

Orsay Virus Infection in *Caenorhabditis elegans*

Lakshmi E. Batachari,¹ Mario Bardan Sarmiento,¹ Nicole Wernet,¹
and Emily R. Troemel^{1,2} 

¹School of Biological Sciences, University of California San Diego, La Jolla, California

²Corresponding author: etroemel@ucsd.edu

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Orsay virus infection in the nematode *Caenorhabditis elegans* presents an opportunity to study host-virus interactions in an easily culturable, whole-animal host. Previously, a major limitation of *C. elegans* as a model for studying antiviral immunity was the lack of viruses known to naturally infect the worm. With the 2011 discovery of the Orsay virus, a naturally occurring viral pathogen, *C. elegans* has emerged as a compelling model for research on antiviral defense. From the perspective of the host, the genetic tractability of *C. elegans* enables mechanistic studies of antiviral immunity while the transparency of this animal allows for the observation of subcellular processes in vivo. Preparing infective virus filtrate and performing infections can be achieved with relative ease in a laboratory setting. Moreover, several tools are available to measure the outcome of infection. Here, we describe workflows for generating infective virus filtrate, achieving reproducible infection of *C. elegans*, and assessing the outcome of viral infection using molecular biology approaches and immunofluorescence. © 2024 The Authors. Current Protocols published by Wiley Periodicals LLC.

Basic Protocol 1: Preparation of Orsay virus filtrate

Support Protocol: Synchronize *C. elegans* development by bleaching

Basic Protocol 2: Orsay virus infection

Basic Protocol 3: Quantification of Orsay virus RNA1/RNA2 transcript levels by qRT-PCR

Basic Protocol 4: Quantification of infection rate and fluorescence in situ hybridization (FISH) fluorescence intensity

Basic Protocol 5: Immunofluorescent labeling of dsRNA in virus-infected intestinal tissue

Keywords: anti-dsRNA immunofluorescence • *C. elegans* • Orsay virus • virus filtrate preparation • virus infection

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INTRODUCTION

Isolated from wild strains of *Caenorhabditis elegans*, the Orsay virus is the only natural virus known to infect this nematode (Félix & Wang, 2019; Félix et al., 2011). Belonging to the *Nodaviridae* family, the Orsay virus is a positive-sense, single-stranded RNA virus with a genome that consists of two segments, RNA1 and RNA2. At ~3.4 kb, the RNA1

segment contains a gene that encodes the RNA-dependent RNA polymerase (RdRP), which controls viral replication. The 2.6 kb RNA2 segment encodes the viral capsid protein as well as a capsid-delta fusion protein (Jiang et al., 2014). Viral replication occurs within intestinal cells, and horizontal transmission from animal-to-animal enables viral spread; no vertical transmission has yet been reported for Orsay virus. As many canonical defense factors are absent in *C. elegans*, the study of immune responses in this nematode host has the potential to uncover novel antiviral defense pathways.

Several factors make *C. elegans* a powerful model for investigating cell-intrinsic as well as systemic (tissue-to-tissue) immune responses to viral infection in an intact animal. With a body plan of ~1000 cells, *C. elegans* has a fully described invariant cell (Corsi et al., 2018). Facilitated by the small size of the organism, a fast generation time, and selfing hermaphrodite reproduction, *C. elegans* genetics enables high-throughput screening and mechanistic studies. The field also has an international, collaborative community of researchers with well-developed protocols, genetic tools, and the Caenorhabditis Genetics Center that catalogues and distributes wild-type and mutant strains to investigators. The *C. elegans* model also provides specific strengths to the study of intestinal immunity. For example, *C. elegans* and mammals share similarities in intestinal cell structure and function, increasing the potential for translational studies (Dimov & Maduro, 2019; Pukkila-Worley & Ausubel, 2012). The intestinal tissue tropism of the Orsay virus enables investigations of the mechanisms that shape intestinal immunity against viral pathogens. Moreover, the absence of known professional immune cells (e.g., macrophages, dendritic cells, T cells, B cells) presents an opportunity to explore how non-professional immune cells, including epithelial cells, coordinate organismal defense against a natural viral pathogen.

Fluorescent labeling of viruses offers a method to assess viral infection levels with spatial and temporal resolution. Unfortunately, it has not yet been possible to genetically encode a fluorescent tag within the Orsay virus genome, which would enable live labeling of the virus. Therefore, current methods for measuring the Orsay virus rely on fixation and fluorescence in situ hybridization (FISH) (Rivera et al., 2022). Although not possible in vivo, FISH labeling provides robust spatial and temporal resolution to assess viral infection. In addition to FISH, qRT-PCR has been used across several laboratories to measure viral load and/or infection rate (Jiang et al., 2014, 2017, 2020; Le Pen et al., 2018; Sandoval et al., 2019; Sowa et al., 2020). Immunofluorescence has also enabled the detection of viral proteins (Franz et al., 2014). However, standardized approaches for performing and measuring infection have not been described in detail. Here, we provide a centralized resource that outlines methods for infecting *C. elegans* with the Orsay virus (Basic Protocols 1 and 2) and quantifying the outcome of viral infection in a reproducible manner (Basic Protocols 3 and 4).

A distinctive feature of single-stranded RNA virus replication is the generation of double-stranded RNA (dsRNA) intermediates. Importantly, dsRNA is considered a ‘non-self’ molecule by the immune system and thus can serve as a key trigger of innate immune responses in mammalian cells (Chen & Hur, 2022), where immunofluorescence can be used to detect dsRNA (Son et al., 2015). A key advantage of *C. elegans* is that immunofluorescence can be used to assess subcellular components in intact animals. Here, we provide a detailed approach that combines FISH with immunofluorescence to simultaneously visualize viral RNA and dsRNA within adult intestinal cells (Basic Protocol 5).

With a biomass equivalent to 82% of total human biomass, nematodes are among the most abundant organisms on this planet (van den Hoogen et al., 2019), yet we have a relatively limited understanding of their antiviral defenses. Findings from multiple laboratories have demonstrated a role for RNA interference (RNAi) in antiviral defense in *C. elegans* (Ashe et al., 2013; Félix et al., 2011; Gammon, 2017; Guo et al., 2013; Lu

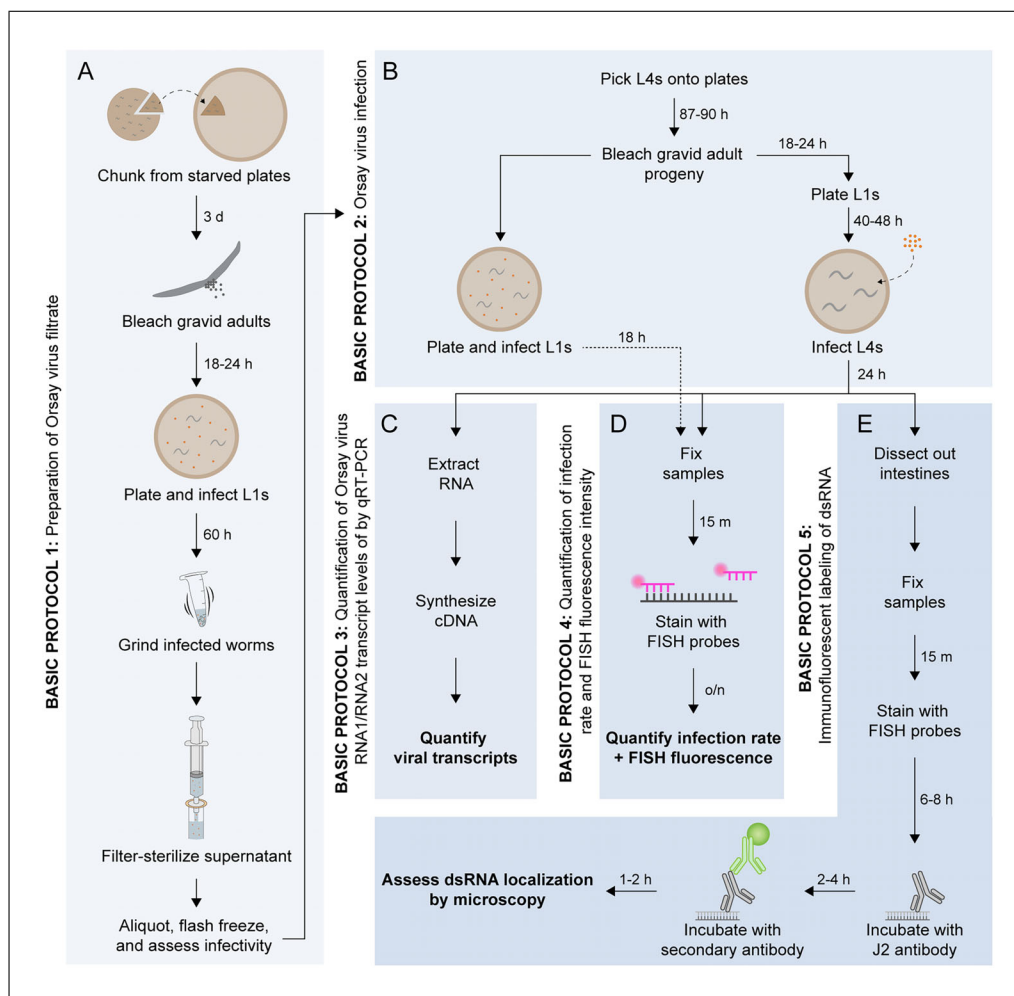


Figure 1 Overview of Basic Protocols 1-5. (A) Basic Protocol 1: Preparation of Orsay virus filtrate. (B) Basic Protocol 2: Orsay virus infection. (C) Basic Protocol 3: Quantification of Orsay virus RNA1 transcript levels by qRT-PCR. (D) Basic Protocol 4: Quantification of infection rate and fluorescence in situ hybridization (FISH) fluorescence intensity. (E) Basic Protocol 5: Immunofluorescent labeling of dsRNA in virus-infected intestinal tissue.

et al., 2009). For example, loss-of-function mutations in the RNAi component RDE-1 results in increased susceptibility to virus. Independent of RNAi-mediated defense, a *C. elegans* terminal uridylyltransferase, CDE-1, promotes antiviral activity through uridylation and subsequent degradation of the Orsay virus genome (Le Pen et al., 2018). Orsay virus infection also induces an antiviral transcriptional immune response, termed the Intracellular Pathogen Response (IPR), that is distinct from RNAi (Bakowski et al., 2014; Reddy et al., 2017; Sowa et al., 2020). However, the relative contributions of these different pathways to antiviral defense remain unclear. We also have a limited understanding of the spatial relationships between host and viral proteins. To address these outstanding questions, the application of the protocols described below will enable further investigation of the *C. elegans* response to viral infection.

