grinding methods. The RT-PCR products (Figure 3) showed a distinct band with the correct size of 716 bp and comparable yields among different RNA preparations, indicating that the RNA prepared by sonication can be used for cDNA synthesis and subsequent RT-PCR.

Microscopic study of plant cells ground in mortars in the presence of liquid nitrogen revealed a lot of broken cell pieces (Figure 4A). However, the cells lysed by sonication looked homogeneously deformed, and very few broken cell pieces were visible (Figure 4B). This result indicates that sonication does not break down the cells. Instead, it may induce cell leakage or damage plasma membranes of plant cells to release cytoplasmic contents.

Sonication has been used to lyse bacterial cells for isolation of proteins (2) and is routinely used in our laboratory to isolate proteins from tobacco suspension cultures (unpublished). As described in this paper, a 60-W sonication with a duration of 0.5–4 min can also be used to isolate total RNA of high quality from tobacco cell suspension cultures and calli and wheat cell suspension cultures on both small and large scales. Furthermore, sonication allowed us to extract RNA from less than 50 mg starting materials (data not shown) using both described protocols, which are interchangeable. The sonication method avoids tedious grinding steps, thereby eliminating the need for preparing RNase-free mortars and pestles and using liquid nitrogen. Because cell lysis and the first RNA extraction step are carried out in the same tube, this method is particularly suitable for processing a large number of samples with limited amounts in microcentrifuge tubes. In parallel, 24 samples can be processed in 5 h. The sonication method is simple and reliable for rapid characterization of gene expression at the transcriptional level using plant calli and suspension cultures.

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Combined Fluorescence In Situ Hybridization and Immunocytochemistry Double Labeling in Whole-Mount *Manduca sexta* Tissue

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Immunohistochemical and in situ hybridization methods are routinely used to localize individual proteins and mRNAs in cells, tissue sections and whole mounts. Variations of these techniques, such as double-label immunocytochemistry and double-label in situ hybridization, are frequently used to co-localize peptides and to visualize similarities and differences in patterns of gene expression in tissues (7). Combined mRNA/protein double-labeling protocols have also been developed and used successfully in tissue sections and *Drosophila* embryos (6), but have been more difficult to perform in other whole-mount tissues. Our interests lie in looking at expression of neuropeptides and their mRNAs in the nervous system of the tobacco hornworm, *Manduca sexta*. Whole-mount histochemical procedures have been used in *M. sexta* to identify distinct cells expressing specific messages and proteins, but combined in situ hybridization/immunocytochemistry has been performed successfully by few investigators (5). The ability to perform mRNA/protein double labeling would enhance and facilitate studies regarding expression and coexpression of peptides and their genes in situ. A recent fluorescence in situ hybridization (FISH) protocol, described for use in whole-mount *Drosophila* embryos (4), discussed the advantages of using fluorescence-labeled probes for in situ and double-labeling methods. These advantages include higher-resolution staining, use of laser-scanning confocal microscopy (LSCM) and the ability to perform double-labeling techniques with fluorescence. We describe a protocol for double labeling for peptides and mRNAs in *M. sexta* whole-mount tissue using the combination of FISH and fluorescence immunocytochemistry.

Benchmarks

The protocol outlined in Table 1 was adapted from several sources (1,5) and recently used to determine the cellular localization of the neuropeptide allatotropin (Mas AT) by Taylor et al. (8). Although in situ hybridization is detected with a fluorescent secondary antibody in this protocol, other conjugates, such as digoxigenin (DIG)-peroxidase or DIG-alkaline phosphatase, can be used reliably with this procedure.

Mas AT mRNA and peptide have been localized in a pair of neurosecretory cells of the frontal ganglion (8). Immunoreactivity against the molluscan peptide FMRFamide is present in similar cells (3). Figure 1 shows co-localization of Mas AT mRNA and FMRFamide-like immunoreactivity in two large cells of the frontal ganglion of day-2 larval *M. sexta* using this combined fluorescence-based procedure (Figure 1, A and B). Faint fluorescence staining throughout the ganglion is due to nonspecific binding of secondary antibodies and not to low levels of mRNA/protein (Figure 1, C and D). Whole-mount tissues were permeabilized with dilute acid and enzyme digestion to allow penetration of the riboprobe without damage to the tissue. The enzyme treatment was shortened

