Video Article

Measuring Attachment and Internalization of Influenza A Virus in A549 Cells by Flow Cytometry

Marie O. Pohl[1], Silke Stertz[1]

[1]Institute of Medical Virology, University of Zurich

Correspondence to: Silke Stertz at stertz.silke@virology.uzh.ch

URL: https://www.jove.com/video/53372
DOI: doi:10.3791/53372

Keywords: Infection, Issue 105, flow cytometry, influenza A virus, attachment, internalization, virus entry, A549 cells, biotinylated virus

Date Published: 11/4/2015

Citation: Pohl, M.O., Stertz, S. Measuring Attachment and Internalization of Influenza A Virus in A549 Cells by Flow Cytometry. *J. Vis. Exp.* (105), e53372, doi:10.3791/53372 (2015).

Abstract

Attachment to target cells followed by internalization are the very first steps of the life cycle of influenza A virus (IAV). We provide here a detailed protocol for measuring relative changes in the amount of viral particles that attach to A549 cells, a human lung epithelial cell line, as well as in the amount of particles that are internalized into the cell. We use biotinylated virus which can be easily detected following staining with Cy3-labeled streptavidin (STV-Cy3). We describe the growth, purification and biotinylation of A/WSN/33, a widely used IAV laboratory strain. Cold-bound biotinylated IAV particles on A549 cells are stained with STV-Cy3 and measured using flow cytometry. To investigate uptake of viral particles, cold-bound virus is allowed to internalize at 37 °C. In order to differentiate between external and internalized viral particles, a blocking step is applied: Free binding spots on the biotin of attached virus on the cell surface are bound by unlabeled streptavidin (STV). Subsequent cell permeabilization and staining with STV-Cy3 then enables detection of internalized viral particles. We present a calculation to determine the relative amount of internalized virus. This assay is suitable to measure effects of drug-treatments or other manipulations on attachment or internalization of IAV.

Video Link

The video component of this article can be found at https://www.jove.com/video/53372/

Introduction

The entry of influenza A virus (IAV) is a multi-step process that starts with the binding of the virus to receptors on the plasma membrane of target cells [1]. The receptor for IAV is sialic acid which is present on a large variety of glycoproteins and glycolipids. The hemagglutinin (HA) protein of IAV which is present in the viral envelope binds to sialic acid and thereby mediates attachment of the viral particles to the plasma membrane of target cells [2]. The virus enters the cells via clathrin-mediated endocytosis but also alternative entry pathways, such as macropinocytosis, have been described [3-6]. The interaction between HA and sialic acid appears to be sufficient for mediating both, attachment and triggering internalization of viral particles [7]. Nevertheless, alternative entry receptors have been proposed and their roles in IAV entry are currently being investigated [1,8,9].

Currently, efforts are made to identify host factors that are involved in the viral life cycle with the aim of developing urgently needed novel antiviral therapies [10-12]. Virus entry would be a favorable step for targeting IAV growth in order to block viral infection at its earliest point. Measuring different stages of IAV entry experimentally is challenging as usually large amounts of virus are required to detect incoming virus. In addition, the detection range for changes in virus entry is only linear due to the lack of viral replication. This underscores the need for assays with high sensitivity.

The assay we present here allows the detection of attached virus at the cell surface as well as detection of internalized virus in relation to the total amount of cell-associated virus. The use of biotinylated wildtype virus enables convenient measurement through staining with STV-Cy3 and readout by flow cytometry. Biotinylated virus is cold-bound to cells to allow attachment but prevent internalization of viral particles. Cells can be fixed, permeabilized and stained with STV-Cy3 to measure attached virus. The signal from extracellular, attached virions can be abrogated if a blocking step is applied before fixation in which the cells are incubated with non-labeled streptavidin (STV). In a next step, following attachment of biotinylated IAV, temperature is shifted to 37 °C and internalization of viral particles is allowed to take place. Internalized particles are protected from binding of STV thereby allowing the discrimination between extra- and intracellular viruses.

