

EMAN2 WORKSHOP

Wednesday 16/03/11, P.M. practical SPT session

Below the *Workshop Tasks* section you can find the *EMAN2 SPT User's Guide* (page 8), which takes you through all you need to know with step-by-step descriptions of the user interface and the commands you'll execute, as well as explanations on how Single Particle Tomography works.

Open the bookmarks tab (left) for easier navigation through the document.

Read as much as you can of the User's Guide BEFORE the Workshop if you want to understand better what you'll be running.

WORKSHOP TASKS by Jesús Galaz

This might consume quite a bit of your computer's memory. Close ALL running applications (text editors, internet browsers, etc) before you start.

TASK1

Step 1: Box one particle (for snapshots and details go to page 9).

1.0) Open the phase-plate data tomogram from the command line:

e2tomoboxer.py e15pp_tomo_bin2.mrc --yshort --inmemory

1.1) Set the box size parameter to 140 in the Main tomoboxer window.

1.2) Select ONE particle by centering the mouse's cursor over it and pressing left-click. (You can tweak the center by holding left-click and dragging)

1.3) Click on *File*, select *Save Box Coord*, and save the coordinates of this particle to a *.txt* file (proposed filename: *e15pp_set1_coords.txt*).

1.4) Click on *File*, select *Save Boxes as Stack*, and save the actual particle to an *.hdf* file (proposed filename: *e15pp_set1_stack.hdf*).

You MUST type in the appropriate format, both when you save a coordinates file or a stack.

1.5) Close *e2tomoboxer*, then check out your boxed particle

e2display.py e15pp_set1_stack.hdf

A GUI will come up showing the volume; middle click on it to bring up a panel with viewing options (you can display the particle at different thresholds, but this might be very slow on certain computers).

Step 2: Check out the icosahedral reference (for details on reference and particle “preparation for alignment” go to page 18)

2.0) Find the prepared symmetric reference with this file name:

e15ref_prep_icos_bin2.hdf

2.1) Make sure it has exactly the same box-size (*nx*, *ny* and *nz* values on the header) and a similar apix (*apix_x*, *apix_y* and *apix_z* values on the header) as the boxed particle. To look at the header of each file, execute these commands:

e2iminfo.py e15ref_prep_icos_bin2.hdf --header

e2iminfo.py e15pp_set1_stack.hdf --header

Step 3: Align one particle to a reference

3.0) This command looks discontinuous here, but it should be ONE single line with all the parameters in a row; there should be a single space separating each option that starts with “--”, for example, --A --B --C ... --N... etc.

There should be NO spaces after double dashes “--” or colons “:”

You can change the value after --parallel=thread to match the number of processors on your computer.

```
e2classaverage3d.py --input=e15pp_set1_stack.hdf --  
output=e15pp_set1_aligned.hdf --ref=e15ref_prep_icos_bin2.hdf --ncoarse=1 -v  
3 --mask=mask.sharp:outer_radius=48 --  
preprocess=filter.lowpass.gauss:cutoff_freq=.025 --  
align=rotate_translate_3d:search=12:delta=8:dphi=8:verbose=1:sym=icos --  
parallel=thread:2 --ralign=refine_3d_grid:delta=3:range=9 --  
averager=mean.tomo --aligncmp=ccc.tomo --raligncmp=ccc.tomo
```

Estimated runtime: Between 3 and 8 minutes (--raling=refine_3d_grid)

You can alternatively use a faster aligner for the fine alignment step, but the answer might not be as good:

Estimated runtime: Between 2 and 6 minutes (--raling=refine_3d)

Step 4: Apply symmetry to the aligned particle

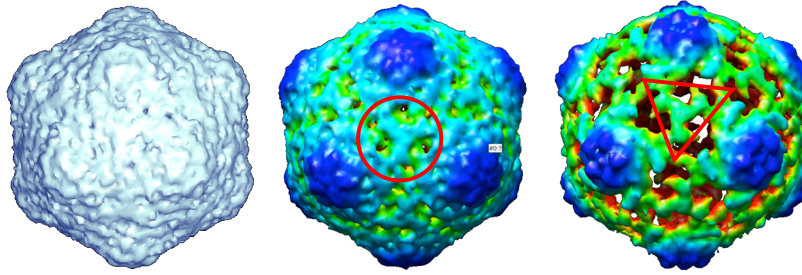
4.0) Apply symmetry to the aligned particle by executing this command:

e2proc3d.py e15pp_set1_aligned.hdf e15pp_set1_aligned_icos.hdf --sym=icos

4.1) Look at the aligned particle after having applied icosahedral symmetry:

e2display.py e15pp_set1_aligned_icos.hdf

It should look similar to this:



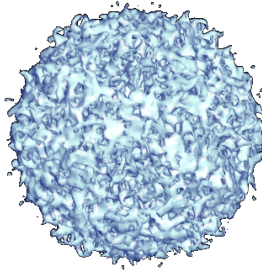
The average looks different at different thresholds (click the middle button on the mouse to get access to the threshold bar), and depending on what particle you picked (some are nicer than others). You can color the volume with Chimera to ease visualization. If you can see the threefold density highlighted by the red circle and triangle, you're doing things right.

Step 5: CONTROL 1

5.0) Apply icosahedral symmetry to the raw boxed particle (before it was aligned).

```
e2proc3d.py e15pp_set1_stack.hdf e15pp_set1_stack_icos.hdf --sym=icos
```

You should get out trash:



Step 6: CONTROL 2

6.0) Go back to the tomogram and box out an “empty particle” with a 140 box-size and save it to an *.hdf* file (proposed name: *empty.hdf*; basically, choose a region of the tomogram where you see no particles; you thus will box only ice).

6.1) Align the garbage “particle” just like the first one (copy the command from the previous alignment and just supply the garbage particle through the *--input* option and select a different output name):

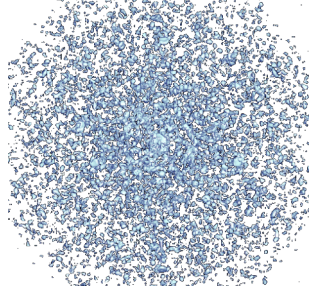
```
e2classaverage3d.py --input=empty.hdf --output=empty_aligned.hdf --  
ref=e15ref_prep_icos_bin2.hdf --ncoarse=1 -v 3 --  
mask=mask.sharp:outer_radius=48 --  
preprocess=filter.lowpass.gauss:cutoff_freq=.025 --  
align=rotate_translate_3d:search=12:delta=8:dphi=8:verbose=1:sym=icos --
```

```
parallel=thread:2 --ralign=refine_3d_grid:delta=3:range=9 --
averager=mean.tomo --aligncmp=ccc.tomo --raligncmp=ccc.tomo
```

6.2) Apply symmetry to the aligned garbage particle:

```
e2proc3d.py empty_aligned.hdf empty_aligned_icos.hdf --sym=icos
```

You should also get trash this time too:



CONCLUSION:

You can't get a decent icosahedral average just out of *anything*. Our aligners work.

TASK2

Step 7: Box a larger set/stack

7.0) Go back to the tomogram and box out 5 particles. Save the coordinates (just as a backup so you don't have to rebox if the computer crashes) and the data as a stack, with .hdf format (proposed file name: *e15pp_set5_stack.hdf*)

Step 8: Align and average a stack

8.0) Set the shrink options to 2 so the alignments go faster. Supply the correct number after "*--parallel=thread:*" to match the number of processors on your computer.

