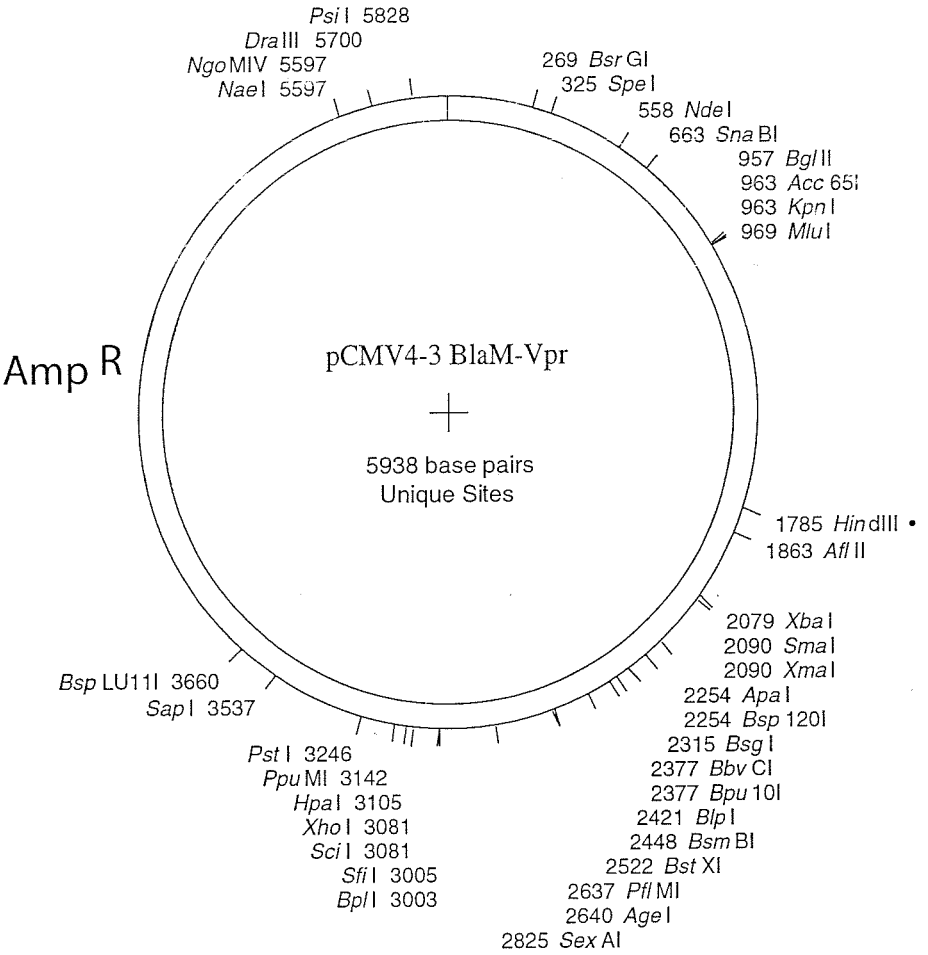

K-Vpr(96aa))ATG of the chimera BlaM-Vpr is at the position 978
The Stop codon is tag at the position 2076
The Chimera BlaM is composed of 366 aa (BlaM(264aa)-GGGGGK-Vpr(96aa))

A sensitive and specific enzyme-based assay detecting HIV-1 virion fusion in primary T lymphocytes

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Published online: 30 September 2002, doi:10.1038/nbt745

As an early event in the viral life cycle, the entry of enveloped viruses into target cells has received considerable attention. Viral fusion to cellular targets has been studied principally with fusion assays in which cells engineered to express the viral envelope are cultured with the target cells[1,2]. These assays yield valuable information but do not fully recapitulate all of the variables governing the fusion of actual virions to their cellular targets. The virion membrane and the plasma membrane, for example, differ strikingly in their lipid and protein compositions[3–5]. Two virion-based fusion assays have been described[6–8]. One is based on the redistribution of a self-quenching fluorophore[6,7], whereas the second depends on photosensitized activation of a hydrophobic probe by a fluorescent lipid loaded into the target membrane[8]. These assays are complex and have not been adapted to study fusion in complex cell populations. We have developed a simple, rapid assay allowing the detection of HIV-1 virion fusion to biologically relevant target cells, including primary $CD4^+$ T lymphocytes. It is based on the incorporation of β-lactamase–Vpr chimeric proteins (BlaM-Vpr) into HIV-1 virions and their subsequent delivery into the cytoplasm of target cells as a result of virion fusion. This transfer is then detected by enzymatic cleavage of the CCF2 dye, a fluorescent substrate of β-lactamase (BlaM), loaded in the target cells. BlaM cleaves the β-lactam ring in CCF2, changing its fluorescence emission spectrum from green (520 nm) to blue (447 nm) and thereby allowing fusion to be detected by fluorescence microscopy, flow cytometry, or UV photometry.

An overview of the assay is shown in Figure 1. To incorporate BlaM specifically in HIV-1, we constructed a protein chimera corresponding to BlaM linked to the N terminus of Vpr separated by a spacer of six glycines. The BlaM-Vpr chimera was incorporated into the HIV virions at high levels, and its enzymatic activity was comparable to that of wild-type BlaM (not shown). Next, we tested whether cleavage of the CCF2 dye could be detected after infection of HeLa-CD4 cells with CXCR4-tropic HIV-1 NL4-3 virions containing BlaM-Vpr (Fig. 2A). Although no blue cells were detected in the mock-infected control cultures (Fig. 2A), ~10% of the cells in cultures infected with HIV-1 NL4-3 displayed a blue color. Consistent with this color change deriving from the fusion of virions to the target cells, essentially no blue cells were detected in virally infected HeLa-CD4 cells pretreated with 250 nM AMD3100, an inhibitor of CXCR4-dependent viral entry[9,10].

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Next, we tested human SupT1 T cells in the virion-based fusion assay and analyzed the results by flow cytometry, using a violet laser for excitation of the CCF2 dye (Fig. 2B). In the mock-infected culture, the SupT1 cells were distributed on a diagonal, reflecting modest differences in dye loading coupled with a low level of spontaneous cleavage of the dye leading to a constant ratio of blue to green fluorescence. After infection with HIV-1 NL4-3 virions containing BlaM-Vpr, ~20% of the SupT1 cells displayed a higher ratio of blue to green fluorescence, indicating active cleavage of CCF2 by BlaM-Vpr. The shift to increased blue fluorescence induced by viral infection was almost completely blocked by pretreatment of the cells with 250 nM AMD3100. This blocking effect appeared specific, in that addition of azidodesoxythymidine (AZT) (10 μM), an inhibitor of the reverse transcriptase reaction that occurs later in the viral life cycle, did not impair the shift to greater blue fluorescence.

