Single-Cell Imaging of HIV-1 Provirus (SCIP)

Cristina Di Primio*, Valentina Quercioli*, Awatef Alouche*, Rik Gijsbers*, Frauke Christ*, Zeger Debyser*, Daniele Arosio*, and Anna Cereseto1,2

*Laboratory of Neurobiology, Scuola Normale Superiore, 56124 Pisa, Italy; 1Unité de Régulation des Infections Retrovirales, Institut Pasteur, 75724 Paris, France; 2Laboratory of Molecular Virology and Gene Therapy, KU Leuven, 3000 Leuven, Flanders, Belgium; 3Istituto di Biofisica, Consiglio Nazionale delle Ricerche, 38123 Trento, Italy; and 4Laboratory of Molecular Virology, Centre for Integrative Biology, 38123 Trento, Italy

Edited* by Stephen P. Goff, Columbia University College of Physicians and Surgeons, New York, NY, and approved February 21, 2013 (received for review September 21, 2012)

Recent advances in fluorescence microscopy provided tools for the investigation and the analysis of the viral replication steps in the cellular context. In the HIV field, the current visualization systems successfully achieve the fluorescent labeling of the viral envelope and proteins, but not the genome. Here, we developed a system able to visualize the proviral DNA of HIV-1 through immunofluorescence detection of repair foci for DNA double-strand breaks specifically induced in the viral genome by the heterologous expression of the I-SceI endonuclease. The system for Single-Cell Imaging of HIV-1 Provirus, named SCIP, provides the possibility to individually track integrated-viral DNA within the nuclei of infected cells. In particular, SCIP allowed us to perform a topological analysis of integrated viral DNA revealing that HIV-1 preferentially integrates in the chromatin localized at the periphery of the nuclei.

Technical developments in imaging-based techniques have greatly improved our understanding of HIV–host cell interactions. HIV-1 virions labeled with fluorophores were pivotal in shedding light onto multiple aspects of the virus–host interplay during all steps of HIV-1 replication cycle (1–13). Nevertheless, few optical approaches have been so far developed to visualize viral particles within the nuclear compartment (14, 15), which limits our comprehension of the interaction between HIV-1 and the nuclear architecture. Moreover, the existing detection tools are based on the visualization of the viral protein complexes or envelope but not of the viral DNA with the only exception of the fluorescence in situ hybridization (FISH) technique. Even though FISH is a powerful technique, it is not very sensitive for HIV-1 detection and moreover disrupts the native architecture of the nuclear compartment as it requires harsh denaturation conditions. In addition, this technique does not allow the discrimination between integrated and nonintegrated viral DNA (16, 17). Here we describe a fluorescent approach to visualize HIV-1 DNA in the nuclear compartment of infected cells. We exploited a site-specific genome engineering technique that represents one of the most promising approaches to detect specific genome regions in modified organism (18) allowing for their spatial localization in the cell (19, 20). This technique couples endogenous repair pathways, induced by rare cutting endonuclease, with immunofluorescence analysis. Rare cutting endonucleases, such as the yeast-homing endonuclease I-SceI, specifically cuts target sequences that cannot be found in the mammalian genome. By engineering DNA to contain the I-SceI cleavage site, it is thus possible to induce endogenous repair mechanism for double-strand breaks (DSBs) at specific genomic positions. DSB repair leads to the formation of distinct subnuclear structures that are generally referred to as “foci” (21). The first sensor of the DSB is the histone H2AX, which becomes massively phosphorylated at serine 139 (γ-H2AX). Foci of DNA repair are thus visible through immunofluorescence by using specific γ-H2AX antibodies. Here, by inserting an I-SceI site in the HIV-1 genome, we show that individual proviral DNA can be efficiently detected at the level of a single infected cell [Single-Cell Imaging of HIV-1 Provirus (SCIP)]. The power of SCIP is the temporal and spatial analysis of HIV-1 in individual nuclei of infected cells, a great benefit over currently used techniques. A 3D topological analysis, performed by SCIP, demonstrated that integrated viral DNA localizes at the periphery of the nuclei revealing important insights in the nuclear biology of HIV-1.

Results

Construction of the HIV–I-SceI Reporter System and Viral DNA Cleavage Validation. DNA DSB leads to the phosphorylation of H2AX molecules and the formation of γ-H2AX foci, which can be visualized by immunofluorescence (IF) technique. The ability of the rare cutting endonuclease I-SceI to induce specific DSB was exploited to detect HIV-1 DNA in infected cells. In specific, we engineered HIV-1 viral particles by inserting an 18-bp target site for the endonuclease I-SceI into viral transfer vectors (pHR-CMVGFPU-SceI, pHCMVΔGFP—I-SceI; Fig. 1B); the cleavage activity of the enzyme on viral DNA was then verified by recombinant I-SceI digestion (Fig. S1).

