Minute kinetics of proapoptotic proteins: BAX and Smac/DIABLO in living tumor cells revealed by homeostatic confocal microscopy

Michał Marek Godlewski*, Magdalena Gorka, Monika Lamparska-Przybysz and Tomasz Motyl

Department of Physiological Sciences, Faculty of Veterinary Medicine, Warsaw Agricultural University, Nowoursynowska 159, 02-776 Warsaw, Poland; (*Author for correspondence; e-mail: mickgodl@hotmail.com; phone/fax: +48-22-847-24-52)

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Abstract

Traditional methods of visualization and analysis based on fixed cell populations treated with the drug for a different time give the limited possibility of time-sequence analysis. In time-lapse microscopy where the whole cell is observed regardless to intracellular structure, precise localization of events and differentiation between colocalization and overlapping of the fluorescence is impossible. Furthermore prolonged experiments with living cells increased the influence of improper environmental conditions. Homeostatic confocal microscopy gives an exceptional insight into minute pattern of changes occurring in the same living cell maintained in stable conditions during whole experimental period. It is built on a confocal system equipped with the homeostatic chamber providing constant, monitored heating and moisturized, CO₂-enriched atmosphere during long period observations. In the present study 2D/time and 4D homeostatic confocal microscopy were applied for analysis of minute pattern of changes occurring at the mitochondria. The release of Smac/DIABLO from mitochondria in tumor cells under the apoptogenic stimulus, consist of two phases: the first immediately after drug administration, and the major second one after 15 min. Furthermore the time-pattern of BAX translocation to the mitochondria and Smac/DIABLO release coincide, suggesting that the release of Smac/DIABLO is correlated with BAX translocation to the mitochondria.

Abbreviations: 2D/time HCM – two-dimensional dynamic homeostatic confocal microscopy; 4D HCM – four-dimensional homeostatic confocal microscopy; CCD – charge-coupled device camera; CPT – camptothecin; DMEM – Dulbecco's modified eagle medium; FCS – fetal calf serum; GB – gigabit; GFP – green fluorescent protein; HCM – homeostatic confocal microscopy; IOD – integrated optical density; MB – megabit; NIM – nimesulide; RFP – red fluorescence protein

Introduction

Mitochondria play a crucial role in triggering apoptosis induced by various stimuli in all cell

types. They are the source of apoptogenic proteins such as cytochrome *c*, AIF, Smac/DIABLO, Omi/HtrA2, procaspase-9 and -3 and endonuclease G. In course of apoptosis these proteins are released

from mitochondrial intermembrane space into the cytosol. This process is facilitated by promoters of apoptosis from the Bcl-2 family such as: BID, BAD, BAX and BAK, which are involved in formation of megachannels in the outer mitochondrial membrane (Susin et al. 1999; Tsujimoto and Shimizu 2000; Grinberg et al. 2002; Roucou et al. 2002). The most potent promoter of apoptosis is BAX, which remains the common link in the chain of reactions leading to tumor cell death induced by various anticancer drugs (Godlewski et al. 2001). Our previous study revealed that BAX (in association with BID) controls the function of VDAC-1 (voltage dependent anion channel 1) and its permeability for apoptogenic factors released from mitochondria in normal and tumor cells stimulated to apoptosis (Godlewski et al. 2002; Kolek et al. 2003). The formation of permeability transition pores in outer mitochondrial membrane and the release of apoptogenic proteins from intermembrane space are regarded as a 'point of no return' in the apoptotic process. The release of Smac/DIABLO from mitochondria could be a reliable marker of apoptosis in cancer cells regardless of the mechanism of death induction. Recent reports indicate that Smac/DIABLO acts as a common mediator of death receptor pathway (Fehlberg et al. 2003; Okano et al. 2003) and mitochondrial pathway (Kashkar et al. 2003; McNeish et al. 2003) of apoptosis in tumor cells.

The response of proapoptotic proteins to apoptotic stimulus is based on: (1) increased expression (at the level of transcript and protein), (2) activation (by proteolytic cleavage e.g. BAX, BID or dephosphorylation – BAD), and (3) subcellular redistribution from cytosol to organellar membranes e.g. mitochondrial (BAX, BID, BAD and BAK) or from compartments of mitochondrial intermembrane space to the cytosol (cytochrome *c*, Smac/DIABLO, AIF). The majority of tumor cells are "primed" to apoptosis, which means that they contain sufficient quantity of proapoptotic proteins to trigger cell death. For this reason, induction of apoptosis in cancer cells depends first of all on the activation and subcellular translocations of proapoptotic proteins. The dynamics of above processes are very difficult to study, since the changes in subcellular protein localization occur within minutes after apoptotic stimulus.