In this article, we describe five main protocols to facilitate studies of Orsay virus infection in *C. elegans* (Fig. 1). Basic Protocol 1 provides detailed steps to generate a large batch of Orsay virus filtrate for use in infection assays. Basic Protocol 2 describes a method for performing viral infections in synchronized first and fourth larval stage (L1 and L4) animals, although all post-embryonic stages of *C. elegans* are susceptible to infection. Basic Protocols 3 and 4 provide standardized approaches to assess the outcome of infection using qRT-PCR and FISH. Finally, Basic Protocol 5 describes steps to combine FISH

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and immunofluorescence to simultaneously visualize viral RNA and dsRNA in intestinal tissue.

PREPARATION OF ORSAY VIRUS FILTRATE

Because *C. elegans* is the only known host of the Orsay virus, and no tissue culture systems exist for this host, the virus must be fed to and amplified inside of *C. elegans* animals that are grown on agar plates seeded with an *Escherichia coli* food source. As an alternate to agar plates, infected worms can be grown in liquid culture to amplify virus (Jiang et al., 2014). After amplification within *C. elegans*, virus filtrate can be extracted, aliquoted, and flash frozen in large batches to ensure reproducible infection across experiments (Fig. 1A). To increase the total number of viral particles generated, filtrate preparations are best performed in animals defective in RNAi and thus exhibiting higher viral load compared to N2 animals. For example, our lab uses RNAi-defective *rde-1* mutant animals. Virus filtrate preparations can also be performed in animals that express the *pals-5p::GFP* reporter (as part of the integrated transgene *jyIs8*). A member of the IPR gene family, *pals-5* is highly upregulated during viral infection. Therefore, the *pals-5p::GFP* reporter provides a visual indicator of infection that can be useful when preparing virus filtrate and determining an optimal viral titer for downstream assays.

Materials

C. elegans strains *jyIs8* and/or *rde-1*; *jyIs8* (*Caenorhabditis* Genetics Center)
Standard 6-well, 6- and 10-cm Nematode Growth Medium (NGM) plates seeded
with *E. coli* OP50-1 (Stiernagle, 2006)
Bleaching solution (see Support Protocol)
M9 buffer (see recipe)
Orsay virus filtrate (OVF)
To obtain virus, contact: Emily Troemel, etroemel@ucsd.edu; Marie-Anne Felix,
felix@bio.ens.psl.eu; or Dave Wang, davewang@wustl.edu.
10× concentrated *E. coli* OP50-1
Grown in LB medium at 37°C and 200 to 300 rpm shaking overnight, concentrated
by pelleting, and removing supernatant.

20°C incubator
Nutator (Labquake Shaker Rotisserie, Model 4125110)
15- and 50-ml conical tubes
Laminar flow hood
10-ml repeater pipette with 5-ml tip
Fluorescence microscope
Glass Pasteur pipette
Benchtop centrifuge
1.5-ml centrifuge tubes
Sterile 1.0-mm diameter silicon carbide beads (BioSpec Products, cat. no.
11079110sc)
Bead beater (Scientific Industries Disruptor Genie, Model SI-D236)
Dissecting microscope
10-ml syringe
0.22-μm filter
Ice
Liquid nitrogen

Obtain a population of developmentally synchronized worms

1. Chunk (i.e., cut pieces of agar containing large numbers of *C. elegans*) from two 6-cm freshly starved plates of *rde-1*; *jyIs8* worms onto ten 10-cm seeded NGM plates.

For a detailed review on worm husbandry, refer to the WormBook chapter titled “Maintenance of C. elegans” (Stiernagle, 2006).

Divide each 6-cm plate into fifths and chunk each fifth onto a 10-cm plate.

2. Incubate at 20°C for 3 days.
3. Bleach gravid adults to release eggs.

See Support Protocol for a detailed protocol on bleaching worms.

4. Incubate embryos in 3 ml M9 buffer at 20°C with rotation on a nutator overnight (18 to 24 hr) to yield a synchronized population of L1 stage animals.

We typically observe a yield of ~600,000 embryos, although this number can vary depending on the number of starved animals on each 6-cm plate in step 1.

Plate and infect L1s

5. In a 50-ml conical tube, prepare an infection master mix containing L1 worms, 10× *E. coli* OP50-1, Orsay virus filtrate (OVF), and M9 buffer. Prepare enough infection mix for at least thirty plates (1 ml per plate).

Each plate should contain 20,000 L1s, 250 µl of 10× E. coli OP50-1, 20 µl undiluted OVF, and M9 in a total volume of 1 ml.

6. In a laminar flow hood, use a repeater pipette to dispense 1 ml of infection mix onto (at least) thirty 10-cm plates. Tilt to spread liquid evenly across the surface of the plate.
7. Allow plates to dry uncovered in laminar flow hood until all liquid disappears.

It should take ~30 min for plates to dry.

8. Cover plates and incubate at 20°C until L1s are gravid adults (~60 hr).

To prevent worms from starving, feed once daily with 500 µl of 100× concentrated E. coli OP50-1, which is made by concentrating the volume of E. coli OP50-1 culture 100-fold. Dry plates uncovered in laminar flow hood after adding 100× E. coli OP50-1. Monitor plates closely and supplement with food as necessary to prevent starvation.

Grind infected worms

9. Confirm infection by fluorescence microscope.

If infection is successful, worms should express pals-5p::GFP that can be visualized using a fluorescence dissecting microscope.

10. Use M9 to collect worms into 15-ml conical tubes, pooling three plates into one tube, as follows:
 - a. Begin by adding 7 ml of M9 to one plate and tilt to collect worms into solution.
 - b. Pour liquid from the first plate onto the second plate and tilt to collect worms into solution.
 - c. Repeat with the third plate.
 - d. Transfer the worms to a 15-ml conical tube by pouring or by using a glass Pasteur pipette (instead of plastic, because worms adhere less to glass than to plastic).
 - e. Repeat the process with an additional 7 ml of M9 to collect any remaining worms on the three plates.
11. Wash worms twice with M9. Pellet worms using a benchtop centrifuge 30 s at 1500 × g, room temperature, remove supernatant to ~2 ml, and resuspend in 10 ml of M9. Repeat steps for the second wash.
12. Pellet worms, resuspend in 1 ml of M9, and transfer to 1.5-ml microcentrifuge tubes.

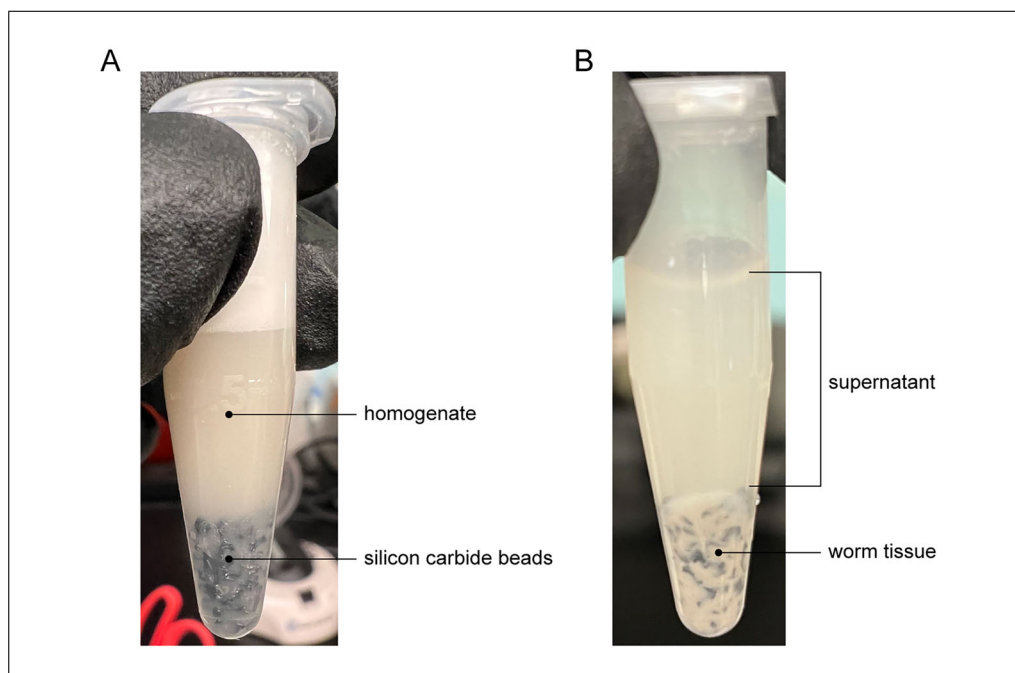


Figure 2 Bead beater homogenization of infected worms. **(A)** After bead beating, the homogenate should be uniform with no large chunks remaining. Silicon carbide beads will settle at the bottom of the tube. **(B)** After separating out remaining worm tissue by centrifugation, the supernatant should then be filter sterilized.

