Title

Generation of naïve blastoderm explants from zebrafish embryos

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Summary

Zebrafish blastoderm explants are generated by isolating embryonic cells from endogenous signaling centers within the early embryo, producing relatively naïve cell clusters that are easily manipulated and cultured *ex vivo*. This article provides instructions for making such explants and demonstrates their utility by interrogating roles for Nodal signaling during gastrulation.

Abstract

Due to their optical clarity and rapid development, zebrafish embryos are an advantageous system in which to examine cell behaviors and developmental processes. However, because of the complexity and redundancy of embryonic signals, it can be challenging to discern the complete role of any one signal during early embryogenesis. By explanting the animal region of the zebrafish blastoderm, relatively naïve clusters of embryonic cells are generated that can be easily cultured and manipulated *ex vivo*. By introducing a gene of interest by RNA injection prior to explantation, one can assess the effect of this molecule on gene expression, cell behaviors, and other developmental processes in relative isolation. Furthermore, cells from embryos of different genotypes or conditions can be combined in a single chimeric explant to examine cell/tissue interactions and tissue-specific gene functions. This article provides instructions for generating zebrafish blastoderm explants and demonstrates that a single signaling molecule - a Nodal ligand - is sufficient to induce germ layer formation and extension morphogenesis in otherwise naïve embryonic tissues. Given their ability to recapitulate embryonic cell behaviors, morphogen gradients, and gene expression patterns in a simplified *ex vivo* system, these explants are anticipated to be of great utility to many zebrafish researchers.

Introduction

A perennial goal of the developmental biology field is to unravel the complexity of developing embryos to better understand the origins of animal form and function. Even early embryos contain a complex medley of signaling molecules, cell and tissue interactions, and mechanical forces, all subject to strict spatial and temporal regulation. For this reason, it is often challenging to pinpoint the precise role of any one signal in a developmental process of interest. By removing embryonic tissues from their endogenous environment, embryo explantation creates a simplified platform in which to discern the developmental roles of individual tissues and

molecules in relative isolation. Explantation techniques are perhaps best known in *Xenopus laevis*, where they have been used to study tissue induction, cell signaling, cell adhesion, and morphogenesis, among other processes [1-4]. So-called animal cap explants, in which the animal-most region of blastula stage *Xenopus* embryos is isolated prior to inductive interactions [5-7], are a particularly common and powerful explant technique. Unmanipulated animal caps are fated to become ectoderm [7,8], but are competent to respond to a number of inductive factors, allowing them to form tissues of all 3 germ layers and undergo tissue-appropriate morphogenetic movements [9-11]. However, limited genetic tools and sub-optimal suitability for live imaging prevent the use of *Xenopus* animal cap explants for many developmental biologists. By explanting blastoderm cells from zebrafish embryos, researchers can combine the utility of the animal cap assay with the optical clarity, abundance of genetic tools, and other experimental advantages of the zebrafish model system.

To date, researchers have made use of two 'flavors' of zebrafish explants: so-called "pescoids" and blastoderm explants. In the pescoid model, the entire blastoderm - including the marginal zone - is isolated from the yolk and allowed to develop *ex vivo* in the absence of the extraembryonic yolk syncytial layer (YSL) [12,13]. In this way, pescoids bear a notable resemblance to the *Fundulus* explants generated decades ago by Jane Oppenheimer and John Trinkaus[14,15]. These explants recapitulate many aspects of embryonic patterning and morphogenesis [12,13]. However, because these isolates contain endogenous signaling centers (the embryonic margin), they are not simplified with respect to their molecular milieu. Alternatively, researchers can generate relatively naïve zebrafish blastoderm explants by excluding the marginal zone [16-21]. Unmanipulated zebrafish blastoderm explants express high levels of bone morphogenetic protein (BMP) morphogens [19] and give rise to non-neural ectoderm and enveloping layer (EVL) when cultured *ex vivo* [18]. However, they recapitulate many aspects of axial patterning and morphogenesis in response to exogenous signaling gradients [19-21], similar to *Xenopus* animal caps. For this reason, blastoderm explants are an advantageous model to study the role of a given morphogen (or morphogens) in germ layer specification, morphogenetic cell movements, and signaling gradients within a simplified signaling environment. Furthermore, blastoderms from embryos of different genotypes or conditions can be combined in a single chimeric explant [19,21] to investigate cell/tissue autonomy and inductive interactions.

