

Monitoring Apoptosis with Fluorescent Zn²⁺-Indicators

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Abstract

Apoptosis, a mechanism of programmed cell death that removes superfluous and harmful cells, is important both during development and in tissue homeostasis. Although Zn^{2+} is believed to be critical in apoptosis, the precise details of its role have yet to be elucidated. The macrocyclic Zn^{2+} ligand dansylamidoethylcyclen [$L^1 \cdot (HCl)_4 \cdot (H_2O)_2$], which is found primarily in a diprotonated form (H_2L^1), is cell-permeable and forms a strongly fluorescent 1:1 Zn^{2+} complex when Zn^{2+} entry into cells is facilitated by the Zn^{2+} ionophore pyrithione. H_2L^1 can be used to readily identify HeLa cells undergoing the early stages of etoposide-induced apoptosis because of the increased level of free Zn^{2+} that occurs at this time. The selectivity of H_2L^1 for the detection of apoptotic cells was verified by a conventional probe for apoptosis, annexin V-Cy3. Here, we describe methods for detecting apoptotic cells with H_2L^1 and for comparing detection of apoptosis with H_2L^1 to detection with annexin V-Cy3 and Zinquin.

Introduction

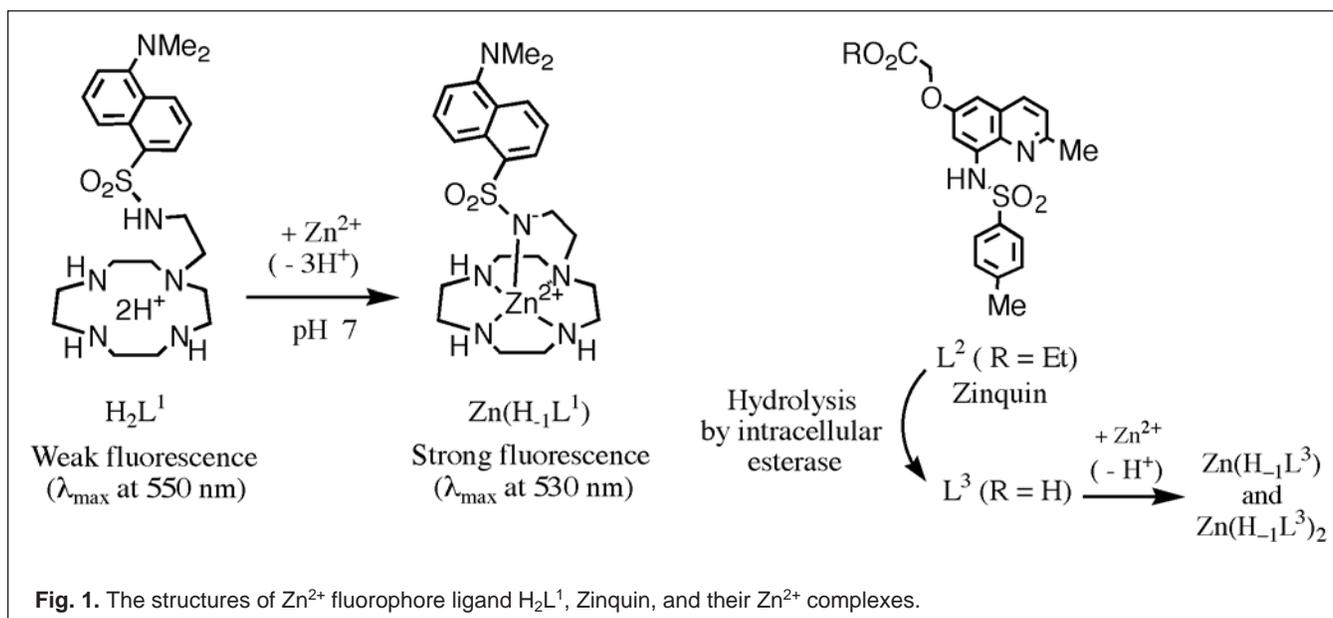
Apoptosis, a type of cell death that is distinctly different from necrosis, involves a unique series of morphologic changes, including cell shrinkage and budding of the cell contents into membrane-enclosed vesicles (blebbing) (1–5). Apoptosis is a normal mechanism for eliminating excess cells during development, or it can be triggered by various agents, including members of the tumor necrosis factor family, cytotoxic lymphocyte granules, or anticancer drugs such as cisplatin and etoposide.

