The use of fluorescent marker dyes for studying intercellular communication in nematode embryos

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ABSTRACT As more and more cases of necessary cell-cell interactions are revealed, the classical view of mosaic development in nematodes has to be replaced by a more dynamic picture showing different types of intercellular communication. To investigate the pattern and function of communication pathways between cells, we have developed different techniques to shunt fluorescent marker dyes into embryos and hatched animals and study their distribution in vivo. During embryogenesis we find that for a long time all somatic cells form a single dye-coupling compartment while transfer into the germline is restricted already at an early stage. Considerable variations between species with respect to the size of communication channels and the time during which these are functional are observed and can be correlated to differences in the developmental program. A different kind of intercellular communication can be visualized with the help of fluorescent dyes: a transfer of yolk proteins in two phases of the life cycle, in the adult hermaphrodite from the gut into the maturing germ cells, and in the embryo from non-gut cells into the gut primordium. Cell-cell interactions in the nematode embryo can be inhibited with polysulfated hydrocarbon dyes (e.g. Trypan Blue) which bind strongly to the plasma membrane. In summary our data indicate that fluorescent marker dyes can be helpful tools to identify and understand the role of intercellular communication and transfer processes in nematode development.

KEY WORDS: pattern formation, soma-germline, gut, micromanipulation, Caenorhabditis elegans

Introduction

The typical representative of the small free-living soil nematode Caenorhabditis elegans is a self-fertilizing hermaphrodite. However, conventional genetics can be performed by mating these with the rare males. Animals and embryos are transparent, so that development can be studied on the cellular level with Nomarski optics (for review see Wood, 1988). C. elegans is the only system in which embryonic and postembryonic cell lineages have been described completely (Sulston and Horvitz, 1977; Deppe et al., 1978; Kimble and Hirsh, 1979; Sulston and White, 1980; Sulston et al., 1983). Based on the early studies on parasitic nematodes (Strasen, 1896, 1959; Boveri, 1899, 1910; Stevens, 1909) their embryogenesis has been considered a classical example for mosaic development. Also in C. elegans aspects of cell-autonomous early development were found (Laule et al., 1980; Strome and Wood, 1983; Edgar and McGhee, 1986; Schierenberg, 1988; Junkersdorf and Schierenberg, 1992). However, during recent years increasing evidence has appeared that on top of cell-autonomous decisions various early cell-cell interactions take place in C. elegans during early stages of development to modulate the underlying basic program of cell specification (Schnabel, 1995). Mutants have been isolated in which either the correct segregation of cytoplasmic factors or potential steps in the signalling pathway are affected (Kemphues et al., 1988; Mello et al., 1992). We are just beginning to understand what kind of signals are involved and how signal transduction is accomplished in C. elegans (Bowerman et al., 1992; 1993; Evans et al., 1994; Mango et al., 1994; Tax et al., 1994).

As a complementary assay to the study of mutants, direct manipulation of blastomeres has proven to be particularly useful to identify cell-cell interactions in the C. elegans embryo. Removal and recombination of blastomeres revealed induction of the gut precursor by the neighboring germ line cell (Schierenberg, 1987; Goldstein, 1992; 1995a,b). Switching the position of certain cells let to a concomitant switch in their differentiation program (Priess and Thomson, 1987; Wood, 1991). Thus, those cells must have been originally equivalent in their developmental potential and depend on positional cues to become different from each other. Based on ablations with a laser microbeam and lineage analysis of the remaining blastomeres it has been suggested that sequential induction steps have to take place to establish asymmetries along the embryonic axes (Hutter and Schnabel, 1994, 1995a,b).

Here we describe the use of fluorescent marker dyes in combination with new micromanipulation methods and contrast-
enhanced microscopy, which allows in vivo visualization of intercellular communication pathways, the observation of transfer processes in individual specimens from early embryogenesis onward up to adulthood and the analysis of development after experimental inhibition of cell-cell interactions.

