United States Environmental Protection Agency

In Vitro Testing of Engineered Nanomaterials in the EPA's ToxCast Program

Keith Houck
National Center for Computational Toxicology

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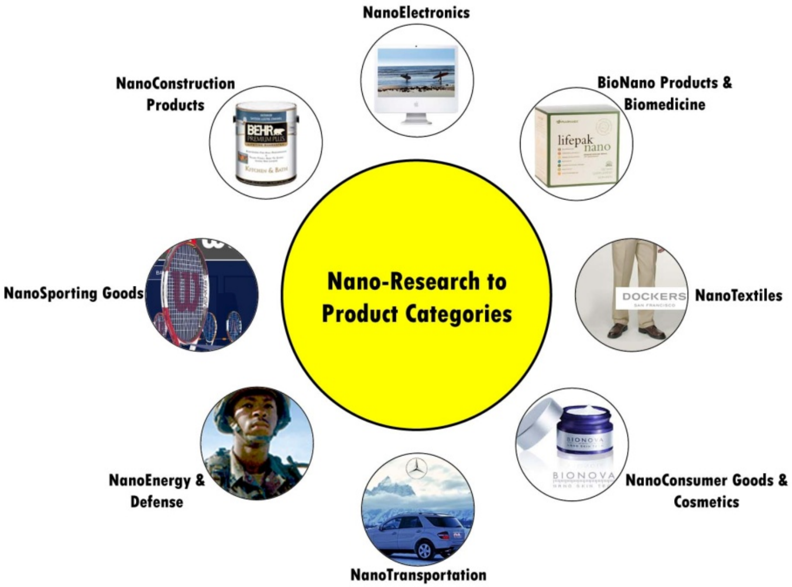
Office of Research and Development
National Center for Computational Toxicology

Many Nanomaterials and Little Bioactivity/Toxicity Data

- Over 2,800 pristine nanomaterials (NMs)[1] and numerous nanoproducts are already on the market
- We have toxicity data for only a small number of them
- Traditional mammalian tox testing for all NM is not practical
  - Estimated $249 million to $1.18 billion for NM already on the market in 2009[2]



http://nrc.ien.gatech.edu/sites/default/files/NanoProductsPostercopy.jpg

1. Nanowerk. Nanomaterial Database Search. Available at: http://www.nanowerk.com/phpscripts/n_dbsearch.php. (Accessed July 26 2012)
2. Choi J-Y, Ramachandran G, Kandlikar M. The impact of toxicity testing costs on nanomaterial regulation. *Environ Sci Technol* 2009, 43:3030-3034.

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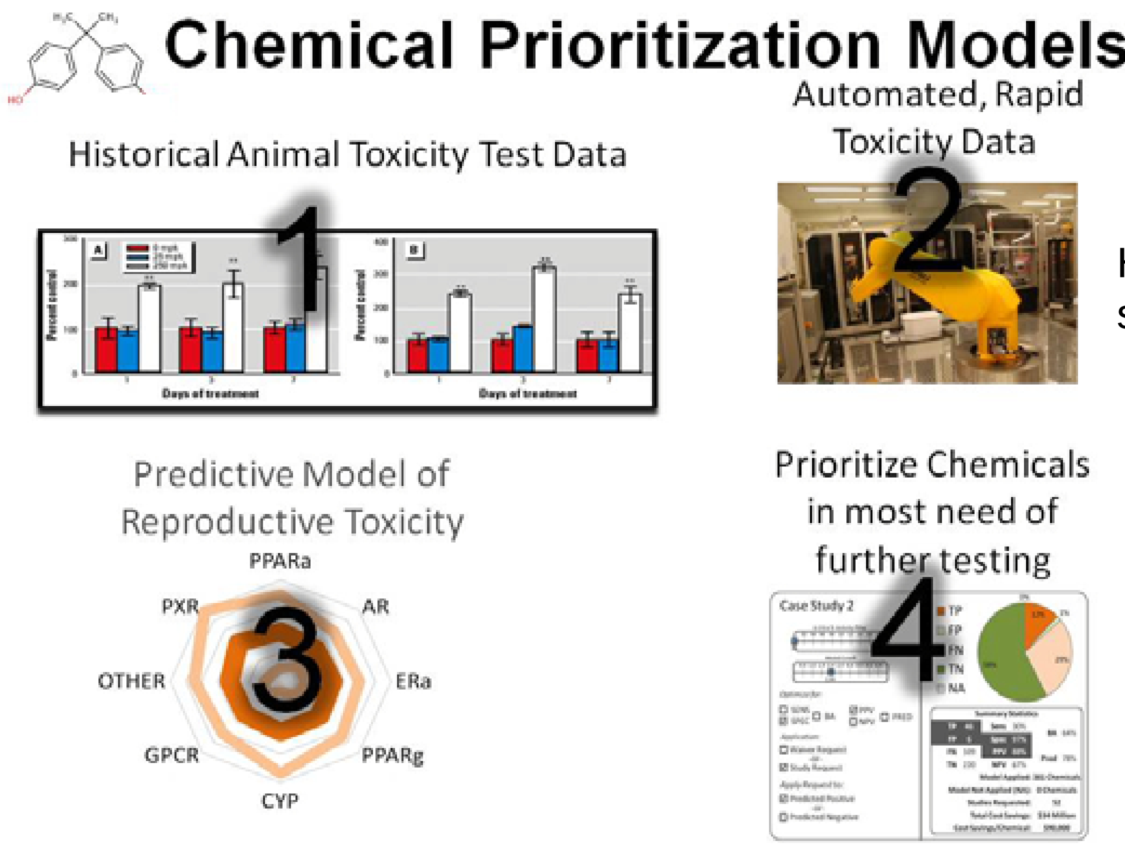
ToxCast - Toxicity Forecaster

- Part of EPA's computational toxicology research
- Initial goal is chemical prioritization
- Find correlations of *in vitro* bioactivity signatures and *in vivo* toxicity endpoints



High-throughput screening (HTS)

NM Testing in ToxCast

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CEINT — Center for the Environmental Implications of Nanotechnology

ORGANISATION FOR ECONOMIC CO-OPERATION AND DEVELOPMENT — OECD

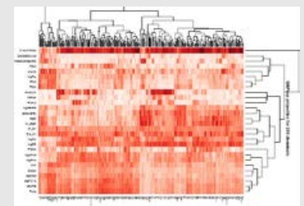
SEVENTH FRAMEWORK PROGRAMME — ENPRA

UC CEIN

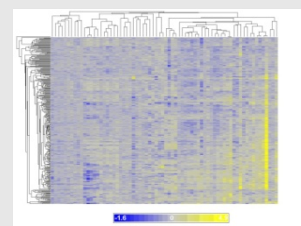
Goals:

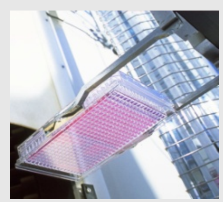
- Evaluate ToxCast HTS assays for screening NMs
  - Compatibility of assays
  - Suitability of endpoints
- Prioritize NMs for further research/hazard identification
- Identify key nanomaterial physicochemical characteristics influencing activities

>1000 chemicals; **~60 NMs** (Ag, Au, TiO_2, SeO_2, ZnO, SiO_2, Cu, etc)



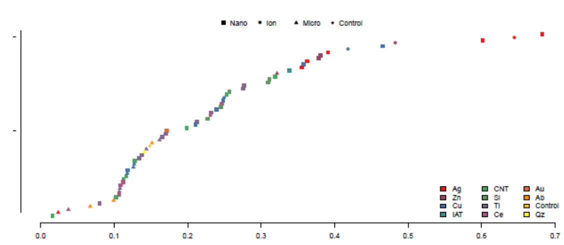
Physical chemical properties of NM

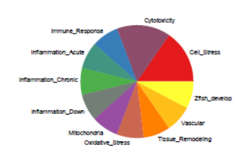




HTS assay results

Profile Matching





Current ToxCast Nano Data

- **HTS of bioactivity** completed for 67 samples (62 unique materials)
  - 6 to 10 concentrations
  - Mammalian cellular assays
  - Zebrafish embryo development assay
- **Characterization/analysis of NM physicochemical properties in progress**

	nano	micro	ion
Ag	5+2*	1	1
Asbestos		3	
Au	1		
SWCNT MWCNT	8		
CeO2	4	1	1
Cu	4+2#	2+1#	2
SiO_2	5	1	
TiO_2	9	4	
ZnO	2	1	1

* IAT NP and IAT NP infused with Ag ion
# purified sample with no/low ions
Not listed: Dispersant of one of the nano-Ag

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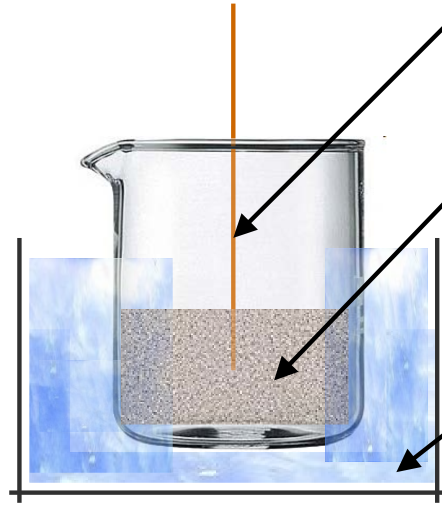
Consistent Handling Protocol: Stock Preparation as an Example



Sonication at 400 W for 16 min

Nanomaterial in ultrapure water + 2% serum

Ice

- All stocks made by one lab
- Used for bioactivity testing within 2 weeks

Adapted from Keld Astrup Jensen developed in FP7 ENPRA (www.enpra.eu)

Testing Concentrations: Based on

Reported potential occupational inhalation exposure → MPPD → Estimated lung retention

Gangwal et al. Environ Health Perspect 119:1539-46, 2011.

