Institute of Experimental Morphology, Pathology and Anthropology with Museum
Bulgarian Anatomical Society

Acta morphologica et anthropologica, 27 (3-4)
Sofia ● 2020

MORPHOLOGY 27 (3)

Original Articles

Fast, Easy Staining Method to Visualize Cell Morphology and Apoptosis

*Anton Kolarov[1], Nikola Mladenov[1], Irina Chakarova[1], Nikolay Ishkitiev[2], Maya Markova[1], Ralitsa Zhivkova[1], Stefka Delimitreva[1], Venera Nikolova[1]**

[1] Department of Biology, Medical Faculty, Medical University of Sofia, Bulgaria
[2] Department of Chemistry and Biochemistry, Medical University of Sofia, Bulgaria

* Corresponding author e-mail: venera.nikolova@abv.bg

The aim of this work was to combine popular fluorescent dyes into a protocol allowing fast application and easy evaluation of the results regarding the morphology of normal and apoptotic cells. A triple staining method was developed, applying simultaneously the blue fluorescent DNA-binding dye Hoechst 33258, the green fluorescent lipophilic dye DiOC6, and the red fluorescent TRITC-phalloidin binding to filamentous actin in cells with sufficient membrane permeability. It was applied to cultured cells, in some of which apoptosis was induced. Hoechst 33258 visualized the apoptosis-related chromatin condensation, DiOC6 revealed the shape of the cell body, and TRITC-phalloidin penetrated and stained only cells at a late stage of apoptosis, outlining a rounded surface with or without blebs. The described staining protocol is easy to use, requires less than an hour, and provides information about overall cell morphology and convenient identification of apoptotic cells.

Key words: Cytochemistry, apoptosis, DiOC6, Hoechst 33258, phalloidin

Introduction

Although microscopic studies of apoptosis have a long tradition, optimized variations of staining methods are still sought to allow fast, easy and accessible visualization of apoptotic cells. There are assays for specific apoptosis-associated changes, such as TUNEL detecting DNA fragmentation [5] and Annexin V binding to detect translocation of phosphatidylserine from the inner to the outer leaflet of cell membrane [7]. In many

instances, however, it is most appropriate to identify apoptotic cells based on the characteristic appearance of their nuclei. The fluorochrome Hoechst 33258 is often used for this purpose because it produces fast and clear staining of DNA, allowing easy identification of the condensed nuclei of apoptotic cells (e.g. [10]). When used alone, however, it does not provide information about the overall morphology of the cells. In this respect, a dye of interest is 3,3′-dihexyloxacarbocyanine iodide (DiOC6). It is lipophilic, green-fluorescent, and suitable for both living and fixed cells. In low concentrations, it binds to mitochondria and the endoplasmic reticulum [4, 6], while in higher concentrations, it stains all intracellular membranes [1].

Another staining reagent that can reveal the morphology of the target cell, and particularly of its surface, is labeled phalloidin. This fungal toxin binds specifically to filamentous actin and, after conjugation to a fluorochrome, is often used to visualize microfilaments. It does not penetrate live cells, with very few exceptions that possess receptors able to bind and internalize it [8]. Moreover, significant amounts of it do not penetrate even fixed cells, unless they have been permeabilized by detergent [9]. It could be assumed, however, that as cell death follows its course, plasma membrane permeability will increase to a degree allowing labeled phalloidin to diffuse inside the cell. To evaluate the potential of Hoechst 33258 and DiOC6 for fast and easy staining to reveal cell morphology, and to test apoptotic cells for their ability to let in TRITC-phalloidin, we developed a triple staining protocol and applied it to control and apoptotic cultured cells.

Materials and methods

Human gingival keratinocytes were isolated as previously described, using gingival tissue acquired during routine dental extractions of healthy third molars [2]. This was done in accordance with legislature and ethical guidelines concerning participation of human subjects, and after obtaining informed consent. Isolated keratinocytes were cultured in 35 mm diameter dishes at 37°C in an atmosphere of 5% carbon dioxide (CO_2) in EpiLife medium (Cascade Biologics, Portland, OR, USA). After formation of colonies, the cells were transferred to coverslips, placed in 6-well plates (TPP, Trasadingen, Switzerland), and cultured until reaching 80% confluence. Experiments were conducted with cells between 2nd and 4th passage.

