Quantitative Imaging of Single, Unstained Viruses with Coherent X Rays

Changyong Song,1 Huaidong Jiang,1 Adrian Mancuso,1 Bagrat Amirbekian,1 Li Peng,2 Ren Sun,2 Sanket S. Shah,3 Z. Hong Zhou,3 Tetsuya Ishikawa,5 and Jianwei Miao1,*

1Department of Physics and Astronomy, University of California, Los Angeles, California 90095, USA
2Department of Molecular and Medical Pharmacology, University of California, Los Angeles, California 90095, USA
3Department of Pathology and Laboratory Medicine, University of Texas-Houston Medical School, Texas 77030, USA
4Department of Microbiology, Immunology & Molecular Genetics, University of California, Los Angeles, California 90095, USA
5RIKEN SPring-8 Center, I-1-1, Kouto, Sayo, Hyogo 679-5148, Japan

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We report the recording and reconstruction of x-ray diffraction patterns from single, unstained viruses, for the first time. By separating the diffraction pattern of the virus particles from that of their surroundings, we performed quantitative and high-contrast imaging of a single virion. The structure of the viral capsid inside a virion was visualized. This work opens the door for quantitative x-ray imaging of a broad range of specimens from protein machineries and viruses to cellular organelles. Moreover, our experiment is directly transferable to the use of x-ray free electron lasers, and represents an experimental milestone towards the x-ray imaging of large protein complexes.

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Since Perutz, Kendrew, and colleagues unveiled the structure of hemoglobin and myoglobin based on x-ray diffraction analysis in the 1950s [1,2], x-ray crystallography has become the primary methodology used to determine the 3D structure of macromolecules. However, biological specimens such as cells, organelles, some viruses, and many important macromolecules are difficult or impossible to crystallize, and hence their structures are not accessible by crystallography. Overcoming this major limitation requires the employment of different techniques. One promising approach currently under rapid development is x-ray diffraction microscopy (or coherent diffractive imaging) where the x-ray diffraction patterns of noncrystalline specimens are measured and then directly phased by the oversampling iterative algorithm [3]. However, due to the absence of the very large signal amplification that occurs in crystals, x-ray diffraction microscopy has so far been limited to the imaging of micron-sized or high-Z specimens [4–19].

Here we, for the first time, recorded and reconstructed x-ray diffraction patterns from single, unstained viruses that have molecular mass about 3 orders of magnitude smaller than those specimens investigated by x-ray diffraction microscopy before. By separating the diffraction pattern of the virus particles from that of their surroundings, we performed quantitative and high-contrast imaging of a single virion with a resolution of 22 nm. The structure of the viral capsid inside the virion was identified. With more brilliant synchrotron radiation sources [20] and future x-ray free electron lasers (X-FELs) [21], much higher resolutions should be achievable. Because of its quantitative capability, high image contrast, and high spatial resolution, we anticipate that x-ray diffraction microscopy will become an important imaging technique for unveiling the structure of a broad range of biological systems including single protein machineries, viruses, organelles, and whole cells.

The samples we studied are single murine herpesvirus-68 (MHV-68) virions. A herpesvirus virion has an asymmetric tegument and envelope outside of the icosahedrally symmetric capsid composed of defined numbers of subunits [22]. However, each virion may have a different size of tegument and envelope, and the viral capsid is not necessary in the center of the virion [23]. While cryoelectron microscopy can determine the capsid structure of herpesviruses by averaging over thousands of virus particles [24], the reconstructions of pleomorphic virions obtained by cryoelectron tomography are limited in low image contrast and high levels of noise [23]. The goal of this study is to perform quantitative and high-contrast imaging of single, unstained virions by using coherent x rays. The MHV-68 virions were inactivated by UV light (500 mJ) and chemically fixed by 3% glutaraldehyde. Unstained virions were suspended in methanol to regulate the concentration to about 20 virions/µL and supported on 30-nm thick silicon-nitride-membranes. Single, isolated virions were located with a high-resolution optical microscope and studied by the x-ray diffraction microscope.

The experiment was carried out on an undulator beam line at SPring-8. Figure 1 shows the schematic layout of the biological x-ray diffraction microscope. Unfocused, monochromatic x rays with an energy of 5 keV were filtered by a 20 μm-diameter-pinhole, placed approximately 1 m upstream of the sample. A Si guard slit with beveled edges was positioned just in front of the sample to block the parasitic scattering from the upstream optical components. Coherent x-ray diffraction patterns were recorded by a liquid-nitrogen-cooled CCD camera with 1340 × 1300
pixels and a pixel size of 20 μm, located a distance of 1 m downstream of the sample. To obtain the diffraction pattern only from single virions, we measured two sets of diffraction intensities with the specimens in and out of the x-ray illumination, and then subtracted the two diffraction patterns. This procedure removed the unwanted scattering from specimens’ surroundings and allowed us to perform quantitative and high-contrast imaging of single virions. The resolution of the diffraction pattern was estimated to be 22 nm based on $q_{\text{max}}$ of the diffracted signal.

