

A novel method to determine specificity and sensitivity of the TUNEL reaction in the quantitation of apoptosis

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Kelly, K. J., Ruben M. Sandoval, Kenneth W. Dunn, Bruce A. Molitoris, and Pierre C. Dagher. A novel method to determine specificity and sensitivity of the TUNEL reaction in the quantitation of apoptosis. *Am J Physiol Cell Physiol* 284: C1309–C1318, 2003. First published December 21, 2002; 10.1152/ajpcell.00353.2002.—Apoptosis is an important mode of cell death under both physiological and pathophysiological conditions. Numerous techniques are available for the study and quantitation of apoptosis in cell culture, but only few are useful when applied to complex tissues. Among these, the terminal transferase-mediated dUTP nick end-labeling (TUNEL) assay remains the most widely used technique. However, its specificity and sensitivity for the detection of apoptosis remain controversial. We developed a technique consisting of staining live cells and tissues with Hoechst 33342 and the vital dye propidium iodide (PI), followed by fixation and the TUNEL reaction. We demonstrate excellent retention of PI in necrotic cells after fixation. We also examined the distribution of TUNEL staining among necrotic and apoptotic cells in various models of cell injury in vitro and in vivo. We show that the sensitivity of the TUNEL varied between 61 and 90% in the models examined. The specificity exceeded 87% in all models but fell to 70% when a predominantly necrotic injury was induced. This novel and simple method will permit the determination of indices of sensitivity and specificity for the TUNEL assay in other tissues and experimental conditions.

necrosis; propidium iodide; two-photon microscopy; ischemia

PROGRAMMED CELL DEATH (PCD) is the highly organized biochemical destruction of unwanted cells in multicellular organisms. Apoptosis, the morphological expression of PCD at the cellular level, has been observed in a myriad of physiological and pathophysiological conditions (1, 13, 17). These include normal development, morphogenesis, and disease conditions such as ischemic injury and degenerative disorders. Conversely, defective PCD has been implicated in the genesis of many neoplastic conditions (9). Therefore, research in the field of apoptosis and PCD has recently reached an unprecedented intensity.

The recognition and quantitation of apoptosis in cell culture are generally straightforward. Nuclear morphology with classic chromatin condensation and frag-

mentation remains one gold standard for identifying apoptotic cells in culture (19, 22). This is traditionally performed on live cells where the concurrent use of cell-permeable stains, like Hoechst, and vital dyes, like propidium iodide (PI), permit the exclusion of necrotic cells with compromised plasma membranes (6). Other widely available techniques are used to complement the morphological studies. These include investigation of plasma membrane changes, mitochondrial potential alterations, and various biochemical assays (19).

In contrast, the study of apoptosis in tissues has been greatly hindered by the structural complexity and cellular diversity of the particular organ under investigation. The evaluation of nuclear morphology in fixed samples, while still feasible, is very difficult and challenging because of the multicellular nature of tissues and their architectural complexity. In addition, the necessity to deal with fixed samples prohibits the use of vital nuclear stains to exclude necrotic cells. The recent application of two-photon microscopy to the study of apoptosis in live tissues is very promising but is not yet widely available (2, 8).

The most commonly used technique to quantify apoptosis in fixed tissues is the terminal transferase-mediated dUTP nick end-labeling (TUNEL) method (21, 24, 25). It takes advantage of the multiple free DNA ends generated by activated endonucleases to insert labeled dUTP that can be later detected by light or fluorescence microscopy. The ease and sensitivity of this technique and its quantitative power have led to its current wide acceptance and use. However, a serious drawback remains in its presumed inability to discriminate apoptotic from necrotic cells given that the latter also have free DNA ends, especially after oxidant and toxic injury. This has led many to question the validity of the TUNEL assay under conditions where apoptosis and necrosis coexist (3, 26).

Here we propose a new approach in which the vital dye PI is delivered to live cells or tissues that are later fixed and stained using the TUNEL reaction. We have demonstrated that the retention of PI after fixation permits the identification of necrotic cells and their exclusion from the TUNEL positive count. We have

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validated the use of this approach in both cell culture and whole tissues using a variety of insults like chemical anoxia, ischemia, reactive oxygen species, staurosporine, and cisplatin. We thus have established indexes of specificity and sensitivity for the TUNEL reaction that permit the quantitation of apoptosis in fixed tissues with a higher degree of confidence.

METHODS

Operational definitions of apoptosis and necrosis. We followed simple yet very useful criteria to define necrosis and apoptosis. Necrosis is defined by the uptake of PI (indicating damage to the cell membrane) and the lack of nuclear condensation and fragmentation. Apoptosis is defined by the lack of PI uptake (indicating the integrity of the cell membrane) and the presence of clear nuclear condensation and/or fragmentation. Secondary necrosis is defined by the presence of nuclear condensation and/or fragmentation along with PI uptake. These are (late) apoptotic cells (and are counted as such) that sustained some terminal damage to the cell membrane before they were cleared. After TUNEL staining, any of the above three categories could be TUNEL positive or TUNEL negative. These are admittedly narrow yet very powerful operational definitions of the complex processes of apoptosis and necrosis. They are well suited for a high-throughput quantitation of cell death in cell culture and tissues that is very reproducible and accurate. They also allow the calculation of parameters like sensitivity (apoptotic TUNEL-positive nuclei/all apoptotic nuclei) and specificity (apoptotic TUNEL-positive nuclei/all TUNEL-positive nuclei).

Cell culture. A4.8 clones of LLC-PK porcine proximal tubule cells were grown in DMEM with 10% FBS (Sigma Chemical, St. Louis, MO) and 5 mM glucose using culture dishes that have a central coverslip area suitable for microscopy. Adherent and floating cells were visualized after staining directly in these culture dishes. Cells were subjected to chemical anoxia with 0.1 μ M antimycin A in depleted media (regular media minus amino acids, glucose, and serum) for 45 min as previously described (6, 15). After media exchange and washing, cells were recovered in standard medium for 2 or 24 h before being stained with 0.1 μ g/ml Hoechst 33342 and 1.5 μ g/ml PI for 20 min. This was done without further washing to avoid loss of floating apoptotic cells. Alternatively, cells were exposed to 1 μ M staurosporine or 1 mM hydrogen peroxide (H_2O_2) for 24 h before being stained with PI and Hoechst 33342.

