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Single Wavelength Shadow Imaging of Caenorhabditis elegans Locomotion including Force Estimates.

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Corresponding Author:	Jenny Magnes, Ph.D. Vassar College Poughkeepsie, NY UNITED STATES
Corresponding Author Secondary Information:	
Corresponding Author E-Mail:	jemagnes@vassar.edu
Corresponding Author's Institution:	Vassar College
Corresponding Author's Secondary Institution:	
First Author:	Alicia Jago
First Author Secondary Information:	
Other Authors:	Alicia Jago
	Tewa Kpulun
	Kathleen Raley-Susman, Ph.D.
Order of Authors Secondary Information:	
Abstract:	This study demonstrates an inexpensive and straightforward technique that allows the measurement of physical properties such as position, velocity, acceleration and forces involved in the locomotory behavior of nematodes suspended in a column of water in response to single wavelengths of light. We demonstrate how to evaluate the locomotion of a microscopic organism using Single Wavelength Shadow Imaging (SWSI) using two different types of examples. The first example is a systematic and statistically viable study of the average descent of C. elegans in a column of water. For this study, we used living and dead wildtype C. elegans. When we compared the velocity and direction of nematode active movement with the passive descent of dead worms within the gravitational field, this study showed no difference in descent-times. The average descent was 1.5 mm/s ± 0.1 mm/s for the both, live and dead worms using 633 nm coherent light. The second example is a case study of select individual C. elegans changing direction during the descent in a vertical water column. Acceleration and force are analyzed in this example. This case study demonstrates the scope of other physical properties that can be evaluated using SWSI while evaluating the behavior using single wavelengths in an environment that is not accessible with traditional microscopes. Using this analysis we estimated an individual nematode is capable of thrusting with a force in excess of 28 nN.

	Our findings indicate that living nematodes exert 28 nN when turning, or moving against the gravitational field. The findings further suggest that nematodes passively descend in a column of water, but can actively resist the force of gravity primarily by turning direction.
Author Comments:	We have revised our manuscript in accordance with the editor's and reviewers' comments. We have also commented on the revisions as appropriate.
Additional Information:	
Question	**Response**

VASSAR COLLEGE
DEPARTMENT OF PHYSICS AND ASTRONOMY

Jenny Magnes, Ph.D.

Phone: 845-437-7081
Email: jemagnes@vassar.edu

16 July 2013

Dear Editor,

I am enclosing our manuscript entitled "*Single wavelength shadow imaging of Caenorhabditis elegans locomotion including force estimations*." The article is original, unpublished, and not being considered for publication elsewhere. This paper describes an easily accessible method to monitor the locomotion of swimming nematodes using single wavelengths. The method is cheap and reveals allows for the measuring of force exerted by microscopic species in select instances. At present, there are no reports of such a method being used in the neuroscience community.

In this manuscript, we numerically reveal the descending time of living nematodes. We also demonstrate how to measure worm thrust and give some numerical examples. This method can be very valuable to the worm community for many types of studies, including studies on worm thrust and gravitational related behavior.

We believe that our results are important and novel. We would be grateful if the manuscript could be reviewed and considered for publication in JoVE.

Best Wishes,

Jenny Magnes, Ph.D.
Assistant Professor of Physics
Vassar College.

BOX 745 · 124 RAYMOND AVENUE · POUGHKEEPSIE · NY 12604-0745
PHONE 845 437-7340 · FAX 845 437-5995

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17 Quincy Street
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AUTHORS:

Jago, Alicia
Physics and Astronomy Department
Vassar College
Poughkeepsie, NY
aljago@vassar.edu

Kpulun, Tewa
Physics and Astronomy Department
Vassar College
Poughkeepsie, NY
tekpulun@vassar.edu

Raley-Susman, Kathleen, M.
Biology Department
Vassar College
Poughkeepsie, NY
kasusman@vassar.edu

Magnes, Jenny
Physics and Astronomy Department
Vassar College
Poughkeepsie, NY
jemagnes@vassar.edu

CORRESPONDING AUTHOR:

Magnes, Jenny
Physics and Astronomy Department
Vassar College
Poughkeepsie, NY
jemagnes@vassar.edu

KEYWORDS:
C. elegans, mechanosensation, nematode, shadow imaging, locomotion, video analysis, force

SHORT ABSTRACT:

The technique presented here measures the path of freely swimming microscopic species using single wavelength exposure. *C. elegans* are used to demonstrate shadow imaging as an inexpensive alternative to costly microscopes. This technique can be adapted to accommodate various orientations, environments and species to measure direction, speed, acceleration and forces.

LONG ABSTRACT:

This study demonstrates an inexpensive and straightforward technique that allows the measurement of physical properties such as position, velocity, acceleration and forces involved in the locomotory behavior of nematodes suspended in a column of water in response to single wavelengths of light. We demonstrate how to evaluate the locomotion of a microscopic organism using Single Wavelength Shadow Imaging (SWSI) using two different types of examples.

The first example is a systematic and statistically viable study of the average descent of *C. elegans* in a column of water. For this study, we used living and dead wildtype *C. elegans.* When we compared the velocity and direction of nematode active movement with the passive descent of dead worms within the gravitational field, this study showed no difference in descent-times. The average descent was *1.5 mm/s ± 0.1 mm/s* for both the live and dead worms using *633 nm* coherent light.

The second example is a case study of select individual *C. elegans* changing direction during the descent in a vertical water column. Acceleration and force are analyzed in this example. This case study demonstrates the scope of other physical properties that can be evaluated using SWSI while evaluating the behavior using single wavelengths in an environment that is not accessible with traditional microscopes. Using this analysis we estimated an individual nematode is capable of thrusting with a force in excess of *28 nN.*

Our findings indicate that living nematodes exert *28 nN* when turning, or moving against the gravitational field. The findings further suggest that nematodes passively descend in a column of water, but can actively resist the force of gravity primarily by turning direction.

INTRODUCTION:

Caenorhabditis elegans is a free-living beneficial soil nematode that is a powerful model organism for studying mechanisms of gene regulation, development and more recently for understanding sensory biology and behavior. Despite having only 302 neurons, *C. elegans* are capable of complex locomotory patterns, reproductive behaviors, navigation, chemotaxis and many other behaviors. *C. elegans* possess mechanoreceptors, chemoreceptors and even detect blue wavelengths of light (Ward et al, 2008)[1]. While much is known about the neural circuitry of sensorimotor function and general locomotory patterns in C. elegans, less is known about

the responses to multiple, concurrent stimuli or more complex environmental conditions than can be modeled under a microscope. A few studies have revealed more complex locomotory patterns that are highly plastic[2,3,4]. Our methodological approach will enable studies of nematodes in solution in real time where we can readily provide multiple environmental conditions simultaneously. This question is difficult to address using conventional microscope-based imaging techniques. We have developed an imaging technique that allows us to place nematodes within a water column to examine locomotory behaviors, as well as determine the capabilities of nematodes to change locomotion in response to different environmental conditions.

Single Wavelength Shadow Imaging (SWSI) is presented in this paper for the first time to address the shortcomings of traditional microscopes. Traditional microscopes are limited to observe species in a horizontal focal plane a few microns in depth[5,6]. Regarding single wavelength studies, most traditional microscopes use color filters to filter white light very broadly – typically, *50 – 100 nm*. Using a laser for SWSI narrows the wavelength selection to less than *1 nm* while maintaining significant light intensity[7]. Similarly, single wavelengths have been used to measure swimming frequencies of *C. elegans* in real time.[8]

For the first demonstration of our method, we monitor the horizontal position, *x*, and the vertical position, *y*, of a freely swimming *C. elegans* in a water column, over a distance of about a centimeter. In particular, we are interested in the vertical movement since gravity also acts vertically. The slope of a linear fit to the vertical position gives the vertical speed, v_y, of the nematode as it descends in the water column:

$$v_y = \frac{dy}{dt} \qquad (1)$$

The root mean square of the error (RMSE)[9] indicates the quality of the fit and indicates whether the descending speed is generally constant. The vertical speeds are then averaged for each species and dead worms. Using these results, the drag, which the worms experience can be estimated.

For the second demonstration of our method, we selected *C. elegans* that did not descend at a constant rate unlike the majority of the worms observed. The selected worms either turned around and swam upwards or hovered for a while before continuing the descent. Physically, this case study shows that the thrust of a swimming microorganism can be calculated. Newton's laws dictate that a body that changes directions accelerates, which implies a net force, $\vec{F}_{Net}$, is acting on that body:[10]

$$\vec{F}_{Net} = \frac{d\vec{p}}{dt}\ , \qquad (2)$$

where $\vec{p}$ is the linear momentum and t is time. The acceleration of the worm is directly proportional to the force acting on the worm since the mass of the worm remains constant. As a result, the vertical net force is:

$$\left(F_{Net}\right)_y = ma_y\ , (3)$$

where m is the mass of a worm and a_y represents the vertical acceleration. The net force in the vertical direction represents then the worm thrust in the same direction. The total thrust can be calculated by taking the horizontal component into account.

