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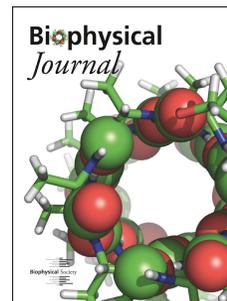
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1 Conformational states control Lck switching between free and 2 confined diffusion modes in T cells

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20 Abstract

21 T cell receptor (TCR) phosphorylation by Lck is an essential step in T cell activation. It is
22 known that the conformational states of Lck control enzymatic activity; however, the
23 underlying principles of how Lck finds its substrate over the plasma membrane remain
24 elusive. Here, single-particle tracking is paired with photoactivatable localization microscopy
25 (sptPALM) to observe the diffusive modes of Lck in the plasma membrane. Individual Lck
26 molecules switched between free and confined diffusion in both resting and stimulated T
27 cells. Lck mutants locked in the open conformation were more confined than Lck mutants in
28 the closed conformation. Further confinement of kinase-dead versions of Lck suggests that
29 Lck confinement was not caused by phosphorylated substrates. Our data supports a model
30 where confined diffusion of open Lck results in high local phosphorylation rates, and
31 inactive, closed, Lck diffuses freely to enable long-range distribution over the plasma
32 membrane.

33 **Statement of Significance**

34 Phosphorylation of the TCR-CD3 complex by the kinase Lck is an essential step in T cell
35 activation, but how membrane-bound Lck finds and phosphorylates its substrates is not well
36 understood. Here, we examined the diffusive behavior of individual Lck molecules by single
37 particle tracking in conjunction with photoactivatable localization microscopy (sptPALM).
38 Our data demonstrate that Lck molecules frequently switch between confined and free
39 diffusion and spent a prolonged time in the confined diffusion mode in stimulated T cells
40 when the kinase is in the open conformation. This may underpin a dual-state search strategy
41 in which open Lck exhibits confined diffusion, resulting in high local phosphorylation rates,
42 and closed Lck diffuses freely to enable wide-range scanning of the plasma membrane.

43 **Introduction**

44 T cell signaling is a tightly controlled process of simultaneous and sequential spatiotemporal
45 events, involving membrane remodeling, and the redistribution of signaling proteins (1).
46 Engagement of the T cell receptor (TCR) with an antigenic pMHC on the surface of an
47 antigen-presenting cell (APC) leads to the formation of immunological synapses (2) and
48 initiates downstream signaling events that lead to T cell activation (3). The Src family kinase
49 Lck plays a crucial role in the signaling cascade. TCR engagement results in the membrane
50 release (4) and phosphorylation of the immunoreceptor tyrosine-based motifs (ITAMs)
51 located in the cytoplasmic tails of the CD3 ζ chain by Lck (5). Phosphorylated sites on the
52 TCR-CD3 complex become docking sites for the zeta chain-associated protein kinase 70
53 (ZAP70), that is further phosphorylated by Lck (6) before recruiting other proteins in the
54 signaling cascade that are necessary for complete T cell activation.

55 The kinase Lck, an essential TCR signaling protein, is a 56 kDa protein comprised of a Src
56 homology (SH) 4 domain at the N-terminus, followed by a unique domain, an SH3 domain,
57 an SH2 domain, a kinase domain, and a short C-terminal tail. Lck is anchored to the plasma
58 membrane through its SH4 domain via post-translational acylation on three specific sites: a
59 myristoylated Gly2 (7), and palmitoylated Cys3 and Cys5. The latter two enable membrane
60 binding, and thus Lck diffusion in the inner leaflet of the plasma membrane (8). Notably, Lck
61 is also found in the cytoplasm, as the palmitoylation is reversible (9), and is recruited to the
62 immunological synapse. The unique domain interacts with the CD3 ϵ subunit in the TCR-
63 CD3 complex (10), as well as the co-receptors CD4 and CD8 (11), via zinc-mediated bonds.
64 However, Lck does not require the co-receptors for recruitment to the immunological
65 synapse, or for TCR triggering (12), suggesting that freely diffusing Lck is sufficient for T
66 cell activation.

67 Lck conformation is regulated by the phosphorylation of two tyrosine residues: Tyr³⁹⁴, whose
68 phosphorylation increases Lck activity, and Tyr⁵⁰⁵, whose phosphorylation reduces Lck
69 activity (13, 14). Intramolecular interactions between the phosphorylated Tyr⁵⁰⁵ (pTyr⁵⁰⁵) and
70 the SH3 and SH2 domains cause rearrangements that keep Lck in an inactive state (15, 16).
71 When dephosphorylated by CD45, Lck exists in an open, primed conformation. When Tyr³⁹⁴
72 is trans-autophosphorylated (14), rearrangements in the activation loop stabilize the active

73 conformation (17). The diffusion behavior (18) and conformational state of Lck (19, 20) are
74 thought to be regulated by the activation state of the cell. The conformational state also
75 influences Lck clustering (21). This means that not only does Lck conformational state
76 regulate Lck enzymatic activity, but also aids in its diffusive search strategy.

77 Whether Lck becomes ‘active’, i.e., converted into the open conformation upon TCR
78 engagement, has been controversial. There is evidence of global changes in relative
79 populations of closed and open Lck in resting *versus* stimulated T cells (19, 20). These
80 studies propose that Lck undergoes conformational changes upon T cell activation, driving it
81 from its closed state to an open state, therefore enhancing its activity. Using biochemical
82 analyses, conformational heterogeneity was observed in resting and stimulated T cells (22),
83 suggesting a “standby-model” in which ~40% of Lck is in the open conformation in both
84 resting and stimulated T cells. Ballek *et al.* challenged these observations in a later report that
85 used different cell lysis conditions (23). Other studies, based on measurements of
86 fluorescence resonance energy transfer (FRET) between fluorescent proteins fused to the N-
87 and C-terminals of Lck, found that 62% of Lck was pre-activated in T cells (24), and
88 concluded there was no significant change in open *versus* closed populations of Lck even
89 after T cell stimulation (25). While different papers report different percentages of open Lck
90 in pre-stimulated cells, constitutively active Lck was also found in CD8⁺ memory T cells, and
91 may account for the enhanced sensitivity to antigen in these cells (26). A pool of active Lck
92 existing prior to T cell stimulation led to the idea that rapid TCR triggering post receptor
93 engagement may be caused by changes in Lck spatial rearrangements as opposed to, or in
94 addition to conformational changes. Using single-molecule localization microscopy in fixed
95 cells, we previously showed that Lck distributed differently on the cell surface depending on
96 its conformational state (21), with open Lck residing preferentially in clusters, and inactive
97 Lck preventing clustering. However, this study only captured the overall distribution of open
98 or inactive Lck, and the movement of Lck clusters, but to understand the search strategy of
99 the membrane-bound kinase, the dynamic behavior of individual molecules needs to be taken
100 into account.

101 The dynamic behavior of Lck was previously mapped with single particle tracking (SPT) in
102 live cells, revealing, for example, the differences in Lck diffusion in stimulated *versus* resting
103 T cells and the formation of microclusters, but without linking dynamics to conformational
104 states (18, 27). Overall changes in diffusion constants were observed, as well as segregation
105 into different confinement zones, attributed to actin and other proteins compartmentalizing
106 the membrane (27, 28), or to the formation of membrane microdomains (18). Recently, Lck
107 compartmentalization upon TCR stimulation was attributed to the formation of close-contact
108 zones between the T cell membrane and the stimulating surface, possibly because of
109 exclusion of CD45, in line with the kinetic segregation model (29). These works, however,
110 did not take into account the conformational change in Lck.

111 In the current study, we utilize SPT using photoactivatable localization microscopy
112 (sptPALM) (30) as a tool to study the diffusion of wild type (WT) and mutated Lck, lacking
113 the tyrosine residues on positions 394 and 505, to measure the dynamics of the inactive and
114 open forms, respectively (19, 20). Lck variants were tagged with photoactivatable monomeric

115 cherry (PAmCherry) (31), expressed in Jurkats E6.1 cells and imaged in resting and
116 activating conditions. Single trajectories were extracted and analyzed in order to find periods
117 when the proteins underwent confined diffusion, and the fraction of confined versus free
118 proteins was determined (32). Measurements of different Lck variants showed that the open
119 form of Lck spent more in confinements compared to the inactive form. Taken together, the
120 data suggest that Lck continuously switched between open and closed states, a process that is
121 likely to determine the probability of productive encounters between Lck and its substrates.

122

123 **Methods and Materials**

124 **Plasmids**

125 Lck and Lck10 were amplified by PCR and inserted within the *Ecot*1 and *Age*1 restriction
126 sites of a pPAmCherry-N1 plasmid. Y394F, Y505F and K273R mutations were further
127 introduced via site-directed mutagenesis.

128 **Sample Preparation**

129 Jurkat cells were cultured in RPMI medium (Gibco) containing phenol-red and supplemented
130 with 10% (vol/vol) FBS, 2 mM L-glutamine (Invitrogen), 1 mM penicillin (Invitrogen) and 1
131 mM streptomycin (Invitrogen). Cell cultures were passaged normally every ~48 hours, when
132 the cell count reached $\sim 8 \times 10^5$ viable cells per ml. The cells were cultured for at least 1 week
133 (3-4 passages) after thawing prior to transfection and imaging. No cells were used after
134 passage 20.

135 Cells were transfected by electroporation (Neon; Invitrogen); briefly, cells were collected
136 before reaching a cell density of 8×10^5 cell/ml and while $\geq 90\%$ viable. The cells were washed
137 twice with 1x PBS in 37°C and resuspended in the resuspension buffer (R-buffer) provided
138 with the Neon kit. Three pulses of 1325 V with 10 ms duration were applied. The cells were
139 allowed to recover in clear RPMI 1640 medium (Gibco) supplemented with 20% HI-FBS for
140 overnight. Prior to imaging, fresh warm (37°C) media with 40 mM HEPES, pH 7.4 was
141 added to achieve a final concentration of 20 mM HEPES.

