Video Article

A Method of Permeabilization of *Drosophila* Embryos for Assays of Small Molecule Activity

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Abstract

The *Drosophila* embryo has long been a powerful laboratory model for elucidating molecular and genetic mechanisms that control development. The ease of genetic manipulations with this model has supplanted pharmacological approaches that are commonplace in other animal models and cell-based assays. Here we describe recent advances in a protocol that enables application of small molecules to the developing fruit fly embryo. The method details steps to overcome the impermeability of the eggshell while maintaining embryo viability. Eggshell permeabilization across a broad range of developmental stages is achieved by application of a previously described d-limonene embryo permeabilization solvent (EPS[1]) and by aging embryos at reduced temperature (18 °C) prior to treatments. In addition, use of a far-red dye (CY5) as a permeabilization indicator is described, which is compatible with downstream applications involving standard red and green fluorescent dyes in live and fixed preparations. This protocol is applicable to studies using bioactive compounds to probe developmental mechanisms as well as for studies aimed at evaluating teratogenic or pharmacologic activity of uncharacterized small molecules.

Video Link

The video component of this article can be found at https://www.jove.com/video/51634/

Introduction

The *Drosophila* embryo continues to be a premier model for investigation of fundamental mechanisms of development[2]. This powerful model is supported by a wide array of molecular genetic tools that permit manipulations of essentially any gene at any time point and within any developing organ. The small size, rapid development, and extensive characterization of morphogenesis of the *Drosophila* embryo make it a model of choice for genetic screens, many of which have uncovered fundamental developmental pathways[3,4]. Numerous phenotypes in the *Drosophila* embryo have been characterized and are easily interpretable, often providing a means to identify underlying molecular genetic mechanisms responsible for an abnormal trait.

Historically, a shortcoming of the fly embryo model has been the difficulty of introducing small molecules to embryonic tissues. This obstacle has posed limitations on: 1) using known bioactive small molecules as probes to interrogate developmental mechanisms and 2) using this established model to evaluate teratogenic or pharmacologic activity of uncharacterized small molecules. As a consequence, the screening potential of the fly embryo has been underutilized in characterization of small molecule activity.

Delivery of small molecules to the fly embryo can be achieved with two methods: 1) permeabilization of the eggshell and 2) microinjection. This article presents advances to the method of permeabilization that are easy to execute in the setting of a conventional *Drosophila* laboratory. It should be noted that recent advances in microinjection methods with microfluidics technology is also contributing to methods of introducing compounds to the embryo[5,6]. Introducing molecules to the embryo is prevented by a waxy layer of the eggshell[7]. The *Drosophila* eggshell consists of five layers. From the inside out they are: the vitelline membrane, the waxy layer, the inner chorionic layer, the endochorion and the exochorion[8]. The three outer chorionic layers can be removed by brief emersion of the embryo in dilute bleach, a step referred to as dechorionation. The exposed waxy layer can then be compromised by exposure to organic solvents, such as heptane and octane[7,9], rendering the dechorionated embryo permeable, while it remains encased in the underlying vitelline membrane. However, use of these solvents introduces complications due to their toxicity and the difficulty in regulating their strong permeabilizing action, both of which have stark negative effects on embryo viability[9,10].

A method of permeabilization using a composition termed embryo permeabilization solvent (EPS) has been previously described[1]. This solvent consists of d-limonene and plant-derived surfactants that enable the solvent to be miscible with aqueous buffers. The low toxicity of d-limonene and the ability to dilute the solvent to desired concentrations has yielded an effective method to generate permeable embryos with high viability[1]. However, two endogenous factors have continued to bring limitations to the application. First, embryos demonstrate heterogeneity in permeability after EPS treatment, even when care is taken to maintain close developmental staging. Second, embryos older than approximately eight hours have proven difficult to permeabilize, consistent with a hardening of the eggshell that occurs after egg laying[11].

Described here are advances in the EPS method that: 1) assist in identifying and analyzing near-identically permeabilized embryos, even after fixation and immunostaining steps have been executed and 2) enable permeabilization of embryos at late developmental time points (>8 hr, stage 12 and older). Specifically, application of a far-red dye, CY5 carboxylic acid, is described that serves as a permeability indicator, which persists in the embryo during development and after formaldehyde fixation. In addition, it is shown that rearing embryos at 18 °C maintains the eggshell in an EPS sensitive state, enabling permeabilization of late stage embryos (stages 12-16).

These advances overcome the previously mentioned limitations to the EPS methodology. This application will therefore provide investigators with a means to introduce small molecules of interest to the embryo at distinct developmental time points while maintaining viability.

