

1 Visual proteomics using whole-lamella 2D template matching

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10 **Abstract**

11 Localization of biomolecules inside a cell is an important goal of biological imaging. Fluorescence microscopy  
12 can localize biomolecules inside whole cells and tissues, but its ability to count biomolecules and accuracy of the  
13 spatial coordinates is limited by the wavelength of visible light. Cryo-electron microscopy (cryo-EM) provides highly  
14 accurate position and orientation information of biomolecules but is often confined to small fields of view inside  
15 a cell, limiting biological context. In this study we use a new data-acquisition scheme called “Defocus-Corrected  
16 Large-Area cryo-EM” (DeCo-LACE) to collect high-resolution cryo-EM data over entire sections (100 – 200 nm thick  
17 lamellae) of neutrophil-like mouse cells, representing roughly 1% of the total cellular volume. We use 2D template  
18 matching (2DTM) to determine localization and orientation of the large ribosomal subunit in these sections, detect  
19 bound small ribosomal subunits and assign ribosomes to polysomes based on their relative orientations to each  
20 other. These data provide “maps” of translational activity across sections of mammalian cells. This new high-  
21 throughput cryo-EM data collection approach together with 2DTM will advance visual proteomics and complement  
22 other single-cell “omics” techniques, such as flow-cytometry and single-cell sequencing.

23 **Introduction**

24 A major goal in understanding cellular processes is the knowledge of the amounts, location, interactions, and  
25 conformations of biomolecules inside the cell. This knowledge can be obtained by approaches broadly divided into

26 label- and label-free techniques. In label-dependent techniques a probe is physically attached to a molecule of  
27 interest that is able to detected with a high signal-to-noise signal, such as a fluorescent molecule. In label-free  
28 techniques the physical properties of molecules themselves are used for detection. An example for this is proteomics  
29 using mass-spectrometry [1]. The advantage of label-free techniques is that they can provide information over  
30 thousands of molecules, while label-techniques offer highly specific information for a few molecules. Especially  
31 spatial information can often only be achieved using label-dependent techniques, such as fluorescence microscopy  
32 [2].

33 Cryo-electron microscopy has the potential to directly visualize the arrangement of atoms that compose biomole-  
34 cules inside of cells, thereby allowing label-free detection with high spatial accuracy. This has been called “visual  
35 proteomics” [3]. Since cryo-EM requires thin samples (<500nm), imaging of biomolecules inside cells is restricted  
36 to small organisms, thin regions of cells, or samples that have been suitably thinned. Thinning can be achieved  
37 either by mechanical sectioning [4] or by milling using a focused ion beam (FIB) [5]. his complex workflow leads to  
38 a low throughput of cryo-EM imaging of cells and is further limited by the fact that at the required magnifications,  
39 typical fields of view (FOV) are very small compared to mammalian cells, and the FOV achieved by label-techniques  
40 such as fluorescence light microscopy. The predominant cryo-EM technique for the localization of biomolecules of  
41 defined size and shape inside cells is cryo-electron tomography [6]. However, the requirement of a tilt series at every  
42 imaged location and subsequent image alignment, severely limits the throughput for molecular localization.

43 An alternative approach is to identify molecules by their structural “fingerprint” in single projection using “2D  
44 template-matching” (2DTM) [7,8,9]. In this method a 3D model of a biomolecule is used as a template to find 2D  
45 projections that match the molecules visible in the electron micrographs. This method requires a projection search  
46 on a fine angular grid, and the projections are used to find local cross-correlation peaks with the micrograph. Since  
47 the location of a biomolecule in the z-direction causes predictable aberrations to the projection image, this method  
48 can be used to calculate complete 3D coordinates and orientations of a biomolecule in a cellular sample [8]

49 Hematopoiesis is the process of generating the various cell types of the blood in the bone marrow. Disregulation  
50 of the process results in diseases like leukemia. Understanding how hematopoietic stem and progenitor cells are  
51 programmed to diffferentiate to the appropriate cell type would be provide new insight how hematopoiesis can be  
52 misregulated. Of special interest is the regulation of translation during hematopoiesis. This is exemplified by the  
53 observation that genetic defects in the ribosome machinery often leads to hematopoietic disease[10]. As such direct  
54 quantification of ribosome location, number and conformational states could lead to new insight into hematopoietic  
55 disease [11].

56 Here we apply 2D-template matching of ribosomes to cryo-FIB milled neutrophil-like murine cells [12]. To in-  
57 crease the amount of collected data and to provide unbiased sampling of the whole lamella, we devised a new  
58 data-acquisition scheme, Defocus-corrected large area cryo-electron microscopy (DeCo-LACE). We characterize

59 aberration cause by the used large beam-image shifts and highly focused beams and find that they can be ad-  
60 equately corrected to enable ribosome detection by 2DTM. The resulting data provide a description of ribosome  
61 distribution in the whole lamellae, which represent roughly 2% of the cellular volume. We find highly heterogeneous  
62 density of ribosome within the cell and can identify discrete clusters of presumably translationally active ribosomes,  
63 by testing for the presence of the small ribosomal subunit. The high accuracy of location and orientation of each  
64 detected ribosome also allows us to cluster ribosome molecules into potential polysomes. Analysis of the throughput  
65 in this method suggests that for the foreseeable future computation will be the bottleneck for visual proteomics.

## 66 Materials and Methods

### 67 Grid preparation

68 ER-HoxA9 cells were maintained in RPMI supplemented with 10% FBS, penicillin/streptomycin, SCF, and estrogen  
69 [12] at 37C and 5% CO<sub>2</sub>. 120h prior to grid freezing cells were washed twice in PBS and cultured in the same  
70 medium, except without estrogen. Differentiation was verified by staining with Hoechst-dye and inspection of  
71 nuclear morphology. Cells were then counted and diluted to 1^106 cells/ml. Grids ( either 200 mesh copper grids,  
72 with a silicone-oxide and 2um holes with a 2um spacing or 200 mesh gold grids with a thin gold film and 2 um  
73 holes in 2um sapcing) were glow-discharged from both sides using a ... for ... . 3.5 ul of cells suspension was added to  
74 grids on the thin-film side and grids were automatically blotted from the back-side using a GP2 cryoplunger (Leica)  
75 for ... s and rapidly plunged into liquid ethane at -185C.

### 76 FIB-milling

77 Grids were loaded into a Acquilos 2 FIB/SEM microscope with a stage cooled to -190C. Grids were sputter-coated  
78 with platinum for 15s at 45 mA and then coated with a layer of platinum-precursor by openin the GIS-valve for  
79 45s. An overview of the grid was created by montaging SEM images and isolated cells at the center of gridsquares  
80 were selected for FB-milling. Lamella were generated automatically using the AutoTEM software, resulting in 6-8  
81 um wide lamella with 150-200 um thickness as determined by FIB-imaging of the lamella edge.

### 82 Data collection

83 Grids were loaded into a Krios Titam TEM operated at 300 keV. The microscope was setup with a cross-grating grid  
84 on the stageby setting the beam-diameter to 900 nm, resulting in the beam being completely visible in the camera.  
85 To establish fringe-free conditions, the “Fine eucentric” procedure of serialEM was used to move a square of the  
86 cross-grating grid to the eucentric position of the microscope. The effective defocus was then set to 2 um, using the  
87 “autofocus” routine of serialEM. The objective focus of the microscope was changed until no fringes were visible.  
88 The stage was then moved in Z until images had a apparent defocus of 2 um. The differnce in stage Z-position  
89 between the eucentric and fringe-free conditions was calculate d and noted to move other areas into fringe-free

90 condition.

