Journal of Visualized Experiments

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--Manuscript Draft--

Article Type:	Invited Methods Collection - JoVE Produced Video
Manuscript Number:	JoVE61136R2
Full Title:	Worm-align and Worm_CP, two open-source pipelines for straightening and quantification of fluorescence image data obtained from C.elegans.
Keywords:	C. elegans. Fluorescence. Quantification. Worm-straightening. Cell profiler. FIJI. Image J.
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Additional Information:	
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.	Cambridge, UK

TITLE:

Worm-align and Worm_CP: Two Open-Source Pipelines for Straightening and Quantification of Fluorescence Image Data Obtained from *Caenorhabditis elegans*

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KEYWORDS:
C. elegans, fluorescence, quantification, worm-straightening, CellProfiler, FIJI, Image J

SUMMARY:
Worm-align/Worm_CP is a simple FIJI/CellProfiler workflow that can be used to straighten and align *Caenorhabditis elegans* samples and to score whole-worm image-based assays without the need for prior training steps. We have applied Worm-align/Worm_CP to the quantification of heat-shock induced expression in live animals or lipid droplets in fixed samples.

ABSTRACT:
An issue often encountered when acquiring image data from fixed or anesthetized *C. elegans* is that worms cross and cluster with their neighbors. This problem is aggravated with increasing density of worms and creates challenges for imaging and quantification. We developed a FIJI-based workflow, Worm-align, that can be used to generate single- or multi-channel montages of user-selected, straightened and aligned worms from raw image data of *C. elegans.* Worm-align is a simple and user-friendly workflow that does not require prior training of either the user or the analysis algorithm. Montages generated with Worm-align can aid the visual inspection of worms, their classification and representation. In addition, the output of Worm-align can be used for subsequent quantification of fluorescence intensity in single worms, either in FIJI directly, or in other image analysis software platforms. We demonstrate this by importing the Worm-align output into Worm_CP, a pipeline that uses the open-source CellProfiler software. CellProfiler's flexibility enables the incorporation of additional modules for high-content screening. As a practical example, we have used the pipeline on two datasets: the first dataset are images of heat shock reporter worms that express green fluorescent protein (GFP) under the control of the promoter of a heat shock inducible gene *hsp-70*, and the second dataset are images obtained from fixed worms, stained for fat-stores with a fluorescent dye.

INTRODUCTION:

A relatively simple organism, the nematode *C. elegans*, is an extremely useful model system for studying human diseases. About 38% of the genes in the *C. elegans* genome have functional counterparts in humans[1,2]. One of the unique characteristics of *C. elegans* is that it is optically transparent, enabling easy access to in vivo information regarding (sub) cellular expression of fluorescent reporters across tissues. This makes *C. elegans* a prime model organism for high-content screens using image-based platforms[3]. However, one issue that often complicates these studies is that when imaging dense populations of worms, they tend to cross and to cluster, making comparisons across individual worms challenging, clouding downstream image analysis and quantitation.

Existing solutions that overcome this issue typically rely on the optimization of the culturing and imaging protocol, such as through the use of micro-fluidics setups[4], allowing single worms to be captured in separate images[5,6]. Others have applied machine-learning algorithms allowing for recognition of single worms, even in a clumped population. An excellent example of the latter is the WormToolbox, which is a modular extension of the open-source image analysis platform, CellProfiler[7]. WormToolbox offers a high-throughput and high-content solution for analysis of *C. elegans*, and clearly benefits from its inclusion in CellProfiler, as additional analysis modules can easily be included. Although WormToolbox comes supplied with a pre-trained model (DefaultWormModel.xml), retraining of the machine-learning algorithm is usually required for each new application. Online tutorials on how to do this are available on Github (https://cp-website.github.io/Worm-Toolbox/). Despite this, installing and using WormToolbox requires a significant time-investment for novice users.

Here, we describe a simple and cost- and time-effective protocol to culture, and image populations of *C. elegans*. To allow the assessment of individual worms in the acquired images we have developed a simple open-source FIJI-based workflow, named Worm-align. Worm-align can be used to generate single- or multi-channel montages of straightened and aligned worms. Firstly, the user must manually select individual worms for analysis by drawing a line from the head to the tail. Worm-align will use this selection to crop selected worms from the overview image, and generate a montage in which selected worms are straightened and aligned to facilitate visual comparison and presentation.

In addition, the output of Worm-align can be used for subsequent quantification of fluorescence intensity in single worms, either in FIJI directly, or in other image analysis software platforms. We demonstrate this by importing the Worm-align output into Worm_CP, a pipeline that uses the open-source CellProfiler software. CellProfiler's flexibility enables the incorporation of additional modules for high-content screening. We have used the Worm_CP pipeline to quantify the heat shock response, a well conserved protective mechanism that refolds proteins that are misfolded due to stressors such as high temperature[8]. Specifically, we applied the pipeline to worms carrying an integrated multi-copy transgene, where the promoter of a heat shock inducible gene, *hsp-70(C12C8.1),* drives green fluorescent protein (GFP)[9]. We have also used the Worm_CP pipeline on fixed animals that have been labelled with a fluorescent dye that visualizes lipid

droplets (LDs), the main fat storage organelle in *C. elegans*[10]. While this workflow does not have the throughput offered by WormToolBox, it is a user-friendly and simple alternative for visual presentation and analysis of image-based *C. elegans* experiments.

PROTOCOL:

1. Fixation of worms for fat-content imaging using a fluorescent dye for lipid droplets (BODIPY)[10]

1.1. Prepare a synchronized population of *C. elegans* by bleaching according to standard procedures[2]. Plate about 1,000 L1-stage worms onto a 9 cm nematode growth media (NGM) plate per condition.

NOTE: More worms are prepared than actually quantified at the end, due to loss of worms when handling.

1.2. To image animals at young adult stage (around 50 h post L1 plating at 20 °C), wash each 9 cm plate with 15 mL of M9 in a conical tube, centrifuge at 252 x *g* for 1 min, and remove the supernatant.

1.3. Repeat the wash and leave 1 mL of M9 over the pellet of worms.

1.4. Transfer 1 mL of M9 containing the worms in a 2 mL low protein binding microcentrifuge tube, using low retention tips.

1.5. Spin down at 252 x *g* in a microcentrifuge for 1 min and remove the supernatant, being careful not to touch the worm pellet at the bottom of the tube.

1.6. For monitoring fat content using 4,4-difluoro-4-bora-3a,4a-diaza-s-indacene (BODIPY) staining[10], fix worms either by adding 0.5 mL of 60% isopropanol to the worm pellet for 3 min[11], or by adding 2 mL of ice-cold methanol for 10 min. For both procedures, invert the tube every 30 s.

CAUTION: If methanol is the chosen fixation reagent, this step must be undertaken in a fume hood due to its toxicity.

1.7. Remove as much fixative as possible without touching the worm pellet and add 1 mL of M9. Let the worms settle at the bottom of the tube for 3−5 min.

1.8. Wash again with 1 mL of M9, let the worms settle and remove the supernatant, leaving about 50 µL in the tube. Secure the tube using a clamp.

1.9. Freeze-crack the worms by plunging the securely closed tube in liquid nitrogen and then into a warm water bath at ~40 °C.

1.10. Repeat step 1.9 one more time.

1.11. Add 0.5 mL of BODIPY diluted in M9 at a final concentration of 1 µg/µL and place on a rotator at room temperature for 1 h.

1.12. After 1 h, let the worms settle at the bottom of the tube for 3−5 min.

1.13. Remove supernatant and add 1 mL of M9.

1.14. Let the worms settle at the bottom and remove supernatant.

NOTE: Alternatively, it is possible to spin down at 252 x *g* for 1 min, as this does not seem to affect the morphology.

1.15. Add 0.5 mL of M9.

NOTE: If the fixative is 60% isopropanol, the worms can only be utilized within 48 h. If the fixative is methanol, the worms should be utilized within 24h.

2. Preparing agarose pads to mount the worms

NOTE: The critical step while preparing an agarose pad is to obtain a pad of regular thickness. Otherwise, worms across the pad will be in different focal planes, making it tricky to get a focused image of a wider field of view.

2.1. Prepare 2% agarose pads by adding 0.2 g of agarose to 10 mL of sterilized H_2O in a beaker or a conical flask. Heat in the microwave for 30 s until the agarose starts boiling.

NOTE: Do not overheat and stop as soon as bubbles appear; otherwise it increases the viscosity of the agarose making it harder to achieve the desired pad thickness.

2.2. Once the agarose is melted, dispense a drop of melted agarose in the center of a microscope glass slide using a 1000 µL pipette tip with its very end cut. Immediately, but delicately, hold another glass slide very close to the agarose drop and release it onto the agarose to form a pad of regular thickness for best microscopy results.

NOTE: Releasing the top slide from a height will not only form bubbles but will result in an uneven pad. Alternatively, it is also possible to use two glass slides flanking the slide with the pad, with a thin layer of tape on each slide.

2.3. After a minimum of 2−3 min, gently remove the top slide. Using the side of the top slide, trim the pad to make it square shaped. Mount the worms within 5 min, to prevent the pad from drying before use.

3. Mounting fixed worms for imaging

3.1. Create a mouth micropipette by extending a thin glass capillary in the flame of a Bunsen burner (**Figure 1A**). After extension in the flame, break the capillary into two pieces (**Figure 1B**). Choose the most adequate, usually the longest, and test whether the end of the capillary is open by trying to aspirate water.

NOTE: If the liquid does not come into the capillary, it is too thin or blocked. Gently cut the end of the capillary with a pair of scissors, avoiding cutting too much as worms will be aspirated if the hole is too big.

3.2. Plug the glass capillary from step 3.1 into the capillary adaptor (**Figure 1C**), linked to the 6 mm silicone tube and plug the other end of the 6 mm silicone tube into a 0.2 µm filter, attached to a 3 mm silicone tube on its other end (**Figure 1C**). Plug a 1 mL filter tip (**Figure 1C**) into the free end of the 3 mm silicone tube to aspirate liquid.

NOTE: The 0.2 µm filter is added between the mouth of the experimenter and the capillary, to ensure maximum safety of the experimenter. A similar solution is used for mouth micropipettes used to handle mouse embryos[12]. Change the filter (usually every few months) if the mouth micropipette is not aspirating liquid anymore.

3.3. Using the mouth micropipette under a dissection microscope, remove as much liquid as possible around the worm pellet of fixed worms (**Figure 2**).

NOTE: Pipetting supernatant out using a manual micropipette can be used as an alternative to the mouth pipettes in steps 3.1 to 3.3 to remove as much supernatant as possible. Add 6 µL of mounting medium. We find that this method does not work as efficiently because excessive liquid in the pads creates a sparse worm density, which makes imaging more time-consuming. Resume step 3.6. Look at the bottom of the tube and make sure that the pipette does not aspirate the worms.

3.4. Quickly add 10 µL of mounting medium (**Table of Materials**) to the bottom of the tube.

3.5. Rinse a 10 µL tip in PBS with traces of detergent (0.01% Triton), to prevent worms from sticking to the sides of the plastic pipette tip. Cut the very end of the tip with a pair of scissors.

3.6. Transfer 8 µL of mounting medium containing the worms onto the agarose pad prepared in step 2.3. Under the microscope, gently shake the slide, to avoid overlap of the worms.

3.7. Cover the drop of mounting medium on the agarose pad with an 18 mm x 18 mm coverslip (**Figure 3**).

NOTE: Smaller coverslips are preferred as they exert less pressure onto the worms. Hold the coverslip with a pair of tweezers and apply one edge of the coverslip against the agarose pad, before gently depositing the coverslip with the tweezers.

4. Mounting live worms for imaging

NOTE: To image live worms, they have to be immobilized on the pad. One way to achieve this is to paralyze them with the nicotinic receptor agonist: levamisole.

4.1. Pipette 3–4 µL of 3 mM levamisole dissolved in M9 onto the agarose pad created in step 2.3.

NOTE: There should be enough liquid volume that the worms are not on top of each other but not too much liquid that the worms are too sparse on the pad. Experienced worm pickers can use 3 µL of levamisole. Less experienced pickers might need to add a larger volume of levamisole, so that the levamisole does not totally evaporate.

4.2. Pick 30–50 worms per condition into the drop of levamisole.

4.3. Cover the drop of levamisole on the agarose pad with an 18 mm x 18 mm coverslip. Image worms within 1 h, as the paralyzing agent will eventually lead to death of the worms.

5. Imaging slides with an epifluorescence microscope

5.1. With an agarose pad of uniform thickness, image all worms on the slide at once using a 6 x 6 or 7 x 7 large image for instance with the 20x objective of a fluorescent microscope. If the thickness of the pad is not even, acquire several smaller 3 x 3 or 4 x 4 images of the same slide.

5.2. Use the same settings for fluorescence intensity and exposure time for all conditions. Adjust each setting to match the slide that has the brightest intensity, ensuring that there is no pixel saturation. For specific instructions on image acquisition, follow the microscope manufacturer's instructions.

6. Creating montage images of aligned single worms using the Worm-align FIJI pipeline

6.1. Install the open-source image analysis software package FIJI[13]/ImageJ[14] from https://imagej.net/Fiji. If a prior installation of FIJI is available, ensure it is updated to version 1.52a or later, as some of the functions used in the macro (e.g., Table functions) require this. Download the Worm-align macro from Github: https://github.com/hannekeo/Worm-align.

6.2. Open FIJI and run the Worm-align macro. Execute Worm-align by clicking **Plugins** | **Macros** | **Run** in the FIJI main menu bar (**Figure S1**). Now locate the **Worm-align.ijm** script on the computer.

6.3. The macro initializes by asking for the location of the images. The pipeline will work on both single-channel and multi-channel fluorescence images (**Figure S2**). Select an input folder and the macro will automatically generate an output folder where all results will be saved (**Figure S3**).

NOTE: It is important that the selected folder contains only image files as the presence of other file formats will cause the macro to crash. Ideally, only group together images that were captured with the same microscope settings. Worm-align will accept many different file formats, including nd2, czi, tif, rgb, jpg and png. The name of the output folder will be the same as the input folder, with '_output' added as a postfix, i.e. if the input folder is 'images', the output will be found in 'images_output'. The output folder will contain four subfolders, in which the data will be saved.

6.4. Allow the macro to proceed to open the first image in the input folder and use it as a representative image to extract a setting to generate the montage.

NOTE: Worm-align will automatically select the first image in the input folder to generate the settings that will be applied to all other images in the folder. If another image is deemed to be more representative of the dataset, ensure this image is selected by saving a copy of the image in the input folder, changing the name so that is now listed at the top of the list (e.g., '0_RepresentativeImage').

6.5. With the **Straight-line** drawing tool, draw a line across the width of a worm (**Figure S4**) and use the length of this line to determine the height of the cropped regions for single worms. For each channel, specify the name, lookup table (LUT), intensity settings (B&C) and whether it should be included in the montage (**Figure S4**).

NOTE: These parameters are recorded and saved in a settings file, **Settings.csv**, located in the **CellProfiler** subfolder of the output folder.

6.6. Once all settings have been captured, the user is shown what the images will look like after application of the settings applied (**Figure S5**). Tick the top box if the settings are sufficient. Select options for montage generation (remove ticks, if not required): 1. Generate a montage of selected worms for each single image, or 2. Generate a combined montage in which all worms selected on all images in the input folder will be combined into a single montage. Click **OK** to execute the rest of the macro.

