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Address correspondence to Dr. Paul Hasty, Department of Molecular Medicine and Institute of Biotechnology, University of Texas Health Science Center, San Antonio, TX 78245-3207, USA. e-mail: hastye@uthscsa.edu

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Greg Donoho and Paul Hasty
Lexicon Genetics
The Woodlands, TX, USA

Direct Comparison of Common Fixation Methods for Preservation of Microtubules in Zebrafish Embryos

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Microtubule arrays in zebrafish (*Danio rerio*) embryos are vital to many developmental processes. Besides their obvious role in mitosis, microtubules are required for epiboly (1-3), furrow formation (4), and the cohesion of post-cytokinesis blastomeres (5). As transport lines for regulatory substances and maternal mRNAs, microtubules are also required for axis determination (6) and symmetric and synchronous cleavage (7). The study of microtubule function in both fixed and live embryos is consequently important for understanding the molecular mechanisms underlying numerous developmental processes.

In fixed preparations, immunofluorescence microscopy allows the comparison of microtubule structures in embryos at different stages or comparison of mutant or developmentally modified embryos to wild-type embryos fixed at the same age. Microtubule arrays in thick samples such as embryos are notoriously difficult to preserve. The dynamic nature of the microtubule polymer and sensitivity to calcium make microtubules extremely labile. Furthermore, fixations that work well to preserve microtubules in one cell type or sample may work very poorly in others.

Several fixation methods are routinely used, and there is only anecdotal support for one method over the other. Most of these methods have been adapted from *Xenopus* protocols, and the anatomy of the zebrafish embryo possesses unique features that may prevent direct extrapolation of these methods. Here we report a comparison of the common fixation methods and make recommendations for the analysis of microtubules in different cell types of the early embryo.

We focus on the visualization of microtubules in three areas of zebrafish embryos: (*i*) the enveloping layer of cells (EVL), (*ii*) the deep cells that lie beneath EVL cells, and (*iii*) the yolk syncytial layer (YSL) (Figure 1). The deep cells of the early embryo will form the tissues of the embryo-proper, while EVL cells are epithelial and form a single-cell covering over the entire embryo. Both the EVL and deep cells sit upon and surround a multinucleated syncytium, the yolk cell. Visualization of these specific domains is valuable in different areas of developmental research.

The embryo develops within a clear sack called the chorion. If the chorion is not removed before fixation, then it must be removed at a later stage for antibody penetration in immunohistochemistry. After fixation, the chorion can be easily removed with forceps. Before fixation, a limited enzymatic pronase digestion can remove the chorion. We tested multiple fixation methods and tested whether removal of the chorion before fixation had any effect.

We examined two common fixation procedures: a simple paraformaldehyde (PFA) fixation and a fixation using microtubule stabilization buffer (MSB). PFA is a widespread fixative used on many different sample types and has been used to prepare zebrafish embryos for immunohistochemistry of many epitopes (5,8), in situ hybridization, and a combination of these two techniques (9). The MSB fixative was originally developed for *Xenopus* (10) and was later used with slight modifications for zebrafish embryos (1,11). We examined these two fixatives both with and without intact chorions during fixation. We also tested embryos that were permeabilized with Triton® X-100 following de-

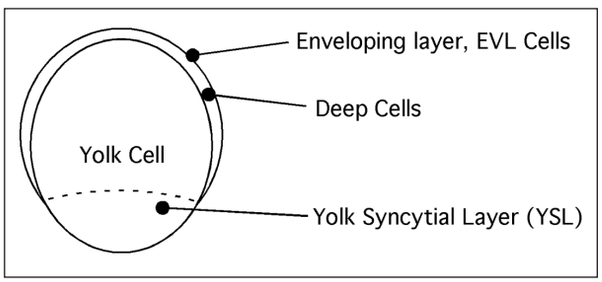

Figure 1. Representation of an 8-h zebrafish embryo, showing the location of the different cell types examined in this study.

