Alpha-BET: Functional labeling of envelope glycoproteins with single domain antibodies for in-virus single molecule imaging

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Abstract

We present Alpha-BET, a structure-guided strategy leveraging AlphaFold to identify optimal ALFAtag insertion sites for minimally disruptive labeling of viral glycoproteins with high-affinity nanobodies. Applied to HIV-1 Env, SARS-CoV-2 S, and Nipah G, Alpha-BET preserves structural integrity and function. For HIV-1 Env, we demonstrate super-resolution DNA-PAINT MINFLUX 3D imaging enabled by tag insertion, showcasing its power for visualizing native trimers in single virions and potential for broader virological applications.

Main

Labeling viral envelope glycoproteins particularly Class I fusogens[1], is crucial for understanding enveloped virus entry and rational vaccine design[2]. These viral glycoproteins undergo complex structural rearrangements that dictate viral entry and immune evasion. For instance, studies on the Nipah virus F glycoprotein have revealed distinct pre-fusion and post-fusion states, highlighting key transitions necessary for viral entry[3]. Similarly, single-molecule Förster resonance energy transfer (smFRET) provided insights into the dynamic conformational changes of the SARS-CoV-2 spike (S) protein[4]. HIV-1 Env has also been extensively studied using smFRET[5] and super-resolution STED microscopy[6], demonstrating its intrinsic heterogeneity and the impact of antibody binding on its conformational landscape. Stop codon AMBER labeling has recently been used in smFRET studies of HIV-1 Env to achieve precise, site-specific tagging without disrupting the protein's function[7]. By introducing an AMBER stop codon at key sites in the gp120 subunit, non-canonical amino acids (ncAAs) carrying bio-orthogonal chemical groups are incorporated, allowing for selective attachment of donor and acceptor fluorophores via click chemistry[7]. Whilst perfect for smFRET studies, these labelling approaches are not suited for super-resolution fluorescence microscopy due to problems related to signal to noise, the effective number of viral particles labeled and notably photobleaching. Single-domain antibodies (sdAbs), also known as ''nanobodies' are attractive candidates for super-resolution imaging labelling due to their small size, ease of production, and their potential for single-site reporter conjugation, e.g. to functionalize the nanobody with a dye or enzyme, etc. A high affinity nanobody-tag pair allows the precision and specificity of genetic labelling with the flexibility of reporter readouts for the same target. Many tagging approaches introduce the epitope sequence to the N- or C-terminal of the target protein. However, these sites may not be accessible or may have important signaling functions. Internal labelling is more complex as it requires positioning the tag so as not to disturb the functionality of either the target protein or the tag. To address this, we present Alpha-BET (AlphaFold2[8] assisted Bio-Enhanced Tagging) (Figure 1a), a structure-guided strategy leveraging the predictive capabilities of AlphaFold2to identify optimal insertion sites for the ALFAtag[9], a small, rationally designed synthetic epitope tag optimized for advanced fluorescence microscopy. Alpha-BET was successfully applied and validated across diverse viral envelope glycoproteins, including HIV-1 Env, SARS-CoV 2 S protein and Nipah F. The workflow consists of five key steps (Figure 1a): A. Production of a list of tagging proposals, B. Generation of structural models with AlphaFold, C. Examination of structural details: malformed tags and changes to host, D. Alignment and/or docking checks for likely nanobody engagement and potential steric clashes and E. Produce, test and validate optimal candidates. In the case of HIV-1 JR-FL Env (a primary isolated Env); Alpha-BET generated five structural models for each of the 26 tagging proposals which were able to assess predict the overall gp120 structure and the specific conformation of the ALFAtag within the v4 loop (Figure 1b). The fitness of each labeling proposal was evaluated based on the ALFAtag's shape and orientation. The crystal structure of the ALFAtag bound to its nanobody (PDB ID: 6i2g) shows the tag as a straight alpha-helix, with three leucine residues exposed and facing the nanobody's binding pocket (Figure 1b and Supplementary Data Figures 1-5). Tagging proposals were therefore favored when their AlphaFold models displayed a straight, alpha-helix and solvent-accessible leucines.

