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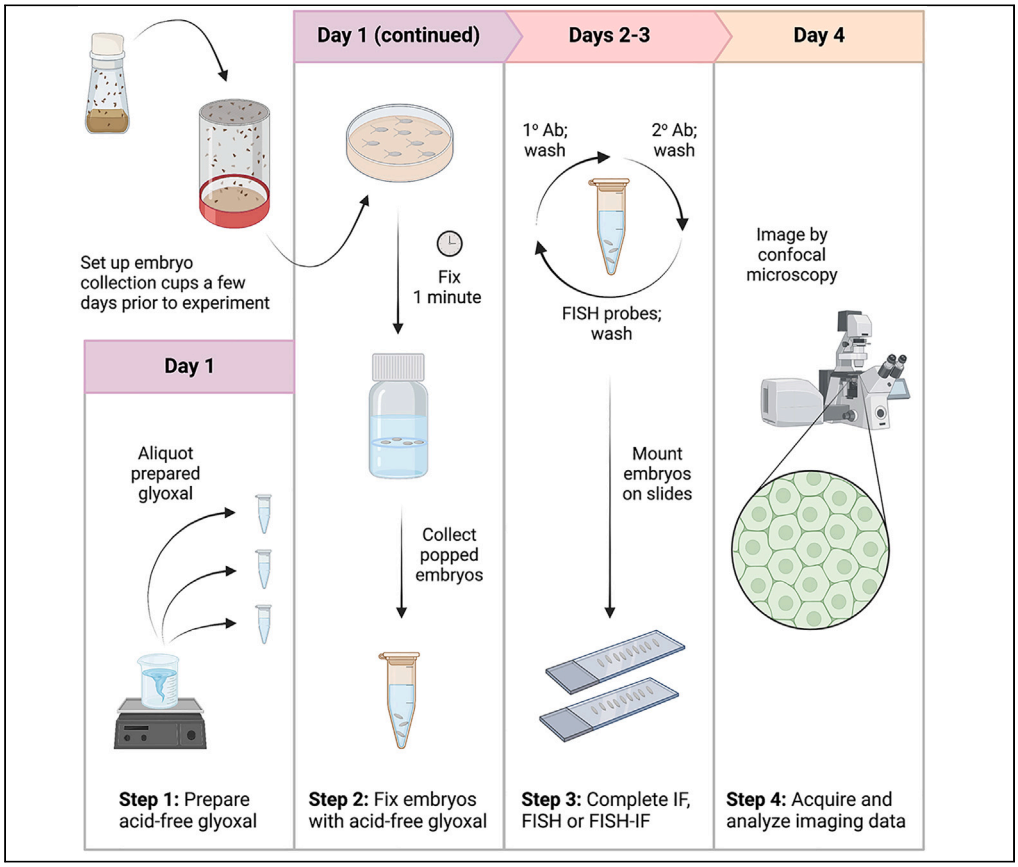
Protocol

Glyoxal-based fixation of *Drosophila* embryos for immunofluorescence staining and RNA *in situ* hybridization



The dialdehyde glyoxal is an alternative chemical fixative that cross-links tissues faster than formaldehyde, retains higher antigenicity, and is less hazardous than either formaldehyde or glutaraldehyde. Here we present a glyoxal-based fixation protocol for use with *Drosophila* embryos. We describe steps to prepare acid-free glyoxal, fix embryos, and then stain with antibodies for immunofluorescence (IF). We also describe methods for RNA fluorescence *in situ* hybridization (FISH) and FISH plus IF (FISH-IF) using glyoxal-fixed embryos.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Glyoxal offers an alternative to formaldehyde for fixation of *Drosophila* embryos

This protocol describes steps to prepare acid-free glyoxal and fix embryos

A method for immunofluorescence staining of glyoxal fixed embryos is detailed

RNA *in situ* hybridization methods are also described

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Glyoxal-based fixation of *Drosophila* embryos for immunofluorescence staining and RNA *in situ* hybridization

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SUMMARY

The dialdehyde glyoxal is an alternative chemical fixative that cross-links tissues faster than formaldehyde, retains higher antigenicity, and is less hazardous than either formaldehyde or glutaraldehyde. Here we present a glyoxal-based fixation protocol for use with *Drosophila* embryos. We describe steps to prepare acid-free glyoxal, fix embryos, and then stain with antibodies for immunofluorescence (IF). We also describe methods for RNA fluorescence *in situ* hybridization (FISH) and FISH plus IF (FISH-IF) using glyoxal-fixed embryos.
This protocol was adapted for *Drosophila* embryos from the methods of Bussolati et al.[1] and Richter et al.[2]

BEFORE YOU BEGIN

We developed a glyoxal-based fixation method for *Drosophila* embryos (Figure 1) due to reports in the literature that glyoxal maintains antigenic sites in proteins better than other fixatives.[2,3,4–6] Our aim was to identify an additional fixation option for fly embryos, which would be compatible with a larger number of primary antibodies for immunostaining. Indeed, we found that some antibodies that failed to stain embryos fixed with formaldehyde, glutaraldehyde, methanol, or heat, did give expected staining patterns in embryos fixed with glyoxal (Figure 2).

For any lab that currently fixes *Drosophila* embryos using standard formaldehyde methods,[7–10] only one additional step is required to fix embryos using glyoxal (Figure 1). Namely, one pre-fixation step is required to deionize the purchased glyoxal to increase its pH to the neutral range. Glyoxal from the manufacturer is acidic, with a pH of 2.0–3.0. At this low pH, glyoxal fixation negatively impacts the

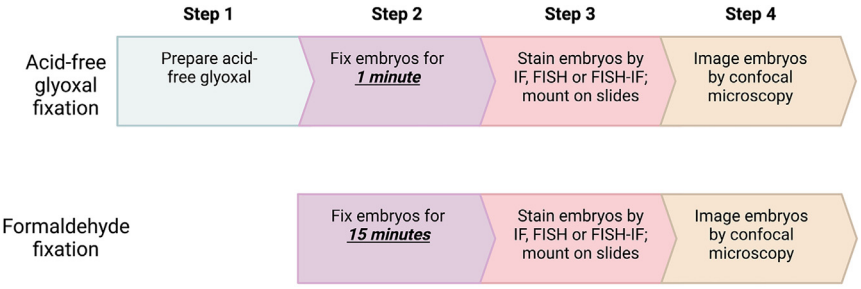

Figure 1. Comparison of steps for embryo fixation using glyoxal or formaldehyde

Embryo fixation with acid-free glyoxal follows the same basic protocol as formaldehyde fixation, with the most notable differences being the addition of an initial step to prepare the glyoxal (Step 1) and a reduction in the fixation time to just 1 min (Step 2).

structural integrity of nucleic acids.[1,11] However, successful deionization with a mixed-bed ion exchange resin results in glyoxal with a pH of ~7.0 that is suitable for nucleic acid preservation.[1,11,12] We found that fixing embryos with this "acid-free" glyoxal maintains nuclear structure and allows better DNA staining with Hoechst (or DAPI), as compared to methods where the pH of the glyoxal was adjusted to either 4.0 or 5.0 by addition of sodium hydroxide[2] (Figure 3).

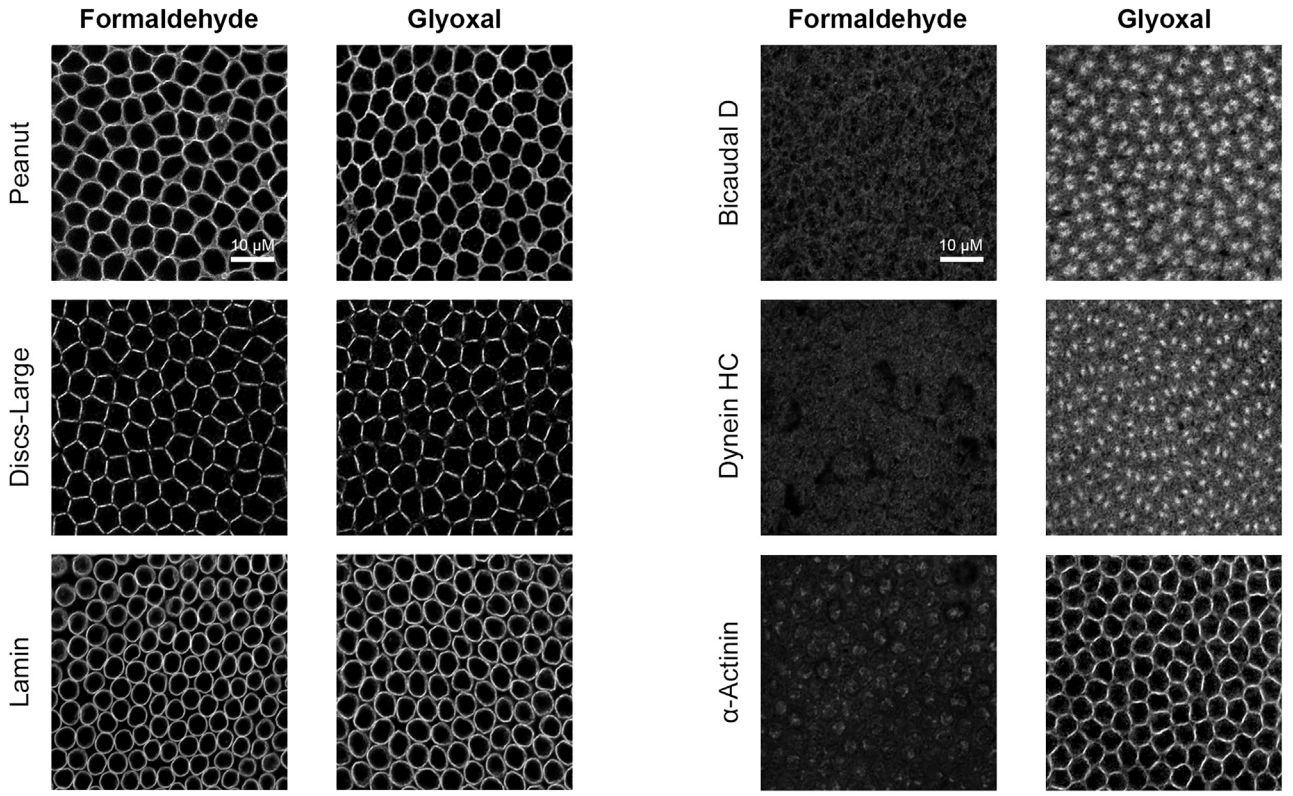

Figure 2. Comparison of IF results for formaldehyde or acid-free glyoxal fixed embryos

Embryos were fixed with formaldehyde for 15 min (Formaldehyde) or acid-free glyoxal for 1 min (Glyoxal) and then immunostained with the indicated primary antibody. In cases where an antibody showed specific staining in formaldehyde fixed embryos, that antibody also showed the same staining pattern in glyoxal fixed embryos (left columns). In some cases, where an antibody failed to show specific staining in formaldehyde fixed embryos, it did show a specific staining pattern in glyoxal fixed embryos (right columns). Peanut, Discs-Large and α-Actinin show the hexagonal organization of plasma membrane furrows in cellularization stage embryos; Lamin shows the nuclear envelope of nuclei; and Bicaudal D and Dynein Heavy Chain (Dynein HC) show centrosomes at the apical side of nuclei. Scale bars 10 microns.

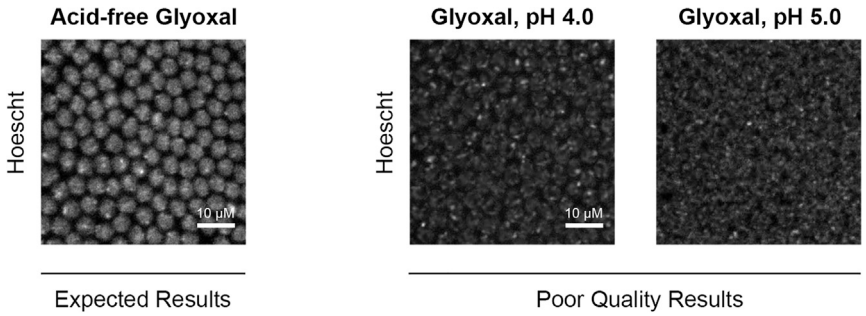

Figure 3. Hoechst staining results following fixation with different preparations of glyoxal

Embryos were fixed with acid-free or acidic glyoxal (pH 4.0 or 5.0) for 1 min as indicated. Acid-free glyoxal preserves DNA structure in a way that supports good quality Hoechst staining, where nuclei appear as distinct compartments of signal. Acidic glyoxal poorly preserves DNA structure such that Hoechst staining is highly variable and nuclear borders are often unclear. Scale bars 10 microns.

