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## Probing cell mechanics with bead-free optical tweezers in the Drosophila embryo

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**TITLE**

# Probing Cell Mechanics with Bead-Free Optical Tweezers in the *Drosophila* embryo

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**KEYWORDS**

Light sheet microscopy, optical tweezers, force measurements, cell mechanics, *in vivo* imaging, *Drosophila* embryo

**SHORT ABSTRACT**

We present a setup of optical tweezers coupled to a light sheet microscope, and its implementation to probe cell mechanics without beads in the *Drosophila* embryo.

**LONG ABSTRACT**

Morphogenesis requires coordination between genetic patterning and mechanical forces to robustly shape the cells and tissues. Hence, a challenge to understand morphogenetic processes is to directly measure cellular forces and mechanical properties *in vivo* during embryogenesis. Here, we present a setup of optical tweezers coupled to a light sheet microscope, which allows to directly apply forces on cell-cell contacts of the early *Drosophila* embryo, while imaging at a speed of several frames per second. This technique has the advantage that it does not require the injection of beads into the embryo, usually used as intermediate probes on which optical forces are exerted. We detail step by step the implementation of the setup, and propose tools to extract mechanical information from the experiments. By monitoring the displacements of cell-cell contacts in real time, one can perform tension measurements and investigate cell contacts' rheology.

## INTRODUCTION

Embryonic development is a highly reproducible process during which the cells and tissues deform to shape the future animal. Such deformations have been shown to require the active generation of forces at the cell level[1,2]. To understand morphogenetic processes during which cells and tissues change their shape, it is therefore key to assess the mechanical properties of the cells involved, and to measure the forces within the tissue during the process[3,4]. Epithelial layers, especially in *Drosophila*, have been widely studied due to their quasi-2D geometry and to their easy manipulation.

A number of techniques have thus been developed by us and others to assess epithelial mechanics *in vivo* during development. We will give a quick overview of the three main techniques used in epithelial tissues. Laser ablation, a widely used method, allows to reveal the local mechanical stress at cell junctions[5-8] or at larger scale[9-11] by performing local cuts that disrupt the mechanical integrity of the target. The dynamics of the opening following the

cut provides information both on the stress prior ablation, and on the rheology of the tissue[12,13]. A drawback of laser ablation is that it is invasive, as it requires the local disruption of the cell cortex. Hence, one can only perform a limited number of ablations if one wants to preserve tissue integrity. Another drawback is that ablations only provide relative estimates of tension at cell contacts, since the opening velocity is dependent on viscous friction, which is generally not known. Magnetic manipulation has also been developed and used in *Drosophila*, involving either the use of ferrofluids[14] or ultramagnetic liposomes[15]. They can provide absolute measurements[16,17], but are also invasive in the sense that they require the injection of probes at the desired location. This can be very tricky depending on the system, which is not always amenable to precise injections. A third technique, fully non-invasive, is force inference[18-20]. Force inference relies on the assumption of mechanical equilibrium at triple points (tricellular junctions, or vertices), and allows to infer tensions at all cell-cell contacts (and possibly, pressures in all cells) by solving an inverse problem. For tensions, each vertex provides two equations (X and Y). This yields a large system of linear equations which can be inverted under some conditions to assess tension at all cell contacts. While this method is very attractive, as it only requires a segmented image and no extra experiment or setup, its accuracy is yet to determine, and again it only provides relative values, unless an absolute calibration measurement is performed.

To overcome some of these limitations, we introduce in this article a setup of optical tweezers coupled to a light sheet microscope to apply controlled forces at the cell scale in the embryonic epithelium of *Drosophila Melanogaster*. Optical tweezers have been used for numerous biological applications including the measurements on single proteins and manipulation of organelles and cells[21]. Here, we report applied forces in the range of a few dozen pN, which is small yet sufficient to induce local deformations of cell contacts and perform mechanical measurements *in vivo*. Typically, we use perpendicular deflection of cell contacts, monitored through the analysis of kymographs, to relate force to deformation. Importantly, our setup does not require the injection of beads at the desired location in the tissue, as optical tweezers are able to directly exert forces on cell-cell contacts. The coupling of the optical tweezers to a light sheet microscope allows one to perform rapid imaging (several frames per second), which is very appreciable for a mechanical analysis at short time scales, and with reduced phototoxicity, since the illumination of the sample is limited to the plane of imaging[22].

Overall, optical tweezers are a non-invasive way to apply controlled forces at cell contacts in vivo in the *Drosophila* embryo, and to extract mechanical information such as stiffness and tension at cell contacts[23], rheological properties[24], and gradient or anisotropy of tension[23].

## PROTOCOL

### 1. Setting-up the Light Sheet Microscope

1.1. Refer to the description of the setup in previous publication[25].

Note: The setup is composed of an upright microscope stage and a light sheet module producing a light sheet in the horizontal plane. A 10X excitation objective lens directs the light sheet into a glass cuvette (**Figure 4**). The detection objective lens has a high NA (1.1), which is necessary for efficient tweezing (see below).

## 2. Setting-up the Optical Tweezers Path

Note: **Figure 1** gives a general scheme of the optical setup.

2.1. Place the 1070 nm laser unit and fix the fiber on the optical table with a V-clamp mount. Ensure that the fiber collimator is parallel to the optical table (hence, horizontal) and that the height selected here will be the height for all the optical components aligned on the optical table.

Note: 100 mm can be a good choice for this height.

2.2. Turn the key of the infrared laser unit and press the **Select** button to turn on the alignment pointer. The light has to be horizontal outgoing of the fiber.

2.3. Place the shutter in the optical path, and fix it to the optical table, so that the infrared laser beam passes through the aperture of the shutter. Ensure that the distance between the optical table and the center of the aperture of the shutter is the height chosen in the step 2.1.

2.4. Turn the key of the shutter controller, select the manual mode with the arrows and press the **Enable** button to open the shutter.

2.5. Place, align and fix the first galvanometer mirror.

Note: The two galvanometer mirrors have to be conjugated to the back aperture of the detection objective, so that when one of the mirrors is rotating, the laser beam does not go out of the back aperture of the objective. Note that the distance between the first galvanometer and the back aperture of the objective has to be calculated to evaluate the position of this galvanometer in relation to the objective (f1+f1+f2+f2+f3+f3+f4+f4 = 30+30+30+30+30+200+200+500+500 = 1550 mm; fi=focal length of lens n°i).

2.6. Turn on the galvanometer power supply. Ensure that the galvanometers are powered for the rest of the alignment protocol.

2.7. Set the galvanometers to zero deflection (with NIMAX or optical tweezer software - see step 3 for galvanometer connections).

2.8. Place, align and fix the relay telescope composed of the two lenses with a focal length of 30 mm.

2.9. Place, align and fix the second galvanometer mirror. Note that the distance between the two galvanometers is 4f = 4 x 30 mm = 120 mm (f = focal length).

2.10. Place and fix the telescope.

Note: The telescope is composed of two lenses that expand the beam to a width at least equal to the diameter of the back aperture of the objective lens. The lens with the smallest focal length should come first.

2.11. Place, align and fix the periscope bringing upwards the optical path closer to the entrance of the dichroic mirror rail.

2.12. Fix the hot mirror in the rail, place the rail in the microscope. Ensure that the rail is sawed on the right side to allow the entrance of the infrared laser (**Figure 2**).

2.13. Check that the laser light is going out of the microscope, first without the objective then with the objective. Correct the position of the laser before the objective with the bottom mirror of the periscope. Correct the position of the laser after the objective with the top mirror of the periscope.

2.14. Put 1 µL of 500 nm fluorescent beads in the glass cuvette and add 10 mL of distilled water. Put the cuvette under the objective in the cuvette holder and adjust the focus of the objective (Z position) so that the objective touches the water surface.

2.15. Start the acquisition homemade software controlling the AOTF, the camera and the piezoelectric stage. A free software such as micromanager can also be used. Turn on the live acquisition mode by pressing the **Live** push-button.

2.16. Adjust the IR laser power to 1 W.

2.17. Put on the goggles (and do not remove it before the end of the experiment) and switch on the laser. A bead should be trapped at the laser focus.

2.17.1. If no bead is trapped at the laser focus, check if the red laser pointer (which is collinear with the IR laser) is coming out of the objective. If not, start again the alignment steps, in particular step 2.13. Or, increase the IR laser power.