Results

Intercellular communication pathways in nematode embryos

Background

Different pathways of signal transduction can be imagined: (1) release of developmental signals by cells in one region of the embryo and transfer through the intercellular space to distant target sites; (2) a ligand/receptor mechanism between two adjacent membranes; and (3) transfer of signal molecules through communication channels, such as gap junctions (GJ). Studies using ions or dyes of known molecular size and charge demonstrated that GJ allow the intercellular exchange of molecules up to 1,000-3,000 Da, depending on the species studied (Flagg-Newton et al., 1979; Finbow and Pitts, 1981; Berdan, 1987). The importance of gap junctional communication for normal development has been documented in a number of different systems (Green, 1988; Warner, 1992). As little is known about this mode of communication in nematodes, we have begun to perform dye-coupling studies in C. elegans and related species. Here we will take into consideration just one of them, Cephalobus spec. Its embryogenesis differs considerably from that in C. elegans in that it develops several times slower and shows an altered division sequence and spatial arrangement of cells (Skiba and Schierenberg, 1992). We wondered whether these differences in the developmental pattern go along with a modified pattern of cell-cell communication.

Distribution of introduced marker dyes can be followed in vivo in the developing embryo

The nematode embryo is surrounded by a prominent chitinous eggshell and an underlying thin vitelline membrane, which is impermeable for most chemicals. One dye which can pass the vitelline membrane is Neutral Red (NR). Incubation of embryos in a NR solution for a few minutes is sufficient to strongly mark all cells (Fig. 3A). As NR eventually accumulates in the lysosomes of the gut primordium, it can be used as a differentiation marker.

More refined techniques are necessary to obtain stage- and cell-specific in vivo staining of the embryo. With the help of a laser microbeam the shell and vitelline membrane can be penetrated at pre-selected time points during embryogenesis to allow the entry of substances into the perivitelline space (Bossinger and Schierenberg, 1992b; Schierenberg and Junkersdorf, 1992). Dyes may either accumulate in the nuclei (Fig. 3B), bind strongly to cell membranes and mark their contours (Fig. 3C), just surround the blastomeres without any apparent binding (Fig. 3D), or pass, like NR, through the cell membranes into the blastomeres (e.g. Acridin Orange, Rhodamin 6G). We found that with the technique of laser-induced penetration substances up to about Mr 90,000 can be shunted into the nematode egg. Larger molecules have to be microinjected which is more difficult but allows labeling of individual cells (Fig. 7B).

When studied under the fluorescence microscope marker dyes may bleach out very rapidly not allowing conventional documentation even with high-speed film. In addition, repeated irradiation – even if only for a few seconds – often damages specimens leading to immediate or later developmental arrest. With video recording as described here, exposure times of 1-2 seconds are sufficient for proper documentation of fluorescent study objects without affecting normal development.
Fig. 3. Different staining properties of marker dyes in the nematode embryo. (A) After incubation with Neutral Red (Mr 289) within a few minutes the lipophilic dye has crossed the eggshell, underlying vitelline membrane, and the cell membrane and marks the cytoplasm of the embryo. (B) After microinjection of RITC-dextran (Mr 10,000) the dye accumulates in the nucleus. (C) After laser-induced penetration of the eggshell in a medium containing Trypan Blue (Mr 960) the dye marks cell membranes. (D) After laser-induced penetration of the eggshell Lucifer Yellow (Mr 457) fills the perivitelline space of an early embryo without entering cells. Epifluorescence, excitation wavelength: 520-560 nm (a,b,c); 436 nm (d). Orientation: anterior, left. Bar, 10 μm.

Communication channels coupling early blastomeres show different properties

We injected Lucifer Yellow (LY) or RITC Dextran (DE) into early blastomeres of C. elegans and Cephalobus (Bassinger and Schierenberg, 1992, 1996a). While LY is a widely used marker for the presence of gap junctions (Stewart, 1981), the passage of DE from one cell to another indicates the presence of cytoplasmic bridges or midbodies (Cartwright and Arnold, 1980; Mahajan-Miklos and Cooley, 1994). After an initial delay (Fig. 4B), from the 4-cell stage onwards until the end of the proliferation phase (see Fig. 2) LY quickly diffuses into all somatic blastomeres (Figs. 4C,D, 5D). DE, however, after injection into one blastomere of a 2-cell stage (after completed cytokinesis) always remains restricted to the descendants of this cell (Fig. 4H-J). In contrast to the findings in C. elegans, in Cephalobus even high molecular weight DE can spread from one somatic cell to another but never enters the germline (Fig. 4K-O). Thus, with respect to dye transfer, from the very beginning germ cells in Cephalobus are different from the soma, while in C. elegans we observed this phenomenon only at a later time (see below).

