Chapter 10

Sample Preparation and Mounting of *Drosophila* Embryos for Multiview Light Sheet Microscopy

Christopher Schmied and Pavel Tomancak

Abstract

Light sheet fluorescent microscopy (LSFM), and in particular its most widespread flavor Selective Plane Illumination Microscopy (SPIM), promises to provide unprecedented insights into developmental dynamics of entire living systems. By combining minimal photo-damage with high imaging speed and sample mounting tailored toward the needs of the specimen, it enables *in toto* imaging of embryogenesis with high spatial and temporal resolution. *Drosophila* embryos are particularly well suited for SPIM imaging because the volume of the embryo does not change from the single cell embryo to the hatching larva. SPIM microscopes can therefore image *Drosophila* embryos embedded in rigid media, such as agarose, from multiple angles every few minutes from the blastoderm stage until hatching. Here, we describe sample mounting strategies to achieve such a recording. We also provide detailed protocols to realize multiview, long-term, time-lapse recording of *Drosophila* embryos expressing fluorescent markers on the commercially available Zeiss Lightsheet Z.1 microscope and the OpenSPIM.

Key words *Drosophila melanogaster*, Embryogenesis, Live imaging, Light sheet microscopy, SPIM, Multiview

1 Introduction

Light sheet fluorescent microscopy (LSFM) is an emerging imaging technique ideally suited for imaging cellular dynamics in intact developing *Drosophila* embryos expressing fluorescent markers. With LSFM it is possible to record the developing embryos from early blastoderm stage until the fully hatched larva. LSFM is using a laser light sheet to illuminate the sample and an objective lens positioned at a 90° angle with respect to the light sheet to image the illuminated plane (Fig. 1a). This arrangement achieves optical sectioning and since only the current acquired section is illuminated leads to very low bleaching and phototoxicity. A digital camera captures the light, with high signal-to-noise ratio and allows very high speed of acquisition [1]. Although several flavors of light sheet microscopy are relevant to *Drosophila* imaging [2], the most commonly used modality is Selective Plane Illumination Microscopy

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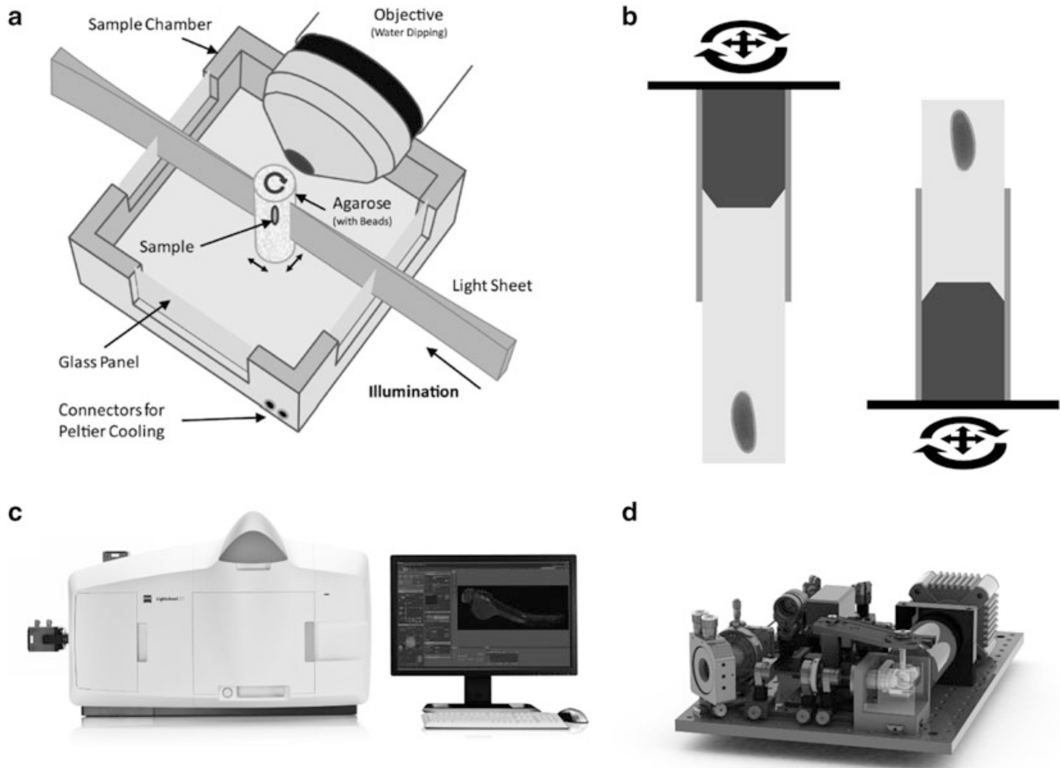

