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Short communication

Application of NucleoCounter for the comprehensive assessment of murine cultured neurons during infection with Equine Herpesvirus type 1 (EHV-1)

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Abstract

The NucleoCounter NC-3000, a portable high-speed cell counting device based on the principle of fluorescence microscopy, provides the alternative method for standard flow cytometry. The main objective of the study was to apply an efficient technique for the assessment of the primary murine neurons culture infected with either neuropathogenic or non-neuropathogenic strains of Equine Herpesvirus type 1 (EHV-1). Using the NucleoCounter NC-3000 we have observed a decrease in mitochondrial potential and reduction in cells viability but we have not observed changes in the cell cycle of cultured neurons infected with all EHV-1 strains.

Key words: NucleoCounter NC-3000, viability, cell cycle, apoptosis, neurons, EHV-1

Introduction

The NucleoCounter NC-3000 system enables to perform automated cell counting and high precision analyses of eukaryotic cells during virus infections. In our study we have chosena few mechanisms to investigate the specific equine herpesvirus – murine neuronal cell relationships. The analysis of apoptosis was chosen, since we have already found that EHV-1 could influence this process (Cymerys et al. 2012). Virus replication and transmission depends upon the existing molecular machinery of an infected cell. Moreover, the latent herpesvirus infections lead to the cell death prevention. Mitochondria play a special role in the apoptosis, so they are the major target for the viral related cell death control. The increased permeabilization of mitochondrial membrane and disturbances in the ions concentrations are the early indicators of apoptosis. The analysis of cell cycle may verify the results obtained from the apoptosis-specific tests during EHV-1 infection of neuronal cell infection.

Materials and Methods

Cell culture and viruses

Primary culture of murine neurons was established as described before (Cymerys et al. 2010). Two neurotropic, non-neuropathogenic strains of EHV-1

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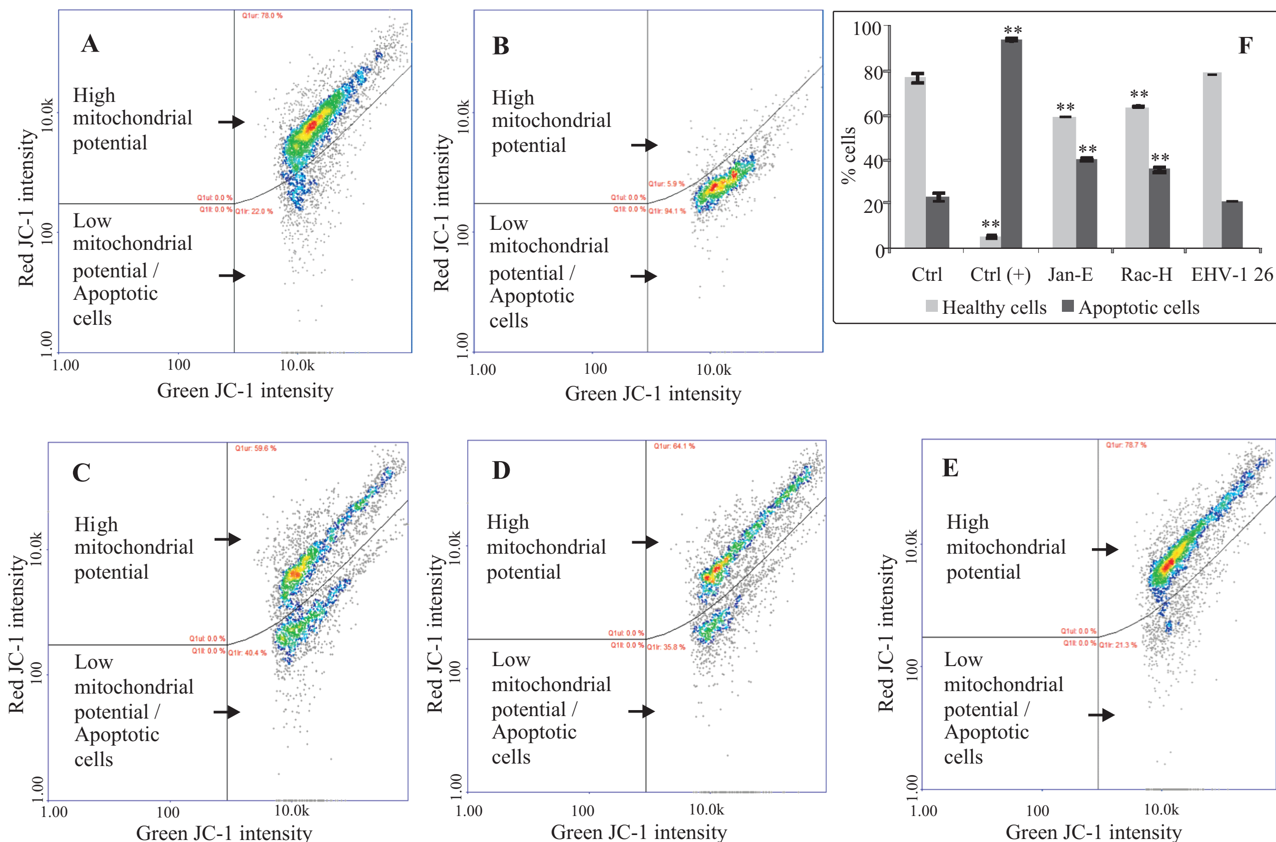

