Flash X-ray diffraction imaging (FXI) acquires diffraction signals from single biomolecules at a high repetition rate from Free X-ray Electron lasers (XFELs), easily obtaining millions of 2D diffraction patterns from a single experiment. Due to the stochastic nature of FXI experiments and the massive volumes of data, retrieving 3D electron densities from raw 2D diffraction patterns is a challenging and time-consuming task.

We propose a semi-automatic data analysis pipeline for FXI experiments, which includes four steps: hit finding and preliminary filtering, pattern classification, 3D Fourier reconstruction, and post analysis. We also include a recently developed bootstrap methodology in the post-analysis step for uncertainty analysis and quality control. To achieve the best possible resolution, we further suggest using background subtraction, signal windowing, and convex optimization techniques when retrieving the Fourier phases in the post-analysis step.

As an application example, we quantified the 3D electron structure of the PR772 virus using the proposed data-analysis pipeline. The retrieved structure was above the detector-edge resolution and clearly showed the pseudo-icosahedral capsid of the PR772.

1. INTRODUCTION

Flash X-ray diffraction imaging (FXI) for single-particle imaging (SPI) is a nascent technology for exploring the structure of individual biological molecules in their natural states without crystallization or cryofixation. Relying on Free X-ray Electron lasers (XFELs), FXI can outrun the radiation damage to biomolecules and capture interpretable 2D diffraction signals at the high repetition rate available at XFELs, thus allowing the study of single-particle structures at femtosecond timescales. In 2011, the first FXI experiment on single mimiviruses was performed [27] at the Linac Coherent Light Source (LCLS). Since then, FXI has succeeded in determining structures of biomolecules in varying shapes and sizes [6, 8, 10, 12, 14, 24, 26, 28].

In FXI experiments the sample particles interact with the X-ray pulses at random, and the dataset therefore consists of diffraction signals of varying quality, including, e.g., misses, single hits, double hits, impurities, and background noise. Furthermore, the data volume of the FXI experiment follows that of the XFEL repetition rate. With LCLS [1], the FXI experiments operate at 120 Hz and can produce over 400,000 diffraction patterns per hour, i.e., more than 1.6 TB per hour or 38 TB per day. The newest facility, the European XFEL [25] operates at up to...
27,000 Hz, i.e., 225 times more than the LCLS, and can produce more than 12.6 million images per hour [3]. The massive volumes of data and the data complexity [6, 10] make a manual analysis impossible, and hence we seek an automatic, robust, high-quality, and fast FXI analysis pipeline which enables the efficient determination of 3D-structures.

In this paper, we design such a data-analysis pipeline and we illustrate its use for a PR772 virus dataset [23]. These results are then complementary to recent work using the same experimental data [12, 14, 24]. Our proposed pipeline, illustrated in Figure 1, is designed to select a limited amount of data frames for determining the 3D Fourier intensities, putting considerable effort in the post-analysis, i.e., phase retrieval, background handling, bootstrap analysis, pattern adjustment, and so on. Requiring a reasonable amount of computing power, the overall design goal is to be able to use our pipeline for handling FXI data on site, possibly even while the experiment is still producing new data.

![Diagram of FXI data-analysis pipeline](image)

**Figure 1.** The proposed FXI data-analysis pipeline. a) The hit finding procedure selects an initial dataset from the raw FXI data. b) From the initial dataset, the classification procedure selects high-quality single-particle diffraction patterns. c) The 3D reconstruction procedure produces a Maximum-Likelihood estimate of the 3D Fourier intensity. d) In the Post analysis step, the 3D real-space structure is estimated together with the overall resolution uncertainty.

Our proposed pipeline consists of four steps:

a) The hit finding/preliminary filtering procedure finds single-particle hits from the raw FXI data. Previous studies on hit-finding by thresholding can be found in [2], or via methods such as spectral clustering techniques [4, 30] and manifold embedding [29].

b) The classification procedure developed by us [17] classifies the initial dataset and selects high-quality diffraction patterns with specific properties, such as icosahedral shape or in a correct size range, and so on. It is possible
to combine this procedure with the hit finding step to reduce the time for selecting high-quality patterns.

c) The 3D reconstruction procedure has been refined by us [7, 16] and assembles a 3D Fourier intensity from the selected 2D diffraction patterns using the Expansion-Maximization-Compression (EMC) algorithm, originally developed in [18].

d) The post analysis step transfers the 3D Fourier intensity into a real-space structure [9, 13, 15, 19, 20]. Before retrieving phases, we may subtract background noise, heal [22] and window the 3D Fourier intensity. The reconstruction uncertainty may be estimated via a bootstrap procedure [16].

