RENAFOBIS 2016

Electron microscopy Practical Tutorial

using EMAN 2.12

Requirements :

- EMAN 2.12 version installed on laptop/computer from website http://blake.bcm.edu/emanwiki/EMAN2
- A 3-button scroll mouse, though there are alternatives using keyboard modifiers for one button mice or trackpads
- Having the beta-galactosidase dataset This dataset is a subset of one of the dataset that you can download on the EMPIAR database. The original paper published with this data achieved a resolution of 2.2 Å. While the full dataset can be processed on a workstation in a few days, it is a bit much for a laptop in a one afternoon tutorial. So, we will be using a subset of the data, which has been down-sampled for faster processing. Even the reduced data set used here is capable of producing about 4 Å resolution, though the final refinement may still be pushing the capabilities of some laptops...

Workflow :

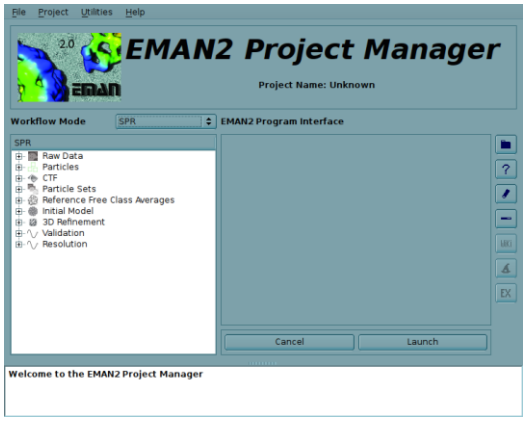
- STEP 0 : Prepare your project folder
- STEP 1 : Evaluate your micrographs
- STEP 2 : Pick your particles
- STEP 3 : Correct your images for CTF modulation
- STEP 4 : Build your set of particles
- STEP 5 : 2D alignment of your particles to sort good/bad particles
- STEP 6 : 3D refinement
- STEP 7 : Molecular modeling
- STEP 8 (Optional) : Publish...

STEP 0 : Prepare your project folder

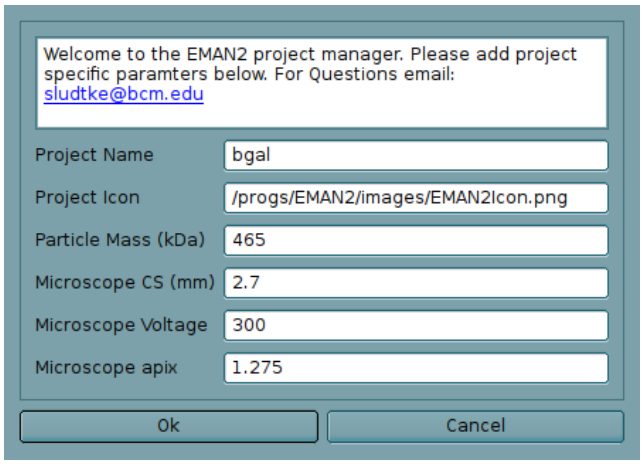
Unzip the sample data archive in a convenient place. This will give you a folder called bgal. Do not run any EMAN2 programs yet. First, from the command line : cd /path/to/bgal. That is , you want to be in the 'bgal' folder, such that typing ls (dir on Windows) will show you the tree of your project.

The folder you are now is called the 'project folder'. You will run virtually all commands from this location. All files associated with this project live in this folder or subfolders inside this folder. The system is designed so all of your data and results are self contained, if you use the GUI in the normal way. The goal is that at the end of the project, you will be able to trace any step you performed to produce any of your results without ambiguity. Any EMAN2 commands you run should be run from this folder, not from subfolders or others locations.

Type on the command line **e2projectmanager.py**. A window should appear that looks roughly like this :



Go to *Project* → *Edit Project* menu item. Enter the following parameters : mass=465, Cs = 2.7, Voltage = 300, apix = 1.275, and then Click OK.



STEP 1 : Evaluate your micrographs

Due to automatic data collection, the microscope will record all the grid squares that were automatically selected (depending on the ice thickness and interesting regions manually selected by you !). Any bad images that will be part of you particles data set will introduce bias and noise in your final reconstruction. A first round of visual inspection is necessary...

Import your micrographs by clicking on *Raw data* and select your mrc files. Look at your images and try to find the images that should be discarded...

STEP 2 : Pick your particles

Pick your particles within each image using the Swarm method.

STEP 3 : Correct your images for CTF modulation

We are now beginning the process of correcting for the Contrast Transfer Function (CTF) of the microscope. It is important to understand that CTF correction cannot be performed completely as a preprocessing step. Programs that claim to do this are likely performing only the subset of CTF correction known as phase-flipping, and ignoring amplitude correction.

CTF correction is broken into a sequence of steps. While the fitting is completely automated, it is a good idea to review at least some of the results manually. This is an important step in quality control of your data, as it gives you another opportunity to eliminate images with low quality particles which could actually damage your reconstruction. If the project simply too many frames to manually review them all, you may consider reviewing the closest and furthest from focus images. These are the most likely areas where errors will occur.