Fig. 1. Mitochondrial membrane potential of cultured neurons. Imaging cytometry was performed using JC-1 to determine the mitochondrial potential and apoptotic cell death rate in primary murine neurons. Cells were stained for 10 minutes at 37°C, washed with PBS and analysed on the NucleoCounter NC-3000 using Mitochondrial Potential Assay. (A) Control cells (non-infected); (B) Positive control (neurons CCCP-treated), (C) Neurons infected with Jan-E strain of EHV-1; (D) Neurons infected with Rac-H strain of EHV-1; (E) Neurons infected with EHV-1 26 strain; (F) Level of apoptotic and normal cells (%), (** $p<0.01$; * $p<0.05$).

were used: Jan-E and Rac-H (collection of the Virology Laboratory, Warsaw University of Life Sciences--SGGW) and one neurotropic, neuropathogenic strain of EHV-1 (EHV-1 26), isolated in Hungary (Malik et al., 2010). Primary murine neurons were infected with either Jan-E, Rac-H or EHV-1 26 strains of the virus (MOI 1.0), and incubated for 24 hours at 37°C with 5% CO_2. A positive control for mitochondrial potential analysis was prepared by adding CCCP (Carbonyl cyanide *m*-chlorophenyl hydrazone; 5 µl/ml cell culture medium). Non-infected neurons served as negative control.

NucleoCounter NC-3000

The NucleoCounter NC-3000, was used for evaluation of apoptosis, viability and cell cycle throughout the study. Cells were stained with JC-1 (for mitochondrial transmembrane potential), with VB-48, AO, PI (for changes in the intracellular level of free thiols, accompanying apoptosis or cell damage), and with DAPI (for DNA content quantification, to determine G1/G0, subG1, S and G2/M cell cycle phases), according to manufacturer's instructions. The results were analyzed using the NucleoView NC-3000 software (details of the NucleoCounter NC-3000 design and capabilities are available at www.chemometec.com).

Results and Discussion

Viability changes in the cultured murine neuronal cells and in their mitochondrial potential during EHV-1 strainds infection were observed. In control, non-infected neurons, we have observed high mitochondrial potential. The negative charge established by the intact membrane potential facilitates the accumulation of JC-1 in the mitochondrial matrix and at high concentrations JC-1 forms red, fluorescent aggregates (Fig. 1A). In positive controls, neurons treated with CCCP, we have observed a low mitochondrial potential and the characteristics of dead cells with dysfunctional mitochondria and JC-1

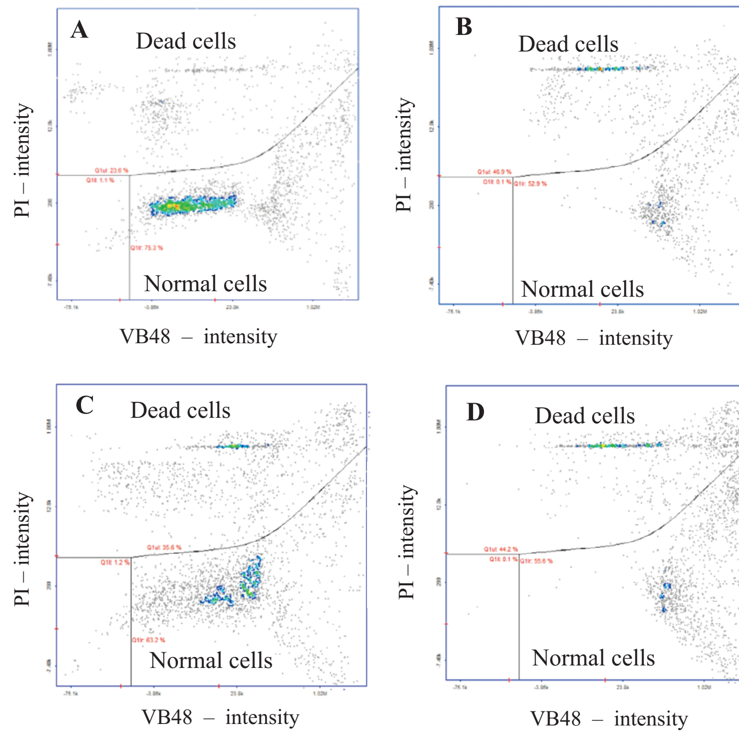

Fig. 2. Neurons stained with VitaBright-48™ (VB-48™), acridine orange (AO) and propidium iodide. (A) The control cells (non-infected); (B) Neurons infected with Jan-E strain; (C) Rac-H strain; (D) EHV-1 26 strain of EHV-1. The cells were stained according to the protocol and analysed on the NucleoCounter NC-3000 using Vitality Assay.

