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Analysis of cell shape and polarity during zebrafish gastrulation

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Summary

Gastrulation is a complex set of cellular rearrangements that establish the overall shape of the body plan during development. In addition to being an essential and fascinating aspect of development, the cells of the gastrulating zebrafish embryo also provides an ideal in vivo system to study the interplay of cell polarity and movement in a native 3D environment. During gastrulation, zebrafish mesodermal cells undergo a series of conversions from initial non-polarized amoeboid cell movements to more mesenchymal and finally highly polarized and intercalative cell behaviors. Many of the cellular behavior changes of these cells are under the control of the RhoA pathway, which in turn is regulated by many signals, including non-canonical Wnts. The goal of this chapter is to provide researchers with the necessary protocols to examine changes in cell polarity and movement in the developing zebrafish embryo.

Keywords

Planar Cell Polarity; Convergent Extension; Convergence and Extension; Wnt; RhoA; Blebbing; Rock; Myosin; Zebrafish; Gastrulation

1. Introduction

Gastrulation is a tightly controlled set of cellular movements required to generate the vertebrate body plan [1]. Convergence and extension (CE; also called convergent extension when the two processes are intimately linked) are two of the primary driving forces of gastrulation [1–4]. During CE, cells migrate toward the dorsal side of the embryo and then intercalate between surrounding cells. This process results in an overall dorsoventral narrowing and anterioposterior lengthening of the embryo [1]. The β -catenin independent Wnt planar cell polarity pathway (Wnt-PCP, also called the non-canonical Wnt pathway) is a major regulator of cell polarity and cell movement in a wide array of developmental and disease systems [5–7]. One context in which the Wnt-PCP has been particularly well studied is its essential role in controlling cell movements during CE [2, 3, 8, 9]. Both gain- and loss-of function of members of the PCP pathway result in severe gastrulation defects in a variety of vertebrates [10–18].

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⁸The 48 cell culture dishes can be washed and reused as it is not essential that they be sterile.

One particularly powerful system for the study of CE is zebrafish. Zebrafish embryos are optically transparent during gastrulation, which makes microscopic observations using Differential Interference Contrast optics (DIC) much simpler than in many vertebrates. Another powerful advantage of zebrafish is the availability of numerous mutants, including several disrupting CE. Finally, gain- and loss-of-function experiments are quite straightforward in zebrafish, as many genes can be over-expressed by injection of mRNA or knocked-down by injection of morpholino oligonucleotides [19, 20].

During zebrafish gastrulation, mesodermal cells undergoing CE undergo a series of stereotypical cellular behavior changes. Early in gastrulation mesodermal cells have little apparent polarity yet are highly migratory using primarily amoeboid cell movement. As gastrulation proceeds, the mesodermal cells become more highly polarized and migrate using a more mesenchymal form of movement [21–23]. Finally, as convergence has largely concluded, the cells reach the dorsal side of the embryo and alter their behavior, switching from primarily migration to cellular intercalation [21]. These dramatic changes in cellular behavior during gastrulation mirror changes seen in metastatic cells, which can invade neighboring tissue using collective cell migration, moving by mesenchymal or amoeboid migration [24, 25].

Many genes have been identified that regulate CE (reviewed in [2, 3]). One common theme for many of these genes is regulation of the RhoA GTPase pathway. RhoA is one of the primary molecular targets of the Wnt-PCP pathway but many Wnt-independent pathways also regulate RhoA [26–33]. However, it seems likely that many more genes involved in this complex process have not been identified. In this chapter we will provide a framework for investigators that wish to characterize the role of novel genes in gastrulation. We will describe a number of techniques to analyze zebrafish at the whole embryo, tissue and cellular level. These protocols can be used to characterize the role of novel genes in gastrulation as well as elucidating the complex regulation of RhoA signaling during gastrulation.

2. Materials

1. Fish Water: 0.3 g/L Instant Ocean® salts in deionized water.
2. Embryo Media (EM): 15 mM NaCl, 0.5 mM KCl, 1.25 mM CaCl₂, 1 mM MgSO₄, 0.12 mM KH₂PO₄, 0.05mM NaHPO₄, 0.8 mM NaHCO₃. A 10X solution can be stored for several months at 4°C without the addition of the NaHCO₃. 1X should be made fresh each week, with freshly added bicarbonate.
3. Use either a gel loading pipette tip (such as VWR 37001-270) or a fine hair (such as an eyebrow hair) glued in a glass capillary as a probe to orient embryos
4. Microinjector (e.g. Picospritzer).
5. Dissecting microscope.
6. Agarose plates: Create a 1% agarose solution in 1X EM, boil in microwave and pour into a 10 cm dish creating a thin layer of agarose. Allow the agarose to solidify and cover with EM.

