5

Single and Double FISH Protocols for *Drosophila*

Sarah C. Hughes and Henry M. Krause

1. Introduction

In situ hybridization within whole-mount *Drosophila* tissues was made routine with the introduction of digoxigenin labeled probes and alkaline phosphatase based detection methods ***(1)***. However, this method of detection until recently has been limited by the required use of alkaline phosphatase conjugated secondary antibodies and chromogenic substrates. The use of alkaline phosphatase substrates and their diffusible products limits the resolution of staining, particularly in thick tissues and deep within the embryo. Without additional probes, double-labeling and interpretation of results is also very difficult.

In this chapter we describe techniques for fluorescent in situ hybridization (FISH) in *Drosophila* tissues. Unlike the products of the alkaline phosphatase reaction, a fluorescent signal is nondiffusible and thus allows higher resolution microscopy ***(2)***. Resolution and sensitivity of probe detection are enhanced even further when coupled with laser scanning confocal microscopy (LSCM), or deconvolution microscopy. Other advantages include the ability to reconstruct three-dimensional images and to peer deep within the specimen. If more than one probe is available, the ability to assess overlaps in patterns of expression is also enhanced ***(3)***. In addition to the increased resolution, overlaps show up as easily discerned novel colors (e.g., red + green = yellow). With LSCM, one can also tell if transcripts do or do not co-localize at the subcellular level.

This chapter describes how to generate multiple probes for double hybridization. Also described are variations of the protocol that allow simultaneous detection of transcripts and proteins and for use of these detection techniques in whole embryos as well as dissected tissues. Underlying the described detection methods is the use of nonradioactive RNA probes and fluorescently conju-

From: *Methods in Molecular Biology, vol. 122: Confocal Microscopy Methods and Protocols*
Edited by: S. Paddock

gated secondary antibodies. Probes labeled with digoxigenin or fluorescein work equally well. Primary antibodies against these molecules are available from Boehringer Mannheim, as are reagents and kits for making the labeled RNA probes. Non-cross-reacting secondary antibodies, conjugated to cyanine fluorochromes, are then used to bind the primary antibodies.

The strength of the signals generated by this procedure, relative to standard chromogenic detection techniques, depends upon the equipment used for detection. We find that the intensities of fluorescent and chromogenic signals are similar when developed using the same RNA probe, and as detected on our Zeiss Axioplan 2E microscope. However, when detected by LSCM, the FISH approach is more sensitive and gives far greater resolution.

2. Materials

2.1. RNA Probe Preparation

1. 1.5-mL Microcentrifuge tubes, autoclaved
2. RNase-free Diethyl Pyrocarbonate (DEPC) treated or double-distilled water
3. 5X T7/T3 Transcription optimized buffer (Promega, Madison WI, USA; Catalog No. P1181)
4. T7 or T3 RNA Polymerase (Promega, Madison WI, USA: Catalog Nos. P2075, P2083; 1000 U)
5. Fluorescein RNA Labeling Mix (Boehringer Mannheim; Catalog No. 1685 619)
6. Digoxigenin RNA Labeling Mix (Boehringer Mannheim; Catalog No. 1277 073)
7. RNAguard (Pharmacia; Catalog No 27-0815-01)
8. 0.5*M* EDTA
9. 4*M* Lithium chloride
10. Absolute ethanol
11. Cold 70% ethanol wash

2.2. Initial Embryo Fixation

1. Chlorine bleach; diluted 1:1 with water
2. 40% Formaldehyde solution (prepared fresh from paraformaldehyde as described below)
3. 10× Phosphate-buffered saline (PBS) solution
4. Heptane
5. Methanol
6. 20-mL disposable glass scintillation vials (Fisher)
7. 1.5-mL microcentrifuge tubes, autoclaved

2.3. Post-Fixation and Hybridization of Whole-Mount Embryos

1. PBT solution: 1× PBS plus 0.1% Tween-20
2. 40% formaldehyde solution prepared that day
3. 20 mg/mL Proteinase K (Sigma). Dissolve in sterile H_2O, divide into 50-µL aliquots, and store at –20°C

