LIST of SUPPLEMENTARY MATERIALS

Mir et al., Dense Bicoid Hubs Accentuate Binding along the Morphogen Gradient

Supplemental Movie S1 (Related to Figure 1). Movies corresponding to the still frames shown in Figure 1A.

Supplemental Movie S2 (Related to Figure 1) Representative data from a 90 second segment of a 100 millisecond exposure time movie acquired at an anterior position (EL (x/L) of 0.1). Top left shows the raw data and top right the corresponding surface plot representation. Bottom left shows a running max projection of the data and bottom right shows a surface plot representation of the same

Supplemental Movie S3 (Related to Figure 2) Representative data acquired at 10 millisecond exposure times for 4 nuclei.

Supplemental Movie S4 (Related to Figures 2 and 3) Temporal dynamics of cluster formation for representative nuclei at Anterior, Middle, and Posterior positions.

Supplemental Table S1 (Related to Figures 1 and 3). Results from 2-exponent model fits to survival probability distributions.

Supplemental Fig. S1 (Related to Figure 1). Lattice Light-Sheet Microscope Implementation **Supplemental Fig. S2** (Related to Figure 1). Single Molecule Imaging of BCD-eGFP at 100 milliseconds to estimate residence times.

Supplemental Fig. S3 (Related to Figure 1). Fits to the survival probability distributions of the 100 millisecond datasets

Supplemental Fig. S4 (Related to Figure 1). Fits to the survival probability distribution of the 500 millisecond dataset

Supplemental Fig. S5 (Related to Figure 1). Analysis of FRAP data

Supplemental Fig. S6 (Related to Figure 2). Analysis of Displacement Distributions

Supplemental Fig. S7 (Related to Figure 2). Cluster identification results from DBSCAN across the A-P axis.

Supplemental Fig. S8 (Related to Figure 2). Fraction of trajectories within clusters across the A-P axis.

Supplemental Fig. S9 (Related to Figure 3). BCD binding in whole and posterior thirds embryos compared to ZLD binding

Supplemental Fig. S10 (Related to Figure 3). BCD and ZLD binding at Zelda peaks

Supplemental Fig. S11 (Related to Figures 2 and 3). Averaged pair-correlation (radial distribution) functions for nuclei in the WT, ZLD-, and simulated cases and representative images.

Captions for Supplemental Movies

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Data Set	A-P Poistion	Non-specific binding time (1/k _{ns} (sec))	Specific Binding time (1/k, (sec))	Corrected Specific Binding time 1/(k _s -k _{bleach}) (sec)	Number of trajectories
		0.175	1.100		
100 ms	Ant	[0.166, 0.184]	[1.013, 1.203]	1.105	17735
		0.160	0.940		
	Mid	[0.152,0.168]	[0.859, 1.038]	0.944	40092
		0.146	0.865		
	Post	[0.141, 0.151]	[0.806, 0.932]	0.868	20823
		0.159	0.959		
	All Together	[0.166, 0.152]	[0.882, 1.050]	0.963	78650
		0.152	0.777		
100 ms (Zelda-)	Ant	[0.146, 0.160]	[0.726,0.836]	0.780	11415
		0.131	0.629		
	Mid	[0.126, 0.137]	[0.587, 0.679]	0.631	7572
		0.112	0.463		
	Post	[0.108, 0.117]	[0.435, 0.495]	0.464	3606
		0.139	0.694		
	All Together	[0.133, 0.145]	[0.647, 0.748]	0.696	22593
		0.265	1.876		
500 ms	All Together	[0.255, 0.277]	[1.69, 2.107]	-	1211

Supplemental Table S1. Results from 2-exponent model fits to survival probability distributions. k_{ns} and k_s are the un-corrected off-rates for the short-lived (non-specific) and longer-lived (specific) populations respectively determined from a two-exponent fits to the survival probability distributions. The binding time (one-over the off rates) are shown with 95% confidence intervals in square brackets. The photo-bleaching corrected binding times are calculated as $1/(k_s-k_{bleach})$ where k_{bleach} is 0.0043 s⁻¹).



Supplemental Figure S1. Lattice Light-Sheet Microscope Implementation

The Lattice light-sheet was built as described by Chen et al, Science, 2014. This simplified schematic shows the major modules of the microscope as follows: (**A**) Laser Module, contains 6 lasers ranging from 405 nm to 639 nm, which are independently expanded, collimated, and input into an Acousto-Optic Tunable Filter (AOTF). (**B**) The patterning module contains a pair of cylindrical lens to expand the input Gaussian beam to a stripe, and a half-wave ($\lambda/2$) plate, a polarizing beam splitter (PBS), and a Spatial Light Modulator (SLM) to perform the patterning. Lens L1 projects the Fourier Transform of the SLM pattern onto an annular mask for spatial filtering (**C**) Lens pair 1 (LP1) is then used to de-magnify the annular mask plane and project it onto the z-scan galvo and LP2 projects the z-galvo plane onto the x-scanning galvo. (**D**) LP3 magnifies the x-galvo plane and projects it onto the back pupil plane of the excitation objective, where it is focused (Fourier transform) to project the final light sheet pattern onto the sample. Emitted fluorescence is collected by the detection objective. (**E**) The tube lens (TL1) focuses an

intermediate image plane which is then magnified using LP4 and projected onto an EMCCD. A dichroic mirror is placed between the first and second lenses of LP4 to split the signal into high and low wavelengths. An emission filter is placed after the dichroic on both sides to select the chromatic bandwidth of the signal reaching each EMMCD independently. Inset shows an image of the EMCCD detection module (**F**) Image of our lattice light sheet microscope. (**G**) Image of the Objective assembly with a dye solution in the sample chamber to visualize the excitation light. (**H**) Image of the sample holder on which the 5 mm coverslip is mounted.



