

# Confocal restricted-height imaging of suspension cells (CRISC) in a PDMS microdevice during apoptosis†

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We have monitored and imaged cell death induced in human leukemic U937 cells over time using three-color confocal imaging. Three different apoptotic inducers, anti-Fas, TNF- $\alpha$  and Etoposide were used. Individual cascaded events such as loss of mitochondrial transmembrane potential, exposure of phosphatidyl-serine, membrane blebbing and permeabilization of the cell membrane have been observed in real time with different individual cells. From the results, an interesting heterogeneity in the apoptotic phenotype has been observed. The CRISC method is easy to use and provides biologist with a powerful additional tool to study in real-time processes of several hours of duration such as apoptosis. We predict that the period of cell viability obtained after protein coating of the PDMS devices (>80 h) will also allow monitoring of other biological processes of longer duration or long onset time, such as mitosis, phagocytosis and differentiation.

## Introduction

Apoptosis is the process by which cells in animal organisms die in order to maintain an appropriate cell number, or as a consequence of pathological situations.<sup>1</sup> This process is highly regulated by activation and deactivation of proteins. The main orchestrators of this process are proteases called caspases, which cleave other proteins, altering the maintenance of cellular structures and metabolism.<sup>2</sup> Caspases can be activated by signals transduced by receptors in the cell membrane such as Fas, TNF- $\alpha$  or TRAIL, or by removal of extracellular survival signals. Another way to activate caspases is by the formation of a multiprotein complex called the apoptosome, which is assembled after some proteins have been released from the mitochondria. The mitochondrion is in this case the cellular organelle that integrates pro- and anti-apoptotic signals from within the cell.<sup>3</sup>

The biochemical events that occur during apoptosis have been extensively studied during the past decade. However, it was only recently that a few studies have revealed the importance of real-time single cell analysis in order to determine how these events are temporally related.<sup>4</sup> As apoptosis is a process that does not happen simultaneously in all the cells of a population, the correct order of events cannot be determined unless these events are observed in a single cell. Also, in whole cell population studies, an event that takes place during a few minutes in an individual cell can be erroneously described to occur during several hours. For instance, the release of cytochrome c from all the mitochondria in a cell occurs in 5 min, as observed by microscopy.<sup>5</sup> However, when the same event was analyzed by an assay involving kinetic studies of several thousand cells, this process seemed to take hours.

The most extensively used method of real time, single-cell analysis is the imaging of fluorescent signals by confocal microscopy. These assays involve following an individual cell (or several cells within a microscope field) during several hours. This method has been successfully used to monitor apoptosis in cells that grow attached to the substrate (adherent cells). However, some adherent cells detach during apoptosis, making the continuous tracking of a cell an impossible task. Also, cells that grow in suspension such as cells from the immune system are difficult to study under these conditions, as they cannot be immobilized for a few hours without a substantial loss of viability.

In the past few years, microfluidic structures have received increasing attention for life-sciences applications, with a recent focus on cell research in particular.<sup>6</sup> Apart from the unique capability to manipulate (sub)nanoliter volumes in a controlled way, microfluidics has been used for cell lysis,<sup>7</sup> electroporation,<sup>8,9</sup> cell culturing,<sup>10</sup> creation of chemical gradients over a single cell<sup>11</sup> and cell immobilization (docking, trapping)<sup>12</sup> among other applications. Recently, microfluidic devices have also been specifically utilized to study apoptosis. Some of the individual events occurring in the apoptotic cascade such as DNA fragmentation,<sup>13</sup> externalization of phosphatidylserine<sup>14</sup> and change of intracellular Ca<sup>2+</sup> concentration<sup>12</sup> have been investigated separately. However, so far there has been no method that allows for continuous monitoring of several of these events in individual cells in real time. In a recent study,<sup>15</sup> we designed and used a microfluidic trap to immobilize cells in order to study kinetics of apoptosis, but that microfluidic device, consisting of silicon anodically bonded to glass, was intended for use in an inverted microscope and did not allow for imaging in a confocal microscope because of the large thickness of the bonded glass used. Therefore, we here propose a simple method that uses shallow (25  $\mu\text{m}$ ) microfluidic channels in polydimethylsiloxane (PDMS) to restrict the height of the area of interest for the microscope, thereby assuring a continuous focusing of

