Journal of Visualized Experiments

Measuring sperm guidance and motility within the Caenorhabditis elegans hermaphrodite reproductive tract

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE59783R2
Full Title:	Measuring sperm guidance and motility within the Caenorhabditis elegans hermaphrodite reproductive tract
Keywords:	fertilization; sperm; chemotaxis; migration; oviduct; C. elegans; guidance; mating; prostaglandin
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Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Open Access (US$4,200)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Birmingham, AL, USA

TITLE:

Measuring Sperm Guidance and Motility within the *Caenorhabditis elegans* Hermaphrodite Reproductive Tract

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KEYWORDS:

fertilization, sperm, chemotaxis, migration, oviduct, *C. elegans*, guidance, mating, prostaglandin

SUMMARY:

Sperm must successfully navigate through the oviduct to fertilize an oocyte. Here, we describe an assay for measuring sperm migration within the *C. elegans* hermaphrodite uterus. This assay can provide quantitative data on sperm distribution within the uterus after mating, as well as on speed, directional velocity, and reversal frequency.

ABSTRACT:

Successful fertilization is fundamental to sexual reproduction, yet little is known about the mechanisms that guide sperm to oocytes within the female reproductive tract. While in vitro studies suggest that sperm of internally fertilizing animals can respond to various cues from their surroundings, the inability to visualize their behavior inside the female reproductive tract creates a challenge for understanding sperm migration and mobility in its native environment. Here, we describe a method using *C. elegans* that overcomes this limitation and takes advantage of their transparent epidermis. *C. elegans* males stained with a mitochondrial dye are mated with adult hermaphrodites, which act as modified females, and deposit fluorescently labeled sperm into the hermaphrodite uterus. The migration and motility of the labeled sperm can then be directly tracked using an epi-fluorescence microscope in a live hermaphrodite. In wild-type animals, approximately 90% of the labeled sperm crawl through the uterus and reach the fertilization site, or spermatheca. Images of the uterus can be taken 1 h after mating to assess the distribution of the sperm within the uterus and the percentage of sperm that have reached the spermatheca. Alternatively, time-lapse images can be taken immediately after mating to assess sperm speed, directional velocity and reversal frequency. This method can be combined with other genetic and molecular tools available for the *C.elegans* to identify novel genetic and molecular mechanisms that are important in regulating sperm guidance and motility within the female reproductive

tract.

INTRODUCTION:

The molecular mechanisms by which spermatozoa (referred to as sperm) navigate through the female reproductive tract toward the oocyte are not well understood, yet are fundamental to sexual reproduction. Sperm motility is highly dynamic and depends on robust communication signals that alter sperm velocity and directional motility[1-12]. *C. elegans* has become a powerful model for studying sperm movement in vivo because the hermaphrodite's transparent epidermis permits the tracking of live sperm at single cell resolution[2,3,8,10]. The purpose of this paper is to provide methods for assessing sperm movement within the *C. elegans* hermaphrodite uterus.

In animal species where sperm and oocyte meet in the external environment (i.e., acquatic environments), sperm respond to chemotactic signals secreted by oocytes. These signals guide the direction of sperm movement, bringing them closer to the signal source[4,6,11]. However, much less is known about sperm movement in species that fertilize internally. A major challenge is the architecture of the female reproductive tract, which is inaccessible to microscopy in most species. In vitro studies in humans, mice, and pigs, for example, provide evidence that subpopulations of sperm can respond to chemoattractants, fluid flow, and thermal gradients[1,5,7,9,12]. With these systems, the inability to visualize and track sperm movement in vivo places serious limitations on strategies to discover the key mechanisms regulating these functions.

To overcome these limitations, we have developed methods using the nematode *C. elegans* to directly visualize sperm after insemination, to measure individual sperm migration parameters in vivo, and to measure the ability of a sperm population to target the fertilization site. These methods, together with the *C. elegans* molecular and genetic toolset, facilitate discovery of the chemical signaling molecules and molecular machinery that regulate sperm motility behaviors. For instance, genetic screens can be conducted in hermaphrodites or males to identify genes that are essential for efficient sperm movement in vivo[13]. Molecules can be injected into the hermaphrodite gonad to test for effects on sperm activation, migration velocity, and directional motility[3]. Additionally, the described methods can be used to monitor rogue sperm migration into ectopic body locations and to evaluate sperm competition[10,14].

C. elegans exist in nature as hermaphrodites and males (see **Figure 1**). The hermaphrodite gonad has two U-shaped arms that are mirror images of each other. During the L4 larval stage, the most proximal germ cells (i.e., the cells near the spermatheca) undergo spermatogenesis. Each primary spermatocyte enters meiosis and produces four haploid spermatids. These spermatids are pushed into the spermatheca along with the first mature oocyte and undergo spermiogenesis[15]. Adult hermaphrodites switch from spermatogenesis to oogenesis. The oocytes mature in an assembly line fashion along the gonad, with the most mature oocyte at the proximal end of the gonad, next to the spermatheca. MSP signals from the sperm is needed to trigger meiotic maturation and ovulation[16,17]. Male *C. elegans*, on the other hand, have a J-shaped gonad that produce only sperm. The spermatids are stored in the seminal vesicle. Upon mating with the hermaphrodite or female, the male inserts the spicules near the tail into the vulva. Spermatids are activated during ejaculation, when they come in contact with the seminal fluid[18]. *C. elegans*

sperm do not swim as they are not flagellated. Instead, they crawl through the reproductive tract, using the pseudopod for locomotion. It is well established that male sperm, which are larger in size, have a competitive advantage over hermaphrodite sperm[14].

In this method, male *C. elegans* act as the sperm donor and are mated to adult hermaprhodites. Adult males are stained with a fluorescent mitochondrial dye to produced labeled sperm. Once deposited through the hermaphrodite vulva, the sperm must crawl around the embryos in the uterus towards the spermatheca, or fertilization site. The transparent epidermis of the *C. elegans* model allows for the direct visualization of each individual sperm as it navigates through the female reproductive tract. In recent years, our lab has successfully used this method to demonstrate the importance of a class of F-series prostaglandins in guiding sperm from the vulva to the spermatheca[19,20]. The molecular mechansims governing its synthesis by the hermaphrodite and response by the sperm are still under investigation. However, this method for assessing sperm motility and migration greatly facilitates the identification of the key players that control sperm and oocyte communication in internally fertilizing animals. The following protocol describes step by step how to perform this assay.

PROTOCOL:

NOTE: All steps in this protocol are performed at room temperature (~20-22 °C) or in constant temperature incubators set to 16 °C or 20 °C. Male and hermaphrodite *C. elegans* are grown using standard culture conditions and NA22 or OP50 *E. coli* as a food source[21,22]. Wild-type N2 hermaphrodites and *fog-2(q71)* males are used in the procedure below.

1. Day 1: Picking L4 stage hermaphrodites for mating

1.1 To obtain consistent results, all hermaphrodites should be synchronized as actively reproducing adults. Pick 20-30 L4 stage hermaphrodites to a 6 cm seeded nematode growth medium (NGM) plate. Incubate the hermaphrodites at 20 °C for 28-30 h.

NOTE: Only 12-15 hermaphrodites will be used for mating. The remaining hermaphrodites are surplus.

2. Day 1: Staining of males with fluorescent mitochondrial dye (mito-dye)

2.1 Make a male staining plate by placing a dot of *E. coli* (food dot) at the center of an unseeded NGM plate. To make the food dot, use the end of a glass stirring rod to scrape *E. coli* from the bacteria lawn of a seeded plate and deposit it on the unseeded plate. The dot should be ~ 5-7 mm in diameter.

2.2 Mix together 2 µL of 1 mM mito-dye (see **Table of Materials**) in DMSO and 10 µL of M9 buffer (3 g of KH_2PO_4, 6 g of Na_2HPO_4, 5 g of NaCl, 1 mL of 1 M $MgSO_4$, H_2O to 1 L. Add $MgSO_4$ after autoclaving). Pipette all of the mito-dye solution onto the food dot on the male staining plate. Let the plate dry in the dark (~ 30 min).

NOTE: Mito-dye is light sensitive. Shield all solution, plates, and worms containing mito-dye from light. Store the 1 mM stock at -20 °C.

2.3 Pick ~100 1-3 day old adult males[23] to the mito-dye stained food dot on the male staining plate. Wrap the plate in aluminum foil and incubate overnight at 16 °C. For mating, use ~50-60 males per 12-15 hermaphrodites. If more than ~100 males are needed, make more staining plates to prevent overcrowding of males.

2.4 Males can also be stained by incubating in a 10 μM mito-dye solution in M9 buffer for 3 h on a watch glass. Keep the worms covered to prevent evaporation and light exposure. After 3 h, use a Pasteur pipet to transfer males onto a 10 cm seeded NGM plate. Transfer as little of the mito-dye solution as possible. Wrap the plate in aluminum foil and incubate overnight in 16 °C.

3. Day 2: Mating

3.1 Pick the stained males from Day 1 onto a new, seeded NGM plate. Leave the plate in the dark until mating. This step ensures that excess mito-dye stained bacteria around the males are removed. Carryover of excessive mito-dye stained bacteria onto the mating plate can stain hermaphrodite tissue.

3.2 Make a mating plate by dropping 2 μL of a thick *E. coli* mixture on an unseeded NGM plate. Let the thick bacteria dry to make the mating dot. Males and hermaphrodites will be transferred onto this dot for mating. To make thick *E. coli*, spin down 3 mL of overnight *E. coli* and resuspend the bacteria pellet in 1 mL of M9. This mixture can be stored at 4 °C and reused for up to 6 months.

