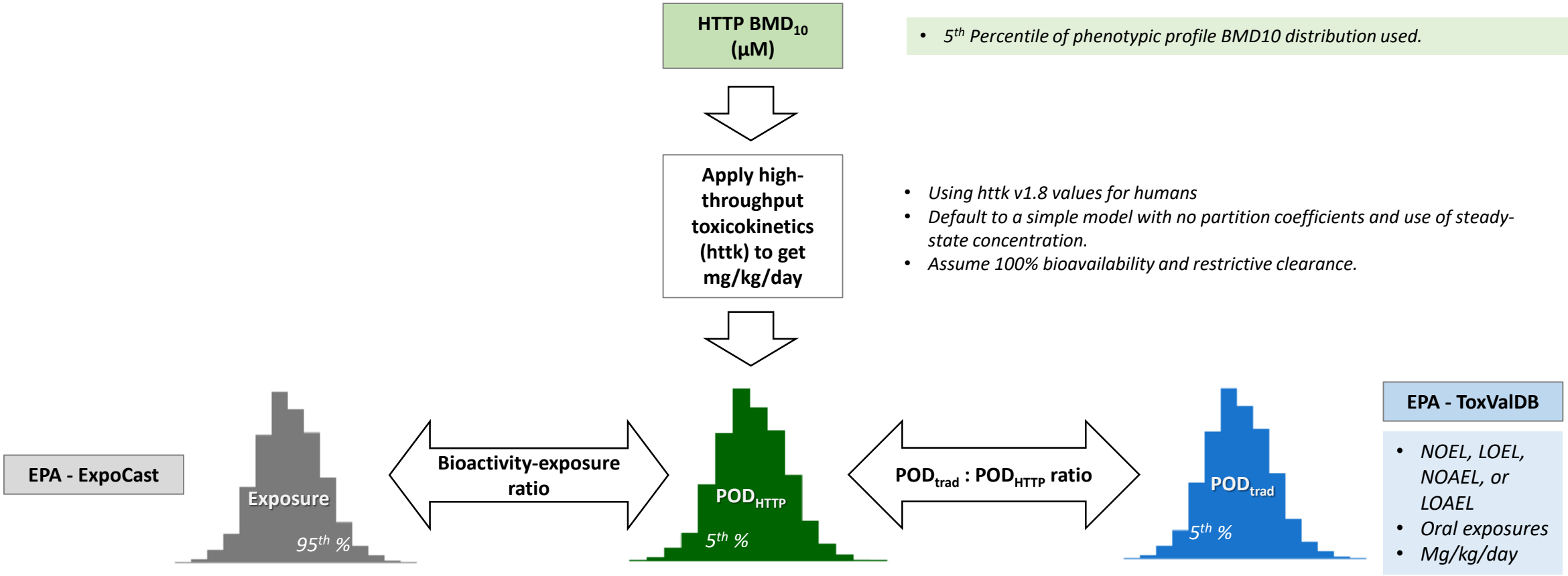


Hoechst / ER / Mito





Bioactivity & Exposure Ratio Comparisons Using Reverse Dosimetry



- *5th Percentile of phenotypic profile BMD10 distribution used.*

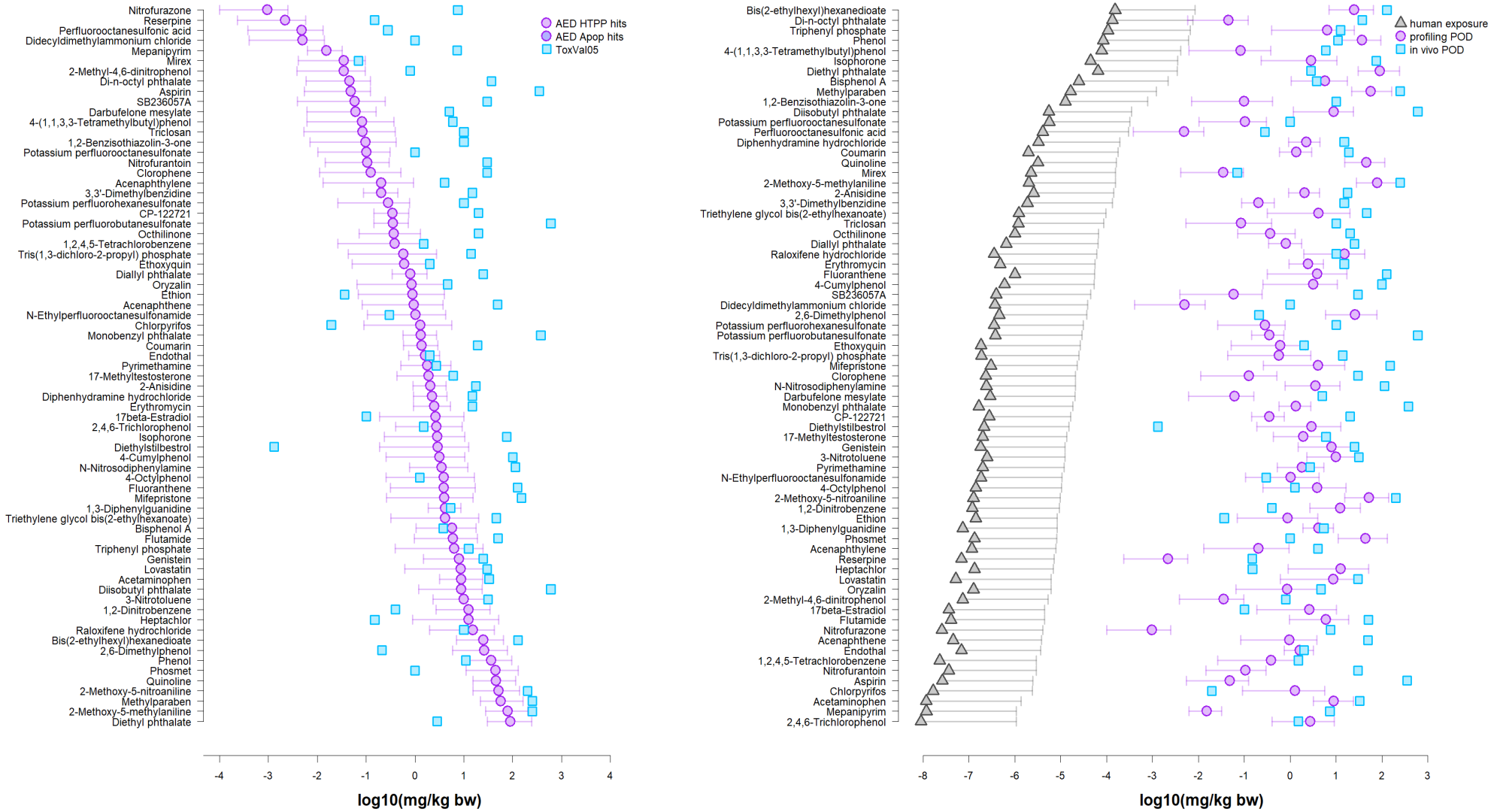
- *Using httk v1.8 values for humans*
- *Default to a simple model with no partition coefficients and use of steady-state concentration.*
- *Assume 100% bioavailability and restrictive clearance.*

- *NOEL, LOEL, NOAEL, or LOAEL*
- *Oral exposures*
- *Mg/kg/day*

- **Reverse dosimetry:** Conversion of a bioactivity value to an *in vivo* steady state concentration using high-throughput toxicokinetic (httk) modeling.
- Facilitates comparisons of biologically active in vitro concentrations to predicted human exposures and/or points-of-departure (PODs) from *in vivo* toxicology studies

Figure Courtesy of Katie Paul-Friedman

BER Preliminary Results



- AEDs for most HTPP BPACs are similar to or below *in vivo* PODs.
- Clear separation between HTPP BPACs and predicted human AEDs.

HTPP Summary

- **Workflow:** Developed a microfluidics-based laboratory workflow for cell plating, chemical screening and fluorescent labeling of cells for measurements of organelle morphology.