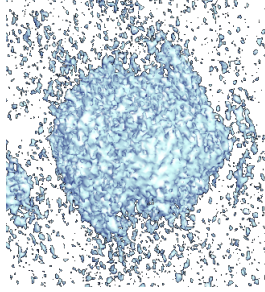
Shrink by 3 if your computer is REALLY slow or you only have 1 processor.

```
e2classaverage3d.py --input= e15pp_set5_stack.hdf --
output=e15pp_set5_shrink2_average.hdf --ref=e15ref_prep_icos_bin2.hdf --
savesteps -- --ncoarse=1 -v 3 --mask=mask.sharp:outer_radius=48 --
preprocess=filter.lowpass.gauss:cutoff_freq=.025 --
align=rotate_translate_3d:search=6:delta=8:dphi=8:verbose=1:sym=icos --
parallel=thread:2 --ralign=refine_3d_grid:delta=3:range=9 --saveali --
averager=mean.tomo --shrink=2 --shrinkrefine=2 --aligncmp=ccc.tomo --
raligncmp=ccc.tomo
```

Estimated runtime: 5 to 9 minutes (using 5 particles, shrinking by 2)

View the average. (It will still be very noisy)

```
e2display.py e15pp_set5_shrink2_average.hdf
```

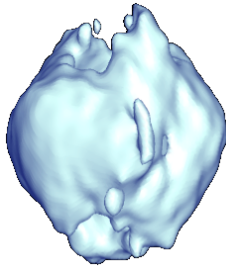
Step 9:

9.0) Filter the average at 100Å and view it.

```
e2proc3d.py e15pp_set5_shrink2_average.hdf  
e15pp_set5_shrink2_average_lp100.hdf --  
process=filter.lowpass.gauss:cutoff_freq=0.01
```

```
e2display.py e15pp_set5_shrink2_average_lp100.hdf
```

It might look something like this (the missing wedge is still heavily affecting it).

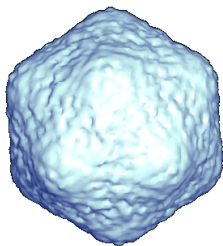


(You could have filtered the average automatically by setting the `--postprocess=` option in `e2classaverage.py`)

9.1) Apply symmetry to the average and look at it again.

```
e2proc3d.py e15pp_set5_shrink2_average.hdf  
e15pp_set5_shrink2_average_icos.hdf --sym=icos
```

```
e2display.py e15pp_set5_shrink2_average_icos.hdf
```



TASK3

Step 10:

Implement an easy trick to minimize the missing wedge effects in the asymmetric 5-particle average (basically, randomize the position of the missing wedge *before* aligning the particles against the symmetric reference).

10.0) Open EMAN2's iPython programming interface:

```
e2.py
```

10.1) Find the number of particles in your stack and save it to a variable ('n'):

```
n = EMUtil.get_image_count("e15pp_set5_stack.hdf")
```

10.2) Import python's *random* module

```
from random import *
```

10.3) Rotate the particles by a random amount in all three angular directions and write them out to a new stack file (the tab in the lines following the "for loop" are a MUST):

```
for i in range(n):  
    a=EMData("e15pp_set5_stack.hdf",i)  
    a.rotate(randint(0,360),randint(0,180),randint(0,360))  
    a.write_image("e15pp_set5_stack_rand.hdf",i)
```

To run the for loop and end it, just press "enter" twice.

Then, to exit the iPython interface type:

```
Exit
```

Then press 'enter'.

Step 11: Repeat steps 8 and 9 for the new "randomized" set.

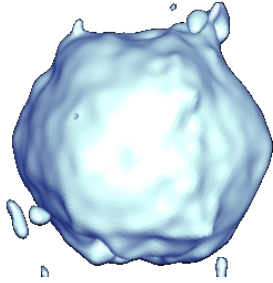
11.0) Align and average the randomized set

```
e2classaverage3d.py --input= e15pp_set5_stack_rand.hdf --  
output=e15pp_set5_rand_average.hdf --ref=e15ref_prep_icos_bin2.hdf --  
savesteps -- --ncoarse=1 -v 3 --mask=mask.sharp:outer_radius=48 --  
preprocess=filter.lowpass.gauss:cutoff_freq=.025 --  
align=rotate_translate_3d:search=6:delta=8:dphi=8:verbose=1:sym=icos --  
parallel=thread:2 --ralign=refine_3d --saveali --averager=mean.tomo --  
shrink=2 --shrinkrefine=2 --aligncmp=ccc.tomo --raligncmp=ccc.tomo
```

11.1) Filter the average to 100Å and view it

```
e2proc3d.py e15pp_set5_rand_average.hdf e15pp_set5_rand_average_lp100.hdf  
--process=filter.lowpass.gauss:cutoff_freq=0.01
```

```
e2display.py e15pp_set5_rand_average_lp100.hdf
```



The missing wedge seems to be completely filled in now, and the icosahedron shape is evident. (You can apply symmetry, but there's not much of a point).

TASK4

Step 12: Asymmetric averaging

12.0) Box some more particles if you wish, with the same box size (proposed filename: *e15pp_set10_stack.hdf*)

12.1) Find the asymmetric reference
e15ref_prep_icos_bin2.hdf

12.2) Align your largest data set against the asymmetric reference.

```
e2classaverage3d.py --input=e15pp_set10_stack.hdf --  
output=set10_average.hdf --ref= e15ref_prep_asym_bin2.hdf --savesteps --  
ncoarse=1 -v 3 --mask=mask.sharp:outer_radius=43 --  
preprocess=filter.lowpass.gauss:cutoff_freq=.0125 --  
align=rotate_translate_3d:search=6:delta=6:dphi=6:verbose=1:sym=c1 --  
parallel=thread:2 --ralign=refine_3d_grid:delta=1.5:range=9 --saveali --  
averager=mean.tomo --shrink=1 --shrinkrefine=1 --aligncmp=ccc.tomo --  
raligncmp=ccc.tomo
```

What you'll get is uncertain. It actually depends on what particles you picked. This data isn't "ideal" in that some e15 particles are too close to other e15 particles; so fancier things might be needed to be able to align the particles asymmetrically and actually have the viral tails match.

EMAN2 Single Particle Tomography

USER'S GUIDE *version β1.0* by Jesús Galaz

NOTE: SPT (single particle tomography) capabilities are relatively new in EMAN2. They were inspired by Michael Schmid's studies on sub-volume averaging. This guide covers (for now) a small fraction of the SPT processing possibilities EMAN2 will eventually offer.



→ "Particle" and "sub-volume" are used interchangeably.
→ There are detailed explanation sections accordingly labeled throughout this document, as well as **tips** like this one (light bulb icon), **warnings** (red crosses), and **strong suggestions** (yellow triangles).

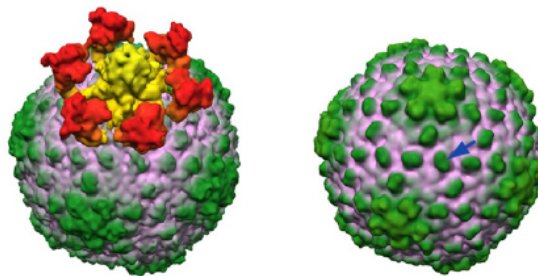
1. PREFACE

In a nutshell, Single Particle Tomography is about extracting sub-volumes from a tomogram to obtain one or more averaged structures from them. The technique is particularly good for imaging heterogeneous specimens (complexes that are shuffling between different conformations or exist at different sizes), and molecules in challenging contexts, such as cellular environments (for example, ribosomes inside a cell).

The User's Guide will take you through using EMAN2 for SPT by running examples on test data available through the *e2spt_data.zip* file on this page of EMAN2's Wiki:

<http://blake.bcm.edu/emanwiki/Ws2011/Spt?highlight=%28spt%29>

If you processed thoroughly *all* the data from the full version of one of the tomograms provided, you should arrive at averages that look like this:



Asymmetric

Icosahedral symmetry applied

Murata et al., 2010; average of Zernike phase-plate data (~130 particles).

