High-throughput In Vitro Assays at NCATS

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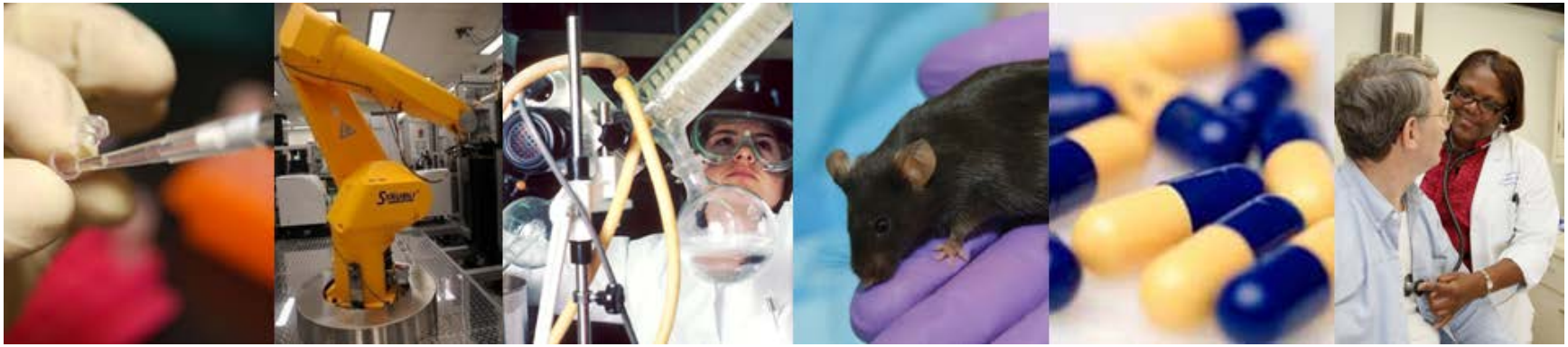
NCATS

National Center for Advancing Translational Sciences

Alternative Approaches for Identifying Acute Systemic Toxicity:
Moving from Research to Regulatory Testing
Bethesda, MD, September 24-25, 2015

Mission of National Center for Advancing Translational Sciences (NCATS)



To catalyze the generation of innovative methods and technologies that will enhance the development, testing and implementation of diagnostics and therapeutics across a wide range of human diseases and conditions.

NIH Chemical Genomics Center (NCGC)

- **Established in 2004**
  - •Part of NIH Roadmap Molecular Libraries Initiative
  - •**National Center for Advancing Translational Sciences** (NCATS, established in Dec, 2011)
  - •>100 staff: Biologists, Chemists, Informatics and Engineers
- **Mission and Robotic HTS facility**
  - •High throughput screening (HTS) to identify lead compounds for therapeutic development and to profile compound libraries including environmental chemicals for their biological and pathophysiological effects
  - •New technologies/paradigms for assay development, screening, informatics, chemistry
  - •Advanced screening robots (capacity: > 0.5 million samples/day) and Compound libraries (high quality with diverse structures)
- **Collaborations**
  - •>200 investigators worldwide

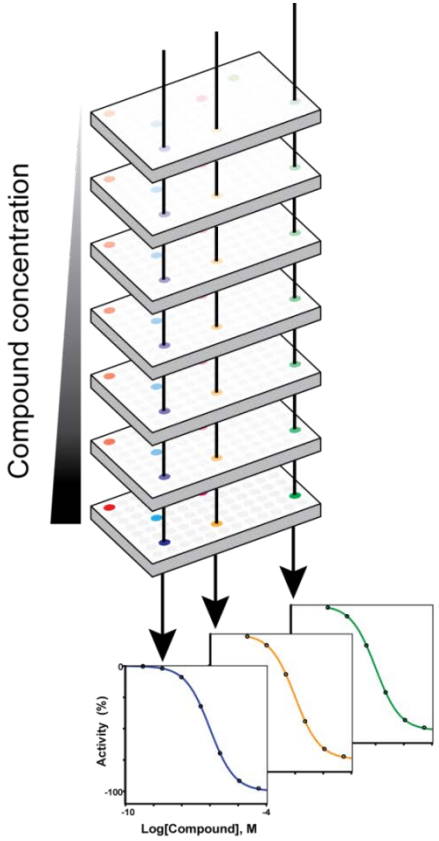
(75% NIH extramural, 10% NIH intramural, 15% Foundations/Research Consortia/Pharma/Biotech)

The Tox21 Community

Goals:

- Identify mechanisms of compound action
- Prioritize chemicals for further in-depth toxicological evaluation
- Develop predictive models for biological response in humans

Quantitative High-Throughput Screening (qHTS)



- Conventional screening done at one concentration
  - Not appropriate for toxicity testing – "dose makes the poison"
- qHTS tests compounds assayed at **multiple** concentrations
  - For Tox21, 15 concentrations over 4 logs (high:~ 100 uM)
- Miniaturized assay volumes 2-8 uL in 1536-well plate
- Informatics pipeline for data processing, curve fitting & classification, extraction of SAR
- Generates *toxicological actives* rather than statistical "hits"
  - Dramatically increases reliability
  - Dramatically reduces false positives and false negatives

Assay Selection and Design – Formats Utilized in HTS

- **Assays selection based on**
  - Biological and toxicological relevance
  - Adaptable to miniaturization and automated screening (a 384 or 1536 well plate format)
- **Readouts** (Detectors – ViewLux, Envision, ImageXpress, ArrayScan)
  - Fluorescence, luminescence, absorbance, no radioactivity
- **Cell types**
  - Mammalian cells
    - Primary cells
    - Embryonic stem cells and IPS derived cells
    - **Established cell lines**
    - **Engineered cells**
      - transiently/stably transfected
      - retroviral infected
  - Chicken cells
  - Bacterial cells and insect cells