91 Low magnification montages were used to find lamella and lamella that were sufficiently thin and free of contamination  
92 were selected for automated data collection. The corners of the lamella were manually annotated in SerialEM  
93 and translated into Beam-Imageshift values using SerialEm calibration. A hexagonal patter of beam-imageshift  
94 positions was calculated that covered the area between he four corners in a serpentine way, with a  $\sqrt{3} * 400$  nm  
95 horizontal spacing and 800 nm vertical spacing. Exposures were then taking at each position with a 30 e/A total  
96 dose. After each exposure that defocus was estimated using the ctffind function of SerialEM and the focus for th  
97 next exposure was corrected by the difference between the estimated focus and the desired defocus of 800 um. Also  
98 after each exposure the deviation of the beam from the center of the camera was measured and corrected using the  
99 “CenterBeamFrom IMage” command of SerialEM.

100 After datacollection a 20s exposure at 2250x magnification of the lamella at 200um defocus was taken for visualiza-  
101 tion purposes.

## 102 **Data pre-processing**

103 Movies were gain-corrected and motion-corrected using a custom version of unblur. To avoid influence of the beam-  
104 edge on motion-correction only a quarter of the movie in the center of the camera was considered for calculation of  
105 the estimated motion. After movie frames were aligned and summed a mask for the illuminated area was calculated  
106 by lowpass filtering the image at ... A, thresholding the image at 10% of the maximal value and then lowpass filtering  
107 the mask at ... A. This mask was then used to replace un-illuminated area with gaussian noise, with the same mean  
108 and standard deviation as the illuminated area. The contrast-transfer function (CTF) was estimated using ctffind,  
109 searching between 0.02 and 2 um defocus.

## 110 **Template matching**

111 The search template was generated from the cryo-EM structure of the mouse large ribosomal subunit (PDB 6SWA).  
112 The ... subunit was deleted from the model and the simulate program of the cisTEM suite was used to calculate an  
113 density map from the atomic coordinates. The match\_template program was used to search for this template in  
114 the preprocessed images, using 1.5 deg angular step in out-of-plane angles and 1.0 deg in-plane. 21 defocus planes in  
115 200nm steps centered around the defocus estimates by ctffind were searches. Matches were defined as peaks above  
116 a threshold calulated according to ... (7.75 for most images).

<sup>117</sup> Data analysis

## <sup>118</sup> Results

### <sup>119</sup> 2DTM detects large ribosomal subunits in cryo-FIB lamella of mammalian cells

<sup>120</sup> To test whether we could detect individual ribosomes in mammalian cells we prepared cryo-lamella of mouse  
<sup>121</sup> neutrophil-like cells. Low-magnification images of these lamellas clearly shows cellular features consistent with a  
<sup>122</sup> neutrophil-like phenotype, mainly a segmented nucleus and a plethora of membrane-organelles, corresponding to the  
<sup>123</sup> granules and secretory vesicles of neutrophils (Fig. 1A). We then proceeded to acquire micrographs on this lamella  
<sup>124</sup> with a defocus of 0.5-1.0 um, 30 e/A<sup>2</sup>/s exposure and 1.5 Å pixelsize. We manually selected multiple locations  
<sup>125</sup> in the lamella and focused using standard low-dose techniques where focusing is performed on a sacrificial area.  
<sup>126</sup> The resulting micrographs showed no signs of crystalline ice and had Thon-rings to resolution, indicating successful  
<sup>127</sup> vitrification.

<sup>128</sup> We used an atomic model of the 60S mouse ribosomal subunit (6SWA) for 2D template matching. In a subset  
<sup>129</sup> of images the distribution of cross-correlation scores significantly exceeded the distribution expected from non-  
<sup>130</sup> significant matching. In the resulting scaled maximum-intensity maps, clear peaks with SNR thresholds up to 10  
<sup>131</sup> were apparent (Fig. S1A). By using the criterion described by for thresholding potential matches we found that in  
<sup>132</sup> images of cytosolic compartments we found evidence of 10-500 ribosomes in the imaged areas. Notably we found  
<sup>133</sup> no matches in images that were taken in the nuclear compartment. In the cytosolic areas we found a drastically  
<sup>134</sup> different number of matches, In some areas we found only ~ 50 matches per image area, while in another area we  
<sup>135</sup> found more than 500 matches . This ten-fold difference in local ribosome concentration , but we realized that current  
<sup>136</sup> data acquistion protocols are limited in that only a small area of the lamella is actually imaged and, due to the  
<sup>137</sup> manual selection of acquisition positions based on the overview image, might be biased towards cellular region that  
<sup>138</sup> appear appealing to the experimentalist. We therefore set out to collect cryo-EM data for 2DTM from mammalian  
<sup>139</sup> lamella in a high-throughput unbiased fashion.

### <sup>140</sup> DECO-LACE for 2D imaging of whole lamella

<sup>141</sup> In order to obtain high-resolution data for complete lamella we used a new approach for data collection. This  
<sup>142</sup> approach uses three key strategies: (1) ensures that every electron that exposes the sample is collected on the  
<sup>143</sup> camera (2) uses beam-image shift to precisely and quickly raster the surface of the lamella and (3) uses a focusing  
<sup>144</sup> strategy that does not rely on a sacrificial area.

<sup>145</sup> To ensure that every electron exposing the sample was captured by the detector, we focused the electron beam so  
<sup>146</sup> that the entire beam was placed on the detector. During canonical low-dose imaging the microscope is configured  
<sup>147</sup> so that the focal plan is identical to the eucentric plane of the specimen stage. This leaves the C2 aperture out

148 of focus, resulting in ripples at the edge of the beam (Figure 2B). While these ripples are low-resolution features  
149 that might not interfere with 2D template matching, which is designed to be robust to low-resolution noise, we also  
150 tested collecting data under a condition where the C2 aperture is in focus (Figure 2C).

151 We then centered a lamella under the electron beam and used beam-image shift of the microscope to systematically  
152 raster the whole surface of the lamella in a hexagonal pattern. Instead of focusing in a sacrificial area, we determined  
153 the defocus after every exposure using a routine implemented in SerialEM modeled after CTFFind. The focus was  
154 then adjusted based on the difference between desired and measured defocus. Since we used a serpentine pattern  
155 for data collection every exposure is close to the previous exposure making drastic changes in the defocus unlikely.  
156 Furthermore we started our acquisition pattern on the platinum deposition edge, so the initial exposure where the  
157 defocus was not yet adjusted did not contain any biologically relevant information.

158 We used this strategy to collect data on 8 lamella, 4 using the eucentric focus condition and 4 using the fringe-free  
159 condition. We were able to highly consistently collect data with a defocus of 8 um (Figure 2D), both in the eucentric  
160 focus and fringe-free focus condition. Together with the nominal defocus of the microscope this data results in a  
161 topological map of the lamella. To ensure that data was collected consistently, we mapped defocus values as a  
162 function of the applied Beam-image shift (Figure 2E). This demonstrated that the defocus was consistent over the  
163 lamella, with outliers only at isolated images and in images containing contamination. We also plotted the measure  
164 objective astigmatism of the lamella and found that it varies with the applied Beam-image shift, become more  
165 astigmatic mostly due to beam-image shift in the X direction. While approaches exist to correct this during the  
166 data-collection, we opted to not use these mechanism for these early experiments and instead rely on computational  
167 correction of these aberrations in order to characterize them.

## 168 **2D-Template matching of cryo-EMILIA data reveals ribosome distribution**

169 We developed customized preprocessing protocol to images obtained by cryo-EMILIA to enable their use for 2D-  
170 template matching. First we restricted calculation of cross-correlation coefficients between individual movie frames  
171 to the central portion of the image to prevent artifacts from the beam edges on estimation of motion [Figure S].  
172 Then we calculated a mask that defined the illuminated area of the micrographs and used it to fill non-illuminated  
173 areas with gaussian noise, matching mean and standard deviation to the illuminated signal (Figure S). The so  
174 processed images were suitable for 2D-template matching and we were able to obtain matches with the same  
175 model used for the data in Figure 1.