- **Concentration-Response Analysis:** Developed a high content image analysis workflow (Harmony) and data analysis pipeline that incorporates concentration-response modeling (R & BMDExpress 2.2).

- **Reference Chemicals:** Replicated profiles described in previous publications and identified candidate chemicals for use as reference controls for screening applications.

- **Sensitivity:** Effects on cell morphology were often observed at concentrations well below the threshold for cytotoxicity both with reference chemicals and a subset of the ToxCast library.

- **Chemical Space:** Screening of 80 ToxCast chemicals in U-2 OS cells produced ~90% hit rate. Comparison with ToxCast and HTTr data indicates that there is a positive association between the number of ToxCast assays affected by a chemical, the number of transcripts affected by a chemical and the number of morphological features affected in HTPP.

NCCT HTTr Project Team

National Center for Computational Toxicology

Joshua
Harrill
Toxicologist

Clinton
Willis
NSSC (JH)

Imran
Shah
Computational Systems Biologist

R. Woodrow
Setzer
Mathematical Statistician

Derik
Haggard
ORISE Fellow

Richard
Judson
Bioinformatician

Russell
Thomas
Director

Acknowledgments

HTTr Project Team:
Imran Shah
Woody Setzer
Derik Haggard
Matt Martin*
Richard Judson
Rusty Thomas

