

Imaging the Pharynx to Measure the Uptake of Doxorubicin in *Caenorhabditis elegans*

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[Abstract] *Caenorhabditis elegans* offers an array of advantages to investigate the roles of uptake transporters. Herein, an epifluorescent microscopy approach was developed to monitor the uptake of the autofluorescent anticancer drug, doxorubicin, into the pharynx of *C. elegans* by organic cation transporters.

Keywords: *C. elegans*, Organic cation uptake transporters, Drug uptake, Doxorubicin, Autofluorescent drug, Epifluorescent microscopy, RNAi feeding bacteria

[Background] Human cells have over 450 solute carrier transporters that are believed to facilitate the uptake of several ions, nutrients, as well as both therapeutic and anticancer drugs (Cesar-Razquin *et al.*, 2015). However, the roles and substrates of a large number of these uptake transporters are not known. *C. elegans* is an inexpensive model organism that offers a multitude of advantages over mammalian cells to rapidly study many biological processes that are highly conserved in nature. During the last decade, this organism has been instrumental in several drug discovery programs to identify novel small molecules, e.g., those that act as antimicrobials and inhibit oxidative stress, although the yield of bioactive compounds has been less striking (Burns *et al.*, 2010; O'Reilly *et al.*, 2014). We reason that the recovery rate could be higher if there is greater and selective influx of the molecules by uptake transporters into the animal cells. To date, only three studies have been performed to understand the roles of uptake transporters in *C. elegans* (Wu *et al.*, 1999; Cheah *et al.*, 2013; Papaluca and Ramotar, 2016). Thus, characterization of the function and substrate specificities of uptake transporters in this organism will be advantageous towards improving the strategies employed to identify novel bioactive molecules. Herein, we outline a method to monitor uptake of the anticancer drug doxorubicin into the pharynx of *C. elegans* (Papaluca and Ramotar, 2016). Doxorubicin autofluorescence can be readily monitored by several widely available detection systems such as the epifluorescent microscope. We note that several benefits can be derived from this approach including a hunt for novel therapeutic substrates of the transporter by competing for doxorubicin uptake.

Materials and Reagents

1. Petri dish 60 x 15 mm (SARSTEDT, catalog number: 82.1194.500)
2. 15 ml conical tube

3. Frosted microscope slides (size: 1 x 3" ; thickness: 1-2 mm) (UltiDent Scientific, catalog number: 170-7107A)
4. Microscope cover glass (size: 22 x 22 #1.5) (Fisher Scientific, catalog number: 12-541B)
5. Platinum wire (Thomas Scientific, catalog number: 1233S71)
6. Pasteur pipet (Fisher Scientific, catalog number: 13-678-20C)
7. *E. coli* bacteria HT115^{DE3} with the plasmid pL4440-empty vector
8. *E. coli* bacteria HT115^{DE3} with the plasmid pL4440-oct-1 (Ahringer's collection). Sequence verified
9. *E. coli* bacteria HT115^{DE3} with the plasmid pL4440-oct-2 (Ahringer's collection). Sequence verified
10. Bristol N2 (wild type) and RB1084 [*oct-1(ok1051)* I] from *Caenorhabditis* Genetic Center
11. Ampicillin (Sigma-Aldrich, catalog number: A9518)
12. Potassium phosphate monobasic (KH₂PO₄) (Bio Basic, catalog number: PB0445)
13. Magnesium sulfate (MgSO₄) (Bio Basic, catalog number: MRB0329)
14. Calcium chloride dihydrate (CaCl₂) (Fisher Scientific, catalog number: C79-500)
15. Cholesterol (Sigma-Aldrich, catalog number: C8503)
16. Ethanol 100% (works from any company)
17. Doxorubicin (for Research from Hôpital Maisonneuve-Rosemont, Montreal, Canada). Stock concentration at 2 mg/ml
18. IPTG (Bio Basic, catalog number: PRB0447)
19. Levamisole hydrochloride (MP Biomedical, catalog number: 155228)
20. Clear nail polish from Wild Shine from Dollarama
21. Tryptone (Bio Basic, catalog number: TG217(G211)) for Luria Broth (LB) media
22. Yeast extract (Wisent Bioproducts, catalog number: 800-150-LG) for LB media
23. Sodium chloride (NaCl) (Wisent Bioproducts, catalog number: 600-082)
24. Bacteriological agar (Wisent Bioproducts, catalog number: 800-010-CG)
25. Peptone (Wisent Bioproducts, catalog number: 800-157-LG) for nematode grown media (NGM)
26. Agarose (Wisent Bioproducts, catalog number: 800-015-CG)
27. Sodium hydroxide (NaOH) (Bio Basic, catalog number: SB0617)
28. Bleach Lavo Pro6 (Lavo Inc, Montreal, Canada)
29. Sodium phosphate dibasic (Na₂HPO₄) (Bio Basic, catalog number: S0404)
30. LB solution (see Recipes)
31. Nematode growth media (NGM) (see Recipes)
32. Agar pad (see Recipes)
33. Alkaline Hypochlorite solution for bleaching the worms (see Recipes)
34. M9 buffer (see Recipes)

