Figure S1: Cross Wavelength Offsets and Alignment:

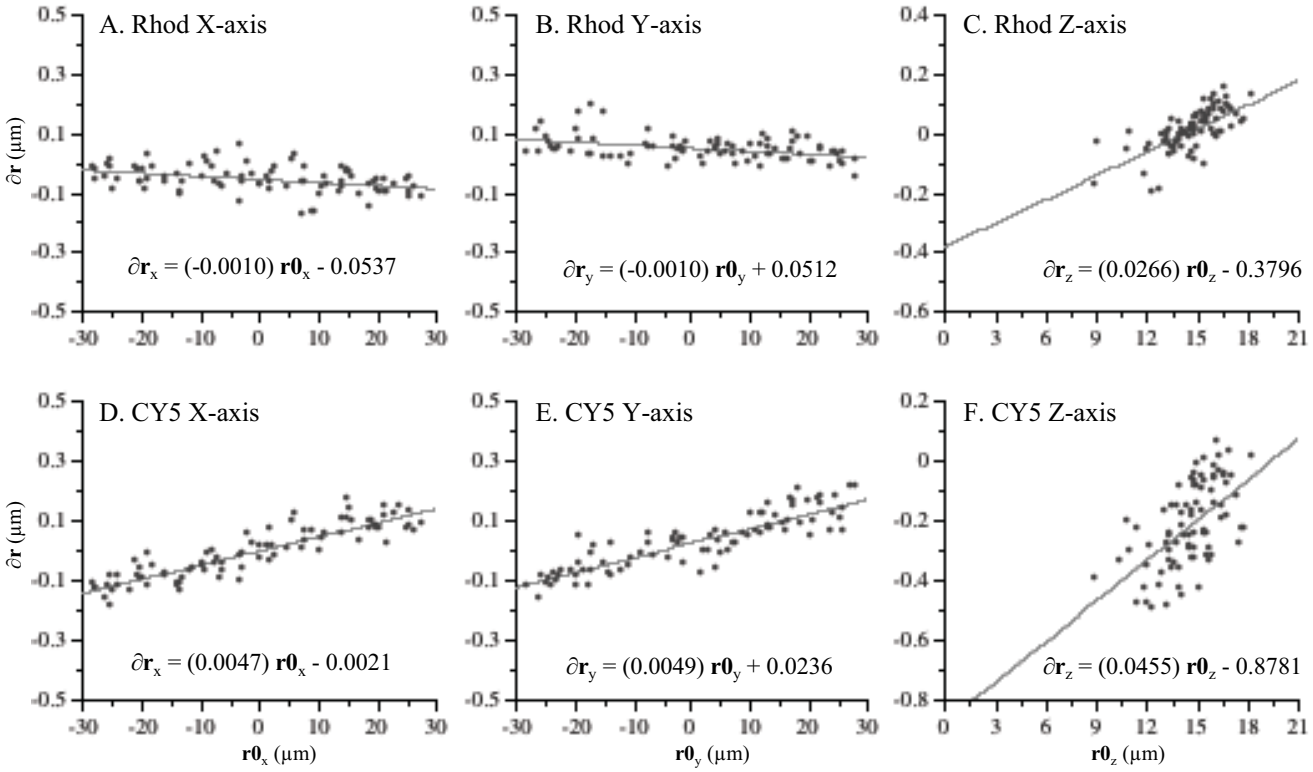


Figure S2: Comparison of Pairwise Distances in Triplet Probe Datasets and Barcode Datasets:

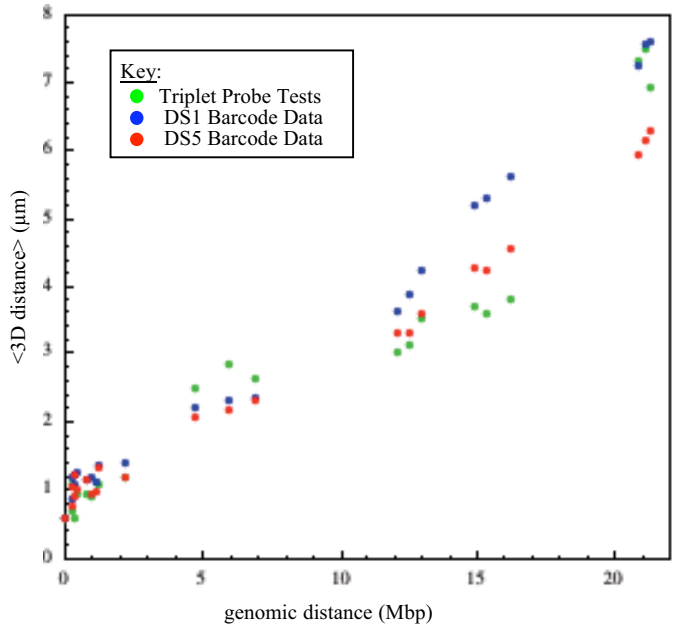
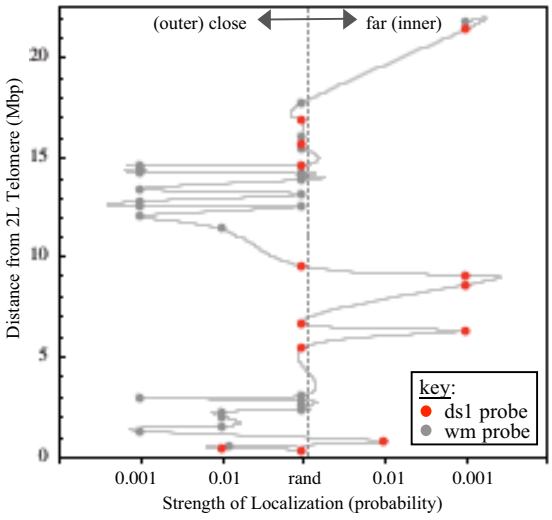


Figure S3: Comparison of Nuclear Localization for Barcode Probes and Those Previously Localized In Drosophila Embryos:



Supplementary Figure S1: Cross-Wavelength Offsets and Alignment: Label 13 (the histone probe) was triple labeled (FITC/RHOD/CY5). The probe positions (throughout the dataset) in the FITC wavelength were used as a reference and the offset position of each probe in the RHOD and CY5 wavelengths were plotted as a function of position within the image relative to the optical axis. X, Y and Z coordinates were processed separately. The center of each XY plane was taken as the optical axis while the top of the datastack was used as the zero point for the Z axis. Linear least-squares were used to derive equations defining offsets as a function of position within the image. The correction factors were then applied to the original positions to correct the position of all RHOD and CY5 labeled probes.

Supplementary Figure S2: Comparison of Pairwise Distances in Triplet Probe Datasets and Barcode Datasets: In the plot shown, data points < 5 Mbp genomic distance are the nearest neighbor probe distances while those >= 5 Mbp are the probe-histone pairs. Overall, the average distances for equivalent probe pairs are very similar in the triplet probe tests and the barcode data. As discussed in the text, nuclei elongate during cycle 14 and pairwise distances increase. While all data shown is from cycle 14 embryos, the triplet data comes from multiple embryos at slightly different points in cycle 14. This explains the small fluctuations in triplet distances relative to the barcode data points which all derive from the same embryo (i.e. all DS5 data points are smaller than equivalent DS1 data points, but the triplet data points fluctuate above and below them).

Supplementary Figure S3: Comparison of Nuclear Localization for Barcode Probes and Those Previously Localized in Drosophila Embryos: The statistical results for DS1 nuclear localization are plotted as a function of chromosomal position. We also include results from a similar study by Marshall *et al.* (1996) and find good agreement for DS1. DS5 which is not plotted did not show as good agreement. Note that although the statistical test is identical, we use the terminology inner/outer whereas Marshall *et al.* (1996) use the terminology far/close.