The system was initially evaluated in U2OS clones obtained by stable transfection of the transfer vector (pHR-CMVGFPU-SceI) and subsequent clonal selection. Representative stable cell clones, U2OS-6 and U2OS-8, containing an average of 6 and 22 integrated DNA copies, respectively, as quantified by quantitative PCR (qPCR), were transfected with the I-SceI endonuclease encoding plasmid (pCBAXsce) and immunostained with specific anti-γ-H2AX antibodies to detect nuclear foci by confocal microscopy. Fig. 1B shows that γ-H2AX foci are clearly detectable in nuclei of U2OS-6 and U2OS-8 cells expressing the I-SceI endonuclease, although few background signals, likely generated by spontaneous DNA strand breaks, are detected in nontransfected cells. Moreover, cells positive for γ-H2AX foci were also expressing the viral reporter gene (GFP; Fig. 1B). Quantification analysis of the γ-H2AX foci revealed that the total number of γ-H2AX foci per nucleus closely correlates with the integrated viral DNA copies measured by qPCR (Fig. S2A and B).

To further prove that the γ-H2AX foci detected by IF correspond to the viral DNA containing the I-SceI site we performed chromatin immunoprecipitation experiments (ChIP) using γ-H2AX antibodies. Chromatin immunoprecipitates from U2OS-8 cells expressing or not expressing the I-SceI endonuclease (Fig. S2C) were amplified by real-time PCR with primer pairs covering the immediate proximity of the I-SceI site (primer pairs A and B) and with primers targeting the viral DNA (MH531–532) (22) (Fig. 1C). We observed an enrichment of γ-H2AX (10–30-fold) with primers specific for the I-SceI region and viral sequences; no enrichment was obtained in control samples (Fig. 1C).

*This Direct Submission article had a prearranged editor.
1To whom correspondence should be addressed. E-mail: cereseto@science.unitn.it.

Author contributions: C.D.P., V.Q., F.C., Z.D., D.A., and A.C. designed research; C.D.P., V.Q., and A.A. performed research; R.G. and A.C. contributed new reagents/analytic tools; D.A. contributed new reagents/analytic tools; D.A. analyzed data; and C.D.P., V.Q., and A.C. wrote the paper.
The authors declare no conflict of interest.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1216254110/-/DCSupplemental.
defective HIV-1 virus (HIV-CMV-GFP-I-SceIΔ116A) bearing the D116A mutation in the integrase active site causing a catalytic defect in integrase. Following infection this mutant virus produces either linear or circularized (2-LTR) nonintegrated DNA. Results in Fig. 2A, Bottom show that even though infected cells produce GFP from nonintegrated forms, no γ-H2AX foci could be detected. To better describe this observation the foci formation in cells infected with HIV-CMV-GFP-I-SceI wild-type or integration-defective (Δ116A) viruses was quantified. The quantification analysis was set up by comparing three approaches in cells infected with

**Similar results were obtained using stable clones infected with** HIV-1 particles containing the I-SceI restriction site (HIV-CMV-GFP-I-SceI) and pseudotyped with the vesicular stomatitis virus G (VSV-G) envelope (Fig. S2 D and E). Taken together, our data proved that the I-SceI target site mediated modification of the HIV-I genome allows visualizing viral DNA at the nuclear level.

**Visualization of Viral DNA in Infected Cells.** The HIV-I-SceI reporter system was then evaluated in the context of viral infection using HIV-CMV-GFP-I-SceI viral particles pseudotyped with the VSV-G envelope, in U2OS cells transfected with the pCBASce plasmid. Cells were immunostained with anti-γ-H2AX antibodies: γ-H2AX foci (in red) viral GFP expression (in green). Scale bars, 10 μm. (C) Chromatin immunoprecipitation (ChIP) analysis of viral DNA associated with γ-H2AX. (Upper) Schematic representation of the positions of the primers used for real-time PCR quantification. (Lower) The γ-H2AX ChIP analysis in the U2OS-8 cell clone expressing or not expressing the I-SceI endonuclease (pCBASce) by transient transfection were immunostained with anti-γ-H2AX antibodies: γ-H2AX foci (red spots) detectable by immunofluorescence. (Bottom) The γ-H2AX ChIP analysis in the U2OS-8 cell clone expressing or not expressing the I-SceI endonuclease. Relative enrichment represents the enrichment of γ-H2AX compared with an IgG control (normalized with a PCR internal control to a locus other than the viral DNA). Error bars represent SDs from at least two independent experiments.

