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Serial Thick Section Gas Cluster Ion Beam Scanning Electron Microscopy

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Focused Ion Beam Scanning Electron Microscopy (FIB-SEM) is used to volume image heavy metal-stained, plastic-embedded biological samples with resolutions below 10 x 10 x 10nm, an ability that is especially important in connectomics [1]. FIB-SEM samples are typically restricted to be <50µm in the direction of the FIB beam because glancing angle milling results in artifacts over longer distances [1]. Removal rate is also restricted due to a current/spot size tradeoff. These limitations are especially problematic when one contemplates combining FIB with the increased speed offered by multibeam SEMs like the 91 beam Zeiss MultiSEM [2]. The MultiSEM's *minimum* field of view is ~180µm, and its imaging rate is approximately two orders of magnitude faster than FIB's milling rate. These considerations appear to preclude the integration of traditional FIB milling with MultiSEM imaging.

To overcome these limitations we chose to develop a broad ion beam milling approach using Gas Cluster Ion Beams (GCIB). GCIB delivers low-energy atoms to a surface and therefor does not require the use of a glancing angle. GCIB has been used for semiconductor polishing and for profiling in mass spectroscopy [3]. We attached a GCIB-10s gun from Ionoptika to a Zeiss Ultra SEM. Using a 10kV beam of Ar2000 (clusters of 2000 argon atoms), we verified that smooth, sub-10nm removal was possible from the surface of 100nm thick tissue sections. In order to obtain surfaces sufficiently smooth to produce quality secondary electron (SE) images (using 1.2kV landing energy and InLens detection meant to mimic MultiSEM conditions) we found it was beneficial to angle the sample so that the GCIB beam made a ~30^{o} angle to its surface, and to rotate the sample during milling. Using this technique we were able to volume image 100nm thick sections of tissue embedded in Durcupan and Spurr's resins but noticed that Epon samples produced surfaces too rough for SE imaging.

Sections thicker than 100nm were insufficiently conductive for quality SE imaging. To overcome this we 'precooked' thicker sections with a high energy electron beam to increase their bulk conductivity [4]. We have verified that sections as thick as 10µm can be GCIB-SEM volume imaged using SE as long as they are precooked with a 30kV electron beam.

For connectomics, GCIB-SEM could be used as follows: A tissue sample would first be cut into thick sections (e.g. 1µm thick using traditional microtomy, >20 µm using a diamond hot knife [5]) which would be laid out on the surface of a silicon wafer and precooked with a high voltage electron flood gun to increase bulk conductivity. This wafer would then be cycled between SEM imaging and GCIB milling acquiring one image of each section's milled surface for every ~10nm of surface removal.

As a demonstration we collected three sequential 1µm thick sections of *Drosophila* brain tissue on silicon. We precooked regions of each section (10,000µm^2 area, 10nA, 4hrs at 10kV, 2hrs at 6kV) to enhance their conductivity. Then we performed ~250 mill/image cycles in order to completely image through all three sections (GCIB: 20nA of Ar2000 at 10kV spread over a 10mm^2 area, 26^{o} angle between beam and plane of surface, 360^{o} rotation, 900s (~4nm removal) per cycle; SEM: 1.2kV, 2nA electron beam, InLens SE detection, 6nm pixels, 2MHz). Figure 1A shows one SEM image of this

dataset which was acquired after many previous rounds of GCIB milling. Neuronal processes and synaptic features are clearly visible. Figure 1B shows a cross section through each of the three 1µm thick sections. The unevenness of the bottoms of each section is a result of GCIB milling rate variance (~10%) between different regions of the same section. This unevenness was computationally flattened prior to stitching the three 1µm thick volumes together into a single volume suitable for connectomic tracing as shown in Figure 2. Since high current GCIB sources are available that can easily keep up with MultiSEM imaging rates we believe that this technique of Serial Thick Section GCIB-SEM may offer a promising approach to large scale connectomics.

References:

[1] CS Xu *et al*, Elife **6** (2017), p. e25916.
[2] T Keman, T Garbowski and D Zeidler, Proc. of SPIE **9658** (2015), p. 965807-1.
[3] I Yamada *et al*, Mater. Sci. Eng. R Rep **34** (2001), p. 231.
[4] L Calcagno, G Compagnini and G Foti, Nucl. Instr. Meth. Phys. Res. B **65** (1992), p. 413.
[5] KJ Hayworth *et al*, Nat. Methods **12** (2015), p. 319.