Here is a basic overview of the normal procedure. Detailed instructions follow:

- Run automatic fitting with 2x oversampling
- Run manual fitting on at least a few images. Do a quick check to make sure determined defocuses are ok. If there are a lot of frames start with a few of the closest to and a few of the furthest from focus. Run structure factor determination on the images you just hand-checked (should cover a range of defocuses.
- Run automatic fitting again (having a structure factor may slightly improve results)
- Run manual fitting. Evaluate your data critically. Adjust quality of any images you suspect may not be as good.
- Generate output with refinebysnr. This:
  - ❖ Fine tunes defocus based on high resolution SSNR optimization (optional)
  - ❖ Performs phase-flipping correction, and optional post-processing
  - ❖ Stores CTF parameters including SSNR in particle headers.

3-1. Automated fitting

Select CTF → Automated Fitting

- check the *allparticles* box. This is an alternative to manually selecting all of the individual images in your particles folder. As a reminder, if *allparticles* is selected, anything in the particles box will be ignored completely.
- *minptcl* allows you to skip any frames with fewer than the specified number of particles. Leave this at 0 for the sample B-gal.
- *minqual* allows you to skip any frames where the user has set the quality below a specified value. Again, you can leave this at 0 for now.
- Set *oversamp* to 2. This will provide better fitting in most cases. If you are working with your own data and the box size is large (256+) you can consider leaving this at 1.
- Select *autohp*. This will modify the SNR curve to eliminate the first sharp peak that appears in virtually all single particle data due to ice gradients and other issues. This peak isn't completely removed, but is just heavily downfiltered, and in most cases it will noticeably improve alignments. However, in some projects, the structure factor is such that this option will filter out too much low resolution information. If you see strange low-resolution artifacts, you may try disabling this.
- Select *curdefocushint*. This will make use of any existing defocus information during fitting. Only uncheck this if you have bad defocus values from earlier fitting attempts and want to start from scratch.
- Do not select astigmatism for now. If trying to correct astigmatism, you should always do a refinement without it first.
- You should see: Cs = 2.7, voltage = 300, apix = 1.275 filled in for you. If not, you missed something in initial steps. Please set the right parameters for the project initial steps.
- *constbfactor* = 75
- Threads - This should be set to the number of processors (cores) on your computer (usually 2 or 4 on a laptop), whenever you see this box. On a cluster, this would be the number of cores on one node. If you have a machine newer than ~2012, you may consider using ~25% more threads than you have processors, for a small additional performance boost.
- *Launch*.

You may consider opening the Task Manager to monitor the progress of this and other running jobs (may not work reliably on some platforms). Otherwise, watch the console where you ran e2projectmanager for the output to finish.

3-2. Interactive Tuning

When automatic fitting is done, then use CTF→interactive tuning

• Select *allparticles* and *sortdefocus*, then press *Launch*.

You will get 4 windows (shown below). You may arrange these windows as you like the first time you run the program. If you run it again in this project, it should put them back in the same location. This analysis is based on particles rather than tiled regions from the micrograph, as used in the earlier micrograph assessment step. Basing the analysis on particles allows us to accurately estimate the SSNR and structure factor (next step) from the particle data. While it is possible to estimate defocus and astigmatism from random boxed out regions of the micrograph, the method used here relies on the fact that each box contains a particle reasonably close to the center of the box. If too many "bad" particles are present, the SSNR and structure factor estimates will be impacted. The control panel window (titled CTF) allows you to select which image to work on, and adjust the various parameters for that image interactively. The particles window shows the average of the first 20 particles (without alignment) and the first 20 particles individually (use up and down arrow in that window). The 2-D FFT window shows the average 2-D power spectrum for the particles in the current image, optionally (if Show 2D Sim is set) the simulated 2-D curve, and optionally (if Show Zeroes is set) the first several zeroes. Finally, the plot window shows several curves. In "Bgsub & Fit" mode, the black curve is the background subtracted, rotationally averaged power spectrum. The dotted black curve is the same thing, but using a different background subtraction scheme which exaggerates the CTF oscillations. The blue curve is the fit based on the parameters shown in the control panel. We do not yet have a structure factor curve, so, while ideally the blue and black curves would match perfectly, at this point we can expect significant deviations at low resolution. Don't bother trying to 'fix' this by adjusting sliders, it is normal for this point in the process. Even later on, there is no need for a perfect fit in amplitudes as long as the zeroes are in the right places.

3-3. Structure Factor

➡ The structure factor we are referring to here is the 1-D structure factor, rather than the 3-D structure factor(s) often referred to in X-ray crystallography. If you had a perfect noise-free 3-D electron density map, took a 3-D Fourier transform, then rotationally averaged its magnitude, that 1-D curve (basically a power spectrum) is what we refer to as the structure factor. Having a good estimate of this curve permits us to do a good job with CTF amplitude correction. Unfortunately, it is difficult to derive an accurate structure factor from the data at high resolution, so what we do instead is determine the low resolution structure factor from the data, typically to a resolution of 15-20 Å, then at resolutions higher than this use a standard protein structure factor with a mix of alpha and beta content. This works well in the majority of situations.