Protocol

1. Preparation of Fly Cultures, Solutions, and Embryo Handling Devices

1. Prepare a cage culture of *Drosophila*. Place 500+ mating flies of the desired strain in a population cage fitted with a 10 cm grape-agar plate and a spot of yeast paste. Maintain culture in a 25 °C humidity controlled incubator. NOTE: Cage cultures require a day or two of conditioning to obtain consistent embryo laying patterns. Grape plates with yeast paste are changed once in the morning and once in the evening during conditioning.
2. Prepare EPS. Warm solutions of surfactants (cocamide DEA and ethoxylated alcohol) at 37 °C. Pipette 18 ml of d-limonene to a glass scintillation vial equipped with a small stir bar. Pipette 1 ml each of the two surfactants (5% final concentration of each) to the d-limonene. Mix thoroughly and remove stir bar. NOTE: This stock solution of EPS is good for approximately 2 months at room temperature. The solution should be warmed at 37 °C and swirled to fully solubilize surfactants prior to use. EPS and d-limonene should be stored in a glass container as they will dissolve some plastics over time.
3. Prepare embryo dechorionation solution, incubation mediums, dye and drug solutions. Mix 25 ml of bleach with 25 ml H_2O and place in shallow dish. Prepare modified basic incubation medium (MBIM) and MBIM-T according to prior recipe[1,12]. Prepare Shields and Sang M3 cell culture medium and PBS according to manufacturer's protocol (see Materials List). Prepare stock solution of permeabilization dye at 10 mM concentration in DMSO.
4. Assemble embryo-handling devices and supplies:
    1. Prepare dechorionation and EPS treatment basket (see **Figure 1A**). Cut off a 3 cm section of a disposable 50 ml polypropylene centrifuge/culture tube with a fine-toothed saw. Make the welding surface flush by rubbing the tube section on sandpaper adhered to a benchtop surface. Weld the tube section to Nitex nylon mesh by melting the rim of the tube section over a flame and pressing on to the mesh on a glass plate. Let cool and trim off extra mesh. NOTE: The sheer sides and bottom allow for rapid and complete rinsing off of residual bleach and EPS at the respective steps. The welding steps should be carried out under a fume hood.
    2. Prepare a development basket. Cut off top portion of a 50 ml centrifuge/culture tube flush with the rim of the cap. Remove and modify the cap by cutting out a central opening and notches along the rim as shown in **Figure 1B**. Screw the cap over the mesh and on the threads of the cut off tube section and trim extra mesh. NOTE: The cap contains notches in the rim that permit diffusion to the bulk medium when positioned in the 60 mm reservoir dish (**Figure 1B'**).
    3. Prepare components of a slide chamber. Cut a square of DO membrane that is larger than the slide chamber opening. Apply a very thin layer of vacuum grease to the inside lip of the opening. Affix the DO membrane into the opening sealing it against the grease with the retainer ring. Trim excess DO membrane by cutting it back close to the retainer ring. NOTE: Specifications for the slide chamber can be found in Kiehart *et al*[13].This chamber is not commercially available and requires custom fabrication by a machine shop.

2. Staging, Dechorionation, and EPS Treatment of Embryos

1. Staging embryos by timed collection. Set a fresh grape/yeast plate to the fly culture cage in the AM. Allow embryos to be laid for 1 hr at 25 °C. Discard this plate and replace with a fresh grape/yeast plate for a subsequent embryo laying of 2 hr at 25 °C. Collect this plate and place in 18 °C incubator for further developmental staging. NOTE: 1 hr of development at 25 °C is equal to 2 hr of development at 18 °C. The effect of aging at 18 °C versus 25 °C on maintaining EPS permeabilization in late stage embryos can be seen in **Figure 2**.
2. Dechorionation. Gently rinse embryos off of grape plate into mesh basket using 25 °C tap water and a paintbrush. Rinse excess yeast away from the embryos in the basket under a gentle stream of tap water. Immerse the basket in 50% bleach for 2 min. Wash embryos thoroughly under a stream of tap water. NOTE: Gently squirt bleach solution on the embryos intermittently using a plastic pipette. While 2 min is usually sufficient for complete dechorionation, this incubation time should be checked by direct examination and adjusted accordingly. Maintain dechorionated embryos in the basket immersed in tap water and proceed immediately to the EPS step.
3. EPS Treatment
    1. Prepare six 60 mm dishes with approximately 10 ml of PBS in each. Prepare EPS dilution by dissolving 75 µl EPS in 2.925 ml MBIM (1:40) in a 50 ml glass beaker with swirling. Note: A white emulsion forms from this mixture.
    2. Blot excess water from the bottom of the mesh basket with a lab wipe. Immerse basket in dilute EPS in beaker and immediately swirl to disperse embryos in the EPS solution in the bottom of the basket. Continue swirling motion for 30 sec. NOTE: EPS dilutions and exposure times can be varied to control permeability. It is recommended that optimal EPS dilutions and treatment times be established empirically with the strains of flies being used. Increasing exposure time to 60-90 sec is favorable for stage 12 and older embryos.
    3. Remove basket, blot away excess EPS with a lab wipe. Proceed with six sequential washes in 10 ml of PBS in the 60 mm dishes. Use a plastic pipette to gently squirt embryos with PBS in each of the six washes. Proceed to dye and drug treatment steps. NOTE: EPS can be disposed of down the sink.