7. Morpholino (Danieau) buffer: 58 mM NaCl, 0.7 mM KCl, 0.4 mM MgSO₄, 0.6 mM Ca(NO₃)₂, 5 mM HEPES, pH 7.6.
8. Morpholinos: Stock kept at 25 mg/ml at RT (or –80°C). Dilutions in Morpholino buffer can be kept at 40°C for several weeks. Some investigators believe heating at 65 C for 3 minutes before dilution produces more consistent results,
9. 1.5, 1.75 and 2.0% methylcellulose in EM: Prewarm 1X EM to 65°C in a water bath, add appropriate amount of powdered methylcellulose and vortex. Cool the solution in an ice bucket until it becomes clear. This can be kept for 2 weeks at room temperature.
10. 1X PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.76 mM KH₂PO₄ pH 7.4.
11. 1X PBST: PBS with 0.1% Tween.
12. Pronase: 4% pronase (Protease, type XIV, Sigma P5147) in EM. (store aliquots at –20°C).
13. 4% Paraformaldehyde in PBS (Make fresh and store in aliquots at –20°C; thaw a fresh aliquot each week).
14. 30%, 50%, 70% Glycerol in PBST.
15. Antibody block: 2% sheep serum, 2mg/ml bovine serum albumin. Store at –20°C.
16. Alkaline phosphatase buffer: 100mM Tris, 50 mM MgCl₂, 100 mM NaCl, 0.1% Tween, pH 9.5. Must be made fresh.
17. Hybridization buffer: 65% formamide, 5X SSC, 500 µg/mL torula (yeast) tRNA, 50 µg/mL heparin, 0.1% Tween-20, 9 mM citric acid, pH 6–6.5.
18. 20X SSC: 3M NaCl, 300 mM trisodium citrate.
19. Methanol 100%.
20. DIG RNA labeling mix (Boehringer 11 277 073 910). Store at –20°C.
21. NBT Stock: 50 mg NBT (Nitrotetrazolium blue chloride; Sigma N6639) in 0.7 ml anhydrous dimethylformamide and 0.3 ml of sterile water. Store at –20°C.
22. BCIP Stock: 50 mg of BCIP (5-Bromo-4-chloro-3-indoyl phosphate disodium salt; Sigma B1026) in 1ml of anhydrous dimethylformamide. Store at –20°C.
23. Wide tip 5¼" Pasteur pipettes for transferring embryos.
24. Water bath set to 65°C.
25. Incubator set to 28.5°C.
26. Depression slide.
27. In situ hybridization probes: *papc* (*paraxial protocadherin*; marker for presomitic mesoderm). Quadfecta probe: mix of *hgg1* (*hatching gland 1*), *shh* (*sonic*

hedgehog), *pax2a* (*paired box gene 2a*) and *dlx3b* (*distal-less homeobox gene 3b*). Store at -20°C

28. Glass bottomed dishes (35 mm): MatTek Cultureware (P35G-1.5–14-C).
29. Microscope for DIC: Time-lapse recordings were performed with a Zeiss Axiovert 200M microscope, DIC optics, and a 40 $\times$ objective. The experiments should be performed at constant temperature, ideally 28°C .
30. Software: AxioVision 4 software was used to collect time lapse movies and measure angles of convergence and extension. ImageJ was used to track cell movements.
31. Caged fluorescein dextran: Currently there are no commercial suppliers of this reagent. However, a protocol for synthesizing this reagent has been developed by James Chen (Stanford) and is available at <http://chen.stanford.edu/documents/CagedFluoresceinDextran.pdf>.
32. Anti-digoxigenin Fab fragments conjugated to alkaline phosphatase (Roche Molecular Biochemicals). Store at 4°C .
33. Rockout: should be maintained in a stock solution in DMSO (Calbiochem 555553). Store at -20°C
34. Blebbistatin: should be maintained in a stock solution in DMSO (Sigma B0560). Store at -20°C .

3. Methods

3.1 Injection and dechorionation of zebrafish embryos

For additional protocols on the husbandry of zebrafish, embryo collection and staging, see the zebrafish book [34], which is available online at <https://wiki.zfin.org/display/prot/ZFIN+Protocol+Wiki>. Morpholino antisense oligonucleotides (MOs) can be easily designed for loss-of-function experiments by either blocking splicing or translation [20] and are commercially available (www.gene-tools.com). If a gene has been hypothesized to play a role in gastrulation it can be knocked down by injection of a MO into the one-cell stage embryo. Although we describe here loss-of-function approaches, the same methods can be used for gain-of-function experiments using synthetic mRNA.

