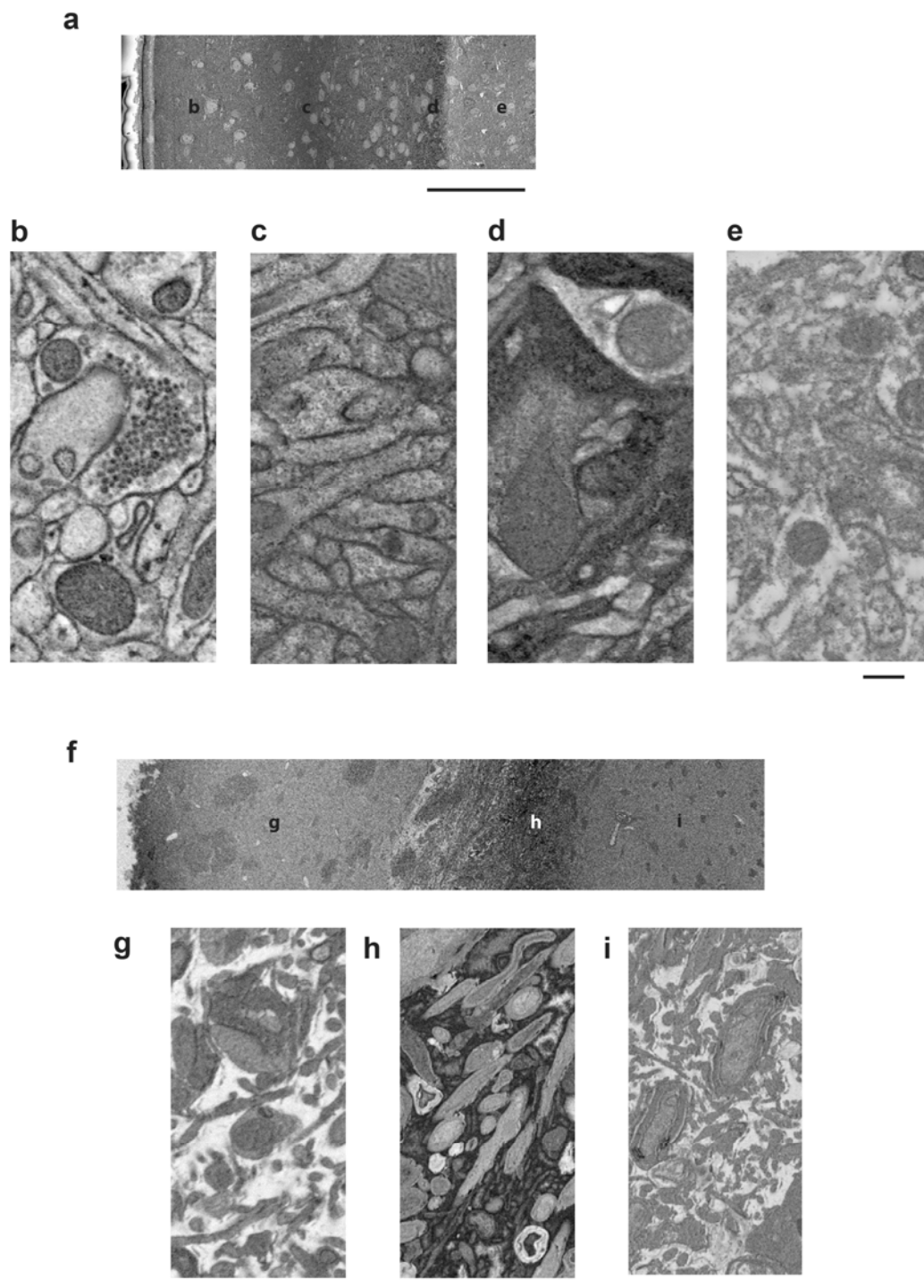
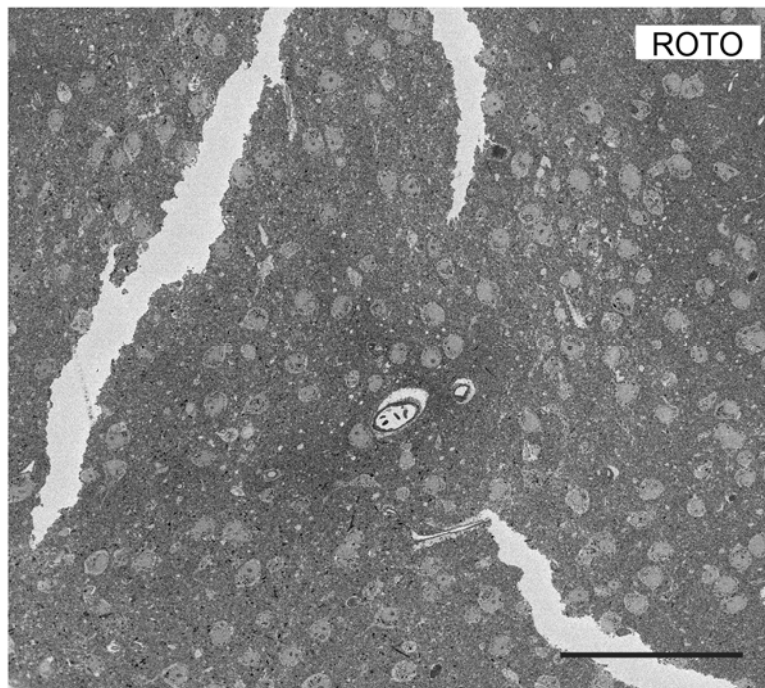




