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TITLE:

Cryo-EM and Single-Particle Analysis with Scipion

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SUMMARY:

Single-particle analysis in cryo-electron microscopy is one of the main techniques used to determine the structure of biological ensembles at high resolution. Scipion provides the tools to create the whole pipeline to process the information acquired by the microscope and achieve a 3D reconstruction of the biological specimen.

ABSTRACT:

Cryo-electron microscopy has become one of the most important tools in biological research to reveal the structural information of macromolecules at near-atomic resolution. In single-particle analysis, the vitrified sample is imaged by an electron beam and the detectors at the end of the microscope column produce movies of that sample. These movies contain thousands of images of identical particles in random orientations. The data need to go through an image processing workflow with multiple steps to obtain the final 3D reconstructed volume. The goal of the image processing workflow is to identify the acquisition parameters to be able to reconstruct the specimen under study. Scipion provides all the tools to create this workflow using several image processing packages in an integrative framework, also allowing the traceability of the results. In this article the whole image processing workflow in Scipion is presented and discussed with data coming from a real test case, giving all the details necessary to go from the movies obtained by the microscope to a high resolution final 3D reconstruction. Also, the power of using consensus tools that allow combining methods, and confirming results along every step of the workflow, improving the accuracy of the obtained results, is discussed.

INTRODUCTION:

In cryo-electron microscopy (cryo-EM), single particle analysis (SPA) of vitrified frozen-hydrated specimens is one of the most widely used and successful variants of imaging for biological macromolecules, as it allows to understand molecular interactions and the function of biological ensembles[1]. This is thanks to the recent advances in this imaging technique that gave rise to the "resolution revolution"[2] and have allowed the successful determination of biological 3D structures with near-atomic resolution. Currently, the highest resolution achieved in SPA cryo-EM was 1.15 Å for apoferritin[3] (EMDB entry: 11668). These technological advances comprise improvements in the sample preparation[4], the image acquisition[5], and the image processing methods[6]. This article is focused on this last point.

Briefly, the goal of the image processing methods is to identify all the acquisition parameters to invert the imaging process of the microscope and recover the 3D structure of the biological specimen under study. These parameters are the gain of the camera, the beam-induced movement, the aberrations of the microscope (mainly the defocus), the 3D angular orientation and translation of each particle, and the conformational state in case of having a specimen with conformational changes. However, the number of parameters is very high and cryo-EM requires using low-dose images to avoid radiation damage, which significantly reduces the Signal-to-Noise Ratio (SNR) of the acquired images. Thus, the problem cannot be unequivocally solved and all the parameters to be calculated only can be estimations. Along the image processing workflow, the correct parameters should be identified, discarding the remaining ones to finally obtain a high-resolution 3D reconstruction.

The data generated by the microscope are gathered in frames. Simplifying, a frame contains the number of electrons that have arrived at a particular position (pixel) in the image, whenever electron-counting detectors are used. In a particular field of view, several frames are collected and this is called a movie. As low electron doses are used to avoid radiation damage that could destroy the sample, the SNR is very low and the frames corresponding to the same movie need to be averaged to obtain an image revealing structural information about the sample. However, not only a simple average is applied, the sample can suffer shifts and other kinds of movements during the imaging time due to the beam-induced movement that need to be compensated. The shift-compensated and averaged frames originate a micrograph.

Once the micrographs are obtained, we need to estimate the aberrations introduced by the microscope for each of them, called Contrast Transfer Function (CTF), which represents the changes in the contrast of the micrograph as a function of frequency. Then, the particles can be selected and extracted, which is called particle picking. Every particle should be a small image containing only one copy of the specimen under study. There are three families of algorithms for particle picking: 1) the ones that only use some basic parameterization of the appearance of the particle to find them in the whole set of micrographs (e.g., particle size), 2) the ones that learn how the particles look like from the user or a pretrained set, and 3) the ones that use image templates. Each family has different properties that will be shown later.

The extracted set of particles found in the micrographs will be used in a 2D classification process that has two goals: 1) cleaning the set of particles by discarding the subset containing pure noise images, overlapping particles, or other artifacts, and 2) the averaged particles representing each class could be used as initial information to calculate a 3D initial volume.

The 3D initial volume calculation is the next crucial step. The problem of obtaining the 3D structure can be seen as an optimization problem in a multidimensional solution landscape, where the global minimum is the best 3D volume that represents the original structure, but several local minima representing suboptimal solutions can be found, and where it is very easy to get trapped. The initial volume represents the starting point for the searching process, so bad initial volume estimation could prevent us to find the global minimum. From the initial volume, a 3D classification step will help to discover different conformational states and to clean again the set of particles; the goal is to obtain a structurally homogeneous population of particles. After that, a 3D refinement step will be in charge of refining the angular and translation parameters for every particle to get the best 3D volume possible.

Finally, in the last steps, the obtained 3D reconstruction can be sharpened and polished. Sharpening is a process of boosting the high frequencies of the reconstructed volume, and the polishing is a step to further refine some parameters, as CTF or beam-induced movement compensation, at the level of particles. Also, some validation procedures could be used to better understand the achieved resolution at the end of the workflow.

After all these steps, the tracing and docking processes[7] will help to give a biological meaning to the obtained 3D reconstruction, by building atomic models de novo or fitting existing models. If

high resolution is achieved, these processes will tell us the positions of the biological structures, even of the different atoms, in our structure.

Scipion[8] allows creating the whole workflow combining the most relevant image processing packages in an integrative way. Xmipp[9], Relion[10], CryoSPARC[11], Eman[12], Spider[13], Cryolo[14], Ctffind[15], CCP4[16], Phenix[17], and many more packages can be included in Scipion. Also, it incorporates all the necessary tools to benefit the integration, interoperability, traceability, and reproducibility to make a full tracking of the entire image-processing workflow[8].

One of the most powerful tools that Scipion allows us to use is the consensus, which means to compare the results obtained with several methods in one step of the processing, making a combination of the information conveyed by different methods to generate a more accurate output. This could help to boost the performance and improve the achieved quality in the estimated parameters. Note that a simpler workflow can be build without the use of consensus methods; however, we have seen the power of this tool[22,25] and the workflow presented in this manuscript will use it in several steps.

All the steps that have been summarized in the previous paragraphs will be explained in detail in the following section and combined in a complete workflow using Scipion. Also, how to use the consensus tools to achieve a higher agreement in the generated outputs will be shown. To that end, the example dataset of the *Plasmodium falciparum* 80S Ribosome has been chosen (EMPIAR entry: 10028, EMDB entry: 2660). The dataset is formed by 600 movies of 16 frames of size 4096x4096 pixels at a pixel size of 1.34Å taken at an FEI POLARA 300 with an FEI FALCON II camera, with a reported resolution at EMDB is 3.2Å[18] .

PROTOCOL:

1. Creating a project in Scipion and importing the data

1.1. Open Scipion and click on **Create Project**, specify the name for the project and the location where it will be saved (**Supplemental Figure 1**). Scipion will open the project window showing a canvas with, on the left side, a panel with a list of available methods, each of them represents one image processing tool that can be used to manage data.

NOTE: **Ctrl+F** can be used to find a method if it does not appear in the list.

1.2. To import the movies taken by the microscope select the **pwem - import movies** on the left panel (or type it when pressing **Ctrl+F**).

1.3. A new window will be opened (**Supplemental Figure 2**). There, include the path to the data, and the acquisition parameters. In this example, use the following setup: **Microscope voltage** 300 kV, **Spherical aberration** 2.0 mm, **Amplitude Contrast** 0.1, **Magnification rate** 50000, **Sampling rate mode** to **From image**, and **Pixel size** 1.34 Å. When all the parameters in the form are filled, click on the **Execute** button.

NOTE: When a method starts, a box appears in the canvas in yellow color labeled as **running**. When a method finishes, the box changes to green, and the label changes to **finished**. In case of an error during the execution of a method, the box will appear in red, labeled as **failed**. In that case, check the bottom part of the canvas, in the **Output Log** tab an explanation of the error will appear.

1.4. When the method finishes, check the results in the bottom part of the canvas in the **Summary** tab. Here, the outputs generated by the method are presented, in this case, the set of movies. Click on **Analyze Results** button and a new window will appear with the list of movies.

2. Movie alignment: from movies to micrographs

2.1. Use the method **xmipp3 – optical alignment** which implements Optical flow[19]. Use the following parameters to fill in the form (**Supplemental Figure 3**): the **Input Movies** are those obtained in step 1, the range in **Frames to ALIGN** is from 2 to 13, the other options stay with the default values. Execute the program.

NOTE: The parameters in bold in a form must be always filled. The others will have a default value or will not be obligatorily required. In the upper part of the form window, the fields where the computational resources are distributed can be found, as threads, MPIs, or GPUs.

2.2. Click on **Analyze Results** to check the obtained micrographs and the trajectory of the estimated shifts (**Figure 1**). For every micrograph seen: look at the power spectral density (PSD), the trajectories obtained to align the movie (one point per frame) in cartesian and polar coordinates, and the file name of the obtained micrograph (clicking on it, the micrograph can be inspected). Notice that the particles of the specimen are much more visible in the micrograph, as compared to a single frame of the movie.

3. CTF estimation: calculating the aberrations of the microscope

3.1. First, use the method **grigoriefflab – ctffind**[15]. The setup is: the **Input Micrographs** are the output of step 2, the **Manual CTF Downsampling factor** is set to 1.5, and the **Resolution** range goes from 0.06 to 0.42. Moreover, in the **Advanced** options (that can be found by selecting this choice in the **Expert Level** of the form), set the **Window size** to 256. The remaining parameters stay with the default values (**Supplemental Figure 4**).

NOTE: In most of the methods in Scipion the **Advanced** option shows more configuration parameters. Use these options carefully, when the program to be launched is completely known and the meaning of the parameters is understood. Some parameters can be difficult to fill without having a look at the data; in that case, Scipion shows a magic wand on the right side that will show a wizard window (**Supplemental Figure 5**). For example, in the **Resolution** field of this form is especially useful, as these values should be selected to approximately cover the

region from the first zero to the last noticeable ring of the PSD.

3.2. Click on **Execute** and on **Analyze Results** (**Figure 2**) when the method finishes. Check that the estimated CTF matches with the experimental one. To that end, look at the PSD and compare the estimated rings in the corner with the ones coming from the data. Also check the obtained defocus values to find any unexpected values and respective micrographs can be discarded or recalculated. In this example, the whole set of micrographs can be used.

NOTE: Use the buttons in the bottom part of the window to make a subset of micrographs (with **Micrographs** red button) and to recalculate a CTF (with **Recalculate CTFs** red button), in case of needing.

3.3. To refine the previous estimation, use **xmipp3 – ctf estimation**[20]. Select as **Input Micrographs** the output of step 2, select the option **Use defoci from a previous CTF estimation**, as **Previous CTF estimation** choose the output of **grigoriefflab – ctffind**, and, in the **Advanced** level, change the **Window size** to 256 (**Supplemental Figure 6**). Run it.

3.4. Click on **Analyze Results** to check the obtained CTFs. With this method, more data is estimated and represented in some extra columns. As none of them show incorrect estimated values, all the micrographs will be used in the following steps.

4. Particle picking: finding particles in the micrographs

4.1. Before starting the picking, carry out a preprocess of the micrographs. Open **xmipp3 – preprocess micrographs**, set as **Input micrographs** those obtained in step 2 and select the options **Remove bad pixels?** with **Multiple of Stddev** to 5, and **Downsample micrographs?** with a **Downsampling factor** of 2 (**Supplemental Figure 7**). Click on **Execute** and check that the size of the resulting micrographs has been reduced.

4.2. For the picking use **xmipp3 – manual-picking (step 1)** and **xmipp3 – auto-picking (step 2)**[21]. The manual picking allows to manually prepare a set of particles with which the auto-picking step will learn and generate the complete set of particles. First, run **xmipp3 – manual-picking (step 1)** with **Input Micrographs** as the micrographs obtained in the previous preprocess. Click on **Execute** and a new interactive window will appear (**Figure 3**).

4.3. In this window a list of the micrographs (**Figure 3a**) and other options is presented. Change **Size (px)** to 150, this will be the size of the box containing each particle. The selected micrograph appears in a bigger window. Choose a region and pick all the visible particles in it (**Figure 3b**). Then, click on **Activate Training** to start the learning. The remaining regions of the micrograph are automatically picked (**Figure 3c**). Check the picked particles and include more by clicking on it, or remove the incorrect ones with shift+clicking, if necessary.

4.4. Select the next micrograph in the first window. The micrograph will be automatically picked. Check again to include or remove some particles, if necessary. Repeat this step with,

approximately, 5 micrographs to create a representative training set.

4.5. Once this is done, click on **Coordinates** in the main window to save the coordinates of all the picked particles. The training set of particles is ready to go to the auto picking to complete the process for all micrographs.

4.6. Open **xmipp3 – auto-picking (step 2)** indicating in **Xmipp particle picking run** the previous manual picking, and **Micrographs to pick** as **Same as supervised**. Click on **Execute**. This method will generate as output a set of around 100000 coordinates.

4.7. Apply a consensus approach, so carry out a second picking method to select the particles in which both methods agree. Open **sphire – cryolo picking**[14] and select the preprocessed micrographs as **Input Micrographs**, **Use general model?** to **Yes**, with a **Confidence threshold** of 0.3, and a **Box Size** of 150 (**Supplemental Figure 8**). Run it. This method should generate also around 100000 coordinates.

4.8. Run **xmipp3 – deep consensus picking**[22]. As **Input coordinates** include the output of **sphire – cryolo picking** (step 4.7) and **xmipp3 – auto-picking** (step 4.6), set **Select model type** to **Pretrained**, and **Skip training and score directly with pretrained model?** To **Yes** (**Supplemental Figure 9**). Run it.

4.9. Click on **Analyze Results** and, in the new window, on the eye icon next to **Select** particles**/coordinates with high 'zScoreDeepLearning1' values**. A new window will be opened with a list of all particles (**Figure 4**). The **zScore** values in the column give an insight into the quality of a particle, low values mean bad quality.

4.9.1. Click on the label **_xmipp_zScoreDeepLearning** to order the particles from highest to lowest **zScore**. Select the particles with **zScore** higher than 0.75 and click on **Coordinates** to create the new subset. This should create a subset with approximately 50000 coordinates.

4.10. Open **xmipp3 – deep micrograph cleaner**. Select as **Input coordinates** the subset obtained in the previous step, **Micrographs source** as **same as coordinates**, and keep **Threshold** at 0.75. Run it. Check in the **Summary** tab that the number of coordinates has been reduced, although in this case, only few coordinates are removed.

NOTE: This step is able to additionally clean the set of coordinates and could be very useful in cleaning other datasets with more movie artifacts as carbon zones or large impurities.

4.11. Run **xmipp3 – extract particles** (**Supplemental Figure 10**). Indicate as **Input coordinates** the coordinates obtained after the previous step, **Micrographs source** as **other**, **Input micrographs** as the output of step 2, **CTF estimation** as the output of the **xmipp3 – ctf estimation**, **Downsampling factor** to 3, and **Particle box size** to 100. In the **Preprocess** tab of the form select **Yes** to all. Run it.

4.12. Check that the output should contain the particles in reduced size of 100x100 pixels and a pixel size of 4.02Å/px.

4.13. Run again **xmipp3 – extract particles** changing the following parameters: **Downsampling factor** to 1, and **Particle box size** to 300. Check that the output is the same set of particles but now at the full resolution.

5. 2D classification: grouping similar particles together

5.1. Open the method **cryosparc2 – 2d classification**[11] with **Input particles** as those obtained in step 4.11 and, in the **2D Classification** tab, the **Number of classes** to 128, keep all the other parameters with the default values. Run it.

5.2. Click on **Analyze Results** and then on the eye icon next to **Display particle classes with Scipion** (**Figure 5**). This classification will help to clean the set of particles, as several classes will appear noisy or with artifacts. Select the classes containing good views. Click on **Particles** (red button in the lower part of the window) to create the cleaner subset.

5.3. Now, open **xmipp3 – cl2d**[23] and set as **Input images** the images obtained in the previous step and **Number of classes** as 128. Click on **Execute**.

NOTE: This second classification is used as additional cleaning step of the set of particles. Usually is useful to remove as much noisy particles as it is possible. However, if a simpler workflow is desired, only one 2D classification method can be used.

5.4. When the method finishes, check the 128 generated classes by clicking on **Analyze Results** and on **What to show: classes**. Most of the generated classes show a projection of the macromolecule with some level of detail. However, some of them appear noisy (in this example approximately 10 classes). Select all the good classes and click on the **Classes** button to generate a new subset with only the good ones. This subset will be used as input to one of the methods to generate an initial volume. With the same selected classes click on **Particles** to create a cleaner subset after removing those belonging to the bad classes.

5.5. Open **pwem – subset** with **Full set of items** as the output of 4.13 (all particles at the full size), **Make random subset** to **No**, **Other set** as the subset of particles created in the previous step, and **Set operation** as **intersection**. This will extract the previous subset from the particles at full resolution.

6. Initial volume estimation: building the first guess of the 3D volume

6.1. In this step, estimate two initial volumes with different methods and then use a consensus tool to generate the final estimated 3D volume. Open **xmipp3 – reconstruct significant**[24] method with **Input classes** as those obtained after step 5, **Symmetry group** as c1, and keep the remaining parameters with their default values (**Supplemental Figure 11**).

Execute it.

6.2. Click on **Analyze Results**. Check that a low resolution volume of size 100x100x100 pixels and a pixel size of 4.02Å/px is obtained.

6.3. Open **xmipp3 – crop/resize volumes** (**Supplemental Figure 12**) using as **Input Volumes** the one obtained in the previous step, **Resize volumes?** to **Yes**, **Resize option** to **Sampling Rate**, and **Resize sampling rate** to 1.34 Å/px. Run it. Check in the **Summary** tab that the output volume has the correct size.

6.4. Now, create the second initial volume. Open **relion – 3D initial model**[10], as **Input particles** use the good particles at full resolution (output of 5.5) and set **Particle mask diameter** to 402Å, keep the remaining parameters with the default values. Run it.

6.5. Click on **Analyze Results** and then in **Display volume with: slices**. Check that a low resolution volume but with the main shape of the structure is obtained (**Supplemental Figure 13**).

6.6. Now, open **pwem – join sets** to combine the two generated initial volumes to create the input to the consensus method. Just indicate **Volumes** as **Input type** and select the two initial volumes in **Input set**. Run it. The output should be a set containing two items with both volumes.

6.7. The consensus tool is the one included in **xmipp3 – swarm consensus**[25]. Open it. Use as **Full-size Images** the good particles at full resolution (output of 5.5), as **Initial volumes** the set with two items generated in the previous step, and be sure that **Symmetry group** is c1. Click on **Execute**.

6.8. Click on **Analyze Results.** Check that a more detailed output volume is obtained (**Figure 6**). Although there is more noise surrounding the structure, to have more details in the structure map will help the following refinement steps to avoid local minima.

NOTE: If UCSF Chimera[26] is available, use the last icon in the upper part of the window to make a 3D visualization of the obtained volume.

6.9. Open and execute **relion – 3D auto-refine**[10] to make a first 3D angular assignment of the particles. Select as **Input particles** the output of 5.5, and set **Particle mask diameter** to 402Å. In **Reference 3D map** tab, select as **Input volume** the one obtained in the previous step, **Symmetry** as c1, and **Initial low-pass filter** to 30Å (**Supplemental Figure 14**).

6.10. Click on **Analyze Results**. In the new window select **final** as **Volume to visualize** and click on **Display volume with: slices** to see the obtained volume. Check also the Fourier shell correlation (FSC) by clicking on **Display resolution plots** in the results window and the angular coverage in **Display angular distribution: 2D plot** (**Figure 7**). The reconstructed volume contains

much more details (probably with some blurred areas in the outer part of the structure), and the FSC crosses the threshold of 0.143 around 4.5Å. The angular coverage covers the whole 3D sphere.

7. 3D classification: discovering conformational states

7.1. Using a consensus approach, if different conformational states are in the data can be discovered. Open **relion – 3D classification**[10] (**Supplemental Figure 15**). As **Input particles** use those just obtained in 6.10, and set **Particle mask diameter** to 402Å. In the **Reference 3D map** tab, use as **Input volume** the one obtained after step 6.10, set **Symmetry** to c1, and **Initial low-pass filter** to 15Å. Finally, in **Optimization** tab, set the **Number of classes** to 3. Run it.

7.2. Check the results by clicking on **Analyze Results**, select **Show classification in Scipion**. The three generated classes and some interesting measures are shown. The first two classes should have a similar number of assigned images (**size** column) and look very similar, whilst the third one has fewer images and a more blurred appearance. Also, the **rlnAccuracyRotations** and **rlnAccuracyTranslations** should be clearly better for the first two classes. Select the two best classes and click on the **Classes** button to generate a subset containing them.

7.3. Repeat steps 7.1 and 7.2 to generate a second group of good classes. Both will be the input of the consensus tool.

7.4. Open and run **xmipp3 – consensus classes 3D** and select as **Input Classes** the two subsets generated in the previous steps.

7.5. Click on **Analyze Results**. The number of coincident particles between classes is presented: the first value is the number of coincident particles in the first class of subset 1 and the first class of subset 2, the second value is the number of coincident particles in the first class of subset 1 and the second class of subset 2, etc. Check that the particles are randomly assigned to classes one or two, which means that the 3D classification method is not able to find conformational changes. Given this result, the whole set of particles will be used to continue processing.

8. 3D refinement: refining angular assignments of a homogeneous population

8.1. Again, apply a consensus approach in this step. First, open and run **pwem – subset** with **Full set of items** as the output of 6.9, **Make random subset** to **Yes**, and **Number of elements** to 5000. With this, a subset of images with a previous alignment to train the method used in the following step is created.

8.2. Open **xmipp3 – deep align**, set **Input images** as the output of good particles obtained in 5.5, **Volume** as the one obtained after 6.10, **Input training set** as the one created in the previous step, **Target resolution** to 10Å, and keep the remaining parameters with the default values (**Supplemental Figure 16**). Click on **Execute**.

8.3. Click on **Analyze Results** to check the obtained angular distribution, where there are no missing directions and the angular coverage slightly improves compared to the one of 6.10 (**Figure 8**).

8.4. Open and execute **xmipp3 – compare angles** and select as **Input particles 1** the output of 6.9 and **Input particles 2** the output of 8.2, make sure that the **Symmetry group** is c1. This method calculates the agreement between **xmipp3 – deep align** and **relion – 3D auto refine**.

8.5. Click on **Analyze Results**, the list of particles, with the obtained differences in shifts and angles, is shown. Click on the bar icon in the upper part of the window, another window will be opened that allows making plots of the calculated variables. Select **_xmipp_angleDiff** and click on **Plot** to see a representation of the angular differences per particle. Do the same with **_xmipp_shiftDiff**. In these figures, approximately in half of the particles both methods agree (**Figure 9**). Select the particles with angular differences lower than 10º and create a new subset.

8.6. Now, open **xmipp3 – highres**[27] to make a local refinement of the assigned angles. First, select as **Full-size Images** the images obtained in the previous step, and as **Initial volumes** the output of 6.9, set **Radius of particle** to 150 pixels, and **Symmetry group** as c1. In the **Angular assignment tab**, set the **Image alignment** to **Local**, **Number of iterations** to 1, and **Max. Target Resolution** as 5Å/px (**Supplemental Figure 17**). Run it.

8.7. In the **Summary** tab check that the output volume is smaller than 300x300x300 pixels and with slightly higher pixel size.

8.8. Click on **Analyze Results** to see the obtained results. Click on **Display resolution plots** to see the FSC, and on **Display volume: Reconstructed** to see the obtained volume (**Supplemental Figure 18**). A good resolution volume close to 4-3.5Å is obtained.

8.9. Click on **Display output particles** and, in the window with the list of particles, click on the bar icon. In the new window, select **Type** as **Histogram**, with 100 **Bins**, select **_xmipp_cost** label, and finally press **Plot** (**Supplemental Figure 19**). This way, the histogram of the **cost** label is presented, which contains the correlation of the particle with the projection direction selected for it. In this case, a unimodal density function is obtained, which is a sign of not having different populations in the set of particles. Thus all of them will be used to continue the refinement

NOTE: In case of seeing a multimodal density function, the set of particles belonging to the higher maximum should be selected to continue the workflow only with them.

8.10. Open and execute again **xmipp3 – highres** with **Continue from a previous run?** to **Yes**, set as **Full-size Images** those obtained after 8.5, and **Select previous run** with the previous execution of Xmipp Highres. In the **Angular assignment** tab, set the **Image alignment** to **Local**, with 1 iteration and 2.6Å/px as target resolution (full resolution).

8.11. Now the output should contain a volume at full resolution (size 300x300x300 pixels). Click on **Analyze Results** to check again the obtained volume and the FSC, which now should be a high resolution volume at around 3Å (**Figure 10**).

9. Evaluation and post-processing

9.1. Open **xmipp3 – local MonoRes**[28]. This method will calculate the resolution locally. Set as **Input Volume** the one obtained after 8.10, set **Would you like to use half volumes?** to **Yes**, and **Resolution Range** from 1 to 10Å. Run it.

9.2. Click on **Analyze Results** and select **Show resolution histogram** and **Show colored slices** (**Figure 11**). The resolution in the different parts of the volume is shown. Most of the voxels of the central part of the structure should present resolutions around 3Å, whilst the worst resolutions are achieved in the outer parts. Also, a histogram of the resolutions per voxel is shown with a peak around (even below) 3Å.

9.3. Open and run **xmipp3 – localdeblur sharpening**[29] to apply a sharpening. Select as **Input Map** the one obtained in 8.10, and as **Resolution Map** the one obtained in the previous step with MonoRes.

9.4. Click on **Analyze Results** to check the obtained volumes. Open the last one, corresponding to the last iteration of the algorithm. It is recommend opening the volume with other tools, such as UCSF Chimera[26], to see better the features of the volume in 3D (**Figure 12**).

9.5. Finally, open the validation tool included in **xmipp3 – validate overfitting**[30] that will show how the resolution changes with the number of particles. Open it and include as **Input particles** the particles obtained in step 8.5, set **Calculate the noise bound for resolution?** to **Yes**, with **Initial 3D reference volume** as the output of 8.10. In **Advanced** options, set the **Number of particles** to "500 1000 1500 2000 3000 5000 10000 15000 20000" (**Supplemental Figure 20**). Run it.

9.6. Click on **Analyze results**. Two plots will appear (**Figure 13**) with the evolution of the resolution, in the green line, as the number of particles used in the reconstruction grows. The red line represents the resolution achieved with a reconstruction of aligned Gaussian noise. The resolution improves with the number of particles and a great difference of the reconstruction from particles compared to the one from noise is observed, which is an indicator of having particles with good structural information.

9.7. From the previous results, a fitting of a model in the post-processed volume could be carried out, which would allow discovering the biological structures of the macromolecule.

REPRESENTATIVE RESULTS:

We have used the dataset of the *Plasmodium falciparum* 80S Ribosome (EMPIAR entry: 10028,

EMDB entry: 2660) to conduct the test and, with the Scipion protocol presented in the previous section, a high resolution 3D reconstructed volume of the macromolecule in this particular example has be achieved, beginning with the information gathered by the microscope that consist of very noisy images containing 2D projections in any orientation of the specimen.

The main results obtained after running the whole protocol are presented in **Figure 10, Figure 11,** and **Figure 12**. **Figure 10** represents the obtained 3D volume before post-processing. In **Figure 10a**, an FSC of 3 Å can be seen, that it is very close to the Nyquist limit (with data with a pixel size of 1.34 Å, the Nyquist limit is 2.6 Å). **Figure 10b** shows some slices of the reconstructed 3D volume with high levels of details and well-defined structures. In **Figure 11** the results after locally analyze the resolution of the obtained 3D volume are presented. It can be seen that most of the voxels in the structure achieve a resolution below 3 Å, mainly those located in the central part of the structure. However, the outer part shows worse resolutions, what is consistent with the blurring appearing in those areas in the slices of **Figure 10b. Figure 12** shows the same 3D map after post-processing that is able to highlight the higher frequencies of the volume, revealing more details and improving the representation, which can be seen especially in the 3D presentation in **Figure 12c**.

In **Figure 14**, Chimera[26] was used to see a 3D representation of the obtained volume (**Figure 14a**), the post-processed (**Figure 14b**), and the resolution map (**Figure 14c**), colored with the color code of the local resolutions. This can give even more information about the obtained structure. This tool is very useful to gain an insight into the quality of the obtained volume, as very small details in the whole 3D context of the structure can be seen. When the achieved resolution is enough, even some biochemical parts of the structure can be found (e.g., alpha-helices in **Figure 14d**. In this figure, it must be highlighted the high resolution achieved in all the central parts of the 3D structure, which can be seen as the dark blue areas in **Figure 14c**.

All the previous results were achieved thanks to a good performance of the whole protocol, but this might be not the case. There are several ways to identify a bad behavior. In the most general case, this happens when the obtained structure has low resolution and it is not able to evolve to a better one. One example of this is presented in **Figure 15**. A blurred volume (**Figure 15c**) results in a low FSC, which can be seen in the FSC curve (**Figure 15a**) and the histogram of the local estimation (**Figure 15b**). This example was generated using a 3D refinement method with incorrect input data, as it was expecting some specific properties in the input set of particles that they do not fulfill. As can be seen, it is always very important to know how the different methods expect to receive the data and prepare it properly. In general, when an output like the one in **Figure 15** is obtained, there might be a problem in the processing workflow or the underlying data.

There are several checkpoints along the workflow that can be analyzed to know if the protocol evolves properly or not. For example, right after picking, several of the methods discussed earlier can rank the particles and give a score for each of them. In the case of having bad particles, these methods allow to identify and remove them. Also, the 2D classification can be a good indicator of having a bad set of particles. **Figure 16** shows an example of such a bad set. In

the **Figure 16a**, good classes containing some details of the structure are shown, while **Figure 16b** shows bad classes, which are noisy or uncentered, in this last case it can be seen that the picking was incorrect and two particles seem to appear together. Another checkpoint is the initial volume estimation, **Figure 17** shows an example of good (**Figure 17a**) and bad (**Figure 17b**) initial estimations. The bad estimation was created using an incorrect setup for the method. It must be taken into account that all the setups should be done carefully, choosing appropriately every parameter according to the data being analyzed. In case of not having a map with some minimal structural information, the following refinement will be unable to obtain a good reconstruction.

When the problem is a bad acquisition, in which the movies do not preserve structural information, it will be impossible to extract good particles from them and get a successful processing. In that case, more movies should be collected to get a high resolution 3D reconstruction. But, if this is not the case, there are several ways to manage problems along the processing workflow. If the picking is not good enough, there are several ways to try to fix it, e.g., repeating the picking, using different methods, or trying to manually pick more particles to help the methods to learn from them. During the 2D classification, if just a few classes are good, consider also to repeat the picking process. In the initial volume estimation, try to use several methods if some of them gave inaccurate results. The same applies to the 3D refinement. Following this reasoning, in this manuscript, several consensus tools have been presented, which could be very useful to avoid problems and continue the processing with accurate data. Thanks to using a consensus among several methods, we can discard data that are difficult to pick, classify, align, etc., which probably is an indicator of poor data. However, if several methods are able to agree in the generated output, probably these data contain valuable information with which to continue processing.

We encourage the reader to download more datasets and try to process them following the recommendations presented in this manuscript and to create a similar workflow combining processing packages using Scipion. Trying to process a dataset is the best way to learn the power of the processing tools available in the state-of-the-art in Cryo-EM, to know the best rules to overcome the possible drawbacks appearing during the processing, and to boost the performance of the available methods in each specific test case.

FIGURE AND TABLE LEGENDS:

Figure 1. Movie alignment result. (a) The main window of the results, with a list of all the micrographs generated and additional information: the power spectral density, the trajectory of the estimated alignment in polar coordinates, the same in cartesian coordinates, the filename of the generated micrograph. **(b)** The alignment trajectory represented in cartesian coordinates. **(c)** The generated micrograph.

Figure 2. CTF estimation with Ctffind result. The main window with the results includes a figure with the estimated PSD (in a corner) along with the PSD coming from the data, and several defocus params.

Figure 3. Manual picking windows with Xmipp. (a) The main window with the list of micrographs to process and some other parameters. **(b)** Manually picking particles inside a region of a micrograph. **(c)** and **(d)** Automatically picked particles to be supervised to create a set of training particles for the Xmipp auto picking method.

Figure 4. Deep consensus picking with Xmipp result. The parameter **zScoreDeepLearning** gives weight to the goodness of a particle and it is key to discovering bad particles. **(a)** The lowest zScores values are associated with artifacts. **(b)** The highest zScores are associated with particles containing the macromolecule.

Figure 5. 2D classification with Cryosparc result. The classes generated (averages of subsets of particles coming from the same orientation) are shown. Several good classes selected in red (with some level of detail) and some bad classes non-selected (noisy and uncentered classes).

Figure 6. 3D initial volume with swarm consensus result. A view of the 3D initial volume obtained after running the consensus tool **xmipp3 – swarm consensus**, using the previous 3D initial volume estimations of Xmipp and Relion. **(a)** The volume is represented by slices. **(b)** 3D visualization of the volume.

Figure 7. Refinement of a 3D initial volume with Relion result. (a) FSC curve obtained, crossing the threshold at a 4.5Å, approximately. **(b)** Angular coverage shown as upper view of the 3D sphere. In this case, as there is no symmetry, the assigned particles should cover the whole sphere. **(c)** Refined volume represented by slices.

Figure 8. 3D alignment based on deep learning with Xmipp result. The results generated by **xmipp3 – deep align** method for 3D alignment. **(a)** The angular assignment for every particle in the form of transformation matrix. **(b)** The angular coverage.

Figure 9. 3D alignment consensus result. (a) List of particles with the obtained differences in shift and angles parameters. **(b)** Plot of the angular differences per particle. **(c)** Plot of the shift difference per particle.

Figure 10. Final iteration of 3D refinement result. (a) FSC curve. **(b)** Obtained volume at full resolution by slices.

Figure 11. Local resolution analysis with Xmipp result. Results of the method **xmipp3 – local MonoRes**. **(a)** Some representative slices colored with the resolution value per voxel, as indicated in the color code. **(b)** Local resolution histogram.

Figure 12. Sharpening with Xmipp result. Results of **xmipp3 – localdeblur sharpening** method. **(a)** List of obtained volumes per iteration. **(b)** 3D volume obtained after the last iteration represented by slices. **(c)** A 3D representation of the final volume.

Figure 13. Validate overfitting tool in Xmipp result. Results of **xmipp3 – validation overfitting**. The green line corresponds to reconstruction from data, the red line from noise. **(a)** Inverse of the squared resolution with the logarithm of the number of particles. **(b)** Resolution with the number of particles.

Figure 14. Several 3D representations of the obtained volume. (a) Pre-processed volume. **(b)** Post-processed volume. **(c)** Local resolution, dark blue voxels are those with higher resolution (2.75Å) and dark red voxels are those with lower resolution (10.05Å). **(d)** Zoom in the post-processed volume where an alpha-helix (red oval) can be seen.

Figure 15. Example of a bad 3D reconstruction. (a) FSC curve with a sharp fall and crossing the threshold at low resolution. **(b)** Local resolution histogram. **(c)** 3D volume by slices.

Figure 16. Example of 2D classes. (a) Good classes showing some level of detail. **(b)** Bad classes containing noise and artifacts (upper part obtained with Xmipp, lower with CryoSparc).

Figure 17. Example of 3D initial volume with different qualities. (a) Good initial volume where the shape of the macromolecule can be observed. **(b)** Bad initial volume where the obtained shape is completely different from the expected one.

SUPPLEMENTARY FILES:

Supplemental Figure 1. Creating a Scipion project. Window displayed by Scipion where an old project can be selected or a new one can be created giving a name and a location for that project.

Supplemental Figure 2. Import movies method. Window displayed by Scipion when **pwem - import movies** is open. Here, the main acquisition parameters must be included to let the movies available to be processed in Scipion.

Supplemental Figure 3. Movie alignment method. Window displayed by Scipion when **xmipp3 – optical alignment** is used. The input movies, the range of frames considered for alignment, and some other parameters to process the movies should be filled.

Supplemental Figure 4. CTF estimation method with Ctffind. The form in Scipion with all the necessary fields to run the program Ctffind.

Supplemental Figure 5. Wizard in Scipion. A wizard to help the user filling some parameters in the form. In this case, the wizard is to complete the resolution field in the **grigoriefflab – ctffind** method.

Supplemental Figure 6. CTF refinement method with Xmipp. The form of **xmipp3 – ctf estimation** with all the parameters to make a refinement of a previously estimated CTF.

Supplemental Figure 7. Preprocess micrographs method. The form of **xmipp3 – preprocess**

micrographs that allows carrying out some operations over them. In this example, **Remove bad pixels** and **Downsample micrographs** is the useful one.

Supplemental Figure 8. Picking method with Cryolo. The form to run the Cryolo picking method using a pretrained network.

Supplemental Figure 9. Consensus picking method with Xmipp. The form of **xmipp3 – deep consensus picking** based on deep learning to calculate a consensus of coordinates, using a pretrained network over several sets of coordinates obtained with different picking methods.

Supplemental Figure 10. Extract particles method. Input and **preprocess** tabs of **xmipp3 – extract particles**.

Supplemental Figure 11. 3D initial volume method with Xmipp. The form of the method **xmipp3 – reconstruct significant** to obtain an initial 3D map. The **Input** and **Criteria** tabs are shown.

Supplemental Figure 12. Resize volume method. The form to make a crop or resize of a volume. In this example, this method is used to generate a full size volume after **xmipp3 – reconstruct significant**.

Supplemental Figure 13. 3D initial volume with Relion result. A view of the obtained 3D initial volume with **relion – 3D initial model** method by slices.

Supplemental Figure 14. Refinement of the initial volume with Relion. The form of the method **relion – 3D auto-refine**. In this example, it was used to refine an initial volume estimated after consensus. The **Input** and **Reference 3D map** tabs are shown.

Supplemental Figure 15. 3D classification method. Form of **relion – 3D classification**. The tabs **Input**, **Reference 3D map**, and **Optimisation** are shown.

Supplemental Figure 16. 3D alignment based on a deep learning method. The form opened for the method **xmipp3 – deep align**. Here it is necessary to train a network with a training set, then that network will predict the angular assignment per particle.

Supplemental Figure 17. 3D refinement method. Form of the **xmipp3 – highres** method. Tabs **Input** and **Angular assignment** are shown.

Supplemental Figure 18. First iteration of 3D refinement result. (a) FSC curve. **(b)** Obtained volume (of a smaller size than the full resolution) represented as slices.

Supplemental Figure 19. First iteration of 3D refinement correlation analysis. A new window appears by clicking on the bar icon in the upper part of the window with the list of particles. In **Plot columns** window a histogram of the desired estimated parameter can be created.

