Published in final edited form as:

J Struct Biol. 2007 December ; 160(3): 324–331.

Towards Automated Screening of Two-dimensional Crystals

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Abstract

Screening trials to determine the presence of two-dimensional (2D) protein crystals suitable for three-dimensional structure determination using electron crystallography is a very labor-intensive process. Methods compatible with fully automated screening have been developed for the process of crystal production by dialysis and for producing negatively stained grids of the resulting trials. Further automation via robotic handling of the EM grids, and semi-automated transmission electron microscopic imaging and evaluation of the trial grids is also possible. We, and others, have developed working prototypes for several of these tools and tested and evaluated them in a simple screen of 24 crystallization conditions. While further development of these tools is certainly required for a turn-key system, the goal of fully automated screening appears to be within reach.

Keywords

TEM; 2D crystallization; automation; membrane proteins; electron crystallography

Introduction

While three-dimensional (3D) reconstructions resulting from single particles or helical arrays have now both reached sub-nanometer resolution, electron crystallography of two-dimensional (2D) crystals is still the highest resolution method for protein structures solved by cryo-transmission electron microscopy (cryoEM) techniques. However, as is also the case for 3D protein crystallography, production of these ordered arrays generally requires screening a range of possible assembly conditions (pH, salt, protein concentration, temperature, lipid choice, lipid to protein ratios, etc.) (Kühlbrandt, 2003). The 2D crystallization conditions are assessed by preparing the specimen using negative staining and then imaging the resulting grid in a transmission electron microscope. During initial coarse screens of crystallization conditions formed by reconstituted membrane proteins, the specimen is examined for the presence of large densely packed proteoliposomes, sheets, or tubular forms, accompanied by minimal amounts of aggregated, precipitated protein. Finer and more refined screens are undertaken to detect the presence of large, well ordered arrays. This is extraordinarily labor-intensive work and, if the parameter space to be explored is large, it quickly becomes unfeasible to undertake without a very high level of automation.

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Over the last few years we, and others, have developed some of the tools and technologies that will be required for a fully automated solution to this tedious task. We present in this manuscript a brief description of the current status of these automation efforts and a discussion of what remains to be completed before fully automated 2D crystallization screening trials become a routine procedure.

Currently available technology for automated 2D crystallization screening trials

The process of 2D protein crystal screening can be roughly divided into four tasks: (1) producing potential crystals; (2) preparing negatively stained TEM grids; (3) loading grids into the electron microscope; (4) acquiring and evaluating images of the specimen. Below we will describe some of the technology that can be used to automate each of these tasks and provide specific examples of our own implementations of these techniques. Wherever relevant, we have also provided a description of the parameters used to prepare the test data that we present as a demonstration of the validity of these techniques.

1. Crystallization trials

Purified proteins are crystallized either as monolayers at air-water interfaces or, more commonly, as a part of proteoliposomes by removing detergent from mixed micelles of protein, lipid, and detergent (Jap *et al.*, 1992; Schmidt-Krey *et al.*, 1998; Kühlbrandt, 2003). Such removal can either be achieved by biobead absorbance (Rigaud *et al.*, 1997) or dialysis across semi-permeable membrane. Since biobeads are normally deposited in the same chamber with the protein sample, the automation of this method should be relatively straight forward. For the dialysis methods, while the choice of the dialysis device is highly influenced by personal preference (Schmidt-Krey, 2007), the device designed by Engel *et al.* (Engel *et al.*, 1988; Jap *et al.*, 1992) that removes the detergent with a continuous flow in a multi-chamber, temperature controlled block is particularly attractive for high throughput screening. The number of trials can be readily scaled up simply by adding more channels to the peristaltic pump, and the chambers are at fixed positions that are amenable to future robotic manipulation.

We have constructed one of these devices according to the design from Andreas Engel's laboratory running on an 8 channel peristaltic pump at 0.3 ml/min. 500 ml of dialysis buffer is used for each channel of the system and the buffer is recycled to its reservoir, in our version, after passing through 4 ml of BioBead SM-2 (Bio-Rad). There are 3 chambers for each channel and each is configured for 10–120 μ l of solution. For the example crystallization screen that we describe through this manuscript, a combination of 2 buffers, 3 lipids, and 4 lipid-to-protein ratios at 25 μ l were included for an initial crystallization trial on a bacterial ligase protein with multiple-membrane-spanning domains that has not been crystallized previously. The dialysis across a Spectra-Por 7 (Spectrum Labs (Rancho Dominguez, CA)) membrane with a 15 kD molecular weight cut-off lasted 3 days at 25°C controlled by a refrigerated water bath. For the trials discussed here, the mixing of the crystallization solution component and the harvest of the trials were done manually, but this step could also be automated for larger scale screens.

