

## Confocal Laser Scanning Microscopy of Morphology and Apoptosis in Organogenesis-Stage Mouse Embryos\*

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### 1. Introduction

In our efforts to use confocal laser scanning microscopy for study of organogenesis-stage rodent embryos, we have developed fixation and clearing methods to allow optical sectioning through embryos with thickness approaching 1 mm (*z*-axis). We have combined fixation and clearing methods with fluorochrome staining for several purposes. In this chapter we present two methods; first, clearing with methyl salicylate (oil of wintergreen) and staining with Nile blue sulfate (NBS) (not used as a vital dye for this protocol) for general morphological assessment, and second, staining live embryos with the vital stain LysoTracker<sup>®</sup> Red (LT), followed by fixation and clearing with benzyl alcohol:benzyl benzoate (BABB) to visualize areas of apoptosis (*see Note 1, ref. 1*). With both protocols, an entire organogenesis-stage rodent embryo can be optically sectioned and reconstructed in three dimensions (3-D) to reveal areas of dye staining.

In the morphological staining procedure, embryos are fixed in 4% paraformaldehyde overnight, dehydrated in a graded methanol series with NBS added to the 95% methanol solution, and cleared in methyl salicylate. Fluorescence appears to come from both the methyl salicylate and the NBS, and staining is uniform and nonspecific, giving good morphological detail. For analysis of apoptosis, embryos were incubated in the LT stain, fixed in 4% paraformaldehyde overnight, dehydrated in a graded methanol series, and cleared in BABB. LT is an aldehyde-fixable lysosomotropic dye that stains large phagolysosomes and apoptotic bodies in a manner that appears to be very similar to several other vital dyes used for this purpose, including acridine orange, NBS, and neutral red. However, LT has the great advantage of being aldehyde fixable, is also quite bright and shows little evidence of bleaching with repeated scans. To test our staining protocol, apoptosis was induced by the chemotherapeutic hydroxyurea or arsenic by adding these compounds to the medium of gestation day (GD) 8 mouse

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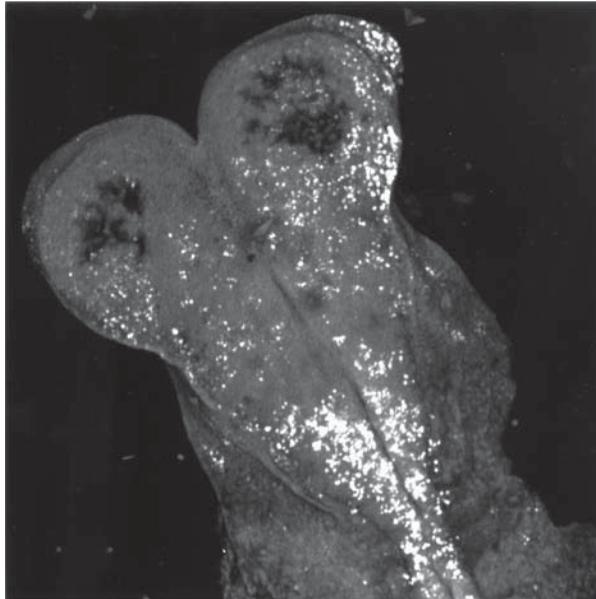


Fig. 1. 3-D reconstruction of an unfixed, uncleared GD 8 mouse embryo treated in vitro with arsenic for 6 h and vitally-stained with 5  $\mu$ M LT for 30 min. The small size and flatness of the embryo at this stage allows for the imaging of this tissue without clearing. However, even here most of the detectable fluorescence originates near the surface. LT is staining regions of lysosomal activity in the embryo (original magnification  $\times 125$ ).

embryos developing in vitro (**Fig. 1**). Alternatively, pregnant mice were given a teratogenic dose of methanol on GD 7; on GD 9, embryos were removed and processed for imaging. For the figures presented in this chapter, mouse embryos were harvested on GD 8 or 9. However, we have used these techniques on mouse embryos from GD 7 through GD 10, as well as on isolated limb buds from GD 14 rat embryos.

