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Tubular Crystals and Helical Arrays: Structural Determination of HIV-1 Capsid Assemblies Using Iterative Helical Real-Space Reconstruction

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Abstract

Helical structures are important in many different life forms and are well-suited for structural studies by cryo-EM. A unique feature of helical objects is that a single projection image contains all the views needed to perform a three-dimensional (3D) crystallographic reconstruction. Here, we use HIV-1 capsid assemblies to illustrate the detailed approaches to obtain 3D density maps from helical objects. Mature HIV-1 particles contain a conical- or tubular-shaped capsid that encloses the viral RNA genome and performs essential functions in the virus life cycle. The capsid is composed of capsid protein (CA) oligomers which are helically arranged on the surface. The N-terminal domain (NTD) of CA is connected to its C-terminal domain (CTD) through a flexible hinge. Structural analysis of two- and three-dimensional crystals provided molecular models of the capsid protein (CA) and its oligomer forms. We determined the 3D density map of helically assembled HIV-1 CA hexamers at 16 Å resolution using an iterative helical real-space reconstruction method. Docking of atomic models of CA-NTD and CA-CTD dimer into the electron density map indicated that the CTD dimer interface is retained in the assembled CA. Furthermore, molecular docking revealed an additional, novel CTD trimer interface.

Keywords

Cryo-EM; Tubular crystals; Helical reconstruction; Real space; Docking; HIV-1 capsid

1. Introduction

Helical structures are often observed in cells and play important functions in different life forms. Examples include actin filaments and microtubules, as part of the cell cytoskeleton, flagella, functioning as a propeller in bacterial locomotion, and the contractile sheath in tailed phages, responsible for injecting genetic material into host cells. Helical structures, such as amyloid fibrils, can also be involved in diseases (1–4). In addition, many proteins, including the integral membrane proteins acetylcholine receptor (5) and Ca^{2+} -ATPase (6), and soluble protein HIV-1 viral capsid protein (7), can be induced to assemble into helical structures, which are favorable for structural determination. Helical structures can be thought of as a special case of two-dimensional (2D) crystals, in which the crystalline sheet has been deformed into a cylinder. Such structures have particular properties that make it relatively straightforward to obtain a three-dimensional (3D) view of the subunit. A single projection image of the object provides all the views of the subunit for 3D reconstruction, thus, eliminating the need for tilting and the attendant missing cone as in the case of 2D crystals. As such, several helical structures have been solved to near-atomic resolution by cryo-electron microscopy (cryo-EM) (5, 8, 9).

The mature HIV-1 capsid is composed of ~1,500 copies of the capsid protein (CA), generally forming conical or tubular assemblies. Based on early EM analysis of native cores and in vitro assemblies, a fullerene cone model of HIV-1 capsid was proposed (10) in which the capsid comprises a hexameric lattice containing a variable number of CA pentamers that allow for closure of tubular or conical structures. HIV-1 CA consists of two domains, a ~150 amino acid N-terminal domain (NTD) and an ~80 amino acid C-terminal domain (CTD), connected by a hinge. The full-length recombinant CA assembles into highly ordered helical structures in vitro. These in vitro assemblies are likely to contain subunit interactions present in the native HIV-1 capsid, since they exhibit features similar to those of native cores (11). A number of atomic structures of individual CA domains (12–18), as well as full-length hexameric CA have been determined by X-ray crystallography (19). In this chapter, we describe the methods used to obtain a cryo-EM structure of HIV-1 CA in the assembled state. With molecular docking, the reconstructed pseudo-atomic model of HIV-1 CA tube indicates that the inter-hexamer interactions in the surface lattice of the capsid are mediated by two distinct CTD–CTD interfaces, a CTD dimer interface that was previously described and a novel CTD trimer interface. We further validate the structural model and demonstrate the significance of the new CTD interface by mutational studies (20). The protocol described here can be easily adapted to other helical specimens for structural determination using cryo-EM. The programs used in this protocol are freely available.