Because, in the early replication steps, the viral DNA species exist as nonintegrated or integrated proviral DNA, we investigated the ability of the HIV-I-SceI visualization system to detect the two different forms. To this aim we infected cells with an integration

![Image](https://via.placeholder.com/150)

**Fig. 1.** Detection of γ-H2AX foci associated with HIV-1 DNA in U2OS cells. (A) Schematic representation of viral constructs and mechanism of γ-H2AX foci formation. The plasmid pHCMV-GFP was modified by inserting the I-SceI target site to produce either the HIV-CMV-GFP-I-SceI virions expressing GFP in infected cells or the HIV-CMVΔGFP-I-SceI GFP deleted. The endonuclease I-SceI (blue spot) exogenously expressed by transfecting infected cells cleaves the I-SceI site producing DNA DSBs. γ-H2AX molecules at the DSB site become phosphorylated forming γ-H2AX foci detectable by immunofluorescence. (B) U2OS-6 and U2OS-8 stable cell clones containing pHCMV-GFP-I-SceI and expressing or not expressing the I-SceI endonuclease (pCBASce) by transient transfection were immunostained with anti-γ-H2AX antibodies: γ-H2AX foci detected in I-SceI expressing cells (red spots) detectable by immunofluorescence. (C) Chromatin immunoprecipitation (ChIP) analysis of viral DNA associated with γ-H2AX. (Upper) Schematic representation of the positions of the primers used for real-time PCR quantification. (Lower) The γ-H2AX ChIP analysis in the U2OS-8 cell clone expressing or not expressing the I-SceI endonuclease. Relative enrichment represents the enrichment of γ-H2AX compared with an IgG control (normalized with a PCR internal control to a locus other than the viral DNA). Error bars represent SDs from at least two independent experiments.

**Fig. 2.** Visualization of viral DNA in infected cells. (A) Detection of γ-H2AX foci and GFP in cells expressing (Top) or lacking (Middle) the exogenous I-SceI endonuclease. Cells were infected by integration competent virus or by integration defective virus (D116A) (Bottom). Scale bars, 10 μm. (B) Quantification of γ-H2AX foci number at 24 and 48 hpi in cells infected with 4 RTU of pHCMV-GFP-I-SceI or pHCMV-GFP-I-SceIΔ116A (means ± SEM from three independent experiments after background subtraction). At least 150 cells per experiment have been analyzed. [P<sub>wt vs. D116A</sub> = 1.332e-15 at 24 hpi, P<sub>D116A vs. bg</sub> = 4.692e-13 at 48 hpi, Kolmogorov–Smirnov test (KS test)]. (See Materials and Methods for background correction.) (C) Quantification of the number of γ-H2AX foci at 48 h and 13 d postinfection. Cells were infected with 0.004 RTU (white bars), 0.4 RTU (gray bars), or 4 RTU (black bars) (means ± SEM from at least two independent experiments after background subtraction). At least 200 cells per experiment have been analyzed. (D) Quantification of γ-H2AX foci number per cell with respect to percentage of GFP positive cells at the three different RTU after background subtraction; (P<sub>D0.4RTU vs. bg</sub> = 0.04542, P<sub>D0.4RTU vs. bg</sub> = 2.7e-5, P<sub>D0.4RTU vs. bg</sub> = 2.9e-7, KS test).
increasing amounts of HIV-CMV-GFP-I-SceI. The first approach, 2D quantification, consisted in counting the number of γ-H2AX foci in the optical section taken in the center of each cell nucleus (Fig. S4A). The second approach measured the total γ-H2AX foci fluorescence intensity in the central optical section of each nucleus (Fig. S4B). The third approach, 3D quantification, consisted in the automated counting of the number of γ-H2AX foci within the entire nuclear volume and was based on the nuclear 3D reconstruction from the Z stacks, by using dedicated imaging software (Imaris BITPLANE Scientific Software, ImageJ NIH). These three approaches showed analogous fold increase at increasing viral titer values (Fig. S4B), thus proving the validity of the HIV-I-SceI reporter system to detect HIV-1 genomes in infected cells. In the further study we applied the 2D quantification approach. We observed that in noninfected control cells the background number of γ-H2AX foci varied among individual experiments (Fig. S4C). For this reason a rigorous procedure was set up for the evaluation of the background number of foci. Background number of foci was quantified in noninfected cells expressing the I-SceI enzyme and subtracted in each individual experiment from the number of foci detected in cells infected with the HIV-CMV-GFP-I-SceI (exemplified in Fig. S4C–E and Materials and Methods).

The foci quantification in cells infected with HIV-CMV-GFP-I-SceI wild-type or integration-defective (D116A) viruses was performed at 24 and 48 h postinfection. We observed that cells infected with wild-type virus were positive for γ-H2AX foci at both time points, but only background values of γ-H2AX foci were detected by using the HIV-CMV-I-SceIΔD116A virus (Fig. 2B and Fig. S5). Therefore, we can conclude that the HIV-I-SceI system specifically detects HIV-1 DNA integrated into the host genome of individual infected cells; accordingly this method was named Single-Cell Imaging of HIV-1 Provirus.

To further explore the correlation of γ-H2AX foci formation with amounts of viral genomes in time and to establish the lowest multiplicity of infection that can be used in SCIP, cells were infected with increasing amounts of HIV-CMV-GFP-I-SceI (0.04, 0.4, and 4 RT units (RTU) as measured in ref. 23) and analyzed at 48 h and 13 d postinfection. To avoid continuous foci formation at both time points the I-SceI endonuclease was transfected at 24 h before immunostaining. The number of foci per nucleus detected at both 48 h and 13 d increased consistently with increased viral titers, thus indicating their correlation with the amounts of integrated DNA and their stability from the time of infection (Fig. 2C and Fig. S5). The analysis performed by measuring the number of foci above background in cells positive for GFP, revealed that as few as 15% of infected cells (GFP positive) can be detected through SCIP (Fig. 2D). However, to obtain maximum sensitivity the following experiments were performed using 4 RTU leading to almost 95% infectivity (Fig. 2D).