## 2. Materials

1. Animals and teratogenic chemicals: CD-1 mice were obtained from Charles River Laboratories (Raleigh, NC). Animals were kept on a 14-h light:10-h dark lighting schedule and provided water and Prolab Rat/Mouse/Hamster 3000 formula (PMI Feeds, St. Louis, MO) ad libitum. Females were housed with males for the last 2 h of the light period, and females with copulatory plugs were considered to be at GD 0. Teratogenic chemicals used for this presentation include hydroxyurea (HU; Sigma, St. Louis, MO) and methanol (Optima grade; Fisher, Pittsburgh, PA).
2. Stains: Morphology: NBS (Fluka, Ronkonkoma, NY). Apoptosis: 1 mM LT, in DMSO (Molecular Probes, Eugene, OR) was obtained in 20 vials (50  $\mu$ L each). A 50- $\mu$ L vial of LT was diluted with 50  $\mu$ L DMSO (Sigma) to make a 500- $\mu$ M stock solution. The LT red stock solution was diluted 100-fold with Dulbecco's phosphate-buffered saline (PBS) (Gibco-BRL, Gaithersburg, MD) to yield a working solution with a final concentration of 5  $\mu$ M.
3. Fixative: Paraformaldehyde (20%; Electron Microscopy Sciences, Fort Washington, PA) was diluted to 4% with PBS and stored frozen at  $-20^{\circ}\text{C}$  in 5-mL aliquots. Use only elec-

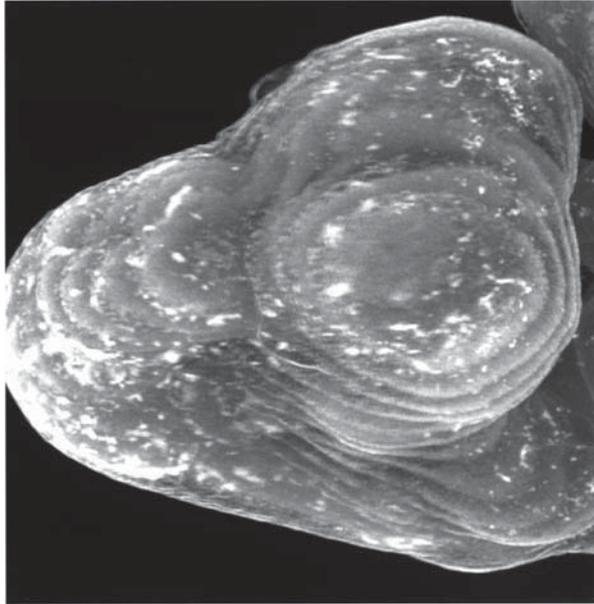


Fig. 2. 3-D reconstruction of 21 sections of a control GD 9 mouse embryo stained with NBS and cleared with methyl salicylate. The depth of the embryo head is greater than 600  $\mu\text{m}$ . The edges of the optical sections can be seen, revealing the stacking of these individual scanned sections (original magnification  $\times 100$ ).

tron microscopy-grade paraformaldehyde that is freshly made or stored frozen at  $-20^{\circ}\text{C}$ . Higher concentrations of paraformaldehyde will yield more autofluorescence. It is advisable to use lower concentrations for probes that excite with 488-nm light and emit in the green range.

4. Methanol dehydration series: After fixation, embryos were washed extensively with PBS to remove the fixative. The embryos were then dehydrated in methanol (#A-412-500; Fisher) with successive 15-min washes of 50, 70, and 95% (v/v) methanol in distilled water and then 2X with 100% methanol. Methanol dehydration is superior to acetone or ethanol dehydration. For NBS staining, NBS powder (1:20,000 w/v) was dissolved in 100% ethanol, and this solution was added dropwise to the 95% methanol dehydrating solution.
5. Clearing agents: A clearing solution consisting of 1:2 (v/v) BABB (Sigma) is used in conjunction with LT staining for apoptosis. The refractive index of the BABB solution is similar to the refractive index of the embryo, yielding an embryo that is nearly transparent (2–4). For Nile blue staining of morphology, methyl salicylate (Fisher or Mallinkrodt [J. T. Baker, Phillipsburg, NJ]) is used for clearing instead of the BABB (Figs. 2–4).
6. Slides, cover slips, sealants: Cleared embryos are transferred to 2-mm thick depression slides (#12-265a; Fisher) and fresh BABB or methyl salicylate is added. Each depression slide is covered with a 20  $\times$  30-mm cover slip (1.5 size), which is sealed with 3–4 successive coats of clear nail polish. It is essential that the depression slides be sealed well, as the BABB and methyl salicylate are potentially damaging to various components of the microscope. The slides can be carefully cleaned with acetone. Acetone will dissolve nail polish so it must be used sparingly and with care. After use, slides can be soaked in acetone to remove cover slips, and depression slides can then be cleaned and reused.