2. Materials

2.1. Equipment

1. FPLC system: AKTA Explorer with Unicorn software, 5 ml Hi-Trap QP column and 5 ml Hi-Trap SP column, and Hi-Load Superdex 75 26/60 column (GE Healthcare, Piscataway, NJ).
2. Vertical electrophoresis system: XCell SureLock Mini-Cell (Invitrogen, Carlsbad, CA).
3. Glow-discharge device model 100X (EMS, Hat field, PA).
4. FEI transmission electron microscopes (FEI Corp., OR): Tecnai T12 with LaB₆ filament, a single-tilt room-temperature specimen holder and a Gatan 2K × 2K CCD camera; Tecnai Polara F30 microscope with a Field Emission Gun, equipped with a Gatan 4 K × 4 K CCD camera.
5. Plunge-freezing devices: Vitrobot Mark III Automated Plunger (FEI Corp., OR) and home-made MRC-type gravity plunger.
6. Nikon super coolscan 9000 ED scanner: resolution of 4,000 dpi (Nikon, Japan).
7. Computers running Linux operating system and image processing and visualization software packages: EMAN (21), IHRSR (22), Spider (23), MRC based helical processing software (24), Real-space helical refinement software (9), Situs (25), Chimera (26).

2.2. Reagents

1. The cDNA encoding gag polyprotein, pr55^{gag} can be obtained from the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH (27).
2. pET21 and Rosetta 2 (DE3) (EMD chemicals, Inc. San Diego, CA).
3. Electron microscope carbon-coated copper grids (SPI Supplies, West Chester, PA), Quantifoil R2/1 200 mesh holey, carbon copper grids (Quantifoil Micro Tools, Jena, Germany).

4. KODAK Electron Image Film SO-163, KODAK D-19 developer, fixer, and PHOTO-FLO solution (Eastman Kodak Co., Rochester, NY).
5. Filter paper type #1: 90 mm diameter (Whatman, Clifton, NJ).
6. DUMONT biology anti-capillary reverse tweezers (Ted Pella, Redding, CA).
7. All other reagents except those listed below are from Sigma (St. Louis, MO) and are of analytical grade or higher.
8. Pre-cast 4–12% SDS PAGE gel, 4x SDS sample buffer and 20x running buffer (Invitrogen, Carlsbad, CA), Amicon concentrators (Millipore, Billerica, MA). Luria-Bertani media (Bacto, NJ),

2.3. Buffer Systems

1. Lysis buffer: 25 mM sodium phosphate, pH 7.0, 0 mM NaCl.
2. Hi-Trap QP column buffer: 25 mM sodium phosphate, pH 7.0, 1 mM DTT, and 0.02% sodium azide, 0 mM NaCl (Low Salt)—1,500 mM NaCl (High Salt).
3. Hi-Trap SP column buffer: 25 mM sodium phosphate, pH 5.8, 1 mM DTT, and 0.02% sodium azide, 0 mM NaCl (Low Salt)—1,000 mM NaCl (High Salt).
4. Hi-Load Superdex 75 26/60 column buffer: 25 mM sodium phosphate, pH 5.8, 100 mM NaCl, 1 mM DTT, and 0.02% sodium azide.
5. CA assembly buffer: 50 mM Tris-HCl, pH 8.0, 1 M NaCl.
6. Dilution buffer: 50 mM Tris-HCl, pH 8.0, 100 mM NaCl.

3. Methods

3.1. Expression and Purification of HIV-1 CA and CA Mutants

1. Amplify and subclone wild-type full-length CA (Pro1-Leu231) into pET21 using *Nde* I and *Xho* I sites.
2. Express full-length CA in *E. coli*, Rosetta 2 (DE3), cultured in Luria-Bertani media and induce with 0.4 mM IPTG. Culture at 23°C for 16 h.
3. Harvest cells 16 h post-induction and lyse by sonication in the lysis buffer.
4. Clear the cell lysate by ultracentrifugation at $100,000 \times g$ for 1 h at 4°C and apply it to a Hi-Trap QP column equilibrated with the lysis buffer.
5. Collect flow through fractions.
6. Adjust the pH of the sample to 5.8 with diluted acetic acid. Add Milli-Q water to lower conductivity to below 2.5 ms/cm. Centrifuge the sample at $25,000 \times g$ with a SLA-1500 rotor for 1 h at 4°C.
7. Apply the resulting supernatant to a Hi-Trap SP column and collect peak fractions. CA should elute at ~10 ms/cm.
8. Concentrate the sample to 15–18 ml and apply it to a Hi-Load Superdex 75 26/60 gel-filtration column.
9. Collect peak fractions and concentrate with Amicon concentrators to 10 mg/ml, based on the extinction coefficient at UV 280.
10. Analyze protein samples by SDS-PAGE and stain with Coomassie Blue to assess protein purity (Fig. 1, lanes marked t).

