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Editorial

Analysis of apoptosis methods recently used in *Cancer Research* and *Cell Death & Disease* publications

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Over several decades, significant advances have been made in implementing several morphological and biochemical criteria to define and characterize apoptosis. In order to appropriately identify apoptosis in cellular cultures or *in vivo* (animal models), with the ultimate aim of discovering novel, useful, specific, and powerful pro-apoptotic antitumoral drugs, it is necessary to accurately validate apoptotic processes through morphological, biochemical, or immunological methods.

Often, apoptosis is detected and/or measured by only one method and the authors frequently use imprecise terminology, such as '% apoptosis', '% cell death', instead of defining the specific method used such as '% cells with condensed chromatin', '% cells TUNEL positive', '% cells Annexin V positive' and others.[1] The Nomenclature Committee on Cell Death (NCCD) recommends and encourages researchers to demonstrate that apoptosis or other forms of cell death take place using more than one assay, as an artifact removal feature, and to avoid the 'confusing and imprecise' nomenclature, such as '% apoptosis', which 'should definitively be abandoned'.[1] Moreover, the NCCD 'urges' all life science journals to join the Cell Death and Differentiation journal in adopting these recommendations.[1]

In order to determine the level of compliance with these recommendations, we analyzed 110 *Cancer Research* articles that detect/measure apoptosis, published between August 15, 2010 and February 15, 2011, and 120 similar research articles published in *Cell Death & Disease* between January 1, 2010 and September 2011. In 21 of the *Cancer Research* and 15 of the *Cell Death & Disease* articles, apoptosis was determined by the authors for different treatments or in different settings using a different combination of apoptosis techniques each time, thus leading the total number of apoptotic determinations (entries) to 137 (*Cancer Research*) and 136 (*Cell Death & Disease*) (see Table 1 and Table 2). The results clearly show that in only 60 of the 137 entries (43.8%) for *Cancer Research* and 96 of the 136 entries (70.58%) for *Cell Death & Disease*, the authors used at least two different methods for apoptosis detection. In addition, we determined that in 5 of the 137 (3.64%) entries from *Cancer Research* and in 34 of the 136 (25%) of the entries from *Cell Death & Disease* the authors use at least three methods for apoptosis detection (Figure 1). Moreover, it is important to emphasize that most, if not all of the apoptosis techniques used to detect apoptosis as single methods may, as shown by some reports, also detect necrosis or other cellular processes. As an example, DNA fragmentation measured by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) (26 of 137 from *Cancer Research*; 26 of 136 from *Cell Death & Disease*) or subG1 DNA content (30 of 137 from *Cancer Research*; 18 of 136 from *Cell Death & Disease*) or Annexin V alone, without PI/7AAD (16 of 137 from *Cancer Research*; 7 of 136 from *Cell Death & Disease)* and other assays can also detect necrosis.[1–6] In addition, caspases can be activated during cellular processes other than apoptosis.[1] Thus, more than one method for apoptosis detection should be used.

In order to investigate the proper use of the specific name for each method used, we analyzed the same entries described above. Our analysis shows similar results for the two journals. While for *Cell Death & Disease*, 83 from 136 entries (61.02%) use the name of the specific method used for apoptosis detection, among the 137 entries from *Cancer Research*, 87 (63.5%) use the name of the specific method used (Figure 1). These results certainly leave space for improvement.

In conclusion, these results clearly suggest the need for improvements and of adequate guidelines for authors, reviewers, and editors regarding the apoptosis (in particular) and cell death (in general) detection, and quantification methods.

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Table 1 Evaluation of the *Cancer Research* experimental articles measuring apoptosis, published between August 15, 2010 and February 15, 2011[7–116]