2. TEM grid preparation

Preparation of negatively stained TEM grids is a fairly delicate operation both because of the fragile nature of the grids as well as the complex procedures involved that include delivery of the sample, removal of excess specimen and undesirable detergent or salt, application of the stain, and blotting of the excess stain. The last step, in particular, must often be optimized for each specific stain.

Since all but the last step of the preparation involves handling of small amounts of liquid, we propose that automation can be achieved with the use of a programmable commercial liquid handling robot. To overcome the complications associated with blotting procedures where filter paper is used to wick away excess wash and stain, we have pursued an alternative staining protocol that uses a pipette to aspirate excess stain from the grid as proposed by Kuhlbrandt (Kuhlbrandt, 2003). In this protocol, we first apply 0.5–1 μ l of specimen suspension to a glow-discharged copper EM grid that is coated with nitrocellulose backed carbon surface. After a brief incubation (30 seconds), 10 μ l of water is pipetted onto the grid and then removed using the same pipette set to the same volume; this procedure is repeated three times. The grid is then negatively stained using 0.5 μ l of 0.5 % methylamine tungstate (sold by Nanoprobe as Nano-W) pipetted onto the grid after which it is allowed to air dry. With some experience, the success rate of manually performing this staining procedure can be as high as 90% (see Figure 1 for examples of specimens stained in this way). In comparison to a standard staining procedure by blotting using filter paper, this procedure tends to give better uniformity of the stain on the grid and a slightly heavier negative staining level, controllable by the concentration of the stain, with less probability of positive staining. We have manually used this staining method successfully on large soluble complexes such as virus capsids, detergent solubilized membrane proteins such as acetylcholine receptor, pure liposomes, as well as 2D crystals of MsbA, an ABC transporter protein with ~50% extramembraneous domain (Figure 1a). Of the 24 grids of the ligase described as a test case in this paper two of them resulted in non-optimal staining; one showed patches of stain droplet, an indication of low hydrophilicity of the grid, and the other contained thick stain crystals, an observation that is often associated with excess liquid left on the grid after washing.

The manual staining protocol described above is compatible with a robotic liquid handling system. We tested the protocol once using a commercially available microplate liquid handling system, the Aurora Biomed Versa 100 (figure 2). The grid trays that we have designed for our robotic grid loading system (see below) are based on a standard 96 well microtiter plate format and are thus already compatible with the micropipetting system. Thus, the grids can remain in the same tray for both staining and imaging. The single head pipette can be programmed to perform both the delivery and the aspiration of the liquid at each step of the protocol with a thorough rinsing of the pipette in between. An example of grids prepared using this robotic system is shown in figure 1b. The example shown is for virus capsids, rather than membrane protein crystals, as the images were obtained using a system loaned to us for testing purposes and we thus chose a straightforward sample to use as a proof of concept. However, we believe that there is no technical difference in applying the automated procedure to crystal and membrane protein staining since we have already proven that this can be achieved using the same protocols and manual staining methods. Our results thus demonstrate that using this device and the Kuhlbrandt method protocol has great potential for a fully automated staining system. However, several improvements will be necessary and we discuss these further in the next section.

3. Robotic exchange of the grids

The next step in a 2D-crystal screening pipeline is the loading of the individual stained grids into a TEM specimen holder and inserting the holder into the electron microscope. Below we describe in some detail our own solution to this problem that uses grid trays based on a 96 well microtiter plate format, a robotic arm and a software system called Leginon (Potter *et al.*, 1999; Suloway *et al.*, 2005), that controls the robotic arm, the microscope and CCD camera, and manages multiscale image acquisition from each grid (Potter *et al.*, 2004). An alternative solution that has recently been described is a commercially available specimen holder that can be manually loaded with up to 100 grids (Lefman *et al.*, 2007).