In this article we present a new application of the confocal microscopy based on the confocal

microscope equipped with the homeostatic chamber for precise evaluation of the minute kinetics of proapoptotic protein aggregation on mitochondria (BAX) and the release from mitochondria to the cytosol (Smac/DIABLO) in the living tumor cells, which have been stimulated to apoptosis. We propose the term homeostatic confocal microscopy (HCM) (Godlewski et al. 2003) in order to underline the maintenance of balanced conditions of cell environment (temperature, CO₂-pressure and humidity) during the investigation process, thus permitting observation of the same living cell and recording changes that occur within it over the period of several hours. Unlike time-lapse microscopy, where it is impossible to differentiate between colocalization and overlapping of the fluorescence, the HCM provides precise localization of the fluorochrome-bind proteins and insight into events occurring on different levels of cell structure.

Materials

Equipment and computer software

FV 500 confocal scanning system, Fluoview software and Microimage analysis system were purchased from Olympus Poland,¹ microscope homeostatic chamber was from Solent Scientific Ltd² and Microcal^{TM} Origin® 5.0 from Microcal Inc.³

Glassware and plastics

Sterile conical flasks (178905), 4-chamber culture slides (155383, 155411) sterile cell scrapers (179693) and sterile disposable pipettes (159609, 159617, 159625, 159633, 159641) were purchased from Nunc Inc.⁴

¹ Olympus Poland Ltd, Swietojerska 5/7, 00-236 Warsaw, Poland.

² Solent Scientific Ltd, Quartermaine Road, Portsmouth PO3 5QT, UK.

³ Microcal Inc., One Roundhouse Plaza, Northampton, MA 01060, USA.

⁴ Nunc Inc., 75 Panorama Creek Drive, Rochester, NY 14625-2385, USA.

Media and reagents

DMEM with GlutaMAX (21885–025), phosphate buffered saline, pH 7.4 (PBS) (21300–017), FCS (10106–169), penicillin-streptomycin (15070–063), fungizone (15290–026), anti-PPLO agent (15220–023), LipofectAMINE reagent (18324–111), kanamycin (15160–047) and geneticin (10131–027) were obtained from Gibco BRL.⁵ pDsRed2 - Mito Vector (6975–1) and pEGFP-N2 Vector (6081–1) were obtained from BD Biosciences Clontech.⁶ PCR reagents (EP0501, EL0335) were from MBI Fermentas.⁷ Camptothecin (C 9911), nimesulide (N 1016) and DMSO (49,442–9) were purchased from Sigma-Aldrich Corp.⁸

Cell lines and bacteria

Human colon adenocarcinoma COLO 205 (CCL-222), human breast cancer MCF-7 (HTB-22) cell lines and *Escherichia coli* strain DH5 α (53868) were obtained from the American Type Culture Collection.⁹

PCR reaction primers

PCR reaction primers Smac1 (5'-ACAATGGCG GCTCTGAAGAGTTGG-3') and Smac2 (5'-ATC CTCACGCAGGTAGGCCTCCTG-3') were constructed by Genset SA.¹⁰

Procedures

Cell culture

Cell cultures were maintained in DMEM supplemented with 10% (v/v) FCS, 50 μ g/ml anti-PPLO agent, 2.5 μ g/ml fungizone, 50 UI/ml penicillin

and 50 μ g/ml streptomycin in an atmosphere of 5% CO₂/95% humidified air at 37 °C, and routinely subcultured every 2 or 3 days.

Drugs

Camptothecin (CPT) and nimesulide (NIM) were diluted in DMSO to create stock solutions which were stored according to manufacturers' suggestions. Drug concentrations were selected on the base of our previous studies (Godlewski et al. 2001, 2002). Final solutions used in experiments were 0.15 μ M for CPT and 1 μ M for NIM.

