RELION

RELION developers

Jan 23, 2025

CONTENTS

CHAPTER

ONE

WHAT'S NEW?

1.1 Release 5.0

Blush regularisation

Dari Kimanius has developed a new method to incorporate more prior knowledge into the cryo-EM refinement process than the one typically used (which merely assumes smoothness in real-space, or limited power in Fourier-space). This method is called Blush regularisation and it uses a denoising convolutional neural network inside the iterative refinement algorithm of Class3D, Refine3D or MultiBody jobs. The effects of this are largest when the signal is weak and standard refinement in RELION would overfit (as for example visible from streaky artefacts in the solvent region). Using Blush reglarisation, Dari successfully refined a data set of a 40 kDa protein:RNA complex to 2.5A. The same data set was intractable in standard RELION or CryoSPARC.

DynaMight for modelling continuous structural heterogeneity

Johannes Schwab developed a method called DynaMight that 'explores protein **Dyna**-mics, and **Might** improve your map'. It is based on a variational auto-encoder that predicts 3D deformations of a Gaussian model for the consensus map, and a deformed backprojection algorithm that attempts to "un-do" these deformations to reconstruct an improved consensus map.

ModelAngelo for automated atomic model building

Kiarash Jamali developed a machine-learning approach for automated atomic model building and identification of unknown proteins in cryo-EM maps. ModelAngelo will build most of your automatically, provided the resolution extends beyond 3.5-4.0 Angstroms. Goodbye to months in the dark graphics room!

Select subsets of filaments using dendrograms

David Li developed a useful utility to select subsets of filament particles that belong to the same structural class. It has been implemented on the Filaments tab of the Subset selection job type. See FilamentTools for more details.

Support for AMD and Intel GPUs (HIP/ROCm and SYCL)

Suyash Tandon from AMD and Jason Do from Intel, together with their colleagues, have contributed code for GPU acceleration of relion-5 in HIP/ROCm and SYCL, respectively. This means that the `relion_refine` program can now also be run efficiently on AMD and Intel GPUs. (The previously existing CUDA implementation and vectorised CPU-acceleration still work too.)

A complete subtomo-gram averaging pipeline

Alister Burt, Euan Pyle, Sjors Scheres and others have developed a new pipeline for sub-tomogram averaging that starts with serialEM mdoc files and raw movies, and potentially ends with automated model building by ModelAngelo. You can access it by launching `relion --tomo` from the command line. However, please do note that this part of the code is not yet well tested and we have not yet been able to write an explanatory tutorial for this, so please be patient. Until we have finished the documentation and testing, you can play with the code already, but we cannot yet provide any feedback…

1.2 Release 4.0

Watch Sjors giving a Structural Studies Colloquium at MRC-LMB about the new features in release 4.0 on YouTube. Note that since then, *Schedules* have been renamed to *Schemes* to prevent confusion with the existing functionality to schedule jobs in the GUI.

A new approach to subtomogram averaging

Jasenko Zivanov and Joaquin (Kino) Oton have implemented a new approach to averaging in cryo-electron tomography, which replaces standard sub-tomograms with the concept of pseudo-sub-tomograms. The new approach leads to better weighting of the individual 2D images that make up a tilt series in `relion_refine` and the single-particle concepts of Bayesian polishing and CTF refinement have now also been implemented for tomography data. A preprint/publication about this work is pending.

The VDAM refinement algorithm

Dari Kimanius has implemented a new, gradient-driven algorithm with implicit regularisation, called Variable-metric Gradient Descent with Adaptive Moments (VDAM). The VDAM algorithm replaces the previously implemented SAGD algorithm for initial model generation, and makes 2D and 3D classification faster, especially for large data sets. A preprint/publication about this work, together with the automated class selection and the execution of workflow, is pending.

Automated 2D class selection

Liyi Dong developed a new algorithm for automatic selection of 2D class average images that combines features that are extracted from RELION's 2D classification metadata with a convolutional neural network that acts on the 2D class averages themselves. The corresponding program, `relion_class_ranker` can be called through the Subset selection job type.

Automatic execution of workflows

Sjors developed a framework for the automated execution of predefined workflows, which is explained in more detail in the section on *On-the-fly processing*.

Tighter integration of the pipeliner with CCP-EM software

The CCP-EM team, mainly Matt Iadanza, Colin Palmer and Tom Burnley, have implemented a python-based pipeliner in the CCP-EM software that mimics the relion pipeliner, but will be extended to include other CCP-EM softwares too. The python interface is convenient for scripting, and can also be called from relion's main GUI, by adding the additional argument `relion --ccpem &`.

1.3 Release 3.1

Aberration corrections and optics groups

One of the major new features in relion-3.1 is a correction for higher-order aberrations in the data, i.e. besides the beamtilt correction already present in relion-3.0, the current version can also estimate and correct for trefoil and tetrafoil, as well as deviations from the nominal spherical aberration (Cs). The corresponding paper can be found on bioRxiv [ZNS20]. The signal to estimate these aberrations is calculated by averaging over particles from multiple micrographs. To allow for multiple subsets of a data set having different Zernike coefficients, relion-3.1 implements the new concept of *optics groups*. Optics groups are defined in a separate table called `data_optics` at the top of a STAR file, which will also contain a table called `data_movies`, `data_micrographs` or `data_particles`, depending on what type of images it refers to. The second table is similar to the content of STAR files in previous releases, but contains a new column called `rlnOpticsGroup`, which is also present in the `data_optics` table. Common CTF-parameters, like `rlnVoltage` and `` _`rlnSphericalAberration ``, but also the new `rlnOddZernike` and `rlnEvenZernike`, can be stored once for each optics group in the `data_optics` table, without the need to store them for each particle/micrograph in the second table.

The same program that handles higher-order aberrations can also be used to refine differences in (anisotropic) magnification between the reference and (groups of) the particles. Besides correcting for anisotropic magnification in the data, this is also useful when combining data from different scopes. As of release 3.1, the program that does 2D/3D classification and 3D refinement (`relion_refine`) can combine particles with different box sizes and pixel sizes in a single refinement, and the magnification refinement can be used to correct small errors in the (calibrated) pixel sizes. The box and pixel size of the input reference (or the first optics group in 2D classification) will be used for the reconstructions/class averages. You may want to check they are on the desired scale before running classifications or refinements!

Upon reading STAR files that were generated in older releases of relion, relion-3.1 will attempt to convert these automatically into the relion-3.1-style STAR files. Therefore, moving a project from an older release to relion-3.1 should be easy.

The External job-type

relion-3.1 allows execution of third-party software within the relion pipeline through the new External job-type. See *this section* for details on how to use this.

***Schedules* for on-the-fly processing**

The python script `relion_it.py` in relion-3.0 has been replaced by a new framework of *Schedules*, which implement decision-based scheduling and execution of relion jobs. This comes with its own GUI interface. See Schedules for details on how to use this.

General tweaks

Several tweaks have been made to enhance user experience:

- The pipeliner no longer looks for output files to see whether a job has finished. Instead, upon successful exit, all programs that are launched from within the relion pipeline will write out a file called `RELION_EXIT_SUCCESS` in the job directory. This avoids problems with subsequent execution of scheduled jobs with slow disc I/O.
- Likewise, when encountering an error, all programs will write out a file called `RELION_EXIT_FAILURE`. The GUI will recognise these jobs and use a red font in the Finished jobs list. Note that incorrectly labeled jobs can be changed using the 'Mask as finished' or 'Mark as failed' options from the Job actions pull-down menu.
- There is an '*Abort running*' option on the Job actions pull-down menu, which will trigger the currently selected job to abort. This works because all jobs that are executed from within the relion pipeline will be on the lookout for a file called `RELION_JOB_ABORT_NOW` in their output directory. When this file is detected, the job will exit prematurely and write out a `RELION_EXIT_ABORTED` file in the job directory. Thereby, users no longer need to kill undesired processes through the queuing or operating system. The GUI will display aborted jobs with a strike-through red font in the Finished jobs list.
- When a job execution has given an error, in previous releases the user would need to fix the error through the input parameters, and then launch a new job. They would then typically delete the old job. relion-3.1 allows to directly overwrite the old job. This is accessible on Linux systems through `ALT+o` or through the `Overwrite continue` option from the 'File menu'. Note that the `run.out` and `run.err` files will be deleted upon a job overwrite.

** Tweaks to helical processing **

Several new functionalities were implemented for helical processing:

- The `relion_helix_inimodel2d` program can be used to generate initial 3D reference maps for helices, in particular for amyloids, from 2D classes that span an entire cross-over (see *this section*).
- The translational offsets along the direction of the helical axis can now be restricted to a single rise in 2D-classification.

- The 3D refinement and 3D classification now can use a prior on the first Euler angle, (`rlnAngleRotPrior`), which was implemented by Kent Thurber from the Tycko lab at the NIH.

CHAPTER

TWO

THE TEAM

Below are the past and present members of the relion team in reverse chronological order of joining.

2.1 Current members

2.1.1 Bogdan Toader

Google Scholar

Bogdan studied Computer Science and Mathematics at the University of Manchester and did a PhD in Mathematics at the University of Oxford. He joined the LMB in January 2024 as a postdoc in both our and Tanmay Bharat's groups to further improve and develop new tools and algoriths for cryo-ET data processing.

2.1.2 Kiarash Jamali

Google Scholar

Kiarash studied Mathematics and Statistics at the University of Toronto, St George. He joined our group as a PhD student in October 2021, but had been working with us a volunteering undergrad student since the COVID19 lockdown in April 2020. Kiarash works on various machine-learning methods for solving cryo-EM structures and beyond.

2.1.3 Johannes Schwab

Google Scholar

Johannes studied Mathematics at the University of Innsbruckm Austria, where he also obtained his PhD. Johannes works on machine-learning approaches to deal with molecular flexibility in cryo-EM reconstruction.

2.1.4 Dari Kimanius

Google Scholar

Dari studied Theoretical Physics at the Royal Institute of Technology (KTH, Stockholm), and did a PhD in Biophysics and Bioinformatics at Stockholm University. Dari joined our group as a post-doc in May 2019. Dari implemented *GPU-acceleration* during his PhD. For his postdoc at LMB, he works on the incorporation of *more informative priors* through deep convolutional neural networks, as well as on the design of more efficient *optimisation algorithms*.

2.1.5 Euan Pyle

Google Scholar

Euan studied Chemistry and Biology at Durham University before going on to complete his PhD at Imperial College London. As a post-doc position in Giulia Zanetti's lab at Birkbeck College/The Francis Crick Institute, he contributed code to the RELION 5.0 tomography pipeline, in close collaboration with Alister Burt.

2.1.6 Takanori Nakane

Google Scholar

Takanori studied as an M.D. at Kyoto University, where he also obtained his PhD in Medicine. In 2014 he also obtained an M.Phil in Computational Biology from the University of Cambridge. Takanori joined our team in October 2017. He works on many facets of single-particle analysis and kindly dedicates a lot of his time to provide support to RELION users worldwide.

2.1.7 Sjors Scheres

Google Scholar

Sjors studied Chemistry at Utrecht University, The Netherlands, where he also obtained his PhD in protein crystallography. He was a post-doc in the group of Jose-Maria Carazo in Madrid, before he started his group at the LMB in 2010. Sjors designed the *regularised likelihood algorithm* and is the *original architect* of relion.

2.2 Past members

2.2.1 Alister Burt

Google Scholar

Alister studied Chemistry at the University at York, UK and did his PhD at the IBS in Grenoble, France. He was a post-doc in the group of David Barford, where he contributed code to the new sub-tomogram avaraging pipeline in relion-5;

2.2.2 David Li

Google Scholar

David studied Electrical Engineering and Computer Science at MIT. He worked in the lab of Prof Feng Zhang on CRISPR systems. David joined our group as an MPhil student in October 2022 to work on in vitro assembly of tau filaments. During his stay with us David also developed new classification software for filaments. David went on to do a PhD at Stanford.

2.2.3 Liyi Dong

Liyi studied Biology at China Agricultural University and did a BSc at Purdue University with Michael Rossmann. Liyi joined our group as a PhD student in November 2017. She worked on *automated selection of 2D class averages*.

2.2.4 Shaoda He

Shaoda studied Biology at Peking University, where he also participated in a programme organized by the Electronic Engineering and Computer Science Department. Shaoda joined our group in October 2014. He developed *helical reconstruction* and *local symmetry averaging*.

2.2.5 Joaquín Otón

Google Scholar

Joaquin (Kino) studied Physics at Universitat de Vàlencia (Spain) and did a PhD in Optical Engineering at Universitat Politècnica de Catalunya. He joined the neighbouring Briggs group in 2018. Together with Jasenko, he co-developed a new approach for *sub-tomogram averaging*.

2.2.6 Jasenko Zivanov

Jasenko studied Computer Science at the University of Basel, Switzerland, where he also obtained his PhD. Jasenko was a postdoc with us for more than three years (during 2017-2020), and continues to work with us from Switzerland. Jasenko developed *Bayesian polishing* and *optical aberration correction* for single-particle analsyis. Together with Kino, he also developed a new approach for *sub-tomogram averaging*.

Note

relion is distributed under a GPLv2 license, i.e. it is completely free, open-source software for both academia and industry.

CHAPTER

THREE

INSTALLATION

The sections below explain how to download and install relion on your computer.

Note that relion depends on and uses several external programs and libraries.

C++ compiler:
RELION 5.0 requires a C++ compiler that fully supports the C++14 standard. For GCC, this means version 5.0 or later. Note that GCC 4.8, which comes with RedHat Enterprise Linux / Cent OS 7.x, is too old. You can obtain newer GCC via devtoolset or use free Intel compiler that comes with oneAPI toolkit (see below).

MPI:
Your system will need MPI runtime (most flavours will do). If you don't have an MPI installation already on your system, we recommend installing OpenMPI.

CUDA, HIP/ROCm, SYCL or oneAPI intel compilers:
If you have GPUs from nvidia, AMD or Intel, you can accelerate many jobs considerably.

By default, relion will build with GPU-acceleration support, for which you'll need cuda. Download it from NVIDIA website. Note that CUDA toolkits support only a limited range of C compilers. Also note that a newer CUDA toolkit requires a newer GPU driver. Carefully read the release note and make sure you have a compatible set of GPU driver, C compiler and CUDA toolkit.

If you want to compile with HIP/ROCm, you will need

- AMD ROCm

If you want to compile with SYCL, you will need

- Intel oneAPI Base Toolkit and HPC Toolkit (All components recommended; this is also recommended if you want to build the CPU acceleration path, see below)
- Intel software for general purpose GPU capabilities
- Intel CPU Runtime for OpenCL(TM) Applications (optional)
- Codeplay oneAPI for NVIDIA GPU (optional)
- Codeplay oneAPI for AMD GPU (optional)

CTFFIND-4.1:
CTF estimation is not part of relion. Instead, relion provides a wrapper to Alexis Rohou and Niko Grigorieff's ctffind 4 [RG15]. Please obtain CTFFIND 4.1.x from their Web site. Note that CTFFIND 5.x is not supported.

Ghostscript:
RELION uses Ghostscript to generate PDF files.

FLTK (only for GUI):
RELION uses FLTK as a GUI tool kit. This will be installed automatically (see below).

X Window system libraries (only for GUI):
RELION needs basic X11 libraries together with Xft for the GUI. Most Linux distributions have packages called `libxft-dev` or `libXft-devel` and `libX11-devel`. Note that you need developer packages if you build your own FLTK.

FFT libraries:
RELION needs an FFT library. The default is FFTW. This will be installed automatically (see below). Depending on your CPU, Intel MKL FFT or AMD optimised FFTW might run faster. See below how to use them.

libtiff:
RELION needs libtiff version >= 4.0. Most Linux distributions have packages called `libtiff-dev` or `libtiff-devel`. Note that you need a developer package.

libpng:
RELION needs libpng. Most Linux distributions have packages called `libpng-dev` or `libpng-devel`. Note that you need a developer package.

pbzip2, xz, zstd:
RELION needs these commands in the `PATH` to read MRC movies compressed by bzip2, xz or ZStandard, respectively. Note that RELION uses `pbzip2`, not `bzip2`. Most Linux distributions provide packages for these utilities.

UCSF MotionCor2 (optional):
relion implements its own (CPU-only) implementation of the UCSF motioncor2 algorithm for whole-frame micrograph movie-alignment [ZPA+17]. If you want, you can still use the (GPU-accelerated) UCSF program. You can download it from David Agard's page and follow his installation instructions. Note that using the UCSF program does not make full advantage of the opportunities provided in Bayesian polishing.

ResMap (optional):
Local-resolution estimation may be performed inside relion's own postprocessing program. Alternatively, one can also use Alp Kucukelbir's resmap [KST14]. Download it from Alp's ResMap website and follow his installation instructions.

In practice, most of these dependencies can be installed by system's package manager if you have the root priviledge.

In Debian or Ubuntu:

```
sudo apt install cmake git build-essential mpi-default-bin mpi-default-dev libfftw3-dev␣
↪libtiff-dev libpng-dev ghostscript libxft-dev
```

In RHEL, Cent OS, Scientific Linux:

```
sudo yum install cmake git gcc gcc-c++ openmpi-devel fftw-devel libtiff-devel libpng-
↪devel ghostscript libXft-devel libX11-devel
```

3.1 Download RELION

We store the public release versions of relion on GitHub, a site that provides code-development with version control and issue tracking through the use of `git`. We will not describe the use of git in general, as you will not need more than very basic features. Below we outline the few commands needed on a UNIX-system, please refer to general git descriptions and tutorials to suit your system. To get the code, you clone or download the repository. We recommend cloning, because it allows you very easily update the code when new versions are released. To do so, use the shell command-line:

```
git clone https://github.com/3dem/relion.git
```

This will create a local Git repository. All subsequent git-commands should be run inside this directory.

To get access to the latest updates for (stable) release of RELION 5.0x, type:

```
git checkout ver5.0
```

The code will be intermittently updated to amend issues. To incorporate these changes, use the command-line:

```
git pull
```

inside you local repository (the source-code directory downloaded). If you have changed the code in some way, this will force you to commit a local merge. You are free to do so, but we will assume you have not changed the code. Refer to external instructions regarding git and merging so-called conflicts if you have changed the code an need to keep those changes.

3.2 Setup a conda environment

To add support for Python modules (e.g. Blush, ModelAngelo and DynaMight) you will have to setup a Python environment with dependencies. We recommend installing via Miniforge to avoid inadvertently installing packages from the restrictively licensed “default” conda repository.

Once you have conda setup, you can install all the RELION Python dependencies into a new environment by running:

```
conda env create -f environment.yml
```

Also code in this environment will be updated intermittently. You can incorporate the latest changes by running:

```
conda env update -f environment.yml
```

Warning

You should **NOT** activate this `relion-5.0` conda environment when compiling and using RELION; RELION activates it automatically only when necessary. Otherwise, system-wide installation of compilers/libraries/MPI runtime might get mixed up with those provided by conda, leading to compilation failures or runtime errors. The same applies to other software packages that provide their own libraries/MPI runtime, such as CCPEM, CCP4, EMAN2, DIALS, PHENIX.

The `cmake` command should automatically detect the `relion-5.0` conda environment created above. If it does not, you can specify `-DPYTHON_EXE_PATH=path/to/your/conda/python`. Additionally, if you intend to make use of automatically downloaded pretrained model weights (used in e.g. Blush, ModelAngelo and class_ranker), it is recommended to explicitly set the destination directory (`TORCH_HOME`) by including the flag `-DTORCH_HOME_PATH=path/to/torch/home`. You have to create this directory before running `cmake`. Otherwise, it will be downloaded to the default location (usually `~/.cache/torch`).

At the moment, the model weights for Blush are stored on MRC-LMB’s FTP server. If your network blocks FTP, please follow instructions here.

3.3 Compilation

relion has an installation procedure which relies on `cmake`. You will need to have this program installed, but most UNIX-systems have this by default. You will need to make a build-directory in which the code will be compiled. This can be placed inside the repository:

```
cd relion
mkdir build
cd build
```

You then invoke `cmake` inside the build-directoy, but point to the source-directoy to configure the installation. This will not install relion, just configure the build:

```
cmake ..
```

The output will notify you of what was detected and what type of build will be installed. Because relion is rich in terms of the possible configurations, it is important to check this output. For instance:

- The path to the MPI library.
- GPU-capability will only be included if a CUDA SDK is detected. If not, the program will install, but without support for GPUs.
- The path to the Python interpreter.
- If FFTW is not detected, instructions are included to download and install it in a local directory known to the relion installation.
- As above, regarding FLTK (required for GUI). If a GUI is not desired, this can be escaped as explained in the following section.

The MPI library must be the one you intend to use relion with. Compiling relion with one version of MPI and running the resulting binary with `mpirun` from another version can cause crash. Note that some software packages (e.g. CCPEM, crYOLO, EMAN2) come with their own MPI runtime. Sourcing/activating their environment might update `PATH` and `LD_LIBRARY_PATH` environmental variables and put their MPI runtime into the highest priority.

The MPI C++ compiler (`mpicxx`) and CUDA compiler (`nvcc`) internally calls a C++ compiler. This must match the compiler `cmake` picked up. Otherwise, the compilation might fail at the linking step.

Following the completion of cmake-configuration without errors, `make` is used to install the program:

```
make -j N
```

, where `N` is the number of processes to use during installation. Using a higher number simply means that it will compile faster.

Take note of any warnings or errors reported. relion will be installed in the `build` directory's sub-directory called `bin`. To make the installation system-wide, see below.

Wherever you install relion, make sure your `PATH` environmental variable points to the directory containing relion binaries. Launching relion with a path like `/path/to/relion` is not the right way; this starts the right GUI, but the GUI might invoke other versions of relion in the `PATH`.

3.4 General configuration

CMake allows configuration of many aspects of the installation, some of which are outlined here. Note that by default, relion is configured to build with CUA acceleration on NVidia GPUs. Instructions for building with CPU, HIP/Rocm (AMD) SYCL (Intel et al) acceleration are given in the next section below.

Most options can be set by adding options to the `cmake` configuration. Under the below subheadings, some example replacement commands are given to substitute the original configuration command. It is also recommended to clean or purge your build-directory between builds, since CMake caches some of previous configurations:

```
cd build
rm -fr *
```

And of course, any of the below options can be combined.

Omitting the GUI:
`cmake -DGUI=OFF ..` (default is ON)

With this option, GUI programs (e.g. `relion`, `relion_manualpick`, `relion_display`) are not be built and FLTK becomes unnecessary.

Using single-precision on the CPU:
`cmake -DDoublePrec_CPU=OFF ..` (default is ON)

This will reduce (CPU but not GPU) memory consumption to about half. This is useful when memory hungry tasks such as motion correction and Polishing run out of memory. This is safe in most cases but please use the default double precision build if CtfRefine produces NaNs.

Using double-precision on the GPU:
`cmake -DDoublePrec_GPU=ON ..` (default is OFF)

This will slow down GPU-execution considerably, while this does *NOT* improve the resolution. Thus, this option is not recommended.

Compiling NVIDIA GPU codes for your architecture:
`cmake -DCUDA_ARCH=52 ..` (default is 35, meaning compute capability 3.5, which is the lowest supported by relion)

CUDA-capable NVIDIA devices have a so-called compute capability, which code can be compiled against for optimal performance. The compute capability of your card can be looked up at the table in NVIDIA website. WARNING: If you use a wrong number, compilation might succeed but the resulting binary can fail at the runtime.

Forcing build and use of local FFTW:
`cmake -DFORCE_OWN_FFTW=ON ..`

This will download, verify and install FFTW during the installation process.

Forcing build and use of AMD optimized FFTW:
`cmake -DFORCE_OWN_FFTW=ON -DAMDFFTW=ON ..`

This will download, verify and install AMD optimized version of FFTW during the installation process. This is recommended for AMD CPUs (e.g. Ryzen, Threadripper, EPYC).

Forcing build and use of Intel MKL FFT:
`cmake -DMKLFFT=ON ..`

This will use FFT library from Intel MKL. In contrast to the FFTW options above, this will *not* download MKL automatically. You have to install MKL and set relevants paths (usually by sourcing the `mkl_vars.sh` script).

Forcing build and use of local FLTK:
`cmake -DFORCE_OWN_FLTK=ON ..`

This will download, verify and install FLTK during the installation process. If any of these are not detected during configuration, this will happen automatically anyway, and you should not have to specify the below options manually.

Specify location of libtiff:
`cmake -DTIFF_INCLUDE_DIR=/path/to/include -DTIFF_LIBRARY=/path/to/libtiff.so.5`

This option is to use libtiff installed in non-standard location.

Specifying an installation location:

To allow relion a system-wide installation use:

```
cmake -DCMAKE_INSTALL_PREFIX=/path/to/install/dir/ ..
make -j N
make install
```

Warning

Do not specify the `build` directory itself as `CMAKE_INSTALL_PREFIX`. This does not work! If you are happy with binaries in the build directory, leave `CMAKE_INSTALL_PREFIX` as default and omit the `make install` step.

3.5 Configuration with CPU acceleration

Enable accelerated CPU code path:

`cmake -DALTCPU=ON`

Note that this is mutually exclusive with GPU acceleration (`-DCUDA=ON`). Intel Classic compilers are recommended for this option (see below).

Use Intel compilers:

There are two Intel compilers: Intel Classic compiler and Intel oneAPI DPC++/C++ compiler. They often generate faster binaries for Intel CPUs, especially when combined with the accelerated CPU code path above. As of 2024 April, the classic compiler generate faster binaries than the DPC++/C++ compiler.

Both compilers used to be available free of chage as part of Intel oneAPI HPC toolkit. Unfortunately, the classic compiler was removed in the 2024 release and only the DPC++/C++ compiler is currently distributed. (Apparently older versions seem to be available via YUM/APT, but we do not know how long they remain.) We recommend you to use the classic compiler if you have older oneAPI toolkit installers at hand.

To use Intel Classic compiler, run below after sourcing the initialization script (*setvars.sh*):

```
mkdir build-cpu
cd build-cpu
cmake .. -DMKLFFT=ON \
-DCMAKE_C_COMPILER=icc -DCMAKE_CXX_COMPILER=icpc -DMPI_C_COMPILER=mpiicc -DMPI_CXX_
↪COMPILER=mpiicpc \
-DCMAKE_C_FLAGS="-O3 -ip -g -xCOMMON-AVX512 -restrict " \
-DCMAKE_CXX_FLAGS="-O3 -ip -g -xCOMMON-AVX512 -restrict "
make -j 24
```

This generates binaries optimized with AVX512 instructions. If your CPU supports only up to AVX256, use `-xCORE-AVX2` instead of `-xCOMMON-AVX512`.

If you do not have Intel Classic compiler, use the DPC++/C++ compiler from the latest oneAPI release. Its performance is being improved. The `cmake` line should be:

```
cmake .. -DMKLFFT=ON \
-DCMAKE_C_COMPILER=icx -DCMAKE_CXX_COMPILER=icpx -DMPI_C_COMPILER=mpiicx -DMPI_CXX_
↪COMPILER=mpiicpx \
-DCMAKE_C_FLAGS="-O3 -qopenmp-simd -xCORE-AVX512 -qopt-zmm-usage=high -qoverride-
↪limits " \
-DCMAKE_CXX_FLAGS="-O3 -qopenmp-simd -xCORE-AVX512 -qopt-zmm-usage=high -qoverride-
↪limits "
```

If your CPU supports only up to AVX256, use `-xCORE-AVX2` instead of `-xCORE-AVX512`.

If you don't want to use Intel MPI, change `DMPI_C_COMPILER` and `DMPI_CXX_COMPILER` variables accordingly. For example, to use OpenMPI with Intel Classic compiler, specify `mpicc` and `mpicxx` after setting environmental variables `OMPI_CC=icc` and `OMPI_CXX=icpc`. If `cmake` still picks up Intel MPI, specify `MPI_HOME`. See OpenMPI FAQ and FindMPI manual for details.

3.6 Configuration with HIP/ROCm acceleration for AMD GPUs

Enable the accelerated HIP/ROCm code path with:
`cmake -DHIP=ON`

Note that this is mutually exclusive with other accelerated code paths (e.g. CUDA, ALTCPU and SYCL). On our system, we build with HIP/ROCm acceleration to use AMD GPUs with the following commands:

```
export LD_LIBRARY_PATH=/opt/rocm/lib:$LD_LIBRARY_PATH
export PATH=/opt/rocm/:$PATH
export ROCM_PATH=/opt/rocm/
mkdir build-amd
cd build-amd
cmake -DCMAKE_BUILD_TYPE=Release -DHIP=ON -DHIP_ARCH="gfx90a,gfx908" -DFORCE_OWN_FFTW=ON␣
↪ -DAMDFFTW=on ..
make -j 24
```

If you get problems finding `omp.h`, make sure you have `openmp-extras-devel` installed on your system too.

3.7 Configuration with SYCL acceleration (Intel GPUs)

Enable accelerated the SYCL code path with:
`cmake -DSYCL=ON`

Note that this is mutually exclusive with other accelerated code paths (e.g. CUDA, ALTCPU and HIP/ROCm). Technically speaking, you can build SYCL for AMD and NVIDIA GPUs to make a single binary that runs on NVIDIA, AMD and Intel GPUs, but this is highly experimental and not tested well.

For now, this way of building RELION is explained here:.

3.8 Set-up queue job submission

The GUI allows the user to submit jobs to a job queueing system with a single click. For this to work, a template job submission script needs to be provided for the queueing system at hand (e.g. TORQUE, PBS, SGE). In this script a set of strings (variables) in the template script is replaced by the values given in the GUI. The following table contains all defined variables:

String	Variable	Meaning
`XXXoutfileXXX`	string	The standard output log file RELION GUI displays.
`XXXerrfileXXX`	string	The standard error log file RELION GUI displays.
`XXXcommandXXX`	string	relion command + arguments
`XXXqueueXXX`	string	Name of the queue to submit job to
`XXXmpinodesXXX`	integer	The number of MPI processes to use
`XXXthreadsXXX`	integer	The number of threads to use on each MPI process
`XXXcoresXXX`	integer	The number of MPI processes times the number of threads
`XXXdedicatedXXX`	integer	The minimum number of cores on each node (use this to fill entire nodes)
`XXXnodesXXX`	integer	The total number of nodes to be requested
`XXXextra1XXX`	string	Installation-specific, see below
`XXXextra2XXX`	string	Installation-specific, see below

The `XXXcommandXXX` variable needs a special care. For non-MPI commands (e.g. `relion_refine`) not only the variable but the whole line is replaced. Thus, `mpirun XXXcommandXXX` will be `mpirun relion_refine_mpi` for an MPI job but `relion_refine` for a non-MPI job. Also note that some jobs consist of multiple lines of commands. See CCPEM threads (1 and 2) for typical pitfalls.

There are two environment variables that control the use of the entry of the 'Minimum number of dedicated cores per node' on the Running tabs of the GUI: `RELION_MINIMUM_DEDICATED` sets its default value (1 if not set); `RELION_ALLOW_CHANGE_MINIMUM_DEDICATED` sets whether the user will be able to change this entry. At LMB, we set the default to 24 and do not allow users to change it. In this way, we enforce that our hyper-threaded 12-core nodes get filled up entirely by individual relion jobs.

By default, the `XXXextra1XXX`, `XXXextra2XXX`, ... variables are not used. They provide additional flexibility for queueing systems that require additional variables. They may be activated by first setting `RELION_QSUB_EXTRA_COUNT` to the number of fields you need (e.g. 2) and then setting the `RELION_QSUB_EXTRA1`, `RELION_QSUB_EXTRA2`, ... environment variables, respectively. This will result in extra input fields in the GUI, with the label text being equal to the value of the environment variable. Likewise, their default values (upon starting the GUI) can be set through environment variables `RELION_QSUB_EXTRA1_DEFAULT`, `RELION_QSUB_EXTRA2_DEFAULT`, etc and their help messages can be set through environmental variables `RELION_QSUB_EXTRA1_HELP`, `RELION_QSUB_EXTRA2_HELP` and so on. But note that (unlike all other entries in the GUI) the extra values are not remembered from one run to the other.

The template job submission script may be saved in any location. By default, the one used at the LMB is present as `gui/qsub.csh` in the relion tar-ball. Upon installation this file is copied to the bin directory. It is convenient for the user if he does not have to select this file each time he opens the relion GUI in a new directory. Therefore, one may set the environment variable `RELION_QSUB_TEMPLATE` to point to the location of the script for the system at hand. This value will be pre-set as default in the GUI. (Note the user still has the liberty to define and use his own template!)

Note

If somehow the job queue submission cannot be set up, relion may still be run in parallel and on a job queueing system. The GUI comprises a Print command button that prints the entire relion command, including all arguments, to the screen. Pasting of this command to a job queue submission script, and manual submission of this script may then be used to submit the parallel job to a queueing system.

3.9 Edit the environment set-up

For relion, we source the following C-shell setup in our `.cshrc` file. You'll need to change all the paths for your own system, and translate the script in case you use a bash shell (which uses *export* instead of *setenv* etc).

```
#!/bin/csh -f

# Setup openMPI if not already done so
if ("" == "`echo $path | grep /public/EM/OpenMPI/openmpi/bin`") then
        set path=(/public/EM/OpenMPI/openmpi/bin $path)
endif
if ("1" == "$?LD_LIBRARY_PATH") then
        if ("$LD_LIBRARY_PATH" !~ */public/EM/OpenMPI/openmpi/lib*) then
                setenv LD_LIBRARY_PATH /public/EM/OpenMPI/openmpi/lib:$LD_LIBRARY_PATH
        endif
else
        setenv LD_LIBRARY_PATH /public/EM/OpenMPI/openmpi/lib
endif

# Setup |RELION| if not already done so
if ("" == "`echo $path | grep /public/EM/RELION/relion/bin`") then
   set path=(/public/EM/RELION/relion/bin $path)
endif
if ("1" == "$?LD_LIBRARY_PATH") then
        if ("$LD_LIBRARY_PATH" !~ */public/EM/RELION/relion/lib*) then
                setenv LD_LIBRARY_PATH /public/EM/RELION/relion/lib:$LD_LIBRARY_PATH
        endif
else
        setenv LD_LIBRARY_PATH /public/EM/RELION/relion/lib
endif

# CUDA for RELION
setenv PATH /public/EM/CUDA/Cuda11.4/bin:$PATH
setenv LD_LIBRARY_PATH /public/EM/CUDA/Cuda11.4/lib64:$LD_LIBRARY_PATH
setenv CUDA_HOME /public/EM/CUDA/Cuda11.4

# Where is qsub template script stored
setenv RELION_QSUB_TEMPLATE /public/EM/RELION/relion-devel/bin/qsub.csh

# Default PDF viewer
setenv RELION_PDFVIEWER_EXECUTABLE evince

# Default MOTIONCOR2 executable
setenv RELION_MOTIONCOR2_EXECUTABLE /public/EM/MOTIONCOR2/bin/MotionCor2_1.0.4

# Default CTFFIND-4.1+ executable
setenv RELION_CTFFIND_EXECUTABLE /public/EM/ctffind/ctffind.exe

# Default ResMap executable
setenv RELION_RESMAP_EXECUTABLE /public/EM/ResMap/ResMap-1.1.4-linux64

# Enforce cluster jobs to occupy entire nodes with 24 hyperthreads
setenv RELION_MINIMUM_DEDICATED 24
# Do not allow the user to change the enforcement of entire nodes
setenv RELION_ALLOW_CHANGE_MINIMUM_DEDICATED 0

# Ask for confirmation if users try to submit local jobs with more than 12 MPI nodes
setenv RELION_WARNING_LOCAL_MPI 12
```

(continues on next page)

(continued from previous page)

```
# Other useful variables
# RELION_MPI_RUN: The mpi runtime ('mpirun' by default)
# RELION_QSUB_NRMPI: The default for 'Number of MPI procs'
# RELION_MPI_MAX: The maximum number of MPI processes available from the GUI
# RELION_QSUB_NRTHREADS: The default for 'Number of threads'
# RELION_THREAD_MAX: The maximum number of threads per MPI process available from the GUI
# RELION_QUEUE_USE: The default for 'Submit to queue?'. "yes" or "no".
# RELION_QUEUE_NAME: The default for 'Queue Name"
# RELION_QSUB_COMMAND: The default for 'Queue submit command'
# RELION_MINIMUM_DEDICATED: The default for 'Minimum dedicated cores per node'
# RELION_ALLOW_CHANGE_MINIMUM_DEDICATED: Whether to allow a user to change the 'Minimum␣
↪dedicated cores per node' field in the GUI
# RELION_SHELL: A shell used to launch CTFFIND in CtfFind jobs ('csh' by default; only␣
↪available from 3.1)
# RELION_SCRATCH_DIR: The default scratch directory in the GUI
# RELION_STACK_BUFFER: The buffer size used for MRC(S) file I/O, potentially useful on␣
↪GPFS or Lustre file system. See https://github.com/3dem/relion/pull/783 for details.
```

CHAPTER

FOUR

SINGLE PARTICLE TUTORIAL

4.1 Introduction

This tutorial provides an introduction to the use of relion-5.0 for cryo-EM structure determination. This tutorial covers the entire single-particle analysis workflow in relion-5.0: beam-induced motion-correction, CTF estimation; automated particle picking; particle extraction; 2D class averaging; automated 2D class selection; VDAM-based initial model generation; 3D classification; high-resolution 3D refinement (including Blush regularisation); CTF refinement and higher-order aberration correction; Bayesian polishing to correct for beam-induced motions in the movies; map sharpening and local-resolution estimation; automated model building with ModelAngelo; and flexibility analysis with DynaMight. Carefully going through this tutorial should take less than a day (if you have a suitable GPU or if you follow our precalculated results). After that, you should be able to run relion on your own data.

This tutorial uses a test data set on beta-galactosidase that was kindly given to us by Takayuki Kato from the Namba group at Osaka university, Japan. It was collected on a JEOL CRYO ARM 200 microscope. The data and our precalculated results may be downloaded and unpacked using the commands below. The full data set is also available at EMPIAR-10204.

```
wget ftp://ftp.mrc-lmb.cam.ac.uk/pub/scheres/relion30_tutorial_data.tar
wget ftp://ftp.mrc-lmb.cam.ac.uk/pub/scheres/relion50_tutorial_precalculated_results.tar.
↪gz
tar -xf relion30_tutorial_data.tar
tar -zxf relion50_tutorial_precalculated_results.tar.gz
```

If you have any questions about relion, first read this entire document, check the FAQ on the relion Wiki and the archives of the CCPEM mailing list. If that doesn't help, subscribe to the CCPEM email list and use the email address above for asking your question.

Caution

Please, please, please, do not send us direct emails, as we can no longer respond to all of those.

4.2 Preprocessing

4.2.1 Getting organised

We recommend to create a single directory per project, i.e. per structure you want to determine. We'll call this the project directory. **It is important to always launch the RELION graphical user-interface (GUI) from the project directory.** Inside the project directory you should make a separate directory to store all your raw micrographs or micrograph movies in MRC, TIFF or EER format. We like to call this directory `Movies/` if all movies are in one directory, or for example `Movies/15jan16/` and `Movies/23jan16/` if they are in different directories (e.g. because they were collected on different dates). If for some reason you do not want to place your movies inside the relion project directory, then inside the project directory you can also make a symbolic link to the directory where your movies are stored.

Warning

Symbolic links must be made by an absolute path (e.g. `/storage/data/15jan16`). Use of relative paths (e.g. `../../storage/data/15jan16`) can cause problems in later steps. Our precalculated example contains a symbolic link from *Movies* to *../Tutorial4.0/Movies/* but please do not follow this practice. Because we don't know where you decompress the archive, we cannot include a link by an absolute path.

Single-image micrographs should have a `.mrc` extension, while movies can have a `.mrc`, `.mrcs`, `.tif`, `.tiff` or `.eer` extension. For EER movies, see *this* for the details. RELION 5.0 can also read *MRC movies compressed by bzip2, xz, zstd or gzip*. When you unpacked the tutorial test data, the (`Movies/`) directory was created. It should contain 24 movies in compressed TIFF format, a gain-reference file (`gain.mrc`) and a `NOTES` file with information about the experiment.

We will start by launching the relion GUI. As said before, this GUI always needs to be launched from the project directory. To prevent errors with this, the GUI will ask for confirmation the first time you launch it in a new directory. Make sure you are inside the project directory, and launch the GUI by typing:

```
relion &
```

and answer `Yes` when prompted to set up a new relion project here.

The first thing to do is to import the set of recorded micrograph movies into the pipeline. Select `Import` from the job-type browser on the left, and fill in the following parameters on the Movies/mics tab:

Import raw movies/micrographs?
: Yes

Raw input files:
: Movies/*.tiff

Are these multi-frame movies?
: Yes

(Set this to `No` if these are single-frame micrographs)

Optics group name:
: opticsGroup1

(This field can be used to divide the data set into multiple optics groups: separately import each optics group with its own name, and then use the `Join star files` jobtype to combine the groups.

MTF of the detector:
: mtf_k2_200kV.star

Pixel size (Angstrom):
: 0.885

Voltage (kV):
: 200

Spherical aberration (mm):
: 1.4

Amplitude contrast:
: 0.1

Beamtilt in X (mrad):

0

Beamtilt in Y (mrad):

0

The MTF file can be obtained from the Gatan Web site. If you are working offline and cannot obtain the file, you can ignore it. The MTF correction does not change the final resolution (but changes the B factor). You can also apply it in PostProcessing.

On the Others tab, make sure the following is set:

Import other node types?

No

You may provide a meaningful alias (for example: *movies*) for this job in the white field named `Current job: Give_alias_here`. Clicking the Run! button will launch the job. A directory called `Import/job001/` will be created, together with a symbolic link to this directory that is called `Import/movies`. Inside the newly created directory a star file with all the movies is created. Have a look at it using:

```
less Import/job001/movies.star
```

If you had extracted your particles in a different software package, then instead of going through the Preprocessing steps below, you would use the same Import job-type to import particles star file, 3D references, 3D masks, etc. Note that this is NOT the recommended way to run relion, and that the user is responsible for generating correct star files.