4. 2 mg/mL glycine in PBS
5. RNA hybridization solution: 50% formamide, 5× SSC, 100 µg/mL heparin, 100 µg/mL sonicated salmon sperm DNA, and 0.1% Tween 20. Filter through a 20-µM filter and store at –20°C in aliquots (stable for at least 6–12 months)
6. Hot block or water bath at 80°C
7. Water bath at 56°C

2.4. Post-Hybridization Washes and Development of the FISH Signal

1. RNA Hybridization buffer
2. PBT Solution: 1× PBS, 0.1% Tween-20
3. PBTB Solution: 1× PBS, 0.1% Tween-20, and 0.5% milk powder
4. Mouse monoclonal anti-fluorescein antibody [IgG; 1:2000 dilution of a 0.1 mg/mL stock solution (*see* **Notes 2** and **3**); Boehringer Mannheim, Laval, QC, Canada; Catalog No. 1426 320]
5. Sheep anti-digoxigenin antibody (IgG; 1:1000 dilution of a 0.2 mg/mL stock solution; Boehringer Mannheim, Laval, QC, Canada; Catalog No. 1333 089)
6. Goat anti-mouse antibody conjugated to CY2 [$F(ab')_2$ fragment of IgG (H+L); 1:2000 dilution of a 1 mg/mL stock solution; Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA; Catalog No. 115-226-062]
7. Donkey anti-sheep antibody conjugated to CY3 [$F(ab')_2$ fragment of IgG (H+L); 1:2000 dilution of a 1 mg/mL stock solution; Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA; Catalog No. 713-166-147]
8. Embryo mountant: 70% glycerol, 2% DABCO (1,4-diazabicyclo [2.2.2.] Octane; Sigma, St. Louis, MO, USA; Catalog No. D-2522)
9. Microscope slides
10. Coverslips (22 × 50 mm)
11. Fluorescence (Zeiss Axioplan 2) and/or LSC microscope (Zeiss)

3. Methods

3.1. RNA Probe Preparation

1. To prepare runoff transcripts, the plasmid template is first linearized to completion (*see* **Note 1**) with the appropriate restriction enzyme, and then the enzyme removed by careful phenol and then chloroform extractions. After removal of all chloroform (heating to 65°C for 15 min helps), precipitate the DNA by adding NaAcetate (pH 5.2) to 0.3*M*, 3 vol of ethanol, and cooling to –70°C for 20 min. Centrifuge 10 min in a cold microcentrifuge and wash with cold 70% ethanol. We generally prepare 5–10 µg of linearized template, resuspended in 20 µL of RNase-free water.
2. RNA probes are prepared as described by Boehringer Mannheim on their digoxigenin and fluorescein RNA labeling spec sheets. On ice add 1 µg of linearized template DNA (3–5 kb), 2 µL of fluorescein or digoxigenin RNA labeling mix, 4 µL 5× transcription buffer (supplied with the RNA polymerase: 0.4 *M* Tris-HCl (pH 8.0); 60 m*M* $MgCl_2$, 100 m*M* dithiothreitol, 20 m*M* spermidine),

1 µL RNase inhibitor (1 U/mL) and sterile, RNase-free water to make the final reaction volume equal to 18 µL. Add 2 µL of the appropriate RNA polymerase (T7 or T3), mix well, and incubate at 37°C for 2 h.

3. Following the transcription reaction (*see* **Note 2**), the labeled probe is precipitated by addition of 1 µL 0.5 *M* EDTA, 2.5 µL 4 *M* LiCl, and 75 µL of absolute ethanol. Chill to –70°C, centrifuge, and wash the pellet as described above. After drying, resuspend the pellet in 100 µL of RNase-free water (*see* **Note 3**). Check the probe by loading and running 4–5 µL on a conventional agarose gel (~1%). The runoff transcript should easily be detected by ethidium bromide staining ***(4)***. Probe should be stored at –20°C. Several freeze–thaw cycles on ice do not impair probe activity.