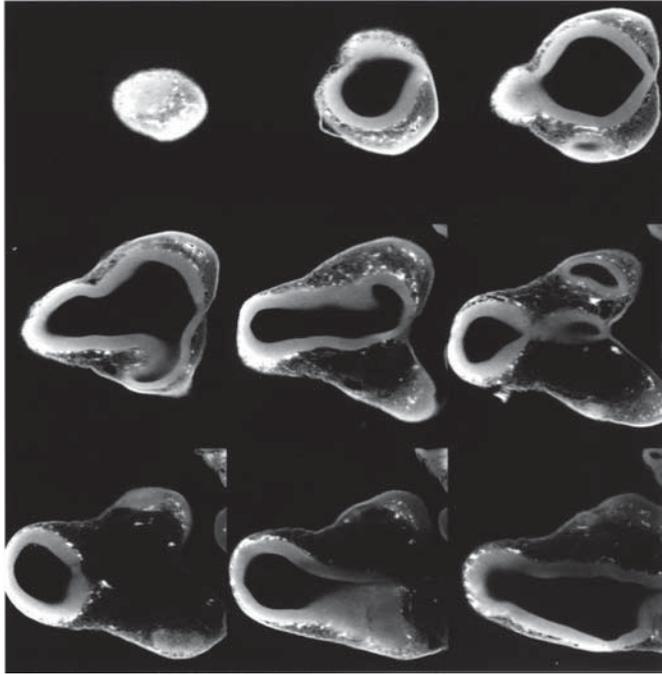


Fig. 3. Optical sections from the GD 9 mouse embryo 3-D reconstruction shown in **Fig. 2**. The individual sections were obtained using 568-nm excitation light, and the emitted light was collected through a 605/32 barrier filter (Chroma). Note the internal morphology of the embryo revealed in the individual sections. For clarity every second section is represented in the figure (original magnification  $\times 100$ ).

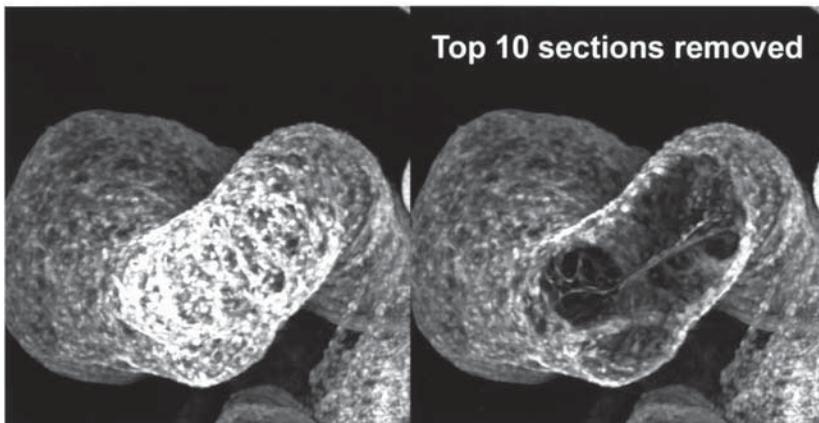


Fig. 4. (A) 3-D reconstruction of 30 sections of a heart from a GD 9 mouse embryo stained with NBS and cleared with methyl salicylate. The section images were obtained using 568 excitation light and emitted light was collected through a 605/32 barrier filter. Here, by choosing a higher number of sections, the individual sections are not apparent and a smooth 3-D image is obtained (i.e., compare to **Fig. 2**). (B) The top 10 sections are deleted from the 3-D reconstruction by the Leica imaging software and the remaining 22 serial sections are converted into a 3-D reconstruction, revealing internal structure (original magnification  $\times 200$ ).

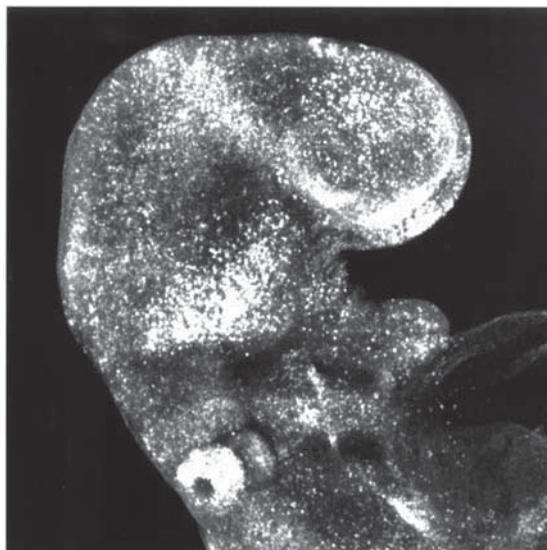


Fig. 5. 3-D reconstruction of 25 sections of a GD 9 embryo stained with LT and cleared with benzyl alcohol/benzyl benzoate to visualize regions of apoptosis after maternal treatment with methanol on GD 7. The fluorescence occurs throughout the embryo with the individual sections showing specificity of staining. The individual section images were obtained using 568 excitation light and the emitted light was collected through a 598/40 barrier filter (Chroma) (original magnification  $\times 100$ ).