Zebrafish blastoderm explants can be used to investigate the role of embryonic signals (for example, Nodal) in morphogenesis and tissue specification during gastrulation. By injecting synthetic *ndr2* RNA (encoding a Nodal ligand) at the single-cell stage, Nodal signaling is activated throughout the blastoderm of the embryo. Explants from these embryos generate Nodal signaling gradients, form all three germ layers, and undergo convergence and extension (C&E) gastrulation movements as seen in intact embryos [20]. Additionally, chimeric explants are used to further illustrate the ability of mesoderm tissues to induce neuroectoderm from uninjected (naïve) blastoderm. This protocol provides instructions for creating zebrafish blastoderm explants and demonstrates their utility in defining the role of Nodal signaling in tissue induction and morphogenesis.

Protocol

1. Prepare reagents and supplies

1.1. Prepare reagents

1.1.1 Prepare 500 mL of **3X** Danieau's solution (Solution Composition table: **Solution 1**).

1.1.2 Prepare 1 L of egg water (Solution Composition table: **Solution 3**).

1.1.3 Prepare a 1.2% solution of agarose in egg water. Melt agarose completely in microwave, then allow to cool to 55°C in a water bath.

1.1.4 Prepare 4 mL explant media (modified slightly from[19,21]) per experimental condition.
***Note:** Remember to account for at least 1 well of explants from uninjected (or control injected) embryos when calculating required volume.

1.1.4.1 Sanitize the workspace with 70% ethanol.

1.1.4.2 Remove cell culture media from 4°C and spray/wipe with 70% ethanol.

1.1.4.3 Make explant media (Solution Composition table: **Solution 4**) and place in the 28.5°C incubator to warm while the embryos are injected.
***Note:** Always include age-matched, intact sibling embryos for staging purposes. Dechorionate these embryos and culture them on agarose-coated plates in **0.3X** Danieau's solution (Solution Composition table: **Solution 2**).

1.1.5 Remove pronase aliquots (1 mL at 20 mg/mL) from -20°C and allow to thaw on ice. Thaw one 1 mL aliquot for every 3 experimental conditions.

1.2 Prepare agarose plates

1.2.1 Make the injection plates.

1.2.1.1 Fill a 100 mm X 15 mm plastic Petri dish half-full with molten agarose in egg water.

1.2.1.2 Gently place the injection mold on top of the molten agarose at a 45° angle and lower it gradually into the agarose, ensuring that no bubbles are trapped underneath. Let cool completely.

1.2.1.3 Remove mold. Use plate immediately or save for later by adding 2 mL of egg water, wrapping the plate, and storing it at 4°C. 15-30 minutes before injection, warm the plate in the 28.5°C incubator.

1.2.2 Make explant cutting plates.

1.2.2.1 Add 3 mL of molten 1.2% agarose in egg water to a 60 mm X 15 mm Petri dish, ensuring the entire bottom of the well is coated. Let cool completely.

1.2.3 Coat culture plates with agarose.

1.2.3.1 For each experimental condition, dispense 1 mL of molten 1.2% agarose in egg water into 1 well of a 6-well plate, ensuring the entire bottom of the well is coated. Let cool completely.

1.2.4 If making chimeric explants, create an explant cutting dish with small wells by adding 12 - 1 mm glass beads to molten agarose in a 60 mm x 15 mm Petri dish. Remove the beads with forceps once the agarose has cooled completely.

2 Inject embryos with RNA

2.1 Handling with gloves, remove an aliquot of synthetic *ndr2* mRNA from storage at -80°C and immediately place on ice.

2.2 Prepare the injection needle.

2.2.1 Fill a pulled glass capillary needle with RNA. Place the filled needle into a micro-manipulator and break the tip of the needle with #5 watch makers forceps.

