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Corresponding Author:	M. Fethullah Simsek, Ph.D. Cincinnati Children's Hospital Medical Center Cincinnati, OH UNITED STATES
Corresponding Author's Institution:	Cincinnati Children's Hospital Medical Center
Corresponding Author E-Mail:	Muhammed.Simsek@cchmc.org
Order of Authors:	M. Fethullah Simsek, Ph.D.
	Ertugrul M. Ozbudak, Ph.D.
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TITLE:

A 3-D Tail Explant Culture to Study Vertebrate Segmentation in Zebrafish

AUTHORS AND AFFILIATIONS:

M. Fethullah Simsek[1,3], Ertugrul M. Özbudak[1,2,3]

[1] Division of Developmental Biology, Cincinnati Children's Hospital Medical Center, Cincinnati, OH
[2] Department of Pediatrics, University of Cincinnati College of Medicine, Cincinnati, OH
[3] Department of Genetics, Albert Einstein College of Medicine, Bronx, NY

muhammed.simsek@cchmc.org
ertugrul.ozbudak@cchmc.org

Corresponding Author:
M. Fethullah Simsek

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Segmentation, Somites, Zebrafish, Vertebrate, Tissue Culture, Live Imaging, Explant, Axis Elongation, Immunostaining

SUMMARY:

Here, we present the protocol for 3-D tissue culture of the zebrafish posterior body axis, enabling live study of vertebrate segmentation. This explant model provides control over axis elongation, alteration of morphogen sources, and subcellular resolution tissue-level live imaging.

ABSTRACT:

Vertebrate embryos pattern their major body axis as repetitive somites, the precursors of vertebrae, muscle, and skin. Somites progressively segment from the presomitic mesoderm (PSM) as the tail end of the embryo elongates posteriorly. Somites form with regular periodicity and scale in size. Zebrafish is a popular model organism as it is genetically tractable and has transparent embryos that allow for live imaging. Nevertheless, during somitogenesis, fish embryos are wrapped around a large, rounding yolk. This geometry limits live imaging of PSM tissue in zebrafish embryos, particularly at higher resolutions that require a close objective working distance. Here, we present a flattened 3-D tissue culture method for live imaging of zebrafish tail explants. Tail explants mimic intact embryos by displaying a proportional slowdown of axis elongation and shortening of rostrocaudal somite lengths. We are further able to stall axis elongation speed through explant culture. This, for the first time, enables us to untangle the chemical input of signaling gradients from the mechanistic input of axial elongation. In future studies, this method can be combined with a microfluidic setup to allow time-controlled pharmaceutical perturbations or screening of vertebrate segmentation without any drug penetration concerns.

INTRODUCTION:

Metameric segmentation of organisms is widely used in nature. These repeated structures are

essential for functionality of lateral organs such as vertebrae, muscles, nerves, vessels, limbs, or leaves in a body plan[1]. As a result of such physiological and geometric constraints of the axial symmetry, most phyla of Bilateria—such as annelids, arthropods, and chordates—exhibit segmentation of their embryonic tissues (e.g., ectoderm, mesoderm) antero-posteriorly.

Vertebrate embryos sequentially segment their paraxial mesoderm along the major body axis into somites with species-specific intervals, counts, and size distributions. Despite such robustness among individual embryos within a species, somite segmentation is versatile in between vertebrate species. Segmentation happens in a vast regime of time intervals (from 25 min in zebrafish to 5 h in humans), sizes (from ~20 µm in tail somites of zebrafish to ~200 µm in trunk somites of mice) and counts (from 32 in zebrafish to ~300 in corn snakes)[2]. More interestingly, fish embryos can develop in a wide range of temperatures (from ~20.5 °C up to 34 °C for zebrafish) while keeping their somites intact with proper size distributions by compensating for both segmentation intervals and axial elongation speeds. Beyond such interesting features, zebrafish stays as a useful model organism to study segmentation in vertebrates due to the external, synchronous and transparent development of a plenitude of sibling embryos as well as their accessible genetic tools. Adversely from a microscopy perspective, teleost embryos develop on a bulky spherical yolk, stretching and rounding the gastrulating tissue around it (**Figure 1A**). In this article, we present a flattened 3-D tissue explant culture for zebrafish tails. This explant system circumvents the spherical constraints of yolk mass, allowing access to high resolution live imaging of fish embryos for somite patterning.

[Insert **Figure 1** here.]

PROTOCOL:

This protocol involves use of live vertebrate embryos younger than 1 day post-fertilization. All the animal experiments were performed under the ethical guidelines of Cincinnati Children's Hospital Medical Center; animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee (Protocol # 2017-0048).

1. Embryo collection

1.1. Set up pairs of zebrafish in crossing tanks the night before the embryo collection day. For precise staging control of embryo development, use barriers between mating pairs.

1.2. Raise barriers before preferred spawning time and collect eggs within 15 minutes in a 100 mm Petri dish.

1.2.1. Clean debris from the Petri dish. If more than 50 embryos are collected from a single clutch, split the clutch into multiple Petri dishes accordingly.

1.3. Incubate embryos in fish system water at 28 °C until they reach 50% epiboly stage (5 hours post-fertilization). A standardized embryo growth medium such as E3 can also be used instead of the aquarium system water until step 3.2.

1.3.1. Remove unfertilized eggs under a stereoscope and move the embryos to a 23.5 °C incubator overnight (O/N). Embryos should be at 8-10 somites stage the morning following collection day.

2. Tool preparation

2.1. Sterilize the microsurgery knife blade, needle tips (used for dissection of the tissue), and glass Pasteur pipette by soaking in 100% ethanol (EtOH) and fire glazing.

2.2. Use two layers of transparent tape (~100-120 µm thickness) on 25 mm x 75 mm microscope slides. Cut ~18 mm x 18 mm square wells in the center of each slide's tape covering with a scalpel.

2.2.1. Wipe the prepared slide chambers with 70% EtOH. These wells will hold ~40 µL of medium.

3. Sample preparation

3.1. Dechorionate embryos using the tip of two needle syringes under a stereoscope. Transfer embryos into a separate Petri dish with fish system water to rinse.

3.2. Using a fire-glazed sterile glass Pasteur pipette, transfer embryos in a 6 cm Petri dish containing dissection medium (Leibovitz-15 cell culture medium with L-Glutamine without Phenol Red, 0.8 mM $CaCl_2$ and 1× antibiotic-antimycotic solution).

NOTE: Continue to use a sterilized glass pipette for all transfers following this step.

3.2.1. Use a glass Petri dish for explanting procedure to avoid polystyrene chips during dissection.

3.3. Put 50 µL of tissue growth medium (dissection medium, and 10% FBS) in the slide chamber.

3.4. Stabilize an embryo for dissection under the stereoscope with a needle tip at the yolk-tissue intersection near the hindbrain.

3.5. Keeping the embryonic tissue stable with a needle, use the microsurgical knife with the blade held at 45^{o} to cut the tissue anterior to the hindbrain apart and peel the yolk off the embryonic tissue starting from the anterior and moving towards the tail bud (**Figure 1A**).

NOTE: Be careful to not lose the skin tissue while cleaning the yolk. The skin would easily peel off as a flanking single layer elastic tissue around the embryo during the dissection, so it is easy to recognize.

3.6. Once the yolk is fully removed from the embryonic body, cut the flanking skin tissue from the tailbud. Keeping the last formed 3-4 somites intact, cut the more anterior tissue out (full-axis explant).

3.6.1. The yolk should come off mainly intact from this procedure. In case of a ruptured yolk, significant granules of yolk can stay attached to the ventral surface of the tissue. If so, use a lash tool to clean off remaining yolk granules gently.

NOTE: An imbalance of skin tissues on the lateral sides of an explant would not allow the tissue to maintain a straight growth direction. The explant instead will bend towards the side of more stretched skin. This imbalance can be corrected under the stereoscope by rupturing the skin layer with the aid of microsurgical knife.

3.6.2. For skinless explants, press on a tip of the skin layer with needle and peel the tissue explant off with the microsurgical knife. These explants will not elongate their body axis in culture.

3.6.3. In addition to the full axis explants, alternative explants can be made at this step. For instance, dissect out already segmented somites using the microsurgical knife (full-PSM explants) or dissect the PSM into its half anteroposterior (half-PSM explants). Please see section 5.1 for an application of such alternative explants.

3.7. Immediately transfer the dissected explant to a 22 mm x 22 mm coverslip on which the imaging will be performed.

3.7.1. Arrange the explant flat on the dorsoventral axis, ventral side touching the coverslip (**Figure 1B**). Gently remove the excess media around the tissue explant using a 20 µL filtered tip pipette.