NOTE: If the top box is not ticked before clicking 'OK' the macro will rerun the setup, so image settings can be optimised.

6.7. The Worm-align pipeline now proceeds to open all images in the input folder. For each image, draw lines on the longitudinal axis of all worms to be included in the montage and/or quantify (**Figure 4** and **Figure S6**) using the 'segmented line' tool (on the FIJI main menu bar). To ensure proper alignment of worm, draw lines consistently from head to tail end (or vice versa), and along the full length of the worm (see examples of "good" and "bad" line drawing in **Figure 4B**).

6.8. Add each line to the **ROI manager**, by clicking Ctrl+T. Worm-align uses the lines added to the ROI manager, in combination with the worm width parameter (set in step 6.4) to generate cropped images of single selected worms. The collection of line ROIs is saved in the 'data' subfolder. This folder also contains a copy of the original image showing the lines, as well as the number of the worm. Lines are color-coded with the Glasbey_on_dark lookup table. The images of straightened worms are saved in the **single_worms** subfolder of the output folder. In addition, if selected in step 6.6 of this protocol, Worm-align produces montages of all worms selected per overview image and/or for all overview images in the input folder (see **Figure 4C** and **Figure S7**). These montages can be found in the 'aligned' subfolder of the output folder.

7. Analyzing single-worm fluorescence intensity using the output from Worm-align in an automated CellProfiler pipeline

7.1. For downstream data processing and analysis of the Worm-align output, download and install the open-source image analysis software package 'CellProfiler[15,16] from https://cellprofiler.org/. Download the **Worm_CP.cpproj** pipeline from GitHub: https://github.com/hannekeo/Worm-align

NOTE: The example pipeline described here was constructed using CellProfiler2.2.0.

7.2. Before starting the **Worms_CP.cpproj** pipeline, ensure that all expected output images are present in the CellProfiler subfolder of the Worm-align output folder: a processed copy of the original image (named: Original_), a binary image mask of the worm population (named: Mask_), a line mask of the lines drawn on selected worms (named: Lines_), and one image representing each of the channels present in the original image (named by channel number).

7.3. To quantify the fluorescence intensity in single worms, click on the **Images input** module and simply drag the **CellProfiler** output folder into the window that says **Drop files and folders here**. If a list of images is present from previous analysis, first remove these by right clicking in the window and selecting **Clear File List**. To exclude the 'Settings.csv' file from the images list, select either 'Images only' or 'Custom' with 'Extension is tif, tiff, ome.tif, or ome.tiff' for the filter settings (**Figure S8**).

7.4. Click on the Metadata input module. In the second extraction method, click on the yellow folder, and locate the **CellProfiler** subfolder in the **WormAlign** output folder (**Figure S9**). Select the **Settings.csv** file and link the settings in the file to the CellProfiler output by matching the metadata in the CSV files to those in the images (Channel metadata).

7.5. Click on the **NamesAndTypes** input module and insert a new image for each channel of the original image to be quantified.

NOTE: Already present in the example pipeline are the worm mask, two color images, the line mask and the original image, and three grey scale images (the green and the red channel). The

list in this module needs to be adjusted if the original images have more or fewer channels than the example images.

7.6. Check that the name of the images in every column label/mask/worms is correct.

NOTE: The names of the images on one line should all correspond to the same initial image to analyze. If a mistake is made during the image analysis process, some of the output images will be missing and the names under the three columns label/mask/worms will not correspond to the same initial image anymore for each line. If this is the case, find where the mistake is.

7.7. Press **View output setting** to select the folder to save the output from Cellprofiler.

7.8. Click **Start Test Mode**. Once in Test Mode, double-check the settings of the pipeline by applying them to the first image in the dataset, by clicking **Run** (which will run through all active modules in the pipeline), or **Step** (which will run through the pipeline one module at a time).

NOTE: While in the Test Mode, the extracted measurements will not be exported, even if an **ExportToSpreadsheet** module is present (and active) in the pipeline.

7.9. Once the pipeline performs the way it should, click **Exit Test Mode** and then press **Analyze Images**. As currently configured, the Worms_CP will (1) use the *Mask_* image to segment a rough worm mask and the *Lines_* image (**Figure S10A**) to single out the selected worms and produce single worm masks (**Figure S10C**), (2) measure the fluorescence intensity of all channels present in the input images in those worms identified and masked. The pipeline can also measure background intensity (**Figure S10B**) and correct all images accordingly, (3) measure the size of the selected worms, and (4) export all measured parameters (**Figure S10D**).

7.9 Open the Worm_CP output consisting of two csv files, **worms.csv** and **lines.csv** that are saved in the selected output folder (see **Figure S11**). These files can be opened in Excel or R (Studio) for post-processing and reporting. If additional output is required, e.g. per image data, this can be selected in the **ExportToSpreadsheet** module in the pipeline.

REPRESENTATIVE RESULTS:

Culturing and imaging *C. elegans* according to the method described in this protocol produces large overview images of worm populations. To facilitate visual inspection and classification of worms from these images we have developed Worm-align. Worm-align is a simple and user-friendly FIJI script that can be used to create montages of straightened and aligned worms. Worms are selected from overview images by drawing a line along the longitudinal axis of the worms. Each selected worm is assigned a number, cropped and straightened, and added to a montage. Montages can be generated per image or combining all images from the original input folder.

As expected, the output of the pipeline largely depends on the quality of the lines drawn on top of the images. To illustrate this point, **Figure 4** shows several line examples, and their output from

Worm-align. A complete line from the head to the tip of the tail produces a properly aligned worm (labelled "good"). **Figure 4** also shows how tracing inaccuracies during the execution of Worm-align affect the output of the alignment. From the annotated montage included in **Figure 4C**, care should be taken to avoid the following errors, as they hinder the proper alignment of worms:

- Inconsistent tracing of the worm from head to tail (labelled "tail to head"). This results in worms being orientated in different directions (i.e., tail-to-head versus head-to-tail) in the alignment.
- Incomplete tracing (labeled "incomplete"). This results in only the part of the worm that was traced to be cropped for the montage.
- Including multiple worms in a single trace (labeled "worm with two heads"). This results in worms plus (significant sections of) their neighbors being inserted in the same panel of the montage.
- Adding random lines to the image (labeled "random"). This results in the insertion of random panels in the montage.

For creation of the montage it is not an issue if two individual lines intersect (labeled "intersecting") or are joined at either end of the worm (labeled "joined"), as long as each line traces the entire length of an individual worm: The Worm-align script individually and sequentially selects each line ROIs, crops and straightens it, so that each panel in the final montage will represent a single line trace. In case of the intersecting worms however, although full length straightened worms appear for worm "intersecting1" and worm "intersecting2" in the montage (see **Figure 5A-B**), it is clearly noticeable that these worms cross each other in the original overview image. Therefore, it can be concluded that visual identification of intersecting lines is possible from the overlay image found in the 'data' subfolder (**Figure 5A**), as well as from the panels of individual worms in the montage (**Figure 5B**). In addition, any overlap of worms/lines can be identified from the quality control (QC) table generated for each processed image by Worm-align, and saved in the data subfolder. This table records three parameters for each of the line ROIs drawn in the image (see **Figure 5C**): Of these, *Length* indicates the length of the line ROI, which is a good indicator of the size of the worm; and the *Worm number* indicates the order in which the lines were drawn on the overview image. Finally, the last column indicates whether there is overlap between the worm/line in question and any of the other worms/lines selected in the image. In case of overlap the number in this column will be different from the one listed in the *Worm number* column. In combination with the overlay image, the QC table should aid the researcher to make learned decisions on which worms should be excluded from the montage and/or quantification.

The output of Worm-align can be used for subsequent quantification of fluorescence intensity in single worms. In FIJI, the line ROIs can be used to measure fluorescence intensity in the original image data, for example by executing the simple FIJI script ' Worm-quant.ijm', which can also be found in the Worm-align repository on Github. Alternatively, the Worm-align output can be imported into third party image analysis software. We demonstrate this by importing the Worm-align output of two datasets into Worm_CP, a pipeline we generated in CellProfiler. Worm_CP uses the files in the 'CellProfiler' subfolder of the Worm-align output folder to fine-tune

segmentation masks of those individual worms selected during execution of the Worm-align macro. Specifically, it uses the line mask (named: Lines_) to isolate selected worms from the worm population seen in the binary mask (named: Mask_). It should be noted that lines that intersect on the overview image (**Figure 5A**), although not a problem for the generation of montages, are problematic for the Worm_CP pipeline. Why this is the case, is illustrated in **Figure 5 D-E**. Worm_CP uses the line mask (**Figure 5D**), and not individual ROIs to aid identification of individual worms. The intersecting line drawn second during the execution of Worm-align is superimposed on the first line and therefore the intensity along this line will be that of ROI2 including in the bit where line 1 and 2 overlap. As a result, CellProfiler will segment line2 as one object, but line1 as two objects that are separated where it intersects with line2. This means that CellProfiler will produce two (half) worm masks for worm 'intersecting1' (**Figure 5E**). The easiest way to exclude these events from the final analysis (if required), is to identify the worm number of intersecting worms from the QC table (data subfolder), and to remove measurements for these worms from the CellProfiler output files. Please note that worms will not necessarily be allocated the same number in FIJI and CellProfiler: To identify the FIJI worm number in the CellProfiler output look at the Intensity_Max_Intensity values in the 'Lines.csv' output file. Any Intensity_Max_Intensity value that appears in the 'Lines.csv' table more than once per image is an indication of that line ROI resulting in a fractured worm mask.

Once individual worm masks are segmented, Worm_CP can measure the fluorescence intensity in the selected worms for all recorded channels. All measurements are taken from the original (raw) image data, although it should be noted that CellProfiler automatically rescales the pixel intensity on a scale of 0-1. This is achieved by dividing the raw pixel intensity value by the maximum possible pixel intensity for the image. In case of 8-bit images this value is 255, and in case of 16-bit images it is 65535. CellProfiler intensity values therefore need to be multiplied by the maximum possible intensity value to regain values equivalent to the raw image data. The Worm_CP output consists of two csv files, 'worms.csv' and 'Lines.csv' that are saved in the selected output folder. While inspecting these files, it is clear that CellProfiler records a large number of parameters related to fluorescence intensity. Of these, the Intensity_IntegratedIntensity corresponds to the total fluorescence per worm (i.e., the sum of the fluorescence intensity within all pixels that construe the mask of an individual worm). The parameter Intensity_MeanIntensity refers to the average fluorescence intensity within an individual worm (i.e., the average fluorescence intensity per pixel for all the pixels contained within an individual worm). Due to the occasional occurrence of (small) errors in segmenting the worm masks it is recommended that MeanIntensity measurements are used when comparing fluorescence measurements of individual worms between two conditions.

We have used the Worm_CP pipeline to quantify fluorescence intensity from fixed animals that have been labelled with a fluorescent dye that incorporates into LDs (**Figure 6**). In order to validate fluorescence quantification from the Worm-align/Worm_CP pipeline, we quantified fluorescence intensity in the same set of worms from the BODIPY dataset by either the Worm-align/Worm_CP pipeline or manual quantification in FIJI/ImageJ. Manual quantification was performed in FIJI/ImageJ by circling each worm as well as a dark background zone in every image. The fluorescence intensity was measured in the ROI manager and the value measured for the

image background was subtracted from the worm fluorescence measurement of each worm, as described[17]. We compared wild type (WT) N2 worms to *dbl-1(nk3)* mutants, which exhibit decreased lipid droplet content[18]. As expected, the green fluorescence intensity is significantly decreased between WT and *dbl-1(nk3)* worms with both methods (**Figure 6A,B**). Examples of aligned worms can be observed in **Figure 6C,D**. The lipid droplet content is decreased by 17% (p-value<0.0001, unpaired t-test) between WT and *dbl-1(nk3)* using manual quantification, and by 12% (p-value=0.0082 unpaired t-test) using the Worm_CP pipeline. The decrease in lipid droplet content observed here in *dbl-1(nk3)* with both quantification methods is in accordance with the literature[18]. This shows that quantification of acquired fluorescence images with the Worm_CP CellProfiler pipeline is comparable to manual quantification.

We have also used Worm_CP to quantify the heat shock response in live worms expressing GFP under control of the heat shock inducible gene *hsp-70(C12C8.1)*[9]. **Figure 7** shows representative images of live *C. elegans* carrying the heat-responsive *hsp-70(C12C8.1)p::*GFP reporter. In the absence of heat stress, the worms do not induce GFP expression (**Figure 7A,B**). However, when worms are exposed to a short heat-shock of 30 min at 34 °C, they induce GFP expression (**Figure 7C,D)**. GFP expression levels with and without heat-shock are quantified in **Figure 7E**.

As it stands, Worm_CP is a very basic pipeline. However, this approach does enable a more accurate segmentation of individual worm masks, which allows for a more accurate quantification of the fluorescence intensity in those worms selected from the image. For this reason, we prefer this approach over a rough quantification in FIJI, using just the line masks. In addition, CellProfiler offers the advantage that additional analysis modules can easily be included in the pipeline. For example, for the dataset that looks at lipid droplet content, insertion of additional modules into the Worm_CP pipeline could investigate lipid droplet numbers and fluorescence intensity of individual droplets in those worms selected in the overview images.

FIGURE AND TABLE LEGENDS:

Figure 1: Generating a mouth micropipette. A glass capillary is extended in the flame of a Bunsen burner (**A**), until it provides thin elongated extremities (**B**). The extended glass capillary is then plugged into the adaptor piece of the mouth micropipette. (**C**) Schematic of a mouth micropipette. The mouth micropipette was assembled with a glass capillary plugged into an adaptor. A 6 mm silicone tube connects the adaptor to a 0.2 µm syringe filter, used for safety. The other end of the filter is attached to a 3 mm silicone tube ending with a 1mL filter tip. The experimenter can aspirate via the filter tip.

Figure 2: Aspiration of the liquid surrounding the worm pellet, using the mouth micropipette.

Figure 3: Adding the coverslip onto the worms laying on the agarose pad

Figure 4: Examples of worm straightening using Worm-align on fluorescence images acquired from live animals carrying the transcriptional reporter *fat-7p::*GFP in the intestine and a red co-injection marker in the pharynx (*myo-2p::tdtomato*). (**A**) Screenshot of a composite image acquired on the fluorescent microscope of live worms at day 3 of adulthood. The image was

opened with Worm_align and lines were drawn along the longitudinal axis of the worms using Worm-align. (**B**) Screenshot of the same image as in (**A**), with examples commented of good and bad drawing of the lines along the axis of the worms, using Worm-align. (**C**) Examples of lines drawn on top of worms that can rise to incorrectly aligned worms. Worm-align output of the worms selected in B. Images were taken with a 20x objective on an inverted widefield microscope (see Materials table) with green fluorescence intensity=1, exposure=60 ms and red fluorescence intensity = 8, exposure=60 ms.

