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Simultaneous Live Imaging of Multiple Insect Embryos in Sample Chamber-Based Light Sheet Fluorescence Microscopes

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Subject: Submission of our revised manuscript
"A light sheet-based fluorescence microscopy protocol for simultaneous live imaging of multiple insect embryos in sample chamber-based setups"

Dear Dr. Werth, dear Dr. Liu, dear Dr. Bajaj, dear editors of *JoVE*,

We submit our revised manuscript *"A light sheet-based fluorescence microscopy protocol for simultaneous live imaging of multiple insect embryos in sample chamber-based setups"* by Julia Ratke, Franziska Krämer and Frederic Strobl for publication in the "Live Imaging" issue of *JoVE*.

First of all, we would like to thank the *JoVE* editors as well as the three reviewers for their comments and inform you that we have addressed all concerns diligently. We have revised some text passages, added a completely new figure (flowchart and sample holder schemes, Figure 1), extended the scope of another figure (embryo collection and preparation, Figure 2) and added another table (lens combinations, Table 1). Changes are annotated within the new manuscript file, and specific answers to the concerns are provided within the "Concerns and Answers" document.

We are still convinced that our protocol is a convenient addition for *JoVE*'s "Methods and protocols for live imaging in development" collection. Since 2014, we are constantly working on improving our protocol towards multiple directions – imaging quality, ease-of-use as well as throughput. Since we present our work at light sheet technology-specific conferences (*e.g. LSFM2019*, Frankfurt, Germany) as well as at developmental biology-specific conferences (*e.g. EuroEvoDevo* 2018, Galway, Ireland), we are well informed about the latest trends on both sides and can ensure that the methodology we develop and constantly improve is up-to-date and relevant.

We sincerely hope that you share our enthusiasm and look forward to your professional opinion.

Best regards,

Julia Ratke, Franziska Krämer and Frederic Strobl

TITLE:

Simultaneous Live Imaging of Multiple Insect Embryos in Sample Chamber-Based Light Sheet Fluorescence Microscopes

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KEYWORDS:

comparative live imaging, simultaneous live imaging, three-dimensional light microscopy, light sheet-based fluorescence microscopy, LSFM, cobweb holder, insect development, embryonic morphogenesis, embryogenesis, *Drosophila melanogaster*, *Tribolium castaneum*, ambient variance

SUMMARY:

Light sheet-based fluorescence microscopy is the most valuable tool in developmental biology. A major issue in comparative studies is ambient variance. Our protocol describes an experimental framework for simultaneous live imaging of multiple specimens and, therefore, addresses this issue pro-actively.

ABSTRACT:

Light sheet-based fluorescence microscopy offers efficient solutions to study complex processes on multiple biologically relevant scales. Sample chamber-based setups, which are specifically designed to preserve the three-dimensional integrity of the specimen and usually feature sample rotation, are the best choice in developmental biology. For instance, they have been used to document the entire embryonic morphogenesis of the fruit fly *Drosophila melanogaster* and the red flour beetle *Tribolium castaneum*. However, many available live imaging protocols provide only experimental frameworks for single embryos. Especially for comparative studies, such approaches are inconvenient, since sequentially imaged specimens are affected by ambient variance. Further, this limits the number of specimens that can be assayed within a given time. We provide an experimental framework for simultaneous live imaging that increases the

throughput in sample chamber-based setups and thus ensuring similar ambient conditions for all specimens. Firstly, we provide a calibration guideline for light sheet fluorescence microscopes. Secondly, we propose a mounting method for multiple embryos that is compatible with sample rotation. Thirdly, we provide exemplary three-dimensional live imaging datasets of *Drosophila*, for which we juxtapose three transgenic lines with fluorescently labeled nuclei, as well as of *Tribolium*, for which we compare the performance of three transgenic sublines that carry the same transgene, but at different genomic locations. Our protocol is specifically designed for comparative studies as it pro-actively addresses ambient variance, which is always present in sequential live imaging. This is especially important for quantitative analyses and characterization of aberrational phenotypes, which result e.g., from knockout experiments. Further, it increases the overall throughput, which is highly convenient when access to light sheet-based fluorescence microscopes is limited. Finally, the proposed mounting method can be adapted for other insect species and further model organisms, e.g., zebrafish, with basically no optimization effort.

INTRODUCTION:

Fluorescence microscopy is one of the most essential imaging techniques in the life sciences, especially in cell and developmental biology. In confocal fluorescence microscopes[1], which are state-of-the-art for three-dimensional fluorescence imaging since the mid-1990s, the same lens is used for fluorophore excitation and emission light detection. The illumination laser beam excites all fluorophores along the illumination/detection axis and the respective out-of-focus signal is discriminated prior to the detection by a pinhole. Hence, for each two-dimensional image, the entire specimen is illuminated. Consequently, for each three-dimensional image, i.e., a stack of spatially consecutive two-dimensional images, the entire specimen is illuminated several dozen to a few hundred times[2], which promotes photobleaching and phototoxicity[3].

Almost twenty years ago, light sheet-based technology[4] emerged as a promising alternative for three-dimensional fluorescence imaging and thus became a valuable tool in developmental biology[5]. In this approach, illumination and detection are decoupled. The illumination lens is used to generate a light sheet with a width of only a few micrometers within the focal plane of the perpendicularly arranged detection lens. Hence, for each two-dimensional image, only a thin planar volume around the focal plane is illuminated. Consequently, for each three-dimensional image, the entire specimen is illuminated only once, which strongly decreases photobleaching and phototoxicity[6]. For this reason, light sheet fluorescence microscopes (LSFMs) offers efficient solutions to study complex processes on multiple biologically relevant scales and is, therefore, of particular value in developmental biology, where specimens as large as several millimeters have to be analyzed at the subcellular level.

Historically, LSFMs have been sample chamber-based[7,8]. In these setups, the illumination (x) and detection (z) axes are usually arranged perpendicularly to the gravity axis (y). Sample chambers offer ample experimental freedom. Firstly, they provide large imaging buffer capacities, which in turn eases the use of a perfusion system to control the environment, e.g., to maintain a specific temperature[9] or to apply biochemical stressors. Further, they support customized mounting methods[10] that are tailored to the respective experimental needs while preserving the three-dimensional, in some instances dynamic[11], integrity of the specimen. Additionally, sample

chamber-based setups are usually equipped with a rotation function that is used to revolve the specimens around the y axis and thus image them along two, four or even more directions. Since the embryos of commonly used model organisms are, in the context of microscopy, relatively large, successive imaging along the ventral-dorsal, lateral, and/or anterior-posterior body axes provides a more comprehensive representation. This allows e.g., long-term tracking of cells that move along complex three-dimensional migration paths[12,13].

Light sheet-based fluorescence microscopy has been applied extensively to study the embryonic morphogenesis of *Drosophila melanogaster*, both systematically[14,15] as well as with a specific focus on the biophysical aspects of development. For instance, it was used to gather high-resolution morphogenetic data in order to detect a biomechanical link between endoderm invagination and axis extension during germband elongation[16] and further to relate the complex cellular flow with force generation patterns during gastrulation[17]. It has also been combined with other state-of-the-art techniques, e.g., with optogenetics to investigate the regulation of Wnt signaling during anterior-posterior patterning in the epidermis[18].

However, studying only one species does not provide insights into the evolution of development. To understand embryogenesis within the phylogenetic context, intensive research has been conducted with alternative insect model organisms. One of the most comprehensively investigated species is the red flour beetle *Tribolium castaneum*, an economically relevant stored grain pest[19], whose embryonic morphogenesis has also already been systematically imaged with LSFM[20]. The embryonic morphogenesis of these two species differs remarkably in several aspects, e.g., the segmentation mode[21], as well as the formation and degradation of extra-embryonic membranes[22]. The latter aspect has already been extensively analyzed using LSFMs. For instance, it has been shown that the serosa, an extra-embryonic tissue that envelops and protects the *Tribolium* embryo from various hazards for the better part of its embryogenesis[23,24] and acts as the morphogenetic "driver" for its own withdrawal process during dorsal closure[25]. Further, it has been demonstrated that during gastrulation, a particular region of the blastoderm remains anchored to the external vitelline membrane in order to create asymmetric tissue movements[26] and, following this observation, that regionalized tissue fluidization allows cells to sequentially leave the serosa edge during serosa window closure[27].

In all *Drosophila*- and *Tribolium*-associated studies cited above, sample chamber-based LSFMs have been used. In most, the embryos were recorded along multiple directions using the sample rotation function. Although not stated explicitly, it can be assumed that they have been recorded individually and thus independent of each other in sequential live imaging assays, similar to our previous work on *Tribolium*[20,28]. In certain scenarios, such an approach is acceptable, but especially in quantitative comparative approaches, ambient variance can distort the results. For instance, it has long been known that the developmental speed of insects is temperature-dependent[29], but a more recent study further suggests that in *Drosophila*, temperature may also affect the concentration of morphogens[30]. Consequently, if certain characteristics of embryogenesis, e.g., the dynamic proportions, division rates and migration velocities of cells, should be precisely quantified, sufficient repetitions without ambient variance are required. This minimizes standard deviations and standard errors, which in turn facilitates juxtaposition with

other, even just marginally divergent experimental conditions.

However, sample chamber based LSFMs are primarily designed for high content rather than for high throughput assays. Unlike confocal microscopes, which are typically equipped with standardized clamp mechanisms for microscopy slides, Petri dishes and well plates, nearly all sample chamber-based LSFMs use cylinder-based clamp mechanisms. These mechanisms are intended for custom-made sample holders that are rotation-compatible as well as non-invasive[10], but usually not designed for more than one specimen[20,31,32]. A framework for simultaneous live imaging of two or more embryos, in which the advantages of sample chamber-based setups are not compromised, addresses the ambient variance issue thereby increasing the value of LSFMs for comparative studies.

In our protocol, we present an experimental framework for comparative live imaging in sample chamber based LSFMs (**Figure 1A**) in which the y axis is used as an option to "stack" embryos. Firstly, we provide a fluorescent microsphere-based calibration guideline for sample chamber-based LSFMs, which is especially important for instruments that lack a calibration assistant. Secondly, we describe a mounting method for multiple embryos based on the cobweb holder[28] (**Figure 1B**) that is compatible with sample rotation and thus allows simultaneous imaging of multiple specimens along multiple directions (**Figure 1C**). Several embryos are aligned on top of a thin agarose film and, after insertion into the sample chamber, moved successively through the light sheet to acquire three-dimensional images. Thirdly, we provide three exemplary live imaging datasets for *Drosophila* as well as for *Tribolium*. For the former, we juxtapose transgenic lines with fluorescently labeled nuclei. For the latter, we compare the performance of transgenic sublines that carry the same transgene, but at different genomic locations. Finally, we discuss the importance of parallelization with regard to comparative live imaging and ambient variance[33], debate the throughput limit of our experimental framework and evaluate adaption of our approach to other model organisms.

PROTOCOL:

1. Preparatory work

1.1 Choose an illumination lens/detection lens/camera combination for the LSFM that suits the scientific question and set up the microscope. The size of the field of view is the quotient of the camera chip size and the magnification of the detection lens. The illumination lens should be chosen so that the entire field of view is covered by a roughly planar light sheet[34]. Three recommended combinations are listed in **Table 1**.

1.2 To prepare agarose aliquots and retrieval dishes, add 2 g low-melt agarose to 200 mL autoclaved tap water and heat the mixture in a microwave oven at 600-800 W until all agarose particles are dissolved. Prepare several 1 mL agarose aliquots in 1.5 mL or 2 mL reaction tubes, then fill several 90-mm Ø Petri dishes 3-5 mm high with agarose. Store solidified aliquots and dishes at 4 °C.

