Spatial Visualization of Apoptosis Using a Whole-Mount In Situ DNA End-Labeling Technique

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Apoptosis is the process by which cells activate a specific program that results in the cell's destruction (1). Many apoptosis detection protocols take advantage of unique cellular events during this progression, the most frequent of which is endonuclease activation and the subsequent DNA cleavage into endonucleosome-sized fragments. The oligonucleosomes can be visualized as either a multimeric DNA ladder after electrophoresis of isolated genomic DNA or on fixed cells or tissue sections by labeling the 3′-OH DNA ends with a hapten-linked nucleotide using the enzyme terminal deoxynucleotidyl transferase (TdT) in the TdT-mediated dUTP nick end-labeling (TUNEL) technique (2).

The spatial distribution of apoptosis within a tissue or embryo can be as crucial to interpretation as quantitation. However, current protocols do not lend themselves to precise identification of small, localized apoptotic populations within a three-dimensional (3-D) context, such as that which occurs during embryogenesis. The regions of interest are frequently too small for surgical excision and DNA extraction, and TUNEL-labeled tissue sections do not always provide clear spatial representation of apoptosis without laborious or computerized reconstruction. Vital dyes (e.g., acridine orange [AcrOr], neutral red, nile blue sulfate) are frequently used to detect programmed cell death within whole tissues (4); however, this technique is not specific for apoptosis. Moreover, the vital dye signal is transient and usually is not retained upon fixation or embedding. We have modified a commercial protocol for in situ DNA end-labeling (ApopTag™; Oncor, Gaithersburg, MD, USA; Reference 5) and combined it with established protocols for the nonisotopic detection of nucleic acids (9). This protocol allows for the 3-D placement of single apoptotic cells within the much larger context of a tissue or embryo.

Whole embryos or tissues no thicker than 3–4 mm are fixed in 1% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min at 4°C; for larger tissues, the fixation time must be determined empirically. Hollow regions (e.g., forebrain, heart) are pierced with a needle to eliminate solvent trapping. Tissues are washed briefly in PBS (10 min at 4°C) and then dehydrated through a graded ethanol/PBS series to make them permeable and improve access for TdT and antibodies; tissues that cannot be immediately analyzed should be stored in 100% ethanol at -20°C. DNA ends are visualized using a modification of the manufacturer's procedures for the ApopTag In Situ Cell Death Kit (Oncor; Reference 5). Embryos or tissues are rehydrated through ethanol/PBS into PBS and then incubated in equilibration buffer (Catalog No. S7110-1; Oncor) for 5 min at room temperature in sufficient volume to cover the tissue (4–5 stage-13 chick embryos at 5-µL volume each required 100 µL total). The solution is removed and replaced with working strength TdT enzyme solution for 2 h at 37°C in a humidified chamber (Catalog Nos. S7110-2 and S7110-3; Oncor; 150 µL per 4–5 embryos). TdT adds a digoxigenin-conjugated dUTP to the 3′-OH ends of the nuclear DNA fragments that are characteristic of early-stage apoptosis (2). Incubation in stop wash buffer (Catalog No. S7110-4; Oncor) for 40 min at 37°C, at 200 µL per 4–5 embryos, terminates the reaction.

To detect the digoxigenin-labeled DNA ends, a modification of the whole-mount in situ hybridization protocol is used (9). Endogenous alkaline phosphatase is inactivated by incubating the tissue twice for 5 min each in Tris-buffered saline (TBST: 0.14 M NaCl, 10 mM KCl, 25 mM Tris-HCl, pH 7.0, 0.1% Tween® 20) and 1 mM levamisol, followed by three washes for 5 min each in Tris-magnesium buffer (NTMT: 0.1 M NaCl, 0.1 M Tris-HCl, pH 9.5, 50 mM $MgCl_2$, 0.1% Tween 20) and 2 mM levamisol. Embryos or tissues are then pre-blocked in TBST containing 10% sheep serum, followed by incubation for 40 min in alkaline phosphatase-conjugated anti-digoxigenin antibody (Boehringer Mannheim, Indianapolis, IN, USA) diluted in NTMT according to the manufacturer's specifications. To remove nonspecific reactivity, the antibody is pre-absorbed with acetone-extracted powder from the tissue being studied, according to established protocol (9). Following extensive washing in TBST, then NTMT (three times for 10 min for each buffer), the bound alkaline phosphatase is visualized after incubation of the tissue in 0.34 mg/mL *p*-nitrotetrazolium blue chloride and 0.18 mg/mL 5-bromo-4-chloro-3-indolyl-phosphate in NTMT.

