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Calcium imaging in freely behaving Caenorhabditis elegans with well-controlled, nonlocalized vibration

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TITLE:

Calcium imaging in Freely Behaving *Caenorhabditis elegans* with Well-Controlled, Nonlocalized Vibration

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KEYWORDS:

C. elegans, calcium imaging, nonlocalized vibration, mechanosensory behavior, neural circuit

SUMMARY:

Reported here is a system for calcium imaging in freely behaving *Caenorhabditis elegans* with well-controlled, nonlocalized vibration. This system allows researchers to evoke nonlocalized vibrations with well-controlled properties at nano-scale displacement and to quantify calcium currents during responses of *C. elegans* to the vibrations.

ABSTRACT:

Nonlocalized mechanical forces, such as vibrations and acoustic waves, influence a wide variety of biological processes from development to homeostasis. Animals cope with these stimuli by modifying their behavior. Understanding the mechanisms underlying such behavioral modification requires quantification of neural activity during the behavior of interest. Here, we report a method for calcium imaging in freely behaving *Caenorhabditis elegans* with nonlocalized vibration of specific frequency, displacement, and duration. This method allows the production

of well-controlled, nonlocalized vibration using an acoustic transducer and quantification of evoked calcium responses at single-cell resolution. As a proof of principle, the calcium response of a single interneuron, AVA, during the escape response of *C. elegans* to vibration is demonstrated. This system will facilitate understanding of neural mechanisms underlying behavioral responses to mechanical stimuli.

INTRODUCTION:

Animals are often exposed to nonlocalized mechanical stimuli such as vibrations or acoustic waves[1,2]. Because these stimuli influence homeostasis, development, and reproduction, animals must modify their behaviors to cope with them[3–5]. However, the neural circuits and mechanisms underlying such behavioral modification are poorly understood.

Mechanosensory behavior in the nematode, *Caenorhabditis elegans*, is a simple behavioral paradigm, in which worms usually change behavior from forward movement to a backward escape response when they encounter nonlocalized vibration[6]. The neural circuit underlying this behavior is composed primarily of five sensory neurons, four pairs of interneurons, and several types of motor neurons[7,8]. Additionally, worms habituate to such mechanical stimuli after spaced training involving repeated stimulation[9–11]. Therefore, this simple behavioral response constitutes an ideal system to investigate neural mechanisms underlying both nonlocalized vibration-evoked behavior and memory. A protocol for calcium imaging in freely behaving worms under the influence of nonlocalized vibrations is illustrated. Compared with previously reported systems, this system is simple in that it does not require an additional camera for tracking; however, it allows us to change the frequency, displacement, and duration of nonlocalized vibration. Because activation of the AVA interneurons induces the backward escape response, worms co-expressing GCaMP, a calcium indicator, and TagRFP, a calcium-insensitive fluorescent protein, under the control of an AVA-specific promoter were used as an example (see **Table of Materials** for details). The protocol demonstrates the activation of AVA neurons as a worm switches from forward to backward movement. This protocol facilitates understanding the neural circuit mechanism underlying mechanosensory behavior.

PROTOCOL:

1. Cultivation of worms until calcium imaging

1.1. Four days before a calcium imaging experiment, streak *Escherichia coli* OP50 in a square pattern (approximately 4 mm x 4 mm) using a cell spreader on a new nematode growth medium (NGM) plate (**Table of Materials**). Transfer two adult ST12 worms onto the plate so that the worm spends most of the time in the bacteria during calcium imaging[12].

1.2. Incubate this NGM plate for 4 days at 20 °C in an incubator (**Table of Materials**).

2. Hardware setup

2.1. Perform calcium imaging using a microscope equipped with a piezoelectric device for

stimulation[13], a 2x objective lens, a high-speed sCMOS camera, image splitting optics, an x-y motorized stage, an LED light source for excitation, and a computer (**Figure 1**). Essential specifications of the sCMOS camera include 2560 x 2160 active pixels, 6.5 µm in size, with a 10-tap camera link as an interface option. Key specifications of the X-Y motorized stage include 110 mm x 75 mm travel range, 250 mm/s max velocity, 2,000 mm/s^2 max acceleration, and 0.25 µm unidirectional repeatability.

2.2. Turn on the precision computer and the x-y motorized stage controller.

2.3. Turn on the amplifier and set the **Volume** adjuster to 10 and the **Bass** and **Treble** adjusters to 8.

2.4. To excite GCaMP and TagRFP, turn on the 488 nm and 560 nm lights of the LED light source. Set the gauges of 470 nm and 550 nm of the control pod in the light source to 5% so that the intensity of the LED light is suitable for imaging.

NOTE: At this LED intensity, photobleaching of GCaMP and TagRFP fluorescence does not occur during recording.

3. Software setup for calcium imaging

3.1. Download and install three software packages in Windows: mouse macro software (software for vibration control), software for running tracking software, and software for data analysis (**Table of Materials**).

3.2. Double click on the tracking software file.

3.3. Push the **Run** button and wait for 5 min to stabilize the software (**Figure 2A**).

3.4. Provide the information on exposure time and binning. 0.033 s exposure time with 2 x 2 binning ensures smooth image acquisition (**Figure 2B**).

NOTE: Only every 10th image acquired is displayed in order to reduce the memory load.

3.5. Provide the information for image acquisition (**Figure 2C**). For example, when 500 images are recorded twice with an interval of 10 s, enter 1,000 in the **Images Total** box, 500 in the **Images** box, and 10 in the **Interval (s)** box.

3.6. Turn on the **Acquisition** button (**Figure 2D**).

3.7. Split the fluorescence image using image splitting optics, and the two images (GCaMP channel, 500–525 nm (**Figure 2E**); TagRFP channel, 584–676 nm (**Figure 2F**)) are projected onto two halves of the sCMOS camera. Calibrate the coordinates of the GCaMP and TagRFP channels (**Figure 2G**). Save the software settings.

