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TECHNIQUES

Imaging Brain Development and Organogenesis in Zebrafish Using Immobilized Embryonic Explants

Tobias Langenberg,[1] Michael Brand,[1] and Mark S. Cooper[2]*

Owing to its optical clarity and rapid rate of development, the zebrafish embryo is an ideal model system for studying the cellular mechanics of organogenesis. Unfortunately, extended time-lapse recordings of zebrafish embryos are often disrupted by the extension and straightening of the embryonic axis, as well as movement artifacts associated with developing musculature. In addition, the embryo's massive yolk cell often prevents optical access to tissues of interest. To circumvent these imaging problems, we have developed a procedure to deflate and mechanically remove the yolk cell. A "paralyzing" agent, AMP-PNP (a membrane-impermeant nonhydrolyzable analog of ATP), is first injected into the embryo's contractile yolk cell. The yolk cell is then removed using sharpened tungsten needles. Deyolked embryos, or organ rudiments explanted from them, are then immobilized on a microscope coverslip using a thin plasma clot. This plasma clot immobilization allows novel mountings of the explants so that ventral, lateral, and even cross-sectional fields of views are possible using high numerical aperture objectives. We show that isolated head rudiments undergo normal morphogenesis and gene expression for at least 1 day after being explanted into organotypic culture. These procedures can be used to study the cellular mechanics of organogenesis in "deyolked" embryos, as well as in tissues explanted from green fluorescent protein transgenic animals. *Developmental Dynamics 228:464–474, 2003.* © 2003 Wiley-Liss, Inc.

Key words: blastoderm; explant; organotypic culture; yolk cell; yolk syncytial layer; confocal imaging; GFP; transgenic fish; stem cells

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INTRODUCTION

The intact zebrafish embryo provides diverse opportunities to examine the cellular mechanics of embryonic morphogenesis. Its relatively small size, transparency, and exceedingly rapid rate of development make it ideal for in vivo studies. For nearly 20 years, morphogenetic movements of fluorescently labeled cells have been tracked in living zebrafish embryos by using various imaging strategies (Kimmel and Law, 1985a–c; Myers et al., 1986). With the advent of green fluorescent protein (GFP) technology and its combination with molecular genetics, there are abundant new reasons to visualize the dynamics of fluorescently labeled cells within the transparent zebrafish embryo.

Visualizing the behavior of labeled cells within intact zebrafish embryos, however, is not free from experimental difficulty. Extended time-lapse recordings of zebrafish embryos are often disrupted by spatial movements associated with the extension and straightening of the embryonic axis, as well as movement artifacts associated with developing musculature. Moreover, the embryo's massive yolk cell often prevents easy optical access to tissues of interest. One direct way of dealing with the above difficulties is to physically re-

The Supplementary Movie referred to in this article can be found at www.interscience.wiley.com/developmentaldynamics/suppmat/index.html.

[1]Max Planck Institute for Molecular Cell Biology and Genetics, and Department of Genetics, University of Dresden, Dresden, Germany
[2]Department of Biology, and Center for Developmental Biology, University of Washington, Seattle, Washington

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*Correspondence to: Dr. Mark S. Cooper, Department of Biology, Box 351800, University of Washington, Seattle, WA 98195-1800. E-mail: mscooper@u.washington.edu

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move the yolk cell and isolate tissues of interest, in the form of embryonic explants.

Embryonic explants have long provided unique opportunities for developmental biologists to examine the structure and behavior of embryonic cells within their native tissue environments. Nearly a century ago, cultured explants of embryonic tissues were first used to examine the outgrowth and motile behavior of individual neurons (Harrison, 1910, 1914). Since that time, explanted embryonic tissues have been widely used to examine patterning, morphogenesis (Schechtman, 1942; Trinkaus and Drake, 1956; Wilson et al., 1989; Wilson and Keller, 1991; O'Rourke et al., 1992, 1995) and the electrophysiology of developing tissues (Gähwiler, 1981, 1984a,b).

In this study, we will describe a new technique to remove the zebrafish yolk cell and to culture the "deyolked" embryo, or portions explanted from it. The procedure is based upon the results of several previous studies (Oppenheimer, 1936; Tung and Tung, 1944; Trinkaus and Drake, 1956; Bozhkova et al., 1994; Simon and Cooper, 1995; te Kronnie et al., 2000), with additional modifications for manipulating the zebrafish embryo. To facilitate microdissections of the zebrafish embryos, we first block Ca^{2+} triggered actomyosin contractility in the zebrafish yolk cell by using a "paralyzing" solution of AMP-PNP, a nonhydrolyzable analog of ATP. Once the yolk cell is paralyzed with AMP-PNP, yolk can be removed from the embryo's yolk cell by using tungsten microneedles. Afterward, the entire embryo, or tissues explanted from it, can be immobilized by using either agarose or plasma clot, and maintained in organotypic culture. We document the functionality of this explant methodology by showing that complex structures of the zebrafish brain develop normally in immobilized explant culture. We also demonstrate that immobilized explants offer improved opportunities to image histologic structures in tissues extirpated from GFP transgenic zebrafish embryos.

