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Multi-Color Electron Microscopy of Cellular Ultrastructure Using 4D-STEM

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Transmission electron microscopy (TEM) is the primary method for acquiring high-resolution images of cellular ultrastructure of biological samples. Alternatively, fluorescence light microscopy techniques are capable of labeling different cellular components with different dyes, and distinguishing them to form multicolor images; however, at a much lower resolution [1]. This serious limitation was recently addressed through the development of "multi-color EM," which uses selective lanthanide ion tagging and electron energy-loss filtered imaging [1,2] to generate data analogous to multi-color fluorescence microscopy, but at ~100× the magnification. While this technique promises to reveal novel structural information, it depends on a very small fraction of the electrons (typically << 1%) that have lost energy due to a core-loss excitation to create the elemental maps. This makes the method extremely tedious and slow to execute, while producing noisy images that sometimes can be difficult to interpret.

To improve the throughput, efficiency, and resolution of multi-color EM, we have developed a new multicolor EM technique based on four-dimensional scanning transmission electron microscopy (4D-STEM), which uses a high-speed pixelated detector to capture signals from the vast majority of the primary electrons that interact with the specimen.

In this study, we used a cellular mitomatrix sample, labeled with Cerium-DAB, Ruthenium tetroxide, and 30 nm gold nanoparticles. The specimen was prepared as previously described [2].

Energy-filtered TEM (EFTEM) was used as a baseline for validation of our the new 4D-STEM based technique. EFTEM was performed with a JEM-3200EF TEM (JEOL, Japan) operating at 300 kV, equipped with an in-column Omega filter and a LaB6 electron source. The EFTEM images of the pre- and post-edges were obtained using a slit of 30 eV width. Conventional bright-field TEM images and EFTEM elemental maps were acquired using a conventional CCD camera. Elemental maps were then generated as previously described [2].

STEM imaging was performed with a Titan Halo (Thermo Fisher, Waltham, MA) operating at 300 kV, equipped with bright-field and HAADF STEM detectors and a high-current XFEG electron source. 4D-STEM data was acquired using a camera length of 150 mm and spot size 8 on a DE-16 direct detection camera (Direct Electron), with hardware frame synchronization with the DE-FreeScan STEM scan generator (Direct Electron). Although the DE-FreeScan is capable of acquiring STEM data using arbitrary or subsampled scan patterns, it was operated in a conventional full raster scanning mode. The DE-16 was operated at 342 frames per second (fps), no hardware binning, and a readout area of 1024 × 1024 pixels.

4D-STEM data was processed using newly-developed DE-4DExplorer GPU-accelerated software (Direct Electron). Briefly, the software loads the 4D-STEM data stack from disk and then performs standard flat-field (dark and gain) correction. Subsequently, the software calculates the sparsity map for each frame,

which is a binary mask corresponding to regions of the frame where the number of primary electrons per pixel is low enough to be processed using electron counting. Based on this sparsity map, the software performs electron counting on sparse regions and scales the intensity of non-sparse regions based on the average pixel intensity per primary electron. Thus, the pixel intensity in the final processed frame approximately corresponds to the actual number of primary electrons incident on each pixel in each frame. Using this data, 4D-STEM analysis was completed by calculating the integrated intensity (total number of primary electrons) within every possible annular disk from the center of diffraction to the edge of the detector. However, we observed that the bright-field disk was not precisely centered on our pixelated detector, moved slightly at different STEM probe positions, and had a slight ellipticity. To correct these diffraction distortions, we averaged the bright-field disk over 16 × 16 STEM pixel areas on the specimen, and then fit an ellipse to the edge of the average bright-field disk. Then, for each frame, the bright-field disk was translationally aligned and its ellipticity was corrected prior to further processing to generate reconstructions of the specimen.

After correcting for distortions in the diffraction patterns, we were able to distinguish the cerium labels and gold nanoparticles based on the radial scattering profile. Results were similar to EFTEM results, but the 4D-STEM results showed higher contrast (Fig. 1). The same data was also used to simultaneously generate bright-field and dark-field images of the specimen at significantly higher resolution than is possible through fluorescence light microscopy. Because all of these reconstructions were generated from a single STEM acquisition, the 4D-STEM-based multi-color EM technique has significantly better throughput than is possible with the previous EFTEM-based multi-color EM technique.

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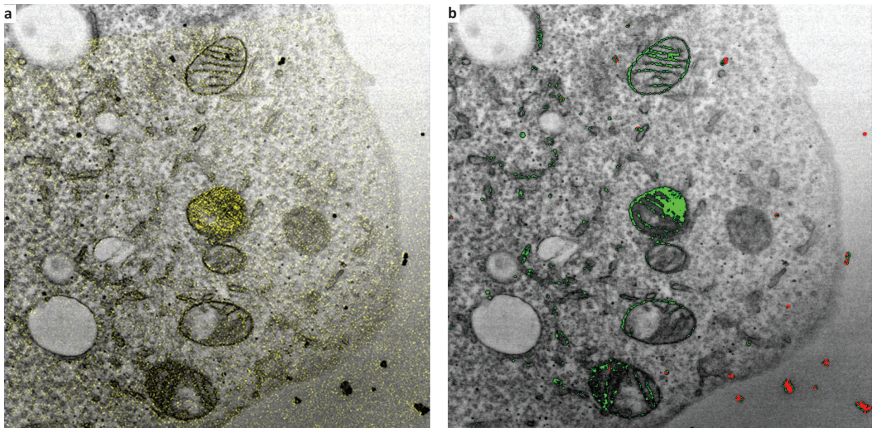

Figure 1. Comparison of EFTEM and single-shot 4D-STEM methods for multi-color EM. (A) An elemental map of cerium (yellow) using EFTEM. (B) The bright-field reconstruction (grayscale) and the cerium locations (green) and gold locations (red) based on our new 4D-STEM method.

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