PROTOCOL:

1. *C. elegans* preparation

1.1) Prepare petri plates of young adult *C. elegans* as described in previous experiments involving suspension of *C. elegans* in a fluid filled cuvette[11].

1.2) On the day of the video analysis, pick live young adult nematodes directly into a cuvette filled with deionized, distilled water using a platinum pick as described in step 2.

1.3) Prepare dead *C. elegans* with chloroform exposure. Continue by following the procedure described for picking live nematodes described in step 2.

2. Optical setup for the video analysis

2.1) Assemble the experimental setup to create shadow images as shown in **Fig. 1.** The camera can be placed at any distance from the screen as long as it is able to capture a frontal view of the screen. A good place is next to the cuvette facing the screen. *[Place Figure 1 here]*

2.1.1) Using at least two mirrors to steer the tunable Helium-Neon laser output into a Galilean beam expander so that the beam is expanded to a diameter of 12 mm.

2.1.2) Place one or two pinholes in the beam path so that the beam passes through the pinholes without being obstructed. Use the pinholes to assure the alignment is maintained.

2.1.3) Direct the expanded beam onto a mounted quartz cuvette that is 10 mm wide, 10 mm deep and 4 cm tall.

2.1.4) Magnify the beam using a plano-convex lens with a positive focal length of 75 mm.

2.1.5) Place a projection screen approximately 120 cm from the lens. Distances greater than this will yield a greater magnification of the shadow image. However, the quality of the image will decrease with a lower light intensity and an increased noticeability of diffraction interference.

2.1.6) Place a high-speed camera, which is capable of at least 60 fps, right next to the cuvette facing the screen.

2.2) Place a dissecting microscope near the optical setup for rapid transfer of nematodes into the cuvette.

2.3) Temporarily secure a transparent ruler with mm divisions to the center of the cuvette holder perpendicular to the expanded beam so that the beam projects a magnified image of the ruler onto the screen. Set the length scale for video analysis.

2.3.1) On the projection screen, draw a 5 cm long line off center from the projected image to avoid interfering with the projection. [Video_1_633nm.MOV]

2.3.2) Record this image and measure the magnification. Remove the ruler from the setup. Repeat this step for other wavelengths as the angle of refraction of light for each wavelength through the lens will vary due to chromatic aberrations.

2.4) Replace the cuvette and fill it to within 1 mm of the rim with distilled water.

2.5) Begin recording while the room light is on so that the 5 cm line is included in the same footage as the projected image. Turn the light off.

2.6) Using a thin, flattened platinum wire pick, move individual young adult *C. elegans* one at a time from the agar plate into the cuvette by touching the pick to the surface of the water. The nematode will become visible in the water column when it enters the beam due to scattered light. [Video_2_CB678 633nm]

2.7) Film the projected images of the worms as they pass through the expanded laser beam. It is important to note that the projected image is inverted and the worms will appear to move in the opposite direction of their actual motion. Worms that are descending with gravity will appear to move upward on the screen (**Fig. 2**). *[Place Figure 2 here]*

3. Video data preparation

3.1) Import the video into the video analysis program.

3.2) Set the scale using the 5 cm line on the screen.

3.3) Track the linear displacement of the head of the shadowed nematode with at least 10 data points over the entire path taken.

3.4) Find the velocity in the vertical direction by taking derivative *("Y", "Time")* and divide this value by the magnification factor determined in step 2.2.

4. Data analysis

4.1) Analyze the linear descent of a *C. elegans*:

4.1.1) Check to ensure that the data points on the vertical position versus time graph generally form a straight line. Some deviations can be ignored due to the head movement of the

nematode. If the data points generally form a straight line, continue to step 4.1.2, otherwise the descent is nonlinear and the analysis should be continued using step 5.1 in this protocol.

4.1.2) Create a linear regression line[12] by fitting a straight line to the data from the 'Analysis' menu. The slope of this line is then the vertical speed of the *C. elegans.* The slope is the change in position divided by the change in time over a particular interval. Determine the descending speed of the live and dead worms in this manner (**Fig. 3**). *[Place Figure 3 here]*

4.1.3) Average the vertical swimming velocities from the nematodes. A sample size of about 50 worms is sufficient. Compare swim velocities in live worms with drift velocities in dead worms.

5.1) Analyze non-linear motion of *C. elegans*:

5.1.1) From Section 3, select an analyzed video file, which shows a nonlinear descent within the water column (**Fig. 4**). A nonlinear descent can be identified using step 4.1.1 in this protocol. *[Place Figure 4 here]*

5.1.2) Select 'Curve Fit' from the 'Analysis' menu in the video analysis software. Select a second order (quadratic) polynomial. Fit the curve.

5.1.3) For a region of interest, adjust the fit on the graph by sliding the brackets on each side of the fit on the graph until the curve is very close to the data points within the fit and/or well within the error bars. The fitting program gives a mathematical expression for the fitted curve, which is vertical position versus time. Consider the fitted curve errors given by the program associated with the physical properties. A relative error of *15 %* or below is usually acceptable.

5.1.4) Add more curve fits to cover additional sections of data. Spline[13] the functions for optimal coverage: make sure that the curve fits overlap and try to align adjacent curves so that the slopes match in the overlapping regions.

5.1.5) Obtain the velocity for a region of interest by taking the derivative of the fitted curve using the mathematical expression obtained in step 5.1.3. Velocities may vary in time. Note that the derivative is the slope of a function. The derivative can be obtained mathematically or graphically. In this case it is practical to use the given mathematical expression.

5.1.6) Take the second derivative of the fitted curves to obtain the acceleration. The acceleration may vary in time.

5.1.7) Multiply the acceleration by the worm mass to calculate the thrust, which the worm exerts. A reasonable estimated mass is *3* µg assuming that the worm consists mostly of water.

REPRESENTATIVE RESULTS:

Steady Descent

The first investigation shows no distinguishable difference in the descending rates of the *C. elegans* during SWSI using *633* nm. The descending rates were found to be constant at *1.5* mm/s ± 0.1 mm/s for both the live and the dead *C. elegans*. A sample size of 50 worms generated a reasonable variance of *7%* for both living and dead worms. There is no acceleration acting on the worms since the descending speed is constant, so that the drag force equals the gravitational force minus the buoyancy force. This implies that the density of the worm is slightly larger than that of water; however, for the estimations below it is still practical to assume that the density of a nematode is roughly that of water.

Changing Direction

The second investigation, a case study, demonstrates that nematodes are capable of changing direction and can swim upwards against gravity. Two curves were fitted to the vertical displacement of the nematode so that the second derivatives of those curves could be splined to graph acceleration versus time (**Fig. 5**). *[Place Figure 5 here]* It is advisable to keep the polynomial order as low as possible while maintaining a good fit. A lower polynomial order indicates less variation in the acceleration over time. The higher order polynomial terms will be negligible, and therefore *unnecessary, if the order of the polynomial is too high. This worm decelerates at a constant rate* of 0.110 mm/s^2 ± 0.002 mm/s^2, turns around and accelerates at the same rate 0.110 mm/s^2 ± 0.002 mm/s^2 until shortly after *the turnaround point*. The *C. elegans* continues to move upwards with a diminishing acceleration of 1.252 – 0.00708 t in mm/s^2 until the acceleration falls to zero. Keeping in mind **Eq. 3**, and an estimated worm mass of 3 µg, the worm undergoes a vertical net force, F_{Ny}, of 0.33 pN until shortly after the turnaround point.

Descent: There are three types of forces acting on the worm until the worm reaches the turnaround point: gravity, drag, buoyancy and thrust (Fig. 6a). *[Place Figure 6 here]* The net force equals the vector sum of all three forces. Here we consider the vertical components only:

$$F_{Ny} = F_{Dy}+F_{Ty}-F_g+F_B \quad (4)$$

where F_B is the buoyancy force. F_g is equal in magnitude but opposite in direction to the buoyancy force assuming that the density of the worm is that of water. **Eq. 4** can then be written in the following way:

$$F_{Ny} = F_{Dy}+F_{Ty} \quad (5)$$

F_{Ny} remains constant during the descent. This implies that $F_{Dy}+F_{Ty}$ remains constant until the nematode reaches the turnaround point. F_{Dy} is largest at the top, which is the beginning of the path, and gradually reduces to zero until the speed is zero at the turnaround point while F_{Ty} must increase to keep F_{Ny} constant.

Turnaround: There is no drag force in the vertical direction at the turnaround point since the vertical speed equals zero at that point. The only forces acting in the vertical direction are gravity, $-F_g$, buoyancy, F_B and the vertical worm thrust, F_{Ty}, as depicted in **Fig. 6b**. At this point, the thrust of the *C. elegans* can be determined:

$$F_{Ty} = F_{Ny}. \quad (6)$$

The thrust at the bottom of the trajectory is then roughly equal to 0.33 pN, which is about 0.001 % of the worm's weight. Taking into account that the estimated weight, F_g of a *C. elegans* is *28* nN*,*

Ascent: Similarly, during the ascent, drag increases but is pointed down (**Fig. 6c**):

$$F_{Ny} = -F_{Dy}+F_{Ty}. \quad (7)$$

The thrust implemented by the worm must now be even larger and equal the sum of the drag force and the weight. The worm is slowing down after beginning to swim up. To swim up at the same rate as the worm descended, the worm would have to exert an upward thrust of at least twice its weight.

