2007

Whole-mount In situ Hybridization of Small Invertebrate Embryos Using Laboratory Mini-columns

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Citation/Publisher Attribution
Irvine S.Q. "Whole-mount in situ hybridization of small invertebrate embryos using laboratory mini-columns," BioTechniques 43:764-768 (2007).

Whole-mount in situ hybridization of small invertebrate embryos using laboratory mini-columns

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BioTechniques 43:764-768 (December 2007)
doi 10.2144/000112617

To facilitate the handling of small invertebrate embryos during whole-mount in situ hybridization, a method of solution exchange using laboratory mini-columns was developed. This protocol speeds time-consuming aspiration of buffers, and avoids accidental loss of the embryos, by gently pushing solutions through the column using air pressure from a syringe after each incubation. The next buffer is then added using a pipettor. Embryos are retained on a filter within the column. As many columns as desired may be processed in parallel for different probes or stages.

Whole-mount in situ hybridization (WMISH) using non-radioactive probes has become a standard technique to determine transcript expression patterns during embryonic development. Traditionally, WMISH is performed with embryos in microcentrifuge tubes or small vials. The many solution exchanges are performed by decanting or aspirating one fluid and adding another. In the case of larger specimens, such as *Drosophila* or mid-later stage mouse embryos, this simple procedure works well. However, the solution exchanges are time consuming. In addition, small embryos, such as those of many marine invertebrates, can be accidentally discarded during the decanting or aspiration steps, especially as embryos may become buoyant in certain buffers. High-throughput WMISH procedures have been developed that ameliorate these problems, such as using multiwell plates with filter bottoms, for example (1); using specialty robots (2); or using specially designed columns [InSitu Chip (3)]. For low-medium throughput, on the other hand, the efficiencies of these high-throughput methods are wasted, and they become expensive per individual preparation. (Refer to Table 1 for a comparison of WMISH formats.) For low-medium throughput applications with small embryos we have used inexpensive laboratory mini-columns to hold the embryos and facilitate rapid buffer exchange (Figure 1).

We illustrate our mini-column strategy by describing its application to WMISH on various-stage embryos of the ascidian *Ciona intestinalis*. We have previously described fixation, dechorionation, and staining in detail (4); here, we provide a brief summary of those steps and explain in detail how the procedure can be adapted to make use of laboratory mini-columns. (See Supplementary Material for a step-by-step protocol, available online at www.BioTechniques.com.)

The initial steps are done in 2 mL microcentrifuge tubes, with embryos for different probes or conditions pooled. Fixed, dechorionated embryos stored in ethanol are rehydrated in a graded ethanol series into PTw (1× PBS, pH 7.5; 0.1% Tween-20) (5 washes total). Embryos are then permeabilized by incubation in 2 µg/mL Proteinase K in PTw for 5–9 min at 37°C. The Proteinase K is quenched by washing two times with 2 mg/mL glycine in PTw, followed by post-fixation in 4% paraformaldehyde in PTw for 30 min at room temperature. Acetylation of embryos is performed by treatment two times for 5 min with fresh 0.25% acetic anhydride in 1% triethanolamine/PTw at room temperature. The specimens are then washed three times with PTw.

At this point the embryos are divided up into Mobicol mini-columns (MoBiTec GmbH, Gottingen, Germany; US distributor, Boca Scientific, Boca Raton, FL, USA)—one for each probe and embryo preparation combination. The embryos are prepared for hybridization by washing with a 1:1 mixture of PTw and hybridization buffer (50% formamide; 5× SSC, pH 4.5; 0.1% Tween-20; 2× Denhardt's solution; 50 µg/mL heparin; 50 µg/mL yeast RNA; 50 µg/mL sonicated herring sperm DNA), then two changes of hybridization buffer and a 2-h prehybridization period at the hybridization temperature of 60°C. The digoxygenin-UTP (Roche, Indianapolis, IN, USA) labeled riboprobe is diluted in hybridization buffer and denatured by heating to 80°C for 5 min. The riboprobe is then added to the mini-column to produce a final hybridization concentration of 30–300 ng/mL. Hybridization at 60°C proceeds overnight.

