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TITLE:

A High-throughput Assay for the Prediction of Chemical Toxicity by Automated Phenotypic Profiling of *Caenorhabditis elegans*

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KEYWORDS:

Chemicals, toxicity, *Caenorhabditis elegans*, image analysis, phenotype, quantification

SUMMARY:

A quantitative method has been developed to identify and predict the acute toxicity of chemicals by automatically analyzing the phenotypic profiling of *Caenorhabditis elegans*. This protocol describes how to treat worms with chemicals in a 384-well plate, capture videos, and quantify toxicological related phenotypes.

ABSTRACT:

Applying toxicity testing of chemicals in higher order organisms, such as mice or rats, is time-consuming and expensive, due to their long lifespan and maintenance issues. On the contrary, the nematode *Caenorhabditis elegans* (*C. elegans*) has advantages to make it an ideal choice for toxicity testing: a short lifespan, easy cultivation, and efficient reproduction. Here, we describe a protocol for the automatic phenotypic profiling of *C. elegans* in a 384-well plate. The nematode worms are cultured in a 384-well plate with liquid medium and chemical treatment, and videos are taken of each well to quantify the chemical influence on 33 worm features. Experimental results demonstrate that the quantified phenotype features can classify and predict the acute toxicity for different chemical compounds and establish a priority list for further traditional chemical toxicity assessment tests in a rodent model.

INTRODUCTION:

Along with the rapid development of chemical compounds applied to industrial production and people's daily life, it is important to study the toxicity testing models for the chemicals. In many cases, the rodent animal model is employed to evaluate the potential toxicity of different chemicals on health. In general, the determination of lethal concentrations (i.e., the assayed 50% lethal concentration [LC50] of different chemicals) is used as the traditional parameter in a rodent (rat/mouse) model in vivo, which is time-consuming and very expensive. Due to the reduce, refine, or replace (3R) principle that is central to animal welfare and ethics, new methods that allow for the replacement of higher animals are valuable to scientific research[1–3]. *C. elegans* is a free-living nematode that has been isolated from soil. It has been widely used as a research organism in the laboratory because of its beneficial characteristics, such as a short lifespan, easy cultivation, and efficient reproduction. In addition, many fundamental biological pathways, including basic physiological processes and stress responses in *C. elegans,* are conserved in higher mammals[4–8]. In a couple of comparisons we and others have made, there is a good concordance between *C. elegans* toxicity and toxicity observed in rodents[9]. All of this makes *C. elegans* a good model to test the effects of chemical toxicities in vivo.

Recently, some studies quantified the phenotypic features of *C. elegans*. The features can be used to analyze the toxicities of chemicals[2,3,10] and the aging of worms[11]. We also developed a method that combines a liquid worm culturing system and an image analysis system, in which the worms are cultured in a 384-well plate under different chemical treatments[12]. An automated microscope stage is used for experimental video acquisition. The videos are processed by a custom-designed program, and 33 features related to the worms' moving behavior are quantified. The method is used to quantify the worm phenotypes under the treatment of 10 compounds. The results show that different toxicities can alter the phenotypes of *C. elegans*. These quantified phenotypes can be used to identify and predict the acute toxicity of different chemical compounds. The overall goal of this method is to facilitate the observation and phenotypic

quantification of experiments with *C. elegans* in a liquid culture. This method is useful for the application of *C. elegans* in chemical toxicity evaluations and phenotype quantifications, which help predict the acute toxicity of different chemical compounds and establish a priority list for further traditional chemical toxicity assessment tests in a rodent model.

PROTOCOL:

The protocol follows the animal care guidelines of the Animal Ethics Committee of the Beijing Center for Disease Prevention and Control in China.

1. Chemical preparation

1.1. Obtain chemicals (**Table 1** and **Table of Materials**).

1.2. Determine the highest and lowest dosage of the individual chemicals for a minimum concentration of 100% lethality (LC100, 24 h) and a maximum concentration of 100% nonlethality (LC0, 24 h) to worms. Use at least six dilutions of the highest concentration (**Table 1**).

NOTE: Conduct a preliminary worm lethality test[9] to explore LC100 and LC0 for a new chemical, to determine the dosage.

1.3. Dilute each chemical with K-medium (**Table of Materials**) to 2x the required concentration. Use K-medium as a control to compare the phenotype alterations caused by the chemicals.

1.3.1. For example, prepare 7 gradient concentrations of cadmium chloride ($CdCl_2$) (**Table 1**). To prepare 2x the highest concentrated aqueous solution (4.64 mg/mL), dissolve 92.8 mg of $CdCl_2$ solid powder in 8 mL of K-medium and fill up to 10 mL after the powder has fully dissolved. Prepare the other concentration levels by dilution with K-medium.

1.4. Prepare eight parallel wells for every concentration in the chemical gradient. Each well contains 50 µL of the 2x chemical solution. Prepare at least three groups of eight parallel wells of K-medium as a control (**Table 2**).

NOTE: In brief, a volume of 500 µL of 2x working solution is necessary for a single dose of each chemical.

2. Worm preparation

2.1. Obtain wild-type N2 worms and *Escherichia coli* OP50 strains from the Caenorhabditis Genetics Center (CGC).

2.2. Obtain synchronized L4 worms.

2.2.1. Pick a single colony of *E. coli* OP50 from the streak plate. Aseptically inoculate the colony in 100 mL of LB broth and grow it overnight at 37 °C.

NOTE: The *E. coli* OP50 solution is now ready for seeding to nematode growth medium (NGM, **Table of Materials**) plates.

2.2.2. Pour NGM into a 90 mm plastic Petri plate. Seed each plate with 300 µL of *E. coli* OP50 solution the day after pouring. Incubate N2 worms on the NGM plates with OP50 at 20 °C for about 2–3 days until most of the worms have reached the adult stage.

2.2.3. Harvest gravid worms into a 15 mL sterile conical centrifuge tube with sterile H_2O. Let the worms settle down for at least 2 min, aspirate the H_2O, and add 5 mL of bleach buffer (**Table of Materials**).

2.2.4. Vortex the tube for 5 min, spin the tube for 30 s (at 1,300 x *g*) to pellet the eggs, and discard the supernatant.

2.2.5. Wash the eggs with 5 mL of sterile H_2O and vortex the tube for 5 s. Centrifuge the tube for 30 s (at 1,300 x *g*), remove the supernatant, and wash again.

2.2.6. Pipette the eggs onto a new NGM plate with OP50. Incubate them at 20 °C. Monitor the hatched L1 worms the next morning; the worms will reach the L4 stage in approximately 40 h.

2.3. Wash the L4 worms off the 90 mm Petri plates with K-medium into a 50 mL sterile conical tube. Adjust the concentration of worms to ~40 animals per 100 µL of K-medium under a stereomicroscope. Add 50 µL (~20 worms) into each well of the 384-well plate. These synchronized worms (L4 stage) are ready for the following treatment by chemicals.

3. Chemical treatment and video capture

NOTE: In a 384-well plate, worms (50 µL in each well) are treated to six to seven dosages of an individual chemical (**Table 1**). Prepare eight parallel wells, each containing 50 µL of the 2x chemical solution for every dosage (eight wells are filled with the same chemical and the same concentration, **Table 2**). All videos are collected using a digital camera attached to an inverted microscope (**Table of Materials**). The chemical treatment experiment lasts for 24 h. Do not add bacterial food to each well during the 24 h chemical treatment experiment.

3.1. Before adding the chemicals, set the 384-well plate with the synchronized worms on the automatic stage and take videos of each well with the programmed acquisition procedure (7 frames per second for 2 s; it takes ~20 min to scan each plate).

3.2. Add 50 µL of the 2x chemical stock prepared according to section 1 for each well (**Table 2**). Set the time as the 0 h point.

3.3. Incubate the 384-well plate at 20 °C and shake it at 80 rpm in an incubator shaker.

3.4. Remove the plate from the incubator and transfer it to an automatic stage. Take videos of each well of the whole plate, at 12 h and at 24 h, to check the phenotypes of the worms for each specific chemical treatment in K-medium. Approximately 25 min are required for one plate screen.