1.3 For *Drosophila*: To prepare fresh rearing vials, cook an adequate amount of custom-made or commercially available *Drosophila* medium, transfer 5-15 mL into wide vials and store them at 4 °C. To prepare egg-laying dishes, add 1 g of low-melt agarose to 50 mL of autoclaved tap water and heat the mixture in a microwave oven at 600-800 W until all agarose particles are dissolved. Allow the mixture to cool down to 45 °C, then add 50 mL fruit juice (preferably apple or red grape) and mix thoroughly. Pour the mixture into 35 mm Ø Petri dishes and store solidified egg-laying dishes at 4 °C.

1.4 For *Tribolium*: To prepare the growth medium, pass whole wheat flour as well as inactive dry yeast through a 710 µm mesh size sieve, then supplement the sieved flour with 5% (w/w) sieved yeast. To prepare egg-laying medium, pass fine wheat flour as well as inactive dry yeast through a 250 µm mesh size sieve, then supplement the sieved flour with 5% (w/w) sieved yeast.

2. Calibration of sample chamber based LSFMs using fluorescent microspheres

NOTE: The purpose of calibration is to align the focal points of the illumination and detection lenses (**Figure 2A**), as this is the premise for clear images. LSFMs should be calibrated regularly, at least once every 3-4 weeks.

2.1 Re-liquefy an agarose aliquot in a dry block heater/mixer at 80 °C, then allow the agarose aliquot to cool down to 35 °C.

2.2 Transfer 50 µL of agarose to a 1.5 mL reaction tube and add 0.5 µL of fluorescent microsphere solution. Mix at 1,400 rpm for 1 min.

2.3 Fill the slotted hole of the cobweb holder with 10 µL of agarose/fluorescent microsphere solution mixture, then aspirate as much agarose as possible until only a thin agarose film remains. Wait 30-60 s for solidification.

2.4 Fill the sample chamber with autoclaved tap water. Insert the cobweb holder slowly into the sample chamber and move the slotted hole with the microtranslation stages in front of the detection lens.

2.5 Rotate the cobweb holder with the rotation stage to a 45° position relative to the illumination (x) and detection (z) axes. The cobweb holder should not be visible in the transmission light channel.

2.6 Switch to the respective fluorescence channel and adjust the laser power as well as the exposure time so that the fluorescent microspheres provide proper signal.

2.7 Specify a volume of view that covers the now transversely oriented agarose film completely. Define the z spacing by calculating the minimally possible axial resolution for the respective illumination lens/detection lens combination[34]. Alternatively, 4 times the lateral resolution can be used as a rough approximation.

2.8 Record a three-dimensional test z stack of the fluorescent microspheres and compare the x, y and z maximum projections to the calibration chart (**Figure 2**). If the microspheres appear blurry, fuzzy, and/or distorted (**Figure 2B,C**), adjust the positions of the illumination and/or detection lens.

3. Collection of *Drosophila* embryos

3.1 Transfer 100-200 adults of the *Drosophila* line of choice to a fresh rearing vial 2-3 days before the imaging assay to establish an egg collection culture. If not yet existent, consider using the old rearing vial to start a progeny culture. Adults should not be older than two weeks, thus replace the embryo collection culture in time with the progeny culture.

3.2 Warm an egg-laying agarose plate to room temperature and add a drop of yeast paste on top of the agar.

3.3 Transfer the adults from the egg collection culture to an empty narrow vial and place it on top of the egg-laying dish. Incubate the egg collection setup at room temperature for 15 min. Avoid anesthesia (cold, CO_2) during this step if possible.

3.4 Return the adults to the rearing vial. Incubate the egg-laying dish at a convenient temperature/time combination, then transfer 10-20 embryos with a small paintbrush from the egg-laying dish to a 100 µm mesh size cell strainer. Discard the egg-laying dish.

3.5 Repeat steps 3.1 to 3.4 for each *Drosophila* line.

4. Collection of *Tribolium* embryos

NOTE: For convenience, a scheme of the *Tribolium* egg collection procedure is provided (**Figure 3A**) to which also the numbers within the brackets within this step refer.

4.1 Transfer 200-300 adults (about 400-700 mg) of the *Tribolium* line of choice to an empty 1 L glass bottle 2-10 days before the imaging assay to establish an egg collection culture (**Figure 3A_01**). Fill the bottle with 50-100 g of fresh growth medium. If not yet existent, consider starting a progeny culture using available larvae and pupae. To ensure adults are no more than 3 months old, replace the egg collection culture in time with the progeny culture.

4.2 Pass the egg collection culture through an 800 µm mesh size sieve (**Figure 3A_02**). Return the growth medium, which contains non-staged embryos, to the initial bottle (**Figure 3A_03**) and transfer adults to an empty 1 L glass bottle (**Figure 3A_04**). Add 10 g of egg-laying medium (**Figure 3A_05**) and incubate the egg collection setup at room temperature for 1 h (**Figure 3A_06,07**).

4.3 Pass the egg collection setup through the 800 µm mesh size sieve (**Figure 3A_08**). Return adults to their initial bottle (**Figure 3A_09**). Depending on the developmental process that should

be imaged, incubate the egg-laying medium, which now contains about 30-100 embryos, at a convenient temperature/time combination (**Figure 3A_10**).

4.4 Pass the egg-laying medium through the 300 µm mesh size sieve (**Figure 3A_11**) and transfer the embryos (**Figure 3A_12**) to a 100 µm mesh size cell strainer. Discard the sieved egg-laying media (**Figure 3A_13**).

4.5 Repeat steps 4.1 to 4.4 for each *Tribolium* line.

5. Sodium hypochlorite-based dechorionation

NOTE: Both *Drosophila* and *Tribolium* embryos are covered by a chorion, a protective and heavily light-scattering protein layer that is not essential for proper development as long as the embryos are kept moist after removal. The dechorionation protocol for the embryos of both species is identical.

5.1 Prepare 6 well plates by filling A1, A2, A3 and B3 wells with 8 mL of autoclaved tap water and the B1 and B2 wells with 7 mL of autoclaved tap water and 1 mL of sodium hypochlorite (NaOCl) solution (**Figure 3B**). Observe the dechorionation process under a stereo microscope, ideally in transmission light.

CAUTION: Sodium hypochlorite is corrosive.

5.2 Insert the embryo-containing cell strainer (Step 3.4 and/or 4.4) into the A1 well and wash the embryos about 30-60 s under gentle agitation.

5.3 Move the cell strainer to the B1 wells and shake the plates vigorously for 30 s, then transfer it to the A2 well and wash the embryos for 1 min under gentle agitation.

5.4 Move the cell strainer to the B2 well and shake the plate vigorously until most embryos are completely dechorionated (**Figure 3C**), then transfer it to the A2 well and wash the embryos for 1 min under gentle agitation.

5.5 Store the cell strainer in the B3 well before proceeding with the mounting procedure.

5.6 Repeat steps 5.1 to 5.5 for each line.

6. Mounting of multiple embryos using the cobweb holder

6.1 Re-liquefy an agarose aliquot in a dry block heater/mixer at 80 °C, then allow the agarose aliquot to cool down to 35 °C.

6.2 Pipet 10 µL of agarose on top of the slotted hole of the cobweb holder. With the pipette tip, spread the agarose over the slotted hole, then aspirate as much agarose as possible until only

a thin agarose film remains. Wait 30-60 s for solidification.

6.3 For each line, carefully pick one or more embryos with a small paintbrush and place them on the agarose film.

6.4 Arrange the embryos along the long axis of the slotted hole, then also align their anterior-posterior axis with the long axis of the slotted hole (**Figure 3D**).

6.5 Stabilize the embryos carefully by pipetting 1-2 µL of agarose into the gap between the embryos and the agarose film. Wait 30-60 s for solidification.

6.6 Insert the cobweb holder with the mounted embryos slowly into the image buffer-filled sample chamber.

7. Comparative live imaging in sample chamber based LSFMs

7.1 Move one of the embryos with the microtranslation stages in front of the detection lens. Ensure that the cobweb holder is in a 45° position relative to the illumination (x) and detection (z) axes (cf. **Figure 1C**).

7.2 In the transmission light channel, move the embryo into the center of the field of view. The cobweb holder should not be visible.

7.3 Move the embryo with the microtranslation stages in z until the midplane of the embryo overlaps with the focal plane, i.e. until the outline appears sharp. Without switching to the fluorescence channel, specify the volume of view by moving 250 µm away from the midplane into both directions.

7.4 Optionally, if imaging along multiple directions is required, rotate the embryo appropriately and repeat steps 7.2 and 7.3. The cobweb holder supports up to four orientations in steps of 90°.

7.5 Repeat steps 7.1 to 7.3 (or 7.4) for all other embryos mounted on the cobweb holder (**Figure 3E**). Ensure that the topmost embryo does not leave the imaging buffer when the bottommost embryo is in front of the detection lens.

7.6 Define the fluorescence channel (laser power, exposure time, detection filter) and time lapse (interval, total duration) parameters and start the imaging process. For indicative values, consult the metadata table of the example datasets (**Supplementary Table 1**). For assays that last several days, consider covering the sample chamber opening at least partially to reduce evaporation.

8. Retrieval and further cultivation of imaged embryos

8.1 When the imaging assay has ended, carefully remove the cobweb holder from the sample chamber.

8.2 Detach the embryos from the agarose film with a small paintbrush and transfer them to an appropriately labeled microscope slide. Place the slide into a retrieval dish and incubate under the respective standard rearing conditions.

8.3 Regarding the experimental modalities, estimate when embryogenesis is completed. As the hatching time point approaches, check the retrieval dishes frequently and transfer hatched *Drosophila* larvae to individual rearing vials and *Tribolium* larvae to individual wells of a 24-well plate. Fill the wells up to the half with growth medium. Incubate under the respective standard rearing conditions.

8.4 Once the observed individuals are adults, provide them with a suitable mating partner and check for progeny after several days.

9. Image data processing and metadata documentation

NOTE: For image data processing, the ImageJ derivate FIJI[35] is recommended (imagej.net/Fiji/Downloads). FIJI does not require installation and 32- as well as 64-bit versions are available. One of the most frequently used formats for LSFM data is the tagged image file format (TIFF), which allows storage of an image stack in form of a TIFF container.

9.1 Calculate the z maximum projections for all z stacks (**Image | Stacks | Z Project**, choose **Max Intensity**). Maximum projections are data simplification approaches that reduce the number of spatial directions from three to two. A FIJI script for batch processing is provided (**Supplementary File 1**).

9.2 Concatenate the respective z maximum projections to create time stacks (t stacks). Save these in one TIFF container. Do this for all recorded embryos as well as the respective directions and respective fluorescence channels if applicable.

9.3 Rotate the z and t stacks around the z axis to align the anterior-posterior axis of the embryos with the x or y image axis (**Image | Transform | Rotate**). Crop the z stacks along all three image axes and the t stack in the x and y image axes so that only minimal buffer space (20-40 pixels along the x and y axes, 5-10 images along the z axis) around the embryo remains (**Image | Adjust | Canvas Size** for the x and y axes, **Image | Stacks Tools | Slice Keeper** for the z axis).

9.3.1. Do this individually for all recorded embryos as well as the respective directions, if applicable. Fluorescence channels, if applicable, should be processed with identical rotation and cropping parameters.

