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Short Contribution

Evaluation of anionic histological dyes as co-injectable cell markers in pre-implantation mouse embryos

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ABSTRACT The enzyme horseradish peroxidase (HRP) is a widely used microinjectable cell marker for studying cell position, lineage, and migration in many kinds of animal embryos. Marked cells are easily identified because they darken when exposed to a chromophore and an HRP substrate such as hydrogen peroxide. This assay, however, requires cytochemical fixation. Thus, when HRP-marked cells need to be identified prior to fixation, visible co-injectants such as dyes and fluorescent substances have been used with HRP. Fluorescent substances have limitations because their excitation could be harmful to the marked cells. Visible but non-fluorescent co-injectants, however, would permit visualization of HRP-marked cells without inflicting such damage. We tested the compatibility of several histological dyes and electrolytic carriers with HRP iontophoresed as a cell marker in 2-cell mouse embryos. The dyes tested were Evans Blue, Cibacron Blue F3GA, Fast Green FCF, and Patent Blue Violet; the electrolytic carriers were KCl, K_2SO_4, CH_3C0_2K, and KH_2PO_4. The combination found most useful was Patent Blue Violet in K_2SO_4. Survival of embryos incubated to the blastocyst stage following injection with HRP + Patent Blue Violet in K_2SO_4 at the 2-cell stage was significantly greater than that of embryos injected with any other dye. Although the proportion of embryos undergoing the 8-cell-to-morula transition was somewhat decreased by this treatment, the proportion of embryos reaching the blastocyst stage was comparable to that in the uninjected (control) group. Our results indicate that Patent Blue Violet is a useful, HRP-co-injectable dye for short-term cell marking in pre-implantation mouse embryos.

KEY WORDS: *mouse embryos, microinjection, histological dyes*

Horseradish peroxidase (HRP) has traditionally been used to delineate neuronal projections *in situ*. This technique has been successfully adapted in recent years for use in cell lineage analysis in both invertebrate and vertebrate embryos (Jacobson and Hirose, 1978; Weisblat *et al.*, 1978, 1980; Hirose and Jacobson, 1979; Balakier and Pedersen, 1982; Kominami, 1983; Nishida and Satoh, 1983; Taghert *et al.*, 1984; Kimmel and Warga, 1986; Technau, 1987). The cell-marking procedure consists of HRP microinjection by pressure or iontophoresis and subsequent introduction of a peroxide substrate combined with an oxidizable chromophore such as diaminobenzidine or a mixture of *p*-phenylenediamine and pyrocatechol (Hanker-Yates reagent; Hanker *et al.*, 1977). This reaction yields a dense, dark-brown reaction product (Saunders *et al.*, 1964; Mesulam, 1982) which reveals the location of the marked cell and/or its descendants. When the position of a marked cell requires precise and immediate verification, HRP has been co-injected with fluorescent conjugates such as rhodamine (Balakier and Pedersen, 1982; Stent *et al.*, 1982; Cruz and Pedersen, 1985; Lawson *et al.*, 1986; Pedersen *et al.*, 1986; Dyce *et al.*, 1987; Winkel and Pedersen, 1988) and fluorescein (Shankland, 1984) dextran or with organic dyes such as Fast Green FCF (FG) (Cruz and Pedersen, 1985; Ho and Weisblat, 1987; Stuart *et al.*, 1989). Co-injection of these readily visible markers is necessary because at the concentrations used, HRP is colorless and therefore undetectable upon injection.

The utility of HRP as a cell marker is considerably enhanced when it is co-injected with a fluorescent conjugate. The cationic conjugate rhodamine dextran has been co-injected with HRP in cell-marking experiments on mouse embryos of various ages (Cruz and Pedersen, 1985; Lawson *et al.*, 1986; Pedersen *et al.*, 1986; Dyce *et al.*, 1987; Winkel and Pedersen, 1988). In these papers, however, concern has been expressed about the possibility of cell injury resulting from exposure of rhodamine-bearing cells to the rhodamine excitation range. Indeed, microinjected fluorescein (Shankland,

Abbreviations used in this paper: CB, Cibacron Blue F3GA; EB, Evans Blue; FG, Fast Green FCF; HRP, Horseradish Peroxidase; PBV, Patent Blue Violet.