NCCT Labs:
Johanna Nyffeler
Clinton Willis

Unilever:
Paul Carmichael
Andy White
Sophie Malcomber
Richard Stark*

National Toxicology Program:
Scott Auerbach

Sciome:
Jason Phillips

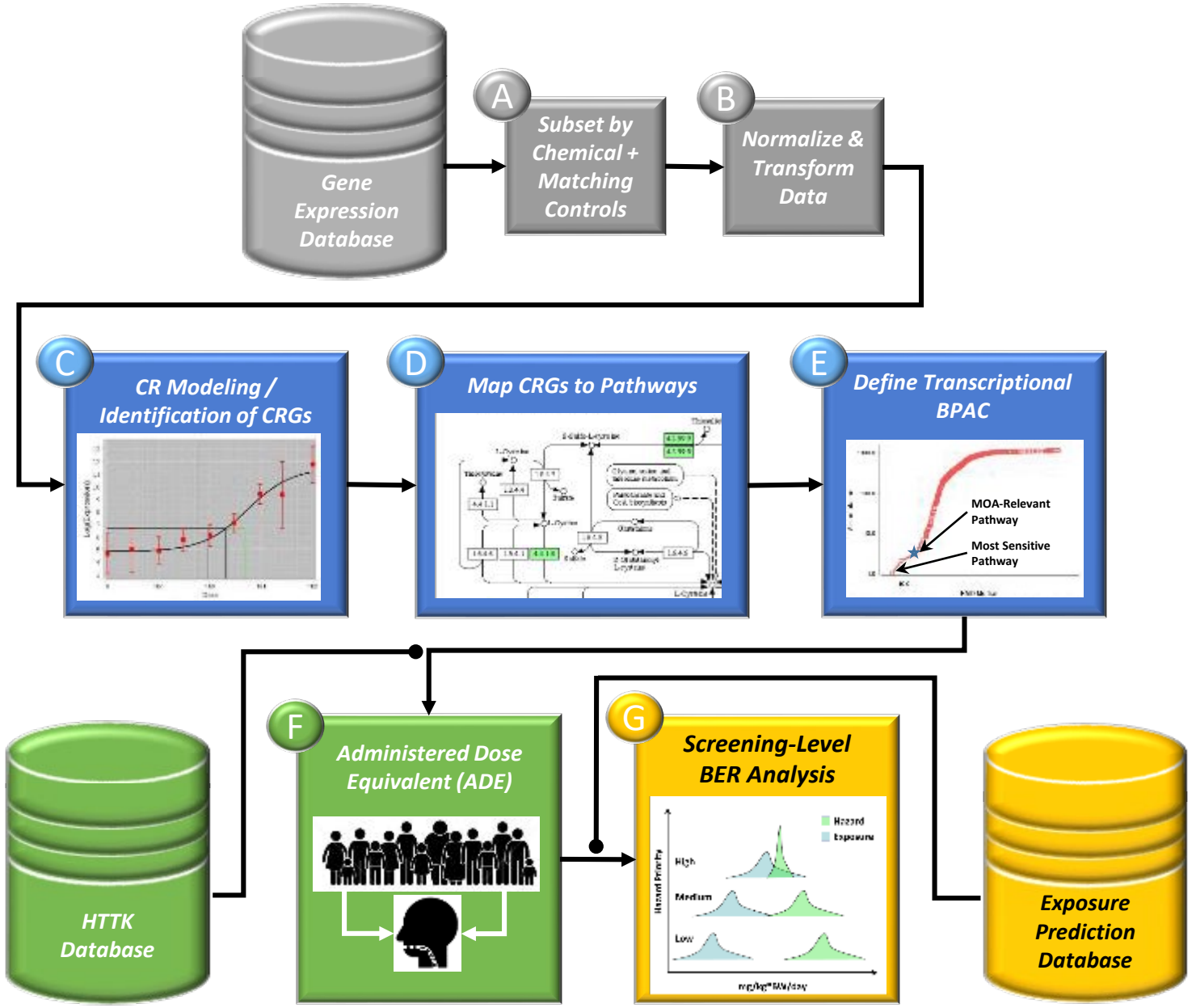
BioSpyder
Joel McComb
Bruce Seligmann
Jo Yeakley
Milos Babic
Pete Shepard
Kyle LeBlanc
Jason Downing