Supplementary Figure 1

ROTO staining and ultrastructural preservation over different depths.

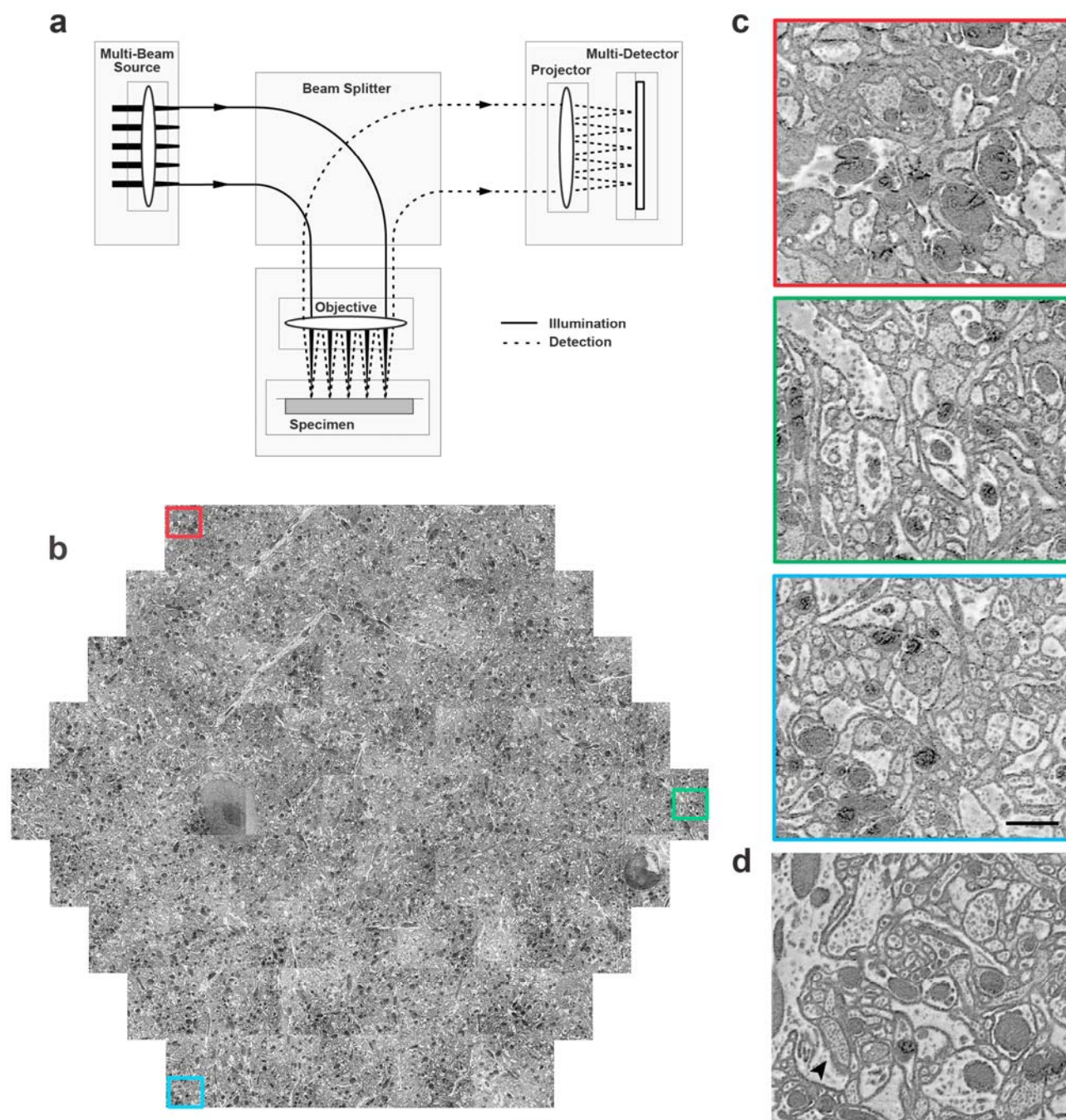
SEM images for ROTO-prepared samples without, (**a-e**), and with, (**f-i**), extracellular space (ECS) preservation. Locations for high-resolution images indicated in the corresponding low-resolution scans. Imaging parameters are 440 nm pixel size, 2.8 kV, 100 pA, and 0.0064 e⁻/nm² in **a** and **f**, 5 nm pixel size, 2.8 kV, 300 pA, and 600 e⁻/nm² in **b-e**, and 10 nm pixel size, 2.8 kV, 100 pA, and 100 e⁻/nm² in **g-i**. Scales bars are 100 μm in **a** and **f**, 250 nm in **b-e** and 500 nm in **g-i**.