3.2. Initial Embryo Fixation

1. Prepare 40% formaldehyde stock solution just prior to embryo dechorionation (*see* **Note 4**). Dissolve 0.92 g of paraformaldehyde in 2.5 mL of water containing 35 µL of 1*N* KOH. Heat the mixture at 80°C until dissolved.
2. Collect and rinse the embryos in water.
3. Dechorionate the collected embryos in a 1:1 mixture of chlorine bleach and water for approximately 90 s. When dechorionated, the embryos will either float to the surface of the bleach solution or stick to the sides of the collection basket. Embryos should be rinsed immediately, as over-dechorionation is apparently detrimental. Rinse the collection basket with plenty of water. Fast flowing tap water can help dechorionate partially dechorionated embryos. An optional rinse with 0.7% NaCl, 0.03% Triton X-100 is helpful for removing residual bleach and for washing embryos down from the sides of the basket.
4. Transfer the embryos to a 20-mL glass scintillation vial (*see* **Note 5**) containing a two-phase mixture of 8 mL of heptane, 2.5 mL of 1× PBS and 250 µL of 40% formaldehyde. Shake for 20 min.
5. Using a 1-mL pipetteman, draw up embryos (which are at the interphase), taking care not to suck up any of the lower aqueous phase (*see* **Note 6**). Transfer to a 1.5-mL microfuge tube containing 0.5 mL of heptane and 0.5 mL of methanol for devitilinization. Shake vigorously until the majority of the embryos sink to the bottom (about 30 s). Carefully remove about 75% of the heptane and methanol and replace with 1 mL of methanol. Shake once more. All or most embryos should have now sunk to the bottom of the tube. Remove all liquid along with any embryos at the interphase, and then rinse two or three times with methanol. Embryos can be stored in methanol at –20°C for several months.

3.3. Post-Fixation and Hybridization of Whole-Mount Embryos

The following steps are optimized for ~50 µL settled embryos in a 1.5-mL microfuge tube.

1. Rinse the embryos once in methanol.
2. Rinse the embryos twice in PBT (1× PBS, 0.1% Tween 20).

3. Post-fix the embryos for 20 min in 0.5 mL of PBT containing 50 µL of freshly prepared 40% formaldehyde. Place tubes on a rocking platform to ensure even fixation.
4. Rinse embryos 3× in PBT. Washes should be approximately 2 min in duration.
5. Add approximately 0.5 mL of PBT containing 50 µg/mL of nondigested proteinase K. Incubate for ~1 to 1.5 min (*see* **Note 7**). Mix by drawing up some of the solution with a pipetteman and gently jetting the embryos back into suspension. Repeat once, allow embryos to settle, and then remove the solution at least 30 s before the end of the incubation period.
6. Stop the proteinase K digestion by immediately adding 1 mL of PBT containing 2 mg/mL of glycine. After about 2 min, remove and rinse for another 2 min in the same solution.
7. Rinse embryos twice in PBT to remove the glycine.
8. Post-fix the embryos once again (as in **step 3**) for 20 min in PBT containing 4% formaldehyde.
9. Wash the embryos extensively in PBT to remove all traces of fixative.
10. Rinse the embryos in 1 mL of 50% PBT, 50% RNA hybridization solution. Replace the mixture with 100% hybridization solution and prehybridize the embryos at 56°C for a minimum of 2 h. If required, embryos can be stored overnight at –20°C in the HYB solution prior to the 2-h heating step.
11. After prehybridization, place the embryos in a sterilized 0.5-mL microfuge tube, remove prehybridization solution and add probe. Optimal probe concentration needs to be determined empirically but generally 1 µL of probe in 100 µL of RNA hybridization solution works well. Diluted probe is heated to 80°C for 3 min, cooled briefly on ice and then added to the embryos.
12. Hybridizations are carried out at 56°C for 12–16 h. Mix embryos two to three times during the course of the incubation, either by quickly inverting the tube, or by using a pipetteman to gently jet the embryos into suspension.

3.4. Post-Hybridization Washes and Development of the FISH Signal

1. Remove any hybridization solution and embryos from the upper walls and cap of the microfuge tube by spinning the tube for ≈10 s at 1500 rpm in a microcentrifuge.
2. Remove the probe solution and rinse the embryos once with 400 µL of prewarmed hybridization buffer. Repeat the wash with another 400 µL of prewarmed hybridization buffer, this time incubating at 56°C for 20–30 min. Invert the tube several times during the course of the wash.
3. Wash embryos for another 20–30 min with a 1:1 mix of hybridization buffer and PBT and then with four 5 min washes of PBT. All washes should be done with preheated solutions at 56°C.
4. Cool to room temperature and incubate for 10 min in 400 µL of PBTB (1× PBS, 0.1% Tween 20, 0.5% milk powder). The use of milk powder in this and subsequent steps helps to reduce background.