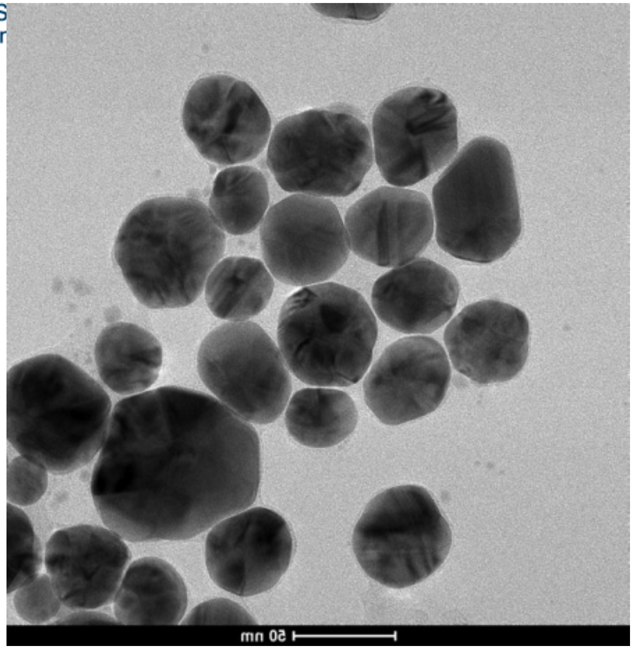
Characterization Data Coverage

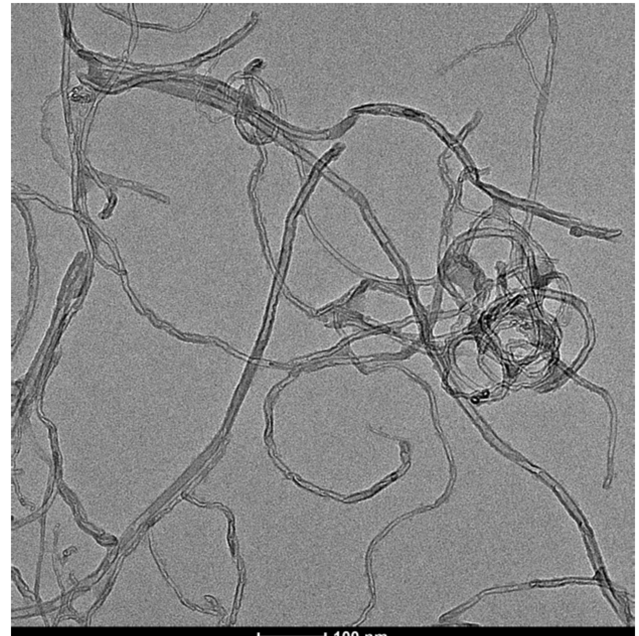
As received		(Re)suspended	
Dry material	Sus- pension	In stock (H_2O+serum)	In 4 testing mediums, 2 conc

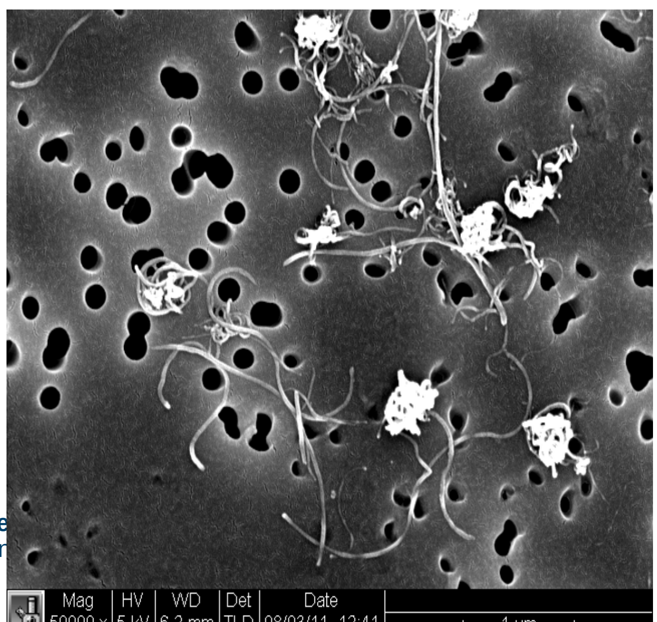
Characterization Data Coverage

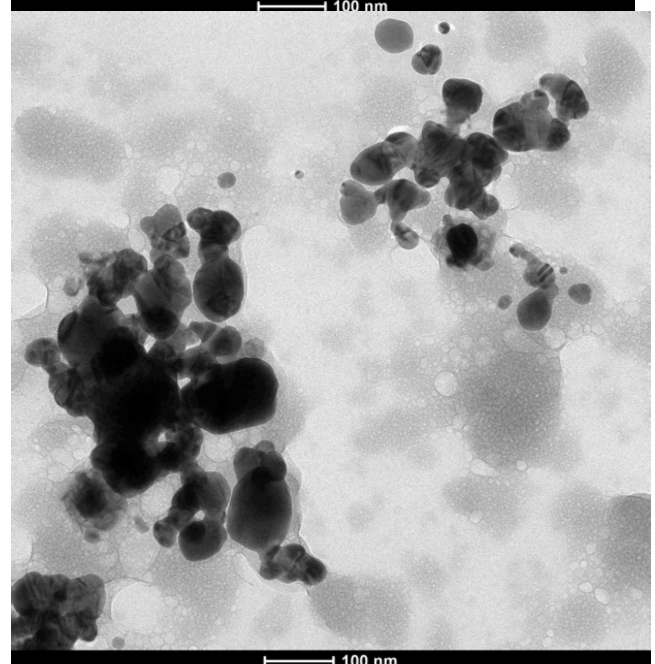
Endpoints	Method (by CEINT, unless specified)	Samples	As received		(Re)suspended	
			Dry material	Sus-pension	In stock (H_2O+serum)	In 4 testing mediums, 2 conc
size distribution and shape	TEM, SEM, DLS	nano and micro	√	√	√	√ (2 time points)
surface area	BET (by NIOSH and NIST), calculate from DLS	nano and micro	√		√	√ (3)
chemical composition	XRD, TOC	all samples	√	√		
crystal form	XRD	applicable samples	√	√		
impurity	XPS	CNT	√			
total metal concentration		metallic samples			√	√ (1)
total non-metal concentration		non-metallic samples			√	
ion concentration	ICP-MS and others	applicable			√	√ (3)
zeta potential, surface charge	zetasizer	nano and micro			√	

Example TEMs









CNTs Have Different Impurities

Weight percent of impurities in CNTs, measured by XPS

	C	Fe	Co	Ni
N010				
N011	97.46	1.09	1.44	0.00
N012	99.31	0.69	0.00	0.00
N013	99.03	0.97	0.00	0.00
N014	99.46	0.00	0.54	0.00
N015	100.00	0.00	0.00	0.00
N016				
N017				

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HTS Assay Coverage

DNA

RNA

Protein

Function/ Phenotype

Main type of result by assay platform	# of endpoint measured	# of direction (time points)	# of potential LEC/AC50 per NM per conc.
• Transcription factor activation	48	NA	48
• Protein biomarker	87	2	174
• Cell growth kinetics	1	1	1
• Toxicity phenotype	19	NA (2)	38
• Developmental malformation	Aggregated to 2	NA	2
Total			**> 260**

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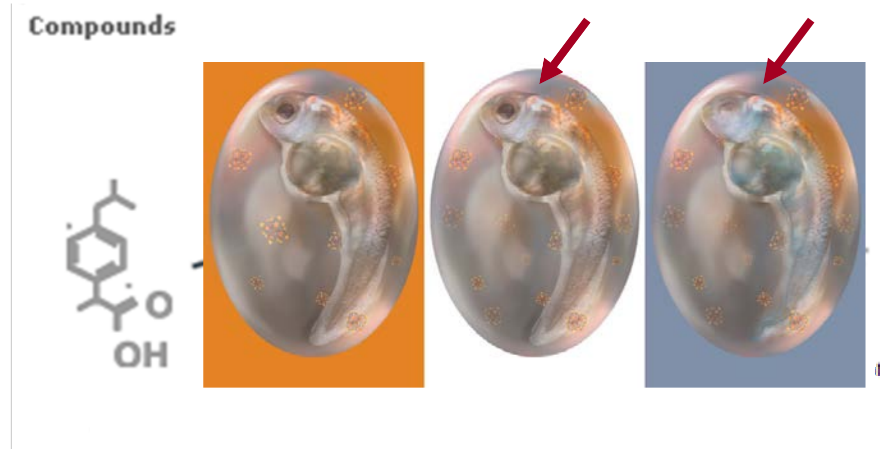
Assay Platforms