CTF→generate structure factor • Either select '*allparticles'* or use the browser to select a subset of the data for this computation. Typically ~10-15 data sets are enough for a good structure factor, and using fewer sets will make it run faster. If you select a subset, generally you should use the images with the largest number of particles, insuring that you cover a range of defocus values. • After pressing Launch, the process will normally only take a couple of minutes. As before you can monitor progress either by looking at the task manager or the terminal where you launched the project manager from.

➡ Once complete, there are 3 additional files in your project folder, strucfac*. The file strucfac.txt contains the actual structure factor as a function of spatial frequency in a 2- column text file. You can

plot it by double-clicking, but you will need to put the Y-axis on a log scale to see anything useful. If present, this file will be used automatically in the remainder of processing. If you have a structure factor curve from some other source, such as an X-ray solution scattering experiment, you can replace this file with whatever curve you like, and it will be used from that point on. If converting a solution scattering curve, however, beware of the differing spatial frequency conventions. In CryoEM we use 's' (the reciprocal of the real-space periodicity) and in solution scattering they normally use q (momentum transfer). i.e. - there is normally a 2π conversion factor.

3-4. Rerun Automatic Fitting

With a decent structure factor in hand, sometimes automatic CTF fitting will be marginally improved. Certainly it should get no worse anyway. There isn't usually much point in iterating this process further, though. That is, you don't need to compute another structure factor after refitting.

Rerun CTF → Automated Fitting

Default options should be correct.

3-5. Interactive Tuning

If you like you can run interactive tuning again and you should see that the blue and black curves are a much better match. In cases where they do not match well, adjusting the B-factor will generally produce an improved fit. If you find that adjusting the B factor to match at low resolution requires sacrificing high resolution oscillations, that means the initial B-factors or the Amplitude Contrast you selected before determining the structure factor may not have been optimal. While you can go back and play with these values and redetermine the structure factor, unless you used extremely inaccurate values (like an %AC of zero), it isn't likely to have a significant impact on your reconstruction. If you find a few images which really don't seem to match well at all, it is very likely that those images have a large fraction of bad particles, or for some reason have a very different particle orientation distribution than the average image.

Now *run CTF→interactive tuning*, again

If you manually adjust parameters at this stage, you should focus on a good fit at high resolution, and not be concerned about any remaining poor low-resolution match, but it should not really be necessary to make adjustments at this stage.

If you would like to assess data quality, you need SSNR curves rather than the basic CTF fit. In the pop-up menu under the list of images, you'll find SNR and Smoothed SNR. For good data, the low resolution SSNR (between s=0.01-0.03, 30-100 Å) should peak higher than ~0.5. Images that do not achieve an SSNR of at least 0.5 at low resolution are unlikely to align properly, and may act as pure noise in your refinement.

You may also look at high resolution and consider whether there is strong enough signal in the image to improve your reconstruction at the desired resolution. Once the SSNR falls much below 0.02 it usually won't contribute much to the reconstruction, even if you have a lot of particles.

If you decide one image is worse than the others, you may use the quality slider, and set it to a lower than the default (5) value. Similarly if you see any unusually good images, you may consider increasing this slider. It is not necessary to press save after adjusting the quality slider.

The actual values you use for quality are arbitrary. They have no direct meaning to EMAN2, but will be displayed to you later in the process when you are deciding which images to include in the reconstruction.

3-6. CTF Generate Output

run *CTF→generate output*

The *refinebysnr* checkbox should normally be checked. While in some cases (low resolution data) it won't do any good, it will rarely do anything harmful either. This option will make a final pass at making very small adjustments to the defocus value to optimize the measured SSNR of your data at high resolution. The other options are used to select what types of output files to generate. New in EMAN2.12, there are some new options which give you much greater flexibility in project management, and efficiently achieving a good structure. It is now possible to have downsampled and filtered versions of your particles present in the same project folder, and to run refinements at different levels of sampling (different A/pix values) within a single project without any conflicts. • You will select one of these two options to produce the phase flipped particles you use for your final high resolution refinements:

phaseflip - this will produce unfiltered phase-flipped particles • *phasefliphp* - This will produce phase flipped particles which have been high-pass filtered to eliminate the very low resolution peak present in the data (ice gradients, etc.). Normally this is a good thing. Sometimes it is not, and can end up filtering out important low-resolution signal. You won't really know until you try. On the GroEL data we're using here, this is a good option to use.

The next two options produce specialized versions of your particle data for specific purposes.

wiener - This will produce Wiener filtered particles. Note that these particles are not normally useful for reconstructions or 2-D averaging. If many Wiener filtered particles are averaged together the result is heavily over filtered (blurrier than they should be). Really the only good purpose for these particles is manually identifying bad particles, and there are other options which can serve this purpose similarly well.

phaseflipsmall - This is a convenience item. It downsamples your data by 2x, does a mild high-pass filter, and low pass filters to ~17 Å. These particles are often useful for generating reference-free class-averages, identifying bad particles, and making initial models. The parameters are all fixed, so if you wish to modify any of them, you will need to use the *phaseflipproc* options below.

