Associate HIV-1 envelope glycoprotein structures with states on the virus observed by smFRET

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The HIV-1 envelope glycoprotein (Env) trimer mediates cell entry and is conformationally dynamic1,4. Imaging by single-molecule fluorescence resonance energy transfer (smFRET) has revealed that, on the surface of intact virions, mature pre-fusion Env transitions from a pre-triggered conformation (state 1) through a default intermediate conformation (state 2) to a conformation in which it is bound to three CD4 receptor molecules (state 3)9,10. It is currently unclear how these states relate to known structures. Breakthroughs in the structural characterization of the HIV-1 Env trimer have previously been achieved by generating soluble and proteolytically cleaved trimers of gp140 Env that are stabilized by a disulfide bond, an isoleucine-to-proline substitution at residue 559 and a truncation at residue 664 (SOSIP.664 trimers)5,11–18. Cryo-electron microscopy studies have been performed with C-terminally truncated Env of the HIV-1JR-FL strain in complex with the antibody PGT15119. Both approaches have revealed similar structures for Env. Although these structures have been presumed to represent the pre-triggered state 1 of HIV-1 Env, this hypothesis has never directly been tested. Here we use smFRET to compare the conformational states of Env trimers used for structural studies with native Env on intact virus. We find that the constructs upon which extant high-resolution structures are based predominantly occupy downstream conformations that represent states 2 and 3. Therefore, the structure of the pre-triggered state 1 conformation of viral Env that has been identified by smFRET and that is preferentially stabilized by many broadly neutralizing antibodies—and thus of interest for the design of immunogens—remains unknown.

To compare the conformational states of gp120 in Env trimers on the surface of virions of the BG505 subtype of HIV-1 (HIV-1BG505) with gp120 in soluble gp140 SOSIP.664 trimers of HIV-1BG505 (hereafter, BHIV-1BG505 gp140 SOSIP664), we used enzymatic and non-natural amino acid strategies to site-specifically introduce donor and acceptor fluorophores in the variable regions V1 and V4 of gp120 at exactly the same positions (Extended Data Fig. 1a). Positions in the HIV-1BG505 Env at which introduced tags do not disrupt Env processing and virus incorporation, infectivity or sensitivity to neutralization by Env-specific antibodies have previously been identified19,20. Tags that were introduced at identical positions into BHIV-1BG505 gp140 SOSIP664 and DS-SOSIP.Mut4 (a further-stabilized variant of BHIV-1BG505 gp140 SOSIP664)20 also had a negligible effect on antigenicity or the ability to assemble into compact trimers (Extended Data Fig. 1b, c). The tags enabled site-specific enzymatic incorporation of donor and acceptor fluorophores into gp120 on the surface of intact viruses2. We prepared complete virus that carried—on average—one double-labelled HIV-1BG505 Env molecule per particle in the context of wild-type HIV-1BG505 Env9 (Fig. 1a), and BG505 gp140 SOSIP.664 trimers that carried—on average—one double-labelled protomer per trimer (Fig. 1b). Labelled viruses or trimers were immobilized within microfluidic sample chambers for total internal reflection smFRET imaging (Methods).

The observed anti-correlated relationship between donor and acceptor intensities, and resulting changes in FRET efficiency, were consistent with discrete motions of the V1 and V4 regions relative to each other within individual gp120 monomers (Fig. 1c, d). Histograms, comprised of smFRET data that were obtained from an ensemble of imaged virus, revealed three FRET states that were indicative of three major conformations of gp120 (Fig. 1e, f). Similar to Env from HIV-1JR-FL and HIV-1NL4-3 isolates8, the native HIV-1BG505 Env predominantly occupied the low-FRET state 1 conformation (Fig. 1e). The native HIV-1BG505 Env spontaneously sampled both state 2 and state 3, which are characterized by high- and intermediate-FRET values, respectively (Extended Data Fig. 4c). By contrast, the BG505 gp140 SOSIP.664 trimers—even though they accessed similar conformations—predominantly occupied state 2 (Fig. 1f). Similar results were observed for BG505 gp140 DS-SOSIP.Mut4, and when using SOSIP664 produced by a different laboratory (Fig. 1g).

Collectively, these data indicate that engineered gp140 SOSIP.664 trimers are stabilized in conformations that more closely resemble the state 2 conformation than the state 1 conformation that predominates on intact virus particles. Cryo-electron microscopy studies of mature HIV-1JR-FL Env lacks the cytoplasmic tail (HIV-1JR-FL Env(ΔCT)), in complex with PGT15119, (Extended Data Fig. 2a) have revealed a structure that is similar to that of the BG505 gp140 SOSIP.664 trimer. We therefore used smFRET to examine the conformational state of HIV-1 Env bound to PGT151. The deletion of the cytoplasmic tail had little or no effect on the conformational landscape of Env (Extended Data Fig. 2b). However, PGT151 induced a notable shift in both HIV-1JR-FL Env and HIV-1BG505 Env from a conformation similar to state 1 to a conformation similar to state 2 (Fig. 1h, Extended Data Fig. 2c–g). Similar results were obtained for variants of HIV-1JR-FL Env and HIV-1BG505 Env that do not rely on the labelling tags (Extended Data Fig. 2h–l). In this case, fluorophores were ‘clicked’ onto unnatural amino acids that were introduced through
the suppression of amber codons21. Thus, the gp120 conformations that are predominantly exhibited by both the BG505 sgp140 SOSIP.664 trimer and the PGT151-bound HIV-1 BG505 Env differ from the predominant state-1 conformation of the virus-resident Env, and instead resemble state 2.

To understand the predominance of the state-2 conformation in BG505 sgp140 SOSIP.664, we introduced the SOSIP changes (that is, the disulfide bond and I559P substitution)—both separately and in combination—into the native HIV-1 BG505 Env (Fig. 2a). Consistent with previous reports22,23, both changes abrogated infectivity without altering Env processing or virus incorporation (Extended Data Fig. 3a, b). smFRET showed that, in combination, the changes stabilize a state-2-like conformation in membrane-bound Env; this effect was largely due to the disulfide bond, both in HIV-1 BG505 Env and in HIV-1 JR-FL Env (Fig. 2a, Extended Data Fig. 3c).

We next tested whether BG505 sgp140 SOSIP.664 retains the conformational plasticity of the native HIV-1 BG505 Env in response to ligands specific to state 3 or state 1. Consistent with previous observations10, the mature pre-triggered HIV-1 BG505 Env transitioned into the state 3 conformation upon addition of soluble dodecameric CD4 (sCD4) + IgG (also called 12×CD4), which consists of domains D1 and D2 of CD4 fused at the C-terminus to the immunoglobulin G (IgG) 1 heavy chain and immunoglobulin A secretory tailpiece) (Fig. 2b, Extended Data Fig. 3d, e, i, 4c, d). The BG505 sgp140 SOSIP.664 similarly transitioned into a state-3-like conformation upon addition of sCD4 + IgG (Fig. 2c, Extended Data Fig. 4a, f, g). This observation is consistent with the large CD4-induced displacements between V1 and V4 that have previously been deduced from structural comparison of BG505 sgp140 SOSIP.664 and B41 sgp140 SOSIP.664 trimers in unliganded states, and in complex with monomeric soluble CD44,5. By contrast, addition of the allosteric inhibitor BMS-378806 failed to re-equilibrate the conformational landscape of BG505 sgp140 SOSIP.664 towards state 1, whereas this compound further stabilized the mature HIV-1 BG505 Env in state 1 (Extended Data Fig. 3h, i, m). BG505 sgp140 SOSIP.664 was observed to exhibit a modest enrichment in a state-1-like conformation only upon addition of the more-potent analogue BMS-626529—at a 1,000-fold excess over its mean 95% inhibitory concentration (Fig. 2b, c, Extended Data Figs. 3j, k, n, 4b, e, h). These results indicate that state 1 is destabilized in BG505 sgp140 SOSIP.664, which probably explains why ligands specific to

