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Fluorescent dyes for cell viability: an application on prefixed conditions

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Abstract In recent years increasing attention has been given to apoptosis for its role in pathologic, organogenetic and homeostatic phenomena. Acridine orange (AO), Hoechst 33342 (HO) and propidium iodide (PI) are among the most used fluorescent dyes used to analyse cell culture viability. In fact, they respectively show specificity for living, apoptotic and late apoptosis/necrosis states. We explored whether HO, AO and PI can be used on prefixed monolayers of three commonly used cell lines. Here we mainly describe the metachromatic effects obtained by fluorescence microscopy with double and triple dye combinations. Furthermore, we propose an easy staining method in which a balanced sequential treatment with HO, AO and PI allows identification of different viability states onto fixed cells by using a long-pass FITC filter. This method extends the spectrum of suitable applications for these dyes in fluorescence viability detection onto previously fixed (prefixed) samples.

Keywords Apoptosis · Viability · HO · AO · PI · Fixed cells

Introduction

Physiological phenomena, such as development and homeostasis, as well as a great number of pathologies, such as cancer, autoimmune, inflammatory and neurodegenerative diseases (Ashkenazi and Dixit 1998; Littlewood and Evan 1998; Savill 1994; Schwartz and Osborne 1993; Steller 1995; Thompson 1995), involve spontaneous or induced cell death. Cell viability changes have been widely studied in vivo and in vitro and the morphological features of apoptotic or necrotic cells are well known. Programmed cell death, also known as apoptosis, is an active, genetically regulated, suicide process, which involves nuclear alterations (chromatin condensation followed by fragmentation into discrete fragments) and, only at its late stage, induces cell membrane blebbing. The metabolic pathway of apoptosis has been recently elucidated at the molecular level (Adams and Cory 1998; Douglas et al. 1995; Li et al. 1998). However, further studies are required to clarify the pathogenetic effects of this phenomenon and its correlation with genetic defects and cell viability alterations.

Necrosis occurs after sudden severe injuries or noxious compound treatment and accounts for many destructive effects that represent only a passive consequence of pathologic damages. Morphologically, dramatic alterations of plasma membrane permeability occur. These events lead to cellular swelling and disintegration followed by mitochondrial disruption, cellular content release and chromatin flocculation. The partial analogy between apoptosis (especially in its late phase) and necrosis contributes to complicate the ascription of structurally altered cells to one or the other phenomenon.

Microscopy cell viability assessment has been widely described. Among the most used published methods or dyes we found TdT-mediated-biotin-dUTP nick-end labelling (TUNEL), propidium iodide (PI), oxazole yellow dimer (YOYO-1), 4′,6′-diamino-2-phenylindole (DAPI), bis-benzimidazole Hoechst 33342 (HO), BrdUTP, fluorescent in situ hybridisation and acridine orange (AO) (Bryson et al. 1994; Davis et al. 1997; Gavrieli et al. 1992; Hardin et al. 1992; Haynes et al. 1990; Matthews et al. 1998; Sailer et al. 1996; Seong et al. 1998; Swe et al. 1996; Telford et al. 1992). Each one of them allows the identification of only one condition at a time.

However, the studies, in which there is the need for differential staining of healthy, apoptotic and/or necrotic cells in the same sample, present the application of two methods or compounds under particular conditions (Bank 1988; Ciancio et al. 1988; Davis et al. 1997; Ertel et al. 1998; Goldberg et al. 1997; Hoorens et al. 1996;

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Liegler et al. 1995; Mpoke and Wolfe 1997; Ormerod et al. 1993; Schmidt et al. 1994; Weber et al. 1997).

In this study we focused our attention on three commonly diffused dyes, HO, AO and PI, which can bind nucleic acids and intercalate into DNA. The feasibility of combining them on the same sample has been particularly analysed.