Per experimental condition, four samples are required: '0 min': The first sample, labeled '0 min', is to measure cold-bound virus at the cell surface. '0 min + STV': The second sample, labeled '0 min + STV', gives the baseline signal intensity of the experiment. Attached virus is blocked with STV and the signal following staining with STV-Cy3 should be much lower compared to the '0 min' sample. '30 min': The third sample, '30 min' contains attached and internalized virus due to the temperature shift to 37 °C for 30 min. '30 min + STV': The fourth sample, '30 min + STV' measures the intracellular fraction of viruses. The STV blocking step is applied after the 30 min incubation period. As a result, viral particles at the cell surface are bound by STV leaving only internalized viruses available for staining with STV-Cy3. The relative amount of

internalized virus can be calculated as the ratio of internalized virus (measured in the '30 min + STV' sample) divided by the total amount of virus (described by the '30 min' sample).

As controls we suggest to include mock-infected cells. The signal following STV-Cy3 staining of mock-infected cells gives the background resulting from the staining protocol. A control for IAV attachment is the pre-treatment of cells with bacterial neuraminidase (NA). NA cleaves off sialic acid from cellular glycoproteins, thereby removing attachment receptors from the cell surface. Sodium azide (SA) is a potent metabolic inhibitor and thereby blocks endocytosis [13]. Cells treated with sodium azide should be positive for virus binding but negative for internalization.

Protocol

Note before start: Use laminar flow hood and appropriate biocontainment when working with live virus. Here, we describe growth conditions suitable to culture influenza virus strain A/WSN/33. Multiplicity of infection (MOI) and incubation times may vary depending on virus strain used.

1. Preparation of Biotinylated A/WSN/33 Virus

1. Seed Madin-Darby canine kindey (MDCK) cells into a T175 cm^2 flask using Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS and 1% penicillin-streptomycin (Pen/Strep). Culture cells at 37 °C until approximately 70% confluency is reached.
2. Before infection, remove medium and wash cells with phosphate-buffered saline (PBS, pH7.0-7.3) by adding a large enough volume to cover the cell monolayer.
3. Remove PBS and infect cells with A/WSN/33 with an MOI of 10^{-4}. Dilute virus in PBS supplemented with 0.02 mg Mg^{2+}, 0.01 mg Ca^{2+}, 0.3% bovine serum albumin (BSA), and 1% Pen/Strep (infection-PBS). Use an inoculum of 4 ml for a T175 cm^2 flask.
4. Incubate cells 1 hr at 37 °C. Remove inoculum by aspiration and pipette 10 ml DMEM supplemented with 0.3% BSA, 20 mM HEPES, and 1% Pen/Strep into the flask.
5. Incubate cells at 37 °C for approximately 3 days until cytopathic effect (CPE) is visible, and then harvest supernatant. CPE can be recognized as rounding up of cells followed by detachment and death of cells [14].
6. Spin supernatant at 450 x g for 5 min to remove cell debris. Transfer supernatant into a new falcon tube and repeat this step one more time. Discard the pellets.
7. Transfer supernatants into ultracentrifuge tubes and underlay supernatants with 5 ml of 30% sucrose diluted in NTE buffer (10 mM Tris [pH 7.4], 100 mM NaCl, 1 mM EDTA). Balance tubes before ultracentrifugation and fill up with PBS to reach an end volume of 30 ml. Spin supernatants at 112,398 x g (corresponding to 25,000 rpm for an SW28 rotor) for 90 min in an ultracentrifuge.
8. Carefully remove supernatant by pipetting and soak virus pellet overnight at 4 °C in 250 µl PBS. Resuspend pellet by pipetting up and down. Measure protein content of purified virus by Bradford assay [15].
9. Use a biotinylation kit to generate biotinylated A/WSN/33. Follow the instruction of the manufacturer.
10. In brief, add the appropriate amount of biotin to the purified virus and incubate for 2h on ice. Transfer virus to an ultracentrifuge tube and fill tube up with PBS to reach an end volume of 30 ml. Spin virus at 112,389 xg for 90 min in an ultracentrifuge. Remove supernatant by pipetting and add 250 µl PBS. Soak pellet over night at 4 °C. Aliquot and store the Biotinylated virus stored at -80 °C where it will be stable for at least 24 months.
    NOTE: It might be helpful to determine the titer of the biotinylated preparation of IAV by standard plaque assay. However, it should be noted that the subsequent protocol will only measure biotinylated virus particles. Remaining unbiotinylated particles due to incomplete biotinylation will contribute to the infectious titer by plaque assay but not to the signal measured in the attachment/internalization assay.