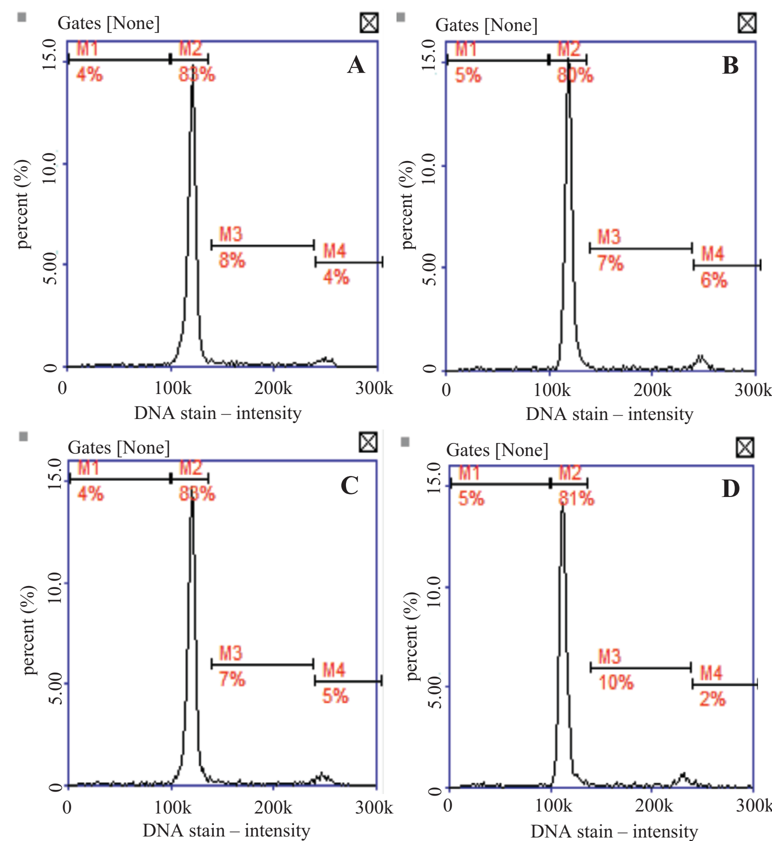

Fig. 3. Comparison of cell cycle phase (G1/G0, S, G2M) and spontaneous apoptosis (SubG1) in murine neurons non-infected (A) and infected by Jan-E strain (B), Rac-H strain (C) and EHV-1 26 strain (D) of EHV-1. Neurons stained with DAPI. M1- subG1 phase, M2- G1/G0 phase, M3- S phase, M4- G2/M phase. The cells were analysed on the NC-3000 system using the 2-step Cell Cycle Assay.

in its monomeric, green fluorescent form in cytosolic localization (Fig. 1B). During infection with all EHV-1 strains we observed a decrease in mitochondrial potential. It was the most significant it was in neurons infected with Jan-E strain of EHV-1 (Fig. 1C). After 24 hours, cells with functionally impaired mitochondria were seen, but there was also increased number of mitochondria with normal potential. We may assume, that mitochondrial potential has stabilized in the late period of infection (Fig. 1 C-E). It should be noted that the red fluorescence was also reduced when cells were submitted to apoptotic cell death (Fig. 1F). It has been found that all used EHV-1 strains reduced viability of cultured murine neurons (Fig. 2A-D). Based on the indicated results, we may conclude that measurement of free thiols levels provides a reliable and fast way to evaluate cells viability, that could be added to the list of cellular phenotypes investigated routinely. We have not observed changes in the cell cycle during EHV-1 neuronal cells infection (Fig. 3A-D). The evaluation of the cell cycle has shown that the highest percentage of infected neurons remained in the G1/G0 phase as in control ones. In conclusion, our results have demonstrated that the NucleoCounter NC-3000 can be used in virological studies and the main advantage was its ability to handle a large number of samples with a high degree of precision in viable cells quantitation. Moreover, this device provides precise automated detection, quantification and analysis of fluorescence at the single cell level and for that reason it could be used in advanced cell analyses of a broad range of mammalian cells.

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