2009 NCCD recommendations	% from 137 entries	Details/comments
Use the specific name of the apoptosis method (A)	87/137 = 63.5%	36.5% of all entries use expressions such as '% apoptosis', '% apoptotic cells', 'relative apoptotic cells (fold change)', '% death' and others, instead of indicating the specific method
At least two methods for apoptosis detection (B)	60/137 = 43.8%	Amongst 77/137 entries only one method is used: 26/79–caspase cleavage/activation; 3/79 only PARP cleavage; 31/79 DNA fragmentation/nuclear condensation; 15/79 TUNEL; 1/79 TUNEL and ELISA; 8/79 subG1; 2/79 subG1 and ELISA; 2/79 subG1 and Hoechst; 1/79 Hoechst; 1/79 Yo-Pro-1; 9/79 Annexin V only, 6/79 Annexin V with PI/7-AAD, 1/79 Citokeratin-18 cleavage; 1/79 only Trypan blue exclusion and clonogenic assay; 1/79 only Calcein/ethidium bromide staining.
Methods		
Caspase activation/Caspase cleavage/ PARP cleavage (1)	84/137 = 61.13%	11/84 PARP cleavage only (without examining caspase activation)
DNA fragmentation/nuclear condensation (2)	66/137 = 48.17%	30/66 subG1; 26/66 TUNEL; 7/66 ELISA-nucleosomal fragmentation/release; 9/66 Hoechst 33342/33258, DAPI, Yo-Pro-1 (condensed/ fragmented chromatin)
Plasma membrane integrity/PS exposure (3)	42/137 = 30.65%	16/42 of entries examine Annexin V positive cells only (not combined with PI/7AAD)
Activation of pro-apoptotic Bcl-2 family members (4)	1/137 = 0.7%	1/6 shows Bax accumulation in the mitochondria; other 5/137 show only total levels of the pro/ anti-apoptotic members
Mitochondrial potential/integrity, release of pro-apoptotic factors (5)	6/137 = 4.38%	3/6 measure MMP and cyt c release together; 1/6 detect MMP only; 2/6 detect the Apo2.7 early apoptotic marker
ROS detection (6)	4/137 = 2.92%	4/4 DCFDA measurement of ROS
Other apoptotic features (blebbing/ floating cells etc) (7)	2/137 = 1.45%	2/2 counting of floating cells (not specific for apoptosis)
No. of entries using methods (1) and (2)	32/137 = 21.89%	15/30 subG1+activation/cleavage of caspases/ PARP (method 1); 10/30 TUNEL+method 1
No. of entries using methods (1) and (3)	24/137 = 17.51%	21/24 Annexin V/PI(or 7-AAD)+method 1; 3/87 Annexin V only (without PI/7-AAD)+method 1
No. of entries using methods (2) and (3)	6/137 = 4.38%	3/6 - subG1+Annexin V/with or without PI (or 7-AAD); 3/6 Hoechst/DAPI+Annexin V with/ without PI or 7-AAD)
No. of entries using at least three of the considered methods	5/137 = 3.65%	Combinations of methods used: 1+2+3; 1+3+5; 1+3+6; 2+4+5+6; 1+2+5+7

Abbreviations: AAD, 7-amino-actinomycin D; Cyt *c*, cytochrome *c*; DAPI, 4, 6-diamidino-2-phenylindole; DCFDA, dichlorofluorescin diacetate; MMP, mitochondrial membrane potential; PARP, poly(ADP ribose) polymerase 1; PI, propidium iodide; PS, phosphatidylserine; ROS, reactive oxygen species; TUNEL, terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling. Note: A higher number of articles were published in *Cancer Research* between August 15, 2010 and February 15, 2011; however, they measured other non-apoptotic cellular processes: non-apoptotic cell death and signaling, mitotic catastrophe, autophagy, proliferation, metastasis, and others[117–162]

Table 2 Evaluation of the *Cell Death & Disease* experimental articles measuring apoptosis, published between January 1, 2010 and September, 2011[163–282]

2009 NCCD recommendations	% from 136 entries	Details/comments
Use the specific name of the apoptotic method (A)	83/136 = 61.03%	38.97% of all entries use expressions such as '% apoptosis', '% apoptotic cells', 'relative apoptotic cells (fold change)', '% death' and others, instead of mentioning the specific method
At least two methods for apoptosis detection (B)	96/136 = 70.58%	40/136 entries use only one method for apoptosis detection: 14/40 caspases cleavage/activation; 1/40 only PARP cleavage detection; 12/40 DNA fragmentation/nuclear condensation (5/40 TUNEL; 3/40 subG1; 3/40 Hoechst; 1/40 nucleosomal fragmentation/release); 10/40 AnnexinV+/−PI/7AAD or To-Pro-3; 2/40–MMP; 1/40 PI only
Methods		
Caspase activation/caspase cleavage/PARP cleavage (1)	94/136 = 69.11%	7/94–PARP cleavage detection only (without examining caspase activation/cleavage); 5/94 zVAD only (without caspase activation/cleavage evaluation)
DNA fragmentation/nuclear condensation (2)	71/136 = 52.20%	26/71 TUNEL; 18/71 subG1; 15/71 Hoechst, 7/71 nucleosomal fragmentation/release, 4/71 DNA ladder, 4/71 nuclear condensation/fragmentation (EM); 3/71 Acridine orange, 2/71 DAPI; 3/71 unspecified method (DNA fragmentation/condensation)
Plasma membrane integrity/PS exposure (3)	57/136 = 41.91%	52/57 Annexin V+/−PI or 7AAD (7/57 use Annexin V only); 2/57 PI/Yo-Pro-1; 2/57 Annexin V/To-Pro-3; 1/57 PI only
Activation of pro-apoptotic Bcl-2 family members (4)	7/136 = 5.14%	7/7 measure Bax, Bak, or Bid activity, localization (Bax and Bak) or cleavage (Bid); other 14/137 entries determine only their total levels
Mitochondrial potential/integrity, release of pro-apoptotic factors (5)	36/136 = 26.47%	21/36 Cyt c release from mitochondria; 19/36 MMP; 4/36 measure both Cyt c release and MMP; 3/36 measure both Cyt c and Smac release
ROS detection (6)	14/136 = 10.29%	DFCDA and DHE measurements of ROS
Other apoptotic features (blebbing/apoptotic bodies) (7)	5/136 = 3.67%	3/5 cell blebbing; 2/5 apoptotic bodies
No. of entries using methods (1) and (2)	45/136 = 33.08%	19/45 TUNEL+activation/cleavage of caspases/ PARP (method 1); 10/45 subG1+method 1; 9/45 Hoechst+method 1
No. of entries using methods (1) and (3)	32/136 = 23.52%	28/32 AnnexinV+PI, 7-AAD or To-Pro-3+method 1; 3/87 Annexin V only (without PI/7-AAD)+method 1; 1/32 PI/Yo-Pro-1+method 1
No. of entries using methods (2) and (3)	19/136 = 13.97%	7/19 subG1+Annexin V with/without PI/7-AAD; 6/87 TUNEL+Annexin V with/without PI /7-AAD
No. of entries using at least three of the considered methods	34/136 = 25%	Combinations of methods used: 5/34 (1+2+3+5; 1+2+5; 1+3+5); 3/5 (1+2+3; 1+4+5); 2/34 (1+2+7; 1+3+6; 1+3+5+6); 1/6 (1+2+6; 1+2+4; 1+5+6; 1+2+4+5; 1+3+5+6; 1+3+4+5; 1+2+4+5+6+7)