Supplementary Figure 2

Mechanical disruption in large ROTO-prepared samples.

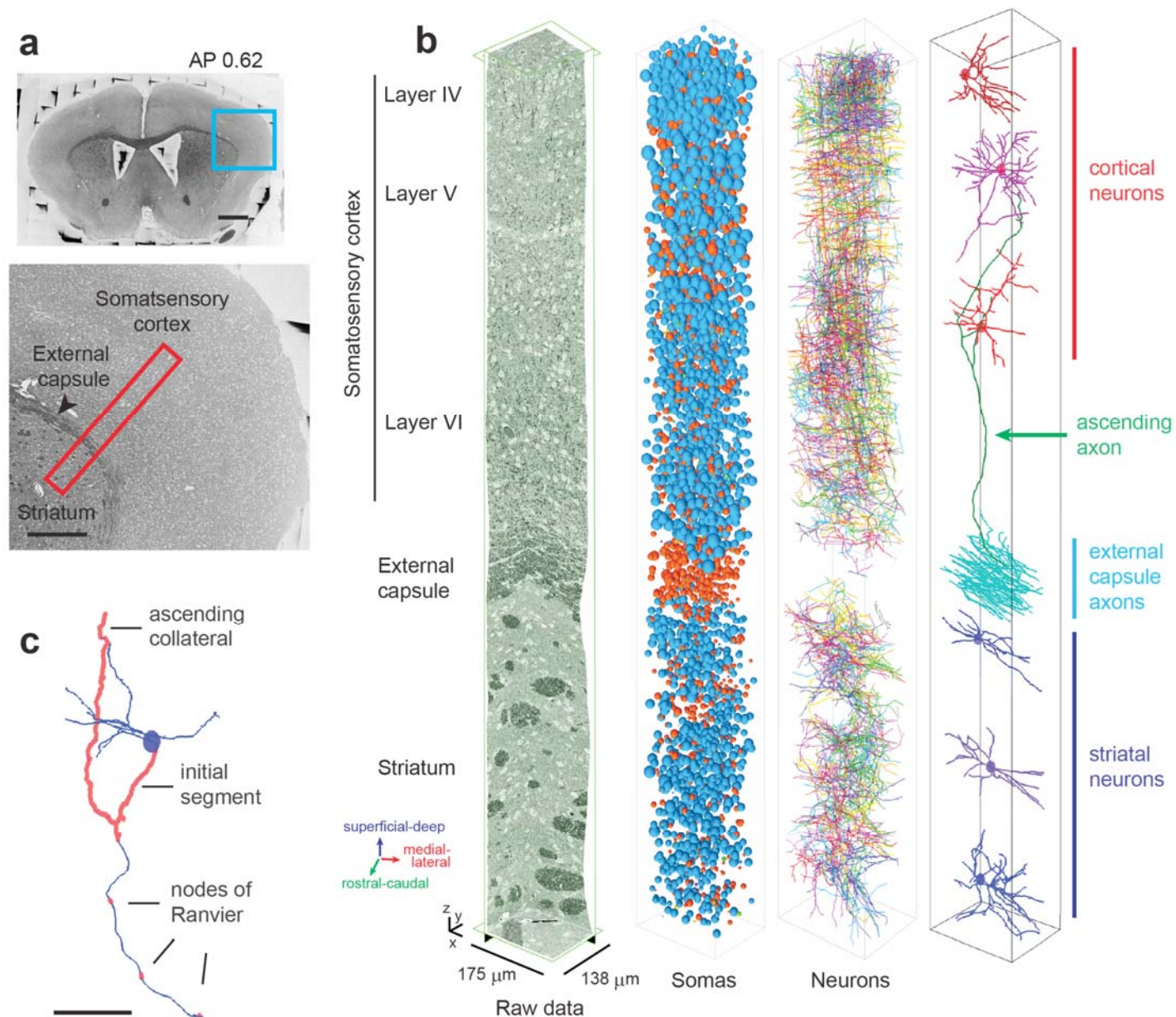
SEM block-face image (220 nm pixel size, 2.8 kV, 100 pA, and $0.0256 \text{ e}^-/\text{nm}^2$) taken from a ROTO sample (with 10% formamide added to the reduced osmium step to increase stain penetration) prepared using an ECS-preserving perfusion medium. The scale bar corresponds to 100 μm .