† Electronic supplementary information (ESI) available: Etoposide-induced apoptosis time-lapse experiment. See [http://www.rsc.org/suppdata/loc/b5/b503770k/](http://www.rsc.org/suppdata/loc/b5/b503770k)  
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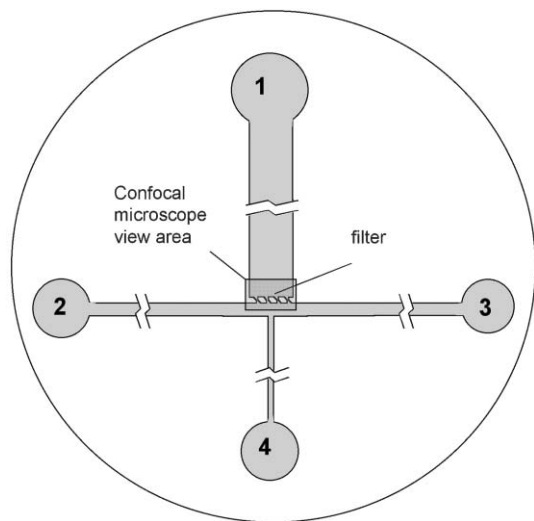
the cells, while still allowing some freedom of movement for suspension cells. The PDMS chips can be attached to standard glass-bottom culture dishes and are thus fully compatible with existing experimental setups to optically monitor cells over time.

## Experimental

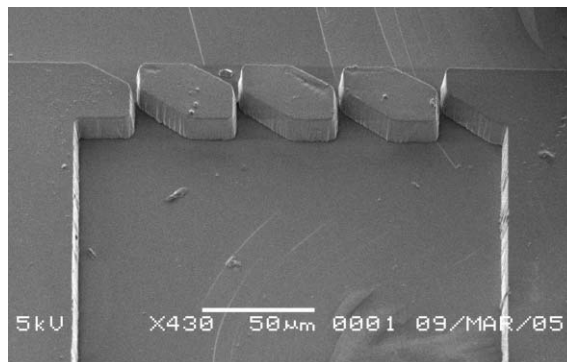
### Chip fabrication

We designed and fabricated PDMS chips containing varying microfluidic channel geometries but with a general scheme as indicated in Fig. 1, and with sizes varying from  $5 \times 20$  mm to  $10 \times 25$  mm. Each chip has four external connections enabling easy filling, rinsing and change of medium, and a filter structure connecting two channels.

Silicon molds used for fabrication of the PDMS chips were realized using DRIE using the Bosch process. Molds with two different etch depths, 15 and 25  $\mu\text{m}$  (Veeco Dektak 8 surface profiler), were fabricated. In Fig. 2 the three-dimensional structure of the 15  $\mu\text{m}$  filter is shown. PDMS chips were made from Sylgard 184 (Dow Corning). A 1 : 10 mixture (hardener : monomer) was degassed in a vacuum chamber for 30 min, and then poured from a small distance over the mold to avoid air bubble inclusion. After a second degassing step the assembly was cured for 2 h at 60  $^{\circ}\text{C}$ . After curing, the PDMS was removed from the mold and subjected to an oxygen plasma treatment during 4 min (Nanotech plasmaprep 100, 30 W). Through holes were mechanically punctured in the PDMS sheet for external access to the channels. Finally the PDMS sheet was sealed to a thin (200  $\mu\text{m}$ ) polypropylene foil to secure long term storage and protection of the microfluidic structures, followed by a moderate heat treatment for improved adhesion. The PDMS chips used for the experiments described in this article were  $10 \times 20$  mm (original size). Prior to use, they were cut from the sheet to fit in the 14 mm diameter glass surface of 35 mm glass-bottom microwell dishes (MatTek Corporation) used for experiments. Typically, the part of the



**Fig. 1** Schematic layout of the PDMS chip. Depending on the requirements of the experiment and the size of the microscope well/dish used, the chip can be cut smaller, and parts of the channels and up to 3 of the vials (2, 3 and 4) can be discarded.