from 30 to 15 min when the in situ procedure was followed by immunocytochemistry to minimize loss of the FMRFamide-like immunoreactivity. Tissues were hybridized with 100 ng of Mas AT cRNA probe at 50°C for 24–48 h; however, as little as 20 ng of probe have been used without affecting the signal. Ganglia were washed under stringent conditions and treated with RNase A (1 mg/mL) and T1 (1 U/mL) (both from Sigma Chemical, St. Louis, MO, USA) to remove unhybridized probe. Tissues were blocked with 2% normal sheep serum for 2 h at room temperature and then incubated overnight with a 1:20 dilution of anti-DIG-rhodamine antibody (Boehringer Mannheim, Indianapolis, IN, USA) in phosphate-buffered saline (PBS) containing Triton® X-100 and 1% normal sheep serum at 4°C in the dark. A less concentrated dilution (1:250) of the secondary antibody is sufficient if a peroxidase or alkaline phosphatase conjugate is used. Nonspecific binding was removed with several washes with PBS and Triton X-100. Tissues were then incubated with FMRFamide antiserum (INCSTAR, Stillwater, MN, USA) at a dilution of 1:1000 for 2 days at 4°C. Tissues were washed with PBS and Triton X-100 for 30 min at room

Figure 1. Co-localization of Mas AT mRNA and FMRFamide-like immunoreactivity in neurosecretory cells in whole-mount frontal ganglion from day-2 fifth instar *Manduca sexta*. (A) Mas AT transcript detected by anti-DIG rhodamine. (B) FMRFamide-like immunoreactivity in same cells detected by anti-rabbit IgG fluorescein. (C and D) Control ganglion (no cRNA probe/no primary antibody).

Benchmarks

Table 1. Combined Whole-Mount FISH/Immunocytochemistry Protocol

A. Probe Synthesis

1. DIG-11-UTP RNA probes are synthesized from 1 µg of linearized plasmid containing cDNA of interest in a standard in vitro transcription reaction using a DIG RNA Labeling Mix (Boehringer Mannheim) and RNA polymerase (Promega, Madison, WI, USA).
2. RNA yield was determined by trichloroacetic acid (TCA) precipitation of a parallel reaction containing ³²P-UTP (2), and transcripts were run on a 6% polyacrylamide gel to assure integrity of the RNA.
3. The probes were added to the hybridization mixture either untreated or hydrolyzed in 0.2 M carbonate buffer (80 mM NaHCO₃/120 mM Na₂CO₃, pH 10.2) to a size of 150–200 nucleotides (1).

B. Tissue Fixation

1. Fix dissected tissues in freshly prepared 4% paraformaldehyde (pH 7.0) made in PBS (1.3 M NaCl, 0.7 M Na₂HPO₄, 0.3 M NaH₂PO₄) for 2–24 h (2 h at room temperature, refrigerated if beyond 2 h).
2. Tissues were rinsed in PBS for 5 min, used immediately or stored in PBS and 0.3% Triton X-100 at 4°C until use.

C. Permeabilization

1. Treat fixed tissues with 0.2 N hydrochloric acid for 20 min at room temperature to permeabilize tissues.
2. Wash acid-treated tissues with sterile water for 5 min at room temperature and remove.
3. Pretreat washed tissues with 2× standard saline citrate (SSC) at room temperature; remove and add 2× SSC warmed to 70°C. Incubate tissue at 70°C for 30 min.
4. Remove 2× SSC and rinse tissue with sterile water for 5 min.
5. Pretreat tissues with room temperature proteinase K buffer (100 mM Tris-HCl, 50 mM EDTA, pH 8.0), remove and add 37°C proteinase K buffer and proteinase K (1 µg/mL; Sigma Chemical); incubate at 37°C for 15–30 min.
6. Transfer tissues to PBS and wash two times for 5 min at room temperature.
7. Remove PBS wash and add 4% paraformaldehyde to tissues. Fix for 20 min at room temperature.
8. Wash out fixative with PBS, wash two times for 5 min.
9. Remove washes and add 0.1 M triethanolamine (TEA) buffer (0.1 M TEA in water, pH to 8.0 with concentrated hydrochloric acid).
10. Treat with freshly prepared acetic anhydride/TEA solution (25 mL of acetic anhydride to 10 mL of 0.1 M TEA buffer); incubate for 10 min at room temperature.
11. Wash tissue twice with PBS for 5 min each at room temperature.

D. Prehybridization and Hybridization

1. Prepare hybridization stock solution (60% formamide, 0.375 M NaCl, 0.025 M Tris-HCl, pH 7.5, 1.25× Denhardt's (1× Denhardt's = 0.02% Ficoll®, 0.02% polyvinylpyrrolidone (mol wt 40 K), 0.02% bovine serum albumin [BSA]), 1.25 mM EDTA, 0.125 M dithiothreitol [DTT], 0.625 mg/mL poly(A) RNA [Sigma Chemical], 0.625 mg/mL yeast tRNA).
2. Remove PBS wash and add 400 µL of prehybridization solution (320 µL hybridization stock solution + 80 µL diethyl pyrocarbonate [DEPC]-treated water); incubate for at least 1 h at room temperature.
3. Transfer sample from prehybridization solution to 400 µL of hybridization solution (320 µL of hybridization stock solution, 80 µL 50% dextran sulfate and DIG-labeled probe (100 ng/mL of hybridization solution) boiled for 5 min prior to adding to tissue; incubate at 50°C for 24–48 h.