Hovering

An example of a worm that slows its descent for about 3 seconds is presented in **Fig. 7**. *[Place Figure 7 here]* A 3rd degree polynomial is a reasonable fit for the overall path. The errors in acceleration and velocity from this fit are less than 15%. The worm starts with a significant upward thrust, slows down and starts to turn around at *68 s*; however, the the upward acceleration decreases continuously (**Fig. 8**) *[Place Figure 8 here]* until the net acceleration equals zero around 68.5 s. This eventually leads to a net acceleration in the downward direction followed by another zero point in the velocity (**Fig. 9**) *[Place Figure 9 here]* and the nematode starts to descend again at 69 s.

It is interesting that the maximum observed vertical acceleration in this case is 2.7 mm/s^2. The acceleration at the turning points are 0.455 mm/s^2 and - 0.455 mm/s^2 respectively; about four times larger than in the case of the nematode that turns around and swims up. Using **Eq. 6**, it can be estimated that the upward thrust is about 1.32 pN at the first turning point. At the second turning point, the net acceleration is negative so that the upward thrust is 1.32 pN.

Figure Legends:

Figure 1: Experimental Setup. The laser, beam expander, lens and screen are essential to the experimental setup. The steering mirrors and pinholes may be omitted, but will make the optical alignment less stable.

Figure 2: Single Wavelength Shadow Image (SWSI). Using *2* mW of 543 nm laser light the shadow of a worm is projected onto a screen. The image is inverted so that the nematode appears to fall upwards.

Figure 3: Vertical Descent of a Single Wildtype *C. elegans* in a Water Column. This nematode was shadowed by 633 nm coherent light. The slope of the linear fit indicates a downward speed of 1.09 mm/s ± 0.01 mm/s.

Figure 4: Displacement Graph of a Single Upward Swimming Wildtype *C. elegans*. Two splined fits trace the overall path of the nematode. The second derivative of the fit reveals the acceleration. The maximum acceleration is easily determined from the first fit: 0.110 mm/s^2 ±

0.002 mm/s^2. Only a few error bars are shown so that the data points and the fit remain visible. This nematode was shadowed by 633 nm coherent light.

Figure 5: Acceleration Graph of a Single Wildtype *C. elegans*. This nematode maintains a constant upward acceleration of 0.110 mm/s^2 ± 0.002 mm/s^2 causing it to slow down and then move upwards. Shortly after the turnaround point the acceleration decreases steadily and the net acceleration diminishes to zero.

Figure 6: Force diagrams for descent, turning point and ascent. Buoyancy and gravity are equal in magnitude and opposite in direction so that the effect of these forces cancel each other out. They are therefore not shown in these diagrams. (a) The worm is descending with drag and thrust pointing up. (b) The worm is at the low point of the trajectory without drag. Thrust is pointing up. (c) The worm is ascending with drag pointing down while thrust is pointing up.

Figure 7: Displacement Graph of Single Hovering Nematode. The worm slows down and starts to turn around at about 68 s but starts descending around 69 s.

Figure 8: Acceleration Graph of a Single Hovering Nematode. This worm starts with an upward acceleration of 2.7 mm/s^2, decreases to zero and eventually has a downward acceleration of -2.6 mm/s^2.

Figure 9: Velocity Graph of a Single Hovering Nematode. This worm comes to a stop at 68 s and starts to head upward, slows down and reaches zero velocity in the vertical direction at 69 s followed by a descent.

Table 1: Average Descent Velocities of N2 *C. elegans*. 50 live and 50 dead nematodes are tracked during their descent. The average velocity for the descents is the same for the live and the dead worms: 1.5 mm/s ± 0.1 mm/s.

DISCUSSION:

The SWSI technique provides an additional way to understand the locomotory capabilities of microscopic organisms like free-living nematodes. With this technique we have distinguished between active locomotion (swimming) and passive drift due to gravity operating on dead nematodes. In addition, when free-swimming nematodes change direction during locomotion in water, we are able to measure the drag forces and angular forces, which are operating on the nematodes and exerted by the nematodes.

Nematodes encounter different environmental conditions within the soil. There are water pockets within soil, as well as solid particles and biological materials of different shapes and textures. In addition, nematodes exist within a gravitational environment that they respond to.[14] Further, nematodes near the surface of the soil are exposed to different wavelengths of light, changes in temperature and humidity, as well as biological variables like bacteria, predatory fungi and other soil organisms. Nematodes must respond to all these different

variables, swimming and crawling in different media, turning and altering navigational strategies. All of these complex computations are carried out by only 302 neurons, a subset of which are involved in locomotion, and 95 body wall muscle cells. Measurements of the sort described by SWSI technique provide important insight into how nematodes accomplish this navigational complexity.

For the first part, we have measured the overall descending rate of wildtype *C. elegans* in 633 nm light. Using these measurements, we can estimate the drag force a worm encounters.

For the case study of an accelerating nematode, the forces involved change continuously since the drag force changes with speed. There are some statements that we are able to make about the forces acting on the worm. As the worm slows down and tries to swim upwards the vertical component of the drag force decreases until it reaches zero at the low point of the nematode's trajectory. At this point, the worm must exert an upward force to swim up.

This method can be modified in several ways. Any microscopic species that navigates in a clear liquid can be tracked using SWSI. Studies can be conducted with any wavelengths that are accessible to digital cameras. Digital cameras will typically pick up wavelengths ranging from the UV to near IR. In addition, horizontal studies can be conducted by directing the laser vertically upward. The species can then be placed on a horizontal transparent surface, like a microscope slide. Adjusting the beam expander or the magnifying lens after the beam expander can sharpen blurry images. The user should be sure to fasten all components to the table to ensure consistent and easy beam alignment.

The method is limited by available laser wavelengths and resolution. In essence the advantages of this method over existing microscopes, which are the flexibility in directions and wavelengths, are also weaknesses since the setup is simple. The unsophisticated optics and speckles of the laser limit the resolution. Some of these drawbacks can certainly be improved in the future by including spatial filter and projecting the image directly onto a CCD camera.

The most critical steps in the protocol can easily be learned as the experiment is performed for the first time. Placing the nematode in the cuvette without creating turbulence is critical. Also, vibrations may disturb the setup and alter the behavior of the worms. Be sure to limit the power, which is used to shadow image. 2 mW for a laser beam that is 1 mm in diameter should be the maximum to avoid heating effects. The setup should be tested for scattering effects when using liquids other than distilled water.

Currently most microscopes operate on a horizontal plane using white light or color filters, which are still very broad in the wavelength range. Microscopes that truly use single wavelengths and have flexibility in the viewing scenario, i.e., horizontal placement, are usually limited to one advantage or the other. Also, these types of microscopes are usually very expensive and still limited to focal planes unlike our method. Our setup can easily be built with an extremely low budget. This method is ready to be used by schools, environmental companies as well as other entities that operate with little funding. In the future, this method

can be used in a very sophisticated setup to study real time effects on locomotion and mechanosensation of microscopic species. This method makes single wavelength studies at a wide range of angles and viewing depths easily available.

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The authors have nothing to disclose.

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*Figure
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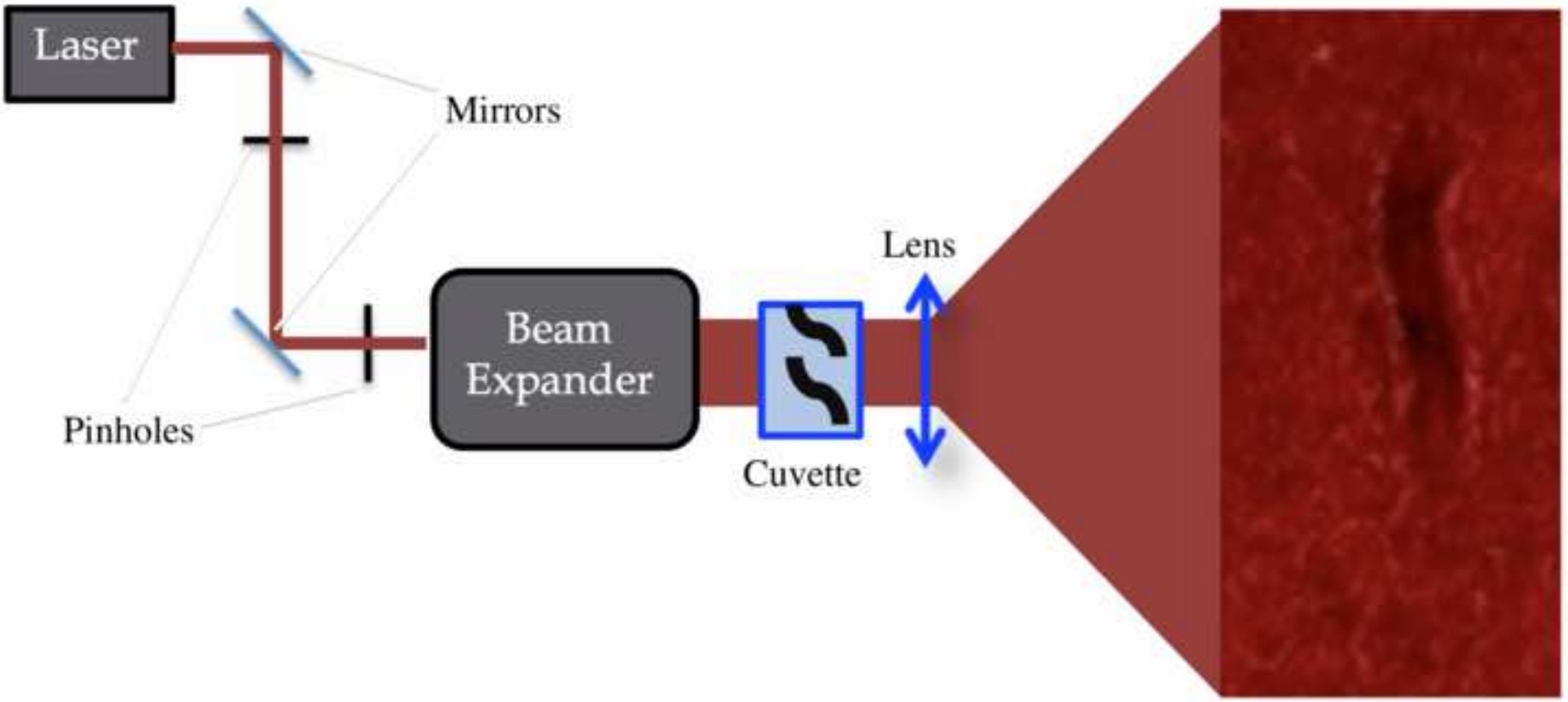