2.2.2 Calibrate injection volume using a micrometer with a drop of mineral oil, adjusting injection time and pressure on the pneumatic injector as necessary. The volume of the bolus will depend on the concentration of the RNA and the desired dose per embryo. For example, if RNA is aliquoted at 10 ng/μL, inject 1 nL to achieve a final dose of 10 pg.

2.2.2.1 Keep RNA needle tip submerged in the oil until ready to inject.

2.3 Load the embryos and begin injections.

2.3.1 Pull dividers in breeding tanks, allow fish to spawn for 10-15 minutes, and collect the embryos using a tea strainer.

2.3.2 Load embryos into the injection plate using a Pasteur pipet and pipet pump, then use a gloved finger to gently press the eggs into the troughs.

2.3.3 Inject 10 pg *ndr2* RNA into the yolk of single-cell embryos until the desired number of embryos is reached or until embryos begin to divide.
***Note**: Do not inject after the single-cell stage to ensure even distribution of the RNA throughout the embryo.

2.3.4 Wash the embryos out of the injection plate into a labeled 100 mm x 15 mm Petri dish with a gentle stream of egg water from a squeeze bottle.
***Note**: Always keep a group of age-matched, uninjected siblings as controls.

2.3.5 Place embryos into the 28.5°C incubator until they reach the 128-cell stage. Remove unfertilized eggs and dead embryos from the dish.

3 Dechorionate embryos

3.1 Once the embryos have reached 128-cell stage, place them into labeled glass Petri dishes and decant as much egg water as possible from them.

3.2 Label glass crystalizing dishes with lab tape (corresponding to small dish names) and fill 2/3 of the way with egg water. Place these dishes next to the dissecting microscope for quick accessibility.

3.3 Add 1 mL pronase stock (20 mg/mL, thawed on ice) to 15 mL **3X** Danieau in a 50 mL conical tube. This is sufficient for up to 3 experimental conditions. Increase volume of pronase and **3X** Danieau's for additional explant conditions.
CAUTION: Pronase is an irritant. Wear gloves when handling.

3.4 Add at least 5 mL of pronase solution to each glass Petri dish containing embryos.

3.5 Agitate the glass dishes in a circular motion, monitoring the progress of dechorionation consistently under a dissecting microscope.

3.6 Once the chorions begin to wrinkle and 1-2 embryos are out of their chorions, carefully dunk the glass Petri dish containing pronase and embryos into the corresponding glass crystalizing dish containing egg water.

3.7 Wash the dechorionated embryos.

3.7.1 Wash the embryos 3 times with egg water by gently adding and then decanting egg water from the dish.

3.7.2 The third and final wash is with **0.3X** Danieau's solution.
***Note:** If the embryos sill have chorions after washing, gently pipet the embryos until the chorions are removed or let them sit in wash (egg water or **0.3X** Danieau's) for a minute or two and gently agitate with circular motions.

3.8 Cover dechorionated embryos with a Petri dish lid and return them to the incubator until they reach 256-cell stage.

4 Cut explants

4.1 Fill agarose-coated 60 mm x 15 mm Petri dish with **3X** Danieau's solution.

4.2 Once the embryos are at 256-cell stage, transfer them into the agarose-coated plate containing **3X** Danieau's, lining them up along the center of the dish.

4.3 Cut the explants using #5 watch makers forceps (**Figure 1**).

4.3.1 Use one pair of forceps, held closed, to stabilize the embryo and use the other to make a cut through the blastoderm at approximately ½ of its height (from margin to animal pole) (**Figure 1A**).

4.3.2 To make the cut, gently squeeze the blastoderm cells with one pair of forceps, then take the stabilizing forceps and run them along the other forceps to slice approximately halfway across the blastoderm (**Figure 1B**).

4.3.3 Rotate the embryo, placing the forceps into the existing cut, then sever the remaining blastoderm orthogonal to the first cut (**Figure 1C**).

4.4 Keep explants in **3X** Danieau's for at least 5 minutes to heal, then transfer them to the well of a 6-well plate coated with agarose and filled with 4 mL of explant media.

