

Short communication

Multiparameter Lab-on-a-Chip flow cytometry of the cell cycle



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ABSTRACT

Multiparameter analysis of apoptosis in relation to cell cycle position is helpful in exploring mechanism of action of anticancer drugs that target specific molecular cogs of the cell cycle. This work demonstrates a new rationale for using microfluidic Lab-on-a-Chip flow cytometry (μ FCM) with a simple 2D hydrodynamic focusing for the multiparameter analysis of apoptosis and DNA ploidy analysis in human hematopoietic cancer cells. The microfluidic system employs disposable microfluidic cartridges fabricated using injection moulding in optically transparent poly(methylmethacrylate). The dedicated and miniaturized electronic hardware interface enables up to six parameter detections using a combination of spatially separated solid-state 473 nm (10 mW) and 640 nm (20 mW) lasers and x - y stage for rapid laser alignment adjustment. We provide evidence that the simple 2D flow focusing on a chip-based device is sufficient to measure cellular DNA content in both fixed and living tumor cells. The feasibility of using the μ FCM system for multiparameter analysis of caspase activation and dissipation of mitochondrial inner membrane potential ($\Delta\Psi_m$ loss) in relation to DNA content is also demonstrated. The data shows that straightforward microfluidic chip designs are sufficient to acquire high quality biological data when combined with sophisticated electronic interfaces. They can be a viable alternative to conventional FCM for multiparameter detection of programmed cell death.

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1. Introduction

High-content analysis is widely recognized as a key component in anti-cancer drug discovery pipelines. In this context, multiparameter flow cytometry (FCM) is one of the most important techniques for rapid quantification of programmed cell death (PCD) (Wlodkowic et al., 2010; Wlodkowic et al., 2011). Measurement of PCD markers such as caspase activation and dissipation of mitochondrial inner membrane potential ($\Delta\Psi_m$) can be directly correlated with the cell cycle phase using FCM (Wlodkowic et al., 2010; Wlodkowic et al., 2011). Concurrent differential labeling of apoptotic markers and DNA content is helpful in exploring the mechanism of action of anticancer drugs that target specific molecular cogs of the cell cycle (Halicka et al., 1997; Wlodkowic et al., 2006, 2007).

The access to conventional FCM is still limited to centralized core facilities due to its inherent high costs and complex

operation (Wlodkowic, 2012; Wlodkowic et al., 2010). The last decade has brought many innovations to the field of cytomics and cytometry including a variety of microfluidic-based flow cytometry technologies (Wlodkowic and Cooper, 2010; Wlodkowic et al., 2009b). Multiple reports have demonstrated chip-based flow cytometers and cell sorters with both electro-osmotic and hydrodynamic-driven flows (Cheung et al., 2010; Hur et al., 2010; Moon et al., 2009; Sugino et al., 2009). Considerable efforts have been made to design advanced three-dimensional (3D) and multilayer microfluidic flow cells (Lee et al., 2009; Scott et al., 2008; Simonnet and Groisman, 2006). Moreover, numerous advances have been made in embedding the optical components directly into chip-based cytometers using a combination of etched fiber optics, miniaturized lenses and solid-state lasers (Chung and Kim, 2007; Gluckstad, 2004; Huh et al., 2005; Wu et al., 2004).

In this work, we demonstrate a radically different approach that exploits a very simple 2D hydrodynamic flow focusing on a planar and disposable plastic chip (cartridge). In contrast to most previously published intricate chip designs we have concentrated, however, our efforts on sophisticated electronic “off-chip” interface that is user-friendly and provides sensitivity comparable to that of conventional, macroscale flow cytometers. To validate our approach we show that the 2D hydrodynamic focusing

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on a planar microflow cytometric chip is sufficient to resolve the profile of DNA content and track the pharmacologically induced activation of caspases and dissipation of mitochondrial inner membrane potential ($\Delta\psi_m$) in relation to the cell cycle in living human tumor cells.

