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Multivariate analysis of apoptotic markers versus cell cycle phase in living human cancer cells by microfluidic cytometry

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Abstract

Measurement of apoptotic markers in tumors can be directly correlated with the cell cycle phase using flow cytometry (FCM). The conventional DNA content analysis requires cell permeabilization to stain nuclei with fluorescent probes such as propidium iodide or use of a costly UV-excitation line for Hoechst 33342 probe. The access to FCM is also still limited to centralized core facilities due to its inherent high costs and complex operation. This work describes development and proof-of-concept validation of a portable and user-friendly microfluidic flow cytometer (μ FCM) that can perform multivariate real time analysis on live cells using sampling volumes as small as 10 microliters. The μ FCM system employs disposable microfluidic cartridges fabricated using injection molding in poly(methylmethacrylate) transparent thermoplastic. Furthermore, the dedicated and miniaturized electronic hardware interface enables up to six parameter detection using a combination of spatially separated solid-state 473 (10 mW) and 640 nm (20 mW) lasers and x-y stage for rapid laser alignment adjustment. We provide new evidence that a simple 2D flow focusing on a chip is sufficient to measure cellular DNA content in live tumor cells using a far-red DNA probe DRAQ5. The feasibility of using the μ FCM system for a dose-response profiling of investigational anti-cancer agents on human hematopoietic cancer cells is also demonstrated. The data show that μ FCM can provide a viable novel alternative to conventional FCM for multiparameter detection of caspase activation and dissipation of mitochondrial inner membrane potential ($\Delta\Psi_m$) in relation to DNA content (cell cycle phase) in live tumor cells.

Keywords

microfluidics; Lab-on-a-Chip; flow cytometry; cell cycle; apoptosis; programmed cell death; cancer

1. INTRODUCTION

Considerable progress is currently being made in our understanding of a diversity of existing modes of programmed tumor cell death.^{1–3} The cell propensity to undergo so called classical apoptosis is a key mechanism in the pathogenesis of many human diseases.^{1–3} The term apoptosis is used here to define the “classical” apoptotic process, initially also called programmed cell death (PCD) which is inclusive of such hallmarks as: (i) mitochondrial changes manifesting by collapse of the transmembrane potential and release of cytochrome c to cytosol, (ii) activation of caspases, (iii) chromatin condensation (pycnosis), (iv) activation of endonuclease(s) followed by internucleosomal DNA cleavage (v), segregation of nucleoli, (vi) fragmentation of nucleus, and (vii) plasma membrane blebbing associated with formation of apoptotic bodies.^{4,5}

In the context of raising clinical relevance of tumor cell death in cancer therapy, there is an ever increasing demand for convenient analytical tools to rapidly quantify and characterize apoptotic cell death.^{4–7} High-content analysis is widely recognized as a key component in the anti-cancer drug discovery pipelines. Multiparameter flow cytometry (FCM) represents one of the most important techniques for rapid quantification of programmed cell death.^{4, 8–11}

Vast majority of classical attributes of apoptosis can be quantitatively examined by flow cytometry.^{4–6} It allows assessment of multiple cellular attributes on a single cell level. The possibility of rapid measurement of individual cells in large cell populations offered by flow cytometry made it the methodology of choice to identify and quantify apoptotic cells as well as to study mechanisms associated with this mode of cell death.^{4–5} By virtue of multiparameter analysis, cytometry allows correlative studies between many cell attributes based on both light scatter and fluorescence measurements.^{4–5} An example of this is that measurements of apoptotic markers in tumors can be directly correlated with the DNA content (cell cycle phase) using FCM.^{12–13} The conventional DNA content analysis requires cell permeabilization to stain nuclei with fluorescent probes such as propidium iodide or use of a costly UV-excitation line for Hoechst 33342 probe.^{4,5} The access to FCM is also still limited to centralized core facilities due to its inherent high costs and complex operation.^{4–6}

This work describes development and proof-of-concept validation of a portable and user-friendly microfluidic flow cytometer (μ FCM) that can perform multivariate analysis on live tumor cells using minimal sampling volumes. We provide new evidence that a simple 2D flow focusing on a chip is sufficient to measure cellular DNA content in live tumor cells using a far-red DNA probe DRAQ5. The feasibility of using the μ FCM system for a dose-response profiling of investigational anti-cancer agents on human hematopoietic cancer cells is also demonstrated. The data show that μ FCM can provide a viable novel alternative to conventional FCM for multiparameter detection of caspase activation and dissipation of mitochondrial inner membrane potential ($\Delta\Psi_m$) in relation to DNA content (cell cycle phase) in live tumor cells.