11. For storage, add 5% glycerol to the sample. Store at -80°C in 25 μl aliquots that have been flash-frozen with liquid N_2 .

3.2. HIV-1 CA Assembly

1. Add 2x assembly buffer (2 M NaCl and 100 mM Tris-HCl pH 8.0) to freshly thawed stock protein to a final CA concentration of 2 mg/ml (see Note 1).
2. Incubate the mixture at 37°C for 1 h. At the end of incubation, transfer the sample to ice for further analysis.
3. For SDS-PAGE analysis of CA assemblies, centrifuge the samples with an Eppendorf centrifuge 5417R (4°C) at $20,000 \times g$ for 15 min. Load the resulting supernatant and pellet samples onto 4–12% gradient SDS-PAGE gel and stain with Coomassie Blue (Fig. 1).

3.3. EM Specimen Preparation

3.3.1. Negatively Stained EM Specimen Preparation—CA assembly conditions have been optimized by screening an array of parameters, such as CA protein concentration, pH value, salt concentration, assembly temperature, etc. The screening is carried out by checking the integrity of negatively stained tubes, assembled under various conditions, on an FEI T12 microscope. The optimized assembly conditions should produce well-ordered, long, straight, separated tubes in reasonable quantities.

1. Glow discharge 200 mesh solid carbon grids under 25 mA for 25 s using a glow discharge device with the HT polarity set to be negative.
2. Lay two droplets ($\sim 40 \mu\text{l}$) of 1% uranyl acetate on a piece of para film for each specimen.
3. Apply a 3–5 μl sample to the freshly glow-discharged grid, which is held by DUMONT anti-capillary reverse tweezers, and allow absorption of the sample for 30 s.
4. Blot the solution with a piece of filter paper and quickly place the carbon side of the grid on top of the first uranyl acetate droplet surface and gently move the grid back and forth for 5 s.
5. Blot away the uranyl acetate, quickly transfer the grid to the second uranyl acetate droplet, and leave it for 30 s. Then, completely blot off the uranyl acetate solution and let the grid air-dry.
6. Examine the grids with a Tecnai 12 electron microscope (Fig. 2).

3.3.2. Frozen-Hydrated EM Specimen Preparation—Embedding biological macromolecules in vitreous ice is the best way to preserve their structure in the hydrated state. During rapid freezing, the molecules can be locked in the state they were just prior to freezing. Thus, high-resolution structural information can be well preserved using this technique (28). These advantages make cryo-EM a powerful method to study protein structure and to study short-lived transitional structures that are hard to catch (29).

Because the HIV-1 CA assemblies are stable only in high salt (1 M NaCl) buffer, which contributes strong background noise in cryo-EM images, we use a rapid dilution and back-

¹For the CA assembly assay, the sequence of mixing reaction components has an impact on the final number of assembled tubes. The most efficient assembly is achieved by adding 2x assembly buffer to the CA protein stock solution followed by the addition of Milli-Q water.

side blotting method to transiently reduce salt concentrations when preparing the frozen-hydrated grid.

1. Glow discharge 200 mesh R2/1 Quantifoil grids under 25 mA for 25 s.
2. Turn on the humidifier for the home-made gravity plunger until the humidity reaches approximately 90% in the humidity chamber.
3. Prepare cold liquid ethane cooled by liquid nitrogen in the FEI plunge freezing dewar (see Note 2).
4. Apply 2.5 μl of CA assembly solution on the carbon side of the grid, which is mounted on forceps, and load the forceps onto the plunger with the carbon side (front side) of the grid facing away from you. Add 3 μl of dilution buffer to the back side of the grid.
5. Immediately blot the grid with a piece of filter paper, placed against the back side of the grid. The grid back surface should be in good contact with the filter paper. After ~6 s, remove the filter paper and release the plunger at the same time (see Note 3).
6. Remove the forceps from the plunger and quickly transfer the grid into a grid storage box.