2.17.2. If the bead is trapped out of focus (of the imaging plane), gently move the position of the last lens of the last telescope (L4 in **Figure 1**) along the optical axis to bring the bead in focus with the imaging plane. If not enough, move the first lens of the last telescope (L3 in **Figure 1**) along the optical axis.

2.17.3. If trapped bead is not centered in the image, use the 2 periscope mirrors to adjust the position of the trap. If the position of the bead is changed by changing the angle of the first mirror, also compensate the direction of the beam by changing the corresponding angle of the second mirror. Correct the X position only (1 screw for each mirror), then correct the Y position (1 screw for each mirror).

2.18. If necessary, adjust the position of the 2$^{nd}$ telescope lens to observe the bead in focus.

2.19. If necessary, adjust the angle of the periscope mirrors to have the bead centered in the image.

## 3. Interfacing the Instrument

Note: **Figure 3** gives a general scheme of the National Instruments (NI) Card connections.

3.1. Insert the output card (NI-9263) in the first slot of the chassis (cDAQ-9178). Note that other models of NI cards with at least 2 analog outputs and 3 analog inputs can be used.

3.2. Insert the input card (NI-9215) in the second slot of the chassis.

3.3. Connect the first galvanometer to the analog output AO0 and the analog input AI0 with BNC cables. Note that a T adapter can be used to connect 2 BNC cables to one. Refer to the galvanometer manual to localize the pins on the galvanometer driver board.

3.4. In the same way, connect the second galvanometer to AO1 and AI1.

3.5. Connect PFI1 to the trigger IN of the shutter (back of the controller).

3.6. Connect PFI0 to the fire of the camera and to AI2.

3.7. Connect AI3 to the trigger out of the shutter (back of the controller).

3.8. Adjust the settings of the shutter controller. Use the arrows to change the parameter values and push the **Select** button to validate the choice and pass to the next parameter. Put the "time open sec" parameter to 000.000, "time closed sec" to 000.000, "mode" to SINGLE, and "trigger" to EXT. Allow the control of the shutter by pushing the **Enable** button.

3.9. Open QtCreator (downloaded from https://www.qt.io/, free version).

Note: QtCreator is the Integrated Development Environment used to develop in C++ the Optical Tweezers software. Qt library is used here to create widgets.

3.10. Open the OT.pro file (provided in the Qt code files). This action will open the project. Change the name of the input and output in the AOGenerator.cpp file according to the NI card(s) used.

3.11. Compile and run the OT software.

## 4. Calibration of the Optical Trap Position with Beads

4.1. Recording of a calibration movie

4.1.1. Put 1 µL of 500 nm red fluorescent beads in the glass cuvette, then fill the cuvette with 10 mL of distilled water. **Figure 4** gives a view of the cuvette in the observation context.

4.1.2. Fix the cuvette on the piezoelectric stage.

4.1.3. Adjust the Z position of the cuvette so that the detection objective dips in the water.

4.1.4. Turn on the infrared laser and set the power to 1 W.

4.1.5. Turn on the image acquisition software.

4.1.6. To acquire time lapse images, select the exposure time, the gain, the time between two images, the number of images to be acquired, and the laser power. Note that for optical tweezer calibration with beads, the illumination laser (561 nm) is not required. The 2-photon effect induced by the infrared laser is enough to excite the fluorescence of a trapped bead.

4.1.7. Start the optical tweezers homemade software.

4.1.8. Set the optical tweezers parameters to trace a circle. Set SamplingRate as 250 samp/s, the bufferSize as 1000, Waveform1 parameters (galvo 1) as Sinusoidal, nbPeriod 1, amplitude 0.5 V, phase 0.0 rad, Offset 0.0 V, Waveform 2 parameters (galvo 2) as Sinusoidal, nbPeriod 1, amplitude 0.5V, phase 1.57 rad, Offset 0.0V.

4.1.9. Check the **AI Parameters** box, and the **Wait for it …** box.

4.1.10. Start the image acquisition (500 or 1000 images with a high frame rate, such as 10 fps).

4.1.11. Switch on the optical tweezers by pressing the **GO!** push button.

4.1.12. Allow the trapped bead to complete at least two full circles and stop the optical tweezers by un-pressing the **GO!** button.

4.1.13. Stop the image acquisition. Note that a tiff movie and a text file with galvanometer voltages are created in the default "C:/TEMP/" folder (unless a custom location is specified). Note that several calibration movies with associated data files can be created if needed.

4.1.13.1. If the circle is not complete, the bead is lost during the circle trajectory. Maybe the speed is too high. Decrease it by decreasing the sampling rate in the OT software. Or, using the red laser pointer of the infrared laser, check if the laser is coming out of the objective during the whole circle trajectory. If not, the galvanometer mirrors may not be conjugated with the back focal plane of the objective lens. Correct the positions of the galvanometers in relation to the back focal plane of the objective, starting again the alignment steps from step 2.5.

4.1.14. Set the amplitudes to zero and switch on the laser to trap a bead at a fix position. Place a landmark at the trap location.

4.2. Position interpolation with image analysis

4.2.1. Open Matlab, go to the calibration folder, and run the "position2tension" script (provided in the Matlab code files). This script computes the interpolation function translating galvanometer voltages to optical trap position.

4.2.2. Choose the number of calibration movies will be used. Several movies can be selected with different trajectories, such as two perpendicular ellipses. Generally, a single calibration movie with a circular trajectory is enough.

4.2.3. Provide the first and last image numbers in the dialog box (one dialog box per movie), sequences with a clear trajectory and good signal to noise ratio.

4.2.4. Provide the path of the text file containing the voltages during the acquisition for each movie. Note that the script reads the file and calculates the mean voltages of the two galvanometers for each image.

4.2.5. Provide the path of the corresponding calibration movie.

Note : The script detects the bead for each frame with a subpixel resolution by fitting a 2D Gaussian to the bead, using custom functions and functions extracted from MTT[26]. It displays a graph showing the bead trajectory.

4.2.6. Eliminate any poorly detected point (outliers) by clicking it.

4.2.7. Check if the interpolation map for x and y laser positions computed and displayed by the script, is complete. If there is a lot of missing values (white regions in the map), repeat the operation with a new calibration movie, with a better signal to noise ratio, or/and with a slower speed of the galvanometers.

Note: These images and the interpolation values are automatically saved in the same folder as the image, with the name convertFct.mat.

5. **Mounting *Drosophila* embryos**[27].

5.1. Collect *Drosophila* embryos from a cage, incubated at 25 °C.

5.2. Remove the yeast, passing the plate contents through a sieve (pore size should be about 100 µm).

5.3. Wash the embryos with 100% bleach (2.6% sodium hypochlorite) for 45 s to remove the chorion.

5.4. Wash the embryos with water for 2 min.

5.5. Put the embryos on an agar pad with a brush.

5.6. Select about 10 embryos according to the desired stage and align the embryos with a pike or a moist brush.

Note: Alignment should be done according to the region of interest (region of interest as close as possible to the detection objective).

5.7. Using a diamond-marking pen, cut a piece of a glass slide of 10 × 20 × 1 mm.

5.8. Add glue on one side of the cut piece, and let it dry for 20 s.

5.9. Turn the cut piece over and place it on the line of embryos, to stick it at the edge of the slide.

5.10. Install this preparation in the sample holder and place it in the cuvette. **Figure 4** gives a view of the cuvette in observation context.

5.11. Put the cuvette on the piezoelectric stage.

## 6. Trapping Experiment *in vivo*

6.1. Trapping of the cell contacts – oscillations (**Figure 5**)

6.1.1. Find the location of interest in the tissue.

Note: Using flies in which cadherins are labeled can be helpful if one needs to work in the plane of adherens junctions.

6.1.2. Move the target junction's midpoint on the trap position (landmark set at step 4.1.14) using the piezo stage.

6.1.3. Set the trap parameters to achieve the oscillations perpendicular to the contact line. Set the phases as zero. Set the amplitudes in X and Y directions to have a movement perpendicular to the contact line. The amplitude should typically be 0.1 V.