Supplemental Figure S2. Single Molecule Imaging of BCD-eGFP at 100 milliseconds to estimate residence times. (**A**) The max projection in time of a 90 second segment of a representative 100 millisecond dataset acquired at an anterior nucleus (EL (x/L) of 0.1), corresponding to the last frame of Video 2. (**B**) Surface plot representation of (A) to illustrate the signal-to-background ratio of single molecule binding events. The smaller peaks likely correspond to slowly diffusing molecules that are not in the imaging volume for the entire exposure time. (**C**) Maximum projection through x-t (kymograph representation) of the data shown in Video 2 from which (A-B) were calculated. Colored circles correspond to those in (A). (**D**) Zoomed in x-t view of the circled regions in (A) and (C), illustrating the transient nature of BCD binding.



Supplemental Figure S3. Fits to the survival probability distributions of the 100 millisecond datasets. The survival probability distributions calculated from the trajectories output by the MTT analysis on the 100 millisecond data set for the Anterior (34 nuclei, 17735, trajectories), Middle (70 nuclei, 40092 trajectories), Posterior (83 nuclei; 20823 trajectories) segments and all the data pooled together (187 nuclei, 78650 trajectories). The solid lines show the fit to a 2 exponent-model and the dashed lines to a 1-exponent model. Insets show the same on a log-log scale to visualize the difference between 1 and 2 exponent fits at longer time scales. All two-exponent fits have an R^2 value>0.99. See Table 1 for a summary of the fit parameters.



Supplemental Figure S4. Fits to the survival probability distribution of the 500 millisecond dataset. The survival probability distribution of residence times calculated from the MTT algorithm on the 500 ms data set of 17 nuclei and 1211 trajectories. The solid lines show the fit to a 2-exponent model and the dotted lines to a 1-exponent model. The insets shows the same on a log-log scale to visualize the difference between 1 and 2 exponent fits. R² value for the 2-exponent fit is 0.99





(A) Averaged Bcd-eGFP FRAP data and single exponential fit results. (B) Double exponential fit results to average Bcd-eGFP FRAP. (C) Comparison of averaged BCD-eGFP (21 nuclei) and

His2AV FRAP data (3 nuclei). (**D**) Double exponential fit to Histone (His2AV) FRAP. (**E**) Representative images from BCD-eGFP and His2AV FRAP experiments, white scale bar is 2µm.



Supplemental Figure S6. Analysis of Displacement Distributions (A) Cumulative distribution functions of the displacement data, pie charts indicate the Bound and Mobile fraction as measured by the CDF value at 0.225 μ m (dashed lines). (B) Probability Density Function of the displacement data (markers) and fits to the two population model (solid lines), pie charts show the mobile and bound fractions from the results of the fit, labels indicate the bound fraction and the error is the standard error associated with the fit parameter. Arrows point to the mobile population in the distributions that is decreasing from anterior to posterior positions. (A-B) For each position the

following number of nuclei and trajectories were included in the analysis Anterior: 30 nuclei, 12923, trajectories, Middle: 67 nuclei, 23640 trajectories, Posterior: 66 nuclei; 8600 trajectories.



Supplemental Figure S7. Cluster identification results from DBSCAN across the A-P axis. Examples of clusters identified along the A-P axis using DBSCAN with the position shown as fraction of embryonic length (EL). Particles included in the same clusters are represented with the same color.



Supplemental Figure S8. Fraction of Trajectories within clusters across the A-P axis. Calculated as trajectories within clusters over total number of trajectories in each nucleus. The median for all nuclei at each spatial position is shown, error bars show standard error.



Supplemental Figure S9. BCD binding in whole and posterior thirds embryos compared to ZLD binding. (**A**) Sum of Normalized BCD ChIP-seq reads divided by the length of the respective CRMs for posterior thirds (blue) and whole embryo (black) vs. Sum of Normalized ZLD ChIPseq reads divided by the length of the respective CRM at annotated cis-regulatory modules (CRMs) of *eve*, *giant*, *hunchback*, *knirps*, *hairy*, *kruppel*, *caudal*, *fushi-tarazu*, *engrailed*, *wingless*, *runt*, and *gooseberry* loci. A total of 293 CRMs from the RedFly database were analyzed. (**A-B**) Sum of Normalized BCD ChIP-seq reads for posterior thirds (blue) and whole embryo (black) vs. Sum of Normalized ZLD ChIP-seq reads over a 500 bp region centered on (**B**) 8331 ZLD peaks and (**C**) 2145 BCD peaks. (**A-C**) Corresponding bar plots show Pearson correlation coefficients and error bars show 95% confidence intervals as determined by bootstrapping.



Supplemental Figure S10. BCD and ZLD binding at Zelda peaks. Heat-map representation of normalized ChIP-seq reads for BCD (1st two panels) and ZLD (3rd panel) in a 500 bp window centered on ZLD peaks sorted according to increasing BCD signal in the posterior embryo data. A total of 8331 peaks are shown.



Supplemental Figure S11. Averaged pair-correlation (radial distribution) functions for nuclei in the normal, ZLD- and simulated cases and representative images. (A) Pair-correlation functions. Error bars indicate the standard error. (B) Representative images corresponding to pair-correlation functions for Normal, ZLD- and randomly distributed points.