Together, these findings indicate that the BlaM-Vpr fusion proteins are effectively incorporated into HIV virions, are not adversely inactivated by the active HIV-1 protease, and are transmitted to both HeLa-CD4 and SupT1 cells during viral infection. Cleavage of the CCF2 dye can be readily detected by fluorescence microscopy or flow cytometry.

Fusion-mediated entry of HIV at the plasma membrane is not the only portal for virion entry into cells. Virion endocytosis represents a very active pathway of uptake. However, in the absence of appropriate viral receptors in the vesicles, this entry pathway does not lead to productive infection[11,12]. To investigate whether the BlaM-Vpr assay discriminates between virion entry by fusion and endocytosis, we incubated human Jurkat T cells expressing or not expressing CCR5 with a R5-tropic strain of HIV-1 (81A) containing BlaM-Vpr (Fig. 2C). In cells lacking CCR5, we readily detected endocytosis of virions by measuring acid-resistant intracellular p24-Gag content (not shown), but we observed no shift to increased blue fluorescence relative to mock-infected control cells. In cells expressing CCR5, however, blue fluorescence increased, indicating that these virions were capable of fusion. Conversely, no shift to increased blue fluorescence was observed in these CCR5-Jurkat T cells when the culture was preincubated with 250 nM TAK-779, an inhibitor of CCR5-dependent entry[13]. We obtained similar results with HeLa-CD4 cells that express CXCR4 but not CCR5. Upon incubation with R5-tropic virus, endocytosis of virions was readily detected by measurement of trypsin-resistant intracellular p24-Gag levels (not shown), but no blue cells were evident (Fig. 2A). These results indicate that the virion-based fusion assay detects entry of virions by fusion but not by endocytosis. This specificity likely derives from the lack of CCF2 substrate availability within the endosome, because the de-esterified CCF2 dye cannot cross biological membranes and remains within the cytoplasm.

Next, we examined whether the level of fusion at 2 h predicted the level of HIV replication at 48 h as reflected by increased production of intracellular HIV Gag (Fig. 2D). SupT1 cells were infected with serial dilutions of NL4-3 virions containing BlaM-Vpr. At 2 h, fusion was analyzed in an aliquot of the cells (left panel). The remaining cells were washed and incubated for two days at 37°C to allow completion of the viral replicative cycle. After 48 h, productive infection was assessed by intracellular immunostaining with monoclonal anti-p24-Gag antibodies (right panel). Each assay displayed dose dependency, and interassay comparisons revealed a clear correlation between the level of fusion and the subsequent level of viral replication. Interestingly, under conditions that progressively favored more than one fusion event per cell, the mean intensity of blue fluorescence (447 nm) increased steadily, suggesting that the depth of color change within individual cells correlates with the number of virions fusing to these cells (see Supplementary Fig. 1 online). These findings underscore the quantitative features

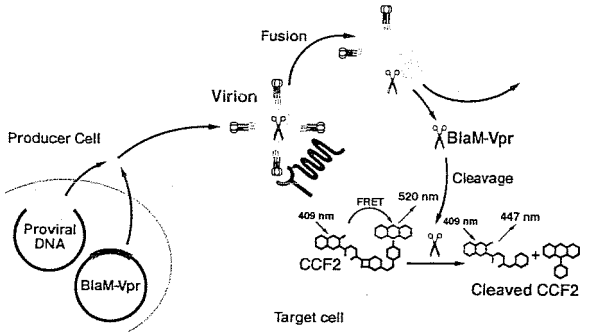

Figure 1. Schematic overview of the HIV-1 virion-based fusion assay. Expression plasmids encoding an HIV provirus and a BlaM-Vpr chimera are coexpressed in producer cells (typically 293T cells). Because Vpr binds to the p6 component of the Gag polyprotein, BlaM-Vpr is specifically incorporated into the virion. BlaM-Vpr is also efficiently incorporated into virions even when the HIV proviral clone retains the Vpr gene, a finding that extends the utility of the assay to many different wild-type HIV proviruses. Target cells are infected with BlaM-Vpr virions and then loaded with CCF2/AM dye, which diffuses passively across the cell membrane. Inside the cell, CCF2 is de-esterified and trapped because of its polyanionic properties. CCF2 contains a cephalosporin ring linking a 7-hydroxycoumarin to fluorescein. In this configuration, excitation of the coumarin at 409 nm leads to fluorescent resonance energy transfer (FRET) to the fluorescein moiety, producing a green emission (520 nm). However, if the β-lactam ring in the cephalosporin moiety is cleaved by BlaM, FRET is blocked, and excitation of the coumarin yields a blue emission (447 nm)[16]. Thus, the changes in the pattern of CCF2 fluorescence reflect the presence of BlaM, introduced by virion fusion, within the target cells.

of the virion-based fusion assay and its correlation with later events in the HIV life cycle.

Because a variety of cells can be loaded with CCF2 dye, we next investigated whether the virion-based fusion assay could be successfully applied to a biologically relevant cell type, primary peripheral blood mononuclear cells (PBMCs) (Fig. 3A). Phytohemagglutinin (PHA)-stimulated PBMCs (2×10^6) were incubated for 2 h with CXCR4-tropic HIV-1 virions containing BlaM-Vpr (100 ng p24-Gag). Fusion was detected in 5.2% of the cells, as indicated by a shift to greater blue fluorescence. Pretreatment with 250 nM AMD3100 inhibited this shift, whereas pretreatment with 10 μM AZT or with 250 nM TAK-779 produced no inhibitory effect. Using higher virus input (500 ng p24-Gag), we detected fusion of R5-tropic 81A virions in ~3% of the cells. These findings indicate that the virion-based fusion assay can be used to study virion fusion in cultures of primary PBMCs.