Fig. 1 SPIM principle, sample mounting, and readily available SPIM set-ups. The sample is illuminated with a thin laser light sheet. The lens is positioned orthogonally in respect to the light sheet. The sample can be moved in x, y, z and can be rotated around its axis (**a**). Mounting with sample suspended from above and below, a metal plunger (dark grey) pushes the agarose (light grey) with the embryo specimen out of a capillary (medium grey) into the sample chamber buffer (**b**). Commercially available SPIM from Carl Zeiss Microscopy—Lightsheet Z.1 (**c**) and DIY Open SPIM (**d**). Figure (**a**) adapted from ref. [13]. Figure (**b**) adapted from ref. [11]. Figure (**c**) © Copyright of Carl Zeiss Microscopy GmbH

(SPIM) that achieves complete coverage of the embryo specimen by imaging it from multiple angles over time [1].

The so-called multiview SPIM imaging of ubiquitously expressed nuclear markers has been used to demonstrate the ability of light sheet microscopy to record developmental anatomy *in toto* [3, 4]. In combination with the plethora of available gene expression reporters [5, 6], SPIM also provides the means to record the dynamics of the gene expression pattern formation during the entire process of *Drosophila* embryogenesis.

The major change SPIM imposes on established imaging protocols are the fundamentally different approaches to sample preparation. In SPIM, the sample is typically embedded in agarose inside a glass capillary. For imaging, the embryos immobilized in agarose are pushed outside of the capillary to enable penetration of the light sheet from the side while avoiding diffraction of the light sheet. The capillary with the agarose column is suspended in a

chamber filled with buffer [7]. This can be done either from above or from below, however most available SPIM set-ups implement the sample suspension from above (Fig. 1b). This sample-mounting paradigm allows moving the sample through the light sheet in the *z*-axis and thus the acquisition of 3D image stacks (series of 2D images produced by optical sectioning with the light sheet). Moreover, the sample can be rotated and multiple stacks (views) of the same specimen from different angles can be imaged. This allows imaging of even very thick or opaque samples in their entirety [1]. Coupled with image processing [8], these different views can be fused and the sample can be reconstructed *in toto* with isotropic resolution. Typically, fluorescent beads are embedded in the agarose together with the embryos to serve as fiduciary markers facilitating the multiview reconstruction and fusion of the views by multiview deconvolution [9, 10].

Principally, two hardware implementations are readily available to achieve multiview imaging: the commercially available Lightsheet Z.1 from Carl Zeiss Microscopy (Fig. 1c) and the "do-it-yourself" (DIY) open access OpenSPIM [11] (Fig. 1d). In both set-ups, a cylindrical lens forms the light sheet. Lightsheet Z.1 offers dual-sided illuminations and pivoting of the light sheet. This reduces the degradation of the light sheet across the field of view and the "stripe" artifacts caused by absorption of the light sheet within the sample [12].

Here, we present a detailed description of the mounting methods for SPIM and the imaging setup employed in our lab, for long-term time-lapse live imaging of *Drosophila* embryogenesis on Lightsheet Z.1. Both the mounting method and the imaging set-up can be easily adapted for imaging with OpenSPIM.

2 Materials

1. Embryo Collection Cages with Stainless steel mesh—97 µm.
2. Phosphate-buffered saline—1× without Mg, Ca.
3. Sodium hypochlorite solution (6–14 % active chlorine)—2.5 l.
4. Sterile Cell Strainer—100 µm mesh size.
5. Piston rod f.Transferpettor Fix+Dig. 10 µl.
6. n-Heptane.
7. Tape—natural rubber.
8. Fluorescent Microspheres—F-Y 050 and F-Z 050 (*see* **Note 1** for selection, **Note 2** for preparation).
9. Low melting point (LMP) agarose; Sea Plaque Agarose (*see* **Note 3** for preparation).
10. Eppendorf tubes—1.5 ml.