The hit finding and the classification procedures are summarized in §2. In §3, we outline the procedure to retrieve the 3D Fourier intensity from the 2D diffraction patterns. In §4, we discuss the phasing problem and how to validate and improve on the results. A concluding discussion is found in §5.

2. Hit Finding and Classification

The bacteriophage PR772 virus diffractive experiment was conducted at the Atomic Molecular Optics (AMO) instrument at the LCLS [23]. The chosen samples, with a size of about 70 nm in diameter, were injected into the XFEL beam with 1.6 keV photon energy and measured by a pnCCD detector in the far field. The resolution of the detector is 11.6 nm at the detector edge and 8.3 nm at the detector corner. The details of the experiment, such as experimental setups, raw data processing, and sample preparation can be found in [23]. In total, the raw PR772 dataset contained 2,976,868 diffraction patterns and a subset of \( N_{14k} = 14,772 \) single-hit patterns [23] was selected via diffusion map embedding. This subset was used as the initial dataset for our reconstruction pipeline, and each image in the subset was converted to photon counts and binned into 256 \( \times \) 256 pixels.

With the EigenImage classifier proposed in [17], the aim is to push a selected and limited number of high quality diffraction patterns to the next step of the analysis pipeline such that the 3D reconstruction step becomes as efficient as possible. To select suitable patterns from \( N_{14k} \), we used a synthetic training dataset constructed by Condor [11], and consisting of 1,000 randomly oriented uniform-density icosahedra, 70 nm in size. The EigenImage classifier trained from only icosahedral patterns performs badly if presented with non-icosahedral patterns. Unfortunately, the dataset \( N_{14k} \) contained a small portion of unwanted particles, such as spherical patterns and multiple-hits patterns. To find those unwanted patterns, we added one 70 nm spherical pattern into the training dataset, and we eliminated the patterns matched with the spherical pattern from the final results. All synthetic patterns used for training were masked with a central 36-pixel in diameter circular mask and a central 5-pixel-width vertical strip mask.

With the EigenImage classifier, we selected two smaller subsets from \( N_{14k} \) based on the pattern distance (the relative Euclidean distance \( e_c \) between the testing pattern and the best matched template [17]), see Figure 2. When selecting, we simultaneously excluded spherical patterns, and weak patterns for which the estimated fluence (the ratio of total photons in the testing pattern to the best matched template) was smaller than 1 (Figure 2(b)), and the estimated particle size was either larger than 72 nm or smaller than 67 nm (Figure 2(c)). By this procedure
we selected in total $N_{1k} = 1,084$ patterns with pattern distance $e_c < 0.48$ and $N_{3k} = 3,140$ frames with $e_c < 0.49$ (Figure 2(a)). On average, the selected particle size was 68.9 nm for the dataset $N_{1k}$ and 69.4 nm for the dataset $N_{3k}$.

![Figure 2](image_url)

**Figure 2.** Statistical measures from the EigenImage classifier. (a): pattern distances (the relative Euclidean distance between the testing pattern and the best matched template) (b): estimated fluence (the ratio of the total photon counts in the test pattern to the best-matched template), and (c): estimated particle sizes. In (a) and (b), we used 1000 equal sized bins, and the bin sizes were $2.062 \times 10^{-4}$ and 0.0381 for (a) and (b), respectively. In (c), we used 12 equal sized bins of size 1.159 nm. Consult [17] for the notation and definitions of pattern distance and estimated fluence.

3. **Maximum Likelihood Imaging**

To reconstruct 3D Fourier intensities, we applied the Expansion-Maximization-Compression (EMC) algorithm as detailed in [7] using the scaled Poissonian probability model [16]. The scaled Poissonian assumes that the $i$th pixel of the $k$th
measured diffraction pattern $K_{ik}$ is Poissonian around the unknown Fourier intensity $W_{ij}$ with the relative fluence $\phi_{jk}$:

$$P(K_{ik} = \kappa | W_{ij}, R_j, \phi_{jk}) = \prod_{j=1}^{\text{M_rot}} \frac{(W_{ij}\phi_{jk})^\kappa e^{-W_{ij}\phi_{jk}}}{\kappa!},$$

(1)

where $i$, $j$, and $k$ are indices to image pixels, rotations (or slices), and images, respectively. $R_j$ is the $j$th sample rotation, and $M_{\text{rot}}$ is the total number of rotation samples, where $M_{\text{rot}} = 50, 100$ is a typical resolution. With both efficiency and robustness in mind we binned the selected diffraction patterns $4 \times 4$, and those binned images were used in the computations until EMC met its stopping criterion [16]. Further, to reduce the smearing effect in the final 3D Fourier intensity, we reran the final compression step using slightly more detailed patterns ($3 \times 3$ binning) with 5-pixels-wide zero paddings around the patterns, yielding a final 3D Fourier intensity of size $96 \times 96 \times 96$ voxels.

