Video Article

High-throughput Screening and Biosensing with Fluorescent *C. elegans* Strains

Chi K. Leung[1], Andrew Deonarine[1], Kevin Strange[2], Keith P. Choe[1]

[1]Department of Biology, University of Florida
[2]Mount Desert Island Biological Laboratory

Correspondence to: Keith P. Choe at kchoe@ufl.edu

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Abstract

High-throughput screening (HTS) is a powerful approach for identifying chemical modulators of biological processes. However, many compounds identified in screens using cell culture models are often found to be toxic or pharmacologically inactive *in vivo*[1-2]. Screening in whole animal models can help avoid these pitfalls and streamline the path to drug development.

C. elegans is a multicellular model organism well suited for HTS. It is small (<1 mm) and can be economically cultured and dispensed in liquids. *C. elegans* is also one of the most experimentally tractable animal models permitting rapid and detailed identification of drug mode-of-action[3].

We describe a protocol for culturing and dispensing fluorescent strains of *C. elegans* for high-throughput screening of chemical libraries or detection of environmental contaminants that alter the expression of a specific gene. Large numbers of developmentally synchronized worms are grown in liquid culture, harvested, washed, and suspended at a defined density. Worms are then added to black, flat-bottomed 384-well plates using a peristaltic liquid dispenser. Small molecules from a chemical library or test samples (e.g., water, food, or soil) can be added to wells with worms. *In vivo*, real-time fluorescence intensity is measured with a fluorescence microplate reader. This method can be adapted to any inducible gene in *C. elegans* for which a suitable reporter is available. Many inducible stress and developmental transcriptional pathways are well defined in *C. elegans* and GFP transgenic reporter strains already exist for many of them[4]. When combined with the appropriate transgenic reporters, our method can be used to screen for pathway modulators or to develop robust biosensor assays for environmental contaminants.

We demonstrate our *C. elegans* culture and dispensing protocol with an HTS assay we developed to monitor the *C. elegans* cap 'n' collar transcription factor SKN-1. SKN-1 and its mammalian homologue Nrf2 activate cytoprotective genes during oxidative and xenobiotic stress[5-10]. Nrf2 protects mammals from numerous age-related disorders such as cancer, neurodegeneration, and chronic inflammation and has become a major chemotherapeutic target[11-13].Our assay is based on a GFP transgenic reporter for the SKN-1 target gene *gst*-4[14], which encodes a glutathione-s transferase[6]. The *gst*-4 reporter is also a biosensor for xenobiotic and oxidative chemicals that activate SKN-1 and can be used to detect low levels of contaminants such as acrylamide and methyl-mercury[15-16].

Video Link

The video component of this article can be found at http://www.jove.com/video/2745/

Protocol

1. Preparation of bacterial worm food

Day 1

1. Add 5 ml of saturated *E. coli* OP50 bacterial culture to 500 ml Terrific broth supplemented with 50 µg/ml streptomycin and grow in a shaking incubator (225 rpm) overnight at 37°C.

Day 2

2. Split the overnight bacterial culture into ten 50 ml tubes and centrifuge bacteria in a refrigerated centrifuge at 2,500 rcf for 20 minutes.
3. Decant off LB broth and resuspend each bacterial pellet in 10 ml of liquid nematode growth media (NGM). Shake horizontally in a floor shaker for 15 minutes to resuspend the bacteria. To make NGM buffer, add 3 g NaCl to 1 L deionized water and autoclave. Cool to 55°C and add in order the following sterile solutions: 1 ml of 1 M $CaCl_2$, 1 ml of 1 M $MgSO_4$, and 25 ml of 1 M potassium phosphate, pH 6.0.
4. Centrifuge the bacterial culture in a refrigerated centrifuge at 2,500 rcf for 20 minutes.
5. Decant off NGM buffer and weigh the bacterial pellet.
6. Add an equal volume of NGM buffer to resuspend the pellets, aliquot 3 ml of bacteria concentrate into 15 ml tubes, and store at -20°C.

2. Large-scale *C. elegans* liquid culture

We use a transgenic line VP596 (dvIs19[pAF15(*gst-4::GFP::NLS*)];vsIs33[*dop-3::RFP*]) carrying two fluorescent constructs: *Pgst*-4::GFP[14] to monitor SKN-1 activity and *Pdop-3::RFP*[17] to serve as a standard for worm number normalization.