7. Confocal microscope: This work requires a point-scanning confocal microscope that has good optical efficiency (*see Note 2*). For these studies, the Leica TCS4D with a Leica inverted DMIRB microscope (Leica Microsystems Inc., Deerfield, IL), and an argon-krypton laser (Omnichrome, Chino, CA) emitting three wavelengths (488, 568, and 647 nm) were used. A  $\times 5$  or  $\times 10$  objective with a high numerical aperture (NA) is desirable. Lenses that we have found to be acceptable include the following: Zeiss  $\times 5$  fluor (NA 0.25); Zeiss  $\times 10$  fluor (NA 0.5); Leica  $\times 10$  Plan APO (NA 0.5) and Leica multi-immersion  $\times 10$  (NA 0.4). A Zeiss lens (Carl Zeiss Inc., Thornwood, NY) will fit on a Leica microscope, but the magnification will be increased by 20%. The working distances of the air lens are in millimeters, whereas the Leica multi-immersion lens is approx 350  $\mu\text{m}$ . Other lenses may also work adequately (*see Notes 6 and 7*).

### 3. Methods

1. Animals and treatments: Mice were mated as described above. For embryo culture, 3–6 somite embryos (GD 8) were removed from the gravid uteri and dissected free of decidua, Reichert's membrane and the parietal yolk sac, leaving the visceral yolk sac intact. Embryos were cultured for 24 h in 75% rat serum:25% Tyrode's by standard procedures. Hydroxyurea was added to the culture medium of treated embryos to give a final concentration of 250  $\mu\text{M}$  for the entire culture period. At the end of the culture period, embryos were divested of the yolk sac and amnion and further processed as detailed below. Animals dosed with methanol were weighed on the morning of GD 7 and given two intraperitoneal injections of methanol 4 h apart, for a total dosage of 4.9 g methanol per kg maternal body weight. On GD 9, dams were killed and embryos removed from the gravid uteri and divested of their surrounding membranes (**Figs. 5 and 6**).

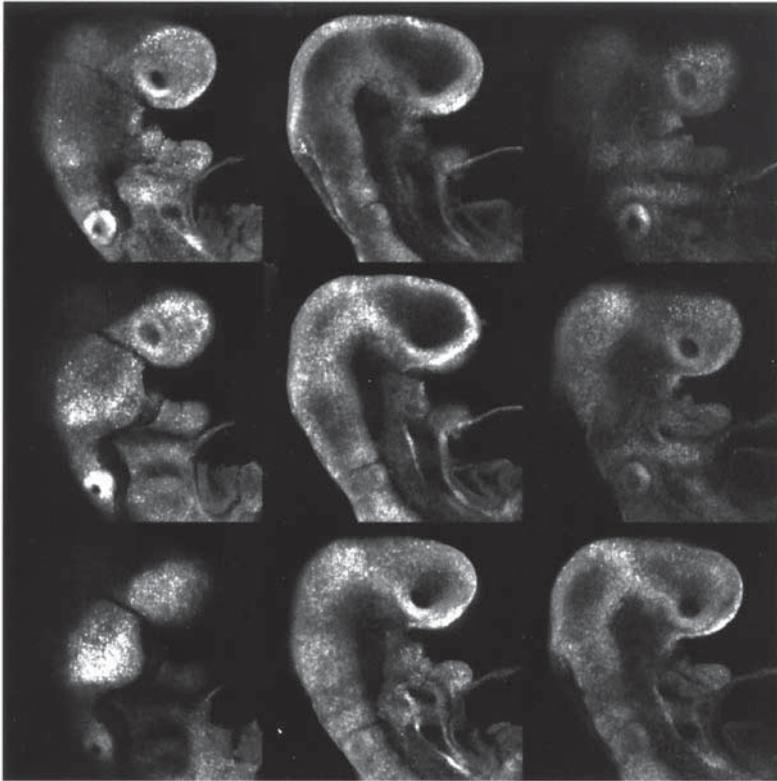


Fig. 6. Optical sections used to derive **Fig. 6**. The neuroepithelium shows increased cell death in the individual tissues. To illustrate the depth of laser penetration the circular structure (otic pit) at the top of the picture becomes bright and then disappears and then reappears as the sections go deeper into the embryo. This represents the future ears of the embryo. The sections above and below the middle sections were removed and every second section in the middle of the embryo is represented as a nine-section display. The sections are approx 20  $\mu\text{m}$  apart with the embryo having a thickness of 500  $\mu\text{m}$ .