3.8. Set the directory to output the data file (**Figure 2G**).

3.9. Turn off the **Acquisition** button (**Figure 2D**).

3.10. Double click on mouse macro system software (**Figure 3A**) and set the vibration properties (**Figure 3B**). Read the **Assay.txt** file with the mouse macro system so that the mouse cursor is controlled based on the coordinates and the schedule in that file (**Figure 3A**). Set the coordinates of the mouse cursor (magenta squares in **Figure 3A**) and wait time until stimulation after starting the recording (magenta in **Figure 3A**). Set the frequency and the amplitude values in the software for vibration control (**Figure 3B**).

4. Preparation of worms for calcium imaging

4.1. Transfer a single worm expressing both GCaMP and TagRFP from the incubated plate to a new fresh NGM plate on which *E. coli* OP50 has been streaked.

4.2. Attach the NGM plate to the piezoelectric acoustic transducer using transparent adhesive tape (**Figure 4**). Do not press the plate onto the actuator because this alters the vibration frequency.

5. Calcium imaging under the control of specific vibration properties

5.1. Turn on the **Acquisition** button (**Figure 2D**).

5.2. Find the worm under bright light and excitation light (488 nm and 560 nm).

5.3. Set the zoom value of microscopy to 2.5x. The field of view is 1.1 mm x 1.1 mm with a resolution of 2.6 µm/pixel.

5.4. Turn off the bright light and turn on the **Homing** button (**Figure 2F**) to track the fluorescent spot of a worm. This homing button starts the movement of the X-Y stage to keep the maximum intensity region of the worm in the middle of the field of view in the TagRFP image.

NOTE: In terms of the flp-18 promoter, the fluorescent intensity of the AVA neurons is strongest and those from other neurons are faint or not detected. Therefore, the low magnification system is not required for tracking and the stage moves throughout the recording.

5.5. Ensure that the values of intensity bars in **Figure 2E** and **2F** are approximately 1,000. If they are not, fine-tune the 470 nm and 550 nm gauges of the control pod in the light source.

5.6. Press the **Run** button in the mouse macro system to allow mouse cursor control according to a schedule described in **Figure 3A**. This starts to record using the tracking software and stimulate by wave gene software while tracking the worm.

5.7. Check whether the output BMP file is appropriately created.

6. Data analysis

6.1. Create a folder on the desktop and name it **CalciumImaging**.

6.2. Create a folder inside the CalciumImaging folder and name it **CIResult**, for the output result files.

6.3. Double click on the **DualViewImaging.nb** file written in data analysis software for Windows.

6.4. Insert the file path to the CIResult folder (e.g., DirectoryName[**/Users/Sugi0911/Desktop/CalciumImaging/CIResult/test.rtf**]) (**Figure 5**).

6.5. Insert the file path to the folder including the BMP file (e.g., SetDirectory**[/Users/Sugi0911/Desktop/XXX/**]) (**Figure 5**), in which **XXX** denotes the folder name including BMP files.

6.6. Push the **Shift** and **Return** keys simultaneously to start an automatic analysis. This analysis uses the value of a maximum intensity region of field of view in the TagRFP image (See the legend of **Figure 6** for detailed calculation procedure in the program).

6.7. Check whether the following output files are appropriately created in the CIResult folder.
XXX-GCaMP.tif (image file illustrating traces of GCaMP intensities for all images)
XXX-GCaMP.xls (spreadsheet file listing GCaMP intensities in all images)
XXX-TagRFP.tif (image file illustrating traces of intensities of TagRFP for all images)
XXX-TagRFP.xls (spreadsheet file listing TagRFP intensities in all images)
XXX-Ratio.tif (image file illustrating traces of the ratio value for all images)
XXX-Ratio.xls (spreadsheet file listing the ratio value in all images)

REPRESENTATIVE RESULTS:

Here, a worm expressing both GCaMP and TagRFP under control of the AVA interneuron-specific promoter is used as an example of calcium imaging in freely behaving *C. elegans*. GCaMP and TagRFP channel data were obtained as a series of images, some of which are shown in **Figure 6** and as a movie (**Supplemental Movie 1**). The displacement of the Petri plate induced by our nonlocalized vibration system (**Figure 7**) was also quantified. The displacement can be controlled by setting the amplitude value in the software for vibration control (**Figure 3B**) and the **Volume** adjuster in the amplifier (**Figure 4**), whereas the frequency is regulated by setting the frequency value in the software (**Figure 4**). In the successful experiments, a transient calcium response of AVA neurons was observed upon stimulation with vibrations having a frequency of 630 Hz and a displacement of approximately 4.5 µm for 1 s, indicating that the AVA neuron was activated during a worm's backward response to the nonlocalized stimulation (**Figure 6**). Baselines of both

GCaMP and TagRFP signals also showed that almost no photobleaching occurred during recording.

FIGURE AND TABLE LEGENDS:

Figure 1: A schematic system configuration. An NGM plate on which a worm freely moves is held on an acoustic transducer. Excitation light (488 nm and 560 nm) is emitted from an LED light source. GCaMP and TagRFP fluorescent emissions are split by image-splitting optics, such that each fluorescent emission is projected onto half of an sCMOS camera. Tracking software controls the x-y motorized stage to track the fluorescent spot of the worm.

Figure 2: Screenshot of the software for tracking a fluorescent spot of a worm. (**A**) The **Run** button for activating the software. (**B**) Boxes for setting image display cycle, exposure time (s), and binning in each channel. (**C**) Boxes for providing information on the image acquisition cycle. (**D**) The **Acquisition** button to initiate live streaming. (**E**) GCaMP image. (**F**) TagRFP image. (**G**) A panel for calibration coordinates between GCaMP and TagRFP images. (**F**) The **Homing** button to start worm tracking.