You won't be able to because we are providing *cropped* tomograms (just a tiny portion of the original tomograms, opposed to the full version) that have been scaled down by a factor of 2 so that the test files aren't aberrantly large and require ridiculous amounts of memory.

The guide addresses the following areas:

- Sub-volume extraction from tomograms using EMAN2's 3D particle-picking tool, *e2tomoboxer.py*
- Detailed description of the particle-picking interface
- Automated "preparation" of extracted particles for alignment, reference-based alignment and averaging with *e2classaverage3d.py*
- Explanation of what "particle preparation" means, and individual command-line alternatives to carry out such preparation "manually".
- SPT processing through EMAN2's workflow, *e2workflow.py*

The *.zip* file on the Wiki (the link in the previous page) contains the following files:

1) e15pp_tomo_bin2.mrc

This first tomogram was reconstructed from a tilt series of epsilon15 virus particles *in vitro*, recorded using Zernike phase-plate technology. [Liu and Murata *et al*, 2010]. It has been binned (shrunk) by a factor of 2.

2) e15n_tomo_bin2.mrc

This tomogram also comes from a tilt series of epsilon15 viruses *in vitro*, but was recorded under "conventional" cryoET imaging conditions. [Liu and Murata *et al*, 2010].

3) e15ref_raw.hdf

A structure of the epsilon15 virus downloaded from the EMDB

4) e15ref_prep_asym_bin2.hdf

The structure above "prepared" to "match" the data (you'll understand later)

5) e15ref_prep_icos_bin2.hdf

The "prepared" structure after applying icosahedral symmetry to it.

Each tomogram will occupy ~500Mb on your hard drive; the other files are small. In this guide, everything is done with the phase-plate tomogram (number 1 on the previous list). You can choose the other one if you want to, but you'll probably get to different results. Hopefully, once you understand the principles of SPT and how it's done in EMAN2 you'll be able to use it to process your own data.

2. BOXING

There are two options for opening a tomogram for the purpose of selecting sub-volumes from it.

- 1) Directly, by typing *e2tomoboxer.py* at the command line, followed by the path to the tomogram (this is what we're going to do, for now).
- 2) A notch less directly, by launching *e2workflow.py* from the command line and accessing a tomogram through the browser in the tomographic reconstruction menu. (This will be described in section 5 of the tutorial, which briefly deals with the workflow).

Opening a tomogram directly with e2tomoboxer.py

Open a Terminal.

To launch the graphical interface that displays tomograms, type the following command (what's in the shaded box) at the command line, using the adequate filename for the tomogram you want to open:

STEP 1A

```
e2tomoboxer.py e15pp_tomo_bin2.mrc --yshort --inmemory
```

EXPLANATION

To specify *--yshort*, or not to

This option will FLIP the orientation of the tomogram, such that the Y-axis becomes the Z-axis, and Z becomes Y (that is, a rotation about the X axis). You should ONLY specify it IF the tomogram has its smallest/slimmest dimension running along the Y-axis, so that it becomes parallel to the Z-axis instead.

WHY? What does this mean?

Some tomograms are built with the slimmest part of the 3D volume (that corresponding to the "ice thickness" in cryoEM) running along the Y-axis. BUT in EMAN2 volumes are displayed such that the Z-axis is perpendicular to the screen. If you open one of the supplied tomograms as is, you'll be looking at it "from the side". Most of the time (and certainly for this workshop), you'll want the slimmest part of the volume to be aligned along Z (NOT Y), so you can see the tomogram "from the top", and look at the entire CCD-captured area in the XY plane, slice by slice, as you go through the volume.

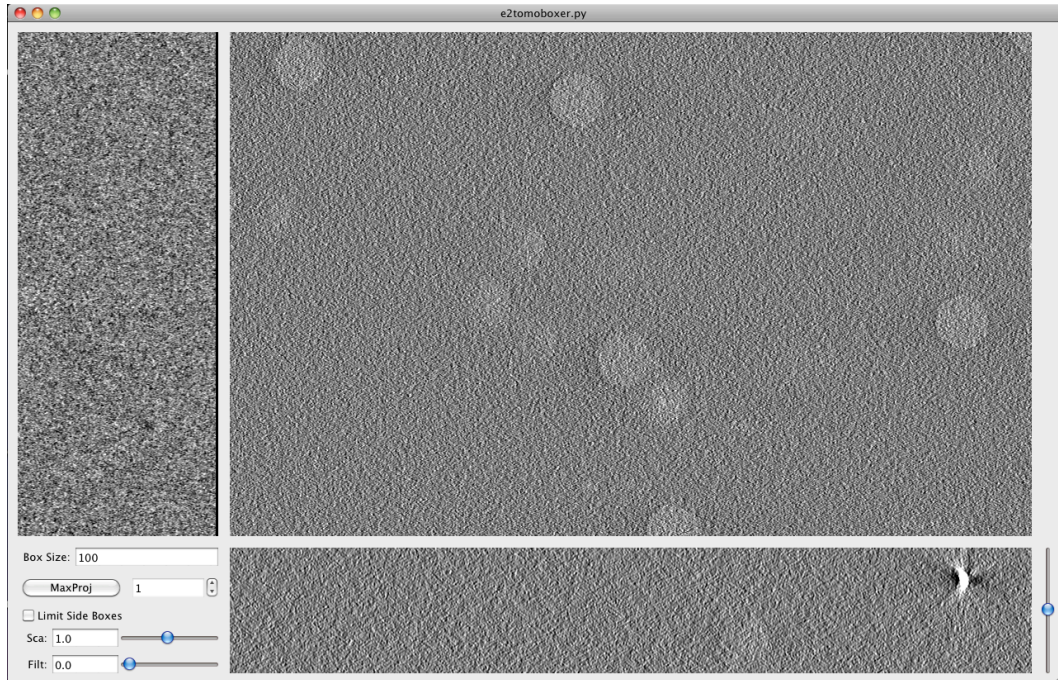
--inmemory

This option pre-loads the tomogram to memory, allowing smoother (faster) viewing and particle extraction, because the computer can read data from memory faster than from disk (your hard drive).



To see the entire list of options for e2tomoboxer.py (not many at this point), and an explanation of what they're for, type *e2tomoboxer.py -h* at the command line. (You can get usage instructions and the list of options for ANY program in EMAN2 in the same way: typing the program's name followed by "-h").

When you open e2tomoboxer.py, the GUI (Graphical User Interface, with nice clickable buttons) below pops up:



From now on, this window will be referred to as **Main Boxer window**.

The **Main Boxer window** is divided into 3 image panels corresponding to top and side view slices of the entire tomogram. If the ice-embedded specimen is nice and yields high contrast, the side-views can be very useful in “tweaking” the center of the boxes selected: Basically, any box created in any one view can be moved in the other two, so you’re literally defining the center of the box in 3D. There’s no point in describing this further; you’ll get a feeling for it as soon as you create a box, see that actually 3 come up (one in each view), and start moving them.

There’s also a small **options panel** at the bottom-left corner of the Main Boxer window (described further below).

First, let’s see what you can do with your mouse and keyboard, with default options.

Boxing:

You can select a region for extraction by doing left-click anywhere in the three views, which triggers the opening of two more windows (described below).