Herein, we describe how to fix early fly embryos using acid-free glyoxal. This fixation method works equally well for embryos that will be processed for immunofluorescence (IF), RNA fluorescence *in situ* hybridization (RNA FISH) or RNA FISH combined with immunofluorescence (FISH-IF) (Figures 2, 4, and 5). Our IF protocol needed no modification for use with glyoxal fixed embryos. Our previously described RNA FISH and FISH-IF protocols[13] did require a few small modifications that are explicitly pointed out in the protocol below.

Prepare embryo collection cups

Timing: 4 days total to pour apple juice agar plates, and set-up and prime embryo collection cups for optimal egg laying on Day 1 of the experiment

1. Prepare apple juice agar plates, which serve as the substrate for embryo collections.
   a. Combine 100 g BD Bacto Agar and 3 L distilled water in a 6 L Erlenmeyer flask. Autoclave for 60 min on the "Slow Exhaust" setting.

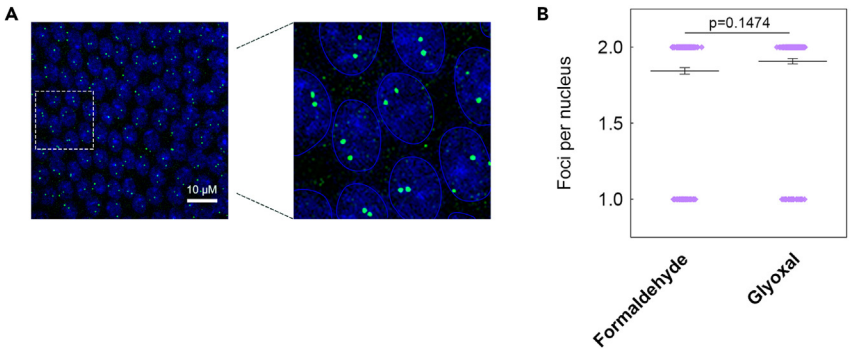

Figure 4. FISH results for acid-free glyoxal fixed embryos

Embryos were fixed with acid-free glyoxal for 1 min and then hybridized with fluorescently-labeled oligonucleotide probes against the zygotically transcribed gene, *serendipity-α* (TAMRA *sry-α*).

(A) The *sry-α* gene is highly expressed at this developmental stage (early cellularization), and foci of nascent transcripts (green) can be seen at the gene inside each nucleus (blue, with outline). Frequently, two foci per nucleus are seen (inset), suggesting transcription from both *sry-α* gene copies. Scale bar 10 microns.

(B) The foci number was compared in stage-matched embryos fixed with formaldehyde for 15 min (Formaldehyde) or acid-free glyoxal for 1 min (Glyoxal). The number of *sry-α* transcriptional foci detected was the same for both fixations. An unpaired two-tailed t-test was used to calculate the p-value (n = 282 nuclei from 2 embryos per fixation type).

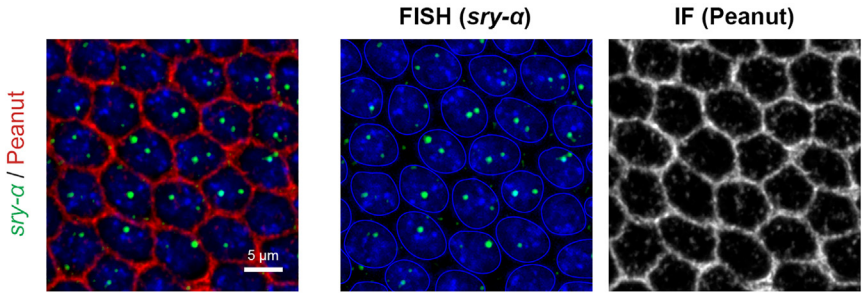

Figure 5. FISH-IF results for acid-free glyoxal fixed embryos

Embryos were fixed with acid-free glyoxal for 1 min and then probed for *sry-α* mRNA (green) by FISH and Peanut protein (red) by IF. Split images show foci of nascent *sry-α* transcripts at the genes inside each nucleus (blue, with outline), and Peanut protein at the furrows of cellularization stage embryos. Scale bar 5 microns.

b. Allow the agar to cool for 10 min in a 50°C water bath.
c. Combine 100 g sucrose, 1 L apple juice and 6 g p-hydroxybenzoic acid in a 2 L Erlenmeyer flask with a magnetic stir bar. Stir and heat to boiling on a hot plate. Boil for a maximum of 2 min or until all the solids are dissolved.
d. Add the apple juice mixture and stir bar carefully to the melted agar. Mix completely on the hot plate.
e. Pour apple juice and agar solution into 60 × 15 mm petri dishes, either by hand or using a peristaltic pump.
f. Cover and allow the plates to cool at room temperature (~22°C) for 3–6 h. Transfer plates in inverted stacks to Rubbermaid containers (shoe box size is convenient). Drape a layer of moist paper towels over the top plates to keep the box humidified. Close the container, and store at 4°C.

2. Two-to-three days prior to the planned fixations, gather and label a small embryo collection cup for each fly genotype that will be used in your experiment.

Note: Cups can either be assembled in house[14] or purchased from Genesee Scientific (see key resources table).

3. Prepare yeast paste by combining approximately 5 g active dry yeast powder to 10 mL distilled water. Mix and add more water as necessary to reach a consistency of wet peanut butter. Store at 4°C in an air-tight container.
4. For each embryo collection cup, prepare an apple juice agar plate by first equilibrating the plate to room temperature (~22°C) and then adding a small dab of yeast paste (1–2 cm diameter) to the center of the plate with your finger.
5. With the prepared apple juice plate at the ready, dump adult flies of desired genotype into a collection cup. Immediately fit with the apple juice plate and secure the plate on the cup using two rubber bands.

Note: If possible, setting up each cup with 100 females and 50 males will provide generous embryo collections.

6. Because it takes the flies some time to acclimate to the cup, replace the apple juice plate with a fresh, yeast-dabbed apple juice plate 2–3 times per day until they are laying well, and you begin your experiment.

Note: To encourage maximum egg laying, make sure that apple juice plates are always equilibrated to room temperature (~22°C) before placing on the cup. Also, set the cups where they

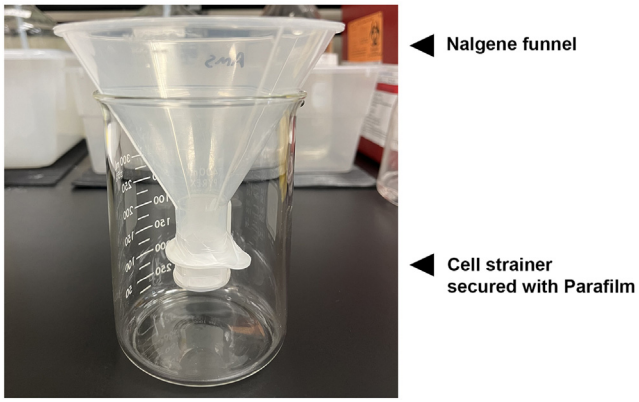

Figure 6. An assembled filtering device for prepping acid-free glyoxal

will be exposed to 12 h light/12 h dark, and ensure that when the light is on, no shadows fall on the cup. (Flies will not lay embryos in the shadowed regions.)

7. After the acclimation period, embryo collections can be made at intervals appropriate to the developmental stage that is desired. For example, a 3-h collection at room temperature (~22°C) should ensure that most embryos in the collection are near or at the cellularization stage.

Prepare acid-free glyoxal

Timing: 2 h, on Day 1

8. Assemble two filtering devices as shown in Figure 6.
   a. For each filtering device, gather an 80 mm Nalgene funnel, a cell strainer and a 1 inch by 6 inch strip of Parafilm.
   b. Affix a strainer tightly to the bottom of each funnel using the Parafilm.
   c. Suspend one filtering device on the edge of a 400 mL glass beaker and the other filtering device on the edge of a 250 mL glass beaker.
9. Weigh out 25 g Amberlite resin and add to the funnel of the filtering device resting on the 400 mL glass beaker.
10. Wash the resin by adding ~50 mL molecular biology-grade water to the filtering device. Allow the water to drain from the funnel and discard water from the beaker.

⚠ CRITICAL: The type of water that is used throughout the deionization is important. We have had consistent success with molecular biology grade water purchased from Corning as described in the key resources table.

11. Prime the resin by adding ~25 mL 1 M NaOH to the filtering device. Allow the NaOH to drain from the funnel and discard NaOH from the beaker.

⚠ CRITICAL: Make the 1 M NaOH fresh with molecular biology grade water on the day of the deionization.

12. Rinse the resin three times with ~50 mL molecular biology-grade water per rinse, allowing the water to fully drain with each wash and disposing water between rinses.

⚠ CRITICAL: The NaOH must be completely washed from the resin. Otherwise, the final pH of the glyoxal will be too basic (up to pH 8.6).

13. Use a spatula to transfer the prepared resin to a 250 mL glass beaker with a small magnetic stir bar.
14. For deionization of glyoxal, pipet 15 mL 40% wt glyoxal solution from the manufacturer's bottle. Add glyoxal to the prepared resin. Cover the beaker with Parafilm and stir on a stir plate at a medium, but not violent speed, for 1 h.
15. Use a spatula to transfer all glyoxal and resin to the second filtering device, resting on the 250 mL glass beaker. Allow the glyoxal to fully drain from the resin.
16. Use a pH meter to check the pH of the glyoxal. The pH should be ~7.0 (troubleshooting 1).

Alternative: It is also possible to ascertain the approximate pH using pH indicator strips.

17. Determine the volume of acid-free glyoxal and dilute with 0.1M Sodium Phosphate Buffer, pH 7.4, to a final concentration of 12.0 wt. % glyoxal. (For example, if 12 mL acid-free glyoxal is retrieved, combine with 28 mL 0.1 M Sodium Phosphate Buffer, pH 7.4.).
18. Make 1.0 mL aliquots of acid-free glyoxal in 1.5 mL microcentrifuge tubes. Store for up to one month in the –20°C freezer.

