Published in final edited form as:

Adv Virus Res. 2019 ; 105: 239–273. doi:10.1016/bs.aivir.2019.07.004.

Illuminating the virus life cycle with single-molecule FRET imaging

Maolin Lu^{*}, Xiaochu Ma, Walther Mothes^{*}

Department of Microbial Pathogenesis, Yale University School of Medicine, New Haven, CT, United States

Abstract

Single-molecule Förster resonance energy transfer (smFRET) imaging has emerged as a powerful tool to probe conformational dynamics of viral proteins, identify novel structural intermediates that are hiding in averaging population-based measurements, permit access to the energetics of transitions and as such to the precise molecular mechanisms of viral replication. One strength of smFRET is the capability of characterizing biological molecules in their fully hydrated/native state, which are not necessarily available to other structural methods. Elegant experimental design for physiologically relevant conditions, such as intact virions, has permitted the detection of previously unknown conformational states of viral glycoproteins, revealed asymmetric intermediates, and allowed access to the real-time imaging of conformational changes during viral fusion. As more laboratories are applying smFRET, our understanding of the molecular mechanisms and the dynamic nature of viral proteins throughout the virus life cycle are predicted to improve and assist the development of novel antiviral therapies and vaccine design.

1. Introduction

Decades of research have led to a detailed understanding of the viral life cycle of most clinically relevant viruses. Molecular virologists have identified the function of individual viral proteins and structural biologists have provided structural snapshots of most viral enzymes, capsids and viral glycoproteins (Das and Arnold, 2013; Harrison, 2008; Mukhopadhyay et al., 2005; Vaney and Rey, 2011). These studies have driven the development of antiviral compounds, many of which have reached the clinic and become available treatments (Cohen et al., 2011; Das and Arnold, 2013). Cell biologists have extensively studied virus entry and egress (Marsh and Helenius, 2006; Sundquist and Krausslich, 2012). For almost all viruses, the cellular receptors have been determined, and genome-wide screens have identified various host dependence factors (Bushman et al., 2009; Carette et al., 2011; Hunter, 1997). Single-particle imaging studies have been powerful in elucidating virus entry, uncoating, nuclear entry, virus assembly and egress, as well as cell-to-cell transmission (Brandenburg and Zhuang, 2007; Campbell and Hope, 2008; Jouvenet et al., 2008; Melikyan, 2014; Sattentau, 2008). Correspondingly, we have a good understanding

^{*}Corresponding authors: maolin.lu@yale.edu; walther.mothes@yale.edu.

Author contributions

M.L. and X.M. generated the figures, and M.L. and W.M. wrote the manuscript.

of the recognition of viral capsids and/or viral genomes by innate sensors and restriction factors, and of the adaptive immune responses, including humoral and cell-mediated immunity (Blanco-Melo et al., 2012; McFadden et al., 2017). While there are a few gaps in the understanding of individual steps of the life cycle in most viruses, two particular areas remain poorly understood. Macroscopically, we know very little about virus dissemination and pathogenesis at the organismal level (Uchil et al., 2019). And microscopically, we know little about the dynamics involved when viral proteins undergo conformational changes to accomplish a specific function throughout the viral life cycle.

Single-molecule Förster resonance energy transfer (smFRET) imaging is uniquely positioned to provide access to the microscopic dimension (Roy et al., 2008), specifically to monitoring the conformational dynamics and conformational changes of viral proteins as they carry out their functions. smFRET detects large conformational changes and domain movements that, along the time scale of biomolecular motions, occur in the milliseconds-to-seconds range (Fig. 1). Theoretically, smFRET measures the distance-dependent energy transfer from an excited donor to a nearby acceptor fluorophore in real time. The energy transfer efficiency (E_{FRET}) depends on the inter-dye distance between donor and acceptor (r) and is described as $E_{\text{FRET}} = (1 + (r/R_0)^6)^{-1}$, where R_0 is the Förster radius when $E_{\text{FRET}} = 50\%$. E_{FRET} can be determined experimentally as the ratio of acceptor intensity to total fluorescence intensity, according to $\text{FRET} = I_A / (I_A + I_D)$, where I_A is the intensity of acceptor fluorescence, and I_D is that of the donor. The cyanine dyes, Cy3, Cy5, and Cy7, are commercially available for smFRET owing to their brightness, photostability, and water solubility. The recent development of self-healing dyes has dramatically improved their lifespan and photon output while maintaining water solubility (Zheng et al., 2014). For smFRET, donor and acceptor fluorophores have to be introduced into the molecules of interest. This has historically been easier for RNA and DNA, which have dominated early studies (Abbondanzieri et al., 2008; Coey et al., 2016; Liu et al., 2008b, 2010). Recent advances in utilizing site specific labeling technologies as well as unnatural amino acids technologies have revolutionized our ability to introduce fluorophores at various positions into proteins of interest (Das et al., 2018; Lu et al., 2019; Plass et al., 2011; Sakin et al., 2017). smFRET imaging is preferentially performed on total internal reflection fluorescence microscopes (TIRFM) for their superior single-molecule sensitivity and high signal to noise ratio. smFRET has proven to be an insightful method for revealing the intrinsically dynamic and heterogeneous nature of biological systems, by identifying previously unknown conformational states and intermediates, as well as delineating the sequence of conformational transitions (Abbondanzieri et al., 2008; Akyuz et al., 2015; Dunkle et al., 2011; Gregorio et al., 2017; Lerner et al., 2018; Lu et al., 2019; Munro et al., 2010, 2014a; Roy et al., 2008; Zhao et al., 2010).

Adhering to the order of the virus life cycle, we will review recent insights gained from applying smFRET to the entry of the human immunodeficiency virus 1 (HIV-1) and influenza A into cells, reverse transcription, genome packaging, and assembly of HIV-1.

2. Viral glycoproteins as entry and immune evasion machines

Enveloped viruses, such as HIV-1 and influenza A, enter host cells by fusion of viral and cellular membranes (Harrison, 2008; Skehel and Wiley, 1998). While membrane fusion of small enveloped viruses with large cells is energetically favored, the electrostatic repulsion between membranes and the initial merging represents an energy barrier (Harrison, 2008). This barrier is overcome by viral fusion machines that bring membranes together for fusion. The lipid merger reaction proceeds through a hemi-fusion intermediate, and then a widening fusion pore that permits content mixing and the delivery of viral capsids into the cytoplasm of the host (Chernomordik and Kozlov, 2008). Viral fusion proteins are often also the only viral protein on the surface of viruses, and as such the target for neutralizing antibodies, which they evolve to evade. This dualism of functioning as viral fusion machines as well as immune evasion machines makes them interesting objects to study, and understanding these machines is vital for the rational design of small molecule inhibitors, immunogens for vaccines, and the development of strategies for a cure.

Enveloped viruses have evolved at least three distinct classes of viral fusion machines that can overcome this barrier and induce fusion (Harrison, 2015). The glycoproteins of HIV-1 and influenza A virus, the envelope glycoprotein (Env) and influenza A hemagglutinin (HA) belong to class 1 fusion machines. They are metastable trimeric proteins that respond to cellular cues, e.g. to receptor CD4 and coreceptor for HIV-1 Env, and to low pH within an endosome for influenza A HA, to initiate a series of conformational changes of loop-to-helix, as well as helix-to-loop transitions that lead to the generation of a highly stable hairpin conformation with a central alpha-helical coil-coiled in the post-fusion conformation (Bullough et al., 1994; Carr and Kim, 1993; Chan et al., 1997; Julien et al., 2013; Lyumkis et al., 2013; Mao et al., 2012; Pancera et al., 2014; Wilson et al., 1981). The energy released during this refolding process is believed to drive the fusion reaction. These metastable fusion proteins are generated from precursor proteins, HIV-1 gp160 trimers, and influenza A HA₀ trimers, and proteolytically primed into the metastable trigger-prone mature fusion proteins, trimers of HIV-1 gp120 and gp41 heterodimers, as well as trimers of influenza A HA₁ and HA₂ heterodimers, respectively. The actual fusion machines reside within the transmembrane subunits HIV-1 gp41 and influenza A HA₂. While structural snapshots of the precursor, mature and postfusion conformations have been generated at low and high resolutions (Bullough et al., 1994; Carr and Kim, 1993; Julien et al., 2013; Kwong et al., 1998; Liu et al., 2008a; Lyumkis et al., 2013; Mao et al., 2012; Ozorowski et al., 2017; Wang et al., 2016; Wilson et al., 1981), smFRET has just recently provided real-time measurements of the conformational changes during fusion for influenza A HA₂ (Das et al., 2018).

In contrast, much of the immune evasion function is mediated by HIV-1 gp120 and influenza HA₁ that initially cover and protect the underlying fusion machines. Both are heavily glycosylated and are undergoing sequence variation. In the case of HIV-1, gp120 subunits are clearly dynamic, concealing the immunogenic functional receptor and coreceptor binding sites from antibody recognition (Barouch, 2008; Crispin et al., 2018; Julien et al., 2012; Kwong et al., 2002; Wilson et al., 1981; Wyatt et al., 1998). These immune evasion strategies are best understood for HIV-1 Env (Kwong et al., 2002, 2013; Wei et al., 2003).

HIV-1 Env has a nearly perfect glycan shield as ~half of its molecular weight is contributed by sugars (Gristick et al., 2016; Stewart-Jones et al., 2016). Vaccine studies with soluble trimers containing systematically placed glycan holes have illustrated how much better the HIV-1 Env trimer is recognized by antibodies the moment holes are introduced into the glycan shield (Crooks et al., 2015; Ringe et al., 2019; Wagh et al., 2018). Due to the high mutation rate of reverse transcriptase (RT) and likely additional APOBEC3 mediated hypermutation, HIV-1 Env also undergoes rapid sequence variation, permitting epitope escape from neutralizing antibodies (Mulder et al., 2008). Last, Env is both structurally flexible and conformationally dynamic, and those features have also been elucidated by smFRET. This dynamic behavior enables concealing of vulnerable functional centers of Env by restricting antibody access to receptor and coreceptor binding sites. Critically for the understanding of HIV-1/AIDS is that the antibodies generated against the more open and highly immunogenic conformational states are ultimately non-neutralizing against primary HIV-1 isolates. In contrast, the broadly neutralizing antibodies (bNAbs) generated by few patients can recognize the glycan-shielded closed conformation. This model is further supported by the Tier classification of HIV-1 isolates (Montefiori et al., 2018). Tier 1 isolates are lab-adapted strains that have not been exposed to innate and adaptive immunity for a long time, and carry more open Env trimers that can be neutralized by “non-neutralizing” antibodies. In contrast, Env trimers from the primary Tier 2 and 3 clinical isolates are more closed and more resistant to most antibodies, but can be recognized by bNAbs (Montefiori et al., 2018). Clearly, structure-based HIV vaccine design that intends to elicit bNAbs focuses on developing immunogens that mimic the structure of glycan-shielded closed conformation of Env. The application of smFRET has begun to elucidate both aspects of these immune evasion and fusion machines (Das et al., 2018; Lu et al., 2019; Munro et al., 2014a).

3. smFRET imaging of viral glycoproteins in the context of intact virions

The establishment of smFRET methods for native virus particles has provided critical new insights into the conformational dynamics of HIV-1 Env and influenza A HA (Munro and Mothes, 2015; Munro et al., 2014a). In the case of Influenza HA, HA was imaged on the surface of HIV-1 particles (Das et al., 2018). The implementation of smFRET for these virus systems is based upon the utilization of site specific labeling as well as unnatural amino acids technologies (Das et al., 2018; Lu et al., 2019; Ma et al., 2018; Munro et al., 2014a). In the case of HIV-1 Env, positions that tolerate the incorporation of 6, 8 or 12 amino acid tags within the variable loops of gp120 were identified. These short peptide tags are site-specifically recognized by enzymes that catalyze the transfer of fluorophore-conjugated substrates onto single residues within these tags (Ma et al., 2018; Munro et al., 2014a). Tagged Env proteins must be validated to retain infectivity, Env synthesis, processing, virus incorporation and sensitivity to trimer specific neutralizing antibodies. For HIV-1 Env, tags were tolerated in V1, V4 and V5 (Munro et al., 2014a). Labeling of viruses must then be accomplished in a way that one virus particle, on average, only carries a single Cy3 and Cy5 pair. The other two gp120 protomers within the same trimer, and all other trimers on the surface of the same single virus, should remain unlabeled. This is accomplished by transfecting an excess of plasmid encoding the untagged wild-type Env over the tagged Env. It should also be noted that this method only works for viruses such as HIV-1, which carry a

limited number of viral glycoproteins. For viruses with higher numbers of viral glycoproteins on their surface, the probability that two separate trimers carry dyes increases to a point where inter-molecular FRET signals can obscure the intra-molecular FRET signals of interest. Viruses purified from culture supernatants are then incubated with the labeling enzymes and their dye-conjugated substrates, purified to remove free dyes, and immobilized on quartz slides for TIRFM smFRET imaging (Fig. 2). If unnatural amino acids are used, clickable amino acids allow copper-free click chemistry to introduce the fluorophores (Das et al., 2018; Lu et al., 2019). The strengths of this approach are that the viral glycoproteins are studied within their natural virus environment using physiological conditions, avoiding the need for protein purification and/or use of detergents.

