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Analyzing Morphogenetic Cell Behaviors in Vitally Stained Zebrafish Embryos

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1. Introduction

Owing to its extremely rapid rate of development, as well as its optical transparency, the zebrafish embryo provides an excellent experimental system for analyzing the cellular dynamics that underlie vertebrate body axis formation. Moreover, the zebrafish (*Danio rerio*) is very amenable to genetic manipulation. Recent large-scale saturation mutagenesis screens have isolated several thousand strains of *Danio rerio* that possess recessive mutant alleles for genes involved in pattern formation or morphogenesis ***(1,2)***. These mutant strains of zebrafish represent a wealth of experimental material from which the patterns of cell division, cell intercalation, cell migration, and coordinate cell shape changes that underlie zebrafish morphogenesis can be analyzed. To help determine the genetic and epigenetic mechanisms that choreograph these events, it is often very useful to follow the morphogenetic behaviors of single cells and cell populations within the living zebrafish embryo.

In this chapter, we discuss how the behavior of cellular populations within intact zebrafish embryos can be analyzed using a combination of vital staining techniques and time-lapse confocal microscopy. In particular, we discuss how a neutral fluorescent dye, Bodipy 505/515, and a related sphingolipid derivative, Bodipy-ceramide, can be used respectively as vital stains for yolk-containing cytoplasm and interstitial space throughout the entire zebrafish embryo. These fluorescent probe molecules allow all of the cells within a living zebrafish embryo to be rapidly stained and then visualized *en masse* using a scanning laser confocal microscope. In addition to Bodipy 505/515 and Bodipy-ceramide, we also discuss a unique fluorescent probe molecule, SYTO-

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11, that can be used to identify the location of the organizer region (i.e., the dorsal marginal zone or DMZ) in late-blastula stage blastoderms ***(3)***.

Finally, we outline experimental parameters that are useful for making single-level and multilevel confocal time-lapse recordings of vitally stained zebrafish embryos. Using these time-compression approaches, one can detect and analyze genetically encoded sequences of cell behaviors that underlie the formation of the zebrafish germ layers and organ rudiments.

1.1. Contrast Enhancement

Confocal imaging of cell movement and cell shape changes within living tissue is intimately linked to the selective placement or accumulation of fluorescent probe molecules (i.e., contrast-enhancing agents) at specific locations within a cell or tissue. The contrast produced by the localization of a fluorescent contrast-enhancing agent can be defined mathematically as:

$$C = \Delta I / I_0, \qquad \text{(Eq. 1)},$$

where DI represents the change in fluorescence intensity at a given region of the specimen with respect to the mean intensity of its background fluorescence, I_0 (i.e., the rest of the cell or tissue). To first approximation, the fluorescence intensity I of a given volume element of cytoplasm is proportional to the concentration of fluorophores located within that specific volume element. Thus, once sufficient numbers of exogenous fluorophores are inserted into specific compartments of a living embryo, these objects of interest stand out from their background and can be easily detected with a confocal microscope. However, to obtain a successful confocal time-lapse recording, the contrast-enhancement mechanism must also be robust, such that multiple images of vitally stained cells or tissues can be acquired over an extended period of time.

Below, we outline an imaging strategy that is based on inserting large numbers of photostable fluorescent probe molecules into specific compartments of the living zebrafish embryo. Once these fluorescent probe molecules have been inserted, the embryo can be repeatedly scanned using moderate laser illumination intensity without compromising the image quality of individual scans during a time-lapse recording.

1.2. General Aspects of Vital Staining

In general, vital staining involves several procedural steps: (1) solubilization of the vital stain in a physiological labeling medium; (2) permeation, intercalation, or absorption of the vital stain into (or onto) the embryo; (3) localization/accumulation of the probe molecule within specific cellular or subcellular compartments; and (4) washout of unbound stain.

Because the "universal solvent" dimethyl sulfoxide (DMSO) has low toxicity on living tissues, it is an excellent choice for solubilizing and applying vital stains to zebrafish embryos. Embryo Rearing Medium (ERM) containing 1–2% DMSO and a variety of vital stains can be applied to zebrafish embryos for up to 1 h without producing toxic or teratogenic effects (3).

Many vital stains become localized in specific cellular compartments of cells and tissues through diffusion-trap mechanisms. As vital stain molecules from the external medium enter a cellular compartment (e.g., the lipid phase of a cell membrane or the lumen of an organelle) by diffusion, physiochemical characteristics of the molecules cause them to become retained (or trapped) within the compartment. For example, the compartment may be hydrophobic (e.g., $diIC_{18}$ partitions into the lipid phase of cell membranes), electronegative (e.g., Rhodamine 123 accumulates inside active mitochondria owing to the large membrane potential across their inner membrane), or possess multiple binding sites for the probe molecule (e.g., Bodipy-ceramide accumulation in *trans*-Golgi elements of vertebrate tissue cells) ***(4,5)***.

Cells and tissues of zebrafish embryos have only weak endogenous fluorescence. Exogenous fluorescent probes molecules must therefore be inserted into zebrafish embryos in preparation for high-resolution confocal imaging. We recommend labeling concentrations on the order of 100 μM when inserting bath-applied fluorescent probes into embryonic zebrafish tissues. The high concentration produces a larger diffusive flux of the vital stain into the embryo, and a faster accumulation of the fluorescent probe into the embryo's constituent tissues. If a fluorescent probe is internalized into cells by endocytosis, submicron endosomes will contain a sufficient concentration of fluorophores to be imaged clearly.

In the next section, we describe the staining characteristics of three complementary vital stains/labels that are quickly and easily applied to zebrafish embryos in preparation for confocal imaging.

1.3. Vital Stains and Vital Labels for Zebrafish Embryos

1.3.1. Bodipy 505/515

Bodipy is an abbreviation that refers to a very versatile set of neutral, boron-containing fluorophores that are derived from a diazaindacene backbone. Bodipy fluorophores have been conjugated to a variety of probe molecules including lipids, hormones, neurotransmitters, dextrans, and the actin-binding probe phallacidin ***(5)***. The notable charge neutrality of Bodipy fluorophores minimizes their effect on the properties of biomolecules to which they are conjugated ***(5)***.