In vivo, HO has access into slightly damaged as well as dramatically altered apoptotic nuclei and prefers binding to poly (d (A-T)) tracts (Hoorens et al. 1996; Weber et al. 1997). By using this dye it is possible to label cells ranging from early to late apoptotic stage. AO can readily enter living cells and shows preference for normal nuclei AT-rich regions. In living systems, in fluorescence blue light, AO has green emission when bound to double-stranded nucleic acids, or red signal when intercalated into single-stranded nucleic acids (Bank 1988). Finally, PI can enter only in late apoptotic and necrotic cultured cells, intercalating nucleic acids every 4–5 bp without sequence preference (Ertel et al. 1998; Lizard et al. 1995; Schmidt et al. 1994). In in vivo systems, cell viability directly correlates with membrane impermeability, contributing to the selective ability of these dyes to reach DNA, while DNA packaging establishes their intercalating capability. Moreover, AO + PI and AO + HO combinations under the fluorescence microscope, and HO + PI combination in flow cytometry, are employed to obtain two-colour staining for testing different viability states in living cell systems.

Routinely applied aldehyde cell fixation involves protein partial denaturation and amine–amine crosslinking, that could lead to intercalating agents specificity loss and to staining effects modifications (Haugland 1996; Haynes et al. 1990). By coupling HO, AO and PI in fluorescence microscopy under different filter types, we examined whether it is possible to optimise a method for studying cellular viability even after samples have been submitted to a fixation procedure. Three cell lines, Chinese hamster ovary (CHO) dhfr-, normal rat kidney (NRK) and mouse pancreatic βTC3 (βTC3) were used as representative testing samples. The staining patterns were observed in apoptotic samples, where cell death was induced by serum deprivation (Hoorens et al. 1996; Takemura et al. 1997), or in necrotic samples, where cells were treated with toxic doses of a chemical agent normally used for clone selection.

Now we propose and describe an HO, AO and PI balanced combination allowing to distinguish different cell viability conditions on the basis of nuclear and cytoplasmic colours obtained in fluorescence microscopy under long-pass FITC filter.

Materials and methods

Materials

All the reagents for cell culture, where not otherwise indicated, were purchased from Gibco BRL (Life Technologies, Paisley, UK) and chemicals were from Sigma (St. Louis, Mo., USA).

Cell cultures

CHO dux-B11 dhfr- cells were cultured in alpha-MEM with nucleosides and deoxynucleosides, 50 U/ml penicillin, 50 µg/ml streptomycin and 10% fetal calf serum (FCS; Euroclone, Life Technologies, Milan, Italy); NRK-52 E were cultured in DMEM with 1% non-essential amino acids and 5% FCS; and finally βTC3 cells (kindly provided by Dr. Shimon Efrat, Albert Einstein College of Medicine, Bronx, N.Y., USA) were cultured in DMEM with 25 mmol/L-glucose, 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 10% FCS. Onto six-well multiplates 3×10^5 cells were plated and apoptosis was induced in NRK and in βTC3 cells by withdrawing FCS from the culture medium when cell confluence was 60–70%. Preliminary experiments were performed in order to determine, for all cell lines, the FCS-free culture time required for apoptosis induction. Necrosis was induced in CHO cells by treatment with 50 nM methotrexate (MTX) for 1 week. Regular cultures in the presence of FCS were always carried out in parallel as control. All the tested cell lines were mycoplasma-free.

Histochemistry

Plated cells were fixed in freshly prepared 2% paraformaldehyde in PBS 0.05 M pH 7.4 for 10 min at room temperature, washed 3 times with PBS and stained either with HO, AO, or PI, or with one of the following combinations: HO + AO, AO + PI, HO + PI or HO + AO + PI.

Paraformaldehyde was chosen because of its broad use as fixative agent. Mild conditions were preferred to stronger ones in order to minimise fluorescence background shown by cells after paraformaldehyde treatment (Fig. 1A, B) and to reduce the interference with dyes and stain alteration, as compared with nonfixed cells.