**Fig. 1** HIV-1 Env on the surface of viruses, or in complexes characterized structurally at high resolution, reside in distinct conformational states. **a, b,** Experimental approach. Membrane-bound HIV-1 trimer on chemically inactivated virus, depicted by cryo-electron tomography at a resolution of about 20 Å (left, side view; right, top view) (a), or Env proteins used to obtain high-resolution structures (BG505 sgp140 SOSIP.664 or HIV-1 JR-FL Env) in complex with PGT15119) (b) were double-labelled in a single protomer in V1 with Cy3 (green) and in V4 with Cy5 (red), and analysed by smFRET. HIV-1 JR-FL Env (ΔCT) in complex with PGT151 and unliganded BG505 sgp140 SOSIP.664 are adapted from RCSB Protein Data Bank accessions 5FUU (Env protomers, orange; PGT151, light blue) and 4ZMJ (magenta), respectively. **c, d,** Example of fluorescence traces of unliganded HIV-1 BG505 Env on the surface of an intact virus (c) and BG505 sgp140 SOSIP.664 (d) that carry fluorophores at identical positions within V1 and V4 of gp120. Top, donor Cy3 in green and acceptor Cy5 in red; bottom, resulting FRET in blue and hidden Markov model idealization in red. Arrows indicate single-step photobleaching events that define the background of our smFRET assay. **e,** Unliganded HIV-1 BG505 Env predominantly resides in state 1. FRET histogram compiled from 180 HIV-1 BG505 Env FRET traces and fitted curve (red) for three confined Gaussian distributions (black) centred at 0.1 (low FRET, state 1), 0.33 (intermediate FRET, state 3) and 0.65 (high FRET, state 2). **f,** BG505 sgp140 SOSIP.664 predominantly samples state-2-like conformations. Experiment as in e, conducted with unliganded BG505 sgp140 SOSIP.664. **g,** BG505 sgp140 SOSIP.664 variants with V3-negative selection (Extended Data Fig. 1d) retain a state 2 dominance that is similar to that of BG505 sgp140 SOSIP.664 (f), and all differ from the HIV-1 Env (e). **h,** Binding of PGT151 at neutralizing concentrations (10 μg ml−1) stabilizes a state-2-like conformational state of HIV-1 BG505 Env. Histograms represent mean ± s.e.m., determined from three independent populations of smFRET traces. Number of FRET traces are indicated. State occupancies and determining parameters are listed in Extended Data Table 1.
Fig. 2 | Stabilizing disulfide bond and I559P mutations stabilize state 2. a, The stabilizing disulfide bond is largely responsible for the stabilization of Env in state 2. Schematic shows the structure-stabilizing modifications A501C and T605C (SOS) and I559P (I559P) that were used in the design of BG505 sgp140 SOSIP.664, when introduced into HIV-1 Env on the surface of virus. FRET histograms of unliganded HIV-1 BG505 Env carrying A501C, T605C and I559P substitutions (top left, HIV-1 BG505 SOS; top right, HIV-1 BG505 I559P) changes, respectively. b, FRET histograms of HIV-1 BG505 Env in the presence of sCD4D1D2–IgGptp (top) and entry inhibitor BMS-626529 (bottom). c, Experiments as in b, for BG505 sgp140 SOSIP.664. FRET histograms represent mean ± s.e.m., determined from three independent groups of smFRET traces. The FRET histograms of liganded HIV-1 BG505 Env and BG505 sgp140 SOSIP.664 in b and c can be compared with those of the unliganded Env in Fig. 1e and Fig. 1f, respectively.

state 1—including both BMS inhibitors—have not been observed to stabilize the Env trimer in conformations that are distinct from the conformation that is observed in their absence.

Broadly neutralizing antibodies directed towards the V2 apex (PG16 and PGT145), the CD4–binding site (VRC01) and the glycan-V3 site (PGT122, PGT128 and 2G12)—as well as the entry inhibitor BMS-626529—have previously been observed to stabilize the Env of HIV-1 NL4-3 and HIV-1 JR-FL virions in a state 1 conformation. We tested additional broadly neutralizing antibodies (3BNC117 and 10-1074) that bind different epitopes on HIV-1 BG505 (Extended Data Fig. 5a–c) and that suppress virus replication in animal models and in individuals infected with HIV-1. The binding of 3BNC117, 10-1074 and PG9 to HIV-1 Env resulted in a dominant state 1 conformation (Fig. 3a–d, Extended Data Fig. 5d). By contrast, the non-neutralizing antibodies F105 and 17b induced a state 3 conformation (Extended Data Fig. 6).

Fig. 3 | Many broadly neutralizing antibodies exhibit a preference for state 1 of HIV-1 Env, whereas cow antibodies exhibit a preference for state 2. a, b, Conformational landscape upon binding of the broadly neutralizing antibody 10-1074 (50 μg ml−1) to HIV-1 BG505 Env. a, Sample trace (arrows defined as in Fig. 1). b, Histogram. FRET histogram of the unliganded HIV-1 JR-FL virus Env is in red.

c, d, Experiments as in a, b, for the broadly neutralizing antibody 3BNC117 (50 μg ml−1) binding to HIV-1 BG505 Env. c, Sample trace (FRET in blue, hidden Markov model idealization in red) and histogram (f) of NC-Cow9 antibody preference for state 2 on HIV-1 (T332N) virus Env. This conformational profile largely resembles that of BG505 sgp140 SOSIP.664, which was used as an immunogen to elicit the NC-Cow9 antibody. FRET histograms represent mean ± s.e.m., determined from three independent populations of smFRET traces.

We next tested the conformational preferences of antibodies elicited in cows using the state-2-like immunogen, BG505 sgp140 SOSIP.664. Interestingly, each of the monoclonal antibodies (NC-Cow1, NC-Cow8, NC-Cow9 and NC-Cow10) isolated from immunized cows shifted the conformational landscape of HIV-1 BG505 Env towards state 2 (Fig. 3e–f, Extended Data Fig. 7a–h). Notably, the conformational landscape of HIV-1 Env bound by the NC-Cow9 antibody was indistinguishable from that of unliganded BG505 sgp140 SOSIP.664 (Fig. 3f). Thus, consistent with a state-2-like conformation of gp120, BG505 sgp140 SOSIP.664—as an immunogen in cows—elicits state-2-specific antibodies.

We next tested whether state 1 or state 2 can be detected using classic antibody staining of cell-expressed HIV-1 Env, followed by flow cytometry. HIV-1 JR-FL, Env(DCT) that was pre-bound with BMS-626529, which stabilizes state 1, exhibited no decrease—or a slight increase—in the binding of PGT122 and 10-1074 antibodies, which prefer state 1 (Extended Data Fig. 7i). By contrast, pre-binding of ligands that prefer state 1 reduced the binding of the antibodies PGT151 and NC-Cow9, which are specific to state 2 (Extended Data Fig. 7i). Conversely, whereas the state-2-specific antibodies NC-Cow9 and PGT151 did not interfere with one another, the pre-binding of ligands specific to state 2 reduced the binding of ligands specific to state 1 (Extended Data Fig. 7j). Therefore, the distinct state 1 or state 2 preferences for Env by ligands can also be detected by conventional bulk measurements.

