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Short communication

A flow-FISH assay for the quantitative analysis of parvovirus B19 infected cells

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A B S T R A C T

Human parvovirus B19 (B19V) replication is a process highly dependent on the cellular environment, therefore methodologies allowing for analysis at single cell level could represent effective tools to understand cell-to cell differences in the replication process and to investigate cell–virus interactions. Fluorescence in situ hybridization (FISH) can be combined with flow cytometry (flow-FISH) to enable the detection of target nucleic acid sequences in thousands of individual cells in a short amount of time. In the present study, a flow-FISH assay based on the use of a digoxigenin-labeled genomic probe has been developed to discriminate B19V infected cells following in vitro infection of UT7/EpoS1 cell line and EPCs (erythroid progenitor cells) generated from peripheral blood mononuclear cells. In B19V infected UT7/EpoS1 and EPCs, viral nucleic acids were detected by the flow-FISH assay starting from 24 hpi up to 48 hpi. The method, used together with quantitative PCR techniques, can be very useful to describe the kinetics of B19V infection within a heterogeneous cell population.

Human parvovirus B19 (B19V) is a widespread virus responsible for an ample range of clinical manifestations (Gallinella, 2013). B19V infection is typically associated with fifth disease in children, arthropathies in adults, transient aplastic crisis in individuals with an underlying hemolytic disorder, pure red cell aplasia, chronic anemia in immunocompromised patients, and hydrops fetalis or intrauterine fetal death during pregnancy. The virus has also been implicated in a growing spectrum of other different pathologies, including myocarditis and rheumatic diseases.

The genome of B19V is a linear 5.6-kb single-stranded DNA, packaged into a 25 nm non-enveloped icosahedral capsid. Replication occurs in the nucleus of infected cells, via a double-stranded replicative intermediate and a rolling hairpin mechanism. The virus shows a marked tropism for erythroid progenitor cells in the bone marrow and their destruction accounts for many of the clinical manifestations associated with B19V infection. In vitro, a well-established cell line system for B19V infection is lacking. UT7/EpoS1 cells are the most commonly used, but the infection is limited to a small number of cells (Bonvicini et al., 2013). An enhanced B19V infection has been obtained in primary human erythroid progenitor cells (EPCs) generated from bone marrow (Wong et al., 2008) or from PBMCs (peripheral blood mononuclear cells) (Filippone et al., 2010), where B19V infects a larger number of cells. However, despite a considerable improvement, even with this cell culture system large amounts of infectious virus were not produced (Wolfisberg et al., 2013).

To analyze the dynamics of viral replicative cycle, qPCR is a very sensitive methodology that can yield information on average results across all the cells in the sample. Considering the variability of cellular environments and the consequent different capacity to support B19V replication process, methods allowing analysis at single cell level could represent additional effective tools to investigate viral replication and cell–virus interactions. Fluorescence in situ hybridization (FISH) is a powerful technique that is used to detect and localize specific nucleic acid sequences within the cells; in order to increase throughput, FISH can be combined with flow cytometry (flow-FISH) to enable the detection of target nucleic acid sequences in thousands of individual cells in a short amount of time. In addition, flow cytometry offers the ability to perform multiparametric analysis allowing for simultaneous measurements of cellular and viral biomarkers.

Flow-FISH assay has been previously used in a number of different applications: telomere length measurement (Baerlocher et al., 2002), analysis of microbial community (Nettmann et al., 2013), identification and enumeration of microorganisms (Hartmann et al., 2005; Friedrich and Lenke, 2006; Liu et al., 2011), detection of cellular RNA (Robertson and Thach, 2009; Bushkin et al., 2015; Wu et al., 2014), and virus-specific nucleic acids in infected cells

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(Hanley et al., 2013; Robertson et al., 2010; Kimura et al., 2009; Narimatsu and Patterson, 2005).

In the present study a flow-FISH assay has been developed to identify and quantify B19V infected cells following in vitro infection of UT7/EpoS1 cells and EPCs. UT7/EpoS1 cells were cultured in IMDM (Cambrex), supplemented with 10% FCS (Cambrex) and 2 U/ml rhu erythropoietin (NeoRecormon, Roche), at 37 °C and 5% CO_2. Cells were kept in culture at densities between 2×10^5 cells/ml and 1×10^6 cells/ml, with medium exchange every three days, and used for infection experiments when at a density of 3×10^5/ml. Erythroid progenitor cells (EPCs) were generated in vitro from peripheral blood mononuclear cells (PBMC) obtained from volunteer staff members. PBMC were isolated by Ficoll-Paque centrifugation then cultured in a medium containing erythropoietic growth factors (Filippone et al., 2010). The cells were maintained at 37 °C in 5% CO_2, after 4 ± 1 days of culture were split and then maintained in fresh medium at a density of $0.5–1 \times 10^6$ cells/ml until day 9 ± 1, when used for infection experiments. EPCs were characterized using flow cytometry (FACSCalibur, Becton Dickinson) with antibodies specific for erythroid differentiation markers (CD36, CD71) and known B19V receptors (globoside, α5β1 integrin), as described (Bonvicini et al., 2015). Anti-isotype antibodies (BD Biosciences) were used in parallel, for specificity control. Data were analyzed using the Cell Quest Pro Software (Becton Dickinson). At 9 ± 1 days of culture, EPCs showed a wide distribution of erythroid differentiation markers (60–70% CD36 and 70–80% CD71) and B19V receptor (50–70% globoside, 55–65% α5β1 integrin).