176 We then matched all individual tiles for ribosomes and assembled a montaged view as described above (Figure  
177 ?? A+G). The resulting montages reveal ribosome distribution throughout the FIB-milled slice of individual cells.  
178 Organelles like the nucleus and mitochondria only show sporadic matches with low SNRs, consistent with the  
179 estimated false-positive rate on one per tile. During the assembly we calculated the Z positions from the individual

180 estimated defocus levels and the focus plane of the microscope during acquisition of each tile. When viewed from  
181 the side the ribosome position therefore show the slope of the lamella in the microscope frame. Furthermore, the  
182 positions are also consistent with an uneven thickness of the lamella, thinner at the leading edge, even though,  
183 lamella were generated using the “overtilt” approach [cite].

184 **Quantitative analysis of translation activity**

185 **Discussion**

- 186 • Elizabeth Wright and Grant Jensen Montage tomography papers
- 187 • Waffle method for higher throughput, automation of fib-milling
- 188 • Throughput and bottlenecks
- 189 • Visual proteomics
- 190 • Granules containing ribosomes?
- 191 • Threshold implications (no matches on most images)
- 192 • K3 not linear

193 **Figures**

194 **References**

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*Nature Methods* (2005-11-18) <https://doi.org/bbpg4n> DOI: 10.1038/nmeth817 · PMID: 16299476
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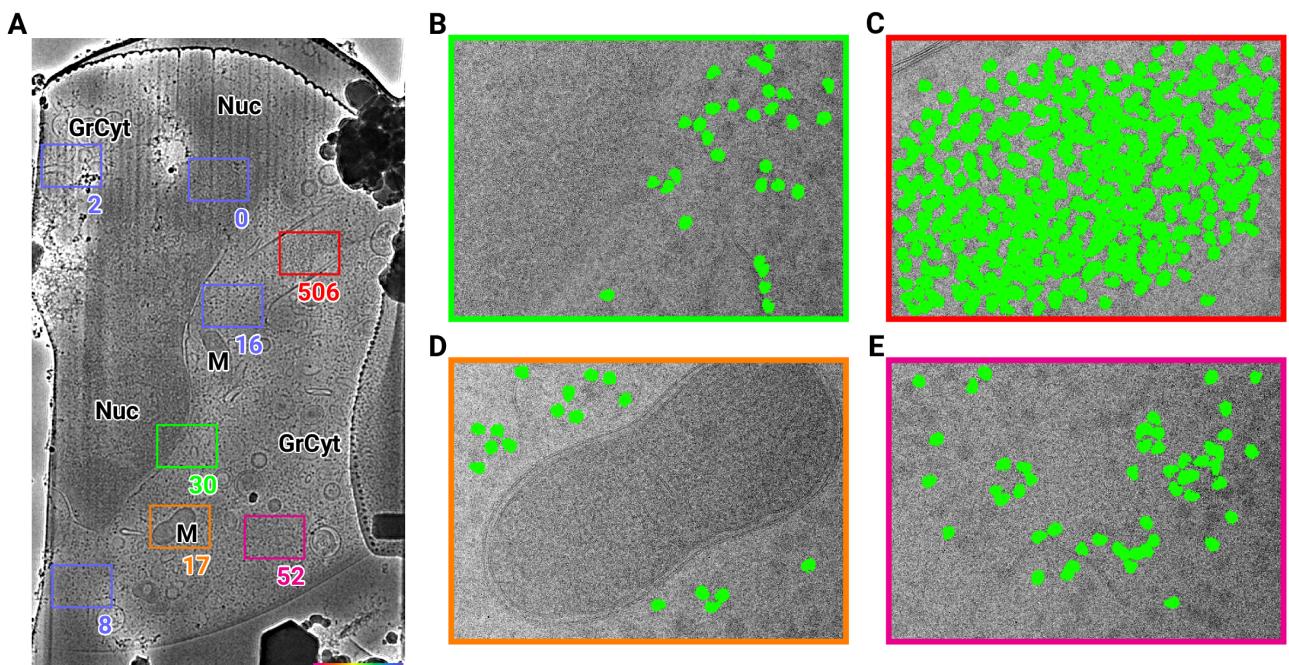


Figure 1: 2D template matching of the large subunit of the ribosome in fib-milled neutrophil-like cells (A) Overview image of the lamella. Major cellular regions are labeled, as Nucleus (Nuc), Mitochondria (M), and granular cytoplasm (GrCyt). FOVs where high-magnification images for template matching were acquired are indicated as boxes with the number of matches indicated on the bottom right. FOVs displayed in Panels B-E are color-coded. (B-E) FOVs with projection of ribosome LSU matches shown in green. (B) Perinuclear region, the only matches are in the cytoplasmic half. (C) Cytoplasmic region with high density of ribosomes (D) Mitochondrion, as expected there are only matches in the cytoplasmic region (E) Cytoplasm, with low density of ribosomes.

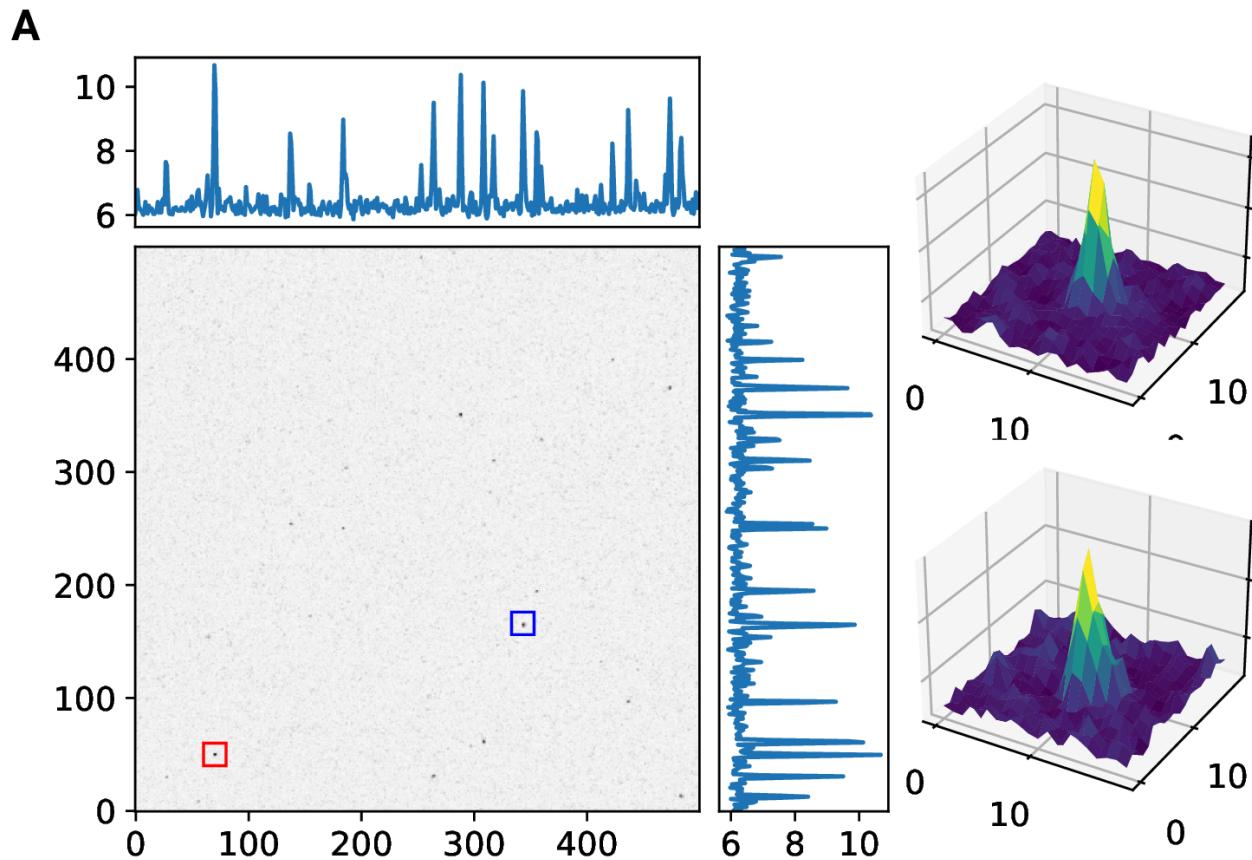


Figure S1: 2D template matching of the large subunit of the ribosome in fib-milled neutrophil-like cells (A) Maximum intensity projection cross-correlation map of micrograph shown in Figure 1 (B+C) 3D plot of MIP regions indicated by color boxes in Panel A