***Note:** Cut explants from uninjected (or control injected) siblings as negative controls. If explants are performed correctly, these explants will neither extend nor express markers of endoderm, mesoderm, or neuroectoderm.

4.5 Place the explant culture plates into the 28.5°C incubator until desired timepoint/stage (determined from intact siblings) is reached.

***Note**: To treat explants with compounds, such as small molecule inhibitors, the desired concentration can be added directly to the explant media within the wells at desired time points. Remember to include the volume of agarose when calculating concentrations. Example: 1 mL agarose + 4 mL explant media = 5 mL total volume per well).

5 Chimeric Explants

5.1 In place of a regular agarose coated plate, cut chimeric explants in a dish with agarose molded into 12 small, shallow wells using 1 mm glass beads (See section 1.2.4). Fill this plate with **3X** Danieau's solution.

5.2 Chimeric explants are generated from blastoderm cells of 2 embryos of different genotypes or conditions. Ensure that these conditions can be distinguished from one another by expression of transgenic or injected fluorescent markers.

5.2.1 Prepare by adding 12 embryos of one genotype/condition to the left side of the plate and 12 embryos of the other genotype/condition to the right side of the plate.

5.2.2 Move one embryo of each condition into the center of the plate, near one of the 12 wells.

5.2.3 Using forceps, cut an explant from each embryo as described for single embryo explants (Step 4.3).

5.2.4 Quickly press the cut edges of the 2 explants together within the shallow well using forceps. This allows the two halves to heal together into a single explant.

5.2.5 Continue with the remaining 12 wells within the plate. Once explants are healed, transfer them to the well of a 6-well plate coated with agarose and filled with 4 mL of explant media. Repeat until the desired number of explants is achieved.

6 Culture, image, and/or fix explants

6.1 Culture explants in the 28.5°C incubator until intact sibling embryos reach the desired stage.

6.2 Live explants can be mounted for continuous time-lapse imaging, imaged periodically throughout the culture period, or imaged live at the experimental end point.

6.3 Fix explants if desired. Once the explants reach the desired endpoint, note the stage of intact embryo siblings and place explants into a glass scintillation vial with 1 mL of 4% paraformaldehyde in PBS. Fix overnight on a shaker at 4°C.
CAUTION: Paraformaldehyde is toxic. Wear gloves when handling this chemical and dispose of via methods approved by each institution.

6.3.1 After fixation, rinse explants 6 times, 15 minutes each with PBS + 0.1% Tween-20 and dehydrate gradually into methanol. Store explants at -20°C for later analysis by whole mount *in situ* hybridization, immunofluorescent staining, etc.

Representative results

Nodal ligands drive germ layer formation and C&E of zebrafish blastoderm explants

Control explants cut from uninjected wild-type (WT) embryos or those injected with 50 pg of mRNA encoding green fluorescent protein (GFP) remained rounded throughout the culture period (Figure 2A-C) and failed to express markers of mesoderm, endoderm, or neuroectoderm (Figure 3C) [20]. Together, these indicate an absence of the morphogenesis and germ layer formation that characterize vertebrate gastrulation. However, explants cut from embryos injected with 10 pg of *ndr2* mRNA became highly elongated after 8-9 hours in culture (Figure 2D). Live time-lapse imaging of these explants by differential interfering contrast (DIC) microscopy revealed that extension onsets at or around 8 hours post fertilization (hpf) (Figure 2F), the same time that C&E morphogenesis begins in intact zebrafish embryos [22]. Explants cut from MZ*oep*-/- embryos, which lack the essential *tdgf1* Nodal co-receptor [23], failed to extend in response to *ndr2* injection (Figure 2E), demonstrating that Nodal activity is critical for this *ex vivo* morphogenesis. In addition, whole mount *in situ* hybridization further demonstrated that *ndr2*-expressing explants express markers of neuroectoderm (*sox2*) and several mesoderm sub-types (*tbxta*, *noto*, *tbx16*)(Figure 2G), as well as endoderm and the embryonic organizer [20].