Finally, to verify the specificity of the γ-H2AX foci signals detected in HIV-CMV-GFP-I-SceI infected cells, coimmunostaining was performed with Rad51 or 53BP1 antibodies recognizing DNA repair focus hallmark proteins. Signal colocalization was observed between γ-H2AX and Rad51 or 53BP1 foci, thus confirming the specificity of the γ-H2AX foci (Fig. S6A).

![Fig. 3. SCIP analysis in transportin-SR2 knockdown cells and in anti-retroviral drug treated cells. (A) Immunofluorescence detection of γ-H2AX foci (yellow spots) in cells transfected with fluorescent siRNA (blue spots) targeting TRN-SR2 mRNA (siRNA-SR2) or a mismatch control siRNA (siRNA-MM) and infected with 4RTU of HIV-CMV-GFP-I-SceI. HIV-CMV-GFP-I-SceI virus was used to avoid the crosstalk between fluorophores. Nuclear middle z stacks are shown. Scale bars, 10 μm. (B) Quantification of γ-H2AX foci in cells transfected by siRNA-MM (black bars) and cells transfected by siRNA-SR2 (white bars) after background subtraction. (pMM-Sr2 vs. siRNA-MM = 7.946e-12, KS test). (C) Immunofluorescence detection of γ-H2AX foci (red spots) in infected cells (4 RTU) either untreated (ctrl-black bar) and in or treated as indicated (white bars) (means ±SEM from at least two independent experiments after background subtraction). Drugs concentrations are indicated. (Pu vs. 100nM AZT = 9.555e-05, Pu vs. 1 μM = 4.485e-07, Pu vs. 10 μM = 4.384e-08, Pu vs. 2 μM = 0.0003, Pu vs. 10 μM = 8.572e-13, Pu vs. 100nM rad < 2.2e-16, Pu vs. 1 μM rad < 2.2e-16, Pu vs. 10 μM CR = 0.0004, Pu vs. 1 μM CR = 3.046e-13, Pu vs. 10 μM CR = 6.122e-10, KS test). (See SI Materials and Methods for background correction.)
These results demonstrate that SCIP is a technique to simultaneously analyze HIV-1 expression and integration in individual cells. This single-cell investigation tool, as opposed to the conventional bulk approaches, is relevant to monitor the expression/integration profiles within a population of infected cells.

SCIP Analysis after Inhibition of Retroviral Replication. To further test the SCIP technique as a tool to analyze HIV-1 proviral DNA at single-cell level, infections were performed in different conditions, where viral replication was inhibited by cofactor knockdown or antivirals.

Transportin-SR2 (TRN-SR2, TNP03) is a cellular import factor of SR-rich proteins that is involved in HIV-1 entry in the nuclei of infected cells (24–31). In fact, after TRN-SR2 knockdown HIV-1 nuclear entry is inhibited and, consequently, the amount of integrated DNA is reduced. Cells depleted for TRN-SR2 (using siRNA-SR2) and expressing the I-SceI endonuclease were infected with the HIV-1-ΔGFP-SceI virus (4 RTU) and quantitatively analyzed for the amounts of γ-H2AX foci. As shown in Fig. 3 A and B and Fig. S5, the number of γ-H2AX foci dropped to background levels in TRN-SR2 knockdown cells thus indicating a lack of viral integration. Because alterations of TRN-SR2 levels may interfere with H2AX phosphorylation, the formation of foci was verified in TRN-SR2 knockdown cells treated with neocarzinostatin (NCS), a strong inducer of DSBs, which inhibits HIV-1 integration by disrupting the binding between integrase and LEDGF/p75, was also used (32, 33).

The quantitative analysis of these experiments demonstrates that both reverse transcriptase and integrase inhibitors decreased the number γ-H2AX of foci (Fig. 3 C, Middle and D; and Fig. S5). The distinct activity of these drugs in HIV-1 replication is indicated by the differential expression of GFP. In fact, GFP expression was greatly reduced in cells treated with reverse transcriptase inhibitors (nevirapine and AZT) as reported both by microscopy (Fig. 3C, Left) and by FACS analysis (Fig. S7) at 36 h postinfection. However, cells treated with integrase inhibitors (raltegravir and CX05045) show unaltered levels of GFP expression (Fig. 3C, Left; and Fig. S7) produced by nonintegrated viral DNA.

Therefore, SCIP has the power to discriminate whether the absence of integration following infection (no γ-H2AX foci) is generated by specific inhibition of viral integration (GFP positive cells) or by upstream viral impairments (GFP negative cells).