NOTE: The thickness of the *E. coli* solution may be adjusted. If the solution is too thin, males may crawl away from the mating dot instead of aggregating on it for mating. Mating dots made from an *E. coli* soluton that is too thick may decrease the mating efficiency.

3.3 While the mating plate from Step 3.2 is drying, mix together 300 μL of 1% (w/v) Tricaine (Tri), 300 μL of 0.1% (w/v) Tetramisole (Tet), and 900 μL of M9.

NOTE: Store 1% (w/v) Tricaine and 0.1% (w/v) Tetramisole as aliquotes at -20 °C. Avoid repeated freeze thaw.

3.4 Transfer 600 μL of the Tet/Tri solution to a watch glass.

3.5 Transfer 12-15 hermaphrodites picked on Day 1 to the Tet/Tri solution in the watch glass. Incubate for 30 min to immobilize the hermaphrodites. Keep the watch glass covered to prevent the Tet/Tri solution from evaporating.

NOTE: It is important that hermaphrodites are anesthetized for at least 30 min. Less time could result in a moving worm during image acquisition, which can interefere with imaging.

3.6 While hermaphrodites are incubating, pick 50-60 stained males from Step 3.1 onto the mating dot (Step 3.2). Store the plate in the dark until Step 3.8.

3.7 After the 30 min incubation in the Tet/Tri solution, use a glass Pasteur pipet to transfer the immobilized hermaphrodites from the watch glass onto an unseeded NGM plate. Remove as much liquid as possible and let the excess liquid dry.

NOTE: Do not let the hermaphrodites dry excessively. As soon as all visible liquid has evaporated, begin the next step.

3.8 Transfer the anesthetized hermaphrodites from the unseeded NGM plate onto the mating dot with the stained males. Incubate in the dark for 30 min to allow the males to mate with the hermaphrodites.

3.9 After mating for 30 min, mount the hermaphrodites immediately for time-lapse imaging or transfer the hermaphrodites onto a new, seeded NGM plate to rest for 1 h before imaging.

NOTE: Time-lapse videos of the hermaphrodite uterus are used to quantify sperm velocity and reversal frequency. Still images of the uterus taken 1 h after mating are used to quantify sperm distribution, or spem guidance.

4. Day 2: Mounting worms for visualization

4.1 Creating a mounting pad with 2% agarose in H_2O

NOTE: 2% agarose can be made in bulk, aliquoted into glass test tubes, and stored at 4 °C. When needed, each aliquot can be microwaved before each use and stored in a heat block to prevent it from solidifying.

4.1.1 To make the mounting pad, align three glass microscope slides side by side with the long edges touching. Place two pieces of masking tape on top of each other on both of the outer slides. These outer glass slides with tape will act as the support so the thickness of the resulting agarose pad will be “two tape deep”.

4.1.2 Place ~75 μL of melted 2% agarose on the center slide (this is the slide without tape). Immediately place a new glass microscope slide on top of the agarose. This top glass slide should be perpendicular to the other slides, with each end resting on the tape of the two support slides.

4.1.3 Let the agarose harden (~ 30 s). Carefully remove the top glass slide by sliding it off the agarose pad.

4.2 Place 10-15 µL of the Tet/Tri solution onto the 2% agarose pad. Transfer the mated hermaphrodites onto the pad. Take care to transfer as little bacteria as possible.

4.3 Place a cover slip over the worms on the agarose pad.

5. Day 2: Image acquisition setup

NOTE: Any upright miscroscope equipped with epi-fluorescence, 10X and 60X objectives, and a digital camera can be used to acquire images for sperm distribution. Software capable of acquiring time-lapsed images are required for assessing sperm speed, directional velocity, and reversal frequency.

5.1 Image acquisition 1 h after mating

5.1.1 Mount the slide onto the microscope stage. Look through the eye pieces to scan for worms on the agarose pad using the 10X objective with the red fluorescence emission filter (TRITC filter). Once a worm has been found, briefly turn on the fluorescence light to see if the worm has mated. If sperm is visible within the uterus, switch to the 60X objective.

NOTE: The pressure created by the 60X objective on the coverslip may damage some fragile worms, causing the intestine or gonad to extrude from the animal. Scanning for successful mating using the 10X objective can minimize the worms' exposure to the added pressure. Do not expose the mated worms to extended periods of fluorescent light.

5.1.2 Using Differential Interference Contrast Microscopy (DIC), position the worm so that both the vulva and one spermatheca are in view. Focus the image by focusing on the center of the spermatheca. Check the exposure for both DIC and TRITC channels. In DIC, internal worm structures should be clearly visible. In TRITC, individual sperm should be visible as distinct puncta.

NOTE: Each image should capture the uterus from the vulva to one of the spermatheca. If the uterus is too long to fit on one image, two separate images can be taken. It is not necessary for all of the images to be taken at the same exposure level. However, it is important that individual sperm can be distinguished and quantified in the fluorescence images.

5.1.3 Acquire DIC and fluorescence images for each uterus.

5.1.4 Repeat Steps 5.1.1-5.1.3 until all mated hermaphrodites have been imaged.

5.2 Capturing time-lapse videos

5.2.1 Scan the agarose pad and locate hermaphrodites that contain labeled sperm within the uterus, as described in Step 5.1.1 and 5.1.2

5.2.2 Configure the software to acquire time-lapse images in DIC and TRITC channels. Generally,

time-lapse images are taken at 15-30 s intervals for 10-20 min per uterus.

6. Quantification

6.1 Quantifying sperm distribution on uterus images taken 1 h after mating

6.1.1 Starting with the vulva on one end and the spermatheca on the other, divide the uterus into thirds. These will represent the three zones. Zone 1 (Z1) contains the vulva and Zone 3 (Z3) contains the spermatheca.

6.1.2 Manually count the number of sperm within each third of the uterus, and report the number in each zone as a percent of the total sperm in the entire uterus. An example is provided below.

$$\%\ Sperm\ in\ Z3 = \frac{Z3}{Z1 + Z2 + Z3}$$

NOTE: Sometimes, the signal intensity of the TRITC channel image needs to be adjusted so that every sperm that has been captured in the image can be visible and quantified.

6.2 Tracking sperm in time-lapse images

NOTE: In this paper, we used the NIS-Elements software for analysis. In the sections below, we give instructions for manually tracking sperm using this software (step 6.2.1) as well as the open source software ImageJ/Fiji (step 6.2.2).

6.2.1 Tracking sperm with NIS-Elements

6.2.1.1 Open the .nd2 file with the time-lapse series to be tracked. To begin tracking, open up the Tracking panel by right clicking in the software and selecting **Analysis Controls | Tracking**.

6.2.1.2 In the tracking panel, select **Define New ROI**. Define each region of interest (ROI) by clicking over each sperm that will be tracked. A colored mark will appear over the selected sperm. Click **Finish** when all ROIs have been selected.

6.2.1.3 Once the ROIs have been identified, move to the next frame in the time-lapse series. Drag the ROI marker to the new position of the sperm in the image. Continue doing this until the sperm can no longer be tracked. Dotted lines will appear connecting each of the locations the ROI marker has been placed through all the frames of the time-lapse image.

NOTE: Only sperm in Zone 2 should be tracked as sperm in Zones 1 and 3 tend to move in a circular pattern even in wild-type animals.

6.2.1.4 Export all quantifiable data (e.g., path length, time, XY position, etc.) from the tracked sperm to an Excel document by clicking **Export** in the Tracking panel.

6.2.2 Tracking sperm with Fiji

6.2.2.1 Convert the TRITC channel images in the time-lapse series to .tif files. Save all files from one series into one folder.

6.2.2.2 Import the images to Fiji by using the BioFormats import function. Import images from one time-lapse series as one hyperstack.

6.2.2.3 Open TrackMate in Fiji[24] via **Plugins | Tracking | Manual tracking with TrackMate**. A diaglogue box will open.

6.2.2.4 Select the TrackMate tool in the Fiji toolbar. Double click on the sperm that will be tracked. A green circle with dashed lines will appear. This circle may be repositioned by clicking inside the circle and dragging to the desired position. The size of this circle may be changed by simultaneously pressing the **ALT** key and scrolling the mouse.

6.2.2.5 Once the size and position of the tracker has been set, click on the circle again. The dashed green lines will turn into a solid green line. Simultaneously hit the **SHIFT** and **L** keys to turn on tracking mode. This will be indicated in the Fiji toolbar.

6.2.2.6 Move to the next frame in the time-lapse series. To set the new location of the tracked sperm in the new frame, hover the mouse over the new point and press the **A** key. The tracker will now appear at the new location, and a line will appear connecting the locations where the tracker has been placed in the previous frames.

6.2.2.7 After the traces have been completed, click **Analyze** in the TrackMate dialogue box to generate the data needed.

6.2.3 To calculate the speed, divide the total path length of the sperm by the elapsed time.

6.2.4 To calculate the vectorial velocity, draw a line through the uterus starting from the vulva pointing toward the spermatheca. Measure the distance the sperm has migrated along this line from the beginning to the end of the trace. Divide this distance by the elapsed time. Negative values indicate the sperm has migrated away from the spermatheca.

6.2.5 To record reversal frequency, count the number of times during which the sperm trace has generated an angle less than 90° during three consecutive time-lapse frames (see **Figure 2N** for a schematic example).