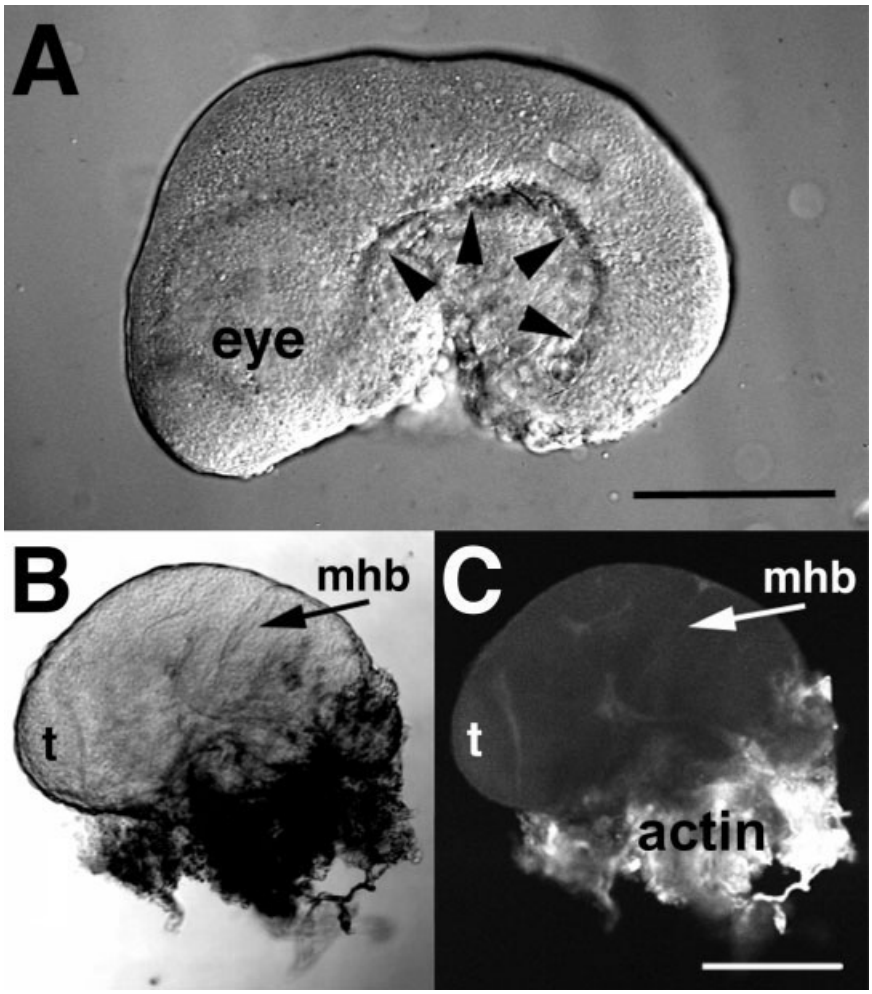

Fig. 1. An explanted head rudiment (isolated without AMP-PNP injection into the yolk cell) curls into a compact mass because of yolk cell contractility. **A:** Head explant of a 24 hr embryo shortly after explantation. Arrowheads delineate a remnant of the yolk cell's actomyosin-filled cortex. **B,C:** Brightfield and epifluorescent confocal images of a similar explant stained with Bodipy-phallacidin. Cortical actin is brightly stained. The telencephalon (t) and midbrain–hindbrain boundary (mhb) are marked for reference. Scale bars = 100 microns in A, in C (applies to B,C).

RESULTS

AMP-PNP Blocks Curling of Explants

The zebrafish yolk cell, like the yolk cell of other teleostean fish embryos (Fink and Trinkaus, 1988), possesses a strongly contractile cortical cytoplasm (Cooper and Kimmel, 1998). This cortical cytoplasm contains a dense actomyosin network (Fig. 1) that becomes activated in response to an elevated Ca^{2+}. Wounding of the yolk cell results in a rapid influx of extracellular Ca^{2+}, which quickly activates a massive contractile response of the cortical cytoskeleton (Fink and Trinkaus, 1988). When trying to mechanically remove the zebrafish yolk cell, this Ca^{2+}-induced contractility results in a pronounced curling of the embryo. This curling of the body tissues not only impedes imaging of tissue structure and dynamics, it also distorts subsequent morphogenesis of tissue explants (Fig. 1). To block Ca^{2+}-induced contractility in the yolk cell, we have developed a procedure that uses a membrane-impermeant inhibitor of ATPase activity, AMP-PNP (adenosine 5′ (β, γ-imido) triphosphate). This nonhydrolyzable membrane-impermeant analog of ATP blocks myosin ATPase activity and, thus, "paralyzes" the contraction of cortical actomyosin networks within the yolk cell.

After yolk cell contractility has

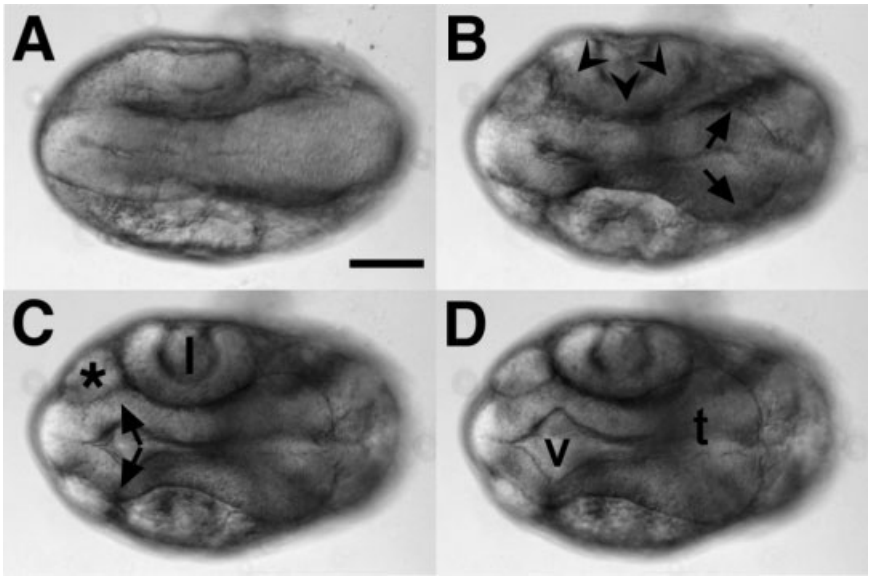

Fig. 2. Stills taken from a movie of a developing head explant. **A:** Explant was isolated (without AMP-PNP injection into the yolk cell) and quickly mounted in low-melting point agarose at room temperature at the 18-somite stage. Dorsal view, anterior is to the left. **B–D:** Explant after 7 hr, 13 hr, and 20 hr of development, respectively. **B:** Optic cup formation (arrowheads) and tectum growth and folding (arrows) are clearly visible. **C:** The lens (l) has formed, as well as the olfactory bulbs (asterisk marks one). Formation of the third ventricle is ongoing (arrows). **D:** The third ventricle (v) has formed, as well as the prominent tectal halves (t). Scale bar = 100 microns in A (applies to A–D).

been paralyzed, yolk can be easily removed from the yolk cell, without inducing a massive curling of the embryonic axis. Hereafter, we refer to removing yolk from the yolk cell as *deyolking* the embryo. Once the embryo has been deyolked, the embryo can be microdissected into small tissue explants for short-term or long-term organotypic culture. Deyolked embryos themselves can also be mounted for similar organotypic culturing.

Embryonic explants separated from yolk cells injected with AMP-PNP remain extended in their natural form. The explant can even be slightly flattened against its natural curvature when it is immobilized in agarose or on a plasma clot. This method provides a fast and effective means of preparing an embryonic explant and maintaining it for hours in culture without major distortions. The use of plasma allows flexible and even reversible mounting of explants in almost any desired position.

We find that blocking of curling by AMP-PNP injection is not absolutely necessary for very small explants, e.g., the head rudiment with a small portion of the trunk (i.e., the first few somites). In these cases, curvature can be overcome by gently pressing the explant down onto a plasma clot or glass surface (in the case of agarose immobilization). AMP-PNP does not seem to harm the embryos and explants, because morphogenesis proceeds normally (see Fig. 2).

Motional Stability of Immobilized Explants

By cutting away most of the embryo, the problem of motional instability is circumvented. The stickiness of the plasma clot or the gelled agarose prevents explanted tissue from floating or curling, allowing extended imaging without the complications of movement artifacts. Ventral-down mountings are easiest to achieve with the plasma clot, because the remaining yolk cell's enveloping layer (EVL)/epidermis will stick to the plasma. Both lateral- and dorsal-down mounts can be done by pushing the explant underneath the clot or by mounting the embryonic explant in low-melting point agarose.

