Supplemental Material for

Biased Brownian ratcheting leads to pre-mRNA remodeling and capture prior to first-step splicing

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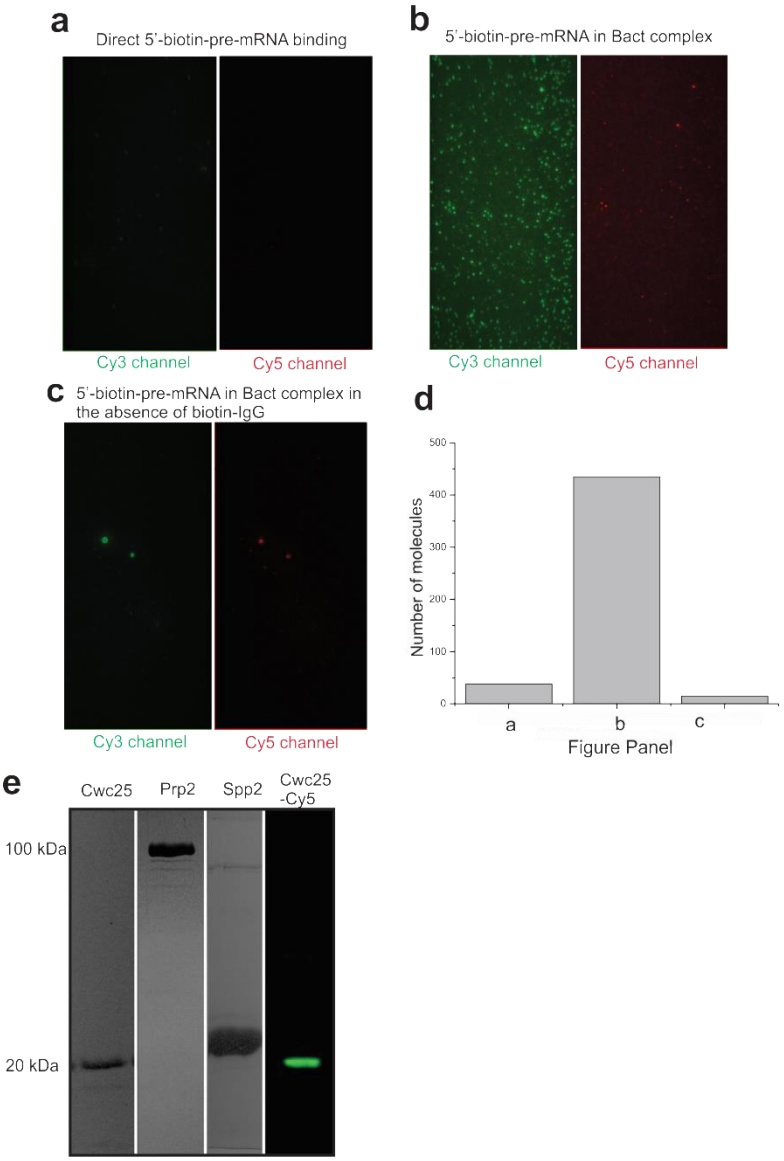
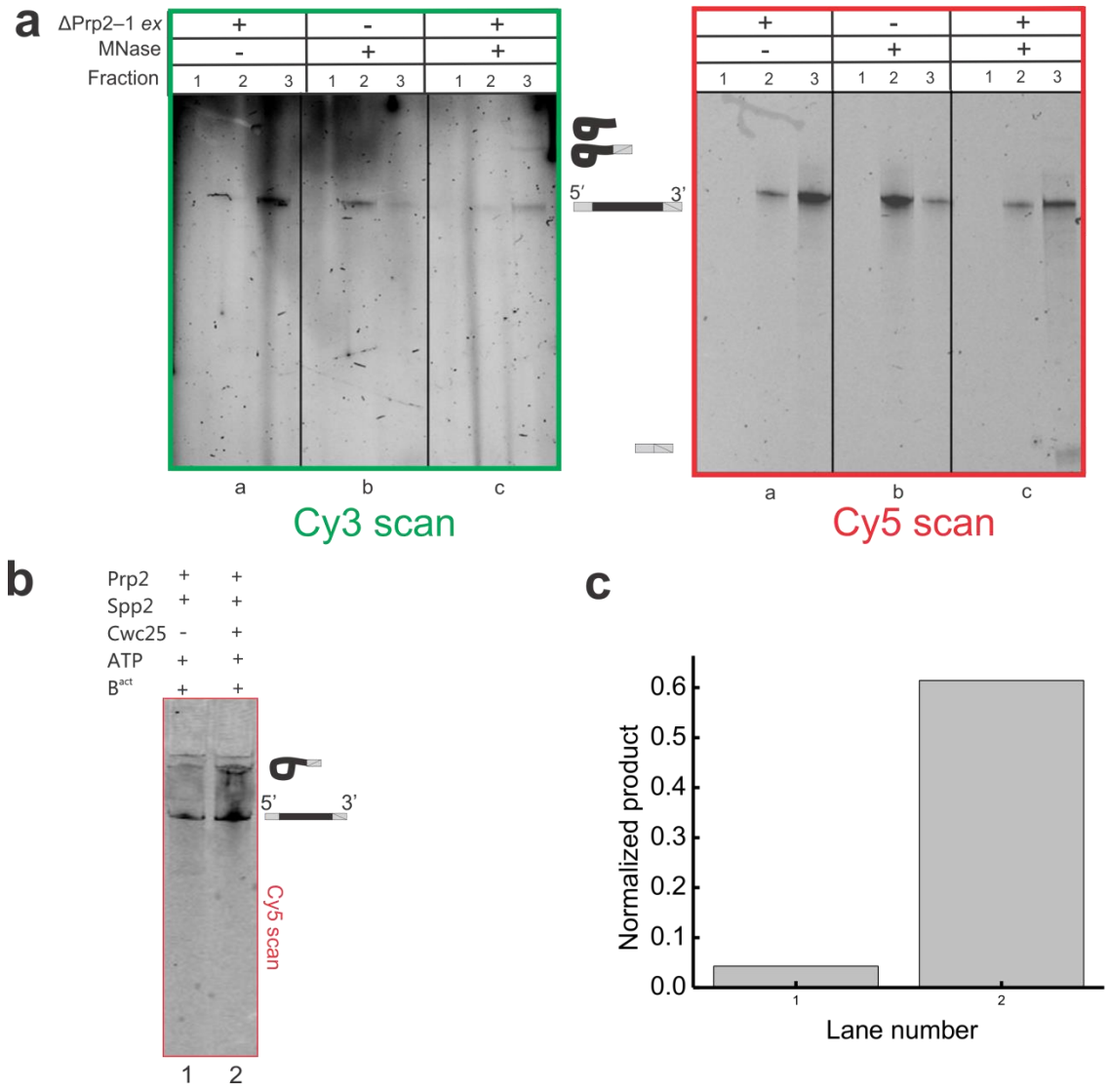
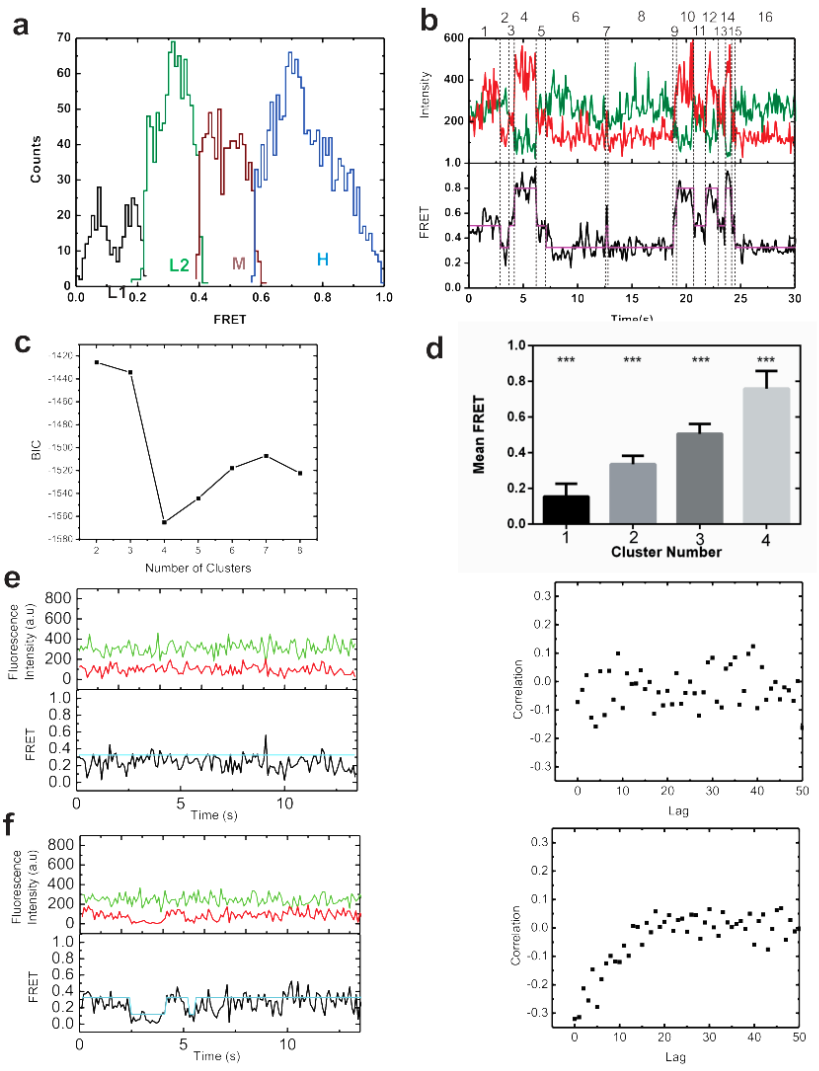