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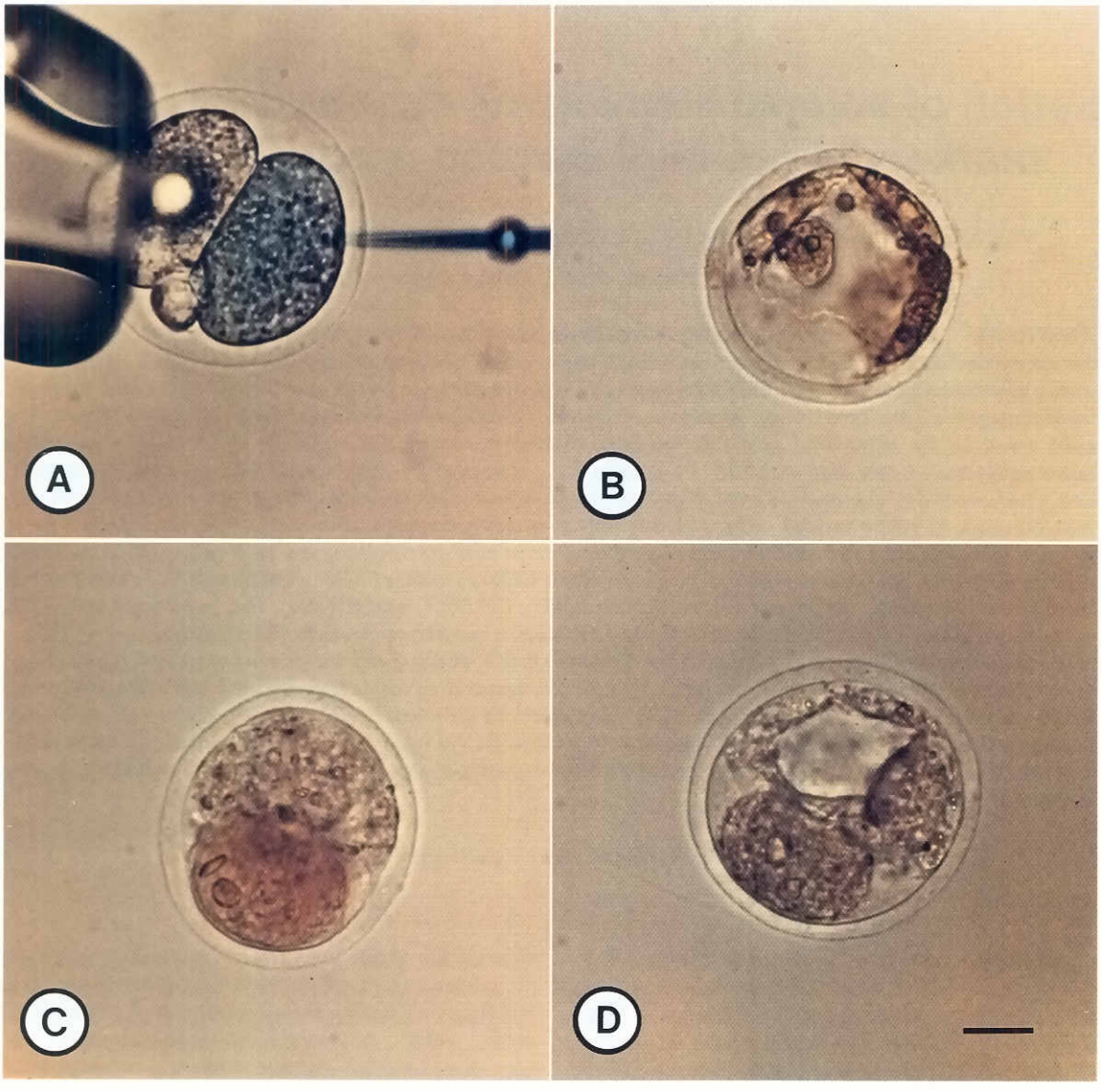