Nodal signaling is not required for neuroectoderm induction by mesoderm

Nodal signaling activity is essential for induction of endoderm and most mesoderm but is dispensable for neuroectoderm specification within zebrafish gastrulae [23,24]. While uninjected zebrafish blastoderm explants did not differentiate into neuroectoderm (Figure 3C, [18]), explants from embryos injected with 10 pg *ndr2* exhibited robust expression of the neuroectoderm marker *sox2* in distinct stripes along the long axis of the explant (Figure 2G), indicating that Nodal activity is required for neuroectoderm formation *ex vivo*. It has long been known that mesodermal tissues can induce neural tissue [25-29], including in zebrafish blastoderm explants [17]. However, it is unclear whether neuroectoderm formation in this explant system requires Nodal signaling directly, or whether exogenous Nodal ligands induce mesoderm that then induces neural tissues secondarily.

Chimeric explants comprised of prospective mesoderm and neuroectoderm portions from 2 different embryos were generated to test whether Nodal signaling is required tissue-autonomously for neuroectoderm specification *ex vivo*. The mesoderm portion of each explant was cut from an otherwise WT embryo expressing a mesoderm-specific transgenic GFP reporter, Tg[*lhx1a:eGFP*] [30], injected with a high dose (100 pg) of *ndr2* (Figure 3A). The putative neuroectoderm portion of each explant was cut from either a control WT embryo or a Nodal signaling deficient MZ*oep*-/- embryo injected only with mRNA encoding the fluorescent nuclear marker H2B-RFP (Figure 3A). Each chimeric explant was generated by combining one

blastoderm from each of these two conditions, which were assayed for expression of tissue-specific markers by whole mount *in situ* hybridization at 12 hpf.

The majority of single-embryo explants from embryos injected with 100 pg *ndr2* expressed little or no *sox2*, and expressed markers of mesoderm – including *tbxta* and the *lhx1a:gfp* reporter - throughout the explant (Figure 3B, G). Uninjected WT blastoderm (of the type that comprise the prospective neuroectoderm portion of chimeric explants) expressed neither mesoderm markers nor *sox2* when cultured as a single explant, indicating a lack of neuroectoderm and mesoderm specification (Figure 3C, H). Single-embryo explants from MZ*oep*-/- embryos similarly lacked expression of both neuroectoderm and mesoderm markers, even when injected with *ndr2* (Figure 3D). However, when uninjected WT blastoderms were combined with mesoderm induced by high doses of Nodal ligands, these chimeric explants expressed both mesoderm markers and *sox2* robustly (Figure 3E, I). These results demonstrate that, as previously observed [17,26,27,29], mesoderm can induce neural fate in cells that would otherwise become non-neural ectoderm. To test whether Nodal signaling is required directly within the prospective neuroectoderm portion of these explants for their neural induction, chimeric explants were created in which WT blastoderms converted to mesoderm by injection of 100 pg *ndr2* were combined with blastoderms from MZ*oep*-/- embryos (Figure 3J). Despite their inability to receive Nodal signals from the neighboring mesodermal portion, these explants expressed *sox2* to a similar degree as WT control chimeras (Figure 3F). This result demonstrates that, consistent with intact embryos in which neural tissues are specified in the absence of Nodal activity, Nodal signaling is not required tissue-autonomously for neuroectoderm induction *ex vivo*.

Figure Legends

Figure 1: Procedure for zebrafish blastoderm explantation (as in Protocol section 4.3).
A) Hold the forceps in the non-dominant hand (orange) closed against the yolk to stabilize the embryo while pinching the blastoderm at approximately ½ of its height using the forceps in the dominant hand (blue). **B**) Run the 'orange' forceps along the edge of the 'blue' forceps that are gripping the embryo to slice through the blastoderm so that the first cut reaches approximately halfway across the blastoderm. **C**) Rotate the embryo 90 degrees, then place the 'blue' forceps inside of (but orthogonal to) the original cut and pinch to sever the remaining blastoderm. **D**) Allow explanted blastoderm cells to heal in **3X** Danieau's solution for approximately 5 minutes before transferring into explant media.

