

# RCT in EMAN2 Tutorial

## Getting Started:

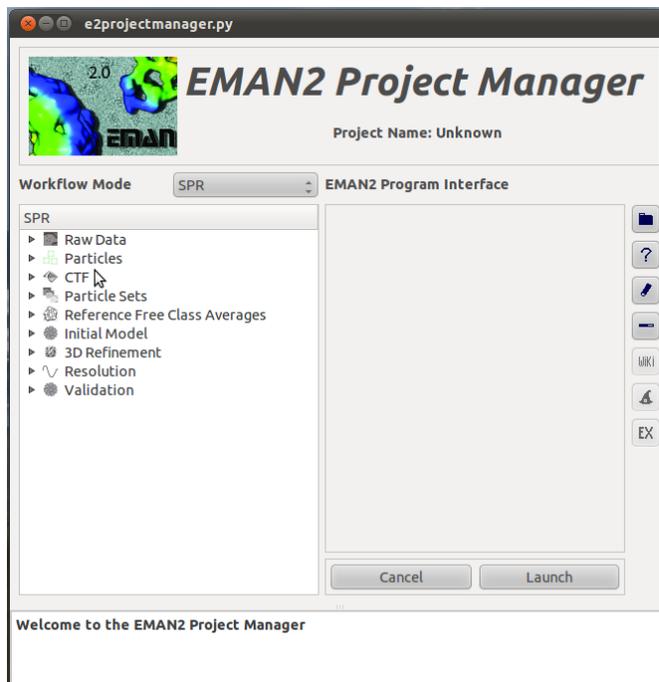
Make sure you have the latest version of EMAN2. For now, you will need the nightly build available for download at <http://blake.bcm.edu>. In the future, EMAN2.1 will be sufficient. You will also need to download the test data associated with this tutorial, and then untar it to get the Ip3R. For this tutorial you will work with Ip3R a 1.3 MDa membrane protein. This data set consists of untilted and tilted micrograph pairs. The tilted micrographs are tilted 45 degrees from the untilted. However for most RCT projects, I recommend collecting 60 degree tilt data.

## Introduction:

EMAN2 is an image processing software package directed towards electron microscopy data. It is composed of a C++ core and a suite of python programs that implement higher-level data processing functions. On top of this infrastructure, there is a GUI based data workflow framework, which will be used and introduced in this tutorial.

## Initializing a new project using e2projectmanager.py

To begin a new project move to your project directory and launch e2projectmanager.py, the new EMAN2 GUI. A window should pop up as shown below:



First you need to set the project parameters, so in the menu bar (the menu bar location is platform dependent) click: Project->Edit  
 A dialog box will pop up. Here set the project parameters (then click OK):

	<b>Ip3R</b>
<b>Project Name</b>	Ip3R validate
<b>Project Icon</b>	Use default
<b>Mass</b>	1,300
<b>CS</b>	2.0
<b>Voltage</b>	200
<b>APix</b>	1.88

### Importing data into your new project:

In the left box of the GUI click on 'raw data' in the SPR workflow tree. This tree box lists the workflow and all options for two types of EM data processing, SPR and tomographic data. For this tutorial we will be using the SPR workflow. If the tomographic workflow is shown, change it to SPR by clicking on SPR in the combobox above the tree. After clicking on 'raw data' tab in the workflow, a list of raw data processing options will appear. You need to click on 'Import raw data'. This will cause a e2program GUI interface to be displayed in the right box of the project manager GUI. Next, in the e2program GUI interface, click on 'Browse', which will display the new EMAN2 file browser. Using this browser, select the files to import (you can multi select by holding shift or range select using control). Once the files to import are selected, click 'Ok' in the file browser. The list of files to import will now be listed in the e2program GUI interface.

