National Center for CryoEM Access and Training

RELION tutorial

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Software requirements

Required: Web browser (tested on Chrome)

AWS Instance Information

Instance type: g4dn.2xlarge	Filesystem: efs
No. of GPUs: 1	Path to projects: /efs/relion
No. of CPUs: 8	Path to test data: /test_data/Movies/
RAM: 32GB	

Accessing RELION

- **Step 1:** In your web browser, enter the URL provided by your instructor or on the Stion dashboard.
- **Step 2:** Sign in with the following information:
  - User: ec2-user
  - Password: test123
  - If your browser warns you about an untrusted certificate, click on Advanced and Proceed to ec2 instance (unsafe).
- **Step 3:** Open the Terminal program.
- **Step 4:** Enter the RELION projects directory and create a directory for yourself using these commands

```
cd /efs/relion
mkdir YOURNAME
cd YOURNAME
```

- **Step 5:** Launch the RELION GUI.

```
relion
```

Movie Preprocessing

Background : This section will cover preprocessing of the data, focusing on three jobs: "Import", "Motion correction", and "CTF estimation".
"Import" will bring our raw movie frames into RELION.
"Motion correction" will take those raw movie frames and align them to account for sample and stage movement, producing an aligned average called a "micrograph."
"CTF estimation" will use the output from our motion correction job and attempt to measure additional parameters that vary from one micrograph to another; astigmatism, defocus, estimated resolution, etc.

- **Step 6:** Select "Import" from the job menu on the left.
- **Step 7:** Fill in the parameters as follows:
  - Import raw movies/micrographs? Yes
  - Raw input files: /test_data/Movies/*tiff
  - Pixel size (Å): 0.495
  - Voltage (kV): 300
  - Spherical abberation (mm): 2.7
  - Amplitude contrast: 0.1
  - **Any parameters not specified should be left at the default value.**
- **Step 8:** Click on the "Run!" button.
- **Step 9:** Select "Motion correction" from the job menu.
- **Step 10:** Under the I/O tab, click the "Browse" button.
  - Navigate to "Import/job001/" and select the "movies.star" file

- **Step 11:** Fill in the parameters as follows:
  I/O tab
  - Dose per frame: 0.929
  - Save non-dose weighted as well? Yes
  Motion tab
  - Number of patches X, Y: 5 5
  - Gain-reference image: /test_data/Movies/gain.mrc
  - Gain rotation: 90 degrees (1)
  - Use RELION's own implementation? No
  - MOTIONCOR2 executable: /home/ec2-user/MotionCor2_1.4.0/MotionCor2_1.4.0_Cuda102
  Running tab
  - MPI procs: 1
  - Number of threads: 1
- **Step 12:** Click "Run!"
- **Step 13:** Select "CTF estimation" from the menu.
- **Step 14:** Click the "Browse" button on the I/O tab
  - Navigate to "MotionCorr/job002" and select the "corrected_micrographs.star" file.
- **Step 15:** Fill in the parameters as follows:
  I/O tab
  - Use micrographs without dose-weighting? Yes
  CTFFIND-4.1 tab
  - Use CTFFIND-4.1? No
  Gctf tab
  - Use Gctf instead? Yes
  - Gctf executable: /home/ec2-user/GCTF_Gautomatch_Cu10.1/GCTF_v1.18_sm30-75_cu10.1
  - Ignore 'Searches' parameters? Yes
  Running tab
  - MPI procs: 1
- **Step 16:** Click "Run!"

Particle picking and 2D Classification

Background: After image preprocessing, the next step is selecting all of the particles in each micrograph. There are many ways to pick particles, with some working better than others for specific datasets. This test dataset is fairly easy to pick (the ice is thin and the particle is large and highly symmetric), but certain automated techniques (i.e. "blob picking") struggle with it due to the lack of density in the center. Thus, we will use a combination of two techniques.
"Manual picking" will be used to select a few particles in order to create an initial average.
"Auto-picking" will take that average and use it to automatically find particles in the images.
The averages obtained after particle picking will be calculated using 2D Classification, which will do two things at once; classify each particle into distinct "classes", and create an average of the particles in each of those classes. This will be used both as a diagnostic to see if our particle picking was accurate, and as a way to remove incorrect particle picks.