Alternative: The protocol can be scaled up to deionize 30 mL 40% wt glyoxal solution using 50 g Amberlite resin.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Goat anti-Mouse IgG (H + L), Alexa Fuor™ 488 (1:1000)	Thermo Fisher Scientific	Cat# A-11001; RRID: AB_2534069
Goat anti-Mouse IgG (H + L), Alexa Fuor™ 546 (1:1000)	Thermo Fisher Scientific	Cat# A-11030; RRID: AB_2534089
Goat anti-Rabbit IgG (H + L), Alexa Fuor™ 488 (1:1000)	Thermo Fisher Scientific	Cat# A-11034; RRID: AB_2576217
Goat anti-Rabbit IgG (H + L), Alexa Fuor™ 546 (1:1000)	Thermo Fisher Scientific	Cat# A-11035; RRID: AB_2534093
Mouse anti-Alpha-Actinin (2G3-3D7; sup) (1:5)	DSHB	RRID:AB_2721943
Mouse anti-Bicaudal D (4C2; conc) (1:5)	DSHB	RRID:AB_2721943
Mouse anti-Discs Large (4F3; conc) (1:100)	DSHB	RRID: AB_528203
Mouse anti-Dynein Heavy Chain (2C11-2; conc) (1:100)	DSHB	RRID: AB_2091523
Mouse anti-γ-Tubulin (1:500)	Millipore Sigma	Cat# T5326; RRID: AB_532292
Mouse anti-Lamin *Dm*0 (ADL84.12; conc) (1:100)	DSHB	RRID: AB_528338
Mouse anti-Peanut (4C9H4; *Dm*Septin; conc) (1:100)	DSHB	RRID: AB_528429
Rabbit anti-Myosin 2 (1:500)	Sokac Lab	Available upon request
Rabbit anti-GFP (1:500)	Abcam	Cat# ab290
Chemicals, peptides and recombinant proteins		
Acetic acid, glacial (aldehyde-free/sequencing)	Fisher Scientific	Cat# BP1185; CAS 64-19-7
Amberlite→ IRA400 Resin, chloride form	Millipore Sigma	Cat# 247669; CAS 60177-39-1
Apple Juice, Mott's, 100% Juice	Amazon	UPC:014800000320
Aqua-Poly/Mount	Polysciences	Cat# 18606
BD Bacto™ Dehydrated Agar, BD214010	Fisher Scientific	Cat# DF0140-01-0
Bleach, Pure Bright, 6% sodium hypochlorite, Ultra	VWR	Cat# 10027-510
Bovine serum albumin, heat shock fraction, pH 7.0, >98%	Millipore Sigma	Cat# A7906; CAS 9048-46-8
Bovine serum albumin, UltraPure™, RNAse free, 50 mg/mL	Thermo Fisher Scientific	Cat# AM2616
BS(PEG)5, PEGylated bis(sulfosuccinimidyl)suberate	Thermo Fisher Scientific	Cat# A35396 CAS 756526-03-1

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
DAPI	Millipore Sigma	Cat# 10236276001; CAS 28718-90-3
Dextran sulfate sodium salt	Millipore Sigma	Cat# D8906; CAS 9011-18-1
E. coli Transfer Ribonucleic Acid (tRNA)	Millipore Sigma	Cat# R1753; CAS 9014-25-9
Ethanol, absolute (200 Proof), mol biol grade	Fisher Scientific	Cat# BP2818; CAS 64-17-5
Formamide, deionized	Thermo Fisher Scientific	Cat# AM9344 CAS 75-12-7
Glyoxal solution, 40 wt.% in H_2O	Millipore Sigma	Cat# 128465; CAS 107-22-2
Hoechst 33342 Solution, 20 mM (12.3 mg/mL)	Fisher Scientific	Cat# PI62249; CAS 23491-52-3
Methanol, Optima™ for HPLC	Fisher Scientific	Cat# A454-1; CAS 67-56-1
Methyl 4-Hydroxybenzoate	Millipore Sigma	Cat# H3647; CAS 99-76-3
n-Heptane, 99%, Optima™ for HPLC and GC	Fisher Scientific	Cat# H360-1; CAS 142-82-5
Paraformaldehyde 16% aqueous solution, EM grade	EMS	Cat# 15710
Phosphate buffered saline (PBS), 1×, w/out calcium and magnesium, pH 7.4	Corning	Cat# 21-040-CM
Phosphate buffered saline (PBS), 10×, Invitrogen™, RNase-free, pH 7.4	Thermo Fisher Scientific	Cat# AM9624
Ribonucleoside Vanadyl Complex	NEB	Cat# S1402S CAS 123334-20-3
RNAse*Zap* RNAse Decontamination Solution	Thermo Fisher Scientific	Cat# AM9780
Sodium hydroxide (pellets/Certified ACS)	Fisher Scientific	Cat# S318; CAS 1310-73-2, 497-19-8
Sodium phosphate dibasic (anhydrous)	Millipore Sigma	Cat# 71640; CAS 7558-79-4
Sodium phosphate monobasic (monohydrate)	Millipore Sigma	Cat# S9638; CAS 10049-21-5
20× SSC, RNAse-free	Thermo Fisher Scientific	Cat# AM9763
Sucrose	Millipore Sigma	Cat# 84097; CAS 57-50-1
6-TAMRA, SE, single isomer	Thermo Fisher Scientific	Cat# C6123; CAS 150810-69-8
Triton X-100	Bio-Rad	Cat# 1610407; CAS 9002-93-1
Tween 20 (Polysorbate 20)	Fisher Scientific	Cat# BP337; CAS 9005-64-5
Water, Invitrogen™, DEPC-treated	Thermo Fisher Scientific	Cat# AM9922
Water, molecular biology grade	Corning	Cat# 46-000-CM
Western Blocking Reagent, 10× Solution, Roche	Millipore Sigma	Cat# 11921673001
Yeast, LeSaffre, Red Star, Active Dry	Amazon	UPC:117929159037
Experimental models: Organisms/Strains		
Fly: OregonR (OreR)	Sokac Lab	N/A
Fly: P{PTT-GA}JupiterG00147 (Jupiter-GFP)	[15]; BDSC	Stock# BL6836
Fly: P{PTT-GB}gish[Spider] (Spider-GFP)	[15]; BDSC	Stock# BL59025
Fly: Myosin RLC-GFP (Spaghetti Squash-GFP)	[16]	N/A
Fly: H2A-GFP (Histone 2A-GFP)	BDSC	Stock# BL24163
Oligonucleotides		
sry-α RNA FISH probe set, 3′ amine modification	Biosearch Technologies	See Table S1 for sequences.
Software and algorithms		
Adobe Illustrator CC	Adobe	https://www.adobe.com/creativecloud
Adobe Photoshop CC	Adobe	https://www.adobe.com/creativecloud

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Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
BioRender	BioRender	https://biorender.com
FIJI/ImageJ	Open Source	https://imagej.net/software/fiji
Other		
BD Hypodermic Needle, 27G$^{1}/_{2}$, BD 305109	Fisher Scientific	Cat# 14-826-48
BD Tuberculin Syringe, 1 mL, BD309602	Fisher Scientific	Cat# 14-823-2F
Bemis™ Parafilm™ M Laboratory Wrapping Film, 4 inch	Fisher Scientific	Cat# 13-374-10
Corning™ Falcon™ Cell Strainers, Corning 352350	Fisher Scientific	Cat# 08-771-2
Disposable Borosilicate Glass Pasteur Pipets, 5.75 inch	Fisher Scientific	Cat# 13-678-20A
Flystuff Embryo Collection Cage-Small, fits 60 mm petri dish	Genesee Scientific	Cat#59-100
Petri dish, plastic, 60 × 15 mm	VWR	Cat# 25384-092
Safe-Lock Microcentrifuge Tubes, 1.5 mL, PCR Clean, Amber (light protection)	Eppendorf	Cat# 022363221
Scintillation vial, 20 mL glass borosilicate with polyethylene linear and urea caps	Fisher Scientific	Cat# 03-337-7
Stainless Steel Mesh 150, 0.0026 inch wire diameter	TWP Inc	T304; SKU# 150X150S0026DISC
Supelco® pH indicator strips, pH 5.0–10.0	Millipore Sigma	Cat# 1.09533; SKU# 1095330001
Supelco® pH indicator strips, pH 0–14.0	Millipore Sigma	Cat# 1.09535; SKU# 1095350001
Supelco® pH indicator strips, pH 4.0–7.0	Millipore Sigma	Cat# 1.09542; SKU# 1095420001
Supelco® pH indicator strips, pH 6.5–10.0	Millipore Sigma	Cat# 1.09543; SKU# 1095430001
Thermo Scientific™ Nalgene™ 80 mm Polypropylene Powder Funnel	Fisher Scientific	Cat# 10–348B
Troemner Class F Test Weight, 200 g, Troemner 1316	Cole-Parmer	Cat# UX-01033-84

MATERIALS AND EQUIPMENT

0.1 M sodium phosphate buffer (pH 7.4)		
Reagent	Final concentration	Amount
$NaH_2PO_4 \cdot H_20$	25.0 mM	3.1 g
Na_2HPO_4 (anhydrous)	76.0 mM	10.9 g
ddH_2O	N/A	to 1.0 L
Total	**N/A**	**1.0 L**
Store at 4°C for up to 6 months.		

⚠ CRITICAL: Pay special attention to the chemical composition of the sodium phosphate powders, as these chemicals are sold in a number of forms (e.g., hexahydrate form), and it is easy to make a mistake that will impact both the concentration and pH of your final solution.

IF PBS·Triton X-100		
Reagent	Final concentration	Amount
Triton X-100	0.1%	50.0 µL
1× PBS, pH 7.4	N/A	to 50.0 mL
Total	**N/A**	**50.0 mL**
This buffer will be the base for all the buffers required for immunofluorescence. Make fresh on the same day that IF staining is initiated. Store at room temperature (~22°C). Discard once the staining is complete.		

Note: The volume can be scaled to accommodate the number of stainings that you are doing. A volume of 50 mL gives sufficient buffer to complete the staining of two tubes of embryos with a single primary antibody or one tube of embryos with two primary antibodies in sequence.

⚠ CRITICAL: Triton X-100 detergent is less stable as a diluted stock, which is why we use undiluted detergent. We recommend always using fresh detergent as we find it to be critical to the quality of the final staining.

Alternative: Tween-20 can be used instead of Triton X-100 for all IF buffers. When optimizing staining for a new antibody, both detergents should be tried and compared to see which gives the best results.

IF wash buffer

Reagent	Final concentration	Amount
BSA	1% wt/vol	0.45 g
PBS·Triton X-100	N/A	to 45.0 mL
Total	**N/A**	**45.0 mL**

Store at room temperature (~22°C) for the duration of the experiment. Discard once the staining is complete.

IF blocking buffer

Reagent	Final concentration	Amount
BSA	10% wt/vol	0.2 g
PBS·Triton X-100	N/A	to 2.0 mL
Total	**N/A**	**2.0 mL**

Store at room temperature (~22°C) for the duration of the experiment. Discard once the staining is complete.

IF antibody buffer

Reagent	Final concentration	Amount
BSA	5% wt/vol	0.15 g
PBS·Triton X-100	N/A	to 3.0 mL
Total	**N/A**	**3.0 mL**

Store at room temperature (~22°C) for the duration of the experiment. Discard once the staining is complete.

FISH PBS·Triton X-100

Reagent	Final concentration	Amount
Triton X-100	0.1%	10.0 µL
10× PBS, (RNase-free)	N/A	1.0 mL
DEPC H_2O	N/A	to 10.0 mL
Total	**N/A**	**10.0 mL**

Make fresh on the same day that RNA FISH or FISH-IF is initiated. Store at room temperature (~22°C). Discard once the staining is complete.

Note: The volume can be scaled to accommodate the number of stainings that you are doing. A volume of 10 mLs gives sufficient buffer to complete staining for one tube of embryos with one set of FISH probes for RNA FISH. For FISH-IF, scale to make 25 mLs total to complete staining for one tube of embryos with one set of FISH probes and one primary antibody.

⚠ CRITICAL: Triton X-100 detergent is less stable as a diluted stock, which is why we use undiluted detergent. We recommend always using fresh detergent as we find it to be critical to the quality of the final staining.

⚠ CRITICAL: For RNA FISH or FISH-IF, take precautions to ensure that reagents and buffers are RNAse-free.

FISH wash buffer

Reagent	Final concentration	Amount
Triton X-100	0.1%	10.0 µL
Formamide (deionized)	20%	1.77 mL
20× SSC (RNase-free)	2×	1.0 mL
DEPC H_2O	N/A	to 10.0 mL
Total	**N/A**	**10.0 mL**

Make fresh on the same day that RNA FISH or FISH-IF is initiated. Store at room temperature (~22°C) for the duration of the experiment. Discard once the staining is complete.

⚠ CRITICAL: Formamide is classified as carcinogenic, mutagenic and/or reprotoxic. Review the manufacturer's Safety Data Sheet, and handle with gloves in a fume hood.

Note: To calculate the final concentration of formamide, we used specific gravity = 1.1334.