4.2.2 Beam-induced motion correction

The Motion correction job-type implements relion's own (CPU-based) implementation of the UCSF motioncor2 program for convenient whole-frame movie alignment, as well as a wrapper to the (GPU-based) motioncor2 program itself [ZPA+17]. Besides executing the calculations on the CPU/GPU, there are three other differences between the two implementations:

- Bayesian polishing (for per-particle motion-correction; see *this section*) can only read local motion tracks from our own implementation;
- The motioncor2 program performs outlier-pixel detection on-the-fly, and this information is not conveyed to Bayesian polishing, which may result in unexpectedly bad particles after polishing;
- Our own implementation can write out the sum of power spectra over several movie frames, which can be passed directly into ctffind 4.1 for faster CTF-estimation.

For these three reasons, we now favour running our own implementation.

On the I/O tab set:

Input movies STAR file:

Import/job001/movies.star

(Note that the Browse button will only list movie star files.)

First frame for corrected sum:

1

Last frame for corrected sum:

-1

(This will result in using all movie frames.)

Dose per frame (e/A2)

1.277

Pre-exposure (e/A2)

0

EER fractionation

32

(This option will be ignored for TIFF files.)

Write output in float16?

Yes

(This will save a factor of 2 in disk space compared to the default of writing in float32. Note that RELION and CCPEM will read float16 images, but other programs may not (yet) do so. For example, Gctf will not work with float16 images. Also note that this option does not work with UCSF MotionCor2. For CTF estimation, use CTFFIND-4.1 with pre-calculated power spectra, by activating the 'Save sum of power spectra' option below.)

Do dose-weighting?

Yes

Save non-dose-weighted as well?

No

(In some cases non-dose-weighted micrographs give better CTF estimates. To save disk space, we're not using this option here as the data are very good anyway.)

Save sum of power spectra?

Yes

Sum of power spectra every e/A2:

4

(This seems to be a good value according to measurements by Greg McMullan and Richard Henderson.)

Fill in the Motion tab as follows:

Bfactor:

150

(use larger values for super-resolution movies)

Number of patches X,Y

5 5

Group frames:

1

Binning factor:

1

(we often use 2 for super-resolution movies)

Gain-reference image:

Movies/gain.mrc

(This can be used to provide a gain-reference file for on-the-fly gain-reference correction. This is necessary in this case, as these movies are not yet gain-corrected.)

Gain rotation:

No rotation (0)

Gain flip:

No flipping (0)

Defect file:

(This can be used to mask away broken pixels on the detector. Formats supported in our own implementation and in UCSF motioncor2 are either a text file in UCSF motioncor2 format (each line contains four numbers: x, y, width and height of a defect region); or a defect map (an image in MRC or TIFF format, where 0=good and 1=bad pixels). The coordinate system is the same as the input movie before application of binning, rotation and/or flipping. **Note that defect text files produced by SerialEM are NOT supported!** However, one can convert a SerialEM-style defect file into a defect map using imod.)

Use RELION's own implementation?

Yes

(this reduces the requirement to install the UCSF implementation. If you have the UCSF program installed anyway, you could also use that one. In that case, you also need to fill in the options below.)

Fill in the Running tab as follows:

Number of MPI procs:

1

(Assuming you're running this tutorial on a local computer)

Number of threads:

12

(As these movies are 24 frames, each thread will do two movie frames)

Submit to queue?

No

(Again, assuming you're running this tutorial on a local computer)

Executing this program takes approximately 5 minutes when using 12 threads on a reasonably modern machine. Note that our own implementation of the motioncor2 algorithm does not use a GPU. This program is multi-threaded. As each thread will work independently on a movie frame, it is optimal to use a number of threads such that the number of movie frames divided by the number threads is an integer number. As these movies have 24 frames, using 12 threads will result in 2 frames being processed by each thread. You can look at the estimated beam-induced shifts, and their statistics over the entire data set, by selecting the `out: logfile.pdf` from the Display: button below the run buttons, or you can look at the summed micrographs by selecting *out: corrected_micrographs.star*. Depending on the size of your screen, you should probably downscale the micrographs (`Scale: 0.3`) and use `Sigma contrast: 3` and few columns (something like `Number of columns: 3`) for convenient visualisation. Note that you cannot select any micrographs from this display. If you want to exclude micrographs at this point (which we will not do, because they are all fine), you could use the Subset selection job-type.

4.2.3 CTF estimation

Next, we will estimate the CTF parameters for each corrected micrograph. You can use the CTF estimation job-type as a wrapper to Alexis Rohou and Niko Grigorieff's ctffind 4.1 to execute efficiently on the CPU. We now prefer ctffind 4.1, as it is the only open-source option, and because it allows reading in the movie-averaged power spectra calculation by relion's own implementation of the motioncor2 algorithm. Support for GCTF was dropped in RELION 5.0. Fill in the settings as follows:

On the I/O :

Input micrographs STAR file:

Motioncorr/job002/corrected_micrographs.star

(You can again use the Browse button to select the *corrected_micrographs.star* file of the Motion correction job.)

Use micrograph without dose-weighting?

No

(These may have better Thon rings than the dose-weighted ones, but we decided in the previous step not to write these out)

Estimate phase shifts?

No

(This is only useful for phase-plate data)

Amount of astigmatism (A):

100

(Assuming your scope was reasonably well aligned, this value will be suitable for many data sets.)

On the CTFFIND-4.1 tab, set:

Use CTFFIND-4.1?

Yes

CTFFIND-4.1 executable:

/wherever/it/is/ctffind.exe

Use power spectra from MotionCorr job?

Yes

(We can use these, as we told relion's own implementation of the motioncor2 algorithm to write these out in the previous section.)

Use exhaustive search?

No

(In difficult cases, the slower exhaustive searches may yield better results. For these data, this is not necessary.)

Estimate CTF on window size (pix)

-1

(If a positive value is given, a squared window of this size at the center of the micrograph will be used to estimate the CTF. This may be useful to exclude parts of the micrograph that are unsuitable for CTF estimation, e.g. the labels at the edge of photographic film.)

FFT box size (pix):

512

Minimum resolution (A):

30

Maximum resolution (A):

5

Minimum defocus cvalue (A):

5000

Maximum defocus cvalue (A):

50000

Defocus step size (A):

500

On the Running tab, use six MPI processes to process the 24 micrographs in parallel. This took less than 10 seconds on our machine. Once the job finishes there are additional files for each micrograph inside the output `CtfFind/job003/Movies` directory: the `.ctf` file contains an image in *MRC* format with the computed power spectrum and the fitted CTF model; the `.log` file contains the output from ctffind; the *.com* file contains the script that was used to launch ctffind.

You can visualise all the Thon-ring images using the Display button, selecting `out: micrographs_ctf.star`. The zeros between the Thon rings in the experimental images should coincide with the ones in the model. Note that you can sort the display in order of defocus, maximum resolution, figure-of-merit, etc. The `logfile.pdf` file contains plots of useful parameters, such as defocus, astigmatism, estimated resolution, etc for all micrographs, and histograms of these values over the entire data set. Analysing these plots may be useful to spot problems in your data acquisition.

If you see CTF models that are not a satisfactory fit to the experimental Thon rings, you can delete the `.log` files for those micrographs, select the `CtfFind/job003` entry from the Finished jobs list, alter the parameters in the parameter-panel, and then re-run the job by clicking the Continue! button. Only those micrographs for which a `.log` file does not exist will be re-processed. You can do this until all CTF models are satisfactory. If this is not possible, or if you decide to discard micrographs because they have unsatisfactory Thon rins, you can use the Subset selection job-type to do this.

4.2.4 Manual particle picking

The next job-type Manual picking may be used to manually select particle coordinates in the (averaged) micrographs. We like to manually select at least several micrographs in order to get familiar with our data. Often, the manually selected particles to calculate reference-free 2D class averages, which will then be used as templates for automated particle picking of the entire data set. However, as of release 3.0, relion also contains a reference-free auto-picking procedure based on a Laplacian-of-Gaussian (LoG) filter. In many cases, this procedure provides reasonable starting coordinates, so that the Manual picking step may be skipped. The pre-shipped *Schemes* for on-the-fly processing in the `relion_it.py` script make use of this functionality to perform fully automated on-the-fly processing. In this tutorial, we will just launch a Manual picking job for illustrative purposes, and then proceed with LoG-based Auto-picking to generate the first set of particles.

Picking particles manually is a personal experience! If you don't like to pick particles in relion, we also support coordinate file formats for Jude Short's ximdisp [Smi99] (with any extension); for xmipp-2.4 [SNRS+08] (with any extension); and for Steven Ludtke's e2boxer.py [TPB+07] (with a `.box` extension). If you use any of these, make sure to save the coordinate files as a text file in the same directory as from where you imported the micrographs (or movies), and with the same micrograph rootname, but a different (suffix+) extension as the micrograph, e.g. `Movies/006.box` or `Movies/006_pick.star` for micrograph `Movies/006.mrc`. You should then use the Import job-type and set `Node type:` to `2D/3D particle coordinates`. Make sure that the `Input Files:` field contains a linux wildcard, followed by the coordinate-file suffix, e.g. for the examples above you **have to** give `Movies/*.box` or `Movies/*_pick.star`, respectively.

On the I/O tab of the Manual picking job-type, use the Browse button to select the `micrographs_ctf.star` file that was created in `CtfFind/job003`, ignore the Colors tab, and fill in the Display tab as follows:

Particle diameter (A):

200

(This merely controls the diameter of the circle that is displayed on the micrograph.)

Scale for micrographs:

0.25

(But this depends on your screen size)

Sigma contrast:

3

(Micrographs are often best display with `sigma-contrast`, i.e. black will be 3 standard deviation below the mean and white will be 3 standard deviations above the mean. The grey-scale is always linear from black to white. See the DisplayImages entry on the RELION wiki for more details)

White value:

0

(Use this to manually set which value will be white. For this to work, `Sigma contrast` should be set to 0)

Black value:

0

(Use this to manually set which value will be black. For this to work, `Sigma contrast` should be set to 0)

Lowpass filter (A):

-1

(Playing with this may help you to see particles better in very noisy micrographs)

Highpass filter (A):

-1

(This is sometimes useful to remove dark->light gradients over the entire micrograph)

Pixel size:

0.885

(This is needed to calculate the particle diameter, and the low- and high-pass filters)

OR use Topaz denoising?:

Yes

(This has been a feature since relion-4.0 and will make a system call to topaz)

Note

As of relion-5.0, Topaz comes pre-installed in the relion-5 conda environment, which should be picked up automatically by the GUI.

Run the job by clicking the Run! button and click on a few particles if you want to. However, as we will use the LoG-based autopicking in the next section, **you do not need to pick any if you don't want to**. If you were going to use manually picked particles for an initial 2D classification job, then you would need approximately 500-1,000 particles in order to calculate reasonable class averages. Left-mouse click for picking, middle-mouse click for deleting a picked particle, right-mouse click for a pop-up menu in which **you will need to save the coordinates!**. Note that you can always come back to pick more from where you left it (provided you saved the star files with the coordinates throught the pop-up menu), by selecting `ManualPick/job004` from the Finished jobs and clicking the Continue! button.

4.3 Particle picking

4.3.1 Select a subset of the micrographs

We will now use a template-free auto-picking procedure based on a Laplacian-of-Gaussian (LoG) filter to select an initial set of particles. These particles will then be used in a 2D classification job to generate 2D class averages. The tutorial up until relion-3.1 would suggest to use the resulting class averages as templates for a second, reference-based Auto-picking job. Since relion-4.0, there is also an integrated topaz wrapper in the Auto-picking job, which will be used instead. In addition, we will use a new automated 2D class average selection procedure to select particles that contribute to good classes without any user interaction. The selected particles will then be used to train the neural network in topaz to specifically pick particles for this data set. Alternatively, one could run topaz picking with their default neural network straight away. In that case, one could skip the jobs of LoG-picking, 2D classification, automated 2D class selection and re-training of the topaz network below, and proceed straight to the last Auto-picking job on this page.

One typically trains the topaz neural network on a relatively small subset of the micrographs. In order to select a subset of the micrographs, go to the Subset selection job, and on the I/O tab leave everything empty, except:

OR select from micrographs.star:
CtfFind/job003/micrographs_ctf.star

Then, on the Subsets tab, set:

OR split into subsets?
Yes

Randomise order before making subsets?
No

Subset size:
10

OR number of subsets:
-1

Then press Run!, which will create star files with subsets of 10 micrographs in the output directory. We will only use the first one `Select/job005/micrographs_split1.star`.

Note that if one would have preferred a more user-interactive way of selecting micrographs for training, one could have also selected certain micrographs in the GUI of the previous Manual picking job, to then save a file called `micrographs_selected.star` inside that output directory.

4.3.2 LoG-based auto-picking

Now, proceed to the Auto-picking job, and on the I/O tab set:

Input micrographs for autopick:
Select/job005/micrographs_split1.star

Pixel size in micrographs (A)
-1

(The pixel size will be set automatically from the information in the input STAR file.)

Use reference-based template-matching?
No

OR: use Laplacian-of-Gaussian?
Yes

OR: use Topaz?
No

On the Laplacian tab, set:

Min. diameter for loG filter (A)
150

Max. diameter for loG filter (A)
180

(This should correspond to the smallest and largest size of your particless projections in Ångstroms.)

Are the particles white?
No

(They are black.)

Maximum resolution to consider
20

(Just leave the default value here.)

Adjust default threshold
0

(Positive values, i.e. high thresholds, will pick fewer particles, negative values will pick more particles. Useful values are probably in the range [-1,1], but in many cases the default value of zero will do a decent job. The threshold is moved this many standard deviations away from the average.)

Upper threshold
5

(Use this to discard picks with LoG values that are this many standard deviations above the average, e.g. to avoid high contrast contamination like ice and ethane droplets. Good values depend on the contrast of micrographs and may need to be interactively explored; for low contrast micrographs, values of ~ 1.5 may be reasonable, but this value is too low for the high-contrast micrographs in this tutorial.)

Ignore the Topaz, References, autopicking and Helix tabs, and run using a single MPI processor on the Running tab. Perhaps an alias like `LoG` would be meaningful? Using a single processor, these calculations take about 15 seconds on our computer.

You can check the results by clicking the `autopick.star` option from the Display: button. One could manually add/delete particles in the pop-up window that appears at this stage. In addition, one could choose to pick more or fewer particle by running a new job while adjusting the default threshold on the Laplacian tab, and/or the parameters for the stddev and avg of the noise on the autopicking tab. However, at this stage we are merely after a more-or-less OK initial set of particles for the generation of templates for a second auto-picking job, so in many cases this is probably not necessary.

4.3.3 Particle extraction

Once you have a coordinate file for every micrograph that you want to pick particles from, you can extract the corresponding particles and gather all required metadata through the Particle extraction job-type. On the corresponding I/O tab, set:

micrograph STAR file:
CtfFind/job003/micrographs_ctf.star

(Use the *Browse* button to select this file. You could also chose the selected micrographs file from the ManualPick directory. It doesn't matter as there are only coordinate files for the three selected micrographs anyway. Warning that coordinates files are missing for the rest of the micrographs will appear in red in the bottom window of the GUI.)

Input coordinates:

AutoPick/job006/autopick.star

(Use the *Browse* button to select this file)

OR re-extract refined particles?

No

(This option allows you to use a `_data.star` file from a 2D cassification, 3D classification or 3D auto-refine job for re-extraction of only those particles in the star file. This may for example be useful if you had previously down-scaled your particles upon extraction, and after initial classifications you now want to perform refinements with the original-scaled particles. As of relion-3.0, this functionality has been extended with an option to 're-center refined coordinates' on a user-specified X,Y,Z-coordinate in the 3D reference used for a 3D classification or 3D auto-refine job. This will adjust the X and Y origin coordinates of all particles, such that a reconstruction of the newly extracted particles will be centered on that X,Y,Z position. This is useful for focused refinements.)

Write output in float16?

Yes

(If set to Yes, this program will write output images in float16 MRC format. This will save a factor of two in disk space compared to the default of writing in float32. Note that RELION and CCPEM will read float16 images, but other programs may not (yet) do so.)

On the extract tab you set the parameters for the actual particle extraction:

Particle box size (pix):

256

(This should always be an even number!)

Invert contrast?

Yes

(This makes white instead of black particles.)

Normalize particles?

Yes

(We always normalize.)

Diameter background circle (pix):

200

(Particles will be normalized to a mean value of zero and a standard-deviation of one for all pixels in the background area.The background area is defined as all pixels outside a circle with this given diameter in pixels (before rescaling). When specifying a negative value, a default value of 75% of the Particle box size will be used.)

Stddev for white dust removal:

-1

Stddev for black dust removal:

-1

(We only remove very white or black outlier pixels if we actually see them in the data. In such cases we would use stddev values of 5 or so. In this data set there are no outlier pixels, so we don't correct for them, and leave the default values at -1 (i.e. don't do anything).

Rescale particles?

Yes

(Down-scaling particles will speed up computations. Therefore, we often down-scale particles in the initial stages of processing, in order to speed up the initial classifications of suitable particles. Once our reconstructions get close to the Nyquist frequency, we then re-extract the particles without down-scaling.)

Re-scaled sized (pixels)?

64

Use autopick FOM threshold?

No

(This option allows to only extract those particles with the highest figure-of-merits from the autopicking procedure. We will use this later on to extract particles picked by topaz.)

As we will later on also use the same job-type to extract all template-based auto-picked particles, it may be a good idea to give this job an alias like `LoG`. Ignore the Helix tab, and run using a single MPI processor.

Your particles will be extracted into MRC stacks (which always have an `.mrcs` extension in relion) in a new directory called `Extract/job007/Movies/`. It's always a good idea to quickly check that all has gone OK by visualising your extracted particles selecting `out:  particles.star` from the Display: button. Right-mouse clicking in the display window may be used for example to select all particles (*Invert selection*) and calculating the average of all unaligned particles (*Show average of selection*).

4.3.4 2D class averaging to select good particles

To calculate templates for the subsequent auto-picking of all micrographs, we will use the 2D classification job-type.

On the I/O tab, set:

Input images STAR file

Extract/job007/particles.star

Continue from here

(Note that any 2D classification, 3D initial model, 3D classification, or 3D auto-refine jobs may be continued in case it stalls, by providing the *_optimiser.star* file from the last completed iteration.)

On the CTF tab set:

Do CTF-correction?

Yes

(We will perform full phase+amplitude correction inside the Bayesian framework)

Ignore CTFs until first peak?

No

(This option is occasionally useful, when amplitude correction gives spuriously strong low-resolution components, and all particles get classified together in very few, fuzzy classes.)

On the Optimisation tab, set:

Number of classes:

`50`

(For cryo-EM data we like to use on average at least approximately 100 particles per class. For negative stain one may use fewer, e.g. 20-50 particles per class. However, with this small number of particles, we have observed a better separation into different classes by relaxing these numbers. Possibly, always having a minimum of 50 classes is not a bad idea.)

Regularisation parameter T:

`2`

(For the exact definition of T, please refer to [Sch12a]. For cryo-EM 2D classification we typically use values of T=2-3, and for 3D classification values of 3-4. For negative stain sometimes slightly lower values are better. In general, if your class averages appear very noisy, then lower T; if your class averages remain too-low resolution, then increase T. The main thing is to be aware of overfitting high-resolution noise.)

Use EM algorithm?

`Yes`

(This is the standard Expectation Maximisation algorithm in relion.)

Number of iterations:

`25`

(For the default EM-algorithm, one normally doesn't change the default of 25 iterations)

Use VDAM algorithm?

`No`

(This is gradient-descent-like algorithm that was introduced in relion-4.0. It runs much faster than the standard EM-algorithm for large data sets, and has been observed to yield better class average images in many cases. It is however slower for data sets with only a few thousand particles, which is the main reason we are not using it here.)

Mask diameter (A):

`200`

(This mask will be applied to all 2D class averages. It will also be used to remove solvent noise and neighbouring particles in the corner of the particle images. On one hand, you want to keep the diameter small, as too much noisy solvent and neighbouring particles may interfere with alignment. On the other hand, you want to make sure the diameter is larger than the longest dimension of your particles, as you do not want to clip off any signal from the class averages.)

Mask individual particles with zeros?

`Yes`

Limit resolution E-step to (A):

`-1`

(If a positive value is given, then no frequencies beyond this value will be included in the alignment. This can also be useful to prevent overfitting. Here we don't really need it, but it could have been set to 10-15A anyway. Difficult classifications, i.e. with very noisy data, often benefit from limiting the resolution.)

Center class averages?

`Yes`

(This will re-center all class average images every iteration based on their center of mass. This is useful for their subsequent use in template-based auto-picking, but also for the automated 2D class average image selection in the next section.)

On the Sampling tab we hardly ever change the defaults. Six degrees angular sampling is enough for most projects, although some large icosahedral viruses or some filamentous structures may benefit from finer angular samplings.

Ignore the Helix tab, and on the Compute tab, set:

Use parallel disc I/O?
: Yes

(This way, all MPI slaves will read their own particles from disc. Use this option if you have a fast (parallel?) file system. Note that non-parallel file systems may not be able to handle parallel access from multiple MPI nodes. In such cases one could set this option to No. In that case, only the master MPI node will read in the particles and send them through the network to the MPI slaves.)

Number of pooled particles:
: 30

(Particles are processed in individual batches by MPI slaves. During each batch, a stack of particle images is only opened and closed once to improve disk access times. All particle images of a single batch are read into memory together. The size of these batches is at least one particle per thread used. The `nr_pooled_particles` parameter controls how many particles are read together for each thread. If it is set to 30 and one uses 8 threads, batches of 30x8=240 particles will be read together. This may improve performance on systems where disk access, and particularly metadata handling of disk access, is a problem. Typically, when using GPUs we use values of 10-30; when using only CPUs we use much smaller values, like 3. This option has a modest cost of increased RAM usage.)

Pre-read all particles into RAM?
: Yes

(If set to Yes, all particle images will be read into computer memory, which will greatly speed up calculations on systems with slow disk access. *However, one should of course be careful with the amount of RAM available.* Because particles are read in double-precision, it will take (N × box_size × box_size × 4 / (1024 × 1024 × 1024)) Giga-bytes to read N particles into RAM. If parallel disc I/O is set to Yes, then all MPI slaves will read in all particles. If parallel disc I/O is set to No, then only the master reads all particles into RAM and sends those particles through the network to the MPI slaves during the refinement iterations.)

Copy particles to scratch directory?

(This is useful if you don't have enough RAM to pre-read all particles, but you do have a fast (SSD?) scratch disk on your computer. In that case, specify the name of the scratch disk where you can make a temporary directory, e.g. `/ssd`)

Combine iterations through disc?
: No

(This way all MPI nodes combine their data at the end of each iteration through the network. If the network is your main bottle-neck or somehow causing problems, you can set this option to No. In that case, all MPI nodes will write/read their data to disc.)

Use GPU acceleration?
: Yes

(If you have a suitable GPU, this job will go much faster.)

Which GPUs to use:
: 0:1

(This will depend on the available GPUs on your system! If you leave this empty, the program will try to figure out which GPUs to use, but you can explicitly tell it which GPU IDs , e.g. 0 or 1, to use. If you use multiple MPI-processors, you can run each MPI process on a specified GPU. Our machine has 2 GPUs, and we will use on MPI process on each GPU in this example. GPU IDs for different MPI processes are separated by colons, e.g. 0:1:0:1 will run MPI process 0 and 2 on GPU 0, and MPI process 1 and 3 will run on GPU 1. GPU IDs for different threads are separated by commas, so when using a single MPI process one could still use multiple GPUs, e.g. 0,1,2,3. Combinations of colons and commas are also possible.)

On the Running tab, specify:

Number of MPI procs

3

(Note that *when using the EM-algorithm*, 2D classification, 3D classification, 3D initial model and 3D auto-refine use one MPI process as a master, which does not do any calculations itself, but sends jobs to the other MPI processors. Therefore, we often run the EM-algorithm using a single worker MPI process on each of the available GPUs, so we specify 3 here to include the master and one workers on each of the two GPUs.)

Number of threads

8

(Threads offer the advantage of more efficient RAM usage, whereas MPI parallelization may scale better than threads for iterations with many particles. Often, you may want to adjust the number of threads to make full use of all the CPU cores on your computer. The total number of requested CPUs, or cores, will be the product of the number of MPI processors and the number of threads.)

Because we will run more 2D classification jobs, it may again be a good idea to use a meaningful alias, for example *LoG*. You can look at the resulting class averages using the Display: button to select *out: run_it025_optimiser.star* from. On the pop-up window, you may want to choose to look at the class averages in a specific order, e.g. based on *rlnClassDistribution* (in reverse order, i.e. from high-to-low instead of the default low-to-high) or on *rlnAccuracyRotations*.

4.3.5 Selecting good 2D classes for Topaz training

Selection of suitable class average images is done in the Subset selection job-type. Up until relion-3.1, this step was always done interactively by the user, who would select good class averages by clicking on them in the GUI. As of relion-4.0, there is also an automated procedure, based on a neural network that was trained on thousands of 2D class averages. This option will be used below.

On the I/O tab, remove the *micrographs.star* file entry from before, and set:

Select classes from job:

Class2D/job008/run_it025_optimiser.star

On the Class options tab, give:

Automatically select 2D classes?

Yes

Minimum threshold for auto-selection

0.21

(The score ranges from 0 for absolute rubbish class average images to 1 for gorgeous ones. We are using a relatively low value here, because we only have a few particles, so the 2D class averages will probably not look very good. On your own data sets, you will probably want to run the program once, sort your class averages on their predicted score and decide what a good value is for those class averages; also see below.)

Select at least this many particles

-1

(If this is value is positive, then even if they have scores below the minimum threshold, select at least this many particles with the best scores.)

OR: select at least this many classes

-1

(If this is value is positive, then even if they have scores below the minimum threshold, select at least this many classes with the best scores.)

Re-center the class averages?

No

(This option allows automated centering of the 2D class averages, but we already did that during 2D class averaging. In particular when using class average images for auto-picking it is important that the are centered, as otherwise all your particle coordinates will become systematically off-centered.)

Regroup the particles?

No

(This option is useful when there are very few (selected) particles on individual micrographs, in which case the estimation of noise power spectra and scale factors become unstable. By default, the latter are calculated independently per micrograph. This option allows to grouping particles from multiple micrographs together in these calcutaions. relion will warn you (in classification or auto-refine runs) when your groups become too small.)

On the Subsets tab, make sure you switch to `No` again the following option:

:OR: split into subsets? No

Ignore the other tabs, and run the job. You can visualise the results of the automated class selection by selecting `rank_optimiser.star` from the Display: button, and sort the images on `rlnClassScore`, in reverse order. Do you want to adjust the threshold for auto-selection?

4.3.6 Re-training the TOPAZ neural network

In older versions of the relion tutorial, one would now use the selected 2D class averages as templates for reference-based auto-picking. Instead, the new wrapper to topaz will be used to first re-train the neural network in topaz and then to pick the entire data set using the retrained network. Note that for this data set one could also have foregone re-training of topaz and just use the pretrained network it comes with. This tutorial is merely showing you the re-training option as it may be relevant for your own data.

On the I/O tab of the Auto-picking job-type, set:

Input micrographs for autopick:

Select/job005/micrographs_split1.star

Pixel size in micrographs (A)

-1

Use reference-based template-matching?

No

OR: use Laplacian-of-Gaussian?

No

OR: use Topaz?
Yes

On the Topaz tab, set:

Particle diameter (A)
180

Perform topaz picking?
No

Perform topaz training?
Yes

Nr of particles per micrograph
300

Input picked coordinates for training
(This option can be used to train on manually selected particles from a Manual picking job. We will use the automatically selected part

OR train on a set of particles?
Yes

Particles STAR file for training
Select/job009/particles.star

Additional topaz arguments

On the autopicking tab, you can ignore everything except the below:

Use GPU acceleration?
Yes

(Topaz picking and training require one GPU)

Which GPUs to use:
0

Ignore the other tabs, and run using a single MPI processor on the Running tab. On our computer, with a Titan V GPU, this step took 10 minutes. Perhaps a good time for a quick cup of coffee?

4.3.7 Pick all micrographs with the re-trained TOPAZ neural network

On the I/O tab of a new Auto-picking job, set:

Input micrographs for autopick:
CtfFind/job003/micrographs_ctf.star

Pixel size in micrographs (A)
-1

Use reference-based template-matching?
No

OR: use Laplacian-of-Gaussian?
No

OR: use Topaz?
    Yes

On the Topaz tab, set:

Particle diameter (A)
    180

Perform topaz picking?
    Yes

Trained topaz model
    AutoPick/job010/model_epoch10.sav

Perform topaz training?
    No

Nr of particles per micrograph
    300

Additional topaz arguments

On the autopicking tab, you can ignore everything except the below:

Use GPU acceleration?
    Yes

    (Topaz picking and training require one GPU)

Which GPUs to use:
    0

On our computer, running with a single process, this step takes approximately 4 minutes. Note that re-training of topaz is not parallelised and should always be performed with a single MPI process. However, picking with topaz has been parallelised and can be run using multiple MPI processes.

The number of particles from default topaz picking will be relatively high, because no threshold to its figure-of-merit will be applied. The figure-of-merits for all picks are stored in the `rlnAutopickFigureOfMerit` column in the output *STAR* files. A minimum threshold of -3 is probably reasonable in many cases. One can visualise the figure of merits by colouring the picks in the micrographs. For that, change the colouring parameters in the Manual picking job-type.

On the following on the Colors tab, set:

Blue<>red color particles?
    Yes

MetaDataLabel for color:
    rlnAutopickFigureOfMerit

STAR file with color label:

Blue value:
    5

Red value:
    -3

and save the settings to the project directory with the *Save job.star* menu item from the top left *Jobs* menu.

Then, select `autopick.star` from the Display: button of the `Autopick/job011` job to launch the GUI. From the `File` menu at the top left of its main window, one can use `Set FOM threshold` to display only picks with a FOM above the threshold A similar option is also available in the per-micrograph viewer, using the right-mouse button pop-up menu. Picks with a high threshold will be blue; picks with a low threshold will be red.

4.3.8 Particle extraction

Finally, one needs to re-extract the final set of picked coordinates by again using the Particle extraction job-type.

On the corresponding I/O tab, set:

micrograph STAR file:
  CtfFind/job003/micrographs_ctf.star

Input coordinates:
  AutoPick/job011/autopick.star

OR re-extract refined particles?
  No

Leave all the other options as they were before, except for the extract tab, where one sets:

Use autopick FOM threshold?
  Yes

Minimum autopick FOM
  -3

Write output in float16?
  Yes

Running this job will generate the initial particle set for further processing. Using four MPI processors, this job takes a few seconds.

4.4 Reference-free 2D class averaging

We almost always use reference-free 2D class averaging to throw away bad particles. Because bad particles do not average well together, they often go to relatively small classes that yield ugly 2D class averages. Throwing those away then becomes an efficient way of cleaning up your data.

4.4.1 Running the job

Most options will remain the same as explained when we were generating templates for the auto-picking in the previous section, but on the I/O tab of the 2D classification job-type, set:

Input images STAR file:
  Extract/job012/particles.star

and on the Optimisation tab, we used:

Number of classes:
  100

  (because we now have more particles, we can allow more classes than before.)

Regularisation parameter T:
  2

(For the exact definition of T, please refer to [Sch12a]. For cryo-EM 2D classification we typically use values of T=2-3, and for 3D classification values of 3-4. For negative stain sometimes slightly lower values are better. In general, if your class averages appear very noisy, then lower T; if your class averages remain too-low resolution, then increase T. The main thing is to be aware of overfitting high-resolution noise.)

Use EM algorithm?

No

(This is the standard Expectation Maximisation algorithm in relion.)

Use VDAM algorithm?

Yes

(This is gradient-descent-like algorithm that was introduced in relion-4.0. It runs much faster than the standard EM-algorithm for large data sets, and has been observed to yield better class average images in many cases.)

Number of VDAM mini-batches

100

(Number of mini-batches to be processed using the VDAM algorithm. Using 200 has given good results for many data sets. Using 100 will run faster, but sometimes at the expense of some quality loss.)

Mask diameter (A):

200

(This mask will be applied to all 2D class averages. It will also be used to remove solvent noise and neighbouring particles in the corner of the particle images. On one hand, you want to keep the diameter small, as too much noisy solvent and neighbouring particles may interfere with alignment. On the other hand, you want to make sure the diameter is larger than the longest dimension of your particles, as you do not want to clip off any signal from the class averages.)

Mask individual particles with zeros?

Yes

Limit resolution E-step to (A):

-1

(If a positive value is given, then no frequencies beyond this value will be included in the alignment. This can also be useful to prevent overfitting. Here we don't really need it, but it could have been set to 10-15A anyway. Difficult classifications, i.e. with very noisy data, often benefit from limiting the resolution.)

Center class averages?

Yes

(This will re-center all class average images every iteration based on their center of mass. This is useful for their subsequent use in template-based auto-picking, but also for the automated 2D class average image selection in the next section.)

The VDAM algorithm cannot be run with MPI parallelisation. Therefore, we ran with the following options on the Compute tab:

Use GPU acceleration?

Yes

Which GPUs to use:

0,1,2,3

(Our machine has 4 GPUs, so we're using them all.)

and on the Running tab, we used:

Number of MPI procs:

1

Number of threads:

12

The job then took just over 3 minutes.

4.4.2 Selecting good particles for further processing

After the 2D classification job has finished, we can launch another Subset selection job (`Select/job014`).

On the I/O tab, set:

Select classes from job:

Class2D/job013/run_it100_optimiser.star

On the Class options tab, set:

Automatically select 2D classes?

Yes

Minimum threshold for auto-selection

0.1

(You may need to run the program twice, perhaps by overwriting the previous run (using the *Overwrite* option from the Jobactions: button, to select the appropriate threshold. Alternatively, you can also selected the classes interactively, while sorting on rlnClassDistribution.)

We got 4598 particles from 34 selected classes.

Note that this procedure of 2D classification and Subset selection may be repeated several times. But if you do so, be careful not to throw away your minority views!

4.4.3 Analysing the Class2D results in more detail

Note

If you are in a hurry to get through this tutorial, you can skip this sub-section. It contains more detailed information for the interested reader.

For every iteration of 2D or 3D classification relion performs, it writes out a set of files. For the last iteration of our 2D class averaging calculation these are:

- `Class2D/job013/run_it100_classes.mrcs` is the MRC stack with the resulting class averages. These are the images that will be displayed in the relion GUI when you select the *_optimiser.star* file from the Display: button on the main GUI. Note that relion performs full CTF correction (if selected on the GUI), so your class averages are probably white on a black background. If the data is good, often they are very much like projections of a low-pass filtered atomic model. The quality of your 2D class averages are a very good indication of how good your 3D map will become. We like to see internal structure within projections of protein domains, and the solvent area around you particles should ideally be flat. Radially extending streaks in the solvent region are a typical sign of overfitting. If this happens, you could try to limit the resolution in the E-step of the 2D classification algorithm.

- `Class2D/job013/run_it100_model.star` contains the model parameters that are refined besides the actual class averages (i.e. the distribution of the images over the classes, the spherical average of the signal-to-noise ratios in the reconstructed structures, the noise spectra of all groups, etc. Have a look at this file using the `less` command. In particular, check the distribution of particles over each class in the table `data_model_classes`. If you compare this with the class averages themselves, you will see that particles with few classes are low-resolution, while classes with many particles are high-resolution. This is an important feature of the Bayesian approach, as averaging over fewer particles will naturally lead to lower signal-to-noise ratios in the average. The estimated spectral signal-to-noise ratios for each class are stored in the `data_model_class_N` tables, where `N` is the number of each class. The table `data_model_groups` stores a refined intensity scale-factor for each group: groups with values higher than one have a stronger signal than the average, relatively low-signal groups have values lower than one. These values are often correlated with the defocus, but also depend on accumulated contamination and ice thickness. For each different optics group, the estimated noise spectra are stored in tables called `data_model_optics_group_N`.
- `Class2D/job013/run_it100_data.star` contains all metadata related to the individual particles. Besides the information in the input `particles.star` file, there is now additional information about the optimal orientations, the optimal class assignment, the contribution to the log-likelihood, etc. Note that this file can be used again as input for a new refinement, as the star file format remains the same.
- `Class2D/job013/run_it100_optimiser.star` contains some general information about the refinement process that is necessary for restarting an unfinished run. For example, if you think the process did not converge yet after 25 iterations (you could compare the class averages from iterations 24 and 25 to assess that), you could select this job in the Finished jobs panel, and on the I/O tab select this file for `Continue from here`, and then set `Number of iterations: 40` on the Optimisation tab. The job will then restart at iteration 26 and run until iteration 40. You might also choose to use a finer angular or translational sampling rate on the Sampling tab. Another useful feature of the optimiser.star files is that it's first line contains a comment with the exact command line argument that was given to this run. As of release-4.0, relion also uses the optimiser.star files as input nodes for different types of subsequent jobs. For example, it replaces the model.star input nodes for Subset selection jobs.
- `Class2D/job013/run_it100_sampling.star` contains information about the employed sampling rates. This file is also necessary for restarting.

4.4.4 Making groups

Note

If you are in a hurry to get through this tutorial, you can skip this sub-section. It contains more detailed information for the interested reader.

relion groups particles together for the estimation of a single-number intensity scale factor that describes differences in overall signal-to-noise ratios between different parts of the data, e.g. due to ice thickness, defocus or contamination.

The default behaviour is to treat all particles from each micrograph as a separate group. This behaviour is fine if you have many particles per micrograph, but when you are using a high magnification, your sample is very diluted, or your final selection contains only a few particles per micrograph, then the estimation of the intensity scale factor (and the noise spectra) may become unstable. We generally recommend to have at least 10-20 particles in each group, but do note that initial numbers of particles per group may become much smaller after 2D and 3D classification.

In cases with few particles per micrograph we recommend to group particles from multiple micrographs together. For this purpose, the GUI implements a convenient functionality in the Subset selection job-type: when selecting a `_optimiser.star` file on the I/O tab, one can use `Regroup particles? Yes` and `Approximate nr of groups: 5` on the Class options tab to re-group all particles into 5 groups. (The actual number may vary somewhat from the input value, hence the *Approximate* on the input field.) This way, complicated grouping procedures in previous releases of relion may be avoided. As the micrographs in this tutorial do contain sufficient particles, we will not use this procedure now.

Please note that the groups in relion are very different from defocus groups that are sometimes used in other programs. relion will always use per-particle (anisotropic) CTF correction, irrespective of the groups used.

4.5 De novo 3D model generation

relion-4.0 uses a gradient-driven algorithm to generate a *de novo* 3D initial reference from the 2D particles. As of release 4.0, this algorithm is different from the SGD algorithm in the CryoSPARC program [PRFB17]. Provided you have a reasonable distribution of viewing directions, and your data were good enough to yield detailed class averages in 2D classification, this algorithm is likely to yield a suitable, low-resolution model that can subsequently be used for 3D classification or 3D auto-refine.

4.5.1 Running the job

Select the `Select/job014/particles.star` file on the I/O tab of the 3D initial reference jobtype. Everything is aready in order on the CTF. Fill in the Optimisation tab as follows (leave the defaults for the angular and offset sampling):

Number of VDAM mini-batches

100

(The VDAM algorithm will loop over mini-batches, which contain only hundreds to thousands of particles each.)

Regularisation parameter T

4

(The default is 4 for 3D runs.)

Number of classes

1

(Sometimes, using more than one class may help in providing a ‘sink’ for sub-optimal particles that may still exist in the data set. In this case, which is quite homogeneous, a single class should work just fine.)

Mask diameter (A)

200

(The same as before).

Flatten and enforce non-negative solvent

Yes

Symmetry

D2

Run in C1 and apply symmetry later?

Yes

(The actual refinement will be run in C1, which has been observed to converge better than running VDAM in higher symmetry groups. After the refinement, the `relion_align_symmetry` program is run automatically to detect the symmetry axes and the symmetry will be applied.)

On the Compute tab, set:

Use parallel disc I/O?
Yes

Number of pooled particles
30

Pre-read all particles into RAM?
Yes

(Again, this is only possible here because the data set is small. For your own data, you would like write the particles to a scratch disk instead, see below.)

Copy particles to scratch directory

Combine iterations through disc?
No

Use GPU acceleration?
Yes

Which GPUs to use
0,1,2,3

On the Running tab, set:

Number of MPI procs
1

(Remember that the gradient-driven algorithm cannot be run with multiple MPI processes.)

Number of threads
12

Using the settings above, this job took 2 minutes on our system.

4.5.2 Analysing the results

You can look at the output map in 2D slices through the 3D map by selecting `InitialModel/job015/initial_model.mrc` from the Display: button. We like looking at 3D maps in 2D slices, as it is a good way to assess artifacts, for example streaks in the solvent region. You may also want to look at your map in 3D, with a 3D viewer like UCSF chimera.

4.6 Unsupervised 3D classification

All data sets are heterogeneous! The question is how much you are willing to tolerate. relion's 3D multi-reference refinement procedure provides a powerful unsupervised 3D classification approach.

4.6.1 Running the job

Unsupervised 3D classifcation may be run from the 3D classification job-type. On the I/O tab set:

Input images STAR file:
Select/job014/particles.star

Reference map:
InitialModel/job015/initial_model.mrc

Reference mask (optional):

(Leave this empty. This is the place where we for example provided large/small-subunit masks for our focussed ribosome refinements. If left empty, a spherical mask with the particle diameter given on the Optimisation tab will be used. This introduces the least bias into the classification.)

On the Reference tab set:

Ref. map is on absolute greyscale:

Yes

(Given that this map was reconstructed from this data set, it is already on the correct greyscale. Any map that is not reconstructed from the same data in relion should probably be considered as not being on the correct greyscale.)

Initial low-pass filter (A):

50

(One should NOT use high-resolution starting models as they may introduce bias into the refinement process. As also explained in [Sch10], one should filter the initial map as much as one can. For ribosome we often use 70 Å, for smaller particles we typically use values of 40-60 Å.)

Symmetry:

C1

(Although we know that this sample has D2 symmetry, **it is often a good idea to perform an initial classification without any symmetry**, so bad particles, which are not symmetric, can get separated from proper ones, and the symmetry can be verified in the reconstructed maps.)

On the CTF tab set:

Do CTF correction?

Yes

Ignore CTFs until first peak?

No

(Only use this option if you also did so in the 2D classification job that you used to create the references.)

On the Optimisation tab set:

Number of classes:

4

(Using more classes will divide the data set into more subsets, potentially describing more variability. The computational costs scales linearly with the number of classes, both in terms of CPU time and required computer memory.)