3.4. Cryo-EM of CA Tubular Assemblies

For flexible HIV-1 CA tubular assemblies, the most common problem encountered during data collection is deformation caused by surface tension, which can flatten or bend the tubes. Such deformation can make the sample unsuitable for 3D reconstruction. Thus, finding an ice area suitable for imaging is critical, not only to maximize the signal to noise ratio under low-dose conditions but also to best preserve the helical symmetry of HIV-1 tubular assemblies.

1. Under low-dose search mode with a dose $<0.01 \text{ e}/\text{\AA}^2$, screen the whole grid at a magnification of ~200 and note the areas with suitable ice in a position file.
2. Further screen these areas at a magnification of 3,900 and select the best sample area for data collection. The best sample area should have a reasonable number of tubes, not too sparse or dense. The ice should be thin. Tubes should be straight and not squashed. A few high-resolution CCD images can be collected to confirm the tube quality. Save all the best areas in a different position file.
3. Switch to exposure mode, insert a 100 μm objective aperture, and adjust objective stigmatism.
4. Identify a good tube at 3,900 magnification and position the tube at the center of the camera's field of view. Switch to the focus mode and adjust the Z-height until

²During the process of preparing a cryo-EM grid, ice contamination can be visible from time to time. The most common source of contamination is contaminated liquid ethane. Ethane should be completely cooled to near solidifying temperature and kept minimally exposed to air to avoid frost. Also, the remaining ethane gas at the bottom of the tank is not suitable for sample preparation.

³Before the sample is vitrified, the sample solution should be blotted away and left with a uniform, thin solution layer that is slightly thicker than the sample size. There are several different ways to blot the sample, depending on the freezing apparatus. FEI mark IV vitrobot and Gatan cryo-plunger both use two-side blotting, whereas the Leica EM GP and most home-made plunge freezing devices deploy single-side blotting. Two-side blotting produces an ice layer with a gradient of thickness, resulting in a band of suitable ice. However, the sample itself sometimes dictates the blotting method. For bacterial cells and tubular crystals such as HIV-1 CA assembly, one-side blotting, particularly from the back-side, is more appropriate, since the contact with the filter paper can result in sample loss or damage. One-side blotting is also useful for rapid buffer exchange before freezing, as many complexes are stable under buffer conditions that are not suitable for cryo-EM. For these samples, a drop (3–5 μl) of dilution buffer can be added to the back side of the grid, and, then, a filter paper can be used to quickly blot away excess solution from the back side. Due to the quick exchange between sample solution and dilution buffer, structural effects on the sample can be kept to a minimum.

the image is completely in focus, meaning no contrast. Switch to the exposure mode, at a nominal magnification of 59,000, set a defocus, normally between 0.5 and 2.5 μm , and an exposure time of 0.3–0.5 s with a dose of 10–15 $\text{e}/\text{\AA}^2$, and collect an image (Fig. 3). When images are collected on a plate camera, the film should be allowed to settle 10 s before an exposure is taken.

5. Move to the next tube and repeat the steps to collect an image.

3.5. Image Processing and 3D Reconstruction

3.5.1. Digitization

1. We digitize the films using a Nikon super coolscan 9000 ED scanner connected to a Mac computer and interfaced with Adobe Photoshop.
2. Place each film onto a clean film holder with emulsion side down, such that it is flush with the stops, and insert the film holder slowly into the holder slot with the arrow facing up and toward the scanner.
3. Launch Nikon scanning software and select settings: Neg (Mono) in the film-type menu, 6×9 in the format size menu, and Grayscale in the color space menu. In the scanner extras palette, choose 16 bit for data size, 215 for manual focusing, and super fine for the CCD scan mode (6.35 $\mu\text{m}/\text{pixel}$).
4. After adjusting the contrast and density range and choosing the area to be scanned in the preview window, click the scan button to start the high-resolution scan.
5. Save the scanned image in a “TIFF Uncompressed” format.

3.5.2. Helical Indexing

Helical Parameters: A helical array may be defined with respect to three parameters, the radius (r), the pitch (P), and the axial distance between neighboring units (p). As illustrated in Fig. 4a, the radius of a helix is simply the distance from the helix axis to all points on the helix. The pitch is the distance of one complete helix turn in the helix axial direction.