Our robotic grid loading system is designed to mimic the action of a microscopist. A commercial robotic arm is programmed to select a grid from a tray, place it into the specimen holder and then load the specimen holder into the microscope. The system is integrated with an FEI Tecnai microscope without requiring any modifications to the microscope and only minimal modifications to the specimen holder. Grids are stored in trays that have a capacity of 96 grids (see Figure 2), based on the format of a standard 96 well microtiter plate. The standardized format avoids confusion during loading by the operator and was designed to be compatible with future plans to automate the staining protocols using the liquid handling robot as described above. Since the trays are relatively inexpensive, grids do not have to be unloaded for each new experiment and can remain temporarily stored in a tray if multiple passes through the grid set are required. This saves time, reduces the wear and tear on the grids, and simplifies the process of finding targets selected from images acquired from a first automated pass through the grids during a subsequent imaging session. The specimen holder rests in a holder workstation that is equipped with two actuators to lift and lower the lever arm clamp. The entire system is suspended from a frame mounted to the ceiling in order to ensure that the microscope remains readily accessible for routine operation and servicing (figure 3a). The system includes a number of built in safety features ranging from a safety stop button, several force, torque, and position sensors, and multiple feedback points in the controlling programs for the benefit of both the operator and the microscope (Potter *et al.*, 2004).

When the Leginon software system requests a grid to be loaded into the microscope a series of coordinated events follow. A grid is selected from the tray, a fiber optic sensor is used to verify successful pick up, the lever arm of the specimen holder is raised using the actuator, the grid is placed precisely into the holder, the lever arm is lowered, the holder is picked up by the robot, and inserted into the microscope with a series of movements coordinated with the vacuum pumping system. Once the imaging session is over, the process is reversed; the holder is removed from the microscope and the grid is returned to its labeled slot in the grid tray. The components of the robotic workstation are controlled using a Visual Basic program integrated with the Leginon automated data collection software, which sends requests to the robotic system to load or unload a grid into the microscope and controls the response of the system in the event of grid pick up failure, vacuum system failure or other unexpected events.

The original prototype of the system was developed in collaboration with SSI Robotics, Inc. Grid pick up was handled using a two fingered actuator notched to provide supports for the grid. This design was later modified to replace the tweezers with a vacuum pickup system (figure 3b). The system uses a small vacuum pump and a fine nozzle to pick up the grid near its edge. This new design has proved to be more robust than the original tweezer pickup system (Potter *et al.*, 2004) in that it is more tolerant to distortions of the EM grids that result from user handling.

The program is designed to skip to the next grid automatically if the grid fails to be picked up during loading. If the grid fails to be removed from the specimen holder after imaging, there are two options for continuing. The first is to send a user alert and wait for the operator to come and manually clear the grid. However, since we normally use the robot through the night or over weekends, when the microscope is typically in less demand, this can severely limit the throughput if grid removal failure occurs even occasionally. Thus, we have implemented an alternative protocol that initiates an automated clearing procedure that simply turns the holder upside down to dump the grid out into a filter paper lined petri dish. Once cleared, the grid imaging can then continue without interruption. While this has the potential to result in confusion if more than one grid is dumped out during a session, in reality, these failures are rare events and if necessary the grids could be sorted out again based on the atlas of the low magnification images that have already been acquired. In our example screen, which required 2 passes through 24 grids, none failed to be picked up from the tray during loading or returned

from the holder after imaging. However, 4 grids were slightly misplaced from their correct position (either seated at a slope in the tray or on the rim of the well) on return and required operator intervention to return them to their precise location.

The cycle time for loading a grid from the tray into the holder and then loading the holder into the microscope is ~3.5 minutes and the unload cycle time to remove the holder from the microscope and then return the grid to the tray is ~2.5 minutes. The loading operation takes longer primarily because of the time necessary to pump the specimen airlock on the microscope. An experienced microscopist performing the same loading operation requires ~1 minute to load and 40 seconds to unload the grid. Our goal for the automated loading is to match the efficiency of a user and we will later discuss some of the modifications to the system that will be necessary in reaching this goal.