The work provides a foundation for development of the next generation μ FCM in drug discovery and personalized therapy. We postulate that microfluidics provides an exceptional evolutionary avenue for development of miniaturized cytometers for testing ultra-low cell samples with superior automation.^{4–6}

2. MATERIALS AND METHODS

2.1 Biological specimen

Human monocytic leukemia THP1 α cells were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in an Advanced RPMI 1640 culture medium (Life Technologies, Carlsbad, CA, USA) supplemented with 5% Fetal Bovine Serum (FBS; Life Technologies) at 37°C under 5% CO₂. During experiments, cells were always in asynchronous and exponential phase of growth.

2.2 Analysis of the DNA content

For analysis of DNA content (cell cycle), THP1 α cells were fixed and permeabilized in 70% ethanol and stained with 20 μ g/ml of stoichiometric DNA stain propidium iodide (PI; Life Technologies) in the presence of RNase A (30 μ g/ml) to remove the RNA.^{8–9} To perform cell cycle analysis, PI fluorescent signals (473 or 488 nm excitation and 585 nm emission) were amplified using linear mode. Analysis of DNA content in living cells was performed by staining with 20 μ M of cell permeable, stoichiometric DNA stain DRAQ5 (640 or 633 nm excitation and 670 nm emission; Biostatus Limited).

2.3 Detection of mitochondrial membrane depolarization in relation to DNA content

To induce intrinsic (mitochondrial) pathway of apoptosis, THP1 α cells were treated with apoptosis stimulators staurosporine (STS; Life Technologies; 0–10 nM), taxol/paclitaxel (TAX; Life Technologies; 0–100 nM), camptothecin (CAM, Merck Millipore; 0–100 nM). For multiparameter analysis of mitochondrial membrane depolarization in relation to DNA content, cells were grown in the presence of 200 nM of $\Delta\Psi_m$ sensitive probe tetramethylrhodamine methyl ester (TMRM; Life Technologies).^{8–9} After stimulation, cells were counterstained with 20 μ M of DRAQ5 probe for 20 min and immediately analyzed on the microflow cytometer. DRAQ5 fluorescent signals (640 or 633 nm excitation and 670 nm emission) were amplified using linear mode whereas TMRM signals (473 or 488 nm excitation and 585 nm emission) using logarithmic mode.

2.4 Detection of caspase activation in relation to DNA content

To induce intrinsic (mitochondrial) pathway of apoptosis, THP1 α cells were treated with apoptosis stimulators staurosporine (STS; Life Technologies; 0–10 nM), taxol/paclitaxel (TAX; Life Technologies; 0–100 nM), camptothecin (CAM, Merck Millipore; 0–100 nM). For multiparameter analysis of caspase activation in relation to DNA content, cells were grown in the presence of a xanthene H-type dimmer caspase substrate PhiPhiLux (OncoImmune Inc, Gaithersburg, MD, USA).¹⁴ After stimulation, cells were counterstained with 20 μ M of DRAQ5 probe for 20 min and immediately analyzed on the microflow cytometer. DRAQ5 fluorescent signals (640 or 633 nm excitation and 670 nm emission) were amplified using linear mode whereas PhiPhiLux signals (473 or 488 nm excitation and 530 nm emission) using logarithmic mode.

2.5 Computational fluid dynamics simulations

Computational fluid dynamics (CFD) technique was utilized to comprehend the performance of the microflow cytometric system. The 3D chip model was designed using SolidWorks 2011 (Dassault Systemes SolidWorks Corp, Concord) CAD package. The geometry was then exported to Gambit 2.3 software (Fluent Inc.) for mesh generation. The mesh file was then exported to the finite-volume based Fluent 6.3 software (Fluent Inc.) to solve the associated differential equations governing the balance of mass, momentum and transport of species. The fluid was considered Newtonian, incompressible and laminar. Boundary conditions consisted of zero pressure at the inlets while a –30 kPa pressure at the

outlets. The no-slip boundary condition was applied at the bottom, top and sidewalls of the system.