Supplemental Figure 20. Validation overfitting tool. Form of **xmipp3 – validate overfitting** method.

DISCUSSION:

Currently, cryo-EM is a key tool to reveal the 3D structure of biological samples. When good data is collected with the microscope, the available processing tools will allow us to obtain a 3D reconstruction of the macromolecule under study. Cryo-EM data processing is able to achieve near-atomic resolution, which is key to understanding the functional behavior of a macromolecule and is also crucial in drug discovery.

Scipion is a software that allows creating the whole workflow combining the most relevant image processing packages in an integrative way, which helps the traceability and reproducibility of the entire image-processing workflow. Scipion provides a very complete set of tools to carry out the processing; however, obtaining high resolutions reconstructions depends completely on the quality of the acquired data and how these data is processed.

To get a high resolution 3D reconstruction, the first requirement is to obtain good movies from the microscope, which preserve structural information to high resolution. If this is not the case, the workflow will not be able to extract high definition information from the data. Then, a successful processing workflow should be able to extract particles that really correspond to the structure and to find the orientations of these particles in the 3D space. If any of the steps in the workflow fails, the quality of the reconstructed volume will be degraded. Scipion allows for using different packages in any of the processing steps, which helps to find the most adequate approach to process the data. Moreover, thanks to having many packages available, consensus tools, that boost the accuracy by finding an agreement in the estimated outputs of different methods, can be used. Also, it has been discussed in detail in the Representative Results section several validation tools and how to identify accurate and inaccurate results in every step of the workflow, to detect potential problems, and how to try to solve them. There are several checkpoints along the protocol that could help to realize if the protocol is running properly or not. Some of the most relevant are: picking, 2D classification, initial volume estimation, and 3D alignment. Checking the inputs, repeating the step with a different method, or using consensus, are options available in Scipion that the user can use to find solutions when issues appear.

Regarding the previous approaches to package integration in the Cryo-EM field, Appion[31] is the only one that allows real integration of different software packages. However, Appion is tightly connected with Leginon[32], a system for automated collection of images from electron microscopes. The main difference with Scipion is that data model and storage are less coupled. In such a way, to create a new protocol in Scipion, only a Python script needs to be developed. However, in Appion, the developer must write the script and change the underlying database. In summary, Scipion was developed to simplify maintenance and extensibility.

We have presented in this manuscript a complete workflow for Cryo-EM processing, using the real case dataset of the *Plasmodium falciparum* 80S Ribosome (EMPIAR entry: 10028, EMDB

entry: 2660). The steps covered and discussed here can be summarized as movie alignment, CTF estimation, particle picking, 2D classification, initial map estimation, 3D classification, 3D refinement, evaluation, and post-processing. Different packages have been used and consensus tools were applied in several of these steps. The final 3D reconstructed volume achieved a resolution of 3 Å and, in the post-processed volume, some secondary structures can be distinguished, like alpha-helices, which helps to describe how atoms are arranged in space.

The workflow presented in this manuscript shows how Scipion can be used to combine different Cryo-EM packages in a straightforward and integrative way to simplify the processing, and obtain more reliable result at the same time.

In the future, the development of new methods and packages will keep growing and software like Scipion to easily integrate all of them will be even more important for the researchers. Consensus approaches will be more relevant even then, when plenty of methods with different basis will be available, helping to obtain more accurate estimations of all the parameters involve in the reconstruction process in Cryo-EM. Tracking and reproducibility are key in the research process and easier to achieve with Scipion thanks to having a common framework for the execution of complete workflows.

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DISCLOSURES:

The authors have nothing to disclose.

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TITLE:
Cryo-EM and Single-Particle Analysis with Scipion

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Cryo-electron microscopy, single particle analysis, Scipion, analysis software, image processing, integration, traceability, processing workflow

SUMMARY:
Single-particle analysis in Cryo-electron microscopy is one of the main techniques used to determine the structure of biological ensembles at high resolution. Scipion provides the tools to create the whole pipeline to process the information acquired by the microscope and achieve a 3D reconstruction of the biological specimen.

ABSTRACT:
Cryo-electron microscopy has become one of the most important tools in biological research to reveal the structural information of macromolecules at near-atomic resolution. In single-particle analysis, the vitrified sample is imaged by an electron beam and the detectors at the end of the microscope column produce movies of that sample. These movies contain thousands of images of identical particles in random orientations. The data need to go through an image processing workflow with multiple steps to obtain the final 3D reconstructed volume. The goal

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of the image processing workflow is to identify the acquisition parameters to be able to reconstruct the specimen under study. Scipion provides all the tools to create this workflow using several image processing packages in an integrative framework, also allowing the traceability of the results. In this article the whole image processing workflow in Scipion is presented and discussed with data coming from a real test case, giving all the details necessary to go from the movies obtained by the microscope to a high resolution final 3D reconstruction. Also, the power of using consensus tools that allow combining methods, and confirming results along every step of the workflow, improving the accuracy of the obtained results, is discussed.

INTRODUCTION:

In Cryo-Electron Microscopy (Cryo-EM), Single Particle Analysis (SPA) of vitrified frozen-hydrated specimens is one of the most widely used and successful variants of imaging for biological macromolecules, as it allows to understand molecular interactions and the function of biological ensembles[1]. This is thanks to the recent advances in this imaging technique that gave rise to the "resolution revolution"[2] and have allowed the successful determination of biological 3D structures with near-atomic resolution. Currently, the highest resolution achieved in SPA Cryo-EM was 1.15Å for apoferritin[3] (EMDB entry: 11668). These technological advances comprise improvements in the sample preparation[4], the image acquisition[5], and the image processing methods[6]. This article is focused on this last point.

Briefly, the goal of the image processing methods is to identify all the acquisition parameters to invert the imaging process of the microscope and recover the 3D structure of the biological specimen under study. These parameters are the gain of the camera, the beam-induced movement, the aberrations of the microscope (mainly the defocus), the 3D angular orientation and translation of each particle, and the conformational state in case of having a specimen with conformational changes. However, the number of parameters is very high and Cryo-EM requires using low-dose images to avoid radiation damage, which significantly reduces the Signal-to-Noise Ratio (SNR) of the acquired images. Thus, the problem cannot be unequivocally solved and all the parameters to be calculated only can be estimations. Along the image processing workflow, the correct parameters should be identified, discarding the remaining ones to finally obtain a high-resolution 3D reconstruction.

The data generated by the microscope are gathered in frames. Simplifying, a frame contains the number of electrons that have arrived at a particular position (pixel) in the image, whenever electron-counting detectors are used. In a particular field of view, several frames are collected and this is called a movie. As low electron doses are used to avoid radiation damage that could destroy the sample, the SNR is very low and the frames corresponding to the same movie need to be averaged to obtain an image revealing structural information about the sample. However, not only a simple average is applied, the sample can suffer shifts and other kinds of movements during the imaging time due to the beam-induced movement that need to be compensated. The shift-compensated and averaged frames originate a micrograph.

Once the micrographs are obtained, we need to estimate the aberrations introduced by the microscope for each of them, called Contrast Transfer Function (CTF), which represents the changes in the contrast of the micrograph as a function of frequency. Then, the particles can be selected and extracted, which is called particle picking. Every particle should be a small image containing only one copy of the specimen under study. There are three families of algorithms for particle picking: 1) the ones that only use some basic parameterization of the appearance of the particle to find them in the whole set of micrographs, e.g. particle size, 2) the ones that learn how the particles look like from the user or a pretrained set, and 3) the ones that use image templates. Each family has different properties that will be shown later.

The extracted set of particles found in the micrographs will be used in a 2D classification process that has two goals: 1) cleaning the set of particles by discarding the subset containing pure noise images, overlapping particles, or other artifacts, and 2) the averaged particles representing each class could be used as initial information to calculate a 3D initial volume.

The 3D initial volume calculation is the next crucial step. The problem of obtaining the 3D structure can be seen as an optimization problem in a multidimensional solution landscape, where the global minimum is the best 3D volume that represents the original structure, but several local minima representing suboptimal solutions can be found, and where it is very easy to get trapped. The initial volume represents the starting point for the searching process, so bad initial volume estimation could prevent us to find the global minimum. From the initial volume, a 3D classification step will help to discover different conformational states and to clean again the set of particles; the goal is to obtain a structurally homogeneous population of particles. After that, a 3D refinement step will be in charge of refining the angular and translation parameters for every particle to get the best 3D volume possible.

Finally, in the last steps, the obtained 3D reconstruction can be sharpened and polished. Sharpening is a process of boosting the high frequencies of the reconstructed volume, and the polishing is a step to further refine some parameters, as CTF or beam-induced movement compensation, at the level of particles. Also, some validation procedures could be used to better understand the achieved resolution at the end of the workflow.

After all these steps, the tracing and docking processes[7] will help to give a biological meaning to the obtained 3D reconstruction, by building atomic models de novo or fitting existing models. If high resolution is achieved, these processes will tell us the positions of the biological structures, even of the different atoms, in our structure.

Scipion[8] allows creating the whole workflow combining the most relevant image processing packages in an integrative way. Xmipp[9], Relion[10], CryoSPARC[11], Eman[12], Spider[13], Cryolo[14], Ctffind[15], CCP4[16], Phenix[17], and many more packages can be included in Scipion. Also, it incorporates all the necessary tools to benefit the integration, interoperability, traceability, and

reproducibility to make a full tracking of the entire image-processing workflow[8].

One of the most powerful tools that Scipion allows us to use is the consensus, which means to compare the results obtained with several methods in one step of the processing, making a combination of the information conveyed by different methods to generate a more accurate output. This could help to boost the performance and improve the achieved quality in the estimated parameters. Note that a simpler workflow can be build without the use of consensus methods; however, we have seen the power of this tool[22,25] and the workflow presented in this manuscript will use it in several steps.

All the steps that have been summarized in the previous paragraphs will be explained in detail in the following section and combined in a complete workflow using Scipion. Also, how to use the consensus tools to achieve a higher agreement in the generated outputs will be shown. To that end, the example dataset of the *Plasmodium falciparum* 80S Ribosome has been chosen (EMPIAR entry: 10028, EMDB entry: 2660). The dataset is formed by 600 movies of 16 frames of size 4096x4096 pixels at a pixel size of 1.34Å taken at an FEI POLARA 300 with an FEI FALCON II camera, with a reported resolution at EMDB is 3.2Å[18] .

PROTOCOL:

1. Creating a project in Scipion and importing the data

    1. Open Scipion and click on **Create Project**, specify the name for the project and the location where it will be saved (**Supplemental Figure 1**).

    2. Scipion will open the project window showing a canvas with, on the left side, a panel with a list of available methods, each of them represents one image processing tool that you can use to manage your data.
    NOTE: **Ctrl+F** can be used to find a method if it does not appear in the list.

    3. To import the movies taken by the microscope select the **pwem - import movies** on the left panel (or type it when you press **Ctrl+F**).

    4. A new window will be opened (**Supplemental Figure 2**). There, include the path to the data, and the acquisition parameters. In this example, use the following setup: **Microscope voltage** 300kV, **Spherical aberration** 2.0mm, **Amplitude Contrast** 0.1, **Magnification rate** 50000, **Sampling rate mode** to **From image**, and **Pixel size** 1.34Å. When all the parameters in the form are filled, click on the **Execute** button.
    NOTE: When a method starts, a box appears in the canvas in yellow color labeled as **running**. When a method finishes, the box changes to green, and the label

changes to **finished**. In case of an error during the execution of a method, the box will appear in red, labeled as **failed**. In that case, check the bottom part of the canvas, in the **Output Log** tab an explanation of the error will appear.

5. When the method finishes, check the results in the bottom part of the canvas in the **Summary** tab. Here, the outputs generated by the method are presented, in this case, the set of movies. Click on **Analyze Results** button and a new window will appear with the list of movies.

2. Movie alignment: from movies to micrographs

    1. The method **xmipp3 – optical alignment** which implements Optical flow[19] will be used. The main parameters to fill in the form are the following (**Supplemental Figure 3**): the **Input Movies** are those obtained in step 1, the range in **Frames to ALIGN** is from 2 to 13, the other options stay with the default values. Execute the program.
    NOTE: The parameters in bold in a form, must be always filled. The others will have a default value or will not be obligatorily required.
    NOTE: In the upper part of the form window the fields where the computational resources are distributed can be found, as threads, MPIs, or GPUs.

    2. Click on **Analyze Results** to check the obtained micrographs and the trajectory of the estimated shifts (**Figure 1**). For every micrograph you can see: the power spectral density (PSD), the trajectories obtained to align the movie (one point per frame) in cartesian and polar coordinates, and the file name of the obtained micrograph (clicking on it, the micrograph can be inspected). Notice that the particles of the specimen are much more visible in the micrograph, as compared to a single frame of the movie.

3. CTF estimation: calculating the aberrations of the microscope

    1. First, the method **grigoriefflab – ctffind**[15] will be used. The setup is: the **Input Micrographs** are the output of step 2, the **Manual CTF Downsampling factor** is set to 1.5, and the **Resolution** range goes from 0.06 to 0.42. Moreover, in the **Advanced** options (that can be found by selecting this choice in the **Expert Level** of the form), set the **Window size** to 256. The remaining parameters stay with the default values (**Supplemental Figure 4**).
    NOTE: In most of the methods in Scipion the **Advanced** option shows more configuration parameters. Use these options carefully, when the program to be launched is completely known and the meaning of the parameters is understood.
    NOTE: Some parameters can be difficult to fill without having a look at the data;

in that case, Scipion shows a magic wand on the right side that will show a wizard window (**Supplemental Figure 5**). For example, in the **Resolution** field of this form is especially useful, as these values should be selected to approximately cover the region from the first zero to the last noticeable ring of the PSD.

2. Click on **Execute** and on **Analyze Results** (**Figure 2**) when the method finishes. Check that the estimated CTF matches with the experimental one. To that end, look at the PSD and compare the estimated rings in the corner with the ones coming from the data. You can check also the obtained defocus values to find any unexpected values and respective micrographs can be discarded or recalculated. In this example, the whole set of micrographs can be used.
   NOTE: Use the buttons in the bottom part of the window to make a subset of micrographs (with **Micrographs** red button) and to recalculate a CTF (with **Recalculate CTFs** red button), in case of needing.

3. To refine the previous estimation **xmipp3 – ctf estimation**[20] will be used. Select as **Input Micrographs** the output of step 2, select the option **Use defoci from a previous CTF estimation**, as **Previous CTF estimation** choose the output of **grigoriefflab – ctffind**, and, in the **Advanced** level, change the **Window size** to 256 (**Supplemental Figure 6**). Run it.

4. Click on **Analyze Results** to check the obtained CTFs. With this method, more data is estimated and represented in some extra columns. As none of them show incorrect estimated values, all the micrographs will be used in the following steps.

4. Particle picking: finding particles in the micrographs

1. Before starting the picking, a preprocess of the micrographs will be carried out. Open **xmipp3 – preprocess micrographs**, set as **Input micrographs** those obtained in step 2 and select the options **Remove bad pixels?** with **Multiple of Stddev** to 5, and **Downsample micrographs?** with a **Downsampling factor** of 2 (**Supplemental Figure 7**). Click on **Execute** and check that the size of the resulting micrographs has been reduced.

2. For the picking use **xmipp3 – manual-picking (step 1)** and **xmipp3 – auto-picking (step 2)**[21]. The manual picking allows to manually prepare a set of particles with which the auto-picking step will learn and generate the complete set of particles. First, run **xmipp3 – manual-picking (step 1)** with **Input Micrographs** as the micrographs obtained in the previous preprocess. Click on **Execute** and a new interactive window will appear (**Figure 3**).

3. In this window a list of the micrographs (**Figure 3 (a)**) and other options is presented. Change **Size (px)** to 150, this will be the size of the box containing each particle. The selected micrograph appears in a bigger window. Choose a region and pick all the visible particles in it (**Figure 3 (b)**). Then, click on **Activate Training** to start the learning. The remaining regions of the micrograph are automatically picked (**Figure 3 (c)**). Check the picked particles and include more by clicking on it, or remove the incorrect ones with shift+clicking, if necessary.

4. Select the next micrograph in the first window. The micrograph will be automatically picked. Check again to include or remove some particles, if necessary. Repeat this step with, approximately, 5 micrographs to create a representative training set.

5. Once this is done, click on **Coordinates** in the main window to save the coordinates of all the picked particles. The training set of particles is ready to go to the auto picking to complete the process for all micrographs.

6. Open **xmipp3 – auto-picking (step 2)** indicating in **Xmipp particle picking run** the previous manual picking, and **Micrographs to pick** as **Same as supervised**. Click on **Execute**. This method will generate as output a set of around 100000 coordinates.

7. A consensus approach is going to be applied, so a second picking method will be carried out to select the particles in which both methods agree. Open **sphire – cryolo picking**[14] and select the preprocessed micrographs as **Input Micrographs**, **Use general model?** to **Yes**, with a **Confidence threshold** of 0.3, and a **Box Size** of 150 (**Supplemental Figure 8**). Run it. This method should generate also around 100000 coordinates.

8. Run **xmipp3 – deep consensus picking**[22]. As **Input coordinates** include the output of **sphire – cryolo picking** (step 4.7) and **xmipp3 – auto-picking** (step 4.6), set **Select model type** to **Pretrained**, and **Skip training and score directly with pretrained model?** To **Yes** (**Supplemental Figure 9**). Run it.

9. Click on **Analyze Results** and, in the new window, on the eye icon next to **Select particles/coordinates with high 'zScoreDeepLearning1' values**. A new window will be opened with a list of all particles (**Figure 4**). The **zScore** values in the column give an insight into the quality of a particle, low values mean bad quality. Click on the label **_xmipp_zScoreDeepLearning** to order the particles from highest to lowest **zScore**. Select the particles with **zScore** higher than 0.75 and click on **Coordinates** to create the new subset. This should create a subset with

approximately 50000 coordinates.

10. Open **xmipp3 – deep micrograph cleaner**. Select as **Input coordinates** the subset obtained in the previous step, **Micrographs source** as **same as coordinates**, and keep **Threshold** at 0.75. Run it. Check in the **Summary** tab that the number of coordinates has been reduced, although in this case, only few coordinates are removed.
    NOTE: This step is able to additionally clean the set of coordinates and could be very useful in cleaning other datasets with more movie artifacts as carbon zones or large impurities.

11. Run **xmipp3 – extract particles** (**Supplemental Figure 10**). Indicate as **Input coordinates** the coordinates obtained after the previous step, **Micrographs source** as **other**, **Input micrographs** as the output of step 2, **CTF estimation** as the output of the **xmipp3 – ctf estimation**, **Downsampling factor** to 3, and **Particle box size** to 100. In the **Preprocess** tab of the form select **Yes** to all. Run it.

12. Check that the output should contain the particles in reduced size of 100x100 pixels and a pixel size of 4.02Å/px.

13. Run again **xmipp3 – extract particles** changing the following parameters: **Downsampling factor** to 1, and **Particle box size** to 300. Check that the output is the same set of particles but now at the full resolution.

5. 2D classification: grouping similar particles together

    1. Open the method **cryosparc2 – 2d classification**[11] with **Input particles** as those obtained in step 4.11 and, in the **2D Classification** tab, the **Number of classes** to 128, keep all the other parameters with the default values. Run it.

    2. Click on **Analyze Results** and then on the eye icon next to **Display particle classes with Scipion** (**Figure 5**). This classification will help us to clean our set of particles, as several classes will appear noisy or with artifacts. Select the classes containing good views. Click on **Particles** (red button in the lower part of the window) to create the cleaner subset.

    3. Now, open **xmipp3 – cl2d**[23] and set as **Input images** the images obtained in the previous step and **Number of classes** as 128. Click on **Execute**.
        NOTE: This second classification is used as additional cleaning step of the set of particles. Usually is useful to remove as much noisy particles as it is possible. However, if a simpler workflow is desired, only one 2D classification method can

be used.

4. When the method finishes, check the 128 generated classes by clicking on **Analyze Results** and on **What to show: classes**. Most of the generated classes show a projection of the macromolecule with some level of detail. However, some of them appear noisy (in this example approximately 10 classes). Select all the good classes and click on the **Classes** button to generate a new subset with only the good ones. This subset will be used as input to one of the methods to generate an initial volume. With the same selected classes click on **Particles** to create a cleaner subset after removing those belonging to the bad classes.

5. Open **pwem – subset** with **Full set of items** as the output of 4.13 (all particles at the full size), **Make random subset** to **No**, **Other set** as the subset of particles created in the previous step, and **Set operation** as **intersection**. This will extract the previous subset from the particles at full resolution.

6. Initial volume estimation: building the first guess of the 3D volume

    1. In this step two initial volumes will be estimated with different methods and then, a consensus tool will generate the final estimated 3D volume. Open **xmipp3 – reconstruct significant**[24] method with **Input classes** as those obtained after step 5, **Symmetry group** as c1, and keep the remaining parameters with their default values (**Supplemental Figure 11**). Execute it.

    2. Click on **Analyze Results**. Check that a low resolution volume of size 100x100x100 pixels and a pixel size of 4.02Å/px is obtained.

    3. Open **xmipp3 – crop/resize volumes** (**Supplemental Figure 12**) using as **Input Volumes** the one obtained in the previous step, **Resize volumes?** to **Yes**, **Resize option** to **Sampling Rate**, and **Resize sampling rate** to 1.34Å/px. Run it. Check in the **Summary** tab that the output volume has the correct size.

    4. Now, the second initial volume will be created. Open **relion – 3D initial model**[10], as **Input particles** use the good particles at full resolution (output of 5.5) and set **Particle mask diameter** to 402Å, keep the remaining parameters with the default values. Run it.

    5. Click on **Analyze Results** and then in **Display volume with: slices**. Check that a low resolution volume but with the main shape of the structure is obtained (**Supplemental Figure 13**).

    6. Now, open **pwem – join sets** to combine the two generated initial volumes to

create the input to the consensus method. Just indicate **Volumes** as **Input type** and select the two initial volumes in **Input set**. Run it. The output should be a set containing two items with both volumes.

7. The consensus tool is the one included in **xmipp3 – swarm consensus**[25]. Open it. Use as **Full-size Images** the good particles at full resolution (output of 5.5), as **Initial volumes** the set with two items generated in the previous step, and be sure that **Symmetry group** is c1. Click on **Execute**.

8. Click on **Analyze Results.** Check that a more detailed output volume is obtained (**Figure 6**). Although there is more noise surrounding the structure, to have more details in the structure map will help the following refinement steps to avoid local minima.
   NOTE: If UCSF Chimera[26] is available, use the last icon in the upper part of the window to make a 3D visualization of the obtained volume.

9. Open and execute **relion – 3D auto-refine**[10] to make a first 3D angular assignment of the particles. Select as **Input particles** the output of 5.5, and set **Particle mask diameter** to 402Å. In **Reference 3D map** tab, select as **Input volume** the one obtained in the previous step, **Symmetry** as c1, and **Initial low-pass filter** to 30Å (**Supplemental Figure 14**).

10. Click on **Analyze Results**. In the new window select **final** as **Volume to visualize** and click on **Display volume with: slices** to see the obtained volume. Check also the Fourier shell correlation (FSC) by clicking on **Display resolution plots** in the results window and the angular coverage in **Display angular distribution: 2D plot** (**Figure 7**). The reconstructed volume contains much more details (probably with some blurred areas in the outer part of the structure), and the FSC crosses the threshold of 0.143 around 4.5Å. The angular coverage covers the whole 3D sphere.

7. 3D classification: discovering conformational states

1. Using a consensus approach, if different conformational states are in the data can be discovered. Open **relion – 3D classification**[10] (**Supplemental Figure 15**). As **Input particles** use those just obtained in 6.10, and set **Particle mask diameter** to 402Å. In the **Reference 3D map** tab, use as **Input volume** the one obtained after step 6.10, set **Symmetry** to c1, and **Initial low-pass filter** to 15Å. Finally, in **Optimization** tab, set the **Number of classes** to 3. Run it.

2. Check the results by clicking on **Analyze Results**, select **Show classification in Scipion**. The three generated classes and some interesting measures are shown.

The first two classes should have a similar number of assigned images (**size** column) and look very similar, whilst the third one has fewer images and a more blurred appearance. Also, the **rlnAccuracyRotations** and **rlnAccuracyTranslations** should be clearly better for the first two classes. Select the two best classes and click on the **Classes** button to generate a subset containing them.

3. Repeat steps 7.1 and 7.2 to generate a second group of good classes. Both will be the input of the consensus tool.

4. Open and run **xmipp3 – consensus classes 3D** and select as **Input Classes** the two subsets generated in the previous steps.

5. Click on **Analyze Results**. The number of coincident particles between classes is presented, i.e., the first value is the number of coincident particles in the first class of subset 1 and the first class of subset 2, the second value is the number of coincident particles in the first class of subset 1 and the second class of subset 2, etc. Check that the particles are randomly assigned to classes one or two, which means that the 3D classification method is not able to find conformational changes. Given this result, the whole set of particles will be used to continue processing.

8. 3D refinement: refining angular assignments of a homogeneous population

    1. Again, a consensus approach will be applied in this step. First, open and run **pwem – subset** with **Full set of items** as the output of 6.9, **Make random subset** to **Yes**, and **Number of elements** to 5000. With this, a subset of images with a previous alignment to train the method used in the following step is created.

    2. Open **xmipp3 – deep align**, set **Input images** as the output of good particles obtained in 5.5, **Volume** as the one obtained after 6.10, **Input training set** as the one created in the previous step, **Target resolution** to 10Å, and keep the remaining parameters with the default values (**Supplemental Figure 16**). Click on **Execute**.

    3. Click on **Analyze Results** to check the obtained angular distribution, where there are no missing directions and the angular coverage slightly improves compared to the one of 6.10 (**Figure 8**).

    4. Open and execute **xmipp3 – compare angles** and select as **Input particles 1** the output of 6.9 and **Input particles 2** the output of 8.2, make sure that the **Symmetry group** is c1. This method calculates the agreement between **xmipp3 –**

deep align and **relion – 3D auto refine**.

5. Click on **Analyze Results**, the list of particles, with the obtained differences in shifts and angles, is shown. Click on the bar icon in the upper part of the window, another window will be opened that allows making plots of the calculated variables. Select **_xmipp_angleDiff** and click on **Plot** to see a representation of the angular differences per particle. Do the same with **_xmipp_shiftDiff**. In these figures, approximately in half of the particles both methods agree (**Figure 9**). Select the particles with angular differences lower than 10º and create a new subset.

6. Now, open **xmipp3 – highres**[27] to make a local refinement of the assigned angles. First, select as **Full-size Images** the images obtained in the previous step, and as **Initial volumes** the output of 6.9, set **Radius of particle** to 150 pixels, and **Symmetry group** as c1. In the **Angular assignment tab**, set the **Image alignment** to **Local**, **Number of iterations** to 1, and **Max. Target Resolution** as 5Å/px (**Supplemental Figure 17**). Run it.

7. In the **Summary** tab check that the output volume is smaller than 300x300x300 pixels and with slightly higher pixel size.

8. Click on **Analyze Results** to see the obtained results. Click on **Display resolution plots** to see the FSC, and on **Display volume: Reconstructed** to see the obtained volume (**Supplemental Figure 18**). A good resolution volume close to 4-3.5Å is obtained.

9. Click on **Display output particles** and, in the window with the list of particles, click on the bar icon. In the new window, select **Type** as **Histogram**, with 100 **Bins**, select **_xmipp_cost** label, and finally press **Plot** (**Supplemental Figure 19**). This way, the histogram of the **cost** label is presented, which contains the correlation of the particle with the projection direction selected for it. In this case, a unimodal density function is obtained, which is a sign of not having different populations in the set of particles. Thus all of them will be used to continue the refinement
   NOTE: In case of seeing a multimodal density function, the set of particles belonging to the higher maximum should be selected to continue the workflow only with them.

10. Open and execute again **xmipp3 – highres** with **Continue from a previous run?** to **Yes**, set as **Full-size Images** those obtained after 8.5, and **Select previous run** with the previous execution of Xmipp Highres. In the **Angular assignment** tab, set the **Image alignment** to **Local**, with 1 iteration and 2.6Å/px as target resolution

(full resolution).

11. Now the output should contain a volume at full resolution (size 300x300x300 pixels). Click on **Analyze Results** to check again the obtained volume and the FSC, which now should be a high resolution volume at around 3Å (**Figure 10**).

9. Evaluation and post-processing

1. Open **xmipp3 – local MonoRes**[28]. This method will calculate the resolution locally. Set as **Input Volume** the one obtained after 8.10, set **Would you like to use half volumes?** to **Yes**, and **Resolution Range** from 1 to 10Å. Run it.

2. Click on **Analyze Results** and select **Show resolution histogram** and **Show colored slices** (**Figure 11**). The resolution in the different parts of the volume is shown. Most of the voxels of the central part of the structure should present resolutions around 3Å, whilst the worst resolutions are achieved in the outer parts. Also, a histogram of the resolutions per voxel is shown with a peak around (even below) 3Å.

3. Open and run **xmipp3 – localdeblur sharpening**[29] to apply a sharpening. Select as **Input Map** the one obtained in 8.10, and as **Resolution Map** the one obtained in the previous step with MonoRes.

4. Click on **Analyze Results** to check the obtained volumes. Open the last one, corresponding to the last iteration of the algorithm. It is recommend opening the volume with other tools, such as UCSF Chimera[26], to see better the features of the volume in 3D (**Figure 12**).

5. Finally, open the validation tool included in **xmipp3 – validate overfitting**[30] that will show how the resolution changes with the number of particles. Open it and include as **Input particles** the particles obtained in step 8.5, set **Calculate the noise bound for resolution?** to **Yes**, with **Initial 3D reference volume** as the output of 8.10. In **Advanced** options, set the **Number of particles** to "500 1000 1500 2000 3000 5000 10000 15000 20000" (**Supplemental Figure 20**). Run it.

6. Click on **Analyze results**. Two plots will appear (**Figure 13**) with the evolution of the resolution, in the green line, as the number of particles used in the reconstruction grows. The red line represents the resolution achieved with a reconstruction of aligned Gaussian noise. The resolution improves with the number of particles and a great difference of the reconstruction from particles compared to the one from noise is observed, which is an indicator of having particles with good structural information.

7. From the previous results, a fitting of a model in the post-processed volume could be carried out, which would allow discovering the biological structures of the macromolecule.

REPRESENTATIVE RESULTS:

We have used the dataset of the *Plasmodium falciparum* 80S Ribosome (EMPIAR entry: 10028, EMDB entry: 2660) to conduct our test and, with the Scipion protocol presented in the previous section, a high resolution 3D reconstructed volume of the macromolecule in this particular example has be achieved, beginning with the information gathered by the microscope that consist of very noisy images containing 2D projections in any orientation of the specimen.

The main results obtained after running the whole protocol are presented in Figures 10, 11, and 12. Figure 10 represents the obtained 3D volume before post-processing. In part (a) of the figure, an FSC of 3Å can be seen, that it is very close to the Nyquist limit (with data with a pixel size of 1.34Å, the Nyquist limit is 2.6Å). Part (b) of the figure shows some slices of the reconstructed 3D volume with high levels of details and well-defined structures. In Figure 11 the results after locally analyze the resolution of the obtained 3D volume are presented. It can be seen that most of the voxels in the structure achieve a resolution below 3Å, mainly those located in the central part of the structure. However, the outer part shows worse resolutions, what is consistent with the blurring appearing in those areas in the slices of Figure 10 (b). Figure 12 shows the same 3D map after post-processing that is able to highlight the higher frequencies of the volume, revealing more details and improving the representation, which can be seen especially in the 3D presentation of part (c) of the figure.

In Figure 14, Chimera[26] was used to see a 3D representation of the obtained volume (a), the post-processed (b), and the resolution map (c), colored with the color code of the local resolutions. This can give even more information about the obtained structure. This tool is very useful to gain an insight into the quality of the obtained volume, as very small details in the whole 3D context of the structure can be seen. When the achieved resolution is enough, even some biochemical parts of the structure can be found, e.g. alpha-helices (d). In this figure, it must be highlighted the high resolution achieved in all the central parts of the 3D structure, which can be seen as the dark blue areas in part (c) of the figure.

All the previous results were achieved thanks to a good performance of the whole protocol, but this might be not the case. There are several ways to identify a bad behavior. In the most general case, this happens when the obtained structure has low resolution and it is not able to evolve to a better one. One example of this is presented in Figure 15. A blurred volume (c) results in a low FSC, which can be seen in the FSC curve (a) and the histogram of the local estimation (b). This example was generated using a 3D refinement method with incorrect input

data, as it was expecting some specific properties in the input set of particles that they do not fulfill. As can be seen, it is always very important to know how the different methods expect to receive the data and prepare it properly. In general, when an output like the one in Figure 15 is obtained, there might be a problem in the processing workflow or the underlying data.

There are several checkpoints along the workflow that can be analyzed to know if the protocol evolves properly or not. For example, right after picking, several of the methods discussed earlier are able to rank the particles and give a score for each of them. In the case of having bad particles, these methods allow to identify and remove them. Also, the 2D classification can be a good indicator of having a bad set of particles. Figure 16 shows an example of such a bad set. In the part (a) of the figure, good classes containing some details of the structure are shown, while part (b) shows bad classes, which are noisy or uncentered, in this last case it can be seen that the picking was incorrect and two particles seem to appear together. Another checkpoint is the initial volume estimation, Figure 17 shows an example of good (a) and bad (b) initial estimations. The bad estimation was created using an incorrect setup for the method. It must be taken into account that all the setups should be done carefully, choosing appropriately every parameter according to the data being analyzed. In case of not having a map with some minimal structural information, the following refinement will be unable to obtain a good reconstruction.

When the problem is a bad acquisition, in which the movies do not preserve structural information, it will be impossible to extract good particles from them and get a successful processing. In that case, more movies should be collected to get a high resolution 3D reconstruction. But, if this is not the case, there are several ways to manage problems along the processing workflow. If the picking is not good enough, there are several ways to try to fix it, e.g., repeating the picking, using different methods, or trying to manually pick more particles to help the methods to learn from them. During the 2D classification, if just a few classes are good, consider also to repeat the picking process. In the initial volume estimation, try to use several methods if some of them gave inaccurate results. The same applies to the 3D refinement. Following this reasoning, in this manuscript, several consensus tools have been presented, which could be very useful to avoid problems and continue the processing with accurate data. Thanks to using a consensus among several methods, we can discard data that are difficult to pick, classify, align, etc., which probably is an indicator of poor data. However, if several methods are able to agree in the generated output, probably these data contain valuable information with which to continue processing.

We encourage the reader to download more datasets and try to process them following the recommendations presented in this manuscript and to create a similar workflow combining processing packages using Scipion. Trying to process a dataset is the best way to learn the power of the processing tools available in the state-of-the-art in Cryo-EM, to know the best rules to overcome the possible drawbacks appearing during the processing, and to boost the performance of the available methods in each specific test case.

FIGURE AND TABLE LEGENDS:

Figure 1. Movie alignment result. (a) The main window of the results, with a list of all the micrographs generated and additional information: the power spectral density, the trajectory of the estimated alignment in polar coordinates, the same in cartesian coordinates, the filename of the generated micrograph. **(b)** The alignment trajectory represented in cartesian coordinates. **(c)** The generated micrograph.

Figure 2. CTF estimation with Ctffind result. The main window with the results includes a figure with the estimated PSD (in a corner) along with the PSD coming from the data, and several defocus params.

Figure 3. Manual picking windows with Xmipp. (a) The main window with the list of micrographs to process and some other parameters. **(b)** Manually picking particles inside a region of a micrograph. **(c)** and **(d)** Automatically picked particles to be supervised to create a set of training particles for the Xmipp auto picking method.

Figure 4. Deep consensus picking with Xmipp result. The parameter **zScoreDeepLearning** gives weight to the goodness of a particle and it is key to discovering bad particles. **(a)** The lowest zScores values are associated with artifacts. **(b)** The highest zScores are associated with particles containing the macromolecule.

Figure 5. 2D classification with Cryosparc result. The classes generated (averages of subsets of particles coming from the same orientation) are shown. Several good classes selected in red (with some level of detail) and some bad classes non-selected (noisy and uncentered classes).

Figure 6. 3D initial volume with swarm consensus result. A view of the 3D initial volume obtained after running the consensus tool **xmipp3 – swarm consensus**, using the previous 3D initial volume estimations of Xmipp and Relion. **(a)** The volume is represented by slices. **(b)** 3D visualization of the volume.

Figure 7. Refinement of a 3D initial volume with Relion result. (a) FSC curve obtained, crossing the threshold at a 4.5Å, approximately. **(b)** Angular coverage shown as upper view of the 3D sphere. In this case, as there is no symmetry, the assigned particles should cover the whole sphere. **(c)** Refined volume represented by slices.

Figure 8. 3D alignment based on deep learning with Xmipp result. The results generated by **xmipp3 – deep align** method for 3D alignment. **(a)** The angular assignment for every particle in the form of transformation matrix. **(b)** The angular coverage.

Figure 9. 3D alignment consensus result. (a) List of particles with the obtained differences in shift and angles parameters. **(b)** Plot of the angular differences per particle. **(c)** Plot of the shift difference per particle.

Figure 10. Final iteration of 3D refinement result. (a) FSC curve. **(b)** Obtained volume at full resolution by slices.

Figure 11. Local resolution analysis with Xmipp result. Results of the method **xmipp3 – local MonoRes**. **(a)** Some representative slices colored with the resolution value per voxel, as indicated in the color code. **(b)** Local resolution histogram.

Figure 12. Sharpening with Xmipp result. Results of **xmipp3 – localdeblur sharpening** method. **(a)** List of obtained volumes per iteration. **(b)** 3D volume obtained after the last iteration represented by slices. **(c)** A 3D representation of the final volume.

Figure 13. Validate overfitting tool in Xmipp result. Results of **xmipp3 – validation overfitting**. The green line corresponds to reconstruction from data, the red line from noise. **(a)** Inverse of the squared resolution with the logarithm of the number of particles. **(b)** Resolution with the number of particles.

Figure 14. Several 3D representations of the obtained volume. (a) Pre-processed volume. **(b)** Post-processed volume. **(c)** Local resolution, dark blue voxels are those with higher resolution (2.75Å) and dark red voxels are those with lower resolution (10.05Å). **(d)** Zoom in the post-processed volume where an alpha-helix (red oval) can be seen.

Figure 15. Example of a bad 3D reconstruction. (a) FSC curve with a sharp fall and crossing the threshold at low resolution. **(b)** Local resolution histogram. **(c)** 3D volume by slices.

Figure 16. Example of 2D classes. (a) Good classes showing some level of detail. **(b)** Bad classes containing noise and artifacts (upper part obtained with Xmipp, lower with CryoSparc).

Figure 17. Example of 3D initial volume with different qualities. (a) Good initial volume where the shape of the macromolecule can be observed. **(b)** Bad initial volume where the obtained shape is completely different from the expected one.