Figure 5: Examples of worm straightening using Worm-align on intersecting worms. (**A**) Screenshot of a composite image acquired on the fluorescent microscope of live worms at day 3 of adulthood animals carrying the transcriptional reporter *fat-7p::*GFP in the intestine and a red co-injection marker in the pharynx (*myo-2p::tdtomato*). Intersecting worms are labelled "intersecting1" and "intersecting2", while non overlapping worms are labelled "good3" and "good4". (**B**) Montage created by Worm-align representing straightened worms selected in (**A**). It is noticeable in the montage that the worms 1 and 2 were intersecting on the original image (worms labelled as "intersecting1" and "intersecting2"). (**C**) Screenshot of the QC table from Worm-align which allows to spot cases where worms intersect. Length: length of the ROI line; worm number: order in which the lines were drawn; last column indicates whether there is an overlap between the worm/line of interest and any other worm. In this example, the worm in the first raw (worm number1) overlaps with worm number 2, as indicated by the number "2" in the last column. (**D**) Screenshot of the lines drawn with Worm-align on the four worms selected in A. (**E**) Screenshot of the masks generated by Worm-align on the four worms selected in A, showing how the masks for the two intersecting worms are rendered. In this case, two masks instead of one are now corresponding to the worm "intersecting1". Images were taken with a 20x objective on an inverted widefield microscope (see Materials table) with green fluorescence intensity=1, exposure=60ms and red fluorescence intensity = 8, exposure = 60 ms.

Figure 6: The Worm_CP pipeline quantifies fluorescence as accurately as would manual quantification. Comparison of fluorescence quantification of young adult worms fixed and stained for lipid droplet content with the green fluorescent dye BODIPY, using either Worm_CP pipeline (**A**) or manual quantification (**B**). The lipid droplet content of WT and *dbl-1 (nk3)* was monitored by fixing and staining animals with BODIPY, that intercalates into fatty acids of lipid droplets (see protocol A). Young adult animals were fixed with 60% isopropanol and stained with BODIPY for 1h. The same set of animals were quantified either using the Worm_CP pipeline (see protocol G) or by manual quantification according to standard procedures[17]. (**A**) Quantification of fluorescence using Worm_CP pipeline. WT: n=22 animals, average fluorescence= 1.016 (A.U) ± 0.188 SD; *dbl-1(nk3*): n=25 animals, average fluorescence= 0.892 (A.U) ± 0.115 SD. Paired t-test. (B) Manual quantification of fluorescence. WT: n=22 animals, average fluorescence = 1.048 ± 0.153 SD; *dbl-1(nk3)*: n=25 animals, average fluorescence = 0.8632 ± 0.109. Paired t-test. (**C**, **D**) Representative example or Worm-Align output for WT (**C**) and *dbl-1(nk3)* (**D**) animals straightened with the Worm-align pipeline. Images were taken with a 20x objective on an inverted widefield microscope (see Materials table) with green fluorescence intensity=2, exposure=60ms.

Figure 7: Example of fluorescence quantification and alignment of live worms from fluorescence images of live worms carrying the transcriptional reporter *hsp-70(C12C8.1)p*::GFP following heat-shock. (**A**) Upon a short heat-shock (30 min at 34 °C), young adult worms were recovered at their cultivation temperature (25 °C) and mounted then imaged 3.5h post heat shock. About 30 worms were quantified following heat shock using the Worm-align pipeline. (**A-B**) Examples of aligned worms that have not been exposed to heat shock. (**C-D**) Examples of heat-shocked worms carrying *hsp-70(C12C8.1)p*::GFP using Worm-align. (**E**) shows the GFP Average intensity of WT young adult worms grown, without heat shock or upon exposure to heat shock (34 °C for 30 min). Images were taken with a 20x objective on an inverted widefield microscope (see Materials table) with green fluorescence intensity=1, exposure=60ms; no HS: n=12, HS: n=32.

Supplemental Figure 1: Location in FIJI where the Worm-align macro can be found, once installed.

Supplemental Figure 2: Selection of the folder containing all images taken with the same settings.

Supplemental Figure 3: Generation of 4 subfolders in the output folder in FIJI.

Supplemental Figure 4: Drawing of a line across the width of the worm and channel settings for the montage.

Supplemental Figure 5: Preview of the image with the applied settings.

Supplemental Figure 6: Drawing of a line along the longitudinal axis of the worms of interest for inclusion in the montage and/or quantification.

Supplemental Figure 7: Montage of the selected worms in the output folder, under "aligned" folder.

Supplemental Figure 8: Clearing previous images from previous analysis in Cell Profiler.

Supplemental Figure 9: Importing metadata into CellProfiler from the Worm-align output folder by selecting the Settings.csv subfolder.

Supplemental Figure 10: The CellProfiler pipeline Worm_CP.cpproj uses the *Lines_* images to single the selected worms (A), and uses the *Mask_* images (B) to create a rough mask of every worm of interest (C). Outlook of all the parameters measured by the pipeline Worm_CP.ccproj which are exported in a csv file (D).

Supplemental Figure 11: Selection of output files in the "ExportToSpreadsheet in the Worm_CP.cpproj pipeline.

DISCUSSION:

Worm-align is a FIJI-based image processing pipeline that readily generates montages of user-selected worms, in which worms are straightened and aligned to aid visual comparison, classification and representation. Although this feature is also offered by some existing tools, notably the WormToolbox module in CellProfiler[7], Worm-align requires comparatively little prior image analysis experience: Users need only to trace those worms they would like to select for the montage (and analysis). Although tracing the worms on the raw image data is an easy process -particularly when a touch-screen computer or tablet is available-, it is paramount, that lines are correctly drawn along the longitudinal axis of the worms. Incomplete lines, that follow only part of the worm, will result in partial worms in the montage (i.e., worms missing heads of tail ends) and partial segmentation masks during CellProfiler analysis. Also, if lines from two individual worms cross, the worms will not be correctly processed in the worm alignment montage as well as for fluorescence quantification. For quality control an overlay image of the line selections on the original image is saved in the data folder, along with a QC table. From these, problematic lines that will lead to incorrectly segmented worms can readily be identified and excluded from montage and/or subsequent analysis.

Although the direct input from the experimenter in the selection of worms perhaps seems a little time-consuming, it presents a clear advantage of the workflow over others in experiments where worms from different developmental stages are present in the same image: Worms can be selected during the "tracing step", by outlining only those worms that are in the right developmental stage. Alternatively, worms can be filtered using the output from Worm_CP based on either the length of the tracing line, or the area of the segmentation mask, both reliable indicators of the length/size of the worms. Arguably, machine-learning algorithms may struggle to recognize worms from different developmental stages, as their size and appearance in the DIC images is so different.

The output of *Worm-align* can be used for subsequent quantification of fluorescence intensity in single worms, either in FIJI directly, or in other image analysis software platforms. We demonstrated this by importing the Worm-align output into a simple CellProfiler pipeline (Worm_CP), which allows the quantification of multi-channel fluorescence intensity in those individual worms that were selected while running the Worm-align pipeline. We chose this approach because of the flexibility of the CellProfiler software: It is straightforward to incorporate additional modules into the pipeline to analyse additional features in individual worms (e.g. measuring the size of lipid droplets, or stress granules, nuclei, mitochondria). In addition, the single worm masks could potentially be used to train a new model for WormToolbox[7].

The main advantages of this method are that it is fast and requires a simple worm mounting set-up. This method is faster as it does not require spending time learning software operation nor running training sets through a machine algorithm[7]. Furthermore, this method works with either live or fixed worms simply mounted on regular agarose pads. There is no need to use complex microfluidic chambers, as developed in other methods[5,6].

ACKNOWLEDGMENTS:

We would like to thank Dr Christian Lanctôt at BIOCEV (Prague, Czech Republic) for teaching us the mouth micropipette technique to mount fixed worms and Dr Fatima Santos and Dr Debbie Drage for sharing safety setup on mouth micropipette. We also thank Francesca Hodge for editing the manuscript, Sharlene Murdoch and Babraham Institute Facilities for their support. OC is supported by ERC 638426 and BBSRC [BBS/E/B000C0426].

DISCLOSURES:

The authors have nothing to disclose.

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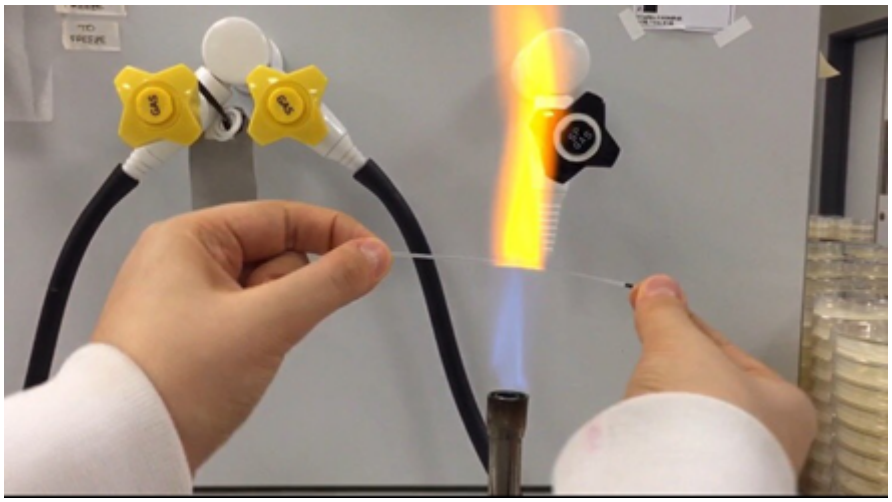

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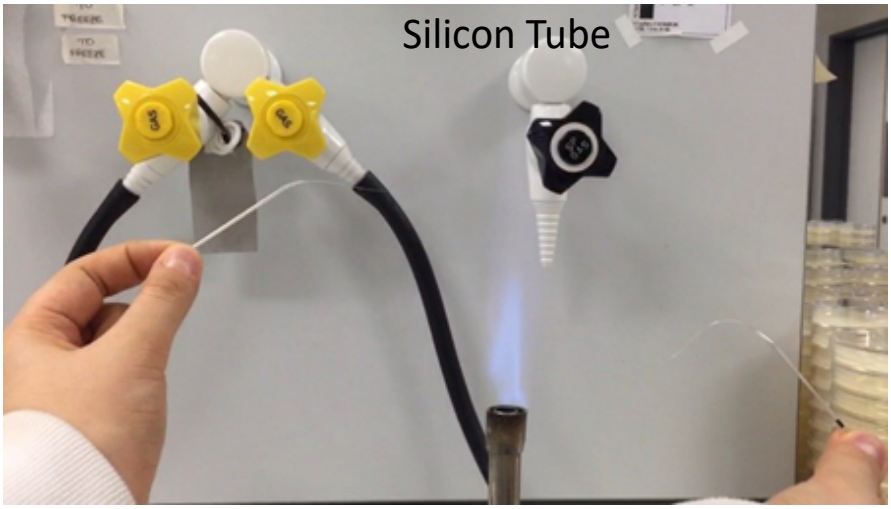

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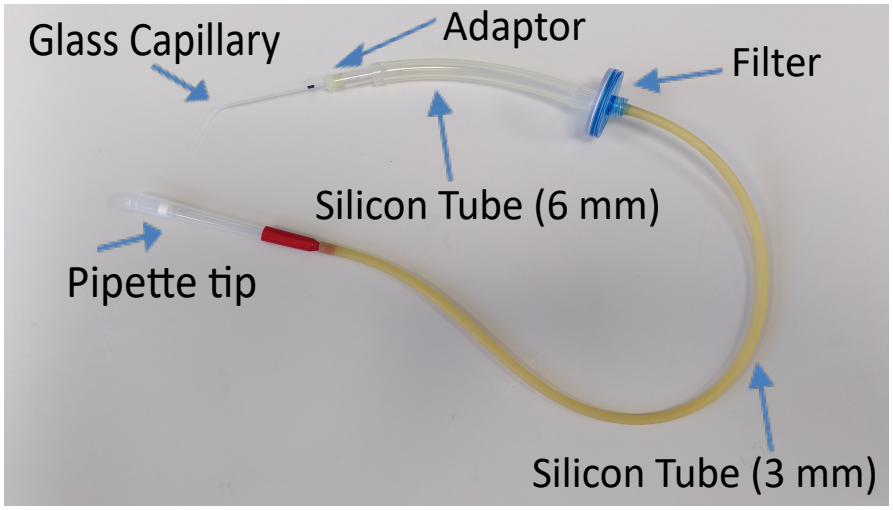

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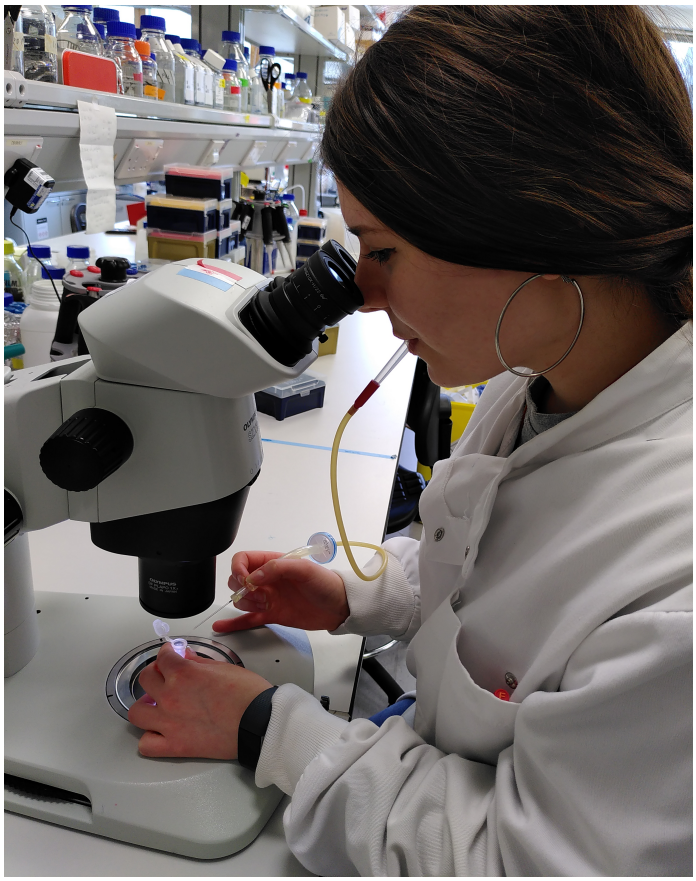

