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ANIMATED-TEM: a toolbox for electron microscope automation based on image analysis

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Abstract – This article presents the ANIMATED-TEM (ANalysis of IMages for Automatic Targeting and Extraction of Data in Transmission Electron Microscopy). This software package is composed of a set of image analysis algorithms for target selection and characterization of biological sample in transmission electron microscopy. Combined with a microscope control software package, it selects automatically regions of interest at appropriate magnification. Acting as a supervisor, ANIMATED-TEM controls the microscope tasks (stage displacement, magnification, etc.), localizes the regions of interest and manages the sample exploration strategy. Data are extracted at different magnifications to assess the grid quality at low magnification, the characteristics of the biological samples at medium magnification (membrane size, shape, and stacking-level), and the crystallinity at high magnification (identification of diffraction peaks). Grid quality and sample features are used to trigger new acquisitions at higher magnifications. These tools have been developed to allow high-throughput screening of 2D-crystallization experiments; the microscope is equipped with a grid-autoloader, allowing the automatic analysis of 96 samples. The toolbox is operational; the testing conducted for several months confirms that the image analysis achieves a full automation with an efficient target selection and a limited computational time for image analysis.

Keywords – Automated image acquisition; Transmission Electron Microscope; Target selection; Specimen characterization; Fully automated electron microscope

1. Introduction

This article presents a software toolbox for the automation of an electron microscope. All the examination steps of a biological sample are entirely

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autonomous. An autoloader system attached to the microscope allows the continuous processing of a set of 96 grids without human intervention.

Each examination step corresponds to the acquisition and analysis of one image. All data, the measured characteristics as well as the images, are stored and managed by a database system that allows the biologist to verify the analysis a posteriori, and if required, to reload and resume the sample observation. A typical 96-grids run acquires about 6000 images, taking 54 hours, corresponding to a mean time of 34 minutes per sample. The run time and the number of image acquisitions are strongly tied to the quality of the sample and the protocol parameters, as will be specified further in the text.

ANIMATED-TEM (ANalysis of IMages for Automatic Targeting and Extraction of Data in Transmission Electron Microscopy) triggers micrographs acquisition and analyses them to evaluate the sample quality, but is also able to fully control the microscope. The main innovation of the ANIMATED-TEM toolbox is the automated online image analysis including the decision steps for grid examination. The automation technique mimics the strategy of a microscopist that selects potentially interesting regions at various magnifications. The objects being generally scarce and scattered randomly on the grid, it is not realistic to pre-define regions randomly nor to examine a grid systematically. The objects must be localized by analyzing the images acquired with the integrated CCD camera.

The design of algorithms for electron microscopy image analysis is a current challenging issue, both for its difficulty and the great potential it shows. Manufacturers offer microscopes interfaced to CCD cameras that are entirely software controlled, with performances suitable for automation. Several recent publications introduce very interesting tools for the automated acquisition of images (e.g., [1-3]). For an entirely autonomous control that is intelligent enough to adapt to each sample, image interpretation must be introduced, at least partial image interpretation. Therefore computer vision for electron microscopy needs to be devised.

Image processing in electron microscopy appeared to be really challenging and led us to develop several original algorithms to solve the problem of localizing objects that are hard to detect, even for the expert eye. The difficulties are caused by the high level of noise in the images, the weak contrast of the biological objects, and the absence of texture or precise characteristics that would identify

1 the objects searched for. As it appears commonly in computer vision tasks, the
2 organization of the image data analysis steps is highly application dependent. The
3 processing chain that we present is dedicated to detecting artificial membranes
4 and testing if these membranes present a periodical structure.
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7 Our algorithms would need to be adapted to visualize other objects in an electron
8 microscopy context. The purpose of the ANIMATED-TEM toolbox is to provide
9 an efficient tool for the study of bi-dimensional crystallization conditions of
10 membrane proteins, part of a European Union project (HT3DEM), and more
11 generally for the testing and validation of an actual TEM automation
12 implementation.
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15 The HT3DEM (High-throughput three-dimensional Electron Microscopy) project
16 resulted in the implementation of a robotic platform for the bi-dimensional
17 crystallization of membrane proteins. This approach uses crystallographic
18 techniques to study the three-dimensional structure of proteins that are
19 reconstituted in the presence of lipids to form artificial membranes. The
20 determination of bi-dimensional crystallization conditions requires a large number
21 of trials that compels automation.
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24 The HT3DEM toolchain includes: i. the DropBox, a device to assess accurately
25 the amount of detergent needed to purify a membrane protein, ii. The Ternary
26 Mixture Robot, a machine mixing automatically the purified protein with various
27 lipids and additives, as membrane proteins are reconstituted in the presence of
28 lipids to form artificial membranes, iii. the 2DX Robot, a crystallization robot
29 based on the neutralization of the detergent by Cyclodextrin, iv. the Staining
30 Robot, a machine preparing the crystalline samples made by the 2DX Robot on
31 special grids suitable for EM screening.
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34 ANIMATED-TEM contributes to the final link of this robotization chain and
35 automates the examination step of each sample with the electron microscope to
36 evaluate crystallization.
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39 The scientific effort towards full TEM automation is overviewed in section 2. The
40 main image processing tools to achieve automation are briefly introduced in
41 section 3, followed in section 4 by the description of the architecture of the fully
42 automated microscope control. Section 5 presents the results and experiments
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conducted on the experimental platform, illustrating the performances achieved by ANIMATED-TEM toolbox.

2. Towards full TEM automation – State of the art

Several recent works illustrate the effort of the scientific community to automate tasks in electron microscopy. The application fields remain somewhat limited, and concentrate on a number of specific fields. Among them, the study of the three-dimensional structure of proteins is the most illustrative example. Indeed, different techniques – tomography, single particles, and crystallization – are the subject of specific and long term efforts for the development of software tools. The evolution of certain software toolboxes over the years shows both the magnitude of the task at hand and the increasing importance played by computer vision.

Today's electron microscopes are microprocessor controlled and can therefore be controlled by external software. The generalization of digital cameras opens the possibility to automate the acquisitions. A complementary step towards autonomy is the recent appearance of loading systems to insert specimens into the microscope. Potter et al.[4] use a robotic arm that reproduces the human grid insertion gesture. Lefman et al. [5] describe a motorized cartridge holder of 100 samples for rapid specimen exchange. In this project, we use a Tecnai T12 equipped with an carousel which can host up to 8 cassettes, for a total of 96 grids [6].

The first software tools for the control of a TEM have been devised for the automation of repetitive data acquisition tasks by executing scripts [7-9] and to create dedicated interfaces for specific techniques like tomography [10, 11]. Image processing has first been used to design auto-tuning methods for accurately setting astigmatism, focus, and alignment of the TEM. Koster [7] introduced a correction of image shifts resulting from tilting the specimen in tomographic series data.

With the evolution of image processing techniques and strong increase and availability of computational power, the interpretation of images becomes of growing importance. It allows to improve auto-tuning techniques (e.g., Mastronarde [12], for correction techniques in tomography) but mostly to address

1 new purposes. Image classification, interpretation or software evaluation become
2 literally a necessity to process the thousands of images recorded by certain semi-
3 automated systems, like the one proposed by Oostergetel et al. [13]. Anderson et
4 al. [14] describe an ambitious project of automated analysis of the neural circuitry
5 reconstruction by assembling thousands of TEM images. The single particle
6 technique [15] is strongly based on computer vision techniques to reconstruct the
7 three-dimensional structure of macromolecules. The principle is to average a large
8 number of identical particles to compensate for the insufficient precision of the
9 electron microscope. However, as the reconstructed resolution approaches the
10 atomic level, hundreds of thousands of particles may be necessary. The manual
11 selection of particles in micrographs becomes too tedious. Detection algorithms
12 have been the subject of much research work compiled in [16].

13 To automate the microscopy tasks entirely, the software must make the decisions
14 in place of the human expert. The interpretation of the image must therefore be
15 done in real time to make choices during the examination of the sample. Although
16 these decisions are often limited, very repetitive and application-dependent, it is
17 still challenging to replace the expert with computational approaches. Two fields,
18 electron tomography and single particles, illustrate well the state of the art, and
19 they are both the subject of important developments.

20 In the field of electron tomography, the automation and integration of software
21 tools in a unified interface is well advanced [3, 17], and manufacturers, like FEI,
22 offer software packages for tomogram acquisition and reconstruction. The
23 sequential tilt-series acquisition is fully automated. Recent software packages
24 enable to chain several series, advancing from one target to another. Three-
25 dimensional reconstructions are produced in real time and user intervention to set
26 markers for reconstruction could be suppressed recently [18]. The automation is
27 therefore almost total, the selection of targets remaining the responsibility of the
28 user. Even though this work is eased by an optimal organization gathering the
29 selections at the beginning of the session and an efficient software assistance [17],
30 it seems not yet possible to replace it by a reliable algorithm.

31 The technique of single particles almost benefits from a full automation when the
32 macromolecules to be detected are localized on a carbon film, with regular holes.
33 The selection procedure determines successively the good squares, then the holes
34 containing suitable and uniform ice layers. Some toolboxes, although offering

1 efficient software assistance, remain semi-automated (e.g. [5, 19]). They require a
2 selection phase where the user picks interesting holes from images. Other
3 toolboxes [1, 2, 20] recently introduced an entirely automated mode using a
4 computational image analysis for the selection task. But their designers remain
5 cautious and do not consider their technique reliable enough. Thus, AutoEM [20]
6 is configured by default in semi-automated mode; JADAS [2] proposes an
7 automated selection after manually setting the image intensity criterion and
8 calibrating parameters as diameter of a hole, the distance between neighboring
9 holes, etc. Even with the success of the automation, Zhang, et al., write that “the
10 presence of trained user or the availability of an intelligent real-time data
11 assessment software is still necessary to assure the data quality”. Stagg, et al. [21],
12 on the other hand, report the satisfactory performance of this selection with the
13 Legigon software package [1].

14 The important noise and the intensity fluctuations represent the main difficulties
15 to extract reliable information from CCD micrographs. The localization of the
16 holes is simplified by the regular geometry of the grid and a fixed hole diameter
17 and their periodical organization. However, the selection of suitable holes is
18 delicate. The cited software packages all use the mean value and the variance of
19 the image intensity within the hole.

20 In many other applications of electron microscopy, the objects of interest are more
21 complex and not localized by a regular structure. The challenge for computer
22 vision is therefore all the more important. The automation of the analysis of bi-
23 dimensional crystal samples is one example [22, 23]. The work presented in our
24 article is the first to integrate a fully automated selection of targets.

25 We close this section with the analysis technique introduced by Kylberg [24] for
26 the first two levels of magnification of an automated virus diagnosis system. The
27 overview images of the grid are first analyzed to precisely localize and select
28 good squares. At higher magnification, an empirical analysis of the problem leads
29 to the formulation of a few simple rules: regions with a higher probability to
30 contain small clusters of viruses are identified by detecting objects that are
31 somewhat circular and of diameter in a given range. The algorithms have not yet
32 been integrated in a control system, but seem promising.

3. Presentation of the image processing tools

The main image processing tools to achieve a full automation of a TEM are briefly introduced in this section. These algorithms are able to adapt to the various types of membranes and to the possible fluctuations of the acquisition parameters, based on a few known characteristics of the samples. All parameters are pre-determined or automatically adjusted, e.g. thresholds, such as the system runs without human intervention at any time.

Automatic analysis of a specimen requires the acquisition of images at different magnifications and their direct processing to determine successively the regions of interest. In this section we present an overview of the tools we developed for the three levels of magnification [25].

3.1 Low magnification image analysis

At low magnification (field of view of at least $300 \times 300 \mu\text{m}$ for 1024×1024 pixel images), images of mesh grids typically used in TEM experiments are analyzed to assess the quality of the overall grid and retain a number of regions for further analysis. In particular, regions where the carbon film is locally broken must be discarded (see Figure 1).