Equipment

1. Incubator at 20 °C, but with a range from 15 to 37 °C (SHEL LAB, model: 2020)
2. 37 °C incubator (Panasonic Healthcare, model: Mir-262)
3. 37 °C shaker (Inforst, model: Multitron Standard)
4. 500 ml glass bottle (Wheaton graduated glass media bottles with lined caps)
5. Microwave (inverter model, Panasonic Healthcare)
6. 55 °C water bath (Precision Scientific, catalog number: 66800)
7. Metal spatula (VWR)
8. Flame
9. Neutrex culture tubes 16 x 15 mm
10. Pyrex Erlenmeyer flask different sizes for bacteria culturing
11. Autoclave
12. Stereomicroscope Leica MZ 8 (Leica 10445538 Plan Microscope Objective Lens 1.0x) (Leica, model: MZ 8)
13. DeltaVision Elite Restoration System (GE Healthcare, model: DeltaVision Elite High Resolution Microscope) and the [DeltaVision imaging system user's manual](#)
14. Fisher Vortex Genie 2 (Fisher Scientific, catalog number: 12-812)
15. Eppendorf 5810 R centrifuge (Eppendorf, model: 5810 R)
16. VWR rocking platform shakers basic (VWR)
17. Ptc-100 Programmable Thermal Controller 96 Well (Bio-Rad Laboratories, model: Ptc-100® Programmable Thermal Controller)
18. Centrifuge (Sigma Centrifuge, model: Sigma 1-14)

Software

1. ImageJ imaging software
2. SoftWorx software

Procedure

A. Preparation of bacteria and media for worm growth

1. Bacteria preparation (*C. elegans* food)
 - a. In 10 ml LB medium, add 10 µl ampicillin (stock 100 mg/ml).
 - b. Inoculate a few colonies of *E. coli* HT115^{DE3} empty vector bacteria into the LB + ampicillin medium.
Note: Alternative bacteria: E. coli OP50.
 - c. Incubate for 6 h at 37 °C in an orbital shaker at 200 rpm.

2. *C. elegans* growth media preparation
 - a. Melt the solid NGM contained in a glass bottle (500 ml) using a microwave (power level 2 for 25 min).
 - b. Keep the melted medium at 55 °C in a water bath for 15-20 min.
 - c. Before usage, add:
 - 12.5 ml of 1 M KH₂PO₄ pH 6
 - 500 µl of 1 M MgSO₄
 - 500 µl of 1 M CaCl₂
 - 500 µl cholesterol (stock 5 mg/ml in 95% ethanol)
 - 500 µl of ampicillin (stock 100 mg/ml), if using knockdown bacteria strain HT115^{DE3} carrying the L4440 vector or the target gene.
 - d. Gently invert the bottle a few times to mix all the ingredients and then pour 6 ml of the medium into each of 60 x 15 mm Petri dish.
 - e. Let it dry at room temperature for around 1 h.
 - f. Once dried, using a sterilized bacterial plating rod spread 70 µl of bacteria prepared in step A1 above to form a lawn. The optical density OD₆₀₀ of the bacteria is approximately 1.0. Alternatively, the bacteria can be added as 3 drops distributed onto the plate.
 - g. Incubate the plate overnight at 37 °C.
3. Worm growth
 - a. From a grown worm plate, cut 4 pieces of approximately 1 x 1 cm agar with a pointy spatula sterilized with 100% ethanol and flame.
 - b. Transfer each agar piece by turning them over onto each of 4 plates prepared in step A2.
 - c. Incubate these worm plates for 2 days at 20 °C.