Table 1. Ratings of Different Aspects of Each Fixation Condition

	(*i*) PFA fixed, chorion intact	(*ii*) MSB fixed, chorion intact	(*iii*) PFA fixed, chorion removed	(*iv*) MSB fixed, chorion removed	(*v*) permea-bilized, then PFA fixed	(*vi*) permea-bilized, then MSB fixed	(*vii*) pH-shift-MSB fixed, chorion removed	(*viii*) methanol fixed, chorion intact	(*ix*) methanol fixed, chorion removed
MT morphology in EVL cells	++	+	++	+++	+	++	++	-	-
MT morphology in deep cells	+	-	+	++	+	+	-	+	-
MT morphology in YSL	++	-	++	++	+++	++	+	+	+
Gross morphology	+	+	+	+	-	-	-	-	-
Overall usefulness	+	-	+	++	+	-	-	-	-

Each aspect was rated qualitatively after observing many pictures from a minimum of 10 embryos fixed using respective techniques.
MT, microtubule.

chorionation but before fixation to see if permeabilization of the embryo would remove soluble tubulin, making assembled microtubules easier to visualize.

Two less common fixation methods were also tested: a pH-shift-MSB fixation modified from a protocol for preserving cytoskeletal antigens in MDCK cells (12) and a simple methanol fixation at -20°C, sometimes used to preserve *Xenopus* oocytes (10). The methanol fixation was tested both with and without intact chorions. We found that embryos subjected to pH-shift-MSB fixation were too fragile following fixation for manual removal of the chorion, so this method was only tested on embryos where the chorion had been removed before fixation. We also tested whether the microtubule stabilizing agent, taxol, enhanced microtubule preservation. PFA and MSB fixations were tested with the inclusion of taxol (0.5 µM). Taxol treatment during fixation had no effect on microtubule preservation.

The nine treatment sets were as follows: (*i*) PFA fixed with intact chorions, (*ii*) PFA fixed after chorion removal, (*iii*) MSB fixed with intact chorions, (*iv*) MSB fixed after chorion removal, (*v*) PFA fixed after chorion removal and permeabilization, (*vi*) MSB fixed after chorion removal and permeabilization, (*vii*) pH-shift-MSB fixed after chorion removal, (*viii*) methanol fixed with intact chorion, and (*ix*) methanol fixed after chorion removal. All steps were performed at room temperature unless otherwise noted. Before proceeding with the fixations, we pretreated all plasticware and glass pipets that would come in contact with the embryos with 0.2% BSA and briefly rinsed them in distilled water to prevent the embryos from sticking. The samples where the chorion was enzymatically removed before fixation were treated for 1 min in a pronase solution (13) to digest the chorions and then rinsed with PBS. To permeabilize the embryos, we treated them for 1 min in permeabilization buffer [PIPES (pH 6.5), 1 mM $MgCl_2$, 5 mM EGTA, 0.2% Triton X-100] before adding fixative (modified from Reference 12).

Fixations were performed as follows: for PFA solution, 4% PFA (Fluka 76240) in PBS (10 mM Na_2PH_4, pH 7.2, 140 mM NaCl) for 2–4 h (4,5); for MSB, 80 mM K-PIPES (pH 6.5), 5 mM EGTA, 1 mM $MgCl_2$, 3.7% PFA, 0.25% glutaraldehyde (Sigma G-5882), and 0.2% Triton X-100 for 2–4 h, followed by quench in 1 mg/mL $NaBH_4$/PBS for 20 min (1,10,11); for pH-shift-MSB, 3% PFA/MSB (see above) (pH 6.5) for 5 min, then 3% PFA, 100 mM $Na_2B_4O_7$ (pH 11.0) for 30 min, and then quenched twice over a period of 15 min with 1 mg/mL $NaBH_4$/PBS (pH 8.0); for methanol, -20°C methanol for 2–4 h.

Following fixation, all embryos were washed three times in PBS. Embryos fixed with chorions intact were manually dechorionated (8,13). All em-

