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Hilzenrat, Geva; Pandži, Elvis; Yang, Zhengmin; Nieves, Daniel J.; Goyette, Jesse; Rossy, Jérémie; Gaus, Katharina

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

3 **Geva Hilzenrat^{1,2}, Elvis Pandžić³, Zhengmin Yang^{1,2}, Daniel J. Nieves^{1,2}, Jesse**
4 **Goyette^{1,2}, Jérémie Rossy⁴, Katharina Gaus^{1,2*}**

5
6 *¹EMBL Australia Node in Single Molecule Science, School of Medical Sciences, University of*
7 *New South Wales, Sydney, Australia*

8 *²ARC Centre of Excellence in Advanced Molecular Imaging, University of New South Wales,*
9 *Sydney, Australia*

10 *³BioMedical Imaging Facility, Mark Wainwright Analytical Centre, University of New South*
11 *Wales, Sydney, Australia*

12 *⁴Biotechnology Institute Thurgau, University of Konstanz, Kreuzlingen, Switzerland*

13
14 *Corresponding author: k.gaus@unsw.edu.au

17 **Abstract**

18 T cell receptor (TCR) phosphorylation by Lck is an essential step in T cell activation. It is
19 known the conformational states of Lck control enzymatic activity; however, the underlying
20 principles of how Lck finds its substrate in the plasma membrane remain elusive. Here,
21 single-particle tracking is paired with photoactivatable localization microscopy (sptPALM) to
22 observe the diffusive modes of Lck in the plasma membrane. Individual Lck molecules
23 switched between free and confined diffusion in resting and stimulated T cells.
24 Conformational state, but not partitioning into membrane domains, caused Lck confinement
25 as open conformation Lck was more confined than closed. Further confinement of kinase-
26 dead versions of Lck suggests that Lck interacts with open active Lck to cause confinement,
27 irrespectively of kinase activity. Our data supports a model that confined diffusion of open
28 Lck results in high local phosphorylation rates and closed Lck diffuses freely to enable wide-
29 range scanning of the plasma membrane.

30 T cell signaling is a tightly controlled process involving both simultaneous and sequential
31 spatiotemporal events, involving membrane remodeling and redistribution of key signaling
32 proteins^{1,2}. Engagement of the T cell receptor (TCR) with an antigenic pMHC on the surface
33 of an antigen-presenting cell (APC) leads to the formation of immunological synapses³ and
34 initiates downstream signaling events that lead to T cell activation⁴. The Src family kinase
35 Lck plays a crucial role in the signaling cascade. TCR engagement results in the membrane
36 release⁵ and phosphorylation of the immunoreceptor tyrosine-based motifs (ITAMs) located
37 in the cytoplasmic tails of the CD3 ζ chain by Lck⁶. Phosphorylated sites on the TCR-CD3
38 complex become docking sites for the zeta chain-associated protein kinase 70 (ZAP70), that
39 is further phosphorylated by Lck⁷ before recruiting other proteins in the signaling cascade
40 that are necessary for complete T cell activation.

41 The role of Lck in T cell activation as a signaling regulator is of particular interest due to its
42 dynamic characteristics. Lck is a 56 kDa protein comprised of a Src homology (SH) 4 domain
43 at the N-terminus, followed by a unique domain, an SH3 domain, an SH2 domain, a kinase
44 domain and short C-terminal tail. Lck is anchored to the plasma membrane through its SH4
45 domain via post-translational acylation on three specific sites: a myristoylated Gly2⁸ and
46 palmitoylated Cys3 and Cys5. The latter two are crucial for membrane binding and biological
47 activity, enabling Lck diffusion in the inner leaflet of the plasma membrane and its
48 recruitment to the immunological synapse⁹. The unique domain interacts with the CD3 ϵ
49 subunit in the TCR-CD3 complex¹⁰ as well as the co-receptors CD4 and CD8¹¹ via zinc-
50 mediated bonds. However, Lck does not require the co-receptors for recruitment to the
51 immunological synapse or for TCR triggering¹², suggesting that freely diffusing Lck is
52 sufficient for T cell activation.