4. smFRET imaging reveals that the unliganded HIV-1 Env is dynamic, having access to three distinct prefusion conformational states

TIRFM imaging of HIV-1 Env carrying fluorophores in V1 and V4 of gp120 revealed that Env from three different isolates (laboratory-adapted NL4-3, clinical isolates JR-FL and BG505) (Ma et al., 2018; Munro et al., 2014a) shows anti-correlative behavior between donor and acceptor fluorescence intensities, indicative of energy transfer between both dyes, and suggesting that the unliganded Env is dynamic. Quantitative analysis showed that Env samples three distinct conformational states characterized by low-FRET, intermediate-FRET, and high-FRET values (Lu et al., 2019; Ma et al., 2018; Munro et al., 2014a) (Fig. 2). The low-FRET conformation was the most populated state in all three cases, and represents the ground-state conformation of the prefusion Env, which is expected to correspond to the most immune evasive closed conformation. Consistent with this observation, the Tier 2 HIV-1 isolates JR-FL and BG505 exhibited greater occupancy of the low-FRET conformational state as compared to the lab-adapted NL4-3, which is known to be more open (Ma et al., 2018). Further statistical analyses of the transitions revealed that Env transitions from the low-FRET to the high-FRET and back, as well as between the high-FRET and the intermediate-FRET state. Transitions between low-FRET and intermediate-FRET states were rare, indicating that the high-FRET state is a necessary intermediate between low-FRET and intermediate-FRET. Based on this biophysical analysis, these three FRET states were designated as pre-triggered State 1 (low-FRET), intermediate State 2 (high-FRET), and State 3 (intermediate-FRET) (Herschhorn et al., 2016; Ma et al., 2018).

5. Ligands identify the nature of HIV-1 Env conformational states observed by smFRET

To identify the nature behind the three observed conformational states, we tested the effects of various ligands including soluble CD4 (sCD4) and numerous antibodies as well as Env mutants (Lu et al., 2019; Ma et al., 2018; Munro et al., 2014a). Interestingly, many bNAbs increased the occupancy of State 1 by reducing that of State 2 and State 3. For example, bNAbs directed against different epitopes of Env including the trimer apex containing the variable loops V1V2 (PG9, PG16 and PGT145), the V3 glycan patch (10-1074, PGT122, PGT128, 2G12), and the CD4 binding site (VRC01, 3BNC117), were either compatible with or stabilized the State 1 conformation of Env (Lu et al., 2019; Munro et al., 2014a). A small

molecule entry inhibitor BMS-626529 (Temsavir®) exhibited a similar conformational signature of a State 1 stabilization (Lu et al., 2019; Munro et al., 2014a).

In contrast, soluble receptor CD4 (sCD4) containing ligands, small molecule CD4 mimetics, as well as coreceptor mimicking antibodies such as 17b, stabilized State 3. Specifically, the potent oligomeric sCD4 ligand 12xCD4 (sCD4_{D1D2}-IgαIg) (Arthos et al., 2002), or the simultaneous presence of the sCD4 (D1D2 domain) and additional presence of the coreceptor-surrogate antibody 17b, which binds to a CD4-induced epitope, all stabilized Env in State 3 conformations (Ma et al., 2018). These experiments allowed an assignment of the observed FRET states as the pre-triggered State 1 conformation, and the three-CD4 bound State 3 open conformation of Env (Ma et al., 2018). As such, the smFRET assay demonstrated that individual Env molecules on native virus particles are dynamic, spontaneously transitioning between three distinct well-populated functional conformational states: a pre-triggered Env (State 1), a default intermediate (State 2), and a three-CD4-bound conformation (State 3) along the Env activation pathway. Thermodynamically, the interaction with CD4 remodels the conformational landscape of Env by stabilizing the State 3 conformation over States 1 and 2, and by lowering the energy barriers for transitions into State 3 (Ma et al., 2018).

6. Stepwise opening of HIV-1 Env by cellular receptor CD4 and coreceptor mimics

With States 1 and 3 identified, the nature of State 2 had initially remained unclear. Differences in the response of HIV-1 isolates to sCD4 suggested that this conformational intermediate might be linked to incomplete CD4 occupancy on the trimer (Ma et al., 2018). To test this hypothesis, Ma et al. engineered trimers that can only bind 1 or 2 CD4 molecules per trimer, and then placed the conformational state monitoring two fluorophores either into the gp120 protomer that can bind CD4, or into the adjacent protomer that cannot bind CD4 (Fig. 3). This was accomplished using the D368R substitution in the CD4 binding pocket that impairs CD4 binding without causing indirect allosteric effects. Two mixed trimers that can only bind a single CD4 molecule were designed. In mixed trimer 1, the Cy3/Cy5 fluorophores were placed into a single protomer that can bind sCD4. The two other protomers within the trimer carried the D368R mutation to prevent CD4 binding. In mixed trimer 2, the Cy3/Cy5 dyes were placed on one of the two CD4-binding incompetent protomers next to the single CD4-binding competent protomer. smFRET imaging revealed that a trimer engaging only a single CD4 protomer is asymmetric. The CD4-bound protomer adopts State 3, while the protomers positioned left and right to the CD4-bound protomer adopt the State 2 conformation. Finally, a trimer with a single CD4 molecule bound was also able to completely open into State 3 if a coreceptor mimicking antibody 17b bound was also introduced (Ma et al., 2018) (Figs. 3 and 4). These data suggest a model for the sequential opening of the HIV-1 Env trimer through an asymmetric trimer intermediate in which only a single CD4 engages the trimer, followed by complete opening into State 3 upon further recruitment of either CD4 or coreceptor (Ma et al., 2018) (Fig. 4). State 3 within gp120 is likely the last conformational state within gp120 along the activation pathway that can be detected by smFRET. From there, a signal must be transmitted to the underlying gp41 fusion

machine to drive membrane fusion (Fig. 4). As we will see below, smFRET analysis of the conformational events within HA2, the influenza A equivalent of the HIV-1 gp41, has revealed conformational changes that correlate with membrane fusion (Das et al., 2018).

7. An asymmetric conformational state of HIV-1 Env highly vulnerable to ADCC

Clinical HIV-1 isolates evade the strong antibody response against the highly immunogenic open conformations by closing into the most immune evasive State 1 conformation. As a consequence, the success of bNAbs likely depends on learning to recognize State 1. However, the apparent need for Env to evade the antibody response means that the so-called “non-neutralizing” antibodies are actually potent in removing the vulnerable open conformations thereby forcing the selection of closed Env conformations. Recent work revealed that mechanistically the antibody-dependent cellular cytotoxicity (ADCC) is largely responsible for depleting Env variants that are more open and thus highly vulnerable (Alsahafi et al., 2019; Bournazos et al., 2014; Prevost et al., 2018; Richard et al., 2018; Veillette et al., 2014, 2015). In ADCC, antibodies bind Env expressed on the surface of infected cells, which can be recognized by Fc-receptor expressing cytotoxic CD8⁺ T cells or NK cells (Forthal and Finzi, 2018). Interestingly, the most ADCC vulnerable conformation of Env allows binding of anti-cluster A antibodies that recognize the inner domain of gp120 (Forthal and Finzi, 2018). This epitope is only accessible in the presence of sCD4 or a small molecule CD4 mimetic (CD4mc), coreceptor mimicking antibodies such as 17b, and anti-cluster A antibodies that recognize the inner domain (Alsahafi et al., 2019; Forthal and Finzi, 2018). Parallel smFRET analysis revealed that Env adopts a fourth conformational state that is connected to State 2, and hence designated State 2A (as shown in Fig. 4) (Alsahafi et al., 2019; Richard et al., 2016). Parallel cryo-electron tomography (cryoET) revealed an asymmetric trimer. Thus, these data suggest that State 2 and State 2A are asymmetric trimer configurations.

These experiments demonstrated the strengths of smFRET and cryo-ET to reveal asymmetric intermediates. Asymmetry is probably more common than previously thought. An asymmetric structural intermediate has also been observed by cryoET for the receptor-bound Env of the Murine Leukemia Virus (Riedel et al., 2017). During HIV-1 fusion, asymmetric activation was observed for the transmembrane gp41 subunit (Khasnis et al., 2016). Finally, molecular dynamic simulation also reveals that HIV-1 Env protomers within a trimer undergo scissoring movements which induce trimer asymmetry (Lemmin et al., 2017).

8. High-resolution structures of HIV-1 Env

Determination of mature cleaved HIV-1 Env trimeric structures have been extremely challenging, mainly due to the weak association between the gp120 and gp41 subunits, the heavy glycan shield, the variable loops, and conformational dynamics. An early breakthrough was the determination of the core gp120 subunit at atomic level in complex with soluble CD4 (sCD4, D1D2 domains) and coreceptor mimicking antibody 17b, followed by the determination of the post-fusion six-helix bundle of gp41 (Buzon et al., 2010; Chan et

al., 1997; Kwong et al., 1998; Weissenhorn et al., 1997). The application of cryoET to complete virus particles provided a low resolution $\sim 20\text{\AA}$ view of Env spikes and gradual opening in response to sCD4 and 17b (Liu et al., 2008a). A critical breakthrough in the characterization of the trimer was possible thanks to ~ 15 years of continued efforts by Rogier Sanders, James Binley and John Moore in collaboration with the groups of Ian Wilson and Andrew Ward that resulted in a stabilized soluble HIV-1 Env trimer (Binley et al., 2000; Ringe et al., 2013; Sanders et al., 2002, 2013). These trimers were designated SOSIPs, SOS for a disulfide bridge that creates a covalent link between the gp120 and the gp41, and IP for an I559P point mutation that destabilizes post-fusion conformations. These soluble trimers were also lacking the transmembrane region (TM), the membrane proximal extracellular region (MPER), were optimized for cleavage efficiency and were engineered using a naturally more stable HIV-1 Env isolate (Sanders et al., 2013). This design allowed the structural characterization of the HIV-1 Env trimer at atomic level by X-ray crystallography and by single-particle cryo-electron microscopy in its ligand-free, or antibody-bound, or small molecules-bound forms (Bartesaghi et al., 2013; Chuang et al., 2017; Julien et al., 2013; Kong et al., 2016; Kwon et al., 2015; Lee et al., 2016, 2017; Lyumkis et al., 2013; Pancera et al., 2014; Scharf et al., 2015; Scheid et al., 2016; Stewart-Jones et al., 2016). Advances in single-particle cryo-electron microscopy allowed the subsequent determination of the structure of the trans-membrane HIV-1_{JR-FL} Env containing the MPER and TM, only lacking the cytoplasmic tail, in complex with the bNAb PGT151 (Lee et al., 2016). Interestingly, all these Env structures determined by various groups and using different methodologies (X-ray crystallography or cryo-EM) revealed similar structural architectures, consolidating the current consensus model of the closed mature HIV-1 Env trimer (Fig. 5). In response to binding to sCD4, with or without the additional presence of 17b Fab fragments, the sg140 SOSIPs open into the CD4-bound conformation (Ozorowski et al., 2017; Wang et al., 2016, 2018). The V1V2 loops undergo a strong outward movement to align near sCD4 (Wang et al., 2016, 2018), and the fusion peptide folds into a new groove (Ozorowski et al., 2017; Wang et al., 2016).

smFRET analyses of the conformational effect of the antibody PGT122 that was used to stabilize the Env trimer by several groups revealed that it stabilized the Env trimer in State 1 (Julien et al., 2013; Pancera et al., 2014). By association, it was believed that these structures represent the pre-triggered closed State 1 conformation of HIV-1 Env seen by smFRET (Munro and Mothes, 2014, 2015; Munro et al., 2014a). On the other hand, several observations had raised questions about the similarity of sg140 SOSIP.664 trimers to the functional Env on the surface of viruses and cells (Alsaifi et al., 2015, 2018; Kesavardhana and Varadarajan, 2014).