3. Dye and Drug Treatment of Permeabilized Embryos

1. Dye Treatment
    1. Add 5 µl of 10 mM CY5 carboxylic acid dye* to 1 ml of MBIM-T (50 µM final concentration) in a 1.5 ml microfuge tube and vortex to mix. NOTE: *Choice of dye depends on downstream analysis. CY5 carboxylic acid is effective for subsequent analyses using fixation and immunostaining. Rhodamine B is useful for analysis of living embryos. The red emission of Rhodamine B, and the green emission of its metabolites[1], can present some complications with downstream applications using fluorescence. Drug or toxin can be added to the dye solution to initiate treatment at this stage, or to limit drug/toxin treatment to a pulse at this stage. Care should be taken to handle and dispose of drugs and toxins according to MSDS and environmental safety standards.
    2. Transfer embryos from the mesh basket to the dye solution using a paintbrush. Cap the tube and invert repeatedly to ensure the embryos float freely in suspension in the dye solution. Place tube on a nutating rocker for 15 min at room temperature.
    3. Remove tube from nutator and let embryos settle. Remove dye solution with a fine pipette and replace with 1 ml of MBIM-T to wash. Invert tube to re-suspend embryos completely. Let embryos settle and repeat with three more MBIM-T washes. Remove all MBIM-T from final rinse and proceed to incubation step.

2. Incubation for Embryo Development
    1. Transfer embryos to one of two chambers: The development basket or the slide chamber. NOTE: The development basket is preferred for longer developmental periods, and is required if subsequent fixation and immunostaining steps are to be executed. The slide chamber is optimal for higher resolution time-lapse imaging and is most effective for short developmental periods (*e.g.*, the early embryonic events). Drug or toxin can be added to the medium at various concentrations and embryo development can be monitored in real-time with live embryos or at an endpoint using standard fixation and immunostaining protocols.
    2. Development in Baskets
        1. Clean a development basket by squirting with 70% ethanol, rinse thoroughly with de-ionized water and blot dry with lab wipe. Prepare 6 ml of incubation medium with desired concentration of drug or toxin. Place the development basket in medium in 60 mm dish taking care to not trap air bubbles under the mesh base. NOTE: Two media are commonly used: MBIM alone, or MBIM/M3 in a 50:50 mixture, the latter being more effective for longer development periods.
        2. Transfer permeabilized embryos to mesh surface in base of basket with a paintbrush. Gently squirt the embryos with media from the surrounding reservoir. Disperse the embryos with the paintbrush so that they are in a monolayer on the mesh. NOTE: Proceed to microscopy imaging and evaluate permeabilization and viability characteristics of the preparation (see step 4).
    3. Development in the Slide Chamber
        1. Invert slide chamber and apply a small bead of vacuum grease on the perimeter of the opening. Place 150 µl of medium with desired amount of drug or toxin on surface of DO membrane within the opening. NOTE: Two media are commonly used: MBIM alone, or MBIM/M3 in a 50:50 mixture, the latter being more effective for longer development periods.
        2. Transfer permeabilized embryos to the drop of media with a paintbrush. Disperse the embryos making them settle on the membrane within the drop.
        3. Carefully apply a 25 mm circular coverslip over the opening thereby flattening the medium. Press down gently along the perimeter of the coverslip to form a seal with the grease. NOTE: Proceed to microscopy imaging to evaluate permeabilization and viability characteristics of the preparation (see step 4).

4. Identification of Permeabilized Viable Embryos

1. Identify permeabilized embryos. Observe embryos under epifluorescence with a microscope equipped with a digital camera. Acquire images (in the blue wavelength to determine the profile yolk autofluorescence) of several fields using fixed microscope and camera settings.
2. Determine permeabilization of embryos based on relative fluorescence intensity. Use a stereomicroscope with a large working distance to accommodate the basket and to allow manipulations of the embryos. The microscope should be equipped with a programmable X-Y-Z stage, epifluorescence illumination and a digital camera. Image the embryos taking care to record exposure and stage position parameters of each embryo image to enable re-evaluation of the same embryos at a later time point (see representative result in **Figure 3**). NOTE: Patterns of dye uptake will vary depending on the dye used, length of exposure and embryo age. A wide range of dye uptake across a single preparation is typical and reflects the variation in the degree of permeabilization.
3. Identify viable embryos. After marking position of permeabilized embryos, continue with embryo development at room temperature or 25 °C. Return basket or slide chamber to microscope. Observe under blue channel fluorescence and acquire image of yolk autofluorescence in previously identified permeabilized embryos. Assess viability according to normal progression of yolk distribution (see Rand *et al.*[1] and representative result in **Figure 3**). NOTE: Other morphological features of the embryo observed with brightfield microscopy can be used to assess viability. It is recommended that establishing the level of permeability that is compatible with viability be determined empirically with each dye and strain of fly used.
4. Evaluate drug or toxin effects in permeabilized viable embryos.
5. Embryos processed with the above protocol are poised for a number of conventional analyses subsequent to drug or toxin exposure. Several of the various types of analyses are considered in the Discussion below and include direct observation of morphogenesis in live embryos as well as post- fixation immunostaining analyses. Both of these approaches are enhanced by use of vital dyes (*e.g.*, GFP) that reveal gene expression patterns and cell lineage and morphology profiles.