The most obvious phenotype of an embryo with severe defects in CE is a shortened and curved body axis at 48 hpf (hours post fertilization). Figure 1 shows a 48 hpf embryo injected with buffer control or a MO targeting *mypt1*, a gene required for both convergence and extension [26]. Loss of some regulators of CE have a dramatically shortened body axis as shown in Figure 1 [15], whereas others have only mild body axis curvature and shortening [10]. Because MOs can have off-target effects [20] that can resemble CE defects, it is important to either show that the MO effect can be rescued by co-injection of a synthetic mRNA for the gene of interest that will not bind to the MO, or to show that two different MOs cause the same effect. It is often useful to test a MO with a 5 bp mismatch to ensure

that the phenotype is specific (we do not use the standard control MO sold by Gene Tools since it seems to be chosen precisely because it has no effects).

1. Allow zebrafish to spawn naturally and collect embryos within 20 minutes of spawning.
2. Place 50 to 100 embryos in an injection tray with a minimal amount of water.
3. Place the desired buffer to be injected into an injection needle. Calibrate the injection time with a reticule to deliver 1 nl of buffer per injection.
4. Inject 100–300 embryos with the MO (diluted in Morpholino buffer). Typical MO range is 1–20 ng, although doses above 5 ng can often cause non-specific effects.
5. After injecting place embryos in petri dish with EM and incubate at 28.5°C until the desired stage is reached.
6. When the embryos approach 50% epiboly prepare to dechorionate them. They can be manually dechorionated with fine forceps, or using pronase as in the following steps.
7. Place 50–100 embryos in 1 ml EM in a small petri dish covered with a thin layer of agarose. Add 1 ml of 4% pronase in EM.
8. Swirl gently and watch for 2–4 minutes for the first sign of embryos falling out of their chorions.
9. Pour the embryos into 300 ml of EM in a 500 ml or 1 L beaker with a thin layer of agarose at the bottom.
10. Allow embryos to fall to the bottom and pour off most of the liquid.
11. Add 300 ml of fresh EM gently and pour off the excess.
12. Repeat once more.
13. Embryos should now be entirely or mostly dechorionated (see Note 1). Remove with a Pasteur pipette and place in another 10 cm plate with an agarose bottom.

3.2 In Situ Probe Synthesis

Note: all water and solutions used from step 2 onward must be nuclease free, either purchased as such or diethyl pyrocarbonate-treated.

1. Digest 10 µg of DNA with the appropriate restriction enzyme. Extract the aqueous solution twice with phenol-chloroform.
2. Ethanol precipitate the linearized DNA and resuspend in 10 µl of water.
3. Place 1 µg of linearized DNA, 1 µl RNase inhibitor, 2 µl of 10X transcription buffer, 2 µl of 10X DIG RNA labeling mix, 1 µl of the appropriate polymerase

¹Dechorionated embryos are extremely fragile and care should be taken in moving them, especially in methylcellulose. Embryos before bud stage should never be exposed to air, this will cause the yolk cell to burst. To orient the embryos use a single gel loading pipette tip, or a fine hair probe to orient the embryos.

(T7, T3 or SP6), bring to a total of 20 μ l with water. Incubate the reaction mix at 37°C for 2 hours.

4. Add 1 μ l RNase-free DNase and incubate at 37°C for 30 minutes.
5. Add 25 μ l of 7.5 M LiCl. Incubate at –20°C for at least 30 minutes.
6. Spin the solution in a microcentrifuge at maximum speed for 15 minutes at 4°C.
7. Remove the supernatant and discard. Add 90 μ l of water, 10 μ l 3M sodium acetate and 240 μ l of ethanol.
8. Precipitate for at least 15 minutes at –20°C. Centrifuge for 5 minutes and discard the supernatant. Wash the pellet with 70% ethanol. Remove all ethanol and air dry pellet for 5 minutes.
9. Resuspend the pellet in 30 μ l nuclease-free water and add 170 μ l of hybridization buffer.

3.3 Phenotypic characterization using in situ hybridization

After examining the phenotype it is essential to carefully characterize the effect at the tissue level. In situ hybridization provides an invaluable tool to measure the extent of CE in developing embryos. Staining bud stage embryos (end of gastrulation) allows for the direct measurement of the length and width of the paraxial mesoderm using a *paraxial protocadherin* (*papc*) probe. Figure 2 shows a control embryo and one defective in CE. Note that the paraxial mesoderm in the CE-defective embryo is dramatically wider and shorter (Figure 2E and F). In addition, both the movement of the prechordal plate and the morphogenesis of the notochord can be monitored in the same experiment. This can be accomplished by creating a mix called the “quadfecta” of *hgg1* (*hatching gland 1*-to mark the prechordal plate), *shh* (*sonic hedgehog* - to mark the midline), *pax2a* (*paired box gene 2a*- to mark the midbrain-hindbrain boundary) and *dlx3b* (*distal-less homeobox gene 3b*- to mark the neural plate). Note the dramatically broader notochord indicating defective CE of the axial mesoderm (Figure 2C and D) with the quadfecta stain. Also, note that the prechordal plate has failed to migrate beyond the midbrain-hindbrain boundary (Figure 2A and B).

