Cytotechnology (2004) 46:139–150
DOI 10.1007/s10616-005-2551-7

Evaluation of a continuous quantification method of apoptosis and necrosis in tissue cultures

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Received 4 July 2005; accepted in revised form 30 August 2005

Key words: Apoptosis, Monitoring, Necrosis, Propidium iodide, Quantification, YO-PRO-1

Abstract

In tissue-engineering and other life sciences, there is a growing need for real-time, non-destructive information on apoptosis and necrosis in 2D and 3D tissue cultures. Previously, propidium iodide was applied as a fluorescent marker for monitoring necrosis. In the current study this technique was extended with a fluorescent apoptosis marker, YO-PRO-1, to discriminate between both stages of cell death. The main goal was to evaluate the performance of YO-PRO-1 and propidium iodide during monitoring periods of up to 3 days. Apoptosis was induced in C2C12 cultures and the numbers of YP-positive and PI-positive nuclei were counted in time. The performance of the dual staining was evaluated with a metabolic measure and a probe intensity study. Cell metabolism was unaffected during the first 24 h of testing. In conclusion, the YP/PI dual staining method was found to be a powerful tool in obtaining real-time spatial information on viability in cell and tissue culture without culture disruption.

Abbreviations: DM – differentiation medium; GM – growth medium; PI – propidium iodide; YP – YO-PRO-1

Introduction

Within the field of tissue engineering, monitoring viability or cell death during *in-vitro* culture is a valuable tool. Other research fields, such as developmental biology, cardiology and oncology, also benefit from such a method (Blankenberg et al. 2000). In those disciplines, such methods can give insight in processes of tissue damage and adaptation under *in-vitro* conditions (e.g., ischemia in heart explants, local (developmental) apoptosis in embryo culture). The ideal probe for evaluation of cell death *in-vitro* should give quantitative information on cell death in space and in time and should be non-destructive to the culture. The development of cell death should be assessable online for several days in the same ongoing culture. In addition, it should differentiate between apoptosis and necrosis to provide understanding of the pathways leading to cell death. Last, quantification should be easy and fast and applicable under *in-vitro* conditions in 2D and 3D cultures.

Several methods are available to assess cell death in engineered tissues and cell cultures but they are either destructive to the culture or unable to differentiate between apoptosis and necrosis in a

spatio-temporal manner (see Park et al. 2000 for a review; Chung 1983; Mosmann 1983; Stadelmann and Lassmann 2000; Rieseberg et al. 2001). Fluorescent cell staining, monitored by conventional fluorescence microscopy or by confocal microscopy, offers advantages over the aforementioned methods. Commercially available fluorescent probes (e.g., calcein/ethidium homodimer-1; Cell Tracker Green/propidium iodide) can be loaded to intact cell cultures and provide spatio-temporal information on cell death development without disrupting the culture (Breuls et al. 2003a; Wang et al. 2004). However, no differentiation between apoptosis and necrosis can be made. Another combination of fluorescent probes, fluorescently labeled annexin to indicate apoptosis and propidium iodide to label necrosis, is able to distinguish both types of cell death. A disadvantage of this method is that the apoptosis cannot easily be quantified, unless the culture is disrupted for flow cytometry.

Different cell death pathways can lead to necrosis and one of them is apoptosis, but necrosis can also occur 'spontaneously.' To be able to discriminate apoptosis and necrosis, an additional probe is required. If apoptosis can also be detected, it is possible to recognize adverse effects on cell viability earlier in the process and to determine if an apoptotic pathway preceded the onset of necrosis.

Until now, a combination of commercially available probes (Molecular Probes, V13243) has been applied to discriminate between both stages of cell death. This technique has been applied to fixed tissue sections (Suzuki et al. 1997), as microplate assay (Wronski et al. 2002), to flow cytometric measurements (Jerome et al. 2001; McGuire et al. 2001), and to 3D tissues (Boffa et al. 2005). One of the probes is a green fluorescent probe, YO-PRO-1 (YP), which can enter cells once their plasma membrane has reached a certain degree of permeability (Idziorek et al. 1995; Liu et al. 2004). The cell membrane becomes slightly permeable during apoptosis and YP can freely enter the cell and bind to its nucleic acids, which greatly enhances its fluorescence intensity. The other probe, propidium iodide (PI), is applied to stain for necrosis with its red fluorescence. This probe can only cross membranes of necrotic cells and not those of apoptotic cells. PI fluorescence increases 20- to 30-fold once it binds to nucleic acids. The cell membrane of viable cells is impermeable to both YP and PI.

In the recent article by Boffa et al. (2005) the application of YP has been shown in 3D quantification of apoptosis at a certain point in time. The goal of the present study was to investigate the performance and effects of YO-PRO-1 and propidium iodide dual staining in continuous monitoring of development of apoptosis and necrosis in a tissue. As a model system C2C12 mouse myoblasts were used in the cultures. In this model system, more insight into the behavior of the dual staining method in ongoing cultures was obtained by monitoring the progression of apoptosis and necrosis after induction of cell death. The influence of time on probe intensity was evaluated, optimal probe concentrations were determined, and the influence of the probes on cell metabolism was quantified.

Materials and methods

Cell and tissue culture

C2C12 Murine skeletal myoblasts (passages 10–15, ECACC, Salisbury, UK) were cultured in growth medium (GM) in an incubator at 37 °C and 5% CO_2. Cells were differentiated from myoblasts into myotubes with differentiation medium (DM) at 80% confluency. GM consisted of 500 ml DMEM high glucose, with L-glutamine, 75 ml FBS, 10 ml HEPES, 5 ml non-essential amino acids, and 2.5 ml gentamicin; whereas DM contained 10 ml HS instead of 75 ml FBS.