EnVision Multilabel Reader

- Absorbance
- Fluorescence
- F.P.
- Luminescence
- TR-FRET
- AlphaScreen
- Top/Bottom reading

ViewLux Multilabel Reader

- **Absorbance**
- **Fluorescence**
- **F.P.**
- **Luminescence**
- **TR-FRET**
- **Top reading**

Molecular Devices ImageXpress Micro

- **HCS**
- **Bottom reading**
- **Slow in speed (20 min - 1 hr/plate)**

Thermo Fisher, ArrayScan

Tox21 Phase I: Assays and Screens

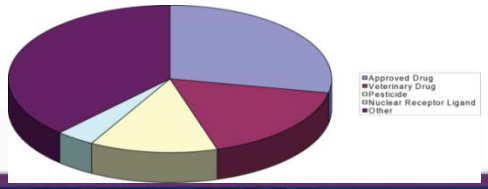
Phenotypic readouts

- Cytotoxicity assays
  - Cell viability assay (measures ATP)
- Apoptosis assays
  - Caspase assays (measure activity of Caspase 3/7, 8, 9)
- Membrane integrity assay
  - LDH and protease release
- Mitochondrial Toxicity assay
  - Mitochondrial membrane potential
- Gene tox assay
  - Differential cytotoxicityDifferential cytotoxicity (DNA damage repair gene deficient lines, DT40 and mouse cell lines)
  - ATAD5 (ELG1)
  - Micronucleus assay
- Phosphlipodosis assay
- Genetic variation: 87 HapMap lines

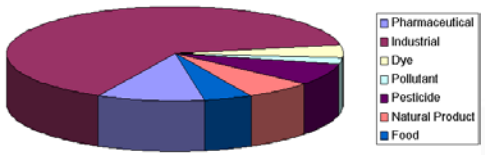
- Pathway-specific toxicological mechanisms
  - Pathway assays (Reporters, e.g., luciferase, β-lactamase): Hypoxia, ER stress, NFkB, P53, ARE, HSE, CREB, AP-1, STAT, NFAT
- Target-specific toxicological mechanisms
  - Nuclear receptor assays: AR, AhR, ERα, FXR, GR, LXR, PPARδ, PPARγ, PXR, RXR, TRβ, VDR, RORα
  - hERG channel assay
  - Cytokine assays: IL-8, TNFα
  - Ca mobilization and influx assays

- Phase I compound collection: 5632 chemicals
  - NTP-1408 A
  - EPA-1408 A
  - NCGC Pharmaceutical Collection (NPC-2816) - Drugs approved in US, EU, Canada, Japan, includes withdrawn

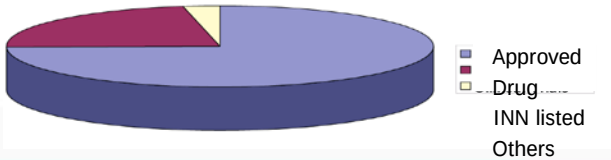
EPA-1408 A



NTP-1408 A



NPC - 2816



Tox21 Phase II: qHTS Screening

- Initial focus
  - Nuclear receptor activation or inhibition (AR, AhR, ER, FXR, GR, LXR, PPAR, PXR, RXR, TR, VDR, ROR)
  - Induction of stress response pathways (e.g., DNA damage, heat shock, hypoxia, inflammation, oxidative)
- Online validation
  - LOPAC + 88 Tox21 compounds
  - Screening 3 times
  - Assay reproducibility and general statistics
- Online screening
  - Three sets of 10k compound collection and each compound in different location of the plate
  - 15 concentrations for each compound, 459 plates per screen
- Compound stability test
  - Chemical QC

