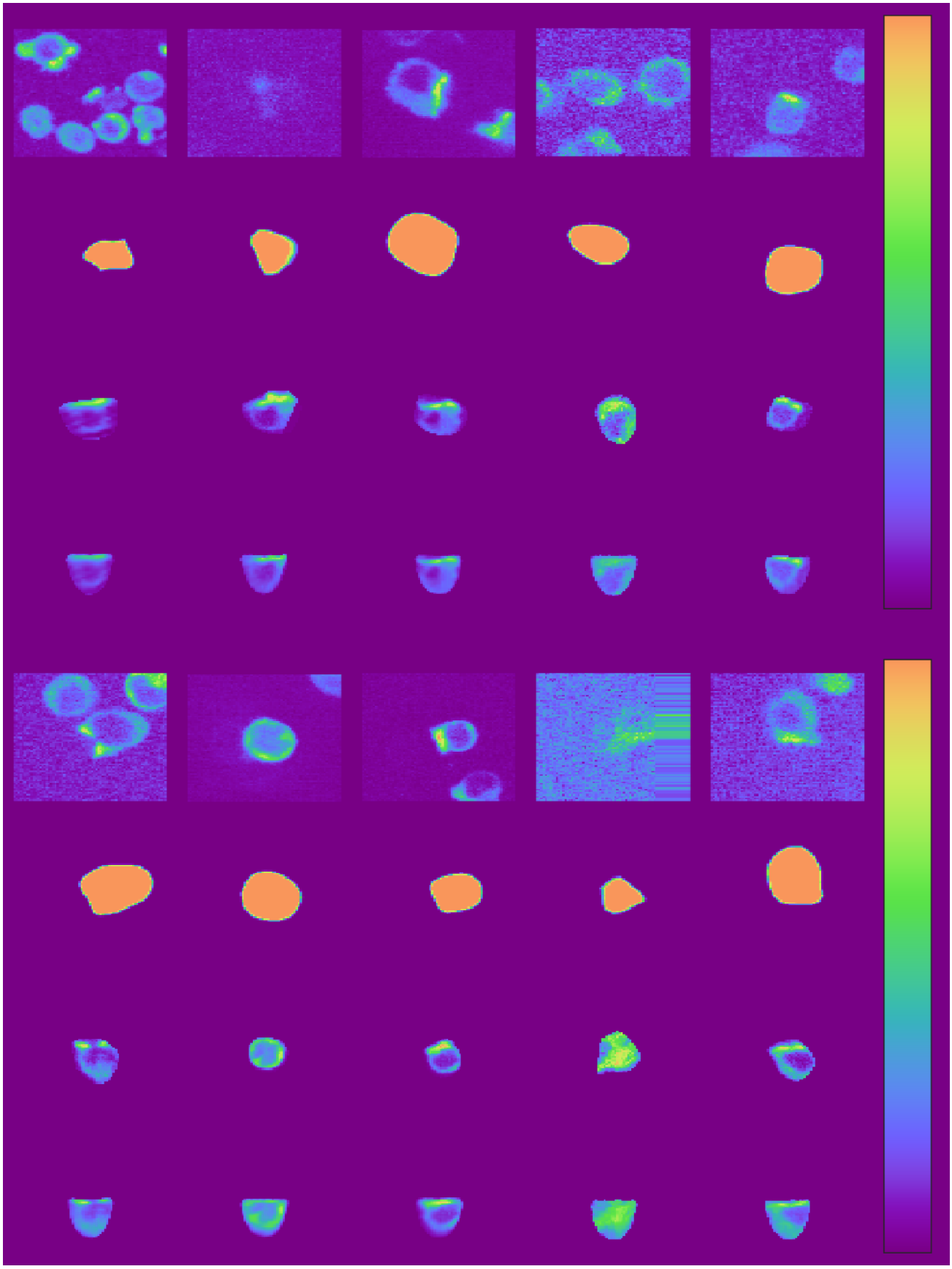


Figure S1. Example images from the segmentation, alignment and morphing pipeline. Images are shown for ten cells. For each cell, the panels from top to bottom are the original fluorescence image, the segmented region, the result after rigid alignment, and the result after morphing.