To quantify the fitness of the datasets to the scaled Poissonian probability model, we studied the most likely rotations for each diffraction pattern, see Figure 3. Let $P_{j\text{k}}$ for $j = 1, \ldots, M_{\text{rot}}$ and $k = 1, \ldots, M_{\text{data}}$ be the normalized rotational probabilities calculated at the Expectation step of the EMC algorithm, for $M_{\text{rot}}$ the number of sampled rotations, and $M_{\text{data}}$ the number of input diffraction patterns. The most likely probabilities are then

$$M_k = \max_j P_{jk},$$

(2)

Since $P$ is normalized ($\sum_j P_{jk} = 1$), the most likely probabilities $M$ range from $M_{\text{rot}}^{-1}$ to one. For a pattern composed of random noise, the expected probability is approximately $1/M_{\text{rot}} = 2 \times 10^{-5}$ with $M_{\text{rot}} = 50, 100$. For a pattern fitted into one specified rotation perfectly, the most likely probability of that pattern will be one. For our selected datasets, the smallest most likely probability (the smallest value in $M$) of $N_{1k}$ and $N_{3k}$ were 0.235 and 0.177, respectively, indicating that the selected patterns fitted well into a 3D intensity using the scaled Poissonian model. Further, more than 43% and 28% patterns from $N_{1k}$ and $N_{3k}$ fitted into only one definite rotation. Moreover, the locations of the most likely rotations were not evenly distributed in 3D space, suggesting an asymmetric structure in the particle electron density in real-space. We also observe that Figure 3(d) is much denser and its isosurface is smoother than Figure 3(c), as the number of frames in $N_{3k}$ are almost 3 times larger than in $N_{1k}$.

We measured the contrast of local intensity extrema [24] to quantify the quality of the obtained 3D Fourier intensity,

$$C = \frac{I_{\text{max}} - I_{\text{min}}}{I_{\text{max}} + I_{\text{min}}},$$

(3)

where $I_{\text{max}}$ and $I_{\text{min}}$ are neighbouring maxima and minima pairs along a line that crossed the particle center. For each reconstruction, we averaged the contrast over all maxima and minima pairs for 100 random lines and and we obtained the average contrasts 0.5792 for $N_{1k}$ and 0.5874 for $N_{3k}$. Notably both contrasts were better than the contrast of the 3D Fourier intensity with background noises in [24] (0.4955).

Our scaled Poisson EMC implementation took up to 40 iterations for recovering the rotational probabilities of both datasets. The computing time was around 10
minutes for $N_{1k}$ and 41 minutes for $N_{3k}$ using 3 Nvidia GeForce GTX 680 GPUs in parallel (see Appendix A for more details of the reconstruction).

4. Post Analysis

In this section, we detail our design of the post analysis workflow with respect to phase retrieval, background noise removal, and the bootstrap analyses in both real and Fourier space. We also provide phasing error statistics and the shape analysis of the retrieved electron electron densities of the PR772 virus for both $N_{1k}$, and $N_{3k}$.

**Figure 3.** The most likely probability $M$ at the final EMC iteration for datasets $N_{1k}$ (a) and $N_{3k}$ (b), cf. (2). The corresponding isosurface plots of Fourier intensities are (c) and (d), respectively, and the red dots show the corresponding rotations of the most likely probabilities.
To phase an EMC intensity model and thus retrieve a 3D electron density distributions, we used a combination of algorithms — 10,000 iterations of the relaxed averaged alternating reflections (RAAR) \cite{15, 19} followed by 2,000 iterations of the Error Reduction (ER) \cite{9} algorithm. The final electron density distribution was computed as an average of 100 phased objects. Note that we reconstruct and phase our Fourier intensities without any symmetry constraints. As suggested in \cite{20}, we took the average of the the absolute values of the phased objects to further reduce the low-order phase errors.

4.1. **Original Object.** The result of the straight-forward phase retrieval is shown in Figure 4. As can be seen, the recovered particle had pseudo-icosahedral capsids with asymmetric interior structures. The particle shape deviated from an ideal icosahedral symmetry as a low density hole existed close to one of the facets, see [Figure 4(f), Figure 4(h)]. Moreover, we observed three concentric layers, which might reflect the character of the Tectiviridae family \cite{21}. However, given the detector edge resolution of 11.6 nm, we judge that the concentric-layers structure is an artefact due to aliasing and noise (see Appendix B for more details). Further, the recovered particle from $N_{3k}$ was smoother, denser, and slightly larger than the one from $N_{1k}$. The resolutions determined by the phase retrieval transfer function (PRTF) \cite{5} were around 10.7 nm for both reconstructions at the $e^{-1}$ threshold, see Figure 9.

