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Visualizing Single Molecule Identity and Sample Integrity *in situ*

Bronwyn A. Lucas[1,2*] and Nikolaus Grigorieff[1,2]

[1.] RNA Therapeutics Institute, University of Massachusetts Chan Medical School, Worcester, Massachusetts, United States.
[2.] Howard Hughes Medical Institute, Janelia Research Campus, Ashburn, Virginia, United States.
* Corresponding author: bronwyn.lucas@umassmed.edu

The combination of increasing resolution in cryo-electron microscopy (cryo-EM) and sample preparation using cryo-focused ion beam (FIB) milling has enabled visualization of macromolecules in their native cellular environment in unprecedented detail. In this paper we describe new methods to assess single molecule identity and structural integrity from 2D images of FIB-milled lamellae.

Cryo-EM has the potential to deliver high-resolution views of cells. However, the density of molecules in cryo-EM images of the cell make identification of individual molecules challenging. Cryo-electron tomography (cryo-ET) has been established as one way to address this issue because overlapping density can be separated by combining images of successive tilts to produce 3D reconstructions [1]–[3]. However, the resolution of tomograms is currently limited to ~15-20 Å [4] and retrieving high-resolution detail requires averaging of many particles, e.g., [5], [6]. Consequently, accurate identification of all but the largest and most abundant molecules in tomograms remains a major challenge. We have described an alternate approach, termed 2D template matching (2DTM) that uses existing molecular models as high-resolution templates to locate molecules in 2D images with high specificity [7]–[9].

2DTM yields SNR values that depend on the similarity between the template and the target molecules in an image. We demonstrate that, when comparing different templates, the SNR values at a single location and orientation indicate the relative similarity of the target molecule to each template. This enables single particle classification *in situ* based on the observed SNR values. By assuming Gaussian distributions of SNR ratios, we calculate the confidence of classification for each detected particle. We apply this approach to locate and distinguish distinct pre-cursors of large ribosomal subunit assembly in the yeast *Sacchromyces cerevisiae* nucleus.

In addition to defining biological populations in cells, 2DTM can also provide a read out of structural integrity. To generate sufficiently thin cellular sections for TEM imaging, a gallium ion beam is typically used to FIB-mill the sample [10]–[12]. The nature and extent of sample damage introduced during cryo-FIB-milling is not well established. We used 2DTM to assess the damage profile of a set of FIB-milled lamellae of frozen *S. cerevisiae* of a range of thicknesses. We observe a measurable decrease in 2DTM SNRs towards the edges of the lamellae to a depth of ~75 nm. *Mycoplasma pneumoniae* cells of similar thickness, which were not FIB-milled, do not display a comparable pattern. We conclude that the observed decrease in 2DTM SNRs reflects sample damage introduced by the gallium beam, about 15-fold greater than previous estimates.

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