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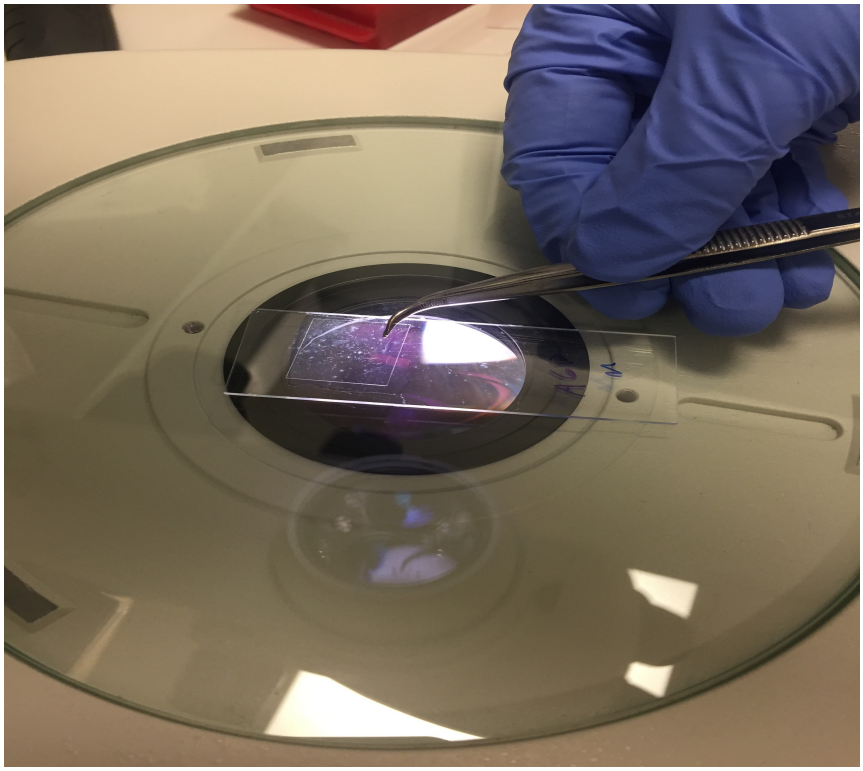

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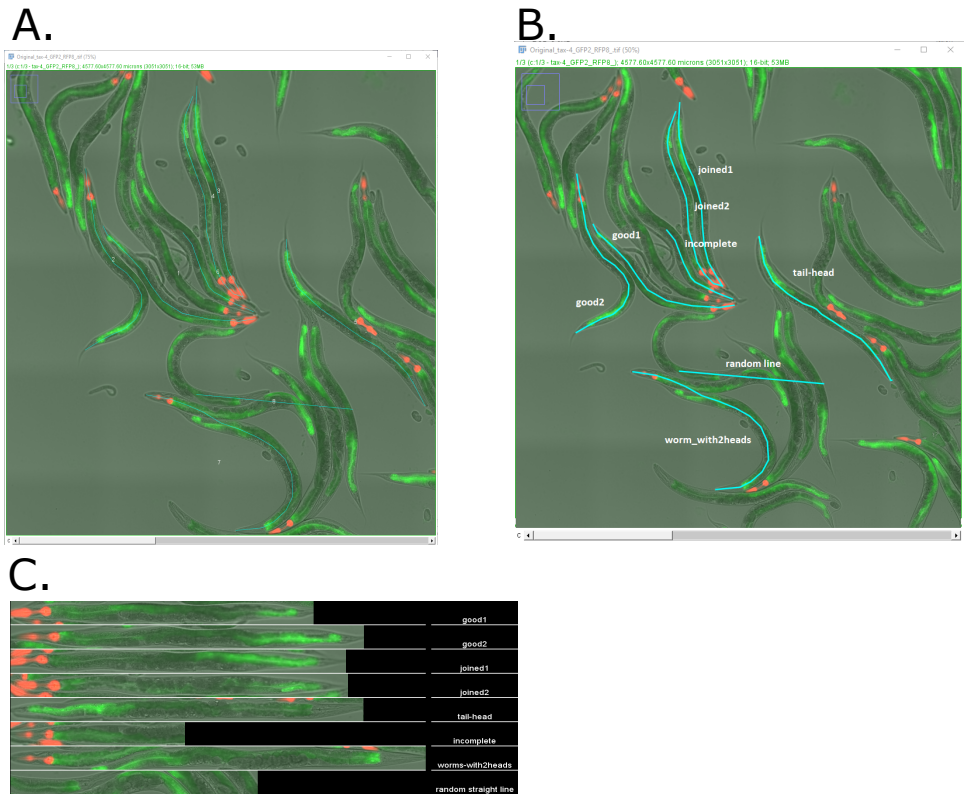

Figure 4.

Figure 5

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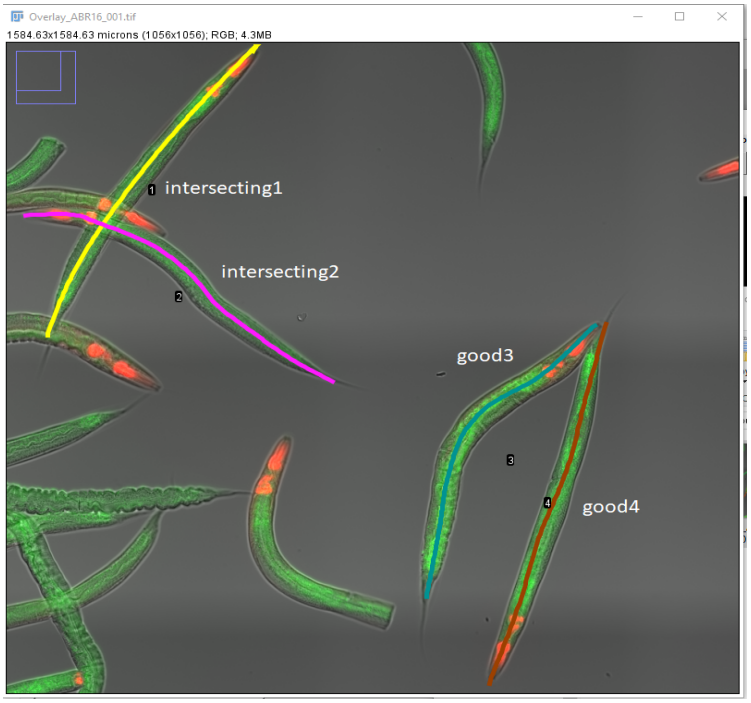

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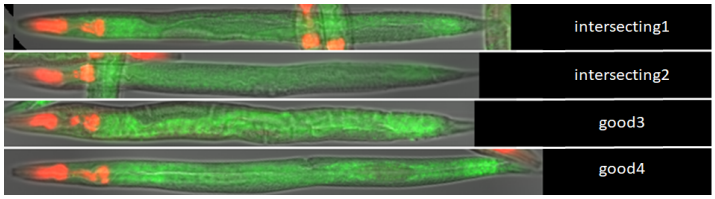

C.

Results

File Edit Font Results

	Label	Length	Worm number	-
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2	mask:0309-0186	440	2	2
3	mask:0521-0545	456	3	3
4	mask:0560-0585	481	4	4

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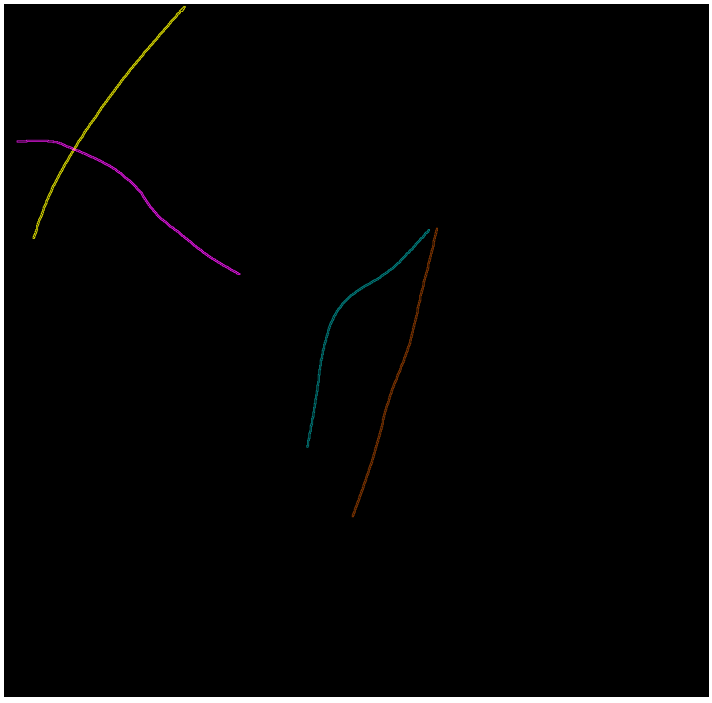

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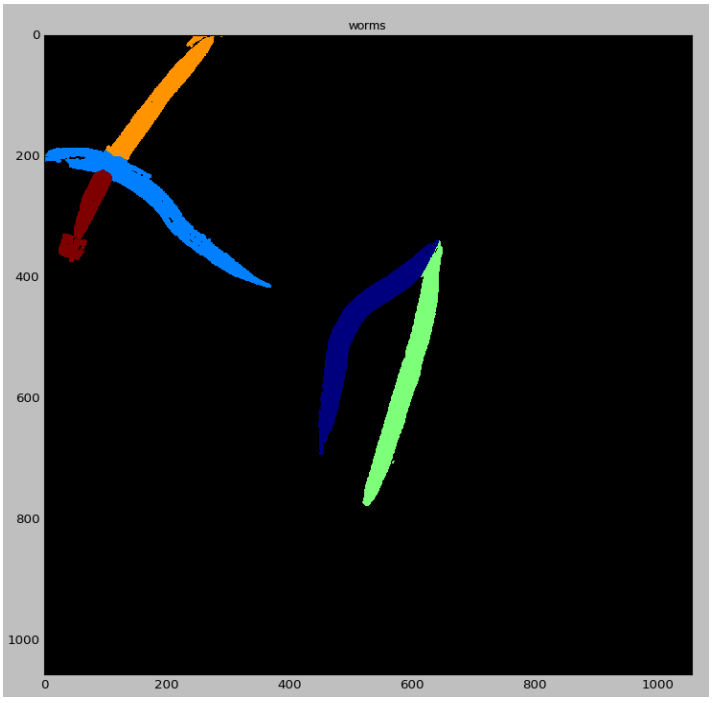
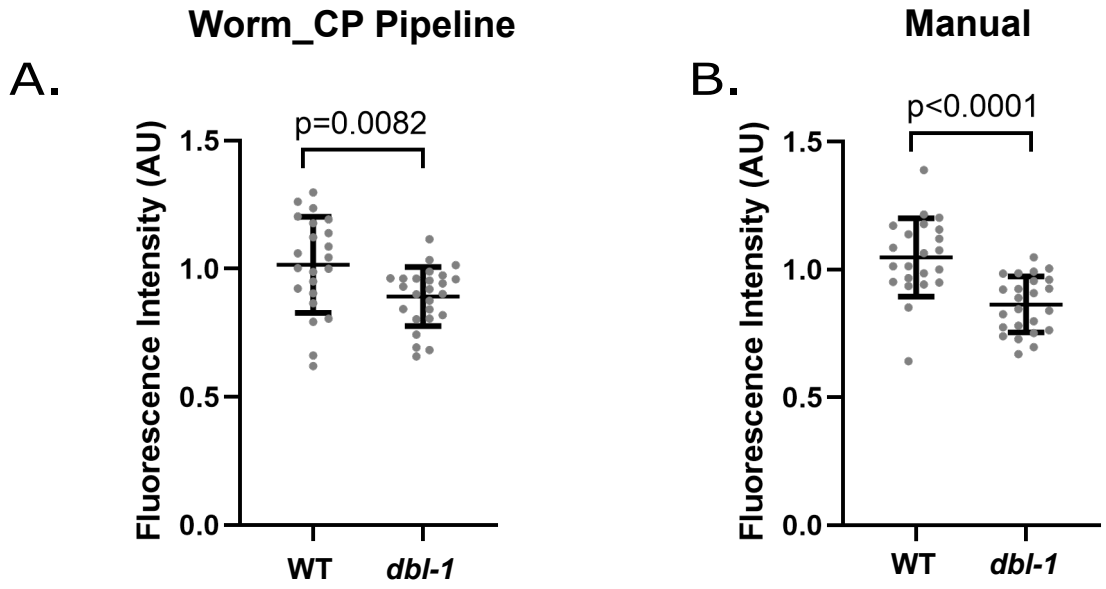
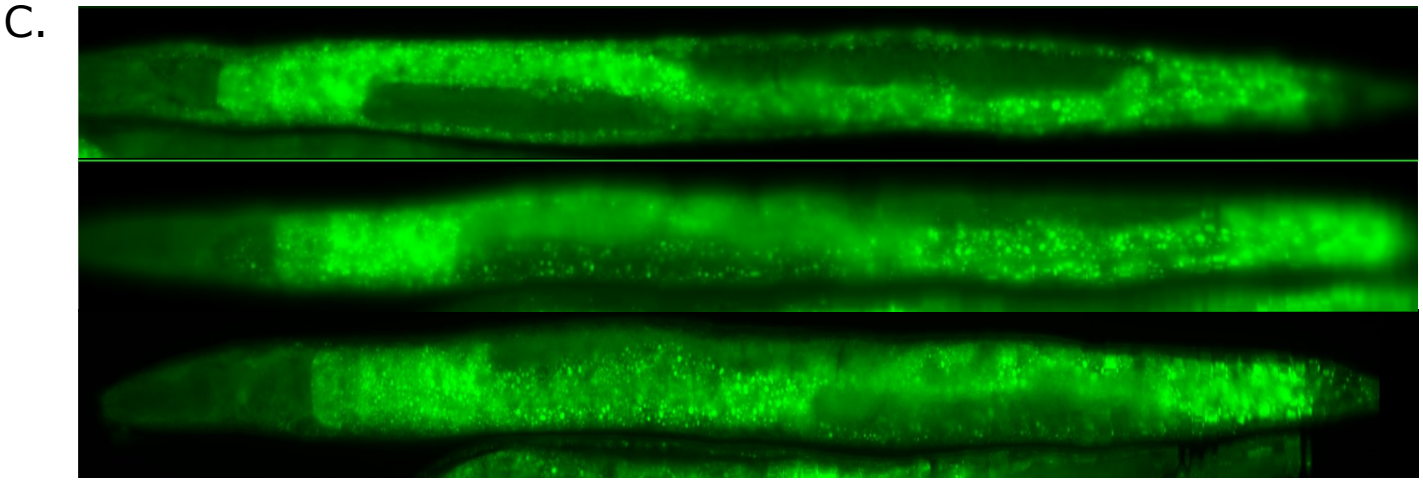


Figure 5.