9.4 Document the metadata as detailed as possible. As a guideline, the metadata table of the example datasets, which are featured in the Representative Results section, can be used

(**Supplementary Table 1**).

10. Data visualization

NOTE: This data visualization guideline focuses primarily on the creation of z maximum projection image matrices that show several recorded embryos along multiple directions and/or over time. The following steps describe the data visualization procedure that was applied to the example datasets for the creation of the figures shown and videos linked in the Representative Results section.

10.1 Combine t stacks of multiple directions (**Image | Stacks | Tools | Combine**) and/or merge multiple fluorescence channels (**Image | Color | Merge Channels**) to visualize the biological structure and/or process of interest.

10.2 Adjust the intensities of the t stacks (**Image | Adjust | Brightness/Contrast**) as needed by using the "**Set**" function. The minimum displayed value should be set slightly above the background signal, the maximum displayed value should result in a convenient contrast. Document both values, as they can be used for a consistent adjustment of the respective z stacks. Imprint adjustments using the "**Apply**" function. Depending on the experimental modalities, consider processing all recorded embryos with identical values.

10.3 Save intensity adjusted t stacks as separate files, do no override the non-adjusted t stacks. Compilate suitable sub stacks from the adjusted t stacks with dedicated image selection functions (e.g., **Image | Stacks | Tools | Slice Keeper**) and use the montage tool (**Image | Stacks | Make Montage**) to create image grids that can be used for figure design.

REPRESENTATIVE RESULTS:

Our protocol describes an experimental framework for comparative fluorescence live imaging in sample chamber based LSFMs. For instance, the framework can be used to juxtapose (i) embryos of two or more species, (ii) embryos of lines in which one or more genes are knocked out plus wild-type controls, (iii) multiple embryos of the same transgenic line, (iv) embryos from different transgenic lines, or (v) embryos from sublines that carry the same transgene, but at different genomic locations. In this section, we provide examples for the last two scenarios.

In our first exemplary application, we show the fluorescence signal dynamics of three embryos that derive from different transgenic *Drosophila* lines (**Table 2**) over a period of about 1 day (**Figure 4**, **Supplementary Movie 1**). For these lines, we expected rather similar fluorescence patterns since all of them express nuclear-localized EGFP/GFP under control of different presumably ubiquitous and constitutively active promoters. However, our comparative live imaging results show that there are strong spatiotemporal differences in the expression patterns that are certainly not secondary effects due to ambient variance.

In our second application example, we compared the performance of three embryos that derive from the AGOC{Zen1'#O(LA)-mEmerald} #1, #2 and #3 (Zen1'LA-mE #1, #2 and #3, respectively)

transgenic *Tribolium* sublines[36]. All of these carry the same piggyBac-based transgene that leads to expression of mEmerald-linked[37] Lifeact[38] under control of the *zerknüllt 1*[39] promoter, a transcription factor involved in serosa specification. Our comparative live imaging results, which illustrate the serosa window closure process during gastrulation, suggest that subline #2 provides a remarkably stronger overall signal than the other two sublines (**Figure 5, Supplementary Movie 2**). This indicates that also in *Tribolium*, the genomic context may have a strong influence on the expression level of transgenes that carry an expression cassette.

Successful retrieval all six embryos was possible as shown in this section as described in Step 8 of our protocol. All of them developed into fully functional and fertile adults (**Supplementary Table 1**, "Retrieval" row), indicating that the overall procedure, from dechorionation over mounting to recording, was non-invasive. This level of quality control is essential whenever wild-type development is expected, e.g., regarding control embryos that are imaged simultaneously with embryos in which one or more genes are knocked down or knocked out.

All data sets that were used to create the representative results are provided as resources that will help especially LSFM novices to evaluate the quality of their own work. The digital object identifier-based download links can be found in the metadata table (**Supplementary Table 1**, "**Data Access**" row).

FIGURE AND TABLE LEGENDS:

Figure 1: Experimental framework and mounting method overview. (**A**) Flowchart of the ten protocol steps with short reminders and an estimation of the required time. Tasks marked with asterisks do not have to be performed during every assay but depending on the circumstances. Green boxes indicate steps associated with *Drosophila* and/or *Tribolium*, blue boxes indicate steps associated with microscopy. (**B**) Detail drawing of the cobweb holder. The presented design is suitable for cylinder-based clamp mechanisms, which are commonly used in sample chamber-based LSFMs. Specifications are in millimeters. The holder can be scaled as long as the working distance of the detection lens is respected. (**C**) Recording sequence for live imaging of multiple embryos along multiple directions. At first, z stacks of the uppermost embryo are recorded along up to four orientations, i.e. 0°, 90°, 180° and 270°. Subsequently, the embryo below is moved in front of the detection lens and the recording/rotation sequence begins anew.

Figure 2: Fluorescent microsphere-based calibration chart for LSFMs. The chart illustrates how fluorescent microspheres appears in the x, y and z maximum projections if the LSFM is correctly calibrated (**A**), or if there is an illumination (**B**) or detection offset (**C**).

Figure 3: The cobweb holder for the mounting and imaging of multiple embryos. (**A**) *Tribolium* embryo collection overview. The illustrations are cited throughout Step 4 according to the numbers in the upper left. (**B**) Preparation and transfer scheme for the 6-well plate used during Step 5. The B1 and B2 wells contained diluted sodium hypochlorite (NaOCl), which induces dechorionation. (**C**) The 100 µm cell strainer which was used to transfer the embryos from one well to another, within the B2 well. The upper detail image shows a close-up of several *Tribolium*

embryos on top of the cell strainer mesh, the lower detail image shows a transmission light stereo microscope image of several *Tribolium* embryos with partially detached chorion. (**D**) Cobweb holder with three mounted *Tribolium* embryos. The embryos, as well as their anterior-posterior axes, were aligned along the long axis of the slotted hole. (**E**) Movement of the cobweb holder within the sample chamber of the LSFM during the recording of three embryos.

Figure 4: Comparative fluorescence live imaging of one embryo each from the #01691, #29724 and #24163 transgenic *Drosophila* lines. Embryos are shown along two (of four recorded) directions over a period of 20:00 h (from a total imaging period of 23:40 h). No dynamic intensity correction over time was performed. Metadata for the *Drosophila* datasets can be found in **Supplementary Table 1**. ZA, Z maximum projection with image adjustment. Scale bar, 100 µm.

Figure 5: Comparative fluorescence live imaging of one embryo each from the Zen1'LA-mE #1, #2 and #3 transgenic *Tribolium* sublines. (**A**) Embryos are shown along four directions during gastrulation, just prior to serosa window closure. (**B**) Embryos are shown ventrally over a period of 01:30 h (from a total imaging period of 118:00 h). Image adjustment was performed with identical minimum and maximum displayed values, no dynamic intensity correction over time was performed. Metadata for the *Tribolium* datasets can be found in **Supplementary Table 1**. Datasets were synchronized to the stage shown in (**A**). ZA, Z maximum projection with image adjustment. Scale bar, 100 µm.

Table 1: Recommended illumination lens/detection lens/camera combinations for live imaging of *Drosophila* and *Tribolium* embryos using LSFM. All combinations have been successfully employed in previous studies (see Reference row). Please note that most chamber-based LSFMs use water-dipping lenses for detection, which are primarily designed for imaging buffers with a refractive index of 1.33 (such as autoclaved tap water or other aqueous media). Imaging buffers with other refractive indices can be used[40], but this changes certain properties of the optical system change, e.g. the working distance.

Table 2: The three transgenic *Drosophila* lines used in the first application example. The "line" column refers to the Bloomington *Drosophila* Stock Center (bdsc.indiana.edu) stock number.

Supplementary Video 1: Comparative fluorescence live imaging of one embryo each from the #01691, #29724 and #24163 transgenic *Drosophila* lines. Each transgenic line expresses nuclear-localized EGFP/GFP under control of a presumably ubiquitous and constitutively active promoter. ZA, Z maximum projection with image adjustment. No dynamic intensity correction over time was performed. ZA, Z maximum projection with image adjustment.

Supplementary Video 2: Comparative fluorescence live imaging of one embryo each from the Zen1'LA-mE #1, #2 and #3 transgenic *Tribolium* sublines. Each transgenic subline expresses mEmerald-labeled Lifeact under control of the *zerknüllt 1* promoter, a transcription factor involved in serosa specification. Please note that the Zen1'LA-mE #1 embryo turns approximately 90° within the serosa right after serosa window closure. Datasets were synchronized to the stage shown in **Figure 4A**. Image adjustment was performed with identical minimum and maximum

displayed values, no dynamic intensity correction over time was performed. Embryos are shown ventrally and laterally over a period of 48:00 h. ZA, Z maximum projection with image adjustment.

Supplementary File 1: FIJI Batch processing script for the automated calculation of z maximum projections from all z stacks within one folder. The script can be opened in FIJI via drag-and-drop. At the start ("Run") only the input folder must be specified. The output subfolder ("Z Maximum Projections") is created automatically.

Supplementary Table 1: Metadata and parameter for the long-term live imaging datasets DS0001-6.

DISCUSSION:

One of the exclusive application areas of LSFMs is developmental biology. In this discipline, it is of importance to look at living specimens, otherwise morphogenetic processes cannot be described in a dynamic manner. An experimental framework for the simultaneous live imaging in sample chamber based LSFMs, as described here, is convenient for two major reasons. Ambient variance, which is unavoidable in sequential live imaging, can be addressed pro-actively. In insect embryo-associated live imaging, we see e.g., the following susceptibilities.

In sequential live imaging, the egg collection cultures may have different ages at the time of embryo collection. In *Drosophila*, it has been shown that this affects the qualities of the progeny[45]. Although working with appropriately desynchronized cultures is an option, simultaneous imaging with synchronized cultures is considerably more convenient. Both the *Drosophila*- and *Tribolium*-based representative results derive from synchronized egg collection cultures, and we can confidently exclude that the different fluorescence characteristics are an unintended effect of parental senescence.

Dechorionation is a crucial step as prolonged exposure to sodium hypochlorite decreases embryo viability. Inconveniently, the ionic strength of sodium hypochlorite decreases over time. Measurements of the ionic strength are possible[46], but laborious and cumbersome. In simultaneous live imaging, a master mix can be used for all lines and/or conditions during the preparation of the dechorionation-associated 6-well plates.

Even in laboratories with efficient air condition, small but irregular temperature fluctuations are inevitable and also affect temperature-controlled[9] sample chamber-based LSFMs. Theoretically, the temperature in the sample chamber can be logged, but an exact repetition is not possible. In simultaneous live imaging, these fluctuations also occur, but they affect all embryos likewise.

From the practical point of view, a protocol for simultaneous imaging of multiple embryos increases the throughput. In live imaging, the duration of the developmental process of interest determines how many assays can be conducted within a given period, which is an important consideration when access to light sheet-based fluorescence microscopes is limited. Prime examples for long-term recording are live imaging assays in which cells are tracked. Such experiments usually range over long development periods. While the embryonic development of

Drosophila takes only about one day at room temperature[47], *Tribolium* takes about seven days[48]. Hence, for this species, parallelization is essential to remain within a reasonable time frame, especially in assays where many conditions are compared.

