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(54) **MULTI-COLOR REPORTER CELLS FOR DETECTING HIV-1**

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(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 278 days.

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(51) **Int. Cl.**

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G01N 33/569	(2006.01)
G01N 33/58	(2006.01)

(52) **U.S. Cl.**

CPC ***C12Q 1/04*** (2013.01); ***G01N 33/56988*** (2013.01); ***G01N 33/582*** (2013.01); *G01N 2333/16* (2013.01)

(58) **Field of Classification Search**

None

See application file for complete search history.

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(57) **ABSTRACT**

Disclosed are methods to measure the extent of viral infection and methods to measure latent viral reservoir in cells harboring or suspected of harboring such a reservoir.

18 Claims, 14 Drawing Sheets

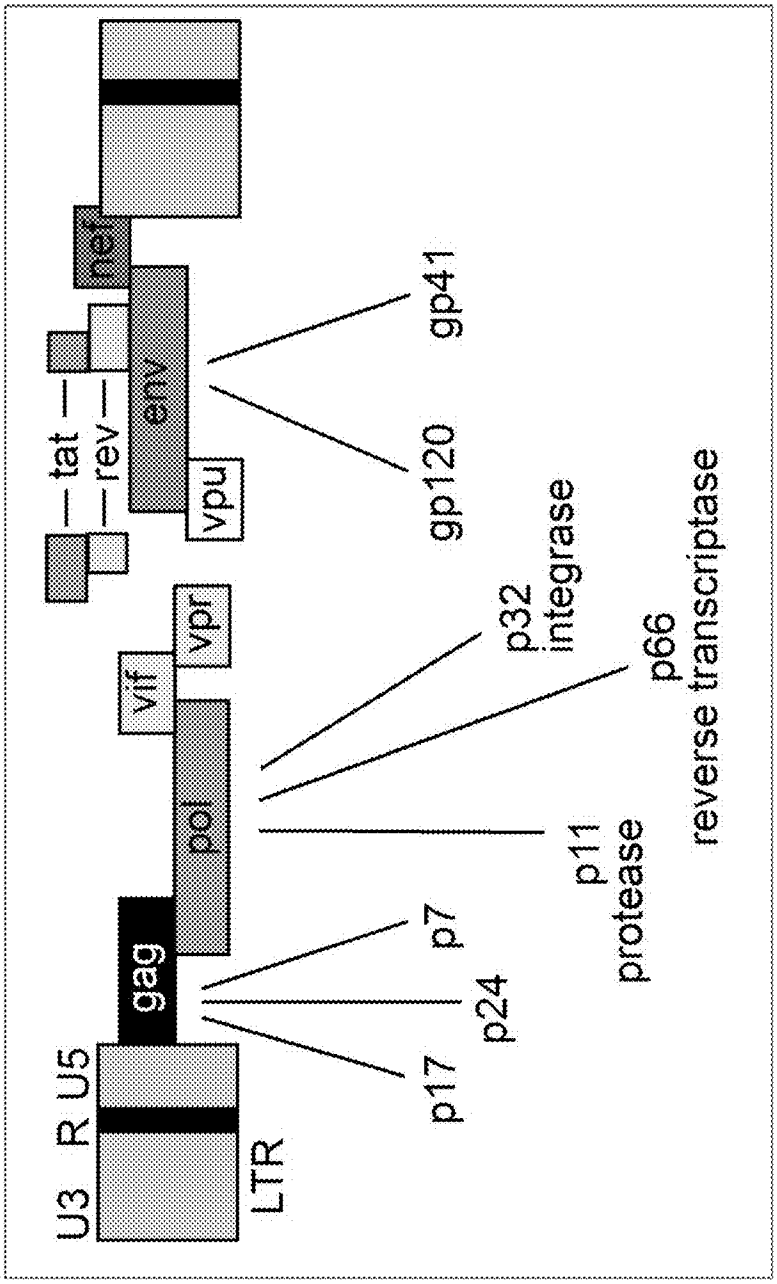
U3
R
U5
LTR
gag
pol
vif
vpr
tat
rev
env
vpu
nef
p17
p24
p7
p11
protease
p32
integrase
p66
reverse transcriptase
gp120
gp41