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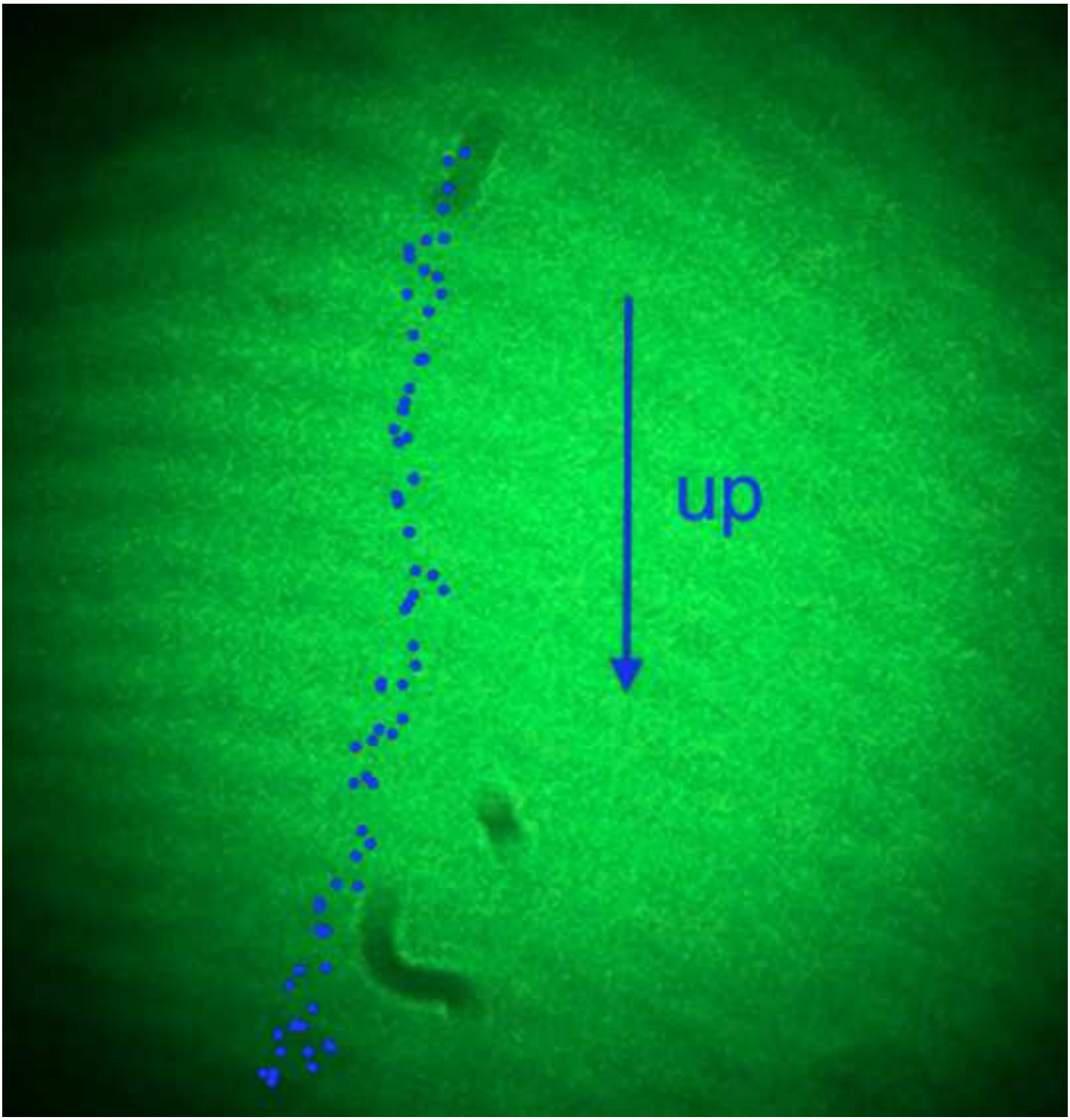

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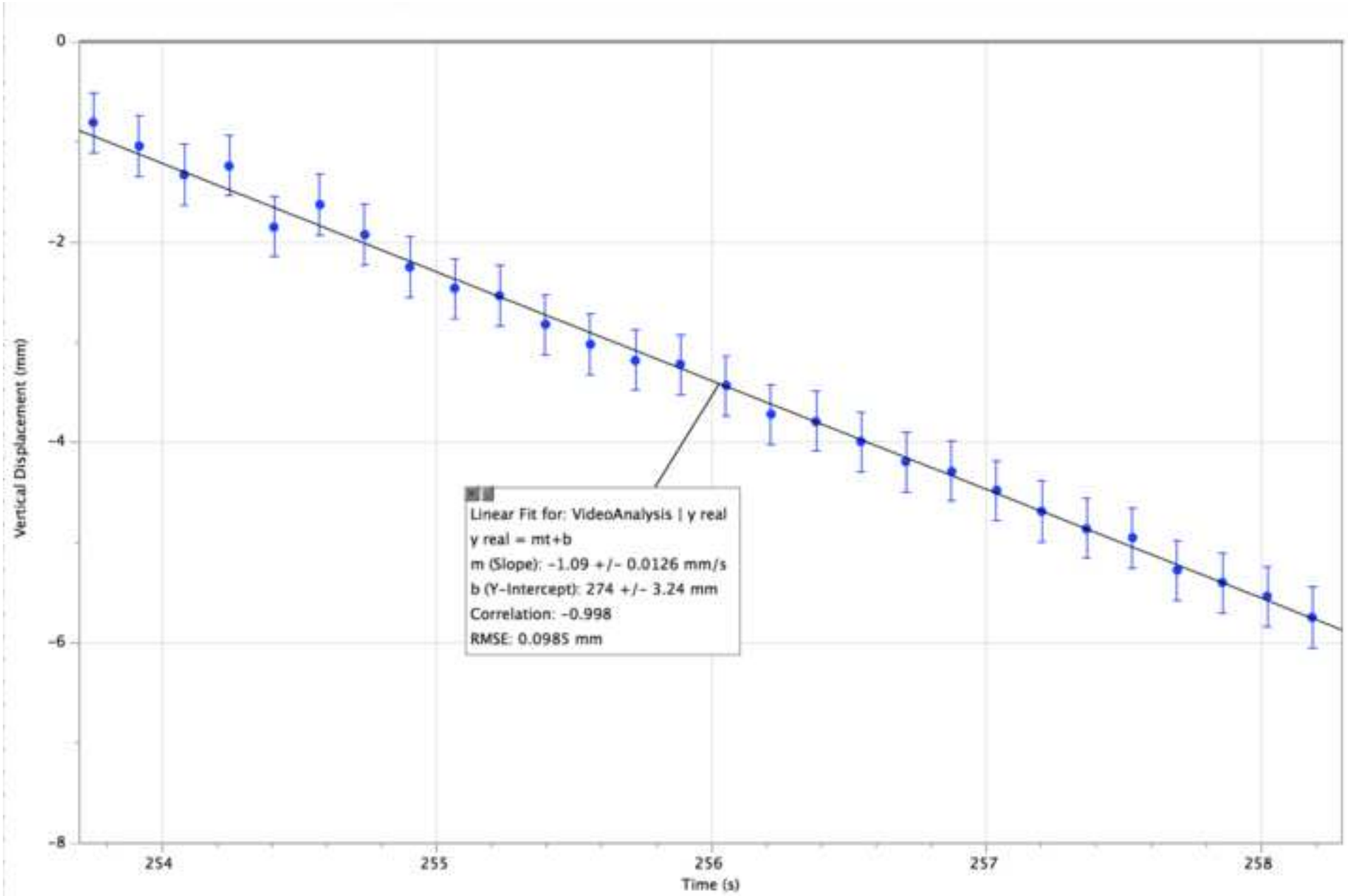