The throughput, i.e., the number of embryos that are imaged simultaneously, is primarily limited by two factors: First is the space available along the y axis within the sample chamber. When the lowermost embryo is about to be imaged, the uppermost embryo should not have left the imaging buffer. Further, the cobweb holder and the associated support system should not be so lengthy that it wobbles and/or jitters noticeably during the z stack recording process. Second, is the intended temporal resolution. The recording time per embryo can be approximated by multiplying the exposure time per two-dimensional image (usually 10-200 ms) with the number of planes per z stack (usually several hundred), the number of channels (usual between one and three), the number of directions (usually between one and eight) as well as a setup-dependent factor for the translation and rotation movements (estimated between 1.2 and 1.5). The ratio of the intended temporal resolution and the recording time per embryo indicates the maximum number of embryos that can be imaged simultaneously. In summary, our protocol is appropriate to advance from sequential live imaging to medium throughput, but not suitable for what is generally regarded as high throughput. For the latter, LSFMs suitable for microscope slides[49–51] or well plates[52] can be considered, but these implementations usually have other limitations.

Depending on the experimental question and hence the illumination lens/detection lens/camera combination in conjunction with the imaging parameters, simultaneously imaged LSFM datasets may require vast storage space in their raw state. This issue can best be illustrated by considering the provided *Drosophila* sample datasets (**Supplementary Table 1**), i.e., DS0001-3. Each two-dimensional raw image had a data volume of about 2.8 Megabytes, each raw z stack consisted of 120 planes, and all three embryos were recorded along four directions a total of 143 times. In consequence, the raw datasets occupied about half a Terabyte. Through image data processing, i.e., cropping along all spatial dimensions (Step 9) in conjunction with ZIP-based compression, the data volume was reduced to 53.3 Gigabyte, which is about 10% of the initial amount. To process datasets in the terabyte regime, the BigDataViewer[53] plugin, which is pre-installed in FIJI (**Plugins | BigDataViewer**), is recommended.

In addition to the five scenarios outlined in the Representative Results section, our protocol can also be used to characterize the effect of RNAi knockdown effects, which is a popular approach in *Tribolium*[54]. However, it is only of limited use for the analysis of compounds (e.g., insecticides or other biochemical stressors) if these should be applied through the imaging buffer – which is the most straightforward approach – as simultaneous imaging of a control embryo is not possible.

We demonstrated our protocol using one of our custom-built microscopes[8,55], but it is applicable to any sample camber-based setup, e.g., OpenSPIM[56] or most commercial LSFMs. This protocol complements the LSFM-associated resource establishment initiative that aims at encouraging novices to actively integrate LSFM into their research plans. Most universities and institutes nowadays operate well-equipped fluorescence imaging facilities[57]. These usually provide access to one or more chamber based LSFMs, but we cannot be expected the staff to have the proper

experimental solutions for every scientific question at hand.

Our results show that our experimental framework is suitable for *Drosophila* and *Tribolium*. We are confident that our comparison-focused protocol can be used to study the embryogenesis of other insect species, e.g., a LSFM-based approach has been applied to compare wild-type and knockdown development in the scuttle fly *Megaselia abdita*[58], and subsequent studies would also benefit from our mounting method. In addition, transgenic lines of the two-spotted cricket *Gryllus bimaculatus* specifically designed for fluorescence live imaging[59] are available, but the only currently available comparison-focused protocol[60] is intended for inverted microscopes and therefore does not address three-dimensional image acquisition or sample rotation. Additionally, it has already been shown that the cobweb holder is a convenient choice for the zebrafish, whose embryos change their shape considerably during embryogenesis[61]. An adaptation of our protocol, which requires basically no optimization effort, would provide the above-described advantages also for this model organism.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

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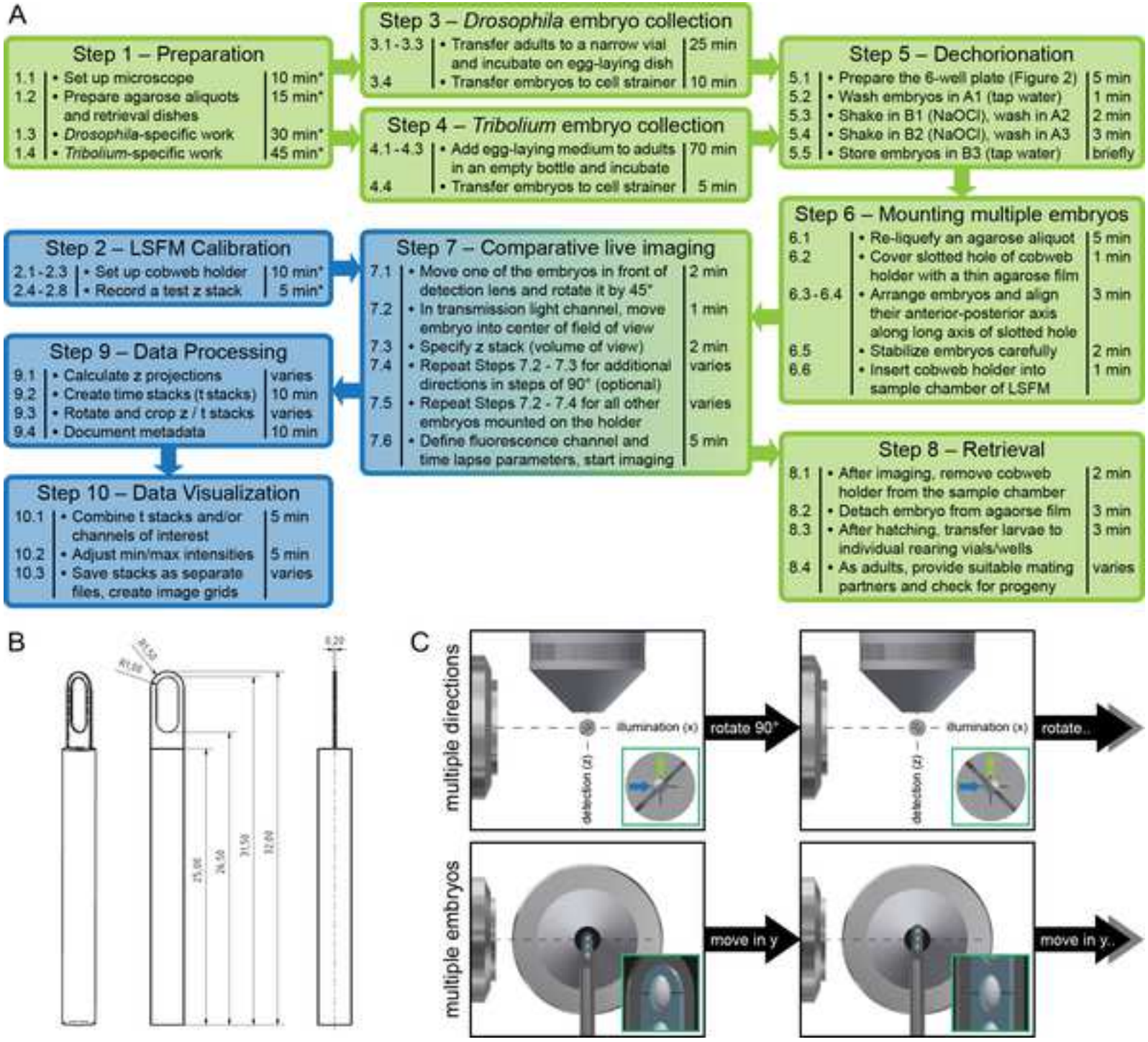

Figure 2

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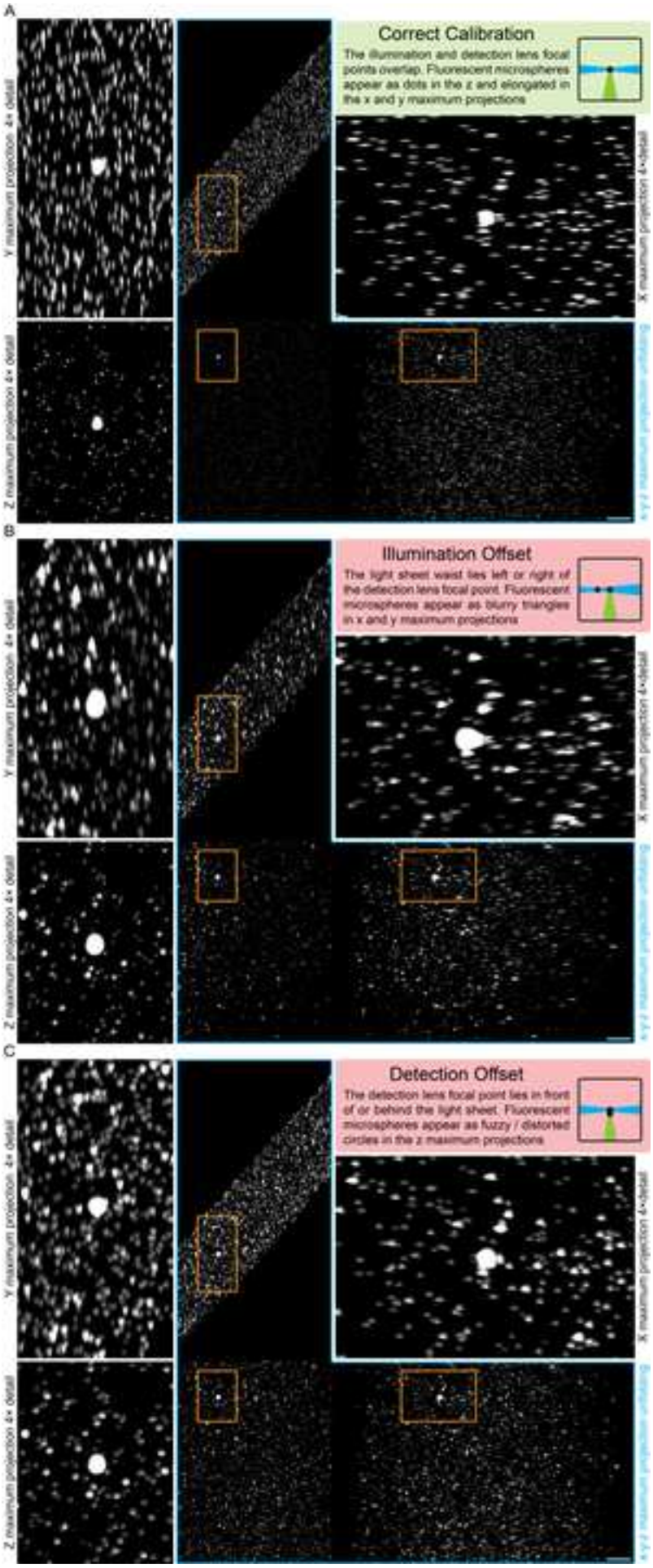