NOTE: Delayed transfer of dissected explants to the coverslip results in deformations of the tissue, as it is relieved from the geometric constraints of the yolk.

3.8. Swiftly and carefully flip the coverslip with the explant over the growth medium filled slide chamber.

3.8.1. To prevent bubble formation, place a side of the square coverslip on the tape chamber and release the other side gently. Take care to not move/deform the explant in this step.

3.8.2. Gently remove excess media bleeding out of the chamber by pressing the slide chamber on a laboratory tissue. The coverslip will sit stably on the slide chamber for live imaging, due to the surface tension of the liquid medium without any sealing.

3.8.3. For long term culturing (>6 hours), use a bigger chamber. 22 mm x 50 mm rectangular coverslips together with two parallel lanes of tape layers on slides can be utilized in such cases. A ~1 mm wide gap can be left in between two tape lanes to facilitate air access into the growth medium.

3.9. Repeat steps 3.3-3.8 to prepare more explants. Prepared explants will elongate their A-P body axis with an average of ~30 µm/h speed and segment their somites with ~40 min intervals at 25 °C (**Figure 2A, Video 1**).

3.9.1. For non-elongating explants, apply gentle pressure to the sides of slide holding the sample in step 3.8.2 while sucking the excess media out on a laboratory tissue. Alternatively explants can be cultured in single tape layer slide chambers. Also, chemically activating the slide chamber surface with Type I Collagen will result in non-elongating explants (**Figure 2B, Video 2**).

3.9.1.1. Perform coating of the chamber with Type I Collagen in advance by fully covering the slide chambers with 15-20 mL of prediluted collagen solution at room temperature for 1 h. Use a laminar flow hood for this protocol to maintain sterility. Carefully rinse chambers with dissection medium at the end.

3.9.2. For embryos older than 15 somite stage, mount the tail explant tissue laterally instead of a flat (dorsoventral) mount (**Video 3**). To prevent muscular twitching, include 0.004% tricaine solution in the culture media as an anesthetic agent[3].

4. Live image acquisition

4.1. Image samples either on a dissection scope for widefield transmitted light imaging of somite segmentation sizes and periods, or with structured illumination/confocal/light sheet microscopy using transgenic reporter fish lines.

4.2. Equilibrate the temperature of tissue explants with the imaging room temperature for at least 15 min.

4.2.1. For more precise temperature control, use a commercial temperature control system mounted on an inverted microscope.

4.3. Set the image acquisition frame intervals to 2 – 10 min depending on the biological process of interest.

NOTE: Zebrafish somite segmentation is a fast process, ranging between 20 – 55 min for viable temperatures of 30 °C to 21.5 °C in whole embryos. Explants will elongate and segment ~30% slower than the whole embryos.

4.3.1. Pay attention to leave enough delay between sets of channel acquisitions to avoid possible phototoxicity to live tissue. Do not expose the tissue to the excitation beam for more than half of the imaging duration and lower the beam intensity as much as possible.

NOTE: Accumulation of reactive oxygen species (ROS) is generally the major cause of phototoxicity in live samples[4]. Ascorbic acid as a ROS scavenger can be supplemented to growth medium at 4 mM concentration to buffer ROS activity and alleviate phototoxicity. Adverse effects of phototoxicity might be hard to notice during live imaging. Tail explants are advantageous in this aspect since some visual markers of phototoxicity such as mitotic arrest, impeded tissue development (i.e., formation of somites, tail elongation), and disintegrating tissue are easier to notice. Please refer to the provided reference[4] for a detailed discussion.

4.4. Use single cell-stage RNA injected embryos to acquire 4-D images intended to be segmented and analyzed in cellular resolution level.

4.4.1. Use 300 pg of RNA from in vitro transcribed membranes and nuclear fluorescent reporter marker plasmids such as pCS-membrane-ceruleanFP (Addgene plasmid #53749) or pCS-memb-mCherry (Addgene plasmid #53750) in combination with pCS2+ H2B-mTagBFP2 (Addgene plasmid #99267) or pCS2+ H2B-TagRFP-T (Addgene plasmid #99271) in injections. For a sample movie with cell membrane and nuclei markers please see **Video 4**.

NOTE: Average cell size of PSM tissue is about ~5 µm in diameter, of which nuclei comprises ~3 – 4 µm. A pixel size of ~0.5 µm and a z-sectioning of ~1 µm should be recorded for proper cell segmentation.

5. Immunostaining of tail explants

NOTE: Tissues grown after various dissection scenarios (elongating, non-elongating, tail bud dissected, half PSM etc.) as flat-mounted tail explants[5] can be recovered from slide chambers for further immunostaining quantifications of proteins of interest. Here, we present the protocol used for di-phosphorylated extracellular signal regulated kinase (ppERK) staining of explants as FGF signaling gradient readout.

5.1. After formation of somites until the desired stage, cautiously shift the coverslip halfway to the corner of the slide chamber without lifting.

5.2. With the help of ~100 µL of supplementary dissection medium in a glass Pasteur pipette, recover the explants from the slide and transfer into a 64-well cell culture plate.

NOTE: Beginning with this step, all solution replacements can be performed under a dissection scope with a separate glass pipette for fixed samples. This will ensure not losing explant tissues in wells or transferring them in between.

5.3. After transferring all explants, suck the excessive medium out of the wells one-by-one and put 100 µL of 4% paraformaldehyde in PBS (PFA) into each well.

CAUTION: PFA is a toxic solution with carcinogenic effects. Proper PPE should be used while handling.

5.4. Fix explants in a 64-well plate at room temperature for 1 h on a shaker.

5.4.1. Tissue explants are more sensitive to deformations than whole embryos. Adjust the shaker speed accordingly.

5.5. Wash the fixative out with 150 µL of PBS-Tw (0.1% Tween20 in PBS) three times. Collect the first wash in a specific "PFA Waste" container.

5.6. Dehydrate explants in 4×5 min steps by replacing ~40 µL of solution each time with 100% methanol (MeOH).

CAUTION: MeOH is a toxic chemical which is volatile and flammable. Work in a well-ventilated space and use proper PPE for handling.

5.7. As the last step of dehydration, remove all solution from wells and replace with 100 µL of MeOH. Incubate at -20 °C for 15 min.

NOTE: Use a specific "MeOH Waste" container to collect the solutions until step 5.11.

5.8. Add 50 µL of MeOH and shake at room temperature for 5 min.

5.9. Rehydrate explants in 4×5 min steps by replacing ~40 µL of solution each time with PBS-T (0.1% Triton-X 100 in PBS). Use a specific "MeOH Waste" container to collect the solutions.

5.10. As the last step of rehydration, remove all solution from wells and replace with 100 µL of PBS-T.

5.11. For tissue permeabilization treat explants with 1.5% Triton-X 100 in PBS for 20 min at room temperature on the shaker.

5.12. Wash samples with MAB-D-T (0.1% Triton-X 100 detergent and 1% dimethyl sulfoxide (DMSO) in 150 mM NaCl 100 mM maleic acid buffer pH 7.5) 3×5 min.

CAUTION: DMSO is flammable and a toxic mutagen. Proper PPE should be used while handling.

5.13. Incubate explants in 100 µL/well serum blocking solution (2% fetal bovine serum in MAB-D-T) for 2 hours at room temperature.

5.14. Replace all the blocking solution with 50-100 µL/well primary antibody solution (1:1000 dilution of monoclonal mouse antibody against ppERK in serum block). Incubate samples O/N (>16 h) at 4 °C on shaker.

5.15. Wash the primary antibody solution with MAB-D-T 5×5 min.

5.16. Incubate samples in secondary antibody solution (Alexa Fluor 597 goat anti-mouse IgG2b (1:200) and Hoechst 33342 (1:5000) in MAB-D-T) O/N at 4 °C on a shaker or for 3 h at room temperature.

NOTE: Beginning at this step, cover the 64-well plate with aluminum foil to avoid light exposure of secondary antibody treated samples.

CAUTION: Hoechst 33342 is a potential carcinogen. Proper PPE should be used while handling.

5.17. Wash the secondary antibody solution with PBS-Tw 3×5 min.

5.18. Fix samples with PFA for 15 minutes at room temperature.

5.19. Wash the fixative with PBS-Tw and equilibrate samples within 60% glycerol. Mount explants on microscope slides with nail polish and 60% glycerol for imaging. For representative immunostaining results please see **Figure 3**.

REPRESENTATIVE RESULTS:

This protocol enables flat geometric culturing of live zebrafish tail explants. Tissue culture presents three major advantages over whole embryos: 1) control of axis elongation speed, 2) control over various signaling (morphogen) sources by simple dissection, and 3) near-objective, high magnification and high NA live imaging.

