Chapter 16

Temporal Heterogeneity in Apoptosis Determined by Imaging Flow Cytometry

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Abstract

Apoptotic process is highly heterogeneous, and a long-standing question is how many parameters define time and reversibility of the apoptotic response at a population and single-cell levels. Cell death analysis applications have greatly expanded since the introduction of flow cytometry. Classical approach for evaluation of apoptosis is en masse analysis of cells treated with different stimuli, but these methods cannot demonstrate heterogeneity in the population. Single-cell heterogeneity is now usually assessed by multicolor fluorescence microscopy; however obtaining reasonable statistics is time consuming and laborious. Therefore we combined flow cytometry, imaging flow cytometry, and fluorescent microscopy to characterize at a single-cell and population level sequence of apoptotic events induced by a variety of treatments (Vorobjev, Barteneva, J Histochem Cytochem 63:494–510, 2015). We show that simultaneous use of membrane potential dye TMRE, caspases 3/7 sensor, Annexin V and nuclear staining along with morphological parameters demonstrate heterogeneity of the whole process and is a valuable method for quantitative study of the apoptosis execution. Imaging flow cytometry allowed us to analyze correlation between TMRE, caspases 3/7, and Annexin V staining and morphological characteristics providing valuable information on the process of apoptotic execution. Importantly, comparisons of different data sets obtained by three methods allowed us to achieve temporal resolution of the whole process superior to that had been obtained by only one method.

Key words Apoptosis, Cell death, Caspase 3/7, Annexin V, Mitochondria, TMRE, Cell volume

1 Introduction

Apoptosis is a programmed form of cell death, triggered by a variety of signals including TNF receptors, p53 activation, mitochondria damage, oxidative stress, DNA damage, etc. Analysis of apoptosis is essential to many fields, and is usually performed on the population level. The key events in the progression and execution of apoptosis include apoptotic volume decrease (AVD), cell shrinkage, phosphatidylserine (PS) externalization on the plasma membrane, nuclear condensation, blebbing, and fragmentation of dying cell. These features are orchestrated by the activation of cysteine

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proteases, namely caspases [1]. Usually morphological changes of apoptotic cells are preceded or supplemented with mitochondria outer membrane permeabilization (MOMP) and occur along with the executioner caspases activation [2–5]. However, the relationship between AVD, MOMP, caspase activation, PS externalization, and morphologically distinguishable events is poorly investigated.

Flow cytometry studies of apoptosis are often limited by use of PI (or other DNA dyes) to study permeability of the plasma membrane and Annexin V staining as a probe for PS externalization (see Rieger and Barreda, this volume for details), while other characteristics like MOMP and AVD are usually studied by microscopy and caspase activation is usually studied by immunoblotting and related methods. Recently we showed that TMRE staining could be successfully used in flow cytometry (with 561 nm excitation laser) and loss of TMRE staining is tightly coordinated with apoptosis execution [6, 7]. Microscopic studies of apoptosis are mainly devoted to the study of MOMP [1], apoptotic volume decrease [8, 9], and rarely—to the activation of caspases [3, 10].