Integrated Viral DNA Localizes at the Nuclear Periphery. HIV-1 preintegration complexes (PICs) preferentially localize at the nuclear periphery, suggesting that integration may preferentially occur at the border of the nuclear compartment (14). To verify this hypothesis we analyzed the localization of the integration spots through SCIP. After measurement of the individual nuclear spots through SCIP, the analysis in U2OS cells revealed that 48 h postinfection in U2OS cells, 55% of distances from the nuclear lamin were established. The analysis in U2OS cells showed that 48 h postinfection in U2OS cells, 55% of the integrated viral DNA positioned within 1.5 μm of distance from the lamin (Fig. 4 A and B, black bars). The same analysis was performed in CEMss T cells, a lymphoid cell line, thus more physiologically relevant in HIV-1 infection. We observed that 62% of HIV-1 proviruses localized in the periphery of the nucleus (Fig. 4 C and D) and were mostly detected within 0.5 μm distance from the lamin. Therefore, compared with U2OS, cells the peripheral positioning was even more pronounced. The viral arrangement along the inner surface of the nuclear lamin of the CEMss-infected cells clearly emerged from the z sectioning of the infected cells (Fig. 4E).

To verify that no bias was introduced by the SCIP approach on the nuclear topology, the analysis was also performed in U2OS and CEMss cells treated with NCS to induce random DNA DSBs. As shown in Fig. 4 B–D (red bars) NCS-induced γ-H2AX foci do not show any preferential nuclear localization by distributing into the whole nuclear compartment. Finally, γ-H2AX foci generated by spontaneous DSBs in U2OS and CEMss cells show no preferential distribution within the nucleus in the absence of infection (Fig. 4E).
We next applied SCIP to cells where HIV-1 integration is retargeted toward heterochromatin and intergenic regions, thus altering the physiological lentiviral integration preference toward gene-rich regions of the chromatin (36), the virus also acquires a specific spatial orientation within the nuclear compartment.

It is remarkable that SCIP does not detect linear or circular nonintegrated viral DNA. This could be due to the conformation of the viral cDNA within the structure of the preintegration complex, where the I-SceI target site would not be accessible to endonucleases because masked by viral and cellular factors (37).

An alternative explanation could be that nonintegrated forms of viral DNA may not be adequately chromatinized to determine γ-H2AX accumulation at I-SceI sites.

To investigate over time the behavior of the HIV-1 provirus in the nuclear compartment, the same topological analysis was also performed 13 d postinfection. HIV-1 proviruses distribution changed remarkably over the explored time interval (Fig. 5A). Moreover, according to the classical nuclear topology analysis where the nucleus can be divided into three concentric zones of equal surface area (34, 35), the outcome of the SCIP analysis indicated a positioning of HIV-1 proviruses at the nuclear periphery: at 48 h postinfection, the majority of the proviral DNA localized in the outer rim of the nuclear compartment, the same topological analysis was also performed 13 d postinfection (red bars) (P = 5.76e-08, KS test). (B) Data are represented in bar graphs as the percentage of γ-H2AX foci in the three concentric zones of equal surface area. The peripheral zone is in black, the middle zone in light gray, and the inner zone in gray.

**Fig. 5.** Analysis of the proviral DNA nuclear localization at different time points after infection. (A) Distribution of distances of proviruses from the nuclear lamin at 48 h (black bars) and 13 d postinfection (red bars) (P = 5.76e-08, KS test). (B) Data are represented in bar graphs as the percentage of γ-H2AX foci in the three concentric zones of equal surface area. The peripheral zone is in black, the middle zone in light gray, and the inner zone in gray.

**Discussion**

Here we report SCIP, an imaging technique to efficiently visualize HIV-1 DNA integrated into the host genome of individual cells. The possibility to analyze viral integrated DNA within structurally intact nuclei of individual cells allowed studying the topology of HIV-1 in the nuclear compartment. This analysis revealed that at 48 h postinfection, HIV-1 is preferentially localized in the nuclear periphery. The observed spatial distribution is consistent with the one obtained by analyzing fluorescently labeled HIV-1 protein complexes at the nuclear level (14). It is interesting to note that the same analysis performed at day 13 from infection revealed a relocalization of proviral DNA toward the center of the nucleus with a random distribution in the nuclear compartment. The localization of PICs and provirus in the periphery of the nucleus at early time points strongly suggests that the virus integrates soon after its transition through the nuclear envelope. Nevertheless, once the HIV-1 genome is stably integrated into the host peripheral chromatin, its radial distribution substantially changes over time probably owing to a rearrangement of the chromosome territory where the integration occurred. We observed that in cells engineered to retarget integration toward nonphysiological integration spots (CBX-LEDGF325-530), proviral DNA does not show any preferential nuclear distribution. These observations clearly indicate that in addition to a linear preference of HIV-1 toward gene-rich regions of the chromatin (36), the virus also acquires a specific spatial orientation within the nuclear compartment.