We considered the potential caveats of the tagging strategies and fluorophores used in the smFRET approach, but we did not observe evidence of abnormal dye behaviour or photophysical effects (Extended Data Fig. 8, Methods, Supplementary Table 1). Control experiments in which the positions of donor and acceptor fluorophores were reversed (Extended Data Fig. 8b–d) and fluorophores were attached...
via unnatural amino acids rather than peptide epitope tags also revealed nearly identical smFRET histograms (Extended Data Fig. 2h–i). We further validated the conclusions of the smFRET approach by assessing HIV-1 Env conformations and dynamics from a distinct structural perspective. Here we measured Env conformational dynamics using a click-labelled unnatural amino acid at residue 542 (Arg542TAG) within gp41, in combination with the A1 enzymatic labelling tag in the gp120 V4 region (V4–A1) (Extended Data Fig. 9a). Compared with the V4V4-labelled HIV-1JR-FL Env—in which the unliganded Env predominantly resides in a low-FRET conformation (Fig. 4a, b)—the unliganded HIV-1JR-FL Env labelled in V4–A1 of gp120 and on Arg542TAG in the α6 helix of gp41 (α6–Arg542TAG) largely exhibited a high-FRET state (Fig. 4c, d). The PGT151 ligand (which is specific to state 2) stabilized an intermediate-FRET state, and the 12×CD4 ligand (which is specific to state 3) stabilized a low-FRET state in the V4–A1, α6–Arg542TAG-labelled populations of smFRET traces. e–j, Temporal progression of the Env conformational landscape, monitored from both perspectives, upon binding of the potent bifunctional eCD4-Ig(Q40A, mim2) ligand at 100 μg ml⁻¹ (Extended Data Fig. 9c). Conceptual presentation of labelling positions (e, f), three-dimensional time-resolved FRET histograms (g, h, two replicates) and relative state occupancies observed over 60 min (i, j) of click-labelled HIV-1JR-FL, V1–Asn136TAG, V4–A1 and V4–A1 α6–Arg542TAG upon addition of eCD4-Ig(Q40A, mim2). FRET data from 15-min intervals were combined into three-dimensional time-correlated histograms. Relative state occupancies (b, d, i, j) are presented as mean ± s.e.m., derived from histograms (a, c, g, h), respectively. The determining parameters are listed in Extended Data Table 1.
These findings collectively suggest that extant high-resolution structures of the HIV-1 Env trimer closely resemble those of the state 2 intermediate, which is only transiently populated on the surface of native virus. The structure of pre-triggered state 1 remains unknown. Although state 2 is on a path to HIV-1 Env activation, state 1 is the predominant Env conformation that is found on the surface of most primary, and transmitted/founder HIV-1 isolates. Importantly, the structurally uncharacterized state 1 Env is a biologically relevant conformation that is preferentially bound by many broadly neutralizing antibodies. Evidence that the BG505 sgp140 SOSIP.664 proteins are in a conformation that is distinct from the native Env is also emerging from cross-linking studies. These findings warrant focused initiatives to define the structure, molecular determinants and capacity of the state-1 conformation of Env to elicit broadly neutralizing antibodies.

Online content
Any methods, additional references, Nature Research reporting summaries, source data, data sets, statements about data availability and associated accession codes are available at https://doi.org/10.1038/s41586-019-1101-y.

Received: 31 December 2017; Accepted: 8 March 2019; Published online 10 April 2019.

Acknowledgements
We thank A. B. Ward, R. W. Sanders and P. J. Bjorkman for discussions, R. Blakemore for assistance with molecular modelling, D. Burton and M. Feinberg for reagents including PG121, PGT122, PGT145, PGT151 and NC-Cow1, NC-Cow8, NC-Cow9 and NC-Cow10 antibodies, and the AIDS Research and the Reference Reagent Program (Division of AIDS, NIAID, NIH) for the antibodies BN3101, 10-1074, PG9 and PQ16. This work was supported by NIH grants GM116654 and U1AI100645 to W.M., R01 GM098859 to S.C.B., R01 AI124982 and R01 AI100645 to J.G.S., K22 AI116262 to J.B.M., CRC Tier 2 RCHS0235 and a CHIR foundation grant 352417 to A.F., R01 GM065650 to W.M., J.G.S., S.C.B. and A.B.III, by a Brown Core Fellowship to M.L., a fellowship from the China Scholarship Council-Yale World Scholars to X.M., by the International AIDS Vaccine Initiative’s (IAVI’s) Neutralizing Antibody Consortium to P.D.K. and by the Intramural Research Program of the Vaccine Research Center (NIAID, NIH) to P.D.K. and A.B.M., and by the SFB1129 and the Emmy-Noether programme (project number 317530061) of the German Research Foundation to E.A.L. and N.-S., respectively.

Reviewer information
Nature thanks David Millar, Alexandra Tkola and the other anonymous reviewer(s) for their contribution to the peer review of this work.

Author contributions
M.L., X.M., L.R.C.-M., J.G.S., W.M. and I.N.-S. contributed equally to this work. M.L., X.M., L.R.C.-M., N.A., J.G., J.B.M., A.F., P.D.K., S.C.B., J.G.S. and W.M. conceived these experiments. A.S., J.A., A.B.S.III, I.N.-S., E.A.L., M.R.G. and M.F. provided reagents. J.B.M. and C.A. performed photophysical measurements and molecular dynamics simulations. A.S., J.A., A.B.S.III, I.N.-S., respectively.

Competing interests
S.C.B. holds an equity interest in Lumindyne Technologies. M.F. is a co-founder of Emmune, a company developing eCD4-lg for clinical use. E.A.L holds the patent WO 2012/104422 A1. S.C.B., J.B.M. and W.M. hold the patent US 9593853 B2.

Additional information
Extended data is available for this paper at https://doi.org/10.1038/s41586-019-1101-y.

Supplementary information is available for this paper at https://doi.org/10.1038/s41586-019-1101-y.

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METHODS

No statistical methods were used to predetermine sample size. The experiments were not randomized and investigators were not blinded to allocation during experiments and outcome assessment.

Preparation of tagged viruses. HIV-1BG505, HIV-1JR-FL and HIV-1NL4-3 viruses with Env that is double-tagged at V1–Q3 and V4–A1 were prepared for smFRET imaging, as previously described. The double-tagged V1–Q3 V4–A1 Env carries a 6-aminocaproic acid (Glu) tag at variable loop V1 and a 12-aminocidoic acid A1 tag in variable loop V4 (Q3, GQQQQL; A1, GDSLDMLEWSLM). HIV-1BG505, HIV-1JR-FL and HIV-1NL4-3 viruses with tagged Env were functionally validated before use.

For smFRET imaging, HIV-1 viruses were prepared as replication-incompetent particles that lack reverse transcriptase (ΔRT). A 40:1 ratio of wild-type gene encoding the tagged Env was used to ensure that on average only one tagged protomer was available for imaging on a single virus particle. Then, enzymatically tagged HIV-1BG505 virus was generated by co-transfecting HEK293 cells with the same amount of the pNL4-3ΔRT plasmid and tagged Env to pseudotype a pNL4-3ΔRT backbone with HIV-1Env (Extended Data Fig. 2h, middle). A 40:1 plasmid ratio of Env expressing wild-type JR-FL gp160 pCAGGS and V1–Q3 and V4–A1 tagged JR-FL gp160 was co-transfected into HEK293 cells, along with the same amount of the pNL4-3ΔEnv ΔRT plasmid. Double-tagged HIV-1JR-FL Env(ΔCT) was generated as above, with plasmids that encode JR-FL Env(ΔCT) and double-tagged JR-FL Env(ΔRT). Tagged HIV-1ΔRT Env mutants that contain A501C and T656C (SO5, 1559P or SO5 and 1559P; and HIV-1ΔRT (Extended Data Fig. 1a) were prepared using the same ratio 40:1 of non-tagged Env SO5 (or 1559P, or SO5 and 1559P) to Env mutant SO5 (or 1559P, or SO5 and 1559P) and double-tagged with V1–Q3 and V4–A1. Double-tagged full-length HIV-1NL4-3 was made by co-transfecting HEK293 cells with a 40:1 ratio of wide-type, full-length HIV-1 NL4-3 ΔRT plasmid to double-tagged HIV-1 V1–Q3 and V4–A1 pNL4-3 ΔRT plasmid.

Labelled HIV-1BG505 and HIV-1JR-FL Viruses that carry dyes at single point substitutions (instead of enzymatic labelling peptides) were generated by suppression of amber (TAG) stop codons to introduce a clickable unnatural amino acid, followed by copper-free click chemistry to introduce the fluorophore13. Our system is based on an amber suppressor tRNA and the Y396A Y384F mutant of the Methanosaurocica mazei pyrroline aminocycl-tRNA synthetase (PyRfSAF) that accepts the clickable unnatural amino acid trans-cycloclooct-2-ene lysine (TCCO*, Sichem)21. In our laboratory, generation of HIV-1 viruses carrying Env with amber-suppressed clickable unnatural amino acids can be accomplished in one of three ways: (1) by using an engineered amberless full-length HIV-1BG505 Q23 genome that lacks all other TAG stop codons (a single TAG amber stop codon is removed from the genome that lacks Env). For example, HIV-1BG505 viruses labelled at Ser401TAG in V4 were produced by transfecting an engineered amberless full-length HIV-1BG505 bi-cistronic plasmid that carries the amber suppressor tRNA and PyRfSAF (1/3 of the amino acid TCO* in the 6 helix of gp41, respectively). The unnatural amino acid TCO* was added to the amber suppression transfection system at 250 μM.Clickable HIV-1JR-FL Virus (V1–A316TAG) was produced by transfecting an amberless Q32 backbone ΔEnv ΔRT and a JR-FL Env mutant that carries a single amber codon at position Ser401TAG, and a bi-cistronic plasmid that carries the amber suppressor tRNA and PyRfSAF (1/3 of the amount of the plasmid that encodes the gene for Env). The unnatural amino acid TCO* was added to the amber suppression transfection system at 250 μM. Clickable HIV-1JR-FL Virus (V1–A316TAG) was produced by transfecting an amberless Q32 backbone ΔEnv ΔRT and a JR-FL Env mutant that carries a single amber codon at position Asn316TAG in V1 and the A1 tag in V4 (Extended Data Fig. 2h), HIV-1JR-FL Virus V4–A1 6–Arg542TAG (Extended Data Fig. 9a) was produced by transfecting an amberless Q32 backbone ΔEnv ΔRT and a JR-FL Env mutant that carries the A1 tag in the gp120 V4 loop and an amber in position Arg542 in the 6 helix of gp41, respectively.