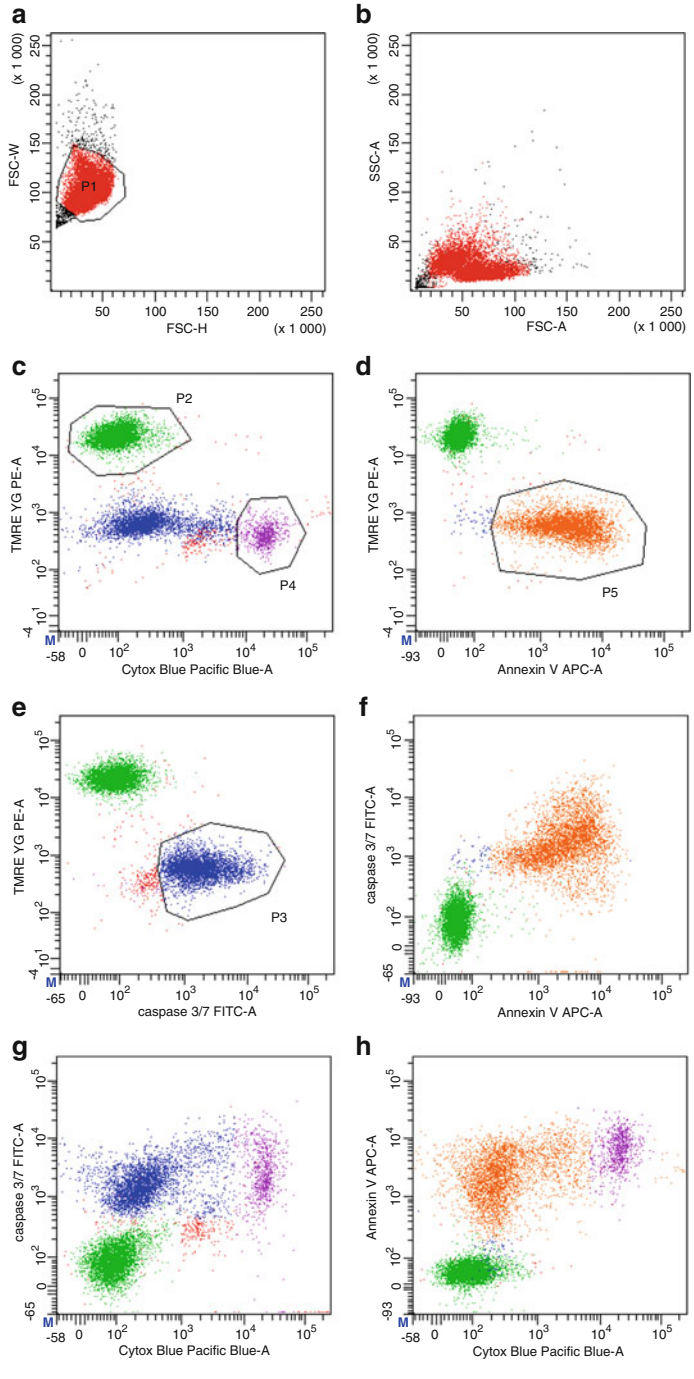
Microscopic studies of apoptosis performed at a single-cell level had been using caspase FRET sensors demonstrated that caspase 3/7 activation can occur immediately after MOMP [3, 11, 12]; however limited statistics was obtained.

To the best of our knowledge no microscopic study of apoptosis evaluated PS externalization along with other events. Thus characterization of apoptosis performed using different methods (microscopy, flow cytometry, immunoblotting) is focused on different markers of the process and thus results obtained are not easy to put into one comprehensive picture.

Because of the technical problems with Annexin V staining—it requires high concentration of calcium and dissociation constant for Annexin V from PS is two orders of magnitude higher than for antibodies—intensive microscopic studies of Annexin V-labeled cells are difficult.

Imaging flow cytometry is a relatively new method allowing to narrow the gap between standard flow cytometry with the lack of information about a given cell and classical microscopy, where obtaining quantitative parameters for cell populations is tedious and time consuming.

In this chapter, we use an ImageStream multi-spectral flow cytometer (Amnis Corporation, EMD Millipore) parallel with conventional flow cytometry and time-lapse fluorescent microscopy to show that multicolor staining of tissue culture cells undergoing apoptosis using caspase 3/7 sensor, TMRE as mitochondria potential probe and Annexin V, conjugated with Alexa 647 as a marker of PS externalization (Fig. 1) allow detailed analysis of apoptosis execution on the population level and analysis of image galleries allows one to make conclusions on the events might or might not happen simultaneously. We show that PS externalization happens always after MOMP, yet before caspase 3/7 activation. Intensity of



Experiment Name: Experiment_001

Population	#Events	%Parent	caspase 3/... Mean	TMRE YG P... Mean
P1	9 450	94,5	1 117	1 1 422
P2	4 840	51,2	94	21 634
P3	4 282	45,3	2 374	588
P4	796	8,4	3 650	413
P5	4 498	47,6	2 229	580

Fig. 1 Dotplots from FACSAria II. THP1 cells treated with TRAIL, 100 ng/ml for 20 h. (**a**) FSC-W versus FSC-H, region *P1* denotes singlet events. (**b**) FSC/SSC plot. (**c**) TMRE/Sytox blue plot, *P2* TMRE positive cells, *P4* Sytox Blue-positive (dead) cells. (**d**) TMRE/Annexin V plot, *P5* Annexin V-positive cells. (**e**) TMRE/caspase 3/7 plot, *P3* caspase-positive cells. (**f**) Annexin V/caspase plot. (**g**) Sytox Blue/caspase 3/7 plot. (**h**) Sytox Blue/Annexin V plot

caspase 3/7 staining does not correlate with Annexin V staining indicating that process of PS externalization and caspase 3/7 activation are largely independent at a single-cell level.

