Video Article

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

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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[1,2,3]. *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[4,5,6,7,8]. 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. Obtain chemicals (**Table 1** and **Table of Materials**).
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.
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. 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.
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

1. Obtain wild-type N2 worms and *Escherichia coli* OP50 strains from the Caenorhabditis Genetics Center (CGC).
2. Obtain synchronized L4 worms.
   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. 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.
   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**).
   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.
   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.
   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.
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.

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

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

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.
3. Add the final result directory in the interface.
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.
5. Add the **Threshold of moved ratio** in the interface.
   NOTE: The ratio used in the experiments is 0.93.
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.

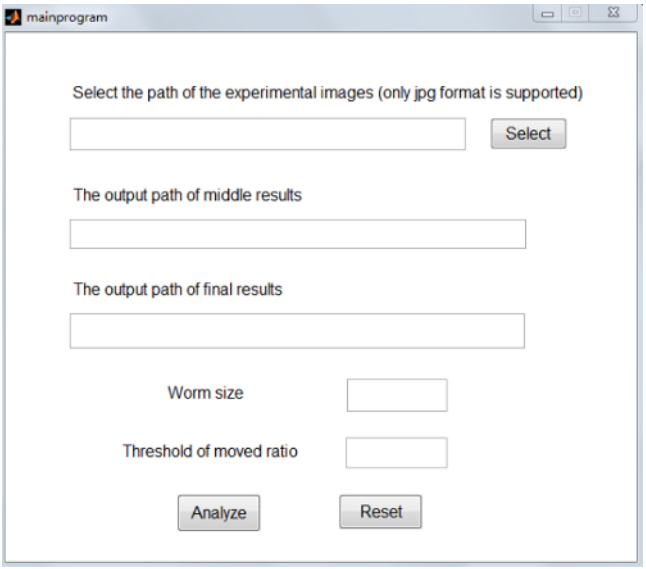

Figure 1: The interface of the software. Please click here to view a larger version of this figure.

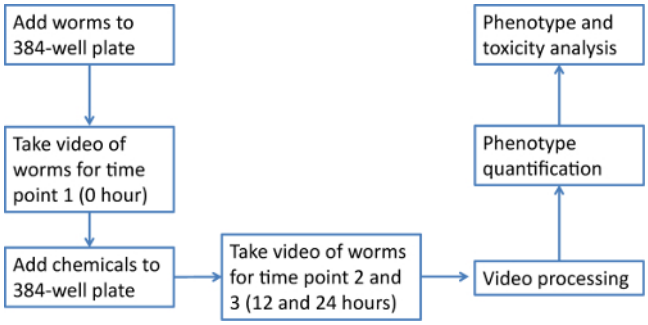

Figure 2: The pipeline of a high-throughput assay for the prediction of chemical toxicity by automated phenotypic profiling of *Caenorhabditis elegans*. Please click here to view a larger version of this figure.

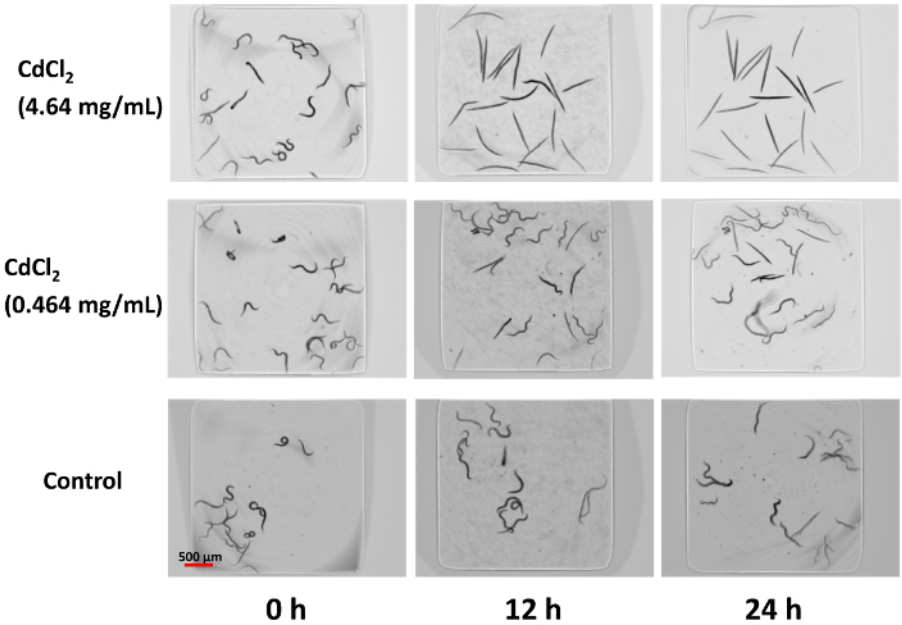

Figure 3: Experimental images of worms under 4.64 mg/mL $CdCl_2$ (upper panel), 0.464 mg/mL $CdCl_2$ (middle panel), and K-medium (bottom panel), at different time points. The images show the status changes of worms under chemical treatment or in a control group in one representative well of the 384-well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

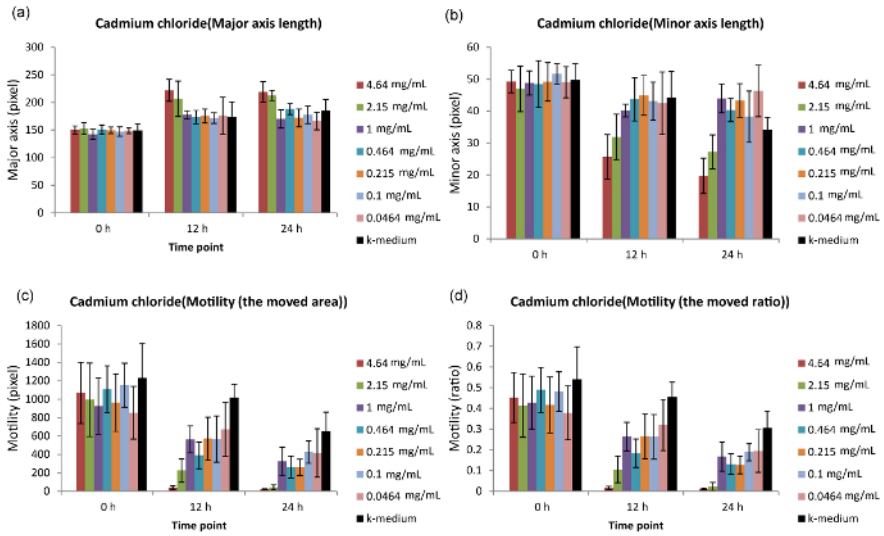

Figure 4: The quantified features of worms under different concentrations of $CdCl_2$. (**a**) The quantified major axis length. (**b**) The quantified minor axis length. (**c**) The quantified motility by the moved area. (**d**) The quantified motility by the moved area/worm size. The bar plots show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

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

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

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 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.

Disclosures

The authors have nothing to disclose.

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