5. Hybridized RNA probes are detected by first incubating the embryos with the appropriate primary antibodies diluted in PBTB. For double-labeling, both anti-digoxigenin and anti-fluorescein antibodies are added. Dilutions (*see* **Note 8**) that were optimal in our hands are given in the Materials section, but batches may vary, as may optimal activity given the many variations that exist in a particular laboratory's reagents, equipment, and methodology. Incubate with primary antibodies for 2 h (optionally overnight at 4°C), with constant mixing on a rocking platform or rotating mixer.
6. Wash for 1–2 h (optionally overnight) with four or five changes of PBTB.
7. Add the appropriate (*see* **Note 9**) secondary antibody(s) diluted in PBTB, and incubate with constant mixing for 2 h. Carry out this step and all subsequent steps in dim light with tubes covered or wrapped in foil.
8. Wash for 2 h with four or five changes of PBTB and then finally with PBT.
9. Resuspend embryos in DABCO-containing mountant. Allow the embryos to settle to the bottom of the tube (1–3 h or overnight at 4°C) before resuspending and mounting.
10. Transfer the embryos to a clean slide in ~80 µL of mountant and cover with a 25 × 50 mm coverslip. Seal the edges with nail polish. Slides can be stored for weeks at 4°C in the dark. Background levels will often decrease over the first few days.
11. Embryos can be viewed by either conventional fluorescence microscopy, LSCM, or deconvolution microscopy. Basic LSCM techniques are discussed elsewhere in this book. An example of double FISH labeling of a *Drosophila* embryo is shown in **Fig. 1A**.

3.5. RNA–Protein Double-Labeling

1. Collect and fix embryos as described previously for FISH, with the exception of the proteinase K step. The proteinase K concentration may have to be lowered to preserve integrity of protein epitopes (*see* **Note 7**).
2. After performing the hybridization and washes, as described, add the primary antibody for the protein of interest, along with the anti-fluorescein or anti-digoxigenin antibody. To obtain non-cross-reacting signals, the protein-specific antibody must have been raised in a host other than the host(s) used for the probe-specific antibodies (i.e., not mouse or sheep).
3. After primary antibody incubation and washes, the primary antibodies are detected using appropriate secondary antibodies (*see* **Note 9**). Wash and mount as with single or double FISH staining. With careful choice of antibodies, triple-staining a combination of transcript and protein targets is possible. However, secondary antibodies conjugated to CY5 are necessary ***(3)***, as are the microscope excitation and detection components required for visualization.

3.6. Performing FISH on Dissected Tissues

1. Dissect tissues such as imaginal disks or salivary glands in PBS. Dissected tissues can be stored briefly (up to 30 min) on ice in a microfuge tube containing PBS while collecting enough tissues for analysis.