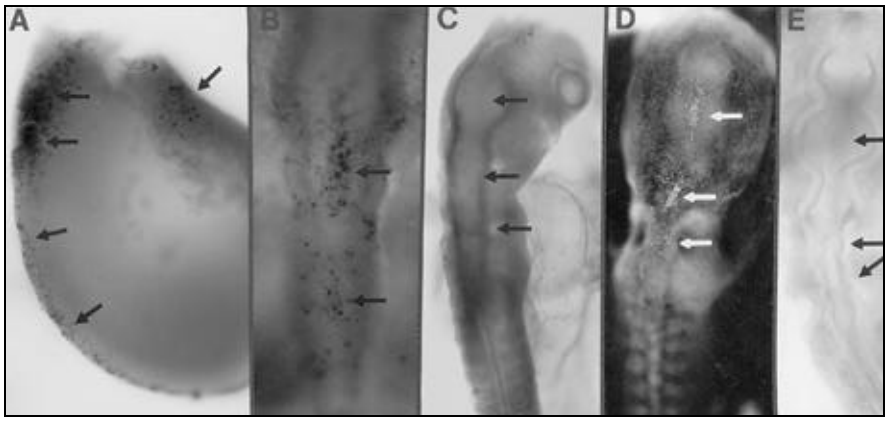

Figure 1. Use of whole-mount TUNEL technique to visualize apoptotic cells within the developing chick embryo. Using the protocol described in the text, the modified ApopTag Kit detected the apoptotic cells within the limb bud anterior necrotic zone and apical ectodermal ridge (A, arrows), as well as the apoptotic neural crest cells within hindbrain segments 3 and 5 (B, arrows). Omission of TdT enzyme served as a negative control and produced no detectable signal in those regions (C). The hindbrain cells that are labeled with the whole-mount TUNEL technique also are detected with AcrOr vital dye staining for programmed cell death (D, arrows), but not with nigrosin dye, which detects necrosis (E, arrows).

Development times average 10 min at room temperature. The reaction is stopped by transferring embryos or tissues into PBS containing 10 mM EDTA. Embryos or tissues are then cleared in a graded series of glycerol-water, and the labeled apoptotic cells are visualized under light microscopy. Negative controls are embryos or tissues either incubated in an irrelevant antibody conjugated with alkaline phosphatase or in which TdT or digoxigenin-dUTP is omitted; as a positive control, tissues can be pretreated with DNase to induce DNA nicking (2).

We have used the whole-mount TUNEL protocol to detect apoptosis in two well-described tissues of the chick embryo: the apoptosis that occurs in the day 4 anterior limb bud margin (6) and that within the day 2 developing hindbrain (3). Figure 1A shows the application of this protocol to detect the small population of mesenchymal cells within the anterior limb margin, which undergoes cell death at day 4 of embryogenesis (6). Whole-mount TUNEL specifically detects these cells and the smaller collections of apoptotic cells within the anterior apical ectodermal ridge and at the proximal base of the limb (Figure 1A, arrows; Reference 7). These cells are traditionally detected using vital dye staining, which detects programmed cell death (4,6) and shows that an apoptotic mechanism is involved in this process.

Apoptosis also occurs within the early hindbrain and specifically within the cranial neural crest cells that localize to hindbrain segments 3 and 5 (3). The whole-mount TUNEL technique detects these individual cells (Figure 1B); no signal is seen in a negative control in which TdT (Figure 1C) or digoxigenin-dUTP (not shown) is omitted. At a slightly later time, these TUNEL-labeled hindbrain populations are detected with the vital dye AcrOr (Figure 1D), which is specific for programmed cell death, thus confirming that the two techniques detect the same event. The fact that these populations also exclude nigrosin dye, which cannot enter cells with intact cell membranes, further confirms that apoptosis and not necrosis is being detected here (Figure 1E). Mitotically active cells contain 3′- OH DNA ends capable of labeling by TdT, but their abundance per cell is below the detection limit of the TUNEL technique (2). As confirmation, we visualized mitotic cells using bromodeoxyuridine (BrdU) incorporation and found that the two techniques detect spatially distinct populations; BrdU-labeled cells are abundantly distributed throughout the rapidly growing embryo (not shown), in contrast to the predominantly hindbrain-restricted TUNEL signal.

Because late stage necrosis can also exhibit DNA fragmentation capable of being end-labeled in the TUNEL technique, it is important to perform control experiments that confirm that early stage apoptosis and not necrosis is being detected. This should include morphological examination of cells to identify pyknosis, kinetic analysis of the cell-death progression, comparison staining with vital dyes and nigrosin and/or, when applicable, inhibition with apoptosis antagonists (such as ICE/*ced3* family protease inhibitors). Immunostaining for incorporated BrdU, either on parallel tissues or in double-labeling, can rule out detection of replicating DNA. The signal's permanence allows tissues to be sectioned for higher resolution analysis; alternatively, confocal microscopy could be used to detect an anti-digoxigenin antibody conjugated with fluorescent label. Sequential hybridization with hapten-conjugated riboprobes could permit simultaneous detection of apoptosis and gene expression, as demonstrated for *Drosophila* (8). Combined with such techniques, the single-cell resolution provided by whole-mount TUNEL will prove useful for investigating spatially distinct apoptotic populations, such as that which occurs during embryogenesis.

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Direct In Situ End-Labeling for Detection of Apoptotic Cells in Tissue Sections

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Apoptosis occurs during development, during normal adult tissue maintenance and during some disease processes. If one wants to study these processes, it is important to detect apoptotic cells from non-dying cells. Since Gavrieli et al. first published their DNA nick end-labeling method (3), many similar, modified versions of terminal deoxyribonucleotidyl transferase (TdT)-mediated dUTP-biotin nick end-labeling (TUNEL) or in situ end-labeling (ISEL) methods involving digoxigenin-11-dUTP have been published (1,2,4,7,8). In all of the above indirect