REPRESENTATIVE RESULTS:

To generate the results depicted in this paper, *fog-2(q71)* males were stained with the mito-dye and mated to wild-type, N2 hermaphrodites. **Figure 3** provides an overall scheme for the method, including worm preparation, mating, and analysis. As **Movie 1** shows, the adult hermaphrodite

reproductive tract has two arms that are mirror images of each other. Upon mating, labeled sperm are deposited in the hermaphrodite uterus through the vulva. The sperm move around the developing embryos within the uterus toward the spermatheca, where they are stored until fertilization. As germ cells in the adult hermaphrodite develop into oocytes, the proximal, most mature oocyte is pushed into the spermatheca via sheath cell contrations. Fertilization occurs while the oocyte is in the spermatheca.

To quantify sperm distribution and migration through the female reproductive tract, the hermaphrodite uterus is divided into three zones (**Movie 1, Figure 4A**). Zone 1 spans the first third of the uterus, starting from the vulva. Zone 2 spans the middle third of the uterus, and Zone 3 spans the last third of the uterus and includes the spermatheca. Proper sperm guidance using wild-type, N2 hermaphrodites and *fog-2(q71)* males should result in approximately 90% of the labeled sperm reaching the spermatheca, or Zone 3 (**Figure 4B**). Matings that result in too few (**Figure 4C,** less than 10-15 sperm) or too many (**Figure 4D,** uterus filled with sperm) sperm in the uterus should not be counted. In matings that result in less than 10-15 sperm, 3-4 rogue sperm may heavily skew the data. Similarly, when the uterus is filled completely with sperm, the sperm cannot migrate appropriately. Sperm may seem scattered throughout the uteri of some mutants that display poor sperm guidance phenotype. However, in this case, sperm should not fill each crevice of the uterus, as seen in **Figure 4D**. Quantification of each arm of the gonad is considered one sample, or one n.

Time-lapse images are taken to quantify sperm velocity and reversal frequency. Only sperm in Zone 2 should be tracked (**Figure 2A**) because sperm in Zones 1 and 3 (**Movie 1**) tend to move in a circular pattern even within wild-type animals. Time-laspe images taken at 15-30 s intervals are usually used to track sperm. Only sperm that can be followed in consecutive frames for more than 2.5-3 min are quantified. In **Figure 2B-M**, the sperm marked by the red and blue dots satisfy this criterion, while the sperm marked by the green dot does not. Therefore, the values defined in **Figure 2N** are quantified for the sperm marked by the red and blue dots (**Figure 2O**), while those for the sperm marked by the green dot were not quantified.

FIGURE AND TABLE LEGENDS:

Figure 1: Cartoon of the adult *C. elegans* hermaphrodite and male. Major reproductive structures are labeled in the figure.

Figure 2: Quantifying sperm velocity and reversal frequency during migration through the uterus. (A). DIC+TRITC merged image of a hermaphrodite uterus containing fluorescent sperm (red). V: vulva, yellow: spermatheca, Z1-Z3: three zones of the uterus, black box: zone 2. (**B-M).** Time-lapse TRITC channel images zoomed in on zone 2 (black box in A). Images were acquired at 20 s intervals. 3 individual sperm were tracked in each image (red, green, and blue dots). Colored dots in panel **M** represents the path of each sperm from **B-L**. Scale bar: 20μm. (**N).** Equations and definitions for sperm speed, vector velocity and reversal frequency. (**O).** Speed, vectorial velocity and reversal frequency of sperm tracked in panels **B-L** by the red and blue dots.

Figure 3: Schematic diagram of sample preparation and data acquisition. Males stained with the mito-dye are mated to synchronized adult hermaphrodites. Time-lapse images of mated hermaphrodites are taken immediately after mating to capture data for sperm velocity and reversal frequency. Still images of mated hermaphrodites are taken 1 h after mating to assess sperm distribution within the uterus. Refer to the text for more details.

Figure 4: Quantifying sperm distribution within the hermaphrodite uterus. (**A).** Schematic of the *C. elegans* hermaphrodite uterus. V: vulva, E: embryo, S: spermatheca, O: oocyte, Z1-Z3: Zones 1-3 used to measure sperm distribution. (**B-D).** DIC+TRITC merged (left panels) and TRITC only (right panels) images of the wild-type hermaphrodite uteri 1 h after mating to *fog-2(q71)* males stained with the mito-dye. Sperm appear red. Yellow outlines indicate the location of the spermatheca. Scale bar: 20 μm. Z1, Z2, Z3 quantification in B represent the percent sperm in each zone ± standard deviation. Images in **C** and **D** represent matings that have resulted in too few (**C**) or too many (**D**) sperm for quantification.

Movie 1: Movie of sperm movement and migration. A wildtype hermaphrodite was mated to *fog-2(q71)* males stained with mito-dye. The movie is a composite of time-lapse images taken at varied time intervals.

DISCUSSION:

The ability of sperm to navigate the convoluted female reproductive tract and find oocytes is critical for sexual reproduction. Recent studies using sperm of internally fertilizing animals suggest they actively respond to various environmental cues, including chemical signals, fluid flow, and temperature gradients[1,5,7,9,12]. However, these observations have largely resulted from in vitro experiments and little is known about sperm behavior and communication within the reproductive tract. One of the main barriers to acquiring in vivo data on sperm migration and motility is the lack of visibility within most female reproductive tracts. The method we have described here using *C. elegans* overcomes this limitation. As the representative results demonstrate, the transparent epidermis allows for direct visualization and tracking of each sperm at single cell resolution in a live, intact organism.

C. elegans has two sexes. The males, with an XO genotype, produce only sperm, and in this method, are used as the sperm donors. The hermaphrodite, with an XX genotype, are modified females. Their gonads first undergo spermatogenesis during the fourth larval stage and switch to oogenesis in adulthood[25]. This assay uses adult hermaphrodites, whose reproductive tissues provide a model for the female reproductive tract. The utilization of both sexes in this assay allows us to identify genetic and molecular pathways in both the male and female that may regulate sperm guidance and motility. Combined with the whole host of genetic and molecular techniques available for *C. elegans*, this method can lead to novel insights into sperm migration and motility as well as sperm and oocyte communication.

A few critical steps in this protocol warrant further consideration, in addition to the details provided in the protocol section.

Worms

fog-2(q71), *him-5(e1490)*, or *him-8(e1489)* mutant males can be used in place of N2 males. These mutations increase the frequency of males in cultures, but do not affect male mating or sperm functions[13]. Females, such as *fog-2(q71)* females, may be used in place of hermaphrodites. However, females must be pre-mated with males to allow proper oocyte development. The presence of fertilized embryos from this pre-mating also ensures that the uterus is long enough to properly assess sperm distribution. If mutant or experimental hermaphrodites are being assessed, include a control group(s). For example, mutant hermaphrodites should be paired with wildtype N2 hermaphrodites as a control for other variables in the assay. Hermaphrodites that have been fed with bacteria containing plasmids for RNA interference assays should also be fed with bacteria containing empty vector control. The distance from the vulva, where sperm are inseminated, to the spermatheca, the fertilization site, can vary depending on the number of eggs in the uterus (i.e., the uterus expands with increasing egg number). If comparisons between genotypes are made, select hermaphrodites whose uteri contain similar numbers of eggs. Do not select hermaphrodites containing hatching embryos or moving larvae. A time course may be performed to identify the optimal age at which hermaphrodites should be assayed.

Picking hermaphrodites and males

It is important that the hermaphrodites picked for this assay are not from overgrown or starved plates. Food and pheromone cues modulate the expression of DAF-7, a TGFß homolog. The DAF-7 pathway has been shown regulate the synthesis of F-series prostaglandins that play important roles in guiding sperm to the spermatheca[20]. Picking hermaphrodites from overcrowded or starved plates may result in poor sperm guidance not related to the target of interest. The density of the plates do not seem to affect the male sperm. However, males that are too young or too old may result in decreased mating efficiency (i.e., the percent of hermaphrodites on the mating plate that have enough sperm in their uteri to quantify). 1-3 day old adult males are optimal for this assay[23].

Anesthetizing hermaphrodites

In our hands, the Tetramisole and Tricaine combination ensures that worms are immobilized, and remain alive during mating and image acquisition. Other anesthetics, such as sodium azide, may also be used. However, sodium azide is highly toxic and conditions need to be standardized. Immobilization techniques using microbeads, agarose, and microfluidics chambers are not recommended as they interfere with mating.

Male staining

The mito-dye used in this manuscript, MitoTrackerCMXRos, has been widely used for labeling sperm, as well as other mitochondria, in *C. elegans*. The sperm labeled with this mito-dye is fully functional, retaining its ability to be activated, migrate, fertilize oocytes, and produce viable progeny[26,27]. Other mitochondrial dyes, such as rhodamine 6G and DiOC6 have been used to stain *C. elegans* mitochondria[28,29]. However, conditions for these dyes need to be standardized for labeling sperm in this assay. In addition to mitochrondrial labeling mechanisms, DNA stains, such as Syto17, may also be used to label sperm for migration assays[30]. While these labeling techniques are relatively easy and quick to perform, transgenic strategies may also be employed

to generate sperm that express fluorescent tags under sperm specific promoters[31,32].

Mating

Mating dots that are too thick may decrease mating efficiency. Care should be taken to transfer as little bacteria as possible when transferring males and anesthetized hermaphrodites onto the mating dot.

Making agarose pads and placing the cover slip

Air bubbles can be generated in the agarose pads. They may refract light during image acquisition or, when large enough, cause worms to fall through, making it impossible to acquire the image(s). Similarly, air bubbles may be created along the hermaphrodites when the cover slip is placed over them on the agarose pad. These bubbles refract light and lead to decreased image quality. Practice will help decrease the occurrence of air bubbles.

