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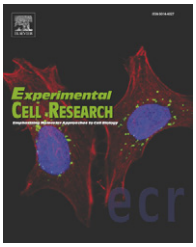

Research Article

Dynamic analysis of apoptosis using cyanine SYTO probes: From classical to microfluidic cytometry

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ABSTRACT

Cell death is a stochastic process, often initiated and/or executed in a multi-pathway/multi-organelle fashion. Therefore, high-throughput single-cell analysis platforms are required to provide detailed characterization of kinetics and mechanisms of cell death in heterogeneous cell populations. However, there is still a largely unmet need for inert fluorescent probes, suitable for prolonged kinetic studies. Here, we compare the use of innovative adaptation of unsymmetrical SYTO dyes for dynamic real-time analysis of apoptosis in conventional as well as microfluidic chip-based systems. We show that cyanine SYTO probes allow non-invasive tracking of intracellular events over extended time. Easy handling and "stain–no wash" protocols open up new opportunities for high-throughput analysis and live-cell sorting. Furthermore, SYTO probes are easily adaptable for detection of cell death using automated microfluidic chip-based cytometry.

Overall, the combined use of SYTO probes and state-of-the-art Lab-on-a-Chip platform emerges as a cost effective solution for automated drug screening compared to conventional Annexin V or TUNEL assays. In particular, it should allow for dynamic analysis of samples where low cell number has so far been an obstacle, e.g. primary cancer stems cells or circulating minimal residual tumors.

Introduction

We are now facing the era of large compound libraries and novel screening platforms, using microfluidic microchip and Lab-on-a-Chip devices. High-content analysis (HCA) is being recognized as a key component in the anti-cancer drug discovery pipelines, and is most commonly used for estimation of drug cytotoxicity [1]. However, dynamic high-throughput analysis of cell death is often limited by excessive probe cytotoxicity [2]. Indeed, supravital analysis of intracellular processes, especially in long-term, requires biomarkers that do not interfere with structure or function of the cell. Indeed, most cell permeant fluorescent probes developed to date, including Hoechst 33342, DRAQ5 or Vybrant DyeCycle Orange, lack this feature [2,3]. We have recently reported that cyanine SYTO probes rapidly diffuse through eukaryotic membranes [1,4], and are applicable for many polychromatic assays in studies of caspase-dependent cell death [4,5]. Here, we introduce innovative SYTO-based assays for kinetic tracking of apoptosis, that meet the following criteria of dynamic and high-throughput analysis, namely: (i) the

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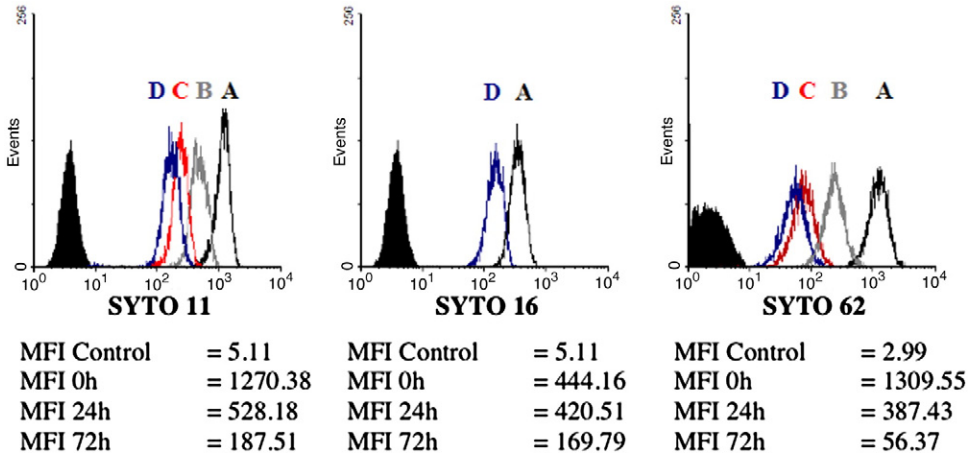