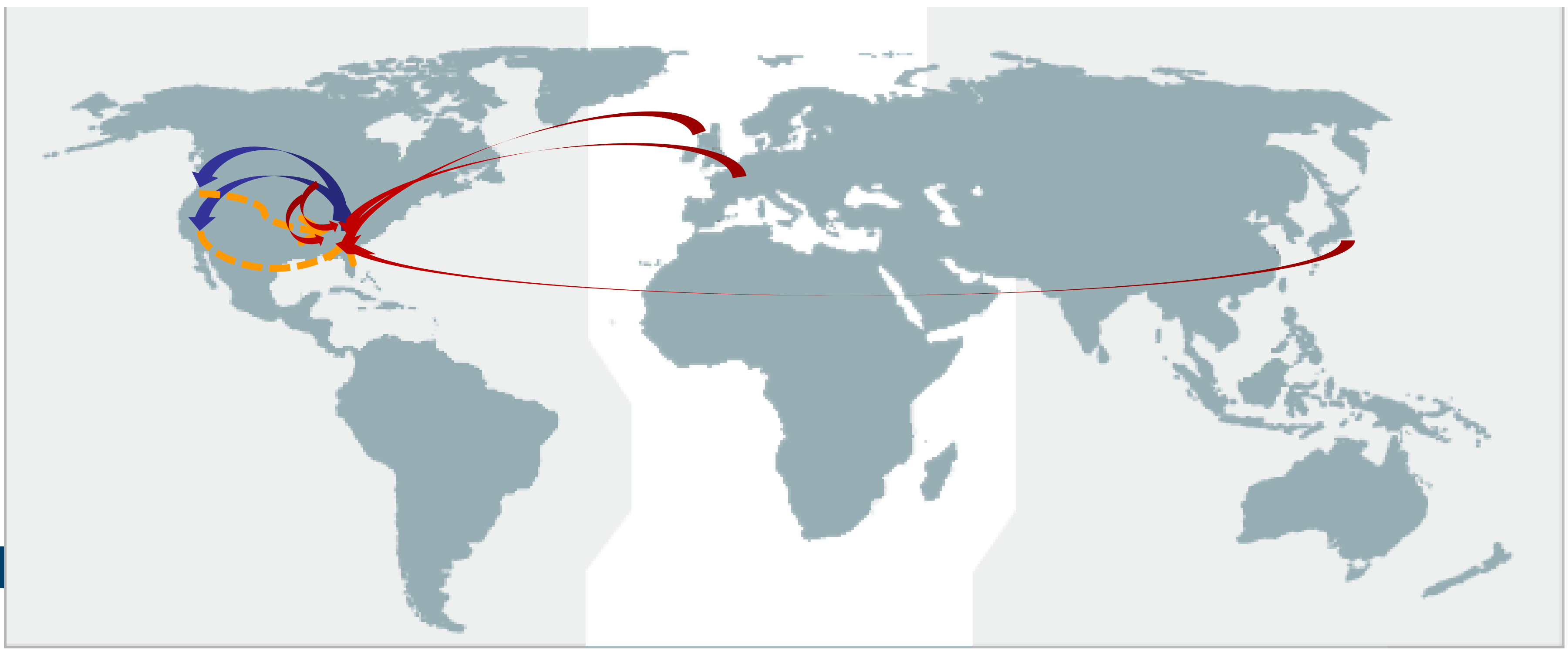
- Selected endpoints
  - Effects on transcription factors in human cell lines (Attagene)
  - Human cell growth kinetics (ACEA Biosciences)
  - Protein expression profiles in complex primary human cell culture models (BioSeek)



  - Toxicity phenotype effects (DNA, mitochondria, lysosomes etc.) in human and rat liver cells through high-content screening/ fluorescent imaging (Apredica)
  - Developmental effects in zebrafish embryos

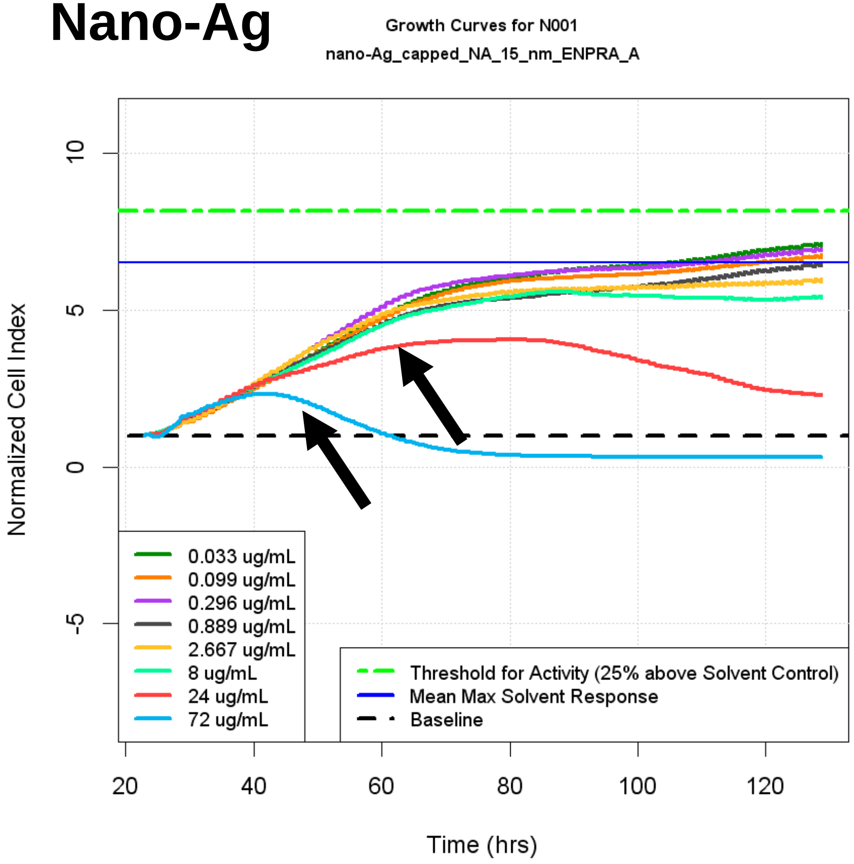
Screening Logistics

- Samples from international sources to EPA
- Samples prepped at Duke University CEINT
- Samples shipped to testing labs: NC (2), CA (3), MA (1)
- Data sent back to EPA
- Physicochemical characterization at CEINT simulating testing conditions

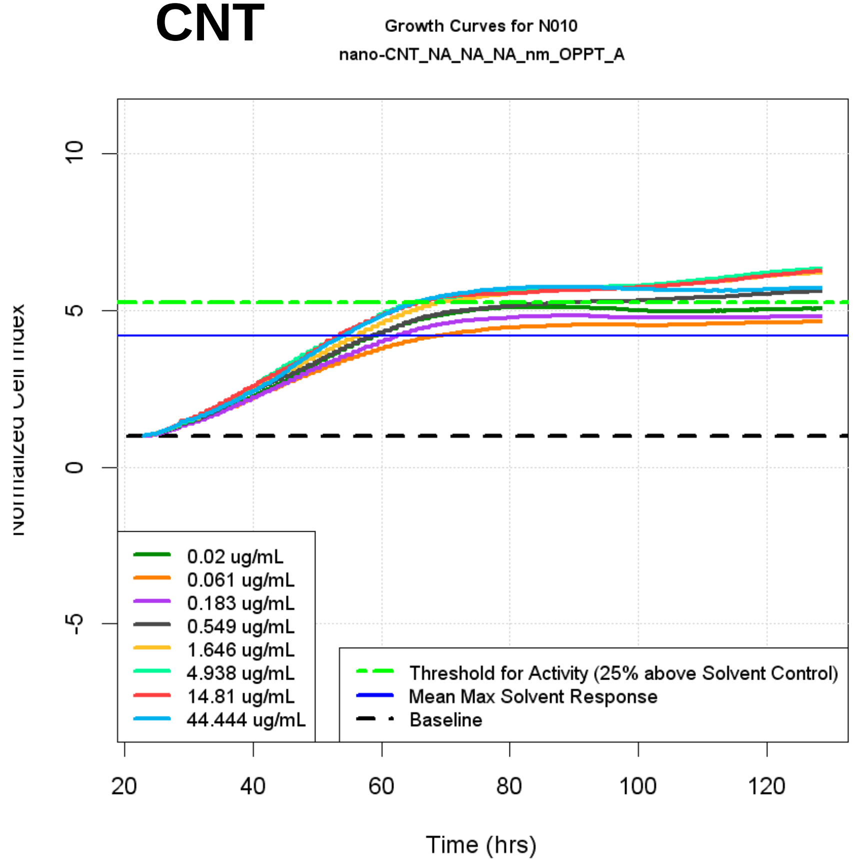


Cell Growth Kinetics in Human Lung Carcinoma Cell Line (A549) (ACEA Biosciences)