C2C12 Cells were seeded onto 12-well plates at a density of 20,000 cells ml^{-1} for the experiments involving monolayers. The monolayers were shifted from GM to DM as indicated in the respective experiments. For the 3D tissue culture, the cells were suspended in a rat-tail collagen I/matrigel mixture, as described previously by Breuls et al. (2003b) and Vandenburgh et al. (1996). In short, 5.10^6 C2C12 myoblasts were suspended in 1 ml of gel mixture and molded between two anchoring points. Matrix remodeling during subsequent culturing resulted in a pre-stress that induced a unidirectional cell configuration between the anchoring points. After 1 day of culture, the cells were shifted to DM to induce the formation of myotubes.

Progression of cell death in 2D and 3D cultures

To evaluate the behavior of YO-PRO-1 and PI as probes for monitoring apoptosis and necrosis in living cultures, positive control experiments, in which apoptosis was induced, were performed. Cell death in these experiments was quantified (by a Matlab computer program) and qualified (from nuclear morphology).

C2C12 Myoblasts were differentiated with DM after 3 days of culture in GM in 12-well plates. At day 8 of culture 2 μl ml^{-1} staurosporine ready-made (Sigma) was added to the cells to induce apoptosis (different concentrations were only applied, where mentioned). This method was described by McArdle et al. (1999), who evaluated the effect of this agent on C2C12 cells by Annexin V staining and TUNEL labeling. They showed that apoptosis can be induced in C2C12 mouse myoblasts within 2 h (phosphatidyl serine exposure) by addition of staurosporine (a protein kinase inhibitor). In the control group no staurosporine was added. In both groups, YO-PRO-1 and propidium iodide were added to the cultures. The cells were placed in an incubator. After 0, 2, 3, 4, 5, 6, 7, 8, 9 and 10 h they were transferred to a confocal laser scanning microscope for a short period of time, to monitor YO-PRO-1 and propidium iodide fluorescence. Confocal images were taken at locations that were memorized by the microscope software. Scans were performed on 6 (first experiment) or 9 (second experiment) locations in 3 wells in each group. The above experiment was conducted twice to determine the reproducibility (reliability) of the procedure. During this experiment also higher magnification images were captured to check for changes in nuclear morphology (e.g., c-shape, nuclear fragmentation) that confirm development of apoptosis.

The above procedure was also carried out for evaluation of cell death progression in the 3D cultures. The 3D tissue was differentiated after 1 day of culture and the experiment was performed on day 4 of culture.

YO-PRO-1 and PI dual staining

YO-PRO-1 and propidium iodide were applied, respectively, for monitoring apoptosis and necrosis in the cell and tissue cultures. YO-PRO®-1 iodide (491/509 nm, Y3603, Molecular Probes) was dissolved in DMSO to a final concentration of 1 mM. YO-PRO-1 and propidium iodide (P3566, 1.0 mg ml^{-1} in water, Molecular Probes) were then diluted in DM. PI was diluted to a final concentration of 10 μg ml^{-1}, as described previously by Breuls et al. (2003a). The optimal concentration of YO-PRO-1 was determined at 1 μM (see 'Probe performance' Sections).

Visualization

The progression of cell death was monitored every hour on a confocal laser scanning microscope (Axiovert 100M, Zeiss, Göttingen, Germany) with a 10× magnification objective. For the detection of YO-PRO-1 fluorescence the probe was excited with a 25 mW Argon laser at 488 nm. Emission was recorded above 510 nm. Propidium iodide staining was measured by excitation with a 1 mW HeNe laser at 538 nm. The microscope was equipped with a motorized stage. The LSM 510 (Zeiss) software enabled memorization of stage positions. In this way, cell death development could be monitored in time on fixed locations. For the 3D cultures, z-stacks were taken at each location in time (an example is shown in Figure 2a). Before quantification, these were projected to render a 2D representation. In the 3D cultures applied here, the projection was allowed as nuclei hardly ever overlapped. The effects of photo bleaching on cell death appeared negligible as evidenced from regular comparison of cell death progression on locations that were not scanned before.

Probe performance

Probe performance was evaluated by determining the optimal concentration of YP that was intense enough for quantification and on the other hand as low as possible to avoid any interference with cell viability. The performance was further evaluated by determining the probe intensity over a 3-day-period. Finally, the probe should be non-toxic to the culture, so the influence of the probe presence in the culture medium was evaluated by an MTT assay.

Optimal YO-PRO-1 concentration and probe intensity in time. The optimal concentration of YP and the probe intensity were evaluated in one experiment. Several concentrations of YO-PRO-1 in GM were added to wells after an attachment period of 24 h: 0.01, 0.1 and 1.0 μM together with 10 μM propidium iodide. The performance of the probe could only be evaluated after induction of apoptosis. Therefore, staurosporine was added to the cells at 24, 48, and 72 h (after adding the fluorescent probes). Cells were only taken out of the incubator for capturing confocal images. Probe intensity was measured at six randomly chosen locations for each concentration on the confocal laser scanning microscope, 7 h after staurosporine addition, at 31, 55, and 79 h. From the same experiment, the optimal YP concentration was determined.

