II

Viruses

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Detection of Infectious Rotaviruses by Flow Cytometry

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1. Introduction

Human rotaviruses are considered the main cause of viral gastroenteritis in infants and young children throughout the world ***(1)***. Their transmission is through the fecal-oral route, mostly after ingestion of contaminated water and food ***(2)***. Since an extremely high number of virus particles are present in the feces during the acute gastroenteritis, methods based on electron microscopy, passive particle agglutination tests, or enzyme-linked immunosorbent assays are readily employed for clinical diagnosis. However, the sensitivity of these procedures is not high enough to detect the low number of viral particles sometimes present in the environment ***(3)***. In the case of environmental samples, amplification of viral nucleic acids by polymerase chain reaction assays coupled to reverse transcription (RT-PCR) has been increasingly applied to detect rotaviruses in water ***(4)*** and shellfish samples ***(5)***. However, procedures based on molecular approaches have to face the drawback that they do not differentiate between infectious and noninfectious particles, which is of major relevance from the public health point of view.

Virus propagation in cell culture prior to detection by immunological or molecular procedures accomplishes the dual purpose of increasing the amount of target material and incorporating an infectivity assay as well.

Wild-type rotaviruses present difficulties in their in vitro replication, although some of them may be adapted to grow in several cell lines such as the monkey kidney cell line MA104 or the human intestinal cell line CaCo-2 ***(6,7)***. More than a decade ago, an assay for the specific detection of infectious rotaviruses in environmental samples, involving an indirect immunofluorescence test (IIF) and optical microscopy (OM) counting of infected foci in infected MA-104 cell monolayers, was described ***(4)***. On the other hand, CaCo-2 cells have been successfully employed in our laboratory for infectivity assays of several fastidious enteric virus strains present in water samples ***(8)***.

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Flow cytometry (FCM) is a method to quantify components or study the structural characteristics of cells, mainly by optical means. The term *flow cytometry* derives from the measurement of single cells as they flow in a fluid stream through a measuring point surrounded by several detectors. FCM involves the use of a beam of light projected through a liquid stream that contains cells. As the cells cross the focused light, they emit signals that are captured by detectors. These signals are then converted for computer storage and data analysis and can provide information about cellular properties. These biophysical properties are then correlated with biological and biochemical properties of interest. To make the measurement of biological properties of concern, the cells are usually stained with fluorescent dyes that specifically bind to cellular constituents. Sorting instruments may also isolate specific cells according to their cytometric profile. FCM can be thus employed for counting and specific cell sorting with several cell types.

The use of FCM for the detection of virally infected cells presents several advantages with respect to OM detection and has been used for different viruses ***(9–11)***. The IIF-OM detection method, although efficient, is cumbersome and requires well-trained personnel, whereas FCM is an automatable procedure that allows the processing of a large number of samples. Additionally, most major hospitals and public health institutions in developed countries possess a flow cytometer. However, standardization of an FCM method requires the study of several critical points, such as fixation (type of fixative, contact time, and so on) or minimization of background noise (lowering the antibody working solution concentration, assaying different blocking solutions, and so on).

2. Materials

2.1. Infectivity Assay

1. Horizontal laminar flow hood.
2. Vertical laminar flow hood.
3. CO_2 incubator.
4. CaCo-2 cell line, a human colon adenocarcinoma cell line, used at passage level 80–100.
5. A cytopathogenic strain of human rotavirus, i.e., Wa or Ito^r P13, used as positive control.
6. Eagle's minimum essential medium (MEM) with Earle's salts; Auto-Pow® (ICN, Costa Mesa, CA, cat. no. 1110024) as the cell culture medium. Dissolve and sterilize according to the manufacturer's instructions, and supplement with the following reagents at final concentrations: 0.15% $NaHCO_3$, 15 m*M* HEPES, 2 m*M* L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin.
7. Fetal calf serum (FCS; BioWhittaker, Walkerville, MD).
8. Phosphate buffer solution (PBS): dissolve 8 g NaCl, 0.2 g KCl, 1.15 g Na_2HPO_4, and 0.2 g KH_2PO_4 in 1000 mL deionized water. After complete dissolution, adjust the pH to 7.1–7.2 by 1 *M* HCl addition. Sterilize by autoclaving and store at 4 ± 1°C.
9. Trypsin for cell passage: dissolve 2.5 g of trypsin (tissue culture grade 1:250; DIFCO, Sparks, MD, cat. no. 0152-13) and 0.2 g of EDTA in 1000 mL PBS. After complete dissolution sterilize by filtration through 0.22-μm GS-type filters (Millipore, Bedford, MA; cat. no. GSWP047S0) and store at –20°C until use.