Representative Results

Embryo handling devices are pictured in **Figure 1** to assist in visualizing the “home-made” devices for manipulation in the above Protocols. Results seen in **Figure 2** illustrate the robust effect of rearing embryos at 18 °C on their ability to be permeabilized by EPS at late stages of

development. This condition is applied in the protocol step 2.1. Efficacy of the CY5 carboxylic acid dye to reveal the various levels of permeability typically seen in EPS treated embryos is seen in **Figure 3**. The developmental dynamics of the dye distribution in the yolk is also seen in **Figure 3**, revealing a criterion used to assess viability, as described in Protocol step 4.2. The utility of the CY5 dye in determining embryo permeabilization subsequent to toxin treatment, formaldehyde fixation and immunostaining is illustrated by the result in **Figure 4**.

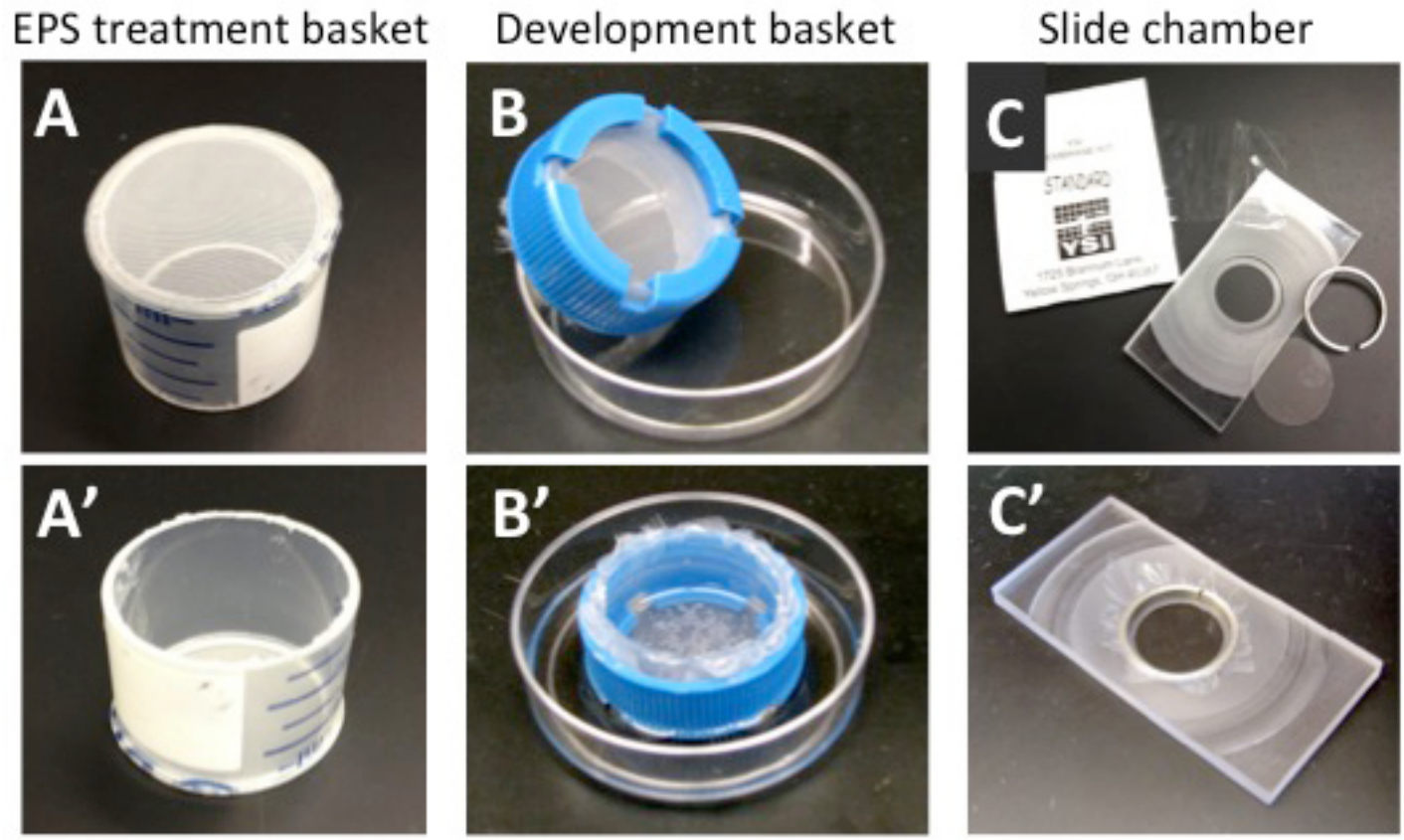

Figure 1. Embryo handling devices for the EPS method. The flat-bottomed basket is used in dechorination and EPS exposure steps (**A**,**A'**). The development basket is used for longer developmental exposures of permeabilized embryo (**B**,**B'**). The slide chamber is used for shorter developmental exposures and higher resolution imaging of live embryos (**C**,**C'**. See text for further description).