Next, we explored the use of this assay with primary lymphoid cells derived from human tonsil or spleen in human lymphoid aggregate cultures (HLACs) composed of dispersed spleen or tonsil tissue[14]. HLACs contain primary cells of mixed type representative of primary lymphoid tissue that grow in the absence of mitogens or cytokines and are permissive for HIV infection without further stimulation. We investigated whether it was possible to couple the HIV fusion assay with cell-surface immunostaining to identify the subset of cells undergoing fusion. To prove feasibility, we conducted the assay with anti-CD3 and anti-CD4 antibodies directly conjugated to different fluorophores. The HLACs were infected with BlaM-Vpr containing NL4-3 viruses. The fusion assay was done as with the PBMCs, except that an immunostaining step with anti-CD3 and anti-CD4 antibodies preceded fixation with paraformaldehyde (Fig. 3B). The CD3 and CD4 antibodies defined three populations within the lymphocyte gate identified by forward and side scattering. The $CD3^-CD4^-$ population was composed principally of B cells, whereas the $CD3^+CD4^+$ population represented $CD4^+$ T cells, and the $CD3^+CD4^-$ cells corresponded principally to $CD8^+$ T lymphocytes. When these populations were examined for evidence of HIV-1 virion fusion, 13% of the $CD3^+CD4^+$ cells displayed a shift to increased blue fluorescence, while almost none of the $CD8^+$ T lymphocytes ($CD3^+CD4^-$) displayed similar changes. These results demonstrate that this virion-based assay can be applied to study fusion in complex cultures of primary lymphocytes derived from

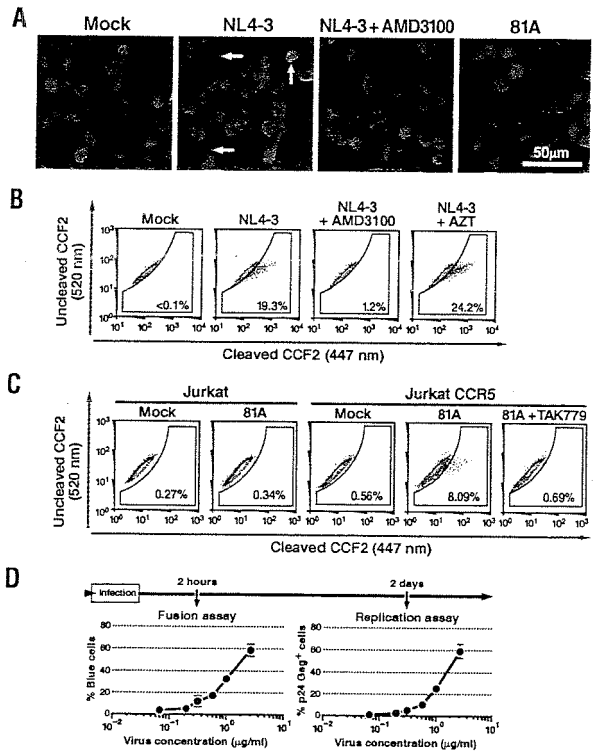

Figure 2. Detection of virion-associated BlaM-Vpr in target cells requires fusion. (A) HeLa-CD4 cells expressing endogenous CXCR4 receptors were mock-infected or infected for 2 h with virions containing BlaM-Vpr (200 ng p24-Gag), corresponding to an X4-tropic (NL4-3) or an R5-tropic (81A) strain of HIV; in one experiment, AMD3100 (250 nM), a specific inhibitor of CXCR4 entry, was added 1 h before infection. The cells were loaded with CCF2/AM dye and analyzed by fluorescence microscopy. The images correspond to an overlay of frames captured at 530 and 460 nm after fluorophore excitation at 405 nm. (B) SupT1 cells (5×10^5) were mock-infected or infected for 2 h with X4-tropic NL4-3 virions (100 ng p24-Gag) containing BlaM-Vpr alone or with AMD3100 (250 nM) or the reverse transcriptase inhibitor AZT (10 μM). After CCF2/AM loading, fusion was analyzed by multiparameter flow cytometry using a violet laser for excitation of CCF2. Percentages are of cells displaying increased blue fluorescence. (C) Jurkat T cells (5×10^5) expressing or not expressing CCR5 were mock-infected or infected with R5-tropic virions (81A) for 2 h. TAK-779 (250 nM), which inhibits entry of R5-tropic virus, was added before infection of the Jurkat-CCR5 cells. (D) SupT1 cells (5×10^5) were infected for 2 h with serial dilutions of NL4-3 virions containing BlaM-Vpr. After 2 h, an aliquot of cells was removed and analyzed in the fusion assay. The remaining cells were incubated for two days at 37°C to allow completion of the HIV replication cycle. To monitor productive HIV infection, cells were subjected to intracellular staining with monoclonal anti-p24-Gag antibodies (KC57) conjugated to fluorescein isothiocyanate (FITC). Each infection was carried out in triplicate; error bars indicate standard deviations.

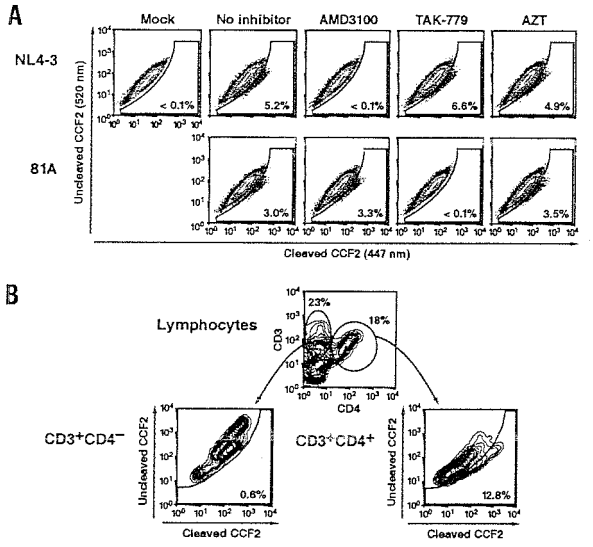

Figure 3. Application of the HIV-1 virion–based fusion assay to biologically relevant primary cells. (A) PBMCs activated with PHA (10 μg/ml) overnight and cultured in the presence of interleukin-2 (20 IU/ml) for five days were mock-infected or infected for 2 h with 100 ng of p24-Gag of X4-tropic NL4-3 virions or 500 ng of p24-Gag from R5-tropic 81A virions containing BlaM-Vpr in the absence of inhibitors or in the presence of AMD3100 (250 nM), TAK-779 (250 nM), or AZT (10 μM). After CCF2/AM dye loading, the BlaM reaction was allowed to develop for 12 h. Virion fusion was analyzed by multiparameter flow cytometry. (B) Primary cultures of dispersed mononuclear cells from human tonsil were cultured overnight and infected for 3 h with X4-tropic NL4-3 virions containing BlaM-Vpr. After CCF2/AM dye loading and incubation for 12 h at room temperature to allow the BlaM cleavage of the substrate, the cells were washed and immunostained with anti-CD4 and anti-CD3 antibodies conjugated to allophycocyanin and phycoerythrin–Texas Red, respectively. Fusion events were analyzed in the $CD3^+CD4^+$ and $CD3^+CD4^-$ subpopulations of lymphocytes. Note that HIV-1 fusion was confined to the $CD4^+$ T cells in these HLACs.

human lymphatic tissues and that the fusion assay can be coupled with immunostaining to identify and characterize the subset of cells undergoing fusion.