Figure 3: Photo of the mouse macro system and software for controlling vibration. (**A**) The magenta square indicates the coordinates for the mouse cursor. The meaning of each script line is explained in magenta letters. (**B**) The GUI (graphical user interface) of software for controlling vibration. Vibration frequency and amplitude values are controlled using this GUI.

Figure 4: Photo of the NGM plate held on the acoustic transducer using transparent adhesive tape. Only a single worm is moving on the plate.

Figure 5: Photo of the data analysis software. Magenta and blue underlines indicate the paths to the CIResult folder and the folder including BMP files, respectively.

Figure 6: Traces of the intensities of GCaMP (A), and TagRFP (B) fluorescence, and the ratio change (C,D). (**A–C**) The representative traces for GCaMP signal, TagRFP signal and ratio changes of a single worm. The ratio change is calculated using the **DualViewImaging.nb** file, as follows. The coordinate in which the intensity of TagRFP fluorescence is maximum after background subtraction is extracted for each image. This TagRFP signal serves to compensate for focus changes caused by the animal's motion. The maximum fluorescence intensity of GCaMP within the extracted coordinate ±10 pixels is calculated as the GCaMP signal intensity for each image. For ratiometry, $((I_{GCaMP} - I_{GCaMP_BG}) - (I_{TagRFP} - I_{TagRFP_BG})) / (I_{TagRFP} - I_{TagRFP_BG})$ is calculated, in which I_{GCaMP} and I_{TagRFP} are the GCaMP and TagRFP signals from the AVA neuron, respectively, and I_{GCaMP_BG} and I_{TagRFP_BG} are the background signals, respectively. This calculation process was applied to all images to quantify the ratio change in a reversal event. The vertical line at 5 s in all traces indicates the stimulation period. (**D**) The average ratio changes for 15 worms. Error bar indicates SEM.

Figure 7: Relationship between parameters. (**A**) Replacements were measured every 100 Hz using a laser doppler vibrometer, in which the amplitude value in the software and the VOLUME adjuster value in the amplifier were fixed to 0 and 10, respectively. The resonance frequency was observed between 100–200 Hz, which was similar to the value described in the specification of the acoustic transducer. (**B**) Frequencies and displacements were controlled by the software (range; 100 to 1,000 Hz) and the amplifier (range: 1–10), respectively and measured using a laser doppler vibrometer. The amplitude value selected by the software is indicated for each frequency.

Supplementary Movie 1: A representative movie for calcium transients in an AVA interneuron of a freely behaving worm in response to a 630 Hz nonlocalized vibration. Stimulation was evoked 10 s after starting the recording. GFP was also expressed in the intestine of the worm as a co-injection marker for GCaMP. This GFP fluorescence does not affect the signal intensity of GCaMP. The scale bar is also indicated in the first image. The direction of the worm is indicated at the top right after the first image. The Windows media player is suitable for playing the AVI file, but it can also be played using some free downloaded media players, such VLC media player in the Mac.

DISCUSSION:

Generally, the quantification of neural activity requires introduction of a probe and/or restraints on animal body movement. However, for studies of mechanosensory behavior, the invasive introduction of a probe and restraints themselves constitute mechanical stimuli. *C. elegans* provides a system to circumvent these problems, because its features are transparent and because it has a simple, compact neural circuit comprising only 302 neurons. Combining these advantages with the previously developed method of evoking nonlocalized vibrations of nanoscale displacement[13], illustrated here is a method for noninvasive calcium imaging of unrestrained *C. elegans* responding to controlled vibration.

Properties of mechanical stimuli are defined by several parameters, such as frequency, displacement, and duration. Recently, biotremology has emerged as a new research field[1]. The main purpose of such studies is to understand mechanisms underlying production, dispersion, and reception of mechanical vibrations by organisms, and the influence of those vibrations on behavior. Many animals, including humans, use vibration and acoustic waves to monitor the surrounding environment. For example, scorpions detect prey through substrate-borne vibration[14]. Therefore, vibration is a tool of communication with the surrounding environment. The method described here can be used to control input parameters and to quantify neural responses at the single-neuron level, leading to a creation of a phase diagram describing the input-output relationship. Based on such phase diagrams, we can gain insights into which parameters affect specific neural circuits, and by what mechanisms animals cope with mechanical vibrations.

Since 2007[15], several systems have been developed for single-neuron calcium imaging in freely moving *C. elegans*. These systems have mainly focused on neuronal responses to temperature[15,16], odorants[16,17], and touch stimuli[17]. Regarding nonlocalized vibrations, the

method of tapping a Petri plate has long been applied for quantifying behavioral responses. However, tapping a Petri plate on a motorized x-y stage caused the fluorescent spot to exit the field of view, leading to failure to track a freely behaving worm. Therefore, the piezoelectric acoustic transducer was embedded, which evokes nonlocalized vibration across the z-axis, in the tracking system. This method allows the performance of reproducible experiments for quantifying neuronal responses to nonlocalized vibration.

The demonstrated methods also offer the possibility of further methodological extension. Since the current protocol can capture responses of only a single neuron in two dimensions, the autofocus system should be equipped in the future to prevent loss of focus. On the other hand, because several volumetric imaging methods have recently been developed using *C. elegans*[18–21], extension of the approach to volumetric imaging should allow noninvasive quantification of multiple neurons[22] or even the whole brain[18–21], in response to vibration having controllable properties. Such a system might be applied to the recently established system to investigate mechanisms underlying collective behavior[23]. Therefore, further biological applications and extensions of the method likely will enable us to understand neural mechanisms underlying mechanosensory behaviors.

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DISCLOSURES:

The authors have no conflicts of interest to disclose.