2 Materials

2.1 Tissue Culture Medium

RPMI-164 supplemented with L-glutamine, antibiotics, and 10 % fetal bovine serum (FBS). Complete culture medium is stored at +4 °C.

2.2 TMRE (Tetramethyl-rhodamine, Ethyl Ester) Solution

Stock solution should be prepared at a concentration of 50 μM in DMSO and could be stored at room temperature. Final concentration of TMRE depends on the particular cell type and is in the range of 10–100 nM.

2.3 Caspase 3/7 Sensor

CellEvent™ Caspase 3/7 Green reagent is purchased from Life Technologies Inc. (Grand Island, NY, USA) and stored at the room temperature (in the dark) (*see* **Note 1**).

2.4 Annexin V

Annexin V conjugated with Alexa-647 is used for the current protocol. Stock solution is stored at +4 °C in the dark (*see* **Note 2**).

2.5 Annexin V Binding Medium

To obtain strong signal from Annexin V high concentration of Ca^{++} is required. It is achieved by adding $CaCl_2$ from stock solution (500 mM) to the culture medium. $CaCl_2$ stock solution is prepared in sterile deionized water and stored at room temperature.

2.6 Sytox Blue (SB)

This dye cannot penetrate plasma membrane so it is only staining nuclei in the cells with disrupted membrane. Sytox Blue can be purchased from different vendors. For our purposes, we used SB from a stock solution of 1 mM in DMSO. Stock solution is kept at +4 °C for indefinitely long time.

3 Methods

This procedure is typically performed in 5 mL polystyrene round-bottom FACS sterile tubes with caps using 0.5–1 ml of cell suspension. For ImageStream analysis 50–70 μl of stained suspension is taken. The optimum concentration for imaging flow cytometry analysis is ~5×10^6 cells per 1 mL volume. If cell concentration is significantly less, larger volume might be recommended. When large files obtained by imaging flow cytometry are necessary, cells are concentrated two- to fivefold immediately before ImageStream analysis. Microcentrifuge tubes are used for ImageStream analysis.

Special staining for ImageStream in 1.5 mL microcentrifuge tubes should be avoided, since small volume does not guarantee cell viability for long enough time (*see* **Note 3**).

3.1 Cell Preparation

1. Harvest cells—use lab-specific procedures for corresponding cell lines or primary cell isolations. Harvesting procedures will vary greatly depending on cell type (e.g., if cell is adherent or non-adherent). Cell suspension is finally prepared in culture medium.
2. Centrifuge samples at $400 \times g$ for 4–5 min at room temperature and decant the supernatant. This speed is sufficient to pellet most suspension cell lines. If the cell of interest requires different centrifugation parameters, modify as required (*see* **Note 4**).
3. Resuspend cells in ~0.5 mL of fresh culture medium to achieve concentration of $1–2 \times 10^6$ cells per ml.

3.2 TMRE and Caspase 3/7 Staining

1. Add TMRE from stock solution at a final concentration 50–100 nM. Typical stock solution is 50–100 μM in DMSO.
2. Add caspase 3/7 reagent—one drop per 0.5 ml is recommended, proper concentration could be titrated.
3. Place vial with cells into CO_2 incubator (37 °C) for 30 min.

3.3 Application of Annexin V Stain

1. Add $CaCl_2$ from the stock solution to the sample to achieve final concentration of 3 mM (*see* **Note 5**).
2. Add Annexin V according to the manufacturer's recommendations (e.g., 5 μL Annexin V-Alexa Fluor 647 if using from Life Technologies). The volume might vary depending on the supplier.
3. Incubate tubes in the dark for 15 min at room temperature (not less than 23 °C) or in CO_2 incubator (37 °C).

3.4 Application of Sytox Blue Stain

1. Add ~0.5 μl Sytox Blue to the sample and take sample for analysis within 2 min.

3.5 Acquisition and Analysis: Flow Cytometry Platform

The analysis procedure described below is based on acquisition with a FACS Aria cells sorter (Becton Dickinson) and analyzed with FACSDiva software. It could be performed in the similar way with any instrument equipped with 405/407, 488, 561, and 635/640 lasers. Consequently caspase 3/7 is viewed in FITC channel (bandpass 530/30), TMRE in PE channel (yellow–green laser; bandpass 575/25), Sytox Blue in Pacific Blue channel (bandpass 450/50), and Annexin V-Alexa 647—in APC channel (bandpass 660/20) (*see* **Note 6**).