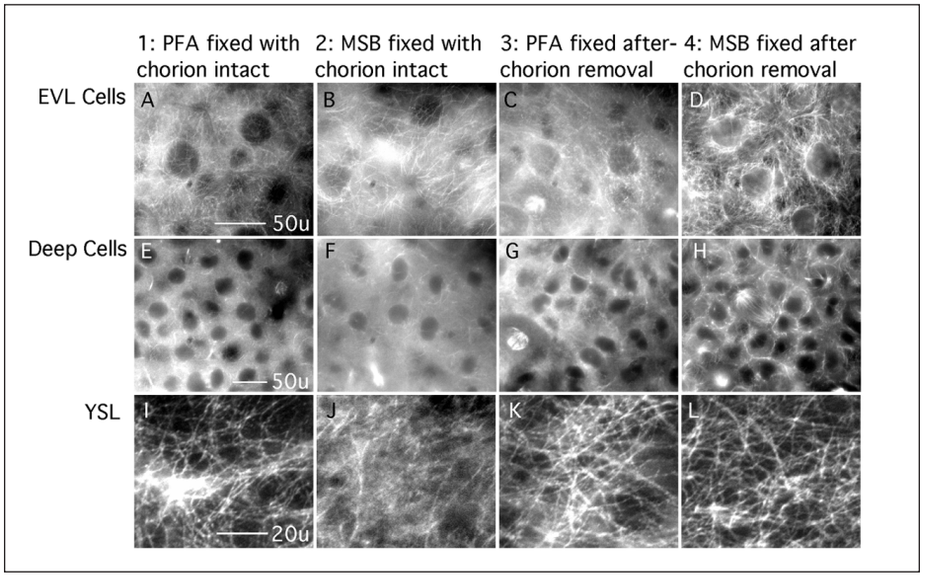

Figure 2. Representative images of microtubules from specific parts of the embryos fixed under different conditions and imaged with wide-field microscopy. Scale bar in A applies to A–D; E applies to E–H; and I applies to I–L.

bryos except those fixed in methanol were dehydrated in a methanol series (10%, 30%, 50%, 70%, 90%, and 100%) for 5 min in each solution, followed by storage at -20°C in methanol for at least 1 h. We also compared samples without any methanol treatment after fixation. Surprisingly, methanol treatment after fixation did not affect microtubules at all; therefore, this step can be omitted while still preserving microtubule morphology.

All embryos were incubated in block solution [10% Normal Goat Serum, 0.5% Triton X-100, 0.1 M Na_2HPO_4 (pH 7.2)] for 1 h, followed by incubation in a 1:500 dilution of monoclonal anti-α tubulin antibody DM1A (Sigma T-9026) in block for 2 h at room temperature. They were washed in block four times over a period of 100 min. Embryos were incubated in secondary antibody Cy3-labeled donkey α-mouse diluted in block for 2 h and washed with wash buffer solution (0.1 M Na_2HPO_4, pH 7.2, and 0.5% Triton X-100) four times over a period of 100 min. To label nuclei, embryos were incubated in 0.5 µg/mL Hoechst stain (Hoechst 33342, Sigma B-2883) in PBS for 2 min with gentle rocking, followed by two rinses in PBS. Embryos were equilibrated in 70% glycerol before mounting as described previously (14). Fluorescent signals were stable, so an anti-fade agent was not used.

Table 1 presents the results of the fixation conditions, while Figure 2 shows representative images from four of the conditions. Of the conditions tested, embryos fixed in MSB with the chorion removed before fixation showed far superior preservation of microtubules in EVL and deep cells (Figure 2, D and H). For observation of the microtubules in the YSL, PFA fixation with an intact chorion gave comparable results to MSB with prior chorion removal (compare Figure 2, I and L).

If the chorion is left intact during fixation (Figure 2, A, B, E, F, I, and J), the PFA fixative yielded superior embryos. In EVL cells, deep cells and in the YSL region, microtubules from the PFA fixation were clearer and better resolved than microtubules from MSB-fixed embryos. Apparently, the chorion impedes the fixation process if MSB is used.

All of the permeabilized samples yielded acceptable microtubule preservation in all areas examined (not shown). Methanol was the least satisfactory. The PFA-fixed and permeabilized embryos specifically showed excellent YSL preservation. However, all of the permeabilized samples had severely compromised integrity. Permeabilized embryos tended to fall apart and were difficult to manipulate and successfully mount. Methanol- and pH-shift-MSB-fixed embryos were worse, falling apart during fixation. It was difficult to identify reproducibly and observe cell types in these severely distorted embryos.

We conclude that PFA fixation is better than the MSB fixation when the chorions are left intact during fixation. However, the best results are produced when the chorions are removed before fixation with MSB. Exposing embryos to a permeabilizing solution to remove unbound tubulin does not improve microtubule structures; in fact, it makes the embryos prone to disintegration. Methanol and pH-shift-MSB fixation procedures produce severely distorted embryos. For observation of microtubule formations in EVL and deep cells, we recommend embryo fixation with MSB after removal of the chorion. For examination of microtubules in the YSL, both PFA fixation with intact chorions and MSB fixation without the chorion gave comparable results. Neither inclusion of taxol during fixation nor post-fixation in -20°C methanol is required for microtubule preservation.

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Sarah McMenamin[1,2], Sigrid Reinsch[2], and Gregory Conway[2]
[1]Mount Holyoke College
South Hadley, MA
[2]NASA Ames Research Center
Moffett Field, CA, USA