The germline forms the first tissue-specific communication compartment

From the 4-cell stage onwards all blastomeres of the early C. elegans and Cephalobus embryo are dye-coupled (Fig. 5B,D). However, this uniform behavior is lost with the beginning of gastrulation (about 26 cells). In Cephalobus the primordial germ cell P4 becomes irreversibly uncoupled from the rest of the embryo at this time (Fig. 5D). In C. elegans (with some delay) P4 still incorporates the marker dye and only its daughters are uncoupled from the soma at a much later stage (about 550 cells, Fig. 5B; Bassinger and Schierenberg, 1992a). Nevertheless, in C. elegans as well as in Cephalobus, the germline forms the first tissue-specific communication compartment, even though at different developmental times.

Somatic communication compartments form at different phases of development in different nematode species

In C. elegans all somatic cells of the embryo are still dye-coupled at the beginning of the morphogenesis phase (550 cells;
Fig. 5. Establishment of communication compartments in nematode embryos. (A,B) In *C. elegans* both daughters of the primordial germ cell P4 get excluded from dye-coupling during the early morphogenesis phase (for developmental stages, see Fig. 2) while all somatic cells still form a single compartment. (C,D) In *Cephalobus* P4 (arrow) is already excluded at the beginning of gastrulation. (E,F) In *C. elegans* the gut primordium forms a dye-coupling compartment during the advanced morphogenesis phase. (G,H) In *Cephalobus* the gut primordium is already uncoupled in the middle of the proliferation phase. Left, Nomarski optics. Right, epifluorescence. Orientation: anterior, left. Bar, 10 μm.

During the following hours we observe the stepwise establishment of different somatic dye-coupling compartments. In the case of the alimentary tract, first a pharynx-gut compartment forms, later the pharynx becomes separated from the gut (Fig. 5F), and subsequently the pharynx is further subdivided into an anterior and a posterior compartment (Bossinger and Schierenberg, 1992a). As for the germline also somatic dye-coupling compartments appear to be established considerably earlier in the *Cephalobus* embryo, e.g. the gut primordium forms a tissue-specific compartment already around the 200-cell stage (Fig. 5H).

Conclusions

The communication channels we have visualized here represent one of several potential ways of signal transduction (see above). In the early nematode embryo communication channels may not be a means for early inductive cell-cell interactions as at least all somatic cells are equally well dye-coupled. The few well enough studied cases suggest that there receptor/ligand mechanisms acting on the cell surface are involved (Roehl and Kimble, 1993; Evans et al., 1994). Therefore, the communication channels visualized here may serve other functions, such as metabolic coupling (Subak-Sharpe et al., 1966). On the other hand we observed that early blastomeres can acquire the potential to differentially accumulate certain marker dyes which initially had been equally distributed during the first cleavage steps (Bossinger and Schierenberg, 1996a). It remains to be tested whether this may reflect a segregation of cytoplasmic components after cell division.

Injection of suitable antibodies into the early embryo which interfere with proper function of GJ as successfully used in other systems (Serras et al., 1988; Bohrmann and Haass-Assenbaum, 1993) and the analysis of mutants with an altered communication pattern should help to better understand function and structure of communication channels in nematodes.

More straightforward is the interpretation of the (variably) early separation of the germ line from the soma in all nematodes we have studied. It has been suggested previously that a separation of germline from soma is necessary to preserve germline potential (King and Beams, 1938; Schierenberg, 1985, 1988; Schlicht and Schierenberg, 1991). Our present data support such a model. Further studies will determine whether the communication channels in *Cephalobus* show more than a superficial similarity to ring channels in the insect gonad, which can be modulated in their diameter (Xue and Cooley, 1993; Robinson et al., 1994).

Early embryonic induction in *C. elegans* can be inhibited by polysulfated hydrocarbon dyes

During embryogenesis of *C. elegans* cellular interactions are necessary to determine the fate of blastomeres. In one of these, taking place in the 4-cell stage, the germline cell P2 induces longitudinal orientation of the cleavage spindle in the neighboring EMS cell. Its unequal division and consequently the establishment of a gut lineage (Schierenberg, 1987; Goldstein, 1992). Another one, the induction of AB-derived pharyngeal muscle cells in the 12-cell stage by MS (Hutter and Schnabel, 1994) appears to involve a surface receptor/ligand interaction (Evans et al., 1994). Application of polysulfated hydrocarbon dyes (Trypan Blue, Evans Blue or Chicago Sky Blue) in the 4- and 12-cell stages following laser-induced penetration of the eggshell inhibits both interactions (Bossinger and Schierenberg, 1996b). In the first case this leads to a transverse division of EMS. In contrast to normal development (see Fig. 2) both daughter cells of EMS behave like MS cells (pharynx and body muscles) and the E-fate (gut) is completely suppressed. In the second case only the pharynx muscle cells coming from MS but not those from AB are generated.