2. Determine the Required Amount of Biotinylated Virus

NOTE: Before the attachment/internalization assay is performed, it is important to determine the amount of biotinylated virus required to infect all cells of a sample.

1. Culture and trypsinize A549 cells according to the manufacturer's instructions. Count the cells using a cell chamber. Pipette 200,000 A549 cells into FACS deepwell tubes. Spin 3 min at 450 x g. Remove supernatant and resuspend pellet with 200 µl PBS. Cool cells down on ice for 10 min.
2. Prepare 5-fold serial dilutions of the biotinylated virus stock in infection-PBS.
3. Spin tubes containing the cells at 4 °C for 3 min at 450 x g. Remove supernatant and resuspend pellet with 100 µl virus-containing infection-PBS. As mock control use infection-PBS without biotinylated virus. Incubate tubes on ice for 1 hr.
4. Spin tubes at 4 °C for 3 min at 450 x g. Remove supernatant and resuspend pellet in 200 µl PBS. Repeat this step one more time.
5. For fixation, spin tubes at 4 °C for 3 min at 450 x g. Remove supernatant and resuspend pellet in 100 µl of 3.7% paraformaldehyde (PFA). Incubate for 10 min at RT. Then, repeat wash step described in 2.4).
6. For permeabilization, spin tubes at 4 °C for 3 min at 450 x g. Remove supernatant and resuspend pellet in 100 µl PBS containing 0.5% Triton X-100. Incubate for 6 min at RT. Then, repeat wash step described in 2.4).
7. Spin tubes at 4 °C for 3 min at 450 x g. Remove supernatant and resuspend pellet in 50 µl 2% BSA diluted in PBS containing STV-Cy3. (Please note, the appropriate concentration of STV-Cy3 should be determined before starting the experiment. Start with a concentration of 1:200 and test 2-fold dilutions up to 1:2,400. In our hands, a dilution of 1:1,000 proved to be optimal.)
8. Incubate 1 hr in the dark at RT. Then, repeat washing step described in 2.4 but resuspend the pellet in 200 µl 2% BSA diluted in PBS.
9. Analyze samples by flow cytometry [16]. Gate on the Cy3-positive population. Choose the lowest concentration of biotinylated virus resulting in the maximum number of Cy3-positive cells in the A549 population.

3. Determine the Required Amount of Streptavidin

NOTE: The efficiency of blocking the signal derived from extracellular virus determines the detection range of the assay. Therefore, it is important to abrogate the STV-Cy3 signal as much as possible. The required amount of STV needed depends on the amount of biotinylated virus stock used (determined in section 2).

1. Follow steps 2.1-2.4, but use virus concentration determined in section 2.
2. Prepare five-fold serial dilutions of STV diluted in PBS containing 2% BSA and 0.1% sodium azide.
3. Spin tubes at 4 °C for 3 min at 450 x g. Remove supernatant and resuspend pellet in 50 µl STV dilution. As mock control use PBS containing 2% BSA and 0.1% sodium azide without STV. Incubate tubes for 30 min on ice. Then, repeat wash step described in 2.4.
4. Follow steps 2.5-2.9. For the internalization assay, choose the lowest concentration of STV resulting in a strong block of the Cy3-signal (compared to the sample in which no STV was present). For this, the percentage of CY3-positive cells or the mean fluorescence intensity of the Cy3 signal can be used. Ideally, the Cy3 signal of STV-treated samples should be as close to the signal from mock-infected cells as possible.

4. Attachment/Internalization Assay

NOTE: Make sure to prepare all reagents before starting the assay. For the three control groups presented in the results section (mock infected cells, neuraminidase pre-treated cells and sodium azide treated cells) small alterations to the protocol are required.