1. Collect injected and control embryos at bud stage and place in 1.5 ml microfuge tube. Fix by placing (25–100) embryos in 4% PFA either for 2 hours at room temperature or overnight at 4°C.
2. Wash the embryos 3 times with 1 ml PBS to remove the PFA and then add 1 ml of 100% methanol (MeOH). These embryos will now dehydrate and can be stored for years at –20°C.
3. To start experiment rehydrate stored embryos by washing for 5 minutes with each of the following: 75% MeOH/25% PBS, 50% MeOH/50% PBS, 25% MeOH/75% PBS, and PBST.
4. Add 0.5 ml of hybridization buffer (HB) and incubate for 2–5 hours at 65°C.

5. Remove hybridization mix and add 0.2 ml of desired probe (diluted 1:200 in HB) and incubate overnight at 65°C.
6. Preheat wash solutions to 65°C in a water bath. Remove the probe mix and return to –20°C. Perform the following washes at 65°C by placing the tubes in the water bath during washes. Wash for 1 minute in HB, 15 minutes in 75% HB/25% 2X SSC, 15 minutes in 50% HB/50% 2X SSC, 15 minutes 25% HB/75% 2X SSC, twice for 10 minutes in 2X SSC and twice for 30 minutes in 0.2X SSC. The following washes are done at room temperature for 5 minutes each: 75% 0.2X SSC/25% PBST, 50% 0.2X SSC/50% PBST, 25% 0.2X SSC/75% PBST and PBST.
7. Add 0.5 ml of antibody block solution and incubate for 1–3 hours at room temperature, without agitation.
8. Remove the block and add 200–500 µl antibody solution (1:5000 anti-digoxigenin Fab fragments conjugated to alkaline phosphatase in block) then incubate overnight at 4°C.
9. Wash the embryos 6 times with PBST for 15 minutes each and 3 times for 5 minutes with alkaline phosphatase buffer (without BCIP/NBT)
10. Prepare a stock of 5 ml of alkaline phosphatase buffer and add 22.5 µl of NBT and 17.5 µl of BCIP. Add 0.5 ml of the alkaline phosphatase buffer with BCIP/NBT to the embryos. Allow the embryos to incubate at room temperature, protected from direct light until strong blue staining is visible but little background has developed (around 1 hour with these probes, but varies and may need to be optimized for each experiment)
11. Stop the reaction by adding 1 ml of PBS, removing the PBS and adding 1 ml of methanol. The embryos can be stored at –20°C for long periods. The methanol step is not necessary but it clears the yolk, which helps imaging the stain.
12. To continue, rehydrate the embryos (as in step 3). Wash once with 30% glycerol in PBS, once with 50% glycerol in PBS and once with 70% glycerol in PBS. Allow each wash to proceed until the embryos sink to the bottom of the tube.
13. Place embryos in depression slide and orient with a gel loading pipette tip and image with a dissecting microscope and digital camera.

3.4 Tracking cell movement (Dye uncaging)

The extent of both convergence and extension can be directly measured in zebrafish embryos [35]. Embryos are loaded with caged dye and grown to the onset of gastrulation, shield stage. At this time the dye is uncaged by a brief pulse of uv radiation to a small area of the embryo. This will causes a small cluster of cells to fluoresce. An initial starting image is then collected, and the embryo is allowed to proceed through gastrulation. At bud stage the embryos are reimaged and the extent of dorsal (convergence) and anterior (extension) migration can be determined. Figure 3 shows the migration of lateral cells in a control embryo.