A three-step algorithm uses the gray-level histogram to automatically select the various thresholds. First, the grid squares are segmented using a global threshold positioned after the first peak of the histogram (representing the copper bars). Second, the background of each grid square, i.e. the brightest region represented by the last peak of the local histogram, is segmented using a local threshold. Each square is classified in three classes: broken carbon film, valid membranes, and unknown. From the first two classes, typical gray-levels of backgrounds are extracted. Third, these gray-level statistics are used to classify squares previously labeled as unknown.

This analysis outputs the proportion of good grid squares for the characterization, and their coordinates for medium-magnification targeting.

3.2 Medium magnification image analysis

At medium magnification (field of view $\approx 15 \times 15 \mu\text{m}$), images of membranes are processed through a chain of algorithms: a segmentation step made of a newly developed contour detection algorithm supplemented by a false edge removal

1 phase [2-28], followed by two labeling steps to identify the foreground region and
2 to characterize its stacking [29]. Once membrane regions are isolated, other
3 characteristics such as size and shape can be extracted. For the automatic
4 targeting, regions of interest (ROI) selected are the coordinates of the largest non-
5 stacked membrane regions.
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9 A thorough and complex image analysis is not always necessary to select the ROI.
10 In [25] we describe a fast procedure to select potentially crystalline regions by
11 simply avoiding background regions (no information) and dark regions (artifacts,
12 important aggregates or stacking, etc.) and selecting ROI inside the objects near
13 background edges. The Partial Edge Detection (PED) process selects ROI near
14 edges detected by a Prewitt filter, as long as the region is not too dark (i.e. above a
15 threshold experimentally set).
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18 In this section, we describe a more precise method to select potentially crystalline
19 regions (cf. [25] for a detailed description).
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22 These target regions being identified by distinct properties (in term of size, shape,
23 etc.), the procedure requires a precise segmentation and characterization of the
24 biological objects present in the image. The chain of algorithms used is presented
25 in Figure 2. The principle of the algorithms is given in the next paragraphs.
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32 *Contour detection*

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36 Because of the nature of the TEM images (very noisy, low-contrasted,
37 heterogeneous gray-levels), a new algorithm based on a multi-scale approach has
38 been devised especially to detect the contours in these difficult images [27].
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41 To identify all types of contrasted edges, gradient images, obtained at different
42 scales of a pyramidal transform, are thresholded using the T-point algorithm [26].
43 This algorithm outputs the threshold of unimodal histogram images in a robust
44 manner, practically insensitive to noise distribution, histogram fluctuations and
45 quantity of edges to segment. The different resulting binary images are combined
46 in one image, called reconstructed gradient-like (RGL), where the gray-level is
47 proportional to the scale at which the edge has been identified. A finer scale
48 corresponds to a higher gray-level, which leads to a more precise positioning of
49 the contour. The final splitting and contour positioning is achieved by applying
50 the watershed algorithm to the RGL image. On the resulting image (Figure 3B),
51 this method allows the identification of most of the regions, even the lowest
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contrasted ones. The downside of the approach is that, even though they are few, generating false edges cannot be avoided.

False Edge removal

The second algorithm will remove the spurious edges introduced by the contour detection method using a statistical analysis of the local contrasts [28]. Each segment of the partitioned image, i.e. each set of contour pixels separating two regions, is analyzed: if the segment is relevant, a regular and coherent gradient perpendicular to this segment can be observed; in the case of a false segment, the gradient is either absent, or it exists only partially, or it is incoherent along the segment. The contour segments are validated using in the following algorithm: i) a mask is created from the orientation evaluation of the contour pixels and the one-dimensional profile of the reference potential contour is extracted according to this mask. ii) a correlation measure between the profile and a reference filter is achieved. iii) the correlation factor, averaged over all segment pixels, is compared to a threshold derived from statistical hypothesis testing to take image noise into account. Segments whose correlation factor is below this threshold are removed (Figure 3C).

Stacking level determination

Once the image is properly partitioned, the specimen can be characterized. In the third step of the chain, regions are labeled: after having identified the background, foreground regions are classified according to the number of superposed objects by the stacking level. The background is temporarily identified as the brightest and largest region [30]. The labeling of the remaining regions is achieved using an iterative algorithm [29]. This algorithm achieves a labeling of the regions according to their stacking level, and it also completes the background detection by detecting smaller background regions. Each iteration of the algorithm is made of two steps, and corresponds to the identification of regions belonging to a given stacking level. To accomplish the classification of the regions, thresholds have to be set. In the first step, using a priori knowledge, we select a set of regions whose probability of belonging to the searched stacking level is high. These regions are used to identify the typical contrast of such regions. In the second step, this typical contrast is used to compute the threshold and refine the classification. This result

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is the labeling of each region according to its relative stacking level within the image (Figure 3D).

Linear and circular shapes

In the last step of the characterization, an algorithm has been developed to locally assess the shapes of the contours of the biological objects [31]. Since biological objects are randomly deposited on the support, they are often adjacent, partially superposed, or stacked. Therefore, the shapes can only be evaluated by analyzing locally the objects in contact with the background. Contours inside the foreground cannot be used to separate adjacent objects, as a contour line may represent a fold inside a folded object and not the object's outline. Shapes of the external contours are divided, when possible, into coherent "linear" or "circular" sections. A recursive method based on shape regression is used to identify if the contours can be approximated locally by lines or arc of circles (Figure 3E).

These characteristics are used to determine the regions that correspond, with the best probability, to well spread out membranes, not superposed, and crystalline, therefore interesting regions to validate the crystallization conditions of the sample. An empirical sorting rule has been devised for region classification (see section 5.2). The primary regions are retained for the next analysis step at high magnification.

3.3 High magnification image analysis

This is the final step where images are acquired at high magnification (around $\times 30,000$, i.e. 0.5 nm/pixels) in order to assess the sample quality. Crystallinity can be automatically checked with a process analyzing the Fourier Transform (FT) of images acquired at high magnification. However, TEM Contrast Transfer Function (CTF) prevents simple thresholding of the FT. CTF generates a heterogeneous background, called the Thon rings, which should be removed first. CTF is assessed by computing the average radial profile of the FT. This profile is then used to reconstruct the 2D CTF and subtract it from the original FT. The obtained corrected FT is finally thresholded to identify diffraction peaks. By default, the threshold is set to identify peaks whose signal-to-noise ratio is above 3.5 (false detections become important below this threshold because of the noise). The user can optionally adjust this value.

4. Fully automated microscope control

The tools presented in section 3 have been assembled and organized to form the ANIMATED-TEM software presented here. It has been developed as a Matlab Toolbox (R2008a), requiring the Image Processing Toolbox. It has been developed to analyze the images acquired and achieves two goals:

(1) Automatic targeting: identify the ROI to be acquired at higher magnification to assess the crystallization experiment. Coordinates of the targeted regions are transmitted to the control system for further acquisition.

(2) Automatic Extraction of Data for sample characterization: images selected and analyzed at different magnifications are used to assess the support (grid's carbon film), the detected membranes, and check their crystallinity through the diffraction pattern.

ANIMATED-TEM has been integrated on a prototype located at the C-CINA, Basel. The on-line automatic control consists of the interaction between three systems: the TEM and two computers, one for the microscope control tools, and one for the analysis of the images achieved by ANIMATED-TEM. The software interacts with the microscope control tools by sending HTTP requests corresponding to the desired action (stage displacement, image acquisition magnification to be set, etc.) The microscope control computer controls physically the devices of the TEM according to the requests received. It also transmits to the image processing computer the images acquired by the CCD camera.

The Tecnai T12 microscope is equipped with a 1kx1k CCD, and with unique autoloader and carousel. The carousel encloses 8 cassettes of 12 grids. Cassettes can be inserted consecutively into the autoloader which then controls the loading of the grid into the microscope, enabling to control the microscope for a fully autonomous acquisition of images from 96 different samples. The microscope control tools achieve the physical command of the microscope (stage displacement, image acquisition, magnification setting...). ANIMATED-TEM processes the images acquired, and decides when, where, and how images should be acquired.

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In the first subsection below, the microscope control tools are briefly presented. The second subsection develops the scenario monitoring the automatic acquisition and introduces the GUIs (Graphical User Interfaces).

4.1 Microscope control

Presentation

The microscope control tools have been developed to interface our image processing tools and the microscope as presented in Figure 4. The software, called JusT12, interacting with the microscope is using the FEI automation servers. An additional server has been developed to control the 12-cassette carousel. COM technology permits to access remotely other computers, but it introduces network latency. Hence, the JusT12 software installed on the microscope computer has been written, and designed to react to HTTP requests. Also, the choice has been made to separate the microscope control code from ANIMATED-TEM to keep the matlab code as clean as possible, and to delegate the microscope control to a dedicated software which made it easier to maintain.

“Semantic” commands are those received from ANIMATED-TEM via an HTTP interface. They include the name of the physical command to be performed, and the required parameters (for instance the coordinates of the new position, the index of the grid to be loaded, the amount of illumination, the value of the stage displacement, the magnification, etc.). We note that during the stage displacement, a backlash correction is achieved by always moving to the desired target from the same direction.

Therefore, the Image Processing part sends HTTP requests to the Microscope Control part that manages TEM devices. If an image acquisition has been requested, the 1k x 1k image acquired with a CCD camera is transferred to the Image Processing computer for analysis and storage.

Carousel Graphical User Interface

A carousel control GUI has been developed to:

- initialize the position of the 8 cassettes within the carousel (Figure 6, right): initialization is one of the first steps to do once the carousel is installed on the

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microscope. The initial positioning adjustment is robust and still valid in our T12 prototype since its initial installation and initialization.

- visualize the state of the machine and the sensors;
- manually control the carousel and the cassette (un)loading: this feature can be used if the user does not use the automatic process, or to take control of the carousel if there is a problem during the automatic process. More than a thousand loadings and unloadings have been automatically and manually performed so far without any problem.

4.2 Automatic run scenario

Presentation

The scenario aims to articulate the different algorithms and allow an on-line image computation of up to 96 grids in a fully automated manner. The scenario has been elaborated to generate the semantic command (acquire image, move microscope, etc.) at the appropriate moment.

The scenario can be decomposed into 4 parts: grid loading (part 1) and specific processing (part 2-4) for each magnification. Figure 6 shows typical acquisitions for this scenario. The flowchart in Figure 7 details each step.

This flowchart has a certain number of loops; the amount of iterations depends on the result of the image processing, and on parameters set by the user before launching the automatic run (as presented in the next section).

As shown in the flowchart, the low-, medium-, and high-magnification steps are made of three parts:

- the managing of the iterations (amount of images acquired).
- the semantic commands to move the stages to the desired targets, and to acquire the images (this latter includes the commands of the exposure time, illumination, defocus): at high and medium magnification, positions are determined by the image processing achieved at the previous magnification. At low magnification, images are acquired according to a circular-like pattern, as shown in Figure 8.
- the processing of the images, using the tools presented in the previous section.

Main Graphical User Interfaces

Two graphical user interface (GUI) are used: one to launch the automatic run and set some parameters, the other to visualize the results.

GUI - Launcher

Through the GUI Launcher, the user can pre-set parameters to meet the requirements of the above described basic scenario. Among the default parameters that can be changed, we note:

- the stop criteria which define how many grid squares should be visited at medium magnification (limit for ΣMM), or the maximum number of low-magnification images to acquire (limit for LM);
- the maximum number r of ROI to identify at medium magnification and the algorithm to use (the PED and/or the multi-resolution-based method);
- the grids g which should be analyzed during the run;
- the acquisition parameters (illumination, exposure time, etc.).

As will be seen in the experimental result section, these parameters greatly influence the time spent by the automatic run.