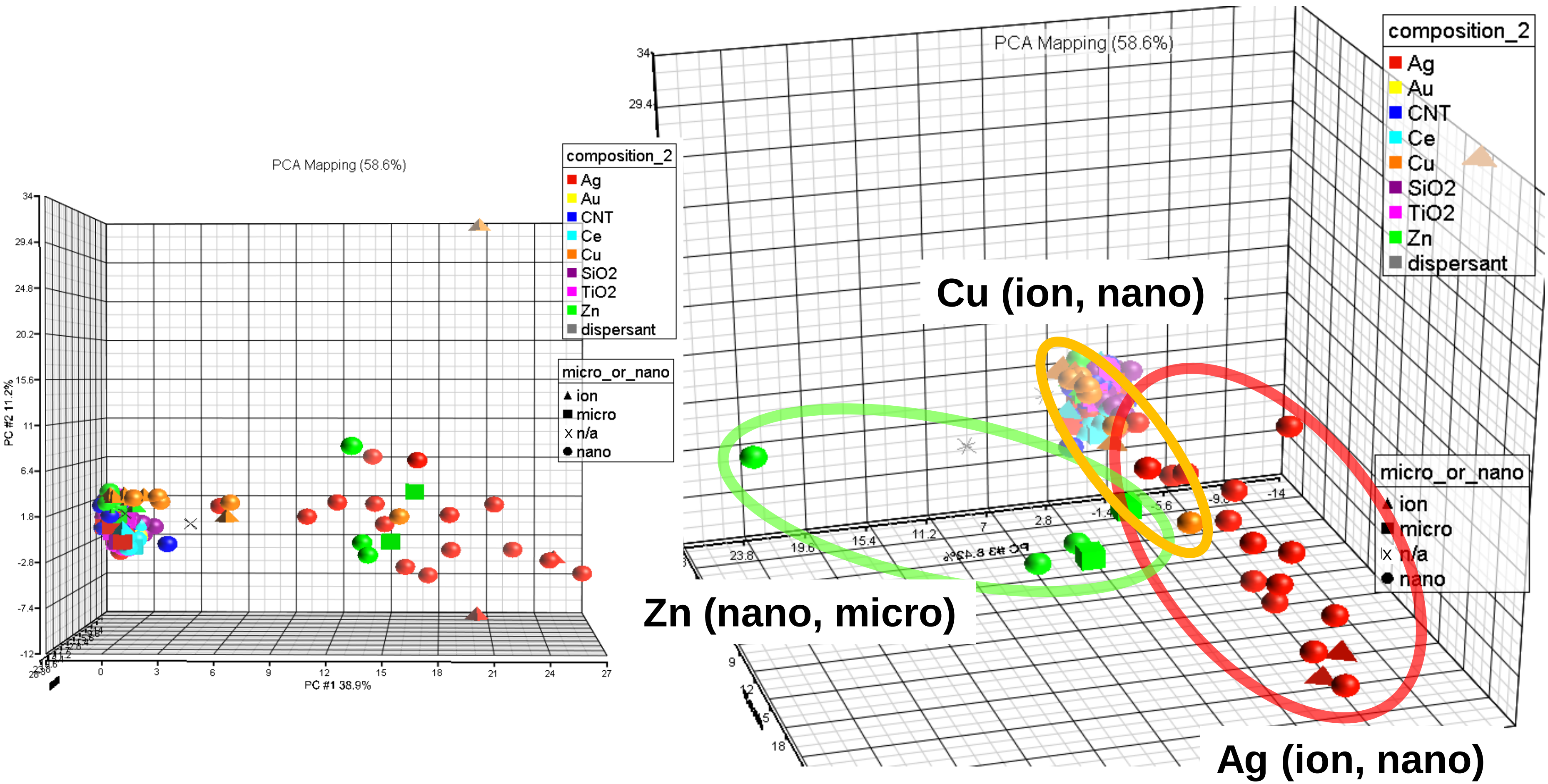
Nano-Ag



CNT



Principle Component Analysis (PCA) of Transcription Factor Activity (Attagene)



Principle component analysis (PCA) mapping of all transcription factor in Cis assay

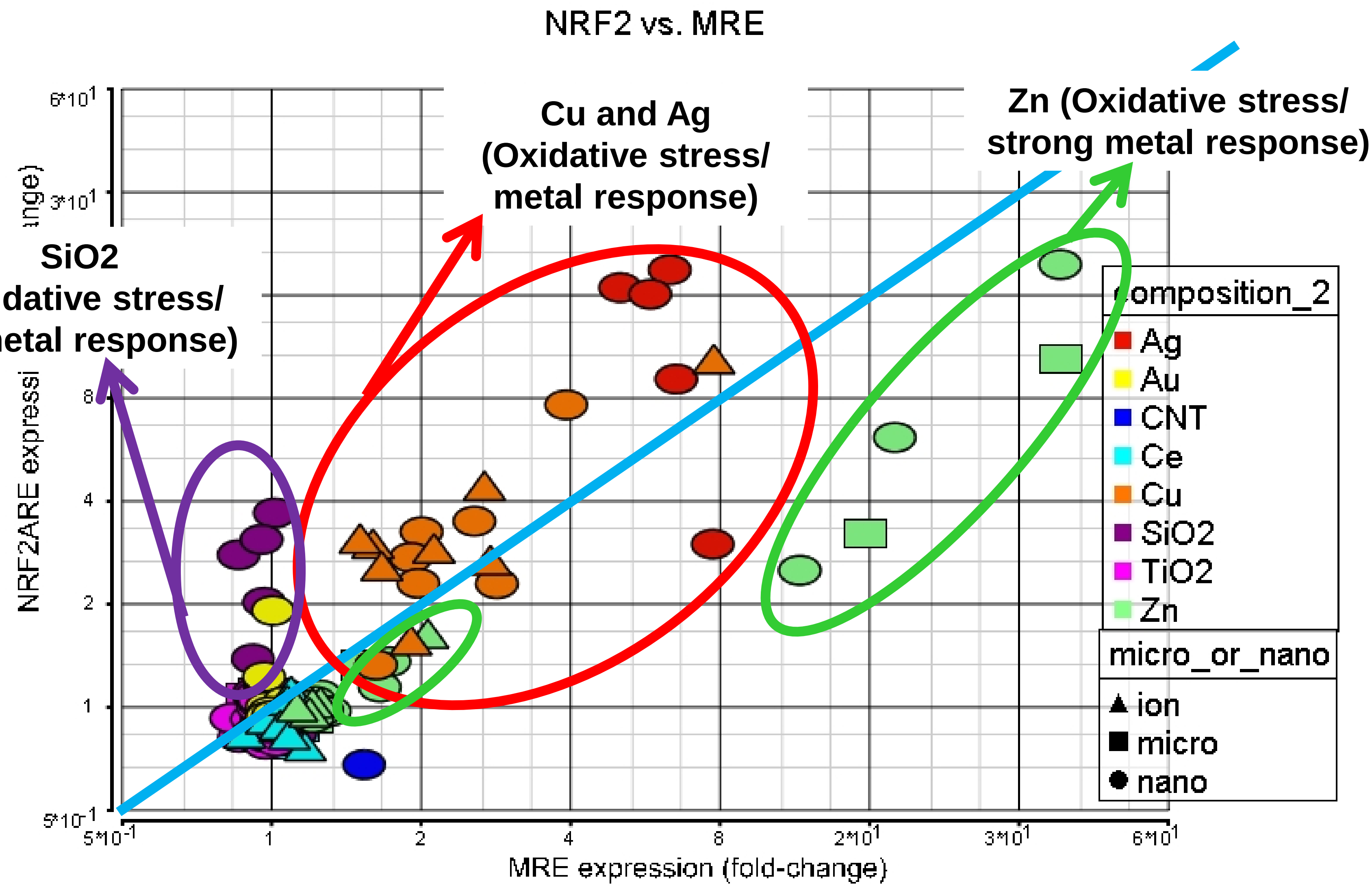
- **Principle component #1: 12 (out of 53) observed variables account for 39% variations**

Pax6
EGR
Xbp1
Oct-MLP
CRE
Sox

Associated with general cellular stress and death

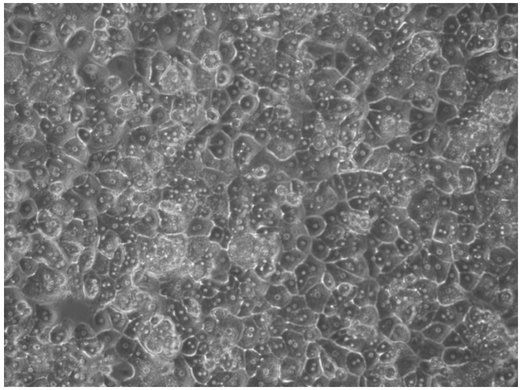
HSE — Heat shock
Sp1
NRF1
MRE — Metal response
C/EBP
NRF2/ARE — Oxidative stress