Fig. 1 – Intracellular retention of green and red fluorescent SYTO probes: Human B-cell lymphoma cells were pre-stained with 100 nM of SYTO 11/16/62 for 20 min at RT. Following incubation, cells were washed and cultured in dye-free medium for the time indicated. Filled histograms denote unstained control cells. A, B, C, and D denote: 0, 24, 48, 72 h of post-staining culture, respectively. Note that apart from retention heterogeneity all probes were sufficiently retained in live cells after 24 h to allow gating of tagged subpopulation. Results are representatives of four independent experiments. Similar data were obtained on U937 and HL60 cells.

straightforward staining and adaptability for automated dispensing; (ii) the prolonged intracellular retention, (iii) the lack of side-effects on cellular viability, proliferation or cell migration; and (iv) the lack of interference with the assay readout.

Materials and methods

Cell culture and reagents

The culture of human B-cell lymphoma and leukemic (HL60, U937) cell lines were as previously described [4,5]. Human osteosarcoma U2OS cells were from ATCC (Manassas, VA, USA). The following inducers of apoptosis were used: dexamethasone (Dex; 1–1000 nM); cycloheximide (CHX; 0–10 μg/ml); staurosporine (STS; 0.1–1 μM); and camptothecin (CAM; 1–10 μM), all from Sigma Chemical Co., St Louis MO, USA).

Staining with SYTO dyes

Labeling with SYTO green (SYTO 11–16) and SYTO red (SYTO 17, 59–64) probes (Molecular Probes, Eugene, OR, USA) was performed as described earlier [1,4,5]. Staining with SYTOX green (30 nM), SYTOX red (10 nM) or YO-PRO 1 (250 nM) was performed as previously described [6,7].

Flow cytometry and cell sorting

Flow cytometry was performed using BD FACSCalibur (BD Biosciences), equipped with a 15 mW Argon-ion and 20 mW red diode lasers served as a reference instrument. Logarithmic amplification scale using following configuration of band-pass (BP) filters was applied: (i) *488 nm excitation line*: FL1 (525 BP for collection of: SYTO green, SYTOX green, YO-PRO 1 fluorescence signals) FL 2 (575 BP for collection of: PI fluorescence signal); and

Fig. 2 – Cytotoxicity of substituted cyanine SYTO probes: (A) Influence of SYTO probes on cell viability. Human B-cell lymphoma cells were pre-loaded with 250 nM of selected dyes as described before. Following incubation, cells were washed and cultured in dye-free medium for 48 h. Cell viability was estimated by staining with SYTOX Green (for SYTO 17–62) or SYTOX Red (for SYTO 11–16) stains. Note that all but SYTO 15 probes do not affect cell viability. Similar results were achieved when cells were continuously challenged with 250 nM of SYTO probes (not shown). Results are representatives of four independent experiments; (B) Influence of SYTO probes on cell cycle progression. Human B-cell lymphoma cells were pre-loaded with selected dyes as described in (A). Cells were then washed and cultured in dye-free medium for 48 h. Cells were subsequently collected and fixed in 70% EtOH for 2 h. Cell cycle analysis was performed using standard PI staining protocol. Note lack of cell cycle disturbances and sub-G1 peak following SYTO pre-loading. Similar results were obtained when cells were continuously cultured in the presence of SYTO dyes (not shown). Results are representatives of four independent experiments; SD values were lower than ±6 for each phase of the cell cycle; (C) Influence of SYTO probes on DNA replication. DNA synthesis (deemed as cell proliferation) was assessed using [methyl-^{3}H]-thymidine incorporation assay 24 h after SYTO staining. Results represent mean (±SD, $n=3$) of [methyl-^{3}H]-thymidine incorporation relative to untreated controls. Note disturbance of DNA replication for only SYTO 15 stain ($p<0.05$). Similar results were achieved when U937 and HL60 cells were continuously cultured in the presence of up to 1 μM of selected SYTO probes (not shown); and (D) The influence of SYTO probes on cell growth. Lymphoma cell lines were treated with indicated SYTO dyes at 250 nM, and the number of viable cells was assessed using standard Trypan Blue assay during the 3-day study. Note cell growth disturbance for SYTO 15 ($p<0.05$). The results represent mean of at least three independent experiments; normalized SD values were lower than ±3.