11. Petri dishes—60 × 15 mm.
12. Plastic Pasteur pipettes—3 ml.
13. Powder free gloves—Nitril gloves.
14. Thermomixer.
15. Vortex.
16. Centrifuge.
17. Microscope slides—76 × 26 mm.
18. Brushes
19. Blunt forceps.
20. Beaker.

3 Methods

3.1 Mounting of Embryos

3.1.1 Mounting in Agarose for Multiview Imaging

In order to perform multiview imaging the sample needs to be accessible to the illumination and detection optics from all sides. For this we embed the embryos in low melting point (LMP) agarose and mount them in a glass capillary. For imaging, the agarose is pushed out of the capillary and the sample is suspended freely in the sample chamber (*see* Fig. 1b). The agarose has a similar refractive index as the immersion medium in the sample chamber of the microscope, thus the light sheet can penetrate the agarose column with minimal refraction or scattering [1].

To computationally reconstruct the sample, the different views need to be registered onto each other. We use fluorescent beads in the surrounding agarose as fiduciary markers to achieve this (*see* **Note 1**). The beads are simply added at an appropriate concentration to the agarose before it polymerizes. After detection of the beads registration overlays the corresponding beads from the different angles. Then the views can be fused either by content-based multiview fusion or multiview deconvolution. This recovers the complete volume of the sample at isotropic resolution [8–10].

1. Prepare beforehand:
   (a) Liquid 2 % LMP agarose in PBS cooled down to min. 37 °C (*see* **Note 3**).
   (b) 1:100 bead dilution of fluorescent beads matching the imaging experiment conditions (*see* **Notes 1** and **2**)
   (c) Prepare capillaries with plungers such that the plunger sticks out a few millimeters at the bottom of the capillary to avoid drawing in air.
   (d) 50 ml 20 % bleach in PBS.
   (e) Timed *Drosophila* embryos.
   (f) Beaker filled with PBS.

2. Remove excess yeast paste from the apple juice plate.
3. Add water to the apple juice plates and loosen the embryos with a brush (*see* Fig. 2a).
4. Transfer the embryos into a sieve by pouring the water into the sieve.
5. Wash the embryos briefly with water (*see* Fig. 2c).
6. Transfer the sieve into a petri dish filled with 20% bleach.
7. During bleaching apply fresh bleach onto the side of the sieve with a plastic Pasteur pipette (*see* Fig. 2d).
8. Bleach embryos under a stereomicroscope for up to 2 min or until properly dechorionated (*see* Fig. 2e for dechorionated

