The Nuclear Area Factor (NAF): a measure for cell apoptosis using microscopy and image analysis

Mark A. DeCoster

Department of Biomedical Engineering and Institute for Micromanufacturing, Louisiana Tech University, Ruston, Louisiana, USA.

Apoptosis has been studied by a number of biochemical and image-based assays that vary widely in complexity, specificity, and cost. Here is described a microscopy and image analysis-based method for measuring what we call the nuclear area factor (NAF). Determination of NAF was based on fluorescent staining of the cell nucleus using DAPI, followed by digital microscopy and measurement of the nuclear area and nuclear circularity using image analysis software. We show that the nuclear area factor can be an early indicator of cell morphological changes occurring during apoptosis. Furthermore, calculation of NAF is relatively straightforward, and may be accomplished with a number of image analysis programs, two of which are compared here.

Keywords: Apoptosis, Neurons, Cell Culture, secreted phospholipase A_2, Image Analysis

1. Introduction

There are a number of biochemical and image-based assays for apoptosis that vary widely in complexity, specificity, and cost. Here I describe a microscopy and image analysis based method for measuring what we call the nuclear area factor (NAF), which was based on fluorescent staining of the cell nucleus using 4',6-Diamidino-2-phenylindole (DAPI), followed by digital microscopy and measurement of the nuclear area and nuclear roundness using image analysis software. We have previously shown that the NAF can be an early indicator of cell morphological changes occurring during apoptosis [1]. Furthermore, calculation of NAF is relatively straightforward, and may be accomplished with a number of image analysis programs, two of which are compared here.

In this chapter I will compare the use of the public image analysis software program Image J and the commercially available program Image Pro Plus. Image J while free, does not include all of the pre-analysis filters and features which are often essential for separating cells before calculating NAF or other measurements. This is especially the case for determining NAF in neurons, which may often aggregate or clump in vitro both under normal conditions, and after apoptotic stimuli. Thus, tools available in Image Pro Plus for separating or "splitting" objects are useful for calculating NAF.

As we described previously, DAPI staining may not label small, fragmented portions of DNA that are sometimes found in later stages of apoptosis [1]. We found significant changes in NAF as early as 4 hours after secreted phospholipase A_2 ($sPLA_2$) treatment was initiated. As long as the cell nucleus remains somewhat intact and the DNA does not become completely fragmented, calculation of NAF should remain a valid marker of apoptotic changes, although the greatest extent of these changes may occur early in the apoptosis process.

In concept, calculation of NAF could as easily be used with another nuclear dye such as Hoechst as with DAPI, which we used here. A benefit of these blue dyes, as we previously described, is that they allow for simultaneous staining of the same cells with independent markers for apoptosis such as FITC-based TUNEL [1]. While access to a good fluorescent microscope with digital camera and image analysis software is necessary for calculation of NAF, this technique does not require complex staining procedures such as TUNEL, and DAPI is a robust, relatively inexpensive nuclear stain. While there is some concern with certain UV-based dyes such as DAPI that plastic culture ware can result in poor digital microscopy images due to background fluorescence or noise, all images presented in this research were from cells grown on plastic. This is feasible in this case due to the robust and bright staining that

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DAPI often provides, as well as using neutral density filters or camera settings that do not result in overexposed halo-type nuclei that appear too bright. In most cases, and especially for UV-excited dyes such as DAPI, digital images usually will be of better quality with better signal-to-noise and less background fluorescence when cells are grown on glass vs. plastic. However, for most cells including neurons, cells adhere poorly on glass compared to plastic, sometimes resulting in loss of cells during the staining procedure, and therefore leading to inaccurate measurements (usually underestimates) of cell damage including apoptosis.

2. Materials and methods

2.1 Cell Culture.

Primary cortical neuronal cultures were prepared from embryonic day 15 (e15) rats and grown in vitro for 48 hours before apoptotic stimulus as previously described [1].

2.2 Cell staining and Microscopy.

After apoptotic stimulus, cells were stained with DAPI and cells examined with a Nikon inverted fluorescence microscope using an excitation wavelength of 355 nm and a 60X objective as described [1]. The main advantages of this technique are that the cells are stained quickly in the culture dish excluding complicated protocols and preparative/handling staining artifacts. Additionally, although there are some concerns with using UV-based fluorescent dyes such as DAPI on plastic culture ware due to background fluorescence, DAPI is a robust nuclear dye and in all of the work reported here, was imaged using cells grown on plastic culture plates.