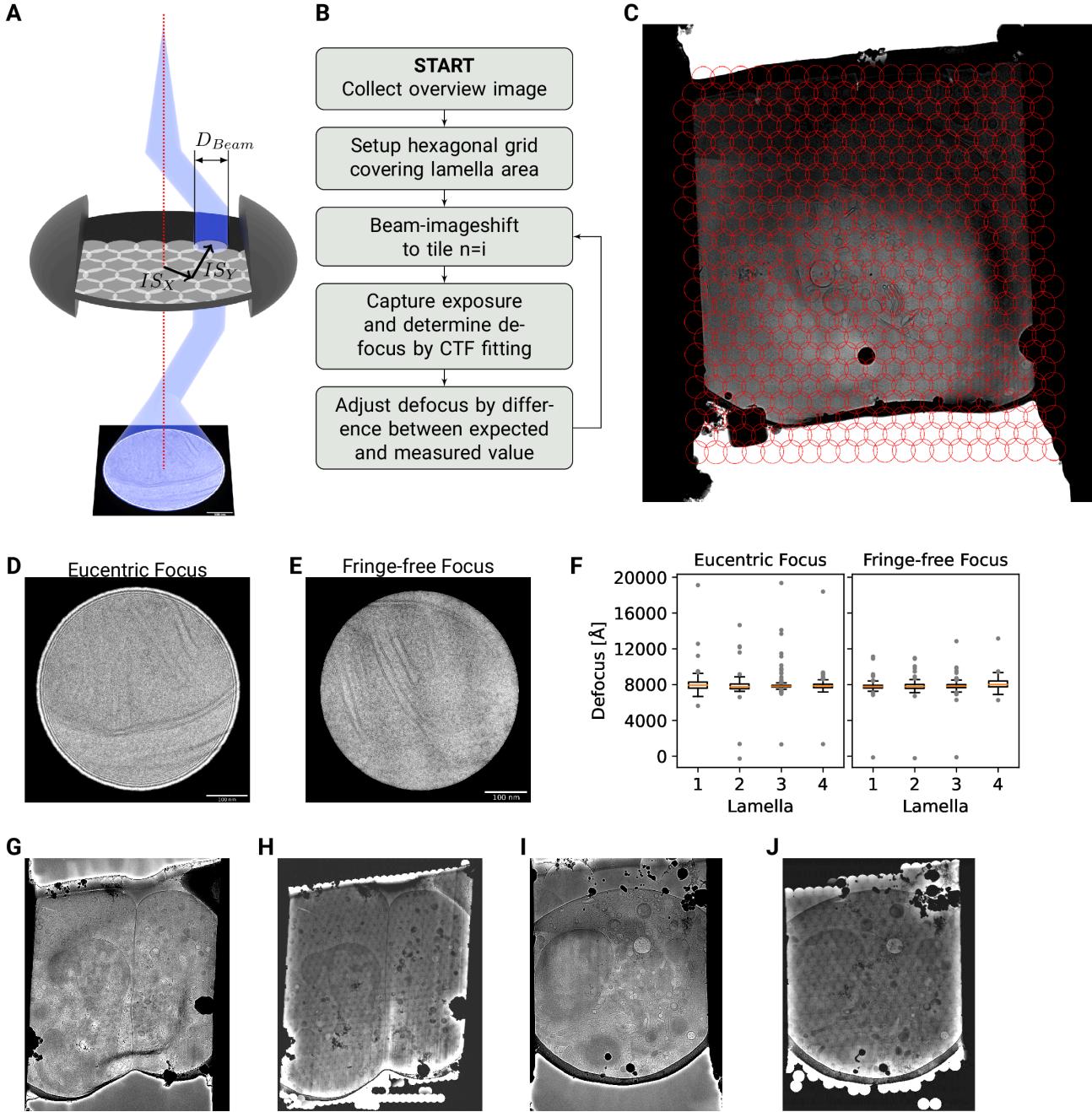


Figure 2: DeCo-LACE approach (A) Graphic demonstrating the data-collection strategy for DeCo-LACE. The electron beam is condensed to a diameter  $D_{Beam}$  that allows captured of the whole illuminated area on the camera. Beam-image shift along X and Y ( $BIS_X, BIS_Y$ ) is used to raster the whole lamella (B) Diagram of the collection algorithm (C) Example overview image of a lamella with the designated acquisition positions and the used beam diameter indicated with red circles (D+E) Representative micrographs taken with a condensed beam at eucentric focus (D) or fringe-free focus (E) (F) Boxplot of defocus measured by ctffind of micrographs taken by the DeCo-Lace approach on 4 lamella images at eucentric focus and 4 lamella imaged with fringe-free focus. (F+G) Lamella overview images of lamella imaged at eucentric focus (F) Overview image taken at low magnification (40 Å pixel size) (G) Overview created by montaging high magnification images taken with the DeCo-Lace approach (1.5 Å pixelsize) (H+I) Lamella overview images of lamella imaged at fringe-free focus (H) Overview image taken at low magnification (40 Å pixel size) (I) Overview created by montaging high magnification images taken with the DeCo-Lace approach (1.5 Å pixelsize)

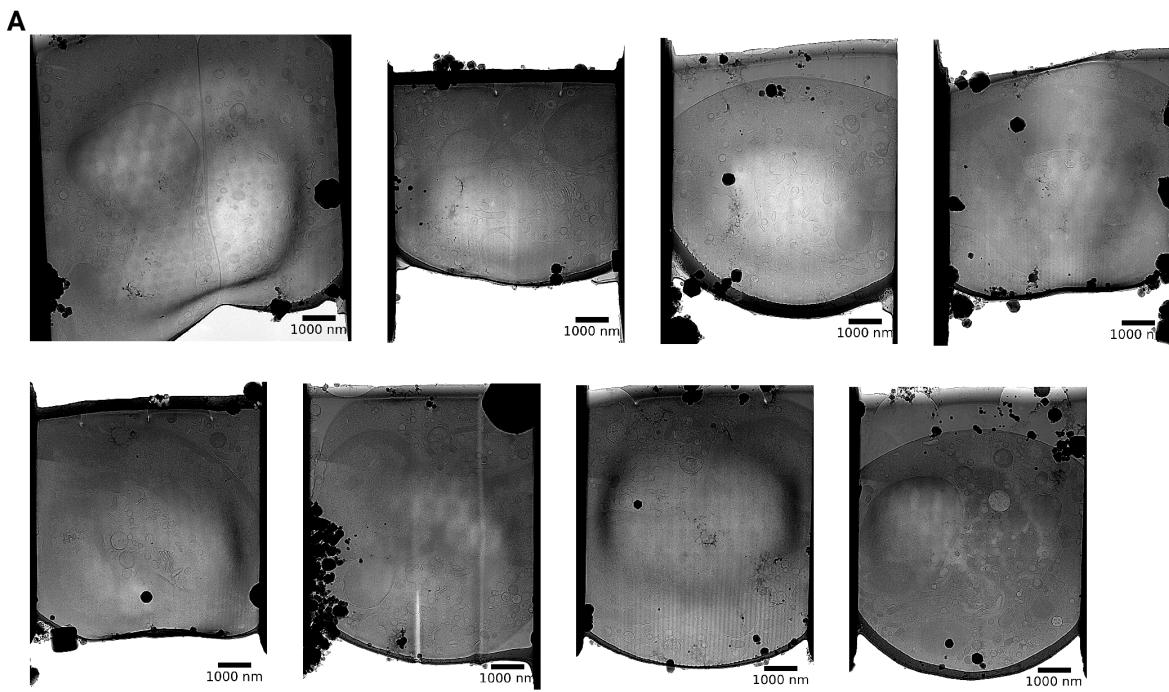


Figure S2: Overview images of lamellae imaged using the DeCo-LACE approach taken at low-magnification