E. Washes

1. Carefully remove hybridization solution and store at -20°C to use again.
2. Add wash buffer [50% formamide/1× salts (0.3 M NaCl, 0.01 M NaPO₄, 0.01 M Tris-HCl, 50 mM EDTA, pH 6.8)]. Wash twice in wash buffer for 1 h each at room temperature.
3. Remove wash buffer and add room temperature RNase buffer (NTE buffer: 0.5 M NaCl, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA).
4. Add 37°C NTE buffer/RNase solution (20 µg RNase A and 1 U RNase T1 per mL of NTE buffer). Incubate at 37°C for 30 min.
5. Wash with 2× SSC twice for 30 min each at room temperature.
6. Treat with 0.1× SSC prewarmed to 50°C. Incubate tissues at 50°C for 15 min.
7. Wash for 30 min at room temperature with 0.1× SSC with agitation.
8. Remove and wash twice with PBS and 0.3% Triton X-100 for 15 min each.
9. Block with 2% normal sheep serum (made in PBS and 0.3% Triton X-100) for at least 1 h.
10. Incubate tissue overnight with a 1:20–1:40 dilution of sheep anti-DIG rhodamine conjugate (Catalog No. 1 207 750; Boehringer Mannheim) in 1% normal sheep serum in PBS and Triton X-100 at 4°C in the dark.
11. Wash three times with PBS and 0.3% Triton X-100 for 30 min each at room temperature in the dark.

F. Immunocytochemistry

1. Incubate tissue in the dark with antiserum at appropriate dilution for 24–48 h at 4°C.
2. Remove antiserum and wash with PBS and 0.3% Triton X-100 three times for 15 min each at room temperature.
3. Incubate with a 1:50–1:100 dilution sheep anti-DIG fluorescein (Catalog No. 1 207 741; Boehringer Mannheim) in 1% normal goat serum made in PBS and 0.3% Triton X-100 overnight at 4°C.
4. Remove secondary antibody and perform three room temperature washes with PBS and Triton X-100 for 15 min each.

G. Mounting

1. Mount in Gel/Mount™ (Biomed, Foster City, CA, USA) and visualize under a fluorescence microscope with the appropriate filter.
2. Specimens were photographed with Kodak® Ektachrome® Color Slide Film ASA 400 on a Nikon® Microflex® UFX-DX camera.

temperature three times. FMRFamide-like immunoreactivity was detected using an anti-rabbit IgG fluorescein conjugate (Jackson ImmunoResearch, Westgrove, PA, USA) used at 1:100 overnight. Tissues were washed for 30 min three times and mounted in an aqueous mounting gel. Rhodamine and fluorescein staining was visualized by epifluorescence.

Using combined FISH/immunocytochemistry, we have been able to show that FMRFamide-like immunoreactivity is present in those cells that express Mas AT mRNA simultaneously. This procedure, as well as other double-labeling techniques, should assist in studying coexpression of various messages and proteins in *Manduca* and other insects.

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Suppression of Irrelevant Signals in Immunoblots by Preconjugation of Primary Antibodies

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Cross-reactivity can pose serious problems in applications using antibodies as detecting tools and substrates, which themselves contain antibodies, such as immunoprecipitates or immune tissues. The optimal solution is to switch to antibodies from a different, non-cross-reactive species. However, such non-cross-reactive antibodies of a particular required specificity are not always available. We present a quick and easy method of general significance, circumventing problems with cross-reactivity.

Examining expression of protein tyrosine phosphatase 1D (PTP-1D) in mouse spleen cells by immunoblotting, we used an unconjugated mouse anti-PTP-1D antibody (750 µg/mL; Transduction Laboratories, Lexington, KY, USA) and a polyclonal rabbit anti-mouse, horseradish peroxidase (HRP)-linked antibody (1.3 mg/mL of immunoglobulin; DAKO, Glostrup, Denmark) as a primary and secondary antibody, respectively. The secondary antibody also reacted against mouse immunoglobulin (Ig) present in the spleen extract, resulting in high background staining and additional signals, potentially disguising a relevant signal (Figure 1, lane 1).

We solved this problem by preconjugating the primary antibody with the secondary antibody before hybridizing it to the blot. Anti-PTP-1D was incubated with anti-mouse HRP overnight at room temperature (RT). Then, an excess of mouse serum was added for 3 h at RT to sequester any secondary anti-mouse HRP antibody not bound to the primary anti-PTP-1D antibody. This detection mixture was hybridized to the blot for 2.5 h at RT. By this procedure, signals from mouse Ig were completely quenched, and only the desired PTP-1D signal was obtained (Figure 1, lanes 2 and 3).

Optimal preconjugation conditions of the primary antibody were empiri-