Figure 3

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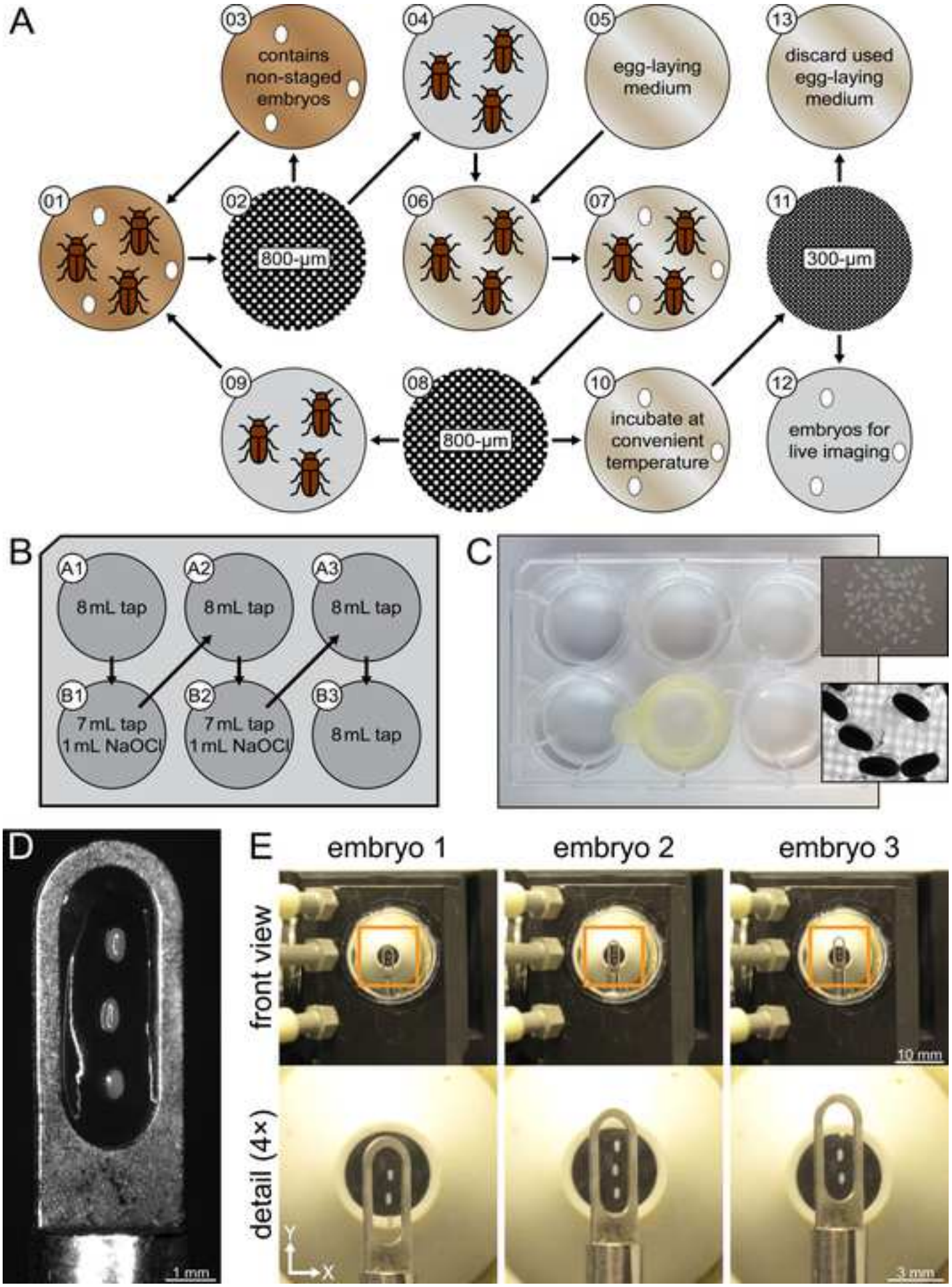

Figure 4

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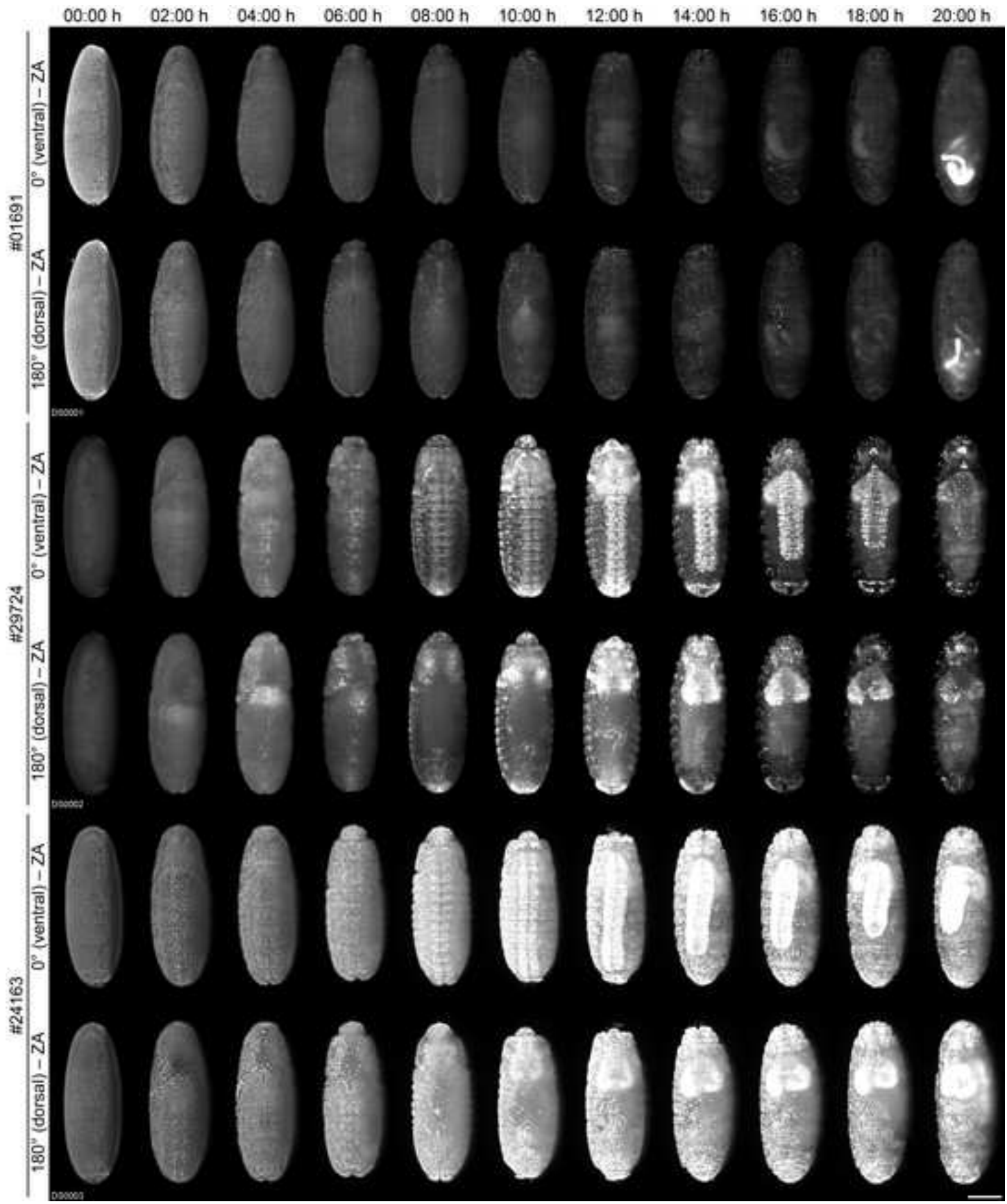

Figure 5

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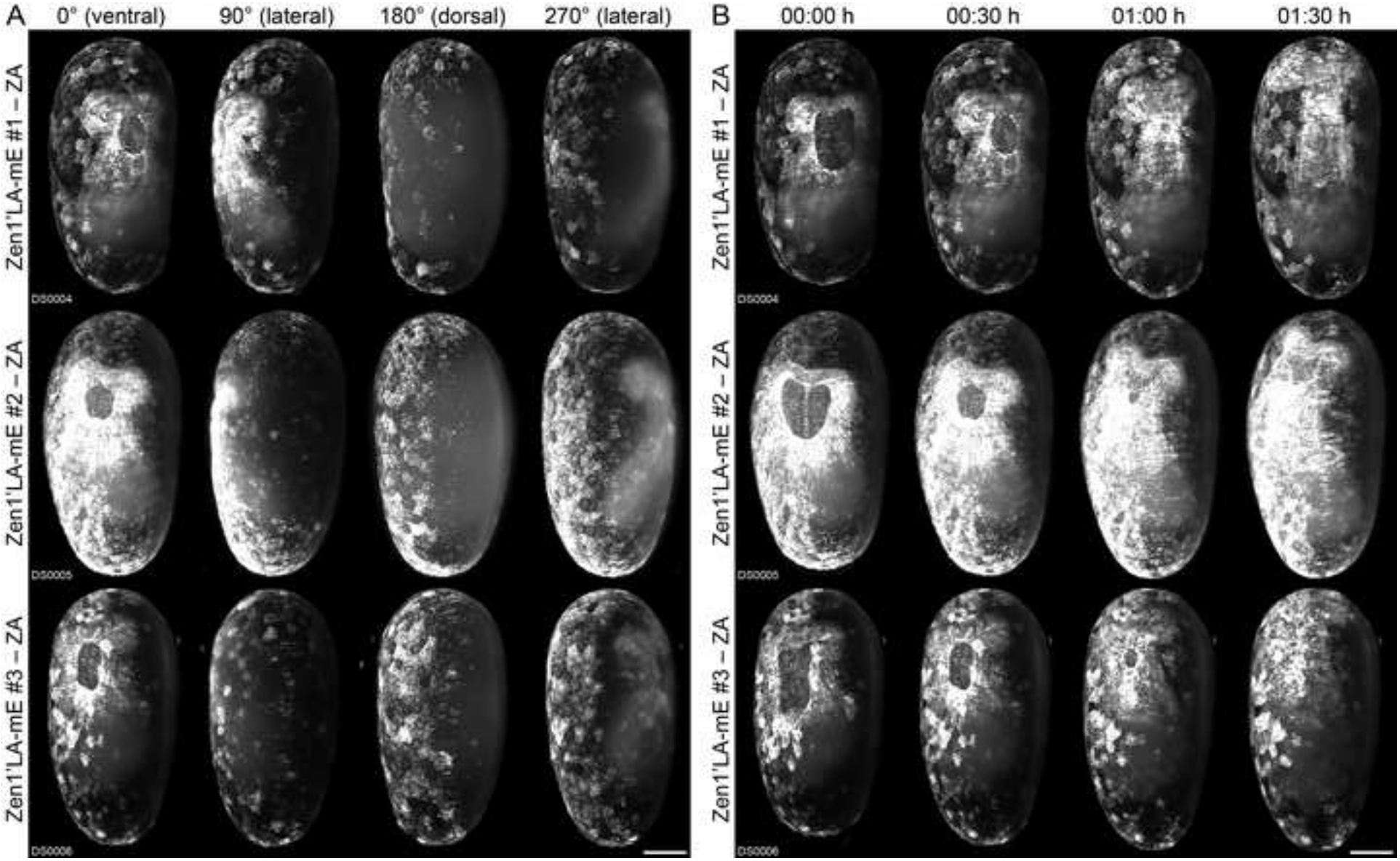

Supplementary Video 1

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Video or Animated Figure

Supplementary Video 1-JQ.avi

Supplementary Video 2

Table 1 Click here to access/download;Table;Table 1.xlsx

Combination	Rationale	Illumination lens	Detection lens	Camera	Reference
#1	entire embryo on the cellular level, large pixel spacing (small data volume)	2.5× magnification Numerical aperture 0.06 (air)	10× magnification Numerical aperture 0.3 (water-dipping)	CCD with 1.4 megapixel (1392×1040, 6.45 µm pitch)	this study, *Drosophila* [41], *Tribolium* [20,41]
#2	entire embryo on the cellular/subcellular level, small pixel spacing (large data volume)	2.5× magnification Numerical aperture 0.06 (air)	20× magnification Numerical aperture 0.5 (water-dipping)	sCMOS with 5.5 megapixel (2560×2160, 6.5 µm pitch)	*Tribolium* [9]
#3	specific regions of the embryo on the subcellular level or specialized applications *e.g.* live imaging of dissected germbands[42,43]	5.0× magnification Numerical aperture 0.16 (air)	40× magnification Numerical aperture 0.75 (water-dipping)	CCD with 1.4 megapixel (1392×1040, 6.45 µm pitch)	*Tribolium* [44]