53 Lck conformation is regulated by the phosphorylation of two tyrosine residues: Tyr³⁹⁴, where
54 phosphorylation increases Lck activity, and Tyr⁵⁰⁵, whose phosphorylation reduces Lck

55 activity^{13,14}. Intramolecular interactions between the phosphorylated Tyr⁵⁰⁵ (pTyr⁵⁰⁵) and the
56 SH3 and SH2 domains cause rearrangements that keep Lck in a closed, inactive conformation
57^{15,16}. When dephosphorylated by CD45, Lck exists in an open, primed conformation. When
58 Tyr³⁹⁴ is trans-autophosphorylated¹⁴, rearrangements in the activation loop stabilize the
59 active conformation¹⁷. Lck's diffusion behavior¹⁸ and conformational state^{19,20} are thought
60 to be regulated by the activation state of the cell. The conformational state also influences
61 Lck clustering²¹. This means that not only does Lck conformational state regulate Lck
62 enzymatic activity but also aids in its diffusive search strategy.

63 Whether Lck becomes 'active', i.e., converted into the open conformation upon TCR
64 engagement, has been controversial. There is evidence of global changes in relative
65 populations of closed and open Lck in resting *versus* stimulated T cells^{19,20}. These studies
66 propose that Lck undergoes conformational changes upon T cell activation, driving it from its
67 closed state to an open state, therefore enhancing its activity. Using biochemical analyses,
68 conformational heterogeneity was observed in resting and stimulated T cells²², suggesting a
69 "standby-model" in which ~40% of Lck is in the open conformation in both resting and
70 stimulated T cells. Ballek et al. challenged these observations in a later report that used
71 different cell lysis conditions²³. Another study, based on measurements of fluorescence
72 resonance energy transfer (FRET) between fluorescent proteins fused to the N- and C-
73 terminals of Lck, concluded there was no significant change in open *versus* closed
74 populations of Lck even after T cell stimulation²⁴. While different papers report different
75 percentages of open Lck in pre-stimulated cells, constitutively active Lck were also found in
76 CD8⁺ memory T cells and may account for the enhanced sensitivity to antigen in these cells
77²⁵. A pool of active Lck existing prior to T cell stimulation led to the idea that rapid TCR
78 triggering post receptor engagement may be caused by changes in Lck spatial rearrangements
79 as opposed to, or in addition to conformational changes. Using single-molecule localization

80 microscopy in fixed cells, we previously showed that Lck distributed differently on the cell
81 surface depending on its conformational state ²¹, with open Lck residing preferentially in
82 clusters and closed Lck preventing clustering, regardless of the cell's activation state.
83 However, this study only captured the overall distribution of open or closed Lck and the
84 movement of Lck clusters, but to understand the search strategy of the membrane-bound
85 kinase the dynamic behavior of individual molecules need to be taken into account.

86 The efficiency of a dual-state search strategies has previously been demonstrated in other
87 systems ²⁶. For Lck, such a strategies would entail that individual molecules oscillate between
88 two distinct states: a confined state that corresponds to high Lck activity and a diffusive state
89 that enables the kinase to scan the membrane for substrates. Such a dual-state search strategy
90 may account for the high fidelity of Lck-mediated phosphorylation of the available TCR-CD3
91 complexes while also retaining high signaling sensitivity when membrane-detached cytosolic
92 tails of the CD3 complex are limited. The former would be mediated by the high enzymatic
93 activity in Lck clusters while the high level of diffusion of Lck in the closed state would
94 enable the latter.

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

105 In the current study, we utilize SPT using photoactivatable localization microscopy
106 (sptPALM)³⁰ as a tool to study the diffusion of wild type (WT) and mutated Lck, lacking the
107 tyrosine residues on positions 394 and 505, to measure the dynamics of the closed and open
108 forms, respectively^{19,20}. Lck variants were tagged with photoactivatable monomeric cherry
109 (PAmCherry)³¹, expressed in Jurkats 1.6E cells and imaged in resting and activating
110 conditions. Single trajectories were extracted and analyzed in order to find periods when the
111 proteins underwent confined diffusion and the fraction of confined versus free proteins was
112 determined³². Measurements of different Lck variants showed that conformation has a key
113 role in Lck's substrate search strategy, with the open form dwelling more in confinements
114 compared to the closed form. Further, we provide evidence of Lck-Lck interaction in the
115 open conformation in stimulated T cells. Taken together, the data suggest that Lck
116 continuously switched between open and closed states, which is likely to determine the
117 probability of productive encounters between Lck and its substrates.