Plasmids

A PCR reaction with Smac1, Smac2 primers and cDNA template (made on RNA material isolated from MCF-7 cells) was performed under the following conditions: 1 $\times$ 94 °C for 5 min, 35 $\times$ (94 °C for 25 s; 60 °C for 15 s; 72 °C for 40 s), 1 $\times$ 72 °C for 10 min. Amplification was performed with the use of the *Pfu* DNA Polymerase. Products of PCR reaction were confirmed by gel electrophoresis and isolated from gel. Obtained Smac/DIABLO fragment was ligated to digested (*Sma*I) pEGFP-N2 Vector before the coding sequence of GFP using T4 DNA Ligase overnight at 16 °C. The resulting plasmid was transformed into *Escherichia coli* strain DH5 α , and transformants were selected on medium containing kanamycin at a concentration of 30 μ g/ml. The correct DNA sequence was confirmed using DNA sequencing analysis. BAX-GFP plasmid was generously gifted by Dr. Bozena Kaminska-Kaczmarek, (The Institute of Experimental Biology, Polish Academy of Science, Warsaw, Poland).

Cell transfection

Transfection solution was prepared with the use of 2 μ g of BAX-GFP plasmid in 0.1 ml serum free medium and 10 μ l lipofectAMINE reagent in 0.1 ml serum free medium. Solutions were then combined and incubated at room temperature for 45 min. Meanwhile COLO 205 and MCF-7 cells were rinsed and overlaid with 2 ml of serum free medium. Then transfection mixture was added

⁵ Gibco BRL, P.O. Box 9418, Gaithersburg, MD 20898, USA.

⁶ BD Biosciences Clontech, 1020 East Meadow Circle, Palo Alto, California 94303, USA.

⁷ MBI Fermentas, V. Graiciuno 8, Vilnius 2028, Lithuania.

⁸ Sigma-Aldrich Corp., P.O. Box 14508, St. Louis, Missouri 63178, USA.

⁹ American Type Culture Collection, P.O. Box 1549, Manassas, Virginia 20108, USA.

¹⁰ Genset SA, Route Nationale 7, 91030 Evry Cedex, France.

dropwise and the cells were incubated in 5% CO₂/95% humidified air at 37 °C for 5 h. Finally transfection medium was exchanged with 10% FCS/DMEM. 72 h following the start of transfection geneticin was added (50 mg/ml) for the selection of positively transfected cells.

MCF-7 cells were transfected using lipofectAMINE reagent and pEGFP-Smac. For transfection solution 1.5 µg of DNA diluted into 0.1 ml serum free medium, and 7 µl of lipofectAMINE reagent diluted into 0.1 ml serum free medium. Both solutions were carefully combined and the obtained mixture was incubated at room temperature for 30 min. The cells were overlaid with 0.8 ml of serum free medium and the transfection mixture was added dropwise and incubated at 37 °C, under 5% CO₂ atmosphere over the next 3 h. Afterwards transfection medium was exchanged with growth medium containing 10% FCS. After 48 h transfected cells were selected using geneticin (400 µg/ml).

After 2 weeks obtained cultures were transfected with pDsRed2 mitovector following identical protocol.

Homeostatic confocal microscopy (HCM)

FV 500 confocal microscope was equipped with special homeostatic chamber for experiments conducted with the use of living cells. This allowed standard growing conditions for the cells over the experiment period (37 °C, 5% CO₂, 95% humidified air). Transfected cells were cultured on coverglass-mounted-4-chamber culture dishes until 70% confluency. Cells were measured with the combinations of excitation/emission: Argon 488 nm laser vs. 505–525 nm filter for GFP, and HeNe 543 nm laser vs. 610 nm filter for RFP. Laser power, voltage of photomultipliers and depth of the crosssections were set during the preliminary study to a level that allowed optimal fluorescence gain with minimal side-effects for the cells observed. Obtained values were: 15% of 10 mW Argon and 25% of 1 mW HeNe laser power, 810 and 690 V for green and red channel PMT, respectively. The gains of acquired images were set to a value of 7.5 for green and 5.5 for red channel with 5% offset for both fluorescence channels. These values were maintained constant during all

conducted experiments. There were no postcapture modifications regarding the intensity of acquired fluorescence. To avoid the cross-talk between the fluorochromes the sequence scanning mode was applied for the experiment. The fluorescence level of control and treatment medium were compared and no changes were observed.

Two-dimensional dynamic homeostatic confocal microscopy (2D/time HCM)

Data were collected from single 3 µm deep cross-section with 2 min time intervals for measurements of double transfected COLO 205 cells (BAX-GFP + mitovector-RFP). After acquiring control images the standard growth medium was replaced with nimesulide (NIM) enriched medium (1 µM) and the same cells were observed over the next 22 min. The IOD values of green fluorescence corresponding to cytosolic BAX-GFP and yellow light coming from simultaneous excitation of red and green fluorescence of BAX-GFP translocated to mitochondria were analyzed using the Micro-image analysis system.

