MpUL-multi: Software for Calculation of Amyloid Fibril Mass per Unit Length from TB-TEM Images

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Structure determination for amyloid fibrils presents many challenges due to the high variability exhibited by fibrils and heterogeneous morphologies present, even in single samples. Mass per unit length (MPL) estimates can be used to differentiate amyloid fibril morphologies and provide orthogonal evidence for helical symmetry parameters determined by other methods. In addition, MPL data can provide insight on the arrangement of subunits in a fibril, especially for more complex fibrils assembled with multiple parallel copies of the asymmetric unit or multiple twisted protofilaments. By detecting only scattered electrons, which serve as a relative measure of total scattering, and therefore protein mass, dark field imaging gives an approximation of the total mass of protein present in any given length of fibril. When compared with a standard of known MPL, such as Tobacco Mosaic Virus (TMV), MPL of the fibrils in question can be determined. The program suite MpUL-multi was written for rapid semi-automated processing of TB-TEM dark field data acquired using this method. A graphical user interface allows for simple designation of fibrils and standards. A second program averages intensities from multiple TMV molecules for accurate standard determination, makes multiple measurements along a given fibril, and calculates the MPL.

Amyloid fibrils are the result of the self assembly of folded, partially folded, or denatured proteins into large filamentous structures in a sequence dependent manner. In vivo formation of amyloid fibrils under physiological conditions is associated with well known disease states such as Alzheimer’s disease, Parkinson’s disease, and Type II Diabetes Mellitus, as well as numerous less common disease states. Fibrils formed from both natural and designed proteins and peptides have also shown promise as platforms for nanoengineered devices.

Determination of amyloid fibril structures by electron microscopy is generally performed using helical reconstruction methods such as iterative real space helical reconstruction (IHRR) or using programs such as SPRING or FREALIX. All such methods require accurate knowledge of the helical symmetry of the fibril in question, and this can be difficult to determine for biological assemblies, owing to the lack of distinct structural features in noisy EM images. This problem is exacerbated when studying amyloid fibrils owing to their typically large helical repeat and structural heterogeneity. Studies aiming to determine amyloid fibril structure therefore typically deduce this information by combining methods such as examination of the Bessel orders present in Fourier transforms of cryo-EM averages or negative stain images, or direct measurements from cryo-EM and/or negative stain EM. Estimates of mass per unit length (MPL) can provide invaluable orthogonal evidence about the helical symmetry of an object and give an independent validation of parameters determined by other methods. They can also provide insight into the arrangement of subunits in the fibril, helping build pseudo-atomic models for fibril architectures using relatively low-resolution EM density. This may be especially interesting for more complex fibrils assembled with multiple copies of the asymmetric unit or multiple twisted protofilaments.

Traditionally, MPL has been determined using scanning transmission electron microscopy (STEM). STEM allows for highly accurate mass determination, without internal standards, using a calibrated detector. This eliminates inaccuracy due to both variation in the standards and error in their measurements. Advanced software exists for processing STEM data, which further improves accuracy by compensating for beam induced mass loss.
and dynamic scattering. MPL measurements by STEM have been used to differentiate fibril morphologies and validate biochemical models for amyloid fibrils composed of a variety of proteins including Calcitonin, Amyloid beta, and IAPP.

Whilst STEM using a calibrated detector remains the "gold standard" for determination of MPL, Chen et al. presented an alternative method using tilted beam transmission electron microscopy (TB-TEM), which can be performed with standard transmission EM. This ability to use standard TEM makes MPL estimation far more accessible. By using dark field imaging, TB-TEM detects only scattered electrons, the number of which is proportional to total scattering and therefore to protein mass. When compared with a standard of known MPL, the MPL of the fibrils in question can be determined. Although not as accurate as MPL determination by STEM, this allows for MPL determination with sufficient accuracy to give a reasonable estimation of the number of monomers present in a given length of fibril and can be used to distinguish some varying fibril morphologies. Here we present a new software tool, MpUL-multi, which allows the rapid semi-automated processing of TB-TEM data with little or no expert knowledge, to allow rapid estimation of MPL for fibrilar biological assemblies.