Apoptotic cell death can be detected in several ways. One method involves visualizing DNA fragmentation in apoptotic cells by agarose gel electrophoresis (6); however, this biochemical method does not allow early apoptotic processes to be monitored and is not suitable for monitoring individual cells undergoing apoptosis. Another method utilizes fluorescently labeled annexin (7) to detect the loss of membrane phospholipid asymmetry that occurs during apoptosis. Annexin has a high affinity for phosphatidylserine, which is exposed on the cell surface of apoptotic cells. However, this method is not selective for cells undergoing apoptotic cell death; annexin labels cells that have died by necrosis as well as apoptosis. Moreover, annexin-staining does not reflect intracellular events.

Changes in the free Zn^{2+} concentration are an important part of the early apoptotic process (8–12). However, it is not clear whether the changes in free Zn^{2+} are a cause or a consequence of apoptosis. Release of Zn^{2+} from endonucleases such as DNase γ (13) in early apoptosis might facilitate DNA fragmentation, so that changes in intracellular Zn^{2+} would contribute to causing apoptosis.

Several cell-permeable Zn^{2+} -sensitive fluorescent probes, such as Zinquin [as the ethyl ester (L^2)] (9, 10), have been described (14–19) (Fig. 1). Zinquin (L^2) is a bidentate ligand, and its hydrolyzed form (L^3) yields a mixture of 1:1 complex (ZnL^3) and 2:1 complex [$Zn(L^3)_2$] under physiologic conditions, which may disturb quantification of Zn^{2+} concentration. Dansylamidoethylcyclen ($L^1 \cdot (HCl)_4 \cdot (H_2O)_2$), a macrocyclic tetra-amine equipped with a dansyl group, which exists as a diprotonated form (H_2L^1) at neutral pH, is a selective fluorescent Zn^{2+} indicator (13, 20–23) that forms a 1:1 Zn^{2+} complex [$Zn(H_1L^1)$] at physiological pH. The emission of H_2L^1 increases by 4.8-fold at 540 nm and by 10-fold at 490 nm (Fig. 1A) through formation of $Zn(H_1L^1)$ with a coordination bond between the deprotonated dansylamide anion and Zn^{2+} . In contrast, the fluorescence emission intensity of the Zn^{2+} -free deprotonated form of L^1 (H_1L^1) at high pH is only 1.2-fold in the absence of Zn^{2+} (20). The dissociation constant of $Zn(H_1L^1)$ is very low ($K_d = 1.4 \times 10^{-10}$ M at pH 7.0 and 5.5×10^{-13} M at pH 7.8), and the detection limit of [Zn^{2+}] by H_2L^1 is 20 nM (20, 22).

Recently, we discovered that the Zn^{2+} fluorophore H_2L^1 is a selective and efficient sensor of apoptosis in cancer cells (24). We compared its utility in detecting apoptosis to that of Zinquin (L^2) and a commercial kit containing annexin V-Cy3. Here, we describe protocols for detecting apoptotic cells with H_2L^1 either alone or in comparison to Zinquin or annexin V-Cy3 or both. The annexin V-Cy3 procedure requires double staining with a vital dye that stains only dead cells to distinguish apoptosis from necrosis because annexin V stains secondary necrotic cells at late stages as well as apoptotic cells at early stages. In contrast, H_2L^1 can be used alone for detection of apoptosis, since it stains only apoptotic cells and does not stain necrotic cells. It should also be noted that emission from $Zn(H_1L^1)$ is more stable than that of Zn^{2+} -Zinquin complexes. Fluorescent probes such as H_2L^1 may also provide a way to investigate the role of Zn^{2+} flux in the apoptotic process.



Materials

Cell Culture Supplies

- 35-mm culture dishes
- 75-cm² cell culture flasks
- Glass cover slips (no coating)
- Human cervix epitheloid carcinoma cells (HeLa cells, Cell Resource Center for Biomedical Research, Tohoku University)

Cell Culture Reagents

- Dulbecco's Modified Eagle Medium (DMEM; Life Technologies)
- Fetal bovine serum (FBS; Life Technologies)
- Hanks' Balanced Salt Solution (HBSS; Life Technologies)
- Penicillin-Streptomycin, liquid (GIBCO #15140-122)
- Phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄•12H₂O, 1.5 mM KH₂PO₄)