Oxidative stress vs. Metal response



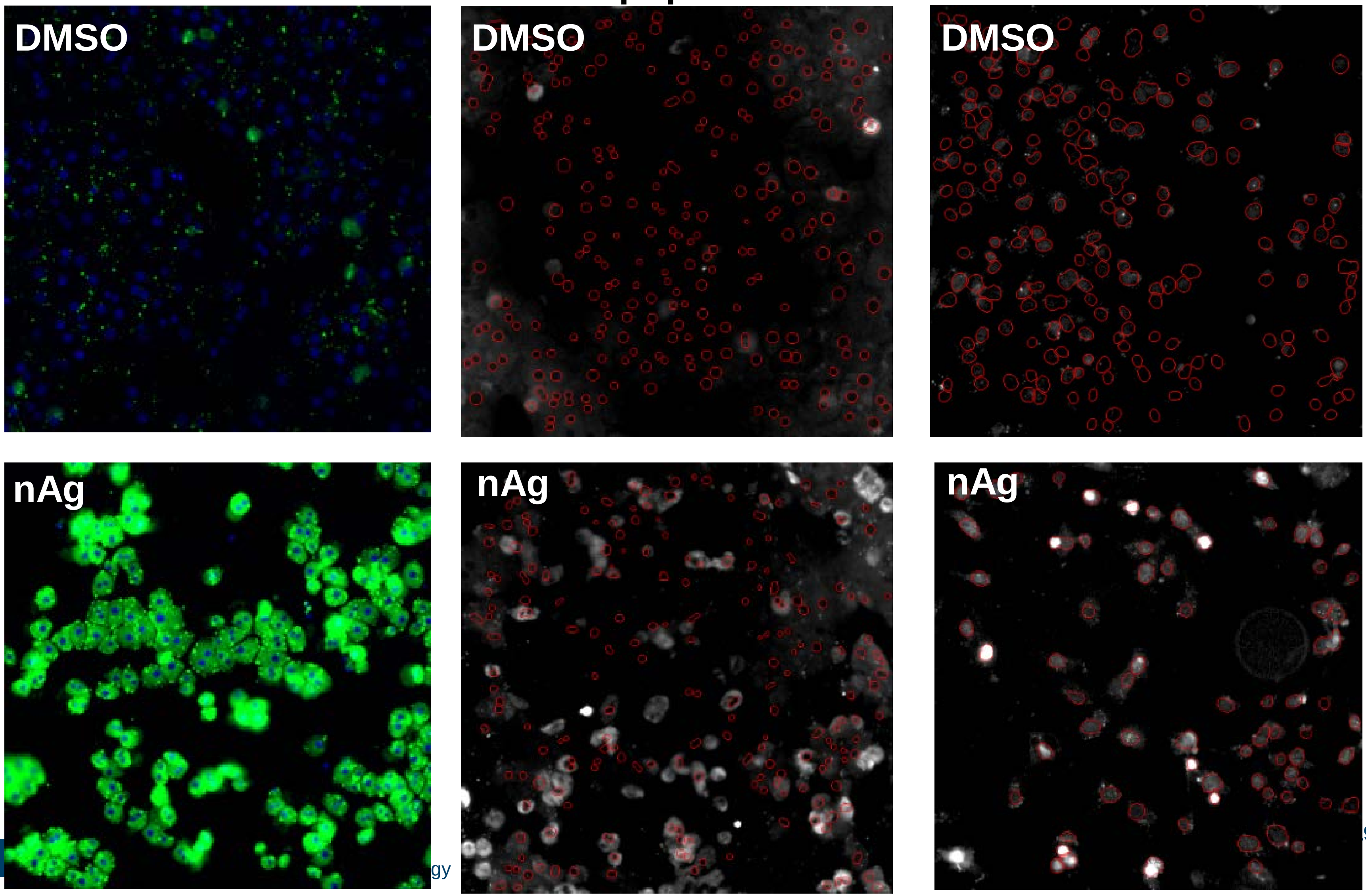
Technology Platform: High-Content Cellular Imaging Toxicity Phenotypes

- Description
  - HepG2 human hepatoma cell line
  - Rat primary hepatocytes
  - Cellular toxicity phenotypes
  - Apredica
- Endpoints (20)
  - Cytotoxicity
  - Oxidative stress
  - DNA damage
  - Mitochondrial function
  - Apoptosis
  - Steatosis
  - Cell cycle
- Result Summary:
  - Cell-selective cytotoxicity (Ag, ZnO, Cu, SiO2)
  - Steatosis (Ag, ZnO, SiO2)
  - Apoptosis (Ag, ZnO, SiO2, Cu)
  - DNA Damage (Ag, ZnO, SiO2, Cu)
  - AC50 > 1ug/ml (except Ag and HepG2 cytotoxicity)
  - Soluble ion and nano effects generally similar

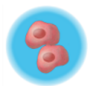
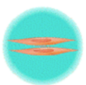
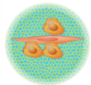


HCS Images

Steatosis | Apoptosis | H2AX/Oxidative Stress



Primary Human Cell Systems (BioSeek)

		3C	4H	LPS	SAg	BE3C	CASM3C	HDF3CGF	KF3CT
BioMAP System									
Primary Human Cell Types		Venular endothelial cells	Venular endothelial cells	Peripheral blood mononuclear cells + Endothelial cells	Peripheral blood mononuclear cells + Endothelial cells	Bronchial epithelial cells	Coronary artery smooth muscle cells	Fibroblasts	Keratinocytes + Fibroblasts
Stimuli		IL-1β + TNF-α + IFN-γ	IL-4+Histamine	TLR4	TCR	IL-1β + TNF-α + IFN-γ	IL-1β + TNF-α + IFN-γ	IL-1β + TNF-α + IFN-γ + EGF + bFGF + PDGF-BB	IL-1β + TNF-α + IFN-γ + TGF-β
# of Endpoints		13	7	11	10	11	14	12	9
Endpoint Types	**Acute Inflammation**	E-selectin, IL-8		E-selectin, IL-1α, IL-8, TNF-α, PGE2	IL-8	IL-1α	IL-8, IL-6, SAA	IL-8	IL-1α
	Chronic Inflammation	VCAM-1, ICAM-1, MCP-1, MIG	VCAM-1, Eotaxin-3, MCP-1	VCAM-1, MCP-1	MCP-1, E-selectin, MIG	IP-10, MIG, HLA-DR	MCP-1, VCAM-1,MIG, HLA-DR	VCAM-1, IP-10, MIG	MCP-1, ICAM-1, IP-10
	Immune Response	HLA-DR		CD40, M-CSF	CD38, CD40, CD69, PBMC Cytotox., T cell Proliferation	HLA-DR	M-CSF	M-CSF	
	Tissue Remodeling					uPAR, MMP-1, PAI-1, TGFb1, SRB, tPA, uPA	uPAR,	Collagen III, EGFR, MMP-1, PAI-1, Fibroblast Proliferation, SRB, TIMP-1	MMP-9, SRB, TIMP-2, uPA, TGFb1
	Vascular Biology	TM, TF, uPAR, EC Proliferation, SRB, Vis	VEGFRII, uPAR, P-selectin, SRB	Tissue Factor, SRB	SRB		TM, TF, LDLR, SMC Proliferation, SRB		
Disease / Tissue Relevance		Vascular Biology, Cardiovascular Disease, Chronic Inflammation	Asthma, Allergy, Oncology, Vascular Biology	Cardiovascular Disease, Chronic Inflammation, Infectious Disease	Autoimmune Disease, Chronic Inflammation, Immune Biology	COPD, Respiratory, Epithelial Biology	Vascular Biology, Cardiovascular Inflammation, Restenosis	Tissue Remodeling, Fibrosis, Wound Healing	Skin Biology,Psoriasis, Dermatitis

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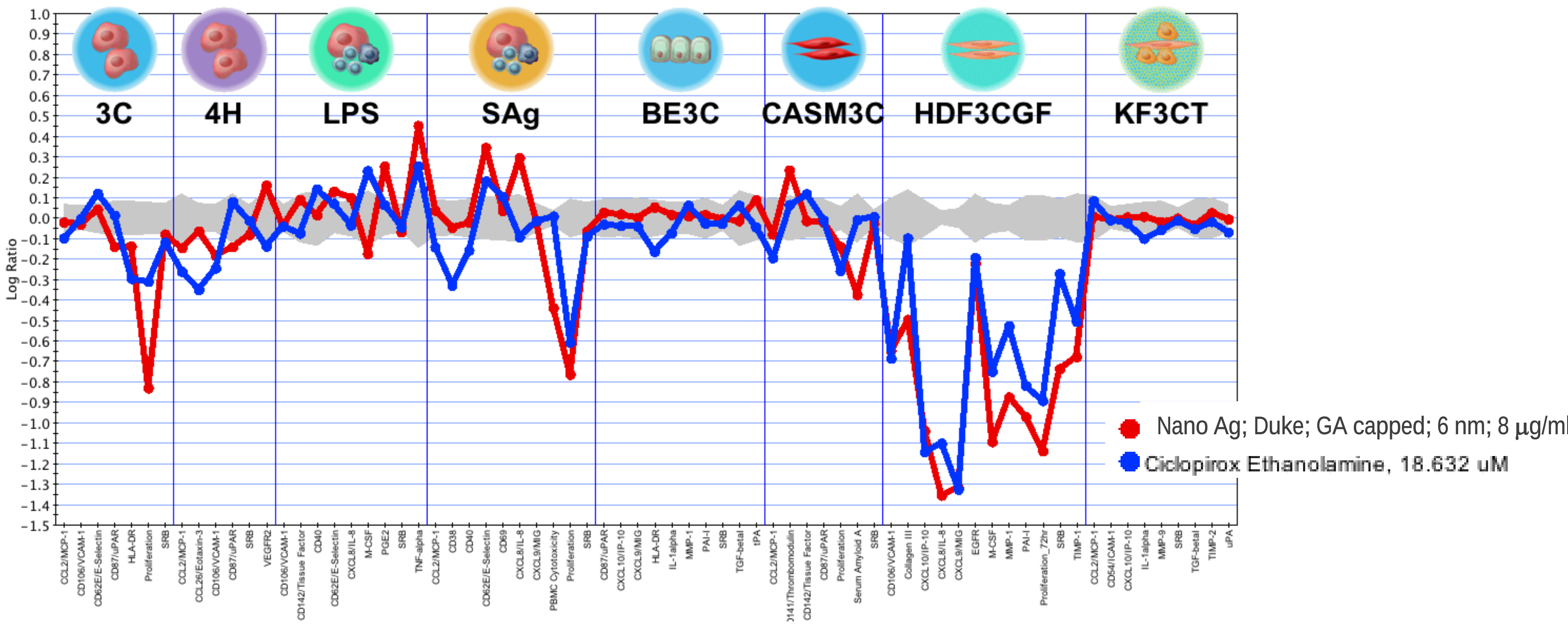
Inferred Mechanism of Toxicity: nano Ag