Normal Morphogenesis in Cultured Explants

To test the viability of the explants, a systematic assay for morphogenetic changes was performed. Embryonic heads were placed into culture at the 18-somite stage (17 hours post-fertilization (hpf)) and incubated for 20 hr at 28°C (Fig. 2). The overall appearance of these explants was normal, tissue integrity was preserved, and the explants continued the normal developmental program. In all of these explants (n = 12), the following morphogenetic changes could be observed. The optic cup invaginated and lens formation took place. The neural tube folded at the level of the diencephalon and midbrain. This folding was accompanied by the typical growth of the midbrain tectum and the formation of the third ventricle in the diencephalon. Anterior to the telencephalon, the olfactory bulbs separated from the rest of the neuroepithelium. Of interest, 90% of the explants formed a ventrally located, beating heart during overnight culture (data not shown).

It should be mentioned that some alterations from a normal wild-type development are apparent. In most cases, the development of the explants seems to be slowed down. Furthermore, pigmentation is either severely reduced or delayed. The explanted heads have a slightly compressed appearance, which can partly be attributed to the smaller size of the ventricles, especially the first two and the fourth, which are reduced by approximately one-third of their size. This compression is more pronounced when the explants are embedded in agarose.

Apoptosis is Normal in Cultured Explants

Figure 3 illustrates that the number of apoptotic cells are normal or only slightly elevated in the explants. To address cell death levels, we performed an acridine orange (AO) staining on explanted heads: Explants (n = 7) were made between the 16- and 18-somite stage (17–18 hpf) and incubated overnight at 28°C. The next morning, after 18 hr in

culture, the medium was exchanged with AO containing (2 μg/ml) L-15 medium and the explants were incubated for another 2 hr. After washing with medium, incorporated AO was detected under 488-nm excitation. Apart from the massive unspecific accumulation of AO in the remnant of the yolk cell, only a few cells were positive for acridine orange in the explants. The punctate staining pattern is comparable to that in a wild-type embryo of an equivalent stage (see Fig. 3) and to the results of a recent study of apoptosis in whole embryos (Cole and Ross, 2001).

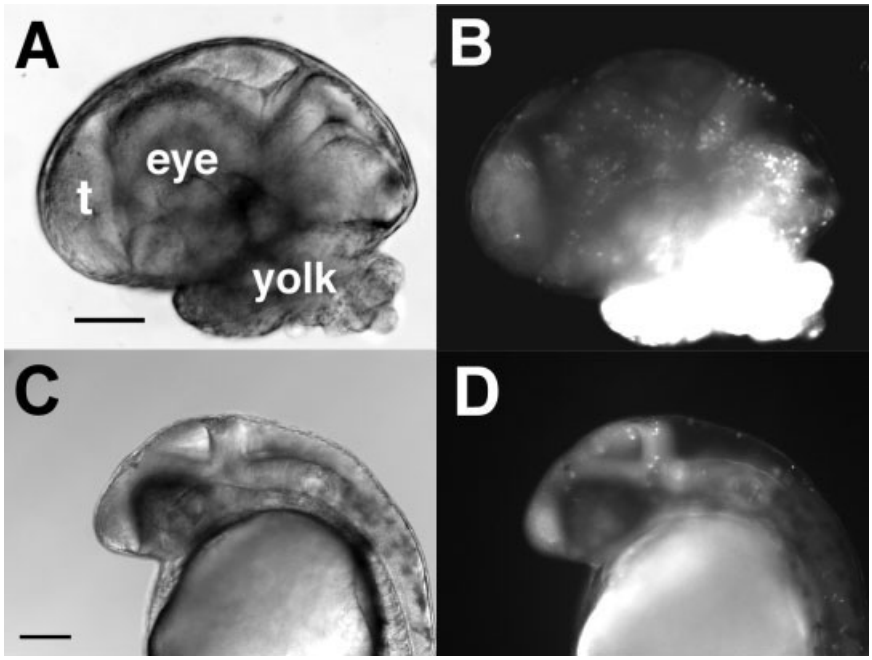

Fig. 3. Cell death levels are not abnormal in head explants. **A:** Differential interference contrast microscopy (Nomarski) image of an agarose-mounted head, explanted at the 16-somite stage, after 20 hr of incubation. **B:** The same head explant under 488-nm epifluorescence excitation, showing acridine orange (AO) incorporation after 2 hr of AO treatment. The remnant yolk has accumulated a high amount of AO, whereas the head tissue only shows slightly elevated apoptotic cell death. (The head explant was isolated without AMP-PNP injection into the yolk cell.) Compare with C,D. Wild-type embryos at 24 hpf were incubated for 2 hr in AO containing medium. At 26 hpf, patches of AO-positive cells can be detected throughout the head. Punctate AO staining is indicative of dead or dying cells. Diffuse AO staining in the telencephalon appears to be nonspecific and is present in the telencephalon of both explants and intact embryos. **C:** Brightfield image. **D:** Epifluorescent images of the same embryo. Scale bars = 100 microns in A (applies to A,B), in C (applies to C,D).

Normal Gene Expression in Explanted Head Rudiments

To address whether explants continue to express important regulatory and patterning genes, we took explants at 18 hpf, incubated them for 20 hr, and stained them for *fgf8* and sonic hedgehog (*shh*) expression by in situ hybridisation. Figure 4 shows that both genes are expressed in all their endogenous expression domains. For fgf8, in situ signal is detectable in the midbrain–hindbrain boundary organizer, the optic stalk, the epiphysis, and in the anterior neural plate. *Shh* can be detected in the floor plate, the hypothalamus, and the zona limitans intrathalamica (ZLI). These patterns of gene expression are similar to those in intact embryos (see Krauss et al., 1993; Reifers et al., 1998). *Shh* expression in the ZLI normally appears around 24 hpf. Thus, *shh* expression occurred after explantation of the head rudiment.

Ventral, Lateral, and Cross-Sectional Imaging of Embryonic Explants

The zebrafish's massive yolk cell makes it very difficult to image ventral organ rudiments in the developing embryo. The bulging yolk cell also reduces working distance when mounting the embryo lateral-side down. Explanting parts of the embryo allows almost any view. High numerical aperture objectives (e.g., a ×40 oil immersion objective with a working distance of less than 100 microns) can be used, because the tissue of interest can be mounted close to the coverslip.

Figure 5 shows a lateral view of the body axis of a deyolked zebrafish embryo vitally stained with Bodipy 505/515. The embryo was immobilized underneath a plasma clot in a manner shown in Figure 6. The vitally stained embryo was then imaged with a confocal microscope across its entire lateral side. The images were photomontaged (Beck et al., 2000) into a composite image, maintaining the high resolution of the individual confocal photomicrographs. In Figure 7, the same photomontaging approach was used to image GFP expression in a flattened explanted head rudiment, isolated from a transgenic zebrafish embryo (Picker et al., 2002).

Starting with deyolked embryos, it is possible to obtain ventral and cross-sectional views of fluorescently labeled zebrafish tissues (Fig. 8). These high-quality ventral and cross-sectional images would be nearly impossible to obtain in intact zebrafish embryos. Cross-sectional images were produced by transecting deyolked embryos by using a tungsten microneedle. The cut end of the embryo was then placed end-down against the plasma clot. The confocal microscope was then focused at a level away from the cut end of the specimen. Fluorescent light, which is transmitted through thick tissues (i.e., through the yolk cell or axial tissues), becomes scattered by cell organelles and cell–water interfaces, seriously degrading luminosity and spatial resolution. By reducing the amount of cell material between the objective and the desired plane of focus, high spatial resolution in the epifluorescence confocal image is preserved.