Quantification

When quantifying sperm distribution, different z-planes through the uterus of a worm may have slight differences in sperm distribution. We find that taking a single image focused on the spermatheca gives us reproducible results that are similar to results obtained by averaging multiple z-sections. We recommend focusing the image on the center of the spermatheca, but the focal planes can be altered slightly based on the experimenter's needs. It is critical, however, that all images are taken in the same manner. Furthermore, it is important that only sperm that are in focus are counted. It is the counter's discretion in determining the criteria for in-focus sperm. However, it is critical that the criteria is applied systematically to every worm that is quantified. For sperm tracking, many software offer automatic tracking capabilities. However, we find that manual tracking outperforms the software's automatic tracking algorithm for two main reasons. 1. The abundance of similarly sized nuclei within the confined space makes it difficult for the software to distinguish between individual sperm and create defined ROIs for each sperm. 2. As sperm go in and out of focus, their intensities shift, making it difficult for the software to keep track of the sperm over extended periods of time.

ACKNOWLEDGMENTS:

We sincerely thank our late mentor, Dr. Michael Miller, for his inspiring and selfless mentorship and creation of this method as a tool to better understand sperm and oocyte communication. His sudden passing has been a tremendous loss for his family, his lab, and the scientific community. This study was supported by the NIH (R01GM085105 to MAM and F30HD094446 to MH). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

DISCLOSURES:

The authors have no conflicts of interest.

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Figure 1

Click here to access/download;Figure;Figure_1_hermaphrodite_male.ai

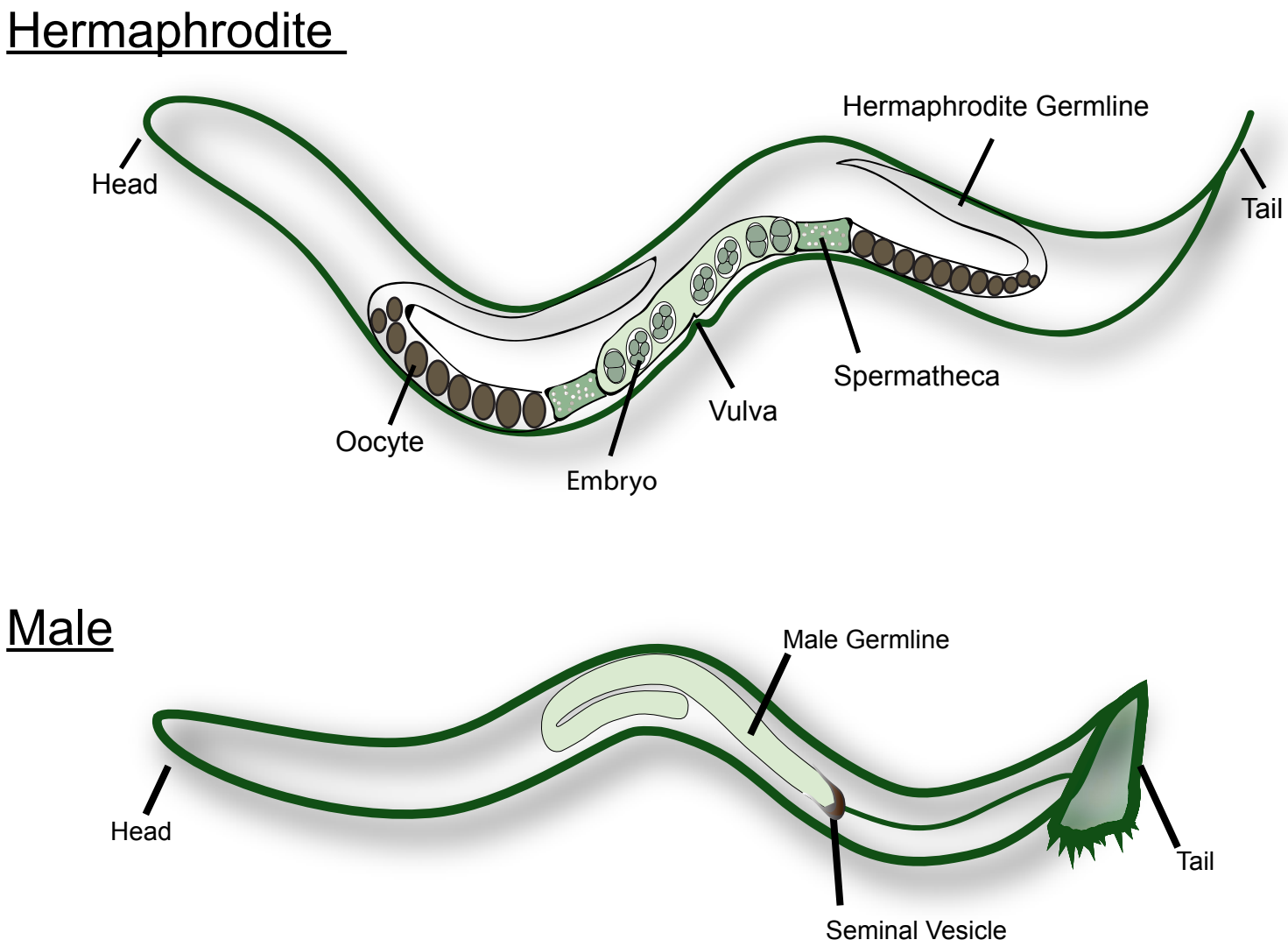
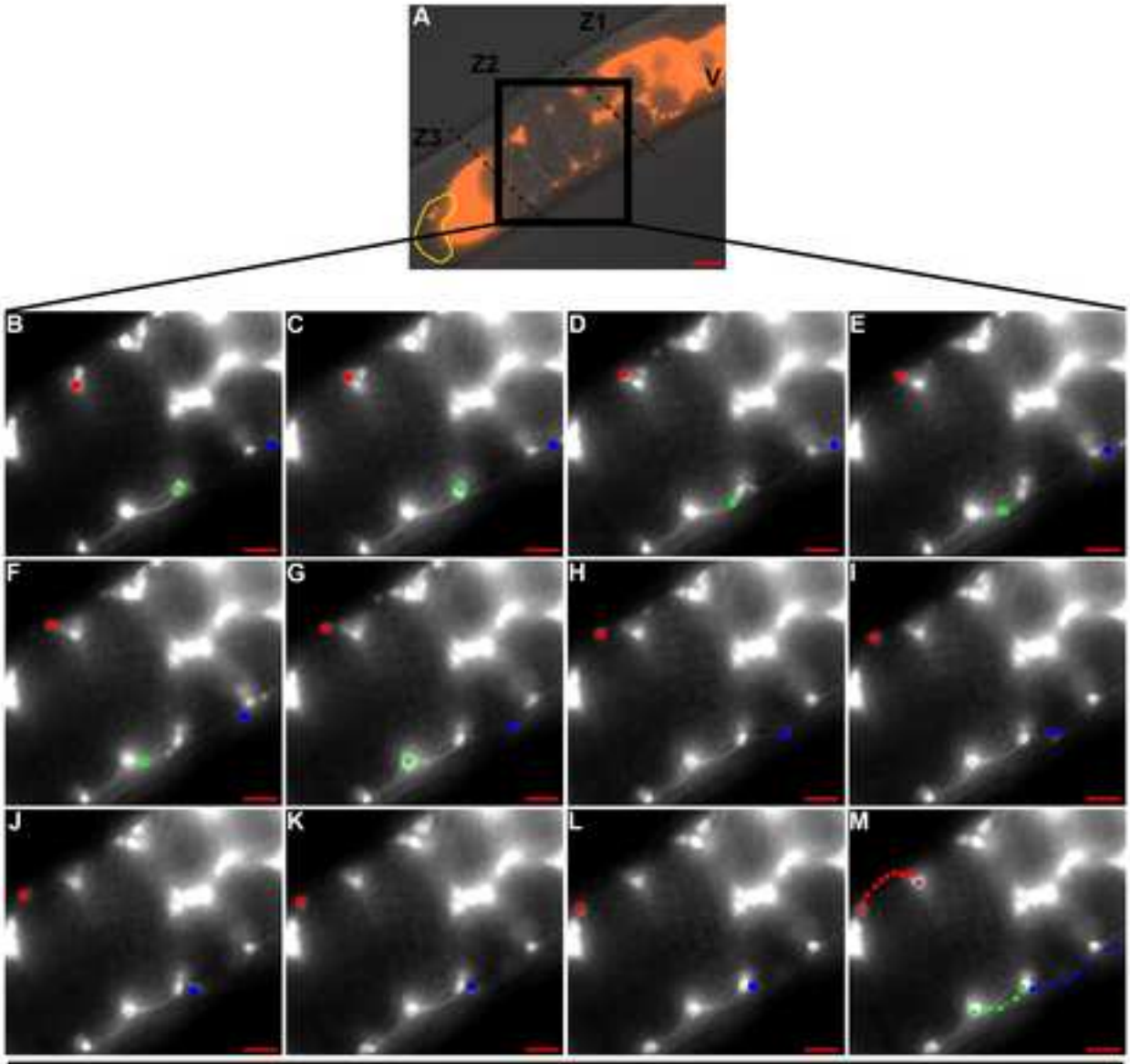


Figure 2

Click here to access/download;Figure;Figure_2_tracking_sperm_edited_flat.ps



N

1. Speed=Total distance Traveled (μm)/time (min)

2. Vectorial velocity=Distance traveled along line from uterus to spermatheca (μm)/time (min)

3. Reversal frequency= Reversals per hour

Reversal= When the angle generated by a sperm in three consecutivetime-lapse frames is less than 90°

O

Calculations for sperm tracked in panels B-L			
Description	Speed (μm min $^{-1}$)	Vectorial Velocity (μm min $^{-1}$)	Reversal frequency (rev per h)
Red	7.5	6.12	0
Blue	9.1	8.9	0