1. During acquisition, set lasers to balance signals from Annexin V, caspase, TMRE, and Sytox Blue so that no overexposure occurs. Usually minimal compensation is required when using 4-laser platform (*see* **Notes 7–9**).
2. Set forward scatter and side scatter parameters to visualize debris. To analyze, gate on cell population based on forward scatter width and height parameters to exclude debris and cell doublets.
3. Data analysis is performed in a standard way for flow cytometry. It is important to notice that intensity of staining (particularly for TMRE and caspase substrate) might be heterogeneous in the entire population and populations with intermediate intensity of staining represent certain stages of apoptotic execution (*see* **Note 10**).

3.6 Acquisition and Analysis Using ImageStream Platform

The analysis procedure described below is based on the ImageStream X Mark II platform and IDEAS software (Amnis Corporation, EMD Millipore). Original (uncompensated) images acquired with an ImageStream™ are stored in a raw image file (*.rif). Single-stained controls are used to produce a compensation matrix (stored as *.ctm file) and, subsequently, compensated image files (*.cif) are generated. Data analysis is performed using *.cif file and saved as *.daf file.

1. Before acquisition, set lasers to balance signals from Annexin V-Alexa 647, caspase 3/7 sensor, TMRE, and Sytox Blue so that no over exposure occurs (*see* **Notes 11** and **12**).
2. Switch off bright-field channel and adjust laser power to achieve a bright signal without saturated pixels for each channel. Acquire at least 200 bright images for each compensation control and create compensation matrix (*.ctm file). Compensation matrixes can be stored and applied to other data files employing the same probes and acquired with same lasers power (*see* **Notes 11** and **13**).
3. Acquire your samples. At least 5000 events should be acquired (10–20 K events is preferable). Recording files containing more than 20,000 pictures should be avoided, since post-acquisition processing of large files is rather slow.
4. After acquisition of the datasets, a compensation matrix has to be applied to correct for spectral overlap. The compensation is performed independently for each of the thousands pixels per image rather than just once for one cellular event in conventional cytometry. This can be done using the wizard that comes standard with IDEAS® (Image Data Exploration and Analysis Software, Amnis Corporation, EMD Millipore) software. The multispectral image compensation in ImageStream system includes nine steps as follows: (1) dark current pixel offset measurement; (2) pixel gain measurement; (3) inter-image

spatial offset measurement; (4) application of dark current corrections; (5) application of spatial alignment corrections; (6) fluorescence crosstalk measurement; (7) bright-field cross talk measurement; (8) application of compensation matrix; (9) application of bright-field pixel gain compensation [13].

5. To start analysis of the data first graph bright field size vs. aspect ratio. Set classifiers to eliminate debris and cell aggregates based on size and "Aspect ratio_M2" of the bright-field channel using linear scale (*see* **Note 14**). This will allow identification of single cells, and eliminate debris from subsequent analyses.
6. In the next step, graph "Brightfield Gradient RMS" and gate on cells that are in focus. Using this population, graph the fluorescence intensity in each channel. It is useful to make a new *.cif and *.daf files including only gated events at this step.
7. Build two-dimensional dotplots in *.cif file and check for the compensation. Whether compensation obtained using IDEAS compensation wizard is not correct, manual compensation could be applied (*see* **Note 15**).
8. To analyze correlation between different staining patterns generate dotplots in Annexin V/TMRE, TMRE/caspase and AnnV/caspase axes in *.daf file.
9. Selection of negative and positive events is performed on the base of image analysis. To put reasonable threshold first gate positive and negative populations on the dot plots. Then look through image galleries generated after gating in each channel. Whether cells with suspicious staining pattern are seen as positive, minimal threshold value should be increased. Useful tips for image analysis are narrowing intensity histogram and enhancing gamma. After selecting the unambiguous threshold value it is applied for statistical analysis.
10. For presentation image galleries, dotplots and histograms are exported and mounted into ppt slides, then scaled and transferred into tiff files using Adobe Photoshop, Paint, or ImageJ software. Example of the output montage is presented in Fig. 2.