142 1.5H coverslips (Marienfeld-Superior) were water bath-sonicated in four 30-minutes stages: 1
143 M KOH, Acetone, EtOH and ultra-pure (18 M Ω .cm) water. The coverslips were then allowed
144 to adsorb 0.01% PLL (Sigma) in ultra-pure water for 15 minutes. Excess solution was later
145 aspirated and the coverslips were baked-dry in 60 °C for 1 hour. Finally, after cooling-down,
146 the coverslips were coated with either 0.01 mg/ml anti-CD3 (OKT3; eBioscience) and 0.01
147 mg/ml anti-CD28 (CD28.2; Invitrogen) for stimulating conditions or 0.01 mg/ml α CD90 for
148 (Thy-1; eBioscience) for resting conditions and let rest in 4°C overnight before imaging. The
149 coverslips were washed 3 times with phosphate buffer saline (PBS) pre-warmed to 37°C
150 before the cells were transferred onto them to interact with the antibodies. For live-cell
151 experiments, imaging took place ~5 minutes after cell-transfer or fixed with 4%

152 paraformaldehyde (P6148; Sigma) in 37°C, followed by 3 washing cycles with PBS for
153 fixed-cell imaging.

154 To verify the activation status of T cells, Jurkat E6.1 cells on resting and stimulating surfaces
155 were fixed with 4% paraformaldehyde and immunostained with a primary antibody against
156 CD3 ζ Y142 conjugated to Alexa Fluor 647 (K25-407.69, BD Biosciences). Fluorescence
157 intensity was determined with TIRF microscopy (Zeiss Elyra) and analyzed as previously
158 described (33). For Ca²⁺ imaging in T cells, cells were loaded with Fluo-4, washed, incubated
159 on the indicated surface for 15 min or kept in solution and imaged with confocal microscopy
160 (Zeiss LSM780). Note, Jurkat cells exhibit Ca²⁺ basal fluxes. To measure CD69 expression,
161 10⁶ Jurkat E6.1 cells were stained with anti-human CD69 Alexa Fluor488 (310916,
162 BioLegend) at 4°C for 30 min, placed on resting or stimulating surfaces or kept in solution,
163 and analysed with flow cytometry (BD bioscience FACSanto II and FlowJo software).

164 **Imaging**

165 For each sptPALM experiment 10,000 frames were acquired in a ~50 frames per second
166 (18 ms exposure time) rate on a total internal reflection fluorescence (TIRF) microscope
167 (ELYRA P1, Zeiss) in 37°C using a 100 \times oil immersion objective (N.A. = 1.46) and a 67.5°
168 incident beam angle. The frame rate was chosen to match mean-square displacement of 160
169 nm for a diffusion of 2 $\mu\text{m}^2/\text{sec}$, which is below the size of a point spread function and fits
170 well with our experiments where diffusion coefficients were < 2 $\mu\text{m}^2/\text{sec}$. PAmCherry fused
171 to Lck variants were continuously photoactivated using a 405 nm laser radiation tuned to 0.5-
172 5 μW (interchangeable during acquisition to maintain a low density) and continuously
173 excited with a 561 nm laser tuned to 2.5 mW. Point density was monitored by using ZEN
174 (Zeiss) online-processing tool.

175 **Data Analysis**

176 All accumulated data are comprised of three biologically independent experiments, i.e., each
177 mutant was imaged in two or more cells (in one of the three repetitions, where a repetition
178 relates to a different transfection) in each cell-activation state (stimulated or resting). We used
179 Diatrack (34) for fitting the point spread functions (PSFs) to a Gaussian with a 1.75 pixel
180 width (1 pixel \approx 0.097 nm) and then to track the particles by setting the search radius to 10
181 pixels. The data was later analyzed by a custom MATLAB (Mathworks) adaptation of the
182 trajectory analysis part of a previously published multi-target tracing (MTT) code (32).
183 Immobile particles (i.e., particles that had trajectories with an end-to-end distance of less than
184 two pixels) and trajectories shorter than 15 frames were excluded from analysis. Stages of
185 confined and free diffusion were detected according to equation 1, with $D_{\text{free}} = 2.15 \mu\text{m}^2/\text{sec}$
186 (Fig. S2b, bottom), $W = 4$ and t_w was the sum of the exposure time and the CCD reading
187 time (~19.7 ms). To detect time spent in confinement, each sequence was segmented to non-
188 overlapping windows of 5 frames and in each block of 5 frames, the ratio of confined:total
189 particles was calculated. Each value of one 5 frames-window is a count in the histogram. The
190 level of confinement, L_{Conf} , was calculated according to:

191
$$(1) L_{Conf} = \frac{D_{free} \times W \times t_w}{var(r)}$$

192 where D_{free} is the diffusion coefficient of freely diffusing Lck in $\mu\text{m}^2 \text{sec}^{-1}$, W is the window
193 size in frames, t_w is the temporal length of the window in seconds and $var(r)$ is the variance
194 in μm^2 , which was determined for each window. All data processing and statistical analyses
195 were performed in MATLAB.

196 **Statistical Tests**

197 To compare between two populations of confinement fractions, that do not normally
198 distribute, we used the Mann-Whitney U test, while the Kruskal-Wellis test was used for
199 multiple datasets followed by a bonferroni post-hoc test. **** and n.s. indicate $p \leq 0.00001$
200 and $p > 0.01$, respectively. Ranges around median and mean values in supplementary text are
201 the 95% confidence intervals calculated from bootstrapping the data by sampling 10,000
202 times.

203 **Results and Discussion**

204 The goal of this study was to determine whether the diffusion properties of individual Lck
205 molecules were influenced by the conformational states of the kinase. Since Lck is found
206 both in a cytosolic pool and attached to the inner leaflet of the plasma membrane (9), we
207 chose sptPALM, since single molecule trajectories under TIRF illumination enable the
208 quantification of only membrane bound Lck. To compare Lck in different conformational
209 states requires the expression of Lck mutants. Because these mutants also impact on Lck
210 activity, and hence the T cell activation status, it was necessary to express Lck in wild-type
211 Jurkat cells that also express endogenous Lck (35). This allowed us, for example, to compare
212 the diffusion of kinase-dead Lck in resting and activated cells since endogenous Lck
213 facilitates T cell activation in cells that also express kinase-dead Lck. Notably, sptPALM
214 experiments do not require high levels of overexpression, so the total levels of Lck can be
215 kept within or close to the physiological range. Finally, we needed to control the T cell
216 activation status. We chose to do this by seeding T cells onto activating and non-activating
217 antibody-coated surfaces. These conditions not only result in controlled T cell activation (33),
218 but also enable the recording of long single molecule trajectories. To determine whether
219 individual Lck molecules switch between diffusion modes and gain statistical certainty, it is
220 necessary to record large numbers of trajectory of sufficiently long durations. Alternative
221 activation protocols such as protein-decorated supported lipid bilayers, are not ideal for SPT
222 experiments as cells move laterally, with different speeds on activating and non-activating
223 bilayers, and severely limit both the number of trajectories per cell and the length of each
224 trajectory that can be recorded. While antibody-coated surfaces result in well-controlled T
225 cell activation statuses (Fig. S1) and enabled sptPALM experiment, it should be noted that
226 TCR clustering and mobility is different in T cells on antibody-coated surfaces compared to T
227 cells on laterally mobile supports, with both protocols resulting in mobile and immobile
228 TCRs under both activating and non-activating conditions (33). In summary, Jurkat E6.1
229 were transfected with either wild-type Lck (wtLck) fused to PAmCherry (wtLck-
230 PAmCherry) or Lck variants, such as a truncated construct of Lck containing only the first

231 ten amino acids that are responsible for Lck anchoring to the membrane (Lck10-
 232 PAmCherry). T cells were incubated for 5 minutes at 37°C on a coverslip coated with anti-
 233 CD3 and anti-CD28 antibodies (stimulated) or anti-CD90 (resting), and then imaged, either in
 234 live-cell conditions, or after chemical fixation.

235 **Identification of free and confined states of Lck in live T cells**

236 For each SPT experiment, we acquired 10,000 frames with an 18 ms exposure for the
 237 duration of ~197 s under TIRF illumination. While imaging was done with continuously
 238 photo-activating and exciting the fluorophores, the rate of photo-activation, and thus density
 239 of emitting fluorophores, was kept deliberately low in order to ensure only individual Lck
 240 molecules were tracked. Because Lck could be cytosolic, or found in fast moving cytosolic
 241 vesicles, which can appear briefly in the TIRF zone, we removed trajectories shorter than 15
 242 frames. Likewise, immobile particles (see Methods) were excluded from analysis to eliminate
 243 Lck in cytosolic vesicles that were docked at the plasma membrane.

244 To address whether individual Lck molecules could switch between different diffusion
 245 modes, we employed a previously described post-tracking analysis that can distinguish
 246 between confined and free diffusion in each trajectory (Fig. S2) (32). Briefly, every trajectory
 247 is first fragmented into overlapping windows. For each window, the normalized variance of
 248 the location of the particle is calculated as a measure of the level of confinement, L_{Conf} ,
 249 according to:

$$250 \quad (2) \quad L_{Conf} = \frac{D_{free} \times W \times t_w}{var(r)}$$

251 where D_{free} is the diffusion coefficient of freely diffusing Lck in $\mu\text{m}^2 \text{sec}^{-1}$, W is the window
 252 size in frames, t_w is the temporal length of the window in seconds and $var(r)$ is the variance
 253 in μm^2 . For the value of D_{free} , we could not choose the diffusion coefficient of full-length Lck
 254 due to the broad distribution in diffusion coefficients, and because the potential protein-
 255 protein interactions, even in resting cells, may mean that full-length Lck may not solely
 256 diffuse freely. Thus, we chose the diffusion coefficient of $2 \mu\text{m}^2 \text{sec}^{-1}$ for Lck10-PAmCherry
 257 (in resting T cells) for all versions of Lck, as Lck10 is membrane anchored and does not
 258 interact with other proteins. The L_{Conf} values for wtLck-PAmCherry in stimulated T cells falls
 259 largely between the L_{Conf} values for Lck10-PAmCherry in resting cells and wtLck-
 260 PAmCherry in fixed cells (Fig. 1A). Assuming that Lck10-PAmCherry in resting cells
 261 diffuses essentially freely, and wtLck-PAmCherry in fixed cells is permanently immobile.
 262 The data is evidence that wtLck-PAmCherry in stimulated T cells either has a homogeneous
 263 diffusion of intermediate speed, has two or more pools of Lck with different diffusion
 264 coefficients, or that individual Lck molecules alter between a fast/free diffusion mode and a
 265 slow/confined diffusion mode.