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 AW McDowall, J-J Chang, R Freeman, J Lepault, CA Walter, J Dubochet  
*Journal of Microscopy* (1983-07) <https://doi.org/bdnzmv> DOI: 10.1111/j.1365-2818.1983.tb04225.x ·  
 PMID: 6350598
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*eLife* (2017-05-03) <https://doi.org/gnq4q4> DOI: 10.7554/elife.25648 · PMID: 28467302 · PMCID:  
 PMC5453696

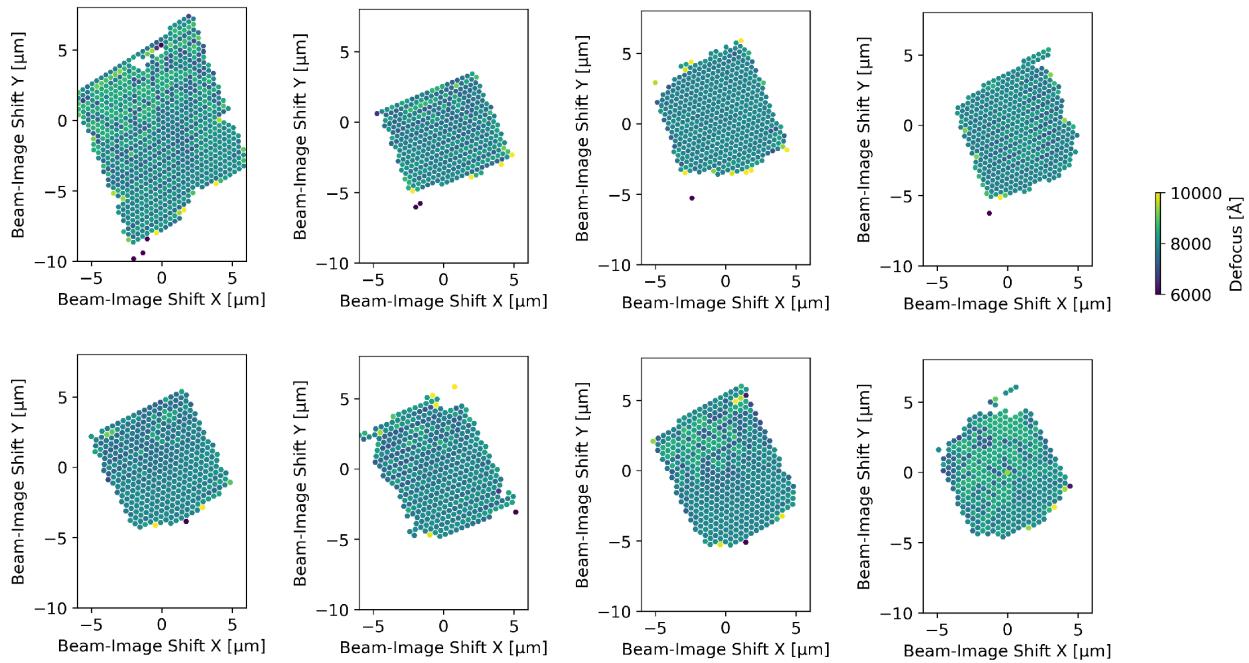
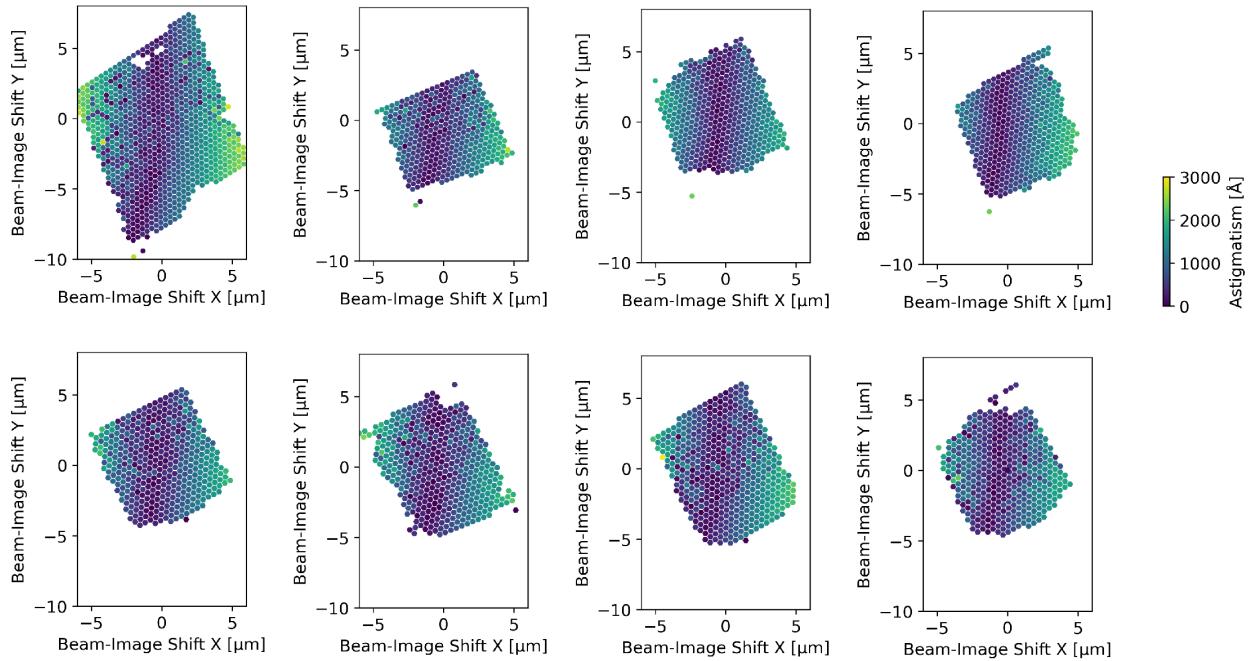
**A****B**

Figure S3: Defocus estimation of individual tiles of DeCo-Lace montages (A) Defocus values of individual micrographs taken using the DeCo-Lace approach plotted as a function of the Beam-Image-Shift values. (B) Defocus astigmatism of individual micrographs taken using the DeCo-Lace approach plotted as a function of the Beam-Image-Shift values.