Tagged viruses were collected 40 h after transfection, filtered and concentrated by centrifugation over a 15% sucrose cushion at 25,000 r.p.m. (SW28, Beckman Coulter) for 2 h and resuspended in labelling buffer (50 mM pH 7.5 HEPES, 10 mM MgCl2, 10 mM CaCl2).

Preparation and validation of tagged BG505 sgp140 SOSIP.664. Positions in native HIV-1BG505 Env at which the enzymatic labelling peptides (Q3, GQQQQL; A1, GDSLDMLEWSLM) are tolerated in the respective V1 and V4 variable regions of gp120 without affecting Env expression, processing, virus incorporation and virus infectivity have previously been identified. The short Q3 and A1 peptides were then replaced by identical positions into V1 and V4 (Q3–A1) of BG505 sgp140 SOSIP.664 or DS-SOSIPMut42 (Extended Data Fig. 1a). For purification and immobilization for total internal reflection fluorescence microscopy, the BG505 sgp140 SOSIP.664 also carried a D7324 affinity tag.
Both BG505 ss gp140 DS-SOSIP664 and ss gp140 DS-SOSIP Mut4 trimers were titrated in serial twofold dilutions, starting from 5 μg/ml. The serially diluted trimers were then transferred (25 μl/well) to the MSD plates above, and incubated for 2 h with shaking at 650 r.p.m. at room temperature. After the plates were washed several times, 2G12 and Sufo-Tag (catalogue number R91A0-1; MSD) at 2 μg/ml were added with shaking (25 μl per well), and further incubated for 1 h with shaking as above. After the washing step, the plates were read using read buffer (catalogue number R912C1-1; MSD) on an MSD Sector Imaging System 2400.

Preparation of fluorescently labelled viruses and BG505 ss gp140 SOSIP664 trimers. The Q3 and A1 double-tagged viruses and BG505 ss gp140 SOSIP664 trimers allowed the incorporation of Cy3B (Cy3B(3S)–cadaverine) and Cy5 (Cy5(M3)–CoA) (LD650–CoA) for the real-time observation of relative movements of V1 and V4 in the gp120 subunit of an individual trimer using smFRET 8,10. For labelling, the sucrose-purified tagged viruses were resuspended in 50 mM HEPES buffer (pH 7.5, 10 mM MgCl₂ and 10 mM CaCl₂) containing Cy3B(3S)–cadaverine (0.5 μM), transglutaminase (0.065 μM, Sigma Aldrich), LD650–CoA (0.5 μM) (Lumidye Technologies) and Acps.53 (5 μM, Abcam) and incubated at room temperature. In Extended Data Fig. 8b–d, Cy3B(3S)–CoA and Cy5(M3)–CoA were used instead of Cy3B(3S)–cadaverine and Cy5(M3)–CoA. For click labelling, Env variants that carry TCO* were clicked using H-tetrazine conjugates of Cy3 or Cy5 (Lumidye Technologies). PEG2000–biotin (Avanti Polar Lipids) was added to the labelling reaction at a final concentration 0.2 mg/ml, and incubated for an additional 30 min before the virus was purified by ultracentrifugation at 40,000 r.p.m. (SW41, Beckman Coulter) over a 6–18% Optiprep (Sigma Aldrich) gradient. The biotin–lipid facilitates the immobilization of the samples on the streptavidin-coated microfluidic sample chambers.

BG505 ss gp140 SOSIP664 proteins with a 20:1 ratio of wild-type/Y14-vagged gp120 were enzymatically labelled with donor and acceptor fluorophores in the labelling buffer described above, at 37 °C for 48 h, and purified away from free dye using Zeba spin desalting columns (Thermo Fisher). For the immobilization on passivated streptavidin-coated microfluidic imaging chambers, the BG505 ss gp140 SOSIP664 protein was incubated with the anti-HIV-1 gp120 D7324 antibody (Aalto Bio Reagents) at 4 °C overnight followed by a 2-h incubation on ice with the secondary biotinylated rabbit anti-sheep IgG (H + L) antibody (Thermo Fisher). smFRET data acquisition and analysis. All smFRET imaging was acquired in a homemade total internal reflection fluorescence microscope, as described 8,34. Microfluidic imaging chambers passivated with a mixture of PEG and biotin–PEG were coated with streptavidin (Invitrogen). Fluorescently labelled virions and BG505 ss gp140 SOSIP664 proteins were then immobilized on passivated streptavidin-coated microfluidic imaging chambers. Donor fluorophores were excited by the evanescent field generated by total internal reflection of a 532-nm diode-pumped solid-state laser (Opus, Laser Quantum). Donor and acceptor fluorescence and single fluorescence bleaching point met our criteria.

The infectivity of HIV-1 BG505 or HIV-1 664 was measured at 48 h after infection using a Gaussia luciferase substrate in MATLAB on the basis of the observation of original FRET signals and hidden Markov modelling 8,10. We used hidden Markov modelling to fit the data with a threestate Markov model that gave a smaller log-likelihood value than a two-state Markov model. Fitting the data into a four-state model did not lower the log-likelihood value. Therefore, at present, a three-state Markov model provides the simplest explanation of the data. For signals obtained from the Env of different HIV-1 strains, and viruses with different tags, the shapes of Gaussian distributions vary slightly. Therefore, the mean of each FRET state of determined for each labelled virus separately (Extended Data Table 1). The occupancy of each state was determined by the area under each Gaussian distribution (Extended Data Table 1). Transition density plots (Extended Data Fig. 4), which display the relative frequency of state-to-state transitions, were idealized using a segmental k-means algorithm 37.

Besides the observations of the same three conformational states from two different structural perspectives (Fig. 4), several other observations support the existence of the state 1 conformation of Env. First, the low-FRET value of V14-labelled Env represents a defined conformational state that is clearly separated from background, as evidenced by discrete photobleaching events 8,10 (Fig. 1d, c, 2a, 3a, c, e, Extended Data Fig. 4a, b). Second, the occupancy of state 1 changes in response to biologically relevant Env ligands (as described here and in previous studies 8,10). Third, the low-FRET state is not simply a consequence of non-specific fluorophore interactions with Env or viral membranes, as neither fluorophore associated with virus preparations in the absence of enzymatic labelling tags 8,10. Dyes in the V1 and/or V4 positions for single- or double-labelled virus, or BG505 ss gp140 SOSIP664, exhibit fluorescence lifetimes that are similar to those of free dyes (Supplementary Table 1). Moreover, molecular dynamics simulations performed for the enzymatic labelling tags, linkers and dyes using known structures revealed that the V1 and V4 positions are distal to the viral membrane (Extended Data Fig. 8a). Fourth, reversing the positions of donor and acceptor fluorophores on the virus revealed nearly identical smFRET histograms for the unliganded Env, as well as similar responses to ligands (Extended Data Fig. 8b–d). Fifth, the predominance of state 1 for the unliganded Env and the stabilization of state 2 in the presence of PGT151 were reproduced when V1 and V4 peptide epitope tags were replaced with labelled unnatural amino acids (Extended Data Fig. 2h–l).