Supplementary Figure 3

Multibeam SEM imaging of a BROPA-prepared sample.

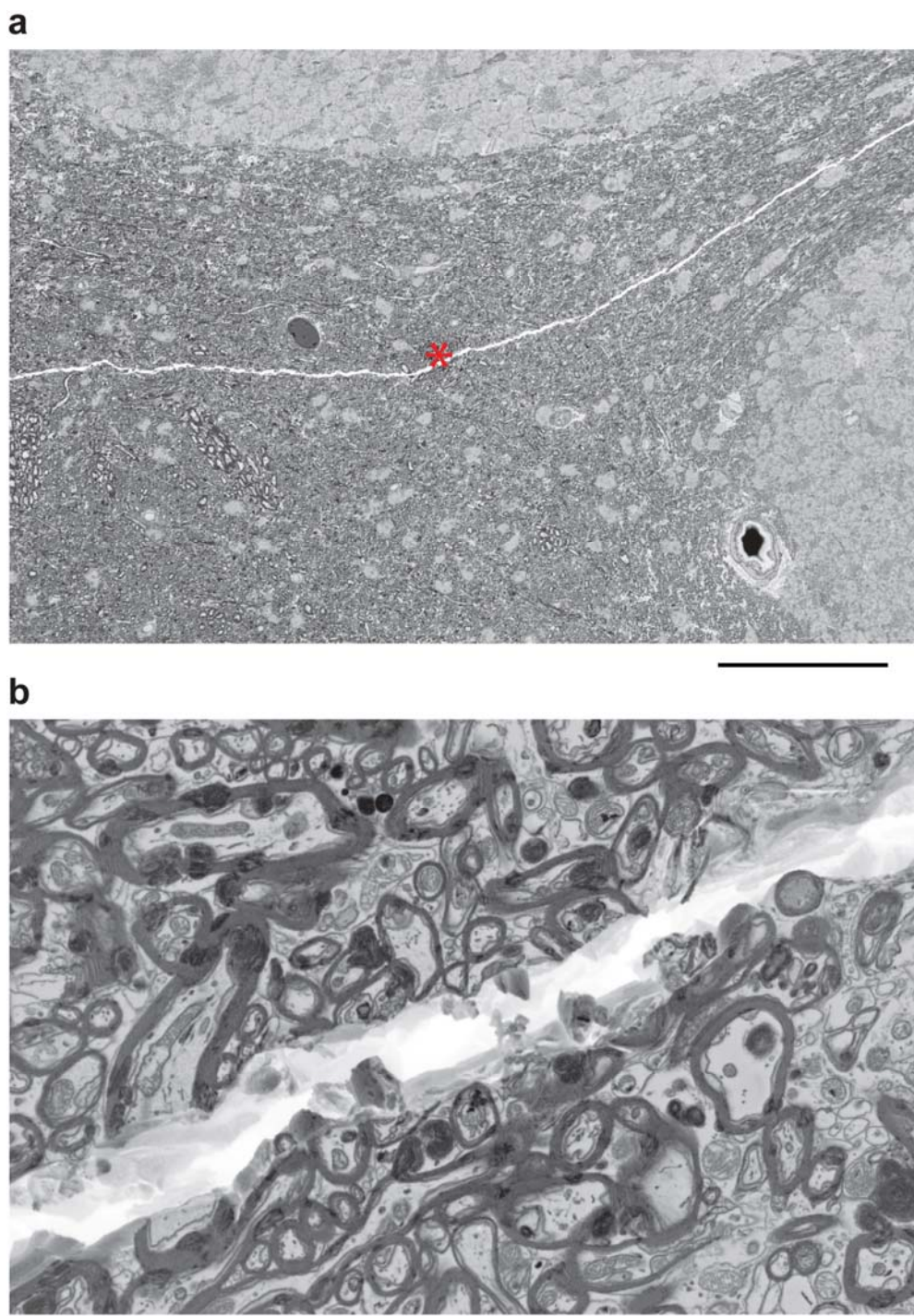
(a) Schematic of the prototype multi-beam microscope. **(b)** Image (3.8 nm pixel size, 430 pA, 100 ns pixel dwell time, 470 MPixel/s effective scan rate, $18.6 \text{ e}^-/\text{nm}^2$) of a block-face coated with a thin film of palladium to avoid charging. Each tile corresponds to an image taken by one of the 61 beams that scan the sample simultaneously. **(c)** Subregions as indicated in **b**. **(d)** Subregion from multi-beam SEM image acquired at high current (4.6 nm pixel size, 3 nA, 50 ns pixel dwell time, 755 MPixel/s effective scan rate, $44.2 \text{ e}^-/\text{nm}^2$). The sample is the same as in **b**. Asymmetric synapse onto a spine head (arrowhead). Scale bars are 10 μm in **b** and 1 μm in **c**. Scale bar in **c** also applies to **d**.



Supplementary Figure 4

Inter-areal SBEM and neurite traceability.

(a) Complete block-face SEM image (top, 440 nm pixel size, 4 kV, 150 pA, $0.0097 \text{ e}^-/\text{nm}^2$) of a BOPA brain and a higher-magnification view of the region of interest (bottom). The red rectangle shows the approximate extent of a continuous SBEM stack. The normal of the stack images is along the long axis of the rectangle. (b) From the left: surface view of aligned stack; manually identified neuronal (blue) and glial (red) nuclei; neurites emerging from 381 randomly-selected nuclei. 6 of 381 neurons (3 each in cortex and striatum) together with 100 randomly selected external-capsule axons. Note that one of them veers into cortex. (c) Single cortical cell with dendrites (thin blue lines), the initial segment and an ascending collateral (thick red, both unmyelinated), and the descending axon (thin blue line) with nodes of Ranvier (red). Scale bars are 1 mm in **a** (top), 500 μm in **a** (bottom), and 40 μm in **c**.



Supplementary Figure 5

Crack in BROPA sample.

(a) Cracks, generally devoid of epoxy, are occasionally observed in epoxy-embedded BROPA samples (see Supplementary Video 4). **(b)** High-magnification image of the red asterisk in **a**. Imaging parameters are 220 nm pixel size, 3.0 kV and 0.29 e⁻/nm² in **a** and 10 nm pixel size, 3.0 kV and 140 e⁻/nm² in **b**. Scale bars are 100 μm in **a** and 5 μm in **b**.

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