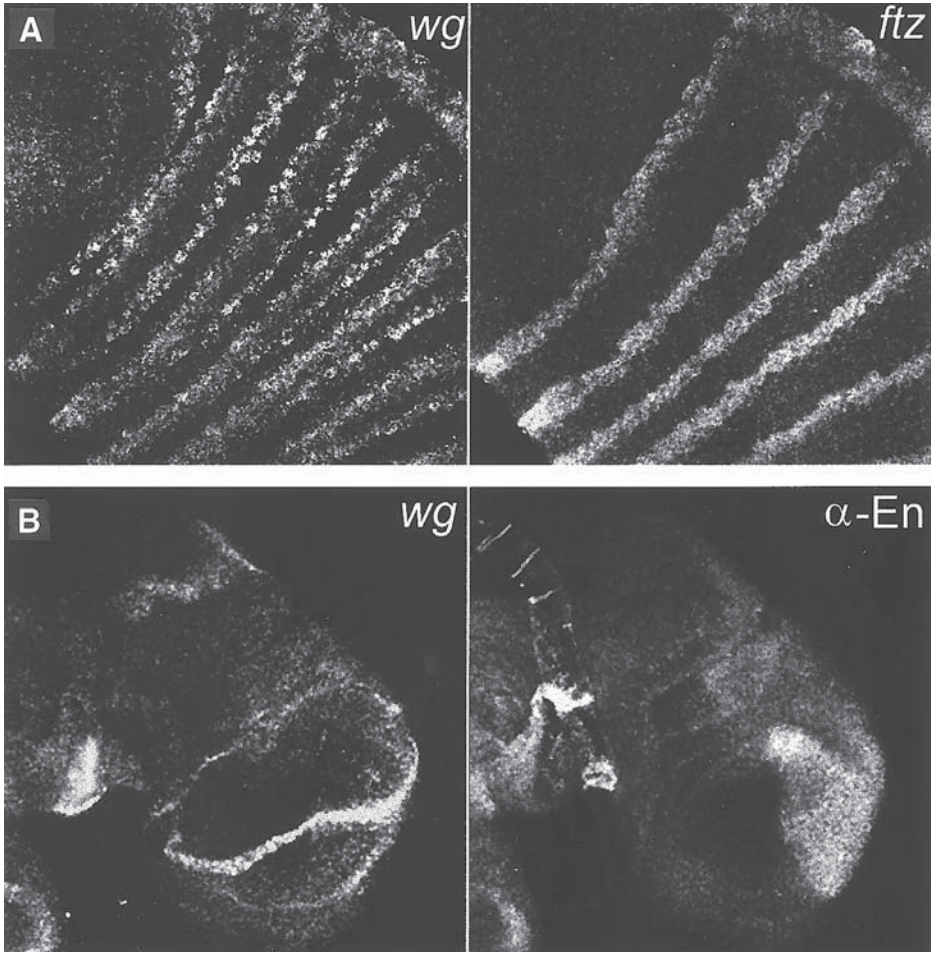

Fig. 1. **(A)** shows the trunk region of a cellular blastoderm embryo with *wg* transcripts detected on the left (CY3 channel) and *ftz* transcripts on the right (CY2 channel). **(B)** A wing imaginal disk with wingless transcripts detected on the left (CY2) and engrailed protein detected on the right (CY2).

An RGB image of the superimposed channels is shown in Color Plate I Examples of double-labeling using FISH. **(A)** A whole-mount embryo at 4 h after egg laying that is double-labeled for *fushi-tarazu* mRNA in green and *wingless* mRNA in red. **(B)** A third instar imaginal wing disk double-labeled for *wingless* mRNA in red and *engrailed* protein (antibody 4D9; *see* **ref. 6**) in green. In both panels, anterior is to the left and ventral is down. (*See* Color Plate I, lower left sequence, appearing after p. 372.)

2. Remove the PBS and add 50 µL of 10× PBS, 325 µL of water, 500 µL of heptane and 125 µL of 40% formaldehyde (freshly prepared as described previously). Shake gently for 45 s.

3. Remove the heptane and most of the fixative, and replace with PBT containing 4% formaldehyde. Continue fixation for another 20 min with gentle mixing.
4. Wash 4× with PBT and proceed to the proteinase K and subsequent steps, as described previously for embryos. Use the appropriate reagents for single or double FISH, or FISH/protein double-staining. **Figure 1B** shows an example of FISH/antibody double labeling in dissected imaginal disks.