For the demo data set we need only phaseflip from the options above.

The next set of options allow you to produce particles with up to 4 arbitrary processors applied to them. This allows for some very powerful filtration, masking and resizing possibilities (see http://blake.bcm.edu/emanwiki/EMAN2/Programs/e2ctf and Interlude #1 for tips).

In this case, we don't want to use the default *'small'* option, but instead will roll our own. That means we will need to launch the generate output stage twice, once for each type of output. First, downsampled by 2 but not lowpass filtered:

- *proctag: small*
- *phaseflipproc: filter.highpass.gauss:cutoff_pixels=2*
- *phaseflipproc2: math.fft.resample:n=2*

Now press *Launch*

When that finishes running, select

Generate Output (e2ctf) again. This time we are going to make another set which is downsampled even more:

- uncheck: *refinebysnr, phaseflip*
- proctag: tiny
- phaseflipproc: filter.highpass.gauss:cutoff_pixels=2
- phaseflipproc2: filter.lowpass.gauss:cutoff_freq=0.07
- phaseflipproc3: math.fft.resample:n=3

Then press *Launch* again

STEP 4 : Build your set of particles

➡ Particle sets allow you to try reconstructions with various subsets of your data without taking large amounts of disk space, while automatically keeping track of exactly what you're doing. For example, if you generate a filtered version of your particles, and mark some of them as bad, all of the other versions of the same particles will also be marked as bad. Sets also allow you to easily try reconstructions with various subsets of your data without clogging your hard drive with multiple copies of the image data.

Particle sets→ build particle sets

- check the *allparticles* and *excludebad* checkboxes
- enter "all" in the setname box (you can use whatever name you like)
- If you gave any micrographs a lower quality value when manually assessing them, you could also enter 5 in the minqual box, which will exclude any images with the quality set below 5.
- Launch

STEP 5 : 2D alignment of your particles to sort good/bad particles

5.1 : Generating reference free class averages

Select Reference free class averages → generate classes

- Input = **sets/all__ctf_flip_tiny.lst**

- Ncls = **20**, iter = **6**, naliref = **12**
- parallel **= thread:N** where N is the number of cores on your computer
- The defaults should be fine for the other options,
- Launch

5-2 : Eliminate some of the bad particles

Eliminate some of the bad particles Once class-averaging has finished, we need to take a critical look at the averages and see if we can identify any classes containing predominantly bad particles. While it is possible to look at class-averages using the browser, and even see the particles within each class by doubleclicking on a class-average, if we wish to mark particles as bad, we need to use e2evalparticles.py instead.

- 2D Analysis → Mark bad particles by class
- There are no options, just press Launch
- This will cause (initially) only one window to open:
- The window is divided into 3 sections:
    - The upper section shows a list of all class averages generated so far in the current project, both reference-free and (if you have run a 3-D refinement) reference based
    - The middle section permits you to modify which class-averages are currently selected for operation
    - The bottom section allows you to perform various operations on the particles associated with the selected class-averages. The only option we will use right now in this section is Mark as Bad. See: http://blake.bcm.edu/emanwiki/EMAN2/Programs/e2evalparticles for other uses.

At present, we need to look at the results of our 2-D reference free classification. In the top section, select r2d_01/allrefs_08.hdf (or whatever the highest number is).

This will cause 3 additional windows to open. Two of these windows, titled: Included Particles and Excluded Particles, will be empty initially. The third will show the classaverages from the selected file. Position these windows for convenient viewing of all three. It would be good to see all of the class-averages at once (maybe with some scaling). The Excluded Particles window can be smaller.

Single-click on any of the class-averages. After a short delay, you will see particles appear in the Included and Excluded particles windows. These correspond to the particles associated with this class-average. The Included particles were used (after alignment) to produce the actual average you selected. The Excluded particles are particles which were members of the class, but looked little enough like the final average that they were excluded from the averaging process. • We now need to identify any class-averages which consist predominantly of “bad” particles, yet do this without eliminating too many “good” particles. Below you will see my classaverages, and the particles associated with average #9.

Hopefully it is obvious that these “particles” are actually just ice contamination. In most cases we won’t have such a clean separation and the particles will consist of some bad and some good particles. We would now like to mark these particles as “bad” so they aren’t used during our 3-D

reconstruction. You should now double-click on any class-averages you think are mostly bad particles, you will see a small blue mark appear in the corner of each.

➡ Generally speaking one bad particle like the ones shown above will do far more damage to a reconstruction than one good particle helps the reconstruction. In fact, particles like this can easily do more damage than 5 good particles would help the structure. So, if you have to sacrifice a fraction of your good particles to get rid of really bad particles, this is usually a worthwhile trade to make.

Note that it is NOT possible to mark individual particles as bad using this interface. If you want to manually mark particles as bad, there is a section in the Appendix which explains how to do this. For now, mark all of the class-averages you wish to exclude with the little blue mark. It will not be the end of the world if you leave a few bad particles in the set. The goal is to eliminate 80-90% of the bad particles, to make our overall population healthier.