**Fig. 6.** Analysis of the proviral DNA retargeting. (A) Distribution of the distances of HIV-1 γ-H2AX foci from the nuclear lamin in HeLa control cells (black bars) and in cells expressing CBX-LEDGF325-530 (blue bars) (P = 2.147e-06, KS test). (B) Data are represented in bar graphs as the percentage of γ-H2AX foci in the three concentric zones of equal surface area as in Fig. 5B.
Nevertheless, the presence of nonintegrated viral forms can be indirectly reported in γ-H2AX negative cells by the presence of the GFP expressed from the nonintegrated viruses. Therefore, by combining GFP and SCIP it is possible to determine three different infectivity conditions: (i) HIV-1 infection leading to complete integration revealed by cells positive for both γ-H2AX and GFP signals, (ii) viral DNA reaches the nuclei of the cells but integration is impaired revealed by cells negative for γ-H2AX and positive for GFP owing to the expression of the nonintegrated DNA, and (iii) absence of viral DNA in the nuclei as a result of replication impairments upstream from nuclear import, revealed by the lack of both GFP and γ-H2AX signals.

An additional important feature of SCIP is the possibility to analyze individual infected cells without culture clonal selection, as opposed to the bulk analysis performed with conventional approaches. This advancement of the SCIP technique revealed that cells are heterogeneously infected showing different densities of HIV-1 genomes per cell (Figs. S4 and S5). This observation is consistent with former reports showing that clones derived from the same infection display variable numbers of proviruses (38, 39).

The power of SCIP to monitor expression/integration in individual cells might be key to investigate the still obscure mechanisms leading to HIV-1 latency (40). Taken together, these data strongly demonstrated that SCIP is a robust and clear-cut readout for understanding HIV-1 replication in details such as cellular conditions or factors affecting HIV-1 nuclear entry and the topography of integration at the individual cell level. The technical advantages and the multiple endpoints detection provided by this system are expected to contribute as a powerful tool also for large-scale analysis. In fact, high content analysis of γ-H2AX foci was reported (41). Similarly, by coupling SCIP to a high-throughput fluorescence microscopy platform, the technique here reported may become a systematic qualitative and quantitative screen for HIV-1 potential cofactors and antiretroviral drugs.

Materials and Methods
For a detailed description, please refer to SI Materials and Methods. It includes detailed procedures for confocal microscopy imaging acquisition and analysis. It also includes description of constructs engineering, virus production, cell culture conditions, and immunofluorescence procedures.

ACKNOWLEDGMENTS. The authors are grateful to the laboratory of W. Thys and M. Giacca for valuable discussion and to A. Calvello for technical assistance. This work was supported by grants from the European Union Seventh Framework Programme (THINC, Health-2008-201032), by the Istituto Superiore di Sanità Italian AIDS Program (Grant 40H90), by the Provincia Autonoma di Trento (COPUND Project, Team 2009 – Incomings), and by FIRB 2008 Futuro in Ricerca (RBFR08BSWG).

Supporting Information

Di Primio et al. 10.1073/pnas.1216254110

SI Materials and Methods

Cells and Viruses. U2OS and HeLa control cells were cultured in DMEM (GIBCO) 10% (vol/vol) FCS; CBX1-L6EDGF325-530 cells were cultured as described in Gjibser et al. (1). CEMs cells were cultured in RPMI (GIBCO) 10% (vol/vol) FCS. Lentiviral vectors HIV-CMVGF-I-SceI, HIV-CMVΔGFP-I-SceI, and HIV-CMVGF were produced by transient transfection of 293T cells by using 150 nM polyethyleneimine (PEI) reagent (Sigma) with the pHR-CMVGF-I-SceI or pHR-CMVΔGFP-I-SceI or pHRCMV-GFP plasmids, together with the Δ8.91 packaging and a VSV-G envelope expressing plasmids. The integration defective virus was produced by using the Δ8.91pND16A packaging plasmid. Viral particles were concentrated by 2 h of ultracentrifugation in 20% (wt/vol) sucrose cushion and vector titers were measured by reverse transcriptase activity (RTU) (2). Infecivity was determined by FACS analysis (FACSCalibur; BD Bioscience) for GFP expression at 36–48 hours post infection.

Transfections and Infections. U2OS cells, 10,000 per well, were seeded in a four-well chamber slide (Lab-Tek) and the day after were transfected using Effectene (QIAGEN) with 200 ng of pCBASce plasmid encoding the I-SceI endonuclease. Six hours posttransfection, cells were infected with different RTUs in Opti-Mem (GIBCO) 1% FCS for 2 h and fixed at indicated time points. To detect foci 13 d postinfection, cells have been transfected with the pCBASce plasmid 48 h before fixation. The pHRCMVGF-I-SceI stable clones cells were produced by infecting with pseudotyped HIV-CMVGF-I-SceI lentiviral particles or by cotransfecting pHRCMVGF-I-SceI and pCDNA3 plasmids and selection with G418 (Roche).