After hybridization, the specimens are washed with a total of 10 buffer changes through a graded series of hybridization buffer/SSC/Tw mixtures at the hybridization temperature. This is followed by another five buffer changes at room temperature through a graded series of SSC/Tw/Ptw mixtures (see Supplementary Material for details). Detection of the hybridization signal is begun by incubation in

Figure 1. Mini-column apparatus for whole-mount in situ hybridization. (A) Mobicol mini-column set up for the WMISH procedure. (B) Four columns set up for use in the hybridization oven. Mobicols are inserted in open-bottom 2 mL microcentrifuge tubes inserted through holes in the tops of 15 mL centrifuge tubes. (C) Cross-section diagram of the mini-column apparatus.

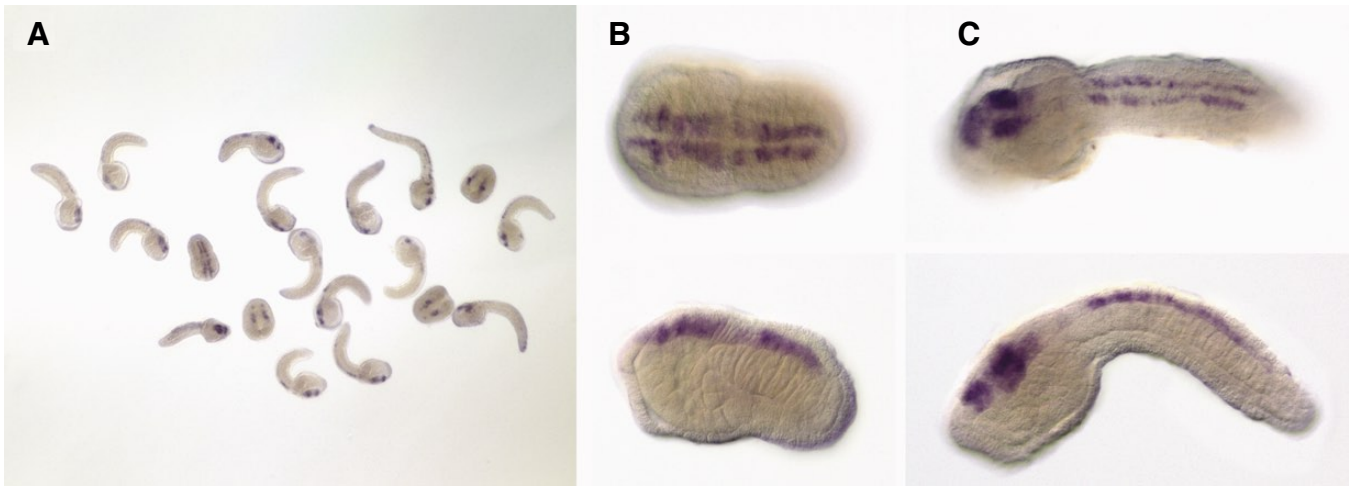

Figure 2. Representative WMISH result processed through the mini-column apparatus. (A) Set of mixed-stage *Ciona intestinalis* embryos hybridized to a *CiPax-6* riboprobe and processed in one mini-column. (B–E) Early tailbud stage (B and C) and mid-tailbud stage (D and E) embryos from the same set shown in panel A. Anterior is to the left in all views. (B and D) are dorsal views, and (C and E) are lateral views of the same respective embryos. Note single-cell resolution of hybridization signal.

two changes of blocking solution [2% Blocking Reagent (Roche) in PTw]. Anti-digoxygenin antibody conjugated to alkaline phosphatase (Roche) in blocking solution is then added to a final concentration of 1:5000 and incubated at 4°C overnight. The antibody is then washed out with three changes of blocking solution and three changes of PTw.

At this point the contents of each column are transferred to individual wells of 12-well plates so that the colorigenic reaction can be monitored. The specimens are washed twice with AP detection buffer (100 mM NaCl; 50 mM MgCl; 100 mM Tris, pH 9.5; 0.1% Tween-20). Nitro blue tetrazolium and 5-Bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) are added (0.66 mg/mL and 0.33 mg/mL, respectively) to detect the hybridization signal, and incubated until the staining reaction is complete (1 h to 2 days). The embryos are then washed with PTw and post-fixed in 4% formaldehyde for 30 min at room temperature. They are then mounted in 70% glycerol for photomicrography.