This principle is demonstrated in Figure 9, which shows neurite dy-

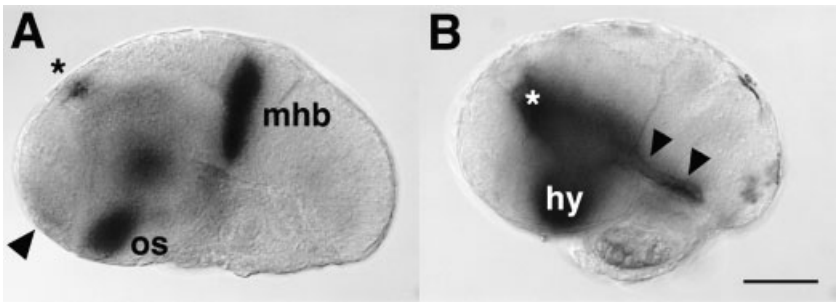

Fig. 4. Head explants stained for *fgf8* (A) and *shh* (B) expression. The head explants were isolated without AMP-PNP injection into the yolk cell. Several explants (n = 7 for *fgf8*, n = 6 for *shh*) were stained after 20-hr overnight incubation. **A:** In all explants, normal expression of *fgf8* was detected in the midbrain–hindbrain boundary (mhb), the optic stalk (os), the epiphysis (asterisk), and the anterior forebrain (arrowhead). **B:** *Shh* is expressed in the floor plate (arrowheads), the hypothalamus (hy), and the zona limitans intrathalamica (asterisk). Scale bar = 100 microns in B (applies to A,B).

namics in an explanted head of a GFP-islet1 transgenic zebrafish embryo (Higashijima et al., 2000). The head explant was partially flattened and immobilized underneath a plasma clot attached to a microscope coverslip. The explanted head was filmed from its ventral surface by using time-lapse confocal microscopy. The dynamics of fine neurite processes, as well as the onset of GFP fluorescence in certain trigeminal neurons, can be easily visualized in the immobilized head explant (see the Supplementary Movie, available at www.interscience.wiley.com/developmentaldynamics/suppmat/index.html.

DISCUSSION

Motional stability is critical for successful three- and four-dimensional imaging of live embryonic tissues. Cells and tissues of interest must be maintained within the field of view during the course of image acquisition, without having the specimen roll, translocate, vibrate, deform, or lose viability. In the past, several technical approaches have been developed to secure embryonic tissues for time-lapse imaging. Kimmel (1972) used agarose to immobilize zebrafish while filming the morphogenesis of sensory neurons with Nomarski (differential interference contrast) optics. A decade later, King et al. (1982) introduced methyl cellulose as a means of immobilizing intact zebrafish embryos for more extended time-lapse recordings. Intact embryos and larvae were maintained for up to 2 weeks in methyl cellulose gels.

In the early 1980s, Gähwiler introduced a method of maintained brain slices in long-term organotypic culture by using clotted plasma to secure the nervous tissues to microscope coverslips. This methodology has been used to film the morphogenesis of fluorescently labeled cortical neurons in neonate mammalian brain slices over the course of several days (O'Rourke et al., 1992, 1995). These epifluorescence time-lapse studies exploited the optical transparency of the plasma clot, as well as its lack of autofluorescence.

Simon and Cooper (1995) used a thin plasma clot to partially immobilize contractile embryonic explants isolated from medaka embryos. Tissue structure, calcium dynamics, and cell motility were examined at the single-cell level in an explanted periderm (i.e., the EVL plus an underlying stellate cell layer). The medaka periderm was loosely immobilized on the surface of the plasma clot, to allow the tissue to rhythmically contract.

In this study, we have demonstrated that thin plasma clots offer a versatile means of securing deyolked zebrafish embryos, as well as embryonic explants extirpated from them. By using the nonhydrolyzable ATP analog AMP-PNP, we also have found a new way of deyolking zebrafish embryos that avoids unwanted yolk cell contractility. Paralysis of the yolk cell with AMP-PNP permits greater flexibility in deyolking, microdissection, and mounting of the zebrafish embryo, in preparation for imaging studies. The immobilized explants provide an extended field of view for immediate viewing, as well as a stable mounting for longer-term organotypic tissue culture. For in situ hybridization and immunohistochemistry staining procedures, being able to remove the yolk cell before fixation should also improve final imaging of the specimen.

Short-term culturing of deyolked fish embryos for 1–2 days has been performed numerous times by others. In several cases, advanced body tissues developed from late stage deyolked embryos (Oppenheimer, 1936; Tung and Tung, 1944; Trinkaus and Drake, 1956; Bozhkova et al., 1994; te Kronnie et al., 2000). Explants from gastrula stage embryos have been used successfully for studying early cell fate specification in the blastoderm (Grinblat et al., 1998, 1999). None of these studies, however, used immobilization media for time-lapse imaging. We have found that tissues develop fairly normally in the cultured explants that are immobilized in either agarose or plasma clot. One noticeable difference, however, is the reduction or lack of pigmentation in cultured explants compared with intact embryos. We speculate that the lack of pigment cell differentiation in the embryonic explants might be connected with the loss of a growth factor normally carried through blood circulation. Reduced ventricle expansion is often observed in immobilized head explants. It is possible that cerebrospinal fluid may be leaking from the cut end of the end of the developing head explant. In addition, when head rudiments are completely gelled within agarose, the ventricle's expansion may be inhibited by the mechanical pressure of immobilization. This problem should be obviated when head rudiments are immobilized on the surface of a plasma clot.

In teleostean fish embryo, the fragility of the yolk cell has hampered the development and application of certain experimental embryologic techniques, such as organ rudiment transplantation or inversion of whole tissue blocks. These tech-

niques have long been used in amphibian and avian embryos (Spemann and Mangold, 1924; Le Douarin, 1973; Stern, 1999; Alvarado-Mallart, 2000; Packard et al., 2000). The ability to mount and culture a deyolked zebrafish embryo by using thin plasma clot immobilization may improve the ability to perform tissue transplantation and other micromanipulations in zebrafish embryos.

Cultured embryonic explants also offer several potential new applications. By placing deyolked embryos in culture medium, it may be possible to nurse mutant embryos through critical periods in their development, when early acting embryonic lethal genes would normally result in the death of the embryo. This technique could allow downstream effects of these genes to be studied at later time points of development. Examples of mutant strains where cultured head explants might be nursed through a critical embryonic lethal time point are *mush for brains* (*mfb*), and *migraine* (*mig*), which possess malformed vascular systems and often exhibit brain hemorrhages and subsequent brain degeneration (Stainier et al., 1996).