Tox21 Phase II - Tox21 Compounds

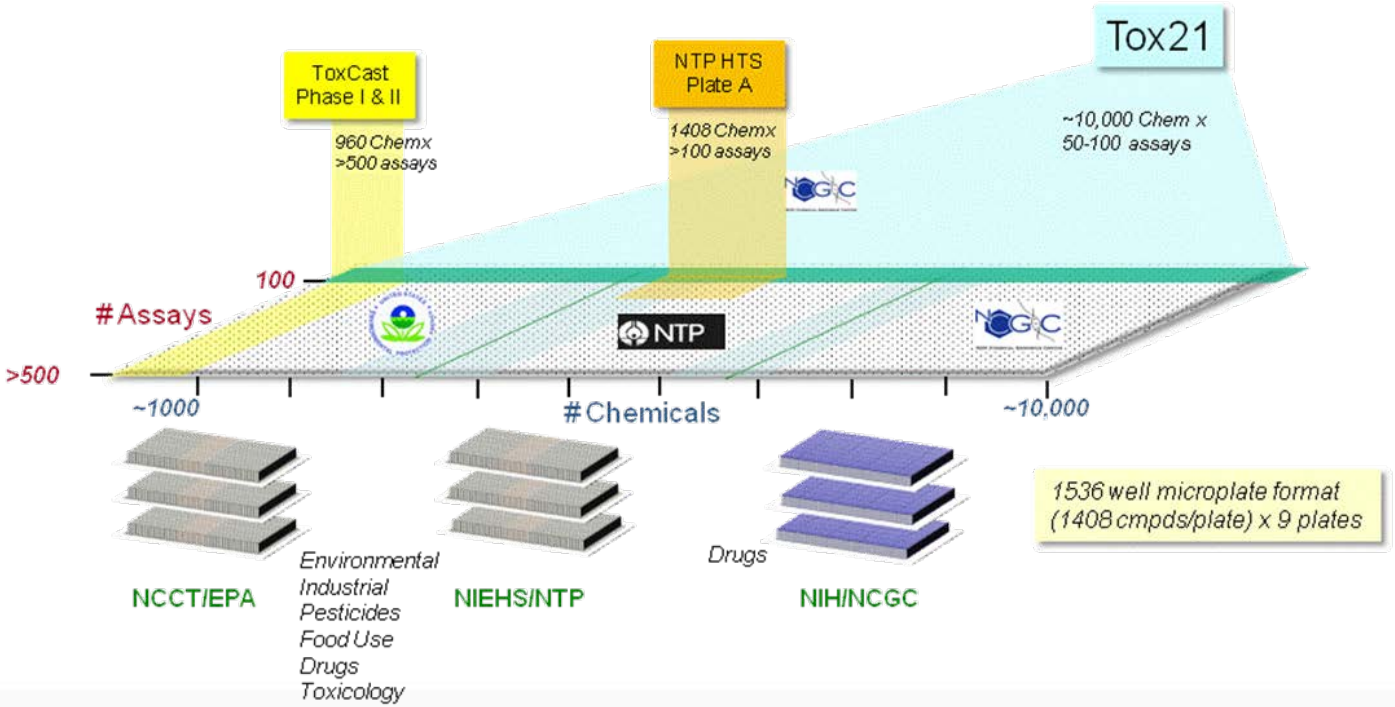
~10,000 compounds in total

Industrial chemicals, sunscreen additives, flame retardants, pesticide additives and their metabolites, plasticizers, solvents, food additives, natural product components, drinking water disinfection byproducts, preservatives, therapeutic agents, synthesis byproducts

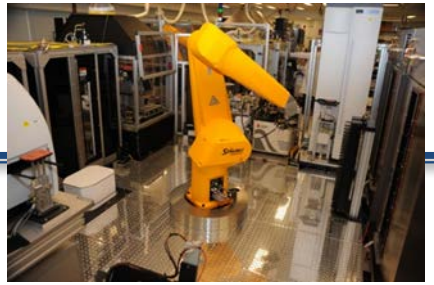
88 duplicate compounds in each plate

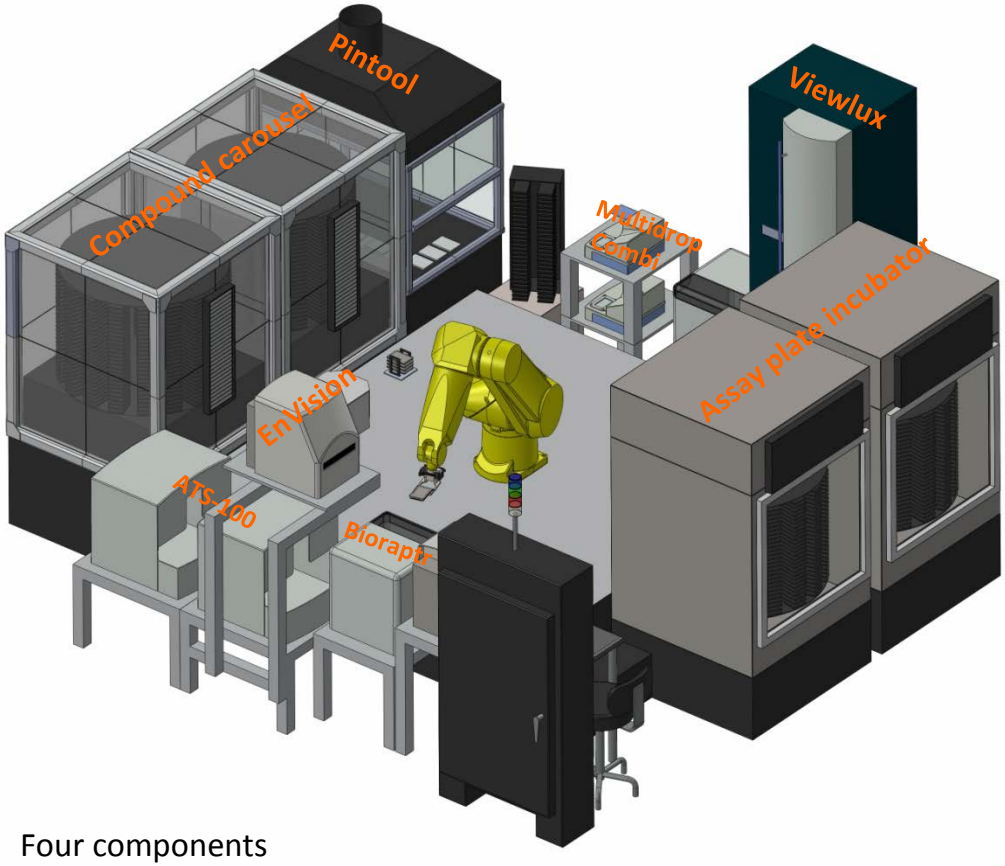
Three sets of this compound collection

All the compounds in different location of the plate



Tox21 Robot System





ViewLux Multilabel Reader

- Absorbance
- Fluorescence
- F.P.
- Luminescence
- TR-FRET
- Top reading

EnVision Multilabel Reader

- Absorbance
- Fluorescence
- F.P.
- Luminescence
- TR-FRET
- AlphaScreen
- Top/Bottom reading