UT7/EpoS1 and EPCs were infected with B19V at a multiplicity of infection of 10^3 viral genomes/cell. The source of virus was represented by a high-titre viremic serum, not containing anti-B19 specific IgM or IgG antibodies detectable by a commercial CLIA test (Diasorin), identified in our laboratory in the course of routine diagnostic analysis and available for research purposes according to Italian privacy law. Cells were incubated in the presence of the viremic serum at a density of 10^7/ml; following adsorption for 2 h at 37 °C, the inoculum virus was removed by washing and the cells were incubated at 37 °C in the respective complete medium at an initial density of 10^6/ml then harvested at 48 h post-infection. The same protocol was used to process mock-infected cells without addition of virus.

A fluorescence in situ hybridization (FISH) assay involves several steps including cell fixation and permeabilisation, nucleic acid target and probe denaturation, hybridization, stringency washes, and hybrid detection. To combine FISH reaction with flow cytometry, all of these steps had to be carried out in suspension without cell loss and preserving the morphology and the light scattering characteristics of the cells. In particular, in the optimization of the flow-FISH assay, a previously established in situ hybridization protocol (Bonvicini et al., 2006a, 2013) was modified with respect to fixation/permeabilisation, target denaturation, and hybridization conditions in order to maintain the cell morphology, suitable for analysis by FACS, the accessibility of probe to the nucleic acid targets and an efficient hybridization.

The optimized flow-FISH assay was performed as follows: 1×10^6 B19V infected and mock-infected cells were fixed in 300 μl

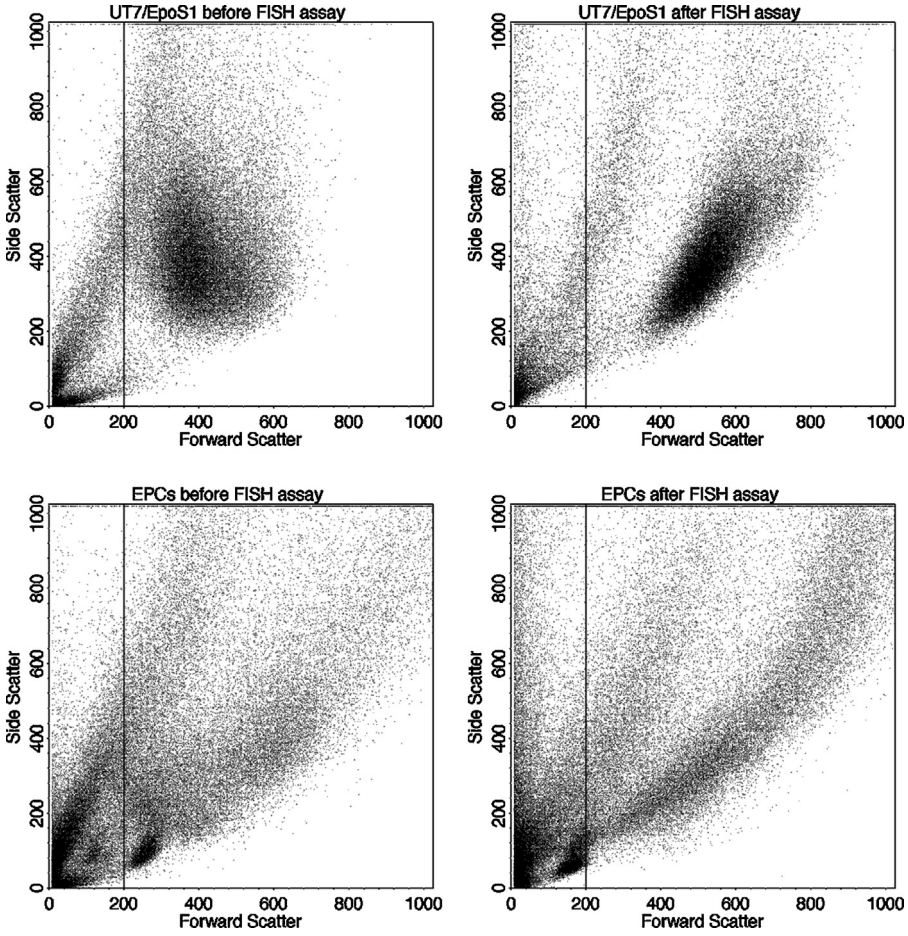

Fig. 1. Flow cytometry dot plots showing the distribution of cell populations with respect to forward and side scatter results, for UT7/EpoS1 and EPCs, acquired before and after flow-FISH assay.