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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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Author Questionnaire

1. **Microscopy**: Does your protocol involve video microscopy, such as filming a complex dissection or microinjection technique? **yes**

   If **Yes**, can you record movies/images using your own microscope camera?

   no

   If **No**, JoVE will need to record the microscope images using our scope kit (through a camera port or one of the oculars). Please list the make and model of your microscope.

   Olympus SZX10

2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **Yes, all done.**

3. **Filming location:** Will the filming need to take place in multiple locations? **no**

protocol: **24 steps, 16 shots for videographer (the rest are SC and already uploaded)**

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Hanneke Okkenhaug:** We describe here a simple open-source FIJI-based workflow, named "Worm-align", which can be used to generate single-or multi-channel montages of straightened worms of interest from raw microscopy images.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. NOTE: This has been filmed and sent directly to JOVE by Hanneke Okkenhaug

1.2. **Hanneke Okkenhaug:** Worm-align/Worm_CP workflow is a simple, cost-effective way to straighten and score fluorescence in user selected worms, without the need for prior training steps. This method is fast and requires a simple worm-mounting set-up from either live or fixed worms.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. NOTE: This has been filmed and sent directly to JOVE by Hanneke Okkenhaug

OPTIONAL:

1.3. **Laetitia Chauve:** Because this workflow relies on user-selected animals of interest, it might be particularly useful when analyzing images from *C. elegans* where the worms are not all at the correct stage or condition.

1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

1.4. **Laetitia Chauve:** It should be noted that the output of Worm-align can be used for subsequent fluorescence quantification with either FIJI or other image analysis software. Here, we demonstrate the quantification of fluorescence using the CellProfiler pipeline Worm_CP.

1.4.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

Protocol

2. Mounting Fixed Worms for Imaging

2.1. Begin by creating a mouth micropipette by **[0]** extending a thin glass capillary in the flame of a Bunsen burner **[1]**. After extension in the flame, break the capillary into two pieces if it did not break into two pieces after extension. If it broke into two pieces, cut the end of the longest one **[2]**. Select the longer piece and test whether the capillary is open by attempting to aspirate water **[3]**. *Videographer: This step is difficult and important!*

2.1.0: Added shot: establishing shot if required

2.1.1. Talent extending a glass capillary in the flame of a Bunsen burner.

2.1.2. Talent extending the capillary in the flame. The capillary got broken into two pieces. The end of the longest one was cut with

2.1.3. Talent attempting to aspirate water with a piece of the pipette.

2.2. Detatch the adaptor from the 3-millimeter silicon tube. Plug the glass capillary into a capillary adaptor linked to a 6-millimeter silicone tube **[1]**, then plug the other end of the 6mm tube into a 0.2-micrometer filter a 3-millimeter silicone tube on its other end **[2-TXT]**. Plug a 1-milliliter filter tip into the free end of the 3-millimeter silicone tube to aspirate liquid **[3]**.

2.2.1. Talent separating the adaptor from the 3 mm tube, plugging the capillary into the adaptor, then puging the adaptor into the 6mm tube attached to the filter. Talent then attaches the other end of the filter (blue) into the 3mm tube and insert a filter tip into the end of the 3mm tube. NOTE: 2.2.1 and 2.3 maybe in one shot. 2.2.1. In the camera log, 2.2.1 is all action as one shot as WIDE, 2.2.2 is all action as one shot as CU This shot was combined with with 2.2.1

2.3. Under a dissection microscope, remove as much liquid as possible around a pellet of fixed worms with the mouth micropipette **[1]**. Quickly add 10 microliters of mounting medium to the bottom of the tube **[2]**. *Videographer: This step is important!*

2.3.0: Added: establishing shot if required

2.3.1. SCOPE: Talent removing liquid from the worm pellet. Author NOTE: This was filmed on the camera of our Nikon macrozoom as it turned out to be better than filming through the eye pieces of our dissection microscope. This video should have been uploaded too by SCIPHY

2.3.2. Talent adding mounting medium to the tube.

2.4. Rinse a 10-microliter tip in PBS with traces of detergent to prevent worms from sticking to the sides of the plastic pipette tip **[1]**, then cut the very end of the tip with a pair of scissors **[2]**.