10. Trypsin for rotavirus activation: dissolve 0.1 g of trypsin grade IX (Sigma, St. Louis, MO; cat. no. T-0134) in 10 mL PBS. Sterilize by filtration. Prepare a stock solution of 1 mg/mL, by subsequent 1:10 dilution with PBS. Store in 1-mL aliquots at –20°C.
11. Sterile plastic ware: 5- and 10-mL plastic pipets, 58 × 17-mm Petri dishes (Nunc, Roskilde, Denmark; cat. no. 150288), sterile 0.2- and 1.0-mL tips, and 10-mL plastic tubes.

2.2. Indirect Immunofluorescence

1. Orbital vertical mixer (Selecta, Barcelona, Spain) or similar.
2. 1 *M* Sucrose solution in deionized water. Sterilize by filtration through 0.22-μm filters and store at room temperature.
3. *p*-Formaldehyde solution (20%): dissolve 20 g *p*-formaldehyde (Merck, cat. no. 1.04005.) in 70 mL deionized water. Heat the mixture for 1 h at 80°C in a thermostatic bath inside an extraction cabinet. Add 2–3 drops of 1 *M* NaOH. After 1 h more of heating, filter the mixture through Whatman filter paper. Bring to a final volume of 100 mL with deionized water and store at –20°C.
4. 1 *M* Dipotassium phosphate solution in deionized water: filter through a 0.45-μm filter and autoclave. Store at room temperature.
5. 1 *M* Sodium phosphate solution in deionized water: filter through a 0.45-μm filter and sterilize by autoclaving. Store at room temperature.
6. 1 *M* Phosphate buffer: adjust the pH of 80 mL 1 *M* dipotassium phosphate solution to pH 7.4 with 1 *M* sodium phosphate solution.
7. 0.2 *M* Phosphate buffer: add 20 mL of 1 *M* phosphate buffer to 80 mL of sterile deionized water.
8. Saline solution: add 0.8 g of NaCl in 100 mL deionized water. Sterilize by autoclaving and store at 4 ± 1°C.
9. Fixative: mix, at room temperature, 3 mL of 20% *p*-formaldehyde solution, 1.2 mL of 1 *M* sucrose, 10 mL of 0.2 *M* phosphate buffer, and 5.8 mL of deionized water.
10. Blocking solution: the day of IIF assay, dissolve 5 g powdered skim milk and 0.2 mL of a 10% Triton X-100 solution in 100 mL of saline solution.
11. Rotavirus positive control serum (Institute Virion, Rüschlikon, Switzerland; cat. no. 3193) as primary antibody: Reconstitute the lyophilized powder with 0.4 mL of deionized water. Transfer the volume to a plastic tube and add blocking solution to reach the working dilution recommended by the manufacturer (between 1:32 and 1:110). Prepare the same day of experiment.
12. Fluorescein isothiocyanate (FITC)-labeled rabbit anti-human IgG (Sigma; cat. no. F-4512) as secondary antibody: Prepare a 1:400 working dilution with blocking solution. Prepare the same day of experiment.

2.3. FCM Detection

1. Coulter Epics XL flow cytometer (Beckman-Coulter, Miami, FL) or similar.

3. Methods

3.1. Infectivity Assay

1. Prepare CaCo-2 cell monolayers in a laminar flow hood by trypsinization at a split ratio of 1:3. Cells are used when they are confluent or near confluence. In normal growing conditions, a maximum of 20 58 × 17-mm Petri dishes may be produced from a 175-cm^2

culture flask after trypsinization (*see* **Subheading 2.1.**, **item 9**). The cells are grown in cell culture medium with 10% (v/v) FCS.
2. Samples (*see* **Note 1**) are pretreated with 10 µg/mL trypsin (*see* **Note 2**) for 30 min at 37°C .
3. Wash the cells monolayers twice, using 5 mL of cell culture medium per Petri dish to remove all traces of FCS (*see* **Note 3**).
4. Remove the washing medium and add 200 µL of each trypsin-treated sample per Petri dish. As positive controls inoculate 200 µL of a viral suspension of a cytopathogenic rotavirus strain with an original titer of 10^3–10^4 infectious units per mL in each of two Petri dishes. As negative controls inoculate 200 µL serum-free medium over each of four Petri dishes.
5. Incubate for 60 min at 37°C, with two or three gentle swirlings of the inoculum over the monolayer.
6. Add 5 mL per plate of postinfection overlay medium consisting of serum-free cell culture medium supplemented with 5 µg/mL trypsin (*see* **Note 4**).
7. Incubate Petri dishes for 4 d at 37°C in an atmosphere of 5% CO_2-air.