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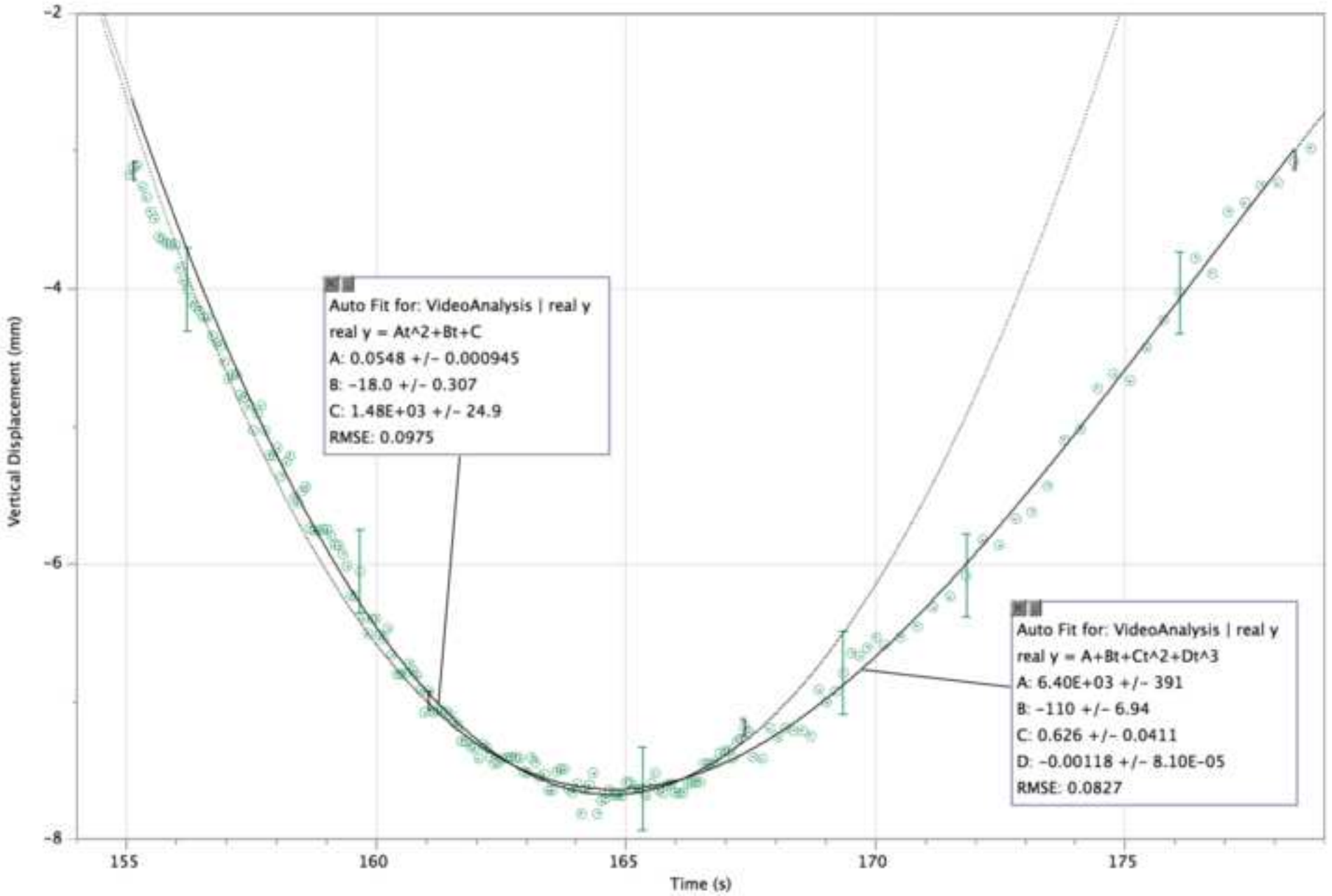

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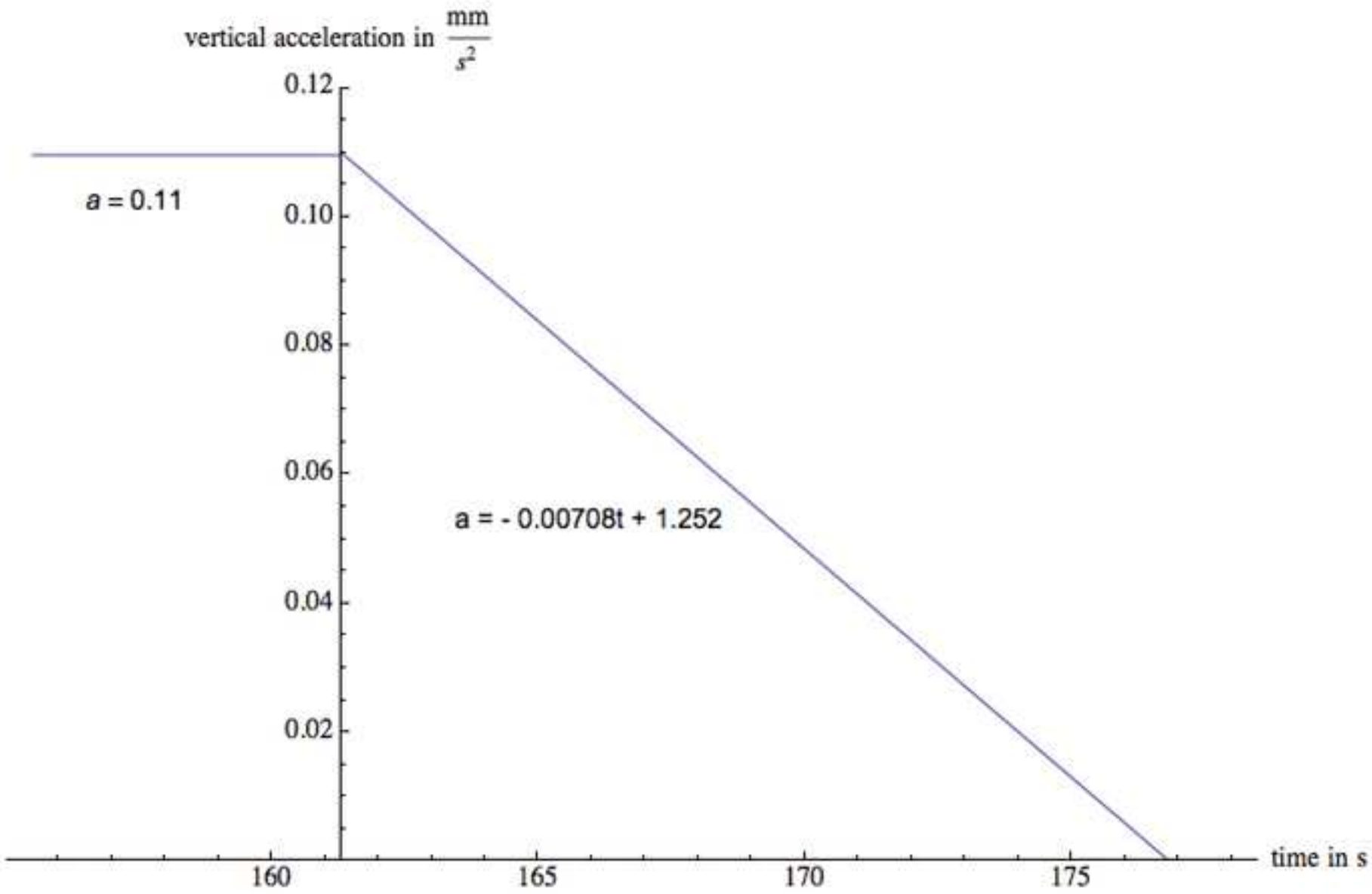

