Real-time Fluorescent Imaging of Apoptosis Using Chip-Based Technologies

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Introduction

Cell-based assays are becoming an important part of the postgenomic biomedical research. The present study was designed to provide a mechanistic rationale for the kinetic assays and to assess their potential to obtain dynamic and single-cell data on the stochastic process of cell death using Lab-on-a-Chip (LOC) technologies.

We propose an innovative approach to dynamically trace cell death in real-time using fluorochromes such as propidium iodide (PI), SYTOX Green, SYTOX Red, YO-PRO 1, Annexin V and TMRM. We also demonstrate that, when used with innovative bioassays, microfluidic dynamic live-cell analysis is a practical alternative for multiparameter studies on a single-cell level.

The adaptation of non-toxic biomarkers that can continuously circulate inside enclosed microculture system will be beneficial for the advancement of up and coming LOC technologies. Our data supports the hypothesis that real-time bioassays in combination with LOC devices allow for rapid and simple analysis of cell death, particularly useful if the death pattern is a stochastic rather than deterministic process. As a result, they provide sensitivity that often cannot be achieved with conventional end-point analysis.

Experimental Setup

Both U937 and HL60 leukaemia and human osteosarcoma U2OS cells were used in the study. For dynamic, real-time quantification of cytotoxicity cells were treated with selected anti-cancer drugs and continuously cultured in the presence of fluorescent probes: SYTOX Green, SYTOX Red, YO- PRO 1, PO-PRO 1, propidium iodide (PI), Annexin V-APC (Molecular Probes, Eugene, OR, USA) and 7-aminoactinomycin D (7-AAD) for up to 5 days (**Fig 1**). Flow cytometry was performed using a BD FACSCalibur (BD Biosciences) analyser. Microfluidic devices were made by casting a PDMS pre-polymer against a negative relief pattern developed in SU-8. A mixture of elastomer base and curing agent (10:1 ratio w/w) was poured and PDMS was thermally cured at 70°C for 1 hour. Microfluidic chips were then sealed to quartz cover slips and interfaced with computer-controlled syringe pumps. Two chip designs were used as outlined in **Fig 2.** Fluorescence images were acquired using a motorized Zeiss Axiovert 200M epifluorescence microscope.

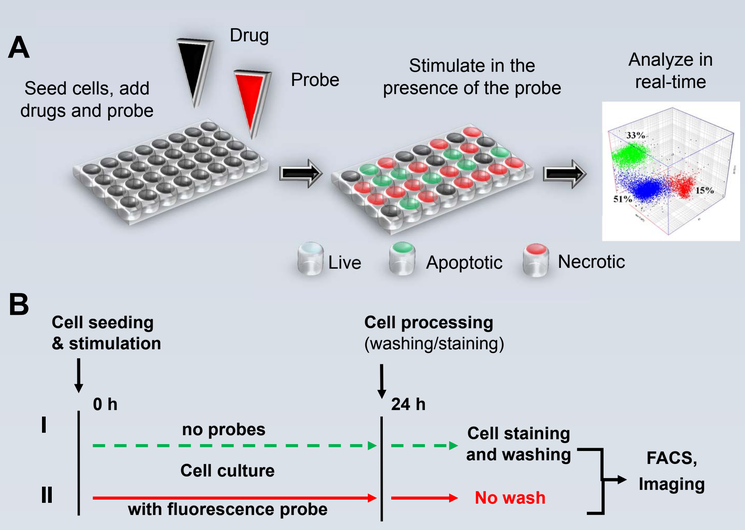

Figure 1. *Dynamic tracking of cytotoxicity using simplified no-wash protocols **A)** Rationale for real-time fluorescence cytotoxicity assays **B)** Workflow of modified no-wash protocol. Note that viability marker is continuously present in the culture medium (lower panel; II) as opposed to standard, end-point staining procedures (upper panel; I).*