In many cases, the ALFAtag maintained its alpha-helical conformation but had the binding-pocket leucine residues facing inward, toward the bulk gp120 structure, which would impede binding. Based on these assessments, for the HIV-1 Env, two tagging proposals with favorable tag insertions (IDs 415d2 and 5d416) and one with an unfavorable insertion (ID 637e9) were selected for further study and validation.

Functional validation involved assessing protein processing, expression, and viral infectivity. Western blot analysis of HEK293T lysates and HIV-1 viruses decorated with either JR-FL WT or JR-FL-v4-ALFAtag (415d2) confirmed that Env cleavage occurred similarly between tagged and WT constructs (Supplementary Data Figure 6). To evaluate infectivity, viral samples expressing JR-FL WT, 415d2, 5d416, and 637e9 constructs were diluted (10-, 100-, and 1000-fold), with and without 5 nM Nb-ATTO 594, and exposed to TZM-bl cells (Supplementary Data Fig. 6). The fraction of infected cells remained unchanged by ALFAtag insertion in the V4 loop or the addition of nanobodies, indicating that these modifications did not impair viral function. Cell-cell fusion assays also showed that the ALFAtag insertion did not impede fusogenic activity (Supplementary Data Figure. 6c). Additionally, fluorescence correlation spectroscopy (FCCS) (Supplementary Data Fig. 7) and confocal colocalization (Supplementary Data Fig. 9) confirmed the proper surface exposure of ALFAtag. FCCS analysis of HIV-1 viruses labeled with Gag-GFP and decorated with either JR-FL-v4-ALFAtag (415d2) or (637e9) revealed that only the 415d2 construct allowed successful docking of labeled NbAtto594, confirming proper ALFAtag surface display. The labeling and validation of NiV F and SARS-CoV S Wuhan also followed these steps (Supplementary Data Figs. 10-13) confirming the effectiveness of Alpha-BET to predict labelling efficiency and maintain functionality.

The successful implementation of Alpha-BET enables robust, function-preserving labeling of viral envelope glycoproteins for high-resolution microscopy. The ALFAtag is particularly well-suited for DNA-PAINT[10], a super-resolution method exploiting transient hybridization of DNA strands to achieve sub-5 nm localization precision—surpassing previous imaging approaches in viruses (Figure 2a)[6,11]. We demonstrate this by imaging Env trimers on HIV-1 viruses decorated with the validated JR-FL-v4-ALFAtag (415d2) construct. Env trimers were labeled with DNA-conjugated, ALFAtag-specific nanobodies, and DNA-PAINT imaging was performed using complementary XX- or Cy3B-labeled imager strands, for MINFLUX[12 13] or total internal reflection (TIRF) excitation, respectively. HIV-1 particles were distinguished from cellular debris through dual Gag-mTurquoise2 labeling. Three-dimensional DNA-PAINT MINFLUX images of individual mature virus-like particles were obtained with an isotropic single-molecule localization precision of 6 nm, as determined by the Cramér-Rao lower bound (CRLB) (Figure 2b). Zoomed-in images revealed tightly clustered Env trimers, consistent with previous STED observations but now visualized with 4-fold improved resolution[14]. Under TIRF, two-dimensional DNA-PAINT achieved CRLB and, nearest-neighbor-based metric (NeNA)[15] precision of 3.6 nm and 4.9 nm, respectively (Figure 2c). This NeNa value represents a resolution of 11.5 nm which sets a new benchmark in HIV imaging enabling for the first time the unambiguous visualization of individual Env trimers (Figure 2d). Each Env trimer comprises three gp120 subunits forming a triangular structure. Cryo-electron

tomography studies[16] reported trimer widths of ~ 13 nm at the widest point, narrowing to ~ 10 nm at the base -dimensions that matched our measurements, validating the approach.