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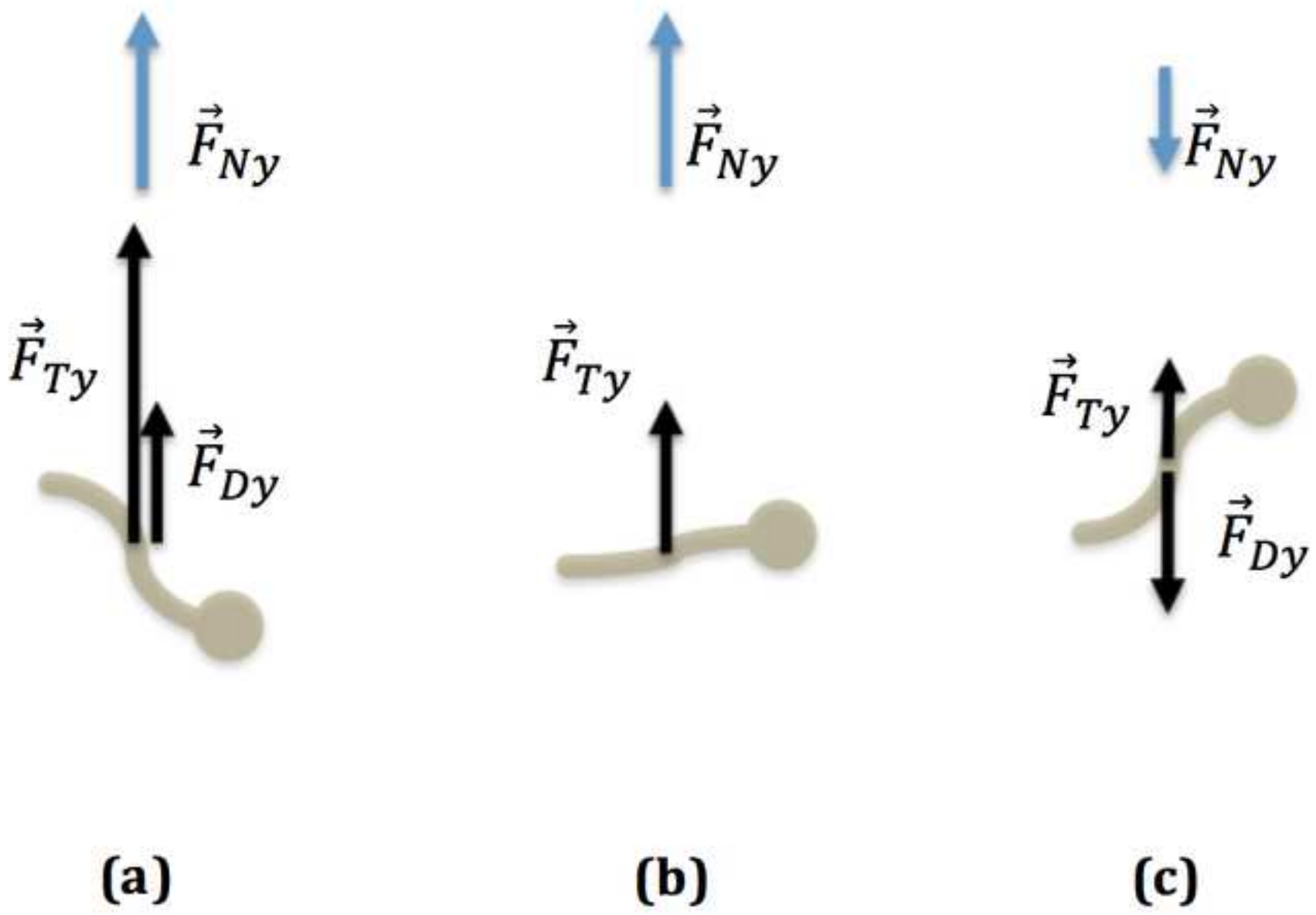

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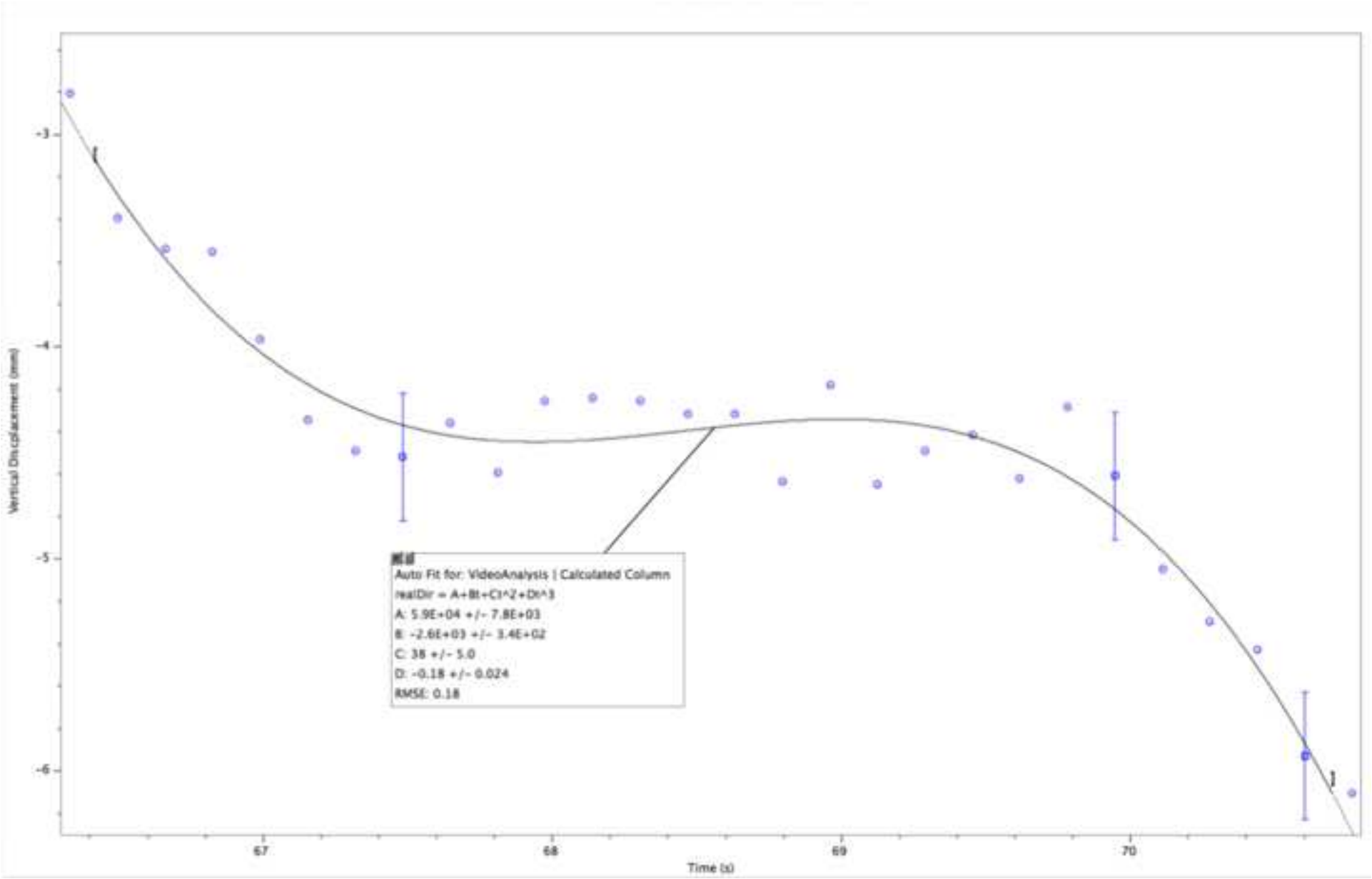

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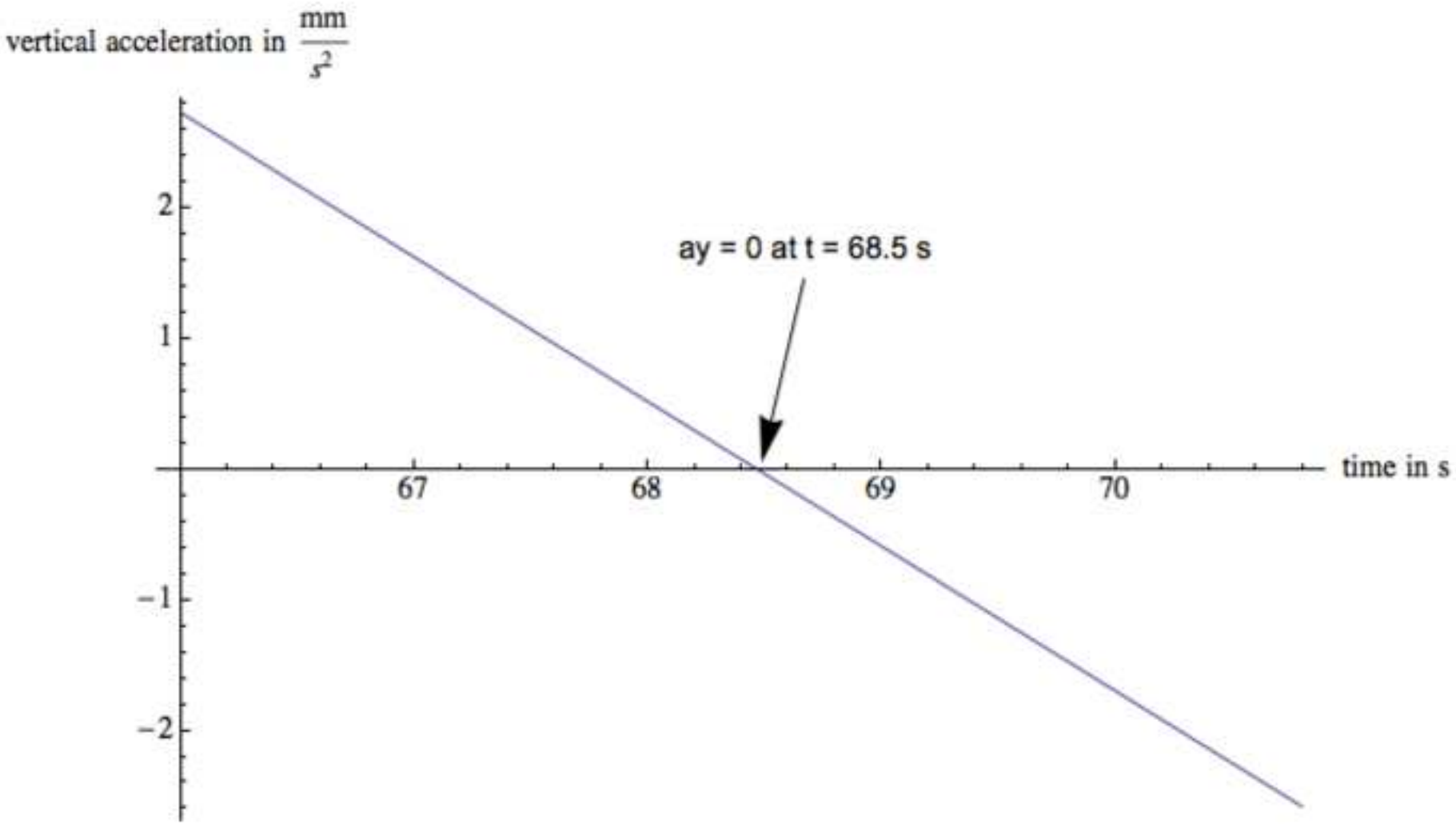