Day 1

1. Mix 150 ml NGM buffer, 1.5 ml LB broth, 150 µl of 5 mg/ml cholesterol (in ethanol), and 75 µl of 100 mg/ml streptomycin. Filter-sterilize the mixture and add to a 1 liter sterile flask.

   NOTE: Adding 1% LB broth to NGM buffer helps to prevent worms from sticking to glassware and plasticware, but this amount is not enough to support bacteria growth.
2. Synchronize worms with the standard hypochlorite procedure[18]. Up to 0.5 ml of gravid worms can be processed in a single 15 ml tube with 5 ml of hypochlorite solution (3.75 ml sterile water, 1 ml household bleach, and 250 µl 10 N NaOH). Wash the released eggs with sterile water and resuspend them in 10 ml of NGM buffer.
3. Dilute a sample of the eggs 100 fold in NGM buffer (e.g., 100 µl in 10 ml) and count the number of eggs in three separate 5 µl aliquots. Multiple the average number by 200,000 (100 dilution factor x 2,000) to estimate the total number of eggs.
4. Add 200,000 to 2 million eggs to the flask with NGM buffer and shake the culture at 100 rpm at 20°C.

Day 2

5. At least 16 h after adding eggs to the flask, use a sterile serological pipet to remove approximately 0.5 ml of the suspended worm culture. Pipet three 5 µl drops onto the sterile lid of a Petri dish. Place the lid in a -20°C freezer for 1-2 minutes to paralyze worms. Count the average number of live hatched worms per 5 µl and then multiple by 30,000 (150 ml/5 µl) to estimate the total number of hatched worms.
6. Thaw a tube of frozen OP50 bacterial culture (Protocol 1.6) and add 3 ml of 50% OP50 into the worm culture per 500,000 hatched worms. Shake the flask at 100 rpm at 20°C. Worms will develop into L4 larvae and young adults in about 51 hours. The OD600 of bacteria should be about 2.200 at the beginning. Monitor the amount of bacteria by absorbance and add more if the OD 600 drops below 0.900.

3. Worm collection and dispensing

Day 4

1. Use a sterile serological pipet to transfer approximately 0.5 ml of the suspended worm culture to a standard NGM agar plate. View the worms with a stereomicroscope to make sure that most are L4 larvae or young adults. L4 larvae will have a ventral clear spot in the middle of the body. Young adults will be slightly larger and will not have a clear spot.
2. Pour the worm culture into sterile 50 ml tubes and place the tubes in a test tube rack on the benchtop. Allow the worms to settle for about 10 minutes. Remove the supernatant by aspiration or pipetting.

   NOTE: This step can be important to remove unhatched eggs or worms that did not develop because they will settle slower than L4 larvae and young adults.
3. Collect all worms into a single 50 ml tube and wash with NGM buffer with 1% LB broth (NGM+LB) 3-4 times to remove bacteria.

   NOTE: This is best done in a swinging-bucket centrifuge by collecting worms at 500 rcf for 30 sec and replacing the supernatant with fresh NGM+LB buffer.
4. After washing, fill the tube with NGM+LB buffer to 50 ml and pour into a custom worm dispensing flask. Add a small stir bar and stir to keep the worms suspended. Count the number of worms in three 5 µl drops as indicated above. Dilute with NGM+LB until you get 12-15 worms per 5 µl drop (˜2.5-3.0 worms /µl).

   NOTE: A minimum of about 20 ml (60,000 worms) is needed to fill the dead space of the dispensing flask and dispenser cassette. Each 384 well plate requires about 35,000 worms or 11.5 ml of suspended worms.
5. Load a 10 µl dispensing cassette and sterilize by priming with 70% ethanol. Rinse out the ethanol by priming with sterile water.
6. Insert the end of the dispensing cassette into the flask so that it is just above the stir bar without disrupting its movement. Make sure that the worms remain suspended throughout the whole flask. Program the dispenser to dispense at low speed with 2 pre-pulses. Prime and run at least 10 ml of suspended worms. Fill plates as needed rapidly to prevent the worms from settling in the tubing.
7. Seal the plates with breathable tape.
8. Place the plates on a shaking platform in an incubator at the appropriate temperature for your assay. Do not stack the plates.