Yolk transfer during nematode development

**Background**

The gut in nematodes serves several functions including the synthesis of yolk. Here we want to focus on the fate of yolk because this gives a different kind of example for intercellular
communication and shows that fluorescent marker dyes can trace the pathway of an essential component during the whole life cycle. In *C. elegans* four major yolk proteins are found, encoded by a single gene family (Sharrock, 1983; Blumenthal et al., 1984). A comparison of proteins synthesized by dissected tissues identified the intestine as the primary site of yolk synthesis. It has been proposed that yolk proteins in *C. elegans* are secreted from the intestine into the body cavity, and taken up from there by the oocytes (Kimble and Sharrock, 1983).

**The transfer of yolk from the intestine into to the oocytes can be followed in vivo**

In other systems it has been shown that yolk proteins can be fluorescently labeled with Lucifer dyes (Danilchik and Gerhart, 1987; Opresko and Karpf, 1987). We confirmed this for *C. elegans* by studying the pattern of fluorescence in stratified embryos (Fig. 6G,H) and by comparing the staining pattern of LY with that of an antibody against yolk proteins (Fig. 7I,J). After feeding worms with Lucifer Yellow (LY) we followed the pattern of fluorescence *in vivo* focusing on the different phases of transport. First, LY elevates the fluorescence in the intestinal cells (Fig. 6B). Soon afterwards we get a signal from the oocyte membrane (Fig. 6D) and then fluorescence appears in the cytoplasm of the oocytes. With increasing maturity oocytes show stronger fluorescence (Fig. 6F), reflecting the massive incorporation of yolk. Our observations indicate that yolk transfer is a very rapid process taking only a few minutes.

**During embryogenesis yolk accumulates in the gut primordium**

After feeding worms with Lucifer Yellow (LY) or microinjection into selected blastomeres, the dye remains visible in the cytoplasm all through embryogenesis. If injected for instance in the AB cell (no gut precursor; see, Fig. 2) of a 2-cell stage, LY quickly binds to yolk granules and remains in the descendants of the injected cell (Fig. 7B,D). Towards the end of the proliferation phase, however, the LY-induced fluorescence increases progressively in the differentiating gut primordium, while disappearing correspondingly from the descendants of the injected cell (Fig. 7F). Well before hatching, LY is essentially restricted to the gut (Fig. 7H). A similar pattern is found after immunostaining with an antibody against yolk proteins (Fig. 7I,J). Our observations suggest that an intercellular transfer of yolk from non-gut cells into the differentiating gut primordium takes place during embryogenesis of *C. elegans* rather than a consumption of yolk in all non-gut cells. This view is supported by our observation that the volume of the gut primordium increases considerably during embryogenesis while the embryo itself does not grow (Bossinger and Schierenberg, 1996b). To further characterize the pathway of yolk into the gut primordium, we penetrated the normally impermeable vitelline layer with a laser microbeam in a medium containing LY. If done in early stages, the dye fills the perivitelline space and surrounds the blastomeres but does not enter the cells (Fig. 8B,D). However, at later stages LY is specifically taken up by the cells of the gut primordium (Fig. 8F). The observed uptake is obviously not a general unspecific process. We found no uptake of dextran (as a marker for pinocytosis) but rapid internalization of transferrin molecules (as a marker for receptor-coupled endocytosis). Thus, gut cells appear to express an intense endocytotic activity as part of their tissue-specific differentiation (Bossinger et al., 1996). We found that also this receptor-coupled endocytosis can be blocked by different inhibitors like Trypan Blue and chlorpromazine (Röhrkasten and Ferenz, 1987; Wang et al., 1993).