Setting up the ANIMATED-TEM toolbox for other microscopes may require only a few adaptations of the parameters (depending on the autoloader of the microscope), and of the instructions used to send the semantic commands (depending on the microscope control tools).

GUI - Browser

A GUI Browser has been developed to view the images automatically acquired. The interface links images to the corresponding acquisition at lower or higher magnification. The user can reload a selected grid and move it to the position where the current image has been acquired.

A pop-up window can be opened to display statistical characteristics concerning the run and the sample. For each grid it gives the time spent for its screening; the number of images acquired; the estimations of the percentage of good-quality grid squares; and the average size of non-stacked membrane regions.

5. Results and experiments

ANIMATED-TEM toolbox is used to control a customized Tecnai 12 microscope equipped with an autoloader and a carousel, which allow to treat automatically up to 96 grids. During the testing phase, over 66,000 images have been treated, corresponding to approximately 1,500 grids analyzed in 230 runs.

This section focuses principally on 2 features: the time required for each step during a run, and the automatic determination of the regions used for next examination, the ROI, which is an essential point of our original automation approach.

5.1 Run time

The overall time spent on an automatic run mainly depends on two parameters: the settings defined by the user (maximum amount of images and regions of interest), and the results of the image processing (actual amount of targets identified, related to the quality of the grid and to the interest of the sample at medium magnification).

Successive acquisitions at low-, medium-, and high-magnification will be processed according to the control strategy defined initially using the GUI.

The processing time can be approximated as follows:

$$T = (N_{LM} \cdot T_{LM} + N_{MM} \cdot T_{MM} + N_{HM} \cdot T_{HM} + N_F \cdot T_F) \cdot N_G + N_C \cdot T_C + N_G \cdot T_G ,$$

with:

- N_{LM} , N_{MM} , and N_{HM} , respectively the amount of images acquired at low, medium, and high magnification;
- T_{LM} , T_{MM} , and T_{HM} , the average times for acquisition and processing;
- N_F , and T_F , the number of executions and the execution time of the autofocus process at high magnification;
- N_G , and T_G , the amount of grids and the loading time of a grid;
- N_C , and T_C , the amount of cassettes and the loading time of a cassette.

Figure 11 gives a chronogram of a grid analysis leading to the acquisition of 10 images. In white, we show the microscope control times, and in black the image processing times. The average times required at low, medium and high magnification are respectively 20s, 27s, and 40s.

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The times displayed here are obtained on an Intel Xeon processor at 2.67 GHz & 6 Gbytes of RAM computer, on large images (1k x 1k images). Processing larger images may require adaptations to cope with the important memory required.

In the example of Figure 11, image 1 allows the selection of two targets (images 2 and 5) that will trigger the next acquisitions at medium magnification. On the contrary, image 7 (low magnification) does not allow a correct target selection, so a new image has to be acquired at low magnification (image 8).

Average times for microscope control and image acquisition

It takes about $T_G \approx 180s$ to insert a grid into the microscope, and about $T_C \approx 300s$ to insert a cassette into the autoloader.

Table 1 shows the times for the execution of the physical commands on the first row. Average times for microscope control and image acquisition include the setting of the acquisition parameters (magnification, exposure time, illumination, and defocus), the stage displacement, and the image acquisition. The last two steps are the most time-consuming. Only the time spent for image acquisition could be reduced by using a faster camera (we estimate a gain of about 5 seconds). The greater distances to travel at low magnification can explain the difference between average times at low and medium magnification. At high magnification, the average time also includes the autofocusing step which are time-consuming (up to $T_F = 50s$). An improved method for autofocusing would reduce this delay. We notice that its influence on the overall time can be reduced by modifying the frequency of the autofocusing measurement. When high-magnification images are acquired consecutively, their positions are close enough to avoid focusing before each acquisition. An option in the GUI Launcher presented previously can be used to decide if the measurement should be done before each high-magnification acquisition or not.

Average time for image processing

The strategy used for the grid analysis is to acquire a low-magnification image in order to assess the quality of the grid and to establish the list of valid grid squares to be examined at higher magnification. At low magnification, more than 99 % of the selected grid squares are non-broken carbon film regions. Such a high result

1 implies two compromises. First, to obtain such a rate of false positives, a higher
2 rate of false negatives was accepted: 81 % of the grids visually considered as valid
3 were indeed selected. Second, the microscope illumination should be
4 homogeneous and properly set to operate with a good contrast: exposure time and
5 illumination should be sufficiently high to achieve this goal, but not too high to
6 avoid over-exposition of the camera. The saturation threshold of the camera has
7 been set to 75%. This value is indicative but not critical as illustrated by the
8 experimental results (see Section 5.2). At this magnification, the average time for
9 the image processing is about 3s.

10 At medium magnification, the chain of algorithms detects edges of membranes,
11 characterizes each region r and selects a list of targets. The computational time is
12 about 15s. The quick ROI selection with the PED algorithm is about 2s. At this
13 magnification, processing time is about 15s for the multi-resolution-based
14 process, e.g. multi-resolution segmentation, contour validation, stacking
15 representation, shape recognition, and ROI selection and about 2s for the PED
16 ROI selection. In our example of Figure 11 two ROI are found in image 2, one in
17 image 5 and none in image 9. Each ROI leads to one up to four high-
18 magnification acquisitions.

19 At high magnification, the average time for image processing corresponds to the
20 computation of the Fourier Transforms for the estimation of the power spectra and
21 the diffraction peak identification. This treatment takes approximately one second.
22 98 % of the Fourier Transforms were properly automatically classified in terms of
23 diffraction peaks. The false classifications are mainly due to bad-quality crystals
24 or crystals presenting diffraction peaks with a SNR below the detection threshold
25 (fixed by default to 3.5).

26 Table 1, second row, presents the average times of image processing for each
27 magnification. Regarding the size of the images and the application, a relatively
28 fast processing is available for on-line TEM image processing and target
29 selection. Execution of the physical commands by the microscope (Table 1, first
30 row) takes more time. The proposed strategy appears therefore to be well adapted
31 for such controls.

32 Other strategies could also have been considered. For instance one could acquire
33 all the images at low magnification, then the ones at medium magnification, and
34 at last the ones at high magnification. However this strategy has been discarded

1 for a number of reasons. First, the low reliability and reproducibility of the stage
2 movements: when moving to a targeted position, it seems better to increase the
3 magnification immediately to the target without moving the stage, as the
4 positioning error tends to get higher when the stage has been moved too much in
5 between. Second, the low flexibility in the stopping commands such as stopping
6 the processing of a grid when a satisfying amount of crystals has been found.
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10 *Conclusion*

11 Table 2 shows the time and the number of images acquired at each magnification
12 for a several runs. The overall time of a run directly depends on the amount of
13 objects in the images, but also on conditions like the maximum number of low-
14 magnification images to be acquired, the number of grid squares to analyze at
15 medium magnification, or the maximum number of ROI for each medium-
16 magnification image, (set by default to 20, 20, and 8 respectively).
17

18 Figure 12 shows the last run of Table 3 in detail. For example, we can see that the
19 carbon film is completely broken on grid 8, cassette 1. Only 20 low-magnification
20 images have been acquired; it takes less than 10 minutes to process such a grid.
21 However, when the grid was interesting, 20 suitable grid squares have been
22 identified, and for each of the medium-magnification images, at most 8 ROI have
23 been identified, each one corresponding to an acquisition at high magnification.
24

25 Finally, in order to reduce these times, several ways are possible, such as doing
26 the stage displacement and launch a new image acquisition at the same time while
27 the previous high-magnification image is processed (Fourier Transform
28 computation). Moreover we notice that by optimizing Matlab code into C-
29 compiled code, these standard times for image processing can be greatly reduced.
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50 **5.2 ROI selection at medium magnification**

51 The ROI selection at medium magnification is a crucial step for the correct
52 development of the automatic analysis of 2D-crystals Therefore, It has to be
53 robust, quick and as close as possible to the choices that would make a biologist
54 during a traditional analysis of the grid. This is shown on an example where the
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automatic selections made by ANIMATED-TEM are compared with the decisions of an expert, in various acquisition conditions.

ROI selection

The selection procedure described by biologists has widely contributed to the choice of the parameters used in the automatic selection of the ROI.

1. Only the lowest-stacked regions are selected. Indeed, the diffraction pattern is easier to study on non-stacked objects.
2. The smallest regions are removed from the selection. In our tests, we considered that small regions (500nm, i.e. smaller than the field of view at high magnification) are less interesting for pattern identification.
3. Experience shows that the crystalline membranes often present some linear edges. The general appearance of a membrane is thus a parameter that has to be considered.

Among all the segmented regions, only those that are considered non-stacked by the stacking algorithm and of size above 500nm are retained as targets. All other regions are discarded. It is then necessary to rank each of these regions in order to select the best ones. Each region is indexed both according to its size and the length of its linear edges. Regions are characterized by both indexes $L[Si_r]=n$ (for the size) and $L[Sh_r]=m$ (for the shape), with $n,m \in \mathbf{N}^*$, corresponding to the rank of the region compared to the other regions of the image. For the widest region $n=1$; in the same way the region having the longest linear edge is characterized by $m=1$. An average rank, \bar{r} , is deduced using:

$$\bar{r} = \frac{a \times L[Si_r] + b \times L[Sh_r]}{a + b},$$

where a and b are weights that can be used to adjust the importance of one of the parameters. ROI having the lowest \bar{r} are considered as the best ones. By default, in our application, only the best two regions are used for acquisitions at high magnification. This limit can be modified by the user. Other criteria may be added to refine the choice of the ROI, considering for example a strong local contrast, meaning that a membrane contour is present rather than artifact-like stain.

Test conditions

Figure 13a shows an image acquired at medium magnification. We asked an expert to select the most appropriate areas at middle magnification to be checked for cristallinity at higher magnification. The result of this manual selection is presented in Figure 13b. Highly interesting regions are represented in black, interesting regions in gray. The main differences in this classification are the size and the shape of the regions. These two parameters come directly from the expert's manual segmentation. This image will be the reference image.

The conditions of image acquisition during a run are not always optimum, as a process to automatically set exposure time and illumination is not available yet. It is interesting to verify the robustness and the reproducibility of the selection by modifying their parameters in large ranges. Membranes of Figure 13a were thus acquired several times with various exposure times and illumination rates within realistic value ranges for that application. Table 3 shows the values of these parameters for each acquisition (acquisitions 1-11). In a second step, acquisitions have been made varying the magnifications (acquisitions 12-16), then the stage position (acquisitions 17-22). Figure 14 shows each of these acquisitions on which the segmentations processed by ANIMATED-TEM are overlaid. The ROI are selected among these segmented regions. Several comments may already be made. First, it can be noted that the segmentation may vary depending on the acquisition conditions. Second, new elements may appear in the images depending on the magnification or the stage position. These parameters have not been considered during the classification done by the expert.