1. Inject at the one cell stage with 1 nl of 0.5 % caged fluorescein dextran (with or without the MO).
2. Grow the embryos at 28.5°C and dechorionate (see section 3.1).
3. Mount a single embryo in a drop of 1.75% methylcellulose in embryo medium at shield stage in a glass-bottomed dish (see Note 2 and Note 3).
4. Carefully orient the embryos with a gel loading tip such that the shield is to the side and the lateral margin faces directly down in a glass bottomed dish (Figure 3A shows the way the embryo should look viewing it with an inverted microscope). Only work with 10–20 embryos at a time, each in their own dish.
5. Gently place the dish on the inverted fluorescent microscope. Find the desired cells using a 5X or 10X objective and then switch to 40X. Close the fluorescence pinhole iris to the narrowest setting. This will allow a narrow beam of light to illuminate only a small portion of the field.
6. Open the DAPI channel for approximately 15 seconds. This UV radiation will uncage the dye only in the portion of the embryo that is illuminated. In order to measure convergence and extension of the lateral mesoderm uncage a region of cells 90° from the dorsal shield and just above the margin (Figure 3A and 3D).
7. Turn off the DAPI channel and zoom out to 5X and open the pinhole (see Note 4).
8. Take a brightfield and a fluorescent image of the embryo showing that a few cells are now fluorescing green (Figure 3D). Acquire images with both a lateral view and animal view of uncaged embryos. This will provide you with an image of the embryo before CE and can be used to confirm that the labeled cells start 90° from dorsal.
9. Place a lid on the dish (important to prevent drying out) and place in air incubator at 28.5°C.
10. Wait approximately 4 hr until gastrulation is completed and the embryos reach bud stage.
11. Photograph at 5X and acquire a dorsal view (with the notochord directly facing down in the dish), a lateral view (with the notochord oriented perpendicular to the bottom of the dish), and an animal view (with the animal pole facing directly down; Figure 3E and F show lateral and dorsal views, and Figure 3C shows a diagram of an animal pole view).

²Embryos left for prolonged time in methylcellulose need to be treated with care. It is essential that the methylcellulose not be allowed to dry out. This will cause the embryo to burst. It is best to leave a lid on the dish with the embryos at all times when not filming.

³The caged fluorescein dextran is not well tolerated by embryos. Great care should be taken to not add too much dye to the embryos; however, using too little dye will result in a weak signal. Unhealthy embryos will produce auto-fluorescence of the dye, and can be used as an indicator of injecting too high a concentration. Dye injection will also produce a noticeable developmental delay and great care should be taken to carefully control all experiments.

⁴The dye can be uncaged by prolonged exposure to visible light and the embryos should be protected from visible light as much as possible. Only a brief exposure to UV light is necessary to uncage the dye, and prolonged exposure can damage the embryo.

12. The lateral view will provide a clear picture of the extent of extension of the embryo. Measure the angle (angles can be easily measured using either Axiovision software or ImageJ) from dorsal to the anterior most fluorescent cells (Figure 3E). This is the angle of extension of the paraxial mesoderm.
13. The animal pole view can be easily used to measure the extent of dorsal convergence. Measure the angle from the middle of the fluorescent cells to dorsal. Immediately after uncaging this angle should be 90°. At bud stage the angle will have been reduced by the migration of the labeled cells. This difference is the angle of convergence of the paraxial mesoderm (Figure 3B and C).
14. The dorsal view is valuable to determine how well the labeled cells have migrated dorsally and approached the notochord (Figure 3F). This angle is, however, difficult to use to determine either convergence or extension (See Note 5).

3.5 Length/width ratios of mesodermal cells

1. Length/width ratios of cells can be easily determined from DIC images (see Note 6) and are a useful assay for cell polarity of cells undergoing gastrulation, especially since disruption of planar-cell polarity signaling can reduce the length-width ratio considerably [13, 15, 36]. Cell shape can be examined at a variety of stages starting at bud stage and continuing through somitogenesis. It is important to compare identical stages when comparing experimentally manipulated and control embryos, because cell shape will change considerably and length-width ratios will increase as development proceeds.
2. Observe a field of cells that contains a clear image of the notochord and the paraxial mesoderm. Collect clear images of 10–25 notochordal cells and 10–25 mesodermal cells at least one cell diameter away from the notochord to analyze. Collect images from at least three different embryos.
3. Measure the length of the cells using the measure function (available in either imageJ or Axiovision software) using the longest part of the cell. Then calculate the width of the cell by measuring the distance across the midpoint of the cell (Figure 4).
4. The length width ratio of paraxial mesodermal cells at bud stage should be approximately 2:1 whereas notochordal cells should have a length-width ratio of approximately 3:1. The notochordal ratio will continue to increase during somitogenesis, reaching 6:1 by the 10-somite stage.

⁵The dye uncaging protocol can also be used to measure extension of the axial mesoderm. The protocol is the same as 3.4 with the exception that the dorsal shield is uncaged instead of lateral cells. At bud stage the angle between the anterior most labeled cell and the midpoint along the anterior-posterior axis on the dorsal side is measured, however, unlike in protocol 3.4 these labeled cells will be in the notochord instead of the paraxial mesoderm.

⁶Best results for time-lapse microscopy are obtained using DIC optics but the cells are sufficiently easy to image that all experiments can be done with bright-field optics.

5. If desired, the length-width ratios can be calculated for ectodermal cell using the same procedure. This can be used to determine if the gene of interest is affecting only mesoderm [33] or multiple germ layers [36].