Bodipy fluorophores are frequently referred to by their excitation and emission maxima. Thus, Bodipy 505/515 indicates that fluorophore is most strongly excited with visible radiation centered at 505 nm (blue light), and emits a spec-

trum of longer wavelength light that peaks at 515 nm. Bodipy 505/515 excitation maximum lies close to the 488 nm line of an argon/krypton laser. In addition, Bodipy 505/515 possesses a high quantum yield of nearly 0.9, and a relatively low photobleaching rate ***(5)***. These fluorescence characteristics make the Bodipy 505/515 fluorophore and its conjugate molecules ideally suited for scanning laser confocal microscopy.

We have found that unconjugated Bodipy 505/515 (4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-s-indacene; mol wt 248) is an excellent vital stain for yolky cytoplasm in zebrafish embryos ***(3)***. Bodipy 505/515 has a high oil–water partition coefficient, which allows it to rapidly cross cell membranes and accumulate within lipidic yolk platelets. These lipidic yolk platelets are distributed throughout the cytoplasm of the blastoderm and nearly completely fill the volume of the zebrafish's yolk cell. In contrast, Bodipy 505/515 does not stain nucleoplasm, nor does it remain within interstitial space. These staining characteristics of Bodipy 505/515 allow individual cell boundaries and cell nuclei to be imaged clearly in time-lapse recordings. Thus, karyokinesis, cytokinesis, and cell rearrangement can be followed in great detail throughout gastrulation, neurulation and organ rudiment formation (**Figs. 1**, **2**, and **3**).

1.3.2. Bodipy-Ceramide

Bodipy-ceramide {Bodipy FL C_5-Cer/C_5-DMB-Cer [N-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-pentanoyl) sphingosine]; mol wt 631} is a fluorescent sphingolipid that has been used for many years as a vital stain for the Golgi apparatus in cultured vertebrate tissue cells ***(4,6,7)***. When applied to intact zebrafish embryo, Bodipy-ceramide stains the plasma membrane, Golgi apparatus and cytoplasmic particles within the superficial envel-

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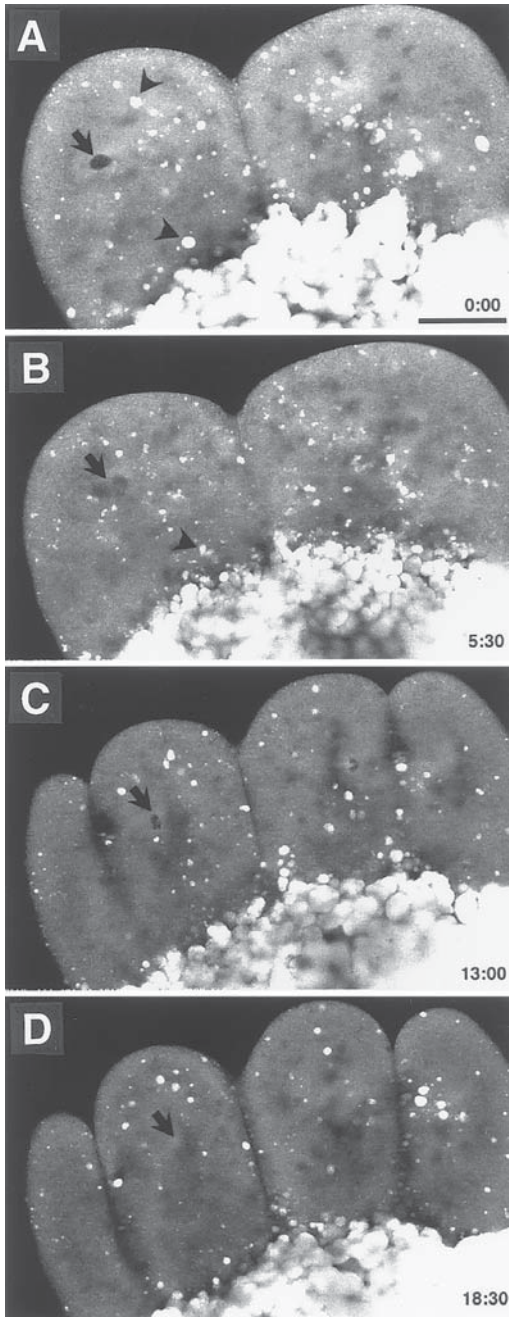
Fig. 1. *(facing page)* Cytoplasmic and nuclear dynamics during early cleavage in a zebrafish embryo, vitally stained with Bodipy 505/515. Shown is a confocal time-series through an animal–vegetal plane of an early cleavage (four-cell) stage zebrafish embryo. Bodipy 505/515 preferentially binds to yolk platelets and yolky cytoplasm, leaving nucleoplasm and interstitial space devoid of the fluorophore. The animal pole is toward the top of the image. Scale bar = 100 μm. **(A)** An interphase cell nucleus (**arrow**) of a specific blastomere is visible in this optical section. Large yolk platelets located in the cytoplasm of the cells are brightly labeled (e.g., **arrowhead**). **(B)** Nuclear membranes break down as blastomeres synchronously enter mitosis. An anaphase spindle is visible (**arrow**). At the onset of prophase, a mass of yolk platelets fragment from the surface of the yolk cell and pass via cytoplasmic bridges into the base of uncellularized blastomeres. Yolk platelets located in the cytoplasm of the cells fragment at the same time (e.g., **arrowhead**). **(C)** Nuclear membranes reform (e.g., **arrow**) as the dividing blastomeres enter telophase. The previously fragmented yolk



platelets begin to refuse**. (D)** The cleavage furrow between the daughter cells is now complete. Nuclear membranes of the blastomeres have broken down in preparation for another round of cell division.