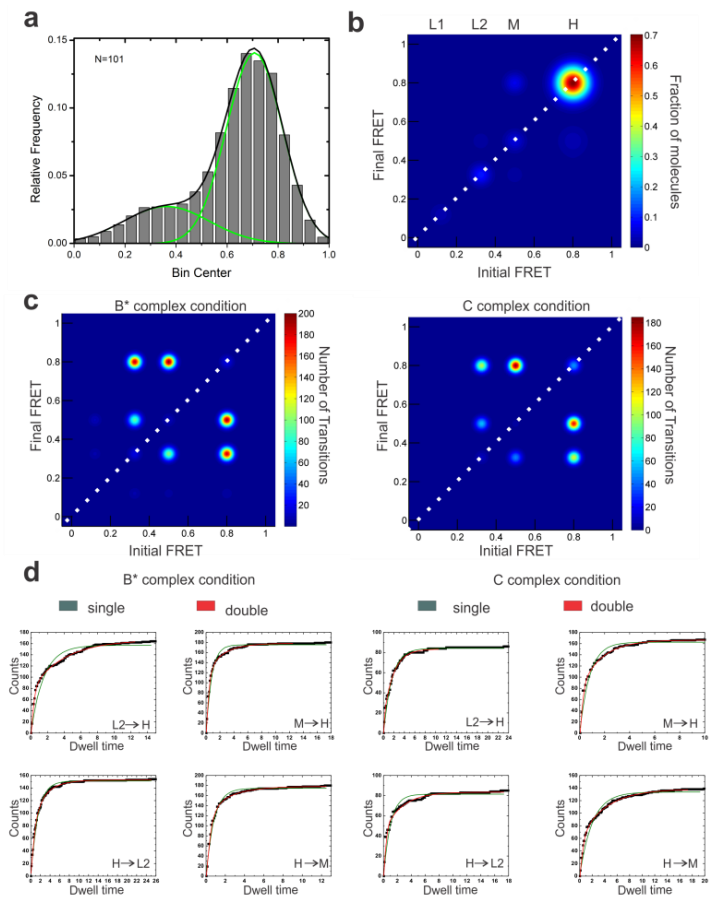
Supplementary Figure 1 Binding specificity of B^{act} complex and purified proteins used for reconstitution (a) Field of view showing direct binding of the 5' biotinylated pre-mRNA to the streptavidin on the slide surface saturated with biotin-IgG and free biotin. (b) Field of view showing the binding of the immunopurified B^{act} spliceosome (with Cef1-TAP) to the streptavidin on the slide surface saturated with free biotin and biotin-IgG. (**c**) Field of view showing the binding of the immunopurified B^{act} spliceosome (with Cef1-TAP) to the streptavidin on the slide surface saturated with free biotin in the absence of IgG-biotin. Left and right panels are the Cy3 and Cy5 channels, respectively. A 532 nm and 635 nm laser was used for excitation under all conditions. (d) Quantification of number of molecules under conditions a-c. (e) Protein expression and purification confirmed by SDS-PAGE analysis. Histidine-tagged Cwc25, Prp2 and Spp2 are shown in lanes 1, 2 and 3, respectively. Cy5-fluorophore labeled single-cysteine Cwc25 is shown in lane 4.