The coverslips with the cultured cells were washed in phosphate buffered saline (PBS), pH 7.2. Some of them were left as controls, while others were irradiated with ultraviolet light for 20 min at 37°C to induce apoptosis, followed by incubation in PBS for 40 min at 37°C to allow the events of apoptosis to take place. Then both the induced and the control cells were fixed with 4% paraformaldehyde in PBS for 20 min at 37°C. After washing 3 times in PBS with 0.02% sodium azide, staining solution was applied for 20 min at 37°C in the dark. The staining solution contained 5 μM DiOC6 (Sigma-Aldrich, Germany), 1 μg/ml Hoechst 33258 and 1 μg/ml TRITC-labeled phalloidin (Sigma-Aldrich, Germany) in PBS.

After staining, cells were washed as before and the coverslips were overlaid over microscopic slides with drops of 100% Mowiol (Sigma-Aldrich, Germany). The slides were observed using fluorescent microscope Axioskop 20 (Zeiss, Germany). Absorption by Hoechst 33258, DiOC6 and TRITC was measured at 365, 490 and 560 nm respectively.

Results

Most control cells were in interphase, with irregularly shaped cell bodies revealed by DiOC6 staining. The chromatin, visualized by Hoechst 33258, had a fine structure. It was characterized by diffuse staining of moderate intensity, often with numerous small regions of more intensive staining. The reaction with TRITC-phalloidin was negative (**Fig. 1**). In addition, some mitotic cells could be observed, as well as occasional cells undergoing spontaneous apoptosis (not shown).

In the irradiated slides, most cells had normal appearance but apoptotic cells with condensed, brightly fluorescent chromatin were a common finding. Some of them, with the high degree of chromatin condensation characteristic for advanced apoptosis, were also positive for TRITC-phalloidin, indicating increased membrane permeability. The red fluorescent staining was most intense at the cell surface, presumably corresponding to cortical microfilaments. The labeled cells had a rounded shape, with or without protruding surface blebs (**Fig. 2**).

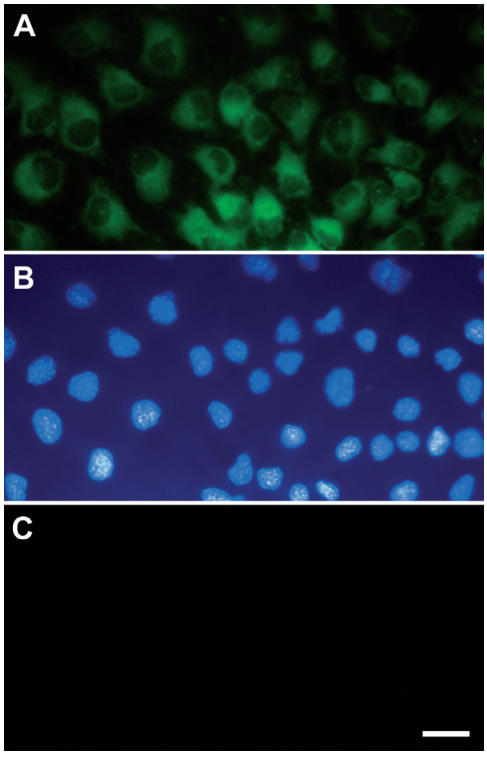

Fig. 1. Control cells stained with DiOC6 (**A**), Hoechst 33258 (**B**), and TRITC-phalloidin (**C**). The cells have irregular shapes, the appearance of chromatin is diffuse, cell membrane is impenetrable to the labeled phalloidin. Bar = 20 μm.