FIG. 1

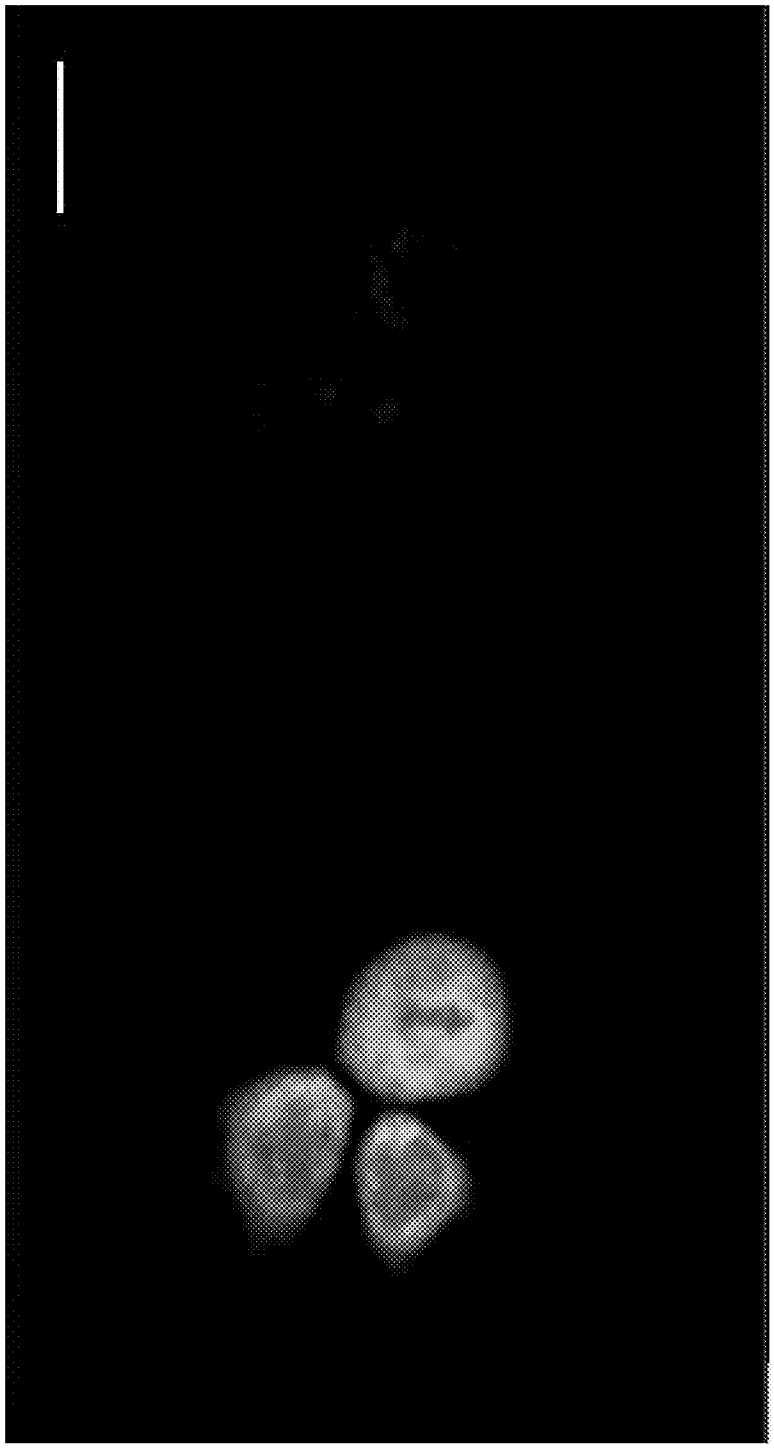

FIG. 2A

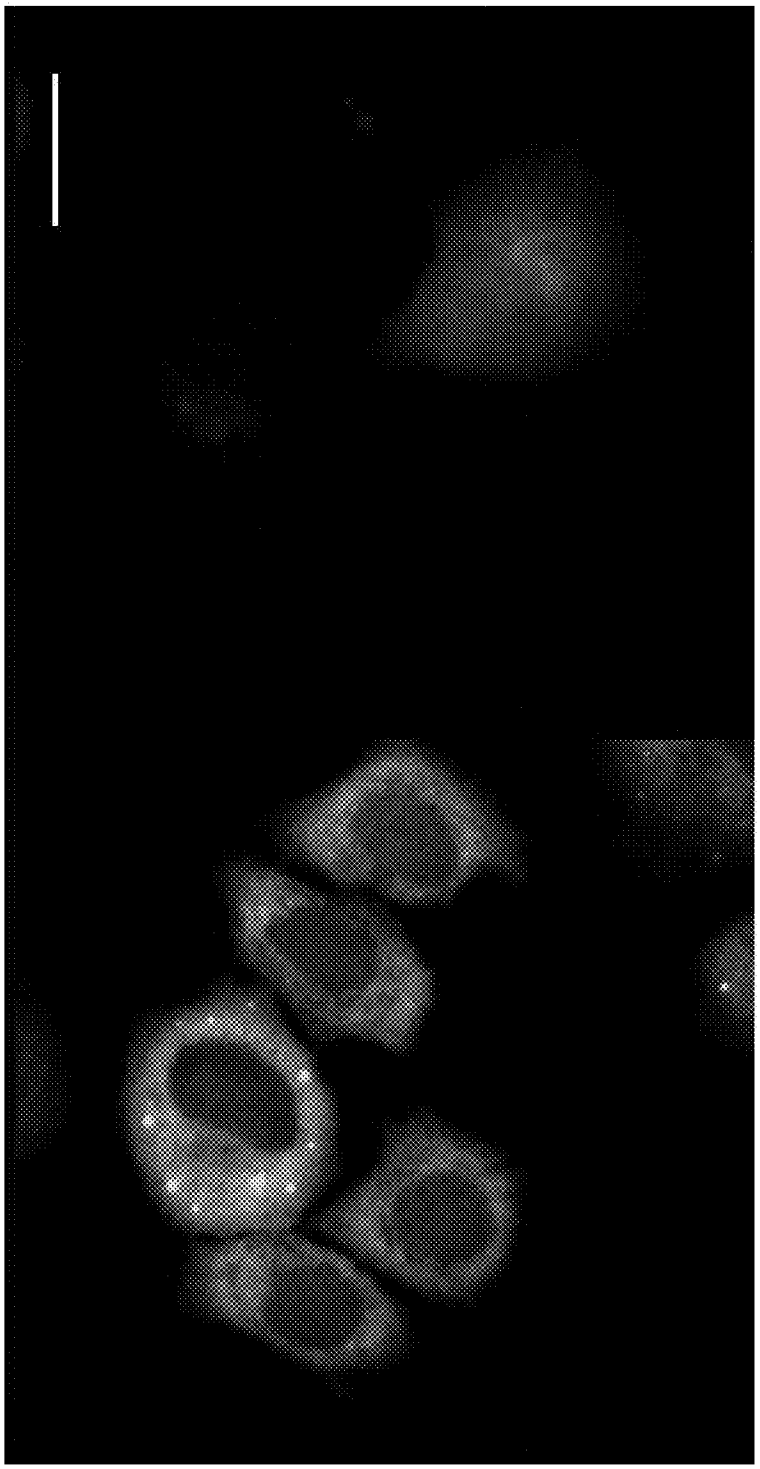

FIG. 2B

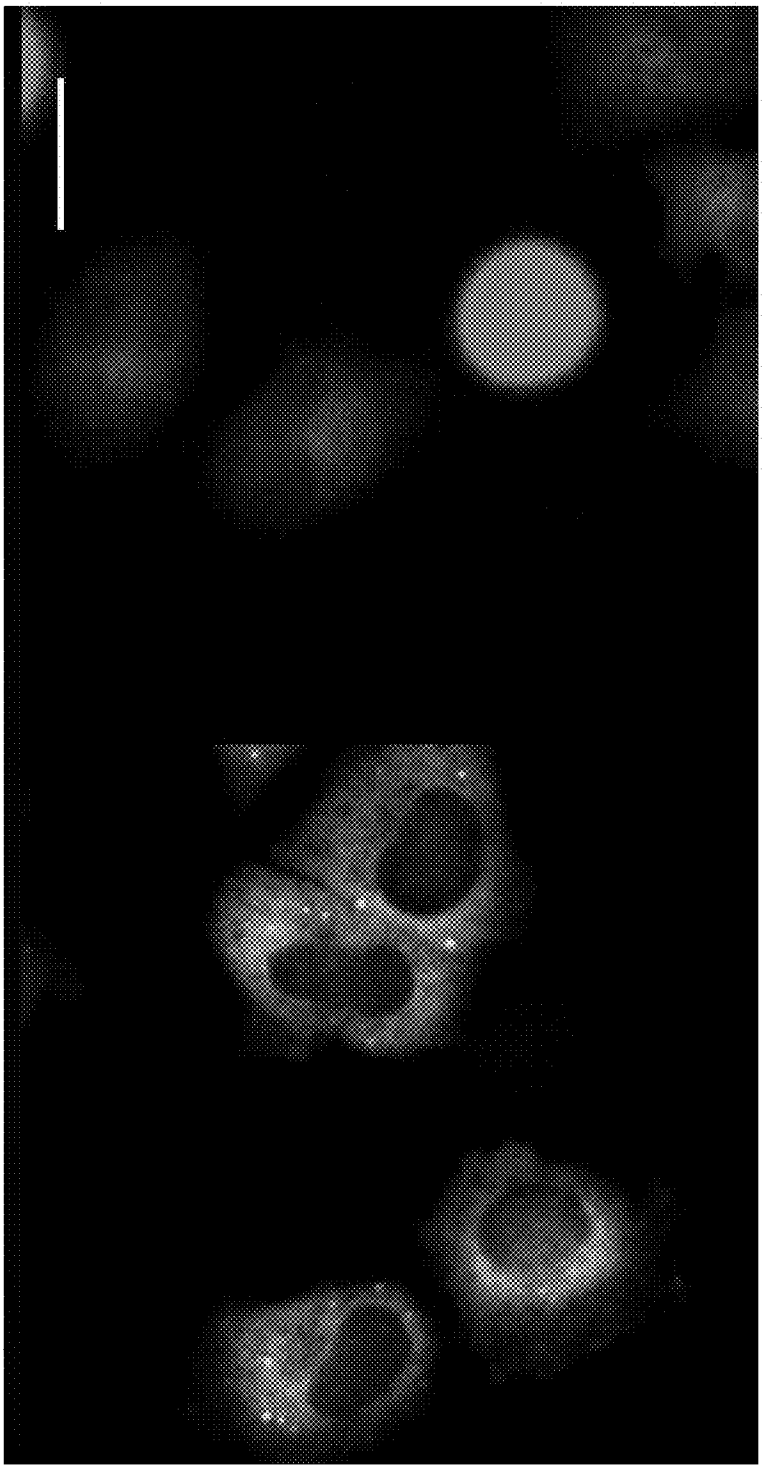

FIG. 2C

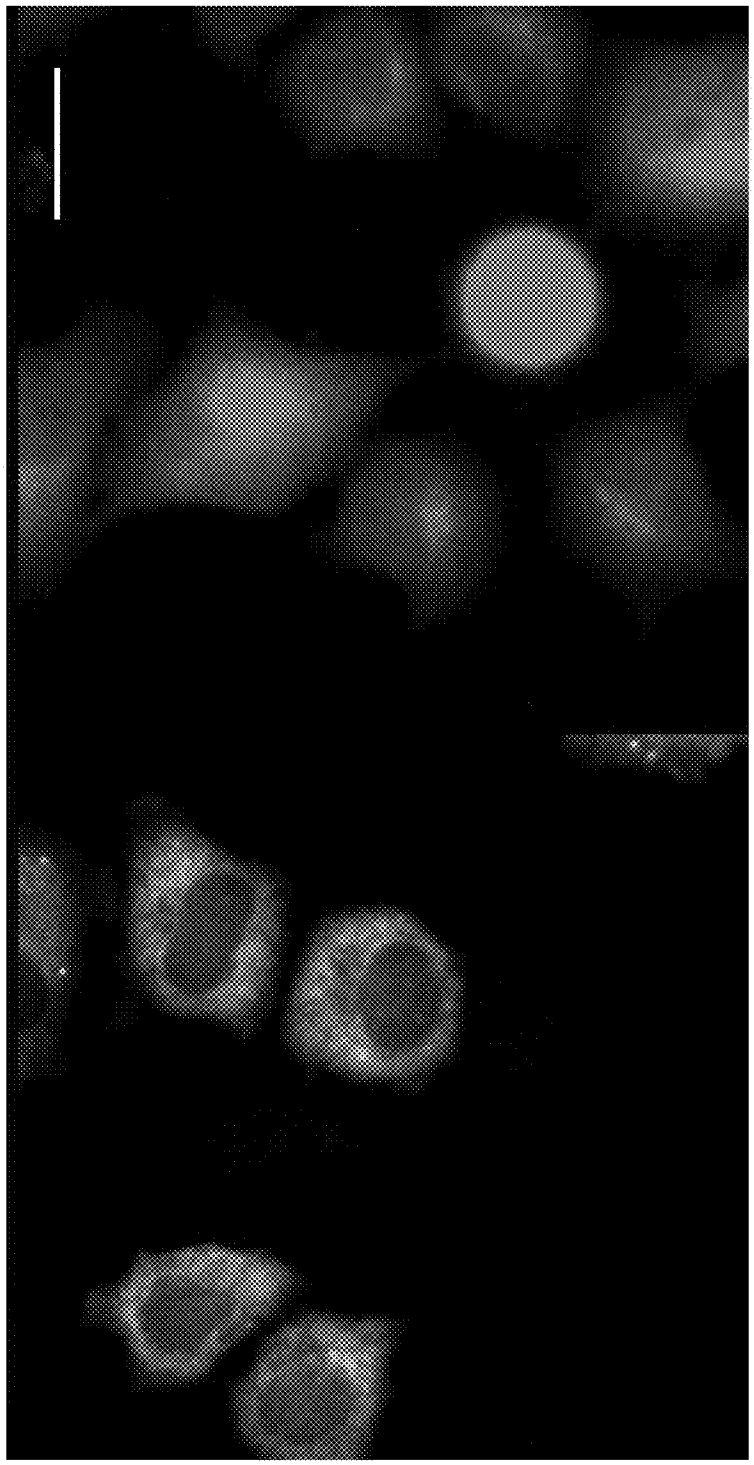

FIG. 2D

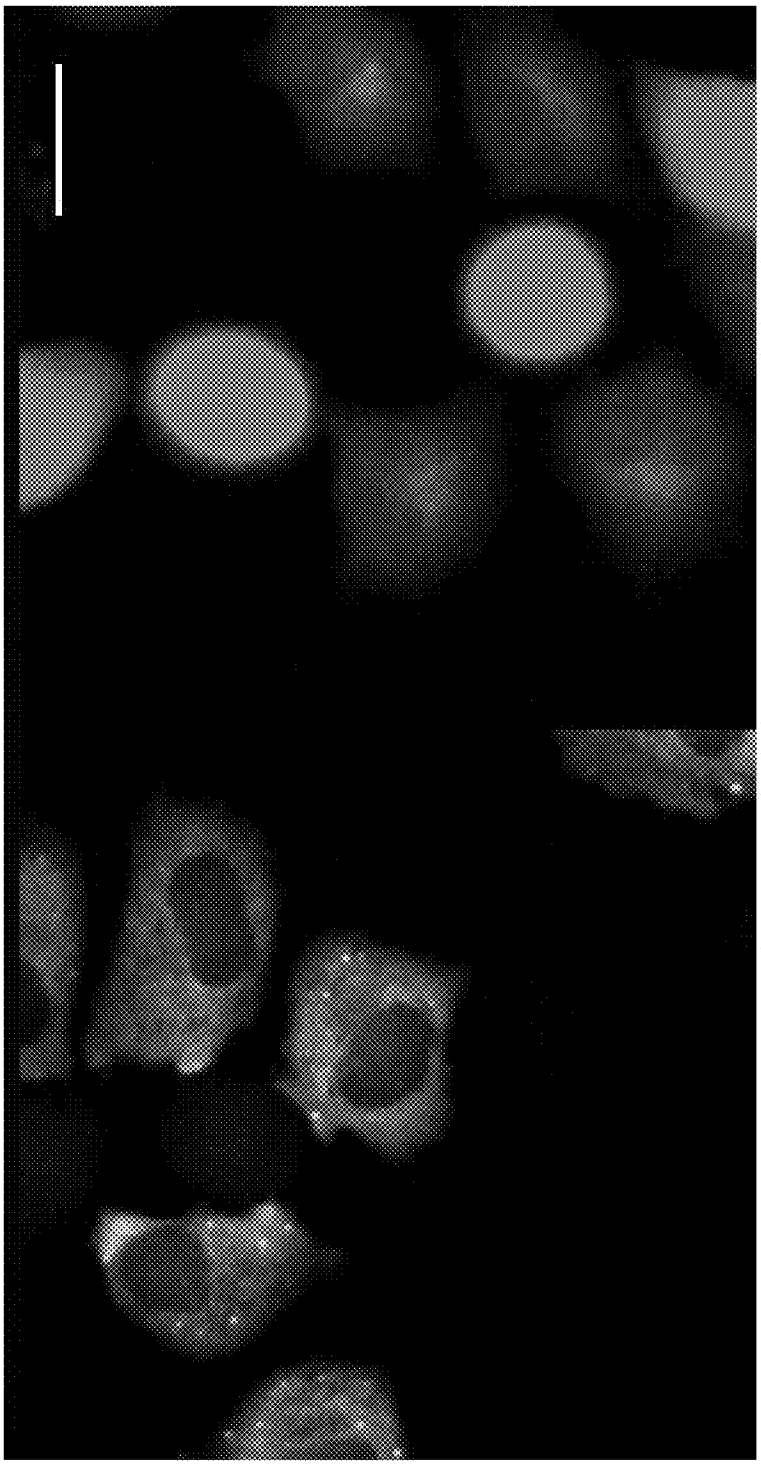

FIG. 2E

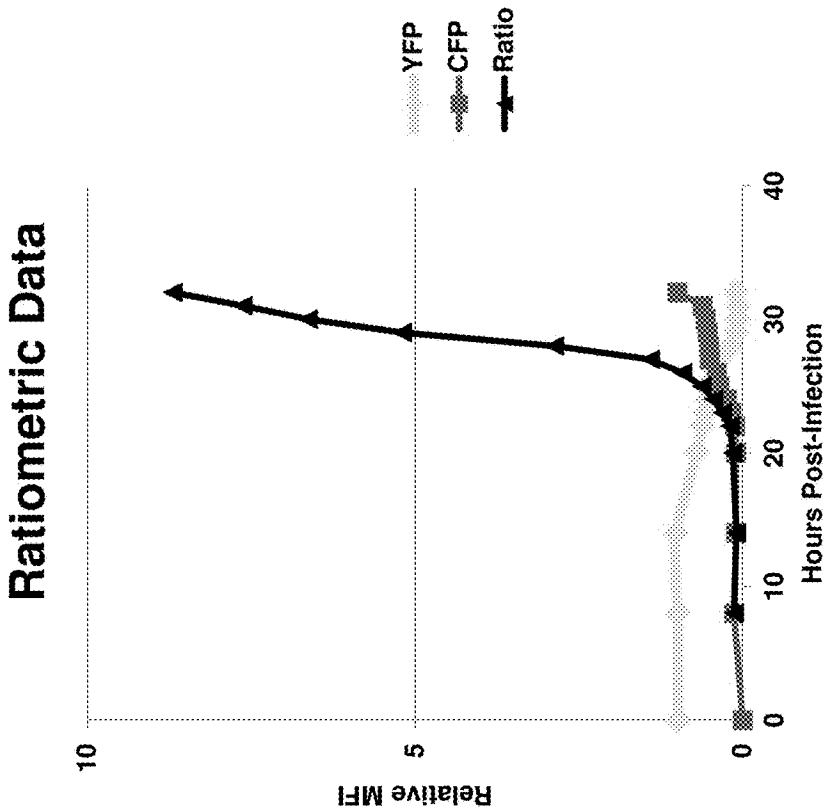
Ratiometric Data
YFP
CFP
Ratio
Relative MFI
10
5
0
0
10
20
30
40
Hours Post-Infection

FIG. 3B

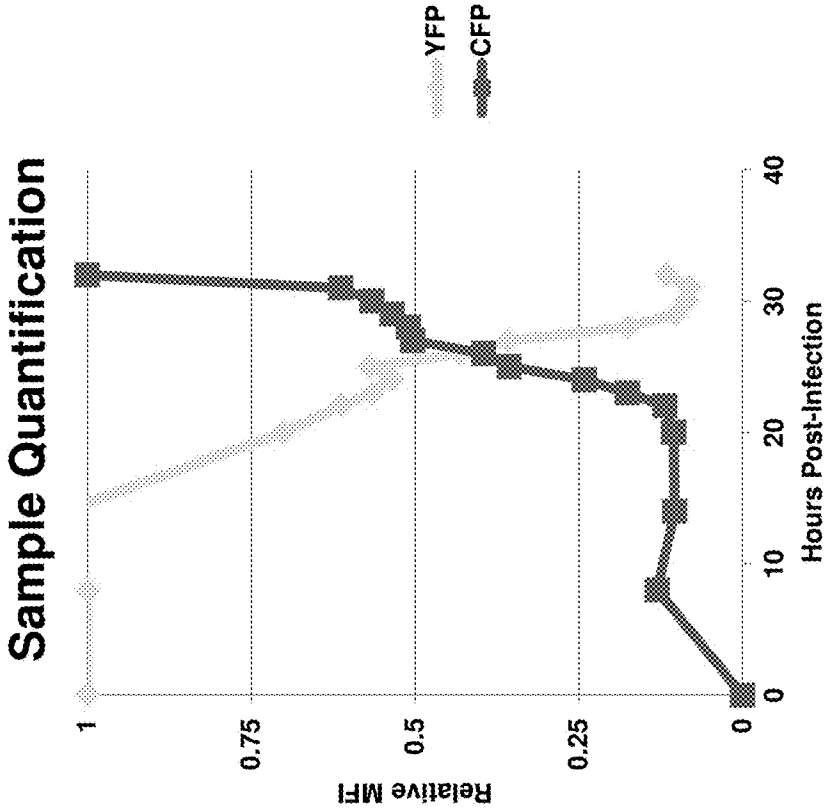
Sample Quantification
YFP
CFP
Relative MFI
1
0.75
0.5
0.25
0
0
10
20
30
40
Hours Post-Infection