SUPPLEMENTARY FILES:

Supplemental Figure 1. Creating a Scipion project. Window displayed by Scipion where an old project can be selected or a new one can be created giving a name and a location for that project.

Supplemental Figure 2. Import movies method. Window displayed by Scipion when **pwem - import movies** is open. Here, the main acquisition parameters must be included to let the movies available to be processed in Scipion.

Supplemental Figure 3. Movie alignment method. Window displayed by Scipion when **xmipp3 – optical alignment** is used. The input movies, the range of frames considered for alignment, and some other parameters to process the movies should be filled.

Supplemental Figure 4. CTF estimation method with Ctffind. The form in Scipion with all the necessary fields to run the program Ctffind.

Supplemental Figure 5. Wizard in Scipion. A wizard to help the user filling some parameters in the form. In this case, the wizard is to complete the resolution field in the **grigoriefflab – ctffind** method.

Supplemental Figure 6. CTF refinement method with Xmipp. The form of **xmipp3 – ctf estimation** with all the parameters to make a refinement of a previously estimated CTF.

Supplemental Figure 7. Preprocess micrographs method. The form of **xmipp3 – preprocess micrographs** that allows carrying out some operations over them. In this example, **Remove bad pixels** and **Downsample micrographs** is the useful one.

Supplemental Figure 8. Picking method with Cryolo. The form to run the Cryolo picking method using a pretrained network.

Supplemental Figure 9. Consensus picking method with Xmipp. The form of **xmipp3 – deep consensus picking** based on deep learning to calculate a consensus of coordinates, using a pretrained network over several sets of coordinates obtained with different picking methods.

Supplemental Figure 10. Extract particles method. Input and **preprocess** tabs of **xmipp3 – extract particles**.

Supplemental Figure 11. 3D initial volume method with Xmipp. The form of the method **xmipp3 – reconstruct significant** to obtain an initial 3D map. The **Input** and **Criteria** tabs are shown.

Supplemental Figure 12. Resize volume method. The form to make a crop or resize of a volume. In this example, this method is used to generate a full size volume after **xmipp3 – reconstruct significant**.

Supplemental Figure 13. 3D initial volume with Relion result. A view of the obtained 3D initial

volume with **relion – 3D initial model** method by slices.

Supplemental Figure 14. Refinement of the initial volume with Relion. The form of the method **relion – 3D auto-refine**. In this example, it was used to refine an initial volume estimated after consensus. The **Input** and **Reference 3D map** tabs are shown.

Supplemental Figure 15. 3D classification method. Form of **relion – 3D classification**. The tabs **Input**, **Reference 3D map**, and **Optimisation** are shown.

Supplemental Figure 16. 3D alignment based on a deep learning method. The form opened for the method **xmipp3 – deep align**. Here it is necessary to train a network with a training set, then that network will predict the angular assignment per particle.

Supplemental Figure 17. 3D refinement method. Form of the **xmipp3 – highres** method. Tabs **Input** and **Angular assignment** are shown.

Supplemental Figure 18. First iteration of 3D refinement result. (a) FSC curve. **(b)** Obtained volume (of a smaller size than the full resolution) represented as slices.

Supplemental Figure 19. First iteration of 3D refinement correlation analysis. A new window appears by clicking on the bar icon in the upper part of the window with the list of particles. In **Plot columns** window a histogram of the desired estimated parameter can be created.

Supplemental Figure 20. Validation overfitting tool. Form of **xmipp3 – validate overfitting** method.

DISCUSSION:

Currently, Cryo-EM is a key tool to reveal the 3D structure of biological samples. When good data is collected with the microscope, the available processing tools will allow us to obtain a 3D reconstruction of the macromolecule under study. Cryo-EM data processing is able to achieve near-atomic resolution, which is key to understanding the functional behavior of a macromolecule and is also crucial in drug discovery.

Scipion is a software that allows creating the whole workflow combining the most relevant image processing packages in an integrative way, which helps the traceability and reproducibility of the entire image-processing workflow. Scipion provides a very complete set of tools to carry out the processing; however, obtaining high resolutions reconstructions depends completely on the quality of the acquired data and how these data is processed.

To get a high resolution 3D reconstruction, first to obtain good movies from the microscope is required, which preserve structural information to high resolution. If this is not the case, the

workflow will not be able to extract high definition information from the data. Then, a successful processing workflow should be able to extract particles that really correspond to the structure and to find the orientations of these particles in the 3D space. If any of the steps in the workflow fails, the quality of the reconstructed volume will be degraded. Scipion allows for using different packages in any of the processing steps, which helps to find the most adequate approach to process the data. Moreover, thanks to having many packages available, consensus tools, that boost the accuracy by finding an agreement in the estimated outputs of different methods, can be used. Also, it has been discussed in detail in the Representative Results section several validation tools and how to identify accurate and inaccurate results in every step of the workflow, to detect potential problems, and how to try to solve them. There are several checkpoints along the protocol that could help to realize if the protocol is running properly or not. Some of the most relevant are: picking, 2D classification, initial volume estimation, and 3D alignment. Checking the inputs, repeating the step with a different method, or using consensus, are options available in Scipion that the user can use to find solutions when issues appear.

Regarding the previous approaches to package integration in the Cryo-EM field, Appion[31] is the only one that allows real integration of different software packages. However, Appion is tightly connected with Leginon[32], a system for automated collection of images from electron microscopes. So the main difference with Scipion is that data model and storage are less coupled. In such a way, to create a new protocol in Scipion, only a Python script needs to be developed. However, in Appion, the developer must write the script and change the underlying database. In summary, Scipion was developed to simplify maintenance and extensibility.

We have presented in this manuscript a complete workflow for Cryo-EM processing, using the real case dataset of the *Plasmodium falciparum* 80S Ribosome (EMPIAR entry: 10028, EMDB entry: 2660). The steps covered and discussed here can be summarized as movie alignment, CTF estimation, particle picking, 2D classification, initial map estimation, 3D classification, 3D refinement, evaluation, and post-processing. Different packages haven been used and consensus tools were applied in several of these steps. The final 3D reconstructed volume achieved a resolution of 3Å and, in the post-processed volume, some secondary structures can be distinguished, like alpha-helices, which helps to describe how atoms are arranged in space.

The workflow presented in this manuscript shows how Scipion can be used to combine different Cryo-EM packages in a straightforward and integrative way to simplify the processing, and obtain more reliable result at the same time.

In the future, the development of new methods and packages will keep growing and software like Scipion to easily integrate all of them will be even more important for the researchers. Consensus approaches will be more relevant even then, when plenty of methods with different basis will be available, helping to obtain more accurate estimations of all the parameters involve in the reconstruction process in Cryo-EM. Tracking and reproducibility are key in the research process and easier to achieve with Scipion thanks to having a common framework for

the execution of complete workflows.

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DISCLOSURES:
The authors have nothing to disclose.

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TITLE:

~~Processing Workflow in Scipion for Single Particle Data in Cryo Electron Microscopy~~Cryo-EM and Single-Particle Analysis with Scipion

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KEYWORDS:

Cryo-electron microscopy, single particle analysis, Scipion, analysis software, image processing, integration, traceability, processing workflow

SUMMARY:

Single-particle analysis in Cryo-electron microscopy is one of the main techniques used to determine the structure of biological ensembles at high resolution. Scipion ~~software~~ provides the tools to create the whole pipeline to process the information acquired by the microscope and achieve a 3D reconstruction of the biological specimen.

ABSTRACT:

Cryo-electron microscopy has become one of the most important tools in biological research to reveal the structural information of macromolecules at near-atomic resolution. In single-particle analysis, the vitrified sample is imaged by an electron beam and the detectors at the end of the microscope column produce movies of that sample. These movies contain thousands of images of identical particles in random orientations. The data need to go through an image

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processing workflow with multiple steps to obtain the final 3D reconstructed volume. The goal of the image processing workflow is to identify the acquisition parameters to be able to reconstruct the specimen under study. Scipion ~~software~~ provides all the tools to create this workflow using several image processing packages in an integrative framework, also allowing the traceability of the results. In this article the whole image processing workflow in Scipion is presented and discussed with data coming from a real test case, giving all the details necessary to go from the movies obtained by the microscope to a high resolution final 3D reconstruction. Also, ~~we discuss~~ the power of using consensus tools that allow combining methods, and confirming results along every step of the workflow, improving the accuracy of the obtained results, is discussed.

INTRODUCTION:

In Cryo-Electron Microscopy (Cryo-EM), Single Particle Analysis (SPA) of vitrified frozen-hydrated specimens is one of the most widely used and successful variants of imaging for biological macromolecules, as it allows to understand molecular interactions and the function of biological ensembles[1]. This is thanks to the recent advances in this imaging technique that gave rise to the "resolution revolution"[2] and have allowed ~~to successfully determining~~the successful determination of biological 3D structures with near-atomic resolution. Currently, the highest resolution achieved in SPA Cryo-EM was 1.15Å for apoferritin[3] (EMDB entry: 11668). These technological advances comprise improvements in the sample preparation[4], the image acquisition[5], and the image processing methods[6]. ~~In t~~This article~~, we are going to~~ is focused on this last point.

~~Shortly~~Briefly, the goal of the image processing methods is to identify all the acquisition parameters to invert the imaging process of the microscope and recover the 3D structure of the biological specimen under study. These parameters are the gain of the camera, the beam-induced movement, the aberrations of the microscope (mainly the defocus), the 3D angular orientation and translation of each particle, and the conformational state in case of having a specimen with conformational changes. ~~Finding them, the acquisition process followed by the microscope could be inverted.~~ However, the number of parameters is very high and Cryo-EM requires using low-dose images to avoid radiation damage, which significantly reduces the Signal-to-Noise Ratio (SNR) of the acquired images. Thus, the problem cannot be unequivocally solved and all the parameters to be calculated~~, giving that we have very noisy data,~~ only can be estimations. Along the image processing workflow, ~~we have to identify~~ the correct ~~ones~~ parameters should be identified, ~~and~~ discarding the remaining ones to finally obtain a high-resolution 3D reconstruction.

The data generated by the microscope are gathered in frames. Simplifying, a frame contains the number of electrons that have arrived at a particular position (pixel) in the image, whenever electron-counting detectors are used. In a particular field of view, several frames are collected and this is called a movie. As low electron doses are used to avoid radiation damage that could

destroy the sample, the SNR is very low and the frames corresponding to the same movie need to be averaged to obtain an image revealing structural information about the sample. However, not only a simple average is applied, the sample can suffer shifts and other kinds of movements during the imaging time due to the beam-induced movement that need to be compensated. The shift-compensated and averaged frames originate a micrograph.

Once the micrographs are obtained, we need to estimate the aberrations introduced by the microscope for each of them, called Contrast Transfer Function (CTF), which represents the changes in the contrast of the micrograph as a function of frequency. Then, the particles can be selected and extracted, which is called particle picking. Every particle should be a small image containing only one copy of the specimen under study. There are three families of algorithms for particle picking: 1) the ones that only use some basic parameterization of the appearance of the particle to find them in the whole set of micrographs, e.g. particle size, 2) the ones that learn how the particles look like from the user or a pretrained set, and 3) the ones that use image templates. Each family has different properties ~~and we will show how several of them work.~~that will be shown later.

The extracted set of particles found in the micrographs will be used in a 2D classification process that has two goals: 1) cleaning the set of particles by discarding the subset containing pure noise images, overlapping particles, or other artifacts, and 2) the averaged particles representing each class could be used as initial information to calculate a 3D initial volume.

The 3D initial volume calculation is the next crucial step. ~~We can see the~~The problem of obtaining the 3D structure can be seen as an optimization problem in a multidimensional solution landscape, where the global minimum is the best 3D volume that represents the original structure, but several local minima representing suboptimal solutions can be found, and where it is very easy to get trapped. The initial volume represents the starting point for the searching process, so bad initial volume estimation could prevent us to find the global minimum. From the initial volume, a 3D classification step will help to discover different conformational states and to clean again the set of particles; the goal is to obtain a structurally homogeneous population of particles. After that, a 3D refinement step will be in charge of refining the angular and translation parameters for every particle ~~with respect~~ to get the best 3D volume possible.

Finally, in the last~~test~~ steps, the obtained 3D reconstruction can be sharpened and polished. Sharpening is a process of boosting the high frequencies of the reconstructed volume, and the polishing is a step to further refine some parameters, as CTF or beam-induced movement compensation, at the level of particles. Also, some validation procedures could be used to better understand the achieved resolution at the end of the workflow.

After all these steps, the tracing and docking processes[7] will help to give a biological meaning to the obtained 3D reconstruction, by building atomic models de novo or fitting existing models. If

high resolution is achieved, these processes will tell us the positions of the biological structures, even of the different atoms, in our structure.

Scipion[8] ~~software~~ allows creating the whole workflow combining the most relevant image processing packages in an integrative way. Xmipp[9], Relion[10], CryoSPARC[11], Eman[12], Spider[13], Cryolo[14], Ctffind[15], CCP4[16], Phenix[17], and many more packages can be included in Scipion. Also, it incorporates all the necessary tools to benefit the integration, interoperability, traceability, and reproducibility to make a full tracking of the entire image-processing workflow[8].

One of the most powerful tools that Scipion allows us to use is the consensus, which means to compare the results obtained with several methods in one step of the processing, making a combination of the information conveyed by different methods to generate a more accurate output. This could help to boost the performance and improve the achieved quality in the estimated parameters. Note that a simpler workflow can be build without the use of consensus methods; however, we have seen the power of this tool[22,25] and the workflow presented in this manuscript will use it in several steps.

All the steps that have been summarized in the previous paragraphs will be explained in detail in the following section and combined in a complete workflow using Scipion. Also, ~~we will show~~ how to use the consensus tools to achieve a higher agreement in the generated outputs will be shown. To that end, the example dataset of the *Plasmodium falciparum* 80S Ribosome has been chosen (EMPIAR entry: 10028, EMDB entry: 2660). The dataset is formed by 600 movies of 16 frames of size 4096x4096 pixels at a pixel size of 1.34Å taken at an FEI POLARA 300 with an FEI FALCON II camera, with a reported resolution at EMDB is 3.2Å[18] .

PROTOCOL:

1. Creating a project in Scipion and importing the data

~~1.~~

1. Open Scipion and click on **Create Project**, ~~you can~~ specify the name for ~~your~~ the project and the location where it will be saved (**Supplemental Figure 1**).

~~1.~~

2. Scipion will open the project window ~~where you will see~~showing a canvas ~~and~~with, on the left side, ~~you will find~~ a panel with a list of available methods, each of them represents one image processing tool that you can use to manage your data.
   NOTE: ~~If you do not find a method in this list, you can use~~ **Ctrl+F** can be used to find a method if it does not appear in the list~~it~~.

3. To import the movies taken by the microscope select the **pwem - import movies** on the left panel (or type it when you press **Ctrl+F**).

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~~3.~~

4. A new window will be opened (**Supplemental Figure 2**)~~.~~ ~~T~~there~~,~~ ~~you have to~~ include the path to ~~your~~ the data, and the acquisition parameters. In ~~our~~ this example, ~~we will~~ use the following setup: **Microscope voltage** 300kV, **Spherical aberration** 2.0mm, **Amplitude Contrast** 0.1, **Magnification rate** 50000, **Sampling rate mode** to **From image**, and **Pixel size** 1.34Å. When all the parameters in the form are filled, click on the **Execute** button.
~~**NOTE:** Gain and dark correction can be carried out by including these files in the form. Both are distortions introduced by the camera that produces a non-uniform readout when a uniform electron illumination is presented.~~
NOTE: When a method starts, a box appears in the canvas in yellow color labeled as **running**. When a method finishes, the box changes to green, and the label changes to **finished**.
~~**NOTE:**~~ In case of an error during the execution of a method, the box will appear in red, labeled as **failed**. In that case, check the bottom part of the canvas, in the **Output Log** tab an explanation of the error will appear.

5. When the method finishes, check the results in the bottom part of the canvas in the **Summary** tab. Here~~,~~ ~~you have~~ the outputs generated by the method are presented, in this case, the set of movies. ~~If you click~~Click on **Analyze Results** button and a new window will appear with the list of movies ~~(**Figure 1**)~~.

2. Movie alignment: from movies to micrographs
~~2.~~

1. ~~We are going to use~~The method **xmipp3 – optical alignment** which implements Optical flow[19] will be used.~~.~~ ~~This method expresses the deformation field of every frame using a high order Taylor expansion.~~The main parameters to fill in the form are the following (**Supplemental Figure 3**): the **Input Movies** are those obtained in step 1, the range in **Frames to ALIGN** is from 2 to 13, the other options stay with the default values. Execute the program.
~~1.~~

~~2. The main parameters to fill in the form are the following (**Supplemental Figure 3**): the **Input Movies** are those obtained in step 1, the range in **Frames to ALIGN** is from 2 to 13, the other options stay with the default values. Execute the program.~~
NOTE: The parameters in bold in a form, must be always filled. The others will have a default value or will not be obligatorily required.
NOTE: In the upper part of the form window ~~you can see~~ the fields where ~~you can distribute~~ the computational resources are distributed can be found, as threads, MPIs, or GPUs.

~~3.~~2. Click on **Analyze Results** to check the obtained micrographs and the

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trajectory of the estimated shifts (**Figure 12**). For every micrograph you can see: the power spectral density (PSD), the trajectories obtained to align the movie (one point per frame) in cartesian and polar coordinates, and the file name of the obtained micrograph (clicking on it, the micrograph can be inspected). Notice that the particles of the specimen are much more visible in the micrograph, as compared to a single frame of the movie.

3. CTF estimation: calculating the aberrations of the microscope

3.

1. First, we are going to use the method **grigoriefflab – ctffind**[15] will be used. The setup is: the **Input Micrographs** are the output of step 2, the **Manual CTF Downsampling factor** is set to 1.5, and the **Resolution** range goes from 0.06 to 0.42. Moreover, in the **Advanced** options (that can be found by selecting this choice in the **Expert Level** of the form), set the **Window size** to 256. The remaining parameters stay with the default values (**Supplemental Figure 4**).
   NOTE: In most of the methods in Scipion the **Advanced** option shows more configuration parameters. Use these options carefully, when the program to be launched is completely known and the meaning of the parameters is understood.
   NOTE: Some parameters can be difficult to fill without having a look at the data; in that case, Scipion shows a magic wand on the right side that will show a wizard window (**Supplemental Figure 5**). For example, in the **Resolution** field of this form is especially useful, as these values should be selected to approximately cover the region from the first zero to the last noticeable ring of the PSD.

2. Click on **Execute** and on **Analyze Results** (**Figure 23**) when the method finishes. Check that the estimated CTF matches with the experimental one. To that end, look at the PSD and compare the estimated rings in the corner with the ones coming from the data. You can check also the obtained defocus values to find any unexpected values and respective micrographs can be discarded or recalculated. In this example, we can continue with the whole set of micrographs can be used.
   NOTE: Use the buttons in the bottom part of the window to make a subset of micrographs (with **Micrographs** red button) and to recalculate a CTF (with **Recalculate CTFs** red button), in case of needing.

3. We are going toTo refine the previous estimation using **xmipp3 – ctf estimation**[20] will be used. Select as **Input Micrographs** the output of step 2, select the option **Use defoci from a previous CTF estimation**, as **Previous CTF estimation** choose the output of **grigoriefflab – ctffind**, and, in the **Advanced** level, change the **Window size** to 256 (**Supplemental Figure 6**). Run it.

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~~3.~~

4. Click on **Analyze Results** to check the obtained CTFs. With this method, more data is estimated and represented in some extra columns ~~(**Figure 4**)~~. ~~We are going to continue processing with all the micrographs, as~~As none of them ~~should~~ show incorrect estimated values, all the micrographs will be used in the following steps.

4. Particle picking: finding particles in the micrographs

~~4.~~

1. Before starting the picking, ~~we are going to~~a preprocess of the micrographs will be carried out~~, to reduce the size and thus accelerate the picking process~~. Open **xmipp3 – preprocess micrographs**, set as **Input micrographs** those obtained in step 2 and select the options **Remove bad pixels?** with **Multiple of Stddev** to 5, and **Downsample micrographs?** with a **Downsampling factor** of 2 (**Supplemental Figure 7**). Click on **Execute** and check that the size of the resulting micrographs has been reduced.

~~1.~~

2. For the picking ~~we are going to~~ use **xmipp3 – manual-picking (step 1)** and **xmipp3 – auto-picking (step 2)**[21]. ~~This is a method based on a classifier that learns from an input provided by the user.~~ The manual picking allows ~~us~~ to manually prepare a set of particles with which the auto-picking step will learn and generate the complete set of particles. First, run **xmipp3 – manual-picking (step 1)** with **Input Micrographs** as the micrographs obtained in the previous preprocess. Click on **Execute** and a new interactive window will appear (**Figure ~~3~~5**).

~~2.~~

3. In this window ~~you will see~~ a list of ~~your~~ the micrographs (**Figure ~~5~~3 (a)**) and other options is presented. Change **Size (px)** to 150, this will be the size of the box containing each particle. The selected micrograph appears in a bigger window. ~~Here you should choose~~Choose a region and pick all the visible particles in it (**Figure ~~5~~3 (b)**). Then, click on **Activate Training** to start the learning. ~~You will see how the~~The remaining regions of the micrograph are automatically picked (**Figure ~~3~~5 (c)**). ~~You can check~~Check the picked particles and include more by clicking on it, or remove the incorrect ones with shift+clicking, if necessary.

~~3.~~

4. Select the next micrograph in the first window. The micrograph will be automatically picked. Check again ~~if you want~~ to include or remove some particles, if necessary. Repeat this step with, approximately, 5 micrographs to create a representative training set.

~~4.~~

5. Once this is done, click on **Coordinates** in the main window to save the coordinates of all the picked particles. The training set of particles is ready to go

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to the auto picking to complete the process for all micrographs.

~~5.~~

6. Open **xmipp3 – auto-picking (step 2)** indicating in **Xmipp particle picking run** the previous manual picking, and **Micrographs to pick** as **Same as supervised**. Click on **Execute**. This method will generate as output a set of around ~~one thousand~~100000 coordinates.

~~6.~~

7. ~~We are going to use a consensus approach, so we want to use another picking method to finally select the particles in which both methods agree, thus removing noisy coordinates.~~ A consensus approach is going to be applied, so a second picking method will be carried out to select the particles in which both methods agree. ~~O~~Open **sphire – cryolo picking**[14] and select the preprocessed micrographs as **Input Micrographs**, **Use general model?** to **Yes**, with a **Confidence threshold** of 0.3, and a **Box Size** of 150 (**Supplemental Figure 8**). ~~Thus, we are skipping the training using a pretrained network.~~ Run it. This method should generate also around ~~one thousand~~100000 ~~particles~~coordinates.

~~7.~~

8. Run **xmipp3 – deep consensus picking**[22]. As **Input coordinates** include the output of **sphire – cryolo picking** (step 4.7) and **xmipp3 – auto-picking** (step 4.6), set **Select model type** to **Pretrained**, and **Skip training and score directly with pretrained model?** To **Yes** (**Supplemental Figure 9**). Run it.

~~8.~~

9. Click on **Analyze Results** and, in the new window, on the eye icon next to **Select particles/coordinates with high 'zScoreDeepLearning1' values**. A new window will be opened with a list of all particles (**Figure ~~46~~**). The **zScore** values in the column give an insight into the quality of a particle, low values mean bad quality~~, so we are going to select the subset of particles with the highest values~~. Click on the label **_xmipp_zScoreDeepLearning** to order the particles from highest to lowest **zScore**. Select the particles with **zScore** higher than 0.75 and click on **Coordinates** to create the new subset. This should create a subset with approximately 50000 coordinates.

~~9.~~

10. Open **xmipp3 – deep micrograph cleaner**. ~~We are going to use this method to additionally clean the selected coordinates by removing those coming from carbon zones or large impurities.~~ Select as **Input coordinates** the subset obtained in the previous step, **Micrographs source** as **same as coordinates**, and keep **Threshold** at 0.75. Run it. Check in the **Summary** tab that the number of coordinates has been reduced, although in this case, only few coordinates are removed.

    NOTE: This step is able to additionally clean the set of coordinates and could be very useful in cleaning ~~the picked coordinates in~~ other datasets with more movie artifacts as carbon zones or large impurities.

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11. Run **xmipp3 – extract particles** (**Supplemental Figure 10**). Indicate as **Input coordinates** the coordinates obtained after the previous step, **Micrographs source** as **other**, **Input micrographs** as the output of step 2, **CTF estimation** as the output of the **xmipp3 – ctf estimation**, **Downsampling factor** to 3, and **Particle box size** to 100. In the **Preprocess** tab of the form select **Yes** to all. Run it.

~~11.~~

12. Check that ~~T~~the output should contain the particles in reduced size of 100x100 pixels and a pixel size of 4.02Å/px. ~~This set will be useful in the following 2D classification, as a reduced size will help to reduce the computational complexity of that step.~~

~~12.~~

13. Run again **xmipp3 – extract particles** changing the following parameters: **Downsampling factor** to 1, and **Particle box size** to 300. Check that ~~T~~the output ~~should be~~is the same set of particles but now at the full resolution ~~(**Figure 7**)~~.

5. 2D classification: grouping similar particles together

~~5.~~

1. ~~We are going to use~~Open the method **cryosparc2 – 2d classification**[11] with **Input particles** as those obtained in step 4.11 and, in the **2D Classification** tab, the **Number of classes** to 128, keep all the other parameters with the default values. Run it.

~~1.~~

2. Click on **Analyze Results** and then on the eye icon next to **Display particle classes with Scipion** (**Figure ~~5~~8**). This classification will help us to clean our set of particles, as several classes will appear noisy or with artifacts. Select the classes containing good views. Click on **Particles** (red button in the lower part of the window) to create the cleaner subset.

~~2.~~

3. Now, open **xmipp3 – cl2d**[23] and set as **Input images** the images obtained in the previous step and **Number of classes** as 128. Click on **Execute**.

NOTE: This second classification is used as additional cleaning step of the set of particles. Usually is useful to remove as much noisy particles as it is possible. However, if a simpler workflow is desired, only one 2D classification method can be used.

~~3.~~

4. When the method finishes, check the 128 generated classes by clicking on **Analyze Results** and on **What to show: classes**. ~~You will see that most~~Most of the generated classes show a projection of the macromolecule with some level of detail. However, some of them ~~will~~ appear noisy (in this example approximately 10 classes). Select all the good classes and click on the **Classes**

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button to generate a new subset with only the good ones ~~(**Figure 9**)~~. This subset will be used as input to one of the methods to generate an initial volume. With the same selected classes click on **Particles** to create a cleaner subset after removing those belonging to the bad classes.

~~4.~~

5. Open **pwem – subset** with **Full set of items** as the output of 4.13 (all particles at the full size), **Make random subset** to **No**, **Other set** as the subset of particles created in the previous step, and **Set operation** as **intersection**. This will extract the previous subset from the particles at full resolution.

6. Initial volume estimation: building the first guess of the 3D volume

~~6.~~

1. In this step two initial volumes will be estimated with different methods and then, a consensus tool will generate the final estimated 3D volume. Open **xmipp3 – reconstruct significant**[24] method with **Input classes** as those obtained after step 5, **Symmetry group** as c1, and keep the remaining parameters with their default values (**Supplemental Figure 11**). Execute it.

~~1.~~

2. Click on **Analyze Results** ~~(**Figure 10**)~~. ~~You should see~~Check that a low resolution volume of size 100x100x100 pixels and a pixel size of 4.02Å/px is obtained.

~~2.~~

3. ~~We are going to resize this initial volume to obtain one at the original sampling rate.~~ Open **xmipp3 – crop/resize volumes** (**Supplemental Figure 12**) using as **Input Volumes** the one obtained in the previous step, **Resize volumes?** to **Yes**, **Resize option** to **Sampling Rate**, and **Resize sampling rate** to 1.34Å/px. Run it. Check in the **Summary** tab that the output volume has the correct size.

~~3.~~

4. Now, ~~we are going to create~~ the second initial volume will be created. Open **relion – 3D initial model**[10], as **Input particles** use the good particles at full resolution (output of 5.5) and set **Particle mask diameter** to 402Å, keep the remaining parameters with the default values. Run it.

~~4.~~

5. Click on **Analyze Results** and then in **Display volume with: slices** ~~to check the generated volume~~. ~~Again, you should see~~Check that a low resolution volume but with the main shape of the structure is obtained (**Supplemental Figure 13**).

~~5.~~

6. Now, ~~we need to use~~open **pwem – join sets** to combine the two generated initial volumes to create the input to the consensus method. Just indicate **Volumes** as **Input type** and select the two initial volumes in **Input set**. Run it. The output should be a set containing two items with both volumes.

~~6.~~

7. The consensus tool is the one included in **xmipp3 – swarm consensus**[25]. Open it.

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Use as **Full-size Images** the good particles at full resolution (output of 5.5), as **Initial volumes** the set with two items generated in the previous step, and be sure that **Symmetry group** is c1. Click on **Execute**. ~~This method will try to generate an output optimizing the correlation between volumes and particles, using swarm optimization.~~

~~7.~~

8. Click on **Analyze Results.** ~~You should see some more details in~~ Check that a more detailed ~~the~~ output volume is obtained (**Figure ~~6~~11**). Although there is more noise surrounding the structure, to have more details in the structure map ~~-~~will help the following refinement steps to avoid local minima.
   NOTE: If ~~you have installed~~ UCSF Chimera[26] is available, ~~you can~~ use the last icon in the upper part of the window to make a 3D visualization of the obtained volume.

9. Open and execute **relion – 3D auto-refine**[10] to make a first 3D angular assignment of the particles. Select as **Input particles** the output of 5.5, and set **Particle mask diameter** to 402Å. In **Reference 3D map** tab, select as **Input volume** the one obtained in the previous step, **Symmetry** as c1, and **Initial low-pass filter** to 30Å (**Supplemental Figure 14**).

~~9.~~

10. Click on **Analyze Results**. In the new window select **final** as **Volume to visualize** and click on **Display volume with: slices** to see the obtained volume. ~~You can c~~Check also the Fourier shell correlation (FSC) by clicking on **Display resolution plots** in the results window and the angular coverage in **Display angular distribution: 2D plot** (**Figure ~~7~~12**). The reconstructed volume ~~should~~ contains much more details (probably with some blurred areas in the outer part of the structure), and ~~an~~ the FSC ~~crossing~~ crosses the threshold of 0.143 around 4.5Å. The angular coverage ~~, represented as an upper view of the 3D sphere, should~~ covers the whole 3D sphere ~~as there is no symmetry in this structure and no missing directions should appear~~.

7. 3D classification: discovering conformational states

~~7.~~

1. Using a consensus approach, ~~we are going to discover if this dataset contains~~ if different conformational states are in the data can be discovered. Open **relion – 3D classification**[10] (**Supplemental Figure 15**). As **Input particles** use those just obtained in 6.10, and set **Particle mask diameter** to 402Å. In the **Reference 3D map** tab, use as **Input volume** the one obtained after step 6.10, set **Symmetry** to c1, and **Initial low-pass filter** to 15Å. Finally, in **Optimization** tab, set the **Number of classes** to 3. Run it.

   ~~1.~~

2. Check the results by clicking on **Analyze Results**, select **Show classification in**

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Scipion. ~~You will see t~~The three generated classes and some interesting measures are shown ~~(**Figure 13**)~~. The first two classes should have a similar number of assigned images (**size** column) and look very similar, whilst the third one has fewer images and a more blurred appearance. Also, the **rlnAccuracyRotations** and **rlnAccuracyTranslations** should be clearly better for the first two classes. Select the two best classes and click on the **Classes** button to generate a subset containing them.

~~2.~~

3. Repeat steps 7.1 and 7.2 to generate a second group of good classes. Both will be the input of the consensus tool.

~~3.~~

4. Open and run **xmipp3 – consensus classes 3D** and select as **Input Classes** the two subsets generated in the previous steps.

~~4.~~

5. Click on **Analyze Results** ~~(**Figure 14**)~~. ~~Here, you will see the~~The number of coincident particles between classes is presented, i.e., the first value is the number of coincident particles in the first class of subset 1 and the first class of subset 2, the second value is the number of coincident particles in the first class of subset 1 and the second class of subset 2, etc. ~~You should see~~Check ~~in these results~~ that the particles are randomly assigned to classes one or two, which means that the 3D classification method is not able to find conformational changes. Given this result, ~~we can continue the processing with~~ the whole set of particles will be used to continue processing~~, considering just one homogeneous population~~.

8. 3D refinement: refining angular assignments of a homogeneous population

~~8.~~

~~1. The goal is to make an accurate 3D angular assignment to the particles, which will give as a result a 3D volume of high resolution. Again, we will rely on a consensus approach. We are going to use **xmipp3 – deep align** to make a new angular assignment. This method uses deep learning and it needs a training set to train the network.~~

1. Again, a consensus approach will be applied in this step. First, ~~O~~open and run **pwem – subset** with **Full set of items** as the output of 6.9, **Make random subset** to **Yes**, and **Number of elements** to 5000. With this, ~~we create~~ a subset of images with a previous alignment to train the method used in the following step~~network~~ is created.

~~2.~~

2. Open **xmipp3 – deep align**, set **Input images** as the output of good particles obtained in 5.5, **Volume** as the one obtained after 6.10, **Input training set** as the one created in the previous step, **Target resolution** to 10Å, and keep the remaining parameters with the default values (**Supplemental Figure 16**). Click on

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Execute.

~~3.~~

3. ~~The output of this method will be the set of particles with the new angular assignments.~~ Click~~ing~~ on **Analyze Results** ~~we have the option~~ to check the obtained angular distribution, where ~~we can check that~~ there are no missing directions and ~~that~~ the angular coverage slightly improves compared to the one of 6.10 (**Figure ~~8~~15**).

~~4.~~

4. ~~Now, we are going to calculate the agreement between **xmipp3 – deep align** and **relion – 3D auto refine**.~~ Open and execute **xmipp3 – compare angles** and select as **Input particles 1** the output of 6.9 and **Input particles 2** the output of 8.~~3~~2, make sure that the **Symmetry group** is c1. This method calculates ~~the differences between the output of both methods in the estimated angles and shifts.~~ the agreement between **xmipp3 – deep align** and **relion – 3D auto refine**.

~~5.~~

5. Click on **Analyze Results**, ~~you will see~~ the list of particles, with the obtained differences in shifts and angles, is shown. ~~If you c~~Click on the bar icon in the upper part of the window, another window will be opened that allows making plots of the calculated variables. Select **_xmipp_angleDiff** and click on **Plot** to see a representation of the angular differences per particle. ~~You can do~~Do the same with **_xmipp_shiftDiff**. In these figures, ~~you can see that~~ approximately in half of the particles both methods agree (**Figure ~~9~~16**). Select the particles with angular differences lower than 10º and create a new subset.

~~6.~~

6. Now, ~~we are going to use~~open **xmipp3 – highres**[27] to make a local refinement of the assigned angles. First, ~~open the form and~~ select as **Full-size Images** the images obtained in the previous step, and as **Initial volumes** the output of 6.9, set **Radius of particle** to 150 pixels, and **Symmetry group** as c1. In the **Angular assignment tab**, set the **Image alignment** to **Local**, **Number of iterations** to 1, and **Max. Target Resolution** as 5Å/px (**Supplemental Figure 17**). Run it.

~~7.~~

~~8.~~7. In the **Summary** tab ~~you will see how~~check that the output volume is smaller than 300x300x300 pixels and with slightly higher pixel size. ~~This is because we asked for a maximum target resolution of 5Å/px, which generated an intermediate result of very good quality to continue processing.~~

~~**NOTE**: This is a common way to proceed when Xmipp Highres is used, the refinement should be done in several steps, going to higher resolutions in every step.~~

8. Click on **Analyze Results** to see the obtained results. Click on **Display resolution plots** to see the FSC, and on **Display volume: Reconstructed** to see the obtained volume (**Supplemental Figure 18**). ~~You should see a~~A good resolution volume

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close to 4-3.5Å is obtained.~~ready to make one final step of refinement.~~

~~9.~~

~~10.~~9. Click on **Display output particles** and, in the window with the list of particles, click on the bar icon. In the new window, select **Type** as **Histogram**, with 100 **Bins**, select **_xmipp_cost** label, and finally press **Plot** (**Supplemental Figure 19**). This way, ~~we will see~~ the histogram of the **cost** label is presented, which contains the correlation of the particle with the projection direction selected for it ~~(**Figure 17**)~~. In this case, ~~we should see~~ a unimodal density function is obtained, which is a sign of not having different populations in the set of particles. Thus~~, we can continue the refinement with~~ all of them will be used to continue the refinement~~.~~

NOTE: In case of seeing a multimodal density function, the set of particles belonging to the higher maximum should be selected to continue the workflow only with them.

10. Open and execute again **xmipp3 – highres** with **Continue from a previous run?** to **Yes**, set as **Full-size Images** those obtained after 8.5~~6~~, and **Select previous run** with the previous execution of Xmipp Highres. In the **Angular assignment** tab, set the **Image alignment** to **Local**, with 1 iteration and 2.6Å/px as target resolution (full resolution).

~~11.~~

~~12.~~11. Now the output should contain a volume at full resolution (size 300x300x300 pixels). Click on **Analyze Results** to check again the obtained volume and the FSC, which now should be a high resolution volume at around 3Å (**Figure 10~~8~~**).

9. Evaluation and post-processing

~~9.~~

1. ~~Now that the obtained 3D volume has high resolution and very well defined features, we are going to further evaluate the volume and post-process it.~~ Open **xmipp3 – local MonoRes**[28]. This method will calculate the resolution locally. Set as **Input Volume** the one obtained after 8.10~~1~~, set **Would you like to use half volumes?** to **Yes**, and **Resolution Range** from 1 to 10Å. Run it.

~~1.~~

2. Click on **Analyze Results** and select **Show resolution histogram** and **Show colored slices** (**Figure 10~~9~~1**). ~~You will see the~~The resolution in the different parts of the volume is shown. Most of the voxels of the central part of the structure should present resolutions around 3Å, whilst the worst resolutions are achieved in the outer parts. Also, a histogram of the resolutions per voxel ~~will be~~is shown~~, where you should see that the~~ with a peak ~~is~~ around (even below) 3Å.

~~2.~~

3. ~~We can post-process our volume by applying a sharpening.~~ Open and run

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xmipp3 – localdeblur sharpening[29] to apply a sharpening. ~~and s~~Select as **Input Map** the one obtained in 8.1~~0~~1, and as **Resolution Map** the one obtained in the previous step with MonoRes.

~~3.~~

4. Click on **Analyze Results** to check the obtained volumes. Open the last one, corresponding to the last iteration of the algorithm. ~~In the representation by slices, it is difficult to see the details of the reconstructed volume after the sharpening, so we~~It is recommend opening the volume~~it~~ with other tools, such as UCSF Chimera[26], to see better the features of the volume in 3D (**Figure 12~~0~~**).

