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Flow Cytometric Analysis of Drug-induced HIV-1 Transcriptional Activity in A2 and A72 J-Lat Cell Lines

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[Abstract] The main obstacle to eradicating HIV-1 from patients is post-integration latency (Finzi *et al.*, 1999). Antiretroviral treatments target only actively replicating virus, while latent infections that have low or no transcriptional activity remain untreated (Sedaghat *et al.*, 2007). A combination of antiretroviral treatments with latency-purging strategies may accelerate the depletion of latent reservoirs and lead to a cure (Geeraert *et al.*, 2008). Current strategies to reactivate HIV-1 from latency include use of prostratin, a non-tumor-promoting phorbol ester (Williams *et al.*, 2004), BET inhibitors (Filippakopoulos *et al.*, 2010; Delmore *et al.*, 2011), and histone deacetylase (HDAC) inhibitors, such as suberoylanilidehydroxamic acid (*i.e.*, SAHA or Vorinostat) (Kelly *et al.*, 2003; Archin *et al.*, 2009; Contreras *et al.*, 2009; Edelstein *et al.*, 2009). As the mechanisms of HIV-1 latency are diverse, effective reactivation may require combinatorial strategies (Quivy *et al.*, 2002). The following protocol describes a flow cytometry-based method to quantify transcriptional activation of the HIV-1 long terminal repeat (LTR) upon drug treatment. This protocol is optimized for studying latently HIV-1-infected Jurkat (J-Lat) cell lines that contain a GFP cassette. J-Lats that contain a different reporter, for example Luciferase, can be treated with drugs as described but have to be analyzed differently.
Keywords: Human immunodeficiency virus-1, Latency, Drug treatment, Transcriptional activation, HIV-1 LTR, Flow cytometry, J-Lat cell lines

[Background] Studies that assess transcriptional activation or repression of the HIV-1 LTR generally use $CD4^+$ T cells containing latent full-length HIV-1, such as NL4-3/E-/GFP-IRES–nef (Kutsch *et al.*, 2002) or R7/E-/GFP (Jordan *et al.*, 2003), which contains a frameshift mutation in the viral *Env* gene to prevent viral spread and expresses GFP in the *Nef* open reading frame allowing separation of actively infected GFP^+ from GFP^- cells (uninfected or latently infected) by cell sorting (Jordan *et al.*, 2003). To specifically investigate transcriptional activation of the HIV-1 LTR, we utilize the J-Lat cell line A72 containing only a latent LTR-GFP construct (Jordan *et al.*, 2003). To determine if drug treatment specifically activates Tat, we utilize a J-Lat cell line harboring a latent lentiviral construct expressing Tat with GFP from the HIV-1 LTR (clone A2; LTR-Tat-IRES-GFP) (Jordan *et al.*, 2003).

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Materials and Reagents

A. A2 and A72 J-Lat cell culture
   1. 75 cm^2 tissue culture flask (Corning, Falcon®, catalog number: 353110)
   2. Tips
      0.1-10 µl (Fisher Scientific, Fisherbrand™, catalog number: 02-681-440)
      1-200 µl (Fisher Scientific, Fisherbrand™, catalog number: 02-707-502)
      101-1,000 µl (Fisher Scientific, Fisherbrand™, catalog number: 02-707-509)
   3. A2 and A72 J-Lat cells (Jordan *et al.*, 2003)
   4. RPMI (Mediatech, catalog number: 10-040-CV)
   5. Fetal bovine serum (FBS) (Gemini Bio-Products, catalog number: 100-106)
   6. L-glutamine (Mediatech, catalog number: 25-005-CI)
   7. 100x penicillin/streptomycin (Mediatech, catalog number: 30-002-CI)