B. Worm synchronisation

1. The 4 plates are expected to be filled with adult worms, visualized through a stereomicroscope, and at which point the synchronization can be done. An alternative method to obtain a huge amount of worms is to grow them in liquid media (NGM medium without agar).
2. Add 2 ml of alkaline hypochlorite solution (bleaching solution) to each plate and rapidly collect the worms by pipetting (about 10 sec per plate) in a sterile 15 ml conical tube. Date and label the tube with the name of the worm strain. Alternatively, harvest the worms with 1 ml of M9 buffer and collect them into 15 ml conical tubes. Centrifuge in the Eppendorf 5810 R centrifuge, using swing bucket rotor (A-4-81) with adapters for 15 ml conical tubes at 1,740 x g for 2 min at 4 °C. Discard the M9 buffer and add 2 ml of the bleaching solution. In this way the worms are washed once.
3. Quickly start to vortex the worms for 7 min at setting 8 on the vortex and check under the stereomicroscope if the adult worms are lysed and only the eggs are present.

Note: Steps B2 and B3 must be carried out with care as they are critical for the eggs to hatch.

4. Without any delay, centrifuge in the Eppendorf 5810 R centrifuge at 1,740 x g for 2 min at 4 °C.

5. Aspirate the supernatant, but leave approximately 1 ml of the solution.
6. Adding 5 ml of M9 buffer and repeat the centrifugation and aspiration as in steps B4 and B5.
7. Repeat 4 more times step B6, in order to remove any trace of the bleaching solution.
8. Incubate the eggs in the final 1 ml of M9 buffer overnight at 20 °C. During this time the eggs will hatch and produce L1 stage worms.

C. Worm under treatment

1. Bacteria preparation
 - a. In 3 different tubes, add 10 ml LB medium and 10 µl ampicillin (100 mg/ml).
 - b. In the 1st tube, add a few colonies of *E. coli* HT115^{DE3}/empty vector L4440.
 - c. In the 2nd tube, add a few colonies of *E. coli* HT115^{DE3}/pL4440-oct-1.
 - d. In the 3rd tube, add a few colonies of *E. coli* HT115^{DE3}/pL4440-oct-2.
 - e. Incubate all the tubes for 5 h in a 37 °C orbital shaker.
2. Drug plate preparation
 - a. Melt a bottle of 500 ml of solid NGM as above (see *C. elegans* growth media preparation above).
 - b. For normal drug plates-no knockdown
 - i. Transfer 30 ml of melted NGM media kept at 55 °C into a small sterile bottle. This will make 3 Petri dishes (60 x 15 mm) each containing ~10 ml of NGM media for triplicate assays.
 - ii. Add the doxorubicin (stock 2 mg/ml) to the desired final concentrations for each 6 ml of NGM media and pour onto the 60 x 15 mm Petri dish. For doxorubicin use between 0.1 to 100 µM depending on the strains. Uptake was observed with as low as 0.5 µM of doxorubicin.
IMPORTANT: Do not forget to prepare a plate without drug.
 - iii. Leave plates for at least 15 min at room temperature for the medium to solidify.
 - iv. Add 70 µl *E. coli* HT115^{DE3}/pL4440-empty vector bacteria.
 - v. Incubate the plates overnight at 37 °C.
 - c. For knockdown drug plates
 - i. Transfer 30 ml of melted NGM medium kept at 55 °C into a small sterile bottle.
 - ii. Add 30 µl of 1 M IPTG, needed to induce the production of dsRNA from the L4440 vector carrying a fragment of the target gene.
 - iii. Add to 6 ml of this media the desired concentrations of doxorubicin as above.
IMPORTANT: Do not forget to prepare a plate without drug.
 - iv. Pour 6 ml of media with and without IPTG and with doxorubicin onto 60 x 15 mm Petri dishes.
 - v. Dry the plates, as above.
 - vi. Add 70 µl *E. coli* HT115^{DE3}/pL4440-oct-1 bacteria for 1st set of 5 plates.
 - vii. Add 70 µl *E. coli* HT115^{DE3}/pL4440-oct-2 bacteria for 2nd set of 5 plates.

viii. Incubate the plates overnight at 37 °C.

3. Now all the plates are ready to add 50 μ l of the L1 synchronized worms.
4. Verify if there are nearly 50 L1-staged worms on the plates under the stereomicroscope.
5. Incubate the plates at 20 °C for 3 days.

D. Microscope visualisation

1. Preparation of slides

- a. On a frosted microscope slide add one drop (~50 μ l) of 3% agarose and squeeze it with another slide to form a sandwich. After 10 sec, remove one of the slide to expose the agarose.
- b. Dry the slide with the agarose at 65 °C around 15-30 min using a Programmable Thermal Controller 96 Well.