FISH hybridization buffer

Reagent	Final concentration	Amount
Dextran Sulfate	100.0 mg/mL	1.0 g
E. coli tRNA	1.0 mg/mL	10.0 mg
200 mM Ribonucleoside Vanadyl Complex	2.0 mM	100.0 µL
50 mg/mL BSA (RNase-free)	0.2 mg/mL	40.0 µL
20× SSC (RNase-free)	2×	1.0 mL
Formamide (deionized)	20%	1.77 mL
DEPC H_2O	N/A	to 10.0 mL
Total	**N/A**	**10.0 mL**

Store as 50 µL aliquots at –20°C for up to 6 months.

FISH SSC·Triton X-100

Reagent	Final concentration	Amount
Triton X-100	0.1%	10.0 µL
20× SSC (RNase-free)	2×	1.0 mL
DEPC H_2O	N/A	to 10.0 mL
Total	**N/A**	**10.0 mL**

Make fresh on the same day that RNA FISH or FISH-IF is initiated. Store at room temperature (~22°C) for the duration of the experiment. Discard once the staining is complete.

FISH-IF Blocking Buffer

Reagent	Final concentration	Amount
Triton X-100	0.1%	5.0 µL
10× PBS, (RNase-free)	1×	0.5 mL
10× Western Blocking Reagent	2×	1.0 mL
200 mM Ribonucleoside Vanadyl Complex	2.0 mM	50.0 µL
DEPC H_2O	N/A	to 5.0 mL
Total	**N/A**	**5.0 mL**

Make fresh on the same day that FISH-IF is initiated. Store at room temperature (~22°C) for the duration of the experiment. Discard once the staining is complete.

Note: The volume can be scaled to accommodate the number of stainings that you are doing. A volume of 5 mLs gives sufficient buffer to complete staining for one tube of embryos with one set of FISH probes and one primary antibody.

Stainless steel mesh basket

To make a basket, cut a circle of 2 cm diameter from a sheet of stainless steel mesh 150 (see key resources table). Poke a finger into the circle and fold the sides up around your finger like a basket. Check that water readily flows through when squirted from a wash bottle into the basket. Baskets can be washed carefully after use and recycled many times. For RNAse-free work, baskets can be soaked in RNAse-Zap and then rinsed thoroughly with RNAse-free water.

STEP-BY-STEP METHOD DETAILS

Section 1: Fix embryos using acid-free glyoxal

Timing: 15 min

This step will yield glyoxal fixed embryos. If you are familiar with standard formaldehyde fixation protocols, then this procedure is similar but with two key differences: First, the fixation time is reduced to 1 min. Second, only the briefest vortexing is required to "pop" glyoxal-fixed embryos out of their vitelline membrane.

1. Prepare a scintillation vial for embryo fixation.
   a. Thaw an aliquot of 12% acid-free glyoxal from the –20°C freezer.
   b. Add 3 mL of 0.1M Sodium Phosphate Buffer, pH 7.4 to a glass scintillation vial. Allow buffer to equilibrate to room temperature (~22°C).
   c. To the scintillation vial, add the thawed 1 mL 12% acid-free glyoxal and 4 mL heptane. Swirl the scintillation vial to mix components.

   Note: The organic and aqueous phases will separate to the top and bottom of the vial, respectively. A clear interface between the phases should be seen.

   Note: This preparation gives a final concentration of 3% glyoxal.

   ⚠ CRITICAL: If the intent is to use the fixed embryos for either RNA FISH or FISH-IF, then take precautions to ensure that reagents, buffers, vials, baskets, tubes, pipets, *et cetera* are RNAse-free. One concise source for tips regarding RNAse-free processing can be found at this ThermoFisher resource link.

2. Retrieve the apple juice plate from an embryo collection cup at the appropriate time for the desired developmental stage, and harvest the embryos as follows.
   a. Squirt fresh bleach onto the apple juice plate, completely covering the surface. Incubate for 30–40 s, swirling occasionally.

   Note: This will release embryos from their outer chorion and a few will float to the top of the bleach near the end of the incubation.

   b. Immediately dump the embryos into a stainless steel mesh basket and rinse vigorously with distilled water. Rinse the apple juice plate with distilled water and dump contents into the basket. Continue washing the embryos in the basket with a generous amount of water.
   c. Wick away the water by dabbing the basket on a clean paper towel. This should be done quickly so that the embryos do not dehydrate.

Figure 7. Demonstration to show how the mesh basket is held at an angle to transfer embryos into the liquid in the scintillation vial

⚠ CRITICAL: Thoroughly rinse the embryos to remove residual bleach. When dabbing the basket on the paper towel, watch carefully for a transient pink color to appear, which indicates the presence of residual bleach. If pink is seen, then continue rinsing the basket with water.

Note: For these steps, it is handy to have the bleach and the distilled water in squeezable wash bottles.

3. Using tweezers, plunge the basket into the heptane/glyoxal mixture in the prepared scintillation vial. Pull out the basket, angling it and shaking carefully so that the embryos remain in the vial (Figure 7).

Note: The embryos will sit at the interface of the organic and aqueous phases.

4. Tightly cap the vial and immediately secure on an orbital shaker. Shake for 1 min at ~250 RPMs.

⚠ CRITICAL: Limit the glyoxal fixation time to 1 min. Longer fixation times lead to a complete loss of antigenicity of proteins in the embryo and failure of subsequent antibody staining by IF.

5. Remove the bottom aqueous phase from the scintillation vial using a glass Pasteur pipet and discard it. Be careful to not aspirate the embryos from the interface.

⚠ CRITICAL: Do remove as much of the bottom phase as possible to limit sample exposure to the glyoxal.

6. Add 4 mL 100% methanol to the embryos and heptane that remain in the scintillation vial.

Note: The heptane and methanol phases will separate to the top and bottom of the vial, respectively. The embryos will remain at the interface.

7. Tightly cap the vial and touch the vial bottom to a vortex set at max for 1 s. This will pop the embryos out of their exterior, vitelline membrane shell. "Popped" embryos will settle to the bottom of the vial.

⚠ CRITICAL: Only touch the vial to the vortex for the popping step. Even if you are used to longer vortexing for formaldehyde fixed embryos, avoid aggressive vortexing here. No embryo debris or clouding of the liquid should be seen (troubleshooting 2).

8. Using a new glass Pasteur pipet, transfer the popped embryos from the bottom of the vial to a 1.5 mL microcentrifuge tube. Some methanol will be transferred with the embryos. Avoid transferring any heptane.

⚠ CRITICAL: Only collect the embryos at the bottom of the vial. These embryos have been released completely from the vitelline membrane. Embryos remaining at the interface retain vitelline membrane, which will act as an impenetrable barrier to antibodies, dyes and FISH probes and so compromise experimental results.

9. Let the embryos settle by gravity to the bottom of the microcentrifuge tube.
10. Remove the methanol and discard. Replace with ~1 mL fresh 100% methanol to wash the embryos. Let the embryos settle by gravity to the bottom of the tube.
11. Repeat the methanol wash three times.
12. Add 1 mL fresh 100% methanol to the embryos and incubate overnight (>8 h) at –20°C to complete the fixation.

Pause point: Embryos can be stained immediately, or they can be stored at –20°C for up to one month for IF (Section 2) or up to one week for RNA FISH or FISH-IF (Section 3 and 4, respectively). If embryo collections are small, then fixed embryos can be pooled to achieve the desired amount before initiating a staining experiment.

Section 2: Immunofluorescence (IF) staining of glyoxal-fixed embryos

Timing: 2–3 days

This step involves the series of washes and antibody incubations required to yield embryos stained for a specific protein. Visualization will follow by confocal microscopy (for example, see Figure 2).

13. Prepare the following four buffers just prior to starting the immunostaining. Recipes are listed in the Materials and Equipment.
    a. IF PBS·Triton X-100, 50 mL.
    b. IF Wash Buffer, 45 mL.
    c. IF Antibody Buffer, 3 mL.
    d. IF Blocking Buffer, 2 mL.

Alternative: In general, Triton X-100 works well for IF. However, if optimizing IF for a primary antibody for the first time, it is advisable to try a side-by-side comparison using either Triton X-100 or Tween-20 as the detergent. Simply replace the Triton X-100 with Tween-20 for all buffers throughout the IF protocol.

14. Using a glass Pasteur pipet, transfer glyoxal fixed embryos to a 1.5 mL amber-colored microcentrifuge tube. Some methanol will be transferred, too.

⚠ CRITICAL: The amber-colored tube is required to preserve the quality of the fluorophores that are conjugated to the secondary antibodies. After secondary antibody incubation, limit exposure of stained embryos to light.

Alternative: If amber-colored tubes are not available or are not preferred, then tubes can be wrapped in aluminum foil to prevent light exposure.

Note: Up to 100 μL volume of embryos can be stained per tube, but fewer will work well, too.

15. Let the embryos settle by gravity to the bottom of the tube. Remove methanol and discard.
16. Rinse the embryos one time as follows:
    a. Add 1 mL IF Wash Buffer.
    b. Allow embryos to settle to bottom of the tube.
    c. Remove and discard buffer.
17. Wash the embryos four times as follows:
    a. Add 1 mL IF Wash Buffer.
    b. Close the tube and incubate for 10 min with gentle nutation (or end-over-end rocking) at room temperature (~22°C).
    c. Allow embryos to settle to bottom of the tube.
    d. Remove and discard buffer.
18. Block the embryos one time as follows:
    a. Add 1 mL IF Blocking Buffer.
    b. Close the tube and incubate for 1 h with gentle nutation at room temperature.
    c. Allow embryos to settle to bottom of the tube.
    d. Remove and discard buffer.
19. Stain the embryos with primary antibody against your target protein as follows:
    a. In a separate tube combine the appropriate volume of primary antibody and IF Antibody Buffer up to 500 μL.
    b. Add the primary antibody mixture to the blocked embryos.
    c. Close the tube and incubate for 4–10 h with gentle nutation at 4°C.
    d. Allow embryos to settle to bottom of the tube.
    e. Remove and discard buffer.

Alternative: If the primary antibody is precious, then the final volume of the antibody incubation can be reduced to 330 μL. After the incubation, the antibody buffer can be collected and saved and used in subsequent IF experiments. Such recycled primary antibody can work well for up to 5–10 subsequent stainings.

Alternative: Faster staining can be achieved by primary antibody incubation for 1 h with nutation at room temperature. However, the faster incubation typically results in more non-specific background staining.

Pause point: The 4–10 h incubation allows the staining to go overnight, offering a good pause point for the experimentalist. On the morning of the following day, simply pick up at Step 19.d.

20. Wash the embryos four times as follows:
    a. Add 1 mL IF Wash Buffer.
    b. Close the tube and incubate for 10 min with gentle nutation at room temperature.
    c. Allow embryos to settle to bottom of the tube.
    d. Remove and discard buffer.
21. Stain the embryos with secondary antibody as follows:
    a. In a separate tube combine 1 μL secondary antibody and IF Antibody Buffer up to 500 μL.

b. Add the secondary antibody mixture to the embryos.
c. Close the tube and incubate for 1 h with gentle nutation at room temperature.
d. Allow embryos to settle to bottom of the tube.
e. Remove and discard buffer.

22. If staining embryos with two primary antibodies, then wash four times as follows, and in Step 22.e, loop back for the second combination of primary/secondary antibody stainings:
    a. Add 1 mL IF Wash Buffer.
    b. Close the tube and incubate for 10 min with gentle nutation at room temperature.
    c. Allow embryos to settle to bottom of the tube.
    d. Remove and discard buffer.
    e. Repeat Steps 19–21 for the second combination of primary/secondary antibodies.
23. After the final secondary antibody incubation, wash the embryos one time as follows:
    a. Add 1 mL IF Wash Buffer.
    b. Close the tube and incubate for 1 min with gentle nutation at room temperature.
    c. Allow embryos to settle to bottom of the tube.
    d. Remove and discard buffer.
24. Stain the DNA/nuclei with Hoechst 33342 as follows:
    a. In a separate tube combine 1.0 μL 20 mM Hoechst dye with 1.0 mL IF Wash Buffer, for a final dilution of 1:1000 Hoechst dye. Mix.
    b. Add 1.0 mL of the diluted Hoechst dye mixture to the embryos.
    c. Close the tube and incubate for 5 min with gentle nutation at room temperature.
    d. Remove and discard buffer.