4. TEM imaging and evaluation

Crystallization trials result in grids that can contain a very varied and diverse range of objects that will be imaged in the electron microscope. These objects might include protein crystals, proteoliposomes, pure lipid vesicles, protein aggregates, dust, stain crystals, puddles of amorphous stain, or pieces of broken carbon support. Other than the last three artifacts, each of these objects provides valuable information for future trials, even if they do not dominate the viewing field. Furthermore, a TEM grid (~4mm²) presents an enormous surface area when imaged at a magnification scale sufficient to distinguish crystalline order (pixel size <~1 nm). Therefore, a brute force approach in which the grid is simply focused and imaged at high magnification at random targets will not provide a useful assessment of the specimen preparation in a reasonable amount of time. An experienced microscopist heavily biases the selection of targets worth investigating to those that resemble 2D crystals of proteins when searching at low magnifications and therefore greatly reduces the number of samples imaged at high magnifications required to evaluate a grid. Since we do not yet have a reliable algorithm to match the object recognition of a human operator, we have adopted a two-pass, multi-scaled imaging scheme as described below (see figure 4) that is designed to minimize the time required for operator intervention. If and when algorithms are developed that can reliably identify potential crystals at low magnifications, these could be readily plugged into the procedure to replace the current manual evaluation steps, reducing the two-pass operation to a single pass and substantially improving the efficiency.

Implementation of the imaging scheme is achieved through the Leginon system that allows applications to be built by assembling a series of modular nodes (Suloway *et al.*, 2005). Two new nodes were written for the 2D screening application, one for relating targets between multiple passes through the grids, and one for selecting and transferring targets between images acquired at multiple scales. The new classes and the improvements required for the screening applications described here are available in version 1.4 and higher of the Leginon distribution (www.legion.org).

(A) 1st Multi-Scale Imaging Grid Survey—The initial survey is designed to systematically screen through the entire set of grids, identifying and sampling areas of suitable stain at one or more intermediate magnifications at a number of targets sufficient to provide a reasonable overall statistical sampling of the grid. For example, in the test case described below, we surveyed at very low magnification (0.7 μ m pixel size), 12 well-spaced areas selected from a circular area of the grid with a diameter of 1.2 mm. From each of the 12 areas, a grid square was selected and images were acquired at 10 and 100 times greater magnifications. The pixel size for the highest magnification images was 6.5 nm, where it is possible to identify the texture of objects as small as 0.5 μ m without adjusting focus. This automated pass achieves the same goal as the low dose search mode in manual operation.

(B) Evaluation and Target Selection—Following the automated first pass survey, the user can browse through the acquired images at any convenient time and manually identify targets of further interest. The targets identified on the intermediate magnification images are transformed to the coordinates on the very low magnification survey images and stored into the Leginon database awaiting a second pass through the set of grids. This evaluation is very efficient; an operator can rapidly evaluate large numbers of images. In our example below, evaluation of all 24 grids (576 intermediate magnification images) took ~1 hr.

(C) 2nd Multi-Scaled Potential-Crystal Imaging—When microscope time becomes available and the application is next activated, the grids that are associated with targets selected by the operator are loaded into the microscope again using the robotic system. Images of the manually selected target areas are then acquired at a magnification sufficient to assess crystalline order (0.7 nm in our example). This imaging requires an adjustment of the target positions and focusing of the microscope (typically 1 μm underfocus) as described next.

An adjustment of targets following this second grid insertion is essential for accurately imaging potential crystals that can be as small as 0.2 μm , at high magnification. The adjustment steps required are similar to those required for updating selected targets on a grid after a period of significant thermal drift (Suloway *et al.*, 2005). An image obtained on the first pass that includes the selected target is compared to a new image obtained on the second pass to determine the relative adjustment, in rotation, shift, and scale. (1) For each grid reloaded into the scope, a log-polar transform is used to evaluate the rotation, shift, and scale changes of the new center image acquired at the same very low magnification relative to that from the first pass. This allows a rough transformation of the targets chosen on the old survey image to the new grid coordinates. (2) For each target, the adjustment is then refined by repeating the same transformation determination in the first step after moving to the location of the target. (3) Targeting is less accurate for larger stage movements, and thus the process of moving to the designated target is iteratively refined until the measured distance to the target is below a set threshold (typically ~0.5 μm). (4) A shift between the optical axis and the stage z-axis (Zheng *et al.*, 2004) results from setting the grid to eucentric height as required for accurate focusing. This shift is measured and the targets adjusted as previously described (Suloway *et al.*, 2005). After these target adjustment steps, our targeting accuracy is in the range of 0.5 to 2 μm for a grid that is successfully returned to the grid tray after the first screening. The error is determined by the accuracy of the stage movement calibrations that are used in the target transformation, the misalignment between image shifts across the magnification scales, and the pixel size of the survey images (0.7 μm) from which the targets are defined. To compensate for the ~2 μm inaccuracy in targeting, we currently acquire a montage of 4 final images to cover an area of $5.7 \times 5.7 \mu\text{m}^2$. This ensures that the target area is imaged.