FIG. 3A

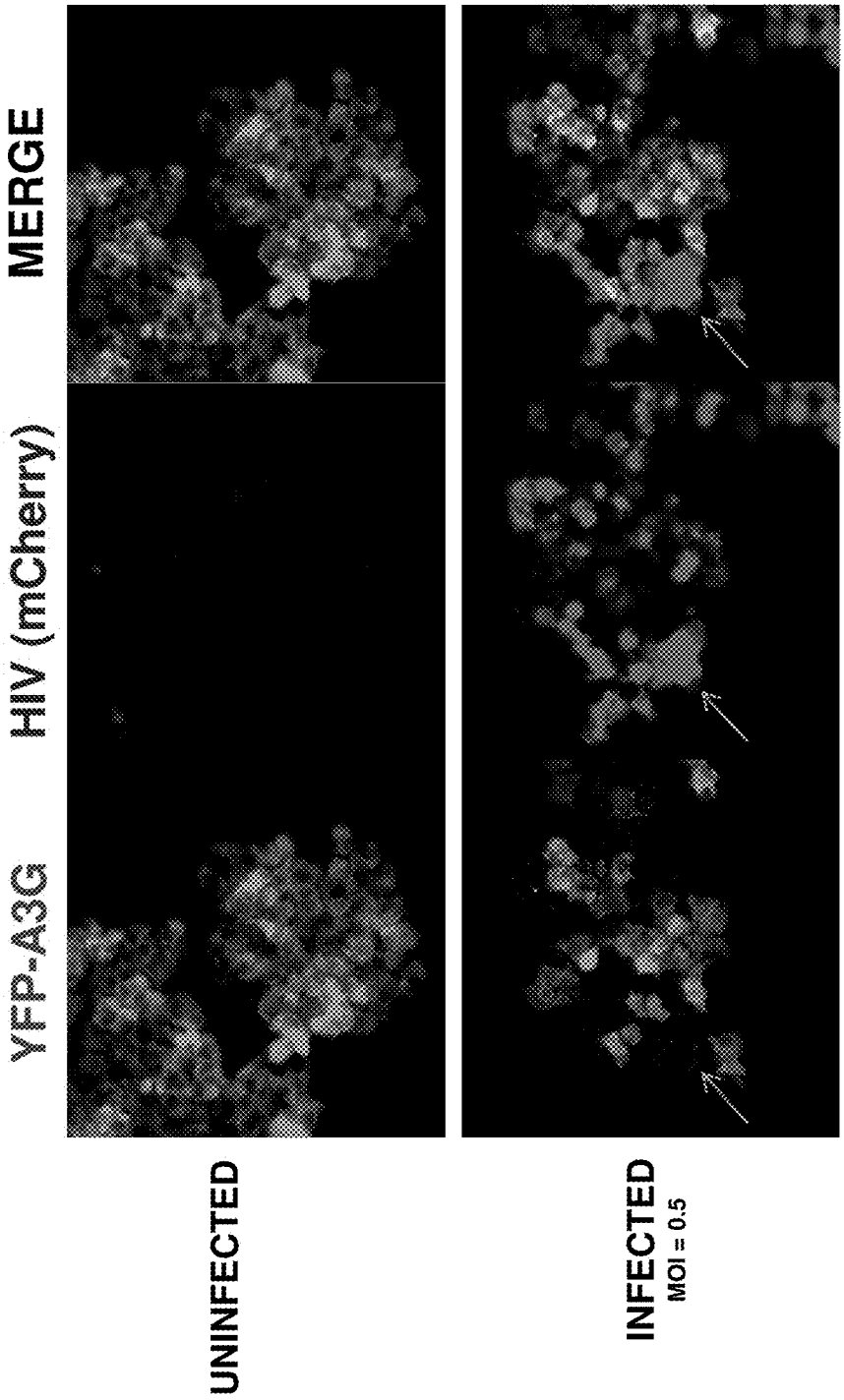

FIG. 4

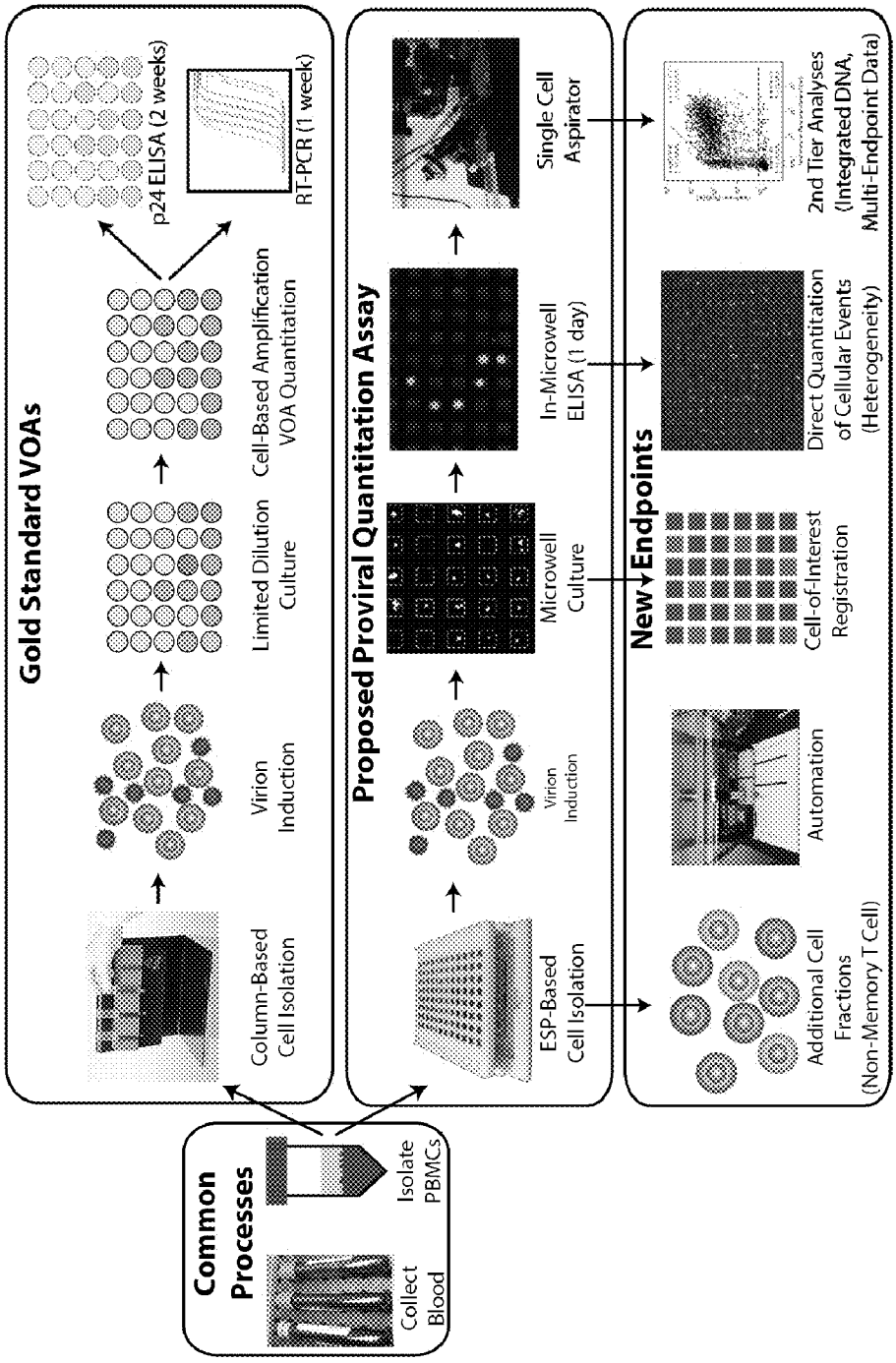
Common Processes
Collect Blood
Isolate PBMCs
Gold Standard VOAs
Column-Based Cell Isolation
Virion Induction
Limited Dilution Culture
Cell-Based Amplification VOA Quantitation
p24 ELISA (2 weeks)
RT-PCR (1 week)
Proposed Proviral Quantitation Assay
ESP-Based Cell Isolation
Virion Induction
Microwell Culture
In-Microwell ELISA (1 day)
Single Cell Aspirator
New Endpoints
Additional Cell Fractions (Non-Memory T Cell)
Automation
Cell-of-Interest Registration
Direct Quantitation of Cellular Events (Heterogeneity)
2nd Tier Analyses (Integrated DNA, Multi-Endpoint Data)

FIG. 5

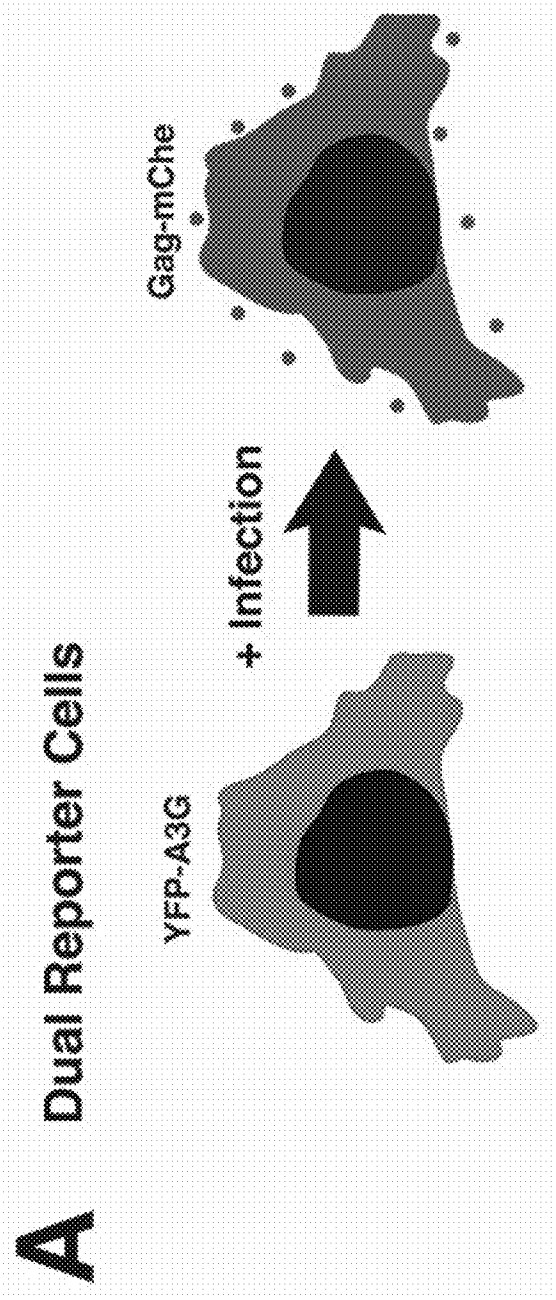

FIG. 6A

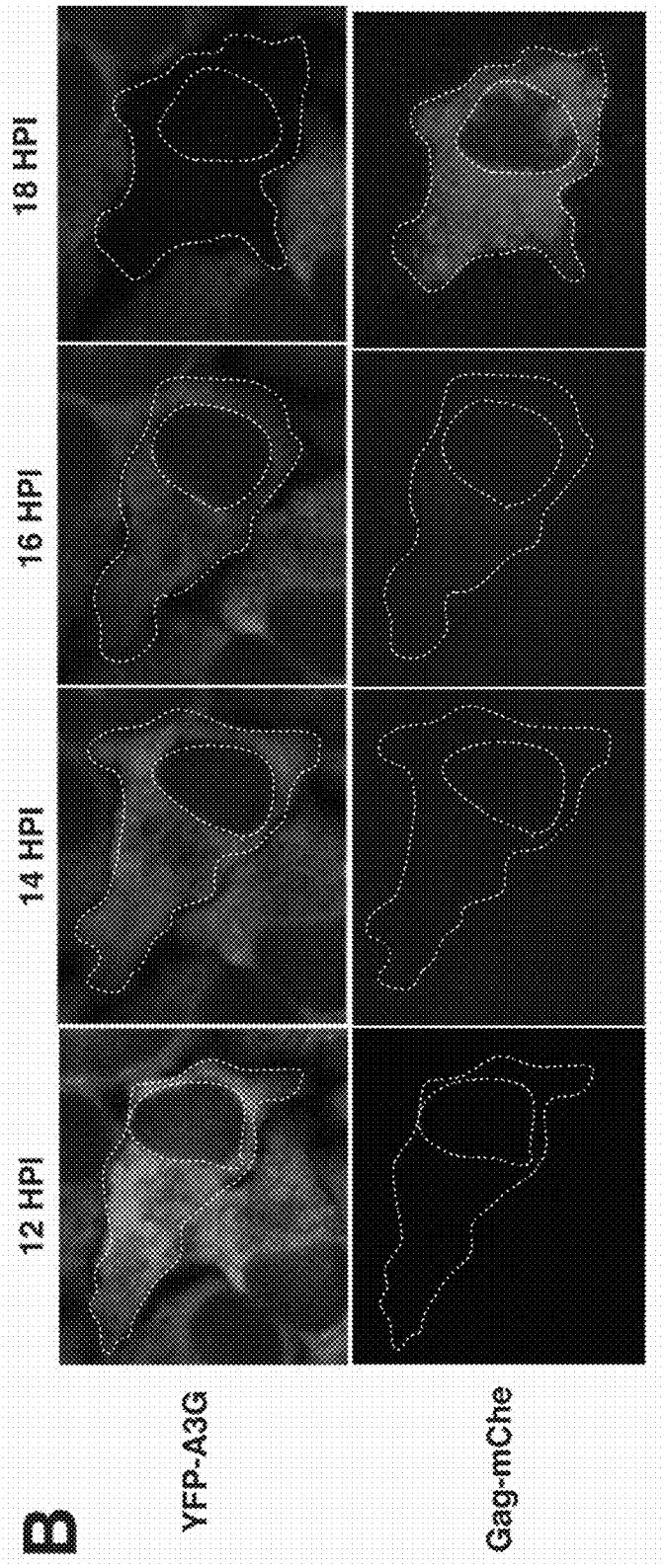

FIG. 6B

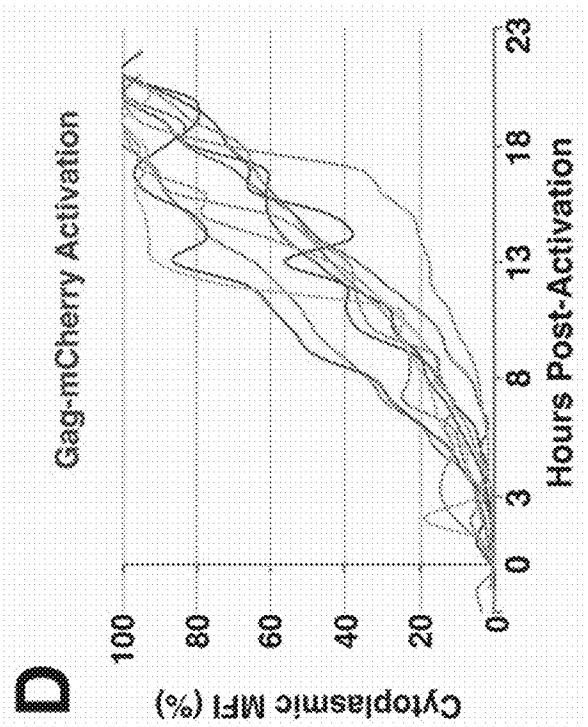
D
Gag-mCherry Activation
Cytoplasmic MFI (%)
100
80
60
40
20
0
0
3
8
13
18
23
Hours Post-Activation

FIG. 6D

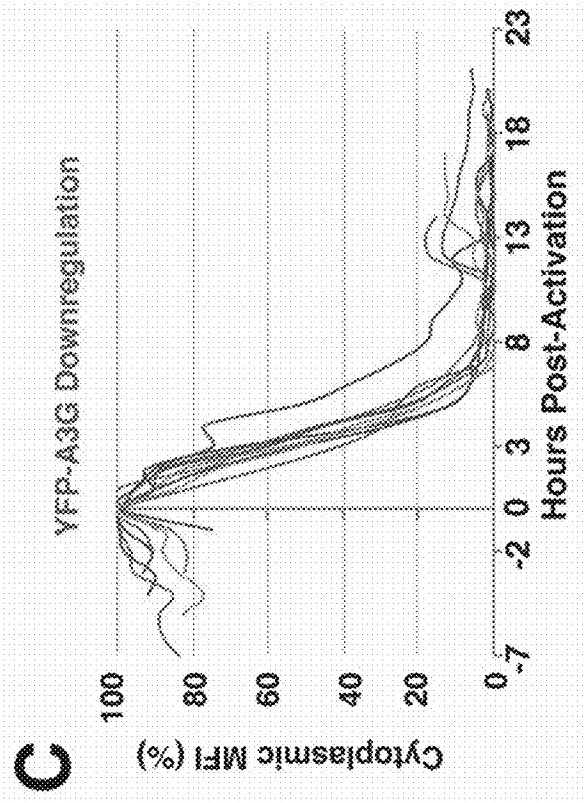
C
YFP-A3G Downregulation
Cytoplasmic MFI (%)
100
80
60
40
20
0
-7
-2
0
3
8
13
18
23
Hours Post-Activation