Figure 2 (Figure and legend modified from [20]): **Nodal ligands promote C&E morphogenesis and germ layer formation in zebrafish blastoderm explants**.
A) Diagram of injection and explantation of zebrafish embryos. **B-E**) Representative bright field images of live blastoderm explants of the indicated conditions/genotypes at the equivalent of 2-4 somite stage. N= number of explants from two to four independent trials. **F**) Time-lapse DIC series of a representative explant from a WT embryo injected with 10 pg *ndr2* RNA. **G**) Representative images of whole mount *in situ* hybridization for the transcripts indicated in explants from WT embryos injected with 10 pg *ndr2* RNA. Scale bars are 200 μm.

Figure 3 (Modified from [31]): **Chimeric explants reveal that neuroectoderm specification does not require tissue-autonomous Nodal signaling *ex vivo***.
A) Diagram of the procedure to generate chimeric zebrafish explants. **B-F**) Whole mount *in situ* hybridization for the mesoderm marker *tbxta* (top) and neuroectoderm marker *sox2* (bottom) in explants from WT embryos injected with 100 pg *ndr2* RNA (B), uninjected WT controls (C), MZ*oep*-/- injected with 10 pg *ndr2* (D), and chimeric explants containing neuroectoderm portions

from WT (E) or MZ*oep*-/- (F) embryos at the equivalent of 2-4 somite stage. Fractions indicate the number of explants with the phenotype shown over the total number of explants examined. **G-J**) Representative images of live Tg[*lhx1a:gfp*] explants from a single embryo (G-H) or combined with H2B-expressing blastoderms (I-J, magenta) of the conditions indicated at the equivalent of 2-4 somite stage. N= number of explants from three independent trials. Scale bars are 200 μm.

Discussion

This article has described how to generate zebrafish blastoderm explants and discussed two practical applications of these explants in addressing the role of Nodal morphogen signaling in gastrulation. This method of cutting and culturing explants provides a "blank slate" of naïve cells that can be manipulated using RNA injections and/or treatment with small molecule compounds to investigate a molecular pathway of interest.

Critical steps

There are four steps in this protocol that are particularly critical for its success. The first is injecting the embryos with the appropriate amount of Nodal. This protocol recommends 10 pg *ndr2* RNA, and although a range of doses promote extension, too much or too little Nodal will prevent optimal explant extension [20]. The second step is dechorionating the embryos. If the embryos remain in pronase for too long, the yolks will burst and the embryos will not be viable to cut. If they are not in the pronase long enough, the chorions will not be loosened by washing and will instead require time consuming manual dechorionation. The third critical step is cutting the explants. Cutting in **3X** Danieau is recommended, as the lower salt content of 0.3X Danieau or egg water does not promote healing and survival of explants. Additionally, the explants must be cut at approximately half the height of the blastoderm to ensure naivety of the cells. If they are cut too close to the yolk, they will contain signals from the margin (including endogenous Nodal) that promote tissue specification and morphogenesis. The fourth and final critical step is the healing of chimeric explants. Two explants will not fuse to form chimeras unless their cut edges are gently pressed together immediately after they are cut.

Modifications and troubleshooting

The critical steps described above provide opportunities for troubleshooting. Some common issues and proposed solutions are presented below.

Problem: explants are not extending in the presence of Nodal signaling.
Possible solutions:
1. Inject embryos at the single-cell stage to ensure that RNA is evenly dispersed throughout the entire embryo.
2. Avoid injecting too much *nodal* RNA by ensuring that the injected volume is correct using a micrometer to measure the injection bolus.
3. Avoid injecting too little *nodal* RNA by measuring its concentration to ensure it has not degraded.
4. Keep some age-matched intact siblings to infer the equivalent stage of the explants. Explants achieve maximum extension when intact siblings reach 2-5 somite stage. If the explants are collected too early, then optimal extension will not be reached.

Problem: the yolks are bursting after dechorionation and the embryos are not viable to cut.
Possible solution: remove the embryos from pronase solution once the chorions begin to crinkle and 1-2 embryos shed their chorion, then rinse immediately in egg water.