Necrotic, PI-positive cells were counted under a Zeiss confocal microscope (LSM 510), equipped with UV, argon, and helium lasers as previously described (6, 15). Cells were then fixed with 4% paraformaldehyde for 25 min. A repeat count of necrotic cells was performed, the cells were kept at 4°C in PBS overnight, and then necrotic cells were again counted. This provided an index of the preservation of the PI stain after fixation.

After permeabilization with 0.2% Triton X-100 for 5 min in PBS, cells were stained with TUNEL reagent (Promega, Madison, WI) for in situ apoptosis detection. In brief, fixed cells were incubated in a nucleotide mixture containing fluorescein-12-dUTP and TdT (terminal transferase) for 45 min following the manufacturer's instructions. Positive controls were pretreated with 1 U/ml DNase, and negative controls were incubated without TdT. Eight to ten fields per dish were examined in each experiment. Nuclei were examined for apoptotic morphology (Hoechst) and staining with PI and TUNEL reagent. A Zeiss confocal microscope was used as

described above. We also randomly collected 0.5- μ m z sections on some nuclei, which allowed three-dimensional (3-D) reconstruction with MetaMorph software (Universal Imaging).

Animal studies. All animal experimentation was conducted in conformity with the "Guiding Principles for Research Involving Animals and Human Beings" (American Physiological Society, Bethesda, MD). Male Sprague-Dawley rats weighing 180–220 g (Harlan, Indianapolis, IN) were administered 100 μ g of PI in 500 μ l of 0.9% saline intravenously over a 10-min period 1 h before ischemia or sham surgery. They were anesthetized with pentobarbital sodium (50–70 mg/kg ip) and placed on a homeothermic table to maintain core body temperature at 37°C. Both renal pedicles were occluded via a midline incision for 30 min as we have described previously (15, 16). During surgery, an additional 200 μ g of PI were infused intravenously over a 1-h period. Sham surgery consisted of an identical procedure with the exception of application of the microaneurysm clamps. An additional 500 μ g of PI were infused in 100- μ g aliquots at 1, 2, 4, 6, and 8 h after surgery. Approximately 22 h after surgery, an additional 100 μ g of PI were given intravenously. Twenty-four hours after surgery, the rats were anesthetized with thiopental sodium (130 mg/kg ip). A final 100- μ g aliquot of PI was administered intravenously. After adequate anesthesia was assured, a 10- to 15-mm lateral incision was made dorsally under sterile conditions, and the kidney was exteriorized.

The animals tolerated the administered PI with no evidence of systemic toxicity over the time course of the experiments. Furthermore, the PI caused no renal toxicity as assessed by histology and creatinine measurement. We did occasionally note some slow uptake of PI in normal kidneys. This was likely due to endocytosis and was easily differentiated from that seen in necrotic kidneys because of its low intensity and homogeneous cellular distribution. A different protocol was attempted to minimize this effect. Only two doses of PI were given, 15 μ g of PI before ischemia and another 5 μ g of PI 1 h after ischemia. This succeeded in restricting PI labeling to necrotic nuclei in the ischemic kidney with no uptake in control kidneys. However, many apparently necrotic nuclei in tubular lumens were unlabeled. With either protocol, the percentage of PI-labeled nuclei was unaffected by fixation, which is the essential point of this technique. Notably, these protocols, while tailored to image the kidney, might need some modifications if other organs or tissues are targeted.

After initial imaging (as described below), 500 μ g of the nuclear dye Hoechst 33342 was administered intravenously over 2 min to allow identification of all cells and examination of nuclear morphology.

In another group of animals, *cis*-diaminedichloroplatinum II (cisplatin; 5 mg/kg ip) was given 72 h before imaging. This was followed by 15 and 5 μ g of PI given at 48 and 36 h before imaging, respectively. At the time of imaging, Hoechst 33342 was administered as described above.

Microscopy. Live animal imaging was performed with the use of a Bio-Rad MRC-1024MP laser scanning confocal/multiphoton scanner (Hercules, CA) attached to a Nikon Diaphot inverted microscope (Fryer, Huntley, IL) using a Nikon \times 60 NA 1.2 water-immersion objective. Fluorescence excitation was provided by a titanium-sapphire laser (Spectraphysics, Mountain View, CA) at 800 nm, which was used for all studies. Laser output was attenuated with neutral density filters to between 3 and 40% so that, after accounting for losses in the optical train of the microscope, we estimate that the power at the surface of the kidney was between 2 and 28

mW. Animals were placed on the stage with the exposed kidney placed in a 50-mm-diameter coverslip-bottomed cell culture dish (Warner Instruments, Hamden, CT) bathed in isotonic saline as we have described previously (8). Sections from the in situ fixed kidneys were preserved in 20% sucrose before 10- μ m frozen sections were obtained. Sections were stained with TUNEL reagent for in situ apoptosis detection using the Zeiss confocal microscope described in *Cell culture*. The TUNEL staining was done as described in *Cell culture*, except that proteinase K (20 μ g/ml) was used instead of Triton X-100 to permeabilize the tissue. Eight to ten fields per section and three to four sections per kidney were examined in each experiment.

RESULTS

Induction of apoptosis and necrosis in LLC-PK cells after chemical anoxia and recovery. Control LLC-PK cells show <0.5% spontaneous apoptosis or necrosis when examined at confluency (6). When subjected to chemical anoxia followed by recovery for 2 h, significant apoptosis (6 \pm 1% of all cells) and necrosis (9 \pm 1% of all cells) were observed. As shown in Fig. 1, apoptotic cells had classic condensation and fragmentation of their nuclei (A and B, insets) yet excluded PI efficiently. Figure 1C, inset, shows the large nucleus of a necrotic cell with dense red PI staining that lacks fragmentation (red and blue channels shown separately). Figure

1D shows, in addition to apoptotic and necrotic cells, the presence of apoptotic cells (dense fragmented nuclei) that are positive for PI (see inset). These are apoptotic cells with secondary necrosis and are frequently seen when plasma membrane breakdown occurs before an apoptotic cell is cleared.