Figure 3

Click here to access/download;Figure;Figure_3_SGA_schematic.ai

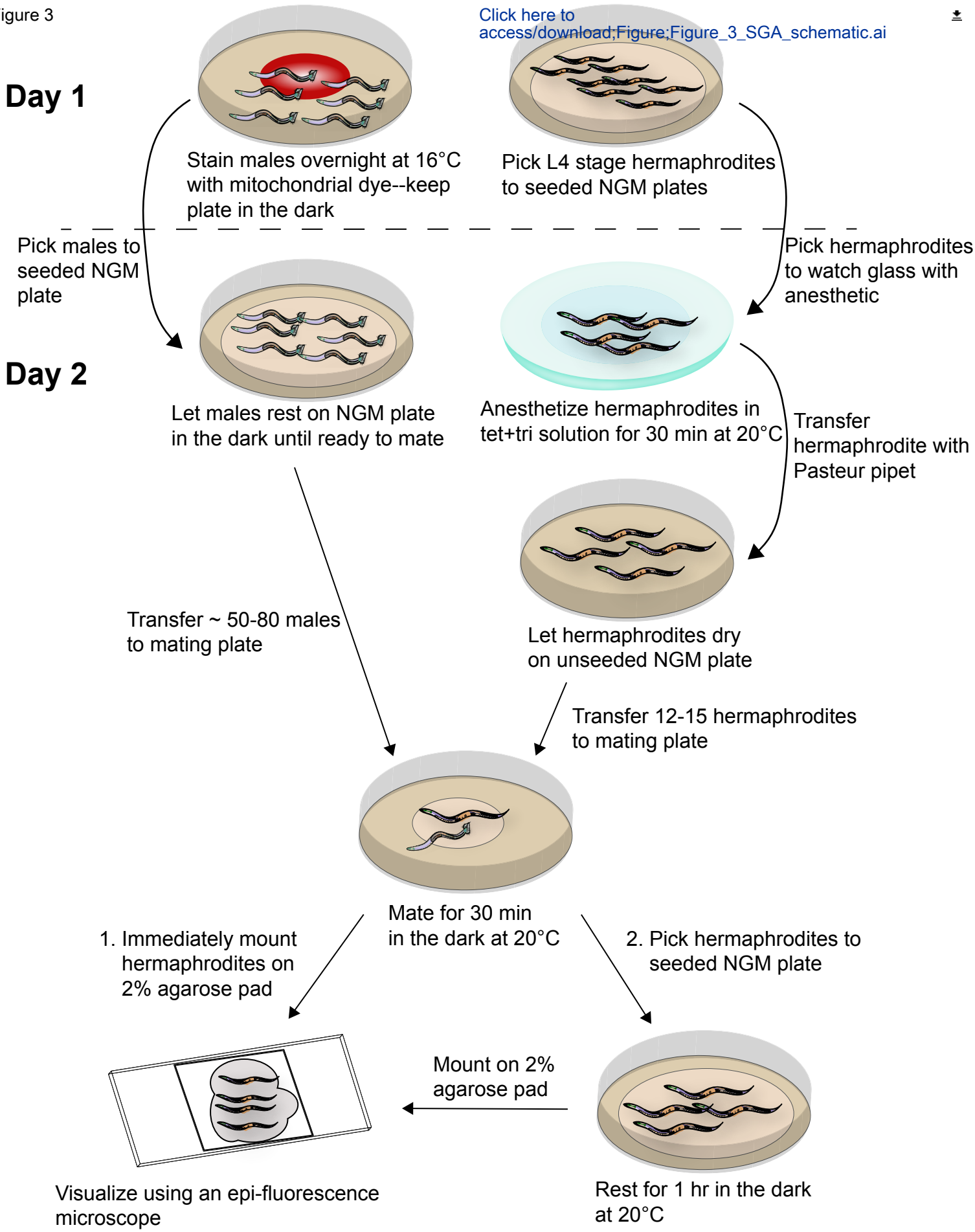
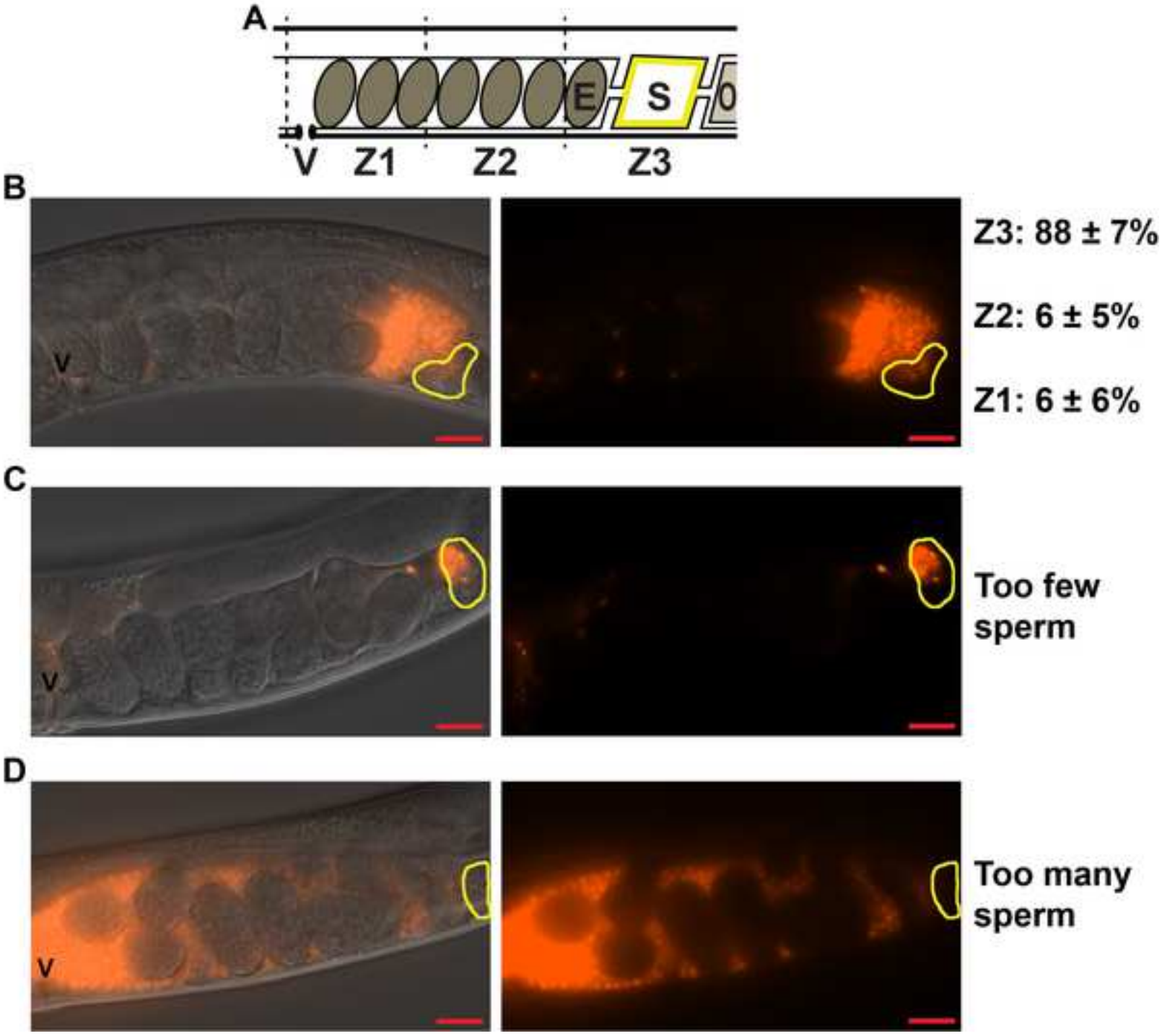


Figure 4



Movie 1

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Video or Animated Figure

Movie_1_final.mp4

Name of Material/ Equipment	Company	Catalog Number
Reagents and Material		
60mm x 15mm Petri Dish	Fisher	FB0875713A
Agar	Fisher	BP1423-500
Sodium Chloride	Fisher	S671-3
Peptone	Fisher	BP1420-500
Cholesterol	Sigma-Aldrich	C8667
LB broth, Miller	Fisher	1426-2
Escherichia coli strain NA22	Caenorhabditis Genetics Center (CGC)	NA22
N2	CGC	N2
fog-2(q71)	CGC	CB4108
Platinum wire 0.25mm dia	Alfa Aesar	10288
5 3/4" Disposable Pasteur pipet	Fisher	13-678-20A
Watch glass	Fisher	02-612A
5mm Dia. Glass rod	Fisher	50-121-5269
MitoTracker CMXRos (Mito-dye)	Fisher	M7512
Monopostassium phosphate	Fisher	P285-500
Disodium phosphate	Fisher	S374-1
Magnesium sulfate	Fisher	M63-500
Dimethyl sulfoxide	Fisher	BP231-1
Aluminum foil	Fisher	01-213-102
Ethyl 3-aminobenzoate methanesulfonate	Sigma	E10521-10G
Tetramisole hydrochloride	Sigma	L9756-5G
Agarose	Fisher	BP1356-100
Coverslips	Fisher	12-548-A
Frosted microscope slides	Fisher	12-552-3

Equipment

16℃ and 20℃ incubators	Fisher	97-990E
Upright Microscope with epi-fluorescence illuminator, camera, and 10x and 60x objectives	Nikon	
Software with image acquisition and tracking capabilities	Nikon	NIS-elements AR
Stereo-microscope	Nikon	SMZ800N

Comments/Description

Either this or OP50 E. coli can be used for C. elegans maintenance and assay. Both may be purchased at the CGC

Shield from light, store at -20°C

DMSO

Tricaine is the common name. Store in aliquotes at -20°C.
Store in aliquotes at -20°C

18 x 18-1

Same model, set at different temperatures.