In summary, we have developed a virion-based assay that specifically detects the fusion of HIV-1 viral particles to target cells. This assay is simple, sensitive, specific, and quantitative. Furthermore, it can be used to study the fusion of HIV-1 to biologically relevant primary cells and coupled with immunostaining for cellular-subset discrimination. Multiple analytical formats can be employed to detect fusion, including fluorescence microscopy, flow cytometry, and UV photometry. Finally, the virion-based fusion assay can also be adapted to study fusion mediated by the envelope proteins from other viruses through the construction of HIV-1 pseudotypes.

Experimental protocol

Construction of the BlaM-Vpr chimera. The coding region for BlaM in GeneBLAzer β-lactamase vector (Aurora Biosciences, San Diego CA) was directionally inserted into pcDNA3.1 as a *Hin*dIII-*Eco*RI fragment to form pcDNA3.1-BlaM. For construction of the BlaM-Vpr chimera, the BlaM gene was linked to the N terminus of Vpr separated by a six-glycine spacer. The plasmid pCMV-BlaM-Vpr was generated by cloning a PCR-generated fragment of BlaM from pCDNA3.1-BlaM into the *Hin*dIII site of pCMV4-HA-Vpr (ref. 15). The BlaM-Vpr chimera was verified by DNA sequencing.

Production of viral particles containing BlaM-Vpr. HIV-1 virions containing the BlaM-Vpr chimera were produced by cotransfecting 293T cells in 175 cm^2 flasks with pNL4-3 proviral DNA (60 μg), pCMV-BlaM-Vpr (20 μg), and pAdVAntage vectors (10 μg) (Invitrogen, Carlsbad, CA). After 48 h at 37°C, the virus-containing supernatant was centrifuged at low speed to remove cellular debris and at 72,000 *g* for 90 min at 4°C. The virion-enriched pellet was resuspended in DMEM and aliquoted for storage at –80°C. For all transfections, calcium phosphate was used to precipitate DNA. Viral stocks were normalized by p24-Gag content measured with an ELISA (Perkin–Elmer, Boston, MA).

Virion-based fusion assay. The virion-based fusion assay is conducted in three sequential steps: (i) incubation of target cells with virions, (ii) loading of target cells with the CCF2/AM dye, and (iii) development and detection of the BlaM reaction. As highlighted in the Aurora manual, the dye-loaded cells must be maintained at room temperature to avoid export of CCF2. Because viral infections are carried out at 37°C, the infection step is done first. HeLa-CD4 cells (3×10^5), Jurkat or SupT1 cells (5×10^5), or primary cells (2×10^6) were incubated with virions containing BlaM-Vpr (50–500 ng p24-Gag) at 37°C for 1–3 h, washed in CO_2-independent medium (GibCo BRL, Rockville, MD), and then loaded with CCF2/AM dye as described by the manufacturer (Aurora Biosciences). Briefly, 2 μl of CCF2/AM (1 mM) was mixed with 8 μl of 0.1% acetic acid containing 100 mg/ml Pluronic-F127R, and 1 ml of CO_2-independent medium was added to constitute the loading solution. Cells were incubated in 100 μl of the loading solution for 1 h at room temperature. After two washes with CO_2-independent medium, the BlaM reaction was allowed to develop for 7 h (unless specified otherwise) at room temperature in 200 μl of CO_2-independent medium supplemented with 10% FBS and 2.5 mM probenecid, a nonspecific inhibitor of anion transport (Sigma Pharmaceuticals, St. Louis, MO). No antibiotics were added to the culture medium. Finally, the cells were washed once in PBS and fixed in a 1.2% solution of paraformaldehyde. The change in emission fluorescence of CCF2 after cleavage by the BlaM-Vpr chimera was monitored by flow cytometry with a three-laser Vantage SE (Becton Dickinson, San Jose, CA). A coherent krypton laser operating at 200 mW and generating light at 406.7 nm was used to excite the CCF2 dye. Blue emission was detected with an HQ455/50 filter, and green emission was detected with an HQ545/90 BP filter; for light splitting, a 505 SP filter was used. These filters were purchased from Chroma Technology (Brattleboro, VT). Data were collected with CellQuest and analyzed with FlowJo software (Treestar, San Carlos, CA). In some experiments, digital images were captured using a Nikon TE300 microscope equipped with a Hammamatsu ORCA II charge-coupled device camera and an XF124 filter set from Omega Optical (Brattleboro, VT). Images were captured and processed with Universal Imaging MetaMorph Version 4.6 and Adobe Photoshop.

Immunostaining. Before fixation in paraformaldehyde, the cells were washed in staining buffer (PBS containing 2% FBS) and incubated for 30 min at room temperature with anti-CD3-ECD and anti-CD4-APC antibodies diluted 1:20 in staining buffer. After two washes, the cells were fixed and analyzed by multiparameter flow cytometry.

Intracellular anti-p24-Gag immunostaining. After two washes in staining buffer, cells were permeabilized by incubation at room temperature for 30 min in PermeaFix (Ortho Diagnostic Systems, Raritan, NJ). After two additional washes, the cells were incubated with monoclonal anti-p24-Gag antibody (KC67; Becton Dickinson) conjugated to FITC (1:20) for 30 min at room temperature. After two washes, the cells were fixed and analyzed by multiparameter flow cytometry.

Note: Supplementary information is available on the Nature Biotechnology website.

Acknowledgments

We thank Dominique Schols for the gift of AMD3100, Michael Miller for informal discussions, Marty Bigos for expertise in the flow cytometry–based experiments, Stephen Ordway and Gary Howard for editorial assistance, and John C.W. Carroll for graphic arts. W.C.G. thanks the J. David Gladstone Institutes, National Institutes of Health (P01 HD40543), the University-wide AIDS Research Program (C99-SF-02), and the James B. Pendleton Charitable Trust for funding support.

Competing interests statement

The authors declare that they have no competing financial interests.

Received 24 May 2002; accepted 19 August 2002

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18

Fluorescence Resonance Energy Transfer-Based HIV-1 Virion Fusion Assay

Marielle Cavrois, Jason Neidleman, Martin Bigos, and Warner C. Greene

Summary

The fluorescence resonance energy transfer (FRET)-based HIV-1 virion fusion assay exploits the incorporation of β-lactamase–Vpr chimeric proteins into HIV-1 virions and their subsequent delivery into the cytoplasm of target cells as a marker of fusion. This transfer can be monitored by the enzymatic cleavage of the CCF2-AM dye, a fluorescent substrate of β-lactamase (BlaM), loaded into the target cells. BlaM cleavage of the β-lactam ring in CCF2-AM prevents the FRET between the coumarin and fluorescein moieties of the dye. This cleavage changes the fluorescence emission spectrum of CCF2-AM from green (520 nm) to blue (447 nm), and thus permits detection of fusion by fluorescence microscopy, flow cytometry, or UV photometry. This assay is simple and rapid to perform, and exhibits high sensitivity and specificity. Importantly, it can be applied to study HIV-1 virion fusion in primary cells and can be combined with immunostaining for subset discrimination in heterogeneous target cell populations. Finally, the assay can also be adapted to study fusion mediated by the envelope proteins from other viruses through the construction of HIV-1 pseudotypes.