Table 2

Line	Genotype
#01691	y[1] w[67c23]; P{w[+mC]=Ubi-GFP.nls}ID-2; P{Ubi-GFP.nls}ID-3
#29724	w[*]; P{w[+mC]=Tub84B-EGFP.NLS}3
#24163	w[*]; P{w[+mC]=His2Av-EGFP.C}2/SM6a

Fluorophore expression
NLS-GFP under control of the *polyubiquitin* promoter
NLS-GFP under control of the *alpha-tubulin 84B* promoter
EGFP-labeled histone 2A variant in all cells

Table of Materials

Click here to access/download;Table of Materials;20_07_11_JoVE_LiveImaging_Materials_Revision.xls

Name of Material/ Equipment	Company	Catalog Number
6-well plate	Orange Scientific	4430500
24-well plate	Orange Scientific	4430300
35-mm Ø Petri dish	Fisher Scientific	153066
90-mm Ø Petri dish	Fisher Scientific	L9004575
100-µm mesh size cell strainer	BD Biosciences	352360
250-µm mesh size sieve	VWR International	200.025.222-038
300-µm mesh size sieve	VWR International	200.025.222-040
710-µm mesh size sieve	VWR International	200.025.222-050
800-µm mesh size sieve	VWR International	200.025.222-051
405 fine wheat flour	Demeter e.V.	SP061006
commercially available *Drosophila* medium	Genesee Scientific	66-115
fluorescent microspheres, 1.0 µm Ø	Thermo Fisher Scientific	T7282
inactive dry yeast	Genesee Scientific	62-108
low-melt agarose	Carl Roth	6351.2
narrow vials	Genesee Scientific	32-109
small paint brush	VWR International	149-2121
sodium hypochlorite (NaOCl), ~12% active Cl	Carl Roth	9062.3
whole wheat flour	Demeter e.V.	SP061036
wide vials	Genesee Scientific	32-110

Comments/Description

Only for live imaging involving *Tribolium*
Only for live imaging involving *Drosophila* .

Only for live imaging involving *Tribolium*
Only for live imaging involving *Tribolium*
Only for live imaging involving *Tribolium*
Only for live imaging involving *Tribolium*
Only for live imaging involving *Tribolium*
Only for live imaging involving *Drosophila* / Custom-made *Drosophila* medium may also be used

Only for live imaging involving *Tribolium*

Only for live imaging involving *Drosophila*

Caution: sodium hypochlorite is corrosive
Only for live imaging involving *Tribolium* / United Kingdom: wholemeal flour
Only for live imaging involving *Drosophila*

Editorial comments #E

Protocol Details #E-1

Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. Please ensure that all specific details (e.g. button clicks for software actions, numerical values for settings, etc) have been added to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Some examples:

Concern #E-1.1

1.1: "decide for the juxtaposition participants" unclear what this means.

Answer #E-1.1

The cited protocol step was removed in accordance with our changes regarding Concern #2-6. Throughout the manuscript, we replaced the term "juxtaposition participants" by either "line", "recorded embryo(s)" or "sublines".

Respective changes are annotated in the manuscript via the "track changes" function.

Concern #E-1.2

3.1: Unclear what the imaging culture is.

Answer #E-1.2

We changed the term "imaging culture" to "egg collection culture" in Step 3.1 (the *Drosophila* embryo collection) as well as in Step 4.1 (the *Tribolium* embryo collection step) for reasons of consistency and rephrased the sentences slightly. Please consider also Concern #3-11 regarding the use of the term "culture".

Respective changes are annotated in the manuscript via the "track changes" function.

Concern #E-1.3

4.1: Do you mean "refresh" the growth medium? What is the medium composition?

Answer #E-1.3

This concern has been addressed by the changes that we made regarding Concern #E-1.3.

Concern#E-2.1

Please adjust the numbering of your protocol section to follow JoVE's instructions for authors, 1. should be followed by 1.1. and then 1.1.1. if necessary and all steps should be lined up at the left margin with no indentations.

Answer #E-2.1

We adjusted the numbering according to the instructions for authors.

Concern #E-2.2

Add a one-line space between each protocol step.

Answer #E-2.2

We added a one-line space between each step.

Concern #E-3.1

After you have made all of the recommended changes to your protocol (listed above), please re-evaluate the length of your protocol section. There is a 10-page limit for the protocol text, and a 3- page limit for filmable content. Please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps.

The highlighting must include all relevant details that are required to perform the step. For example, if step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be included in the highlighting.

Answer #E-3.1

After all adjustments to our protocol, there are now nearly exactly 2.5 pages highlighted for filming.

Concern #E-3.2

The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.

Answer #E-3.2

We (all three authors as well as one not directly involved colleague) have ensured that the sections to be filmed provide a coherent narrative.

Concern #E-3.3

Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when calculating the final highlighted length.

Answer #E-3.3

Done as requested.

Concern #E-3.4

Notes cannot be filmed and should be excluded from highlighting.

Answer #E-3.4

Done as requested.

Concern #E-4

JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 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.

Answer #E-4

We are sure that we cover all mentioned points adequately, especially troubleshooting (calibration guideline), limitations (minimum interval, analysis of compounds, very high throughput), significance (increased throughput without large sacrifices, addressing the ambient noise problem) and future applications (RNAi knockdown embryos, other species).

Concern #E-5

Fig 3: Scale bar on fig 3 is hard to see.

Answer #E-5

The scale bars on all microscopy images have been enlarged and were moved slightly further away from the edge of the images. The labels were removed and scale bar dimensions are now annotated in the respective figure legends.

Concern #E-6

Please spell out journal names.

Answer #E-6

Journal names in the reference section are spelled out now.

Commercial Language #E-7

JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Examples of commercial sounding language in your manuscript are Nutrifly.

Answer #E-7

We replaced the “Nutrifly” term within the manuscript (and the Materials table) by “custom-made or commercially available *Drosophila* medium”. These changes also address Concern #3-9.

Respective changes are annotated in the manuscript via the “track changes” function.

Table of Materials #E-8

Sort the list alphabetically.

Answer #E-8

Done as requested. In addition, we added a comment for each consumable that is only required for either *Drosophila* or *Tribolium* to simplify the protocol for laboratories that are only interested in either species.

Reviewer #1

Concern #1-1 (Minor)

In a certain way, this manuscript is a complement of a previous publication from the same author (Strobl, Klees & Stelzer 2017) in JoVE. I have to mention that points 4, 5 and 8 from the Protocol section are almost the same that appear in this paper. I' am not totally sure about the need to repeat this part in this manuscript except to maintain the integrity of the protocol in each publication.

Answer #1-1

Live imaging in light sheet-based fluorescence microscopes is a vast and complex topic, and this protocol was written with a considerably different focus so that new and thus complementary aspects are spotlighted (2017: technical background of LSFM, multiple mounting methods, stained embryos – 2020: microscope calibration, comparative live imaging, *Drosophila* and *Tribolium*, sample data).

We believe that a protocol must be self-contained (and assume that this is also in the interests of JoVE), thus a slight overlap is unavoidable. However, as our methods are constantly under improvement, the respective workflows became more streamlined over the last 3-4 years and hence, the descriptions also became more advanced/concise.

Concern #1-2 (Minor)

Introduction.
Line-120 Only a commentary: It is surprising not to mention that one of the main differences between Drosophila and Tribolium is that the fly presents a long germ development and this beetle develops as short germ embryos.

Answer #1-2

We focused on the extra-embryonic membranes here because our *Tribolium* sample data is based on a transgenic line in which exactly this tissue is labeled. But we agree with Reviewer #1 here that it would be argumentatively convenient to mention more differences. We do now briefly mention the differences in segmentation.

Respective changes are annotated in the manuscript via the "track changes" function.

Concern #1-3 (Minor)

Protocol.
1. Preparation work.
Given that you recommend choosing illumination lens/detection lens/camera combination as well as the number of embryos (participants) considering the scientific question, it would be very helpful to mention some recommendations regarding these decisions. In addition, you can explain/justify your decisions in the case of the examples presented both with Drosophila and Tribolium. Do you think it can be possible to mount dissected Tribolium embryos? Please comment.

Answer #1-3

This is an important concern, but the JoVE protocol guidelines do recommend not too many statements/sentences per step. We consider the addition of a table (new Table 1, old Table 1 is now Table 2) as the only convenient solution here.

We have not tested in how far LSFM and dissected embryos synergize, but we know from previous studies that such an approach works well with confocal microscopes and that the germbands still elongate for several hours (see Sarrazin *et al.* 2012, *Science* and Macaya *et al.* 2016, *Development Genes and Evolution*).

We agree that it is a good idea to inform the reader of this approach, but we are limited in text space here. We have therefore decided to mention this approach in the new Table 1 as an example case for the usage of high magnification (with the two references mentioned above).

We also turned the "Fill the sample chamber…" sentence (last sentence of Step 1.1) into an individual step (now Step 1.2).

Concern #1-4 (Minor)

6. Mounting of multiple embryos using the cobweb holder.
Do you think that the incorporation of some compounds (drugs, small molecules, etc.) into the mounting medium (agarose/buffer) can reach embryos' cells during live imaging in order to exert their function? Any comment about this?

Answer #1-4

In principle, this is possible, but unlike in RNAi or knockdown assays for instance, no elegant control embryo can be simultaneously imaged. We consider this a limitation that is worth discussing and added a brief paragraph to the discussion.

Respective changes are annotated in the manuscript via the "track changes" function.

Concern #1-5 (Minor)

8. Retrieval and further cultivation of imaged embryos.
Can you explain when is it important to recover the imaged embryos, estimate their full embryogenesis, rear them until adulthood and check for progeny?

Answer #1-5

Reviewer #1 raises an important concern here, and we agree that this had not been explained sufficiently in our first submission. We added another brief paragraph to the Representative Results section that explains the importance of embryo retrieval with regard to our sample data.

Respective changes are annotated in the manuscript via the "track changes" function.

Reviewer #2

Concern #2-1 (Minor)

I suggest that the authors highlight why it is difficult to image several embryos in parallel on a light sheet microscope, as compared to a confocal, for example. This prepares the novice users of light sheet microscopy to the difficulties ahead and highlights the value of the study.

Answer #2-1

Reviewer #2 points out correctly that this may not be directly obvious for LSFM novices. We added a respective explanation to the second-to-last paragraph of the introduction. These changes also address Concern #2-5.

Respective changes are annotated in the manuscript via the "track changes" function.

Concern #2-2 (Minor)

At the beginning of the Protocol section, would be better to add a protocol overview before delving into the details.

Answer #2-2

This minor concern aligns with the major concern of Reviewer #3. Please see Answer #3-1.

Concern #2-3 (Minor)

Line 114: "Drosophila is mainly of academic interest". What does this mean? Is imaging embryonic development currently of interest to industry? If so, would be nice to give examples.

Answer #2-3

Please see Concern and Answer #3-7.

Concern #2-4 (Minor)

Line 133-134: "similar to our previous Tribolium-associated studies methodology". A complex sentence. Maybe better: similar to our previous work on Tribolium.

Answer #2-4

We agree with Reviewer #2 here and changed the sentence accordingly.

Respective changes are annotated in the manuscript via the "track changes" function.

Concern #2-5 (Minor)

Line 134-135: "built around the specimen". Unclear to non-experts.

