**Supplementary Figure 1.** Influence of the spatial frequency range used in MAPFoSt analysis. A given through-focus series of experimental images analyzed using (a) spatial frequencies $|k| < 0.06 \kappa_{Nyq}$, (b) $|k| < 0.25 \kappa_{Nyq}$, (c) $|k| < 0.95 \kappa_{Nyq}$, (d) all spatial frequencies.
Supplementary Figure 2. Initial focal spot size and spot size achieved by MAPFoSt after up to five iterations. (a) Mouse brain sample imaged at 80-nm-pixel size and (b) 20-nm-pixel size. (c) Tin-on-carbon resolution target imaged at 80-nm-pixel size. (d) Gold-on-carbon resolution target, analyzed using improved astigmatism scaling for faster convergence.

Supplementary Figure 3. Modal wavefront sensing provides a reference for microscope parameters corresponding to zero aberrations. Consecutive modal scans of (a) working distance, (b) astigmatism $x$, and (c) astigmatism $y$ allow removal of the residual astigmatism $x$ present after the first iteration of 20-nm MAPFoSt in Figure 7b, as evidenced by a second iteration of modal scans (d) working distance, (e) astigmatism $x$, and (f) astigmatism $y$. All six subfigures use the same $y$ scaling for the metric.