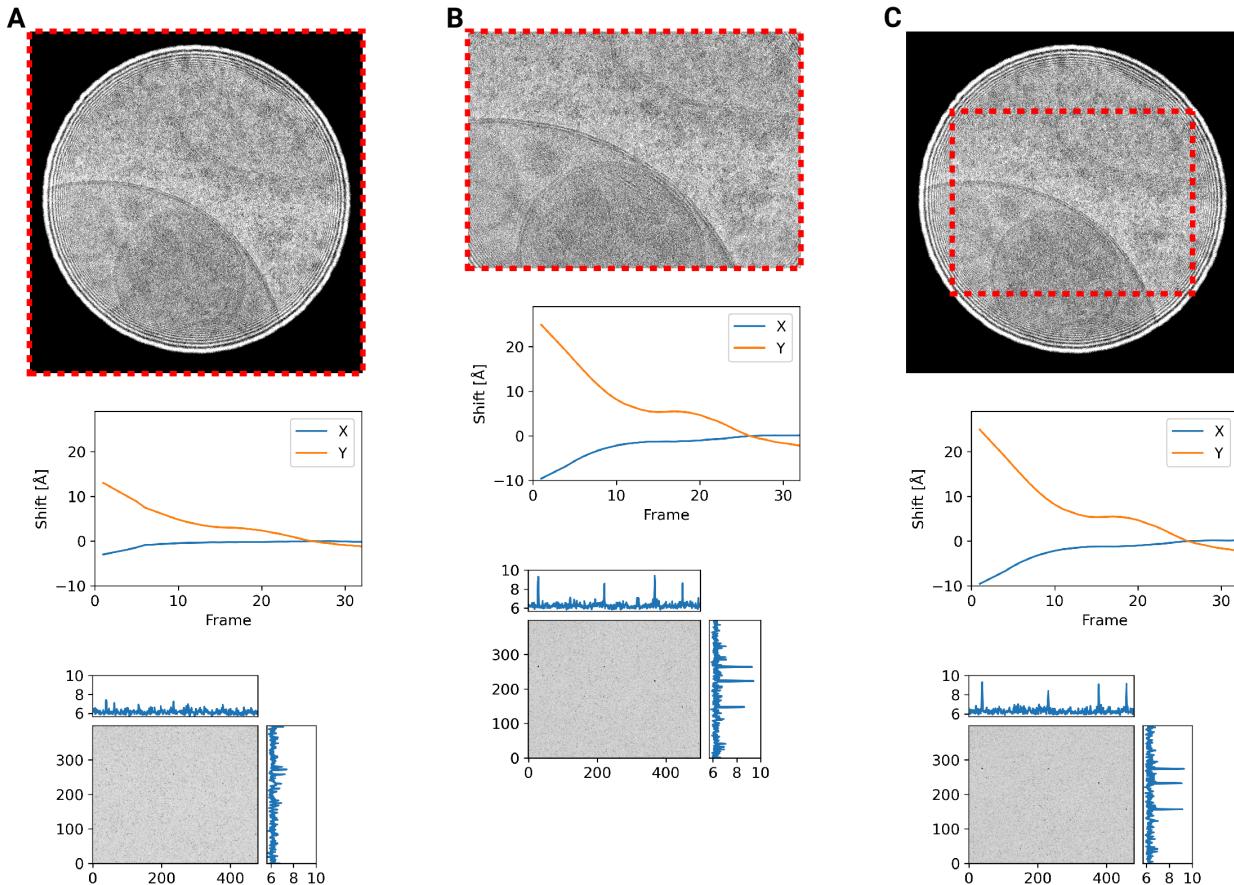


Figure S4: Motion correction of movies with condensed beams. At the top of each panel is an average of the movie that was motion-corrected with a red dashed box indicating the region that was used to estimate shifts. Below is a graph indicating the estimated shifts of the individual frames of the movie. Below this is the MIP of 2DTM using the large subunit of the mouse ribosome. (A) Motion correction of the whole movie (B) Motion correction of a cropped region of the movie that eliminates the beam edges (C) Motion correction of the whole movie, using only the cropped region to estimate the shifts

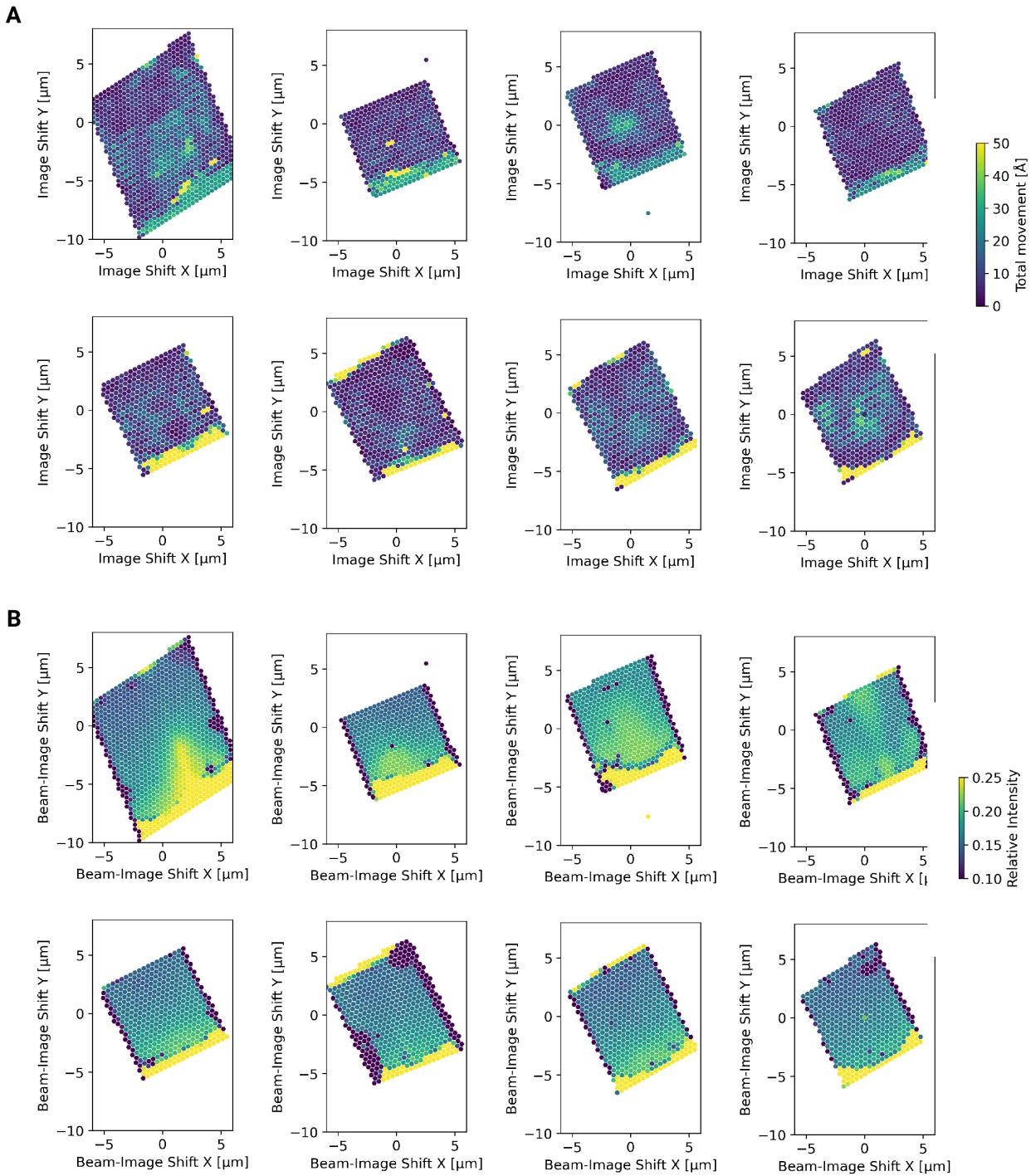


Figure S5: Motion correction of individual tiles imaged using the DeCo-LACE approach (A) Total estimated motion of individual micrographs taken using the DeCo-Lace approach plotted as a function of the Beam-Image-Shift values. (B) Electron intensity of individual micrographs taken using the DeCo-Lace approach plotted as a function of the Beam-Image-Shift values.