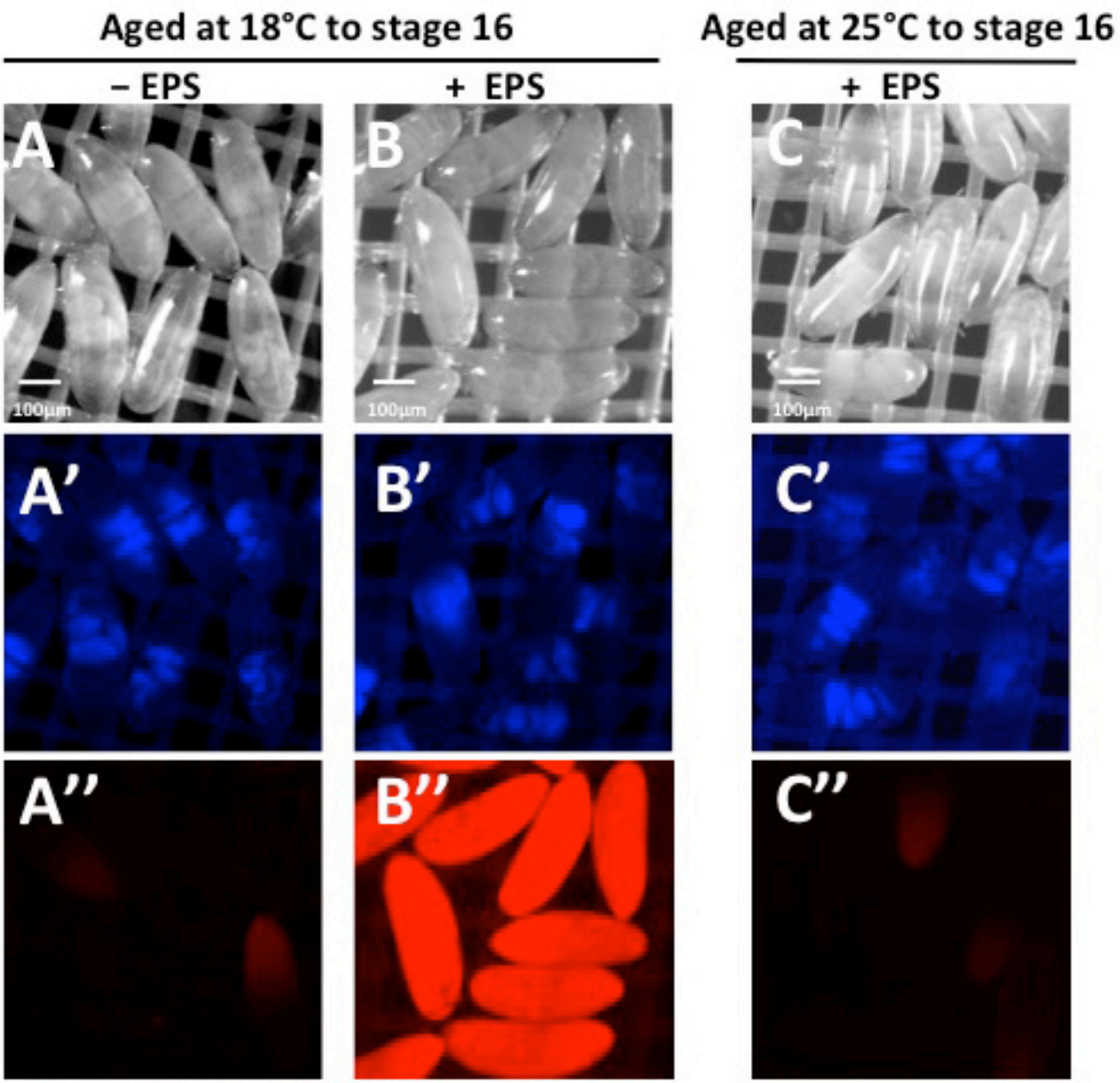

Figure 2. Effect of aging at 18 °C on EPS efficacy in late stage embryos. Embryos were collected for two hours followed by aging at 18 °C for 20 hr (**Panels A-A"**, **B-B"**) or at 25 °C for 10 hr (**Panel C-C"**). Embryos at 18 °C were then dechorionated and divided into two samples. The first sample was treated directly with 1 mM Rhodamine B dye in MBIM-T for 5 min, washed and visualized under brightfield and blue and red fluorescence channels (Panel A-A"). The second sample was treated with EPS (1:10 in MBIM for 1 min), washed and then treated with 1 mM Rhodamine B for 5 min and washed before visualization (**Panel B-B"**). Embryos raised at 25 °C were dechorionated and treated directly with EPS (1:10 in MBIM for 1 min), washed and then treated with 1 mM Rhodamine B for 5 min before visualization (**Panel C-C"**). Embryos were determined to be at stage 14 by the folds in the gut revealed by yolk autofluorescence in the blue channel (**Panel A', B', C'**). Embryos raised at 18 °C are impermeable prior to EPS treatment as seen by absence of Rhodamine B uptake (**Panel A"**). EPS treatment of 18 °C embryos yields a high degree of permeability as seen by Rhodamine B uptake (**Panel B"**). Embryos raised at 25 °C remain impermeable even with EPS treatment as seen by exclusion of Rhodamine B (**Panel C"**).