3.7 Acquisition and Analysis by Fluorescent Microscopy

The analysis procedure described below is based on acquisition with an Axiovert 200 fluorescent microscope (Zeiss) equipped with following filter cubes: DAPI, FITC, TRITC/Cy-3, Cy5, and SlideBook software. Similar analysis could be done on any other fluorescent microscope.

1. Protocol for attached cells: Subculture cells into 96-well glass-bottom dish at a density of 10,000–20,000 cells per well (in

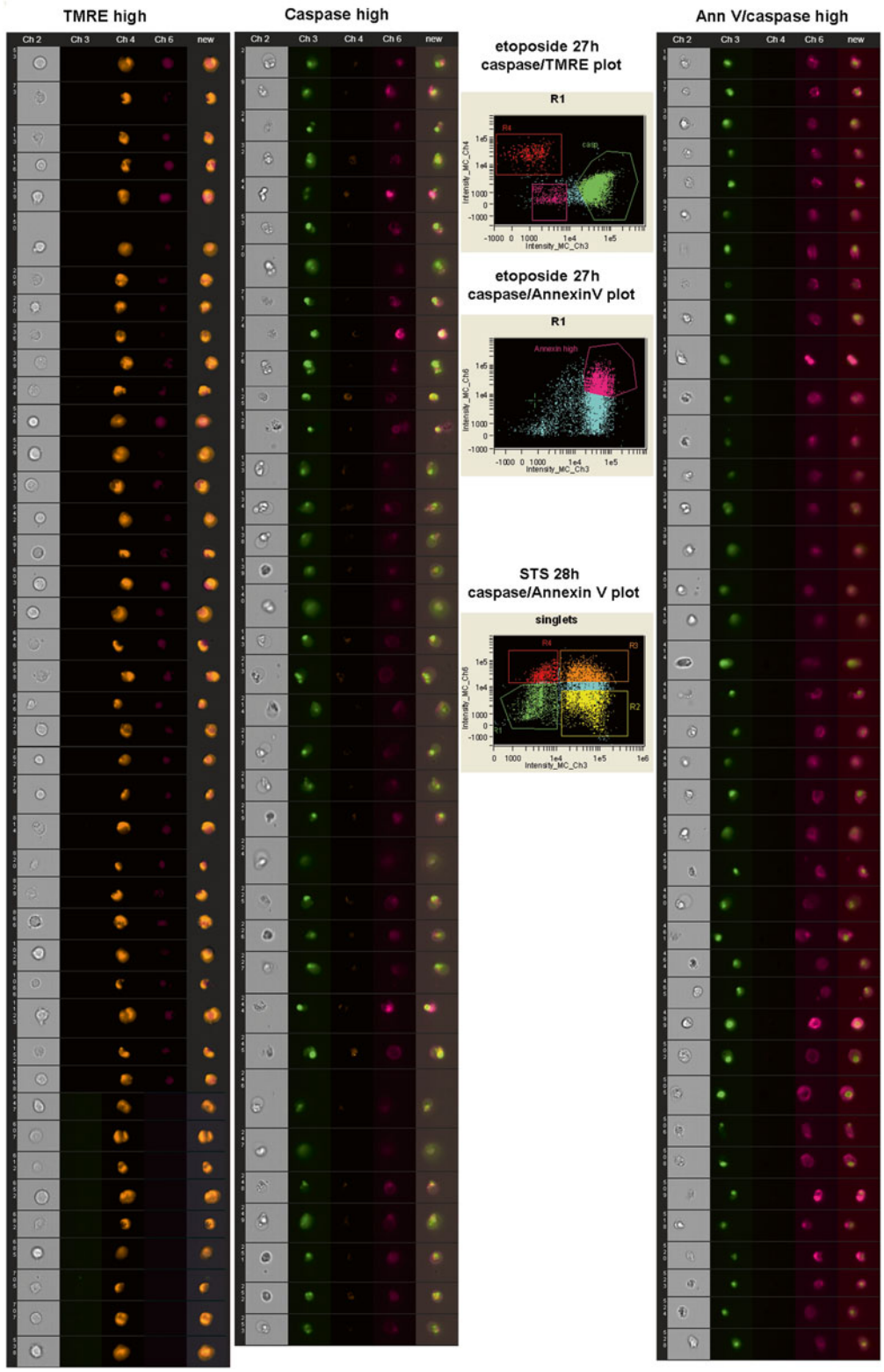


Fig. 2 ImageStream output (Jurkat cells treated with etoposide, 50 μM for 27 h). Images correspond to cells that are TMRE positive (*left column*); caspase positive/Annexin dim (*middle column*), and Annexin V positive (*right column*). Regions are taken from corresponding dotplots (caspase sensor—Intensity Ch3, TMRE—Intensity Ch4, Annexin V intensity–Ch6)