4. Notes

1. Template DNA should be chosen and linearized such that runoff transcripts correspond to unique portions of the gene's coding region. So far, we've found that runoff transcripts ranging from about 0.4 to 1 kb work well as probes. Cutting to completion generally takes 2–4 h, and should be confirmed by agarose gel electrophoresis.
2. Removal of template with DNaseI subsequent to the transcription reaction was found to be unnecessary. Precipitation of the probe with LiCl removes most unincorporated nucleotides.
3. Previous protocols (e.g., Protocol 6) used carbonate degradation to reduce the size of probe RNA. In our hands this was found to be unnecessary and in fact was usually detrimental.
4. Freshly prepared formaldehyde appears to be required with the high temperatures used for RNA hybridization. Commercially prepared formaldehyde solutions, even ultrapure, generally yield ruptured embryos.
5. Vessel sizes used here are optimized for small collections (<250 µL of settled embryos). For greater collection sizes, larger vessels should be used, keeping approximately to the same relative ratios. 50-mL Falcon tubes work well for fixing and devitillinizing settled embryo volumes from ~0.25 to 2 mL. Care should be used as some tubes or plastics appear to interfere with fixation and devitillinazation (e.g., Sarstedt polystyrene tubes).
6. Aqueous solution interferes with the efficiency of the subsequent devitillinzation step. This has likely occurred if the devitillinization solution is cloudy and less than 80% of embryos have moved from the interphase to the bottom of the tube. Care should be taken to minimize uptake of the lower aqueous phase when drawing up embryos from the fixative into the pipet tip. Quite often, if this occurs, the phases will separate in the tip, and the lower aqueous phase can be returned to the scintillation vial. If transfer of aqueous solution has already occurred, the devitillinization step can be repeated as necessary by removing as much heptane and methanol as possible, replacing with fresh heptane and methanol and shaking again.
7. The extent of proteinase K digestion is a very important consideration. In general, proteinase K digestion enhances probe accessibility and hence the strength of the signal. However, overdigestion results in poor embryo morphology and ruptured embryos. Also, when double-labeling for proteins, proteinase K digestion can destroy the epitope. This can be remedied by lowering the working concentration of proteinase K as required. In fact, some in situ probes work very well with little or no proteinase K digestion. Newly prepared proteinase K stock solutions should be tested at several dilutions and/or digestion times. Prepare a 20 mg/mL

stock of proteinase K by dissolving in sterile water and storing at –20°C in 20–50 µL aliquots. Repeated freeze–thaw cycles appear to increase the activity of the enzyme.

8. The antibodies used here come lyophilized. For uniformity and convenience, we resuspend the powders in 50% glycerol and then aliquot and store at –70°C. One of the aliquots can be kept at –20°C for convenience (does not freeze and is relatively stable).
9. Antibodies described here have been chosen with usefulness in double-labeling in mind. The primary antibodies are whole IgGs raised in different hosts. Similarly, secondary antibodies are selected so that they are unlikely to crossreact with the other primary and secondary antibodies. Jackson laboratories, from which the recommended secondary antibodies were obtained, provide information, suggestions, and many products that make choosing and obtaining the appropriate antibodies relatively easy. Secondary antibodies most suitable for multiple-labeling are designated "ML." These are generally comprised of the $F(ab')_2$ portion of IgG antibodies that recognize both heavy and light (H and L) chains of their target antibodies. ML antibodies are also preabsorbed against multiple host sera. For this reason, and because the antibodies contain light-sensitive molecules, we do not bother to preabsorb them against embryos. However, if background is obtained, this may help. Cyanine-conjugated secondaries were chosen because of their strong emission spectra and resistance to photo-bleaching. The latter is particularly important with the high energy lasers used for LSCM.

Acknowledgments

We would like to thank Andrew J. Simmonds for his assistance with the imaginal disk staining and confocal microscopy. The *en* 4D9 antibody was a gift from T. Kornberg. This technology was developed with support from the National Cancer Institute of Canada (NCIC).

References

1. Tautz, D. and Pfeiffle, C. (1989) A non-radioactive in situ hybridization method for the localization of specific RNAs in *Drosophila* embryos reveals translational control of the segmentation gene *hunchback*. *Chromosoma* **98,** 81–85.
2. Harlow, E. and Lane, D. (1988) *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 396–399.
3. Paddock, S. W., Langeland, A., DeVries, P. J., and Carroll, S. B. (1993) Three-colour immunofluoresence imaging of *Drosophila* embryos by laser scanning confocal microscopy. *BioTechniques* **14,** 42–47.
4. Hughes, S. C. and Krause, H. M. (1998) Double labeling with FISH in *Drosophila* whole-mount embryos. *BioTechniques* **24,** 530–532.
5. Lehmann, R. and Tautz, D. (1994) *In situ* hybridization to RNA, in *Methods in Cell Biology*, Vol **44**, (L. S. B. Goldstein and E. A. Fyrberg, eds.), Academic Press, San Diego, pp. 575–598.
6. Patel, N. H., Martin-Blanco, E., Coleman, K. G., Poole, S. J., Ellis, M. C., Kornberg, T. B., and Goodman, C. S. (1989) Expression of *engrailed* proteins in arthropods, annelids, and chordates. *Cell* **58,** 955–968.