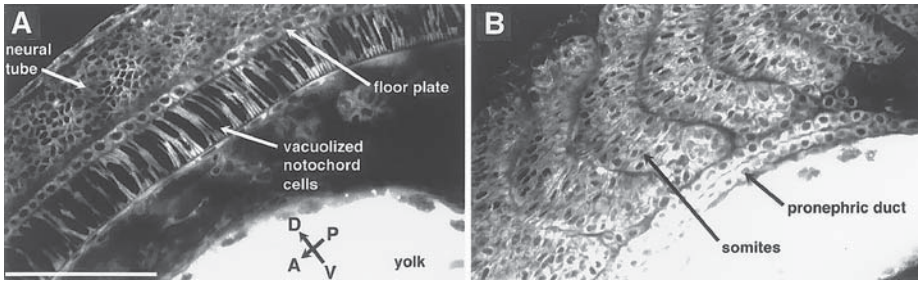


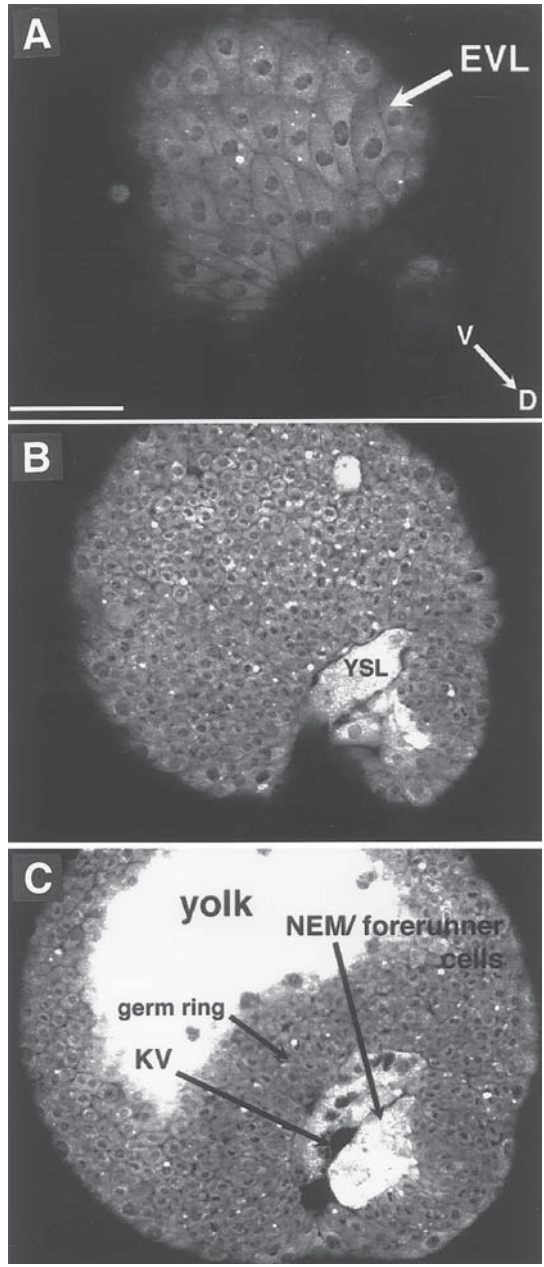
Fig. 2. Cellular detail of organ rudiments in the trunk region of a 24-h zebrafish embryo vitally stained with Bodipy 505/515. **(A)** Sagital sections through the median plane and **(B)** a lateral plane, respectively. Dorsal–ventral (D–V) and anterior–posterior (A–P) axes are shown. The embryo was imaged with a 40x/1.3 NA oil objective. Scale bar = 100 μm.

oping layer (EVL) cells of the embryos. However, once the fluorescent lipid percolates through the enveloping layer epithelium, the fluorescent lipid remains localized within the interstitial fluid of the embryo (hereafter referred to as the interstitum) and freely diffuses between cells (**Fig. 4**).

The insertion of large numbers of fluorophores into the interstitum allow hundreds of cells to be imaged simultaneously, as well as large-scale tissue movements to be observed. Because Bodipy-ceramide is able to diffuse through the interstitum, photobleached molecules are quickly exchanged with unbleached molecules, thus replenishing fluorescence in the scanned field of view. The large reservoir of mobile fluorescent lipid present in the embryo's segmentation cavity (part of the interstitum) allows single-level or multilevel time-lapse recordings to be acquired over extended periods of time (up to 10 h).

Interestingly, there is very little partitioning of Bodipy-ceramide into deep cells. In particular, the Golgi apparatus in deep cells remains essentially devoid of the lipid probe. To explain the lack of permeation of Bodipy-ceramide inside the embryo, we hypothesize that there may be high density of lipid-carrier proteins within the interstitum that can bind endogenous lipoproteins, as well as exogenously inserted fluorescent probe lipids. Binding of Bodipy-ceramide to mobile, impermeant lipid-carrier proteins could thus account for the apparent

Fig. 3. *(facing page)* Optical sections of the vegetal pole of a 100% epiboly stage zebrafish embryo showing the position of the forerunner cells in relation to the germ ring and developing body axis. The embryo was double-stained with SYTO-11 and Bodipy 505/515 at 50% epiboly. Scale bar = 100 μm. **(A)** Surface view showing Bodipy-labeled EVL cells. **(B)** A deeper plane of focus (15 μm) into the embryo reveals deep cells and the yolk syncytial layer (YSL). **(C)** 45 μm deeper into the embryo.



Forerunner cells, brightly labeled with SYTO-11, as well as the germ ring are prominent features. Kupffer's vesicle (KV) is forming between the YSL and the NEM/forerunner cells. The NEM/forerunner cell cluster is located at the posterior limit of the embryo's dorsal midline.