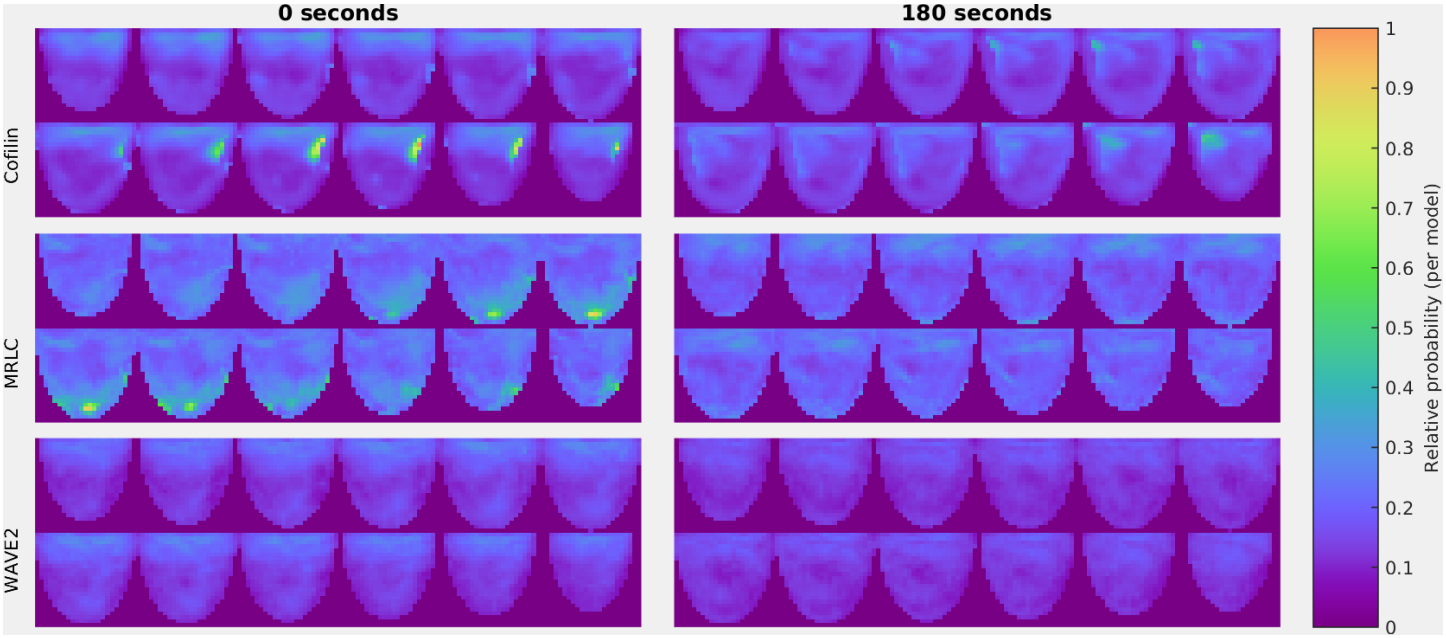


Figure S2. Illustrations of the standard deviations of the spatiotemporal models. The standard deviations of the models for cofilin, MRLC, and WAVE2 shown in Figure 3B are shown. Each panel contains slices perpendicular to the synapse of the full model at 0 or 180 seconds after synapse formation for each sensor. Within a panel, the slices start at the upper left corner and move vertically through the model to the upper right, then wrap to the lower left corner and continue to move vertically towards the lower right slice. The full scale for this figure is 1.32 times higher than Figure 3B.