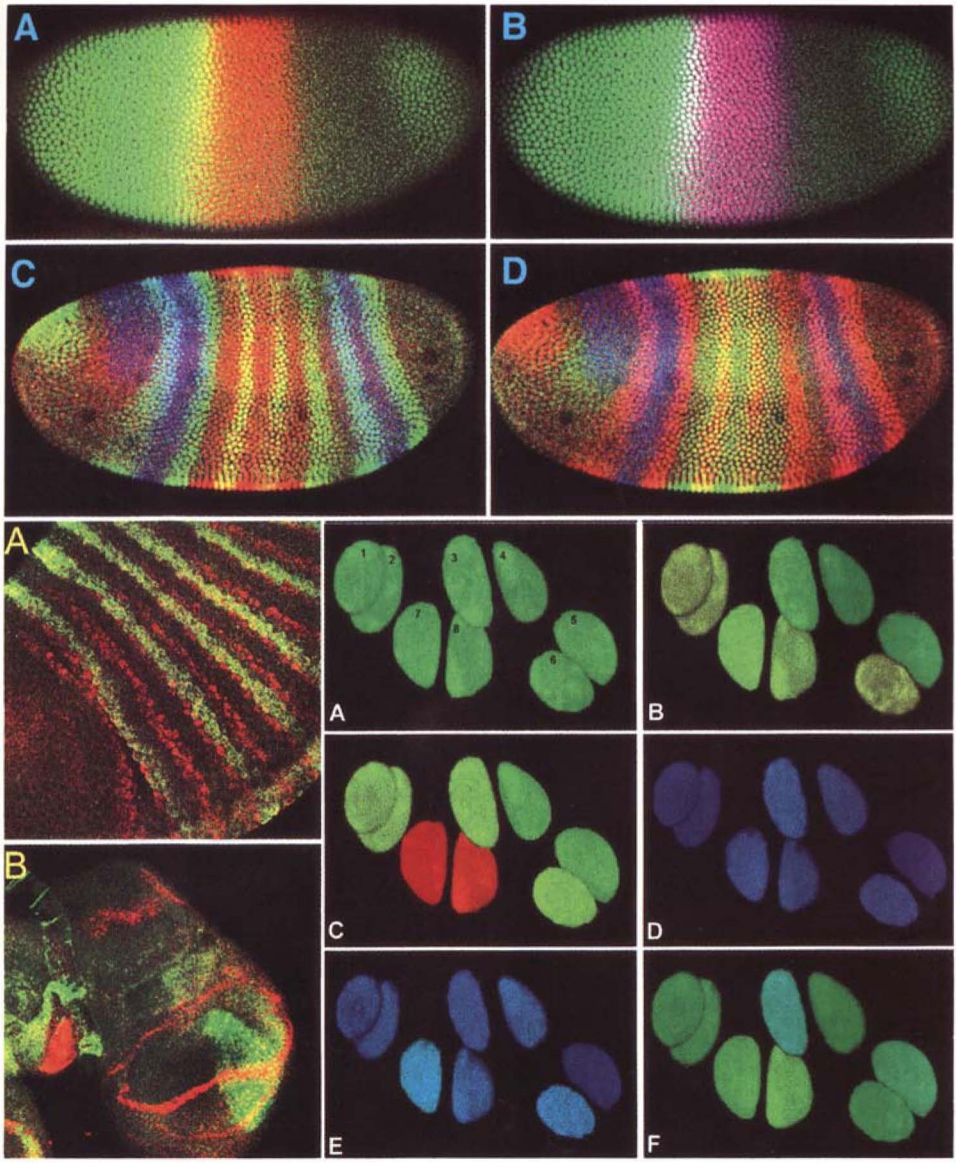

Plate I (*Top Sequence:* **[A-B]** Fig. 21-2 A-B, *see* full caption on p. 379; **[C-D]** Fig. 21-1 B, D, *see* full caption on p. 377 and discussion in Chapter 21. Lower Left Sequence: **[A-B]** Fig. 5-1 A-B, *see* full caption on p. 99 and discussion in Chapter 5. Lower Right Sequence: **[A-F]** Fig. 18-13 A-F, *see* full caption on p. 336 and discussion in Chapter 18).