13. Add a scoop of sterile silicon carbide beads to each 1.5-ml tube.

The scoop size should be equivalent to the size of a pencil eraser.

Other protocols call for ~10 individual beads, but the density of worms using this protocol requires more beads to keep the grinding time reasonable.

Ensure that the addition of beads does not overfill the tube.

14. Transfer tubes to the bead beater and shake for ~30 min at full speed (3000 rpm). Check for homogeneity under a dissecting microscope. Not all worm tissue will be dissolved but ensure that no large chunks remain (Fig. 2A).

Leave worms in bead beater until homogenized. The timing may vary depending on the worm density.

Filter supernatant

15. Spin tubes in benchtop centrifuge 2 min at maximum speed ($\sim 21,000 \times g$), room temperature.
16. Transfer supernatant from 1.5-ml tubes into a 50-ml conical tube (Fig. 2B).
17. Use a 0.22- μm filter and 10-ml syringe to filter-sterilize 10 ml of the supernatant into a fresh 50-ml conical tube on ice.

Be cautious of knocking the conical tube over, as vacuum pressure may make it difficult to push liquid through the filter.

18. In a sterile environment and on ice, aliquot 100 μl of filtrate into each 1.5-ml tube.

The use of a repeater pipette is recommended here.

Flash freeze aliquots

19. Flash freeze aliquots in liquid nitrogen.

Ensure that tubes do not spend more than a few seconds in liquid nitrogen, as lids may pop open.

20. Once frozen, place tubes into a box labeled with the lot number, date of preparation, and initials. Store at -80°C .

Virus has been shown to remain viable after 8 years of storage at -80°C (Dave Wang, personal communication).

Keep box on dry ice until transferring to -80°C .

Assess infectivity of virus filtrate

Perform infections at the L4 stage and assess infectivity at 24 hr post-inoculation (hpi).

21. Seed 6-well NGM plates with 200 μl of $10\times$ *E. coli* OP50-1 per well. Incubate at room temperature for 2 days. Store at 4°C until L1s are ready to be plated.

22. Pick 20 to 40 L4 stage worms onto 10-cm NGM plates seeded with *E. coli* OP50-1.

Worms must be grown without undergoing starvation for at least one generation prior to plating.

23. Incubate worms at 20°C for 87 to 90 hr until progeny become gravid adults.

24. Bleach gravid adults to release eggs.

See Support Protocol for a detailed protocol on bleaching worms.

25. Incubate eggs at 20°C with rotation overnight (18 to 24 hr) to yield a synchronized population of L1s.

26. Plate 200 L1s in each well of the seeded 6-well NGM plates. Incubate at 20°C for 40 to 48 hr until worms reach the L4 stage.

Set up at least three 6-well plates for each strain.

27. Set up an infection dosage curve by performing serial 10-fold dilutions of the virus filtrate across seeded 6-well NGM plates. Each well should contain 50 μl of $10\times$ *E. coli* OP50-1, 3 μl virus filtrate, and 47 μl M9 in a total volume of 100 μl . Set up triplicates for each of the following OVF dilutions: 100, 10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} , and 0.

28. Add 100 μl of infection mix to each well containing L4 animals.

29. Incubate at 20°C for 24 hr.

30. Using a fluorescence microscope, determine the percentage of animals expressing *pals-5p::GFP* across the different virus filtrate dilutions.

*A recommended working dilution is one that results in $\sim 50\%$ of animals expressing *pals-5p::GFP*.*

31. To determine the infectivity of the virus filtrate, proceed to Basic Protocols 3 and 4 to carry out qRT-PCR and fluorescence in situ hybridization (FISH), respectively.

*For qRT-PCR measurements and FISH staining, perform infections with the dilution of virus filtrate that results in $\sim 50\%$ of animals expressing *pals-5p::GFP*.*

SYNCHRONIZE *C. ELEGANS* DEVELOPMENT BY BLEACHING

The steps below describe how to synchronize a population of *C. elegans* animals at the L1 larval stage. In this protocol, gravid adults are exposed to bleach to dissolve the worm cuticle. By limiting the duration of bleach exposure, this technique results in the release

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of eggs without killing the embryos, as the eggshells provide protection against bleach. Eggs are then allowed to hatch in minimal media overnight to obtain starved L1 animals. Once exposed to food, L1s resume synchronous growth.

Materials

L4 stage worms (see Basic Protocol 1)
Standard 10-cm NGM plates seeded with *E. coli* OP50-1 (Stiernagle, 2006)
10× concentrated *E. coli* OP50-1
M9 buffer (see recipe)
5.65% to 6% sodium hypochlorite (Fisher Chemical, cat. no. SS290)
5 M sodium hydroxide (NaOH) (Fisher Chemical, cat. no. SS256)

20°C incubator
Glass Pasteur pipette
15-ml conical tubes
Clinical centrifuge (Thermo Scientific Sorvall ST 8R Centrifuge, cat. no. 75007203)
Dissecting microscope
Nutator (Labquake Shaker Rotisserie, Model 4125110)
Glass slides

NOTE: Bleach loses potency over time due to oxidation. If the bleach is old, worms may need to be exposed to bleach for a greater duration than recommended below in order to dissolve the cuticle.

Bleach gravid adults

1. Pick 20 to 40 L4 stage worms onto standard 10-cm NGM plates seeded with *E. coli* OP50-1. Supplement plates with 1 ml of 10× *E. coli* OP50-1.

Worms must be grown without undergoing starvation for at least one generation prior to plating.

The number of worms plated may vary depending on brood size.

2. Incubate plates at 20°C for 87 to 90 hr until most of the progeny become gravid adults.
3. Wash gravid adults off the plate with 15 ml of M9 buffer and transfer worms into a 15-ml conical tube using a sterile glass Pasteur pipette.

Because worms adhere to plastic, using glass pipettes, instead of plastic tips, minimizes the loss of worms during the transfer.

4. Pellet worms in clinical centrifuge 30 s at 1500 × *g*, room temperature.
5. Remove supernatant until there is ~2 ml liquid remaining in the tube.
6. Add 800 µl of 5.65% to 6% sodium hypochlorite and 200 µl of 5 M NaOH to the worm suspension. Shake or vortex vigorously. Observe worms with a dissecting microscope until they break apart and release eggs.

Worms should be lysed but not dissolved.

The amount of time required to break worms open may vary depending on several factors (e.g., worm strain, the number of worms, reagents). A recommended starting point is 90 s.

Over-bleaching affects the health of the eggs, and thus the hatched L1s, so proceed to next step once worms have broken apart, but undissolved corpses are still present. Experimental results will be impacted if bleaching results in unhealthy L1s.

7. Add M9 to a volume of 14 ml. Perform this step quickly.
8. Spin down sample 30 s at $1500 \times g$, room temperature.
9. Remove supernatant by decanting.

Ensure that pellet remains intact while pouring out liquid. Inverting the tube once will not disturb the pellet, but the pellet will be resuspended as soon as the tube is righted again. Do not invert the tube at this point.
10. Repeat steps 7 to 9 for a total of 5 washes.
11. Resuspend eggs in 3 to 5 ml M9.
12. Incubate eggs at 20°C with rotation using a nutator overnight (18 to 24 hr) to yield a synchronized population of L1s.

Eggs will hatch into starved L1s that are best used the day after bleaching. While it is possible to use L1s on the second day after bleaching, worms will be less healthy, exhibit delays in restoring development and thus will be less synchronized.
13. After 18 to 24 hr, determine the concentration of L1s in M9 by pipetting 5 μ l of the L1 suspension onto a glass slide. Count the number of L1s in the drop. Repeat two more times and calculate the total number of L1s per μ l. If the L1 density is too low, concentrate L1s by spinning at 30 s at $1500 \times g$, room temperature. If the density is too high, dilute with M9.

ORSAY VIRUS INFECTION

Orsay virus infections are performed by feeding *C. elegans* a mixture of *E. coli* OP50-1 as a food source together with virus filtrate, which can be prepared by following Basic Protocol 1. While infections can be performed at any developmental stage, the protocol below describes the steps for performing infections at both L1 and L4 stages (Fig. 1B). Advantages of infecting L1s, compared to infecting animals at later stages, are that worms can be used immediately after hatching and can be plated simultaneously with food and virus. Thus, L1 infections do not require pre-seeded plates. An advantage of performing L4 infections is the ability to compare the outcome of viral infection with the outcome of infection by other pathogens, especially as bacterial infections are commonly performed at the L4 stage. Additionally, L4 infections are ideal if downstream analysis includes qRT-PCR, as L4s and adults yield greater amounts of RNA than younger animals. Depending on the experimental setup, infections can be performed using various plate formats. Here, we describe protocols for setting up infections on 6-well, 6-cm, and 10-cm plates.

Materials

Unseeded 6-well, 6-cm, or 10-cm NGM plates (Stiernagle, 2006)
 10 $\times$ concentrated *E. coli* OP50-1
 Standard 10-cm NGM plates seeded with *E. coli* OP50-1
 Bleaching solution (see Support Protocol)
 Orsay virus filtrate (OVF) (see Basic Protocol 1)
 M9 buffer (see recipe)

Laminar flow hood
 20°C incubator
 Nutator (Labquake Shaker Rotisserie, Model 4125110) Laminar flow hood
 15-ml conical tubes

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Seed NGM plates with *E. coli* OP50-1 (L4 infections only)

Seeded plates will be used for performing L4 infections (see steps 11 to 17 in the 'Perform infection' section below).