- **Step 17:** Once CTF estimation has completed, select Manual picking.
- **Step 18:** Fill in the job parameters:
  - Input micrographs: CtfFind/job003/micrographs.star
  Display tab
  - Particle diameter (Å): 128
- **Step 19:** Click "Run!"
- **Step 20:** Click the "pick" button next to a micrograph.
- **Step 21:** Manually select ~50 particles using the left mouse button.
  - Shift + left click removes a particle pick
- **Step 22:** Right click and select "Save STAR with coordinates" or click CTRL + s
  - Close the micrograph window. Feel free to pick particles in other micrographs and save the selections using the File menu.
  - For this dataset, far fewer than 50 particles would be fine. For other datasets, 50 may be far too few.
  - For non-symmetric particles, it is important to try and pick as many different orientations of

the particle as possible. It is also generally advised to pick particles from a variety of different micrographs (different defocus values, different ice thicknesses, etc.)

- **Step 23:** Select the "Particle extraction" job
- **Step 24:** Fill in the job parameters and Run:
  - Input micrographs: CtfFind/job003/micrographs_ctf.star
  - Input coordinates: ManualPick/job004/coords_suffix_manualpick.star

Extract tab

  - Particle box size (pix): 416
  - Rescale particles? Yes
  - Re-scaled size (pixels): 208

Running tab

  - MPI procs: 4
  - The details behind finding the right box size are beyond this tutorial, but a good starting point is given by the following equation (for 300 kV, at 200 kV the multiplier is 20):

$$Box[\text{Å}] = \frac{MaxDefocus[\text{Å}]}{25 * BestPossibleResolution[\text{Å}]}$$

    This recommendation may be too small if your particle is very large.
  - Re-scaling the box size to 208 bins the particles by 2x to speed up processing
  - This doubles our Nyquist frequency, but for this small dataset that will not impact the resolution we get.
- **Step 25:** Select "2D classification" from the menu.
- **Step 26:** Fill in the job parameters and Run:
  - Input images star file: Extract/job005/particles.star

Optimization tab

  - Number of classes: 2
  - Mask diameter (Å): 300

Compute tab

  - Number of pooled particles: 30
  - Pre-read all particles into RAM? Yes
  - Use GPU acceleration? Yes
  - Which GPUs to use: 0:0

Running tab

  - MPI procs: 2
  - Threads: 1
  - In this case, we only need 2 classes, and likely all/most of the particles will end up in a single class. The usual rule of thumb is ~100 particles/class, with no more than ~200. Processing time scales with this number.
- **Step 27:** Select "Subset selection"
  - This job will let us select the best-looking class for auto-picking
- **Step 28:** Fill in the job parameters and Run:
  - Select classes from model.star: Class2D/job006/run_it025_model.star
- **Step 29:** On the Display GUI, use the following parameters and click the Display! button:
  - Scale: 1
  - Sigma contrast: 5
- **Step 30:** Find the best-looking class and select it with left click. Then, right click and click "Save selected classes"
- **Step 31:** Close the class window and Display GUI
- **Step 32:** Select "Auto-picking" from the menu.
- **Step 33:** Fill in the job parameters and Run:
  - Input micrographs for autopick: CtfFind/job003/micrographs_ctf.star
  - 2D references: Select/job007/class_averages.star
  - Use GPU acceleration? Yes
  - You should end up with ~2,200 particles
- **Step 34:** Extract the picked particles from the micrographs using "Particle extraction"
  - Micrograph STAR file: CtfFind/job003/micrographs_ctf.star
  - Input coordinates: AutoPick/job008/coords_suffix_autopick.star
  - Particle box size: 416
  - Rescale particles? Yes
  - Re-scaled size: 208
  - MPI procs: 4
- **Step 35:** Repeat 2D classification as per Step 26, this time with 2-4 classes

- **Step 36:** Click the "File" button near the top of the GUI and select "Display".
- **Step 37:** Navigate to Class2D/job010 and scroll to the bottom. Select the file "run_it025_model.star" and click OK. Use the same parameters as Step 28 and click Display!
- **Step 38:** IF Step 37 revealed a class that looks like background noise, create another Subset selection job and select the classes that look good.

3D Reconstruction

Background: Now that we have a clean stack of particles, as confirmed by 2D classification, we move on to the final phase; obtaining a 3D reconstruction. This will happen in two stages; Initial modeling where an initial low-resolution map is obtained, followed by 3D auto-refine where that map is refined to high resolution. Much more can be said about 3D reconstruction, but given the high level of homogeneity in this sample, it will easily reach high resolution with only two fairly quick jobs.

- **Step 39:** Select 3D initial model from the job menu, with the "input images STAR file" being the "particles.star" output from either job010 (Step 34), if your 2D classes all looked like clean particles, or job011 (Step 38), if there was one or more classes that looked like background noise.
- **Step 40:** Change the 3D initial model job parameters as follows, then run the job.