Once you are satisfied with your blue marks, press the Mark as Bad button, and confirm that you want to do this in the dialog that appears. Do not exit e2evalparticles yet, though! 14. Selecting Good Class-averages To make an initial model, we need a set of 15-20 good class-averages representing different views of our particle. The more different views you have, the more likely it is that your starting model will be good, and lead to a quick reconstruction.

➡ The lower your particle's symmetry, the more averages you will want to use. For GroEL, with a preferred orientation you may get away with as few as 10. For ribosomes, you might want 20-30. While larger is better, note that the amount of time the initial model generation takes is proportional to the number of averages you use, so if you are in a hurry, you may not want to go crazy and use 50 or 100 averages.

While some of our class-averages represented bad particles, the rest should be very nice averages we can use to generate an initial model, we just need to extract them:

Middle-click on the window showing the class-averages to bring up the control panel. In the control panel press the Sets button under the histogram, then also select the Sets tab (next to Main).

You will see one set shown: evalptcl. We need to make another set for our good classaverages. Press the New button and type good in the box that appears. You should now have a green colored set with this title. Click on it (the green word good) to make it active. Now double click on class-averages you think are good. You will see a green mark appear instead of a blue mark.

Once you have selected all of the good class-averages, press the Save button in the lower right corner of the control panel (not the one in the upper right). This will save the particles in the highlighted class to a specified file. Name the file good_classes.hdf

You can now close e2evalparticles

5-3 : Making an initial reconstruction

There is a lot of controversy in the cryo-EM community on this point. Some people feel that initial model generation is the most critical step in refinement, and you need to use difficult and time-consuming experimental methods to get an initial model before you can proceed. In the vast

majority of cases, we have shown that the simple approach used in EMAN2 can give a completely reliable initial model with no additional experiments required. However, there are a few important caveats here:

➡ When you are uncertain about the quaternary structure of your molecule, tilt validation is a critical test. You collect pairs of images at 0 degrees and typically 10-20 degrees tilt, box out tilt pairs of particles, then run them through a tilt validation procedure against your final 3-D map. This method is fully implemented in EMAN2, but we will not cover it during this workshop (there is a tutorial for this: http://blake.bcm.edu/emanwiki/OxfordWs2012).

➡ Heterogeneity is a potential issue. If you have a particle that is highly heterogeneous, the EMAN initial model strategy is likely to fail to produce a unique answer (since there isn't one). Single particle tomography may offer the best solution towards understanding the heterogeneity in your specimen. Once you understand the heterogeneity, EMAN2 includes e2refinemulti.py and e2classifyligand.py for purposes of processing such data sets using normal single particle data.

➡ Poor angular distribution. If your particles have a single, strongly preferred orientation, especially if this is combined with a low symmetry, there may not be enough information to produce an unambiguous starting model. However, it is also important to note that in this situation, even if you get a good starting model, refinement will also tend to degrade rather than improving the model. To perform a proper 3-D reconstruction, you must have a reasonable number of particles in orientations covering at least one great circle around the unit sphere.

➡ If you do have a difficult structure, single particle tomography is one good solution (http://blake.bcm.edu/emanwiki/SPT/Spt). Random Conical Tilt is another possibility (http://blake.bcm.edu/emanwiki/RctTutorial). Despite all of the lengthly caveats and descriptions, the actual initial model generation program is quite easy to use. Just select: Initial Model→Make Initial Model

This program is effectively a Monte-carlo. That is, it tries a bunch of random starting models then refines each against the class-averages. At the end, it sorts the results based on how well they agree with the inputs. In most cases ~10 tries is sufficient to get a good starting model, but sometimes you get unlucky or have a particularly tricky structure, and may need additional attempts.

Since we are under time constraints at the workshop, I suggest starting with 10 initial models, then if you don't get one you like, run another 10. If your laptop is particularly slow, you may start with even fewer and hope for the best.

- Input = good_classes.hdf, sym = D2, iterations = 8
- parallel= "thread:N" (again, N is the number of cores on your machine)
- Use the default values for the other options, and Launch

➡ This program will take a few minutes to run. What it does is fairly straightforward. It treats the class-averages you provided as input as particles, generates a randomized blob as a starting model, and runs a highly optimized version of the normal EMAN2 refinement procedure to refine a structure. It does this models times, and in the end sorts the various output maps in order of apparent quality.

➡ For most structures, there are a number of 'local minima' in the energy space. What that means is, there are a number of incorrect structures which can still appear to agree fairly well (but not as well as the correct structure) with the input data. So, some fraction of the answers you get out are likely to be bad starting models. On the bright side, such bad starting models are usually quite obvious. The severity of this problem varies considerably with the shape of the molecule and the amount of orientation coverage you have. The Bgal structure is reasonably good in this respect, but there are still some wrong answers you will see. Interestingly, particles like ribosomes, generally viewed as 'difficult' have virtually no local minima, and will produce a usable starting model most of the time.