6.1.4. Start the acquisition (with a fast frame rate, such as 10 fps).

6.1.5. Switch on the optical tweezers pressing the **GO!** push button.

6.1.6. When done, switch off the tweezers and stop image acquisition. A movie and a text file containing galvanometer voltages during acquisition should now appear in the specified folder.

6.2. Trapping of the cell contacts - pull-release (**Figure 6**)

6.2.1. Find the location of interest in the tissue.

6.2.2. Move the target junction using the piezo stage so that its midpoint is typically 1 µm away from the trap position (landmark set at step 4.1.14).

6.2.3. Set the amplitudes to 0 to have a steady trap.

6.2.4. Start the image acquisition (with a fast frame rate, such as 10 fps).

6.2.5. Switch on the optical tweezers pressing the **GO!** push button.

6.2.6. After the desired time, switch off the trap and wait for relaxation to occur.

6.2.7. Stop the image acquisition. A movie and a text file containing galvanometer voltages during acquisition should now appear in the specified folder.

6.3. Semi-automatic kymograph generation and detection of the interface position.

6.3.1. In the Matlab command window, load the interpolation map generated during the calibration procedure (4b): load('convertFct.mat'). This provides the fx and fy variables.

6.3.2. In the Matlab command window, run autokymo(fx,fy), provided in the Matlab code files. The purpose of this function is to generate a kymograph along the laser trajectory, and to detect the position of the contact line with subpixel resolution for each frame.

6.3.3. Select the prompted text file path containing the voltage values.

6.3.4. Select the first and last frame numbers of the movie to be analyzed.

6.3.5. Select the tiff movie file. Two new figures will be displayed: The first one is an image of the kymograph. The second one is a graph of the detection of the contact line and laser positions, versus the image number.

Note: The function saves the graph representing the position of the sample and the position of the laser versus the image number (matlab figure, jpg), the kymograph (tiff image) and the kymograph with the laser position (tiff image). For steady trap experiments, the kymograph has to be done manually, for instance using ImageJ. The detection of the interface from the kymograph can then be done using the same algorithm.

## 7. Mechanical Measurements

7.1. Stiffness and tension measurements

7.1.1. Perform an oscillation experiment (step 6.1) and the corresponding analysis (step 6.3) of the time lapse movie.

7.1.2. Plot the interface position $x_m$ as a function of the trap position $x_t$ using Matlab plot function or any data plotting software.

Note: This should be approximately linear.

7.1.3. Perform a linear fit, using Matlab “ezfit” free toolbox or any software allowing fits. The inverse of the fit’s slope provides the average ratio $x_t/x_m$.

7.1.4. Perform estimates of the apparent bending stiffness of the interface, which, assuming small deformations and a quadratic potential for trapping, is given by $k_m = k_t \left(\frac{x_t}{x_m} - 1\right)$. $k_t$ is the trap stiffness (see step 8 for trap stiffness calibration).

7.1.5. Perform estimates of tension, which can be defined as[23]:
$T = \frac{k_t l_0}{4}\left(\frac{x_t}{x_m} - 1\right) = \frac{k_m l_0}{4}$.

7.2. Rheological measurements

7.2.1. Perform pull release experiments (step 6.2) and the corresponding analysis (step 6.3)

7.2.2. Fit the resulting curve with the custom rheological model.

## 8. Calibration of the Trap Stiffness

Note: The determination of the absolute forces requires the knowledge of the trap stiffness on interfaces. This can be accessed using beads through a two-step procedure.

8.1. Determination of the cytosol viscosity

8.1.1. Place the embryos in halocarbon oil and inject them using a microinjection setup with fluorescent polystyrene beads (1:1000 stock dilution, 0.46 µm diameter)[23,27].

8.1.2. Place the injected embryos under the microscope.

8.1.3. Track the movement of single beads found in the cytosol. Extract the mean square displacement of beads (use a large number of trajectories > 100 beads). Determine the bead diffusion constant from the slope of the mean square displacement. Relating the diffusion constant to viscosity by the Stokes-Einstein equation, deduce the effective viscosity of the cytosol. In the early embryo, a viscosity of approximately 3.5 Pa.s is reported[23].

8.2. Determination of trap stiffness on beads

8.2.1. Trap single beads in the cytosol with optical tweezers.

8.2.2. Move the bead in a stepwise fashion between two trap positions separated by 0.5 µm

8.2.3. Determine the trap stiffness on beads, as the ratio between the drag coefficient and the bead relaxation time from one trap position to the other.

Note: The drag coefficient is $6\pi\eta R$, where $\eta$ is the viscosity determined in (8.1) and R the bead radius. The relaxation time is obtained from exponential fits of the bead position, using Matlab or any software allowing fits. Typical values of the trap stiffness are 120 ± 50 pN.µm$^{-1}$ at 200 mW laser excitation.

8.3. Determination of trap stiffness on cell interface

8.3.1. Focus the laser on a contact line, and deflect it (as detailed in step 6). Measure the deformation amplitude. This should be repeated on several contact lines to obtain a representative average.

8.3.2. Compare the produced deformation with that induced by beads pushed against contact lines. Since the deformation is inversely proportional to the trap stiffness, deduce the trap stiffness on cell contacts.

Note: The trap stiffness on cell contacts was typically found 2- to 3-fold smaller than that on beads (0.46 µm diameter).

## REPRESENTATIVE RESULTS

**Figure 5** shows experimental data obtained by imposing a sinusoidal movement to the trap. The trap produces a deflection of the interface, as exemplified by the 3 snapshots showing 3 successive interface positions (**Figure 5A**)[23]. Recorded movies are used to generate a kymograph (**Figure 5B**) and are further analyzed to determine the position of the interface with subpixel resolution, using a Gaussian fit along the x direction for each frame. In the regime of small deformations, the trap and interface positions are proportional (**Figure 5C**). The amplitude of the interface deflection relative to that of the trap (**Figure 5D**) gives access to the interface tension or stiffness (see step 7.1).

**Figure 6** shows experimental data obtained by performing pull release experiments. The optical trap is switched on approximately 1 µm away from the midpoint of the interface between two cells, which causes the interface to deflect towards the trap position (**Figure 6A**)[24]. The trap is then switched off after the desired amount of time. The position xm of the interface (**Figure 6B**) is obtained from kymographs (**Figure 6C**), again using Gaussian fits along the x direction for each frame. The resulting graph can be compared to hypothesized rheological models, for instance, a Maxwell model (**Figure 6D**).

## FIGURE AND TABLE LEGENDS

**Figure 1: Schematic of the optical tweezers (red path) setup combined with the light sheet microscope**. This figure has been modified from Bambardekar, K. *et al.*[23]. The light sheet microscope, composed by the illumination unit and the detection unit, is described previously[25]. The optical tweezers correspond to the red part of the scheme: The infrared laser passes through an optical shutter and 2 galvanometers which control the position of the trap in the sample. A 1-fold telescope is placed between the 2 galvanometers to keep the conjugation between them. Then, a telescope increases the size of the beam by 2.5-fold and the periscope brings it to the height of the microscope. The infrared laser enters the detection objective of the microscope thanks to a dichroic mirror. Important distances between optical elements are given directly in the figure. Distance between the last lens (focal length 500 mm) and the back aperture of the objective is 500 mm.

**Figure 2: Homemade modified rail holding the dichroic mirror and the hot mirror.** The dichroic mirror rail of the microscope has been cut on the left side to allow the entrance of the infrared laser. The dichroic mirror reflects the infrared light and transmits the visible light (fluorescence).

**Figure 3: Connection of the instruments to the NI Cards.** AO = analog output, AI = analog input, AO0 and AI0 are connected to galvo1, AO1 and AI1 are connected to galvo2, PFI0 is connected to the fire of the camera, to AI2 and PFI1 is connected to the trigger in of the shutter and AI3 is connected to the trigger out of the shutter.

**Figure 4: Sample holder in the observation context**. This figure has been modified from Chardès, C., *et al*.[25]. The embryos are immobilized on the glass coverslip. The slide is held by the sample holder. The sample holder is inserted in the glass cuvette, held by the holder fixed to the piezoelectric stage. The light sheet is horizontal and illuminates the embryos from the side. The detection objective lens is vertical, above the sample, and dips into the cuvette.