(D) Evaluation and Scoring—Evaluation of the final high magnification images acquired during the second pass currently requires input from a human operator but, as with the first pass screening, can be performed quite efficiently as the images are available via a web based graphical user interface (Suloway *et al.*, 2005) that can be configured to show both the real space image and its power spectrum. The user interface provides for a method of scoring the targets according to a variety of criteria specified by the operator, and for adding operator comments for specific images. A knowledge base built from the accumulated information entered by the operators will be critical for the development of a fully automated algorithm for identifying potential 2D crystals.

Results and Discussion

Our initial experience with using a liquid handling robot for automated negative staining, indicates that it will be necessary to develop a mechanism for clamping the grids down as the

aspiration process occasionally unseats the grid from the tray. In addition, the buffer used for washing sometimes wicks up the Teflon grid holder well, resulting in inefficient aspiration of the washing liquid and a negative impact on the stain concentration and drying speed. We propose that both of these problems can be easily resolved by the use of tagged grids that are held down in a modified holder with a cover that clamps the tag and has minimal contact with the remainder of the grid. With these modifications we believe that an automated staining protocol can be as efficient and reliable as manual staining.

Although none of the 24 crystallization conditions that we screened using the semi-automated procedures yielded crystals, we include them here as they provide a reasonable test case to illustrate each step of the screening process. Table 1 shows the time required for the screening of 24 negatively stained grids. There were three types of grids examined during the trials: (i) those with potential crystals, (ii) those that contain objects that are obviously not an indication of crystallization or even reconstitution, and (iii) ones that are simply not suitable for imaging such as grids filled with aggregates. The semi-automated imaging procedures described above requires a total of ~1.5 hr to evaluate the first type of grid, which requires two imaging passes; 21 min to evaluate a grid of the second type, which only requires a single imaging pass; and 10 min to survey a grid of the third type as no intermediate magnification images are acquired. In total hours this is considerably less efficient than manual grid screening where an experienced human operator might spend 30, 15, and 5 minutes, respectively, on these three types of grid. However, the total operator time required is much less for the automated screening, where the operator only needs to concentrate on the evaluation of results from the automated imaging. Thus, for a grid of the first type the operator time can be reduced from 30 minutes to ~5 minutes as a result of these automated methods.

There are a number of improvements that can be implemented in order to reduce the time required for the automated imaging, at least two of which are fairly straightforward. First, since the robot is idle during imaging, adding a second grid loading station will enable the next grid from the holding tray to be loaded to a second microscope specimen holder while the current grid is being imaged. This would cut ~2 minutes from the current 6 minutes of time required to exchange a grid. Second, we can improve the accuracy of target relocation so that we do not need to acquire a montage of images at each target in order to ensure that the targeted area is covered; this would have reduced the time for the second pass by a total of ~4 hours for the 24 grid screen detailed in Table 1. The targeting accuracy can be improved by a straightforward change to the algorithm that transforms the chosen targets from one pass to the next. Once implemented, the targeting accuracy should only be limited by the accuracy of the calibrated stage goniometer movements (20–50 nm) or by the threshold we impose in the iterative targeting location algorithm.

We also intend to make further improvements to the current method for grid handling. Grids are still occasionally mishandled on pick up, which results in failure to load or unload the grid or not centering the grid properly in the holder. This will be improved by modifying the pick up tool from the current single vacuum pickup needle to one that provides 8 contact points with the grid. The 8 contact points will be located on the outer rim of the grid, balancing the pickup force and minimizing any damage to the carbon film.

As mentioned earlier, the final evaluation and scoring of the results is still in its infancy. The experience will help in developing the most effective and efficient system. Automated scoring will likely include an evaluation of the number, quality and resolution of the reflections in a diffraction pattern of any potential crystal using methods previously described (Potter *et al.*, 1999). Additional scores could be based on statistics on the sampling schemes for each grid (e.g. number of high magnification targets selected, number of diffracting objects identified etc.) Other metrics would include comments or ratings added by the user.