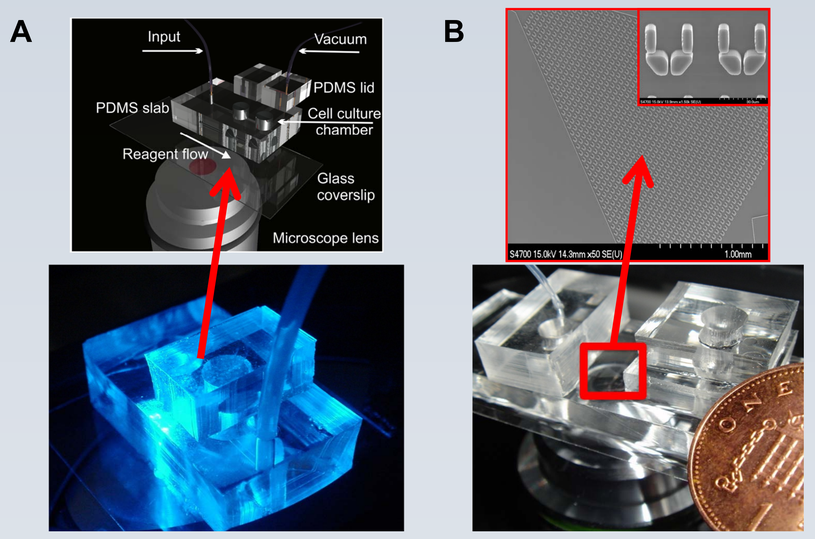

Figure 2. A) *Microfluidic flow through chip-based cell culture system The device comprised two microculture chambers (3 mm in diameter) connected by a 250 µm wide perfusion channel. **B)** The microfluidic array cytometer consisted of a microfluidic chip containing 440 mechanical traps. The dimensions of each cell trap were 18 µm (w), 20 µm (l) and 10 µm (d).*

Results

It is generally assumed that commonly used fluorescent markers, such as SYTOX Green, SYTOX Red, TO-PRO , YO-PRO and PI are toxic to cells. As a consequence, these dyes are predominantly used in end-point assays to discriminate between the cells with preserved plasma membrane integrity (i.e. live cells) and those with impaired membranes (i.e. dead cells). The present study was designed to investigate the cytotoxic profiles of cell impermeant fluorescent probes and subsequently to assess their potential to obtain dynamic and single-cell data on the stochastic process of cell death **(Fig 3 and 4)**.

We provide evidence that even when fluorescent probes are applied at concentrations higher than these used in cytotoxicity assays, for up to 72 hrs, there is no evidence of adverse effects on proliferating cells. Plasma membrane integrity remains preserved and no significant perturbation of the cell cycle progression is apparent **(Fig 3 and 4).**

The low-dosage, continuous labelling procedure (**Protocol II**) not only provided similar results to the standard end-point staining protocol (Protocol I), but also provided a proof of principle that these assays could be adapted for a high-throughput dynamic screening protocol (HTS) **(Figure 5)**. For example, cells incubated in the presence of SYTOX Green were viable even after 72hrs providing the potential for a non-invasive and long term-tracking of cell viability, either in a flow cytometry or a microfluidic format **(Figure 5)**. Dynamic analysis provided a stratified cell death/survival curves. Importantly the stochastic nature of cell death and dissimilar kinetic profiles of STS and BFA action were easily visualized **(Figure 5)**. Finally, real-time assays were exploited to unravel stochastic patterns of caspase-dependent apoptosis at a single-cell level using innovative microfluidic array cytometer **(Figure 6).**