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Figure 1

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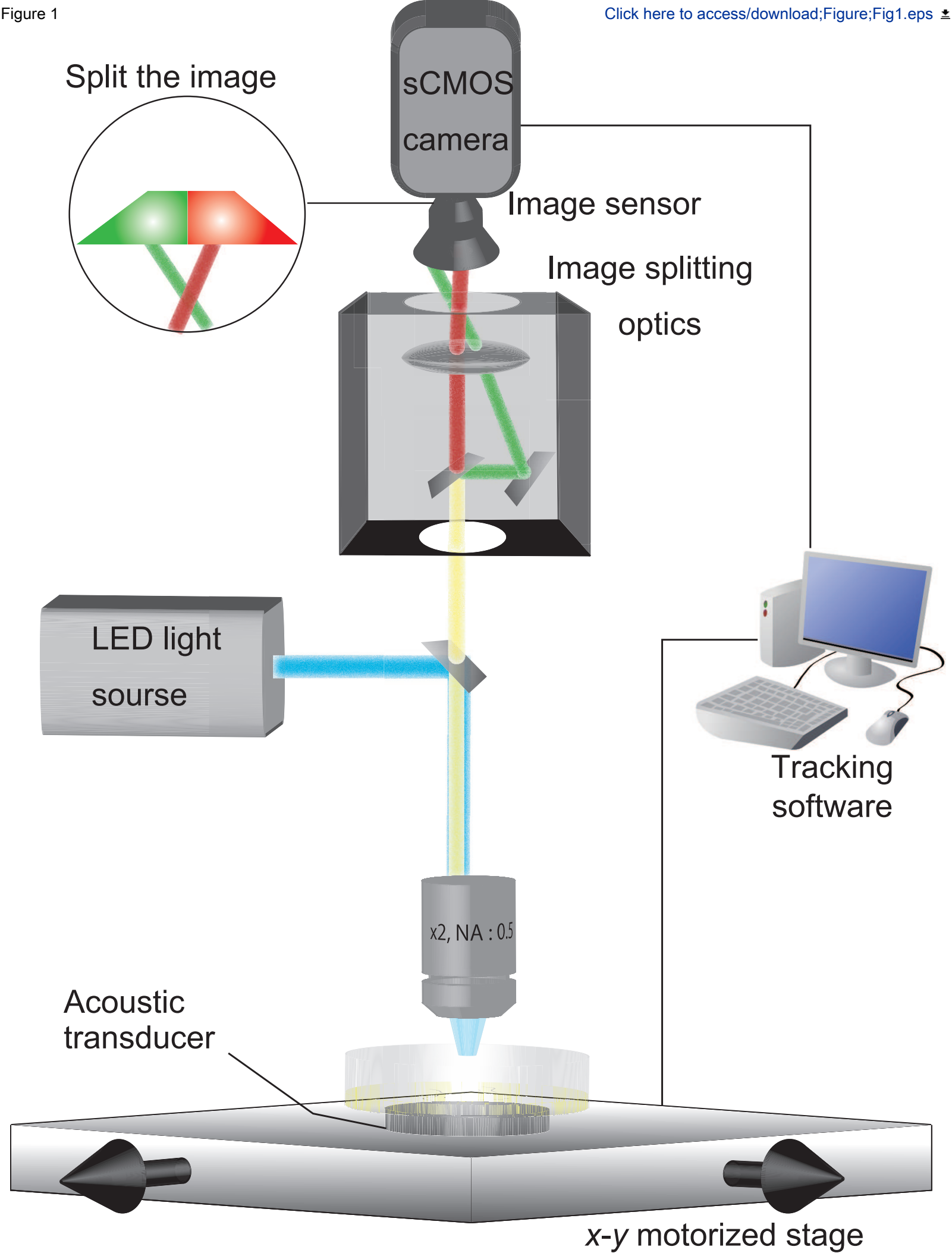

Figure 2

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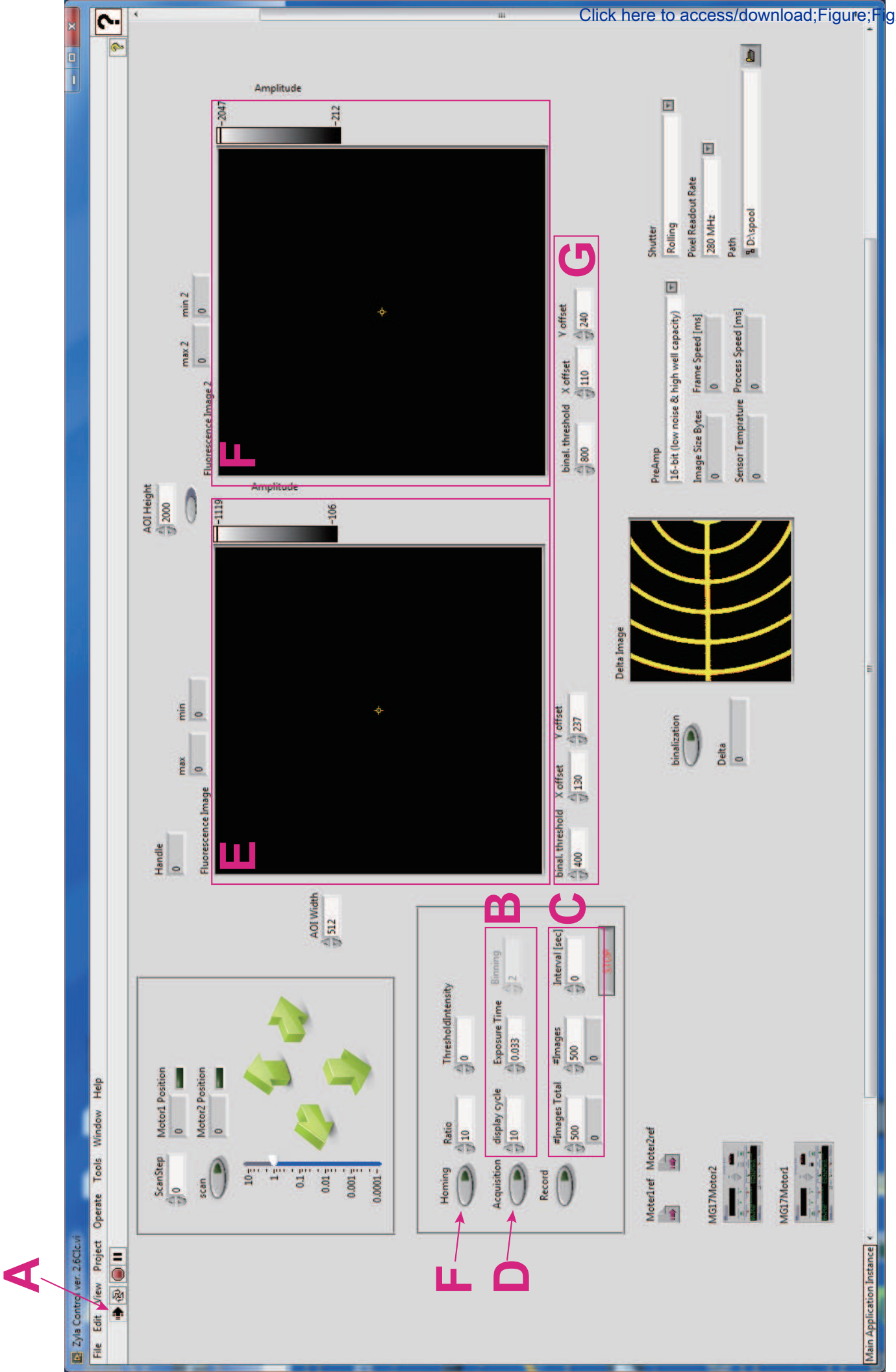