2.3 Image processing using Image J software.

In Image J ver 1.37v, the formula for circularity is 4 pi (area/perimeter^2). A value of 1.0 indicates a perfect circle, which can be associated with apoptosis, at least for neurons under the conditions reported here. Note that the circularity field was first added to ImageJ ver. 1.35e. As the circularity value approaches 0.0, it indicates an increasingly elongated polygon, which reflects for the purposes of the current work, the values for control neurons in culture (before apoptosis). The user notes for ImageJ software states that measuring circularity may lead to invalid values for very small particles. These aberrant readings can be eliminated by excluding particles that are below a specific minimum area.

2.4 Image processing using Image Pro Plus software.

In Image pro plus ver 6.1 the formula for roundness is (perimeter^2)/(4*pi*area), with 1.0 indicating a perfect circle and larger values indicating oblong and non-circular objects.

2.5 Calculation of Nuclear Area Factor (NAF).

NAF was calculated as the product of object area (in pixels2)*roundness. For calculating NAF from Image J data, since roundness was not an available function, circularity was used. Since the formula for circularity in Image J and roundness in Image Pro Plus are the inverse of each other, the reciprocal of the circularity measurement from Image J was used to obtain the roundness, and then used to calculate NAF.

3. Results and Discussion

Figure 1 shows the phase, fluorescence, and merged images of neurons stained with DAPI under control conditions (Figs. 1A-C) and after 38 hours of exposure to 25 ng/ml of $sPLA_2$ (Figs. 1D-F).

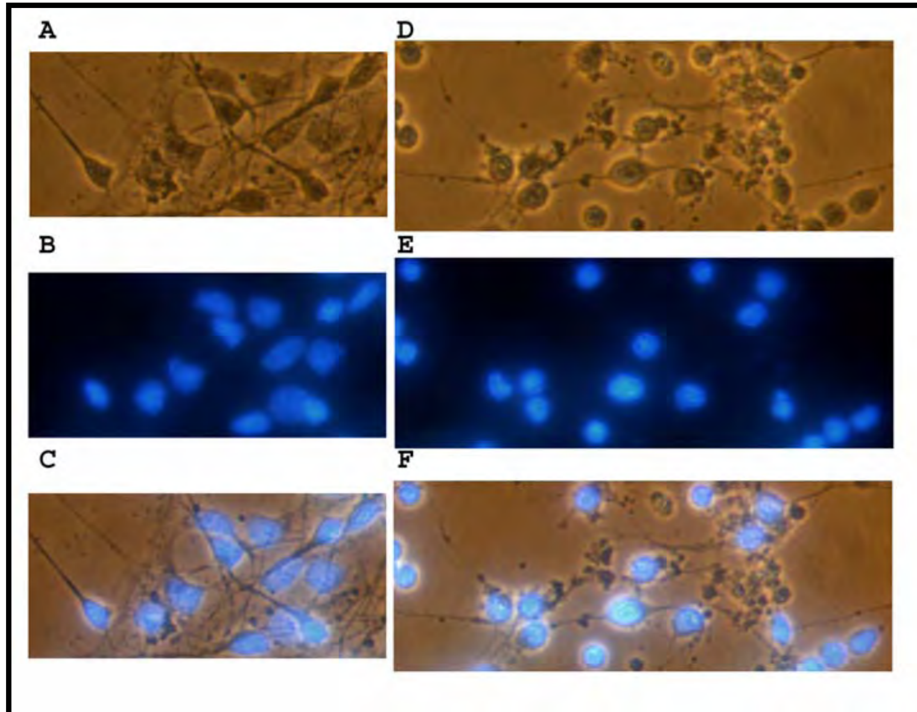

Figure 1. Phase contrast, fluorescence, and merged digital microscope images of neurons in culture. In the left column, control neurons are shown in phase, fluorescence with DAPI staining, and merged (panels A-C, respectively). In the right column, neurons treated with 25 ng/ml of $sPLA_2$ for 38 hours are shown in phase, fluorescence, and merged (panels D-F, respectively). Original magnification of all images= 600 X.

Figure 2 shows the DAPI stained images only of these same fields for control (Figure 2A) and $sPLA_2$-treated neurons (Fig. 2B) after image analysis by Image Pro Plus software. Note that the software numbers and outlines each counted object. In control cells (Fig. 2A), one large object near the bottom was excluded from the count because it was observed to consist of a neuron on top of an astrocyte, thus the two nuclei could not be separated by epifluorescence.