B. Analysis of HIV-1 LTR transcriptional activation by flow cytometry
   1. 96-well V-bottom tissue culture plates and lids (Thermo Fisher Scientific, Thermo Scientific™, catalog numbers: N249570 and N163320)
   2. Tips
      0.1-10 µl (Fisher Scientific, Fisherbrand™, catalog number: 02-681-440)
      1-200 µl (Fisher Scientific, Fisherbrand™, catalog number: 02-707-502)
      101-1,000 µl (Fisher Scientific, Fisherbrand™, catalog number: 02-707-509)
   3. RPMI (Mediatech, catalog number: 10-040-CV)
   4. Fetal bovine serum (FBS) (Gemini Bio-Products, catalog number: 100-106)
   5. L-glutamine (Mediatech, catalog number: 25-005-CI)
   6. 100x penicillin/streptomycin (Mediatech, catalog number: 30-002-CI)
   7. TNFα (PeproTech, catalog number: 300-01A)
   8. JQ1 (Cayman Chemical, catalog number: 11187)
   9. Prostratin (Sigma-Aldrich, catalog number: P0077)
   10. Suberoylanilide hydroxamic acid (SAHA) (Sigma-Aldrich, catalog number: SML0061)
   11. Dimethyl sulfoxide (DMSO) (Sigma-Aldrich, catalog number: D8418)
   12. 1x PBS (Mediatech, catalog number: 21-031-CV)
   13. MACSQuant Running buffer (Miltenyi Biotech, catalog number: 130-092-747)
   14. ApoTox-Glo™ Triplex Assay (Promega, catalog number: G6320)
   15. RPMI medium (see Recipes)
   16. TNFα stock solution (see Recipes)
   17. JQ1 stock solution (see Recipes)
   18. Prostratin stock solution (see Recipes)
   19. Suberoylanilide hydroxamic acid (SAHA) stock solution (see Recipes)

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Equipment

1. Pipette
2. Biosafety cabinet level 2
3. CO_2 tissue culture incubator (Thermo Electron, model: Forma™ Steri-Cult™ CO_2 Incubators, catalog number: 3307)
4. Tabletop centrifuge (Beckman Coulter, model: Allegra X-14R) for 96-well plates
5. MACSQuant VYB FACS analyzer (Miltenyi Biotech, model: MACSOuant® VYB, catalog number: 130-096-116)
6. SpectraMax MiniMax™ 300 Imaging Cytometer (Molecular Devices, model: SpectraMax MiniMax 300)

Software

1. FlowJo 9.9 or never (Tree Star)

Procedure

A. A2 and A72 J-Lat suspension cell culture
   1. A2 and A72 cells are cultured in RPMI medium (supplemented with 10% FBS, 1% L-glutamine and 1% penicillin/streptomycin) in 75 cm^2 tissue culture flasks in 40 ml medium. The flasks are placed horizontally to increase the surface area in which the cells are grown.
   2. Cells are split every 1-2 days and should be kept at a concentration of 2 x 10^5/ml-1.5 x 10^6/ml.
   3. One day before drug treatment split A2 or A72 cells to 5 x 10^5/ml.

B. Analysis of HIV-1 LTR transcriptional activation by flow cytometry
   1. Count the cells and adjust cell number with new media. About 2 x 10^5/well of A2 or A72 cells are plated in 96-well tissue culture plates in 195 µl RPMI medium (supplemented with 10% FBS, 1% L-glutamine and 1% penicillin/streptomycin). For each condition perform three replicates.
   2. Cells are stimulated with 0.016-10 ng/ml of TNFα, 100 nM-1 µM JQ1, 100 nM-2.5 µM Prostratin, 330 nM-2.5 µM SAHA, DMSO control, or left untreated. If treated with TNFα use PBS or water as control, depending on what was used as diluent. All drugs and controls are pipetted in 5 µl to reach a total volume of 200 µl/well. Mix cells and drugs by pipetting up and down at least 3 times.
   3. Culture in a CO_2 incubator at 37 °C for 18-48 h.
   4. Take the plate from the incubator and centrifuge at room temperature for 2 min at 1341 *x g* (2,400 rpm) (Tabletop centrifuge, Beckman).
   5. Then proceed directly to flow cytometry analysis. Cells do not have to be washed or fixed.