Any stereo-microscope that can be used to visualize *C. elegans* may be used with this protocol

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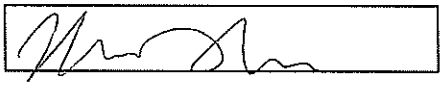
Signature:	
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We thank all the reviewers for taking the time to give us such detailed and constructive comments. The revisions to the text and figures in response to the comments have been added to the updated manuscript and figures. Specific responses to the reviewers are below.

Reviewer 1—Minor concerns:

Comment 1:
The authors mention using fog-2 males for the crosses, but should also consider mentioning the use of fog-2 'females' as an option if it is desirable not to have hermaphrodite sperm.

Author Response:
We agree that utilizing *fog-2(q71)* females is a great way to assess the effect of mutant sperm on sperm guidance. However, because sperm signaling is required for proper oocyte maturation, females should be pre-mated with males and allowed to rest before mating in the sperm guidance assay. The presence of fertilized embryos from this pre-mating also ensures that the uterus is long enough to properly assess sperm distribution within the three zones. The following clarification has been added in the text.

- Line 113-117: “Females, such as *fog-2(q71)* females, may be used in place of hermaphrodites. However, females must be pre-mated with males to allow proper oocyte development. The presence of fertilized embryos from this pre-mating also ensures that the uterus is long enough to properly assess sperm distribution.”

Comment 2:
In 1.1 the authors provide suggestions for the best age of hermaphrodites to provide as mating partners. I personally think 28-30 hours after L4 is too old. In order to avoid the multiple egg issue, younger hermaphrodites (eg. just past the L4 lethargus) could be used.

Author Response:
We find that sperm guidance decreases in wild-type hermaphrodites that are near the end of their reproductive life span (i.e. hermaphrodites that contain unfertilized oocytes in the uterus). However, hermaphrodites that are too young pose their own set of problems. In our hands, young hermaphrodites seem to have decreased mating efficiency, and the shorter uteri in younger hermaphrodites sometimes make it difficult to quantify sperm distribution within three clear cut zones. We find that wild-type hermaphrodites that are 28-30 h post L4 at 20 ºC gives us the most consistent results. We do clarify that a time course can be done to find the most optimal time point for the experimenter.

- Line 134-135: “A time course may be performed to identify the optimal age at which hermaphrodites should be assayed.”

Comments 3-6:

3. **Reviewer There are some instances of 'u' rather than the micro symbol.**
4. **5.1 Since there are many different software packages, the details on how to use NIS-Elements might not be that generally useful**
5. **5.2 "Scales bar" should be scale bar.**
6. **Line 418 provide specific guidance on the best aged male (day 1 adult, I think).**

.

Author Response:

3. We have changed the “u” to “µ” where appropriate.
4. We agree that many software are available for this type data analysis. Since we used the NIS-Elements software for our data, we feel that the instructions on using this software on sperm tracking may provide some guidance for people who already use this software, but may be new to the tracking function. However, in order for this method to apply to a broader audience, we added instructions for tracking sperm using the free, open source software in Fiji/ImageJ (341-361). We have also changed the language regarding the image acquisition steps to be more applicable to more upright, epi-fluorescence microscopes.
5. We have changed it to “scale bar”.
6. The following change has been added to the text.
    - Line154 and 480: “1-3 day old adult males are optimal for this assay.”

Reviewer 2—Minor concerns

Comment 1:
Line 149: Do you really use 2 microliters of food on the mating plates? This seems like a very small amount of food. I just wanted to make sure that there is not a missing zero there.

Author Response:
We understand the concern. However, we do use 2 microliters of the thick bacteria for the mating plates. The goal is to increase the chances that a male comes in contact with a hermaphrodite in the 30 min mating period. The small surface area from 2 microliters of bacteria ensures that most of the males stay in one concentrated area.

Comment 2:
From reading the protocol, it seems that all of the analysis is done on an image(s) taken in a single plane (i.e. not a maximum projection from a Z stack). I don't have a problem with this, but I believe it is worth discussing this and mentioning that there will be some amount of sperm that are not accounted for in this technique.

Author response:
We have quantified sperm distribution in single z-planes and compared with averages from multiple images within a z-stack. We find that that the percent distribution of the sperm between these two methods are comparable. We discuss this in the discussion section.

- Line 497-503: “When quantifying sperm distribution, different z-planes through the uterus of a worm may have slight differences in sperm distribution. We find that taking a single image focused on the spermatheca gives us reproducible results that are similar to results obtained by averaging multiple z-sections. However, it is important that only sperm that are in focus are counted. It is the counter’s discretion in determining the criteria for in-focus sperm. However, it is critical that the criteria is applied systematically to every worm that is quantified.”

Reviewer 3—Major Concerns:

Comment 1:
Since researchers often make substitutions that compromise a protocol, it would be

helpful for the authors to discuss the advantages of their anesthetic protocol over azide or other common choices.

Author Response:
We have added the following information in the text.

- Line1878-192: “Note: Store 1% (w/v) Tricaine and 0.1% (w/v) Tetramisole as aliquotes at -20 °C. Avoid repeated freeze thaw. The Tetramisole and Tricaine combination ensures that worms are immobilized, and remain alive, during mating and image acquisition. Other anesthetics, such as sodium azide, may also be used. However, sodium azide is highly toxic and conditions need to be standardized. Immobilization techniques using microbeads, agarose, and microfluidics chambers are not recommended as they interfere with mating.”

Comment 2:
Detailed software instructions are provided for one model of Nikon microscope. Citations to guides for Zeiss and Leica would be helpful.

Author Response:
We realize that our initial instructions for image acquisition and data analysis may be too limited for general use. We therefore changed the instructions to be generally applicable to most upright, epi-fluorescence microscopes. Additionally, we have added instructions for sperm tracking using the free ImageJ/Fiji software (lines: 341-367). However, we still feel that the instructions for sperm tracking using the NIS-elements may be useful for people who already use this software, but may be unfamiliar with the tracking functions.

Comment 3:
On lines 346-7 the authors write "Matings that result in too few (Figure 2C) or too many (Figure 2D) sperm in the uterus should not be counted." This point needs to be expanded upon to ensure that the criterion are clear and do not lead to users 'massaging' their data.

Author Response:
The following clarification has been made in the text. Figure 2 is now the new Figure 3.

- Line 397-403: “Matings that result in too few (Figure 3C, less than 10-15 sperm) or too many (Figure 3D, uterus filled with sperm) sperm in the uterus should not be counted. In matings that result in less than 10-15 sperm, 3-4 rogue sperm may heavily skew the data. Similarly, when the uterus is filled with sperm, the sperm cannot migrate appropriately. Sperm may seem scattered throughout the uteri of some mutants that display poor sperm guidance phenotype. However, in this case, sperm should not fill each crevice of the uterus, as seen in Figure 3D.

Comment 4:
On line 26 the authors write "migration velocity, directional velocity," and they repeat this formulation throughout the manuscript. It looks to me like the authors are trying to compare "speed" and "velocity" here. If not, it's not clear what they mean. The text should be revised appropriately.

Author Response:
We agree with the reviewer. “Migration velocity” has been changed to “speed” wherever it appears in the text and figures.

Reviewer 3—Minor Concerns:
Line 35: Replace "clear" with "transparent"
Line 38: Delete "animal"
Line 41: Replace "has" with "have"
Line 53: Replace "to study" with "for studying"
Line 198: Replace "aliquote" with "aliquot"
Line 241: Change "may damage the worm can cause the intestine or gonad to extrude" to "may damage the worm, causing the intestine or gonad to extrude"
Line 247: Change "Conctrast" to "Contrast"
Line 252: Change "spermatheca." To "spermathecae."
Line 319: Change "velocity," to "speed,"
Line 335: Change "sperm is" to "sperm are"
Line 397: Change "the lack of visibility of most female reproductive tracts" to "the lack of visibility within most female reproductive tracts"
Line 402: Change "with a XO" to "with an XO"
Line 405: Change "transforms" to "switches"

Reviewer Response:
All of the above suggestions have been edited and the changes are tracked within the text.

Reviewer 4:

Comment 1:
The amoeboid motility and its unique MSP cytoskeleton for worm sperm should be mentioned in the introduction and discussion.

Author Response:
We have included a more detailed description of male and hermaphrodite germline development and mating in the introduction. This description also includes details about the sperm's amoeboid motility.

- Lines: 78-92: "*C. elegans* exist in nature as hermaphrodites and males (see Figure 1). The hermaphrodite gonad has two U-shaped arms that are mirror images of each other. During the L4 larval stage, the most proximal germ cells (i.e. the cells near the spermatheca) undergo spermatogenesis. Each primary spermatocyte enters meiosis and produces four haploid spermatids. These spermatids are pushed into the spermatheca along with the first mature oocyte and undergo spermiogenesis[15]. Adult hermaphrodites switch from spermatogenesis to oogenesis. The oocytes mature in an assembly line fashion along the gonad, with the most mature oocyte at the proximal end of the gonad, next to the spermatheca. MSP signals from the sperm is needed to trigger meiotic maturation and ovulation[16,17]. Male *C. elegans*, on the other hand, have a J-shaped gonad that produce only sperm. The spermatids are stored in the seminal vescicle. Upon mating with the hermaphrodite or female, the male inserts the spicules near the tail into the vulva. Spermatids are activated during ejaculation, when they come in contact with the seminal fluid[18]. *C. elegans* sperm do not swim as they are not flagellated. Instead, they crawl through the reproductive tract, using the pseudopod for locomotion. It is well established that male sperm, which are larger in size, have a competitive advange over hermaphrodite sperm[14]."