4. Experiment video processing

NOTE: A program for experimental video and images processing was written and packaged. It can be freely downloaded (see **Table of Materials**). The experimental video is stored in the form of an image frame sequence, and the frame sequence of each video is stored in a specific directory. The program can recognize worms and quantify phenotypes automatically.

4.1. In the graphical user interface (GUI, **Figure 1**), add the parameters, such as the frame sequence directory, the output directory, the worm size parameter, and the movement threshold parameter. Click the **Analyze** button to process the experimental images.

4.1.1. Click the **Select** button to choose the source images directory.

4.1.2. Add the middle result directory in the interface.

NOTE: The middle results include the segmented images. These middle results are useful for the visual observation of the processed images.

4.1.3. Add the final result directory in the interface.

4.1.4. Add the average worm size parameter in the **Worm Size** textbox in the interface.

NOTE: The size parameter used in the experiments is 2,000.

4.1.5. Add the **Threshold of moved ratio** in the interface.

NOTE: The ratio used in the experiments is 0.93.

4.1.6. Click the **Analyze** button to start the image processing. Click the **Reset** button to clear the added parameters.

NOTE: There are 33 features defined and quantified for worms. All the defined phenotypes are sorted by categories (listed in **Table 3**). These features can be quantified from experimental images. A quantitative comparison among different chemicals, which have different toxicities, can be done by comparing these features.

REPRESENTATIVE RESULTS:

We have tested the phenotypes of worms exposed to different concentrations of more than 10 chemicals[12]. In the test, 33 distinct features were quantified for each chemical compound at three

time points (0 h, 12 h, and 24 h). Previously, a comparison between a manual and an automatic analysis of a lifespan assay was done[11,12]. In this assay, we found that chemicals and concentrations can influence the worm phenotypes. An overview of this method is shown in **Figure 2**.

The results (**Figure 3** and **Figure 4c,d**) showed that the worms died quickly as the chemical concentration increased. At higher concentrations, the worms became straighter and less curved than at lower concentrations or in control groups (**Figure 3** and **Figure 4b**). In the beginning (at 0 h), there was no significant difference between the control (K-medium) and chemical treatments for all phenotypes. After 12 h of treatment with a given chemical dosage, the phenotypes of worms showed different degrees of differences among control and different concentration groups. For example, the major axis length increased as time increased. There is also a gradient trend from lower to higher chemical concentrations. The gradient trend of different chemical concentrations was also significant in the minor axis length (**Figure 4a,b**).

In this assay, the worm's motility was calculated in two ways, based on the area the worm moved in and the motility ratio (**Figure 4c,d**). Motility results of both ways showed similar patterns. There were no significant differences of the worm motility among different concentrations and control groups at the beginning (at the 0 h time point). As time passed, the worms in the control groups showed a stable decrease in motility. At 12 h, the worms that underwent chemical treatments at different concentrations showed significant differences in motility compared with control groups. In addition, the worms under higher concentration treatments showed weak motility compared to the worms under lower concentration treatments. This indicates that worms under higher concentration treatments became less motile and died quicker (**Figure 4c,d**). These results suggest that the designed method is useful for chemical toxicity assessments, and the quantified phenotypes of *C. elegans* are useful markers for chemical toxicity identification.

FIGURE AND TABLE LEGENDS:

Figure 1: The interface of the software.

Figure 2: The pipeline of a high-throughput assay for the prediction of chemical toxicity by automated phenotypic profiling of *Caenorhabditis elegans*.

Figure 3: Experimental images of worms under 4.64 mg/mL $CdCl_2$ (upper panel), 0.464 mg/mL $CdCl_2$ (middle panel), and K-medium (bottom panel), at different time points. The images show the status changes of worms under chemical treatment or in a control group in one representative well of the 384-well plate throughout time.

Figure 4: The quantified features of worms under different concentrations of $CdCl_2$. (**a**) The quantified major axis length. (**b**) The quantified minor axis length. (**c**) The quantified motility by the moved area. (**d**) The quantified motility by the moved area/worm size. The bar plots show the average quantification for each feature on single worms. The error bars denote ± standard deviation (SD). The concentration unit = mg/mL.

Table 1: Exposure concentration of 10 chemicals for the 384-well-plate *C. elegans* acute toxicity test.

Table 2: A schematic of the 384-well plate layout.

Table 3: Defined phenotypes of worms.

DISCUSSION:

The advantages of *C. elegans* have led to its increasing usage in toxicology[9], both for mechanistic studies and high-throughput screening approaches. An increased role for *C. elegans* in complementing other model systems in toxicological research has been remarkable in recent years, especially for the rapid toxicity assessment of new chemicals. This article provides a new assay of high-throughput, quantitative screening of worm phenotypes in a 384-well plate for the automatic identification and assessment of chemical toxicity. This assay is ideal for acute toxicity testing of chemicals within 24 h, and it could be applied to subacute toxicity testing as well when more time points of data are collected and food source (OP50) is supplied for the worms.

The medium used for diluting the chemicals can vary; we chose K-medium in the assay by referring to Sofie et al.[13]. Worms were cultured in K-medium in both the control and chemical treatment groups. An artificial freshwater solution or a soil solution with low ionic strength could be alternatives to K-medium.

Chemicals with different toxicities can alter the phenotypes of *C. elegans* in different patterns. Chemicals used in this test were chosen from the third to sixth categories of the Globally Harmonized System of Classification and Labeling of Chemicals (GHS). *C. elegans* were exposed to chemicals at six or more dosage levels, which covered the 0%–100% mortality dosage range. For those chemicals with low water solubility, DMSO is recommended to promote the chemical dissolution in water. As a high concentration of DMSO may affect worm development and lifespan[14], no more than 0.2% DMSO should be used for aquatic tests.

The automatically quantified features show significant difference among different toxicities, which demonstrates that these quantified phenotypes of worms are very useful in identifying the toxicity of chemicals. It indicated that phenotypic profiling revealed conserved functions to classify and predict the toxicity of different chemicals using nematode *C. elegans* as an in vivo model organism.

The US National Toxicology Program (NTP) established the Tox21 community through a memorandum of understanding with the U.S. Environmental Protection Agency (EPA) and the National Institutes of Health (NIH) Chemical Genomics Center, now the National Center for Advancing Translational Sciences (NCATS). Tox21 uses high-throughput in vitro screening and in vivo alternative animal model testing to identify mechanisms of toxicity, to prioritize chemicals for additional in vivo toxicity testing, and to develop predictive models of human toxicological responses. As part of that effort, *C. elegans* was used to screen the EPA's ToxCast Phase I and

Phase II libraries, which contain 292 and 676 chemicals, respectively, for chemicals leading to decreased larval development and growth[15]. The COPAS (Complex Object Parametric Analyzer and Sorter) platform has also been used for the worm toxicological screening studies[2]. However, the COPAS platform only quantifies few features, such as worm width, worm length, and the fluorescence intensity. This method is an improvement to current methods using worms to rapidly prescreen the toxicity of new chemicals.