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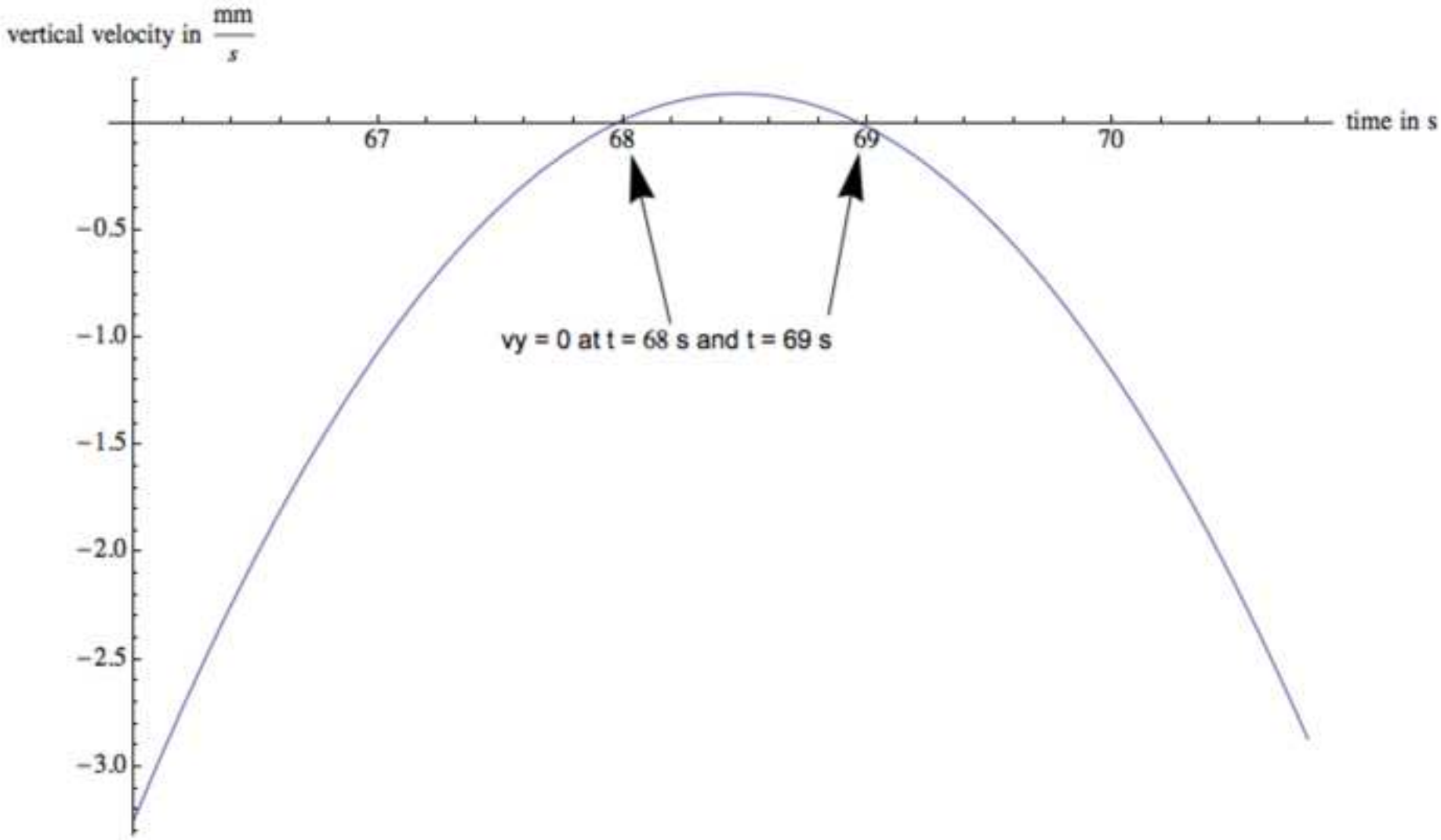

Table

Click here to download Table: Table1_N2 DeadandLive red.pdf

Dead	**Live**	**Dead**	**Live**
Vertical velocity (mm/s)	Vertical velocity (mm/s)	*Table continued*	
1.40	1.16	1.96	1.58
1.90	1.42	1.64	1.83
1.76	1.22	0.85	1.79
0.87	0.96	1.48	1.72
1.66	2.10	1.25	1.46
1.37	0.94	1.43	1.73
1.60	1.55	0.86	1.78
1.32	1.23	1.30	1.60
1.91	0.97	2.17	1.49
1.65	1.77	1.94	0.90
1.30	1.73	1.38	1.06
1.88	1.67	0.87	1.49
1.78	2.01	2.06	1.63
0.97	1.74	1.63	1.31
1.49	1.08	1.52	1.74
1.77	1.60	1.59	1.89
1.31	1.14	1.64	1.63
1.53	1.49	1.43	1.56
2.13	1.62	0.80	1.72
1.30	1.82	1.40	1.84
1.83	1.00	1.15	1.52
1.08	0.95	1.97	0.99
1.83	1.70	0.84	1.08
1.68	1.96	2.16	1.88
1.76	1.41	1.43	1.39
	Average	1.52	1.50
	Variance	0.14	0.11

*Excel Spreadsheet- Table of Materials/Equipment
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Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Tunable Helium-Neon laser	Research Electro-Optic	30602	Four wavelengths can be selected between 543 nm and 633 r
2 Front Surface Aluminum Mirrors	Thorlabs	PF10-03-F01	
High Speed Exilim Camera	Casio		
Quartz Cuvette	Starna Cells	21/G/5	
LoggerPro (Software)	Vernier		http://www.vernier.com/products/software/lp/
Mathematica 8	Wolfram		http://www.wolfram.com/
5x – 10x variable zoom Galilean beam expander	Thorlabs	BE05-10-A	
Plano-convex lens with a positive focal length of 75 mm	Thorlabs	LA1257	

ım.

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Author(s): Alicia Jago, Tewa Kpulun, Kate Raley-Susman, Jenny Magnes

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AUTHOR:

Name: Jenny Magnes

Department: Physics and Astronomy

Institution: Vassar College

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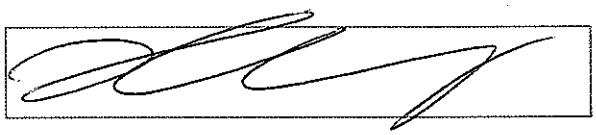
Signature:  Date: 16 June 2013

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1. Please remove the instructional grey and blue text from the manuscript template.

Response: instructional grey and blue text removed.

2. The abstract in your editorial manager account and the one in your manuscript do not match, please be sure that when you re-upload your revised manuscript that you update the abstract in your editorial manager file.

Response: It was not clear if you want the long or the short abstract here. Let me know if I do not have the right one now.

3. Please remove the parenthesis in-text citation "(Ward et al, 2008)" from the Introduction. JoVE follows recommended formatting for release to PubMedCentral, for specific reference questions please see the following: http://www.ncbi.nlm.nih.gov/staff/beck/citations/citationtags.html
Please also revise your "References" section such that the numbers in the reference list are not superscripted.

Response: In text citation removed. Superscript on references removed.

4. Please adjust the formatting of your protocol section so that all text is aligned to the left margin with no indentations.

Response: Text aligned to left margin.

5. Please revise step 1.1 to avoid the use of the pronoun "our."

Response: Deleted the pronoun “our.” The sentence still makes sense.

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Response: Commercial language has been removed from the text and appropriately replaced with generic terms.