Fig. 1. Embryos labeled with horseradish peroxidase plus dye. (A) *Two-cell embryo injected with HRP + CB in K_2SO_4. A similar injection procedure was used for the other treatments.* **(B)** *Blastocyst with approximately half its blastomeres staining positively for HRP. These stained cells are descended from the one blastomere which received HRP-containing injectant at the 2-cell stage.* **(C)** *Embryo with uninjected blastomeres successfully forming a morula. Large, undivided, HRP-positive blastomere has been excluded from the small but otherwise normal morula.* **(D)** *Embryo with uninjected blastomeres successfully forming a blastocyst. The two large blastomeres, which died after the second cleavage, are weakly HRP-positive. Scale bar, 25 μm.*

1984) and fluorescein dextran amine (Gimlich and Braun, 1985; Stuart *et al.*, 1989) have been used to sensitize leech blastomeres to laser-mediated photoablation. Fluorescent emission by fluorescein is apparently accompanied by formation of singlet oxygen radicals, which leads to cell lysis (Braun and Stent, 1989a, b). While fluorescein and rhodamine fluoresce at different excitation ranges (~450 - 510 nm and ~510 - 560 nm, respectively) and are only briefly excited when used as cell markers, the possibility of cell damage cannot be completely eliminated. In certain circumstances therefore, fluorescent cell markers have limited use.

We wanted to devise a cell-labeling protocol for use with pre-implantation mouse embryos which would allow us to ascertain the position of HRP-injected blastomeres but would not entail the use of fluorescent markers. Our previous experience with HRP-FG co-injections suggested that either FG or the negative current required for its iontophoresis results in increased mortality in mouse blastocysts (Cruz and Pedersen, 1985). We therefore set out to test other histological dyes and electrolytic carriers for suitability as microinjectable cell markers. Our results indicate that Patent Blue Violet iontophoresed in K_2SO_4 carrier is a potentially useful short-term cell marker in pre-implantation mouse embryos.

The effects on survival of iontophoretically microinjecting 1) HRP

TABLE 1

DEVELOPMENT OF MOUSE EMBRYOS MICROINJECTED WITH HRP IN DIFFERENT CARRIERS IONTOPHORESED WITH A TANDEM PULSE OF HYPERPOLARIZING/DEPOLARIZING CURRENT

Stage*	Unexposed control**	Control	Injected with HRP in			
			KCl	CH_3CO_2K	KH_2PO_4	K_2SO_4
2-cell***	103	47	71	68	65	57
4-cell	91.3a	93.6a	70.4ab	70.6ab	60.0b	71.9ab
8-cell	79.6a	70.2ab	36.6cde	47.1bdf	32.3cf	47.4be
morula	73.8a	61.7ab	29.5bd	36.8b	18.5cd	38.5b
blastocyst	45.6a	40.4ab	9.8c	10.3cd	13.8bd	15.8b
blastocyst from uninjected blastomere	-	-	4.2a	7.4a	4.6a	7.0a

* For any stage, values not sharing a common letter suffix differ significantly ($P<0.001$).
** Embryos were not exposed to microinjection arena.
*** Number of embryos. All other values are expressed as % of initial numbers of incubated 2-cell embryos.

in different carriers, 2) HRP + K_2SO_4 with a single pulse of depolarizing or a tandem pulse of hyperpolarizing/depolarizing current, and 3) HRP + K_2SO_4 + different dyes into one of the two blastomeres in 2-cell mouse embryos are presented in Tables 1-3. Embryos that lysed during or immediately after injection were discarded. Only embryos in which the injected blastomere became colored and sustained no additional visible changes were incubated for further observation (Fig. 1). Control embryos were placed in the injection arena although they were not injected. Because control embryos received the same handling and exposure to ambient temperature as injected embryos, mortality in each injected group could be directly and specifically attributed to the injectant used. Embryos were incubated until the blastocyst stage and assayed for the presence of HRP. Most blastocysts had several HRP-positive blastomeres, indicating that the blastomere injected with HRP at the two-cell stage survived and divided (Table 2). When the injected blastomere did not survive, the uninjected survivor nevertheless formed a blastocyst (Fig. 1D). This is reported as "blastocyst from uninjected blastomere" in Tables 1-3.