There are several critical steps within the protocol: the worm culture in a 384-well plate, the chemical treatment, the experimental image capture, and the phenotype quantification. Compared to traditional toxicity evaluation methods, this protocol can quantify some phenotypes of worms that are difficult to calculate manually and useful to reflect the toxicities of every chemical, such as the worm motility, worm width, worm size, and gray intensity. Clearly, this high-throughput assay for the prediction of chemical toxicity will be a valuable toxicity model approach and could be used for the prescreening of chemicals before rodent animal experiments.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

Click here to access/download;Figure;fig1.tif

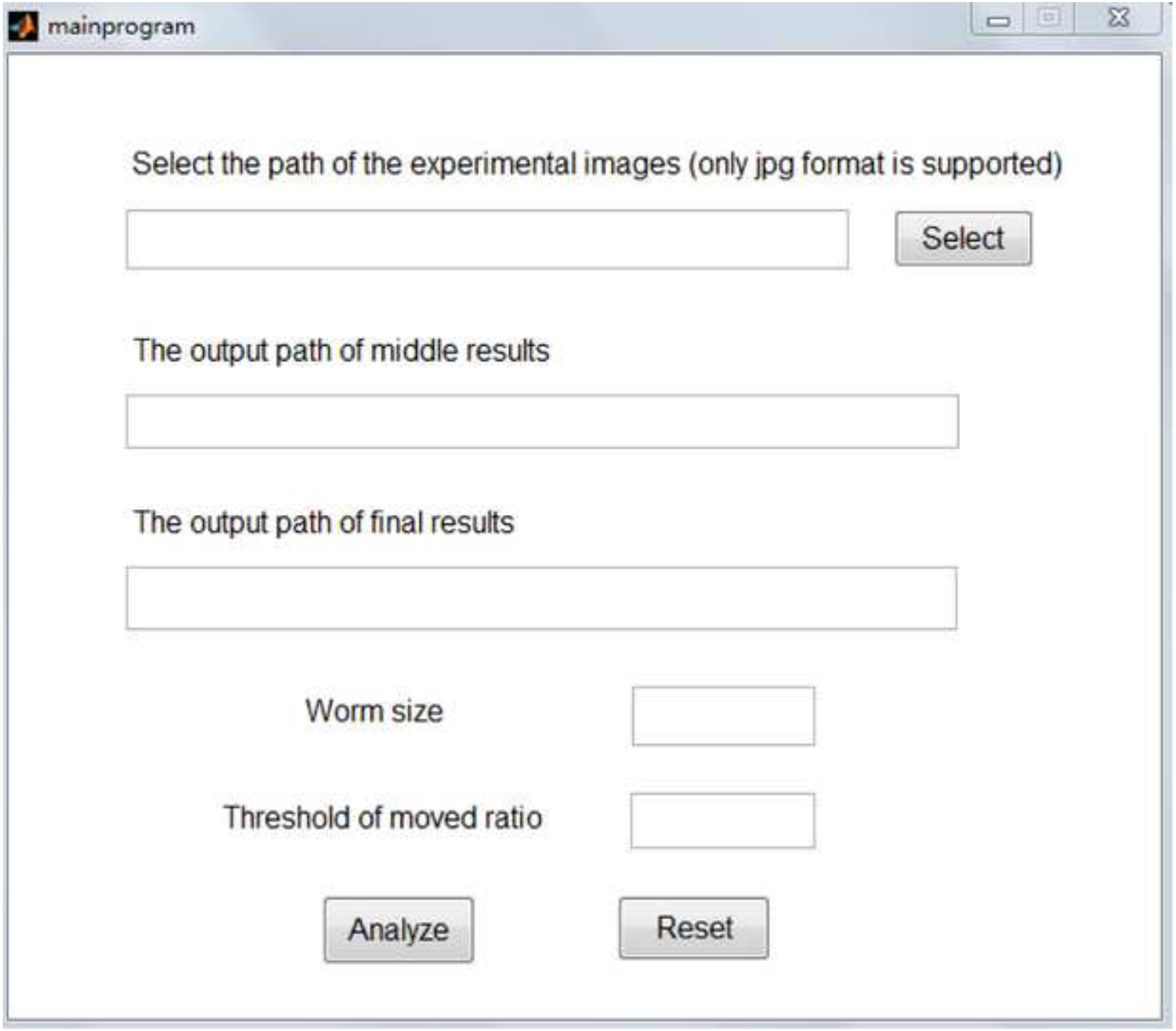

Figure 2

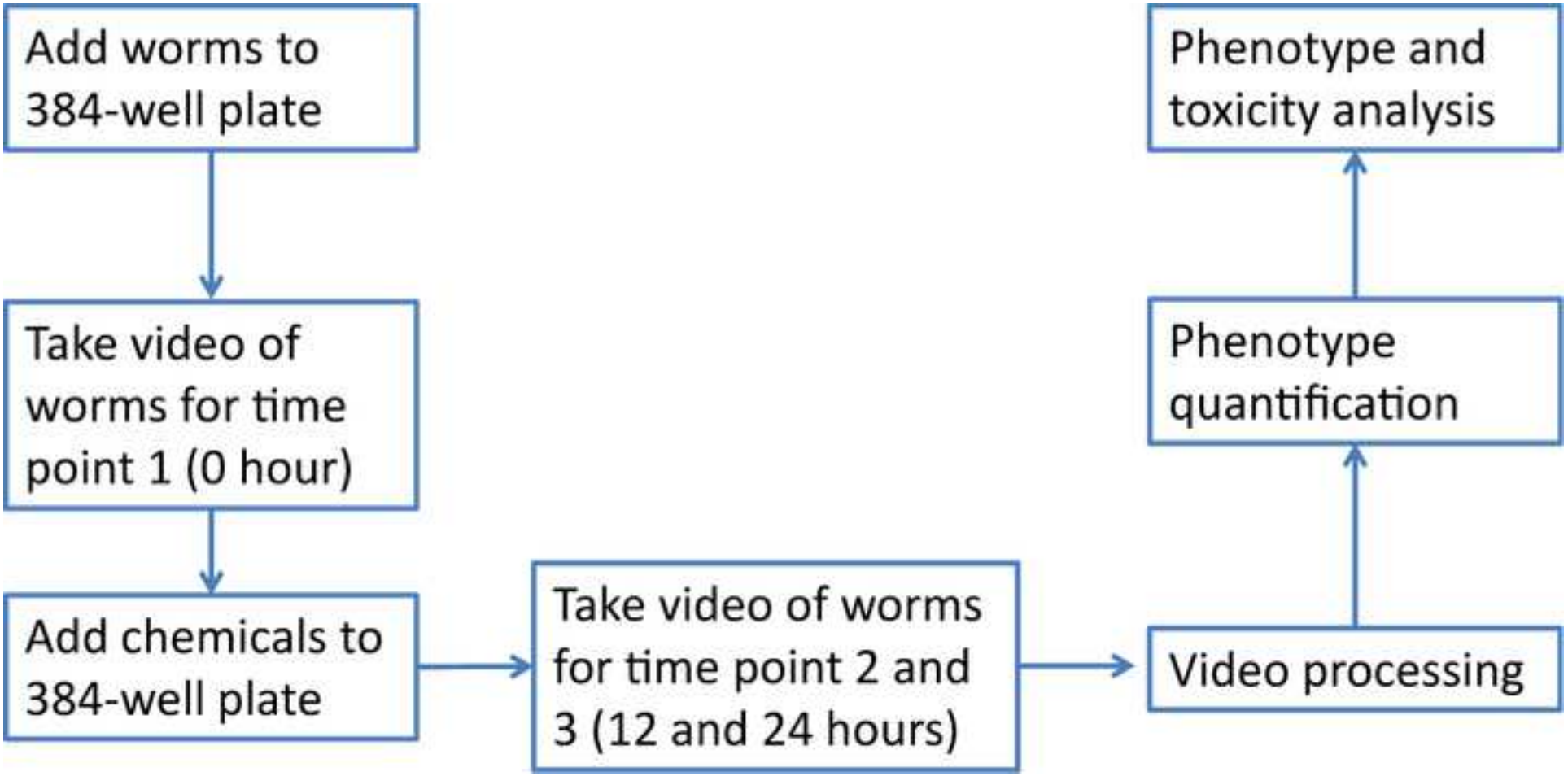

Figure 3

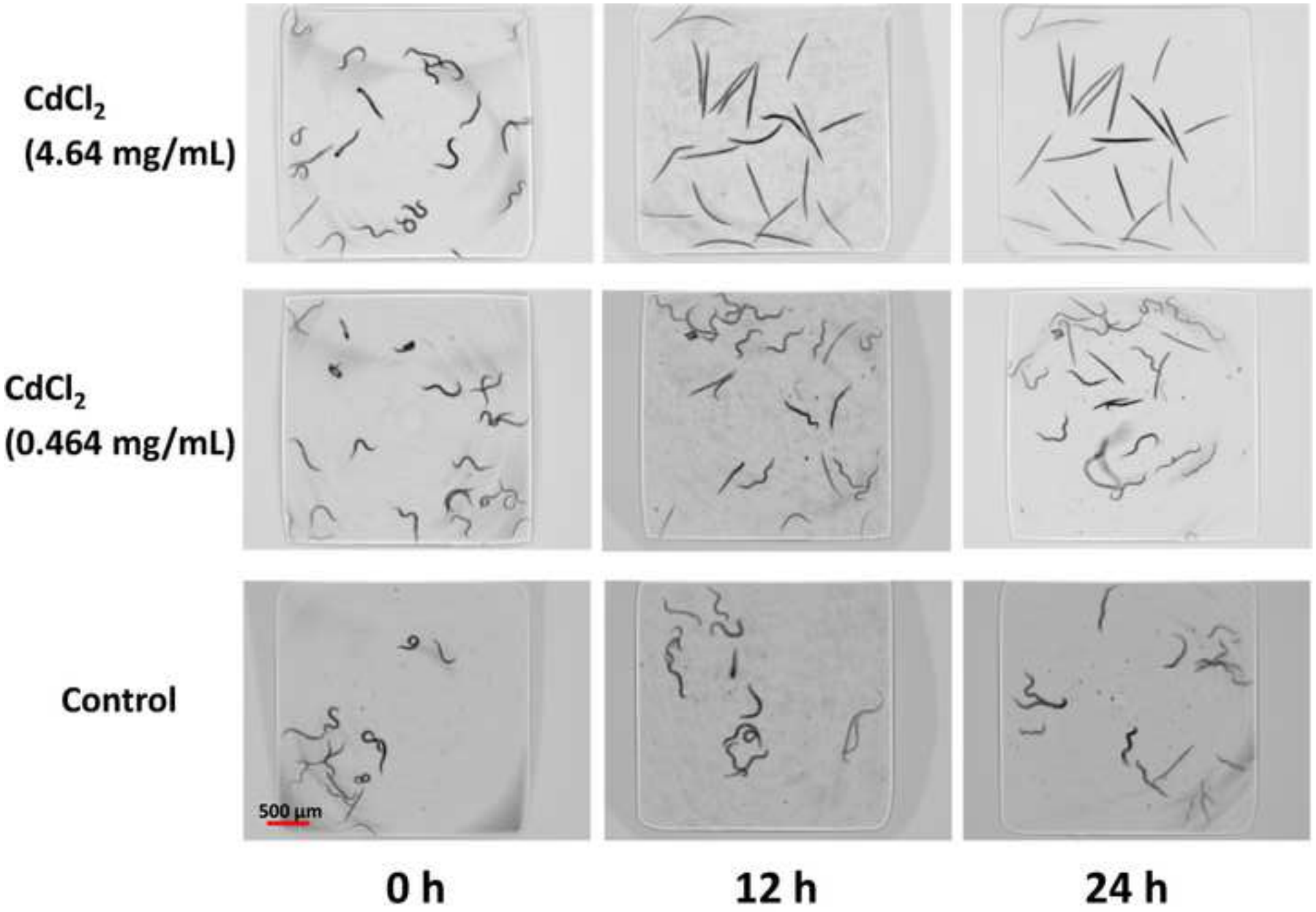

Figure 4

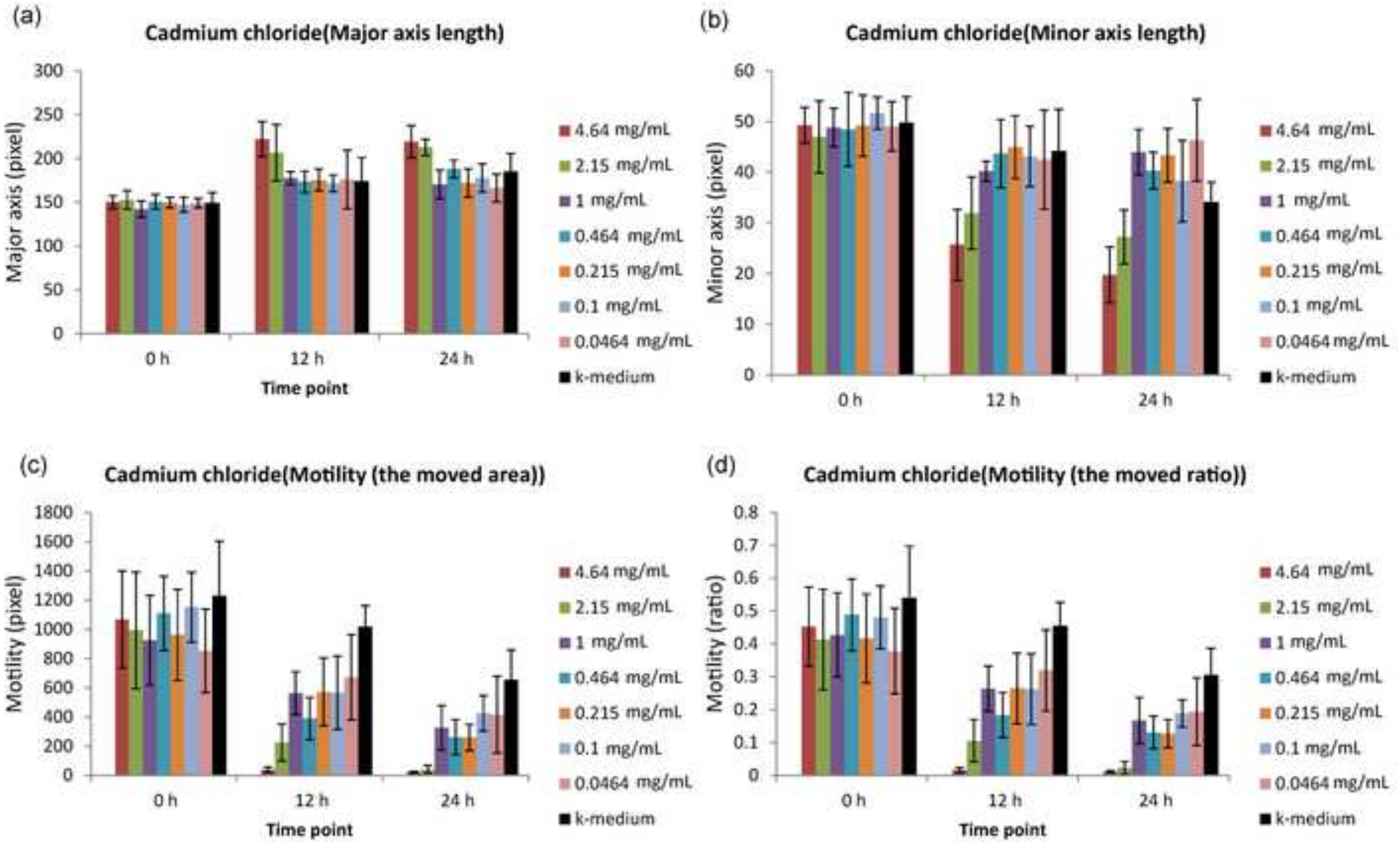

Table 1. Exposure concentration of chemicals for 384 well-plate *C. elegans* acute toxicity test.