Optimal concentrations and incubation times, investigated in preliminary experiments in order to maximise single staining detection in our preparations, were found to be as follows: 20 µM, 20 min at 37°C for HO; 0.67 nM, 10 min at 37°C for AO; 3 nM, 5 min at 37°C for PI. Moreover, tests were performed to verify whether dye application sequence influenced stain results. Consequently we applied HO, followed by AO and then by PI. All of them were added to the last washing step PBS at the end of the fixation procedure. Results of double and triple staining on FCS-deprived or MTX-intoxicated cells were examined in detail. HO + AO + PI application results, on prefixed vs non-fixed cell monolayers, were also analysed. All the experiments were repeated at least 3 times. Cells were observed at an inverted microscope (IX50; Olympus, Milan, Italy), equipped with a rhodamine fluo-

Fig. 1 **A, B** A field of fixed but unstained Chinese hamster ovary (CHO) cells as it appears in brightfield and in blue light, respectively; autofluorescence background absence resulted in **B** in non-assessable cells. **C–H** Effect of multiple labels on paraformaldehyde prefixed cells in blue light. **C, D** CHO cells treated with HO + AO or with HO + PI, respectively. In **C**, *arrow* points to one condensed nucleus, while in **D** *arrows* indicate a few groups of condensed nuclei. In **E**, normal rat kidney (NRK) cells stained with AO + PI, (*arrow* indicates bright yellow apoptotic cell); notice yellow nucleoli inside the green nuclei of the viable cells. **F, G** HO + AO + PI label of βTC3 and NRK cells FCS deprived for 9 days. In **F**, notice the abundance of condensed yellow nuclei (*arrowheads* indicate late apoptotic cells). In **G**, bubbles are evident in NRK apoptotic cell cytoplasms (*arrows*). **H** MTX-treated, HO + AO + PI stained CHO monolayer, where necrotic (field's focused plan) and healthy cells are contemporarily present (*arrow* indicates necrosis). **I, J** Same NRK cells field as **G**, as observed in green and grey light, respectively. In **I** the staining is blurred; in **J** condensed nuclei appear slightly brighter than normal ones and cytoplasm is unstained. **K** CHO cells MTX-treated and in vivo stained with HO + AO + PI (*arrows* indicate bubbled cells undergoing necrosis). Compare **K** to **H**. *Bar* 10 µm