Effects of fixation and TUNEL staining on LLC-PK cells after chemical anoxia and recovery. These cells were fixed with paraformaldehyde and then stained with the TUNEL reagent. The percentage of necrotic cells before and after fixation and TUNEL staining was 12.3 \pm 1.9 and 12.1 \pm 2.8%, respectively. Thus fixation and TUNEL staining did not alter PI staining. We next examined the distribution of TUNEL staining among necrotic and apoptotic cells. As shown in Fig. 2, green TUNEL staining labeled primarily apoptotic cells (A and C, insets). Conversely, TUNEL staining was almost completely negative in necrotic cells (Fig. 2B, inset). We also noted apoptotic cells (Fig. 2E, inset) and apoptotic cells with secondary necrosis (Fig. 2D, inset) that were TUNEL negative. Finally, Fig. 2F, inset, shows an example of an apoptotic cell with secondary necrosis that is TUNEL positive. Therefore, in this model system, the TUNEL assay showed 99% specificity but only ~64% sensitivity (~35% apoptotic cells

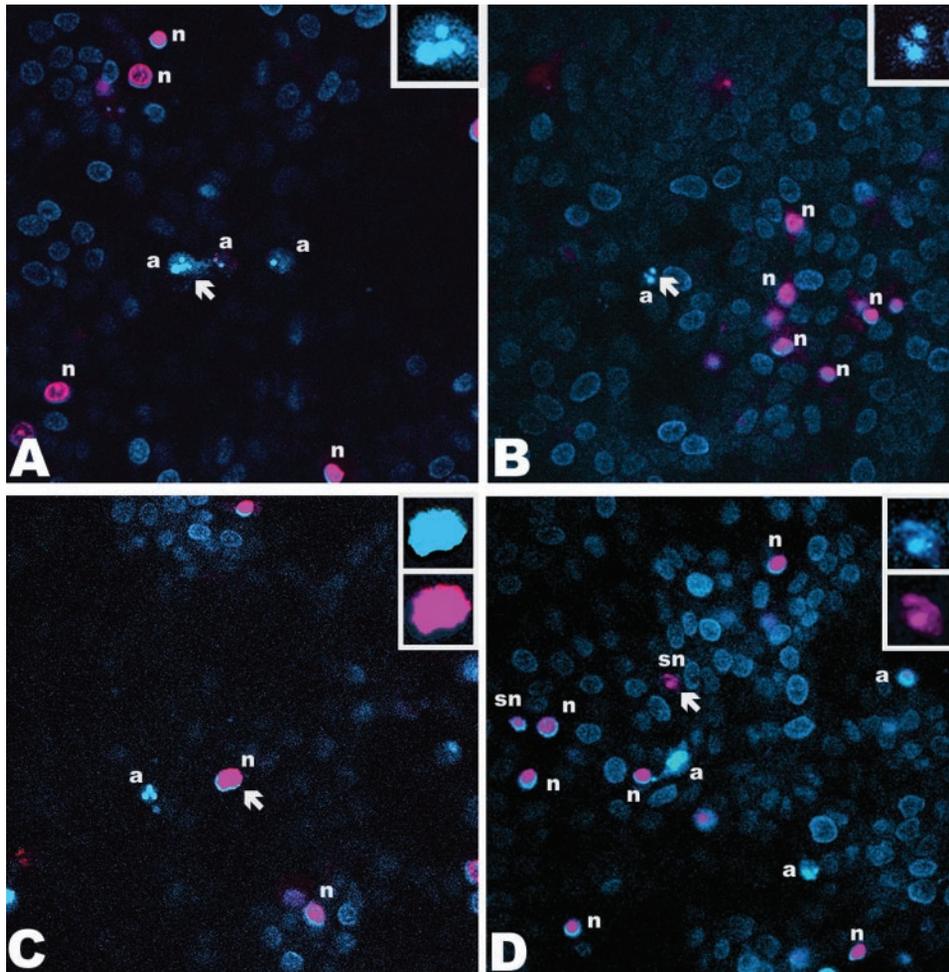
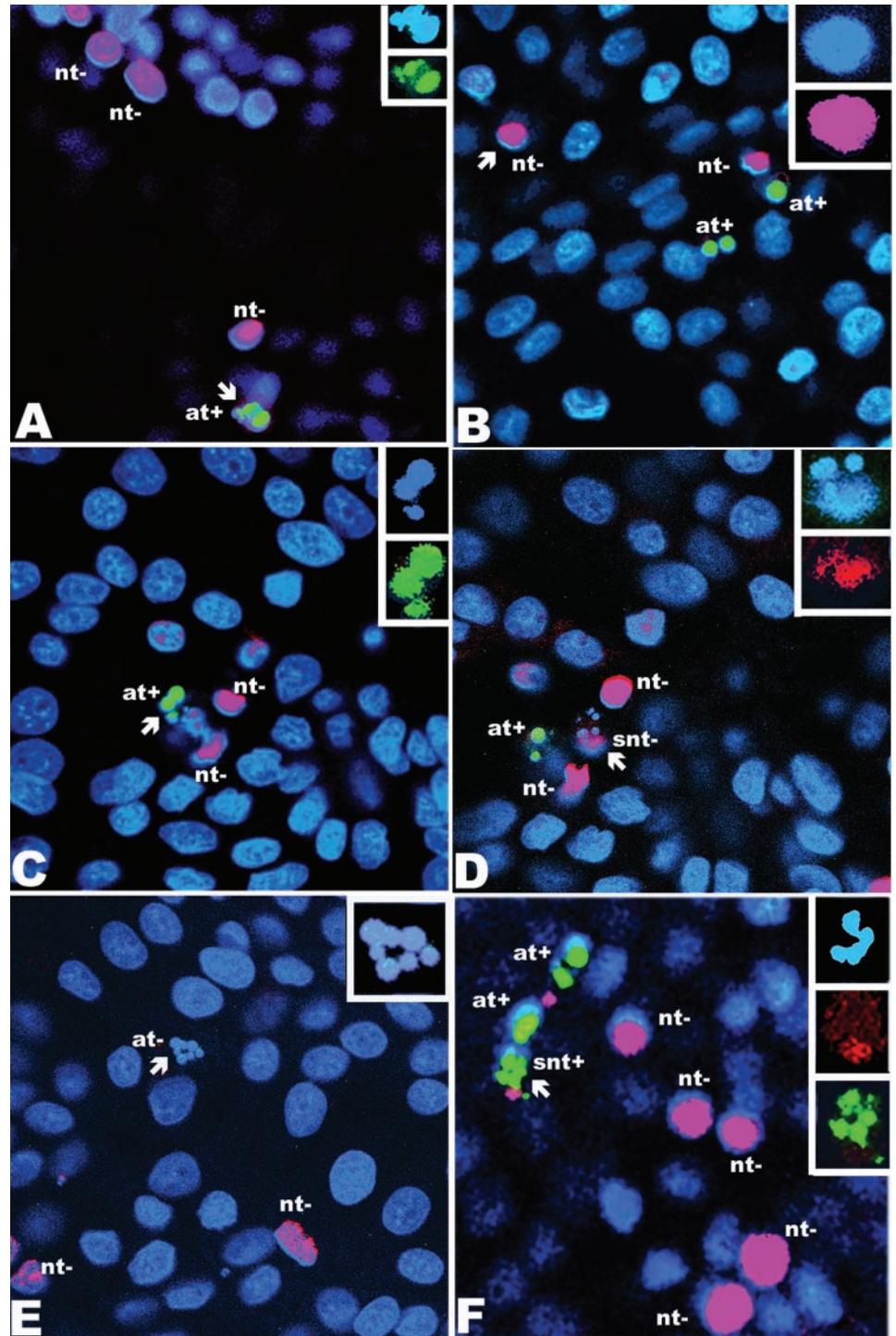