RNA Interference and Antiretroviral Drugs. U2OS cells (200,000) were seeded in a six-well plate 24 h prior transfection with siRNA labeled with 3′ Alexa Fluor 546 (QIAGEN) against TRN-SR or mismatch by using Gene Silencer kit (Genelantis). At 24 h posttransfection, cells were detached and seeded in chamber slides (Lab-TekTM) for transfection with pCBASce plasmid. Six hours posttransfection with pCBASce, plasmid cells were infected with HIV-I-SceI viral particles. Medium was replaced 2 hpi and cells were fixed 24 hpi. Antiretroviral drugs dissolved in DMSO have been added to cells during and after the infection.

Immunofluorescence and Foci Quantification. Cells were fixed with 4.0% (wt/vol) paraformaldehyde in PBS 1× for 5 min at room temperature at the indicated time postinfection. After permeabilization with PBS 1× containing 0.2% Triton-X100 for 10 min, samples were blocked overnight with 3% (wt/vol) BSA at 4 °C. The slides were incubated with the primary antibody 1 h at room temperature and with secondary antibodies fluorophore-conjugated 1 h at room temperature. Slides were mounted with Vectashield mounting medium (Vector Laboratories). Nuclear fluorescence signal from γ-H2AX foci was acquired with the TCS SL laser-scanning confocal microscope (Leica Microsystems) equipped with galvanometric stage using a 63×/1.4 NA HCX PL APO oil immersion objective and processed by image software for automated object counting (ImageJ, NIH). The γ-H2AX foci have been counted from single optical sections taken in the central regions of the nuclei or quantified in the whole volume of the nucleus, reconstructed by Imaris (Bitplane) from 3D stacks of the cells acquired with a z step of 0.3 μm. Total fluorescence of γ-H2AX foci has been measured in nuclear compartment from 2D sections of the nucleus after subtraction of the nuclear background. For each experiment at least 100 cells were captured and every experiment was repeated at least three times.

Antibodies. Primary antibodies for Western blot analysis were: polyclonal anti-I-SceI and monoclonal anti-α-Tubulin (Santa Cruz Biotechnology). Secondary antibodies HRP conjugated anti-mouse and anti-rabbit IgG were purchased from Santa Cruz Biotechnology. Primary antibodies for IF were: anti-γ-H2AX (Millipore), 53BP1 (SIGMA), RAD51 (Abnova), and LamB1 (Abcam). Secondary antibodies fluorophore conjugated were anti-mouse and anti-rabbit Alexa Fluor 633 and 488 conjugated IgG (Molecular Probes).

ChIP Assay. pHR-CMVGF-I-SceI stable cell clones plated in 150-mm dishes were transfected by pCBASce plasmid using Effectene (QIAGEN). At 48 h posttransfection, each cell plate was treated with 1% formaldehyde for 10 min to crosslink proteins to DNA. Glycine 125 mM was added to quench the reaction. Cells were collected using a cell scraper and processed by EZ-ChIP Kit (Millipore). Internal controls as anti-rRNA pol II and normal mouse IgG have been provided by EZ-ChIP Kit (Millipore).

Real-Time Quantitative PCR. The copy numbers of the integrated HIV-1 vector (pHR-CMVGF-I-SceI) in U2OS stable cell clones were quantified by real-time quantitative PCR using late RT primers MH531 and MH532 and LTR-P probe as previously described (3). A kinetic PCR assay for human β-globin DNA was used to normalize the total DNA as previously described (4). The primer pairs close to the I-SceI restriction site are: FwdA201 CTCGAGACCTAGAAAAAATGGAGACCA, RevA33 GTGGCTAAAGATCTAGAGCTGGCT, FwdB191 AACCATCTACGCTGGACCCCA, RevB 23 TACCTGTGACGGGAAGATCACTTGCG. Real-time quantitative PCR of ChIP samples were carried out by using SYBR green Master Mix (BIORAD). GAPDH primers have been provided by EZ-ChIP Kit (Millipore).

Statistical Data Analysis and Background Correction. Differences in the distributions of the number of foci were assessed using the nonparametric two-tailed Kolmogorov–Smirnov (KS) test, in which the maximum value of the absolute difference (D) is evaluated between the two cumulative distribution function of the number of foci. Results and comparison were based on three independent biological samples and sample sizes exceeded n > 100, unless otherwise indicated (Figs. S5 and S6). The primary analysis and the P values are reported in the manuscript figure legends. Background number of γ-H2AX foci was quantified in noninfected cells expressing the I-SceI enzyme and subtracted in each individual experiment from the number of foci quantified in cells infected with the HIV-CMVGF-I-SceI. In experimental conditions of low infectivity, the background subtraction may generate negative values (Fig. S5). To avoid misinterpretations we omitted the negative values from the graph and noted with (*) in Figs. 2B and 3D. The omitted values are as follows: in Fig. 2B: 24 hpi = -0.69 ± 0.55; in Fig. 3D: AZT 10 μM = -0.17 ± 0.66, Raltegravir 100 nM = -0.32 ± 1.1. Raltegravir 1 μM = -0.06 ± 0.62, CX05045 10 μM = -1.94 ± 0.77.