9. Associating the conformational states observed by smFRET with high-resolution structures of HIV-1 Env

To determine how the observed smFRET states relate to these high-resolution structures, donor and acceptor fluorophores were placed at exactly the same positions on both BG505 sg140 SOSIP.664 and wild-type BG505 Env on the surface of viral particles (Fig. 5) (Lu et al., 2019). Surprisingly, smFRET revealed that the conformational landscape of sg140

SOSIP.664 differs from the State 1 Env conformation observed on the surface of virus particles. Instead, BG505 sgp140 SOSIP.664 closely resembles the downstream State 2 conformation (Fig. 5) (Lu et al., 2019). This large shift in the conformational landscape was confirmed for additional BG505 sgp140 SOSIP.664 proteins including further stabilized immunogens (Lu et al., 2019). Moreover, PGT151 used as a ligand in cryo-EM structures with the membrane-bound HIV-1_{JR-FL} Env (Lee et al., 2016), also stabilized the State 2 on virion resident Env. These observations were verified for a second perspective where a dye was introduced into gp41 using amber suppression click labeling and the distance between gp41 and V4 in gp120 monitored by smFRET (Lu et al., 2019). Collectively, all data indicate that existing high-resolution structures are similar to the State 2 conformation of Env (Fig. 5). The high-resolution structure of State 1 remains unknown. These data are consistent with findings in other laboratories that have also observed differences between HIV-1 virus Env and sgp140 SOSIP.664. For instance, the processing of HIV-1 Env glycans differs (Cao et al., 2018; Go et al., 2017). And a recent report applying cross-linking followed by mass spectrometry also detected dramatic differences between both proteins, specifically within the trimer association domain, the C-terminus of gp120 subunit, and in the heptad repeat 1 (HR1) of gp41 (Castillo-Menendez et al., 2019).

The interpretation that existing high-resolution structures correspond to the State 2 seen by smFRET is further supported by comparing the changes in distances between unliganded and CD4-bound sgp140 SOSIP with the corresponding FRET values (Stadtmueller et al., 2018). In the sgp140 SOSIP structures, the V1 and V4 positions where the fluorophores reside are very close. This is consistent with the observed high-FRET signal (Stadtmueller et al., 2018). Upon binding of sCD4, the V1V2 loops move out to align parallel to the bound sCD4 consistent with the lower FRET value (distance between fluorophores increasing, reducing energy transfer) (Ozorowski et al., 2017; Stadtmueller et al., 2018; Wang et al., 2016). Altogether, these observations are consistent with current sgp140 proteins being in State 2.

The smFRET approach revealed that soluble sgp140 SOSIP trimers including trimers further stabilized for immunogen design (Sanders and Moore, 2017), reside in a conformation that resembles State 2 (Lu et al., 2019). Importantly, many such stabilized sgp140 SOSIP proteins are now entering clinical vaccine trials for safety evaluation and to evaluate their potential to elicit bNAbs in patients. We thus tested the conformational preference of antibodies elicited using sgp140 SOSIP in earlier experimental vaccine studies in animals such as cows (Sok et al., 2017). Intriguingly, the sgp140 SOSIP elicited cow antibodies exhibited a preference for State 2 (Fig. 5). This preference is unlike many bNAbs such as 3BNC117 and 10–1074 (Caskey et al., 2017; Scheid et al., 2016) developed in some HIV-1-infected individuals fighting the native virus that exhibit a preference for the State 1 conformation (Lu et al., 2019; Munro et al., 2014a). These data suggest that State 2 specific immunogens elicit State 2-specific antibodies. Going forward, the characterization of a putative structure of State 1 becomes of great importance. A high-resolution structure would facilitate the design of State 1-specific immunogens that should be tested in vaccine settings.

10. smFRET analysis of conformational changes during membrane fusion for the influenza A hemagglutinin fusion glycoprotein (HA₂)

smFRET application has elucidated the conformational dynamics of influenza A HA and provided dynamic insights into the fusion reaction of another class I fusion protein (Das et al., 2018). Similar to HIV-1 Env trimer, HA is synthesized as a precursor HA₀, then cleaved by cellular proteases into a metastable trimer of HA₁/HA₂ heterodimers: the receptor sialic acid binding domain HA₁ and transmembrane domain that harbors the fusion machine HA₂. In contrast to HIV-1 Env, influenza A HA₂ is activated by low pH within an endosome (Marsh and Helenius, 2006; White and Whittaker, 2016). Current models for the activation of HA₂ are based on structural characterizations of the uncleaved precursor, the mature protein and the low-pH activated form (Bullough et al., 1994; Carr and Kim, 1993; Stevens et al., 2006; Wilson et al., 1981). In this model, low pH induces a loop-to-helix conformational change within HA₂ that leads to the exposure and subsequent insertion of the fusion peptide into the target membrane, followed by a helix-to-loop transition that brings both membranes together for fusion (Skehel and Wiley, 2000). However, direct real-time measurements of single conformational changes during fusion have never been directly observed. Using unnatural amino acids (Sakin et al., 2017), Das et al. introduced donor and acceptor fluorophores before (near the fusion peptide) and toward the end of the switch regions within the influenza A fusion machine HA₂ (Das et al., 2018). At neutral pH, smFRET analysis revealed that the HA trimer is conformationally dynamic, sampling at least three conformational states: a most populated high-FRET (75% occupancy), an intermediate-FRET, and a low-FRET (Fig. 6). Lowering pH gradually shifts the conformational equilibrium of HA₂ from the prefusion high-FRET conformation to the low-FRET through a short-lived intermediate-FRET state (suggested to be a peptide-released intermediate in which the fusion peptide is positioned out of the hydrophobic pocket in HA₂). For a few minutes (up to 15), this conformational change is reversible and HA₂ can fully transition back to the high-FRET if the pH is neutralized. Interestingly, the presence of receptor sialic acid on target membranes was shown to increase the transitions into the low-FRET conformation even at neutral pH, and to accelerate the low pH induced conformational changes. The distinct features of the reversible low-FRET intermediate and the final irreversible low-FRET conformation suggest that two distinct conformational states are behind this FRET state, and that the pre-hairpin intermediate was not detectable due to the predicted long distance between the dyes (Fig. 6). Thus, smFRET indicates that HA₂ is activated through two structural intermediates and that these transitions are initially reversible. Reversibility would explain how HA is transported through the acidic Golgi lumen in infected cells without activation, and that only receptor and low pH on target cells most effectively activate HA₂ for fusion (Benhaim and Lee, 2018; Das et al., 2018). Going forward, monitoring additional perspectives to resolve these two conformational states, and correlating conformational changes within HA with the lipid mixing reaction that proceeds through hemi-fusion, flickering and widening fusion pores will result in additional crucial insights (Chernomordik and Kozlov, 2003, 2008; Melikyan, 2014; Melikyan et al., 2005).

11. Conformational dynamics during HIV-1 reverse transcription

Following virus entry, capsid uncoating and reverse transcription are the next steps in the viral life cycle of retroviruses. During reverse transcription, the reverse transcriptase (RT) reverse transcribes the viral RNA (vRNA) into double-stranded DNA. RT is a multifunctional enzyme that contains the polymerase (Pol) and ribonuclease H (RNase H) subunits. Structurally, RT is a heterodimer of p51 and p66 which share both a common amino terminus and four polymerase subdomains (fingers, palm, thumb, and connection) (Huang et al., 1998; Kohlstaedt et al., 1992; Sarafianos et al., 2009). RT begins with the formation of the HIV-1 reverse transcriptase initiation complex that includes the RT bound to primer (tRNA)-template vRNA complex (RT/tRNA/vRNA). The early phase of reverse transcription is a slow, non-processive process. It involves slow DNA polymerization, fast disassociation of RT and RNA complex, and frequent pauses of RT at different locations (Lanchy et al., 1996). While the first strand of DNA is synthesized, the RNA is cleaved from the RNA/DNA hybrid intermediate by the RNase H subunit. Leftover tighter bound RNA stretches then functions as primers for the synthesis of the second strand of DNA. It has been speculated that the initiation complex changes progressively in structural/conformational configurations along the RT catalytic pathway; however, it has been technically difficult to capture those dynamic features.

Several smFRET studies on RT have revealed surprising insights into the underlying mechanism of how RT (Pol and RNase H) interacts with the RNA and DNA templates using RNA and DNA primers to initiate Pol and RNase H activities, respectively. smFRET studies of labeling the duplex substrates with a dye in combination with another dye on RT p66 subunit at different locations (Fig. 7) enabled direct observations of enzyme-substrate associating/disassociating events of RT catalysis in real time (Abbondanzieri et al., 2008; Liu et al., 2008b, 2010). Using this elegant experimental design, orientational and translational dynamics of RT were further uncovered (Fig. 7) (Abbondanzieri et al., 2008; Liu et al., 2008b, 2010). RT was observed to adopt two horizontally-flipped binding orientations on nucleic acid duplexes substrates depending on whether a RNA or DNA primers were bound, directing its DNA synthesis or RNA hydrolysis activity, respectively (Fig. 7). RT switching between those two orientations was regulated by nucleotides and non-nucleoside reverse transcriptase inhibitors, a major drug class of anti-retroviral therapy (Abbondanzieri et al., 2008). Extensive smFRET assay further revealed that those opposite orientations of RT are closely related to polymerization-competent and RT-paused polymerization-incompetent configurations of initiation complex in early phase of reverse transcription, respectively (Liu et al., 2010).

Besides this large-scale orientational motion (180° flipping), RT was also able to undergo long-range sliding on nucleic acid duplexes substrates, rapidly shuttling between two termini of substrates (Fig. 7) (Liu et al., 2008b). The sliding motion of RT play critical roles in multiple phase of reverse transcriptase: it shuttles RT to the tRNA terminus for DNA polymerization, translocates RT to multiple sites of RNA/DNA hybrid for vRNA degradation, and displaces non-template strand (Liu et al., 2008b). Flipping and sliding go hand-by-hand to effectively help RT carry out the catalysis of vRNA to double-stranded DNA.

RT is not the only component of RT/tRNA/vRNA complex involved in reverse transcription that is constantly dynamic. Indeed, tRNA/vRNA together or alone are also dynamic in real time. The combination of bulk NMR and smFRET allowed the observation of tRNA/vRNA hybrids adopting two or more conformations, suggesting conformational heterogeneity formed by conserved RNA sequences within HIV-1 reverse transcription initiation complex (Coey et al., 2016).

These smFRET applications highlighted the existence of multiple dynamically exchanging structures within the RT/tRNA/vRNA complex, and provide a framework of how various RT inhibitors shift the equilibrium between different states. And as in many areas, combining smFRET techniques with other structural methods such as single-particle cryo-electron microscopy is predicted to be particularly powerful. One such successful study describes the initiation complex determined by single-particle cryo-electron microscopy (Larsen et al., 2018). Strikingly, helices of highly dynamic primer/template (tRNA/vRNA) become visible at high-resolution. The overall structure (8Å) shows RT being in an inactive polymerase conformation with open finger and thumb subdomain (4.5Å), whereas the primer/template RNA complex moves away from the active sites (Fig. 7). Parallel smFRET studies helped to define the structures of highly dynamic and disordered parts of the complex. Altogether, smFRET applications in combination with additional structural techniques have allowed a detailed scrutiny into the slow initiation phase of RT, exposing its vulnerability to inhibition by RT inhibitors.