1. Prepare 16 deepwell tubes each containing 200,000 A549 cells. Next to the experimental set consisting of 4 tubes, there are 3 control conditions, each requiring 4 tubes: mock-infected cells, neuraminidase (NA)-pretreated cells and sodium azide-treated cells. Each group consists of 4 tubes labelled as '0 min', '0 min + STV', '30 min', '30 min + STV'.
2. Spin tubes at 4 °C for 3 min at 450 x g. Remove supernatant and resuspend pellet in 200 µl PBS.
3. Spin tubes at 4 °C at 450 x g. Remove supernatant and resuspend pellet in 200 µl DMEM containing 0.3% BSA, 20 mM HEPES and 1% penicillin-streptomycin (incubation medium). Incubate for 30 min at 37 °C.
   NOTE: For the neuraminidase control: Use bacterial neuraminidase (*e.g.* sialidase from Vibrio cholera) at a concentration of 200 mU/ml diluted in incubation medium to resuspend the cells.
4. Spin tubes at 4 °C for 3 min at 450 x g. Remove supernatant and resuspend pellet in 200 µl PBS. Repeat this step one more time, then cool tubes down on ice for 10 min.
5. Spin tubes at 4 °C for 3 min at 450 x g. Remove supernatant and resuspend pellet in 100 µl biotinylated virus (use the virus concentration determined in section 2 diluted in infection-PBS). Incubate 1 hr on ice. Then, repeat wash step described in 2.4.
   NOTE: For the mock-infected control: Use infection-PBS without virus. For the sodium azide control: Use virus diluted in infection-PBS supplemented with 0.1 % sodium azide.
6. Processing of the samples after the infection on ice:
   1. For the '0 min' samples, follow step 2.5 and store samples at 4 °C until the other samples are fixed.
   2. For the '0 min + STV' samples, spin tubes at 4 °C for 3 min at 450 x g. Remove supernatant and resuspend pellet in 50 µl STV (use the concentration determined in section 3) diluted in PBS containing 2% BSA and 0.1% sodium azide (to prevent internalization while handling the sample). Incubate 30 min on ice. Follow step 2.4-2.5 and store samples at 4 °C.
   3. For the '30 min' samples, spin tubes at 4 °C for 3 min at 450 x g. Remove supernatant and resuspend pellet in 200 µl PBS containing 2% BSA. Incubate 30 min at 37 °C. Follow step 2.4-2.5 and store samples at 4 °C.
      NOTE: For the sodium azide control: Use PBS supplemented with 2 % BSA and 0.1 % sodium azide for the 30 min incubation at 37 °C.
   4. For the '30 min + STV' samples spin tubes at 4 °C for 3 min at 450 x g. Remove supernatant and resuspend pellet in 200 µl PBS containing 2% BSA. Incubate 30 min at 37 °C. Then, spin tubes at 4 °C for 3 min at 450 x g. Remove supernatant and resuspend pellet in 50 µl STV (use the concentration determined in section 3) diluted in PBS containing 2% BSA and 0.1% sodium azide (to prevent internalization while handling the sample). Incubate 30 min on ice. Follow step 2.4-2.5 and store samples at 4 °C.
      NOTE: For the sodium azide control: Use PBS supplemented with 2 % BSA and 0.1 % sodium azide for the 30 min incubation at 37 °C.
7. Follow steps 2.6-2.8 and analyze samples by flow cytometry. Determine the percentage of Cy3-positive cells in each sample.
8. Calculate the percentage attached virus and the relative amount of internalized virus.
   1. To calculate the amount of attached virus, use the percentage of Cy3-positive gated cells or the mean fluorescence intensity of the Cy3 signal. For comparison, set untreated cells to 100 % (**Figure 4B**).
      NOTE: The percentage of attached virus is described by the "0 min" sample.
   2. To calculate the amount of internalized virus, take the ratio of the "30 min + STV" sample to the "30 min" sample (**Figure 5B**).
      NOTE: As mentioned above, the percentage of Cy3 positive gated cells or the mean fluorescence intensity of the Cy3 signal can be used.

Representative Results

A cartoon describing the four different experimental conditions is shown in **Figure 1**. Results of a representative experiment are presented in **Figures 2**-**5**. In the "0 min" sample, biotinylated virus is cold-bound to target cells which can be visualized by STV-Cy3 staining (**Figures 1** and **2**). When a blocking step (in the "0 min + STV" sample) is applied, virus at the cell surface can no longer be detected by STV-Cy3 staining. As a result, the signal intensity in the "0 min + STV" sample is strongly reduced compared to the "0 min" sample (**Figures 1** and **2**). Upon raising the temperature to 37 °C, attached virus is taken up into the cells ("30 min" sample). Internalized virus is not sensitive to blocking with unlabeled STV which results in a less dramatic drop in signal intensity in the "30 min + STV" samples relative to the "30 min" sample in which no STV blocking was applied **(Figures 1** and **2**).