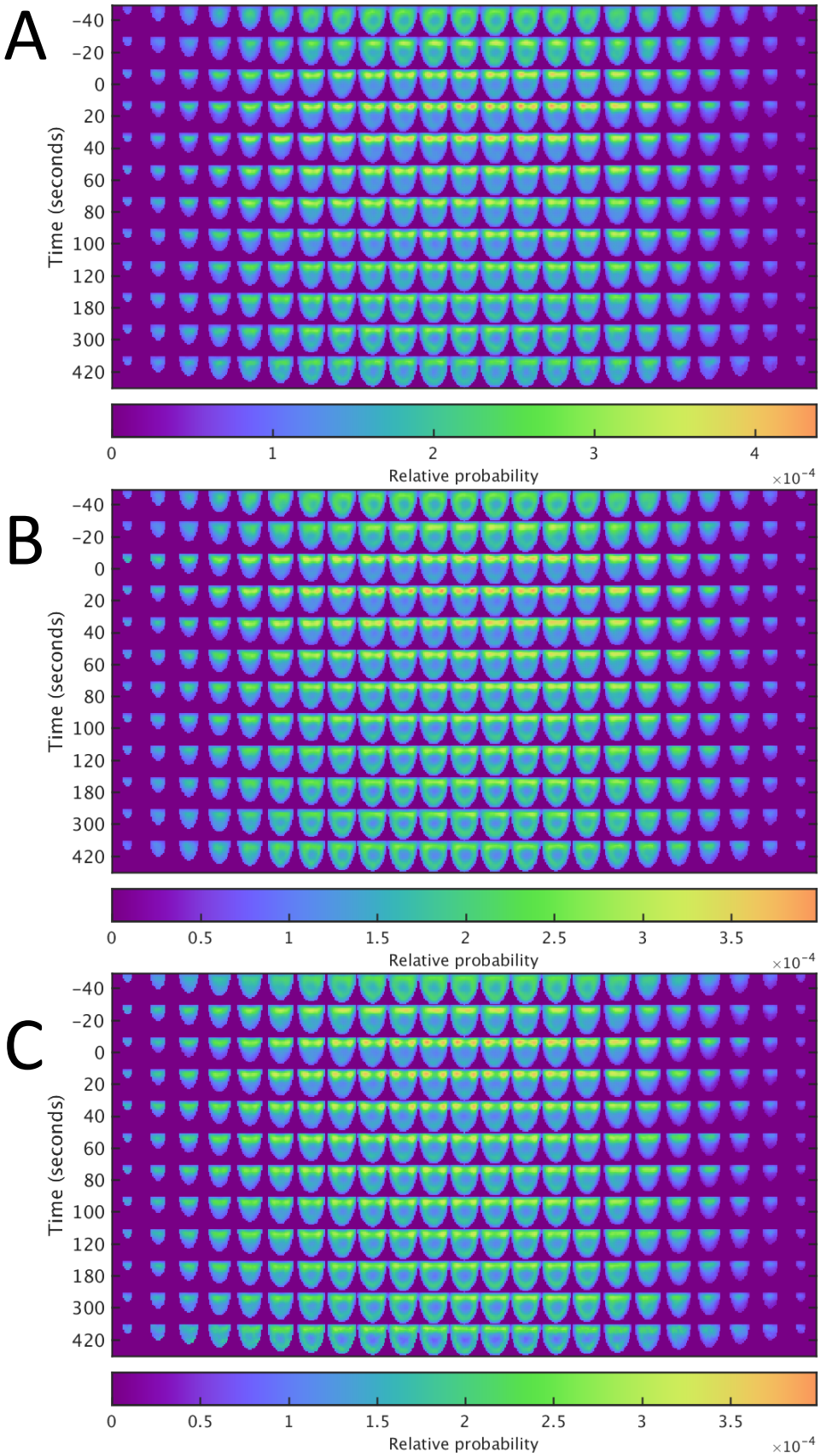


Figure S3. Spatiotemporal models for actin distribution using two-point annotation. Actin images were processed after annotation using a line across the synapse to improve alignment of the synapse plane. **A**) Full stimulus. **B**) Costimulation blockade. **C**) Costimulation blockade in the presence of active Rac and Cofilin.

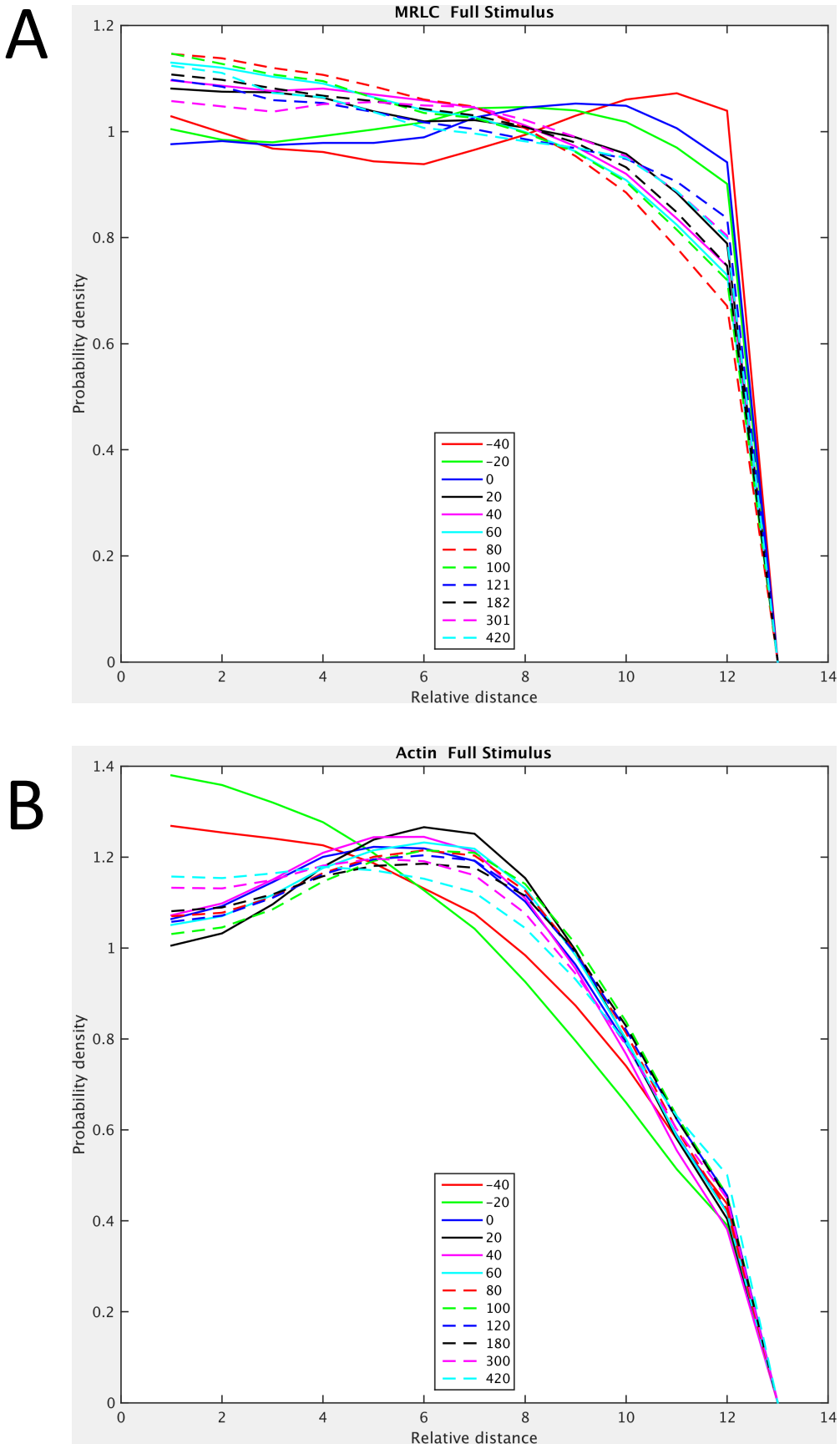


Figure S4. Analysis of radial distributions confirms differences between myosin and actin. The models for myosin regulatory light chain (**A**) and actin (**B**) at various time points were integrated for the synapse region as a function of the radial distance from the center of the synapse. The model for actin was created using two point synapse annotations to provide improved radial resolution.