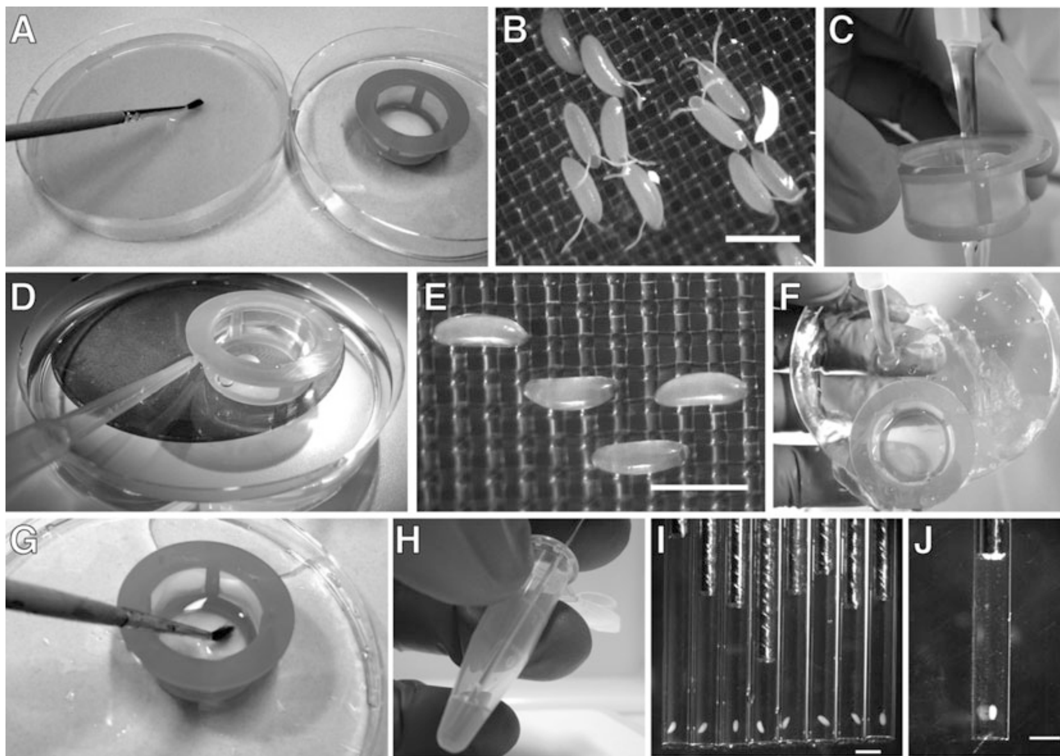

Fig. 2 Agarose mounting of embryos. The embryos are loosened with a brush from the apple juice plate (**a**). *Drosophila* embryos with intact chorion, note the dorsal appendages (**b**). After transfer into the sieve the embryos are washed briefly (**c**) and dechorionated under a stereomicroscope (**d**). Dechorionated *Drosophila* embryos with removed dorsal appendages, the surface is glossier and the embryos are more transparent (**e**) as compared to (**b**). After bleaching the embryos are washed briefly with ddH_2O, apply water onto the outside of the sieve (**f**). The embryos are transferred into an eppendorf tube with agarose using a brush (**g**). The agarose with an embryo is drawn into a capillary (**h**). Several embryos are mounted and a suitable sample is selected under the stereomicroscope (**i**). A suitable mounting with 1 embryo length of agarose below the sample and several millimeters above (note the end of the metal plunger). The embryo is upright with the AP axis roughly aligned with the axis of the glass capillary or in the desired orientation (**j**). Scale bars: 500 μm (**b** and **e**) and 1 mm (**i** and **j**)

embryos and compare to Fig. 2b showing embryos with intact chorion).

9. Immediately wash the embryos with ddH_2O, by applying ddH_2O onto the side of the sieve, not directly on the embryos (*see* Fig. 2f).
10. Place the sieve into a petri dish filled with 1× PBS to avoid drying the embryos.
11. Mix 125 μl of 2 % agarose with 112.5 μl of PBS.
12. Vortex the 1 % agarose mixture briefly and put back on max. 37 °C.
13. Vortex 1:100 bead dilution for 1 min.
14. Add 12.5 μl of the bead dilution (final conc. of beads 1:2000) to the 1 % agarose (*see* **Note 4**).
15. Vortex agarose bead mixture for 1 min.
16. Transfer 100 μl of agarose bead mixture into a fresh 1.5 ml eppendorf tube.
17. Place the remaining 1 % agarose mixture back on max. 37 °C.
18. Transfer embryos with a brush into the 1 % agarose bead mixture (*see* Fig. 2g).
19. Draw one embryo in each capillary and prepare 10 or more capillaries (*see* **Note 5** and Fig. 2h).
20. Place the capillaries carefully into a beaker filled with PBS (*see* **Note 6**).
21. Check capillaries and select a sample that fulfils the following criteria (*see* Fig. 2i, j and **Note 5**):
    (a) Embryo of the proper stage and morphology.
    (b) Embryo properly aligned for imaging.
    (c) No bubbles near the sample or close to the plunger.
    (d) No debris of the chorion near the sample.
22. Repeat until one or more suitable samples for live imaging are found.
23. Store mounted embryos in PBS at room temperature until imaging.

3.1.2 Mounting on Capillaries for Multi-sample Imaging

Mounting the embryos in agarose does not lend itself easily toward imaging multiple samples at the same time. Mounting one *Drosophila* embryo in the desired orientation in agarose can be a challenge because of its shape. Mounting several of them after each other in the same orientation is therefore not practical. Additionally, extending the agarose too much will lead to an increase of swinging of the column during imaging and increases the risk of drift of the agarose column out of the capillary. Thus only a limited

number of embryos can be mounted robustly in one experiment using the agarose mounting. This limits the throughput of SPIM imaging significantly.