3.2. Indirect Immunofluorescence

1. Recover the cells by vigorously pipeting up and down the postinfection medium, and transfer the whole volume to a 10-mL plastic tub.
2. Centrifuge at low speed, around 900*g* for 10 min.
3. Carefully discard the supernatant.
4. Resuspend cell pellet in 1 mL of saline.
5. Transfer the cell suspension to a 1.5-mL microtube.
6. Centrifuge microtubes in an Eppendorf 5415C microcentrifuge (or similar) at 10,000*g* for 90 s, at room temperature (*see* **Note 5**).
7. Discard the supernatant by aspiration using a micropipet and resuspend again the cell pellet with another 1 mL of saline.
8. Spin the microtube again at the same conditions described above.
9. Discard the supernatant and resuspend cell pellet with 1 mL of freshly prepared (the same day) fixative solution.
10. Incubate in gentle agitation for 30 min at room temperature.
11. Centrifuge at 10,000*g* for 90 s, at room temperature. Carefully discard the fixative and add 1 mL of saline to each microtube (*see* **Note 6**).
12. Perform two more washes with saline as described above (1 mL of saline solution per microtube, centrifuge at previous described conditions, discard the supernatant, add 1 mL more of saline solution, centrifuge another time, and pipet out the supernatant).
13. Permeabilize the fixed cells by a 15-min treatment with 0.1% Triton X-100 at room temperature, with constant gentle agitation.
14. Add 300 µL of primary antibody solution to the cell pellet of each microtube, with gentle mixing (*see* **Note 7**). Place the microtubes in an orbital vertical mixer.
15. Keep in agitation for 45 min at room temperature (*see* **Note 8**).
16. Centrifuge the microtubes at 10,000*g* for 90 s, and discard the primary antibody solution by aspiration with a pipet.
17. Wash the cellular pellet five times with the blocking solution, using for each wash 1 mL of blocking solution per microtube (*see* **Note 7**), centrifuge after each wash at 10,000*g* for 90 s, and gently discard the supernatant by careful aspiration with a pipet.

18. Add 300 µL of the FITC-labeled secondary antibody solution. Place the microtubes in an orbital vertical mixer.
19. Keep in agitation for 45 min in the dark at room temperature (*see* **Note 8**).
20. Centrifuge at 10,000*g* for 90 s, and discard the secondary antibody solution by careful aspiration with a pipette.
21. Wash the cell pellet at least four times with saline solution, using for each wash 1 mL per microtube (*see* **Note 7**), centrifuge after each wash at 10,000*g* for 90 s, and gently discard the supernatant by careful aspiration with a pipet.
22. Resuspend the cell pellet in 1 mL of saline.
23. Store at 4 ± 1°C in dark conditions to await the FCM assay (*see* **Note 9**).

3.2. FCM Detection

The cellular suspensions are analyzed with a Coulter Epics XL flow cytometer equipped with the standard 488-nm argon-ion laser at 15 mW power.

1. Select logarithmic forward angle light scatter (FSC), logarithmic side-angle light scatter (SS), logarithmic green fluorescence (FL1), and time as the acquisition parameters.
2. Adjust cytometer settings according to the values given in **Table 1**.
3. Define an FS log vs SS log dotplot (resolution: 128 × 128 channels) (**Fig. 1A**) and a 1024-channel histogram using the FL1 parameter (**Fig. 1B**).
4. Check instrument stability, analyzing 10-µm calibration beads (Flowcheck, Coulter). Record the channel position of these beads for FSC, SSC, and FL1 as a daily quality control of the instrument.
5. Acquire a minimum of 100,000 cells. To avoid coincidences, injection flow rate must be adjusted to keep total events/s below 800.
6. Draw a polygonal region on the cellular population of the FSC vs SS dotplot. FSC is used to select cell size, and SS is used to select shape and structure in order to restrain the readings to the population of intact eukaryotic cells and not the cell debris.
7. Create a gate using the polygonal region of the FSC/SS dotplot. Apply the gate on the fluorescence histogram (**Fig. 1 B**) in order to represent fluorescence of the cells inside this region. Fluorescence intensity (*x*-axis) is expressed in log scale, which means that in the higher channels small differences in channel number represent large differences in the amount of dye per cell.
8. Define a cursor on the FL1 histogram from 59.7 to 1024. This cursor is used to quantify positive events. The XL analysis software give us the percentage of cells under the cursor. The position of this cursor is established after analysis of a pool of negative control samples ***(11)***. For quality assurance, a large number (30–50 *n*) of mock-infected samples should be processed before attempting the detection of wild-type rotaviruses in natural environmental samples. An arbitrary cursor (A) is drawn at the right end of their fluorescence curves (channels 10–1024). This cursor included some of the negative cell population counts. The mean fluorescence of each of the A cursors from the negative controls is calculated. These mean values follow a normal distribution. The mean and standard deviation of this latter curve are figured, and a second cursor (B) is then defined starting at the point obtained by adding 2 standard deviations to the mean fluorescence (channel 60), and ending at channel 1024. The ratio of cells present in cursor B in respect to the total counted cells is calculated for each negative sample, and the mean plus 2 standard deviations of these ratios in the negative samples is established as the threshold of positivity .