2. Experimental methods

Human histocytic U937 and monocytic THP1 α leukemia cell lines were obtained from American Type Culture Collection (ATCC, USA) and grown in an advanced RPMI 1640 culture medium supplemented with 5% FBS as described previously (Włodkowiec et al., 2009a; Włodkowiec et al., 2008). Computational fluid dynamics (CFD) was used to simulate the performance of the microflow cytometric system. The 3D chip model was designed using the SolidWorks 2011 (SolidWorks Corp., USA) package and a finite-volume based model was run on the Fluent 6.3 software (Fluent, Lebanon, NH, USA) to solve the associated Navier–Stokes differential equations. The microfluidic cartridges (chips) were fabricated in optically transparent poly(methyl methacrylate) (PMMA) using the injection molding technique (On-Chip Biotechnologies Co. Ltd., Japan). The hardware interface included (i) microcontroller-driven syringe pump providing adjustable pressurization between 5–30 kPa; (ii) two spatially separated, solid-state laser excitation sources (473 nm and 640 nm); (iii) two photodiodes for forward and side light scatter detection; and (iv) four photomultiplier tubes (PMT) with band-pass filters: FL1 (530 ± 21 nm), FL2 (585 ± 20 nm), FL3 (692 ± 20 nm), and FL4 (775 ± 23 nm). A typical run used a sample with $2.5\text{--}5 \times 10^3$ cells. Data acquisition was performed using native Fishman R software (On-chip Biotechnologies Co. Ltd.). A typical run used samples with 5×10^3 cells. Data files were subsequently converted to FCS 3.0 standard using a Fishman-R data converter and analyzed on the FCS Express 4 RUO flow cytometry software with a cell cycle fitting module (De Novo Software, USA).

3. Results and discussion

The planar flow cytometry chip-based device was designed to provide a 2D hydrodynamic flow focusing. It featured three 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 $75 \mu\text{m} \times 50 \mu\text{m}$; and (iii) the output port with collection chambers that mirrored the input port (Fig. 1A and B). Electronic air syringe pump was used for flow control. The air-over-liquid system used air pressure value as an index for the sample speed. We found that this device 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 (Fig. 1B and C). In spite 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 (Fig. 1C and D). The fluid flow rate was adjustable between 1 and 20 μ l/min by changing the pressure applied to the input port. The electronic hardware interface enabled up to four-color detections using a combination of spatially separated solid-state 473 (10 mW) and 640 nm (20 mW) lasers and the micrometer x – y axis stage for user-friendly laser alignment adjustments (Fig. 1D). 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). The system also included two photodiodes for true forward light scatter (FSC; a representative of cell size) and side light scatter (SSC; a representative of cell complexity/granularity) detection. The electronic detection interface acquired analog signal from a PMT (Hamamatsu Photonics KK, Hamamatsu City, Japan) detector. The signal then underwent I/V (current–voltage) and A/D (analog–digital) conversion using an A/D converter operating at 16 bit, 1 MHz sampling and with capability to simultaneously process data streams from six independent channels. Moreover, the system included an innovative real-time oscilloscope mode for detection and evaluation of fluorescence signals. The embedded system was run under Windows XP operating system and allowed for real-time control of the system parameters together with data collection using one integrated user interface.

The SSC parameter was collected using a specially designed reflective edge of the chip and named SLER (Side scattered Light detection using Edge Reflection of chip) technology (Fig. 1D). The SLER system utilized a specially designed slope in the side wall of the microfluidic chip. This structure was formed by injection molding during the chip fabrication. Roughness of the edge slope depended on roughness of the metal mold and in this example the roughness was optical grade. The detection of the signal coming out of the SLER module used an optical guide block for collecting the side-scattered light. The width of the optical guide block was 10 times larger than that of edge SLER slope. As a result this detection system was not sensitive for up to 0.1 mm order change in x – y position.

We validated that a 2D hydrodynamic flow focusing on a planar chip is sufficient to measure cellular DNA content in fixed human leukemic cells (Fig. 2A and B). Cellular DNA content represents a distribution of cells in three separate phases of the cell cycle (G1, S, and G2/M). Cell cycle process is required to perform synthesis and duplication of the DNA before commencing the process of cell division (mitosis).

Following staining of cells with a DNA-specific and stoichiometric fluorescent stain the characteristic cell cycle profile can be recognized by flow cytometry. This is performed by plotting fluorescence intensity on the x -axis and number of cells on the y -axis, and this is commonly referred to as a DNA content frequency histogram. The DNA content frequency histogram allows to quickly analyze the distribution of cells in different phases of the cell cycle in a mixed cell population. In this regard, the first peak denoted as G1 represents diploid (2N) resting cells whereas the second peak denoted as G2/M cells that have duplicated their DNA content (4N) and are preparing for or undergoing mitosis (M). The region denoted S that is located between G1 and G2/M peaks corresponds to cells actively replicating their DNA content (Fig. 2).