2. Mounting the worms

- a. Add 7 μ l of 1 M levamisol onto the surface of the dried agar on the slide.
- b. To each slide, add 10-15 young adult worms from a single treatment condition by using a platinum wire to transfer the worms.
- c. Sterilize the platinum wire using a flame before transferring the next 10-15 worms from a different treatment condition onto a new slide.
- d. Add the coverslip and seal around the slide and coverslip with clear nail polish.

3. Microscopy visualisation

The slide containing the worm is ready to be visualised. Place the slide on the stage of an epifluorescence microscope.

1st Adjust the objectives

2nd Select the filters and adjust all the necessary parameters

3rd Once everything is ready, start capturing the images

4th Save the images

Below is a detail description to visualize the worms using a DeltaVision microscope.

- a. Add a small drop of DeltaVision Immersion oil 1.534 onto the slide in order to use objective 40x.
- b. Place the mounted slide on the microscope by inverting it.
- c. Adjust the eyepiece filter roll to POL filter.
- d. Open the SoftWorx software . See GE Healthcare manual (GE Healthcare, 2014).
- e. Press on the microscope.

3 windows will be opened (Figure 1).

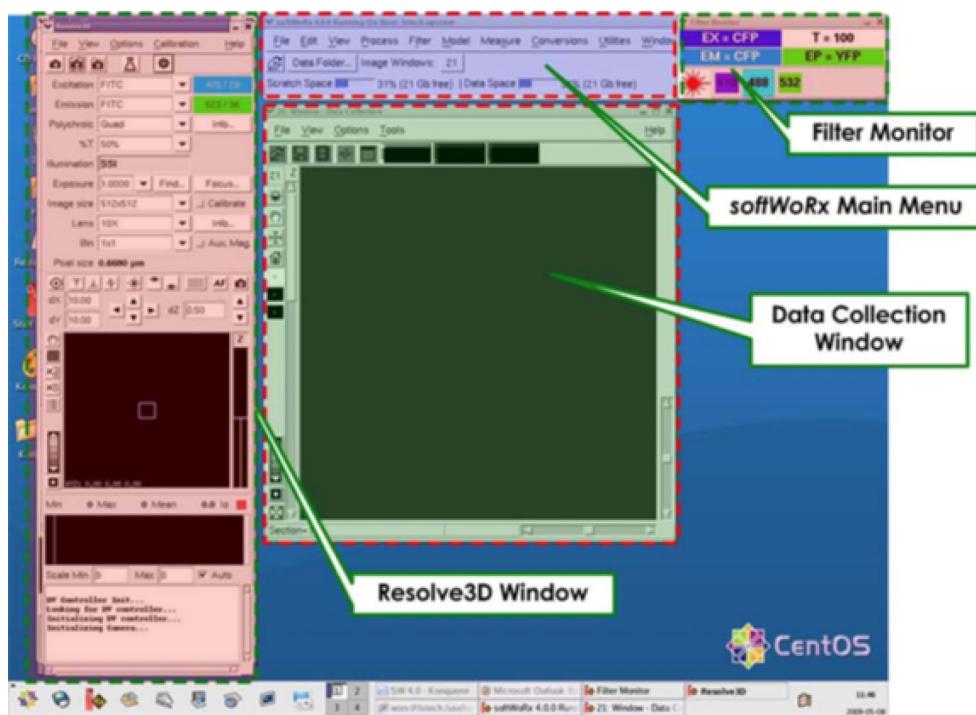


Figure 1. SoftWorx desktop display. The Resolve3D window includes acquisition parameters and controls for moving the stage, the Data Collection window displays images as they are acquired, and the Filter Monitor displays the filters currently selected.

- f. On the window called Resolve3D (Figure 1).
- g. Press on the gear wheel (setting) to open Resolve3D setting window (Figure 2).