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Notes:

a. *For flow cytometry, MACSQuant VYB FACS analyzer was used to run 96-well plates. However, other flow cytometers such as Calibur or LSRII are also suitable for these experiments.*

b. *If the analysis of cell viability by forward scatter (FSC) vs. side scatter (SSC) is not sufficient for accurate assessment of drug toxicity in drug studies, one of a variety of dyes/stains; for example 7AAD, Propidium iodide or one of the Zombie viability dyes can be used according to manufacturer's instructions in the flow cytometric analysis in this protocol.*

6. Viability, cytotoxicity and apoptosis was measured with ApoTox-Glo™ Triplex Assay (Promega) according to manufacturer's instructions using a SpectraMax MiniMax™ 300 Imaging Cytometer (Figure 1).

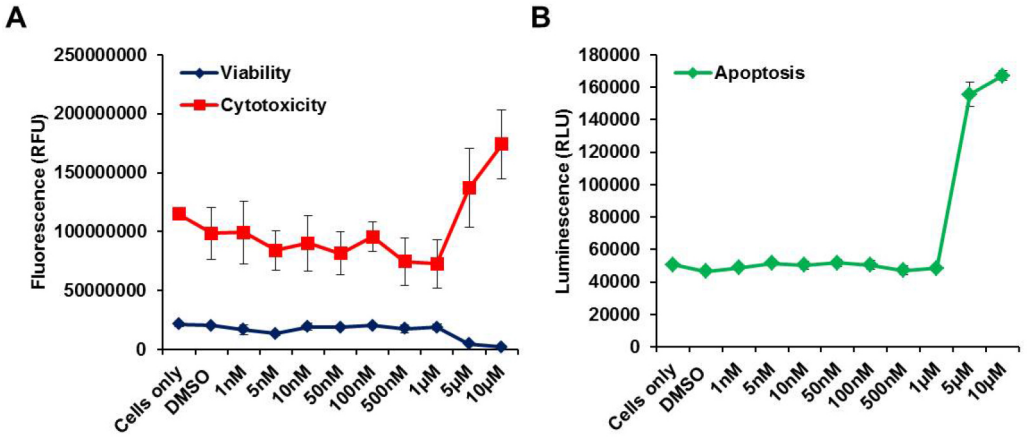

Figure 1. Measurement of Viability, Cytotoxicity and Apoptosis of drug treated cells. ApoTox-GloTM Triplex Assays (Promega) were performed in drug-treated A72 J-Lat cells. A. Cytotoxicity and Viability; B. Apoptosis; All measurements were repeated at least three times and average of three technical replicates (± SD) is shown.

Data analysis

Analysis of HIV-1 LTR transcriptional activation by flow cytometry (Figure 2)

1. First, set the gate on live J-Lat cells. Cell viability is monitored by forward (FSC-Area) and side scatter (SSC-Area) analysis (Figure 2A).
2. Gate on singlets (FSC-Height vs. FSC-Area) (Figure 2B).
3. Set the gate on SSC-Area and GFP/FITC-Area to identify the amount of GFP^+ cells (Figure 2C).
4. Each sample is usually analyzed in triplicate and the experiment is performed with cells coming from at least 3 independent experiments (Figure 3). Three replicates are averaged by calculating (GFP^+ cells Experiment 1 + GFP^+ cells Experiment 2 + GFP^+ cells Experiment 3)/3. Also calculate standard deviation (STDEV) for error bars.