118 **Results**

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

120 In order to characterize the diffusion patterns of Lck, we applied single particle tracking on
121 image sequences from different experimental conditions and decomposed each trajectory into
122 free and confined segments. Jurkat E6.1 cells were transfected with either wild-type Lck
123 (wtLck) fused to PAmCherry (wtLck-PAmCherry) or a truncated construct of Lck containing
124 only the first ten amino acids that are responsible for Lck anchoring to the membrane (Lck10-
125 PAmCherry). T cells were stimulated for ~5 minutes at 37°C on a coverslip coated with anti-
126 CD3 and anti-CD28 antibodies and imaged either in live-cell conditions or after chemical
127 fixation. In each experiment we acquired 10,000 frames with an 18 ms exposure for the
128 duration of ~197 s. Imaging was done while continuously photo-activating and exciting the

129 fluorophores. Trajectories shorter than 15 frames and immobile particles (particles with a low
130 RMSD, as described in the Methods section) were excluded from analysis.

131 We decomposed trajectories by adopting a previously described post-tracking analysis³².
132 Briefly, every trajectory is first fragmented into overlapping windows. For each window, the
133 normalized variance of the location of the particle is calculated as a measure of the level of
134 confinement, L_{Conf} , according to:

135
$$(1) L_{Conf} = \frac{D_{free} \times W \times t_W}{var(r)}$$

136 where D_{free} is the diffusion coefficient of freely diffusing Lck in $\mu\text{m}^2 \text{sec}^{-1}$, W is the window
137 size in frames, t_W is the temporal length of the window in seconds and $var(r)$ is the variance
138 in μm^2 . We chose the diffusion coefficient of Lck10-PAmCherry as the value for D_{free} for all
139 versions of Lck as Lck10 is membrane anchored but does not interact with other proteins. We
140 then defined a threshold of L_{Conf} above which particles are considered confined. For this
141 threshold, we chose the most abundant L_{Conf} value for wtLck-PAmCherry in stimulated T
142 cells, following the procedure published previously³² (dotted line in Fig. 1A). This threshold
143 was suitable because the majority of values for Lck10-PAmCherry in resting cells were
144 below this threshold and all values for wtLck-PAmCherry in fixed cells were above the
145 threshold (Fig. 1A). In order to ensure that Lck molecules were in fact confined, rather than
146 just temporarily slowing down, we only regarded a molecule as confined if it has an L_{Conf}
147 value above the threshold for three or more consecutive windows. Trajectories were then
148 segmented into confined and free periods (Fig. 1B), depending on whether L_{Conf} was above or
149 below the threshold (Fig. 1C). From this analysis it was evident that molecules that diffused
150 slowly for 3 or more consecutive states were found to be confined (Fig. S1). This analysis
151 was applied to all trajectories recorded in a cell (Fig. 1D). As is evident from this sptPALM
152 analysis, individual wtLck-PAmCherry molecules in live cells switched between free and

153 confined diffusive states (Fig. 1D) while in fixed cells, only confined or immobile molecules
154 were observed (Fig. 1D).

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

156 Previous studies provided evidence that T cell activation decreases the overall diffusion of
157 Lck^{18,27}. In our experiments, resting T cell data was generated by placing T cells expressing
158 wtLck-PAmCherry onto coverslips coated with anti-CD90 antibodies. This resulted in good T
159 cell adhesion, but not TCR signaling or T cell activation³³. Our measurements confirmed the
160 decrease in diffusion (Fig 2A; Movie S1: resting - right, stimulated - left), with diffusion
161 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
162 cells, respectively (Fig. 2A, Fig. S2a; Movie S1). We wanted to assess whether this
163 slowdown is caused by enhanced spatial compartmentalization in the membrane. Thus, we
164 conducted the L_{Conf} analysis for wtLck-PAmCherry in resting and stimulated cells. When
165 comparing the L_{Conf} histogram of wtLck in stimulated T cells (Fig. 2B, blue) *versus* resting T
166 cells (Fig. 2B, orange), it is noticeable that the values in activated cells are shifted to higher
167 values, resulting in a mean L_{Conf} value of 32.9 in stimulated cells and 29.1 in resting cells.