TABLE 2

DEVELOPMENT OF MOUSE EMBRYOS MICROINJECTED WITH K_2SO_4 ALONE OR HRP IN K_2SO_4 CARRIER IONTOPHORESED WITH A PULSE OF DEPOLARIZING (d) OR A TANDEM PULSE OF HYPERPOLARIZING/DEPOLARIZING (h/d) CURRENT

Stage*	Control	Injected with			
		K_2SO_4 (d)	K_2SO_4 (h/d)	HRP (d)	HRP (h/d)
2-cell**	70	63	83	85	40
4-cell	91.4a	92.0a	83.1a	89.4a	77.5a
8-cell	84.2a	82.5a	79.5a	81.1a	72.5a
morula	77.1a	73.0a	60.2ac	42.3bc	20.0b
blastocyst	55.7a	46.0a	30.1a	20.0b	15.0b
blastocyst from uninjected blastomere***	-	-	-	27.0a	25.0a

* For any stage, values not sharing a common letter suffix differ significantly ($P<0.001$).
** Number of embryos. All other values are expressed as % of initial numbers incubated at the 2-cell stage.
*** This value could not be unequivocally determined for the K_2SO_4-only injections.

Effect of Carrier

The carriers tested were KCl, CH_3CO_2K, KH_2PO_4, and K_2SO_4. K_2SO_4 was clearly superior as it caused no significant mortality at any stage from the 2-cell to the blastocyst (Table 1). CH_3CO_2K ranked second to K_2SO_4, causing significant mortality during the blastocyst stage only. KCl significantly decreased embryonic survival in two instances: the 2-to-4-cell and the morula-to-blastocyst transitions. KH_2PO_4 consistently decreased embryonic survival through the pre-blastocyst stages, but did not cause significant mortality during the morula-to-blastocyst transition.

TABLE 3

DEVELOPMENT OF MOUSE EMBRYOS INJECTED WITH ANIONIC HISTOLOGICAL DYES AND HRP IN K_2SO_4 CARRIER IONTOPHORESED WITH A TANDEM PULSE OF HYPERPOLARIZING/DEPOLARIZING CURRENT

Stage*	Control	HRP only	HRP+ Cibracron Blue	HRP+ Evans Blue	HRP+ Fast Green	HRP+ Patent Blue Violet
2-cell**	64	60	59	63	65	65
4-cell	91.2a	78.3a	77.9a	66.6a	67.6a	75.3a
8-cell	84.3a	71.6a	72.8a	65.0a	61.5a	70.7a
morula	81.2a	20.0bc	18.6c	14.2c	15.3c	49.2b
blastocyst	53.1a	15.0bc	16.9bc	6.3bc	15.3bc	33.8a
blastocyst from uninjected blastomere	-	25.0a	6.7b	28.6a	26.1a	7.6b

* For any stage, values not sharing a common letter suffix differ significantly ($P<0.001$).
** Number of embryos. All other values are expressed as % of initial numbers of incubated 2-cell embryos.

Effect of HRP and Injection Current

Iontophoresis of K_2SO_4 carrier alone had no detrimental effects on embryonic survival to the blastocyst stage. This was true whether a single depolarizing pulse or a hyperpolarizing/depolarizing tandem pulse of current was used for iontophoresis (Table 2). When HRP was added to K_2SO_4, however, embryonic survival decreased significantly regardless of injection current used. This decline, which was notably absent with K_2SO_4-alone injections, began at the 8-cell and continued through the blastocyst stage.