1. Proceed to step 4 for L1 infections or step 2 for L4 infections.
2. If using 6-well or 6-cm plates, add 200 μ l of 10 $\times$ *E. coli* OP50-1 to plates and incubate at room temperature for 2 days. If using 10-cm plates, add 500 μ l of 10 $\times$ *E. coli* OP50-1 to plates and incubate at room temperature for 5 days.

Allow plates to dry uncovered in laminar flow hood until all liquid disappears.

3. After incubation, use plates immediately or store at 4°C for up to 1 month.

Bring plates to room temperature prior to plating worms.

Obtain developmentally synchronized populations of worms

4. Pick 20 to 40 L4 stage worms onto 10-cm NGM plates seeded with *E. coli* OP50-1.

Worms must be grown without undergoing starvation for at least one generation prior to plating.

The number of worms plated may vary depending on brood size.

5. Incubate worms at 20°C for 87 to 90 hr until progeny become gravid adults.

The incubation time may vary depending on the growth rate of the worm strain.

6. Bleach gravid adults to release eggs.

See Support Protocol for a more detailed protocol on bleaching worms.

7. Incubate eggs at 20°C with rotation using a nutator overnight (18 to 24 hr) to yield a synchronized population of L1s.

Perform infection

Bring 10 $\times$ *E. coli* OP50-1 to room temperature prior to making up master mix. The working dilution for Orsay virus filtrate will depend on the preparation described in Basic Protocol 1.

8. Proceed to step 9 for L1 infections or step 11 for L4 infections.
9. For L1 infections, make up an infection master mix containing L1 worms, 10 $\times$ *E. coli* OP50-1, Orsay virus filtrate, and sterile M9 buffer in a 15-ml conical tube. Additionally, make up an uninfected control master mix by replacing Orsay virus filtrate with sterile M9.

The viral titer can be adjusted depending on the goal of the experiment.

*If using 6-well plates, each well should contain 400 L1s, 50 μ l of 10 $\times$ *E. coli* OP50-1, 3 μ l Orsay virus filtrate, and sterile M9 in a total volume of 100 μ l.*

*If using 6-cm plates, each plate should contain 1200 L1s, 150 μ l of 10 $\times$ *E. coli* OP50-1, 10 μ l Orsay virus filtrate, and sterile M9 in a total volume of 300 μ l.*

*If using 10-cm plates, each plate should contain 3600 L1s, 450 μ l of 10 $\times$ *E. coli* OP50-1, 30 μ l Orsay virus filtrate, and sterile M9 in a total volume of 900 μ l.*

10. Add 100 μ l per well (6-well plate), 300 μ l (6-cm plate), or 900 μ l (10-cm plate) of infection mix to each plate and tilt to spread liquid evenly across the surface of the plate. Proceed to step 15.

Spreading out the liquid across the surface of the plate reduces the drying time and provides a more even distribution of viral particles across the plate.

11. For L4 infections, plate the following number of worms onto each pre-seeded plate: 200 L1s per well (6-well plates), 700 L1s (6-cm plates), or 2000 L1s (10-cm plates). See steps 2 to 3 for seeding plates.
12. Incubate plates at 20°C for 40 to 48 hr until worms reach the L4 stage.
13. In a 15-ml conical tube, make up an infection master mix containing 10× *E. coli* OP50-1, Orsay virus filtrate, and sterile M9. Additionally, make up an uninfected control master mix by replacing Orsay virus filtrate with sterile M9.

The viral titer can be adjusted depending on the goal of the experiment.

*If using 6-well plates, each well should contain 50 µl of 10× *E. coli* OP50-1, 3 µl Orsay virus filtrate, and sterile M9 in a total volume of 100 µl.*

*If using 6-cm plates, each plate should contain 50 µl of 10× *E. coli* OP50-1, 10 µl Orsay virus filtrate, and sterile M9 in a total volume of 300 µl.*

*If using 10-cm plates, each plate should contain 150 µl of 10× *E. coli* OP50-1, 30 µl Orsay virus filtrate, and sterile M9 in a total volume of 900 µl.*

14. Add 100 µl per well (6-well plate), 300 µl (6-cm plate), or 900 µl (10-cm plate) of infection mix to each plate and tilt to spread liquid evenly across the surface of the plate.

Spreading out the liquid across the surface of the plate reduces the drying time and provides a more even distribution of viral particles across the plate.

15. Allow plates to dry uncovered in laminar flow hood until all liquid disappears.

Drying time can range from 30 min to 1 hr. Once liquid is no longer visible, cover plates to prevent over-drying. Aim to keep drying time consistent between experiments.

16. Incubate plates at 20°C for 18 hr (L1 infections) or 24 hr (L4 infections).

For L4 infections with N2 animals, the infection rate is generally higher at 24 hr post-inoculation (hpi) when compared to the infection rate at 48 hpi or 72 hpi.

17. Assess infection outcomes (see Basic Protocols 3 and 4).

QUANTIFICATION OF ORSAY VIRUS RNA1/RNA2 TRANSCRIPT LEVELS BY QRT-PCR

The outcome of viral infection can be quantified using several different approaches, including qRT-PCR (Fig. 1C) and/or fluorescence in situ hybridization (FISH) (Fig. 1D). Choosing which method(s) to use for analysis depends on the expected magnitude of the effect, although this magnitude may not be known prior to performing the experiment. In Basic Protocol 3, we describe how to measure Orsay virus RNA1 and RNA2 transcript levels with qRT-PCR. Analysis by qRT-PCR is a direct measurement of viral load and provides useful information when large differences are expected between samples.

Materials

Infected *C. elegans* in 10-cm plates (from Basic Protocol 2)
 M9 buffer (see recipe)
 RNase-free surface decontaminant (Apex BioResearch Products, cat. no. 10-228)
 TRI reagent (MRC, cat. no. TR118)
 BCP phase separation reagent (MRC, cat. no. BP151)
 Isopropanol (Sigma, cat. no. I9516-500ML)
 100% ethanol (Volu-Sol, cat. no. VAE-016)
 H₂O, HyPure molecular biology grade (GE Life Science, cat. no. SH30528.03)
 iScript cDNA synthesis kit (Bio-Rad, cat. no. 1708890)

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Table 1 qRT-PCR Primers

Target	Primer name	Sequence	Source
RNA1	GW194	ACCTCACAAGTCCATCTACA	Félix et al. (2011)
	GW195	GACGCTTCCAAGATTGGTATTGGT	
RNA2	GW303	CCGGCGACAATGTGTACCA	Jiang et al. (2014)
	GW304	CCAGCCCTCCGTTGACAA	

- iQ SYBR Green Supermix (Bio-Rad, cat. no. 1708880)
- qPCR primers (see Table 1)
- Glass Pasteur pipette and bulb
- Sterile 15-ml conical tubes
- Clinical centrifuge (Thermo Scientific Sorvall ST 8R Centrifuge, cat. no. 75007203)
- Filter pipette tips
- Sterile 1.5-ml microcentrifuge tubes
- Mini centrifuge
- NanoDrop spectrophotometer
- 0.2-ml PCR tubes
- PCR thermocycler
- 96-well qPCR plate
- Microseal ‘B’ PCR plate sealing film (Bio-Rad, cat. no. MSB1001)
- Real-time PCR system

CAUTION: TRI reagent is highly toxic. Avoid exposure to the skin and airways.

CAUTION: BCP phase separation reagent is a flammable liquid and is toxic if ingested or inhaled. Use in a well-ventilated area and keep away from open flames.

Extract RNA

This protocol is optimized for measuring mRNA transcript levels in L4s and adults. 1000 L4 animals yield ~10 µg total RNA. Always use filter pipet tips when performing RNA extractions. Keep RNA tubes and reagents separate from other reagents. RNA is most vulnerable once it has been extracted from cells.

1. Add 15 ml M9 buffer to a 10-cm plate containing infected worms (from Basic Protocol 2).
2. Use a glass Pasteur pipette to transfer worms to a 15-ml conical tube.
Because worms adhere to plastic, using glass pipettes, instead of plastic tips, minimizes the loss of worms during the transfer.
3. Pellet worms by centrifuging 30 s at 1500 × g, room temperature, and aspirate supernatant to 100 µl.
4. Add 15 ml M9, pellet worms 30 s at 1500 × g, room temperature, and aspirate supernatant to 100 µl.
If supernatant is still cloudy, perform an additional wash.
5. Wipe down work area, reagents, and equipment with RNase-free surface decontaminant.

6. Using filter pipet tips, add 1 ml TRI reagent to 100 μ l of packed worms and homogenize by slowly pipetting up and down 10 times. Transfer mixture to a 1.5-ml microcentrifuge tube.

We do not recommend vortexing samples, as this may lead to nuclear DNA contamination.

7. Store homogenate at room temperature for 10 to 30 min.

Optional: Sample can be frozen at this point and remaining steps can be continued at a later time.

8. Add 100 μ l BCP to each sample, press down against the lid of the tube to prevent leaking, and shake vigorously for 15 s.
9. Store mixture at room temperature for 2 to 15 min.
10. Spin 15 min at $12,000 \times g$, 4°C .
11. Transfer the aqueous phase (clear layer) to a new tube without disturbing the interphase (cloudy layer), which contains genomic DNA.

Avoid DNA contamination by leaving $\sim 20\%$ of aqueous phase behind. Spin tubes again if necessary.