Figure 6





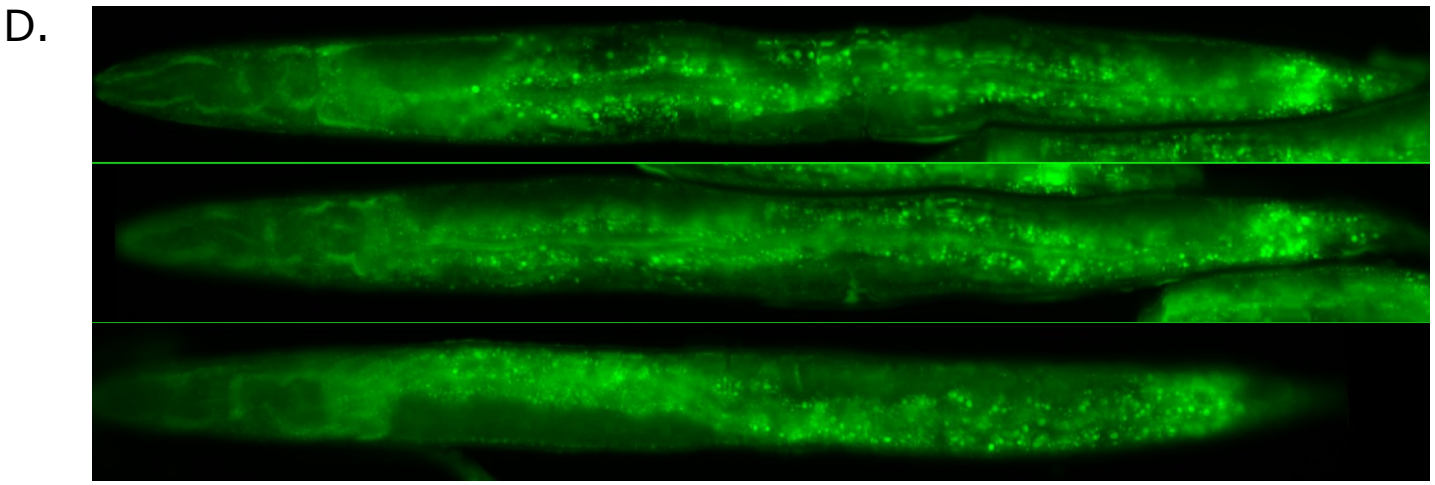


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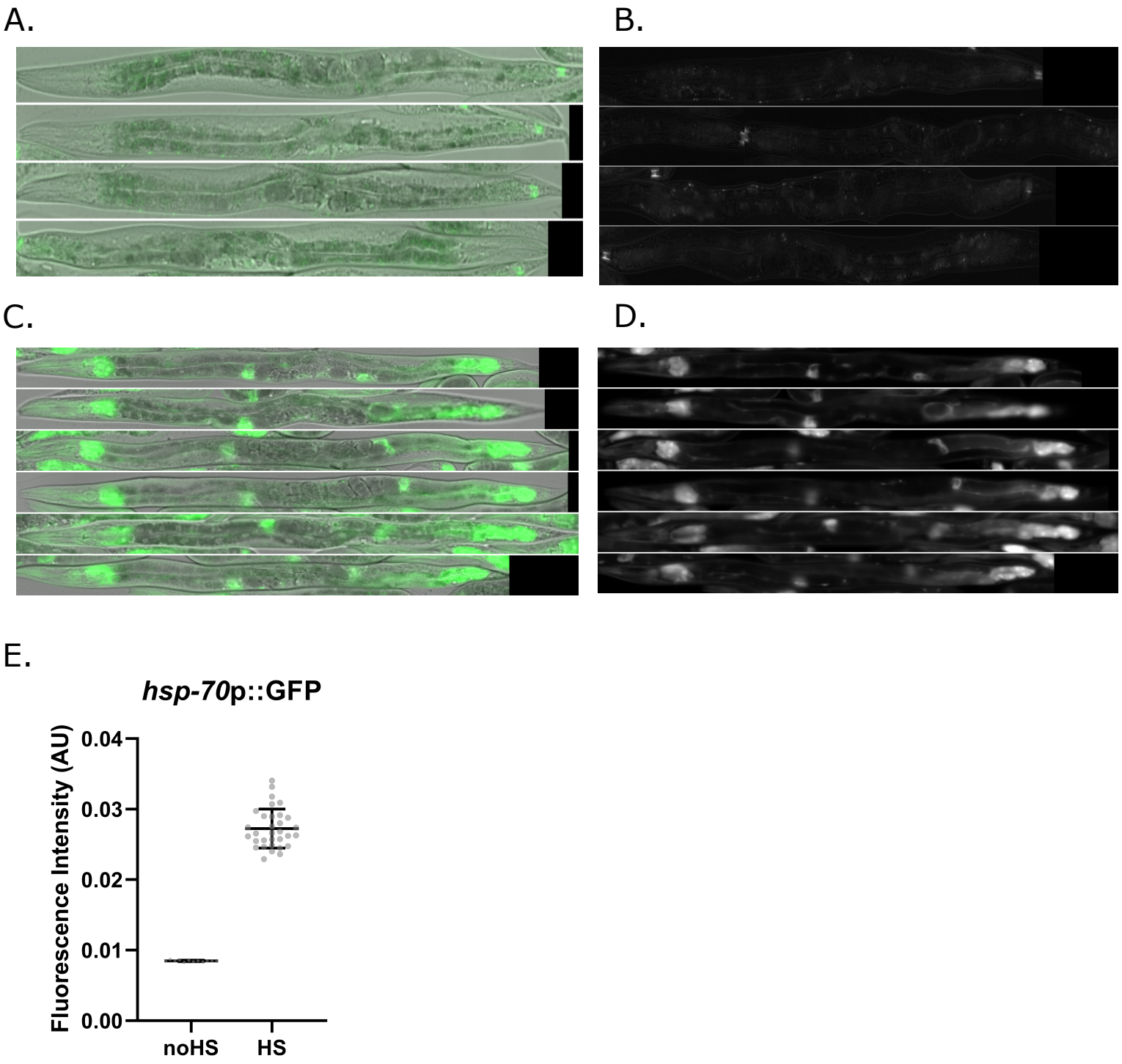
Figure 7



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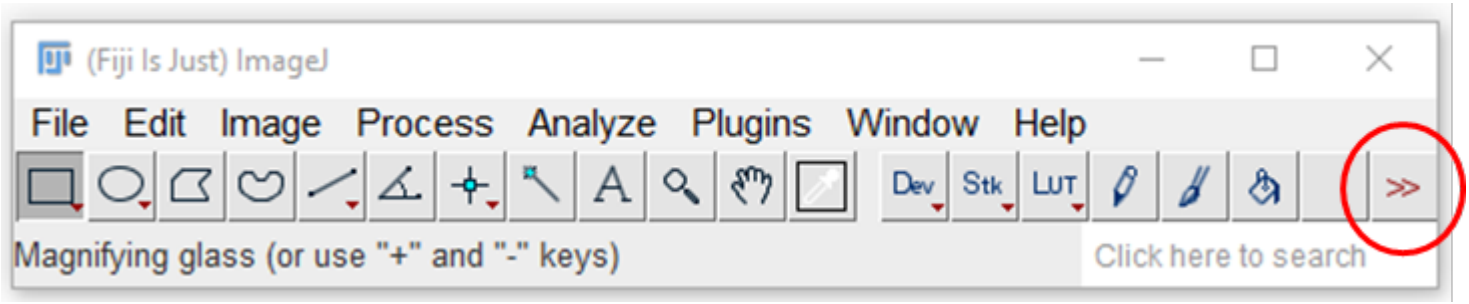

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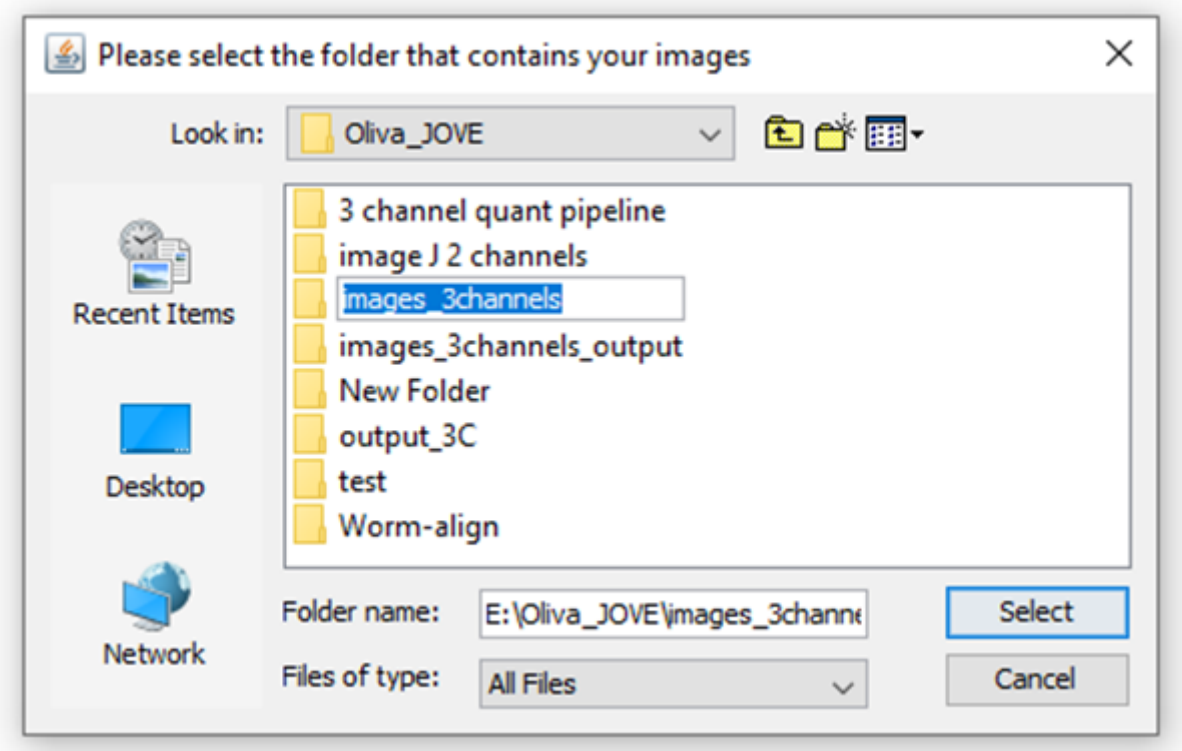

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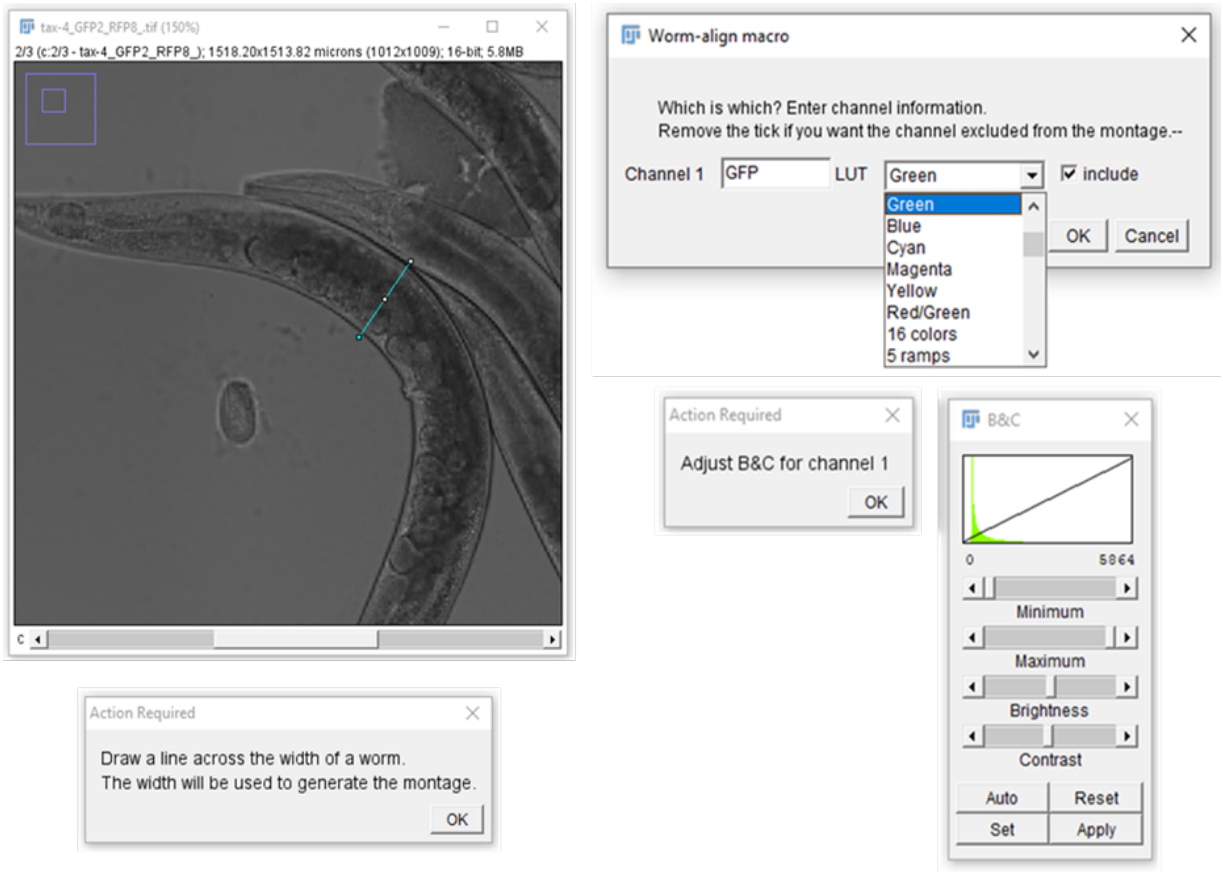

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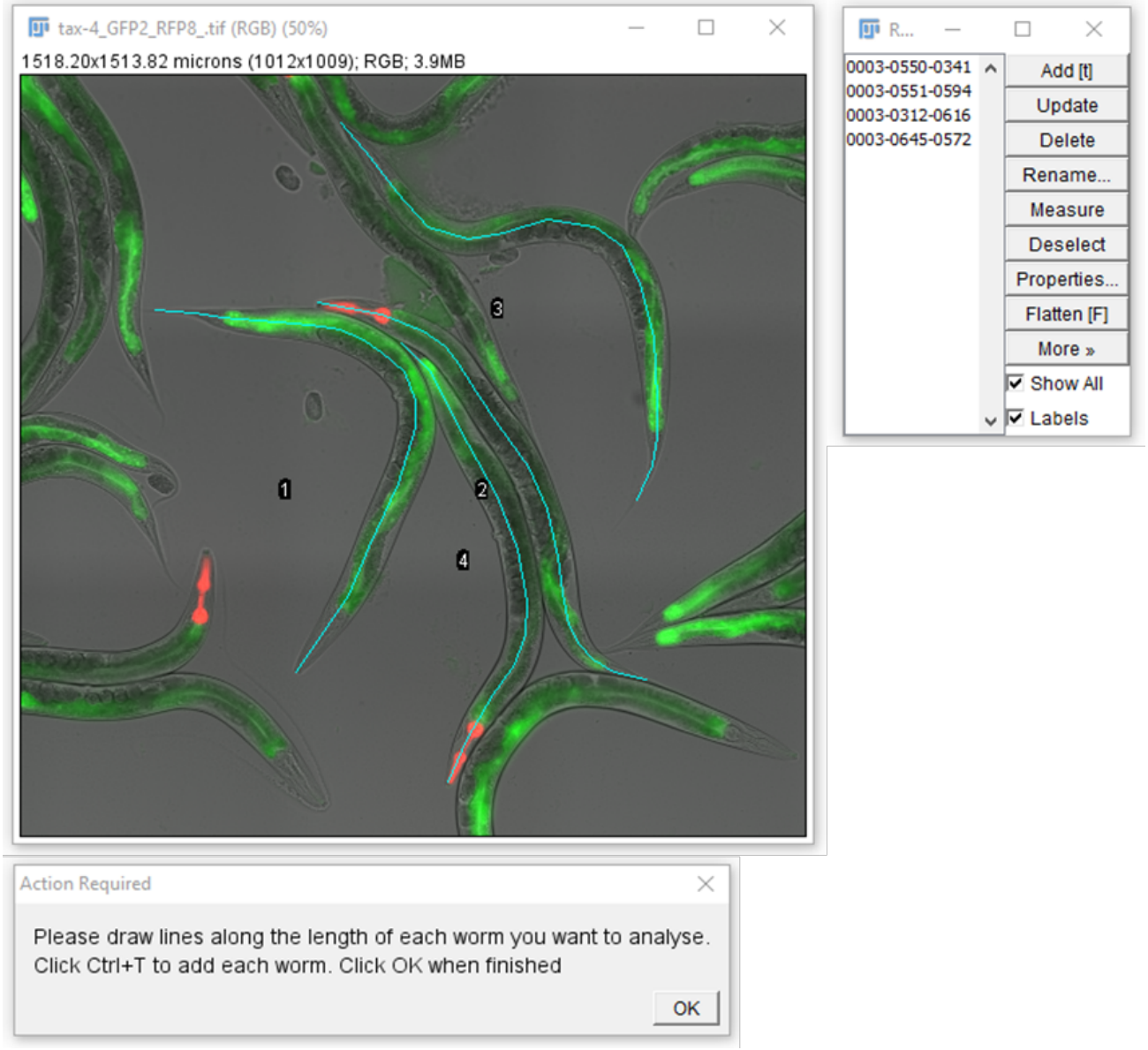

