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Quantitation of HIV-1 entry with β-Lactamase-loaded virions

I Overview:

-**Day 1:**
-Seed target cells
- **Day 2:**
- Infect cells with HIV-1/BlaM-VPR 4-5 h 37°C
- Transfer plate to RT
- Load cells with substrate CCF2-AM 16-18 h, incubator 25°C/RT
- **Day 3:**
- Wash substrate away
- Fix cells with paraformaldehyd
- Analysis on BD-LSR flow-cytometer

II Prepare reagents:

a) **Serum free medium** containing 10 mM HEPES
b) **Solution A**: dissolve 200 μg CCF2-AM (= 185 nmol), a lyophilized white powder (GeneBLazer Loading Kit, Panvera (previously Aurora Biosciences, La Jolla) by adding 185 μl dry DMSO (GeneBLazer Loading Kit), store stock solution at -20°C in the dark, make aliquots a 20 μl, prevent repeated thawing and freezing, good for several month.
c) **Solution B**: 100 mg/ml Pluronic-F127 (GeneBLazer Loading Kit), store at RT, good for 12 months.
d) **Probenecid**: 250 mM Probenecid (e.g. Sigma, P-8761), a non-specific anion exchange blocker, dissolves in 250 mM NaOH (100x stock), aliquot and store at -20°C.

III Entry assay:

Day 1:
e) Seed cells for infection: HOS $1x10^5$/ 6-well dish
Day 2:
f) Infect in 1-2 ml, using HIV/BlaM-VPR MOI 0.5 or higher, 4-5 h 37°C. (Instead infecting $2x10^5$ fibroblasts/6-well dish, you can infect $4x10^5$ suspension cells/24-well dish.)

g) Prepare loading solution in the following order: add 2 µl of solution A to 8 µl of solution B and mix. Add the resulting solution to 1ml of serum free medium containing 10 mM HEPES and mix (= loading buffer). Add probenecid stock solution: 10 µl to 1ml loading buffer (=1% probenecid).

h) Adjust cells to RT, remove medium from cells, wash cells 1x with PBS or serum-free medium, replace with 1ml loading solution, incubate 16-18 h at 25°C**,** 5% CO_2**.**

Day 3:

i) After 16-18 h loading: remove loading solution, wash cells 1x with PBS, detach the cells with trypsin, inactivate trypsin with medium containing serum, wash and fix (final 1% PFA/PBS), keep on ice until analysis.

j) Analyze samples on a BD LSR 3 analytical flow cytometer (Becton Dickinson), at the Salk Institute, with a UV laser and 470 nm long-pass dichroic filter exciting at 325 nm. Cleaved substrate is detected as blue fluorescence with a 424/44 nm bandpass filter; uncleaved substrate is detected with a 516/20 nm bandpass filter. Analyze in linear mode, no compensation needed, adapt the voltage to get a strong green- and blue-signal, without loosing the most positive cells and keeping negative (unloaded) cells in the corner of a green : blue plot. Plot the results as the ratio of the blue/green to minimize differential dye loading.

IV Principle of the assay **(Merck, Aurora):**

HIV reporter virions containing β-lactamase are generated by cotransfecting 293T cells with the HIV plasmid DNA and BlaM-VPR vector, pMM310. HIV/BlaM virions are harvested from the supernatants of the transfeted cells and used to infect traget cells expressing CD4 and coreceptor. The cells are incubated at 37° C for 4-5 hours to allow virus binding and entry. The cells are then loaded with the cell-permeant, fluorescent β-lactamase substrate ester, CCF2-AM (Aurora Biosciences) and incubated an additional 16-18 h (25°C or RT) to allow β-lactamase cleavage of the substrate. In the CCF2 molecule, the cephalosporin core links a 7-hydroxycoumarin to a fluorescein. In the intact molecule, excitation of the coumarin results in fluorescence resonance energy transfer (FRET) to the fluorescein, which emits green light. Cleavage of CCF2 by β-lactamase results in spatial separation of the two dyes, disrupting FRET such that excitation of the coumarin now gives rises to blue fluorescence. Fluorescence is quantified by flow cytometer with UV or violet laser or a fluorometer. Cells in which viral entry has occurred are blue because the β-lactamase contained in the viral particle cleaves the fluorescent CCF2 substrate. Cells in which no viral entry has occurred will remain green because CCF2 substrate is still intact.