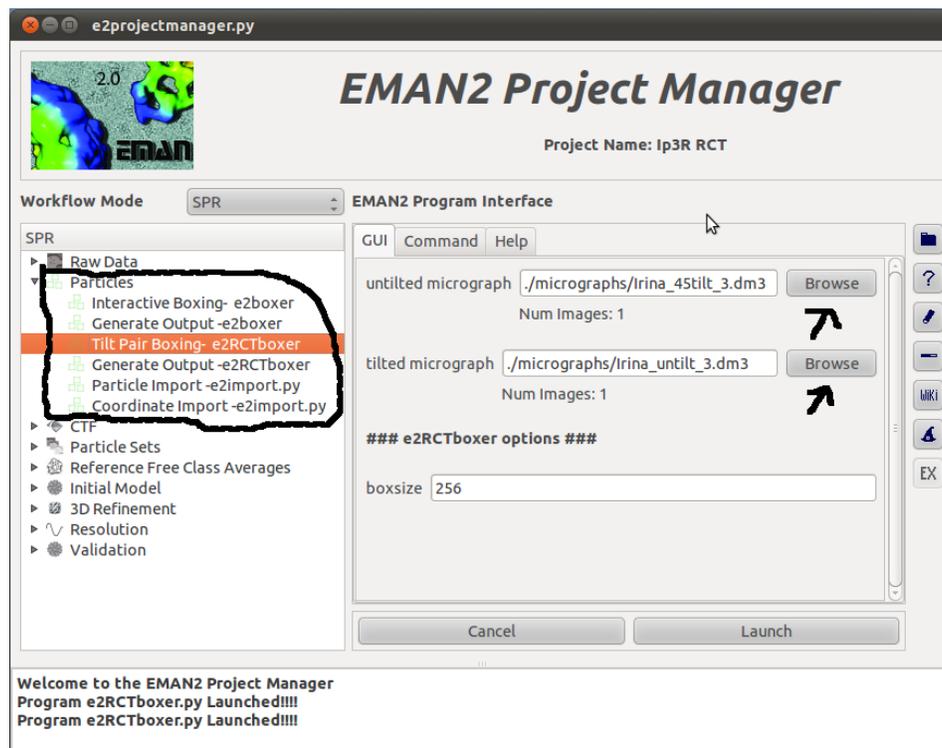


In addition to importing the particles, this program also preprocesses the micrographs. You can invert, remove X-ray pixels, change file format, etc. The recommended output format is HDF for all EMAN2 projects.

For all e2programs that projectmanager can run, in addition to displaying a GUI interface for the e2programs, you can also click on the command tab in the e2program interface, and the command that will be run is displayed. You can edit this command to tailor the e2program, that will be launched after clicking 'launch'. You can also click on the help tab to list e2program specific help information. Once you have the selected files for import, click on 'Launch', this will cause e2projectmanager.py to run the e2program. In this case files will be imported to your project.

### Picking particle pairs using e2RCTboxer.py

Once you have imported your particles, you need to extract particle pairs. This is accomplished using e2RCTboxer.py. To use e2RCTboxer.py with e2projectmanager.py, click on the particles tab in the SPR workflow tree. This will display a list of e2programs that can be used for particle extraction. Click on 'Tilt Pair Boxing' to load the e2RCTboxer.py GUI interface into e2projectmanager.py.

