Gap Junctional Communication in the Early Xenopus Embryo

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Abstract. In the Xenopus embryo, blastomeres are joined by gap junctions that allow the movement of small molecules between neighboring cells. Previous studies using Lucifer yellow (LY) have reported asymmetries in the patterns of junctional communication suggesting involvement in dorso-ventral patterning. To explore that relationship, we systematically compared the transfer of LY and neurobiotin in embryos containing 16–128 cells. In all cases, the junction-permeable tracer was coinjected with a fluorescent dextran that cannot pass through gap junctions. Surprisingly, while LY appeared to transfer in whole-mount embryos, in no case did we observe junctional transfer of LY in fixed and sectioned embryos. The lack of correspondence between data obtained from whole-mounts and from sections results from two synergistic effects. First, un.injected blastomeres in whole-mounts reflect and scatter light originating from the intensely fluorescent injected cell, creating a diffuse background interpretable as dye transfer. Second, the heavier pigmentation in ventral blastomeres masks this scattered signal, giving the impression of an asymmetry in communication. Thus, inspection of whole-mount embryos is an unreliable method for the assessment of dye transfer between embryonic blastomeres. A rigorous and unambiguous demonstration of gap junctional intercellular communication demands both the coinjection of permeant and impermeant tracers followed by the examination of sectioned specimens. Whereas LY transfer was never observed, neurobiotin was consistently transferred in both ventral and dorsal aspects of the embryo, with no apparent asymmetry. Ventralization of embryos by UV irradiation and dorsalization by Xwnt-8 did not alter the patterns of communication. Thus, our results are not compatible with current models for a role of gap junctional communication in dorso-ventral patterning.

Key words: Gap junctions • dye transfer • dorso-ventral axis • Xwnt-8 • UV irradiation • Lucifer yellow • neurobiotin • cytoplasmic bridges • Xenopus

Introduction

Gap junctional intercellular communication (GJIC) in early development has been most extensively explored in the amphibian Xenopus laevis using fluorescein (M_r = 332) and Lucifer yellow (LY; M_r = 457) as reporter molecules (Guthrie, 1984; Warner et al., 1984; Cardellini et al., 1988). A marked asymmetry of dye movement between presumptive dorsal and ventral sides of the animal pole was noted as early as the 16-cell stage (Guthrie et al., 1988). Later studies examined the effect of conditions that alter dorso-ventral patterning. Experimental conditions that dorsalize embryonic development, such as lithium treatment (Nagajski et al., 1989) and injections of RNA coding for wnt-1 (Olson et al., 1991; Olson and Moon, 1992; Guger and Gumbiner, 1995; Kruka et al., 1998). Experimental conditions that dorsalize embryonic development, such as lithium treatment (Nagajski et al., 1989) and injections of RNA coding for wnt-1 (Olson et al., 1991; Olson and Moon, 1992), activin B (Olson and Moon, 1992), and b-catenin (Guger and Gumbiner, 1995), resulted in the increased intercellular transfer of LY between presumptive ventral blastomeres in 32-cell stage embryos. Conversely, manipulations that ventralize embryonic development, such as UV irradiation (Nagajski et al., 1989) and depletion of b-catenin (Kruka et al., 1998), result in decreased dye transfer between the presumptive dorsal blastomeres of 32-cell stage embryos. Together, these findings suggest a requirement for GJIC, as measured by LY transfer, in early patterning events.
For the most part, the previous studies have used whole-mounts of unsectioned embryos after injection of LY for the assessment of dye transfer. Whereas the whole-mount method is widely accepted, it is difficult to construct an accurate map of the patterns of dye movement. Therefore we analyzed the patterns of GJIC during early Xenopus embryonic development using fixed, sectioned embryos injected with a variety of small (<1,000 D) tracers. Three tracers were fluorescent (LY, α-lexa 350, α-lexa 488), while a fourth, neurobiotin, was localized with a fluorescent avidin or streptavidin reagent. To ensure that any apparent cell–cell transfer was due to GJIC, we coinjected 10-KD fixable fluorescent dextran that was too large to pass through junctional channels. We followed cleavage patterns of Xenopus embryo blastomeres and compared the fluorescence of dyes in whole-mount embryos with dye transfer observed in paraffin sections. Surprisingly, we were unable to demonstrate any intercellular transfer of LY without accompanying fluorescent dextran within the time scales generally used for these studies. We found that the fluorescent signal emanating from an injected dorsal blastomere can reflect and be scattered within the whole-mount embryo, creating the impression of GJIC. In addition, the higher levels of pigment in the ventral blastomeres masked fluorescence, creating the impression of lower levels of GJIC on the ventral-animal pole of whole-mount Xenopus embryos. We also found evidence for the presence of patent intercellular cytoplasmic bridges beyond the 8-cell stage. In contrast to LY, neurobiotin was widely transferred among all animal blastomeres up to the 128-cell stage and among vegetal blastomeres up to stage 8. However, we found no obvious asymmetry in the pattern of neurobiotin transfer. Most importantly, neurobiotin intercellular transfer was not detectably affected by conditions that altered dorso-ventral patterning such as Wnt expression or UV treatment, conditions reported to continue development to insure that the dye injection did not interfere with subsequent cell cleavages (see Fig. S6, A-D, control panels).