V Analysis of the samples:

The FACS measures the ratio of blue/green intensity for each cell. The ratio is calculated in analog mode in the machine's hardware before analog-to digital conversion is done, making it more precise. So you get a population of points because the cells differ one to another. If you would have a clearly defined population to measure the percentage of, it ought to be okay to use the percentage of blue cells in a green: blue dot plot. If on the other hand you have a "shifted population" without a clear distinction between negative and positive, you may want to worry about where you set the analysis marker when calculation the results, and what effect changes in things like dye loading efficiency (at least that's the big advantage for things like Indo-1). The ratio of the emission intensity at 447-nm (blue) to that at 520-nm (green) is a concentration independent measure of the extent of the reaction.

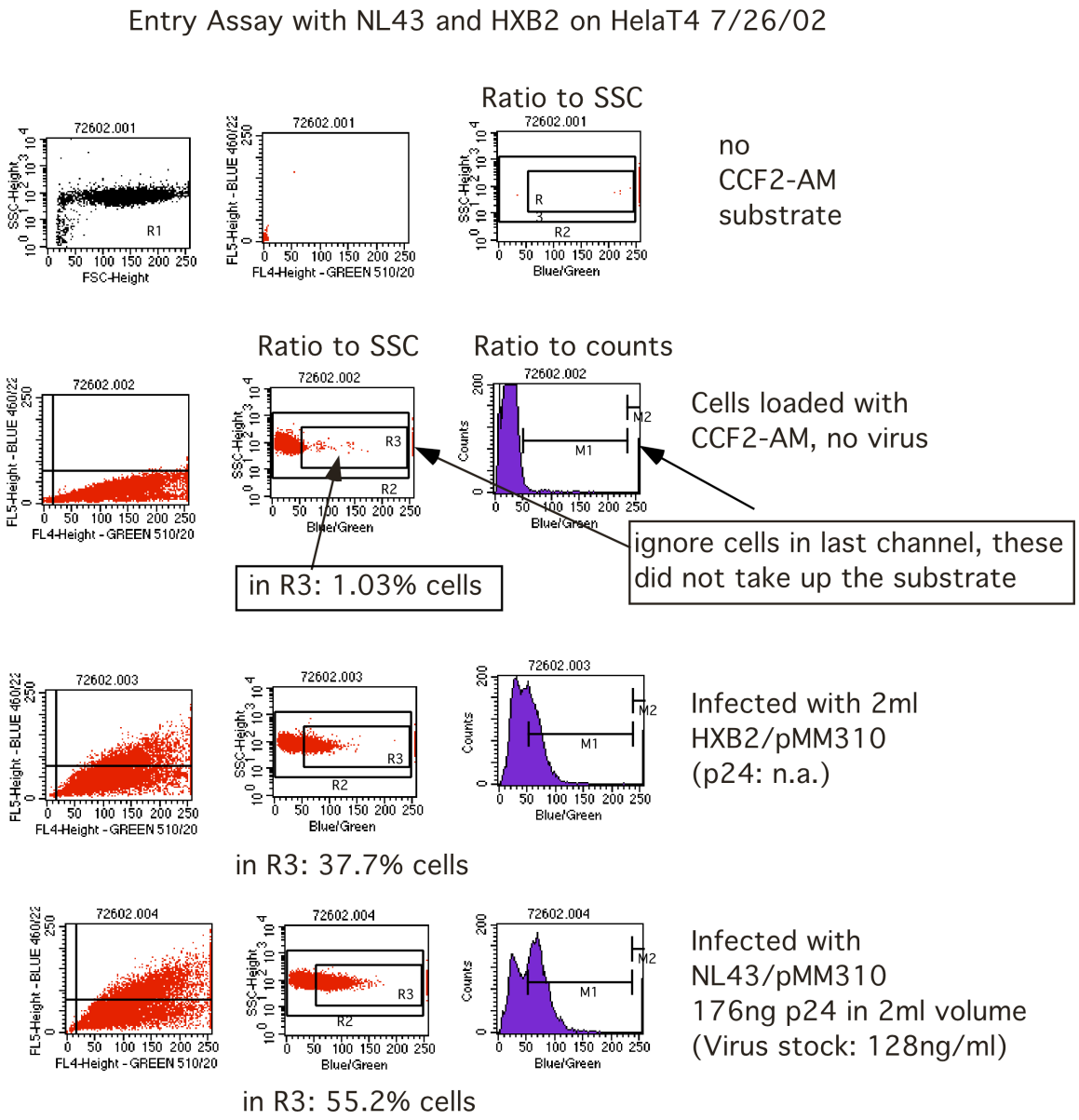

Fig. 1:Shows typical results of the viral entry assay, analysed on a BD-LSR with UV laser. HelaT4 cells were infected with either HXB2-BlaM-VPR (pMM310) or NL4.3/BlaM-VPR. Note: Cells unloaded with CCF2-AM should not have a significant fluorescence for either green or blue.