12. smFRET monitoring of conformational changes in HIV-1 genomes and capsid protein during HIV-1 Gag assembly

HIV-1 assembly is mediated by HIV-1 Gag polyprotein. The Gag polyprotein is synthesized as a Gag precursor that contains the matrix (MA), capsid (CA), nucleocapsid (NC) and p6 domain. The N-terminal MA domain targets Gag to the plasma membrane through its specificity for *phosphatidylinositol 4,5-bisphosphate* (PIP₂) (Jouvenet et al., 2008; Mucksch et al., 2017; Ono et al., 2004). CA drives Gag multimerization into the immature viral core, NC recruits a dimeric RNA genome into an assembling particle through binding to the packaging signal (Psi), which is located in the 5'UTR of the RNA (Keane and Summers, 2016; Lu et al., 2011a) and the p6 domain recruits the endosomal sorting machinery to promote the release of the immature virus particle (Bell and Lever, 2013; Freed, 2015; Ganser-Pornillos et al., 2012; Mattei et al., 2016; Sundquist and Krausslich, 2012). Live cell imaging has revealed that assembly and genome packaging are coordinated whereby the genome stimulates the nucleation of capsid assembly (Jouvenet et al., 2009). Both Gag and RNA are conformationally dynamic and smFRET studies have provided insights into both processes (Beerens et al., 2013; Brigham et al., 2019; Coey et al., 2018; Jouvenet et al., 2009; Munro et al., 2014b).

Gag has been proposed to undergo major conformational changes, from a compact conformation in solution to the extended rod-like conformation observed within the final immature capsid (Datta et al., 2007; Ganser-Pornillos et al., 2012; Mattei et al., 2016; Sundquist and Krausslich, 2012). smFRET has allowed direct measurements of the

conformational state of individual Gag molecules in solution as well within virus particles (Munro et al., 2014b). By site-specifically placing two dyes on different domains of the Gag polyprotein, smFRET analysis demonstrated that Gag monomers undergo transitions between a compact conformation and an extended linear conformation (Munro et al., 2014b). These two distinct Gag conformations form viral-like particles (VLPs) with different spherical morphology: in the compact conformation, Gag forms VLPs with an approximately ~30nm in diameter in the presence of nucleic acid; with the presence of additional inositol hexaphosphate, Gag exhibits an extended rod-like conformation, and forms VLPs of ~100nm in diameter (Munro et al., 2014b). Parallel fluorescence correlation spectroscopy (FCS) measurements combined with smFRET confirmed that the compact-to-extended conformational transition happens early when the Gag oligomers are still very small (Munro et al., 2014b). These experiments represent the first measurement of the conformational state of Gag within complete virus-like particles. Also, at that time, we speculated that inositol hexaphosphate may mimic the role of phosphoinositides in the plasma membrane to trigger this conformational change, but today we know that inositol hexaphosphate plays an integral role in stabilizing the HIV capsid (Dick et al., 2018; Mallery et al., 2018).

The packaging of a specifically dimeric RNA genome is incompletely understood. A dimerization initiation site (DIS) lies within the 5'UTR of the Psi packaging signal on the genomic RNA. The transition from RNA monomer to dimer is regulated by the U5 region. In the monomer, the U5 pairs with the DIS, thereby preventing dimer formation, while U5 pairing with a stem loop harboring the AUG Gag start codon, frees up the DIS for dimerization. The RNA is highly heterogeneous and competing models have been proposed for how monomers undergo conformational changes to form dimers that are specifically packaged into particles (Lu et al., 2011b). In the case of the murine leukemia virus, NC has a higher affinity for the dimerized genome (Miyazaki et al., 2010). In the case of HIV, smFRET and bulk in-gel FRET experiments have recently helped to elucidate how tRNA and NC promote transition to an extended dimer (Brigham et al., 2019). Intra-molecular conformational transitions within a single monomer were measured with fluorophores positioned at the termini of a 238-nucleotide RNA segment, which contains the Psi packaging signal as well as the primer binding site for the tRNA. Inter-molecular interactions between two RNA molecules were measured with fluorophores placed into separate RNA molecules. smFRET measurements indicated that the monomeric Psi can spontaneously sample the dimerization-competent and -incompetent forms. The annealing of tRNA to the monomer promoted the formation of the DIS exposed conformation, thereby shifting the equilibrium toward the kissing loop dimerization. NC binding then promoted the formation of the extended dimer, a conformation that facilitates packaging (Brigham et al., 2019). Thus, these experiments suggest that genome packaging proceeds through a series of events involving primer annealing, genome dimerization and packaging.

13. Concluding remarks

smFRET has previously provided critical insights into the dynamics of viral proteins throughout their life cycle (Abbondanzieri et al., 2008; Coey et al., 2016; Liu et al., 2008b, 2010). Here we focused on recent applications particularly in the Mothes as well as the

Munro laboratories that have extended these applications to physiologically relevant conditions such as complete HIV virions (Brigham et al., 2019; Das et al., 2018; Lu et al., 2019; Ma et al., 2018; Munro et al., 2014a,b). smFRET identified a new conformational Env state on the surface of HIV-1 (Lu et al., 2019), detected and characterized novel structural intermediates in the activation of both HIV-1 and influenza A viral glycoproteins (Das et al., 2018; Ma et al., 2018), and identified a new conformational state of Env that is highly vulnerable to ADCC (Alsaifi et al., 2019). Going forward, monitoring additional perspectives to provide better structural overviews, and correlating conformational changes within envelope glycoproteins with the membrane fusion reaction that proceeds through hemi-fusion, flickering and widening fusion pores will result in additional crucial insights (Chernomordik and Kozlov, 2008; Melikyan, 2014). Since it is emerging that reverse transcription is also coupled to the uncoating of the viral capsid (Hulme et al., 2011), great insights can be expected from expanding RT studies to complete virions. Finally, most powerful approach would be a seamless integration of dynamic methods such as smFRET and NMR with single-particle and cryo-electron microscopy to provide a comprehensive characterization of the structure and dynamics of biological processes.

Of great help are recent advances in unnatural amino acid and click chemistry labeling technologies that are expanding our ability to place fluorophores into sterically restricted regions of proteins that don't tolerate enzymatic labeling tags or free cysteines. This technology has allowed access into conformational changes within tight viral fusion machines (Das et al., 2018; Lu et al., 2019). The generation of membrane permeable dyes will also make unnatural amino acid and click chemistry available to imaging in living cells, which at some point can be expected to permit smFRET studies in living cells. The continued application of smFRET methods to increasingly physiologically relevant experimental conditions will enrich our knowledge of the conformational events during the viral life cycle, and inform the design of antiviral inhibitors, as well as immunogens for vaccine design.

Acknowledgments

We thank Andres Finzi, Jonathan R. Grover and James B. Munro for the critical reading of the manuscript, and James B. Munro for sharing unpublished results. Work in this area in the Mothes laboratory is supported by NIH grants RO1 GM116654, P01 AI150471, and P50 AI150464 to W.M., and by a Brown Coxe Fellowship to M.L.

Abbreviations

5'UTR	5' untranslated region
ADCC	antibody-dependent cellular cytotoxicity
AIDS	acquired immunodeficiency syndrome
APOBEC3	apolipoprotein B editing complex
bNAbs	broadly neutralizing antibodies
CA	capsid

CD4mc	CD4 mimetics
cryoET	cryo-electron tomography
CT	cytoplasmic tail
DIS	dimerization initiation site
Env	envelope glycoprotein
FCS	fluorescence correlation spectroscopy
gp41	glycoprotein 41, transmembrane domain of HIV-1 Env
gp120	glycoprotein 120, receptor/coreceptor binding domain of HIV-1 Env
gp160	glycoprotein 160, the precursor of HIV-1 Env
HA	influenza A hemagglutinin
HA₀	the precursor of HA
HA₁	the receptor sialic acid binding domain of HA
HA₂	the transmembrane domain of HA
HIV-1	human immunodeficiency virus 1
HR1, HR2	heptad repeat 1, heptad repeat 2
MA	matrix
MPER	membrane proximal extracellular region
NC	nucleocapsid
NMR	nuclear magnetic resonance
PIP₂	phosphatidylinositol 4,5-bisphosphate
Pol	polymerase
Psi	packaging signal
RNase H	ribonuclease H
RT	reverse transcriptase
smFRET	single-molecule Förster resonance energy transfer
SOSIP.664	SOS for a disulfide bridge, IP for an I559P mutation, 664 for the truncation side at residue 664
TIRFM	total internal reflection fluorescence microscope
TM	transmembrane region

tRNA	transfer RNA
VLPs	viral-like particles
vRNA	viral RNA

References

- Abbondanzieri EA, Bokinsky G, Rausch JW, Zhang JX, Le Grice SF, Zhuang X, 2008 Dynamic binding orientations direct activity of HIV reverse transcriptase. *Nature* 453, 184–189. [PubMed: 18464735]
- Akyuz N, Georgieva ER, Zhou Z, Stolzenberg S, Cuendet MA, Khelashvili G, Altman RB, Terry DS, Freed JH, Weinstein H, Boudker O, Blanchard SC, 2015 Transport domain unlocking sets the uptake rate of an aspartate transporter. *Nature* 518, 68–73. [PubMed: 25652997]
- Alsahafi N, Debbeche O, Sodroski J, Finzi A, 2015 Effects of the I559P gp41 change on the conformation and function of the human immunodeficiency virus (HIV-1) membrane envelope glycoprotein trimer. *PLoS One* 10, e0129405. [PubMed: 26010978]
- Alsahafi N, Anand SP, Castillo-Menendez L, Verly MM, Medjahed H, Prevost J, Herschhorn A, Richard J, Schon A, Melillo B, Freire E, Smith AB III, Sodroski J, Finzi A, 2018 SOSIP changes affect human immunodeficiency virus (HIV-1) envelope glycoprotein conformation and CD4 engagement. *J. Virol* 92, e01080–18. [PubMed: 30021898]
- Alsahafi N, Bakouche N, Kazemi M, Richard J, Ding S, Bhattacharyya S, Das D, Anand SP, Mothes W, Lifson J, Sodroski J, Smith AB, Rouiller I, Finzi A, Munro JB, 2019 An asymmetric opening of HIV-1 Env is required for anti-cluster A antibody binding. *Cell Host Microbe* 25 (4), 578–587. [PubMed: 30974085]
- Arthos J, Cicala C, Steenbeke TD, Chun TW, Dela Cruz C, Hanback DB, Khazanie P, Nam D, Schuck P, Selig SM, Van Ryk D, Chaikin MA, Fauci AS, 2002 Biochemical and biological characterization of a dodecameric CD4-Ig fusion protein: implications for therapeutic and vaccine strategies. *J. Biol. Chem* 277, 11456–11464. [PubMed: 11805109]
- Barouch DH, 2008 Challenges in the development of an HIV-1 vaccine. *Nature* 455, 613–619. [PubMed: 18833271]
- Bartesaghi A, Merk A, Borgnia MJ, Milne JL, Subramaniam S, 2013 Prefusion structure of trimeric HIV-1 envelope glycoprotein determined by cryo-electron microscopy. *Nat. Struct. Mol. Biol* 20, 1352–1357. [PubMed: 24154805]
- Beerens N, Jepsen MD, Nechiporuk-Zloy V, Kruger AC, Darlix JL, Kjems J, Birkedal V, 2013 Role of the primer activation signal in tRNA annealing onto the HIV-1 genome studied by single-molecule FRET microscopy. *RNA* 19, 517–526. [PubMed: 23404895]
- Bell NM, Lever AM, 2013 HIV Gag polyprotein: processing and early viral particle assembly. *Trends Microbiol.* 21, 136–144. [PubMed: 23266279]
- Benhaim M, Lee KK, 2018 Single-molecule analysis of a viral fusion protein illuminates a fusion-active intermediate state. *Cell* 174, 775–777. [PubMed: 30096307]
- Binley JM, Sanders RW, Clas B, Schuelke N, Master A, Guo Y, Kajumo F, Anselma DJ, Maddon PJ, Olson WC, Moore JP, 2000 A recombinant human immunodeficiency virus type 1 envelope glycoprotein complex stabilized by an intermolecular disulfide bond between the gp120 and gp41 subunits is an antigenic mimic of the trimeric virion-associated structure. *J. Virol* 74, 627–643. [PubMed: 10623724]
- Blanco-Melo D, Venkatesh S, Bieniasz PD, 2012 Intrinsic cellular defenses against human immunodeficiency viruses. *Immunity* 37, 399–411. [PubMed: 22999946]
- Bournazos S, Klein F, Pietzsch J, Seaman MS, Nussenzweig MC, Ravetch JV, 2014 Broadly neutralizing anti-HIV-1 antibodies require Fc effector functions for in vivo activity. *Cell* 158, 1243–1253. [PubMed: 25215485]
- Brandenburg B, Zhuang X, 2007 Virus trafficking—learning from single-virus tracking. *Nat. Rev. Microbiol* 5, 197–208. [PubMed: 17304249]