## 2. Staining:

- a. Morphological staining with NBS: NBS was used to stain embryos to aid in general morphological assessment. NBS staining is accomplished in fixed embryos at the 95% methanol dehydration stage. NBS staining conditions were derived empirically. NBS powder is dissolved in 100% alcohol (approx 1:20,000 w/v) and was added dropwise to the 95% dehydrating solution to yield a light blue color. The embryo should become dark blue after a 1–2 h staining time. The final intensity of stain in the embryo is decreased during the clearing procedure, resulting in a lightly stained embryo that facilitates handling and observation on the microscope stage and enhances fluorescence.
- b. Vital staining of apoptosis with LysoTracker red: LT was used at a final concentration of 5  $\mu\text{M}$  in Hank's Balanced Salt Solution (HBSS). It is advisable to use a nonbuffered salt solution for vital staining with LT, as specific staining depends on a pH gradient across membrane-bound compartments. Between 1 and 3 embryos were incubated in this medium for 30 min at 37°C. The dye concentration used was decided on after preliminary experiments were carried out using 1, 2, or 5  $\mu\text{M}$  LT for either 30-min or

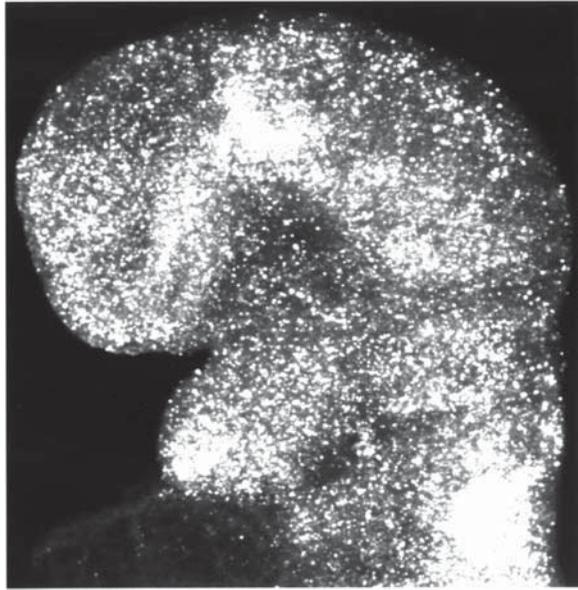


Fig. 7. 3-D reconstruction of 25 sections of a GD 9 mouse embryo that was treated with 250  $\mu\text{M}$  hydroxyurea for 6 h *in vitro*. The bright spots represent LT staining and indicate regions of high lysosomal activity. The areas containing bright spots are located throughout the embryo but concentrations can be seen in the neural tube and otic pit (ear) regions (original magnification  $\times 100$ ).

1-h incubation. Consistent staining was achieved with all combinations of LT concentration and time, but the best resolution with the highest signal-to-noise ratio, and best tissue conditions were achieved using 5  $\mu\text{M}$  LT for 30 min (**Figs. 7 and 8**). Embryo heartbeats were usually maintained through the end of the 30-min incubations.

3. Fixation: Paraformaldehyde (20%; Electron Microscopy Sciences) was diluted to 4% with PBS and stored frozen at  $-20^{\circ}\text{C}$ . Fresh embryos were fixed immediately with ice-cold 4% paraformaldehyde for 24 h. Embryos stained with LT were washed with PBS after the 30-min stain period and fixed in the same manner. Embryos fixed overnight at  $4^{\circ}\text{C}$  were then stored at the 70% methanol dehydration stage (*see #4*) until further processing.
4. Dehydration: Embryos were dehydrated in methanol with successive 15-min washes of 50, 70, and 95% (v/v) methanol in distilled water and then 100% methanol. For NBS staining, the stain is added to the 95% methanol dehydrating solution, and embryos are left in this solution for 1–2 h (*see 2a*). Two additional 100% methanol washes were made to ensure that the embryo was completely dehydrated. When the dehydrating solutions were changed, the solutions were removed carefully with a transfer pipet and new solutions were added to the same glass tube containing the embryos rather than transferring the embryo into a different tube. In this way, potential pipetting damage to the embryo was minimized.
5. Clearing (*see Note 3*): For uniform morphological staining, NBS and methyl salicylate were used. Embryos were successively placed in 50% and 75% methyl salicylate in methanol, followed by two changes of 100% methyl salicylate, each step for 15 min. For staining of apoptosis with LT, the BABB clearing protocol was used. The BABB protocol produced an embryo that was more transparent than the one produced by the methyl salicylate protocol, allowing better discrimination of specific staining for apoptosis. Care