Key Words

CCF2-AM, entry, flow cytometry, fluorescence resonance energy transfer, fusion, HIV-1, HIV-1-pseudotyped virions, β-lactamase, peripheral blood mononuclear cells, Vpr.

1. Introduction

The fluorescence resonance energy transfer (FRET)-based HIV-1 virion fusion assay is based on the incorporation of β-lactamase–Vpr chimeric proteins into HIV-1 virions and their subsequent delivery into the cytoplasm of target cells as a marker of fusion *(1)*. This transfer can be monitored by the enzymatic cleavage of the CCF2-AM dye, a fluorescent substrate of β-lactamase

From: *Methods in Molecular Biology: Flow Cytometry Protocols, 2nd ed.*
Edited by: T. S. Hawley and R. G. Hawley © Humana Press Inc., Totowa, NJ

(BlaM), loaded into the target cells. BlaM cleavage of the β-lactam ring in CCF2-AM prevents the FRET between the coumarin and fluorescein moieties of the dye. This cleavage changes the fluorescence emission spectrum of CCF2-AM from green (520 nm) to blue (447 nm) and thus permits detection of fusion by fluorescence microscopy, flow cytometry, or UV photometry.

Heterologous proteins can be incorporated into the viral particle as chimera with Vpr *(2–5)*. To incorporate BlaM into the HIV-1 virion, we constructed a chimera of BlaM linked to the N-terminus of Vpr. Because Vpr binds to the p6 component of the Gag polyprotein, BlaM-Vpr is specifically incorporated into the virions (*see* **Note 1**). Because fewer than 100 molecules of BlaM can be readily detected within a cell *(6)*, the incorporation of several hundred BlaM-Vpr molecules into a virion is theoretically sufficient to allow the detection of the fusion of a single virion to the target cell.

An overview of the FRET-based HIV-1 virion fusion assay is shown **Fig. 1**. HIV-1 virions containing BlaM-Vpr are produced by cotransfection of proviral DNA and BlaM-Vpr expression vectors. These viral preparations are then used to infect target cells. The transfer of BlaM-Vpr during the fusion is revealed by monitoring the degradation of CCF2-AM dye loaded into the target cells. Because fusion is analyzed by flow cytometry, the assay can be combined with immunostaining for subset discrimination in heterogeneous target cell populations.

The fusion of the NL4-3 strain of HIV-1 to $CD4^+$ T cells from peripheral blood lymphocytes (PBLs) will be used to illustrate the methods for production of virions containing BlaM-Vpr, the FRET-based HIV-1 virion fusion assay, and the analysis by flow cytometry (*see* **Note 2**).

2. Materials

2.1. Viral Production

1. pAdVAntage (Promega, Madison, WI).
2. pCMV4-BlaM-Vpr (available on request from Dr. Greene, Gladstone Institute of Virology and Immunology, UCSF, San Francisco, CA).
3. pNL4-3 proviral DNA (AIDS Reagent Program, NIH, Bethesda, MD).
4. 293T cells (American Type Culture Collection, Manassas, VA).
5. 2X HEPES-buffered saline (HBS): 280 m*M* NaCl, 10 m*M* KCl, 1.5 m*M* Na_2HPO_4, 12 m*M* dextrose, 50 m*M* *N*-(2-hydroxyethylpiperazine)-*N'*-(2-ethanesulfonic acid) (HEPES), pH 7.05. Store at –20°C.
6. 2 *M* $CaCl_2$.
7. Dulbecco's modified Eagle medium (DMEM) culture media: DMEM, 10% fetal bovine serum, 100 U/mL of penicillin, and 100 µg/mL of streptomycin.
8. Ultra-Clear polyallomer centrifuge tubes (25 × 89 mm) (Beckman Instruments, Palo Alto, CA).

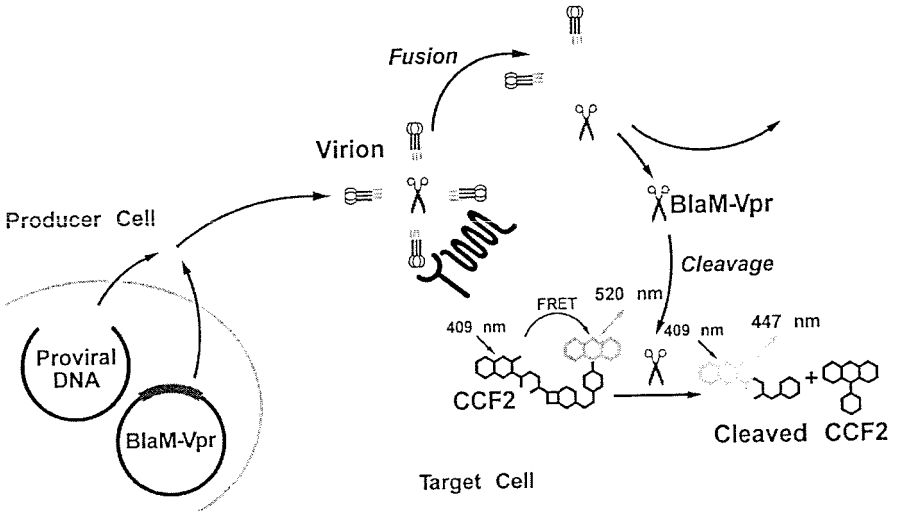


Fig. 1. Overview of the FRET-based HIV-1 virion fusion assay. Expression plasmids encoding an HIV provirus and a BlaM-Vpr chimera are coexpressed in 293T cells. Because Vpr binds to the p6 component of the Gag polyprotein, BlaM-Vpr is specifically incorporated into the virion. Target cells are infected with BlaM-Vpr virions and then loaded with CCF2-AM dye, which diffuses passively across the cell membrane. Inside the cell, CCF2-AM is de-esterified and trapped because of its polyanionic properties. CCF2-AM contains a cephalosporin ring linking a 7-hydroxycoumarin to fluorescein. Excitation of the coumarin at 409 nm causes FRET to the fluorescein, producing a green emission (520 nm). However, if the β-lactam ring in the cephalosporin moiety is cleaved by BlaM, FRET is blocked, and excitation of the coumarin yields a blue emission (447 nm) *(6)*. Thus, the changes in CCF2-AM fluorescence reflect the presence of BlaM, introduced by virion fusion, within the target cells.