Alternative: DAPI can be used instead of Hoechst dye. Combine 5 μL 5mg/mL DAPI in 0.5 mL IF Wash Buffer, for a final dilution of 50 μg/mL DAPI. Nutate with embryos for 10 min at room temperature.

25. Wash the embryos four times as follows:
    a. Add 1 mL IF Wash Buffer.
    b. Close the tube and incubate for 10 min with gentle nutation at room temperature.
    c. Allow embryos to settle to bottom of the tube.
    d. Remove and discard buffer.
26. Mount embryos immediately on a microscope slide in AquaPolymount as follows:
    a. Add 200 μL IF Wash Buffer to stained embryos.
    b. Transfer embryos to a slide using a Pasteur pipet.
    c. Wick away excess buffer with the edge of a paper towel.
    d. Add 1 drop of AquaPolymount and arrange embryos as desired using a 27G$^{1}/_{2}$ needle (troubleshooting 2).
    e. Allow AquaPolymount to harden overnight (>8 h).

⚠ CRITICAL: Protect stained embryos from light to the greatest extent possible. Mounting will necessarily occur in the light; but after mounting, slides should be stored in the dark.

Note: It is convenient to secure the 27G$^{1}/_{2}$ needle on the end of a 1 mL syringe and hold the syringe like a pencil to push individual embryos around on the slide.

Alternative: Embryos can also be stored overnight at 4°C in IF Wash Buffer and mounted on a slide on the following day.

27. Apply a coverslip to the prepared slide as follows:
    a. Place the slide embryo side up on a paper towel.
    b. Make an "X" of AquaPolymount from corner to corner of a coverslip of appropriate thickness for the microscope objective that will be used for imaging.

c. Flip the coverslip over so the AquaPolymount is facing down and position directly over the slide.
d. Gently drop the coverslip straight onto the slide, making sure that it does not slide sideways on the surface of the mounted embryos.
e. Cut a paper towel with dimensions just slightly larger than the slide. Place the paper towel on the coverslip, being careful to not move the coverslip.
f. Lower a 200 G metal analytical weight straight onto the sample. Excess AquaPolymount will seep out of the slide/coverslip sandwich and be soaked up by the paper towels.
g. Let the AquaPolymount harden for > 1 h.

⚠ CRITICAL: To avoid distorting or damaging the embryos, limit slippage of the coverslip sideways on the surface of the slide.

Pause point: Once embryos are mounted they are stable for up to a few months. However, best imaging results are achieved with freshly stained embryos.

28. Proceed to imaging (troubleshooting 3; troubleshooting 4).

⚠ CRITICAL: Before imaging, make sure that the coverslip and slide are stable, and the coverslip should be cleaned of residual AquaPolymount to avoid damage to microscope objectives. AquaPolymount can be wiped away using a Kimwipe moistened with water.

Section 3: RNA FISH staining of glyoxal-fixed embryos

Timing: 2–3 days

This step involves the series of washes and oligo hybridization required to yield embryos stained for a specific RNA transcript. Visualization will follow by confocal microscopy (for example, see Figure 4).

29. Prepare the following three buffers just prior to starting the FISH experiment. Recipes are listed in the Materials and Equipment. Also, confirm that an aliquot of FISH Hybridization Buffer is available in the –20°C freezer. Otherwise, that buffer must also be prepared.
    a. FISH PBS·Triton X-100, 10 mL.
    b. FISH Wash Buffer, 10 mL.
    c. FISH SSC·Triton X-100, 10 mL.

Alternative: Triton X-100 works well for RNA FISH. However, if optimizing your protocol for a probe set for the first time, it is advisable to try a side-by-side comparison using either Triton X-100 or Tween-20 as the detergent. Simply replace the Triton X-100 with Tween-20 for all buffers throughout the RNA FISH protocol.

⚠ CRITICAL: Take precautions to ensure that reagents, buffers, tubes, pipets, *et cetera* are RNAse-free.

30. Using a glass Pasteur pipet, transfer glyoxal fixed embryos to a 1.5 mL amber-colored microcentrifuge tube. Some methanol will be transferred, too.

⚠ CRITICAL: The amber-colored tube is required to preserve the quality of the fluorophores that are conjugated to the probes. After hybridization, limit exposure of stained embryos to light.

Alternative: If amber-colored tubes are not available or are not preferred, then tubes can be wrapped in aluminum foil to prevent light exposure.

Note: Up to 50 μL volume of embryos can be stained per tube, but fewer will work well, too.

31. Let the embryos settle by gravity to the bottom of the tube. Remove methanol and discard.
32. Rinse the embryos one time as follows:
    a. Add 1 mL FISH PBS·Triton X-100.
    b. Allow embryos to settle to bottom of the tube.
    c. Remove and discard buffer.
33. Wash the embryos four times as follows:
    a. Add 1 mL FISH PBS·Triton X-100.
    b. Close the tube and incubate for 10 min with gentle nutation (or end-over-end rocking) at room temperature (~22°C).
    c. Allow embryos to settle to bottom of the tube.
    d. Remove and discard buffer.
34. Rinse the embryos one time as follows:
    a. Add 1 mL FISH Wash Buffer.
    b. Allow embryos to settle to bottom of the tube.
    c. Remove and discard buffer.
35. Pre-hybridize the embryos two times as follows:
    a. Add 1 mL FISH Wash Buffer.
    b. Close the tube and incubate for 10 min with gentle nutation (or end-over-end rocking) at room temperature.
    c. Allow embryos to settle to bottom of the tube.
    d. Remove and discard buffer.
36. Hybridize the embryos with fluorophore labeled FISH probes against your target transcript as follows:
    a. In a separate tube combine 1 μL 10 μM FISH probes with 49 μL FISH Hybridization Buffer. If using FISH Hybridization Buffer from the freezer, pre-warm to room temperature.
    b. Add the FISH probe/Hybridization Buffer mixture to the embryos.
    c. Close the tube and incubate for ~16 h with gentle nutation at 27°C.
    d. Allow embryos to settle to bottom of the tube.
    e. Remove and discard buffer.

Alternative: If optimizing your protocol for a probe set for the first time, it is advisable to try a side-by-side comparison using 1 μL of FISH probe at a working concentration of 2, 5, 10 and 20 μM.

Note: Hybridization at 27°C deviates from our published protocol for FISH,[13] where the hybridization temperature used was 30°C. However, we found that 30°C promotes clumping of glyoxal fixed embryos, whereas embryos do not clump at the slightly lower temperature.

Pause point: The 16 h incubation allows the staining to go overnight, offering a good pause point for the experimentalist. On the morning of the following day, simply pick up at Step 36.d.

37. Rinse the embryos one time as follows:
    a. Add 1 mL FISH Wash Buffer.
    b. Allow embryos to settle to bottom of the tube.
    c. Remove and discard buffer.
38. Wash the embryos two times as follows:
    a. Add 1 mL FISH Wash Buffer.
    b. Close the tube and incubate for 30 min with gentle nutation at 27°C.
    c. Allow embryos to settle to bottom of the tube.
    d. Remove and discard buffer.

39. Do a serial dilution of Hoechst 33342 dye and stain the DNA/nuclei as follows:
    a. In a separate tube combine 1 μL 20 mM Hoechst dye with 1 mL FISH Wash Buffer, for a final dilution of 1:1000 Hoechst dye. Mix.
    b. In a separate tube combine 100 μL 1:1000 Hoechst dye with 0.9 mL FISH Wash Buffer, for a final dilution of 1:10,000 Hoechst dye. Mix.
    c. Add 1 mL of the diluted Hoechst dye mixture to the embryos.
    d. Close the tube and incubate for 30 min with gentle nutation at 27°C.
    e. Allow embryos to settle to bottom of the tube.
    f. Remove and discard buffer.
40. Wash the embryos two times as follows:
    a. Add 1 mL FISH SSC·Triton X-100.
    b. Close the tube and incubate for 10 min with gentle nutation at room temperature.
    c. Allow embryos to settle to bottom of the tube.
    d. Remove and discard buffer.
41. Mount embryos immediately on a microscope slide in AquaPolymount as follows:
    a. Add 200 μL FISH SSC·Triton X-100 to stained embryos.
    b. Transfer embryos to a slide using a Pasteur pipet.
    c. Wick away excess buffer with the edge of a paper towel.
    d. Add 1 drop of AquaPolymount and arrange embryos as desired using a 27G$^{1}/_{2}$ needle (troubleshooting 2).
    e. Allow AquaPolymount to harden overnight (>8 h).

⚠ CRITICAL: Protect stained embryos from light to the greatest extent possible. Mounting necessarily occurs in the light; but after mounting, slides should be stored in the dark.

Alternative: Embryos can also be stored overnight at 4°C in FISH SSC·Triton X-100 and mounted on a slide on the following day.

42. Apply a coverslip to the prepared slide as follows:
    a. Place the slide embryo side up on a paper towel.
    b. Make an "X" of AquaPolymount from corner to corner of a coverslip of appropriate thickness for the microscope objective that will be used for imaging.
    c. Flip the coverslip over so the AquaPolymount is facing down and position directly over the slide.
    d. Gently drop the coverslip straight onto the slide, making sure that it does not slide sideways on the surface of the mounted embryos.
    e. Cut a paper towel with dimensions just slightly larger than the slide. Place the paper towel on the coverslip, being careful to not move the coverslip.
    f. Lower a 200 G metal analytical weight straight onto the sample. Excess AquaPolymount will seep out of the slide/coverslip sandwich and be soaked up by the paper towels.
    g. Let the AquaPolymount harden for >1 h.

⚠ CRITICAL: To avoid distorting or damaging the embryos, limit slippage of the coverslip sideways on the surface of the slide.

Pause point: Once embryos are mounted they are stable for up to a few weeks. However, best imaging results are achieved with freshly stained embryos.

43. Proceed to imaging (troubleshooting 3; troubleshooting 4).

⚠ CRITICAL: Before imaging, make sure that the coverslip and slide are stable, and the coverslip should be cleaned of residual AquaPolymount to avoid damage to microscope objectives. AquaPolymount can be wiped away using a Kimwipe moistened with water.

Section 4. FISH-IF staining of glyoxal-fixed embryos

Timing: 3 days

This step involves the series of washes, antibody incubations and oligo hybridization required to yield embryos stained for both a specific protein and RNA transcript. Visualization will follow by confocal microscopy (for example, see Figure 5). A post-fixation step is included after the IF staining and prior to FISH.

44. Prepare the following five buffers just prior to starting the FISH-IF experiment. Recipes are listed in the Materials and Equipment. Also, confirm that an aliquot of FISH Hybridization Buffer is available in the –20°C freezer. Otherwise, that buffer must also be prepared.
    a. FISH PBS·Triton X-100, 25 mL.
    b. FISH-IF Blocking Buffer, 5 mL.
    c. 1× PBS, 5 mL.
    d. FISH Wash Buffer, 10 mL.
    e. FISH SSC·Triton X-100, 10 mL.

    Alternative: Triton X-100 works well for FISH-IF. However, if optimizing your protocol for a probe set for the first time, it is advisable to try a side-by-side comparison using either Triton X-100 or Tween-20 as the detergent. Simply replace the Triton X-100 with Tween-20 for all buffers throughout the FISH-IF protocol.

    ⚠ CRITICAL: Take precautions to ensure that reagents, buffers, tubes, pipets, *et cetera* are RNAse-free.

45. Using a glass Pasteur pipet, transfer fixed embryos to a 1.5 mL amber-colored microcentrifuge tube. Some methanol will be transferred, too.

    ⚠ CRITICAL: The amber-colored tube is required to preserve the quality of the fluorophores that are conjugated to the probes. After hybridization, limit exposure of stained embryos to light.

    Alternative: If amber-colored tubes are not available or are not preferred, then tubes can be wrapped in aluminum foil to prevent light exposure.

    Note: Up to 50 µL volume of embryos can be stained per tube, but fewer will work well, too.