Deleting boxes:

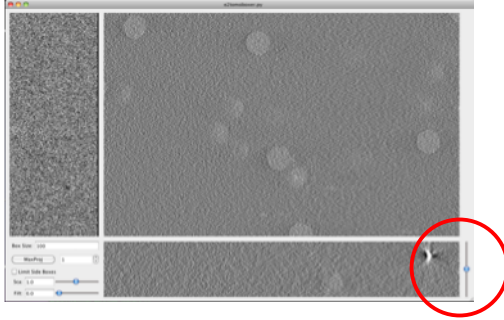
Hold shift and left-click with the mouse on the box you want to delete, in any of the 3 views on the Main Boxer window. You can also delete particles with these same buttons (shift + left click) from the **Particle List window**.

Zooming:

Zoom in and out from all three views simultaneously by scrolling with the middle button of the mouse.

Slicing:

Go through slices along the Z direction by holding shift while scrolling with the middle button (this might not work if you're on a Mac computer), or by moving the bar at the bottom-right corner of the Main Boxer window.



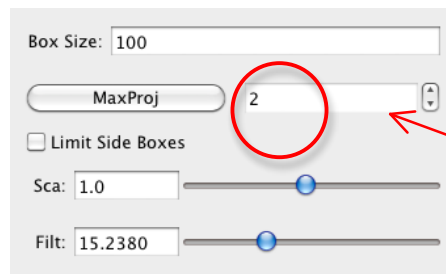
Dragging:

Drag the tomogram slice displayed (translations in 2D) by pressing and holding the right-button on the mouse, and sliding the mouse as desired. (You might also be able to drag the views with the up, down, left and right keys on your keyboard).



Boxing as accurately as possible *is* actually IMPORTANT (you don't have to be OCD about it though). If the boxes are severely off-center, "preparation" of the particles for alignment can cut off chunks of density from them and/or it might not be possible to center them correctly during alignment. This can be disastrous for your reconstruction. (Take it as a dogma, or read the explanation in section 3)

Default display and boxing options can be changed from the panel at the bottom-left corner of the Main Boxer window, which looks like this:



Spin box

Box size:

Defines the side-length (in pixels) for the cubical box that will be extracted from the tomogram. Usually, you want to center the box on putative particles of interest.



Select a reasonable box-size. Alignments usually run faster if it's even, and a multiple of 8. Not a golden rule. To actually find out what the best sizes to use are, go to this page on EMAN2's Wiki:

<http://blake.bcm.edu/emanwiki/EMAN2/BoxSize>

Make the box-size ~2x the diameter of the particles. This "padding" is a MUST for alignment to work. If you box unwisely, you'll end up running failed alignments and becoming utterly unhappy.

Spin box:

When set to more than 1, it displays the average of neighboring slices in each of the 3 tomogram's views. For example, if it's set to 5, that means that 5 adjacent slices will be averaged as you scroll through the tomogra. This can enhance contrast and facilitate boxing.

MaxProj:

It only works when the spin box value is set to more than 1. When MaxProj is pressed, instead of seeing the average of a number of neighboring projections, you see the "maximum value". The image of the averaged slices will contain $n_x * n_y$ pixels in the top view, and $n_x * n_z$ and $n_y * n_z$ for the side views, right? Well, the value of each pixel comes from the maximum value for that pixel among all the slices-to-average. In some cases, this can also make the particles easier to find.

Limit Side Boxes:

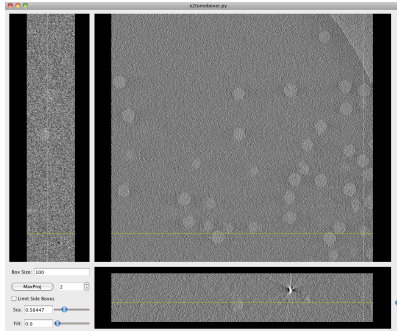
Shows on the side views only the boxes that are in the vicinity of the XZ and YZ slices being shown. Keep in mind that the "top view" or largest view in the Main Boxer window is a slice parallel to the XY plane, whereas the side views are slices parallel the XZ and YZ planes. So when you've boxed many particles, their boxes start to overlap. This quickly crowds the side views (because they're so narrow) if the boxes selected were shown through *the entire* tomogram, as you scroll through the slices. This option avoids such crowding. There's a visual demonstration coming up ~2 pages ahead (snapshots).

Sca:

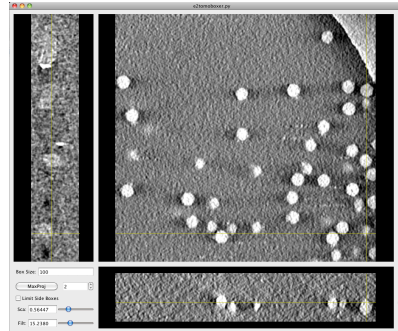
"Scale". The same as described in "Zoom".

Filt:

Applies a Gaussian low-pass filter to the slices displayed in the tomogram's views. It is extremely powerful in facilitating boxing. Lo and behold!:



Unfiltered tomogram slice



Filtered tomogram slice



Turning filtration on WILL slow down your computer because it is applied on the fly. (Not recommended for already frustrated grad students and impatient boxers with high blood pressure, such as senior PIs). The slowing down effect might not be noticeable on small tomograms, or futuristically fast computers.



Alternatively, you can “manually” filter an entire tomogram before loading it to the tomoboxer by typing the following as one continuous line at the command line (no spaces after “--”):

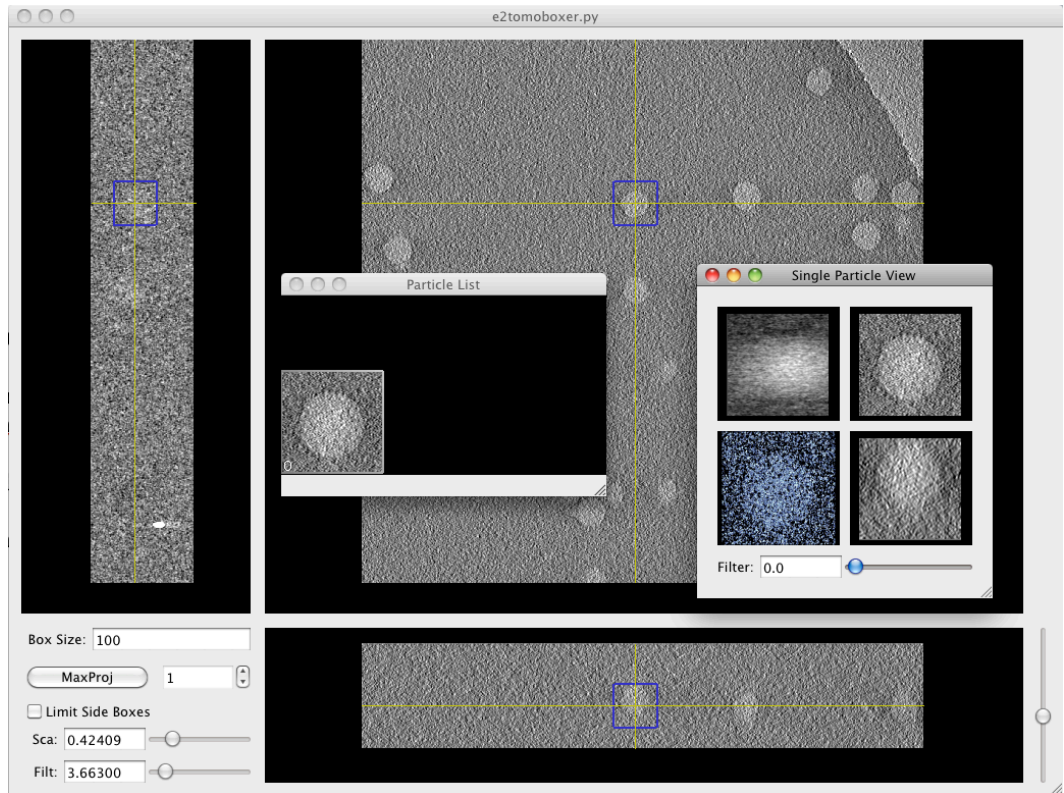
```
e2proc3d.py <input> <output> --  
process=filter.lowpass.gauss:cutoff_freq=0.01
```

You have to provide the reciprocal of the resolution you want to filter at (1/resolution) after “cutoff_freq”. For example, 0.01 filters to 100Å, and 0.02Å filters to 50Å...