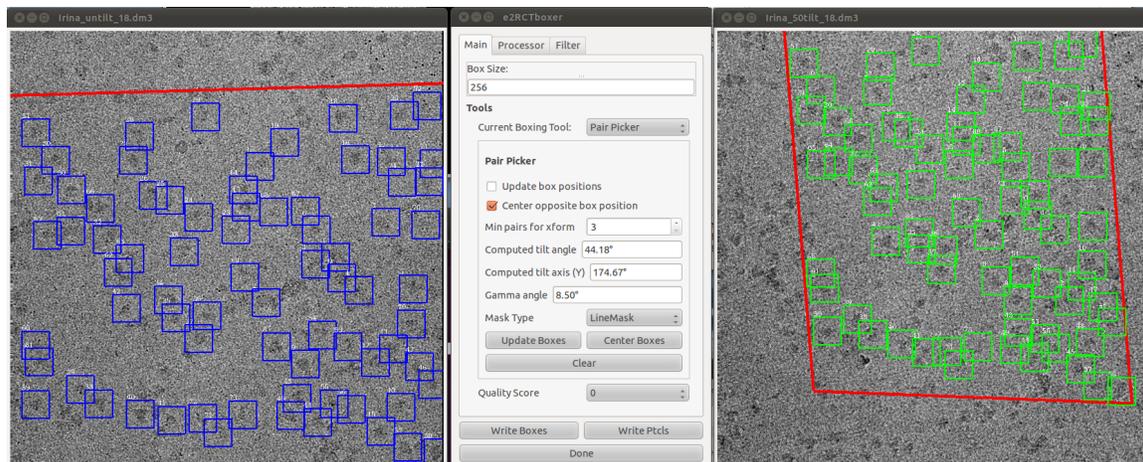


As before, click on browse to load filenames to be processed. Click on browse, to obtain to untitled micrograph using the file browser. As before click 'ok' in the file browser. Next click on browse to obtain the tilted micrograph using the file browser.

Once you have selected two micrographs for tilt pair extraction, set the boxsize to 256(for Ip3R), and click on 'Launch' to start e2RCTboxer.py.

e2RCTboxer.py will show four windows: a untilted micrograph window, a tilted micrograph window, a GUI control window, and a particles window. e2RCTboxer has two modes, Manual mode (default) and Pair picker mode). You should use Pair Picker mode, by clicking on the combo box and selecting this (see diagram below).

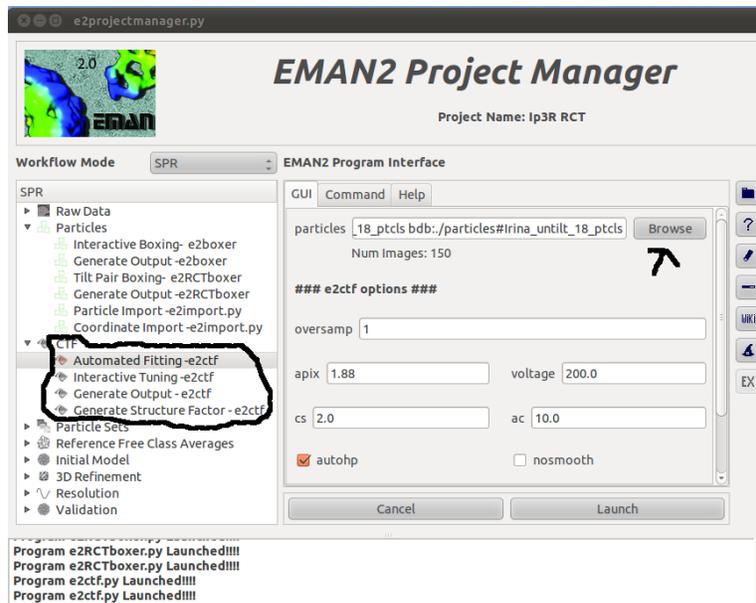
Before semiautomatic particle selection can begin, you need to select 3 anchor pairs, so starting on the untilted micrograph, pick any particle and then in the tilted micrograph pick this same particle. Repeat 3 times. The numbers at the upper left corner of the particles denote particle pairs. For example 1, and 1 are the same particle at different tilts. Now pick a particle in the untilted micrograph, and the corresponding particle will be automatically picked, though it might be a little off center, so you'll need to manually center it. Continue until you have picked all particle pairs. One useful tip is to change the 'mask type' in e2RCTboxer GUI to Line Mask, which will demarcate the areas the two micrographs share.