Four-dimensional homeostatic confocal microscopy (4D HCM)

Four-dimensional HCM (3D reconstruction + time-dependence) was applied for measurements of double transfected MCF-7 cells (BAX-GFP or Smac/DIABLO-GFP together with mitovector-RFP). Series of 3 µm deep cross-sections from single MCF-7 cell were gathered at 3 min time intervals. After acquiring control images the standard growth medium was replaced with camptothecin (CPT) enriched medium (0.15 µM) and the same cell was observed over the next 45 min. Afterwards the 3D reconstruction of cell was created from the stack of cross-sections using the Fluoview program and changes in fluorescence values occurring during the experiment were observed. The IOD values of yellow light coming from simultaneous excitation of red and green fluorescence of mitochondria-localized proteins and green fluorescence of cytosol-localized proteins were analyzed using the Microimage analysis system.

Statistical evaluation

The results were statistically evaluated by ANOVA and Tukey's multiple range tests using Microcal^{TM} Origin® 5.0. $p \leq 0.05$ was regarded as significant.

Results and discussion

Two-dimensional dynamic homeostatic confocal microscopy (2D/time HCM)

Experiments were conducted with the use of BAX-GFP and mitovector-RFP-transfected COLO 205

Figure 1. 2D/time HCM experiment, conducted with the use of living COLO 205 cells transfected with BAX-GFP (green fluorescence) and Mito-RFP encoding RFP conjugated with mitochondria localization sequence (red fluorescence). Cells were observed with 2 min intervals. In non-treated cells no pattern of colocalization can be observed between BAX and mitochondria related Mito-RFP fluorescence. Afterwards NIM (1 μ M) was added to the medium and the same cells were observed for the next 24 min. Rapid increase in colocalization pattern between BAX and mitochondria (visible as a gain of yellow light from simultaneous excitation of both green and red fluorochrome) can be observed since 16 min of experiment.

cells to reveal the dynamic pattern of BAX translocation to mitochondria after the apoptosis induction by NIM. NIM is a nonsteroid anti-inflammatory drug possessing apoptogenic properties in human colon adenocarcinoma COLO 205 cells (Godlewski et al. 2002). The pattern of BAX aggregation on mitochondria in two COLO 205 cells cross-section visualized in 2 min time intervals was presented on Figure 1. Cells observed in control medium (10% FCS/DMEM) revealed almost no pattern of colocalization between green fluorescence of BAX-conjugated GFP and red fluorescence of mitovector-RFP (IOD of yellow light ranging from 1.66 ± 0.08 to 1.81 ± 0.07) (Figures 1 and 2 – CTRL). After the medium was supplemented with NIM ($1 \mu\text{M}$) the slight increase in yellow light from simultaneous excitation of colocalized BAX-GFP and mitovector-RFP was observed. This continued until the 14 min of observation (IOD at 4.42 ± 0.17) and was followed by the dramatic increase in yellow light, which IOD doubled to 10.39 ± 0.39 at 16 min and was steadily climbing to 24.55 ± 2.39 at 24 min. Interestingly, while the intensity of BAX-related green fluorescence increased significantly from 35.45 ± 9.78 (control average) to 70.19 ± 4.66 during the first 2 min of experiment, no further significant changes in green IOD were observed (Figures 1 and 2). For IOD evaluation

the data from five different experiments were acquired.

Four-dimensional homeostatic confocal microscopy (4D HCM)

BAX translocation to the mitochondria.

Two-dimensional/time HCM gave us insight into processes going in the living cell, but there are some limitations to the method. It only allows a view of the single cross-section of the cell, which may be, but not necessarily, representative to the entire cell. To overcome this obstacle the 4D HCM was implemented providing the superb insight into pattern of changes occurring in every single slice and, after the 3D reconstruction, in the cell as a whole. The series of crosssections from MCF-7 cell presented in Figure 3 were gathered in 5 min time-intervals. Cell image from every time-point was then reconstructed with the use of the Fluo-view software. In a non-treated cell (Figure 3 CTRL) the majority of BAX-related green fluorescence is localized outside of the mitochondria (separate green and red fluorescence). During CPT-treatment the increasing amount of yellow fluorescence coming from simultaneous excitation of colocalized mitochondria-bound red and green fluorescence is visible (Figure 3 CPT). The

Figure 2. Minute pattern of BAX translocation to mitochondria in non-treated cells (CTRL) and in the same cells treated with NIM ($1 \mu\text{M}$). Translocation of BAX to mitochondria is represented by an increase in yellow light coming from simultaneous excitation of BAX-related GFP and mitochondria-localized RFP (empty dots). Series of 2D/time HCM images were quantitatively analyzed using Microimage system. Results are expressed as integrated optical density (OD $\times$ area) $n=5$.