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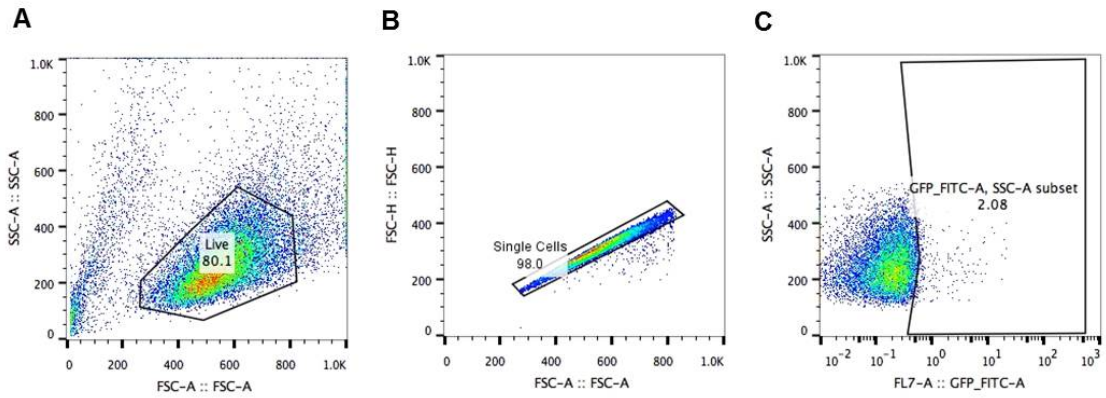

Figure 2. Analysis of HIV-1 LTR transcriptional activation by flow cytometry. Gating strategy to analyze A2 or A72 J-Lat cells: A. gating on live J-Lat cells based on size (FSC-Area) and granularity (SSC-Area); B. singlets gate (FSC-Height vs. FSC-Area); C. gating on GFP$^+$ J-Lat cells (SSC-Area vs. GFP-FITC-Area).

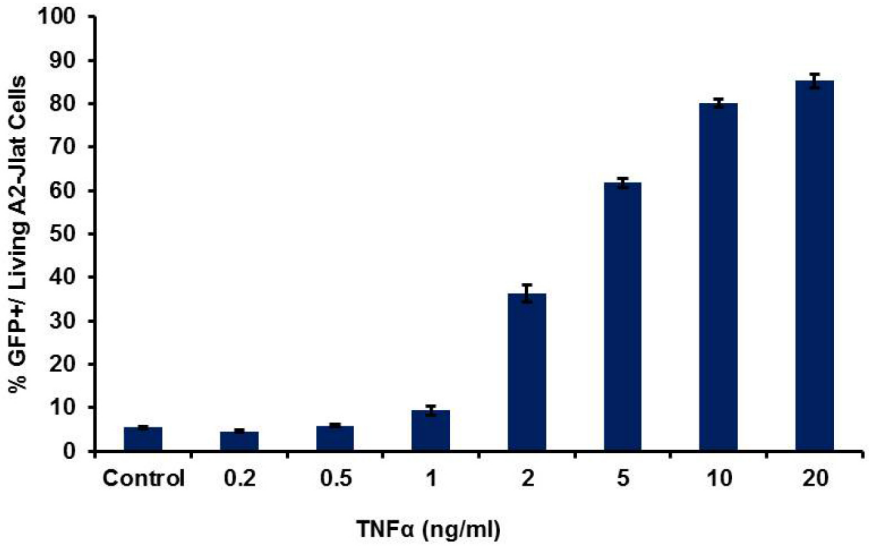

Figure 3. HIV-1 LTR transcriptional activation by flow cytometry. Typical results obtained for 18 h treatment with TNFα, with dose dependent response using A2 J-Lat cells. Average for percentage of GFP$^+$ cells from three replicates (± SD) is shown.

Recipes

1. RPMI medium
   RPMI supplemented with,
   10% FBS
   1% L-glutamine
   1% penicillin/streptomycin
   Store at 4 °C

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2. TNFα stock solution
   100 ng/µl in sterile water
   Store at -80 °C
3. JQ1 stock solution
   10 mM in DMSO
   Store at -80 °C
   Note: Avoid repeated freeze-thaws!
4. Prostratin stock solution
   5 mM in sterile water
   Store at -20 °C
5. Suberoylanilide hydroxamic acid (SAHA) stock solution
   10 mM in DMSO
   Store at -20 °C

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