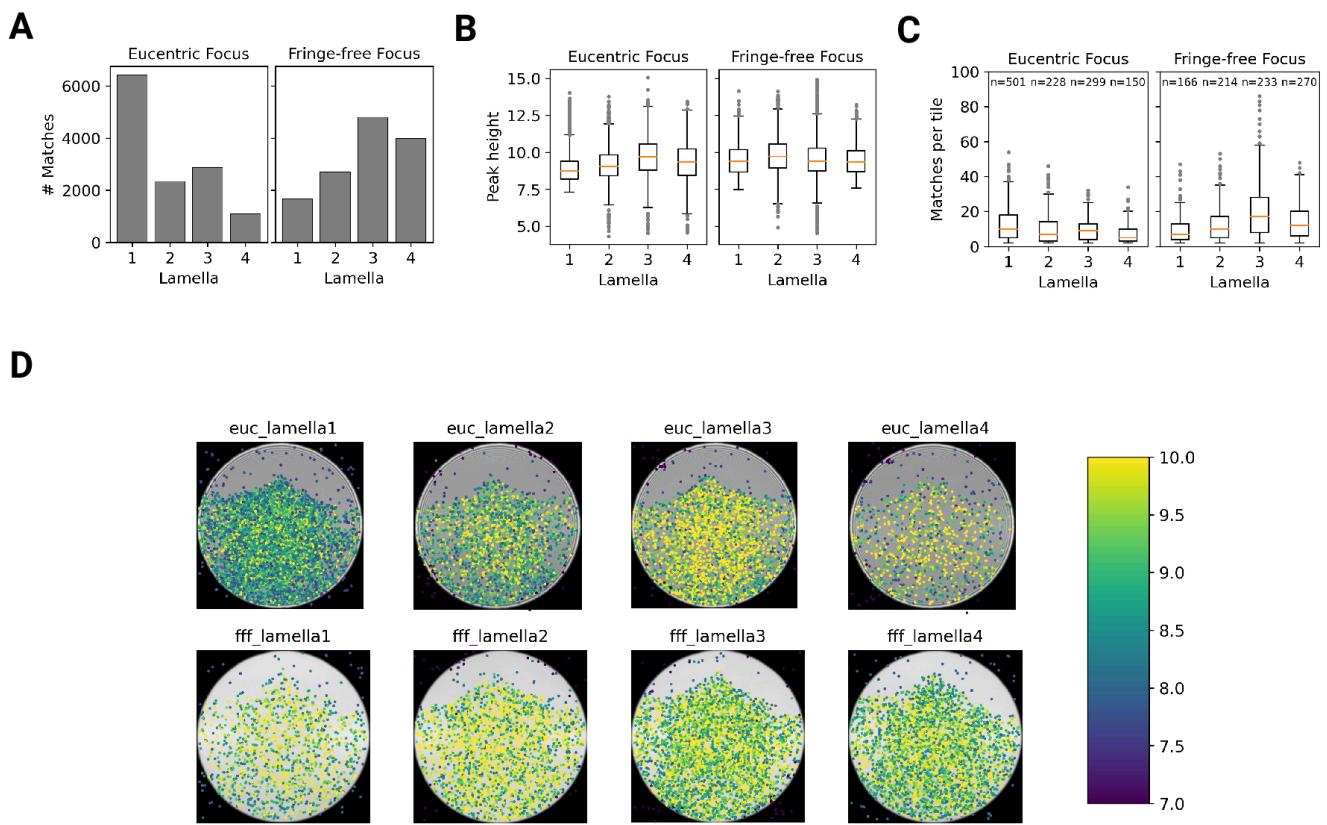


Figure 3: This is an example-figure

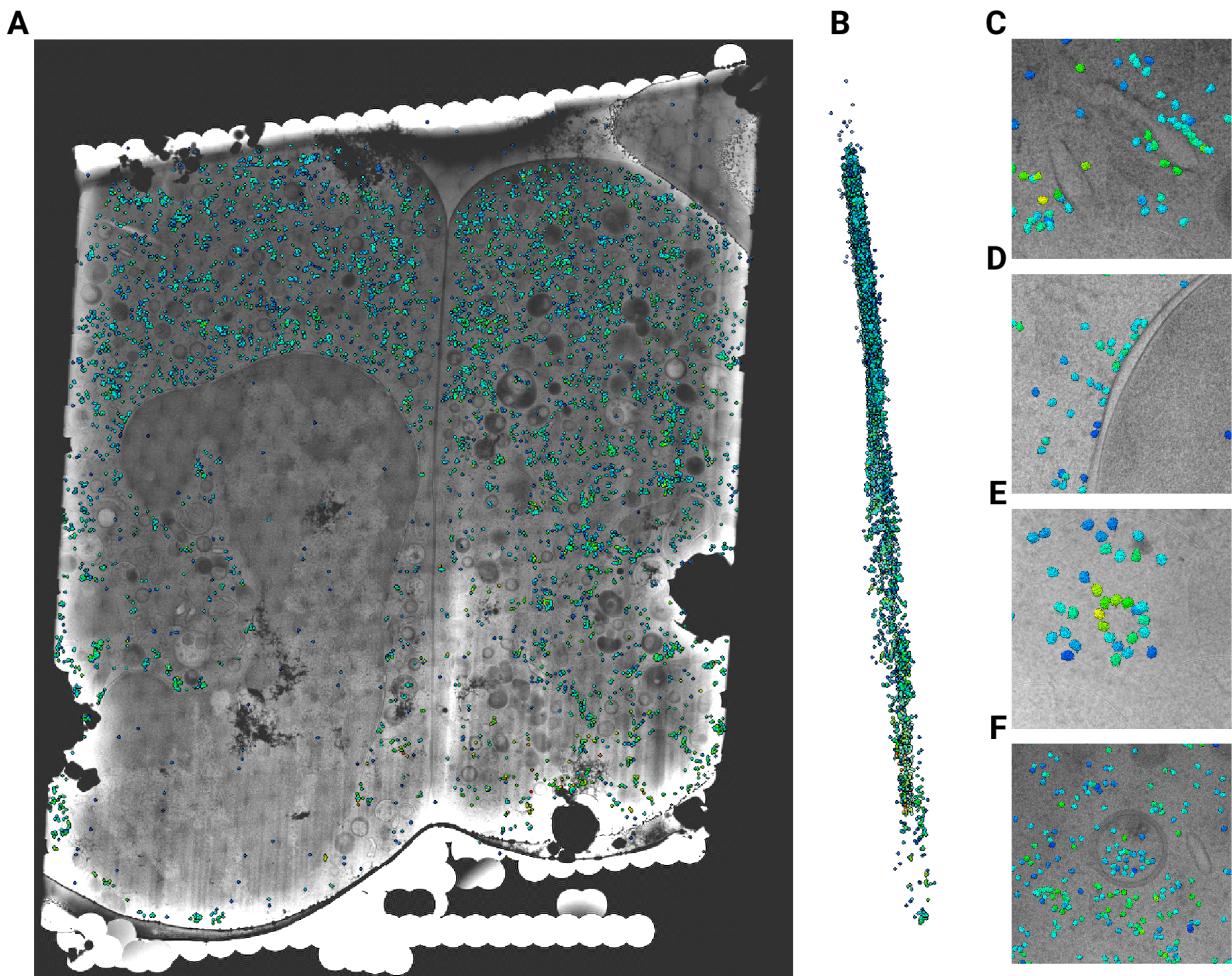


Figure 4: Template matching in lamella imaged using the DeCo-Lace approach at eucentric focus