12. Add 500 μ l isopropanol, mix by inverting tubes, and store at room temperature for 5 to 10 min to precipitate the RNA.
13. Spin 15 min at $12,000 \times g$, 4°C . Remove the supernatant by pipetting.
14. Add 1 ml of 75% ethanol to wash the RNA pellet.

The pellet should appear whiter and larger after adding ethanol.

15. Centrifuge 5 min at $7500 \times g$, 4°C .
16. Remove the ethanol first with a large-bore pipet tip (i.e., 1000 μ l). Perform a quick spin down of tubes using a mini centrifuge at room temperature and remove any remaining ethanol with a smaller pipet tip (i.e., 200 μ l or 10 μ l), making sure not to disturb the pellet. A white pellet should be visible if RNA yield is $> 10 \mu\text{g}$.
17. Leave tubes uncovered and monitor until the pellet becomes translucent, which indicates that all ethanol has evaporated.

White indicates the presence of ethanol. Do not allow pellet to over-dry, as this makes it difficult to re-solubilize.

18. As soon as the pellet becomes translucent, resuspend in 20 to 30 μ l nuclease-free water and incubate at room temperature for 10 min to dissolve pellet. Store RNA samples at -80°C .

The incubation step can be performed at a higher temperature (i.e., $\sim 50^{\circ}\text{C}$) to promote re-solubilization of the RNA pellet.

Storing the RNA samples at -80°C for at least 1 hr reduces the variability when measuring the concentration using a NanoDrop.

Synthesize cDNA

cDNA synthesis is performed with the iScript cDNA synthesis kit.

19. Measure the concentration of RNA on a NanoDrop.
20. Make up a master mix containing iScript reverse transcriptase, $1 \times$ iScript buffer, and nuclease-free water.

Each cDNA synthesis reaction should contain 0.5 μ l iScript reverse transcriptase, 2 μ l of 5 $\times$ iScript buffer, and 2.5 μ l nuclease-free water.

- 21. In a 0.2-ml PCR tube, add 5 μ l iScript master mix, 500 ng RNA, and nuclease-free water to a total volume of 10 μ l.
- 22. Run the reaction protocol on a thermocycler following the kit instructions.

Run qRT-PCR

- 23. Make up a master mix containing iQ SYBR Supermix and primers targeting Orsay virus RNA1 and/or RNA2 (Table 1). Make up an additional master mix using primers targeting the housekeeping gene. Include a minimum of duplicate reactions per sample.

Each reaction should contain 5 μ l iQ SYBR Green Supermix, 1 μ l forward primer, 1 μ l reverse primer, and 3 μ l cDNA. It is recommended to use a 1:100 dilution of cDNA from step 10.

It is recommended to use *snb-1* as the housekeeping gene.

- 24. In a 96-well qPCR plate, pipet 10 μ l of reaction mix into each well. Seal plate with film.
- 25. Run the reaction protocol on a real-time PCR system.

**BASIC
PROTOCOL 4**

**QUANTIFICATION OF INFECTION RATE AND FISH FLUORESCENCE
INTENSITY**

This protocol describes how to measure the percentage of infected worms within a population by FISH staining (Part 1) and how to quantify FISH fluorescence intensity as a proxy for pathogen load (Part 2). By assessing individuals within a population, FISH analyses are better able to distinguish small differences in viral resistance. Analysis by qRT-PCR analysis can lack sensitivity in discriminating modest differences, as qRT-PCR measures the viral load of an entire sample without accounting for individual heterogeneity within the sample. For example, several heavily infected worms within a population can skew the viral load measurement, thus obscuring the effects of uninfected worms, which is more clearly detected by FISH.

Materials

- C. elegans* strains
- Orsay virus RNA1 and RNA2 FISH probes (Biosearch Technologies) (Table 2)
- 16% paraformaldehyde (Electron Microscopy Sciences, cat. no. 15710)
- VECTASHIELD antifade mounting medium (Vector Laboratories, cat. no. H-1000-10), optional
- Agarose
- FISH hybridization and wash buffers (see recipes)

- Glass microscope slides
- 22-mm $\times$ 22-mm cover slip

Table 2 FISH Probes

Target gene	Fluorophore	Sequence	Source
RNA1	Cal Fluor Red 610 (CF610)	GACATATGTGATGCCGAGAC	Biosearch Technologies
	Quasar 670 (Q670)		
RNA2	Cal Fluor Red 610 (CF610)	GTAGTGTTCATTGTAGGCAGC	
	Quasar 670 (Q670)		

Fluorescence microscope
Glass Pasteur pipette
Fiji (see Internet Resources)

CAUTION: Paraformaldehyde is a carcinogenic substance that becomes toxic when exposed to the skin and airways. Any steps involving paraformaldehyde should be performed in a fume hood or well-ventilated area.

Hybridize FISH probes

1. Perform FISH staining of *C. elegans* following the protocol detailed in Rivera et al. (2022). Use probes targeting Orsay virus RNA1 and RNA2 genomic segments (Table 2).

We recommend fixing samples in 4% paraformaldehyde (PFA) for 15 min; longer fixation times may quench the fluorescence of proteins like GFP.

To increase FISH signal, perform staining with both RNA1 and RNA2 probes, or use multiple probes (also known as “single-molecule FISH probes”). A recommended final concentration for each probe is 10 ng/μl.

2. Proceed to Part 1 to measure the infection rate and Part 2 to quantify FISH fluorescence intensity.

Part 1: Quantification of the percentage of worms with any evidence of infection by FISH staining

3. Add ~5 μl of sample onto a glass microscope slide and place a 22-mm × 22-mm cover slip on top.

Optional: Add 1 to 2 μl Vectashield antifade mounting medium to worms.

4. Under a fluorescence microscope, manually score animals and record the percentage of animals that show positive FISH staining. As a control for positive staining, it is recommended to include a condition in which highly susceptible animals, e.g., *rde-1* mutants, are infected with virus. As a negative control, FISH staining should also be performed on uninfected animals.

We recommend scoring a minimum of 100 animals to ensure an accurate representation of the population.

Part 2: Quantification of FISH fluorescence intensity

5. Make a 5% agarose pad on a glass microscope slide as follows:

- a. Dissolve agarose in water to make a 5% agarose solution.
- b. Using a glass Pasteur pipette, add a drop of the agarose solution to a glass microscope slide.
- c. Immediately place another glass slide on top of the drop to create a circular pad.
- d. Once the agarose has dried, carefully separate both slides while leaving the agarose intact on one of the slides.

6. Add ~5 μl of sample onto the agarose pad and place a 22-mm × 22-mm cover slip on top of the sample.

Ensure that worms do not overlap with one another.

Optional: Add 1 to 2 μl of Vectashield antifade mounting medium directly to sample on the agarose pad prior to placing the coverslip over sample.

Worms can also be imaged in 96-well plates using an automated imaging platform (e.g., ImageXpress Nano automated imaging system, Molecular Devices) (Nyaanga et al., 2021).

7. Acquire images on a fluorescence microscope.

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8. Using Fiji software, quantify background-corrected mean fluorescence intensity (MFI) as follows:
 - a. Open images for all channels by dragging and dropping image files into Fiji.
 - b. Merge transmitted light (TL) and fluorescent channels by selecting “Image” > “Color” > “Merge Channels...”.
 - c. To set measurement parameters, select “Analyze” > “Set Measurements...”.
 - d. Select “Mean gray value” > “OK”.
 - e. Within the image stack, switch over to the fluorescence channel corresponding to the fluorescent probe used for FISH staining.
 - f. Use the polygon selection tool to trace around the border of the worm.
Only trace worms that are fully visible and remained motionless during imaging. Do not trace overlapping worms or those that touch the border of the well.
 - g. Select “Analyze” > “Measure” to record the mean gray value, or mean fluorescence intensity (MFI), of the worm.
 - h. To measure background fluorescence, left click and drag the polygon shape to an empty space in the well next to the worm. Select “Analyze” > “Measure”.
 - i. Calculate background-corrected fluorescence by subtracting the background fluorescence from the worm fluorescence using the equation below.

$$\text{MFI}_{\text{corrected}} = \text{MFI}_{\text{worm}} - \text{MFI}_{\text{background}}$$

BASIC PROTOCOL 5

IMMUNOFLUORESCENT LABELING OF DSRNA IN VIRUS-INFECTED INTESTINAL TISSUE

Performing immunohistochemistry in adult *C. elegans* intestinal cells is not straightforward due to challenges in efficiently delivering antibody across the worm cuticle and into cells, in addition to the high levels of autofluorescence in adult intestinal cells. Here, we describe how to dissect out the intestine in order to provide increased antibody permeability and perform immunofluorescence in the intestinal tissue of adult *C. elegans* using the J2 antibody, which targets dsRNA. In addition, we describe how to combine dsRNA immunofluorescence with FISH staining to enable visualization of dsRNA in virus-infected cells (Fig. 1E).