9. Ultracentrifugation equipment.
10. Alliance HIV-I p24 ELISA Kit (PerkinElmer Life Sciences, Boston, MA).

2.2. FRET-Based HIV-1 Fusion Assay

1. Peripheral blood lymphocytes.
2. RPMI culture media: RPMI, 10% fetal bovine serum, 100 U/mL of penicillin, and 100 µg/mL of streptomycin.
3. 96-Well V-bottom plate (Corning Incorporated, Corning, NY).
4. CCF2-AM substrate and β-lactamase loading solutions (PanVera, Madison, WI).
5. CO_2-independent media (Gibco™–Invitrogen Corporation, Carlsbad, CA).
6. Probenecid (Sigma, St. Louis, MO).

7. Fluorescence-activated cell sorting (FACS) staining buffer: 1X phosphate-buffered saline (PBS) and 2% fetal bovine serum. Store at 4°C.
8. Mouse antihuman CD3 conjugated to APC, mouse antihuman CD4 conjugated to PE-Cy7, and mouse antihuman CD8 conjugated to APC-Cy7 (BD Biosciences, San Jose, CA).
9. 16% Paraformalehyde (Electron Microscopy Sciences, Ft. Washington, PA).

2.3. Analysis by Flow Cytometry

1. Flow cytometer: The FRET-based HIV-1 virion fusion assay alone requires a flow cytometer that incorporates violet laser excitation (violet-enhanced krypton or violet diode) and two measurement parameters on signals excited by that laser (*see* **Note 3**). Measurement of immunophenotypes requires an additional red laser capable of exciting allophycocyanin (APC) and/or APC-Cy7 and the associated detection system. For this assay, we used a FACSVantage SE with three-laser option (BD Biosciences) including: Innova I-70 argon laser (run at 200 mW, 488 nm), an Innova Spectrum krypton laser (200 mW, 647 nm), and Innova I-302 violet-enhanced krypton laser (200 mW, 407 nm) (all from Coherent, Santa Clara, CA). The detection system employed the following bandpass filters: CCF2-AM blue = HQ445/50, CCF2-AM green = HQ545/90, PE-Cy7 = 740 LP, APC = HQ695/65, and APC-Cy7 = 740 LP (all from Chroma Technology, Rockingham, VT).
2. Software for data analysis: FlowJo 4.3 software (Tree Star, San Carlos, CA).

3. Methods

The methods described in the following subheadings outline: (1) the production of HIV-1 virions containing BlaM-Vpr, (2) the FRET-based HIV-1 virion fusion assay, and (3) the analysis by flow cytometry.

3.1. Production of NL4-3 Virions Containing BlaM-Vpr

This section outlines the optimal procedure for producing NL4-3 virions containing BlaM-Vpr.

3.1.1. Transfection of 293T Cells With Proviral DNA, pCMV4-BlaM-Vpr, and pAdVAntage

The transfections are performed using calcium phosphate for precipitation of DNA.

1. Plate 1.5×10^7 293T cells in 40 mL of DMEM culture media in a T175-cm^2 tissue culture flask and culture overnight at 37°C in a 5% CO_2 humidified incubator.
2. Generate the DNA precipitate.
   a. Prepare 1.75 mL of H_2O containing 60 µg of pNL4-3 proviral DNA, 20 µg of pCMV-BlaM-Vpr, and 10 µg of pAdVAntage vectors (*see* **Note 4**).
   b. Slowly add 2 mL of 2X HBS and mix gently by pipetting up and down.
   c. Add 250 µL of 2 *M* $CaCl_2$ drop by drop.
   d. Incubate 30 min at room temperature to precipitate the DNA.

3. Replace the 293T cell culture media with 40 mL of fresh DMEM culture media prewarmed to 37°C.
4. Slowly add 4 mL of DNA precipitate and incubate for 16 h at 37°C.
5. Replace the media with 40 mL of fresh DMEM culture media prewarmed to 37°C.
6. Incubate for 24 h at 37°C.

3.1.2. Harvest Viral Supernatant and Concentrate the Viral Preparation by Ultracentrifugation

To allow more flexibility in the use of the FRET-based HIV-1 virion fusion assay, viral preparations are concentrated by ultracentrifugation (*see* **Note 5**).

1. Harvest the supernatant of the transfected 293T cells into a 50-mL Falcon tube.
2. Centrifuge the supernatant at 800*g* at room temperature for 10 min to remove the cellular debris.
3. Transfer 36 mL of virion-containing supernatant to Ultra-Clear centrifuge tubes.
4. Place the tube in the bucket of the SW28 rotor and balance the tube with DMEM culture media if necessary.
5. Ultracentrifuge (72,000*g*, 90 min) at 4°C without using brakes.
6. Remove the supernatant, resuspend the viral pellet in 1 mL of DMEM, divide into 100-µL aliquots, and store at –80°C (*see* **Note 6**).

3.1.3. Quantify $p24^{Gag}$ Content of the Viral Preparation by Enzyme-Linked Immunosorbent Assay

The $p24^{Gag}$ content of the viral preparations is quantified using the Alliance HIV-I p24 ELISA Kit according to the manufacturer's instructions. Viral production after ultracentrifugation ranges from 20–50 µg of $p24^{Gag}$/mL.

3.2. FRET-Based HIV-1 Virion Fusion Assay

The virion-based fusion assay is performed in three steps: (1) incubation of target cells with NL4-3 virions containing BlaM-Vpr, (2) loading of target cells with the CCF2-AM substrate and development of the BlaM reaction, and (3) immunostaining and fixation.

3.2.1. Incubation of Target Cells With NL4-3 Virions Containing BlaM-Vpr

1. Wash the PBLs with RPMI culture media.
2. Count PBLs and make a suspension 2×10^7 cells/mL in RPMI culture media.
3. Divide the cell suspension in aliquots of 100 µL (2×10^6 cells) per condition to be tested in a V-bottom 96-well plate. Include four additional wells for compensation when the FRET-based HIV-1 virion fusion assay is performed in conjunction with immunostaining (*see* **Subheading 3.2.3.**).
4. Add a quantity of NL4-3 virions containing BlaM-Vpr equivalent to 400 ng of $p24^{Gag}$ and incubate for 2 h at 37°C.