Supplementary Figure 2 Confirmation of B^{act} complex specificity and activity (a) 15% Urea-polyacrylamide gel scanned with a variable mode Typhoon imager. The intron and intron-lariat products are observed in the Cy3 scan (left) and the mature mRNA product is visualized in the Cy5 scan (right). Lanes 1, 2 and 3 represent fractions wash, unbound and bound, respectively. Conditions a and c represent wild-type Ubc4 pre-mRNA assembled in B^{act} complex and immobilized on magnetic beads with biotin-IgG. Condition b is wild-type pre-mRNA assembled in the absence of extract. Bound molecules were reconstituted with or without Micrococcal Nuclease (MNase) treated extract. (b) 6% Urea-polyacrylamide gel scanned using a variable mode Typhoon imager. Affinity purified B^{act} complex formed with Cy5-actin pre-mRNA supplemented with Prp2, Spp2 and 2 mM ATP (lane 1) and Prp2, Spp2, Cwc25 (lane 2). (c) Quantification of lanes 1 and 2 from panel b.



Supplementary Figure 3 Single molecule clustering and cross correlation analysis (a) Histogram of HMM-idealized states for each of the K-means derived clusters L1, L2, M and H obtained by clustering single molecule trajectories from all the experimental conditions (B^{act}, B* and C). (b) Representative molecule showing the FRET states assigned by HMM upon K-means clustering. (c) The number of states for clustering was selected through the use of the Bayesian Information Criterion (BIC). (d) The mean and standard deviation of the FRET states from the four K-means derived clusters is shown. *** indicates an extremely significant ($p<0.001$) difference between all pairwise comparisons as determined by the Tukey test. (e) Sample static trajectory from the B^{act} condition with the raw donor (Cy3, green), acceptor (Cy5, red), FRET (black) trajectories, and idealized HMM models (cyan).The corresponding cross correlation analysis of donor and acceptor trajectories with time lags from 0-50 is shown to the right of each panel. (f) Sample dynamic trajectory from the B^{act} condition and corresponding cross correlation analysis.