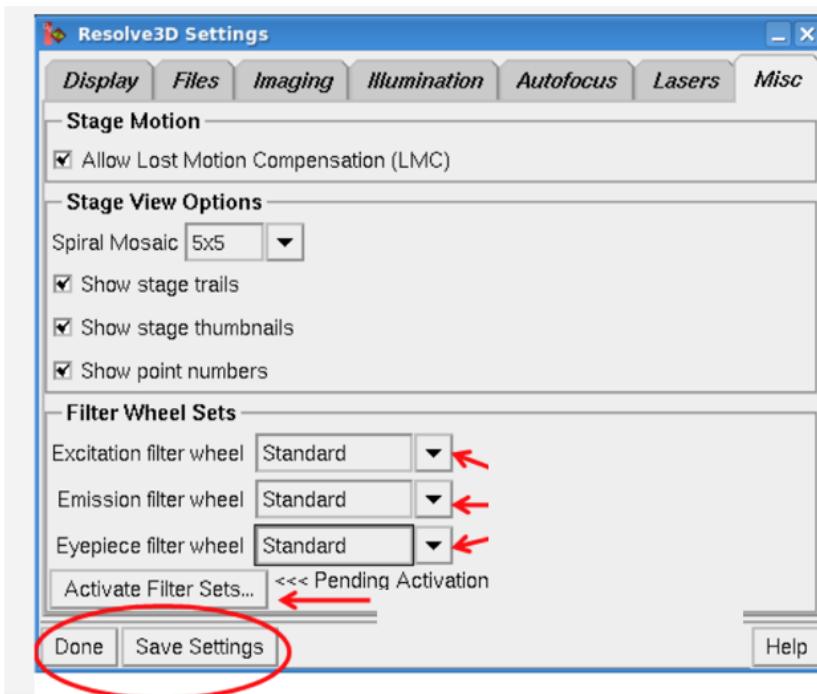


Figure 2. Resolve3D window setting

- i. Under MISC.
- ii. Select the filters.
- iii. Save settings.
- iv. Under FILES (Figure 3).

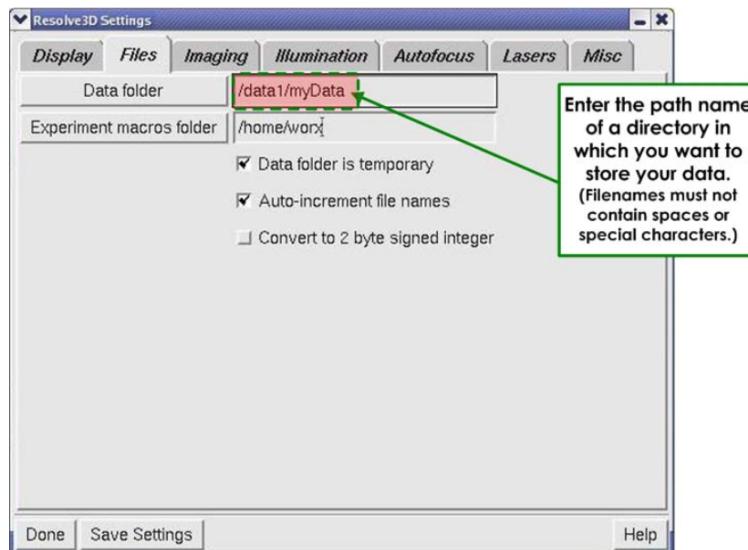


Figure 3. Display of the Files tab

- v. In the Data folder field (Figure 3), enter the directory in which to save the image.
- vi. In the Experiment macros folder field (Figure 3), enter the same directory.
- vii. Save settings.
- viii. Done.

h. In the Resolve3D window (Figure 1), set the following parameters in Figure 4.

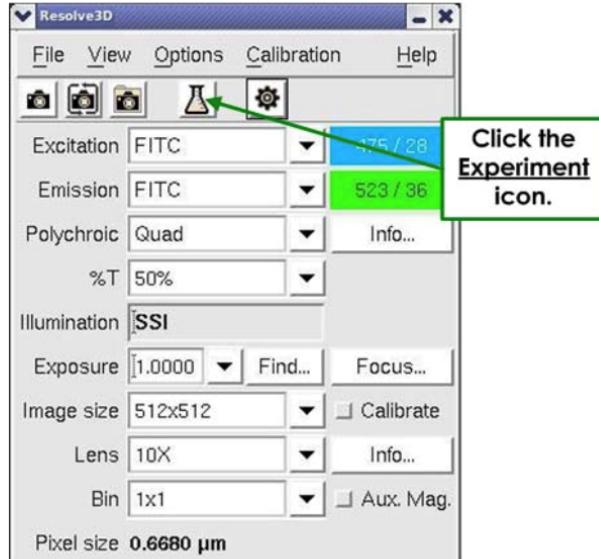


Figure 4. Setting the parameters

- i. Excitation: POL.
- ii. Emission: POL.
- iii. % T: 32%.
- iv. Exposure: 0.025.
- v. Lens: 40x.