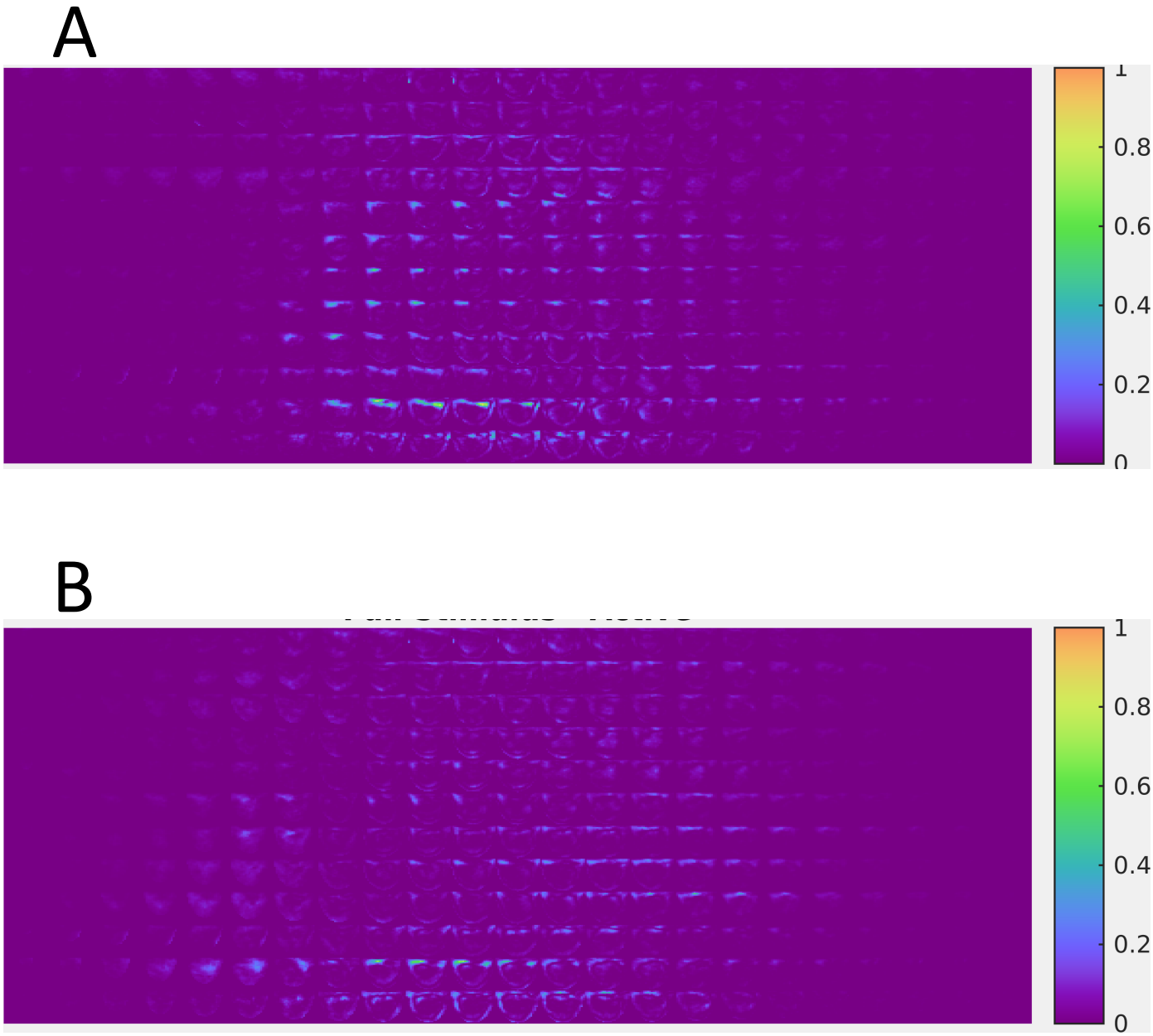
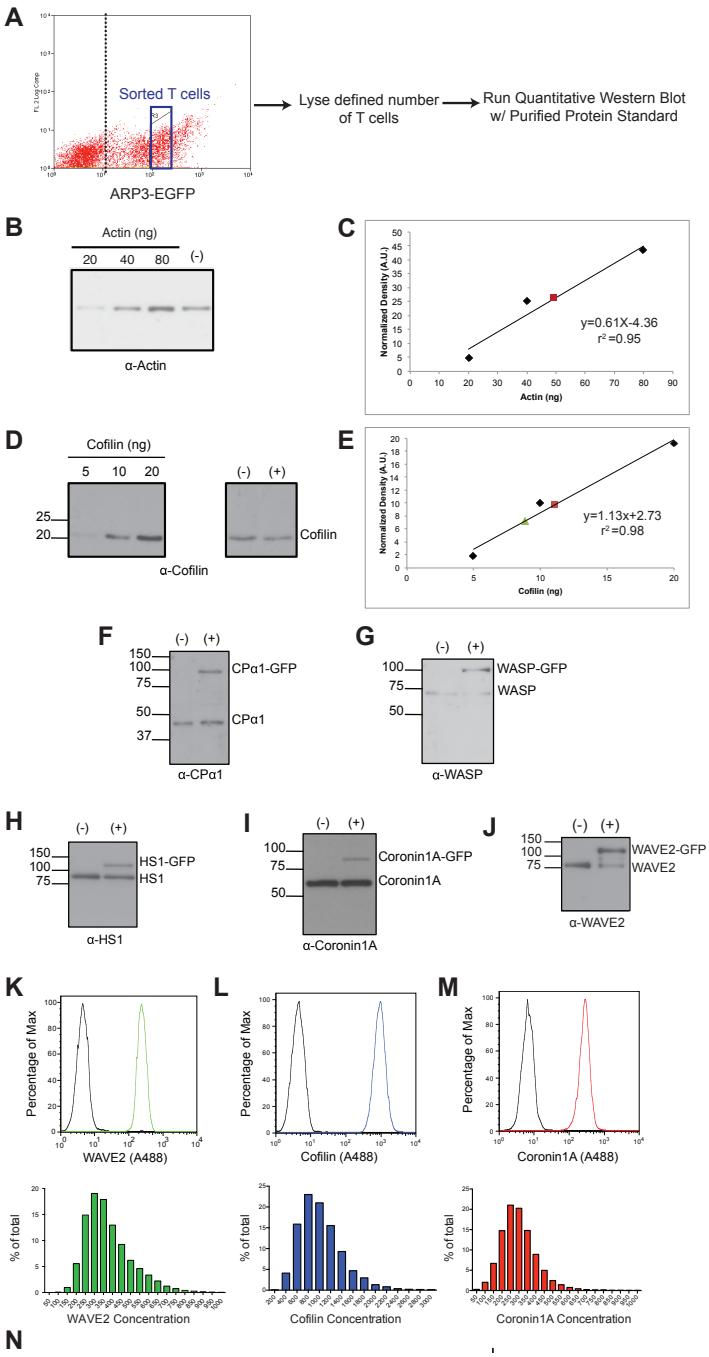