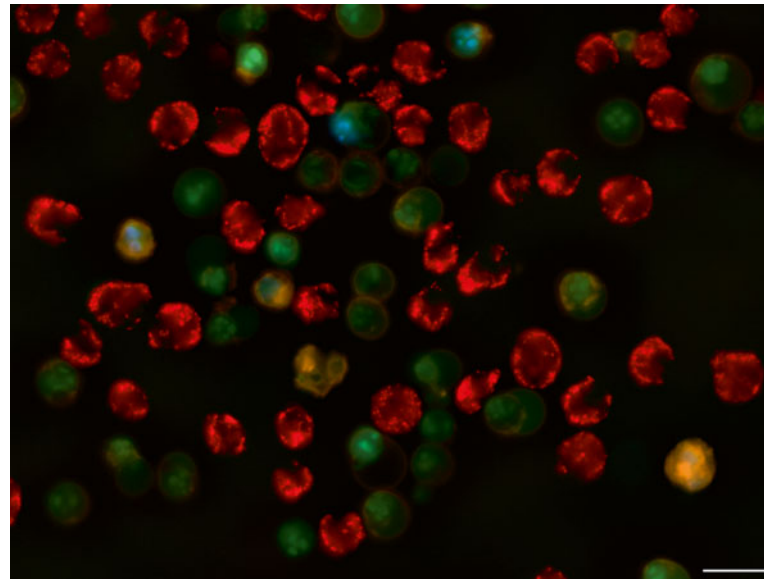


Fig. 3 THP-1 cells treated with TRAIL, 100 ng/ml, 24 h. Quadruple staining, microscopy, objective lens ×40/1.3. *Red*—TMRE staining, *green*—caspase 3/7 staining, *yellow*—Annexin V-Alexa 647 staining, *blue*—Sytox Blue staining. Scale bar—20 μm

0.3 ml volume). Experiment could be started next day after plating. Staining is performed in the following sequence: TMRE, caspase 3/7 substrate, then Annexin V (with additional $CaCl_2$), and finally Sytox Blue.

2. Pictures are taken using DIC and each fluorescent channel. Exposure time should be determined to achieve signal for the positive cells at least 20 % of the dynamic range of camera (*see* **Note 16**).
3. Perform compensation by subtracting images from neighboring channels in the same way as it is recommended for the flow cytometry. Prepare color images using multichannel option and subtracted channels (under View knob—User-defined colors) in SlideBook software. Colors have to be selected from the palette. Example of such image is given on Fig. 3.
4. Protocol for suspended cells. Plate cells into 96 well glass bottom dish at a density of 20,000–50,000 cells per well (in 0.3 ml volume). Staining is performed as described for the flow cytometry. Alternatively, cells already stained with TMRE and caspase sensor could be plated into the dish from the tubes.
5. Allow cells to sediment (it takes not less than 20 min) in CO_2 incubator.

6. Stain cells either in appliance with complete protocol (Subheadings 3.2–3.4) or if TMRE/caspase staining were performed in the tube with Annexin V and then with Sytox Blue as described in Subheadings 3.3–3.4.
7. Pictures are taken 2–3 min after addition of Sytox Blue using DIC and each fluorescent channel. Exposure time should be determined to achieve signal for the positive cells at least 20 % of the dynamic range of camera (*see* **Note 17**).