In conclusion, Alpha-BET enables structurally informed, site-specific tagging compatible with advanced imaging workflows. The resulting enhancement in labeling precision and functionality paves the way for dissecting protein organization at the nanoscale—not only in viral systems but across a broad range of membrane-associated protein complexes.

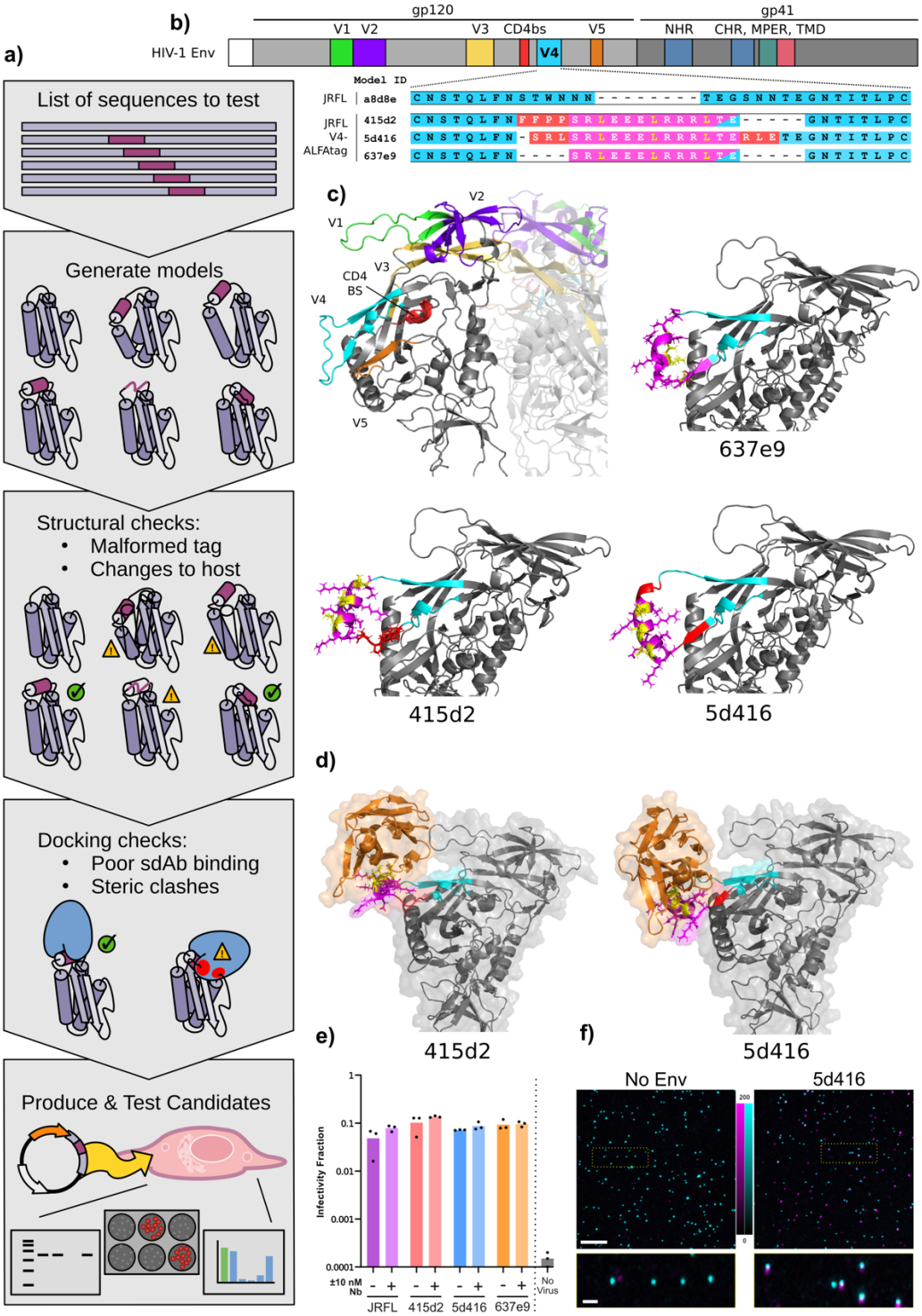

Figure 1 A general approach to screening tag inserts using AlphaFold, a) a schematic of the major steps involved, b) major features of HIV-1 Env, and the context of three ALFAtag labeling candidates 415d2, 5d416, and 637e9, c) structural model of JR-FL, with variable loops highlighted and AlphaFold2 models of the three candidate structures; highlighted regions: V4 loop (cyan), ALFAtag (magenta), binding-pocked leucine residues (yellow), and padding residues (red), d) ALFAtag