**Materials and Methods**

**General**

Assays using Xenopus oocytes and embryos were all done at a constant temperature of 18°C. Fertilized Xenopus eggs and embryos were kept in 0.1 M MM R (Peng, 1991) and were transferred into 0.1 M MM R, 5% Ficol (pH 7.4) before injections. The volume of dye (tracers and fluorescent conjugates) injected varied, depending on the size of the injected blastomere. Typically, ~2 nl at the 16-cell embryo, 0.5–2 nl at the 32-cell embryo, 0.5–1 nl at the 64-cell embryo and 0.1–0.5 nl at the 128-cell embryo. For each experiment, a cohort of the injected embryos was allowed to continue development to insure that the dye injection did not interfere with subsequent cell cleavages (see Fig. S6, A–D, control panels).

**Tracers and Fluorescent Conjugates**

The following tracers and conjugates were used: Neurobiotin, mol wt = 322.85 (Vector Laboratories); LY, lithium salt, mol wt = 457.24 (Molecular Probes); α-lexa 350 hydrazide, sodium salt, mol wt = 349.29 (Molecular Probes); α-lexa 488 hydrazide, sodium salt, mol wt = 570.48 (Molecular Probes); dextran-fluorescein, lysine fixable, mol wt = 10,000 (Molecular Probes); dextran-rhodamine, lysine fixable, mol wt = 10,000 (Molecular Probes); dextran-biotin, lysine fixable, mol wt = 10,000 (Molecular Probes); avidin-rhodamine conjugate (Pierce Chemical Co.); and streptavidin α-lexa 488 conjugate (Molecular Probes).

**Tracer Mixtures**

Free, unbound fluorescent molecules were removed from the dextran tracer solutions on Biomax-5K, 15 µl M illipore filters. The following mixtures were prepared: 7.5% neurobiotin, 2% dextran-rhodamine, and 50 mM H epes, pH 7.8; 7.5% neurobiotin, 2% dextran-fluorescein, and 50 mM H epes, pH 7.8; 2% LY, 2% dextran-rhodamine, and 50 mM H epes, pH 7.8; 2% α-lexa 350, 2% dextran rhodamine, and 50 mM H epes, pH 7.8; 2% α-lexa 488, 2% dextran-rhodamine, and 50 mM H epes, pH 7.8; and 2% dextran-biotin, 2% dextran-rhodamine, and 50 mM H epes, pH 7.8.

**Histology**

10 min after dye injection, embryos were fixed for 2–6 h in 4% formaldehyde (EM grade; Electron Microscopy Sciences). A pan analysis of whole-mount embryos was done either during or at the end of the fixation time. This early analysis was aimed to make sure that all injections were successfully done, that blastomeres of all embryos were not damaged, and that no leaks resulted either during injection or fixation. This also allowed whole-mount analysis of transfer of LY, α-lexa 350, and α-lexa 488. A 24 h fixation, embryos were embedded in paraffin and ~70, 12 µm serial sections were cut through each embryo.

Sections of embryos injected with mixtures containing neurobiotin and dextran biotin were deparaffinized through graded steps of xylene and alcohol and immediately processed for the detection of biotin. Sections of embryos injected with mixtures containing LY, α-lexa 355 and α-lexa 488 were deparaffinized and then were either mounted in Gurr (a xylene-based medium) or were gradually passed through alcohol to PBS and mounted in Vectashield (Vector Laboratories), a glycerol-based medium.

**Detection of Neurobiotin and Biotin**

Slides were incubated for 15 min in blocking solution (1% gelatin solution from fish skin in PBS). Each slide was then covered with either avidin rhodamine conjugate (10 µg/ml) or streptavidin α-lexa 488 conjugate (50 µg/ml) and incubated for 45 min. Then slides were washed twice for 10 min in PBS and mounted in Vectashield.