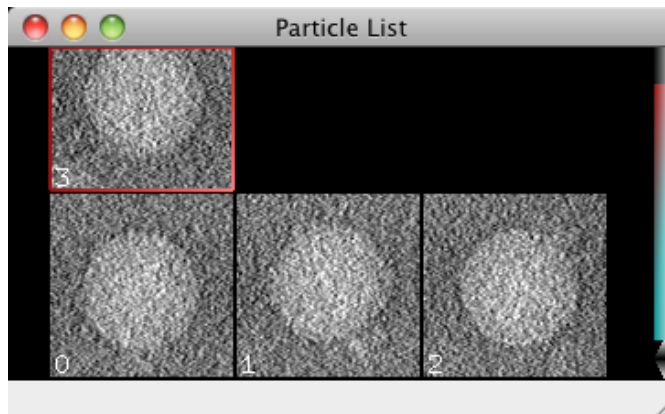
[If the tomogram is too large for the computer’s memory, a “segmentation error” will come up].

Once you box particles from the filtered tomogram, you can save the box-coordinates and load them into the raw tomogram for sub-tomogram extraction through the *File menu*, as described below.

As mentioned before, selecting a box opens two windows: **Particle List** and **Single Particle View**:

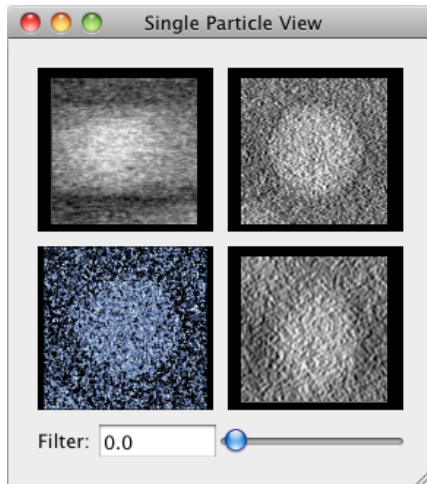


The **Particle List** window shows the XY projection of all the sub-volumes boxed.

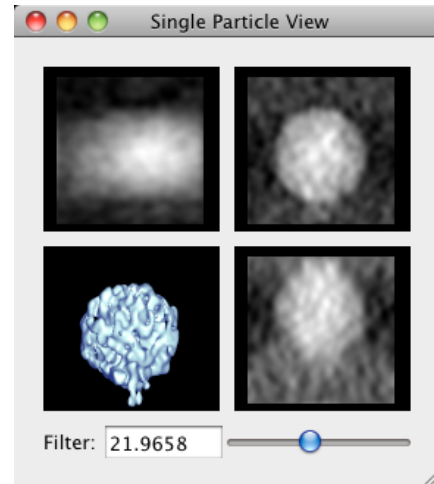


The **Single Particle View** window is *awesome* (provided your specimen isn't tiny): It shows the 3D isosurface and 3 orthogonal projections for the sub-volume that is currently selected (that is, the last you boxed).

It has a *very cool* filtering bar, which applies a Gaussian low pass filter to all 4 displays at the resolution specified:

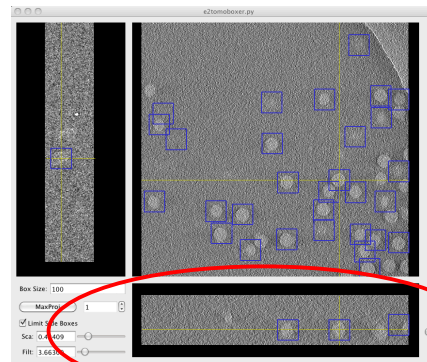
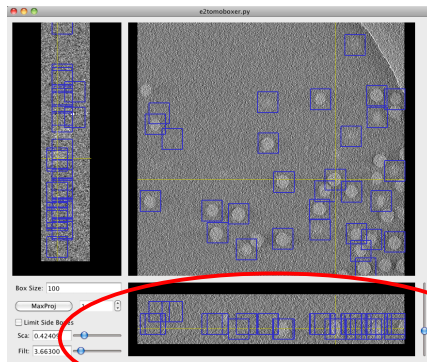


Unfiltered views

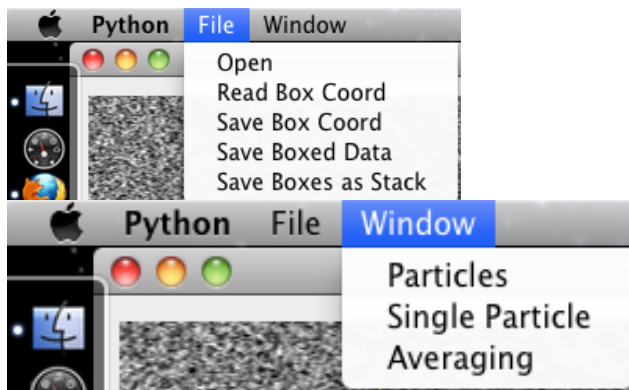


*Doesn't that just look *neat*?*

And below is the promised visual demonstration that the “Limit Side Boxes” option in the Main Boxer window can relieve crowding of boxes in the side views:



You might have noticed the **File and Window menus** at the left of the apple bar if you're on a Mac (way upper-left corner of the screen), or attached to the Main Boxer window otherwise.



The **File menu** lets you save a text file with the coordinates of the sub-volumes selected (Save Box Coord), the sub-volumes themselves as individual files (Save Boxed Data) or as a stack (Saved Boxed as Stack), which means you would only see one file listed in the directory where you save the stack, but it actually contains ALL the sub-volumes you've extracted (and EMAN2 can tell); finally, you can load a coordinates file (Read Box Coord) from a previous boxing session or generated by other means.

Note: You have to EXPLICITLY supply the format for the files you save. Use .hdf for boxed particles saved as separate files, or as a stack.

The **Window menu** lets you re-open any of the windows previously described if you happen to close them. Remember that they'll also open automatically whenever you select a sub-volume.

When you're done exploring e2tomoboxer in its GUI form, follow step 002:

STEP 1B

1.A) You've presumably opened the phase-plate data tomogram:

e2tomoboxer.py e15pp_tomo_bin2.mrc --yshort --inmemory

1.1) Set the box size parameter to 140 in the Main tomoboxer window.

1.2) Select ONE particle with your mouse's left click, not too close to others or the edge of the tomogram. (You can tweak the center by holding left click and dragging)

1.3) Click on *File*, select *Save Box Coord*, and save the coordinates of this particle to a .txt file (proposed filename: *e15pp_set1_coords.txt*).

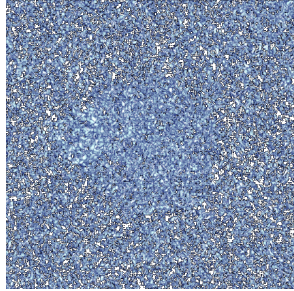
1.4) Click on *File*, select *Save Boxes as Stack*, and save the actual particle to an .hdf file (proposed filename: *e15pp_set1_stack.hdf*).

You MUST type in the appropriate format, both when you save a coordinates file or a stack.

1.5) Check out your boxed particle

e2display.py e15pp_set1_stack.hdf

A GUI will come up showing the volume. It WILL look awfully noisy. This is normal. Do middle click on it to bring up a panel with viewing options (you can display the particle at different thresholds).