2.6 Microfluidic flow cytometry system

The microfluidic, disposable chips were fabricated in optically transparent thermoplastic poly(methyl methacrylate) (PMMA) using proprietary injection molding and UV bonding technology (On-Chip biotechnologies Co Ltd, Tokyo, Japan) (Figure 1). The chip design featured three integrated modules: (i) the input port with integrated cell sample (200 μ l) and sheath fluid (2 ml) reservoirs; (ii) a 2D cell focusing manifold with a channel 75 μ m $\times$ 50 μ m; and (iii) the output port with collection chambers that mirrored the input port (Figure 1). The off-chip hardware analysis was equipped with two solid-state lasers (blue; 473 nm and red; 640 nm). Four photomultiplier tube-based (PMT) detection channels with band-pass (BP) filters: FL1 (530 $\pm$ 21 nm), FL2 (585 $\pm$ 20 nm), FL3 (692 $\pm$ 20 nm), and FL4 (775 $\pm$ 23 nm) (Figure 2). The use of spectrally dissimilar probes excited by two separate lasers eliminated the need for compensation of the fluorescent signals. The air-over-liquid system with positive pressure generated in the input port using the microcontroller-driven syringe pump was used to actuate the microfluidic chip-based devices inside the Fishman R cytometer. The fluid flow rate was adjustable between 1 to 20 μ l/min by changing the pressure applied to the outlet port. Despite of high pressure (up to 30 kPa) the Reynolds number based on the hydraulic diameter of the detection channel was 41.7 corresponding to the laminar characteristics of the flow inside the device. A typical run used a sample with 1–2.5 $\times$ 10³ cells.

2.7 Reference, conventional flow cytometry

Flow cytometry was performed using a BD FACSCalibur (Becton Dickinson, Franklin Lakes, NJ, USA) analyzer, equipped with a 15 mW Argon-ion (488 nm) and 20 mW red diode (633 nm) lasers. Acquisition of FCS2.0 data files was performed in 1024 channels resolution scale using CellQuest Pro software (Becton Dickinson). A typical run used a sample with 5 $\times$ 10³ cells.^{8–10}

3. RESULTS AND DISCUSSION

3.1 Chip design, fabrication and operation

The microfluidic chip-based cytometer featured three integrated modules: (i) the input port with sample and sheath fluid reservoirs; (ii) cell focusing and interrogation manifold with a channel 75 μ m $\times$ 50 μ m; and (iii) the output port with collection reservoirs for sample and sheath fluid (Figure 1). The chip was designed to provide a simple 2D hydrodynamic flow focusing principle (Figure 2). It was suitable for focusing cells into a single file by using an air-over-liquid system with positive pressure generated in the input port using the microcontroller-driven syringe pump (Figure 2). The fluid flow rate was adjustable between 1 to 20 μ l/min by changing the pressure applied to the outlet port. The indirect actuation of the flow ensured no carryover and lack of any liquid/sample contact. This eliminated any potential cross-contamination.

The electronic hardware interface enabled up to four-color detection using a combination of spatially separated solid-state 473 (10 mW) and 640 nm (20 mW) lasers and micrometer x-y axis stage for use-friendly laser alignment adjustments (Figure 3). Conventional photomultiplier tubes (PMTs) were used for fluorescent signal collection in area, height and width parameters for all four fluorescent channels. The estimated sensitivity of the system was within specifications of conventional flow cytometers (FITC < 600 MESF, PE < 200 MESF; Molecules of Equivalent Soluble Fluorochrome) (Figure 3).