Reagents for Imaging

- 2-Mercaptopyridine *N*-oxide sodium salt (pyrithione) (Aldrich, #63844)
- Annexin V-Cy3 Apoptosis Detection Kit Plus (BioVision, #K120-25)
- Dansylamidoethylcyclen as L¹•(HCl)₄•(H₂O)₂ [Dojindo Laboratories, #D480, Kumamoto, Japan (<http://www.dojindo.co.jp>)]
- Dimethylsulfoxide (DMSO)
- Etoposide (Sigma-Aldrich, #E1383)
- Propidium iodide (PI) (Aldrich, #28707-5)
- Zinquin as the ethyl ester [Dojindo Laboratories, #Z215, Kumamoto, Japan (<http://www.dojindo.co.jp>)]

Zn²⁺-free deionized and redistilled water

Note: Deionized and redistilled water can usually be assumed zinc-free without further treatment.

ZnSO₄•7H₂O (Kanto Chemical, #48056-30)

Equipment

Inverted fluorescence microscope equipped with 150-W Xenon arc lamp and dichroic mirrors.

Standard cell culture equipment: Laminar flow hood, humidified incubator at 37°C, 5% CO₂

Recipes

Recipe 1: Cell Culture Medium

<i>Reagent</i>	<i>Amount</i>	<i>Final Concentration</i>
FBS	50 ml	10% (v/v)
Penicillin-Streptomycin, liquid	5 ml	Penicillin 100 U/ml, Streptomycin 100 µg/ml

Add these three reagents to 500 ml of DMEM under sterile conditions and store at 4°C; incubate at 37°C for 30 min immediately before use.

Recipe 2: Zn²⁺-Treatment Solution

<i>Reagent</i>	<i>Amount</i>	<i>Final Concentration</i>
ZnSO ₄ •7H ₂ O, 25 mM	10 µl	25 µM
Pyrithione, 20 mM in DMSO	10 µl	20 µM

Prepare in 10 ml of HBSS with DMSO (containing 0.1% DMSO). Use immediately.

Recipe 3: Zn²⁺-Detection Solution 1

Add 3.2 mg of L¹•(HCl)₄•(H₂O)₂ to 5 ml of HBSS for a final concentration of 100 µM. Store at 4°C.

Recipe 4: Zinquin Stock

Dissolve 1 mg of Zinquin in 500 µl of DMSO for a final concentration of 5 mM. Store at -20°C for 1 week.

Recipe 5: Zn²⁺-Detection Solution 2

Add 50 µl of Zinquin Stock (Recipe 5) to 5 ml HBSS to obtain a final concentration of 50 µM. Use immediately.

Recipe 6: Etoposide Solution

Add 20 µl of 25 mM etoposide to 5 ml of Cell Culture Medium (Recipe 1) for a final concentration of 100 µM. Use immediately.

Recipe 7: Apoptosis-Detection Solution 1

Reagent	Amount	Final Concentration
L ¹ •(HCl) ₄ •(H ₂ O) ₂	3.2 mg	100 μM
PI, 10 mM	15 μl	30 μM

Add to 5 ml of HBSS. Store at 4°C.

Recipe 8: Apoptosis-Detection Solution 2

Reagent	Amount	Final Concentration
L ¹ •(HCl) ₄ •(H ₂ O) ₂	3.2 mg	100 μM
PI	1.0 mg	30 μM
Annexin V-Cy3	50 μl	1:100

Prepare in 5 ml of the binding buffer included in the Annexin V-Cy3 kit. Use immediately.

Instructions

We include instructions for three procedures. The first describes how to detect increases in [Zn²⁺]_i with zinc fluorophores in the presence of a zinc ionophore and serves as a control to confirm that, under your conditions, H₂L¹ and Zinquin specifically detect increases in [Zn²⁺]_i. If this is the case, the two fluorophores will show enhanced fluorescence following exposure to the ionophore. The second procedure describes how to use H₂L¹ to detect the early stages of apoptosis in HeLa cells and illustrates the changes in morphology that take place during apoptosis. The third procedure describes how to compare staining obtained with H₂L¹ to that obtained with annexin V-Cy3. This procedure can be used to confirm that H₂L¹ stains apoptotic cells at earlier stages than annexin-V and does not stain dead cells. These procedures may be useful to study the role of Zn²⁺ in apoptotic processes (25) and to observe time-dependent events in apoptotic cells.