"raw" e15 sub-volume

As a quick test, we will align one particle against a symmetrical reference. Then we will apply symmetry to the particle and see if it looks like a healthy icosahedron.

3. READY! GET SET! ... Don't go (yet) - Preparation



For a myriad of reasons, it is not recommendable to align and average sub-volumes directly after extracting them, without "preparing" them first.

Particle preparation for alignment involves several steps, most of which will be taken care of for you 'automatically'. One of these steps is finding and preparing a suitable reference for 'reference based alignment', which can often be critical. This is a branch point at which EMAN2 users have two options (not true for our first test though):

1) *Do not provide a reference*

In this case, a reference will be auto-generated from the boxed data itself, at the alignment step (the mechanism of such reference generation is explained later).



Reference auto-generation is NOT expected to easily yield the best results, specially if your data set is small. It is PROBABLY safer and faster to use a known 'good reference' (a model that can nudge your data in the right direction).

2) *I'm scared by the intimidating warning and feel psychologically coerced to use a reference:*

2a)

STEP 2

2.0) Find the prepared symmetric reference with this file name:

e15ref_prep_icos_bin2.hdf

2.1) Make sure it has exactly the same box-size (*nx*, *ny* and *nz* values on the header) and a similar apix (*apix_x*, *apix_y* and *apix_z* values on the header) as the boxed particle. To look at the header of each file, execute these

commands:

```
e2imininfo.py e15ref_prep_icos_bin2.hdf --header  
e2imininfo.py e15pp_set1_stack.hdf --header
```

2b) If you're feeling adventurous, you can prepare the raw reference for alignment yourself (the file called **e15ref_raw.hdf**). The way to do so is described below.

3.1. "Manual" preparation of a reference for alignment (Skip explanation if using a prepared reference and don't want these details now)

EXPLANATION

The apix and box-size of the reference should match those of the data you want to align to it. Find out what these values are from the "header" of the reference and the header of your boxed particle(s) (the 'header' constitutes a bunch of *metadata* associated with a file; in this case it's information about the EM maps; for example, their box-size and apix).

Displaying values on the header of an EM file

You can look at the header of the reference by typing this on the command line:

```
e2imininfo.py e15ref_prep_icos_bin4.hdf --header
```

Substitute the reference for the filename of your particles to look at their header.

Find *apix_x*, *apix_y* and/or *apix_z* (they should all be the same). The value of those parameters is the apix you need to remember (write it down if juvenile Alzheimer's is affecting you).

Also find *nx*, *ny* and/or *nz*. These are the x, y and z dimensions of the box (they should all be the same too, at least for the boxed particles because e2tomoboxer can only extract cubical boxes; the numbers might not be all the same for the raw reference).

Scaling a reference to match your data's apix

Calculate the scaling factor to apply to the reference by computing (on a piece of paper, a calculator, or your head; this is NOT a command for the command line):

$$\text{scale_factor} = \text{apix_of_data} / \text{apix_of_reference}$$


The cautious (and the paranoid) SPTers (people who do SPT) shall NEVER blindly trust the apix stored on the header of EM files for scaling purposes. You should always confirm, visually, that the reference and the data seem to be

scaled reasonably well. This might imply scaling by a factor slightly different than the one derived from the apix values.

Then run this at the command line, filling in adequate filenames and values:
e2proc3d.py <input> <output> --scale=scale_factor

Clipping the box of the reference to match your data's box

The *--clip* option in *e2proc3d.py* shrinks the data in a volume but it does not shrink the box *itself* (the “space” where the data is contained).

To make the box-size decent, run this command:

e2proc3d.py <input> <output> --clip=box_size_of_data

Applying a threshold to EM data

You can apply a threshold to the reference if you want to delete aberrant densities that come up at low or high-density thresholds (this step is rarely necessary):

e2proc3d.py <input> <output> --process=threshold.belowtozero:minval=value

Remember that EMAN2's modular approach lets you use any threshold processor available. Just change the part that says “threshold.bewlotozero” for the name of another threshold processor. Take a look at all the existent processors by typing the following at the command line (you'll have to determine which ones are threshold processors either from their name, their description, or by asking someone who knows):

e2help.py processors --verbose=10

But..., how to determine what threshold value to use? Open the reference with *e2display.py <reference_file>* from the command line. A nice GUI (window with helpful clickable buttons) will come up, displaying the volume. Click the middle click of the mouse to bring up a visualization controller. Find the threshold bar and see how the densities in the model change as you move it. Determine, visually, by looking at what number the threshold-bar is at, at what value you see reasonable densities only. If you have no experience with visualizing 3D volumes of decent reconstructions and know nothing about your specimen, or have very bad data, the term “reasonable” might seem unreasonable. In that case, I can't help you.

You can (if you want to) also apply to the reference any ‘preparation’ operations that you apply on the data itself, which you'll learn about in the next sub-section. Actually, this is the default behavior in EMAN2 for now (and you can't change it).

3.2. “Manual” preparation of raw particles for alignment (Note EMAN2 can automatically prepare the particles for you)

EXPLANATION



Keep a copy of the raw particles untouched if you want to attempt to do this manually. The prepared particles are used during alignment, but averaging should *always* be done on the raw particles.

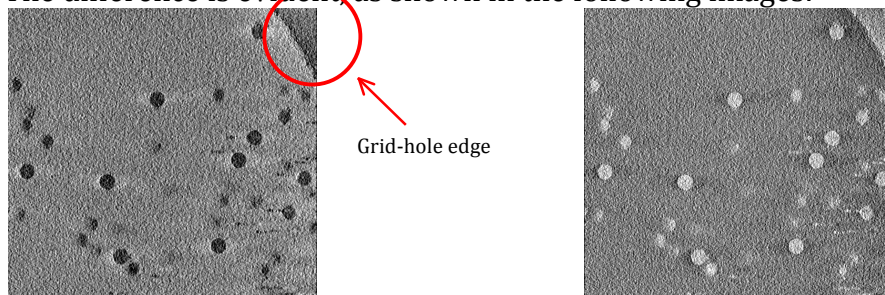
The order in which you apply the preparation steps *matters*. You might have to apply some of them several times to get things “right” if you do things in a sub-optimal order. If you get them wrong altogether, your alignments WILL be wrong. The order here presented is the one we’ve found to make the most sense; follow it if you’re doing things “manually”:

1) Contrast reversal, 2) pad (if needed), 3) mask, 4), normalize 5), the same mask *again*, 6) filter, 7) shrink

Contrast reversal

Some tomographic reconstruction programs yield tomograms with “black densities” corresponding to the specimen and “white densities” to the background. This is expected given the nature of electron microscopy data (the specimen scatters electrons from the beam more strongly than the background and thus fewer electrons hit the CCD camera wherever the specimen gets in the way, compared to where it doesn’t). EMAN2 likes, and MUST have, white densities for protein (not that it’s a racist program; it’s really just what makes sense: “white” pixel values correspond to high-intensity values (large numbers), which imply the presence of high-density material).

The difference is evident, as shown in the following images:



“Black” protein

“White” protein

Notice that the edge of the grid hole can also tell you whether you’re working with “white” or “black” densities.

If you bring a random tomogram from elsewhere with the “wrong” contrast, you can reverse it very easily from the command line by doing:

`e2proc3d.py <input> <output> --mult=-1`

Padding

The box-size of the extracted sub-volumes should be ~2x the diameter of the particles to avoid “aliasing effects” in Fourier space, and to provide a clear background during inter-particle comparisons. If you haven’t defined the box-size properly during the boxing step, or if an unwise collaborator gives you boxed-out data for you to process, you can always fix the box by padding it with the following command:

`e2proc3d.py <input> <output> --clip=50`

Substitute the number after “clip” as needed; it is the length of the new box-size, in all three directions, x, y and z. Any new voxels added to the box (when you make it bigger) will be filled in with zeros.