Figure 3. 4D HCM analysis of minute pattern of BAX translocation to the mitochondria in a non-treated cell (CTRL) and in the same cell treated with CPT (0.15 μ M). MCF-7 cell is presented in subsequent images as a 3D-reconstruction from different time-points. BAX translocation to the mitochondria is represented by an increase in yellow light coming from simultaneous excitation of BAX-related GFP and mitochondria-localized RFP.

increase in yellow fluorescence is noticeable in subsequent images in areas pointed to by arrows. To estimate semiquantitatively the changes in fluorescence IOD the data from three different experiments were analyzed by Microimage system (Figure 4). In non-treated cells (Figure 4 CTRL) the cytosol BAX-related green fluorescence IOD oscillated between 47.47 ± 0.49 and 67.62 ± 0.87 , and the yellow IOD was around 4. During the CPT-treatment IOD of cytosol localized BAX-related green fluorescence increased gradually from 56.34 ± 0.53 to 152.32 ± 2.42 at 15 min, which was most probably the effect of proteolytic activation of BAX and

its release from inactivating complexes with other proteins from BCL-2 family. Between 15 and 20 min of CPT incubation the amount of unbound, cytosol-localized BAX-related green fluorescence decreased from 152.32 ± 2.42 to 99.9 ± 0.93 (Figure 4 CPT). This process was accompanied by a simultaneous increase in yellow fluorescence of mitochondria-localized BAX from the 25.13 ± 0.55 to 81.44 ± 2.42 at 15 and 20 min, respectively. The level of cytosol-localized BAX was stable during the following 10 min then it gradually increased to 160.01 ± 2.02 at 40 min of incubation. The amount of mitochondria-localized

Figure 4. Minute pattern of BAX translocation to mitochondria in non-treated cells (CTRL) and in the same cells treated with CPT (0.15 μ M). Translocation of BAX to mitochondria is represented by an increase in yellow light coming from simultaneous excitation of BAX-related GFP and mitochondria-localized RFP (empty dots). Series of 4D HCM images were quantitatively analyzed using Microimage system. Results are expressed as integrated optical density (OD $\times$ area) $n=3$.

BAX (yellow fluorescence) increased slowly until the IOD reached 104.21 ± 3.6 at 30 min then steadily dropped during following 10 min to the level of 64.54 ± 3.6 .

Smac/DIABLO release from the mitochondria

The 3D reconstruction of a nontreated and CPT-treated MCF-7 cell double-transfected with Smac/DIABLO-GFP and mitovector-RFP is shown in Figure 5. On the following panels the same cell is presented in 3 min time lapses, rotated 9° in Y dimension. In control conditions (10% FCS/DMEM) Smac/DIABLO-GFP and mitovector-RFP colocalize almost completely and no changes in that pattern can be observed (Figures 5 and 6 – CTRL). After the CPT was added (0.15 μ M) there was an immediate increase in green IOD from 2.19 ± 0.02 to 15.26 ± 0.91 associated with slight decrease of yellow IOD. This suggested slight preliminary efflux of Smac/DIABLO from mitochondria. Over the next 12 min no changes were observed. Time between 15 and 18 min of CPT treatment seems to be crucial for the events occurring in mitochondria. Green IOD increased from 17.69 ± 0.9 to 28.78 ± 1.81 while intensity of yellow light decreased from 10.71 ± 1.01 to 4.66 ± 0.61 (Figure 6). It is also clearly visible on the confocal image as a decrease in yellow light

area after 18 min of observation (Figure 5). Following this moment there was an evident increase of green IOD up to 43.61 ± 2.05 , and accompanied by a slight but stable decrease in yellow IOD down to 1.68 ± 0.07 after 42 min (Figure 6). Also visible was an almost complete evanescence of yellow light at 45 min of cell observation (Figure 5). IOD analysis was performed on data from 10 different experiments.