As controls we employed mock infection, NA treatment and SA treatment. **Figure 3** displays the percentages of Cy3-positive cells for all experimental conditions described in section 4. Mock-infected cells give the background signal intensity for the experiment and act as negative control for virus attachment (**Figure 4**). An additional control for virus attachment is treatment with bacterially expressed NA. NA removes sialic

acid, the receptor for IAV attachment, from glycoproteins on the cell surface and thereby reduces binding of the virus to target cells [17]. As shown in **Figure 4**, treatment with NA strongly abrogates IAV attachment by approximately 90% compared to untreated cells. Regarding internalization, we used SA as control. SA treatment promptly depletes cellular ATP levels thereby inhibiting energy consuming processes such as endocytosis [13]. Incubation of cells with SA during and after infection inhibited internalization of virus particles. The relative amount of internalized virus (depicted in **Figure 5**) was raised from approximately 10% to 45% by incubation at 37 °C for 30 min in untreated cells. Following SA treatment however, the percentage of relative internalized virus was around 15% for both, cells incubated for 0 and 30 min at 37 °C. This indicates that indeed, SA reduces the effective uptake of viral particles into cells.

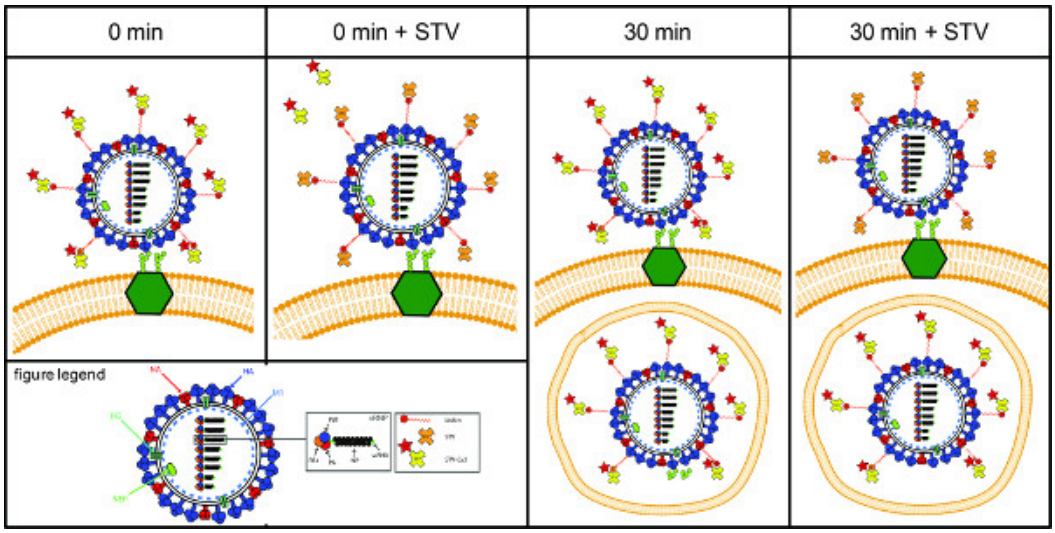

Figure 1. Principle of the assay. Cartoon explaining the principle of the attachment/internalization assay. Please click here to view a larger version of this figure.