2.4.1. Talent rinsing the tip in PBS.

2.4.2. Talent cutting the end of the tip.

2.5. Transfer 8 microliters of mounting medium with the worms onto a previously prepared agarose pad **[1]**. While observing the slide under the microscope, gently agitate it to avoid overlap of the worms **[2]**. Then, cover the drop of mounting medium on the agarose pad with an 18 by 18-millimeter coverslip **[3]**.

2.5.1. Talent transferring the worms onto an agarose pad. Author NOTE: filmed on the camera of our Nikon macrozoom as it turned out to be better than filming through the eye pieces of our dissection microscope. This video should have been uploaded too by SCIPHY but has been labelled 2.5.2 instead of 2.5.1 in the camera log and in its file name

2.5.2. SCOPE: Talent agitatingthe slide. NOTE: This is labelled as 2.5.3 in the camera log

2.5.3. Talent putting a coverslip on the mounting medium. NOTE: This is labelled as 2.5.4 NEW SHOT into the camera log

2.6. To mount live worms, pipette 3 to 4 microliters of 3 millimolar levamisole dissolved in M9 onto an agarose pad **[1]**. Pick 30 to 50 worms per condition into the drop of levamisole **[2]**, then cover the drop an 18 by 18-millimeter coverslip and image the worms within 1 hour **[3]**. *Videographer: This step is important!*

2.6.1. Talent pipetting levamisole onto the agarose pad.

2.6.2. SCOPE: Talent adding worms to the levamisole. Author NOTE: This was filmed on the camera of our Nikon macrozoom as it turned out to be better than filming through the eye pieces of our dissection microscope. This video should have been uploaded too by SCIPHY

2.6.3. Talent covering the slide with the coverslip.

3. Creating Montage Images of Aligned Single Worms Using the Worm-align FIJI Pipeline

3.1. Install the open-source image analysis software package FIJI from ImageJ **[1]** or, if it is already installed on the computer, verify that it is version 1.52A or later **[2]**. Then, download the Worm-align repository from Github *(pronounce 'git-hub')* and save it on the computer **[3]**.

3.1.1. SCREEN: 61136_6.1a.mp4. 0:33 – 0:43.

3.1.2. SCREEN: 61136_6.1b.mp4. 0:20 – 0:27.

3.1.3. SCREEN: 61136_6.1c.mp4. 0:15 – 0:33.

3.2. Once all pipeline components have been downloaded and installed, open FIJI and execute the Worm-align macro by clicking **Plugins**, **Macros**, and **Run** in the main menu bar. Locate the **Worm-align.ijm** script and click **open [1]**.

3.2.1. SCREEN: 61136_6.2.mp4. 0:24 – end.

3.3. Navigate to the input folder with the images to be analysed and click **Select**, making sure that the selected folder only contains image files. The macro will automatically generate an output folder where all results will be saved. The name of the folder will be the same as the input folder, with "output" as a postfix **[1]**.

3.3.1. SCREEN: 61136_6.3.mp4. 0:07 – 0:40.

3.4. Allow the macro to proceed to open the first image in the input folder and use it as a representative image to extract the settings required to generate the montage, including the width of the worms, the brightness, and the contrast **[1]**.

3.4.1. SCREEN: 61136_6.4.mp4.

3.5. Using the **Straight-line** drawing tool, draw a line across the width of a worm and click **Ok**. 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, and whether it should be included in the montage, then click **Ok [1]**.

3.5.1. SCREEN: 61136_6.5.mp4. 1:02 – 1:54.

3.6. Next, adjust the brightness and contrast settings for the channels using the sliders. Repeat this process for the remaining channels **[1]**.

3.6.1. SCREEN: 61136_6.5.mp4. 1:54 – 2:25.

3.7. Once all settings have been configured, they will be recorded in a settings table and saved to the cell profiler subfolder of the output folder. An image is generated to show what all images will look like after application of the settings **[1]**.