Results

The ROI have been selected on each of the 22 acquisitions of Figure 14. The results are presented in Figure 15. For each acquisition (abscissa), the percentage of ROI found by ANIMATED-TEM is represented according to the classification by the expert: first class in black, second class in dark gray, regions not selected by the expert in lighter gray, and regions not even present in the expert's image in pale gray (acquisition 12 to 22). Stars and little circles represent for each category the number of ROI found by ANIMATED-TEM, i.e. the number of non-stacked regions of over 500nm. Two stars for an acquisition correspond to the best two selected regions leading to acquisitions at high magnification. For example in

1 acquisition 7, 12 ROI have been found, 5 of them according to the first class of
2 the expert (41,6%), 4 to the second one (33,3%) and 3 have not been retained by
3 the expert (25%).
4

5 The main difference between first and second class lies in the size of the ROI.
6 These two classes allow therefore to verify the crystallinity of a sample. We can
7 notice that segmentation in regions within the different acquisitions is very
8 variable, and what is considered by the expert as one sole zone can be broken
9 down in several regions by ANIMATED-TEM.
10

11 When the acquisition parameters change for the same location (acquisition 1-11),
12 we notice that in average 80% of the selections made by ANIMATED-TEM
13 correspond to the selection of the expert. Moreover, if we only consider the two
14 retained regions, only one of the ROI of acquisition 11 does not match with a
15 choice of the expert, and for this acquisition the experimental conditions are far
16 off the nominal values (Table 3). In 64% of cases, both selected targets match
17 with regions of first class of the expert and in 91% of cases they are part of the
18 choice of the expert (first or second class).
19

20 The goal of the application is to validate experiences of 2D crystallization. If the
21 experimental protocol is correct, there will be plenty of occasions to observe
22 crystalline membranes on the grid. Consequently, even if a few high-
23 magnification acquisitions are useless because of a wrong choice of targets, it will
24 not have a dramatic effect on the final result. There will be statistically enough
25 observations allowing the characterization of the studied experimental protocol.
26

27 When the magnification is changed or when the stage is moved (acquisitions 12 to
28 22), the elements composing the image are not the same anymore. Yet the
29 selection of the targets remains relevant. Except for acquisition 12 where the
30 membrane we used as reference is too small and less interesting than other objects
31 within the image, many regions being identified as interesting by the expert also
32 appeared to be so by ANIMATED-TEM.
33

34 Conditions of acquisition have to be as good as possible to allow image
35 processing tools to be efficient. Despite this, ANIMATED-TEM is capable of
36 selecting ROI in a very robust manner, according to what a biologist expert would
37 do, even when the conditions are not optimal.
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Conclusion

In this article we have presented the first fully automated system for sample analysis without human intervention. In recent months, several test runs have been made, where about 1500 grids have been analyzed automatically, confirming the efficiency of the overall system. The image processing time is much less than the microscope control commands and can easily be optimized in future development. The proposed control strategy handling the acquisition procedure achieves the processing of a grid in an average of 34 minutes.

This first success of a full TEM automation opens the way for the development of image processing tools for electron microscopy.

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Figure Captions

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3 Fig. 1 Selection (white squares) of valid membranes grid squares

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5 Fig. 2 Structure of the image processing chain for medium-magnification images

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7 Fig. 3 Illustrative example of an image processing chain for medium-magnification images. A/
8 Initial image; B/ Result of contour detection displayed on the initial image; C/ Segments to be
9 removed (in red) after statistical analysis of the local contrasts; D/ Labeling of the stacking
10 (lowest-stacked regions in red, bi-stacked regions in green; three-stacked regions in magenta;
11 multi-stacked regions in cyan); E/ Labeling of the shapes of the contour (linear portions in red;
12 circular portions in cyan; portions not associated with any of these two shapes in black)

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16 Fig. 4 Detailed architecture of the microscope control and interaction with the TEM and the image
17 processing computer

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19 Fig. 5 Carousel GUI Left: visual and manual control of the state of the carousel; Right: pop up
20 window to initialize the position of the 8 cassettes within the carousel

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23 Fig. 6 Typical example of an automatic run scenario at 3 magnification levels. Selected grid
24 squares appear in red at low magnification; Selected targets appear, at medium magnification, in
25 yellow (PED method) and red (multi-resolution-based method); identified diffraction peaks appear
26 in red ($4.5 \leq \text{SNR}$), blue ($4 \leq \text{SNR} < 4.5$) and green ($3.5 \leq \text{SNR} < 4$)

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30 Fig. 7 Flowchart of the major steps of the scenario (ΣMM is the amount of images acquired at
31 medium magnification on the whole grid)

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34 Fig. 8 Example of 20 low-magnification images acquired during a run; numbers show the order in
35 which the images have been acquired; each image is processed immediately after its acquisition

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37 Fig. 9 GUI to set the parameters for an automatic run (above), and two pop-up windows to select
38 the grids to be analyzed (left), and to adjust the default parameters used by image processing
39 algorithms (right)

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42 Fig. 10 GUI to browse through the result and the corresponding pop-up window displaying
43 statistical results for each of the grid analyzed

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45 Fig. 11 Example of the chronology of events in an automatic run

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47 Fig. 12 Pop-up window showing a summary of an automatic run executed with 96 grids

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49 Fig. 13 a) Initial image at medium magnification and b) ROI selection by an expert

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52 Fig. 14 Results of the automatic partition obtained on the same object acquired under different
53 conditions

54
55 Fig. 15 Comparative results of the automated selection at medium magnification, for images
56 acquired under different conditions

Figure 1

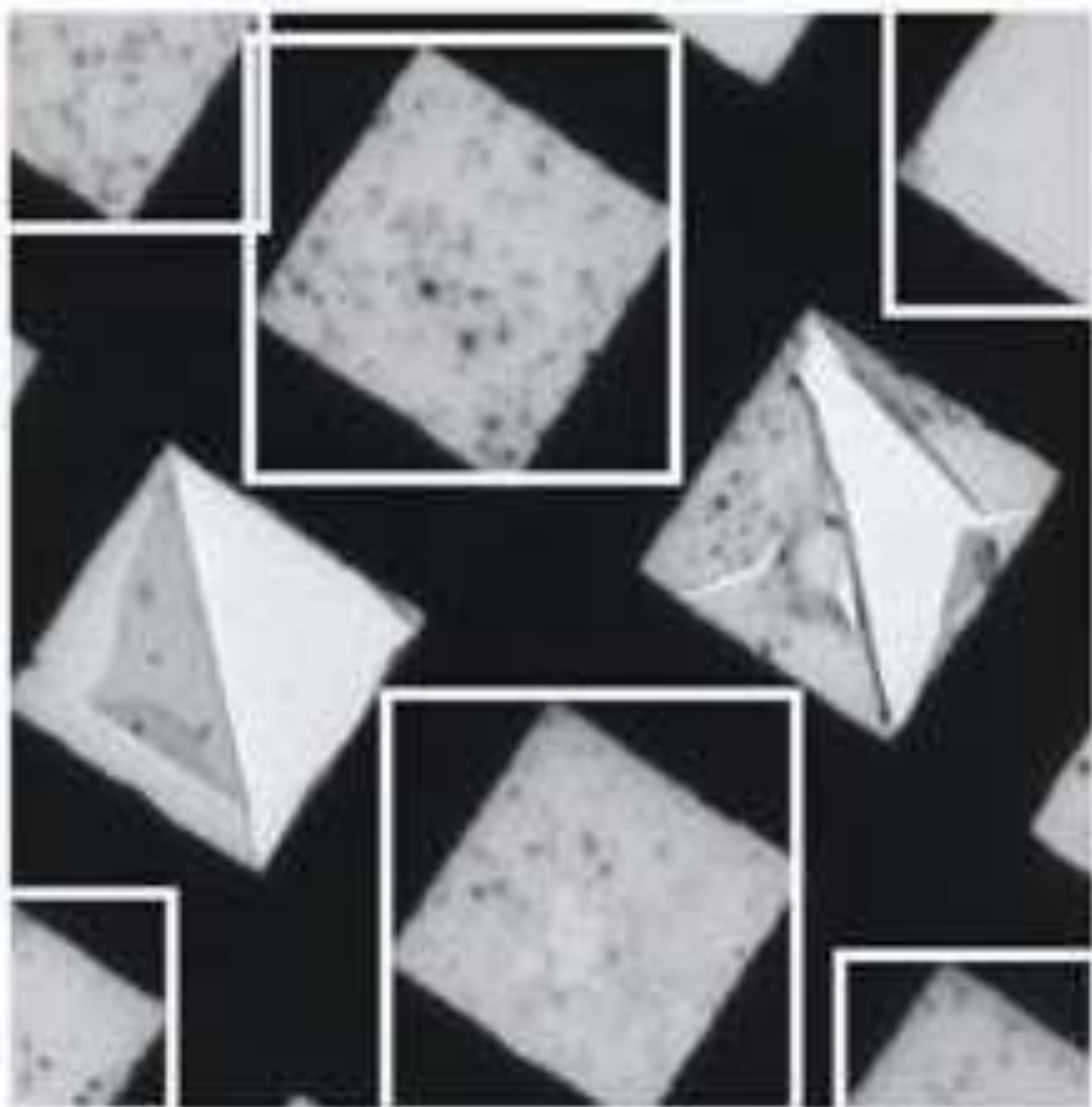


Figure 2

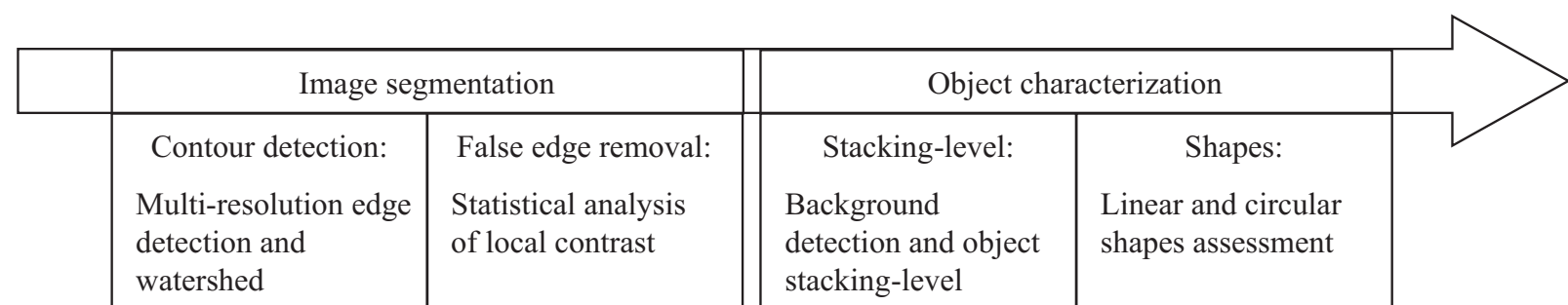


Figure 3
[Click here to download high resolution image](#)