For this purpose, the THP1 α and U937 cells were fixed and permeabilized in 70% ethanol and stained with 20 μ g/ml of stoichiometric DNA stain propidium iodide (PI) in the presence of RNase A to remove the RNA (Halicka et al., 1997). Alternatively living human tumor cells were labeled with a novel cell-permeable and far-red fluorescence DNA binding probe DRAQ5 (Smith et al., 1999; Zhao et al., 2009). The latter probe enters living cells and stoichiometrically labels DNA content albeit at lower resolution when compared to PI (Smith et al., 1999; Zhao et al., 2009). Upon staining, the cells were loaded onto the microfluidic device and analyzed using the linear amplification mode at the flow rate of 20 μ l/min. We have found that results obtained on the microflow cytometry chip correlated well with the conventional gold standard of flow cytometry, as shown in Fig. 2A and B. Quantification of peaks representing G1, S and G2/M phases of the cell cycle was carried out with automatic DNA fitting using Dean and Jett's algorithm (Fig. 2B and D). For fixed

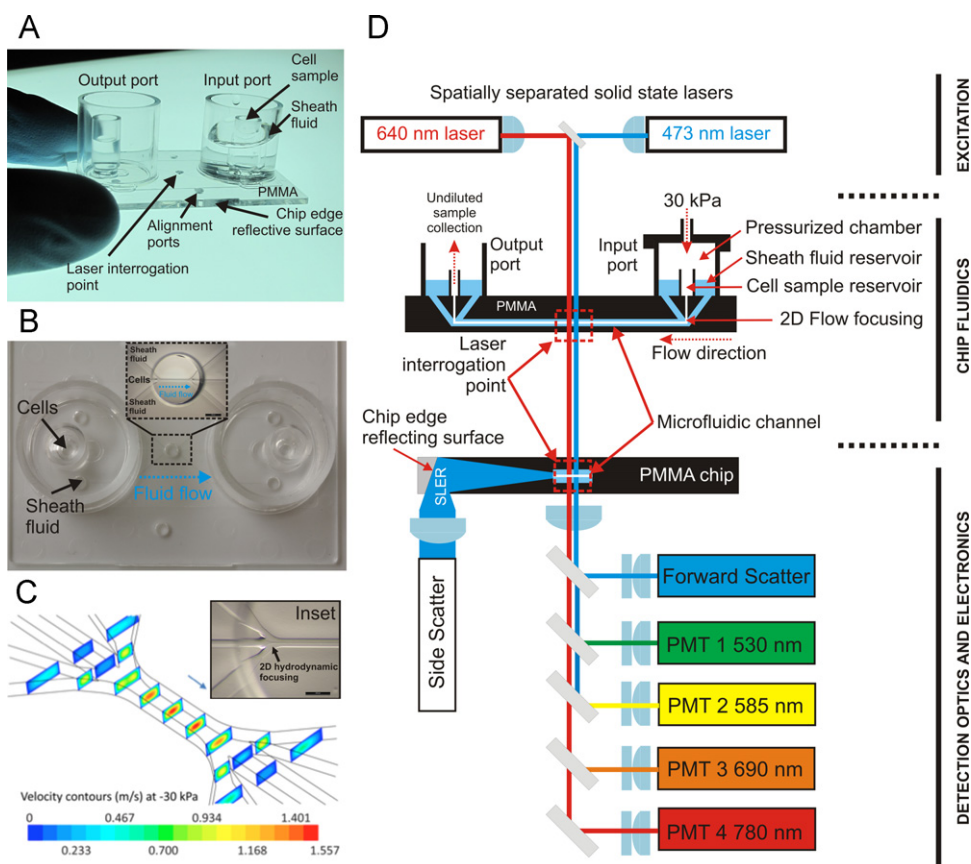


Fig. 1. Lab-on-a-Chip flow cytometry system. (A) Disposable microfluidic chip with integrated sheath and cell sample reservoirs. (B) Layout of the chip-based microflow cytometer. Inset: microphotograph depicts the magnified section of the chip (marked with dotted lines). (C) Contours of fluid flow velocity (m/s) obtained by computational fluid dynamic (CFD) simulations. Inset: phase-contrast microphotograph showing the 2D hydrodynamic focusing inside the chip-based cytometer. (D) Overview of the subcomponent system design with solid-state laser excitation, integrated air-over-liquid pressurization of the chip and the optical path with true forward and side light scatter detection performed using the on-chip SLER (Side scattered Light detection using Edge Reflection) technology.

cancer cells labeled with PI this yielded 42%, 39.6%, 18.4% on a reference flow cytometer versus 40.1%, 43.2%, 16.7% on a microflow cytometer for G1/S/G2-M, respectively ($p < 0.05$, ANOVA). Alternatively, for living cells labeled with DRAQ5 this yielded 47.5%, 32.2%, 20.3% on a reference flow cytometer versus 45.9%, 35.6%, 18.4% on a microflow cytometer for G1/S/G2-M, respectively ($p < 0.05$, ANOVA).