Discussion

HCM presented in this work fills the niche in the research methods used for studying the kinetics of proteins involved in the regulation of apoptosis. Up to now, the analysis of BAX aggregation on mitochondria or Smac/DIABLO release from them, were based on Western blot, fluorescence microscopy, traditional confocal imaging and immuno-electron microscopy (Adrain et al. 2001; Dirsch et al. 2003; Justo et al. 2003; Saito et al. 2003). These methods allowed only analysis of fixed cells, different for different time points. The application presented in this work – the HCM – was designed specially for assessment of proteins aggregating on mitochondria (BAX) and released from them (Smac/DIABLO). It enables continuous

Figure 5. 4D HCM analysis of minute pattern of Smac/DIABLO efflux from mitochondria in non-treated cell (CTRL) and in the same cell treated with CPT ($0.15 \mu\text{M}$). MCF-7 cell is presented as a 3D-reconstruction, rotated by 9° in subsequent images. Smac/DIABLO efflux from mitochondria is represented by an increase in green light coming from cytosolic localization of Smac/DIABLO and decrease in yellow light coming from simultaneous excitation of Smac/DIABLO-related GFP and mitochondria-localized RFP.

Figure 6. Minute pattern of Smac/DIABLO efflux from mitochondria in non-treated cells (CTRL) and in the same cells treated with CPT (0.15 μ M). Smac/DIABLO efflux from mitochondria is represented by an increase in green light coming from cytosolic localization of Smac/DIABLO (full dots) and decrease in yellow light coming from mitochondrial Smac/DIABLO (empty dots). 4D HCM images were quantitatively analyzed using Microimage system. Results are expressed as integrated optical density (OD $\times$ area) $n = 10$.

observation of processes occurring in same single living cell undergoing the apoptotic stimulation (Figures 1, 3 and 5). Furthermore, the HCM method was combined with fluorescence IOD analysis performed in sequence of time intervals. This allowed semiquantitative analysis of protein kinetics and graphic presentation of these processes (Figures 2, 4 and 6). Two variations of HCM method were presented: 2-D analysis in time (2D/time HCM) (Figure 1) and 3-D reconstruction in time (4D HCM) (Figures 3 and 5). 2D/time method was designed for analysis of quick processes occurring in short time-intervals, but it is less precise than 4D HCM. In the 4D HCM the data are gathered from the whole cell, thus allowing different point view around the examined cell and comparison between different layers of the same cell (compare Figure 1 with Figures 3 and 5). It is noticeable that fluorescence-IOD values measured by 4D HCM doubled those measured by 2D/time HCM (compare Figure 2 with Figure 4). The 4D HCM seems to be the method of choice for analysis of pattern of changes of weak fluorescence and fluorescence that is unevenly distributed within the cell. The only setback is a long data-acquiring time, which disallows measurements of processes occurring in less than 3 min time-intervals. In contrast to common method of time-

lapse microscopy the HCM provides not only the overview of processes occurring within the cell, but also their precise localization on cell structure. A brief comparison and possible fields of research activity for time-lapse microscopy and two variations of HCM are presented in Table 1. The time-lapse microscopy seems sufficient for observation of cell movement, changes in cell morphology and rough visualization of ion or protein distribution. More precise localization of measured processes within the cell can only be acquired with the use of either 2D/time or 4D homeostatic confocal microscopy. These two methods allow measurement data to be acquired from thin cross-sections of the cell and (in case of 4D HCM) 3D reconstruction of the scanned cell. The variety of applications in 2D/time HCM extends to measurements of channel opening and other fields requiring ultra-fast (millisecond) timing. On the other hand, 4D HCM seems to be a method of choice for all measurements concerning dynamics of protein production, activation, distribution and redistribution, interaction and degradation. Furthermore changes in cell structure and dynamics of ion pools can be observed and precisely localized. The long time required for data collection (minutes per time point) is considered the main setback of this method.

Table 1. Comparison between time-lapse microscopy, 2D/time HCM and 4D HCM. Advantages, setbacks and proposed fields of research.

	Time-lapse microscopy		2D/time HCM	4D HCM
Visualization	Whole cell, regardless to intracellular localization		Single layer of the cell	Each and every layer of the cell, 3D and 4D reconstruction of the cell
Minimal time interval between scans	Depending on scan type and area from milliseconds to seconds		Depending on scan type and area from milliseconds to seconds	1–5 min
Equipment ^a	Fluorescent, contrast-phase or relief microscope with homeostatic chamber, CCD and frame-grabber		Confocal microscope with homeostatic chamber	Confocal microscope with homeostatic chamber
Size on hard disk	MB		MB	GB
Advantages	Cheap, quick, popular		Quick, precise, ideal for short-time experiments, allows observation of very dynamic processes	Precise measurement of processes occurring in entire cell, different depths and areas of the cell
Setbacks	No precise localization and connection between occurring processes and cell ultrastructure		Difficulties in maintaining the same layer of cell under measurement especially during longer experiments	Time-consuming method, relatively long time interval between scans
Proposed fields of research	Cell movement, ion distribution and redistribution		Neuronal activity, ion distribution and redistribution, channel opening	Protein production, activation, degradation, translocation and redistribution, protein-protein interactions, changes in intracellular ion pools, cytoskeleton changes, interactions between proteins and cell organelles

^aAll methods require an image-analysis system for quantitative evaluation of acquired data.