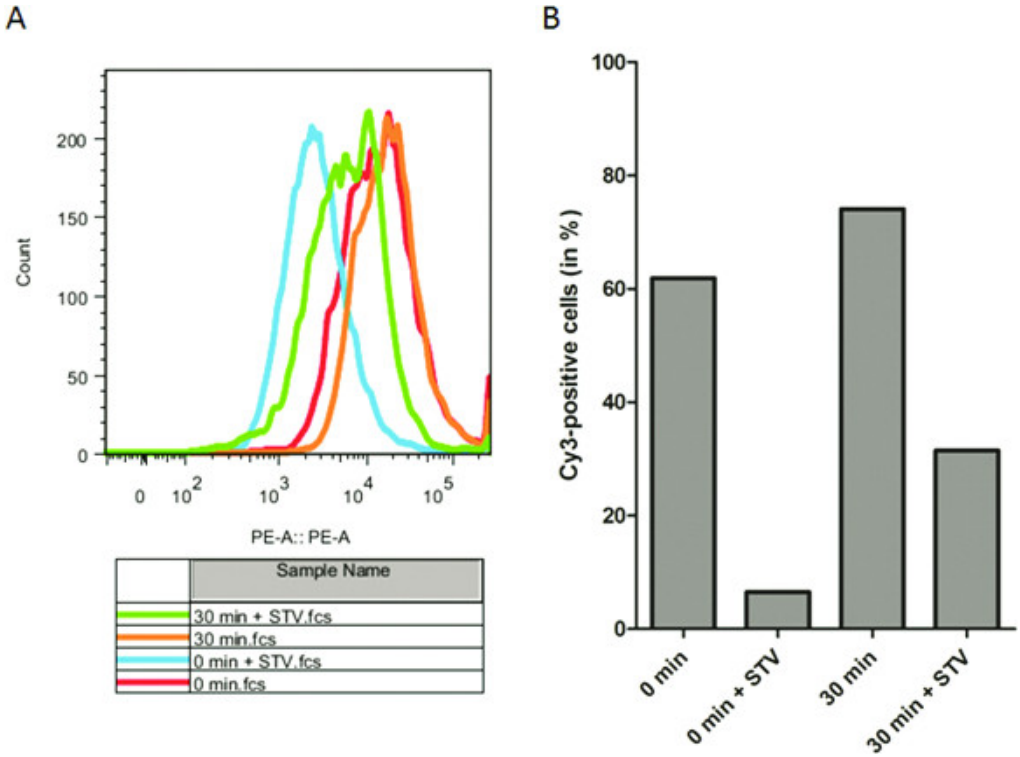

Figure 2. Representative result for IAV attachment and internalization. (**A**) Histogram showing the signal intensity of the Cy3 signal for untreated cells. Shown are the "0 min", "0 min + STV", "30 min" and "30 min + STV" sample. (**B**) Percentage of Cy3-positive cells from A. Please click here to view a larger version of this figure.

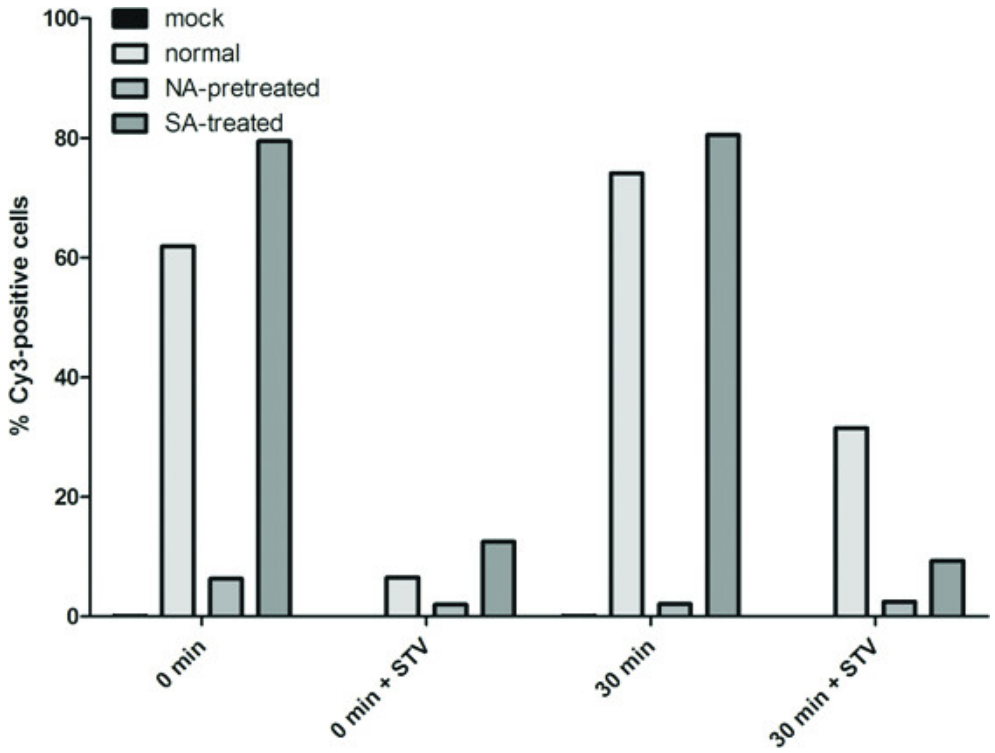

Figure 3. Representative result for attachment and internalization including controls. Percentage of Cy3-positive A549 cells following indicated treatment. Shown are mock-infected, untreated, NA-treated and SA-treated cells. Please click here to view a larger version of this figure.