To control for the degradation of the junction-impermeant tracers in the context of the blastomere cytoplasm, a mixture of 2% dextran-biotin and 2% dextran-fluorescein was injected into one dorsal-ventral blastomere of 64-cell stage embryos. The embryos were fixed, sectioned and both the biotin and fluorescein visualized (see Fig. S6, G and H, control panels). Colocalization of the green and the red fluorescence in a single cell demonstrated that both dextran conjugates were stable and that no free biotin or fluorescein was released during the experimental procedures.

**Fluorescence Microscopy**

Whole embryos and sections were viewed under 4× or 10× objectives on a Nikon E阐释 E 800 microscope equipped with Nikon fluorescent filter cubes. Each of the following dyes was visualized by using a specific filter: LY: exciter cube no. 96153 exciter 400–440, barrier 480 nm; rhodamine: exciter cube no. 96157 exciter 528–533, barrier 560–660 nm; fluorescein and α-lexa 488: exciter cube no. 96170 exciter 460–500, barrier 510–560 nm; or α-lexa 350: cube no. 96130 exciter 330–380, barrier 420 nm.

**Image Capture and Processing**

Using Image Pro-plus capturing program (Media Cybernetics), images were digitally captured by a Spot camera (Diagnostic instruments, Inc.). Exposure time was adjusted to capture data that most faithfully reflected...
the visual images. Data were subsequently arrayed figures using Canvas 5 (Deneba Systems).

**Synthetic RNA**

RNA was transcribed from linearized template Xwnt-8 (Sokol et al., 1991) using the mMESSAGE mMACHINE from Ambion.

2 pg of Xwnt-8 RNA was injected into the marginal zone of two ventral blastomeres of 4-cell stage embryos. 10 pg of Xwnt-8 RNA was injected into the vegetal hemisphere of fertilized eggs.

**Online Supplemental Materials**

Six additional supplemental figures (Figs. S1–S6) discussed throughout this manuscript are available at http://www.jcb.org/cgi/content/full/150/4/929/DC1.

Figs. S1 and S2. There is no evidence for junctional transfer of LY in sectioned embryos coinjected with LY and dextran-rhodamine (injection into one dorsal animal blastomere). Fig. S1 shows sections from four different embryos. Fig. S2 shows a whole mount and serial sections of a single embryo.

Fig. S3. Cytoplasmic bridges allow distribution of dyes at the 16- and 32-cell stage embryos. The colocalization of LY and dextran-rhodamine indicates no gap junctional transfer of LY.

Fig. S4. Gap junctional transfer of neurobiotin among vegetal blastomeres of normal embryos at the 64-cell stage and at stage 8.

Fig. S5. UV irradiation does not block gap junctional transfer of neurobiotin.

Fig. S6. Control panels show lineage tracing of dye injected cells, controls for the detection of neurobiotin and the results of alterations of the dorsal-ventral axis of embryos whose siblings were analyzed in dye transfer assays.

**Results**

**LY Transfer in Early Xenopus Embryos**

To assess the levels of intercellular communication while controlling for the presence of cytoplasmic bridges, each blastomere was injected with a mixture of intercellular channel-permeant (<1 kD) and -impermeant (>10 kD) molecules. The first set of experiments used LY and dextran-rhodamine (DR), the latter extensively dialyzed to eliminate unbound rhodamine. One animal cell, tier 1 of 32-cell stage embryos, was injected, incubated 10 min then fixed and examined by fluorescence microscopy. As reported in earlier studies, ventral injections produced little evidence of LY transfer (Fig. 1 A), whereas dorsally injected embryos showed variable levels of apparent transfer (Fig. 1 C). However, LY and DR were always coincident (compare Fig. 1, A and B with C and D) suggesting that an extensive network of cytoplasmic bridges, rather than gap junctions, accounted for the movement of the markers. To examine this possibility, serial sections of 36 embryos were analyzed. Fig. 2 compares the whole-mount and sectioned appearance of a single dorsally injected 32-cell stage embryo. Surprisingly, serial sectioning revealed that LY and DR were present in only two cells (Fig. 2, C–H), not in a significant number of cells as suggested by the whole-mount appearance (Fig. 2, A and B). Note that the concentrations of LY and DR are not identical in the sections of each of the cell pairs in Fig. 2, indicating that the cytoplasmic bridge was narrow or transient enough to partially restrict the intercellular transfer of both fluorescent molecules. A limited LY and DR codistribution was consistently observed in all sectioned embryos. A limited comparisons between sectioned and whole-mounted embryos may be seen in Figs. S1 and S2.