When the program is done, open the browser ( ). You will see a new folder called initial_models. The program shows you all of the initial models it generated, not just the best one. For each, 4 files will be produced in the initial_models folder. We will only look at two of them in the tutorial: model_NN_MM and model_NN_MM_aptcl. The first time you run the program NN will be 00, and MM will indicate the number of the attempt. If you need to run the program again, new results will have 01 for NN.

model_00_01.hdf ostensibly contains the best 3-D refined initial model, but looking at it is not the best way to detect whether it is bad or good. Instead, start by looking at model_00_01_aptcl.hdf. Here are two possible results, the left good, and the right bad:

Each of these files contains adjacent pairs of images. The first image is one of the classaverages you provided and the second image is a projection of the initial model it created. If you have a good initial model, the class-averages and projections should agree with each other very well (aside from noise). In the left (good) case above, you will see that this is the case every class is a good qualitative match to the corresponding projection, with the possible exception of 0-1.

In the right (bad) case, while several of the pairs agree well, such as 38-39, many of them do not. In a good initial model, agreement should be good for ALL of the class averages.

If one or more do not match, there are 3 possibilities: 1) a bad initial model, 2) a bad classaverage, 3) heterogeneity in the particle population. In this case 0,1 in the left model is likely just not a very good class average, as other than this, agreement is excellent. Clearly having seen the left case, we know that the right case is just a (random) bad initial model.

If your best model doesn't agree for more than 1 or 2 class-averages, you should try running the program again. If you persistently fail to get completely self-consistent results, the conclusion would be that you have some structural heterogeneity in your particles. We will deal with that situation in the second tutorial on heterogeneity. This B-gal data set should not have significant enough heterogeneity to be an issue.

Once you identify a starting model with a good _aptcl file, go ahead and open the corresponding model_NN_MM.hdf file in 3-D and have a look at it. This will be the starting model we use for refinement. Note that the handedness of this model will be arbitrary. There is a 50/50 chance that it is correct. We deal with that after refinement. 16. Building sets - redux Above we went through a lot of hassle to identify bad particles. However, so far all we have done is put a mark on the bad particles. At present they are all still included in the sets/*.lst files which we use for our refinements.

This is done intentionally, so you can retain the original set of particles, in case you want to go back and test what effect eliminating the bad particles had. So, to process the data without bad particles we need to build new sets excluding the newly marked bad particles.

Particle sets→ build particle sets

check the allparticles and excludebad checkboxes

enter all-bad1 in the setname box, this name indicates to me that I have made one pass at removing bad particles, but otherwise the set includes everything. If I made another pass and excluded even more bad particles, I would call it all-bad2.

Launch We'll make one more set containing less of the data for our initial refinement (for speed)

Particle sets→ build particle sets

- Uncheck the *allparticles* box
- Press the Browse button. Click on the snr-hi column to sort, and select ~1/2 of the images with the highest snr-hi values, then click OK.
- enter best-bad1 in the setname box.
- Launch

➡ If you aren't pressed for time, you could consider going back and repeating steps 12-13, using the all-bad1 set as input. You can then see if you are able to eliminate a few additional bad particles. Each time you repeat the process it should become more difficult to find bad particles to eliminate (diminishing returns). If you are trying to keep up with the workshop, you don't need to do this again, a single pass is perfectly adequate for a very nice reconstruction.

STEP 6 : 3D refinement

➡ e2refine_easy has a built in system of heuristics to automatically select the best options for your refinement. These are not just default values. The program actually looks at all of the information it has about your project to decide the best set of options and parameters to use. It describes the options it selected and the reasoning behind it in the refinement report it produces as described below. You can override any of these options from the commandline as required, but we strongly recommend trying the automatic system first, before making arbitrary modifications.

➡ e2refine_easy computes a 'gold standard' resolution as part of the refinement process, for each iteration of the refinement algorithm, and uses this information with the structure factor we already computed to near-optimally CTF correct and filter the final 3-D maps. The 'gold standard' does not eliminate noise bias, but it simply prevents noise bias from entering the resolution calculation, which in turn influences the filter EMAN uses. In our first refinement we will use the data we downsampled by 2x, to make the refinement run faster. We are also using the smaller set with only ~1/2 of the particle data. The goal of this first refinement is to take the very coarse starting model (which was 3x downsampled) and quickly get something at ~15 Å or better based on the particle data directly, rather than a few classaverages. We will then immediately do a second run targeting ~7 Å with the

downsampled data, followed finally by a refinement with our complete data set with full sampling, targeting ~4.5 Å resolution. The first 2 refinements should be easily possible on your laptops. The final refinement might take a full day or more on some laptops.

6-1 : 3D refinement

3D refinement → Single map refinement (e2refine_easy).

- input → sets/best-bad1__ctf_flip_small.lst
- inputavg → leave empty
- model → select your good starting model (model_00_01.hdf usually)
- targetres→15, sym→d2, iter→2, mass→465, speed→7, apix→0
- fill in parallel and threads as previously
- Launch

e2refine_easy creates an HTML (web page) report as it runs explaining the various decisions it makes and the parameters it used, as well as summarizing the results. Browse into the refine_01/report folder and select index.html. You can press the "Firefox" button (or just browse to the file in your web-browser if that doesn't work on your machine).

The report is updated continuously, including resolution and convergence plots. You will need to reload the index.html page to display the new content. With these parameters, on a machine with a total speed factor of ~4.0, the refinement will take ~1 hour to complete (ie - 4 CPU-hr).