Materials

C. elegans strains
 M9 buffer (see recipe)
 100 μM levamisole (MP Biomedicals, cat. no. 155228)
 16% paraformaldehyde (Electron Microscopy Sciences, cat. no. 15710)
 10 $\times$ PBS (Current Protocols, 2006)
 Tween 20 (Thermo Scientific, cat. no. AC233362500)
 Blocking solution (see recipe)
 Mouse anti-dsRNA antibody clone rJ2 (Sigma, cat. no. MABE1134-25UL)
 Goat anti-mouse IgG(H+L) cross-adsorbed secondary antibody, Alexa Fluor 488 (Invitrogen, cat. no. A-11001)
 Agarose (Sigma-Aldrich, cat. no. A9539)

15-ml conical tube
 Benchtop centrifuge
 Glass microscope slide
 25G needle
 1.5-ml microcentrifuge tubes
 Glass Pasteur pipette
 Heat block, 47°C

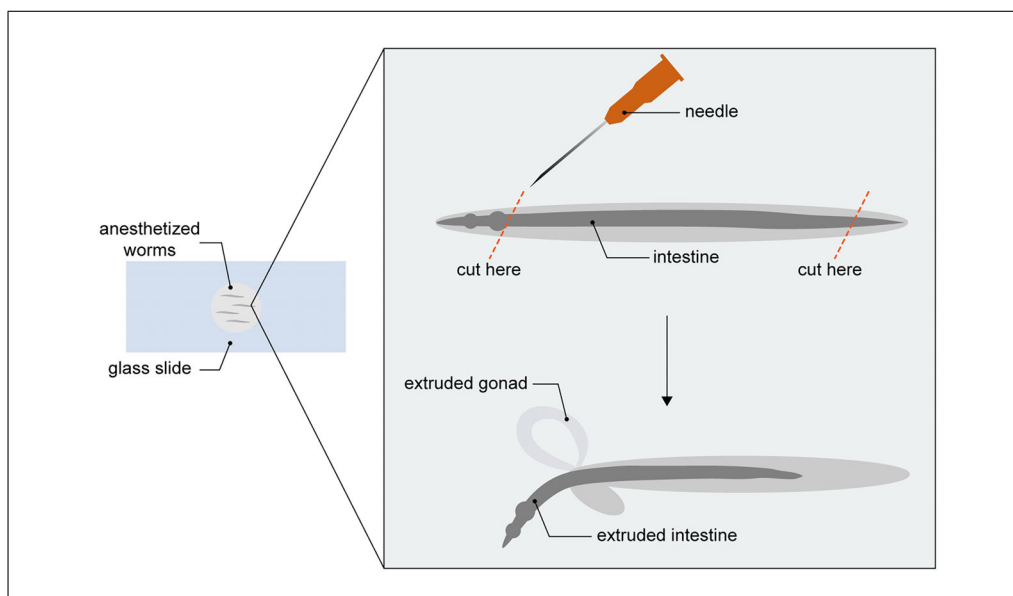


Figure 3 Schematic of *C. elegans* intestinal dissection. Add worms to a glass slide and anesthetize with levamisole. Position the needle at an angle and cut at either or both of the indicated cut sites (orange dashed lines) by touching the worm with the sharp edge of the needle. A single touch should be sufficient to cause extrusion of the intestine (and gonad).

22-mm × 22-mm cover slip

Confocal microscope

CAUTION: Paraformaldehyde is a carcinogenic substance that becomes toxic when exposed to the skin and airways. Any steps involving paraformaldehyde should be performed in a fume hood or well-ventilated area.

CAUTION: The blocking solution used in this protocol contains sodium azide, which is a hazardous compound that forms a toxic gas when mixed with water or acid. Work in a fume hood when handling sodium azide.

Infect worms with virus

This protocol is optimized for staining intestines from adult animals.

1. Perform infections at the L4 stage for 24 hr following steps outlined in Basic Protocol 2.

Infect 2000 worms on a 10-cm plate.

Remove intestines by dissection

2. For each treatment condition, wash worms off of plates with 15 ml M9 and transfer to a 15-ml conical tube.
3. Pellet worms by centrifuging 1 min at $2000 \times g$, room temperature, and then aspirate supernatant.
4. Wash samples by adding 15 ml M9 to each tube. Pellet worms as in step 3 and aspirate supernatant. Repeat wash if supernatant is cloudy.
5. Pipet 50 μ l worms and 5 μ l of 100 μ M levamisole onto a microscope slide.

Pipet directly from worm pellet.

6. Dissect out 100 to 200 intestines using a 25G needle (Fig. 3). Holding the needle at an angle, use the sharp edge to cut off the head and/or tail of each worm. The internal pressure of the worm will cause the intestine and gonad to ooze out.

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7. Transfer dissected intestines to 1.5-ml microcentrifuge tubes using a glass Pasteur pipette.

If necessary, add 50 μ l M9 to the glass slide to collect any remaining intestines and transfer the liquid to the 1.5-ml tube using a glass pipette.

8. Centrifuge tubes 1 min at $2000 \times g$, room temperature, and carefully aspirate supernatant without disturbing dissected intestines. Resuspend in a total volume of 100 μ l M9.

Fix samples and stain with FISH probes

9. Add 33 μ l of 16% PFA to 100 μ l of sample to achieve a final concentration of 4% PFA. Incubate at room temperature for 15 min.

10. Wash samples twice with $1 \times$ PBS + 0.1% Tween 20. For each wash, add 1 ml of $1 \times$ PBS + 0.1% Tween 20, centrifuge tubes 1 min at $2000 \times g$, room temperature, and remove supernatant down to $\sim 100 \mu$ l.

Make up $1 \times$ PBS by diluting $10 \times$ PBS stock solution in water and adding 0.1% (v/v) Tween 20.

11. Perform FISH staining as described in Rivera et al. (2022).

Incubate with FISH probe at 47°C using a heat block for 6 to 8 hr or overnight.

Add primary and secondary antibodies

12. Add 200 μ l blocking solution to each sample and incubate overnight at 4°C .

13. Centrifuge tubes 1 min at $2000 \times g$, room temperature, and aspirate supernatant.

Remove supernatant down to $\sim 100 \mu$ l.

14. Add mouse anti-dsRNA J2 antibody and incubate at room temperature for 1 to 2 hr.

The amount of antibody may need to be adjusted, but a recommended starting point is a 1:400 dilution of J2 antibody in blocking solution.

15. Wash samples 4 times with $1 \times$ PBS + 0.1% Tween 20. For each wash, add 200 μ l of $1 \times$ PBS + 0.1% Tween 20, centrifuge tubes 1 min at $2000 \times g$, room temperature, and remove supernatant down to $\sim 100 \mu$ l.

16. Add 4 to 10 $\mu\text{g/ml}$ goat anti-mouse IgG(H+L) Alexa Fluor 488 secondary antibody and incubate in the dark at room temperature for 1 to 2 hr.

It is possible to add anywhere from 4 to 10 $\mu\text{g/ml}$ secondary antibody depending on the outcome of staining. A recommended starting concentration is 10 $\mu\text{g/ml}$ of secondary antibody diluted in blocking solution.

17. Wash samples 5 times with 200 μ l $1 \times$ PBS + 0.1% Tween 20, performing centrifugation as in step 15.

18. Add 100 μ l of $1 \times$ PBS + 0.1% Tween 20 and store samples at 4°C .

Acquire confocal images

19. Make a 5% agarose pad on a glass microscope slides as follows:

- a. Dissolve agarose in water to make a 5% agarose solution.
- b. Using a glass Pasteur pipette, add a drop of the agarose solution to a glass microscope slide.
- c. Immediately place another glass slide on top of the drop to create a circular pad.
- d. Once the agarose has dried, carefully separate both slides while leaving the agarose intact on one of the slides.

20. Pipet ~5 μ l of sample onto the agarose pad and place a cover slip on top of the sample.
21. Acquire images on a confocal microscope.

REAGENTS AND SOLUTIONS

Blocking solution

100 ml of 1 $\times$ PBS (Current Protocols, 2006)
 0.5 ml of 0.5% Tween 20 (Thermo Scientific, cat. no. AC233362500)
 5 g of bovine serum albumin (BSA) (5%) (Sigma-Aldrich, cat. no. A3294)
 0.05 g NaN₃ (0.05%) (Sigma-Aldrich, cat. no. 71289)
 Filter sterilize using a 0.22- μ m filter
 Store up to 6 months at 4°C

FISH hybridization buffer

180 μ l of 5 M NaCl (Current Protocols, 2006)
 20 μ l of 1 M Tris pH 7.5 (Current Protocols, 2006)
 800 μ l ddH₂O
 2 μ l of 10% sodium dodecyl sulfate (SDS) (Current Protocols, 2006)
 Prepare fresh for each experiment otherwise precipitation will occur
This recipe makes 1 ml of buffer and 200 μ l buffer is needed per sample. Scale up accordingly.

FISH wash buffer

180 μ l of 5 M NaCl (Current Protocols, 2006)
 20 μ l of 1 M Tris pH 7.5 (Current Protocols, 2006)
 10 μ l of 0.5 M EDTA (Current Protocols, 2006)
 790 μ l ddH₂O
 2 μ l of 10% SDS (Current Protocols, 2006)
 Prepare fresh for each experiment otherwise precipitation will occur
This recipe makes 1 ml of buffer and 200 μ l buffer is needed per sample. Scale up accordingly.

M9 buffer

For 1 L:
 5.8 g Na₂HPO₄ (Current Protocols, 2006)
 3.0 g KH₂PO₄ (Current Protocols, 2006)
 0.5 g NaCl (Current Protocols, 2006)
 1.0 g NH₄Cl
 1 L ddH₂O
 Sterilize by autoclaving
 Store up to 9 months at room temperature

COMMENTARY

Background Information

In non-mammalian hosts, the study of antiviral defense has led to landmark discoveries, including CRISPR and RNA interference (RNAi). Until the discovery of the Orsay virus in 2011 (Félix et al., 2011), the absence of a known viral pathogen capable of infecting and undergoing its entire life cycle inside *C. elegans* presented a limitation to the use of *C. elegans* as a model organism for studying host-virus interactions. In early work, studies of antiviral defense were carried out using genetic

(Lu et al., 2005), ex vivo (Schott et al., 2005; Wilkins et al., 2005), or artificial approaches (Liu et al., 2006) to model viral infection in *C. elegans*. The isolation of the Orsay virus and the development of standardized protocols, including those presented above, expands the potential of *C. elegans* as an experimental system for studying host-virus interactions.