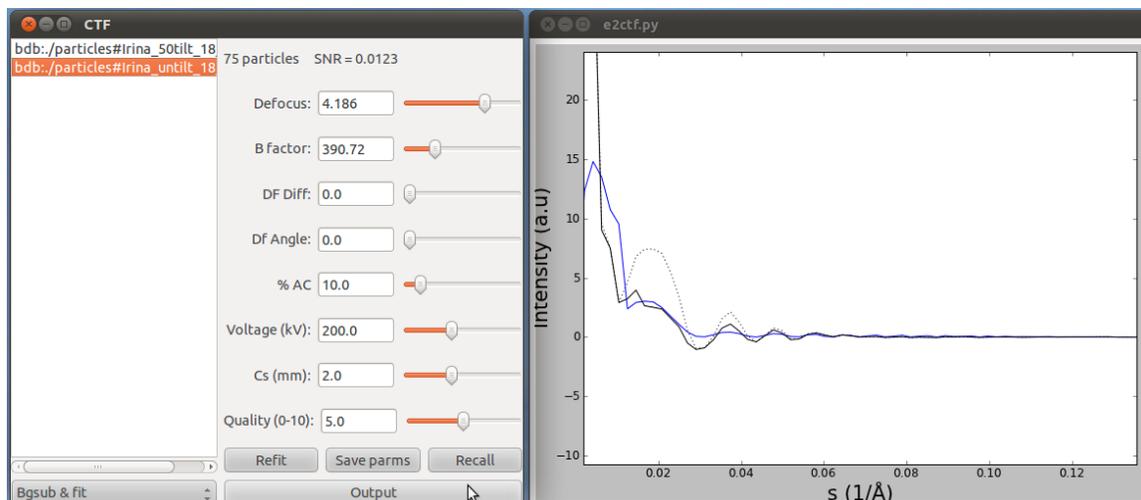
After you have finished picking particle pairs. Click on 'Generate-Output-e2RCTboxer' in the particles tab of the e2projectmanager (SPR workflow mode). This will load a GUI interface into projectmanager to extract particle pairs from the micrographs using the stored box coordinates (ords are stored automatically). In addition, to particle extraction, particle contrast can be inverted, particle density can be normalized, and the particles can be shrunk in size. For the tutorial, you'll need to ensure that the invert contrast check box is checked, so that the particle's contrast is inverted. Also set shrink to 2 to speed later processing. Click 'Launch' as usual to run this e2program.

### **CTF correction of the untilted particle stacks**

We only CTF correct the untilted particles. Because the tilt angle is so high, it is not possible, at this point, to CTF correct the tilted particles. This means the final resolution of the RCT reconstruction will never be better than the first zero of the CTF (40-25Å). To begin CTF correcting in EMAN2, start by auto fitting. Click 'Automated Fitting'. This will bring up a GUI for e2rct.py. Click browse to load files, and then check to ensure the data collection parameters are correct (defocus, voltage, cs, etc). You may want to use oversampling to improve your results (set to 2). Finally click the 'Launch' button.



After you auto fit, visually check the fitting results by clicking 'Interactive tuning'. The particle stacks should already be listed, so just click 'Launch'. If the fit (blue curve) does not match the data (black curve), then adjust the defocus and or B factors using the sliders. Shown below is an example of an interactive tuning session.



Finally you need to generate CTF corrected images. For the purposes of RCT, only phaseflipped data is needed since the actual recon will not be CTF corrected at all (uses tilted image for this)! The only purpose of the CTF corrected phase flipped images is to perform reference free classification and determination of the inplane azimuthal angle. To create phaseflipped images, click '*Generate Output*'. Only check 'phaseflip'. If anything else is checked, uncheck it. Click '*Launch*'

### **Building particle stacks**

Click the Particle Sets tab, listing options to build particle stacks for processing. Click '*Build particle Sets*', loading the program GUI to build stacks for processing.

Click browse to search for stacks to build. The file browser will launch. Select all the stacks corresponding to the untilted images, chose a name for this stack, and click '*Launch*'. Next do the same but this time for the tilted particle sets. Be sure to choose the same number of particle stack as you did in the untilted AND in the same order. It is critical to maintain the 1-to-1 relationship (in set theory speak) of the untilted to tilted particles. If this is messed up your RCT recon(s) will be junk.