- Brigham B, Kitzrow J, Reyes JP, Musier-Forsyth K, Munro JB, 2019 Intrinsic conformational dynamics of the HIV-1 genomic RNA 5' UTR. *Proc. Natl. Acad. Sci. U. S. A* 116 (21), 10372–10381. [PubMed: 31068467]
- Bullough PA, Hughson FM, Skehel JJ, Wiley DC, 1994 Structure of influenza haemagglutinin at the pH of membrane fusion. *Nature* 371, 37–43. [PubMed: 8072525]
- Bushman FD, Malani N, Fernandes J, D'Orso I, Cagney G, Diamond TL, Zhou H, Hazuda DJ, Espeseth AS, Konig R, Bandyopadhyay S, Ideker T, Goff SP, Krogan NJ, Frankel AD, Young JA, Chanda SK, 2009 Host cell factors in HIV replication: meta-analysis of genome-wide studies. *PLoS Pathog.* 5, e1000437. [PubMed: 19478882]
- Buzon V, Natrajan G, Schibli D, Campelo F, Kozlov MM, Weissenhorn W, 2010 Crystal structure of HIV-1 gp41 including both fusion peptide and membrane proximal external regions. *PLoS Pathog.* 6, e1000880. [PubMed: 20463810]
- Campbell EM, Hope TJ, 2008 Live cell imaging of the HIV-1 life cycle. *Trends Microbiol.* 16, 580–587. [PubMed: 18977142]
- Cao LW, Pauthner M, Andrabi R, Rantalainen K, Berndsen Z, Diedrich JK, Menis S, Sok D, Bastidas R, Park SKR, Delahunty CM, He L, Guenaga J, Wyatt RT, Schief WR, Ward AB, Yates JR, Burton DR, Paulson JC, 2018 Differential processing of HIV envelope glycans on the virus and soluble recombinant trimer. *Nat. Commun* 9, 3693. [PubMed: 30209313]
- Carette JE, Raaben M, Wong AC, Herbert AS, Obernosterer G, Mulherkar N, Kuehne AI, Kranzusch PJ, Griffin AM, Ruthel G, Dal Cin P, Dye JM, Whelan SP, Chandran K, Brummelkamp TR, 2011 Ebola virus entry requires the cholesterol transporter Niemann-Pick C1. *Nature* 477, 340–343. [PubMed: 21866103]
- Carr CM, Kim PS, 1993 A spring-loaded mechanism for the conformational change of influenza hemagglutinin. *Cell* 73, 823–832. [PubMed: 8500173]
- Caskey M, Schoofs T, Gruell H, Settler A, Karagounis T, Kreider EF, Murrell B, Pfeifer N, Nogueira L, Oliveira TY, Learn GH, Cohen YZ, Lehmann C, Gillor D, Shimeliovich I, Unson-O'Brien C, Weiland D, Robles A, Kummerle T, Wyen C, Levin R, Witmer-Pack M, Eren K, Ignacio C, Kiss S, West AP Jr., Mouquet H, Zingman BS, Gulick RM, Keler T, Bjorkman PJ, Seaman MS, Hahn BH, Fatkenheuer G, Schlesinger SJ, Nussenzweig MC, Klein F, 2017 Antibody 10–1074 suppresses viremia in HIV-1-infected individuals. *Nat. Med* 23, 185–191. [PubMed: 28092665]
- Castillo-Menendez LR, Nguyen HT, Sodroski J, 2019 Conformational differences between functional human immunodeficiency virus envelope glycoprotein trimers and stabilized soluble trimers. *J. Virol* 93, e01709–e01718. [PubMed: 30429345]
- Chan DC, Fass D, Berger JM, Kim PS, 1997 Core structure of gp41 from the HIV envelope glycoprotein. *Cell* 89, 263–273. [PubMed: 9108481]
- Chernomordik LV, Kozlov MM, 2003 Protein-lipid interplay in fusion and fission of biological membranes. *Annu. Rev. Biochem* 72, 175–207. [PubMed: 14527322]
- Chernomordik LV, Kozlov MM, 2008 Mechanics of membrane fusion. *Nat. Struct. Mol. Biol* 15, 675–683. [PubMed: 18596814]
- Chuang GY, Geng H, Pancera M, Xu K, Cheng C, Acharya P, Chambers M, Druz A, Tsybovsky Y, Wanninger TG, Yang Y, Doria-Rose NA, Georgiev IS, Gorman J, Joyce MG, O'Dell S, Zhou T, McDermott AB, Mascola JR, Kwong PD, 2017 Structure-based design of a soluble prefusion-closed HIV-1 Env trimer with reduced CD4 affinity and improved immunogenicity. *J. Virol* 91, e02268–16. [PubMed: 28275193]
- Coey A, Larsen K, Puglisi JD, Viani Puglisi E, 2016 Heterogeneous structures formed by conserved RNA sequences within the HIV reverse transcription initiation site. *RNA* 22, 1689–1698. [PubMed: 27613581]
- Coey AT, Larsen KP, Choi J, Barrero DJ, Puglisi JD, Puglisi EV, 2018 Dynamic interplay of RNA and protein in the human immunodeficiency virus-1 reverse transcription initiation complex. *J. Mol. Biol* 430, 5137–5150. [PubMed: 30201267]
- Cohen MS, Chen YQ, McCauley M, Gamble T, Hosseini-pour MC, Kumarasamy N, Hakim JG, Kumwenda J, Grinsztejn B, Pilotto JH, Godbole SV, Mehendale S, Chariyalertsak S, Santos BR, Mayer KH, Hoffman IF, Eshleman SH, Piwowar-Manning E, Wang L, Makhema J, Mills LA, de Bruyn G, Sanne I, Eron J, Gallant J, Havlir D, Swindells S, Ribaud H, Elharrar V, Burns D, Taha

- TE, Nielsen-Saines K, Celentano D, Essex M, Fleming TR, Team HS, 2011 Prevention of HIV-1 infection with early antiretroviral therapy. *N. Engl. J. Med* 365, 493–505. [PubMed: 21767103]
- Crispin M, Ward AB, Wilson IA, 2018 Structure and immune recognition of the HIV glycan shield. *Annu. Rev. Biophys* 47, 499–523. [PubMed: 29595997]
- Crooks ET, Tong T, Chakrabarti B, Narayan K, Georgiev IS, Menis S, Huang X, Kulp D, Osawa K, Muranaka J, Stewart-Jones G, Destefano J, O'Dell S, LaBranche C, Robinson JE, Montefiori DC, McKee K, Du SX, Doria-Rose N, Kwong PD, Mascola JR, Zhu P, Schief WR, Wyatt RT, Whalen RG, Binley JM, 2015 Vaccine-elicited tier 2 HIV-1 neutralizing antibodies bind to quaternary epitopes involving glycan-deficient patches proximal to the CD4 binding site. *PLoS Pathog.* 11, e1004932. [PubMed: 26023780]
- Das K, Arnold E, 2013 HIV-1 reverse transcriptase and antiviral drug resistance. Part 1. *Curr. Opin. Virol* 3, 111–118. [PubMed: 23602471]
- Das DK, Govindan R, Nikic-Spiegel I, Krammer F, Lemke EA, Munro JB, 2018 Direct visualization of the conformational dynamics of single influenza hemagglutinin trimers. *Cell* 174, 926–937. [PubMed: 29961575]
- Datta SA, Curtis JE, Ratcliff W, Clark PK, Crist RM, Lebowitz J, Krueger S, Rein A, 2007 Conformation of the HIV-1 Gag protein in solution. *J. Mol. Biol* 365, 812–824. [PubMed: 17097677]
- Dick RA, Zadrozny KK, Xu C, Schur FKM, Lyddon TD, Ricana CL, Wagner JM, Perilla JR, Ganser-Pornillos BK, Johnson MC, Pornillos O, Vogt VM, 2018 Inositol phosphates are assembly co-factors for HIV-1. *Nature* 560, 509–512. [PubMed: 30069050]
- Dunkle JA, Wang L, Feldman MB, Pulk A, Chen VB, Kapral GJ, Noeske J, Richardson JS, Blanchard SC, Cate JH, 2011 Structures of the bacterial ribosome in classical and hybrid states of tRNA binding. *Science* 332, 981–984. [PubMed: 21596992]
- Forthal DN, Finzi A, 2018 Antibody-dependent cellular cytotoxicity in HIV infection. *AIDS* 32, 2439–2451. [PubMed: 30234611]
- Freed EO, 2015 HIV-1 assembly, release and maturation. *Nat. Rev. Microbiol* 13, 484–496. [PubMed: 26119571]
- Ganser-Pornillos BK, Yeager M, Pornillos O, 2012 Assembly and architecture of HIV. *Adv. Exp. Med. Biol* 726, 441–465. [PubMed: 22297526]
- Go EP, Ding H, Zhang S, Ringe RP, Nicely N, Hua D, Steinbock RT, Golabek M, Alin J, Alam SM, Cupo A, Haynes BF, Kappes JC, Moore JP, Sodroski JG, Desaire H, 2017 Glycosylation benchmark profile for HIV-1 envelope glycoprotein production based on eleven Env trimers. *J. Virol* 91, e02428–16. [PubMed: 28202756]
- Gregorio GG, Masureel M, Hilger D, Terry DS, Juette M, Zhao H, Zhou Z, Perez-Aguilar JM, Hauge M, Mathiasen S, Javitch JA, Weinstein H, Kobilka BK, Blanchard SC, 2017 Single-molecule analysis of ligand efficacy in beta2AR-G-protein activation. *Nature* 547, 68–73. [PubMed: 28607487]
- Gristick HB, von Boehmer L, West AP Jr., Schamber M, Gazumyan A, Golijanin J, Seaman MS, Fatkenheuer G, Klein F, Nussenzweig MC, Bjorkman PJ, 2016 Natively glycosylated HIV-1 Env structure reveals new mode for antibody recognition of the CD4-binding site. *Nat. Struct. Mol. Biol* 23, 906–915. [PubMed: 27617431]
- Harrison SC, 2008 Viral membrane fusion. *Nat. Struct. Mol. Biol* 15, 690–698. [PubMed: 18596815]
- Harrison SC, 2015 Viral membrane fusion. *Virology* 479–480, 498–507.
- Henzler-Wildman K, Kern D, 2007 Dynamic personalities of proteins. *Nature* 450, 964–972. [PubMed: 18075575]
- Herschhorn A, Ma X, Gu C, Ventura JD, Castillo-Menendez L, Melillo B, Terry DS, Smith AB 3rd, Blanchard SC, Munro JB, Mothes W, Finzi A, Sodroski J, 2016 Release of gp120 restraints leads to an entry-competent intermediate state of the HIV-1 envelope glycoproteins. *mBio* 7, e01598–16. [PubMed: 27795397]
- Huang H, Chopra R, Verdine GL, Harrison SC, 1998 Structure of a covalently trapped catalytic complex of HIV-1 reverse transcriptase: implications for drug resistance. *Science* 282, 1669–1675. [PubMed: 9831551]