4. Fluorescence analysis

1. Place the target plate into a microplate reader to measure the fluorescence intensity of each well with the appropriate emission and excitation wavelength (Filters for our assay: GFP 485/20ex 528/20em; RFP 540/25ex 590/35em).
2. Calculate the ratio of GFP/RFP and normalize with the readings of the control wells (without activating compound) to determine the exact fold difference of the fluorescence intensity derived from individual treatments.

5. Representative Results:

Our SKN-1 assay uses a chromosomally integrated dual reporter strain (VP596). As shown in Figure 1, the number of worms is well correlated to volume dispensed. As shown in Figures 2A and 2B, total GFP and RFP fluorescence per well is highly reproducible from well to well across a 384 well plate. As shown in Figure 2C, un-induced *Pgst-4::GFP* fluorescence is linearly correlated to *Pdop-3::RFP* across 384 wells. When expressed as a ratio of GFP/RFP, fluorescence becomes highly reproducible from well to well across a 384 well plate (compare coefficient of variation from Figure 2D to 2A) demonstrating the ability of the *Pdop-3::RFP* reporter to reduce variability. As shown in Figure 3, the induction of GFP relative to RFP with a SKN-1 activating xenobiotic (38 µM juglone) is robust and highly reproducible across a 384 well plate.

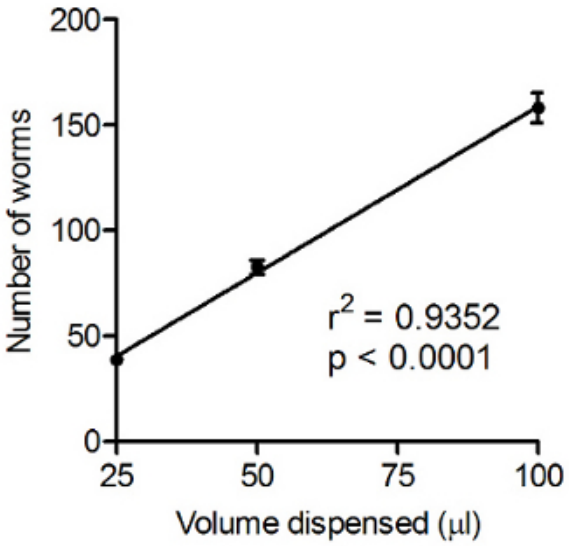

Figure 1. Number of worms versus volume dispensed. Worms were diluted to approximately 2/µl and dispensed into a 24 well plate for manual counting with a stereomicroscope. N = 8 wells per volume.