2D/time HCM – two-dimensional dynamic homeostatic confocal microscopy; 4D HCM – four-dimensional homeostatic confocal microscopy; CCD – charge-coupled device camera; MB – megabit; GB – gigabit.

Observations of kinetics of BAX aggregation on mitochondria and Smac/DIABLO released from mitochondria were conducted on different cell lines with different apoptogenic stimuli (COLO 205 with NIM and MCF-7 with CPT, respectively). It proved that HCM method is competent for many different experimental setups, also for the apoptosis-unrelated studies.

Obtained results indicate that BAX and Smac/DIABLO subcellular redistributions are quick and dynamic processes in tumor cells. The constant readiness of tumor cells to switch-on the death program is based probably on high contents of death promoters, mediators and executors within the cell. In these cells the apoptosis induction works through activation and subcellular redistribution of death-promoting proteins, rather than their *de-novo* synthesis. BAX activation, based on BH3 death domain exposure, is facilitated by m-calpain-mediated proteolytic cleavage at the N-terminus (Suzuki et al. 2000), or conformational changes in protein structure mediated by BID (Eskes et al. 2000). Active BAX, together with other proteins e.g. BID and VDAC-1, creates heteromultimeric complexes on outer mitochondrial membrane. These complexes form megachannels (permeability transition pores) involved in release of mitochondrial apoptogenic factors (cytochrome *c*, Smac/DIABLO, Omi/Hrt A2, AIF, procaspase-3 and -9, endonuclease G) (Shimizu et al. 2000). Activation of Smac/DIABLO is based on N-terminal cleavage of mitochondria-localization domain. Observations included in this article suggest that BAX aggregation on mitochondria, as well as Smac/DIABLO release from them in tumor cells under the apoptogenic stimulus, consist of two phases. First phase – immediately after drug administration, and second phase occurring after 15 min. While the second phase is probably associated with the formation of the permeability transition pores in the outer mitochondrial membrane, the origins of the first phase of BAX and Smac/DIABLO activity remain difficult to understand. Increase in BAX-GFP-related and Smac/DIABLO-related green fluorescence occurring immediately after the drug administration might be a result of their activation or, in case of BAX, release from inactivating complexes with other proteins e.g. BCL-2. We can not exclude the possibility that physicochemical changes occur in cellular microenvironment, but the supplemented

drugs were always diluted in the growth medium, where cells normally live. It is interesting that the kinetics of the second phase of BAX aggregation, and Smac/DIABLO release from mitochondria are similar, regardless of the type of tumor cell lines (COLO 205 and MCF-7) and apoptogenic stimuli (NIM vs. CPT) used in the experiments. This coincidence suggests that the release of Smac/DIABLO is correlated with BAX translocation to the mitochondria.

In conclusion, the homeostatic confocal microscopy is a potent tool for analysis of protein kinetics in living cells, as well as cells stimulated undergo to apoptosis.