Optimisation tab

  - Number of classes: 1
  - Mask diameter (Å): 150

SGD tab

  - Number of initial iterations: 25
  - Number of in-between iterations: 100
  - Number of final iterations: 25
  - Initial resolution (Å): 30
  - Final resolution (Å): 8

Compute tab

  - Number of pooled particles: 30
  - Pre-read all particles into RAM? Yes
  - Use GPU acceleration? Yes
  - Which GPUs to use: 0:0
  - MPI procs: 2
  - Threads: 4
  - The main changes here to the defaults are halving the number of iterations and allowing initial modelling to go to much higher resolution. This can be done because of the high quality of the data. For most data, try the default parameters first and then adjust if needed.
  - NOTE: This will take about ~20 minutes. A good time to grab a coffee.

- **Step 41:** Navigate back to the terminal and use the following commands to visualize the results of 3D initial modelling.

```
cd /efs/relion/YOURNAME
chimera InitialModel/job012/run_it150_class001.mrc
```

  - With any luck, you should be able to see alpha helices. If not, try to repeat your 3D initial modelling with a final resolution of 6 or 7Å.

- **Step 42:** Our test sample has octahedral symmetry, and thus we will get the best results if we tell RELION to impose O symmetry. First, we must align the symmetry axis of our protein to the XYZ axes RELION uses. This can be done via the command line:

```
relion_align_symmetry \
--i InitialModel/job012/run_it150_class001.mrc \
--o InitialModel/job012/run_it150_class001_alignO.mrc \
--sym O
# Open the alignO.mrc file in Chimera to make sure the symmetry axis is in-plane, then continue
relion_image_handler \
--i InitialModel/job012/run_it150_class001_alignO.mrc \
--o InitialModel/job012/run_it150_class001_symO.mrc \
--sym O
```

- **Step 43:** With a suitable initial model, we can now run 3D auto-refine.

- Input images STAR file: Extract/job010/particles.star **-OR-** Select/job011/particles.star if you had to throw out junk classes
- Reference map: InitialModel/job012/run_it150_class001_symO.mrc

Reference tab

- Initial low-pass filter (Å): 50
- Symmetry: O

CTF tab

- Has reference been CTF-corrected? Yes

Optimization tab

- Mask diameter (Å): 150

Auto-sampling tab

- Initial angular sampling: 7.5 degrees
- Local searches from auto-sampling: 1.8 degrees

Compute tab

- Number of pooled particles: 30
- Pre-read all particles into RAM? Yes
- Use GPU acceleration? Yes
- Which GPUs to use: 0:0:0
- MPI procs: 3
- Number of threads: 1
- This should take 15-20 minutes, depending on how long it takes to converge.

Post-processing

Background: Since no masking was used in 3D auto-refine, the resolution it produced is likely to be lower than what it truly is. Post-processing will allow us to apply a mask, which we must create, and sharpen our map to obtain high resolution features.

- **Step 44:** Select "Mask creation" from the job menu. Set the parameters below and Run.
  - Input 3D map: Refine3D/job013/run_class001.mrc
  - Initial binarization threshold: 0.007
  - NOTE: open the input map in Chimera and find the threshold value where noise is no longer present and use that. It may be different than the number above.
  - Extend binary map this many pixels: 5
  - Add a soft-edge of this many pixels: 6
- **Step 45:** Open the map from your 3D auto-refine job and the mask you just created in Chimera.

```
chimera MaskCreate/job014/mask.mrc Refine3D/job013/run_class001.mrc
```

- **Step 46:** Adjust the threshold of the mask so that it is at 1 (slider all the way to the right). At this point, every voxel in the mask has a value of 1. Make sure that there is no density from your refined map extending out of the mask. If it is, re-run Mask creation and extend the mask further.
- **Step 47:** Select "Post-processing" from the menu to calculate the masked FSC value and sharpen the map.
  - One of the 2 unfiltered half-maps: Refine3D/job013/run_half1_class001_unfil.mrc
  - Solvent mask: MaskCreate/job014/mask.mrc

Visualization

Background: Now that we have our sharpened map, it's time to visualize it in Chimera.

- **Step 48:** Open the sharpened map in Chimera.

```
chimera PostProcess/job020/postprocess.mrc
```

  - Change the step size and threshold to visualize the features
  - Check the pitch of the α-helices of the map. It is possible that cryoSPARC got the wrong handedness, which can be corrected with the command vop zflip #0.
- **Step 49:** Run the following command to fetch an atomic model that should fit well into this density

```
open 6S61
```

- **Step 50:** Use Chimera's FitMap command to dock the model into the density.