Table 1
XL Cytometer Settings

Signal	Volts	Gain	Total gain
FS	0	1.0	1.00
SS	0	1.0	1.00
FL1	500	1.0	
Discriminator: Sensor SS, value 7			

FS, forward scatter; SS, side scatter; FL1, logarithmic green fluorescence.

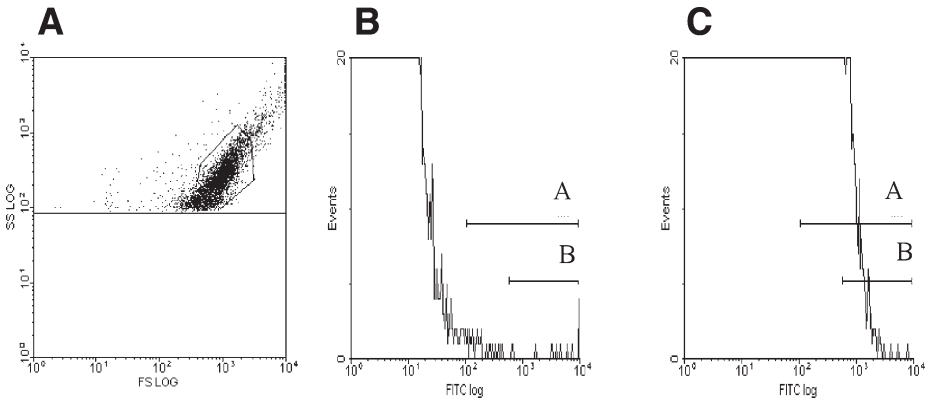

Fig. 1. (**A**) Dot plot of FSC vs SS, used to identify intact cells (inside the polygonal region) and to exclude debris and aggregates. (**B**) Green fluorescence histogram of a negative control. (**C**) Green fluorescence histogram of a positive sample. Events included inside the polygonal region are gated and represented in these two histograms according to its fluorescence.

The procedure must be ascertained using 10-fold dilutions of a cell-adapted rotavirus strain to infect CaCo-2 cell monolayers. Different infectious doses are assayed and each of them determined at least in triplicate. When wild-type rotaviruses are assayed, negative and positive control samples should be included in each assay.

4. Notes

1. In environmental studies, a water concentration step is frequently required to reduce the sampled volume to an amount able to be analyzed in the laboratory. Additionally, we regularly lyophilyze (freeze-dry) 2 mL of the water concentrate and resuspend the lyophilyzate in 200 µL of saline solution, which adds a further 10X concentration factor. This procedure contributes to detoxification of the sample prior to its inoculation onto the cell monolayers.
2. Trypsin treatment enhances rotavirus infectivity by cleaving capsid protein VP4 ***(12)***.

3. Alternatively, cell monolayers may be kept overnight with the serum-free cell culture medium at 37°C to remove all traces of fetal bovine serum, which is inhibitory to rotavirus infectivity.
4. Addition of different concentrations of trypsin (1–20 μg/mL) to media overlays is generally employed for the propagation of rotavirus in cell cultures. However, the CaCo-2 cell line is somewhat sensible to this enzyme, and in our studies the maximum permissive concentration was 5 μg/mL. Moreover, although the cells may detach from the plates, they keep alive and show no deleterious effect on virus infectivity.
5. This centrifugation speed usually allows us to recover all the cells from the suspension. Sometimes, however, the cell pellet is somewhat fluffy. In this case, it is recommended to repeat this centrifugation step before discarding the saline.
6. Do not exceed the fixation time. A contact time between 25 and 30 min does not affect the final outcome of fixation. The fixation works best on monodispersed cells.
7. When any solution is added, cells have to be dispersed by gentle agitation prior to centrifugation. This is specially crucial in the final washing step, to reduce the presence of the blocking agent (skim milk) and FITC-labeled secondary antibody. The visual aspect of the final cell suspensions should be near transparence. If not, perform additional washes. Too much blocking agent can affect proper sample processing by the flow cytometer.
8. One of the most critical points in IIF is the contact time of antibodies and cell suspension. From our experience, it is not recommended to exceed 45 min of contact time, including centrifugation time. Longer contact time, particularly in secondary antibody incubation, result in high backgrounds, which causes problems in discriminating between negative and positive samples.
9. Although it is better to perform the flow cytometry detection the same day as the immunofluorescence staining, no significant loss in fluorescence signal has been observed 2 wk after immunofluorescent staining.

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