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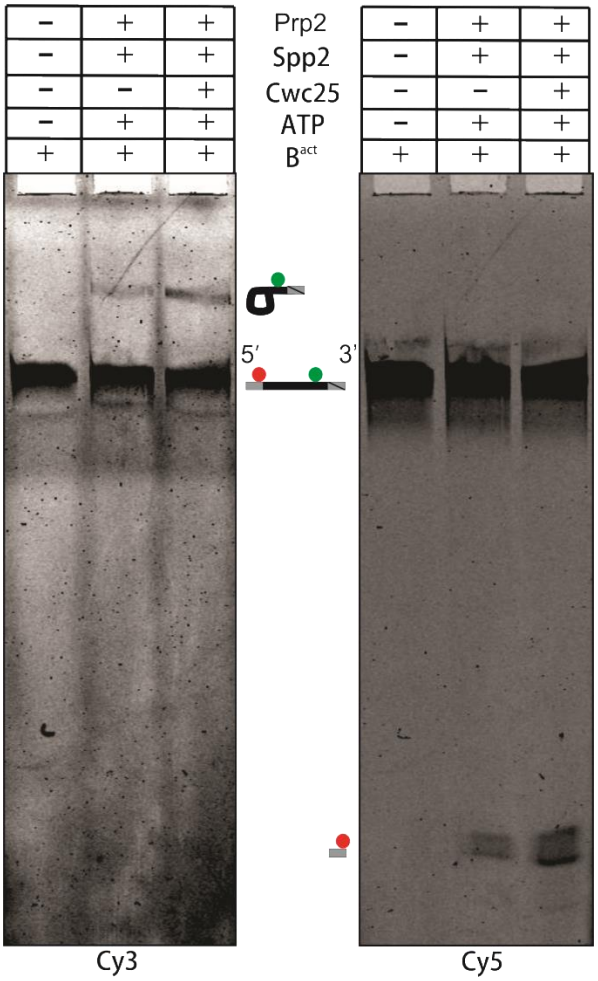
	L2 -> H		H -> L2		M -> H		H -> M	
Parameter	B*	C	B*	C	B*	C	B*	C
A1	90.50 +/- 1.32	70.53 +/- 1.10	65.60 +/- 4.30	40.58 +/- 1.23	61.00 +/- 3.03	91.65 +/- 2.08	54.90 +/- 4.14	80.33 +/- 1.18
t_1 (s)	3.92 +/- 0.14	1.84 +/- 0.05	2.82 +/- 0.11	2.54 +/- 0.10	2.33 +/- 0.13	1.65 +/- 0.05	2.40 +/- 0.18	4.09 +/- 0.09
A2	94.40 +/- 2.75	27.76 +/- 5.61	89.70 +/- 4.01	55.51 +/- 4.01	135.84 +/- 3.24	105.75 +/- 3.40	140.41 +/- 4.10	68.46 +/- 1.59
t_2 (s)	0.29 +/- 0.02	0.08 +/- 0.03	0.78 +/- 0.04	0.21 +/- 0.02	0.35 +/- 0.02	0.16 +/- 0.01	0.43 +/- 0.02	0.45 +/- 0.02
t_w (s)	2.07	1.34	1.64	1.19	0.96	0.85	0.98	2.42
$k_{w,observed}$ (s^{-1})	0.48	0.74	0.61	0.84	1.04	1.17	1.01	0.41
$k_{w,actual}$ (s^{-1})	0.42	0.68	0.55	0.78	0.98	1.11	0.95	0.35

Supplementary Figure 4 Post-first step splicing signature and single molecule kinetic analysis (a) FRET probability distribution for molecules stalled by the addition of Prp16 (K379A) dominant negative (DN) mutant. (b)TODP for Prp16DN mutant. (c) Transition Density Plots (TDPs) for the B* and C complex molecules scaled to the number of transitions determined by HMM. (d) Cumulative distribution plot of dwell times extracted for the indicated transition and fit with either a single- or double-exponential rate equation. (e) Parameters for the double-exponential equations fitted to the dwell time data. To reduce the dimensionality of the data, a weighted average rate constant k_w was calculated by utilizing the amplitudes associated with each time constant as weighting factors. k_w was used for K_{eq} calculations and rate comparisons between B* and C complex conditions.



Supplementary Figure 5 Confirmation of B^{act} complex activity using recombinant proteins. 15% polyacrylamide gel scanned with a variable mode Typhoon imager. Ubc4 pre-mRNA assembled in B^{act} complexes and supplemented with or without recombinant proteins Prp2, Spp2, Cwc25. This represents the uncropped unedited form of the gel presented in Figure 1 (main text).

UBC4 Wildtype (WT)	5’-GAACUAAGUGAUC**(5-N-U)**AGAAAG*GUAUGUCUAAAGU UAUGGCCACGUUUCAAAUGCGUGCUUUUUUUUUAAAACU UAUGCUCUUAUUUACU**A**ACAAAA**(5-N-U)**CAACAUGCUAUUG AACUA**G**A**G**AUCCACCUACUUCAUGUUT-3’*
UBC4 3’Splice Site mutant (3’SS)	5’-GAACUAAGUGAUC**(5-N-U)**AGAAAG*GUAUGUCUAAAGU UAUGGCCACGUUUCAAAUGCGUGCUUUUUUUUUAAAACU UAUGCUCUUAUUUACU**A**ACAAAA**(5-N-U)**CAACAUGCUAUUG AACUA**C**A**C**AUCCACCUACUUCAUGUUT-3’*
DNA splint (dSplint)	5’-GTTGATTTTGTTAGTAAATAAG(SP9)GTTTTAAAAAAAAAGCACGC-3’