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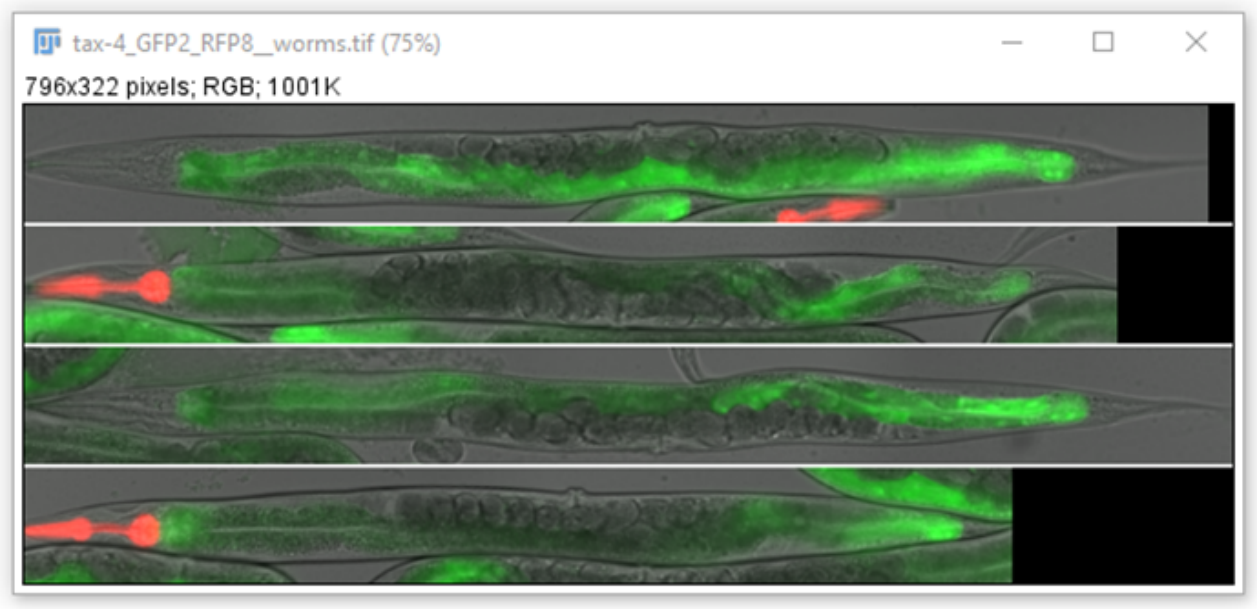

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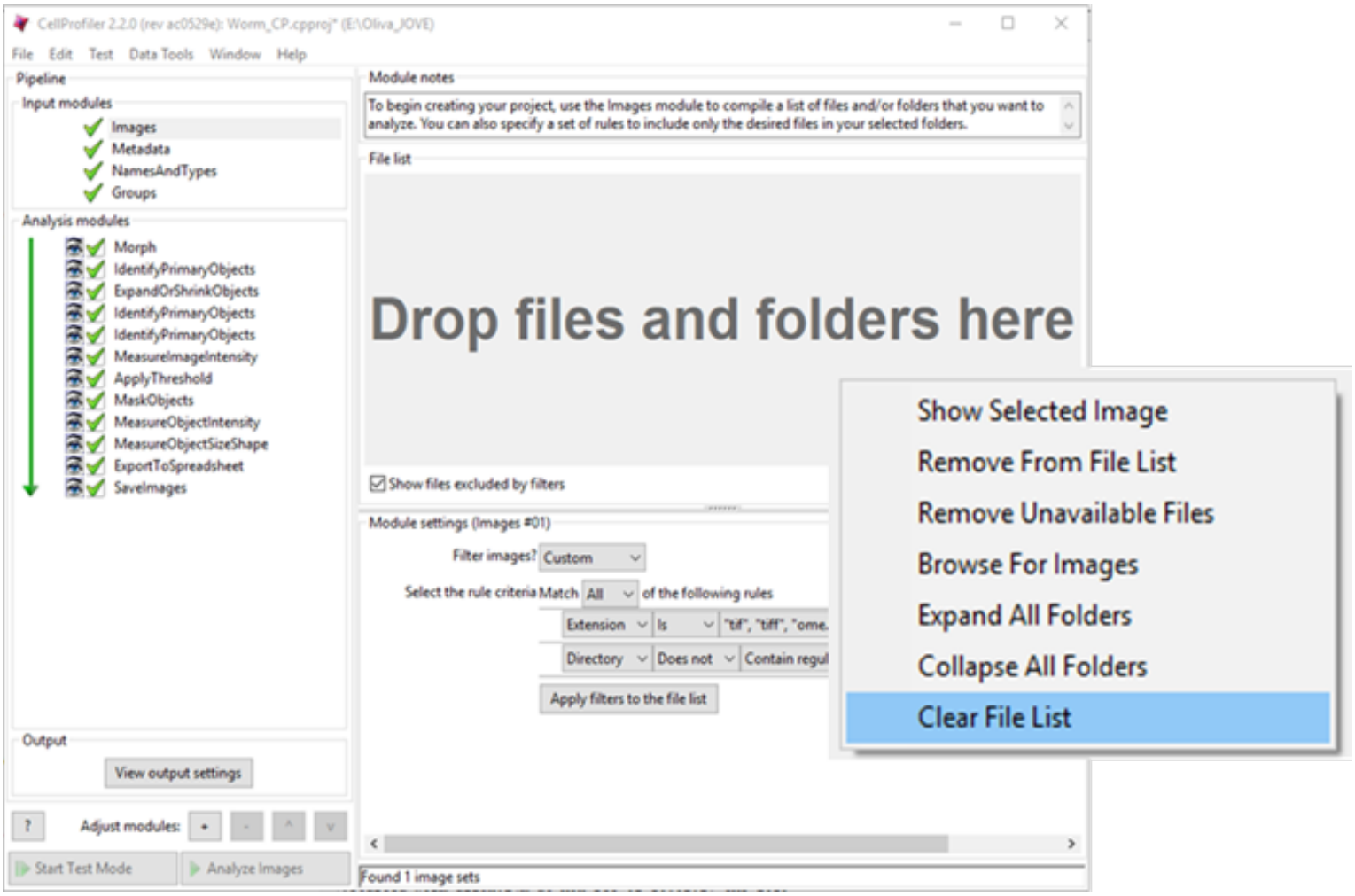

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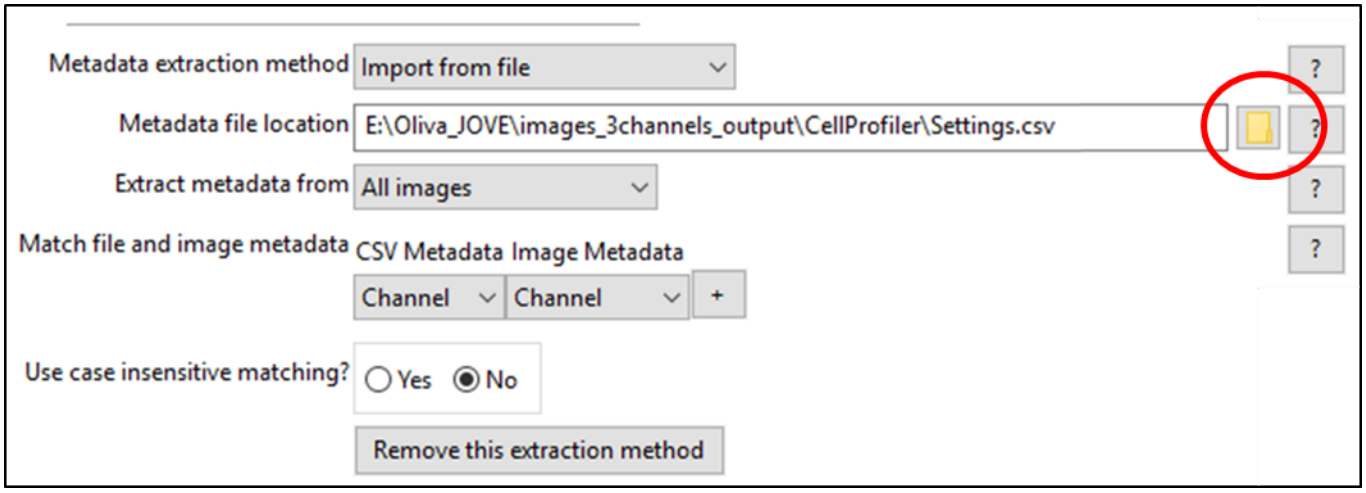

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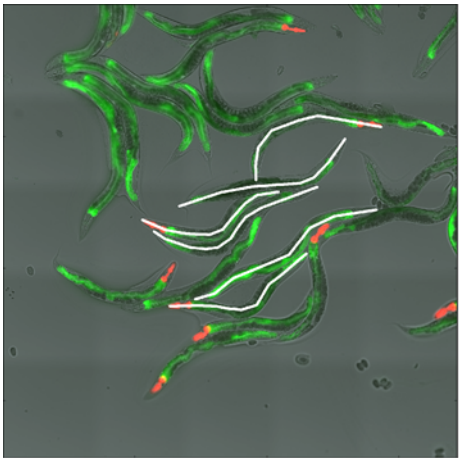

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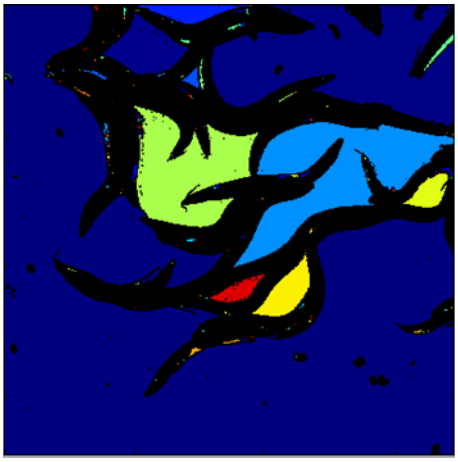

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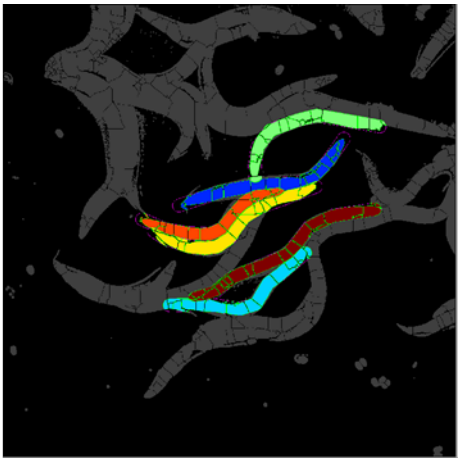


D.

MeasureObjectIntensity #13, image cycle #1

File Tools Subplots Help

Image	Object	Feature	Mean	Median	STD
green	worms	IntegratedIntensity	490.25	415.788	168.508
green	worms	MeanIntensity	0.016	0.016	0.004
green	worms	StdIntensity	0.01	0.01	0.003
green	worms	MinIntensity	0.007	0.007	0.0
green	worms	MaxIntensity	0.065	0.069	0.01
green	worms	IntegratedIntensityEdge	48.934	40.333	16.145
green	worms	MeanIntensityEdge	0.013	0.012	0.003
green	worms	StdIntensityEdge	0.008	0.007	0.004
green	worms	MinIntensityEdge	0.007	0.007	0.0
green	worms	MaxIntensityEdge	0.055	0.056	0.011
green	worms	MassDisplacement	22.407	21.768	13.76
green	worms	LowerQuartileIntensity	0.009	0.009	0.0
green	worms	MedianIntensity	0.012	0.012	0.002
green	worms	MADIntensity	0.004	0.003	0.002
green	worms	UpperQuartileIntensity	0.02	0.019	0.007
green	worms	CenterMassIntensity_X	972.079	979.401	120.632
green	worms	CenterMassIntensity_Y	808.351	831.608	191.024
green	worms	MaxIntensity_X	1014.333	1035.5	238.707
green	worms	MaxIntensity_Y	798.0	833.0	218.388
green_threshold	worms	IntegratedIntensity	289.164	233.911	146.904

Figure S10.

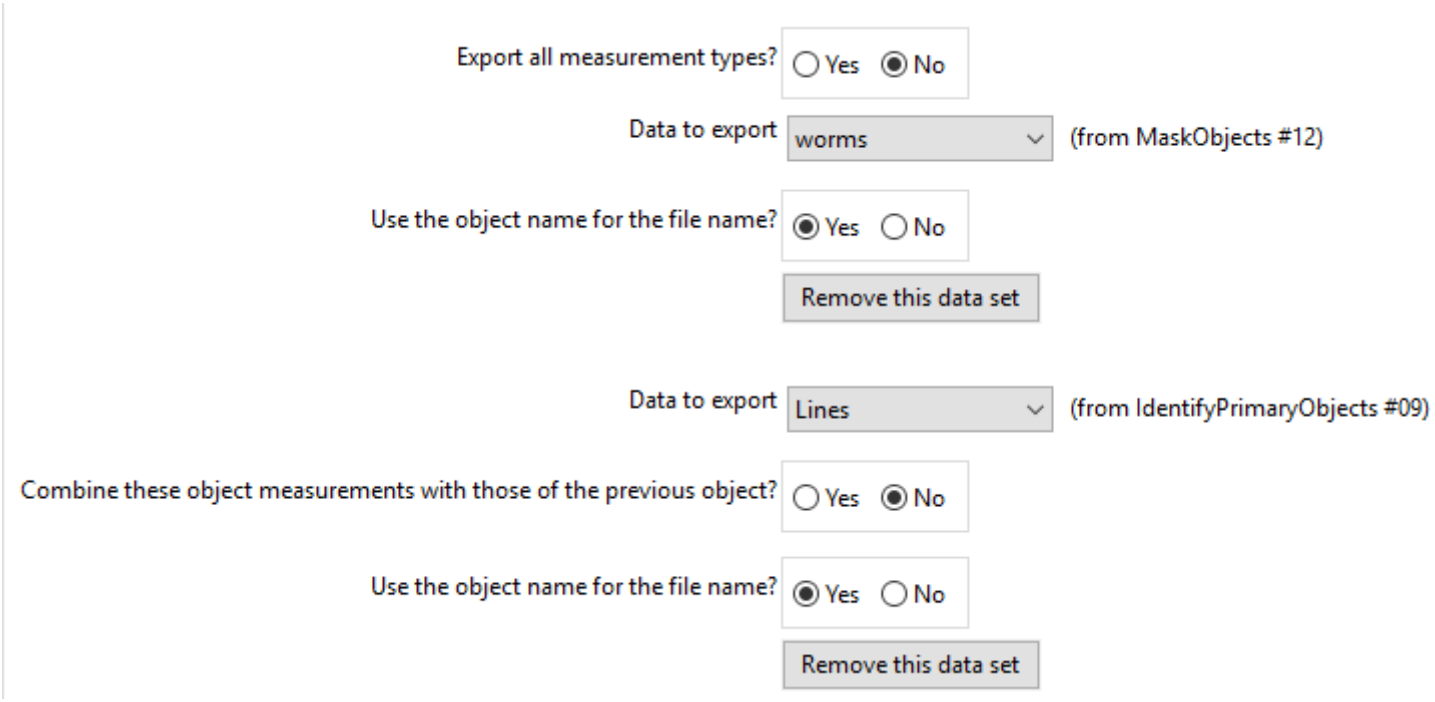


Figure S11.