Fig. 1. Induction of apoptosis and necrosis in LLC-PK cells after chemical anoxia and recovery. Representative photomicrographs (20–30 fields per experiment, $n = 4$) are presented of live LLC-PK cultured renal tubular cells subjected to chemical anoxia with antimycin A for 45 min and 2 h of recovery. Cells were stained with propidium iodide (PI) and counterstained with Hoechst 33342 to examine nuclear morphology. Morphologic evidence of apoptosis was present in ~6% of all nuclei (A, B, and D, with insets). Primary (C, with inset) and secondary necrosis (D, with inset) is shown by uptake of PI. Insets show individual channels for Hoechst (blue) and PI (red) fluorescence. a, Apoptosis; n, necrosis; sn, secondary necrosis. Arrows indicate nuclei shown in insets. For clarity in this and subsequent figures, only some examples of each type of nuclear staining are labeled.

Fig. 2. Effects of fixation and TUNEL staining on LLC-PK cells after chemical anoxia and recovery. Representative photomicrographs (20–30 fields per experiment, $n = 4$) are presented of LLC-PK cells subjected to chemical anoxia and recovery, staining with PI and Hoechst 33342, fixation with 4% paraformaldehyde, and then staining with the TUNEL reaction. TUNEL-positive cells also had nuclear morphology characteristic of apoptosis (A, C, F, with insets). Necrotic cells remained PI positive after fixation (B, with inset) and were TUNEL negative. Secondary necrosis was seen in some of the apoptotic cells (D and F, with insets). Approximately 35% of the nuclei with condensation characteristic of apoptosis were TUNEL negative (D and E, with insets). Insets show individual channels for Hoechst (blue), PI (red), and TUNEL reaction (green) for a given cell. nt-, Necrosis, TUNEL negative; at+, apoptosis, TUNEL positive; at-, apoptosis, TUNEL negative; snt-, secondary necrosis, TUNEL negative; snt+, secondary necrosis, TUNEL positive. Arrows indicate nuclei shown in insets.

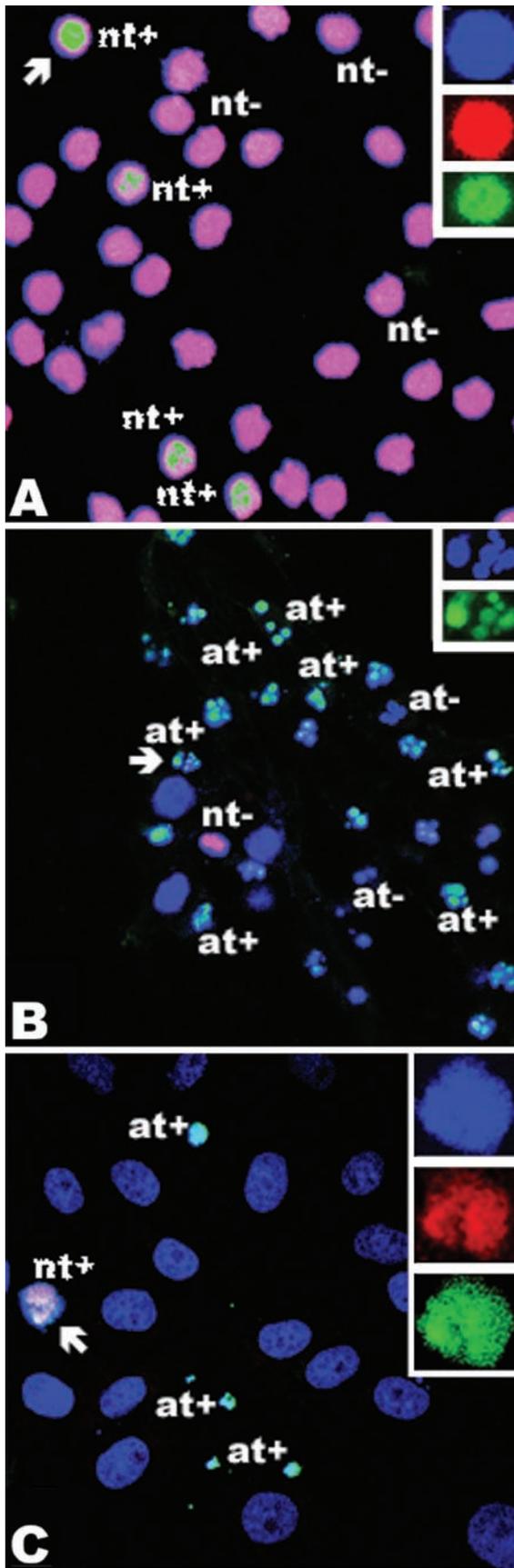


were TUNEL negative, including apoptotic cells with secondary necrosis).