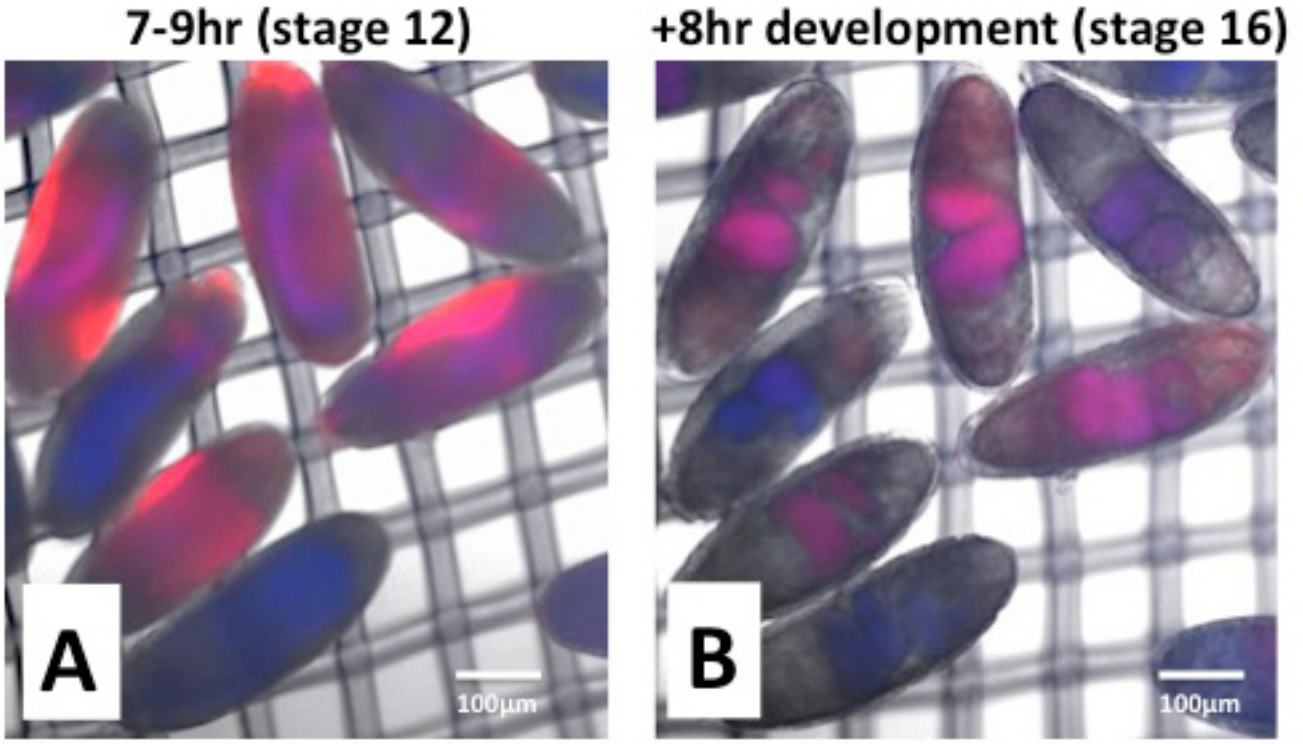

Figure 3. Incorporation of CY5 in permeable and viable embryos. Embryos were collected at 25 °C for 2 hr and aged for 14 hr at 18 °C (equivalent to 7-9 hr embryos at 25 °C, stage 12). After dechorination, EPS treatment was done (1:40 in MBIM for 1 min) followed by incubation in CY5 dye (50 µM in MBIM-T for 15 min). Embryos were washed three times in MBIM-T and transferred to development basket with MBIM in the reservoir. Development was allowed to proceed for 8 hr at room temperature. Uptake of CY5 (Red) is imaged in the far-red channel immediately after dye treatment and washing (**Panel A**) and after 8 hr development (**Panel B**). Distribution of the yolk is seen by autofluorescence in the blue channel. Dye uptake, hence permeability, is seen to vary from embryo to embryo. CY5 dye (red) is seen to localize to the yolk (blue), which becomes concentrated to the lumen of the gut at stage 16 (purple, Panel B).