- i. Press on the Erlenmeyer.

- j. A window called Design/Run Experiment window will be opened (Figure 5).

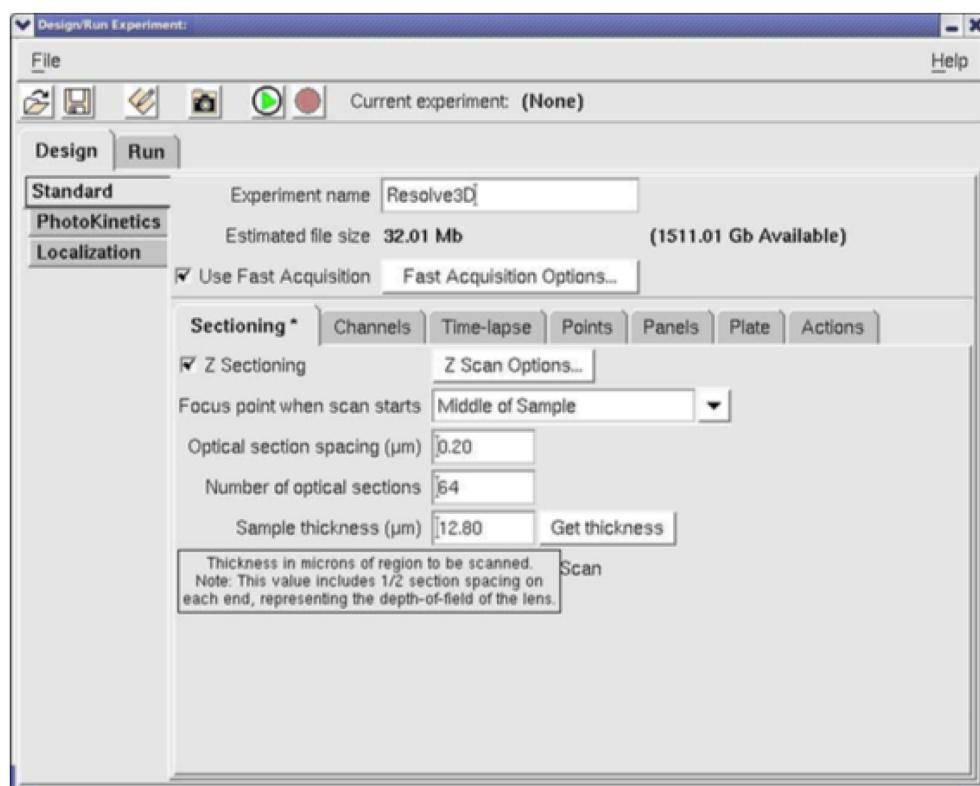


Figure 5. Design and Run experiment window

- i. Under design
 - 1) Experimental name field: write your file name (Figure 5).
 - 2) Under Sectioning
Remove the check beside the Z sectioning.
 - 3) Under channels (Figure 5).