- **Ciclopirox – inhibitor of Na+ K+ ATPase**
- **Toxicity of silver is associated with inhibition of Na+K+ATPase (PMID: 6240533)**

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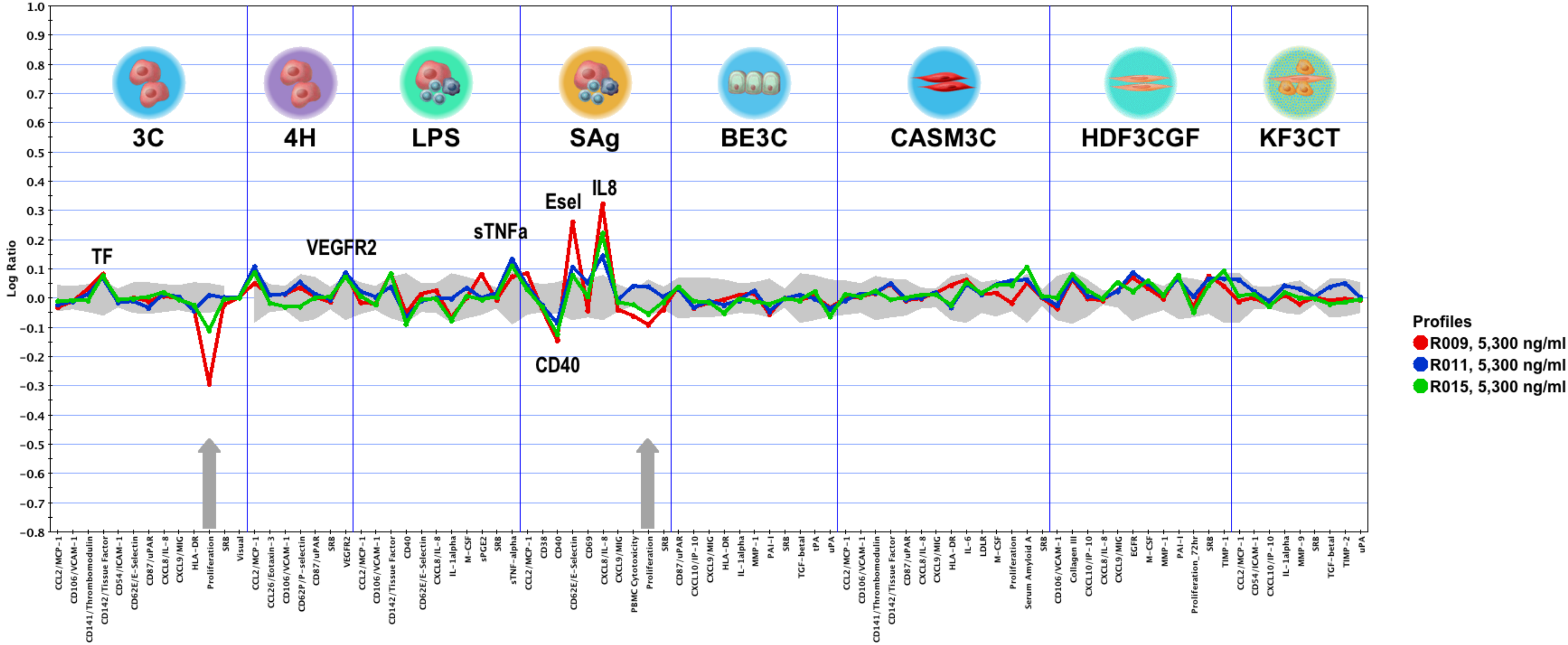
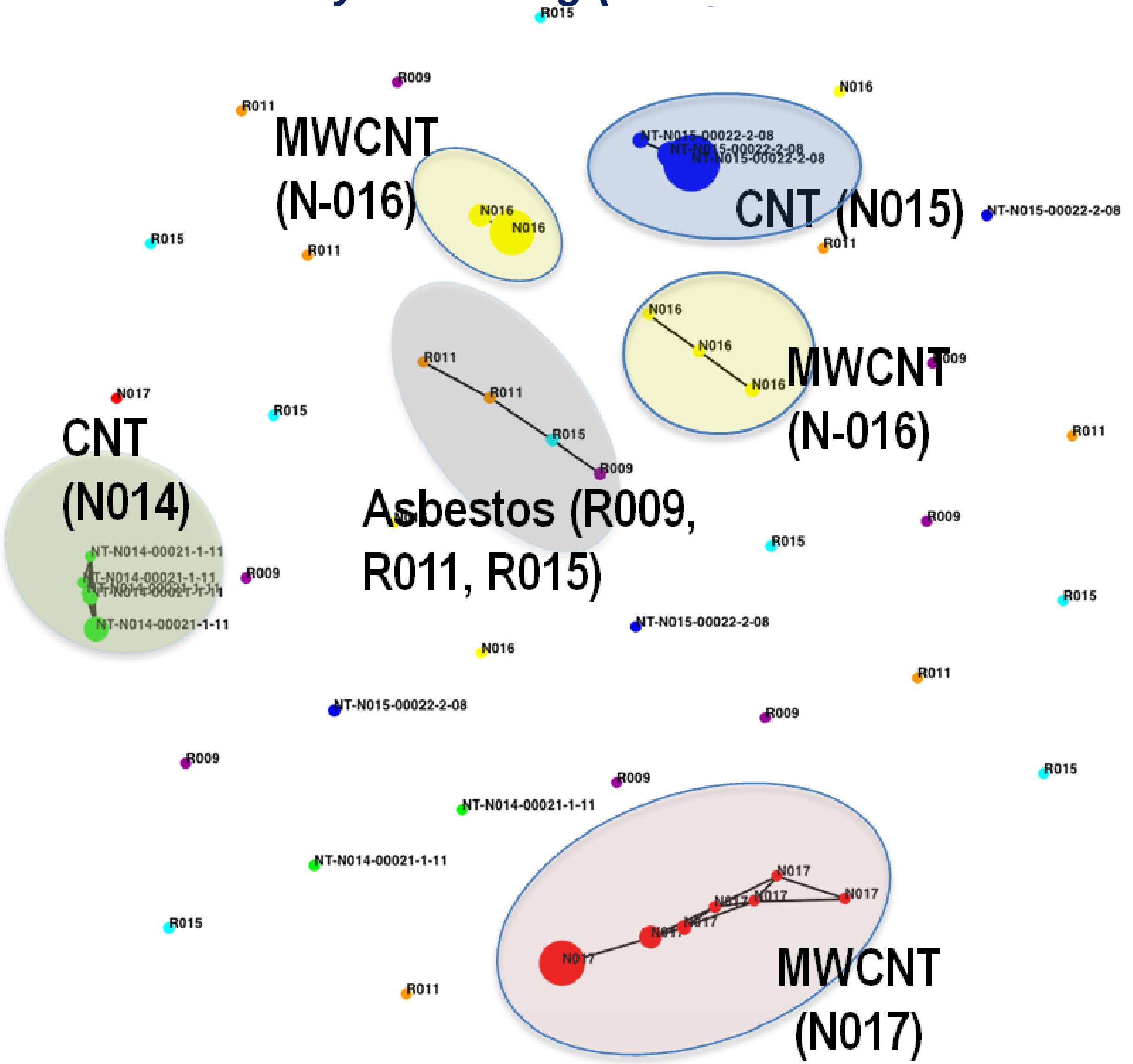
Similarity of Asbestos Inflammation Profiles



- R009 (micro amosite), R011 (micro tremolite) and R015 (micro amphibole) had highly similar profiles and were primarily active in epithelial cell-containing BioMAP systems (3C, 4H, LPS, SAg)