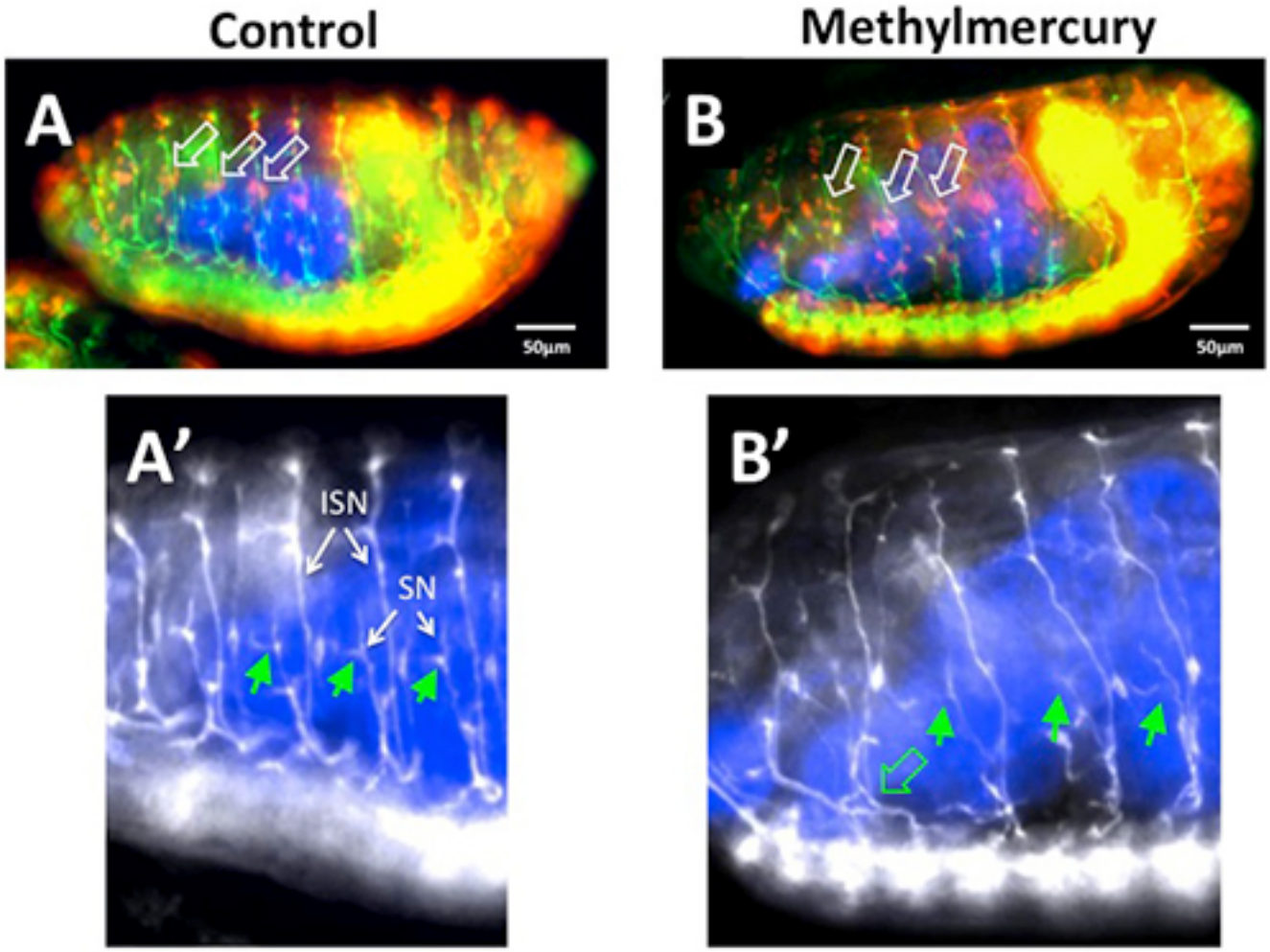

Figure 4. Determination of permeabilization and methylmercury effects in fixed and immunostained embryos. Embryos were collected at 25 °C for 2 hr and aged for 14 hr at 18 °C (equivalent to 7-9 hr embryos at 25 °C, stage 12). After dechorination, EPS treatment was done (1:40 in MBIM for 1 min) followed by incubation in CY5 dye (50 µM in MBIM-T for 15 min) together with methylmercury (50 µM MeHg, **Panel B**) or DMSO solvent control (0.1% final concentration, **Panel A**). Embryos were washed with MBIM-T and placed in a development basket with MBIM:M3 medium in the reservoir and aged for an additional 8 hr at room temperature. Embryos were then fixed in a two-phase 4% paraformaldehyde-heptane preparation by a standard protocol[14]. Staining was performed with anti-Fasciclin II (green in **A**,**B** and white in **A'**,**B'**) to label motor neurons and anti-elav antibodies (red in **A**, **B**) to label all neuron cell bodies. CY5 dye is revealed by direct fluorescence, which requires extended exposure due to diminished fluorescence intensity due to fixation (CY5 is pseudo-colored blue in all panels). The effects of MeHg are seen in the irregular patterning and clustering of the lateral chordotonal neuron cell bodies (elav-positive, labeled in red and denoted with white arrows in **B** versus **A**). In addition, a characteristic branching of the segmental (SN) (solid green arrows in **A'**) is seen to be highly variable with MeHg exposure (solid green arrows in **B"**) consistent with previously reported effects of MeHg on the embryo[15]. Projection of the intersegmental and segmental nerves at their roots are seen to be displaced posteriorly with MeHg exposure (open green arrow in **B'**). Note: Methylmercury is a potent neurotoxin. Care should be taken to wear gloves and eye protection when handling. Disposal should be done through an institutional environmental safety facility and service.