Time-lapse analysis of fluorescently labeled stem cells integrated into host tissues is another potential application for long-term cultured embryonic explants. Similarly, it should also be possible to follow the behavior of fluorescently labeled pathogens in immobilized cultured explants, as has been demonstrated recently in the intact zebrafish embryos and larvae (Davis et al., 2002). Cultured cross-sectional slices of embryos may also provide unique opportunities for studying organogenesis and histodifferentiation in developing zebrafish tissues.

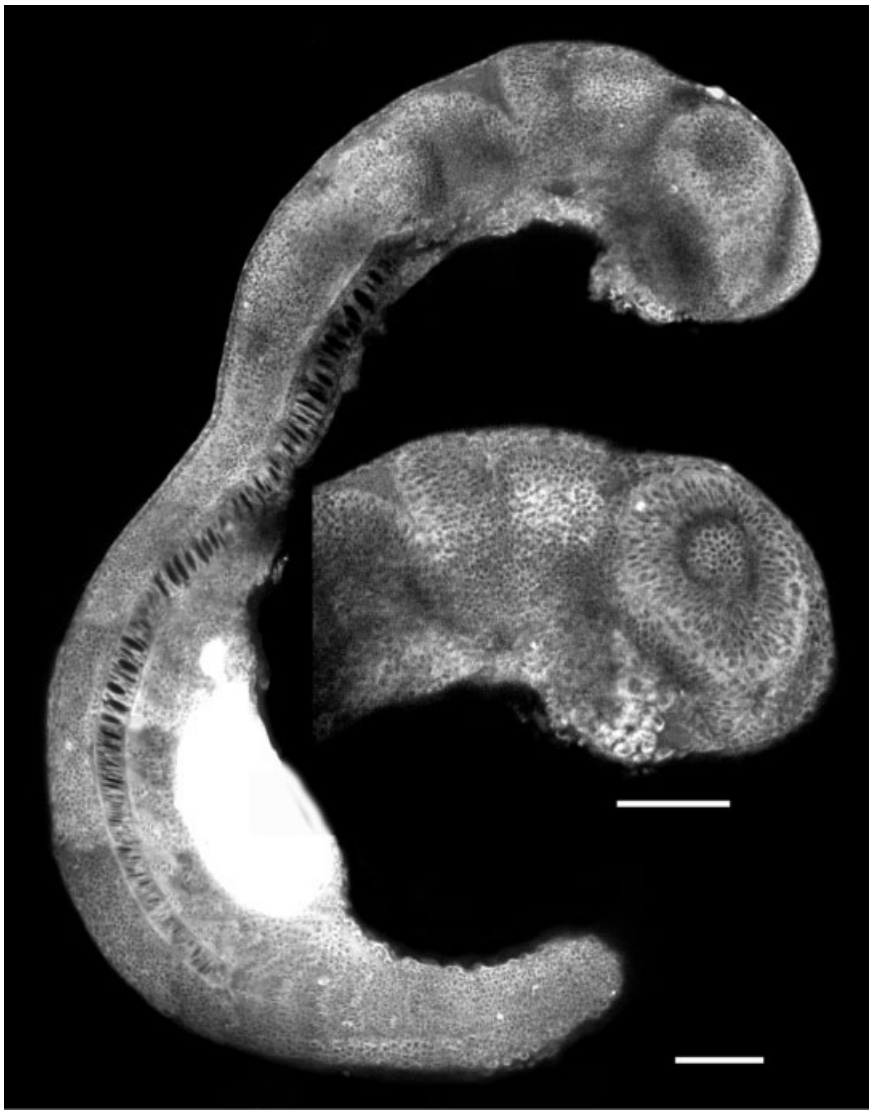

Fig. 5. An extended lateral view of a live 24-hr zebrafish embryo vitally stained with Bodipy 505/515. This image is a seamless photomontage made from 19 separate confocal images, acquired with a ×40/1.0 numerical aperture dry objective. Inset shows the head at higher electronic magnification (i.e., zoom), demonstrating the photomontage contains high-resolution spatial information. The explanted embryo was immobilized underneath a plasma clot (see Fig. 6) before imaging. The embryo's yolk cell was removed after the yolk cell had been injected with AMP-PNP. Scale bars = 100 microns.

EXPERIMENTAL PROCEDURES

In past imaging studies, fish embryos or explanted embryonic fish tissues have been immobilized by using either methylcellulose, agarose, or plasma clot (Kimmel, 1972; King et al., 1982; Simon and Cooper, 1995). Below, we describe modifications to agarose and plasma clot immobilization procedures that are appropriate for short- and long-term organotypic culture of fluorescently labeled zebrafish embryos and tissues. Both methods of immobilization are also amenable for high-resolution confocal imaging.

Culture Media Preparation

A penicillin/streptomycin/antimycotic mixture (GibcoBRL) is added to L-15 amphibian culture medium (GibcoBRL) to a final activity of 100 U/ml for the penicillin. The medium is then partitioned into 50-ml aliquots and stored at 4°C. Others have used L-15 diluted to 67% strength in sterile water to culture zebrafish embryonic cells (Peppelenbosch et al., 1995). All of our experiments were performed in full-strength L-15.

Preparation of Vital Stains

Embryos were vitally stained with the fluorescent dye Bodipy 505/515 by using the procedures outlined by (Cooper et al., 1999).