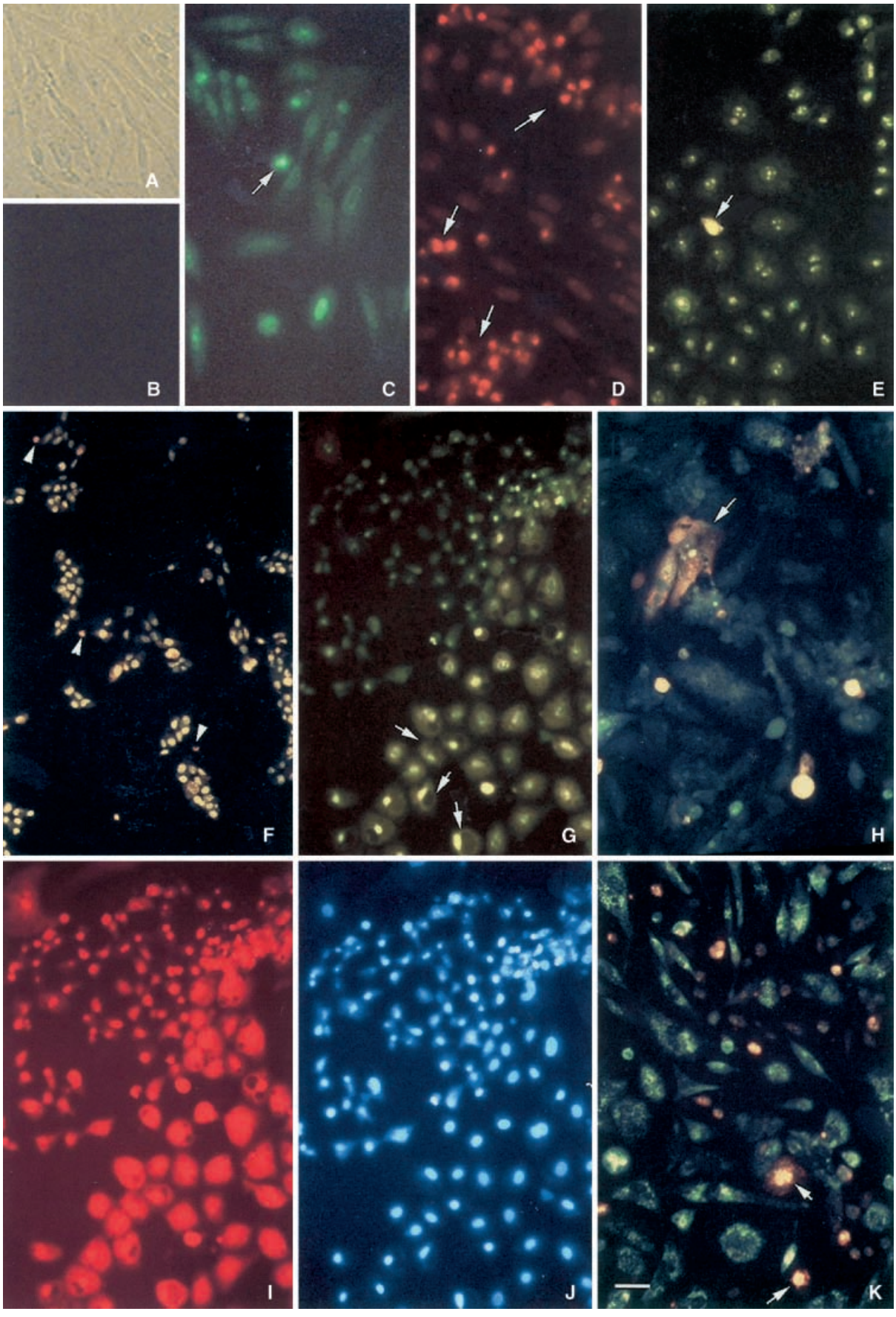
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Table 1 Effects in fluorescence microscopy of single staining with Hoechst 33342 (HO), acridine orange (AO) or propidium iodide (PI) on cell monolayers; prefixed cells. Morphological evaluation: healthy = regular nuclear contour and normal cell shape; early apoptotic = nuclear condensation in presence or absence of cytoplasm alteration; late apoptotic = nuclear fragmentation and cytoplasm alteration. (*ND* Labelling signal not detectable)

Dyes	Blue light	Green light	Grey light
AO	Green nuclei and weak green cytoplasm in healthy cells; ND other cells	ND	ND
HO	Green/yellow nuclei and weak green cytoplasm in apoptotic cells; ND other cells	ND	Blue nuclei and weak blue cytoplasm in all the cells present
PI	Orange shadow in all the cells present	Red nuclei and cytoplasm in all the cells present	ND

Table 2 Effects in fluorescence microscopy of single staining with HO, AO or PI on cell monolayers; non-fixed cells. Morphological evaluation: healthy = regular nuclear contour and normal cell shape; mitotic = nuclear condensation consistent with mitotic chromosome assembly during mitotic phases; early apoptotic = nuclear condensation in presence or absence of cytoplasm alteration; late apoptotic = nuclear fragmentation and cytoplasm alteration; necrotic = nuclear shape diffuse or irregular and altered cell shape. (*ND* Labelling signal not detectable)

Dyes	Blue light	Green light	Grey light
AO	Green nuclei in viable cells; ND other cells	ND	ND
HO	Green nuclei in mitotic and apoptotic cells; ND other cells	ND	Bright blue nuclei of apoptotic and mitotic cells; azur nuclei in healthy cells; ND necrotic cells
PI	Red late apoptotic nuclei and necrotic nuclei	Red late apoptotic nuclei and necrotic nuclei	ND

rescence cube (excitation = 530–550 nm, dichromatic lamina = 570 nm, barrier filter = 590 nm), i.e. green light, DAPI fluorescence filter cube (excitation = 360–370 nm, dichromatic lamina = 400 nm, barrier filter = 420 nm), i.e. grey light, and FITC/GFP long-pass filter cube 4 (excitation = 455–495 nm, dichromatic lamina = 505 nm, barrier filter = 510 nm), i.e. blue light.