Staining Intact, Zn²⁺ Ionophore-Treated Cells with H2L1 or Zinquin

This procedure, which describes how to detect increases in [Zn²⁺]_i with either H₂L¹ or Zinquin in response to exposure of cells to the zinc ionophore pyrithione, serves as a control to confirm specificity of the fluorophores in detecting changes in [Zn²⁺]_i (Fig. 2). It is assumed that the protonated form (H₂L¹) is cell membrane-permeable at neutral pH and forms a thermodynamically and kinetically stable 1:1 Zn²⁺ complex [Zn(H₁L¹)], which emits an enhanced blue-shifted fluorescence.

1. Suspend 0.5×10^7 HeLa cells in 10 ml of Cell Culture Medium (Recipe 1).
2. Add 1 ml of cell suspension (0.5×10^6 cells/ml) to a 35-mm cell culture dish containing a coverslip.
3. Incubate dishes at 37°C, 5% CO₂ overnight.
4. Replace medium with 1 ml of Zn²⁺-Treatment Solution (Recipe 2).
5. Incubate for 10 min at 37°C, 5% CO₂.
6. Rinse three times with 1 ml of HBSS to remove extracellular Zn²⁺.
7. Replace medium with 1 ml of Zn²⁺-Detection Solution 1 (Recipe 3) and incubate for 30 min at 37°C, 5% CO₂.

Note: To compare fluorescence obtained with H2L1 to that obtained with Zinquin, Zn²⁺-Detection Solution 2 (Recipe 5) can be used in place of Zn²⁺-Detection Solution 1 (Recipe 3).

8. Rinse once with 1 ml of PBS.
9. Observe the cells by phase contrast and UV fluorescence microscopy (excitation at 330 to 385 nm, emission at 500 nm).