Injection Solution

AMP-PNP (adenosine 5′ (β, γ-imido) triphosphate, lithium salt; Calbio-

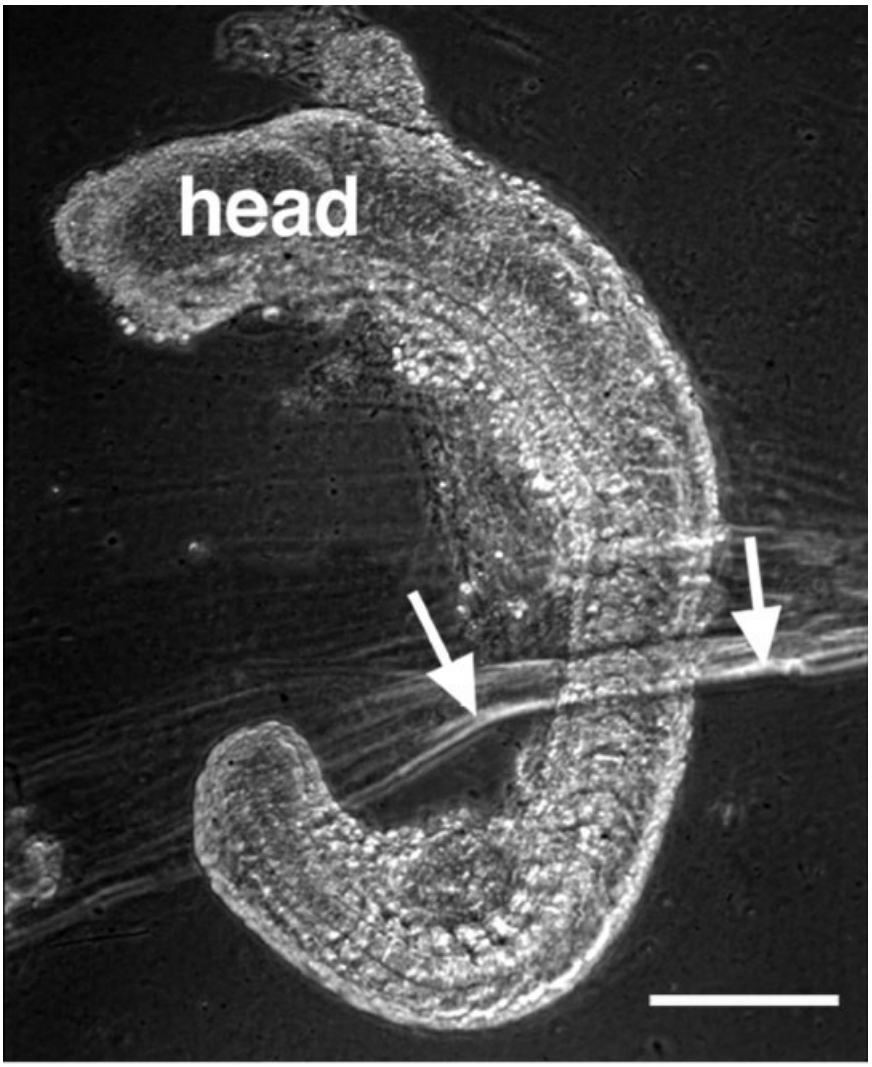

Fig. 6. Whole body explant (18 hr) of a deyolked zebrafish embryo, immobilized underneath a plasma clot. The edge of the clot is marked by arrows. This mounting technique allows extended lateral imaging of the body axis. The yolk cell was removed with microforceps and a tungsten microneedle after it had been injected with AMP-PNP. Scale bar = 250 microns.

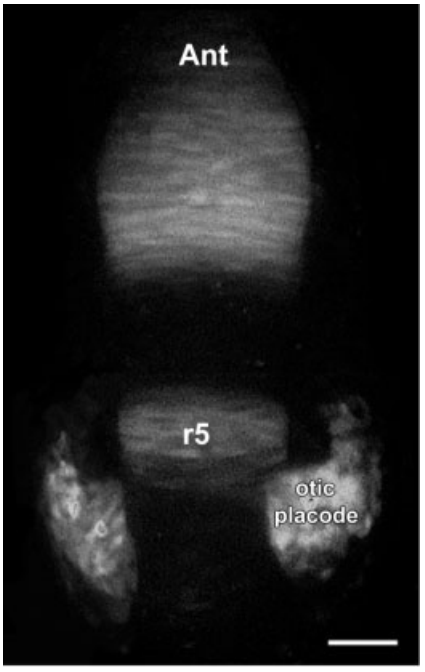

Fig. 7. Immobilized explants allow an extended field of view of green fluorescent protein (GFP) -labeled tissues. A confocal photomontage of GFP expression in the explanted head of a pax 2.1-GFP transgenic zebrafish embryo (15 hr). Dorsal view, anterior up (Ant). Developmental activation of the pax 2.1 promotor is driving GFP expression in the ear anlage, rhombomere 5 (r5), as well as the midbrain–hindbrain region (Picker et al., 2002). Vertical stacks of confocal images (i.e., z-series) from four contiguous regions of the developing brain were projected and fused into a seamless photomontage by using the method of Beck et al. (2000). Photomontaging allows high-resolution images to be obtained over an extended field of yield. For motional stability, the head explant was immobilized in gelled agarose before imaging. Scale bar = 50 microns.

chem, San Diego, CA) is diluted to a final concentration of 40 mM in Millipore water. Owing to its chemical lability, AMP-PNP should be quickly partitioned and frozen at −20°C. A total of 2 μl of the AMP-PNP solution is backfilled into microcapillary micropipettes. Typically, an 8-nl bolus of the 40 mM AMP-PNP solution is injected into the yolk cell.

Preparation of Tungsten Needles and Eyelash Tools

Tungsten needles were electrolytically sharpened by using a modification of the procedure outlined by Cutter (1967). Briefly, a 20-gauge syringe needle is fastened to the end of a 1-cc tuberculin syringe. The tip of the needle is then cut off by using wire cutters. The end of the needle is reopened with needle nose pliers. A tungsten wire is inserted into the syringe opening. The needle is then crimped to hold the wire in place. An alligator electrical connector is attached to the base of the needle, and the syringe plus needle is secured in a chemical stand and electrolytically sharpened according to the method described by Cutter (1967). Eyelash and hairloop micropositioning tools are prepared according to the procedures of Grinblat et al. (1999).

Agarose Immobilization of Whole Embryos

To immobilize whole embryos, it is very convenient to use normal agarose at 0.4–0.5%, melted in a water bath or heating block at 42°C. The embryo is then pipetted into the agarose, quickly taken out with some molten agarose, then poured onto a mounting device for imaging. We recommend an imaging chamber described by Concha and Adams (1998), consisting of a glass ring (Fisher Scientific) sandwiched between two glass coverslips and sealed with silicone grease. The embryo has to be oriented quickly with a eyelash poker (or equivalent), be-

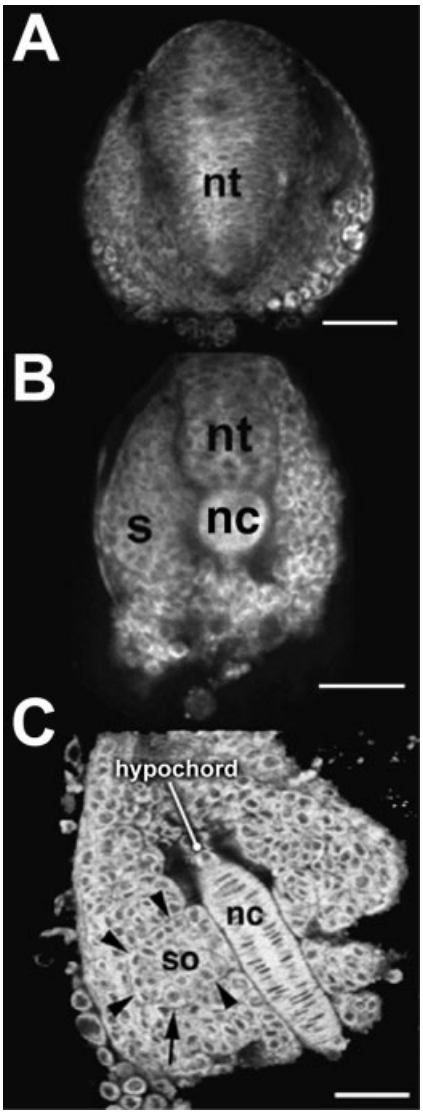

Fig. 8. Cross-sectional and ventral confocal imaging of live explants (×40/1.0 numerical aperture objective). **A,B:** An 18-hour embryo; s, somitic mesoderm; nt, neural tube; nc, notochord. (A) Midbrain. (B) Trunk. **C:** Ventral view of an explanted tail rudiment (14-somite stage) mounted ventral side down using a plasma clot immobilization. The tissues were vitally stained with Bodipy 505/515 before explantation from the embryo. The notochord and a neighboring somite (so, arrowheads) are clearly visible. An individual somitic mesodermal cell marked (arrow) is in mitotic prophase. Scale bars = 50 microns in A–C.