**Figure 5: Interface deflection imposed by sinusoidal movement of the trap.** This figure has been modified from Bambardekar, K. *et al* [23]. (**A**) The trap is moved sinusoidally perpendicular to the interface. The trap and interface positions are xt and xm, respectively. The right panels show three images of the interface at different positions. The laser trap position is marked by a yellow arrowhead. Interface are labelled with a membrane marker (GAP43::mcherry). (**B**) Kymograph along the axis defined by the direction of trap movement (perpendicular to the interface) (period: 5 s). (**C**) Representative plot of deflection versus time showing both trap (red solid line) and interface positions (black solid line). (**D**) Interface position as a function of trap position during few cycles of laser oscillation (amplitude: 0.5 µm, period: 2 s).

**Figure 6: Interface deflection in pull-release experiments.** This figure has been modified from Serge, A., *et al*.[24]. (A) The trap is switched on at a distance from the midpoint of the interface, then switched off again. (B) The trap and interface positions are xt and xm, respectively. Kymographs are generated along the x direction, perpendicular to the contact line's midpoint. (C) Kymograph of a pull-release experiment. Cell contacts are labelled with Utrophin::GFP. (D) Representative plot of deflection versus time showing both trap (dotted red/green lines) and interface position (black solid line). The solid red line shows a fit obtained using a Maxwell-like rheological model[24].

**Supplementary Movie 1**: Tweezing experiment. Pixel size = 194 nm, 10 fps, trap oscillation period = 2 s, labelling: Gap43::mCherry.

## DISCUSSION

Optical tweezers allow to perform absolute mechanical measurements directly in the developing embryonic epithelium in a non-invasive manner. In that sense, it presents advantages over other methods such as laser ablation, which are invasive and provide relative measurements, magnetic forces, which require injections, or force inference, which relies on strong assumptions and also provide relative measurements.

The protocol includes a few critical steps. First, as the objective lens may show chromatic aberrations and that the laser trap pushes the object "downstream", it is important to check that the IR laser traps the bead in the imaging plane, and eventually correct for it (step 2.18). Second, the method relies on the measurements of the cell contacts' position. It is thus crucial to use a high-contrast fluorescent marker.

The quality of the objective lens and the laser beam are critical to effective trapping. The numerical aperture of the objective lens should exceed 1.0. If trapping is ineffective, make sure that the laser beam fills the rear aperture of the objective lens.

Our method comes with several limitations. First, it is not clear what exactly provides the support for optical trapping. Although a mismatch of refractive index is detected, its origin remains to be determined. Second, the calibration process, if one is interested in absolute measurements, can be a bit tedious, as it requires passive microrheology experiments, and calibration of the trap stiffness on beads. It is important to recognize that the calibration of the stiffness on cell contacts is subject to experimental uncertainty: it relies on measurements of the trap stiffness on beads, which can be measured only in the cytosol, but not near cell contacts. Third, it is unclear how versatile optical tweezers can be. Although they are able to deform the cells in the *Drosophila* embryo, other tissues might present higher tension or more difficult access (such as going through a cuticle) and therefore be less amenable to optical tweezing.

Optical forces are small (< few tens of pN) and may be thus insufficient to deform stiff or highly tensed structures. Magnetic tweezers on large particles would probably be more effective in this case.

We described here the coupling of optical tweezers to a light sheet microscope, but optical tweezers can be coupled to other types of microscopes, such as an epifluorescence or a confocal spinning disk microscope. The introduction of the IR trapping laser into the microscope depends on the microscope configuration. It mainly requires the possibility to add a dichroic mirror to combine the paths for imaging and optical manipulation. Most microscopy companies propose modular illumination systems, with a two-layer module, which allow this combination.

Several directions exist to improve or upgrade the technique. A possibility is to split the laser residence time among several positions or to use more advanced holographic techniques, to produce several traps. This could allow to create more complex force patterns on target cells or cell contacts. Another improvement could be to design a real-time feedback between the deflection caused and the position of the trap. This could allow proper creep experiments in which the force applied is maintained constant throughout the experiment.

**ACKNOWLEDGMENTS**

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**DISCLOSURES**

The authors have nothing to disclose.

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Figure1

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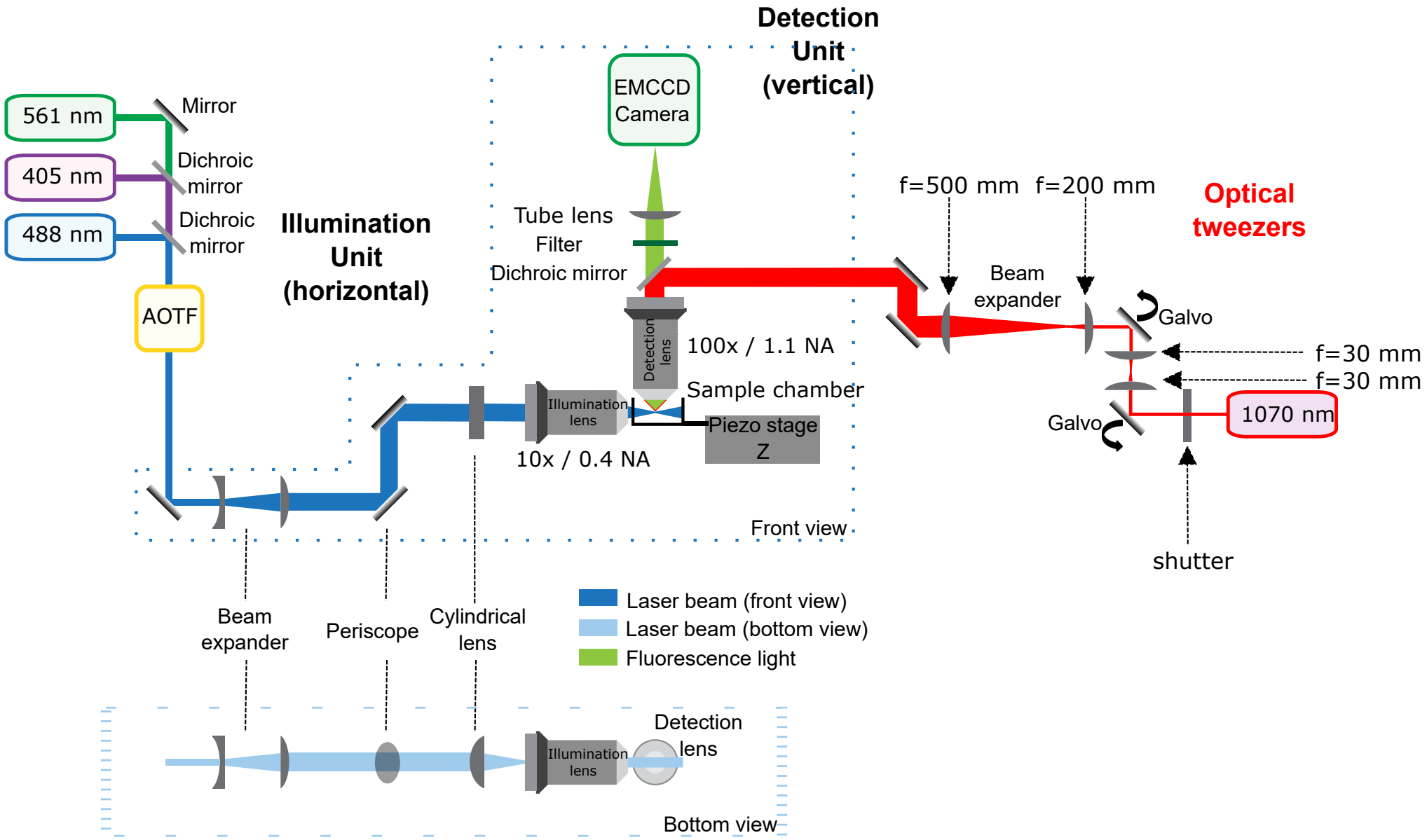