168 Next we examined whether the decrease in local displacement variance is due to a
169 redistribution of wtLck-PAmCherry into confinements that would result in an increase in the
170 number of consecutive steps that fall above the L_{Conf} threshold value. Thus, we segmented the
171 total video into segments of five frames (Fig. S3), in which we asked how many particles, out
172 of the total number of particles imaged, were confined. Histograms obtained for stimulated
173 and non-stimulated cells (Fig. 2C) were collected. There was a clear difference in peak value
174 for the two populations as well as a larger tail of high values for wtLck-PAmCherry in
175 stimulated cells. As a consequence, the populations were statistically different (Fig. 2C) when
176 tested against the null hypothesis according to which the samples are drawn from the same

177 population, using the rank sum test, with different medians and non-overlapping 95%
178 confidence intervals with the values of 27.27% (26.67-27.78) and 22.22% (21.82-22.73) for
179 stimulated and resting cells, respectively. The percentage of confined wtLck-PAmCherry
180 were 30.97% (30.63-31.3) and 26.4% (26.14-26.68) in stimulated and resting cells,
181 respectively.

182 Overall, these results show that wtLck-PAmCherry diffused slower in stimulated cells
183 compared to resting cells, suggesting that in addition to a global reduction in diffusion, a
184 redistribution of Lck into confinements had occurred. These results are in agreement with an
185 increase in wtLck-PAmCherry clustering in fixed stimulated *versus* fixed resting T cells ²¹.

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

187 Lck confinement may be attributed to the formation of membrane domains, i.e., changes in
188 membrane order, as a result of TCR triggering ³⁴. If that is the case, a truncated version of
189 Lck, Lck10, that includes the first ten amino acids that are responsible to Lck anchoring to
190 the membrane as it contains the post-translational lipid modifications, is expected to
191 experience the same slowdown and confinement as full-length Lck. However, we did not
192 observe such a scenario (Fig. 3A; Movie S2), as the diffusion coefficients found for Lck10-
193 PAmCherry in stimulating and resting conditions remained high (Fig. S2b). The overall level
194 of confinement of Lck10-PAmCherry was almost identical for both resting and stimulated
195 cells, with a peak L_{Conf} value of 7.2 and 7.74, respectively (Fig. 3B). These values were
196 significantly different from the ones found for wtLck-PAmCherry, with most of the
197 probability function having a value below the threshold described above. A histogram of
198 confinement events (Fig. 3C) shows comparable peak values for both stimulated and resting
199 cells. No statistically significant difference was found between the two samples (Fig. 3C, top
200 panel), as shown by median of 9.62% (9.43-9.8) and 9.68% (9.52-10.00) for Lck10-

201 PAmCherry expressed in stimulated and resting cells, respectively. Further, the mean fraction
202 of confined particles was slightly higher in resting cells, with values of 13.96% (13.74-14.18)
203 and 14.73% (14.45-15.00) for stimulated and resting cells, respectively. These values were
204 lower than those found for wtLck-PAmCherry, suggesting Lck10-PAmCherry was far less
205 confined than wtLck-PAmCherry, even in stimulated cells.

206 Taken together, the data strongly suggest that the increased confinement observed for full-
207 length wtLck-PAmCherry was not due to global changes in membrane organization or
208 membrane domains¹⁸ as confinement of Lck10 in resting and stimulated T cells was similar.

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

210 Previously, we reported that Lck clustering was regulated by the kinase's conformational
211 state²¹. We thus quantified the influence of conformation on confinement of Lck in live cells.
212 First, we introduced a Tyrosine-to-Phenylalanine mutation at position 505 in Lck (Lck^{Y505F}).
213 The mutation prevents the binding of Lck pTyr⁵⁰⁵ to its own SH2 domain. This mutation is
214 well known as 'constitutively open'^{19-21,24,35} and 'hyperactive'¹³. An overall change in the
215 diffusion constants due to cell activation (Fig. 4A; Movie S3) was observed, with values of
216 $0.65 \mu\text{m}^2 \text{s}^{-1}$ (0.64-0.66) and $0.95 \mu\text{m}^2 \text{s}^{-1}$ (0.94-0.96) in stimulated and resting cells,
217 respectively (Fig. S2c). Further, L_{Conf} values for Lck^{Y505F}-PAmCherry were higher than that
218 of wtLck-PAmCherry (Fig. 4B), with peak values of 39.28 and 42.53 in stimulated and
219 resting cells, respectively, with <50% of $\log_{10}(L_{\text{Conf}})$ events above the confinement threshold.
220 In contrast to wtLck-PAmCherry, the L_{Conf} distributions of Lck^{Y505F}-PAmCherry were similar
221 in resting and stimulated T cells. This similarity was also observed in the histograms of the
222 confined fractions (Fig. 4C), with a large population of Lck^{Y505F} molecules falling into the
223 right tail of the distribution. Importantly, unlike in the corresponding data for wtLck-
224 PAmCherry, these values were not significantly different from each other (Fig. 4C, top), with