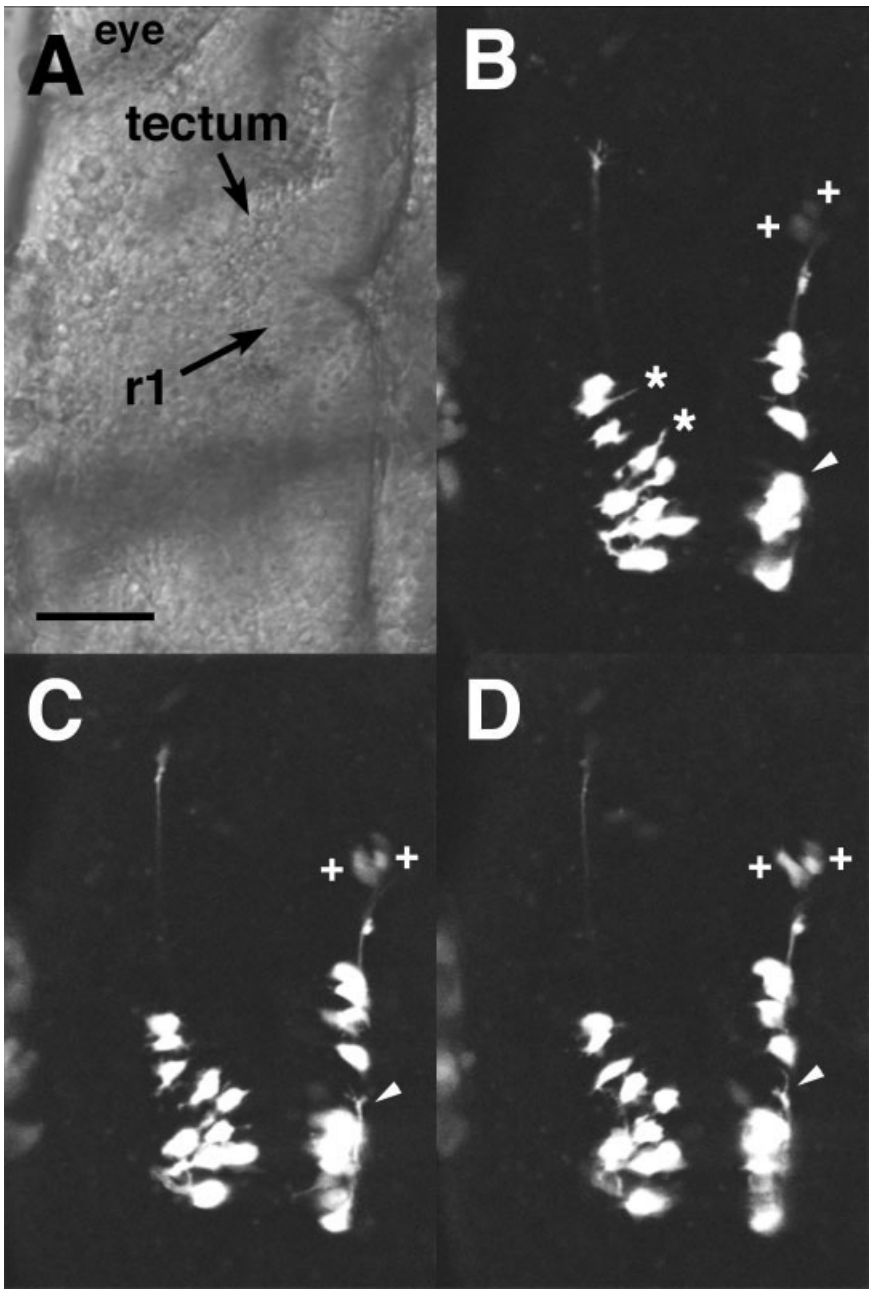

Fig. 9. Neurite activity in an immobilized head explant of a green fluorescent protein (GFP) -islet1 transgenic zebrafish embryo (Higashijima et al., 2000). **A:** Nomarski (differential interference contrast microscopy (DIC)) image of a head explant that was isolated from an embryo whose yolk cell had been paralyzed with an injection of AMP-PNP. The head explant was partially flattened and immobilized underneath a thin plasma clot and imaged from its ventral side using an inverted microscope configured for simultaneous DIC/epifluorescence confocal imaging (Simon and Cooper, 1995). **B–D:** The 2-hr time-lapse sequence shows dynamics of neurites extending (arrowheads) and retracting (asterisks) from GFP-labeled trigeminal neurons in the zebrafish hindbrain. The time series demonstrates that neurite activity can be visualized in an immobilized explant, using a low magnification objective (×20/0.75 numerical aperture), over a relatively large field of view. The time series also shows the onset of GFP fluorescence in two trigeminal neurons (crosses), illustrating that the temporal dynamics of GFP reporter genes can be monitored in cultured immobilized explants (see also Supplementary Movie, available at www.interscience.wiley.com/developmentaldynamics/suppmat/index.html). Scale bar = 50 microns in A.

cause the agarose will gel within 10–20 sec, firmly holding the embryo in place.

For long-term time-lapse imaging, a hole for the body axis to elongate should be cut into the agarose (Fig. 10). This hole allows the body to extend normally, while maintaining immobilization of the head. The hole can be cut by using tungsten needles. Embryos survive in agarose without medium exchange for at least 24 hr. Low melting point (LMP) agarose (Invitrogen, Karlsruhe, Ger-