CNT and Asbestos Differences in Inflammatory Response Profiles

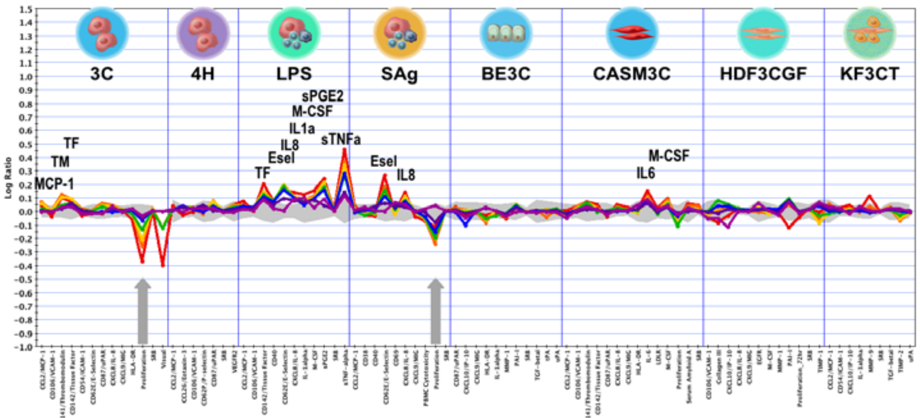
Similarity clustering (Pearson's correlation coefficient > 0.7)



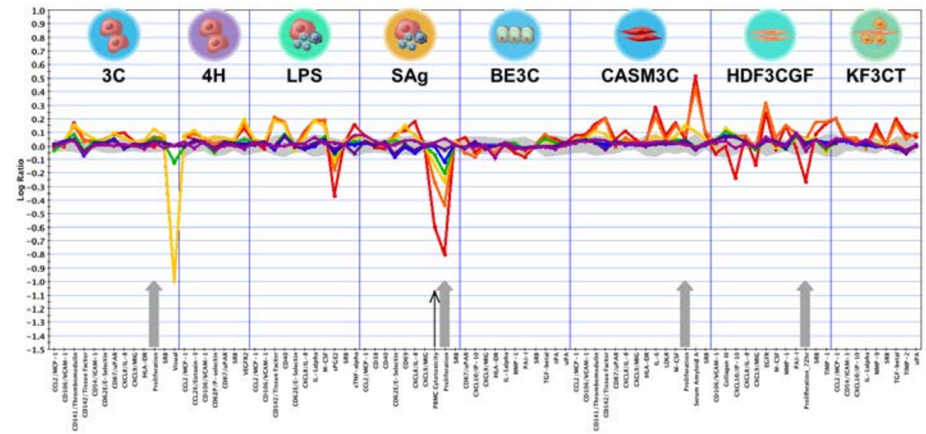
- Asbestos at highest test concentrations had similar profiles
- Same CNT at different concentrations, had similar profiles
- CNT and asbestos did not appear similar in BioSeek assays

CNTs Showed Sample-Specific Response Profiles

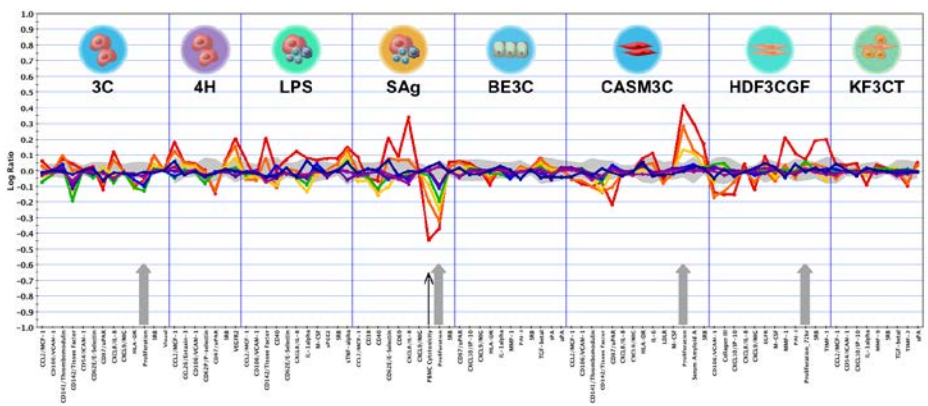
N014



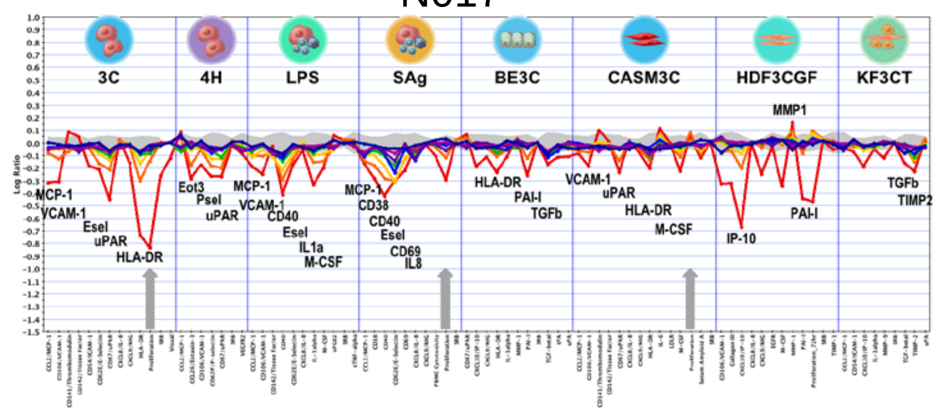
N015



N016



N017



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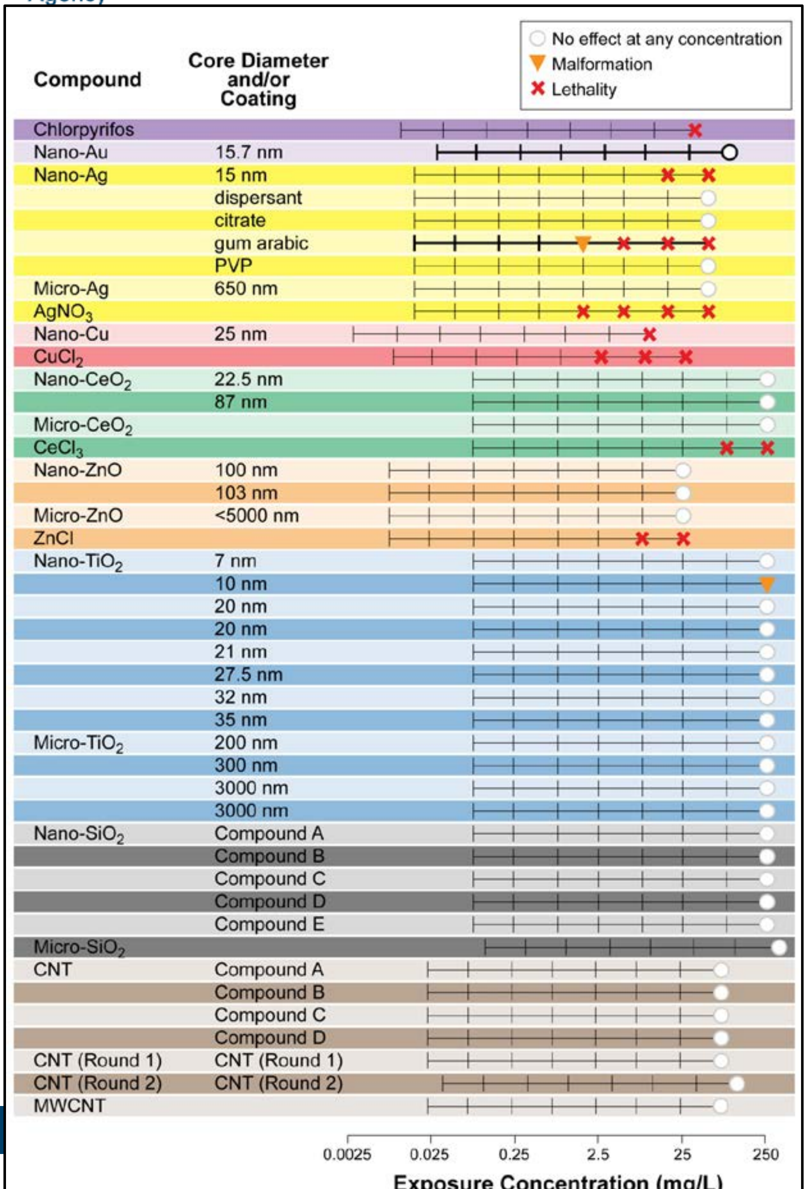
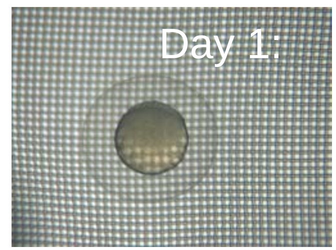
Zebrafish Embryo Developmental Assay