We estimate that the cost of developing an entire automated pipeline for 2D crystal screening as described here will be substantially below the costs of purchasing an electron microscope and peripheral equipment required for 2D crystallography (it is difficult to provide more accurate cost estimates as some of these devices are prototypes). Nevertheless, since it is unlikely that every laboratory will be equipped with an automated robotic grid handling and imaging system, we anticipate that remote collaborations will be common in which the operator evaluates the results both from the 1st and 2nd pass from another part of the country or world. Therefore, we will continue to develop web-based evaluation tools that can be accessed easily through the internet.

An automated method for identifying potential targets during the 1st screening pass would have a major impact on the overall efficiency. While it is unlikely that any single algorithm will be capable of reliable automated target selection in a general case, we plan to build up a library of useful algorithms that can be adapted to individual specimens on a case by case basis. An approach like this was used by the Fujiyoshi laboratory where targets were identified based on the contrast of the identified object relative to the carbon background as well as the overall shape. Learning-based approaches that have been successfully used for particle detection (Mallick *et al.*, 2004) are another promising avenue to pursue.

Conclusions

We are convinced that automated screening is an essential component of routine high-throughput electron crystallography. While we are still quite far from a fully automated system that rivals those used in 3D crystal screening, developments from several labs are slowly fitting together to provide an initial working prototype for a semi-automated system that substantially reduces the time requirements and burden on the operator, while at the same time still retaining the unprecedented skills of a human operator during the screening process. The dialysis device and blot-free grid staining are ready for automation, while technology for robotic handling of the grids for microscopic imaging has matured. Although the total time for imaging and evaluation using the automated methods is still greater than would be required by an experienced microscopist to complete the same tasks, the reduction in operator time is already substantial and will only increase as the process scales up. A knowledge base developed from this semi-automated system will in turn enable more automation in the future and result in more efficient use of microscope time.

Acknowledgements

We thank Ron Milligan, Sheila Mulligan, Marianne Manchester Joseph Lam, and Priyanka Abeyrathne for providing test samples. Some of this research was conducted at the National Resource for Automated Molecular Microscopy, which is supported by the National Institutes of Health through the National Center for Research Resources' P41 program (RR17573).

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Figure 1. Grids prepared using a negative staining protocol suitable for automation using a liquid handling robot. (a) Patches of Msb A, an ABC transporter, two-dimensional crystals prepared manually. (b) Virus capsids stained using the liquid handling robot. Scale bar = 50 nm

Figure 2.
The Aurora Biomed Microplate Pipetting system loaded with a microtiter plate (A) and one of our robotic grid trays (B). Inset shows specimen being transferred to a grid by the system.

Figure 3. Robotic specimen handling system. (a) Overview of the robot installed at the electron microscope. (b) Vacuum pickup system transferring an EM grid (indicated by the arrow).

Figure 4.

Semi-automated TEM imaging and evaluation of negatively stained 2D crystallization trials of the ligase. The colored crosses and squares link the targets between multi-scaled imaging. (a) 1st pass acquires a grid survey atlas and samples the grid at three scales. (b) As an example, on the right, an object at the highest of the three multi-scale images is identified and marked as a target (red) and transformed to a grid coordinate in the surveying, low magnification image (yellow). (c) 2nd pass acquires images at the transformed target with multiple targeting adjustments (see text for details). The flow for adjustment of the transformed yellow target in (b) is shown in magenta symbols. In the case shown, the target adjustment in the second pass requires a small rotation and a significant shift. Additional required target adjustments are not illustrated. (d) Evaluation of the final image is achieved via a web browser based graphical user interface.

Table 1

Time Line for a Semi-Automated 2D-Crystal EM Screening Trial.

Process	Time for each process	Number of processes	Total time
1st pass screen grid loading 3 mag. imaging grid unloading	17 min 3.5 min 12 × 0.9 min 2.5 min	24 grids	6.8 hr
Evaluation and target selection	1–8 sec (avg. ~ 6 sec)	576 images	1 hr
2nd pass imaging focusing final imaging target adjusting	9 min 2.1 min 4 × 1 min 2.9 min	73 targets	11 hr
2nd pass grid exchange grid loading target adjusting grid unloading	7 min 3.5 min 1 min 2.5 min	18 grids	2.1 hr
Evaluation	2–30 sec (avg. ~20 sec)	292 pairs of final images	1.6 hr