3.7.1. SCREEN: 61136_6.6a.mp4. 0:05 – 0:32.

3.8. If the image is satisfactory, tick the top box **[1]**. The second and third box of the **checking all settings** panel specify the options for montage generation. Leave both boxes ticked and click **OK** to execute the rest of the macro. Failure to tick the top box will cause the macro to re-run the setup **[2]**.

3.8.1. SCREEN: 61136_6.6a.mp4. 0:32 – 0:37.

3.8.2. SCREEN: 61136_6.6a.mp4. 0:20 – 0:45.

3.9. The macro then 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 by using the segmented or the freehand line tool **[1]**.

3.9.1. SCREEN: 61136_6.7.mp4. 0:25 – 0:45.

3.10. Draw lines consistently from head to tail, or vice versa, along the full length of the worm and add each line to the **ROI manager** by clicking Control and T **[1]**.

3.10.1. SCREEN: 61136_6.8a.mp4. 0:00 – 0:23.

3.11. Once all desired worms have been added, click **Ok**. Worm-align will then generate cropped images of single selected worms, which will be saved in the **single_worms** subfolder of the output folder. Montages of all worms selected will be saved in the **aligned** subfolder **[1]**.

3.11.1. SCREEN: 61136_6.8c.mp4. 0:05 – as long as needed to cover VO.

4. Analyzing Single-worm Fluorescence Intensity Using the Output from Worm-align in an Automated CellProfiler Pipeline

4.1. Download and install Cellprofiler 2.2.0 by clicking the link for previous Cellprofiler releases on the download page and selecting the required operating system **[1]**. Before starting the Worm CP pipeline, ensure that all expected output images are present in the CellProfiler subfolder of the Worm-align output folder **[2]**.

4.1.1. SCREEN: 61136_7.1.mp4. 0:33 – 1:05.

4.1.2. SCREEN: 61136_7.2.mp4. 0:10 – 0:17.

4.2. A processed copy of the original image, a binary image mask of the worm population, a line mask of the lines drawn on selected worms, and one image representing each of the channels in the original image should be present **[1]**.

4.2.1. SCREEN: 61136_7.2.mp4. 0:17 – 0:46.

4.3. Open CellProfiler, then click on the **Images input** module and drag the **CellProfiler** output folder into the window that says **Drop files and folders here [1]**. If a list of images is present from a previous analysis, first remove these by right clicking in the window and selecting **Clear File List [2]**.

4.3.1. SCREEN: 61136_7.3c.mp4. 0:12 – 0:36.

4.3.2. SCREEN: 61136_7.3d.mp4.

4.4. Click on the Metadata input module and, on the second extraction method, click on the yellow folder and navigate to the CellProfiler subfolder of the Worm-align output folder. Select the Settings file, then click **Update [1]**.

4.4.1. SCREEN: 61136_7.4.mp4.

4.5. Next, click on the **NamesAndTypes** input module and make sure that all images required for the pipeline are present **[1]**. Before running the pipeline, select a destination for the output results in the **Output** module **[2]**.

4.5.1. SCREEN: 61136_7.5.mp4. 0:00 – 0:20.

4.5.2. SCREEN: 61136_7.7.mp4. 0:17 – 0:35.

4.6. Use the test mode to see how the pipeline performs by clicking **Start Test Mode** and allow it to run through the first image in the folder **[1]**. When satisfied with the performance of the pipeline, click **Exit Test Mode** and **Analyze Images [2]**.

4.6.1. SCREEN: 61136_7.8.mp4. 0:10 – 0:20.

4.6.2. SCREEN: 61136_7.9a.mp4.

4.7. Before beginning analysis, make sure that all eyes in front of the analysis modules are closed **[1]**. When analysis is complete, open the Worm_CP output folder that consists of two csv files, **worms.csv** and **lines.csv [2]**.