Figure S5. Comparison of LAT spatiotemporal dynamics reveals restoration of costimulation by active Rac and Cofilin. Spatiotemporal maps were created using two-point annotations as described in the Methods. Maps of the absolute value of the difference between maps are shown for full stimulation and B7 blockade (**A**) and full stimulation and reconstitution (**B**). Each row shows a sequence of slices perpendicular to the synapse for a different time point (with time increasing from top to bottom). Note the major differences in the synapse region when full stimulus and costimulation blockade are compared (especially from 0 to 100 seconds after synapse formation), and that differences are much smaller between full stimulation and reconstitution.



N

Concentration Ranges (μM)								
WAVE2			Cofilin			Coronin1A		
5th Percentile	Mean	95th Percentile	5th Percentile	Mean	95th Percentile	5th Percentile	Mean	95th Percentile
3.65	6.44	10.43	2.66	5.56	9.28	40.78	85.71	141.16

Figure S6. Quantification of actin regulatory proteins in primary mouse T cells. (A) A representative sort of transduced 5C.C7 T cells for quantitative analysis of actin regulator levels is shown. **(B-E)** Actin regulator expressing 5C.C7s (+) and non-transduced controls (-) were sorted and run against protein standards. Standard curves are provided with calculated unknown values plotted for controls (-) red square, transduced endogenous (+) green triangle, and GFP-tagged purple circle. **(F-J)** Actin regulators for which purified proteins were not available were quantified based on the density measurement of the GFP tagged protein band (2.6μM). **(K)** 5C.C7 T cells were stained for endogenous levels of WAVE2, **(L)** Coronin1A, and **(M)** Cofilin and single cell levels were assessed by flow cytometry. Frequency distribution of single cell protein levels is given with the coefficient of variation (CV). **(N)** Based on the frequency analysis, the 5th to 95th percentile molar concentrations were determined and are given.