Diffraction patterns of a helical array (Fig. 4b) are composed of a set of layer lines, which follow the “helical selection rule”, $l = tn + um$. Here l and n are layer line indices. m and u are integers, and t and u are constants of the helix. For any given helix, there may be exactly u units in exactly (or very closely) t complete turns. The helical repeat distance (c) is defined as $c = Pt = pu$.

Indexing Helical Parameters in a Fourier Transform: For helical indexing, we have borrowed the notation from two-dimensional (2D) lattices, since the surface of a helical object is basically a 2D lattice. Thus, the layer line indices l and n can be associated with the 2D lattice vectors, noted as $n_{(1,0)}$ and $l_{(1,0)}$ and $n_{(0,1)}$ and $l_{(0,1)}$ (Fig. 4c, d). Since the intensity of the n th layer line is determined by the Bessel function, $J_n(2\pi Rr)$, the first maximum, R_{max} , measured from the meridian can be used to calculate n , as approximated by the following Eq. 1 (30), in which,

$$2\pi R_{\text{max}} r \approx J_n \approx 1.1|n| + 0.9 \quad (1)$$

1. Using the program “heliboxer” in EMAN, frame a straight and long tube with uniform diameter and save the boxed area in MRC format.
2. Determine the helical repeat distance, c , using a cross-correlation based program, “imgccf”, in the MRC package.

3. Calculate the Fourier transform with a new box length that is an integral of the repeat distance c (see Note 4).
4. Measure the radius of the tube, r , and the height and radius of the two principal layer lines in Fourier transform, $l_{(1,0)}$, $R_{\max(1,0)}$, $l_{(0,1)}$, $R_{\max(0,1)}$, respectively.
5. Calculate $n_{(1,0)}$ and $n_{(0,1)}$ according to Eq. 1 (see Note 5). The handedness of the helix needs to be determined separately (see Note 6). A right-handed helix assumes a positive n and a left-handed helix has a negative n . For instance, the estimated helical symmetry for the tube in Fig. 4c is $n_{(1,0)} = -12$ and $n_{(0,1)} = 10$.
6. Test some pairs of n numbers to find the correct helical symmetry using Eq. 1 and the programs for 3D reconstruction, IHRSR (Subheading “Segmentation”). For example, we tested $n_{(1,0)} = -12$ and $n_{(0,1)} = 10$, $n_{(1,0)} = -11$ and $n_{(0,1)} = 10$ and $n_{(1,0)} = -13$ and $n_{(0,1)} = 11$ and found that only $n_{(1,0)} = -13$ and $n_{(0,1)} = 11$ gave us a stable converged reconstruction. Thus, the correct helical symmetry for the tube in Fig. 4c is $n_{(1,0)} = -13$ and $n_{(0,1)} = 11$.

3.5.3. Determining the Screw Symmetry—The screw symmetry of a helical object is described by two real numbers: the rotation between subunits ($\Delta\phi$) and the axial rise (Δz) as well as a possible point group symmetry, which would be an n -fold rotation about helix axis (22). If the helical object can be completely described by a rotation and axial rise of an asymmetric unit along a 1-start helix ($n = 1$), the point group symmetry does not need to be set. Since the helical arrays obey the selection rule, Δz and $\Delta\phi$ can be calculated according to the selection rule.

In practice, we use a simple script to calculate the screw symmetry by using the $l_{(1,0)}$, $l_{(0,1)}$, $n_{(1,0)}$, and $n_{(0,1)}$ values and by testing a range of m values. For instance, given $l_{(1,0)} = 27$, $l_{(0,1)} = 45$, $n_{(1,0)} = -13$, $n_{(0,1)} = 11$, $-50 < m < 50$, and a repeat distance of 5,721.15 Å, we have $\Delta z = 6.4854$ Å, $\Delta\phi = 165.306^\circ$.

3.5.4. Reconstruction Using IHRSR Programs—The iterative real-space helical reconstruction (IHRSR) method (22) treats helical objects as a string of single particles to resolve their three-dimensional (3D) structure using cryo-EM. It is especially useful for partially disordered helical objects when Fourier-Bessel helical reconstruction falls short. The IHRSR algorithm involves determining the helical screw symmetry, the rotation between subunits ($\Delta\phi$) and axial rise (Δz), that best fits the reconstructed volume at each cycle, as well as a point group symmetry. The procedure is not sensitive to the choice of an initial reference volume, and even a solid cylinder can be used quite effectively as an initial reference as long as a reasonable starting symmetry is used.