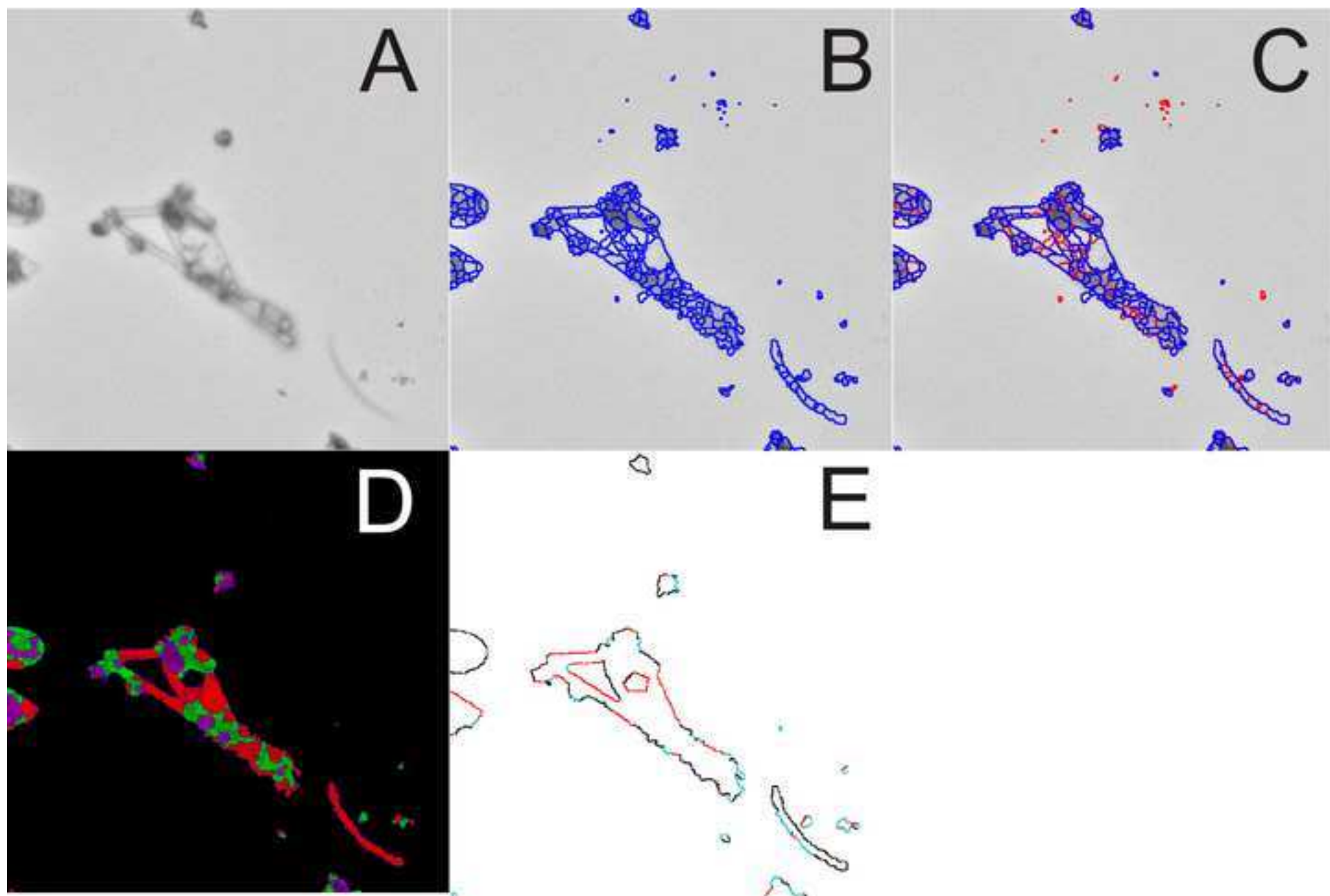


Figure 4

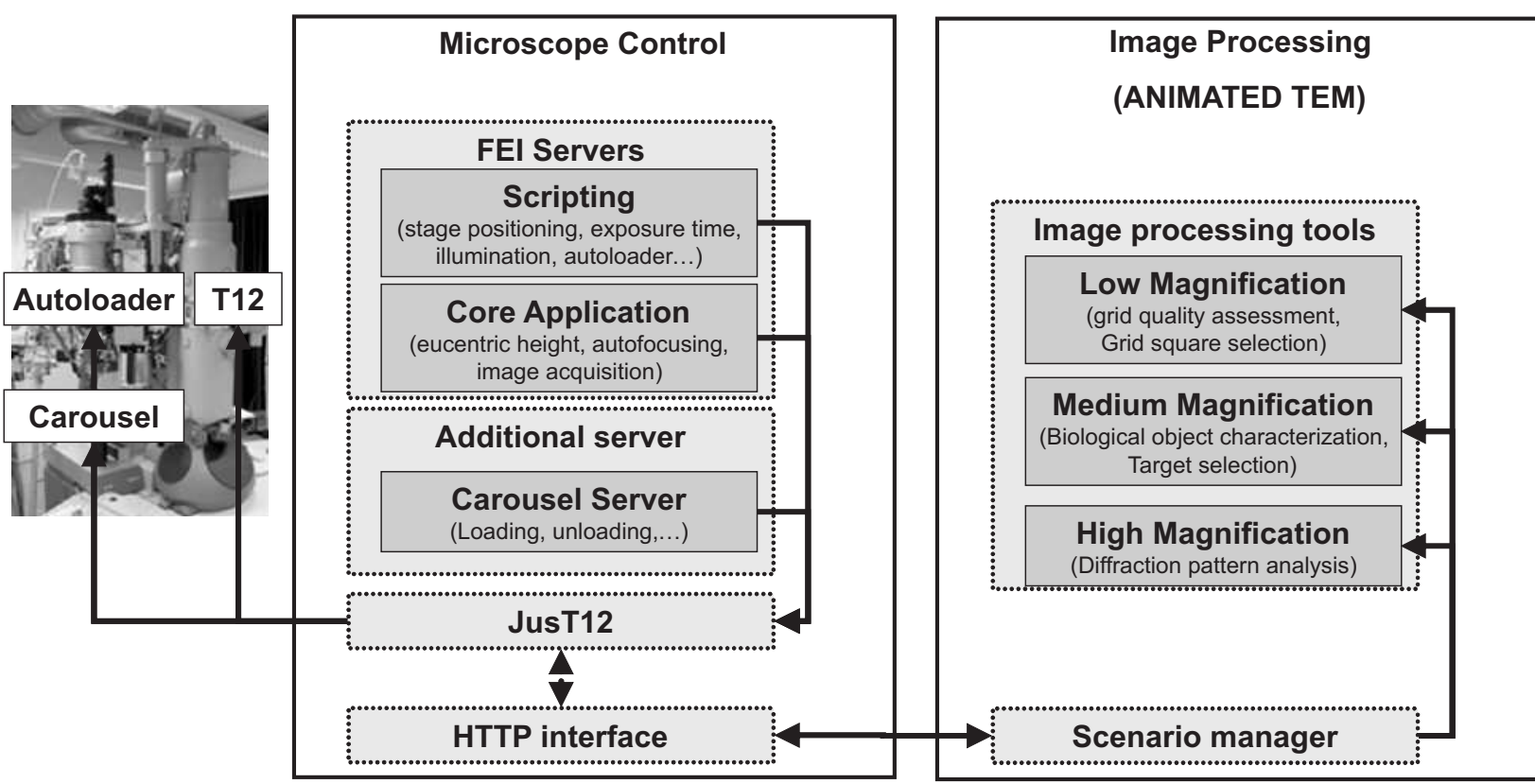


Figure 5

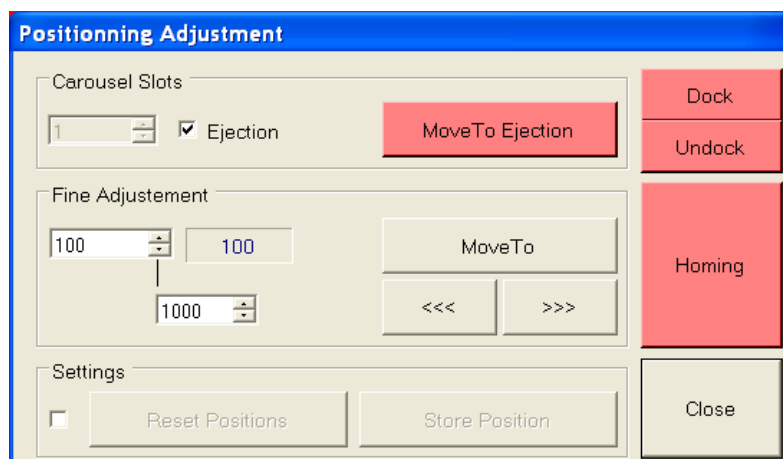
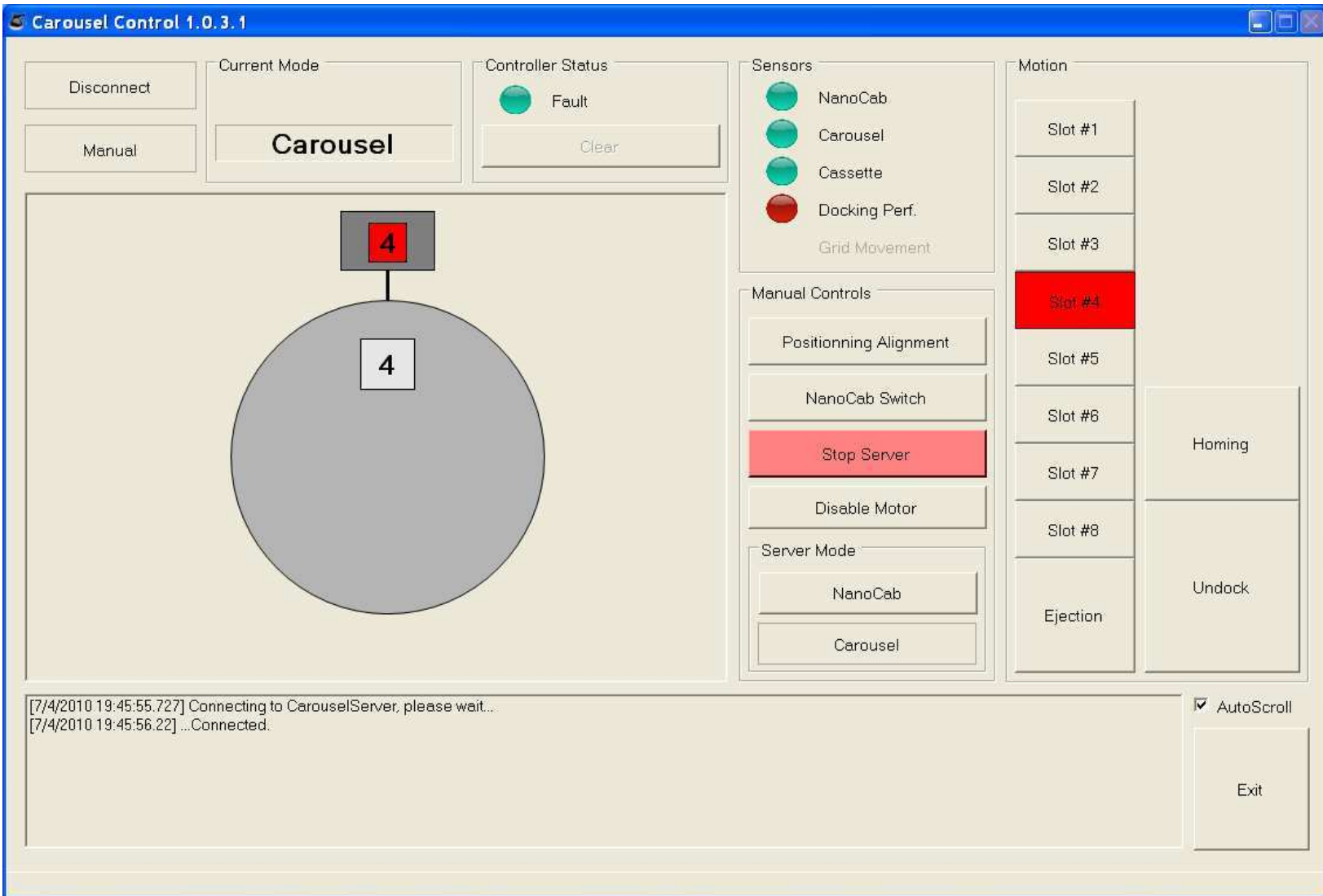
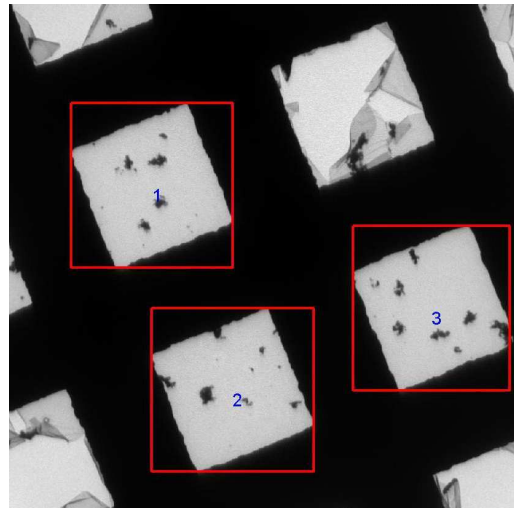