The initial refinement should not take very long to run. As soon as it finishes, without even looking at the results, go ahead and start the second refinement running. Make the following changes to the last run:

model → refine_01/threed_02.hdf

- targetres→7, iter→4, speed→5
- Launch

Now that you have a real refinement running, let's quickly go over what all of these options are and what they mean:

input - this is simply the set (.lst file) containing the phase-flipped particles to be refined.

inputavg - if specified, this permits you to use a different stack of particle images for reconstruction and alignment. input is used for alignment, inputavg is used for the final class-averages and 3-D reconstruction. This is used in cases such as direct-detector movie mode processing when you want to use high-dose particles to determine orientation, but low dose (minimal radiation damage) particles for the final map.

model - Perhaps an unfortunate historical term. We refer to the 3-D map used to seed the iterative refinement as the initial model. Model is also often used to describe the PDB model derived from a high resolution map. That is not the meaning here. This file must be a 3-D map file with a valid A/pix value in the header. You do not need to resize/rescale the map. This is handled automatically.

startfrom - this is used instead of all 3 parameters above. If you have already completed a refinement run, and wish to continue without changing the input particle set, but perhaps wish to specify a higher target resolution, specify the name of an existing refine_XX folder, and it will pick up where that refinement left off. It is not possible to change the input particle stack with this option, as that would violate the assumptions for the 'gold standard' method.

targetres - This is the resolution (in Å) you are trying to achieve in this particular refinement run. If you select a much higher resolution than you can actually achieve, it will make the refinement take much longer, and in extreme cases may even make the refinement somewhat worse (not typical). If you set the resolution much lower than the provided data can achieve, it will not sample sufficiently, and you may get some odd resolution curves with apparently exaggerated resolution. If your refinement ever achieves a resolution better than your targetres, you need to rerun the refinement with a higher resolution target to have a valid result.

sym - the enforced symmetry of your structure. Your initial model must be in a symmetryaligned orientation matching the specified value here. See: http://blake.bcm.edu/emanwiki/EMAN2/Symmetry

iter - Number of refinement iterations to run. Linearly related to time required for the job to run. With a decent starting model, most EMAN2 jobs will converge after ~3 iterations, and we often run 4 just to make sure we have achieved pseudoconvergence. If you are starting with a very poor starting model, such as trying to refine a ribosome from a featureless ellipsoid, you may need to run 10-12 iterations to converge to something sensible, much like the initial model generator.

treeclassify - don't use this at present. It is an experimental new option.

breaksym - A powerful option for studying pseudosymmetric objects, such as icosahedral viruses with portal complexes or multimeric proteins with highly homologous subunits. This will determine the orientation of each particle first with the stated symmetry. It will then search that orientation in each of the N asymmetric units for the best match. Converges much more quickly if a symmetry broken starting model is provided, but after enough iterations you can even start with a symmetrized starting map.

m3dold - Not normally used. Reverts the 3-D reconstruction to an older version of the code. If you experience any strange artifacts in your 3-D reconstruction you could try turning this on. If it helps, please report the situation to sludtke@bcm.edu.

mass - in kDa. However, that isn't really what this parameter means. The goal is to set 1.0 to be a reasonable isosurface threshold for the map. Historically this was often done by assuming a mean protein density of 1.35, and using the mass to compute a volume which should be enclosed by an isosurface (at a threshold of 1). However, if you were to visualize a 4 Å resolution map using this method, you would see a solid density in space that was impossible to interpret. An isosurface where the backbone and sidechains are visible is mostly open space, and has perhaps 1/2 the volume you would normally assume. So in a project targeting high resolution this mass value should normally be significantly less than the true mass of the target molecule.

automaskexpand - A 'solvent flattening' procedure is a standard part of most single particle refinement procedures. That is, a mask is constructed just outside what is believed to be the protein

density, the mask is softened with a gaussian falloff to the mean solvent level. This improves both refinements as well as resolution values by eliminating pure noise. However, if you have a ligand with weak fractional association, or a dynamic component on the exterior of the molecule or somesuch, it can create a weak density just outside the main molecule, which can sometimes get sliced off by this automatic masking. This parameter allows you to specify an additional number of 1 voxel shells to expand the mask beyond the normal value. Doing this may degrade the resolution slightly, but will include weak peripheral densities that would otherwise have been excluded.

classkeep - the fraction of particles in each orientation class which should be included in the class-average. When making class-averages, the particles are combined, then each is compared against the just-created average. If this value is, for example 0.9, then the 10% of the particles that look least like the average will be excluded from the classaverage.

m3dkeep - similar to classkeep, but the fraction of class-averages to include when building the 3-D reconstruction. Classes are already automatically weighted based on number of particles or SSNR, but if you have some class-averages in underpopulated orientations which are really simply bad, this allows you to exclude them from the reconstruction.

classrefsf - causes the class-averages to be filtered to have a similar structure factor as the projections. This doesn't actually influence the information content in the classaverage, but makes them easier to visually compare with the projections.

classautomask - an experimental option for performing 2-D masking of the classaverages during iterative class-averaging.

prethreshold - An experimental option for applying a threshold to the 3-D map before using it as a reference for the next round of refinement.

m3dpostprocess - allows you to specify a manual processor to be applied to the 3-D map at the end of each iteration. Normally not necessary as the maps are automatically filtered.