Because images were collected with a confocal microscope, single planes are shown in all Figs. 1–6. To ascertain that different staining did not occur in other planes, we took z sections (0.5 μm apart) of multiple nuclei randomly. This allowed 3-D reconstruction of whole nuclei and confirmed the findings in single

planes. Please refer to the Supplementary Material¹ for this article (published online at the *American Journal of Physiology-Cell Physiology* web site) to view video images of such 3-D nuclei.

¹Supplemental material to this article (video images) is available online at <http://ajpcell.physiology.org/cgi/content/full/284/5/C1309/DC1>.



Effects of fixation and TUNEL staining in other models of injury to LLC-PK cells. We next examined the sensitivity and specificity of TUNEL staining in different models of injury, where the amount of apoptosis and necrosis varied widely. The models included H_2O_2 (a predominantly necrotic injury) or staurosporine (a predominantly apoptotic injury) exposure as well as antimycin A-induced chemical anoxia followed by 24-h recovery (mixed apoptosis and necrosis). In all these models, PI was well retained after fixation with no significant change in the percentage of PI-labeled cells before and after fixation. Figure 3 shows the appearance of necrotic TUNEL-positive cells (false positive, nt+), predominantly in the H_2O_2 -model (A). Thus the specificity of the TUNEL reaction is reduced in this predominantly necrotic model of cell injury. Conversely, the sensitivity of the TUNEL reaction is improved when applied to predominantly apoptotic models of cell death like staurosporine or prolonged recovery from antimycin A exposure (Fig. 3, B and C, respectively). This improved sensitivity was not accompanied by any significant loss of specificity. These data are summarized in Table 1.

Induction of apoptosis and necrosis in tubular cells after renal ischemia in vivo. Rats were given PI and Hoechst intravenously, subjected to renal artery occlusion, and imaged live as described in METHODS. A field from a control nonischemic kidney is shown in Fig. 4A. There is homogeneous staining of nuclei with Hoechst and no PI staining. Fields from ischemic kidneys are shown in Fig. 4, B–D. Many PI-positive necrotic cells are shown (Fig. 4B, inset). The PI uptake in cells resulted in different shades of color (red, pink, and white) that reflect the variable uptake of PI relative to Hoechst and the effect of saturation. Apoptotic cells (Fig. 4C, inset) and apoptotic cells with secondary necrosis (Fig. 4D, inset) were also noted. In addition, extensive tubular damage, abundant cast formation in tubular lumina, and patchy loss of normal architecture were evident after ischemia.

Effects of fixation and TUNEL staining on kidney sections from ischemic rats. As with LLC-PK cells, fixation and TUNEL staining of sections from the ischemic kidneys did not alter the frequency or distribution of PI staining. As shown in Fig. 5, the same variety of nuclear staining was observed, including TUNEL-negative apoptotic cells (A, with inset), TUNEL-negative necrotic cells (C, with inset), TUNEL-positive apoptotic cells (E, with inset), and TUNEL-positive apoptotic cells with secondary necrosis (B, D, and F, with insets). As with LLC-PK cells after chemical anoxia, there was a remarkable absence of TUNEL-positive

Fig. 3. Effects of fixation and TUNEL staining in different models of injury to LLC-PK cells. Representative photomicrographs (20–30 fields per experiment, $n = 3$) are presented of LLC-PK cells subjected to 1 mM H_2O_2 (A), 1 μ M staurosporine (B), or 0.1 μ M antimycin A followed by 24-h recovery (C). Cells were stained with PI and Hoechst 33342, fixed with 4% paraformaldehyde, and then stained with the TUNEL reaction. nt+, necrosis TUNEL positive.

Table 1. The frequency of apoptosis, necrosis and the TUNEL staining in various models of injury to LLC-PK cells

	H ₂ O ₂	Staurosporin	AA (2-h recovery)	AA (24-h recovery)
Total apoptosis	23 ± 6%*	72 ± 7%*	6 ± 1%	12 ± 2%*
TUNEL (+) apoptosis	17 ± 5%	63 ± 7%	4 ± 1%	11 ± 2%
Total Necrosis	34 ± 18%*	11 ± 4%	9 ± 1%	8 ± 4%
TUNEL (+) necrosis	6 ± 4%	0 ± 0%	0 ± 0%	2 ± 2%
Sensitivity	76 ± 7%	87 ± 4%	64 ± 5%	90 ± 4%
Specificity	71 ± 2%	100 ± 0%	99 ± 1%	87 ± 10%

Values are means ± SE and represent percentages with respect to total number of nuclei. LLC-PK cells were treated with 0.1 μM antimycin A (AA) followed by recovery for 2 h (20 to 30 fields, $n = 4$) or 24 h (20 to 30 fields, $n = 3$). Alternatively, cells were treated with 1 mM H₂O₂ for 24 h or 1 μM staurosporin for 24 h (20 to 30 fields, $n = 3$). Apoptotic cells with secondary necrosis were counted as apoptotic. * $P < 0.05$ when total apoptosis or total necrosis in the various conditions is compared to that in AA/2 h. Sensitivity, apoptotic TUNEL-positive nuclei/all apoptotic nuclei; specificity, apoptotic TUNEL-positive nuclei/all TUNEL positive nuclei.

necrotic cells along with the presence of some TUNEL-negative apoptotic cells. Averaging the results from 30 to 40 fields taken from $n = 5$ rats, the overall specificity and sensitivity of the TUNEL assay were 99 ± 1 and $61 \pm 3\%$, respectively. Thus the TUNEL assay shows high specificity but only moderate sensitivity in the detection of apoptosis in this in vivo model of renal ischemia. Of note is the complex and diverse nuclear morphology that exists in tissues. Thus, unlike with cell culture, studying nuclear morphology to determine apoptosis can be a daunting task. This underscores the usefulness of the TUNEL as validated in our experiments.