Figure 3

A

LMouse Down (58,752)
LMouse Up (58,752)
Press and release of recording button of GUI in Zyla Control ver.2.6CI.vi software

5000 ← Waiting time until stimulation (5 s)

LMouse Down (1428,49)
LMouse Up (1428,49)
Press and release of run button of GUI in WaveGene software for starting stimulation

1000 ← Stimulation time (1 s)

LMouse Down (1454,47)
LMouse Up (1454,47)
Press and release of stop button of GUI in WaveGene software for ending stimulation

B

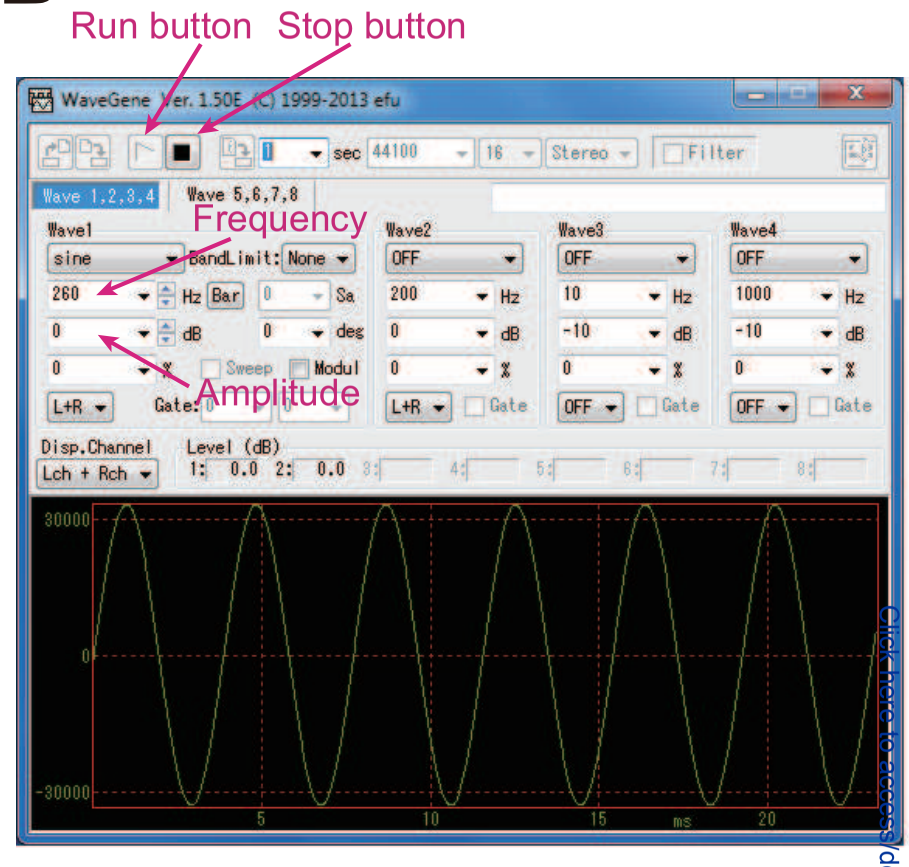

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Figure 4

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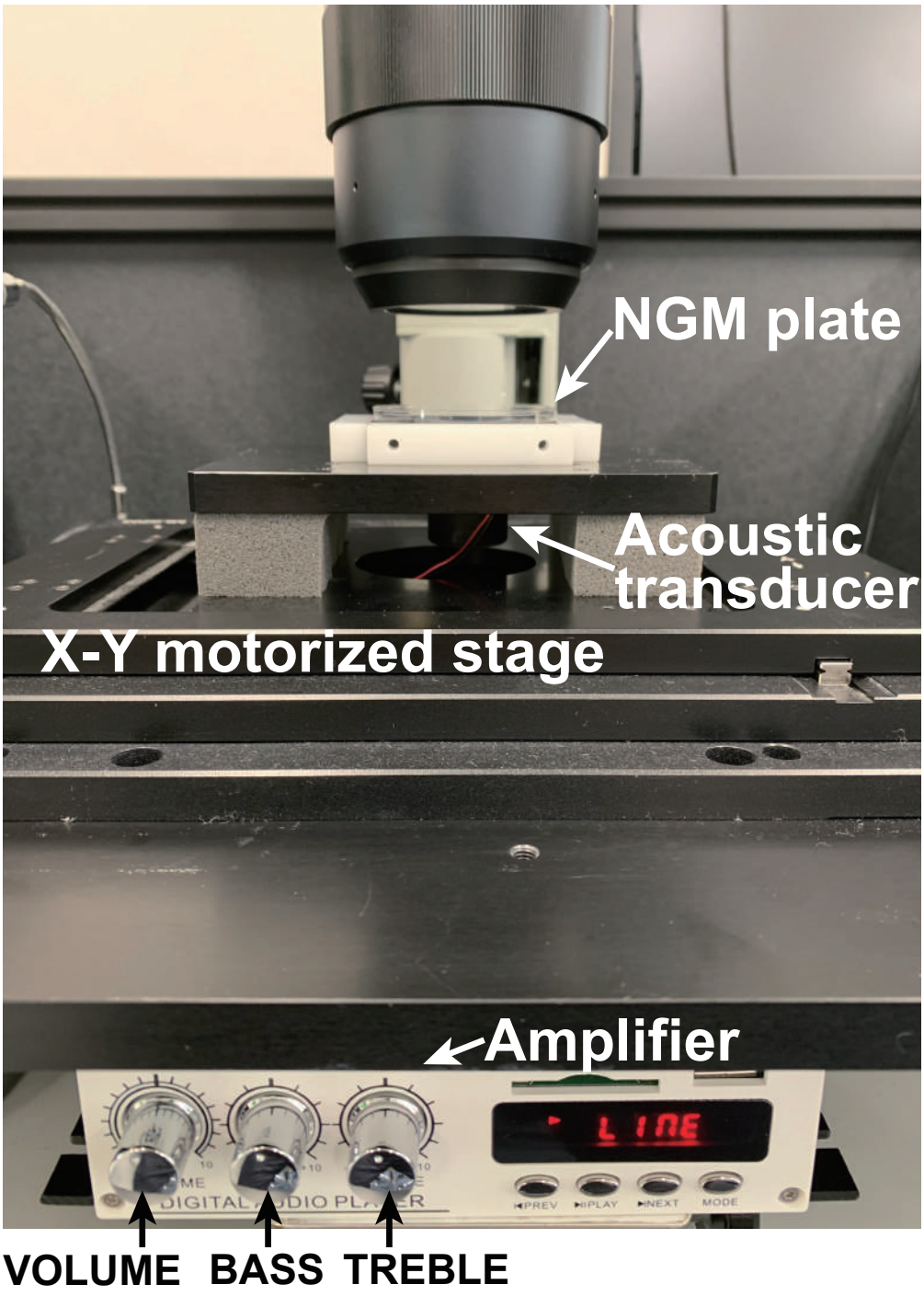