We addressed this problem by developing a mounting method for *Drosophila* embryos that allows imaging multiple embryos in the same recording. This method is based on gluing the embryos to the side of a glass capillary (*see* **Note 7**). Only the range of the stage in the *y*-axis and the time resolution limits the number of embryos one is able to image in a single experiment. This method thus is ideal for screening and testing of imaging conditions.

1. Prepare beforehand:
   (a) Capillaries coated with glue (*see* **Note 8**).
   (b) Mounting setup: A microscopy slide is fixed on the cover of a petri dish using double-sided tape. The capillary is fixed with plasticine next to the slide with the coated side on top. Adjacent to the capillary a piece of double-sided tape is placed on the microscopy slide (*see* Fig. 3a)
   (c) Staged *Drosophila* embryos.
2. Take up embryos with blunt forceps (*see* Fig. 3b)
3. Place the embryos on the double-sided tape next to the capillary (*see* Fig. 3c).

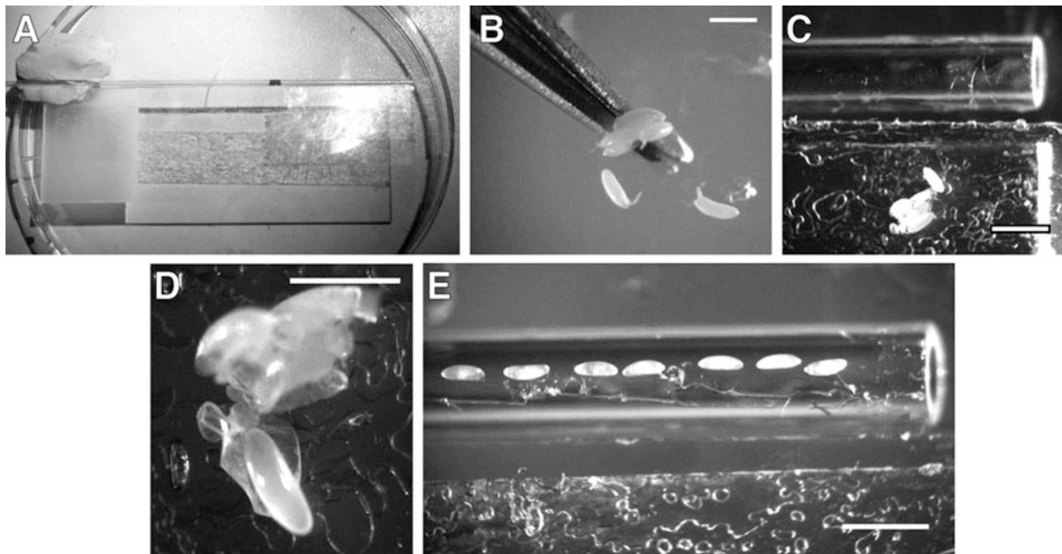

Fig. 3 Multi-sample mounting of embryos. A slide is fixed on the lid of a petri dish with double-sided tape. The capillary is fixed adjacent to the slide with plasticine. Double-sided tape is on the slide adjacent to the capillary (**a**). Embryos are transferred from the apple juice plate onto the double-sided tape with blunt forceps (**b** and **c**). The embryos are dechorionated by gently rolling them on the tape (**d**). The embryos are mounted on the coated glass capillary in the desired orientation (**e**). Scale bars: 500 μm (**b** and **d**) 1 mm (**c** and **e**)

4. Roll each embryo carefully on the tape until the embryo pops out of the chorion (*see* Fig. 3d).
5. Transfer the embryo with its intended orientation onto the coated capillary (*see* Fig. 3e).
6. Submerse the capillary in PBS after enough embryos are mounted.

3.2 Live Imaging Experiment Using Lightsheet Z.1

First, we will describe the microscope setup for both multiview and multi-sample imaging (*see* **Note 9**). Specific conditions for each of the methods are found in their respective sections.

3.2.1 Light Sheet alignment

The key step for successful LSFM imaging is the alignment of the center of the light sheet in the focus of detection objective (*see* **Note 9**). We are using the symmetry of point-spread functions (PSF) of fluorescent beads as readout for the correctness of the alignment. Alternatively, one can also use the intensity and sharpness of the sample for alignment [14, 15].