Results

Single staining

A first set of experiments has been performed to assess whether alterations in DNA accessibility and binding, caused by cell fixation, could give advantage to HO, or AO, or PI single stains. Eventually, little distribution modifications have been considered acceptable, as compared to in vivo patterns, while the staining capability preservation was essential.

Tables 1 and 2 show the cell labelling pattern for each dye when applied alone at concentrations and conditions as described in Materials and methods: Table 1 shows results after the cell-fixing procedure and Table 2 presents non-fixed cells. In fixed samples, each dye and light produce the staining of both cytoplasm and nucleus; this is the most relevant difference between fixed and non-fixed cells. Moreover, in prefixed vs non-fixed conditions some slight differences are also observed in viability-specific wave length-dependent properties (compare Tables 1 and 2).

Double staining

By applying HO + AO onto living avian chondrocytes, Mpoke and Wolfe (1997) have shown a peculiar metachromasia in blue light, allowing to distinguish between apoptotic and healthy cells. Nevertheless, the authors underlined their incapability to detect necrosis with this method and the partial specificity loss of these labels when applied onto fixed cells. The problem they described mainly concerns non-nuclear nucleic acid stains in fixed cells in blue light. But we considered that intracellular acidic vesicles labelling data overcomes the purposes of this work.

In prefixed cells first treated with HO and then with AO we observe viability-specific colour in blue light (Fig. 1C) and in grey light (data not shown). No labelling is observed in green light, as shown in Table 3. Table 3 also summarises the effects of HO + PI (see also Fig. 1D) or AO + PI in blue (see also Fig. 1E), grey and green light. Of note, after fixation, inter- and intracellular PI diffusion (Levelt and Eichmann 1994) does not occur in AO + PI applications, possibly because of an AO quenching of PI.

In blue light, the AO + PI incubation after fixation resulted in a particularly interesting staining pattern (Table 3). Healthy cells show weak green nuclei with yellow nucleoli, whereas apoptotic cells have bright yellow nuclei: yellow stain denotes condensed chromatin presence (Fig. 1E). Although we were unable to obtain

Table 3 Effects in fluorescence microscopy of double staining on cell monolayers; prefixed cells. Morphological evaluation: healthy = regular nuclear contour and normal cell shape; early apoptotic = nuclear condensation in presence or absence of cytoplasm alteration; late apoptotic = nuclear fragmentation and cytoplasm alteration; necrotic = nuclear shape diffuse or irregular and altered cell shape. (*ND* Labelling signal not detectable)

Dyes	Blue light	Green light	Grey light
HO+AO	Green nuclei and weakly coloured cytoplasm in viable and apoptotic cells; ND necrotic cells	ND	Light blue nuclei (brilliant in apoptotic condensed) and blue cytoplasm in all cells
HO+PI	Orange nuclei (strongest in apoptotic condensed) and weaker to dark cytoplasm in all the cells present	Red nuclei and red cytoplasm in all the cells present	Light blue nuclei (brilliant in apoptotic condensed) and blue cytoplasm in all cells
AO+PI	Green nuclei, yellow nucleoli and dull musked cytoplasm in healthy cells; bright yellow nuclei and musk/yellow cytoplasm in apoptotic cells; ND necrotic cells	Red nuclei and red cytoplasm in all the cells present	Azur cellular shadows; weak azur to pink nuclei in damaged cells

Table 4 Effects in fluorescence microscopy of double staining on cell monolayers; non-fixed cells. Morphological evaluation: healthy = regular nuclear contour and normal cell shape; early apoptotic = nuclear condensation in presence or absence of cytoplasm alteration; late apoptotic = nuclear fragmentation and cytoplasm alteration; necrotic = nuclear shape diffuse or irregular and altered cell shape. (*ND* Labelling signal not detectable)