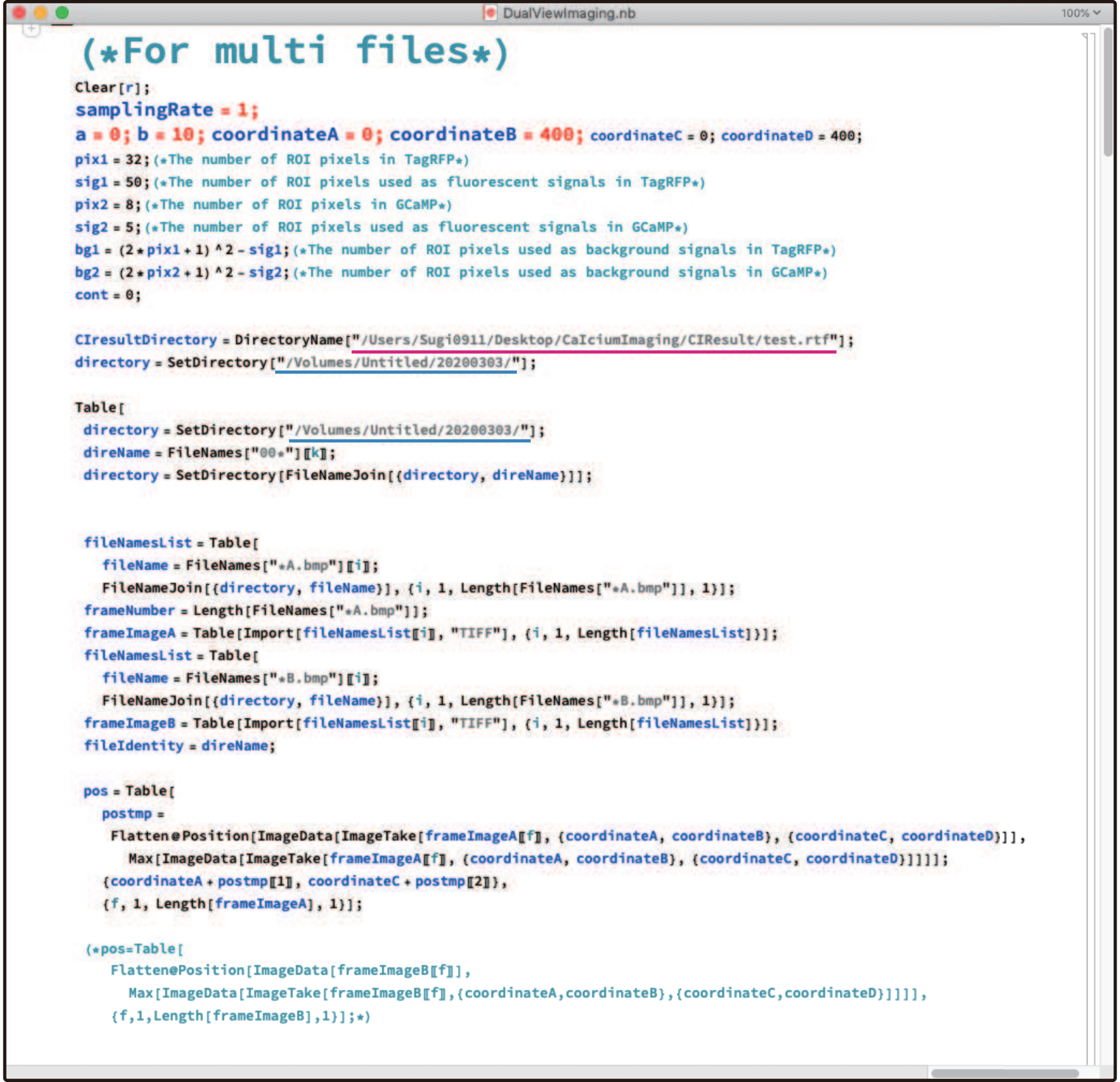

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sig1 = 50;(*The number of ROI pixels used as fluorescent signals in TagRFP*)
pix2 = 8;(*The number of ROI pixels in GCaMP*)
sig2 = 5;(*The number of ROI pixels used as fluorescent signals in GCaMP*)
bg1 = (2*pix1+1)^2-sig1;(*The number of ROI pixels used as background signals in TagRFP*)
bg2 = (2*pix2+1)^2-sig2;(*The number of ROI pixels used as background signals in GCaMP*)
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    Flatten@Position[ImageData[ImageTake[frameImageA[[f]], {coordinateA, coordinateB}, {coordinateC, coordinateD}]],
      Max[ImageData[ImageTake[frameImageA[[f]], {coordinateA, coordinateB}, {coordinateC, coordinateD}]]]];
   {coordinateA + postmp[[1]], coordinateC + postmp[[2]]},
   {f, 1, Length[frameImageA], 1}];