Next, we treated the THP1 α cells with pan-kinase inhibitor Staurosporine (STS; 1 and 10 nM) and mitotic inhibitor Taxol/Paclitaxel (TAX; 100 nM) to evaluate microflow cytometry capabilities to quantify drug-induced cell cycle disturbances. As expected, cells underwent G2/M cell cycle arrest upon exposure to 100 nM of STS and TAX, as shown in Fig. 2E. Representative results from the microflow cytometer correlated well with the conventional gold standard of flow cytometry (Fig. 2E). Although the chip-to-chip variation exceeded the variations obtained by the conventional flow cytometer, the excellent correlation between the two platforms lends further support to the notion that 2D hydrodynamic focusing can be efficiently employed for DNA histogram measurements.

The knowledge on cell cycle specificity of apoptosis induction is of clinical importance as it provides clues to design multidrug treatment protocols (Halicka et al., 1997; Huang et al., 2005; Wlodkowiec et al., 2006). We therefore set out to demonstrate that it is possible to perform concurrent differential labeling of apoptotic markers and DNA content using the Lab-on-a-Chip flow cytometer in living leukemic cells. For this purpose, we measured the dissipation of mitochondrial inner membrane potential ($\Delta\Psi_m$ loss; using $\Delta\Psi_m$ sensitive probe tetramethyl rhodamine methyl

ester, perchlorate (TMRM)) and cell cycle profile (using DRAQ5 probe; Fig. 3A). 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 (Wlodkowiec et al., 2006). The TMRM probe accumulates bright fluorescence only in energized mitochondria and its uptake is governed by the Nernst equation (Wlodkowiec et al., 2006, 2007). Following mitochondrial depolarization during programmed cell death, the $\Delta\Psi_m$ loss is observed as a reduction of TMRM fluorescence. After 24-hour treatment of THP1 cells with a topoisomerase I inhibitor camptothecin, the multiparameter microcytometric analysis uncovered substantial accumulation of cells with intact mitochondrial inner membrane potential in S phase (S phase cell cycle arrest) at lower dose of camptothecin (Fig. 3A). Interestingly, although camptothecin caused $\Delta\Psi_m$ loss irrespective of the cell cycle position, the S phase arrested cells exhibited a substantial resistance to higher doses of camptothecin as evidenced by preservation of the mitochondrial inner membrane potential (Fig. 3A). These cells accounted for nearly 99% of surviving cells.

Finally, we demonstrated a tri-color differential labeling with two independent apoptotic markers such as fluorogenic caspases substrate PhiPhiLux and $\Delta\Psi_m$ sensitive probe TMRM in relation to DNA content in living leukemic cells (Fig. 3B and C). The PhiPhiLux system utilizes xanthene dyes forming H-type dimers that exhibit fluorescence quenching associated with their formation (Packard and Komoriya, 2008; Telford et al., 2002). Upon cleavage of the linker by the activated caspases, both rhodamine molecules are released and become highly fluorescent (Packard and Komoriya, 2008; Telford et al., 2002). After 24 h of