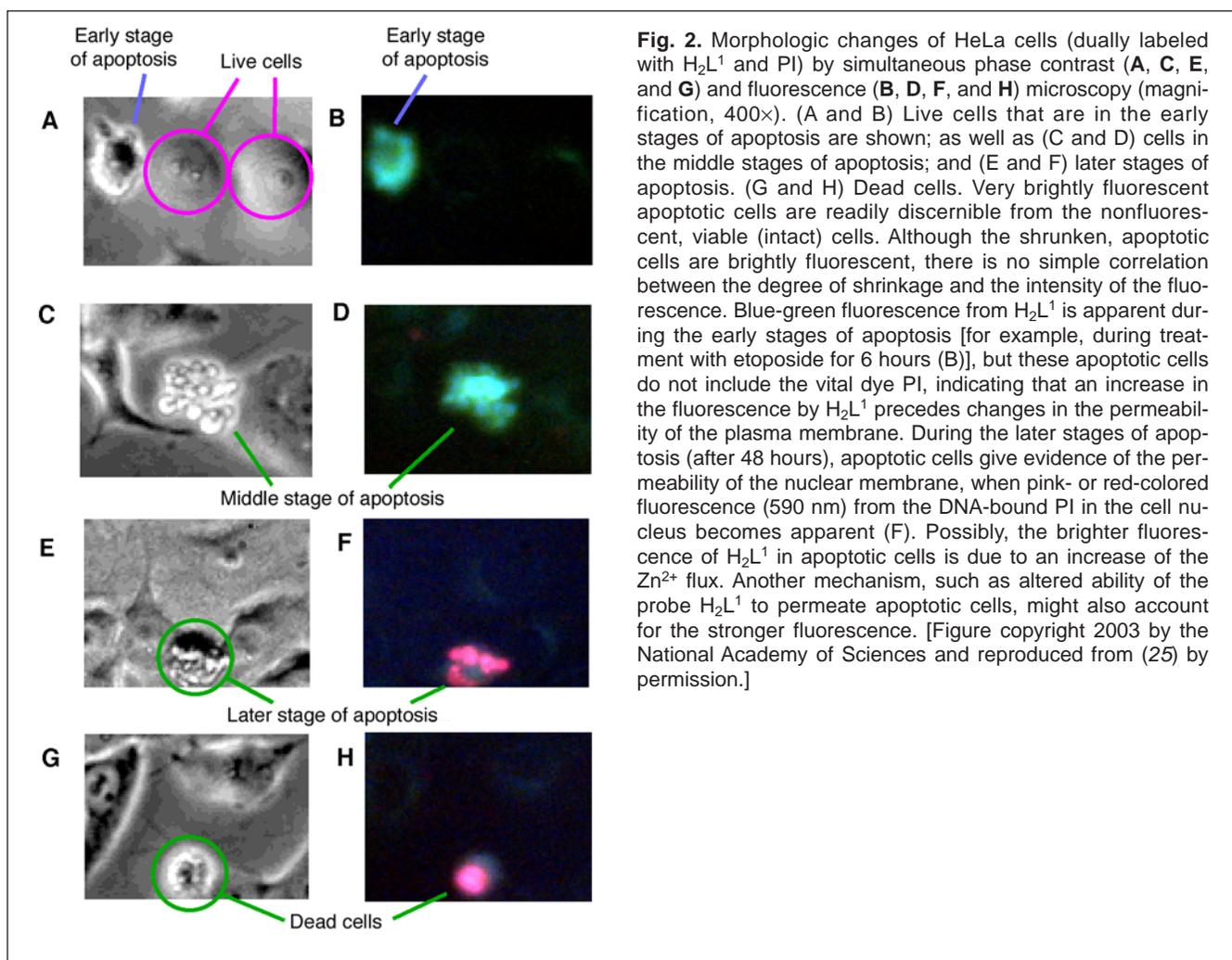


Fig. 2. Morphologic changes of HeLa cells (dually labeled with H₂L¹ and PI) by simultaneous phase contrast (A, C, E, and G) and fluorescence (B, D, F, and H) microscopy (magnification, 400×). (A and B) Live cells that are in the early stages of apoptosis are shown; as well as (C and D) cells in the middle stages of apoptosis; and (E and F) later stages of apoptosis. (G and H) Dead cells. Very brightly fluorescent apoptotic cells are readily discernible from the nonfluorescent, viable (intact) cells. Although the shrunken, apoptotic cells are brightly fluorescent, there is no simple correlation between the degree of shrinkage and the intensity of the fluorescence. Blue-green fluorescence from H₂L¹ is apparent during the early stages of apoptosis [for example, during treatment with etoposide for 6 hours (B)], but these apoptotic cells do not include the vital dye PI, indicating that an increase in the fluorescence by H₂L¹ precedes changes in the permeability of the plasma membrane. During the later stages of apoptosis (after 48 hours), apoptotic cells give evidence of the permeability of the nuclear membrane, when pink- or red-colored fluorescence (590 nm) from the DNA-bound PI in the cell nucleus becomes apparent (F). Possibly, the brighter fluorescence of H₂L¹ in apoptotic cells is due to an increase of the Zn²⁺ flux. Another mechanism, such as altered ability of the probe H₂L¹ to permeate apoptotic cells, might also account for the stronger fluorescence. [Figure copyright 2003 by the National Academy of Sciences and reproduced from (25) by permission.]

Observing Apoptotic Morphology in HeLa Cells and Detecting Apoptosis with H2L1

The morphologic features of apoptosis that are apparent by phase microscopy include blebbing of membrane, decrease of cell volume, and presence of pyknotic (shrunken) nuclei. Most cells show marked shrinkage ($36 \pm 6\%$) at 48 hours, and all dying cells shrink after longer periods. Here, apoptosis is induced by the DNA-damaging agent etoposide and detected by H₂L¹. This procedure provides a means of detecting early stages of apoptosis with a single fluorophore and of comparing these changes in fluorescence with the various morphological changes typical of the apoptotic response.

1. Suspend 0.5×10^7 HeLa cells in 10 ml of Cell Culture Medium (Recipe 1).
2. Add 1 ml of cell suspension (0.5×10^6 cells/ml) to a 35-mm cell culture dish containing a coverslip.
3. Incubate dishes at 37°C, 5% CO₂ overnight.
4. Replace medium with 1 ml of Etoposide Solution (Recipe 6).
5. Place dishes in an incubator at 37°C, 5% CO₂ for 6 to 48 hours.
6. Replace medium with 1 ml of Apoptosis-Detection Solution I (Recipe 7).
7. Incubate the cells at 37°C, 5% CO₂ for 30 min.
8. Rinse once with 1 ml of PBS.
9. Observe the cells using both phase contrast and UV fluorescence microscopy (excitation at 330 to 385 nm, emission at 500 nm for H₂L¹; excitation at 460 to 490 nm, emission at 590 nm for PI) (Fig. 2).