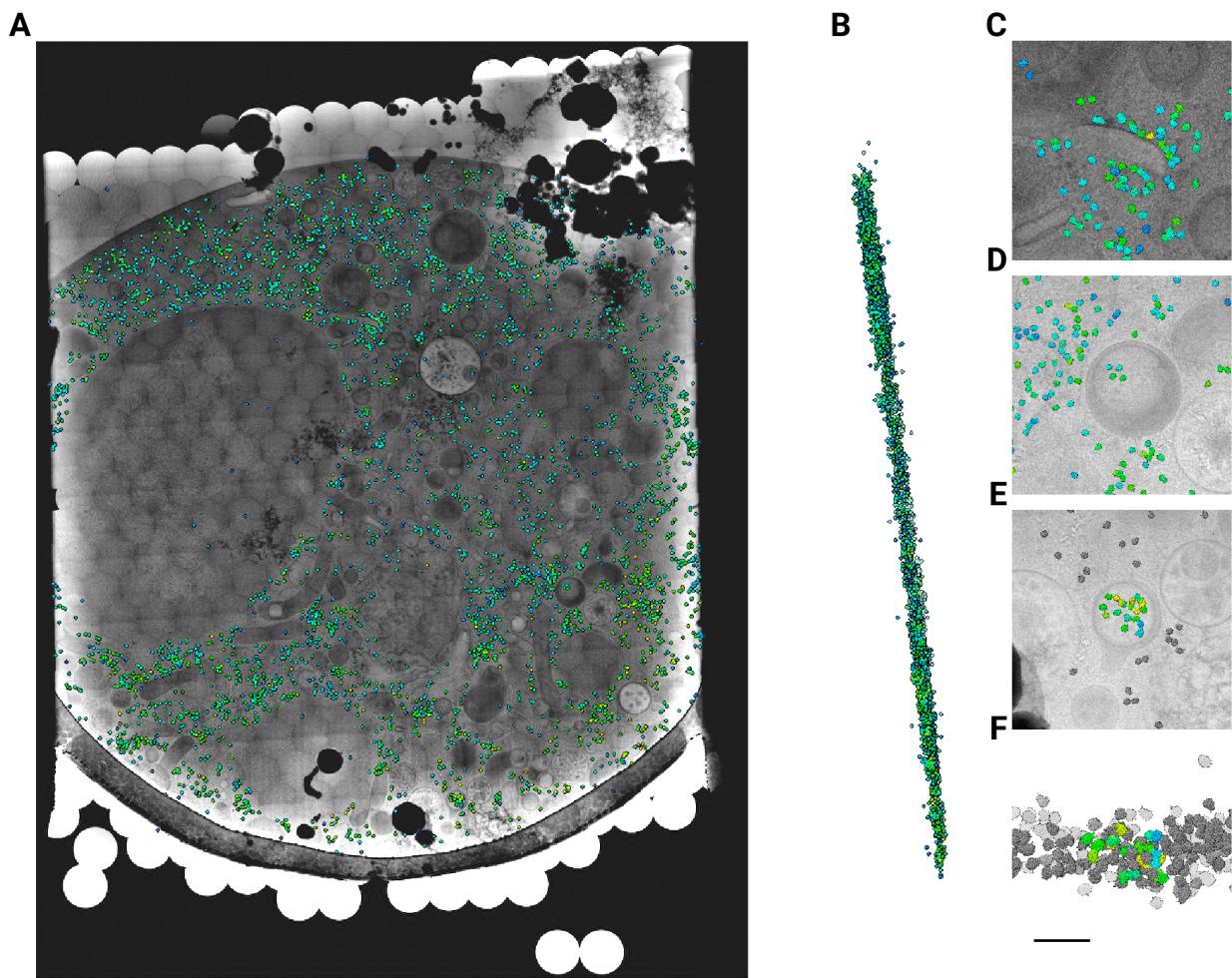


Figure 5: This is an example-figure

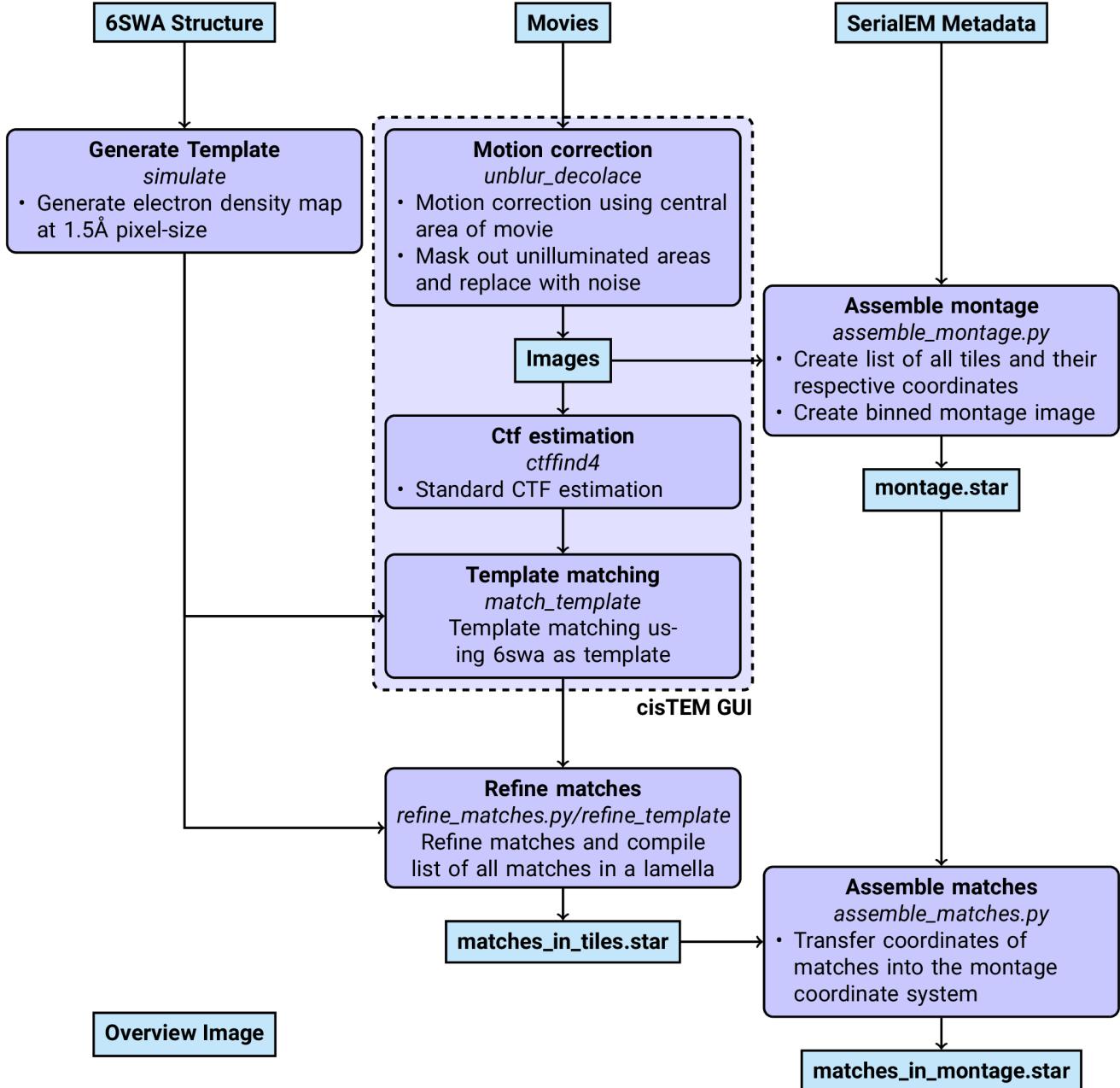


Figure 6: Workflow of DeCo-Lace processing

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PMC7360335