**Conclusions**

Based on our findings reported above we suggest that the inferred transfer of yolk in nematode embryos is accomplished in two steps: (1) exocytosis from non-gut cells into the perivitelline
Fig. 7. From non-gut cells into the gut primordium: the way of fluorescently labeled yolk during embryogenesis of C. elegans. (A-D) After injection of Lucifer Yellow into the AB cell of a 2-cell embryo, the dye segregates only to the descendants of the AB cell. (E-H) During the morphogenesis phase the dye accumulates in the intestine and disappears from the AB descendants. (I,J) An antibody against yolk proteins marks the gut regions of an early and a late morphogenesis stage. Nomarski optics. (B,D,F,H,I,J) Epifluorescence. Orientation: anterior; left. Bar, 10 μm (modified after Bossinger and Schierenberg, 1992a).

Discussion

We have shown that fluorescent dyes can not only mark various structures in the nematode worm and embryo, but in addition allow us to follow intercellular transfer processes. Our observations together with those demonstrating inductive interactions (see above) indicate that nematodes are much more dynamic systems than it was imagined in the past. Communication pathways are activated and closed during specific phases of development allowing transport in and out of cells. So far, we do not know much about the developmental significance of transfer processes during nematode development and the underlying mechanisms (Spieth et al., 1985). Therefore, we attempt to suppress the function of communication channels with drugs (Ramon and Riveria, 1986) and study subsequent development. The detailed knowledge of the division and differentiation program in C. elegans permits detection of even subtle abnormalities. We have also started to examine mutants in which soma-germline differentiation is defective (Strome et al., 1995) and our preliminary results indicate that at least in some of these the typical early dye exclusion of the germline is lost. The intestine of nematodes, though simple in structure, is quite complex in function. It not only digests food, but also appears to take up and release metabolites, and to permanently store waste products (Davis et al., 1982). Thus, this organ may turn out to be the central part in a communication system connecting different cells or tissues. To further study the pathway of substances in and out of the gut we have started to screen for mutants which are not able to properly transfer the fluorescently marked yolk during embryonic or postembryonic development.

We found distinct variations with respect to intercellular communication between different nematode species. A comparative assay correlating this pattern with other parameters like cell line- eage and necessary interactions revealed by blastomere isolations and inhibition experiments may help us to better appraise the significance of such differences for development and give additional clues for the delineation of phylogenetic relationships among nematodes.

Materials and Methods

Nematode strains and maintenance

C. elegans (var. Bristol) strain N2, and Cephalobus spec. (laboratory designation: ES 501) isolated from soil probes (Skiba and Schierenberg, 1992) were raised on nutrient agar plates with a uracil-deficient strain of E. coli (OP 50) as a food source at 16–25°C, essentially as described by Brenner (1974).

Microscopy

C. elegans eggs were dissected out of gravid adults with a scalpel in a drop of distilled water on a microscope slide. Embryos of Cephalobus (which are laid before first cleavage) were rinsed directly from agar plates. Early embryos were identified under the dissecting microscope and transferred with a drawn-out glass mouth pipette to a second microscope slide or a coverslip (for microinjection, see below), where they were stuck to a thin polylysine layer (Cole and Schierenberg, 1986). Development of embryos was observed with Nomarski optics and epifluorescence to visualize fluorescent dyes (excitation: 340-380 nm, 436 nm, 520-560 nm; barrier: 415 nm, 490 nm, 580 nm) using a Leitz Diavert microscope equipped with an Olympus 40x APO UV oil-immersion objective.
**Centrifugation**

One-cell embryos were transferred into a drop of distilled water on a polylysine-coated microscope slide. While slowly settling down they can be oriented with the drawn-out tip of the transfer pipette. Alternatively, embryos were stuck in random orientation and appropriate specimens were selected afterwards. As unprotected microscope slides easily break in the centrifuge, Schlicht and Schierenberg (1991) designed a holder which prevents damage of the slide. The plastic holder sitting in the metal centrifuge beaker was filled with culture medium and then the slide was placed into the slot. Embryos were centrifuged for 15 min at 18000 g in a swing-out rotor. The temperature during centrifugation was about 12°C. After centrifugation embryos attached to the microscope slides were covered with a coverslip sealed with Vaseline.