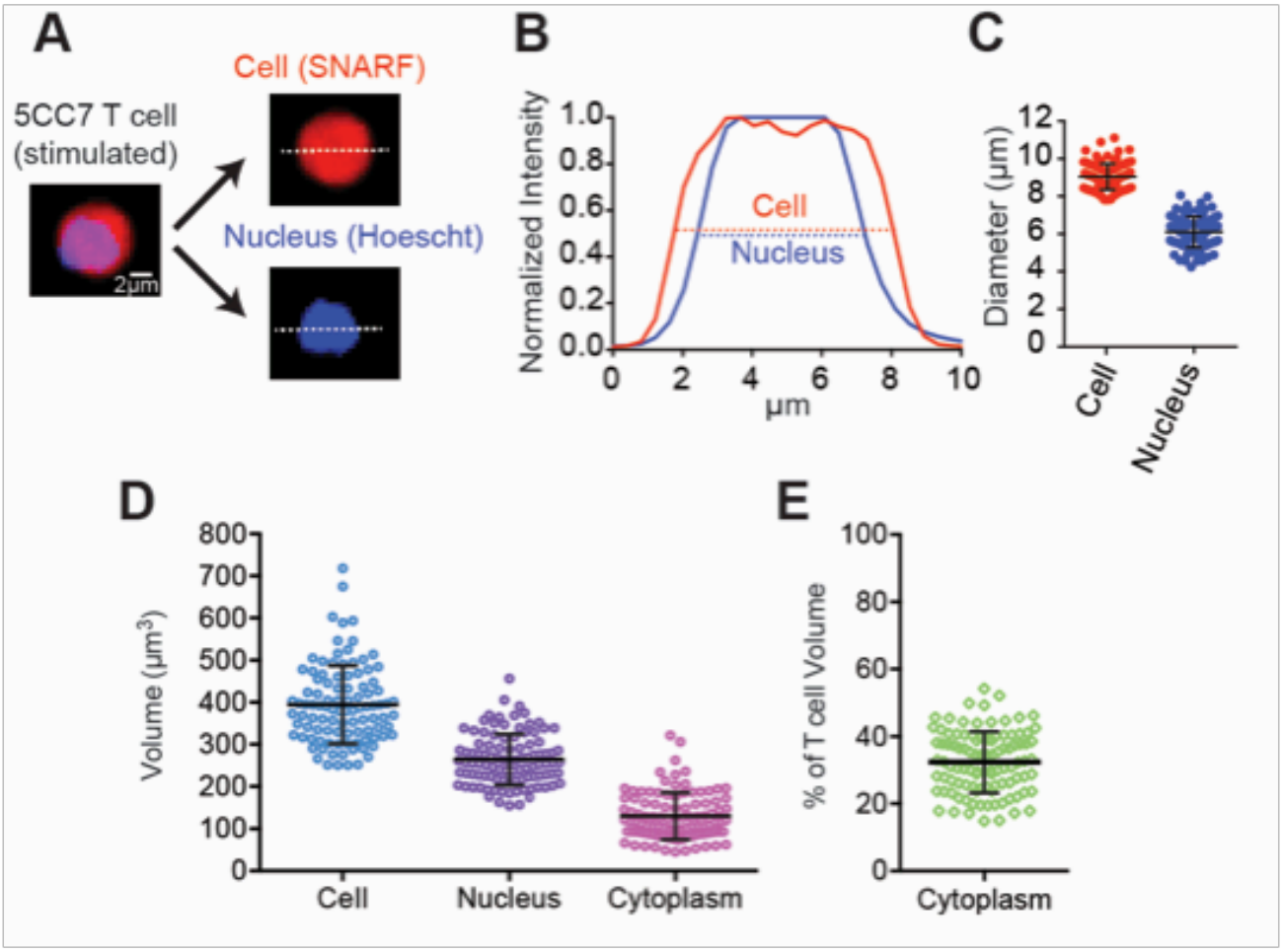


Figure S7. Volume measurements of primary mouse T cells. A) A representative primed 5C.C7 T cell is shown labeled with SNARF-1 (whole cell stain) and Hoechst (nuclear stain). **(B-C)** Diameters of the whole cell and nucleus were measured based on linescans and calculation of the full width at half maximum of the intensity distribution (n=100). **(D)** Volume measurements for the whole T cell, nucleus, and cytoplasm are given along with **(E)** the percentage of the T cell that is cytoplasm. All volumes were calculated from diameters in panel C and error bars are standard errors of the mean.