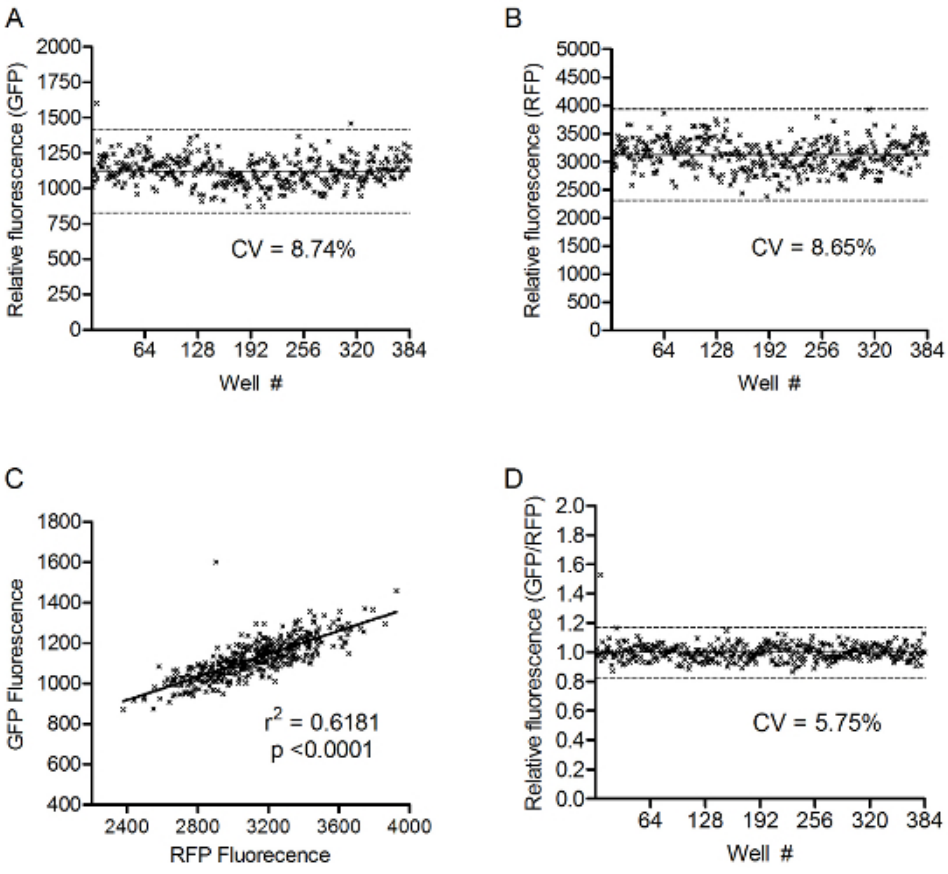

Figure 2. Total fluorescence of worms dispensed into a 384 well plate is reproducible. Worms were diluted to approximately 2.5/µl and 30 µl was dispensed into every well of a 384 well plate. GFP (A) and RFP (B) fluorescence of all wells had a coefficient of variation below 9%. (C) GFP fluorescence is highly correlated to RFP fluorescence. (D) Calculating the ratio of GFP to RFP reduced the coefficient of variation to below 6% (A, B, and D) Solid lines indicate the means and broken lines indicate three standard deviations above or below the mean.

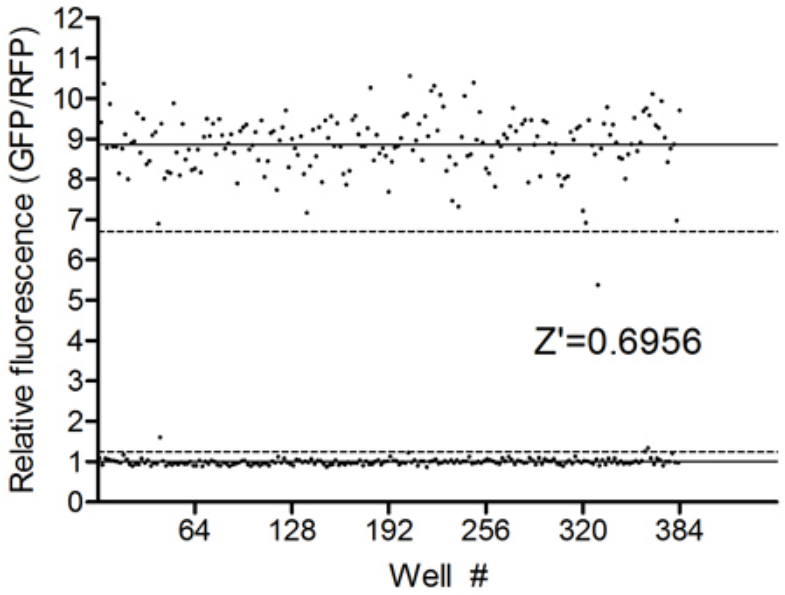

Figure 3. Activation of *Pgst-4::GFP* is robust and consistent. Approximately 75 L4 worms were dispensed into all wells of a 384 well plate and 38 μM juglone was added in every other column. GFP and RFP fluorescence was measured after 21 h of incubation. The mean relative fluorescence ratios of all control (1.0) and juglone wells (8.9) are marked with solid lines. Three standard deviations above the control mean and below the juglone mean are marked with broken lines.

Discussion

We present a method for culturing and dispensing large numbers of transgenic nematodes. The equipment used for culturing worms is standard for laboratories performing molecular cloning and the liquid handling and fluorescence equipment is standard for laboratories processing large numbers of microplates. Other methods of dispensing large numbers of live *C. elegans* require expensive particle sorting equipment[19]. The *Pgst-4::GFP* assay can be used to screen for small molecule modulators of cap 'n' collar transcription factors and to detect xenobiotic and oxidant contaminants in environmental and food samples[14,16]. Robust inducible transgenic GFP reporters are available for a wide-range of pathways and environmental stimuli[4], and therefore our method should be applicable to developing modulators of many pathways and to detect a broad spectrum of contaminants.

Disclosures

No conflicts of interest declared.

Acknowledgements

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