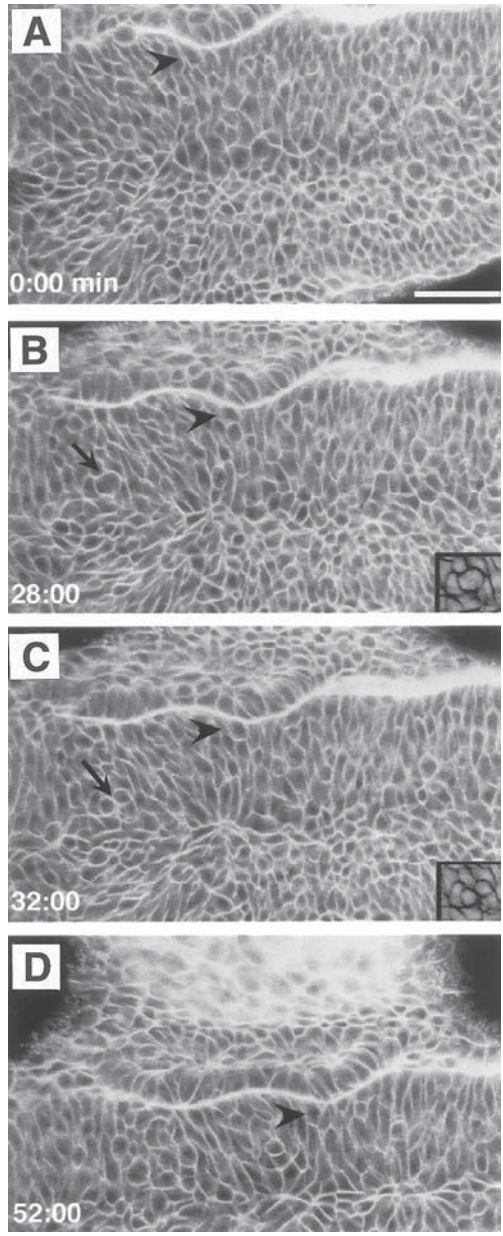


Fig. 4. Patterns of cellular convergence and infolding in the neuroepithelium of the hindbrain region of a five-somite stage (11.6-h) embryo. The embryos has been vitally stained with Bodipy-ceramide. Bodipy-ceramide molecules remain localized to the interstitial space of the embryo, outlining the boundaries of deep cells. Anterior is to the left. Scale bar = 50 μm. **(A)** Lateral folds of the neural plate converge toward the

change in permeation characteristics of Bodipy-ceramide molecules after they enter the embryo's interstitum.

1.3.3. SYTO-11

During the late-blastula stage, a unique group of cells with increased endocytic activity appears at the presumptive dorsal margin (organizer region) of the zebrafish blastoderm ***(3)***. The longitudinal position of this cellular domain in late blastula stage embryos (30–40% epiboly) accurately predicts the site of embryonic shield formation at the onset of gastrulation (50% epiboly) (**Fig. 5**). Unlike other blastomeres around the circumference of the blastoderm, these marginal cells do not involute during germ ring formation or blastoderm epiboly. Instead, this group of noninvoluting endocytic marginal (NEM) cells remains at the border where the dorsal EVL and dorsal yolk syncytial layer (YSL) come into contact (**Fig. 4**). During mid- to late epiboly, deep cells within the NEM cell cluster move to the leading edge of the dorsal blastoderm. At this point, these cells are referred to as "forerunner cells" ***(3,8)***.

NEM/forerunner cells can be easily visualized in late blastula to late gastrula stage embryos because they exhibit accelerated endocytic activity and can be selectively labeled by applying membrane-impermeant fluorescent probes, such as SYTO-11 (methane, sulfinylbis; ~400 mol wt), to pre-epiboly and early epiboly embryos ***(3)***. Because this particular vital labeling method can identify the location of the organizer region in late blastula stage embryo, it is potentially useful for experimental studies of dorsal cell fate specification and embryonic axis formation.

[Because SYTO-11 is internalized into zebrafish embryos by endocytosis ***(3)***, as opposed to passive permeation, it is more appropriate to refer to SYTO-11 as a vital label, rather than a vital stain].

2. Materials

1. Bodipy 505/515, Bodipy-C_5-ceramide, or SYTO-11 (Molecular Probes, Eugene, OR).
2. Anhydrous DMSO.

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neural midline. An arrowhead points to a cell at the posterior margin of the fifth rhombomere. The cell remains at this marginal location during convergence and infolding (A–D). Rounded cells (e.g., arrow) within the neural plate are preparing to divide. **(B,C)** A rounded cell in the neural plate undergoes mitosis (**arrow**). The division plane is at 45° with respect to the embryonic axis. **(D)** The right otic vesicle of the embryo is centrally positioned at the lateral margin of the fourth and sixth rhombomeres of the developing hindbrain. The cell at the lateral margin of the neural fold (marked by the **arrowhead**) has converged further toward the neural midline. Note that the neural midline has moved toward the lower portion of the field of view. This is due to a torsional rotation of the embryo.