Segmentation: One important parameter for segmentation is the size of the segments, which can only be determined empirically. Segments should be extensively overlapped (~90%) when cutting these from the helical images. Thus, 10% of the box size along a helical axis is typically used as a shift for each box.

⁴When box length is an integral of the repeat distance (c), the layer line signals fall on the exact pixels instead of between pixels, thus producing sharp layer lines.

⁵As with any two-dimensional lattice, all the lattice vectors are linear combinations of two basic vectors $a_{(1,0)}$ and $b_{(0,1)}$ with $c_{(h,k)} = h^* a_{(1,0)} + k^* b_{(0,1)}$. We may express $n_{(h,k)}$ of any other layer lines in terms of $n_{(1,0)}$ and $n_{(0,1)}$: $n_{(h,k)} = h^* n_{(1,0)} + k^* n_{(0,1)}$. $R_{\max}$, combined with an estimate of the radius r of a helix, gives the correct n according to Eq. 1. In practice, however, $R_{\max}$ value may be smaller due to out-plane tilt (31) or the effects of thickness (32), leading to an underestimation of r . Typically, there will be a range of r values estimated from each set of helices, so the best estimate of n will be from a range of possible values.

⁶The handedness of a helix can be determined prior to image reconstruction using freeze-etching, followed by rotary shadowing method (33). It can also be verified post-reconstruction, as 3D atomic models of individual components should fit well into the density map when a correct handedness is assumed. Otherwise, the opposite handedness should be chosen.

1. Use the boxer program in the EMAN package to open a graphical user interface and to select helical objects from micrographs. In the control panel of boxer, choose “Helix” and set the parameters for boxing: the size of the box should be larger than the diameter of tube and a value for “Olap” should be ~90% of box size.
2. After selecting two boxes on either end of the helical object, click on the image to automatically generate a series of particle boxes along the tube length.
3. Save boxed particles as well as their coordinates.

Generating the First Reconstruction

1. Invert the contrast of the cryo-EM images and apply low pass filtering (optional) prior to processing with the iterative helical real space reconstruction method (IHRSR).
2. Use the program “Generator” to open the graphical interface, and provide it with the name and path of the raw images that will be used by IHRSR to generate the reconstruction script, b25.spi. Use a solid cylinder as an initial reference and allow the procedure to cycle until there are no changes in the helical symmetry. The helical symmetry usually converges after a few cycles (Fig. 5a).
3. When $\Delta\phi$ and Δz values are stabilized after several iterations, an initial 3D reconstruction is generated in the last cycle (Fig. 5b).

3.5.5. CTF Correction—The images generated by cryo-electron microscopes are not true projections of the specimen. They suffer from a set of artifacts, including the contrast transfer function (CTF) and the envelope function of the microscope. In addition, noise is present from a variety of sources. The CTF in particular can cause serious artifacts in a 3D reconstruction. The severity and type of these effects, of course, depends on many factors, the most significant of which is the defocus of the data used in the reconstruction. Therefore, defocus determination and CTF correction is crucial for reconstructing a reliable 3D density map.

1. Use the segments from one micrograph to calculate the power spectrum and determine an initial defocus value using *ctfit* in the EMAN package (Fig. 6).
2. Further refine the defocus values using programs *CTFFIND3* and *CTFTILT* and determine an average defocus value.
3. The CTF correction involves the use of a Wiener filter (see Note 7). This operation corrects both phases and amplitudes. With a Wiener filter, the Fourier transform of an image, F_i , is first multiplied by the CTF, which serves as phase reversals, then divided by the sum of the squares of the CTF plus a noise-to-signal term, shown in Eq. 2. The f term effectively serves as a trap to avoid boosting noises.

$$S_{WF} = \frac{F_1 * CTF_1 + F_2 * CTF_2 + \dots + F_i * CTF_i}{\sum_{i=1}^n CTF_i^2 + f} \quad (2)$$

⁷Wiener filtering is used in image processing for both phase and amplitude correction in such a way as to reduce the level of noise amplification. It is more powerful when multiple projection sets are available, each with a different value of the contrast transfer function. In this case, inverse filtering can be used to recover some of the frequencies for which at least one of the CTF values is not zero.