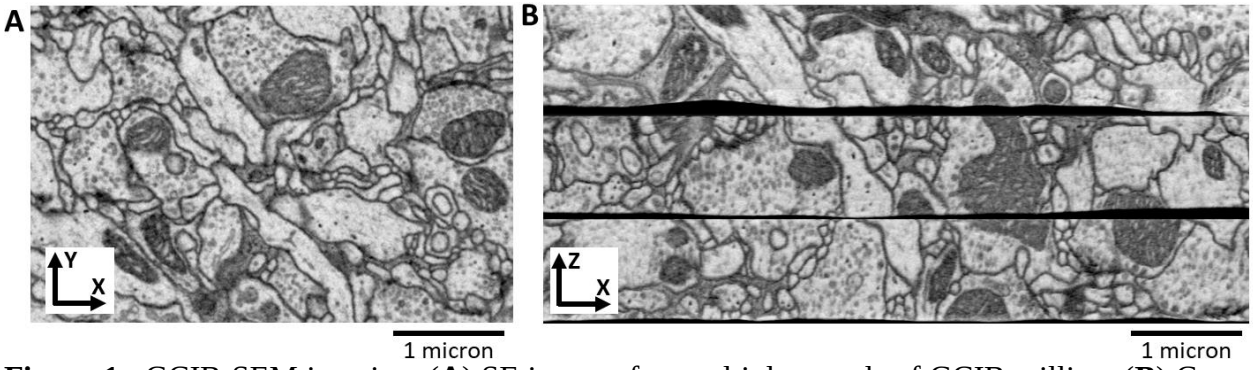

Figure 1. GCIB-SEM imaging. (**A**) SE image after multiple rounds of GCIB milling. (**B**) Cross section through dataset of three consecutive 1µm thick sections prior to computational flattening.

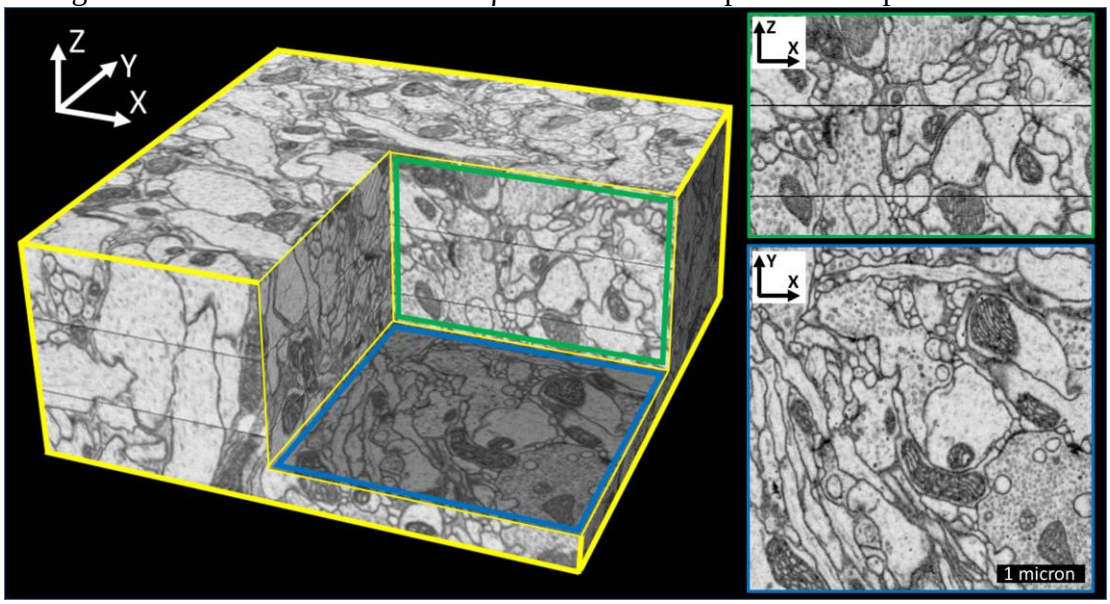

Figure 2. Final GCIB-SEM dataset after computationally flattening and volume-stitching the three consecutive 1µm thick sections together.

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