Comment 2:

Though it is easy to perform the experiment, the shortcoming for this methods obviously exists. The authors need to discuss the next generation of sperm tracking with the aid of CRISPR CAS-9 knock-in of a marker, for example H2B tagged with GFP or mCherry driven by sperm specific promotor.

Author Response:
We have included other alternatives to staining males with MitoTracker CMXRos in the discussion section. References included in this section show examples of possible transgenic approaches to labeling sperm.

- Line: 480-489: "**Male staining**: The mito-dye used in this manuscript, MitoTrackerCMXRos, has been widely used for labeling sperm, as well as other mitochondria, in *C. elegans*. The sperm labeled with this mito-dye is fully functional, retaining its ability to be activated, migrate, fertilize oocytes, and produce viable progeny[26,27]. Other mitochondrial dyes, such as rhodamine 6G and DiOC6 have been used to stain *C. elegans* mitochondria[28,29]. However, conditions for these dyes need to be standardized for labeling sperm in this assay. In addition to mitochrondrial labeling mechanisms, DNA stains, such as Syto17, may also be used to label sperm for migration assays[30]. While these labeling techniques are relatively easy and quick to perform, transgenic strategies may also be employed to generate sperm that express fluorescent tags under sperm specific promoters[31,32]."

Reviewer 5—Major Concerns:

Comment 1:
In general, it might be helpful to emphasize more strongly the importance of keeping mitotracker plates and labeled worms in the dark whenever possible.

Author Response:
We have noted the importance of keeping mitotracker containing solution, plates, and worms in the dark in the following places.

- Line: 151-152: "Note: Mito-dye is light sensitive. Shield solution, plates, and worms containing mito-dye from light. Store the 1mM stock at -20 ºC."
- Line 154-155: "2.3 Pick ~ 100 adult males to the mito-dye stained food dot on the male staining plate. Wrap the plate in aluminum foil and incubate overnight at 16 ºC."
- Line: 168-169: "3.1 Pick the stained males from Day 1 onto a new, seeded NGM plate. Leave the plate in the dark until mating."
- Line: 213-215: "3.8 Transfer the anesthetized hermaphrodites from the unseeded NGM plate onto the mating dot with the stained males. Incubate in the dark for 30 min to allow the males to mate with the hermaphrodites."
- Line: 266-267: "Do not expose the mated worms to extended periods of fluorescent light because the signal from the stained sperm bleaches quickly."

Comments 2-3:
2. Do tricaine and tetramisole solutions need special storage or handling?
3. Lines 137-140. Is it necessary to take measures to avoid evaporation of liquid?

Author Response:
The storage and handling instructions for tricaine and tetramisole have been added to the text.

- Line187-188: “Note: Store 1% (w/v) Tricaine and 0.1% (w/v) Tetramisole as aliquotes at -20 °C. Avoid repeated freeze thaw.”

Instructions for covering the watch glass to prevent evaporation has been added to the text.

- Line 161-162: “Keep the watch glass covered in the dark to prevent evaporation and light exposure.”

Comment 4:

Lines 246-257 (step 5.1.2); also Lines 289-90. More detail should be provided about how to select a focal plane for imaging. The spermatheca and uterus are at least ~20 microns thick and different focal planes typically contain different numbers of sperm. For example, should a focal plane with maximal sperm be selected? One in the midpoint of the spermatheca, assessed by focusing up and down and choosing a center point? Also, be more specific about what constitutes an" appropriate" exposure - this is presumably one in which signals from individual spermatozoa can be distibguished from one another without oversaturating the signal. Finally, it does not seem necessary to specify the specific method for selecting the XY position using NIS software; or a note could be added to clarify that this can be done manually.

Author Response:

We have specified in Line 270 that the focal plan should be the center of the spermatheca. However, we specify in the discussion section that it is more important that images are taken in a consistent manner (Line 500-502).

We have provided further explanation for what constitutes as an “appropriate” exposure.

- Line 271-272: “In DIC, internal worm structures should be clearly visible. In TRITC, individual sperm should be visible as distinct puncta.”
- Line 277-278: “However, it is important that the TRITC channel images are not overexposed, so that individual sperm can be distinguished and quantified.

We have changed the language for image acquisition and data analysis to be more generally applicable to many fluorescence microscopes.

Comment 5:

Figure 3A. The gonad should be labeled with Z1, Z2, Z3 and spermatheca.
It was necessary to download the high-resolution image to see the red, green, and especially blue tracking dots. It might be helpful to add arrows as well, especially to the beginning and end time points.

Author Response:

We have added the labels Z1, Z2, and Z3 to Figure 3A (now Figure 4A). The spermatheca is demarcated by the yellow outline. In order to make the tracing dots easier to see, we have made them bigger in the image.

Reviewer 5—Minor Concerns:

Comment 1:

Line 210. In our experience it is not necessary to allow agarose to harden more than ~20-30 sec. Also, indicate whether the top slide should be removed by sliding or by some other method.

Author response:
We agree with the reviewer and have changed the time to ~30 s. Futhermore, we have specified that the top slide should be removed by sliding off the agarose pad.

- Line 241-242: “4.1.3 Let the agarose harden (~ 30 s). Carefully remove the top glass slide by sliding it off the agarose pad.”

Comment 2:
Line 107. Should more information be provided about the nature of appropriate controls, or is this left up to the user?

Author Response:
We have added some examples of what appropriate controls may entail.

- Line 127-130: For example, mutant hermaphrodites should be paired with wildtype N2 hermaphrodites as a control for other variables in the assay. Hermaphrodites that have been fed with bacteria containing plasmids for RNA intereference assays should also be fed with bacteria containing empty vector control.”

Comment 3:
Lines 225-231. It does not seem necessary to list parameters for filter cubes, especially those not used to visualize mitotracker dye. What is the journal's standard for this type of equipment?

Author Response:
We have removed the specifications for the scope.

Comment 4:
The filter set is referred to as Texas Red in the list of cubes, and TRITC in the protocol steps. TRITC is a specific dye, so different wording should be used, e.g. "using epifluorescence" "mitotracker channel" "red channel" or whatever is appropriate.

Author response:
We referenced other published journals and have found the wording “TRITC filter” to be widely used. Furthermore, the filter set on our scope refers to this red channel as the “TRITC filter”. However, we have clarified that our definition of the “TRITC filter” refers to the red fluorescent emission filter (Line 259).

Comment 5:
Materials list: For NGM reagents, cholesterol should be added to this list.

Author Response:
We have added cholesterol to our materials list.

Suggestions for clarifying the text:
Line 32. Should be "surroundings"
Line 39. Delete "can"
Line 44. Delete "model"
Line 57-8. Suggest replacing "rivers, lakes, or seas" with "aquatic environments"
Line 58. Suggest replacing "change" with "influence" or "guide" depending on how strongly the authors want to word this.
Line 114. Should be Fisher or Thermo Fisher (not sure how this company is cited

currently)
Line 140. Replace "in" with "at"
Line 170. Add comma after "acquisition"
Line 221. Delete "and Fluorescence Cubes"
Line 222. "Imaging Workstation software"
Line 235 "eyepieces"
Line 241. "by causing"
Line 341. Replace "their ability… spermatheca" with "migration through the uterus/female reproductive tract"
Line 361. Suggest title "Schematic diagram of sample preparation and data acquisition"
Line 374. Replace "quantification" with "quantifying localization"
Line 376-77. Suggest title: "Quantification of sperm velocity and reversal frequency during migration through the uterus/female reproductive tract"
Line 378-9. Should be B-L
Line 379-80. Suggest "Images were acquired at 20s intervals"
Line 409. available for C. elegans
Line 428. "Practice and repetition" seems redundant.
Line 76. A citation for Hansen, Chavez, Stanfield (2015) eLife should be added along with ref. 10.

Author Response:
We have changed the above suggestion in the text.

Suggestions for clarifying the text:
Line 36. Suggest deleting "which act as modified females" - this detail seems distracting rather than helpful.
Line 51. Delete "robust". Also, simply saying "highly dynamic" seems rather uninformative - suggest rephrasing, for example, "The speed and directionality of sperm motility can be highly dynamic" - since these parameters are what the method addresses.
Line 72. Delete "in hermaphrodites
Line 94. Delete "Male and hermaphrodite"or males"

Author Response:
We feel that our wording for the above suggestions provide some added information/clarification to the text. Therefore, we have not made edits to the above suggested changes.

Reviewer 6—Major concerns:

Comment 1:
Figure 3: sperm tracking

A. **Though figure 2 explains not to use track sperms in uteri which have too many sperms, this figure shows tracking of sperm in the uterus of an animal which seems to have excess sperm!**
B. **All the tracking dots (red, green & blue) are barely visible.**
C. **Scale bars are also not visible.**
   The authors may want to either (preferably) replace the figure or make dots or scale bar clearer.