In addition to advancing what is known about antiviral RNAi, which has been extensively studied, the investigation of Orsay virus infection within the last decade has revealed

additional genes and pathways that participate in *C. elegans* antiviral defense. For example, mutagenesis and RNAi screens have uncovered host factors (*sid-3*, *viro-2*, *nck-1*, and *hipr-1*) that regulate the outcome of viral infection at early, prereplication life stages of the virus (Jiang et al., 2017, 2020). Follow-up studies have demonstrated that these proteins share conserved function with their mammalian homologs (Jiang et al., 2019, 2020). Recent studies have also implicated a role for structural proteins (i.e., actin, collagen) and the endoplasmic reticulum in mediating antiviral defense (Efsthathiou et al., 2022; Zhou et al., 2024). Curiously, several of the collagen components identified as antiviral factors in *C. elegans* are homologs of human proteins involved in innate immune responses (Zhou et al., 2024). These findings lend support to the potential of the *C. elegans*-virus model for discovering novel antiviral factors that are conserved in mammals. Future work can clarify the functional role of these proteins in *C. elegans* antiviral defense.

The study of viral infection in *C. elegans* has facilitated the discovery of novel host immune responses, such as the Intracellular Pathogen Response (IPR), which is induced by infection with intracellular pathogens, including the Orsay virus. While the IPR is distinct from the well-studied antiviral RNAi response, the regulation of both the IPR and RNAi during infection involves DRH-1, a mammalian RIG-I-like receptor (RLR) homolog. In mammals, RLRs function as cytosolic RNA sensors that detect viral infection and promote signaling to induce type I interferons. RLR-mediated regulation is one of several similarities shared by the type I interferon response and the IPR (Lažetić et al., 2023). However, downstream components of the canonical RLR pathway are absent in *C. elegans*, presenting an opportunity for the discovery of non-canonical signaling factors that regulate transcriptional responses to viral infection. Taken together, these findings highlight exciting avenues for further exploration into the evolution of host immune responses and the fundamental principles of antiviral immunity.

The techniques presented above offer several advantages for investigating antiviral immunity. One of the central advantages includes the use of microscopy-based tools to visualize viral infection in the context of an entire organism. Furthermore, the rapid life cycle of *C. elegans*, along with the rapid spread of Orsay virus infection, enables experiments to be per-

formed on a relatively short timescale. Further streamlining infection workflows, Orsay virus filtrate can be generated and stored in large batches, and infection can be easily achieved through feeding. Despite the strengths of this model system, we acknowledge that there are limitations to the protocols described in this article. Namely, the inability to label Orsay virus with a genetically encoded fluorescent tag remains a major obstacle in understanding viral dynamics, which is challenging to assess by FISH due to the need for sample fixation. Another limitation is the lack of a tissue culture system that supports amplification and characterization of the Orsay virus.

While immunofluorescence methods for *C. elegans* have been previously described, our workflow (Basic Protocol 5) enables simultaneous staining of dsRNA and viral RNA in the intestine of adult worms. Earlier work pioneered the use of immunofluorescence to target viral proteins in the tissue of intact animals (Franz et al., 2014). This study was the first to report the localization of viral proteins in a natural *C. elegans* infection model. Findings from this study defined the tissue tropism of the Orsay virus as well as the subcellular localization of viral proteins within an intact animal. Viral proteins were visualized in larvae, as the authors noted that performing immunofluorescence on adult animals was challenging due to considerable levels of non-specific staining. Here, we optimize an immunofluorescence assay for staining dsRNA in adult intestinal cells with high specificity, in combination with FISH staining for viral RNA. A limitation of Basic Protocol 5 is the need to dissect intestines for performing immunofluorescence, which increases the duration of the protocol and can introduce unintended effects caused by manipulation of the tissue. While it can be labor-intensive, dissection increases the efficiency of antibody uptake into intestinal cells, allowing for more robust staining. Applications of the immunofluorescence workflow described in Basic Protocol 5 are not limited to dsRNA staining, as this workflow can be adapted for use with an antibody against any desired target.

Critical parameters

For Orsay virus infections, several experimental parameters might influence the infection outcome, including lot-to-lot variation of virus filtrate preparations, handling of virus filtrate, feeding rate, bacterial food source, and overbleaching embryos. Prior to setting up an experiment, the optimal dose of virus

Table 3 Troubleshooting Guide for RNA Extraction

Problem	Possible cause	Solution
Low RNA yield	Insufficient starting sample	Collect RNA from at least 1000 L4s or adults per sample for optimal yields; adults yield more RNA compared to L4s
	Incomplete resolubilization of RNA pellet	After obtaining RNA pellet, perform 10 min incubation at a higher temperature (i.e., ~50°C) and store at -80°C overnight to promote resolubilization

Table 4 Troubleshooting Guide for FISH Staining

Problem	Possible cause	Solution
High background fluorescence	Insufficient removal of FISH probe	Increase duration and/or number of washes following hybridization step (e.g., wash 3× with 500 µl wash buffer)

Table 5 Troubleshooting Guide for Visualization of dsRNA by Immunofluorescence

Problem	Possible cause	Solution
Little to no sample remaining at the end of protocol	Loss of sample during wash steps	Increase centrifugation time, ensure pellet is visible after each wash
		Increase sample size (i.e., number of dissected intestines)
High background fluorescence	Antibody concentrations too high	Set up antibody titration to determine concentration that yields optimal signal-to-noise ratio
	Antibody incubation time too long	Decrease incubation time (i.e., 1 hr at room temperature)
	Insufficient blocking	Prepare fresh blocking buffer
	Insufficient removal of unbound secondary antibody	Increase number of washes following secondary antibody incubation

filtrate should be empirically determined. Experiments should be performed using the same lot of virus filtrate. Of note, virus filtrate loses infectivity with each freeze-thaw cycle. To maintain infectivity of Orsay virus filtrate preparations, the filtrate should be divided into small aliquots for a single use (Basic Protocol 1). Discard the aliquot once it has been thawed. As Orsay virus infection is established through feeding, any changes to the feeding rate of the *C. elegans* strain or treatment group may impact the rate of inoculation. Similarly, changes to bacterial food sources, which can influence the feeding rate, can also affect the infection rate. Other factors that can influence the outcome of infection are overbleaching gravid adults and/or incomplete removal of the bleaching solution once eggs are released.

Prolonged exposure to bleach can result in the death of the released embryos. Similarly, residual bleach left overnight can kill hatched larvae or cause asynchronous development, introducing variability to infection assays.

Troubleshooting

The tables below list potential solutions for problems that may occur when performing RNA extraction (Table 3), FISH staining (Table 4), and dsRNA immunofluorescence (Table 5).

Understanding Results

For each virus filtrate preparation (Basic Protocol 1), a dose-response curve should be performed to determine the optimal dose of virus filtrate for downstream experiments