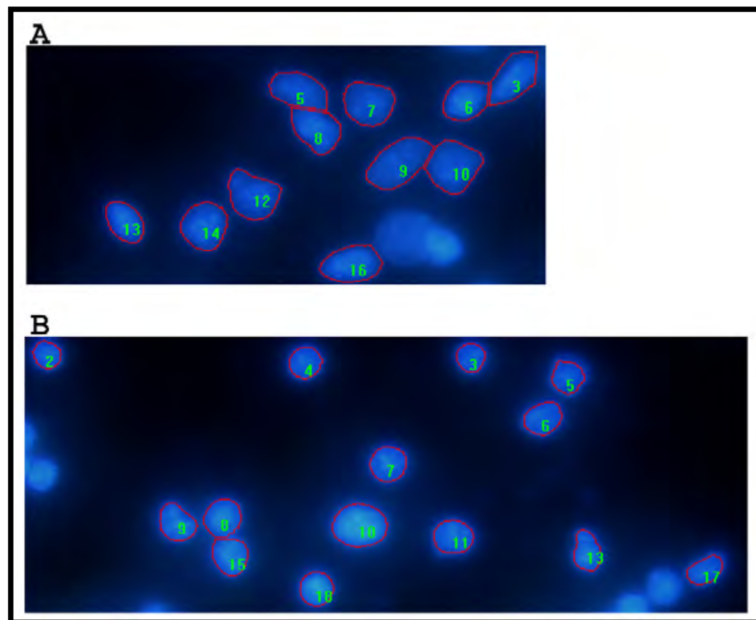

Figure 2. Image Pro Plus analysis of neurons in culture. Neurons stained with DAPI were digitally imaged under UV-fluorescence. Top panel (A) shows control cells and bottom (B) shows cells treated with $sPLA_2$ as shown in Figure 1. Polygon outlines and numbering of cells was carried out by the Image Pro Plus software.

Theoretically with confocal images using the same methodologies described here, nuclei at different planes in the cell culture could be separated and counted. This has been done recently for example to separate nuclei using DAPI staining of 3D cultures [2] . It is also important to note that as shown in $sPLA_2$-treated cultures (Fig. 2B), objects on the edge of the image or cut by the perimeter of the captured image can be excluded by the software and thus are not counted. This ensures that partial cells are not included in the analysis.

Figure 3 shows the analysis of the same control and $sPLA_2$-treated cells as carried out by Image J ver 1.35e. This software is more limited (but is freely available on the Web), and does not allow particle analysis of color images. Therefore, for our data, images were converted to 8-bit greyscale, and then thresholded, as shown in Figures 3A and C.

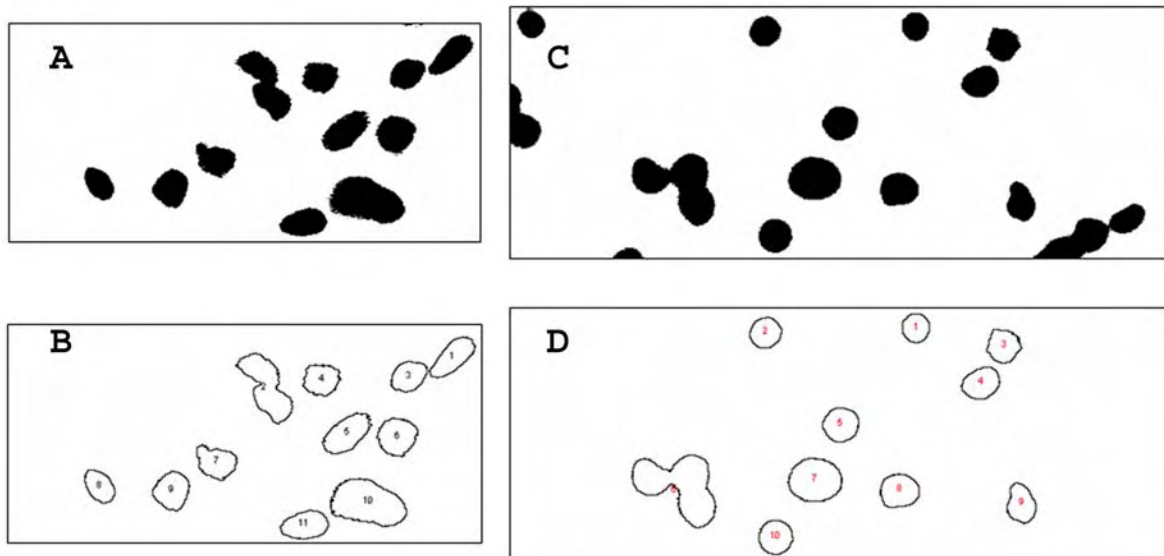

Figure 3. Image J analysis of neurons in culture. Neurons stained with DAPI were digitally imaged under UV-fluorescence. Left column (panels A+B) shows control neurons thresholded and then counted by the program, respectively. Right panel shows neurons treated with $sPLA_2$ as for Figure 1, thresholded (panel C) and outlined and counted by the program (panel D).