3.5.6. Reconstruction with Iterative refinement

1. Refinement cycles need to be carried out to determine the relative orientation of each segment, namely Euler angles. This can be done using a multi-reference alignment procedure, as illustrated in the flow chart (Fig. 7). We use the image processing package SPIDER (23) for many of the steps of refinement cycles. A series of operations can be controlled with SPIDER scripts (9), which are user-created batch control files containing sequences of operations and parameter values.
2. The 3D map generated by the IHRSR programs (Subheading “Generating the First Reconstruction”) can be used as an initial reference. During refinement, the helical symmetry is fixed with the values of $\Delta\phi$ and Δz , determined from IHRSR (Subheading “Generating the First Reconstruction”).
3. Prior to 3D reconstruction, CTF phase and amplitude correction are applied to the segments with Eq. 2. Projection matching is then performed by comparing projections of the reference volume with the CTF-corrected images using multi-reference alignment. Projections of the reference are generated in 1° increments around the helical axis. The variation of the out-of-plane tilt angle is limited and sampled in 1° steps. Constraints are imposed to the alignment parameters of each segment. Only those segments that satisfy constraints such as high correlation coefficients, near 0° or 180° in-plane angles, and limited x -shifts, are kept.
4. After each iterative refinement cycle, a 3D density map is generated by back projection followed by imposing the helical symmetry. The iterative refinement is stopped when the resolution of the new 3D map doesn't have any improvement (see Subheading 3.5.7 for resolution estimation). The final 3D map is shown in Fig. 8a–d.

3.5.7. Resolution Estimation—The resolution measures used in single particle reconstruction are based on comparisons of averages calculated for subsets of the data. These measures are the differential phase residual and the Fourier ring correlation (FRC). “RF 3” in SPIDER can be used to calculate the resolution of our 3D density map. The resolution of the map is 16 Å at FSC = 0.5, or at a differential phase residual of 65° (Fig. 8e).

3.6. Molecular Docking

We reconstructed pseudoatomic models of CA hexamers by docking the following models (PDB codes 1gwp for CA-NTD and 2kod for CA-CTD) into the tubular EM density map using an automated map-fitting feature implemented in Chimera (Fig. 9). A new CTD–CTD trimer interface was identified in the reconstructed pseudoatomic model (Fig. 10).

1. Open pdb models and a 3D density map with Chimera (26).
2. Align the model and the map manually. Move and rotate the model so it is similarly oriented and superimposed on the map.
3. In the Chimera Window, click Tools > Volume Data > Color Zone, select surface to be colored and coloring radius. Then click “Color”.
4. Optimize the fit of the model in the map using the tool, “Fit in Map”.
5. The docking was further refined using the program “colacor” in Situs package (25). Two parameters can be adjusted to give the highest correlation. These parameters are density cutoff and Laplacian filter (on or off).

3.7. Conclusion

We used cryo-EM and iterative helical real-space reconstruction methods described above and obtained a 3D density map of helically assembled HIV-1 CA hexamers at 16 Å resolution. Docking of the atomic models of CA domains into the 3D density map allowed us to construct a pseudo-atomic model of assembled HIV-1 CA. Our cryo-EM structure model of assembled CA revealed a novel CTD–CTD interface at the pseudo-threefold axis that has not been characterized previously. This new CTD trimer interface plays a critical role in capsid assembly, disassembly, and stability, as evidenced by the available mutational data and by additional novel mutations that we have generated based on our structural data. We further validated the trimer interfaces in CA tubular assemblies and in HIV-1 virions by engineering intermolecular cross-linking of cysteine residues, P207C and T216C, at the trimer interface. The methods demonstrated here are applicable not only to HIV-1 capsid assemblies but also to many other helical objects for structure determination at medium resolution.

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Fig. 1.

Coomassie Blue-stained SDS-PAGE of purified HIV-1 CA and CA mutants before assembly (t) and following assembly and centrifugation (s and p). Assembled CA tubes are present in the pellet (p) after high-speed centrifugation.