Effect of Dye

Four anionic dyes in the green-to-violet range were tested using K_2SO_4 as carrier: Cibacron Blue F3GA (CB), Evans Blue (EB), Fast Green FCF (FG), and Patent Blue Violet (PBV) (Table 3). Each dye was co-injected with HRP using a tandem pulse of hyperpolarizing/depolarizing current to iontophorese, first the dye, then HRP. The survival of injected embryos was comparable to that of controls until the 8-cell-to-morula transition. After this time, all the dyes caused

TABLE 4

CHARACTERISTICS OF HISTOLOGICAL DYES USED AS MICROINJECTABLE CELL MARKERS

Name	Molecular Weight	Color Index Number
1. Cibacron Blue F3GA	840.1	61211
2. Evans Blue	960.8	23860
3. Fast Green FCF	808.9	42053
4. Patent Blue Violet	566.7	undesignated*

* Isosulfan Blue is listed by the manufacturer as a proposed name for this dye (cat. no. P-1888, Sigma Chemical Co.). Its properties are similar to those of dyes with Color Index Numbers 42045 and 42051.

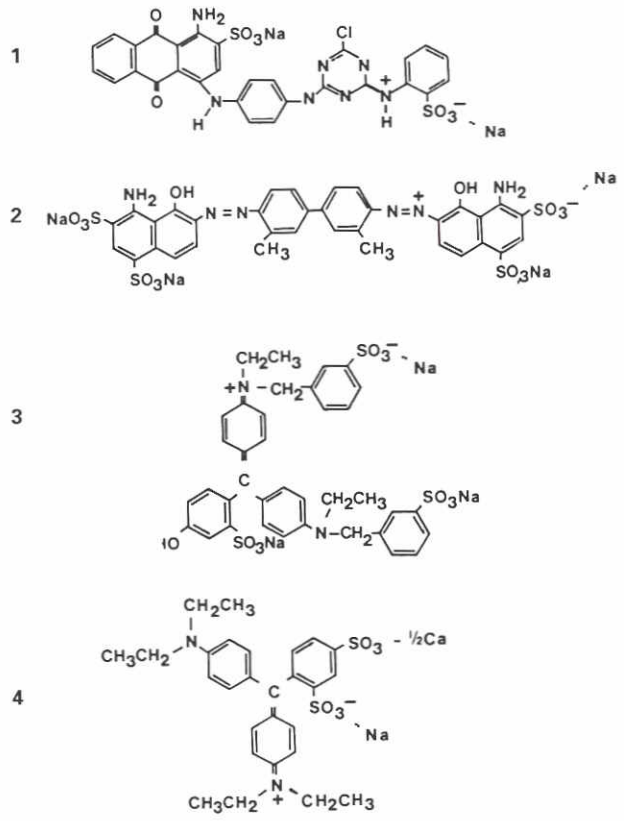
a progressive decline in survival, although embryos injected with PBV sustained no further decline at the blastocyst stage. Each dye was visible in the cytoplasm of the injected blastomere for variable periods (5 min to 2 hr) after injection.

Our results indicate that certain anionic, non-fluorescent dyes may be co-injected with HRP as short-term cell markers in mouse pre-implantation embryos. In particular, Patent Blue Violet co-iontophoresed with HRP in K_2SO_4 carrier was determined to be superior to other anionic dyes of similar molecular weight in the green-to-violet range.

Cell markers iontophoresed at the 2-cell stage generally decreased embryonic survival, but did so only after a delay of at least one cell cycle. Thus, only at the 8-cell stage was the decline in survival detected. This decline coincides with compaction and the initial formation of cell junctions in the mouse embryo (Ducibella and Anderson, 1975; Lo and Gilula, 1979; Wiley, 1987; reviewed in Cruz, in press) and suggests that intercellular coupling precedes, but does not presage, the onset of degenerative changes. A more detailed study of these changes is required to establish precisely how they arise and how they may be eliminated. The present results suggest, however, that iontophoresis of visible dyes as cell markers is a useful method of cell lineage analysis in the short term.

The choice of carrier is important when iontophoresis is required to introduce cytoplasmic cell markers. Unlike Cl^- ions, the concentration of cytoplasmic K^+ ions is generally high. Thus, a cell is more likely to withstand a small influx of iontophoresed K^+ ions than Cl^-. Since the dyes tested were anionic, it was important to select a carrier that did not contain Cl^- ions (Cruz and Pedersen, 1985). The present results indicate that iontophoresed SO_4^{-2} ions do not significantly increase embryonic mortality at any stage evaluated. Thus, K_2SO_4 is more suitable than the widely used electrolytic carrier, KCl, for marking cells by iontophoresis of anionic substances.