**Fig. 2** SEM picture showing filter structure.

chips containing vials 2 and 4 (Fig. 1) was discarded, so that the final structure contained the channels from the filter to vials 1 and 3, and only the most internal part of the other two channels (2–4 mm from the filter). After removing the sealed polypropylene sheet, the single PDMS chips were attached to the glass bottom plate by applying a slight mechanical force. In certain cases, PDMS chips were reused after thoroughly rinsing the chip with ethanol and allowing it to dry in air.

### Coating the PDMS chips

PDMS chips were coated with either fetal bovine serum (FBS) (Omega) or a solution of  $100 \mu\text{g ml}^{-1}$  human plasma fibronectin (Chemicon International). In both cases, the coating solution was aspirated into the chip by connecting vacuum tubing to one of the connection vials and covering the other three with a droplet of coating solution. After filling the chip with the coating solution, it was put in the cell-culture incubator for 30 min.

### Filling the PDMS chips with cells

After aspiration of the coating solution out of the microchannels, the attached PDMS chips were filled and thoroughly rinsed with a mixture of culture medium, dyes and apoptotic inducer, but no cells. Then a droplet of the same medium mixture plus cells ( $\approx 1\text{--}5 \times 10^6 \text{ ml}^{-1}$ ) was added to vial 1 (see Fig. 1). We used the 25  $\mu\text{m}$  filters for the experiments described in this article, as U937 cells are 15–20  $\mu\text{m}$  in diameter. Cells were allowed to slowly migrate towards the filter structure. In some cases additional aspiration through vials 1–3 was required to move the cells towards the filter structure. After the filter area (confocal microscope view area in Fig. 1) was filled with the appropriate amount ( $\approx 10$ ) cells, superfluous cell solution was removed from vial 1 with tissue paper and the whole assembly was covered immediately with 2 ml of media with dyes and inducers, thereby equalizing pressure differences between the different vials and thus effectively eliminating any residual flow or cell movement in the microfluidic device. We found during filling of the PDMS chip that even with relatively small pressure differences some cells were drawn through the filter, while others remained stuck in it. Usually, those cells were somehow damaged and prone to die quickly. Fig. 3 shows a differential image contrast (DIC, Zeiss Axiovert 200M) image illustrating filter structure and U937 cells.

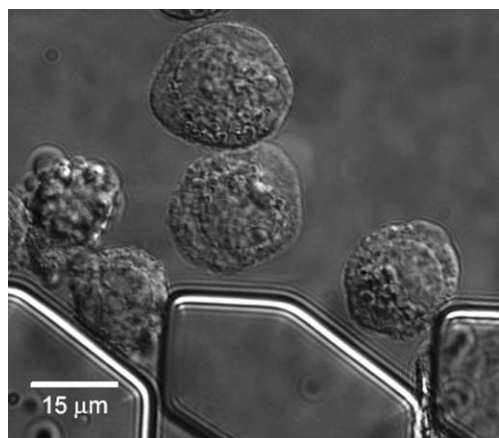


Fig. 3 DIC picture of U937 cells in filter chamber.

### Cell culturing

Human U937 myeloid leukemic cells were maintained in RPMI 1640 medium (Mediatech Inc.) containing 10% fetal bovine serum, 2 mM glutamine, 200  $\mu\text{g ml}^{-1}$  penicillin and 100  $\mu\text{g ml}^{-1}$  streptomycin sulfate at 37 °C in a humidified 5%  $\text{CO}_2$ , 95% air incubator.

### Staining and apoptosis induction

Exponentially growing cells were centrifuged and resuspended in fresh pre-warmed media for every experiment. For apoptosis studies, the culture media was supplemented with the appropriate inducers: etoposide 50  $\mu\text{M}$  (Sigma), anti-Fas 50  $\text{ng ml}^{-1}$  (CH11 antibody, MBL Co, Ltd), human Tumor Necrosis Factor- $\alpha$  50  $\text{ng ml}^{-1}$  (TNF- $\alpha$ , PeproTech Inc). For treatments with anti-Fas and TNF, 2-deoxy-D-glucose 5 mM (Sigma) was added to the media.

For microscopy experiments, the media was also supplemented with 20 mM HEPES (pH 7.3) for pH buffering, 55  $\mu\text{M}$  2-mercaptoethanol for minimization of phototoxicity, and 2.5 mM  $\text{CaCl}_2$  for facilitation of Annexin-V binding. Dyes (all from Molecular Probes) were added at the following concentrations: tetramethylrhodamine ethyl ester (TMRE) 20 nM, YO-PRO-1 500 nM, Annexin V Alexa Fluor<sup>®</sup> 647 conjugate 0.5% (v/v).

