**Supplementary Data 1.** FSaqOTO mPrep capsules and containers used for freeze-substitution of plant materials at room temperature and shown here without reagents. **A.** Top view (start upper left and follow counterclockwise) of specimen cup lid, specimen cup with Drierite™ desiccant, Teflon™ substitution fluid container, mPrep capsule CPD holder, mPrep capsule bottom with HPF planchette (and specimen) inside, and the second mPrep/s capsule used as a top. **B.** Top view of assembled specimen cup with mPrep CPD holder within Teflon™ substitution fluid container in specimen cup. **C.** Same as B, but with a bottom mPrep/s capsule to hold HPF planchette for processing. **D.** Configuration of final mPrep capsule assembly to entrap the sample with HPF planchette (and specimen) between two mPrep/s capsules.
**Supplementary Data 2.** Sample preparation steps from freeze-substitution to resin.

| Sample | Fixation Buffer | pH | pH Neutralization | Fixation Protocols | Resin Protocol | Glue | Deproteinization | Fixation Protocols | Resin | Glue | Deproteinization |
|--------|-----------------|----|------------------|--------------------|---------------|------|------------------|--------------------|-------|------|------------------|
| H. vulgare | Leics EM Ice HPF | 1% OsO4 | 97% acetone + 3% water | QFS to 2% OsO4 in 0.1M Na cacodylate | Yes | 2.5% in water | 1% in water | 1% in water | 50°C | 1% in water | 4°C/50°C | Acetone/Propylene Oxide | Quetol 651 hard formulation |
| C. elegans | Leics EM Ice HPF | 2% OsO4 | 97% acetone + 3% water | Manual to 2% OsO4 in 0.1M Na cacodylate | Yes | 2% in water | X | X | X | X | X | Acetone PolyBed hard formulation |
| S. cerevisiae | Leics EM Ice HPF | 1% OsO4 + 1% UAc | 90% acetone + 5% methanol + 5% water | QFS to 2% OsO4 in 0.1M Na cacodylate | Yes | 2.5% in water | 1% in water | 1% in water | 50°C | 1% in water | 4°C/50°C | Acetone Quetol 651 hard formulation |
| S. cerevisiae | Leics EM Ice HPF | 1% OsO4 + 1% glut | 97% acetone + 3% water | None | No | X | X | X | X | X | X | Acetone PolyBed hard formulation |

AFS - Automated Freeze-Substitution, QFS = Quick Freeze-Substitution, KeFeCN = potassium ferrocyanide, TCH = thiocarbohydrazide, OsO4 = osmium tetroxide, Pb = lead, UAc = uranyl acetate.
### Supplementary Data 3

Sample and image conditions table providing major acquisition parameters (SBF-SEM, FIB-SEM, STEM and TEM) for all included images of barley root and anther (*Hordeum vulgare ssp. vulgare*), yeast (*S. cerevisiae*) and nematode (*C. elegans*) samples.

| Sample Type | Freeze-substitution Protocol | Imaging Platform | eTM Platform/Mode | FCC N₂ Settings (%) | X-Y resolution (slice thickness) | Z-Resolution (mm) | Image Pixel Resolution | Voltage (kV) | Beam Current (pA) | Dwell/Image-Time |
|-------------|-------------------------------|------------------|-------------------|----------------------|----------------------------------|------------------|------------------------|--------------|-------------------|------------------|
| 2A Barley Root | Manual FSaqOTO | Zeiss GeminiSEM300 | S/View 2P w FCC | 10 | 130nm | NA | 3000x-4000 | 5 | 1pA | 3µs |
| 2B Barley Root | Manual FSaqOTO | Zeiss GeminiSEM300 | S/View 2P w FCC | 10 | 5nm | 50nm | 10000x-10000 | 5 | 1pA | 3µs |
| 2C Barley Root | Manual FSaqOTO | Zeiss GeminiSEM300 | S/View 2P w FCC | 10 | 5nm | 50nm | 10000x-10000 | 5 | 1pA | 3µs |
| 2D Barley Root | Manual FSaqOTO | Zeiss GeminiSEM300 | S/View 2P w FCC | 25 | 130nm | NA | 4000x-4000 | 5 | 1pA | 4µs |
| 3A Barley Anther | Manual FSaqOTO | Zeiss GeminiSEM300 | S/View 2P w FCC | 35 | 5nm | 50nm | 10000x-10000 | 5 | 1pA | 3µs |
| 3B Barley Anther | Manual FSaqOTO | Zeiss GeminiSEM300 | S/View 2P w FCC | 35 | 5nm | 50nm | 10000x-10000 | 5 | 1pA | 3µs |
| 3C Barley Anther | Manual FSaqOTO | Zeiss GeminiSEM300 | S/View 2P w FCC | 35 | 5nm | 50nm | 10000x-10000 | 5 | 1pA | 3µs |
| 3D Barley Anther | Manual FSaqOTO | Zeiss GeminiSEM300 | S/View 2P w FCC | 35 | 5nm | 50nm | 10000x-10000 | 5 | 1pA | 3µs |
| Suppl_Video 1 Barley Anther | Manual FSaqOTO | Zeiss GeminiSEM300 | S/View 2P w FCC | 35 | 5nm | 50nm | 10000x-10000 | 5 | 1pA | 3µs |
| Suppl_Video 2 Yeast | QFSaqOTO | Zeiss CrossBeam550 | CypressFIBSEM.KLASS | NA | 5nm | 12nm | 2661x626 | 1.5 | 1.5µA | 3µs/1 line average |
| 4A Nematode | QFSaqOTO | Zeiss CrossBeam550 | Brightfield STEM | NA | 10mm | 70nm | 2048x2048 | 30 | 600pA | 3µs/1 line average |
| 4B Nematode | AFSaqOTO | Zeiss CrossBeam550 | Brightfield STEM | NA | 3mm | 70nm | 2048x2048 | 30 | 600pA | 3µs/1 line average |
| 4C Nematode | QFSaqOTO | Zeiss CrossBeam550 | Brightfield STEM | NA | 10nm | 70nm | 2048x2048 | 30 | 600pA | 3µs/1 line average |
| 4D Nematode | AFSaqOTO | Zeiss CrossBeam550 | Brightfield STEM | NA | 3mm | 70nm | 2048x2048 | 30 | 600pA | 3µs/1 line average |
| 5A Yeast | QFSaqOTO | Hitachi HT7500 | STEM | NA | 3mm | 70nm | 2048x2048 | 30 | 600pA | 3µs/1 line average |
| 5B Yeast | AFSaqOTO | Hitachi HT7500 | STEM | NA | 3mm | 70nm | 2048x2048 | 30 | 600pA | 3µs/1 line average |
| 5C Yeast | QFSaqOTO | Hitachi HT7500 | STEM | NA | 3mm | 70nm | 2048x2048 | 30 | 600pA | 3µs/1 line average |
| 5D Yeast | AFSaqOTO | Hitachi HT7500 | STEM | NA | 3mm | 70nm | 2048x2048 | 30 | 600pA | 3µs/1 line average |
Supplementary Data 4. 3D Sensor Deep learning model parameters used with ORS Dragonfly segmentation of barley root (Fig 2E).

Supplementary Data Video 1. SBF-SEM stack of high-pressure frozen barley root prepared by FSaqOTO.

Supplementary Data Video 2. FIB-SEM stack of high-pressure frozen yeast (S. cerevisiae) by FSaqOTO.