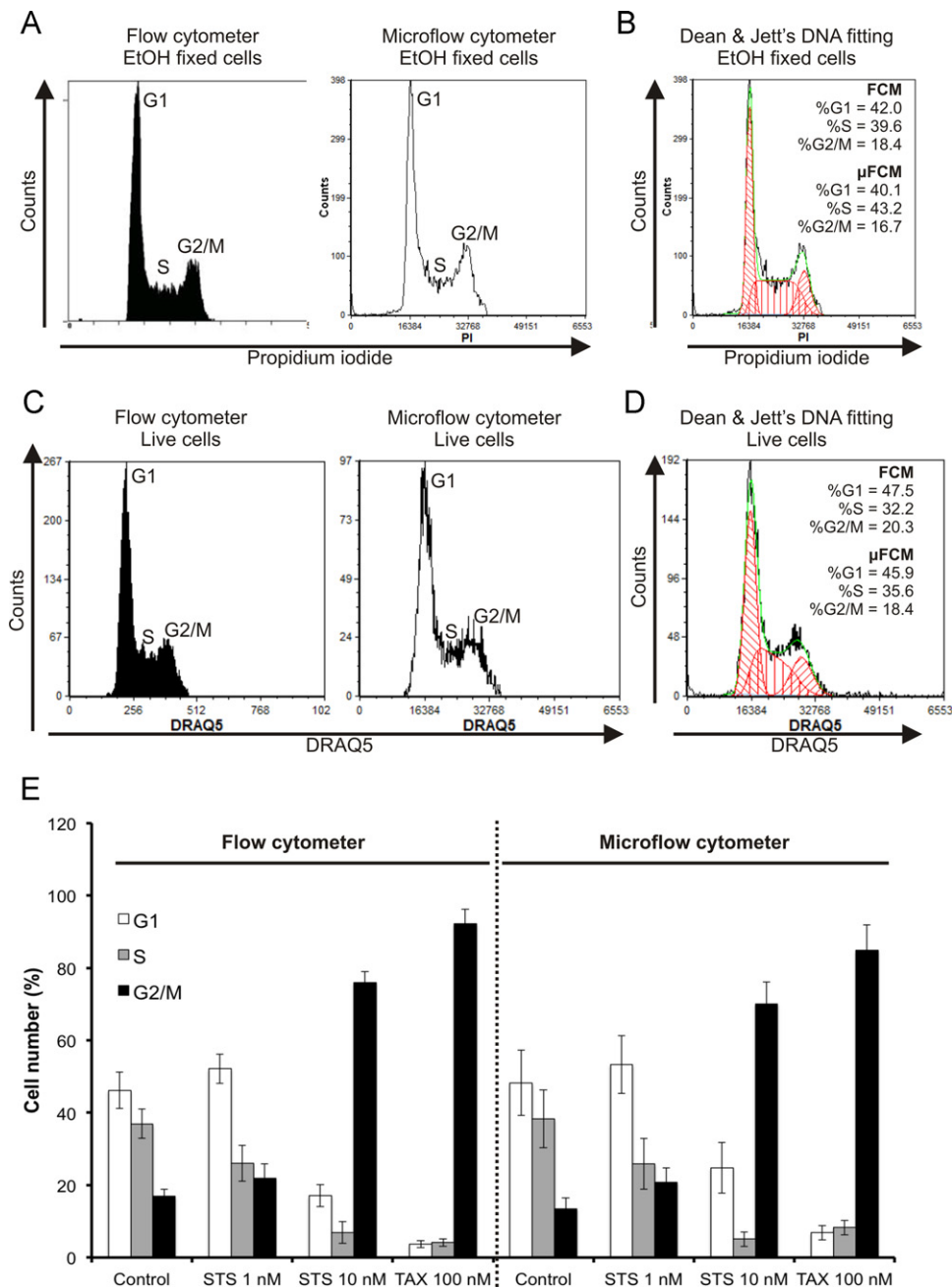


Fig. 2. Lab-on-a-Chip flow cytometry of the cell cycle. (A) Comparison of DNA histogram profiles obtained on fixed and permeabilized THP1 α cells using the conventional and microflow cytometers. Propidium iodide fluorescent signals (Ex/Em 473/575 nm) were amplified using linear mode. (B) Automatic DNA content histograms fitting and cell cycle phase detection obtained in A. (C) Comparison of DNA histogram profiles obtained on living THP1 α cells using the conventional and microflow cytometers. DRAQ5 fluorescent signals (Ex/Em 640/690 nm) were amplified using linear mode. (D) Automatic DNA content histograms fitting and cell cycle phase detection obtained in B. (E) Comparative live cell analysis of the cell cycle perturbations during exposure to a pan-kinase inhibitor Staurosporine (STS; 1 and 10 nM) and microtubule poison Taxol/Paclitaxel (TAX; 100 nM). DNA histograms in living cells were obtained using a live cell permeant probe DRAQ5 as depicted in C and D. A typical run used a sample with 5×10^3 cells.

exponential in vitro culture, the majority (66%) of spontaneously dying human THP1 cells were characterized by concomitant TMRM loss and caspase activation as evidenced by PhiPhiLux labeling in Fig. 3B. Interestingly, spontaneous apoptosis was triggered without any preference to a particular phase of the cell cycle (Fig. 3B). Upon treatment with transcription inhibiting polypeptide antibiotic Actinomycin D (Act D) for 24 h, the tri-color assay revealed that THP1 cells undergo apoptosis mainly from S phase, and to a lesser extent from G1-phase (Fig. 3C). Most of the cells with $\Delta\Psi_m$ loss were characterized by concomitant caspase activation. Interestingly, however, nearly 33% of cells

with the intact mitochondrial inner membrane potential were labeled positive with the caspase activation marker PhiPhiLux. This remarkable data provide evidence that Act D-induced initiation of programmed cell death may proceed independently of mitochondrial disruption as a primary event.