Fig. 2. EM of negatively stained CA wt and CA mutant assemblies. **(a)** CA wt assemblies show bundled tubes. **(b)** CA E45A assembles more efficiently, but with multiple layers. **(c)** CA A92E assembles single, long, and well-ordered tubes. Scale bars, 100 nm.

Fig. 3. CryoEM micrographs of recombinant HIV-1 CA tubular assemblies. **(a)** CA wt tubes. **(b)** CA A92E tubes. Scale bars, 100 nm. (Reproduced from ref. 20 with permission from Elsevier Science).

Fig. 4. Indexing of HIV-1 CA helical tubes. **(a)** The geometry of a discontinuous helical structure. A motif (*circle*) is repeated with a vertical spacing p along a helix of pitch P . **(b)** The diffraction pattern of the helical structure shown in **(a)**. Sets of cross-like intensities repeated with a vertical spacing of $1/p$. There are two principal spacings: $1/P$ within any single X pattern and $1/p$ between the centers of separate X patterns. All layer lines are separated by $1/c$. **(c)** An HIV-1 CA A92E tube image. Scale bars, 40nm. **(d)** The Fourier transform of **(c)** with helical indices. The *arrow* at the top points to the layer lane at 20 Å resolution.

Fig. 5. Initial reconstruction using IHRSR. **(a)** Helical symmetry determination for each iterative cycle. $\Delta\phi$ and Δz , starting from the initial values, converge to the stable values after ten iterative refinement cycles. **(b)** The initial map using IHRSR.

Fig. 6. CTF determination using ctfit program. The parameters of the CTF in each micrograph were determined by fitting of the intensity of CTF to the power spectrum calculated from segmented tubes. Shown are a power spectrum (*thick line*) and fitted CTF (*thin line*) of tubes recorded at a defocus value of 2.5 μm .

Fig. 7.

Flow chart for the refinement of the 3D map. The CTF-corrected segments are aligned against the reference projections and back-projected to generate a 3D volume. The helical symmetry was then imposed onto the 3D volume, which is then used as the new reference for the next refinement cycle. The cycle (*dashed box*) is iterated until a stable reconstruction is obtained.

Fig. 8.

The 3D density map of the A92E CA tubes from the $(-13, 11)$ helical family. **(a – c)** The density map of CA tubes is displayed as three orthogonal slices: parallel to the tube axis and close to the surface **(a)**, perpendicular to the tube axis **(b)**, and parallel to and through the tube axis **(c)**. Scale bars, 10 nm. **(d)** Surface rendering of the 3D density map contoured at 2.3σ (*solid*) and 1.3σ (*transparent*) enclosing 65% and 100% volume, respectively. *Dashed lines* connect hexamers in the three distinct helical arrangements denoted as $n = -2, 11$, and -13 helices; n is the Bessel order and the sign indicates the handedness of the helix ($-$ sign as left hand, and $+$ sign as right hand). **(e)** Fourier shell correlation and phase residual plots of the 3D density map reconstructed from 197 tubular segments. The resolution of the map is 16 Å at FSC = 0.5, or at phase residue of 65°. (Reproduced from ref. 20 with permission from Elsevier Science).

Fig. 9.

Molecular Docking of NTD and CTD domain models independently into the tubular density map (contour enclosing 90% volume). **(a)** An overlay of docked pseudo-atomic model and 3D density map viewed from the tube surface. **(b, c)** Slab views to show the model fitting at NTD region **(b)** and the CTD region **(c)**. Three docked CA-hexamers are displayed. The local threefold axis is marked by *asterisk*. (Reproduced from ref. 20 with permission from Elsevier Science).

Fig. 10.

Novel CTD-CTD interactions at the local threefold axis in CA tubular assemblies. **(a)** A pseudo-atomic model of three CA-hexamers in the assembled tube. *Boxed region* encloses the new CTD-CTD interface at the local threefold axis (indicated by *asterisk*). **(b)** A detailed view of the boxed region illustrating the interactions at the interface. Side chains of K203, P207, E213, T216, and Q219 are shown in ball and stick representation. Mutations of these residues are known to affect in vitro assembly and capsid stability. A pair of spatially close residues, P207/T216, were tested by cysteine cross-linking (20). (Reproduced from ref. 20 with permission from Elsevier Science).