225 median values and overlapping 95% confidence interval of 26.55% (26.32-26.67) and
226 26.39% (26.14-26.67) for stimulated and resting cells, respectively. The means of Lck^{Y505}
227 were 29.85% (29.59-30.11) and 29.97% (29.74-30.22) in stimulated and resting cells,
228 respectively.

229 These data show that when Lck is locked in the open state, it is also driven into a more
230 confined diffusive behavior, which is comparable with wtLck-PAmCherry in stimulated cells
231 (Fig. S4). Although the diffusion coefficient found for Lck^{Y505F}-PAmCherry is lower, in
232 terms of confinement, open Lck was insensitive to the T cell activation with Lck^{Y505F}-
233 PAmCherry confinement levels being similar in both stimulated and resting cells. This
234 indicates that Lck confinement is driven by the open conformation of the kinase and supports
235 that a higher proportion of Lck is in the open conformation in stimulated T cells^{19,20,36}.

236 **Closed Lck is as confined as wild-type Lck in resting cells**

237 To further investigate the hypothesis that Lck conformation regulates Lck diffusive behavior,
238 we expressed a closed form of Lck in Jurkat cells. A mutation in position 394 converting a
239 tyrosine into phenylalanine (Lck^{Y394F}) prevents the formation of the activation loop and
240 results in reduced-activity¹⁴ or an inactive Lck¹³ because of the hyper-phosphorylated tyr⁵⁰⁵
241²² that constitutively closes the enzyme¹⁹.

242 As with the wtLck and Lck^{Y505F}, Lck^{Y394F}-PAmCherry did undergo a decrease in diffusion
243 coefficient due to stimulation (Fig.5A; Movie S4), from 1.24 $\mu\text{m}^2 \text{s}^{-1}$ (1.22-1.26) in resting
244 cells to 0.88 $\mu\text{m}^2 \text{s}^{-1}$ (0.87-0.89) in stimulated cells (Fig. S2d). We applied the same
245 sptPALM analysis to Lck^{Y394F}-PAmCherry and lower L_{Conf} values were obtained with peak
246 values of 32.93 and 30.36 in stimulated and resting cells, respectively (Fig. 5B). Histograms
247 of the fraction of confined Lck^{Y394F}-PAmCherry showed the populations were skewed
248 towards lower values (Fig. 5C). Similarly to Lck^{Y505F}-PAmCherry, Lck^{Y394F}-PAmCherry

249 showed no statistically significant difference between stimulated and resting cells (Fig. 5C,
250 top panel) and medians of 22.22% (21.88-22.58) and 21.95% (21.43-22.22) for Lck^{Y394F}-
251 PAmCherry in stimulated and resting cells, respectively. The mean confinement fractions
252 were 26.09% (25.85-26.33) and 26.24% (25.92-26.55) for Lck^{Y394F}-PAmCherry in stimulated
253 and resting cells, respectively.

254 The confinement fraction values we found for the closed Lck were smaller than the ones
255 found for the open Lck (Fig. S4), illustrating the significance conformational states have on
256 Lck diffusion. Indeed closed Lck has a similar level of confinement as wtLck in resting cells
257 while open Lck was similarly confined as wtLck in activated cells (Fig. S4). Thus, the data
258 confirms that confinements are regulated by the conformational state of Lck with open Lck
259 being more confined and closed Lck being less confined.