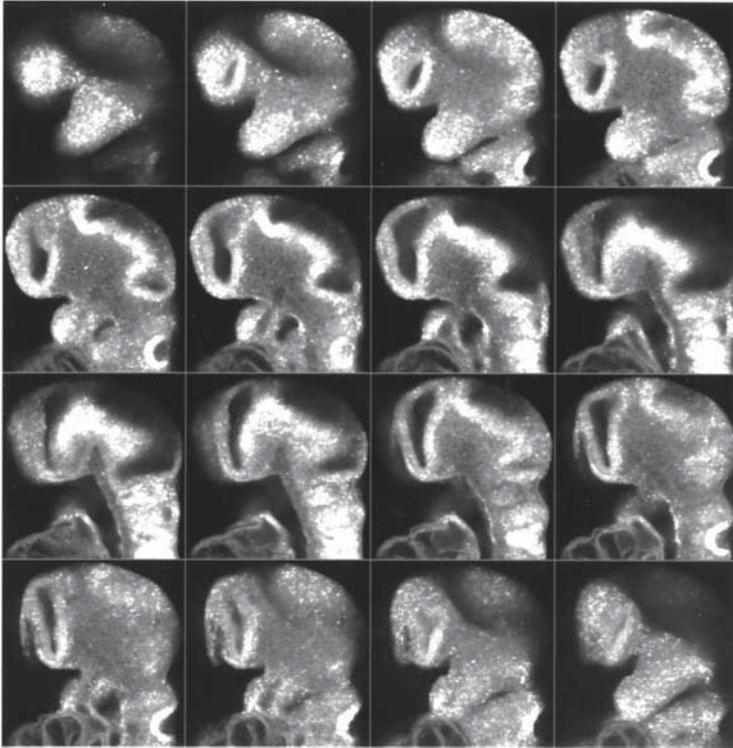


Fig. 8. Individual optical sections of the GD 9 hydroxyurea-treated embryo represented in **Fig. 7**. The sections are separated by about 20  $\mu\text{m}$ . The sections above and below these sections were removed to present a 16-section display. There is an increased amount of fluorescence throughout the embryo compared to control embryos, but individual sections show specificity of label. Both the left and right otic pits can be observed, demonstrating the depth of scanning with resolution and brightness of fluorescence maintained. The neuroepithelium shows dramatic hydroxyurea-induced cell death in the individual sections.

must be taken during the clearing procedure because the embryos become totally transparent and could be damaged or lost. The refractive index of the BABB solution is similar to the refractive index of the embryo, yielding an embryo that is transparent (1,5,6). Embryos were first put into a solution of 1:1 MEOH/BABB for 30 min and then the solution was removed and 100% BABB was added for at least 30 min. After clearing with either methyl salicylate or BABB, most of the clearing agent was carefully decanted and the embryo at the bottom of the tube was transferred into a large glass depression slide with some of the clearing agent.

6. Mounting and cover-slipping of embryos (*see Notes 4 and 5*): Methyl salicylate- or BABB-cleared embryos: After carefully removing the methyl salicylate or BABB from the large glass depression slide with a transfer pipet, the transparent embryos were visualized by surface light reflection. The embryos were then transferred to 2-mm-thick depression slides (Fisher 12-265a) and fresh methyl salicylate or BABB was added. The depression slide was then covered with a 20  $\times$  30-mm cover slip (1.5 size) and was sealed with 3–4 successive coats of “Sally Hard as Nails” nail polish. It is essential that the depression slide be sealed, as the methyl salicylate and BABB are potentially damaging to various components of the microscope.

7. Confocal microscopy (*see* **Notes 2 and 8–10**): The embryo contained in the sealed depression slide was imaged using a Leica laser scanning confocal microscope (TCS4D). The 568 line excited the LT dye and a BP-TRITC filter was used to measure the emitted light. The sample was line-averaged at 16 or 32 scans per line, and a total of 20–30 sections per embryo were found to be sufficient to reconstruct the entire thickness of the embryo in 3-D. Embryos as thick as 700  $\mu\text{M}$  have been completely sectioned optically by this technique with good resolution of internal staining both in individual optical sections and in 3-D reconstructions. The stack of confocal images was combined using a Leica 3-D program and the resultant composite, 256 gray-level TIFF file was transferred to a Pentium PC operating under Windows NT 4.0. Thumbs Plus (Cerrious Software, Charlotte, NC) was used to display and categorize the images obtained from the confocal microscope. Image Pro Plus (Media Cybernetics LP, Silver Spring, MD) was used to label the images prior to digital printing. Digital printing was done using a NP1600 (Codonics, Middleburg Heights, OH) dye sublimation printer ( $\gamma = 2.0$ , contrast = 15). In order to represent a composite image form a stack of images, a few of the top and bottom images having minimal information were eliminated. The resultant image consisted of 16 individual TIFF images instead of the entire stack of approx 25 images.

