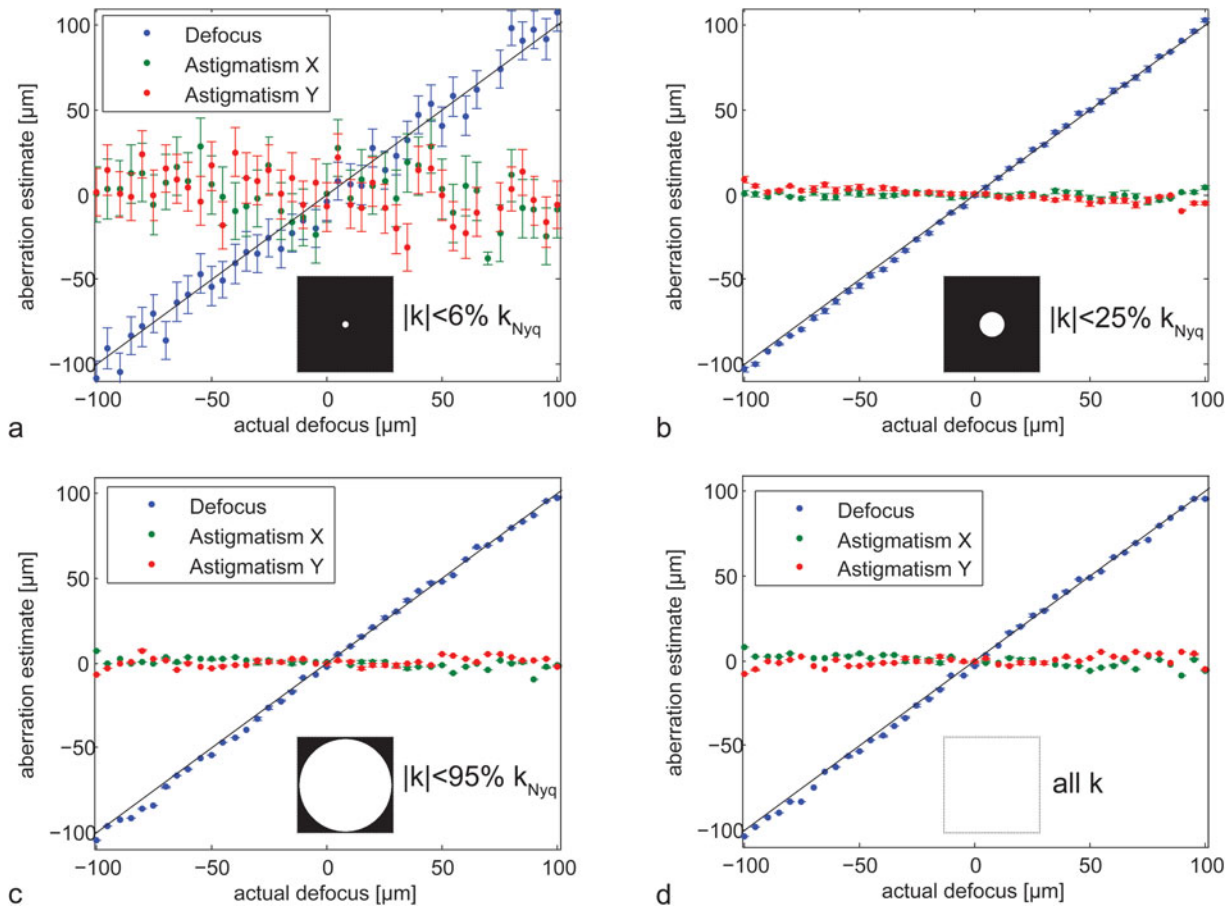
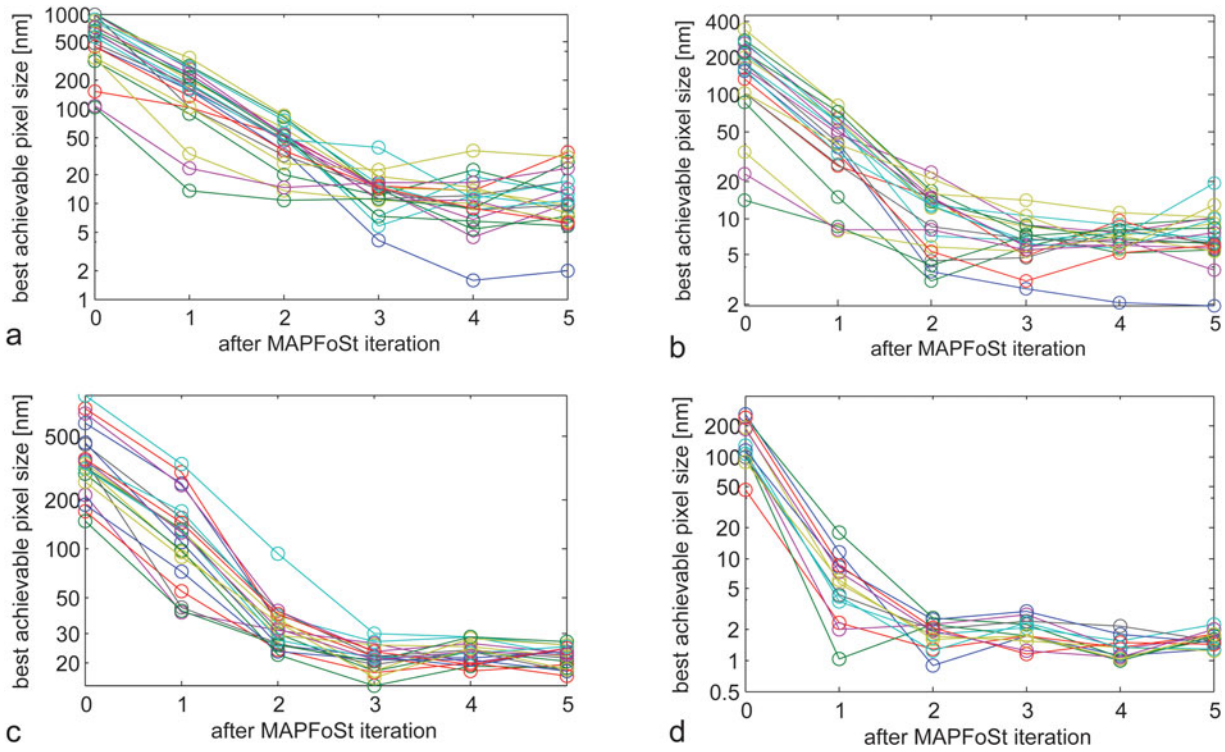
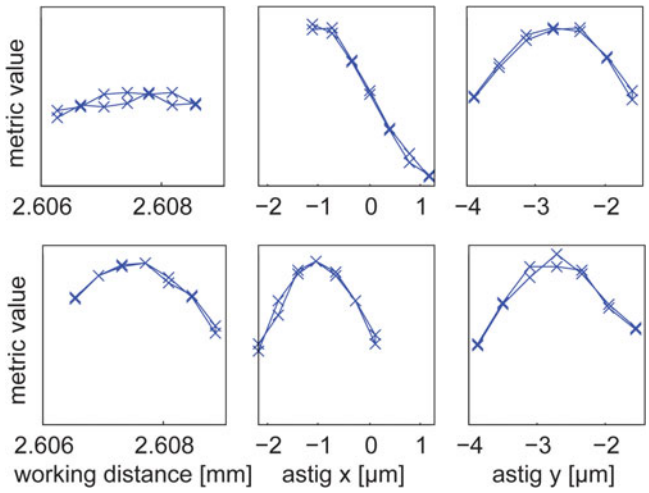


Supplementary Figure 1. Influence of the spatial frequency range used in MAPFoSt analysis. A given through-focus series of experimental images analyzed using (**a**) spatial frequencies $|\mathbf{k}| < 0.06\ k_{Nyq}$, (**b**) $|\mathbf{k}| < 0.25\ k_{Nyq}$, (**c**) $|\mathbf{k}| < 0.95\ k_{Nyq}$, (**d**) 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.