Cell metabolism. The effects of the dual staining on cell metabolism were studied by an MTT assay over a period of 3 days in GM. In short, four groups were formed ($n = 3$) in 12-well plates: control cells in GM, cells under 1 μM YO-PRO-1 in GM, cells in 10 μM propidium iodide in GM, cells in both YP and PI in GM. After seeding, the cells were allowed to attach for 24 h in GM. Thereafter, the respective groups were formed and metabolic activity was assessed after 24, 48, and 72 h of exposure. The MTT assay involved a washing step in PBS before addition of 250 μl GM and 20 μl MTT (5 mg ml^{-1} in PBS) to each well. The cells were incubated in this MTT mix for 30 min to allow crystal formation in metabolically active cells. Subsequently, the MTT mix was removed and replaced with isopropanol containing 10% Triton-X100 and HCl (Sigma, M-8910) to dissolve the purple crystals on a shaking table for 10–15 min (the blank reading was performed on the solubilization solution). Absorbance was measured at 570 nm, after transfer into a 96-well plate.

Quantification of cell death in time

A custom-made program in MATLAB® was written for intensity threshold-based detection of pixels that either belonged to a green or red stained nucleus. First, background noise is removed by defining 95% of the first image of a series (time point 0) as noise. This noise can subsequently be removed from the successive images on the same location. Based on visually pre-determined threshold values, the program calculates the number of areas that have an intensity exceeding the threshold. The program can also separate overlapping nuclei. The program's results were verified by comparison with results from manual quantification. A small difference (maximum 5%) between results from both methods was found.

Statistical analysis

Differences in mean of the (raw) cell metabolism data were evaluated by one-way ANOVA. *Post-hoc* analysis was performed by applying Fisher's least significant difference (LSD) procedure. The numbers of apoptotic and necrotic nuclei, in the 2D and 3D cultures, were compared by their medians for each time point between the groups (with or without staurosporine). For this, Mann–Whitney (Wilcoxon) W tests were performed. All statistical calculations were carried out in STATGRAPHICS Plus 5.1.

Results

Progression of cell death in 2D and 3D cultures

Images of the development of cell death in time as a result of staurosporine addition in a monolayer were recorded (Figure 1a). The amounts of YP-positive nuclei and PI-positive nuclei were quantified in time for both the control group (no staurosporine) and the experiment group (staurosporine added) (Figure 1b), applying the Matlab program. The first signs of apoptosis as observed by YP staining of nuclei occurred between 2 and 3 h after staurosporine addition. After 8 h, part of the nuclei that stained positive for apoptosis had become necrotic, as evidenced from a positive propidium iodide signal. A convenient property of the dual staining method was observed: the propidium iodide probe replaced the YO-PRO-1 nuclear staining completely, once necrosis developed (Figure 1a, arrowheads).

The reproducibility of the method was derived from the repeated experiments. In both experiments, elevated amounts of apoptotic nuclei (in the staurosporine treated cultures) were evident