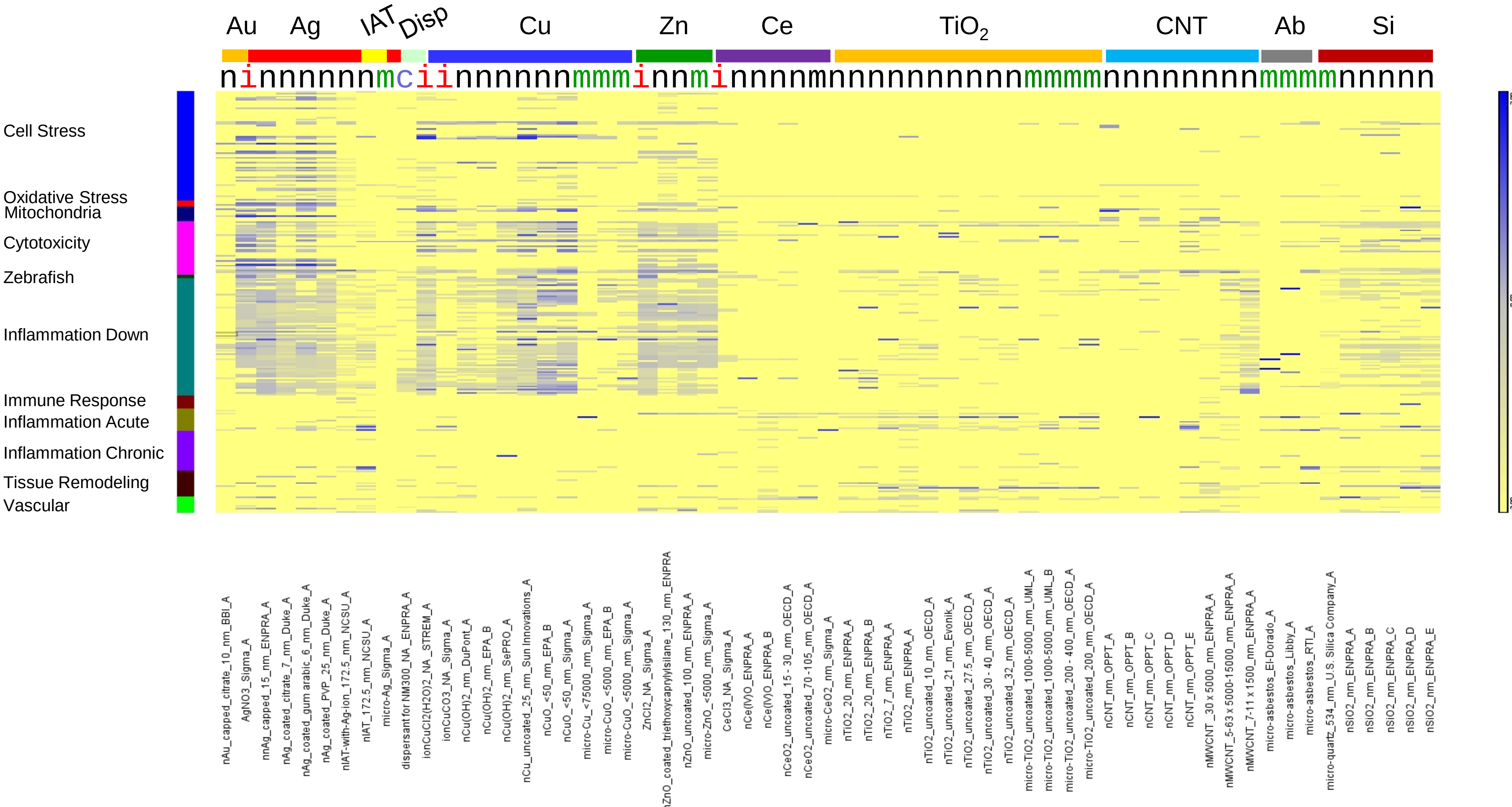
No effect at any concentration

Malformation

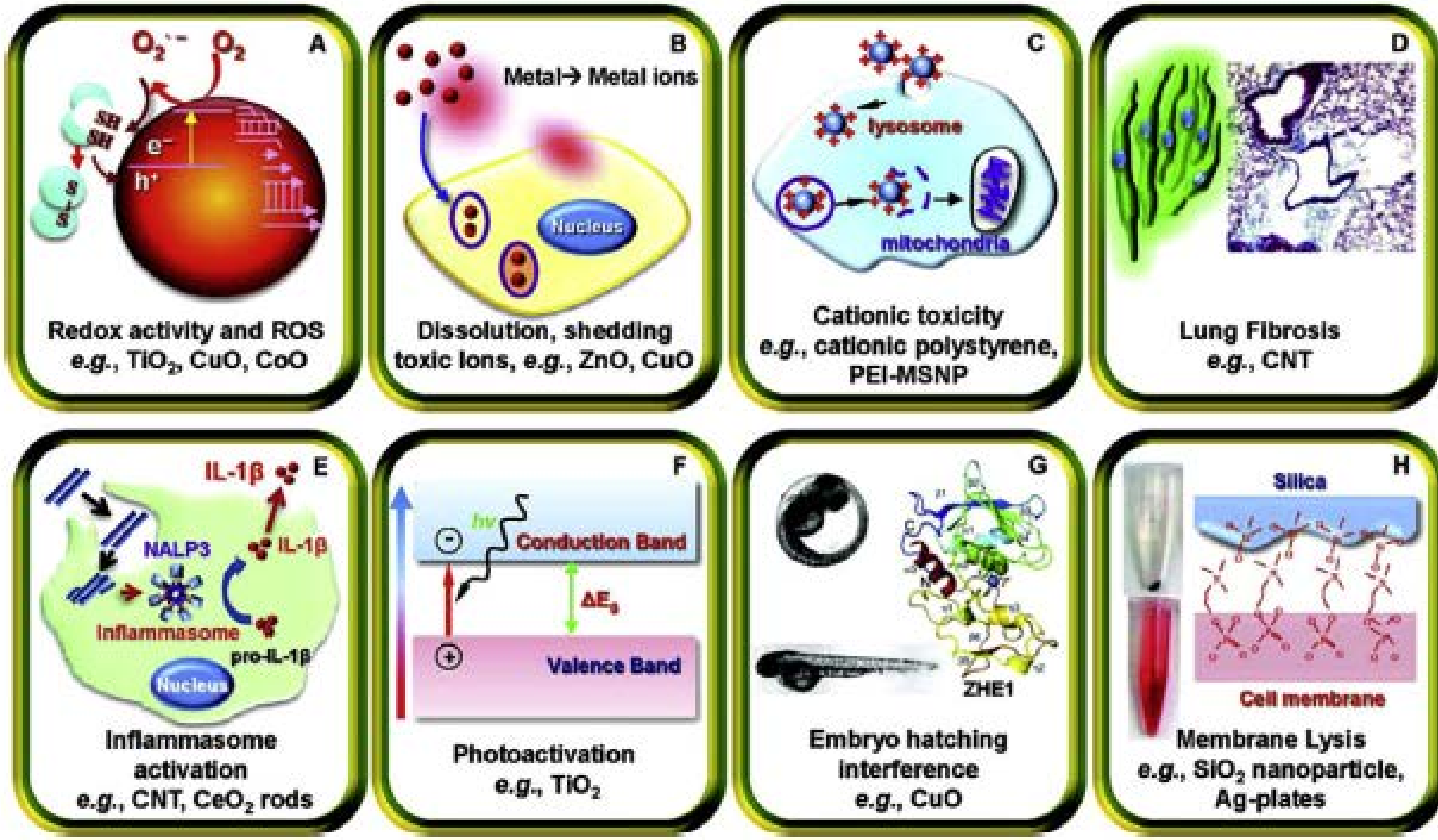
Lethality



NM Screening Results



Proposed Nanomaterial Mechanisms of Toxicity



Nel *et al*., Acc. Chem. Res. 46, 607-621, 2013.

Summary of Screening

- Ag, Cu, Zn much more active than other materials
  - Primarily cell stress/oxidative stress and cytoxicity
  - Ion and nano had very similar behavior; micro generally lower activity
  - Supports ion shedding as mechanism of toxicity of these metal nanomaterials
- CNTs, SiO2, TiO2 had lower levels of activity
  - Wider range of individual sample variation
  - Primarily inflammatory endpoints upregulated
  - Low cytotoxicity
- Au, Ce, additional CNTs, SiO2, TiO2 had very low activity
  - Little to no cytotoxicity or cell stress markers induced
  - Few inflammatory markers induced

Summary of Challenges

- Characterization of NM physicochemical properties is limited by available technology and time
- Testing materials were not selected specific for testing structure-activity relationship
- Assay predicting power is unknown
  - For predicting chronic effects: most assays are 24 hr exposure
  - Assay model may not be appropriate: e.g. lung effects may depend on macrophages phagocytizing NMs
  - Very limited *in vivo* data available

Conclusions

- HTS for profiling NMs is feasible
- Critical to couple physicochemical analysis to HTS testing (which may be rate-limiting)
- What is dose?
  - Aggregation
  - Sedimentation
  - Dissolution
  - Cell permeability
- Could design to address specific questions, e.g. SAR for ROS generation with modified experimental design
- Probably much more significant *in vitro* to *in vivo* extrapolation problems than soluble chemicals due to poor modeling of ADME *in vitro*
  - How to disperse?
  - Flow needed?
  - 3D and/or co-cultures needed?

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