266 To test which of the three scenarios apply to wtLck-PAmCherry, we defined a threshold for
 267 L_{Conf} . We followed the published procedure (32) of choosing the most common L_{Conf} value
 268 (dotted line in Fig. 1A) of the protein of interest as the threshold. Importantly, the L_{Conf}
 269 threshold is defined in the ensemble measurement (i.e., the L_{Conf} histogram, Fig. 1A) and then

270 applied to individual Lck trajectories (Fig. 1B). To distinguish between confined Lck
271 molecules and Lck molecules that temporarily slowed down, we only regarded a molecule as
272 confined (yellow areas in Fig. 1B-C) if it has an L_{Conf} value above the threshold for three or
273 more consecutive windows. If Lck diffusion is homogenous with an intermediate diffusion
274 coefficient, this criterium would not be fulfilled, and no confinement zones would be detected
275 in individual trajectories. If two pools of Lck exist that have different diffusion modes, but
276 Lck molecules do not switch between diffusion modes while being tracked, the entire length
277 of the trajectory would either be contained within or excluded from the confinement zone,
278 i.e., there would be two types of trajectories. If, and only if, a molecule switched diffusion
279 mode would part of the trajectory lay within a confinement zone and part outside the
280 confinement zone (Fig. 1B-C). The latter was observed for almost all mobile trajectories of
281 wtLck-PAMCherry in stimulated T cells (Fig. 1B-E), providing strong evidence that
282 individual wtLck-PAMCherry molecules in live cells on activating antibody-coated surfaces
283 switched between free and confined diffusive states (Fig. 1D-E). In contrast, in fixed cells,
284 only confined or immobile molecules were observed (Fig. 1D-E).

285 **Wild-type Lck was more confined in stimulated than resting T cells**

286 Our data strongly suggest that individual Lck molecules frequently switched between at least
287 two diffusion modes, a more confined diffusion mode and a free diffusion mode. Since
288 previous studies provided evidence that T cell activation decreases the overall diffusion of
289 Lck (18, 27), we asked whether T cell activation altered the Lck diffusion mode overall, or
290 altered the time spend in either diffusion mode. In our experiments, resting T cell data was
291 generated by placing T cells expressing wtLck-PAMCherry onto coverslips coated with anti-
292 CD90 antibodies. This resulted in good T cell adhesion (Fig. 2A), but not TCR signaling or T
293 cell activation (Fig. S1) (33). On antibody-coated surfaces, T cell shape and contact size is
294 broadly comparable for resting and stimulating conditions, with T cells often spreading more
295 on the activating surface, but with considerable cell-cell variability under both conditions.
296 Importantly, good cell adhesion meant that a high number of trajectories could be recorded
297 under both cell conditions (Table 1). To visually compare the two conditions, we color-coded
298 each trajectory according to their initial diffusion coefficient (Fig. 1A left with color code
299 below) and applied the L_{Conf} threshold value to each trajectory (Fig. 1B right, same color
300 code as Fig. 1). It can be seen that Lck overall diffused faster in resting cells with diffusion
301 coefficients of $1.16 \mu\text{m}^2 \text{s}^{-1}$ (1.15-1.17) to $0.69 \mu\text{m}^2 \text{s}^{-1}$ (0.68-0.7) for resting and stimulated
302 cells, respectively (Fig. 2A, Fig. S3a; Movie S1: resting - right, stimulated - left). Further,
303 fewer confinement zones were detected for wtLck-PAMCherry in resting than stimulated
304 cells (Fig. 1A). When comparing the L_{Conf} histogram of wtLck in stimulated T cells (Fig. 2B,
305 blue) *versus* resting T cells (Fig. 2B, orange), it is noticeable that the values in activated cells
306 are shifted to higher values, resulting in a mean L_{Conf} value of 32.9 in stimulated cells and
307 29.1 in resting cells.

308 Next, we examined whether the decrease in local displacement variance is due to a
309 redistribution of wtLck-PAMCherry into confinements that would result in an increase in the
310 number of consecutive steps that fall above the L_{Conf} threshold value. Thus, we segmented the
311 total video into segments of five frames (Fig. S4), in which we asked how many particles, out

312 of the total number of particles imaged, were confined. Histograms obtained for stimulated
313 and non-stimulated cells (Fig. 2C) were collected. There was a clear difference in the peak
314 value for the two populations, as well as a larger tail of high values for wtLck-PAmCherry in
315 stimulated cells. As a consequence, the populations were statistically different (Fig. 2C) when
316 tested against the null hypothesis according to which the samples are drawn from the same
317 population, using the rank sum test, with different medians and non-overlapping 95%
318 confidence intervals with the values of 27.27% (26.67-27.78) and 22.22% (21.82-22.73) for
319 stimulated and resting cells, respectively. The percentage of confined wtLck-PAmCherry
320 were 31.0% (30.6-31.3) and 26.4% (26.1-26.7) in stimulated and resting cells, respectively.

321 Overall, these results show that wtLck-PAmCherry diffused slower in stimulated cells
322 compared to resting cells because individual Lck molecules spent more time in confinement
323 zones. These results are in agreement with an increase in wtLck-PAmCherry clustering in
324 fixed stimulated *versus* fixed resting T cells (21). Confinement of Lck could be caused by
325 lipid rafts (36, 37), microdomains (38, 39) and/or because Lck became trapped in protein
326 clusters (18), such as TCR clusters (40). In our experiments, potential Lck binding partners
327 were not fluorescent, and the low laser intensity made it unlikely that neighboring Lck
328 molecules were photo-converted in the same imaging frame. Thus, it is not possible to
329 classify detected Lck molecules as belonging to a Lck cluster, bound to the TCR, or other
330 signaling proteins. Because we also observed that individual Lck molecules experience
331 confinement in resting T cells, it is unlikely that all confinement events were caused by TCR
332 signaling clusters since the TCR phosphorylation levels in resting T cells on antibody-coated
333 surfaces was hardly detectable (Fig. S1). It should be noted that we previously found that
334 Lck clusters in stimulated cells on antibody-coated surfaces were constantly remodeled, and
335 not positionally stable (21), but it is possible that Lck confinement is caused by Lck
336 clustering in stimulated T cells.

337 **Membrane anchoring alone is not contributing to Lck confinement**

338 Lck confinement may be attributed to the formation of membrane domains, i.e., changes in
339 membrane order, as a result of TCR triggering (41). We used a truncated version of Lck,
340 Lck10, that contained the first ten amino acids that Lck anchors to the membrane via post-
341 translational lipid modifications. If membrane domains are responsible for the slowdown and
342 confinement of full-length Lck, Lck10 should also exhibit different diffusion in resting and
343 activated T cells. Thus, we repeated the sptPALM experiments with Lck, again plotting the
344 initial diffusion coefficient and confinement analysis for individual Lck 10 trajectories (Fig.
345 3A). Particularly when compared to full-length Lck (Fig. 2A), there was noticeably less
346 difference in Lck10 diffusion in resting and stimulated T cells (Fig. 3A; Movie S2), although
347 the latter appeared fully spread and activated. Indeed, the diffusion coefficients for Lck10-
348 PAmCherry were very high, and similar in stimulating and resting conditions (Fig. S3b and
349 Table 1). The overall level of confinement of Lck10-PAmCherry was almost identical for
350 both resting and stimulated cells, with a peak L_{Conf} value of 7.2 and 7.7, respectively (Fig.
351 3B). These values were significantly different from the ones found for wtLck-PAmCherry,
352 with most of the probability function having a value below the threshold. A histogram of
353 confinement events (Fig. 3C) shows comparable peak values for both stimulated and resting

354 cells. No statistically significant difference was found between the two samples (Fig. 3C, top
355 panel), as shown by median of 9.62% (9.43-9.8) and 9.68% (9.52-10.00) for Lck10-
356 PAmCherry expressed in stimulated and resting cells, respectively. Further, the mean fraction
357 of confined particles was also similar in stimulating and resting cells, with values of 14.0%
358 (13.7-14.2) and 14.7% (14.5-15.0), respectively. These values were lower than those found
359 for wtLck-PAmCherry, suggesting Lck10-PAmCherry was far less confined than wtLck-
360 PAmCherry, even in stimulated cells. Our data is in agreement with previous work by
361 Kusumi and colleagues that also showed different diffusions between a full-length Lck and
362 Lck10 (27). Taken together, the data strongly suggest that the increased confinement
363 observed for full-length wtLck-PAmCherry was not due to global changes in membrane
364 organization or membrane domains (18), as confinement of Lck10 in resting and stimulated T
365 cells was similar.

366 **Open Lck is highly confined in stimulated and resting cells**

367 Next, we quantified the influence of conformation on confinement of Lck in live cells. First,
368 we introduced a Tyrosine-to-Phenylalanine mutation at position 505 in Lck (Lck^{Y505F}). The
369 mutation prevents the binding of Lck pTyr⁵⁰⁵ to its own SH2 domain. This mutation is well
370 known as ‘constitutively open’ (19-21, 25, 42) and ‘hyperactive’ (13). It should be noted
371 overexpression of Lck^{Y505F} can lead to spontaneous, antigen-independent triggering of the
372 TCR. To keep our experiments consistent throughout this study, we thus expressed Lck^{Y505F}-
373 PAmCherry to a similar level as wtLck-PAmCherry in Jurkat cells. The same sptPALM
374 analysis as above yielded images pseudo-colored for initial diffusion and confinement (Fig.
375 4A; Movie S3). Resting cells still had a smaller surface contact zone than stimulated T cells
376 despite the over-expression of Lck^{Y505F}-PAmCherry.

377 An overall change in the diffusion constants due to cell activation was observed, with values
378 of $0.65 \mu\text{m}^2 \text{s}^{-1}$ (0.64-0.66) and $0.95 \mu\text{m}^2 \text{s}^{-1}$ (0.94-0.96) in stimulated and resting cells,
379 respectively (Fig. S3c and Table 1). Further, L_{Conf} values for Lck^{Y505F}-PAmCherry were
380 higher than that of wtLck-PAmCherry (Fig. 4B), with peak values of 39.28 and 42.53 in
381 stimulated and resting cells, respectively, with $< 50\%$ of $\log_{10}(L_{\text{Conf}})$ events above the
382 confinement threshold. In contrast to wtLck-PAmCherry, the L_{Conf} distributions of Lck^{Y505F}-
383 PAmCherry were similar in resting and stimulated T cells despite the shoulder at low L_{Conf}
384 values in resting cells. In both resting and activated T cells, Lck^{Y505F}-PAmCherry was more
385 confined than wtLck-PAmCherry. This was also observed in the histograms of the confined
386 fractions (Fig. 4C), with a large population of Lck^{Y505F} molecules falling into the right tail of
387 the distribution. Importantly, unlike in the corresponding data for wtLck-PAmCherry, these
388 values were not significantly different from each other (Fig. 4C, top), with median values and
389 overlapping 95% confidence interval of 26.55% (26.32-26.67) and 26.39% (26.14-26.67) for
390 stimulated and resting cells, respectively. The fractions of confined Lck^{Y505F}-PAmCherry were
391 29.9% (29.6-30.11) and 30.0% (29.7-30.2) in stimulated and resting cells, respectively.