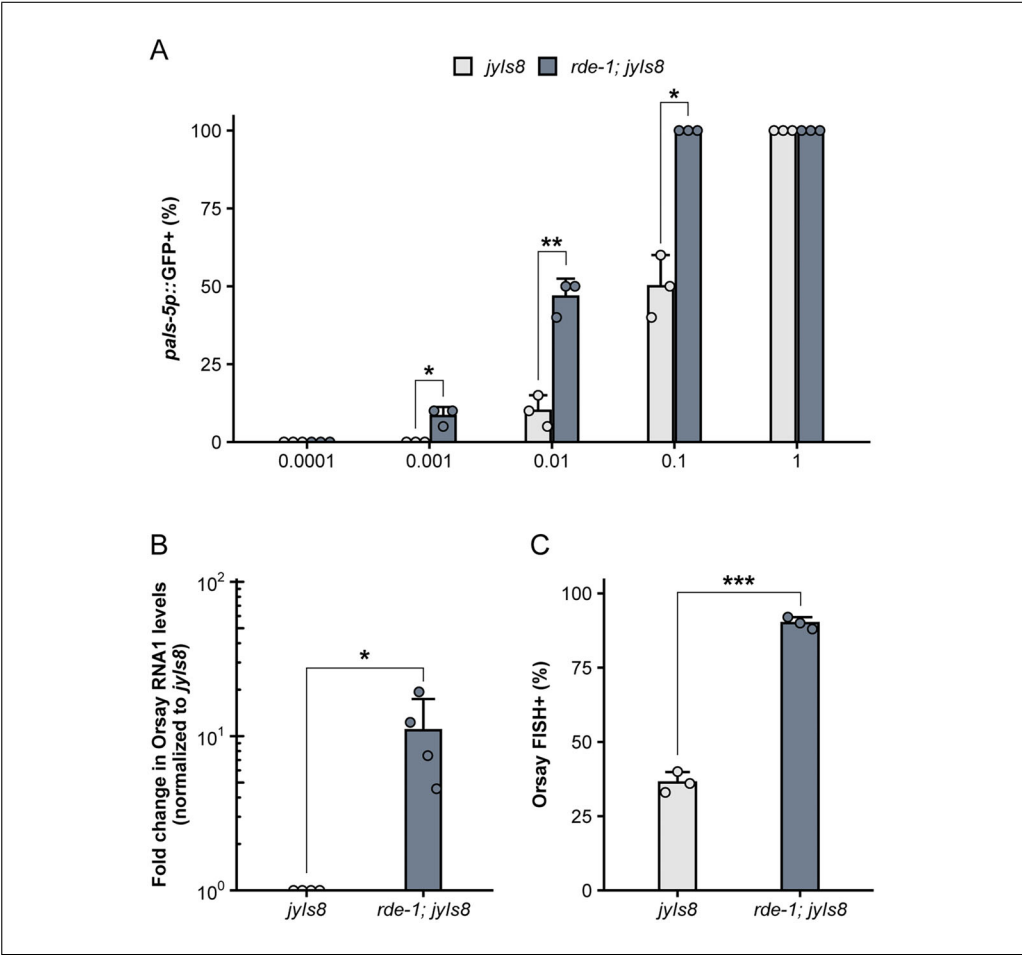


Figure 4 Assessing outcomes of viral infection. **(A)** Percentage of animals expressing *pals-5p::GFP* following inoculation with different dilutions of virus filtrate. A two-tailed *t*-test was used to calculate *p*-values; **p* < .05, ***p* < .01. **(B)** qRT-PCR analysis of viral load in *rde-1; jyls8* and *jyls8* animals at 24 hpi. Animals were infected at the L4 stage. *rde-1* mutants exhibit increased viral RNA1 transcript levels compared to *rde-1* wild-type animals. Fold change in gene expression was determined relative to *jyls8*. A one-tailed *t*-test was used to calculate *p*-values; **p* < .05. **(C)** Percentage of animals that show Orsay FISH staining at 18 hpi following L1 infection. The percentage of infected animals is significantly increased in *rde-1* mutants compared to *rde-1* wild-type animals. A two-tailed *t*-test was used to determine statistical significance; ****p* < .001. For all plots, each dot represents an experimental replicate; 3-4 independent experimental replicates were performed. Bars represent the mean across experimental replicates; error bars indicate standard deviation.

(Fig. 4A). The magnitude of the immune response, as measured by *pals-5p::GFP*, is dependent on the starting concentration of virus. An optimal dynamic range for infection assays (Basic Protocol 2) can be achieved by determining a working dilution of virus filtrate that results in ~50% *pals-5p::GFP* activation in a wild-type population. For example, a dilution of 0.1 results in ~50% wild-type *pals-5p::GFP* induction in the virus filtration preparation shown in Figure 4A. At this dilution, the sensitivity of the assay is sufficient to detect differences between the percentage of *pals-5p::GFP*+ animals when comparing wild-type (*jyls8*) and susceptible (*rde-1; jyls8*) mutants. When animals are treated

with a higher concentration of virus, the difference in *pals-5p::GFP* activation between wild-type and susceptible animals is no longer detected (Fig. 4A).

Differences in viral load and infection rate can be quantified by qRT-PCR (Basic Protocol 3) and FISH staining (Basic Protocol 4). Consistent with increased *pals-5p::GFP* activation, the level of Orsay virus RNA1 transcripts is elevated in *rde-1* mutants compared to wild-type animals (Fig. 4B). Furthermore, the percentage of animals exhibiting Orsay FISH staining is also significantly increased in *rde-1* mutants relative to wild-type animals (Fig. 4C). In addition to measuring infection rate within a population, FISH

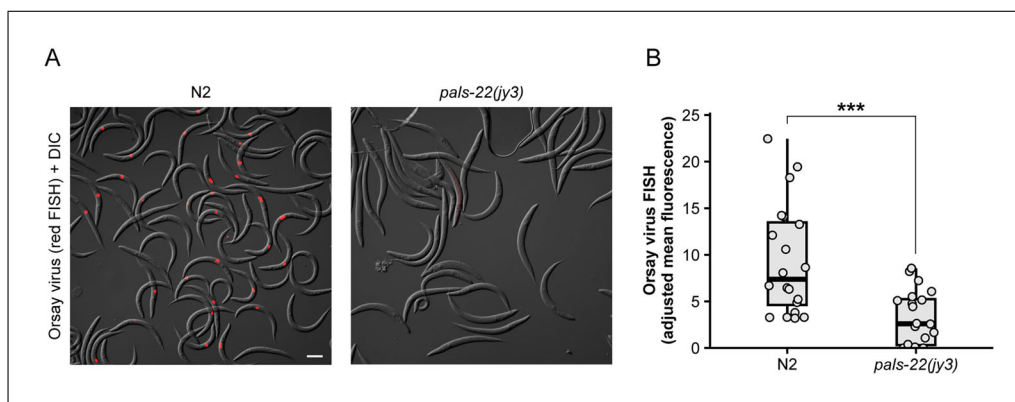


Figure 5 Quantification of fluorescence by FISH staining. **(A)** Representative images of WT and *pals-22(jy3)* animals stained with CF610-conjugated Orsay FISH probes (red) targeting the RNA1 and RNA2 viral genomic segments. Animals were infected at the L1 stage, and images were taken at 18 hpi. Scale bar = 100 μ m. **(B)** Quantification of background-corrected mean fluorescence in **(A)**. Each dot represents an individual animal; 20 animals were analyzed for each strain. Horizontal lines in box-and-whisker plots represent median values, and the box reflects the 25th to 75th percentiles. A Mann-Whitney *U* test was used to determine statistical significance; ****p* < .001.

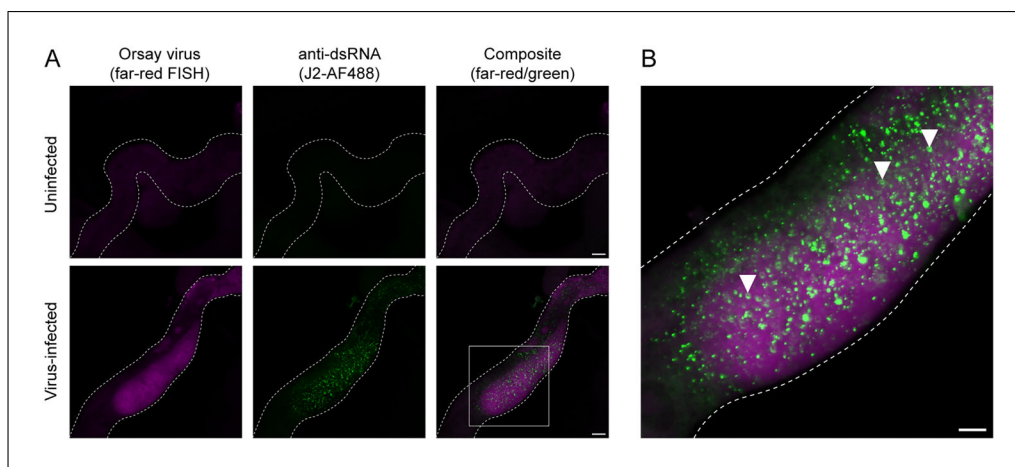


Figure 6 Viral FISH and dsRNA staining in dissected intestines. **(A)** Representative images of intestines (white dotted outline) from uninfected or virus-infected animals. Viral RNA was visualized using Quasar 670-conjugated (far-red) FISH probes targeting the Orsay virus RNA1 and RNA2 genomic segments. Samples were incubated with the J2 antibody to visualize dsRNA (green). Scale bar = 25 μ m. **(B)** Magnification of region outlined by white box in **(A)**. White arrowheads indicate dsRNA forming ring-like structures in cells that exhibit Orsay FISH staining. Scale bar = 25 μ m.

fluorescence can also be used as a readout for infection levels within individual animals. Previous work has demonstrated that *pals-22* mutants exhibit a significant decrease in the infection rate, or percentage of FISH+ animals, relative to wild-type animals (Fig. 5A) (Reddy et al., 2019). Here, we demonstrate how mean FISH fluorescence can also be used to quantify the increased resistance in *pals-22* mutants (Fig. 5B) and provide a distribution of fluorescence levels within a population.

In combination with FISH, immunohistochemistry enables the visualization of both viral RNA and dsRNA at subcellular resolution

(Basic Protocol 5). Infected intestinal tissue exhibits bright dsRNA puncta, which are not present in uninfected intestinal cells (Fig. 6) (Batachari et al., 2024). Distributed throughout the cytosol, dsRNA foci are ubiquitous and form ring-like structures. In infected animals, dsRNA is predominantly observed in viral FISH+ cells, whereas neighboring FISH- cells exhibit minimal or no dsRNA fluorescence (Fig. 6), supporting a model in which dsRNA is a product of viral replication. Collectively, Basic Protocols 3 to 5 provide both quantitative and spatial approaches to assessing the outcome of Orsay virus infection.

Time Considerations

The preparation of Orsay virus filtrate (Basic Protocol 1) can be completed in 8 days. Assessing the infectivity of the filtrate may take up to an additional 2 weeks depending on which assays are performed. Performing Orsay virus infections (Basic Protocol 2) may take anywhere from 1 to 2 weeks depending on whether infections are performed at the L1 or L4 larval stage. If combining infections with RNAi, the duration of the assay depends on whether RNAi is performed for one or more generations prior to infections. Time estimates should also account for the growth rate of the *C. elegans* strain used in the experiment. Assays for measuring viral RNA1 and RNA2 transcript levels by qRT-PCR (Basic Protocol 3), FISH fluorescence intensity (Basic Protocol 4), and infection rate (Basic Protocol 4) can be completed in 2 days. Combining FISH staining with dsRNA immunofluorescence can take 2 to 3 days (Basic Protocol 5).

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Author Contributions

Lakshmi E. Batachari: Conceptualization; data curation; formal analysis; investigation; methodology; visualization; writing—original draft; writing—review and editing. **Mario Bardan Sarmiento:** Investigation; methodology; writing—review and editing. **Nicole Wernet:** Methodology; writing—review and editing. **Emily R. Troemel:** Conceptualization; funding acquisition; resources; supervision; writing—original draft; writing—review and editing.

Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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