 (*pos=Table[
    Flatten@Position[ImageData[frameImageB[[f]]],
      Max[ImageData[ImageTake[frameImageB[[f]],{coordinateA,coordinateB},{coordinateC,coordinateD}]]]],
    {f,1,Length[frameImageB],1}];*)
```

Figure 5

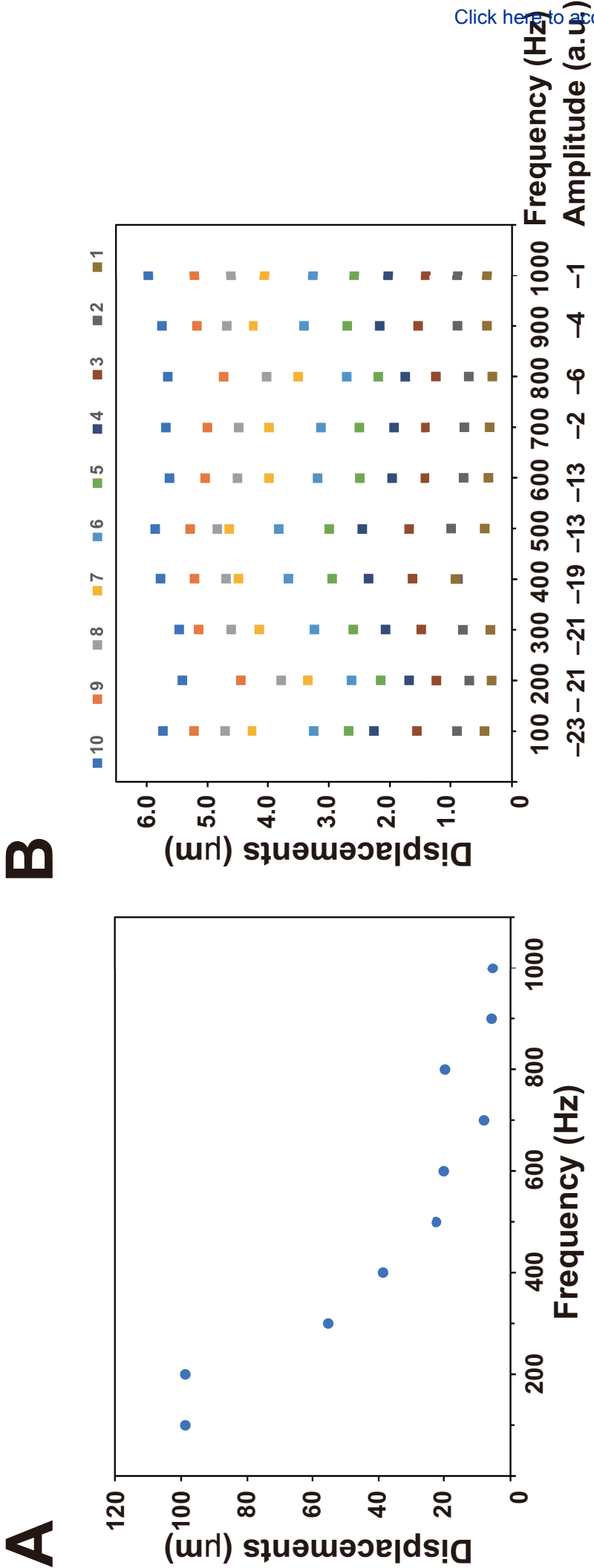
Click here to access/download;Figure;Fig5.eps

Figure 6

Click here to access/download;Figure;Fig6.eps

Figure 7

Click here to access/download;Figure;Fig7.eps



Click here to access/download;Table of Materials;Table of Materials- 61626R2.xlsx

Name of Reagent/ Equipment	Company	Catalog Number	Comments/Description
Data anaylsis software			
DualViewImaging.nb	author		For analysis of acquired data
Mathematica12	Wolfram		For running data anaysis software DualViewImaging
***Escherichia coli* and *C. elegans* strains**			
E. coli OP50	*Caenorhabditis* Genetics Center	OP50	Food for *C. elegans* . Uracil auxotroph. *E. coli* B.
lite-1(ce314) strain	*Caenorhabditis* Genetics Center	KG1180	Light-insensitive mutant
lite-1(ce314) strain expressing NLS-GCaMP-NLS and TagRFP under the control of the AVA-speciric promoter	author	ST12	*lite-1(ce314)* mutant carrying the genes expressing NLS-GCaMP5G-NLS (NLS; nuclear localization signal) and TagRFP under the control of the *flp-18* promoter as an extrachoromosomal arrays
Laser Doppler vibrometer			
Lase Doppler vibrometer	Polytec Japan	IVS-500	For quantifying frequency and displacement generated by the accoustic transducer
Mouse macro system			
Assay.txt	Author		Script for temporally and specially controlling mouse cursol in Windows
HiMacroEx	Vector	https://www.vector.co.jp/download/file/winnt/util/fh667310.html	Free download software for controling mouse cursor based on a script
Nematode growth media plate			
Agar purified, powder	Nakarai tesque	01162-15	For preparation of NGM plates

Bacto pepton	Becton Dickinson	211677	For preparation of NGM plates
Calcium chloride	Wako	036-00485	For preparation of NGM plates
Cholesterol	Wako	034-03002	For preparation of NGM plates
di-Photassium hydrogenphosphate	Nakarai tesque	28727-95	For preparation of NGM plates
LB broth, Lennox	Nakarai tesque	20066-95	For culture of *E. coli* OP50
Magnesium sulfate anhydrous	TGI	M1890	For preparation of NGM plates
Potassium Dihydrogenphosphate	Nakarai tesque	28720-65	For preparation of NGM plates
Sodium Chloride	Nakarai tesque	31320-05	For preparation of NGM plates
Petri dishes (60 mm)	Nunc	150270	For preparation of NGM plates
Nonlocalized vibration device			
Amplifier	LEPY	LP-A7USB	For stimulation with controllable vibration
Acoustic transducer	MinebeaMitsumi	LVC25	For stimulation with controllable vibration
WaveGene Ver. 1.5	Thrive	http://efu.jp.net/soft/wg/down_wg.html	Free download software for controling vibration property
Noninvasive calcium imaging			
2-Channel benchtop 3-phase brushless DC servo controller	Thorlabs	BBD202	Compatible controller for MLS203-1 stages
479/585 nm BrightLine dual-band bandpass filter	Semrock	FF01-479/585-25	For acquisition of two channel images (GCaMP and TagRFP)
505/606 nm BrightLine dual-edge standard epi-fluorescence dichroic beamsplitter	Semrock	FF505/606-Di01-25x36	For acquisition of two channel images (GCaMP and TagRFP)
