

**Supplementary information**

---

# Synaptic-resolution connectomics: towards large brains and connectomic screening

---

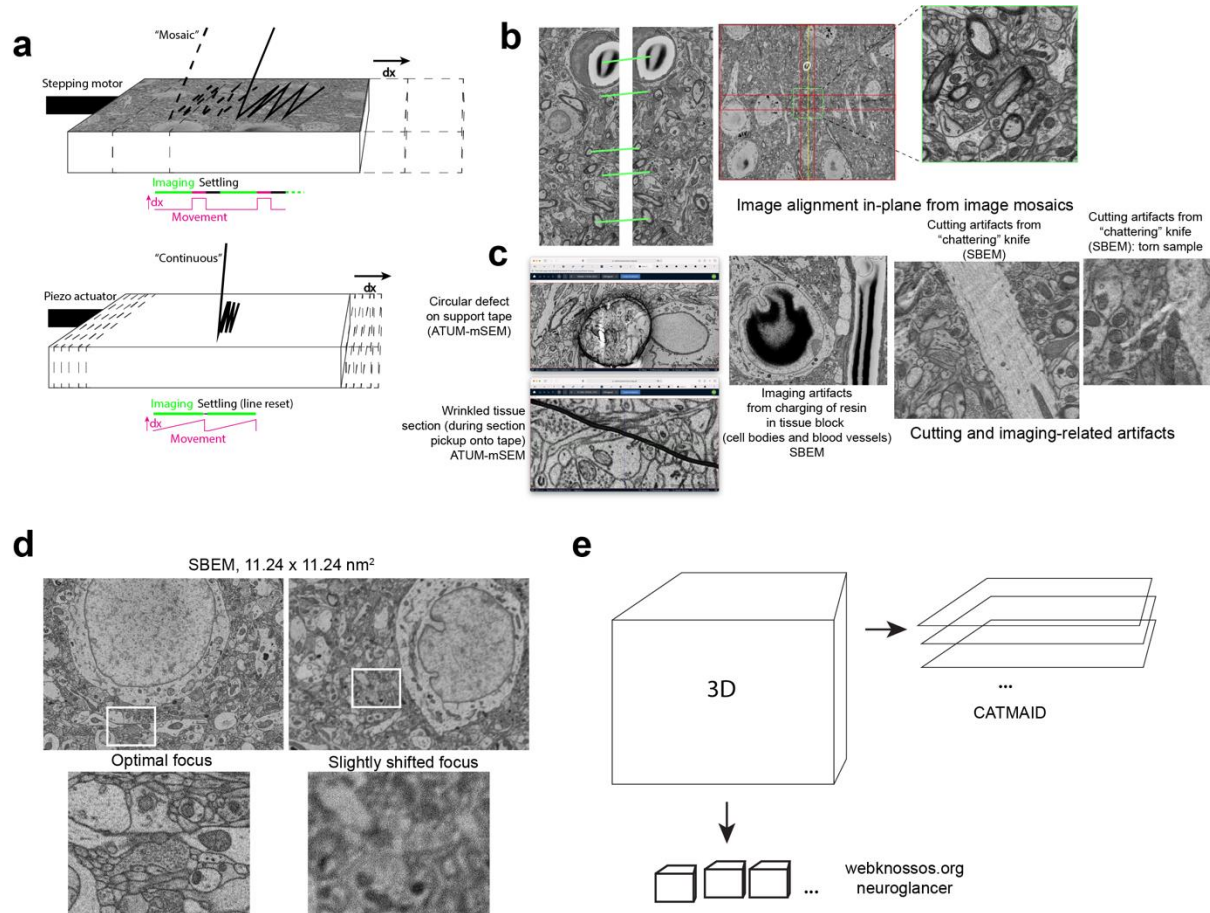
In the format provided by  
the authors and unedited

# Supplementary Material

for

## Synaptic-resolution connectomics: towards large brains and connectomic screening

### Supplementary Box. 1: Typical imaging artifacts in 3D EM



**3D EM imaging methods, resolution requirements, image alignment and typical imaging artifacts.** (a) SBEM, FIB-SEM and multiSEM currently operate in mosaic mode: image acquisition is interleaved with stage movement to reach the next sample location. However, this approach introduces substantial pauses for stage movement and, importantly, settling of the rather heavy stage. As a result, effective imaging speed is often 5-10 fold lower than the nominal scanning speed that the EM can provide. Continuous imaging approaches (bottom), in which the sample is continuously moved along one dimension under the scanning electron beam, abolish these inefficiencies, introduce novel challenges of repeated and precise positioning<sup>1</sup>. For the most ambitious connectomic projects (connectomic frontier, Fig. 5a), it is expected that the additional speedup from continuous imaging is required. (b) Image alignment and registration by overlapping image regions (typically 0.5-1  $\mu\text{m}$  per dimension) in-plane, and by cross-plane matching. Image alignment becomes a substantial computational burden for petascale datasets, non-rigid alignments are required, especially for dealing with artifacts (c). (c) Typical imaging artifacts induced by cutting or tape defects, or by reduced conductivity of the tissue sample leading to charge accumulation in cell nuclei and resin-filled blood vessels. (d) Focus stability is critical in 3D EM, especially for blockface methods in which re-imaging of problematic regions is not possible due to the destruction during the image-cutting/ablation cycle. Already slight defocus yields non-reconstructable data, even when acquired at nominally sufficient voxel size (compare left and right; note large processes are similarly well identifiable, but thin axons are not). (e) Conversion of 3D image volume into data structures available for efficient data annotation and browsing (split into 3D minimal cubes, webKnossos.org and neuroglancer, or 2D planes, catmaid). Images courtesy of V. Ganghadharan, M. Sievers and A. Khalifa.