### Time lapse experiments

For confocal time-lapse analysis, cells were placed in the microchannels, and the chips were covered with media as described above. Media was then overlaid with 2 ml mineral oil (Sav-On, Buena Park CA) in order to avoid excessive evaporation during experiments. The dish was placed in a Leiden Micro-Incubator coupled to a Bipolar Temperature Controller (Harvard Apparatus) (Fig. 4). Image acquisition started 10 min after the temperature was stable at 37 °C (typically 90 min after resuspension of the cells in media).

### Confocal microscopy

Fluorescence was monitored using a Nikon Eclipse TE 300 microscope coupled to a Biorad MRC 1024 confocal head

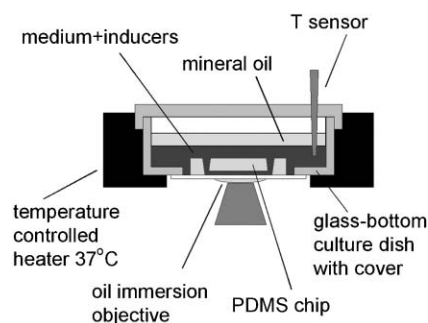


Fig. 4 Thermostated setup used in the confocal microscope.

and a 15 mW krypton/argon (Kr/Ar) laser unit. YO-PRO-1, TMRE and Annexin-647 were excited sequentially using the 488, 568 and 647 nm lines (respectively) of the laser. Laser intensity was typically attenuated at 98–94% (YO-PRO-1 and TMRE) and 88–94% (Annexin-647). Fluorescence was detected by the photomultipliers after transmission of a 522DF35 bandpass filter (YO-PRO-1), a 585EFLP filter (TMRE) or a 680DF32 filter (Annexin). Images were taken every 4 min with a 60 $\times$  oil objective.

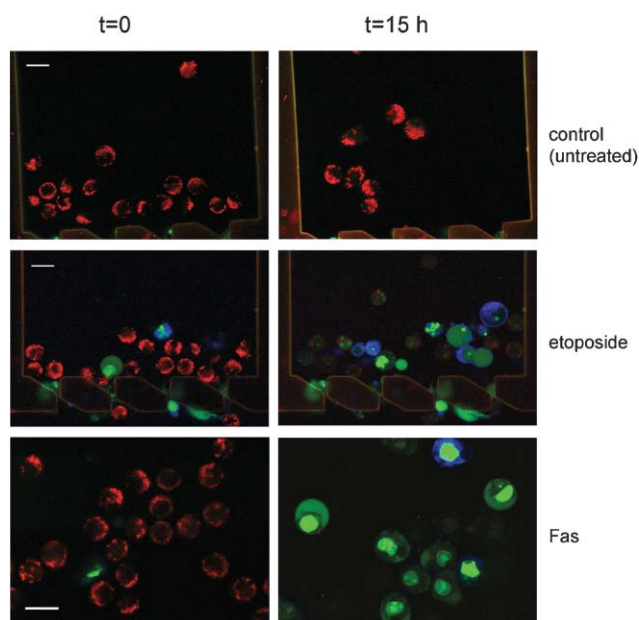
## Results and discussion

### Medium/long term culturing using coated chips

PDMS has been successfully employed as a substrate for growth of adherent cells.<sup>16</sup> However, there is no data about the growth of suspension cells in confined PDMS chips. We loaded new chips, without coating, with U937 cells, as described in the Experimental section, and cultured them at 37 °C. The survival of the cells under these conditions, as observed by cell morphology under the microscope, was low: over 90% of the cells appeared dead in 15 h (data not shown). We then prepared chips by coating them with protein-rich solutions, as described above: Fetal Bovine Serum and a fibronectin solution. The survival of the cells in coated chips after 90 h was comparable to normal viability rates in culture (below 10% death).