1. Prepare beforehand:
   (a) Bead sample without biological sample (*see* **Note 9**)
   (b) Prepare the sample chamber and the microscope according to the company protocol.
   (c) Set the correction collar of the objective lens to 1.33 (refractive index of water).
2. Set up the basic parameters for your imaging experiment in the ZEN black software of the Lightsheet Z.1 microscope:
   (a) Activate the necessary laser lines.
   (b) Select the correct objective in the software (*see* **Note 10**).
   (c) Choose the correct laser-blocking filter for your lasers.
   (d) Choose suitable emission filters.
   (e) Set the zoom: for the 20×/1.0 lens, a zoom of 0.8x is sufficient to cover the entire *Drosophila* embryo. Only for the alignment: Select single-sided illumination, untick *online dual side fusion* and *pivot scan*.
   (f) Set the frame size to 1200 × 1920 px (for an upright embryo).
   (g) Laser setting for 488 nm laser: 8 mW 561 nm laser: 15 mW, exposure time: 30 ms (*see* **Note 11**).
3. For the first alignment use the automatic light sheet alignment of the software or move the manual setting such that the beads are in focus and appear in their highest intensity (*see* Fig. 4c for aligned light sheet and compare to Fig. 4a for misaligned light sheet).

4. Acquire a stack of the agarose column closest to the objective using the lowest possible interval size. A stack of 20 μm is sufficient.
5. Evaluate the axial symmetry of the PSF using the *ortho* tool in ZEN black (*see* Fig. 4b for aligned light sheet and compare to Fig. 4d for misaligned light sheet).
6. Move the light sheet and repeat the stack until an optimal symmetry is reached for one side of the light sheet (*see* Fig. 3d).
7. Repeat for the second light sheet.
8. Optional: reset the correction collar on the objective to reduce spherical aberrations and then readjust the light sheet to achieve again a symmetrical PSF.
9. After the light sheets are adjusted, switch to dual-sided illumination, tick *online dual side fusion* and *pivot scan*.

3.2.2 Setup for Multiview Imaging

1. Insert the capillary into the sample holder from the top.
2. The red mark of the capillary should be at the bottom of the sample holder (*see* Fig. 4e arrowhead).
3. Place the sample holder into the stage of the microscope.
4. Move the capillary into the field of view of the objective.
5. Push out the agarose until the embryo is visible.