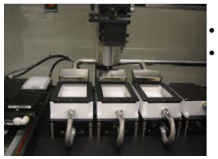
BioRAPTR FRD Workstation

- Transfer size: 0.2 - 10 ul
- 0.5 ml dead volume
- 4 reagents

Multidrop Combi

- Transfer size: 2 - 10 u
- 10 ml dead volume
- 1 reagent

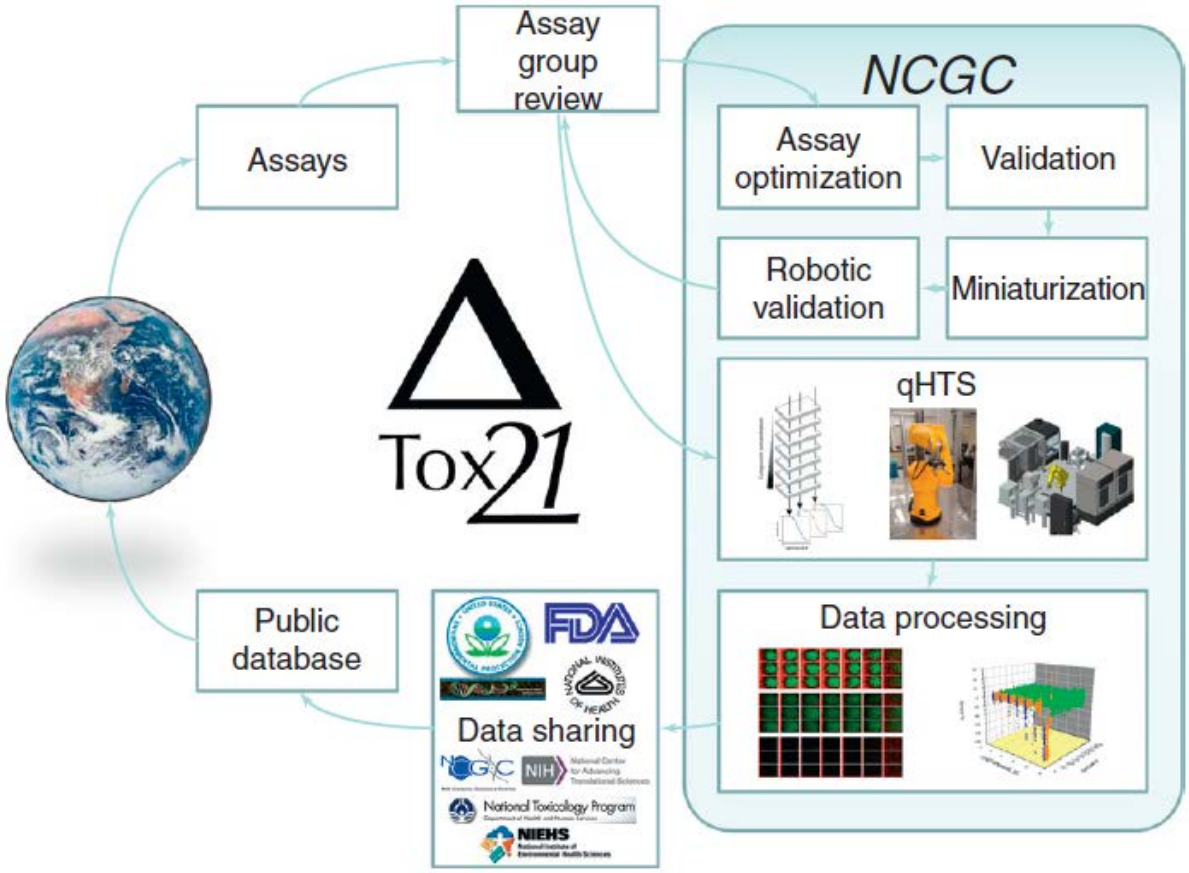
Pintool Station



- Transfer size: 20 nl
- Pins washed in 3 solvents

Four components
(1) Cell incubators & compound storage
(2) Liquid handling: Pintool station and acoustic dispenser for nanoliter compound transfer, and reagent dispensers (BioRPTR, Multidrop)
(3) Plate detection: ViewLux and EnVision plate readers
(4) Robot arms & software controlling system

Tox21 Screening Process



Validation

- Positive controls
- Time course
- Signal to background

Miniaturization

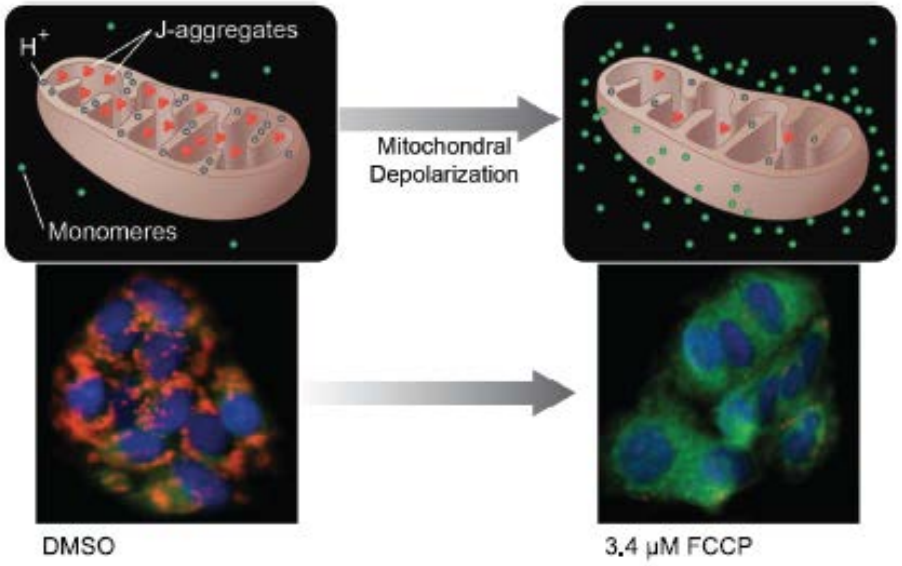
- Cell density per well
- Positive controls
- Signal to background ≥ 3
- CV <10%
- Z factor > 0.5