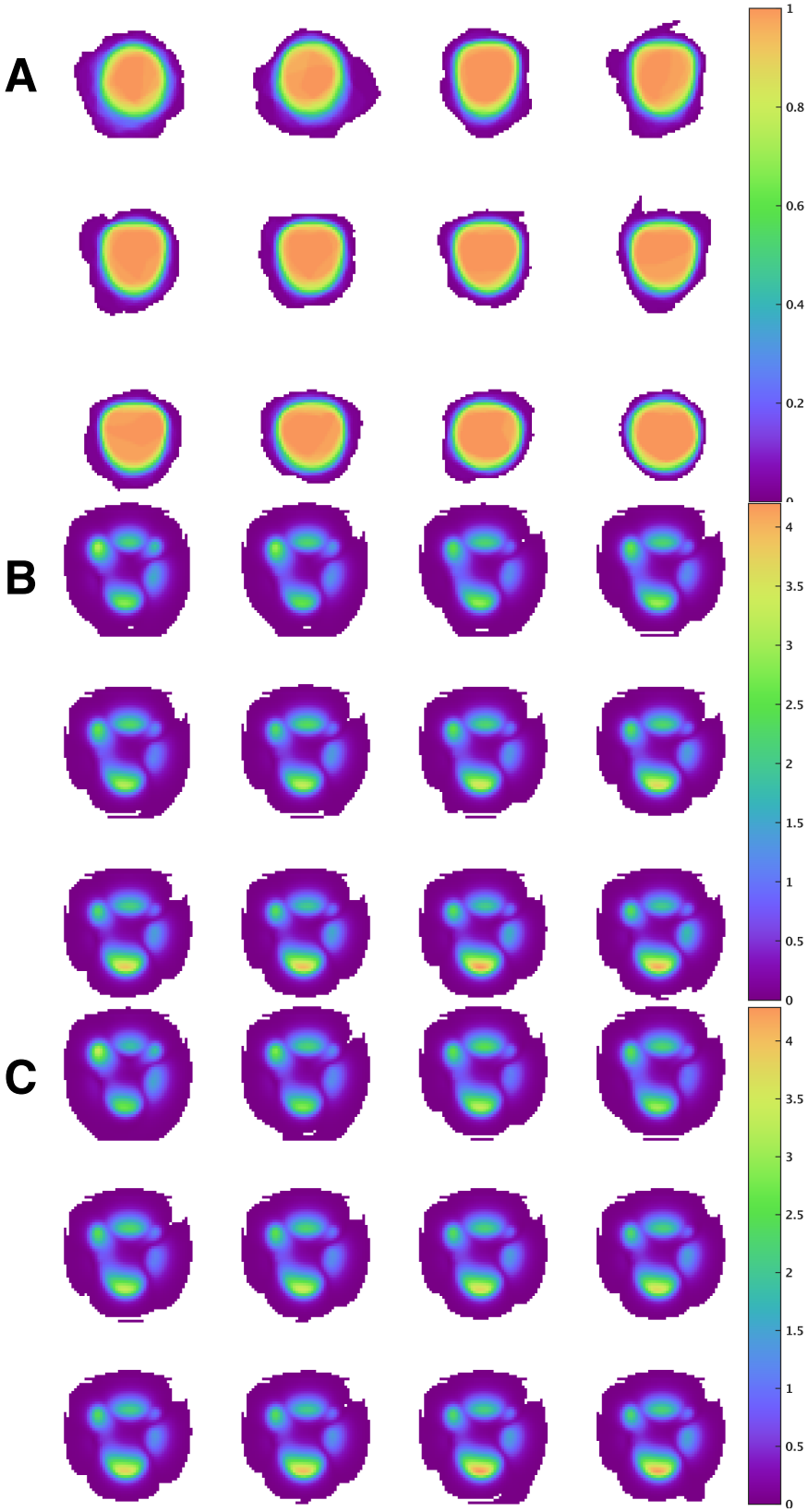


Figure S8. Variation in cell shape and morphing across time points and conditions. A) Variation in unmorphed cell shape for the central slice at various time points under full stimulus conditions. The color of each pixel shows the fraction of cells that included that pixel in their rigidly-aligned cell shape (orange indicates those pixels common to all cells). **B**) The average morph vector over all cells was taken for each voxel of the central slice, and the magnitude of that vector is shown for each time point for the full stimulus (B) or B7 blockade (C) conditions. The colorbar shows the amount of linear displacement (in voxels in the 3D map) corresponding to each pixel color.

Sensor	Number of imaging fields		Number of cell couples		Number of synapse time points marked	
	Full stimulus	**B7 blockade**	**Full stimulus**	**B7 blockade**	**Full stimulus**	**B7 blockade**
ARP3	5	5	110	95	1177	990
Actin	6	17	105(91)	160(86)	1130(962)	1708(903)
CPalpha1	6	7	127	101	1327	1093
Cofilin	7	5	134	107	1217	1019
Coronin1A	5	8	100	91	1053	953
HS1	7	7	96	70	1069	730
LAT	5	7	57(57)	37(37)	610(610)	397(397)
MRLC	5	3	49	38	508	392
WASP	4	5	86	59	909	641
WAVE2	5	5	77	67	807	707

Table S1. The number of imaging fields and manually marked synapses used to construct the spatiotemporal model of each condition-sensor combination. Each synapse was marked at up to 12 time points (numbers in parentheses indicate the number of synapses marked at two points on the synapse). There were a total of 124 fields, 1766 individual cell couple movies, and 18,437 marked time points. In addition, 12, 93 and 55 cell couples were imaged for WAVE2, Actin and LAT (respectively) under reconstitution conditions.

Parameter name	Coarse stage	Fine stage
α	0.15	0.15
β	0.1	0.1
γ	0.2	5
δ	0.1	0.1
$\varkappa$	1	10
λ	0.95	0.75
Iterations	240	240
GIterations	0	5
W_{line}	-5	-5
W_{edge}	0	0
σ_1	1	1
σ_2	2	1
σ_3	2	1

Table S2. The manually tuned parameters used for both stages of segmentation by the active contour method. Parameters are named to correspond to our implementation, which is a modified version of http://www.mathworks.com/matlabcentral/fileexchange/28149-snake-active-contour.