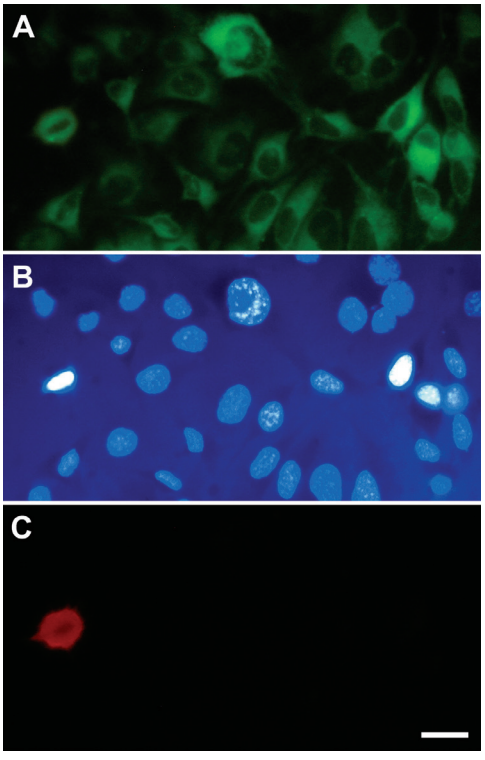

Fig. 2. Cells stained with DiOC6 (**A**), Hoechst 33258 (**B**), and TRITC-phalloidin (**C**) after induction of apoptosis. The Hoechst staining reveals condensed, brightly fluorescent nuclei characteristic of apoptosis both at left and at right. The cell with the most condensed nucleus (left) is also positive for TRITC-phalloidin. Bar = 20 μm.

Discussion

Experiments studying the influence of various factors on cell populations often require comparison of cohorts including vast numbers of cells. In such cases, fast staining methods that allow easy estimation of cell morphology and viability status are very useful. The lipophilic dye DiOC6 already has a specific application in apoptosis research: because its binding to mitochondria is voltage-dependent, it is used on fresh unfixed cells to detect the mitochondrial membrane potential (delta psi) changes associated with apoptosis [3, 10]. In our setting, it was used simply to stain the intracellular membranes in order to reveal the overall morphology of the cell body and so to supplement the information obtained for the nucleus by Hoechst 33258 staining. The simultaneous application of the two dyes allows the morphology and apoptosis status of the observed cell population to be estimated at a glance.

In the present study, we included TRITC-phalloidin because we were interested in the permeability transition that would allow it to penetrate apoptotic cells without detergent treatment. We found that it stained only cells with highly condensed nuclei and rounded cytoplasm indicating a relatively late stage in the course of apoptosis. Staining by TRITC-phalloidin could be omitted from the protocol and, given the high cost of this reagent, in many instances it would be appropriate to skip it. However, its specificity for dying cells and the opportunity to use it simultaneously with the other dyes allows quick evaluation of the effects of apoptosis-inducing factors, even by an inexperienced observer. It should be noted that the membrane disruption associated with necrotic cell death is also expected to let TRITC-phalloidin inside the cell, therefore nuclei of stained cells should always be examined to see whether their morphology (revealed by the Hoechst 33258 staining) corresponds to apoptosis or to necrosis.

Conclusion

The described protocol of simultaneous triple staining of cells with Hoechst 33258 (DNA-binding, blue-fluorescent), DiOC6 (lipophilic, green-fluorescent) and TRITC-phalloidin (actin-binding, red-fluorescent, with low penetrating ability), requiring less than an hour, allows easy estimation of cell morphology and apoptotic status using a fluorescent microscope. Nuclear morphology of phalloidin-stained cells should be examined to distinguish between apoptotic and necrotic cell death.

Acknowledgements: This work was supported by Grants No. D-97/24.06.2020 and D-215/12.12.2018 of the Medical University of Sofia and by the Bulgarian Ministry of Education and Science under the National Program for Research “Young Scientists and Postdoctoral Students”.

References

1. **Buch, C., R. Lindberg, R. Figueroa, S. Gudise, E. Onischenko, E. Hallberg.** An integral protein of the inner nuclear membrane localizes to the mitotic spindle in mammalian cells. – *J. Cell Sci.*, **122**, 2009, 2100-2107.
2. **Calenic, B., N. Ishkitiev, K. Yaegaki, T. Imai, Y. Kumazawa, M. Nasu, T. Hirata.** Magnetic separation and characterization of keratinocyte stem cells from human gingiva. – *J. Periodontal Res.*, **45**, 2010, 703-708.