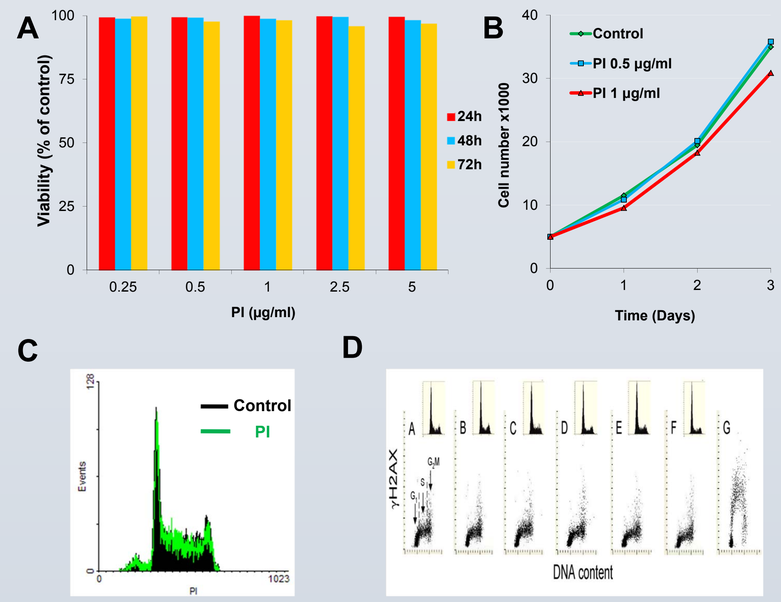

Figure 3*. Continuous exposure to PI has no effect on physiology of human HL60 and A549 cells. **A)** PI presence does not imply loss of HL60 cell viability when cultured for up to 24 hours, **B)** PI does not disturb HL60 cell proliferation as assessed using standard Trypan Blue assay during the 3-day study, **C)** Exposure to PI does not disturb cell proliferation. HL60 cells were continuously challenged with 0.5 µg/ml of PI for up to 48 hours. Cells were then fixed and their DNA content profile analysed by flow cytometry, **D)** DNA damage (phosphorylation of histone H2AX) in A549 cells growing in the presence of PI. The bivariate distributions (DNA content vs expression of γH2AX) of cells growing in the absence of PI (A, 24 h; B, 48 h) or presence of 1.5 µM PI for 24 h (C) and 48 h (E) or 7,5 µM PI for 24 (D) and 48 h (F). DNA content histograms are shown in the insets. Positive control - 50 J/m² of UV B light exposure (F).*

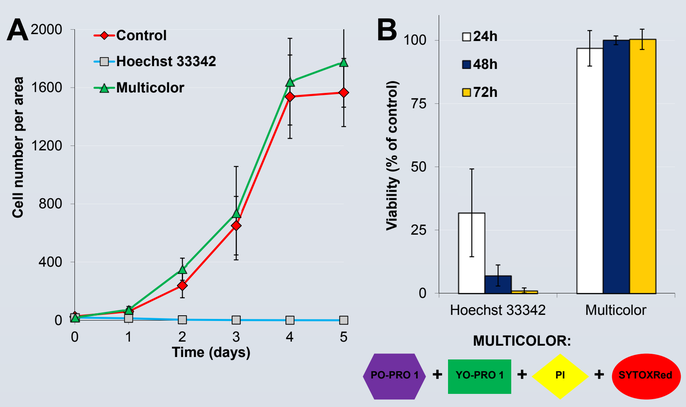

Figure 4*. LIVE/DEAD probes are non-toxic even in multiplexed applications. Neither U937 cell proliferation **A)** nor cell viability **B)** is affected during the continuous culture in the presence of combination of four cyanine fluorescent probes for up to 4 days. Hoechst 33342 – positive control (DNA damaging probe)*