392 These data show that when Lck is locked in the open state, it is also driven into a more
393 confined diffusive behavior, which is comparable with wtLck-PAmCherry in stimulated cells
394 (Fig. S5). Given that Lck in the open conformation exhibited confined diffusion and

395 hyperactivity (13, 14), it is highly likely that the confined diffusion state results in high local
396 phosphorylation rates. Open Lck may become preferentially trapped in protein clusters that
397 form upon TCR triggering (18, 40), resulting in slower overall diffusion, and more
398 confinement. However, open Lck was also strongly confined in resting T cells suggesting that
399 protein clusters *per se* are not necessary to confine Lck. Confinement may be caused by
400 enhanced protein-protein interactions including Lck self-association (21). If the level of
401 confinement is indicative of the fraction of Lck in the open conformation, our data support
402 the notion that TCR triggering results in a higher proportion of wild-type Lck in the open
403 conformation (19, 20).

404 **Inactive Lck is as confined as wild-type Lck in resting cells**

405 To further investigate the hypothesis that Lck conformation regulates Lck diffusive behavior,
406 we expressed an inactive form of Lck in Jurkat cells. A mutation in position 394 converting a
407 tyrosine into phenylalanine (Lck^{Y394F}) prevents its trans-autophosphorylation that is necessary
408 for Lck activity and results in reduced-activity (14) or an inactive Lck (13), likely because of
409 the hyper-phosphorylated Tyr505 that constitutively closes the enzyme (19). Despite the
410 over-expression of Lck^{Y394F}-PAmCherry, T cells adhered well on non-activating surfaces.
411 Visual examination of Lck^{Y394F}-PAmCherry suggests that closed Lck was rarely confined
412 even in T cells on stimulating surfaces (Fig. 5A; Movie S4).

413 As with the wtLck and Lck^{Y505F}, Lck^{Y394F}-PAmCherry did undergo a decrease in diffusion
414 coefficient due to stimulation, from $1.24 \mu\text{m}^2 \text{s}^{-1}$ (1.22-1.26) in resting cells to $0.88 \mu\text{m}^2 \text{s}^{-1}$
415 (0.87-0.89) in stimulated cells (Table 1, Fig. S3d). We applied the same sptPALM analysis to
416 Lck^{Y394F}-PAmCherry, and lower L_{Conf} values were obtained with peak values of 32.93 and
417 30.36 in stimulated and resting cells, respectively (Fig. 5B). Histograms of the fraction of
418 confined Lck^{Y394F}-PAmCherry showed the populations were skewed towards lower values,
419 and also contained the shoulder at low L_{Conf} values (Fig. 5C). Similar to Lck^{Y505F}-
420 PAmCherry, Lck^{Y394F}-PAmCherry showed no statistically significant difference between
421 stimulated and resting cells (Fig. 5C, top panel), and medians of 22.22% (21.88-22.58) and
422 21.95% (21.43-22.22) for Lck^{Y394F}-PAmCherry in stimulated and resting cells, respectively.
423 The mean confinement fractions were 26.1% (25.9-26.3) and 26.2% (25.9-26.6) for Lck^{Y394F}-
424 PAmCherry in stimulated and resting cells, respectively. It is possible that the K273R
425 mutation in Lck prevents the rearrangements in the activation loop that prevent interaction
426 with other proteins, thus, limiting confinement (13). Our data are consistent with Wan *et al.*
427 who also report a slower diffusion rate for Lck^{Y394F} than wild-type Lck due to less Lck-Lck
428 interactions (24).

429 The confinement fraction values we found for the inactive Lck were smaller than the ones
430 found for the open Lck (Fig. S5), suggesting that each Lck activity or conformational state
431 regulates Lck diffusion. Indeed, inactive Lck had a similar level of confinement as wtLck in
432 resting cells while open Lck was similarly confined as wtLck in activated cells (Fig. S5).
433 Thus, the data supports the notion that confinements are regulated by the conformational state
434 of Lck with open Lck being more confined than inactive Lck.

435 Lck conformation and activity determine confinement

436 To delineate Lck activity from Lck conformational state, we expressed a constitutively
437 inactive Lck variant in which the lysine in position 273 in the kinase domain is replaced with
438 Arginine (Lck^{K273R}-PAmCherry, Fig. 6, Fig. S6), which has been shown to render Lck
439 kinase-dead (43). Images of resting and activated T cells expressing Lck^{K273R}-PAmCherry
440 (Fig. 6A) and the L_{Conf} histogram (Fig. 6B) looked similar to images of T cells expressing
441 wild-type Lck only, with the histogram again containing the shoulder at low L_{Conf} values.
442 Different diffusion coefficients of 0.82 $\mu\text{m}^2 \text{s}^{-1}$ (0.81-0.83) and 1.13 $\mu\text{m}^2 \text{s}^{-1}$ (1.12-1.15) were
443 observed for Lck^{K273R}-PAmCherry in stimulated and resting cells, respectively (Table 1, Fig.
444 S3e). However, similar L_{Conf} histograms, with values of 34.80 for stimulated and 37.58 for
445 resting cells, were obtained (Fig. 6B, blue and orange) with no significant difference
446 observed in the fraction of time spent confined (Fig 6C, blue and orange). Lck^{K273R}-
447 PAmCherry spent 25.8% (25.6-26.1) and 25.6% (25.4-25.9) of time confined in stimulated
448 and resting cells, respectively (Fig 6C). Thus, the level of confinement of kinase-dead Lck
449 did not depend on the T cell activation status as it did for wild-type Lck (Fig. S6), suggesting
450 that trans-autophosphorylation of wild-type Lck (13) contributes to confinement.

451 To further test this hypothesis, we expressed a constitutively open, kinase-dead mutant
452 Lck^{K273R, Y505F}-PAmCherry. The images (Fig. 6A) and the L_{Conf} histogram (Fig. 6B) were
453 similar to Lck^{K273R}-PAmCherry. Lck^{K273R, Y505F}-PAmCherry had slower diffusion coefficients
454 of 0.41 $\mu\text{m}^2 \text{s}^{-1}$ (0.41-0.42) and 0.51 $\mu\text{m}^2 \text{s}^{-1}$ (0.5-0.51) in stimulated and resting cells,
455 respectively (Fig. 6A; Fig. S3f; Movie S5), values that were slower than those obtained for
456 Lck^{K273R}-PAmCherry (Fig. S3e, f). Further, Lck^{K273R, Y505F}-PAmCherry had higher L_{Conf}
457 values in stimulated cells (Fig. 6C, purple and yellow) compared to resting cells (44.78 and
458 35.09, respectively). When comparing total trajectories, Lck^{K273R, Y505F}-PAmCherry in
459 stimulated cells was more confined than in resting cells and more than Lck^{K273R} in both cell
460 activation statuses (Fig. S6). These data support the notion that open, but not necessarily
461 enzymatically active Lck confined the kinase in distinct zones in the plasma membrane.
462 Lck^{K273R, Y505F}-PAmCherry was more confined in stimulated cells (27.0% (26.8-27.2)) than
463 resting cells (23.3% (23.1-23.5)). Moreover, the lowered confinement for the K273R-Y505F
464 mutant in resting cells compared to stimulated cells excludes the possibility of confinement
465 due to increase in hydrodynamic radius of the enzyme (Fig. S6).

466 Finally, we compared the level of confinement of open and inactive Lck mutations (Fig. S5).
467 It should be noted that these mutants were expressed in wild-type Jurkat cells and thus
468 contained endogenous, untagged wild-type Lck. It is possible that wild-type Lck affected the
469 diffusion of mutant Lck. Taken together, our data suggest that diffusion behavior could be
470 regulated by the conformational state of the enzyme. Lck^{Y394F}-PAmCherry i.e. inactive Lck
471 was less confined than wtLck-PAmCherry in stimulated cells and Lck^{Y505F}-PAmCherry i.e.
472 open Lck in stimulated and resting cells. Further, Lck^{Y394F}-PAmCherry demonstrated similar
473 confinement to that of wtLck-PAmCherry in resting cells. The values obtained for the open
474 mutant, both in stimulated and resting cells were closer to the value that we obtained for
475 wtLck-PAmCherry in stimulating conditions. Taken together, our data support the notion that
476 the open conformational state of Lck may result in Lck confinement. Thus, a model emerges

477 in which the enzyme switches between open and closed conformation, which may result in a
478 dual-state search strategy where open and active Lck is confined and closed and inactive Lck
479 diffuses freely (Fig. 7).

480 **Conclusions**

481 Phosphorylation of the TCR-CD3 complex by the kinase Lck is an essential step in T cell
482 activation (44). While the link between phosphorylation state and activity in Lck is
483 reasonably well established (45), how membrane bound Lck finds and phosphorylates its
484 substrates is not well understood. Here we provide evidence that individual Lck molecules
485 frequently switched between confined and free diffusion in resting and stimulated T cells. A
486 possible driver for the switch in diffusion modes could be the conformational states of Lck, as
487 open Lck exhibited more confined diffusion, and inactive or closed Lck exhibited more free
488 diffusion. As it has been shown that auto-inhibited Src cannot bind substrates (46), this is
489 consistent with a dual-state search strategy that enables Lck to redistribute over large areas of
490 the membrane in its closed state, and high local activity to efficiently phosphorylate TCR-
491 CD3 complexes at numerous sites in the open state. Lck interactions with other proteins (47-
492 50) and lipids (51) could also contribute to the temporary confinement of Lck. While the
493 mechanism or mechanisms for Lck confinement remain unknown, Lck conformation may
494 control the probability of interactions with binding partners to modulate T cell signaling
495 activity, particularly if confined Lck is predominately in the open and enzymatically active
496 state.