Since published reports of LY transfer are based on studies of both 16- and 32-cell stage embryos, we also analyzed 16-cell stage embryos. Guthrie et al. (1988) concluded that during the 16-cell stage, dye freely moved between blastomeres on both the presumptive ventral and dorsal embryonic poles. At the 32-cell stage, 50–70% of dorsal injections and only 35% of ventral injections resulted in LY transfer (Guthrie et al., 1988). To reinvestigate these observations, we collected a population of 16-cell stage embryos (n = 150) and injected a cohort of them...
every 5 min with the LY/DR mixture. Injections were continued until the embryos were well into the 32-cell stage. None of the serial sections of the embryos in this experiment evidenced LY gap junctional transfer (see Fig. S3).

We have repeated these experiments with two additional negatively charged tracers, Alexa 350 hydrazide (Mr = 349.29) and Alexa 488 hydrazide (Mr = 570.48). As in the LY experiments, embryos were injected in each case with a mixture of the small tracer together with a large dextran complex, and were studied in serial sections. Both dyes failed to indicate junctional transfer (24 injected embryos with each tracer were analyzed; not shown).

Analysis of dye transfer in whole-mount could produce false positive results due to reflection and scattering of the fluorescent signal. Individual, round blastomeres could act as crude lenses, focusing emitted light into adjacent, yolk-filled cells, which then scatter the light and appear to contain dye. This lens effect could account for the asymmetry in dye transfer between ventral and dorsal blastomeres reported previously (Guthrie, 1984; Guthrie et al., 1988; Olson et al., 1991; Olson and Moon, 1992; Guger and Gumbiner, 1995; Krufka et al., 1998). On the ventral surface, the increased pigment would tend to mask the lens effect, making the ventral blastomeres appear to be less capable of transferring dye.

Figure 2. No evidence for junctional transfer of LY was detected in sectioned embryos coinjected with LY and dextran-rhodamine. An embryo in which LY appeared to transfer to adjacent cells (Fig. 2, A and B) was serially sectioned. ~70 cross sections along the vegetal to animal axis were analyzed. Colocalization of the dyes was always seen, indicating that there was no junctional transfer. (C–F) The dyes were unevenly distributed between the injected cell (very bright green or red cell) and a second cell, which is marked by an asterisk. (G–H) Both dyes remained colocalized in one cell in a plane of section that did not include the injected cell. The presence of dextran-rhodamine in asterisked cells indicates the presence of persistent cytoplasmic bridges with the more brightly stained injected cells. Bars: (A) 250 μm; (C) 200 μm.
To demonstrate the lens effect, dextran-fluorescein (DF) alone was injected into one dorsal or one ventral blastomere in tier 1 of 32-cell stage embryos (Fig. 3). Whereas DF is too large to pass through gap junctions, an apparent transfer of the tracer to many cells in the dorsally injected embryo can be seen (Fig. 3A). In the ventrally injected embryo, which was injected with an identical volume of DF, the higher pigment content not only makes the injected cell appear to contain less tracer, but it also shields the lens effect (Fig. 3B). Sections through these embryos (Fig. 3, C and D) revealed that after either injection, the junction-impermeable DF is confined, as expected, to those cells that are joined by cytoplasmic bridges.

Summarizing our LY experiments, the apparent distribution of the junction-permeable marker LY in whole-mounts was very different from that in serial sections. In addition, we were unable to find any evidence for junctional transfer of LY using either approach. Our inability to demonstrate LY transfer was completely inconsistent with published studies (Guthrie, 1984; Warner et al., 1984; Guthrie et al., 1988; Nagajski et al., 1989; Olson et al., 1991; Olson and Moon, 1992; Guger and Gumbiner, 1995; Krufka et al., 1998; Levin and Mercola, 1998).