Figure 2(a) shows the diffraction pattern of a single, unstained virion which was added up from three independent diffraction patterns, each having a radiation dose of $3.5 \times 10^7$ Gy. Careful examination of the three diffraction patterns indicated that the radiation dose made minimum appreciable structure changes to the virion at this resolution. To significantly improve the signal-to-noise ratio (SNR) of the diffraction pattern, we integrated the diffraction intensities by binning 13 pixels into 1 pixel, and performed deconvolution to the integrated pattern [25]. The deconvolution process removed the effects of the finite pixel size of the CCD on the phase retrieval [25]. Figure 2(a) shows the characteristic ring structures of the diffraction pattern, reflecting the general round shape of the virion. Based on the diameter of the rings, the size of the virion was estimated to be $\sim 200$ nm. Intensity variation along the azimuthal angle is also visible in Fig. 2(a), which is due to the internal structure of the virion.

The phase retrieval of the diffraction pattern was carried out by the guided hybrid-input-output algorithm (GHIO) [14,26]. The GHIO started with 16 independent reconstructions of the diffraction pattern with different random phase sets as the initial input. Each reconstruction was iterated back and forth between real and reciprocal space while positivity and zero-density constraints were enforced. The iterative algorithm was guided towards minimizing the $R$ value, i.e., the difference between the measured and calculated Fourier modulus. The algorithm was terminated when the $R$ value could not be further improved and all the 16 independent reconstructions became very consistent. Figure 2(b) shows the average of the five best images with the smallest $R$ values. To examine the reliability of the reconstruction, we performed another GHIO run of the x-ray diffraction pattern. Figures 3(a) and 3(b) show the final images from two independent GHIO runs. The reconstruction error ($R_{\text{err}}$) was calculated by

$$R_{\text{err}} = \frac{\sum_{x,y} |\rho_1(x,y) - \rho_2(x,y)|}{\sum_{x,y} |\rho_1(x,y) + \rho_2(x,y)|},$$  \hspace{1cm} (1)$$

where $\rho_1(x,y)$ and $\rho_2(x,y)$ represent the two final reconstructed images. $R_{\text{err}}$ between Figs. 3(a) and 3(b) was estimated to be $\sim 2.3\%$, indicating the robustness of the phase retrieval. We also took a scanning electron microscopy (SEM) image of the same virion [Fig. 2(c)], on which
a 5 nm thick Au film was deposited to remove the surface charging effect. While the SEM image only provides the surface morphology of the virion, the overall shape is in good agreement with the x-ray image. To characterize the internal structure, we took negative stain TEM images of similar virions, shown in Fig. 2(d). The bright circular region in the TEM image represents the viral capsid which is tightly packed of the viral genome. The high-density region in the x-ray image [i.e., the darker area in Fig. 2(b)] shows features similar to the capsid structure of the TEM image. Compared with the thin-film-deposited SEM and negative stain TEM images, the x-ray diffraction image of a single, unstained virion shows the highest contrast as the background and surroundings of the virion were completely removed.

To quantify the x-ray image, we calculated the absolute electron density of the virion by

\[ I(0, 0) = I_0 r_e^2 |F(0, 0)|^2 \frac{\Delta s}{r^2}, \]

where \( I(0, 0) \) represents the number of diffracted x-ray photons in the forward direction (i.e., within the centroid pixel of the CCD camera), \( I_0 \) is the incident x-ray flux per unit area, \( r_e \) is the classical electron radius, \( |F(0, 0)| \) represents the total number of electrons in the virion, \( \Delta s \) is the area of the centroid pixel, and \( r \) is the distance from the sample to the CCD camera. Although \( I(0, 0) \) cannot be experimentally measured due to a beam stop, our recent results have shown that as long as the missing data are confined within the centrospeckle, they can be reliably recovered from the measured diffraction intensities alone [27]. Figure 4(a) shows the absolute electron density of the virion, where the yellow region represents the highest electron density with a size of ~100 nm. Figure 4(b) shows an AFM image of a similar virion, which was used to estimate the thickness profile of the virions. After taking the thickness into account, the electron density within the contour line was estimated to be approximately 1.3 times higher than the average density of the virion. It is reasonable to conclude that the high-density region represents the viral capsid of the single, unstained virion. To quantify the contrast of the viral capsid, we took a lineout across the capsid shown in Fig. 5. Because of the high-contrast ability of the x-ray diffraction microscope, the absolute electron density variations inside the capsid are visible (Fig. 5), which may be due to the packing of the viral genome.

Presently, the resolution of x-ray diffraction microscopy is limited by the coherent x-ray flux. With more brilliant synchrotron radiation sources [20], the resolution is ultimately limited by radiation damage to biological specimens [28,29]. While cryogenic cooling of biological specimens can alleviate the radiation damage problem [30–32], recent studies have shown that the highest resolution attainable for imaging the 3D pleomorphic structure of biological specimens is ~5 nm [28,29]. For the imaging of large protein molecules having identical copies, the resolution may be further improved by employing extremely intense and ultrafast x-ray pulses such as X-FELs [21]. Computer modeling and experimental results have both indicated that significant damage occurs only after an ultrafast x-ray pulse (≤ 25 fs) traverses a specimen [13,34]. By
Arguably, the major driving force for these techniques is the potential of imaging single biomolecules. The first recording and reconstruction of x-ray diffraction patterns from single, unstained virions hence represents an experimental milestone towards the ultimate goal of imaging large protein complexes [33, 34].

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*Corresponding author.
miao@physics.ucla.edu.