Chemically untreated slide chambers allow the tail explant to elongate its major axis (**Figure 2A**) by the skin ectoderm wrapping around the tissue beneath. When we cultured the explants on the chemically activated slide chambers (with Type I Collagen), the skin ectoderm stretched and adhered to the slide chamber, which halted the axis elongation of the explant. Despite this, the somites continued to segment (**Figure 2B, Supplementary Movies S1 and S2**). As described in the protocol, axis elongation can also be halted directly by applying physical pressure during the mounting process or mounting explants in a shallower slide chamber. Quantification of axis elongation speed under such physical restraints can be found in our previously published work[5].

[Insert **Figure 2** here.]

Secondly, explants can be cultured by dissecting out the sources of morphogens to identify instructive information they provide for developmental processes. Here we present three exemplary images showing the effect of dissections on ppERK signaling levels (**Figure 3**). In the PSM tissue an FGF signaling gradient is established from posterior to anterior (read out by ppERK levels). Only the tail bud tissue actively transcribes *fgf8* [8] and forms a source for this gradient with the help of FGF ligand diffusivity[5]. Tail explants missing the tail bud portion of the tissue after dissection (**Figure 3C**) results in a shorter ppERK gradient (**Figure 3B,3D**). Opposingly, a retinoic acid gradient is established from the anterior to the posterior in both the PSM and dorsal neural tissue. Recently formed somites and the anterior end of the PSM express retinoic acid (RA) synthesizing enzymes and act as a source for the RA gradient[7]. When we dissected out the anterior PSM tissue in the explants (**Figure 3E**), we still observed a normal extent of the ppERK gradient (**Figure 3F**) as visualized by immunostaining. A detailed utilization of this strength of explant method can be found in our recent study[5].

Thirdly, flat-mounted zebrafish explants are optimal for high resolution live observation of tissue morphogenesis. Here we present a movie (**Video 4**) taken with a transgenic explant expressing EGFP as a cell membrane marker (false colored with red) and stained with a far-red cell nuclei marker (false colored with cyan). Without further quantification, many processes such as ingression of neuromesodermal progenitors into the tail bud, higher motility of posterior PSM cells as compared to anterior, and epithelialization of somitic boundary cells can directly be observed in the movie.

[Insert **Figure 3** here.]

FIGURE AND TABLE LEGENDS:

Figure 1: Slide Chamber Explant System for Zebrafish Embryos. (**A**) Zebrafish embryos have advantages for live imaging, such as the transparency of gastrulating embryonic tissue (blue), but the tissue forms around a bulky spherical yolk mass (yellow) which prevents near-objective, high-resolution imaging in intact embryos. Tail explants can be dissected starting with a microsurgical knife (brown) cut from the tissue anterior of somites (red) and continuing at the border with the yolk posteriorly. (**B**) Dissected tail explants can be placed on a coverslip (light blue) dorsoventrally; keeping neural tissue (light gray) on top and notochord (dark gray) at the bottom.

Figure 2: Control of Axis Elongation in Explants. (**A**) Explants cultured on a regular slide chamber (left) elongate axially as they keep segmenting new somites. Transmitted light (left, grayscale) and transgenic nuclear marker (right, red) snapshots are shown for 2 h of culture duration. (**B**) Chemically activating the slide chamber with Type I Collagen before culturing stalls the axial elongation but does not affect somite segmentation speed. Scale bar is 100 µm.

Figure 3: Immunostaining of Tail Explants. (**A**) Full PSM explants with intact signaling gradients along the PSM as a control. (**B**) Regular ppERK gradient is observed in full PSM explants with immunostaining. (**C**) Tail bud dissected explants are missing a major part of posterior FGF signaling source. (**D**) A very posteriorly restricted ppERK signal is observed in tail bud dissected explants. (**E**) Anterior PSM and somitic tissue can be dissected out to remove the sources for

possible anterior PSM signaling factors such as RA signaling. (**F**) Anterior PSM removal does not change the normal extent of ppERK gradient. Tissues were fixed 2 h after explanting for immunostaining protocol. Scale bar is 100 µm.

Movie 1: Axis Elongation and Somite Segmentation in 3D Explants. Widefield transmitted light (top) and nuclear localized GFP (false colored red) epifluorescence (bottom) time lapse images of a regular slide chamber flat-mounted explant. Tail tissue was explanted from embryo at 13 somites stage. Image acquisition is performed on an inverted microscope with 3 min frame intervals. Scale bar is 100 µm.

Movie 2: Stalled Axis Elongation on Chemically Activated Slide Chamber. Widefield transmitted light (top) and nuclear localized GFP (false colored red) epifluorescence (bottom) time lapse images of a flat-mounted explant. A 11 somites stage embryo explant was mounted on a slide chamber coated with rat tail collagen solution for 30 min before mounting. Image acquisition is performed on an inverted microscope with 3 min frame intervals. Scale bar is 100 µm.

Movie 3: Lateral Mounting of Late-Stage Embryo Explants. Widefield transmitted light (left) and nuclear localized GFP (false colored red) epifluorescence (right) time lapse images of a regular slide chamber lateral-mounted explant. Tail tissue was explanted from embryo at 15 somites stage and tricaine solution was used as anesthetics. Image acquisition is performed on an inverted microscope with 3 min frame intervals. Scale bar is 100 µm.

Movie 4: Single Cell Resolution Imaging of Tail Explants. Time-lapse confocal imaging of an explant expressing EGFP as membrane marker (false colored red) and far red stained for nuclei in live (false colored cyan). Average intensity projection from 5 z-layers (10 µm) are shown in the movie over 1 hour. Image acquisition is performed on a GaAsP detector inverted confocal microscope with a 40× apochromatic λS DIC-water immersion 1.15 NA objective lens, with 4 min frame intervals. Scale bar is 100 µm.

DISCUSSION:

This article presents a detailed protocol of a tissue culture explant technique we developed and used recently[5] for zebrafish embryos. Our technique builds on the previous explant methods in chick[8] and zebrafish[9,10,11] model organisms. Tail explants prepared with this protocol can survive as long as >12 h in a simple slide chamber, continuing to elongate its major body axis and segmenting somites, until the end of somitogenesis.

Care should be given to keep explant tissue healthy and successfully elongating for long durations. First, the tissue explant should be dissected without damaging the intactness of the posterior tissues. We observed that the skin cells are providing a pouch for neuromesodermal progenitor cells ingressing into the posterior tailbud. If the skin of explants gets peeled off these highly motile cells, leave the tailbud tissue and migrate posteriorly over the coverslip beyond the tissue limits. Second, mediolaterally imbalanced tension of the skin tissue can result in divergent axis elongation, which can bend the axis of the PSM and notochord. A short-slit cut made to the flanking skin cells on both sides of the explant helps to alleviate that concern. Besides the skin-

relevant concerns, extra attention should be paid to maintaining the sterility of the growth media and dissection tools for longer duration cultures.

With proper care, the explant culture recapitulates the healthy growth of whole embryos. We observed muscular twitches in the explants beginning from 20 somites stage like in whole embryos[12]. Although we focused on the PSM tissue for study of somite segmentation, adjacent tissues such as skin ectoderm, neural keel (later neural rod and neural tube), notochord, intermediate and lateral plate mesoderm also remain intact under the described culturing conditions (**Figure 1B**). This is particularly advantageous for tissues obscured by the yolk that can be imaged in high resolution at ventrally mounted explants, such as notochord, Kupffer's vesicle and other tissues developing at segmentation stages[12]. It should be emphasized that the explant system does not grow as fast as whole embryos, and do not segment somites at the same pace as whole embryos. This limitation for the explant system can also alter temporal dynamics of other events unobserved here.

Here, we presented representative results that highlight three major advantages of the tail explant system over whole embryo experiments. We recently utilized this method to untangle instructive roles of axis elongation from morphogen gradients for somite segmentation[5]. Late advances in light sheet microscopy have made whole embryo live imaging possible[13] for several model organisms. But most of these methods still lack proper subcellular resolution and are barely accessible to the broader research community. The tail explant model described here makes subcellular resolution tissue-level live imaging accessible with simple inverted or confocal microscopes. Aside from methodological advantages, such live imaging can provide insights on segmentation of the posterior vertebrate body axis. Keeping the protocol as direct and accessible as possible, the explant system can also benefit the broader zebrafish developmental biology field.

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DISCLOSURES:

The authors have nothing to disclose and declare no conflicts of interest.