National Center for Computational Toxicology

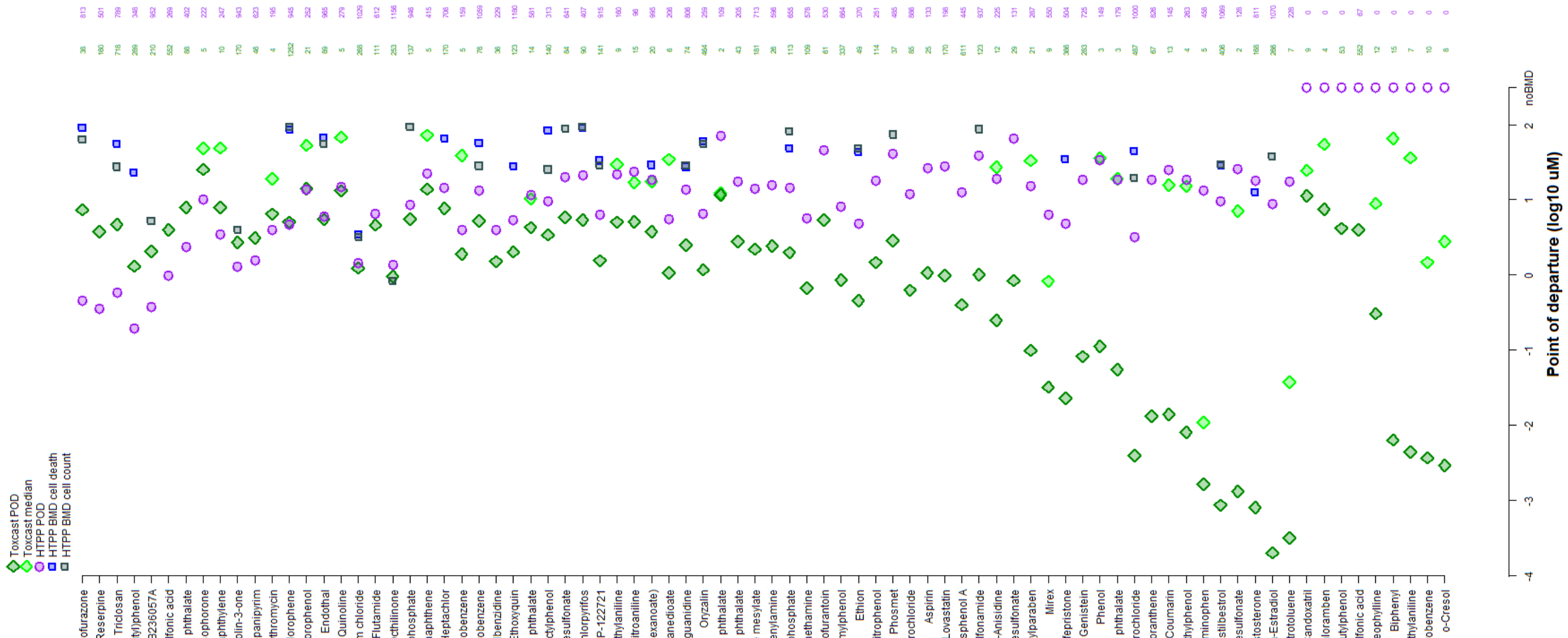
EPA United States Environmental Protection Agency

