We next determined whether the processing of datasets representing highly multiplexed images of the genome would benefit from Deconwolf. For this, we turned to image datasets produced by OligoFISSEQ and analyzed by an “every-pixel” chromosome tracing pipeline (PMID: 32719531). OligoFISSEQ leverages the FISH-based Oligopaints technology to target regions of chromosomal DNA with user designed oligomers (oligos), the 5’ and 3’ nongenomic sequences of which enable functions such as amplification, labeling, detection, and barcoding. Specifically, in OligoFISSEQ, barcodes are sequenced in situ, enabling high levels of multiplexing (PMID: 32719531, PMID: 23236188). We reasoned that images from ligation-based OligoFISSEQ (O-LIT), which can result in a very high density of signals, would benefit from Deconwolf. Visual inspection immediately demonstrated the capacity of Deconwolf to improve the resolution of crowded raw OligoFISSEQ signals (Fig. Xa). Indeed, it significantly improved the decoding of five rounds of sequencing at each of the 46 regions along the X chromosome that had been targeted by the ChrX-46plex Oligopaint library (PMID: 32719531). This translated into an increase in the efficiency of target detection from 73.9 ± 2.1%, with Richardson-Lucy algorithms (ref), to 97.1 ± 1.0% (n = 168 cells from seven replicates) after the application of Deconwolf (Fig. Xb and Extended Data Fig. XX). The increased detection efficiency resulted in more complete chromosome tracing (Fig. Xc), further demonstrating the benefits gained from integrating Deconwolf with multiplexed genome imaging analysis.

**Figure X | Deconwolf improves OligoFISSEQ target detection. a**, Targets of ChrX-46plex

and nuclei after the first round of ligation-based OligoFISSEQ (O-LIT). Images are from

maximum-intensity z-projection. RL = Richardson-Lucy, 20 iterations. DW = Deconwolf, 50

iterations. **b**, Tier 2 target detection efficiency of ChrX-46plex across seven replicates (n =

168 cells) after five rounds of O-LIT with RL (orange; average of 73.9 ± 2.1%) or DW (blue;

average of 97.1 ± 1.0%) deconvolved images. Detection efficiencies for individual cells are

plotted. Error bars represent the 95% bootstrap CI of the mean. **c**, Chromosome traces and

ball-and-stick visualization of the nucleus from **a** after tier 2 analysis (70% after RL and 98%

after DW) and interpolation of missing targets (black spheres).

**Figure XX | Deconwolf improves OligoFISSEQ ChrX-46plex target detection.** Tier 2

target detection efficiency of ChrX-46plex across seven replicates (n = 168 cells) after five rounds of O-LIT with RL (orange; average of 73.9 ± 2.1%) or DW (blue; average of 97.1 ± 1.0%) deconvolved images. Error bars represent the 95% bootstrap CI of the mean.