### **Generating reference free class averages using e2refine2d.py**

Next you need to generate reference free class averages using the untilted images. Click '*Generate Classes*', then click browse. There should only be two particle stacks listed, the tilted and untilted particle stack. Each stack should have the same number of particle, if not you will need to rebuild your stacks. CHOOSE THE UNTILED particle stack. Next set the following parameters:

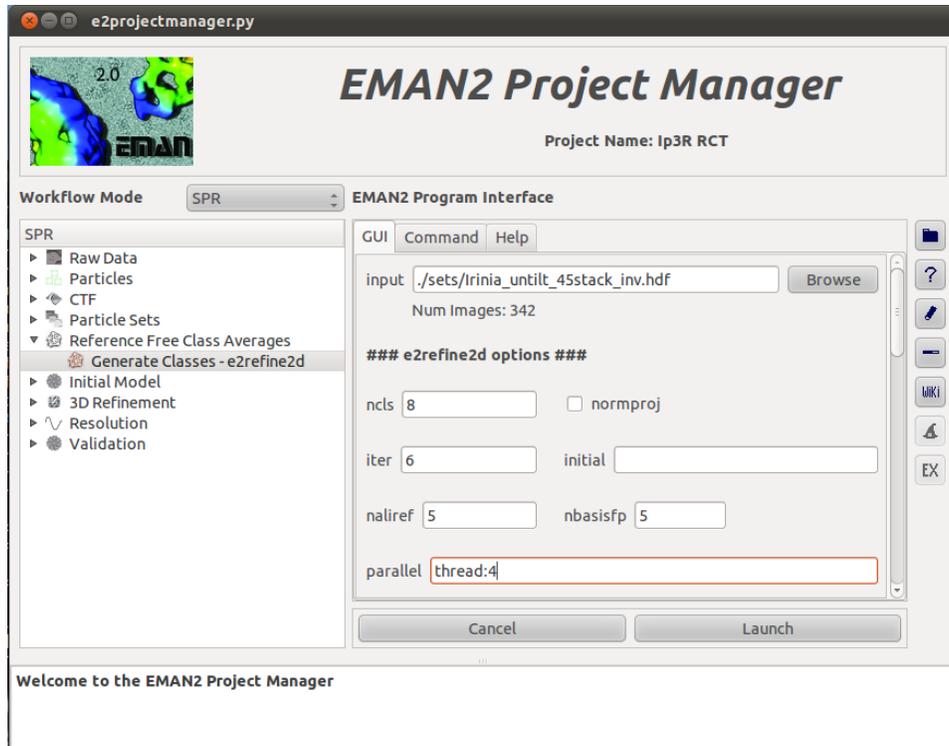
ncls=8, this specifies that we wish to sort the images into 8 classes. For a project with more views or heterogeneity you'll need to increase this number.  
maxshift=30, this specifies how much inplane translation is allowed during image alignment. If you images are off center or you have a larger boxsize you'll need to increase this parameter. Conversely you should decrease it for small boxsizes.  
classkeep=0.9, this specifies that we want to keep 90% of the particles and toss 10% because they are 'bad'. You can fiddle with this parameter depending on how the data quality is.

iter=6, creation of reference free averages is an iterative process due to the need for inplane azimuthal rotation and translation alignment prior to classification via MSA and K-means clustering. Six iterations is a good choice to achieve convergence.