Regularisation parameter T:

4

For the exact definition of T, please refer to [Sch12a]. For cryo-EM 2D classification we typically use values of T=1-2, and for 3D classification values of 2-4. For negative stain sometimes slightly lower values are better. In general, if your class averages appear noisy, then lower T; if your class averages remain too low resolution, then increase T. The main thing is to be aware of overfitting high-resolution noise.

Number of iterations:

25

(We typically do not change this.)

Use fast subsets (for large data sets)?:

No

(This option will significantly speed up calculations for data sets of hundreds of thousands pf particles. However, sometimes performance is affected too. For small data sets like this one, we do not recommend using this option.)

Mask diameter (A):

200

(Just use the same value as we did before in the 2D classification job-type.)

Mask individual particles with zeros?

Yes

Limit resolution E-step to (A):

-1

(If a positive value is given, then no frequencies beyond this value will be included in the alignment. This can also be useful to prevent overfitting. Here we don't really need it, but it could have been set to 10-15A anyway.)

Use Blush regularisation?

Yes

(Blush regularisation is a new feature in relion-5.0. It switches from the default smoothness prior in Fourier space to a new prior in the form of a denoising convolutional neural network that was trained on many EMDB half-maps. It is particularly powerful when the signal-to-noise ratios in the images is low, e.g. because of small complexes, and standard 3D classification, 3D auto-refinement (see next section of this tutorial), or multi-body refinement suffers from overfitting. This tutorial data set has relatively high signal-to-noise ratios, so using Blush regularisation is probably not necessary. We used it here anyway to make you aware of the option. To run this option, the relion-5 conda environment should be installed and you need CUDA-enabled GPUs to run `pytorch` on. If you cannot run this, don't worry and just set the option to `No`.)

On the Sampling tab one usually does not need to change anything (only for large and highly symmetric particles, like icosahedral viruses, does one typically use a 3.7 degree angular sampling at this point). Ignore the Helix tab, and on the Compute tab set:

Use parallel disc I/O?

Yes

Number of pooled particles:

30

Skip padding?

No

Pre-read all particles into RAM?

Yes

(Again, this is only possible here because the data set is small. For your own data, you would like write the particles to a scratch disk instead, see below.)

Copy particles to scratch directory

Combine iterations through disc?
No

Use GPU acceleration?
Yes

Which GPUs to use
0:1:2:3

On the Running tab, set:

Number of MPI procs
5

Number of threads
6

3D classification takes more memory than 2D classification, so often more threads are used. However, in this case the images are rather small and RAM-shortage may not be such a big issue. On our computer with 4 GPUs, we used 5 MPIs and 6 threads, and this calculation took approximately 6 minutes without Blush. Switching Blush on changed this to 13 minutes. The relatively difference will be much smaller for larger data sets that spend most of their time during their E-step, whereas Blush regularisation happens only in the M-step.

When analysing the resulting class reconstructions, it is useful to also look at them in 2D slices, not only as a thresholded map in for example UCSF chimera. In the slices view you will get a much better impression of unresolved heterogeneity, which will show up as fuzzy or streaked regions in the slices. Slices also give a good impression of the flatness of the solvent region. Use the Display: button and select any of the reconstructions from the last iteration to open a slices-view in relion; you can also see a central slice through all classes simultaneously by selecting the `run_it025_optimiser.star` option from the Display: button. We then often reverse sort them on `rlnClassDistribution`.

When looking at your rendered maps in 3D, e.g. using UCSF chimera, it is often a good idea to fit them all into the best one, as maps may rotate slightly during refinement. In chimera, we use the `[Tools]-[Volume Data]-[Fit in Map]` tool for that. For looking at multiple maps alongside each other, we also like the `[Tools]-[Structure Comparison]-[Tile Structures]` tool, combined with the `independent` center-of-rotation method on the `Viewing` window.

4.6.2 Selecting good particles for further processing

After the 3D classification job has finished, we can launch another Subset selection job.

On the I/O tab, set:

Select classes from job:
Class3D/job016/run_it25_optimiser.star

Because automated class selection has not been implemented for 3D classification, on the Class options tab, we now need to set:

Automatically select 2D classes?
No

We selected a single good class, discarding particles from 3 suboptimal classes. We retained over 4400 particles. This well-behaved tutorial data set is relatively homogeneous…

Note that this procedure of 3D classification and Subset selection may be repeated several times.

4.6.3 Analysing the results in more detail

Note

Again, if you are in a hurry to get through this tutorial, you can skip this sub-section.
It contains more detailed information for the interested reader.

The output files are basically the same as for the 2D classification run (we're actually using the same code for 2D and 3D refinements). The only difference is that the map for each class is saved as a separate MRC map, e.g. *run_it025_class00?.mrc*, as opposed to the single MRC stack with 2D class averages that was output before.

As before, smaller classes will be low-pass filtered more strongly than large classes, and the spectral signal-to-noise ratios are stored in the `data_model_class_N` tables (with $N = 1, \ldots, K$) of the `_model.star` files. Perhaps now is a good time to introduce two handy scripts that are useful to extract any type of data from star files. Try typing:

```
relion_star_printtable Class3D/job016/run_it025_model.star
  data_model_class_1 rlnResolution rlnSsnrMap
```

It will print the two columns with the resolution (`rlnResolution`) and the spectral signal-to-noise ratio (`rlnSsnrMap`) from table `data_model_class_1` to the screen. You could redirect this to a file for subsequent plotting in your favourite program. Alternatively, if *gnuplot* is installed on your system, you may type:

```
relion_star_plottable Class3D/job016/run_it025_model.star
  data_model_class_1 rlnResolution rlnSsnrMap
```

To check whether your run had converged, (as mentioned above) you could also monitor:

```
grep _rlnChangesOptimalClasses Class3D/job016/run_it???_optimiser.star
```

As you may appreciate by now: the star files are a very convenient way of handling many different types of input and output data. Linux shell commands like `grep` and `awk`, possibly combined into scripts like `relion_star_printtable`, provide you with a flexible and powerful way to analyze your results.

4.7 High-resolution 3D refinement

Once a subset of sufficient homogeneity has been selected, one may use the 3D auto-refine procedure in relion to refine this subset to high resolution in a fully automated manner. This procedure employs the so-called **gold-standard** way to calculate Fourier Shell Correlation (FSC) from independently refined half-reconstructions in order to estimate resolution, so that self-enhancing overfitting may be avoided [SC12]. Combined with a procedure to estimate the accuracy of the angular assignments [Sch12b], it automatically determines when a refinement has converged. Thereby, this procedure requires very little user input, i.e. it remains objective, and has been observed to yield excellent maps for many data sets. Another advantage is that one typically only needs to run it once, as there are hardly any parameters to optimize.

However, before we start our high-resolution refinement, we should first re-extract our current set of selected particles with less down-scaling, so that we can potentially go to higher resolution. To do this, go to the Particle extraction jobtype on the GUI, and on the I/O tab give:

micrograph STAR file:
CtfFind/job003/micrographs_ctf.star

(This should still be there.)

Coordinate-file suffix:

(Leave this empty now.)

OR re-extract refined particles?

Yes

Refined particles STAR file:

Select/job017/particles.star

(Now, we will use only the refined subset of selected particles.)

Reset the refined offsets to zero?

No

(This would discard the translational offsets from the previous classification runs.)

OR: re-center refined coordinates?

Yes

(This will re-center all the particles according to the aligned offsets from the 3D classification job above.)

Recenter on - X, Y, Z (pix)

0 0 0

(We want to keep the centre of the molecule in the middle of the box.)

Write output in float16?

Yes

And on the extract tab, we keep everything as it was, except:

Particle box size (pix)

360

(we will use a larger box, so that de-localised CTF signals can be better modeled. This is important for the CTF refinement later on.)

Rescale particles?

Yes

(to prevent working with very large images, let's down-sample to a pixel size of 360*0.885/256=1.244 Å. This will limit our maximum achievable resolution to 2.5 Å, which is probably enough for such a small data set.)

Re-scaled size (pixels):

256

In addition, we will need to rescale the best map obtained thus far to the 256-pixel box size. This is done from the command-line, but make sure you select the correct class (check the file `Class3D/job016/run_it025_model.star` to see which class is the largest)!:

```
relion_image_handler --i Class3D/job016/run_it025_class002.mrc \
 --angpix 3.54 --rescale_angpix 1.244 --new_box 256 \
 --o Class3D/job016/run_it025_class002_box256.mrc
```

4.7.1 Running the auto-refine job

On the I/O tab of the 3D auto-refine job-type set:

Input images STAR file:

Extract/job018/particles.star

Reference map:

Class3D/job016/run_it025_class002_box256.mrc

(Note this one is not directly available through the Browse button, as it was not part the relion pipeline yet.)

Reference mask (optional):

(leave this empty for now)

On the Reference tab, set:

Ref. map is on absolute greyscale?

No

(because of the different normalisation of down-scaled images, the rescaled map is no longer on the correct absolute grey scale. Setting this option to `No` is therefore important, and will correct the greyscale in the first iteration of the refinement.)

Initial low-pass filter (A)

50

(We typically start auto-refinements from low-pass filtered maps to prevent bias towards high-frequency components in the map, and to maintain the *gold-standard* of completely independent refinements at resolutions higher than the initial one.)

Symmetry

D2

(We now aim for high-resolution refinement, so imposing symmetry will effectively quadruple the number of particles.)

Parameters on the CTF, Optimisation tabs remain the same as they were in the 3D classification job, but let's skip Blush regularisation for this data set with its relatively high signal-to-noise particles.

On the Auto-sampling tab, one can usually keep the defaults. Note that the orientational sampling rates on the Sampling tab will only be used in the first few iterations, from there on the algorithm will automatically increase the angular sampling rates until convergence. Therefore, for all refinements with less than octahedral or icosahedral symmetry, we typically use the default angular sampling of 7.5 degrees, and local searches from a sampling of 1.8 degrees. Only for higher symmetry refinements, we use 3.7 degrees sampling and perform local searches from 0.9 degrees. The only thing we will change here is to set:

Use finer angular sampling faster?

Yes

(This will be more aggresive in proceeding with iterations of finer angular sampling faster. This will therefore speed up the calculations. You might want to check that you're not loosing resolution for this in the later stages of your own processing, but during the initial stages it often does not matter much.)

On the Compute tab, we also replicate the settings of the 3D classification:

Use parallel disc I/O?

Yes

Number of pooled particles:

30

Skip padding?

Yes

(By default, relion will pad the 3D maps with zeros, to allow better interpolations in Fourier space. This requires 8x more computer memory (RAM). By switching this off for this early refinement, we will save RAM and increase speed. One risk is that aliasing artefacts fold in at the edges of the 3D reconstruction, so be careful with this option if your box is very tight.)

Pre-read all particles into RAM?
Yes

(Again, this is only possible here because the data set is small. For your own data, you would like write the particles to a scratch disk instead, see below.)

Copy particles to scratch directory

Combine iterations through disc?
No

Use GPU acceleration?
Yes

Which GPUs to use
0:1:2:3

As the MPI nodes are divided between one master (who does nothing else than bossing the others around) and two sets of slaves who do all the work on the two half-sets, it is most efficient to use an odd number of MPI processors, and the minimum number of MPI processes for 3D auto-refine jobs is 3. Memory requirements may increase significantly at the final iteration, as all frequencies until Nyquist will be taken into account, so for larger sized boxes than the ones in this test data set you may want to run with as many threads as you have cores on your cluster nodes.

On our computer with 4 GPUs, we used 5 MPIs and 6 threads, and this calculation took approximately 7 minutes.

4.7.2 Analysing the results

Also the output files are largely the same as for the 3D classification job. However, at every iteration the program writes out two `run_it0??_half?_model.star` and two `run_it0??_half?_class001.mrc` files: one for each independently refined half of the data. Only upon convergence a single *run_model.star* and `run_class001.mrc` file will be written out (without `_it0??` in their names). Because in the last iteration the two independent half-reconstructions are joined together, the resolution will typically improve significantly in the last iteration. Because the program will use all data out to Nyquist frequency, this iteration also requires more memory and CPU.

Note that the automated increase in angular sampling is an important aspect of the auto-refine procedure. It is based on signal-to-noise considerations that are explained in [Sch12b], to estimate the accuracy of the angular and translational assignments. The program will not use finer angular and translational sampling rates than it deems necessary (because it would not improve the results). The estimated accuracies and employed sampling rates, together with current resolution estimates are all stored in the *_optimiser.star* and `_model.star` files, but may also be extracted from the stdout file. For example, try:

```
grep Auto Refine3D/job019/run.out
```

4.8 Mask creation & Postprocessing

After performing a 3D auto-refinement, the map needs to be sharpened. Also, the gold-standard FSC curves inside the auto-refine procedures only use unmasked maps (unless you've used the option *Use solvent-flattened FSCs*). This means that the actual resolution is under-estimated during the actual refinement, because noise in the solvent region will lower the FSC curve. relion's procedure for B-factor sharpening and calculating masked FSC curves [CMF+13] is called `post-processing`. First however, we'll need to make a mask to define where the protein ends and the solvent region starts. This is done using the Mask Creation job-type.

4.8.1 Making a mask

On the I/O tab, select the output map from the finished 3D auto-refine job: `Refine3D/job019/run_class001.mrc`.

On the Mask tab set:

Lowpass filter map (A):

15

(A 15 Å low-pass filter seems to be a good choice for smooth solvent masks for many proteins.)

Pixel size (A):

-1

(This value will be taken automatically from the header of the input map.)

Initial binarisation threshold:

0.01

(This should be a threshold at which rendering of the low-pass filtered map in for example chimera shows no noisy spots outside the protein area. Move the threshold up and down to find a suitable spot. Remember that one can use the command-line program called `relion_image_handler` with the options `--lowpass 15 --angpix 1.244` to get a low-pass filtered version of an input map. Often good values for the initial threshold are around 0.002-0.02.)

Extend binary map this many pixels:

03

(The threshold above is used to generate a black-and-white mask. The white volume in this map will be grown this many pixels in all directions. Use this to make your initial binary mask less tight.)

Add a soft-edge of this many pixels:

8

(This will put a cosine-shaped soft edge on your masks. This is important, as the correction procedure that measures the effect of the mask on the FSC curve may be quite sensitive to too sharp masks. As the mask generation is relatively quick, we often play with the mask parameters to get the best resolution estimate.)

Ignore the Helix tab.

To speed things up, on the Running tab, make sure to use multiple threads, e.g.:

Number of threads

12

You can look at slices through the resulting mask using the Display: button, or you can load the mask into UCSF chimera. The latter may be a good idea, together with the map from the auto-refine procedure, to confirm that the masks encapsulates the entire structure, but does not leave a lot of solvent inside the mask. You can continue the same job with new settings for the mask generation multiple times, until you have found a mask you like.

4.8.2 Post-processing

Now select the Post-processing job-type, and on the I/O tab, set:

One of the 2 unfiltered half-maps:

Refine3D/job019/run_half1_class001_unfil.mrc

Solvent mask:

MaskCreate/job020/mask.mrc

Calibrated pixel size (A):

1.244

(Sometimes you find out when you start building a model that what you thought was the correct pixel size, in fact was off by several percent.

Inside relion, everything up until this point was still consistent (at least up to ~2A resolution), so you do not need to re-refine your map and/or re-classify your data. All you need to do is provide the correct pixel size here for your correct map and final resolution estimation.)

On the Sharpen tab, set:

Estimate B-factor automatically:

Yes

(This procedure is based on the classic Rosenthal and Henderson paper [RH03], and will need the final resolution to extend significantly beyond 10 Å. If your map does not reach that resolution, you may want to use your own `ad-hoc` B-factor instead.)

Lowest resolution for auto-B fit (A):

10

(This is usually not changed.)

Use your own B-factor?

No

Skip FSC-weighting?

No

(This option is sometimes useful to analyse regions of the map in which the resolution extends **beyond** the overall resolution of the map. This is not the case now.)

MTF of the detector (STAR file)

mtf_k2_200kV.star

Original detector pixel size

0.885

(This is the original pixel size (in Angstroms) in the raw (non-super-resolution!) micrographs.)

Run the job (no need for a cluster, as this job will run very quickly). Using the Display button, you can display slizes through the postprocessed map and a PDF with the FSC curves and the Guinier plots for this structure. You can also open the `PostProcess/job021/postprocess.mrc` map in chimera, where you will see that it is much easier to see where all the alpha-helices are then in the converged map of the 3D auto-refine procedure. The resolution estimate is based on the phase-randomization procedure as published previously [CMF+13]. Make sure that the FSC of the phase-randomized maps (the red curve) is more-or-less zero at the estimated resolution of the postprocessed map. If it is not, then your mask is too sharp or has too many details. In that case use a stronger low-pass filter and/or a wider and more softer mask in the Mask creation step above, and repeat the postprocessing.

4.9 CTF and aberration refinement

Next, we'll use the CTF refinement job-type to estimate the asymmetrical and symmetrical aberrations in the dataset; whether there is any anisotropic magnification; and we'll re-estimate per-particle defocus values for the entire data set. Running this job-type can lead to further improvements in resolution at a relatively minor computational cost, but it all depends on how flat your ice was (for per-particle defocus estimates), and how well you had aligned your scope (for the aberrations). It runs from a previous 3D auto-refine job as well as a corresponding Post-processing job. Let's start with the higher-order aberrations, to see whether this data suffered from beamtilt or trefoil (which are asymmetric aberrations), or from tetrafoil or an error in spherical aberration (which are symmetric aberrations).

4.9.1 Higher-order aberrations

On the I/O tab of CTF refinement job-type on the GUI the set:

Particles (from Refine3D)
Refine3D/job019/run_data.star

Postprocess STAR file:
PostProcess/job021/postprocess.star

On the Fit tab set:

Estimate (anisotropic magnification
No

(We will do this later, see below.)

Perform CTF parameter fitting?
No

(We will do this later, see below.)

Estimate beamtilt?
Yes

(Despite the observation that most microscopists perform coma-free alignment schemes prior to data acquisition, there are still many data sets with a significant amount of beamtilt. That's why in this example we're first looking for beamtilt, and do the anisotropic magnification and the per-particle defocus later. In general, one would try to first estimate the source of the largest errors.)

Also estimate trefoil?
Yes

(This will allow more higher-order Zernike polynomials in the fitting of the asymmetric aberrations.)

estimate 4th order aberrations?
Yes

(This is done mostly for illustrative purposes here. One would not expect a big improvement at the current resolution of 3 Å.)

Minimum resolution for fits (A):
30

(Just leave the default.)

This program is only implemented on the CPU. Using 1 MPI and 12 threads, on our computer, this job finished in approximately one minute.

You can analyse the accumulated averages for the asymmetrical and symmetrical aberrations, as well as their models, by selecting the `logfile.pdf` file from the Display: button on the GUI. You'll see that this data actually suffered from some beamtilt: one side of the asymmetrical aberration images is blue, whereas the other side is red. You can find the values (approximately -0.2 mrad of beamtilt in the Y-direction) in the optics table of the output STAR file:

```
less CtfRefine/job022/particles_ctf_refine.star
```

There was also a small error in the spherical aberration, as the symmetrical aberration image shows a significant, circularly symmetric difference (the image is blue at higher spatial frequencies, i.e. away from the center of the image). Importantly, for both the asymmetric and the symmetric aberaations, the model seems to capture the aberrations well.

If the data had suffered from trefoil, then the asymmetric aberration plot would have shown 3-fold symmetric blue/red deviations. If the data had suffered from tetrafoil, then the symmetric aberration plot would have shown 4-fold symmetric blue/red deviations. Examples of those are shown in the supplement of our 2019 publication on Tau filaments from the brain of individuals with chronic traumatic encephalopathy (CTE).

4.9.2 Anisotropic magnification

Next, let's see whether these data suffer from anisotropic magnification. On the I/O tab of CTF refinement job-type on the GUI, use the output from the previous CTF refinement job as input to this one:

Particles (from Refine3D)
CtfRefine/job022/particles_ctf_refine.star

Postprocess STAR file:
PostProcess/job021/postprocess.star

And this time, on the Fit tab set:

Estimate (anisotropic magnification
Yes

(This will deactivate most of the other options, as simultaneous magnification and aberration refinement is unstable.)

Minimum resolution for fits (A):
30

(Just leave the default.)

Using 1 MPI and 12 threads, on our computer, this job finished in approximately one minute.

Again, the relevant images to analyse are in the `logfile.pdf`. There seem to be some blue-red trends, but the actual anisotropy is very small, as assessed from the `_rlnMagMat??` elements of the (2x2) transformation matrix in the optics table of the output STAR file, which are close to the 0 and 1 values of the identity matrix:

```
less CtfRefine/job023/particles_ctf_refine.star
```

4.9.3 Per-particle defocus values

Lastly, let's re-estimate the defocus values for each particle. Again, use the output from the previous job as input for this one (although we could have just as well kept using the output from the aberration correction, as the magnification anisotropy was very small):

Particles (from Refine3D)
CtfRefine/job023/particles_ctf_refine.star

Postprocess STAR file:
PostProcess/job021/postprocess.star

And this time, on the Fit tab set:

Estimate (anisotropic magnification
No

Perform CTF parameter fitting?
Yes

Fit defocus?
Per-particle

(Provided the resolution of the reference extends well beyond 4 Å, per-particle defocus estimation seems to be relatively stable. It will account for non-horizontal ice layers, and particles at the top or bottom of the ice layer.)

Fit astigmatism?

Per-micrograph

(Provided the resolution of the reference extends well beyond 4 Å, and there are enough particles on each micrograph, estimating astigmatism on a per-micrograph basis seems to be relatively stable. Doing this on a pre-particle basis would require particles with very strong signal.)

Fit B-factor?

No

Fit phase-shift?

No

(This is useful for phase-plate data.)

Estimate beamtilt?

No

estimate 4th order aberrations?

No

Minimum resolution for fits (A):

30

(Just leave the default.)

Using 1 MPI and 12 threads, on our computer, this job finished in four minutes on our computer.

Per-particle defocus values are plotted by colour for each micrograph in the `logfile.pdf`. Can you spot micrographs with a tilted ice layer?

It is probably a good idea to re-run 3D auto-refine and Post-processing at this stage, so we can confirm that the new particle STAR file actually gives better results.

For the 3D auto-refine, we left all options as before, except on the I/O tab:

Input images STAR file

CtfRefine/job024/particles_ctf_refine.star

Reference map

Refine3D/job019/run_class001.mrc

and on the Reference tab:

Ref. map is on absolute greyscale?

Yes

After another Post-processing job, the resolution improved to 3.0 Å.

4.10 Bayesian polishing

relion implements a Bayesian approach to per-particle, reference-based beam-induced motion correction. This approachs aims to optimise a regularised likelihood, which allows us to associate with each hypothetical set of particle trajectories a prior likelihood that favors spatially coherent and temporally smooth motion without imposing any hard constraints. The smoothness prior term requires three parameters that describe the statistics of the observed motion. To estimate the prior that yields the best motion tracks for this particular data set, we can first run the program in 'training mode'. Once the estimates have been obtained, one can then run the program again to fit tracks for the motion of all particles in the data set and to produce adequately weighted averages of the aligned movie frames.

4.10.1 Running in training mode

Using 24 threads in parallel, this job took almost 2 hours on our computer... If you do not want to wait for this, you can just proceed to the next section and use the sigma-values from our precalculated results, which are already given in that section. For many data sets the default parameters on the GUI ($\sigma_{\text{vel}} = 0.2; \sigma_{\text{div}} = 5000; \sigma_{\text{acc}} = 2$) will also perform well, so people often skip training for Bayesian polishing.

If you do want to run the training job yourself, on the I/O tab of the Bayesian polishing job-type set:

Micrographs (from MotionCorr):
MotionCorr/job002/corrected_micrographs.star

Particles (from Refine3D or CtfRefine):
Refine3D/job025/run_data.star

(These particles will be polished)

Postprocess STAR file
PostProcess/job026/postprocess.star

(the mask and FSC curve from this job will be used in the polishing procedure.)

First movie frame:
1

Last movie frame:
-1

(Some people throw away the first or last frames from their movies. Note that this is **not recommended** when performing Bayesian polishing in relion. The B-factor weighting of the movie frames will automatically optimise the signal-to-noise ratio in the shiny particles, so it is best to include all movie frames.)

Extraction size (pix in unbinned movie)
-1

(This option can be used to extract polished particles in a box with a different size than the ones from the input refinement above. It is irrelevant for a training job.)

Re-scaled size (pixels)
-1

(This option can be used to re-scale the polished particles to a different size than the ones from the input refinement above. It is irrelevant for a training job.)

Write output in float16?
Yes

On the Train tab set:

Train optimal parameters?
Yes

Fraction of Fourier pixels for testing:
0.5

(Just leave the default here)

Use this many particles:

3000

(That's almost all we have anyway. Note that the more particles, the more RAM this program will take. If you run out of memory, try training with fewer particles. Using much fewer than 4000 particles is not recommended.)

On the Polish tab make sure you set:

Perform particle polishing?

No

Note that the training step of this program has not been MPI-parallelised. Therefore, make sure you use only a single MPI process. We ran the program with 24 threads to speed it up. Still, the calculation took more than 1 hour.

4.10.2 Running in polishing mode

Once the training step is finished, the program will write out a text file called `Polish/job027/opt_params.txt`. To use these parameters to polish your particles, click on the job-type menu on the left to select a new Bayesian polishing job. Keep the parameters on the I/O tab the same as before, and on the Train tab, make sure you switch the training off. Then, on the Polish tab set:

Perform particle polishing?

Yes

Optimised parameter file:

Polish/job027/opt_params_all_groups.txt

OR use your own parameters?

No

Minimum resolution for B-factor fit (A):

20

Maximum resolution for B-factor fit (A):

-1

(just leave the defaults for these last two parameters)

Alternatively, if you decided to skip the training set, then you can fill in the Polish tab with the sigma-parameters that we obtained in our run:

Perform particle polishing?

Yes

Optimised parameter file:

(leave this empty to use the optimal parameters we got as per below.)

OR use your own parameters?

Yes

Sigma for velocity (A/dose)

0.45

Sigma for divergence (A)

1290

Sigma for acceleration (A/dose)

2.66

Minimum resolution for B-factor fit (A):

20

Maximum resolution for B-factor fit (A):

-1

(just leave the defaults for these last two parameters)

This part of the program is MPI-parallelised. Using 3 MPI processes, with 8 threads each, our run finished in less than six minutes. We could have used multiple MPI processes to speed this up, although disk access may become limited.

The Bayesian polishing job outputs a STAR file with the polished particles called *shiny.star* and a PDF logfile. The latter contains plots of the scale and B-factors used for the radiation-damage weighting, plus plots of the refined particle tracks for all included particles on all micrographs. Looking at the plots for this data set, it appeared that the stage was a bit drifty: almost all particles move from the top right to the bottom left during the movies. The B-factors and scale factors look like expected: a slight dip for the first frames, which comes from a small amount of initial fast beam-induced movement, and then going down linearly as a result of radiation damage.

4.10.3 Re-running refinement and post-processing

After polishing, the signal-to-noise ratio in the particles has improved, and one should submit a new 3D auto-refine job and a corrsponding Post-processing job. We chose to run the 3D auto-refine job with the shiny particles using the following option on the I/O tab:

Input images STAR file:

Polish/job028/shiny.star

Reference Map:

Refine3D/job025/run_half1_class001_unfil.mrc

(By giving one of the half-maps as reference, both half-maps will actually be read in for gold-standard refinement. This prevents overfitting by reading in a joint reconstruction, and therefore one can start the refinement from higher initial resolutions.)

Reference mask (optional):

MaskCreate/job020/mask.mrc

(this is the mask we made for the first Post-processing job. Using this option, the solvent will be set to zero for all pixels outside the mask. This reduces noise in the reference, and thus lead to better orientation assignments and thus reconstructions.)

Because we are providing a half-map, overfitting shouldn't be a problem and on the Reference tab we can set:

Initial low-pass filter (A)

8

On the Optimisation tab, we set:

Use solvent-flattened FSCs?

Yes

(Using this option, the refinement will use a solvent-correction on the gold-standard FSC curve at every iteration, very much like the one used in the Post-processing job-type. This option is particularly useful when the protein occupies a relatively small volume inside the particle box, e.g. with very elongated molecules, or when one focusses refinement on a small part using a mask. The default way of calculating FSCs in the 3D auto-refinement is without masking the (gold-standard) half-maps, which systematically under-estimates the resolution during refinement. This is remediated by calculating phase-randomised solvent-corrected FSC curves at every iteration, and this generally leads to a noticeable improvement in resolution.)

Also, on the Auto-sampling tab, we now set:

Use finer angular sampling faster?

No

(This was OK in the earlier stages to speed up calculations, but at this stage we want to get the highest resolution we can, so we will opt for the slower, but safer options.)

And on the Compute tab, we now set:

Skip padding?

No

(For the same reason as above: skipping padding speeds up, but some aliasing artefacts can fold back in. As we do have the required computer memory for the padded reconstruction, let's go safe for this last refinement.)

As you can see in the pre-calculated results, after a final Post-processing job, we obtained an overall resolution of 2.9 Å. Not bad for 3GB of data, right?

4.10.4 When and how to run CTF refinement and Bayesian polishing

Both Bayesian polishing and CTF refinement, which comprises per-particle defocus, magnification and higher-order aberration estimation, may improve the resolution of the reconstruction. This raises a question of which one to apply first. In this example, we first refined the aberrations, the magnification, and then the per-particle defocus values. We then followed up with polishing, but we could have also performed the polishing before any of the CTF refinements. Both approaches benefit from higher resolution models, so an iterative procedure may be beneficial. For example, one could repeat the CTF refinement after the Bayesian polishing. In general, it is probably best to tackle the biggest problem first, and some trial and error may be necessary.

Moreover, we have seen for some cases that the training prodcedure of Bayesian polishing yields inconsistent results: i.e. multiple runs yield very different sigma values. However, we have also observed that often the actual sigma values used for the polishing do not matter much for the resolution of the map after re-refining the shiny particles. Therefore, and also because the training is computationally expensive, it may be just as well to run the polishing directly with the default parameters ($\sigma_{\text{vel}} = 0.2$; $\sigma_{\text{div}} = 5000$; $\sigma_{\text{acc}} = 2$), i.e. without training for your specific data set.

4.11 Local-resolution estimation

The estimated resolution from the post-processing program is a global estimate. However, a single number cannot describe the variations in resolution that are often observed in reconstructions of macromolecular complexes. Alp Kucukelbir and Hemant Tagare wrote a nifty program to estimate the variation in resolution throughout the map [KST14]. relion implements a wrapper to this program through the Local resolution job-type. Alternatively, one can choose to run a post-processing-like procedure with a soft spherical mask that is moved around the entire map. In the example below, we use the latter.

4.11.1 Running the job

On the I/O tab set:

One of the two unfiltered half-maps:

Refine3D/job029/run_half1_class001_unfil.mrc

User-provided solvent mask:
MaskCreate/job020/mask.mrc

Calibrated pixel size:
1.244

(Sometimes you find out when you start building a model that what you thought was the correct pixel size, in fact was off by several percent. Inside relion, everything up until this point was still consistent (provided you aren't at resolutions beyond 2 Angstrom). so you do not need to re-refine your map and/or re-classify your data. All you need to do is provide the correct pixel size here for your correct map and final resolution estimation.)

On the ResMap tab set:

Use ResMap?
No

On the Relion tab set:

Use Relion?
Yes

User-provided B-factor:
-30

(This value will be used to also calculate a locally-filtered and sharpened map. Probably you want to use a value close to the one determined automatically during the Post-processing job.)

MTF of the detector (STAR file):
mtf_k2_200kV.star

(The same as for the Post-processing job.)

4.11.2 Analysing the results

Running with 8 MPI processes, this job took approximately 7 minutes. The output is a file called `LocalRes/job031/relion_locres.mrc` that may be used in UCSF chimera to color the *Postprocess/job030/postprocess.mrc* map according to local resolution. This is done using `[Tools]-[Volume data]-[Surface color]`, and then select `by volume data value` and browse to the `resmap` file.

Unique to the relion option is the additional output of a locally-filtered (and sharpened map), which may be useful to describe the overall variations in map quality in a single map. This map is saved as `LocalRes/job031/relion_locres_filtered.mrc` and can be visualised directly in UCSF chimera (and optionally also coloured by local resolution as before).

4.12 Checking the handedness

Careful inspection of the map may indicate that the handedness is incorrect, e.g. because the α-helices turn the wrong way around. Remember that it is impossible to determine absolute handedness from a data set without tilting the microscopy stage. The SGD algorithm in the 3D initial model jobtype therefore has a 50 % chance of being in the opposite hand. In our precalculated results, this was not the case. One may flip the handedness of the postprocessed map from the command line as follows:

```
relion_image_handler --i PostProcess/job030/postprocess.mrc  \
  --o PostProcess/job030/postprocess_invert.mrc --invert_hand
```

The same command could also be run on any of the other maps. If one realises earlier on in the image processing procedure that the hand is wrong, one could of course also switch to the other hand earlier on. For relion itself it doesn't matter, as both hands cannot be distinguished, but it may be more convenient to flip the hand as soon as you notice it.

Once in the correct hand, you might want to load the map into UCSF chimera and superimpose it with an atomic model for β-galactosidase. You could try fetching one straight from the PDB using PDB-ID 5a1a.

4.13 ModelAngelo: atomic modelling

As of release 5.0, relion comes with a machine-learning approach for automated atomic model building called `ModelAngelo` [JKZ+24]. ModelAngelo is a graph neural network that combines information from the cryo-EM map with sequence information of the proteins that are present in the map to build an atomic model.

Because we know that the sample in this data set is beta-galactosidase from *E.coli*, we can provide the protein sequence as a `FASTA` file. You can download it from `uniprot` through this link. Save it as `betagal.fasta` in the project directory.

4.13.1 Running the job

Using the ModelAngelo building job type, we set on the I/O tab:

B-factor sharpened map:
PostProcess/job030/postprocess_masked.mrc

FASTA sequence for proteins:
betagal.fasta

FASTA sequence for DNA:

(There is no DNA in this complex.)

FASTA sequence for RNA:

(There is no RNA in this complex.)

Which GPUs to use:
0,1,2,3

On the Hmmer tab make sure you this is set:

Perform HMMer search?
No

4.13.2 Analysing the results

Running with 4 GPUs (relatively old 1080s), this job took approximately 18 minutes. The output is a coordinate file called `ModelAngelo/job032/job032.cif` that may be used in UCSF chimera together wit the `Postprocess/job030/postprocess.mrc` map. Note that although `ModelAngelo` did a very good job on this relatively easy test case, you should always check its results carefully in a program like `coot` [ELSC10] . You will also need to perform a stereochemical refinement of the coordinates. For this, we like `servalcat` [YPBM21].

4.13.3 Discovering unknown proteins

One does not always know the identity of all proteins that are present in a cryo-EM reconstruction. One can also run `ModelAngelo` without providing a `FASTA` file with the known protein sequence. It will still try to model protein chains in the map, but will perform less well without the knowledge of the sequence. But, one can then perform a Hidden Markov Model search using the full probabilities for all 20 amino acids for every residue, for example against a FASTA file that contains the entire genome for that organism. In that way, one can identify unknown proteins in the map.

Download the *E.coli* genome (K-12 substrain) from this link to NCBI. Use the `Send to` dropdown menu on the top right to select `Coding Sequences`, select the `FASTA protein` format, and click `Create File`. Save the resulting file as `Ecoli.fasta` in your project directory.

Using the same ModelAngelo building job type, this time we set on the I/O tab:

B-factor sharpened map:
PostProcess/job030/postprocess_masked.mrc

FASTA sequence for proteins:

FASTA sequence for DNA:

(There is no DNA in this complex.)

FASTA sequence for RNA:

(There is no RNA in this complex.)

Which GPUs to use:
0,1,2,3

On the Hmmer tab, we now set:

Perform HMMer search?
Yes

Library with sequences for HMMer search:
Ecoli.fasta

Alphabet for HMMer search:
amino

And we leave the rest of the HMMSearch parameters at their defaults.

4.13.4 Which one is my protein?

Running with 4 GPUs (again our relatively old 1080s), this job took approximately 12 minutes: running without the sequence is faster than running with the sequence. However, without knowledge of the sequence, ModelAngelo has trouble building a single chain, as you can see by visualising `ModelAngelo/job033/job033.cif` in UCSF chimera. The subsequent HMM search easily identifies beta-galactosidase, as you can see in `ModelAngelo/job033/best_hits.csv`. Cool, huh?

4.14 DynaMight: exploring motions

As of release 5.0, relion comes with a machine-learning approach for the analysis of molecular motions and flexibility called `DynaMight` [SKB+23]. DynaMight will fit molecular motions for each experimental particle image as a 3D deformation field, which is learnt using a variational auto-encoder. It also implements functionality to calculate a pseudo-inverse 3D deformation field that can then be used in a deformed weighted backprojection algorithm to obtain an improved 3D reconstruction of the consensus structure.

Beta-galactosidase is not a floppy protein, therefore running DynaMight on this data set will not be exciting! The below just walks you through the steps, so you learn how to run DynaMight jobs for your own data set.

4.14.1 Estimating the motions

We will use the DynaMight flexibility job type. On the I/O tab set:

Input images STAR file:
Refine3D/job029/run_data.star

Consensus map:
Refine3D/job029/run_class001.mrc

Number of Gaussian:
8000

(Describing flexibility at higher resolutions will require more Gaussians. As a rule of thumb, use 1-2 Gaussians per amino acid or nucleotide in your complex. The beta-galactosidase tetramer has just over 4x1042 amino acids, so we will use 8000 Gaussians here. The more Gaussians you use, the longer this will take… so you may want to run smaller tests too. Note that running with more than 30,000 Gaussians will be difficult in terms of memory unless you have a very large GPU.)

Initial map threshold (optional):
0.015

(This is a threshold at which flexible regions still show in 3D visualisation tools like UCSF chimera, but you should avoid the appearance of noisy density islands in the solvent region at this threshold.)

Regularization factor:
1

Which (single) GPU to use:
0

(For now, DynaMight only uses one GPU at a time.)

Preload images in RAM?
Yes

(As we only have a few thousand, we can read all particles in RAM and save time.)

For nowm ignore the Tasks tab; on the Running tab set:

Number of threads:
4

4.14.2 Visualising the deformations

On our, relatively old, 1080 GPU, estimating the deformations took less than onehour and a half. To visualise the flexibility in the beta-galactosidase complex, we use a continuation of the DynaMight flexibility job, where on the Tasks tab we set:

Checkpoint file:
DynaMight/job034/forward_deformations/checkpoints/checkpoint_final.pth

Do visualization?

Yes

Half-set to visualize:

1

(You can visualize either halfset 1 or halfset 2. There is another option where you visualize halfset 0. In that case, you can visualize error estimates on the 3D deformations for a validation set of 10% of your particles)

Do inverse-deformation estimation?

No

Do deformed backprojection?

No

Running this by pressing the Continue! button will launch a viewer that was written in `napari`. It is quite a heavy-handed tool, so you will need to run this viewer locally, while also needing a GPU (specified on the I/O tab, to perform some of the calculations…

By clicking on the colourful representation of latent space on the right, the 3D viewer will display the corresponding deformed state of the consensus structure. On the bottom right, by selecting `trajectory` under the `action` menu, one can draw a line through latent space using the middle-mouse button. This will create a movie of the states in the 3D viewer, which you can play with the triangle button underneath the 3D viewer. As said before, this looks underwhelming for beta-galactosidase, which basically has very little flexibility. You can use the right-mouse to zoom in on the tips, where perhaps you can see some imnute movements?

You can save maps or movies using the button on the bottom right. The individual states of such a movie would be saved as maps in MRC format here: `DynaMight/job138/movies/movie01_half1/`. You can then load all these maps into a 3D visualisation program, like UCSF **|chimera|**, and recreate the movie there.

4.14.3 Estimating inverse deformations

The 3D deformations are defined from the consensus positions of the Gaussians to their new position. For use in deformed weighted backprojection, we first need estimates for the inverse deformations at every point in the 3D Cartesian space. For this, DynaMight also uses a neural network. We can train it by using another continuation of the same DynaMight flexibility job, where on the Tasks tab we now set:

Checkpoint file:

DynaMight/job034/forward_deformations/checkpoints/checkpoint_final.pth

Do visualization?

No

Do inverse-deformation estimation?

Yes

Number of epochs to perform

200

Store deformations in RAM?

No

(If set to Yes, dynamight will store all deformations in the GPU memory, which will speed up the calculations, but you need to have enough GPU memory to do this. We could not do this on our small 1080s.)

Do deformed backprojection?

No

Running this by pressing the Continue! button took a bit over an hour on one of our 1080s.

4.14.4 Deformed backprojection

Finally, the resulting inverse deformations can then be used for a deformed backprojection that attempts to reconstruct an improved version of the consensus map, where densities for each particle are warped into non-straight lines in the back-projection in an attempt to "un-do" their deformations. Again, we use a continuation of the same DynaMight flexibility job, where on the Tasks tab we set:

Checkpoint file:
DynaMight/job034/forward_deformations/checkpoints/checkpoint_final.pth

Do visualization?
No

Do inverse-deformation estimation?
No

Do deformed backprojection?
Yes

Backprojection batchsize:
2

(Batches are processes in parallel on the GPU. Again, because our 1080s don't have a lot of memory, we could only run with a batchsize of 2. If you have a larger GPU, you could try and use more, e.g. 5 or 10.)

This took approximately 45 minutes on our system. The resulting half-maps can be used for a standard Post-processing job. In this case, nothing really interesting happens as there are hardly any deformations. The resolution actually decreases by about 0.5 Angstrom, probably because the deformed backprojection algorithm is not as good as good as the Fourier inversion algorithm for structurally homogeneous data sets. More interesting examples of deformed backprojection reconstructions are in the DynaMight paper [SKB+23].

4.15 Wrapping up

4.15.1 Cleaning up your directories

In order to save disk space, relion has an option to clean up job directories. There are two modes of cleaning: 'gentle' cleaning will only delete intermediate files from the job directory being cleaned; 'harsh' cleaning also deletes files that may be necessary to launch a new job that needs input from the job being cleaned. For example, harsh cleaning will remove averaged micrographs from a MotionCorr job, or extracted particles stacks from a Particle extraction job, while gentle cleaning will remove all files from itermediate iterations of 2D classification, 3D classification or 3D auto-refine jobs. You can clean individual jobs from the Job actions button; or you can clean all jobs from the 'Jobs' pull-down menu at the top of the GUI. We used the 'Gently clean all jobs' option from that menu before making a tarball of the project directory that we distributed as our precalculated results. You might want to gently clean your project directory before you put your data in long-term storage.