GJIC between Blastomeres in the Xenopus Embryo Revealed by Neurobiotin

To ascertain if intercellular communication is present in the early embryo, we employed neurobiotin (NB), which can detect intercellular communication in situations where LY does not (Peinado et al., 1993; White et al., 1998). NB was injected in a mixture with either FR or DF and detected in serial sections with either streptavidin–Alexa 488 conjugate or avidin–rhodamine. In the first set of experiments, the neurobiotin mixture was injected into one dorsal-animal tier-1 cell of embryos. NB transfer was evident in 16-cell (Fig. 4A and B) and 32-cell stage embryos (Fig. 4C and D). In those examples, both gap junctional transfer (Fig. 4A and C, cells marked 1 and 2) and passage through a cytoplasmic bridge (Fig. 4C and D, the cell marked with an asterisk) were detected. As development proceeded, the levels of GJIC increased. This could be clearly seen among animal pole cells in embryos at the 64- (Fig. 4E and F) and 128-cell stage (Fig. 4G–K). At the 128-cell stage, junctional transfer from animal pole cells (Fig. 4G) to vegetal cells (Fig. 4I) was apparent. In the second set of experiments, neurobiotin mixture was injected into one central-most vegetal blastomere in a second group of embryos and junctional transfer was evident as well (Fig. S4).

Neurobiotin Does Not Reveal Asymmetry in Gap Junctional Communication

To reinvestigate the possibility of dorso-ventral asymmetry in intercellular communication, we injected NB and DF into a ventral animal cell in tier 1 of 64-cell stage embryos (n = 48) and analyzed serial sections. Dye transfer could be seen in the plane of the injected cell (Fig. 5A and B), as well as in adjacent sections towards the marginal zone where the injected cell was not seen (Fig. 5C and D). This pattern of dye transfer was indistinguishable from the data obtained when a single dorsal animal cell was injected in the same batch of embryos (see Fig. 4E and F). To control for fidelity in the identification of the dorso-ventral embryonic poles, LY was injected into one ventral or one dorsal blastomere of embryos that were analyzed at the tadpole stage (see Fig. S6E and F, control panels).

Quantitated data from 12 dorsally injected and 12 ventrally injected embryos are shown in Table I. Fluorescent cell profiles were examined on each section for both NB and DF, and only NB-containing cells were scored positively. Thus, the injected cell and all cells joined to the in-
jected cell by cytoplasmic bridges were omitted from the totals. Comparison of the resulting numbers of cells revealed no asymmetry in neurobiotin transfer between dorsal and ventral sides of the embryo. The numbers shown in Table I are not the absolute numbers of cells receiving the dye, as all blastomeres were present in multiple sections and were counted more than once. We conclude that GJIC, as demonstrated by neurobiotin transfer, is similar among ventral and dorsal blastomeres.

**UV-irradiated and Xwnt-8–injected Embryos**

It has been reported that intercellular communication was sensitive to manipulations affecting the dorso-ventral fate of the embryo (Nagajski et al., 1989; Olson et al., 1991; Olson and M. oon, 1992; Guger and Gumbiner, 1995; Krufka et al., 1998). To determine if such treatments changed the patterns of NB transfer, we examined embryos ventralized by UV irradiation or dorsalized by exogenous expression of Xwnt-8. Unirradiated and UV-irradiated embryos from the same batch were then injected with a mixture of NB and FD. Analysis of the serial sections from 64-cell stage embryos (n = 48) showed unperturbed dye transfer between dorsal blastomeres in UV-irradiated embryos (Fig. S5).

Determination of Guidelines for Dye Transfer Experiments

Our attempts to study GJIC in the *Xenopus* embryo revealed that LY does not transfer between embryonic blastomeres through gap junctions. Furthermore, the analysis of whole-mount embryos using any fluorescent dye is subject to optical artifacts. While whole-mount specimens showed apparent transfer of junction permeant and impermeant molecules, transfer was not observed in paraffin embedded and in frozen (not shown) sectioned specimens. The explanation for this discrepancy is that the intense emission from a single, highly fluorescent cell can be internally reflected within the embryo, illuminating adjacent cells whose light-scattering organelles make them also ap-
pear fluorescent. In addition, the asymmetric pigmentation of the Xenopus embryo produces an apparent asymmetry in this light-scattering effect. Thus, inspection of whole-mount embryos is an unreliable method for the assessment of dye transfer between embryonic blastomeres. A rigorous and unambiguous demonstration of gap junctional intercellular communication demands both the coinjection of permeant and impermeant tracers and the examination of sectioned specimens.