260 **Lck self-associates with other Lcks, depending on its conformation and activity**

261 An open Lck that is also phosphorylated in Tyrosine 394 is known to be active, while studies
262 done on Lck^{Y505F} showed hyperactivity^{13,14}. By expressing a constitutively inactive Lck, we
263 could assess whether Lck confinement relies on enzymatic activity. We tested an Lck variant
264 in which the lysine in position 273 in the kinase domain is replaced with Arginine (Lck^{K273R}-
265 PAmCherry, Fig. 6, Fig. S5), which has been shown to render Lck kinase-dead³⁷. Different
266 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 observed
267 for Lck^{K273R}-PAmCherry in stimulated and resting cells, respectively (Fig. S2e). However,
268 similar L_{Conf} histograms, with values of 34.80 for stimulated and 37.58 for resting cells, were
269 obtained (Fig. 6B, blue and orange) with no significant difference observed in the fraction of
270 time spent confined (Fig 6C, blue and orange). Additionally, Lck^{K273R}-PAmCherry spent
271 25.80% (25.56-26.07) and 25.62% (25.38-25.86) of time confined in stimulated and resting
272 cells, respectively (Fig 6C). Thus, the level of confinement kinase-dead Lck did not depend

273 on the T cell activation status as it did for wtLck (Fig. S5). Assuming that the K273R
274 mutation disables Lck activation via autophosphorylation, as hypothesized previously¹³, the
275 finding suggest that confinement of wtLck in stimulated T cells is regulated by Lck
276 activation.

277 To further test this hypothesis, we expressed a constitutively-open kinase-dead mutant
278 Lck^{K273R, Y505F}-PAmCherry. This mutant had slower diffusion coefficients of $0.41 \mu\text{m}^2 \text{s}^{-1}$
279 ($0.41\text{-}0.42$) and $0.51 \mu\text{m}^2 \text{s}^{-1}$ ($0.5\text{-}0.51$) in stimulated and resting cells, respectively (Fig. 6A;
280 Fig. S2f; Movie S5), values that were slower than those obtained for Lck^{K273R}-PAmCherry
281 (Fig. S2e, f). Further, Lck^{K273R, Y505F}-PAmCherry had higher L_{Conf} values in stimulated cells
282 (Fig. 6C, purple and yellow) compared to resting cells (44.78 and 35.09, respectively).

283 Conducting the same analysis to quantify confinement fractions, we found a large fraction of
284 kinase-dead mutant in the open conformation was highly confined in stimulated cells (Fig.
285 6C, purple and yellow). When comparing total trajectories, Lck^{K273R, Y505F}-PAmCherry in
286 stimulated cells was more confined than in resting cells and more than Lck^{K273R} in both cell
287 activation statuses (Fig. S5). These data confirm the conclusion that open, but not necessarily
288 enzymatically active Lck confined the kinase in distinct zones in the plasma membrane.

289 Lck^{K273R, Y505F}-PAmCherry was more confined in stimulated cells (26.97% (26.76-27.18))
290 than resting cells (23.30% (23.08-23.52)). It is possible that the open, kinase-dead variant of
291 Lck interacts with endogenous Lck in Jurkat cells that were already shown to be confined in
292 stimulated cells (Fig. 2). This would suggest that open Lck is confined in activated T cells by
293 Lck-Lck interaction. Moreover, the lowered confinement for the K273R-Y505F mutant in
294 resting cells compared to stimulated cells excludes the possibility of confinement due to
295 increase in hydrodynamic radius of the enzyme (Fig. S5). Taken together, the experiments
296 with the kinase-dead version of Lck confirmed the finding that it was the open conformation

297 that caused the Lck confinement. Thus it is likely that the enzyme switches between open and
298 close conformation, which results in a dual-state search strategy where open and active Lck is
299 confined, and closed and inactive Lck diffuses freely (Fig. 7).