46. Let the embryos settle by gravity to the bottom of the tube. Remove methanol and discard.
47. Rinse the embryos one time as follows:
    a. Add 1 mL FISH PBS·Triton X-100.
    b. Allow embryos to settle to bottom of the tube.
    c. Remove and discard buffer.
48. Wash the embryos four times as follows:
    a. Add 1 mL FISH PBS·Triton X-100.
    b. Close the tube and incubate for 10 min with gentle nutation (or end-over-end rocking) at room temperature (~22°C).
    c. Allow embryos to settle to bottom of the tube.
    d. Remove and discard buffer.
49. Block the embryos one time as follows:
    a. Add 1 mL FISH-IF Blocking Buffer.
    b. Close the tube and incubate for 1 h with gentle nutation at room temperature.
    c. Allow embryos to settle to bottom of the tube.

d. Remove and discard buffer.

50. Stain the embryos with primary antibody against your target protein as follows:
    a. In a separate tube combine the appropriate volume of primary antibody and FISH-IF Blocking Buffer up to 500 μL.
    b. Add the primary antibody mixture to the blocked embryos.
    c. Close the tube and incubate for 4–10 h with gentle nutation at 4°C.
    d. Allow embryos to settle to bottom of the tube.
    e. Remove and discard buffer.

Alternative: If the primary antibody is precious, then the final volume of the antibody incubation can be reduced to 330 μL. After the incubation, the antibody buffer can be collected and saved and used in subsequent immunofluorescence experiments. Such recycled primary antibody can work well for up to 5–10 subsequent stainings.

Alternative: Faster staining can be achieved by primary antibody incubation for 1 h with nutation at room temperature. However, the faster incubation typically results in more non-specific background staining.

Pause point: The 4–10 h incubation allows the staining to go overnight, offering a good pause point for the experimentalist. On the morning of the following day, simply pick up at Step 50.d.

51. Wash the embryos four times as follows:
    a. Add 1 mL FISH PBS·Triton X-100.
    b. Close the tube and incubate for 10 min with gentle nutation at room temperature.
    c. Allow embryos to settle to bottom of the tube.
    d. Remove and discard buffer.
52. Stain the embryos with secondary antibody as follows:
    a. In a separate tube combine 1 μL secondary antibody and FISH-IF Blocking Buffer up to 500 μL.
    b. Add the secondary antibody mixture to the embryos.
    c. Close the tube and incubate for 1 h with gentle nutation at room temperature.
    d. Allow embryos to settle to bottom of the tube.
    e. Remove and discard buffer.
53. Wash the embryos four times as follows:
    a. Add 1 mL FISH PBS·Triton X-100.
    b. Close the tube and incubate for 10 min with gentle nutation at room temperature.
    c. Allow embryos to settle to bottom of the tube.
    d. Remove and discard buffer.
54. Post-fix the embryos as follows:
    a. Add 350 μL of 5 mM BS(PEG)5 in 1× PBS.
    b. Close the tube and incubate for 45 min with gentle nutation at room temperature.
    c. Remove and discard buffer.

Note: Post-fixation can be performed with alternative fixatives. However, we found that BS(PEG)5 gave better results regarding quality of the immunostaining.[17,18]

55. Pre-hybridize the embryos two times as follows:
    a. Add 1 mL FISH Wash Buffer.
    b. Close the tube and incubate for 10 min with gentle nutation at room temperature.
    c. Allow embryos to settle to bottom of the tube.
    d. Remove and discard buffer.

56. Hybridize the embryos with fluorophore labeled FISH probes against your target transcript as follows:
    a. In a separate tube combine 1 μL 10 μM FISH probes with 49 μL FISH Hybridization Buffer.

    Note: If using FISH Hybridization Buffer from the freezer, pre-warm to room temperature.

    b. Add the FISH probe/Hybridization Buffer mixture to the embryos.
    c. Close the tube and incubate for ~16 h with gentle nutation at 27°C.
    d. Allow embryos to settle to bottom of the tube.
    e. Remove and discard buffer.

    Alternative: If optimizing your protocol for a probe set for the first time, it is advisable to try a side-by-side comparison using 1 μL of FISH probe at a working concentration of 2, 5, 10 and 20 μM.

    Note: Hybridization at 27°C deviates from our published protocol for FISH-IF, where the hybridization temperature used was 30°C. However, we found that 30°C promotes clumping of glyoxal fixed embryos, whereas embryos do not clump at the slightly lower temperature.

    ⏸ **Pause point:** The 16 h incubation allows the staining to go overnight, offering a good pause point for the experimentalist. On the morning of the following day, simply pick up at Step 56.d.

57. Rinse the embryos one time as follows:
    a. Add 1 mL FISH Wash Buffer.
    b. Allow embryos to settle to bottom of the tube.
    c. Remove and discard buffer.
58. Wash the embryos two times as follows:
    a. Add 1 mL FISH Wash Buffer.
    b. Close the tube and incubate for 30 min with gentle nutation at 27°C.
    c. Allow embryos to settle to bottom of the tube.
    d. Remove and discard buffer.
59. Do a serial dilution of Hoechst 33342 dye and stain the DNA/nuclei as follows:
    a. In a separate tube combine 1 μL 20 mM Hoechst dye with 1 mL FISH Wash Buffer, for a final dilution of 1:1000 Hoechst dye. Mix.
    b. In a separate tube combine 100 μL 1:1000 Hoechst dye with 0.9 mL FISH Wash Buffer, for a final dilution of 1:10,000 Hoechst dye. Mix.
    c. Add 1 mL of the diluted Hoechst dye mixture to the embryos.
    d. Close the tube and incubate for 30 min with gentle nutation at 27°C.
    e. Allow embryos to settle to bottom of the tube.
    f. Remove and discard buffer.
60. Wash the embryos two times as follows:
    a. Add 1 mL FISH SSC·Triton X-100.
    b. Close the tube and incubate for 10 min with gentle nutation at room temperature.
    c. Allow embryos to settle to bottom of the tube.
    d. Remove and discard buffer.
61. Mount embryos immediately on a microscope slide in AquaPolymount as follows:
    a. Add 200 μL FISH SSC·Triton X-100 to stained embryos.
    b. Transfer embryos to a slide using a Pasteur pipet.
    c. Wick away excess buffer with the edge of a paper towel.
    d. Add 1 drop of AquaPolymount and arrange embryos as desired using a 27G$^{1}/_{2}$ needle (troubleshooting 2).
    e. Allow AquaPolymount to harden overnight (>8 h).

⚠ CRITICAL: Protect stained embryos from light to the greatest extent possible. Mounting necessarily occurs in the light; but after mounting, slides should be stored in the dark.

Alternative: Embryos can also be stored overnight at 4°C in FISH SSC·Triton X-100 and mounted on a slide on the following day.

62. Apply a coverslip to the prepared slide as follows:
    a. Place the slide embryo side up on a paper towel.
    b. Make an "X" of AquaPolymount from corner to corner of a coverslip of appropriate thickness for the microscope objective that will be used for imaging.
    c. Flip the coverslip over so the AquaPolymount is facing down and position directly over the slide.
    d. Gently drop the coverslip straight onto the slide, making sure that it does not slide sideways on the surface of the mounted embryos.
    e. Cut a paper towel with dimensions just slightly larger than the slide. Place the paper towel on the coverslip, being careful to not move the coverslip.
    f. Lower a 200 G metal analytical weight straight onto the sample. Excess AquaPolymount will seep out of the slide/coverslip sandwich and be soaked up by the paper towels.
    g. Let the AquaPolymount harden for >1 h.

⚠ CRITICAL: To avoid distorting or damaging the embryos, limit slippage of the coverslip sideways on the surface of the slide.

Pause point: Once embryos are mounted they are stable for up to a few weeks. However, best imaging results are achieved with freshly stained embryos.

63. Proceed to imaging (troubleshooting 3; troubleshooting 4).

⚠ CRITICAL: Before imaging, make sure that the coverslip and slide are stable, and the coverslip should be cleaned of residual AquaPolymount to avoid damage to microscope objectives. AquaPolymount can be wiped away using a Kimwipe moistened with water.

Alternative: If optimizing your protocol for an antibody/probe set pair for the first time, it is advisable to try (i) doing the IF and FISH in the order described here but skipping the post-fixation step; and (ii) reversing the order so that FISH precedes IF.

EXPECTED OUTCOMES

We found that embryo fixation using acid-free glyoxal gives equivalent or superior results in IF, RNA FISH and FISH IF experiments compared to embryo fixation with formaldehyde (Figures 2, 4, and 5). For example, in all cases where we tested an antibody known to give specific IF staining patterns in formaldehyde fixed embryos, we saw the same staining pattern in glyoxal fixed embryos (Figure 2, Peanut, Discs Large and Lamin). In addition, some antibodies that failed in IF staining of formaldehyde fixed embryos yielded expected localization patterns in glyoxal fixed embryos (Figure 2, Bicaudal D, Dynein Heavy Chain and α-Actinin). Glyoxal and formaldehyde fixation also gave similar results when identifying transcriptional foci in nuclei in RNA FISH experiments (Figure 4B), with glyoxal fixation tending to show a higher signal to noise ratio. Finally, we found that glyoxal-based crosslinking reliably preserved tissue structure, as judged by comparing protein localization in live versus glyoxal fixed embryos (Figure 8).

We also found that glyoxal fixation offers some additional advantages. First, glyoxal is considered less toxic than other common fixatives, including formaldehyde or glutaraldehyde.[1,2,19,20] In contrast to formaldehyde, glyoxal is not yet classified as a carcinogen (see ToxFAQs at the

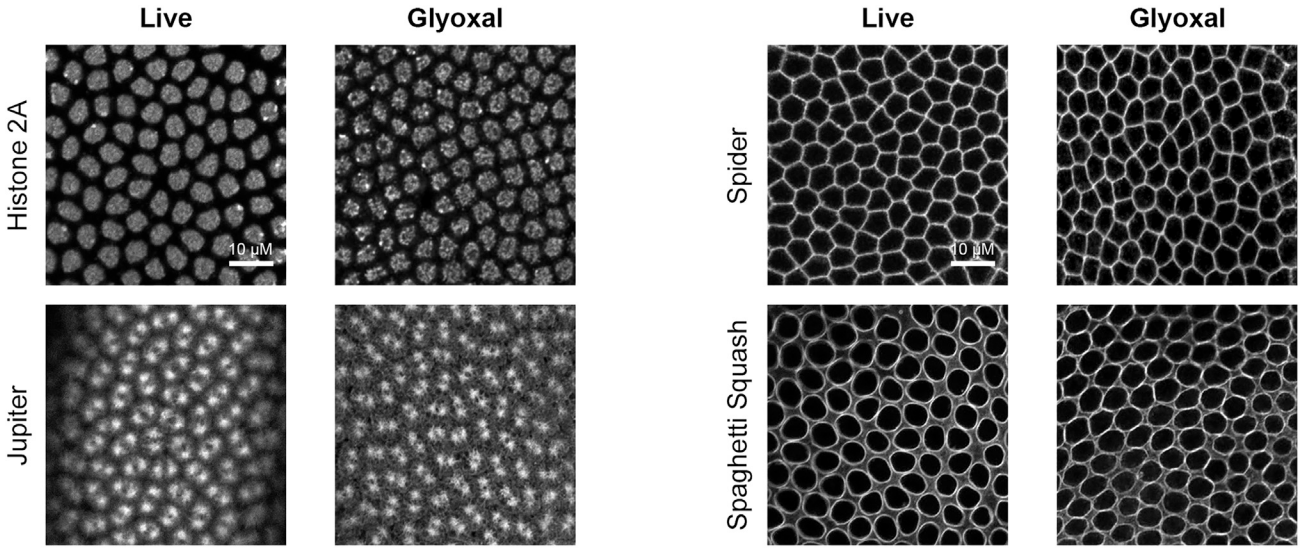

Figure 8. Comparison of tissue structure in live or acid-free glyoxal fixed embryos

Embryos expressing GFP fusion proteins were either imaged live (Live) or were fixed with acid-free glyoxal (Glyoxal) for 1 min and then immunostained with anti-GFP primary antibody. Fusion proteins label multiple structures, including DNA (Histone 2A-GFP), centrosomally-enriched microtubules (Jupiter-GFP), plasma membrane furrows (Spider-GFP; *D.m.* Casein Kinase 1γ), and actomyosin contractile rings (Spaghetti Squash-GFP; *D.m.* Myosin Regulatory Light Chain). Tissue structure looks similar in live and fixed embryos, suggesting reliable preservation with acid-free glyoxal. Scale bars 10 microns.