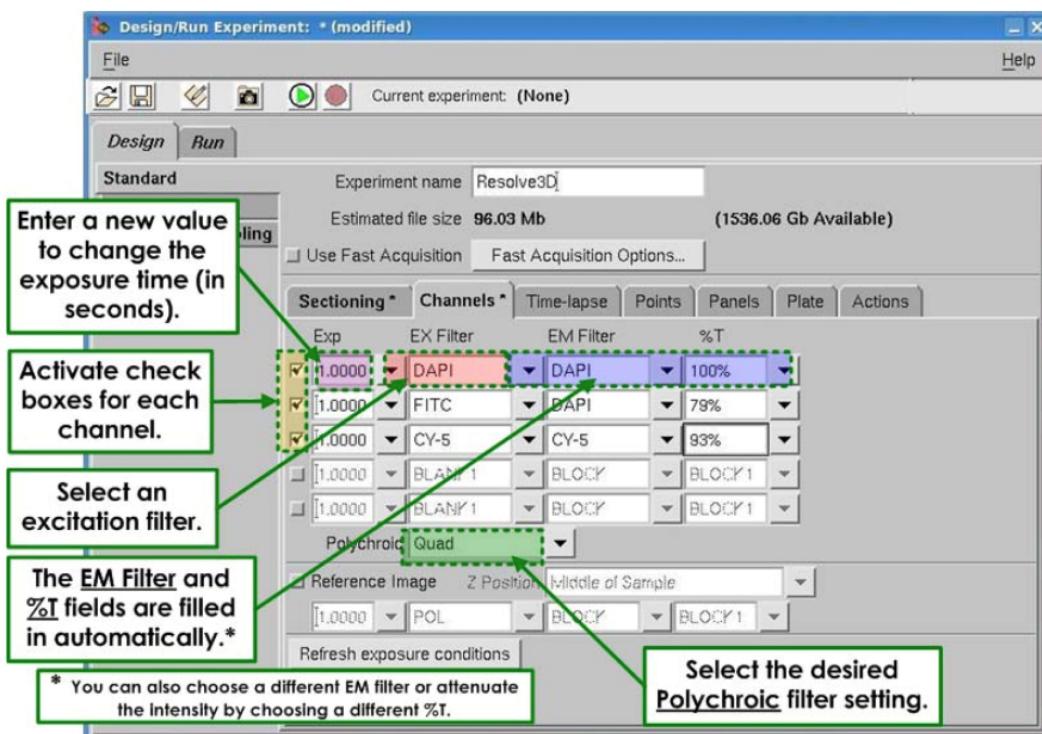


Figure 6. The Channels in the Design and Run experiment window

Check 1st box (Figure 6)

Under EX filter: POL

Automatically EM filter will be modifying

Under% T: choose 32%

Check 2nd box (Figure 6)

Under EX filter: FITC

Automatically EM filter will be modified

Under% T: choose100%

Check 3rd box (Figure 6)

Under EX filter: mCherry

Automatically EM filter will be modified

Under% T: choose 100%

ii. Under Run before starting to acquire image

Image file name field, write the same name you wrote under the design/Experimental name (Figure 5).

iii. Now you are ready to acquire image by pressing on the start button  as shown in Figure 7. You can adjust the image by looking on the Resolve3D window which contains the stage trails in the stage View window. This way you can focus at the pharynx level.

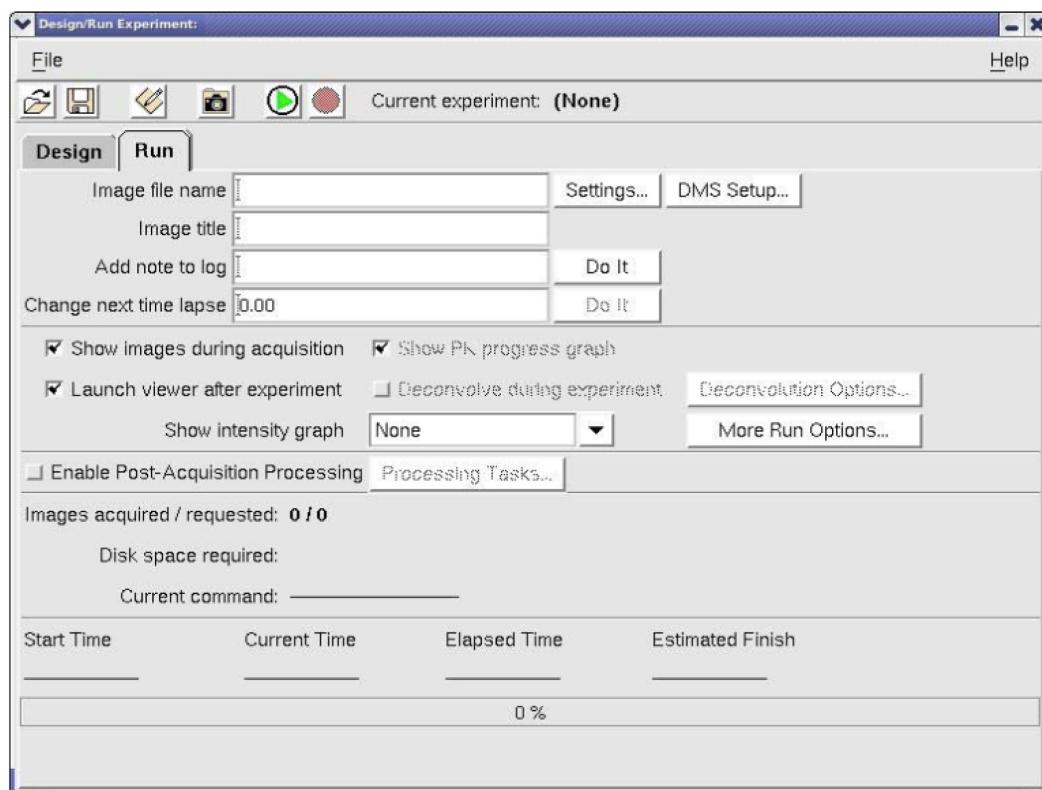


Figure 7. The start button in the Design and Run experiment window

4. Result analysis
 - a. Software used: ImageJ
 - i. Drag the file to imageJ window.
 - ii. Under image → Transform → rotate the image to get an image of the worm placed anterior to posterior.
 - iii. Under image → Stacks → Stack to images.
 - iv. Under image → Color → merge channels.
Select the image which is appropriate to the desired color.
 - v. Create montage: under image → Stacks → make montage.
 - vi. Create the 3D picture based on the intensity.
Take the merged image.
Under Plugins → 3D → Interactive 3D surface plot.