Although useful in cell lineage studies, iontophoresis of HRP contributed to the mortality observed among embryos grown *in vitro*. Our results indicate that iontophoresis of HRP in K_2SO_4 decreased embryo survival from 40%-56% to 15%-16% (Tables 1-3). These figures represent a more drastic decline than those reported by Balakier and Pedersen (1982) (66% to 48%), who used culture media identical to ours. The discrepancy between these two sets of results may be due to differences in how the control embryos were handled, the carrier and current used, and mouse strains involved. We allowed our control embryos the same handling and brief exposure to room temperature sustained by injected embryos. Balakier and Pedersen (1982) do not indicate whether their control



Structural formula of histological dyes used as microinjectable cell markers. (1) *Cibacron Blue F3GA.* **(2)** *Evans Blue.* **(3)** *Fast Green FCF.* **(4)** *Patent Blue Violet.*

embryos were placed alongside the treated embryos as these were being microinjected, or incubated without further exposure to room temperature. However, a comparison of the survival of uninjected embryos placed in the microinjection arena (40.4%, Control) with that of uninjected embryos immediately incubated after removal from the uterus (45.6%, Unexposed Control) indicates that embryonic survival to blastocyst stage is unaffected by mere exposure to the microinjection arena (Table 1) but severely diminished by microinjection with HRP, HRP + dye, or HRP + dye + carrier other than K_2SO_4 (Tables 1-3). Since Balakier and Pedersen (1982) used KCl as carrier, depolarizing injection current only, and a different mouse strain, a direct comparison of their data with ours is not possible.

Co-injection of dyes with HRP permitted instant visual verification of the position of the injected cell. Unlike dyes in the red-to-black range preliminarily screened for use in this study, the dyes were clearly visible at the concentration used (0.5 *M*). Although their anionic character required injection with tandem pulses of hyperpolarizing/depolarizing current, we reasoned that since iontophoresed SO_4^{-2} ions appeared to have no detrimental effects on embryonic development (Table 2), we would be able to attribute any decline in embryonic survival to HRP or to the HRP-dye combination.

The dyes chosen for this study are described in Table 4. All of them were readily soluble in the carrier (K_2SO_4), releasing an SO_3^{-2}-bearing color moiety (Gurr, 1960, 1966). Normally used as general histological stains, some of these dyes have specific uses. For instance, EB has been used not only for estimation of blood

volume and vital staining of human malignant tumors (Gurr 1960, 1966) but also for retrograde labeling of axons (van der Kooy and Kuypers, 1979). EB has also been reported to elicit DNA repair in hamster hepatocytes (Kornbrust and Barfknecht, 1984). FG was originally proposed as a food dye (Johnson and Staub, 1927) and today continues to be a common food additive (Kornbrust and Barfknecht, 1985). It has also been used as a microinjectable neuronal marker (Parnas and Bowling, 1977) and is routinely used for cell lineage analysis in leech embryos as an HRP-co-injectable marker (see, for example, Ho and Weisblat, 1987; Stuart *et al.*, 1989). Dyes in the yellow-to-red range, although cationic, were not visible at low concentrations, nor were the blue dyes Nile Blue Sulfate, Aniline Blue, and Chicago Sky Blue. Black or brown dyes such as Sudan Black and Nigrosin were also excluded, as they were either highly insoluble in the carrier, or were likely to release heavy metals when dissolved.