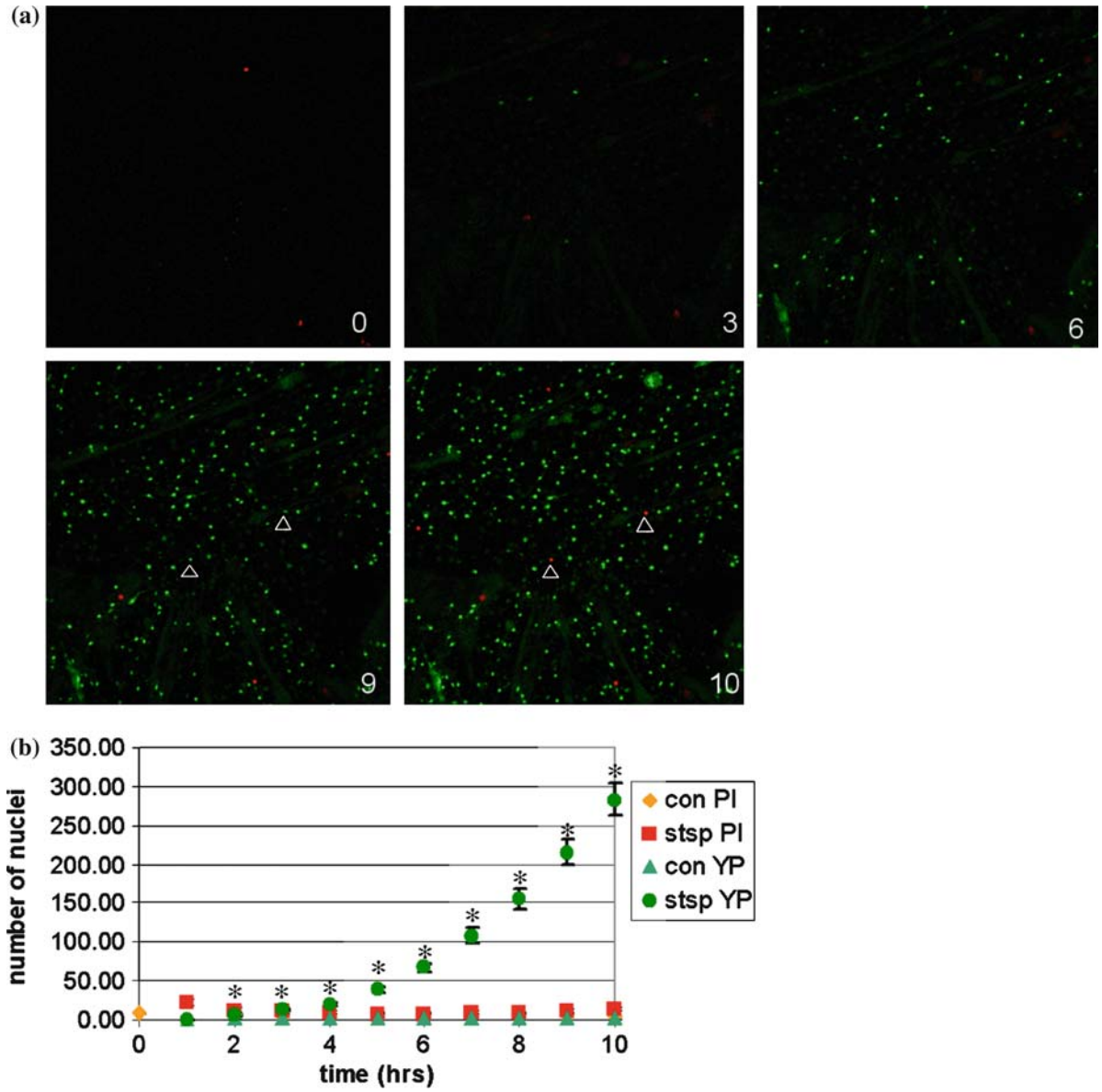

Figure 1. Development of apoptosis and necrosis after staurosporine addition to a C2C12 murine myoblast and myotube monolayer (differentiated for 5 days). (a) Time series showing apoptotic (green) and necrotic (red) nuclei in a monolayer culture at a fixed location in time, at 0, 3, 6, 9 and 10 h after staurosporine addition to a C2C12 culture (100× magnification). Note the color shift of the nucleus at the arrowheads. (b) Number of apoptotic (green, YP) and necrotic (red, PI) nuclei in time, for both control cells (con) and cells affected by staurosporine (stsp) (*$p < 0.05$).

within 2–3 h. The rates of increase in apoptotic nuclei and the maximum number after 10 h were comparable as well.

In the 3D cultures, a comparable progression of cell death in time was observed (Figure 2b). An increase in the number of apoptotic nuclei after 2–3 h as observed in the 2D culture could not be confirmed (only after 4 h), as no images were taken at these time points. After 6 h, the number of apoptotic cells seemed to have reached a constant value. At this moment an increase in the number of necrotic cells commenced, as well as a slight increase in the number of necrotic cells in the control.