4.2. **Background Noise.** The recovered intensity in Figure 4 is strongly concentrated in the central rings, which might partially be due to background noise. By subtracting 50% of the minimum values from the Fourier intensities at each frequency (see Appendix C for details), we improved the contrast of the Fourier intensities (eq. (3)) to 0.67 and 0.71 (from 0.58 and 0.59 with background) for $N_{1k}$ and $N_{3k}$, respectively, and the retrieved density distributions were less concentrated, yet the concentric layer structure was maintained, see Figure 5. The sizes of the retrieved particle became slightly smaller than 69 nm after background subtraction, and the PRTF analysis gave a resolution of 9.5 nm and 8.4 nm, respectively, for $N_{1k}$ and $N_{3k}$.

4.3. **Windowed Signal.** The finite capturing time for FXI experiments and the discrete digital detector may lead to spectral leakage in diffraction patterns, which will consequentially lead to artefacts in the phased object in real space, such as very low intensities in the particle center and aliasing effects. To compensate the potential energy leakage, we applied a square 3D Hann window to the Fourier intensities before phasing them. The phased objects were much smoother in both the outer capsid and the interior structure in Figure 6, comparing with the ones in Figure 4 and Figure 5. Moreover, the three concentric layers vanished after applying the Hann window. As expected, after applying Hann windows, the intensities of the retrieved particles with background noises were still more concentrated at their centers, while the intensities of the ones with background subtraction spread out. With background noises, the obtained resolutions were around 10 nm for both $N_{1k}$ and $N_{3k}$ datasets. With background subtraction, the resolutions were 11.2 nm and 8.7 nm for the $N_{1k}/N_{3k}$ datasets, respectively. Note that the Hann window efficiently removes aliasing effects, but it may also reduce the resolution of the object, due to its effect on the higher frequencies. Further, the window function enlarged the size of the phased object by 0.5 pixels. The estimated sizes
The 3D electron density distributions of the PR772 virus. (a) and (c) are the isosurface plots at 10% values of the maximum electron density for $N_{1k}$ and $N_{3k}$, respectively. (b) and (d) are the corresponding interior structures as isosurface plots at 10%, 50%, 79% and 89% values of the maximum electron density. In the second row, (e) and (g) are projection images from (a) and (c), and (f) and (h) are cross-section slices through the particle center. The black scale bars denote 70 nm. The resolution was 10.7 nm according to a PRTF analysis, which was better than the detector edge resolution (11.6 nm), see also Figure 9. The particle size was determined to be 68.9 nm and 69.4 nm, respectively, for $N_{1k}$ and $N_{3k}$.

of the obtained objects were around 69 nm after we deducted 0.5 pixels from our size calculation. We suggest that subtracting background noise and applying a square 3D Hann window before phasing improves the resolution without introducing aliasing effects.

4.4. Constrained Support. The alternating phasing algorithms, i.e., RAAR and ER, solve a concave optimization problem using convex optimization iteratively, and hence may produce local optima. By applying the Convex Optimization of Autocorrelation with Constrained Support (COACS) [22] to the 3D Fourier intensity we may instead get global optimas and hopefully achieve a higher resolution. Since COACS also uses windows, we may also get less aliasing effects and avoid the low-intensity center.

Figure 7 shows the phasing results of the COACS healed Fourier intensity for $N_{1k}$. Compared with the original phasing results for $N_{1k}$ in Figure 4, the COACS healed results were much smoother and are slightly larger, due to the Hann window used in COACS. Similar to the Hann window results in Figure 6, the healed results also removed the 3-layers structure, and the central low intensity hole. It also gave
Figure 5. The 3D electron density distributions of the PR772 virus with background subtraction. See Figure 4 for a detailed description. The resolution was 9.5 nm from a PRTF analysis for \( N_{1k} \) and 8.4 nm for \( N_{3k} \), and the particle size was determined to be 68.6 nm and 68.9 nm, respectively.