497 For technical reasons, we used coverslips coated with antibodies for our sptPALM
498 experiments, and this format may have impacted on the mobility of the TCR-CD3 complex
499 under activating conditions. We have previously conducted a detailed analysis of the
500 dynamics of the TCR-CD3 complex in T cells, and compared TCR mobility in T cells on
501 supported lipid bilayers and activating antibodies (as used here) (33). Surprisingly, we found
502 no differences in the percentage of mobile to immobile TCR complexes and clusters, but
503 subtle changes with respect to cluster remodeling during movement. In T cells on supported
504 lipid bilayers, TCR clusters moving towards the cell center and are increased in molecular
505 density, while TCR clusters moving away from the cell center exhibited a loss in molecular
506 density (33). This correlation was not readily observed in T cells on antibody-coated
507 surfaces. It is possible that the movement and remodeling of TCR-CD3 complexes in T cells
508 on immobilized antibodies impacted on Lck diffusion and confinement. In T cells activated
509 on support lipid bilayers, only short sptPALM trajectories could be recorded that could not be
510 used for a quantitative analysis of the diffusion modes of single Lck molecules. New imaging
511 technology, such as lattice light-sheet microscopy, may reveal insights in Lck behavior when
512 a T cell is in contact with antigen-presenting cell.

513 Dual-state search strategies have previously been demonstrated in other systems (52). For
514 Lck, this strategy could entail a confined state that corresponds to high Lck activity while
515 probing the local environment for substrates and a diffusive state that enables the kinase to
516 distribute quickly over the entire membrane. Such a dual-state search strategy may account
517 for the high fidelity of Lck-mediated phosphorylation of the available TCR-CD3 complexes

518 while also retaining high signaling sensitivity when membrane-detached cytosolic tails of the
519 CD3 complex are limited. The former would be mediated by the high enzymatic activity in
520 Lck clusters while the high level of diffusion of Lck in the closed state would enable the
521 latter. In conclusion, a dual-state search strategy facilitated by the behavior of individual Lck
522 molecules may be a regulatory mechanism in T cell activation.

523

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530 **Author Contributions**

531 GH performed experiments, modified analysis, analyzed data, and wrote manuscript. EP
532 established analysis and helped write the manuscript. ZY was responsible for the generation
533 of Lck constructs. DJN and JG aided in writing and drafting of the manuscript. JR provided
534 guidance with experiments. KG designed the project, interpreted the data and wrote the
535 manuscript.

536 **Conflicts of interests**

537 The authors declare no conflicts of interests.

538 Supplementary Materials

539 **Fig. S1** TCR phosphorylation, calcium fluxes and CD69 expression in resting and activated T
540 cells.

541 **Fig. S2** Relationship between Lck diffusion coefficient and confinement

542 **Fig. S3** Diffusion coefficients histograms of wtLck, Lck10, LckY505F, LckY394F,
543 LckK273R and LckK273R, Y505F in stimulated and resting Jurkat cells

544 **Fig. S4** Illustration of confinement ratio analysis

545 **Fig. S5** Comparison of confinement analysis result

546 **Fig. S6** Comparison of confinement analysis result of wtLck-PAmCherry, LckK273R-
547 PAmCherry and LckK273R, Y505F-PAmCherry in stimulated and resting cells

548 **Movie S1**

549 **Movie S2**

550 **Movie S3**

551 **Movie S4**

552 **Movie S5**

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	Resting T cells	Stimulated T cells
wtLck-PAmCherry	D = 1.16 $\mu\text{m}^2 \text{s}^{-1}$ (1.15-1.17) Confined: 26.4% (26.1-26.7) $N_{\text{total}} = 34,309$ $N_{\text{immobile}} = 2,929$ (8.54%)	D = 0.69 $\mu\text{m}^2 \text{s}^{-1}$ (0.68-0.7) Confined: 31.0% (30.6-31.3) $N_{\text{total}} = 21,065$ $N_{\text{immobile}} = 2,508$ (11.91%)
Lck10-PAmCherry	D = 2.15 $\mu\text{m}^2 \text{s}^{-1}$ (2.14-2.17) Confined: 14.7% (14.5-15.0) $N_{\text{total}} = 53,357$ $N_{\text{immobile}} = 805$ (1.51 %)	D = 2.08 $\mu\text{m}^2 \text{s}^{-1}$ (2.07-2.09) Confined: 14.0% (13.7-14.2) $N_{\text{total}} = 67,352$ $N_{\text{immobile}} = 1,526$ (2.27 %)
Lck ^{Y505F} -PAmCherry	D = 0.95 $\mu\text{m}^2 \text{s}^{-1}$ (0.94-0.96) Confined: 30.0% (29.7-30.2) $N_{\text{total}} = 41,127$ $N_{\text{immobile}} = 3,650$ (8.87%)	D = 0.65 $\mu\text{m}^2 \text{s}^{-1}$ (0.64-0.66) Confined: 29.9% (29.6-30.11) $N_{\text{total}} = 32,024$ $N_{\text{immobile}} = 4,467$ (13.95%)
Lck ^{Y394F} -PAmCherry	D = 1.24 $\mu\text{m}^2 \text{s}^{-1}$ (1.22-1.26) Confined: 26.2% (25.9-26.6) $N_{\text{total}} = 21,309$ $N_{\text{immobile}} = 1,350$ (6.34%)	D = 0.88 $\mu\text{m}^2 \text{s}^{-1}$ (0.87-0.89) Confined: 26.1% (25.9-26.3) $N_{\text{total}} = 32,477$ $N_{\text{immobile}} = 2,921$ (8.99 %)
Lck ^{K273R} -PAmCherry	D = 1.13 $\mu\text{m}^2 \text{s}^{-1}$ (1.12-1.15) Confined: 25.6% (25.4-25.9) $N_{\text{total}} = 31,025$ $N_{\text{immobile}} = 2,251$ (7.26 %)	D = 0.82 $\mu\text{m}^2 \text{s}^{-1}$ (0.81-0.83) Confined: 25.8% (25.6-26.1) $N_{\text{total}} = 26,059$ $N_{\text{immobile}} = 2,479$ (9.51 %)
Lck ^{K273R, Y505F} -PAmCherry	D = 0.51 $\mu\text{m}^2 \text{s}^{-1}$ (0.5-0.51) Confined: 23.3% (23.1-23.5) $N_{\text{total}} = 35,712$ $N_{\text{immobile}} = 4,945$ (13.85%)	D = 0.41 $\mu\text{m}^2 \text{s}^{-1}$ (0.41-0.42) Confined: 27.0% (26.8-27.2) $N_{\text{total}} = 50,734$ $N_{\text{immobile}} = 9,168$ (18.07%)

707

708 **Table 1: Diffusion coefficients and confinement of wild-type Lck, Lck10 and Lck mutations**
709 **obtained by single particle tracking.** Average diffusion coefficient, D, and percentage of
710 confinement were extracted from the single particle analysis for Lck and Lck10 in Jurkat cells on
711 resting and stimulating surfaces. 95% confidence values are listed in brackets. N_{total} refers to the total
712 number of trajectories detected prior to analysis (including immobile trajectories) and N_{immobile} is the
713 number of immobile particles that were excluded from the analysis. The percentage is $N_{\text{immobile}}/N_{\text{total}}$.

714 **Figure Legends**

715 **Fig. 1 Lck switches between free and confined states.** (A) L_{Conf} acquired for Lck10-
 716 PAmCherry in resting Jurkat cells (purple), wtLck-PAmCherry in stimulated Jurkat cells
 717 (orange) and wtLck-PAmCherry in fixed cells (cyan), normalized to peak value. The dashed
 718 vertical line marks the threshold where a particle was to be considered confined, i.e., if it had
 719 three or more consecutive steps with an L_{Conf} value greater than that threshold. (B) An
 720 experimental trajectory decomposed to free (magenta) and confined (cyan) states, with the
 721 confinements highlighted in yellow circles. (C) Time evolution of L_{Conf} values for the
 722 trajectory in (B) with the threshold marked with an orange dashed line and the confined
 723 periods with a yellow shade. (D) Trajectory decomposition maps of wtLck-PAmCherry in a
 724 stimulated live cells (left) and fixed Jurkat cells (right) Free periods are colored magenta,
 725 whereas confined periods are colored cyan. Scale bar = 5 μm . (E) 5 μm by 5 μm zoomed-in
 726 regions of interest in (D) (top – live, bottom - fixed). Scale bar = 1 μm .

727 **Fig. 2 wtLck-PAmCherry is more confined in stimulated cells.** (A) Representative
 728 stimulated and resting Jurkat E6-1 cells expressing wtLck-PAmCherry. The left panels show
 729 bright field images of the cells with detected trajectories overlaid, color-coded according to
 730 their initial diffusion. The right panels show the free (magenta) and confined (cyan) modes of
 731 diffusion. Scale bar = 5 μm . Bottom: diffusions histogram corresponding to the cells above,
 732 sharing mutual color-coding. (B) L_{Conf} histograms for wtLck-PAmCherry in resting (orange)
 733 and stimulated (blue) cells. (C) Histograms of the fraction of confined wtLck-PAmCherry
 734 molecules obtained for 13 stimulated (blue) and 17 resting (orange) Jurkat cells. Box plot
 735 shows the median. Notch 95% confidence interval, box edges first and third quartile, lines
 736 Tukey's fences, **** $p \leq 0.00001$.

737 **Fig. 3 Lck10-PAmCherry demonstrates free-diffusion in resting and stimulated calls.**
 738 (A) Representative stimulated and resting Jurkat E6-1 cells expressing Lck10-PAmCherry.
 739 The left panels show bright field images of the cells with detected trajectories overlaid, color-
 740 coded according to their initial diffusion. The right panels show the free (magenta) and
 741 confined (cyan) modes of diffusion. Scale bar = 5 μm . Bottom: diffusions histogram
 742 corresponding to the cells above, sharing mutual color-coding. (B) L_{Conf} histograms for
 743 Lck10-PAmCherry in resting (orange) and stimulated (blue) cells. (C) Histograms of the
 744 fraction of confined Lck10-PAmCherry molecules obtained for 19 stimulated (blue) and 15
 745 resting (orange) Jurkat cells. Box plot shows the median. Notch 95% confidence interval, box
 746 edges first and third quartile, lines Tukey's fences, n.s. $p > 0.01$.