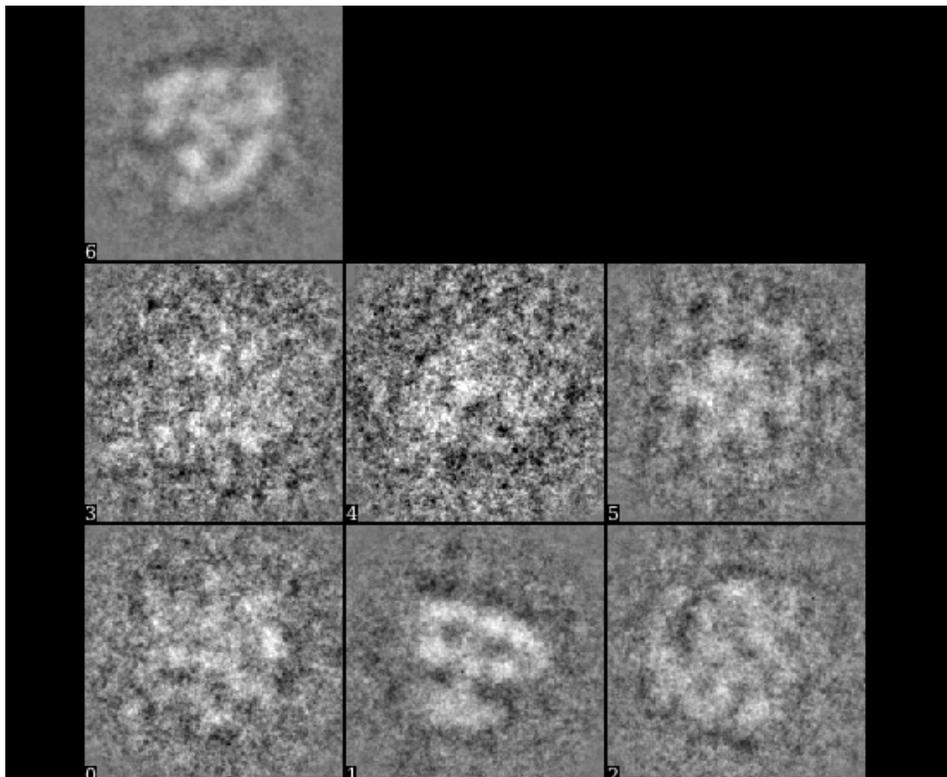
naliref=5, this specifies the number of references to azimuthally and translationally align to. Like ncls, increase this number to reflect increased heterogeneity.

Parallel=thread:4, on my machine I have 4 cores. Change 4 to however many cores you have on your machine.

Finally click, *Launch!*



After the job finishes, you should have class averages looking like the figure below. You can check the status by opening the status window, (4<sup>th</sup> tool down from the top of the toolbar on the right hand side of the PM).



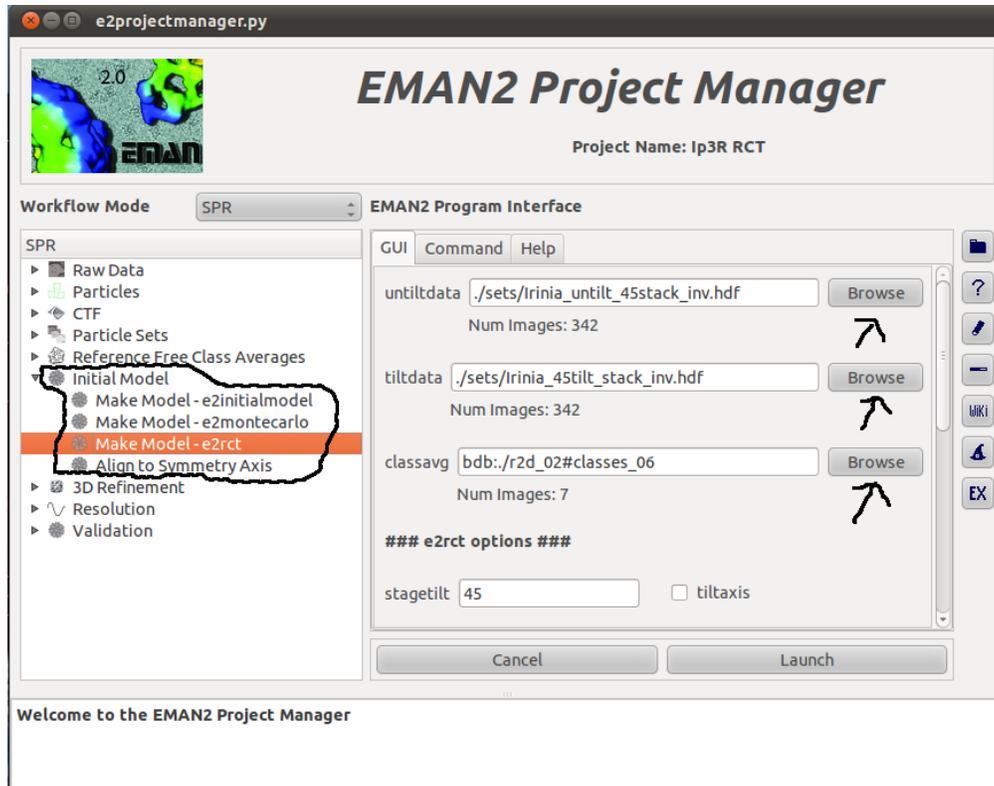
## Computing RCT recons from each of the class averages