4.5. Uncertainty Analysis. We have previously proposed a bootstrap procedure to estimate the uncertainties of the EMC Fourier intensities in [16], and in this section, we extend the uncertainty-estimation procedure into real-space, see Figure 8. By doing \( B = 100 \) bootstrap runs for the dataset \( N_{1k} \) and \( N_{3k} \) using the standard bootstrap procedure from [16], we obtained \( B \) EMC Fourier intensities \( \mathcal{W}_r, r = 1, \ldots, B \), from \( B \) random initial guesses, and the bootstrap mean of \( \mathcal{W}_r \) was \( \mathcal{W}_m \). By analyzing \( \mathcal{W}_r \) by the method in [16], we obtained the Fourier uncertainties, see Figure 8(j). Our uncertainty analysis evolve around the error limits \( R_{50} = 0.248 \) and \( R_{100} = 0.485 \), respectively, determined as follows. From synthetic data, \( R_{50} \) was the average radial difference between a certain 3D ground truth and a 3D Fourier intensity, obtained by inserting 1,500 frames (50%) in their correct rotations and the remaining 1,500 frames in random rotations. The latter value \( R_{100} \) was the difference between the 3D truth and a Fourier intensity obtained from 3,000 patterns inserted in random rotations.

We also measured the uncertainties in the real domain, see Figure 8(k). Let \( O_a \) be the average phased structure in the real domain for the dataset \( N_{1k} \) (or \( N_{3k} \)). Let \( A_a \) be the 3D Fourier intensity computed from \( O_a \). Let \( O_r, r = 1, \ldots, B \) be \( B \) phased structures from \( B \) EMC Fourier intensities, and \( A_r \) be the corresponding Fourier intensity computed from \( O_r \). We also denote the average of \( A_r \) by \( A_m \), and hence, the real-space uncertainty can be defined as follows:

\[
R_{total}^2 = R_{bias}^2 + R_{std}^2 = |A_a - A_m|^2 + Var(A_r),
\]
Figure 6. The windowed 3D electron density distributions for $N_{1k}$ (left two columns) and $N_{3k}$ (right two columns), respectively. The top two rows are from reconstructions with background noises and the bottom two are windowed electron density from reconstructions with subtracted background noise. The Hann windows made the retrieved intensities much smoother and effectively removed the three concentric layers, comparing with Figure 4 and Figure 5. Further, the intensities heavily concentrated at the particle centers with background noises, and by removing the background noises, the intensities spread out in the retrieved particles. The resolution was 10 nm from a PRTF analysis with threshold $e^{-1}$. [(j) – (q)]: the corresponding results with background removed. The resolution from $N_{1k}$ was here 11.2 nm and 8.7 nm for $N_{3k}$. The combination of Hann window and background subtraction made the retrieved particle smooth and of comparably high-resolution.
Figure 7. Phasing results of COACS healed \( N_{3k} \). (a) shows the interior structures as isosurface plots at 10%, 50%, 79% and 89% values of the maximum electron density, and here the electron density was phased without applying any constraint. [(b) – (d) are three orthogonal slices through the particle center of (a)].

where \( Var \) is the variance. Note that all data are aligned into the same orientation during the analysis.

Figure 8 illustrates the results from the bootstrap analysis in both real-space and Fourier space. Both the phased bootstrap mean \( O_m \) and the mean of \( O_r \) gave similar results as \( O_u \), see Figure 8(a) and Figure 8(b) (or Figure 8(c) and Figure 8(d)). As expected, the Fourier bootstrap analysis showed a sharp increase outwards the detector edge and crossed \( R_{50} \) at a resolution of 10 nm. The real-space bootstrap analysis gave a similar results. However, the uncertainty from the dataset \( N_{1k} \) crossed \( R_{50} \) at 12.5 nm, which may be due to the smaller number of frames in \( N_{1k} \). We obtained similar resolution from the PRTF analysis, see Figure 9. Again, PRTF showed that the resolution from \( \mathbb{W}_r \) of \( N_{3k} \) was 10.7 nm and 12.8 nm for \( N_{1k} \). The phased objects from the bootstrap analysis maintained the concentric capsid layer structure, and the low intensity area close to the facets. However, the central ring got a higher intensity and the phased particle was much smoother compared with the original particle (see Figure 4).