747 **Fig. 4 Lck^{Y505F}-PAmCherry is equally confined in stimulated and resting cells.** (A)
 748 Representative stimulated and resting Jurkat E6-1 cells expressing Lck^{Y505F}-PAmCherry. The
 749 left panels show bright field images of the cells with detected trajectories overlaid, color-
 750 coded according to their initial diffusion. The right panels show the free (magenta) and
 751 confined (cyan) modes of diffusion. Scale bar = 5 μm . Bottom: diffusions histogram
 752 corresponding to the cells above, sharing mutual color-coding. (B) L_{Conf} histograms for
 753 Lck^{Y505F}-PAmCherry in resting (orange) and stimulated (blue) cells. (C) Histograms of the
 754 fraction of confined Lck^{Y505F}-PAmCherry molecules obtained for 14 stimulated (blue) and 18

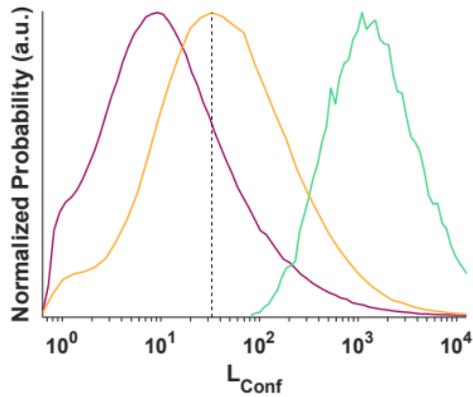
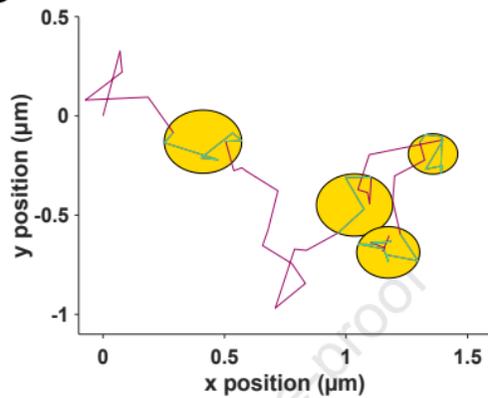
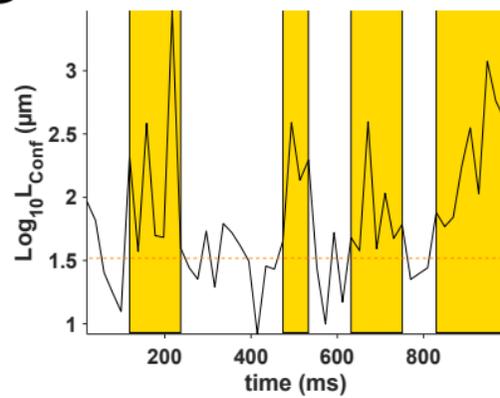
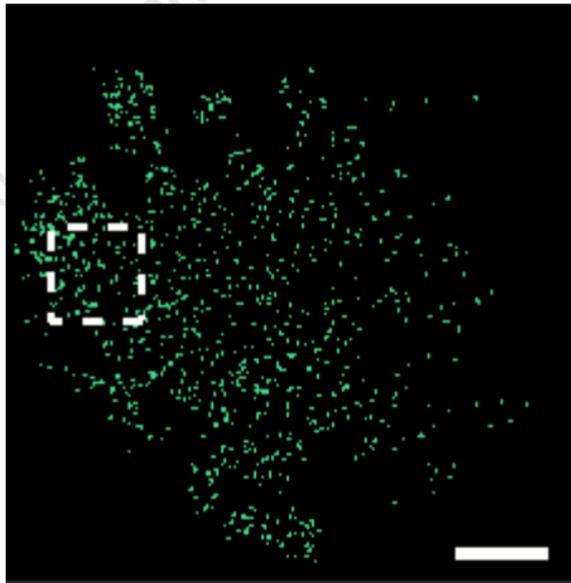
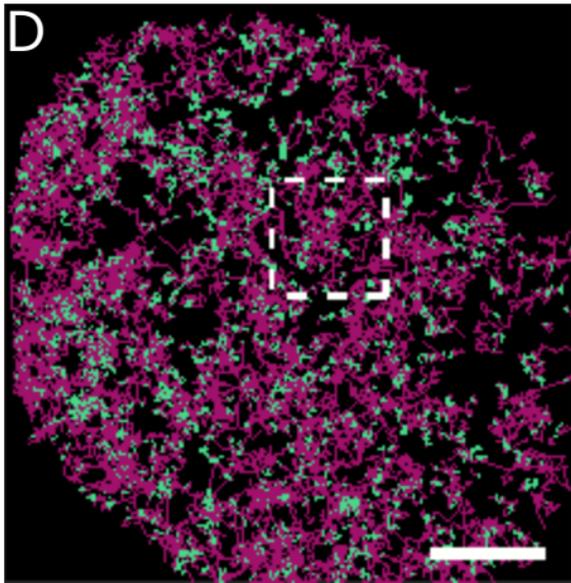
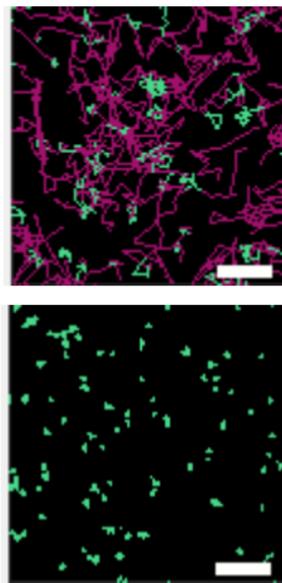
755 resting (orange) Jurkat cells. Box plot shows the median. Notch 95% confidence interval, box
 756 edges first and third quartile, lines Tukey's fences, n.s. $p>0.01$.

757 **Fig. 5 Lck^{Y394F}-PAmCherry is equally confined in stimulated and resting cells.** (A)
 758 Representative stimulated and resting Jurkat E6-1 cells expressing Lck^{Y394F}-PAmCherry. The
 759 left panels show bright field images of the cells with detected trajectories overlaid, color-
 760 coded according to their initial diffusion. The right panels show the free (magenta) and
 761 confined (cyan) modes of diffusion. Scale bar = 5 μm . Bottom: diffusions histogram
 762 corresponding to the cells above, sharing mutual color-coding. (B) L_{Conf} histograms for
 763 Lck^{Y394F}-PAmCherry in resting (orange) and stimulated (blue) cells. (C) Histograms of the
 764 fraction of confined Lck^{Y394F}-PAmCherry molecules obtained for 16 stimulated (blue) and 14
 765 resting (orange) Jurkat cells. Box plot shows the median. Notch 95% confidence interval, box
 766 edges first and third quartile, lines Tukey's fences, n.s. $p>0.01$.

767 **Fig. 6 Confinement analyses for Lck^{K273R}-PAmCherry and Lck^{K273R, Y505F}-PAmCherry**
 768 **in stimulated and resting cells.** (A) Representative stimulated and resting Jurkat E6-1 cells
 769 expressing Lck^{K273R}-PAmCherry and Lck^{K273R, Y505F}-PAmCherry. The left panels show bright
 770 field images of the cells with detected trajectories overlaid, color-coded according to their
 771 initial diffusion. The right panels show the free (magenta) and confined (cyan) modes of
 772 diffusion. Scale bar = 5 μm . Bottom: diffusions histogram corresponding to the cells above,
 773 sharing mutual color-coding. (B) L_{Conf} histograms for Lck^{K273R}-PAmCherry and Lck^{K273R,}
 774 ^{Y505F}-PAmCherry in and stimulated cells. (orange, blue, purple and yellow, respectively). (C)
 775 Histograms of the fraction of confined Lck^{K273R}-PAmCherry molecules obtained for 12
 776 stimulated (blue) and 14 resting (orange) Jurkat cells and histograms of the fraction of
 777 confined Lck^{K273R, Y505F}-PAmCherry obtained for 8 stimulated (yellow) and 8 resting (purple)
 778 Jurkat cells. Box plot shows the median. Notch 95% confidence interval, box edges first and
 779 third quartile, lines Tukey's fences, , **** $p\leq 0.00001$, n.s. $p>0.01$.

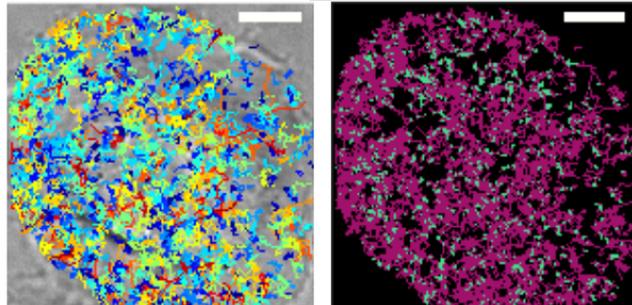
780 **Fig. 7. Lck molecules switch between a confined and free diffusion modes.** Lck
 781 (illustrated in blue) exists in two main conformations: a closed conformation characterized by
 782 low catalytic activity and mediated by intramolecular interactions; and an open conformation
 783 characterized by high catalytic activity and free SH2 and SH3 domains. Our data propose that
 784 the closed conformation diffuses unimpeded (purple line), whereas the open conformation
 785 interacts with other membrane proteins (illustrated in green) via SH2 and SH3 domain
 786 mediated interactions and becomes confined (yellow circles) through rapid rebinding (teal
 787 line). This may result in a dual-stage search strategy where free diffusion allows Lck to
 788 relocate over large membrane areas while confinement in the open conformation enables high
 789 substrate phosphorylation rates.