#### 4. Notes

1. A fixable vital stain and subsequent clearing procedure has been developed to study cell death in murine embryos at mid-gestation by confocal laser scanning microscopy, enabling the visualization of structures inside the embryo that are several hundreds of micrometers below the surface. The fluorescent dye, LT Red, allowed visualization of known areas of programmed cell death in normal embryos and increased staining in embryos treated with chemicals (hydroxyurea, MEOH) that correlated to lysosomal activity and apoptosis. It has been reported previously in embryos that dyes that stain lysosomes (Nile blue, neutral red, or acridine orange) were correlated to lysosomal activity, phagocytosis, and apoptosis (**6–16**). LT Red was used to observe these regions of high lysosomal activity, which related directly to engulfment of apoptotic bodies and indirectly to cell death.
2. There are many technical difficulties associated with imaging of an object greater than 50  $\mu\text{m}$  (**1,5,17,18**). One approach used to solve this imaging problem is to cut serial sections of the object, image a thin slice, and then reconstruct the object with software. Serial section analysis is a time-consuming, tedious process in which errors can be introduced by sectioning the embryo and then realigning the measured sections. Maintaining the embryo as an intact structure can eliminate many problems associated with the reconstruction of serial sections. The whole mount provides a 3-D view of stained components located inside the embryos. However, imaging whole mounts also has technical difficulties. By using a confocal laser microscope instead of a normal fluorescent microscope, the laser light can penetrate deeper into the tissue for better visualization. However, because the tissue is dense and opaque (not transparent), the light becomes scattered, reflected, refracted, and absorbed when entering the tissue and then when passing back through the tissue into the objective (**17,18**). This results in a reduction in the intensity of laser light and decreased optical quality as increased penetration into the tissue is attempted. Thus, depending on the light gathering characteristics of the objective and the power of the laser light, imaging of an embryo can be severely affected.
3. To overcome the opaqueness of the tissue, a clearing procedure previously applied to histological sections and amphibian eggs was used. The clearing procedure involves extracting water from the tissue and then replacing it with a solution that has a similar refractive index as the tissue has (**2–4**). Previous clearing agents used include the follow-

ing: potassium hydroxide/glycerol, methyl salicylate (artificial oil of wintergreen), carbon disulfide, glycerol, and xylene. The clearing solution [1:2 mixture of benzyl alcohol (refractive index = 1.54035) and benzyl benzoate (refractive index = 1.5681)] (BABB) used in this study was derived by Murray and Kirschner (2). Although the BABB mixture was designed for amphibian eggs and embryos, it appears to work equally well with mammalian embryos. Experiments varying the percentages of the components did not seem to increase the image quality of the mammalian embryo image. In choosing a clearing agent to render the tissue transparent, one must consider its compatibility with the staining reagents used, compatibility with aqueous solutions, and effects on the fixed tissue.