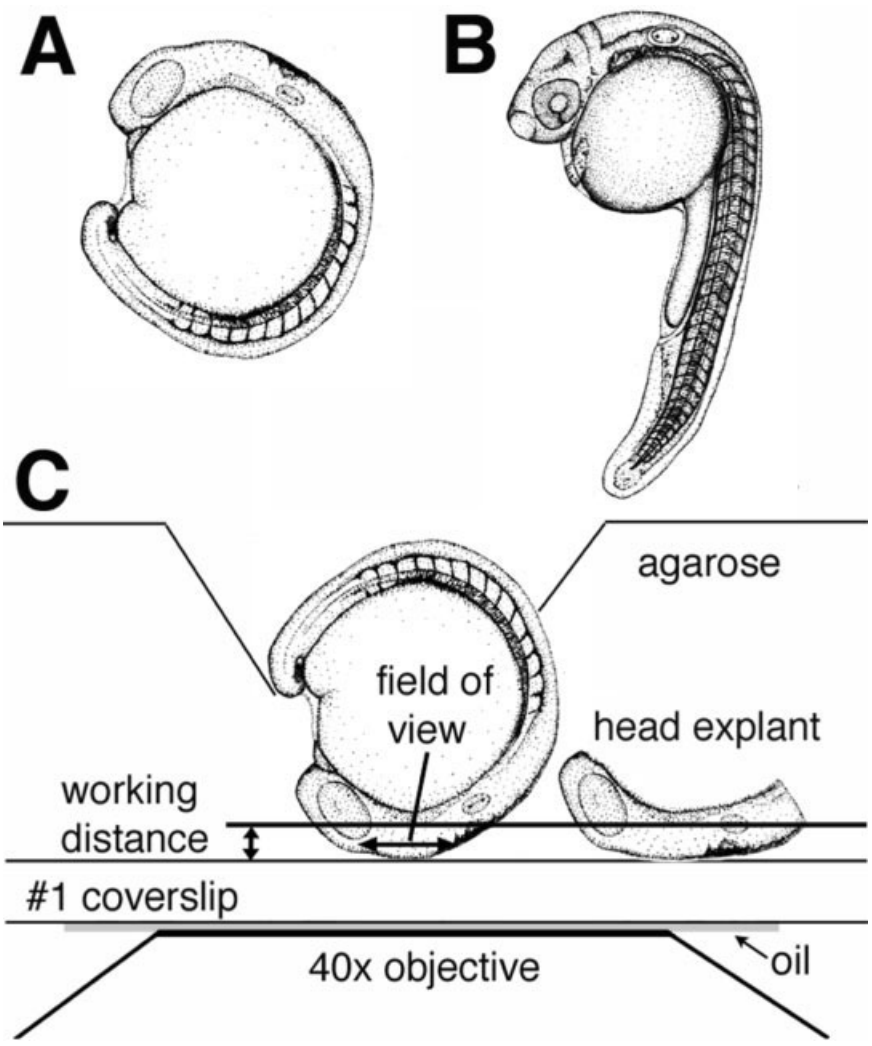

Fig. 10. Imaging brain development in immobilized zebrafish embryos and head explants. **A,B:** As the zebrafish embryo develops from the 15-somite stage (16.5 hours postfertilization (hpf)) to the 24-hr stage, the body axis undergoes substantial straightening. The head region also undergoes changes in its curvature. Spontaneous twitching of the body musculature begins at 19 hpf. These morphogenetic and motor movements each produce difficulties in imaging brain development at the cellular level. **C:** These difficulties can be circumvented by partially immobilizing the embryo in agarose, allowing open space in the agarose for body axis extension. However, effective imaging is still limited, because the curved embryonic head extends beyond the working distance and field of view of the high numerical aperture, oil immersion objective. Larger areas of interest can be potentially imaged in head explants that have been partially flattened and immobilized, in a medium such as agarose or plasma clot. Images from contiguous regions of the specimen can then be acquired and assembled into seamless photomontages (e.g., Figs. 5, 8).

many) is preferable for very young embryos (up to the tail bud stage, 10 hpf), because it can be added at lower temperatures. This way, yolk cell rupture is less likely to occur. LMP agarose takes much longer to gel than regular agarose and, thus, should be used at higher concentrations (e.g., 0.75–1.5%).

Removal of the Yolk Cell

To retard yolk cell contractility in response to wounding, AMP-PNP is microinjected into the yolk cell before dissection of the embryo. To efficiently block curling, we injected an 8-nl bolus of 40 mM AMP-PNP dissolved in distilled water. Within 1 min after injection, the yolk cell's cortex becomes paralyzed. AMP-PNP is membrane impermeant and, thus, will not leave the yolk cell and enter the blastoderm. Cytoplasmic bridges between the yolk cell and the blastoderm are completely lost after the sphere stage of development (Cooper and D'Amico, 1996).

Once the yolk cell's actomyosin networks have been paralyzed, much of the yolk mass within the yolk cell can be extruded by gentle pressure applied from a blunt metal microneedle. After the yolk has been extruded, the ventral epidermis covering the yolk cell can be split open along the anterior–posterior axis using a tungsten needle, and most of the yolk cell's enveloping layer and underlying epidermis is cut away.

Plasma Clot Preparation

To mount explanted pieces of embryonic tissue, we modified the plasma clot technique developed by Gähwiler (Gähwiler, 1981, 1984a,b). Lyophilized bovine plasma (Sigma) is first reconstituted in Millipore water, partitioned, and frozen at −20°C. Thrombin (Sigma) is diluted to 100 U/ml stock concentration, partitioned and frozen at −20°C. A drop (~20 μl) of reconstituted bovine plasma is spread over a 1-cm^2 area on the surface of a microscope coverslip. Excess plasma should be removed to make a uniform thin layer (Simon and Cooper, 1995). The coverslip is then placed under an incandescent desk lamp to dry the plasma and promote its absorption to the coverslip. We have found that this drying step is very helpful in promoting prolonged adherence of plasma clots to glass coverslips.

An aqueous solution of thrombin (100 units/ml) is quickly applied to the dried plasma layer for several seconds, then quickly removed using a Pasteur pipette. The thrombin will quickly catalyze a clotting of the plasma layer that is adsorbed to the coverslip. The coverslip can now be covered with culture medium. Deyolked embryos or embryonic explant can be transferred to the coverslip with a heat-polished Pasteur pipette, making sure to avoid contact of the tissues with air–water interfaces. The deyolked embryos or explanted tissue rudiments can be positioned and secured on top of, or underneath, the plasma clot layer (see below). Deyolking can also be accomplished on top of a plasma clot, underneath culture media. This

approach avoids the step of transferring a deyolked embryo or embryonic explant by means of a Pasteur pipette from one solution to another.

Explant Preparation and Mounting

Desired sections of the zebrafish embryo can be extirpated by using sharp tungsten tools or the *Gastromaster* microdissecting device (model GST-1, Xenotek Engineering, Belleville, IL; www.gastromaster.com). Removal of the EVL, which covers the embryo proper, must be avoided. Explants quickly lose their natural morphology without the EVL and epidermis. The cut edges of the enveloping layer and epidermis often help embryonic explants stick to the plasma clot.

By using a fire-polished glass Pasteur pipet, individual embryonic explants can be transferred onto the plasma clot. The explant can then be oriented with a blunt metal poker (sewing needle mounted in a holder), the end of fine watchmaker's forceps, or an eyelash tool. Once the explant is manipulated into the desired orientation, it is gently pressed down against the plasma layer to secure it. Additional plasma can be used to further stabilize the explant. Residual thrombin from the first plasma layer will clot the added plasma. Excessive plasma should be avoided, because it will restrain the explant and prevent normal morphogenesis. To circumvent this, plasma can be diluted before adding. If needed, additional culture medium can be added once the explant is immobilized.

Lateral-side down and dorsal-side down explants are made somewhat differently. A small hole is opened in the plasma layer using microforceps and/or a tungsten microneedle. The plasma clot layer is then lifted up until the clot becomes slightly detached from the cover slip. The explant can then be moved underneath the plasma layer. Once released, the plasma clot will gently press the explant down against the coverslip (Fig. 5). Additional plasma can be injected under the clot to further stabilize the explant. An alternative securing medium is low-melting point agarose. A low concentration of 0.75% agarose is preferable.

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