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Agarose	MeLford Biolaboratories Ltd	MB1200	
Aspirator tube	Sigma-Aldrich	A5177	to create mouth micro-pipette (see protocol step)
Beaker			
BODIPY 493/593	Invitrogen	D3922	stock solution prepared in DMSO at 1mg/mL
Centrifuge	MSE MISTRAL 1000		
Conical flask			
Cover Slip	VWR	631-0120	
Filter 0.2 µm for Syringe	Sartrius	16534-K	Filter, to create mouth micro-pipette (see protocol step)
Isopropanol			
Levamisole hydrochloride	Sigma-Aldrich	BP212	3mM solution prepared by dissolving levamisole in M9
Liquid Nitrogen			Liquid Nitrogen facility
Low Retention Tip 1000µL	Starlab	S1182-1830	
Methanol	VWR chemicals	20847.307	
Microscope Slides, MENZEL GLASSER	Thermo Scientific	BS7011/2	
Microscope	Nikon		Eclipse Ti
Microwave Oven	Delongi		
M9			prepared in the lab according to[15]
9cm NGM Plates			prepared in the lab according to[15]
PBS			prepared in the lab
Protein LoBind Tube 2ml	Eppendorf	22431102	
Triton	SIGMA	T9284-500ML	
Ring Caps	SIGMA-ALDRICH	Z611247-250EA	glass micro-capillary tubes to create mouth micro-pipette (see protocol step)
Rotator	Stuart Scientific		
Silicone tubine translucent	Scientific Laboratory Suppliers	TSR0600200P	6.0 mm x 2.0 mm wall - to create mouth micro-pipette (see protocol step)
Sterilized H2O			MilliQ water autoclaved in the lab
10µL Tips	Starlab	S1120-3810	
200µL Tips	Starlab	S1120-8810	
1000µL Tips	Starlab	S1122-1830	
15mL Centrifuge Tube	CORNING	430791	
Vecta Shield	VECTOR	94010	antifade mounting medium (H-1000) without DAPI

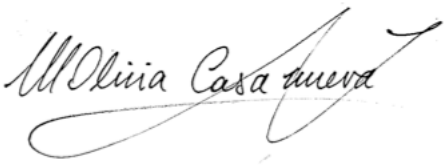
Babraham Institute

Babraham Institute.
26th/02/2020

Dear Dr. Cao:

We would like to submit a revised JoVE61136 entitled manuscript "Worm-align and Worm_CP, two open-source pipelines for straightening and quantification of fluorescence image data obtained from *Caenorhabditis elegans*". We want to thank the editorial team and the reviewers for bringing up important issues related to both the manuscript and the pipeline. We have made substantial changes to the manuscript and added 2 new figures to address all concerns. We thank reviewers as we believe that our protocol and pipeline have improved compared to the first submission.

Best regards,



Olivia Casanueva

Group leader

Editorial comments:

We have addressed all editorial comments. The changes have been highlighted in the manuscript.

- We have proof-read the manuscript and eliminated all commercial language.
- We have eliminated all personal pronouns, colloquial phrases, we have used imperative tense when appropriate.
- We have revised the text of the notes.
- (4.1.2) We have clarified that after centrifugation, the supernatant eliminated.
- (5.5.2) We have specified the microscopy settings
- We have highlighted the critical steps to be filmed.
- (Figure 4.14) We have added the screen shots in the supplementary material.
- We have sorted materials alphabetically in the Table of materials.

Reviewer#1:
Summary:
In "Worm-align, and Worm_CP, two open-source pipelines for straightening and fluorescence image data obtained from Caenorhabditis elegans.", Okkenhaug et al. describe a FIJI macro that can be used to untangle immobilized or fixed C. elegans individuals without the need for deep-learning based training datasets. In addition, the authors also import their straightened results into Worm_CP, a Cell Profiler pipeline to allow for quantification of fluorescence intensity in individual worms. The results described in this paper should be helpful for high-throughput quantification of worm biology. I believe this paper is suitable for publication in JOVE, however I believe some revisions are required before publication. In particular, I had some concerns about the output of the Worm-align program shown in Figure 15C and Figure 17B and Figure 17C.

Major Concerns:

*1A. I found the straightened worm images in **Figure 17**, panels B and C confusing, and think that some revision to this figure is needed before publication. I had two particular issues. The first issue is the ease of matching up the two-channel (GFP + transmitted light) straightened worm images with the GFP channel straightened images in Panels B and C. For example, in panel B, the middle-left side of the top GFP image appears to show faint pharyngeal labeling from two different animals, while the corresponding top two-channel image only shows one animal. Other parts of panels B and C are similarly hard to interpret. I would suggest outlining the worm body with dashes or some other contrasting marker in the GFP images to make the relationship to the two-channel images clearer.*

We thank the reviewer for noticing this issue. The discrepancies described resulted from a mistake we made when compiling Figure 17: The top (GFP channel + transmitted light) and bottom (GFP

channel only) panels were not produced during the same run of Worm-align. We have reviewed **figure 17** (**now it corresponds to Figure 7**) to best represent the output of the pipeline.

*1B. My second issue with **Figure 17** involves the presence of two apparent head/pharyngeal regions in a single straightened worm. For example, in Panel B, the fourth two-channel (GFP + transmitted light) straightened worm (counting down from top) clearly shows two head regions. It's not clear to me how this result could happen if you are straightening worms using a single nose-to-tail line drawn down an individual animal, and suggests a significant difference between the output of the WormAlign program, and the output the reader expects based on the text. I think the authors need to clarify how this type of output could be produced before publication.*

*2. **Figure 15** has a similar "two head regions" problem for worms 3, 10, and 12 in Panel C (counting down from top).*

We thank the reviewer for highlighting this issue. We have added two new Figures (**Figure 4 and 5**) and a paragraph in the results section (**lines 437-473**) that shows that the output of the pipeline depends on the quality of the lines drawn on top of the images. A complete line from the head to the tip of the tail will produce a properly aligned worm. We have updated **Figure 4** to highlight how worm tracing mistakes during the execution of Worm-align affect the output of the alignment. From the annotated montage now included, it is clear that care should be taken to avoid the following errors, as they could present issues for the proper alignment of worms:

- Tracing of the worm from tail to head (5): This results in the worm being reversed in the alignment
- Incomplete tracing (6): This results in only the part of the worm that was traced to be cropped for the montage
- Including multiple worms in a single trace (7): This results in worms plus their neighbors being inserted in the same panel of the montage
- Adding random lines to the image (8): This results in the insertion of random panels in the montage.

For creation of the montage it should not be an issue if two lines either intersect or join, even if that is over a significant part of their length, as line ROIs are individually selected, cropped and straightened by the workflow. For the CellProfiler analysis however, these occurrences would be problematic, as the Worm_CP pipeline uses the line mask, and not individual ROIs to aid identification of individual worms. We have added a new figure (**Figure 5**) to illustrate this problem. To facilitate identification of "bad worms" we suggest the use of the overlay image and the ROI collection saved in the data subfolder. This image shows the selected lines plus their labels. We have explained this step in the results and discussion sections.

*3. Several of the straightened worm images in the figures (EG **figure 10, figure 17)** show portions of other worms in the straightened images. I would like to see some discussion of how this could affect fluorescence quantitation vs. images with only a single worm - does the additional signal lead to higher measured fluorescence, and is there an easy way to control for this?*

We thank the reviewer for bringing this up. The primary application of Worm-align is to straighten and align worms, and to produce a montage that facilitates visual comparison of different worm populations. The output of the Worm-align macro however also includes line and whole population masks, which in combination with the original raw image data can be used to quantify fluorescence intensities in those worms selected. To avoid problems that arise when incomplete worm masks are segmented, either due to incomplete tracing of worms in Worm-align, or due to segmentation errors in the pipeline itself, it is advised that MeanIntensity values are reported, and not Integrated (Total) Intensities. This is because Mean Intensity values are corrected by the total area measured.

However, to make the pipeline more robust, we have added an optional checkpoint, where the user can observe the outline of the worm mask generated by cell profiler overlaid on the original image, this will show if the worm was properly segmented. The worms are numbered and any incorrect segmented worm can be eliminated from the final output. This has been illustrated in **Figs 4 and 5.**

Minor Concerns:

1. In the Table of Materials, I would list the mouth pipette as individual components, eg Aspirator Tube, Sigma-Aldrich, A5177 (or whatever source the authors used). It could be argued that these are common equipment, but I would suggest that more detail is better here especially for new labs

We have changed the mouth pipette to make the protocol safer (detailed in answer to reviewer #3), and are including a 0.2 μm syringe filter for safety in the making of the mouth micropipette We are detailing in the Materials table, the information about each component of the micropipette. We have also updated the images in supplemental **Figures 1** and **2** with the new mouth micropipette design. The changes have been made to **lines 197 to 206**.

2. In Table of Materials, I would also suggest listing company and catalog number for all commercially available like Bopidy and Levamisole.

We have added the catalog numbers to chemicals, as requested.

3. As described, the WormAlign procedure seems like it may have some issues dealing with different stages of animals (e.g. as L4 and younger animals have a smaller diameter than adults, the user may run into problems if they analyze different stages in the same images.) I would suggest discussing this and noting that the user should stick to analyzing a single stage in each image. This is implied in the text but not explicit.

This is a valid point if an experiment will be run with different developmental stages. Quantitation across genotypes should be done on synchronized animals. If synchronization is not possible, this pipeline has an advantage over fully automated pipelines such as WormTool Box, because Worm_CP allows visual inspection of the samples, so the outlines can be drawn in DIC images of worms at the desired developmental stages. We have added a paragraph in the discussion to highlight this point (**lines 646** to **653**).

4. The text suggests that manual alignment is faster than generating training models for deep learning-based approaches. I would like to see some more quantification or discussion of this - is there a limit to manual analysis where using training datasets are just as fast?

Cell profiler is an open source highly resourceful program that is ideal for high-content screening. Proficiency on the use of the software requires, however, a significant time investment. Tutorials are available that are very helpful in this process. However, the advantage of Worm-align/worm CP, is that it is very easy to use. For simple visualization and quantitation, we think that it is preferable to use this pipeline, however, WormTool Box presents many options for users and if high content screening is required, the choice would be to get training on WormTool Box. We have added a paragraph in the introduction and in the discussion to guide users to the right choice (**lines 65-73** and **666** to **670)**.

5. Clarify where to download the Worm-Align Macro during the install process. As written, the paper seems to suggest that the Worm-Align macro is included in the FIJI macros folder with the standard FIJI distribution. I would suggest a more explicit statement to the effect of "Download the Worm Align Macro from our included link, then paste into the FIJI startup macros file."

Worm-align is not included as a FIJI package, we have added the Macros as supplementary material and also added them to GITHUB. https://github.com/hannekeo/Worm-align.

Reviewer#2:

Summary:

Okkenhaug et al present a workflow to take images of a population of worms on a slide, which may be curved and overlapping, segment and computationally straighten individuals, and quantify fluorescence. The workflow appears to be a useful tool for quantifying data from small-to-medium size datasets, but some more details would improve the manuscript.

Major_Concerns:

In the introduction, the authors outline the rationale for developing their worm-align/worm_CP pipeline. An existing pipeline, WormToolbox, uses a pre-trained model to segment worms, and may require retraining when the experimental setup is modified. The worm-align/worm_CP pipeline in turn does not depend on a trained model, but the experimenter must draw the skeletons of each worm so that they can be segmented. A quantification of the time taken to process small, medium and large datasets, as well as a comparison to the time taken to process the same data using WormToolbox (including retraining) is required for readers to judge which tool best suits their needs. The WormToolbox authors suggest 50 training worms were sufficient to train the model, which seems quite a manageable investment when setting up a large experiment

The reviewer is correct in that the worm-align/worm_CP is not automatic and that it requires the user to draw a line of the worms. This process is time consuming but it halves the time compared to manually drawing an outline around each animal to quantify fluorescence using FIJI alone.

We have changed the text to reflect this point in the discussion and in the introduction.

Compared to Worm_CP, WormToolbox is an automatic solution because in principle, all that it takes from the user is to pre-train the system with a subset of the samples. Although this is correct, in practice the community -unfortunately-does not use it so often. In PubMed only 50 studies have cited the open-source software WormtoolBox in the 8 years of availability. WormTool Box provides a great number of features and capabilities that allow high-content screening. The use of WormTool or CellProfiler, however, requires an investment of time to learn how to use it. Although there are tutorials available online and anyone who needs an automated high-content phenotyping capability should consider investing this time, Worm_align/Worm_CP does only require opening the macro on Fiji or CellProfiler and following simple instructions. Worm align was developed to visualize worms to produce quickly a montage of straightened worms for improved visualization. The analysis side of the pipeline is secondary. The simplicity of the pipeline may suit some users better, but it does not provide the same number of capabilities accessible through WormToolbox.

We have added a paragraph in both the introduction and in the discussion to guide users in their choice of pipeline (**lines 65-73 and 666 to 670**).

Due to imperfect drawing of the worm skeleton, the straightened worms are often distorted. Also, some parts of the worm are being stretched or compressed during straightening. Whether this could affect the resulting quantification should be discussed. For example, if a fluorescent patch lies along the bend of the animal and the corresponding pixels are stretched or distorted during straightening, would it affect the quantification of fluorescence intensity? To what degree? This could be measured by comparing measurements from individual worms before and after straightening.

As explained to Reviewer 1, the straightening of the worms does not affect quantitation. Moreover, we have added an image that shows all lines superimposed with the montage, to any worm that is incorrectly traced should be eliminated from the final quantitation. (**Figures 4 and 5, lines 437-473)**

Minor_Comments:
We have updated the text to reflect changes related to all concerns by reviewer 2.

- We have added Carpenter, 2006; Rueden 2017 and Schindelin 2012 papers (refs 13,14,15)
- Lines 165-6: We have used Beaker instead of Becher and conical flask instead of Erlenmeyer.