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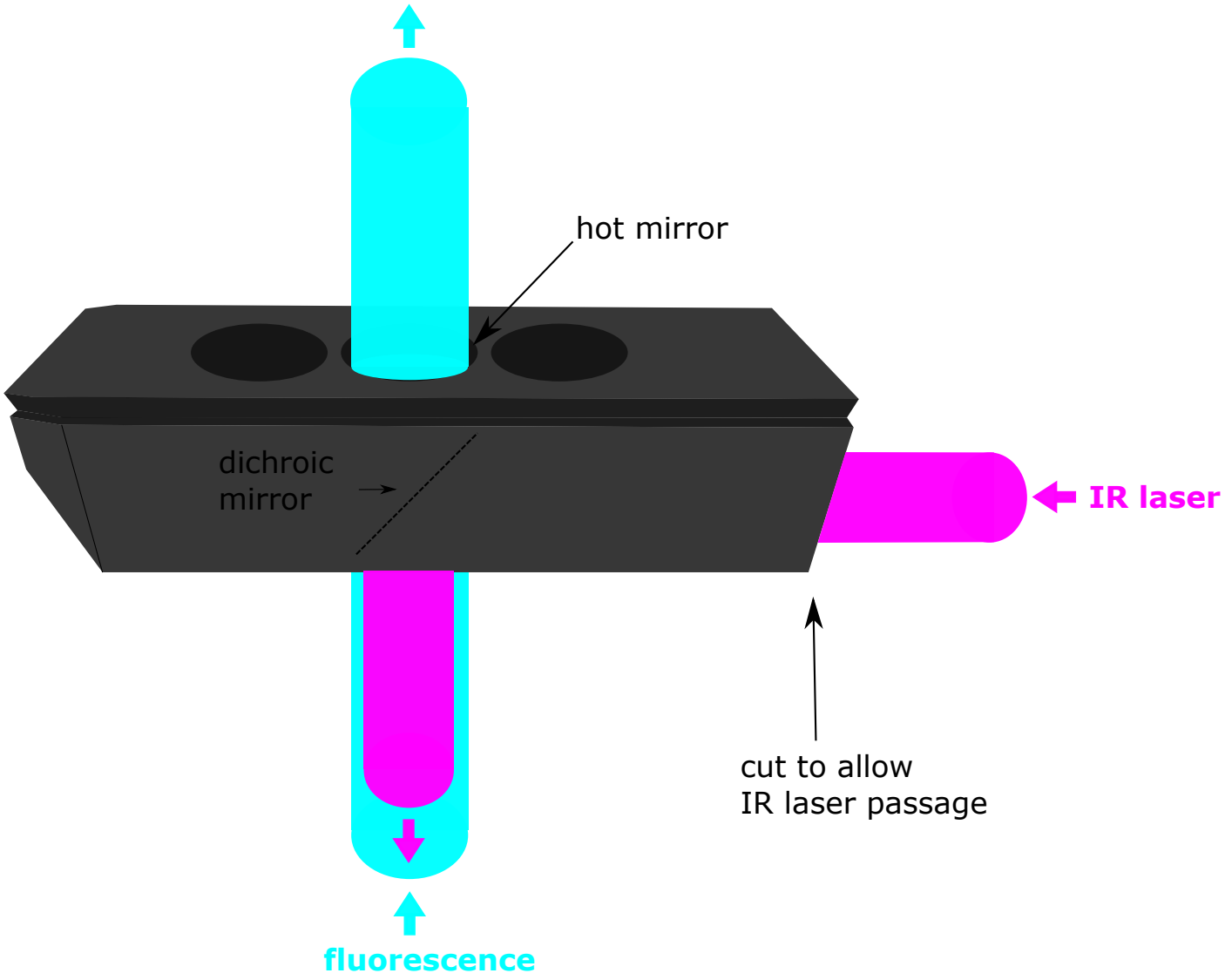

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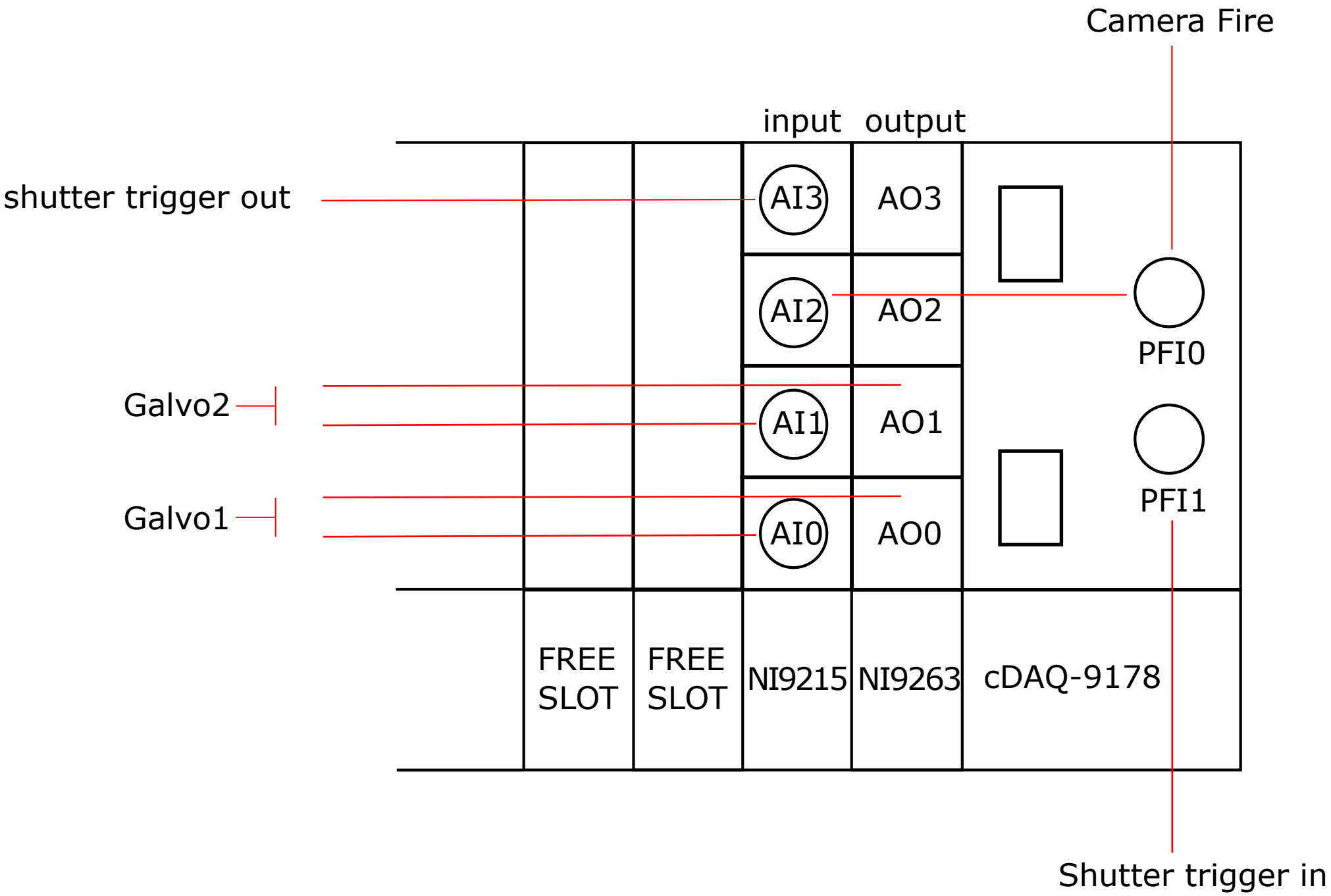