PBV was clearly the most promising HRP-co-injectable cell marker tested. The survival of PBV-injected embryos to the blastocyst stage compares favorably with that of uninjected controls (Table 3). Our results indicate PBV is superior to FG, an anionic dye that has been used with some success to study cell lineage in leech (Blair, 1982, 1983; Blair and Weisblat, 1982, 1984; Shankland and Weisblat, 1984; Ho and Weisblat, 1987; Shankland, 1987; Stuart *et al.*, 1989) and mouse (Cruz and Pedersen 1985) embryos. PBV should thus be a viable substitute for FG. Unlike the other dyes tested here for use with 2-cell mouse embryos, PBV had no adverse effect on embryonic survival except during the 8-cell-to-morula transition. This effect was surprising, as it suggests that co-injecting PBV with HRP alleviated the decrease in survival attributed to HRP injection with a tandem pulse of hyperpolarizing/depolarizing current (Table 2). Although we are unable to explain this unusual result, we observed it consistently in our experiments. It is therefore unlikely to be an artifact.

Experimental Procedures

Embryos

Six- to twelve-week-old female Swiss Webster mice (Hilltop Laboratory Animals, Inc., Scottdale, PA) were induced to superovulate with intraperitoneal injections of 5 IU each of pregnant mare's serum gonadotropin (a gift from Teikoku Zoki Co., Tokyo, Japan) and human chorionic gonadotropin (HCG) (Sigma Chemical Company, St. Louis, MO) administered 48-52 h apart. Each female was caged overnight with a male and examined for a copulation plug the next morning. All mice were maintained on a photoperiod of 14:10 (L:D) in a room kept at 24°C. Two-cell embryos were obtained 40-42 h after HCG injection by flushing explanted oviducts with modified Hanks' balanced salt solution (Spindle, 1980). The embryos were transferred with a sterile, finely drawn glass pipet to an organ culture dish (Falcon Plastics, Oxnard, CA) with 1 ml modified Eagle's medium (Spindle, 1980) and incubated 1-2 h before injection at 37°C in a saturated atmosphere of 5% (v/v) CO_2 in air.

Dyes

A mixture of 2.5% HRP (Type VI, mol wt 40,000) and one of the following dyes was used: Cibacron Blue (CB) (Aldrich Chemical Co., Milwaukee, WI), Evans Blue (EB), Fast Green FCF (FG) (Fisher Scientific Co., Firlawn, NJ), and Patent Blue Violet (PBV). Instead of calculating concentrations on a w/v basis, we used a standard 0.05 *M* concentration for all the dyes tested. This figure closely approximates what has been reported for FG (Cruz and Pedersen, 1985) and allowed us to evaluate their effects at equivalent concentrations. The carriers used were 0.05 *M* aqueous solutions of KCl, K_2SO_4, KH_2PO_4, and CH_3CO_2K. All injectants were obtained from Sigma Chemical Co., (St. Louis, MO) unless otherwise indicated.

Microinjection

Embryos were injected using the technique of Cruz and Pedersen (1985). Briefly, this procedure involved constant-current (4-6 x 10^{-6} A) iontophoresis for 15 sec with a single depolarizing pulse, or a tandem pulse of hyperpolarizing/depolarizing current (15 sec each) monitored with an electrometer and bridge (Winston Electronics, San Francisco, CA). An inverted microscope (Nikon Diaphot) fitted with stage-mounted hydraulic micromanipulators (Narishige Scientific Instrument Laboratory, Tokyo, Japan) and microelectrode holders (W-P Instruments, Inc., New Haven, CT) was used.

Culture and Observations

All embryos were incubated to the blastocyst stage prior to assay. HRP was histochemically detected using Hanker-Yates reagent (Polysciences, Warrington, PA) as chromophore and 1% H_2O_2 as substrate in 0.1 *M* Tris buffer (Hanker *et al.*, 1977). Embryos were then fixed and stored in 2.5% glutaraldehyde (Tousimis Research Corp., Rockville, MD) in 0.1 *M* phosphate buffer, pH 7.2. Fixed embryos were refrigerated until scored for HRP staining by direct visualization at 200X under Nomarski optics. Each embryo was rotated several times during visual examination with a manually pulled glass micropipette heat-polished in a Narishige microforge. Photographs were taken with a Nikon UFX microscope camera on Kodak Ektachrome film, 160 ASA.

Statistical Analysis

Predetermined pairs of treatments were compared using a 2-sample chi square test.

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