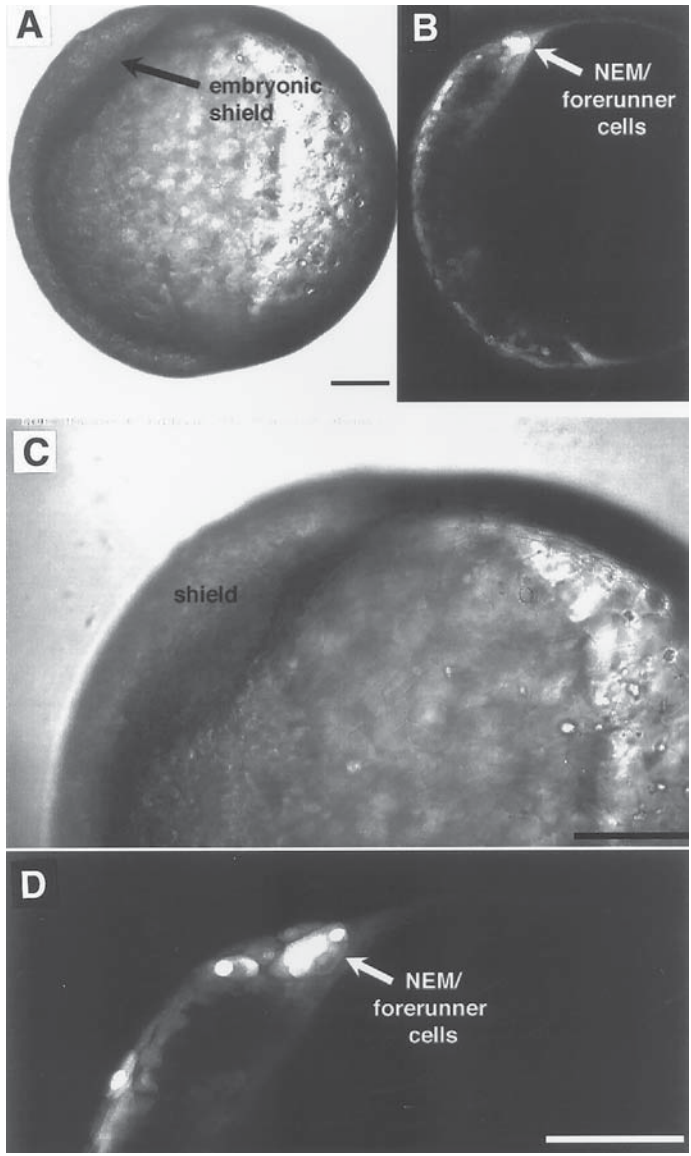

Fig. 5. The position of NEM/forerunner cells (labeled with SYTO-11) in early gastrula stage embryos correlates with the site of embryonic shield formation. Scale bars = 100 μm. **(A,B)** A Nomarski and confocal image pair of an early gastrula stage zebrafish embryo showing that the brightly labeled NEM/forerunner cell cluster is located at the leading edge of the dorsal marginal zone (DMZ). **(C,D)** Higher magnification of the DMZ and the NEM/forerunner cell cluster.

3. 35-mm Plastic culture dishes and/or 1.5-mL microfuge tubes.
4. Drierite.
5. Embryo Rearing Medium (ERM), buffered with HEPES.
6. Fire-polished Pasteur pipets (with bulbs) for transferring embryos.
7. 10- or 20-mL micropipets (VWR Scientific) for molding agarose.
8. Hairloop mounted on a Pasteur pipet.
9. Agarose.
10. Dechorinated zebrafish embryos [see *The Zebrafish Book* (Westerfield, M., ed.)], for manual and enzymatic methods. These methods can also be accessed on the WWW at the Fish Net Website: http://zfish.uoregon.edu/zf_info/zfbook/zfbk.html).
11. Embryonic Rearing Medium for Applying Vital Stains ***(9)***: 1.0 mL of Hank's Stock no. 1, 0.1 mL of Hank's Stock no. 2, 1.0 mL of Hank's Stock no. 4, 95.9 mL of double-distilled (dd) H_2O, 1.0 mL of Hank's Stock no. 5, 1.0 mL of fresh Hank's Stock no. 6. Add 0.24 g of HEPES [N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)] to obtain a pH buffer capacity of 10 m*M* in the final solution (modification of original recipe).
    Use 1*M* NaOH to pH 7.2.
    Hank's Stock no. 1: 8.0 g of NaCl, 0.4 g of KCl, in 100 mL of dd H_2O.
    Hank's Stock no. 2: 0.358 g of Na_2HPO_4 anhydrous, 0.60 g of $K_2H_2PO_4$, in 100 mL of dd H_2O.
    Stock no. 4: 0.72 g of $CaCl_2$ in 50 mL of dd H_2O.
    Stock no. 5: 1.23 g of $MgSO_4 \cdot 7H_2O$, in 50 mL dd H_2O.
    Stock no. 6: 0.35 g of $NaHCO_3$, 10.0 mL of dd H_2O.

3. Methods

3.1. Vital Staining

As a general procedure, zebrafish embryos are placed in a vital staining solution for 30 min, then washed 3x with HEPES-buffered ERM, in order to remove excess or unbound fluorescent probe molecules. The intensity of staining can be varied by changing the duration of staining. Detailed steps for this procedure are given below for vitally staining embryos with Bodipy 505/515. (This same procedure, with slight modifications, is used for vital staining with Bodipy-ceramide or SYTO-11; *see* **Note 5**).

1. A stock solution of the unconjugated fluorophore Bodipy 505/515 (4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3*a*,4*a*-diaza-s-indacene) was made by dissolving the fluorescent dye in anhydrous DMSO to a stock solution concentration of 5 m*M* (*see* **Note 1**). Aliquots (20-µL) of the stock solution are placed into microfuge tubes and stored in a light-tight container with Drierite at –20°C. Bodipy 505/515 aliquots are usually stable for up to 6 mo.
2. Preparation of chambers for vital staining. All labeling chambers should be covered with agarose to prevent the dechorionated embryos from sticking to plastic

surfaces. Molded agarose chambers can also be created to minimize the volume of staining solution needed to vitally stain a group of embryos. For very small volumes (50–100 µL) of labeling solution, a labeling chamber can be constructed from the detached cap of a 1.5-mL microfuge tube. Molten agarose can be dropped into the cap using a Pasteur pipet until the agarose completely covers the bottom of the cap.