Chemical	GHS grade	*C1	C2	C3	C4	C5	C6	C7
Cadmium chloride	III	0.0464	0.1	0.215	0.464	1	2.15	4.64
Sodium Fluoride	III	0.1	0.215	0.464	1	2.15	4.64	
Copper(II) sulfate pentahydrate	IV	0.01	0.0215	0.0464	0.1	0.215	0.464	1
Atropine sulfate	IV	0.464	1	2.15	4.64	10	21.5	46.4
Potassium chloride	V	2.15	4.64	10	21.5	46.4	100	
Ethanol	VI	2.15	4.64	10	21.5	46.4	100	215
Ethylene glycol	VI	4.64	10	21.5	46.4	100	215	464
Anhydrous two propanol	VI	2.15	4.64	10	21.5	46.4	100	215
Glycerol	VI	4.64	10	21.5	46.4	100	215	464
Sodium Hypochlorite	VI	1	2.15	4.64	10	21.5	46.4	

*： C stands for concentration (mg/mL)

GHS is an acronym for the Globally Harmonized System of Classification and Labeling of Chemicals (by the United Nations)

Table 2. A schematic of the 384-well plate layout

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A																								
B				X1	X1	X1	X1	X1	X1	X1	X1			W1	W1	W1	W1	W1	W1	W1	W1			
C				X2	X2	X2	X2	X2	X2	X2	X2			W2	W2	W2	W2	W2	W2	W2	W2			
D				X3	X3	X3	X3	X3	X3	X3	X3			W3	W3	W3	W3	W3	W3	W3	W3			
E				X4	X4	X4	X4	X4	X4	X4	X4			W4	W4	W4	W4	W4	W4	W4	W4			
F				X5	X5	X5	X5	X5	X5	X5	X5			W5	W5	W5	W5	W5	W5	W5	W5			
G				X6	X6	X6	X6	X6	X6	X6	X6			W6	W6	W6	W6	W6	W6	W6	W6			
H				X7	X7	X7	X7	X7	X7	X7	X7			W7	W7	W7	W7	W7	W7	W7	W7			
I				Y1	Y1	Y1	Y1	Y1	Y1	Y1	Y1			V1	V1	V1	V1	V1	V1	V1	V1			
J				Y2	Y2	Y2	Y2	Y2	Y2	Y2	Y2			V2	V2	V2	V2	V2	V2	V2	V2			
K				Y3	Y3	Y3	Y3	Y3	Y3	Y3	Y3			V3	V3	V3	V3	V3	V3	V3	V3			
L				Y4	Y4	Y4	Y4	Y4	Y4	Y4	Y4			V4	V4	V4	V4	V4	V4	V4	V4			
M				Y5	Y5	Y5	Y5	Y5	Y5	Y5	Y5			V5	V5	V5	V5	V5	V5	V5	V5			
N				Y6	Y6	Y6	Y6	Y6	Y6	Y6	Y6			V6	V6	V6	V6	V6	V6	V6	V6			
O				Y7	Y7	Y7	Y7	Y7	Y7	Y7	Y7			V7	V7	V7	V7	V7	V7	V7	V7			
P																								

Note:

- 100 µL of water (do not need to load worms, without picture capture)
- 100 µL of K-medium (control group)
- **X** chemical"X"
- **Y** chemical"Y"
- **W** chemical"W"
- **V** chemical"V"
- **number 1-7:** seven gradient concentrations of each chemical (from lowest to highest)

Table 3. Defined phenotypes.

Category	Phenotype	Category	Phenotype
Worm number	The single worm number in each well;	**Worm body size**	The average size of single worms;
	The living single worm number in each well;		The average length of single worms;
	The smoothed living worm number, this operation is used to calculate the survival rate;		The average width of single worms;
	Survival rate;		The average perimeter of single worms;
Motility	The motility of single worms, it is computed by the average moved area of single worms;		The average size of living single worms;
	The motility of single worms, it is the ratio between the moved area and worm size;		The average length of living single worms;
	The motility of living single worms, it is computed by the worm moved area;		The average width of living single worms;
	The motility of living single worms, it is the ratio between the moved area and worm size;		The average perimeter of living single worms;
Worm distribution	The worms' disperse situation in each well, it is the ratio between the single worm number and The total worm number in each well;	**Worm body shape**	The major axis length of single worms, it is the major axis of the ellipse that has the same normalized second central moments as the worm body region;
	The mean distance of all worms' centroid in each well;		The major axis length of living single worms;
	The standard deviation of all worms' centroid distances;		The minor axis length of single worms, it is the minor axis of the ellipse that has the same normalized second central moments as the worm body region;
Gray intensity	The average gray intensity of single worms;		The minor axis length of living single worms;
	The standard deviation of gray intensity of single worms;		The ratio between the single worms' minor axis length and major axis length, this feature more close to 1 more close to one circle;
	The average gray intensity of living single worms;		The ratio between the living single worms' minor axis length and major axis length;
	The standard deviation of gray intensity of living single worms;		The eccentricity of single worms, it is computed from the ellipse of the worm's body region;
Worm body orientation	The orientation of single worms, it is the angle between the major axis of the worm's body covered ellipse and the x-axis;		The eccentricity of living single worms;
	The orientation of living single worms;		

Table of Materials

Click here to access/download;Table of Materials;Materials59082.xlsx

Name of Material/ Equipment	Company	Catalog Number
2-Propanol	Sigma-Aldrich	59300
384-well plates	Throme	142761
Agar	Bacto	214010
Atropine sulfate	Sigma-Aldrich	PHL80892
Bleach buffer		
Cadmium chloride	Sigma-Aldrich	202908
Calcium chloride	Sigma-Aldrich	21074
CCD camera	Zeiss	AxioCam HRm
Cholesterol	Sigma-Aldrich	C8667
Copper(II) sulfate	Sigma-Aldrich	451657
Ethanol	Sigma-Aldrich	24105
Ethylene glycol	Sigma-Aldrich	324558
Glycerol	Sigma-Aldrich	G5516
K-Medium		
LB Broth		
Magnesium sulfate heptahydrate	Sigma-Aldrich	63140
NGM Plate		
Peptone	Bacto	211677
Potassium chloride	Sigma-Aldrich	60130
Potassium phosphate dibasic	Sigma-Aldrich	795496
Potassium phosphate monobasic	Sigma-Aldrich	795488
PPB buffer		
shaker	ZHICHENG	ZWY-200D
Sodium chloride	Sigma-Aldrich	71382
Sodium fluoride	Sigma-Aldrich	s7920
Sodium hydroxide	Sigma-Aldrich	71690
Sodium hypochlorite solution	Sigma-Aldrich	239305
The link of program		
Tryptone	Sigma-Aldrich	T7293
Yeast extract	Sigma-Aldrich	Y1625
Zeiss automatic microscope	Zeiss	AXIO Observer.Z1

Comments/Description
0.5 mL of 10 M NaOH, 0.5 mL of5% NaClO, 9 mL ofultrapure water
Zeiss microscopy GmbH
3.04 g of NaCl and 2.39 g of KCl in 1 L ultrapure water
10 g/L Tryptone, 5 g/L Yeast Extract, 5 g/L NaCl
3 g ofNaCl, 17 g ofagar, 2.5 g ofpeptone in 1 L of ultrapure water, after autoclave add 1 mL of cholesterol (5 mg/mL in ethanol), 1 mL of MgSO4 (1 M), 1 mL of CaCl2 (1 M), 25 mL of PPB buffer
35.6 g of K2HPO4, 108.3 g of KH2PO4 in 1 L ultrapure water
https://github.com/weiyangc/ImageProcessForWellPlate
Zeiss automatic microsco with peproprietary software Zen2012 and charge coupled device(CCD) camera

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Title of Article: A high-throughput assay for prediction of chemical toxicity by automated phenotypic profiling of Caenorhabditis elegans

Author(s): Shan Gao, Weiyang Chen, Wenjing Zhang, Haiming Jing, Nan Zhang, Matthew Flavel, Markandeya Jois, Yingxin Zeng, Jing-Dong J. Han, Bo Xian, Guojun Li

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Article Title:	A high-throughput assay for prediction of chemical toxicity by automated phenotypic profiling of Caenorhabditis elegans
Signature:	Bo Xian
Date:	2018.09.10

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Response to Reviewers

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Comments from the editors and reviewers:

Editorial comments:

Changes to be made by the Author(s) regarding the written manuscript:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We have carefully edited our manuscript for word usage and grammar.

2. Please note that numbering of institutional affiliation should follow the order of authors. First author gets 1, next author with different affiliation gets 2, etc., following from first to last.

We have updated the numbering of institutional affiliations in the revised manuscript.

3. Please provide an email address for each author.

We added the email address for each author in the revised manuscript.

4. Please rephrase the Introduction to include a clear statement of the overall goal of this method and include Information that can help readers to determine if the method is appropriate for their application.

We have rephrased the Introduction.

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We have removed all commercial language from our manuscript.

6. Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.

We have included an ethics statement.

"The protocol follows the animal care guidelines of the Animal Ethical Committee of Beijing Center for Disease Prevention and Control in China."

7. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets, dashes, or indentations.

We have updated the numbering of the Protocol.

8. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).
We have revised the protocol text.

9. Please revise the protocol to contain only action items that direct the reader to do something (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible.
We have revised the protocol to contain only action items.

10. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below:
Line 80: Please reference Table 1 here for the chemicals tested in this protocol
We have referenced Table 1 there, and added more details to the protocol steps.

Line 81: Please reference Table 1 here for the concentration levels tested in this protocol. Please briefly mention how to prepare chemical solutions. What solvent is used for solid chemicals?
We have referenced Table 1 here. And we added an example of describing how to prepare chloride ($CdCl_2$) solutions.
“Take Cadmium chloride ($CdCl_2$) for example, seven gradient concentrations were designed (Table 1). The highest concentration was 4.64 mg/mL, 2X of the highest concentration aqueous solution was prepared, 92.8 mg $CdCl_2$ solid powder was diluted by 8mL of K-Medium, and made up to 10 mL after fully dissolved. The other concentration levels were diluted gradiently by K-Medium.”

Line 84: Listing an approximate volume to prepare would be helpful.
We have added the preparation in the revised manuscript.
“1.4 Set eight parallel wells for every specific point of the concentration gradients of each chemical, each well containing 50 µL of 2X chemical solution. Set at least 3 groups of eight parallel wells of K-medium as control (**Table 2**).

NOTE: In brief, a volume of 500 µL of 2X working solution is necessary for a single dosage of each chemical.”

Lines 90-91: Please describe how these are done. For example, how are the plates seeded with E. coli? How is bleaching done? How long does it take to reach L4 stage?
We have added the description in the revised manuscript.

Line 93: 100 ul of what? K-medium? Are the worms transferred from the plate to a tube, or a new plate? It is unclear.
We have made it clear in the revised manuscript.

Line 99: Please write the sentence in imperative tense and include it in the step. How to carry out 8 parallel tests? How many (8?) wells are filled with the same chemical but with different concentrations?
We have modified the sentence, and the 8 parallel tests are for each concentration we carry out 8 parallel tests (8 wells are filled with the same chemical and same concentration).

Line 105: Are the wells of plates labelled for different chemical treatment?
Yes, we numbered each well and recorded the tested chemical and concentration in each well.

Line 107: Are the plates taken out from the incubator and transferred to the automatic stage for taking videos? Please specify.
Yes, the plates were taken out from the incubator and transferred to the automatic stage for taking videos.

Lines 110-117: Software must have a GUI (graphical user interface) and software steps must be more explicitly explained ('click', 'select', etc.). Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc.) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.
We have added more specific details of software.

Lines 120-121: Please describe in imperative tense how to perform phenotype quantification and toxicity analysis.
We have modified the description.

11. Please include single-line spaces between all paragraphs, headings, steps, etc.
We have modified the line space.

12. Table 1: Please define GSH.
GSH is an acronym for Globally Harmonized System of Classification and Labeling of Chemicals (by United Nations).

13. Please upload each Figure individually to your Editorial Manager account as a .png, .tiff, .pdf, .svg, .eps, .psd, or .ai file.
We have uploaded each figure individually.

14. Please remove the titles and Figure Legends from the uploaded figures. The information provided in the Figure Legends after the Representative Results is sufficient.
We have removed the titles and Figure Legends from the uploaded figures.

15. Figures 2 and 3: Please change “ml” to “mL” and the unit of the scale bar “um” to “µm”. Please include a space between all numbers and their units (i.e., 0 h, 12 h, 24 h, 500 µm).
We have modified according to your suggestions.

16. Figure 2 legend: Please describe the figure in more detail. For example, what are the top and bottom panels? What is in the control well?
We have added more details accordingly.

17. Figure 3 legend: Please label and explain the different panels.
We have labeled and explained the different panels.

18. JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique
We have revised the discussion.

19. For in-text references, the corresponding reference numbers should appear as superscripts after the appropriate statement(s) in the text (before punctuation but after closed parenthesis). The references should be numbered in order of appearance.
We have modified accordingly.

20. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al. See the example below:
Bedford, C.D., Harris, R.N., Howd, R.A., Goff, D.A., Koolpe, G.A. Quaternary salts of 2-[(hydroxyimino)methyl]imidazole. Journal of Medicinal Chemistry. 32 (2), 493-503 (1998).
We have modified accordingly.

21. References: Please do not abbreviate journal titles.
We have modified accordingly.

Reviewers' comments:

Please note that novelty is not a requirement for publication and reviewer comments questioning the novelty of the article can be disregarded.

Please note that the reviewers raised some significant concerns regarding your method and your manuscript. Please thoroughly address each concern by revising the manuscript or addressing the comment in your rebuttal letter.
We thank reviewers for these suggestions. We have now addressed reviewers' comments point-by-point (below), and revised the manuscript accordingly.

Reviewer #1:

Manuscript Summary:
The Manuscript "A high-throughput assay for prediction of chemical toxicity by automated phenotypic profiling of Caenorhabditis elegans", by Xian Bo et al, provides insights into a novel protocol for C. elegans phenotypical screening. While of potential interest for the community, a few points needs to be considered before it is accepted for publication.

Major Concerns:
Line 110. My only major concern regarding the protocol are the steps 5 and 6. While steps 1-4 are described into enough detail and will allow the user to reproduce the method, a much more detailed description of the Experiment video processing (Point5) and phenotypes definition (Point6) will be required if another user would like to use this method. The current description provide only a general explanation on how the software works, but does not give any specific indication on how to use the software and how to tune its parameters prior to analysis. A description of the type of output that the software provides will be necessary in order to facilitate data analysis.
We have added more details according to this suggestion.

It was also not clear to me if the software is open source or not. In case it is, a link to download should be provided. If this was already provided in the paper referenced as [11], this should be indicated in the text.
We have added the link to download the software.

In Line 124. It was also not clear to me if these data referred to are the same data reported in the reference [11], or another, or if they are original data. If these data were previously reported, the appropriate reference should be included to avoid confusion for the reader.
We included the appropriate reference.

Figure 3 should be reformatted with a clearer colour code. Especially given that the k-medium is used as a positive control, this should be clearly distinct from the other compound concentrations, such as 2.15 mg/ml.
We reformatted this figure according to this suggestion.

Providing a comparison of the phenotypical screening with a manual paralysis or lifespan assay will be helpful to prove the validity of the method and its advantage

over manual analysis (which it was not clear to me if it was the sensitivity or the throughput). If this comparison was already provided in the previous works, providing the reference will be helpful.
This comparison was already provided in the previous works. We now have added the reference of our previous paper (reference [11]). In this paper, we did the comparison between manual analysis and automatically analysis results.

Minor Concerns:
Line 80. Please specify which "chemicals" are required.
We have listed the chemicals in the “table of materials”.

Line 81. Please provide an indication of a specific range of concentrations that can be tested in the text.
We added more detailed description according to this suggestion.
“Design the highest and lowest dosages of individual chemical at its minimum concentration of 100% lethality (LC100, 24h) and maximum concentration of 100% non-lethality (LC0, 24h) to worms, with totally at least six serial diluting from the highest concentration (**Table 1**).”

Line 90. Which temperature do you raise the worms, is it 20C? Please provide a reference for the bleaching step if details are not provided in the text, as the readers may not be familiar with it.
We added more details accordingly.

Line95. The Authors suggest that this protocol can be used 24h after the L4 stage. Is it possible of applying it for more time-points in the lifetime of the worms or is it limited to a D0 screening?
This method is ideal for acute toxicity test within 24 hours. It is possible for applying more time-points with food source for worms.

Line 102. Please provide more details for the user for this step to use this automated procedure
We have added more details.

Line 106. Is this step done inside and incubator?
This step was done in an incubator shaker.

Line 108. An indication on the time required to finish a screening of 384 wells will be helpful
We added the indication “ 7 frames per second for 2s, it takes ~20 min for scanning each plate ”

Reviewer #2:

Manuscript Summary:
The paper of Xian Bo et al described a high throughput methods for screening the

effects of chemicals on survival and behavioural trait of the nematode Caenorhabditis elegans. C. elegans is certain likely to prove a useful model system for chemical screening and prioritisation, so developing higher throughput testing and screening methods with this species is a worthwhile pursuit. That said the work presented is not entirely novel, as similar method for screening chemical toxicity with C. elegans have already been developed and used with the EU-EPA Tox21 program. Screening in Tox21 is done by quantifying population growth rate, rather than individual survival and/or movement as done here. However, as survival and population growth are linked (not necessarily linearly), it could be argued the method presented complements that used in Tox21. Despite the potential usefulness of the method, I have a number of concerns about whether the method develop is suitable as a universal chemical screening tool, based on concerns about dosing and representative exposure as stated below.

Major Concerns:

1. The author do a good job of promoting the advantages of C. elegans for toxicological screening. However, this is rather a cherry picked view. Disadvantages are not mentioned. For example, comparative studies have shown that C. elegans lack up to 40% of all human drug targets. Assumption of a similar proportion of missing for specifically acting chemical toxicants (e.g. pesticide receptors etc.), that would indicate that a significant portion of more toxic chemical would not be identified based on screening in C. elegans alone. There are also difference in anatomy, metabolism and organ structures that may mean different effects in C. elegans and mammalian studies. These will need to be considered in any scheme that uses studies in C. elegans as a toxicological screening tool.
We added some discussions about these disadvantages.
"Clearly, this high-throughput assay for prediction of chemical toxicity will be a valuable toxicity model approach and could be used to the pre-screening of chemicals before the rodent animal experiments."

2. The author state that for the experimental design, 4-8 treatments should be used. Based on describing effects with logistic concentration-response models that may contain 3 or 4 parameters, four treatments is too few. The minimum should be at least six treatments for more reliable model fitting.
We have change the statement and recommend users to design more than six treatments.

3. Replication levels are not mentioned clearly in the text. The text at the start of results section 3. "Chemical treatment and video capture" mentioned single dosage and 8 parallel tests, yet it is hard to see how this maps on to the design with multiple treatments mentioned early. The replication used needs to be clarified.
We clarified the replication.

4. Promotion of a single chemical supplier (Sigma-Aldrich) should be removed. Here instead details of the purities of substances used or ways to report on the composition of any mixtures tested (e.g. formulations, oils etc.) should be included.

We modified the description accordingly.

5. K-medium is not appropriate for use when conducting toxicity tests. The high ionic strength and high chloride content, result in potentially unrealistic chemical behaviour in toxicity tests undertaken in this solution. An alternative to K-medium with low ionic strength should be recommended for the assay (e.g. artificial freshwater or soil solution).
We appreciate reviewer's suggestion and add it in the discussion. We have added the recommendation in the discussion.

6. In discussing approaches to dosing the authors do not mention what to do with poorly water soluble chemicals. Many chemicals have low water solubility and so cannot simply be easily dissolved in water and so present a challenge for aquatic tests. Yet these chemical can and do present a hazard. Method to use solvents and to conduct passive dosing are already published for aquatic tests. These need to be integrated here to show how testing for C. elegans can be conducted with more lipophilic chemicals, otherwise the test system is not applicable to a large tranche of substances on the market today.
We added more details accordingly.

7. The program for analysis of the phenotyping data needs to be released with the paper. If the authors wish to keep this as proprietary software for planned commercial exploitation, this is fine. However, in that case the paper should be presented for promotional purposes to support software release etc., not published as a peer review article that focusses on the method itself
We have released the software freely and added a link for downloading by users.

Reviewer #3:

Manuscript Summary:
Authors show a method to assay toxicity in C. elegans. They named high-throughput because they use 384 well-plates, but they use only 19 compounds only.
They have an automatized system to take pictures from well, and a customized software to score phenotypes that can be relaetd to toxicity.

Major Concerns:
There are many other methods to study toxicity of compounds in C. elegans in a large-scale manner. Surprisingly, authors do not mention any of those but they claim that this method "is an improvement of current methods". The discussion section is just a summary of settings and results, and do not comment on their method in any context. Why somebody should see their video and no others?. I have not found an answer in the manuscript.
We added more details according to suggestions.

There are other concerns, as the solubility of molecules. They used 19 compounds but for a high-throughput assay that would find molecules with low solubility in the media they used (K-medium)
We added more descriptions about the solubility of molecules.

There is not info about the matlab pipeline or script that they use.
We added more information about the matlab pipeline or script.

Minor Concerns:
They mention the use of a Nikon microscope, but later the refer to a Zeiss.
We have modified accordingly.

Do you need the 33 features to label a compound as toxic, or would be sufficient with less?
We listed all the features that we can quantify. It provided more optional parameters are provided to different users.

Why do you recommend chemicals from Sigma, and not from other suppliers?
We removed all commercial language from our manuscript.

Reviewer #4:

Manuscript Summary:
The authors describe an automated method for descriptive and kinematic analysis of C. elegans in 384 well plates. The method was used by the authors to analyze phenotypes resulting from the exposure of worms to CdCl2 after 12 and 24 hours of exposure in the absence of food. Worms showed a dose and time dependent decrease in many movement parameters.

Major Concerns:
It would seem to me that in this submission, Fig. 1, Table 1, and Table 2 have already been published in the author's recent BMC Pharm and Tox paper. Fig. 1 in this submission is roughly equivalent to Fig. 5 in the BMC paper. Table 1 in this submission is equivalent to Supplemental Table 1 in the BMC paper. Likewise Table 2 in this submission is also listed in the BMC paper 'Methods'. While this information is not presented in EXACTLY the same format in the two papers, the exact same information is communicated.
We added the previous paper as reference in related sections.

Minor Concerns:
Many awkward and unclear statements throughout - needs extensive proofreading for clear English
We have carefully edited our manuscript for word usage and grammar.

Why does the fact that the worm genome is fully sequenced make it a good model for testing chemical toxicity? Other organisms have fully sequenced genomes as well. Please clarify

We modified the description accordingly.

The MATLAB code needs to be made available in some way with the publication

We added the link to download the software.

L82 - Can the authors describe or cite a reference to explain 'the degree of saturation and Horn method'

We added the reference accordingly.

Please acknowledge the CGC and its funding information in the 'Acknowledgements' section.

We now have acknowledged the CGC and its funding information in the 'Acknowledgements' section.

Response to editor

Please note that the editor's concerns are listed one by one below (black characters), followed by our responses (blue characters) and, when relevant, the corresponding changes in the text (green characters).

Comments from the editor:

1. Please shorten it to no more than 50 words.
We have shortened the SUMMARY to no more than 50 words.

2. Please remove the square brackets that enclose the reference numbers.
We have removed the square brackets that enclose the reference numbers.

3. What is LD50?
We have removed the 'LD50'.

4. Can the authors describe or cite a reference to explain 'the degree of saturation and Horn method'?
We have removed this description.

5. What are the criteria for selecting the highest concentration?
We set the highest dosage of individual chemical at it minimum concentration of 100% lethality (LC100, 24h)

6. In the JoVE Protocol format, "Notes" should be concise and used sparingly. They should only be used to provide extraneous details, optional steps, or recommendations that are not critical to a step. Any text that provides details about how to perform a particular step should either be included in the step itself or added as a sub-step. Please consider moving some of the notes about the protocol to the discussion section.
We have modified according to this suggestion.

7. Please specify what this stands for. Step 1.2 mentions 4-8 gradient concentration levels.
We have modified this section and described as "Design the highest and lowest dosages of individual chemical at it minimum concentration of 100% lethality (LC100, 24h) and maximum concentration of 100% non-lethality (LC0, 24h) to worms, with at least 6 serial diluting among them (Table 1)."

8. In the JoVE Protocol format, "Notes" should be concise and used sparingly. They should only be used to provide extraneous details, optional steps, or

recommendations that are not critical to a step. Any text that provides details about how to perform a particular step should either be included in the step itself or added as a sub-step. Please consider moving some of the notes about the protocol to the discussion section.
We have modified according to this suggestion.