FIG. 6C

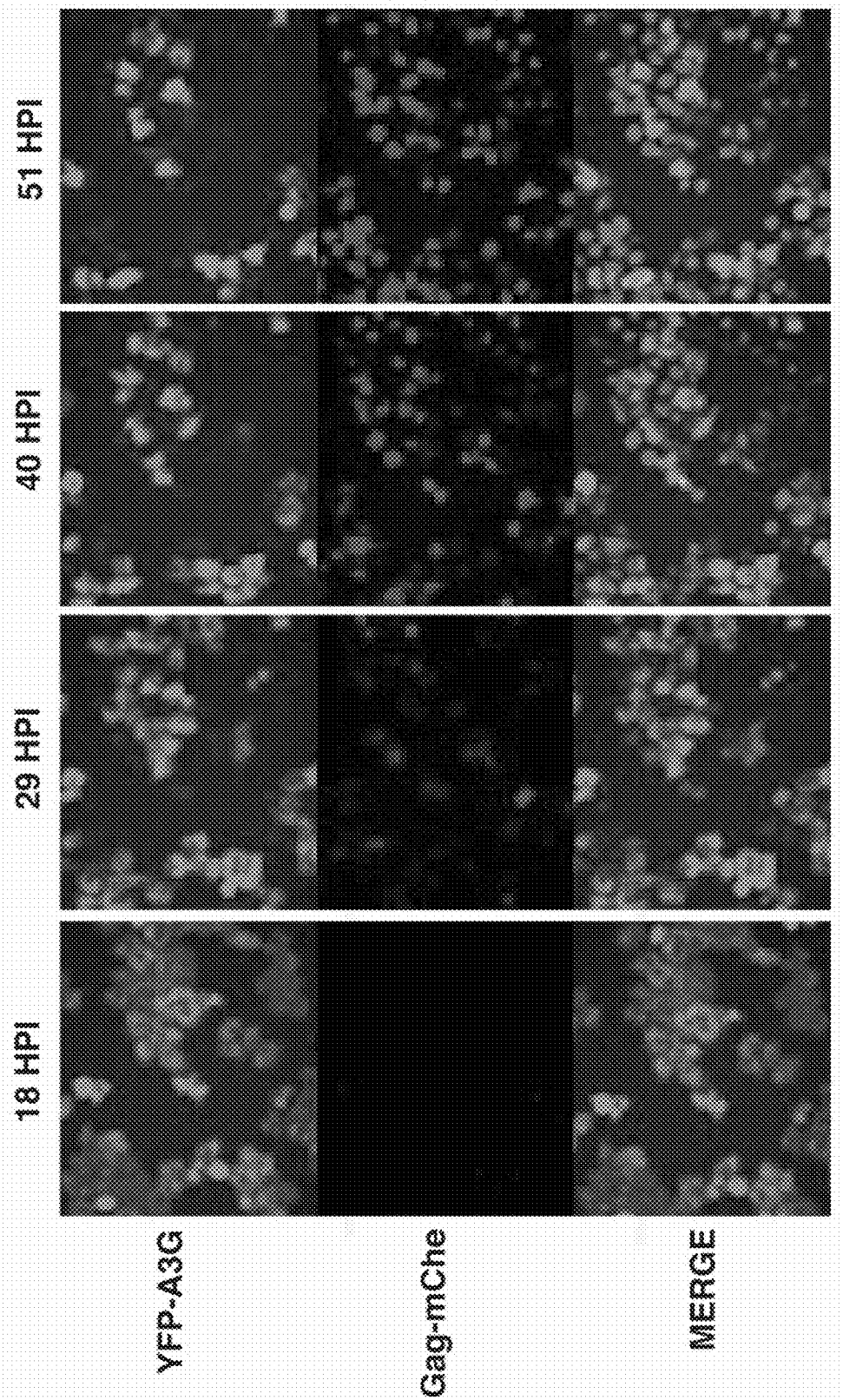

FIG. 7A

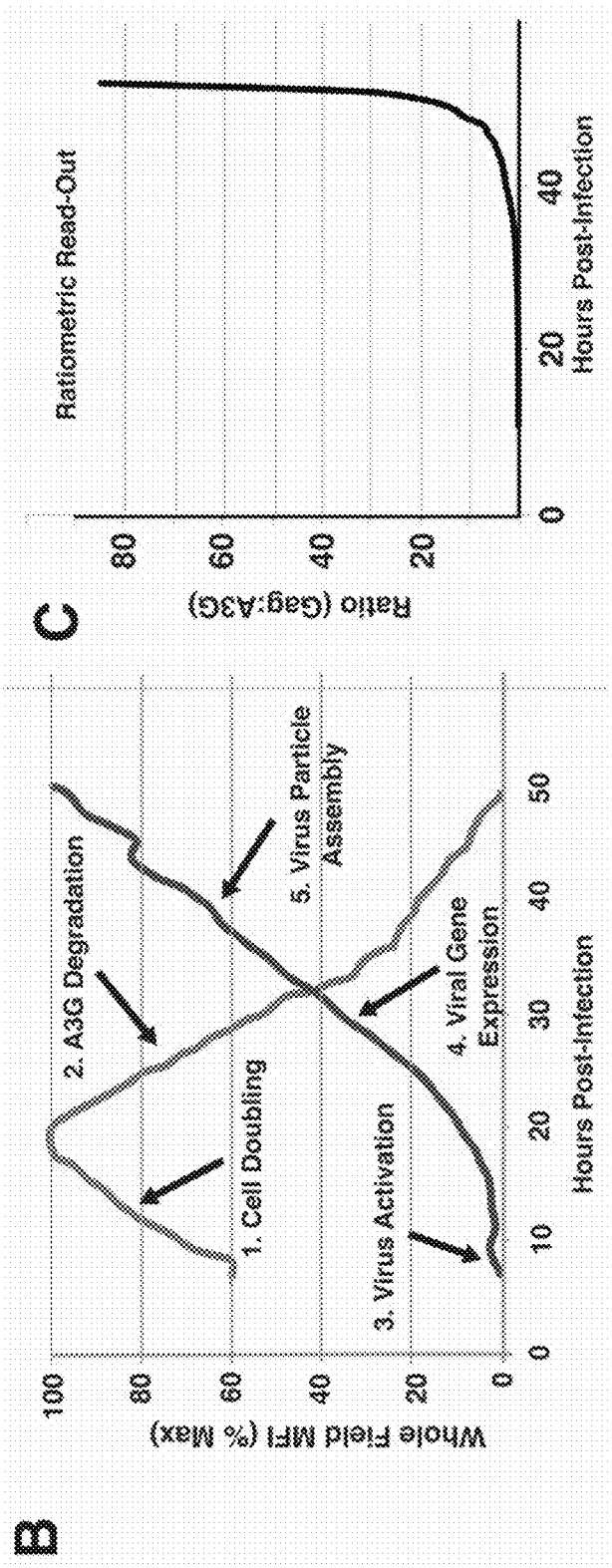
B
Whole Field MFI (% Max)
100
80
60
40
20
0
0
10
20
30
40
50
Hours Post-Infection
1. Cell Doubling
2. A3G Degradation
3. Virus Activation
4. Viral Gene Expression
5. Virus Particle Assembly
C
Ratio (Gag:A3G)
80
60
40
20
0
20
40
Hours Post-Infection
Ratiometric Read-Out

FIG. 7B

FIG. 7C

MULTI-COLOR REPORTER CELLS FOR DETECTING HIV-1

CROSS-REFERENCE TO RELATED APPLICATIONS

Priority is hereby claimed to provisional application Ser. No. 62/369,908, filed Aug. 2, 2016, which is incorporated herein by reference.

FEDERAL FUNDING STATEMENT

This invention was made with government support under AI110221 awarded by the National Institutes of Health. The government has certain rights in the invention.

BACKGROUND

The human immunodeficiency type 1 (HIV-1) infects more than 34 million people worldwide and contributes significantly to global morbidity due to HIV/AIDS. HIV-1 establishes a lifelong infection for which there is no cure. Like all viruses, HIV-1 spreads infection among cells, tissues, and people in the form of nanometer-scale particles known as virions. While virions can transmit infection in cell-free form, it has been demonstrated that the spread of retroviral infection is much more efficient when infected cells can form tight, physical cell-to-cell interfaces known as virological synapses (VS's). VS-mediated spread may be the predominant mode of HIV spread in vivo.

A persistent problem in treating HIV invention and perhaps eventually eradicating the virus is that the virus can exist in a latent form, known as the latent HIV reservoir or simply the viral reservoir. In short, HIV-1 is able to persist in a non-virulent, non-pathogenic form by integrating its genome into the host cell DNA. Integration allows the viral genome to remain in the cell as it replicates (from generation to generation). Because there is no production of HIV virions during the latent stage, the dormant state is able to evade host immune responses that require epitopes/antigens to initiate their cascade. The integrated viral genome is replicated along with the cellular genome when the infected cell divides. The integration of the HIV-1 viral genome allows viral reproduction to initiate again later (such as when anti-retroviral chemotherapy is discontinued). The latent HIV reservoir thus consists of infected cells (typically memory T cells) in which HIV is able to persist in the latent phase even when the patient is on anti-retroviral therapy.

The current methods to measure quantitatively the latent HIV reservoir are difficult to perform, time-consuming, and expensive. The two most common assays are the quantitative viral outgrowth assay (QVOA) and Tat/Rev Induced Limiting Dilution Assay (TILDA). These two assays have been described in the scientific literature and will not be described in any detail here. Regarding QVOA, see Finzi et al (14 Nov. 1997) "Identification of a reservoir for HIV-1 in patients on highly active antiretroviral therapy," Science 278(5341):1295-1300. Regarding TILDA, see Procopio et al. (27 Jun. 2015) "A Novel Assay to Measure the Magnitude of the Inducible Viral Reservoir in HIV-infected Individuals," EBioMedicine 2(8):874-83.

These difficult assays are due to a host of factors inherent in the HIV infection process. For one, there are no known extracellular markers that are associated with latently infected cells. Additionally, the reservoir is established in the very early stages of HIV infection, but the exact timing remains unknown. See Palmer S. (2014) "HIV Cure 101: Challenges in identifying and targeting the HIV reservoir." *AIDS* 2014 20th International AIDS Conference. Latency occurs within infected cells prior to the onset of anti-retroviral chemotherapy; the reservoir once established is not eliminated by anti-retroviral chemotherapy. Chemotherapy does block reactivation of the latent reservoir by blocking the virion reproduction process. However, once chemotherapy is discontinued, the virus can and does reactivate. A means to detect and quantify the reservoir quickly, cheaply, and accurately is critical to evaluating the efficacy of new treatment protocols and putative cures. There is a long-felt and unmet need for a method to detect the presence of replication-competent HIV-1 provirus.

At least one fluorescent assay has been described in the literature for measuring when HIV-1 virions have entered a cell. See Campbell, Perez, Melar, and Hope (10 Apr. 2007) "Labeling HIV-1 virions with two fluorescent proteins allows identification of virions that have productively entered the target cell," *Virology*. 360(2): 286-293. In this approach, HIV-1 virions were dual-labeled with GFP. Briefly, a fluorescent protein fusion was targeted to the plasma membrane by the addition of the N-terminal 15 amino acid sequence of c-Src (S15). This construct was efficiently packaged into HIV virions. Using fluorescent proteins fused to this sequence, the authors generated virions dually labeled with S15-mCherry and GFP-Vpr. The loss of the S15-mCherry membrane signal can be tracked following fusion. That is, after infection with labeled HIV virions, the authors found a measurable, specific loss of membrane label during infection. This loss of fluorescence was not observed when fusion is prevented using bafilomycin A. The assay thus discriminates between non-productively endocytosed virions and those HIV virions actively undergoing steps in the infectious process.

SUMMARY

Disclosed herein is a method of using multicolor fluorescent reporter cells in a simple, cost-effect, HIV-1 quantitative viral outgrowth assay (Q-VOA). The assay reliably monitors latent virus reservoir size in HIV-1-infected human subjects and non-human primate models of HIV/AIDS. The method uses cells and viruses labeled with at least two, at least three, at least four, at least five, or more than five different fluorescent reporter molecules whose respective fluorescence signals are generated and/or extinguished differentially. By examining the ratios of signals generated by the two or more reporter molecules, the progress of HIV-1 assembly and propagation can be followed via microscopic video. The multicolor fluorescence-based cell readouts respond to HIV-1 infection with high sensitivity and without the need for chemical substrates, thus representing a significant improvement over existing HIV-1 GFP- or chemiluminescent-based single reporter lines. A fundamental benefit of the method derives from coupling at least one first fluorescent marker that turns "on" (i.e., a fluorescent marker that is activated) in response to HIV-1 infection (for example, via a response to HIV-1 Rev or Tat) with at least one second fluorescent marker that is turned "off" (i.e., a fluorescent marker that is deactivated) by an HIV-1 immune modulatory protein (for example Vif, Vpu, or Nef). "Off" markers include fluorescent versions of host proteins APOBEC3G, BST2/Tetherin, and CD4 that are rapidly degraded by HIV-1 Vif, Vpu, and Nef, respectively. Calculating net "on/off" ratios over time for up to five independent fluorescent signals provides for extremely high sensitivity. The method is capable of identifying viral isolates that are prone to

latency; that is, viral isolates with reduced Rev or Tat activities. The method is also capable of identifying viral isolates that exhibit suppressor phenotypes (e.g., suboptimal Vif, Vpu, or Nef activities). The HIV-1-responsive reporters may be modified so as to amplify signals more rapidly due to virus-induced cell-cell signaling (paracrine reporters).