Finally, we create a RCT reconstruction for each class average using `e2rct.py`. To load the GUI click *'Make Model - e2rct'* from the Initial model tab. Next, load the untilted image stack and the tilted image stack from the sets directory. Then choose the class averages you wish to use from an `rd2_??` directory (these directories hold the results from `e2refine2d.py`). Choose the best class averages where you can clearly see features and not blobs. In the preceding figure, the second and last class averages are the best.

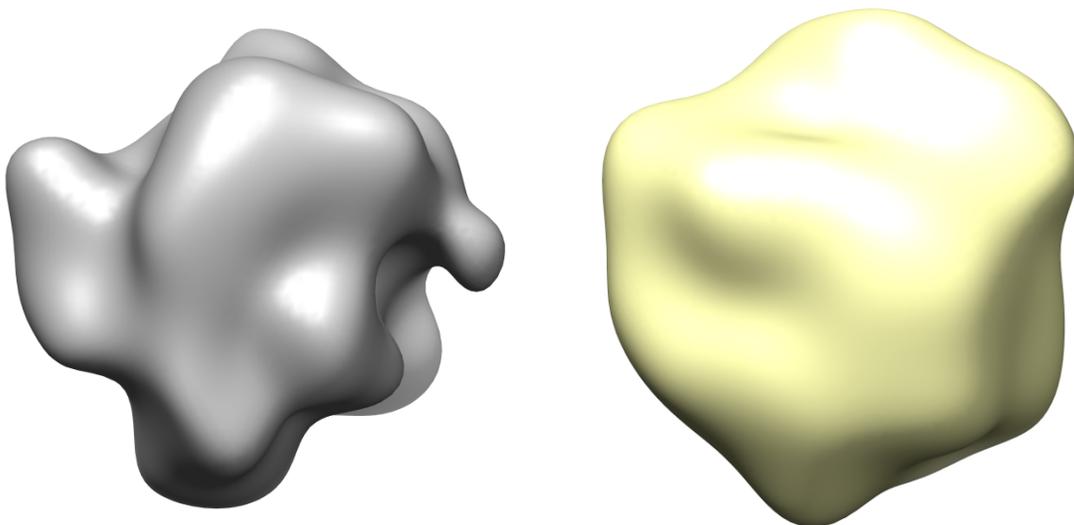
`e2rct.py` has the following options:

- `stagestilt`, set this value to 45 for Ip3R, the amount of tilting in degrees that the untilted vs. tilted images were collected at.
- `tiltaxis`, you can check this box to do a tiltaxis correction, this only works if you picked particle pairs with `e2rct.py`, if you have imported them for somewhere also leave this box unchecked. For this tutorial leave this box unchecked
- `careject`, list bad class averages you don't want RCT reconstructed. For Ip3R set this list (comma delimited) to: 0,3,4
- `minproj`, set the minimum number of particles required to compute a RCT recon. Obviously 1 or 2 particles will not produce a decent 3D volume, so I recommend setting this value to 10
- `align`, check this box if your tilted particles need to be centered. Centering works by computing an average, translational aligning to this average, and then iterating the process. This may not work well for highly elongated particles. For Ip3R check this box.
- `maxshift`, set the maximum translation shift allowed during tilted particle centering. For Ip3R I set this to 4 pixels.
- `avgrcts`, after creating an RCT for each class average, the RCT recons are aligned and averaged to produce an optimal RCT recon, averaging away the missing cone. For Ip3R check this box.
- `reference`, without a reference, the RCTs are aligned to each other, however if you have a reference model, you can get improved results. For Ip3R, use the Ip3R I have provided with the datasets.
- `sym`, specify the symmetry of your structure. You should only use this option if you provide a reference AND this reference is aligned to the symmetry axis. If you don't have a reference, you can run `e2RCT` with no reference and set `sym` to `'c1'`. Then you can align your RCT recon to its symmetry axis using `e2symsearch.py`. Use this as your reference and set `sym` to the correct symmetry. Set this to `c4`, the symmetry of Ip3R.
- `aligngran`, the fineness of 3D alignment, usually 10 degrees is fine.
- `weightrecons`, check this box to perform a weighted average using particle population as the weights. Leave this unchecked for Ip3R.