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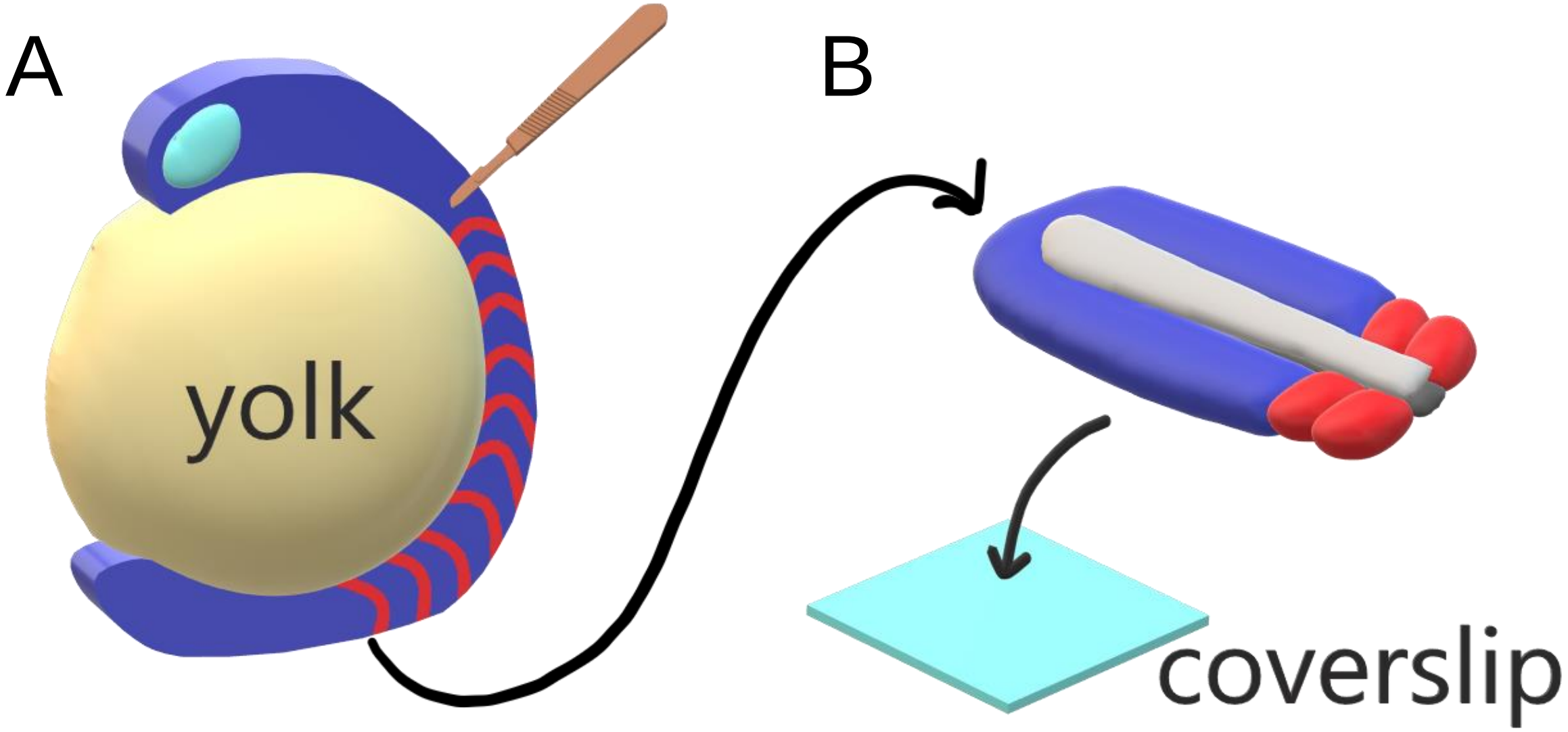
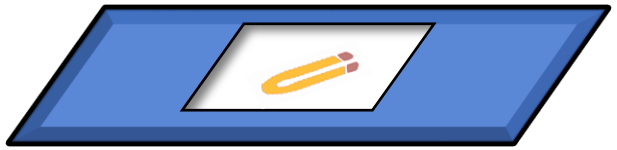
Figure1