Data analysis

The result is representative of a single worm from an experiment where 10 worms were placed onto the slide (Figure 8). The experiment was repeated three times with identical data. Fluorescence posterior to the pharynx is autofluorescence detected from the intestine (Figure 8). The data were quantified using ImageJ according to Papaluca and Ramotar (2016).

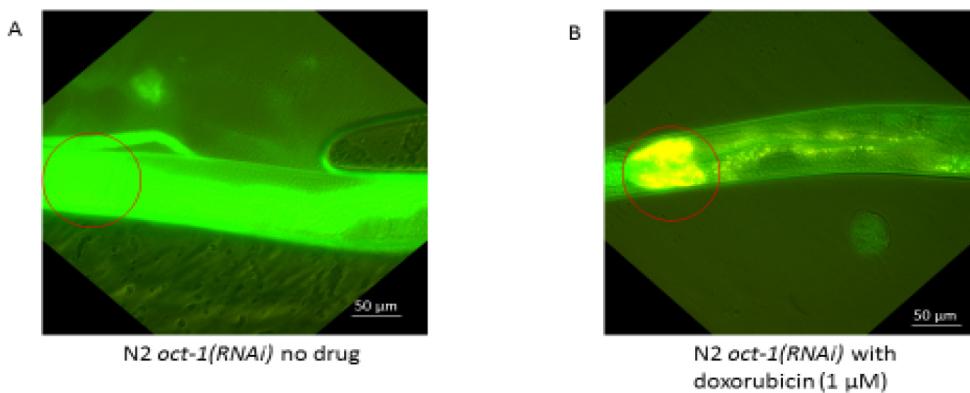


Figure 8. Uptake of doxorubicin in the N2 wild type animals downregulated for *oct-1* that causes upregulation of OCT-2. Previously, we showed that downregulation stimulated expression of *oct-2*, which drives uptake of drugs into *C. elegans* (Papaluca and Ramotar, 2016). A. Untreated; the absence of doxorubicin is illustrated by the lack of yellow fluorescence and the green background is autofluorescence from ingestion of food by the animal. B. Treated with doxorubicin (1 μM). Doxorubicin uptake is visible as yellow due to the merge of the green autofluorescence and red fluorescence from the drug. Circles show the location of the pharynx. Scale bars = 50 μm. See Data analysis for additional details.

Notes

1. Instead of using frozen bacteria as worm food, we typically use fresh culture of the bacteria.
2. Always work under sterile conditions. Be on the watch for fungus (white to black like spider net) and rarely mushroom (brownish spots). If this is the case, the best solution will be to bleach the worms and collect the eggs for new larvae.

Recipes

1. LB solution
5 g tryptone
2.5 g yeast extract
5 g NaCl
ddH₂O up to 500 ml
Add 7.5 g of agar, if making solid media plates
2. Nematode growth media (NGM)
1.25 g peptone
1.5 g NaCl
8.75 g agar
ddH₂O up to 500 ml
Autoclave

When the media is between 50 to 55 °C, add the following constituents before pouring the plates:

12.5 ml of 1 M KH_2PO_4 pH 6

500 μl of 1 M MgSO_4

500 μl of 1 M CaCl_2

500 μl cholesterol (stock 5 mg/ml in 95% ethanol)

500 μl of ampicillin (stock 100 mg/ml), if using the plasmid containing bacteria HT115^{DE3}

3. Agar pad

3% agarose in MilliQ water

Melt when needed

4. Alkaline hypochlorite solution (bleaching solution) (make fresh monthly)

8.25 ml ddH₂O

3.75 ml 1 N NaOH

3.00 ml Bleach (Javel)

5. M9 buffer (1 L)

6 g Na_2HPO_4

3 g KH_2PO_4

5 g NaCl

0.25 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$

Sterilized by autoclaving

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