300

301 **Discussion**

302 Phosphorylation of the TCR-CD3 complex by the kinase Lck is an essential step in T cell
303 activation ³⁸. While it is relatively well documented that the conformational states control
304 enzymatic activity, how membrane-bound Lck finds and phosphorylates its substrates is not
305 well understood. For example, the link between phosphorylation state and activity is well
306 established ³⁹, as well as some interactions of Lck with other proteins ⁴⁰⁻⁴³ and lipids ⁴⁴. Most
307 studies so far have focused on whether or not T cell stimulation results in an ‘activation’ of
308 Lck itself, i.e., whether there is an overall increase of Lck molecules in the open
309 conformation and whether a stable pool of open Lck already exists in resting T cells.
310 However, it is also possible that in the dynamic environment of the inner leaflet of the plasma
311 membrane, Lck switches between open and closed states, as many other types of enzymes do
312 ⁴⁵⁻⁴⁷. Utilizing single molecule localization microscopy (SMLM) techniques, our group
313 showed that open Lck clusters were bigger and denser than closed Lck clusters ²¹. In SMLM,
314 re-excitation of the same molecule can lead to overestimation of clustering ⁴⁸. Thus, we
315 investigated here whether Lck switches between confined and free diffusion modes. By
316 tracking single Lck molecules, we were indeed able to set a threshold to distinguish a
317 population that diffuses freely from one that exhibited restricted diffusion. We found that
318 wild-type Lck (wtLck-PAmCherry) transitioned between free and confined states in both
319 resting and stimulated T cells, strongly suggesting that the kinase has a sophisticated search
320 strategy.

321 In a study employing immunofluorescence staining, a pre-existing pool of constitutively
322 active Lck was used to explain the readiness of Lck to phosphorylate the TCR immediately
323 after T cell stimulation ²², while showing no difference in the fraction of open Lck when
324 comparing stimulated to resting cells. Therefore, it was speculated that Lck undergoes re-
325 distribution upon T-cell stimulation, while maintaining the same overall fraction of Lck in the
326 open and closed conformation. By examining the diffusion modes of Lck, as a function of
327 conformational status, we can provide an alternative explanation of how the kinase can be
328 efficient at both searching for substrates and phosphorylating the TCR complex. Firstly, we
329 found that T cell stimulation significantly changed the behavior of wtLck, promoting Lck
330 molecules to spend more time in confinements, compared to resting cells. Further our results
331 showed that in resting cells, wtLck behaved like the closed Lck mutant in both activating and
332 resting conditions. In contrast, in stimulated cells, wtLck demonstrated a diffusion pattern
333 that was similar to that of the open Lck mutant in both conditions. These observations led us
334 to the conclusion that T cell activation leads to a higher proportion of open Lck, supporting
335 the recent findings that were obtained with a fluorescence resonance energy transfer (FRET)
336 Lck biosensor ¹⁹. Our findings do not exclude the possibility of a pre-existing pool of open
337 Lck.

338 Comparing the level of confinement of open and closed Lck mutations (Fig. S4) clearly
339 showed that diffusion behaviour dependent on the conformational state of the enzyme.
340 Lck^{Y394F}-PAmCherry i.e. closed Lck was confined than wtLck-PAmCherry in stimulated
341 cells and Lck^{Y505F}-PAmCherry i.e. open Lck in stimulated and resting cells. Further,
342 Lck^{Y394F}-PAmCherry demonstrated similar confinement to that of wtLck-PAmCherry in
343 resting cells. The values obtained for the open mutant, both in stimulated and resting cells
344 were closer to the value that we obtained for wtLck-PAmCherry in stimulating conditions.
345 Taken together, our data support that notion that the open conformational state of Lck is

346 responsible for Lck confinement and that T cell activation resulted in converting some of the
347 wtLck molecules into the open state^{19,20}.

348 All Lck variants demonstrated some level of confinement in resting conditions. As these
349 results were obtained by expressing Lck variants in Jurkat cell lines, this confinement may be
350 an outcome of self-association with endogenous active Lck, and may be related to a pre-
351 existing pool of opened Lck in resting cells. Other mechanisms such as Lck's SH4 domain
352 interaction with lipid rafts^{49,50} and microdomains^{51,52} were previously suggested. Such
353 scenarios should have, however, also affected Lck10-PAmCherry, as this segment is
354 responsible for anchoring Lck to the membrane and should have resulted in slower diffusion
355 in stimulated cells. However this was not the case; similarly to closed Lck (Lck^{Y394F}-
356 PAmCherry) we found no difference in confinement of Lck10-PAmCherry in resting and
357 stimulated T cells. Further, one may hypothesize that Lck confinement is indirectly related to
358 membrane domains, by interacting with other proteins that are lipid raft-associated. However,
359 from our results with open Lck (Lck^{Y505F}-PAmCherry) we could not find support for this
360 idea, as Lck^{Y505F} was similarly confined in resting and stimulated cells.