~~4.~~

5. Finally, ~~we are going to use another~~ open the validation tool included in **xmipp3 – validate overfitting**[30] that will ~~tell us how~~show how the resolution changes with the number of particles. Open it and include as **Input particles** the particles obtained in step 8.5~~6~~, set **Calculate the noise bound for resolution?** to **Yes**, with **Initial 3D reference volume** as the output of 8.1~~0~~1. In **Advanced** options, set the **Number of particles** to "500 1000 1500 2000 3000 5000 10000 15000 20000" (**Supplemental Figure 20**). Run it.

~~5.~~

6. Click on **Analyze results**. Two plots will appear (**Figure 13~~2~~1**) with the evolution of the resolution, in the green line, as the number of particles used in the reconstruction grows. The red line represents the resolution achieved with a reconstruction of aligned Gaussian noise. ~~We can see how the~~The resolution improves with the number of particles and ~~the~~ a great difference of the reconstruction from particles compared to the one from noise is observed, which is an indicator of having particles with good structural information.

~~6.~~

7. From the previous results, ~~we can try to make~~ a fitting of a model in the post-processed volume could be carried out, which would allow discovering the biological structures of the macromolecule.

REPRESENTATIVE RESULTS:

We have used the dataset of the *Plasmodium falciparum* 80S Ribosome (EMPIAR entry: 10028, EMDB entry: 2660) to conduct our test and,

~~W~~ with the Scipion protocol presented in the previous section, ~~you will be able to achieve~~ a high resolution 3D reconstructed volume of the macromolecule ~~under study~~in this particular example has be achieved, beginning with the information gathered by the microscope that consist of very noisy images containing 2D projections in any orientation of the specimen.

The main results obtained after running the whole protocol are presented in Figures 1~~8~~0, 1~~9~~, and 12~~0~~. Figure 1~~0~~8 represents the obtained 3D volume before post-processing. In part (a) of

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the figure, ~~we can see that the~~an FSC ~~achieved was~~of 3Å can be seen, that it is very close to the Nyquist limit (with data with a pixel size of 1.34Å, the Nyquist limit is 2.6Å). Part (b) of the figure shows some slices of the reconstructed 3D volume with high levels of details and well-defined structures. In Figure 11~~9~~ ~~we have~~ the results after locally analyze the resolution of the obtained 3D volume are presented. ~~We have found~~It can be seen that most of the voxels in ~~our~~ the structure achieve a resolution below 3Å, mainly those located in the central part of the structure. However, the outer part shows worse resolutions, what is consistent with the blurring appearing in those areas in the slices of Figure 1~~8~~0 (b). Figure 12~~20~~ shows the same 3D map after post-processing that is able to highlight the higher frequencies of the volume, revealing more details and improving the representation, which can be seen especially in the 3D presentation of part (c) of the figure.

In Figure 14~~22~~, ~~we have used~~ Chimera[26] was used to see a 3D representation of the obtained volume (a), the post-processed (b), and the resolution map (c), colored with the color code of the local resolutions. This can give ~~us~~ even more information about the obtained structure. This tool is very useful to gain an insight into the quality of the obtained volume, as ~~as you can see~~ very small details in the whole 3D context of the structure can be seen. When the achieved resolution is enough, even some biochemical parts of the structure can be found, e.g. alpha-helices (d). In this figure, ~~we should~~it must be highlighted the high resolution achieved in all the central parts of the 3D structure, which can be seen as the dark blue areas in part (c) of the figure.

All the previous results were achieved thanks to a good performance of the whole protocol, but this might be not the case. There are several ways to identify a bad behavior. In the most general case, this happens when the obtained structure has low resolution and it is not able to evolve to a better one. One example of this is presented in Figure 15~~23~~. A blurred volume (c) results in a low FSC, which can be seen in the FSC curve (a) and the histogram of the local estimation (b). This example was generated using a 3D refinement method with incorrect input data, as it was expecting some specific properties in the input set of particles that they do not fulfill. As can be seen, it is always very important to know how the different methods expect to receive the data and prepare it properly. In general, when an output like the one in Figure 15~~23~~ is obtained, ~~we know that~~ there might be a problem in the processing workflow or the underlying data.

There are several checkpoints along the workflow that can be analyzed to know if the protocol evolves properly or not. For example, right after picking, several of the methods discussed earlier are able to rank the particles and give a score for each of them. In the case of having bad particles, these methods allow ~~us~~ to identify and remove them. Also, the 2D classification can be a good indicator of having a bad set of particles. Figure 16~~24~~ shows an example of such a bad set. In the part (a) of the figure, ~~it shows~~ good classes containing some details of the structure are shown, while part (b) shows bad classes, which are noisy or uncentered, in this last case ~~you will see~~it can be seen that the picking was incorrect and two particles seem to

appear together. Another checkpoint is the initial volume estimation, Figure 17~~25~~ shows an example of good (a) and bad (b) initial estimations. The bad estimation was created using an incorrect setup for the method. It must be taken into account that all the setups should be done carefully, choosing appropriately every parameter according to the data being analyzed. In case of not having a map with some minimal structural information, the following refinement will be unable to obtain a good reconstruction.

When the problem is a bad acquisition, in which the movies do not preserve structural information, it will be impossible to extract good particles from them and get a successful processing. In that case, more movies should be collected to get a high resolution 3D reconstruction. But, if this is not the case, there are several ways to manage problems along the processing workflow. If the picking is not good enough, there are several ways to try to fix it, e.g., repeating the picking, using different methods, or trying to manually pick more particles to help the methods to learn from them. During the 2D classification, if just a few classes are good, consider also to repeat the picking process. In the initial volume estimation, try to use several methods if some of them gave inaccurate results. The same applies to the 3D refinement. Following this reasoning, in this manuscript, ~~we have presented~~ several consensus tools have been presented, ~~that~~ which could be very useful to avoid problems and continue the processing with accurate data. Thanks to using a consensus among several methods, we can discard data that are difficult to pick, classify, align, etc., which probably is an indicator of poor data. However, if several methods are able to agree in the generated output, probably these data contain valuable information with which to continue processing.

We encourage the reader to download more datasets and try to process them following the recommendations presented in this manuscript and to create a similar workflow combining processing packages using Scipion. Trying to process a dataset is the best way to learn the power of the processing tools available in the state-of-the-art in Cryo-EM, to know the best rules to overcome the possible drawbacks appearing during the processing, and to boost the performance of the available methods in each specific test case.

FIGURE AND TABLE LEGENDS:

~~**Figure 1. Import movies result. (a)** A list with the file path and other parameters of every imported movie. Clicking on one of them, the movie is opened in a new window **(b)** where each frame can be inspected.~~

Figure 1~~2~~. Movie alignment result. (a) The main window of the results, with a list of all the micrographs generated and additional information: the power spectral density, the trajectory of the estimated alignment in polar coordinates, the same in cartesian coordinates, the filename of the generated micrograph. **(b)** The alignment trajectory represented in cartesian coordinates. **(c)** The generated micrograph.

Figure 2~~3~~. CTF estimation with Ctffind result. The main window with the results includes a figure with the estimated PSD (in a corner) along with the PSD coming from the data, and several defocus params.

~~**Figure 4. CTF refinement with Xmipp result.** The results window shows the PSD and several figures with the estimated CTF. This method is able to additionally estimate more parameters representing the CTF behavior.~~

Figure 3~~5~~. Manual picking windows with Xmipp. (a) The main window with the list of micrographs to process and some other parameters. **(b)** Manually picking particles inside a region of a micrograph. **(c)** and **(d)** Automatically picked particles to be supervised to create a set of training particles for the Xmipp auto picking method.

Figure 4~~6~~. Deep consensus picking with Xmipp result. The parameter **zScoreDeepLearning** gives weight to the goodness of a particle and it is key to discovering bad particles. **(a)** The lowest zScores values are associated with artifacts. **(b)** The highest zScores are associated with particles containing the macromolecule.

~~**Figure 7. Extract particle result.** The obtained particles after using the extract particles method. This window shows a grid with all of them.~~

Figure ~~8~~5. 2D classification with Cryosparc result. The classes generated (averages of subsets of particles coming from the same orientation) are shown. ~~Here you can see several~~Several good classes selected in red (with some level of detail) and some bad classes non-selected (noisy and uncentered classes).

~~**Figure 9. 2D classification with Xmipp CL2D result.** A window with the 128 classes generated. Most of them are good classes, selected in red. Some bad classes are non-selected (mainly due to noise).~~

~~**Figure 10. 3D initial volume with Xmipp result.** A view by slices of the initial volume generated by **xmipp3 – reconstruct significant** method. We can see a low resolution volume that starts to contain some information about the shape of the structure.~~

Figure 6~~11~~. 3D initial volume with swarm consensus result. A view of the 3D initial volume obtained after running the consensus tool **xmipp3 – swarm consensus**, using the previous 3D initial volume estimations of Xmipp and Relion. **(a)** The volume is represented by slices. **(b)** 3D visualization of the volume.

Figure 7~~12~~. Refinement of a 3D initial volume with Relion result. (a) FSC curve obtained, crossing the threshold at a 4.5Å, approximately. **(b)** Angular coverage shown as upper view of

the 3D sphere. In this case, as there is no symmetry, the assigned particles should cover the whole sphere. **(c)** Refined volume represented by slices.

~~**Figure 13. 3D classification result.** The three generated classes are shown with some parameters with the accuracy of every generated class. Two of the classes look very similar, but the last one contains too much noise.~~

~~**Figure 14. Consensus classes 3D result.** The number of coincident particles among every combination by pairs of classes shows that the 3D classification method was not able to distinguish classes in this dataset.~~

Figure 8~~15~~. 3D alignment based on deep learning with Xmipp result. The results generated by **xmipp3 – deep align** method for 3D alignment. **(a)** The angular assignment for every particle in the form of transformation matrix. **(b)** The angular coverage.

Figure 9~~16~~. 3D alignment consensus result. (a) List of particles with the obtained differences in shift and angles parameters. **(b)** Plot of the angular differences per particle. **(c)** Plot of the shift difference per particle.

~~**Figure 17. First iteration of 3D refinement correlation analysis result.** The histogram of the correlation between particles and the assigned projection direction. Unimodal distribution shows that the method cannot distinguish different populations in the data.~~

Figure 10~~8~~. Final iteration of 3D refinement result. (a) FSC curve. **(b)** Obtained volume at full resolution by slices.

Figure 11~~9~~. Local resolution analysis with Xmipp result. Results of the method **xmipp3 – local MonoRes**. **(a)** Some representative slices colored with the resolution value per voxel, as indicated in the color code. **(b)** Local resolution histogram.

Figure 12~~20~~. Sharpening with Xmipp result. Results of **xmipp3 – localdeblur sharpening** method. **(a)** List of obtained volumes per iteration. **(b)** 3D volume obtained after the last iteration represented by slices. **(c)** A 3D representation of the final volume.

Figure 13~~21~~. Validate overfitting tool in Xmipp result. Results of **xmipp3 – validation overfitting**. The green line corresponds to reconstruction from data, the red line from noise. **(a)** Inverse of the squared resolution with the logarithm of the number of particles. **(b)** Resolution with the number of particles.

Figure 14~~22~~. Several 3D representations of the obtained volume. (a) Pre-processed volume. **(b)** Post-processed volume. **(c)** Local resolution, dark blue voxels are those with higher resolution (2.75Å) and dark red voxels are those with lower resolution (10.05Å). **(d)** Zoom in the

post-processed volume where an alpha-helix (red oval) can be seen.

Figure 15~~23~~. Example of a bad 3D reconstruction. (a) FSC curve with a sharp fall and crossing the threshold at low resolution. **(b)** Local resolution histogram. **(c)** 3D volume by slices.

Figure 16~~24~~. Example of 2D classes. (a) Good classes showing some level of detail. **(b)** Bad classes containing noise and artifacts (upper part obtained with Xmipp, lower with CryoSparc).

Figure 17~~25~~. Example of 3D initial volume with different qualities. (a) Good initial volume where the shape of the macromolecule can be observed. **(b)** Bad initial volume where the obtained shape is completely different from the expected one.

SUPPLEMENTARY FILES:

Supplemental Figure 1. Creating a Scipion project. Window displayed by Scipion where ~~you can select~~ an old project can be selected or ~~create~~ a new one can be created giving a name and a location for that project.

Supplemental Figure 2. Import movies method. Window displayed by Scipion when **pwem - import movies** is open. Here, ~~you must include~~ the main acquisition parameters must be included to let the movies available to be processed in Scipion.

Supplemental Figure 3. Movie alignment method. Window displayed by Scipion when **xmipp3 – optical alignment** is used. The input movies, the range of frames considered for alignment, and some other parameters to process the movies should be filled.

Supplemental Figure 4. CTF estimation method with Ctffind. The form in Scipion with all the necessary fields to run the program Ctffind.

Supplemental Figure 5. Wizard in Scipion. A wizard to help the user filling some parameters in the form. In this case, the wizard is to complete the resolution field in the **grigoriefflab – ctffind** method.

Supplemental Figure 6. CTF refinement method with Xmipp. The form of **xmipp3 – ctf estimation** with all the parameters to make a refinement of a previously estimated CTF.

Supplemental Figure 7. Preprocess micrographs method. The form of **xmipp3 – preprocess micrographs** that allows carrying out some operations over them. In this example, ~~we are interested in~~ **Remove bad pixels** and **Downsample micrographs** is the useful one.

Supplemental Figure 8. Picking method with Cryolo. The form to run the Cryolo picking method using a pretrained network.

Supplemental Figure 9. Consensus picking method with Xmipp. The form of **xmipp3 – deep consensus picking** based on deep learning to calculate a consensus of coordinates, using a pretrained network over several sets of coordinates obtained with different picking methods.

Supplemental Figure 10. Extract particles method. Input and **preprocess** tabs of **xmipp3 – extract particles**.

Supplemental Figure 11. 3D initial volume method with Xmipp. The form of the method **xmipp3 – reconstruct significant** to obtain an initial 3D map. The **Input** and **Criteria** tabs are shown.

Supplemental Figure 12. Resize volume method. The form to make a crop or resize of a volume. In this example, ~~we use~~ this method is used to generate a full size volume after **xmipp3 – reconstruct significant**.

Supplemental Figure 13. 3D initial volume with Relion result. A view of the obtained 3D initial volume with **relion – 3D initial model** method by slices.

Supplemental Figure 14. Refinement of the initial volume with Relion. The form of the method **relion – 3D auto-refine**. ~~We use it~~In this example, it was used to refine an initial volume estimated after consensus. The **Input** and **Reference 3D map** tabs are shown.

Supplemental Figure 15. 3D classification method. Form of **relion – 3D classification**. The tabs **Input**, **Reference 3D map**, and **Optimisation** are shown.

Supplemental Figure 16. 3D alignment based on a deep learning method. The form opened for the method **xmipp3 – deep align**. Here ~~we need~~it is necessary to train a network with a training set, then that network will predict the angular assignment per particle.

Supplemental Figure 17. 3D refinement method. Form of the **xmipp3 – highres** method. Tabs **Input** and **Angular assignment** are shown.

Supplemental Figure 18. First iteration of 3D refinement result. (a) FSC curve. **(b)** Obtained volume (of a smaller size than the full resolution) represented as slices.

Supplemental Figure 19. First iteration of 3D refinement correlation analysis. A new window appears by clicking on the bar icon in the upper part of the window with the list of particles. In **Plot columns** window ~~we can create~~ a histogram of the desired estimated parameter can be

created.

Supplemental Figure 20. Validation overfitting tool. Form of **xmipp3 – validate overfitting** method.

DISCUSSION:

Currently, Cryo-EM is a key tool to reveal the 3D structure of biological samples. When ~~we are able to have~~ good data is collected with the microscope, the available processing tools will allow us to obtain a 3D reconstruction of the macromolecule under study. Cryo-EM data processing is able to achieve near-atomic resolution, which is key to understanding the functional behavior of a macromolecule and is also cru~~t~~cial in drug discovery.

Scipion is a software that allows creating the whole workflow combining the most relevant image processing packages in an integrative way, which helps the traceability and reproducibility of the entire image-processing workflow. Scipion provides a very complete set of tools to carry out the processing; however, obtaining high resolutions reconstructions depends completely on the quality of the acquired data and how these data is processed.

To get a high resolution 3D reconstruction, first ~~we need~~ to obtain good movies from the microscope is required, which preserve structural information to high resolution. If this is not the case, the workflow will not be able to extract high definition information from the~~se~~ data. Then, a successful processing workflow should be able to extract particles that really correspond to the structure and to find the orientations of these particles in the 3D space. If any of the steps in the workflow fails, the quality of the reconstructed volume will be degraded. Scipion allows for using different packages in any of the processing steps, which helps to find the most adequate approach to process ~~your~~ the data. Moreover, thanks to having many ~~tools~~ packages available, ~~we can use~~ consensus tools, ~~–~~that boost the accuracy by finding an agreement in the estimated outputs of different methods, can be used. Also, ~~we have~~it has been discussed in detail in the Representative Results section several validation tools and how to identify accurate and inaccurate results in every step of the workflow, to detect potential problems, and how to try to solve them. There are several checkpoints along the protocol that could help to realize if the protocol is running properly or not. Some of the most relevant are: picking, 2D classification, initial volume estimation, and 3D alignment. Checking the inputs, repeating the step with a different method, or using consensus, are options available in Scipion that the user can use to find solutions when issues appear.

Regarding the previous approaches to package integration in the Cryo-EM field, Appion[31] is the only one that allows real integration of different software packages. However, Appion is tightly connected with Leginon[32], a system for automated collection of images from electron microscopes. So the main difference with Scipion is that data model and storage are less

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coupled. In such a way, to create a new protocol in Scipion, only a Python script needs to be developed. However, in Appion, the developer must write the script and change the underlying database. In summary, Scipion was developed to simplify maintenance and extensibility.

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We have presented in this manuscript a complete workflow for Cryo-EM processing, using the real case dataset of the *Plasmodium falciparum* 80S Ribosome (EMPIAR entry: 10028, EMDB entry: 2660). The steps covered and discussed here can be summarized as movie alignment, CTF estimation, particle picking, 2D classification, initial map estimation, 3D classification, 3D refinement, evaluation, and post-processing. ~~We have used d~~Different packages haven been used and consensus tools were applied in several of these steps. The final 3D reconstructed volume achieved a resolution of 3Å and, in the post-processed volume, ~~we were able to distinguish~~ some secondary structures can be distinguished, like alpha-helices, which helps to describe how atoms are arranged in space.

The workflow presented in this manuscript shows how Scipion can be used to combine different Cryo-EM packages in a straightforward and integrative way to simplify the processing, and obtain more reliable result at the same time.

In the future, the development of new methods and packages will keep growing and software like Scipion to easily integrate all of them will be even more important for the researchers. Consensus approaches will be more relevant even then, when plenty of methods with different basis will be available, helping to obtain more accurate estimations of all the parameters involve in the reconstruction process in Cryo-EM. Tracking and reproducibility are key in the research process and easier to achieve with Scipion thanks to having a common framework for the execution of complete workflows.

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DISCLOSURES:
The authors have nothing to disclose.

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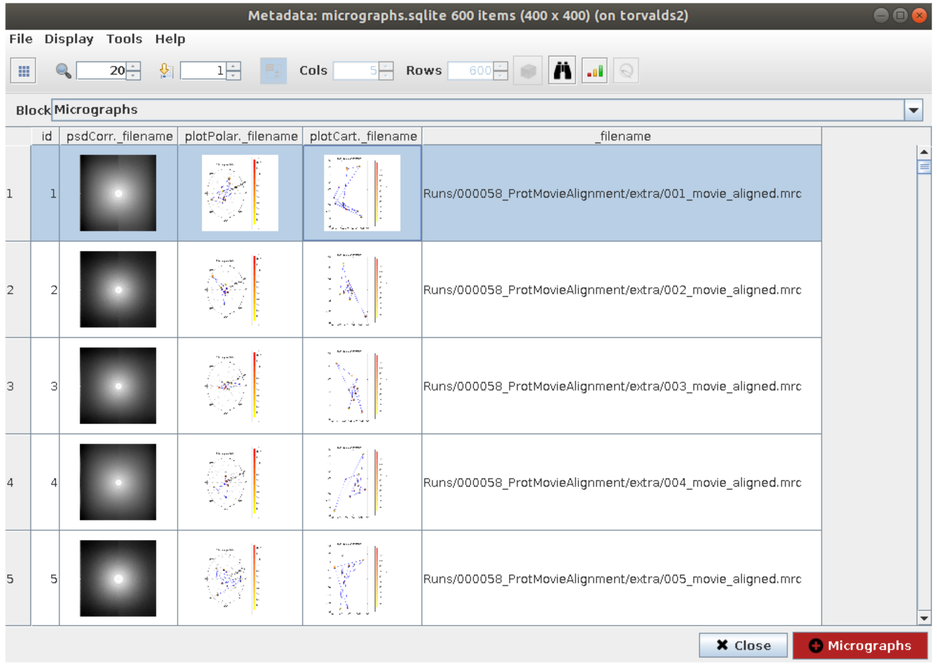

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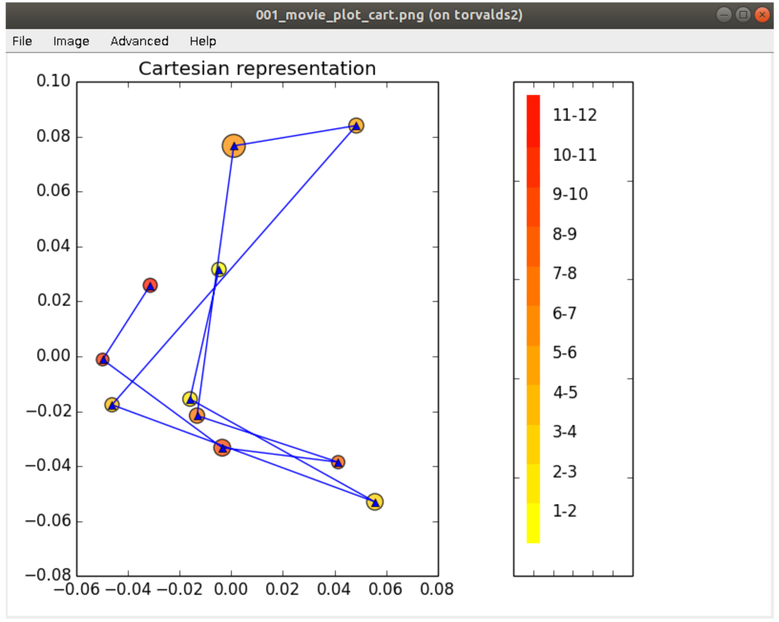

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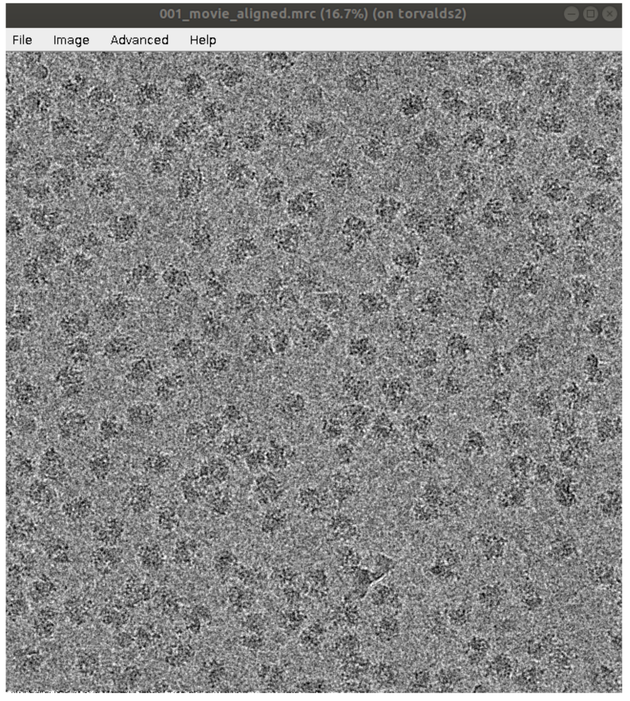

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Figure2

Click here to access/download;Figure;Figure2.pdf

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File Display Tools Help

50 1 Cols 8 Rows 600

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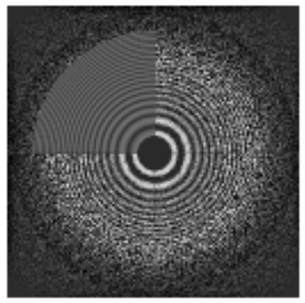
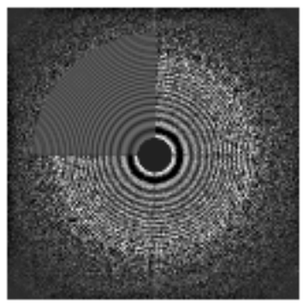
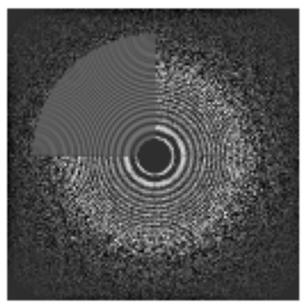
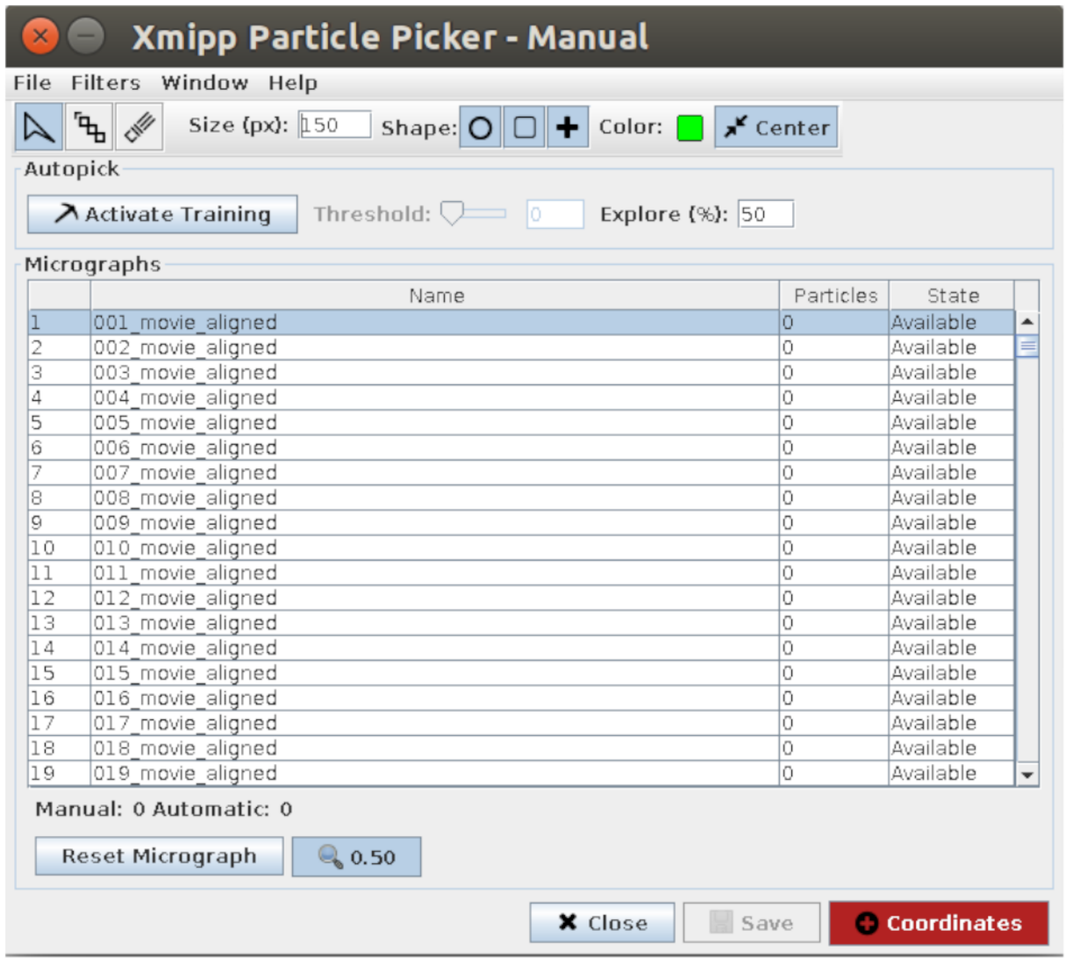
1	1	☑		23469.4004	23327.1309	134.9592	1.0061	Runs/000058_ProtMovieAlignment/extra/001_movie_aligned.mrc
2	2	☑		26284.0996	26071.6797	122.9434	1.0081	Runs/000058_ProtMovieAlignment/extra/002_movie_aligned.mrc
3	3	☑		31894.4199	31646.6699	128.0674	1.0078	Runs/000058_ProtMovieAlignment/extra/003_movie_aligned.mrc

Close Recalculate CTFs Micrographs

Figure3

Click here to access/download;Figure;Figure3.pdf



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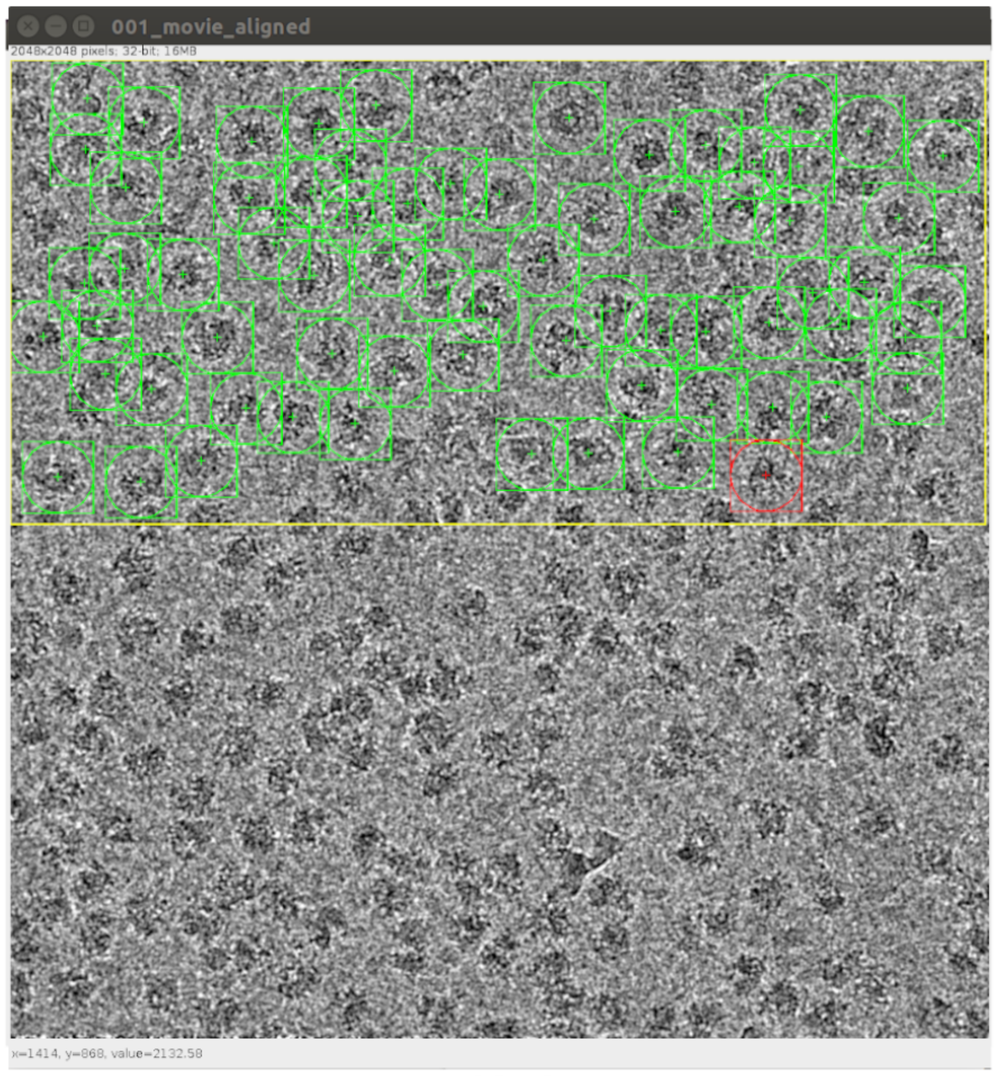

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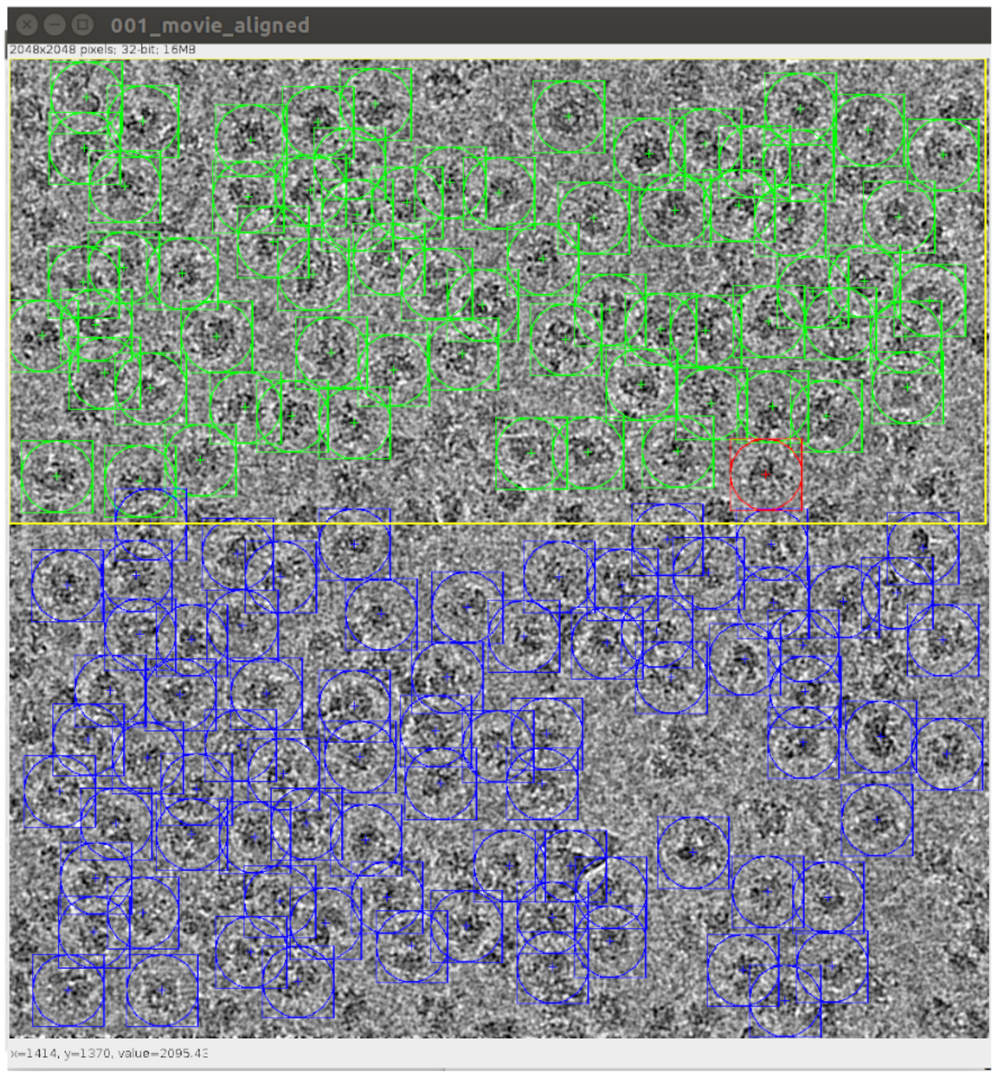

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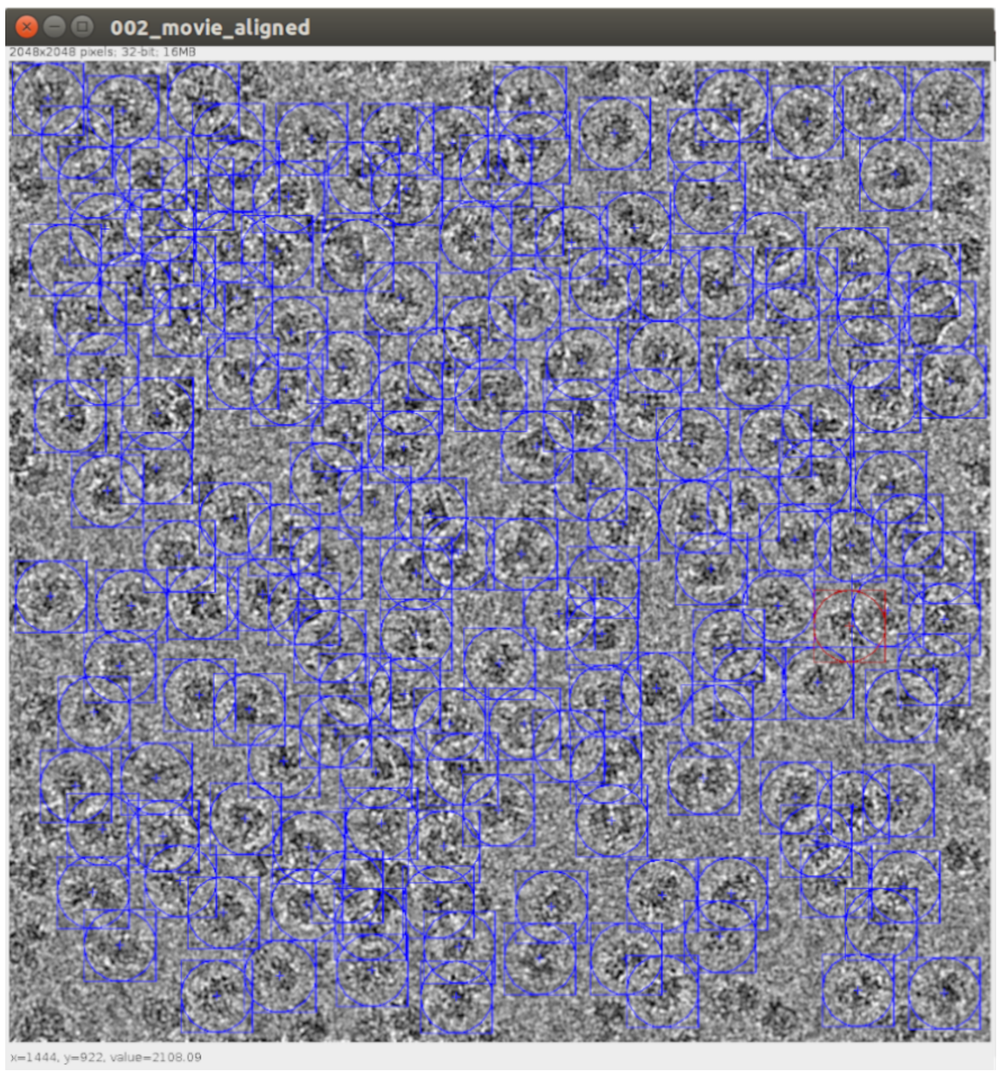