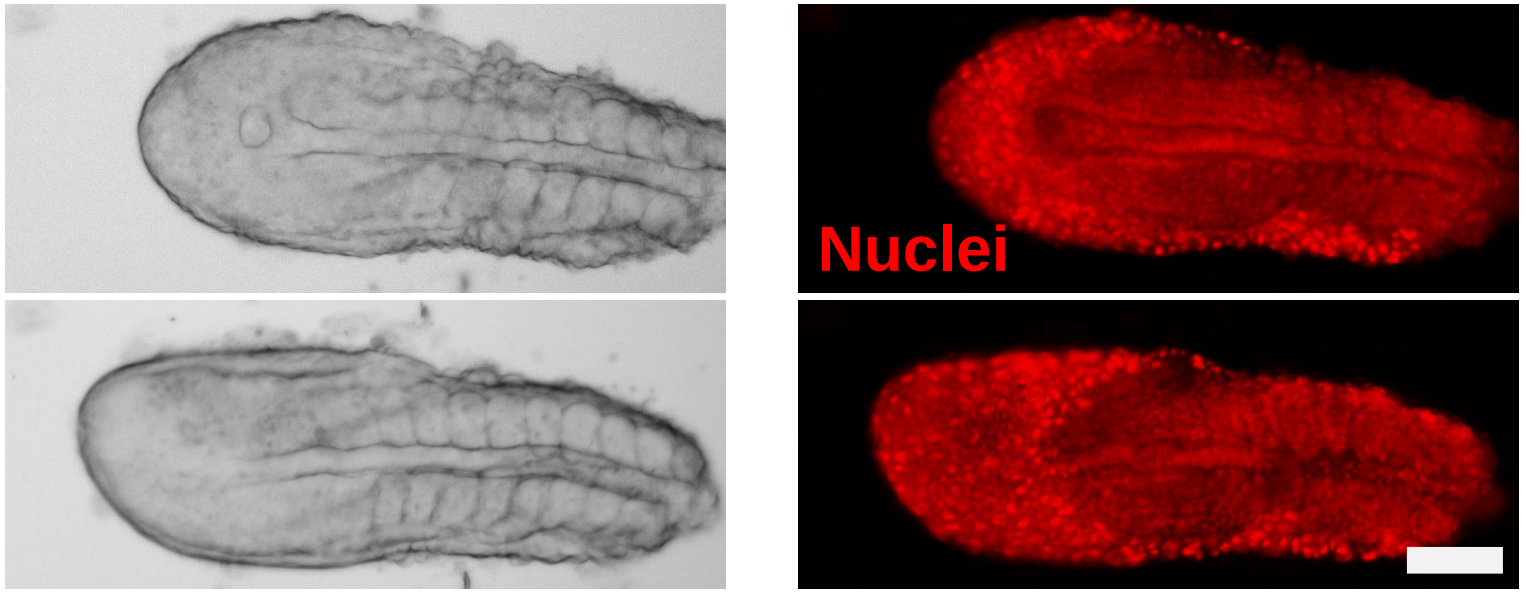


A

Normal Growth

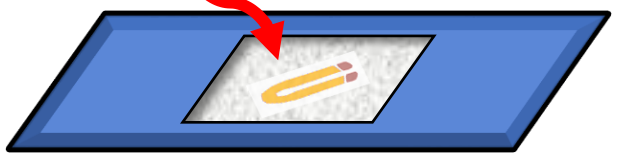
Slide Chamber



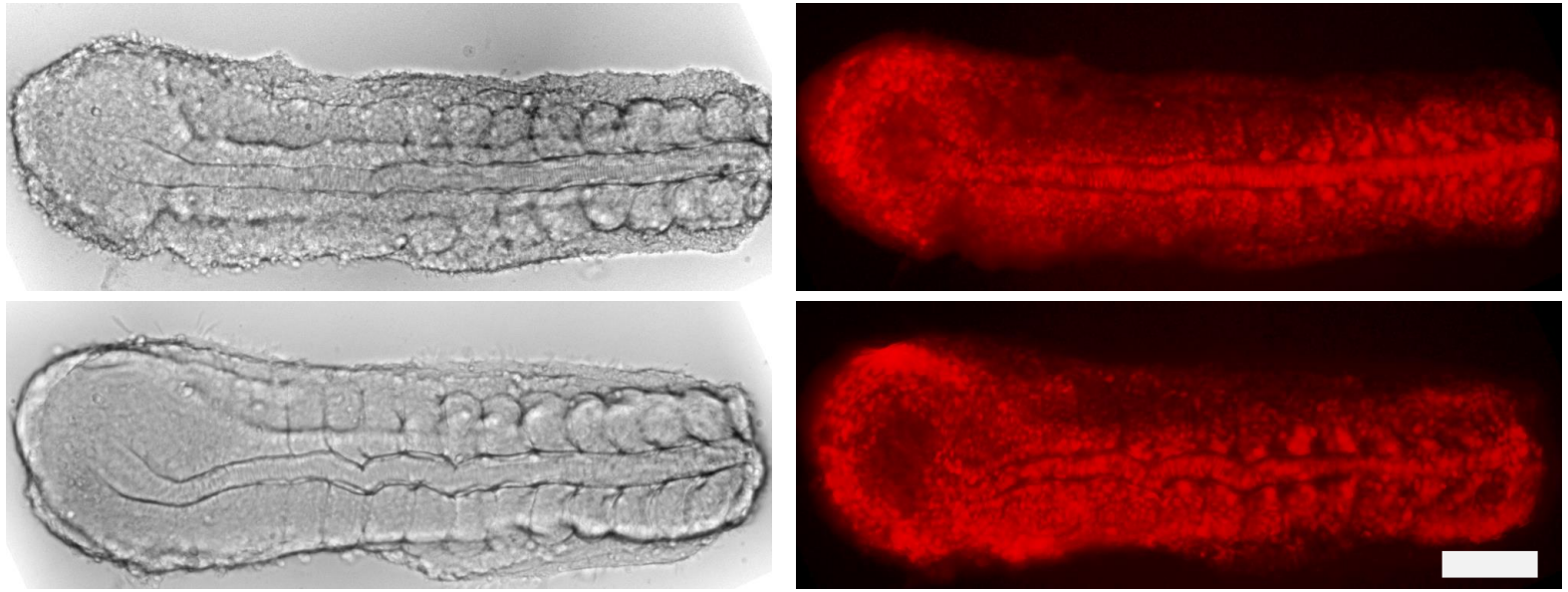


B

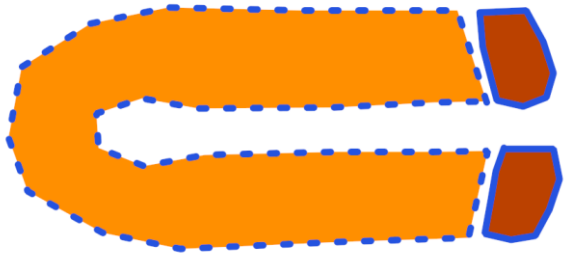
Chemically activated surface



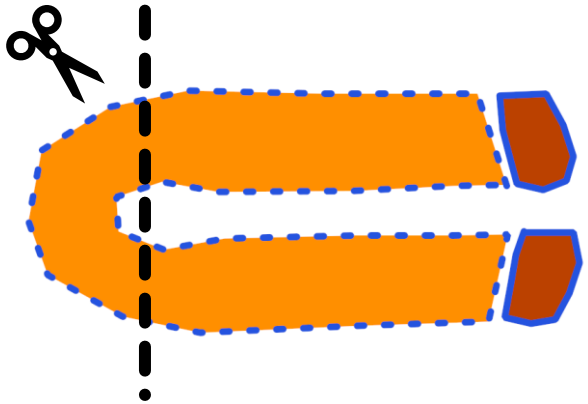
Stalled Axis Elongation



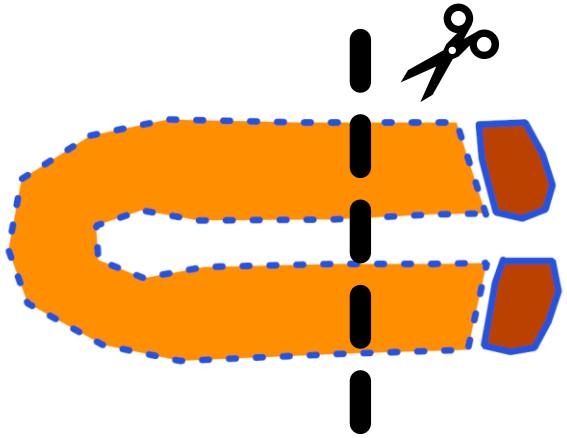
A Full PSM Explant



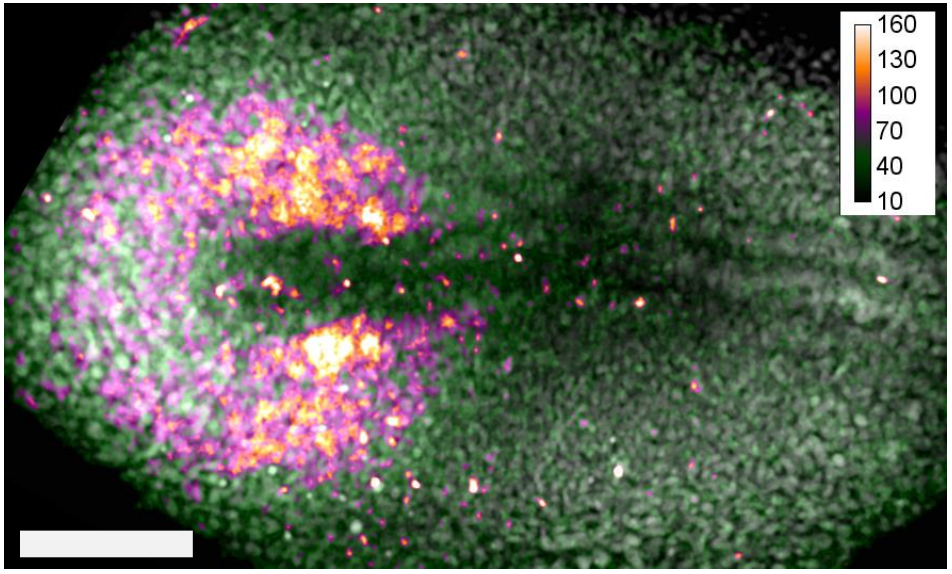
C Tail Bud Dissected



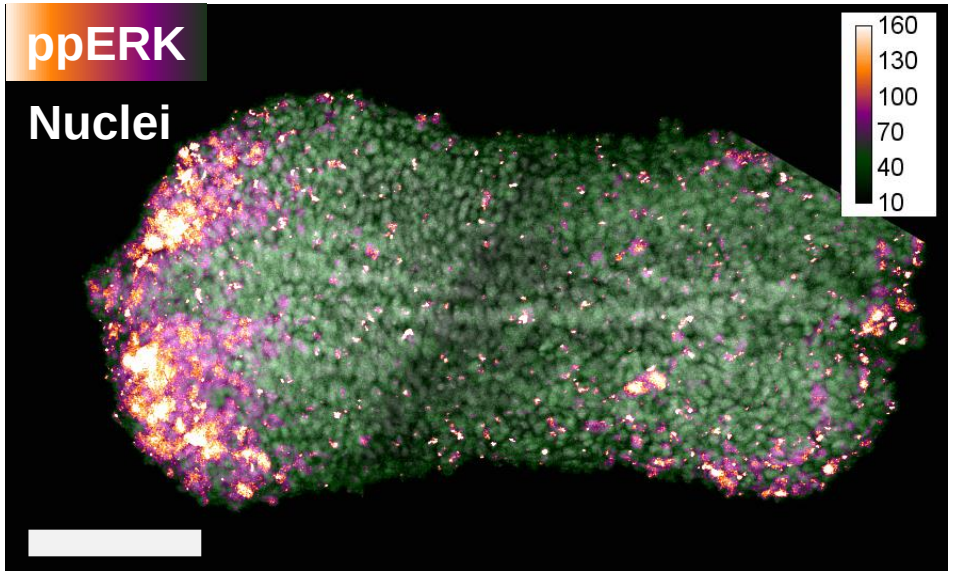
E Half PSM Dissected



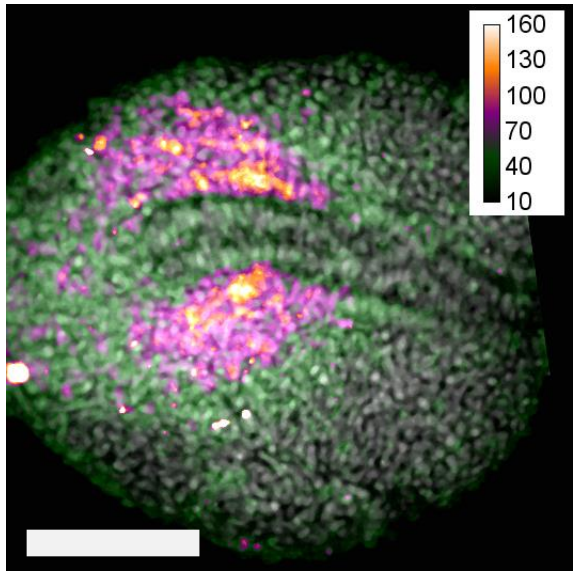
B



D



F



Video 1

Click here to access/download

Video or Animated Figure

Video1.mov

Video 2

Click here to access/download

Video or Animated Figure

Video2.mov

Video 3

Click here to access/download

Video or Animated Figure

Video3.mov

Video 4

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Video or Animated Figure

Video4.mov

Table of Materials

Click here to access/download;Table of Materials;JoVE_Table_of_Materials_3DExplants_MFS_Updated.xlsx