Agency for Toxic Substances and Disease Registry, CDC, USA). Unlike either formaldehyde or glutaraldehyde, glyoxal is non-volatile and does not emit hazardous fumes during use.[1,21] While care must still be taken with glyoxal because it is an irritant (see PubChem at the National Library of Medicine, NIH, USA), risks to both immediate lab members and the environment are somewhat mitigated when it is used in place of other aldehydes for fixation.

Second, we found that glyoxal fixation saves time since embryo yields per fixation are higher due to more efficient methanol popping when compared to formaldehyde fixation (Figure 9). The popping step removes the embryo's impermeable, wax-coated, vitelline membrane shell.[22,23] Popping is required so that antibodies and probes gain maximal access to embryos during staining.[24,25] For formaldehyde fixation, the methanol popping step can lead to loss of up to half of the embryos in a sample.[8,9] This loss is even worse for some genotypes or when the number of embryos being fixed is small.[10] In contrast, embryo yields for methanol popping during glyoxal fixation were almost 100% and showed no dependence on starting embryo numbers (Figure 9B). This improved yield means that fewer embryo collections and fixations are needed to reach adequate embryo numbers for staining experiments.

Given our results, we recommend adding glyoxal fixation to the panel of conditions that are tested during optimization of staining for any antibody and/or FISH probe set. For antibody applications where acid free glyoxal gives unsatisfactory results, it is worthwhile to try acidic glyoxal fixations at pH 4.0 and 5.0 [3], because we occasionally saw some antibodies perform better at lower pH. Alternatively, if co-staining with two primary antibodies is hindered in an IF experiment due to incompatible fixation methods (e.g., formaldehyde versus heat), glyoxal might be tested as a condition that is suitable for both antibodies.

LIMITATIONS

Our method does have some limitations: First, the fixation time must be scrupulously monitored because staining results rapidly deteriorate with fixation times exceeding 1 min (Figure 10). This is particularly counterintuitive if you are used to working with formaldehyde, where some fixation times for fly embryos can extend to 30 min. However, glyoxal penetrates and crosslinks molecules faster

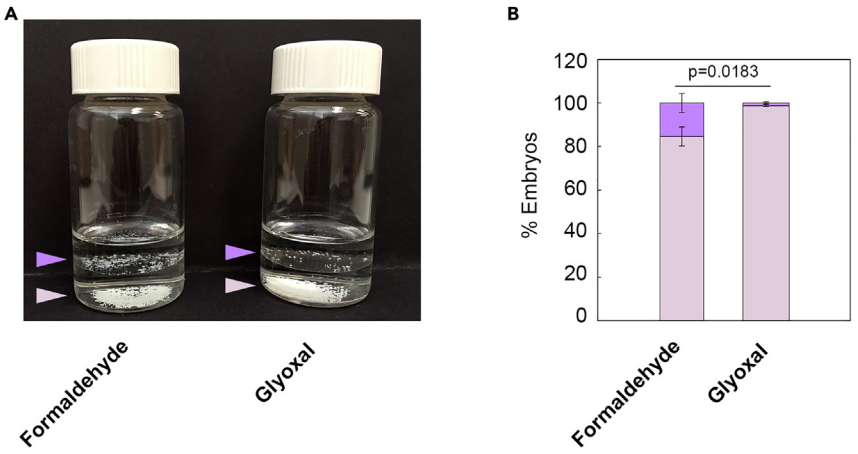

Figure 9. Comparison of popping efficiency for formaldehyde or acid-free glyoxal fixed embryos

Embryos were fixed with formaldehyde for 15 min (Formaldehyde) or acid-free glyoxal for 1 min (Glyoxal) and then methanol popped to remove the vitelline membrane.

(A) Image shows scintillation vials just after popping where embryos retaining their vitelline membrane continue to float at the interface between the heptane and methanol layers (darker purple arrowheads), whereas embryos that were released from the vitelline membrane settle to the bottom (light purple arrowheads). Embryos at the bottom of the vial can be harvested and stained, while unpopped embryos should be discarded.

(B) Quantification shows that the proportion of popped embryos (light purple) to unpopped embryos (darker purple) is greater following glyoxal fixation. An unpaired two-tailed t-test was used to calculate the p-value for unpopped embryos (n > a total of 7000 embryos from four independent poppings per fixation type).

than formaldehyde.[2,3] So, fixation times that work well for standard formaldehyde-based methods are too long and cause over-fixation with glyoxal.[26–28] The result will be diffuse non-specific staining with your antibody or probe (Figure 10).

Second, perhaps due to the stronger fixation activity that has been attributed to glyoxal,[2] the tissue can be somewhat brittle causing the posteriorly protruding pole cells in early embryos to be lost during fixation. However, this problem can be entirely avoided if vortexing during the methanol popping step is limited to just a few seconds.

Third, the quality of DNA staining in glyoxal fixed embryos is very dependent on the experimenter's technique. Glyoxal at low pH negatively impacts fixed nucleic acid integrity,[1,11] and our results for

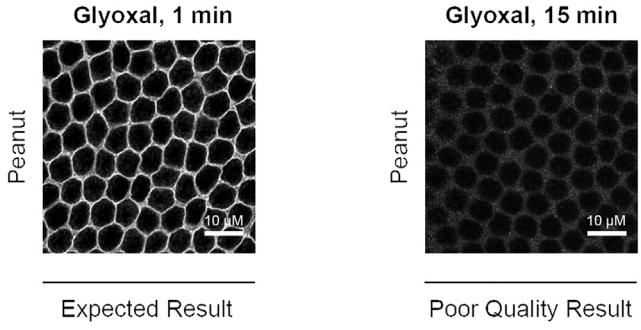

Figure 10. Comparison of IF results following fixation with acid-free glyoxal for different times

Embryos were fixed with acid-free glyoxal for 1 or 15 min (min) as indicated and then immunostained with anti-Peanut primary antibody. Fixation for 1 min maintains antigenicity of Peanut protein and supports good quality staining, where signal concentrates on plasma membrane furrows as expected in cellularizing embryos. Longer fixation results in loss of antigenicity: Fixation for 15 min shows failed Peanut staining, where diffuse signal fills the cytoplasmic space and no specific furrow signal is detected. Scale bars 10 microns.

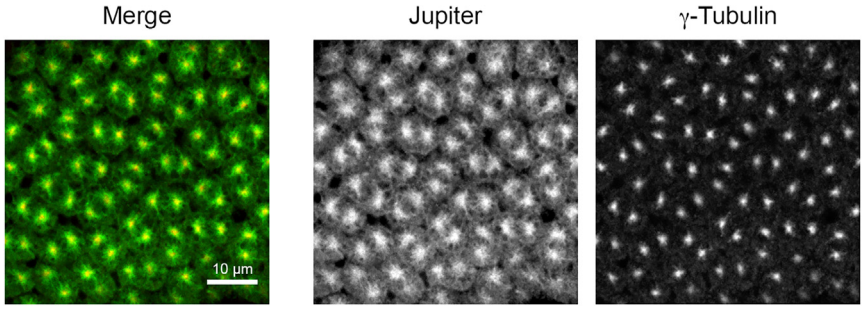

Figure 11. Results for IF of centrosomal proteins in acid-free glyoxal fixed embryos

Embryos expressing Jupiter-GFP were fixed with acid-free glyoxal for 1 min and then immunostained with anti-GFP (green) and anti-γ-Tubulin (red) primary antibodies. Merged image shows overlap of Jupiter foci with γ-Tubulin, which is a component of centrosomes. Scale bar 10 microns.

embryos fixed with acidic glyoxal gave very poor Hoechst staining results (Figure 3). This led us to neutralize the glyoxal prior to use by deionization over the mixed bed ion exchange resin as described.[1,12] Under these conditions, Hoechst staining was dramatically improved to the extent required for our purposes. Discrete nuclei were visible with strong signal intensity (Figure 3).

Fourth, despite our best efforts, we were not able to optimize the glyoxal protocol to preserve microtubule structures. Our inability to preserve microtubules with glyoxal is consistent with results reported by others.[2] We tried to optimize for microtubule preservation by fixing with glyoxal at different concentrations, at pH 4.0 or 5.0, and with the microtubule stabilizer, Taxol. Nevertheless, we did not find an improved condition for microtubule preservation. Interestingly, we did find that centrosomes, within microtubule organizing centers (MTOC), were preserved by glyoxal fixation. Specifically, we were able to immunostain centrosomes, using γ-Tubulin antibodies, as well as other proteins concentrating at MTOCs, including Jupiter (Figure 11), the microtubule minus-end directed motor Dynein, and its cargo adapter Bicaudal-D, with results that surpassed formaldehyde fixation (Figure 2).[29,30]

Fifth, glyoxal fixed embryos can be hand-peeled rather than methanol popped for actin filament staining using phalloidin, but only with the greatest of care. Embryos are easily damaged and yields are very low after peeling. Since we have not yet developed a solution to this problem, we do not regularly hand-peel glyoxal fixed embryos.

One final word of caution: We optimized our glyoxal fixation method for use with *Drosophila* embryos at early developmental stages 1–5.[31] We have not exhaustively tested the effectiveness of the method in later developmental stages when staining is notably more difficult.[32] However, we have successfully completed IF in later stage embryos (Figure 12) and observed protein localization patterns that were expected based on previously published IF results in formaldehyde fixed embryos.[33–35]

TROUBLESHOOTING

Problem 1

The pH of the glyoxal is lower than 7.0 following incubation with Amberlite resin (before you begin/ Step 9).

Potential solution

The deionization was incomplete. The glyoxal can be re-incubated with a newly prepared batch of resin, starting from the beginning of the protocol. If the low pH problem persists, then we strongly recommend changing the water source. We get consistently good results with the molecular biology grade water from Corning (see, key resources table).

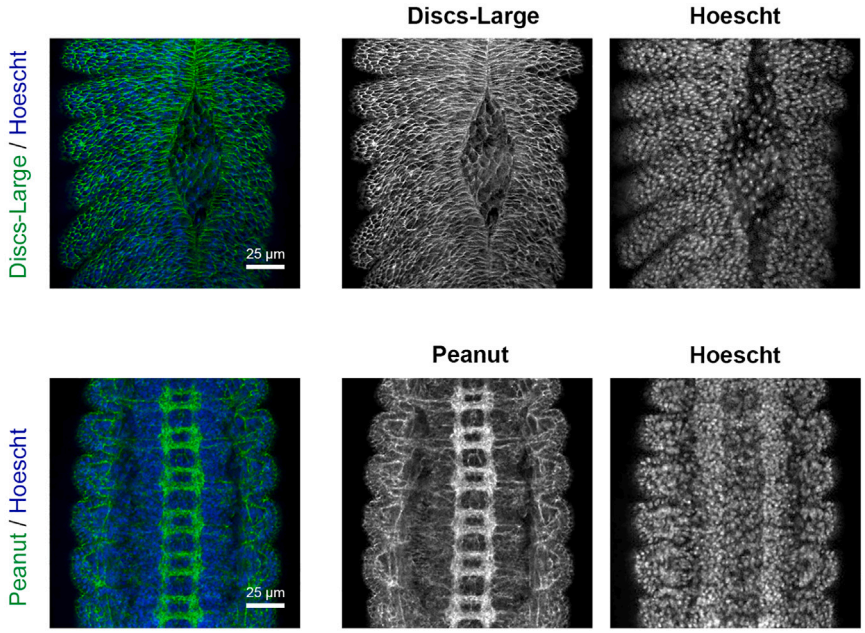

Figure 12. Results for IF in acid-free glyoxal fixed embryos at later development stages

Post-Stage 5 embryos were fixed with acid-free glyoxal for 1 min and then immunostained with anti-Discs-Large (top, green) or anti-Peanut (bottom, green) primary antibodies. Nuclei were stained with Hoechst (blue). Discs-Large was seen at cell surfaces and Septin was seen in neurons, as described previously from stainings in formaldehyde fixed embryos. Scale bars 25 microns.