- CV (coefficient of variation) = standard deviation (SD) of compound area/median of compound area
- Z factor = 1-[3*(SD of compound area + SD of basal)/(median of compound area – median of basal)]

Attene-Ramos et al., 2013, Drug Discovery Today 18:716-723

Case Study: Screening for Environmental Chemicals that Decrease MMP using qHTS

- To screen and identify chemical compounds that decrease mitochondrial membrane potential (MMP)
- Prioritization of actives for further in-depth evaluation in animal models
- Development of models to predict mitochondrial toxicity potential of untested chemicals



MMP – one of the most widely assessed parameters for mitochondrial toxicity

Mito-MPS (JC-10, m-MPI, modified JC-1) – a lipophilic, cationic dye accumulating into Mitochondrial membrane
matrix space in inverse proportion to MMP

Healthy cells – Mito-MPS accumulates in the mitochondria
as aggregates with red fluorescence

FCCP treated cells – Mito-MPS remains in cytoplasm as monomeric form showing green fluorescence

Sakamuru et al., 2012, Physiological Genomics 44:495-503

FCCP, mesoxalonitrile 4-trifluoromethoxyphenylhydrazone

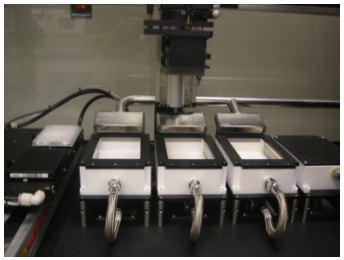
MMP Assay Protocol

Add 2000 HepG2 Cells/well into 1536-well plate, 5 µl total

Multidrop Combi

Incubate assay plates overnight at 37°C, 5% CO2 incubator

Pintool



Add 23 nl compounds (2.9 nM to 46 µM titration series)
or positive controls (17.5 nM to 11.5 µM titrations, FCCP)

Incubate assay plates at 37°C, 5% CO2 incubator for 1 or 5 h

BioRAPTR FRD

Add 5 µl of Mito-MPS dye (Incubate 30 min at 37°C, 5% CO2 incubator)

Read fluorescence signal (Ex/Em=485/535 nm;
Ex/Em=540/590 nm) on Envision plate reader

EnVision Multilabel Reader

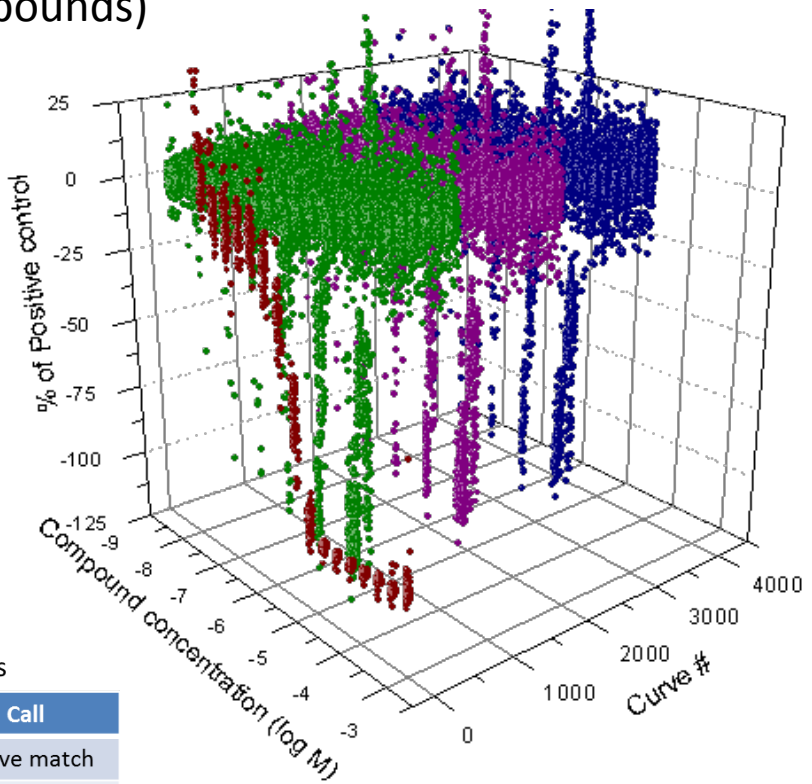
- Absorbance
- Fluorescence
- F.P.
- Luminescence
- TR-FRET
- AlphaScreen
- Top/Bottom reading

Online Validation – General Statistics and Reproducibility

- 1,368 compounds (LOPAC + 88 Tox21 compounds)
- 7 concentrations (3 nM to 46 uM), 3 times
- Assay reproducibility and general statistics

MMP	Mean ± SD
CV (%)	7.925 ± 0.373
Z factor	0.774 ± 0.008
S/B	9.405 ± 0.161

• CV (coefficient of variation) = standard deviation (SD) of compound area/median of compound area
• Z factor = 1-[3*(SD of compound area + SD of basal)/(median of compound area – median of basal)]



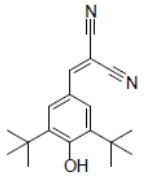
Reproducibility	
Active match	9.84%
Inactive match	69%
Mismatch	0.34%
Inconclusive	20.81%
AC50 fold change	1.19