Masking

When particles are crowded, you might end up having more than one in a given box after sub-volume extraction. The box should be centered on one of the particles so that the central particle is left intact after “zeroing out” (masking) whatever is beyond the radius of that particle. This would get rid of the other unwanted, invasive, intruding densities in the box. Choose your mask carefully, as you can end up zeroing out *wanted* densities from the desirable particle. To apply a spherical mask, do:

`e2proc3d.py <input> <output> --process=mask.sharp:outer_radius=123:value=456`

Because of EMAN2’s modular approach, you can use many different masks. You’re not constrained to using “mask.sharp”. This can be substituted for any other processor that you see in the list that comes up when you enter this on the command line:

`e2help.py processors -verbose=10`

The “verbose option” will give you detailed information regarding the parameters/options that each listed processor requires or can accept. (You’d probably want to focus on masking processors only, for this step).

Normalization

Makes the standard deviation of the pixel values in a given volume equal to one and the mean equal to zero. This makes particles “comparable” by compensating for changes in illumination (particles from one tomogram might look brighter than those from another, because of differences in dose, ice thickness, etc.).

You can apply this operation from the command line as follows:

`e2proc3d.py <input> <output> --process=normalize`

Repeat the *same* masking you did the first time

Filtering


```
e2proc3d.py <input> <output> --process=filter.lowpass.gauss:cutoff_freq=0.01
```

Applying a low pass filter to an extracted sub-volume “smooths out” the noise in it. It positively affects alignment based on coarse features. Recall you had already learned to filter tomograms using this option in section 2 –if you read the corresponding tip-box. This is exactly the same thing, except that a sub-tomogram, as deduced from the name itself, is smaller.

Again, keep in mind you can apply *any* filter listed in the processors list, which you can get by typing `e2help.py processors -verbose=10` at the command line.

Shrinking

```
e2proc3d.py <input> <output> --scale=0.5
```

Provide a shrink factor smaller than 1 to shrink. For example, if you want to shrink a volume to half of its original size, specify `scale=0.5`; you can guess that `--scale=0.3` would shrink the side length of the volume to 1/3 of their original size, etc...

Shrinking can provide a great boost in speed. Just think about it: If you shrink each side of a volume by a factor 2, you actually end up with 1/8 of the original volume, as clearly depicted in the diagram below:



I hope it isn't necessary to explain why having 1/8 of the original number of voxels to compare during alignment and to process during averaging would speed things up dramatically.

Another advantage from shrinking is that averaging neighboring pixels enhances the signal (noise is “random” and thus averages out to zero, whereas signal is consistent, and should average coherently to non-zero values).

The caveat with shrinking is that you end up losing high-resolution information: sampling (apix value) is proportionally reduced. For example, if a particle originally has `apix=4.5` (how sloppy to leave out the units!), and you shrink the sub-volume by a factor of 2, it will end up with an `apix` of 9. `Apix` literally means Å/pixel (units to the rescue): The number of angstroms of the specimen one pixel ‘represents’ or ‘contains’. In short, an image/volume with `apix=4.5` has information down to that resolution, but no higher.

4. ALIGNMENT AND AVERAGING

Presently, EMAN2 only officially supports reference-based alignments. This step is so automated now that it is trivial to run, but subtle considerations might be the rice to tip the scale between getting *Natureable* results vs. publishing in JMB. An intelligent choice of parameters is highly dependent on what data you're working with, and what questions you are seeking to answer.



You might want to care about understanding the details if you're working on an SPT project and want to graduate. (If you're a postdoc or a research-assistant, continuing to receive a paycheck is probably sufficient motivation, but a reminder can't hurt).

The program that does the automated particle-preparation, alignment and averaging is *e2classaverage3d.py*. You can see all the options/parameters it accepts and what they're for by typing, at the command line:

```
e2classaverage3d.py -h
```

If the particle stack only has one particle, the program will return the aligned particle (because there's nothing to average).

For now, run the monstrously long command below, being careful to provide the correct file name for the stack of raw particles through "*--input=*" and for the reference through "*--ref=*" (you can also choose through the "*--output=*" option what file name you want the final average to have. It MUST be an HDF file; for example, "*--output=beautiful_avg_please.hdf*") :

STEP 3

3.0) This command looks discontinuous but should be ONE single line with all the parameters in a row, with a single space separating each option that starts with "--", for example: *--A --B --C ... --N...* etc.

```
e2classaverage3d.py --input=e15pp_set1_stack.hdf --output=e15pp_set1_aligned.hdf --ref=e15ref_prep_icos_bin2.hdf --savesteps --ncoarse=1 -v 3 --mask=mask.sharp:outer_radius=48 --preprocess=filter.lowpass.gauss:cutoff_freq=.025 --align=rotate_translate_3d:search=12:delta=8:dphi=8:verbose=1:sym=icos --parallel=thread:2 --ralign=refine_3d_grid:delta=3:range=9 --saveali --averager=mean.tomo --aligncmp=ccc.tomo --raligncmp=ccc.tomo
```

To commands above have profound *Meaning*. Details below:

EXPLANATION

Why use a reference

This was supplied through *--ref=* in the commands above.

Sub-volumes are very noisy and have a huge missing wedge, so it's very hard to align them accurately against each other. On the other hand, a perfect reference has no-missing wedge and virtually no noise. Aligning your data to It would give it a "nudge" in the right direction. If you don't provide a

reference, it will be self-generated with a “binary tree” approach. This means that particle 1 will be aligned to particle 2 and averaged with it, particle 3 will be aligned to and averaged with 4, etc.

If you had 10 particles, one cycle of this would lead to 5 “new” particles. The program continues to do cycles like these until all the particles in the stack have been merged into one average, which will be used as the “**initial reference**”.

What does “refinements” mean, and why they’re useful

This refers to `--iter=` in the commands above.

Regardless of what reference is used, you generally want to run several rounds of “refinement” on the data. What this means is that after you generate a **final average** from your data, you use that as the reference for a second round of alignment: you align the raw data all over again to this new reference. When you’re using a nice model (from the PDB or EMDB or wherever) as a reference, refinements help to take care of model bias, and often improves the average too, specially when the initial model/reference used did not *exactly* correspond to the biochemical specimen that constitutes your data. For example, if you use a chaperonin in the “closed state” to align particles that are in the “open state”, the average you’ll get after the first round of alignment might look pretty “closed”. Subsequent rounds of refinements *should* lead to an average resembling more the “open” state, if your data is any good.

When you do not provide a reference and a self-generated one is used, refinements help you improve the average you’re getting out from the data. This lacks the initial “nudge” in the right direction that some specimens appear to require sometimes, so with this approach there’s the risk of doing endless refinements and never improving at all.

`--align` specifies what aligner you want to use for the “coarse alignment” step. The different available aligners differ in terms of speed and accuracy. To get a list of all the aligners and a description of what they do, type this at the command line:

e2help.py aligners

Once you have “more or less” found the correct orientation of one sub-volume respect to another (for example a reference), then you can afford to do a “fine alignment” around the orientation you found in the coarse step. Actually, the coarse step can find as many “approximate” answers as you want, and the fine alignment step will search for a precise answer around all of them.

`--ncoarse` is the number of best answers you want to keep from the coarse alignment step, and send for fine alignment to the fine alignment step.

`--ralign` lets you choose an aligner for the fine alignment step.