The method may be structured in any suitable format, including as a micro-fluidic addressed array.

Without being limited to any underlying mechanism or biological phenomena, the inventors hypothesize that the efficiency of spreading infection reflects the coordination of viral, cellular and extracellular matrix (ECM) factors that regulate the efficiency of virological synapse formation and turnover. The present method thus provides a novel and inventive, quantitative, live-cell imaging platform based on video microscopy and fluorescent retroviruses and/or reporter cells. The method enables direct, real-time monitoring of the biogenesis and transmission of HIV-1 at single cell resolution and over multiple rounds of viral replication. The method is capable of measuring, quantitatively, the latent HIV-1 reservoir in HIV-1-infected humans and in non-human primate models of HIV/AIDS.

Measuring the latent HIV-1 reservoir size is a critical first step in evaluating whether any putative treatment actually reduces the reservoir size. Large reservoirs lead to rapid viral rebound with even brief antiviral therapy interruptions. HIV chemotherapy management is difficult; treatment failure occurs often despite intensive patient monitoring. It is estimated that half of the new HIV infections in the US are from patients who know they are HIV-positive but underestimate their own transmissability. Measuring the latent HIV reservoir in these individuals will reduce the incidence of new HIV infections. Actually reducing the HIV-1 latent reservoir will result in better long-term outcomes for patients, reduce the odds of spreading the infection, and, ideally, provide a baseline for engineering an effective cure strategy.

The latent reservoir size varies several logs between patients. Thus, a method that accurately measures the latent reservoir is key. The reservoir can shrink markedly during drug-based antiretroviral therapy. The latent viral reservoir of one HIV patient (Timothy Ray Brown, known as The "Berlin Patient") has been successfully eradicated. See Hütter, G; Nowak, D; Mossner, M; et al. (2009) "Long-Term Control of HIV by CCR5 Delta32/Delta32 Stem-Cell Transplantation," *New England Journal of Medicine* 360(7):692-8. In short, advances in reservoir monitoring are needed so that patient-specific measurements can guide decision-making.

Numerical ranges as used herein are intended to include every number and subset of numbers contained within that range, whether specifically disclosed or not. Further, these numerical ranges should be construed as providing support for a claim directed to any number or subset of numbers in that range. For example, a disclosure of from 1 to 10 should be construed as supporting a range of from 2 to 8, from 3 to 7, from 1 to 9, from 3.6 to 4.6, from 3.5 to 9.9, and so forth.

All references to singular characteristics or limitations of the present method shall include the corresponding plural characteristic or limitation, and vice-versa, unless otherwise specified or clearly implied to the contrary by the context in which the reference is made.

All combinations of method or process steps as used herein can be performed in any order, unless otherwise specified or clearly implied to the contrary by the context in which the referenced combination is made.

The methods disclosed herein can comprise, consist of, or consist essentially of the elements and limitations of the method described herein, as well as any additional or optional ingredients, components, or limitations described herein or otherwise useful in biochemistry and the imaging of live cells using confocal microscopy and fluorescent reporter molecules.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** is a schematic diagram of the genome of HIV-1.

FIGS. **2**A, **2**B, **2**C, **2**D, and **2**E are a series of time-lapse photomicrographs depicting ratiometric/multicolor HIV-1 reporter cells that combine reporter activation and reporter degradation to achieve a high signal-to-noise read-out of infection. As reporter 1 (blue) is activated by Tat/Rev, reporter 2 (green) is deactivated (degraded) by Vif, Elapsed time between FIG. **2**A and FIG. **2**E is 12 hours. Images generated from VSVg pseudotyped HIV-1 cyan fluorescent protein (CFP) expressed from Nef position.

FIG. **3**A is a graph showing the deactivation of reporter 2 superimposed upon the activation of reporter 1 over time. Yellow diamonds are reporter 2 (yellow fluorescent protein; YFP); blue squares are reporter 1 (cyan fluorescent protein; CFP). The graph thus depicts the rate of YFP-A3G (yellow) downregulation and HIV-1 (blue) upregulation over 35 hours of infection. FIG. **3**B is a corresponding graph that presents the same data as in FIG. **3**A, with the addition of a trace showing the ratio between the signal from reporters 1 and 2 (black triangles). As shown in FIG. **3**B, using the ratio of activation:degradation fluorescence provides ~10-fold increase to sensitivity of the assay.

FIG. **4** is a pair of photomicrographs comparing the assay results in uninfected cells (top panel) and 24 hours after exposure to HIV in infected cells (bottom panel). As shown in the bottom panel of the figure, at 24 hours post-infection, the activation signal (reporter 1; red) is robust, while the degradation signal (reporter 2; green) is near background. The HIV-1-infected cells (which fluoresce brightly red) are clearly differentiated from the uninfected cells (which do not fluoresce red). In other words, when uninfected (top panel), cells are green due to YFP-APOBEC3G expression. Upon infection, the cells turn on a mCherry reporter (red) that responds to Rev and Tat, while the YFP-A3G (green) signal disappears due to Vif activity.

FIG. **5** is a schematic diagram contrasting conventional quantitative viral outgrowth assays (top panel) versus the method disclosed herein.

FIG. **6**A is a schematic diagram of a HIV-susceptible HeLa or Jurkat cell constitutively expressing YFP-tagged APOBEC3G (YFP-A3G; green) and encoding an HIV-responsive reporter gene expressing HIV Gag fused to mCherry (Gag-mChe; red).

FIG. **6**B is a series of photomicrographs depicting productive infection of HeLa cells. As shown in the top series of photos, infection leads to the downregulation of YFP-A3G (green) due to expression of the viral Vif protein that degraded YFP-A3G. Simultaneously, as shown in the bottom series of photos, infection leads to activation of Gag-mCherry (red) gene expression due to HIV Tat and Rev activity. "HPI" means "hours post-infection."

FIG. **6**C is a graph depicting quantification of YFP-A3G downregulation kinetics for 10 cells based on measurements of cytoplasmic mean fluorescence intensity (MFI) over time.

FIG. **6**D is a graph depicting quantification of viral activation kinetics for the same cells shown in FIG. **6**C and tracking measurements of Gag-mChe expression.

FIG. 7A is a series of photomicrographs showing whole-field read-outs using multicolor reporter cells. Shown here are HeLa reporter cells constitutively expressing YFP-tagged APOBEC3G (YFP-A3G; green) and encoding an HIV-responsive reporter gene expressing HIV Gag fused to mCherry (Gag-mChe; red) tracked over 51 hours post-infection ("HPI'). Approximately 50% of the cells were infected and turned from green to red.

FIG. 7B is a graph depicting whole-field mean fluorescence intensity (MFI) as a function of time (hours post-infection). Useful features of the cells are that they provide dynamic, whole-field measures of YFP-A3G and Gag-mChe fluorescence signatures allowing for direct measurements of: (1) cell doubling rate; (2) A3G downregulation kinetics; (3) viral gene activation; (4) rates of viral late gene expression; and (5) onset of virus particle production (based on changes to cell granularity).

FIG. 7C is a graph depicting the ratio Gag:A3G. This graph illustrates another useful feature of the cells is that signal-to-noise can be reduced through ratioing the relative measurements of YFP-A3G and Gag-mCherry signals.

DETAILED DESCRIPTION

Disclosed herein are reporter cell lines having two or more differentially detectable fluorescent labels or reporter molecules for detecting and studying patient-derived viruses. These highly sensitive and specific reporter cell lines are suitable for automated detection of HIV in a microfluidic chamber. Accordingly, disclosed herein are 2-, 3-, 4, 5- and more than 5-color fluorescence based cell read-outs that respond robustly to HIV-1 infection. The cells and the method that uses them are thus useful both for tracking the spread of HIV-1 infection but are also ideal for implementation into an automated Q-VOA assay.

The reporter cells are based on coupling fluorescent markers that are turned "on" in response to HIV infection by viral trans-acting factors (e.g., Rev and Tat) to markers turned "off" by the virus (e.g., YFP-tagged versions of the antiviral proteins APOBEC3G or BST2/Tetherin that are rapidly degraded by the viral proteins Vif or Vpu). Calculating net "on/off" ratios over time, relative to standards, allows for high sensitivity and favorable signal-to-noise. The ability to amplify response signals with minimal background and without the need for chemical substrates thus represents a significant improvement over existing HIV-1 GFP- or chemiluminescent-based single reporter lines.

Thus, a first version of the method is a method of tracking viral infection in a cell. The method comprises providing a cell susceptible to infection by a virus in which the cell contains at least two differentially labeled proteins: a first protein labeled with a first fluorophore whose signal is up-regulated upon infection of the cell by the virus and a second protein labeled with a second fluorophore that is down-regulated upon infection of the cell by the virus. The cell is exposed to the virus under conditions where the virus can infect the cells. Fluorescent signals generated by the first fluorophore and the second fluorophore are measured. The extent of infection of the cell by the virus is determined by comparing the fluorescent signals measured in the test cells with corresponding signals generated in a corresponding control cell not exposed to the virus. Significant changes in the signals generated by the fluorophores indicates infection of the cells by the virus.

The virus may be an HIV virus, such as HIV-1 virus or a related non-human primate virus, such as variants of simian immunodeficiency virus (SIV). The cell may be a human T-cell or a non-human primate cell. For example, the cell may be a HeLa cell or a Jurkat cell.

The first protein may be a reporter responsive to HIV Tat (trans-activator of transcription) or HIV Rev. The second protein may be any or all of A3G (apolipoprotein B mRNA editing enzyme), BST2 (bone marrow stromal antigen 2, also known as tetherin), CD4 (cluster of differentiation 4), and SERINC5 (serine incorporator 5).

Optionally, the cell may contain a third protein labeled with a third fluorophore and wherein the third fluorophore generates a fluorescent signal that is distinct from and can be detected independently of the signals generated by the first and second fluorophores, and wherein fluorescent signals generated from the third fluorophore are detectably altered upon infection of the cell with the virus.

Optionally, the cell may contain a fourth protein labeled with a fourth fluorophore and wherein the fourth fluorophore generates a fluorescent signal that is distinct from and can be detected independently of the signals generated by the first, second, and third fluorophores, and wherein fluorescent signals generated from the fourth fluorophore are detectably altered upon infection of the cell with the virus.

Optionally, the cell may contain a fifth protein labeled with a fifth fluorophore and wherein the fifth fluorophore generates a fluorescent signal that is distinct from and can be detected independently of the signals generated by the first, second, third, and fourth fluorophores, and wherein fluorescent signals generated from the fifth fluorophore are detectably altered upon infection of the cell with the virus.

Another version of the method is a method of quantitating a latent viral reservoir of a cell suspected of being infected with a latent virus. Here, the method comprises providing a cell suspected of harboring a latent viral reservoir in which the cell contains at least two differentially labeled proteins: a first protein labeled with a first fluorophore whose signal is up-regulated upon activation of virus in the latent viral reservoir and a second protein labeled with a second fluorophore that is down-regulated upon activation of virus in the latent viral reservoir. The cell is exposed to an agent capable of inducing activation of the latent virus. Fluorescent signals generated by the first fluorophore and the second fluorophore are measured. The latent viral reservoir in the cell is quantitated by comparing the fluorescent signals measured in step (c) with corresponding signals generated in a corresponding cell known not to contain a latent viral reservoir and exposed to the same agent capable of inducing activation of the latent virus.

The latent viral reservoir may comprise a latent HIV virus, such as latent HIV-1 virus.

The cell may be a human T-cell. The cell may be a non-human primate cell.

The first protein may be a reporter responsive to Tat (trans-activator of transcription) or a reporter responsive to Rev. The second protein may be A3G, BST2, CD4, and SERINC5.

Optionally, the cell contains a third protein labeled with a third fluorophore and wherein the third fluorophore generates a fluorescent signal that is distinct from and can be detected independently of the signals generated by the first and second fluorophores, and wherein fluorescent signals generated from the third fluorophore are detectably altered upon activation of the latent viral reservoir.

Optionally, the cell contains a fourth protein labeled with a fourth fluorophore and wherein the fourth fluorophore generates a fluorescent signal that is distinct from and can be detected independently of the signals generated by the first, second, and third fluorophores, and wherein fluorescent

signals generated from the fourth fluorophore are detectably altered activation of the latent viral reservoir.

Optionally, the cell contains a fifth protein labeled with a fifth fluorophore and wherein the fifth fluorophore generates a fluorescent signal that is distinct from and can be detected independently of the signals generated by the first, second, third, and fourth fluorophores, and wherein fluorescent signals generated from the fifth fluorophore are detectably altered upon activation of the latent viral reservoir.

Delving specifically into the machinations of retroviruses, in addition to AIDS, the retroviruses human immunodeficiency virus type 1 (HIV-1) and human T lymphotropic virus type 1 (HTLV-1) also cause human cancers. These retroviruses exploit cell-cell contacts to enhance the spread of persistence of infection in lymphocytes See, for example, Mothes W, Sherer N M, Jin J, Zhong P. (September 2010) "Virus cell-to-cell transmission," *J Virol.* 84(17):8360-8368. All retroviruses encode three polyproteins required for infectious virion production: Group specific antigen (Gag), Polymerase (Pol) and Envelope (Env). See Petropoulos C. "Retroviral Taxonomy, Protein Structures, Sequences, and Genetic Maps," © 1997, Cold Spring Harbor Laboratory Press (Cold Spring Harbor, N.Y., USA). Available online at http://www.ncbi.nlm.nih.gov/books/NBK19417. HIV-1 and other complex retroviruses such HTLV-1 also encode additional proteins that regulate viral gene expression and antagonize the host immune response. See Malim M H, Bieniasz P D. (May 2012) "HIV Restriction Factors and Mechanisms of Evasion," *Cold Spring Harb Perspect Med.* 2(5):a006940; Nekhai S, Jeang K-T (December 2006) "Transcriptional and post-transcriptional regulation of HIV-1 gene expression: role of cellular factors for Tat and Rev," *Future Microbiol.* 1(4):417-26; and Bindhu M, Nair A, Lairmore M D (September 2004) "Role of accessory proteins of HTLV-1 in viral replication, T cell activation, and cellular gene expression," *Front Biosci J Virtual Libr.* 1; 9:2556-2576.