Abbreviations: AAD, 7-amino-actinomycin D; Cyt *c*, cytochrome *c*; DCFDA, dichlorofluorescin diacetate; DHE, dihydroethidium; EM, electron microscopy; MMP, mitochondrial membrane potential; PARP, poly(ADP ribose) polymerase 1; PI, propidium iodide; PS, phosphatidylserine; ROS, reactive oxygen species. Note: A higher number of articles were published in *Cell Death & Disease* between January 1, 2010 and September, 2011; however, they measured other non-apoptotic cellular processes: non-apoptotic cell death and cell signaling, mitotic catastrophe, autophagy, proliferation, metastasis, and others[283–317]

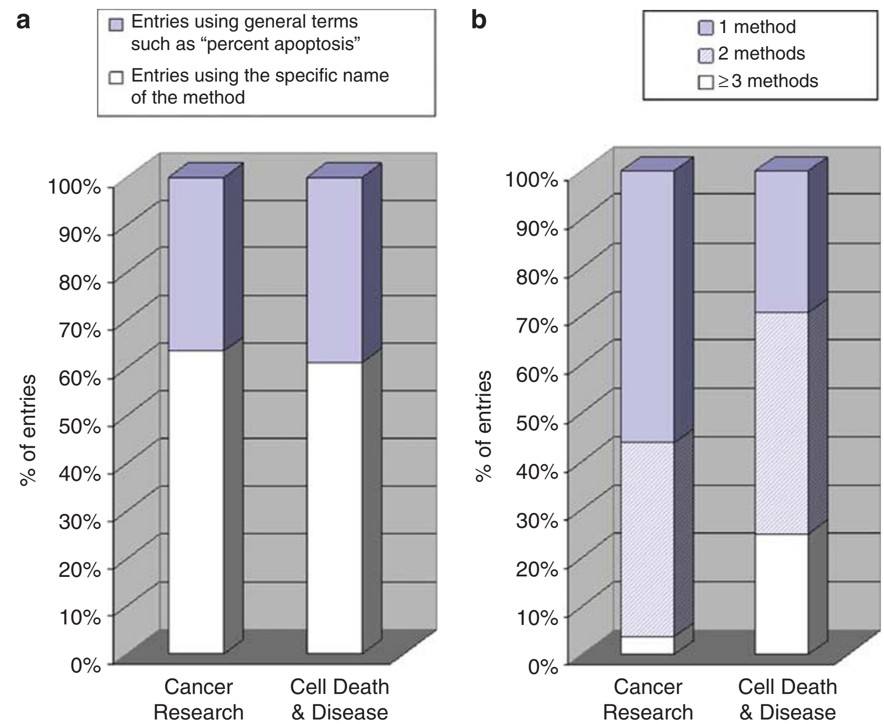

Figure 1 Evaluation of the *Cancer Research* and *Cell Death & Disease* articles. (**a**) Percentage of entries using the specific name of the apoptosis method used (87 of 137 = 63.5% for *Cancer Research*; 83 of 136 = 61.03% for *Cell Death & Disease*), instead of using the general terms such as '% apoptosis'. (**b**) Percentage of entries using one, two (55 of 137 = 40.14% for *Cancer Research*; 62 of 136 = 45.58% for *Cell Death & Disease*), or at least three methods (5 of 137 = 3.65% for *Cancer Research*; 34 of 136 = 25% for *Cell Death & Disease*) for apoptosis detection/quantification

Conflict of Interest

The authors declare no conflict of interest.

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