9. Please reorganize the steps/notes here so that the protocol can be followed in chronological order.
We have modified according to this suggestion.

10. Please provide the composition of NGM. If it is purchased, provide product information in the Table of Materials and cite the Table of Materials here.
We have added the details of NGM in the Table of Materials and cited the Table of Materials.

11. In the JoVE Protocol format, “Notes” should be concise and used sparingly. They should only be used to provide extraneous details, optional steps, or recommendations that are not critical to a step. Any text that provides details about how to perform a particular step should either be included in the step itself or added as a sub-step. Please consider moving some of the notes about the protocol to the discussion section.
We have modified according to this suggestion.

12. Please describe how to settle down the worms.
We have added “let the worms settle down for at least 2 minutes”

13. What is used to wash the eggs? And what volume?
We have added “wash the eggs by 5mL sterile H_2O at least 2 times”.

14. Please provide the program information in the Table of Materials and move the weblink to Table of Materials.
We have provided the program information in the Table of Materials and move the Weblink to Table of Materials.

15. Software steps must be more explicitly explained ('click', 'select', etc.). Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc.) to your protocol steps.
We have rewritten this section according to the suggestion.

16. Please revise the protocol to contain only action items that direct the reader to do something (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Any text that cannot be written in the imperative tense may be added as a “Note.”
We have modified according to this suggestion.

17. Please specify the method here.

We have modified according to this suggestion.

18. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al. See the example below:

Bedford, C.D., Harris, R.N., Howd, R.A., Goff, D.A., Koolpe, G.A. Quaternary salts of 2-[(hydroxyimino)methyl]imidazole. Journal of Medicinal Chemistry. 32 (2), 493-503 (1998).

We have reformatted the references.

Response to Editor

Please note that the editor's concerns are listed one by one below (black characters), followed by our responses (blue characters) and, when relevant, the corresponding changes in the text (purple characters).

Comments from the editor:

1. The description suggests 8 concentrations while Table 1 shows only 7 concentrations. Or do you mean 6 serial diluting from the highest concentration?
Yes, we mean "6 serial diluting from the highest concentration" for the description. We have modified the manuscript accordingly, "... with totally at least six serial diluting from the highest concentration", which is consistent with Table 1.

2. Please provide a reference for how to perform this test.
We have added reference 9 for this test.

3. Do you mean the eight parallel wells all contain the same chemical with the same concentration? Also, Table 1 shows only 7 concentrations. Does one well contain K-medium only? A schematic showing the plate layout would be helpful.
Yes, for each individual chemical, there are at least 6 concentration levels to test. For each concentration level, there are eight parallel wells (containing the same chemical, same concentration). Also there are at least 3 groups of eight parallel wells of K-medium as control. We have added Table 2 as a schematic for showing the 384-well plate layout.

4. Please specify the volume.
100 mL. We have modified the manuscript accordingly.

5. Please specify the temperature and other conditions.
We have added " Raise N2 worms on the NGM plates with OP50 lawn in 20 °C for about 2-3 days when most of worms are reached adult stage. "

6. I rearranged the steps based on my understanding. Please confirm that the intended meaning has been retained.
We accept the rearrangement and modified some descriptions. Thanks.

7. When are the worms harvested?
About 2-3 days when most of worms are reached adult stage, the worms were harvested. This has been indicated in the modified 2.2.2.

8. Is supernatant discarded after centrifugation? Please specify.

Yes, we discarded the supernatant after centrifugation. We have modified the manuscript accordingly.

9. Is water removed after each wash? Please specify.
Yes, we removed the supernatant and add water to rewash the egg each time. We have modified the manuscript accordingly.

10. Step 1 describes the preparation of chemicals with different concentrations. The results also show the effect of different concentrations. Therefore, in the treatment here, please include how the test with different concentrations is performed.
We have modified the manuscript accordingly. "In 384-well plates, worms (50 µL in each well) are treated by individual chemical at 6-7 dosages (Table 1). Set eight parallel wells each containing 50 µL of 2X chemical solution for every dosage (8 wells are filled with the same chemical and same concentration)."

11. There are 384 wells. So, in this protocol, do you fill other wells with chemical at different concentrations and different chemicals? Please specify.
Yes, we have added Table 2 as a schematic for showing the 384-well plate layout.

12. Why is this needed?
The middle results include the segmented images. These middle results are useful for the visual observation of the processed images.

13. Please specify the size parameter used.
The size parameter used in the experiments is 2000.

14. Please specify.
We have modified accordingly.

15. Please specify the ratio used.
The ratio used in the experiments is 0.93.

16. Please specify.
We have modified accordingly.

Response to Editor

Please note that the editor's concerns are listed one by one below (black characters), followed by our responses (blue characters) and, when relevant, the corresponding changes in the text (orange characters).

Comments from the editor:

1. The language in the manuscript has been signficantly edited for coherence. Please review.
We accepted and appreciated the modification.

2. In the Table of Materials, the composition of the NGM plate is not very clear (mL/L??). Please revise:
3 g/L NaCl, 17 g/L agar, 2.5 g/L peptone, 1 mL/L cholesterol (5 mg/methyl alcohol), 1 mL/L 1 M MgSO4, 1 mL 1 M CaCl2, 25 mL/L PPB buffer
We 've revised this as "3 g NaCl, 17 g agar, 2.5 g peptone in 1L ultrapure water, after autoclave add 1 mL cholesterol (5 mg/mL in ethanol), 1 mL MgSO4 (1M), 1 mL CaCl2 (1M), 25 mL PPB buffer"

3. Please upload the program files here as Supplementary Coding Files.
Now we have uploaded the program files as Supplementary Coding Files.

4. Additional comments are in the attached manuscript.
We have modified according to the comments.

5. Figure 4 needs units to define the panel legend on the right: 4.64, 2.15, etc. need units for context.
We have added the units on Figure 4.

6. Please obtain explicit reprint permissions for previously published figures and tables.
The figures and tables we used in this manuscript are different from those published previously.

[A1] These are not 6 serial dilutions. Do you make three different solutions and then dilute solution 1:10?
How do you get a 2.15 mg/mL solution from 4.64 mg/mL solution cleanly?
We diluted the solution 2.15 times from the higher concentration.

[A2] Please revise for clarity. Movement threshold?
We have modified accordingly.

[A3] Again, please revise for clarity. This is not the same language in Figure 1 as well.
We have modified accordingly.

[A4] Where are the lower concentrations?
We show the quantified phenotypes of all the used concentrations in Figure 4, we have changed the reference from Figure3 to Figure 4.
We have added 3 representative pictures of worms under 0.464 mg/mL $CdCl_2$ (middle panel) at 0, 12 and 24 hours in Figure 3.

[A5] ??? Where are the other concentrations? Only the highest (4.64 mg/mL) and the control are shown.
We show the quantified phenotypes of all the used concentrations in Figure 4, we have changed the reference from Figure3 to Figure 4.
We have added 3 representative pictures of worms under 0.464 mg/mL $CdCl_2$ (middle panel) at 0, 12 and 24 hours in Figure 3.

[A6] Figure 3 or Figure 4? Please specify the panels.
We have modified.

[A7] Redundant with previous sentence?
We have deleted this sentence.

[A8] Please revise for clarity.
We have modified accordingly.

[A9] Please specify the panels.
We have specified the figures.

[A10] Please revise for clarity.
We have modified as "This assay is ideal for acute toxicity testing of chemicals within 24 hours, and it could be applying for subacute toxicity testing as well when more time-points of data were collected and food source (OP50) is supplied for worms."

[A11] ???
We have modified the text.

[A12] Table 1 says GSH. Which is it?
It should be GHS. We have corrected it in the table1.

[A13] ????
We have deleted this sentence.

[A14] Citation?

We have added reference 15 and modified the text.

[A15] Define this abbreviation.
The COPAS is the abbreviation of Complex Object Parametric Analyzer and Sorter. We have modified in the text.

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Supplemental Coding Files

SupplementaryCodingFiles.zip