`--preprocess` lets you supply any filter and it will be applied to the “prepared” version of the particles during alignment. For example, to low pass filter the particles to 50Å, you would supply:

`--preprocess=filter.lowpass.gauss:cutoff_freq=0.02`

--postprocess: This applies a filter to the final average, and is supplied in the same format as *--preprocess*.

STEP 4

You are practically done!

If the stack you aligned had contained more than one particle, you would have gotten back an average (with one particle you just get back the particle in the right orientation respect to the reference).

Now wait (patiently, if possible) for your job to run. Afterward, follow these last instructions:

4.0) Apply symmetry to the aligned particle by executing this command:

```
e2proc3d.py e15pp_set1_aligned.hdf e15pp_set1_aligned_icos.hdf --sym=icos
```

4.1) Look at the aligned particle after having applied icosahedral symmetry:

```
e2display.py e15pp_set1_aligned_icos.hdf
```

If you actually want to get an average, repeat steps 1 through 4. Just box more particles, choose a sensible filename for the stack, such as *e15pp_set10.hdf*, and proceed to align and average the stack using *e2classaverage3d.py*.

Try following the activities proposed in Appendix A (the last section in this User's Guide).

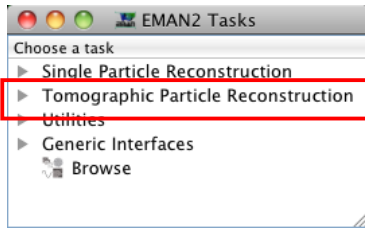
5. USING THE WORKFLOW FOR SPT

The workflow provides a holistic GUI approach to *everything* here described (except reference preparation). Use it if you don't know that when you see *<input>* in the instructions of a tutorial you actually need to substitute it for the name (or path) to a file, and that whenever you see *<output>* it's an opportunity for you to define the name of the file where you want to save your results (for example, the final average after several rounds of refinement during reference-based alignment of subtomographic volumes).

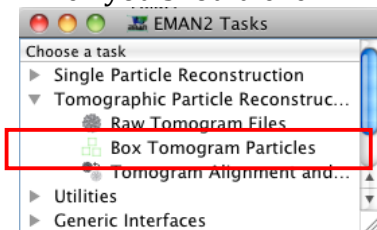
Don't despair. The workflow shall save you. You will have to make the effort to enter one command at the command line though:

```
e2workflow.py
```

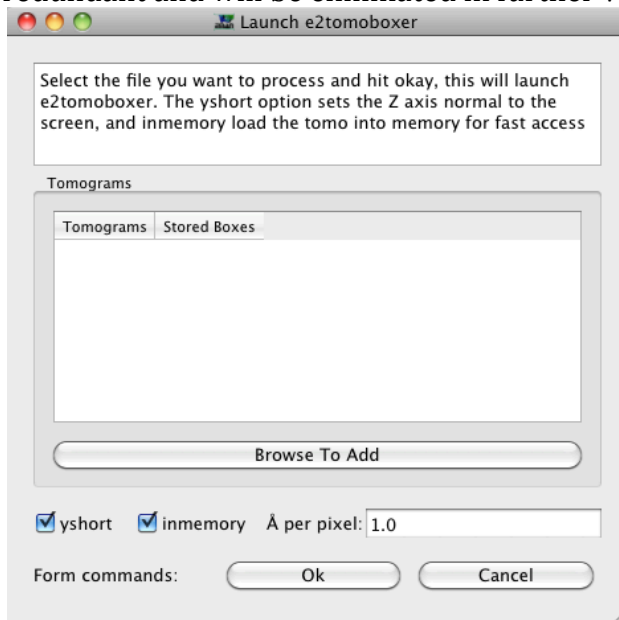
You can breath. There are buttons 😊.



For now, ignore everything, except the Tomographic Particle Reconstruction menu, which you should click.



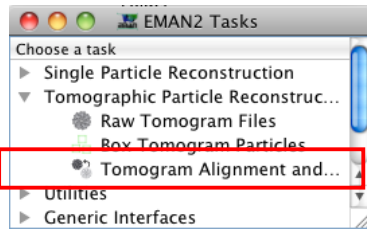
Go directly to *Box Tomogram Particles* (ignore the *Raw Tomogram Files* option; it is redundant and will be eliminated in further versions).



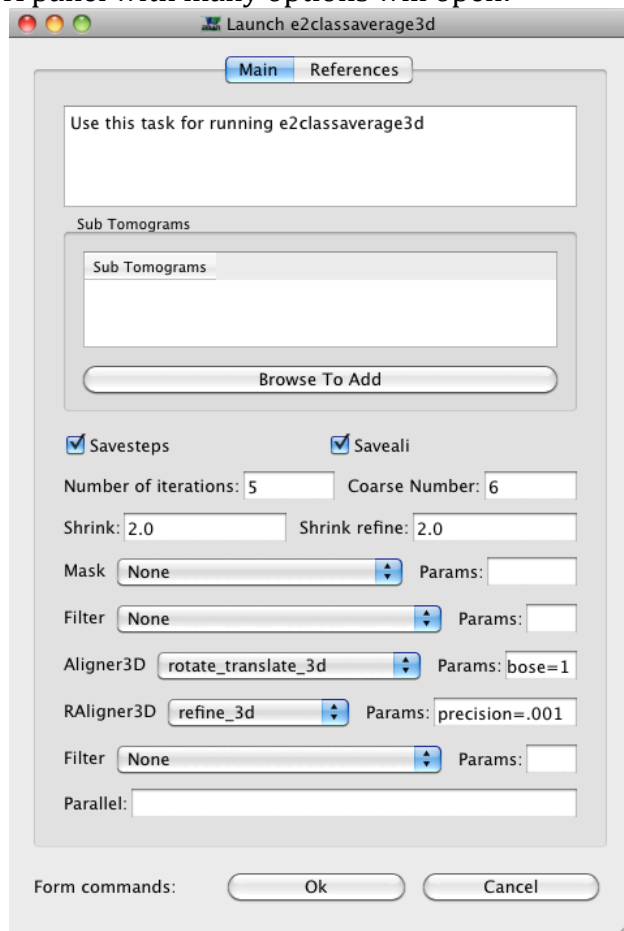
Once you've browsed for a tomogram, enter the apix value to insure it will be displayed properly and click Ok (apix=10.9 if you're using the epsilon15 data downloaded from the EMAN2 Wiki; go back to section 2 for descriptions of the *yshort* and *inmemory* options).

This triggers the opening of *e2tomoboxer.py*, a program whose Graphical User Interface is fully described in section 2.

When you're done boxing, click on *Tomogram Alignment and Averaging* in the workflow.



A panel with many options will open:



Supply a reference via the *References* button. If *Saveali* is checked the aligned particles right before averaging will be saved as a stack inside the EMAN2DB directory, as *class_ptcl.bdb*. The *Number of Iterations* means the number of refinements that will be run on the data (the explanation of what this and everything that follows means is in section 4). If *Savesteps* is checked, the average obtained after each iteration will be appended to a stack file called *class_00.bdb* inside the EMAN2DB directory. *Coarse Number* specifies the number of solutions you want to keep from the coarse alignment to further “tweak” in hopes of finding the right solution during fine alignment. Shrink, Mask and Filter are self-descriptive processors (if not, read about them in section 4). *Aligner3D* lets you pick an aligner for the coarse alignment step, while *RAligner3D* specifies the aligner to use for the fine alignment step. *Params* lets you enter the parameters that the processor or

aligner to its left accepts (notice that each aligner and processor has its own *Params* box). *Parallel* takes the number of processors on your computer you want to use to run the job.

When you're done entering all the parameters, click "Ok" and patiently wait for the results.

APPENDIX A

(TASKS - At the beginning, for the workshop)