BONUS SLIDES

Potential Application for HTTr in Screening Level BER Analysis

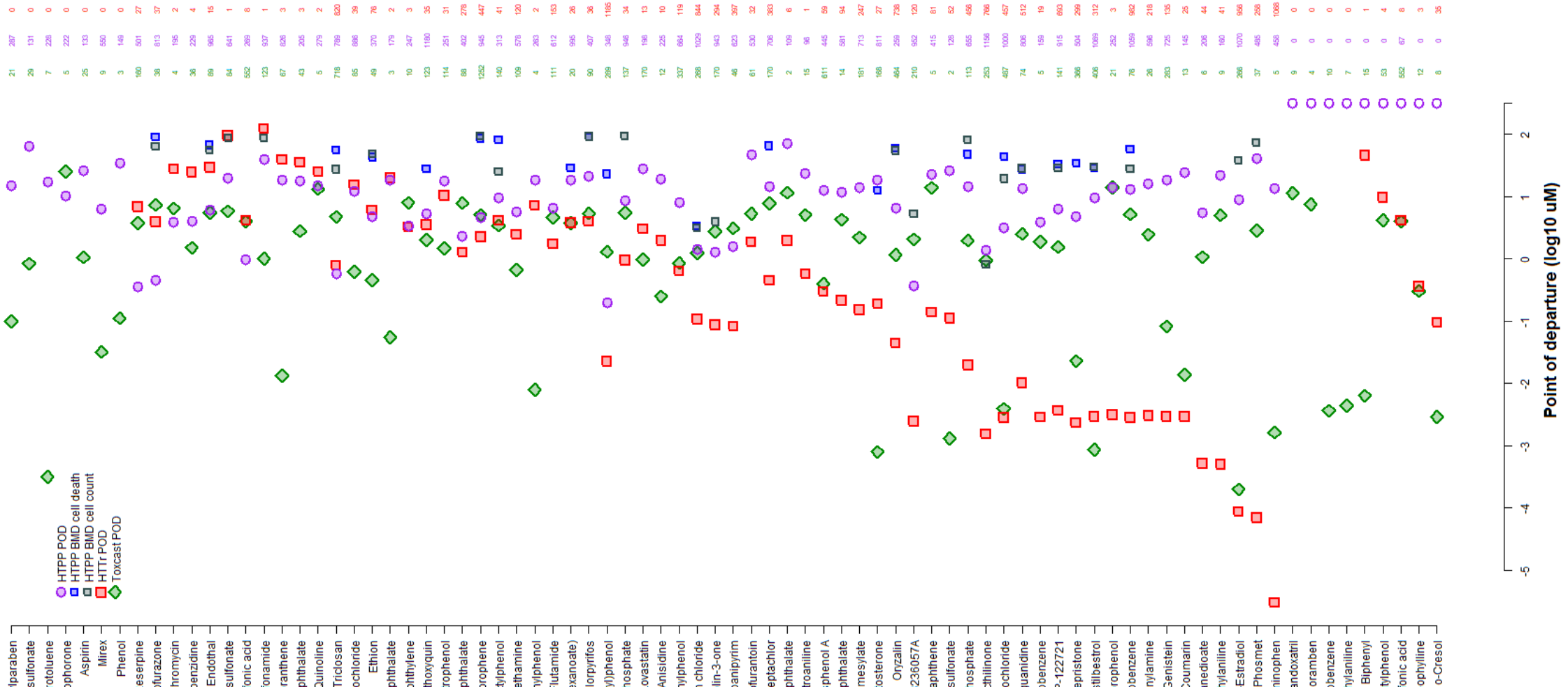


Preliminary Comparison with ToxCast Data



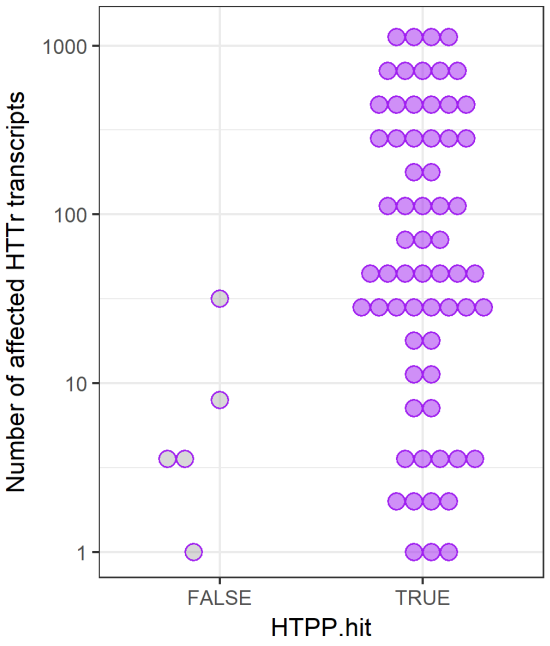
Preliminary Data – DO NOT CITE OR QUOTE

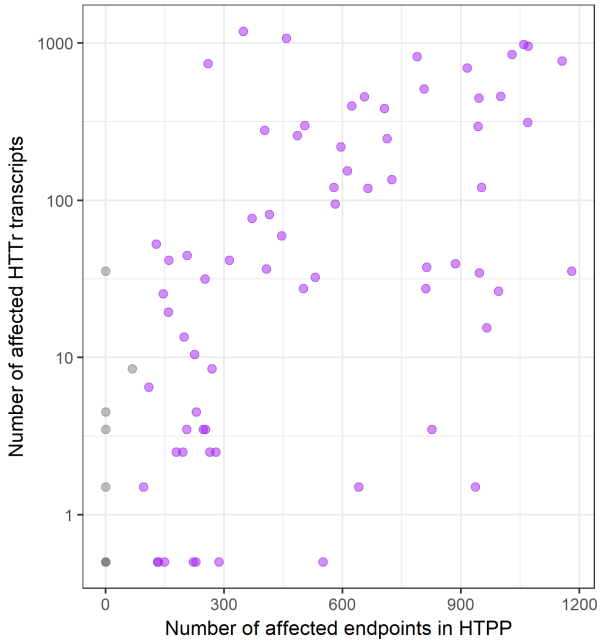
Preliminary Comparison with HTTr Results