Relative time	Actin	ARP3	Cofilin	Coronin1A	CPalpha1	HS1	MRLC	WASP	WAVE2	average
Full Stimulus										
-2	0.150	0.163	0.141	0.145	0.152	0.152	0.252	0.148	0.146	0.161
-1	0.148	0.154	0.132	0.152	0.138	0.151	0.219	0.124	0.136	0.150
0	0.136	0.143	0.120	0.141	0.128	0.147	0.220	0.116	0.130	0.142
1	0.137	0.143	0.124	0.134	0.131	0.140	0.194	0.118	0.133	0.139
2	0.139	0.144	0.122	0.137	0.140	0.145	0.189	0.117	0.132	0.141
3	0.136	0.142	0.124	0.137	0.134	0.142	0.175	0.116	0.130	0.137
4	0.141	0.146	0.126	0.141	0.137	0.145	0.172	0.116	0.129	0.139
5	0.136	0.143	0.126	0.135	0.134	0.136	0.156	0.119	0.126	0.135
6	0.140	0.146	0.126	0.137	0.129	0.136	0.160	0.115	0.126	0.135
7	0.138	0.143	0.128	0.134	0.131	0.140	0.149	0.122	0.122	0.134
8	0.138	0.148	0.122	0.136	0.127	0.142	0.131	0.131	0.135	0.134
9	0.140	0.145	0.121	0.137	0.134	0.140	0.144	0.128	0.124	0.135
B7 Blockade										*average*
-2	0.167	0.154	0.163	0.159	0.162	0.143	0.256	0.155	0.176	0.171
-1	0.159	0.147	0.151	0.150	0.160	0.150	0.201	0.149	0.155	0.158
0	0.154	0.139	0.143	0.143	0.160	0.148	0.173	0.142	0.156	0.151
1	0.142	0.138	0.143	0.134	0.159	0.141	0.173	0.147	0.151	0.148
2	0.148	0.144	0.141	0.144	0.157	0.133	0.187	0.139	0.158	0.150
3	0.148	0.140	0.143	0.146	0.150	0.139	0.173	0.146	0.140	0.147
4	0.146	0.142	0.140	0.141	0.156	0.139	0.165	0.139	0.141	0.145
5	0.141	0.138	0.138	0.144	0.152	0.134	0.158	0.141	0.138	0.143
6	0.140	0.139	0.142	0.140	0.147	0.135	0.173	0.134	0.144	0.144
7	0.142	0.144	0.146	0.146	0.148	0.133	0.145	0.141	0.128	0.141
8	0.140	0.141	0.153	0.147	0.155	0.137	0.142	0.150	0.141	0.145
9	0.143	0.144	0.149	0.149	0.154	0.131	0.143	0.137	0.142	0.144

Table S3. Average amount of morphing required for each sensor at each time point. The values represent the average of the integral of the diffeomorphic function that transforms each cell to the template.

	Time relative to synapse formation (s)											
Sensor	-40	-20	0	20	40	60	80	100	121	181	300	420
ARP3	5.28E+00	**2.60E-02**	**2.30E-08**	**5.41E-05**	**9.81E-05**	**3.45E-04**	**1.78E-05**	**2.08E-04**	**4.24E-04**	6.40E-02	**4.64E-03**	**2.95E-04**
Actin	5.05E+00	5.28E+00	1.24E+00	1.49E+00	1.73E+00	2.95E+00	1.86E+00	1.10E+00	1.86E-01	5.66E+00	1.05E+00	5.05E+00
CPalpha1	5.51E+00	2.00E+00	9.24E-01	2.24E+00	6.40E-02	**1.07E-02**	**3.45E-04**	**3.19E-03**	**4.30E-02**	**5.39E-04**	**4.46E-05**	5.65E-02
Cofilin	2.82E+00	6.87E-01	1.74E-01	**2.63E-03**	**4.52E-04**	**1.81E-05**	**1.80E-05**	**1.11E-04**	**5.45E-06**	**3.59E-03**	**3.93E-03**	5.89E-01
Coronin1A	2.89E+00	5.39E+00	**6.82E-03**	8.50E-01	**7.38E-03**	**1.28E-02**	**1.67E-03**	**2.05E-02**	**9.64E-03**	6.10E-02	**4.82E-04**	**4.05E-03**
HS1	5.91E+00	**1.44E-02**	**4.68E-07**	**1.67E-05**	**4.64E-03**	**8.03E-03**	8.85E-02	1.11E-01	5.66E+00	3.22E+00	1.74E+00	5.52E+00
MRLC	3.90E+00	6.08E+00	1.04E+00	2.18E+00	5.85E+00	2.53E+00	4.59E+00	6.27E+00	1.06E+00	2.20E+00	5.51E+00	6.27E+00
WASP	4.86E+00	6.08E+00	2.35E-01	1.08E+00	1.85E+00	1.02E+00	2.24E+00	1.01E+00	5.28E+00	5.77E-01	3.84E+00	1.86E+00
WAVE2	2.95E+00	**5.40E-04**	**1.16E-04**	**2.87E-05**	**1.98E-05**	**5.08E-06**	**1.33E-07**	**1.00E-06**	**1.88E-07**	**2.33E-03**	**3.76E-02**	1.06E+00

Table S4. Statistical testing of differences in sensor enrichment in the immunological synapse region between the full stimulus condition and costimulation blockade. For each sensor and time point, the enrichment in the region shown in Figure 5A was calculated for all cells (for both conditions), and the lognormal distributions of those values were compared for the two conditions using Welch's t test. The table shows the resulting p-values after Bonferroni-Holmes correction for multiple hypothesis testing. Sensors and time points with statistically significant differences at the 0.05 level are shown in bold.