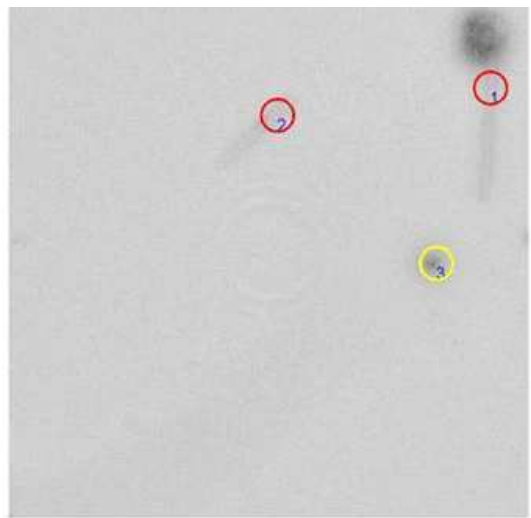
Figure 6

Low
Magnification



For all selected grid squares

Medium
Magnification



For all selected ROIs

High
Magnification

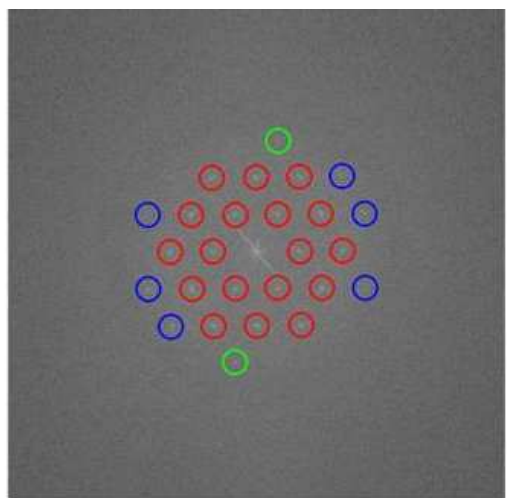
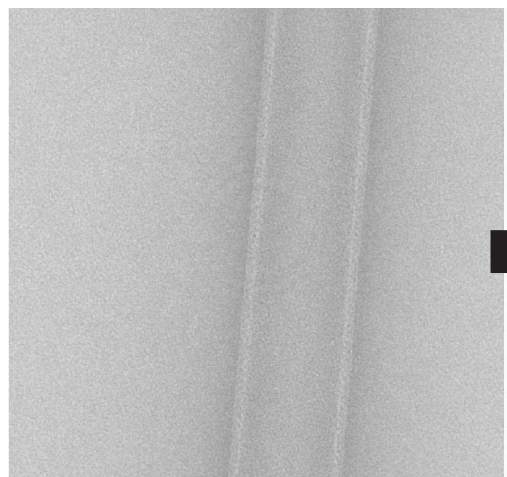


Figure 7

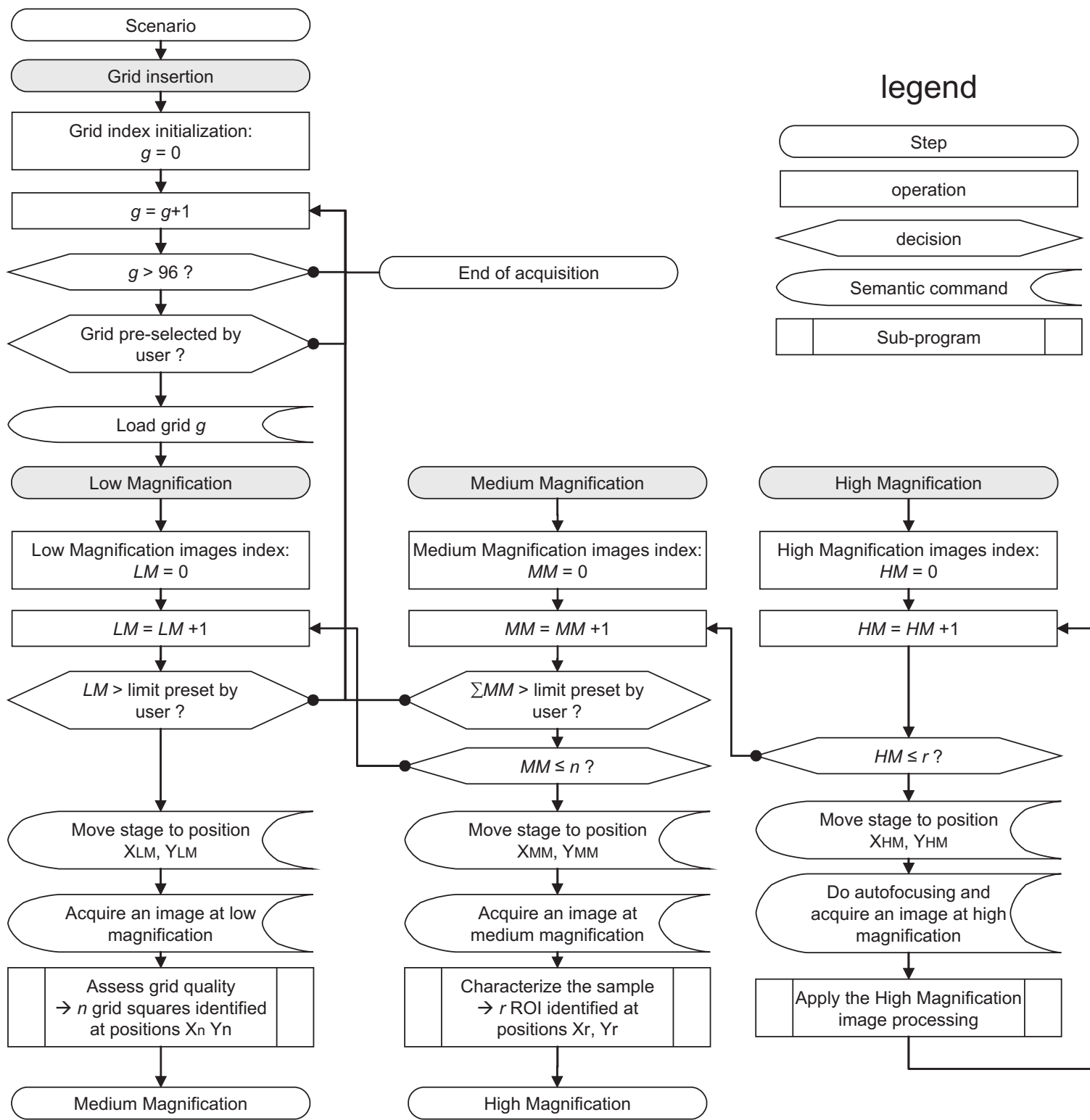
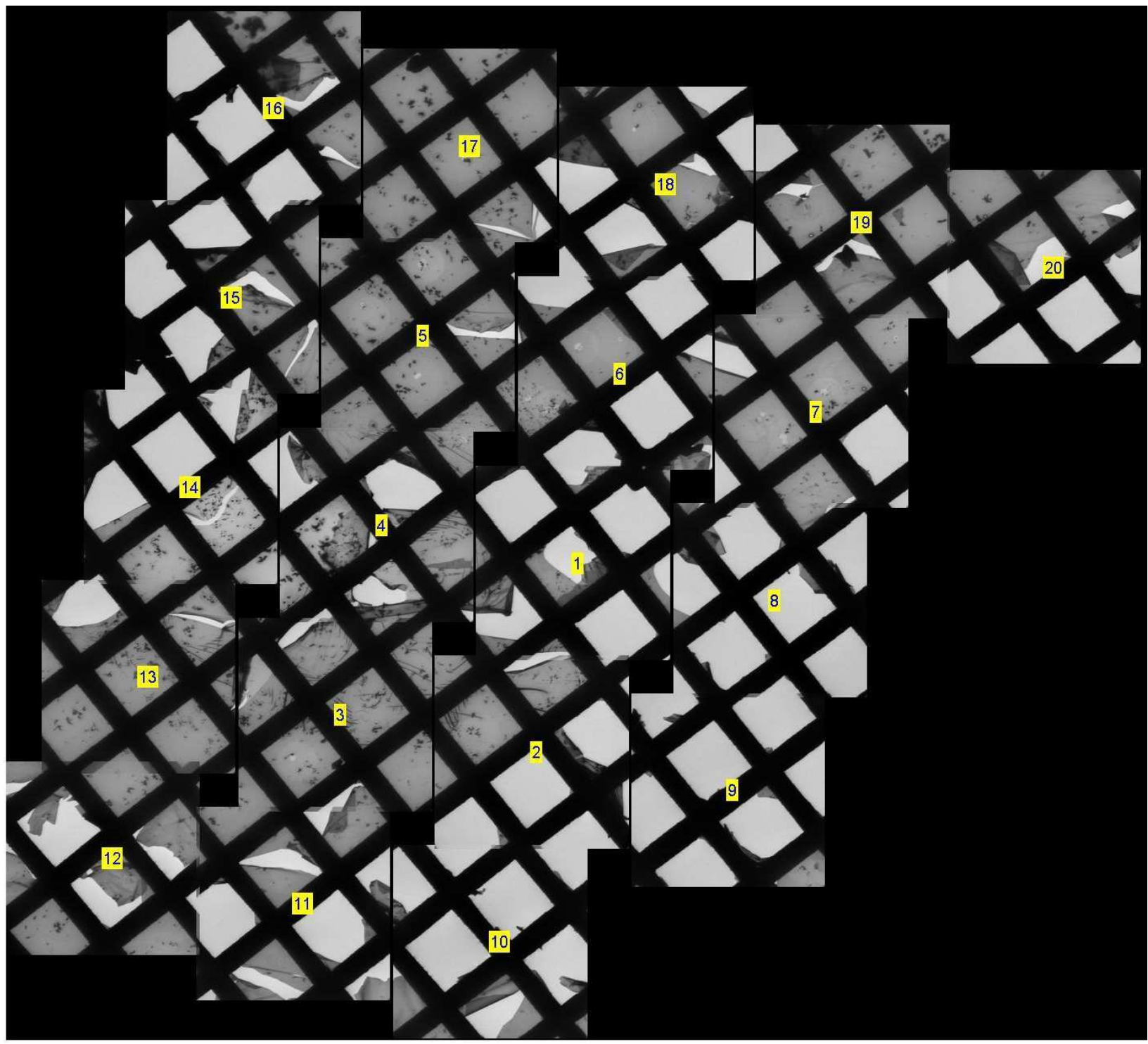