Figure4

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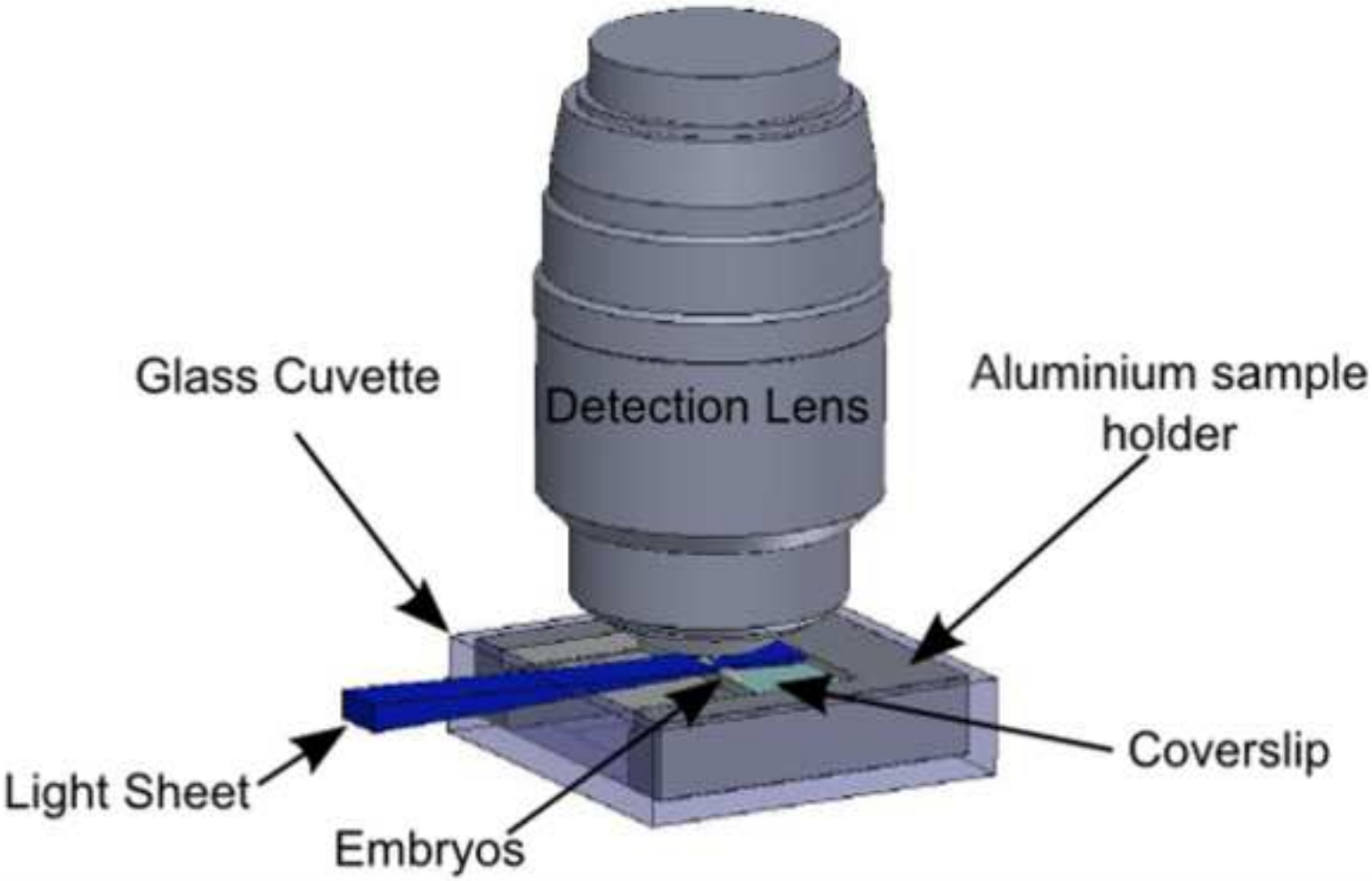

Figure5

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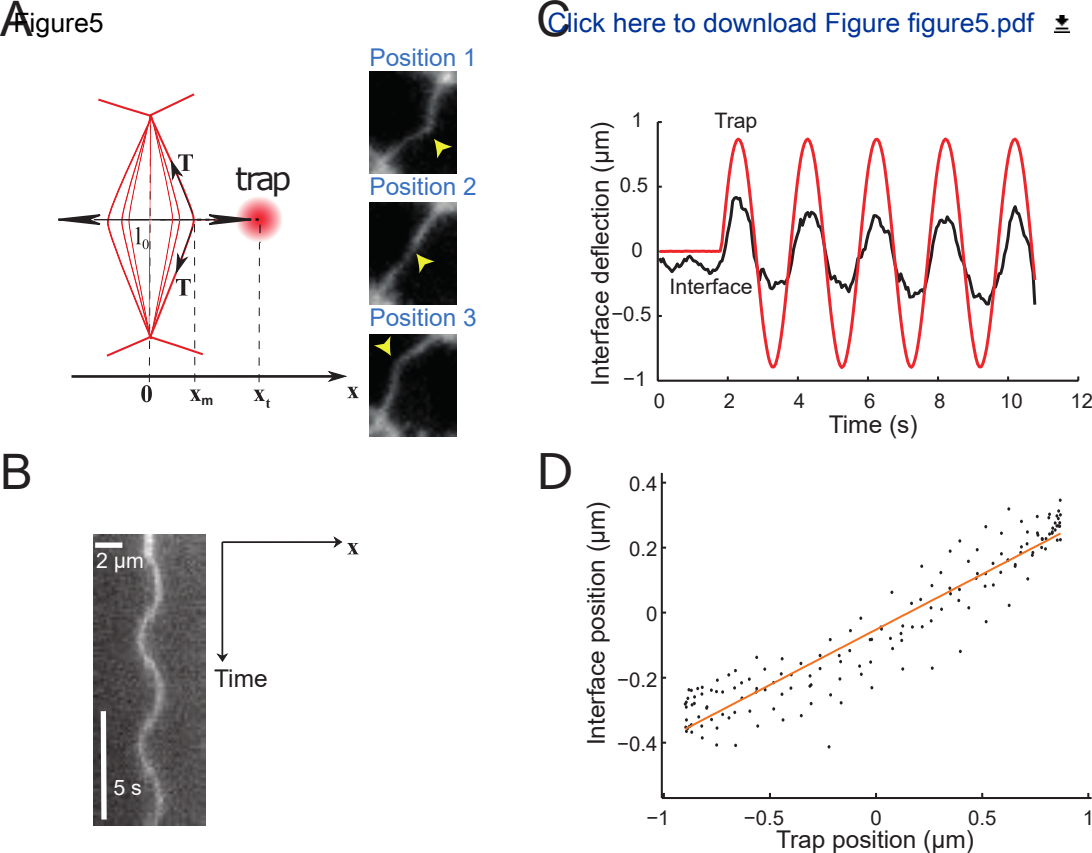

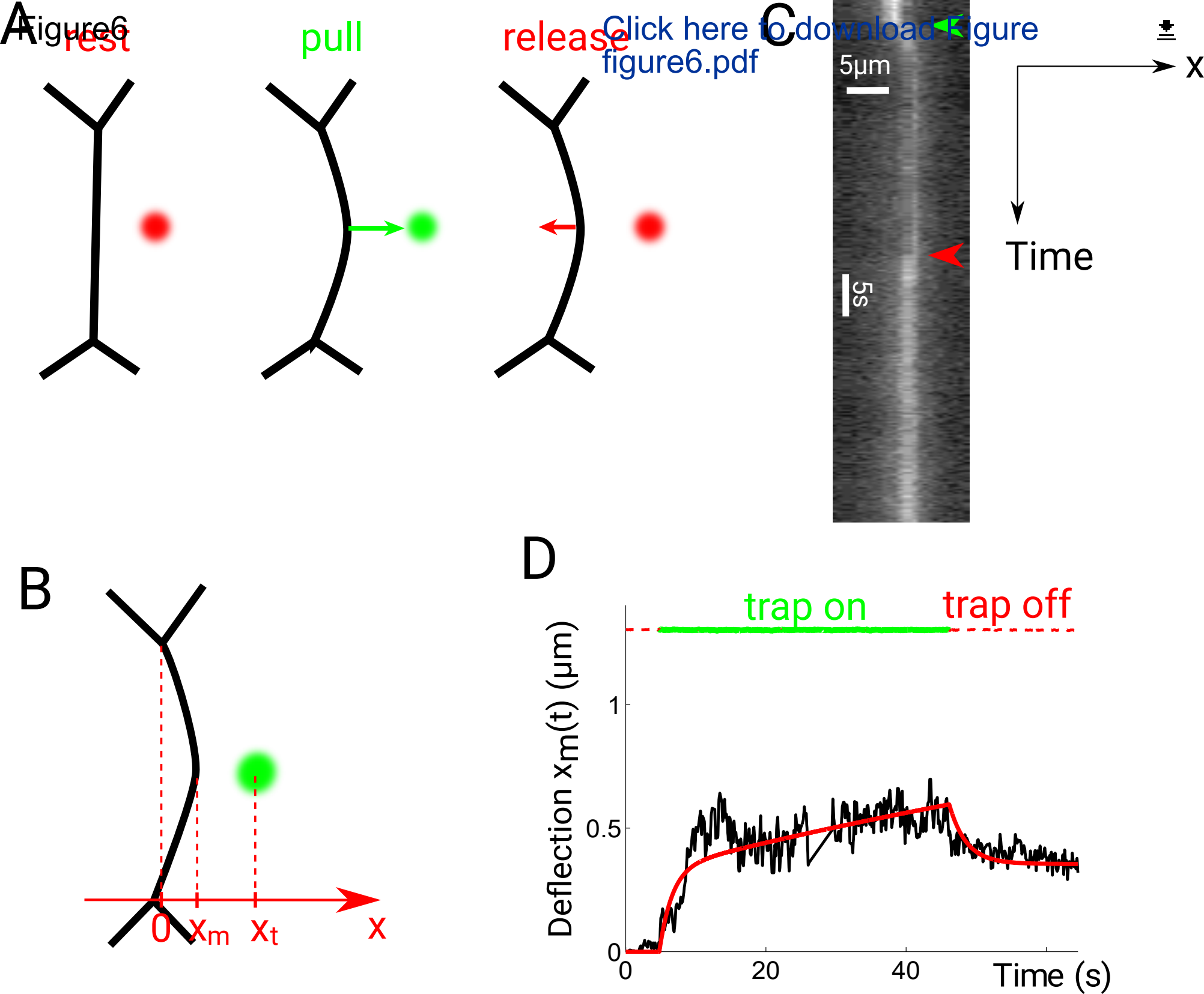
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C
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x
Time
B
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$x_t$
x
D
trap on
trap off
Deflection $x_m(t)$ (μm)
1
0.5
0
0
20
40
Time (s)