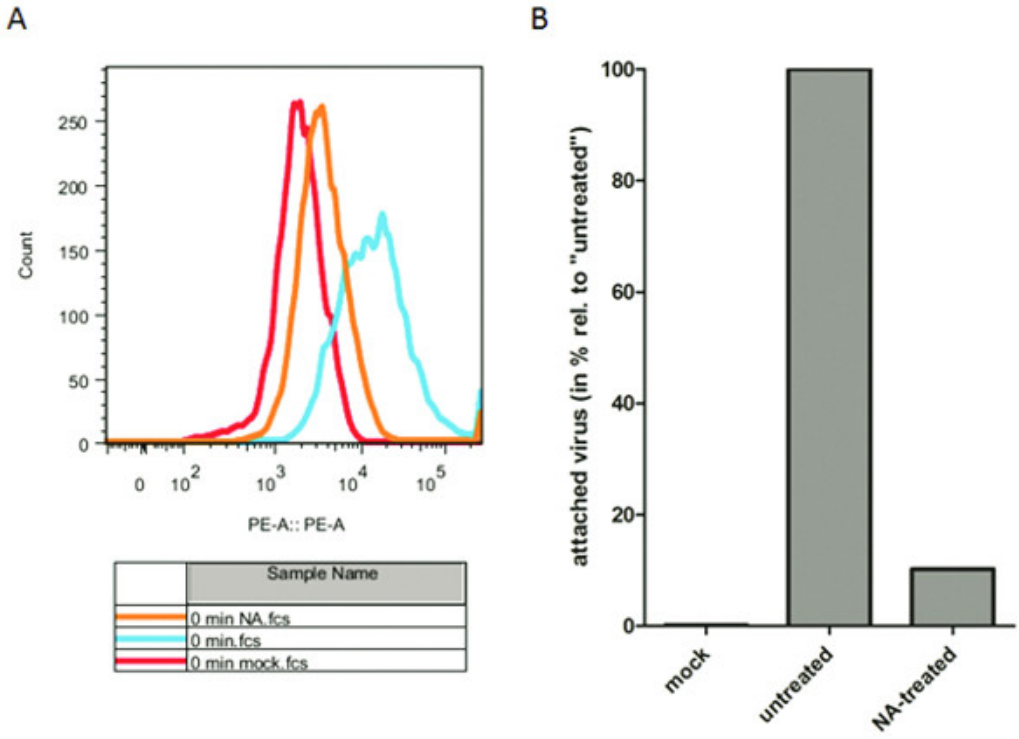

Figure 4. Inhibition of virus attachment by neuraminidase. (**A**) Histogram showing the Cy3 signal intensity of "0 min" samples. Displayed are untreated cells (in blue) and two control samples: mock-infected and NA-treated cells (in red and orange, respectively). (**B**) Percentage of Cy3-positive cells from A. Shown are mock, untreated and NA-treated cells from the "0 min" sample. The value for untreated cells was set to 100%. Please click here to view a larger version of this figure.