The off-chip interface was equipped with two photodiodes for a forward light scatter (FSC; a representative of cell size) and side light scatter (SSC; a representative of cell complexity/granularity) detection. The latter parameter was collected using a specially designed reflective edge of the chip and named SLER (Side scattered Light detection using Edge Reflection of chip). SLER used a slope in the sidewall of the microfluidic chip formed by injection molding during the chip fabrication. The detection of the signal coming out of the SLER module used an optical guide block for collecting the side-scattered light. The data processing acquired analog signals from PMTs (Hamamatsu Photonics KK, Hamamatsu City, Japan) detectors and performed I/V (current to voltage) and A/D (analog to digital) conversion using A/D converter operating at 16 bit, 1 MHz sampling. The system had capability to simultaneously process data stream from 6 independent data channels. It also included an innovative real-time oscilloscope mode for detection and evaluation of fluorescence signals.

3.2 DNA frequency histograms using planar microflow cytometry system

Analysis of DNA content refers to ploidy of the cells and the position in the cell cycle phase. It is of utmost importance in diagnostic oncology and treatment monitoring. By virtue of multiparameter analysis, flow cytometry allows correlative studies between many cell attributes based on both light scatter and fluorescence measurements.⁴⁻⁵ An example of this is quantification of apoptotic markers in tumors that can be directly correlated with the DNA content using FCM. The conventional DNA content analysis requires, however, cell permeabilization to stain nuclei with fluorescent probes such as propidium iodide or use of a costly UV-excitation line for cell permeable Hoechst 33342 probe.⁴⁻⁵ The capability to perform rapid and supravital analysis of cell cycle on ultra-low samples of living tumor cells would offer a considerable analytical advantage.^{4-6, 15}

In this regard, we are first to provide evidence that straightforward 2D hydrodynamic flow focusing on a disposable and planar chip-based device can unravel DNA content in living tumor cells using fluorescence detection. Our data indicated that the sufficient resolution of the DNA histogram could be achieved on fixed and permeabilized THP1 leukemic cells labeled with propidium iodide with unmistakably distinguishable G1, S and G2/M cell cycle phases (Figure 4).

We also examined the capacity to measure the cellular DNA content in living tumor cells and study their responses to drugs perturbing the cell cycle phase distribution (cell cycle arrest) (Figure 5). The microflow cytometric platform combined with staining of leukemic cells with a new cell permeant, live-cell dye DRAQ5 allowed to analyze the cell G2/M cell cycle arrest induced by a pan-kinase inhibitor staurosporine and microtubule toxin taxol/paclitaxel (Figure 5). The data provide proof-of-concept that microflow cytometry can acquire DNA frequency histogram data at sufficient resolution to produce statistically robust information on small samples of living tumor cells. When combined with innovative off-chip opto-electronic interfaces the simple chip design can indeed provide statistical power comparable to that of conventional flow cytometric measurements.

3.3 Multiparameter detection of mitochondrial depolarization in relation to the cell cycle phase

Concurrent differential labeling of apoptotic markers and DNA content provides a convenient assay to correlate induction of programmed cell death with the cell cycle phase.⁸⁻⁹ The mitochondrion stands at the nexus of sensing and integrating diverse incoming stress signals, and mitochondrial disturbances often occur long before any marked morphological symptoms of apoptosis.^{8-9, 16} The cytometric detection of $\Delta\psi_m$ loss is a sensitive marker of early apoptotic events.^{4-5, 8-9} Procedures are based on lipophilic cationic

probes such as tetramethylrhodamine methyl ester perchlorate (TMRM) that are readily taken up by live cells and accumulate in mitochondria according to the Nernst equation.^{4-5, 8-9} In this work, we demonstrated that it is possible to perform two-color labeling of mitochondrial depolarization in living tumor cells in relation to DNA content using cell permeable probe TMRM and stoichiometric DNA probe DRAQ5, respectively (Figure 6). In this assay, cell cycle position is measured for both apoptotic and surviving cells, allowing direct determination of cell cycle specificity of the pharmacological agent. Following mitochondrial depolarization during pharmacologically induced programmed cell death using transcription inhibiting polypeptide antibiotic actinomycin D (Act D), the $\Delta\Psi_m$ loss is observed as a reduction of TMRM fluorescence. Our data provide proof-of-concept that microflow cytometric system was adequate to discern the DRAQ5 fluorescent signals amplified using linear mode and plot them against the TMRM signals using logarithmic amplification mode (Figure 6).