In closing, our data provide collective evidence that a planar microfluidic chip-based cytometer 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. Our work shows that simple

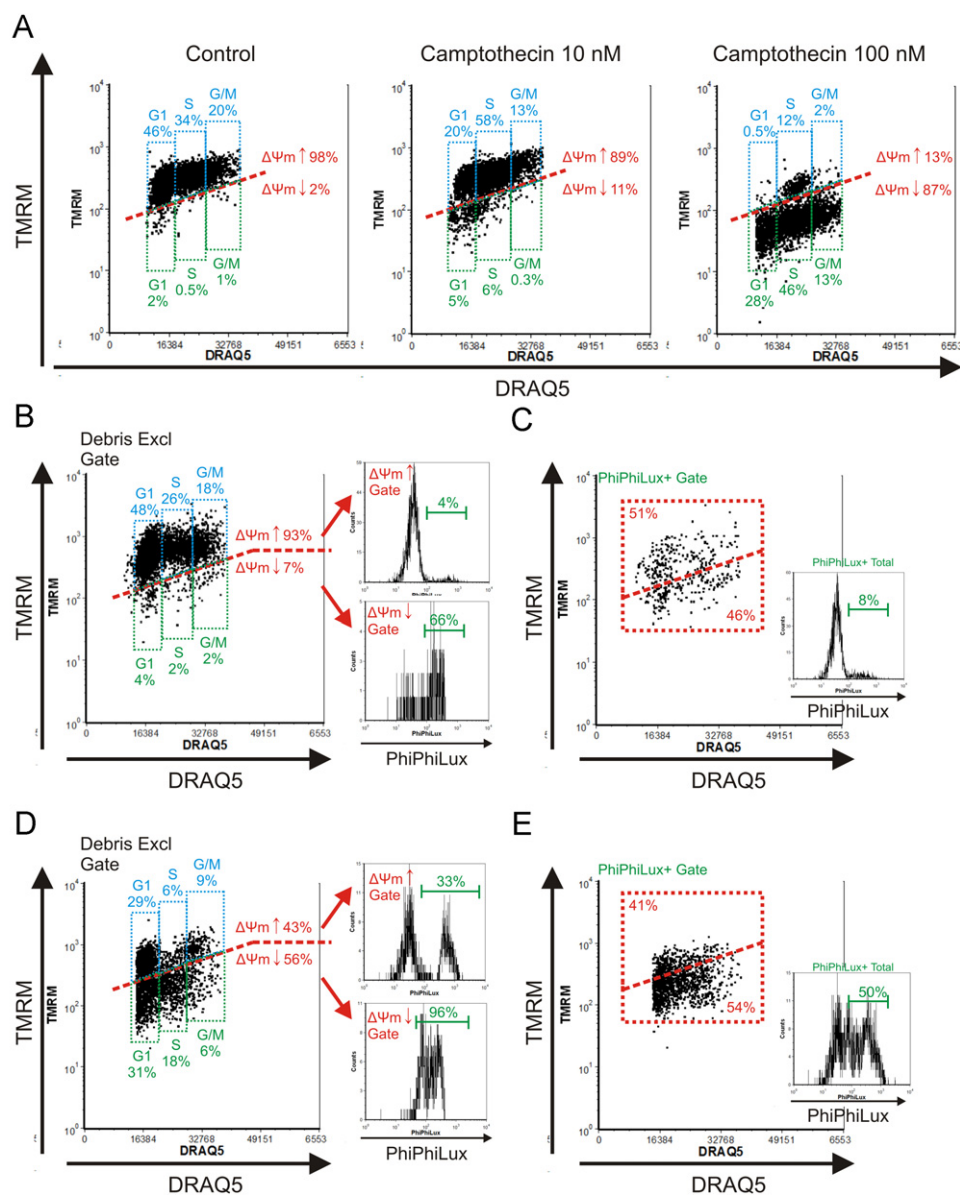


Fig. 3. Multiparameter labeling of apoptotic markers and DNA content using Lab-on-a-Chip flow cytometry. (A) Two-color differential labeling of living leukemic THP1 α cells with a $\Delta\Psi_m$ sensitive probe TMRM and stoichiometric DNA probe DRAQ5. Cells were treated with a topoisomerase I inhibitor camptothecin. (B) Tri-color differential labeling with two independent apoptotic markers such as fluorogenic caspases substrate PhiPhiLux and $\Delta\Psi_m$ sensitive probe TMRM in relation to DNA content (DRAQ5 staining) in exponentially growing living leukemic THP1 α cells. The insets denote the labeling of cells with energized mitochondria ($\Delta\Psi_m\uparrow$ gate, top panel) and de-energized mitochondria ($\Delta\Psi_m\downarrow$ gate, lower panel) with fluorogenic caspases substrate PhiPhiLux. Note that in the unstimulated sample majority of cells with $\Delta\Psi_m\uparrow$ exhibit only residual caspase activation as denoted by the lack of staining with PhiPhiLux probe. (C) The bivariate dot plot representing the staining with $\Delta\Psi_m$ sensitive probe TMRM in relation to DNA content (DRAQ5) as seen in B but gated on PhiPhiLux+ subpopulation (activated caspases) only. Inset denotes the histogram distribution of PhiPhiLux staining in ungated cells. (D) Tri-color differential labeling with PhiPhiLux and TMRM probes in relation to DNA content in living leukemic THP1 α cells upon treatment with transcription inhibiting polypeptide antibiotic Actinomycin D (Act D) for 24 h. The insets denote the labeling of cells with energized