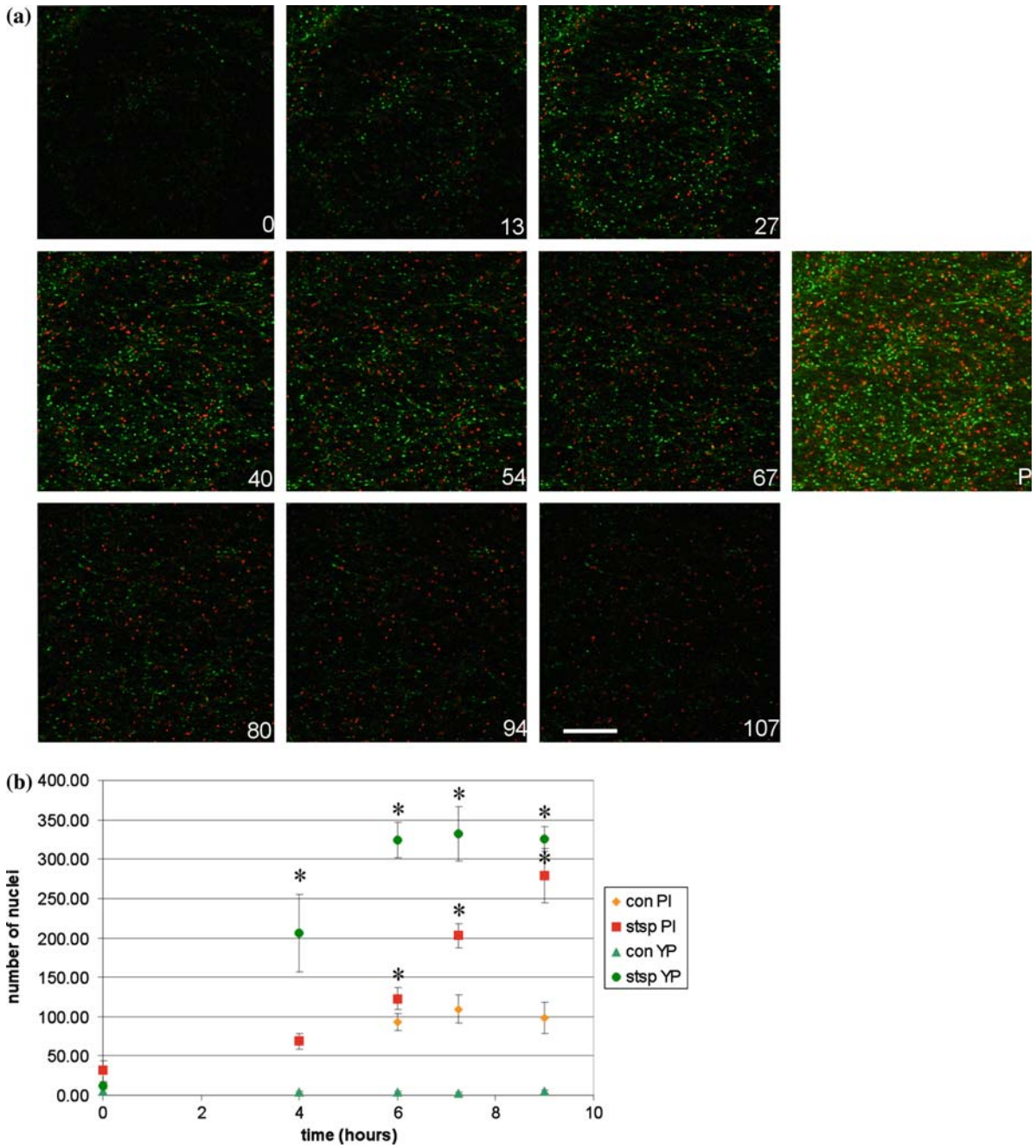

Figure 2. Projection of 3D data is demonstrated and results of the subsequent quantification. (a) A selection of slices through an engineered muscle construct is presented at 0, 13, 27, 40, 54, 67, 80, 94 and 107 μm of height, respectively. On the right, P shows the projection of the complete dataset, which can be analyzed by a computer program. (b) Progression of cell death in time in 3D muscle cultures after addition of staurosporine (stsp) or without staurosporine (con). PI nuclei represent the necrotic nuclei population, and YP shows numbers of apoptotic nuclei in time (*$p < 0.10$).

Nuclear morphology

Changes in nuclear morphology were monitored in time at fixed locations. Once cell permeability had increased by apoptosis, YO-PRO-1 could enter the cell and stain the nucleus. In time, an increase in intensity of the green staining was observed. Upon completion of the apoptotic process, necrosis initiated and the green nuclear stain was completely replaced by the red fluorescent propidium iodide, as mentioned before. The change from green fluorescent to red fluorescent labeling of the nuclei occurred within an hour. Therefore, the transitional period, the period in which the nucleus exhibited both green and red fluorescence, was rarely found in the images.

The increased cell membrane permeability was not only observed by influx of the fluorescent probes. Apoptotic cells had an intact cell membrane, whereas necrotic nuclei had no distinguishable membrane (Figure 3). YO-PRO-1 staining of the nuclei during development of apoptosis facilitated monitoring of nuclear morphology. Characteristics of apoptotic nuclei include chromatin condensation, fragmentation, and formation of c-shaped figures. In Figure 4a, c-shaped figures and nuclear fragmentation are indicated by arrowheads. Early and late necrosis could be distinguished by an increasing size of the nucleus and decreasing intensity, caused by nuclear disintegration.

Probe performance

Optimal YO-PRO-1 concentration and probe intensity in time. The lowest concentration at which the probe still stained the nuclei of apoptotic cells was determined by incubation with staurosporine. Sufficient staining was seen at 1.0 μl ml^{-1} and this concentration was applied in all other experiments. At this concentration, the probe was still capable of marking apoptotic cells after 3 days (79 h). An increase in background signal was noticed with time. The difference in signal intensity between apoptotic nuclei and background staining was too low at 0.1 μl ml^{-1} YP to distinguish them properly. At 0.01 μl ml^{-1} of YP the probe concentration was found to be too low to distinguish apoptotic nuclei from the background even just after adding the probe to the cells (i.e., when the background staining is lowest).

Cell metabolism. The effects of YP, PI, and both YP and PI on cell metabolism are summarized in Figure 5. The blank measurement was subtracted from the absorption values for the control group (GM) and the three experiment groups (Figure 5a). In the control group a maximum increase

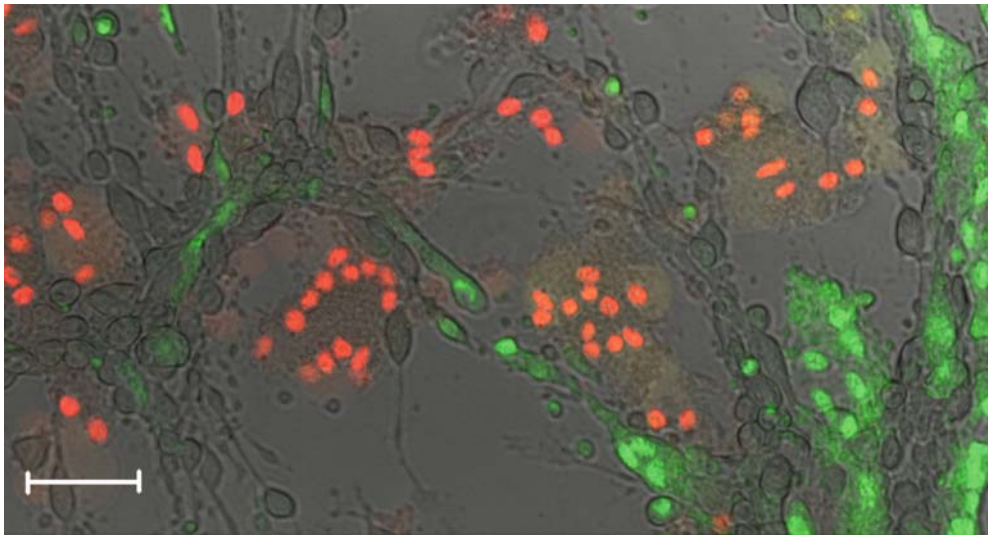

Figure 3. This image shows the intact plasma membranes in viable and apoptotic cells (C2C12, see Figure 1), whereas those of necrotic cells were hardly distinguishable. The image was captured 5 h after induction of apoptosis (staurosporine 5 μl ml^{-1}). Bar denotes 50 μm.