- Hulme AE, Perez O, Hope TJ, 2011 Complementary assays reveal a relationship between HIV-1 uncoating and reverse transcription. *Proc. Natl. Acad. Sci. U. S. A* 108, 9975–9980. [PubMed: 21628558]
- Hunter E, 1997 Viral entry and receptors In: Coffin JM, Hughes SH, Varmus HE (Eds.), *Retroviruses*. Cold Spring Harbor, New York.
- Jouvenet N, Bieniasz PD, Simon SM, 2008 Imaging the biogenesis of individual HIV-1 virions in live cells. *Nature* 454, 236–240. [PubMed: 18500329]
- Jouvenet N, Simon SM, Bieniasz PD, 2009 Imaging the interaction of HIV-1 genomes and Gag during assembly of individual viral particles. *Proc. Natl. Acad. Sci. U. S. A* 106, 19114–19119. [PubMed: 19861549]
- Julien JP, Lee PS, Wilson IA, 2012 Structural insights into key sites of vulnerability on HIV-1 Env and influenza HA. *Immunol. Rev* 250, 180–198. [PubMed: 23046130]
- Julien JP, Cupo A, Sok D, Stanfield RL, Lyumkis D, Deller MC, Klasse PJ, Burton DR, Sanders RW, Moore JP, Ward AB, Wilson IA, 2013 Crystal structure of a soluble cleaved HIV-1 envelope trimer. *Science* 342, 1477–1483. [PubMed: 24179159]
- Keane SC, Summers MF, 2016 NMR studies of the structure and function of the HIV-1 5'-leader. *Viruses* 8, 338.
- Kesavardhana S, Varadarajan R, 2014 Stabilizing the native trimer of HIV-1 Env by destabilizing the heterodimeric interface of the gp41 postfusion six-helix bundle. *J. Virol* 88, 9590–9604. [PubMed: 24920800]
- Khasnis MD, Halkidis K, Bhardwaj A, Root MJ, 2016 Receptor activation of HIV-1 Env leads to asymmetric exposure of the gp41 trimer. *PLoS Pathog.* 12, e1006098. [PubMed: 27992602]
- Kohlstaedt L, Wang J, Friedman J, Rice P, Steitz T, 1992 Crystal structure at 3.5 Å resolution of HIV-1 reverse transcriptase complexed with an inhibitor. *Science* 256, 1783–1790. [PubMed: 1377403]
- Kong R, Xu K, Zhou TQ, Acharya P, Lemmin T, Liu K, Ozorowski G, Soto C, Taft JD, Bailer RT, Cale EM, Chen L, Choi CW, Chuang GY, Doria-Rose NA, Druz A, Georgiev IS, Gorman J, Huang JH, Joyce MG, Louder MK, Ma XC, McKee K, O'Dell S, Pancera M, Yang YP, Blanchard SC, Mothes W, Burton DR, Koff WC, Connors M, Ward AB, Kwong PD, Mascola JR, 2016 Fusion peptide of HIV-1 as a site of vulnerability to neutralizing antibody. *Science* 352, 828–833. [PubMed: 27174988]
- Kwon YD, Pancera M, Acharya P, Georgiev IS, Crooks ET, Gorman J, Joyce MG, Guttman M, Ma X, Narpala S, Soto C, Terry DS, Yang Y, Zhou T, Ahlsen G, Bailer RT, Chambers M, Chuang GY, Doria-Rose NA, Druz A, Hallen MA, Harned A, Kirys T, Louder MK, O'Dell S, Ofek G, Osawa K, Prabhakaran M, Sastry M, Stewart-Jones GB, Stuckey J, Thomas PV, Tittley T, Williams C, Zhang B, Zhao H, Zhou Z, Donald BR, Lee LK, Zolla-Pazner S, Baxa U, Schon A, Freire E, Shapiro L, Lee KK, Arthos J, Munro JB, Blanchard SC, Mothes W, Binley JM, McDermott AB, Mascola JR, Kwong PD, 2015 Crystal structure, conformational fixation and entry-related interactions of mature ligand-free HIV-1 Env. *Nat. Struct. Mol. Biol* 22, 522–531. [PubMed: 26098315]
- Kwong PD, Wyatt R, Robinson J, Sweet RW, Sodroski J, Hendrickson WA, 1998 Structure of an HIV gp120 envelope glycoprotein in complex with the CD4 receptor and a neutralizing human antibody. *Nature* 393, 648–659. [PubMed: 9641677]
- Kwong PD, Doyle ML, Casper DJ, Cicala C, Leavitt SA, Majeed S, Steenbeke TD, Venturi M, Chaiken I, Fung M, Katinger H, Parren PWLH, Robinson J, Van Ryk D, Wang LP, Burton DR, Freire E, Wyatt R, Sodroski J, Hendrickson WA, Arthos J, 2002 HIV-1 evades antibody-mediated neutralization through conformational masking of receptor-binding sites. *Nature* 420, 678–682. [PubMed: 12478295]
- Kwong PD, Mascola JR, Nabel GJ, 2013 Broadly neutralizing antibodies and the search for an HIV-1 vaccine: the end of the beginning. *Nat. Rev. Immunol* 13, 693–701. [PubMed: 23969737]
- Lanchy JM, Ehresmann C, Le Grice SF, Ehresmann B, Marquet R, 1996 Binding and kinetic properties of HIV-1 reverse transcriptase markedly differ during initiation and elongation of reverse transcription. *EMBO J.* 15, 7178–7187. [PubMed: 9003793]

- Larsen KP, Mathihran YK, Kappel K, Coey AT, Chen DH, Barrero D, Madigan L, Puglisi JD, Skiniotis G, Puglisi EV, 2018 Architecture of an HIV-1 reverse transcriptase initiation complex. *Nature* 557, 118–122. [PubMed: 29695867]
- Lee JH, Ozorowski G, Ward AB, 2016 Cryo-EM structure of a native, fully glycosylated, cleaved HIV-1 envelope trimer. *Science* 351, 1043–1048. [PubMed: 26941313]
- Lee JH, Andrabi R, Su CY, Yasmeen A, Julien JP, Kong L, Wu NC, McBride R, Sok D, Pauthner M, Cottrell CA, Nieusma T, Blattner C, Paulson JC, Klasse PJ, Wilson IA, Burton DR, Ward AB, 2017 A broadly neutralizing antibody targets the dynamic HIV envelope trimer apex via a long, rigidified, and anionic beta-hairpin structure. *Immunity* 46, 690–702. [PubMed: 28423342]
- Lemmin T, Soto C, Stuckey J, Kwong PD, 2017 Microsecond dynamics and network analysis of the HIV-1 SOSIP Env trimer reveal collective behavior and conserved microdomains of the glycan shield. *Structure* 25, 1631–1639. [PubMed: 28890362]
- Lerner E, Cordes T, Ingargiola A, Alhadid Y, Chung S, Michalet X, Weiss S, 2018 Toward dynamic structural biology: two decades of single-molecule Forster resonance energy transfer. *Science* 359, eaan1133. [PubMed: 29348210]
- Liu J, Bartesaghi A, Borgnia MJ, Sapiro G, Subramaniam S, 2008a Molecular architecture of native HIV-1 gp120 trimers. *Nature* 455, 109–113. [PubMed: 18668044]
- Liu S, Abbondanzieri EA, Rausch JW, Le Grice SF, Zhuang X, 2008b Slide into action: dynamic shuttling of HIV reverse transcriptase on nucleic acid substrates. *Science* 322, 1092–1097. [PubMed: 19008444]
- Liu S, Harada BT, Miller JT, Le Grice SF, Zhuang X, 2010 Initiation complex dynamics direct the transitions between distinct phases of early HIV reverse transcription. *Nat. Struct. Mol. Biol* 17, 1453–1460. [PubMed: 21102446]
- Lu K, Heng X, Garyu L, Monti S, Garcia EL, Kharytonchyk S, Dorjsuren B, Kulandaivel G, Jones S, Hiremath A, Divakaruni SS, LaCotti C, Barton S, Tummillo D, Hosic A, Edme K, Albrecht S, Telesnitsky A, Summers MF, 2011a NMR detection of structures in the HIV-1 5'-leader RNA that regulate genome packaging. *Science* 334, 242–245. [PubMed: 21998393]
- Lu K, Heng X, Summers MF, 2011b Structural determinants and mechanism of HIV-1 genome packaging. *J. Mol. Biol* 410, 609–633. [PubMed: 21762803]
- Lu M, Ma X, Castillo-Menendez LR, Gorman J, Alsaifi N, Ermel U, Terry DS, Chambers M, Peng D, Zhang B, Zhou T, Reichard N, Wang K, Grover JR, Carmen BP, Gardner MR, Nikic-Spiegel I, Sugawara A, Arthos J, Lemke EA, Smith AB, Farzan M, Abrams C, Munro JB, McDermott AB, Finzi A, Kwong PD, Blanchard SC, Sodroski J, Mothes W, 2019 Associating HIV-1 envelope glycoprotein structures with states on virus observed by smFRET. *Nature* 568 (7752), 415–419. [PubMed: 30971821]
- Lyumkis D, Julien JP, de Val N, Cupo A, Potter CS, Klasse PJ, Burton DR, Sanders RW, Moore JP, Carragher B, Wilson IA, Ward AB, 2013 Cryo-EM structure of a fully glycosylated soluble cleaved HIV-1 envelope trimer. *Science* 342, 1484–1490. [PubMed: 24179160]
- Ma XC, Lu ML, Gorman J, Terry DS, Hong XY, Zhou Z, Zhao H, Altman RB, Arthos J, Blanchard SC, Kwong PD, Munro JB, Mothes W, 2018 HIV-1 Env trimer opens through an asymmetric intermediate in which individual protomers adopt distinct conformations. *Elife* 7, e34271. [PubMed: 29561264]
- Mallery DL, Marquez CL, McEwan WA, Dickson CF, Jacques DA, Anandapadamanaban M, Bichel K, Towers GJ, Saiardi A, Bocking T, James LC, 2018 IP6 is an HIV pocket factor that prevents capsid collapse and promotes DNA synthesis. *eLife* 7, e35335. [PubMed: 29848441]
- Mao Y, Wang L, Gu C, Herschhorn A, Xiang SH, Haim H, Yang X, Sodroski J, 2012 Subunit organization of the membrane-bound HIV-1 envelope glycoprotein trimer. *Nat. Struct. Mol. Biol* 19, 893–899. [PubMed: 22864288]
- Marsh M, Helenius A, 2006 Virus entry: open sesame. *Cell* 124, 729–740. [PubMed: 16497584]
- Mattei S, Schur FK, Briggs JA, 2016 Retrovirus maturation-an extraordinary structural transformation. *Curr. Opin. Virol* 18, 27–35. [PubMed: 27010119]
- McFadden MJ, Gokhale NS, Horner SM, 2017 Protect this house: cytosolic sensing of viruses. *Curr. Opin. Virol* 22, 36–43. [PubMed: 27951430]