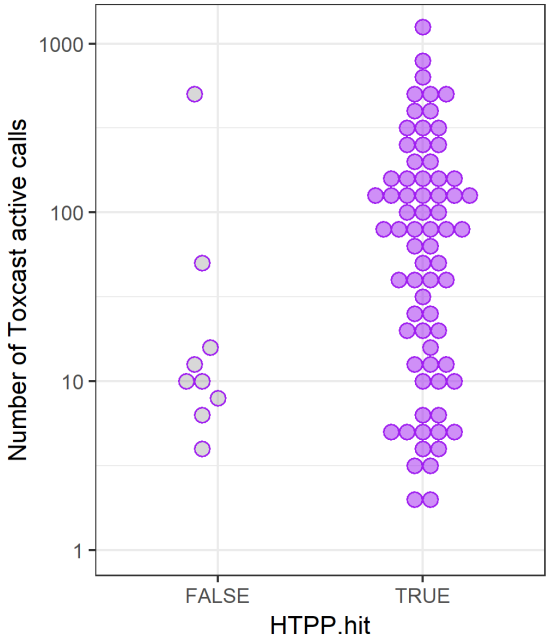


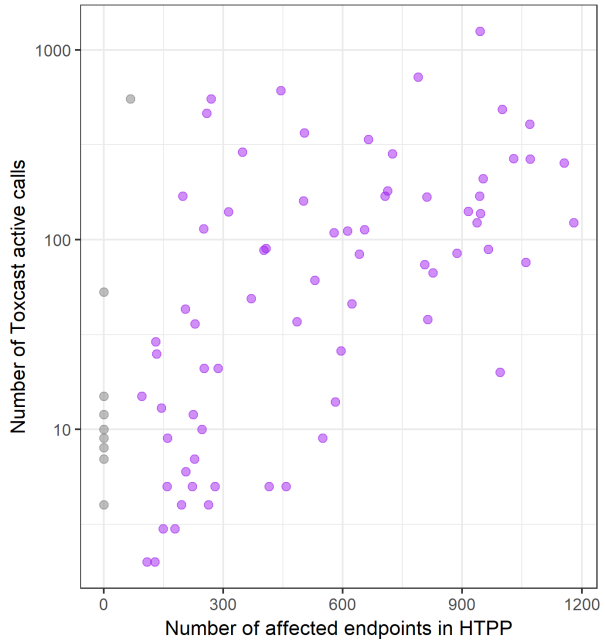
Preliminary Data – DO NOT CITE OR QUOTE

Correlation Between HTPP, HTTr and ToxCast









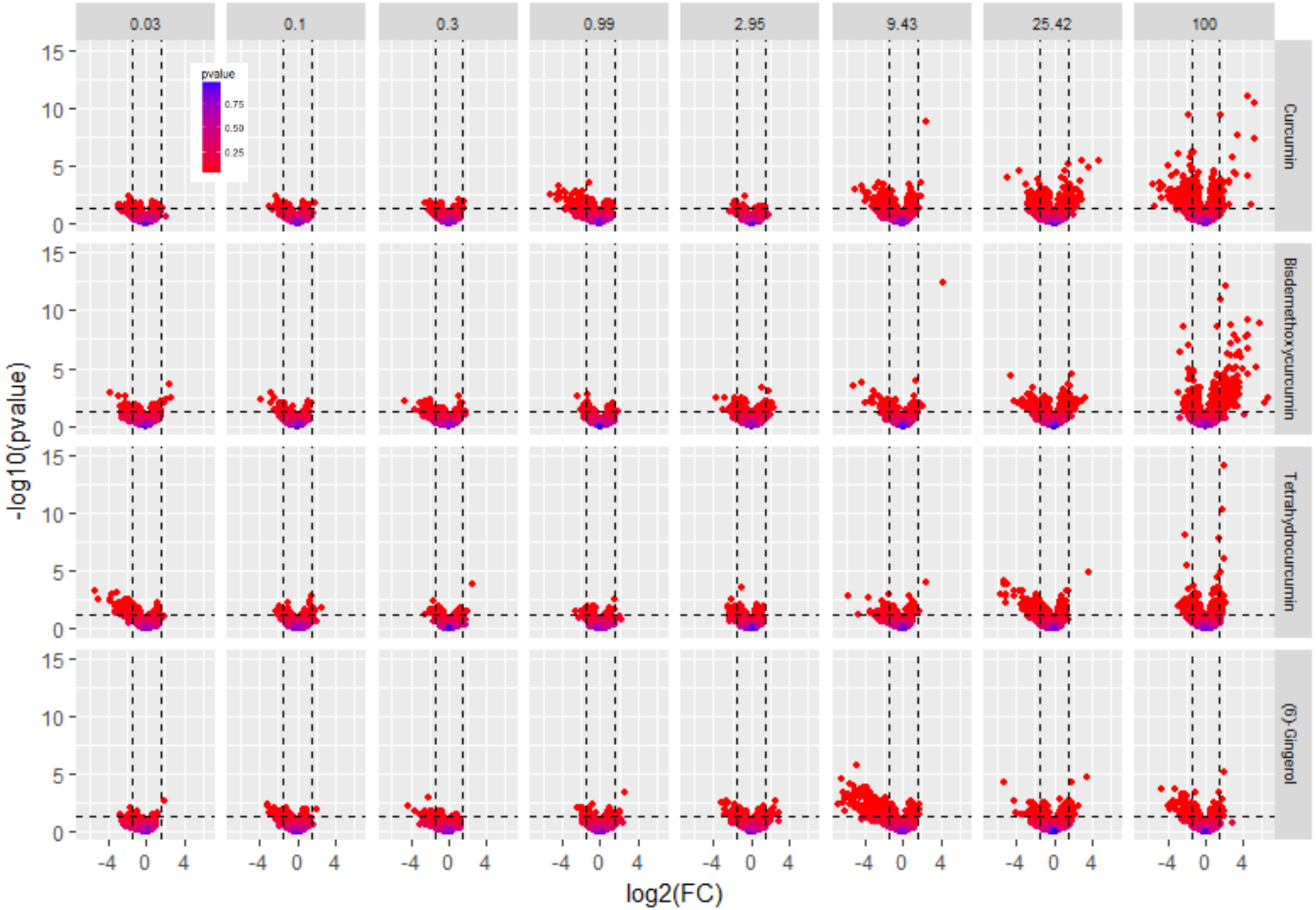
- There a positive correlation between the number of ToxCast hits, the number of transcripts affected in HTTr and the number of morphological endpoints affected by HTPP.
- Chemicals with apparent mechanistic promiscuity have a high number of hits in each data type.