Answer #2-5

This concern has been addressed by the changes that we made regarding Concern #2-1.

Concern #2-6 (Minor)

Line 159: unnecessary.

Answer #2-6

In accordance with the confusion about the term “juxtaposition participants” (see Concern and Answer #E-1.1), we removed Step 1.1 entirely.

Respective changes are annotated in the manuscript via the “comment” function.

Concern #2-7 (Minor)

Lines 161-163: Better dividing to two sentences: The size of the field of view is the quotient of the camera chip size and the magnification of the detection lens. The illumination lens should be chosen so that the entire field of view is covered by a roughly planar light sheet.

Answer #2-7

Done as suggested.

Respective changes are annotated in the manuscript via the “track changes” function.

Concern #2-8 (Minor)

Line 165 "To prepare agarose aliquots and retrieval dishes". That came suddenly without mentioning what retrieval dishes are what are they used for. This could be fixed by mentioning this in a protocol overview at the beginning.

Answer #2-8

With the addition of the suggested protocol overview (please see Concerns and Answers #2-2 and #3-1), this step should now be self-explanatory.

Concern #2-9 (Minor)

Maybe expand the LSFM preparation step (Lines 160-164) if there are any differences between your protocol and standard LSFM protocols.

Answer #2-9

This is a little bit tricky. The LSFM field, with its many custom-built instruments, is much more diverse than for example the confocal microscopy field with its many commercial solutions. In our opinion, there are no standard protocols and also never will be due to the traditional LSFM-associated “customize-it” idea (→ "built around the specimen"). Such

claims would also be against the current spirit within the LSFM community. With agreement of Reviewer #2 and the editor, we would refrain from citing protocols as "standard" or referring to our protocol as the new "standard".

Concern #2-10 (Minor)

Line 183: Better to mention somewhere beforehand in the protocol why calibration is important and what you are calibrating. Again, writing an overview of the protocol is highly recommended.

Answer #2-10

We added a brief note to Step 2 that explains why calibration is important. About the overview, please see Concern #3-1.

Concern #2-11 (Minor)

Line 189: Explain what the cobweb holder is and why it is used.

Answer #2-11

The cobweb holder is now described in Subfigure B and C of the new Figure 1.

Concern #2-12 (Minor)

Lines 295-298: Would be nice to suggest some parameter values or ranges to start with, if possible.

Answer #2-12

Parameter values depend on many different criteria (scientific question, irradiation tolerance of the specific species and transgenic line) and also cannot be chosen completely arbitrary, as a high laser power only functions with a long interval between the time points. Hence, we decided to reference the imaging metadata-associated supplementary table.

Respective changes are annotated in the manuscript via the “track changes” function.

Concern #2-13 (Minor)

Line 308: "As this time point approaches" ?

Answer #2-13

We changed the sentence to “As the hatching time point approaches”.

Respective changes are annotated in the manuscript via the “track changes” function.

Concern #2-14 (Minor)

Line 368: "two latter scenarios". Better: the last two scenarios.

Answer #2-14

We adopted the suggestion regarding the sentence structure as proposed.

Respective changes are annotated in the manuscript via the "track changes" function.

Concern #2-15 (Minor)

Line 372: I think you mean Figure 3.

Answer #2-15

We are grateful to Reviewer #2 for pointing out this typo.

Respective changes are annotated in the manuscript via the "track changes" function.

Concern #2-16 (Minor)

Lines 372-374: better to mention that these are different promoters.

Answer #2-16

We changed singular to plural throughout the whole sentence and added the term "different".

Respective changes are annotated in the manuscript via the "track changes" function.

Reviewer #3

Concern #3-1 (Major)

There is a lack of flow within the protocol, specifically with the overview of the steps necessary to complete the imaging approach (i.e. sample preparation / collection, calibration of the LSFM, collection of samples, imaging, and post-imaging processing). Inclusion of an outline / organizational chart would greatly help the reader in the steps necessary towards understanding the protocol.

Answer #3-1

We agree with Reviewer #3 that more visual information is needed. We added a new figure (Figure 1), which includes a comprehensive overview of our protocol. The overview is color coded, contains concise bullet point-style reminder text and roughly indicates the time required for every steps.

Concern #3-2 (Major)

The authors present an approach to conduct comparative studies with an emphasis on eliminating experimental variation between samples. Although the experimental approach outlined does address the problem of experimental variation between sequential samples, the experimental design does not support a framework for quantitative comparative studies. For use in quantitative comparative studies, the authors should consider emphasizing the imaging of 3 embryos of the same strain, which would provide a comparative framework for quantitative analysis. While, the authors touch upon this possibility in the results section, it may be more useful to include this in the abstract or introduction, as many readers will likely be interesting in using this protocol to facilitate quantitative analysis.

Answer #3-2

We are glad that Reviewer #3 sees potential for our protocol in terms of quantitative comparative studies. We followed his suggestions and included a brief remark in the abstract in the introduction.

Respective changes are annotated in the manuscript via the "track changes" function.

Concern #3-3 (Major)

Each section possess a difficulty in understanding how the steps work together. Inclusion of diagrams, especially in the calibration of sample chamber-based LSFMs using fluorescent microspheres, Collection of Tribolium embryos, and Sodium hypochlorite-based dechorionation, could really use diagrams to help the reader follow the steps.

Answer #3-3

Besides the overview (see Concern #3-1), we added two more schemes (Figure3A and B), one for the *Tribolium* embryo collection procedure and one for the sodium hypochlorite-based dechorionation process (which also includes one large and two small photographs, Figure 3C).

Concern #3-4 (Major)

In the Image data processing and metadata documentation, the authors contend that these images can be opened using FIJI, however a serious detractor in use of LSFM is the size of the data sets. The authors should include some discussion of the size of the data sets and ways to reduce or deal with large sizes, as large file sizes tend to crash FIJI.

Answer #3-4

This concern is important, but also rather dependent on *e.g.* the available hardware (especially available RAM). We added another paragraph to the discussion that addresses this and Concern #3-5 sufficiently so that the reader/viewer gets a basic overview, but a comprehensive elaboration lies beyond the scope of this protocol. It would be worth thinking about another JoVE video that exclusively covers LSFM assembly, calibration, performance evaluation and data management/storage.

Respective changes are annotated in the manuscript via the "track changes" function.

Concern #3-5 (Minor)

Since you are imaging multiple embryos, it would be helpful to know more about the acquisition settings. How many stacks are produced? What is the time interval between stacks? How long does it take to image all 3 embryos? How much storage in terms of memory are necessary for one experiment? These are important considerations for the reader to know about so that they may adapt this protocol to suit their biological question.

Answer #3-5

This concern is now answered throughout the whole manuscript. (i) We do now, quite often, refer to Supplementary Table 1, which contains a lot of numbers which provides especially LSFM novices with a certain impression of the "magnitudes" in the LSFM world. (ii) The third paragraph of the discussion suggest on how to estimate the time required to image one/all embryos and thus the minimal possible interval between two time points (just answer the question the other way round: how many embryos can be imaged when the interval is fixed?). (iii) The new fourth paragraph of the discussion exemplifies how to estimate raw dataset size and mentions the storage saving potential via cropping and compression.

Concern #3-6 (Minor)

Sparse telecentric arrangements is mentioned twice in abstract and introduction; however, it is not well covered why sparse telecentric arrangements are beneficial or essential for this protocol. To this end, it may not be necessary to mention detail regarding the optical engineering behind the imaging modality.

Answer #3-6

We agree with Reviewer #3 and as we stated I Answer #1-1, we do not focus on the technical background of LSFM in this article. Hence, we rephrased the sentences and removed the term "sparse telecentric arrangement".

Respective changes are annotated in the manuscript via the "track changes" function.

Concern #3-7 (Minor)

Line 114 - 117 "Drosophila is mainly of academic interest, and working with only one species does not provide insights into the evolution of development. To embed the vast "fly knowledge" into the phylogenetic context, intensive research has been conducted with alternative insect model organisms." The phrasing here implies that research is done just to

transfer "fly knowledge" onto other organisms, which is largely not how "fly knowledge" is transferred or applied to other organisms. Consider rephrasing with an emphasis for the questions phylogenetic studies are asking - such as the evolution of certain proteins and their functions.

Answer #3-7

The intention was more to point out that some insect species (*Tribolium* for example) have become scientific model organisms due to their classification as pests. We have rephrased the two first sentences of the respective paragraph.

Respective changes are annotated in the manuscript via the "track changes" function.

Concern #3-8 (Minor)

Line 163-64: use of imaging buffer - autoclaved tap water is recommended, however does the buffer need to match the NA of the objectives? Most LSFM systems use objectives that are water based, but a mention of this would be helpful, as imaging of embryogenesis will sometimes use buffers that contain a different refractive index.

Answer #3-8

We added a brief "water-dipping objective/refractive index" annotation to the table description of the new Table 1 since we think that this is the most suitable location.

Concern #3-9 (Minor)

Line 170: use of Nutrifly media - why nutrifly? Can any Drosophila media be used?

Answer #3-9

This concern has already been addressed.

Please see Answer #E-7.

Concern #3-10 (Minor)

Line 199: There is a mention of beads, not sure what is meant here. What are these beads?

Answer #3-10

In the field of fluorescence microscopy, "beads" is a slightly more informal term for fluorescent microspheres. For consistency reasons alone, we changed from the former to the latter term.

Respective changes are annotated in the manuscript via the "track changes" function.

Concern #3-11 (Minor)

Line 209: There is a reference to imaging cultures, does the authors mean the parental fly lines that are crossed to collect embryos? In discussion of transgenic fly lines, please refer to them as lines, as cultures indicates an aqueous - cell culture based approach and can confuse the reader.

Answer #3-11

We understand the slight confusion here, but our terminology is as follows: A fly/beetle laboratory can have one or more (transgenic) *Drosophila* and/or *Tribolium* lines (=stocks). Of these lines, one or more cultures may exist: a "backup culture" (usually kept at lower temperatures to slow down development), an "imaging culture" (primarily to collect embryos for live imaging assays e.g. in the morning), an "in situ" culture (primarily to collect embryos for in situ hybridizations e.g. in the afternoon) and a "progeny" culture that mainly consists of larvae and pupae that will be used in the near future to replace aged cultures.

We adopted our terminology from the Bloomington Drosophila Stock Center standard that can be found on their Care & Information Website (https://bdsc.indiana.edu/information/fly-culture.html). For "Live Culture Stockkeeping", they write:

"Most stocks can be successfully cultured by periodic mass transfer of adults to fresh food. Bottles or vials are tapped on the pounding pad to shake flies away from the plug, the plug is rapidly removed and the old culture inverted over a fresh bottle or vial. Flies are tapped into the new vessel, or some shaken back into the old one, as necessary, and the two are rapidly separated and replugged."

as well as

"If the quality of your fly food is unreliable it is wise to have at least two cultures for each stock, staggered to assure the use of different batches of medium (at least until you find a new cook)."

Also, Ashburner and Roote, who wrote the "Maintenance of a Drosophila Laboratory: General Procedures" Cold Spring Harbor Laboratories protocol (doi:10.1101/pdb.ip35) use the term "culture" similar to us.

Hence, we would therefore prefer to retain the term, but are also quite dispirited by this inconsistent use.

Concern #3-12 (Minor)

Line 253: ...embryo containing cell strainer. Is this the same as used in above (Line 238) in collection of tribolium embryos? Please clarify.