Molecular dynamics simulations. Atomic coordinates for the BG505 ss gp140 SOSIP664 trimer were obtained from the Protein Data Bank (accession code 4ZMJ). Coordinates for the Q3 and A1 peptides, and for the missing amino acids in the V4 loop, were generated in PyMol (Schrodinger), and inserted into one gp120 domain in the 4ZMJ BG505 ss gp140 SOSIP model, using LeaP in the AmberTools software package. The modified BG505 ss gp140 SOSIP trimer was charge-neutralized, and solvated in explicit water using the TIP3P solvent model with periodic boundary conditions in LeaP. The protein was parameterized with the amber force field (ff14SB). The system was then energy-minimized and equilibrated in the NPT ensemble, using NAMD version 2.12. Only the V1 and V4 loops, the Q3 and A1 peptides, and linkers were generated in PyMol. The geometry of the fluorophores was first optimized at the AM1 level of theory, using the sqm program in AmberTools. Further geometry optimization and electrostatic potential calculations were then performed at the HF/6-31G(d) level of theory in Gaussian 9 (Gaussian). Partial atomic charges were then determined by restrained electrostatic potential fitting in antechamber in AmberTools. Atom types and force-field parameters were taken from the generalized amber force field (GAFF2). The fluorophores and linkers were then bound to the Q3 and A1 peptides in the BG505 ss gp140 SOSIP664 model using LeaP. Three unique starting positions for each fluorophore were generated by randomizing the torsion angles of the fluorophore linkers. Again, the system was charge-neutralized, and solvated, and minimized and equilibrated in the NPT ensemble. Only the V1 and V4 loops, the Q3 and A1 peptides, the linkers, the fluorophores and the solvent were allowed to move; the positions of all core SOS and I599P trimer atoms were fixed. Temperature and pressure were maintained at 300 K and 1 atm, using Langevin dynamics and the Nose–Hoover Langevin piston method, respectively. Subsequently, 50 ns simulations were run using NAMD on the Stampede2 machine at the Texas Advanced Computing Center.

Infectivity and titration measurements. The infectivity of HIV-1BG505 or HIV-1 664 with wild-type or mutant Env that carries SOSIP, I599S or SOSIP and I599P mutations—or amber-suppressed Env—was determined using a vector containing an HIV-1 long terminal repeat that expresses a Gaussia luciferase reporter4,38. In brief, HIV-1BG505 viruses that carry wild-type or mutant Env were generated by transfecting 60–80% confluent HEK293 cells with full-length HIV-1BG505 Q323 and an intron-regulated luciferase reporter plasmid (HIV-1-InGluC) at a ratio of 6:1 using Fugene 6 (Promega). Transfection of HIV-1BG505 or HIV-1 664 with Env that carries amber TAG codons included plasmids that encode the amber suppressor protein in the context of a co-factor (for Env) and 250 μM TCO*. Culture supernatant was collected 40 h after transfection, filtered through a 0.45-μm filter (Pall) and titred on TZMbl cells. Gaussia luciferase activity was measured at 48 h after infection using a Gaussia luciferase substrate.
Neutralization assays were conducted by incubating viruses with ligands or antibodies at the indicated concentrations for 30 min at room temperature, before addition to TZMbl cells. The level of infection relative to that seen in the absence of ligand or antibody is reported.

**Flow cytometry.** The binding preferences of ligands and antibodies for Env conformational states of wild-type HIV-1 JR-FL Env(ΔCT) were assessed by flow cytometric analysis. Antibody combinations (first pre-bound + second probe) (Extended Data Fig. 7i, j) with known Env binding-site competition were not included into the analysis. In our experiments, $3 \times 10^5$ HEK293T cells were transfected by the calcium phosphate method with the HIV-1 JR-FL Env(ΔCT)-expressing plasmid along with a pIRES-GFP vector, at a ratio of 2 μg of pcDNA3.1 HIV-1 JR-FL Env(ΔCT) to 0.5 μg of GFP-expressing plasmid. Then, 16 h after transfection, cells were washed with fresh medium (DMEM) and epitope exposure was evaluated 24 h later. Alternatively, transfected HEK293T cells were incubated for 1 h at room temperature with increasing concentrations of first-pre-bound BMS-529 (0–200 μM) before detection with second-probe anti-HIV-1 Env monoclonal antibodies (1 μg/ml PGT122, 10-1074, 3BNC117, PGT151 and NC-Cow9 antibody isolated from BG505 sgp140 SOSIP.664-immunized cows) that were conjugated using cyanine-based far-red fluorescence dye (Mix-n-Stain CF-647 Antibody labelling kit, Sigma). The largely conformation-independent anti-gp120 outer domain 2G12 antibody was used to monitor Env expression on the cell surface. For antibody competition assays, transfected cells were incubated with increasing concentrations of first-pre-bound anti-HIV-1 Env monoclonal antibodies 3BNC117, 10.1074, PGT122, PGT151 and NC-Cow9 (0, 1, 5 or 10 μg/ml) for 1 h at room temperature, followed by a 30-min incubation at room temperature with second-probe anti-HIV-1 Env antibodies coupled to C-F 647. Antibody binding was detected by gating on GFP-positive cells with an LSRII cytometer (BD Biosciences). Data analysis was performed using FlowJo v.X.0.7 (Tree Star). Mean fluorescence intensity, indicating the level of second-probe binding, was normalized to that seen in the absence of first-pre-bound ligand. Data are from experiments that were repeated at least five independent times, and averaged.

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this paper.

**Data availability**

The data that support the findings of this study are available from the corresponding authors upon reasonable request.