790

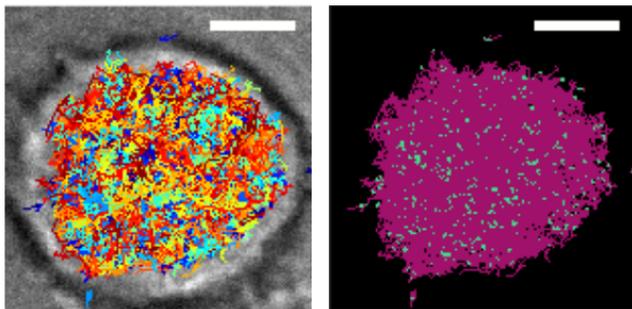
A**B****C****D****E**

A

Stimulated

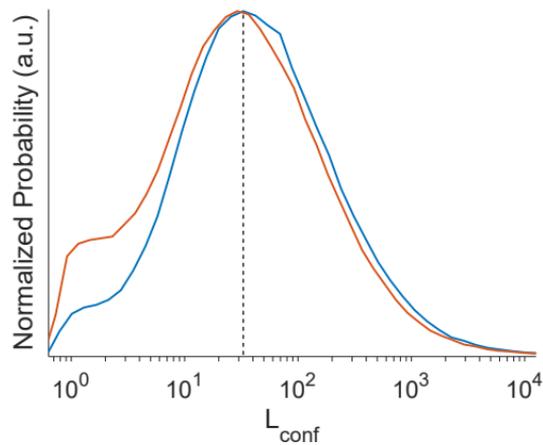


Resting

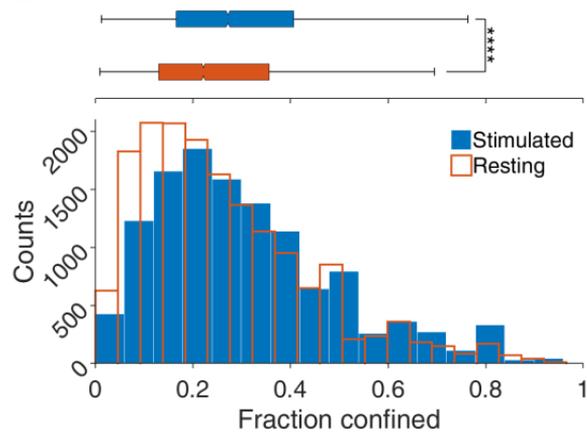
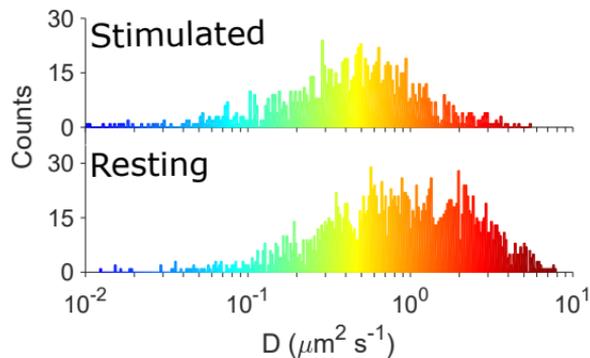


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B

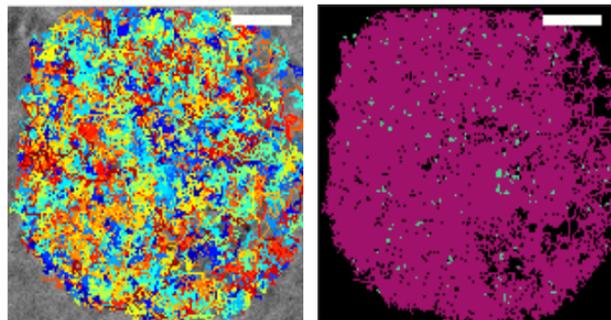


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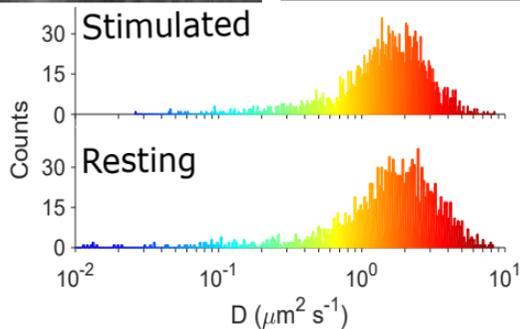
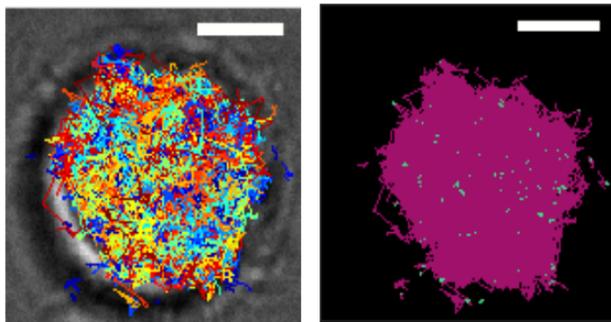


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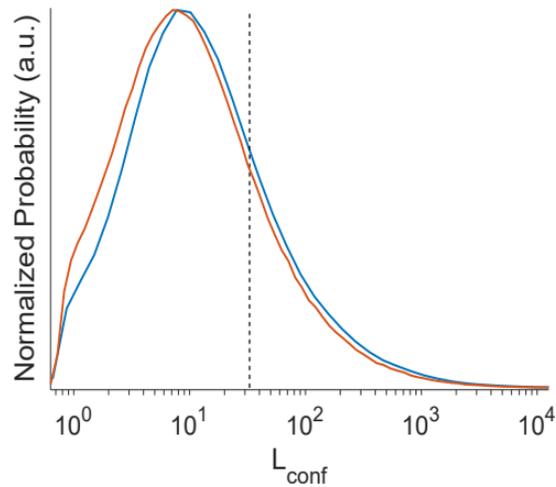
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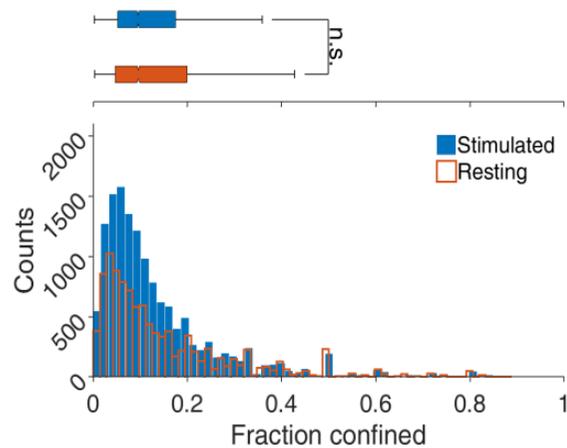
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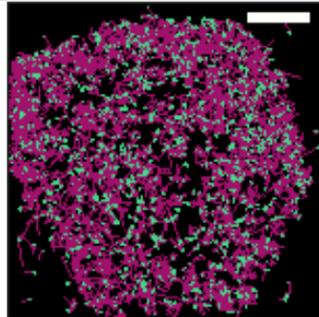
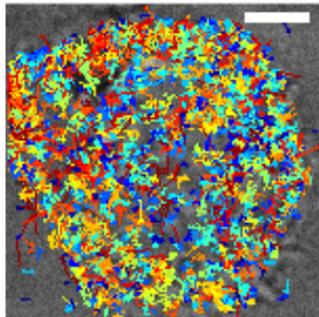


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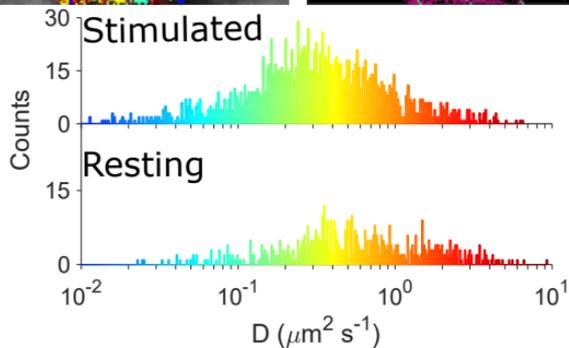
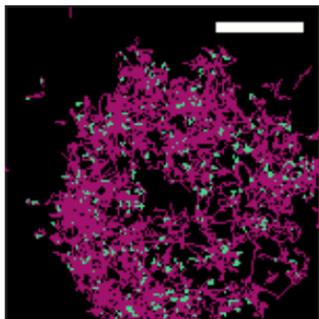
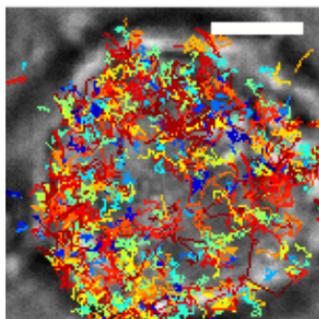


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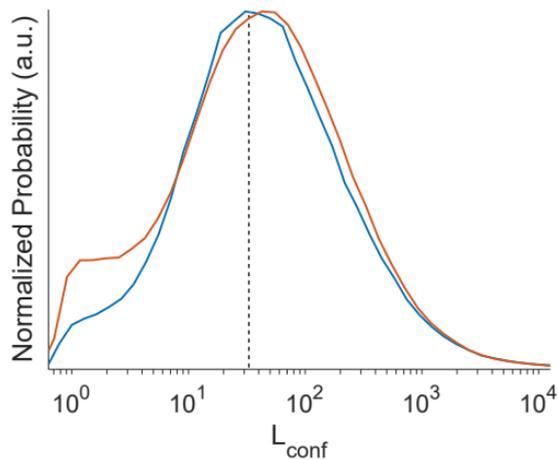


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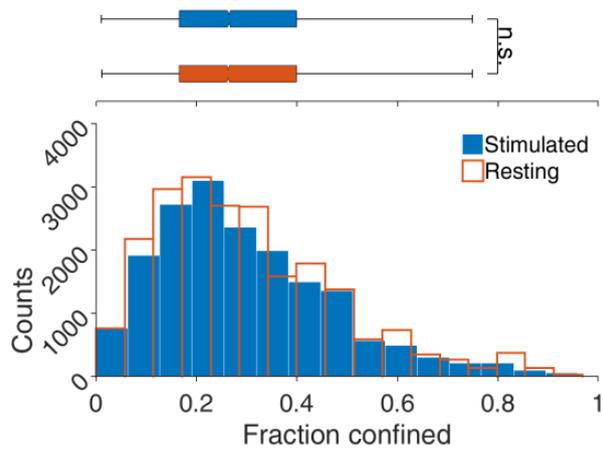


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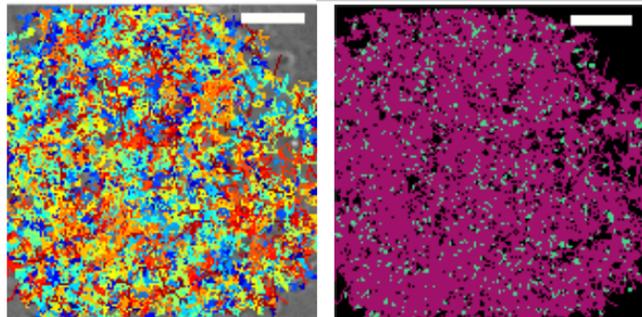