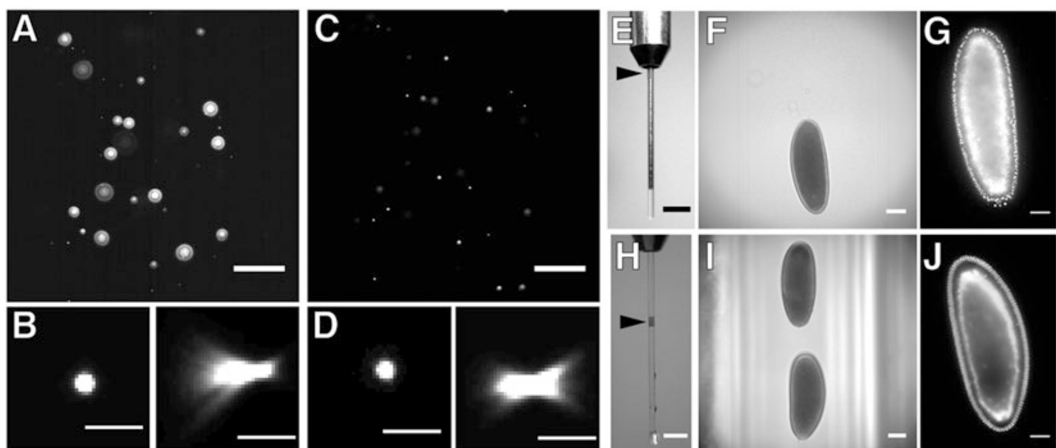

Fig. 4 Light sheet alignment. Diffraction rings of out of focus beads in a *xy*-slice through a stack acquired with a misaligned light sheet (**a**). *xy*- and *yz*-slices of a PSF of a bead acquired with a misaligned light sheet (**b**). Beads appear sharp in a *xy*-slice through a stack acquired with an aligned light sheet (**c**). *xy*- and *yz*-slices of the PSF of a bead acquired with an aligned light sheet. The PSF appears symmetrical in *yz* (**d**). Sample holder for multiview mounting, the red marker on the capillary (*arrowhead*) is positioned below the bottom of the sample holder (**e**). The agarose is pushed 2–3 embryo lengths out of the capillary, such that the capillary is out of the field of view of the microscope (**f**). Expression of His-YFP imaged with multiview agarose mounting (**g**). Sample holder for multi-sample mounting, the red marker (*arrowhead*) is positioned 1 cm below the sample holder (**h**). *Drosophila* embryos imaged using multi-sample mounting (**i** and **j**). Scale bars: 50 μm (**a, c, g,** and **j**), 5 μm (**b** and **d**), 5 mm (**e** and **h**) and 100 μm (**f** and **i**)

6. Further push out the agarose, until the embryo is 2–3 embryo lengths away from the bottom of the glass capillary (*see* Fig. 4f).
7. Wait until the agarose is stable (*see* **Note 12**).
8. Move the sample to the position were the first view should be. Read out the angle of the first position from the *specimen navigator* of the software and calculate the angles for the rest of the positions (*see* **Note 13**).
9. For each stack move the sample into the centre of the field of view. Set the first slice 10–15 μm on top of the embryo and the last one well past the waist of the embryo and set the step size to 1.5 μm (*see* **Note 14**).
10. When the first and the last slice of the stack are determined add the positions in the *multiview setup*.
11. Type in the next angle into the angle field of the *specimen navigator*.
12. Move the embryo again into the centre of the field of view and set the stack for this view and add it to the *multiview setup*.
13. After all views are added, set the number of cycles and the interval of the time-lapse in the *time series* tab and start the recording (*see* **Note 15**).
14. Observe the first couple of time points.
15. If there is dramatic movement of the agarose adjust the *multiview setup* and restart the time-lapse or replace the sample.

3.2.3 Setup for Multi-sample Imaging

1. Insert the capillary from below into the sample holder.
2. Between the bottom of the sample holder and the red mark should be about 1 cm, since there will be no agarose (*see* Fig. 4h arrowhead).
3. Carefully mount the sample holder in the stage. Do not touch the stage with the capillary.
4. Move the capillary into the field of view.
5. Locate the embryos and rotate them such that they are now facing the objective (*see* Fig. 4i).
6. Since there is no agarose that can move, directly start to prepare the recording.
7. Rotate the sample until the first embryo is in the correct position and such that the capillary does not reflect the light sheet (*see* **Note 7**).
8. Set the first stack 10–15 μm above the embryo and the last stack until the image quality is too degraded or the capillary starts to reflect the light sheet (*see* **Note 16**).
9. Set the interval, we recommend using the optimal setting suggested by the software.

10. After the first and the last slice are set, add the position to the *multiview setup*.
11. Move to the next embryo, adjust the stack and add it to the *multiview setup*
12. Choose *new* in the group tab, this assigns a new group to this embryo.
13. After each group has been set up, select the number of cycles in the time series tab and start the recording.
14. Observe the first few time points and determine if the entire embryo is acquired for each group.

4 Notes

1. Choose and test the fluorescent beads based on the laser lines, laser power, and filter settings used in the imaging experiment. For example for single channel imaging, using a 488 nm laser and a longpass filter we use the Estapor fluorescent microspheres F-Y 050. For single channel imaging, using a 561 nm laser and a longpass filter we use the Estapor fluorescent microspheres F-Z 050 beads. For dual channel experiments it is recommended to use beads that are visible in both imaged channels since different wavelengths have different effects on the registration. Sometimes this is not practical, either no beads can be found that are visible in all imaged channels or the beads would dominate the image too much in certain channels. To address this problem, suitable beads in one channel can be imaged and the registration of this channel is then applied to the others.
2. Prepare a 1:100 dilution from the stock solution: Vortex the bead stock for 1 min. Add 10 μl of the bead stock to 990 μl of sterile ddH_2O and store at 4 °C until use. The bead dilution should be sterile and in ddH_2O, contamination or use of buffers leads to the formation of bead clumps. In general vortexing before use is enough to dissociate the beads but sonication can also be helpful. Never heat up the Estapor fluorescent latex microspheres since they will lose their dye. Always vortex the bead dilution before use!
3. Preparation of 2% LMP agarose: Weigh in 30 mg of LMP agarose in 1.5 ml of 1× PBS. Heat up to 70 °C at 900 rpm on a thermomixer until the agarose is fully dissolved. Spin in a centrifuge for 5 min at max speed at 37 °C and transfer supernatant into a new tube to get rid of any undissolved agarose. Keep on 37 °C 900 rpm on a thermomixer until use or store at RT and heat up before use. The buffer in the agarose should

match the buffer used for the experiment to avoid shrinking or swelling of the agarose.