3.2.2. Loading of Target Cells With the CCF2-AM Substrate and Development of the BlaM Reaction

1. Prepare 1 mL of loading solution according to the PanVera protocol summarized below.
   a. Resuspend CCF2-AM in dimethylsulfoxide to generate a stock solution (1 m*M* CCF2-AM). Divide into aliquots and store in the dark at –80°C.
   b. Mix by vortex-mixing 1 µL of 1 m*M* CCF2-AM with 9 µL of a solution containing 100 mg/mL of Pluronic-F127 and 0.1% acetic acid (solution B provided by Panvera with the CCF2-AM).
   c. Add 1 mL of CO_2-independent media and vortex again.
2. Prepare 1 mL of development media (2.5 m*M* probenecid, 10% fetal bovine serum in CO_2-independent media).
   a. Prepare a stock solution of probenecid (250 m*M*) in 250 m*M* NaOH. Divide into aliquots and store at –20°C.
   b. Dilute 10 µL of the stock solution of probenecid in 1 mL of CO_2-independent media.
   c. Add 100 µL of fetal bovine serum.
   d. Vortex-mix.
3. Collect the cells by centrifugation at 800*g* for 5 min at room temperature.
4. Wash the cells once with 200 µL of CO_2-independent media and centrifuge at 800*g* for 5 min at room temperature.
5. Resuspend the pellet in 100 µL of CCF2-AM loading solution and incubate for 1 h at room temperature in the dark.
6. Collect the cells by centrifugation at 800*g* for 5 min at room temperature.
7. Wash the cells with 200 µL of development media and centrifuge at 800*g* for 5 min at room temperature.
8. Resuspend the pellet in 200 µL of development media and incubate the cells at room temperature for 16 h in the dark.

3.2.3. Immunostaining and Fixation

The FRET-based HIV-1 virion fusion assay can be combined with immunostaining to further characterize cellular populations in which HIV-1 fusion has occurred (*see* **Note 7**). This immunostaining step can be skipped when HIV-1 fusion is analyzed in cell lines or in purified cellular populations. As an example, the FRET-based HIV-1 fusion assay will be followed by immunostaining with antihuman CD3 conjugated to APC, antihuman CD4 conjugated to PE-Cy7, and antihuman CD8 conjugated to APC-Cy7. The compensations are set on uninfected samples, which are loaded with CCF2-AM and singly stained with each antibody.

1. Collect the cells by centrifugation at 800*g* for 5 min at 4°C.
2. Wash the cells once by addition of 200 µL of FACS staining buffer.

3. Collect the cells by centrifugation at 800*g* for 5 min at 4°C and resuspend the pellet in 50 µL of immunostaining solution (in FACS staining buffer) containing a 1:100 dilution of anti-CD3-APC, a 1:50 dilution of anti-CD4-PE-Cy7, and a 1:50 dilution of anti-CD8-APC-Cy7. For the four compensation controls, use FACS staining buffer alone, or with a 1:100 dilution of anti-CD3-APC, a 1:50 dilution of anti-CD4-PE-Cy7, or a 1:50 dilution of anti-CD8-APC-Cy7 (*see* **Note 8**).
4. Incubate for 30 min at 4°C.
5. Collect the cells by centrifugation at 800*g* for 5 min at 4°C.
6. Wash the cells twice with 200 µL of FACS staining buffer.
7. Fix the cells in 1.2% paraformaldehyde for 2 h at 4°C (*see* **Note 9**).

3.3. Data Collection by Flow Cytometry and Analysis With FlowJo Software

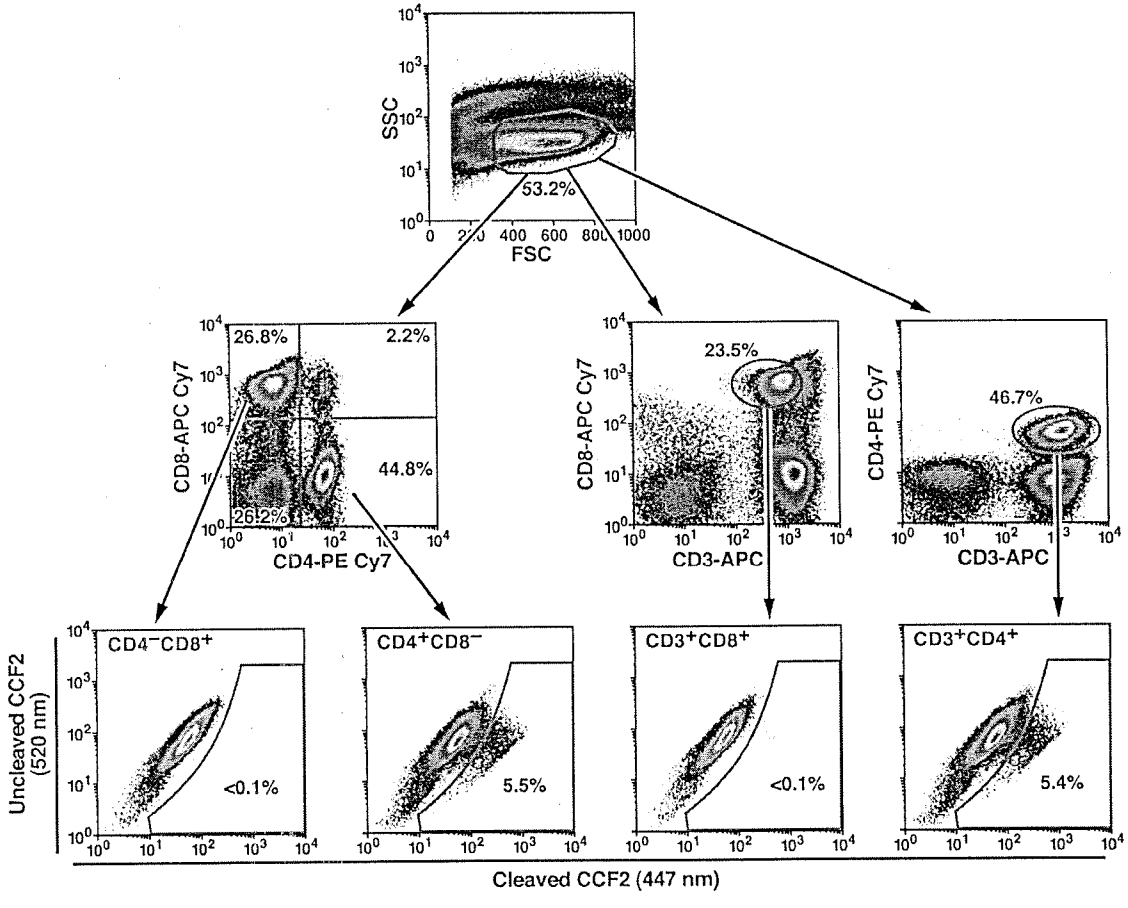
The FACSVantage SE was configured as follows. The FL1 and FL2 electronic measurement channels were connected to the third laser (violet krypton) detectors to record CCF2-AM blue and green signals. The FL6 electronic measurement channel was connected to the first laser (488-nm argon) detector to measure PE-Cy7. FL4 and FL5 were used in the standard second laser (647-nm krypton) position to measure APC and APC-Cy7. All fluorescence detection uses log amplification. Forward scatter from the first laser is used to trigger the instrument, and the combination of forward scatter and side scatter from the first laser is used to delineate the cells of interest.