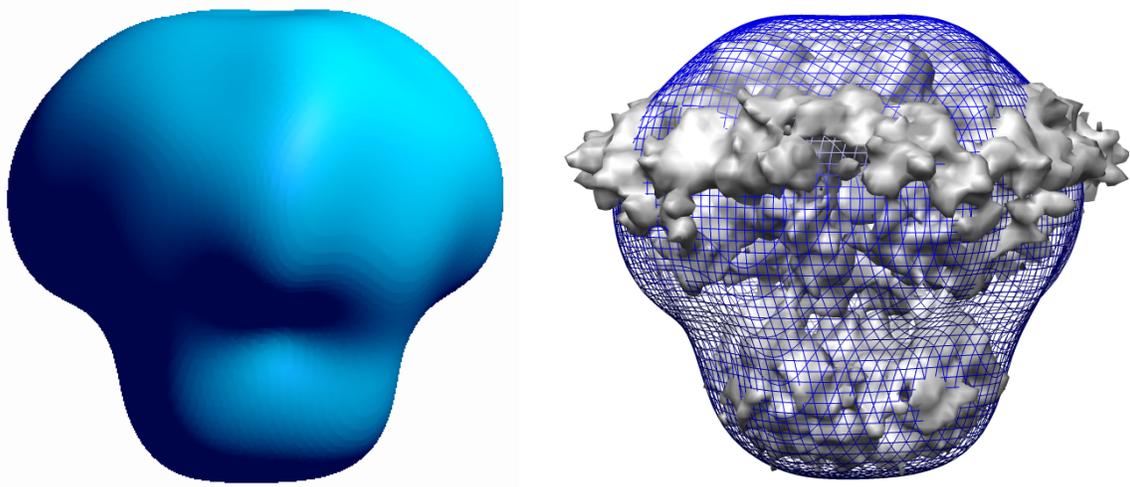
When you have set all the options, click *'launch'*.



After the job finished your results will be in `./rct` in the EMAN2 database. The RCT reconstructions will be listed as `rectrecon_01`, `rectrecon_02`, etc. These RCT recons are unfiltered, so you will probably need to filter them (they will look very noisy by themselves). This can be done using `e2proc3d.py`. For example: `e2proc3d.py 'bdb:rct/rctrecon_01' rctrecon_filt01.hdf -process=filter.lowpass.gauss:cutoff_abs=0.05`  
 For Ip3R, do this filtering for `rctrecon_01` and `rct_recon_06`. You should get something looking like the figures below.



Although these individual RCT recons do not look so great, the results can be vastly improved by 3D alignment, averaging and application of c4 symmetry. In this case a major reason for the poor quality is because we have used 45 degree tiltdata, which means half the structure factors are missing. If you have not done so already, run `e2rct.py` using `avgrcts` (check this box) and the `Ip3R` structure bundled with the data for this tutorial as a reference, you should get something looking like the figure below (left). The averaged RCT recon will be named `rctrecon_avg` or `rctrecon_symavg` if you are using symmetry. This recon will be in the database in the `./rct` directory. On the right, in the figure below, is the RCT recon compared to the `Ip3R` model from SPR, as you can see they look quite similar, except that the RCT recon is of much lower resolution.



**That's is, you have completed the RCT tutorial in EMAN2 ☺**