Specifically relevant to the present method is that HIV-1 encodes two gene regulatory factors, Rev and Tat, and four immunomodulatory factors: Vif, Vpr, Vpu, and Nef. See FIG. **1**, which is a schematic diagram of the HIV-1 genome. Gag is the viral assembly machine that, for "C-type" retroviruses such as HIV-1, HTLV-1 and the simple onco-retrovirus murine leukemia virus (MLV), drives virus particle biogenesis at the plasma membrane. In this process, Gag encapsidates two copies of the viral genomic RNA (gRNA) and acquires a protective, cell-derived lipid envelope bilayer. Ono A. (2009) "HIV-1 Assembly at the Plasma Membrane: Gag Trafficking and Localization," *Future Virol.* 4(3):241-57. Although Gag expression is sufficient to generate virus-like particles in the absence of other viral factors, Pol, Env, and two copies of gRNA must be incorporated into virions in order for them to be infectious. Pol is delivered to virion assembly sites in the form of Gag-Pol fusion proteins that, during virion maturation, are cleaved to generate the viral enzymes Protease, Reverse Transcriptase and Integrase. See Hill M, Tachedjian G, Mak J. (January 2005) "The packaging and maturation of the HIV-1 Pol proteins," *Curr HIV Res.* 3(1):73-85. Envelope (Env) glycoproteins are type I transmembrane proteins that mediate viral entry. Env is cleaved in the endoplasmic reticulum to generate transmembrane (TM) and surface (SU) subunits prior to delivery to assembly sites through the secretory pathway in the form of SU-TM trimers. Checkley M A, Luttge B G, Freed E Q. (July 2011) "HIV-1 envelope glycoprotein biosynthesis, trafficking, and incorporation," *J Mol Biol.* 410(4):582-608.

This provides an opportunity to tag two or more of the proteins involved with HIV-1 infection and propagation to be tagged, and thereby to follow the fate of the viral genomic RNA. The method is capable of directly monitoring how gRNAs, Gag, and Env are coordinately trafficked to the plasma membrane. After virion assembly, budding, and release, Env trimers must bind to both CD4 receptors and a chemokine co-receptor (CCR5 or CXCR4) found on the surface of susceptible T cells and macrophages. Coreceptor binding triggers conformational changes in Env resulting in virion-cell membrane fusion and delivery of viral capsids to the target cell cytoplasm prior to reverse transcription of a viral cDNA and integration of the DNA provirus into the host cellular chromatin. For many retroviruses, proviral integration leads to gene dysregulation associated with the evolution of cancer. HIV-1's integration into resting memory T cells serves as the basis for establishing the long-term latent viral reservoir that leads to AIDS. See Finzi D, Blankson J, Siliciano J D, Margolick J B, Chadwick K, Pierson T, et al. (1999): "Latent infection of CD4+ T cells provides a mechanism for lifelong persistence of HIV-1, even in patients on effective combination therapy," *Nat Med.* 5(5):512-7.

Most retroviruses are able to spread infection via cell-free virions, wherein infectious virions are released from cells to diffuse through the extracellular milieu prior to binding to uninfected target cells bearing appropriate entry receptors. However, spread of infection can be up to 10,000-fold more efficient when infected cells can form physical contacts with uninfected cells referred to as "virological synapses" (VS's). For HIV-1, VS formation is driven, in part, through interactions between viral Env glycoproteins on the infected cell surface and CD4 receptors on the uninfected cell. VS-mediated spread is likely advantageous to HIV-1 and other retroviruses for several reasons. First, contacts directly couple the processes of viral egress and entry in space and time, thereby providing a kinetic advantage. Second, large numbers of virions are transferred simultaneously from cell to cell at the VS (i.e., there is high multiplicity infection), providing a mechanism by which the virus may be able to overwhelm select antiviral factors. Third, some retroviruses have adapted more sophisticated, signal-based mechanisms of exploiting cell-cell contacts to enhance the spread of infection. For example, it has been shown that MLV Env glycoproteins, in addition to their role as fusion machines, function as cell surface adhesion factors that signal virion assembly to occur preferentially at the site of cell-cell contact. See Jin J, Sherer N M, Heidecker G, Derse D, Mothes W. (July 2009) "Assembly of the murine leukemia virus is directed towards sites of cell-cell contact," *PLoS Biol.* 7(7):e1000163. In preliminary data (not shown), the present inventors have demonstrated that the same is likely true for HIV-1 Env, although the mechanism is not yet understood.

Without being limited to any underlying mechanism, the present inventors hypothesize that Env has, for multiple viruses, evolved to serve as a critical "sensor" for detecting neighboring receptor-expressing cells, thus ensuring a rapid, directional flow of infectious material.

It is likely that HIV-1 exploits several different modes of transmission during acute infection in order to efficiently access susceptible CD4+ T cells in targeted lymphoid tissues including the gut-associated lymphoid tissue (GALT), spleen, and lymph nodes. These modes include cell-free transmission, VS-mediated transfer from infected and uninfected T cells, and trans-infection, a mode of spread wherein virions associate with the plasma membrane of migratory,

uninfected cells (typically dendritic cells) that subsequently deliver infectious virions to unsuspecting target cells during instances of transient cell-cell contact.

Ex vivo, the VS can protect HIV-1 transmission from neutralizing antibodies, antiviral drugs, or host restriction factors. However, the in vivo relevance of the VS to HIV-1 spread and persistence is still unclear. That said, there is growing evidence that ongoing replication continues in dense lymphoid tissues of infected individuals despite long-term therapy. Lorenzo-Redondo R, Fryer H R, Bedford T, Kim E-Y, Archer J, Kosakovsky Pond S L, et al. (February 2016) "Persistent HIV-1 replication maintains the tissue reservoir during therapy," *Nature*. 4; 530(7588):51-6. Moreover, multiple groups have shown that VS-mediated spread and trans-infection can occur in vivo using intravital imaging in infected mouse models.

Thus, disclosed herein is a quantitative virus outgrowth assay (Q-VOA) capable of detecting and measuring viral reservoirs in infected people. To test and optimize the cell lines for such an assay, de-identified CD4+ T cells isolated from long-term HIV-1 infected individuals who are on or off antiretroviral therapy, as well as from HIV-negative donors are obtained. These cells are then incubated with YFP-A3G/HIVmCherry reporter HeLa and/or Jurkat cells (see FIG. **5**) in 24-well glass bottomed dishes. Because a subset of cells may be latently infected (and quantitatively measuring the extent of the latent infection is the goal of the method), a subset of these cells will be treated with histone deacetylase (HDAC) inhibitors to activate latent virus prior to washing and adding reporter cells. Cultures will be monitored using an automated microscope with whole well images acquired every 24 hours to measure changes to mCherry/YFP expression ratios over time. The sensitivity of the assay is determined in co-cultures using spiked HIV-1 infected cells at quantities from one donor cell per 100,000 target cells to one donor per 20 million T cells. A cell line model for latency (e.g., ACH-2 that carries a single integrated provirus and is inducible with PMA to produce infectious HIV-1) will be used as a positive control. This reagent is available from the NIH AIDS Reagent Program.

The method described herein uses differential labeling with at least two different fluorophores to generate distinct signals in response to HIV-1 infection of a cell. The signals may be diametrically opposed (i.e., one is deactivated as the other is activated), but this is not required. The signals, however, must be distinguishable from one another, either by whether they are activated or deactivated in response to HIV-1 infection of the test cells or via the wavelengths at which they absorb and emit photons. It is preferred that the excitation and emission wavelengths are in the visible region, although this is not strictly required. Fluorophores that absorb or emit at wavelengths above and below the visible spectrum may be used. A very large number of suitable fluorophores are known and commercially available. The term "fluorophore" is used broadly herein to encompass any and all of them. An exemplary, non-limiting list fluorophores that may be used in the present method are contained in Table 1:

TABLE 1

Fluorophores that may be used in the present method:

Fluorophore	Absorption	Emission	Other info
1,5 IAEDANS	336	490	
1,8-ANS	372	480	
4-Methylumbelliferone	385	502	
5-carboxy-2,7-dichlorofluorescein	504	529	
5-Carboxyfluorescein (5-FAM)	492	518	
5-Carboxynapthofluorescein (pH 10)	512/598	563/668	Ratio Dye, pH
5-Carboxytetramethylrhodamine (5-TAMRA)	542	568	
5-FAM (5-Carboxyfluorescein)	492	518	
5-HAT (Hydroxy Tryptamine)	370-415	520-540	
5-Hydroxy Tryptamine (HAT)	370-415	520-540	
5-ROX (carboxy-X-rhodamine)	578	604	
	567	591	
5-TAMRA (5-Carboxytetramethylrhodamine)	548	552	
	542	568	
6-Carboxyrhodamine 6G	518	543	
6-CR 6G	518	543	
6-JOE	520	548	
7-Amino-4-methylcoumarin	351	430	
7-Aminoactinomycin D (7-AAD)	546	647	
7-Hydroxy-4-methylcoumarin	360	449, 455	
9-Amino-6-chloro-2-methoxyacridine	412, 430	471, 474	
ABQ	344	445	
Acid Fuchsin	540	630	
ACMA (9-Amino-6-chloro-2-methoxyacridine)	412, 430	471, 474	
Acridine Orange + DNA	502	526	
Acridine Orange + RNA	460	650	
Acridine Orange, both DNA & RNA	440-480	520-650	
Acridine Red	455-600	560-680	
Acridine Yellow	470	550	
Acriflavin	436	520	
Acriflavin Feulgen SITSA	355-425	460	
Aequorin (Photoprotein)		466	Photoprotein
AFPs-AutoFluorescent Protein-(Quantum Biotechnologies) see sgGFP, sgBFP			
Alexa Fluor 350 ™	346	442	
	342	441	

TABLE 1-continued

Fluorophores that may be used in the present method:

Fluorophore	Absorption	Emission	Other info
Alexa Fluor 430 ™	431	540	
Alexa Fluor 488 ™	495, 492	519, 520	
Alexa Fluor 532 ™	531, 532	553, 554	
Alexa Fluor 546 ™	556, 557	572, 573	
Alexa Fluor 568 ™	577,578	603	
Alexa Fluor 594 ™	590, 594	617, 618	
Alexa Fluor 633 ™	632	650	
Alexa Fluor 647 ™	647	666	
Alexa Fluor 660 ™	668	698	
Alexa Fluor 680 ™	679	702	
Alizarin Complexon	530-560, 580	580, 624-645	
Alizarin Red	530-560	580	
Allophycocyanin (APC)	630, 645	655, 660	
AMC, AMCA-S	345	445	
AMCA (Aminomethylcoumarin)	345	425	
	347	444	
AMCA-X	353	442	
Aminoactinomycin D	555	655	
Aminocoumarin	346	442	
	350	445	
Aminomethylcoumarin (AMCA)	345	425	
	347	444	
Anilin Blue		600	
Anthrocyl stearate	360-381	446	
APC (Allophycocyanin)	630, 645	655, 660	
APC-Cy7	625-650	755	
APTRA-BTC = Ratio Dye, Zn2+	466/380	520/530	Ratio Dye, Zn2+
APTS	424	505	
Astrazon Brilliant Red 4G	500	585	
Astrazon Orange R	470	540	
Astrazon Red 6B	520	595	
Astrazon Yellow 7 GLL	450	480	
Atabrine	436	490	
ATTO-TAG ™ CBQCA	465	560	
ATTO-TAG ™ FQ	486	591	
Auramine	460	550	
Aurophosphine G	450	580	
Aurophosphine	450-490	515	
BAO 9 (Bisaminophenyloxadiazole)	365	395	
BCECF (high pH)	492, 503	520, 528	
BCECF (low pH)	482	520	
Berberine Sulphate	430	550	
Beta Lactamase	409	447, 520	
BFP blue shifted GFP (Y66H) Blue Fluorescent Protein	381, 382, 383	445, 447, 448	blue shifted GFP (Y66H) Blue Fluorescent Protein
BFP/GFP FRET			
Bimane	398	490	
Bisbenzamide	360	461	
Bisbenzimide (Hoechst)	360	461	
bis-BTC = Ratio Dye, Zn2+	455/405	529/505	Ratio Dye, Zn2+
Blancophor FFG	390	470	
Blancophor SV	370	435	
BOBO ™-1	462	481	
BOBO ™-3	570	602	
Bodipy 492/515	490	515	
Bodipy 493/503	533	549	
Bodipy 500/510	509	515	
Bodipy 505/515	502	510	
Bodipy 530/550	528	547	
Bodipy 542/563	543	563	
Bodipy 558/568	558	569	
Bodipy 564/570	564	570	
Bodipy 576/589	579	590	
Bodipy 581/591	584	592	
Bodipy 630/650-X	625	642	
Bodipy 650/665-X	647	665	
Bodipy 665/676	605	676	
Bodipy Fl	504, 505	511, 513	
Bodipy FL ATP	505	514	
Bodipy Fl-Ceramide	505	511	
Bodipy R6G SE	528	547	
Bodipy TMR	542	574	
Bodipy TMR-X conjugate	544	573	
Bodipy TMR-X, SE	544	570	

TABLE 1-continued

Fluorophores that may be used in the present method:

Fluorophore	Absorption	Emission	Other info
Bodipy TR	589	617	
Bodipy TR ATP	591	620	
Bodipy TR-X SE	588	616	
BO-PRO ™-1	462	481	
BO-PRO ™-3	544	570	
Brilliant Sulphoflavin FF	430	520	
BTC-Ratio Dye Ca2+	464/401	533/529	Ratio Dye Ca2+
BTC-5N-atio Dye, Zn2+	459/417	517/532	Ratio Dye, Zn2+
Calcein	494	517	
Calcein Blue	373	440	
Calcium Crimson ™	588, 589	611, 615	
Calcium Green	501, 506	531	
Calcium Green-1 Ca2+ Dye	506	531	Ca2+ Dye
Calcium Green-2 Ca2+	506/503	536	Ca2+
Calcium Green-5N Ca2+	506	532	Ca2+
Calcium Green-C18 Ca2+	509	530	Ca2+
Calcium Orange	549	575	
		576	
Calcofluor White	385, 395, 405	437, 440, 445	
Carboxy-X-rhodamine (5-ROX)	576	601	
Cascade Blue ™	377	420	
	398	423	
	399		
Cascade Yellow	399	550	
	400	552	
Catecholamine	410	470	
CCF2 (GeneBlazer)			
CFDA	494	520	
CFP-Cyan Fluorescent Protein	430, 433, 436, (453)	474, 475, 476, (501)	Cyan Fluorescent Protein
CFP/YFP FRET			
Chlorophyll	480	650	
Chromomycin A	436-460	470	
Chromomycin A	445	575	
CL-NERF (Ratio Dye, pH)	504/514	540	Ratio Dye, pH
CMFDA	494	520	
Coelenterazine Ca2+ Dye, bioluminescence	(429)	465	Ca2+ Dye, bioluminescence, native molecule
Coelenterazine cp (Ca2+ Dye,)	(430)	442	Ca2+ Dye, bioluminescence
Coelenterazine f	(437)	473	Ca2+ Dye, bioluminescence
Coelenterazine fcp		452	Ca2+ Dye, bioluminescence
Coelenterazine h	(437)	464	Ca2+ Dye, bioluminescence
Coelenterazine hcp	(433)	444	Ca2+ Dye, bioluminescence
Coelenterazine ip		441	Ca2+ Dye, bioluminescence
Coelenterazine n	(431)	467	Ca2+ Dye, bioluminescence
Coelenterazine O	460	575	
Coumarin Phalloidin	387	470	
C-phycocyanine			
CPM Methylcoumarin	384	469	Methylcoumarin
CTC	400-450	602	
CTC Formazan			
Cy2 ™	489	506	
Cy3.1 8	554	568	
Cy3.5 ™	581	598	
Cy3 ™	514	566	
	552	570	
	554		
Cy5.1 8	649	666	
Cy5.5 ™	675	695	
Cy5 ™	649	666	
		670	
Cy7 ™	710, 743	767, 805	
Cyan GFP	433 (453)	475	
		(501)	
cyclic AMP Fluorosensor (FiCRhR)	500	517	
CyQuant Cell Proliferation Assay	480	520	Cell Proliferation Assay

TABLE 1-continued

Fluorophores that may be used in the present method:

Fluorophore	Absorption	Emission	Other info
Dabcyl	453		
Dansyl	340	578	
Dansyl Amine	337	517	
Dansyl Cadaverine	335	518	
Dansyl Chloride	372	518	
Dansyl DHPE	336	517	
Dansyl fluoride	356	none	
DAPI	359	461	
Dapoxyl	403	580	
Dapoxyl 2	374	574	
Dapoxyl 3	373	574	
DCFDA	504	529	
DCFH (Dichlorodihydrofluorescein Diacetate)	505	535	
DDAO	463	607	
DHR (Dihydorhodamine 123)	505	534	
Di-4-ANEPPS	496	705	
Di-8-ANEPPS (non-ratio)	488	605	
	498	713	
DiA (4-Di-16-ASP)	456	591	
Dichlorodihydrofluorescein Diacetate (DCFH)	505	535	
DiD-Lipophilic Tracer	644	665	Lipophilic Tracer
DiD (DiIC18(5))	644	665	
DIDS	341	415	
Dihydorhodamine 123 (DHR)	505	535	
DiI (DiIC18(3))	549, 551	565	
Dinitrophenol	349		
DiO (DiOC18(3))	484, 487	501, 502	
DiR	748	780	Lipophilic Tracer
DiR (DiIC18(7))	750	779	
DM-NERF (high pH)	497/510	540	Ratio Dye, pH
DNP	349		
Dopamine	340	490-520	
DsRed	558	583	Red fluorescent protein
DTAF	494	520	
DY-630-NHS	621	660	Hemicyane label for proteins and DNA
DY-635-NHS	634	664	Hemicyane label for proteins and DNA
EBFP	383	447	Enhanced Blue Fluorescent Protein
ECFP	436	474	Enhanced Cyan Fluorescent Protein
EGFP	488, 498	507, 516	Enhanced Green Fluorescent Protein
ELF 97	345	530	
Eosin	524	545	
Erythrosin	529, 532	554, 555	
Erythrosin ITC	529	555	
Ethidium Bromide	510, 523	595, 605	
Ethidium homodimer-1 (EthD-1)	528	617	
Euchrysin	430	540	
EukoLight			
Europium (III) chloride			
EYFP	513, 520	527, 532	Enhanced Yellow Fluorescent Protein
Fast Blue	360	440	
FDA	494	520	
Feulgen (Pararosaniline)	570	625	
FT (Formaldehyd Induced Fluorescence)	405	433	
FITC	490, 494	520, 525	
FITC Antibody	493	517	
Flazo Orange	375-530	612	
Fluo-3	480-506, 506	520, 527	

TABLE 1-continued

Fluorophores that may be used in the present method:

Fluorophore	Absorption	Emission	Other info
Fluo-4	494	516	
Fluorescein (FITC)	490, 494	520, 525	
Fluorescein Diacetate	494	520	
Fluoro-Emerald	495	524	
Fluoro-Gold (Hydroxystilbamidine)	361	536	
Fluor-Ruby	555	582	
FluorX	494	520	
FM 1-43 ™	479	598	
FM 4-46	515	640	
Fura Red ™ (high pH)	572	657	
Fura Red ™/Fluo-3			
Fura-2, high calcium	335	505	Excitation ratio dye
Fura-2, low calcium	363	512	Excitation ratio dye
Fura-2/BCECF			
Genacryl Brilliant Red B	520	590	
Genacryl Brilliant Yellow 10GF	430	485	
Genacryl Pink 3G	470	583	
Genacryl Yellow 5GF	430	475	
GeneBlazer (CCF2)			
GFP (S65T)	498	516	
GFP red shifted (rsGFP)	498	516	
GFP wild type, non-UV excitation (wtGFP)	475	509	
GFP wild type, UV excitation (wtGFP)	395	509	
GFPuv	385	508	
Gloxalic Acid	405	460	
Granular Blue	355	425	
Haematoporphyrin	530-560	580	
Hoechst 33258	345	487	
Hoechst 33342	347	483	
Hoechst 34580	392	440	
HPTS	355	465	
Hydroxycoumarin	325-360	386-455	
Hydroxystilbamidine (FluoroGold)	361	536	
Hydroxytryptamine	400	530	
Indo-1, high calcium	330	401	Emission ratio dye
Indo-1, low calcium	346	475	Emission ratio dye
Indodicarbocyanine (DiD)	644	665	
Indotricarbocyanine (DiR)	748	780	
Intrawhite Cf	360	430	
JC-1	514	529	
JO-JO-1	530	545	
JO-PRO-1	532	544	
LaserPro	795	812	
Laurodan	355	460	
LDS 751 (DNA)	543	712	
LDS 751 (RNA)	590	607	
Leucophor PAF	370	430	
Leucophor SF	380	465	
Leucophor WS	395	465	
Lissamine Rhodamine	572, 577	591, 592	
Lissamine Rhodamine B	577	592	
LIVE/DEAD Kit Animal Cells	494	517	for more details
Calcein/Ethidium homodimer	528	617	refer to www.probes.com
LOLO-1	566	580	
LO-PRO-1	568	581	
Lucifer Yellow	425, 428	528, 536, 540	
Lyso Tracker Blue	373	422	
Lyso Tracker Blue-White	466	536	
Lyso Tracker Green	504, 534	511, 551	
Lyso Tracker Red	490	516	
Lyso Tracker Yellow	551	576	
LysoSensor Blue	374	424	
LysoSensor Green	442	505	
LysoSensor Yellow/Blue	384	540	
Mag Green	507	531	
Magdala Red (Phloxin B)	524	600	
Mag-Fura Red	483/427	659/631	Ratio Dye, Mg2+

TABLE 1-continued

Fluorophores that may be used in the present method:

Fluorophore	Absorption	Emission	Other info
Mag-Fura-2	369/329	508	Ratio Dye Ca2+
	369/330	511/491	Ratio Dye Mg2+
Mag-Fura-5	369/330	505/500	Ratio Dye, Ca2+
	369/332	505/482	Ratio Dye, Mg2+
Mag-Indo-1	349/328	480/390	Ratio Dye, Ca2+
	349/330	480/417	Ratio Dye, Mg2+
Magnesium Green	506, 507	531	
Magnesium Orange	550	575	
Malachite Green	628		
Marina Blue	362	459	
Maxilon Brilliant Flavin 10 GFF	450	495	
Maxilon Brilliant Flavin 8 GFF	460	495	
Merocyanin	555	578	
Methoxycoumarin	360	410	
Mitotracker Green FM	490	516	
Mitotracker Orange	551	576	
Mitotracker Red	578	599	
Mitramycin	450	470	
Monobromobimane	398	490	
Monobromobimane (mBBr-GSH)	398	500	
Monochlorobimane	380	461	
MPS (Methyl Green Pyronine Stilbene)	364	395	
NBD	466	539	
NBD Amine	450	530	
Nile Red	515-555, 559	590, 640	
Nitrobenzoxadidole	465	510-650	
Noradrenaline	340	490-520	
Nuclear Fast Red	289-530	580	
Nuclear Yellow	365	495	
Nylosan Brilliant Iavin E8G	460	510	
Oregon Green	503	522	
Oregon Green 488-X	494	517	
Oregon Green ™	503	522	
Oregon Green ™ 488	490, 493	514, 520	
Oregon Green ™ 500	497	517	
Oregon Green ™ 514	506	526	
Pacific Blue	405	455	
Pararosaniline (Feulgen)	570	625	
PBFI	340/380	420	Excitation ratio dye
PE-Cy5	488	670	
PE-Cy7	488	755, 767	
PerCP	488	675	
PerCP-Cy5.5	488	710	
PE-TexasRed [Red 613]	488	613	
Phloxin B (Magdala Red)	524	600	
Phorwite AR	360	430	
Phorwite BKL	370	430	
Phorwite Rev	380	430	
Phorwite RPA	375	430	
Phosphine 3R	465	565	
PhotoResist	365	610	
Phycoerythrin B [PE]	546-565	575	
Phycoerythrin R [PE]	565	578	
PKH26 (Sigma)	551	567	
PKH67	496	520	Chroma
PMIA	341	376	
Pontochrome Blue Black	535-553	605	
POPO-1	433	457	
POPO-3	533	574	
PO-PRO-1	435	455	
PO-PRO-3	539	567	
Primuline	410	550	
Procion Yellow	470	600	
Propidium Iodid (PI)	(305), 536, 538	617	
PyMPO	412, 415	561, 564, 570	
Pyrene	360	387	
Pyronine	410	540	
Pyronine B	540-590	560-650	
Pyrozal Brilliant Flavin 7GF	365	495	
QSY 7	560		
Quinacrine Mustard	440	510	
Red 613 [PE-TexasRed]	488	613	
Resorufin	571	584, 585	
RH 414	532	716	

TABLE 1-continued

Fluorophores that may be used in the present method:

Fluorophore	Absorption	Emission	Other info
Rhod-2	552	576	
Rhodamine	550	573	
Rhodamine 110	496, 497	520	
Rhodamine 123	507	529	
Rhodamine 5 GLD	470	565	
Rhodamine 6G	525	555	
Rhodamine B	540	625	
Rhodamine B 200	523-557	595	
Rhodamine B extra	550	605	
Rhodamine BB	540	580	
Rhodamine BG	540	572	
Rhodamine Green	502	527	
Rhodamine Phallicidine	558, 542	575, 565	
Rhodamine Phalloidine	542	565	
Rhodamine Red	570	590	
Rhodamine WT	530	555	
Rose Bengal	525, 540	550-600	
R-phycocyanine			
R-phycoerythrin (PE)	565	578	
rsGFP	473	509	red shifted GFP (S65T)
S65A	471	504	
S65C	479	507	
S65L	484	510	
S65T	488	511	
Sapphire GFP	395	511	
SBFI	340/380	420	Excitation ratio dye
Serotonin	365	520-540	
Sevron Brilliant Red 2B	520	595	
Sevron Brilliant Red 4G	500	583	
Sevron Brilliant Red B	530	590	
Sevron Orange	440	530	
Sevron Yellow L	430	490	
sgBFP ™	387	450	
sgBFP ™ (super glow BFP)	387	450	Quantum's SuperGlo ™ GFP AFPs
sgGFP ™	474	488	
sgGFP ™ (super glow GFP)	474	509	Quantum's SuperGlo ™ GFP AFPs
SITS	336	436	Ion Channels
SITS (Primuline)	395-425	450	
SITS (Stilbene Isothiosulphonic Acid)	365	460	
SNAFL calcein	506/535	535/620	Ratio Dye, pH
SNAFL-1	508/540	543/623	Ratio Dye, pH
SNAFL-2	514/543	546/630	Ratio Dye, pH
SNARF calcein	552/574	590/629	Ratio Dye, pH
SNARF1	576/548	635/587	Excitation and emission ratio dye
Sodium Green	506, 507	532	Na+, K+
SpectrumAqua	433,/53	480/55	Vysis
SpectrumGreen	497/30, 509/31	538/44, 524/56	Vysis
SpectrumOrange	559/38, 560	588/48	Vysis
Spectrum Red	587, 587/35	612, 612/51	
SPQ (6-methoxy-N-(3-sulfopropyl)quinolinium)	344	443	
Stilbene	335	440	
Sulphorhodamine B can C	520	595	
Sulphorhodamine G Extra	470	570	
SYTO 11	508, 510	527, 530	Dye for DNA, RNA
SYTO 12	499, 500	522, 519	Dye for DNA, RNA
SYTO 13	488, 491	509, 514	Dye for DNA, RNA
SYTO 14	517, 521	549, 547	Dye for DNA, RNA
SYTO 15	516, 518	546, 555	Dye for DNA, RNA
SYTO 16	488, 494	518, 525	Dye for DNA, RNA

TABLE 1-continued

Fluorophores that may be used in the present method:

Fluorophore	Absorption	Emission	Other info
SYTO 17	621	634	Dye for DNA
SYTO 18	490, 493	507, 527	Dye for DNA, RNA
SYTO 20	512	530	Dye for DNA
SYTO 21	494	517	Dye for DNA
SYTO 22	515	535	Dye for DNA
SYTO 23	499	520	Dye for DNA
SYTO 24	490	515	Dye for DNA
SYTO 25	521	556	Dye for DNA
SYTO 40	420	441	Dye for DNA
SYTO 41	430	454	Dye for DNA
SYTO 42	433	460	Dye for DNA
SYTO 43	436	467	Dye for DNA
SYTO 44	446	471	Dye for DNA
SYTO 45	452	484	Dye for DNA
SYTO 59	622	645	Dye for DNA
SYTO 60	652	678	Dye for DNA
SYTO 61	628	645	Dye for DNA
SYTO 62	652	676	Dye for DNA
SYTO 63	657	673	Dye for DNA
SYTO 64	599	619	Dye for DNA
SYTO 80	531	545	Nucleic Acid Stain
SYTO 81	530	544	Nucleic Acid Stain
SYTO 82	541	560	Nucleic Acid Stain
SYTO 83	543	559	Nucleic Acid Stain
SYTO 84	567	582	Nucleic Acid Stain
SYTO 85	567	583	Nucleic Acid Stain
SYTOX Blue	445	470	Nucleic Acid Stain
SYTOX Green	504	523	Nucleic Acid Stain
SYTOX Orange	547	570	Nucleic Acid Stain
Tetracycline	390-425	525-560	
Tetramethylrhodamine (TRITC)	555	576	
Texas Red ™	595	620	
Texas Red-X ™ conjugate	595	615	
Thiadicarbocyanine (DiSC3)	651, 653	674, 675	
Thiazine Red R	596	615	
Thiazole Orange	510	530	
Thioflavin 5	430	550	
Thioflavin S	430	550	
Thioflavin TCN	350	460	
Thiolyte	370-385	477-488	
Thiozole Orange	453	480	
Tinopol CBS (Calcofluor White)	390	430	
TMR	550	573	
TO-PRO-1	515	531	
TO-PRO-3	644	657	
TO-PRO-5	747	770	
TOTO- 1	514	531, 533	
TOTO-3	642	660	
TriColor (PE-Cy5)	(488) 650	667	
TRITC TetramethylRodamineIsoThioCyanate	550	573	
True Blue	365	425	
TruRed	490	695	
Ultralite	656	678	
Uranine B	420	520	
Uvitex SFC	365	435	
wt GFP	395 (475)	508	wild type GFP
WW	781	605	639
X-Rhodamine	580	605	
XRITC	582	601	
Xylene Orange	546	580	
Y66F	360	508	
Y66H	360	442	
Y66W	436	485	

TABLE 1-continued

Fluorophores that may be used in the present method:

Fluorophore	Absorption	Emission	Other info
Yellow GFP	513	527	Yellow shifted Green Fluorescent Protein
YFP	513, 520	527, 532	Yellow Fluorescent Protein
YO-PRO-1	491	506	
YO-PRO-3	613	629	
YOYO-1	491	508, 509	
YOYO-3	612	631	

The fluorophores listed in Table 1, along with many others, are commercially available from worldwide suppliers, including Molecular Probes, a division of ThermoFisher Scientific, Eugene, Oreg., USA.

Methods of attaching fluorophores to proteins and polypeptides are exceedingly well known and will not be discuss in any detail. For a complete overview of the subject with an extensive list of relevant literature citations, see Modesti (2011) "Fluorescent labeling of proteins," *Methods Mol Biol.* 783:101-20. See also Michael Z. Lin, Lei Wang (12 Jun. 2008) "Selective Labeling of Proteins with Chemical Probes in Living Cells," *Physiology* 23(3), 131-141. See also Sauer, Hofkens, and Enderlein, "Handbook of Fluorescence Spectroscopy and Imaging: From Ensemble to Single Molecules 1st Edition," © 2011, Wiley-VCH (Weinheim, Germany), ISBN-13: 978-3527316694.

FIG. **1** is a schematic diagram of the HIV-1 genome. As noted above, HIV expresses auxiliary proteins that activate phases of gene expression. Tat activates efficient transcription of HIV-1 pre-mRNA. Rev activates nuclear export of partially and unspliced viral mRNA. HIV also encodes HIV encodes accessory proteins that degrade cellular proteins in order to increase infectivity. Vif induces the degradation of the APOBEC3G restriction factor. Vpu induces the degradation of BST2/Tetherin restriction factor. Nef induces the degradation of SERINC5 restriction factor. Vpu, Nef, and Env induce the degradation of the CD4 receptor protein.

FIGS. **2**A, **2**B, **2**C, **2**D, and **2**E are a series of time-lapse photomicrographs taken from a full motion picture depicting ratiometric/multicolor HIV-1 reporter cells that combine reporter activation and reporter degradation to achieve a high signal-to-noise read-out of infection. As reporter 1 (blue) is activated by Tat/Rev, reporter 2 (green) is deactivated (degraded) by Vif, Elapsed time between FIG. **2**A and FIG. **2**E is 12 hours. Images generated from VSVg pseudotyped HIV-1 cyan fluorescent protein (CFP) expressed from Nef position.

FIG. **3**A is a graph depicting signal intensity from the test run shown in FIGS. **2**A through **2**E. Here, the deactivation of reporter 2 (the green cells in FIGS. **2**A-**2**E) superimposed upon the activation of reporter 1 (the blue cells in FIGS. **2**A-**2**E) over time. Yellow diamonds in FIG. **3**A are fluorescent values for reporter 2 (yellow fluorescent protein; YFP); blue squares in FIG. **3**A are fluorescent values for reporter 1 (cyan fluorescent protein; CFP). The graph thus depicts the rate of YFP-A3G (yellow) downregulation and HIV-1 (blue) upregulation over 35 hours of infection.

FIG. **3**B is a corresponding graph that presents the same data as in FIG. **3**A, with the addition of a trace showing the ratio between the signal from reporters 1 and 2 (black triangles). As shown in FIG. **3**B, using the ratio of activation:degradation fluorescence provides ~10-fold increase to sensitivity of the assay. By using the ratio of the two signals, the time point at which HIV infection and replication begins to occur can also be precisely pinpointed.

FIG. **4** is a pair of photomicrographs comparing the assay results in uninfected cells (top panel) and 24 hours after exposure to HIV in infected cells (bottom panel). As shown in the bottom panel of the figure, at 24 hours post-infection, the activation signal (reporter 1; red) is robust, while the degradation signal (reporter 2; green) is near background. The HIV-1-infected cells (which fluoresce brightly red) are clearly differentiated from the uninfected cells (which do not fluoresce red). In other words, when uninfected (top panel), cells are green due to YFP-APOBEC3G expression. Upon infection, the cells turn on a mCherry reporter (red) that responds to Rev and Tat, while the YFP-A3G (green) signal disappears due to Vif activity.

FIG. **5** is a schematic diagram contrast conventional quantitative viral outgrowth assays (top panel) versus the method disclosed herein. As shown in the bottom panel of FIG. **5**, the method disclosed herein can be formatted as microfluidics-based quantitative virus outgrowth assay (Q-VOA) for detecting HIV-infected cells from infected people and measuring levels of productive infection.

Any cell subject to viral infection can be used in the assay. HeLa cells and Jurkat cells are preferred. HeLa cells are hardy, reliable, and readily form monolayers. This makes them excellent for image acquisition and analysis. Jurkat cells are likewise hardy and reliable. They are also exhibit migratory (seek and find) behaviors and may be superior for detecting virus in tissues.

By way of a working example, the method has been implemented and shown to function as described. The basic functionality of the method is shown schematically in FIG. **6**A. Here is depicted a schematic diagram of a HIV-susceptible HeLa or Jurkat cell constitutively expressing YFP-tagged APOBEC3G (YFP-A3G; green) and encoding an HIV-responsive reporter gene expressing HIV Gag fused to mCherry (Gag-mChe; red). Pre-infection, as shown in the left side of FIG. **6**A, the cell yields no signal from the first fluorophore, which generates a first fluorescent signal—the red reporter Gag-mChe. This first fluorescent signal is upregulated only after productive infection of the cell. The second fluorophore (YFP-A3G; green), generates a robust signal in the left portion of FIG. **6**A. This second fluorescent signal is down-regulated after productive infection of the cell. Thus, as shown in FIG. **6**A, when the cell is infected, the first fluorescent signal (the red signal generated by

Gag-mChe) is turned on; the second fluorescent signal (the green signal generated by YFP-A3G) is turned off.

This is shown in real time in FIG. **6**B, which shows the course of the up-regulation of the first fluorescent signal (red) and the simultaneous down-regulation of the second fluorescent signal (green) over the course of 18 hours post-infection (HPI) in a single HeLa cell. FIG. **6**B is a series of photographs depicting productive infection of a HeLa cell with HIV in which the cells was modified to contain a first fluorophore and a second fluorophore as described herein. As shown in the top series of photos, infection led to the downregulation of YFP-A3G (green, the second fluorphore) due to expression of the viral Vif protein that degraded YFP-A3G. Simultaneously, as shown in the bottom series of photos, infection leads to activation of Gag-mCherry (red, the first fluorophore) gene expression due to the increased activities of HIV Tat and Rev.

FIGS. **6**C and **6**D are graphs showing the mean fluorescent intensity in ten (10) individual cells for YFP-A3G down-regulation kinetics (FIG. **6**C) and up-regulation for Gag-mChe expression (FIG. **6**D). These two graphs very neatly show the clean down-regulation of YFP-A3G as the infection progresses, and the simultaneous up-regulation of Gag-mChe over the same time period. At around 23 hours post-infection, the signals from YFP-A3G have bottomed out, while the signals from Gag-mChe have reached maxima.

The method is equally useful when analyzing large collections of cells, rather than single cells. FIG. **7**A is a series of photomicrographs showing whole-field read-outs using the same multicolor reporter cells as used in FIG. **6**B. Shown in FIG. **7**A are HeLa reporter cells constitutively expressing YFP-tagged APOBEC3G (YFP-A3G; green) and encoding an HIV-responsive reporter gene expressing HIV Gag fused to mCherry (Gag-mChe; red) tracked over 51 hours post-infection ("HPI"). Approximately 50% of the cells were infected and turned from green to red. In the same fashion as described previously, the green signal was down-regulated upon infection, while the red signal was up-regulated upon infection.

FIG. **7**B is a graph depicting whole-field mean fluorescence intensity (MFI) as a function of time (hours post-infection) for both the green signal and the red signal. A great many useful metrics can be gleaned from the graph shown in FIG. **7**B. Plotting both signals on a single grid allow for the direct measurement of (1) cell doubling rate; (2) A3G downregulation kinetics; (3) viral gene activation; (4) rates of viral late gene expression; and (5) onset of virus particle production (based on changes to cell granularity).

FIG. **7**C is a graph depicting the ratio of Gag signal to the A3G signal (Gag:A3G). This graph illustrates another useful feature of the cells, namely the signal-to-noise can be reduced by calculating the ratio of the relative measurements of YFP-A3G and Gag-mCherry signals. By using this ratio, the signal is unmistakable from any noise.

What is claimed is:

1. A method of tracking viral infection of a cell, the method comprising:

(a) providing a cell susceptible to infection by a virus in which the cell comprises:

a first nucleic acid encoding a gene that constitutively expresses a first fusion protein, wherein the first fusion protein comprises (i) a first domain susceptible to degradation by a protein from the virus, and (ii) a second domain comprising a first protein fluorophore; and

a second nucleic acid encoding a second fusion protein, wherein the second fusion protein comprises (i) a first domain operably linked to a promoter that is responsive to infection by the virus, and (ii) a second domain comprising a second protein fluorophore;

wherein a fluorescent signal from the first protein fluorophore is down-regulated upon infection of the cell by the virus, and a fluorescent signal from the second protein fluorophore is up-regulated upon infection of the cell by the virus;

(b) exposing the cell to the virus under conditions where the virus can infect the cell;

(c) measuring the fluorescent signals generated by the first protein fluorophore and the second protein fluorophore; and

(d) determining the extent of infection of the cell by the virus by comparing the fluorescent signals measured in step (c) with corresponding signals generated in a corresponding cell not exposed to the virus.

2. The method of claim **1**, wherein the cell is a human T-cell.

3. The method of claim **1**, wherein the cell is a HeLa cell or a Jurkat cell.

4. The method of claim **1**, wherein the virus is an HIV virus.

5. The method of claim **4**, wherein the cell is a human T-cell.

6. The method of claim **4**, wherein the cell is a HeLa cell or a Jurkat cell.

7. The method of claim **1**, wherein the virus is an HIV-1 virus.

8. The method of claim **7**, wherein the cell is a human T-cell.

9. The method of claim **7**, wherein the cell is a HeLa cell or a Jurkat cell.

10. The method of claim **1**, wherein the second fusion protein comprises Tat (trans-activator of transcription) or Rev.

11. The method of claim **1**, wherein the first fusion protein comprises a protein selected from the group consisting of A3G (apolipoprotein B mRNA editing enzyme), BST2 (bone marrow stromal antigen 2, CD4 (cluster of differentiation 4), and SERINC5 (serine incorporator 5).

12. The method of claim **1**, wherein:

The second fusion protein comprises Tat (trans-activator of transcription) or Rev; and

the first fusion protein comprises a protein selected from the group consisting of A3G, BST2, CD4, and SERINC5.

13. The method of claim **12**, wherein the cell is a human T-cell, a HeLa cell, or a Jurkat cell.

14. The method of claim **13**, wherein the virus is an HIV virus.

15. The method of claim **13**, wherein the virus is an HIV-1 virus.

16. The method of claim **1**, wherein in step (a) the cell contains a third nucleic acid encoding a gene that constitutively expresses a third fusion protein, wherein the third fusion protein comprises (i) a first domain susceptible to alteration by a protein from the virus, and (ii) a second domain comprising a third protein fluorophore; and wherein the third protein fluorophore generates a fluorescent signal that is distinct from and can be detected independently of the fluorescent signals generated by the first and second protein fluorophores, and wherein the fluorescent signal generated from the third protein fluorophore is detectably altered upon infection of the cell with the virus.

17. The method of claim 16, wherein in step (a) the cell contains a fourth nucleic acid encoding a gene that constitutively expresses a fourth fusion protein, wherein the fourth fusion protein comprises (i) a first domain susceptible to alteration by a protein from the virus, and (ii) a second domain comprising a fourth protein fluorophore; and wherein the fourth protein fluorophore generates a fluorescent signal that is distinct from and can be detected independently of the signals generated by the first, second, and third protein fluorophores, and wherein the fluorescent signal generated from the fourth protein fluorophore is detectably altered upon infection of the cell with the virus.

18. The method of claim 17, wherein in step (a) the cell contains a fifth nucleic acid encoding a gene that constitutively expresses a fifth fusion protein, wherein the fifth fusion protein comprises (i) a first domain susceptible to alteration by a protein from the virus, and (ii) a second domain comprising a fifth protein fluorophore; and wherein the fifth protein fluorophore generates a fluorescent signal that is distinct from and can be detected independently of the signals generated by the first, second, third, and fourth protein fluorophores, and wherein the fluorescent signal generated from the fifth protein fluorophore is detectably altered upon infection of the cell with the virus.

* * * * *