**Code availability**

The full source code of SPARTAN$^{34}$, which was used for all analysis of smFRET data, is available at http://www.scottblanchardlab.com/software.
Extended Data Fig. 1 | Tagged BG505 sgp140 SOSIP.664 proteins largely retain their known immunogenic features and preferentially sample state-2-like conformations. a, Schematics for wild-type (WT) BG505 Env and BG505 sgp140 SOSIP.664 with D7324 affinity tag; V1–Q3 peptide in green, V4–A1 peptide in red. b, Validation of tagged BG505 sgp140 SOSIP.664. Top, antigenic profile of 100% untagged (WT), 100% double-tagged V1V4 (V1–Q3 V4–A1), and 20:1 of untagged to double-tagged BG505 sgp140 SOSIP.664. Binding by the indicated VRC01, 17b, PG9, 19b, PGT151 and 902090 antibodies was assessed from two independent ELISA assays in hexaplets and displayed as percentage of 2G12 binding (mean ± s.d.). The epitope for the antibody 902090 was more exposed in the 100%-tagged BG505 sgp140 SOSIP.664 than in the untagged BG505 sgp140 SOSIP.664, although this was not the case for the 2:10 tagged/wild-type trimers used for our smFRET analyses. The insertion of the Q3 tag into all three V1 regions of Env may exert local effects on the V2 β-barrel that contains the 902090 epitope (residues 171–177). Bottom, reference-free negative-staining electron microscopy two-dimensional class averages with representative trimeric density map of the BG505 sgp140 SOSIP.664 (wild type: V1V4-tagged at a 20:1 ratio) used for smFRET imaging. A Fourier shell correlation is also provided. c, Antigenic characteristics of BG505 sgp140 DS-SOSIP.664 (left) and 100% V1V4-tagged BG505 DS-SOSIP.Mut4 (right), determined by MSD. Antibodies are labelled. CD4bs, CD4 binding site; CD4i, CD4-induced; V1V2, V1V2-directed; V3, V3 glycan site-directed; gp120/gp41, interface between gp120 and gp41. Antigenic profiles of BG505 DS-SOSIP.664 (left) and 100% V1V4-tagged DS-SOSIP.Mut4 (right) after V3-negative selection were assessed by a panel of CD4-induced antibodies (17 and 48b, with and without sCD4), CD4 binding site antibodies (VRC01, VRC03, b12 and weakly neutralizing F105), V1V2-directed antibodies (PGT145 and VRC26.25), V3 glycan site-directed antibodies (2G12, PGT121, PGT128) and weakly neutralizing V3-directed antibodies (447-52D, 3074 and 2557, with and without soluble CD4), gp41–gp120 interface antibodies (PGT151, 35O22, 8ANC195 and VRC34.01) and the negative-control antibody CR9114 (an influenza virus antibody that does not recognize HIV-1 Env). ECL, electrochemiluminescence. d, The indicated BG505 sgp140 SOSIP.664 variants exhibit predominantly state-2-like conformations. FRET histograms for V1V4-tagged BG505 sgp140 SOSIP.664 with molecules after V3-negative selection (left), and for the stabilized BG505 sgp140 SOSIP.664 variant DS-SOSIP.Mut420 (right) (see Methods).Histograms represent mean ± s.e.m., determined from three independent populations of smFRET traces.
Extended Data Fig. 2 | Binding of PGT151 stabilizes a state-2-like conformational state of HIV-1 Env. a, Structure of HIV-1JR-FL Env(ΔCT) in complex with PGT151 [19]. Two PGT151 antigen-binding fragments are distant from the positions of the gp120 variable loops (V1 and V4) that carry the fluorophores. b, Population FRET histograms of unliganded HIV-1JR-FL Env(ΔCT), and HIV-1JR-FL Env(ΔCT) in the presence of 10 μg ml⁻¹ sCD4D1D2–Igα. c–f, Addition of PGT151 at neutralizing concentrations (10 μg ml⁻¹) shifts the conformational landscapes for enzymatically labelled HIV-1JR-FL (c, e) and HIV-1BG505 (d, f) from the unliganded preference towards state 1 (red solid lines) to a preference for state 2 (blue solid lines). g, The addition of PGT151 to BG505 sgp140 SOSIP.664 did not alter the dominance of the state-2-like conformation exhibited in the absence of PGT151. h, Schematic of use of amber-suppressor tRNAs to introduce unnatural amino acids that can be clicked with fluorophores (h, top; see Methods), and schematic comparison between the Q3 and A1 double tag used for enzymatically labelling and click-labelling of V1 and V4 of HIV-1JR-FL (h, middle) and HIV-1BG505 Env (h, bottom). To introduce the unnatural amino acid TCO*, Asn136 in the V1 loop of HIV-1JR-FL or Ser401 in the V4 loop of HIV-1BG505 was genetically altered to an amber (TAG) stop codon. i–l, Experiment as in c–f, characterizing the conformational landscape upon binding of PGT151 to click-labelled HIV-1JR-FL V1–Asn136TAG V4–A1 (i, k), and HIV-1BG505 V1–Q3 V4–Ser401TAG (j, l). Neutralization data (mean ± s.d.) are averaged from three independent experiments in triplicates (c, d, i, j). FRET population histograms represent mean ± s.e.m., determined from three independent populations of smFRET traces.
Extended Data Fig. 3 | SOS and I559P effects on infectivity and conformational plasticity of sgp140 SOSIP.664. a, SOS and/or I559P (IP) changes introduced into native HIV-1 BG505 Q23 Env do not influence Env processing or virus incorporation. Env expression, processing and virus incorporation for HIV-1 BG505 Q23 carrying SOS, I559P and SOS and I559P (SOS&IP) changes were tested by centrifugation of viruses from cell culture supernatants, followed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis in the presence of dithiothreitol, and western blotting using the antiserum to HIV-1 gp120 (NIH AIDS reagent no. 288) and HIV-1 p24 monoclonal antibody (NIH AIDS reagent no. 3537). Experiments were repeated twice. b, The structure-stabilizing modifications A501C and T605C (SOS) and I559P used in the design of BG505 sgp140 SOSIP.664 abort HIV-1 infectivity. Infectivity of HIV-1 BG505 SOS and I559P was measured by a Gaussia Luciferase assay, and normalized to that of wild-type HIV-1 BG505 Q23. c, FRET histogram of HIV-1 JR-FL Env carrying SOS, confirming similar data for HIV-1 BG505 Env that the SOS change is largely responsible for the state 2 stabilization of Env on virus. d, e, FRET histograms of HIV-1 BG505 Env in the absence (unliganded, d) or the presence of sCD4D1D2–Igαtp (e). f, g, Experiments as in d, e for BG505 sgp140 SOSIP.664. h–k, FRET histograms of HIV-1 BG505 Env and BG505 sgp140 SOSIP.664 in the presence of the entry inhibitors BMS-378806 (h, i) and BMS-626529 (j, k). l–m, Neutralization of HIV-1 BG505 by sCD4D1D2–Igαtp (l), BMS-378806 (m) and BMS-626529 (n). Red arrows indicate concentrations used in smFRET experiments. Histograms correspond to those in the main figures: in d (Fig. 1e), e (Fig. 2b, top), f (Fig. 1f), g (Fig. 2c, top), h (Fig. 2b, bottom) and k (Fig. 2c, bottom). Infectivity and neutralization curves represent mean ± s.d. from three replicates in triplicates. FRET population histograms represent mean ± s.e.m., determined from three independent populations of smFRET traces.
Extended Data Fig. 4 | Conformational remodelling of HIV-1BG505 and BG505 sgp140 SOSIP.664 by sCD4<sub>D1D2</sub>–Igα<sub>tp</sub> and BMS-626529.

a, b, Examples of fluorescence traces of BG505 sgp140 SOSIP664 in the presence of 10 μg ml<sup>−1</sup> sCD4<sub>D1D2</sub>–Igα<sub>tp</sub> (a), and 100 μM BMS-626529 (b). Arrows indicate single-step photobleaching events that define the background of our smFRET assay.

c–e, Transition density plots of HIV-1BG505 in the absence (c) or presence (d) of sCD4<sub>D1D2</sub>–Igα<sub>tp</sub>, or in the presence of BMS-626529 (e). Transition density plots that indicate the relative frequency of state-to-state transitions were generated from individual traces (180 traces in Fig. 1e, 147 traces in Fig. 2b (top), and 116 traces in Fig. 2b (bottom)). n, number of total transitions observed.