4.6. Shape Analysis and Error Validation. Phase retrieval algorithms such as RAAR \cite{15, 19} and ER \cite{9}, are iterative algorithms with concave Fourier constraints, and hence we validate our phasing procedure with Fourier error \( E_f \) and real error \( E_r \),

\[
E_f = \sqrt{\frac{\sum_{i=1}^{M_{\text{pix}}}(|\tilde{h}_i| - \sqrt{I_i})^2}{\sum_{i=1}^{M_{\text{pix}}} I_i}},
\]

and

\[
E_r = \sqrt{\frac{\sum_{i \in S}(|h_i|)^2}{\sum_{i \in S \cup \bar{S}} |h_i|^2}},
\]

where \( S \) is the object support, \( \bar{S} \) is the area outside of \( S \). Further, \( h \) is the recovered real-space intensity, while \( \tilde{h} \) is the recovered wave in Fourier space, \( M_{\text{pix}} \) is the number of pixels of the detector, and \( I_i \) the \( i \)-th pixel value of a diffraction pattern. We also explored the particle shapes and sizes of the phased objects as follows.
Figure 8. Bootstrap analysis. [(a) and (b)]: Isosurface at 10%, 50%, 77% and 87% values of the maximum electron density of the average phased object of \( W_r \) and the phased object of \( W_m \) for \( N_{1k} \). [(c) and (d)]: Isosurface from the phased objects of \( W_r \) and \( W_m \) for \( N_{3k} \). [(e) and (h)]: a slice through the particle center of [(a) and (d)]. [(j) and (k)]: the estimated uncertainties in the Fourier space and the real-space, respectively.

For calculating sizes, we chose an intensity threshold of 10% of the maximum value, and the object diameter \( D_r \) is defined as

\[
D_r = 2m \left( \frac{3V}{4\pi} \right)^{-\frac{3}{2}},
\]

where \( V \) is the number of pixels with intensities above the intensity threshold, \( m \) is the pixel size in the real space, and \( m \approx 5.2 \) nm for our retrieved objects. We also investigated the maximum \( D_{max} \), the minimum \( D_{min} \) and the mean \( D_{mean} \) distances of 300 opposite points pairs of the virus capsid. With these values, we may quantitatively analyse the particle shape and phasing errors in Table 1.

The volume of a regular icosahedron, whose distance between two vertices is 69 nm is about \( 1.04 \times 10^5 \) voxels, which is equivalent to the volume of a sphere of diameter 58.36 nm. However, the estimated diameter \( D_r \) of the retrieved particle
Figure 9. PRTF analysis for different reconstruction of dataset $N_{1k}$ and $N_{3k}$.

Table 1. Shape and error analysis for the phased objects from the dataset $N_{1k}$ and $N_{3k}$. The measured sizes ($D_r, D_{\text{mean}}, D_{\text{max}}, D_{\text{min}}$) are in nanometers and so are the resolutions (from a PRTF analysis with a threshold of $e^{-1}$, see Figure 9a and Figure 9b). The object diameter was defined in (7). The phasing errors $E_r$ and $E_f$ were calculated in the last phase-retrieval iteration by (6) and (5), respectively. The abbreviations Win., w/o, bg., and Res. mean windowed signal, without, background and resolution, respectively.

|       | $D_r$ | $D_{\text{mean}}$ | $D_{\text{max}}$ | $D_{\text{min}}$ | $E_r$    | $E_f$    | Res   |
|-------|-------|-------------------|-------------------|-------------------|----------|----------|-------|
| $N_{1k}$ Original | 64.8 | 63.2 | 68.9 | 58.5 | 6.25e-7 | 3.4e-3 | 10.6 |
| Win. | 64.7 | 64.6 | 69.1 | 58.5 | 9.9e-7 | 3.2E-3 | 10   |
| w/o bg. | 65.7 | 63.4 | 68.6 | 57.0 | 8.0E-6 | 5.5E-3 | 9.5  |
| Win. w/o bg. | 65.8 | 63.6 | 68.7 | 58.0 | 6.1E-6 | 4.0E-3 | 11.2 |
| Bootstrap | 65.3 | 63.0 | 69.1 | 58.1 | 5.4E-6 | 3.1E-3 | 12.8 |
| Bootstrap mean | 65.2 | 63.0 | 68.5 | 57.8 | 1.1E-6 | 1.5E-3 | 10.7 |
| COACS | 65.1 | 64.3 | 68.4 | 59.3 | 4.3E-6 | 1.7E-4 | 9.1  |

|       | $D_r$ | $D_{\text{mean}}$ | $D_{\text{max}}$ | $D_{\text{min}}$ | $E_r$    | $E_f$    | Res   |
|-------|-------|-------------------|-------------------|-------------------|----------|----------|-------|
| $N_{3k}$ Original | 64.9 | 63.2 | 69.4 | 58.6 | 6.3E-6 | 3.2E-3 | 10.7 |
| Win. | 64.4 | 64.4 | 69.2 | 58.5 | 9.9e-5 | 3.4E-3 | 10   |
| w/o bg. | 64.7 | 64.4 | 68.9 | 56.6 | 4.6E-5 | 5.1E-3 | 8.4  |
| Wind. w/o bg. | 65.2 | 64.0 | 69.0 | 58  | 4.6E-5 | 5.0E-3 | 8.7  |
| Bootstrap | 65.3 | 66.5 | 69.2 | 57.1 | 5.4E-6 | 2.7E-3 | 10.7 |
| Bootstrap mean | 65.3 | 63.3 | 69.4 | 57.8 | 5E-6 | 1.5E-3 | 10.7 |