Answer #3-12

We now reference the respective steps (Step 3.4 and 4.4) of the *Drosophila*/*Tribolium* egg collection procedure.

Respective changes are annotated in the manuscript via the "track changes" function.

Supplementary Table 1

Dataset (DS)	**DS0001**	**DS0002**	**DS0003**
Species	*Drosophila melanogaster* (Meigen) Arthropoda → Insecta → Diptera → Drosophilidae		
Line	Bloomington #01691	Bloomington #29724	Bloomington #24163
Line Genotype	y[1] w[67c23]; P{w[+mC]=Ubi-GFP.nls}ID-2; P{Ubi-GFP.nls}ID-3	w[*]; P{w[+mC]=Tub84B-EGFP.NLS}3	w[*]; P{w[+mC]=His2Av-EGFP.C}2/SM6a
Stock	~100-200 adults, less than 2 weeks old		
Stock Medium	Nutri-Fly Bloomington Formulation (66-112, Dominique Dutscher SA, Brumath, France)		
Stock Conditions	12:00 h light / 12:00 h darkness at 25°C and 70% relative humidity (DR-36VL, Percival Scientific, Perry, IA, USA)		
Egg Laying Period	00:15 h at room temperature (23±1°C)		
Egg Laying Medium	apple juice (254615, REWE Markt GmbH, Köln, Germany) dishes with 1% (w/v) agarose		
Pre-imaging Incubation	00:30 h at room temperature (23±1°C)		
LSFM Type	mDSLM (monolithic digital scanned laser light sheet-based fluorescence microscope) based on DSLM		
Laser Lines	488 nm / 20 mW diode laser (PhoxX 488-20, Omicron Laserprodukte GmbH, Rodgau-Dudenhofen, Germany)		
Excitation Objective	2.5× NA 0.06 EC Epiplan-Neofluar objective (422320-9900-000**,** Carl Zeiss, Göttingen, Germany)		
Emission Objective	10× NA 0.3 W N-Achroplan objective (420947-9900-000, Carl Zeiss, Göttingen, Germany)		
Emission Filters	525/50 single-band bandpass filter (FF03-525/50-25, Semrock/AHF Analysentechnik AG, Tübingen, Germany)		
Camera	High-resolution CCD (Clara, Andor, Belfast, United Kingdom), 14 bit, 1040×1392 pixel (pitch 6.45 µm)		
Dataset File Type	TIFF, 16 bit grayscale (planes saved as Z stacks in ZIP-compressed container files, indicated as PL(ZS))		
Dechorionation	~30-60 s in 14% (vol/vol) sodium hypochlorite (425044-250ML, Sigma Adlrich, Taufkirchen, Germany) in autoclaved tap water		
Mounting Method	Cobweb holder (embryos are glued to a thin agarose film spanning a slotted hole)		
Parallel Imaging	With DS0002 and DS0003	With DS0001 and DS0003	With DS0001 and DS0002
Mounting Agarose	1% (w/v) low-melt agarose (6351.2, Carl Roth, Karlsruhe, Germany) in autoclaved tap water		
Imaging Buffer	autoclaved tap water		
Imaging Temperature	room temperature (23±1°C)		
Retrieval	developed to healthy adult, produced fertile progeny	developed to healthy adult, produced fertile progeny	developed to healthy adult, produced fertile progeny
Comment	-	-	-

Dataset (DS)	**DS0001**	**DS0002**	**DS0003**
Dataset Size	16.1 Gigabyte (TIFF)	14.4 Gigabyte (TIFF)	22.8 Gigabyte (TIFF)
Figures	Figure 3	Figure 3	Figure 3
Supplementary Videos	Supplementary Movie 1	Supplementary Movie 1	Supplementary Movie 1
Dataset DOI (Zenodo)			
Time Points (TP)	**143 (TP0001-TP0143)**	**143 (TP0001-TP0143)**	**143 (TP0001-TP0143)**
TP Interval	00:10 h	00:10 h	00:10 h
Total Time (TP×TP Interval)	23:40 h	23:40 h	23:40 h
Directions (DR)	**4 (DR0001-DR0004)**	**4 (DR0001-DR0004)**	**4 (DR0001-DR0004)**
DR Orientations	0°, 90°, 180°, 270°	0°, 90°, 180°, 270°	0°, 90°, 180°, 270°
Channels (CH)	**1 (CH0001)**	**1 (CH0001)**	**1 (CH0001)**
CH0001 Excitation	488 nm	488 nm	488 nm
CH0001 Power	135 µW (close to the embryo)	135 µW (close to the embryo)	135 µW (close to the embryo)
CH0001 Exposure Time	50 ms	50 ms	50 ms
CH0001 Emission Filter	525/50 single-band bandpass filter	525/50 single-band bandpass filter	525/50 single-band bandpass filter
Planes (PL)	**85 (PL0001-PL0085)**	**85 (PL0001-PL0085)**	**85 (PL0001-PL0085)**
Z Spacing	2.58 µm	2.58 µm	2.58 µm
Z Distance (PL×Z Spacing)	219.3 µm	219.3 µm	219.3 µm
X-Dimensions (XD)	**500 pixels (cropped)**	**500 pixels (cropped)**	**500 pixels (cropped)**
X Spacing	0.645 µm	0.645 µm	0.645 µm
X Length (XD×X Spacing)	322.5 µm	322.5 µm	322.5 µm
Y-Dimensions (YD)	**900 pixels (cropped)**	**900 pixels (cropped)**	**900 pixels (cropped)**
Y Spacing	0.645 µm	0.645 µm	0.645 µm
Y Length (YD×Y Spacing)	580.5 µm	580.5 µm	580.5 µm
Data Access	10.5281/zenodo.3932189	10.5281/zenodo.3932193	10.5281/zenodo.3932195

Supplementary Table 1

Dataset (DS)	**DS0004**	**DS0005**	**DS0006**
Species	*Tribolium castaneum* (Herbst) Arthropoda → Insecta → Coleoptera → Tenebrionidae		
Line	AGOC{Zen1' #O(LA)-mEmerald} #1 subline	AGOC{Zen1' #O(LA)-mEmerald} #2 subline	AGOC{Zen1' #O(LA)-mEmerald} #2 subline
Line Genotype	one insert (mC/mC) homozygous	one insert (mC/mC) homozygous	one insert (mC/mC) homozygous
Stock	~200-300 adults, less than 3 month old		
Stock Medium	full grain wheat flour (113061006, Demeter, Darmstadt, Germany) supplemented with 5% (wt/wt) inactive dry yeast (62-106, Flystuff, San Diego, CA, USA)		
Stock Conditions	12:00 h light / 12:00 h darkness at 25°C and 70% relative humidity (DR-36VL, Percival Scientific, Perry, IA, USA)		
Egg Laying Period	01:00 h at room temperature (23±1°C)		
Egg Laying Medium	405 fine wheat flour (113061036, Demeter, Darmstadt, Germany) supplemented with 5% (wt/wt) inactive dry yeast (62-106, Flystuff, San Diego, CA, USA)		
Pre-imaging Incubation	15:00 h at 25°C and 70% relative humidity in darkness, 01:00 h at room temperature (23±1°C)		
LSFM Type	mDSLM (monolithic digital scanned laser light sheet-based fluorescence microscope) based on DSLM		
Laser Lines	488 nm / 20 mW diode laser (PhoxX 488-20, Omicron Laserprodukte GmbH, Rodgau-Dudenhofen, Germany)		
Excitation Objective	2.5× NA 0.06 EC Epiplan-Neofluar objective (422320-9900-000**,** Carl Zeiss, Göttingen, Germany)		
Emission Objective	10× NA 0.3 W N-Achroplan objective (420947-9900-000, Carl Zeiss, Göttingen, Germany)		
Emission Filters	525/50 single-band bandpass filter (FF03-525/50-25, Semrock/AHF Analysentechnik AG, Tübingen, Germany)		
Camera	High-resolution CCD (Clara, Andor, Belfast, United Kingdom), 14 bit, 1040×1392 pixel (pitch 6.45 µm)		
Dataset File Type	TIFF, 16 bit grayscale (planes saved as Z stacks in ZIP-compressed container files, indicated as PL(ZS))		
Dechorionation	~30-60 s in 14% (vol/vol) sodium hypochlorite (425044-250ML, Sigma Adlrich, Taufkirchen, Germany) in autoclaved tap water		
Mounting Method	Cobweb holder (embryos are glued to a thin agarose film spanning a slotted hole)		
Parallel Imaging	With DS0005 and DS0006	With DS0004 and DS0006	With DS0004 and DS0005
Mounting Agarose	1% (w/v) low-melt agarose (6351.2, Carl Roth, Karlsruhe, Germany) in autoclaved tap water		
Imaging Buffer	autoclaved tap water		
Imaging Temperature	room temperature (23±1°C)		
Retrieval	developed to healthy adult, produced fertile progeny	developed to healthy adult, produced fertile progeny	developed to healthy adult, produced fertile progeny
Comment	-	-	-

Dataset (DS)	**DS0001**	**DS0002**	**DS0003**
Dataset Size	57.1 Gigabyte (TIFF)	60.0 Gigabyte (TIFF)	49.6 Gigabyte (TIFF)
Figures	Figure 4	Figure 4	Figure 4
Supplementary Videos	Supplementary Movie 2	Supplementary Movie 2	Supplementary Movie 2
Dataset DOI (Zenodo)			
Time Points (TP)	**237 (TP0001-TP0237)**	**237 (TP0001-TP0237)**	**237 (TP0001-TP0237)**
TP Interval	00:30 h	00:30 h	00:30 h
Total Time (TP×TP Interval)	118:00 h	118:00 h	118:00 h
Directions (DR)	**4 (DR0001-DR0004)**	**4 (DR0001-DR0004)**	**4 (DR0001-DR0004)**
DR Orientations	0°, 90°, 180°, 270°	0°, 90°, 180°, 270°	0°, 90°, 180°, 270°
Channels (CH)	**1 (CH0001)**	**1 (CH0001)**	**1 (CH0001)**
CH0001 Excitation	488 nm	488 nm	488 nm
CH0001 Power	135 µW (close to the embryo)	135 µW (close to the embryo)	135 µW (close to the embryo)
CH0001 Exposure Time	50 ms	50 ms	50 ms
CH0001 Emission Filter	525/50 single-band bandpass filter	525/50 single-band bandpass filter	525/50 single-band bandpass filter
Planes (PL)	**150 (PL0001-PL0150)**	**150 (PL0001-PL0150)**	**150 (PL0001-PL0100)**
Z Spacing	2.58 µm	2.58 µm	2.58 µm
Z Distance (PL×Z Spacing)	387.0 µm	387.0 µm	387.0 µm
X-Dimensions (XD)	**600 pixels (cropped)**	**600 pixels (cropped)**	**600 pixels (cropped)**
X Spacing	0.645 µm	0.645 µm	0.645 µm
X Length (XD×X Spacing)	387.0 µm	387.0 µm	387.0 µm
Y-Dimensions (YD)	**1000 pixels (cropped)**	**1000 pixels (cropped)**	**1000 pixels (cropped)**
Y Spacing	0.645 µm	0.645 µm	0.645 µm
Y Length (YD×Y Spacing)	645.0 µm	645.0 µm	645.0 µm
Data Access	10.5281/zenodo.3932197	10.5281/zenodo.3932199	10.5281/zenodo.3932201

All datasets can be downloaded at www.physikalischebiologie.de/bugcube.

Supplementary File 1

Click here to access/download

Supplemental Coding Files

Supplementary File 1 - FIJI Batch Processing Script.zip