Figure 8



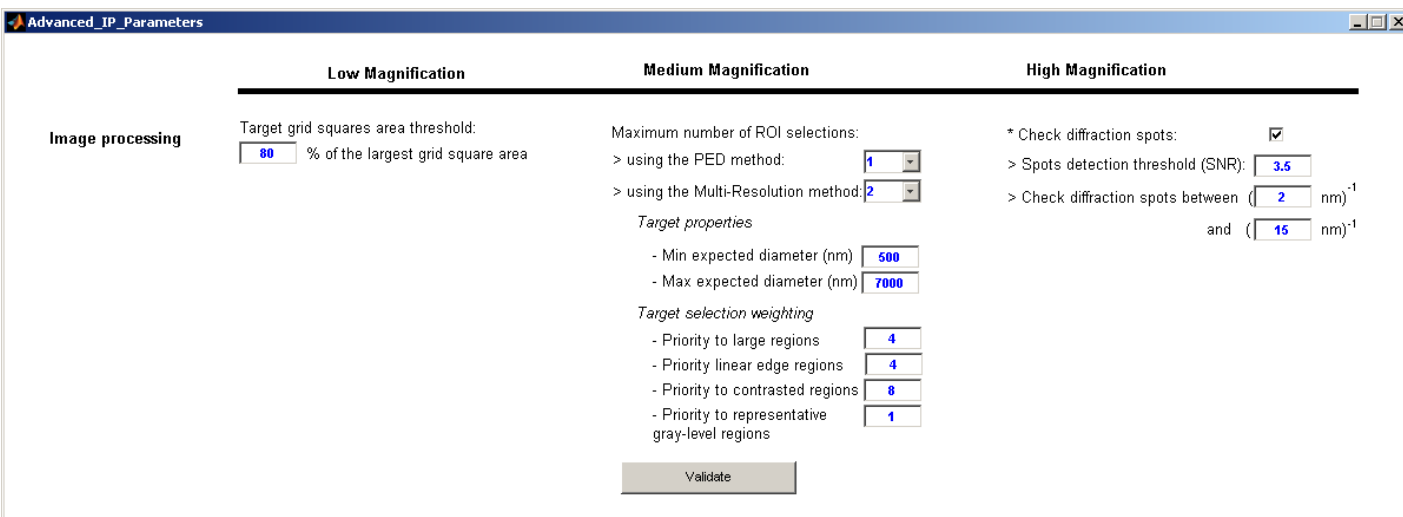
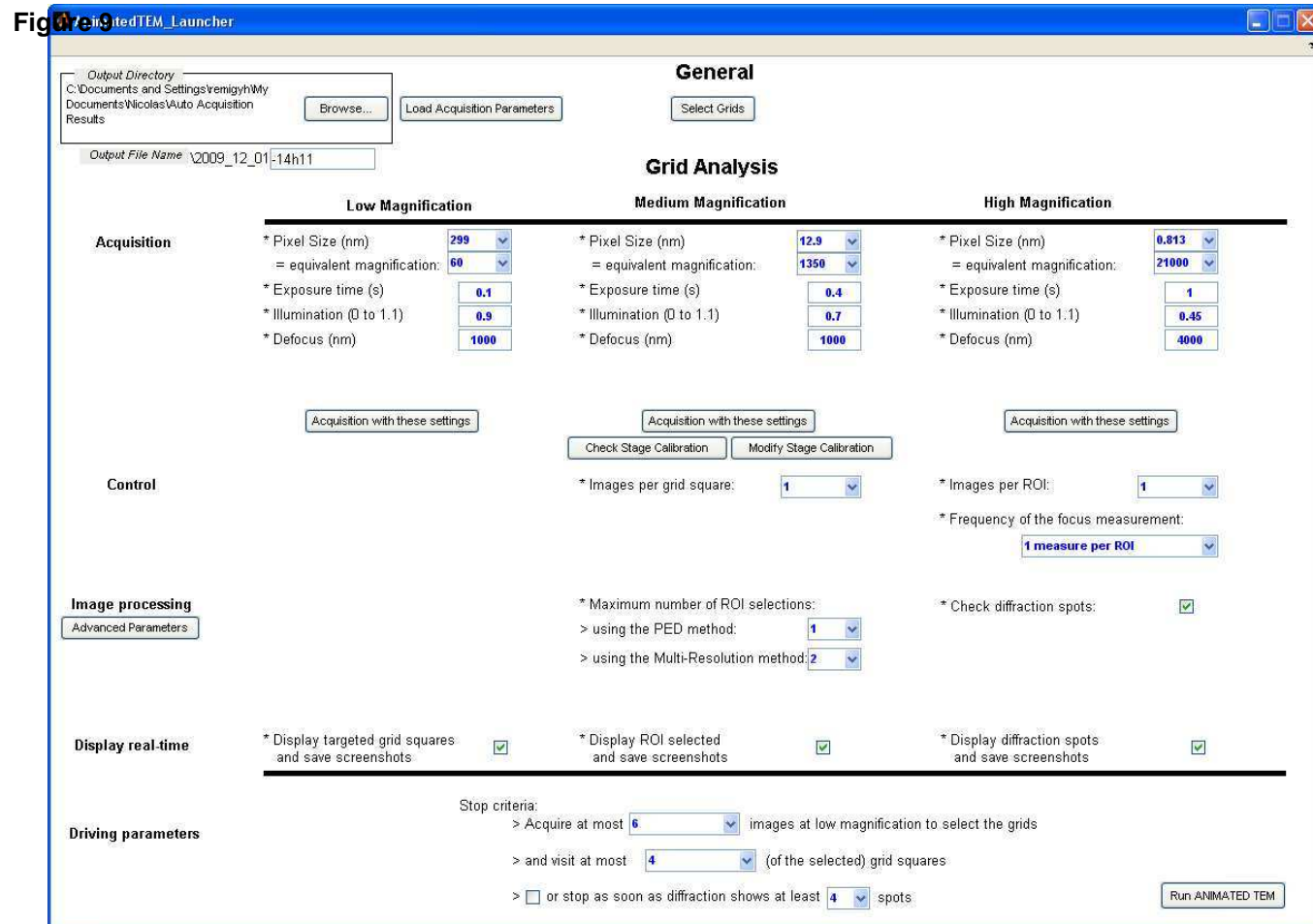


Figure 10

GUIinterfaceBD1

MonterSel

- 2009_9_8-14h6
- 2009_9_3-14h29
- 2009_9_3-11h26
- 2009_9_2-15h59
- 2009_9_2-14h48
- 2009_8_27-13h57
- 2009_10_08-Stacking-3
- 2009_10_08-Stacking-2
- 2009_10_08-Stacking-1
- 2009_10_08-Stacking
- 2009_10_02-test_r
- 2009_10_02-both_Autofo
- 2009_10_02-both_Autofo
- 2009_10_02-both_Autofo
- 2009_10_02-1_4_1_2aut
- 2009_09_23-9h15
- 2009_09_21-10h6
- 2009_09_14-17h0
- 2009_09_11-17h32
- 2009_09_09-14h42
- 2009_09_09-13h18
- 2009_09_03-HighMag Co
- 2009_09_02-Francoise
- 2009-07-29-Images

Low Magnitude

3/9 LowMag_3

1/10 MedMag_3_1

1/2 /

Overview

Next overview

FFT

04-2

03-0

01-0

Load Grid Get Image Cassette 1, Grid 1.

Grid_N_Cassette_Overview

Directory
C:\Documents and Settings\cina-tec\My Documents\Francoise\2010_03_23-16h23

Chose Grid Summary: Grid quality

Estimated percentage of convenient grid squares

Grid Indexes	1	2	3	4	5	6	7	8
12		34%	0%	37%	21%	1%		
		21%	0%	8%	32%	1%		
10		34%	3%	63%	16%	0%		
		57%	13%	57%	5%	12%		
8		15%	0%	3%	27%	0%		
		85%	4%	5%	6%	6%		
6		56%	5%	10%	31%	0%		
		10%	6%	6%	48%	5%		
4		87%	11%	3%	89%	9%		
		44%	11%	0%	21%	5%		
2		27%	0%	1%	29%	13%		
		53%	4%	0%	30%	10%		

Cassette Indexes

Figure 11

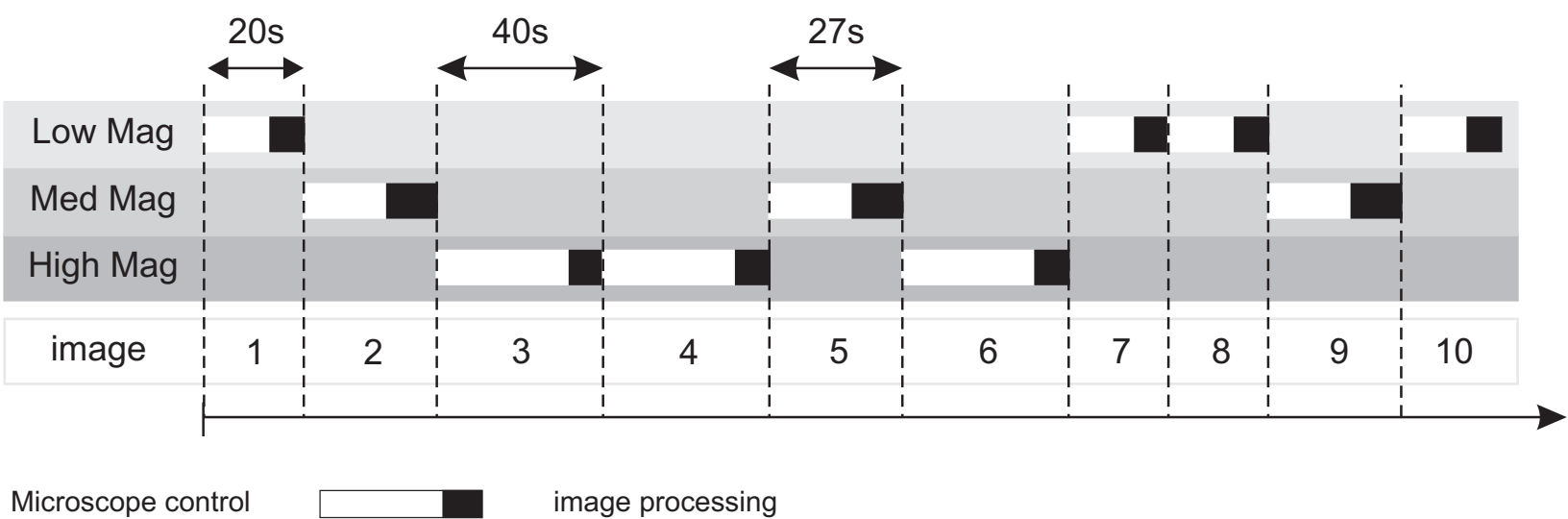
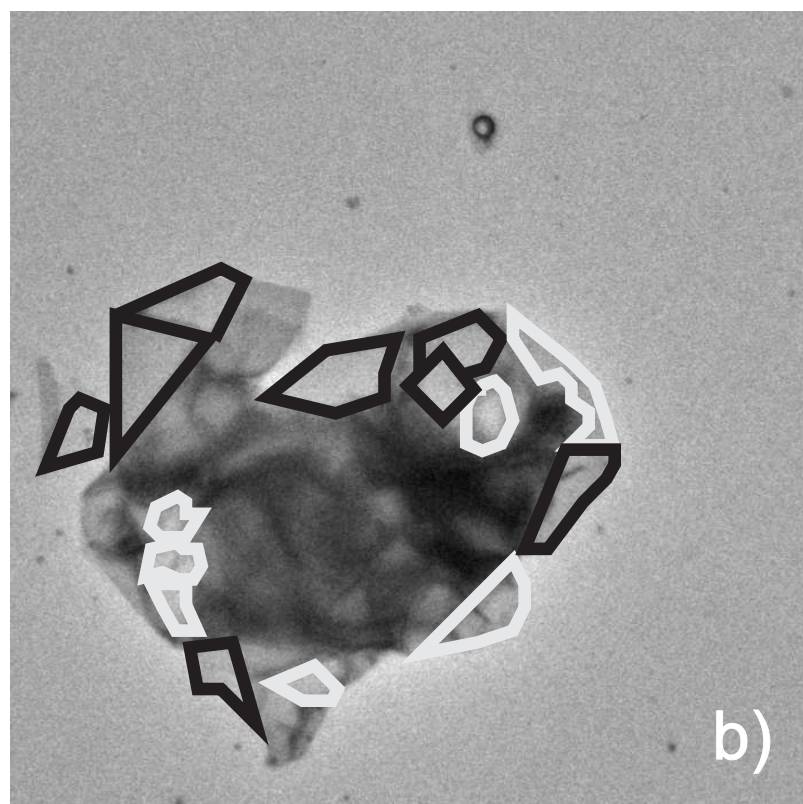
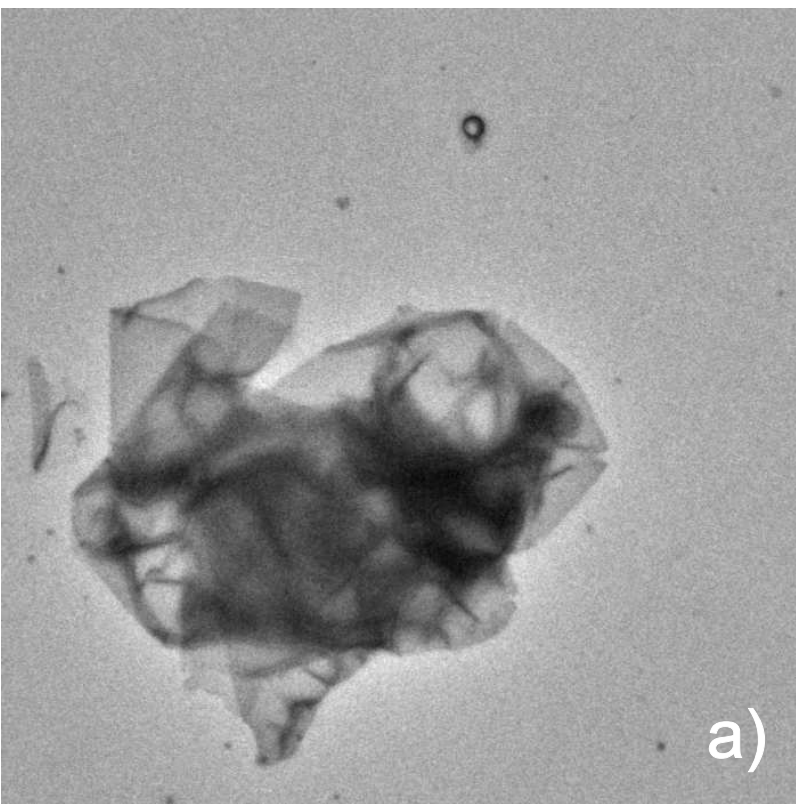