**Program**

MpUL-multi uses an extension of the method described by Chen et al. to determine sample and background intensity for a standard protein of known MPL and the fibril of interest. The inputs for MpUL-multi are TB-TEM dark field images that contain the fibrils of interest and a standard of known MPL, such as tobacco mosaic virus (TMV). After performing background subtraction, the intensity of the fibrils is compared with the intensity of the standard, allowing for estimation of the MPL of the unknown fibril. The program allows for the averaging of values from multiple standards to reduce variability due to differences in the intensity measured from the individual TMV molecules.

TMV standards and multiple areas along a fibril are defined by specifying the start and end coordinates for lines along the fibril long axis, and defining its width using the GUI (Fig. 1). Pixel values along one-pixel wide lines perpendicular to the fibril axis are then measured, reporting a fibril intensity and two background intensity measurements. These values are then used with the measurements from the TMV standards in the same image to determine the MPL for the fibril as:

\[
MPL = K \left( \frac{I_f - B_f}{I_{tmv} - B_{tmv}} \right)
\]

Where \(I_f\) and \(I_{tmv}\) are the uncorrected intensity measurements for the fibril and TMV standard, \(B_f\) and \(B_{tmv}\) are corresponding background intensity measurements for each intensity measurement, and \(K\) is the MPL of TMV, taken form the literature as 131 kDa nm\(^{-1}\). The individual 1 px wide measurements from all subregions of the fibrils are then combined to calculate the mean MPL.

The program does not require an exact determination of the width of the fibrils being measured, but instead requires only that the defined width be wider than the actual fibril width and is robust in regard to small errors.
novo raphy. Monomeric protein was dissolved in 20 mM TRIS HCl (pH 7.4) with 100 mM NaCl, filtered through a recombinantly in consisting of multiple twisted fibrils were excluded. In all cases fibrils that appeared to be of the lowest order were selected for measurements. Higher-order structures containing 10 mM MOPS (pH 7.0), 50 mM KCl, 1 mM MgCl2, and 1 mM EGTA as in27. UltraScan US1000XP CCD (Gatan Inc, USA.) using a 10 second exposure (Fig. 3B). adjusted to give even illumination and maximum intensity over the entire image. Images were recorded on an focused using bright field illumination (Fig. 3A). The electron beam was then tilted to Technai TEM operated at 80 kV . Regions containing both TMV and fibrils were identified and the microscope Image processing. The images were converted to 8 bit TIF format with ImageJ and processed using MpUL-multi. All available molecules of TMV in each image were selected to serve as the MPL standard. Individual WT α-syn, α-syn A30P, α-syn A53T, F-actin, and β2m fibrils were measured in separate micrographs. In all cases fibrils that appeared to be of the lowest order were selected for measurements. Higher-order structures consisting of multiple twisted fibrils were excluded. Program availability. MpUL-multi and the MpUL-GUI are distributed under the GNU General Public License33 and available (with test data and comprehensive instructions) from the Collaborative Computational Project for Electron cryo-Microscopy (CCP-EM; http://www.ccpem.ac.uk/download.php) The programs are written in python 2.7.1 and require the “math”, “TkInter”, “os”, “numpy”, and “sys” standard modules. A local copy of ImageJ (or FIJI) is also required.
Results and Discussion

To benchmark the accuracy of the MpUL-multi programs we determined the mean MPL for two samples for which MPL estimates already exist. (Table 1) The MPL of F-actin was calculated as $17 \pm 5 \text{kDa.nm}^{-1}$ ($n = 7$ fibrils, 814 measurements) which corresponds well to the theoretical value of $15.4 \text{kDa.nm}^{-1}$ derived from an atomic model of the F-actin fibril and MPL of $15.4 \text{kDa.nm}^{-1}$ determined using STEM28. The MPL of $\beta_2$m amyloid fibrils have been calculated previously using STEM data. MpUL-multi determined an MPL of $56 \pm 4 \text{kDa.nm}^{-1}$ ($n = 31$ fibrils, 14067 measurements; Fig. 4A), corresponding well to the previous estimate of $53 \pm 3 \text{kDa.nm}^{-1}$ for two-protofilament “type I” and “type II” $\beta_2$m fibrils13.