3. Transfer Pasteur pipets for moving embryos between solutions are made by removing the tip, as well as most of the shank, of the glass pipet (a diamond scribe is useful for this procedure). The remaining 3–5 mm diameter opening is fire-polished using an alcohol flame. When moving embryos between solutions, avoid having the embryos contact the air–water interface at the mouth of the transfer pipette. This is done by tilting the transfer Pasteur pipet to a more horizontal position once embryos have been drawn into the pipet.
4. A hairloop Pasteur pipet for moving and positioning embryos is made by first heating the middle shank of a Pasteur pipet in an alcohol flame. As the glass softens, the pipet is drawn out of the flame. Once the shank is out of the flame, the two ends of the pipet are then quickly pulled in opposite directions, thus stretching and narrowing the middle shank. The middle shank is then broken off. The two ends of a human hair (5–7 cm long) are placed into the tapered pipet and pushed inward until a 0.5 cm diameter loop of hair remains outside. A dab of molten paraffin applied to the pipet tip will secure the hairloop in place.
5. Thaw an aliquot of Bodipy 505/515. The dye is then diluted into HEPES-buffered ERM to a labeling concentration of 100 µ*M*. The final concentration (v/v) of DMSO in the labeling solution is 2%.
6. Embryos are transferred to a labeling chamber and stained with a 100 µ*M* Bodipy 505/515 staining solution for 30 min (*see* **Note 2**). During vital staining, the solution in the labeling chamber should be circulated by gentle swirling every 10 min (*see* **Note 3**). The yolk cell of each embryo will become visibly green if the vital staining is working (*see* **Note 4**). As a precaution during the staining procedure, we recommend that the staining chamber be covered with aluminum foil, to keep vitally stained embryos from being exposed to excess light. However, we have found that exposure to room light for up to 30 min does not harm or overly photobleach vitally stained embryos (*see* **Note 5**).
7. After vital-staining, the embryos are passed through three successive washes of HEPES-buffered ERM, using a fire-polished transfer Pasteur pipet. These washes are performed in separate agarose-coated 35-mm culture dishes. After washing, the embryos are ready to be mounted for observation under the confocal microscope (*see* **Notes 6** and **7**).

3.2. Mounting Embryos for Imaging

3.2.1. Imaging Chambers

Confocal time-lapse recordings of zebrafish embryos require that the vibration of the specimen be minimized. Live embryos are most easily imaged using an inverted microscope mounted on an airtable, because gravity pulls the

embryo toward the coverslip and helps maintain its position with respect to the objective. A useful chamber to image teleost embryos is a 0.7 cm thick piece of plexiglass with a 24 mm diameter hole in its center. A 30 mm diameter circular glass coverslip (no. 1 thickness) can be secured with high-vacuum silicone grease (Dow Corning, Midland, MI) to serve as the bottom of the bath well. During time-lapse recordings, a plastic culture dish lid can be placed over the well to prevent air currents from producing displacements of the culture medium and unsecured embryos.

3.2.2. Stabilizing the Spatial Orientation of Embryos

To prevent rolling from occurring during extended time-lapse recordings, we have found it useful to place fluorescently labeled embryos in an agarose holding well made from ERM plus agarose (Type IX) (Sigma, St. Louis, MO) (modified from the procedure in Westerfield, 1995) (*see* **Notes 8** and **9**).

1. Apply a thin coat of molten solution of 1.2% agarose in ERM to the coverslip. Let the agarose cool and harden to form a thin layer of agarose gel on the coverslip.
2. To make holding wells in the agarose layer, start with a 10- or 20-µL glass micropipet in which the end has been pulled out using an alcohol flame. After breaking off the tip of the pipet, heat the remaining end until it melts into a small bead of glass, approximately 0.8 mm in diameter. Use the rest of the pipette as a handle. After heating this glass bead/ball with the alcohol flame, quickly plunge it into the agarose to melt a hole. The holes should be small enough so that the embryo will not be able to roll, but large enough so that epiboly of the blastoderm is not impeded. Because an embryo will be secured inside this hemispherical-shaped hole, the hole should be as round and smooth as possible.
3. Use room temperature ERM or water to wash out any melted agarose so that it does not refill the holes.
4. Repeat this procedure to create multiple holes in the agarose layer. You may have to make a new glass bead, as repeated heating will cause the bead to increase in size as more glass melts.
5. After the agarose wells are made, add ERM buffer to cover the agarose hole and then add the embryos. Gently position the embryos into the agarose holes using a nonsticky implement, such as a hairloop mounted on the end of a Pasteur pipet with a dab of molten paraffin.
6. Carefully position the embryo so that the area of interest is facing toward the objective lens. This operation is most easily accomplished on an inverted microscope with an open holding chamber. However, repositioning the embryos once they are in the wells is difficult and must be done with care. It is useful to secure 5–10 embryos in a given agarose sheet in preparation for a time-lapse recording. This increases the likelihood that a well-stained embryo, in an appropriate orientation, can be located for imaging (*see* **Note 8**).

3.3. Imaging Procedures

3.3.1. Selection of Optics

The choice of objective for imaging zebrafish embryos is determined by several considerations: (1) numerical aperture (NA); (2) working distance; and (3) magnification. The NA must be large enough to adequately collect fluorescent light from the specimen. In addition, the working distance of the objective lens must be great enough to reach the desired plane of focus within the embryo. To examine cell behaviors in zebrafish embryos, we have found a versatile set of objective lenses to be: (1) a dry 20x/0.75 NA; (2) a dry 40x/0.85 NA; (3) an oil 40x/1.3 NA. A 10x/0.5 NA objective is also useful for locating the organizer region (dorsal marginal zone) of zebrafish embryos labeled with SYTO-11.

Appropriate filter sets for the confocal microscope are determined from the excitation and emission spectra of the fluorophores. In **Figs. 1–5**, embryos stained either with Bodipy 505/515, Bodipy-ceramide or SYTO-11 were imaged using an excitation wavelength of 488 nm. Fluorescent light collected from the specimen was filtered by a 515 nm long-pass filter before it was transmitted to the confocal microscope's photomultiplier tube.

3.3.2. Illumination Intensity, Optimization of Gain, and Offset

To generate large numbers of photons for high-quality confocal images with single scans, it is necessary to illuminate the embryo with moderately intense laser light. To obtain optimum contrast for time-lapse recordings, system gain should be increased until saturation begins to occur. At this point, offset is added to bring these pixels below a value of pure white (i.e., 255 on a 0-255 grayscale). A "pleasing image" generally contains a spectrum of gray values that span the entire grayscale. With offset (or black level) adjustments, the background should set at the equivalent of a slightly negative grayscale value. With this offset adjustment, the background will remain pure black throughout the recording. When imaging a zebrafish embryo with Bodipy 505/515, the yolk cell will often be extremely bright (*see* **Note 10**). To achieve optimum contrast of blastomeres, the highly fluorescent yolk platelets in the yolk cell will appear pure white (i.e., fully-saturated on the video monitor).