Table II provides a summary of the studies which used LY transfer to demonstrate asymmetric communication in the Xenopus embryo. None of these studies meet the criteria we have established. Some of these studies did not include injection of impermeant tracers while others injected the impermeant markers independently, in sibling embryos. In all previous studies, quantitative data were collected by whole-mount analysis, although in a few cases, a subset of sibling embryos were sectioned. However, our data show that sibling controls are unreliable because of the transient and asynchronous presence of cytoplasmic bridges. A synchronous division of equivalent blastomeres in a cohort of embryos results in an equivalent lack of synchrony in the completion of cytokinesis and a variable persistence of cytoplasmic bridges. In addition, due to the rapidity of the cell cycle, not only may dye-injected cells cleave at different times than their counterparts in sibling embryos but a cell may also enter a new cycle before it has finished cytokinesis. Therefore, a gap junctional impermeant tracer must always be coinjected with a permeant molecule in each embryo in order to distinguish cytoplasmic bridges from transfer through gap junctions.

Junctional Communication between Xenopus Blastomeres

In contrast to LY, transfer of NB is evident at all embryonic stages tested. Different rates of LY and NB transfer have been observed in the ocular lens and postnatal neocortex (Peinado et al., 1993; White et al., 1998), likely due to the well-documented dependence of channel permeability on connexin composition (Elfgang et al., 1995; Cao et al., 1998). Our data show that gap junctions between Xenopus blastomeres are either impermeable to LY or transfer NB too slowly to be detectable on a time scale compatible with the rapid cell divisions. Our failure to detect LY transfer is subject to technical limitations. Whereas published studies have shown that fixation and embedding do not result in dye redistribution, about half of the injected dye may be lost during these processes.
If the rate of LY transfer were very low, just on the edge of detection, and if half were lost during processing for sectioning, than such a transfer may not be detectable. Regardless, LY transfer in Xenopus embryos does not occur at the levels and in the asymmetrical patterns previously reported.

Our studies leave open the question of the role of junctional communication in embryonic patterning. NB fluorescent dextran mixtures clearly revealed communication within and between blastomeres of the animal and vegetal poles. However, communication levels among presumptive dorsal blastomeres were similar to those among ventral blastomeres as visualized by NB. Neither UV irradiation nor exogenous Xwnt-8 expression had any effect on these patterns of cell–cell communication. Thus, our data do not support previously published correlative studies suggesting that communication affects dorso-ventral axis determination in Xenopus. However, we observed significant increases in the levels of communication during early cleavage stages using neurobiotin as a probe and it is possible that other probes would reveal asymmetries that would implicate junctional communication in early patterning events.

We are grateful for helpful discussions with Drs. A.E. Warner, R.T. Moon, B.M. Gumbiner, J. Heasman, and R.G. Johnson. We are also grateful to Drs. A.E. Warner, R.T. Moon, B.M. Gumbiner, J. Heasman, and R.G. Johnson. We are also grateful to Drs. M. Levin and M. Mercola for helpful discussions and sharing of Lucifer yellow reagents.

This work was supported by grant GM 18974 (D.A. Goodenough) and GM 37751 (D.L. Paul).

Submitted: 13 March 2000
Revised: 30 May 2000
Accepted: 6 July 2000

References


table II. Comparison Lucifer Yellow Protocols to Study GJIC in Early Xenopus Embryos

<table>
<thead>
<tr>
<th>Study</th>
<th>Measured Lucifer transfer at stages</th>
<th>How passage via cytoplasmic bridges was excluded</th>
<th>All data were collected from</th>
<th>Control for reflection and scattered light</th>
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</thead>
<tbody>
<tr>
<td>Warner et al., 1984</td>
<td>32</td>
<td>No fluorescent dextran injections</td>
<td>Whole-mounts</td>
<td>Few sibling embryos were analyzed on frozen or plastic sections</td>
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<td>Guthrie, 1984</td>
<td>32</td>
<td>Lucifer yellow was injected into one group of embryos and fluorescent dextran was injected into a separate group of embryos</td>
<td>Whole-mounts</td>
<td>No sections</td>
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<td>Whole-mounts</td>
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<td>Krufta et al., 1998</td>
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<td>Whole-mounts</td>
<td>No sections</td>
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<tr>
<td>Levin and Mercola, 1998</td>
<td>16–128</td>
<td>Fluorescent dextran was injected in a mixture solution with Lucifer yellow</td>
<td>Whole-mounts</td>
<td>No sections</td>
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<tr>
<td>This study</td>
<td>16–128</td>
<td>Fluorescent dextran was injected in a mixture solution with Lucifer yellow</td>
<td>Paraffin or frozen sections</td>
<td>All embryos were analyzed on either 12-μm paraffin or 14-μm frozen sections</td>
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All embryos were analyzed on either 12-μm paraffin or 14-μm frozen sections.