3.6 Analyzing cell shape and movement

A great strength of the zebrafish system is the ability to directly watch the cell behaviors and track cell movements in real time. Investigators are not limited to only observing the phenotype after gastrulation has finished. Zebrafish mesodermal cells undergo a variety of cellular behaviors as they undergo CE including non-polarized amoeboid movement and more polarized migratory and intercalative behaviors. Mesodermal cells are capable of a several types of membrane protrusions during gastrulation, including blebs, lamellipodia and filopodia [26, 33]. During normal gastrulation protrusive activity is dramatically reduced late in gastrulation as cells switch from migratory to intercalative behaviors. This protocol will give investigators the ability to create time lapse images which will allow them to observe these changes in cellular behavior.

1. Mount 1 to 5 dechorionated embryos in a drop of 1.5% methylcellulose in EM in a glass-bottomed dish.
2. Carefully orient the embryos with a gel-loading pipette tip under a dissecting microscope such that dorsal is facing directly downward and both the notochord and paraxial mesoderm are clearly visible.
3. Gently place embryos on the stage of a microscope with DIC optics.
4. Find the desired cells using the 5 or 10X objective. Switch to the 40X objective and focus through the enveloping layer cells, which have an epithelial shape, until you reach the more mesenchymal mesodermal cells. It is important to be consistent with the stage, location and layer of the cells to be imaged. The notochord provides a valuable landmark at bud stage whereas the margin and shield are useful at earlier stages.
5. Once you have located the desired cells prepare a time-lapse recording. In order to track cell migration imaging every 30 sec to 1 minute for 30 minutes is sufficient and will help reduce file size. In order to assay cell protrusive activity a faster frame rate is required, usually 3 second frames for 15 minutes is sufficient.
6. The time lapse movie will be quite large and will need to be compressed for further manipulation. In order to quantify membrane protrusions chose 20 to 40 mesodermal cells per embryo from at least 3 embryos. Simply follows the cells through the course of the movie and score them for the number of membrane protrusions. DIC optics provides excellent resolution of blebs, lamellipodia and filopodia in these cells (Figure 5).

3.7 Pharmacological inhibition of Rho-Rock Signaling

In many systems, including gastrulation, one of the key pathways that regulates membrane blebbing is the Rho-Rock-myosin pathway. Commercially available pharmacological inhibitors of Rock and Myosin are available to block membrane blebbing.

1. Remove dechorionated embryos from the incubator.
2. Prepare a 48 well culture plate by placing a few drops of melted 1% agarose in EM in the bottom of several wells.
3. Prepare 0.5–1 ml treatment solutions of 50 μ M myosin inhibitor Blebbistatin and 200 μ M Rockout (Rho kinase inhibitor III) or a DMSO control in EM in microfuge tubes.
4. Pipette embryos with a minimal amount of water directly into the microfuge tubes containing 1 ml of the drug in embryo media. Then transfer the embryos and media to an agarose coated well in a 48 well plate (see Notes 7–9).
5. Both drugs will affect cell shape and behavior within approximately 20 minutes.
6. After 30 minutes embryos are transferred to methylcellulose (with the same concentration of drug) and placed in a glass bottomed dish and imaged as in protocol 3.6

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References

1. Keller R. Shaping the vertebrate body plan by polarized embryonic cell movements. *Science*. 2002; 298:1950–1954. [PubMed: 12471247]
2. Hammerschmidt M, Wedlich D. Regulated adhesion as a driving force of gastrulation movements. *Development*. 2008; 135:3625–3641. [PubMed: 18952908]
3. Solnica-Krezel L. Gastrulation in zebrafish -- all just about adhesion? *Curr Opin Genet Dev*. 2006; 16:433–441. [PubMed: 16797963]
4. Wallingford JB, Fraser SE, Harland RM. Convergent extension: the molecular control of polarized cell movement during embryonic development. *Dev Cell*. 2002; 2:695–706. [PubMed: 12062082]
5. Mlodzik M. Planar cell polarization: do the same mechanisms regulate *Drosophila* tissue polarity and vertebrate gastrulation? *Trends Genet*. 2002; 18:564–571. [PubMed: 12414186]
6. Veeman MT, Axelrod JD, Moon RT. A second canon. Functions and mechanisms of beta-catenin-independent Wnt signaling. *Dev Cell*. 2003; 5:367–377. [PubMed: 12967557]
7. Fanto M, McNeill H. Planar polarity from flies to vertebrates. *J Cell Sci*. 2004; 117:527–533. [PubMed: 14730010]
8. Kodjabachian L, Dawid IB, Toyama R. Gastrulation in zebrafish: what mutants teach us. *Dev Biol*. 1999; 213:231–245. [PubMed: 10479444]
9. Roszko I, Sawada A, Solnica-Krezel L. Regulation of convergence and extension movements during vertebrate gastrulation by the Wnt/PCP pathway. *Semin Cell Dev Biol*. 2009; 20:986–997. [PubMed: 19761865]
10. Veeman MT, Slusarski DC, Kaykas A, Louie SH, Moon RT. Zebrafish prickles, a modulator of noncanonical Wnt/Fz signaling, regulates gastrulation movements. *Curr Biol*. 2003; 13:680–685. [PubMed: 12699626]

⁷Rockout will often precipitate if added directly to an aqueous solution without sufficient mixing. For this reason we often place the stock solution in a new tube first and then vigorously add the buffer when making a dilution.