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Figure4

Click here to access/download;Figure;Figure4.pdf

Metadata: particles.sqlite 109188 items (128 x 128)

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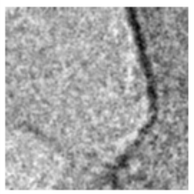
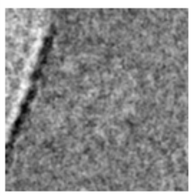
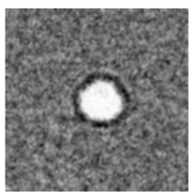
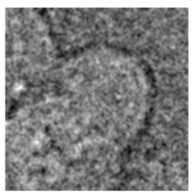
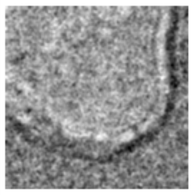
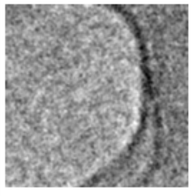
1	58179	☑	165		0.0000
2	13003	☑	205		0.0000
3	91725	☑	50		0.0001
4	57648	☑	118		0.0001
5	52493	☑	163		0.0001
6	29526	☑	88		0.0001

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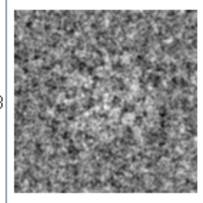
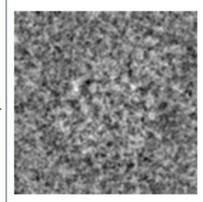
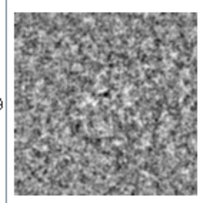
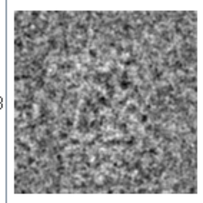
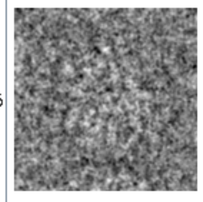
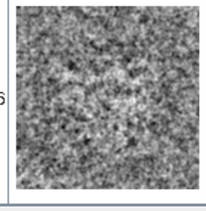
1	24506	☑	113		0.9922
2	66749	☑	201		0.9920
3	51823	☑	89		0.9914
4	28363	☑	168		0.9911
5	44974	☑	95		0.9909
6	44975	☑	96		0.9909

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Figure5

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File Display Tools Help

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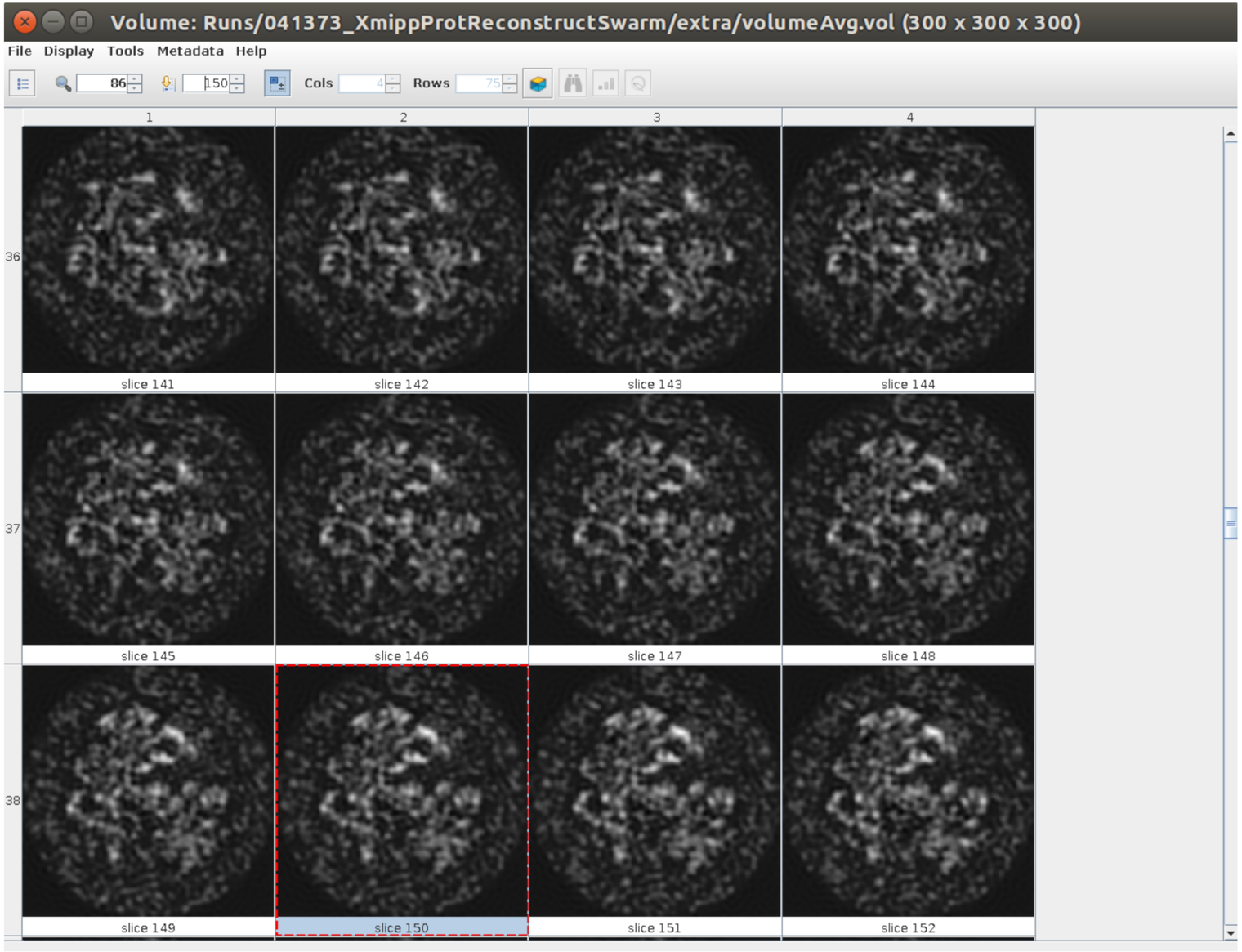

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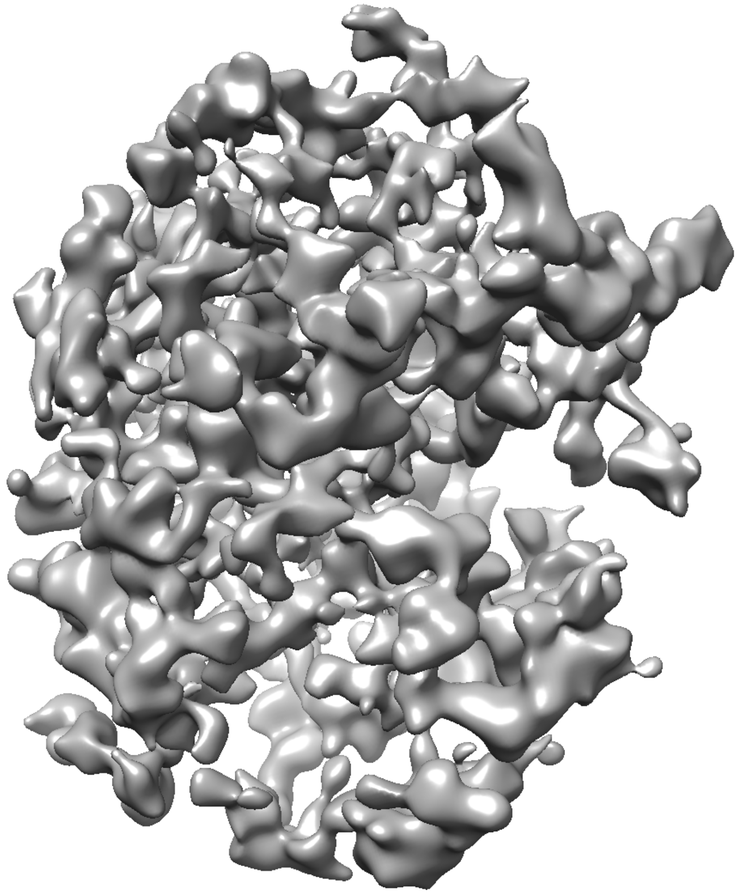

(b)

Figure7

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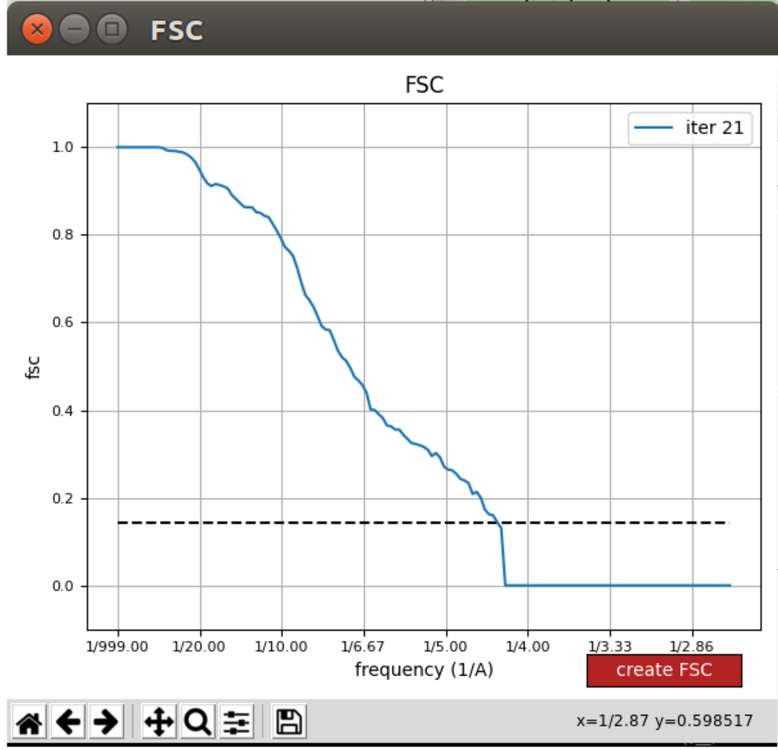
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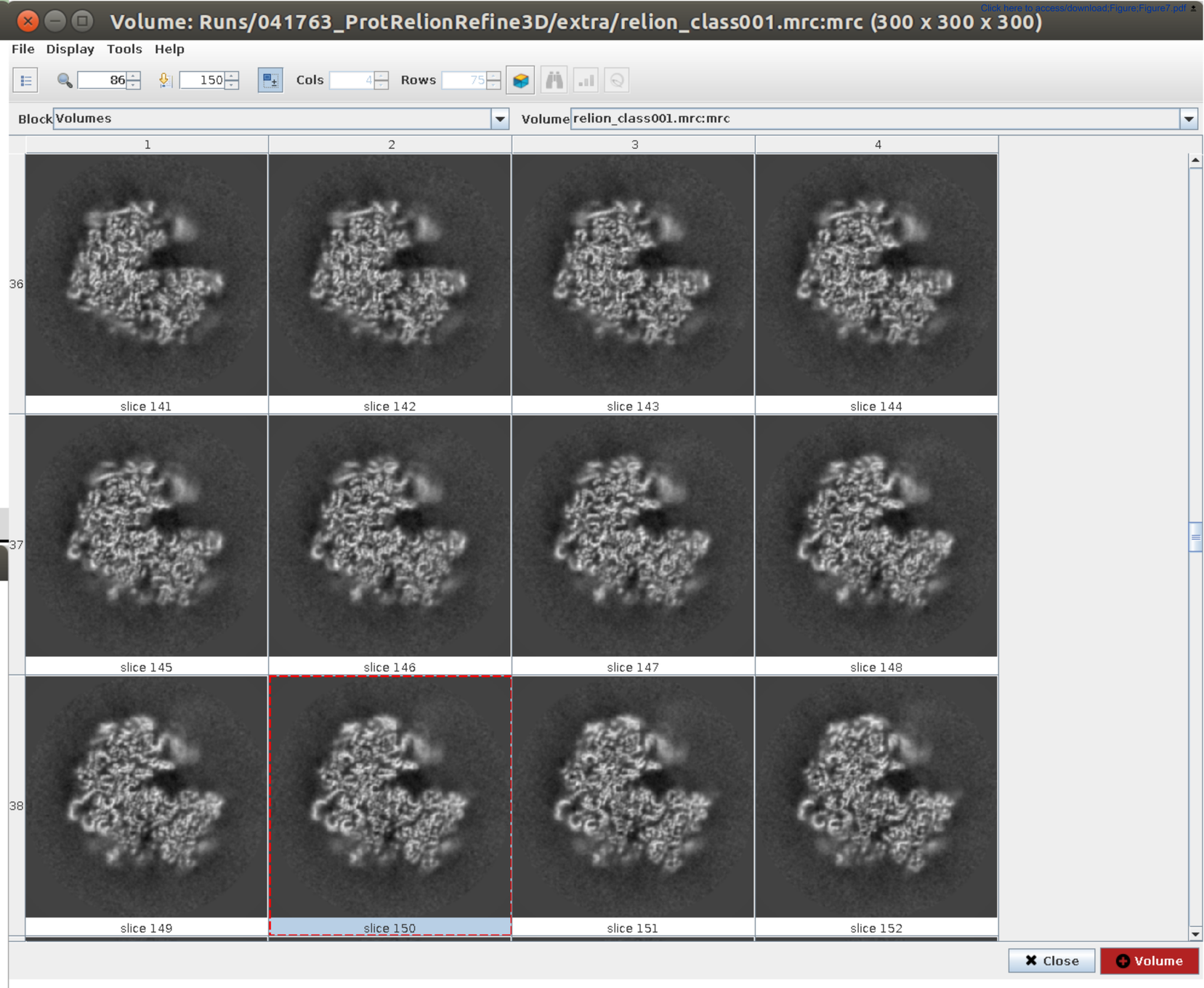
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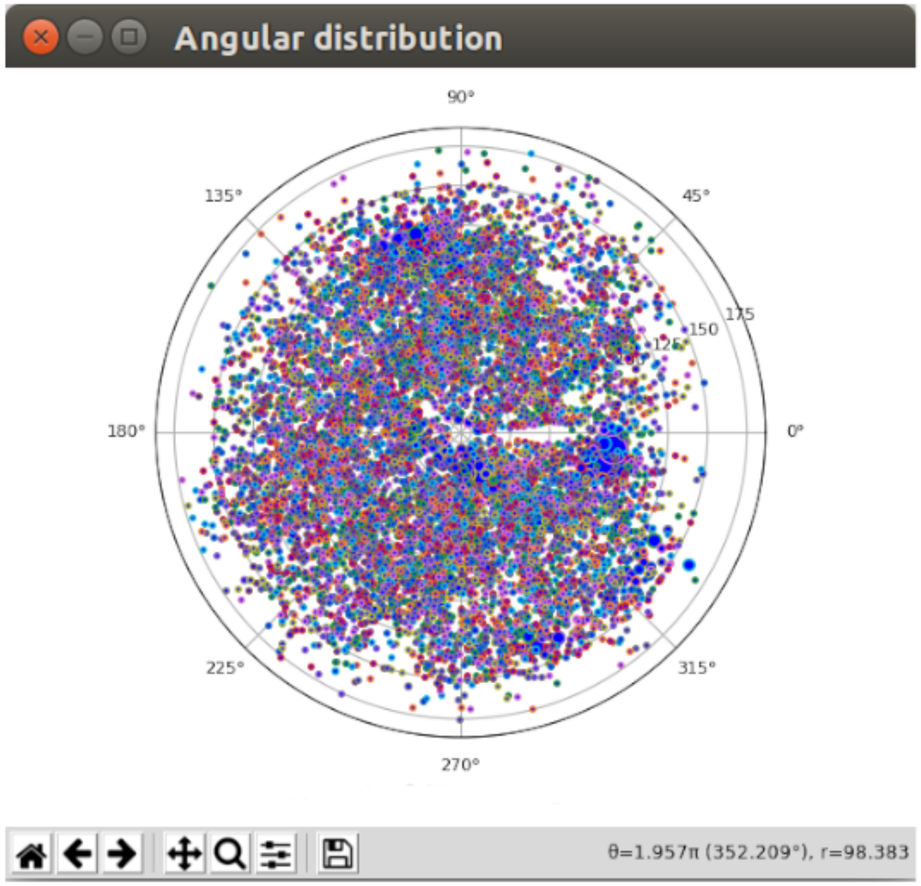

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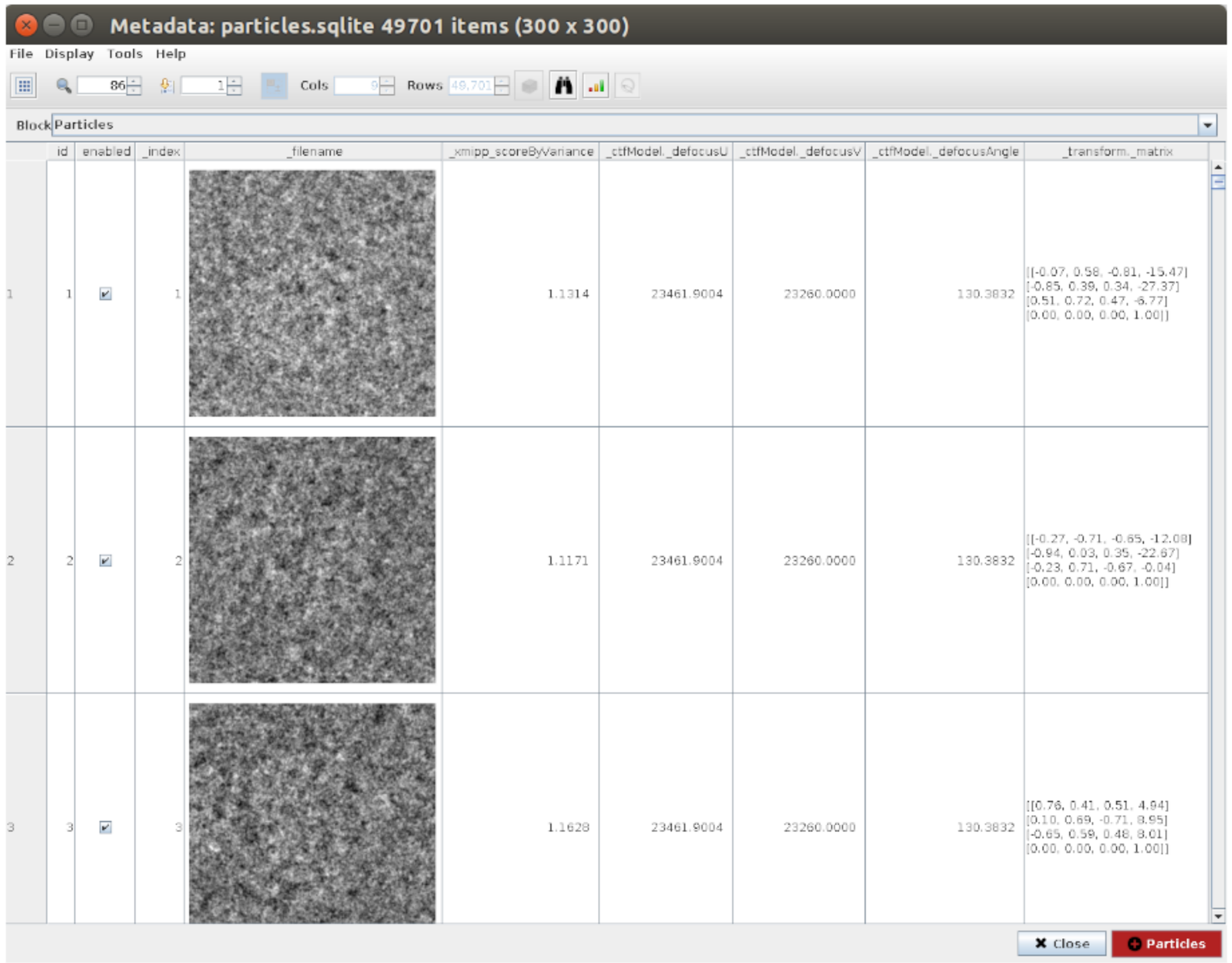

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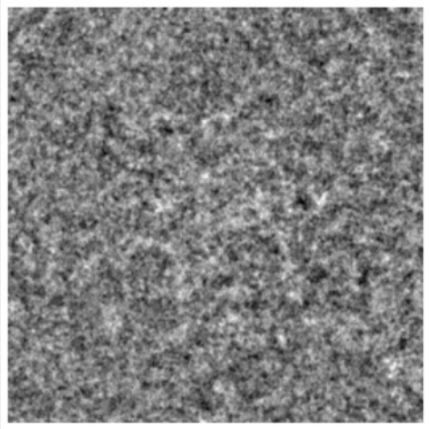
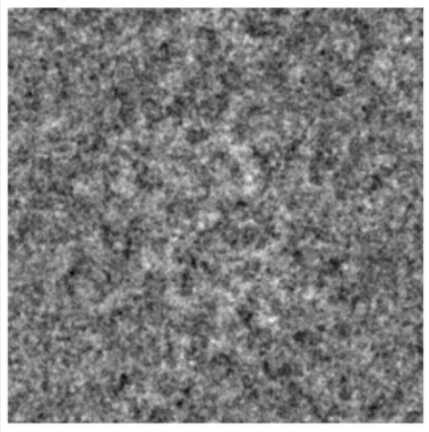
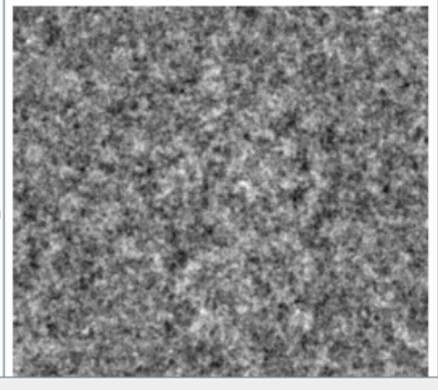
1	32576	☑	37		1.2697	0.0175
2	46903	☑	30		0.4238	0.0315
3	26270	☑	10		0.8290	0.0558

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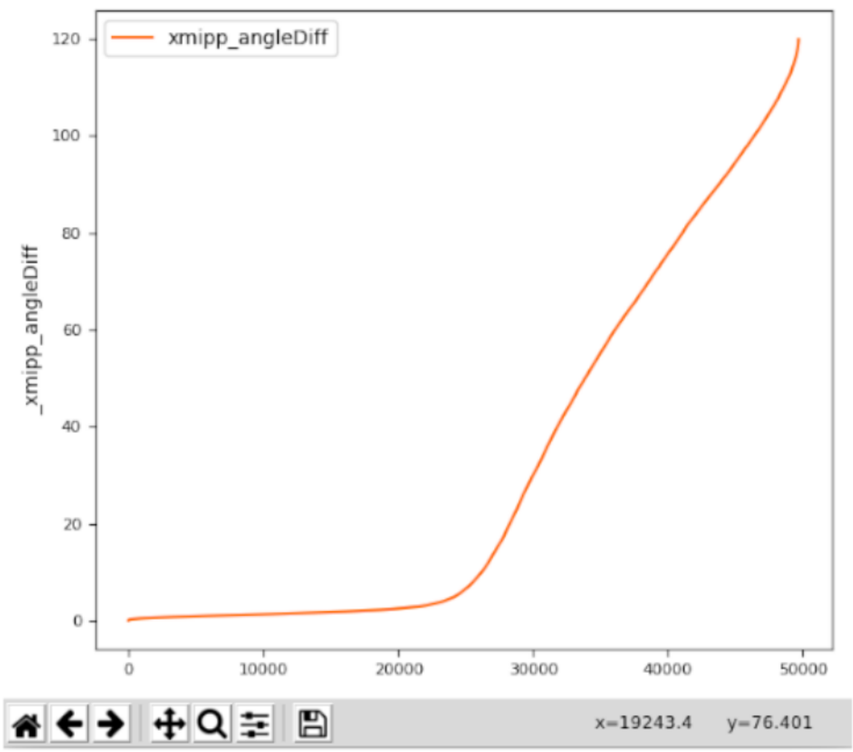

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Figure 2

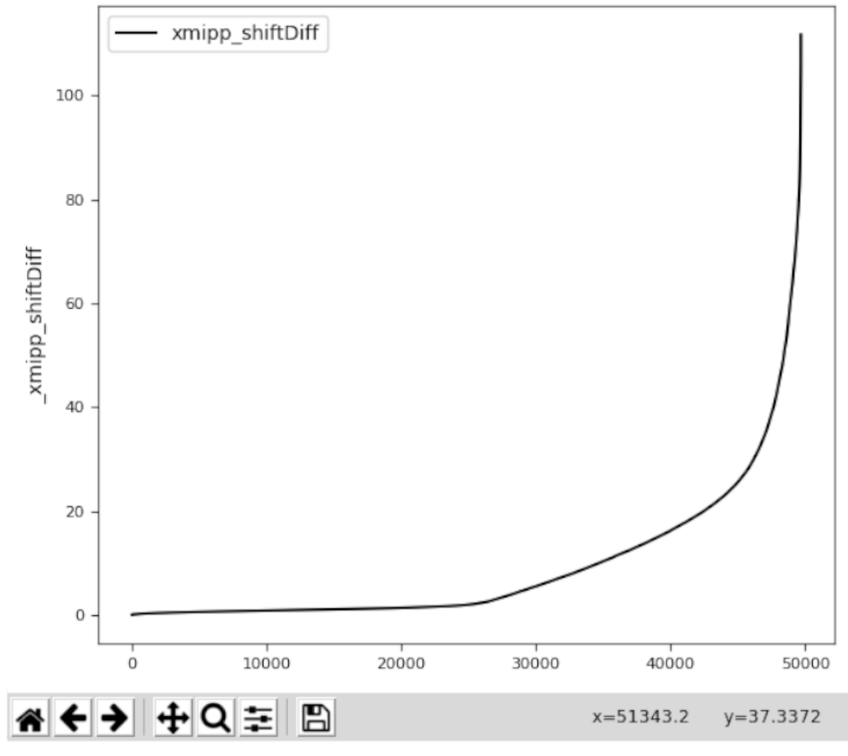

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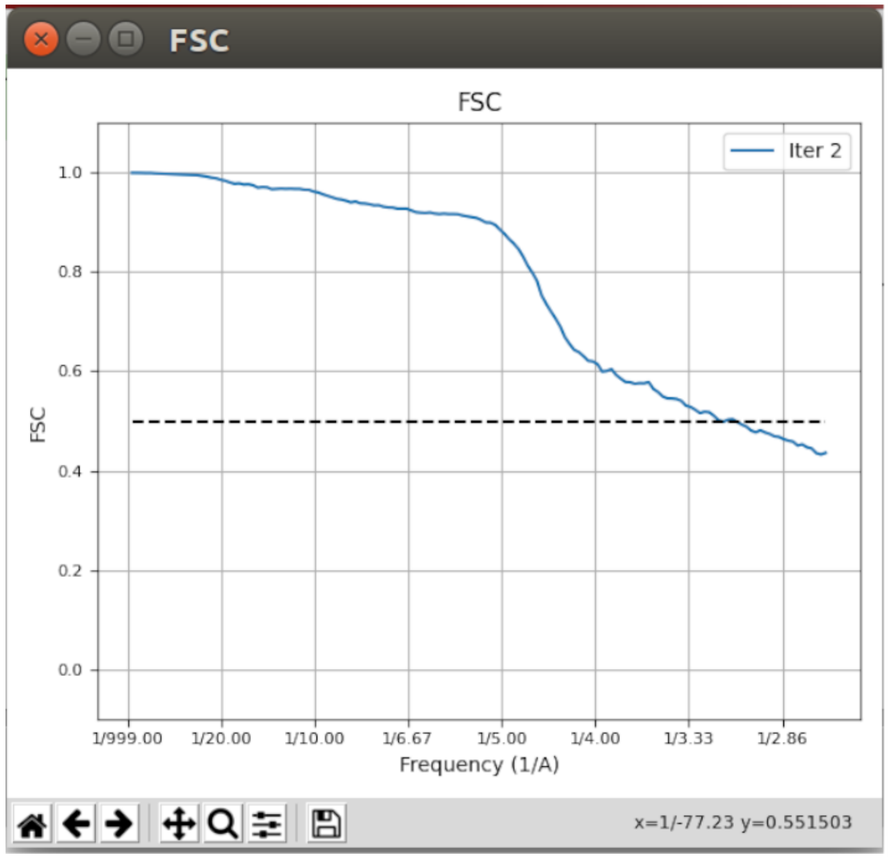
Figure10

Click here to access/download;Figure;Figure10.pdf



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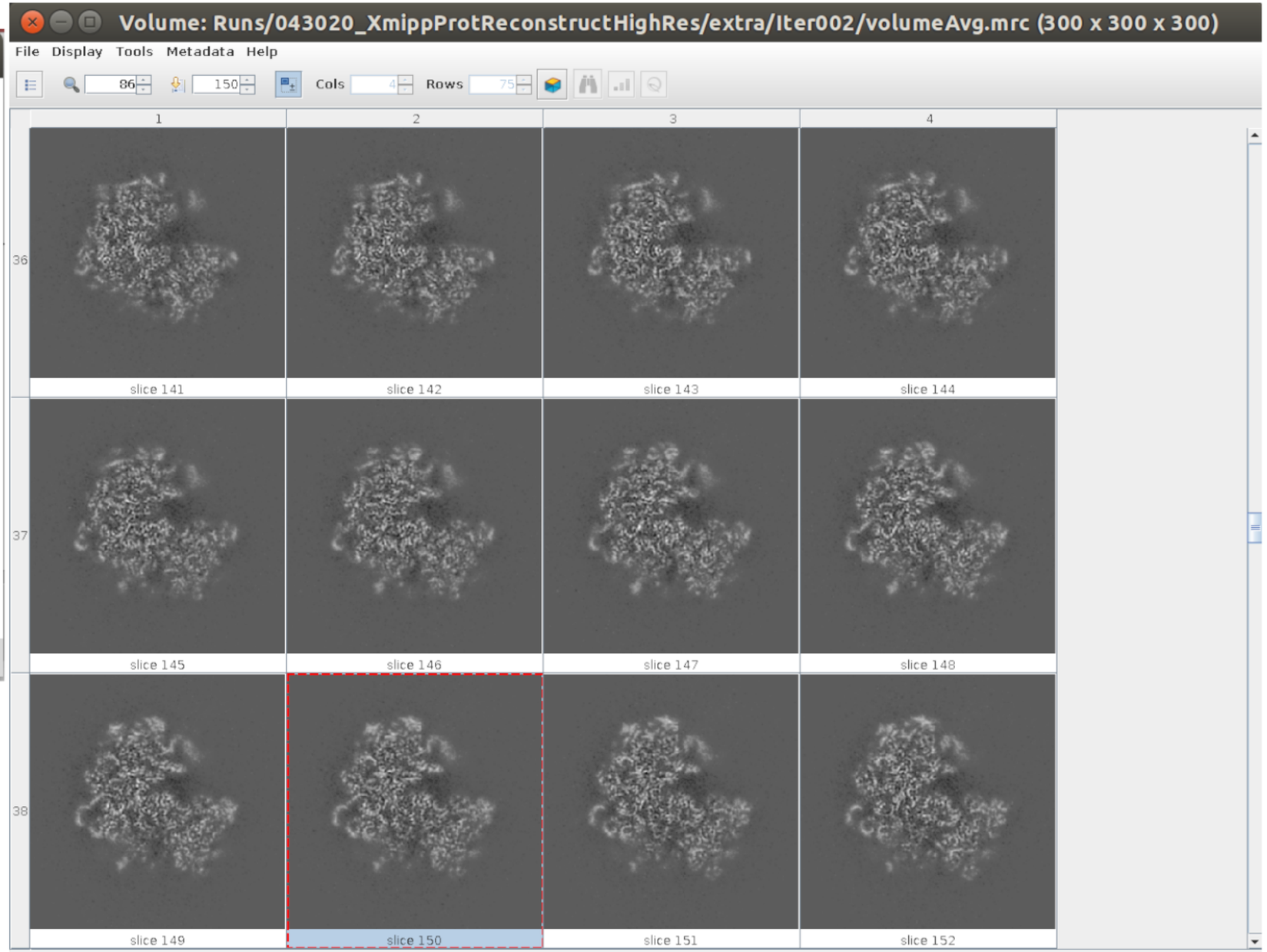

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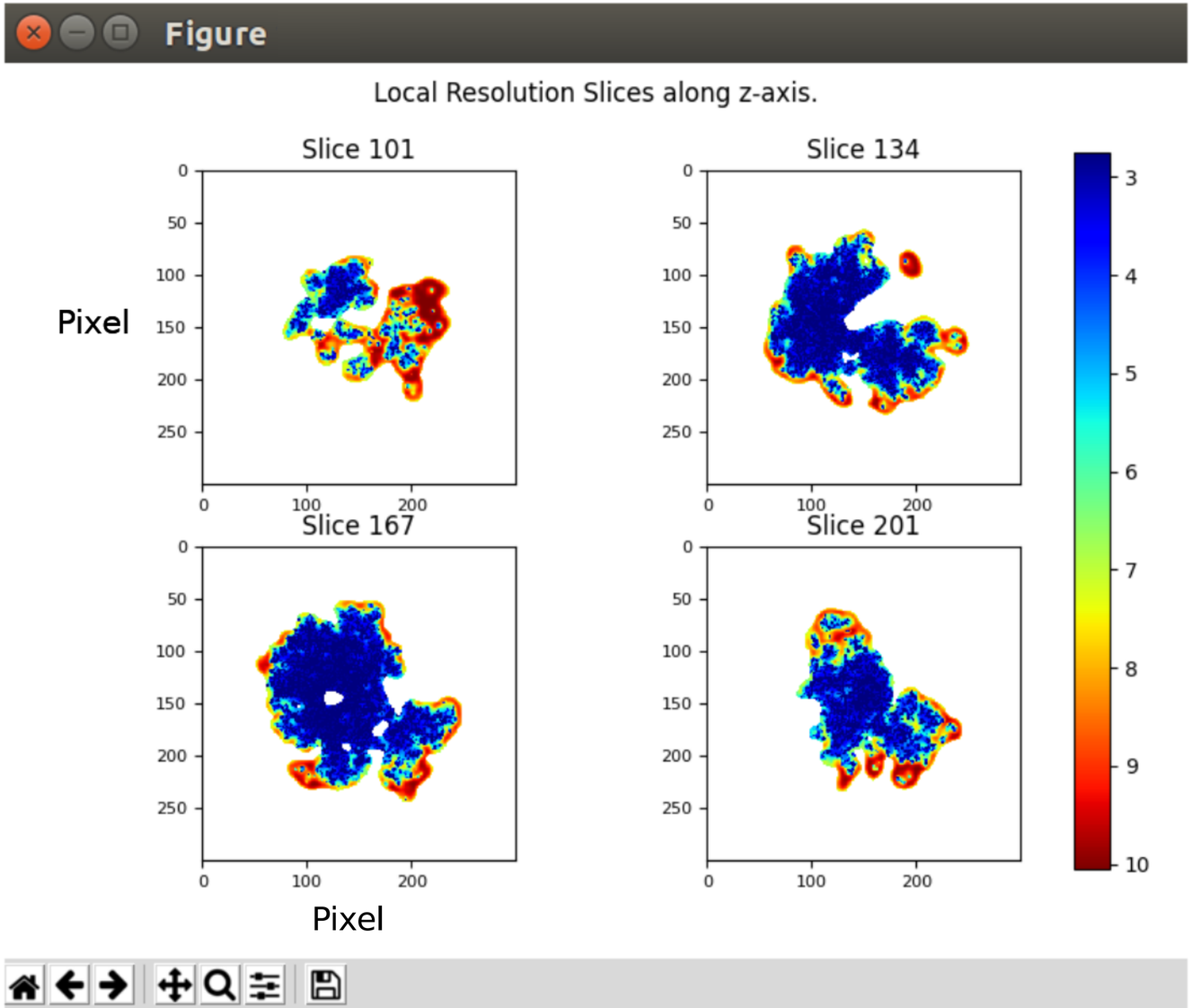

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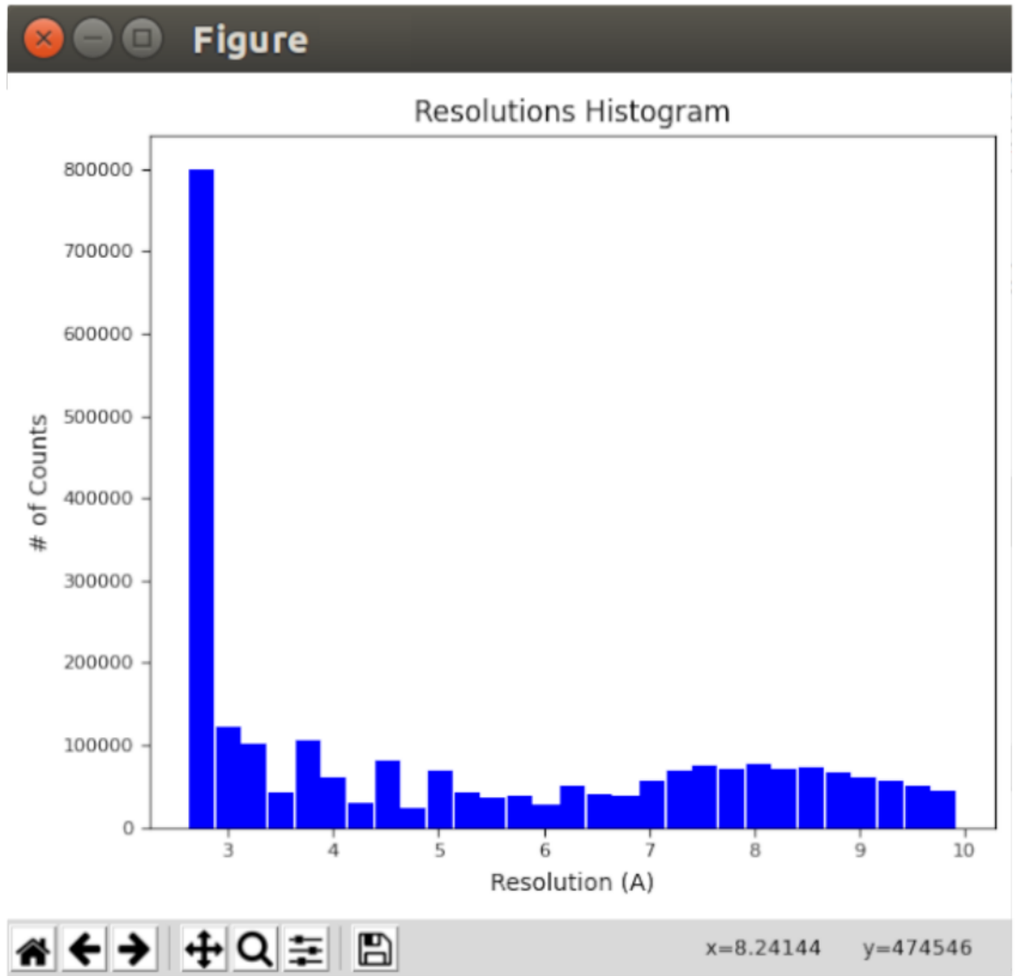