Author Response:

A. The still image in Figure 3A (now Figure 4A due to the addition of a *C. elegans* schematic) was extracted from a time-lapse series in which the sperm is actively crawling through the uterus. Therefore, the uterus does have sperm near the spermatheca as well as the vulva. Moreover, the red fluorescence in Figure 4A is also over exposed in the image to make the red more prominent in the merged image.
B. We have made the tracking dots bigger.
C. We have made the scale bars thicker

Reviewer 6—Minor Concerns:

1. **Line 80: replace "to produced labeled sperm" with "to produce labeled sperms".**
2. **Lines 95-97: Rewrite these as the first lines suggest that Fog-2(q71) males are used and then next line indicates that fog-2 and other mutation males can also be used in place of N2!**
3. **Line 97-98: May want to provide any reference that these mutations do not affect male mating or sperm functions.**
4. **Line 131: How old the males should be? Two days or older males have lower mating efficiency. Ideally one should be picking L4 males on day one, which would give, roughly synchronized and healthy & young males on next day.**
5. **Line 144: Should males be transferred using pick or be washed off of the plate?**
6. **Figure 1: Day 1 male staining -- consider adding "keep plate in dark"**
7. **Figure 2:**
   A. **Whereas D shoes a hermaphrodite, which has recently mated and thus almost all the sperms are in zone 1 of the uterus, B and C depict uteri of hermaphrodites which have all the sperms moved into or in close proximity of spermatheca. Authors may want to be consistent here and choose figures similar to D, for both B and C OR add one more figure, which will clearly show as to what to expect for a optimally mated hermaphrodite.**
   B. **Scale bar is barely visible.**
8. **Line 369: Change "...DIC+TRITC (left panel) merged and TRICT (right panel)..."**
9. **Line 398: Change "described using here" to "described here using"**

Author Response:

1. We feel that “sperm” can be used to denote singular or plural forms. Therefore, we did not change “sperm” to “sperms”.
2. We have switched these sentences in the text.
   - Line 110-113: “*fog-2(q71)*, *him-5(e1490)*, or *him-8(e1489)* mutant males can be used in place of N2 males. These mutations increase the frequency of males in cultures, but do not affect male mating or sperm functions[13]. Wild-type N2 hermaphrodites and *fog-2(q71)* males are used in the procedure below.”
3. Reference number 13, which shows that all three mutants males produce sperm that migrate like wild-type N2 sperm, has been added to the text.
4. We have added that 1-3 day old males should be picked and stained. We have also included the reference (number 14) for the mating fitness of males.
5. We changed the text to specify that the males should be picked onto the plate.
6. We added “keep plate in the dark” to Figure 1 (now Figure 2).
7. Figure 2D (now figure 3D) is used to showcase a uterus that has been completely filled with sperm. The image in the D is similar to the images in B (an optimally mated hermaphrodite uterus) and C (one the has too few sperm). However, due to the complete congestion of sperm, they are unable to travel down to the spermatheca. We have increased the thickness of the scale bar.

Reviewer 7:

Comment 1:

The Introduction would benefit from the inclusion of a diagram of the C. elegans germline and somatic structures needed for mating and reproduction and that are referred to in the text.

Author Response:

We have added a new figure (the new Figure 1), that shows a cartoon of the adult hermaphrodite and male. The major reproductive tissues listed in the text have been labeled.

Comment 2:

Some discussion of the unique properties of spermatogenesis and fertilization in this system would be helpful for general readers. For example, general readers might be reminded that nematode sperm possess a pseudopod and move by crawling instead of using a flagellum.

Author Response:

We added the following description regarding hermaphrodite and male reproductive development, germ cell development, and mating to the introduction. The corresponding references are also added to the text.

- Line 78-92: *"C. elegans* exist in nature as hermaphrodites and males (see Figure 1). The hermaphrodite gonad has two U-shaped arms that are mirror images of each other. During the L4 larval stage, the most proximal germ cells (i.e. the cells near the spermatheca) undergo spermatogenesis. Each primary spermatocyte enter meiosis and produce four haploid spermatids. These spermatids are pushed into the spermatheca along with the first mature oocyte and undergo spermiogenesis[15]. Adult hermaphrodites switch from spermatogenesis to oogenesis. The oocytes mature in an assembly line fashion along the gonad, with the most mature oocyte at the proximal end of the gonad next to the spermatheca. MSP signals from the sperm is needed to trigger meiotic maturation and ovulation[16,17]. Male *C. elegans*, on the other hand, have a J-shaped gonad that produce only sperm. The spermatids are stored in the seminal vesicle. Upon mating with the hermaphrodite or female, the male inserts the spicules near the tail into the vulva. Spermatids are activated during ejaculation, when they come in contact with the seminal fluid[18]. *C. elegans* sperm do not swim as they are not flagellated. Instead, they crawl through the reproductive tract, using the pseudopod for locomotion. It is well established that male sperm, which are larger in size, have a competitive advantage over hermaphrodite sperm[14]."

Comment 3:

Line 104. The text should recommend that hermaphrodites of several different ages be used (i.e., it would be preferable to conduct time-course studies).

Author Response:

We have added this recommendation to the text.

- Line: 134-135: "A time course may be performed to identify the optimal age at which hermaphrodites should be assayed."

Comment 4:

Line 107. "RNAi hermaphrodites" is jargon and should be better explained. Also, please specify the nature of the optimal "control group(s)."

Author Response:
We have added examples of control groups for certain experimental scenarios to the text.

- Line 127-130: "For example, mutant hermaphrodites should be paired with wildtype N2 hermaphrodites as a control for other variables in the assay. Hermaphrodites that have been fed with bacteria containing plasmids for RNA intereference assays should also be fed with bacteria containing empty vector control."

Comment 5:
Use of MitoTracker CMXRos. I couldn't find data in the literature about the vitality of sperm labeled in this manner on a per sperm basis. In single mating tests, what is the brood size of a female mated with a labeled male vs. an unlabeled male? The text might include other means of labeling sperm (e.g., DiOC6(3), Wolke et al., 2007).

Author Response:
We have included references to literature that show MitoTracker CMXRos labeled sperm are not only able to migrate along the reproductive tract, but also fertilize the oocytes to generate viable embryos. To further test the sperm fitness, we mated stained and unstained *fog-2(q71)* males with virgin *fog-2 (q71)* females. We watched to make sure that each female was only mated once with one male. After 30 hrs at 20 ºC, the females mated with stained and unstained males generated comparable numbers of progeny (88 +/- 5, n=5 matings each). This suggest to us that the stained sperm function similar to unstained sperm.

We have included some other potential methods for labeling males in the discussion section.

- Line 480-489: "**Male staining**: The mito-dye used in this manuscript, MitoTrackerCMXRos, has been widely used for labeling sperm, as well as other mitochondria, in *C. elegans*. The sperm labeled with this mito-dye is fully functional, retaining its ability to be activated, migrate, fertilize oocytes, and produce viable progeny[26,27]. Other mitochondrial dyes, such as rhodamine 6G and DiOC6 have been used to stain *C. elegans* mitochondria[28,29]. However, conditions for these dyes need to be standardized for labeling sperm in this assay. In addition to mitochrondrial labeling mechanisms, DNA stains, such as Syto17, may also be used to label sperm for migration assays[30]. While these labeling techniques are relatively easy and quick to perform, transgenic strategies may also be employed to generate sperm that express fluorescent tags under sperm specific promoters[31,32]."

Comment 6:
Line 186. Is mating monitored to ensure that hermaphrodites are mated to a single male? If not, is this a variable in the study?

Author Response:
We do not watch to make sure that hermaphrodites are mated to a single male. In our hands, we find that our method is highly reproducible. With any given experiment, using different batches of hermaphrodites or sperm, we find that approximately 90% of wild-type sperm reach the spermatheca. The variability comes with how much sperm is deposited in each uterus. As shown in the new Figure 3, some uterus may contain too many sperm while others contain too few. However, these occasions seem to be rare when the right ratio of males and hermaphrodites are used for the mating.

Comment 7:
A concern is that the protocol for image acquisition and analysis is specific for proprietary software from Nikon. It would be preferable to provide instructions for data analysis using open source software.

Author response:
We have included instructions for sperm tracking using the open source ImageJ/Fiji software (Line 341-367)

Comment 8:
An issue for the image acquisition is that only a single focal plan appears to be imaged. Would it not be preferable to acquire a z-series?

Author Response:
We have compared sperm distribution in single z- plane and averages of multiple z-planes. We find the results to be comparable. However, we do note in the discussion that the experimenter may take multiple z-series and average the sperm distribution between the different focal planes.

Comment 9:
In the movie, it was not immediately clear how to distinguish between sperm movement by crawling and displacement caused by contraction/movement of somatic cells of gonad and the reproductive tract as well as fluid flow. Is there a way to visualize the pseudopod and its leading edge during movement?

Author Response:
Unfortunately, we are not able to visualize the pseudopod in this method. However, the hermaphrodites are heavily anesthetized. Therefore, we do not feel that the robust sperm migration is an artifact of somatic gonad contractions or the resulting fluid flow.

Comment 10:
Please put a scale bar in Figures 2 and 3 and display fluorescent images according to the convention that dorsal should be up, ventral down, and anterior to the left.

Author Response:
We have edited the figure accordingly.

Dear Editor,

Thank you for the following editorial comments. We have addressed each comment below and have made the corresponding changes in the manuscript.

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

Author Response:
We have carefully read through the entire manuscript and have checked for spelling and grammar issues to the best of our abilities. Mistakes that were found have been corrected and tracked in the manuscript.

Comment 2:
The highlighted protocol steps are over the 2.75 page limit (including headings and spacing). Please highlight fewer steps for filming.

Author Response:
We have removed some of the text from the highlighted section. The highlighted text should be approximately 2.75 pages long.

Comment 3:
Please do not abbreviate journal titles for all references.

Author Response:
We have edited all of the references to contain the full names of the journal.

Comment 4-5:
4. Please do not use more than 1 note per step.
5. Please avoid long steps/notes (more than 4 lines).

Author Response:
We have removed sections where there is more than one note per step. For all steps and notes that are more than 4 lines long, we have shortened it by moving the content to the discussion section.