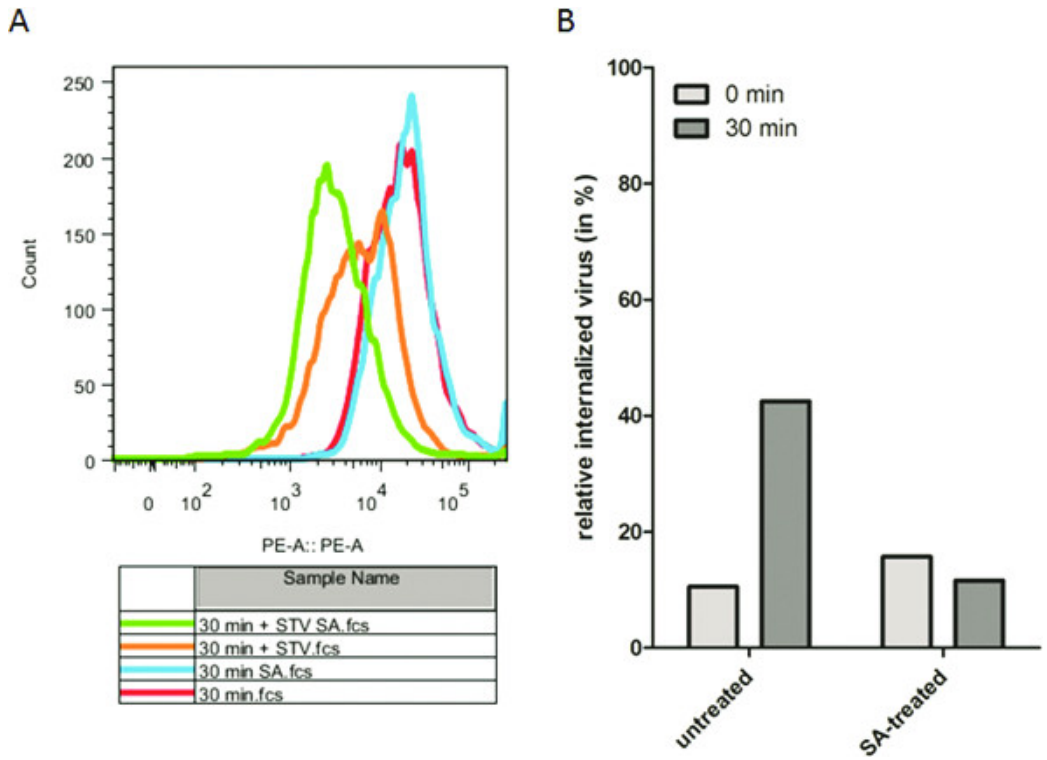

Figure 5. Inhibition of virus internalization by sodium azide (SA). (**A**) Histogram showing the Cy3 signal intensity of the "30 min" and "30 min + STV" samples from untreated (in red and orange, respectively) and SA-treated (in blue and green, respectively) cells. (**B**) Percentage of relative internalized virus from untreated and SA-treated cells after 0 and 30 min. Please click here to view a larger version of this figure.

Discussion

Our protocol describes an easy way of measuring virus attachment and internalization by flow cytometry. It allows the use of labeled wildtype virus which mimics more closely virus infections compared to the use of virus-like particles (VLP). While our protocol has been optimized to measure attachment and internalization of IAV it can easily be adapted for other viruses. In addition, as flow cytometry is used for readout, co-stainings can be easily added to the protocol *e.g.* to test expression levels following knockdown or overexpression of a protein of interest. Thus, the assay allows for modifications of the protocol depending on the individual needs. Of note, the assay can also be performed for a series of time points to measure virus internalization over time and obtain kinetic information. This might be particularly important if the assay were to be adapted for a different virus with unknown internalization kinetics.

However, it should be noted that the window of detection is relatively small as differences in attachment or internalization occur on a linear scale due to the absence of viral replication. To increase confidence and to reduce noise we recommend including controls (*e.g.* the controls describe above) and several replicates. While the results shown depict a representative experiment, we observed good consistency across different experiments: For example, for attachment, the percentage of Cy3-positive cells ranged from 60-80%; for internalized virus we obtained values ranging between 40-60%.

Before the assay is started, it is important to titrate the working concentrations of the used reagents such as STV, STV-Cy3 and the amount of biotinylated virus. Please note that the amount of reagents and incubation times can vary depending on the cell line and virus strain used. To maximize the window of detection, the percentage of cells positive for virus attachment should be as high as possible in the "0 min and "30 min" sample (depending on the amount of biotinylated virus used) and as low as possible in the "0 min +STV" sample (depending on the amount of STV used). In addition, noise can be reduced by keeping the cells at 4 °C during centrifugation and by washing with ice-cold PBS.

Disclosures

The authors have nothing to disclose.

Acknowledgements

This work was supported by a grant from the Swiss National Science Foundation (31003A_135278) to SSt. MOP is the beneficiary of a doctoral grant from the AXA Research Fund. We thank Patricia Nigg for help with the design of **Figure 1**.

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