nanobody (orange) aligned to the ALFAtag (magenta), e) functional test by infectivity of ALFatagged Env in the presence of nanobody, and f) functional test by imaging of ALFatagged Env (magenta) in viral particles (cyan).

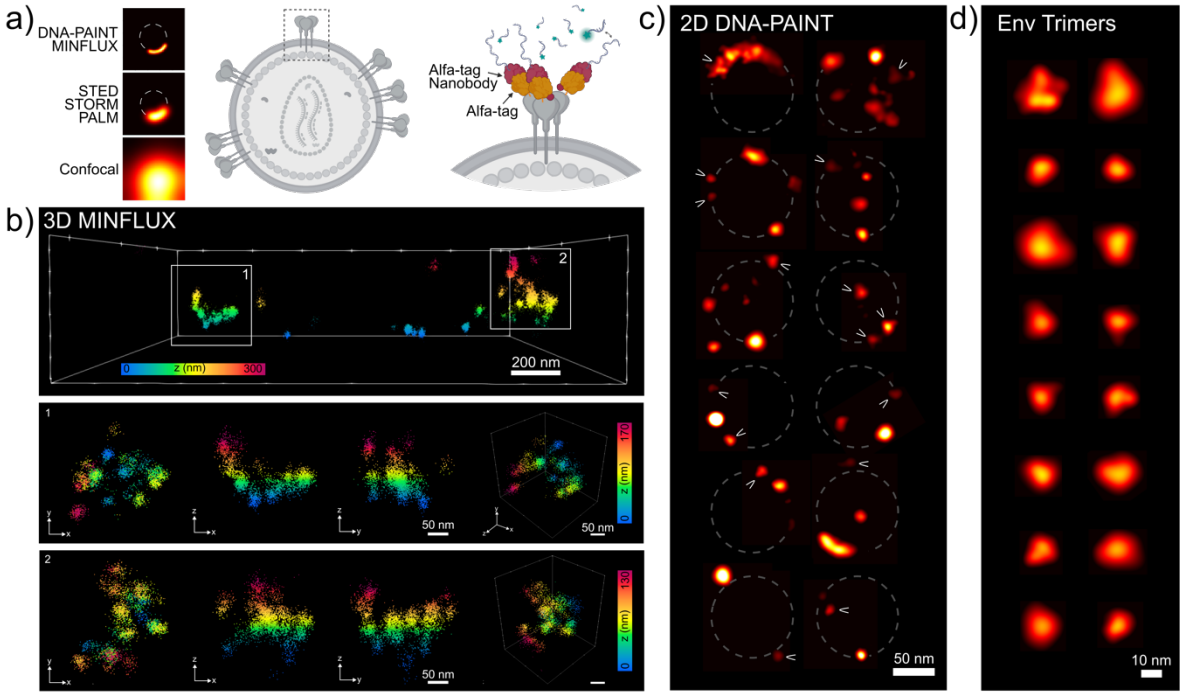

Figure 2. Super resolution imaging enabled by Alpha-BET: (a) Schematic of the resolution capabilities of various fluorescence microscopy techniques for visualizing Env clusters on a viral particle. (b) 3D DNA-PAINT MINFLUX imaging of individual mature virus-like particles, with two zoomed-in examples shown from different orientations. (c) Representative 2D DNA-PAINT images, obtained under TIRF illumination, of single mature virus-like particles; arrows indicate individual Env trimers. (d) Magnified view of the highlighted Env trimers shown in panel (c).

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