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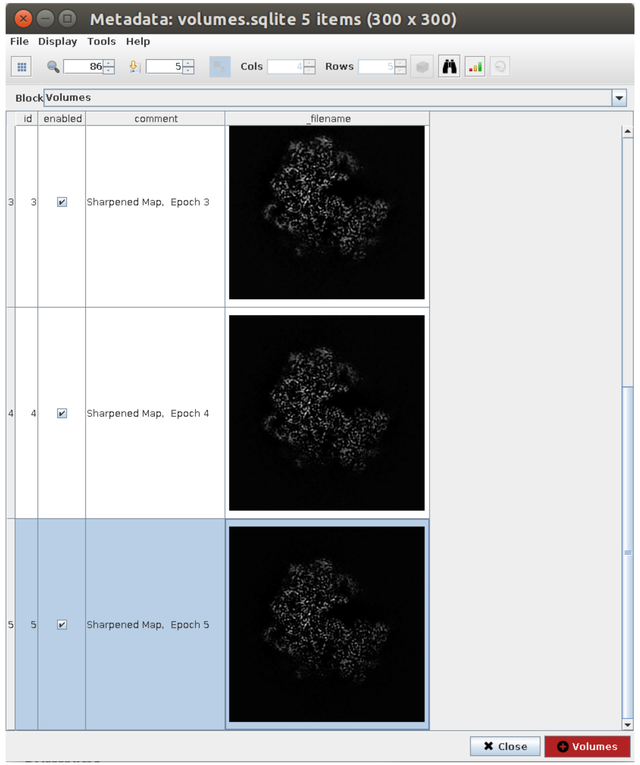

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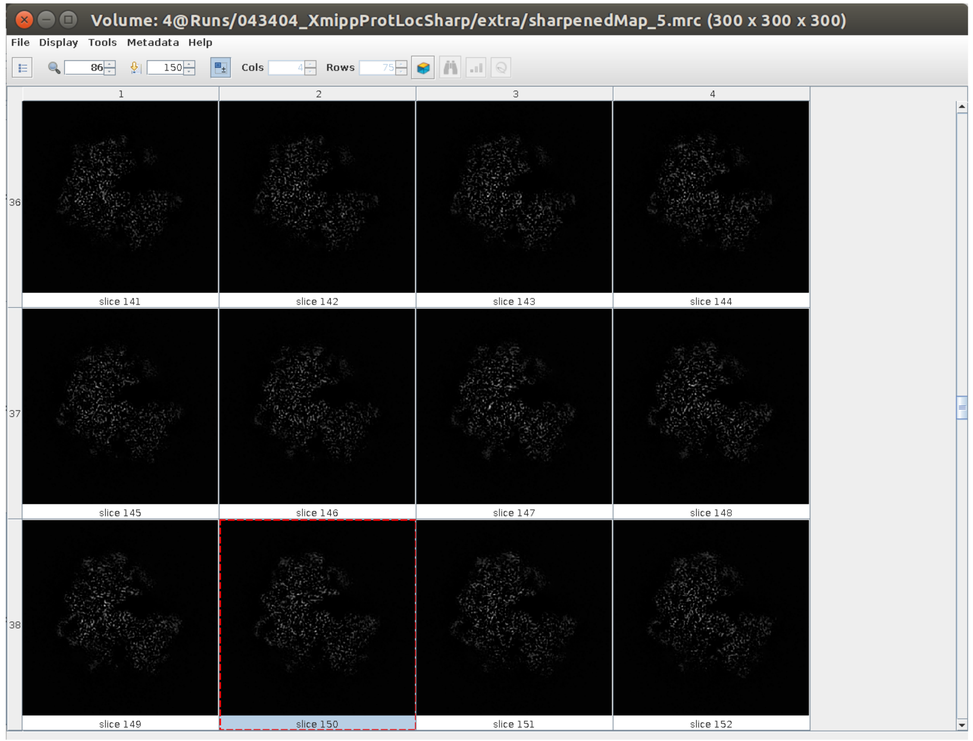

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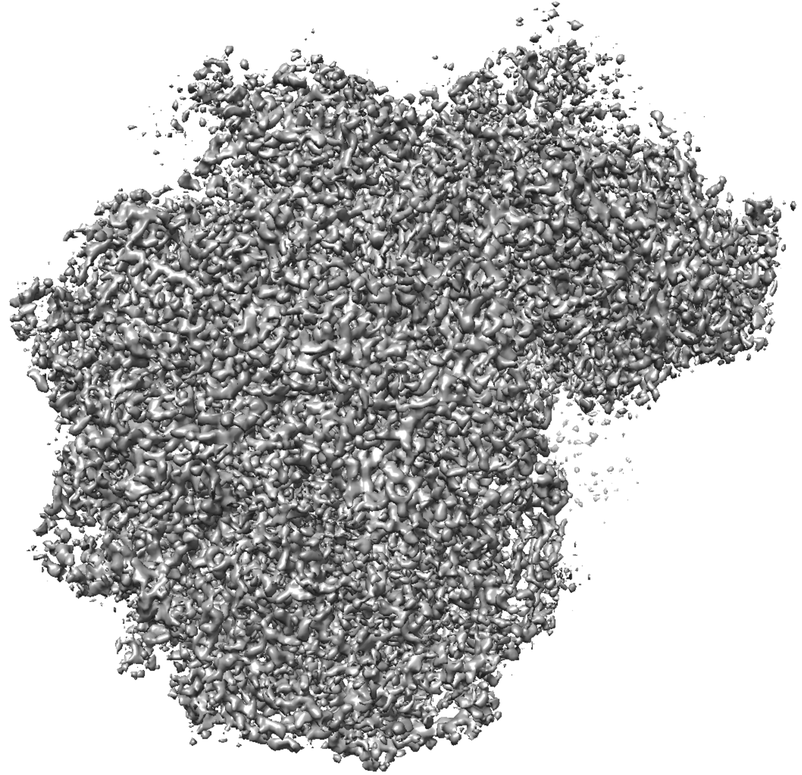

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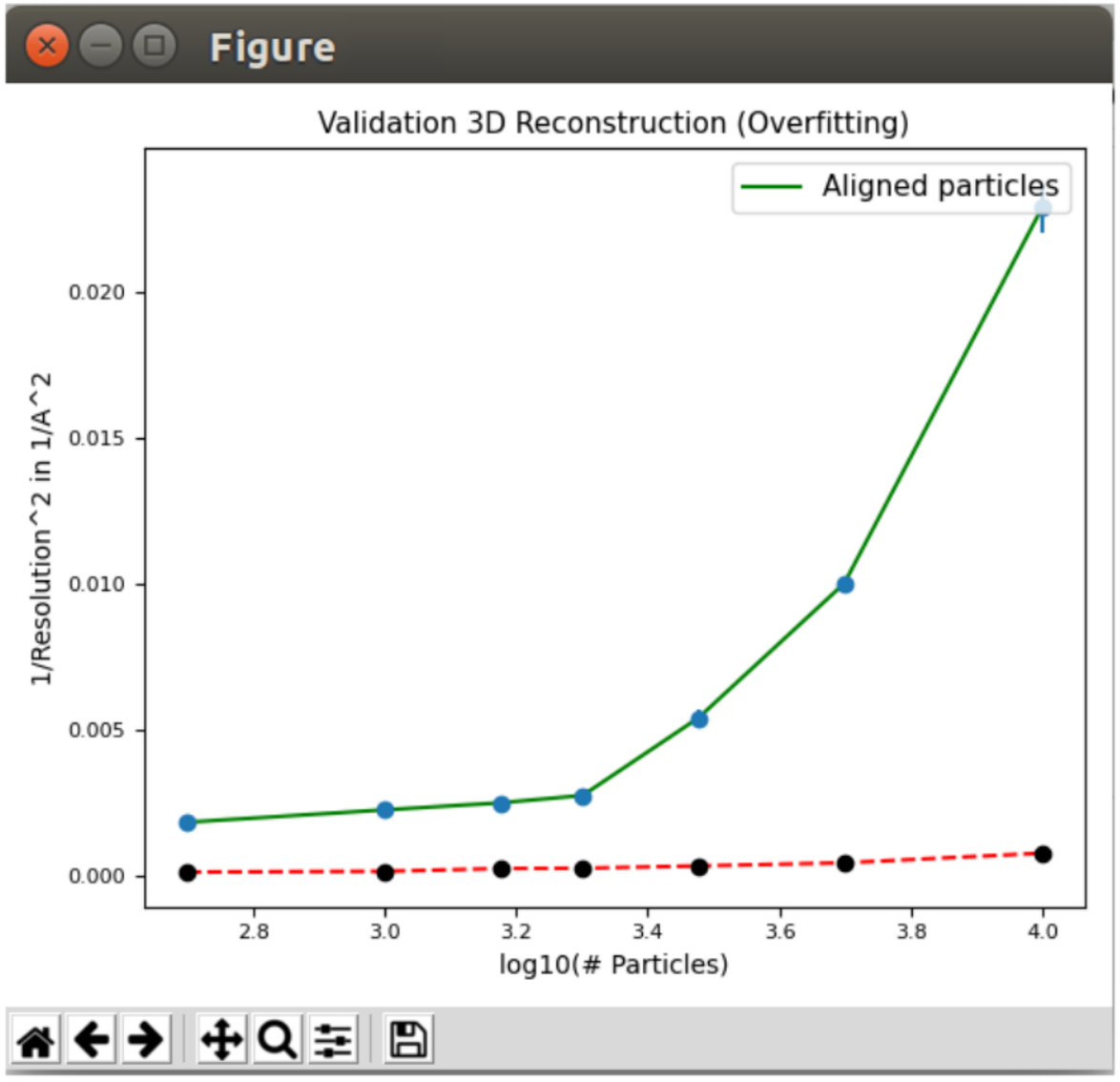
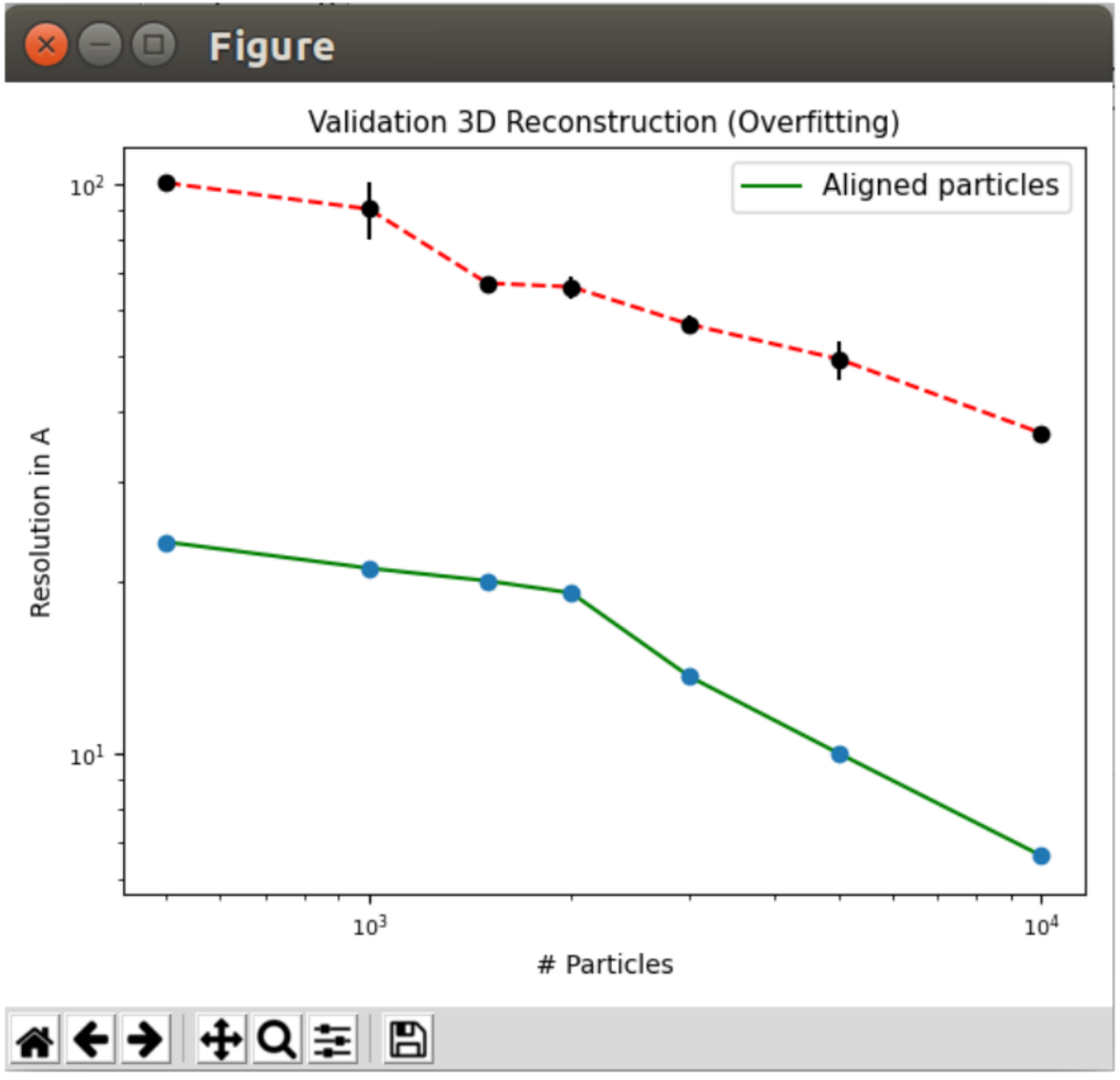
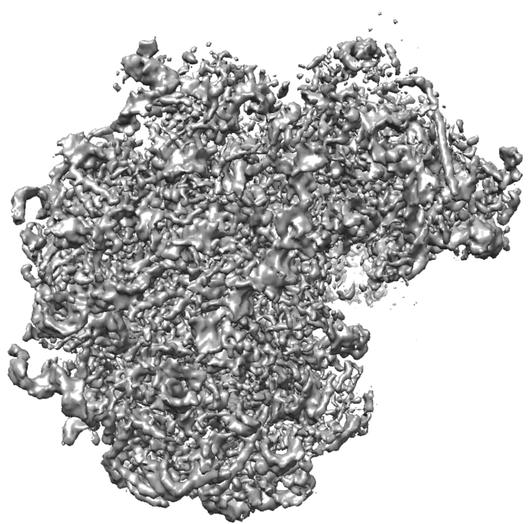


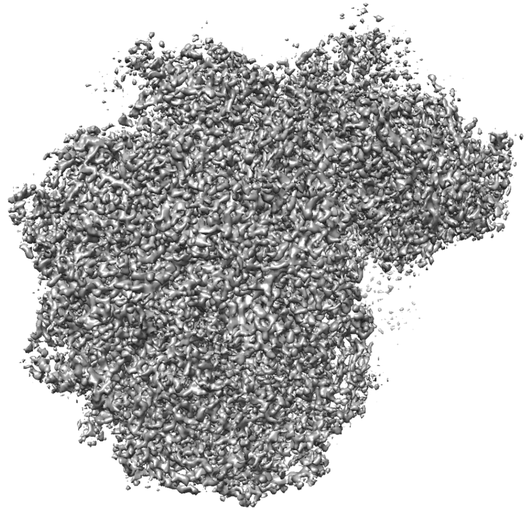
(a)



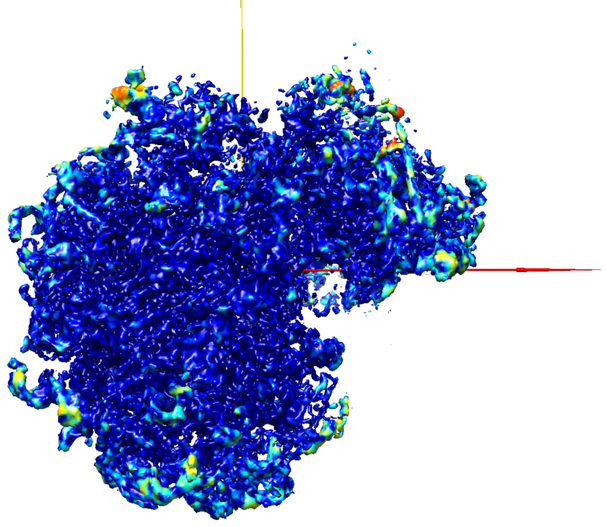
(b)



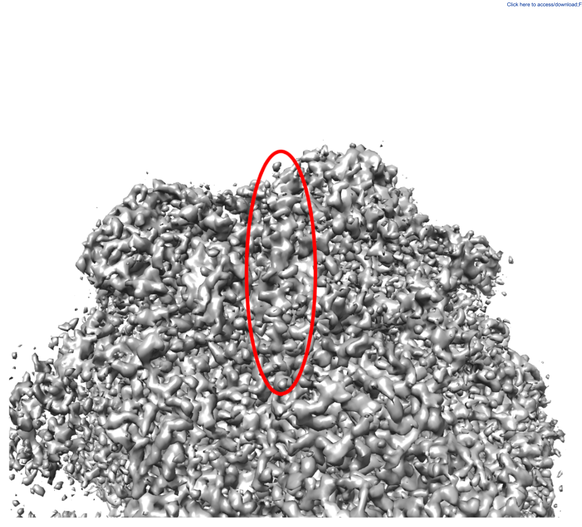
(a)



(b)

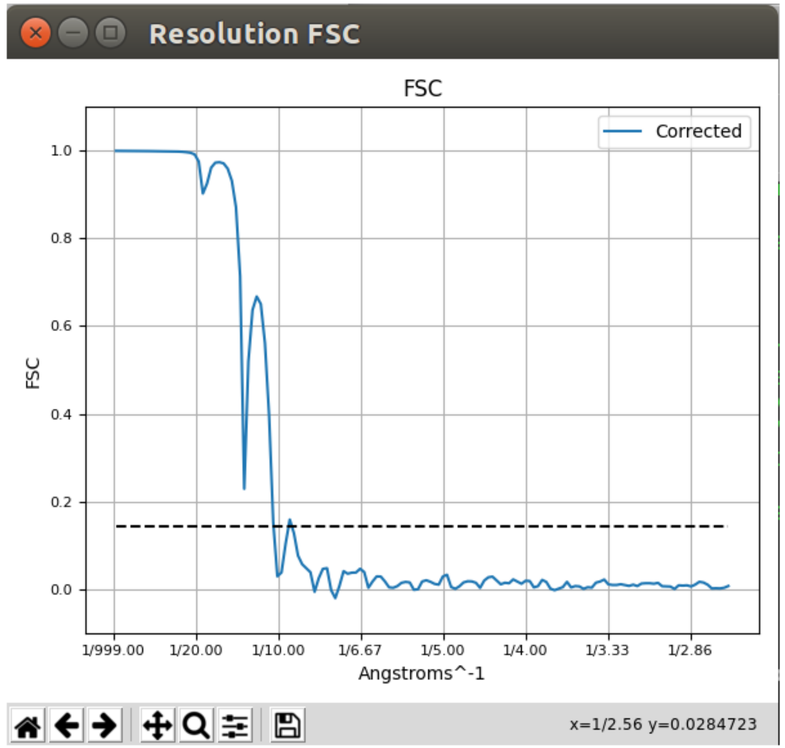


(c)

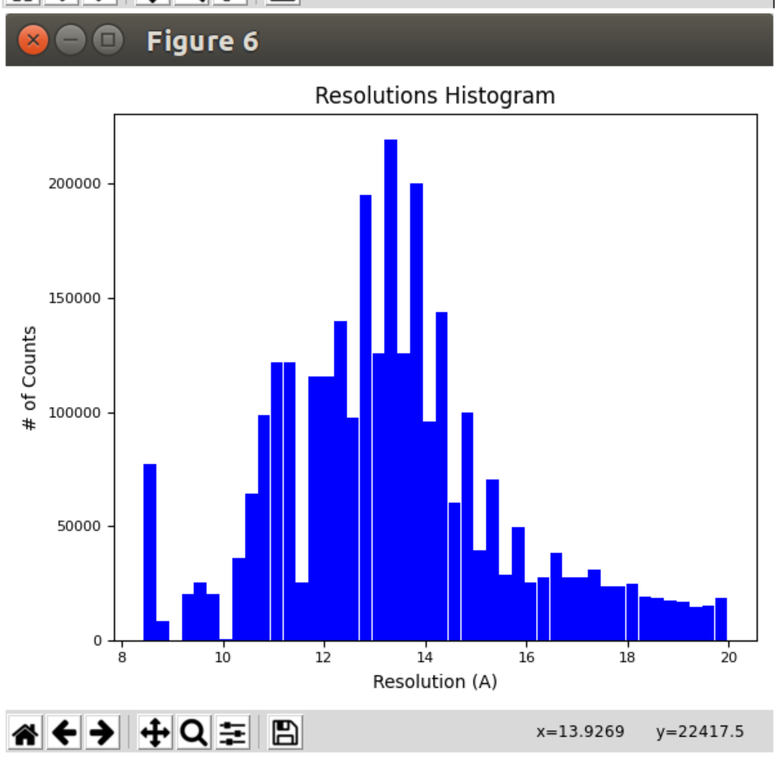


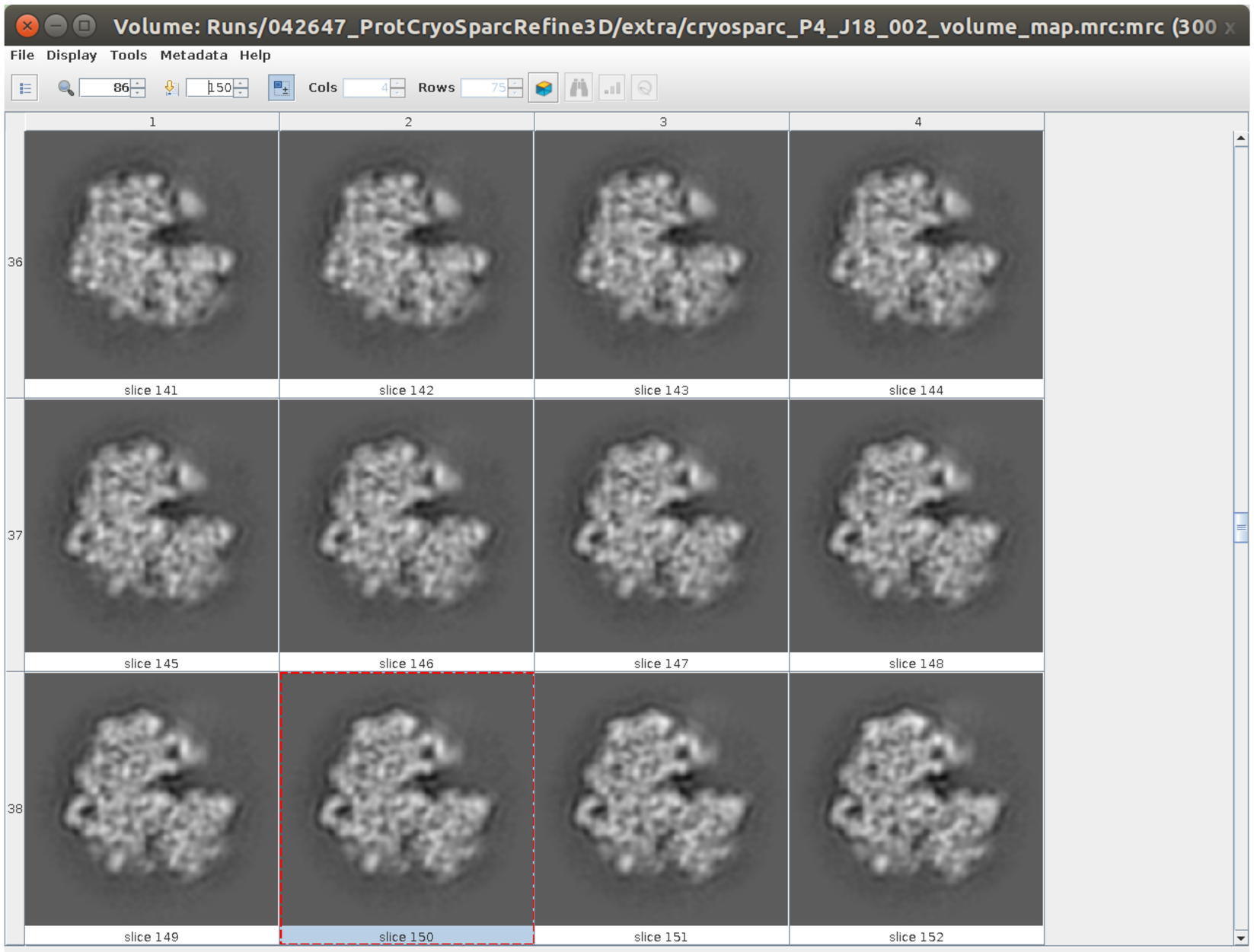
(d)

(a)

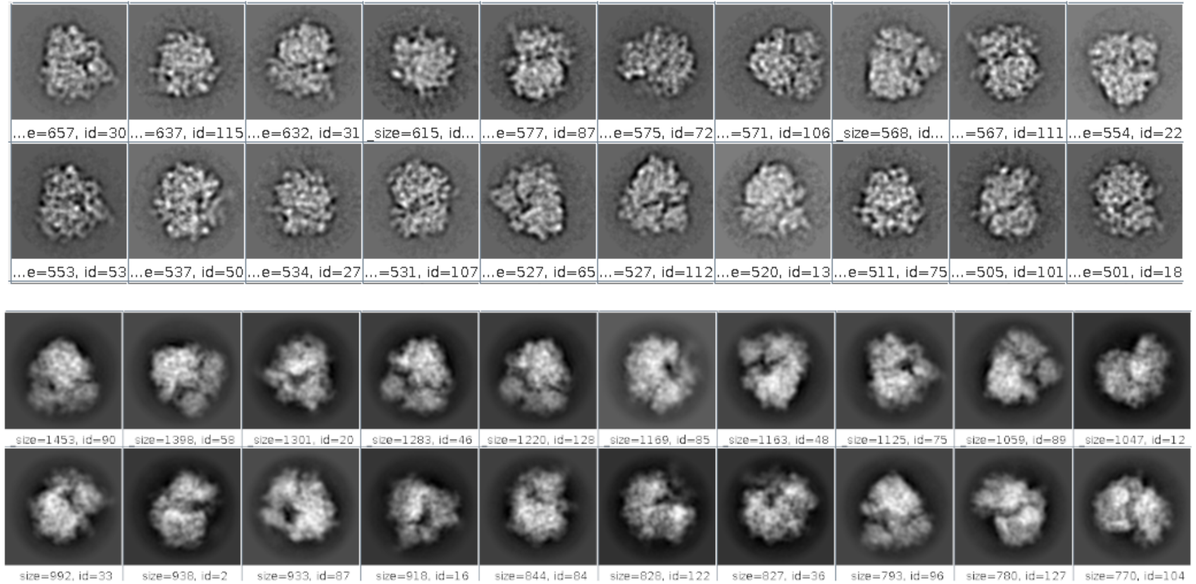


(b)

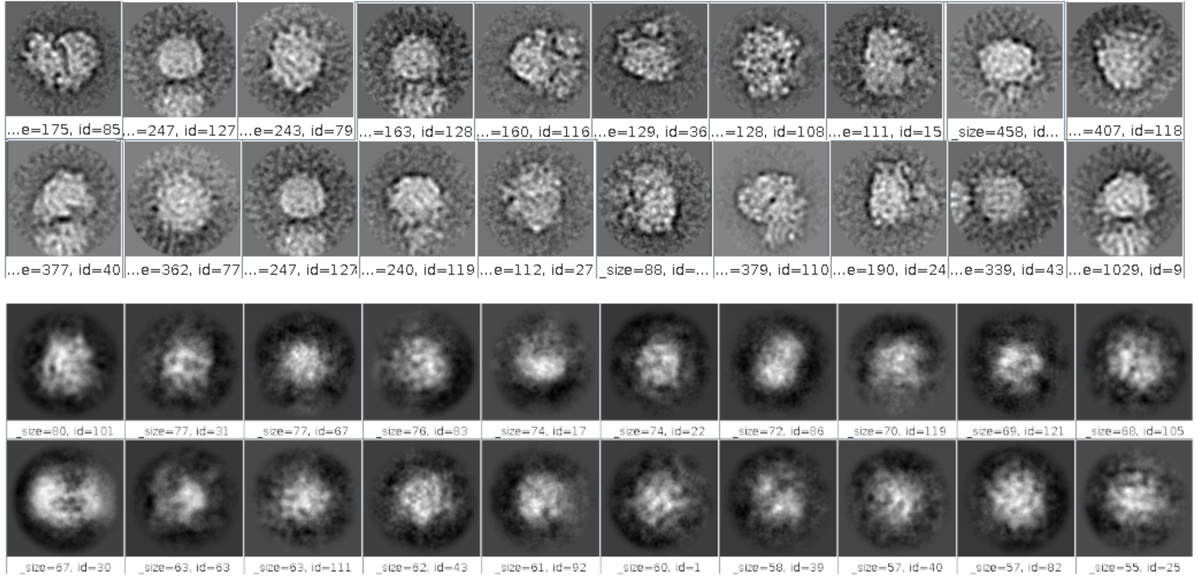




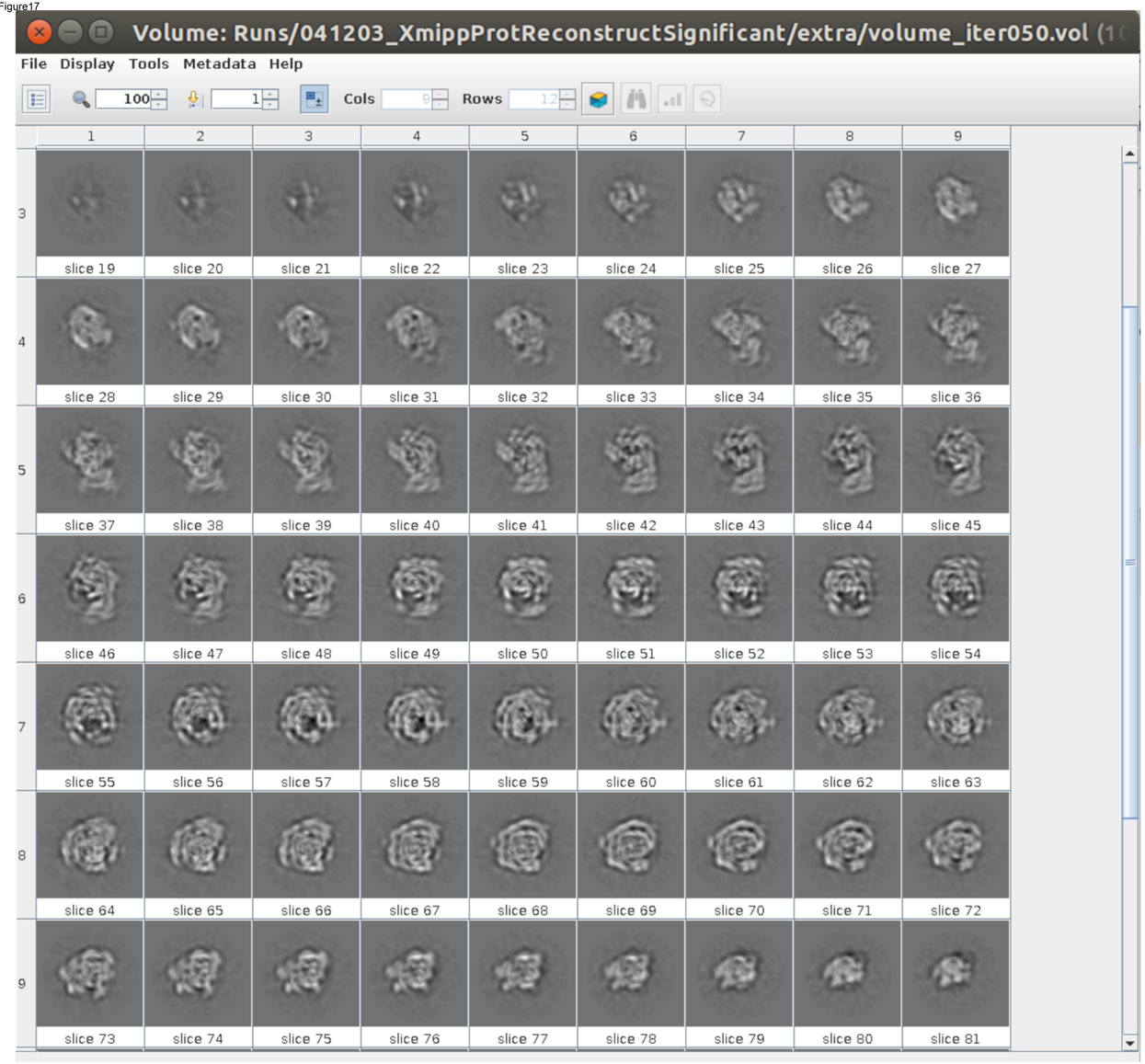
(c)



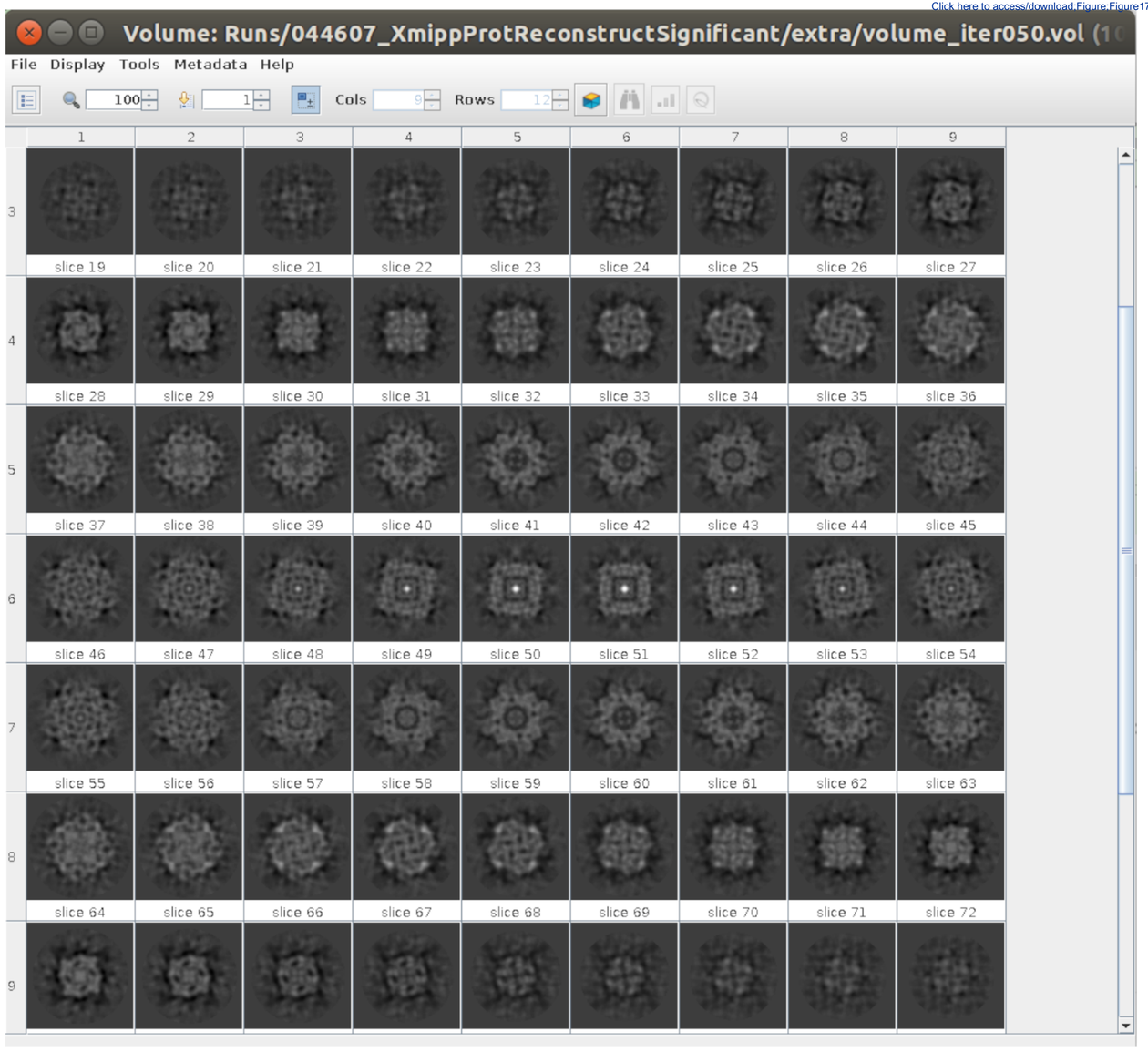
(a)



(b)



(a)



(b)

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
no material is used in this article	-	-	-

Dear Dr. Maluenda,

Your manuscript, JoVE62261 "Processing Workflow in Scipion for Single Particle Data in Cryo-Electron Microscopy," has been editorially and peer reviewed, and the following comments need to be addressed. Note that editorial comments address both requirements for video production and formatting of the article for publication. Please track the changes within the manuscript to identify all of the edits.

After revising and uploading your submission, please also upload a separate rebuttal document that addresses each of the editorial and peer review comments individually.

Your revision is due by Jan 07, 2021.

To submit a revision, go to the JoVE submission site and log in as an author. You will find your submission under the heading "Submission Needing Revision". Please note that the corresponding author in Editorial Manager refers to the point of contact during the review and production of the video article.

Best,

Vidhya Iyer, Ph.D.
Review Editor
JoVE
vidhya.iyer@jove.com
617.674.1888
Follow us: Facebook | Twitter | LinkedIn
About JoVE

Dear PhD Vidhya Iyer,

thank you very much for your efforts as editor of our manuscript. We have changed the manuscript following the editorial recommendations and those of the reviewers. We hope these changes could improve it.
In the following lines, we have answered all the comments.

Regards

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
2. Please provide an institutional email address for each author.
3. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).
4. Please define all abbreviations before use (CTF, FSC).
5. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol.

Any text that cannot be written in the imperative tense may be added as a "Note." However, notes should be concise and used sparingly.
6. The Protocol should contain only action items that direct the reader to do something. Please move the discussion about the protocol to the Discussion.
7. Please include a one line space between each protocol step. Then, revise the highlighting to be 3 pages or fewer to ensure that the videography can occur in a single day.

We have followed all these recommendations. The text has been carefully reviewed to assure that it follows the recommended style.

8. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

The Discussion has been modified to include these points.