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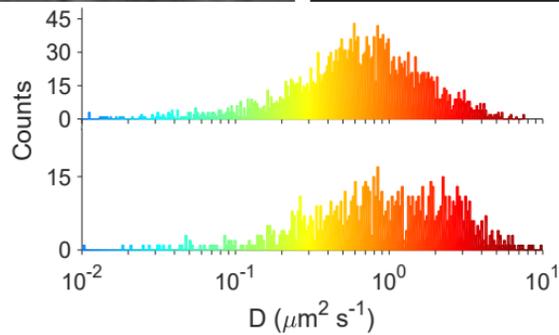
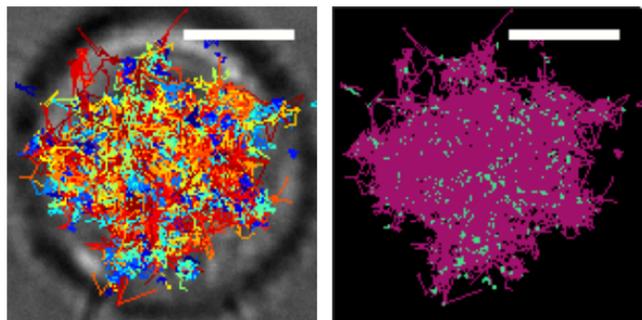


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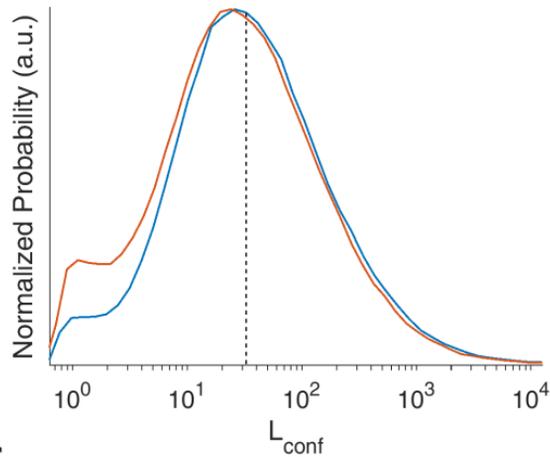
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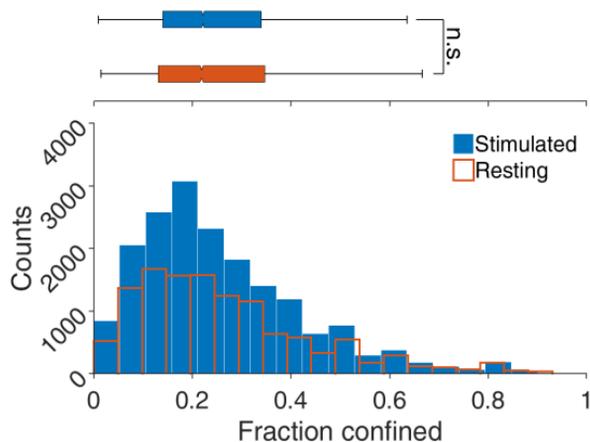
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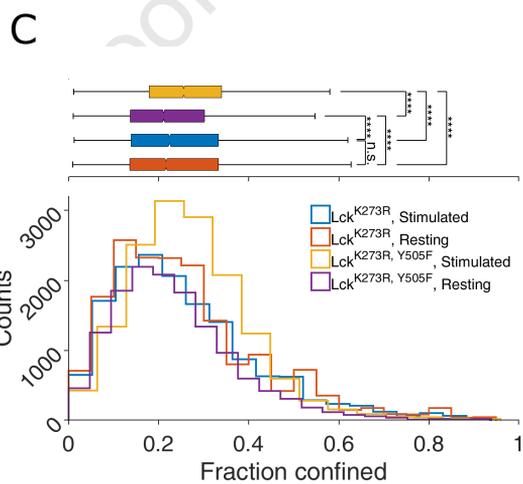
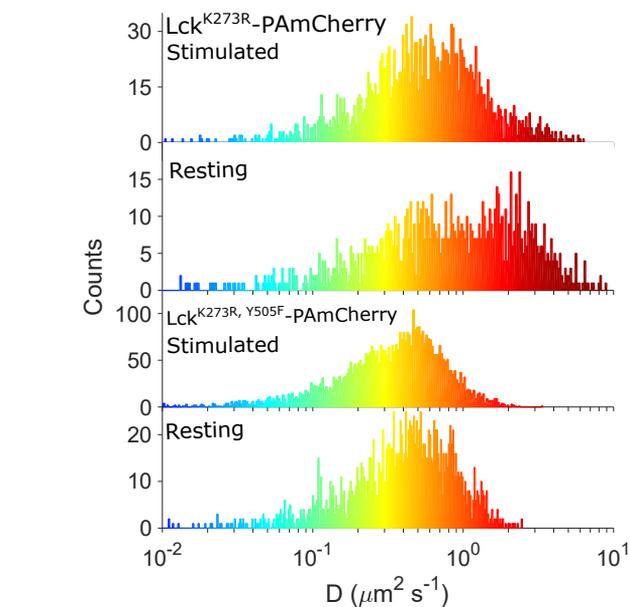
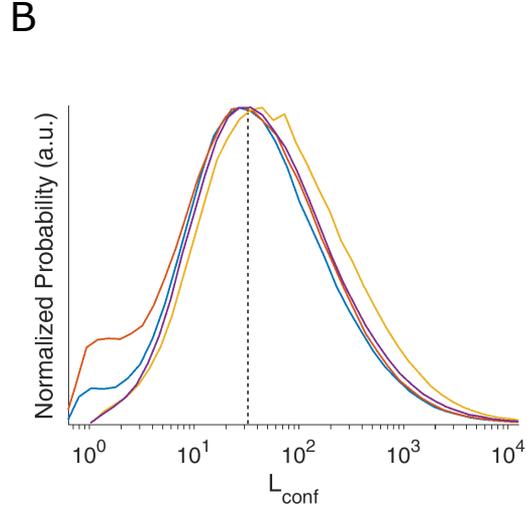
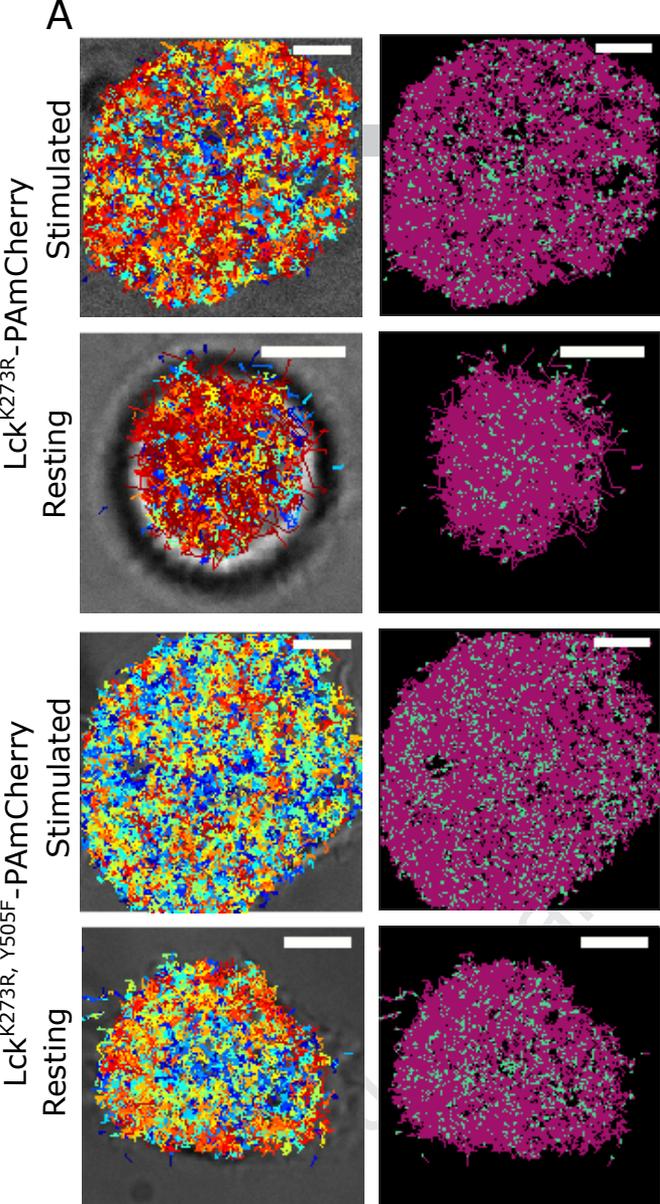


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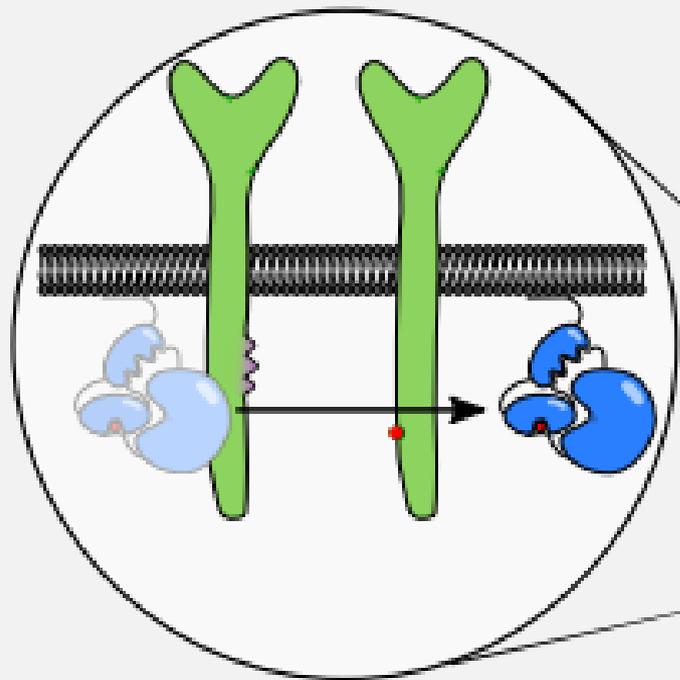


C





"closed" Lck



"open" Lck