Dyes	Blue light	Green light	Grey light
HO+AO	Green nuclei in viable and apoptotic cells; ND necrotic cells	ND	Brilliant blue staining of apoptotic nuclei; azur nuclear shadows of other cells
HO+PI	Weak yellow nuclei in apoptotic cells; yellow-orange nuclei in necrotic cells; ND healthy cells	Red nuclei in late apoptotic and in necrotic cells; ND healthy cells	Brilliant blue staining of apoptotic nuclei; azur nuclear shadows of other cells
AO+PI	Green nuclei with dark cytoplasm and brilliant green granules in healthy cells; red nuclei in late apoptotic and necrotic cells	Red nuclei in late apoptotic and in necrotic cells; ND healthy cells	ND

Table 5 Triple combination labelling effects on paraformaldehyde-fixed or living cell monolayers. Morphological evaluation: healthy = regular nuclear contour and normal cell shape; early apoptotic = nuclear condensation in presence or absence of cytoplasm alteration; late apoptotic = nuclear fragmentation and cytoplasm alteration; necrotic = nuclear shape diffuse or irregular and altered cell shape. (*ND* Labelling signal not detectable)

Fixation	Blue light	Green light	Grey light
Present	Healthy cells: green nuclei, yellow nucleoli and green to rust-coloured cytoplasms; early apoptotic cells: green-yellow to brilliant yellow nuclei and dark rust-yellow cytoplasms; necrotic/late apoptotic cells: yellow-orange nuclei and orange cytoplasms	Red nuclei and red cytoplasms in all the cells present	Azur cellular shadows; weak azur to pink nuclei in unhealthy cells
Absent	Green living cells with brilliant cytoplasmic granules; reddish necrotic cells with orange cytoplasmic granules and red/yellow nuclei	Red nuclei in late apoptotic and in necrotic cells; ND healthy cells	Brilliant blue staining of apoptotic nuclei azur nuclear shadows of other cells

any additional information useful to discriminate between apoptotic and necrotic cells, the informations obtained with this staining combination are viability dependent (compare Tables 3 and 4).

Triple staining

Blue light double-labelling observations encouraged us to try a triple combination of the three dyes, to improve the differentiation not only between healthy and damaged cells, but even between apoptotic and necrotic ones. Briefly, as HO + AO produce a monochromatic stain of healthy and apoptotic cells, and HO combined with PI strongly modified the PI single effect, the possibility that HO superimposition could affect the AO + PI signal was assayed. Preliminary experiments suggested the use of HO as the first dye in multiple labelling, to preserve its stain capability (the presence of signal in grey light was used as positive stain index). Moreover a reproducible staining is obtained in all the considered samples only when AO is applied before PI.

Table 5 schematises HO + AO + PI triple-staining effects. By analysing the prefixed samples in blue light a strong analogy between HO + AO + PI (Fig. 1F, H) and AO + PI (Fig. 1E) staining was found. However a deeper observation of HO + AO + PI staining allows to detect not only nuclear but also cytoplasmic alterations, and to easily discriminate cell injury severity. This method allows distinguishing between apoptotic and necrotic cells (compare Fig. 1F, G with Fig. 1H). Of note is that every signal in green light gives similar results when PI was applied alone or in combination with the other two considered agents; analogous observations can be done for HO in grey light; triple staining is shown as representative (Fig. 1I, J).

Triple combination applied to non-fixed cell cultures (Table 5) shows analogous results. Healthy cells presented brilliant green intracellular granulation that turns to yellow in apoptotic cells (data not shown) and to orange-red in necrotic cells (Fig. 1K). On living cultures also, the coupling of three dyes together seems not to affect the stain peculiarities of HO in grey light and of PI in green light, as assessable when a single labelling has been performed (compare Tables 5 and 2).