3.4 Multiparameter detection of caspases activation in relation to the cell cycle phase

One of the hallmarks of classical apoptosis is the activation of unique cysteine aspartyl-specific proteases having a conserved QACXG consensus site containing active cysteine, called caspases (from cysteinyl aspartate-specific proteases).¹⁷⁻¹⁹ Under normal physiological conditions caspases are constitutively expressed in the cytoplasm as zymogens with very low intrinsic activity. They become activated upon transcatalytic cleavage followed by dimerization. Once activated, caspases function in an orchestrated proteolytic cascade leading to self-amplification, cleavage of vital cell substrates and ultimate cell disassembly.

Here we also demonstrated that it is possible to perform two-color labeling of caspase activation in living tumor cells in relation to DNA content using cell permeable fluorogenic caspases substrate PhiPhiLux and stoichiometric DNA probe DRAQ5, respectively (Figure 7). In this assay, cell cycle position is measured for both cells with activated caspases (deemed apoptotic) and inactive caspases (deemed live), allowing direct determination of cell cycle specificity of the investigational pharmacological agent. The PhiPhiLux system utilizes xanthenes dyes forming H-type dimers that exhibit fluorescence quenching associated with their formation.^{4-5, 14} Upon cleavage of the linker by the activated caspases, both rhodamine molecules are released and become highly fluorescent.^{4-5, 14} Most importantly, the microfluidic cytometer was fully capable to discern the DRAQ5 fluorescent signals amplified using linear mode and plot them against the PhiPhiLux signals using logarithmic amplification mode (Figure 7).

4. CONCLUSIONS

There is an ever-increasing demand for convenient analytical tools to rapidly quantify and characterize diverse tumor cell demise modes.⁴⁻⁶ Since cell death is a stochastic process, high-throughput single-cell analysis platforms are often of essence to deliver meaningful insights into intrinsically heterogeneous cell populations.²⁰⁻²¹ In this context, we provide new evidence that a simple 2D flow focusing on a chip is sufficient to measure cellular DNA content in live tumor cells using both propidium iodide and far-red DNA probe DRAQ5. Our work demonstrates that planar 2D focusing microfluidic chip-based cytometer can provide a viable novel alternative to conventional FCM for multi-parameter detection of caspase activation and dissipation of mitochondrial inner membrane potential ($\Delta\Psi_m$) in relation to DNA content (cell cycle phase).

Our work shows that simple microfluidic chip designs are sufficient to acquire high quality biological data and provide statistical power comparable to that of conventional flow cytometric measurements when combined with sophisticated off-chip electronic interfaces.

The work provides a foundation for development of the next generation Lab-on-a-Chip flow cytometers in drug discovery and personalized therapy capable testing ultra-low cell samples with superior automation. We anticipate that the Lab-on-a-Chip technologies warrant a major “quantum leap” in studies of cell death at both single cell and population levels.

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Figure 1. Microfluidic flow cytometry system A) Disposable plastic chip inside chip interface (alignment holder) B) Microphotograph of the chip-based cytometry showing its layout and main modules.

Figure 2.
2D hydrodynamic focusing on the planar microflow cytometric system.

Figure 3.
Schematics of the off-chip interface depicting major optical path components.

Figure 4.

DNA frequency histogram deconvolution on fixed and permeabilized THP1 leukemic cells labeled with propidium iodide A) Comparative histogram analysis; B) Quantification of the statistical data obtained from histogram deconvolution.

Figure 5. DNA frequency histogram deconvolution on living THP1 leukemic cells labeled with supravital live-cell stain DRAQ5 A) Comparative histogram analysis; B) Quantification of the statistical data obtained from histogram deconvolution.

Figure 6. Multiparameter analysis of mitochondrial depolarization in relation to the cell cycle phase. A) Dot plot analysis with DRAQ5 fluorescent signals amplified using linear mode plotted against the TMRM signals using logarithmic amplification mode; B) Comparative analysis of the cumulative DNA content between conventional and microfluidic flow cytometer.

Figure 7.
Multiparameter detection of caspases activation (PhiPhiLux+) in relation to the cell cycle phase.