was around 64.9 nm, suggesting that the retrieved particle was smoother than an ideal icosahedron, and it is hard to observe vertices in some directions, see the cross-section images in Figure 10. As references, we compared our retrieved virus with (smoothed/non-smoothed) regular icosahedra. To generate smoothed icosahedron we convoluted two Gaussian kernels (window size was $3 \times 3 \times 3$ pixels and the standard deviation was 1 pixel) over an 69 nm regular icosahedron. We argue that,
at the current resolution, we can not observer clear features in the retrieved object as the object was blurred, and therefore further investigation of higher resolution diffraction patterns is necessary to determine any interior features.

Figure 10. **Top row**: Cross-section images of the retrieved particles from the dataset $N_{3k}$ in Figure 4. **Middle row**: Cross-section images from a smooth icosahedron object at the same rotations. To create the smooth icosahedron, we first generated an ideal icosahedron, whose outer radius was 12 pixels, and then smoothed it using two Gaussian convolution kernels. [(i)–(k)]: The surfaces of a ideal icosahedron object at the same rotations. The retrieved particle (the top row) matched well to the smoothed icosahedron (the middle row), but the vertices were much smoother than vertices of an ideal icosahedron (the bottom row).

5. Conclusion

We have proposed a working data-analysis pipeline for FLASH X-ray single particle imaging experiments. This pipeline works with the raw FXI diffraction
patterns, and retrieves high-resolution 3D electron density of the sample particles together with the associated uncertainty analysis. The workflow consists of several steps, including a) selection of single hit diffraction data, b) classification, c) 3D reconstruction in Fourier space, and d) post analysis with reconstruction of the particle electron density by phase retrieval methods, ‘healing’ the 3D Fourier intensity, handling background noise, bootstrapping analysis and shape/size analysis.

As an applied demonstration we used the data from the AMO beamline at LCLS [23]. We used the results of the diffusion map embedding in [23], as the collection of single hit diffraction data, and used a template-based classification method [17], the EigenImage classifier, to select high-quality patterns. As a result of this classification, we pushed slightly more than 1000 and, respectively, 3000 diffraction patterns into the reconstruction procedure, based on a scaled Poissonian model [16]. With 3×Nvidia GeForce GTX 680, we were able to align our datasets into 3D Fourier intensities within the hour.

At the last step, we performed the 3D phase retrieval to reveal the electron density of the virus. As expected, the size of the retrieved particle was around 69 nm with asymmetric shape and the obtained resolution was in between the detector-edge resolution and the detector-corner resolution. We conclude that the proposed data-analysis pipeline is able to handle raw FXI data properly and obtain 3D electron densities at the design resolution. We also argue that increasing the scattering angle will further improve the resolution. In addition to performing phase retrieval directly on the Fourier intensities, we also suggested a 3D background removal procedure, the use of 3D Hann window, and the use of the Convex Optimization of Autocorrelation with Constrained Support (COACS) method [22] for achieving better resolution and lower aliasing effects. Further, we also employed a previously suggested bootstrap procedure to reconstruct and measure Fourier/real intensities in a more robust way with a consistent uncertainty analysis.

The newer XFEL facilities, such as the European XFEL and the LCLS II, can produce a sufficient amount of single hits at larger diffraction angles with higher photon fluences. We may thus obtain several billions of diffraction patterns for one FXI experiment in the near future, and hence a proper data analysis pipeline is absolutely necessary to determine the 3D electron density of sample particles reliably, rapidly, and robustly. With our proposed pipeline the 3D electron density may be practically obtained during the FXI experiment along with the appropriate uncertainty analysis.

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Appendix A. Orientation determination

The orientation determination was performed for both data sets $N_{1k}$ and $N_{3k}$ using the scaled Poissonian model. In order to make the orientation determination robust with background noise and with possible detector saturation, a mask of 39 pixels in diameter, see Figure 11(a), was used for computing the rotational probability. In the final iteration, a smaller mask, which covered only the gap between two detectors, was used in merging the 3D diffraction volume, see Figure 11(b).