Unlike fibrils of F-actin or $\beta_2$m, no measurements of MPL for fibrils grown from full-length, wild type $\alpha$-syn are currently available. We therefore also used MpUL-multi to make such measurements, yielding a MPL of $70 \pm 2 \text{kDa.nm}^{-1}$ ($n = 23$ fibrils, 20091 measurements; Fig. 4B) (Table 1).

An analysis of fluctuations in background intensity for the WT $\alpha$-syn fibrils was performed as in21 to estimate the contribution to uncertainty in the MPL measurements. Background fluctuations were measured as:

$$\frac{B_1 - B_2}{I_{\text{tmv}}}$$

where $B_1$ and $B_2$ are subsequent background measurements, one pixel apart. The standard deviation of the Gaussian distribution of background measurements was 10.5 compared to 16.1 for the background-subtracted fibrils. This is similar to values reported in Chen et al.21 but does suggest some contributions from other sources of error, which could include heterogeneity in the fibril structure along with other factors such as variability in the standards and effects during data collection such as beam induced mass loss.

We also determined the MPL of two variants of $\alpha$-syn (A53T and A30P) that are known to occur naturally in individuals with early-onset Parkinson’s disease, and have been previously suggested to have fibril morphologies that vary from the WT fibrils (Table 1)29,30. $\alpha$-syn A53T showed a MPL of $60 \pm 4 \text{kDa.nm}^{-1}$ ($n = 29$ fibrils, 11588 measurements), while $\alpha$-syn A30P showed a MPL of $43 \pm 3 \text{kDa.nm}^{-1}$ ($n = 25$ fibrils, 14025 measurements). These estimates are consistent with very recent measurements of MPL for three different polymorphs of $\alpha$-syn made using STEM31. Given the molecular mass of an $\alpha$-syn monomer (14.4 kDa), this corresponds to $4.8 \pm 0.2$, $4.1 \pm 0.3$, and $2.9 \pm 0.2$ monomers per nm respectively, suggesting that these fibrils do indeed have different protofilament arrangements and/or architectures, information that can be used to guide model building in ongoing structural studies.

Table 1. MPL values calculated for 5 fibular protein samples. 1White et al., 200913. 2Steinmetz, et al., 199828.
Vilar et al.32 proposed models for α-synuclein based of EM and solid-state NMR analysis of fibrils composed of the core α30–110 fragment. Fibrils in this model are assembled from combinations of protofilaments having 2 protein molecules per β-sheet spacing. The estimated MPL values suggest the α-syn and α-syn A53T fibrils examined in this study would be single protofilaments of the Vilar et al. model. This may suggest the N- and/or C-terminal regions of α-syn play a role in the assembly of protofilaments into larger fibrillar aggregates.

The MpUL-multi program and GUI are designed to expedite the determination of fibril MPL and allow groups that do not have access to STEM to make rapid MPL estimations. TB-TEM dark field imaging is relatively easy to implement and does not require any special equipment beyond a standard TEM. The TB-TEM method is however, sensitive to ion contamination during grid preparation, especially from buffer salts. The TMV standard used should preferably be stored in pure water rather than a salt-containing buffer, water should be as pure as possible, and ions generated by interactions between the buffer and grids can be mitigated for by the use of inert titanium or gold grids. Freeze drying the sample to the grid may be preferable to air-drying as the sample may be sensitive to surface tension forces generated by the drying process. It is inherently less accurate than STEM measurements. However, it does allow estimates of mass per unit length to be made on in-house EM resources, and the MpUL provided GUI simplifies the process of selecting fibrils and standards. Finally, MpUL-multi performs image analysis and generates plain text measurement outputs for analysis as desired. Although not as accurate as MPL measurement by STEM, this allows for rapid ‘working estimates’ of fibril MPL within ~15%, allowing for identification of gross morphological differences that can be investigated by orthogonal biochemical and/or microscopy methods.

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Author Contributions
M.G.I., S.E.R. and N.A.R. conceived the project, designed experiments, and prepared the manuscript. M.P.J. designed fibril growth conditions and prepared fibril samples. M.G.I. prepared samples, collected micrographs, and wrote the software.

Additional Information
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