➡ Clearly we won't be running any jobs on Linux clusters at the workshop, but if you continue to do single particle analysis, eventually it is almost inevitable that you will need to do this. The final refinement below with speed=1 can take ~150 CPU-hr to finish. With the full data set that has 10x as many particles and even finer sampling, we'd be talking something closer to 1000 CPU-hr. Even if you had a powerful 24 core workstation, this is still more than a day to finish the job. On a cluster you could bring the time down to a couple of hours. There is fairly extensive documentation on the topic in the Wiki (http://blake.bcm.edu/emanwiki/EMAN2/Parallel) where you can find many of the necessary details. If you have problems, don't be shy about asking!

6-2 : Final refinement

If everything went well, you should now have a refinement which has achieved at least ~8 Å resolution. If the resolution curve has gone beyond this point due to the high quality of the data, then you can't completely trust the resolution value (ie - the resolution value can only be trusted to a bit past the target resolution you specify), and you need to run another refinement with a better target resolution. In this case we will go ahead and switch to our final refinement targeting ~4 Å resolution. This will be similar to the last run, but we will use the complete data set with full sampling

and target higher resolution. Doubling the box size will increase the computational load by at least 4, probably more like 6 or 8 times. Doubling the number of particles will increase the time by a factor of 2. Increasing the resolution target is probably only a 20% increase. So if your previous refinement took 2 hours, we're looking at something like a full day to complete this refinement on a laptop. We're still talking something like 100 CPU-hours here, so in normal cluster computing terms, which is often measured in thousands or tens of thousands of CPU-hr, this is still a pretty small project. While you can try running this overnight, if you want to look at the final results without the wait, we have made a copy of the refinement folder containing the full project described here available for download (but not at the workshop site). Of course this will have results of the specific choices I made rather than the choices you made, but it will give you some idea of what to expect. I included a final refinement with both speed=5 and speed=1 so you can see the range.

3D refinement → Single map refinement (e2refine_easy)

- Change model to refine_02/threed_04.hdf
- Change input to sets/all-bad1__ctf_flip.lst
- Change targetres to 4.0
- speed - you have a choice here. If you use 5, you should be able to hit 4.5 Å reasonably quickly (~15 CPU-hr/iteration), and the structure will look reasonably nice. If you go all the way down to 1, you should readily hit 4 Å, and should have nicely resolved beta-strands with good helical pitch, but the refinement will take ~3x longer to run (~40 CPU-hr/iteration).
- iter can be set to 3 or 4 depending on how long you're willing to wait. Note that you will make good progress after even 1 or 2 iterations.
- Launch. If you monitor progress, make sure to look at the report in the refine_02 folder.

This will take longer to run.

If you want to really optimize the refinement, you can set speed to 1 and may play with some of the other options. If you really want to push resolution, though, you will need to download the full challenge data set, and work with that.

➡ Note that setting *start from* to an existing refine_xx folder is NOT equivalent to setting model to the final map from the same refine_xx folder. If you specify a single input model, it will be phase-randomized two times to relatively low resolution as starting models for the even/odd half refinements (that is, you take a step backwards if you do this). If you use *start from*, the existing even/odd references are used, and the refinement can progress naturally, with no model bias, but you are not able to change the input data set.

➡ If your resolution curves are not “healthy”. That is, they have strange peaks, don’t fall all the way to zero, or have other artifacts, then some sort of bias still managed to creep its way into your project. If this happens, I’d suggest asking me about it. Sometimes you will see this in the tightly masked curve, but the curve with the normal mask will be fine. This simply indicates that the automatic tight mask was a little too aggressive, and you should ignore that curve. Particularly if you observe any anisotropy in the resolution (if the map looks smeared or blurry in some particular direction), it is a good idea to take a look at the orientation distribution of your map. If you run Validation and Analysis → Run e2eulerxplor, it will give you a window showing the distribution of particle orientations within one asymmetric unit. As usual, middleclicking on the orientation display

window will give a control panel allowing you to select which iteration of which refinement to look at.

STEP 7 : Molecular modeling

Look at the final refined map (refine_03/threed_XX.hdf). You can open it in Chimera and compare it to PDB map 3j7h. If you have gotten to 4 Å resolution you should be able to resolve a lot of the strands in the beta-sheets, and the alpha helical pitch (there are only a few short helices) should be visible. If you refined a little less aggressively, you should still see the beta-sheets as planar structures with the helices as clear rods. If you can't get the maps to line up properly (using Chimera's 'fit in map' tool), you may need to flip the handedness of the final map. As mentioned earlier, the handedness of a single particle reconstruction without a tilt experiment is arbitrary. To do this, you can open the final map in e2filtertool and use xform.flip with axis=z to invert (then save the modified map using the appropriate menu item). Similarly you could use the command-line.

STEP 8 (Optional) : Publish...