VI Comments:

Transfections:

Co-transfection (1:1) of 293T cells with a HIV expression plasmid and pMM310 (BlaM-VPR) results in a significant reduction of the p24 yield. VPR expression of the HIV plasmid has only a minimal influence. To compensate the lower yield we produce BlaM containing virions by lipofectamin 2000 transfection. Freeze virus aliquots like regular virus, store -70°C.

Cells:

Best results were obtained with $2x10^5$ fibroblast cells (e.g. HOS, NIH3T3, FrHL-2) in a 6-well dish. Seeding $2x10^5$ in 12-well dish reduces loading of the substrate. Using lower number of cells will complicate the subsequent analysis with the flow-cytometer, because of to low cell numbers (During dye loading cells are not diving much and some cells will detach). This assay works fine with T-cell lines. But not all cells like to take up the substrate equally, e.g. we were never able to load CV-1 cells with CCF2-AM. We do not know if PBMC take up the substrate and keep the cleavage product.

Cell loading:

If the plates containing the loading solutions are not incubated at the 16-18 h time period in a 25°C 5% CO_2 incubator, you can keep them in a hood (seal the plate and increase the HEPES to 25mM). The temperature has a significant influence on the results; the cells are exporting CCF2-AM more efficiently out of the cells with higher temperatures.

Controls:

Controls for every experiment: uninfected cells +/- substrate. Optional: 123C6-cells +/- substrate, supT1 +/- substrate. (123C6 cells (obtained from Merck) are SupT1 cells expressing beta-lactamase and CCR5, growth in RPMI, 500μg/ml G418 (beta-lactamase selection) and 0.45μg/ml puromycin (CCR5 selection))

Virus:

The amount of virus you should use is MOI 0.5 or higher. MOI 0.1 can clearly be detected, below MOI 0.1 the values are not reproducible and to close to the background fluorescence. This was tested on a 325-nm UV laser and might be different using a violet laser. Virus pseudotyped with VSV-G is also detected in this assay, but cells infected with equal MOIs show more entry with particles carrying HIV envelope. We do not know why VSV-G pseudotyping interferes slightly with the BlaM entry assay.

Flow Cytometry Lasers:

We are doing the entry experiments using the BD-LSR (3 lasers, six colors) system and use as excitation source an 8-mW 325-nm helium-cadmium laser (UV). According to the flow-cytometry experts from Aurora, the CCF2-AM shows minimal to moderate response to this laser. It is OK to use it, but only with controls. Best excitation source is a violet laser 407-nm or 413-nm (krypton). Next best is a mode-lock UV laser (argon-krypton) 356-nm to 362-nm. Best detection with the filters 460/50-nm, 535/40-nm and a 500-nm long-pass dichroic filter. Since 2002, BD is offering the BD-LSR II (4 lasers, 15 colors), which offers now in addition to the 488-nm argon-ion and the 635-nm helium-neon lasers, a variety of UV/violet lasers: a 405-nm coherent VioFlame violet laser, a 355-nm mode-lock UV laser, and/or a 325-nm helium-cadmium UV laser.

Caution:

A repeated problem using the SALK BD LSR was based on misaligned filters in the LSR. this was corrected by a BD engineer. Check that the filters are in the correct positons!!!