Effects of fixation and TUNEL staining in a cisplatin model of renal injury in vivo. As with the ischemia model, cisplatin induced a mixture of necrotic and apoptotic injury that could be easily studied in the live animal. Figure 6, A and B, shows examples of apoptotic (A, inset) and necrotic (B, inset) cells. After fixation, the percentage of PI-positive cells was not significantly affected by fixation. When the TUNEL stain was applied to the fixed tissues (Fig. 6, C and D), apoptotic TUNEL-positive (C, inset) and necrotic TUNEL-negative nuclei (D, inset) could be identified. TUNEL-negative apoptotic nuclei were also observed. Overall, the specificity and sensitivity of the TUNEL were 98 ± 5 and $87 \pm 3\%$, respectively.

DISCUSSION

The quantitation of apoptosis in solid tissues remains a very challenging problem. This is due in part to the short half-life of apoptotic cells but also to the lack of a reliable and quantitative method for identifying these cells. Routine light and electron microscopic examination remain one gold standard for identifying the fingerprint of an apoptotic cell. However, both techniques fail when a high throughput and quantitative count of apoptosis is needed. The simultaneous staining with Hoechst and PI takes advantage of the exclusion of the latter from normal or early apoptotic cells with intact plasma membranes. Hoechst staining is then examined to determine the nuclear morphology by fluorescence microscopy. This approach is very powerful but necessitates live cells and can be applied to

tissues only under in vivo conditions (19, 22). This requires the use of two-photon imaging, an emerging technique not yet widely available (8).

The TUNEL reaction is the most widely used technique to quantitatively study apoptosis in fixed tissues. However, it remains plagued with criticism regarding its specificity (3, 11, 12, 26). The nonspecific activation of endonucleases during necrosis is thought to generate free DNA ends that could possibly result in a TUNEL-positive reading. Still, this issue has not been examined systematically and relies mostly on electron microscopic examination of only a few cells in a given field. Furthermore, the issues of secondary necrosis and non-caspase-mediated apoptosis have not been excluded (7, 18, 23). Finally, the electron microscopic features of apoptotic nuclei fall in a spectrum that depends on the insult and the specific apoptotic pathway utilized by the cells (7, 10). This makes the identification of these apoptotic cells by electron microscopy less certain than previously assumed. Therefore, a thorough assessment of the specificity of the TUNEL reaction is still lacking.

We have validated the novel use of PI and the TUNEL reaction in a sequential way. We have utilized simple operational definitions of necrosis and apoptosis that rely on the integrity of the plasma membrane and on nuclear morphology. A similar technique utilizing trypan blue and the TUNEL assay was previously reported (20). However, in that report, there was no validation of the criteria of necrosis and apoptosis by nuclear morphology, as we have documented extensively. More importantly, ours is the first demonstration of the applicability of this method to live animals. Indeed, our study shows that PI, when delivered to tissues in vivo, is a marker of necrotic cells that is retained very well after fixation. The administration of PI to live animals was well tolerated by the animals and has been reported by others (14). The application of the TUNEL reaction to these tissues after fixation provides a means by which necrotic, PI-positive cells are easily identified and possibly excluded from the TUNEL-positive count.

As noted in RESULTS, the TUNEL reaction showed a very high degree of specificity, exceeding 87% in all injury models except H₂O₂. Indeed, our systemic exam-