9. Please reduce the number of figures presented. Some of the figures may be unnecessary with the video of the protocol. Please upload the figures that represent the output of your specific protocol and include the discussion of those representative figures in the Representative Figures section. If the figures are only to help the scriptwriter visualize the computational steps, then they can be uploaded as Supplemental Files.
10. Please consider removing the screen shot appearance from the figures presented in representative results.
11. Figure 19: Please remove the figure numbers in the black box represented on the top of the figure. Please include details of the X and Y axis (Figure 19 a).
12. Figure 21: Please remove the figure numbers in the black box represented on the top of the figure.
13. Figure 22: Please define the color code presented in the Figure Legends. Please mention the red oval (d) in the Figure Legends.

The number of Figures have been reduced and the issues solved.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:
Image processing protocols in the integrative Scipion package are described. This is an excellent contribution that I find highly suitable for publication in JoVE. I have the following minor points that the authors may wish to address before publication (see below).

Major Concerns:
None

Minor Concerns:

Line 3: I find the title "Processing Workflow in Scipion for Single Particle Data in Cryo-Electron Microscopy" a bit oddly formulated. Maybe "Cryo-EM and single-particle analysis with Scipion" would suffice? Or "Dynamic workflows for cryo-EM and single-particle analysis with Scipion" to emphasize that Scipion provides many alternative image processing tools as part of the workflow.

Line 23: Single-particle analysis in Cryo-electron microscopy. I believe the authors mean "Cryo-EM and single-particle analysis".

Line 24: Scipion software provides… Given that the title already establishes what Scipion is, I think it is sufficient to say "Scipion provides the computational tools…"

Line 36: Scipion software -> Scipion

Line 51: successfully determining biological 3D structures with near-atomic resolution. I believe the authors mean "successful determination of biological 3D structures at near-atomic resolution".

Line 57: Shortly -> Briefly

Lines 61-67: I believe that the authors try to describe that we are dealing with an inverse problem that is ill-posed and has many nuisance variables. This description needs to be re-written for improved clarity.

Line 116: Scipion software -> Scipion

Thanks for your recommendations. We have followed all of them.

Reviewer #2:

The authors present a workflow for using the new Scipion 3, including several deep learning integrations and consensus tools. The workflow appears to be fine and should be turned into a proper JoVE video tutorial. I have no major comments.

Minor edits:

-Line 106: "Finally, in the last steps, …" ?
-Line 550: "workflow or"
-Line 586: "Trying"
-Line 778: "crucial"

Thanks for your recommendations. We have solved all these issues.

Reviewer #3:

The authors describe a protocol for single particle analysis of cryoEM data in Scipion. As a particular test case, they use cryoEM data of the malaria parasite ribosome. The introduction describes the essentials of a generic single particle averaging workflow. Scipion is then

introduced, with emphasis on its so called consensus tools that allow checking for consistency between results from different programs. The protocol gives an in-depth description how to determine a high-resolution map of the ribosome from data that is publicly available. Finally, the authors discuss how to handle typical problems.

The paper is a useful contribution to cryoEM field and will help both novice and expert users to carry out cryoEM data processing workflow in Scipion and to use its many different tools for data visualisation and validation. I have only a few suggestions, one regarding putting possibly less emphasis on consensus methods (or alternatively giving more details how useful they actually are), and few more regarding the workflow itself that seems overly complicated in places. The rest of the comments could help to further clarify the text.

The authors could still consider what to emphasise in the introduction. Traceability and reproducibility, ability to easily combine different methods, abstraction of different EM data objects (separation from files, formats and their locations) are all key benefits of Scipion. Emphasising just the consensus tools may not be entirely warranted / balanced. Why would a consensus result between one superior method and several lesser methods be better than than using one superior method alone? Have the benefits or some examples published? If yes, please add some references and discussion to justify this emphasis. If not, and if this is just assumed to be a good strategy, it could be that the consensus methods are emphasised too much. It is of course possible that in some cases these tools are beneficial, and it is nice that Scipion offers this option. It is thus appropriate to demonstrate different consensus tools in the protocol, but perhaps they could be marked as optional in relevant places (if they were indeed not crucial for the outcome) to not overcomplicate the presented workflow.

We have focused on the consensus method as we think it's a more novel point to highlight. Traceability, reproducibility, the combination of methods, etc. are also mentioned in the manuscript but these points have been previously covered[1] and we think it's valuable to highlight now the new consensus tools. However, we have given more weight in the introduction of the reviewed manuscript to these points also.
We have published several papers that prove in detail the benefits of the consensus[2,3]. These citations now are more highlighted in the manuscript to show to the reader where to find stronger proof of the consensus value. Also, we are preparing two more manuscripts in this regard[4,5].
Moreover, in the text we have detailed now that these tools are optional, and that a simpler workflow could be built.

Lines 112-114: Would be useful to briefly mention how the map gives positions of atoms. I.e. building of atomic models de novo or fitting of existing models.

Line 183: Please check that grigoriefflab - ctffind is correct (instead of cistem – ctffind4)

Line 219: Particle picking: I find it confusing that two separate methods both produce 1000 particles and the consensus method 50000. Please clarify this.

Line 282: Consider changing "movie artefacts" to "carbon areas" or some other more descriptive term. Isn't the main purpose of this tool to discard areas that show carbon, ice contamination or similar problematic areas in the micrographs?

Thanks for the comments, we have made these reviews.

Line 306: Why is a different method suddenly used for 2D classification? This might seem pretty random to a general reader / user. If the justification is to demonstrate Scipion's ability

to mix different protocols (which can be sometimes useful), you could say "for demonstration purposes we use a different classifier this time, namely...". Or is there some other, for example scientific justification to first use one and then another? Typically such mixing is not required, rather the entire workflow can be done in e.g. CryoSparc or Relion, so again it would be good to not complicate things and possibly confuse a novice user to make them think that this particular combination of methods was required.

We have now included a note explaining that the second classifier can help to eliminate more noisy particles, but it's an optional step.

Lines 352-356: The statement here regarding noisy reference is most likely factually in correct. As I am sure the authors are aware, noise in the reference map may lead to alignment of noise (over fitting). Typically high frequency noise is removed by low pass filtering the initial map. If such low pass filter is used (or 30 A as used here), does it really matter which method here was used to create the initial model? Again, I feel that while it's nice to demonstrate different methods and how to integrate them, the problem could be that general reader just gets confused. Perhaps some parts of the initial model generation could be marked as optional.

In the introduction we have clarified that the consensus are optional tools. The advantage of using it in this step is that the swarm consensus is able to generate a more detailed initial volume, although slightly noisy in the background. Generally, having more details in the structure and reducing the relevance of the background using a mask is beneficial for the following refinement algorithms.

Lines 514-517: This promises a bit too much. Achieving high resolution depends on many other factors than the software used.

Line 519: It would be good to remind the reader here what the test case used to calculate the representative results was.

Thanks, for the comments. We were referring to this particular case. Now this has been clarified in the text.

1 de la Rosa-Trevín, J. M. *et al*. Scipion: A software frameowrk toward integration, reproducibility and validation in 3D electron microscopy. *Journal of Structural Biology*. **195** 93-99, (2016).

2 Sorzano, C. O. S. *et al.* Swarm optimization as a consensus technique for Electron Microscopy Initial Volume. *Applied Analysis and Optimizatio*n. **2** 299-313, (2018).

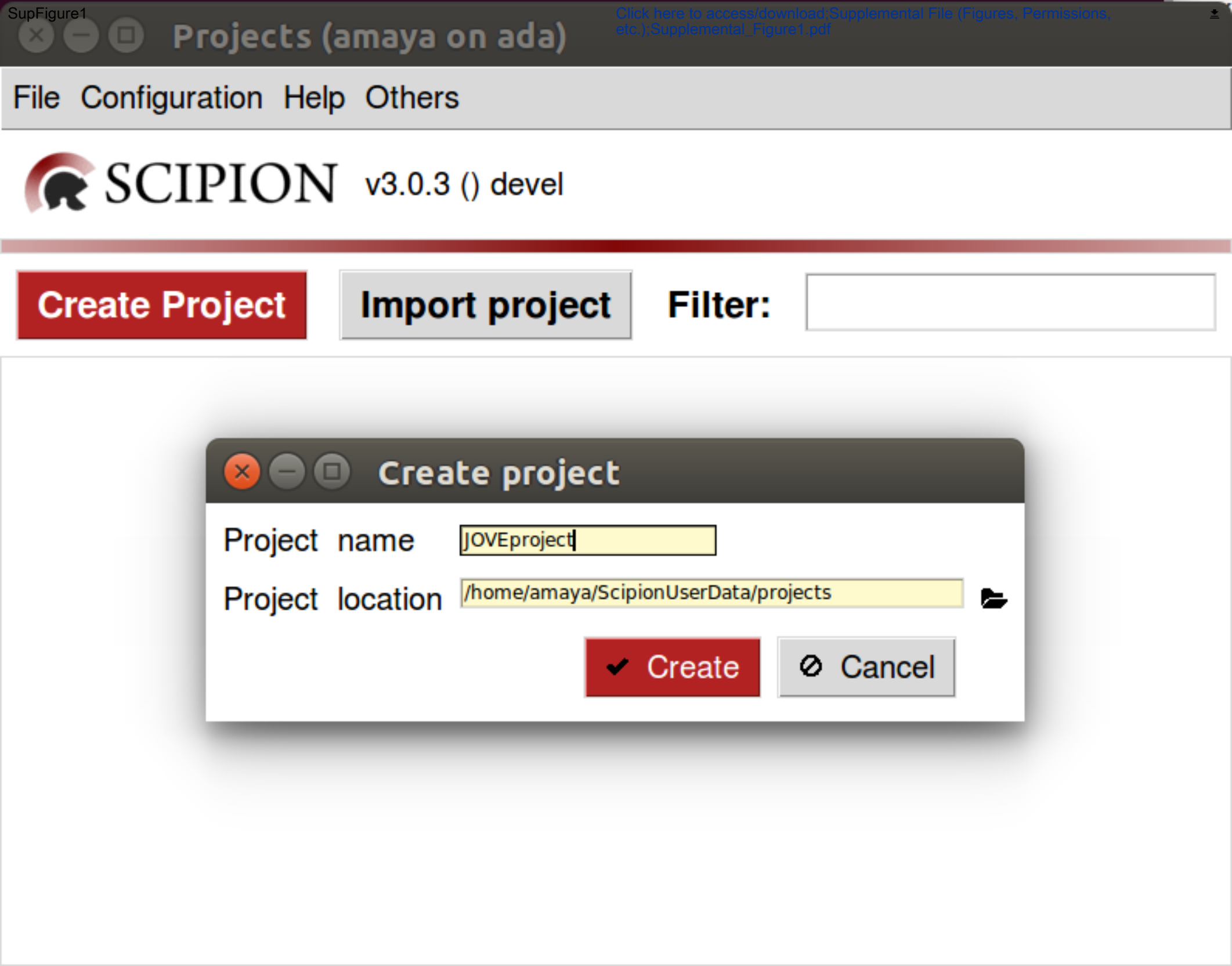
3 Sánchez-García, R., Segura, J., Maluenda, D., Carazo, J. M. & Sorzano, C. O. S. Deep Consensus, a deep learning-based approach for particle pruning in cryo-electron mmicroscopy. *IUCrJ*. **5** 854-865, (2018).

4 Jiménez-Moreno, A., Strleak, D., Filipovic, J., Carazo, J. M. & Sorzano, C. O. S. DeepAlign, a 3D alignment method based on regionalized deep learning (submitted).

5 Sorzano, C. O. S. *et al.* On bias, variance, overfitting, gold standard and consensus in Single Particle Analysis by Cryo-electron microscopy (submitted).

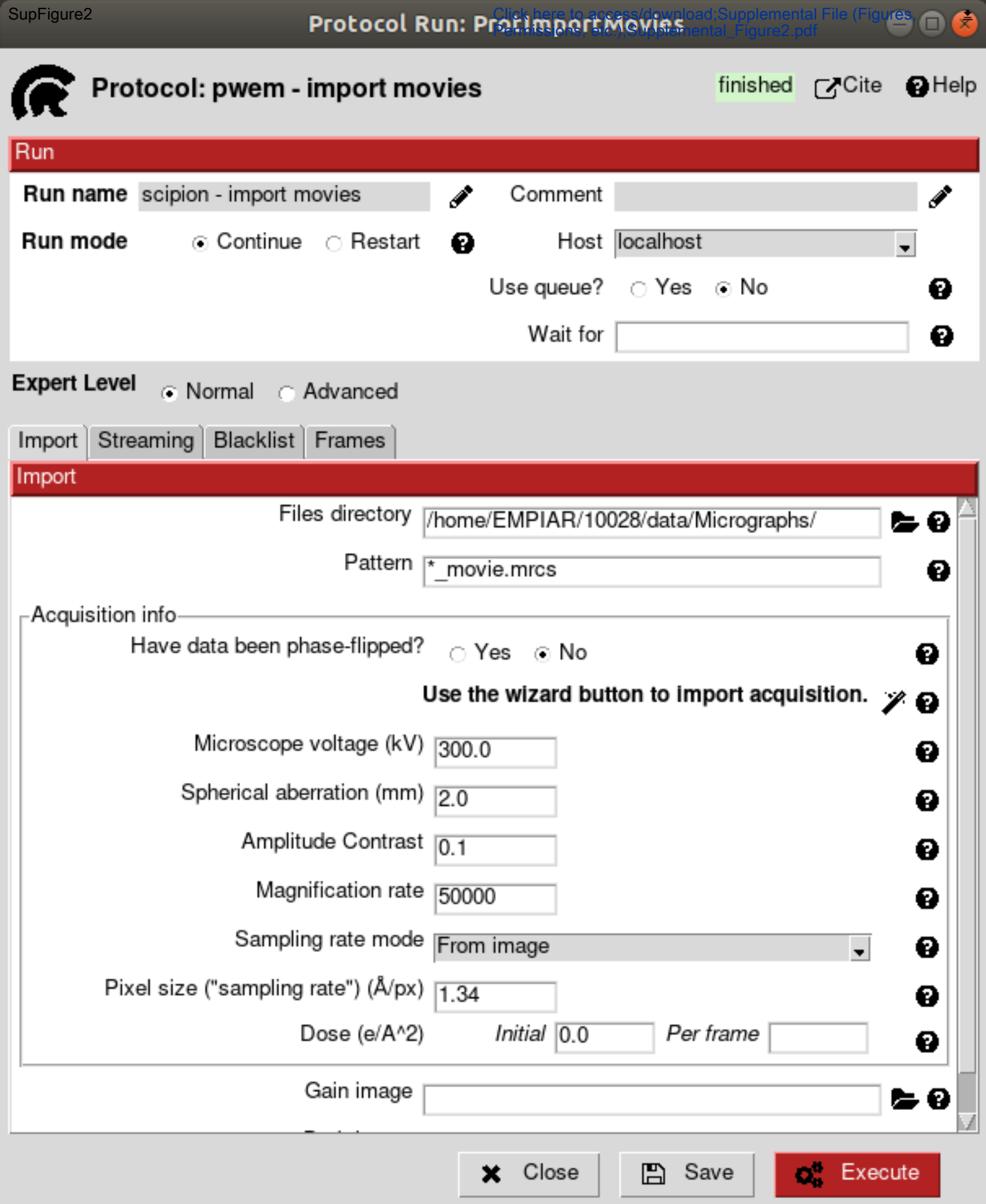
SupFigure1

Click here to access/download;Supplemental File (Figures, Permissions, etc.);Supplemental_Figure1.pdf

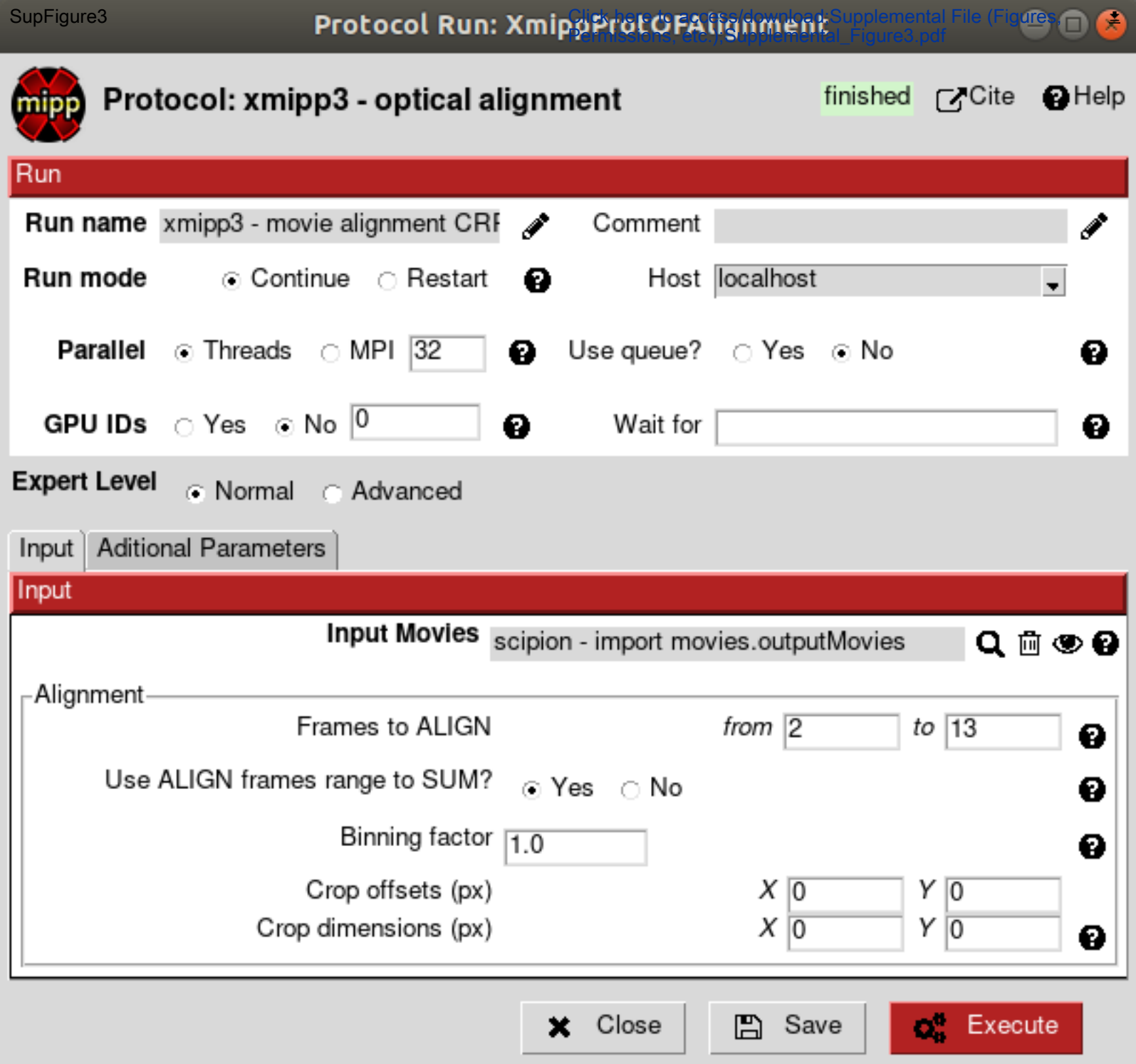


SupFigure2

Click here to access/download;Supplemental File (Figures, Permissions, etc.);Supplemental_Figure2.pdf



SupFigure3



SupFigure4

Click here to access/download;Supplemental File (Figures, Permissions, etc.);Supplemental_Figure4.pdf

Protocol Run: ProtCTFFind

Protocol: grigoriefflab - ctffind4

finished | Cite | Help

Run

Run name grigoriefflab - ctffind | Comment

Run mode (•) Continue ( ) Restart | Host localhost

Parallel (•) Threads ( ) MPI 20 | Use queue? ( ) Yes (•) No

Wait for

Expert Level ( ) Normal (•) Advanced

CTF Estimation | Streaming

CTF Estimation

Input Micrographs xmipp3 - movie alignment CRR+OF.outputMicrogr

Automatic Downsampling Factor ( ) Yes (•) No

Manual CTF Downsampling factor 1.5

Use ctffind4 to estimate the CTF? (•) Yes ( ) No

Expected (tolerated) astigmatism (A) 100.0

Find additional phase shift? ( ) Yes (•) No

Resample micrograph if pixel size too small? (•) Yes ( ) No

Slower, more exhaustive search? (•) Yes ( ) No

Resolution *Lowest* 0.06 *Highest* 0.42

Defocus search range (microns) *Min* 0.25 *Max* 4.0

Window size 256

Close | Save | Execute

SupFigure5

Click here to access/download;Supplemental File (Figures, Permissions, etc.);Supplemental_Figure5.pdf

Wizard

Object
001_movie_aligned.m
002_movie_aligned.m
003_movie_aligned.m
004_movie_aligned.m
005_movie_aligned.m
006_movie_aligned.m
007_movie_aligned.m
008_movie_aligned.m
009_movie_aligned.m
010_movie_aligned.m
011_movie_aligned.m
012_movie_aligned.m
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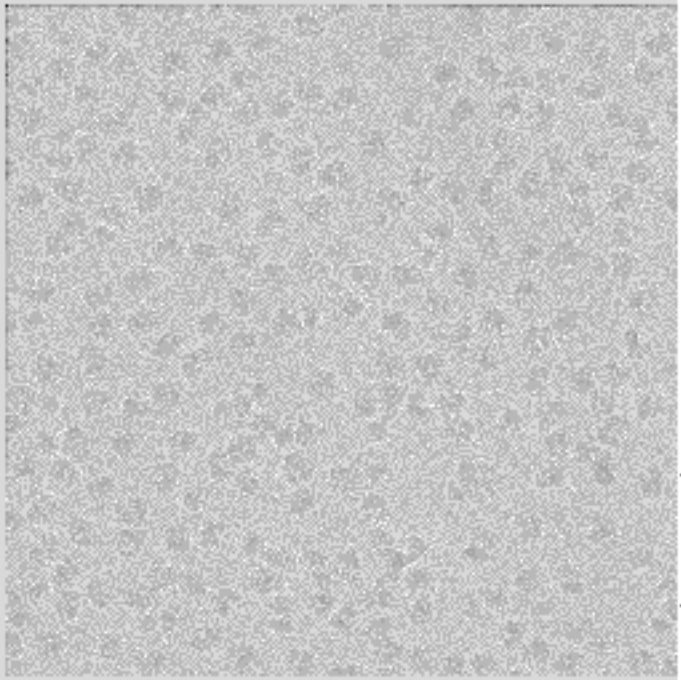
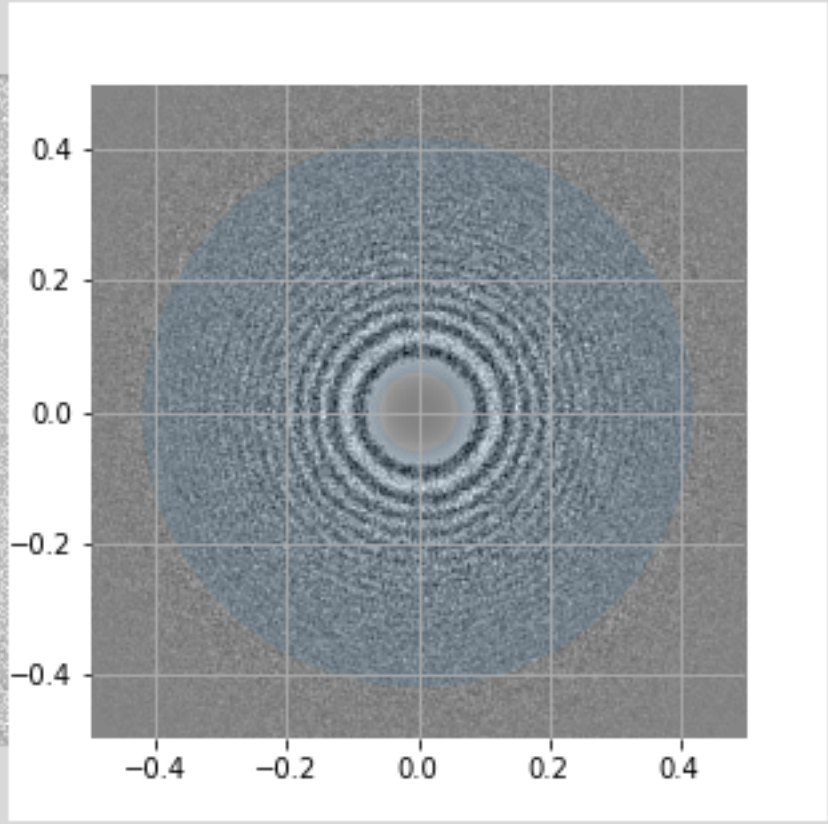
014_movie_aligned.m
015_movie_aligned.m



Micrograph



PSD

Downsample 1.5 Preview

Frequencies

Low freq 0.06 22.333 A

High freq 0.42 3.190 A

Select Cancel

SupFigure6

Protocol Run: XmippProtCTFMicrographs

Protocol: xmipp3 - ctf estimation

finished | Cite | Help

Run

Run name xmipp3 - ctf estimation | Comment

Run mode (•) Continue ( ) Restart | Host localhost

Parallel (•) Threads ( ) MPI 20 | Use queue? ( ) Yes (•) No

Wait for

Expert Level ( ) Normal (•) Advanced

CTF Estimation

CTF Estimation

Input Micrographs	xmipp3 - movie alignment CRR+OF.outputMicrographs
Automatic Downsampling Factor	(•) Yes ( ) No
Use defoci from a previous CTF estimation	(•) Yes ( ) No
Previous CTF estimation	grigoriefflab - ctffind.outputCTF.
Find additional phase shift?	( ) Yes (•) No
Automatic CTF downsampling detection	(•) Yes ( ) No
Allow amplitude constrast refinement	( ) Yes (•) No
Skip borders	( ) Yes (•) No
Resolution	*Lowest* 0.05 *Highest* 0.35
Defocus search range (microns)	*Min* 0.25 *Max* 4.0
Window size	256

Close | Save | Execute

SupFigure7

Protocol Run: XmippProtPreprocessMicrographs

Protocol: xmipp3 - preprocess micrographs

finished Cite Help

Run

Run name xmipp3 - preprocess micrograp Comment

Run mode ◉ Continue ○ Restart Host localhost

Parallel ◉ Threads ○ MPI 32 Use queue? ○ Yes ◉ No

Wait for

Expert Level ◉ Normal ○ Advanced

Preprocess

Preprocess

Input micrographs xmipp3 - movie alignment CRR+OF.outputMicrographs

Operations are performed in the order shown below

Crop borders? ○ Yes ◉ No

Take logarithm? ○ Yes ◉ No

Remove bad pixels? ◉ Yes ○ No

Multiple of Stddev 5

Invert contrast? ○ Yes ◉ No

Downsample micrographs? ◉ Yes ○ No

Downsampling factor 2.0

Denoising ○ Yes ◉ No

Gaussian filter ○ Yes ◉ No

Highpass filter ○ Yes ◉ No

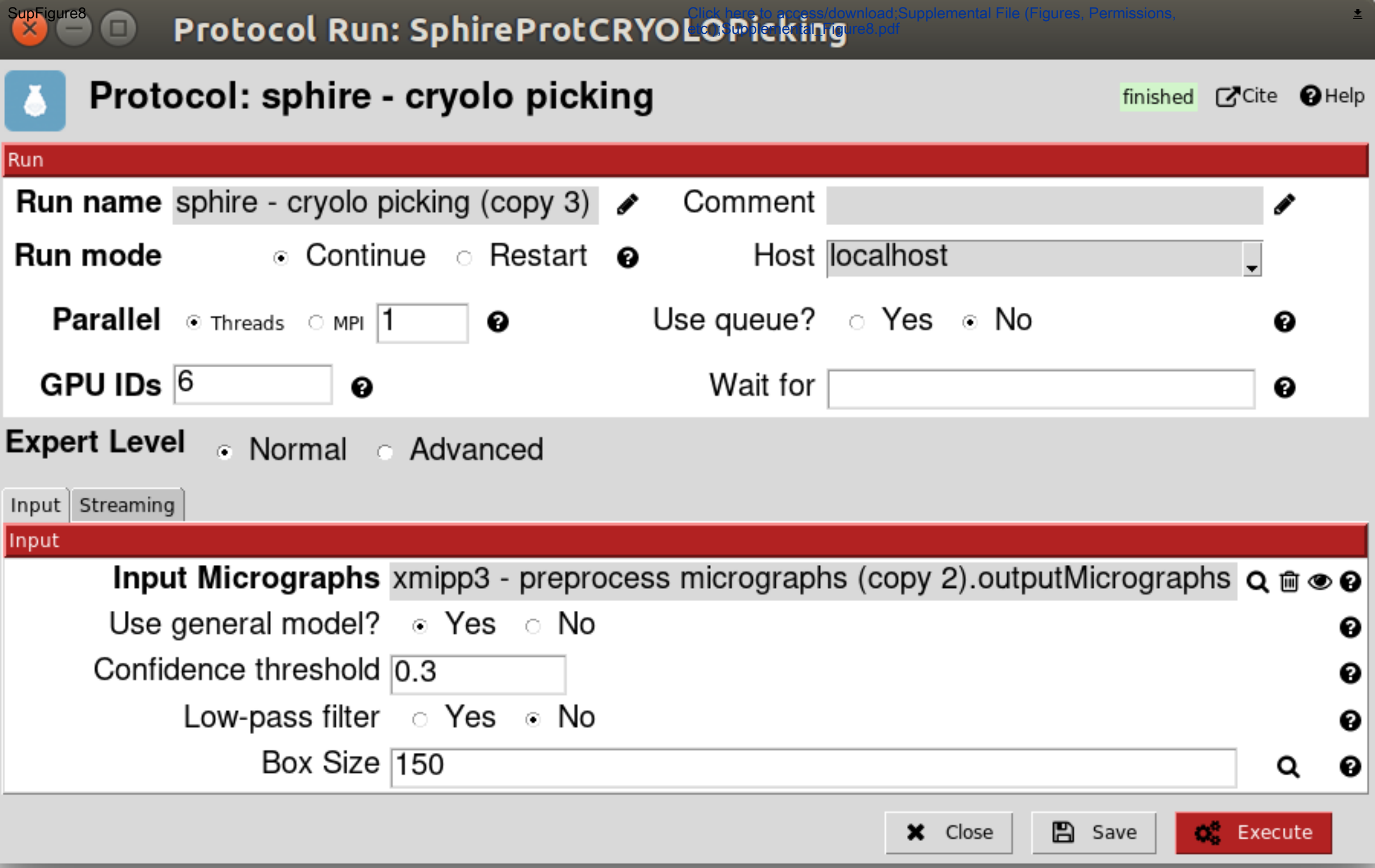
Lowpass filter ○ Yes ◉ No

Normalize micrograph? ○ Yes ◉ No

Close Save Execute

SupFigure8

Click here to access/download;Supplemental File (Figures, Permissions, etc);Supplemental_Figure8.pdf



SupFigure9

Click here to access/download;Supplemental File (Figures, Permissions, etc.);Supplemental_Figure9.pdf

Protocol Run: XmippProtScreenDeepConsensus

Protocol: xmipp3 - deep consensus picking

finished Cite Help

Run

Run name xmipp3 - deep consensus pickir Comment

Run mode ◉ Continue ○ Restart Host localhost

Parallel Threads 2 Use queue? ○ Yes ◉ No

GPU IDs ◉ Yes ○ No 6 Wait for

Expert Level ◉ Normal ○ Advanced

Input | Preprocess | Training | Additional training data

Input

Select model type: Pretrained

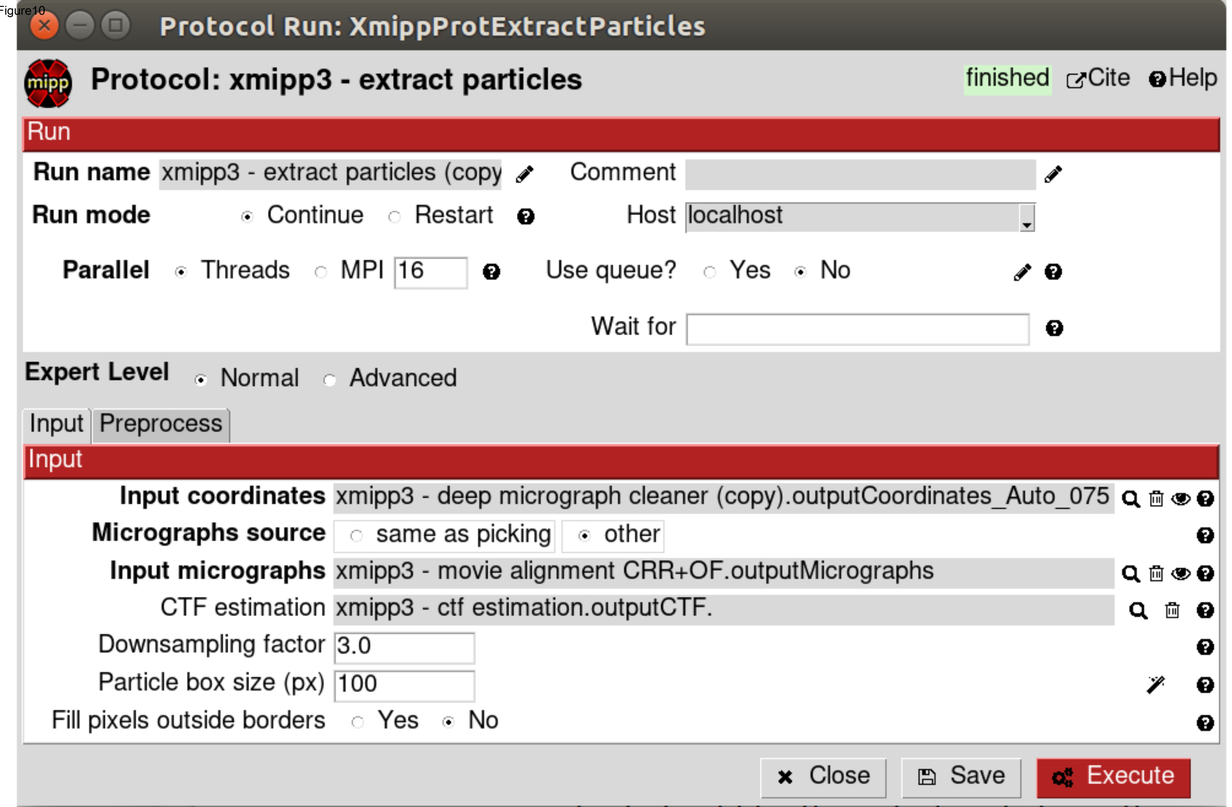
Skip training and score directly with pretrained model? ◉ Yes ○ No

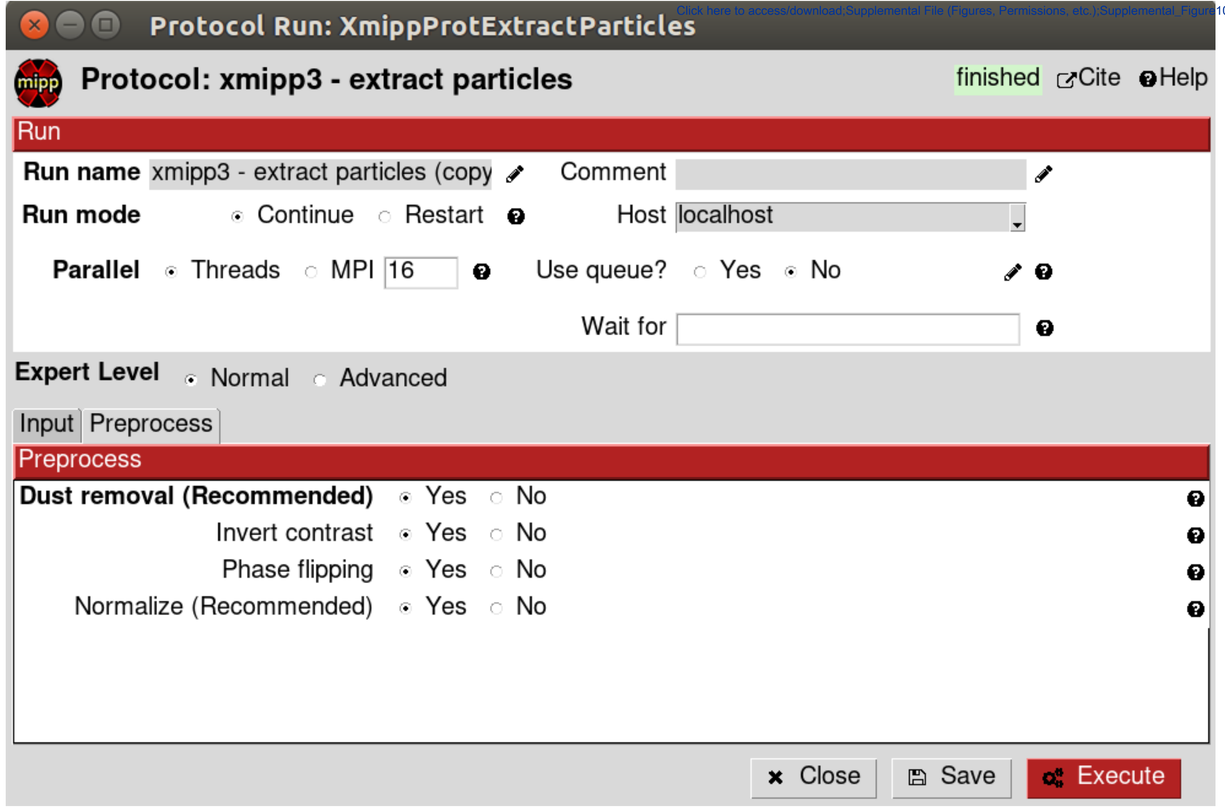
Input coordinates

Object	Info
xmipp3 - auto-picking (step 2) (2)	SetOfCoordinates (93(
sphire - cryolo picking (copy 3).ou	SetOfCoordinates (10'