4.15.2 Asking questions and citing us

That's it! Hopefully you enjoyed this tutorial and found it useful. If you have any questions about relion, please first check the FAQ on the relion Wiki and the CCPEM mailing list. If that doesn't help, use the CCPEM list for asking your question.

If relion turns out to be useful in your research, please do cite our papers and tell your colleagues about it.

4.15.3 Further reading

The theory behind the refinement procedure in relion is described in detail in:

- S.H.W. Scheres (2012) “relion: Implementation of a Bayesian approach to cryo-EM structure determination” **J. Struc. Biol.**, 180, 519-530.
- S.H.W. Scheres (2012) “A Bayesian view on cryo-EM structure determination” **J. Mol. Biol.**, 415, 406-418.

A comprehensive overview of how to use relion for all types of classifications is described in:

- S.H.W. Scheres (2016) “Processing of structurally heterogeneous cryo-EM data in relion” **Meth. Enzym.**, 579, 125-157.

This tutorial does not cover multi-body refinement, which is useful to describe continuous motions in relatively large complexes. You can find a manuscript with specific instructions on how to perform multi-body refinement on the RELION Wiki

CHAPTER

FIVE

SUBTOMOGRAM TUTORIAL

5.1 Introduction

This tutorial will walk you through the tomography and sub-tomogram averaging pipeline of relion-5.0 . This semi-automated pipeline is designed to carry out all of the steps in a typical subtomogram averaging project from your raw tilt series movies to your final subtomogram average. We will use a test data set composed of 5 tomograms of immature HIV-1 dMACANC VLPs, which is available at EMPIAR-10164.

5.1.1 Requirements

1. Your raw tilt series data as individual movies, or as individual motion-corrected images, but not as combined tilt series stacks.
2. The mdoc files containing the metadata for each tilt series.

5.1.2 Getting Organised

We recommend creating a single directory per project. We'll call this the project directory.

To start processing, make your own project directory and download the raw data (frames and mdoc files) corresponding to the five tomograms (`TS_01`, `TS_03`, `TS_43`, `TS_45`, `TS_54`) from the EMPIAR link above. A RELION project directory containing precalculated results of all the processing steps of this dataset can be downloaded from:

We will start by launching the relion GUI. **It is important to always launch the RELION graphical user-interface (GUI) from the project directory**. To prevent errors with this, the GUI will ask for confirmation the first time you launch it in a new directory. Make sure you are inside the project directory, and launch the GUI by typing:

```
relion --tomo&
```

and answer `Yes` when prompted to set up a new relion project here.

5.2 Import

We will now import the raw data into RELION. In the GUI, select Import from the jobt-type browser on the top left and fill in the following parameters on the General tab:

Tilt image files

frames/*.mrc

Movies already motion corrected?

No

(These are 8-frame movies that have not yet been motion-corrected.)

mdoc files
mdoc/*.mdoc

Optics group name
optics1

(All imported tomograms will belong to the same optics group with this name. When left empty, each tomogram will be its own optics group, with the tomogram name as the optics group name.)

Prefix
""

Pixel size (Angstrom):
0.675

Voltage (kV):
300

Spherical aberration (mm):
2.7

Amplitude contrast:
0.1

On the Tilt series tab:

Dose rate per tile-image
3

Is dose rate per movie frame?
No

Tilt axis angle (deg)
85

(This is the nominal value for the tilt-axis orientation wrt to the Y-axis (positive is CCW from Y))

MTF file
""

Invert defocus handedness?
Yes

(Specify Yes to flip the handedness of the defocus geometry; the default, Yes, leads to a value of -1 in the STAR file, which is the correct one for the tutorial dataset.)

We skip the Coordinates tab for now (we will use it in the *Import coordinates* section to import particle coordinates obtained from other programs), and on the Running tab set:

Submit to queue?
No

You may provide a meaningful alias (for example: *tilt_series*) for this job in the white field named `Current job: Give_alias_here`. Clicking the Run! button will launch the job.

A directory called `ImportTomo/job001/` will be created, together with a symbolic link to this directory that is called `ImportTomo/tilt_series`. Inside the newly created directory a `tilt_series.star` file is created. It contains a table with an entry for each tilt series. For each tilt series, a separate starfile contains relevant metadata that has been extracted from the input images and mdoc files. Have a look at these by typing:

```
less Import/job001/tilt_series.star
less Import/job001/tilt_series/TS_01.star
```

5.3 Motion correction

As the raw data in EMPIAR-10164 are movies, we need to carry out motion correction. If your own raw data are already motion-corrected, you can skip this section provided you have selected 'Yes' under 'Movies already motion corrected?' in the Import

To start, click the Motion correction job type in the job-type browser and fill in the following parameters on the I/O tab:

Input tilt series

ImportTomo/job001/tilt_series.star

(RELION will use this star file to locate the movies for each tilt series.)

EER fractionation

32

(This dataset was not saved in the EER format. Therefore, we can ignore the EER fractionation field; but don't leave it empty. If your own data are collected in the EER format, the fractionation should be adjusted as described here.)

Write output in float16?

Yes

(We recommend writing images in float16 format to save disk space. If you select 'Yes' to writing in float16, you must also select 'Yes' to 'Save sum of power spectra?', because CtfFind4 will not be able to read float16 images.)

Save images for denoising?

Yes

(The tutorial dataset is very high quality with good contrast and, consequently, the tomograms produced probably do not need to be denoised. However, to demonstrate RELION's denoising protocol later, we will select 'Yes' in the 'Save images for denoising?' field. If, for your own data, you have limited hard drive space and you are confident your tomograms will be high contrast and easily interpreted, you can select 'No'. However, for most datasets we recommend saving images for denoising, just in case it may be needed at a later stage.)

Save sum of power spectra?

Yes

(Sums of non-dose weighted power spectra provide better signal for CTF estimation. The power spectra can be used by CTFFIND4. This option is not available for UCSF MotionCor2. You must use this option when writing in float16, as CtfFind4 cannot read images in float16 format.)

Sum power spectra every n frames

4

(McMullan et al. Ultramicroscopy, 2015 suggest that summing power spectra every 4.0 e/A2 gives optimal Thon rings. One frame will be 3 e/A2.)

On the Motion tab:

Bfactor

150

(For your own data, you may need to increase this value, if the SNR is particularly low. For super resolution movies, increasing the B factor may also help.)

Number of patches

1 1

(As there is so little dose in each movie frame, it is better not to use patch-based motion correction.)

Group frames

1

Binning factor

2

(The raw images were collected in super-resolution mode and have not yet been binned. This will bin the images to 1.35 Å.)

Gain-reference image

“”

(The tutorial data has already been gain corrected so does not need a ‘Gain reference image’ and so this field should be left blank.)

Gain rotation

No rotation (0)

(This will be ignored as we are not doing gain correction.)

Gain flip

No flip (0)

(This will be ignored as we are not doing gain correction.)

Defect file

“”

(We don’t have a file that describes the camera defects for this data set. For your own data, the defect file can be used to mask away broken pixels on the detector. Formats supported in our own implementation and in UCSF motioncor2 are either a text file in UCSF motioncor2 format (each line contains four numbers: x, y, width and height of a defect region); or a defect map (an image in MRC or TIFF format, where 0=good and 1=bad pixels. The coordinate system is the same as the input movie before application of binning, rotation and/or flipping. Note that defect text files produced by SerialEM are NOT supported! However, one can convert a SerialEM-style defect file into a defect map using imod.).

Use RELION’s own implementation

Yes

If you prefer to use UCSF MotionCor2, select No, provide the path to the executable, the GPU ID(s) of the GPUs you wish to use, and any other MotionCor2 arguments in their respective fields. Note that MotionCor2 cannot save images in float16 yet, nor does it write out summed power spectra of movie frames for subsequent CTF estimation.

On the Running tab:

Number of MPI procs:

32

(We used 32 parallel processes on our computer.)

Number of threads:

4

Submit to queue?

No

(We used a local machine, but this will depend on your setup.)

Clicking the Run! button will launch the job. Your motion corrected particles will be output into the `MotionCorr/job002/` directory. The output star file containing all necessary metadata for input into other jobs is saved as `MotionCorr/job002/corrected_tilt_series.star`. You can again have a look at the star files it refers to, to see accumulated metadata about the motion correction by typing:

```
less MotionCorr/job002/tilt_series/TS_01.star
```

5.4 CTF estimation

Next, we will estimate the CTF parameters for each motion-corrected image of the five tilt series. We will use Alexis Rohou and Niko Grigorieff's CTFFIND-4.1 because it gives excellent results and allows reading in the movie-averaged power spectra calculation by RELION's own implementation of the MotionCor2 algorithm.

Select the CTF estimation job type in the job-type browser and fill in the following parameters on the I/O tab:

Input tilt series

MotionCorr/job002/corrected_tilt_series.star

Estimate phase shifts?

No

(Estimating phase shifts option is only useful for phase plate data)

Amount of astigmatism (A)

100

(This number is suitable for most data sets on reasonably well aligned microscopes.)

On the CTFFIND-4.1 tab:

CTFFIND-4.1 executable

path/to/ctffind

(You will need to have CTFFIND-4.1 installed somewhere. Copy the filename of the executable here.)

Use power spectra from MotionCorr job?

Yes

(This estimates the CTF parameters using the power spectra that were saved during motion correction.)

Use exhaustive search

No

Estimate CTF on window size(pix)

-1

FFT box size (pix)

512

Minimum resolution (A)

50

(This is the lowest resolution that is used for CTF estimation. For you own data, you may need to change these values.)

Maximum resolution (A)

5

(This is the highest resolution that is used for CTF estimation, in the image with the least dose. For you own data, you may need to change these values.)

Minimum defocus value (A)

5000

(Note that this value will be ignored as long as we use the 'Nominal defocus search range' field below.)

Maximum defocus value (A)

50000

(Note that this value will be ignored as long as we use the 'Nominal defocus search range' field below.)

Defocus step size (A)

500

Nominal defocus search range (A)

10000

(If a positive value is given, the defocus search range will be set to +/- this value (in A) around the nominal defocus value from the input STAR file. The nominal defocus would have been extracted from the mdoc files. When using this option make sure that the correct values are present in the input star files for each tilt series. If a zero or negative value is given for this field, then the overall min-max defocus search ranges above will be used instead.)

Dose-dependent Thon ring fading (e/A2)

100

(If a positive value is given, then the maximum resolution for CTF estimation is lowerered by exp(dose/this_factor) times the original maximum resolution specified above. Remember that exp(1)~=2.7, so a value of 100 e/A^2 for this factor will yield a 2.7x higher maxres number (i.e. 2.7x lower resolution) for an accumulated dose of 100 e/A^2; Smaller values will lead to faster decay of the maxres. If zero or a negative value is given, the maximum value specified above will be used for all images.)

On the Running tab:

Make sure you use a suitable number of parallel MPI processes for your computational setup, possibly submit to a queue, and click the Run! button to launch the job.

The output will be saved in the `CtfFind/job003/` directory. The output star file containing all necessary metadata for input into other jobs is saved as `CtfFind/job003/tilt_series_ctf.star`. You can again have a look at added metadata for each tilt series image by looking into their star files:

```
less CtfFind/job003/tilt_series/TS_01.star
```

Check the defocus values for a few tilt series by examining their star files in the tilt_series directory of the CtfFind job. The `rlnDefocusU` and `rlnDefocusV` columns specify the estimated defocus values.

In addition, the `logfile.pdf` file contains plots of useful parameters, such as defocus, astigmatism, estimated resolution, etc for all micrographs, and histograms of these values over the entire data set. Analysing these plots may be useful to spot problems in your data acquisition.

Lastly, you can also see the power spectrum of the estimated CTF of each tilt image in a tilt series using the `relion_dislay` command:

```
relion_display --gui --i CtfFind/job003/tilt_series/TS_01.star
```

Compared to single-particle data, tilt series images tend to have low SNR, particularly at higher tilts. Consequently, the defocus values may vary at higher tilts. Later in this tutorial, we will perform reference-based CTF refinement, during which we can determine better defoci, so as long as the estimated values aren't too far off, we can hope to improve them later on and we need not worry too much at this stage.

5.5 Exclude tilt-images

Often, tilt series contain some images that are not suitable for tomogram reconstruction or further image processing, for example because they are partially or all black because of grid bars blocking the field of view. We can exclude these images from further processing using the Exclude tilt-images job.

On the I/O tab, set:

Input tilt series

CtfFind/job003/tilt_series_ctf.star

Number of cached tilt series

5

(This will store 5 tilt series in memory and minimise loading times between tilt series. If your computer struggles with loading so many, you can change this to 1.)

Clicking the Run! button will launch the Napari. **Note that Napari is notoriously slow when displaying over a network, so make sure you are running this job from the same computer as you are sitting behind!**

Before doing anything, wait for the tilt series to load. The loading of the tilt series is indicated in the bottom left corner. Once loading is finished, we can examine the first tilt series. In the Images panel on the far right of the GUI, click the top image and scroll through the tilt series using the arrow key on your keyboard. For `TS_01`, the first image (`TS_01_039_-60_0.mrc`) is blank but the rest of the tilt series looks good. Therefore, that first image should be excluded from further calculations. Do this by unticking the box next to its name and then clicking Save tilt-series STAR file in the bottom right corner. Then, move onto the next tilt series (`TS_03`) by clicking Session_1_TS_03 in the tilt series panel in the bottom right. Repeat the same for all tilt series. We recommend clicking Save tilt-series STAR file after each tilt series, just in case Napari crashes. If Napari does crash, you can start a new Exclude tilt-images job, where the input tilt series file on the I/O tab is the `selected_tilt_series.star` from the `ExcludeTiltImages/` directory of the crashed job.

When finished, click Save tilt-series STAR file and close Napari.

5.6 Align tilt-series

Before tomogram reconstruction, each tilt series must be aligned. To do this, RELION 5 implements a wrapper to IMOD or AreTomo. For the tutorial dataset, we will use IMOD's fiducial-based alignment as the raw data contains (10 nm) gold beads as fiducial markers. For your own data, you may want to use various tilt series alignment methods and then compare the quality of the tomograms that each method produces (see next step).

Start by selecting the Align tilt-series job, and in the I/O tab set:

Input tilt series

ExcludeTiltImages/job004/selected_tilt_series.star

On the IMOD tab, set:

Use IMOD's fiducial based alignment?

Yes

Fiducal diameter (nm)

10

Use IMOD's patch-tracking for alignment?

No

(For your own data, select Yes if you want to use IMOD's patch-tracking instead of fiducial-based alignment.)

On the AreTomo tab, make sure this is still set:

Use AreTomo

No

Then, run the job, which has not been parallelised (yet), by clicking the Run! button.

You can again view the accumulated metadata for each tilt series by typing:

```
less AlignTiltSeries/job005/tilt_series/TS_01.star
```

The tutorial data is very good and automated alignment is not a problem. This may not be the case for your own data. You can however go into each of the individual IMOD directories (e.g. `AlignTiltSeries/job005/external/TS_01`) and re-run IMOD for individual tilt series by tweaking the parameter files. This will obviously require some skills in IMOD, which may be useful to acquire anyway. You can find the IMOD documentation here. First delete the corresponding `AlignTiltSeries/job005/tilt_series/TS_XX.star` file (if it exists in the first place) and then continue the Align tilt-series job from the GUI. The job will then read in the new results and generate an updated star file.

5.7 Reconstruct tomograms

We can now generate our tomograms. Click on the Reconstruct tomograms job from the job types, and on the I/O tab set:

Input tilt series

AlignTiltSeries/job005/aligned_tilt_series.star

On the Reconstruct tab:

Unbinned tomogram width (Xdim)

4000

(This is the X-dimension of the reconstructed tomogram, in voxels. We're using a slightly larger tomogram volume than the actual size of the images (which is 3710 x 3838) so that if they rotate, all pixels will still be in the tomogram. Because we use a large binned pixel below, the costs on disk space are not too high, but you could tweak this to have a bit smaller tomograms.)

Unbinned tomogram height (Ydim)

4000

(As above for the Y-dimension.)

Unbinned tomogram thickness (Zdim)

2000

(This is the Z-dimension of the tomogram, in voxels. For the tutorial data, 2000 voxels encapsulates the signal for all five tomograms. For your own data, you may want to test a few values here to make sure the tomogram thickness is not too small to contain your entire sample. If you intend to denoise your tomograms later, it is better not to pick a tomogram thickness that is much greater than the thickness of your sample, because the denoising protocol randomly extracts subtomograms from your tomograms and you don't want too many without signal.)

Binned pixel size (A)

10

(A binned pixel size of 10 Angstrom will suffice for particle picking and denoising. Typically, the larger the pixel size, the faster the tomogram reconstruction and the less space the tomograms occupy on disk.)

Generate tomograms for denoising?

Yes

(The quality of the tutorial tomograms is very good and they don't need to be denoised; however, we set this to `Yes` to demonstrate denoising at the next step. This setting requires setting `Save images for denoising?` to `Yes` in the Motion correction job.)

Tilt angle offset (deg)

0

(This option allows you to reconstruct all your tomograms according to a pre-set offset for the tilt angle. This may be useful, for example when you have FIB-milled all you lamellae under a know tilt angle.)

Reconstruct only this tomogram

""

(This option allows you to reconstruct only one tomogram from the input series, for example because you have tweaked its alignment parameters or because you want to run a quick test. Just specify the rlnTomoName, e.g. TS_01)

On the Running tab choose the approriate number of MPIs and Threads and then click on the Run! button. With 5 MPI and 12 threads this step took less than 3 minutes on our computer. But denoising tomograms will take more time.

The output tomograms are called `Tomograms/job006/tomograms/rec_TS_01.mrc` etc. if `Generate tomograms for denoising?` was set to `No` or `Tomograms/job006/tomograms/rec_TS_01_half<1/2>.mrc` otherwise. You can visualise them in your favourite viewer, including IMOD's 3dmod or Napari. The main objective of these tomograms is to assess the quality of your sample and to allow particle picking. They do not need to contain high-resolution information at this point.

5.8 Denoise tomograms

For denoising tomorgams, the Denoise tomograms is a wrapper of `Cryo-CARE` [BJPJ19].

The first step is to train a neural network on patches from the odd and even frame tomograms using the NOISE2NOISE approach. Therefore, denoising only works if both `Save images for denoising?` in the Motion correction job and `Generate tomograms for denoising?` in the Reconstruct tomograms were both set to `Yes`. The second step is to apply the trained neural network to patches in the noisy tomograms to obtained the denoised versions. Each of the two steps will be carried out as a separate Denoise tomograms job, as described below.

5.8.1 Train

For the training step, select the Denoise tomograms job type, and on the I/O tab, set:

Input tomograms.star:

Tomograms/job006/tomograms.star

Directory with cryoCARE execuables:

“...”

(The path to the cryoCARE directory on your local machine)

Which GPU to use:

0

On the CryoCARE: Train tab, set:

Train denoising model:

Yes

Tomograms for model training:

TS_01:TS_03:TS_43:TS_45:TS_54

Number of sub-volumes per tomogram:

1200

Sub-volume dimensions (px):

72

On the CryoCARE: Predict tab, set:

Generate denoised tomograms:

No

and press the Run button to run the job.

On our machine, this job took under 1.5 hours to run.

5.8.2 Predict

For the actual denoising step, select a new Denoise tomograms job, and set the fields on the I/O tab to the same values as in the previous Denoise tomograms job. On the CryoCARE: Train tab, set:

Train denoising model:

No

and on the CryoCARE: Predict tab, set:

Generate denoised tomograms:

Yes

Path to denoising model:

Denoise/job007/denoising_model.tar.gz

(The model trained in the previous Denoise job.)

and leave the other fields set to their default values. Running this job is much faster than the training job and in our workspace it took around one minute.

5.8.3 Analysing the results

The *tomogram set* resulting from the `Predict` step (in our case the file `Denoise/job008/tomograms.star`) contains the additional column `rlnTomoReconstructedTomogramDenoised` pointing to the denoised tomograms, which can be visualised, for example, using IMOD or Napari. As mentioned in the *Reconstruct tomograms* section, the denoised tomograms, just like the initial reconstructed odd/even frame tomograms, are used for visualisation and particle picking only, and will not be used for further processing in the sub-tomogram averaging pipeline once the particles have been annotated.

5.9 Particle picking

Particle picking is probably the most difficult part of the subtomogram averaging pipeline. It is important that you pick particles as accurately as possible, so RELION is not trying to align and average particles without the appropriate signal. Alister Burt has written a Napari plug-in to pick particles directly in your tomograms. His picker can be used to pick individual particles, or particles that are randomly sampled from geometric shapes (curved lines for filaments, spheres for capsids or vesicles, or surfaces for membranes or other larger objects. (Please do not use the surface picker – it is not functional yet).

To launch the Napari picker, **again make sure you are working on the computer you are actually sitting behind, as Napari performs poorly over remote connections**, and select the Pick tomograms job type. On the I/O tab, set:

Input tomograms.star
Denoise/job008/tomograms.star

Picking mode
spheres

(The Napari picker has 4 modes of picking: particles, spheres, filaments and surfaces. For the tutorial data we use spheres, because HIV-VLPs are more-or-less that shape. Besides randomly picking particles with the distance specified below, this mode of picking also provides prior angles on the particles that will mean that with a tilt angle of 90 degrees, they will be oriented with their Z-axis along the normal to the sphere surface. In what follows, this prior information will be used in refinements and classification of the subtomograms. Filaments are picked as multiple points forming a (curved) line, with priors that result in tilt=90 angles orienting particles with their Z-axis along the helical axis. Individual particle picking does not provide any priors. Surface picking is not functional yet.)

Particle spacing (A)
60

(This is the inter-particle distance with which particle positions will be randomly sampled from on the sphere. HIV capsid hexamers are approximately 75Å apart. By over-sampling the sphere we reduce the number of missed particles.)

Input particles.star (optional)
“”

(We do not use this field at this point in the pipeline. It can be used to load the particles in a given star file as annotations on the tomogram by providing such a star file in this field together with setting **Picking mode** to `particles`.)

On the Running tab set:

Submit to queue
No

And click on the Run! button to launch the Napari GUI.

Napari will automatically open the first tomogram on the GUI. Keep down (drag) the left mouse button (`lef-mouse + drag`) to rotate the scene; use the scroll wheel to zoom in/out; use `Shift + lef-mouse + drag` on the tomogram plane to move the visualised plane along the Z axis (normal to plane). The `contrast limits` slide bar in the top left side of the screen can be used to adjust contrast in the visualised tomogram plane.

Sphere annotation:
The first step is to locate the centre of the sphere-like object in the tomogram. In the tutorial data, HIV-VLPs

are more-or-less distorted spheres. Move along the Z-dim of a chosen capsid to find the maximum diameter and locate the middle plane of that capsid. Then use `Alt + left-click` or (`Ctrl + Alt + left-click` on some Linux flavours) at that centre to place a small sphere. To resize the sphere press letter `r` and the sphere will be resized to point where the mouse is pointing. You'll need to play a bit to get the right radius of the sphere. The right sphere size is when the surface of the sphere cuts through the middle of the capsid membrane. Then go to a new capsid and repeat the above process. To move on to a new capsid press the letter `n`. To delete or move an existing sphere, select the `n3d spheres` layer on the left side of the screen, then left-click on the centre of the sphere. The sphere can then be moved by drag-and-drop of its centre, or deleted by pressing the `x` button on the top-left of the screen (under `layer controls`). Once you finished annotating all the spheres in one tomogram, press the `save spheres` button. Next you can choose the second tomogram and so on. Once you finished annotating all the tomograms, close the Napari window.

Filaments annotation:
If you're picking filaments, find the start of one and use `(Ctrl +) Alt + left-click` to add a point to its (curved) line; keep adding as many points as you need to describe the curvature. To move on to a new filament press the letter `n`. Once you finished annotating all the filaments in one tomogram press the button `save filaments` and move to the next tomogram.

Surface annotation:
This is not functional yet. Please stay tuned.

Individual particle annotation:
To pick a new indiviual particle, use `(Ctrl +) Alt + left-click`. Napari will show this location with a small blue sphere. To delete or move any particle picked, first select the `n3d points` layer on the Napari GUI (the layer list is on the left of the screen), then select the particle by left-clicking on it. Pressing the `x` button on the top left side of the screen (under `layer controls`) deletes the particle, while pressing the third button (`Select points`) allows you to select a new particle, which can be deleted in the same way, or moved by drag-and-drop. Once you finished picking all the particles in one tomogram, click on the `save particles` button and move to the next tomogram. picking all the tomograms just close the Napari GUI.

After the Napari picker is closed, a python script will save the selected annotations to individual annotation files `*_spheres.star`, `*_filaments.star` or `*_particles.star`, one per tomogram, to the `Picks/job009/annotations` directory, and then write out a file called `Picks/job009/particles.star` with all particles from all tomograms. This star file contains the coordinates of your particles in 3 dimensions under the headers `rlnCoordinate<X/Y/Z>`, which are in (unbinned) pixels starting from (1,1,1) in the top corner of the tomogram. We are currently working to write out these coordinates as `rlnCenteredCoordinate<X/Y/Z>Angst`, which wil be in Angstroms from the centre of the tomogram. Please bear with us as this work is being finished during beta-code development.

For your own data, you may also want to try other particle pickers such as TomoTwin, DeePiCt, DeepFinder, CrYOLO, or others. We strongly recommend only picking in tomograms generated in `ReconstructTomograms` (or `Denoise`) jobs, unless you can verify that the coordinates that you picked in tomograms generated outside of RELION match the coordinates of the RELION tomograms perfectly. Future developments in the ccp-em tomography pipeline will hopefully make using third-party pickers easier.

5.10 Extract subtomos

Now that we have 3D particle coordinates, the Extract job extracts the relevant cropped areas of the tilt series images for each individual particle and saves them as CTF-premultiplied extracted 2D image stacks (or as 3D volumes) on the disk for further processing using the *relion_refine* program. As these are not really boxed out of a 3D tomogram, we call these particles pseudo-subtomograms.

The pseudo-subtomograms can be either 2D stacks or 3D volumes (where Fourier slices of the 2D images are combined in 3D). While both options are available in Extract, we strongly recommend working with 2D stacks, as they take less disk space and, in the case of reconstruction and refinement, this saves an interpolation step and therefore avoids artifacts related to pseudo-subtomogram construction.

Pseudo-subtomogram particles are described by a *particle set* star file, as well as a *tomogram set* star file. In addition, if Bayesian polishing has been performed, the pseudo-subtomograms are also described by a *trajectory set* star file. For convenience, a single *optimisation set* star file provides links to the corresponding three star files. After a Bayesian polishing and/or CTF refinement job, a new set of updated pseudo-subtomos particles should be extracted prior to further refinement or classification by *relion_refine*.

We will start by extracting pseudo-subtomograms in relatively large boxes with a large (binned) pixel size to speed up the initial calculations for obtaining a *de novo* initial model. Select the Extract subtomos jobtype, and on the IO tab set:

Input optimisation set

Picks/job009/optimisation_set.star

(You can see how this file refers to the inidividual *particle set* and *tomogram set* star files using `cat Picks/job008/optimisation_set.star`.)

OR: use direct entries?

No

Input particle set

""

Input tomogram set

""

Input trajectory set

""

On the Reconstruct tab, we set:

Binning factor

6

(This will result in a pixel size of 8.1 Angstroms…)

Box size (binned pix)

192

(This is the box size for the original cropping of the particle from the tilt series images. This box is often set larger than the cropped box size below, because a larger box will allow more of the high-frequency signal to be captured by pre-multiplication of the CTF.)

Cropped box size (binned pix)

96

(This is the final box size of the particles. If this value is smaller than the original box size above, then a second cropping operation is performed after the CTF pre-multiplication.)

Maximum dose (e/A^2)

50

(Tilt series frames with a dose higher than this maximum dose (in electrons per squared Angstroms) will not be included in the 3D pseudo-subtomogram, or in the 2D stack. For 2D stacks, this will reduce disc I/O operations and increase speed.)

Minimum nr. frames

1

(Some particles are outside the field of view (i.e. invisible) in high-tilt images. When set to a positive value, each particle needs to be visible in at least this number of tilt series frames with doses below the maximum dose to be written out as a pseudo-subtomogram.)

Write output as 2D stacks?

Yes

(This is new as of relion-5 and the preferred way of generating pseudo-subtomograms. If set to No, then relion-4 3D pseudo-subtomograms will be written out. Either can be used in subsequent refinements and classifications, but 2D stacks take up less disk space and give slightly better results in 3D refinements. In some cases, we have noticed that 3D pseudo-subtomograms behave better in the VDAM algorithm of 3D initial reference jobs, which is why one can still write in this format too.)

Write output in float16?

Yes

(This will save a factor of 2 in disk space.)

On the Running tab, we aim to saturate the processes on our 112-core CPU node by setting:

Number of MPI procs

5

Number of threads

12

Note that the MPI versions of this program (and those of Reconstruct particle, CTF refinement and Bayesian polishing are parallelized at the level of individual tomograms. Therefore, the `Number of MPI processes` should not exceed the number of tomograms.

Using the settings above, this job took around 17 minutes on our system.

Your pseudo-subtomogram 2D stacks will be stored into MRC files in a new directory called `Extract/job010/Subtomograms/TS_01/1_stack2d.mrcs` etc. The program will also write out an updated *particle set* as `Extract/job010/particles.star` and a new *optimisation set* as `Extract/job010/optimisation_set.star`.

5.11 Import coordinates

While we will not use this functionality in this tutorial, here we describe briefly how one can import coordinates of particles picked outside RELION, for example by using picking software such as `crYOLO` [WMS+19].

We assume, for illustration purpose, that the coordinate files are one text file per tomogram, containing particle coordinates that have been picked in the tomograms obtained from a Reconstruct tomograms or Denoise tomgorams job, with the file names containing the tomogram name (e.g. `rec_TS_01.coords`) and are located in a directory `PARTICLE_COORDS`.

Select Import from the jobt-type browser on the left and fill in the following parameters on the Coordinates tab:

Or Import coordinates instead?

Yes

Input coordinates?

PARTICLE_COORDS/rec_TS_*

(Alternatively, if the particle files are in RELION STAR file format, then the input coordinate file should be a 2-column STAR file with columns `rlnTomoName` and `rlnTomoImportParticleFile` for the tomogram names and their corrsesponding particle coordinate files.)

Remove substring from filenames:

rec_

(This field and the one below allow the import program to find the tomogram name in the file name.)

Second substring to remove

.coords

Text files contain centered coordinates?

No

Multiply coords in text files with:

7.407407

(If the coordinates are with respect to the binning/voxel size in the tomograms in `Denoise/job008/tomograms/`, this is the number that they will be multiplied with so that the resulting coordinates correspond to the pixel size in the motion-corrected tilt series. In this case, this is found in the `rlnTomoTomogramBinning` column of the `Denoise/job008/tomograms.star` file.)

Add this to coords in text files:

0

Inside the newly created directory, you will find the imported *particle set* `particles.star` file. This should then be used as input to a new Extract subtomos job together with the *tomogram set* `tomograms.star` file containing the details of the tomograms used for picking outside RELION:

Input optimisation set:

“”

OR: use direct entries?

Yes

Input particle set:

Import/jobXX/particles.star

Input tomogram set:

Denoise/job008/tomograms.star

This will generate new 2D stacks or 3D pseudo-subtomograms that can be used in the next refinement steps. The resulting particles can be visualised using the Napari plugin in a new Pick tomograms job with `Picking mode` set to `particles`, as explained in the *Visualising the remaining particles* section.

5.12 De novo 3D model generation

relion-5.0 uses a gradient-driven algorithm to generate a *de novo* 3D initial reference from the pseudo-subtomograms. This algorithm is different from the SGD algorithm in the CryoSPARC program [PRFB17]. Provided you have a reasonable distribution of angular directions, this algorithm is likely to yield a suitable low-resolution model that can subsequently be used for 3D classification or 3D auto-refine.

The sample and the sphere picking procedure used in this tutorial make it possible to obtain initial orientations normal to the spheroidal surface during the picking process, which can be used to obtain an initial reference using the Reconstruct particle job, as explained in the *Reconstruct particle* section. The map obtained in this way will be used as a reference for the first 3D auto refine job at bin 6, which will be explained in the *Initial 3D refinement* section.

However, here we will show how to obtain a *de novo* model without any prior knowledge, using the VDAM algorithm. We have noticed that in some cases VDAM generates better initial models using 3D pseudo-sobtomograms rather than 2D stacks, so you may want to try both approaches for your own dataset. To generate 3D stacks, a new Extract subtomos job should be run with the **Write output as 2D stacks?** option set to `No` (see the *Extract subtomos* section).

5.12.1 Running the job

Select the `Extract/job010/optimisation_set.star` file on the I/O tab of the 3D initial reference jobtype. Everything is already in order on the CTF tab. Fill in the Optimisation tab as follows:

Number of VDAM mini-batches

100

(The number of iterations of VDAM. The algorithm will loop over mini-batches, which contain only hundreds to thousands of particles.)

Regularisation parameter T

4

(Values greater than 1 for this regularisation parameter (T in the JMB2011 paper) put more weight on the experimental data. Values around 2-4 have been observed to be useful for 3D initial model calculations.)

Number of classes

1

(Sometimes, using more than one class may help in providing a 'sink' for sub-optimal particles that may still exist in the data set. In this case, which is quite homogeneous, a single class should work just fine.)

Mask diameter (A)

500

Flatten and enforce non-negative solvent

Yes

Symmetry

C6

Run in C1 and apply symmetry later

Yes

(If set to yes, the actual refinement will be run in C1, which has been observed to converge better than performing it in higher symmetry groups. After the refinement, the `relion_align_symmetry` program is run to automatically detect the symmetry axes and the symmetry will be applied.)

Prior width on tilt angle (deg)

10

(Since the picking gives tilt angles so that the particles are normal to surface of the pseudo-spheres, we enforce this prior knowledge here.)

On the Compute tab, set:

Use parallel disc I/O?

Yes

Number of pooled particles:

30

Pre-read all particles into RAM?

No

Copy particles to scratch directory

""

Combine iterations through disc?

No

Use GPU acceleration?

Yes

Which GPUs to use

0

On the Running tab, set:

Number of MPI procs

1

(Remember that the gradient-driven algorithm does not scale well with MPI.)

Number of threads

8

Using the settings above, this job took 90 minutes on our system. If you didn't get that coffee before, perhaps now is a good time too…

5.12.2 Analysing the results

You could look at the output map from the gradient-driven algorithm (`InitialModel/job011/run_it100_class001.mrc`) with a 3D viewer like UCSF chimera. If **Run in C1 and apply symmetry later** was set to `yes`, you should probably confirm that the symmetry point group was correct and that the symmetry axes were identified correctly. If so, the symmetrised output map (`InitialModel/job011/initial_model.mrc`) should look similar to the output map from the gradient-driven algorithm.

5.13 Reconstruct particle

The Reconstruct particle job generates a reference map from a particle set by averaging the particles using positions and orientations given in the input particles file. This reference map will then be used for further processing in 3D auto-refne, 3D classification, CTF refinement or Bayesian polishing.

Continuing the tutorial, we will now generate a first 3D reference map using the particles obtained by the previous Extract subtomos job at bin 6.

5.13.1 Running the job

Select the IO tab from the Reconstruct particle jobtype.

Input optimisation set:

Extract/job010/optimisation_set.star

OR: use direct entries:

No

Input particle set:

Input tomogram set:

Input trajectory set:

On the Average tab, make sure the following is set to reconstruct particles with a binning factor of 6:

Binning factor:
6

Box size (binned pix):
192

Cropped box size (binned pix):
96

Wiener SNR constant:
0

Symmetry:
C6

On the Running tab, set:

Number of MPI procs:
5

Number of threads:
12

Note that reconstructing from 2D tilt series *relion_tomo_reconstruct_particle* program has 3 thread arguments. The number of threads provided here sets both `--j` and `--j_out` arguments so, to avoid exceeding the available memory resources, the `--mem` argument should also be set using around 80-90% to keep a safety margin. In our system, we ran 1 MPI process per cluster node (64Gb) so we added:

Additional arguments:
--mem 50

Using the settings above, this job took less than 5 minutes on our system.

5.13.2 Analysing the results

You can look at the output map `Reconstruct/job011/merged.mrc` with a 3D viewer like UCSF chimera. When random halfsets are specified in the input particles file via the `rlnRandomSubset` field, the two halfmaps `half1.mrc` and `half2.mrc` are also generated.

5.14 Initial 3D refinement

Once we have an initial reference map, one may use the 3D auto-refine procedure in relion to refine the dataset to high resolution in a fully automated manner. This procedure employs the so-called **gold-standard** way to calculate Fourier Shell Correlation (FSC) from independently refined half-reconstructions in order to estimate resolution, so that self-enhancing overfitting may be avoided [SC12]. Combined with a procedure to estimate the accuracy of the angular assignments [Sch12b], it automatically determines when a refinement has converged. Thereby, this procedure requires very little user input, i.e. it remains objective, and has been observed to yield excellent maps for many data sets. Another advantage is that one typically only needs to run it once, as there are hardly any parameters to optimize.

However, as the pseudo-subtomogram files require more memory resources compared to SPA, we suggest running this procedure in several steps, from high binning factors to 1, to improve processing time. Since the first set of pseudo-subtomograms have been extracted at binning factor 6, we will start the 3D refinement using those same particles.

5.14.1 Running the auto-refine job at bin 6

On the I/O tab of the 3D auto-refine job-type set:

Input optimisation set:

Extract/job010/optimisation_set.star

(If an optimisation set file is provided, the input particles and tomograms STAR files are set based on its content.)

OR: use direct entries?

No

(Since we provide an optimisation set file in the field above, we will not be providing a particle set, tomogram set or trajectory set.)

Input particle set:

“”

(Note this is blank as it is extracted from the optimisation set file.)

Input tomogram set:

“”

(This is also blank as it is given in the optimisation set file.)

Input trajectory set:

“”

(This is blank as we have not run a Bayesian polishing job yet.)

Reference map:

Reconstruct/job011/merged.mrc

Reference mask (optional):

“”

(We’re not using a mask at this point, so leave this empty for now.)

On the Reference tab, set:

Ref. map is on absolute greyscale?

Yes

Resize reference if needed?

Yes

Initial low-pass filter (A)

60

(We typically start auto-refinements from low-pass filtered maps to prevent bias towards high-frequency components in the map, and to maintain the *gold-standard* of completely independent refinements at resolutions higher than the initial one.)

Symmetry

C6

On the CTF tab set:

Do CTF correction?

Yes

Ignore CTFs until first peak?

No

On the Optimisation tab set:

Mask diameter (A):

500

and keep the defaults for the remaining options.

Note that the box size at bin 6 is 96 x 8.1Å = 777.6Å, so setting a large mask diameter of 500Å (remember the HIV capsid hexamers are 75Å apart) in the first 3D auto-refine job at bin 6 allows us to use more information in the low-resolution images to obtain a first round of particle alignments and a map that will then be further refined with a smaller mask of diameter 230Å and a smaller binning factor (i.e. higher resolution).

On the Auto-sampling tab, one can usually keep the defaults. Note that the orientational sampling rates on the Auto-sampling tab will only be used in the first few iterations, from there on the algorithm will automatically increase the angular sampling rates until convergence. Therefore, for all refinements with less than octahedral or icosahedral symmetry, we typically use the default angular sampling of 7.5 degrees, and local searches from a sampling of 1.8 degrees. Only for higher symmetry refinements we use 3.7 degrees sampling and perform local searches from 0.9 degrees.

The last two fields on the Auto-sampling tab are set as follows:

Use finer angular sampling faster?

No

(If set to yes, the refinement is more aggresive in proceeding with iterations of finer angular sampling. This will speed up the calculations at the potential cost of suboptimal convergence. Therefore, if using this option, you might want to check that you are not obtaining suboptimal alignments in the early refine jobs and not losing resolution in the later stages of your own processing.)

Prior width on tilt angle (deg)

10

(This field has the same purpose as in the 3D initial reference job: enforcing priors on the tilt angle of the particles. Since we know from the sphere picking procedure that the particles are normal to the surface of the spheres, we can use this knowledge to speed-up convergence.)

Ignore the Helix tab, and on the Compute tab set:

Use parallel disc I/O?

Yes

Number of pooled particles:

30

Skip padding?

No

Pre-read all particles into RAM?

No

Copy particles to scratch directory

“”

Combine iterations through disc?

No

Use GPU acceleration?

Yes

Which GPUs to use

(Set the id sequence of the GPU cards separated by colon (`0:1:2`) or leave blank to automatically use all configured cards)

On the Running tab, set:

Number of MPI procs

5

Number of threads

6

As the MPI nodes are divided between one leader (who does nothing else than bossing the others around) and two sets of followers who do all the work on the two half-sets, it is most efficient to use an odd number of MPI processors, and the minimum number of MPI processes for 3D auto-refine jobs is 3. Memory requirements may increase significantly at the final iteration, as all frequencies until Nyquist will be taken into account, so for larger sized boxes than the ones in this test data set you may want to run with as many threads as you have cores on your cluster nodes.

Before pressing the Run! button, we give this job the alias `bin6` so we can refer to it easily later.

On our computer with 2 GPUs, this calculation took approximately 3.5 hours.

5.14.2 Analysing the results

At every iteration the program writes out two `run_it0??_half?_model.star` and two `run_it0??_half?_class001.mrc` files: one for each independently refined half of the data. Only upon convergence a single `run_model.star` and `run_class001.mrc` file will be written out (without `_it0??` in their names). Because the two independent half-reconstructions are joined together in the last iteration, the resolution will typically improve significantly. This iteration also requires more memory and CPU, as the program will use all the data up to Nyquist frequency.

Note that the automated increase in angular sampling is an important aspect of the auto-refine procedure. It is based on signal-to-noise considerations that are explained in [Sch12b], to estimate the accuracy of the angular and translational assignments. The program will not use finer angular and translational sampling rates than it deems necessary (because it would not improve the results). The estimated accuracies and employed sampling rates, together with current resolution estimates, are stored in the `_optimiser.star` and `_model.star` files, but may also be extracted from the stdout file. For more information, check the SPA tutorial *high-resolution 3D refinement* step.

The program also writes an optimisation set `run_optimisation_set.star` file, updated with `run_data.star` (i.e. the particles file) and the tomograms and trajectories files (given as input to the 3D auto-refine job). This `run_optimisation_set.star` file should not be confused with the `_optimiser.star` files used regularly by *relion_refine*.

This job will have likely reached Nyquist frequency so, to go to higher resolution, we will need a new set of pseudo-subtomo particles at a smaller binning factor, 2 or directly 1.

5.14.3 Pseudo-subtomograms at bin 2

We will now perform 3D refinement at binning factor 2, which will lead to a higher resolution features than the previous binning factor. To do this, we first need to extract a new set of pseudo-subtomograms at binning factor 2. Go to the Extract subtomos jobtype on the GUI, and on the I/O set:

Input optimisation set:

Refine3D/job012/run_optimisation_set.star

On the Reconstruct tab, make sure the following is set to extract particles with a binning factor of 2:

Binning factor:
2

Box size (binned pix):
256

Cropped box size (binned pix):
128

The other parameters are the same as in the previous Extract subtomos job:

Maximum dose (2/A^2)
50

Minimum nr. frames
1

Write output as 2D stacks?
Yes

Write output in float16?
Yes

5.14.4 Obtaining a 3D reference at bin 2

Having extracted a new set of particles at binning factor 2, we will now obtain a 3D reference map at the same binnig factor. Select the Reconstrcut particle jobtype on the GUI, and set in the I/O tab:

Input optimisation set:
Extract/job013/optimisation_set.star

OR: use direct entries?
No

and on the Average, set:

Binning factor:
2

Box size (binnied pix):
256

Cropped box size (binned pix):
128

Symmetry:
C6

Then run the job with the same settings on the Running tab as in the previously run Reconstruct particle job. With the newly extracted bin 2 particles and 3D reference, we will now proceed to the bin 2 3D auto-refine job.

5.14.5 Running the auto-refine job at bin 2

On the I/O tab of the 3D auto-refine job-type set:

Input optimisation set:
Extract/job013/optimisation_set.star

OR: use direct entries?

No

(Note that the input particle set, input tomogram set and input trajectory set are empty as this information is extracted from the optimisation set file.)

Reference map:

Reconstruct/job014/half1.mrc

(Here we use the resulting map from the bin 6 3D auto-refine job.)

On the Reference tab, set:

Ref. map is on absolute greyscale?

Yes

Resize reference if needed?

Yes

Initial low-pass filter (A)

20

(We set the low-pass filter slightly below the reached resolution in the previous step. In this case, it is the Nyquist resolution at binning factor 6.)

Symmetry

C6

On the CTF tab set:

Do CTF correction?

Yes

Ignore CTFs until first peak?

No

On the Optimisation tab set:

Mask diameter (A):

230

On the Auto-sampling tab, to resume the refinement from the current resolution, we could adjust the angular sampling below the angular resolution given the initial low-pass filter argument and mask diameter. A coarse estimation can be obtained by $\arctan(\frac{resolution*2}{diameter})$. In our case:

Initial angular sampling:

7.5 degrees

Use finer angular sampling faster?

No

On our computer with 2 GPUs, we used 5 MPIs and 6 threads, and this calculation took approximately 7.5 hours. Again, the 3D refinement will have reached Nyquist resolution.

Before doing further refinement at binning factor 1, we need to eliminate the duplicate particles that would lead to an overestimated resolution, as well as the bad particles that do not have sufficient information for high-resolution refinement. We will do this in the next two sections.

5.15 Duplicate particles removal

Before continuing with higher-resolution refinement of the structure we have so far, now is a good time to discard any unwanted particles, which are either duplicates or low quality. In this section, we address the problem of duplicate particles.

As the initial particle picking consists of random sampling of positions on the annotated spheres, these positions are gradually refined into positions of particles in the tilt series that resemble projections of the structure being subtomogram averaged. Consequently, it is likely that multiple particle positions converge to the same, or very close, positions in the tilt series (and equivalently in the tomogram). This is problematic because, in addition to unnecessarily refining the same particle multiple times in each refinement cycle (and therefore wasting time and computational resources), the same particle can appear in both random halfsets during refinemt, which leads to overestimated resolution estimates, since the gold-standard FSC criterion is no longer satisfied. Therefore, duplicate particles must be removed from the particle set.

To do this, go to the Subset selection job-type, and on the I/O tab, write the path of the previous job's output particles file in the appropriate field:

Select classes from job:
    “”

OR select from micrographs.star:
    “”

OR select from particles.star:
    Refine3D/job015/run_data.star

Leave all the other fields at their default values, except on the Duplicates tab:

OR: remove duplicates?
    Yes

Minimum inter-particle distance(A)
    30

(Since the distance between two adjacent hexamers is approximately 75Å, we can reasonably assume that any particles closer than 30Å will eventually converge to the same particle, and therefore should be considered duplicates.)

We give this job the alias `remove-dups` and press the Run! button. The duplicated particles are then removed and the resulting duplicate-free particle set is in `Select/remove-dups/particles.star`.

In our workspace, this job removed 8937 duplicated particles from the initial 30596 particles.

5.16 3D classification

After removing the duplicate particles from the particle set, we will now perform 3D classification in order to filter out bad particles that will prevent us from obtaining a high-resolution structure in the subsequent refinement steps at binning factor 1.

As the particles were initially picked at random on the spherical annotations, while the HIV capsids are not strictly spherical, it is likely that some of the particles we have refined up to this point either do not correspond to actual hexamers at all (in the case when the spherical annotation and the capsid do not coincide in the tomogram) or were sampled from regions where the data itself does not contain high-resolution information or has artifacts.

Therefore, assuming we have obtained a good low-resolution reference map, we will now classify the particles based on how well they align with the reference and discard those that align poorly, which will show as blurry, or completely incorrect, 3D classes.

5.16.1 Running the 3D classification job

Select the 3D classification job-type, and on the I/O tab, set the appropriate files:

Input optimisation set:

“”

OR: use direct entries?

Yes

(Since we ran the Subset selection job to remove the duplicate particles in the previous step, we will now use its output particles file as the particle set instead of the previously used optimisation set. The tomogram set will be the same as the one in the previous optimisation set file.)

Input particle set:

Select/job016/particles.star

Input tomogram set:

Denoise/job008/tomograms.star

Reference map:

Refine3D/job015/run_class001.mrc

On the Reference tab, ensure that the following two fields are set as follows:

Initial low-pass filter (A):

60

(We low-pass filter the reference map at a low enough resolution in order not to bias the refined classes towards high-resolution features.)

Symmetry:

C1

(At this point, the aim is to find the bad particles in the particle set rather than to obtain the best reconstruction, so we perform the classification without enforcing the symmetry, since the bad particles are not necessarily symmetric. The good (and symmetric) particles will end up assigned to a class that hopefully results in a symmetric map.)

Leave the fields on the CTF tab with their default values, then in the Optimisation tab leave the default values for all fields except:

Number of classes:

9

(There is no right number of classes, and in your own run of the pipeline it may be useful to experiment with different numbers to ensure that the largest number of good particles is selected. Note that the computational cost of 3D classification scales linearly with the number of classes, both in terms of CPU time and memory.)

Mask diameter (A):

230

On the Sampling tab, change the fields:

Perform image alignment?

Yes

Angular sampling interval:

1.8 degrees

Perform local angular searches?

Yes

(As the purpose of this classification step for this particular dataset is to see which particles align well with the reference structure we have so far, rather than to discover heterogeneity in the data, we only perform local alignment of the individual particles.)

Prior width on tilt angle (deg)

10

Ignore the Helix tab and set the fields on the Compute and Running tabs to the same values as in the 3D auto-refine jobs in the *Initial 3D refinement* section:

Number of pooled particles:

30

Use GPU acceleration?

Yes

Number of MPI procs:

5

Number of threads:

6

On our computer with 2 GPUs, this job took around 2 hours for 25 iterations and 21659 particles.

5.16.2 Analysing the results of the 3D classification

For an overview of the the resulting classes in the final iteration, use the Display: button on the 3D classification job that has just completed and select `out:run_it025_optimiser.star`, which will open a Relion Display GUI. This allows you to see central slices through all classes side-by-side. Before pressing the Display! button on the Relion Display GUI, it is useful to set a few fields as follows.

The `Min` and `Max` fields set the intensity range of the displayed images, and setting them to non-zero values allows you to see all class images in the same intensity range. This is useful to see which classes have low intensity (i.e. little mass inside the volume), which otherwise would be difficult to assess on a normalised scale for each image. The specific values to set depend on the resulting intensities after each run; in our data for this particular 3D classification run, the following values were useful:

Min

-0.01

Max

0.04

Another useful field to tick is

Sort images on:

rlnClassDistribution

as well as the `Display label?` field. Finally, press the Display! button and the class images will be shown in the order of the fraction of the total number of particles assigned to each class, with the fraction shown as the label of each image. Right-clicking on individual images and selecting `Show metadata this class` will open a new window with more information about the class, such as the number of particles in it.

It is also possible to see 2D slices through each class map by going back to the main Relion GUI and clicking on Display: and `out:run_it025_class00X.mrc` for any class index `X`. This will open the Relion Display GUI and pressing Display! will open a new window with the individual slices through the map. Alternatively, the individual map files are located in `Class3D/job017/run_it025_class001.mrc` and can be opened with a 3D volume viewer such as UCSF chimera.

5.16.3 Discarding the bad particles

Next, we want to only keep in our dataset the particles belonging to the best classes obtained in the 3D classification and discard the rest. To do this, go to the Subset selection job-type and in the I/O tab input the `_optimiser.star` file from the previous 3D auto-refine job into the appropriate field:

Select classes from job:
Class3D/job017/run_it025_optimiser.star

Leave all the other fields in all tabs set to their default values (set the `remove duplicates?` field in the Duplicates tab to `No` if it was set otherwise from the previous job) and press the Run! button. This will open the Relion Display GUI, and after setting the fields to the same values as explained above, press the Display! button, which will open the window showing all classes side-by-side.

Select the good classes by clicking on the individual class images – the selected classes will have a red border, then right-click on one of the selected images and select `Save selected classes`. The output of the job in the main Relion GUI will show a message indicating that the `particles.star` file has been saved and the number of selected particles it contains. You can now safely close the Relion Display GUI windows, and the new particles file containing only the particles in the selected classes is `Select/job018/particles.star`.

In our workspace, the resulting `particles.star` file now contains 9442 particles.

5.16.4 Visualising the remaining particles

To visualise the particles we have left as 3D annotations on the tomograms, we can launch the Napari picker with `picking mode: particles` (also see *Particle picking*), with the additional particles file given as the optional input.

First, make sure that the Relion GUI is running locally, as the Napari plugin is slow over the network. Then, select the Pick tomograms job type and set the following fields on the I/O tab:

Input tomograms.star:
Denoise/job008/tomograms.star

Picking mode:
particles

Particle spacing(A):
“”

(Since we selected the `particles` picking mode above, this field will be ignored.)

Input particles.star (optional):
Select/job018/particles.star

(The particle set file that we want to visualise.)

Running the job will generate the particle annotation files `Picks/job019/annotations/TS_XX_particles.star` from the input `particles.star` file and start the Napari plugin, where you can visualise the particle annotations and manipulate them as explained in the *Particle picking* section. After clicking the `save particles` button and closing the Napari window, new `particles.star` and `optimisation_set.star` files will be created, which can be used in subsequent steps.

The procedure to perform 3D classification using pseudo-subtomograms is the same as in the single particle analysis pipeline, so you may find it useful to also read the description in the *SPA tutorial*.

5.17 High-resolution 3D refinement

At this point in the tutorial, we have obtained a refined map at binning factor 2, we have removed any duplicate particles, as well as any bad particles that do not align well with our map. We are now ready to perform 3D refinement at binning factor 1.

5.17.1 Pseudo-subtomograms and reference map at bin 1

First, we need to generate new pseudo-subtomograms and a reference map at binning factor 1. The order of running the Extract subtomos and Reconstruct particle jobtypes is irrelevant as long as they are run consecutively to keep track of the *optimisation set* file. In addition, since the previous job we ran was a Subset selection job, we must use its output particles file as the input particle set to the next job (either Extract subtomos or Reconstruct particle):

Input optimisation set:
: “”

OR: use direct entries?
: Yes

Input particle set
: Select/job018/particles.star

Input tomgoram set
: Denoise/job008/tomograms.star

Input trajectory set
: “”

For 3D refinement at binning factor 1, make sure the following options are properly set on Extract subtomos Reconstruct tab and Reconstruct particle Average tab:

Binning factor:
: 1

Box size (binned pix):
: 512

Cropped box size (binned pix):
: 192

5.17.2 Masks for high-resolution refinement and FSC estimation

Next, we need to generate appropriate masks to help the refinement process and FSC assessment. In the particular case of the HIV capsid, we provide the following masks:

1. `mask_align.mrc`, which we will use in the `Reference mask` field of the 3D auto-refine job: Since the refined region in our structure so far contains both the HIV capsid and the matrix, we need to make sure that we only focus on the capsid. Moreover, this mask is nearly as wide as the 230Å diameter mask applied to the images during refinement, ensuring that we use as much of the information available in the volume as possible to align the particles.
2. `msk_fsc.mrc`, which we will use in the `Solvent mask` field of the Post-processing job (see *Post-processing* below). Since we use a wide mask to improve the alignment of particles during refinement, the volume we refine will consist of more than one hexamer. In order to asssess the reconstruction quality of the central hexamer only, we use a tighter mask around it for FSC calculations.

The above masks can be created externally using tools like UCSF chimera or *dynamo_mask_GUI* from the dynamo package. For general mask creation, refer to SPA tutorial *mask description*.

In order to use the provided masks, we suggest recentering the reference map to ensure that the masks and the reference are aligned (so that the masks focus on the capsid). You could look at the output reference map from the previous step (`Reconstruct/job025/merged.mrc`) and the mask (`masks/mask_align.mrc`) with a 3D viewer like IMOD 3dmod to estimate the Z offset between both maps, in pixels. In our case, it is 2.75 pixels but this could be different as it depends on the initial *de novo* model. Thus, recentering the particles can be done from the command-line:

```
relion_star_handler --i Extract/job020/particles.star \
--o Extract/job024/particles2.75.star --center --center_Z 2.75
```

To check that the capsid within the reference map is aligned with the mask, we can reconstruct it using the Reconstruct particle job-type, described in *Reconstruct particle*.

5.17.3 Running the auto-refine job at bin 1

On the I/O tab of the 3D auto-refine job-type set:

Input optimisation set:
Extract/job020/optimisation_set.star

OR: use direct entries:
No

(If a new particles file has been generated in the previous step during recentering, this option should be set to `Yes` and the correct input particle set and tomogram set files should be used.)

Reference map:
Reconstruct/job021/half1.mrc

(Once we reach a high enough resolution in the refinement process, it is important to keep the two halfsets entirely separate in order to obtain a gold-standard reconstruction. Halfmap files should be used as reference maps for each halfset processed by *relion_refine*, keeping the 3D refinement of both halfsets independent along the whole workflow. To that end, when the reference map file name contains either `*half?*.mrc` template, each halfmap is automatically assigned to its halfset.)

Reference mask (optional):
mask_align.mrc

On the Reference tab, set:

Ref. map is on absolute greyscale?
Yes

Resize reference if needed?
Yes

Initial low-pass filter (A)
5.5

(We set the low-pass filter slightly below the reached resolution in the previous step. In this case, it's Nyquist resolution at binning factor 2.)

Symmetry
C6

On the CTF tab set:

Do CTF correction?
Yes

Ignore CTFs until first peak?
No

On the Optimisation tab set:

Mask diameter (A):
230

Mask individual particles with zeros?
Yes

Use solvent-flattened FSCs?
Yes

(This option enables the computation of the resolution during refinement using masked halfmaps using the provided `mask_align.mrc` mask file.)

Use Blush regularisation?
No

On the Auto-sampling tab set:

Initial angular sampling:
1.8 degrees

On our system, using 2 GPU cards, this job took around 1.5 hours to run.

We now remove duplicates again by running Subset selection with a minimum inter-particle distance of 50Å to ensure that no other particles converged to the same positions, and then generate a new reference map with Reconstruct particle.

5.17.4 Post-processing

Finally, the procedure to sharpen a 3D reference map and estimate the gold-standard FSC curves for subtomogram averaging is the same as described in the *SPA tutorial*.

Select the Post-processing jobtype, and on the I/O tab, set:

One of the 2 unfiltered half-maps:
Reconstruct/job025/half1.mrc

Solvent mask:
mask_fsc.mrc

(This is the tight mask that only includes the central hexamer.)

We leave the other fields as they are and run the job. The resulting final resolution we see in our workspace is 4Å.

At this point, this is the best alignment we could reach without applying any specific tomo refinement, as shown in the *Tomo refinement cycle* section.

5.18 Tomo refinement cycle

We will now describe the specific tomo refinement steps that will refine the particle-specific CTF parameters, tilt angles and beam-induced deformations, which will lead to higher resolution in our subtomogram-averaged structure.

The tomo refinement jobs are CTF refinement and Bayesian polishing, and one tomo refinement cycle would include both. Moreover, for better improvements, we recommend running multiple tomo refinement cycles.

In the current section, we give an overview of how such a tomo refinement cycle would look.

5.18.1 Reference map and FSC data

Before running each of the tomo refinement jobs (CTF refinement and Bayesian polishing), the following are required:

1. Reference halfmaps obtained by running Reconstruct particle. The implementation of the Reconstruct particle job is consistent with the the implementation of the tomo refinement jobs (e.g. range of the intensities), so it is important to use this reference map rather than maps obtained from 3D auto-refine.
2. Post-processed FSC data for the reference halfmaps obtained by running Post-processing. This is required to estimate the SNR. If not provided, these programs internally calculate it without phase randomization, so SNR will be slightly optimistic.
3. Alignment mask file at binning facor 1
4. *optimisation set*

Note that, independently of the binning factor of the data in the previous steps, CTF refinement and Bayesian polishing protocols process data in the original pixel size (binning 1). Therefore, the reference map should always be reconstructed at this binning level and proper reference and FSC masks should be used.

5.18.2 Running the jobs

The order of CTF refinement and Bayesian polishing is not important, as long as we keep track of the optimisation set file. In our workspace, in each cycle we ran the two jobs as follows:

1. CTF refinement, described in detail in the *Tomo refinement 1: CTF refinement* section, and
2. Bayesian polishing, described in detail in the *Tomo refinement 2: Bayesian polishing* section.

5.18.3 3D refinement

After running both tomo specific refinement steps, it is still recommended to run a new 3D auto-refine job to take advantage of the improved tomograms and particles. To this end, we need to construct a new set of pseudo-subtomos and reference maps as described in the *Pseudo-subtomograms and reference map at bin 1* subsection. For the new 3D auto-refine job, the same parameters as in the *Running the auto-refine job at bin 1* section apply, except for:

On the Reference tab, set:

Initial low-pass filter (A):

4

On the Auto-sampling tab set:

Initial angular sampling:

0.9 degrees

This new 3D refinement step took just under 2 hours on our system (2 GPU cards) and, after Reconstruct particle and Post-processing with the tight mask, we reached a resolution of 3.6Å, completing the first tomo refinement cycle. After another four full tomo refine cycles, we reached 3.3Å, and depending on the quality of the picked particles, it is also possible to obtain 3.2Å.

5.19 Tomo refinement 1: CTF refinement

In this step we will show how to apply the refinement of the different parameters related to the CTF estimation such as tilt series projection defocus and contrast scale. For a full description of the arguments, check the *relion_tomo_refine_ctf* program.

If you are running this job without following the tutorial, please note the requirements for the reference map and FSC data as described in *Reference map and FSC data*.

5.19.1 Running the job

Since we ran both Reconstruct particle and Post-processing after the bin 1 refinement step at the end of the *High-resolution 3D refinement* section, we already have the required reference halfmaps (`Reconstruct/job025/half<12>.mrc`) and post-processing FSC data (`PostProcess/job026/postprocess.star`).

In addition, we can use the *optimisation set* file from a previous job or, in this case, the explicit *particle set* from the duplicate removal step and the separate *tomogram set* (which would otherwise be included in the optimisation set).

Lastly, the reference mask file used in the previous 3D auto-refine at binning factor 1 is also required by the tomo refinement jobs, which in our case is `mask_align.mrc`.

Select the CTF refinement job-type and set on the I/O tab:

Input optimisation set:

“”

OR: use direct entries?

Yes

(Because the previous job run was Subset selection to remove duplicate particles, we will use the resulting particles file as the input particle set. Otherwise, we could have used the optimisation set file directly from the previous 3D auto-refine job.)

Input particle set:

Select/job024/particles.star

Input tomogram set:

Denoise/job008/tomograms.star

Input trajectory set

“”

(This is empty in the first tomo refinement cycle, unless Bayesian polishing is run first, in which case we would include the generated `motion.star` file, unless it already is included in the optimisation set file.)

One of the 2 reference half-maps:

Reconstruct/job025/half1.mrc

Reference mask:

mask_align.mrc

Input postprocess STAR:

PostProcess/job026/postprocess.star

On the Defocus tab, set:

Box size for estimation (pix)

512

Refine defocus?
Yes

Defocus search range (Å)
3000

Do defocus regularisation?
Yes

Defocus regularsation lambda
0.1

Refine constrast scale?
Yes

Refine scale per frame?
Yes

Refine scale per tomogram?
No

On the Running tab, set:

Number of MPI procs:
5

Number of threads:
12

With these parameters, the job should take around 10 minutes to run.

5.19.2 Analysing the results

The output folder `CtfRefine/job027` contains a new `tomograms.star` file with the refined parameters. To assess the result, run new Reconstruct particle and Post-processing jobs using the generated `CtfRefine/job027/optimisation_set.star` file. In our workspace, we see a slight improvement in the resolution to 3.87Å.

These reference map and postprocess files will also be used as inputs for the next Bayesian polishing run.

5.20 Tomo refinement 2: Bayesian polishing

relion has also implemented the analogous to *Bayesian polishing* for tomography. This procedure refines the projections that map 3D space onto the images of the tilt series. Optionally, the beam-induced motion trajectories of the particles and deformations can also be estimated. For a complete description of the arguments, check the *relion_tomo_align* program.

If you are running this job without following the tutorial, please note the requirements for the reference map and FSC data as described in *Reference map and FSC data*.

5.20.1 Running the job

Select the Bayesian polishing job-type and set on the I/O tab:

Input optimisation set:
CtfRefine/job027/optimisation_set.star

OR: use direct entries?
No

One of the 2 reference half-maps:
    Reconstruct/job028/half1.mrc

Reference mask:
    mask_align.mrc

Input postprocess STAR
    PostProcess/job029/postprocess.star

On the Polish tab, set:

Box size for estimation (pix)
    512

Max position error (pix)
    5

Align by shift only?
    No

Alignment model

    (Does not apply)

On the Motion tab, set:

Fit per-particle motion?
    Yes

Sigma for velocity (Å/dose)
    0.2

Sigma for divergence (Å)
    5000

Use Gaussian decay
    No

On the Running tab, set:

Number of MPI procs:
    5

Number of threads:
    12

Note that the per-particle motion estimation increases the processing time significantly. On our system it took around 2 hours.

5.20.2 Analysing the results

In the output folder `Polish/job030` you will find new `tomograms.star` and `particles.star` files including the corrected tilt series alignment and particle positions and a *trajectory set* file `motion.star` with particle trajectories. To assess the result, we generate new particles with the Extract subtomos job using the resulting `optimisation_set.star` file, followed by Reconstruct particle and Post-processing. Compared to the previous FSC estimation, we observe a clear improvement and a resolution of 3.65Å.

5.21 Model building with ModelAngelo

Building an atomic model using `ModelAngelo` [JKZ+24] is done in the same way as in the *SPA tutorial*. First, download the protein sequence as a FASTA file from this link (`Download Files -> FASTA Sequence`).

5.21.1 Running the job

Select the ModelAngelo building job type and, on the I/O tab, set:

B-factor sharpened map:
PostProcess/job079/postprocess_masked.mrc

FASTA sequence for proteins:
rcsb_pdb_5L93.fasta

After inputing the path to the `ModelAngelo` executable and the GPUs to use and making sure that `Perform HMMer search?` is set to `No` on the Hmmer tab, we run the job.

5.21.2 Analysing the results

On our machine with 2 GPUs, this job took 3 minutes to run. The output is a coordinate file called `ModelAngelo/job080/job080.cif` that may be used in UCSF chimera together with the `Postprocess/job079/postprocess.mrc` map. Note that although `ModelAngelo` did a very good job on this relatively easy test case, you should always check its results carefully in a program like `coot` [ELSC10] . You will also need to perform a stereochemical refinement of the coordinates. For this, we like `servalcat` [YPBM21].

CHAPTER

SIX

ON-THE-FLY PROCESSING

As of relion-4.0, on-the-fly processing is based on the *Schemes* functionality. This has been implemented together with a small *Tkinter* GUI that was written by Colin Palmer from CCPEM for the `relion_it.py` python script that was distributed with relion-3.0 and 3.1. The new program still carries the same name, and can be launched from your Project directory from the command line:

```
relion_it.py &
```

This will launch the GUI, which contains several sections that need to be filled in by the user.

Note

This script depends on pre-configured `Schemes/prep` and `Schemes/proc` *Schemes* that are distributed inside the *scripts* directory on the relion source code. To find these, the environment variable `RELION_SCRIPT_DIRECTORY` needs to be set to point to this directory. You can also use this variable to point towards your own modified version of the *Schemes*.

6.1 Computation settings

This section specifies what calculations will be performed.

Do MotionCorr & CTF?

v

(If selected, a *Scheme* called `Schemes/prep` will be lanuched that will loop over all movies (as defined in the section *Preprocessing settings* to run Import, Motion correction and CTF estimation. By default this will be done for a maximum of 50 movies at a time.)

micrographs_ctf.star:

Schemes/prep/ctffind/micrographs_ctf.star

(This option is only used if *Do MotionCorr & CTF?* is not selected. In that case, the user can provide the output STAR file from a previous CTF estimation job to perform the rest of the processing on.

Do Autopick & Class2D?

v

(If selected, a second *Scheme* called `Schemes/proc` will be lanuched that will automatically pick particles, as specified on the *Particle settings* and *Processing settings* sections, and then perform 2D classification and automated class selection through the Subset selection job. This is repeated on a loop, while the number of particles extracted is still increasing.)

Do Refine3D?

v

(If selected, the iteration of the second *Scheme* will also comprise a 3D auto-refine job.)

3D reference:

None

(If set to *None*, or anything else that does not exist as a file, then an 3D initial model job will be launched before the auto-refine job. Note that in the case of symmetry, as defined in the *Particle settings* section, the initial model calculation is still performed in C1, but this calculation is followed by an alignment of the symmetry axes and the application of the symmetry to the model.)

GPUs (comma-separated):

0,1

(This option is used to specify the IDs of the GPU devices you want to run on. Since the motion correction and CTF estimation are CPU-only, this option is only used by the second *Scheme*.)

6.2 Preprocessing settings

This section specifies information about where the movies are and how they were recorded.

Pattern for movies:

Movies/*.tiff

(This specifies where the movies are. If you have recorded movies in the `.eer` format, it is recommended that you convert them to `.tiff` format during the copying process from the microscope computer to your processing computer.)

Gain reference (optional):

Movies/gain.mrc

(Only use this option if your movies have not been gain-corrected yet, otherwise leave empty.)

Super-resolution?

(Click this if your movies are in super-resolution. Note that we do not recommend recording movies in super-resolution, and that they will be binned during the Motion correction job.)

Voltage (kV):

300

Cs (mm):

2.7

Phase plate?

v

(Click this if you have collected your images with a phase plate. In that case, the CTF estimation job will also estimate the phase shift.)

(Super-res) pixel size (A):

(Provide the pixel size in the movies. If they are in super-resolution, then provide the (smaller) super-resolution pixel size.)

Exposure rate (e-/A2/frame):

1.2

(This is the accumulated dose in a single movie frame.)

6.3 Particle settings

Symmetry:

C1

Longest diameter (A):

180

(The longest diameter will be used to automatically determine the box size below, as well as for LoG and topaz picking.)

Shortest diameter (A):

150

(This will only be used for LoG picking. This value should be smaller or equal than the longest diameter above, and is useful to pick elongated particles.)

Mask diameter (A):

198.0

(This is used for Auto-picking jobs, as well as 2D classification, 3D initial model and 3D auto-refine

Box size (px):

246

(The box size in the original micrograph.)

Down-sample to (px)

64

(To speed up all calculations in the `proc` *Scheme*, all particles will be downsampled to this box size.)

Calculate for me:

v

(This will generate automated suggestions for the mask diameter, the box size and the down-sampled box size. We often use these.)

6.4 Processing settings

Min resolution micrographs?

6

(Only micrographs with an estimated CTF resolution beyond this value will be selected. Set to 999 not to throw away any micrographs.)

Retrain topaz network?

v

(If this is selected, then the `proc` *Scheme* will first use the below specified number of particles for an initial 2D classification and automated class selection in Subset selection. The selected particles are then used to re-train the neural network in topaz for this data set. Once the re-training is finished, the entire data set will be picked using topaz.

Nr particles for Log picking:

10000

(The number of particles used for LoG picking.)

LoG picking threshold:

`0`

(The threshold to LoG pick particles.

LoG class2d score:

`0.5`

(The threshold to automatically select 2D class averages from the LoG picked particles. A value of 0 means rubbish classes; a value of 1 means gorgeous classes.)

Topaz model:

`Schemes/proc/train_topaz/model_epoch10.sav`

(If one does not retrain the topaz network, then this option can be used to provide a pre-trained network. If this option is left empty, then the default general network inside topaz is used.)

Nr particles per micrograph:

`300`

(The expected number of particles per micrograph, which is used both for topaz training and picking.)

Topaz picking threshold:

`0`

(The topaz threshold to select particle. Using negative values, e.g. -3, will pick more particles.)

Topaz class2d score:

`0.5`

(The threshold to automatically select 2D class averages from the LoG picked particles. A value of 0 means rubbish classes; a value of 1 means gorgeous classes.)

Finally, the GUI has two action buttons:

The Save options button will save the currently selected options to a file called `relion_it_options.py`. This (together with any other options files) can be read in when launching the GUI a next time from the command line:

```
relion_it.py relion_it_options.py [extra_options2.py ....] &
```

The Save &run button will also save the options, and it will actually launch the *Schemes* and open the normal relion GUI, from which the progress can be monitored, as explained on the *Schemes* reference page.

6.5 Intervening

Once the *Schemes* are running, you will see new jobs popping up in the normal relion GUI. As soon as you start seeing some results, you may find that you want to change some of the parameters. To make stopping and restarting a *Scheme* easier, there is another GUI: `relion_schemegui.py`. It needs to be launched for each running *Scheme* separately. The Save &run button above, will have launched one for both the `prec` and `proc` *Scheme*, but you can also launch it from the command line:

```
relion_schemegui.py proc &
```

This GUI will look for the hidden directory (`.relion_lock_scheme_proc`) that locks this *Scheme* to see whether it is running or not, and it will update the `Current` entry to indicate at what job or operator the *Scheme* currently is.

To stop a running *Scheme*, press the Abort button and wait for the underlying jobs and the schemer to receive the abort signal. Depending on what the *Scheme* is executing, this may take a bit of time. Once it has been aborted, you can then change options to specific jobs through the `Set Job option` section, or change variables in the *Scheme* through the `Set Scheme variable` section. (The GUI still needs some work here to make this easier and more error-resistant).

After changing variables to any job, it's status will be reverted to `has not started`, meaning that a new relion job will be launched next time the *Scheme* comes across it. For any `continue` type of job (like Motion correction, CTF estimation, Auto-picking or Particle extraction), a new job will only be launched if that job's options were changed, or if the options were changed for any job that came before that job. Otherwise, the job will just continue, and thereby already performed calculations will not be repeated.

To start the *Scheme* again, press the Restart button. The *Scheme* will be executed from the job or operator specified on the `Current` entry. If you want, you can change this from the point where it was aborted. If you want to restart the *Scheme* all the way from the beginning, then press the Reset button, before pressing Restart.

Sometimes, a *Scheme* dies because of an error, not because of it finishing or being aborted. In that case, the lock directory (`.relion_lock_scheme_proc`) needs to be deleted, before the *Scheme* can be used again. Press the Unlock button to print instructions on how to do that. (TODO: implement this through a popup window from the GUI…)

6.6 Control more options

Not all options of all relion jobs, or all of the parameters of the *Schemes* themselves can be controlled from the `relion_it.py` GUI. You can still control all of these through manually editing the `relion_it_options.py` file. For this, use double underscores to separate `SCHEMENAME__JOBNAME__JOBOPTION` for any option. Some options are already in the default file, but any other options can be added.

E.g. to change the number of 2D classes (`nr_classes`) in the `class2d_ini` job of the the `proc` Scheme, you can add the following line to the `relion_it_options.py` file:

```
'proc__class2d_ini__nr_classes', '200',
```

Likewise, use `SCHEMENAME__VARIABLENAME` for variables in the *Schemes* themselves, e.g. to set de `do_at_most` variable, which determines the maximum number of micrographs that are processed in one cycle of the `prep` *Scheme*, edit this line:

```
'prep__do_at_most', '100',
```

You can also save options for the relevant settings for your local setup in a second options file, e.g. `relion_it_options_LMB-Krios1.py`, and then call `relion_it.py` with those, e.g.:

```
relion_it.py relion_it_options_LMB-Krios1.py relion_it_options.py &
```

If the same option is specified in multiple options files, the value in the last file on the command line will be used.

One could even make a specific command for each microscopy setup by using an alias like:

```
alias relion_it_krios1.py 'relion_it.py relion_it_options_LMB-Krios1.py'
```

6.7 Site-specific setup

At the very least, you will need to change the position of the executables for ctffind 4.1 or *Gctf* and topaz, but you may also want to tweak the default settings for number of threads or MPI processors for the different jobs. So, you local setup options file will likely include options like:

```
{
'prep__ctffind__fn_ctffind_exe' : '/wherever/ctffind/ctffind.exe',
'prep__ctffind__fn_gctf_exe' : '/wherever/Gctf/bin/Gctf',
'proc__inipicker__fn_topaz_exe' : '/wherever/topaz/topaz',
'proc__restpicker__fn_topaz_exe' : '/wherever/topaz/topaz',
```

(continues on next page)

(continued from previous page)

```
'proc__train_topaz__fn_topaz_exe' : '/wherever/topaz/topaz',
'prep_motioncorr__nr_threads' : '16',
'proc_restpicker__nr_mpi' : '4',
'proc_extract_ini__nr_mpi' : '4',
'proc_extract_rest__nr_mpi' : '4',
'proc_class2d_ini__nr_threads' : '12',
'proc_class2d_rest__nr_threads' : '12',
'proc_inimodel3d__nr_threads' : '12',
'proc_refine3d__nr_threads' : '8',
'proc_refine3d__nr_mpi' : '3'
}
```

Remember it is also possible to edit the `job.star` and `scheme.star` files inside your own copy of the `Schemes/prep` and `Schemes/proc` directories, and use the environment variable `$RELION_SCRIPT_DIRECTORY` to point towards the modified scripts. That provides an alternative that would no longer rely on specifying an extra options file, and allows maximum flexibility in adopting the schemes to your specific needs.

CHAPTER

SEVEN

REFERENCE PAGES

7.1 Movie Compression

This page describes how to compress CryoEM movies to save disk spaces and reduce I/O loads.

7.1.1 Falcon3 / Falcon4

Falcon3 and Falcon4 output images in 16-bit integers. Gain normalization is always applied and cannot be disabled.

In the counting mode, an electron is not rendered as a single 1 but seems to be placed as a blob of 3x3 pixels that sum to 100. This is probably to reduce noise aliasing from frequencies higher than the Nyquist frequency.

Below is an example from Falcon3. You can see three electrons. Two electrons have overlapping tails:

```
0   0   0   0   0   0   0   0   0   0   0
0   0   3   7   1   0   0   0   0   0   0
0   0  15  42  11  16   4   0   0   0   0
0   0   6  15  14  44  11   3   7   1   0
0   0   0   0   2   7   2  15  42   7   0
0   0   0   0   0   0   0   6  15   3   0
0   0   0   0   0   0   0   0   0   0   0
```

Because of this feature, Falcon3 and Falcon4 movies do not compress well. Typically, one can reduce the size to about 50 to 60 % of the original movie by TIFF with the **deflate filter** (also called zip filter). The LZW filter is faster but gives larger files.

By converting to TIFF from EER, you can avoid this blob convolution. Keep reading below.

7.1.2 Falcon4 EER

EER (Electron Event Representation) is a new movie format for Falcon4. It records electron events at the detector's physical frame rate (248 Hz). The file size is typically smaller than MRC, but can be larger than fractionated TIFF from Falcon4.

The location of events is stored in a 4x super-resolution grid (i.e. 16K x 16K pixels). RELION renders an electron as a single dot in a 4K or 8K grid. By rendering in a 8K grid and then Fourier cropping to a 4K grid, you can remove noise beyond the (physical) Nyquist frequency. If you directly render in a 4K grid, the noise aliases back and slightly lowers the DQE. However, this effect is very tiny in practice and the resolution rarely changes. One possibility is to start processing at 4K and coarse slicing and then switch to 8K and finer slicing in Polish to save processing time and memory (see below).

Note

As of RELION 3.1.1, 8K rendering can create artifacts around defect lines. Thus, we do not recommend 8K rendering.

To process an EER dataset, proceed as follows. This feature needs RELION >= 3.1.1.

1. Decide how many (internal) frames to group into a fraction.

   For example, if you have 1000 internal frames and group them by 30, you will get 33 fractions. The remaining 10 (= 1000 - 30 * 33) frames will be ignored. Too fine slicing leads to very slow processing and out of memory errors.

2. Calculate how many e/A2 each fraction has.

   Fractionate such that each fraction has about 0.5 to 1.25 e/A2. You can change this later.

3. Import EER files as usual.

   Use the physical pixel size for 4K rendering. **For 8K rendering, the pixel size should be half of the physical size.**

4. Run motion correction.

   - Specify the value decided in the step 1 to `EER fractionation`.
   - Specify the dose rate calculated in step 2.
   - Specify the gain reference.
   - `Group frames` in the GUI **must be 1** regardless of what you choose in step 1.
   - **`Binning factor` should be 2 to bring a 8K super resolution grid into a 4K physical grid by Fourier cropping.**
     Otherwise, set it to 1.
   - Add `--eer_upsampling 2` if you work in a 8K grid. The default is 4K, that is, `--eer_upsampling 1`.

If you want to change the rendering mode (4K or 8K) and/or the fractionation before Polish, you have to modify the trajectory STAR files produced by a MotionCorr job. Use `scripts/eer_trajectory_handler.py`:

```
python3 /path/to/eer_trajectory_handler.py --i MotionCorr/job002/corrected_micrographs.
↪star --o up2_gr8 --resample 2 --regroup 8
```

will change the sampling to 8K and the fractionation to 8 frames. The output will be `MotionCorr/job002/corrected_micrographs_up2_gr8.star`, which should be specified to a Polish job. Note that the extraction box size for Polish is in pixels of the rendered movie.

The gain reference for EER has confusing convention due to historical reasons. When the movie is in the EER format and the gain reference is in the MRC format, RELION will **divide** raw pixel values with the provided gain. In contrast, if the gain reference is given in the TIFF format with the `.gain` extension (as written by newer versions of the Falcon software), RELION will **multiply** raw pixel values with the provided gain (as is done for K2/K3). In any case, when the gain is zero, the pixel is considered as defective. The gain reference can be 8K x 8K or 4K x 4K. If the size of the gain reference and the size requested by `--eer_upsampling` do not match, the gain reference is upsampled / downsampled.

If memory usage is a concern, consider building RELION in CPU single precision (`cmake -DDoublePrec_CPU=OFF`).

Another useful tool is `relion_convert_to_tiff`, which renders an EER movie into a compressed integer TIFF. You can render in 4K or 8K (`--eer_upsampling`) with specified fractionation (`--eer_grouping`). This can further reduce file sizes by sacrificing spatial and temporal resolutions to a reasonable value. Due to the different meanings of the gain reference for EER and TIFF, you have to take the inverse of the EER gain reference before processing the resulting TIFF files. The tool performs this conversion when the EER gain reference is specified in the `--gain` option.

Note

Old versions of EPU display the one-seventh of the real number of frames in EER, as reported in CCPEM. This issue has been fixed in the latest EPU release. In case of doubt, check the real number of frames by running `relion_convert_to_tiff` on one of the movies. The command will print "Found X raw frames."

7.1.3 Gatan K2 / K3 in counting or super-resolution mode

Gain non-normalised movies

Always use the gain **non-normalised** mode. Then the images represent electron counts and consist of mostly 0s, a few 1s, fewer 2s, even fewer 3s and so on. Below is an example:

```
1   0   0   0   0   0   0   0
0   1   0   3   1   1   0   2
1   0   0   1   0   1   0   0
2   1   0   0   0   0   0   1
0   2   0   1   0   0   0   0
```

Such images compress extremely well by TIFF with the **LZW filter**. The deflate filter is much slower and gives larger files.

SerialEM can directly write compressed TIFF movies. EPU cannot write movies in TIFF but in integer (8 bit for counting mode, 4 bit for super-resolution mode) MRC, which can be converted to TIFF later.

Update: According to Grigory Sharov at MRC-LMB, TIFF output is available in EPU >=2.4 for K3 and EPU >=2.9 with TEM server >=7.6 (requires Windows 10) for K2.

Gain normalised movies

The gain reference looks like below:

```
1.149962  1.083618  1.198896  1.140650  1.159426  1.063172  1.204020  1.145287
1.075346  1.122473  1.173919  1.149962  1.159426  1.051271  1.178831  1.071257
1.043484  1.009823  1.109215  1.100549  1.183784  1.100549  1.126962  0.978266
1.193816  1.047363  1.246640  1.214399  1.193816  1.067199  1.051271  0.999080
1.131488  1.159426  1.304355  1.051271  1.035811  1.131488  0.974881  0.988563
```

If you save movies in the gain normalised mode, the electron counts are multiplied by this to yield:

```
1.149962  0.000000  0.000000  0.000000  0.000000  0.000000  0.000000  0.000000
0.000000  1.122473  0.000000  3.449886  1.159426  1.051271  0.000000  2.142514
1.043484  0.000000  0.000000  1.100549  0.000000  1.100549  0.000000  0.000000
2.387632  1.047363  0.000000  0.000000  0.000000  0.000000  0.000000  0.999080
0.000000  2.318852  0.000000  1.051271  0.000000  0.000000  0.000000  0.000000
```

Now, values are 32-bit floating points, which hardly compress.