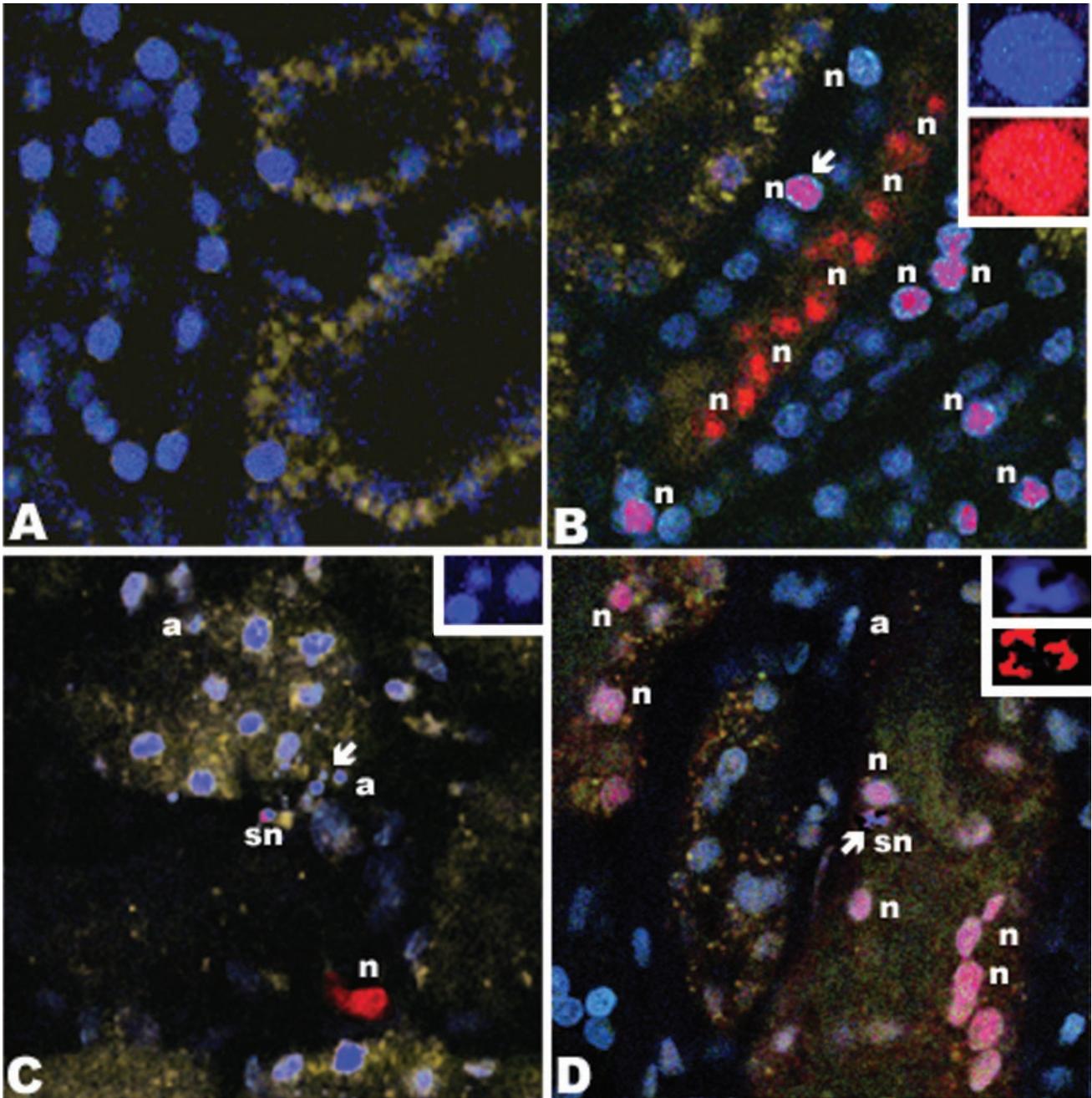
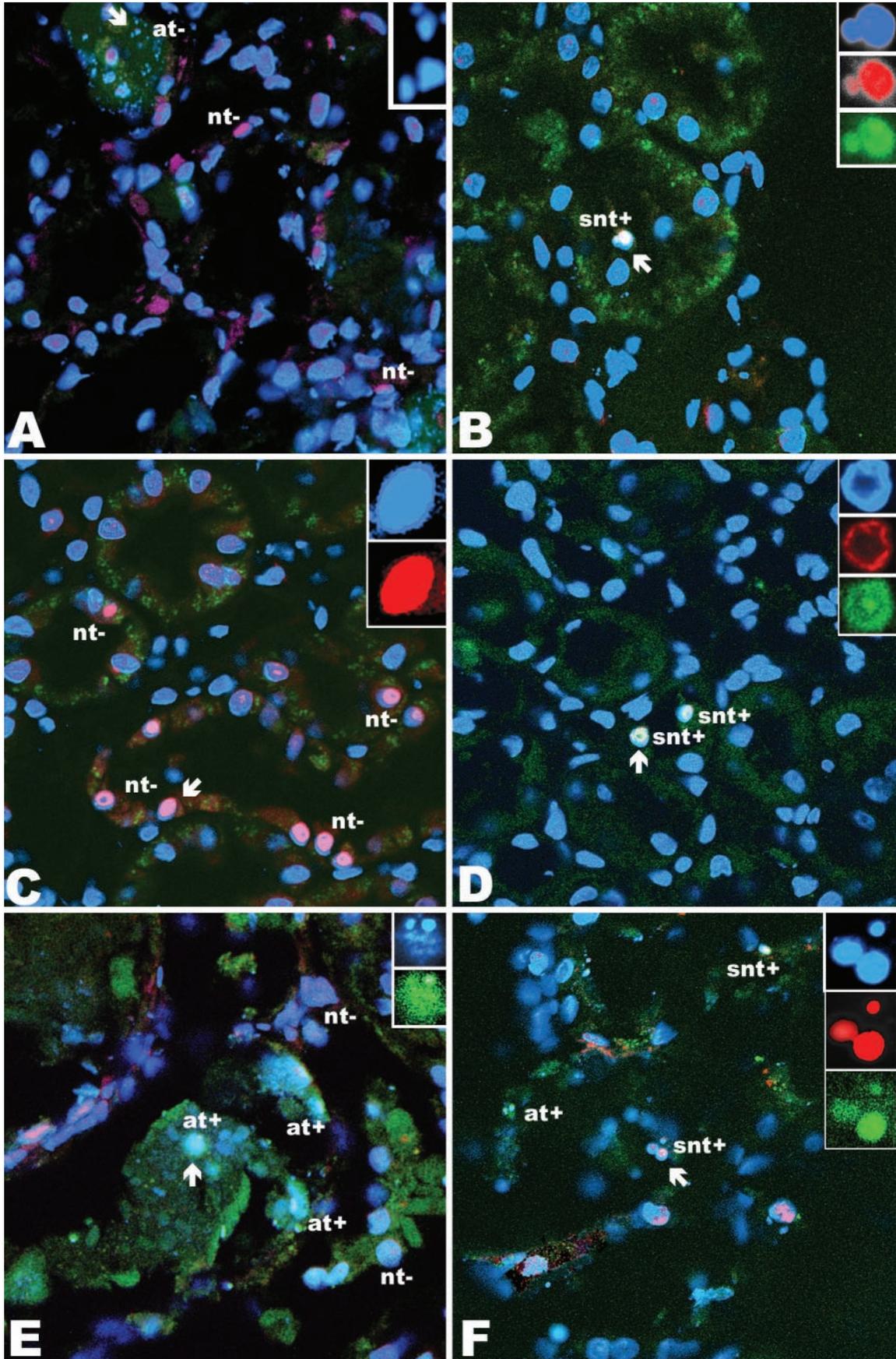


Fig. 4. Induction of apoptosis and necrosis after renal ischemia in vivo. Representative 2-photon images of kidneys from live animals obtained 24 h after renal ischemia and administration of PI and Hoechst are shown. A field from a control nonischemic kidney is shown in *A*. No PI staining is observed. In ischemic kidneys, necrosis is evident in many cells, demonstrating the uptake of both PI and Hoechst (*B*, with inset; *C* and *D*) with the final color of the nucleus determined by the relative saturation of each. Red necrotic nuclei are shown in tubular lumina, where they may be inaccessible to the Hoechst given immediately before imaging. In addition, cells with nuclear morphology consistent with apoptosis are shown (*C*, with inset, and *D*). Many of these apoptotic cells demonstrated PI uptake consistent with secondary necrosis (*D*, with inset, and *C*). Fields shown are representative of ~30–40 fields per experiment ($n = 5$).

ination of nuclear morphology and PI uptake strongly supports our conclusions. Thus all TUNEL-positive cells counted as true positives had condensed and fragmented nuclei by fluorescence microscopy, the telltale of apoptosis. A few of them also showed some PI uptake, and these were clearly apoptotic cells undergoing terminal secondary necrosis. We believe these apop-

totic cells with secondary necrosis to be the basis (at least in part) for the confusion regarding the specificity of the TUNEL assay. However, staining with Hoechst clearly reveals the apoptotic morphology of their nuclei, which is quite different from that of primary necrotic cells. This high specificity of the TUNEL assay confirms and supports our previous electron micro-