4. In the BABB protocol, the embryos were put first into a solution of 1:1 MEOH and BABB and then into a solution of 100% BABB. The refractive index of this solution is similar to the refractive index of the embryo, yielding an embryo that is nearly transparent (2,3). After the final step, the medium was carefully decanted and the embryo contained in the bottom of the tube was transferred into a large glass depression slide. After carefully removing the medium, the embryo could be observed by light refraction. The embryos from both procedures were then transferred into a thick depression slide and fresh methyl salicylate or BABB was added and covered with a 20 × 30-mm cover slip. The cover slip was sealed with 3–4 successive coats of clear nail polish. It is essential that the depression slide be sealed, as the BABB cocktail is toxic to microscopes. Although the embryos are stable for a long time, the clearing agents will gradually dissolve the seal. It is sometimes useful to reseal the slides prior to using them on the confocal microscope to ensure that no spills occur.
5. The BABB clearing procedure has some disadvantages. Because the clearing agent is not compatible with aqueous solutions, the water in the tissue sample must be totally displaced by alcohol prior to the addition of the clearing agent. We have been successful in using fixable dyes like ethidium bromide or LT Red, but have been unsuccessful in using dyes like Hoechst 33342, which are removed by the alcohol dehydration procedure. Another disadvantage with this clearing agent is that the embryos become very brittle during the procedure. Care must be used in sealing the slide to immobilize the embryo. In addition, care must be taken to remove excess clearing agent to eliminate the possibility that the solution come into contact with the microscope or its objectives.
6. The proper choice of objectives is critical for this work. Most confocal microscopy applications use lenses with high power and high numerical apertures (NA) to image cells (5,17,18). However, to image parts of embryos, it is necessary to use low-power objectives. In our applications it was important to observe large regions of the embryo to determine the cell death patterns. To obtain these images, it was necessary to use low-power objectives (×5 and ×10) with relatively high NAs to allow sufficient visible fluorescent wavelengths to be transmitted.
7. In our experiments, the use of depression slides yielded the best results with dry objectives having high NAs. Both sides of the chamber can be scanned with almost equal resolution. Although an oil lens will give better resolution than a dry lens, the oil lens will introduce a series of problems that include shallow depth of focus (usually less than 300 μm), possible contamination with clearing agent residues on the cover glass surface, and possible exertion of mechanical stress on the cover slip and thus the seal. Removal of the oil from the cover glass may also break the nail polish seal on the depression slide. We have added a ×5 Zeiss Fluor objective (.25 NA) to the Leica microscope, based on its higher NA and its ability to effectively transmit more fluorescence at lower power. The use of the Zeiss lens on the Leica infinity-corrected microscope results in a 20% increase in magnification. Thus, the effective magnification of the ×5 Zeiss lens is ×6.2 on the Leica sys-

tem. Leica has just released a  $\times 10$  multi-immersion lens (0.4 NA) and a  $\times 10$  dry (0.4 NA) that have depth of field of 350  $\mu\text{m}$  making them extremely useful in observing entire small embryos or limbs. Although other objectives may be used for these applications, it is important to consider the NA, magnification, and the working distance of the lens.

8. Image quality can be affected by a number of controls on the confocal microscope. These include the selection of dichroic bandpass filter, averaging, laser power, and bleaching of the dye. It was observed that a narrower bandpass instead of a long-pass filter decreased the reflections. Methyl salicylate also yielded a clear uniform embryo that did not contain reflections observed with the BABB clearing procedure. It is important that the embryo not have saturation pixels. For the current applications, image quality was superior when bandpass filters were used instead of conventional Schott filters.
9. This procedure is rapid and flexible, although sample preparation variables including staining, fixation, dehydration, and clearing must be carefully controlled for optimum resolution. Image quality will also be affected by the choice of laser line, specific filter selections, and specific confocal microscope controls (*I*). It appears from our studies using LT Red that sufficient fluorescence occurs to allow the pinhole size to be decreased, the laser power increased, and the photomultiplier (PMT) to be operated below their saturation points. These factors combined with the minimal bleaching observed with the LT Red dye have produced clear images and a straightforward way to monitor apoptosis and morphology in the embryo. In fact, if enough sections are obtained (60) and the z-distance between sections is reduced to approx 2X the x/y distance, the embryo can be visualized by rotating it 360° using 3-D software (Voxblast, Vaytec, Fairfield, IA).
10. Although this assay has been done primarily on embryos, it should be applicable to a broad range of tissues and other fixable dyes. In order to achieve fluorescence with less-efficient systems than the Leica TCS4D point scanner, it may be necessary to incorporate other more efficient dyes into the embryo. We have successfully used nucleic acid stains (ethidium bromide, YO-PRO, and BO-PRO) prior to MEOH dehydration. Final concentrations of the nucleic acid stains in PBS were: ethidium homodimer, 25  $\mu\text{g}/\text{mL}$ ; YO-PRO, 5  $\mu\text{M}$ ; and BO-PRO, 5  $\mu\text{M}$ . Ethidium bromide, YO-PRO, or BO-PRO staining is done after fixation. We stained the samples at 37°C for up to 24 h to ensure that the stain penetrated the entire tissue. The excess stain is then washed out of the sample with PBS and dehydration is initiated with the 50% MEOH stage. The YO-PRO dye was excited with a 488 line using a BP-FITC bandpass filter. Ethidium bromide was excited with the 488-nm line and a BP-TRITC filter was used in the detection. BO-PRO was excited with a 568 line and a BP-TRITC or BP 598/40 filter (Chroma, Brattleboro, VT) was used in the detection. These different filters may be used to increase the amount of transmitted light.

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