4 Notes

1. For caspase sensor there is a single vendor—Life Technologies (catalogue # R37111). The big advantage is that caspase substrate can be stored at room temperature. In our hands proper dilution was 5 μL of caspase sensor for 300 μL of cell suspension.
2. Other fluorescent conjugates of Annexin V could be used (with APC etc.), however according to our experience the best results are obtained with Alexa-647 conjugate purchased from Life Technologies. Stock solution of Annexin V conjugate cannot be stored for prolonged period—according to our experience it should be used within 1–2 months upon purchase. After this time intensity of staining of apoptotic cells and reproducibility of results go down.
3. Cell loss may result from staining procedure and thus we recommend that each sample consist of $2–4 \times 10^6$ cells at the start of the procedure. For standard flow cytometry platforms, a lower cell concentration is possible. In our experience 1×10^6 cells is enough when using a BD FACSAria II platform.
4. Apoptotic cells often become fragile, thus centrifuge acceleration should be minimized not to damage cells at late stages of apoptosis. After centrifugation cells should be resuspended gently.
5. Buffers and culture media have different calcium concentrations. Low concentration of calcium might result in a lack of staining with Annexin V of externalized phosphatidylserine or staining is short-living. To our experience when complete culture media like DMEM with calf serum is used Annexin V staining is detected ambiguously. However, once cells are stained and further kept in the presence of >3 mM Ca^{2+}, Annexin V binding to externalized phosphatidylserine is strong and unambiguous.
6. If using three laser platform (488; 407 and 640 nm) excitation of TMRE is rather low and compensation between FITC and

PE channels should be performed using single-stain controls of untreated cells stained with TMRE and cells at late apoptotic stages stained with caspase 3/7 sensor.

7. For FITC and Pacific Blue channels strong autofluorescence might be present (depends on the cell type)—unstained controls are essential.
8. Relative intensity of TMRE, caspase sensor, Annexin V and Sytox Blue staining usually is different from standard signals obtained with conjugated antibodies viewed in the same channels. PMT voltage and compensation table needs to be modified to optimize signals for positive and negative populations in all four channels. For proper compensation single stained probes are required, since emission spectra of these TMRE and caspase sensor differ from standard control beads.
9. It is important to notice that positive staining for caspase and Annexin V could be obtained only on the cells in late apoptotic stages, while TMRE staining is stronger in the untreated cells.
10. On our experience relatively large TMRE intermediate population often appears after staurosporine treatment and smaller populations after other treatments. Intensity of caspase staining could vary in the range of two decades. Dead cells are determined by high intensity of SytoxBlue staining, SytoxBlue intermediate cells might be alive.
11. Since $TMRE^{bright}$ cells dominate in control specimen, while caspase $3/7^{bright}$ and $SytoxBlue^{bright}$ cells are found at the late stages of apoptosis, at least two types of specimens—control (untreated) and late apoptotic probes—should be used for calibration of laser settings.
12. Intensity of TMRE staining depends on the cell type and this dye should be titrated prior to experiment. Usually TMRE gives very strong signal compared to the other fluorophores and 488 nm laser should be set to a minimal intensity (~20 mW). Whether TMRE signal is saturating less concentration of TMRE should be used. Using 561 nm laser for TMRE excitation might give excessive signal even at minimal power.
13. The problems in spectral compensation matrixes can happen because of a number of reasons such as high autofluorescence background and inclusion of saturated pixels. The compensation matrix can be manually adjusted and edited by manually changing crosstalk matrix values in increments and using Preview option. Detailed information is provided in IDEAS manual. The post-acquisition compensation is critical and is one of major obstacles for development of cell sorting option for imaging flow cytometer, which would require a spectral compensation of images available during a sorting.

14. Elimination of cell clamps is better performed manually, since narrowing aspect ratio will exclude a lot of single cell events from subsequent analysis.
15. Our experience is that data obtained through IDEAS compensation wizard (*.ctm file) are usually undercompensated. Manual compensation should be done cautiously by gradually increasing coefficients in compensation matrix not to obtain overcompensated data. During compensation emphasis should be put on the brightest events for each channel. After building proper compensation it is useful to make another *.ctm file that can be used in analysis of other files in the current and similar experiments.
16. To obtain reasonable statistics high-magnification objectives are not useful. Our experience is that the best objective to be used is PlanApo 20×/0.8. Analysis of the pictures taken with 10× objective is possible, but often time consuming.
17. Since cells are not attached to the substrate Brownian motion will blur the image. Thus minimal exposure will give sharper pictures. To obtain reasonable statistics high-magnification objectives are not useful. Our experience is that the best objectives to be used are PlanApo 20×/0.8 (preferable) or PlanApo 40/1.3, but not dry ×40 objective. Analysis of the pictures taken with 10× objective is possible; however objective with high numerical aperture and the same magnification is preferable.

Acknowledgments

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