Avoid trace amounts of bleach.:

A word of cautions: even minimal amounts of bleach in your sample, will result in a dramatic increase of blue fluorescence. Carefully wash the nozzle of the flow-cytometer, the previous user might bleached it

VII Quantitation by Alternative methods:

Green and blue fluorescence can be analyzed also with other systems than flow-cytometers: Fluorescence spectrometer, fluorescence plate reader and fluorescence microscopes:

Fluorescence plate reader (Merck)

The β-Lactamase filter set for use in a 96-well micro plate reader is available from Chroma Technologies (800-824-7662) (set #APR1)
Set # APR1 - For ratio metric readout using a fluorescence micro plate reader:
Excitation filter: HQ405/20x (405 ± 10 nm)
Emission filter: HQ460/40m (460 ± 20 nm)
Emission filter: HQ530/30m (530 ± 15 nm)
You need to specify the type of instrument you are using to get the correct size filter.
Typically Chroma is familiar with the requirements for most common plate readers and microscopes. If you use a BMG fluorometer (e.g., FluoStar, PolarStar), order the special β-lactamase filter set manufactured by BMG. It is recommended to use culture medium (-)phenol-red and black multiwell plates with clear bottom Note: The cells must be washed to remove unincorporated CCF2-AM,

Microscopy (Merck)

Recommended filter sets for observing β-lactamase activity are described below and are available from Chroma Technologies (800-824-7662) Chroma Set # 41031
Excitation filter: HQ405/20x (405 ± 10)
Dichroic mirror: 425 DCXR
Emission filter: HQ435LP
Filter sizes vary for specific microscopes and need to be specified by you when you order the filters**.** For epifluorescence microscopes, a long-pass dichroic mirror is needed to separate excitation and emission light and should be matched to the excitation filter (that is, maximally block the excitation light around 405 nm, yet allow good transmission of the emitted light).

VIII Recent changes from Panvera

The manufacturer Panvera changed some recommendations for preparing the loading solutions. We never tested these protocols.

<u>Standard Loading Protocol</u>

Solution A: 1mM CCF2/AM in dry DMSO
Solution B: 100 mg/ml Pluronic-F127 in DMSO with 0.1% acetic acid
Solution C: 24% w/w PEG 400, 18% TR40 by volume in water

Serum-free media containing 25 mM HEPES

Preparation of 1ml 6x Loading buffer and Cell Loading

1) Remove media from cells and replace with serum-free media or buffer containing HEPES (25 mM).

2) In a small tube, ad 12µl of solution A to 120 µl of solution B.

3) Add 2ml solution C to the mixture of solution A+B above with vigorous agitation (Vortex). This solution (mixture of solutions A+B+C) now constitutes the 6x loading buffer.

4) Pipette 6x loading buffer gently over the cells (do not mix; 6x loading buffer will sink over cells) to a 1x final concentration (e.g. add 20 µl of 6x loading buffer to 100µl of cells in buffer). NOTE: Discard 6x loading buffer that has not been used within 1 hour.

5) Incubate cells in the dark for 1 hour.

Enhanced Loading Protocol

Solution A: 1mM CCF2/AM in dry DMSO
Solution B: 100 mg/ml Pluronic-F127 in DMSO with 0.1% acetic acid
Solution C: 24% w/w PEG 400, 18% TR40 by volume in water
Soution D: 200 mM probenecid dissolved in 200 mM NaOH

Serum-free media containing 25 mM HEPES

Preparation of 1ml 6x Loading buffer and Cell Loading

1) Remove media from cells and replace with serum-free media or buffer containing HEPES (25 mM).

2) In a small tube, ad 24µl of solution A to 120 µl of solution B.

3) Add 1850µl solution C to the mixture of solution A+B above with vigorous agitation (Vortex).

4) Add 150µl of solution D to the mixture of solutions A+B+C above and mix. This solution (mixture of solutions A+B+C+D) now constitutes the 6x loading buffer.

5) Pipette 6x loading buffer gently over the cells (do not mix; 6x loading buffer will sink over cells) to a 1x final concentration (e.g. add 20 µl of 6x loading buffer to

100µl of cells in buffer). NOTE: Discard 6x loading buffer that has not been used within 1 hour.

6) Incubate cells in the dark for 1 hour.

VIX References:

Literature:

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Zlokarnik G, Negulescu PA, Knapp TE, Mere L, Burres N, Feng L, Whitney M, Roemer K, Tsien RY. **Quantitation of transcription and clonal selection of single living cells with beta-lactamase as reporter.** Science. 1998 Jan 2; 279(5347): 84-8.

Cavrois M, De Noronha C, Greene WC. **A sensitive and specific enzyme-based assay detecting HIV-1 virion fusion in primary T lymphocytes**. Nat Biotechnol. 2002 Nov; 20(11): 1151-4.

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Web resources:

AURORA biosciences: www.aurorabio.com (Recently, *Aurora* was bought by *Panvera*, URL might change)