**Introduction of marker dyes into embryos or animals**

**Feeding animals**

Specimens were either incubated with the dye on nutrient agar plates overnight or in cell culture medium complemented with the dye and a low concentration of E. coli as a food source for a few hours at room temperature. In both cases the dye was taken up orally together with the bacteria. Animals were exposed to the following final concentrations: Rhodamin 6G (10^{-5}%, Serva, Mr 477), Acidin Orange (1x10^{-4}%, Merck, Mr 302), Neutral Red (5x10^{-3}%, Riedel de Haen, Mr 289), Lucifer Yellow VS (1x10^{-2}%, Sigma, Mr 550).

**Laser microsurgery**

Embryos were attached to a microscope slide as described above and briefly preincubated with a Trypan Blue solution (14 mg/ml, Mr 961, Serva). The dye adheres to the eggshell and allows absorption of the laser microbeam by the normally transparent eggshell. Then the Trypan Blue solution was replaced by cell culture medium containing 1% of Lucifer Yellow VS (Mr 550, Sigma). With a N2-pumped dye laser (Lambda Physics, Göttingen, Germany) coupled to a microscope the eggshell and underlying vitelline membrane were perforated with brief pulses using the laser dye Rhodamine 6G (Lauffer and von Ehrenstein, 1981; Bossinger and Schierenberg, 1992b) to allow entry of marker dyes. After 15 minutes, the LY-containing medium was exchanged for the same medium without dye. No LY leaked out from the perivitelline space after that, probably because the vitelline membrane had resealed.

**Microinjection**

After mounting embryos were first covered with a drop of cell culture medium (Cole and Schierenberg, 1986) and then sealed with a drop of fluorocarbon oil to avoid evaporation while permitting oxygen diffusion. Penetration of embryos was performed with a piezotranslator (PM10, Bachofen, Pletningen). The dyes were delivered into cells with a microelectrode-amplifier (LIM-1, List-electronic, Darmstadt, Germany) which was pulled on a microelectrode puller (Brown-Flaming P-97A, Sutter Instruments, San Francisco, USA). The microelectrode was backfilled with a 3-5% aqueous solution of the dye (dye-coupling studies: Lucifer Yellow CH, Mr 457 and RITC-Dextran, Mr 10,000; 70,000; Sigma; yolk labeling: Lucifer Yellow VS, Mr 550; Sigma). A platinum wire was used as reference electrode and placed into the cell culture medium surrounding the embryo. Dye was injected under video observation using a 0,5-2 nA hyper- or hypopolarizing constant current depending on the charge of the dyes. The amount of injected dye was monitored using a remote-controlled shutter between the mercury bulb and the microscope to minimize harmful irradiation of the embryos. As the amount of injected dye is very critical for normal further development, only minimal amounts were injected, such that fluorescence became clearly visible after electronic enhancement (see below).

**Immunostaining**

Egg preparations were made as described for microscopy and microscope slides frozen in liquid nitrogen. Afterwards the coverslip was removed and the sticking frozen embryos fixed with methanol (10 min) and acetone (20 min). Embryos were incubated for 2 hr at room temperature with primary antibodies OIC1 and PIA3 against C. elegans yolk proteins (kindly provided by S. Strome, Bloomington) and then by an FITC-labeled secondary antibody for at least 2 hr, following the procedure given in Wood (1988).

**Video recording, electronic enhancement and documentation**

Injected embryos were recorded on a time lapse video recorder (Panasonic AG-6720-E) with a video camera (Panasonic WV-BC700 with infrared filter removed). After activation of the electronic "Sensitivity Up" mode of the camera control unit (Panasonic WV-CU204), the appropriate level of sensitivity was selected (accumulation of 2-32 frames). With an image processor (Hamamatsu, Argus-10) the quality was further improved. To document localization of fluorescent dyes, the embryos were exposed to brief fluorescence excitation and recorded simultaneously. Selected images of the recorded specimens were either photographed from the video screen at shutter speed 0.25 sec or printed directly with a video copy processor (Mitsubishi, P66E).

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**References**


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**Fig. 8. Stage-dependent uptake of Lucifer Yellow from the perivitelline space into the gut primordium of C. elegans.** (A-D) After laser-induced penetration Lucifer Yellow surrounds the cells of the early embryo. (E, F) During the morphogenetic phase LY disappears from the perivitelline space and accumulates in the gut primordium. Left, Nomarski optics. Right, epifluorescence. Orientation: anterior, left. Bar, 10 μm (modified after Bossinger and Schierenberg, 1992a).