## Mosaic vs. continuous EM imaging

When imaging millimeter-scale sections of brain tissue, the field of view in the electron microscope has to be moved under the electron beam. Usually, this is implemented as a stepwise mosaic: field of views sized typically 40-60  $\mu\text{m}$  on a side are sequentially imaged, with some overlap between the images to allow their proper alignment into one continuous image plane. However, this process of interleaved imaging and stage movement creates substantial overhead time during which no imaging can take place. Most problematic is the settling time of the (often heavy) stage after the high accelerations and decelerations involved in the movement to the next mosaic position. Practically, because of these and other overheads, nominal imaging speed (in voxel per second that can be acquired with enough electrons detected from a given voxel) is often reduced 5-to-10 fold. For large-scale imaging projects, this can drastically increase the resource needs. For example, the nominal imaging speed of a 61-beam multiSEM operated using 50 ns dwell time per voxel is 1.2 GVx/s, but realistic acquisition is around 200 MVx/s (similarly, the effective imaging rate in single-beam scanning EM is often only below 2 MVx/s including overheads<sup>2,3</sup> even when operated at 5-10 MHz nominal scan rate). One approach to solving this problem is to avoid the mosaicking step and instead equip the EM for continuous imaging<sup>1</sup>: here, using piezo actuators, the sample stage is continuously pulled under the scanning EM beam, such that in one dimension in-plane, no mosaic movements are needed. The remaining shifts between imaging “columns” is then a much smaller fraction of the imaging time. Alternative approaches could involve the detection of stage movement during the settling time, allowing to resume imaging earlier after stage movement, and correcting for the induced movement artifacts *post-hoc*.

## Supplementary Methods

### Estimation of connectomic and genomic configuration space size

For the graph in Fig. 1b, the following approximate calculations were used (computed in MATLAB R2023a, MathWorks). All numbers  $y$  are reported as  $y = \log_{10}(\log_{10}(Z))$ , with  $Z$  the size of the (genomic, connectomic) configuration space. The size of the genomic configuration space  $Z_g$  was computed as  $Z_g = 4^{(n_{bp})}$  with  $n_{bp}$  the number of base pairs in the species genome, thus  $y_g = \log_{10}(n_{bp} * \log_{10}(4))$ . The size of the connectomic configuration space was computed using Stirling's formula for obtaining the logarithm of the number of possibilities to draw  $k$  synaptic connections from  $n^2 - n$  possible entries in the directed connectome (excluding self-connections), with  $n$  the number of neurons and  $k$  the number of synaptically connected neuron pairs in a brain/circuit.  $k$  was estimated as the total number of synapses in a brain/circuit divided by the approximate average number of synapses used per neuron-to-neuron connection (see Fig. 1e). The size of the connectomic configuration space for cell-type based connectivity  $Z_{ct}$  was computed as

the number of binary cell type connectomes (excluding cell-type self connections):  $Z_{ct} = 2^{(n_{ct}^2 - n_{ct})}$  with  $n_{ct}$  the number of cell types in a given brain/circuit.

## Display of 3D EM datasets

The EM data in Fig. 1d is from the dataset H5\_L23\_multiSEM published in ref<sup>4</sup>. Briefly, the data was acquired from a sample of human STG cortex using a 61-beam multiSEM (ZEISS, Oberkochen) at 50ns dwell time, 4x4x35 nm<sup>3</sup> voxel size. The SBEM data in Fig. 3a is from the dataset published in ref<sup>5</sup>. Briefly, the data was acquired from a sample of mouse S1 cortex layer 4 using a single-beam SBEM setup at 11x11x28 nm<sup>3</sup> voxel size. The multiSEM data in Fig. 3a is from the dataset in ref<sup>6</sup>. Briefly, the data was acquired from a sample of mouse S1 cortex using a 61-beam multiSEM at 50ns dwell time, 4x4x35 nm<sup>3</sup> voxel size. The display items in Fig. 4a are raw 3D EM data, CNN output and watershed segmentation and agglomeration results from dataset ex145 published in ref<sup>5</sup>. Briefly, the dataset was acquired from a sample of mouse S1 cortex layer 4 using a SBEM setup at 11x11x28 nm<sup>3</sup> voxel size. CNN, watershed and agglomeration routines were as described in the methods of ref<sup>5</sup>.

## References

- 1 Schmidt, H. *et al.* Axonal synapse sorting in medial entorhinal cortex. *Nature* **549**, 469-475, doi:10.1038/nature24005 (2017).
- 2 Briggman, K. L., Helmstaedter, M. & Denk, W. Wiring specificity in the direction-selectivity circuit of the retina. *Nature* **471**, 183-188, doi:10.1038/nature09818 (2011).
- 3 Kornfeld, J. *et al.* EM connectomics reveals axonal target variation in a sequence-generating network. *Elife* **6**, e24364 doi:10.7554/eLife.24364 (2017).
- 4 Lombar, S. *et al.* Connectomic comparison of mouse and human cortex. *Science* **377**, eabo0924, doi:10.1126/science.abo0924 (2022).
- 5 Motta, A. *et al.* Dense connectomic reconstruction in layer 4 of the somatosensory cortex. *Science* **366**, doi:10.1126/science.aay3134 (2019).
- 6 Sievers, M. *et al.* Connectomic reconstruction of a cortical column. *bioRxiv*, doi:10.1101/2024.03.22.586254 (2024).