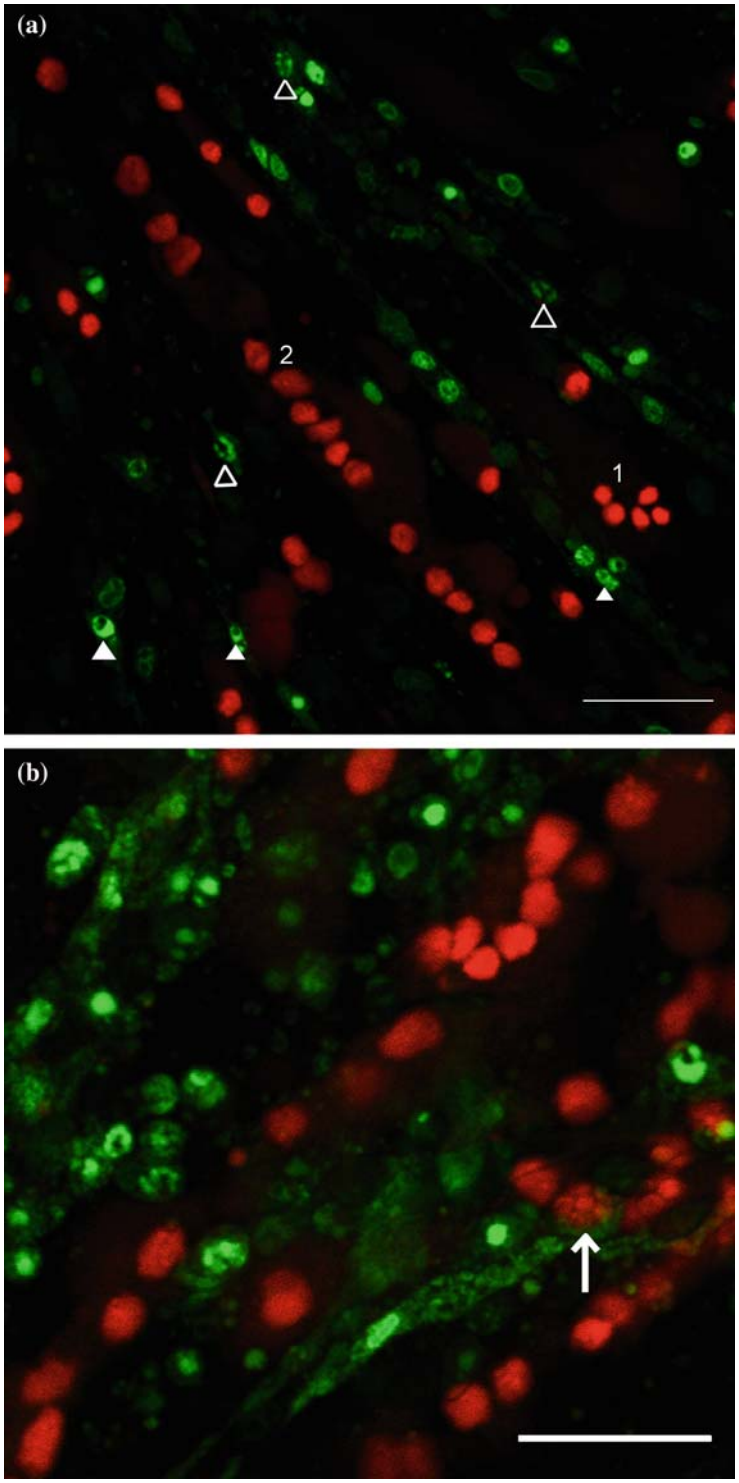

Figure 4. The effects on nuclear morphology of C2C12 cells (see Figure 1) after staurosporine addition, are shown. (a) Approximately 6.5 h after addition of 2 μl ml^{-1} staurosporine, nuclear fragmentation (open arrowheads) and crescent-shaped figures (closed arrowheads) were observed in C2C12 cells. Necrotic nuclei were marked by propidium iodide in red and showed no clear nuclear morphology in early (1) or late (2) necrosis. (b) The arrow shows a necrotic nucleus with apoptotic appearance. Bars denote 50 μm.

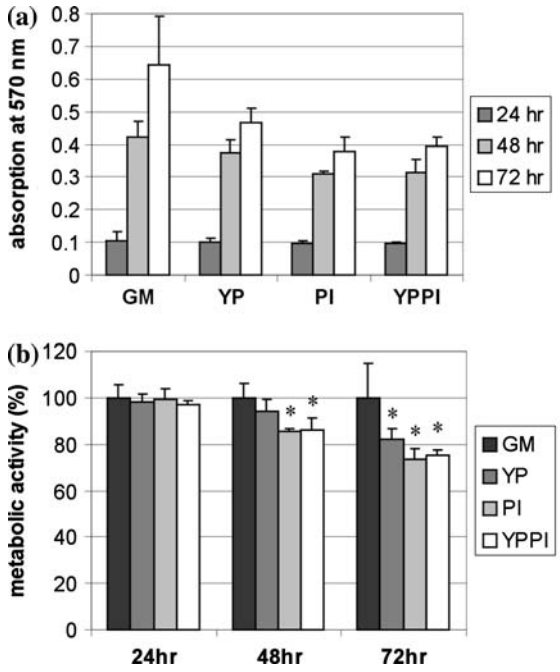

Figure 5. Absorption and metabolic activities of C2C12 cells (see Figure 1) for 3 days and three medium groups are shown. (a) Absorption values at 570 nm (corrected for blank value) are shown for the medium groups (GM, YP, PI, YP + PI) at 24, 48 and 72 h. Within one medium group, all time groups are significantly different from both others. (b) Metabolic activity of all medium groups is shown, relative to the metabolic activity of the control group (GM) (*$p < 0.05$).

in metabolic activity as a result of proliferation is observed. The experimental groups show a more moderate increase in metabolism. The absorption values were normalized to the control values from the cells in GM (Figure 5b). Different effects were observed for the presence of YP and PI (or both) in the growth medium after 24, 48, or 72 h of culture. Addition of YP to culture medium resulted in a significant decrease in activity after 3 days. Presence of PI in the medium resulted in declined metabolic activity of the cells after 48 h. This decrease was similar to the one observed for addition of both, YP and PI. So, the addition of both probes did not render a synergistic effect.