4. We found that a dilution of 1:2000 in the final sample works best with our imaging setup and gives a robust registration. Depending on your multiview setup, the size of the stacks, and the overlap between the different views, you may want to vary this concentration.
5. We found that it is best to have only a small column of agarose. One or two embryo lengths below the sample and about 2–3 mm above is best. At most there should be a half a cm of agarose column in total. This prevents that the agarose column falls out of the capillary during imaging, reduces swinging of the agarose column during imaging and reduces the initial swelling and shrinking of the agarose.
6. Avoid dropping the capillaries into the beaker. If the room temperature exceeds 25 °C the capillaries need to be placed briefly at 25 °C before putting them into the buffer in order for the agarose to solidify, otherwise the embryos fall out of the capillary.
7. Please note that the glass capillary is restricting the angle of imaging by blocking or scattering the light sheet, thus true multiview is not possible with this method and only a limited number of views can be acquired reliably. This drawback is offset by the increased throughput allowing imaging of multiple embryos with high speed. Also the software allows using different imaging conditions (i.e. laser power) and thus multiple different setups can be explored efficiently.
8. Cut pieces of double-sided tape and transfer them into a bottle with n-Heptane. Incubate at room temperature overnight before use, to allow the glue to dissolve from the tape and store at room temperature. Prepare glass capillaries by dispensing and distributing n-heptane glue with a tip onto the capillaries.
9. We generally recommend setting up the microscope before starting the mounting procedure, since the embryos are already developing. We use an agarose bead sample without biological specimen for the alignment and we recommend using beads that are visible in all imaged channels. This not only allows judging proper alignment of the light sheet in all channels but also ensures that the channels are aligned to each other. The alignment is then based on beads in the centre of the field of view and in the top section of the agarose close to the objective. The PSFs of beads in this part are the least influenced by optical aberrations introduced by the agarose or the objective.

10. The calibration contains the voxel size in *xy* and *z*. These parameters are stored in the metadata of the czi format. The *xy* size depends on the magnification of the lens and the selected zoom. Whereas the *z* size is determined by the step size of the motors in the *z* direction. It is very important to select the correct lens in the program otherwise the *xy* to *z* ratio is incorrectly stored in the metadata of the dataset and the processing will fail.
11. We in general use the smallest exposure time available and rather adjust the laser intensity settings, very fast and dynamic processes would otherwise get distorted or blurred. The embryos are most sensitive to 488 nm irradiation and are more tolerant against 561 nm.
12. In the beginning, the agarose will expand or shrink in the capillary. Wait until the agarose is equilibrated or alternatively readjust the stacks during imaging. We usually observe that the agarose is shrinking and thus moving up into the capillary, but it is also possible that the agarose is expanding out further. Equilibration usually takes 15–45 min, in rare cases this can take even longer. Thus plan your experiment around this and start the mounting accordingly and prepare multiple samples or adjust the multiview setup during the recording.
13. We recommend designing the principle multiview setup before starting the experiment to avoid exposing the sample unnecessarily. Decide on the number of views and the angles between the views. Then calculate the angles for each view once you determined the first view.
14. For us 130.5 μm work best, we also recommend setting each stack to the same size. We use a step size of 1.5 μm, this allows faster acquisition of each time point.
15. Before beginning the recording, we recommend to save the multiview setup and make a screenshot of the window. We also recommend splitting the .czi files by time points and views in the *separate files* tab.
16. Usually a stack of 140 μm is possible, we recommend setting each stack to the same size. Test the time it takes to acquire one stack. This will determine the time resolution one can achieve by multiplying this number by the number of groups. For example if one stack takes 30 s, 5 groups will take about 2 min and 30 s to acquire. Set this number with a bit extra time to account for the movement of the stage in the time-lapse interval.

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