Supplementary protocol 1

Application 1: High yield automated cryo-EM data acquisition of large particles

During an automated single particle acquisition using SerialEM, we usually employ the following procedure:

Grid map

We acquire a low magnification (e.g. 100×, 6×6) montage that covers the entire holey carbon TEM grid. In this montage, grid squares of interest are identified.

Grid square maps

For each identified grid square, we acquire a montage ("grid square map") at medium magnification (e.g. 2300×, 2×2) to provide more visible detail (Fig. 1b).

Selecting acquisition areas

Larger particles (e.g. filaments or viruses) can be identified in these montages. The distribution of smaller particles can be screened at higher magnification. For all holes containing evenly distributed large or small particles, Navigator points are added either manually or using SerialEM's capability to generate an evenly-spaced grid of points.

Automated acquisition

Automatic acquisition is run by executing a script on each previously selected Navigator point. By controlling the microscope stage and moving the sample, the script centers the FOV to the hole at medium-high magnification (e.g. 15 000×) acquisition by aligning to the template image of a centered hole that the user stores in a dedicated alignment buffer. It then performs optional autofocus and

drift measurements by acquiring multiple images in a regular circular pattern around the center of the hole at high magnification (e.g. 100 000×) using image shift. This script is available at the online repository (http://serialemscripts.nexperion.net/script/25).

Problematic samples

For many samples, particle concentration and spreading can be a problem. Acquiring such samples using a predefined acquisition template for each hole results in a low yield of useable images for further processing.

When such problematic samples contain large particles on continuous or lacey carbon grids that are visible in the grid square maps at medium magnification, one conventional option is to use SerialEM's *Realign to Item* before running the high-magnification acquisition template. The large field of view of the medium magnification images however leads to overlap of imaged regions. This increases the total applied exposure dose and thus cause radiation damage and reduces the quality of the final high magnification images.

The second conventional option is to manually select all particles of interest in the medium magnification maps and acquire another round of single images at medium-high magnification (15 000×) in *Low-Dose View* mode, saved as maps. In these maps, particles are then selected once again and automated acquisition is started using these maps for the Realign to Item procedure. This workflow is efficient for both holey and lacey grids but requires additional setup and

significant mapping time and also introduces extra dose at each position.

Solution for problematic samples or when using lacey grids

After adding Navigator points for particles of interest in the medium magnification
grid square maps and marking them for acquisition, the py-EM script
maps_aquire.py is run on the Navigator file. The script is available at
https://git.embl.de/schorb/pyem/tree/master/applications where we also provide a
version that supports multi-threading. It uses IMOD to merge the grid square
maps that contain the selected Navigator points if necessary. It then creates
Virtual Maps at each position. It uses the parameters from a medium-high
magnification template map taken with the SerialEM Low-Dose View
parameters. It crops the respective image areas from the medium magnification
grid square maps for each selected Navigator point and transforms the extracted
images. The script accounts for both the relative scaling and rotation between the
grid square map and the medium-high magnification template and thus makes the
extracted image suitable for direct correlation with an image acquired at medium-
high magnification (Fig. 1c).

The cryo-EM workflow then becomes:
- Acquire grid map. (low mag)
- Acquire grid square maps. (medium mag)
- Select acquisition areas by adding Navigator points in the grid square maps.
- Setup SerialEM Low-Dose, acquire one single Low-Dose View image, save it
  as a reference/template map.

- Run py-EM script *maps_aquire.py* on the saved Navigator file.

- Load the new Navigator file generated by *maps_aquire.py*.

- Start automated acquisition using a SerialEM script.

  (http://serialemscripts.nexperion.net/script/46)

Using py-EM for the creation of medium-high magnification virtual maps directly
from the grid square maps enables automated high-yield acquisition of cryo-EM
data with minimal dose on samples with low concentrations of large particles,
using either continuous, holey or lacey carbon grids.