Reproducibility Calls

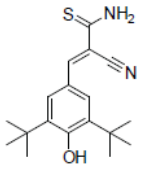
Run #1	Run #2	Call
Active	Active	Active match
Inactive	Inactive	Inactive match
Active	Inactive	Mismatch
Inactive	Active	Mismatch
Activation	Inhibition	Mismatch
Inhibition	Activation	Mismatch
Other	Other	Inconclusive

Tyrphostin Compounds

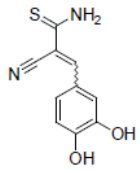
- 20 tyrphostin analogs were identified
- Potency depends on # of hydroxyl groups and the tertiary butyl groups



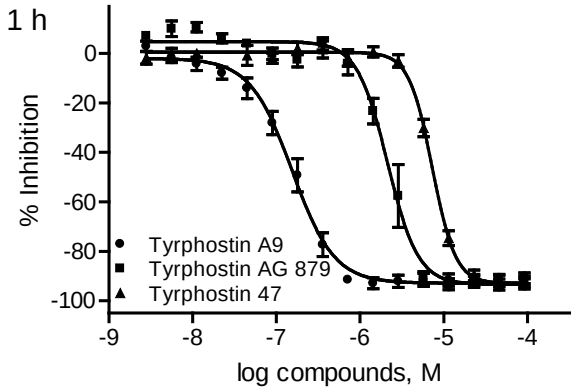
● Tyrphostin A9
IC_{50} = 0.15 μM, 1h
IC_{50} = 0.18 μM, 5h

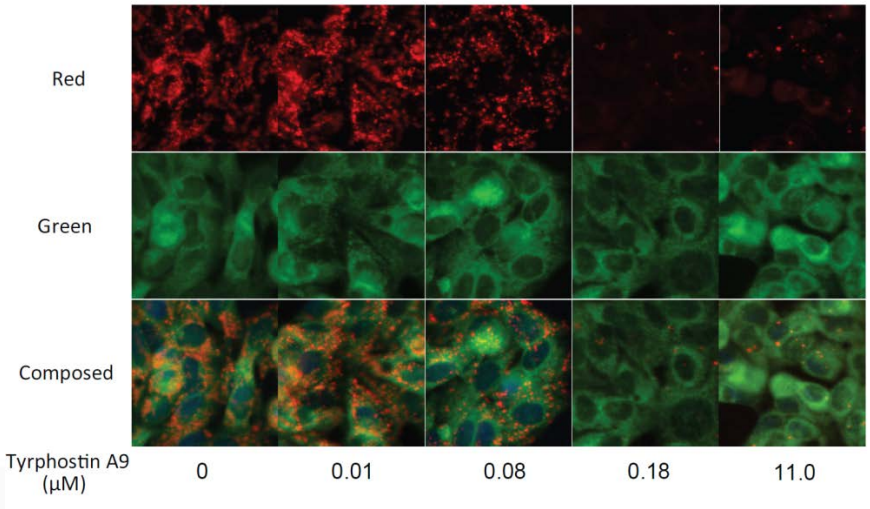


■ Tyrphostin AG 879
IC_{50} = 2.13 μM, 1h
IC_{50} = 3.71 μM, 5h



▲ Tyrphostin 47
IC_{50} = 7.29 μM, 1h
IC_{50} = 20.2 μM, 5h





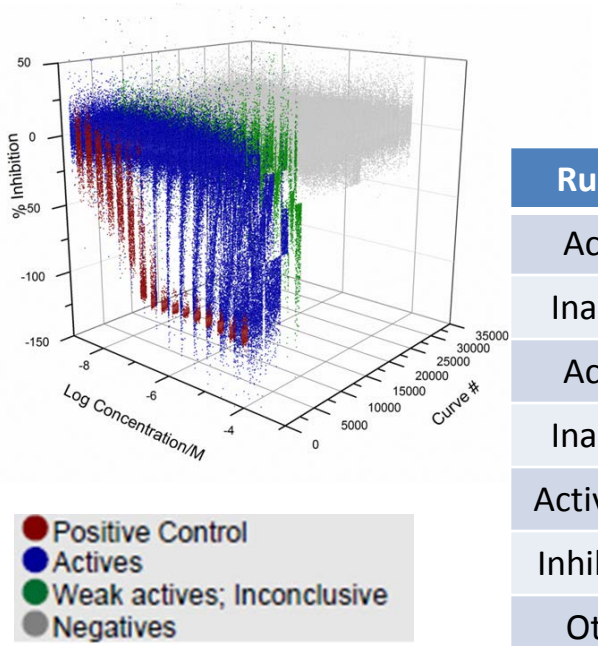
Molecular Devices ImageXpress Micro

- **HCS**
- **Bottom reading**
- **Slow in speed (20 min - 1 hr/plate)**

Images acquired in ImageXpress Micro using a 20x objective. While red fluorescent aggregates are localized in the mitochondria, green fluorescent monomers are mainly in cytosol. The composed images were the merger of red and green fluorescence.