- Lines 168-9: 'negatively impacts the pad's thickness' - I know what you mean, but the way it is written I had to think about it. Could be more precise, such as 'makes it harder to achieve the desired pad thickness'
- Line 171: we have changed 'agar' for agarose
- Line 183-4: We have written 'Mount the worms within 5 min' - followed by 'to prevent the pad from drying out before use'
- Line 190-1: instead of 'try to aspirate H2O with it', could make it be clearer by saying 'test whether the end of the capillary is open by trying to aspirate water'
- Lines 220-1: might be useful to give rationale for detergent - presumably to prevent worms from sticking to the sides of the plastic pipette tip
- Line 221: 'with a scissor' should be with a pair of scissors
- Line 226: English: 'shake a little bit the slide' should be 'gently shake the slide'
- We agree that Protocol G is not easy to follow by reading, however, we think that the video will be very helpful. We have made the manuscript open source, so that everyone can access the video.
- Unfortunately, the misplaced numbers that indicate individual worms cannot be changed, as they are generated automatically from FIJI, however, it is quite obvious which number belongs to which worm.
- We have italicized *dbl-1* in **Figure 16 (now figure 6)**.
- We have updated **Figure 16 (now figure 6)** and **Figure 17 (now figure 7)** and their respective legends.
- We have updated the table of materials. Table of materials: in the comments/description column, references need to be inserted ('prepared in the lab according to ref').
- We are providing an example dataset for potential users to test the workflow on their own computer setup. We have added it to GitHub.

Reviewer#3:

Summary:

The manuscript "Worm-align and Worm_CP, two open-source pipelines for straightening and quantification of fluorescence image data obtained from Caenorhabditis elegans" by Okkenhaug et al. presents computation tools for manipulation and quantitation of fluorescent images. The tools should be useful for the C. elegans community, which heavily uses fluorescent reporters to monitor proteostasis and other biological phenomena. Overall, this manuscript will be a nice addition to collection.

Major_Concerns:

In the abstract, the authors claim that their methodology represents a high-throughput solution that can be adapted to high-content screening with additional modules. However, I am concerned that this is overstated since the method requires manually drawn lines for every worm in each image to be analyzed. I would classify this approach as low-throughput and I do not anticipate that it would be suitable for high-content screening.

We apologize and thank the reviewer for pointing this out, the high-throughput comment was related to WormTool Box, but we agree that this can cause confusion to the readers. We have changed the text in the abstract.

I have serious reservations about the mouth pipetting described in Protocol C. Mouth pipetting is prohibited in most laboratories due to safety concerns and readily available alternatives. At a minimum, the authors must explain why other methodology is unsuitable and must describe safety precautions for this technique. Furthermore, the technique itself is not clearly described. It is not clear whether the capillary is broken after extending in the flame and the nature of the adaptor, silicone tubing, and the assembly of the pipette are all unclear.

We thank the reviewer for taking into consideration safety. We have updated the preparation of custom-made mouth micropipettes, according to the safety standards in mouse labs, which use this type of micropipettes routinely to isolate mouth embryos[12]. In particular, a 0.2 μm syringe filter is now being inserted along the silicone tube as a safety check. We have added a picture of this in **Figure 1C**. We also propose an alternative method for mounting the worms, if the experimenter does not want to use a mouth micropipette (**lines 211-216**).

The heat shock experiments described and shown in Figure 17 have two issues. First, in order to compare the reporter induction under the two different conditions, it would be useful to include a control set of images with worms that are not heat shocked.

We thank the reviewer for pointing out an important missing control. We have now included in **Figure 16 (now figure 6 A-B)**, images of strain AM446 carrying *hsp-70*p::GFP in the absence of heat-shock. We have also quantified them in **Figure 6E**.

Second, it has been documented in other systems that the amount of temperature change is an important determinant of the degree of induction, but that lower growth temperatures result in stronger heat shock responses (see Abravaya et al. Attenuation of the heat shock response in HeLa cells is mediated by the release of bound heat shock transcription factor and is modulated by changes in growth and in heat shock temperatures, Genes and Development, 1991). Therefore, the result that the authors present is quite unexpected, and thus the experiment warrants a better description, requires motivation/justification, and also it needs to be put into the proper context. Specifically, why was this experiment done? What do these results mean? When would a reader of the manuscript need this information? Finally, do the error bars represent SD or SEM, what is the N value, and how was the p-value calculated?

We have added the missing information regarding errors bars, n value, and statistics in the legend of **Figure 6.** We have performed this experiment in the context of a research project in the lab, investigating the influence of temperature acclimation on the heat shock response (data unpublished yet). This experiment provides an example of slight change in a transcriptional reporter, which can be quantified using the Worm_CP method. We report data from one experiment here, but we have observed this result several times.

We thank Reviewer #3 for putting this experiment in a broader context, and it is true that this result seems to contradict the conclusion of Abravaya *et* al, 1991, where HeLa cells that were incubated at a slightly lower growth temperature exhibited stronger activation of HSF-1. It should be noted that in this experiment, the heat shock is sustained (4h at 42°C degrees), whereas in our case, it is a short heat shock (30 min at 34°C degrees). We have been looking for similar experiments in a wide range of organisms, but did not find many reports on the consequences of temperature acclimation on the heat shock response. There is another report in mice showing that the heat shock response is stronger if the housing temperature is lower (Eng *et al*., housing temperature influences the pattern of heat shock protein induction in mice following mild whole-body hyperthermia, 2014). This report seems to corroborate Abravaya *et al*. results in mammalian cells.

However, this is less clear in ectotherms. Although there is evidence that lines of *D. melanogaster* evolved to grow at slightly higher temperatures (still within the range of acceptable temperatures) for several years are more resistant to thermal stress (Gilchrist *et al*, thermal sensitivity of *Drosophila melanogaster:* evolutionary responses of adults and eggs to laboratory selection at different temperatures, 1997), we did not find reports in *D. melanogaster* of temperature acclimation experiments on the scale or one generation. In a study on heat shock response in intertidal gastropods, 2 out of 3 species investigated (*T. funebralis* and *T. montereyi*) exhibited higher *hsp-70* heat-induction after acclimation at higher temperature (Tomanek, Variation in the heat shock response and its implication for predicting the effects of global climate change on species biogeographical distribution ranges and metabolic costs, 2009). We wonder whether the link between temperature acclimatization and heat shock response could be different in endotherms such as mammals versus in ectotherms organisms such as *C. elegans*. We have not discussed this point of this manuscript, since it is not relevant to this publication *per se* and we have only kept animals grown at 25°C, eliminated the animals grown at 15°C, to avoid any confusion.

Minor_Concerns:

- Line 93 - We have changed fixated to fixed.
- Line 107-108 We have added a note regarding the following point: “why are 1000 worms needed for fixing if only 20-30 are analyzed by immobilization. This is probably due to large loss of worms when handling but should be more clearly indicated for someone who is not familiar with this type of manipulation”.
- Line 105 -W have checked the diameter of our plates (9cm). “standard sizes for petri dishes are 6cm and 10cm, please verify that your plates are 9cm”.
- Line 111 - We have changed the term falcon tube by conical tube.
- Line 113 - We have changed "repeat wash one more time" to just "repeat wash".
- Line 115- The tube has been described as a microcentrifuge tube.
- Line 121-122 and throughout the protocol - We have replaced Bodipy by BODIPY. It is true that two kinds of BODIPY dyes exist: BODIPY 493/503 and BODIPY 581/591 C11. We have mentioned BODIPY 493/503 in the Materials Table. In the text, we refer to BODIPY, which is the abbreviation of *4,4-difluoro-4-bora-3a, 4a-diaza-s-indacene.* We cannot refer to in the text as BODIPY 493/503 as it is a trademark. On the other hand, we did not want to be too vague by referring to it as lipid droplets binding dye, as several dyes of that sort exist (e.g. Nile red, ORO) and it could be confusing.

- Line 123 - Isopropanol is not capitalized.
- Line 128-129 -has been grammatically fixed.
- Line 139 - the temperature has been specified to be about 40 degrees.
- Line 152 - the time and force of the spin has been indicated to be 1500 rpm
- Line 154 - We have removed this note. “this note should be removed because it is better described in the line 136 note”.
- Line 171-177 - We have used the technique based on tape on flanking slides to make pads. In our experience, we obtained better results with the technique described here. We mention now in the protocol the tape on flanking sides as an alternative method. 2.2 - “it is typical to use a piece of tape as a spacer when forming agarose pads - was tape used here?”
- Line 482 -we have replaced GFP with fluorescent dye that incorporates into LDs. “GFP is incorrect here”.
- Line 497 - The macro can be easily updated to include more channels, but we 2 channels plus DIC is what is used most commonly in labs, so we have kept the first version simpler.
- We have corrected this - “Figure 12 is incorrectly labelled as Figure 11”
- We have corrected this: “Figure 16 is incorrectly labelled as Figure 14”

Figure 16 (now figure 6) - it would be helpful to know how a 12% decrease in signal compares to the published literature and/or other methods that have been used to quantitate this effect, especially since this seems like a modest effect.

In ref 18 (Clark *et al*., 2018), the decrease observed in lipid droplet content is stronger (about 35% decrease). However, the lipid droplet content is monitored with another dye in this study (ORO staining) and on another allele of *dbl-1(wk70),* whereas we are investigating *dbl-1(nk3)*. We observe the same trend as Clark and colleagues, however the discrepancies in the amplitude of the decrease could be due to methods or genetic background differences. Therefore, we did not compare directly the amplitude of our result to the one observed by Clark and colleagues.

Reviewer#4:
Summary:
This paper describes software to easily quantify fluorescence of C. elegans. Fluorescence quantification is such a regularly used technique for C. elegans, that this protocol will provide a valuable reference for essentially all labs doing C. elegans research. The protocol is very well described and illustrated.

Major Concerns:
The text that refers to figure 16 mentions measuring GFP fluorescence, but I think this should be BODIPY fluorescence. If GFP is correct, the figure legend will have to be modified. Figure 16 is labeled figure 14.

We thank the reviewer for pointing this out, we have altered the text to reflect that wavelength used.

Minor_Concerns:
Section 2.2: If uniform thickness is important, why not use flanking layers of tape on the lower slide to provide pads of a reproducible and uniform thickness? This is the standard technique.

We have used the standard technique based on tape on flanking slides to make pads. In our experience, we obtained better results with the technique described here. However, we also mention in the protocol the tape on flanking sides as an alternative method (**lines 171-177**).

If you modify section 2.2 as I have suggested, you can then simplify section 5.1.

We have kept section 5.1, as we kept the description as our pad method along the description of the standard technique using tapes on flanking slides.

In the first note under section 6.3, you should indicate acceptable file formats.

The protocol now indicates acceptable file formats, nd2, czi, tif, rgb, jpg and png (**lines 286-287**).

section 76.3 was disorienting. You state that something is second, but nowhere do you state what is first. If this is second, you need to explicitly state what is first. [You may be referring to a menu item that is second in a list? If so, write as if you are navigating a menu.

We have rewritten parts of section 7.3 (**lines 369-370**).

Please see more specific comments/corrections in the attached pdf.

We have addressed all the comments mentioned in the pdf document.

Dear Dr. Casanueva,

Your manuscript, JoVE61136R1 "Worm-align and Worm_CP, two open-source pipelines for straightening and quantification of fluorescence image data obtained from C.elegans.," has been editorially reviewed and the following comments need to be addressed. Please track the changes to identify all of the manuscript edits. After revising the submission, please also upload a separate document that addresses each of the editorial comments individually with the revised manuscript.

Your revision is due by **Mar 12, 2020**.

To submit a revision, go to the JoVE submission site and log in as an author. You will find your submission under the heading "Submission Needing Revision". Please note that the corresponding author in Editorial Manager refers to the point of contact during the review and production of the video article.

Best,

Xiaoyan Cao, Ph.D.
Review Editor

We thank the editor for the comments on the manuscripts. Below is the list of edits:

Line 97-98: Editioral comment: Please convert centrifugation force to x g. Do you remove supernatant after centrifugation?
We added the centrifuge speed in g and kept the speed in rpm in brackets through the manuscript. We also added that the supernatant needs to be removed.

Line 108-111: Editioral comment: Please note that I simplified this step to highlight the actions being performed here.

We have noted.

Line 136-137: Editioral comment: Is this an alternative step?
Yes, spinning down is an alternative step and we have reworded this phrase in that sense.

Line 156-163: Editioral comment: I rephrased this step and its note. Please review for accuracy.
We have reviewed this step for accuracy.

Line 180-183: Editioral comment: The glass capillary from step 3.1? Do you mean the pipette tip shown in Figure 1C? I added this step based on information provided in the Note. Please review for accuracy.
We have now added that the glass capillary comes from step 3.1, and the pipette tip is the one shown in figure 1C. We have reviewed the edited step for accuracy.

Line 208: Editioral comment: Please upload Figure 3.
We have now uploaded figure 3

Lines 282-290 (section 6.6):
This section has been edited to explain: The macro will not proceed beyond the setup until the top box has been ticked (= user is happy with settings). WRT to the montages, both boxes are ticked by default, but can be deselected if not required. The text has been modified to match that in the macro GUI. In addition two user actions were highlighted in yellow, as they are important for execution of the Worm-align macro.

Editorial comment: Are the same as those shown in Figure S5 (Montage of worms from single image, combined montage)? If so, please use the same language as shown in the figure.
Boxes for generation of montages are ticked by default, but can be removed if not required. If removed, the macro will still proceed to generate straightened image crops of single worms (stored in 'single_worms' subfolder, but these will not be combined into montages

Line 283: Editorial comment: Are both boxes selected? Please specify.Boxes now explained better: the macro will not proceed beyond the setup until the top box has been ticked (= user is happy with settings).

Line 302-320 (previous version), now lines 299-307: Editorial comment: The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please remove such introductory information from the protocol if possible. In addition, some folders are already described in the protocol text (see lines 298-300, 340-343). In these cases, they should be removed to avoid repetition.
Section 6.8 has been shortened and the highlighted paragraph has in part been moved to the Results section (where relevant).

Line 340-343 of previous manuscript: This note was removed and (in part) incorporated in section 7.2 as it is more relevant here.

Line 370 of previous manuscript: Deleted the phrase 'Click 'Next Image Set' to skip to the next image.' as this is not something that is necessary.

Line 314-315 : In section 7.1, we added: Download the Worm_CP.cpproj pipeline from GitHub: https://github.com/hannekeo/Worm-align.

Line 383-396 (of previous manuscript): Editorial comment: The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please move this paragraph to introduction, results, discussion as appropriate.
This note was rephrased and moved to the Results section.

Line 402-411: We changed the order of the Results section. In the current submission it should flow more logically.

Line 435: Editorial comment: Which panel?
We have precised that this deals with figure 5 panel D-E.We rephrased some of this paragraph to make it clearer how CellProfiler fine-tunes the mask of the worm population to generate individual worm masks of those worms that were selected during execution of Worm-align. This also makes it clearer why although not a problem for generation of montages, lines that intersect are problematic for the Worm_CP CellProfiler pipeline. We have also added comments on how such problematic masks (fragmented worm masks) can easily be identified in the CellProfiler output and excluded from the final analysis.