⁹Although Y27632 is widely used to inhibit Rock in a variety of systems it does not work as well as Rockout in zebrafish.

11. Ohkawara B, Yamamoto TS, Tada M, Ueno N. Role of glypican 4 in the regulation of convergent extension movements during gastrulation in *Xenopus laevis*. *Development*. 2003; 130:2129–2138. [PubMed: 12668627]
12. Carreira-Barbosa F, Concha ML, Takeuchi M, Ueno N, Wilson SW, Tada M. Prickle 1 regulates cell movements during gastrulation and neuronal migration in zebrafish. *Development*. 2003; 130:4037–4046. [PubMed: 12874125]
13. Jessen JR, Topczewski J, Bingham S, Sepich DS, Marlow F, Chandrasekhar A, Solnica-Krezel L. Zebrafish trilobite identifies new roles for Strabismus in gastrulation and neuronal movements. *Nat Cell Biol*. 2002; 4:610–615. [PubMed: 12105418]
14. Winklbauer R, Medina A, Swain RK, Steinbeisser H. Frizzled-7 signalling controls tissue separation during *Xenopus* gastrulation. *Nature*. 2001; 413:856–860. [PubMed: 11677610]
15. Topczewski J, Sepich DS, Myers DC, Walker C, Amores A, Lele Z, Hammerschmidt M, Postlethwait J, Solnica-Krezel L. The zebrafish glypican knypek controls cell polarity during gastrulation movements of convergent extension. *Dev Cell*. 2001; 1:251–264. [PubMed: 11702784]
16. Wallingford JB, Rowning BA, Vogeli KM, Rothbacher U, Fraser SE, Harland RM. Dishevelled controls cell polarity during *Xenopus* gastrulation. *Nature*. 2000; 405:81–85. [PubMed: 10811222]
17. Heisenberg CP, Tada M, Rauch GJ, Saude L, Concha ML, Geisler R, Stemple DL, Smith JC, Wilson SW. Silberblick/Wnt11 mediates convergent extension movements during zebrafish gastrulation. *Nature*. 2000; 405:76–81. [PubMed: 10811221]
18. Djiane A, Riou J, Umbhauer M, Boucaut J, Shi D. Role of frizzled 7 in the regulation of convergent extension movements during gastrulation in *Xenopus laevis*. *Development*. 2000; 127:3091–3100. [PubMed: 10862746]
19. Nasevicius A, Ekker SC. Effective targeted gene ‘knockdown’ in zebrafish. *Nat Genet*. 2000; 26:216–220. [PubMed: 11017081]
20. Eisen JS, Smith JC. Controlling morpholino experiments: don’t stop making antisense. *Development*. 2008; 135:1735–1743. [PubMed: 18403413]
21. Yin C, Kiskowski M, Pouille PA, Farge E, Solnica-Krezel L. Cooperation of polarized cell intercalations drives convergence and extension of presomitic mesoderm during zebrafish gastrulation. *J Cell Biol*. 2008; 180:221–232. [PubMed: 18195109]
22. Concha ML, Adams RJ. Oriented cell divisions and cellular morphogenesis in the zebrafish gastrula and neurula: a time-lapse analysis. *Development*. 1998; 125:983–994. [PubMed: 9463345]
23. Sepich DS, Calmelet C, Kiskowski M, Solnica-Krezel L. Initiation of convergence and extension movements of lateral mesoderm during zebrafish gastrulation. *Dev Dyn*. 2005; 234:279–292. [PubMed: 16127722]
24. Charras G, Paluch E. Blebs lead the way: how to migrate without lamellipodia. *Nat Rev Mol Cell Biol*. 2008; 9:730–736. [PubMed: 18628785]
25. Wolf K, Friedl P. Molecular mechanisms of cancer cell invasion and plasticity. *Br J Dermatol*. 2006; 154:11–15. [PubMed: 16712711]
26. Weiser DC, Row RH, Kimelman D. Rho-regulated myosin phosphatase establishes the level of protrusive activity required for cell movements during zebrafish gastrulation. *Development*. 2009; 136:2375–2384. [PubMed: 19515695]
27. Zhu S, Liu L, Korzh V, Gong Z, Low BC. RhoA acts downstream of Wnt5 and Wnt11 to regulate convergence and extension movements by involving effectors Rho kinase and Diaphanous: use of zebrafish as an in vivo model for GTPase signaling. *Cell Signal*. 2006; 18:359–372. [PubMed: 16019189]
28. Habas R, He X. Activation of Rho and Rac by Wnt/frizzled signaling. *Methods Enzymol*. 2006; 406:500–511. [PubMed: 16472682]
29. Zhu S, Liu L, Korzh V, Gong Z, Low BC. RhoA acts downstream of Wnt5 and Wnt11 to regulate convergence and extension movements by involving effectors Rho Kinase and Diaphanous: Use of zebrafish as an in vivo model for GTPase signaling. *Cell Signal*. 2005