f–h, Transition density plots of BG505 sgp140 SOSIP664 under the same experimental conditions as those shown in c–e.
Extended Data Fig. 5 | Many bNAb neutralize and exhibit preference for the state 1 conformation of HIV-1. a–c, Neutralization of native HIV-1_{BG505} by bNAb that recognize different Env epitopes: V3 glycan site-directed bNAb 10-1074, PGT121 and PGT122 (a); CD4 binding site bNAb 3BNC117, VRC01 and VRC03 (b); and V1V2 glycan bNAb PG9, PG16 and PGT145 (c). Only bNAb that potently neutralize HIV-1_{BG505} or HIV-1_{JR-FL} and allowed smFRET imaging at an antibody concentration 5 times above the 95% inhibitory concentration were analysed further (Fig. 3b, d). Neutralization data (mean ± s.d.) were averaged from three independent experiments in triplicate. d, FRET histogram that shows that HIV-1_{BG505} Env remains in state 1 in the presence of PG9 (50 μg ml⁻¹). FRET population histograms represent mean ± s.e.m., determined from three independent populations of smFRET traces.
Extended Data Fig. 6 | Conformational preferences of non-neutralizing antibodies for HIV-1 Env on virus. a, b, FRET histograms and overlaid landscapes of HIV-1NL4-3 in the presence of 100 μg ml⁻¹ 17b (a) and 100 μg ml⁻¹ F105 (b) acquired after 0 min, 30 min and 60 min of incubation. c, d, FRET histograms and overlaid landscapes of HIV-1BG505 in the presence of 17b (c) and F105 (d), acquired after 0 min, 30 min and 60 min of incubation. Non-neutralizing antibodies have preference for the state 3 conformation of Env. Note that in contrast to the tier 1 HIV-1 isolate NL4-3, the tier 2 isolate BG505 does not respond to 17b. FRET population histograms represent mean ± s.e.m., determined from three independent groups of smFRET traces.
Antibodies isolated from cows immunized using BG505 sgp140 SOSIP.664 immunogens exhibit a preference for state 2. a, FRET histogram of HIV-1BG505(T332N). b, Neutralization curves of HIV-1BG505 by NC-Cow1, NC-Cow8, NC-Cow9 and NC-Cow10 antibodies. Data are presented as mean ± s.d. determined from three independent experiments in triplicate. c–e, FRET histograms of native HIV-1BG505 in the presence of 10 μg ml⁻¹ NC-Cow1 (c), NC-Cow8 (d) and NC-Cow10 (e). f, The FRET histogram of HIV-1BG505 that carries the T332N substitution in Env is overlaid with that of wild-type HIV-1BG505. The T332N substitution in HIV-1BG505 Env does not detectably change the conformation of the Env. g, h, Cow antibodies (NC-Cow1, NC-Cow8, NC-Cow9 and NC-Cow10) shift the conformational landscape of native Env on the virus from state 1 towards that of BG505 sgp140 SOSIP.664 (state 2). FRET population histograms (a, c–e) represent mean ± s.e.m., from three independent populations of smFRET traces. i, j, Ligand preferences for states 1 and 2 probed by antibody staining of cell-expressed HIV-1JR-FL Env(ΔCT). Increasing amounts of the first ligand were pre-bound to cells for 1 h. The cells were washed, incubated with the second dye-labelled probe for 30 min, and the binding was quantified by flow cytometry. The ratio of measured mean fluorescence intensity (MFI) was normalized to that seen in the absence of pre-bound ligand (Methods). Matched combinations (state 1 and state 1 or state 2 and state 2) and non-matched combinations (state 1 and state 2 or state 2 and state 1) at the highest concentration of pre-bound first ligand were compared, and statistical significance was evaluated using a paired Student’s two-sided t-test. *P < 0.05. Note that the strong interference between 3BNC117 and PGT151 is due to a steric clash between the two antibodies, and was included as a control.
Extended Data Fig. 8 | Validating the behaviour of dyes used for smFRET. a, The 50-ns molecular dynamics simulations of fluorophore tumbling on the BG505 sgp140 SOSIP.664 trimer (4ZMJ) shows that dyes in V1 and V4 are far from the viral membrane. Molecular dynamics simulation was performed to account for movements of loops, enzymatic labelling tags, linkers and dyes to describe the possible dye tumbling space within 50 ns. The sampled space was docked into the approximately 20 Å structure of the HIV-1 virus Env spike determined by cryo-electron tomography. A 50-ns molecular dynamics simulation is not temporally comparable to the time resolution of single-molecule imaging at 40 ms, or the timescale of observed conformational changes of Env (milliseconds to seconds). b–d, Conformational properties of the HIV-1BG505 Env remain highly similar when the dyes are flipped. b, Reference FRET histograms of HIV-1BG505 that carries Cy3B in V1 and Cy5 in V4, in unliganded form (from Fig. 1e), in the presence of PGT151 (from Fig. 1h) or in the presence of sCD4D1D2–Igαtp (from Fig. 2b). c, FRET histograms of HIV-1BG505 Env that carries Cy5 in V1 and Cy3B in V4 (see Methods), in the absence and in the presence of 10 μg ml⁻¹ PGT151 or 10 μg ml⁻¹ sCD4D1D2–Igαtp, respectively. d, Overlaid conformational landscapes of HIV-1BG505 Env labelled as in c with flipped dyes (green), compared to HIV-1BG505 Env labelled as in b (red). FRET population histograms represent mean ± s.e.m., determined from three independent populations of smFRET traces.
Extended Data Fig. 9 | Suppressed HIV-1JR-FL that carries amber positions in gp120 and gp41 enables smFRET imaging of Env from two distinct perspectives. a, Schematic of tagged sites in HIV-1JR-FL Env that were used for enzymatic labelling and amber stop codon (TAG)-suppressed incorporation of unnatural amino acids for click labelling. HIV-1JR-FL V1–Q3 V4–A1 carries the Q3 peptide in the V1 loop and the A1 peptide in the V4 loop. HIV-1JR-FL V1–Asn136TAG V4–A1 carries a TAG at position Asn136 in V1 and the A1 peptide in V4. HIV-1JR-FL V4–A1 α6–Arg542TAG carries the A1 tag in gp120 V4 and a TAG at Arg542 in the α6 helix of gp41. b, c, Neutralization of HIV-1JR-FL wild type, 100%-peptide-tagged V1–Q3 V4–A1, 100%-amber-suppressed V1–Asn136TAG V4–A1 and V4–A1 α6–Arg542TAG by sCD4D1D2–Igαtp (b), and eCD4-Ig(Q40A, mim2) (c). Neutralization curves (b, c) represent mean ± s.d. from three replicates in triplicates.
Extended Data Table 1 | Relative occupancies and determining parameters in each of three observed FRET states

| HIV-1g505 | Curve fitting R-squared | State 1 | State 2 | State 3 |
|-----------|--------------------------|---------|---------|---------|
| Unliganded | 0.9949 | 45% +/- 6 | 24% +/- 5 | 31% +/- 8 |
| + PGT151 | 0.9573 | 17% +/- 6 | 60% +/- 1 | 23% +/- 15 |
| + sCD4<sub>tgD</sub>-lgtp | 0.9860 | 20% +/- 8 | 30% +/- 11 | 50% +/- 13 |
| + BMS-378806 | 0.9933 | 50% +/- 13 | 22% +/- 7 | 28% +/- 7 |
| + BMS-626529 | 0.9796 | 58% +/- 8 | 18% +/- 11 | 24% +/- 14 |
| + 38INC117 | 0.9915 | 52% +/- 9 | 23% +/- 9 | 25% +/- 12 |
| + PG9 | 0.9760 | 49% +/- 4 | 25% +/- 6 | 26% +/- 4 |
| + 17b | 0.9903 | 48% +/- 4 | 19% +/- 6 | 33% +/- 14 |
| + F105 | 0.9754 | 20% +/- 4 | 38% +/- 10 | 42% +/- 6 |
| SOS & IP unliganded | 0.9755 | 25% +/- 6 | 49% +/- 7 | 26% +/- 10 |
| SOS unliganded | 0.9553 | 23% +/- 12 | 54% +/- 10 | 23% +/- 11 |
| IP unliganded | 0.9604 | 40% +/- 12 | 30% +/- 8 | 29% +/- 15 |
| T332N Unliganded | 0.9844 | 46% +/- 7 | 25% +/- 8 | 28% +/- 10 |
| + Cow 1 | 0.9788 | 25% +/- 8 | 47% +/- 7 | 28% +/- 12 |
| + Cow 8 | 0.9867 | 41% +/- 7 | 44% +/- 8 | 35% +/- 12 |
| + Cow 9 | 0.9758 | 8% +/- 4 | 68% +/- 6 | 24% +/- 8 |
| + Cow 10 | 0.9700 | 29% +/- 9 | 41% +/- 5 | 30% +/- 12 |
| Flipped dyes unliganded | 0.9780 | 50% +/- 11 | 24% +/- 7 | 26% +/- 13 |
| + PGT151 | 0.9338 | 20% +/- 4 | 49% +/- 7 | 31% +/- 6 |
| + sCD4<sub>tgD</sub>-lgtp | 0.9877 | 19% +/- 7 | 37% +/- 8 | 44% +/- 12 |

| HIV-1g505 | Curve fitting R-squared | State 1 | State 2 | State 3 |
|-----------|--------------------------|---------|---------|---------|
| Unliganded | 0.9940 | 53% +/- 9 | 18% +/- 11 | 29% +/- 14 |
| + PGT151 | 0.9387 | 23% +/- 13 | 51% +/- 9 | 26% +/- 15 |

| HIV-1g505 | Curve fitting R-squared | State 1 | State 2 | State 3 |
|-----------|--------------------------|---------|---------|---------|
| Unliganded | 0.9737 | 13% +/- 6 | 62% +/- 4 | 25% +/- 7 |
| + PGT151 | 0.9606 | 22% +/- 13 | 58% +/- 10 | 20% +/- 15 |
| + sCD4<sub>tgD</sub>-lgtp | 0.9973 | 7% +/- 1 | 44% +/- 1 | 49% +/- 12 |
| + BMS-378806 | 0.9860 | 10% +/- 4 | 61% +/- 10 | 29% +/- 11 |
| + BMS-626529 | 0.9676 | 26% +/- 7 | 48% +/- 8 | 26% +/- 10 |
| V3 exposure eliminated | 0.9201 | 16% +/- 10 | 56% +/- 11 | 26% +/- 10 |
| DG-SOSIPMut4 | 0.9254 | 16% +/- 10 | 54% +/- 9 | 31% +/- 12 |

| HIV-1<sub>FL</sub> | Curve fitting R-squared | State 1 | State 2 | State 3 |
|-------------------|--------------------------|---------|---------|---------|
| Unliganded | 0.9821 | 53% +/- 11 | 19% +/- 6 | 28% +/- 13 |
| + PGT151 | 0.9434 | 29% +/- 11 | 49% +/- 10 | 22% +/- 12 |
| + sCD4<sub>tgD</sub>-lgtp | 0.9726 | 24% +/- 10 | 27% +/- 14 | 49% +/- 15 |
| + 10-1074 | 0.9984 | 60% +/- 10 | 20% +/- 7 | 20% +/- 5 |
| SOS unliganded | 0.9836 | 23% +/- 4 | 50% +/- 8 | 27% +/- 8 |
| ΔCT unliganded | 0.9776 | 50% +/- 9 | 23% +/- 13 | 27% +/- 14 |
| + sCD4<sub>tgD</sub>-lgtp | 0.9712 | 23% +/- 4 | 25% +/- 10 | 52% +/- 12 |