Sakamuru et al., 2012, Physiological Genomics 44:495-503

Reproducibility of Tox21 10K Compound Screen



Reproducibility Calls

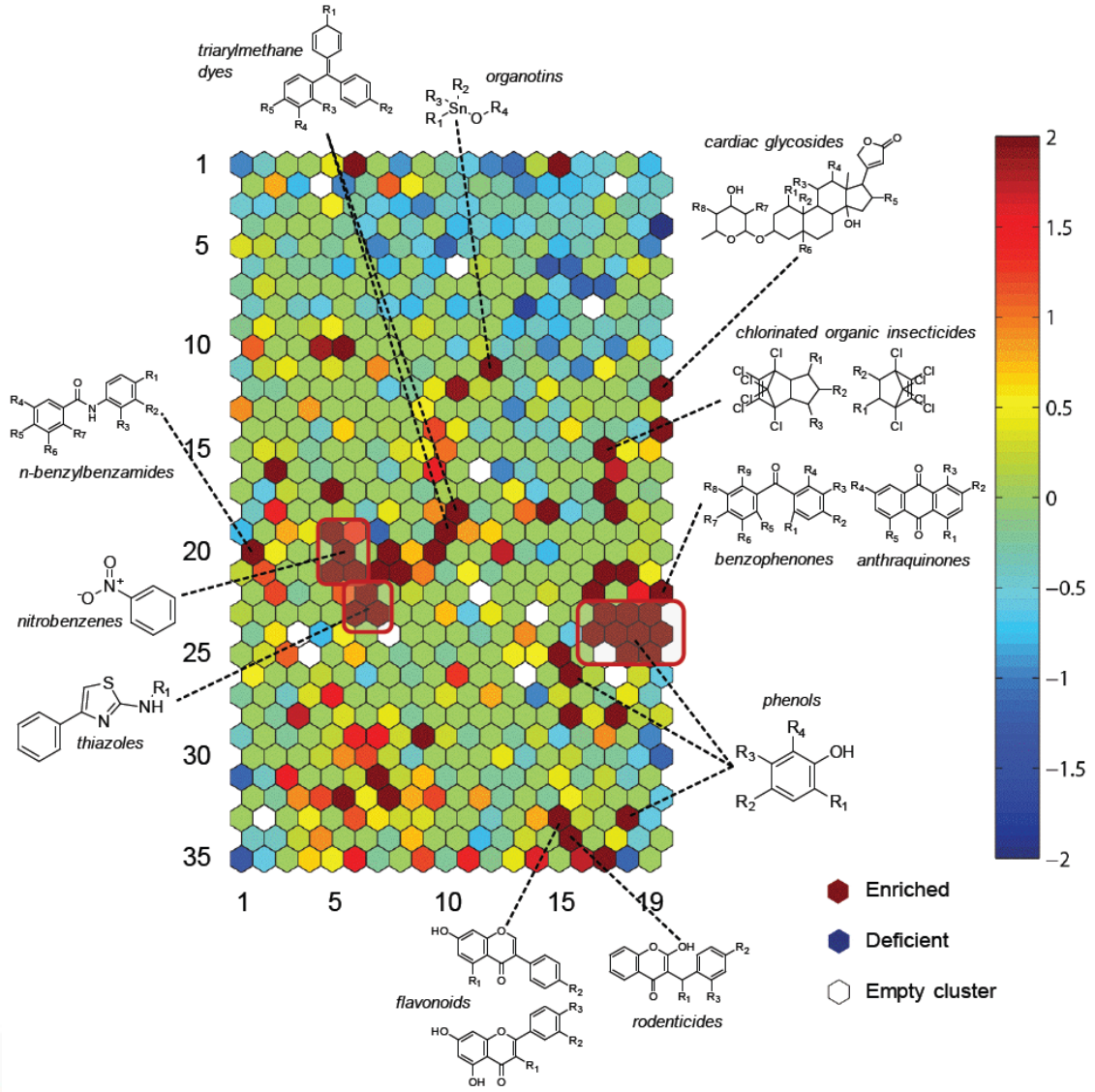
Run #1	Run #2	Call
Active	Active	Active match
Inactive	Inactive	Inactive match
Active	Inactive	Mismatch
Inactive	Active	Mismatch
Activation	Inhibition	Mismatch
Inhibition	Activation	Mismatch
Other	Other	Inconclusive

Activity Definitions

- Active
  - Class 1.1, 1.2, 2.1
  - Class 2.2 (efficacy>50%)
- Inactive
  - Class 4
- Inconclusive
  - All other cases
- Triplicate run outcome
  - Active
    - Active in ≥2 runs
    - 3rd run Curve class
      - Non-class 4
      - Agonist mode: >0
      - Antagonist mode: <0
  - Inactive
    - Inactive in ≥2 runs
    - 3rd run not active
  - Inconclusive
    - All other cases

Assay Reproducibility	Active Match	Inactive Match	Inconclusive	Mismatch	IC50 fold change
MMP	17.57%	67.52%	14.33%	0.55%	1.53