Discussion

The present study shows the feasibility of YO-PRO-1/propidium iodide dual staining as a tool for spatial monitoring of apoptosis and necrosis in an ongoing culture. Apoptosis and necrosis can be quantified and distinguished in living cell cultures and engineered tissues in a non-destructive manner.

Induction of apoptosis in 2D and 3D cultures

From the results it can be concluded that C2C12 apoptosis, induced by staurosporine, can be monitored by YP staining, at least 5–6 h before definite cell death (defined from PI labeling) commences. Thus, by applying the dual staining method presented in this study, it was possible to differentiate between two consecutive stages of cell death.

Previously, McArdle et al. (1999) induced apoptosis in C2C12 cultures by addition of 2 μM staurosporine. They observed phosphatidyl exposure through annexin V binding from 2 h after addition until 4 h. From this, it can be assumed that YP stains apoptotic nuclei just after and concomitantly with early detectable events in apoptosis after staurosporine treatment. Also, labeling persisted during later apoptotic phenomena, such as cell shrinkage, nuclear condensation, and procaspase activation, and positive TUNEL labeling, as reported by McArdle's group as well. Twelve hours after induction, they found DNA laddering before cell detachment was completed

(18 h). After 24 h the plasma membrane and nuclear membranes were fragmented, and the mitochondria were swollen and disrupted.

The development of apoptosis in the monolayer experiments started in both experiments 2–3 h after addition of staurosporine. Thereafter, the number of nuclei undergoing apoptosis increased with time in a comparable, almost linear fashion. Also, the time point, at which the rise in the amount of necrotic nuclei occurs, is similar. The performance of the dual staining method was found to be reproducible.

The markedly different course of cell death development in the 3D culture could be explained by a different passage number of the cells and the extended culture time. Another factor influencing the culture's behavior was the 3D environment. Due to the collagen gel mixture, dead cells were trapped and shown on the initial cell death measurements as an increased number of necrotic cells. In monolayers, these dead cells normally detach from a culture substrate and float in the medium (out of the field of view) until washed away during medium replacement. Detached cells floated in the medium, above the focal plane of the confocal microscope and thus stayed 'undetected.'

Nuclear morphology

YP-Staining marked the presence of nuclear shapes characteristic of apoptosis. The shapes confirmed the execution of apoptosis in the cells exhibiting YO-PRO-1 staining. Crescent c-shaped figures and condensed fragmented nuclei were observed. The study by McArdle et al. (1999) confirmed the execution of apoptosis after addition of staurosporine to C2C12 cell culture. Compared to the annexin V staining applied in their work, YP staining exhibited a prolonged period of apoptotic cell identification. At the final stages of apoptosis, upon the influx of PI, the bonds of YP to the nucleic acids were terminated, as the PI replaced the YP staining of the nucleus completely within a short time span. The nuclear morphology, characteristic of apoptosis, was no longer seen in this stage of cell death. Besides a distinction based on nuclear labels (green vs. red), the nuclear morphology can be applied to distinguish the type of cell death as well. Confusion may arise because the transition from apoptosis to necrosis may occur at any time point during the development of apoptosis. This is dependent on the energy level of the cell. In case of ATP (the cell's energy source) depletion, the process of apoptosis cannot be completed and necrosis will commence. This may result in nuclear morphology, typical for apoptosis with a necrotic (PI) staining (Figure 4b).

Probe performance

Probe performance was evaluated by determining the optimal probe concentration, intensity of the probe in time, and probe toxicity.

Optimal YO-PRO-1 concentration and probe intensity in time. Since the best results were obtained with the maximum amount of YP in GM that was tested, one might expect that adding larger concentrations of the probe will further increase the distinguishing capability of the probe. However, in this study the minimally required amount of the probe for sufficiently marking apoptotic cells was determined. The intensity of the probe was sufficient up to 3 days after probe addition. In experiments exceeding this period, one might consider to reload YP/PI into the medium. However, the effects on cell metabolism need to be considered.

After 3–4 h of incubating the cells with YO-PRO-1, an increased background staining of the cells was detected. This can be attributed to the presence of RNA in the cytoplasm of the cells (Suzuki et al. 1997). In online monitoring of cell viability it is not desirable to apply RNase for removal of this background staining. The background signal intensity of the RNA-bound YO-PRO-1 was lower than that of DNA-bound YO-PRO-1. This might be caused by the more condensed structure of the DNA in the nucleus as compared to the more diffuse appearance of the RNA and DNA in the cytoplasm. In the presently used two-laser scanning system with YP and PI, in which no additional channel is available for e.g., cell monitoring, the RNA staining can be applied to track the cells in the culture by increasing the gain of the emission signal. In this way, no extra channel is required for detection of the cells. However, the use of YP for cell tracker purposes exclusively is not recommendable.