Name of Material/Equipment	Company
1 mL Sub-Q Syringe with PrecisionGlide Needle	Becton, Dickinson and Co.
200 Proof Ethanol, Anhydrous	Decon Labs
Antibiotic Antimycotic Solution (100×)	Sigma-Aldrich
Calcium Chloride Anhydrous, Powder	Sigma-Aldrich
Dimethylsulfoxide	Sigma-Aldrich
Disposable Scalpel, #10 Stainless Steel	Integra-Miltex
Ethyl 3-aminobenzoate methanesulfonate salt (Tricaine)	Sigma-Aldrich
Fetal Bovine Serum (FBS)	ThermoFisher
Goat anti-Mouse IgG2b, Alexa Fluor 594	Invitrogen
L-15 Medium with L-Glutamine w/o Phenol Red	GIBCO
Methanol	Sigma-Aldrich
Microsurgical Corneal Knife 2.85 mm Angled Tip Double Bevel Blade	Surgical Specialties
Mouse monoclonal anti-ppERK	Sigma-Aldrich
NucRed Live 647 ReadyProbes Reagent	Invitrogen
Paraformaldehyde Powder, 95%	Sigma-Aldrich
Rat Tail Collagen Coating Solution	Sigma-Aldrich
Stage Top Incubator	Tokai Hit
Transparent Tape 3/4''	Scotch
Triton X-100	Sigma-Aldrich
Tween 20	Sigma-Aldrich
Zebrafish: Tg(actb2:2xMCP-NLS-EGFP)	Campbell et al., 2015
Zebrafish: Tg(Ola.Actb:Hsa.HRAS-EGFP)	Cooper et al., 2005

Catalog Number	Comments/Description
REF 309597	for dechorionating embryos and manipulations
2701	for immunostaining
A5955	for tissue dissection media
499609	for tissue dissection media
D5879	for immunostaining
MIL4-411	for preparing tape slide wells
886-86-2	(optional) for anesthesizing tissues older than 20 somites stage
A3160601	additional for tissue culture media
Cat#A-21145; RRID: AB_2535781	secondary antibody for immunostaining
21083-027	for tissue dissection media
179337	for immunostaining
72-2863	for tissue dissection
Cat#M8159; RRID:AB_477245	for ppERK immunostaining
R37106	(optional) for live staining of cell nuclei
158127	for fixation of samples for immunostaining
122-20	(optional) for chemically activating slide chambers
tokai-hit-stxg	(optional) for temperature control during live imaging
S-9782	for preparing tape slide wells
X100	for immunostaining
P1379	for immunostaining
ZFIN: ZDB-TGCONSTRCT-150624-4	transgenic fish with nuclear localized EGFP
ZFIN: ZDB-TGCONSTRCT-070117-75	transgenic fish with cell membrane localized EGFP

Dear Editor(s),

Thank you for carefully handling our submitted manuscript “A 3-D Tail Explant Culture to Study Vertebrate Segmentation in Zebrafish” through the peer-review process. Please find below our responses to the editorial/production and reviewer comments. We indexed the comments for ease of access one by one and addressed them separately. We kept comments on the production of video separated at the end in **green** color. We used **red** color for our responses, and **inline** texts for the quotations from the manuscript. We **underlined** the parts of those quotes which had gone through changes. We are looking forward to see our manuscript in your “Modern Approaches to Vertebrate Segmentation” collection.

With regards,

M. Fethullah Simsek, Ph.D.

On behalf of authors M.F.S. and E.M.Ö.

Editorial comments:

Comment E1:

Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Response to Comment E1:

We went over the manuscript in detail and corrected some typos and grammar mistakes in line numbers 45, 54, 76, 246, 276, 286, and 313.

Comment E2:

As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

Response to Comment E2:

We included two more paragraphs to the discussion, paraphrasing critical steps and troubleshooting ways provided during the protocol, in detail as follows:

> Care should be given to maintain explant tissue healthy and successfully elongating for long durations. First, the tissue explant should be dissected without damaging the intactness of the posterior tissues. We observed that the skin cells are providing a pouch for neuromesodermal progenitor cells ingressing into the posterior tailbud. If the skin of explants gets peeled off these highly motile cells leave the tailbud tissue and migrate posteriorly over the coverslip beyond the tissue limits. Second, mediolaterally imbalanced tension of the skin tissue can result divergent axis elongation which might result bent axis of the PSM and the notochord. A short-slit cut made to the flanking skin cells on both sides of the explant would help to alleviate that concern. Besides the skin-relevant concerns, extra attention should be given to have the growth media and

dissection tools sterile for longer duration cultures. With proper care, our explant culture recapitulates the healthy growth of whole embryos.

We also mentioned a possible significance of our technique for other tissues as also suggested by Reviewer #3, and we included the limitations of the method provided as follows:

This is particularly advantageous for tissues obscured by the yolk which can be imaged in high resolution at ventrally mounted explants, such as Kupffer's vesicle and pronephric duct developing at segmentation stages[Error! Reference source not found.]. It should be emphasized that explant system does not grow as fast as whole embryos, and do not segment somites at the same pace with whole embryos. This limitation for the explant system can also alter temporal dynamics of other events unobserved here.

We also provided a reference for a highly impactful light sheet microscopy paper in zebrafish (Ref. #13) in the "The significance with respect to existing methods" part of discussion.

Reviewers' comments:

Reviewer #1:

Comment R#1-1:

After mounting in chamber slides of their own making, the authors demonstrate that explanted tissues continue to grow, extend, and segment somites similarly to (although at a reduced rate compared with) live intact embryos. The protocol thoroughly describes the technique and would likely allow for recreation of their results.

Response to Comment R#1-1:

We thank the Reviewer for their appreciation of the protocol for full replicability of our method.

Comment R#1-2:

1. As a whole, I found the introduction section confusing. Some examples are below:

- "The gut tract… or pith of vascular plants would not lose their functionality if extended indefinitely." Do the authors mean to say that these tissues aren't segmented and don't need to be?

Response to Comment R#1-2:

We thank the Reviewer for alerting us a potential confusion. We removed this sentence.

Comment R#1-3:

- "In result of such… constraint of reciprocal symmetry…". Do the authors mean "as a result of"? What is meant by reciprocal symmetry?
- "exhibit sequential/simultaneous segmentation of their ectoderm/mesoderm…". How can segmentation be both sequential and simultaneous? Is the ectoderm of chordate embryos segmented? (Posterior to the rhombomeres, I mean).

Response to Comment R#1-3:

Taking into consideration reviewer's suggestions, we now changed the sentence as follows:

As a result of such physiological/geometric constraint of axial symmetry, most phyla of Bilateria such as annelids, arthropods, and chordates exhibit segmentation of their embryonic tissues such as ectoderm or mesoderm antero-posteriorly.

Comment R#1-4:

- "somite segmentation is versatile, happening in a vast regime of time intervals…". Do the authors mean that segmentation is variable? And that it happens across a range of time intervals?

Response to Comment R#1-4:

Per reviewer's request, here we clarified the quoted part by emphasizing the versatility of the segmentation system comes from comparison among various species:

> Despite such robustness among individual embryos within a species, somite segmentation is versatile in between vertebrate species. Segmentation happens in a vast regime of time intervals (from 25 min in zebrafish to 5 h in humans), sizes (from ~20 µm in tail somites of zebrafish to ~200 µm in trunk somites of mice), and counts (from 32 in zebrafish to ~300 in corn snakes)**Error! Reference source not found.**.

Comment R#1-5:

- The authors refer to zebrafish development as "ex-utero", a term which refers to development of embryos after removal from the uterus, not embryos that develop externally.

Response to Comment R#1-5:

Although ex-utero term is used in teleost community to clarify both the fertilization of the egg and the development of the fertilized egg happens after the egg is laid, hence left the uterus (some examples of such usage can be found at Peterson et al., 2000 (PMID: 11087852), Webb et al., 2013 (PMID: 236373583), and Richardson et al., 2017 (PMID: 27612182)), we decided to change "ex-utero" to "external" as requested.

Comment R#1-6:

2. The authors stress how important the skin is to proper elongation of the tailbud explants, but they don't tell or show us how to recognize the skin or know when it has been explanted and mounted correctly. This information would be helpful for successful recreation of their results.

Response to Comment R#1-6:

We thank the reviewer for bringing this up to our attention. We now added following sentence to the Protocol step 3.5:

> Skin would easily peel off as a flanking single layer elastic tissue around the embryo during the dissection, so is easy to recognize.