Supplementary Table 1 Sequence information of oligonucleotides used in this study. The Ubc4 intron is italicized, and the allyl-amine modified uridines are denoted as (5-N-U). The red and green colors represent positioning of the Cy5 and Cy3 fluorophores, respectively. In the 3’SS mutant, the two bold and underlined cytosines replace guanines in the wildtype 3’ splice site. The bold and underlined A is the BP adenosine. dSplint is the DNA splint used for templated ligation to synthesize the pre-mRNA as described[34]. Sp9 denotes a 9-carbon linker.

Segment	μ_{FRET}	HMM state	$\mu_{ACP}-\mu_{DNR}/(\mu_{ACP}+\mu_{DNR})$	Cluster
1	0.51	0.5	0.039	M
2	0.31	0.325	-0.37	L2
3	0.51	0.5	0.030	M
4	0.82	0.8	0.628	H
5	0.47	0.5	-0.075	M
6	0.32	0.325	-0.352	L2
7	0.56	0.5	0.123	M
8	0.32	0.325	-0.353	L2
9	0.55	0.5	0.111	M
10	0.76	0.8	0.525	H
11	0.50	0.5	0.012	M
12	0.72	0.8	0.454	H
13	0.52	0.5	0.061	M
14	0.87	0.8	0.747	H
15	0.45	0.5	-0.084	M
16	0.31	0.325	-0.366	L2

Supplementary Table 2 K-means clustering parameters used on the HMM assigned FRET states: Segment number (first column) the mean raw FRET value (second column), HMM state (third column), and normalized difference of mean acceptor and mean donor intensities for each segment (fourth column).

Initial FRET	Final FRET	Fraction of molecules			Prp16 DN
		Bact	B*	C	
0.12	0.12	0.11	0.00	0.00	0.03
0.12	0.33	0.11	0.02	0.00	0.00
0.12	0.50	0.02	0.01	0.00	0.00
0.12	0.80	0.01	0.02	0.00	0.00
0.33	0.12	0.12	0.02	0.01	0.00
0.33	0.33	0.52	0.11	0.06	0.09
0.33	0.50	0.06	0.12	0.08	0.02
0.33	0.80	0.01	0.39	0.29	0.01
0.50	0.12	0.01	0.01	0.00	0.00
0.50	0.33	0.05	0.11	0.08	0.02
0.50	0.50	0.09	0.06	0.03	0.06
0.50	0.80	0.02	0.26	0.31	0.07
0.80	0.12	0.01	0.02	0.00	0.00
0.80	0.33	0.01	0.37	0.30	0.01
0.80	0.50	0.01	0.24	0.29	0.03
0.80	0.80	0.04	0.12	0.32	0.70

Supplementary Table 3 TODP quantification for all data sets. Molecules with at least one occurrence of the FRET transition given by the Initial and Final FRET states in columns one and two are counted and divided by the total number of molecules in that transition. Molecules that only occupy one state are accounted for in rows where the Initial and Final FRET states are equal.

Condition	Number of molecules	Average photobleaching time (Seconds)
B^{act} complex	297	11.8±7.0
B* complex	157	15.9±10.9
C complex	154	16.5±13.5

Supplementary Table 4 Comparison of average photobleaching times and number of molecules per condition.

Fraction	Pre-Cwc25 addition	Post-Cwc25 addition	Classification
0.11	High FRET with long dwell	High FRET with long dwell	Catalysis of 1st step
0.39	Various behaviors (excluding high FRET with long dwell)	High FRET with long dwell	Cwc25 mediated enhancement
0.33	Fast Switching	Slow/No switch (no stable high FRET)	Prp2 unbound
0.08	Fast Switching	Fast Switching	Cwc25 not bound
0.09	N/A	Elevated Noise	N/A

Supplementary Table 5 Classification of molecules from the observation of the same molecule chased from the B* to the C complex with the inclusion of a dark period during Cwc25 addition.