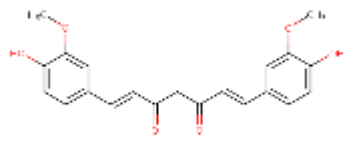
EPA United States Environmental Protection Agency

Searching MSigDB Using ER Signature

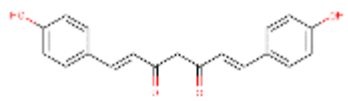
category_code	jaccard	olap	organism	standard_name
H	0.127329193	41	human	HALLMARK_ESTROGEN_RESPONSE_EARLY
C2	0.094972067	34	human	DUTERTRE_ESTRADIOL_RESPONSE_6HR_UP
H	0.093373494	31	human	HALLMARK_ESTROGEN_RESPONSE_LATE
C2	0.087378641	18	human	MASSARWEH_RESPONSE_TO_ESTRADIOL
C2	0.083067093	26	human	NAGASHIMA_NRG1_SIGNALING_UP
C2	0.07826087	18	human	STEIN_ESR1_TARGETS
H	0.077151335	26	human	HALLMARK_TNFA_SIGNALING_VIA_NFKB
C2	0.076666667	23	human	PHONG_TNF_RESPONSE_VIA_P38_PARTIAL
C6	0.075301205	25	human	RAF_UP.V1_DN
C2	0.074712644	26	human	BHAT_ESR1_TARGETS_NOT_VIA_AKT1_UP
C2	0.07266436	21	human	PODAR_RESPONSE_TO_ADAPHOSTIN_UP
C2	0.069686411	20	human	BROCKE_APOPTOSIS_REVERSED_BY_IL6
C6	0.068452381	23	human	LTE2_UP.V1_DN
C2	0.065822785	26	human	MASSARWEH_TAMOXIFEN_RESISTANCE_DN
C2	0.065759637	29	human	CREIGHTON_ENDOCRINE_THERAPY_RESISTANCE_4
C2	0.064748201	27	human	BHAT_ESR1_TARGETS_VIA_AKT1_UP
C2	0.063829787	12	human	FRASOR_RESPONSE_TO_ESTRADIOL_UP
C2	0.0625	13	human	NAGASHIMA_EGF_SIGNALING_UP
C2	0.06031746	19	human	ELVIDGE_HYPOXIA_UP
C2	0.059259259	16	human	KAN_RESPONSE_TO_ARSENIC_TRIOXIDE