If we *knew* the gain reference, we could divide pixel values by the gain to bring them back to integers. However, the gain reference is not written in the gain normalised mode. Fortunately, there is a way to reliably estimate the gain reference.

Consider a particular pixel and look at the values over many frames. The values should be integer multiples of its gain, for example:

```
0.000000  2.21843  0.0000000  1.109215  0.000000  0.000000  1.109215  3.327645  0.000000␣
↪...
```

Thus, we can estimate the gain by finding the greatest common divisor among these values. Since the dose rate of counting mode movies is very low (otherwise, you will have coincidence losses), it is highly probable that the list contains an observation corresponding to one electron. Thus, one simply needs to find the smallest **positive** value and use it as the gain for this pixel. In this case, it is 1.109215.

There is one complication. Digital Micrograph applies defect correction when working in the gain normalised mode. Values of such pixels are no longer integer multiples of their gain and the above trick does not work. For such pixels, one can keep the original values and set the gain to 1.000000. Then gain multiplication does not modify such pixels. The output remains 32bit floating point numbers, not integers, but since most values are 0.000000, 1.000000, 2.000000, 3.000000, etc except for defect pixels, the entropy is smaller than the input and compression is more efficient.

7.1.4 relion_convert_to_tiff

The command `relion_convert_to_tiff` implements above compression schemes.

For Falcon3, Falcon4, gain non-normalised K2/K3 images, the usage is very simple:

```
relion_convert_to_tiff --i movies.star --o Converted/
```

The STAR file needs only the `rlnMicrographMovieName` column. You can also specify a list file `.lst` that contains movie names without any STAR headers.

When the input is from Falcon detectors, judged by the width being 4096 pixels, it applies deflate compression at level 6. Otherwise, LZW compression is performed. This default can be overridden by `--compression` and `--deflate_level` arguments. By default, `relion_convert_to_tiff` treats all rows in a frame as one TIFF strip to improve the compression ratio. This can be disabled by `--line_by_line` option.

`--only_do_unfinished` allows conversion of only new files. The program writes to a temporary file and renames it to `.tif` only after all frames have been written. Thus, killing a program in the middle is safe.

In contrast to mrc2tif command from the IMOD suite, `relion_convert_to_tiff` does not support thread parallelization to compress one movie with many cores. However, one can use MPI parallelization as:

```
mpirun -np 24 relion_convert_to_tiff_mpi --i movies.star --o Converted/ # 24 processes
```

to process many movies simultaneously.

Note

In MRC-LMB computer cluster, you should run the above command after booking a full CPU node by `qlogin -l dedicated=24`. Note that our cluster nodes cannot access `/teraraid*`. If your movies are there, you have to run conversion on `max`, `hex` or `hal` (be considerate to others by reducing the number of processes!).

Gain estimation

To compress gain normalised K2 movies, one has to first estimate the gain *used during data collection*. Note that this gain is different from what `relion_estimate_gain` estimates.

```
relion_convert_to_tiff --i movies.star --o Converted/ --estimate_gain
```

This prints a row per frame:

```
Processing Original/FoilHole_7230495_Data_7226082_7226083_20181209_0240-40759.mrc
 Original/FoilHole_7230495_Data_7226082_7226083_20181209_0240-40759.mrc Frame 000
↪#Changed    7673083 #Mismatch          0, #Negative          0, #Unreliable   14238980␣
↪/   14238980
 Original/FoilHole_7230495_Data_7226082_7226083_20181209_0240-40759.mrc Frame 001
↪#Changed    4549992 #Mismatch      80676, #Negative          0, #Unreliable   14238980␣
↪/   14238980
 Original/FoilHole_7230495_Data_7226082_7226083_20181209_0240-40759.mrc Frame 002
↪#Changed    2743457 #Mismatch      89580, #Negative          0, #Unreliable   14238980␣
↪/   14238980
 Original/FoilHole_7230495_Data_7226082_7226083_20181209_0240-40759.mrc Frame 003
↪#Changed    1670936 #Mismatch      77997, #Negative          0, #Unreliable   14238980␣
↪/   14238980
 Original/FoilHole_7230495_Data_7226082_7226083_20181209_0240-40759.mrc Frame 004
↪#Changed    1028044 #Mismatch      59783, #Negative          0, #Unreliable   14238980␣
↪/   14238980
 Original/FoilHole_7230495_Data_7226082_7226083_20181209_0240-40759.mrc Frame 005
↪#Changed     638637 #Mismatch      44309, #Negative          0, #Unreliable   14238980␣
↪/   14238980
 Original/FoilHole_7230495_Data_7226082_7226083_20181209_0240-40759.mrc Frame 006
↪#Changed     399216 #Mismatch      30629, #Negative          0, #Unreliable   14238980␣
↪/   14238980
 Original/FoilHole_7230495_Data_7226082_7226083_20181209_0240-40759.mrc Frame 007
↪#Changed     251807 #Mismatch      21201, #Negative          0, #Unreliable   14238980␣
↪/   14238980
 Original/FoilHole_7230495_Data_7226082_7226083_20181209_0240-40759.mrc Frame 008
↪#Changed     159379 #Mismatch      15021, #Negative          0, #Unreliable   14238980␣
↪/   14238980
 Original/FoilHole_7230495_Data_7226082_7226083_20181209_0240-40759.mrc Frame 009
↪#Changed     101211 #Mismatch      10315, #Negative          0, #Unreliable   14238980␣
↪/   14238980
 Original/FoilHole_7230495_Data_7226082_7226083_20181209_0240-40759.mrc Frame 010
↪#Changed      64619 #Mismatch       7191, #Negative          0, #Unreliable   14238980␣
↪/   14238980
 Original/FoilHole_7230495_Data_7226082_7226083_20181209_0240-40759.mrc Frame 011
↪#Changed      41322 #Mismatch       5089, #Negative          0, #Unreliable   14238980␣
↪/   14238980
 Original/FoilHole_7230495_Data_7226082_7226083_20181209_0240-40759.mrc Frame 012
↪#Changed      26191 #Mismatch       3789, #Negative          0, #Unreliable   14238980␣
↪/   14238980
 Original/FoilHole_7230495_Data_7226082_7226083_20181209_0240-40759.mrc Frame 013
↪#Changed      16901 #Mismatch       2901, #Negative          0, #Unreliable   14238980␣
↪/   14238980
 Original/FoilHole_7230495_Data_7226082_7226083_20181209_0240-40759.mrc Frame 014
↪#Changed      10994 #Mismatch       2284, #Negative          0, #Unreliable   14238980␣
↪/   14238980
 Original/FoilHole_7230495_Data_7226082_7226083_20181209_0240-40759.mrc Frame 015
↪#Changed       7170 #Mismatch       1885, #Negative          0, #Unreliable   14238980␣
↪/   14238980
 Original/FoilHole_7230495_Data_7226082_7226083_20181209_0240-40759.mrc Frame 016
↪#Changed       4538 #Mismatch       1613, #Negative          0, #Unreliable   14238980␣
↪/   14238980
 Original/FoilHole_7230495_Data_7226082_7226083_20181209_0240-40759.mrc Frame 017
```

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```
↪#Changed       2980 #Mismatch       1446, #Negative          0, #Unreliable   14238980␣
↪/   14238980
Original/FoilHole_7230495_Data_7226082_7226083_20181209_0240-40759.mrc Frame 018
↪#Changed       1913 #Mismatch       1349, #Negative          0, #Unreliable   14238980␣
↪/   14238980
Original/FoilHole_7230495_Data_7226082_7226083_20181209_0240-40759.mrc Frame 019
↪#Changed       1273 #Mismatch       1293, #Negative          0, #Unreliable   14238980␣
↪/   14238980
Original/FoilHole_7230495_Data_7226082_7226083_20181209_0240-40759.mrc Frame 020
↪#Changed        859 #Mismatch       1256, #Negative          0, #Unreliable   14238980␣
↪/   14238980
Original/FoilHole_7230495_Data_7226082_7226083_20181209_0240-40759.mrc Frame 021
↪#Changed        554 #Mismatch       1206, #Negative          0, #Unreliable   14238980␣
↪/   14238980
Original/FoilHole_7230495_Data_7226082_7226083_20181209_0240-40759.mrc Frame 022
↪#Changed        344 #Mismatch       1232, #Negative          0, #Unreliable   14238980␣
↪/   14238980
Original/FoilHole_7230495_Data_7226082_7226083_20181209_0240-40759.mrc Frame 023
↪#Changed        243 #Mismatch       1188, #Negative          0, #Unreliable   14238980␣
↪/   14238980
Original/FoilHole_7230495_Data_7226082_7226083_20181209_0240-40759.mrc Frame 024
↪#Changed        169 #Mismatch       1189, #Negative          0, #Unreliable   14238980␣
↪/   14238980
Original/FoilHole_7230495_Data_7226082_7226083_20181209_0240-40759.mrc Frame 025
↪#Changed        107 #Mismatch       1195, #Negative          0, #Unreliable   14238980␣
↪/   14238980
Original/FoilHole_7230495_Data_7226082_7226083_20181209_0240-40759.mrc Frame 026
↪#Changed         79 #Mismatch       1182, #Negative          0, #Unreliable   14238980␣
↪/   14238980
Original/FoilHole_7230495_Data_7226082_7226083_20181209_0240-40759.mrc Frame 027
↪#Changed         60 #Mismatch       1206, #Negative          0, #Unreliable   14238980␣
↪/   14238980
Original/FoilHole_7230495_Data_7226082_7226083_20181209_0240-40759.mrc Frame 028
↪#Changed         53 #Mismatch       1187, #Negative          0, #Unreliable   14238980␣
↪/   14238980
Original/FoilHole_7230495_Data_7226082_7226083_20181209_0240-40759.mrc Frame 029
↪#Changed         39 #Mismatch       1177, #Negative          0, #Unreliable   14238980␣
↪/   14238980
Original/FoilHole_7230495_Data_7226082_7226083_20181209_0240-40759.mrc Frame 030
↪#Changed         37 #Mismatch       1165, #Negative          0, #Unreliable   14238980␣
↪/   14238980
Original/FoilHole_7230495_Data_7226082_7226083_20181209_0240-40759.mrc Frame 031
↪#Changed         30 #Mismatch       1199, #Negative          0, #Unreliable   14238980␣
↪/   14238980
Original/FoilHole_7230495_Data_7226082_7226083_20181209_0240-40759.mrc Frame 032
↪#Changed         23 #Mismatch       1185, #Negative          0, #Unreliable   14238980␣
↪/   14238980
```

As explained above, the program finds smallest positive numbers for each pixel over many frames. `#Changed` is the number of pixels whose minimum value is updated. `#Mismatch` is the number of pixels whose value in the frame is not an integer multiple of the current gain estimate. This happens when (1) the pixel is defective and Digital Micrograph applied correction or (2) the estimated gain is not correct (for example, the current minimum corresponds to two electrons and the frame contains three electrons).

`#Unreliable` is the number of pixels whose gain estimate is still unreliable. A pixel is considered to be reliable when values which are integer multiples of the current gain estimate were observed at least `--thresh` times (default 50) without being interrupted by mismatch.

After processing several hundreds frames, the values should become stable. The number of mismatches fluctuates. The number of unreliable pixels is usually 1000 to 5000 in most K2 detectors.

```
Processing Original/FoilHole_7232574_Data_7226091_7226092_20181209_2221-42294.mrc
 Original/FoilHole_7232574_Data_7226091_7226092_20181209_2221-42294.mrc Frame 000
↪#Changed          0 #Mismatch       1216, #Negative          0, #Unreliable      1346␣
↪/   14238980
 Original/FoilHole_7232574_Data_7226091_7226092_20181209_2221-42294.mrc Frame 001
↪#Changed          0 #Mismatch       1203, #Negative          0, #Unreliable      1346␣
↪/   14238980
 Original/FoilHole_7232574_Data_7226091_7226092_20181209_2221-42294.mrc Frame 002
↪#Changed          0 #Mismatch       1199, #Negative          0, #Unreliable      1346␣
↪/   14238980
 Original/FoilHole_7232574_Data_7226091_7226092_20181209_2221-42294.mrc Frame 003
↪#Changed          0 #Mismatch       1199, #Negative          0, #Unreliable      1346␣
↪/   14238980
 Original/FoilHole_7232574_Data_7226091_7226092_20181209_2221-42294.mrc Frame 004
↪#Changed          0 #Mismatch       1192, #Negative          0, #Unreliable      1346␣
↪/   14238980
 Original/FoilHole_7232574_Data_7226091_7226092_20181209_2221-42294.mrc Frame 005
↪#Changed          0 #Mismatch       1210, #Negative          0, #Unreliable      1346␣
↪/   14238980
 Original/FoilHole_7232574_Data_7226091_7226092_20181209_2221-42294.mrc Frame 006
↪#Changed          0 #Mismatch       1186, #Negative          0, #Unreliable      1346␣
↪/   14238980
 Original/FoilHole_7232574_Data_7226091_7226092_20181209_2221-42294.mrc Frame 007
↪#Changed          0 #Mismatch       1219, #Negative          0, #Unreliable      1346␣
↪/   14238980
 Original/FoilHole_7232574_Data_7226091_7226092_20181209_2221-42294.mrc Frame 008
↪#Changed          0 #Mismatch       1224, #Negative          0, #Unreliable      1346␣
↪/   14238980
 Original/FoilHole_7232574_Data_7226091_7226092_20181209_2221-42294.mrc Frame 009
↪#Changed          0 #Mismatch       1197, #Negative          0, #Unreliable      1346␣
↪/   14238980
 Original/FoilHole_7232574_Data_7226091_7226092_20181209_2221-42294.mrc Frame 010
↪#Changed          0 #Mismatch       1202, #Negative          0, #Unreliable      1346␣
↪/   14238980
 Original/FoilHole_7232574_Data_7226091_7226092_20181209_2221-42294.mrc Frame 011
↪#Changed          0 #Mismatch       1176, #Negative          0, #Unreliable      1346␣
↪/   14238980
 Original/FoilHole_7232574_Data_7226091_7226092_20181209_2221-42294.mrc Frame 012
↪#Changed          0 #Mismatch       1197, #Negative          0, #Unreliable      1346␣
↪/   14238980
 Original/FoilHole_7232574_Data_7226091_7226092_20181209_2221-42294.mrc Frame 013
↪#Changed          0 #Mismatch       1196, #Negative          0, #Unreliable      1346␣
↪/   14238980
 Original/FoilHole_7232574_Data_7226091_7226092_20181209_2221-42294.mrc Frame 014
↪#Changed          0 #Mismatch       1204, #Negative          0, #Unreliable      1346␣
↪/   14238980
```

Now you can stop the program by pressing `Ctrl-C`. The program updates `gain_estimate.bin` and gain_estimate_reliability.bin every movie.

To perform actual compression, specify gain_estimate.bin as --gain option:

```
relion_convert_to_tiff --i movies.star --o Converted/ --gain Converted/gain_estimate.bin
```

The program writes not only TIFF movies but also gain-reference.mrc, which should be used for subsequent data processing.

Practical considerations

If you updated the gain reference in Digital Micrograph during data collection, you have to divide your dataset into two and estimate gain separately.

Some pixels are cold pixels and emit 0 most of the time. Thus, it is very rare to observe values corresponding to one electron. If you terminate gain estimation too early, such pixels are flagged as unreliable. This is safe, because values of unreliable pixels are always written as they are with the gain value of 1.0000.

If the program never observes an event corresponding to one electron but only events corresponding to two or four electrons during gain estimation, the program mistakenly considers the value for two electrons as the gain and still flags the pixel as reliable. If the program encounters an event corresponding to one or three electrons during compression, which is not multiple of the estimated gain, the program emits an error and terminates. In this case, you have to re-run gain estimation from more frames and repeat compression **from the beginning**. Fortunately, such situation is highly unlikely; because the pixel values are Poisson distributed and the dose rate is low, you observes an event corresponding to one electron frequently. When the dose rate is high, the probability for one-electron events is lower, but the distribution becomes also wider. This means that you observe neighbouring values (e.g. two-, three- and four-electron events) with similar frequencies. In other words, it is unlikely to observe many two- and four-electron events without observing any three-electron events. Because three is not divisible by two, this pixel remains flagged as unreliable. The --ignore_error option forces the program to continue by rounding non-conforming values but this leads to change of pixel values.

Defective pixels do not carry much information. If we round them to nearest integers, the output can be saved as integers, not floating point numbers, and the compression ratio will improve. Since the number of defects are very small (1000 to 5000 out of 14 million pixels in K2) and their values are not very accurate anyway, such a slightly-lossy compression scheme probably do not hurt the resolution. Implementation and verification of such a strategy is on our TODO list.

7.1.5 Compressed MRC files

Some movies, especially Falcon 3 or Falcon 4 movies (non-EER), can be compressed significantly better by bzip2 than deflate TIFF. RELION 4.0.1 and newer support MRC movies compressed by bzip2, xz or ZStandard in RELION's own motion correction and Bayesian Polish for SPA. Other RELION features, including tomography, relion_movie_reconstruct and relion_image_handler, do NOT support compressed MRC files (yet).

To read compressed MRC files, RELION needs pbzip2 (not bzip2), xz and zstd command in your PATH for bzip2, xz and ZStandard, respectively.

These formats do not allow random access; in other words, RELION has to decompress all the N - 1 preceeding frames only to read the N-th frame. This is not a big issue for tools that read all frames anyway (e.g. motion correction and Polish), but poses significant inefficiency for others. Fortunately, LZW-TIFF achieves similar (or better) compression for K2/K3 movies and Falcon 3/4 movies converted from EER. Thus, we hope the compressed MRC format is necessary only for archiving old Falcon 3/4 MRC movies.

7.1.6 Examples

Compression rates depend on dose. Fewer electrons typically lead to better compression.

Falcon 3 counting

FoilHole_24156969_Data_24154827_24154828_20170425_0847_Fractions.tif from EMPIAR-10309 (A2a receptor). The deposited file is already in TIFF, but decompressed to 16 bit integer MRC for testing. 4096 x 4096 pixels, 75 frames, 16 bit integer, mean = 36.644 (i.e. 0.36 e/px/frame)

- 16 bit integer MRC: 2,516,583,424
- IMOD mrc2tif, lzw: 1,583,972,550 (62.9 %)
- IMOD mrc2tif, zip level 6: 1,432,846,496 (56.9 %)
- IMOD mrc2tif, zip level 9: 1,432,820,192 (56.9 %)
- relion_convert_to_tiff, auto = zip level 6: 1,337,873,325 (53.2 %)
- bzip2: 1,067,277,634 (42.4 %)

Note that bzip2 gives a smaller file.

K2 counting, gain normalised from EPU

FoilHole_12404830_Data_12400523_12400524_20181213_1058-251321.mrc from EMPIAR-10317 (ABC transporter). 3838 x 3710 pixels, 40 frames, 32 bit floating point from EPU, mean = 1.45

- 32 bit floating point MRC: 2,278,237,824
- IMOD mrc2tif, lzw: 1,554,985,144 (68.3 %)
- IMOD mrc2tif, zip level 6: 1,274,226,678 (55.9 %)
- bzip2: 739,204,755 (32.4 %)
- relion_convert_to_tiff after gain estimation: 270,856,741 (11.9 %)

1502 pixels were marked as unreliable.

Also note that it would have been 569560224 bytes (25 %) in gain non-normalised 8-bit integer MRC even before compression.

K2 counting, gain non-normalised from EPU

FoilHole_2491648_Data_2484494_2484495_20190505_2224-167458.mrc from EMPIAR-10340 (tau filaments). 3838 x 3710 pixels, 48 frames, 8 bit integers from EPU, mean = 0.96

- 8 bit integer MRC: 683,472,064
- IMOD mrc2tif, zip level 6: 205,103,218 (30.0 %)
- IMOD mrc2tif, lzw: 193,826,068 (28.4 %)
- relion_convert_to_tiff, auto = lzw: 190,200,465 (27.8 %)
- bzip2: 189,773,107 (27.8 %)

By saving in the gain non-normalised mode, the integer MRC file is one forth the size of the floating point MRC file (32 / 8 = 4). LZW compression further reduces the size.

Falcon 4 EER

FoilHole_13722039_Data_13716084_13716086_20200315_0111_Fractions.mrc.eer from EMPIAR-10500 (GABAA receptor). The deposited file is in the EER format. The file is converted to LZW-TIFF at different temporal resolutions (frame grouping) and spatial resolutions (4K physical grid or 8K super-resolution grid). 4096 x 4096 pixels, 0.725 Å/px, 1113 detector frames, mean = 20.275 (i.e. 0.0182 e/px/frame)

- Original EER: 498,516,028
- LZW-TIFF, 4K, group by 24 (0.44 e/px/fraction, 0.83 e/Å/fraction): 146,363,549 (29.4 %)
- LZW-TIFF, 4K, group by 16 (0.29 e/px/fraction, 0.55 e/Å/fraction): 175,643,049 (35.2 %)
- LZW-TIFF, 4K, group by 12 (0.22 e/px/fraction, 0.41 e/Å/fraction): 198,052,039 (39.7 %)
- LZW-TIFF, 4K, group by 8 (0.15 e/px/fraction, 0.28 e/Å/fraction): 233,619,295 (46.9 %)
- LZW-TIFF, 8K, group by 24 (0.83 e/Å/fraction): 257,857,873 (51.7 %)
- LZW-TIFF, 8K, group by 16 (0.55 e/Å/fraction): 296,274,173 (59.4 %)
- LZW-TIFF, 8K, group by 12 (0.41 e/Å/fraction): 325,821,955 (65.4 %)
- LZW-TIFF, 8K, group by 8 (0.28 e/Å/fraction): 373,938,153 (75.0 %)

Although EER files are smaller than Falcon MRC files written by EPU, they can be made even smaller by fractionation to a reasonable temporal resolution. By converting to LZW-TIFF from EER, you can avoid 3x3 blob convolution and get smaller files than converting from MRC. Since electrons are rendered as dots in EER (as in K2/K3), LZW filter is faster and produces smaller files than the deflate filter.

7.2 Pixel size issues

7.2.1 What should I do if the pixel size turned out to be wrong?

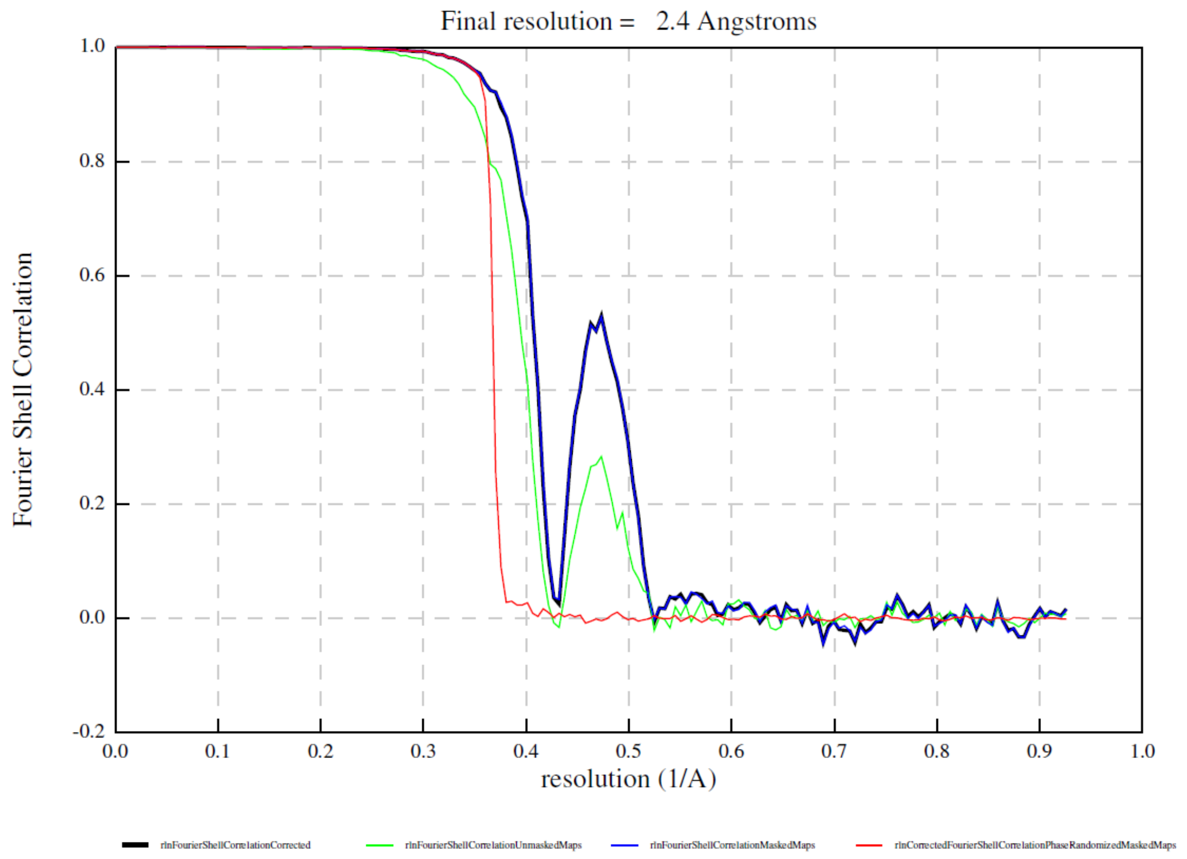
If the error is small (say 1-2 %) and the resolution is not very high (3 Å), you can specify the correct pixel size in the PostProcess job. This scales the resolution in the FSC curve. When the error is large, the presence of spherical aberration invalidates this approach. Continue reading.

Warning

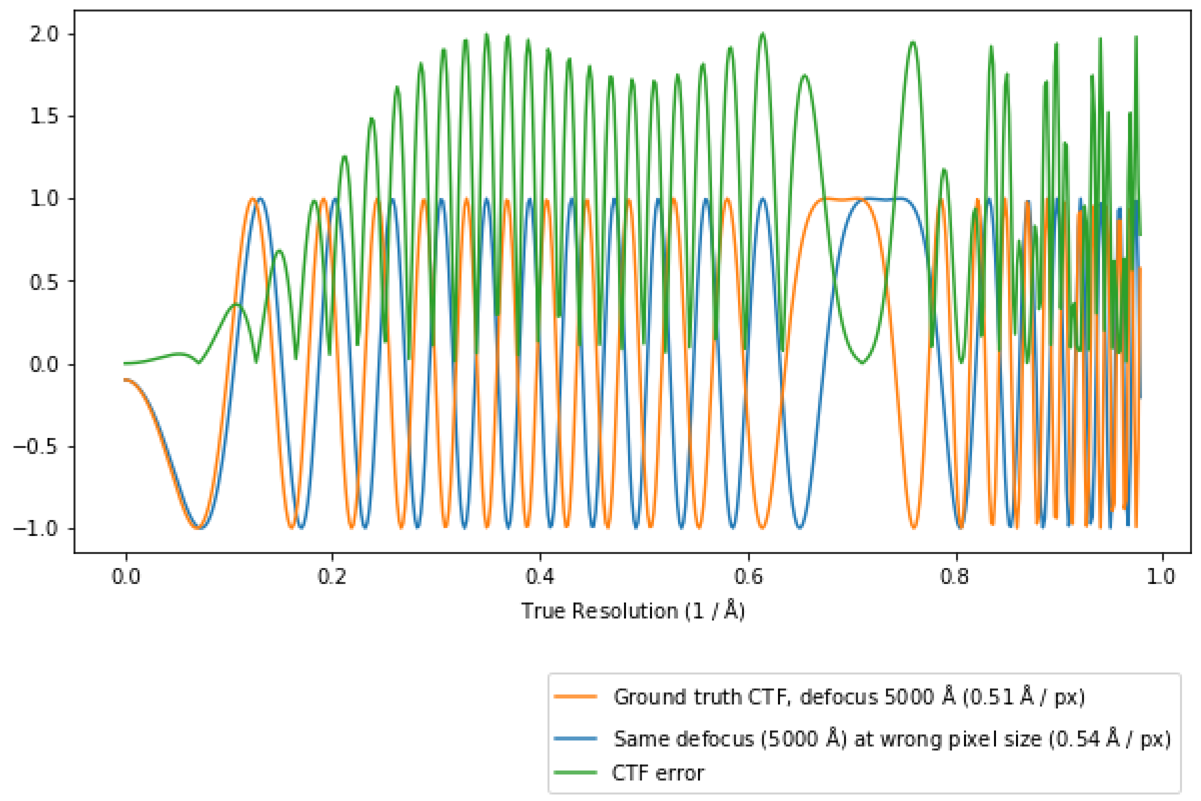
You **should not** edit your STAR files because your current defocus values are fitted against the initial, slightly wrong pixel size. Also, you **should not** use "Manually set pixel size" in the Extraction job. It will make metadata inconsistent and break Bayesian Polishing. Thus, this option was removed in RELION 3.1.

7.2.2 Cs and the error in the pixel size

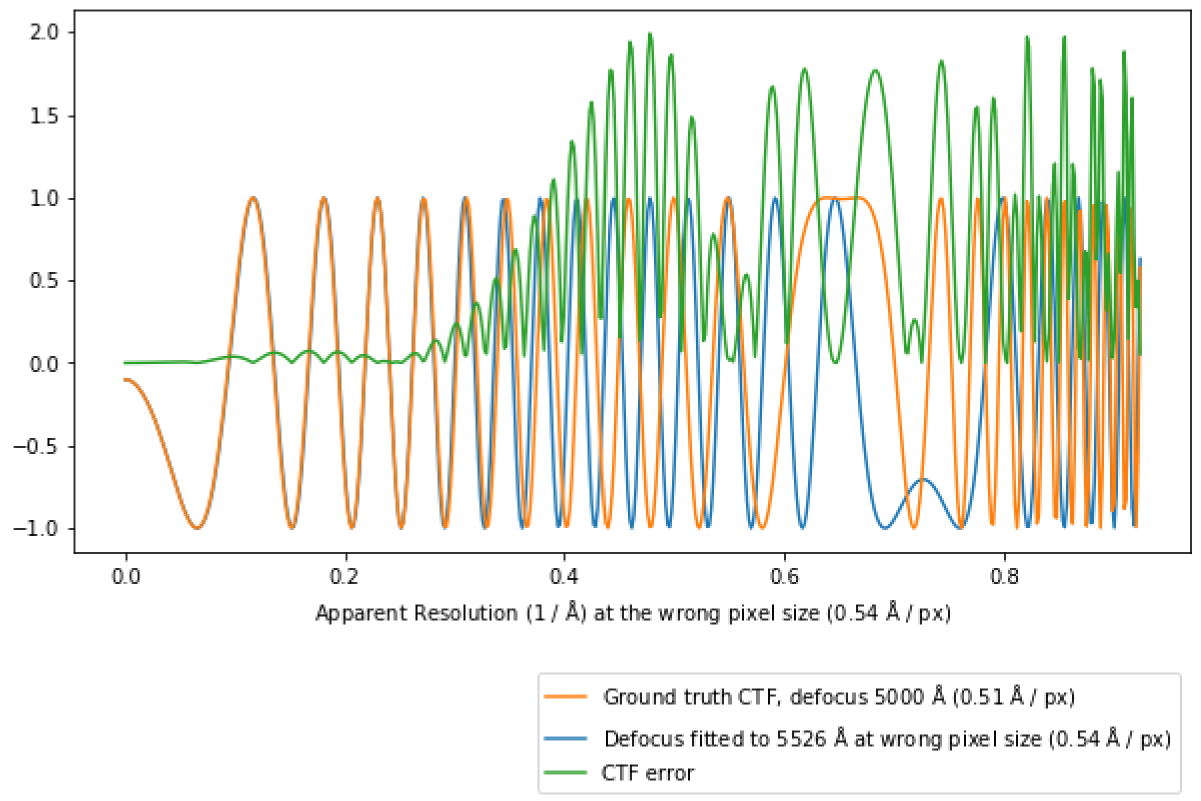
Recall that the phase shift due to defocus is proportional to the square of the wave number (i.e. inverse resolution), while that due to spherical aberration is proportional to the forth power of the wave number. At lower resolutions, the defocus term dominates and errors in the pixel size (i.e. errors in the wave number) can be absorbed into the defocus value fitted at the nominal pixel size. At higher resolution, however, the Cs term becomes significant. Since Cs is not fitted but given at the correct pixel size, the error persists. As the two terms have the opposite sign, the errors sometimes cancel out at certain resolution shells, leading to a strange bump in the FSC curve. See an example below contributed from a user. Here the pixel size was off by about 6 % (truth: 0.51 Å/px, nominal: 0.54 Å/px).



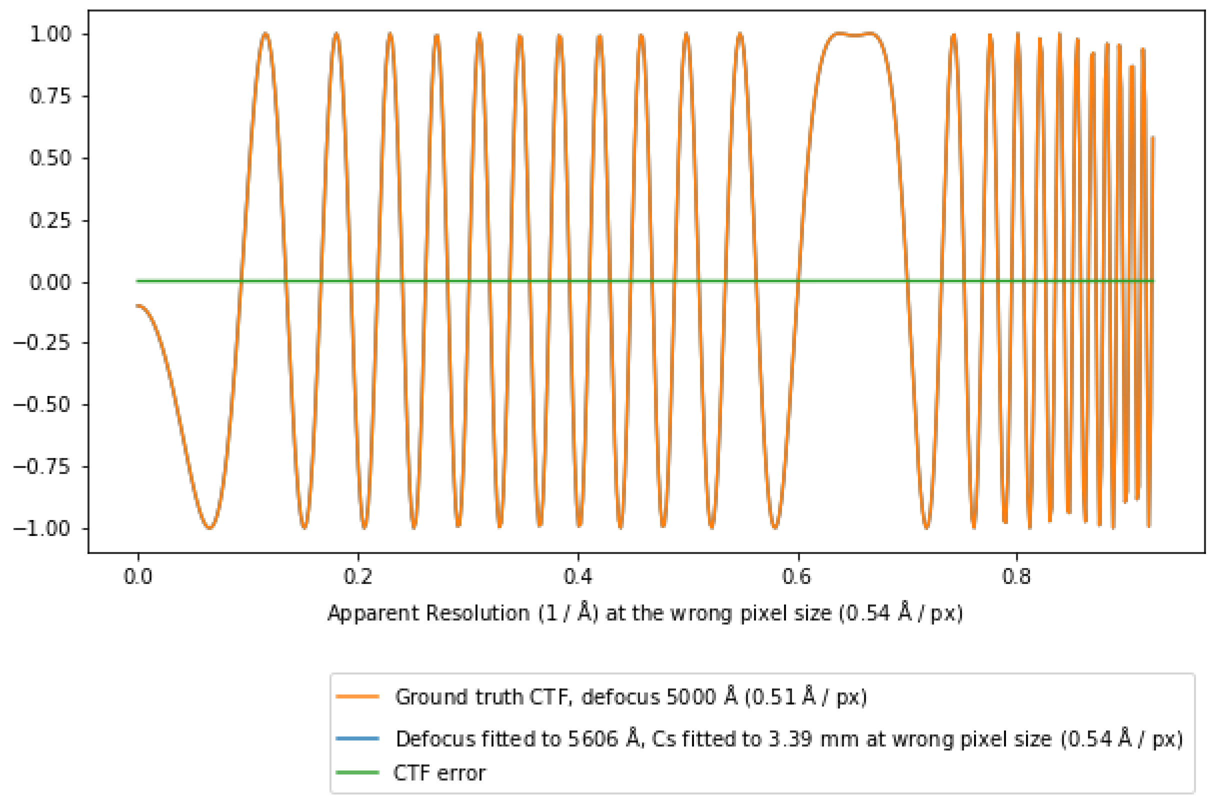
Below is a theoretical consideration. Let's consider a CTF at defocus 5000 Å, Cs 2.7 mm at 0.51 Å/px. This is shown in orange. If one thought the pixel size is 0.54 Å/px, the calculated CTF (blue) became quite off even at 5 Å (0.2).



However, the defocus is fitted (by CtfFind, followed by CtfRefine) at 0.54 Å/px, the nominal pixel size. The defocus became 5526 Å, absorbing the error in the pixel size. This is shown in blue. The fit is almost perfect up to about 3.3 Å. In this region, you can update the pixel size in PostProcess. Beyond this point, the error from the Cs term starts to appear and the two curves go out of phase. This is why the FSC drops to zero. However, the two curves came into phase again at about 1.8 Å (0.55)! This is why the FSC goes up again.



If you refine Cs and defocus simultaneously, the error in the pixel size is completely absorbed and the fit becomes perfect. Notice that the refined Cs is 3.39, which is 2.7 * (0.54 / 0.51)^4. Also note that the refined defocus 5606 Å is 5000 * (0.54 / 0.51)^2.



In practice, one just needs to run `CtfRefine` twice: first with `Estimate 4th order aberrations` to refine Cs, followed by another run for defocus. Note that `rlnSphericalAberration` remains the same. The error in Cs is expressed in `rlnEvenZernike`. You **should never** edit the pixel size in the STAR file!

Now everything is consistent at the nominal pixel size of 0.54 Å/px. In PostProcess, one should specify 0.51 Å/px to re-scale the resolution and the header of the output map.

7.2.3 How can I merge datasets with different pixel sizes?

First of all: it is very common that one of your datasets is significantly better (i.e. thinner ice) than the others and merging many datasets does not improve resolution. First process datasets individually and then merge the most promising two. If it improves the resolution, merge the third dataset. Combining millions of bad particles simply because you have them is a very bad idea and waste of storage and computational time!

For RELION 3.0, please see an excellent explanation posted to CCPEM by Max Wilkinson.

From RELION 3.1, you can refine particles with different pixel sizes and/or box sizes. Suppose you want to bring polished (shiny) particles from the dataset2 project into the dataset1 project.

First, go to the `Polish` directory within the dataset1 project. Make a symbolic link to dataset2's `Polish/jobXXX` by **a full path**. A link by a relative path sometimes causes unexpected problems in RELION when it tries to expand paths.

If you are unlucky and the job number `jobXXX` is already present in the `Polish` directory of the dataset1 project, you have to make a link by another name. In this case, you have to replace the file names in dataset2's `shiny.star`.

Next, make sure they have different `rlnOpticsGroupName`. For example:

dataset1's shiny.star:

```
data_optics

```

(continues on next page)

(continued from previous page)

```
loop_
_rlnOpticsGroup #1
_rlnOpticsGroupName #2
_rlnAmplitudeContrast #3
_rlnSphericalAberration #4
_rlnVoltage #5
_rlnImagePixelSize #6
_rlnMicrographOriginalPixelSize #7
_rlnImageSize #8
_rlnImageDimensionality #9
            1  dataset1    0.100000     2.700000   300.000000     1.000000     1.000000␣
↪         140             2
```

dataset2's shiny.star:

```
data_optics

loop_
_rlnOpticsGroup #1
_rlnOpticsGroupName #2
_rlnAmplitudeContrast #3
_rlnSphericalAberration #4
_rlnVoltage #5
_rlnImagePixelSize #6
_rlnMicrographOriginalPixelSize #7
_rlnImageSize #8
_rlnImageDimensionality #9
            1  dataset2    0.100000     2.700000   300.000000     1.100000     1.100000␣
↪         128             2
```

Then use JoinStar. You don't have to import; just go above the `.Nodes` directory to see the file from dataset2.

The result should look like:

```
data_optics

loop_
_rlnOpticsGroup #1
_rlnOpticsGroupName #2
_rlnAmplitudeContrast #3
_rlnSphericalAberration #4
_rlnVoltage #5
_rlnImagePixelSize #6
_rlnMicrographOriginalPixelSize #7
_rlnImageSize #8
_rlnImageDimensionality #9
            1  dataset1    0.100000     2.700000   300.000000     1.000000     1.000000␣
↪         140             2
            2  dataset2    0.100000     2.700000   300.000000     1.100000     1.100000␣
↪         128             2
```

Note that the dataset2's `rlnOpticsGroup` has been re-numbered to 2.

If two datasets came from different detectors and/or had very different pixel sizes, you might want to apply MTF correction during refinement. To do this, add two more columns: `rlnMtfFileName` to specify the MTF STAR file (the path is relative to the project directory) and `rlnMicrographOriginalPixelSize` to specify the detector pixel size (i.e. before down-sampling during extraction).

Refine this combined dataset. For a reference and mask, you must use the pixel size and box size of the first optics group (or use `--trust_ref_size` option). The output pixel size and the box size will be the same as the input reference map. After refinement, run `CtfRefine` with `Estimate anisotropic magnification: Yes`. This will refine the **relative** pixel size difference between two datasets. In the above example, the nominal difference is 10 %, but it might be actually 9.4 %, for example. Then run Refine3D again. The **absolute** pixel size of the output can drift a bit. It needs to be calibrated against atomic models.

You can do similar things for other job types as long as you make all relevant paths consistent. For example, to run Polish in a new project directory, you have to make sure the paths to raw movies, the gain reference and the motion correction job are valid as well as paths to the particles and the references. For Polishing, do **NOT** merge MotionCorr STAR files. First, run Polishing on one of the MotionCorr STAR files with `run_data.star` that contains all particles. This will process and write only particles from micrographs present in the given MotionCorr STAR file. Repeat this for the other MotionCorr STAR files. Finally, join two `shiny.star` files from the two jobs. The `--only_group` option is not the right way to do Polishing on combined datasets.

7.2.4 How can I estimate the absolute pixel size of a map?

Experimentally and ideally, one can use diffraction from calibration standards.

Computationally, one can compare the map and a refined atomic model. However, if the model has been refined against the same map, the model might have been biased towards the map. Also note that bond and angle RMSDs are not always reliable when restraints are too strong.

If you are sure that Cs_{true} given by the microscope manufacturer is accurate, which is often the case, AND the acceleration voltage is also accurate, you can optimize the pixel size such that the apparent Cs fitted at the pixel size becomes Cs_{true}.

First, find out the Z_4^0 coefficient. This is the 7th number in `rlnEvenZernike`. The contribution of the spherical aberration to the argument of the CTF's sine function is $1/2\pi\lambda^3k^4Cs$, where k is the wave-number and λ is the relativistic wavelength of the electron (0.0196 Å for 300 kV and 0.0251 Å for 200 kV). $Z_4^0 = 6k^4 - 6k^2 + 1$. By comparing the k^4 term, we find the correction to the Cs is $12Z_4^0/(\pi\lambda^3)$. Note that $-6k^2 + 1$ terms are cancelled by the lower-order Zernike coefficients and can be ignored. Thus,

$Cs_{\text{apparent}} = Cs_{\text{nominal}} + 12Z_4^0 * 10^{-7}/(\pi\lambda^3)$.

10^{-7} is to convert Cs from Å to mm. Cs_{nominal} is what you used in CTFFIND (e.g. 2.7 mm for Titan and Talos).

The real pixel size can be calculated as NominalPixelSize$(Cs_{\text{true}}/Cs_{\text{apparent}})^{1/4}$.

7.2.5 Examples

In RELION 3.1, `relion_refine` (Refine3D, Class3D, MultiBody) stretches, shrinks, pads and/or crops input particles so that the output has the same nominal pixel size and the box size as the input reference. Let's study what happens in various cases.

One optics group

Suppose your optics group says 1.00 Å/px. If the header of your reference also says 1.00 Å/px, particles are used without any stretching or shrinking. The output remains 1.00 Å/px.

Let's assume that the nominal pixel size of 1.00 Å/px turns out slightly off and the real pixel size is 0.98 Å/px. As discussed above, you should **never change the pixel size in the optics group table**, since all CTF parameters, coordinates and trajectories are consistent with the nominal pixel size of 1.00 Å/px. When the header of your reference is 1.00 Å/px, the output map header says 1.00 Å/px, but it is actually 0.98 Å/px. Thus, you should specify 0.98 Å/px in

PostProcess. The post-processed map will have 0.98 Å/px in the header and the X-axis of the FSC curve will be also consistent with 0.98 Å/px.

You **must not** use this 0.98 Å/px map from PostProcess in future Refine3D/MultiBody/Class3D jobs. Otherwise, `relion_refine` stretches nominal 1.00 Å/px particles into 0.98 Å/px. Thus, the output map will have the nominal pixel size of 0.98 Å/px but the actual pixel size will be 0.98 * 0.98 / 1.00 = 0.96 Å/px!

Multiple optics groups

Suppose your have two optics groups, one at 1.00 Å/px and the other at 0.95 Å/px. If the header of your reference is 1.00 Å/px, the particles in the first group are used without any stretching or shrinking, while those from the second group are shrunk to match 1.00 Å/px. The output map is at 1.00 Å/px.

Let's assume that the real pixel size of the first group is exactly at 1.00 Å/px, while that of the second group is actually 0.90 Å/px. After stretching by the ratio between the nominal pixel size and the pixel size in the reference header, the true pixel size for the second group corresponds to 0.90 / 0.95 * 1.00 = 0.947 Å/px. Thus, the reconstruction is a mixture of particles at 1.00 Å/px and 0.947 Å/px. Depending on the number and signal-to-noise ratio of particles in each group, *the real pixel size of the output can be any value between 0.947 and 1.00 Å/px*, while the output header says 1.00 Å/px.

Anisotropic magnification refinement in CtfRefine finds the **relative** pixel size differences between particles and the reference. Suppose the actual pixel size of the output from Refine3D is 0.99 Å/px and there is no anistropic magnification. CtfRefine finds the particles in the first group look smaller in real space than expected and puts `0.9900 0 0 0.9900` in `rlnMatrix00, 01, 10, 11` (0.9900 = 0.99 / (1.00 * 1.00 / 1.00)). The particles in the second group look larger in real space than expected and `rlnMatrix00` to `rlnMatrix11` will be `1.045 0 0 1.045` (= 0.99 / (0.90 / 0.95 * 1.00)).

Notice that these numbers **do not give you the absolute pixel size** (e.g. 0.95 / 1.045 = 0.9091 $\neq$ 0.900). However, you can work out the pixel size of the reconstruction and other optics groups if you are confident with the pixel size of one optics group. You can also work out the pixel size of all optics groups if you calibrate the pixel size of the reconstruction with atomic models. Carefully follows two streching/shrinking steps done in `relion_refine`: one to match the nominal pixel size of an optics group to the nominal pixel size of the reference and the further correction by `rlnMatrix`.

7.3 Automation: *Schemes*

Schemes were introduced to relion-3.1, where they were called *Schedules* but to prevent confusion with scheduled jobs in the main GUI, they were renamed to *Schemes* in relion-4.0 and their functionality was further improved. *Schemes* aim to provide a generalised methodology for automatic submission of relion jobs. This is useful for creating standardised workflows, for example to be used in on-the-fly processing. The `relion_it.py` script that was introduced in relion-3.1 has been re-written to work with the *Schemes*.

The *Schemes* framework is built around the following key concepts: a directed graph that represents the logic of a series of subsequent relion job-types is encoded in *Nodes* and *Edges*. *Nodes* can be either a relion job or a so-called *Operator*; *Edges* form the connections between *Nodes*. In addition, *Schemes* have their own *Variables*.

All information for each *Scheme* is stored in its own subdirectory of the `Schemes/` directory in a relion project, e.g. `Schemes/prep`. Within each *Scheme*'s directory, the `scheme.star` file contains information about all the *Variables*, *Edges*, *Operators* and *Jobs*.

7.3.1 Variables

Three different types of *Variables* exist: *floatVariables* are numbers; *booleanVariables* are either True or False; and *stringVariables* are text. Each Variable has a *VariableName*; a so-called *VariableResetValue*, at which the value is initialised; and a *VariableValue*, which may change during execution of the *Scheme* through the actions of Operators, as outlined below.

One special *stringVariable* is called `email`. When this is set, upon completion or upon encountering an error, the *Scheme* will send an email (through the Linux `mail` command) to the value of the *email stringVariable*.

7.3.2 Jobs

Jobs are the first type of *Node*. They can be of any of the jobtypes defined in the relion pipeliner, i.e. Import, Motion correction, etc, including the new External. Any *Variable* defined in the *Scheme* can be set as a parameter in a Job, by using two dollar signs on the GUI or in the *job.star* file. For example, one could define a *floatVariable* `voltage` and use `$$voltage` on the corresponding input line of an Import job. Upon execution of the job inside the *Scheme*, the `$$voltage` will be replaced with the current value of the `voltage` *floatVariable*.

Jobs within a *Scheme* each have a *JobName* and a *JobNameOriginal*. The latter is defined upon creation of the job (see next section); the former depends on the execution status of the *Scheme*, and will be set to the executed relion job's name, e.g. `CtfFind/job004`. In addition, each job has a *JobMod* and a *jobHasStarted* status. There are two types of *JobMode*:

`new`
: regardless of *jobHasStarted*, a new job will be created, with its own new *JobName*, every time the *Schemer* passes through this Node.

`continue`
: if *jobHasStarted* is False, a new job, with its own new *JobName*, will be created. If *jobHasStarted* is True, the job will be executed as a continue job inside the existing *JobName* directory.

When a *Scheme* executes a Job, it always sets *jobHasStarted* to True. When a *Scheme* is reset, the *jobHasStarted* status for all jobs is set to False.

7.3.3 Operators

Operators are the second type of *Node*. Each operator within a *Scheme* has a unique name and a type. Operators can also have an output Variable: *output*, on which they act, and up to two input Variables: *input1* and *input2*. Most, but not all operators change the value of their *output* Variable.

The following types of operators act on an *output* that is a *floatVariable*:

`float=set`
: *output* = *floatVariable input1*

`float=plus`
: *output* = *floatVariable input1* + *floatVariable input2*

`float=minus`
: *output* = *floatVariable input1* - *floatVariable input2*

`float=mult`
: *output* = *floatVariable input1* × *floatVariable input2*

`float=divide`
: *output* = *floatVariable input1* / *floatVariable input2*

`float=round`
: *output* = ROUND(*floatVariable input1*)

`float=count_images`
: sets *output* to the number of images in the STAR file with the filename in *stringVariable input1*. *stringVariable input2* can be *particles*, *micrographs* or *movies*, depending on what type of images need to be counted.

`float=count_words`
: sets *output* to the number of words in *stringVariable input1*, where individual words need to be separated with a , (comma) sign.

`float=read_star`
: sets *output* to the value of a double or integer that is read from a STAR file. *stringVariable input1* defines which variable to read as: *starfilename,tablename,metadatalabel*. If *tablename* is a table instead of a list, then *floatVariable input2* defines the line number, with the default of zero being the first line.

`float=star_table_max`
: sets *output* to the maximum value of a column in a starfile table, where *stringVariable input1* specifies the column as *starfilename,tablename,metadatalabel*.

`float=star_table_min`
: sets *output* to the minimum value of a column in a starfile table, where *stringVariable input1* specifies the column as *starfilename,tablename,metadatalabel*.

`float=star_table_avg`
: sets *output* to the average value of a column in a starfile table, where *stringVariable input1* specifies the column as *starfilename,tablename,metadatalabel*.

`float=star_table_sort_idx`
: a sorting will be performed on the values of a column in a starfile table, where *stringVariable input1* specifies the column as *starfilename,tablename,metadatalabel*. *stringVariable input2* specifies the index in the ordered array: the lowest number is 1, the second lowest is 2, the highest is -1 and the one-but-highest is -2. Then, *output* is set to the corresponding index in the original table.

The following types of operators act on an *output* that is a *booleanVariable*:

`bool=set`
: *output* = *booleanVariable input1*

`bool=and`
: *output* = *booleanVariable input1* AND *booleanVariable input2*

`bool=or`
: *output* = *booleanVariable input1* OR *booleanVariable input2*

`bool=not`
: *output* = NOT *booleanVariable input1*

`bool=gt`
: *output* = *floatVariable input1* > *floatVariable input2*

`bool=lt`
: *output* = *floatVariable input1* < *floatVariable input2*

`bool=ge`
: *output* = *floatVariable input1* >= *floatVariable input2*

`bool=le`
: *output* = *floatVariable input1* <= *floatVariable input2*

`bool=eq`
: *output* = *floatVariable input1* == *floatVariable input2*

`bool=file_exists`
: *output* = True if a file with the filename stored in *stringVariable input1* exists on the file system; False otherwise

`bool=read_star`
: reads *output* from a boolean that is stored inside a STAR file. *stringVariable input1* defines which variable to read as: *starfilename,tablename,metadatalabel*. If *tablename* is a table instead of a list, then *floatVariable input2* defines the line number, with the default of zero being the first line.

The following types of operators act on an *output* that is a *stringVariable*:

`string=set`
: *output = stringVariable input1*

`string=join`
: *output* = concatenate *stringVariable input1* and *stringVariable input2*

`string=before_first`
: sets *output* to the substring of *stringVariable input1* that occurs before the first instance of substring *stringVariable input2*.

`string=after_first`
: sets *output* to the substring of *stringVariable input1* that occurs after the first instance of substring *stringVariable input2*.

`string=before_last`
: sets *output* to the substring of *stringVariable input1* that occurs before the last instance of substring *stringVariable input2*.

`string=after_last`
: sets *output* to the substring of *stringVariable input1* that occurs after the last instance of substring *stringVariable input2*.

`string=read_star`
: reads *output* from a string that is stored inside a STAR file. *stringVariable input1* defines which variable to read as: *starfilename,tablename,metadatalabel*. If *tablename* is a table instead of a list, then *floatVariable input2* defines the line number, with the default of zero being the first line.

`string=glob`
: *output* = GLOB(*stringVariable input1*), where input1 contains a Linux wildcard and GLOB is the Linux function that returns all the files that exist for that wildcard. Each existing file will be separated by a comma in the *output* string.

`string=nth_word`
: *output* = the Nth substring in *stringVariable input1*, where N=*floatVariable* *input2*, and substrings are separated by commas. Counting starts at one, and negative values for *input2* mean counting from the end, e.g. *input2=-2* means the second-last word.

The following types of operators do not act on any variable:

`touch_file`
: performs `touch input1` on the file system

`copy_file`
: performs `cp input1 input2` on the file system. *stringVariable input1* may contain a linux wildcard. If *stringVariable input2* contains a directory structure that does not exist yet, it will be created.

`move_file`
: performs `mv input1 input2` on the file system. *stringVariable input1* may contain a linux wildcard. If *stringVariable input2* contains a directory structure that does not exist yet, it will be created.

`delete_file`
: performs `rm -f input1` on the file system. *stringVariable input1* may contain a linux wildcard.

`email`
: sends an email, provided a *stringVariable* with the name *email* exists and the Linux command *mail* is functional. The content of the email has the current value of *stringVariable input1*, and optionally also *stringVariable input2*.

`wait`
: waits *floatVariable input1* seconds since the last time this operator was executed. The first time it is executed, this operator only starts the counter and does not wait. Optionally, if *output* is defined as a *floatVariable*, then the elapsed number of seconds since last time is stored in *output*.

exit_maxtime
: terminates the execution of the *Scheme* after the number of hours have passed since its start as stored in *float-Variable input1*.

exit
: terminates the execution of the *Scheme*.

7.3.4 Edges

Two types of *Edges* exist. The first type is a normal *Edge*, which connects an *inputNode* to an *ouputNode*, thereby defining their consecutive execution.

The second type is called a *Fork*. A Fork has one *inputNode*, an *outputNode*, an *outputNodeIfTrue*, and an associated *booleanVariable*. Whether one or the other output Node is executed depends on the current value of the booleanVariable that is associated with the Fork. The fork with lead from the *inputNode*, an *outputNode* if the *booleanVariable* is *False*. The fork will lead from the *inputNode*, an *outputNodeIfTrue* if the *booleanVariable* is *True*. Thereby, Forks are the main instrument of making decisions in *Schemes*.

7.3.5 Create a Scheme

The combination of the *Variables*, *Nodes* and *Edges* allows one to create complicated sequences of jobs. It is probably a good idea to draw out a logical flow-chart of your sequence before creating a *Scheme*. Then, use your favourite text editor to manually edit the files `Schemes/SCHEMENAME/scheme.star` and all the files `Schemes/SCHEMENAME/JOBNAMES/job.star` for all the jobs in that *Scheme*. Following the `prep` and `proc` examples in the `scripts` directory of your relion installation is probably the easiest way to get started.

In the `Schemes/SCHEMENAME/scheme.star` file, first add all the different variables and operators that you will need.

Note that any variable names that contain a *JobNameOriginal* of any of the *Jobs* inside any *Scheme* that is present in the *ProjectDirectory*, will be replaced by the current *JobName* upon execution of an operator. For example, a *stringVariable* with the value `Schemes/prep/ctffind/micrographs_ctf.star` will be replaced to something like `CtfFind/job003/micrographs_ctf.star` upon execution of the job that uses it, assuming that the current *JobName* of that job is `CtfFind/job003/` in the `Schemes/prep/scheme.star` file.

Then, add your jobs. You can use the normal relion GUI to fill in all parameters of each job that you need and then use the `Save job.star` options from the `Jobs` menu to save the `job.star` file in the corresponding `Schemes/SCHEMENAME/JOBNAMES/` directory. Jobs use the same mechanism as described for the *Variables* above. So, if an Auto-picking job depends on its micrographs STAR file input on a CTF estimation job called *ctffind*, and this CTF estimation job is part of a *Scheme* called `prep`, then the micrographs STAR file input for the Auto-picking job should be set to `Schemes/prep/ctffind/micrographs_ctf.star`, and this will be converted to `CtfFind/job003/micrographs_ctf.star` upon execution of the job. In addition, a corresponding edge will be added to the `default_pipeliner.star` upon execution of the *Scheme*. Also note that parameters in `job.star` files may be updated with the current values of *Variables* from the *Scheme* by using the `$$` prefix, followed by the name of the corresponding *Variable*, as also mentioned above.

In addition, the *JobMode* needs to be chosen from options: `new` or `continue`. Typically, in on-the-fly-processing procedures that iterate over ever more movies, jobs like Import, Motion correction, CTF estimation, Auto-picking and Particle extraction are set as `continue`, whereas most other jobs are set as `new`.

Finally, once all the *Variables*, *Operators* and *Jobs* are in place, one should define all the *Edges* between them.

The *Scheme* will be initialised (and reset) to the left-hand *Node* of the first defined *Edge*. If the *Scheme* is not an infinite loop, it is recommended to add the `exit` *Operator* as the last *Node*.

Once a *Scheme* has been created, it may be useful for more than one relion project. Therefore, you may want to store it in a tar-ball:

```
tar -zcvf preprocess_scheme.tar.gz Schemes/preprocess
```

That tar-ball can then be extracted in any new relion project directory:

```
tar -zxvf preprocess_scheme.tar.gz
```

Executing a *Scheme*

Once you have created the Scheme/name/ (sub)directories (with "name" being the name of your scheme), you can launch a separate GUI using:

```
relion_schemegui.py name
```

You can start, stop, change parameters, and restart the scheme from there. You can also look into this python script to see the actual calls it makes to relion_schemer, which is the command line program that executes the scheme. While it runs, you can then follow the generation of new jobs in the normal relion GUI.

7.4 Helical reconstruction

Shaoda He, a PhD-student in the Scheres group, implemented a workflow for the processing of helical assemblies. This involves additional tabs to the parameter-panels of the Auto-picking, Particle extraction, 2D classification, 3D classification, 3D auto-refine, Particle polishing and Mask create job-types. We do not have a separate tutorial for processing helical assemblies. The general principles remain the same as for single-particle analysis, which is covered in this tutorial. Therefore, users intending to use relion for helical processing are still encouraged to do this tutorial first. For a detailed description of the helical options, the user is referred to the corresponding pages on the RELION wiki, or to Shaoda's paper[HS17]. We are aware that a tutorial on helical processing is probably overdue, but due to time constraints we haven't got to doing that yet. Sorry…

7.4.1 Initial model generation for amyloids

The `relion_helix_inimodel2d` program is a new feature in relion-3.1. It allows generation of an initial 3D reference for helical reconstruction, in particular for amyloids. It is run from the command line, and takes a selection of suitable 2D class averages as input. It will try to align these class averages with respect to each other to form a continuously changing density that spans an entire cross-over. At the heart of the iterative refinement process lies a *tomographic* 2D reconstruction with all 1D pixel columns from the cross-over. Details of this program, together with a more elaborate documentation of its functionality remain to be published. Possible usage is:

```
relion_helix_inimodel2d --i Select/job056/class_averages.star \
  --crossover_distance 800  --angpix 1.15 --maxres 9 --search_shift 3 \
  --mask_diameter 250 --j 6 --iter 5 --o IniModel/run1

relion_helix_inimodel2d --i IniModel/run1_it005.star \
  --iniref 1@IniModel/run1_it005_reconstructed.mrcs  --angpix 1.15 \
  --maxres 6 --search_angle 2 --step_angle 0.5 --mask_diameter 250 \
  --j 6 --iter 5  --o IniModel/run2
```

We like xmipp-2.4 for live-updated display of the following images during the optimisation:

```
xmipp_show -img rec.spi after_reproject.spi before_reproject.spi -poll &
```

7.5 Subtomogram Averaging

7.5.1 Data Types

The custom data types used in tomography programs in relion are:

Tomogram set

The tomogram set describes all the tomograms (i.e. tilt series) defined in a project, and is usually named `tomograms.star`. It contains a single table, named `global`, which defines the properties pertaining to each tomogram. Each row corresponds to one tomogram, and it lists the following properties:

- The **tomogram name** (`rlnTomoName`): a unique identifier used to refer to this tomogram within the entire project (i.e. within the tomogram set, the particle set and the trajectory set)
- The **tilt series file name** (`rlnTomoTiltSeriesStarFile`): the path to the star file corresponding to each tomogram/image stack
- The **tomogram size** (`rlnTomoSize<X/Y/Z>`): the extent of the tomogram region in 3D space
- The **handedness** (`rlnTomoHand`): this value is either +1 or -1, and it describes whether the focus increases or decreases as a function of depth (projected Z coordinate). It cannot be known a priori and has to be determined experimentally. It is typically identical across the entire data set.
- The **pixel size of the raw micrographs**, given in Å (`rlnMicrographOriginalPixelSize`)
- The **pixel size of the tilt series**, given in Å (`rlnTomoTiltSeriesPixelSize`), corresponding to the dimensions given by `rlnTomoSize<X/Y/Z>`, after the binning applied in the Motion correction job
- The **tomogram binning** (`rlnTomoTomogramBinning`): the binning applied to the tilt series in `rlnTomoTiltSeriesStarFile` to reconstruct the tomogram in `rlnTomoReconstructedTomogram`
- The **reconstructed tomogram file** (`rlnTomoReconstructedTomogram`): the path to the reconstructed tomogram mrc file, at the binning in `rlnTomoTomogramBinning`
- The **Etomo file** (`rlnEtomoDirectiveFile`)
- The **optics group name** (`rlnOpticsGroupName`)
- The **voltage**, given in kV (`rlnVoltage`)
- The **spherical aberration**, given in mm^-1 (`rlnSphericalAberration`)
- The **amplitude contrast** (`rlnAmplitudeContrast`)

The star file for each tomogram, to which `rlnTomoTiltSeriesStarFile` points, contains a single table with the same name as the tomogram. In it, each row corresponds to one tilt image and includes the following properties, among others:

- The **tilt image rotation angles and shifts** (`rlnTomo<X/Y>Tilt`, `rlnTomoZRot` and `rlnTomo<X/Y>ShiftAngst`)
- The **astigmatic defocus** (`rlnDefocus<U/V>` and `rlnDefocusAngle`)
- The **intensity scale factor** related to effective ice thickness at each tilt angle (`rlnCtfScalefactor`)
- The **cumulative radiation dose** (`rlnMicrographPreExposure`)
- The **frame count** (`rlnTomoTiltMovieFrameCount`)

Particle set

The particle set (typically named `particles.star`) lists all the particles in a data set. It is roughly equivalent to the corresponding particles file used by the SPA programs in relion, and it serves as the binding element between the tomography programs and the ones used for the general refinement.

Like an SPA particle set, the tomography particle set consists of an optics table and a particles table. Unlike in an SPA particle set, the coordinates of the particles are given in 3 dimensions (`rlnCenteredCoordinate<X/Y/Z>Angst` and `rlnOrigin<X/Y/Z>Angst`), and each particle also requires a tomogram name (`rlnTomoName`) that identifies the particle's tomogram of origin, as defined in the *tomogram set*. Note that in Relion 5, the pixel coordinates rlnCoordinate<X/Y/Z> have been replaced by the absolute and centered coordinates rlnCenteredCoordinate<X/Y/Z>Angst, which are in Angstroms from the centre of the tomogram.

Also unlike in SPA, the tomography particles should not be thought of as pixel data stored on disk, but instead as abstract entities defined in this file. They are represented on the disk either as 2D stacks of CTF-premultiplied extracted images (the preferred way in Relion 5) or as 3D volumes where Fourier slices of the 2D images are combined in 3D. The particles that are used for refinement (i.e. the pseudo-subtomograms) can be created using the program *relion_tomo_subtomo*, but they should be regarded as transient data that become invalid once any of the other properties of their tomograms have been updated. Therefore, during the processing of a tomography data set, the pseudo-subtomograms are typically created multiple times.

Another difference to a SPA 2D particle set is that tomography particles can optionally possess two different orientations. In tomography, the approximate orientation of a particle is often dictated by the geometric context (e.g. the orientation of a membrane in which they are embedded). Therefore, the subtomogram alignment can be greatly accelerated by defining the initial orientation of each subtomogram in relation to that geometry (using the fields rlnTomoSubtomogram<Rot/Tilt/Psi>) and then applying strong priors during refinement to constrain the angles that describe the orientation of the particle itself with respect to its subtomogram (rlnAngle<Rot/Tilt/Psi>). Since only the latter angles are considered by relion_refine, the priors will only affect those angles. Typically, this means that the rlnAngleTilt angle will be constrained, while the other two are usually unknown, even in tomography.

Trajectory set

The trajectory set (typically named motion.star) contains the motion trajectories of all particles in a corresponding *particle set*. The trajectories can be estimated using the program *relion_tomo_align*, and they will be considered by any relion tomography program that requires particle positions.

The trajectory file consists of P+1 tables, where P is the number of particles in the corresponding particle set. The first table only contains the number of particles, while every successive table, named using the particle name (the same as the value of rlnTomoParticleName in the *particle set* file) lists the trajectory of each particle. The trajectories are given in Å, and they are relative to the position of the particle in the chronologically first tilt image (i.e. the image with the lowest cumulative dose).

Optimisation set

The optimisation set is the central data type in a relion tomography project, and it only contains paths to other files. It is usually named optimisation_set.star.

Specifically, it can point to the following data files:

- A *tomogram set* listing all tomograms.
- A *particle set* listing all particles.
- A *trajectory set* describing the motion of all particles.

All of those entries are optional, and different programs require different inputs. All programs take an optimisation set as input, and most of them also write one out. If a data file is created or updated by a program, it is added to the optimisation set that the program writes out. This frees the user from having to track which programs update which data files. For example, *relion_tomo_refine_ctf* will only update the *tomogram set*, because the defoci are only defined once for each tilt image, while *relion_tomo_align* will update the *tomogram set*, the *particle set* and the *trajectory set*.

When running a program, the individual data files can also be specified separately, in which case the individual files will override the ones listed in the optimisation set. This allows the user to easily perform the same procedure with specific data files exchanged. If all data files required by a program have been specified individually, then an optimisation set is not required at all.

There are 3 ways to create an initial optimisation set:

- Use the utility program *relion_tomo_make_optimisation_set*.
- Run any program and specify the individual data files. The program will then output a new optimisation set.
- Write one manually.

7.5.2 Programs

These are the most relevant tomography-related programs in relion:

relion_tomo_reconstruct_particle

This program constructs high-resolution 3D reference maps from a given *optimisation set*. It is similar to `relion_reconstruct`, except that it does not require pre-extracted particle images. Instead, it extracts square windows of arbitrary size (`--b` argument) directly from the tilt series and it uses those for the reconstruction (using Fourier inversion). It considers the defoci of the individual particles, as well as particle motion and higher-order aberrations.

The output consists of a pair of maps (one for each half set) as well as a merged map. In addition, the numerator (data term) and denominator (weight term) (see Eq. 3 in the |RELION| paper) of all 3 maps are also written out to facilitate further processing.

Relevant program arguments:

- `--i` and/or `--p`, `--t` and `--mot`: input optimisation-set and/or its components (see *optimisation set*).
- `--b`: box size of the reconstruction. Note that this is independent of the box size used to refine the particle. This allows the user to construct a 3D map of arbitrary size to gain an overview of the structure surrounding the particle. A sufficiently large box size also allows more of the high-frequency signal to be captured that has been delocalised by the CTF.
- `--crop`: cropped box size. If set, the program will output an additional set of maps that have been cropped to this size. This is useful if a map is desired that is smaller than the box size required to retrieve the CTF-delocalised signal.
- `--bin`: downsampling (binning) factor. Note that this does not alter the box size. The reconstructed region instead becomes larger.
- `--SNR`: apply a Wiener filter with this signal-to-noise ratio. If omitted, the reconstruction will use a heuristic to prevent divisions by excessively small numbers. Please note that using a low (even though realistic) SNR might wash out the higher frequencies, which could make the map unsuitable to be used for further refinement.
- `--sym`: name of the symmetry class (e.g. C6 for six-fold point symmetry).
- `--mem`: (approximate) maximum amount of memory to be used for the reconstruction (see below).
- `--j`: number of threads used for the non-reconstruction parts of the program (e.g. symmetry application or gridding correction). This should be set to the number of CPU cores available.
- `--j_out`: number of threads that compute partial reconstructions in parallel. This is faster, but it requires additional memory for each thread. When used together with the `--mem` argument, this number will be reduced to (approximately) maintain the imposed memory limitation.
- `--j_in`: number of threads to be used for each partial reconstruction. This is a slower way to parallelise the procedure, but it does not require additional memory. Unless memory is limited, the `--j_out` option should be preferred. The product of `--j_out` and `--j_in` should not exceed the number of CPU cores available.
- `--o`: name of the output directory (that will be created).

Program output:

After running the program, the output directory (`--o` argument) will contain the following items:

- **<half1/half2/merged>.mrc**: the reconstructed cropped maps.
- **<half1/half2/merged>_full.mrc**: the reconstructed full-size maps, in case the full size (`--b` argument) differs from the cropped size (`--crop` argument).
- **data_<half1/half2/merged>.mrc**: the reconstructed data term at full size.
- **weight_<half1/half2/merged>.mrc**: the reconstructed weight term at full size.
- **note.txt**: a text file containing the command issued to run the program.

relion_tomo_subtomo

This program creates *pseudo subtomograms* that can be fed into `relion_refine` to perform subtomogram alignment. Those pseudo subtomograms are not meant to represent the actual density distributions of the particles, but instead abstract 3D image terms that allow `relion_refine` to approximate the cost function that would arise if the alignment were to be performed on the stack of corresponding 2D images instead. Specifically, each pseudo subtomogram consists of a sum of CTF-weighted observations (data term) as well as the corresponding sum of CTF^2 terms (weight term). In addition, the output weight term also contains - concatenated along the Z axis - a 3D multiplicity-image (number of Fourier slices contributing to each Fourier voxel) that allows `relion_refine` to estimate a spherically symmetrical noise distribution.

The reconstructed pseudo subtomograms consider the motion of the particles as well as higher-order aberrations, if either have been estimated. The program will output a new *particle set*, as well as a new *optimisation set* in which the initial particle set has been replaced by the newly generated one. That particle set can be fed into `relion_refine`.

If a subtomogram orientation has been defined (see *particle set*), then the subtomogram will be constructed in that coordinate system. If the approximate orientation of the particle is known a-priori from the surrounding geometry, this allows angle priors to be used in `relion_refine` to avoid searching through implausible orientations. In particular, for particles sampled from a manifold (using *relion_tomo_sample_manifold*), the Z axis of the subtomogram coordinate-system is always perpendicular to the local manifold. A strong tilt-angle prior can then be applied to only search through orientations that do not tilt the particle too far away from that orientation, while leaving the remaining two angles unconstrained (i.e. the direction of tilt and the rotation around the Z axis).

Relevant program arguments:

- `--i` and/or `--p`, `--t` and `--mot`: input optimisation-set and/or its components (see *optimisation set*).
- `--b`: initial box size of the reconstruction. A sufficiently large box size allows more of the high-frequency signal to be captured that has been delocalised by the CTF.
- `--crop`: cropped output box size. After construction, the resulting pseudo subtomograms are cropped to this size. A smaller box size allows the (generally expensive) refinement using `relion_refine` to proceed more rapidly.
- `--bin`: downsampling (binning) factor. Note that this does not alter the initial or the cropped box size. The reconstructed region instead becomes larger.
- `--cone_weight`: downweight a cone in Fourier space along the Z axis (as defined by the coordinate system of the particle). This is useful for particles embedded in a membrane, as it can prevent the alignment from being driven by the membrane signal (the signal of a planar membrane is localised within one line in 3D Fourier space). Note that the coordinate system of a particle is given by both the subtomogram orientation (if defined) *and* the particle orientation (see *particle set*). This allows the user to first obtain a membrane-driven alignment, and to then specifically suppress the signal in that direction.

- `--cone_angle`: the (full) opening angle of the cone to be suppressed, given in degrees. This angle should include both the uncertainty about the membrane orientation and its variation across the region represented in the subtomogram.
- `--j`: number of threads used to reconstruct pseudo subtomograms in parallel. Each thread will require additional memory, so this should be set to the number of CPU cores available, unless the memory requirements become prohibitive.
- `--o`: name of the output directory (that will be created).

Program output:

After running the program, the output directory (`--o` argument) will contain the following items:

- **particles.star**: a new *particle set* containing the file names of the reconstructed subtomograms. This file can be understood by `relion_refine`
- **optimisation_set.star**: a new *optimisation set* pointing to the new particle set.
- **Subtomograms/<**`particle_name`>_data.mrc**: 3D images representing the data terms of all subtomograms.
- **Subtomograms/<** `particle_name` **>_weights.mrc**: the weight terms of all subtomograms. Each 3D image contains, concatenated along Z, an image describing the sums over all CTF^2 and an image describing the multiplicities.
- **note.txt**: a text file containing the command issued to run the program.

relion_tomo_align

This program refines the projections that map 3D space onto the images of the tilt series, as well as (optionally) also the beam-induced motion trajectories of the particles. Each projection is optimised using the full 5 degrees of freedom: the assumption of a common tilt axis is abandoned. Even if no beam-induced motion is estimated, the (in that case static) 3D particle-positions are also optimised by the program. This is because those 3D positions cannot be assumed to be known in advance, since they have only been inferred from the observed 2D particle-positions in the individual tilt images. Therefore, this program always looks for the optimal set of 3D positions *and* projections.

If the particle motion is also being estimated (using the `--motion` argument), then the 3D positions of the particles are allowed to differ between tilt images, albeit only in a limited and spatially smooth manner. This is done analogously to Bayesian polishing in 2D (implemented in `relion_motion_refine`), except that the trajectories are estimated in 3D, and that no acceleration constraint is imposed. Because part of the particle motion is rigid, the motion estimation also always includes a rigid alignment of the frames.

Please note that estimating beam-induced motion significantly increases the runtime of the program, so it should only be done in the final rounds of optimisation.

The estimated particle trajectories are written into the output *trajectory set*, the projection matrices (containing the rigid part of the motion as well as the alignment of the tilt series - these cannot be distinguished) into the output *tomogram set* and the estimated (initial) particle positions into the output *particle set* which replace the given trajectory, tomogram and particle sets in the output *optimisation set*.

General program arguments:

- `--i` and/or `--p`, `--t`, `--mot`, `--ref<1/2>`, `--mask` and `--fsc`: input optimisation-set and/or its components (see *optimisation set*).
- `--b`: box size to be used for the estimation. Note that this can be larger than the box size of the reference map. A sufficiently large box size allows more of the high-frequency signal to be captured that has been delocalised by the CTF.

- `--r`: maximal assumed error in the initial 2D particle-positions (distances between the projected 3D positions and their true positions in the images), given in pixels.
- `--j`: number of threads to be used. This should be set to the number of CPU cores available.
- `--o`: name of the output directory (that will be created).

Motion-related arguments:

- `--motion`: perform motion estimation simultaneously with the tilt-image alignment.
- `--s_vel`: the expected amount of motion (i.e. the std. deviation of particle positions in Å after 1 electron per $Å^2$ of radiation).
- `--s_div`: the expected spatial smoothness of the particle trajectories (a greater value means spatially smoother motion).
- `--sq_exp_ker`: assume that the correlation of the velocities of two particles decays as a Gaussian over their distance, instead of as an exponential. This will produce spatially smoother motion and result in a shorter program runtime.

Deformation-related arguments:

- `--deformation`: perform 2D deformation estimation for each tilt-image.
- `--def_w`: Number of horizontal sampling points for the deformation grid.
- `--def_h`: Number of vertical sampling points for the deformation grid.
- `--def_model`: Type of model to estimate deformations (linear, spline or Fourier).
- `--def_reg (0.0)`: Value of the deformation regulariser
- `--per_frame_deformation`: Model separate 2D deformations for each tilt-image instead of the whole tilt series.

Program output:

After running the program, the output directory (`--o` argument) will contain the following items:

- **motion.star**: a new trajectory set
- **tomograms.star**: a new *tomogram set* containing the newly estimated projection matrices (`rlnTomoProj<X/Y/Z/W>`)
- **particles.star**: a new *particle set* containing the newly estimated particle positions in 3D (`rlnCoordinate<X/Y/Z>`)
- **optimisation_set.star**: a *optimisation set* pointing to the new trajectory, tomogram and particle sets.
- **Trajectories/<** `tomogram_name` **>_x<1/8>.ply**: meshes of 3D trajectories in ply format, scaled by a factor of 1 or 8, respectively. Note that only the trajectories themselves are scaled - their positions are those of the particles. They can be viewed in relation to (low-magnification) tomograms as produced by *relion_tomo_reconstruct_tomogram* in a viewer that supports both meshes and 3D images (such as e.g. Paraview).
- **note.txt**: a text file containing the command issued to run the program.

relion_tomo_refine_ctf

This program estimates the astigmatic defoci of the individual tilt images, the ice thickness (which determines the overall signal intensity) and higher-order optical aberrations.

Defocus: In tomography, the relative depth distances between particles are known from the 3D positions of the particles. Therefore, only one defocus value is estimated for all the particles in each tilt image. Because of the often large number of particles in each tomogram, this value can typically be estimated to greater precision than in single-particle analysis, where the defocus of each particle has to be estimated independently.

Ice-thickness: A thicker sample permits fewer electrons to pass through, which reduces the scale of the signal. In addition to the actual variation in sample thickness, the effective thickness of the ice also increases as the sample is tilted. This program allows the user to estimate the signal intensity either independently for each tilt image, or by fitting the base thickness, the initial beam luminance and the surface normal of the sample assuming Beer-Lambert's Law. In the latter case, only one surface normal and base thickness are estimated for an entire tilt series, which allows for a more stable fit from tomograms with fewer particles.

Higher-order optical aberrations: This algorithm works analogously to `relion_ctf_refine` for single-particle analysis. As in single-particle analysis, the aberrations are estimated per optics group. This allows the user to group particles that are expected to share the same aberrations, either by image region or by subset of tilt series, or both. Both symmetrical (even) and antisymmetrical (odd) aberrations are supported. A detailed description of the aberrations estimation algorithm can be found in the relion aberrations paper.

The estimated defocus and ice-thickness values are written into the output *tomogram set* and the aberrations into the *particle set*, both of which replace the given sets in the output *optimisation set*.

General program arguments:

- `--i` and/or `--p`, `--t`, `--mot`, `--ref<1/2>`, `--mask` and `--fsc`: input optimisation-set and/or its components (see *optimisation set*).
- `--b`: box size to be used for the estimation. Note that this can be larger than the box size of the reference map. A sufficiently large box size allows more of the high-frequency signal to be captured that has been delocalised by the CTF.
- `--j`: number of threads to be used. This should be set to the number of CPU cores available.
- `--o`: name of the output directory (that will be created).

Defocus-estimation arguments:

- `--do_defocus`: instructs the program to estimate the defoci.
- `--do_reg_defocus`: applies defocus regularisation. High-tilt images do not offer enough signal to recover the defocus value precisely. The regularisation forces the estimated defoci to assume similar values within a given tilt series, which prevents those high-tilt images from overfitting.
- `--lambda`: defocus regularisation strength.
- `--d0` and `--d1`: minimal and maximal defocus offset (from the initial value) to scan in the initial part of the defocus estimation. This scan allows the algorithm to escape a local minimum in case it has been intialised in one. Afterwards, a local astigmatic-defocus refinement is performed. Both values are assumed to be given in Å.
- `--ds`: number of steps between `--d0` and `--d1`.
- `--kmin`: Lowest spatial frequency to consider, in Å.
- `--reset_to_common`: Reset the CTFs of all tilts to a common one prior to local refinement.

Ice-thickness estimation arguments:

- `--do_scale`: instructs the program to estimate the signal scale or ice thickness.
- `--per_frame_scale`: estimate the signal-scale parameter independently for each tilt. If not specified, the ice thickness, beam luminance and surface normal are estimated instead. Those three parameters then imply the signal intensity for each frame. Due to the smaller number of parameters, the ice thickness model is more robust to noise. By default, the ice thickness and surface normal will be estimated per tilt-series, and the beam luminance globally.
- `--per_tomogram_scale`: estimate the beam luminance separately for each tilt series. This is not recommended.

Aberrations-estimation arguments:

- `--do_even_aberrations`: instructs the program to estimate the even (i.e. symmetrical) aberrations. These deform the shape of the CTF.
- `--do_odd_aberrations`: instructs the program to estimate the odd (i.e. anti-symmetrical) aberrations. These rotate the phases of the observed images.
- `--ne`: number of Zernike bands used to fit the even aberrations. A greater number can quickly lead to overfitting. The user is advised to keep this value at 4 - this will allow for a correction to the spherical aberration term, as well as four-fold and first- and second-order two-fold astigmatism.
- `--no`: number of odd Zernike bands. The user is advised to keep this value at 3 - this will allow for beam tilt and three-fold astigmatism.

Program output:

After running the program, the output directory (`--o` argument) will contain the following items:

- **tomograms.star**: a new *tomogram set* containing the newly estimated defoci (`rlnDefocus<U/V>` and `rlnDefocusAngle`) and ice thickness values. Note that only the signal scale (`rlnCtfScalefactor`) implied by the ice thickness is used by other programs.
- **particles.star**: a new *particle set* containing the estimated aberrations.
- **optimisation_set.star**: a new *optimisation set* pointing to the new tomogram and particle sets.
- **note.txt**: a text file containing the command issued to run the program.
- **group_<** `optics_group` **>_<even/odd>_phase_per-pixel.mrc**: visualisations showing the optimal phase-shift for each pixel. Inspecting this allows the user to ensure that the estimated pattern is not mere noise.
- **group_<** `optics_group` **>_<even/odd>_phase_nonlinear-fit.mrc**: visualisations of the fits using Zernike polynomials, which will be used by the other programs.

relion_tomo_local_particle_refine

This program optimises the viewing angles and 3D positions of all particles. This is done by following the local gradient of the L2 error function downhill, so it is significantly faster than a full refinement using `relion_refine`. Currently, the results are slightly worse than those from `relion_refine`, though.

The estimated particle angles and positions are written into the output *particle set* which replaces the given particle set in the output *optimisation set*.

Relevant program arguments:

- `--i` and/or `--p`, `--t`, `--mot`, `--ref<1/2>`, `--mask` and `--fsc`: input optimisation-set and/or its components (see *optimisation set*).
- `--b`: box size to be used for the estimation. Note that this can be larger than the box size of the reference map. A sufficiently large box size allows more of the high-frequency signal to be captured that has been delocalised by the CTF.
- `--j`: number of threads to be used. This should be set to the number of CPU cores available.
- `--o`: name of the output directory (that will be created).

Program output:

After running the program, the output directory (`--o` argument) will contain the following items:

- **particles.star**: a new *particle set* containing the newly estimated angles and positions.
- **optimisation_set.star**: a new *tomogram set* pointing to the new particle set.
- **note.txt**: a text file containing the command issued to run the program.

relion_tomo_fit_blobs_3d

This program refines spherical manifolds to better fit the double membranes of vesicles, producing spheroidal manifolds. The resulting manifold will run along the outer interface of the outer membrane, where the signal is the strongest. These spheroidal manifolds can then be used to sample random particles at a specific depth and with an appropriate initial orientation using the program *relion_tomo_sample_manifold*.

The initial spherical manifolds need to be part of the input manifold set that can be created using the program *relion_tomo_add_spheres*. The estimated spheroids are written into the output manifold set which replaces the given one in the output *optimisation set*. Note: the user is recommended to first detect all fiducials in the input tomograms, since their strong signal can disturb the membrane estimation. This can be done using the program *relion_tomo_find_fiducials*.

Relevant program arguments:

- `--i` and/or `--t` and `--man`: input optimisation-set and/or its components (see *optimisation set*).
- `--ms`: approximate membrane separation in Å.
- `--rr`: the expected variation of the vesicle radius as a multiple of the initial sphere radius.
- `--frad`: approximate radius of the fiducial markers (required for their deletion).
- `--bin`: binning level at which to perform the membrane estimation. This needs to allow multiple pixels of separation between the two membranes.
- `--n`: number of Spherical Harmonics bands used to represent the spheroids. A greater number will allow for a more precise membrane fit, but it will also make overfitting more likey. A value between 5 and 9 is recommended.
- `--j`: number of threads to be used. This should be set to the number of CPU cores available.
- `--o`: name of the output directory (that will be created).

Program output:

After running the program, the output directory (`--o` argument) will contain the following items:

- **manifolds.star**: a new manifold set containing the spheroidal membranes. It will *not* contain the initial spheres, nor any other initial manifolds.

- **optimisation_set.star**: a new *optimisation set* pointing to the new manifold set.
- **Meshes/<** `tomogram_name` **>.ply**: visualisations showing the fitted spheroids for each tomogram in ply format. They can be viewed in relation to (low-magnification) tomograms as produced by *relion_tomo_reconstruct_tomogram* in a viewer that supports both meshes and 3D images (such as e.g. Paraview).
- **note.txt**: a text file containing the command issued to run the program.

relion_tomo_reconstruct_tomogram

This program creates a tomogram (i.e. a 3D image approximating the scattering potential within a cryo-EM sample) from a tilt series defined in a given *tomogram set*. Please note that these tomograms are *not* intended for further processing. Instead, their purpose is only to allow for the inspection of the sample and the detection of specific structures, such as manifolds or individual particles. In order to perform subtomogram averaging, the detected particles should be written into a|particle_set|, from which *pseudo subtomograms* should be constructed using the program program_subtomo. These pseudo subtomograms can then be refined using `relion_refine`.

This program is also not intended for the reconstruction of full-size tomograms, since the resulting 3D image needs to fit into memory.

Relevant program arguments:

- `--i` or `--t`: input optimisation-set or tomogram set (see *optimisation set*).
- `--tn`: `tomogram_name` of the tomogram to reconstruct (see *tomogram set*).
- `--no_weight`: reconstruct an unweighted tomogram, as opposed to applying a weighting in 3D using a Wiener filter. If the purpose of the tomogram is for a human to slice through it along the viewing direction, then an unweighted tomogram offers the most contrast. It will introduce streaking artifacts along the viewing direction, however.
- `--pre_weight`: apply a pre-weighting in 2D instead of a 3D weighting. This is significantly more efficient for large tomograms.
- `--SNR`: the signal-to-noise ratio assumed by the Wiener filter. Note: a realistic SNR might lead to an excessive dampening of the high frequencies. If the purpose of the tomogram is only for humans to inspect it, then this dampening might make the image clearer.
- `--noctf`: do not modulate the input images by a (spatially constant) CTF.
- `--keep_mean`: do not subtract the mean from (i.e. do not zero the DC component of) every 2D image prior to reconstruction.
- `--td`: taper distance. Do not backproject image regions closer than this to the edge of a 2D image.
- `--tf`: taper falloff. Fade out the contribution of each 2D image along this distance to the edge of the effective image. If a value has been specified for the `--td` argument, then this falloff begins at that edge. These two options are useful if the images show artifacts near the edge, and the resulting tomogram is to be used for automated detection.
- `--<x0/y0/z0>`: the origin of the 3D region to be reconstructed. Note: to maintain consistency with other processing software, the default origin (corresponding to the one used by IMOD) is equal to 1. Therefore, 1 is also the default value for these arguments.
- `--<w/h/d>`: the size of the 3D region to be reconstructed (in bin-1 pixels). If not specified, the size indicated in the tomogram set will be used, which should correspond to the region defined in IMOD.
- `--bin`: the binning level of the reconstruction. Note: the default value for this is 8, in order to prevent accidentally reconstructing a bin-1 tomogram.
- `--j`: number of threads to be used. This should be set to the number of CPU cores available.

- `--o`: name of the output 3D image. This is the only file created by this program. If the file name contains subdirectories, then those will also be created.

relion_tomo_import_tomograms

This program imports a set of tilt series to form a *tomogram set*. It is typically the first program to be used when creating a relion tomography data set. It imports an existing alignment from IMOD and the initial CTF parameters from either CTFFind or CtfPlotter. Additional parameters, such as the fractional electron dose and the chronological sequence of frames have to be supplied by the user.

The tilt series alignment effectively defines an arbitrary 3D coordinate system, as well as a set of projections that map those 3D coordinates to the individual 2D images of the tilt series. The aim of this program is to translate that alignment into a format readable by relion. If used successfully, this will produce an equivalent 3D coordinate system inside relion that will allow particle positions already defined in an IMOD tomogram to be used unchanged.

Optimally, the tilt series would be imported into relion right after alignment, and the particles would be picked in a low-magnification tomogram generated by *relion_tomo_reconstruct_tomogram*. That way, the particle positions would be guaranteed to be correct.

The primary input to *relion_tomo_import_tomograms* is a `.star` file containing one row for each tilt series and at least the following columns:

- **rlnTomoImportImodDir**: path to the IMOD directory. That directory has to contain the command files used to run IMOD's `tilt` and `newst` programs. Typically, those are named `tilt.com` and `newst.com`. If those are not their names, then the actual names can be specified using the `--tc` and `--nc` arguments, respectively. Additional IMOD files that are referenced inside those `.com` files also need to be present inside the IMOD directory.
- **rlnTomoImportCtfFindFile** or **rlnTomoImportCtfPlotterFile**: path to the initial CTF estimate from either CTFFind or CtfPlotter, respectively.
- **rlnTomoTiltSeriesName**: path to the actual tilt series. Note: if the tilt series ends with `.st`, this needs to be specified with an `.st:mrc` ending to tell relion to interpret it as an mrc file.

The following additional columns may be present. If they are not, then the corresponding command line arguments will be used. In that case, they will be identical for all tilt series.

- **rlnTomoName**: the tomogram name. The user is advised to specify a short and meaningful name here, since it will make the data more readable and simplify the resulting directory structure. Names like e.g. `TS_001` are recommended. If not specified, the entire path to the tilt series (i.e. the value of `rlnTomoTiltSeriesName`) will be used instead.
- **rlnTomoImportFractionalDose**: the electron dose corresponding to one tilt image. If omitted, the value of the `--fd` argument will be used.
- **rlnTomoImportOrderList**: path to a two-column text file specifying the *chronological* order in which the images were acquired. That file has to contain two numbers per line separated by a comma (and no spaces), where the first number counts up from 1, while the second describes the sequence of tilt angles. To determine the sequence of tilt images, the program will look for the closest tilt angle for each image (as stored in the `.tlt` file). This approach can deal with frames missing from the tilt series. If not specified in the `.star` file, the `--ol` input argument will be used.
- **rlnOpticsGroupName**: an arbitrary name for an optics group. This allows the set of tilt series to be separated into subsets that share the same optical aberrations. This is useful if the data have been collected in multiple sessions that might exhibit different aberrations. If omitted, all tilt series will be assigned to the same default optics group.
- **rlnTomoImportOffset<X/Y/Z>**: an arbitrary offset to the 3D coordinate system. This is useful if particles have already been picked in tomograms that have been cropped after reconstruction by IMOD. If the IMOD-internal `SHIFT` command has been used to apply offsets, then this will be handled internally and does *not* need to be

specified here. If omitted, then the values of the `--off<x/y/z>` command line arguments will be used instead (which default to 0).

- **rlnTomoImportCulledFile**: output file name for a new tilt series with the excluded frames missing. This is only needed if tilt images have been excluded using IMOD's `EXCLUDE`, `EXCLUDELIST` or `EXCLUDELIST2` commands. In that case, this becomes a mandatory parameter.

General program arguments:

- `--i`: path to the input .star file.
- `--t`: path to an optional input *tomogram set* . If specified, the imported tomograms will be added to that set.
- `--hand`: the handedness of the tilt geometry. The value of this parameter is either +1 or -1, and it describes whether the focus increases or decreases as a function of Z distance. It has to be determined experimentally. In our experiments, it has always been -1.

Optics arguments:

- `--angpix`: the pixel size in Å.
- `--voltage`: the voltage in kV.
- `--Cs`: the spherical aberration in mm.
- `--Q0`: the amplitude constrast.

Electron dose arguments:

- `--ol`: the file name of a frame-order list file. Corresponds to the `rlnTomoImportOrderList` column in the input .star file.
- `--fd`: The fractional (i.e. per tilt-image) electron dose. Corresponds to the `rlnTomoImportFractionalDose` column in the input .star file.

IMOD import arguments:

- `--flipYZ`: if the IMOD tomogram has been flipped along Y and Z (i.e. using clip flipyz) after an IMOD reconstruction and before the particles have been picked, this will apply the same transformation to the relion coordinate system. In case a rotation around X was performed (i.e. using clip rotx) this parameter should be used together with `--flipZ`. This will allow relion to use particle positions defined in the X-rotated tomogram unchanged.
- `--flipZ`: same as above, in case the Z axis has been flipped. This can be used together with the `--flipYZ` option .
- `--flipAng`: indicates that all angles in the IMOD `.tlt` file have also been flipped. This is rare.
- `--off<x/y/z>`: applies an offset to the 3D coordinate system. Equivalent to the `rlnTomoImportOffse<X/Y/Z>` column in the input .star file.

Final note: not all IMOD options are supported by this program. For example, the X-axis tilt option is not supported. In case it has been used, we recommend ignoring it and refining the resulting alignment using the program *relion_tomo_align*.

relion_tomo_import_particles

This program imports a set of 3D coordinates and (optionally) Euler angles to construct a new *particle set*.

The 3D coordinates can be provided either in one big .star file, or as a set of shorter files, one for each tomogram.

If all the coordinates are provided as one .star file, it has to contain at least the following columns:

- **rlnTomoName**: name of the tomogram to which a particle belongs.
- **rlnCoordinate<X/Y/Z>**: the 3D coordinates within that tomogram.

If the input file also contains the particle angle columns **rlnAngle<Rot/Tilt/Psi>**, or any other column, they are also imported.

In case the other format is chosen, then the input file only needs to contain the following columns: **rlnTomoName** and **rlnTomoImportParticleFile**. The latter column points to a set of files (one for each tomogram) that contain the coordinates (**rlnCoordinate<X/Y/Z>**) and (optionally) angles (**rlnAngle<Rot/Tilt/Psi>**).

Program arguments:

- `--i`: Input `.star` file containing a set of particle coordinates or a set of names of files containing those.
- `--t`: Input *tomogram set* which particles belong.
- `--o`: Output directory.

relion_tomo_make_optimisation_set

This program creates or, updates, an *optimisation set* file.

Program arguments:

- `--i` and/or `--p`, `--t`, `--mot`, `--ref<1/2>`, `--mask` and `--fsc`: input optimisation-set and/or its components (see *optimisation set*).
- `--o`: name of the output optimisation set file.

relion_tomo_make_reference

This program runs `relion_postprocess` on the pair of reference half maps set in the input *optimisation set* or generated by *relion_tomo_reconstruct_particle* so they can be used by the different refinement programs more easily.

This program creates an output *optimisation set* file and a `Postprocess` folder within the output directory.

Optimisation set arguments:

- `--i` and/or `--p`, `--t`, `--mot` and `--man`: input optimisation-set and/or its components (see *optimisation set*).
- `--opt_mask`: Mask to be used for subsequent optimisation procedures. This argument updates the output *optimisation set* file.

General program arguments:

- `--rec`: Reconstruction path of a *relion_tomo_reconstruct_particle* job (The `--o` argument). If this argument is set, half maps in input *optimisation set* file are ignored.
- `--mask`: Mask to be used for FSC computation. This argument *does not* updates the output *optimisation set* file.
- `--o`: name of the output directory (that will be created).

relion_tomo_find_fiducials

To be completed.

relion_tomo_add_spheres

To be completed.

relion_tomo_sample_manifold

To be completed.

Relion 1.4 Subtomogram averaging

For sub-tomogram averaging, which was implemented with help from Tanmay Bharat, a former postdoc in the Lowe group at MRC-LMB, the same holds as for helical processing. Many general concepts remain the same as for single-particle analysis, and users intending to perform sub-tomogram averaging in relion are encouraged to go through this tutorial first. For a detailed description of the sub-tomogram averaging procedures, the user is referred to the corresponding pages on the :textsc:`relion Wiki`, or to Tanmay's paper[BRL+15]. Please note that we are still actively working on making the sub-tomogram averaging pipeline more convenient to use and better. This work is done in close collaboration with the group of John Briggs, also at MRC-LMB. Meanwhile, please be advised that the sub-tomogram averaging pipeline is considerably less stream-lined than the single-particle one, and users should be prepared to do some scripting outside the relion pipeline for many cases.

Core programs

- *relion_tomo_reconstruct_particle* : creates high-resolution 3D maps from a given data set.
- *relion_tomo_subtomo* : creates pseudo subtomograms that can be fed into `relion_refine`.

Refinement programs

These programs use an existing particle set and a pair of high-resolution 3D reference maps to estimate or improve different properties:

- *relion_tomo_align* : aligns the images of a tilt series and estimates beam-induced motion to better fit a set of particles.
- *relion_tomo_refine_ctf* : refines the astigmatic defocus, signal intensity (i.e. ice thickness) and higher-order aberrations.
- *relion_tomo_local_particle_refine* : offers a rapid way to refine the angles and positions of individual particles.
- *relion_tomo_fit_blobs_3d* : fits a set of spherical manifolds representing vesicles to the observed membranes.

Utility programs

- *relion_tomo_import_tomograms* : imports new tilt series to create or extend a tomogram set.
- *relion_tomo_import_particles* : imports new particles.
- *relion_tomo_reconstruct_tomogram* : creates a (typically low-magnification) tomogram.
- *relion_tomo_make_optimisation_set* : ties a set of disparate data elements into an *optimisation set*.
- *relion_tomo_make_reference* : runs `relion_postprocess` on a pair of reference half maps.
- *relion_tomo_add_spheres* : creates a manifold set from a set of spheres given in `.cmm` format.
- *relion_tomo_find_fiducials* : detects the fiducial markers in a given data set.
- *relion_tomo_sample_manifold* : samples a set of particle positions and orientations from a set of manifolds.

7.5.3 Previous pipeline

- *RELION 1.4 subtomogram averaging pipeline*.

7.6 Using RELION

7.6.1 The GUI

A pipeline approach

The GUI serves a central role in it's pipelined approach, details of which have been published in the 2016 Proceedings of the CCP-EM Spring Symposium [FLS17]. We recommend to create a single directory per project, i.e. per structure you want to determine. We call this the project directory. It is important to always launch the relion graphical user-interface (GUI), by typing the command `relion`, from the project directory.

The GUI keeps track of all jobs and how output from one job is used as input for another, thereby forming a workflow or pipeline. Each type of job has its own output directory, e.g. `Class2D/`, and inside these job-type directories, new jobs get consecutive numbers, e.g. `Class2D/job010`. Inside these individual job directories, output names are fixed, e.g. `Class2D/job010/run`. To provide a mechanism to have more meaningful names for jobs, a system of job *aliases* is used, which are implemented as symbolic links to the individual job directories on the filesystem. All info about the pipeline is stored in a file called `default_pipeline.star`, but in normal circumstances the user does not need to look into this file. In case this file gets corrupted, one can copy back a backup of this file from the last executed job directory.

The upper half: jobtype-browser and parameter-panel

On the left of the upper half of the GUI is the jobtype-browser: a vertical list of jobtypes, e.g. 2D classification. On the right is a panel with multiple tabs, where parameters to the different types of jobs may be input. On the top left of the GUI are three different menu's, providing a range of functionalities. The Scheme and Run! buttons can be used to scheme jobs for future execution, or to execute them now. The former is particularly useful in preparing fully automated `pipelines` that can be run iteratively, for example in real-time as data is being collected. See *Schemes* for more details. By clicking in the jobtype-browser on the left-hand side of the GUI, a new job (with a Run! button) will be loaded in the parameter-panel on the right.

The lower half: job-lists and stdout/stderr windows

The lower half of the GUI contains lists of jobs that are still running (Running jobs), have already finished (Finished jobs), or are schemed for later execution (Schemed jobs). By clicking jobs in these lists, the parameters of that job will be loaded in the parameter-panel, and the Run! button will change color and turn into continue now! . Upon clicking the latter, no new output job-directory will be made, but the job will be continued according to the parameters given in the parameter-panel. 2D classification, 3D classifications and 3D auto-refine jobs will need a *_optimiser.star* file to continue from, and will have filenames with the iteration from which they were continued, e.g. *run_ct23*. Other types of jobs may continue from the point until they were executed before, e.g. Motion correction, CTF estimation, Auto-picking and Particle Extraction will continue by running only on those micrographs that weren't done before. The Input to this job and Output from this job lists link jobs together and can be used to browse backwards or forwards in the project history.

At the bottom of the lower half of the GUI, the standard output (stdout) and standard error (stderr) of the selected (finished or running) job will show in black and red text, respectively. The stderr should ideally be empty, any text here is usually worth inspection. These text displays get updated every time you click on a job in the job-lists. Double-clicking on the stdout or stderr displays will open a pop-up window with the entire text for more convenient scrolling.

The Display button

The Display: button below the run and scheme buttons serves to visualise the most important input and output files for each job. When a job from the job-lists in the lower half of the GUI is selected, clicking this button will pop-up a menu with all the input and output of this job that can be displayed (for example, particles, micrographs, coordinates, PDF files, etc). A more general functionality to display any (e.g. intermediate) file can be accessed through the *Display* option of the *File* menu on the top left of the GUI.

The Job actions button

The Job actions button opens up a little menu with options for the selected (running, finished or schemed) job. Here, you can access a file called *note.txt* (that is saved in every individual job directory and which may be used to store user comments); you can change the alias of the job; you can mark a job as finished (in case it somehow got stuck); you can make a flowchart of the history of that job (provided LaTeX and the *TikZ* package are installed on your system, also see *this section*); or you can delete or clean a job to save disk space (see below).

Clean-up to save disk space

Deletion of jobs moves the entire job directory from the project directory into a directory called `Trash/`. You can empty the Trash folder from 'File' menu on the top left of the GUI to really free up the space. Until you do so, you can still *undelete* jobs using the corresponding option from the *Jobs* menu on the top left.

To save disk space, you can also *clean* jobs, which will move intermediate files to the Trash folder, e.g. the files written out for all intermediate iterations of refine jobs. There are two cleaning options: `gentle clean` will leave all files intact that could be used as input into another job, while `harsh clean` may also remove those. Evidently, `harsh` cleaning can free up more space, in particular directories with particle stacks or micrographs may become large, e.g. from Motion correction, Particle extraction, Movie refinement and Particle polishing job types. One can also clean all directories in the project with a single click using the corresponding options from the *Jobs* menu on the top left of the GUI. You can protect specific directories from `harsh` cleaning by placing a file called `NO_HARSH_CLEAN` inside them, e.g. you may want to protect your final set of polished particles from deletion by executing:

```
touch Polish/job098/NO_HARSH_CLEAN
```

7.6.2 Optimise computations for your setup

GPU-acceleration

Dari Kimanius and Bjoern Forsberg from the group of Erik Lindahl (Stockholm) have ported the most computationally expensive parts of relion for the use of GPUs. Because they used the cuda-libraries from nvidia to do this, GPU-acceleration in relion only works with nvidia cards. These need to be of compute capability 3.5 or higher. Both single and double precision cards will work, so one is not restricted to the expensive double-precision cards, but can use the cheaper gaming cards as well. Details of their implementation can be found in their eLife paper[KFSL16].

Two different relion programs have been GPU-accelerated: *relion_autopick* (for Auto-picking) and *relion_refine* (for 2D classification, 3D classification and 3D auto-refine jobs). Both the sequential and the MPI-versions of these programs have been accelerated.

Disk access

With the much improved speed of image processing provided by the GPU-acceleration, access to the hard disk increasingly becomes a bottle neck. Several options are available on the relion GUI to optimise disk access for your data set and computer setup. For 2D classification, 3D initial model, 3D classification and 3D auto-refine one can choose to *use parallel disc I/O*. When set to *Yes*, all MPI processes will read particles simultaneously from the hard disk. Otherwise, only the master will read images and send them through the network to the slaves. Parallel file systems like gluster of fhgfs are good at parallel disc I/O. NFS may break with many slaves reading in parallel.

One can also set the *number of pooled particles*. Particles are processed in individual batches by MPI slaves. During each batch, a stack of particle images is only opened and closed once to improve disk access times. All particle images of a single batch are read into memory together. The size of these batches is at least one particle per thread used. This parameter controls how many particles are read together in a batch by each thread. If it is set to 3 and one uses 8 threads, batches of 3x8=24 particles will be read together. This may improve performance on systems where disk access, and particularly metadata handling of disk access, is a problem. It has a modest cost of increased RAM usage.

If one has a relatively small data set (and/or a computer with a lot of RAM), then one can *pre-read all particles into RAM* at the beginning of a calculation. This will greatly speed up calculations on systems with relatively slow disk access. However, one should of course be careful with the amount of RAM available. Because particles are read in float-precision, it will take $\frac{N \times boxsize \times boxsize \times 4}{1024 \times 1024 \times 1024}$ gigabytes to read N particles into RAM. For 100,000 particles with a 200-pixel boxsize that becomes 15GB, or 60 GB for the same number of particles in a 400-pixel boxsize.

If the data set is too large to pre-read into RAM, but each computing node has a local, fast disk (e.g. a solid-state drive) mounted with the same name, then one can let each MPI slave copy all particles onto the local disk prior to starting the calculations. This is done using the `Copy particles to scratch directory`. If multiple slaves will be executed on the same node, only the first slave will copy the particles. If the local disk is too small to hold the entire data set, those particles that no loner fit on the scratch disk will be read from their original position. A sub-directory called `relion_volatile` will be created inside the specified directory name. For example, if one specifies `/ssd`, then a directory called `/ssd/relion_volatile` will be created. If the `/ssd/relion_volatile` directory already exists, it will be wiped before copying the particles. Then, the program will copy all input particles into a single large stack inside this directory. If the job finishes correctly, the `/ssd/relion_volatile` directory will be deleted again. If the job crashes before finishing, you may want to remove it yourself. The program will create the `/ssd/relion_volatile` directory with writing permissions for everyone. Thereby, one can choose to use `/ssd`, i.e. without a username, as a scratch directory. That way, provided always only a single job is executed by a single user on each computing node, the local disks do not run the risk of filling up with junk when jobs crash and users forget to clean the scratch disk themselves.

Finally, there is an option to `combine iterations through disc`. If set to `Yes`, at the end of every iteration all MPI slaves will write out a large file with their accumulated results. The MPI master will read in all these files, combine them all, and write out a new file with the combined results. All MPI salves will then read in the combined results. This reduces heavy load on the network, but increases load on the disc I/O. This will affect the time it takes between the progress-bar in the expectation step reaching its end (the mouse gets to the cheese) and the start of the ensuing maximisation step. It will depend on your system setup which is most efficient. This option was originally implemented to circumvent bugs on the network cards on our old cluster at LMB. Nowadays, we prefer not to use this option, as it tends to be very slow when refinements reached high resolutions.

7.6.3 Interaction with other programs

Although, in principle, relion can use particles that have been extracted by a different program, this is NOT the recommended procedure. Many programs change the particles themselves, e.g. through phase flipping, band-pass or Wiener filtering, masking etc. All these are sub-optimal for subsequent use in relion. Moreover, gathering all the required metadata into a correctly formatted relion-type star file may be prone to errors. Because re-extracting your particles in relion is straightforward and very fast, the procedure outlined below is often a much easier (and better) route into relion.

Also, several implementations of wrappers around relion have now been reported (e.g. in eman2, scipion and appion). Although we try to be helpful when others write these wrappers, we have absolutely no control over them and do not know whether their final product uses relion in the best way. Therefore, in case of any doubt regarding results obtained with these wrappers, we would recommend following the procedures outlined in this tutorial. The recommended way of executing external programs from within the relion pipeline itself is outlined in the next section.

7.6.4 The External job-type

User interaction through the GUI

The External job-type on the relion-3.1 GUI provides a way to execute any third-party program from within the relion pipeline. The interaction with the user is as follows:

On the Input tab set:

External executable:

myscript.py

(This is the filename of an executable script, which will call the external program.)

Input movies:

Input micrographs:

Input particles:

Input coordinates:

Input 3D reference:

Input 3D mask:

The user provides at least one of the input entries to tell relion from which other jobs the input nodes come from, and what type of input this is. This will therefore allow to maintain an intact directional graph inside the pipeliner. On the Params tab, the user can then provide up to ten (optional) free parameters that will be passed onto the executable script. Finally, the Running tab allows multi-threaded execution, queue submission, and any other arguments to be passed through the *Additional arguments* entry.

The GUI will then create and execute the following command, either locally or through a queueing system:

```
myscript.py --o External/jobXXX/ --in_YYY ZZZ --LABELN VALUEN --j J
```

where

- `XXX` is the current jobnumber in the pipeline.
- `YYY` is the type of the input node: *movies*, *mics*, *parts*, *coords*, *3dref*, or *mask*,
- `ZZZ` is the name of the corresponding input node. Note that more than one input node may be given, each with its own `--in_YYY` argument.
- `LABELN` is the label of a free parameter, as defined on the Params tab of the GUI. Note that up to ten different labels may be used.
- `VALUEN` is the corresponding value of the free parameter. This is optional: not every label needs a value.
- `J` is the number of threads defined on the running tab.

Functionality of the executable script

It is the responsibility of the executable script (*myscript.py*) to handle the command line parsing of the generated command. In addition, there are a few rules the script needs to adhere to:

- All output needs to be written out in the output directory, as specified by the `--o External/jobXXX/` option. In addition, for jobs that emulate relion job-types like Motion correction or Auto-picking, the output should be organised in the same directory structure as the corresponding relion job-type would make.
- When completed, the script should create an empty file called `RELION_JOB_EXIT_SUCCESS` in the output directory. This will tell the pipeliner that the task has finished successfully. Aborted or failed runs may optionally be communicated by creating files called `RELION_JOB_EXIT_ABORTED` and `RELION_JOB_EXIT_FAILURE`.
- The job should output file called `RELION_OUTPUT_NODES.star`, which should have a table called *data_output_nodes*. This table should have two columns with the names and types of all the output nodes of the job, using the `_rlnPipeLineNodeName` and `_rlnPipeLineNodeType` metadata labels. See the `data_pipeline_nodes` table in the `default_pipeline.star` file of any relion project directory for examples. Output nodes defined here will lead to the creation of edges between jobs in the directional graph of the pipeliner; and output nodes will be available for convenient displaying by the user using the Display: button on the GUI.
- The following may not be necessary or relevant, but the GUI has a Job actions button, which allows users to abort running jobs. This button will create a file called `RELION_JOB_ABORT_NOW` in the output directory. If this functionality is to be used, the script should abort the job when this file is present, create the `RELION_JOB_EXIT_ABORTED` file, remove the `RELION_JOB_ABORT_NOW` file, and then exit.

Example: a particle-picker

If your external program is a particle picker, e.g. Topaz [BMR+19], then you would give on the Input tab:

External executable:
run_topaz.py

Input micrographs:
CtfFind/job005/micrographs_ctf.star

(This file would be visible through the Browse button next to the input entry, which would only show star files of micrographs that exist in the current project.)

On the Params tab, one would provide any necessary arguments to be picked up by the script, for example:

Param1 label, value:
threshold 0.1

Param2 label, value:
denoise_first

Upon pressing the Run! button, this would execute the following command:

```
run_topaz.py --o External/job006/ \
  --in_mics CtfFind/job005/micrographs_ctf.star \
  --threshold 0.1 --denoise_first --j 1
```

The executable *run_topaz.py* is then responsible for correctly passing the command line arguments to *topaz*, and to make sure the rules in the previous section are adhered to. For picking jobs, the directory structure of the input movies (or micrographs) should be maintained inside the output directory, and each micrograph would have a STAR file with the picked coordinates that has the same rootname as the original micrograph, but with a `_PICKNAME.star` suffix. The `PICKNAME` is a free string. One could use the name of the particle-picking program, for example `topaz`. Therefore, if a movie was originally imported as `Movies/mic001.tif`, its corresponding STAR file with the picked coordinate would be placed in `External/job006/Movies/mic001_topaz.star`. In addition, in the output directory, the script should create a text file called `coords_suffix_PICKNAME.star` (i.e. `coords_suffix_topaz.star`). This file should contain at least one line of text, which is the name of the input micrographs STAR file given on the Input tab, i.e. `CtfFind/job005/micrographs_ctf.star`. The output node (the `coords_suffix_topaz.star` file) should also be listed in the `RELION_OUTPUT_NODES.star` file. This file would therefore look like this:

```
data_output_nodes
loop_
_rlnPipeLineNodeName #1
_rlnPipeLineNodeType #2
External/job006/coords_suffix_topaz.star            2
```

7.7 Conventions

7.7.1 Image formats

relion reads the following image file formats:

- **MRC images** (with extension `.mrc`)
- **MRC stacks** (with extension `.mrcs`)
- **SPIDER images** (with extension `.spi`)
- **SPIDER stacks** (with extension `.spi`)
- **TIFF images or movies** (with extensions `.tif` and `.tiff`)
- **EER movies** (with extension `.eer`)

For compatibility with other EM programs (e.g. UCSF MotionCor2, IMOD), TIFF images are flipped along the slow axis when being read into memory or written to a file. This happens regardless of the `TIFFTAG_ORIENTATION` value in the header.

relion writes individual images and image stacks in the MRC format . Because there is no distinction between 3D maps or stacks of 2D images in MRC format, 3D maps are indicated with a `.mrc` extension, and stacks with a `.mrcs` extension. Individual images in stacks are indicated by an integer number (ranging from 1 to the number of images in the stack) followed by an "@" sign and the filename of the stack. Thereby, the first three images in a stack called my_images.mrcs are called:

```
1@my_images.mrcs
2@my_images.mrcs
3@my_images.mrcs
```

RELION supports the following MRC modes.

0:
signed 8-bit integer

1:
signed 16-bit integer

2:
32-bit floating point

6:
unsigned 16-bit integer

12:
16-bit floating point (RELION extension)

101:
packed 4-bit integer (IMOD extension)

The mode 12 is RELION's extension proposed in 2021 to save disk space. It uses IEEE754, binary16 encoding. Motion Correction (with RELION's own implementation, not UCSF MotionCor2), Extract, Polish and Subtract jobs can write them with the `--float16` option. All RELION programs can read mode 12 MRC files. External programs that use recent versions of mrcfile Python package can read them but other programs might not be able to read them.

As of 2021 March,

- GCTF does not support float16.
- CTFFIND does not support float16 but RELION's motion correction job always writes power spectra in float32, which can be used in CTFFIND.
- Topaz does not support float16 but RELION's wrapper performs preprocessing from float16 to float32. Thus, as long as you use Topaz via RELION's wrapper, training and picking work fine.

7.7.2 STAR format

relion uses the *STAR* (Self-defining Text Archiving and Retrieval) format (Hall, Allen and Brown, 1991) for the storage of label-value pairs for all kinds of input and output metadata. The STAR format is an alternative to XML, but it is more readable and occupies less space. The STAR format has been adopted by the crystallographic community in the form of CIF (Crystallographic Information Framework), and Bernard Heymann's BSOFT package was the first to use STAR in the field of 3D-EM.

relion's implementation of the STAR format has the following rules (partially copied from BSOFT's manual):

- The file name must end in a ".star" extension.
- Each file must have one or more data blocks. The start of a data block is defined by the keyword `data_` followed by an optional string for identification (e.g., "data_images").
- Multiple values associated with one or more labels in a data block can be arranged in a table using the keyword `loop_` followed by the list of labels and columns of values. The values are delimited by whitespace (i.e., blanks, tabs, end-of-lines and carriage returns). The loop must be followed by an empty line to indicate its end.
- Label names always starts with an underscore ("_"). Each label may only be used once within each data block.
- Data items or values can be numeric or strings of characters. A string is interpreted as a single item when it doesn't contain spaces
- Comments are strings which can occur in three places: * File comments: All text before the first `data_` keyword * Data block comments: Strings on their own lines starting with "#" or with ";" as the first character in the line. * Item comments: Strings on the same line as and following tag-value items, also indicated by a leading "#".
- String values that contain spaces can be quoted by `"`. An empty string becomes `""`.

Metadata label definitions

relion has its own set of defined metadata labels. The following command will print a list of the definitions of all of them:

```
relion_refine --print_metadata_labels
```

7.7.3 Optics Groups

relion 3.1 introduced optics groups. See [ZNS20] for an introduction.

Movies, micrographs or particles are in the `movies`, `micrographs` and `particles` tables, respectively, and refer to an optics group entry in the `optics` table via the `rlnOpticsGroup` column. `rlnOpticsGroupName` strings are used when merging two STAR files. Optics groups with different names are considered different and `rlnOpticsGroup` IDs will be re-numbered.

relion 3.1+ automatically upgrades old-style STAR files from relion 3.0 and earlier. You can also manually convert them by `relion_convert_star`. Downgrading to the relion 3.0 format is not officially supported but you might find this ccpem mailing list post useful.

7.7.4 Orientations

Orientations (`rlnAngleRot`, `rlnAngleTilt`, `rlnAnglePsi`) in a STAR file rotate the reference into observations (i.e. particle image), while translations (`rlnOriginXAngstrom` and `rlnOriginYAngstrom`) shifts observations into the reference projection. For developers, a good starting point for code reading is `ObservationModel::predictObservation()` in the src/jaz/obs_model.cpp.

In compliance with the Heymann, Chagoyen and Belnap (2005) standard relion uses a right-handed coordinate system with orthogonal axes X, Y and Z, where right-handed rotations are called positive, and Euler angles are defined as:

- The first rotation is called `rlnAngleRot` and is around the Z-axis.
- The second rotation is called `rlnAngleTilt` and is around the new Y-axis.
- The third rotation is called `rlnAnglePsi` and is around the new Z axis

As such, relion uses the same Euler angles as XMIPP, SPIDER and FREALIGN.

The center of rotation of a 2D image of dimensions xdim x ydim is defined by `((int)xdim/2, (int)(ydim/2))` (with the first pixel in the upper left being (0,0). Note that for both xdim=ydim=65 and for xdim=ydim=64, the center will be at (32,32). This is the same convention as used in SPIDER and XMIPP. Origin offsets reported for individual images translate the image to its center and are applied BEFORE rotations.

Particle translations used to be in pixels (`rlnOriginX` and `rlnOriginY`) but this changed to Angstroms (`rlnOriginXAngstrom` and `rlnOriginYAngstrom`) in relion 3.1.

The unit of particle coordinates in a micrograph (`rlnCoordinateX` and `rlnCoordinateY`) is pixel in the aligned and summed micrograph (possibly binned from super-resolution movies). The origin is the first element in the 2D array of an MRC file. The origin is displayed at the upper-left corner in relion (other programs might display in other ways).

7.7.5 Contrast Transfer Function

CTF parameters are defined as in ctffind 4.1, also see [MG03].

Higher order aberrations

`rlnOddZernike` contains coefficients for asymmetric (antisymmetric) Zernike polynomials $Z_1^{-1}, Z_1^1, Z_3^{-3}, Z_3{-}1, Z_3^1, Z_3^3, \cdots$ in this order. `rlnEvenZernike` contains coefficients for symmetric Zernike polynomials $Z_0^0, Z_2^{-2}, Z_2^0, Z_2^2, Z_4^{-4}, Z_4^{-2}, Z_4^0, Z_4^2, Z_4^4 \cdots$ in this order. Thus, the 7-th item in the `rlnEvenZernike`, $Z_4{}^0$, is related to an error in the spherical aberration coefficient.

Look at the table in Wikipedia but ignore square root terms, as the coefficients are not normalised in relion. For example, $Z_3^{-1} = (3r^3 - 2r)\sin\theta = 3(k_x^2 + k_y^2)k_y - 2k_y$, where k_x and k_y are wave-numbers in the reciprocal space (1 / Å).

Anisotropic magnification corrections

Transformation by anisotropic magnification brings the reference into observations (i.e.particle images) in real space. Note that stretching in real space is shrinking in reciprocal space and vice versa.

`rlnMagMatrix_00` to `rlnMagMatrix_11` represent the matrix `M` in the section 2.4 of [ZNS20]. The values become larger when the observed particle in the real space *looks larger* than the reference projection at the nominal pixel size. This also means that the true pixel size is *actually smaller* than the nominal pixel size.

7.7.6 Symmetry

Symmetry libraries have been copied from XMIPP. As such, with the exception of tetrahedral symmetry, they comply with [JBH05]:

Group	Notation	Origin	Orientation
Asymmetric	C1	User-defined	User-defined
Cyclic	C<n>	On symm axis, Z user-defined	Symm axis on Z
Dihedral	D<n>	Intersection of symm axes	principle symm axis on Z, 2-fold on X
Tetrahedral	T	Intersection of symm axes	3-fold axis on Z (deviating from Heymann et al!)
Octahedral	O	Intersection of symm axes	4-fold axes on X, Y, Z
Icosahedral	I<n>	Intersection of symm axes	**

** Multiple settings of the icosahedral symmetry group have been implemented:

I1:
No-crowther 222 setting (=standard in Heymann et al): 2-fold axes on X,Y,Z. With the positive Z pointing at the viewer, the front-most 5-fold vertices are in YZ plane, and the front-most 3-fold axes are in the XZ plane.

I2:
Crowther 222 setting: 2-fold axes on X,Y,Z. With the positive Z pointing at the viewer, the front-most 5-fold vertices are in XZ plane, and the front-most 3-fold axes are in the YZ plane.

I3:
52-setting (as in SPIDER?): 5-fold axis on Z and 2-fold on Y. With the positive Z pointing at the viewer and without taken into account the 5-fold vertex in Z, there is one of the front-most 5-fold vertices in -XZ plane

I4:
Alternative 52 setting: with the positive Z pointing at the viewer and without taken into account the 5-fold vertices in Z, there is one of the front-most 5-fold vertices in +XZ plane.

In case of doubt, a list of all employed symmetry operators may be printed to screen using the command (for example for the D7 group):

```
relion_refine --sym D7 --print_symmetry_ops
```

7.8 Developer Guide

Note

This page describes information for RELION developers and code contributors. General users do not need information here.

7.8.1 Coding styles in C++

Indentation should be done with tabs, while alignment should be done with spaces.

New lines should be as follows. Note that due to limitation of rST syntax, I used spaces instead of tabs.

```
// Use this
void test(int a, int b)
{
```

(continues on next page)

(continued from previous page)

```
    if (!flag)
    {
        A();
        B();
    }
    else
    {
        C();
        D();
    }
}

// Not this
void test(int a, int b) {
    if (!flag) {
        A();
        B();
    } else {
        A();
        B();
    }
}
```

7.8.2 How to compile this documentation

To compile this documentation into HTML, type:

```
make html
```

For PDF, perform:

```
make latex
cd build/latex
pdflatex relion.tex # you might have to install several style files
```

If your system has `latexmk`, this can be done in one line:

```
make latexpdf
```

If above commands complain about missing Python packages, try:

```
python3 -m pip install --upgrade sphinx sphinxcontrib-bibtex
```

7.8.3 Coding styles in reStructuredText (rST)

For Å, α, β in the main text, just type as is using Unicode. Don't use TeX commands.

To make `git diff` easier to understand, each sentense should use a new line.

```
A sentence.
Next sentence.

A new paragraph.
```

(continues on next page)

(continued from previous page)

```
``Keyword``, *emphasis by italic* and **emphasis by bold** are marked like this.
You can cite a paper :cite:`Zivanov_estimation_2020`.
A link to `rST documentation <https://www.sphinx-doc.org/en/master/usage/
↪restructuredtext/basics.html>`_.
Note the underline.
You can use LaTeX math :math:`\sum x_i^2`.

We have custom tags for :button:`buttons`, :joblist:`joblists`, :jobtype:`jobtypes` and␣
↪:guitab:`guitabs`.
|RELION| prints RELION in smallscaps.
We also have |MotionCor2| and |CTFFIND4.1| defined.

This paragram is a bad example. Two sentences are in one line.

::

    A block quote for commands is an indented block following two
    colons and an empty line. Please use FOUR SPACES, not tabs for indentation.

-   When using a list, be careful about indentation.
    The second sentence must be aligned.
-   The second item in the list.

:Field list: suggested value

    (Explanation sentence 1.
    Note the indentation of this line!
    Also note the following blank line.)

:Another option without a value: \

    (Please put `\` at the end of the line to the field that should be left empty.)
```

The above example is rendered as:

A sentence. Next sentence.

A new paragraph. `Keyword`, *emphasis by italic* and **emphasis by bold** are marked like this. You can cite a paper [ZNS20]. A link to rST documentation. Note the underline. You can use LaTeX math $\sum x_i^2$.

We have custom tags for buttons, joblists, jobtypes and guitabs. relion prints RELION in smallscaps. We also have motioncor2 and ctffind 4.1 defined.

This paragram is a bad example. Two sentences are in one line.

```
A block quote for commands is an indented block following two
colons and an empty line. Please use FOUR SPACES, not tabs for indentation.
```

- When using a list, be careful about indentation. The second sentence must be aligned.
- The second item in the list.

  Field list

  suggested value

  (Explanation sentence 1. Note the indentation of this line! Also note the blank lines.)

Another option without a value

(Please put \ at the end of the line to the field that should be left empty.)

7.9 Bibliography

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