Di Primio et al.  www.pnas.org/cgi/content/short/1216254110 1 of 7

**Fig. S1.** Viral DNA digestion. Digestion of pHRCMVΔGFP, pHRCMVΔGFP-I-SceI, and pHRCMVΔGFP-I-SceI plasmids by I-SceI enzyme and the single cutter Nhel. This demonstrates that I-SceI, as Nhel, cuts a single site in the indicated plasmids.
Transfected clones

Fig. S2. Stable clones characterization and I-SceI expression. (A) Quantification by qPCR of pHR-CMVGFPI-SceI DNA copies integrated into stable U2OS cell clones produced by transfection of the pHR-CMVGFPI-SceI plasmid (means ±SEM from at least three independent experiments). (B) Quantification by IF of the number of γ-H2AX foci in U2OS transfected stable cell clones after overexpression of I-SceI endonuclease (means ±SEM from at least three independent experiments). (C) Expression of I-SceI endonuclease in U2OS-8 clone 48 h posttransfection with pCBASce plasmid. (D) Quantification by qPCR of HR-CMVGFPI-SceI DNA copies integrated into stable U2OS cell clones produced by antibiotic selection after infection of VSV-G pseudotyped HR-CMVGFPI-SceI viral supernatants (means ±SD from two independent experiments). (E) Quantification by IF of the number of γ-H2AX foci in U2OS stable cell clones after overexpression of I-SceI endonuclease (means ±SD from two independent experiments).
Fig. S3.  Foci detection in control experiments. Additional controls related to Fig. 2A. The γ-H2AX foci detection in cells infected with pHR-CMV GFP virus either transfected or not transfected with pCBASce plasmid. Nuclear middle z stacks are shown. Scale bars, 10 μm.

Fig. S4. Analysis for HIV-1 γ-H2AX foci quantification. (A) The z sectioning of an infected nucleus; γ-H2AX foci are in red, nuclear lamin is in blue. Scale bar is 10 μm. The middle slice is white squared. (B) Comparative analysis of 2D quantification (γ-H2AX foci scoring in the middle slice of the nuclear z stack), 3D quantification (nuclear volume reconstruction and automated γ-H2AX foci scoring), and total γ-H2AX foci fluorescence (measuring the fluorescence of the middle slice of the nuclear z stack). Quantification (fold increase) was performed in U2OS cells infected with 0.04 RTU (white bars), 0.4 RTU (gray bars), or 4 RTU (black bars) of HIV-CMV GFP-I-SceI vector. (C) Box plot (spanning the interquartile range) of raw data from 2D quantification of three different experiments (A–C). Number of γ-H2AX foci in control cells (I-SceI+, noninfected) and in cells infected with 0.04–0.4–4 RTU (I-SceI+). (D) Box plot of the same data in C after subtraction of the relative background from each individual experiment. (E) Cumulative box plot combining the three independent data set (normalized for background) from D.
Fig. S5. Raw data. (A) Box plot (spanning the interquartile range) of raw data related to Fig. 2 B and C. Background γ-H2AX foci are indicated as bg. (B) Box plot of raw data related to Fig. 3 B and D. Background γ-H2AX foci are indicated as bg.
Fig. S6. (A) Colocalization of HIV-1 γ-H2AX foci (red spots) and Rad51 (green spots, Upper) and 53BP1 (green spots, Lower) in nuclei of infected cells. Nuclear middle z stacks are shown. Scale bars, 10 μm. (B) The γ-H2AX foci in transportin-SR2 knockdown cells. Transportin-SR2 knockdown does not interfere with H2AX phosphorylation after DNA damage. Transportin-SR2 knockdown cells (siRNA-SR2, Right) and control cells (siRNA-MM, Left) were treated by NCS to induce DNA DSBs. Fluorescent siRNA-SR2 and siRNA-MM are labeled in blue and γ-H2AX foci in yellow. Nuclear middle z stacks are shown. Scale bars, 10 μm.

Fig. S7. Quantification of GFP positive cells in cells treated with antiretroviral drugs and analyzed by FACS. Percentage of GFP expressing cells are normalized on infected untreated cells (means ±SEM from at least three independent experiments).
Analysis of the spontaneous foci nuclear localization. (A) Distribution of the distances of HIV-1 γ-H2AX foci and background foci (bg) from the nuclear lamin in U2OS cells; \([P = 3.119e-08]\) Kolmogorov–Smirnov test (KS test). (B) Distribution of the distances of HIV-1 γ-H2AX foci and background foci (bg) from the nuclear lamin in CEMss cells; \([P = 4.078e-05]\) KS test. (C) Distribution of the distances of HIV-1 γ-H2AX foci and background foci (bg) from the nuclear lamin in HeLa control cells; \([P = 4.778e-10]\) KS test. (D) Distribution of the distances of HIV-1 γ-H2AX foci and background foci (bg) from the nuclear lamin in CBX-LEDGF\(_{325-530}\) cells; \([P = 0.0068]\) KS test.