mitochondria ($\Delta\Psi_m\uparrow$ gate, top panel) and de-energized mitochondria ($\Delta\Psi_m\downarrow$ gate, lower panel) with fluorogenic caspases substrate PhiPhiLux. Note that in Act D stimulated sample over 1/3 of the cells with $\Delta\Psi_m\uparrow$ exhibit caspase activation as denoted by strong peak in PhiPhiLux staining. Nearly all cells with $\Delta\Psi_m\downarrow$ exhibit caspase activation. (E) The bivariate dot plot representing the staining with $\Delta\Psi_m$ sensitive probe TMRM in relation to DNA content (DRAQ5) as seen in D but gated on PhiPhiLux+ subpopulation (activated caspases) only. Inset denotes the histogram distribution of PhiPhiLux staining in ungated cells. A typical run used a sample with 5×10^3 cells.

microfluidic chip designs are sufficient to acquire high quality biological data when combined with sophisticated electronic interfaces.

4. Conclusions

Our work demonstrates that high quality and multiparameter FCM data can be in fact obtained on very small quantities using very basic planar chip designs. When combined with innovative micro-opto-electronic interfaces such simple chip

designs provide statistical power comparable to that of conventional flow cytometric measurements. We postulate that rather than complicating the complexity of microfluidic chip designs, more emphasis is needed in the field of intelligent and miniaturized opto-electronic off-chip interfaces for the flow cytometry to become as ubiquitous as PCR thermal cyclers. By providing an alternative to expensive flow cytometers that are unaffordable to small research- or clinical-laboratories, these simple yet robust microflow cytometers can be widely available in the near future. This will benefit healthcare especially in underprivileged countries.