- Melikyan GB, 2014 HIV entry: a game of hide-and-fuse? *Curr. Opin. Virol* 4, 1–7. [PubMed: 24525288]
- Melikyan GB, Barnard RJO, Abrahamyan LG, Mothes W, Young JAT, 2005 Imaging individual retroviral fusion events: from hemifusion to pore formation and growth. *Proc. Natl. Acad. Sci. U. S. A* 102, 8728–8733. [PubMed: 15937118]
- Miyazaki Y, Garcia EL, King SR, Iyalla K, Loeliger K, Starck P, Syed S, Telesnitsky A, Summers MF, 2010 An RNA structural switch regulates diploid genome packaging by Moloney murine leukemia virus. *J. Mol. Biol* 396, 141–152. [PubMed: 19931283]
- Montefiori DC, Roederer M, Morris L, Seaman MS, 2018 Neutralization tiers of HIV-1. *Curr. Opin. HIV AIDS* 13, 128–136. [PubMed: 29266013]
- Mucksch F, Laketa V, Muller B, Schultz C, Krausslich HG, 2017 Synchronized HIV assembly by tunable PIP2 changes reveals PIP2 requirement for stable Gag anchoring. *eLife* 6, e25287. [PubMed: 28574338]
- Mukhopadhyay S, Kuhn RJ, Rossmann MG, 2005 A structural perspective of the flavivirus life cycle. *Nat. Rev. Microbiol* 3, 13–22. [PubMed: 15608696]
- Mulder LC, Harari A, Simon V, 2008 Cytidine deamination induced HIV-1 drug resistance. *Proc. Natl. Acad. Sci. U. S. A* 105, 5501–5506. [PubMed: 18391217]
- Munro JB, Mothes W, 2014 The HIV-1 Env trimer in HD. *Structure* 22, 935–936. [PubMed: 25007222]
- Munro JB, Mothes W, 2015 Structure and dynamics of the native HIV-1 Env trimer. *J. Virol* 89, 5752–5755. [PubMed: 25762739]
- Munro JB, Altman RB, Tung CS, Cate JHD, Sanbonmatsu KY, Blanchard SC, 2010 Spontaneous formation of the unlocked state of the ribosome is a multistep process. *Proc. Natl. Acad. Sci. U. S. A* 107, 709–714. [PubMed: 20018653]
- Munro JB, Gorman J, Ma X, Zhou Z, Arthos J, Burton DR, Koff WC, Courter JR, Smith AB 3rd, Kwong PD, Blanchard SC, Mothes W, 2014a Conformational dynamics of single HIV-1 envelope trimers on the surface of native virions. *Science* 346, 759–763. [PubMed: 25298114]
- Munro JB, Nath A, Farber M, Datta SA, Rein A, Rhoades E, Mothes W, 2014b A conformational transition observed in single HIV-1 Gag molecules during in vitro assembly of virus-like particles. *J. Virol* 88, 3577–3585. [PubMed: 24403576]
- Ono A, Ablan SD, Lockett SJ, Nagashima K, Freed EO, 2004 Phosphatidylinositol (4,5) bisphosphate regulates HIV-1 Gag targeting to the plasma membrane. *Proc. Natl. Acad. Sci. U. S. A* 101, 14889–14894. [PubMed: 15465916]
- Ozorowski G, Pallesen J, de Val N, Lyumkis D, Cottrell CA, Torres JL, Copps J, Stanfield RL, Cupo A, Pugach P, Moore JP, Wilson IA, Ward AB, 2017 Open and closed structures reveal allostery and pliability in the HIV-1 envelope spike. *Nature* 547, 360–363. [PubMed: 28700571]
- Pancera M, Zhou T, Druz A, Georgiev IS, Soto C, Gorman J, Huang J, Acharya P, Chuang GY, Ofek G, Stewart-Jones GB, Stuckey J, Bailer RT, Joyce MG, Louder MK, Tumba N, Yang Y, Zhang B, Cohen MS, Haynes BF, Mascola JR, Morris L, Munro JB, Blanchard SC, Mothes W, Connors M, Kwong PD, 2014 Structure and immune recognition of trimeric pre-fusion HIV-1 Env. *Nature* 514, 455–461. [PubMed: 25296255]
- Plass T, Milles S, Koehler C, Schultz C, Lemke EA, 2011 Genetically encoded copper-free click chemistry. *Angew. Chem. Int. Ed. Engl* 50, 3878–3881. [PubMed: 21433234]
- Prevost J, Richard J, Ding S, Pacheco B, Charlebois R, Hahn BH, Kaufmann DE, Finzi A, 2018 Envelope glycoproteins sampling states 2/3 are susceptible to ADCC by sera from HIV-1-infected individuals. *Virology* 515, 38–45. [PubMed: 29248757]
- Richard J, Pacheco B, Gohain N, Veillette M, Ding SL, Alsahafi N, Tolbert WD, Prevost J, Chapleau JP, Coutu M, Jia MX, Brassard N, Park J, Courter JR, Melillo B, Martin L, Tremblay C, Hahn BH, Kaufmann DE, Wu XL, Smith AB, Sodroski J, Pazgier M, Finzi A, 2016 Co-receptor binding site antibodies enable CD4-mimetics to expose conserved anti-cluster A ADCC epitopes on HIV-1 envelope glycoproteins. *EBioMedicine* 12, 208–218. [PubMed: 27633463]
- Richard J, Prevost J, Alsahafi N, Ding S, Finzi A, 2018 Impact of HIV-1 envelope conformation on ADCC responses. *Trends Microbiol.* 26, 253–265. [PubMed: 29162391]

- Riedel C, Vasishtan D, Siebert CA, Whittle C, Lehmann MJ, Mothes W, Grunewald K, 2017 Native structure of a retroviral envelope protein and its conformational change upon interaction with the target cell. *J. Struct. Biol* 197, 172–180. [PubMed: 27345930]
- Ringe RP, Sanders RW, Yasmeen A, Kim HJ, Lee JH, Cupo A, Korzun J, Derking R, van Montfort T, Julien JP, Wilson IA, Klasse PJ, Ward AB, Moore JP, 2013 Cleavage strongly influences whether soluble HIV-1 envelope glycoprotein trimers adopt a native-like conformation. *Proc. Natl. Acad. Sci. U. S. A* 110, 18256–18261. [PubMed: 24145402]
- Ringe RP, Pugach P, Cottrell CA, LaBranche CC, Seabright GE, Ketas TJ, Ozorowski G, Kumar S, Schorcht A, van Gils MJ, Crispin M, Montefiori DC, Wilson IA, Ward AB, Sanders RW, Klasse PJ, Moore JP, 2019 Closing and opening holes in the glycan shield of HIV-1 envelope glycoprotein SOSIP trimers can redirect the neutralizing antibody response to the newly unmasked epitopes. *J. Virol* 93, e01656–18. [PubMed: 30487280]
- Roy R, Hohng S, Ha T, 2008 A practical guide to single-molecule FRET. *Nat. Methods* 5, 507–516. [PubMed: 18511918]
- Sakin V, Hanne J, Dunder J, Anders-Osswein M, Laketa V, Nikic I, Krausslich HG, Lemke EA, Muller B, 2017 A versatile tool for live-cell imaging and super-resolution nanoscopy studies of HIV-1 Env distribution and mobility. *Cell Chem. Biol* 24, 635–645. [PubMed: 28457706]
- Sanders RW, Moore JP, 2017 Native-like Env trimers as a platform for HIV-1 vaccine design. *Immunol. Rev* 275, 161–182. [PubMed: 28133806]
- Sanders RW, Vesanen M, Schuelke N, Master A, Schiffner L, Kalyanaraman R, Paluch M, Berkhout B, Maddon PJ, Olson WC, Lu M, Moore JP, 2002 Stabilization of the soluble, cleaved, trimeric form of the envelope glycoprotein complex of human immunodeficiency virus type 1. *J. Virol* 76, 8875–8889. [PubMed: 12163607]
- Sanders RW, Derking R, Cupo A, Julien JP, Yasmeen A, de Val N, Kim HJ, Blattner C, de la Pena AT, Korzun J, Golabek M, de Los Reyes K, Ketas TJ, van Gils MJ, King CR, Wilson IA, Ward AB, Klasse PJ, Moore JP, 2013 A next-generation cleaved, soluble HIV-1 Env trimer, BG505 SOSIP.664 gp140, expresses multiple epitopes for broadly neutralizing but not non-neutralizing antibodies. *PLoS Pathog.* 9, e1003618. [PubMed: 24068931]
- Sarafianos SG, Marchand B, Das K, Himmel DM, Parniak MA, Hughes SH, Arnold E, 2009 Structure and function of HIV-1 reverse transcriptase: molecular mechanisms of polymerization and inhibition. *J. Mol. Biol* 385, 693–713. [PubMed: 19022262]
- Sattentau Q, 2008 Avoiding the void: cell-to-cell spread of human viruses. *Nat. Rev. Microbiol* 6, 815–826. [PubMed: 18923409]
- Scharf L, Wang H, Gao H, Chen S, McDowall AW, Bjorkman PJ, 2015 Broadly neutralizing antibody 8ANC195 recognizes closed and open states of HIV-1 Env. *Cell* 162, 1379–1390. [PubMed: 26359989]
- Scheid JF, Horwitz JA, Bar-On Y, Kreider EF, Lu CL, Lorenzi JC, Feldmann A, Braunschweig M, Nogueira L, Oliveira T, Shimeliovich I, Patel R, Burke L, Cohen YZ, Hadrigan S, Settler A, Witmer-Pack M, West AP Jr., Juelg B, Keler T, Hawthorne T, Zingman B, Gulick RM, Pfeifer N, Learn GH, Seaman MS, Bjorkman PJ, Klein F, Schlesinger SJ, Walker BD, Hahn BH, Nussenzweig MC, Caskey M, 2016 HIV-1 antibody 3BNC117 suppresses viral rebound in humans during treatment interruption. *Nature* 535, 556–560. [PubMed: 27338952]
- Skehel JJ, Wiley DC, 1998 Coiled coils in both intracellular vesicle and viral membrane fusion. *Cell* 95, 871–874. [PubMed: 9875840]
- Skehel JJ, Wiley DC, 2000 Receptor binding and membrane fusion in virus entry: the influenza hemagglutinin. *Annu. Rev. Biochem* 69, 531–569. [PubMed: 10966468]
- Sok D, Le KM, Vadnais M, Saye-Francisco KL, Jardine JG, Torres JL, Berndsen ZT, Kong L, Stanfield R, Ruiz J, Ramos A, Liang CH, Chen PL, Criscitiello MF, Mwangi W, Wilson IA, Ward AB, Smider VV, Burton DR, 2017 Rapid elicitation of broadly neutralizing antibodies to HIV by immunization in cows. *Nature* 548, 108–111. [PubMed: 28726771]
- Stadtmueller BM, Bridges MD, Dam KM, Lerch MT, Huey-Tubman KE, Hubbell WL, Bjorkman PJ, 2018 DEER spectroscopy measurements reveal multiple conformations of HIV-1 SOSIP envelopes that show similarities with envelopes on native virions. *Immunity* 49, 235–246. [PubMed: 30076100]

- Stevens J, Blixt O, Tumpey TM, Taubenberger JK, Paulson JC, Wilson IA, 2006 Structure and receptor specificity of the hemagglutinin from an H5N1 influenza virus. *Science* 312, 404–410. [PubMed: 16543414]
- Stewart-Jones GB, Soto C, Lemmin T, Chuang GY, Druz A, Kong R, Thomas PV, Wagh K, Zhou T, Behrens AJ, Bylund T, Choi CW, Davison JR, Georgiev IS, Joyce MG, Kwon YD, Pancera M, Taft J, Yang Y, Zhang B, Shivatare SS, Shivatare VS, Lee CC, Wu CY, Bewley CA, Burton DR, Koff WC, Connors M, Crispin M, Baxa U, Korber BT, Wong CH, Mascola JR, Kwong PD, 2016 Trimeric HIV-1-Env structures define glycan shields from clades A, B, and G. *Cell* 165, 813–826. [PubMed: 27114034]
- Sundquist WI, Krausslich HG, 2012 HIV-1 assembly, budding, and maturation. *Cold Spring Harb. Perspect. Med* 2, a006924. [PubMed: 22762019]
- Uchil PD, Haugh KD, Pi R, Mothes W, 2019 In vivo imaging driven approaches to study virus dissemination and pathogenesis. *Annu. Rev. Virol* 6, 19.1–19.24.
- Vaney MC, Rey FA, 2011 Class II enveloped viruses. *Cell. Microbiol* 13, 1451–1459. [PubMed: 21790946]
- Veillette M, Desormeaux A, Medjahed H, Gharsallah NE, Coutu M, Baalwa J, Guan Y, Lewis G, Ferrari G, Hahn BH, Haynes BF, Robinson JE, Kaufmann DE, Bonsignori M, Sodroski J, Finzi A, 2014 Interaction with cellular CD4 exposes HIV-1 envelope epitopes targeted by antibody-dependent cell-mediated cytotoxicity. *J. Virol* 88, 2633–2644. [PubMed: 24352444]
- Veillette M, Coutu M, Richard J, Batrville LA, Dagher O, Bernard N, Tremblay C, Kaufmann DE, Roger M, Finzi A, 2015 The HIV-1 gp120 CD4-bound conformation is preferentially targeted by antibody-dependent cellular cytotoxicity-mediating antibodies in sera from HIV-1-infected individuals. *J. Virol* 89, 545–551. [PubMed: 25339767]
- Wagh K, Kreider EF, Li Y, Barbican HJ, Learn GH, Giorgi E, Hraber PT, Decker TG, Smith AG, Gondim MV, Gillis L, Wandzilak J, Chuang GY, Rawi R, Cai F, Pellegrino P, Williams I, Overbaugh J, Gao F, Kwong PD, Haynes BF, Shaw GM, Borrow P, Seaman MS, Hahn BH, Korber B, 2018 Completeness of HIV-1 envelope glycan shield at transmission determines neutralization breadth. *Cell Rep.* 25, 893–908. [PubMed: 30355496]
- Wang H, Cohen AA, Galimidi RP, Gristick HB, Jensen GJ, Bjorkman PJ, 2016 Cryo-EM structure of a CD4-bound open HIV-1 envelope trimer reveals structural rearrangements of the gp120 V1V2 loop. *Proc. Natl. Acad. Sci. U. S. A* 113, E7151–E7158. [PubMed: 27799557]
- Wang H, Barnes CO, Yang Z, Nussenzweig MC, Bjorkman PJ, 2018 Partially open HIV-1 envelope structures exhibit conformational changes relevant for coreceptor binding and fusion. *Cell Host Microbe* 24, 579–592. [PubMed: 30308160]
- Wei X, Decker JM, Wang S, Hui H, Kappes JC, Wu X, Salazar-Gonzalez JF, Salazar MG, Kilby JM, Saag MS, Komarova NL, Nowak MA, Hahn BH, Kwong PD, Shaw GM, 2003 Antibody neutralization and escape by HIV-1. *Nature* 422, 307–312. [PubMed: 12646921]
- Weissenhorn W, Dessen A, Harrison SC, Skehel JJ, Wiley DC, 1997 Atomic structure of the ectodomain from HIV-1 gp41. *Nature* 387, 426–430. [PubMed: 9163431]
- White JM, Whittaker GR, 2016 Fusion of enveloped viruses in endosomes. *Traffic* 17, 593–614. [PubMed: 26935856]
- Wilson IA, Skehel JJ, Wiley DC, 1981 Structure of the haemagglutinin membrane glycoprotein of influenza virus at 3 Å resolution. *Nature* 289, 366–373. [PubMed: 7464906]
- Wyatt R, Kwong PD, Desjardins E, Sweet RW, Robinson J, Hendrickson WA, Sodroski JG, 1998 The antigenic structure of the HIV gp120 envelope glycoprotein. *Nature* 393, 705–711. [PubMed: 9641684]
- Zhao YF, Terry D, Shi L, Weinstein H, Blanchard SC, Javitch JA, 2010 Single-molecule dynamics of gating in a neurotransmitter transporter homologue. *Nature* 465, 188–U173. [PubMed: 20463731]
- Zheng Q, Juetter MF, Jockusch S, Wasserman MR, Zhou Z, Altman RB, Blanchard SC, 2014 Ultra-stable organic fluorophores for single-molecule research. *Chem. Soc. Rev* 43, 1044–1056. [PubMed: 24177677]