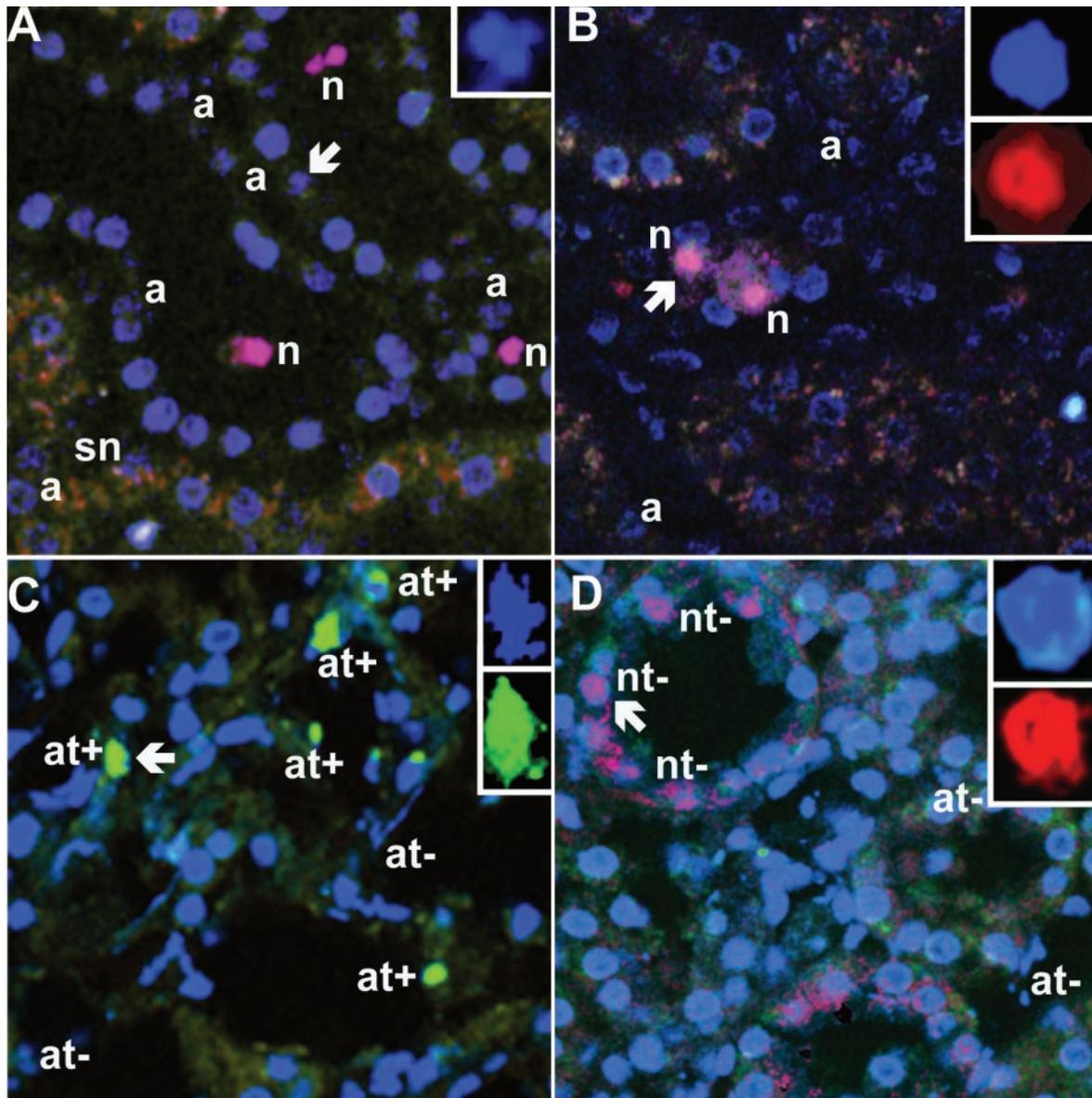


Fig. 6. Induction of apoptosis and necrosis in a cisplatin model of renal injury. Animals were treated with cisplatin along with PI and Hoechst 33342 injections as detailed in METHODS. The kidney was imaged in the live animal with 2-photon microscopy (A and B). As with the ischemia model, a mixture of apoptosis, apoptosis with secondary necrosis, and primary necrosis can be seen. Insets show examples of apoptosis (A) and necrosis (B). Kidneys were then fixed and stained with the TUNEL reagent. As shown in D, PI was discretely retained in individual cells. C and D also show the presence of TUNEL-positive (C, inset) and TUNEL-negative apoptotic cells. Fields shown are representative of ~30–40 fields per experiment ($n = 2$).

scopic analysis of TUNEL-positive nuclei revealing their apoptotic features at high resolution (15).

Finally, our method revealed the presence of apoptotic cells that failed to stain with the TUNEL. Thus the TUNEL reaction does lack some sensitivity and, as such, can underestimate apoptosis. The lack of high

sensitivity could be related to the specific TUNEL reaction utilized, given that various commercial kits are available (5, 15, 25). It could also be due to subtle differences in DNA degradation among various apoptotic cells or the initiation of different apoptotic pathways in different models of injury (4, 7, 10). The

Fig. 5. Effect of fixation and TUNEL staining on PI staining in postischemic kidneys. Representative sections taken 24 h after ischemia and administration of PI are shown. Sections were fixed in 4% paraformaldehyde and then subjected to the TUNEL reaction. PI staining of necrotic cells was retained after fixation and TUNEL staining (A, C, and E). Cells with nuclear morphology consistent with apoptosis were both TUNEL negative (A, with inset) and TUNEL positive (B, D, E, and F, with insets), and many demonstrated secondary necrosis (B, D, and F, with insets). Fields shown are representative of ~30–40 fields per experiment ($n = 5$).

method of sequential application of PI and TUNEL reagent can easily be used in other models of cell death and apoptosis. Therefore, the sensitivity and specificity of the TUNEL should be determined in the particular tissues and specific conditions under investigation.

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