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References

- Adrain C., Creagh E.M. and Martin S.J. 2001. Apoptosis-associated release of Smac/DIABLO from mitochondria requires active caspases and is blocked by Bcl-2. *EMBO J.* 20(23): 6627–6636.
- Dirsch V.M., Muller I.M., Eichhorst S.T., Pettit G.R., Kamano Y., Inoue M., Xu J.P., Ichihara Y., Wanner G. and Vollmar A.M. 2003. Cephalostatin 1 selectively triggers the release of Smac/DIABLO and subsequent apoptosis that is characterized by an increased density of the mitochondrial matrix. *Cancer Res.* 63(24): 8869–8876.
- Eskes R., Desagher S., Antonsson B. and Martinou J.C. 2000. Bid induces the oligomerization and insertion of Bax into the outer mitochondrial membrane. *Mol. Cell Biol.* 20(3): 929–935.
- Fehlberg S., Gregel C.M., Goke A. and Goke R. 2003. Bisphenol A diglycidyl ether-induced apoptosis involves Bax/Bid-dependent mitochondrial release of apoptosis-inducing factor (AIF), cytochrome *c* and Smac/DIABLO. *Br. J. Pharmacol.* 139(3): 495–500.
- Godlewski M.M., Motyl M.A., Gajkowska B., Wareski P., Koronkiewicz M. and Motyl T. 2001. Subcellular redistribution of BAX during apoptosis induced by anticancer drugs. *Anticancer Drugs* 12(7): 607–617.
- Godlewski M.M., Gajkowska B., Lamparska-Przybysz M. and Motyl T. 2002. Colocalization of BAX with BID and VDAC-1 in nimesulide-induced apoptosis of human colon adenocarcinoma COLO 205 cells. *Anticancer Drugs* 13(10): 1017–1029.
- Godlewski M.M., Gorka M., Jezierska A., Gajkowska B. and Motyl T. 2003. Homeostatic Confocal Microscopy gives an

- insight into minute events occurring in mitochondria in cells undergoing apoptosis. 1st International Workshop Slide-Based Cytometry (SBC) 15 – 17.05, Leipzig, Germany, 12 pp.
- Grinberg M., Sarig R., Zaltsman Y., Frumkin D., Grammatikakis N., Reuveny E. and Gross A. 2002. tBID Homooligomerizes in the mitochondrial membrane to induce apoptosis. *J. Biol. Chem.* 277(14): 12237–12245.
- Justo P., Sanz A., Lorz C., Gomez-Garre D., Mezzano S., Egido J. and Ortiz A. 2003. Expression of Smac/Diablo in tubular epithelial cells and during acute renal failure. *Kidney Int. Suppl.* 86: S52–S56.
- Kashkar H., Haefs C., Shin H., Hamilton-Dutoit S.J., Salvesen G.S., Kronke M. and Jurgensmeier J.M. 2003. XIAP-mediated caspase inhibition in Hodgkin's lymphoma-derived B cells. *J. Exp. Med.* 198(2): 341–347.
- Kolek O., Gajkowska B., Godlewski M.M. and Motyl T. 2003. Co-localization of apoptosis-regulating proteins in mouse mammary epithelial HC11 cells exposed to TGF-beta1. *Eur. J. Cell Biol.* 82(6): 303–312.
- McNeish I.A., Bell S., McKay T., Tenev T., Marani M. and Lemoine N.R. 2003. Expression of Smac/DIABLO in ovarian carcinoma cells induces apoptosis via a caspase-9-mediated pathway. *Exp. Cell Res.* 286(2): 186–198.
- Okano H., Shiraki K., Inoue H., Kawakita T., Saitou Y., Enokimura N., Yamamoto N., Sugimoto K., Fujikawa K., Murata K. and Nakano T. 2003. Over-expression of Smac promotes TRAIL-induced cell death in human hepatocellular carcinoma. *Int. J. Mol. Med.* 12(1): 25–28.
- Roucou X., Montessuit S., Antonsson B. and Martinou J.C. 2002. Bax oligomerization in mitochondrial membranes requires tBid (caspase-8-cleaved Bid) and a mitochondrial protein. *Biochem. J.* 368(Pt 3): 915–921.
- Saito M., Sakagami H. and Fujisawa S. 2003. Cytotoxicity and apoptosis induction by butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT). *Anticancer Res.* 23(6C): 4693–4701.
- Shimizu S., Shinohara Y. and Tsujimoto Y. 2000. Bax and Bcl-xL independently regulate apoptotic changes of yeast mitochondria that require VDAC but not adenine nucleotide translocator. *Oncogene* 19(38): 4309–4318.
- Susin S.A., Lorenzo H.K., Zamzami N., Marzo I., Snow B.E., Brothers G.M., Mangion J., Jacotot E., Costantini P., Loeffler M., Larochette N., Goodlett D.R., Aebersold R., Siderovski D.P., Penninger J.M. and Kroemer G. 1999. Molecular characterization of mitochondrial apoptosis-inducing factor. *Nature* 397(6718): 441–446.
- Suzuki M., Youle R.J. and Tjandra N. 2000. Structure of Bax: coregulation of dimer formation and intracellular localization. *Cell* 103(4): 645–654.
- Tsujimoto Y. and Shimizu S. 2000. VDAC regulation by the Bcl-2 family of proteins. *Cell Death Differ.* 7(12): 1174–1181.