Evaluating Structurally Related Chemicals

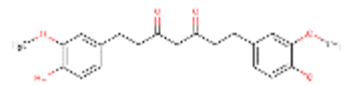




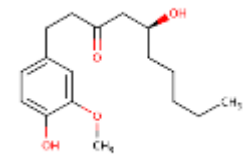
Curcumin | DTXSID8031077



Bisdemethoxycurcumin | DTXSID00872663

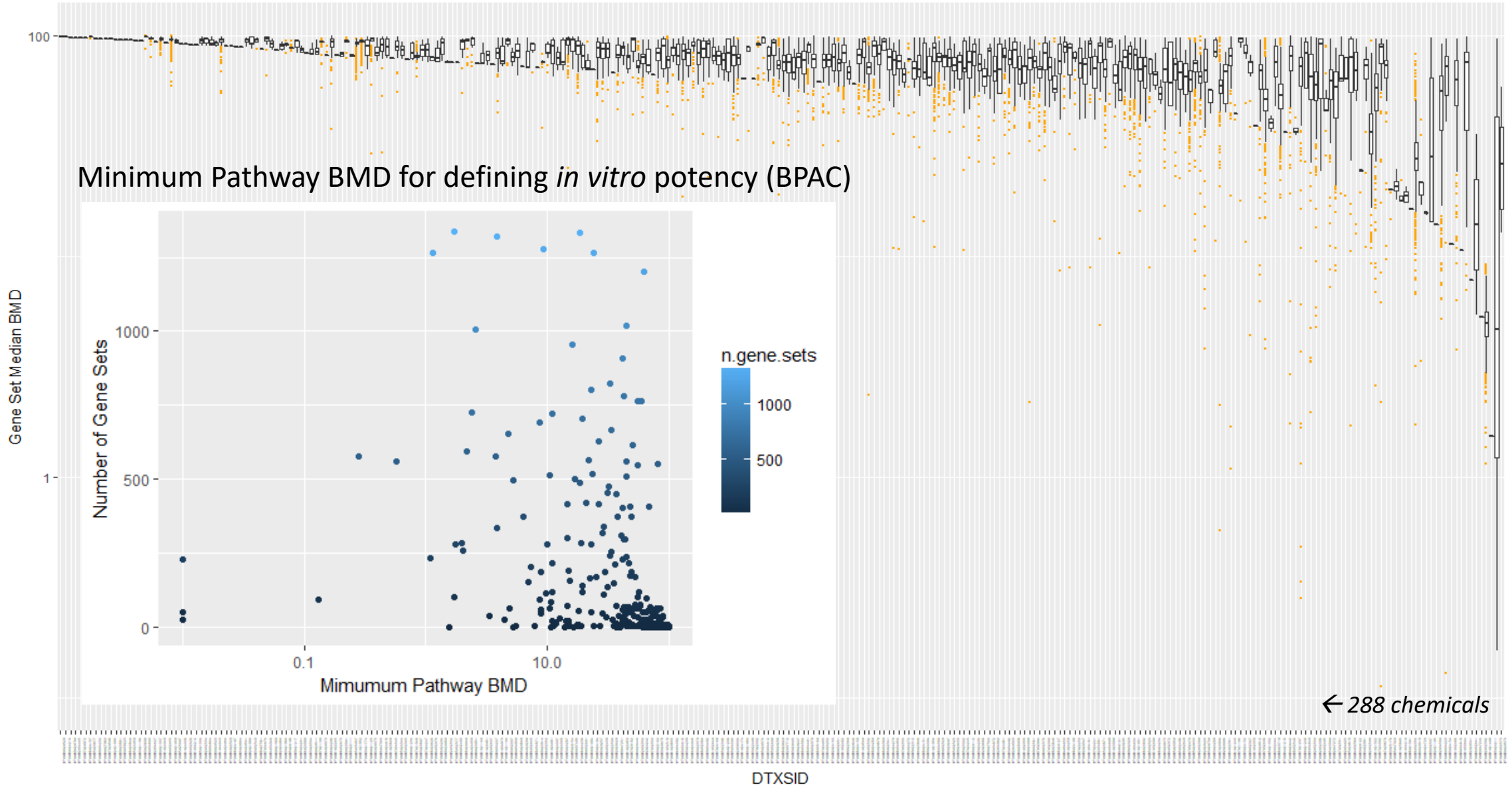


Tetrahydrocurcumin | DTXSID30865801



(6)-Gingerol | DTXSID3041035

Gene Set Analysis Summary (1)



EPA United States Environmental Protection Agency

Searching ER Signature against MCF-7 TempO-Seq Data

Most genistein (reference chemical) Profiles match the signature

Many hits with chemicals that have Not been curated

target	chem	conc	jaccard	n_d	n_u	ola
ABCB1|ESR2|CYP3A4|ESR1	Tamoxifen	50	0.0582386	300	300	41
Other	Dehydroepiandrosterone	25.42373	0.056338	300	300	40
Other	HMR1171 trifluoroacetate (1:1)	35	0.0548523	300	300	39
Other	SSR146977	25	0.0546218	300	300	39
ESR2|ESR1	Fulvestrant	0.014967	0.0527778	300	300	38
KCNH2|HRH1	Astemizole	25.42373	0.0518934	300	300	37
Other	Estradiol cypionate	0.299461	0.0516039	300	300	37
ESR2|ESR1	Fulvestrant	0.04997	0.0516039	300	300	37
NR3C2|NR3C1|NR1I2|ABCB1	Dexamethasone	25.42373	0.0511757	300	300	37
Other	Niclosamide	12.71186	0.0504909	300	300	36
ESR2|ESR1	Fulvestrant	1.473477	0.0499307	300	300	36
Other	dl-Norgestrel	9.433962	0.0499307	300	300	36
ALOX15|ALOX5|ALOX12	Nordihydroguaiaretic acid	25.42373	0.0498615	300	300	36
Other	SSR 240612	11.44068	0.0497238	300	300	36
Other	Diethylstilbestrol dipropionate	25.42373	0.0495868	300	300	36
Other	SSR69071	25	0.0492264	300	300	35
Other	SSR 240612	45	0.0486787	300	300	35
ESR2|ESR1	Fulvestrant	12.71186	0.0486787	300	300	35
ESR2|ESR1	Fulvestrant	4.716981	0.0486111	300	300	35
Other	Disulfiram	25.42373	0.0476858	300	300	34
ESR1	Raloxifene hydrochloride	25.42373	0.0469613	300	300	34
Other	Cyclosporin A	9.433962	0.0469613	300	300	34
ESR2|ESR1	Fulvestrant	0.149731	0.0469613	300	300	34
Other	Emamectin benzoate	25.42373	0.0464135	300	300	33
Other	Fluorometholone	0.299461	0.0460251	300	300	33
Other	Fluorometholone	2.946955	0.0458971	300	300	33
AHR	Benzo(k)fluoranthene	9.433962	0.0458333	300	300	33
Other	Flurandrenolide	25.42373	0.0457064	300	300	33
Other	Hydramethylnon	25.42373	0.0453297	300	300	33
Other	Methyl Violet	25.42373	0.0450704	300	300	32
Other	Antimony trichloride	25.42373	0.0448808	300	300	32
Other	Rhodamine 6G	2.946955	0.0448179	300	300	32
Other	Sodium (2-pyridylthio)-N-oxide	25.42373	0.0448179	300	300	32
Other	Tributyltin chloride	2.946955	0.0446927	300	300	32
Other	1-Chloro-2,4-dinitrobenzene	25.42373	0.0445682	300	300	32
NR3C1	Methylprednisolone	0.09994	0.0443828	300	300	32
Other	Dibenz(a,h)anthracene	8.018868	0.0443213	300	300	32
Other	Cyclosporin A	25.42373	0.0443213	300	300	32
ESRRG|ESRRB|ESR2|ESR1	Diethylstilbestrol	0.029935	0.04426	300	300	32
ESR2|ESR1	Fulvestrant	50	0.0441989	300	300	32