| HIV-1<sub>FL</sub> N130TAD-V4A1 | Curve fitting R-squared | State 1 | State 2 | State 3 |
|-----------------------------|--------------------------|---------|---------|---------|
| Unliganded | 0.9809 | 52% +/- 12 | 20% +/- 14 | 28% +/- 15 |
| + PGT151 | 0.9446 | 20% +/- 10 | 57% +/- 7 | 23% +/- 13 |
| + sCD4<sub>tgD</sub>-lgtp | 0.9651 | 41% +/- 10 | 28% +/- 8 | 31% +/- 12 |
| + eCD4-IgQ400a-mim2 15 min | 0.9079 | 29% +/- 13 | 37% +/- 8 | 34% +/- 20 |
| + eCD4-IgQ400a-mim2 30 min | 0.9550 | 14% +/- 15 | 23% +/- 11 | 63% +/- 20 |
| + eCD4-IgQ400a-mim2 45 min | 0.7715 | 6% +/- 2 | 35% +/- 9 | 59% +/- 13 |

The FRET histograms were carefully fitted into the sum of three Gaussian distributions with defined means and variances for each state, based on observation of original FRET signals and determined using hidden Markov modelling. μ, the mean or expectation of the Gaussian distribution; σ, s.d. of the Gaussian distribution. For FRET data obtained from different HIV-1 Env strains, or the same strains with different labelling positions or that contain or lack labelling peptides, the shapes of Gaussian distributions may vary slightly. These HIV-1 Env variants were therefore analysed and grouped separately. Relative state occupancies are presented as mean +/- s.e.m., determined from three independent measurements. R² values were calculated to evaluate the goodness of fit.
Reporting Summary

Nature Research wishes to improve the reproducibility of the work that we publish. This form provides structure for consistency and transparency in reporting. For further information on Nature Research policies, see Authors & Referees and the Editorial Policy Checklist.

Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

☐ The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement

☐ A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly

☐ The statistical test(s) used AND whether they are one- or two-sided

☐ Only common tests should be described solely by name; describe more complex techniques in the Methods section.

☐ A description of all covariates tested

☐ A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons

☐ A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)

☐ For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted

Give P values as exact values whenever suitable.

☐ For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings

☐ For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes

☐ Estimates of effect sizes (e.g. Cohen's d, Pearson's r), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection

Single-molecule data collection: custom-built prism-based total internal reflection (TIR) microscope operated by LabView (64-bit, National Instruments); HIV-1 infectivity data collection: TriStar LB 941 (Berthold Technologies); Env binding data collection: LSRII cytometer (BD Biosciences, Mississauga, ON, Canada) and FlowJo vX.0.7 (Tree Star, Ashland, OR, USA).

Data analysis

Single-molecule analysis: customized Matlab (Mathworks) program SPARTAN (publically available: https://www.scottcblanchardlab.com/software); HIV-1 Infectivity and Env binding: GraphPad Prism; Visualization and generation of Env trimer Structure: PyMOL (TM) 2.0.6; Molecular dynamic running: AmberTools (The Amber project, AmberTools) and NAMD on the Stampede2 machine at the Texas Advanced Computing Center.

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

The full source code of SPARTAN, which was used for all analysis of smFRET data, is publicly available (http://www.scottcblanchardlab.com/software).
Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

- Life sciences
- Behavioural & social sciences
- Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see https://nature.com/documents/nr-reporting-summary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

| Sample size | No statistical methods were used to determine sample size. All experiments were repeated several times giving similar results. |
| Data exclusions | No data was excluded from the analysis. |
| Replication | At least two, up to five independent measurements were performed. Assay reproducibility is described in each Figure legend. With respect to smFRET imaging, behavior is stochastic at the single molecule level. Therefore, analyses are based on at least ~100 FRET traces, numbers provided in each graph. |
| Randomization | Experiments were not randomized. Groups are not relevant to this work. |
| Blinding | The investigators were not blinded during data collection or during analysis. Groups are not relevant to this work. |

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

| Materials & experimental systems | Methods |
|---|---|
| Antibodies | Involved in the study |
| Eukaryotic cell lines | ChIP-seq |
| Palaeontology | Flow cytometry |
| Animals and other organisms | MRI-based neuroimaging |
| Human research participants | |
| Clinical data | |

Antibodies

All antibodies used were requested either from NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH (MD, USA), provided by P.D.K., or commercially purchased from authorized vendors. 3BNC117 (Catalog #12474, lot #150165, NIH AIDS Reagent, MD, USA). PG9 (Catalog #12149, lot #180007, NIH AIDS Reagent, MD, USA). PG16 (Catalog #12150, lot #130229, NIH AIDS Reagent, MD, USA). PGT145 (Catalog #12703, lot #170023, NIH AIDS Reagent, MD, USA). 10-1074 (Catalog #12477, lot #150162, NIH AIDS Reagent, MD, USA). Antiserum to HIV-1 gp120 (Catalog #288, lot #DV-012, NIH AIDS Reagent, MD, USA). Anti-HIV-1-gp120 D7324 antibody (Catalog #D7324, Aalto Bio Reagents, Dublin, Ireland). Rabbit anti-sheep IgG (H+L) antibody (Catalog #31480, Thermal Fisher, IL, USA). All other antibodies were provided by P.D.K. (VRC/NIAID/NIH, MD, USA).

Validation

Each antibody was tested in HIV-1 neutralization assays and/or binding of gp120 by ELISA, FACS or MSD-electrochemiluminescence immunoassay, prior to use in smFRET. For anti-HIV-1-gp120 D7324 antibody, based on Aalto Bio Reagents, the serological activity of the antibodies was checked by ELISA, was further confirmed by our immobilization assay. Rabbit anti-sheep IgG (H+L) antibody was successfully used in Western blot, IF, ICC, IHC, IP and FACS applications from Thermofisher Scientific website, and was tested by Western blot in this study.

Eukaryotic cell lines

Policy information about cell lines

| Cell line source(s) | HEK293 and HEK293T cells from The American Type Culture Collection (ATCC), HeLa TZMbl cells from NIH AIDS Reagent, 293 Freestyle cells from ThermoFisher. |
| Authentication | ATCC, which carries out Cell Authentication using Short Tandem Repeat Profiling before distribution. We maintain a large... |
Authentication
batch of frozen stocks from early passage. Each cell line was tested for virus infectivity assay, antibody neutralization, protein expression, ELISA, western blot, or flow cytometry.

Mycoplasma contamination
All cell lines introduced into our laboratories from external sources are tested using MycoAlert™ Mycoplasma Detection Kit.

Commonly misidentified lines
(See ICLAC register)
None

Flow Cytometry

Plots
Confirm that:
☒ The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
☒ The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a ‘group’ is an analysis of identical markers).
☒ All plots are contour plots with outliers or pseudocolor plots.
☒ A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation
HEK293T cells were used to produce cell-expressed HIV-1 Env

Instrument
Antibody binding was detected by gating on GFP-positive cells with an LSRII cytometer (BD Biosciences, Mississauga, ON, Canada). Data analysis was performed using FlowJo vX.10.4 (Tree Star, Ashland, OR, USA).

Software
Antibody binding was detected by gating on GFP-positive cells with an LSRII cytometer (BD Biosciences, Mississauga, ON, Canada). Data analysis was performed using FlowJo vX. 10.4 (Tree Star, Ashland, OR, USA).

Cell population abundance
Sixteen hours post-transfection, cells were washed with fresh medium (DMEM) and epitope exposure was evaluated 24 h later

Gating strategy
Cyanine-based far-red-Fluor 647 (CF-647)-conjugated primary antibodies were used for detection and Aqua Vivid as a vital dye to distinguish live and dead cells. Mean Fluorescence Intensity (MFI) of CF-647 staining was measured on single/live/GFP+ cells.

☒ Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.