Discussion

Identification of apoptotic or necrotic cells in a living population represents a puzzling question from a long time. The use of intercalating agents in fluorescence-based analysis, supported by a lot of published methods, is widely known and they enter in the formulation of viability determination commercial kits. In vivo, intercalating dyes selectively label some different cell viability stages, but their specificity is deeply influenced by experimental conditions.

Fixation and/or permeabilisation represent two important parameters in viability evaluation experiments; consequently compound choice as well as fixation protocols are crucial for final results.

A fixation negative effect of the intercalating agents specificity, because of affecting the differential stain of single- and double-stranded DNA, has been underlined by other authors (Levelt and Eichmann 1994; Mpoke and Wolfe 1997). Haynes et al. (1990) mentions the paraformaldehyde membrane permeability alteration, showing an aspecific diffusion of PI signal. In all cases the main problem is represented by cell compartmentalisation changes following fixative application.

Conversely, a cytometry study of apoptosis in murine lymphocytes and in spleen cells performed by Douglas et al. (1995) demonstrated that, following paraformaldehyde procedure, PI staining is quantitatively comparable to data obtained after ethanol fixation or by applying the TUNEL method. Furthermore these authors verified the surface proteins preservation to antibodies binding in paraformaldehyde-prepared cell samples. This information suggests the existence of a balance between paraformaldehyde-dependent increased membrane permeability and easy dye access to nucleic acids inside the cells, and between diminished membrane selectivity and aspecific dye staining. In our work we use a mild paraformaldehyde treatment onto single-stained monolayers to enhance HO, AO and PI diffusion inside cells without dramatically affecting dye specificity. These results suggest the possibility to obtain a balanced dye combination which can be used to discriminate among healthy, apoptotic and necrotic cells.

To our knowledge, other authors described only in vivo applications of two out of the three compounds here considered, obtaining a specific labelling of healthy or apoptotic or necrotic cells at a time (Bank 1988; Ciancio et al. 1988; Hoorens et al. 1996; Mpoke and Wolfe 1997; Ormerod et al. 1993; Schmidt et al. 1994).

Conversely, we optimise a method, which allows colour-selective visualisation of all cell viability stages at the same time, which is of utmost importance. Observations on single and double labels, concerning the effects of cell fixation on cell chromasia, suggest that grey light and green light are useless for this purpose, while blue light is the most suitable. In this work we describe an easy to perform HO + AO + PI application, that is assessable by means of a common fluorescence microscope equipped with a long-pass FITC filter system. One possible explanation resides in the “general specificity” of our method: we employ substances that bind DNA tracts and label it in dependence of nucleic acid accessibility but are independent from DNA breaks existence, while other methods, like TUNEL, stain the fragmented DNA only (Gavrieli et al. 1992).

In triple staining the partial diffusion of dyes, occurring only after fixation, allows to observe cell cytoplasmic bubbling and shape changes, ameliorating the differential screening of damaged cells.

We focused our attention on fixed samples on the basis of sample availability; living cultures have been expressly inserted to grossly compare our results on fixed monolayers to the “in vivo” data and to verify the correspondence between the latter and the literature data, where possible. By employing living samples we have the advantage to give a real picture of cell viability conditions and to have a follow-up without any cell loss or pitfall problems. In spite of this, we have to deal with an evolving system, having a physiological equilibrium, which can also be affected by prolonged analysis. Conversely, the fixation process could lead to a partial loss in cell number, especially affecting the late apoptotic and necrotic cells that are physiologically losing the connections to the substrate. Furthermore, this procedure allows only the study of a particular cell lifetime. However these disadvantages can be compensated by sample storing as well as by the opportunity to analyse samples not expressly prepared for a viability study, but suitable for further immunohistochemical approaches. It is important to point out that the slight but existing difference in cell viability label in blue light with triple combination onto prefixed and living cells, cultured in similar conditions, leads to two comparable, but not superimposable systems.

In conclusion, the development of this easy and suitable procedure for cell viability screening on prefixed conditions (also applicable to living samples) provides a useful tool for studying cell response to different treatments or for morphometric analysis of apoptosis.

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