3.3.3. Parameters for Time-Lapse Recordings

Time-lapse recordings of developing zebrafish embryos are best made from single slow scans of the specimen, as opposed to time-averaged images (e.g., Kalman averages). Because the scanning laser beam of the confocal microscope passes rapidly over the sample, a stroboscopic illumination of the specimen is produced. This greatly reduces the motional blurring of fluorescent objects that are being displaced within cells by active transport or by diffusion

(11). At a slow scan rate, enough photons are collected in a single pass over the specimen to generate adequate signal-to-noise level in the image. By adjusting neutral density filters, it is useful to use as intense laser light as possible without producing substantial photobleaching in the specimen over a desired time-lapse interval. For an embryo labeled with Bodipy 505/515 or Bodipy ceramide, 100–300 frames can be generally recorded with a 300–700-µm field of view over a 1–10-h time period. A minimum of 50 frames is usually needed to determine the cellular dynamics that comprise a morphogenetic event (*see* **Note 11**).

3.3.4. Single-Level Time-Lapse Recordings

Most confocal microscopes now have internal macros for obtaining and transferring single-level and multilevel time-lapse recordings to their host computer's hard disk. Although a computer hard disk is a fast and convenient means for immediate data storage, an excellent high-capacity, random-access storage medium for time-lapse recordings is an optical memory disk recorder (OMDR).

A QuickBasic program and a Bio-Rad macro necessary to activate a Panasonic OMDR in time-lapse form have been published ***(12)***. This program, called "OMDR," is compiled into an executable (.exe) file using Microsoft QuickBasic.

```
OMDR
open "com2:2400,n,2,1" for random as #1
com(2) on
print #1, chr$(2) + command$ + chr$(3)
close
```

This function can then be adapted into a macro for the collection of Z-series to an OMDR.

The following macro or "command file" (.cmd) for the Bio-Rad confocal microscope can be used to make a time-lapse recording with a Panasonic OMDR via a serial port connection.

```
TLAPSE.CMD
for i = 1, %2
wait %1
clear
collect 1
$omdr gs
next i
```

Before the above program initiates scanning and recording, the program queries the user for two external inputs, %1 and %2. %1 is the number of seconds between the collection of images, whereas %2 is the number of images to be

collected. The "gs" command (after $omdr) signals the Panasonic OMDR to record a single video frame using the OMDR.exe file.

3.3.5. Multilevel Time-Lapse Recordings

If desired, algorithms for multilevel time-lapse recordings can also be implemented using a stepping-motor coupled to the focusing apparatus of the confocal microscope. The following program has been modified slightly from that published previously ***(12)***.

```
MLTLAPS.CMD
echo -
$cls
$omdr on
box size 1 hor
clear
print MACRO FOR A MULTILEVEL TIME-LAPSE RECORDING
print
input reps, NUMBER OF MULTILEVEL SAMPLES (Z-SERIES)
input sects, NUMBER OF SECTIONS PER Z-SERIES
input lapse, TIME INTERVAL BETWEEN MULTILEVEL SAMPLES (Z-SERIES)
input vstep, VERTICAL STEP SIZE, (number of motor steps)
input rewind, MOTOR REWIND AMOUNT, (number of motor steps)
print
motor off on
for k = 1, #reps
motor inc #vstep
for i = 1, #sects
clear
print COLLECTING IMAGE #i OF MULTILEVEL SAMPLE #k
collect
$omdr gs
motor step
next i
wait 0
wait #lapse
motor inc #rewind
motor step
next k
motor off
echo +
```

3.3.6. Analysis of Time-Lapse Recordings

Many optical memory disk recorders (OMDRs) allow a 100-fold range of speeds over which the time-lapse recording can be sped up or slowed down.

Because the visual perception of human observers is limited in bandwidth, this flexibility in OMDR playback rates greatly facilitates data-to-brain coupling. To trace the trajectory of individual cells, or to digitize individual OMDR images (analog video), the video output of the OMDR can be routed to the input of a video frame buffer. A variety of software programs can then be used for either digitizing analog OMDR images or performing morphometric/kinematic analyses of cells in the time-lapse recording.

Saving confocal time-lapse images as digital files is the best procedure to preserve their spatial resolution. However, one must load these digital image files into a suitable program for viewing on a computer. NIH Image is a useful program, as it will import Bio-Rad .PIC files using a specific macro (NIH Image and Bio-Rad macros can be downloaded from the following URL: http://rsb.info.nih.gov/nih-image/more-docs/docs.html). One can load a Z-series and scan through the data set using arrow keys. If desired the scan-through can be animated and saved.

Finally, acquired confocal time-lapse recordings can be compressed into digital movie files in either a QuickTime or a MPEG format for distribution over the Internet. Examples of such compressed time-lapse recordings from our laboratory, showing zebrafish embryos stained with Bodipy 505/515 and Bodipy-ceramide, are located on the WWW at the following URL: http://weber.u.washington.edu/~fishscop/.