![Figure 11](image)

**Figure 11.** Masks used in the EMC algorithm. (a): The masks used for computing the rotational probability in each EMC iteration. (b): The mask used in the final EMC compression step. (c) is a random pattern from our selected dataset $N_{1k}$. (d) is the background scattering estimated from dark runs. (e) is an estimated background with extra scattering. (f) is a synthetic pattern with the small mask in (b).

We stopped the EMC algorithm when the sum absolute differences of the two adjacent models was less than 0.001, and hence 41 EMC iterations were performed for $N_{3k}$ and 30 iterations for $N_{1k}$. Figure 12 illustrated the sum absolute differences of the two adjacent 3D Fourier intensities. As can be seen, a peak appeared around the fifth EMC iteration for both datasets, suggesting the EMC algorithm has formed an icosahedron, and further iterations perfected the model. For synthetic dataset, EMC stopped after around 15 iterations.
Figure 12. The sum absolute differences of two EMC models from two adjacent iterations.

Appendix B. Retrieved objects from Synthetic data

Let $W_j$ be the ground truth of a 2D diffracted pattern in the $j$th rotation, i.e. $W_j$ can be simulated as a slice of a Fourier transformed particle through the particle center at the $j$th rotation. If photon counting is a Poisson process, we may write the 2D diffraction patterns without background noise as $(K^*_j)^M_{j=1} = \text{Poisson}(W_j)$, where $M$ is the number of sampled rotations, where $M = 3000$ was used in our simulations. Considering a varying photon fluence, we write $K_f = \text{Poisson}(W_\phi)$, where $\phi$ is constant for all pixels in one pattern, but differs from shot to shot. For our synthetic data we took $\phi$ a uniform random number in the range $[0.9, 1.1]$. We also added a background signal, related to the X-ray beam configuration, i.e. $(K^b_j)^M_{j=1} = \text{Poisson}(W_j \phi_j + K_b)$. Note that $K_b$ is a 2D pattern that is the same for all patterns under the same beam configuration, and we illustrate $K_b$ in Figure 11(d). Moreover, an extra source of scattering, was considered as $(K^3_j)^M_{j=1} = \text{Poisson}(W_j \phi_j + K_b + B(p))$, where $B$ takes the value 1 with probability $p = 1000^{-1}$, and zero otherwise, and we illustrate an example of $K_b + B(p)$ in Figure 11(e).

Different real-space objects reconstructed from different datasets are shown in Figure 13.

Appendix C. Background Subtraction

For given discrete shells $(s_u)_{u=1}^U$, let $S = (S_u)_{u=1}^U$ be the radial shells of a 3D Fourier intensity $\mathbb{W}$. The $u$th shell is given by $S_u = \{ s = (x, y, z); s_u \leq \|s\| < s_{u+1} \}$, where $s$ is a point (voxel) at position $(x, y, z)$, and $\|s\|$ is the Euclidean norm. The background noise in shell $u$ is then estimated by

$$B_u = \beta \min_{s \in S_u} (\mathbb{W})_s,$$

where $\beta$ is a scaling factor.
Figure 13. (a) illustrates interior structures as isosurface plots at 10%, 50%, 79% and 89% values of the maximum electron density for a synthetic Poissonian dataset $K$, and (b) is a random cross-section image from (a). [(c), (d)], [(e), (f)] and [(g), (h)] are the corresponding interior structures and cross-section images from the synthetic datasets $K^f$, $K^b$, and $K^e$, respectively. As can be seen, aliasing effects existed in all retrieved electron intensities, since a uniform-intensity icosahedron was used for generating the diffraction patterns. The background noise and the extra scattering contributed to the high-intensity central ring as shown in (f) and (h).

and we chose $\beta = 0.5$ for our EMC reconstructions. We denote the average shell of a 3D Fourier intensity by

$$D_u = |S_u|^{-1} \sum_{s \in S_u} (W)_s,$$

where $|S_u|$ is the number of voxels in the $u$th shell. Note that $D_u$ can be also considered as the average power spectral density (PSD). The results of background subtraction showed larger contrasts and clear boundaries, see Figure 14.

The PSD profiles of $N_{1k}$ and $N_{3k}$ were similar, however, the Fourier intensity for $N_{1k}$ was coarser in the high frequencies, due to the lack of data to fill in the space, see Figure 14(e) and Figure 14(f).
Figure 14. The left column are results from the dataset $N_{1k}$, and the right column are from $N_{3k}$. (a) and (c) were the cross-section plots of the EMC intensity at the final EMC iteration for $N_{1k}$ and $N_{3k}$, respectively. (b) and (d) were the corresponding background corrected data. The intensities and the background values were illustrated in (e) and (f).

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