Figure 12



Figure 13



highly interesting regions



interesting regions

Figure 14

[Click here to download high resolution image](#)

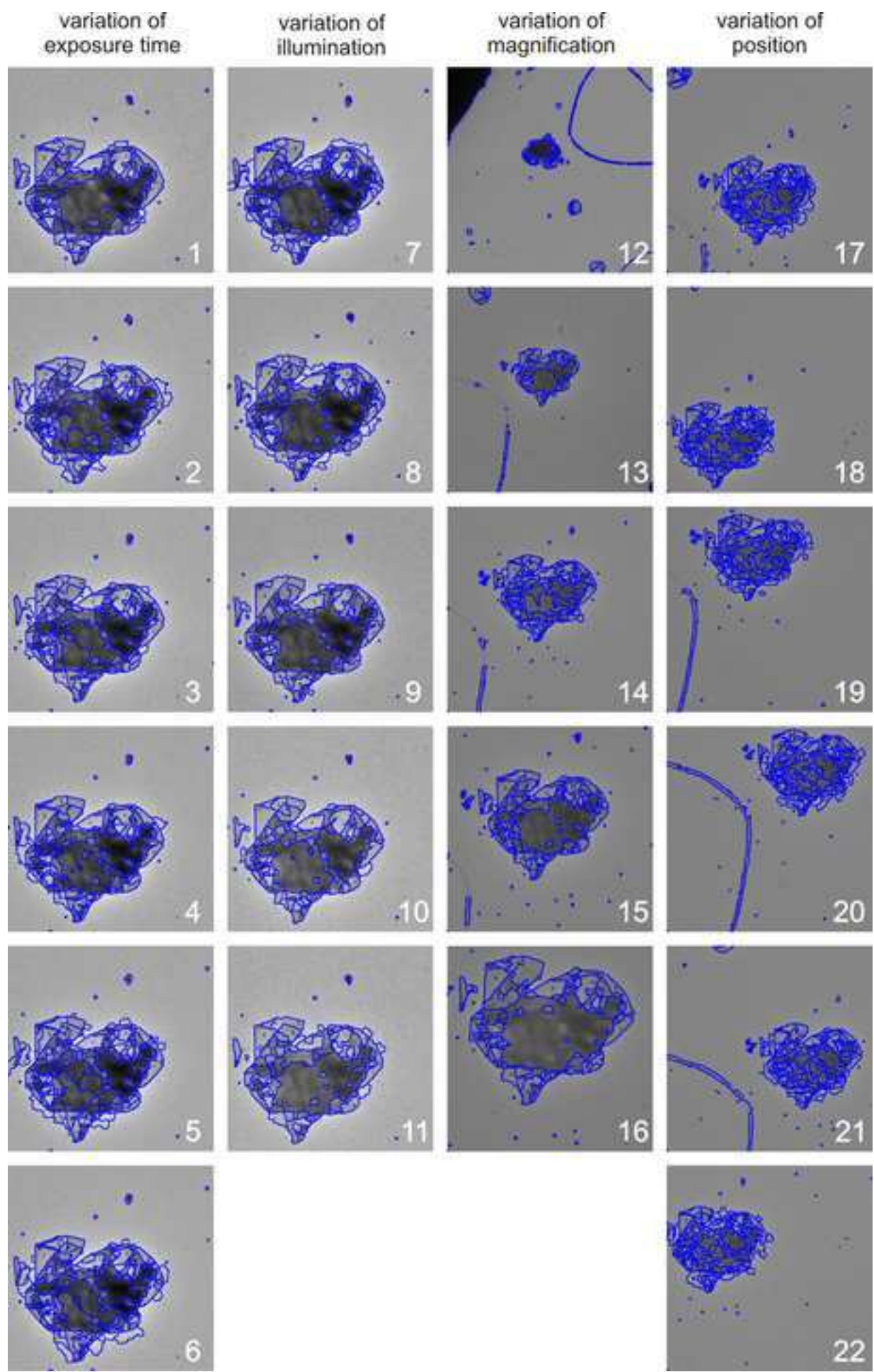


Figure 15

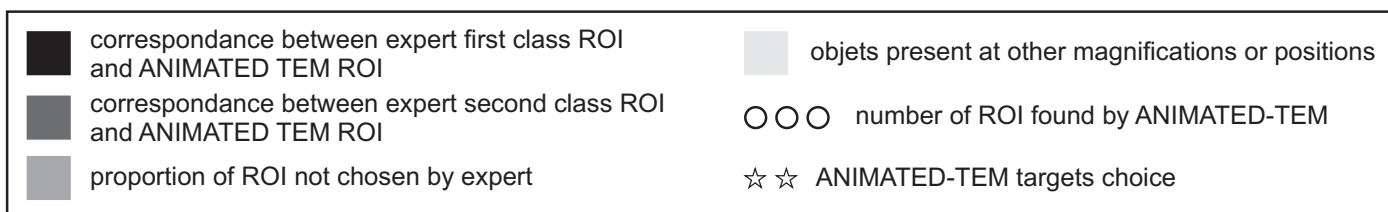
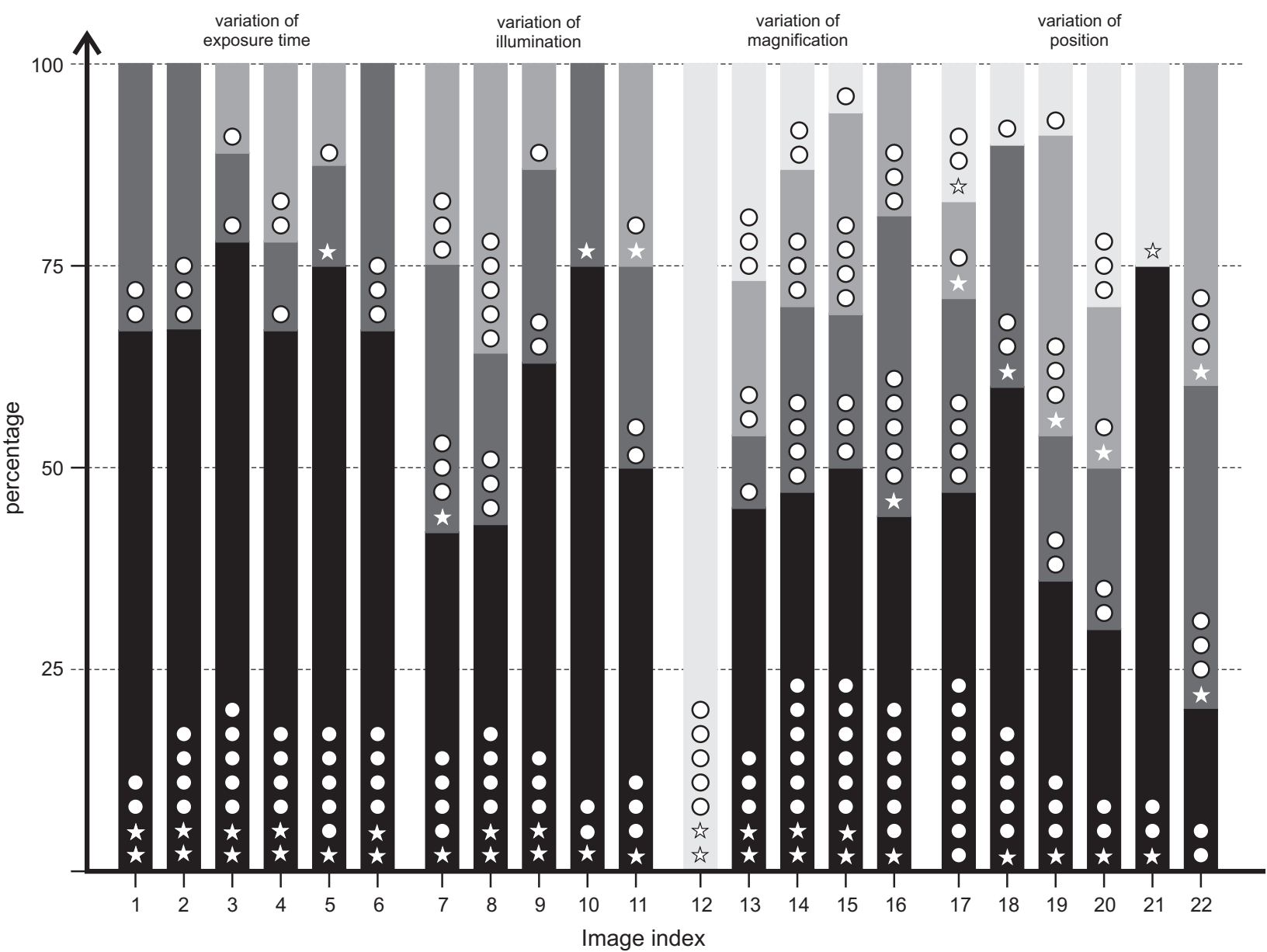


Table 1 Average processing times

Magnification	Low Magnification	Medium Magnification	High Magnification
Standard time for microscope control and image acquisition	~17s	~12s	~39s
Standard time for image processing	~3s	~15s	~1s

Table 1 Examples of automatic run experimental results

Number of grids	Number of images				Time
	Low magnification	Medium magnification	High magnification	Total	
96	1651	1351	3012	6014	50h25
58	400	569	1018	1987	21h09
96	1004	1305	3278	5587	59h17
55	592	936	1623	3151	37h43
41	454	696	1067	2217	22h23
96	1254	1751	3229	6234	59h08

Table 1 Acquisition conditions used for the tests

Image index	Exposure time	Illumination	Magnification
1	0.2	0.4	1350*
2	0.3	0.4	1350*
3	0.4	0.4	1350*
4	0.5	0.4	1350*
5	0.6	0.4	1350*
6	0.7	0.4	1350*
7	0.7	0.6	1350*
8	0.7	0.7	1350*
9	0.7	0.8	1350*
10	0.7	0.9	1350*
11	0.7	1.0	1350*
12	0.7	0.4	560
13	0.7	0.4	890
14	0.7	0.4	1350
15	0.7	0.4	1700
16	0.7	0.4	2200
17 to 22	0.7	0.4	1350