### Observation of apoptosis in single cells

In order to monitor cell death in the confocal microscope, we used 3 fluorescent dyes: TMRE, Annexin-V Alexa-Fluor 647 and YO-PRO-1. TMRE stains mitochondria, and its accumulation is dependent on mitochondrial potential.<sup>17</sup> Thus, a loss of TMRE fluorescence will indicate mitochondrial depolarization, which is an apoptotic event occurring as a consequence of caspase activation.<sup>18</sup> Annexin-V binds phosphatidylserine. This lipid can be normally found in the inner leaflet of the plasma membrane. However, during apoptosis there is a translocation of phosphatidylserine to the outer side of the plasma membrane.<sup>19,20</sup> YO-PRO-1 is a DNA dye, which is normally not cell-permeant. However, apoptotic cells become permeable to YO-PRO-1.<sup>21</sup> In Fig. 5 we show staining of the cells with the dyes mentioned above. The left side of the picture shows the cells at the beginning of the time-lapse experiment. The right hand side shows the cells after 15 h of incubation with two apoptotic inducers in the chips.

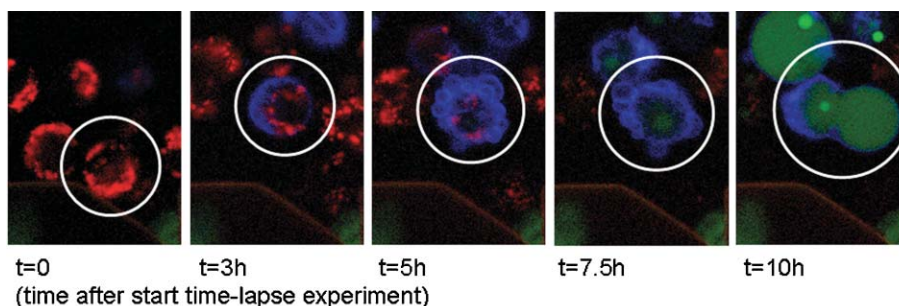


**Fig. 5** Snapshots taken from timelapse experiments at the start of the movie and after 15 h of incubation. Fluorescent signal from TMRE is shown in red, from YO-PRO-1 in green, and from Annexin V in blue. Bars = 20  $\mu\text{m}$ .

#### Etoposide-induced death

Etoposide is a chemotherapeutic drug used to treat tumors. U937 cells are very sensitive to etoposide. We treated the cells with etoposide, and monitored the death of the cells. The first and last picture of a time lapse experiment are shown in Fig. 5. At time = 0, we observed the presence of cells that had lost mitochondrial potential and thus do not appear red under the microscope. Many of these cells were YO-PRO-1 permeant but not Annexin-positive, suggesting that these cells had presumably lost the plasma membrane during the process of loading the chips.

A few hours later ( $t = 3$  h in Fig. 6), some cells started to lose mitochondrial potential, and acquired blue fluorescence in the membrane, indicating the translocation of the lipid phosphatidylserine to the outer leaflet of the plasma membrane. In some cells, we observed the appearance of blebs in the membrane; this is a common feature of apoptosis. 2–3 h later (as in the case of the cell shown in Fig. 6,  $t = 5$  h and 7.5 h), the cell became slightly permeable to YO-PRO-1. As the



**Fig. 6** Snapshots from the etoposide-induced apoptosis time-lapse experiment. Red, TMRE signal; green, YO-PRO-1 signal; blue, Annexin V. The width of the area shown for every snapshot is 45  $\mu\text{m}$ .

final step observed, there was a sudden (<8 min) massive permeabilization of the membrane, and the whole nucleus was stained with YO-PRO-1. This event is probably related to the phenomenon of secondary necrosis that happens to cells in culture after apoptosis and it is not supposed to happen *in vivo*, as apoptotic cells are phagocytosed quickly. A protrusion from the cell, filled with nucleic acids, was observed. The whole sequence of events is shown in a compressed movie (see ESI†).