3. **Kepp, O., L. Senovilla, I. Vitale, E. Vacchelli, S. Adjemian, P. Agostinis, L. Apetoh, F. Aranda, V. Barnaba, N. Bloy, L. Bracci, K. Breckpot, D. Brough, A. Buqué, M. G. Castro, M. Cirone, M. I. Colombo, I. Cremer, S. Demaria, L. Dini, A. G. Eliopoulos, A. Faggioni, S. C. Formenti, J. Fučíková, L. Gabriele, U. S. Gaipl, J. Galon, A. Garg, F. Ghiringhelli, N. A. Giese, Z. S. Guo, A. Hemminki, M. Herrmann, J. W. Hodge, S. Holdenrieder, J. Honeychurch, H.-M. Hu, X. Huang, T. M. Illidge, K. Kono, M. Korbelik, D. V. Krysko, S. Loi, P. R. Lowenstein, E. Lugli, Y. Ma, F. Madeo, A. A. Manfredi, I. Martins, D. Mavilio, L. Menger, N. Merendino, M. Michaud, G. Mignot, K. L. Mossman, G. Multhoff, R. Oehler, F. Palombo, T. Panaretakis, J. Pol, E. Proietti, J.-E. Ricci, C. Riganti, P. Rovere-Querini, A. Rubartelli, A. Sistigu, M. J. Smyth, J. Sonnemann, R. Spisek, J. Stagg, A. Q. Sukkurwala, E. Tartour, A. Thorburn, S. H. Thorne, P. Vandenabeele, F. Velotti, S. T. Workenhe, H. Yang, W.-X. Zong, L. Zitvogel, G. Kroemer, L. Galluzzi.** Consensus guidelines for the detection of immunogenic cell death. – *Oncoimmunology*, **3**, 2014, e955691.
4. **Koning, A. J., P. Y. Lum, J. M. Williams, R. Wright.** DiOC6 staining reveals organelle structure and dynamics in living yeast cells. – *Cell Motil. Cytoskeleton*, **25**, 1993, 111-128.
5. **Negoescu, A., C. Guillermet, P. Lorimier, E. Brambilla, F. Labat-Moleur.** Importance of DNA fragmentation in apoptosis with regard to TUNEL specificity. – *Biomed. Pharmacother*., **52**, 1998, 252-258.
6. **Sabnis, R. W., T. G. Deligeorgiev, M. N. Jachak, T. S. Dalvi.** DiOC6(3): a useful dye for staining the endoplasmic reticulum. – *Biotech. Histochem*., **72**, 1997, 253-258.
7. **Schutte, B., R. Nuydens, H. Geerts, F. Ramaekers.** Annexin V binding assay as a tool to measure apoptosis in differentiated neuronal cells. – *J. Neurosci. Methods*, **86**, 1998, 63-69.
8. **Thiede, B. R., J. T. Corwin.** Permeation of fluorophore-conjugated phalloidin into live hair cells of the inner ear is modulated by P2Y receptors. – *J. Assoc. Res. Otolaryngol.*, **15**, 2014, 13-30.
9. **Ujfalusi-Pozsonyi, K., G. Hild, P. Gróf, Z. Gutay-Tóth, Z. Bacsó, M. Nyitrai.** The effects of detergents on the polymerization properties of actin. – *Cytometry A*, **77**, 2010, 447-456.
10. **Zhang, J. Y., H. Y. Wu, X. K. Xia, Y. J. Liang, Y. Y. Yan, Z. G. She, Y. C. Lin, L. W. Fu.** Anthracenedione derivative 1403P-3 induces apoptosis in KB and KBv200 cells via reactive oxygen species-independent mitochondrial pathway and death receptor pathway. – *Cancer Biol. Ther.*, **6**, 2007, 1413-1421.