Fig. 1.

smFRET imaging allows for real-time observation of conformational dynamics of biomolecules. (A) Along the timescale of biomolecular dynamics, large domain motions and global motions of proteins lie within the range of millisecond to a few seconds (Henzler-Wildman and Kern, 2007). (B) Diagrams depicting the distance dependence of the energy transfer from donor to acceptor fluorophore (left), and an example smFRET analysis of conformational dynamics for a labeled HIV-1 Env molecule (right). (Left) Energy transfer efficiency (FRET) between donor and acceptor depends on the distance. When they are close to each other, energy transfer efficiency is high, or vice versa. By recording donor (such as Cy3) and acceptor (such as Cy5) fluorescence, corresponding energy transfer efficiency is derived to monitor the relative distance changes between two labeled sites on a biomolecule of interest. At single-molecule level, distinct conformational states and the transitions between distinct states are directly visible based on FRET value and FRET trace in real time, respectively. (Right) For instance, single HIV-1 gp120 molecules within native trimers are dynamic in time observed from a FRET trace, and it constantly samples three conformational states characterized as low-FRET, intermediate-FRET, and high-FRET (Lu et al., 2019).

Fig. 2.

smFRET imaging of native HIV-1 Env on the surface of intact viruses reveals three distinct conformational states. (A) Individual HIV-1 virus carrying single Cy3/Cy3 FRET dyes labeled gp120 within a trimer in the context of otherwise wild-type trimers was immobilized and imaged via total internal reflection microscopy. High-resolution structure of Env trimer shows the positions where dyes are labeled on gp120 (cyan) within other wild-type gp120/gp41 (blue/gray) trimer (structure was adapted from PDB: 4NCO). (B) Observation of the opening and further closing of HIV-1 Env trimer via FRET population histograms. Native functional HIV-1 Env (HIV-1 isolates NL4-3, JR-FL, and BG505) predominately resides in pre-triggered State 1, and has inherent access to State 3, through one necessary intermediate (State 2). HIV-1 Env opens into State 3 upon binding of the potent 12xCD4 (left panel).

bNAbs PG16, PGT145 (NL4–3), PGT128, PGT122 (JR-FL), and entry inhibitor BMS reduce the occupancy of State 2 and State 3, further stabilizing State 1 (right panel). *Adapted from Ma, X.C., Lu, M.L., Gorman, J., Terry, D.S., Hong, X.Y., Zhou, Z., Zhao, H., Altman, R.B., Arthoe, J., Blanchard, S.C., Kwong, P.D., Munro, J.B., Mothes, W., 2018. HIV-1 Env trimer opens through an asymmetric intermediate in which individual protomers adopt distinct conformations. eLife 7, e34271; Munro, J.B., Gorman, J., Ma, X., Zhou, Z., Arthos, J., Burton, D. R., Koff, W.C., Courter, J.R., Smith 3rd, A.B., Kwong, P.D., Blanchard, S.C., Mothes, W., 2014. Conformational dynamics of single HIV-1 envelope trimers on the surface of native virions. Science 346, 759–763, with permission from AAAS.*

Fig. 3.

Experimental scheme utilizing mixed trimers to test possible existence of asymmetric HIV-1 Env trimer intermediates. Env carrying the D368R substitution are incapable of binding CD4 (Ma et al., 2018). Taking advantage of D368R substitutions, trimers were engineered to engage none, a single CD4, two CD4, or three CD4 molecules, respectively. Within the unliganded trimer, all three gp120 protomers are in State 1 resulting in the trimer configuration (1, 1, 1). In contrast, the three CD4-bound trimer resides in a trimer configuration (3, 3, 3). In the case of single CD4-bound trimer, experimental data indicated that gp120 protomers binding the single CD4 adopted State 3. In contrast, protomers adjacent to the single bound CD4, adopted State 2. Thus, the Env trimer is in asymmetric configuration (3, 2, 2). Adding to this, when trimer binds a second CD4 molecule, all three gp120 subunits open into State 3, that is, the trimer is configured as (3, 3, 3). *Adapted from Ma, X.C., Lu, M.L., Gorman, J., Terry, D.S., Hong, X.Y., Zhou, Z., Zhao, H., Altman, R.B., Arthoe, J., Blanchard, S.C., Kwong, P.D., Munro, J.B., Mothes, W., 2018. HIV-1 Env trimer*

opens through an asymmetric intermediate in which individual protomers adopt distinct conformations. eLife 7, e34271.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Fig. 4.

A smFRET studies-derived model for the activation of HIV-1 Env by receptor and coreceptor. HIV-1 Env predominately resides in pre-triggered closed State 1 conformation, and opens through an asymmetric State 2 in which initially only a single CD4 molecule engages the trimer, to finally completely open into the three CD4-bound State 3 conformation in response to binding of two or three CD4 molecules or coreceptors. One off-pathway event is the formation of asymmetric State 2A that is vulnerable to ADCC. *Adapted from Ma, X.C., Lu, M.L., Gorman, J., Terry, D.S., Hong, X.Y., Zhou, Z., Zhao, H., Altman, R.B., Arthoe, J., Blanchard, S.C., Kwong, P.D., Munro, J.B., Mothes, W., 2018. HIV-1 Env trimer opens through an asymmetric intermediate in which individual protomers adopt distinct conformations. eLife 7, e34271.*

Fig. 5. Existing high-resolution structures of HIV-1 Env trimer and derived immunogens resembles a State 2-like conformation. (A) High-resolution crystal and single-particle electron microscopy structures (PDB codes: 4ZMJ, 4NCO, 3J5M, 4TVF, 5I8H, 5CJX, and 8V8L) were aligned and show similar structural architectures. (B) Schemes for comparing soluble gp140 SOSIP.664 with wild-type HIV-1 BG505 Env. Trimer stabilizing modifications SOS, IP, and gp41 truncations are illustrated in sgp140 SOSIP.664. (C and D) Representative FRET trajectories and FRET histograms of wild-type Env on virus surface and soluble gp140 SOSIP.664 indicating predominant State 1 or State 2 occupancies, respectively. (E and F) Immunizing cows with soluble gp140 SOSIP.664 as immunogens elicit cow antibodies that exhibit a preference for State 2. Note that the conformational landscape of HIV-1 Env on virus in the presence of antibody Cow-9 is indistinguishable to the State 2-dominated conformational landscape as that of immunogen itself SOSIP.664. In contrast, most bNAbs developed by patients against the native virus exhibit a preference for State 1. Adapted from Lu, M., Ma, X., Castillo-Menendez, L.R., Gorman, J., Alsahafi, N., Ermel, U., Terry, D.S., Chambers, M., Peng, D., Zhang, B., Zhou, T., Reichard, N., Wang, K., Grover, J.R., Carmen, B.P., Gardner, M.R., Nikic-Spiegel, I., Sugawara, A., Arthos, J., Lemke, E.A., Smith, A.B., Farzan, M., Abrams, C., Munro, J.B., McDermott, A.B., Finzi, A., Kwong, P.D., Blanchard, S.C., Sodroski, J., Mothes, W., 2019. Associating HIV-1 envelope glycoprotein structures with states on virus observed by smFRET. *Nature* 568 (7752), 415–419, with permission from Nature.

Fig. 6. smFRET imaging of influenza virus HA₂ reveals the step-wise conformational changes in HA₂ during receptor-binding, low pH triggering, and membrane fusion. (A) Low pH triggers conformational changes in HA₂. Conformational landscapes show large shifts from prefusion (high-FRET) to post-fusion (irreversible low-FRET) structures of HA₂ in response to lowering the pH. (B) Proposed model of HA-mediated influenza virus membrane fusion based on smFRET analysis. Facilitated by binding to sialic acid receptor, HA₂ initially reversibly transits from high-FRET pre-fusion state, through intermediate-FRET (peptide-released intermediate I), to reversible low-FRET intermediate II (steps 1 and 2). Accelerated by the combined presence of receptor and low pH, HA₂ transitions from the reversible low-FRET intermediate II to the irreversible low-FRET post-fusion state. *Adapted from Benhaim, M., Lee, K.K., 2018. Single-molecule analysis of a viral fusion protein illuminates a fusion-active intermediate state. Cell 174, 775–777; Das, D. K., Govindan, R., Nikic-Spiegel, I., Krammer, F., Lemke, E.A., Munro, J.B., 2018. Direct visualization of the*

conformational dynamics of single influenza hemagglutinin trimers. Cell 174, 926–937, with permission from Elsevier.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Fig. 7.

Conformational variabilities in HIV-1 reverse transcriptase (RT) and RNA complex revealed by smFRET analysis. (A) Single-molecule FRET experimental design for monitoring the dynamics of RT. The overall structural density map of HIV-1 RT bound to a DNA/DNA duplex substrate. RT is labeled with Cy3, and primer/template duplex (DNA/DNA, RNA/DNA, DNA/RNA) are labeled with Cy5 dyes. Labeling sites for Cy3 on RT are located at either Fingers (F) or RNase H domains (H), highlighted by green stars. (B) RT exhibits orientational and sliding dynamics on primer/template nucleic acid substrates. smFRET reveals that RT is able to flip its orientation by engaging different substrates for adjusting its multifunctional catalysis, and RT can slide on nucleic acid substrates to facilitate polymerization site targeting during reverse transcription. (C) Single-particle cryo-EM high-resolution structure of HIV-1 RT initiation complex (RT/tRNA/vRNA). EM density map reveals that vRNA and tRNA are highly dynamic in conformations. *Adapted from Abbondanzieri, E.A., Bokinsky, G., Rausch, J.W., Zhang, J.X., Le Grice, S.F., Zhuang, X., 2008. Dynamic binding orientations direct activity of HIV reverse transcriptase. Nature 453, 184–189; Larsen, K.P., Mathiharan, Y.K., Kappel, K., Coey, A.T., Chen, D.H., Barrero, D., Madigan, L., Puglisi, J.D., Skiniotis, G., Puglisi, E.V., 2018. Architecture of an HIV-1 reverse transcriptase initiation complex. Nature 557, 118–122; Liu, S., Abbondanzieri, E.A.,*

Rausch, J.W., Le Grice, S.F., Zhuang, X., 2008. Slide into action: dynamic shuttling of HIV reverse transcriptase on nucleic acid substrates. Science 322, 1092–1097, with permission from AAAS and Nature.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript