NTP Medium-throughput Toxicity Screen using *C. elegans* - WormTox

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Advantages of Alternative Species

- EPA requiring multiple species in toxicological test
- Most agencies are encouraging the use on non-vertebrate species
- Fewer or no animal welfare concerns
- Genetics/transgenics
- Rapid assays
- Lower cost

Typical Rodent Study

- Animals: 10,000 – 20,000
- Time: 1 - 2 years
- Cost: $2,000,000 – 3,000,000

Typical *C. elegans* Study

- Animals: 100 – 200,000*
- Time: 3 – 5 days
- Cost: $100's

What can you monitor in medium throughput format?

- Growth
- Movement
- Feeding
- Reproduction
- Size
- Shape
- Gene expression
- Development
  - Whole organism
  - Specific cells

- Screen using
  - Wild-type *C. elegans*
  - Genetic mutations
  - Transgenic nematodes
  - Knockout Library

Characteristics of *C. elegans*

- Non-parasitic nematode
- ~ 1 mm in length
- Transparent
- Easily grown in the laboratory
- Animals synchronously develop through four distinct larval stages into adults
- 10 day life span
- 3.5 day developmental cycle
- *C. elegans* can be grown in sufficient quantities for biochemical studies
- Cell and developmental biology are understood in exceptional detail.
- Cell lineage's are known for the entire developmental program
- Amenable to classic and molecular genetic analysis
- Small genome (2 x10^8 base pairs)
- 21,700 predicted ORFs
- *C. elegans* genome completely sequenced
- Transgenic technology

Conservations Between *C. elegans* and Mammals

- Neurotransmitters
  - Dopamine
  - Acetylcholine
  - GABA
  - glutamate
  - serotonin
  - nitric acid
- Diseases
  - Cancer
  - ALS
  - Lysosomal storage disease
  - Polycystic kidney disease
  - Huntington's disease
  - Parkinson's disease
- Basic metabolic proteins
- Stress response
- Cell cycle control
- Signal transduction pathways
  - Insulin
  - Retinoic Acid
  - MAPK/Ras
  - Toll
  - p53
  - TGF
  - WNT

Project Tasks

Task 1. Develop methods to measure the toxicity of developmental and neurological toxicants This task involves the development of computer and image analysis software for monitoring growth, size, reproduction and movement. It also requires development of a 96-well format for growth, dosing and toxicity testing.

Task 2. Expose *C. elegans* to at least 200 known or suspected developmental and/or neurological toxicants and determine changes in phenotypic characteristics (survival, size, growth, reproduction and movement).

Task 3. Create and/or obtain GFP-based, stress-responsive transgenic *C. elegans* for improving sensitivity and specificity of toxicity screens. This task will also include the development of multi-dimensional (3-D, 4-D) computer imaging software to quantitatively measure the effects of toxicant exposure on nervous system development.

Task 4. Use *C. elegans* microarray analysis and test a subset of chemicals from Task 2.

Task 5. Adapt methods for high throughput analysis to assess the toxicological responses in *C. elegans* in which each gene has been inactivated using RNA interference.

Infrastructure

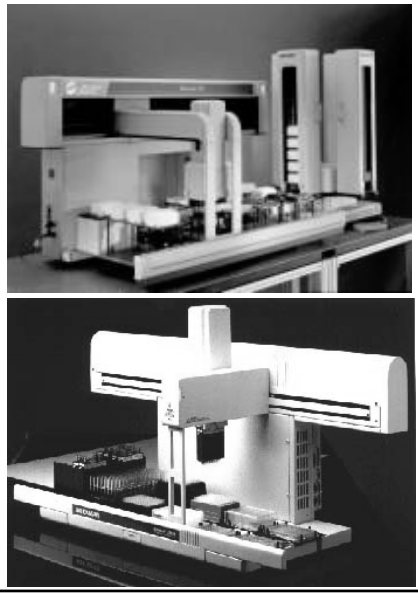
Titertek MAP C2 Agar Dispenser

- Fill 96-well plates with precise volumes (± 1%)
  - Agar
  - Liquid growth medium
  - Bacteria (*C. elegans* food)

96- and 24-well sample preparation

Liquid Handling Robots

- Biomek FX
  - Toxicant addition
  - Bacterial colony replication
- Biomek 2000
  - Toxicant dilution
  - Master plate preparation

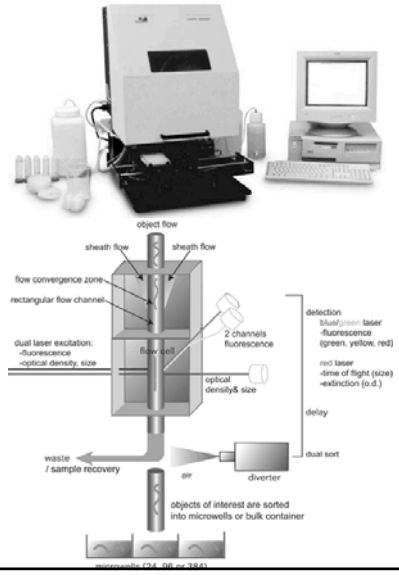


COPAS Biosort

(Complex Object Parametric Analyzer and Sorter)

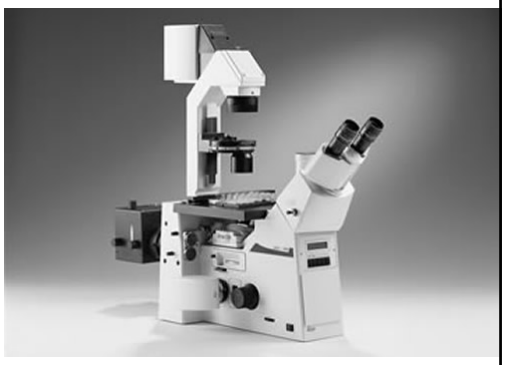
- Dispense *C. elegans* (exact numbers at specific developmental stages)
- Count/Sort nematodes
  - 96-well format
  - Live versus dead
  - Developmental stage
  - GFP-expressing versus non-expressing

- Mutant screens
- Growth rates
- Population distributions
- Level of stress-responsive gene expression



Microscopy

- Microscopes
  - –Inverted motorized
  - –GFP dissecting
  - –Zeiss Confocal



- Automated, multi-well measurement
- Motion tracking
- Size distribution
- Z-series

- 3-D rendering
- Phenotype characterization

Toxicological Tests

Current Assays

- Growth
- Feeding
- Reproduction/Fecundity

Standard Protocol

- 12 concentrations, geometric dose-response
- n = 7 for each concentration
- Each assay is repeated a minimum of three times

- One month per chemical
- Six chemicals per month

Chemicals Tested

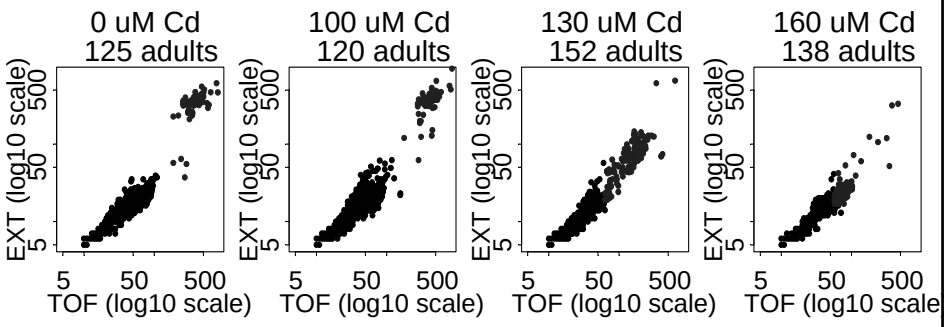
Ref #		CAS #	NTP studies
1	Acetaminophen*	103-90-2	T,C,R,G
2	Acetic Acid	64-19-7	G
3	a-Cyclodextrin	10016-20-3	D
4	AgNo3	7761-88-8	N/A
5	AlCl3	10124-27-3	N/A
6	All-trans Retinoic Acid	302-79-4	D
7	Ascorbic acid**	50-81-7	T,C,R,G
8	AsNaO2	7784-46-5	I
9	b-Cyclodextrin	7585-39-9	N/A
10	bCyclodextrin hydrate	68168-23-0	N/A
11	Caffeine	58-08-2	T,R,D,G
12	Carbaryl	63-25-2	D
13	CdCl2	10108-64-2	I,G
14	Chlorpyrifos	2921-88-2	R,I,G
15	CoCl2	7646-79-9	N/A
16	CrO3	1333-82-0	N/A
17	CuSO4	7758-99-8	T,G
18	Demeton-S-methylsulfone	17040-19-6	N/A
19	Dichlorvos	62-73-7	T,C,G
20	Diphenylhydantoin	57-41-0	T,C,G
21	Diquat	6385-62-2	T
22	DMSO	67-68-5	G
23	EMS	62-50-0	D,G
24	ENU	759-73-9	D,G
25	Ethephon	16672-87-0	N/A
26	EtOH	64-17-5	C,R,D,G
27	Fipronil	120068-37-3	N/A
28	Fumonisin	116355-83-0	T,C,D,G
29	Glyphosate*	1071-83-6	T,G

Ref #		CAS #	NTP studies
30	Juglone	481-39-0	G
31	Lindane	58-89-9	T,C,G
32	Mercuric Chloride	7487-94-7	T,C,G
33	Metam Sodium	6734-80-1	N/A
34	Methadone hydrochloride	1095-90-5	I
35	Methanol	67-56-1	G
36	Methyl cellulose	9004-67-5	N/A
37	Methyl Mercury	16056-34-1	N
38	Methyl Parathion	298-00-0	T,C,G
39	Methylisothiocyanate	556-61-6	N/A
40	MMS	66-27-3	D,G
41	MNNG	70-25-7	D,G
42	Monocrotophos	6923-22-4	N/A
43	MoO3*	1313-27-5	T,C,G
44	Na2SeO3	10102-18-8	T
45	Nicotine	54-11-5	G
46	NiSo4 heptahydrate	10101-98-1	T,C,G
47	Paraquat	1910-42-5	G
48	Parathion	56-38-2	T,C,G
49	Pb(CH3COO)2•3H2O*	6080-56-4	R
50	PbNO3*	10099-74-8	N/A
51	PCB mixture*	TEFPCBMIX	C
52	PEG-60	25322-68-3	G
53	Pyridine	110-86-1	T,C,G
54	Tamoxifen*	54965-24-1	R
55	Tebuconazol	107534-96-3	D
56	Thimerosal	54-64-8	G
57	V2O5*	1314-62-1	T,C,I,G
58	Valproic acid	99-66-1	G

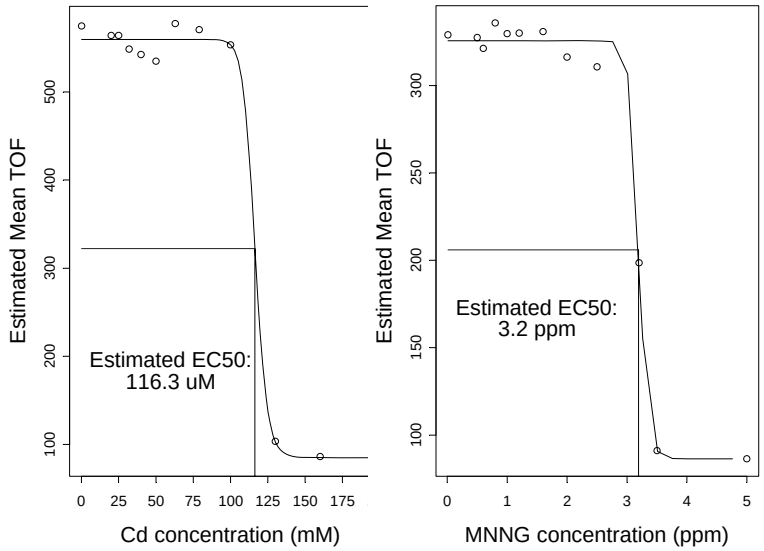
Protocol for Growth

1. Prepare 96-well plate with the Biomek 2000
   - K-medium, toxicant, and *E. coli*
2. Load 25 L1 stage nematodes to each well using COPAS Biosort. Read OD.
3. Incubate at 20 C for 72 hours. Read OD.
4. Analyze nematodes using the COPAS Biosort.

Effect of Cadmium on Growth



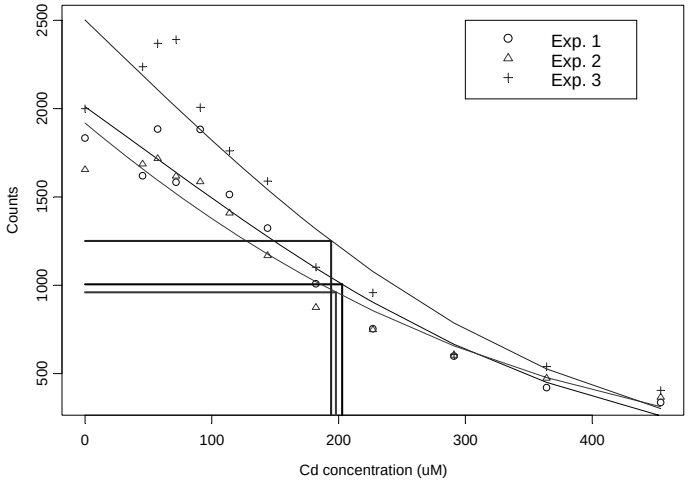
Growth: Dose - Response



Reproduction Protocol

1. Prepare 96-well plate with the Biomek 2000
   - K-medium, toxicant, and *E. coli*
2. Load 5 L4 stage nematodes to each test well using COPAS Biosort. Read OD.
3. Incubate at 20 C for 48 hours. Read OD.
4. Count nematodes using the COPAS Biosort.

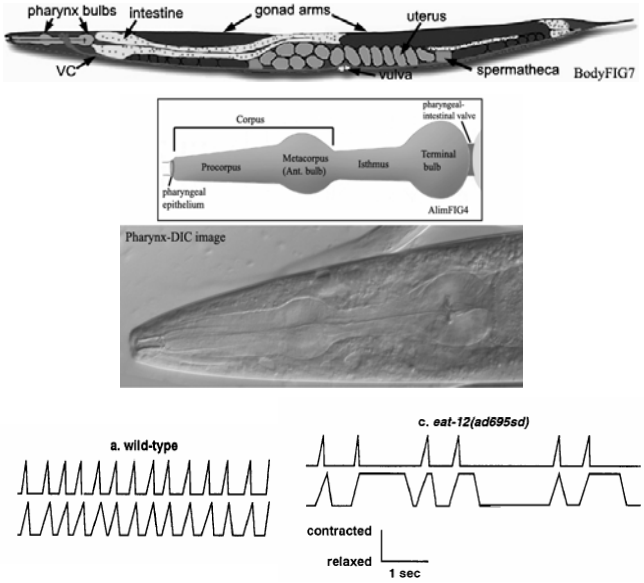
Effect of Cadmium on Reproduction



Protocol for Feeding

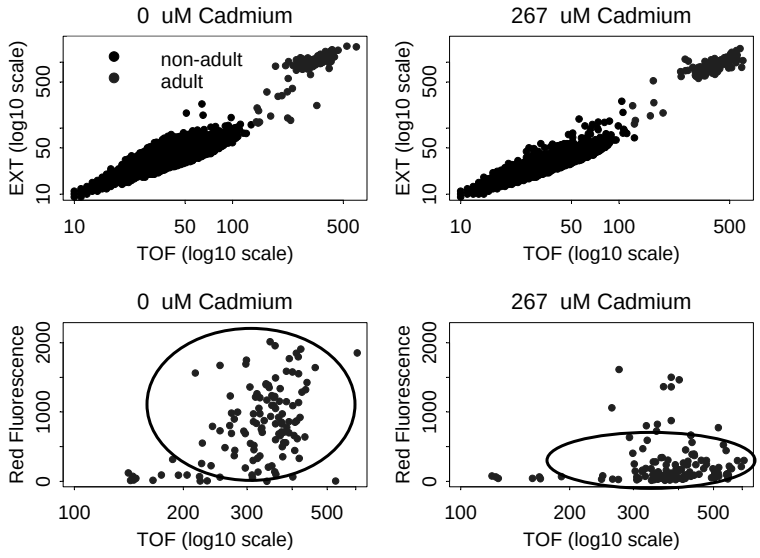
1. Prepare 96-well plate with the Biomek 2000
   - K-medium, toxicant, and *E. coli*
2. Load 25 adult nematodes to each test well using COPAS Biosort. Read OD.
3. Incubate at 20 C for 24 hours. Read OD.
4. Add red microspheres to each well.
5. Incubate for 15 minutes.
6. Add sodium azide to inhibit further feeding.
7. Count nematodes and measure the fluorescence using the COPAS Biosort.

Contraction of corpus and terminal bulb

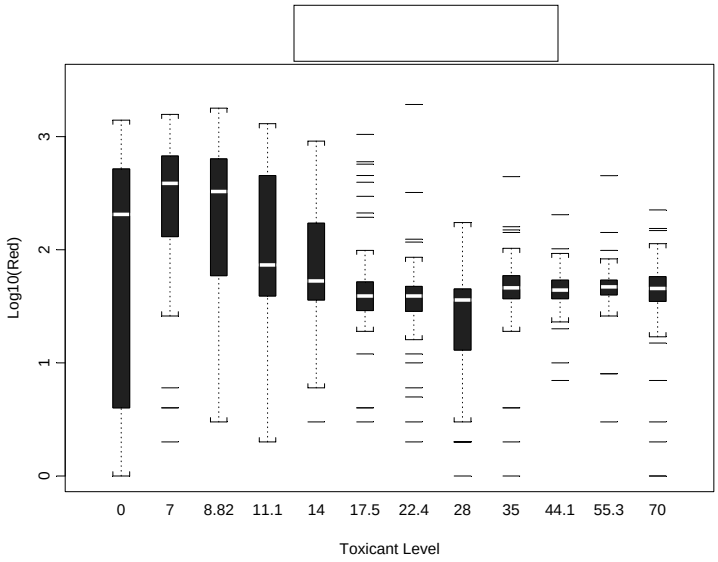


Avery 1993

Effect of Cadmium on Feeding



Effect of MMNG on Feeding



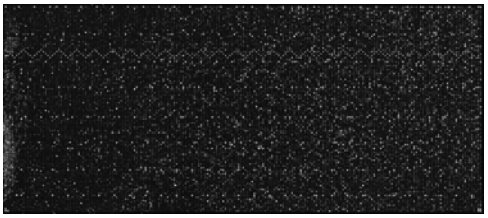
Transgenic Nematodes (GFP-based)

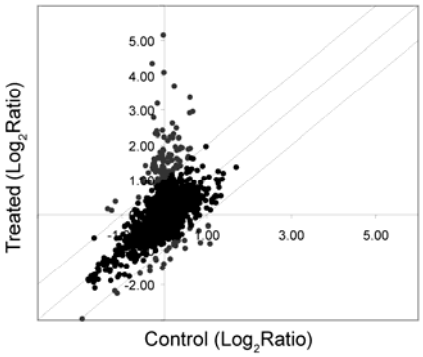
- Metal responsive: cdr-1, mtl-1, mtl-2
- Phase I and II Biotransformation
  - CYPs: 80 CeCYPs (e.g. cyp-35(A1-A5,B1-2,C1), cyp-31A, cyp-34A, cyp-29A)
  - Cyt b5: vem-1
  - GSTs: 36 CeGSTs
  - UDPGTs: 23 CeUDPGTs
  - Carboxylesterases: 17 Ce
- Apoptosis: egl-1, ced-3, csp-1, csp-2, csp-3
- Heat Shock Protiens: 22 CeHSPs
- Vitellogenins: vit-1 to vit-6
- Acetylcholinesterases: ace-1, ace-2, ace-3, ace-4
- MAP Kinases: mek-1, pmk-1

Genomics

- Agilent *C. elegans* custom microarrays
  - Based on ~21K predicted OFRs

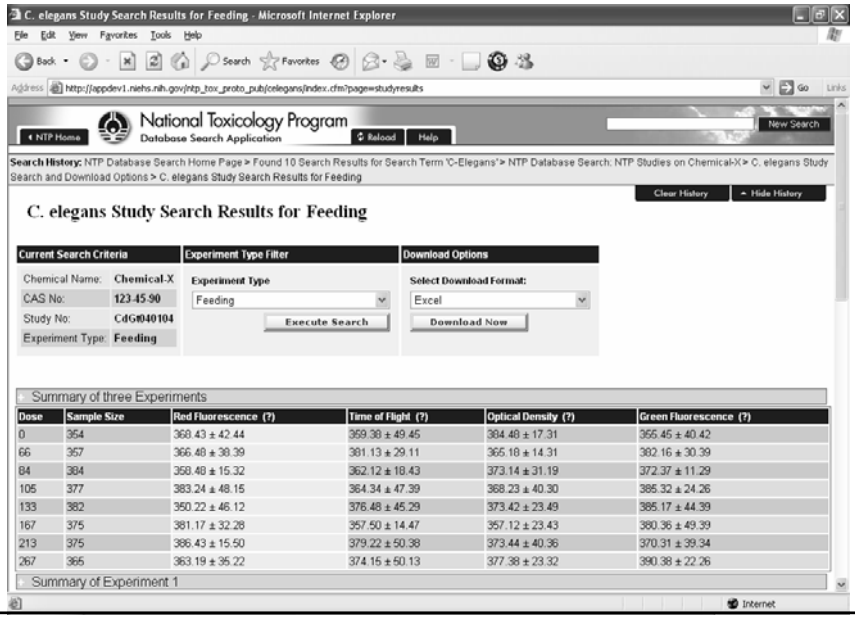
- Cadmium
- NMMG
- Diquat
- Fumonisin



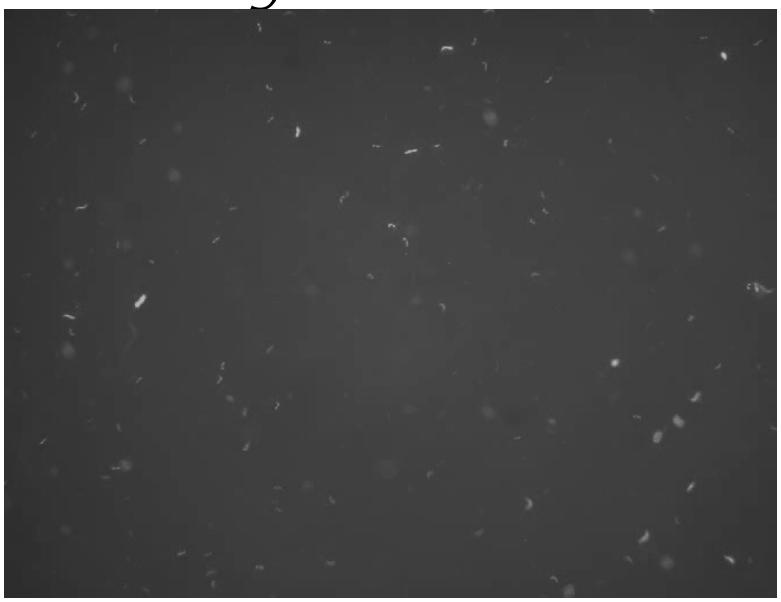


Current Developments

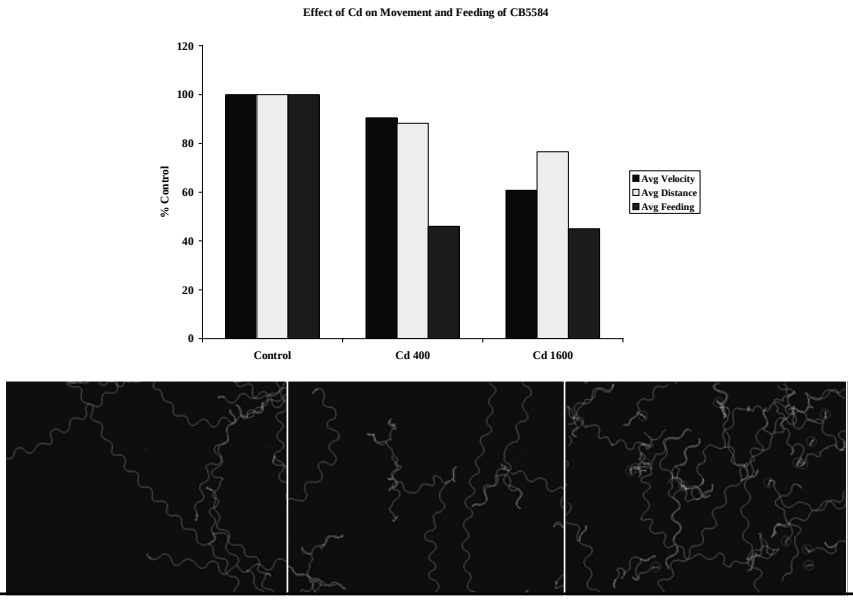
Public Accessible Database



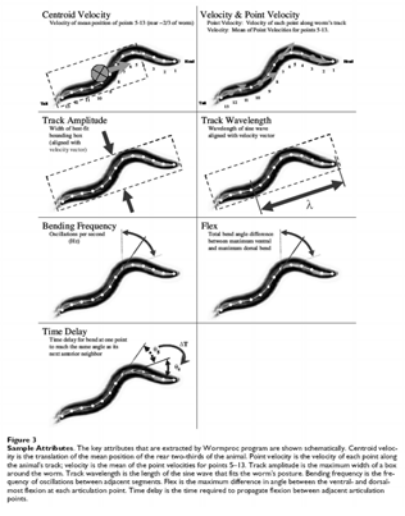
C. elegans in Motion

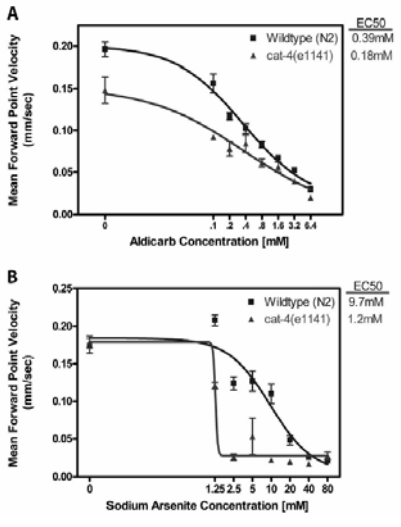


Effect of Cadmium on Movement



Alternative Tracking Software





Cronin et al. 2005

Alternative tracking software…

Table 1: Features measured by the automated system. List of the behavioral and morphological features. Detail algorithms to out these features are found in supplemental data. 144 statistical results (mean, maximum and minimum where applicable) of these features are output into Microsoft Access, while the values of these feature at each time when an image is grabbed are saved in Micro Excel.

Features	Comments	Catalog
Area	Total area of worm	Body morphology
BendingFrequency	Frequency of body bends	Wave form/speed
CmpctFactor	Compactness factor	Body posture
ElgFactor	Elongation factor	Body posture
ElfMAxis	Best-fit ellipse, major axis	Body posture
ElfRatio	Best-fit ellipse, major axis/minor axis	Body posture
EqvElfRatio	Equivalent ellipse ratio	Body posture
Fatness	Area/Length	Body morphology
Flex	Maximum skeleton point angle difference	Wave form
Foraging	Frequency of sideways (foraging) head movements	Specific behavior (feeding)
Foraging angle	Angle of foraging movements	Specific behavior (feeding)
Foraging distance	Distance moved by head during foraging	Specific behavior (feeding)
FRE	Frequency of angle change between skeleton points	Wave form/speed
GlbMvScope	Maximum distance moved from starting point	Global movement
GlbSpd	Speed of the animal's centroid movement	Global movement
HdTlRatio	Ratio of head to tail movement speed	Head movement
Heywood	Heywood circularity factor	Body morphology
Hydraulic	Hydraulic radius	Body morphology
IXX	Inertia XX	Body morphology
IXY	Inertia YY	Body morphology
IYY	Inertia XY	Body morphology
LclSpd	Local movement speed	Local speed
Length	Distance from head to tail	Body morphology/posture
LengthToPixel	Length/number of pixels in skeleton	Body posture
Loop	Percentage of time worms coils their body	Specific behavior (coil)
MaxInterCept	Max intercept	Body morphology
MeanIntcptPpdcl	Mean perpendicular intercept	Body morphology
MostPopularArea	Mode of area	Body morphology
MostPopularSpeed	Mode of speed	Global speed
PercentageMPArea	Frequency of occurrence of modal area	Body morphology
PercentageMPSpeed	Frequency of occurrence of modal speed	Global speed
Pushing	Local body movement speed/centroid speed	Locomotion wave efficiency
RectBigSide	Minimum enclosing rectangle (MER) length	Body posture
RectRatio	Minimum enclosing rectangle length/width ratio	Body posture
Reversal	Percentage of time that a worm performs reversals	Specific behavior (escape)
ReversalCount	Number of reversal sessions that a worm performs	Specific behavior (escape)
ReversalDisAve	Average distance traveled backward in reversals	Specific behavior (escape)
SktAgAve	Mean value of skeleton point vector angle	Body bending
SktCmpctFactor	Length of worm / oriented MER area	Body posture
SktElgFactor	Skeleton enlongation factor	Body posture
SktHght	Oriented MER height	Body posture
SktXX	Sum of x coordinate times x coordinates of skeleton points	Body posture
SktXY	Sum of x coordinate times y coordinates of skeleton points	Body posture
SktYY	Sum of y coordinate times y coordinates of skeleton points	Body posture
SktvAgAve	Average angle between skeleton points and centroid	Body bending
SktvAgMax	Max angle between skeleton points and centroid	Body bending
SktvDisAveToLength	Average distance between skeleton points and centroid/length	Body bending
SktvDisMaxToLength	Max distance between skeleton points and centroid/length	Body bending
SktvDisMinToLength	Min distance between skeleton points and centroid/length	Body bending
SktWidth	Oriented MER width	Body posture
Theta	Direction of centroid movement	Global movement
Thickness	Thickness of worm	Body morphology
TotalTravelDistance	Total distance traveled by a worm	Global movement
TrackAmplitude	Amplitude of waves in worm's track	Wave form
TrackWavelength	Wavelength of waves in worm's track	Wave form
Transparency	Transparency of worm body	Body morphology
Turn	Percentage of time that a worm performs a sharp turn	Specific behavior (search)

Feng et al. 2004

96-Well HTS Repro Study Plate Setup

	1	2	3	4	5	6	7	8	9	10	11	12
A	W	C1	W	C9	W		C17	W	C25	W		
B	W	C2	W	C10	W		C18	W	C26	W		
C	VC	C3	W	C11	W	+C	C19	W	C27	W	VC	
D	W	C4	W	C12	W	W	C20	W	C28	W	W	
E	VC	C5	W	C13	W	VC	C21	W	C29	W	+C	W
F	W	C6	W	C14	W	W	C22	W	C30	W	W	W
G	+C	C7	W	C15	W	VC	C23	W	C31	W	VC	W
H	W	C8	W	C16	W	W	C24	W	C32	W	W	W

6 vehicle control wells
3 positive control wells
32 chemical wells

Summary of HTS Results

- Completed two runs of the 1408
  - Three months for completion
  - 61 hits for both replicates
  - 182 hits for one replicate
- Future Development
  - Start HTS at 10 μM???
  - One more rep at 100 μM???
  - Generate dose-response curves for hits

100 μM HTS - Top 25 Hits

Chemical_Name	Chem Num	Counts	Perc	Pval	Counts	Perc	Pval
p-Toluenesulfonamide	1089	5	-0.974	0.131	14	-0.932	0.167
Actein	1102	7	-0.963	0.124	4	-0.981	0.137
Chlorpheniramine maleate	457	10	-0.952	0.141	5	-0.971	0.358
Isobutyl alcohol	997	15	-0.946	0.125	9	-0.96	0.129
Ninhydrin	951	13	-0.944	0.133	27	-0.857	0.146
N-Acetyl-p-toluidine	323	10	-0.942	0.13	22	-0.893	0.374
Cyclohexene oxide	1182	11	-0.941	0.118	13	-0.936	0.131
Hexyl cinnamic aldehyde	167	16	-0.933	0.319	20	-0.891	0.327
1,3-Dimethyl-4-nitrobenzene (4-Nitro-m-xylene)	579	18	-0.928	0.135	18	-0.903	0.137
Colchicine	1221	14	-0.922	0.125	9	-0.942	0.153
5-Methyl-2-nitroaniline	644	18	-0.922	0.141	21	-0.912	0.148
Adiponitrile	372	18	-0.915	0.125	94	-0.513	0.32
Cobaltocene	942	20	-0.914	0.138	19	-0.899	0.144
Lauryl chloride	1014	20	-0.911	0.145	62	-0.777	0.152
Glutaraldehyde (Glutaric dialdehyde)	945	17	-0.91	0.128	23	-0.901	0.137
Sodium lauryl sulfate	1200	20	-0.901	0.135	25	-0.883	0.136
a-Solanine	1207	21	-0.901	0.161	13	-0.935	0.161
boron trifluoride dihydrate	171	26	-0.891	0.33	16	-0.913	0.331
2-Amino-4-phenylthiazole HBr H2O	746	24	-0.889	0.158	86	-0.661	0.224
n-Octylamine	185	21	-0.885	0.324	15	-0.937	0.338
Triton X-100	1224	23	-0.872	0.136	28	-0.821	0.139
2-Nitropropane	456	27	-0.871	0.14	40	-0.771	0.314
7-Methylquinoline	720	35	-0.865	0.138	87	-0.674	0.212
1,3-Dinitronaphthalene	537	29	-0.855	0.13	35	-0.812	0.195
Pentachlorophenol	378	34	-0.84	0.14	131	-0.321	0.352

Data Analytic Challenges in WormTox

- Goal: Model nematode populations using a 4-dimensional distribution
  - Time of Flight (TOF): length
  - Extinction (EXT): optical density
  - Fluorescence: green, red or yellow
- Develop statistical algorithms to:
  - Classify nematodes into discrete growth stages
  - Characterize statistical properties of nematode populations at different developmental stages and toxicant exposures
  - Rapidly analyze large volumes of data

Manuscripts using WormTox

- Meyer, J.N., Boyd, W.A., Azzam, G.A., Haugen, A.C., Freedman, J.H., and Van Houten, B. Decline of nucleotide excision repair capacity in aging *Caenorhabditis elegans*. (Submitted Genome Biol.)
- Alper, S., McBride, S.J., Lackford, B., Freedman, J.H., Schwartz, D.A. Specificity and Complexity of the *C. elegans* innate immune response. (Submitted. Proc. Nat. Acad. Sci. U.S.A.)
- Cui,Y., Boyd, W.A., McBride, S.J., and Freedman, J.H. Functional Analysis of Cadmium Responsive Transcription in *Caenorhabditis elegans*. (To be submitted Genes & Develop.)
- Boyd, W.A., McBride, S.J., Rice, J.R., Snyder, D.W., and Freedman, J.H. Development of a feeding assay for medium-throughput toxicant screening using *C. elegans*. (To be submitted to Nat. Biotech.)

Future Directions

Chemicals

- EPA Priority Chemicals (David Dix, EPA)
- EPA DNT Chemicals (Kevin Crofton, EPA)
- Marine toxins
- Ionic liquids (Michelle Hooth, NTP)
- Nano-materials (Nigel Walker, NTP)
- NTP 1408 (ver. 2)

New Transgenic Strains

SCIENTIFIC FRONTIERS IN DEVELOPMENTAL TOXICOLOGY AND RISK ASSESSMENT
Committee on Developmental Toxicology
Board on Environmental Studies and Toxicology
Commission on Life Sciences
National Research Council

New Transgenic Strains

- Wnt pathway via β–catenin and JNK
- Receptor serine/threonine kinase (TGF-β receptor) pathway
- Receptor tyrosine kinase pathway (small G-protein [Ras] linked)
- Nuclear hormone receptor pathway
- Notch-Delta pathway
- Stress responses and checkpoints for DNA damage and replication.
- Receptor-linked cytoplasmic tyrosine kinase (cytokine) pathway
- Integrin pathway
- Cadherin pathway
- Gap junction pathway
- Ligand-gated cation channel pathway
- A stress response: The unfolded protein response (UPR)
- G-protein coupled receptor (large G-protein) pathway
- Apoptosis pathway (cell death pathway)
- Receptor protein tyrosine phosphatase (RPTPs) pathway
- Receptor guanylate cyclase pathway

High Throughput Screening

- Goal: identify "hits" using vehicle control and positive control information
- Issues
  - Variability of reproduction counts
    - Day to day, plate to plate, within plate
  - Balance between # vehicle control wells & chemical reps
  - Choice of number of nematodes,
  - Exposure time
  - Positive controls: single concentration or dose-response curve
  - Carryover rates and number of rinse wells

Long Term Directions

- Continued support for DIR and NTP studies
- Transcriptome analysis
  - Currently all transcriptomics are DIR-based research projects
  - Should WormTox collect microarray data for other chemicals
- The function of WormTox in the NTP
  - Is it "Research and Development" or "Manufacturing"
  - Put out contract request for others to do WormTox activities

The WormTox Group

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