4.7.1. SCREEN: 61136_7.9b.mp4. 0:05 – 0:10.

4.7.2. SCREEN: 61136_7.9c.mp4. 0:08 – 0:22.

Results

5. Results: Fluorescence Quantification with the Worm_CP Pipeline

5.1. Culturing and imaging *C. elegans* according to the method described in this protocol produces large overview images of worm populations. The output of the pipeline largely depends on the quality of the lines drawn on top of the images. Several line examples and their output from Worm-align are shown here **[1]**.

5.1.1. LAB MEDIA: Figure 4.

5.2. When using the Worm-align script **[1]**, visual identification of intersecting worms is possible from the overlay image found in the 'data' subfolder **[2]**, as well as from the panels of individual worms in the montage **[3]**. The QC table can also be used to identify overlapping worms **[4]**.

5.2.1. LAB MEDIA: Figure 5 A – C.

5.2.2. LAB MEDIA: Figure 5 A – C. *Video Editor: Emphasize A.*

5.2.3. LAB MEDIA: Figure 5 A – C. *Video Editor: Emphasize B.*

5.2.4. LAB MEDIA: Figure 5 A – C. *Video Editor: Emphasize C.*

5.3. However, intersecting lines are problematic for the Worm_CP pipeline **[1]**. This is because Worm_CP uses the line mask, not regions of interest, to help identify individual worms **[2]**. Therefore, CellProfiler will segment one of the worms as two objects **[3]**.

5.3.1. LAB MEDIA: Figure 5 D – E.

5.3.2. LAB MEDIA: Figure 5 D – E. *Video Editor: Emphasize the 2 intersecting lines in D.*

5.3.3. LAB MEDIA: Figure 5 D – E. *Video Editor: Emphasize the same intersecting worms in E.*

5.4. The Worm_CP pipeline has been used to quantify fluorescence intensity from fixed animals labelled with a fluorescent dye that incorporates into lipid droplets **[1-TXT]**. The lipid droplet content is decreased by **[2]** 17% between wild type and mutant *dbl-1 (pronounce 'D-B-L-1')* animals using manual quantification **[3]**, and by 12% using the Worm_CP pipeline **[4]**.

5.4.1. LAB MEDIA: Figure 6 C and D. *Video Editor: Label the images in C "***WT***" and the images in D "****dbl-1(nk3)****".*

5.4.2. LAB MEDIA: Figure 6 A and B.

5.4.3. LAB MEDIA: Figure 6 A and B. *Video Editor: Emphasize A.*

5.4.4. LAB MEDIA: Figure 6 A and B. *Video Editor: Emphasize B.*

5.5. Worm_CP was also used to quantify the heat shock response in live worms expressing GFP under control of a heat shock inducible gene **[1]**. In the absence of heat stress, the worms do not induce GFP expression **[2]**. When the worms are exposed to a short heat-shock, GFP expression is induced **[3]**.

5.5.1. LAB MEDIA: Figure 6.

5.5.2. LAB MEDIA: Figure 6. *Video Editor: Emphasize A and B and the no HS data in E.*

5.5.3. LAB MEDIA: Figure 6. *Video Editor: Emphasize C and D and the HS data in E.*

Conclusion

6. Conclusion Interview Statements

6.1. **Hanneke Okkenhaug**: The output of Worm_CP depends largely on the quality of the lines drawn using Worm-align. Make sure that the lines are not touching or overlapping and draw all lines in the same direction. Checking the QC table can help identify touching or overlapping lines to exclude those cases from the analysis.

6.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 3.10.1.* NOTE: This has been filmed and sent directly to JOVE by Hanneke Okkenhaug

6.2. **Hanneke Okkenhaug:** The output of Worm-align can be used for subsequent quantification either in FIJI or in other Image Analysis software. Other analysis modules could be incorporated into the Worm_CP pipeline, for instance to count the number of lipid droplets or quantify intensity of individual lipid droplets within one worm.

6.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. NOTE: This has been filmed and sent directly to JOVE by Hanneke Okkenhaug

6.3. **Laetitia Chauve:** One of the advantages of this technique is the possibility to quantify fluorescence from individual worms rapidly. Our lab has an interest in inter-individual variability using fluorescent reporters and we use Worm-align and Worm_CP routinely for this purpose.

6.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.