Problem 2

The pH of the glyoxal is higher than 7.0 following incubation with Amberlite resin (before you begin/ Step 9).

Potential solution

The rinses were insufficient to remove the 1M NaOH from the Amberlite resin. Both the used resin and glyoxal should be discarded and the deionization repeated with fresh reagents.

Problem 3

After vortexing to achieve embryo popping in the fixation step, embryo debris is evident in the scintillation vial and/or many of the embryos are fragmented (Step 7). Alternatively, at the embryo mounting step for IF, RNA FISH and FISH-IF, you observe that the pole cells broke away from the embryos (Steps 26, 41 and 61, respectively).

Potential solution

Reduce the vortexing time for embryo popping during fixation. Just touching the vial to the vortex for 1 s will be sufficient to pop most of the embryos.

Problem 4

Only diffuse staining or bright vitelline envelope staining is observed when imaging IF, RNA FISH and FISH-IF embryos (Steps 28, 43 and 63, respectively).

Potential solution

Poor staining quality or non-specific staining patterns can have many explanations, so must be navigated methodically and systematically with proper controls. However, one likely explanation to consider when working with glyoxal fixation for the first time is that the embryos were fixed too

long in the acid-free glyoxal. To address this issue, simply limit embryo fixation time to 1 min as described, and work quickly to reduce exposure time of the embryos to glyoxal crosslinking activity.

Problem 5

Hoechst signal appears uniform rather than showing discrete nuclei when imaging IF, RNA FISH and FISH-IF embryos (Steps 28, 43 and 63, respectively).

Potential solution

Confirm that the glyoxal pH is neutral and not acidic; and use the specific Hoechst dilutions described for each protocol. Note that the dilutions differ for IF versus RNA FISH and FISH-IF.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Anna Marie Sokac (asokac@illinois.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate new unique datasets or code.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2023.102385.

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AUTHOR CONTRIBUTIONS

A.M.S. and X.W. initiated method development; S.A., X.W., M.B., V.B., A.D., S.M., A.M.S., and S.Z. tested antibody compatibility; S.A. and T.M. completed RNA-FISH analysis; S.A. optimized FISH-IF; X.W. and V.B. completed embryo popping analysis; A.M.S. performed live imaging; A.M.S. supervised the work; A.M.S., S.A., M.B., and T.M. prepared the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

1. Bussolati, G., Annaratone, L., Berrino, E., Miglio, U., Panero, M., Cupo, M., Gugliotta, P., Venesio, T., Sapino, A., and Marchiò, C. (2017). Acid-free glyoxal as a substitute of formalin for structural and molecular preservation in tissue samples. PLoS One *12*, e0182965. https://doi.org/10.1371/journal.pone.0182965.

2. Richter, K.N., Revelo, N.H., Seitz, K.J., Helm, M.S., Sarkar, D., Saleeb, R.S., D'Este, E., Eberle, J., Wagner, E., Vogl, C., et al. (2018). Glyoxal as an alternative fixative to formaldehyde in immunostaining and super-resolution microscopy. EMBO J. *37*, 139–159. https://doi.org/10.15252/embj.201695709.

3. Dapson, R.W. (2007). Glyoxal fixation: how it works and why it only occasionally needs antigen retrieval. Biotech. Histochem. *82*, 161–166. https://doi.org/10.1080/10520290701488113.

4. Paavilainen, L., Edvinsson, A., Asplund, A., Hober, S., Kampf, C., Pontén, F., and Wester, K. (2010). The impact of tissue fixatives on morphology and antibody-based protein profiling in tissues and cells. J. Histochem. Cytochem. *58*, 237–246. https://doi.org/10.1369/jhc.2009.954321.

5. Tubbs, R.R., Hsi, E.D., Hicks, D., and Goldblum, J. (2004). Molecular pathology testing of tissues fixed in prefer solution. Am. J. Surg. Pathol. *28*, 417–419. https://doi.org/10.1097/00000478-200403000-00022.

6. Umlas, J., and Tulecke, M. (2004). The effects of glyoxal fixation on the histological evaluation of breast specimens. Hum. Pathol. *35*, 1058–1062. https://doi.org/10.1016/j.humpath.2004.04.013.

7. Narasimha, M., and Brown, N.H. (2006). Chapter 8 - confocal microscopy of *Drosophilia* embryos. In Cell Biology, Third Edition, J.E. Celis, ed. (Academic Press), pp. 77–86. https://doi.org/10.1016/B978-012164730-8/50136-2.

8. Rothwell, W.F., and Sullivan, W. (2007). Fixation of *Drosophila* embryos. CSH Protoc. *2007*. pdb.prot4827. https://doi.org/10.1101/pdb.prot4827.

9. Rothwell, W.F., and Sullivan, W. (2007). *Drosophila* embryo collection. CSH Protoc. *2007*. pdb.prot4825. https://doi.org/10.1101/pdb.prot4825.

10. Theurkauf, W.E. (1994). Immunofluorescence analysis of the cytoskeleton during oogenesis and early embryogenesis. Methods Cell Biol. *44*, 489–505. https://doi.org/10.1016/s0091-679x(08)60928-0.

11. Lassalle, S., Hofman, V., Marius, I., Gavric-Tanga, V., Brest, P., Havet, K., Butori, C., Selva, E., Santini, J., Mograbi, B., and Hofman, P. (2009). Assessment of morphology, antigenicity, and nucleic acid integrity for diagnostic thyroid pathology using formalin substitute fixatives. Thyroid *19*, 1239–1248. https://doi.org/10.1089/thy.2009.0095.

12. Maniatis, T., Fritsch, E.F., and Sambrook, J. (1982). Molecular Cloning: A Laboratory Manual (Cold Spring Harbor Laboratory).

13. Xu, H., Sepúlveda, L.A., Figard, L., Sokac, A.M., and Golding, I. (2015). Combining protein and mRNA quantification to decipher transcriptional regulation. Nat. Methods *12*, 739–742. https://doi.org/10.1038/nmeth.3446.

14. Figard, L., and Sokac, A.M. (2011). Imaging cell shape change in living *Drosophila* embryos. J. Vis. Exp. https://doi.org/10.3791/2503.

15. Morin, X., Daneman, R., Zavortink, M., and Chia, W. (2001). A protein trap strategy to detect GFP-tagged proteins expressed from their endogenous loci in *Drosophila*. Proc. Natl. Acad. Sci. USA *98*, 15050–15055. https://doi.org/10.1073/pnas.261408198.

16. Royou, A., Field, C., Sisson, J.C., Sullivan, W., and Karess, R. (2004). Reassessing the role and dynamics of nonmuscle myosin II during furrow formation in early *Drosophila* embryos. Mol. Biol. Cell *15*, 838–850. https://doi.org/10.1091/mbc.e03-06-0440.

17. Cheng, Y., Liu, M., Hu, M., and Wang, S. (2021). TAD-like single-cell domain structures exist on both active and inactive X chromosomes and persist under epigenetic perturbations. Genome Biol. *22*, 309. https://doi.org/10.1186/s13059-021-02523-8.

18. Takei, Y., Yun, J., Zheng, S., Ollikainen, N., Pierson, N., White, J., Shah, S., Thomassie, J., Suo, S., Eng, C.H.L., et al. (2021). Integrated spatial genomics reveals global architecture of single nuclei. Nature *590*, 344–350. https://doi.org/10.1038/s41586-020-03126-2.

19. Buesa, R.J. (2008). Histology without formalin? Ann. Diagn. Pathol. *12*, 387–396. https://doi.org/10.1016/j.anndiagpath.2008.07.004.

20. Titford, M. (2001). Safety considerations in the use of fixatives. J. Histotechnol. *24*, 165–171. https://doi.org/10.1179/his.2001.24.3.165.

21. Betterton, E.A., and Hoffmann, M.R. (1988). Henry's law constants of some environmentally important aldehydes. Environ. Sci. Technol. *22*, 1415–1418. https://doi.org/10.1021/es00177a004.

22. Margaritis, L.H., Kafatos, F.C., and Petri, W.H. (1980). The eggshell of *Drosophila* melanogaster. I. Fine structure of the layers and regions of the wild-type eggshell. J. Cell Sci. *43*, 1–35. https://doi.org/10.1242/jcs.43.1.1.

23. Zalokar, M. (1971). Transplantation of nucle in Drosophila melanogaster. Proc. Natl. Acad. Sci. USA *68*, 1539–1541. https://doi.org/10.1073/pnas.68.7.1539.

24. Mitchison, T.J., and Sedat, J. (1983). Localization of antigenic determinants in whole *Drosophila* embryos. Dev. Biol. *99*, 261–264. https://doi.org/10.1016/0012-1606(83)90275-0.

25. Rand, M.D., Kearney, A.L., Dao, J., and Clason, T. (2010). Permeabilization of *Drosophila* embryos for introduction of small molecules. Insect Biochem. Mol. Biol. *40*, 792–804. https://doi.org/10.1016/j.ibmb.2010.07.007.

26. Eltoum, I., Fredenburgh, J., and Grizzle, W.E. (2001). Advanced concepts in fixation: 1. Effects of fixation on immunohistochemistry, reversibility of fixation and recovery of proteins, nucleic acids, and other molecules from fixed and processed tissues. 2. Developmental methods of fixation. J. Histotechnol. *24*, 201–210. https://doi.org/10.1179/his.2001.24.3.201.

27. Eltoum, I., Fredenburgh, J., Myers, R.B., and Grizzle, W.E. (2001). Introduction to the theory and practice of fixation of tissues. J. Histotechnol. *24*, 173–190. https://doi.org/10.1179/his.2001.24.3.173.

28. Melan, M.A. (1994). Overview of cell fixation and permeabilization. Methods Mol. Biol. *34*, 55–66. https://doi.org/10.1385/0-89603285-x:55.

29. Schejter, E.D., and Wieschaus, E. (1993). Functional elements of the cytoskeleton in the early *Drosophila* embryo. Annu. Rev. Cell Biol. *9*, 67–99. https://doi.org/10.1146/annurev.cb.09.110193.000435.

30. Sharp, D.J., Brown, H.M., Kwon, M., Rogers, G.C., Holland, G., and Scholey, J.M. (2000). Functional coordination of three mitotic motors in *Drosophila* embryos. Mol. Biol. Cell *11*, 241–253. https://doi.org/10.1091/mbc.11.1.241.

31. Bownes, M. (1975). A photographic study of development in the living embryo of *Drosophila* melanogaster. J. Embryol. Exp. Morphol. *33*, 789–801. https://doi.org/10.1242/dev.33.3.789.

32. Manning, L., and Doe, C.Q. (2017). Immunofluorescent antibody staining of intact *Drosophila* larvae. Nat. Protoc. *12*, 1–14. https://doi.org/10.1038/nprot.2016.162.

33. Neufeld, T.P., and Rubin, G.M. (1994). The Drosophila peanut gene is required for cytokinesis and encodes a protein similar to yeast putative bud neck filament proteins. Cell *77*, 371–379. https://doi.org/10.1016/0092-8674(94)90152-X.

34. Fares, H., Peifer, M., and Pringle, J.R. (1995). Localization and possible functions of *Drosophila* septins. Mol. Biol. Cell *6*, 1843–1859. https://doi.org/10.1091/mbc.6.12.1843.

35. Bonnello, T.T., Choi, W., and Peifer, M. (2019). Scribble and Discs-large direct initial assembly and positioning of adherens junctions during the establishment of apical-basal polarity. Development *146*, 1–18. https://doi.org/10.1242/dev.180976.