30. Kwan KM, Kirschner MW. A microtubule-binding Rho-GEF controls cell morphology during convergent extension of *Xenopus laevis*. *Development*. 2005; 132:4599–4610. [PubMed: 16176947]
31. Jopling C, den Hertog J. Fyn/Yes and non-canonical Wnt signalling converge on RhoA in vertebrate gastrulation cell movements. *EMBO Rep*. 2005; 6:426–431. [PubMed: 15815683]
32. Tahinci E, Symes K. Distinct functions of Rho and Rac are required for convergent extension during *Xenopus* gastrulation. *Dev Biol*. 2003; 259:318–335. [PubMed: 12871704]
33. Weiser DC, Pyati UJ, Kimelman D. Gravin regulates mesodermal cell behavior changes required for axis elongation during zebrafish gastrulation. *Genes Dev*. 2007; 21:1559–1571. [PubMed: 17575056]
34. Westerfield, M. *The Zebrafish book : a guide for the laboratory use of zebrafish (Brachydanio rerio)*. Eugene. Or: University of Oregon Press; 1993.
35. Sepich DS, Solnica-Krezel L. Analysis of cell movements in zebrafish embryos: morphometrics and measuring movement of labeled cell populations in vivo. *Methods Mol Biol*. 2005; 294:211–233. [PubMed: 15576915]
36. Marlow F, Topczewski J, Sepich D, Solnica-Krezel L. Zebrafish Rho kinase 2 acts downstream of Wnt11 to mediate cell polarity and effective convergence and extension movements. *Curr Biol*. 2002; 12:876–884. [PubMed: 12062050]

Figure 1. CE is required for body axis elongation in zebrafish

(A) Dorsal views of 48 hpf zebrafish embryos injected with (A) morpholino buffer or (B) 1 ng *mypt1* MO. Knock-down of Mypt1 causes dramatic reduction in both convergence and extension [26], leading to a dramatically shortened and curved body axis.

Figure 2. Disruption of CE leads to characteristic morphological defects in zebrafish embryos
 Embryos at bud stage stained with (A–D) *hgg1* (to mark the prechordal plate), *shh* (midline), *pax2a* (midbrain-hindbrain boundary) and *dlx3b* (neural plate) or (E, F) *papc* (presomitic mesoderm). The narrow bracket marks the width of the notochord and the wide bracket marks the width of the presomitic mesoderm. MO indicates embryos injected with 1 ng *mypt1* MO.

Figure 3. CE can be directly measured using dye uncaging

(A) A diagram demonstrating the correct lateral orientation for dye uncaging. A shield stage embryo with the margin, shield (gray) and the desired area to uncage (black dot) are all labeled. (B) A diagram of an animal view of an embryo immediately after uncaging. A cluster of cells 90° from dorsal have been uncaged. (C) A diagram of an animal view of a bud stage embryo after the labeled cells have undergone CE. The labeled cells have migrated toward the dorsal side. The angle between their original lateral position and their final position is the angle of convergence. (D–F) The fluorescent dye was uncaged at shield stage (D, white arrow), and examined at bud stage in lateral (E) and dorsal (F) views with the animal pole at top. In (F) the white arrowheads indicate the position of the notochord. (E) Anterior extension is defined as the angle between the anterior-most labeled cell and the mid-point along the anterior-posterior axis on the dorsal side (lateral view, anterior at top).

Figure 4. Mesodermal cell polarity

The cell polarity of presomitic and notochordal mesodermal cells was determined by calculating the length/width ratio. Wild-type mesodermal cells with the long axis length and the short axis width are shown.

Figure 5. Mesodermal cell shape changes dramatically during zebrafish gastrulation
 Membrane protrusive activity of mesodermal cells at bud stage was determined by time-lapse microscopy. Behavior of dorsal mesodermal cells in control embryos (A) and *mypt1* morphants (B) are shown at bud stage. White arrows indicate bleb-like protrusions (s=seconds).