We further discussed the skin near the end of the article as follows:

> First, the tissue explant should be dissected without damaging the intactness of the posterior tissues. We observed that the skin cells are providing a pouch for neuromesodermal progenitor cells ingressing into the posterior tailbud. If the skin of explants gets peeled off these highly motile cells leave the tailbud tissue and migrate posteriorly over the coverslip beyond the tissue limits. Second, mediolaterally imbalanced tension of the skin tissue can result divergent axis elongation which might result bent axis of the PSM and the notochord. A short-slit cut made to the flanking skin cells on both sides of the explant would help to alleviate that concern.

Comment R#1-7:

3. In protocol section 5.1, the authors describe various dissection scenarios such as tail bud-dissected and half PSM, but do not describe how to make these types of explants. All types of dissections should be described in detail in section 3.6.

Response to Comment R#1-7:

We now clarified the "full-axis explant" nomenclature in protocol step 3.6 and further provided a sub-step in the relevant part of the protocol (3.6.3) as follows:

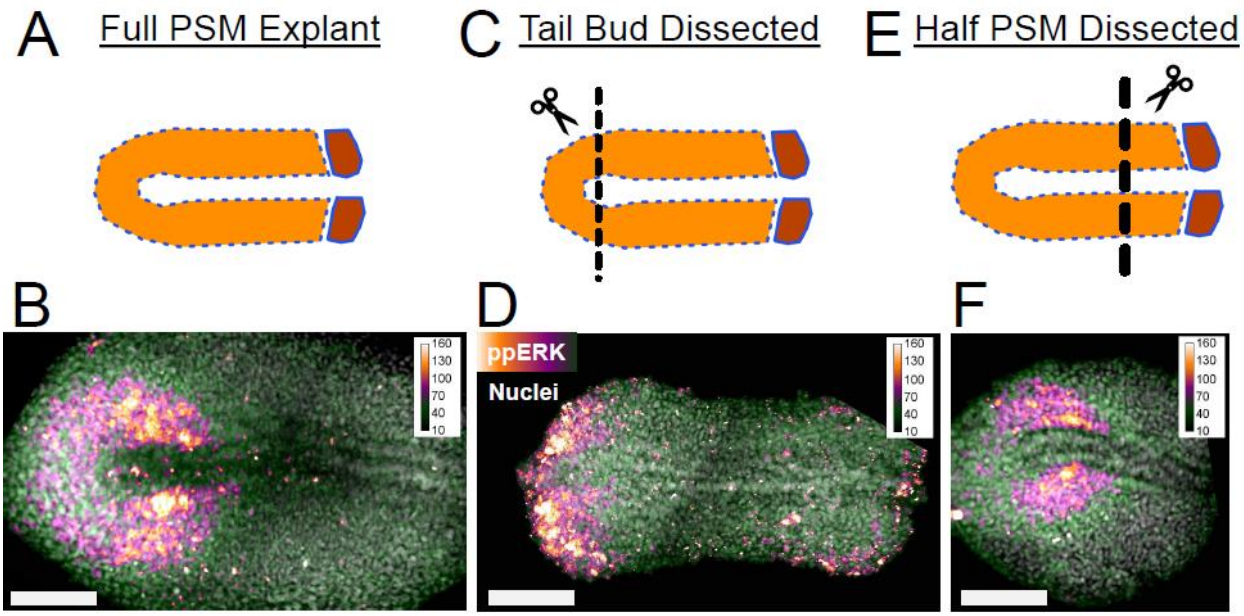
In addition to the full axis explants, alternative explants can be made at this step. For instance, already segmented somites can be dissected out using the microsurgical knife (full-PSM explants) or the PSM can be dissected into its half anteroposteriorly (half-PSM explants). Please see section 5.1 for an application of such alternative explants.

Comment R#1-8:

4. In line 255, the authors describe how removal of the tail bud from explants results in a shorter ppERK gradient, but in Figure 3 they do not show ppERK staining in a complete tail explant for comparison.

Response to Comment R#1-8:

We apologize for the omission of proper control data in Figure 3 and thank the Reviewer for reminding us. We now updated the Figure 3 to include a Full PSM explant as two additional panels A and B. We also updated the figure legends and callouts accordingly.



Comment R#1-9:

1. In protocol section 3.1, the authors describe using injection needles to dechorionated embryos. Many zebrafish researchers use glass needles for injection, which is not what is shown in the video. Can the authors please explain the types of needles they are using?

Response to Comment R#1-9:

The needles used were regular needle syringes. We fixed that mistake as follows:

3.1. Dechorionate embryos using the tip of two needle syringes under a stereoscope.

We also added the tool to the equipment list spreadsheet.

Comment R#1-10:

2. In section 3.9, the authors state that gentle pressure can be applied to the coverslip to prevent tail explant elongation in culture. How is such pressure applied?
They also refer to chemically activating slide chambers with Type I collagen. Should a detailed description of this step should be included in the protocol as well?

Response to Comment R#1-10:

The pressure is applied to the sides of the slide, at the step slide chamber with sample and coverslip is flipped on a Kimwipe (3.8.2). We clarified that step as follows:

> 3.9.1. For non-elongating explants, a gentle pressure can be applied to the sides of slide holding the sample in Step 3.8.2 while sucking the excess media out on a Kimwipe…

We also added the following quick protocol for Collagen coating of slide chambers:

> Coating of the chamber with Type I Collagen can be performed in advance by fully covering the slide chambers with 15-20 mL prediluted collagen solution at room temperature for 1h. Use a laminar flow hood for this protocol to maintain sterility. Carefully rinse chambers with dissection medium at the end.

Comment R#1-11:

3. In section 4.3 the Authors advise to leave enough delay between channel acquisitions to avoid phototoxicity. How does the reader know how long of a delay is enough? Similarly, in section 5.5 they advise that shaker speeds should be adjusted to avoid deformation of explanted tissues. What speeds are too fast?

Response to Comment R#1-11:

We realized the need for a thorough discussion on phototoxicity in Section 4.3 after the valuable comments from the Reviewer. We added the following sentence:

> A good practice is not exposing the tissue to excitation beam more than half of the imaging duration and lowering the beam intensity as much as possible.

We also updated the end of 4.3.1. as follows:

> Adverse effects of phototoxicity might be hard to notice during live imaging. Tail explants are advantageous in that aspect since some visual markers of phototoxicity such as mitotic arrest, impeded tissue development (i.e., formation of somites, tail elongation), and disintegrating tissue are easier to notice. Please refer to the provided reference **Error! Reference source not found.** for a detailed discussion.

Comment R#1-12:

4. In section 5.16, we are told to incubate samples in secondary antibody solution O/N at 4 degrees for 3 hours at room temperature. Which one is it?

Response to Comment R#1-12:

We apologize for the omission typo there. The sentence is updated as follows:

> …O/N at 4 °C on shaker or for 3h at room temperature.

Reviewer #2:

Comment R#2-1:

The technique will be useful to others and is therefore of significant value. The data images are of good quality and show the efficacy of the method.

Response to Comment R#2-1:

We thank the Reviewer for agreeing with us on the usefulness of presented tail explant technique.

Reviewer #3:

Comment R#3-1:

This methodology gives the field additional tools to image tissue dynamics using common microscopy techniques that are limited in angles of observation. This protocol also expands upon previous methods in describing options for pharmacological inhibition, stalling axis elongation, and immunostaining analysis of tail explants.

Response to Comment R#3-1:

We thank the Reviewer for appreciating the presented strengths of the tail explant technique.

Comment R#3-2:

-An additional advantage of this method could be its ability to image tissues otherwise difficult to observe, such as the ventral tissues that would normally be obscured by the yolk. Some consideration of this could be mentioned in the text.

Response to Comment R#3-2:

We thank the Reviewer for that comment. In discussion, we had briefly mentioned possible utility of the technique for imaging tissues other than the presomitic mesoderm. We now expanded that discussion and emphasized the yolk obstruction problem in whole embryos as follows:

> Although we focused on the PSM tissue for study of somite segmentation, adjacent tissues such as skin ectoderm, neural keel (later neural rod and neural tube), notochord, intermediate and lateral plate mesoderm also remain intact under described culturing conditions (Figure 1B). This is particularly advantageous for tissues obscured by the yolk which can be imaged in high resolution at ventrally mounted explants, such as notochord, Kupffer's vesicle and intermediate mesoderm tissues such as pronephric duct developing at segmentation stages[12].

Comment R#3-3:

-No citations are listed when discussing the retinoic acid/FGF gradient in the text. The authors should cite articles to support those details, which could include (Begemann et al., 2001) (Sorrell & Waxman, 2011) (Cunningham et al., 2011) (Berenguer et al., 2018).