7. Step 2.1 contains too many actions and is not written in imperative tense using step-by-step instructions.

A) Please simplify this step so that individual steps contain only 2-3 actions per step. We recommend that you split your longer steps into sub-steps numbered accordingly, i.e. step 1 is followed by sub-step 1.1 then 1.1.1 as necessary.

B) Please also re-write this step in imperative tense.

Response: The steps have been broken down and re-written.

8. Please also divide step 2.2 into sub-steps containing 2-3 actions per step.

Response: The steps have been broken down and re-written.

9. Several of your steps could use more detail to help viewers complete the protocol. When you

use terms such as, "Create a linear regression line" or "take the derivative" particularly, please answer the "how" question, i.e. is this step done through a computer program? If so, can you please provide step-by-step instructions for how to complete this operation using the computer program as you have done in step 4.2.2.

> *Response:* I have elaborated on all the indicated points. I am not sure about the extent needed here. Should explain how to take the derivative of a polynomial? Any scientist knows how to do that. Please advise if the changes are not sufficient.

10. Please do not underline any text in the manuscript (representative results). You may bold this text for emphasis.

> *Response:* Changed the underlined words to bold.

11. Please modify your discussion to cover the following in detail and in paragraph form: 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.

> *Response:* As requested a couple of paragraphs have been included to address concerns 1-5. However, item 3 is the last paragraph in the text.

Your revision is due by **Jul 30, 2013**.

To submit a revision, go to the *JoVE* submission site and log in as an author. You will see a menu item called 'Submission Needing Revision'. You will find your submission record there.

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Dear Editor,

We made the editorial changes you requested. We also thank the reviewers for their work.

Below are our responses in *green italics*:

Reviewer 1

We see the value in a labeled diagram and made changes accordingly.

- 64-65: ?.should read something like "for both the live and dead worms?.."
  - *inserted article 'the' as suggested and adjusted comma.*
- 117: ?.should read something like "this case study shows that the thrust?"
  - *change made*
- 118: first two commas should be taken out.
  - *change made*
- 121 - 126 Perhaps make two sentences here?
  - *Suggested change made*
- 160: Reference is made to video 1 in 2.3.1. I didn't understand at first why one needs a video here, but later (2.5) it became clear that it illustrates how the 5 cm line disappears when the light is turned off. Perhaps some additional explanatory note is in order.
  - *The video note is as directed by the editors of JoVE. This will stay as unless the editor directs other wise.*
- 179: Is Logger Pro the video analysis program?
  - *Yes, it is listed in the 'supplies list' as directed by JoVE we are not allowed to mention commercial products in the article.*
- 198: no comma after "file"
  - *We disagree with the reviewer here. A comma is placed since we are referring directly to the video file.*
- 203: This section (5.1.3) can only be understood by someone familiar with the operation of the software.
  - *We agree with the reviewer. The editor wanted specific instructions on how to complete this task on this software without us mentioning the specific commercial product. We are not sure how to resolve this without acting against the reviewer's request; however, the software is specified in the supplies list.*
- 225: replace "each," with "both".
  - *Change made*
- 273 - 276 The sentence that follows needs to be rewritten:

  The worm starts with a significant upward thrust, slows down to start turning around at 68 s; however, the continuous decrease of upward acceleration (Fig. 8) [Place Figure 8 here] until the net acceleration equals zero around 68.5 s.
  - *Yes, we see the issue with this sentence and changed it.*
- 313: Presumably they mean that there is no drag, and that the thrust is pointing up.
  - *Yes, we made a change but are not sure what the reviewer wanted changed here.*
- 322: "eventually has" rather than "has eventually"
  - *change made*
- 325: This caption needs clearer punctuation.
  - *change made*
- 339: don't need comma after "forces".
  - *We disagree with the reviewer here. A comma is placed since we are referring directly to the forces.*

- **Reviewer 2**

This manuscript describes a method for tracking the movements of C. elegans in a liquid environment using laser illumination and image analysis. The authors measure the descent rate of live and dead worms and estimate the forces exerted during swimming.

I have two concerns about this manuscript: First, there are major mathematical and conceptual errors in the analysis. For example, the estimate of mass is incorrect by about 3 orders of magnitude,

Our calculation could be rounded up one order of magnitude depending on the assumptions made. The reviewer does not explain or give a reference how he/she arrives at the indicated mass error. We arrived at the mass estimate by assuming that the worm is cylindrical, roughly 1mm long and 100 microns in diameter. This assumption leads to a worm volume of about 1 x 10^{-8} m^3. Assuming that the worm consists mainly of water, the density can be assumed to be 1 kg/decimeter^3. This gives a mass of 10^{-8} kg, which is 10^{-5} g or 0.01 mg or 10 micrograms, which is cannot even be considered an error of one order of magnitude since our initial estimate was 3 micrograms. We clearly state that this is an estimate and not the focus of this work. We do not think that this is worth expanding past the allowable length of the manuscript; however, we are clarifying in the text that we are assuming that the density of the worm is roughly that of water.

and the discussion of force balance neglects the buoyancy of the water, resulting in another error of about 2 orders of magnitude. These are, however, errors which could be corrected.

We recognize that buoyancy should be addressed explicitly. The reviewer does not offer an explicit reasoning for his/her conclusions. We have made the adjustments and necessary corrections in the manuscript as we see it.

A more serious problem is that method is poorly motivated and does not seem very useful. The emphasis on single wavelength imaging is perplexing, as multiple wavelengths do the job equally well, if not better. The discussion of the limitations of "conventional microscopes" is highly misleading (as detailed below). In fact, everything described in the manuscript could be done much more easily and inexpensively using a simple dark-field illuminated cuvette and a webcam-quality video camera. It seems unlikely to me that any scientists will find this technique helpful for their research.

Detailed comments:

"This study demonstrates an inexpensive and straightforward technique that allows the measurement of physical properties such as position, velocity, acceleration and forces involved in the locomotory behavior of nematodes suspended in a column of water in response to single wavelengths of light. "

This sentence seems to imply that nematode locomotion occurs in response to laser illumination. This doesn't make sense and I don't think it was intended.

*The reviewer is mistaken in his/her assumptions. We did mean to suggest that this method is a cheap and easily accessible method to conduct measurements in **various types of orientations** in response to **highly selective wavelengths**. We challenge the reviewer to provide the make and model of even one commercially available microscope under $100,000.- that is capable of using any commercially available laser wavelength.*

"It is currently unknown how C. elegans respond to different types of environments stimulating various types of locomotory patterns"

This overly broad statement neglects a large body of literature connecting mechanical, thermal, olfactory, and other sensory stimuli with behavioral responses in C. elegans. The authors should reference some of the many relevant papers on worm behavioral tracking.

We acknowledge that our statement seems to overstate things by being too general. We have modified our statement to be more specific to the goals of our project.

"While much is known about the neural circuitry of sensorimotor function and general locomotory patterns in C. elegans, less is known about the responses to multiple, concurrent stimuli or more complex environmental conditions than can be modeled under a microscope. A few studies have revealed more complex locomotory patterns that are highly plastic (Pierce-Shimomura et al, 2008; Berri et al, 2009; Vidal-Gadea et al, 2012). Our methodological approach will enable studies of nematodes in solution in real time where we can readily provide multiple environmental conditions simultaneously."

"Single Wavelength Shadow Imaging (SWSI) is presented in this paper for the first time to address the shortcomings of traditional microscopes."

It is not demonstrated how single wavelengths are helpful here. If anything the long coherence length of the laser source seems to create unnecessary fringes and noise in the image.

The reviewer is completely ignoring the remainder of the paragraph and the references to which he/she is referring. There are behavioral studies which have studied locomotory response with limited wavelengths using conventional light microscopy.

"Traditional microscopes are limited to observe species in a horizontal focal plane a few microns in depth"

Depth of field is determined by the numerical aperture of the imaging system, and can be as large as several centimeters with appropriate optics. For a relatively large field of view considered here, it is not necessary to use microscope optics. A conventional camera lens does quite well and is capable of a large depth of field.

The reviewer is partially correct here neglecting that there is a trade off for depth of field. Also, conventional cameras don't allow for single wavelength studies.

"A reasonable estimated mass is 3 <mu>g." Typical dimensions of a young adult worm are 1 mm length and 60 micron diameter. Assuming a density close to water, a worm has a mass of about 3 ng, three orders of magnitude smaller than stated.

Following the calculations presented earlier in this rebuttal, the reviewer made a calculation error even assuming a 60 micron width.

The buoyant force due to water displacement is neglected in the force balance. If worms had a density equal to that of water, there would be no net force due to gravity. For estimates of worm density the authors may see a recent paper from George Whitesides' lab.

We are grateful for pointing out this error and made appropriate corrections in this manuscript.

"The first investigation shows no distinguishable difference in the descending rates of the C. elegans during SWSI using 633 nm"

The authors need to perform a statistical test to justify this conclusion.

We are presenting a method here and not an extensive biological study. The data presented are not without value and is representative of the method as required by JoVE.

Figure 1: Where is the camera positioned?

We have included explicit instructions in procedure item 2.1.

Materials: The camera is described in the text as Nikon Coolpix and in the appendix as Casio Exilim. If two different cameras were used, please state this.

This issue was corrected during an earlier editorial review.