Movie1

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MaterialsAndEquipments.xls

Feuille1

| **Name of Material/ Equipment** | **Company** |
|---|---|
| Ytterbium Fiber Laser LP, 10 W, CW | IPG Laser |
| Ø1/2" Optical Beam Shutter | Thorlabs |
| Small Beam Diameter Galvanometer Systems | Thorlabs |
| 1D or 2D Galvo System Linear Power Supply | Thorlabs |
| 2 lenses f = 30mm | Thorlabs |
| Lens f = 200mm | Thorlabs |
| Lens f = 500mm | Thorlabs |
| Right-Angle Kinematic Elliptical Mirror Mount with Tapped Cage Rod Holes | Thorlabs |
| Laser Safety Glasses, Light Green Lenses, 59% Visible Light Transmission, Universal Style | Thorlabs |
| 45° AOI, 50.0mm Diameter, Hot Mirror | Edmund Optics |
| Multiphoton-Emitter HC 750/S | AHF |
| CompactDAQ Chassis | National Instruments |
| C Series Voltage Output Module | National Instruments |
| C Series Voltage Input Module | National Instruments |
| FluoSpheres Carboxylate-Modified Microspheres, 0.5 µm, red fluores | ThermoFisher Scientific |
| C++ (Qt) home made optical tweezers software | |

| **Catalog Number** |
|---|
| YLM-10-LP-SC |
| SH05 |
| GVS001 |
| GPS011 |
| LB1757-B |
| LB1945-B |
| LB1869-B |
| KCB1E |
| LG1 |
| #64-470 |
| HC 750/SP |
| cDAQ-9178 |
| NI-9263 |
| NI-9215 |
| F8812 |
| |

| **Comments/Description** |
| --- |
| including collimator LP : beam D=1.6 mm and red guide laser |
| |
| 1 for X displacement, 1 for Y displacement |
| galvanometers power supply |
| relay telescope between 2 galva |
| 2.5X telescope |
| 2.5X telescope |
| Periscope |
| |
| |
| |
| |
| Analog output module |
| Analog input module |
| calibration beads |
| developed by Olivier Blanc and Claire Chardès. Alternative solution: labview |

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a) Critical steps within the protocol
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c) Any limitations of the technique
d) The significance with respect to existing methods
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**Dear Editor,**

**We have answered all the points that you and the reviewers raised. Our answers to the reviewers' comments are in bold below. We hope that the manuscript now meets the criteria to be published in JOVE. We thank you for your help with this manuscript.**

**Reviewers' comments:**

**Reviewer #1:**
Manuscript Summary:
This manuscript describes setup consisting of a custom lightsheet microscope and an optical tweezer. The authors describe how to align and couple the optical tweezers to the custom microscope, and how to perform experiments on living specimen to probe mechanical properties, such as intracellular contact, stiffness and tension in Drosophila melanogaster. Furthermore, the authors share both the control and the analysis softwares needed for steering the optical tweezers and for quantifying the measurements which can be obtained by such a setup.

Major Concerns:
The manuscript seems to describe a setup which is tailored to the author's custom lightsheet. However, the technique described is independent of the type of microscope used for imaging. I therefore encourage the authors to put some more effort into generalizing the description of how to couple an optical tweezers to a general microscope.

**It is true and said in the discussion that the optical tweezers setup can be adapted to other microscopes such as an epi-fluorescence or a spinning-disk microscope. The introduction of the IR trapping laser into the microscope depends on the microscope configuration. This mainly requires the possibility to add a dichroic mirror to combine the paths for imaging and optical manipulation. Most microscopy companies propose modular illumination systems, with a two-layer module, which allow this combination. We have added this information in the main text.**

A troubleshooting section is needed: the authors should try and give helpful hints about what to do or which parameters to check in case a user cannot reproduce what is written in the text. As an example, what are the components/settings to be checked if no bead can be stably trapped?

**Thank you for the suggestion. We have added for few steps of the protocol a troubleshooting section. We hope it will help the reader to overcome possible difficult steps within the protocol.**

Minor Concerns:
The description about how to align and couple the optical tweezers optical elements with each other and finally to the microscope is somehow too crude: importantly, a figure reporting the accurate distances and relative positions of all the optical elements is missing.

The authors do report the theoretical values for setting up 4f systems (lines 118-119 and 125) but without knowing where the lenses and mirrors need to be placed, the whole procedure might be hard to grasp for the general reader.

**Distances are now provided in the figure 1.**

Some passages in the protocol might not lead to an optimal alignment if performed according to what the authors write: for example, when describing how to setup the tweezing path the authors write to place and align the two galvanometers and the periscopes and to check whether the IR laser reaches the objective (lines 112 onward, points 5 to 17), but omit to say that the mirrors should be powered and set to zero deflection for best results. Optimizing the optical path when the galvanometers are in a random position might result in sub-optimal light coupling.

**This is true, and we modified the protocol point 2.6 and 2.7.**

It is not clear which input/output of the galvanometer need to be connected with the board analog inputs and outputs (line 173). Please clarify which signal pin of the galvanometer mirror should be connected to the board input and which to the output

**Pins of the driver board of the galvanometer can be localized thanks to the Thorlabs manual. We added a note in the protocol.**

The objective used for imaging and trapping is referred to as having an NA of 1.0 (line 94), but Figure 1 reports it as a 100x/1.1 NA. Which one is correct?

**The numerical aperture of the objective is 1.1. We corrected this in the protocol.**

**Reviewer #2:**

Manuscript Summary:

The authors describe a procedure and instrumentation for applying forces using optical tweezers to Drosophila embryos, and imaging the resulting dynamics using light sheet fluorescence microscopy.

The paper is well motivated. The mechanics that govern the development of animals are poorly understood, and methods to probe these systems are few in number, and are often invasive or difficult to implement. The paper is clearly written. Technically, the authors' work is impressive - this is a difficult project, and the system the authors have developed is admirable. However, the paper has significant flaws, described below.

Major Concerns:

1 A central claim of the work is the ability to measure forces noninvasively. The method, however, does not do this, and I do not think the argument the authors provide makes sense. The authors quite nicely move intercellular junctions and observe their motion. To extract a force from this requires knowledge of the stiffness of the junctions, or their optical properties, neither of which are known. The authors calibrate their force measurement using a three-step procedure. First, they track bead displacements to determine the local viscosity; this assumes a Newtonian fluid, and assumes purely thermal motions (i.e. no active motions), neither of which are in general true for the cytoplasm. Second, they determine the trap stiffness using beads. This is fine, but because the stiffness is itself a consequence of the index of refraction of the beads relative to their surroundings, it is valid only for a "free" bead in the cytosol. Third, the authors tug on a cellular contact line and move it, and then move "beads pushed against contact lines," comparing the displacement between the two to determine the trap stiffness for contact lines relative to the stiffness for the beads. However:

the fact that the contact line can be moved implies that its local index of refraction is different than that of the cytoplasm, so bringing a bead near the contact line changes the optical properties around the bead, so the trapped-bead-stiffness will not he the same as what was measured in part 2. Overall, I do not believe that the authors are able to absolutely determine forces, and they certainly don't make a clear case that the numbers they extract are "real." I agree that they can provide relative measurements.