512/25 nm BrightLine single-band bandpass filter	Semrock	FF01-512/25-25	For acquisition of two channel images (GCaMP and TagRFP)
630/92 nm BrightLine single-band bandpass filter	Semrock	FF01-630/92-25	For acquisition of two channel images (GCaMP and TagRFP)

Computer	Dell	Precision T7600	Windows7 with Intel Xeon CPU ES-2630 and 8 GB of RAM
High-speed x-y motorized stage	Thorlabs	MLS203-1	Fast XY scannning stage
Image splitting optics	Hamamatsu photonics	A12801-01	For acquisition of two channel images (GCaMP and TagRFP) generated by W-VIEW GEMINI Image spliting optics
LED light source	CoolLED	pE-4000	For generating 470 nm and 560 nm excitation light
Microscope	Olympus	MVX10	
sCMOS camera	Andor	Zyla	
x 2 Objective lens	Olympus	MVPLAPO2XC	Working distance 20 mm and numerical aperture 0.5
Plasmid			
pKDK66 plasmid	author	pKDK66	Co-injection marker
pTAK83 plasmid	author	pTAK83	Plasmid for expression of TagRFP under the control of the *flp-18* promoter
pTAK144 plasmid	author	pTAK144	Plasmid for expression of NLS-GCaMP5G-NLS under the control of the *flp-18* promoter
Tracking software			
homingback.vi	author		SubVi file for tracking a fluoresent spot of a worm through feedback control of sCMOS camera and x-y motorized stage
LabVIEW	National instruments		For running tracking software
Zyla Control ver.2.6CI.vi	author		For tracking a fluoresent spot of a worm through feedback control of sCMOS camera and x-y motorized stage

Rebuttal Letter
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Reviewer #3:

Manuscript Summary:

This article describes methodology for quantifying calcium currents in a single C. elegans interneuron during the escape response evoked by vibration. The authors also describe the use of a piezoelectric acoustic transducer to evoke nonlocalized vibration of a specific amplitude, duration, and frequency. They show that this method is useful for studying the neural mechanisms underlying nematode response to mechanical stimuli. Because they were able to record calcium response at a single cell resolution in freely behaving worms the imaging method should be generally applicable to other behavioral studies. The protocol described is clear and other researchers should be able to duplicate it.

Major Concerns:

none

Minor Concerns:

Lines 39-40. "This method allows to produce well-controlled,.." Should be 'This method allows one to produce well-controlled..." or 'This allows the production of well-controlled..."

lines 304-305 " This allows to perform..." Should be 'This allows one to perform...' or 'This allows the performance of...'

Editor revised both sentences. Thank you for reviewing our manuscript.

Reviewer #5:

Manuscript Summary:

In this revision, the author has done an impressive job addressing almost all concerns of the reviewers. I do not have any further problem with the manuscript and I recommend the paper to be published. It provides a useful system for researchers in the field of neuronal imaging and mechanosensation.

Major Concerns:

None

Minor Concerns:

None

Thank you for reviewing our manuscript.