Heatmap of Structure Clusters



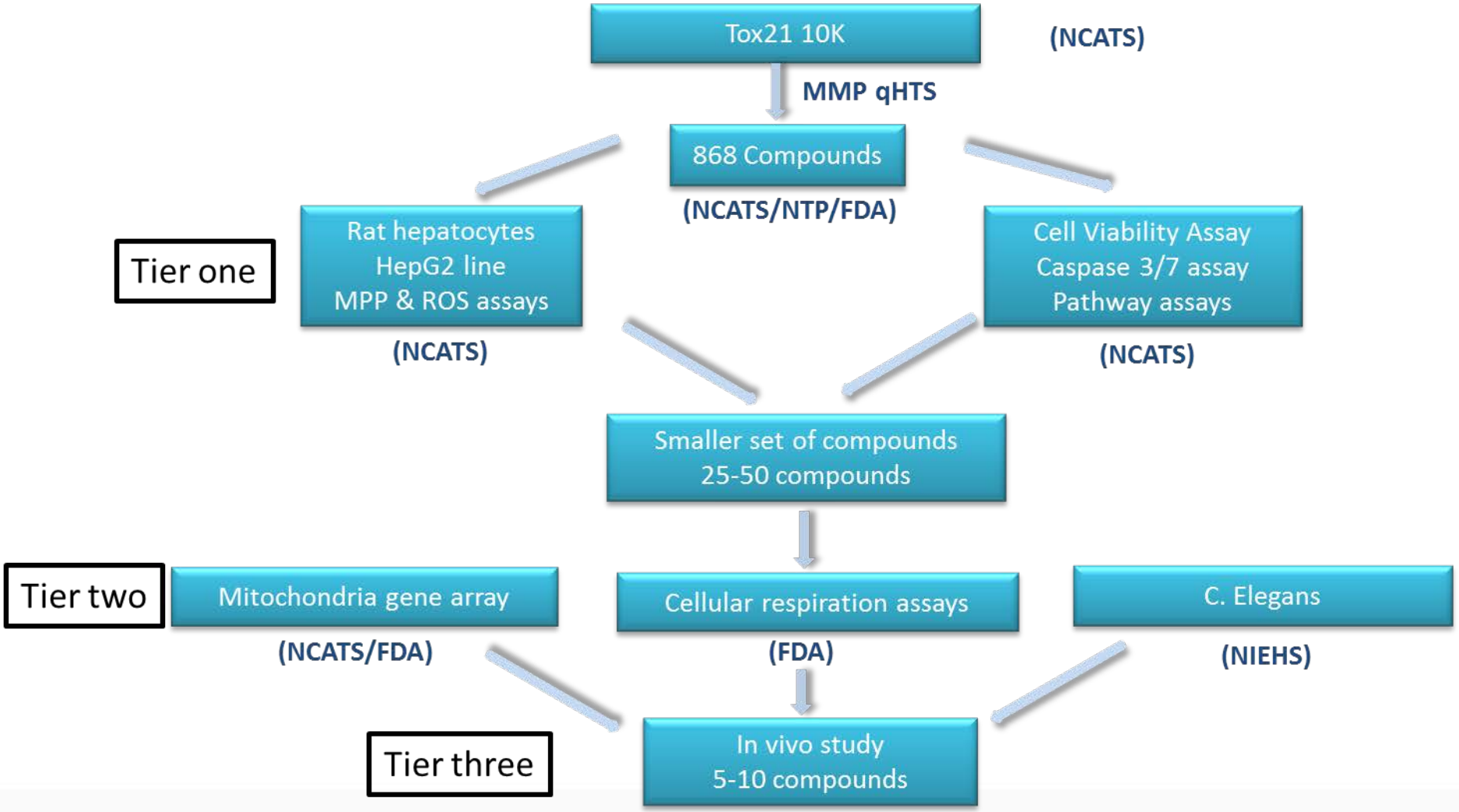
- ~ 650 clusters from Tox21 10k library

using SOM (self-organizing map algorithm)

- 76 clusters (~11%) decreased MMP ($p<0.05$)

ttene-Ramos et al., 2015, Environ Health Perspect 123:49-56

Compound Prioritization Workflow



Challenges from Tox21 qHTS

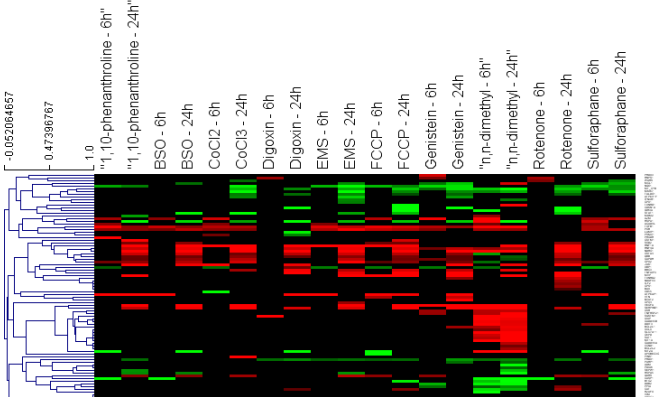
- Lack of xenobiotic metabolic capability
- Limited pathway coverage
- Reliance on engineered transformed and immortal cell lines
- Focus on single compounds
- Limited to acute exposure scenarios
- Limited availability of “BIG” data analysis tools

Tox21 Phase III Focus

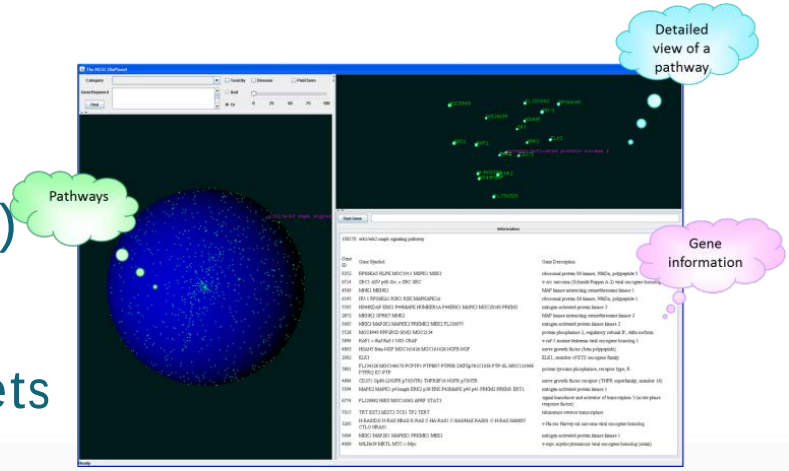
- Incorporate xenobiotic metabolic capability into the cell systems
  - Use more physiologically relevant cells (e.g. differentiated cells, primary cells and stem/IPS-derived cells)
- Increase pathway coverage
  - Gene expression platform (>1000 genes)

    RASL-seq technology
  - Identify key pathway perturbations

    BioPlanet web tool



- Explore compound mixtures and fruit/vegetable extracts (NIEHS and EPA nominated)



- Build predictive models using Tox21 datasets
  - Tox21 Challenge crowdsourcing program

Acknowledgement

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