**We agree that the determination of the absolute forces is critical and as we state, we can only estimate them through a three-step procedure, that was already described in Bambardekar, Clément et al. The first step utilizes single bead tracking in the cytosol to determine local viscosity. We are aware that our approach is only valid for Newtonian fluid and for a regime dominated by thermal fluctuations. As explained in Bambardekar, Clément et al, bead trajectories were acquired at a frequency (38 Hz) for which active fluctuations are considered to be small compared to thermal ones (Mizuno et al, Science 2007). This assumption has been directly assessed in the cytoplasm of the early Drosophila embryo by microrheology measurements (Wessel et al, Biophys J. 2015: 21, 1899-907). These measurements have also shown that the cytoplasm is viscously dominated.**
**We agree with the reviewer that the refractive index difference at the cell contacts is likely to modify the trap stiffness on the bead. Thus the estimation of the trap stiffness is subject to an experimental error, but, unfortunately, we haven't found a better strategy of calibration. To clarify this point, we have added a sentence to avoid confusion: "It is important to recognize that the calibration of the trap stiffness, and thus of the forces on cell contacts is subject to experimental uncertainty: it relies on measurements of the trap stiffness on beads, which can be measured only in the cytosol, but not near cell contacts."**

2 Though the setup is technically challenging and impressive, there is nothing novel about it. The construction of optical tweezers is well described in many places. Light sheet microscopes, though less common, are also well described elsewhere, and the authors' don't discuss the light sheet design much anyway. The authors have put two well-established techniques together. Though this is technically challenging, I do not see what experimental advance the paper provides. Put differently: if I wanted to build an optical trap on a light sheet microscope, I'd look at how to build an optical trap (if I had never built one), and build it onto a light sheet microscope; there's no "conflict" between the two methods that makes this paper necessary. Again, I appreciate the practical challenge of doing this, but I do not see the conceptual challenge.

**We agree that the two parts of the setup, the light sheet microscope and the optical tweezers, are not novel, and we don't claim they are. Here we describe a setup, which is the combination of two known techniques and a protocol to probe mechanics in the *Drosophila* embryo. We think that this may be useful to the scientific community. We believe that our protocol enters in the category "innovative applications of existing techniques" (JOVE) and that a text protocol and a video demonstration will be useful. Following the referee's suggestion, we now also refer to existing protocols for optical tweezers (Svoboda, K., & Block, S. M. 1994).**

3 The technical description the authors provide gives lots of details particular to the exact equipment the authors use, but gives almost no discussion of the general principles. Suppose one uses a different Daq card - what are the relevant features to keep in mind? Why a 1070 nm laser? What is QT Creator? Etc.

**We described the setup as we built and used it. Of course, some elements can be changed and we tried to tell it when possible. For example, other NI cards can be used, and we notified in step 3.1 the required numbers of analog outputs and inputs. QtCreator is a Integrated Development Environment, allowing C++ programming and the use of Qt, a widget library. This IDE is needed for people who want to use the code that we provide. We added a note in step 3.10.**

4 The authors focus 200 mW of IR light onto embryos for their trap. This is a lot of power, and they need to demonstrate that this does not alter the local mechanical properties, and does not damage the embryos.
**In Bambarderkar, Clément et al, we monitored the cell response at different IR laser powers. In particular, we showed the linearity of contacts' deflection for powers ranging from 50 mW to 300 mW. We noted damaging effects for powers larger than 400 mW. Therefore, we decided to use 200 mW, which induces quite large deflection without damage. Yet, to fully address this point, we would need to compare our measurements with other mechanical non-invasive measurements. Magnetic tweezers may be an alternative to optical tweezers but this would require an extensive study, which goes beyond the scope of the present manuscript.**

**Reviewer #3:**
Manuscript Summary:
the manuscript is a protocol following two research papers (21 and 22). the idea of the protocol should be to make it easier for other scientists to follow and replicate their experimental set-ups. Unfortunately, so much details is left vage that this is not possible.

Major Concerns:
Many parts are describes sloppily without sufficient detail for the user to copy. The authors should carefully go through all aspects making sure that the descriptions are precise, correct and contain the important detailes, incl manufacturing numbers.

**We provide a list of the main optical and mechanical elements that we use with their reference numbers. This list can be found in a separate table, so that the readers can easily find the pieces of equipment to replicate the set-up. The small optical elements that are not listed are standard and have been purchased from Thorlabs; we added a sentence to make this clear.**

A few examples:
1) In the tweezer path setup, it needs to be mentioned that for maximal trap stiffness the IR beam should just over-illuminate the back-focal aperture of the 100x objective. If the beam is small edge rays get lost and the trap will be weaker and if the NA is only 1.1 won't be very strong.

**We thank you the reviewer for this comment; we have now clarified this point by stating: "Note that the IR beam should just overfill the back-focal aperture of the objective lens to maximize the focalization of the IR beam and thus the trap stiffness."**

2) "1ul of pink beads in glass cuvette, fill with water" is really unscientific, saying nothing about the bead concentration, the material, source and little about the fluorescence loading, what glass covette (the on shown in fig 2), what volume

**We added the reference for fluorescent beads in the materials table and now precise the amount of water required. Note that the concentration of beads is far from being critical.**

3) "turn the acquisition software on" - what software? acquisition of what? I assume it's for the camera, but really needs to be mentioned

**We refer to the QT acquisition software, described in the protocol. It controls the whole microscope including the optical tweezers and the camera. We tried to make it clearer (everytime the software is mentioned) which part of the software is used.**

4) "Adjust the settings of the shutter controller" - what shutter? camera? IR laser?
... there are many, too many of these 'sloppy' description for me to list. They have to be more specific to make this a useful protocol

**The protocol describes the optical setup and mentions the different elements including the shutter and its controller, the camera and the IR laser. Following the reviewer's suggestion, we also now specify these elements both in the Figure 1 and the table that provides a comprehensive list of the main optical and hardware devices. The video that will be made for JOVE will also specify these elements; we hope that both the text and the video will make this a useful protocol.**

while the abstract and title promise bead free measurements, they protocol only mentions calibration with a bead injected into the cytosol, then ref. 6 is cited and only little detail is given, ref. 21 and 22 seem more important.
More information needs to be given to this process. In the original publication, the authors claim that pushing the beads agains the cell wall, they know the force. However, now that they should the optical trap can directly move the cell wall, it is clear that the optical properties are such they distort the optical trap profile and the force on the bead agains the wall is not same the as the force on the bead in the cytoplasm. also a protein corona will build on the bead affecting the force as well.

**In the original publication (Bambardekar, Clément et al), we already reported the two possibilities of optical manipulation, either using trapped bead to push the cell contacts and or by direct tweezing of the cell contacts. We agree with the reviewer that the refractive index difference at the cell contacts is likely to modify the trap stiffness on the bead. Thus the estimation of the trap stiffness is subject to an experimental error, but, unfortunately, we haven't found a better strategy of calibration. To clarify this point, we have added a sentence to avoid confusion: "It is important to recognize that the calibration of the trap stiffness, and thus of the forces on cell contacts is subject to experimental uncertainty: it relies on measurements of the trap stiffness on beads, which can be measured only in the cytosol, but not near cell contacts."**

Minor Concerns:
Large sections are highlighted yellow, not clear why.
several links in the reference do not work as they are two links in a row

**These are the sections that will be used to prepare the script of the movie.**
**We corrected the links.**

Links for figure reprint :

Bambardekar et al PNAS, 2015

http://www.pnas.org/page/about/rights-permissions

Clement et al, Current Biology 2017

http://www.cell.com/current-biology/authors

Supplemental Coding Files

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