Discussion

The above method outlines a means to obtaining viable *Drosophila* embryos that are accessible to small molecule treatments across a wide developmental range. This method introduces the novel and simple finding that aging embryos at 18 °C enables permeabilization of late stage embryos with the same efficacy as previously seen only in early stage embryos. In addition, use of the far-red dye CY5 carboxylic acid as a permeability indicator has proven effective in post-fix applications and does not interfere with conventional red and green fluorescent markers that can be used to reveal developmental phenotypes. These findings significantly advance the efficacy and utility of the EPS method.

This method is amenable to analyses of both living and fixed embryo preparations. Using brightfield microscopy and the slide chamber set up, typical features to score in the first half of embryo development are morphogenetic movements such cellularization of the blastoderm, cephalic furrow formation, germband elongation and germband retraction[1]. With GFP or RFP reporters more specific endpoints can be discerned, *e.g.* early segmentation patterns and formation of neural structures in later development[1]. GFP and RFP reports also enable observation of developmental events in living permeabilized embryos in both the slide chamber and developmental basket preparations. A simple method

to determine gross toxicity of an applied drug or chemical is to monitor the pattern of yolk protein auto-fluorescence in the blue channel to determine a delay or cessation of development[1].

The EPS methodology also has great potential for broadening the investigative tools for non-model insects, in particular the mosquito, which shares a similar architecture of the eggshell. Application of small molecules to embryos of other insect species would open up an avenue of investigation where standard genetic approaches to functional studies are currently lacking. Embryos developed in baskets can be processed for formaldehyde fixation, therefore opening up analyses to the wide array of immunostaining reagents available for *Drosophila* studies. However, this step requires that a post-fix determination of permeabilization be feasible to correlate phenotypes with embryos that had been made accessible to the drug. Application of CY5 carboxylic acid dye has proven highly effective for this approach. CY5 carboxylic acid is efficiently taken up in permeabilized embryos (**Figure 3A**). During development CY5 is concentrated in the yolk, which is ultimately sequestered in the lumen of the forming gut at stage 14 and later (**Figure 3B**). After fixation, CY5 fluorescence is markedly decreased, yet reliably detectable in the gut and serves as a marker of those embryos that were effectively permeabilized at the outset (see representative result **Figure 4**). It should be noted that CY5 detection at this stage requires longer camera exposures (*e.g.*, 1-4 seconds) for detection. Thus, scoring of phenotypes with immunostaining patterns can proceed using the CY5 signal to confirm similarly permeabilized embryos together with tissue specific markers (*e.g.*, neural specific antibodies seen in **Figure 4**).

The biggest challenge in this method is the sensitivity of embryo viability to the permeabilization process, something that has long troubled prior attempts to develop this method[9,10,16]. Viability subsequent to permeabilization is starkly age-dependent, and increases dramatically the older the embryo is upon permeabilization[9]. Yet, as we have shown previously, permeability becomes increasingly difficult with age[1]. A recent report now demonstrates the ability to permeabilize late stage embryos (stage 14) by re-invoking heptane as a solvent in conjunction with d-limonene[17]. The broad utility of this latter method is not clear as only one drug effect was characterized (nocodazole) and application to earlier stage embryos was not described[17]. Furthermore, application of the 18 °C development step outlined above yields late stage permeability and further avoids use of toxic organic solvents. The investigator who is new to the EPS protocol will experience variability in permeabilization and viability outcomes upon initial attempts. The steps outlined here give the investigator the tools to systematically vary conditions of permeabilization treatments and subsequent incubation steps to optimize conditions for specific strains of *Drosophila* they are working in their own laboratory setting.

An additional challenge with the method is the variability in chemical uptake seen from embryo to embryo. This variability is reflected in the heterogeneity of CY5 dye uptake seen in embryos immediately after dye treatment (**Figure 3A**). In contrast, Rhodamine B dye is more rapidly and evenly dispersed across the embryonic tissues than CY5 dye (**Figure 2B"**). Thus, some embryo-to-embryo variability may be harbored in the distribution properties of the chemical, drug or toxin of interest and is inherent to the method. Where quantification of dose is critical, it is recommended that uptake of the drug or toxin of interest is characterized through an alternative analytical method. Nonetheless, the ease of the above protocol, and the ability to screen hundreds of embryos, allows the investigator to evaluate dose responses and score characteristic phenotypes with little investment of resources, making for a powerful first approach to characterizing drugs or toxins in this highly developed model system.

Disclosures

The authors have nothing to disclose.

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