4. Notes

1. Bodipy 505/515 will appear yellow-green when solvated in DMSO. Once the DMSO solution is dispersed into aqueous ERM, the color of Bodipy 505/515 often changes to orange-red. This solution is appropriate for vital staining, unless large particles of Bodipy 505/515 start to precipitate out of solution. If this occurs, one should remake a new Bodipy 505/515 stock solution using anhydrous DMSO. Because DMSO is hydroscopic, it is possible that Bodipy 505/515 aliquots may accumulate H_2O in the –20°C freezer over time, causing the DMSO in the Bodipy 505/515 aliquot to lose its solvating activity.
2. The yolk cell of zebrafish embryos becomes visibly green within several minutes after the embryo is placed in an aqueous labeling solution containing 100 μ*M* Bodipy 505/515 and 2% DMSO. The yolk cell continues to accumulate dye as the embryo remains in the labeling solution, and becomes intensely green after 30 min of staining. To the naked eye, the blastoderm of the Bodipy-labeled embryo appears colorless. However, when these embryos are examined under the scanning laser confocal microscope, the embryo's blastomeres are well labeled with Bodipy 505/515 (**Fig. 1**). In young embryos (< 24 h), Bodipy 505/515 is retained within the yolk cell and the yolk-containing cytoplasm of cells.
3. In primordia-stage embryos, as in younger embryos, Bodipy 505/515 does not stain the nucleoplasm of cells. The dye is also absent from fluid-filled organelles,

such as vacuoles within the extending notochord. Initially, Bodipy 505/515 is absent from both cerebral-spinal fluid and the bloodstream. However, in later stage embryos (> 24 h), Bodipy 505/515 becomes highly concentrated in the blood and cerebrospinal fluid (data not shown). We speculate that this change in staining pattern results from Bodipy 505/515 remaining bound to lipoproteins as they are exported from the yolk cell into the bodily fluids of the embryo.

4. Another small, neutral fluorophore, Bodipy 564/591, represents an alternative fluorescent vital stain for yolky cytoplasm in zebrafish embryos ***(3)***. Because Bodipy 564/591 can be excited with the yellow 568 nm line of the argon/krypton laser, this fluorophore can be used for dual-labeling purposes with other vital stains, such as SYTO-11 (*see* **Fig. 3** and **Note 4.6**). Bodipy 564/591 can be obtained from Molecular Probes (Eugene, OR) on a custom synthesis basis. Embryos can be labeled with Bodipy 564/591 using the same procedure as Bodipy 505/515.
5. Embryos can be vitally stained with either Bodipy-ceramide or SYTO-11 using the same procedure used for staining with Bodipy 505/515. Recommended labeling concentrations (and labeling times) for Bodipy-ceramide and SYTO-11 are 100 μ*M* (30 min), and 75 μ*M* (15 min), respectively.
6. Costaining with Bodipy fluorophores. Using a sequential application of the standard vital staining procedure (*see* **Fig. 3**), embryos can be first vitally stained with Bodipy 505/515, and then vitally-labeled with SYTO-11. Dual-staining with these two fluorescent probes allows NEM/forerunner cells to be observed along with all other blastomeres. Cell nuclei and endosomes labeled with SYTO-11, or cellular cytoplasm labeled with Bodipy 564/591, can be viewed independently using 488 nm and 568 nm excitation wavelengths, respectively. When desired, both fluorophores can be viewed simultaneously using dual-wavelength excitation (488 nm and 567 nm) and 585 nm long-pass emission.
7. Bodipy 505/515, Bodipy 564/591, Bodipy-ceramide, and SYTO-11 do not appear to have any teratogenic effects on developing zebrafish embryos. Normal somitogenesis and neurulation proceed on schedule. Hatched fry do not exhibit any noticeable morphological malformations or behavioral abnormalities.
8. pH stabilization: One of the most important experimental variables to control in making time-lapse recordings is the pH of the embryonic or tissue culture medium. Bicarbonate-containing media, in particular, are subject to extreme changes in pH with temperature, as carbon dioxide exchange with the atmosphere alters the carbonic acid/bicarbonate equilibrium of the medium. CO_2, pH, and HCO_3^- are interrelated through the following equilibrium:

$$CO_2 + H_2O = H_2CO_3 = H^+ + HCO_3^- \qquad \text{(Eq. 2)}$$

Atmospheric CO_2 tension will alter the concentration of dissolved CO_2 in a temperature-dependent fashion ***(10)***. Increased HCO_3^- concentration pushes the reaction to the left. Equilibrium will be reached only in conditions where atmospheric CO_2 is stable at a given temperature. In the absence of CO_2 and temperature control, it is necessary to add a buffering agent at twice the concentration of HCO_3^- to achieve pH stabilization of the experimental solution. Therefore, 10–

20 m*M* HEPES should be added to all experimental salines or media to stabilize their pH during extended time-lapse recordings.

9. Tricane (MS-222) Anesthesia: To prevent muscle twitching during time-lapse recordings, embryos at 20-somite stage or later can be anesthetized with 0.1 mg/mL of Tricaine (also known as MS-222; Sigma, St. Louis, MO) dissolved in ERM ***(9)***.
10. Light absorption in vitally stained embryos: Owing to absorption by superficial layers of fluorescently labeled cells, the excitation light is attenuated as it passes deeper into the embryo. This results in a progressive loss of fluorescent light emanating from deeper optical sections of embryos labeled with Bodipy 505/515. Darkness occurs because of light absorption by labeled cells and/or organ rudiments in the path of the scanning laser beam. However, because many of the cellular movements that produce body axis formation take place on the surface of the yolk cell, embryos can be usually rotated into an appropriate orientation for viewing an area of interest.
11. Compensations for photobleaching. The rate of photobleaching that occurs during a time-lapse recording is inherently linked to the choice of fluorophore, as well as laser illumination intensity. Even when using a fairly photostable fluorophore, such as Bodipy 505/515, it is inevitable that some photobleaching will take place over the duration of the recording. One can add extra gain at the beginning of a recording, anticipating that a certain rate of photobleaching will occur. Alternatively, one can gradually increase the imaging system's gain by hand during the recording. This helps keep the average black level of the image constant during the recording. The imaging tradeoff produced by this mode of compensation is that a gradual increase in pixel noise will occur during the recording, as the gain of the imaging system is steadily increased. It is sometimes useful to employ this mode of compensation when recording cellular dynamics with Bodipy 505/515. This mode of compensation for photobleaching is not usually necessary when using Bodipy-ceramide, as Bodipy-ceramide is able to diffuse through interstitial fluid and replenish fluorescent molecules in scanned regions where they have been bleached.

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