From these thresholded images, Image J then outlines and numbers each object as shown in Figures 3B and D. As for Image Pro Plus, Image J also has the function of "cleaning edges", thus cells on the edge of the image and cut by the image border can be excluded from the final analysis (compare Figure 3C and D). However, unlike Image Pro Plus, Image J does not have a "split" function used to separate closely touching cells. Thus, cells that cannot be separated by thresholding remain as larger groups, and also need to be excluded from the final analysis (Examples include object 2 in the upper center of Figure 3B, and object 6 in the lower left of Figure 3D).

Table 1 presents the software analysis of the counted objects. Object #, area, roundness, and nuclear area factor (NAF) are shown, along with averages for control and $sPLA_2$-treated cells, and the analysis using the two programs is compared.

Table 1 Nuclear Area Factor (NAF) values from area and roundness data calculated from Image Pro Plus and Image J software.

Image Pro Plus- $sPLA_2$ treated cells				Control Cells			
Obj#	Area	Roundness	NAF	Obj#	Area	Roundness	NAF
2	483	1.22	591	3	1427	1.50	2140
3	528	1.17	619	5	1310	1.47	1932
4	646	1.13	731	6	1190	1.29	1530
5	655	1.43	940	7	1422	1.30	1850
6	765	1.18	905	8	1322	1.44	1909
7	818	1.13	925	9	1797	1.43	2562
8	898	1.25	1124	10	1707	1.48	2533
9	831	1.25	1040	12	1518	1.33	2025
10	1504	1.23	1856	13	939	1.27	1193
11	859	1.21	1036	14	1388	1.23	1701
13	701	1.27	891	16	1417	1.33	1884
15	864	1.13	981				
17	595	1.26	749				
18	741	1.14	848				
Averages							
	778	1.22	945		1403	1.37	1933

Image J- $sPLA_2$ treated cells				Control Cells			
Obj#	Area	Roundness	NAF	Obj#	Area	Roundness	NAF
1	585	1.07	626	1	940	1.52	1433
2	704	1.12	792	3	772	1.28	986
3	799	1.35	1080	4	825	1.57	1293
4	855	1.16	992	5	1132	1.64	1856
5	900	1.12	1012	6	1060	1.37	1450
7	1717	1.28	2196	7	901	1.62	1460
8	979	1.20	1178	8	660	1.22	805
9	795	1.31	1043	9	958	1.25	1201
10	851	1.12	952	11	954	1.32	1257
Averages							
	909	1.19	1097		911	1.42	1304

The larger number of available functions for cell counting in Image Pro Plus results in more objects that can be included in the count. For example, as described above, the “split” function allows one to separate closely touching cells, and once separated, the area and roundness data for the liberated cells are then generated. This was used to separate objects 8 and 15 as shown in Figure 2B. In Image J, these two objects are still grouped within the larger object #6 (see Figure 3D), and thus was excluded from the table.

While objects can be theoretically separated using thresholding in Image J, this is a destructive process, which makes all objects smaller as the thresholding is increased. Thus, in an attempt to separate the cells under control conditions (see Figures 3A+B), the image was thresholded resulting in cells with an average area of 911 pixels^2, which was smaller than that using techniques using Image Pro Plus (1403 pixels^2, see Table 1).

For calculations using Image Pro Plus, both the average area and roundness of neurons treated with $sPLA_2$ decreased compared to control cells, resulting in a dramatic reduction of NAF from 1403 in

control cells to 778 in $sPLA_2$-treated cells. Using Image J software, roundness and area of $sPLA_2$-treated cells decreased compared to control cells, although average area decreased only slightly. However, since NAF is the product of area and roundness, a decrease in NAF was observed using Image J analysis from 1304 to 1097 in control and $sPLA_2$-treated cells, respectively.

The techniques reported here may also have utility for measuring cell morphological changes such as those occurring in cancer. For example, the nuclear morphometry measures of mean nuclear area, mean nuclear elongation factor, and mean nuclear regularity factor were used as potential predictors for clinical outcomes in localized renal carcinoma [3]. However, in a consensus report of prognostic factors in prostate cancer, it was deemed that factors such as nuclear roundness and nuclear area required further studies before routine use could be recommended [4].

In summary, calculation of the Nuclear Area Factor (NAF) for cells in culture appears to be a useful morphological indication of the apoptotic process, especially as shown here, for neurons. Since apoptosis is a complex, and multi-step process, calculation of NAF should be combined with other assays to determine the extent and timecourse of the apoptotic process. Using such fluorescent dyes as DAPI, staining of the cells in culture is relatively straightforward and inexpensive. If one has access to digital microscopy, nuclear area and roundness can be calculated using free software from the Web, as well as more powerful commercially available software as I have discussed here. Using these techniques, a quantitative measure of apoptosis can be obtained that is linked to individual cell responses as reflected by morphological changes.

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