Problem: the explants appear “bubbly” around the edges.
Possible solutions:
1. Cut explants only within a specific timeframe of development. Although explants cut at any stage from 128- to 1000-cell can survive and extend in culture, those cut at 256- to 512-cell stages tend to be the most robust.
2. Ensure that explants are cut in **3X** Danieau’s to ensure proper healing.
3. Cut explants cleanly but gently. Avoid stretching or pulling the cells apart during the cutting process.

Problem: uninjected control explants are extending.
Possible solution: explants were likely cut too close to the yolk. For explants to be naïve, ensure that the cuts are made halfway between the yolk and the top of the blastoderm.

Problem: chimeric explants fail to fuse.
Possible solution: once cut, the tendency of explants in **3X** Danieau’s solution is to round up and heal over the cut edge. To ensure that 2 blastoderms heal to each other rather than to themselves, press them together immediately after cutting. Use forceps to apply gentle pressure to the newly joined blastoderms within the agarose well to encourage them to heal together.

Limitations
While these explants are an advantageous tool to study the role of a given morphogen (or other molecule of interest) in relative isolation, observations made in any *ex vivo* model must be interpreted with care. Explants exhibit C&E morphogenesis that is very similar to that observed *in vivo* [20], but they do not recapitulate all aspects of gastrulation, for example: epiboly movements. They also lack many other regulatory factors and signaling molecules that are present within an intact embryo. While this is a major experimental advantage of explants, it can also lead to conclusions that do not hold true *in vivo*. For example, because explants that do not receive exogenous Nodal ligands fail to express neuroectoderm markers, one might conclude from explants alone that Nodal signaling is required for neuroectoderm specification. However, neuroectoderm is indeed formed within intact embryos lacking all Nodal signaling [23,24], demonstrating the important role of other signaling molecules in neural specification [32]. Explants can tell us what a morphogen is capable of in an isolated environment, but all such findings should be confirmed in / compared with intact embryos for results to be interpreted fully. In other words, explants cannot take the place of a developing embryo, but rather, they are a supplementary tool to identify the role and relationship of a morphogen with the surroundings. With these limitations in mind, zebrafish blastoderm explants are a valuable tool for many research questions.

Significance with respect to existing methods
With renewed interest in the field of synthetic embryology, a number of *ex vivo* and *in vitro* approaches are regularly employed to model aspects of embryonic development. For example, 2- and 3-dimensional “gastruloids” composed of mouse or human embryonic / induced pluripotent stem cells can be coaxed, through the application of exogenous signaling molecules, to recapitulate some of the patterning and/or morphogenetic events of gastrulation, segmentation, and neurulation [33-37]. Although powerful, these methods require laborious and prolonged culture methods to both continuously maintain pluripotent stem cells and to grow gastruloids, which take many days to reach ‘gastrulation stages’. By contrast, zebrafish explants require no maintenance of stem cells cultures, as embryos are simply collected as needed. They are relatively simple to generate and reach ‘gastrulation stages’ within hours, the same as zebrafish embryos. This highlights another advantage of zebrafish explants - their intact

'developmental clock'. Because the developmental age of embryonic and induced pluripotent stem cells can be variable and highly debated, embryonic explants are perhaps better suited to investigate temporal regulation of development. Finally, while "pescoid" zebrafish explants (which contain the embryonic margin) similarly extend in culture [12,13], they do so in response to endogenous signaling centers. The explants described here instead enable researchers to investigate molecules of interest with relatively little interference from such embryonic signals.

Potential future applications

Here, explants were used to demonstrate that Nodal signaling is necessary and sufficient for C&E morphogenesis, but it is anticipated that they can and will be used to discern the role of many different molecules in many different developmental processes. For example: regulation of gene expression, signaling gradients, and additional morphogenetic programs. Additionally, because these explants are viable until at least 24 hpf [19], it can be expected that their utility will extend beyond gastrulation into processes such as segmentation and organogenesis – any process in which researchers desire a developmental "blank slate".

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Disclosure

The authors declare that they have no competing financial interests.

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