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References

- Cheung, K.C., Di Berardino, M., Schade-Kampmann, G., Hebeisen, M., Pierzchalski, A., Bocsi, J., Mittag, A., Tarnok, A., 2010. Microfluidic impedance-based flow cytometry. *Cytometry A* 77 (7), 648–666.
- Chung, T.D., Kim, H.C., 2007. Recent advances in miniaturized microfluidic flow cytometry for clinical use. *Electrophoresis* 28 (24), 4511–4520.
- Gluckstad, J., 2004. Microfluidics: sorting particles with light. *Nature Materials* 3 (1), 9–10.
- Halicka, H.D., Seiter, K., Feldman, E.J., Traganos, F., Mittelman, A., Ahmed, T., Darzynkiewicz, Z., 1997. Cell cycle specificity of apoptosis during treatment of leukaemias. *Apoptosis* 2 (1), 25–39.
- Huang, X., Halicka, H.D., Traganos, F., Tanaka, T., Kurose, A., Darzynkiewicz, Z., 2005. Cytometric assessment of DNA damage in relation to cell cycle phase and apoptosis. *Cell Proliferation* 38 (4), 223–243.
- Huh, D., Gu, W., Kamotani, Y., Grotberg, J.B., Takayama, S., 2005. Microfluidics for flow cytometric analysis of cells and particles. *Physiological Measurement* 26 (3), R73–98.
- Hur, S.C., Tse, H.T., Di Carlo, D., 2010. Sheathless inertial cell ordering for extreme throughput flow cytometry. *Lab on a Chip* 10 (3), 274–280.
- Lee, M.G., Choi, S., Park, J.K., 2009. Three-dimensional hydrodynamic focusing with a single sheath flow in a single-layer microfluidic device. *Lab on a Chip* 9 (21), 3155–3160.
- Moon, S., Keles, H.O., Ozcan, A., Khademhosseini, A., Haeggstrom, E., Kuritzkes, D., Demirci, U., 2009. Integrating microfluidics and lensless imaging for point-of-care testing. *Biosensors and Bioelectronics* 24 (11), 3208–3214.
- Packard, B.Z., Komoriya, A., 2008. Intracellular protease activation in apoptosis and cell-mediated cytotoxicity characterized by cell-permeable fluorogenic protease substrates. *Cell Research* 18 (2), 238–247.
- Scott, R., Sethu, P., Harnett, C.K., 2008. Three-dimensional hydrodynamic focusing in a microfluidic Coulter counter. *Review of Scientific Instruments* 79 (4), 046104.
- Simonnet, C., Groisman, A., 2006. High-throughput and high-resolution flow cytometry in molded microfluidic devices. *Analytical Chemistry* 78 (16), 5653–5663.
- Smith, P.J., Wiltshire, M., Davies, S., Patterson, L.H., Hoy, T., 1999. A novel cell permeant and far red-fluorescing DNA probe, DRAQ5, for blood cell discrimination by flow cytometry. *Journal of Immunological Methods* 229 (1–2), 131–139.
- Sugino, H., Ozaki, K., Shirasaki, Y., Arakawa, T., Shoji, S., Funatsu, T., 2009. On-chip microfluidic sorting with fluorescence spectrum detection and multiway separation. *Lab on a Chip* 9 (9), 1254–1260.
- Telford, W.G., Komoriya, A., Packard, B.Z., 2002. Detection of localized caspase activity in early apoptotic cells by laser scanning cytometry. *Cytometry* 47 (2), 81–88.
- Wlodkowic, D., 2012. The case for multiparameter cytometry in neurobiology. *Cytometry Part A: The Journal of the International Society for Analytical Cytology* 81 (7), 544–545.
- Wlodkowic, D., Cooper, J.M., 2010. Tumors on chips: oncology meets microfluidics. *Current Opinion in Chemical Biology* 14 (5), 556–567.
- Wlodkowic, D., Faley, S., Zagnoni, M., Wikswo, J.P., Cooper, J.M., 2009a. Microfluidic single-cell array cytometry for the analysis of tumor apoptosis. *Analytical Chemistry* 81 (13), 5517–5523.
- Wlodkowic, D., Skommer, J., Darzynkiewicz, Z., 2010. Cytometry in cell necrobiology revisited: recent advances and new vistas. *Cytometry A* 77 (7), 591–606.
- Wlodkowic, D., Skommer, J., Faley, S., Darzynkiewicz, Z., Cooper, J.M., 2009b. Dynamic analysis of apoptosis using cyanine SYTO probes: from classical to microfluidic cytometry. *Experimental Cell Research* 315 (10), 1706–1714.
- Wlodkowic, D., Skommer, J., Hillier, C., Darzynkiewicz, Z., 2008. Multiparameter detection of apoptosis using red-excitable SYTO probes. *Cytometry A* 73 (6), 563–569.
- Wlodkowic, D., Skommer, J., Pelkonen, J., 2006. Multiparametric analysis of HA14-1-induced apoptosis in follicular lymphoma cells. *Leukemia Research* 30 (9), 1187–1192.
- Wlodkowic, D., Skommer, J., Pelkonen, J., 2007. Brefeldin A triggers apoptosis associated with mitochondrial breach and enhances HA14-1- and anti-Fas-mediated cell killing in follicular lymphoma cells. *Leukemia Research* 31 (12), 1687–1700.
- Wlodkowic, D., Telford, W., Skommer, J., Darzynkiewicz, Z., 2011. Apoptosis and beyond: cytometry in studies of programmed cell death. *Methods in Cell Biology* 103, 55–98.
- Wu, W.K., Liang, C.K., Huang, J.Z., 2004. MEMS-based flow cytometry: microfluidics-based cell identification system by fluorescent imaging. *Conference Proceedings of IEEE Engineering in Medicine and Biology Society*, 4, 2579–2581.
- Zhao, H., Traganos, F., Dobrucki, J., Wlodkowic, D., Darzynkiewicz, Z., 2009. Induction of DNA damage response by the supravital probes of nucleic acids. *Cytometry A* 75 (6), 510–519.