Cell metabolism. The cell metabolism was clearly affected by addition of either YP or PI to the

medium, or both. After 24 h, the maximal decrease in metabolism was approximately 10%. However, this decrease was not found to be statistically significant. One might consider this as a tolerable influence on the culture. After 48 h, the negative effect was significant as compared to the control group for addition of either PI alone, or YP and PI. Addition of YP still did not significantly influence the cell metabolism. Extension of the experiment duration rendered a maximal decrease in metabolic activity of 40% after 72 h. So, in experiments of periods exceeding 24 h, involving both of the probes, these effects should be taken into account. The decrease in metabolism can be explained as decreased metabolic activity of the cells or a (partial) impairment of cell proliferation and therefore decreased total metabolic activity. Also, metabolic decrease can be caused by cell death. So, if an experiment exceeding 24 h is performed, one might consider to more deeply study the effects on the cell culture to be able to differentiate the effects of the staining method or the experimental conditions on cell viability.

Despite the effects of YP and PI on cell metabolism during extended periods of time (>1 day), the dual staining method is a convenient, non-destructive method for real-time quantification of apoptosis and necrosis on a local level in 2D and 3D cultures. The ability to distinguish, quantify and localize both kinds of cell death can contribute in elucidating cell death pathways in all research focusing on cell death development in monolayer cultures or (engineered) tissues. And finally, the dual staining facilitates online assessment of ultrastructural changes in the appearance of dying nuclei.

In future, the dual staining method will be applied in our lab to quantify the development of cell death in engineered muscle tissue. Cell death will be induced by tissue compression and ischemia to study the pathway of cell death development in (deep) pressure ulcers.

Acknowledgements

Financial support by The Dutch Technology Foundation (STW, Grant EFB.6003) is gratefully acknowledged.

References

Blankenberg F.G., Tait J.F. and Strauss H.W. 2000. Apoptotic cell death: its implications for imaging in the next millennium. Eur. J. Nucl. Med. 27: 359–367.

Boffa D.J., Waka J., Thomas D., Suh S., Curran K., Sharma V.K., Besada M. and Muthukumar T. et al. 2005. Measurement of apoptosis of intact human islets by confocal optical sectioning and stereologic analysis of YO-PRO-1-stained islets. Transplantation 79: 842–845.

Breuls R.G., Bouten C.V., Oomens C.W., Bader D.L. and Baaijens F.P. 2003a. Compression induced cell damage in engineered muscle tissue: an *in vitro* model to study pressure ulcer aetiology. Ann. Biomed. Eng. 31: 1357–1364.

Breuls R.G., Mol A., Petterson R., Oomens C.W., Baaijens F.P. and Bouten C.V. 2003b. Monitoring local cell viability in engineered tissues: a fast, quantitative, and non-destructive approach. Tissue Eng. 9: 269–281.

Chung K.T. 1983. The significance of azo-reduction in the mutagenesis & carcinogenesis of azo dyes. Mutat. Res. 114: 269–281.

Idziorek T., Estaquier J., De Bels F. and Ameisen J.C. 1995. YOPRO-1 permits cytofluorometric analysis of programmed cell death (apoptosis) without interfering with cell viability. J. Immunol. Methods 185: 249–258.

Jerome K.R., Chen Z., Lang R., Torres M.R., Hofmeister J., Smith S., Fox R., Froelich C.J. and Corey L. 2001. HSV and glycoprotein J inhibit caspase activation and apoptosis induced by granzyme B or Fas. J. Immunol. 167: 3928–3935.

Liu X., Van Vleet T. and Schnellmann R.G. 2004. The role of calpain in oncotic cell death. Annu. Rev. Pharmacol. Toxicol. 44: 349–370.

McArdle A., Maglara A., Appleton P., Watson A.J., Grierson I. and Jackson M.J. 1999. Apoptosis in multinucleated skeletal muscle myotubes. Lab. Invest. 79: 1069–1076.

McGuire T.F., Trump D.L. and Johnson C.S. 2001. Vitamin D(3)-induced apoptosis of murine squamous cell carcinoma cells. Selective induction of caspase-dependent MEK cleavage and up-regulation of MEKK-1. J. Biol. Chem. 276: 26365–26373.

Mosmann T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods 65: 55–63.

Park J.C., Hwang Y.S. and Suh H. 2000. Viability evaluation of engineered tissues. Yonsei Med. J. 41: 836–844.

Rieseberg M., Kasper C., Reardon K.F. and Scheper T. 2001. Flow cytometry in biotechnology. Appl. Microbiol. Biotechnol. 56: 350–360.

Stadelmann C. and Lassmann H. 2000. Detection of apoptosis in tissue sections. Cell. Tissue Res. 301: 19–31.

Suzuki T., Fujikura K., Higashiyama T. and Takata K. 1997. DNA staining for fluorescence and laser confocal microscopy. J. Histochem. Cytochem. 45: 49–53.

Vandenburgh H., Del Tatto M., Shansky J., Lemaire J., Chang A., Payumo F., Lee P., Goodyear A. and Raven L. 1996. Tissue-engineered skeletal muscle organoids for reversible gene therapy. Hum. Gene Ther. 7: 2195–2200.

Wang Y.N., Bouten C.V., Lee D.A. and Bader D.L. 2004. Compression induced damage in a muscle cell model '*in vitro*'. Proc. Inst. Mech. Eng. [H] 219: 1–12.

Wronski R., Golob N., Grygar E. and Windisch M. 2002. Two-color, fluorescence-based microplate assay for apoptosis detection. BioTechniques 32: 666–668.