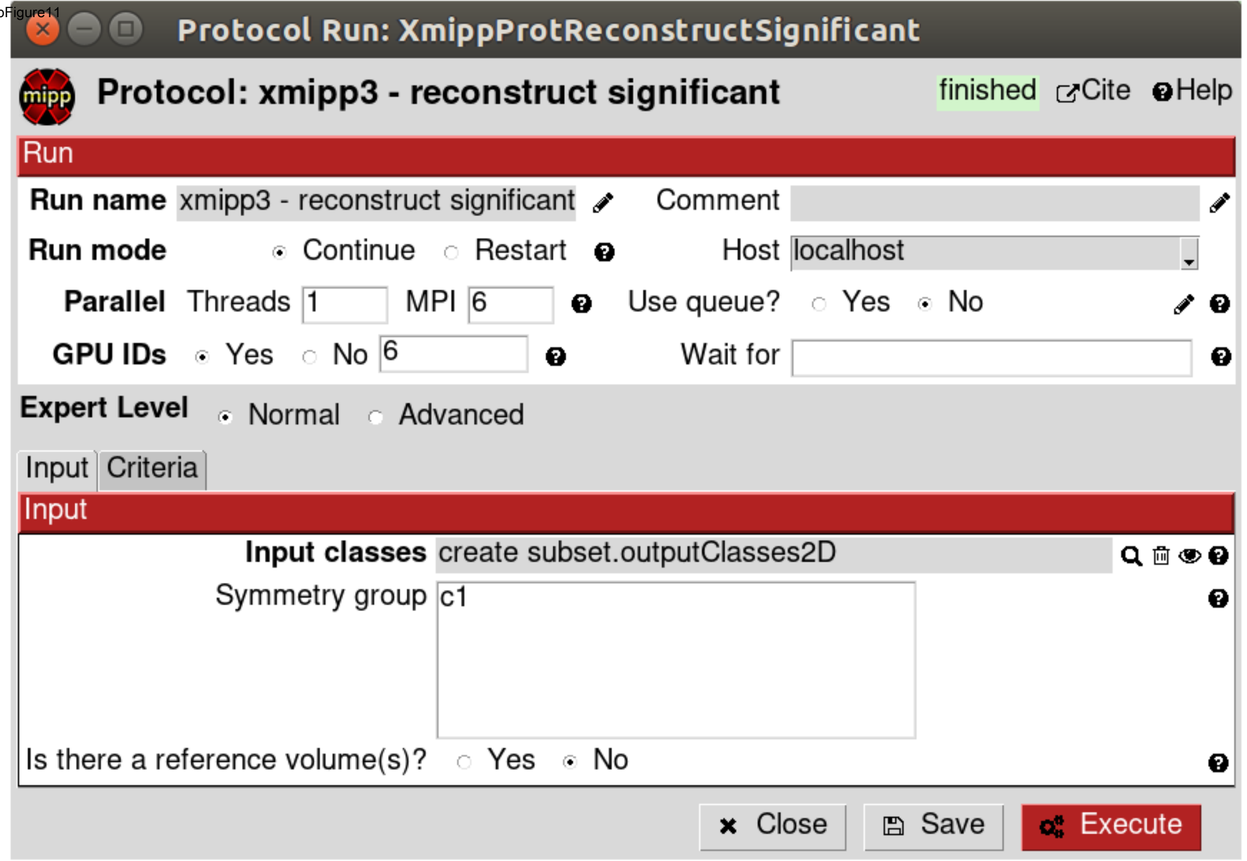
Close Save Execute

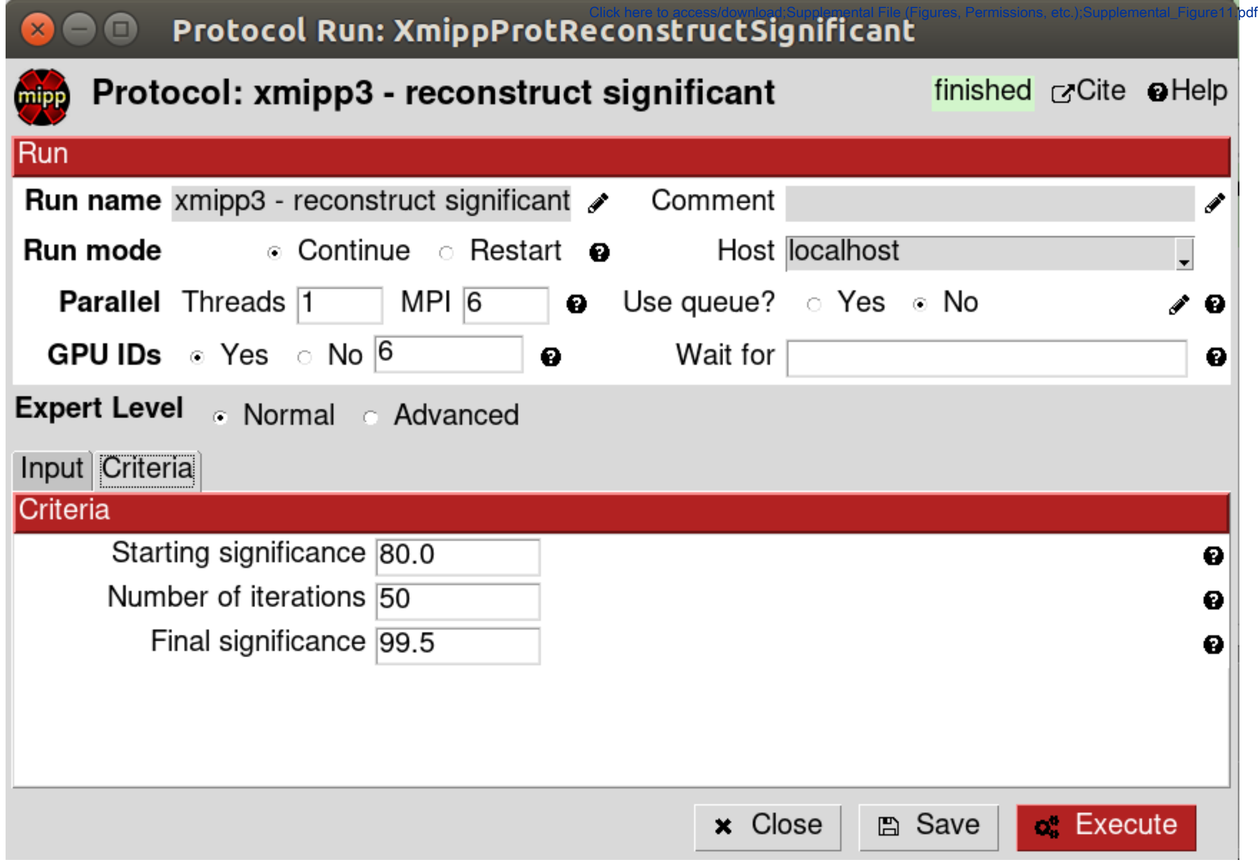
SupFigure10





SupFigure11





SupFigure12

Protocol Run: XmippProtCropResizeVolumes

Protocol: xmipp3 - crop/resize volumes

finished Cite Help

Run

Run name xmipp3 - crop/resize volumes (

Comment

Run mode Continue Restart

Host localhost

Parallel Threads 2 MPI 1

Use queue? Yes No

Wait for

Expert Level Normal Advanced

Input

Input

Input Volumes xmipp3 - reconstruct significant (2) (copy 2).outputVolume

Resize volumes? Yes No

Resize option Sampling Rate

Resize sampling rate (Å/px) 1.34

Apply a window operation? Yes No

Close Save Execute

SupFigure13

Volume: Runs/040969_ProtRelionInitialModel/extra/relion_it300_class001.mrc:mrc (300 x 300 x 300)

File Display Tools Help

86 150 Cols 4 Rows 75

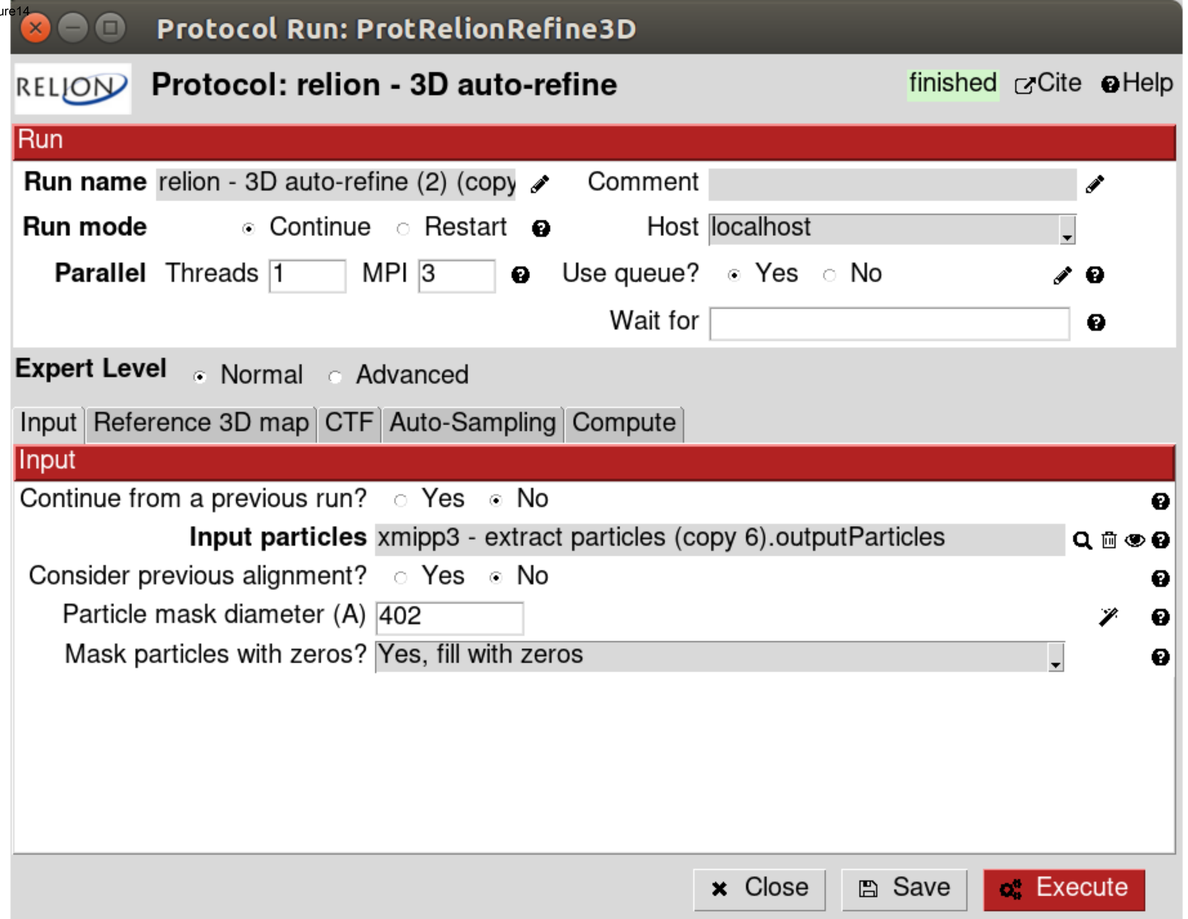
Block Volumes

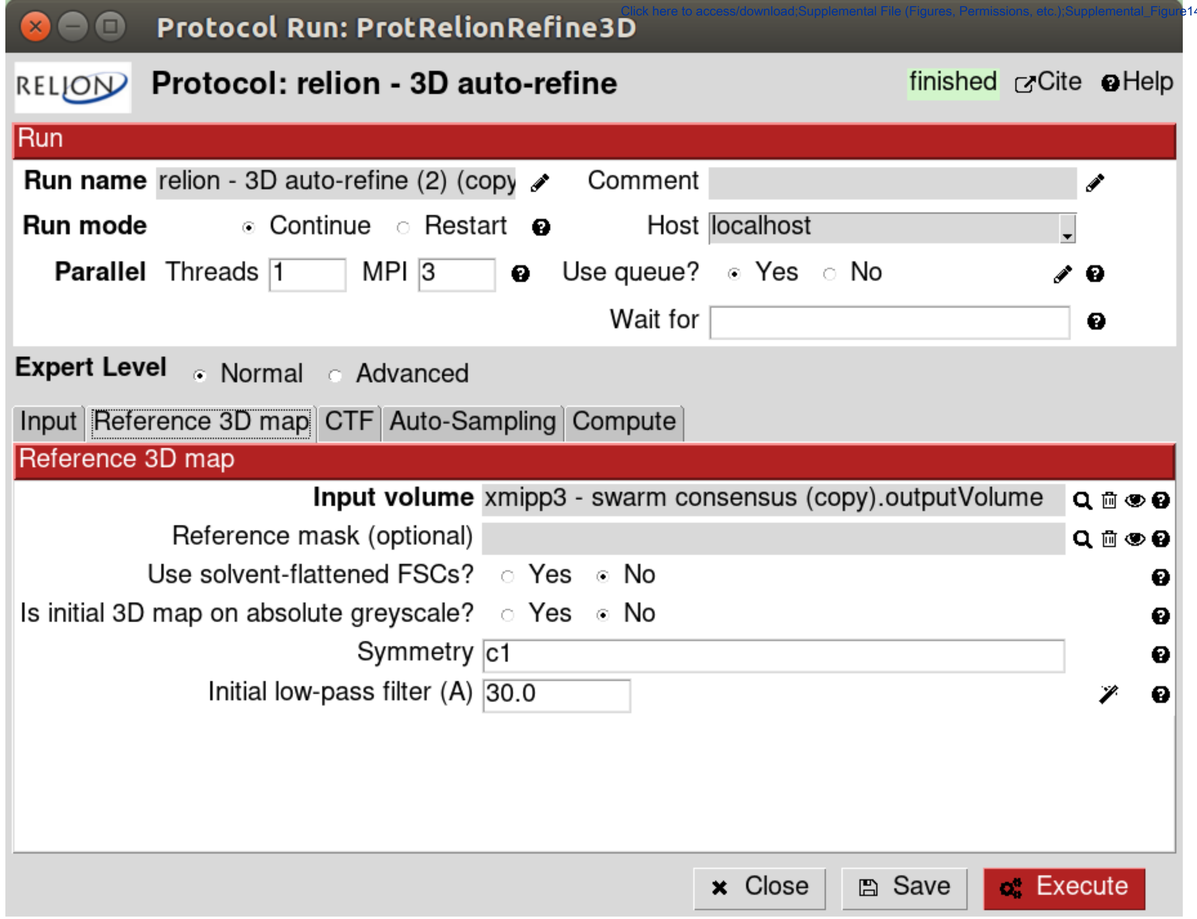
	1	2	3	4
36	slice 141	slice 142	slice 143	slice 144
37	slice 145	slice 146	slice 147	slice 148
38	slice 149	slice 150	slice 151	slice 152

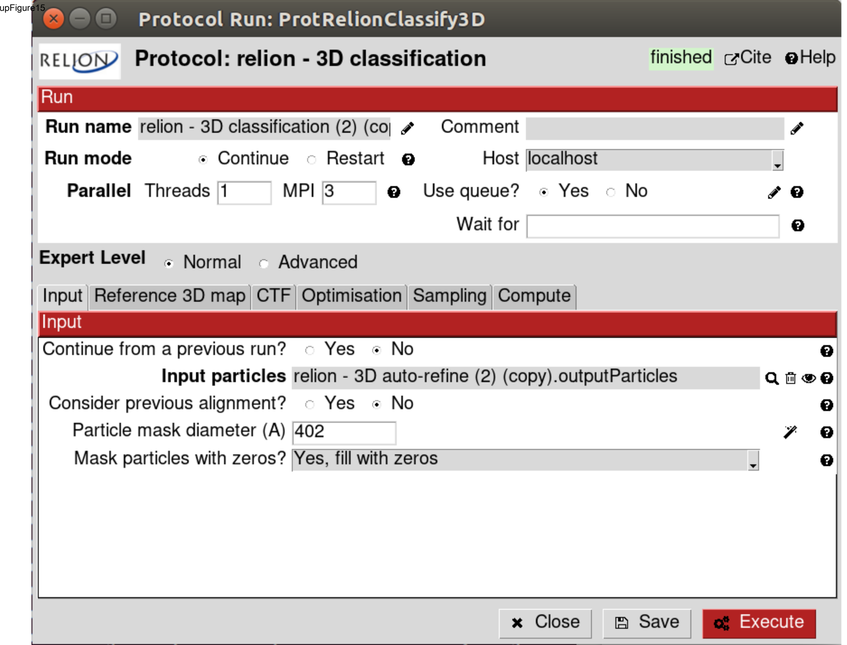
Close Volume

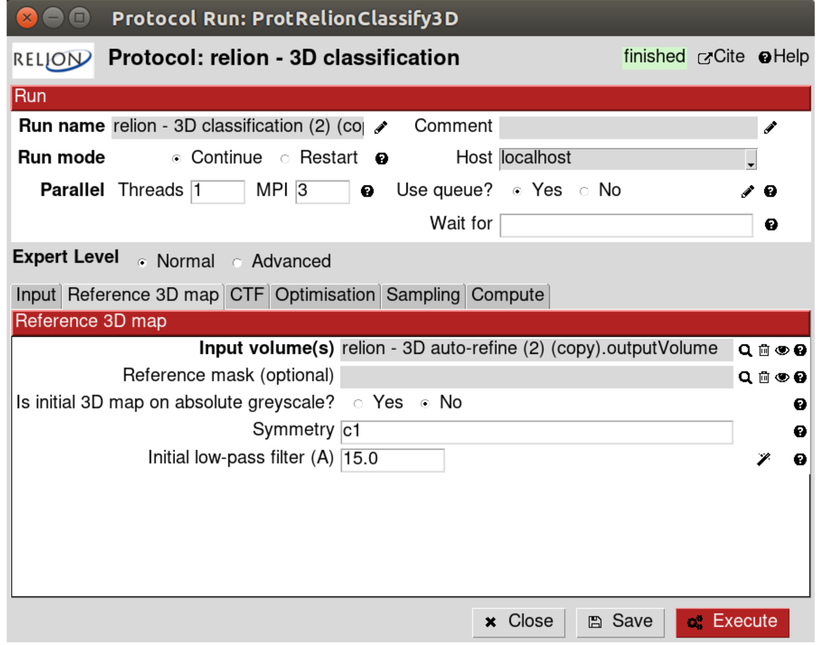
SupFigure14

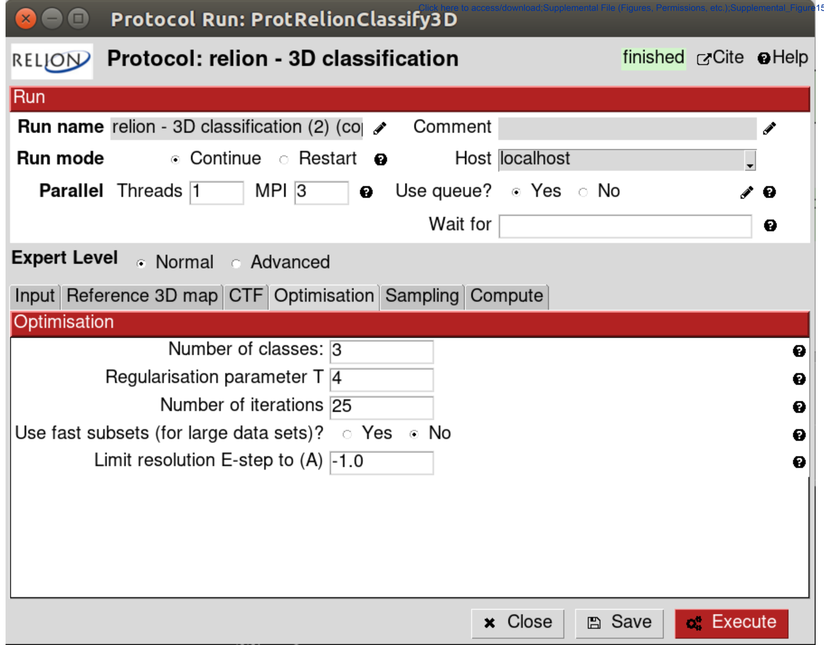
Click here to access/download;Supplemental File (Figures, Permissions, etc.);Supplemental_Figure14.pdf











SupFigure16

Protocol Run: XmippProtDeepCones3DGT_2

Protocol: xmipp3 - deep cones3D highres GT 2

finished | Cite | Help

Run

Run name xmipp3 - deep cones3D highres | Comment

Run mode ◉ Continue ○ Restart | Host localhost

Parallel ◉ Threads ○ MPI 8 | Use queue? ○ Yes ◉ No

GPU IDs 4,5,6 | Wait for

Expert Level ◉ Normal ○ Advanced

Input

Input

Input images: xmipp3 - extract particles (copy 6).outputParticles

Volume: relion - 3D auto-refine (2) (copy).outputVolume

Choose your if you want to use pretrained models: ○ Yes ◉ No

Input training set: scipion - subset (3) (copy).outputParticles

Target resolution: 10.0

Symmetry group: c1

Number of epochs for training: 10

Distance between cone centers: 30.0

Number of selected cones per image: 2

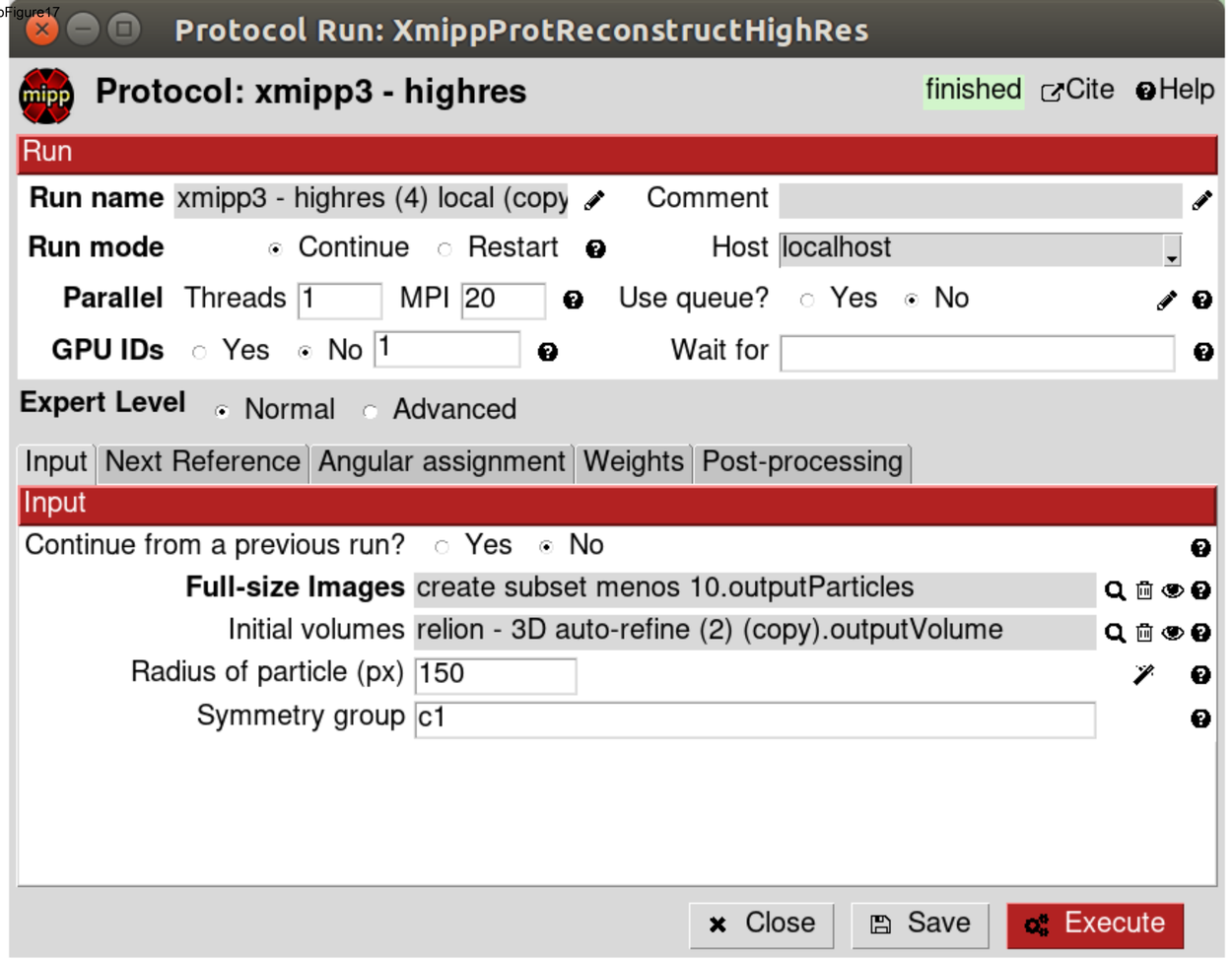
Correct CTF: ○ Yes ◉ No

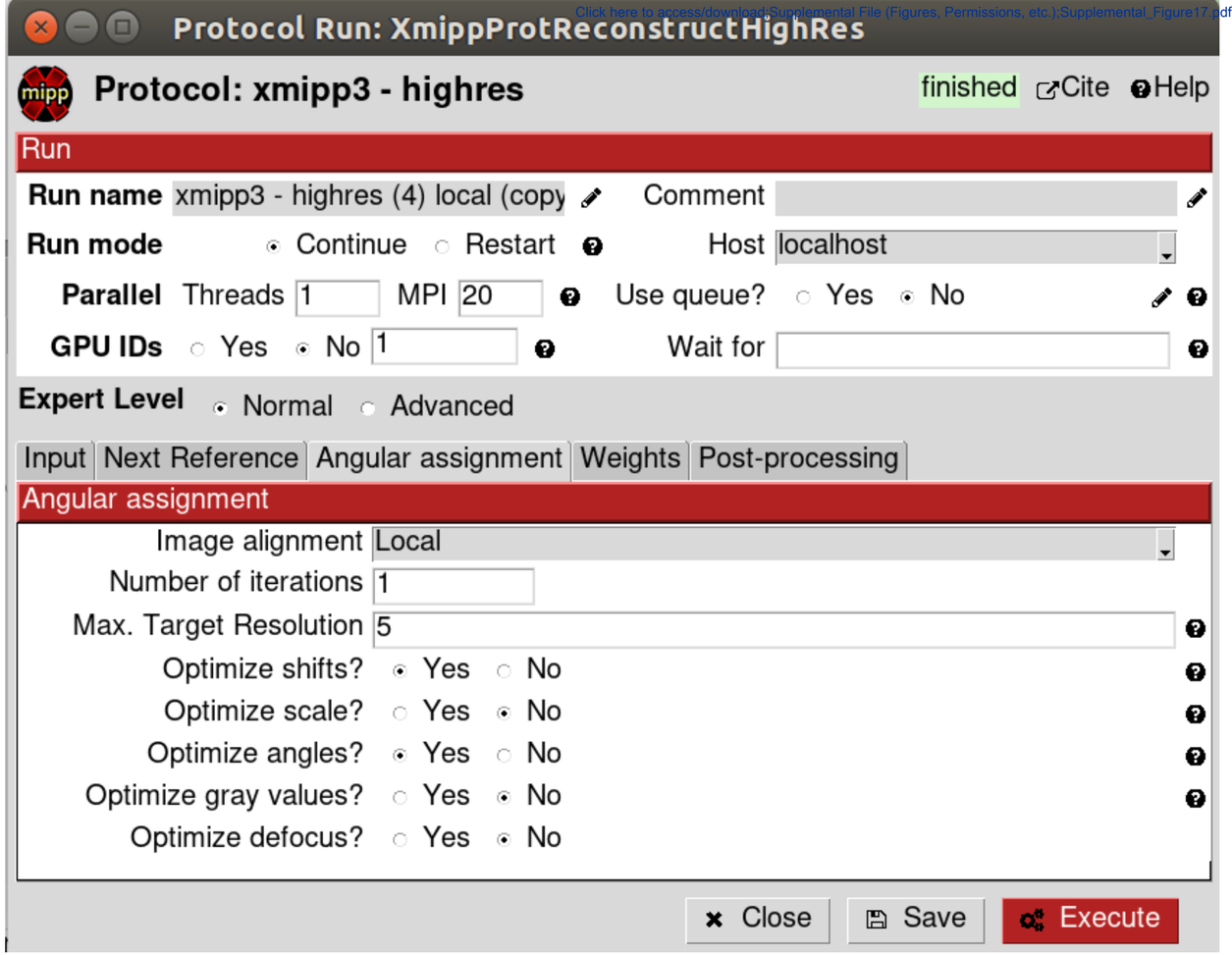
Use GPU alignment: ◉ Yes ○ No

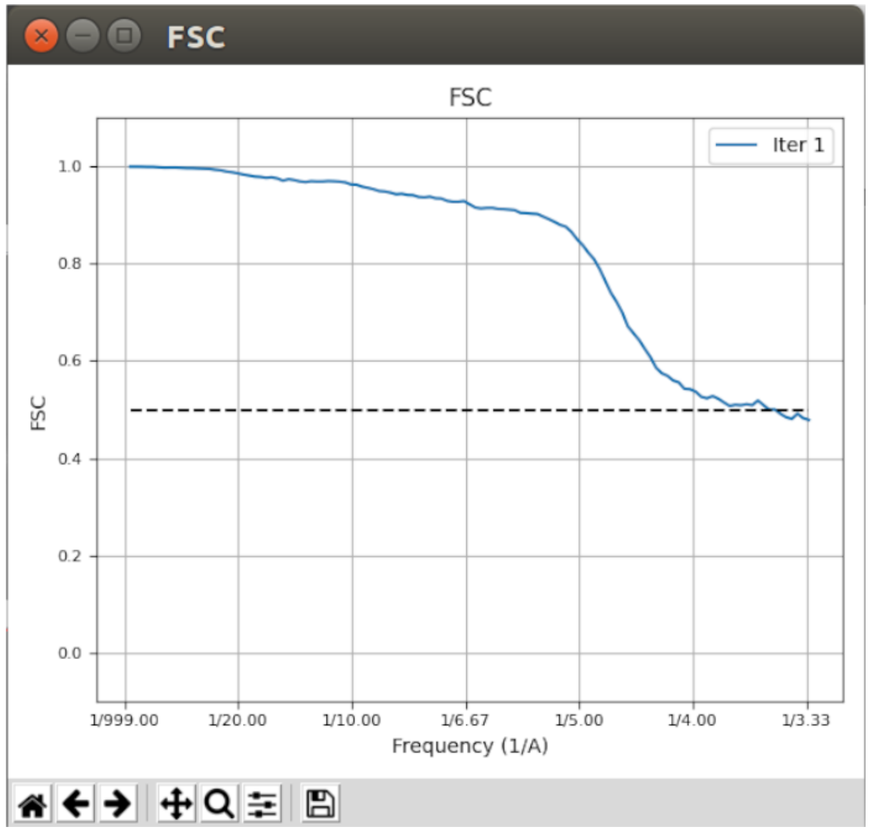
XMipp MPIs: 8

Close | Save | Execute

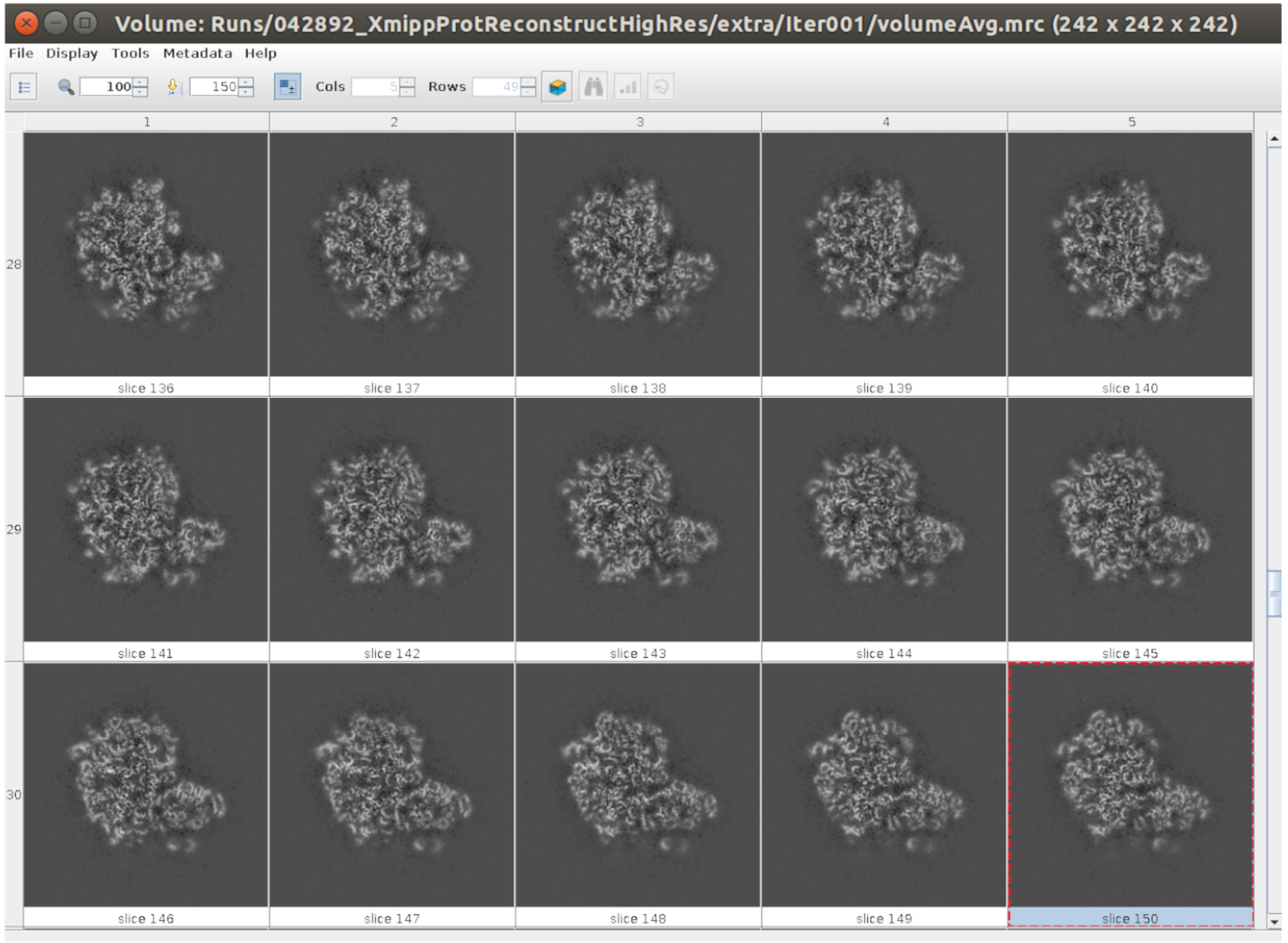
SupFigure17







(a)



(b)

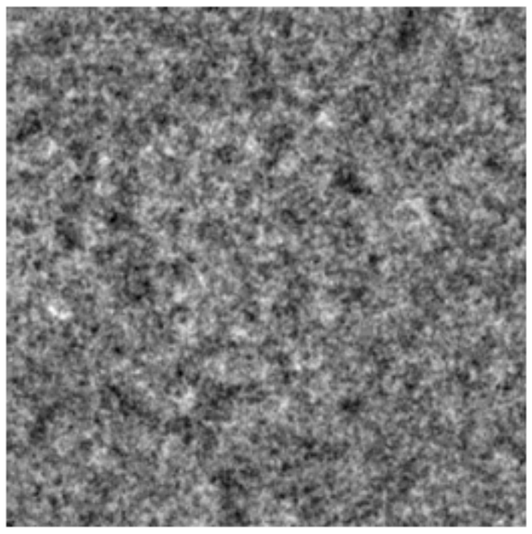
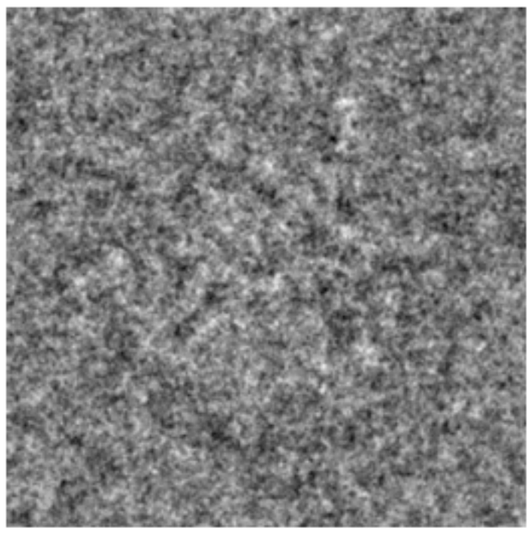
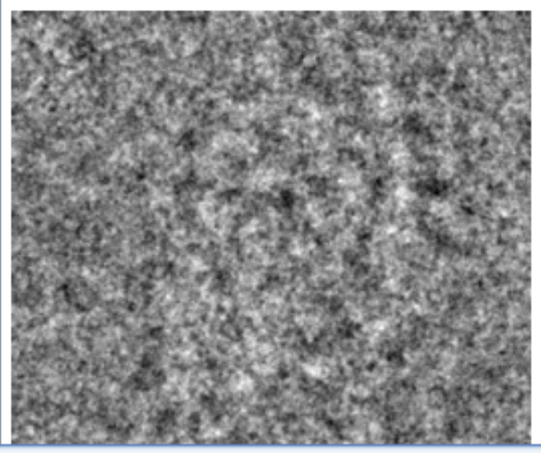
SupFigure19

Metadata: particles.sqlite 26093 items (300 x 300)

File Display Tools Help

86 1 Cols 12 Rows 26,093

Block Particles

	id	enabled	_filename	_ctfModel._defocusU	_ctfModel._defocusV	_xmipp_shiftX	_xmipp_shiftY	_xmipp_weight	_xmipp_cost	_xmipp_weightContinuous2	_>
1	3	✔		23461.9004						0.7660	
2	4	✔		23461.9004						0.5461	
3	5	✔		23461.9004	23260.0000	-6.8685	8.0239	0.7410	0.3467	0.8178	

Plot columns

Title:

X label:

Y label:

Type: Histogram Bins 100

X Axis:

Labels

Label	Plot	Color	Line style	Marker
id	☐		solid	none
enabled	☐		solid	none
_ctfModel._defocusU	☐		solid	none
_ctfModel._defocusV	☐		solid	none
_xmipp_shiftX	☐		solid	none
_xmipp_shiftY	☐		solid	none
_xmipp_weight	☐		solid	none
_xmipp_cost	✔		solid	none
_xmipp_weightContinuous2	☐		solid	none
_xmipp_angleDiff	☐		solid	none
_xmipp_costPerc	☐		solid	none
_coordinate._xmipp_goodRegi...	☐		solid	none
_index	☐		solid	none

Plot Cancel

Close Particles

SupFigure20

Click here to access/download;Supplemental File (Figures, Permissions, etc.);Supplemental_Figure20.pdf

Protocol Run: XmippProtValidateOverfitting

Protocol: xmipp3 - validate overfitting

finished Cite Help

Run

Run name xmipp3 - validate overfitting (co Comment

Run mode (•) Continue ( ) Restart Host localhost

Parallel Threads 1 MPI 8 Use queue? ( ) Yes (•) No

GPU IDs (•) Yes ( ) No 4,5 Wait for

Expert Level ( ) Normal (•) Advanced

Input

Input

Input particles: create subset menos 10.outputParticles

Resize input particles and volume? ( ) Yes (•) No

Calculate the noise bound for resolution? (•) Yes ( ) No

Initial 3D reference volume: xmipp3 - highres (4) local (copy 6).outputVolume

Symmetry group: c1

Number of particles: 500 1000 1500 2000 3000 5000 10000 15000 20

Number of times the randomization is performed: 10

Maximum resolution (dig.freq): 0.5

Angular sampling rate: 5.0

Close Save Execute