Table 1. Comparison of WMISH Formats

Format	Advantages	Disadvantages
Microcentrifuge tubes	Readily available	Solution changes laborious Embryos easily lost Low throughput only
"Home-made" mesh-bottom buckets	Prevent embryo loss Faster solution changes	Need to be fabricated by researcher High reagent volumes required Low throughput only
96-well filter plates (Reference 1)	May be semi-automated Very rapid solution changes High throughput No embryo loss	Plates are expensive Requires special vacuum manifold Not ideal for low throughput Embryos may be damaged by vacuum
InSitu Chip (Reference 3)	May be semi-automated Low-high throughput Rapid solution changes No embryo loss	"Chips" not currently available outside Japan May require high reagent volumes
Commercial automated systems (e.g., Reference 2)	Labor saving automation Medium-high throughput	Apparatus extremely expensive
Mini-columns (this protocol)	Low-medium throughput Rapid solution changes Low reagent volumes No embryo loss Columns readily available and inexpensive Relatively "foolproof" technique	Not suitable for very high throughput Not readily amenable to automation

As can be seen from this summary, the protocol requires at least 48 buffer changes, with 26 of those changes occurring while the embryos are in the mini-columns. If desired all steps may be done in the mini-columns, but in our hands it is more convenient to process large numbers of embryos in the initial steps in 2 mL microcentrifuge tubes. These embryos are then split up into the mini-columns after preparation. Likewise, the detection steps are performed in 12-well plates to facilitate monitoring of the color reaction in a dissecting microscope.

As described above, 1 mL Mobicol laboratory mini-columns are used during the middle parts of the protocol (Figure 1). Ten micrometer filters available with the Mobicols are inserted into the columns using the supplied tools. Before use we treat the columns by washing with RNase-Away (Molecular BioProducts, San Diego, CA, USA) and rinse thoroughly with DEPC-treated water, to eliminate RNases. The columns are pre-charged with PTw and embryos are transferred after acetylation. The columns are placed into 2 mL microcentrifuge tubes with the bottoms removed and this assembly in turn is placed into a 15 mL centrifuge tube with its cap drilled out to accept the 2 mL tube

(Figure 1C). The 15 mL tubes are placed in a test tube rack in a hybridization oven for the prehybridization, hybridization, and hybridization temperature washing steps (Figure 1B). The rack is removed for subsequent room temperature washes.

Buffer changes are accomplished by gently pushing the buffer through the filters using a syringe attached to plastic tubing and a tubing adapter available from the column manufacturer. The elution is stopped just above the level of the embryos sitting on the top filter, so that they do not dry out between buffers, or are not damaged by surface tension. The next buffer is added using a conventional 100–1000 µL pipettor through the top fill tube. The bottom plug and top cap are only used during the overnight hybridization step and overnight antibody incubation step. For all other washes, surface tension is sufficient to maintain the fluid in the column during the length of the incubation. We have used fresh mini-columns for each preparation. However, it should be possible to reuse the columns without any negative effects, upon washing with water and treatment with RNase-Away (Molecular BioProducts) or the equivalent. Autoclaving is also possible at 120°C if filters are removed, or 110°C with existing filters left in place.

This method using laboratory mini-columns has had advantages over performing WMISH in individual tubes. Our results are comparable to those we have obtained using the microcentrifuge tube or homemade mesh-bottom bucket formats. (Refer to Figure 2 for examples using the mini-column format.) Buffer changes using the syringe method are rapid—saving time and making it easier to keep the embryos at hybridization temperature. The mini-columns hold only 0.5 mL, so they are economical in reagent use. The mini-columns also eliminate loss of embryos, especially in situations we have encountered of embryos becoming buoyant in the hybridization buffer and being sucked out when changing solutions. Because the buffers are pushed through the filter the embryos rest on, there is no chance of accidentally discarding them. Despite the slight pressure on the embryos during solution changes we have not seen damage in the embryos after the procedure. Also, perhaps due to the detergents in all of the solutions used during the procedure, we have not experienced any sticking of the embryos to the column or filters. Several preparations may be done at one time, without waste of the expensive filter-bottom plates used in high-throughput methods. While we have performed this WMISH protocol using mini-columns only on ascidian embryos (150–500 µm long), the method is broadly applicable to WMISH on any multicellular organism with embryos smaller than a few millimeters in size.

ACKNOWLEDGMENTS

The author acknowledges the able technical assistance of Michael A. Zompa and insightful suggestions from three anonymous reviewers. The work was supported by National Institutes of Health grant 1R15GM07373701 from the National Center for General Medical Sciences, and grant P20 RR016457 from the Biomedical Research Infrastructure Networks/ Idea Network of Biomedical Research Excellence (BRIN/INBRE) Program of the National Center for Research Resources.

COMPETING INTERESTS STATEMENT

The author declares no competing interests.

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Received 16 July 2007; accepted 26 September 2007.

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