#### Fas- and TNF- $\alpha$ -induced death

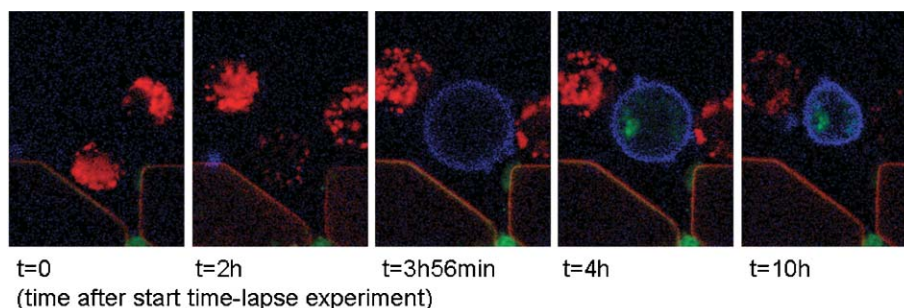
U937 cells die by apoptosis when Fas or TNF- $\alpha$  receptors are triggered in the membrane of the cells. These cells are particularly sensitive to a combination of 2-deoxyglucose and anti-Fas antibody or TNF.<sup>22</sup> In Fig. 5, we show how cells in the chip look after 15 h after treatment with anti-Fas. Cells treated with both inducers lost mitochondrial potential and became permeable to the DNA dye YO-PRO-1 (shown for Fas in Figs. 5 and 7). Some of the cells became Annexin-V positive before they were highly permeable to the DNA dye. The DNA staining was sudden (less than 4 min), as shown in Fig. 7 ( $t = 3$  h 56 min and  $t = 4$  h), indicating a sudden permeabilization to YO-PRO-1. The morphology of the dying cells, however, was different than the morphology of cells treated with etoposide. As an example, the cell shown in Fig. 7, maintained spherical morphology during death, as opposed to the irregular morphology that cells acquired during apoptosis induced by etoposide. These results suggest that, as has been described, these inducers trigger different biochemical cascades of protease activation during apoptosis.

#### Untreated cells

In order to verify that the changes that we observed were due to the presence of apoptosis inducers, and not to an effect of the confinement of the cells or the effect of the laser, we monitored the behaviour of untreated cells. During the 15 h monitored, we did not observe any death of the cells. The mitochondrial potential was maintained, and cells were negative for Annexin staining, and impermeable to YO-PRO-1.

#### Discussion

From the time-lapse experiments described above we conclude that the shallow confinement chambers enable a continuous



**Fig. 7** Snapshots from the Fas-induced apoptosis time-lapse experiment. Red, TMRE signal; green, YO-PRO-1 signal; blue, Annexin V. The width of the area shown for every snapshot is 45  $\mu\text{m}$ .

focusing on the cells in the confocal microscope over a long period of time. Besides the physical limitation of the vertical movement of the cells we believe that the restricted height also prevents the development of convective fluid flows in the chamber as a result of thermal gradients. However, in several cases, and in particular when cells were left untreated in the channels (Fig. 5, top panel), some cells moved laterally in and out of the field. The filter structure serves in this case as a physical barrier, limiting the movement. However, we are currently developing more advanced filter structures with designs that will allow a closer confinement of the cells within a microscope field.

Preparation of the samples for the time-lapse experiment is easy and fast. The crossed-channel's design and the filter structure permit a slow deposition of the cells around the filter area, thus favouring the concentration of many cells in a small volume, irrespective of the initial concentration of the cell solution. It also allows the replacement of the media at any given time during the experiment. Freedom in microchannel geometry allows more complex setups enabling e.g. co-culturing and other techniques that require confinement of cells.

A point of further optimization is the adhesion of the PDMS to the glass plate. In some cases the PDMS chip detached from the glass after several hours. We expect that appropriate chemical treatment of the glass and/or PDMS will resolve this problem. On the other hand, moderate attachment of the PDMS allows for an easy release of the chip enabling cleaning and reuse of it. Moreover, the CRISC method allows the chips to be used in combination with any existing glass-bottom plate device for optical imaging.

## Conclusions

We have developed an easy method to study apoptosis of cells in confinement. This method allows the continuous monitoring of several cells simultaneously and thus will permit the study of death of cells that can normally not be observed unless they are artificially fixed to a substrate. The restricted height of the channels enables the confocal analysis, avoiding the loss of focus that is observed when cells move out of the focal plane. This is a frequent problem caused by convection in the case of suspension cells but also occurring during apoptosis

of adherent cells due to detachment from the surface. We observed that the cells, when untreated, maintained the viability for long periods of time, suggesting that this method will be probably very useful for the analysis of cellular events that require prolonged continuous observation of the cells.

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