3.3.1. Data Collection by Flow Cytometry

1. Adjust forward and side scatter to place the cells of interest on scale.
2. Adjust the PMT voltages of the green and blue (third laser) detectors so that these cells are roughly mid-scale. This is done using the uninfected samples loaded with CCF2-AM only. The population is organized on a diagonal reflecting the constant ratio of blue to green fluorescence. When immunophenotyping is done as well, the autofluorescence of the cells in the PE-Cy7, APC, and APC-Cy7 measurement channels is set to encompass the first decade of the log scale. Then, the CCF2-AM loaded samples and immunostained anti-CD3-APC, anti-CD8-APC-Cy7, or anti-CD4-PE-Cy7 alone are run to set the compensation between FL4 and FL5, and to provide controls for software compensation between PE-Cy7 and APC-Cy7.
3. Finally, analyze the infected samples. Upon infection, the ratio of blue to green increases and part of the population shifts toward a higher ratio of blue to green fluorescence.

3.3.2. Analysis With FlowJo Software

The data can be analyzed with FlowJo software. If required, the software allows compensation between APC-Cy7 and PE-Cy7. **Figure 2** shows the typ-


SSC
FSC
53.2%
CD8-APC Cy7
CD4-PE Cy7
26.8%
2.2%
44.8%
26.2%
CD8-APC Cy7
CD3-APC
23.5%
CD4-PE Cy7
CD3-APC
46.7%
Uncleaved CCF2 (520 nm)
Cleaved CCF2 (447 nm)
CD4⁻CD8⁺
<0.1%
CD4⁺CD8⁻
5.5%
CD3⁺CD8⁺
<0.1%
CD3⁺CD4⁺
5.4%

ical data obtained with NL4-3 containing BlaM-Vpr used to infect PBLs, and suggests a gating strategy for analysis of this data.

4. Notes

1. BlaM-Vpr is efficiently incorporated into virions even when the HIV proviral clone retains the Vpr gene. This finding extends the use of the FRET-based HIV-1 fusion assay to many different wild-type HIV proviruses.
2. Multiple cell lines and various primary cell types, including macrophages and HUVEC, have been successfully used in the assay. However, certain cell lines, for example MAGI cells, exhibit endogenous BlaM activity and thus are not suitable for analysis. One of the vectors used to express a transgene in this cell line likely encodes an enzyme that degrades the CCF2-AM substrate. In such case, a portion of the cells is already shifted to high ratio of blue to green of fluorescence in absence of viral infection. Therefore, to rule out endogenous BlaM activity, new cell types should be tested by loading the cells with CCF2-AM and following the protocol described in **Subheading 3.2.2.**
3. The original papers describing CCF2-AM *(6,7)* used UV excitation for CCF2-AM fluorescence readout. However, there was a limitation of this approach due to excitation mismatch between the laser line (355 nm) and CCF2-AM, and the high autofluorescence of cells generated by the UV light. Using a krypton 406.7-nm line provides optimal excitation and a lower autofluorescence signal from cells. However, this laser is quite expensive and not commonly available. Subsequent tests have demonstrated that excitation with a relatively inexpensive 20-mW solid-state violet diode laser in a cuvet flow system that is now commercially available yields equivalent results.
4. The assay can also be conveniently adapted to study fusion mediated by the envelope proteins from other viruses through the construction of HIV-1 pseudotypes. The pseudotyped HIV-1 virions containing BlaM-Vpr are produced as described in **Subheadings 3.1.1.–3.1.3.** with minor modifications. The DNA precipitate is prepared with 40 µg of Δenv pNL4-3 proviral DNA, 20 µg of an envelope encoding construct (VSV-G, A-MLV or other), 20 µg of pCMV-BlaM-Vpr, and 10 µg of pAdVAntage vectors. Fusion of these pseudotyped virions will occur according to the pathway dictated by the envelope and will be sensitive to the inhibitor of fusion corresponding to this envelope.

Fig. 2. *(see opposite page)* Analysis of HIV-1 fusion to PBLs by the FRET-based HIV-1 virion fusion assay. PBLs (2×10^6 cells) were infected for 2 h with NL4-3 virions containing BlaM-Vpr (400 ng of $p24^{Gag}$). After CCF2-AM dye loading and incubation for 16 h at room temperature to allow the BlaM cleavage of the substrate, the cells were immunostained with anti-CD3 antibodies conjugated to APC, anti-CD8 antibodies conjugated to APC-Cy7 and anti-CD4 conjugated to PE-Cy7. Fusion events were analyzed in the $CD4^-CD8^+$, $CD4^+CD8^-$, $CD3^+CD4^+$, and $CD3^+CD8^+$ subpopulations of lymphocytes. Percentages represent cells displaying increased blue fluorescence.

5. Viral supernatant from transfected 293T cells can be used directly to infect the target cells. However, because the FRET-based HIV-1 virion fusion assay is a single-round infection assay, fusion will occur in fewer target cells. The infection rates could be further improved by using the "spinoculation" protocol *(8)*.
6. Frozen viral preparations are stable for at least 2 yr at –80°C. They can be thawed and frozen three times without significant loss of infectivity. However, to compare the fusion proprieties of different viral preparations, we recommend using virions that were produced in parallel and subjected to equivalent cycles of thawing/freezing.
7. As pointed out, CCF2-AM is a tandem conjugate of coumarin and fluorescein. The fluorescein component of the substrate excites quite well with the standard 488-nm argon line used in most flow cytometers. Because of the amount of substrate typically loaded in a cell, the resulting fluorescence from fluorescein is bright in the PE, PE-TR, and PE-Cy5 measurement channels. Fluorochromes excited by a red laser (APC, APC-Cy5.5, APC-Cy7, and red-emitting Alexa dyes) are not affected by this problem.
8. When fusion is analyzed in T-cell lines, the shift toward a high ratio of blue fluorescence is more pronounced when the cells have been maintained in 1.2% paraformaldehyde for 6 h. Further incubation in paraformaldehyde had no additional effect. The shift remains the same for at least 24 h.
9. The specified antibody dilutions were optimal for our conditions. However, reagents from different lots and different manufacturers may vary. The optimal dilution should be determined beforehand by performing a titration experiment for each reagent *(9)*.

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