Response to Comment R#3-3:

Motivated by the Reviewer's comment we now included two citations for how FGF and RA signaling gradients are established in the presomitic mesoderm as Dubrulle and Pourquié, 2004 and Diez del Corral, et al., 2003 respectively. We made underlined changes to following sentences for accuracy of description:

> Only the tail bud tissue actively transcribes *fgf8* **Error! Reference source not found.** and in result, with the help of FGF ligand diffusivity[5], forms a source for this gradient…
>
> Recently formed somites and the anterior end of the PSM express retinoic acid (RA) synthesizing enzymes and act as a source for the RA gradient[7]…

Comment R#3-4:

-It is unclear, in Figure 3C, if the retinoic acid (RA) gradient was truly eliminated by dissecting the anterior PSM. It would be best to restrict the claim to separating the anterior PSM from potential posterior PSM morphogens, unless the authors can provide a retinoic acid reporter to confirm loss of RA.

Response to Comment R#3-4:

Here we do not necessarily claim that RA signaling is fully eliminated in half PSM explants, but just present a snapshot on how the FGF signaling might be affected in the absence of any anteriorly sourced signaling factors including RA signaling. Our recent work (Simsek and Ozbudak, 2018) had presented a

detailed discussion for the interpretation of tissue dissection experiments in regards of FGF and RA signaling gradients. This study was not cited in one of the suggested references (Berenguer et al., 2018) probably due to nearby publication dates, but later cited in a more recent review by the same group (Ghyselinck and Duester, 2019). We added a reference to that work as follows:

> A detailed utilization of this strength of explant method can be found in our recent study[5].

We also changed figure legend description of relevant panels followingly for clarity of the issue:

> (**E**) Anterior PSM and somitic tissue can be dissected out to remove the sources for possible anterior PSM signaling factors such as RA signaling. (**F**) Anterior PSM removal does not change the normal extent of ppERK signal gradient.

Comment R#3-5:
-The authors may want to clarify the quality of the fish system water they develop the embryos in. Many fish systems in use across the field are different and are not standardized. It may be useful to confirm if this methodology works for embryo development in clearly dictated embryo growth media, such as E3.

Response to Comment R#3-5:

We thank the Reviewer for notifying us on the variety of practices among different zebrafish labs. Our facility maintains a standardized and continuously monitored aquarium water system managed by Veterinary Services, conditioning the purified water by addition of synthetic sea salts, and magnesium chloride and calcium chloride. This composition is matching with the E3 medium recipes. Per recommendation we added the following sentence to step 1.3:

> A standardized embryo growth medium such as E3 can also be preferred instead of the aquarium system water until step 3.2.

Comment R#3-6:
-Materials and equipment section should be formatted to keep catalog numbers in line with each item.

Response to Comment R#3-6:

Unfortunately, we are not able to address that comment. The version of materials and equipment spreadsheet we uploaded to the submission system and the one we downloaded from the system both present the catalog numbers in line with each item already. We will try to clarify this issue with editors.

Comment R#3-7:
-Tricaine, PFA, and rat tail collagen are listed as materials used in the text, but are not listed in the Materials section. It is recommended to list all the materials mentioned in the protocols, including the immunostaining protocol.

Response to Comment R#3-7:

We thank the reviewer for letting us know the reagents missing in the list. We now included them. We also clarified the materials used in immunostaining protocol, in description column.

Comments on the Video:

Production Comments:

Changes to be made by the Author(s) regarding the video:
1. Please increase the homogeneity between the video and the written manuscript. Ideally, the narration is a word for word reading of the written protocol.
2. The introduction and conclusion segments have a lot of background noise and it is difficult to hear the speaker. We recommend re-recording this section in a quieter environment will multiple takes to choose

the best and clearest performance.
3. For the protocol segments, consider muting the audio tracks of the video clips so that we only hear the narration. The sound from the lab is background noise and is not needed to understand the procedure.
4. A ""jump cut"" is a type of edit where the camera does not move but the action changes instantly, like at this time point. This type of edit is distracting and jarring for the viewers, and we suggest using a quick dissolve (or fade) to move quickly between similar shots. Some other instances of where using dissolves instead of a jump cut are listed below:
○ 00:39
○ 01:09
○ 01:53 In this instance, you can just cut out the proceeding shots which don't show much and start on this shot where the actual action begins.
○ 02:06
○ 02:31
○ 03:32
○ 03:44
○ 04:16
○ 04:33
○ 04:52
○ 05:00
○ 06:09
○ 06:20
○ 07:07
○ 07:55
○ 08:42
5. 00:05 Consider removing this schematic image and going directly to the shot of M. Fethullah Simsek @00:08. It appears suddenly and should be used later.
6. 00:10 The text for M. Fethullah Simsek's name caption is very small. Consider reformatting this text object so it will be easily readible on smaller screens.
7. 00:18 The audio here cuts out abruptly, consider fading it out.
8. 02:17 A lot of this section can probably be cut down. Simply add an instruction to sterilize the tools with the burner and keep the graphic of the tools, but cut down on the repetitive or redundant footage.
9. 05:20 Similar solution here- instead of fixing the jump cuts, just cut out the first shot ot the unscrewing of the pipette and just skip to the next shot. We don't need to see the whole thing, as most of us know how to use the equipment. You would be skipping to the shot that starts here: 05:29 (the actual pipetting)
10. 10:39 The time lapse movie is interesting. Consider repeating it and holding another moment.
11. 11:06 Consider holding on this last Results image another moment before going straight into the Conclusion chapter title card
12. 11:09 Unfortunately, this edit is not going to work "The method we have...[cut to long shot]. Please pick one shot to use.
13. 11:29 Fade this final shot out and end on black or on a title card.

Please upload a revised high-resolution file here:
https://www.dropbox.com/request/LfP1LDBlPYJcvkG5eo6y?oref=e

Response to Production Team Comments:

We thank the production team for carefully going over the video and highlighting all the jump cut moments together with providing detailed suggestions. We included fade-in and fade-outs in all jump cut moments identified. We complied with the rest of the edit suggestions provided above as well. We repeated the time-lapse movie with slightly slowed down speed for clarity and increased the last results image’s duration by 3 more seconds before conclusion. We also eliminated the background noise throughout the tracks and lowered the background music volume. We overlayed the intro and conclusion remarks with higher quality recorded voices, which we had recorded previously during the shots but had not used.

Reviewer #1 Comments on Video:

Comment R#1-V1:

Major Concerns:

Author-submitted video
1. All of the protocol steps performed on a microscope are either shown from the side (sometimes with the author's hands blocking the view) or on a monitor positioned next to the microscope. As a result, all of these steps, which are the most critical to success of the procedure, cannot be seen well.

Response to Comment R#1-V1:

We thank the reviewer for the valuable comment. Unfortunately, due to standard laboratory setting with facing benches and shelves throughout the department, most of the time, best angle available to shoot the protocols was from sideways. We tried to shoot from two separate angles or through widescreen monitor whenever was necessary, such as tape chamber preparation steps, explant dissection steps, and microscopy acquisition steps. We apologize for that restriction.

Comment R#1-V2:
2. At 6:43, the narrator says to "trim the leftover tissue off"… but how do we know which tissues are "leftover" and which are important to keep?

Response to Comment R#1-V2:

We now added a snapshot from the procedure as a still image in the relevant moment of the video file (06:17-06:20). Here we highlighted all the tissue names relevant for discerning leftover tissues properly.

Comment R#1-V3:
Minor Concerns:
Author-submitted video
1. At the very beginning and very end of the video when the author/researcher is speaking directly to the camera, his voice is much quieter than the narrator voice throughout the majority of the video.

Response to Comment R#1-V3:

We now fixed the background noise and audio recording quality issues for these sections of the video.

Reviewer #2 Comments on Video:

Major Concerns:
My concerns are with parts of the video. I have a hard time hearing the commentary in the intro and the outro - the sound quality is very poor, especially at the end, the music is intrusive, and the speaker talks too quickly. Also, the text slides go past too quickly to actually read. The video is often filmed from partly behind the researcher, so that their arm blocks one's view, or on a bad angle that makes it hard to see what is going on. For collecting embryos and making solutions I don't care too much, as these are standard - and probably don't even need to be shown. However, for setting up the chambers on the slides, using tape, it is frustrating not to have a better angle of view. The parts under the microscope are however much better - and these are the most interesting and important parts. I would have liked to see a third specimen but it is not really necessary as they show two nice ones.

Response to R#2 Comments on Video:

We now fixed the background noise and audio recording quality issues for the intro and concluding remarks of the video. We also lowered the music volume throughout the whole video. We tried to lengthen durations of most text markups in the video. We apologize for the shooting angle issues of the video but due to standardized bench setup of laboratories we were only able to shoot from these two angles, trying to capture steps as detailed as possible.