Triple Staining with H₂L¹, Annexin V-Cy3, and PI

Detection of apoptosis by H₂L¹ can be compared with detection by annexin V-Cy3 (7), which correlates with apoptotic nuclear morphology and DNA fragmentation and relies on a different chemical principle to stain apoptotic cells. In this procedure, cells are induced to undergo apoptosis by etoposide, then apoptotic cells are detected with both annexin V-Cy3 and H₂L¹. Detection of apoptosis with annexin V-Cy3 requires double staining with a vital dye such as PI to distinguish apoptosis from necrosis, because annexin V binds to dead cells as well as apoptotic ones. In contrast, H₂L¹ can be used alone. Moreover, the 1:1 complex of H₂L¹ with Zn²⁺ [Zn(H₂L¹)] is membrane impermeable, and its emission remains detectable for several hours.

1. Suspend 0.5×10^7 HeLa cells in 10 ml of Cell Culture Medium (Recipe 1).
2. Add 1 ml of cell suspension (0.5×10^6 cells/ml) to a 35-mm cell culture dish containing a coverslip.
3. Place dishes at 37°C, 5% CO₂ overnight.
4. Replace medium with 1 ml of Etoposide Solution (Recipe 6).
5. Place dishes in an incubator at 37°C, 5% CO₂ for 6 to 48 hours.
6. Replace medium with 1 ml of Apoptosis-Detection Solution 2 (Recipe 8).
7. Stain for 1 hour at 37°C, 5% CO₂.
8. Observe the cells by both phase contrast and UV fluorescence microscopy (excitation at 330 to 385 nm, emission at 500 nm for H₂L¹; excitation at 460 to 490 nm, emission at 590 nm for PI and annexin V-Cy3) (Fig. 3).

Troubleshooting

Incubation times longer than 36 hours in the presence of etoposide may cause cells to detach from coverslips. Morphology of the cells should be checked occasionally to verify that they remain attached to the coverslip.

Notes and Remarks

Although prolonged exposure (more than 48 hours) to H₂L¹ (100 μM) alone causes apoptosis, a short exposure (for instance, 2 to 3 hours) does not cause substantial damage to HeLa cells.

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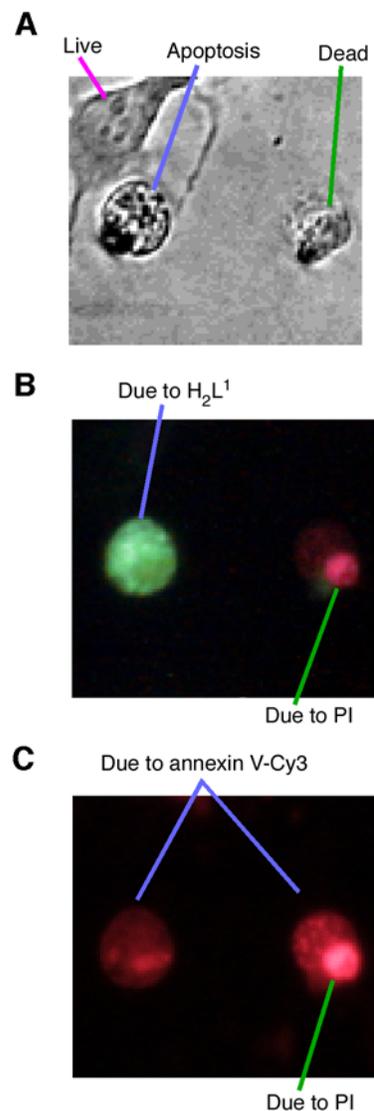


Fig. 3. (A) Phase contrast image, (B) merged fluorescent image by irradiation with UV (330 to 385 nm) and visible light (460 to 490 nm), and (C) fluorescent image by irradiation with visible light (460 to 490 nm) of apoptotic HeLa cells triply stained with H₂L¹, annexin V-Cy3, and PI. In (B), blue-green fluorescent apoptotic cells are stained by H₂L¹ (irradiated at 330–385 nm) and red-fluorescent dead cells are stained by PI (irradiated at 460–490 nm). In (C), red-fluorescent apoptotic cells are stained by annexin V-Cy3 with red-fluorescent dead cells stained by PI. The same apoptotic cells were stained by annexin V-Cy3 and by H₂L¹. [Figure copyright 2003 by the National Academy of Sciences and reproduced from (25) by permission.]

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