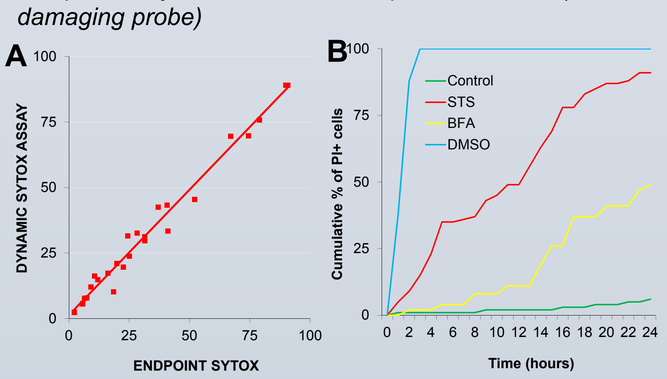

Figure 5*. Dynamic analysis of drug induced cytotoxicity using microculture chip-based system. **A)** Excellent agreement of data obtained using dynamic vs. end-point SYTOX assays. **B)** Different kinetic profiles of cell death induced by investigational anti-cancer drugs Staurosporine (STS) and Brefeldin A (BFA) revealed using dynamic labelling with PI. DMSO – positive control.*

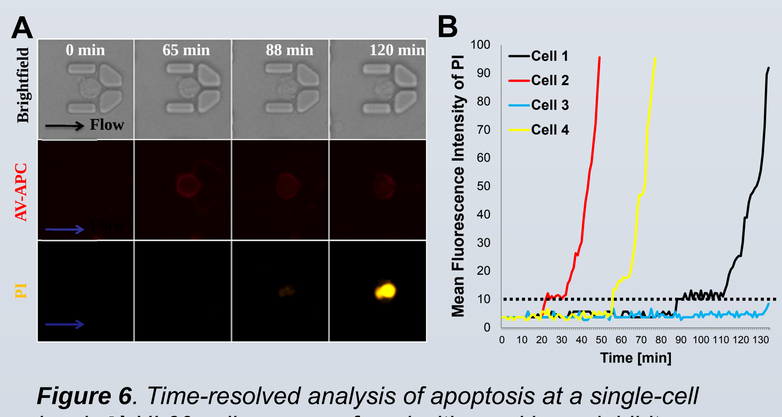

Figure 6*. Time-resolved analysis of apoptosis at a single-cell level. **A)** HL60 cells were perfused with pan-kinase inhibitor staurosporine (STS; 2 µM) for up to 2 hours in the presence of fluorescence probes Annexin V (APC conjugate; red) and propidium iodide (PI; yellow). Note the stochastic nature of anti-cancer drug action. Gradual increase in staining with Annexin V marks the externalization of phosphatidylserine (PS) residues characteristic of early apoptotic stages (red) whereas gradual plasma membrane permeability to PI represents progressive destabilization of plasma membrane structure (yellow). **B)** Four representative cells were selected and their fluorescence following incorporation of PI assessed as a Mean Fluorescent Intensity (MFI). Note the stochastic response to a pan-kinase inhibitor Staurosporine with a profound variability between cells in population.*

Conclusions

We show that fluorescent viability probes do not adversely affect integrity of plasma membrane, cell cycle progression, cell proliferation nor induce DNA-damage when used in continuous perfusion experiments. As a result, instead of being used solely in end-point assays they can be conveniently applied to multiplexed assays for dynamic real-time analysis of drug induced cytotoxicity. Vast reduction of sample consumption achieved with these protocols is an important consideration for high-throughput screening routines.

References

- Wlodkowic, D.; Faley, S.; Zagnoni, M.; Wikswo, J. P.; Cooper, J. M. *Anal Chem* **2009**, *81*, 5517.
- Wlodkowic, D.; Skommer, J.; McGuinness, D.; Faley, S.; Kolch, W.; Darzynkiewicz, Z.; Cooper, J. M. *Anal Chem* **2009**, *81*, 6952
- Wlodkowic, D.; Faley, S.; Skommer, J.; McGuinness, D.; Cooper, J. M. *Anal Chem* **2009**, *81*, 9828
- Wlodkowic, D.; Skommer, J.; Faley, S.; Darzynkiewicz, Z.; Cooper, J. M. *Exp Cell Res* **2009**, *315*, 1706.
- Zhao, H.; Oczos, J.; Janowski, P.; Trembecka, D.; Dobrucki, J.; Darzynkiewicz, Z.; Wlodkowic, D. *Cytometry A* **2010**, *77*, 399.

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