361 The kinase-dead mutant, Lck^{K273R}-PAmCherry, was found to be minimally-confined in
362 resting and stimulated cells. It is possible that the K273R mutation in Lck prevents the
363 rearrangements in the activation loop that prevent self-association of Lck^{K273R}, or interaction
364 with other proteins, thus, limiting confinement¹³. Relying on our results obtained for wtLck-
365 PAmCherry, and thus assuming that a greater population of endogenous Lck was in the open,
366 confined state in stimulated Jurkat cells compared to resting cells, Lck^{K273R, Y505F}-PAmCherry
367 was found to be highly confined in stimulated cells, supporting the hypothesis that Lck self-
368 associated with other active Lck, therefore, promoting a more confined population. This is
369 consistent with a previous report on Lck self-association in the open conformation⁴³. Given

370 that Lck in the open conformation exhibited confined diffusion and hyperactivity^{13,14}, it is
371 highly likely that this state results in high local phosphorylation rates.

372 In conclusion, we provide evidence that the conformation of Lck was the main driver of Lck
373 diffusion modes with open Lck causing confined diffusion and closed Lck enabling free
374 diffusion. Individual Lck molecules can switch between confined and free diffusion in resting
375 and stimulated T cells. This is consistent with a dual-state search strategy that enables Lck to
376 scan large areas of the membrane in the closed state, but efficiently phosphorylate TCR-CD3
377 complexes at numerous sites in the open state.

378

379 **Methods and Materials**

380 **Plasmids**

381 Lck and Lck10 were amplified by PCR and inserted within the Eco1 and Age1 restriction
382 sites of a pPAmCherry-N1 plasmid. Y394F, Y505F and K273R mutations were further
383 introduced via site-directed mutagenesis.

384 **Sample Preparation**

385 Jurkat cells were cultured in RPMI medium (Gibco) containing phenol-red and supplemented
386 with 10% (vol/vol) FBS, 2 mM L-glutamine (Invitrogen), 1 mM penicillin (Invitrogen) and 1
387 mM streptomycin (Invitrogen). Cell cultures were passaged normally every ~48 hours, when
388 the cell count reached $\sim 8 \times 10^5$ viable cells per ml. The cells were cultured for at least 1 week
389 (3-4 passages) after thawing prior to transfection and imaging. No cells were used after
390 passage 20.

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

398 1.5H coverslips (Marienfeld-Superior) were waterbath-sonicated in four 30-minutes stages: 1
399 M KOH, Acetone, EtOH and ultra-pure (18 M Ω) water. The coverslips were then allowed to
400 adsorb 0.01% PLL (Sigma) in ultra-pure water for 15 minutes. Excess solution was later
401 aspirated and the coverslips were baked-dry in 60 °C for 1 hour. Finally, after cooling-down,

402 the coverslips were coated with either 0.01 mg/ml anti-CD3 (OKT3; eBioscience) and 0.01
403 mg/ml anti-CD28 (CD28.2; Invitrogen) for stimulating conditions or 0.01 mg/ml α CD90 for
404 (Thy-1; eBioscience) for resting conditions and let rest in 4°C overnight before imaging. The
405 coverslips were washed 3 times with phosphate buffer saline (PBS) pre-warmed to 37°C
406 before the cells were transferred onto them to interact with the antibodies. For live-cell
407 experiments, imaging took place ~5 minutes after cell-transfer, or fixed with 4%
408 paraformaldehyde (P6148; Sigma) in 37°C, followed by 3 washing cycles with PBS for
409 fixed-cell imaging.

410 **Imaging**

411 For each sptPALM experiment 10,000 frames were acquired in a ~50 frames per second (18
412 ms exposure time) rate on a total internal reflection fluorescence (TIRF) microscope
413 (ELYRA, Zeiss) in 37 °C using a 100× oil immersion objective (N.A. = 1.46) and a 67.5°
414 incident beam angle. PAmCherry fused to Lck variants were continuously photoactivated
415 using a 405 nm laser radiation tuned to 0.5-5 μ W (interchangeable during acquisition to
416 maintain a low density) and continuously excited with a 561 nm laser tuned to 2.5 mW. Point
417 density was monitored by using ZEN (Zeiss) online-processing tool.

418

419 **Data Analysis**

420 All accumulated data are comprised of three biologically-independent experiments, i.e. each
421 mutant was imaged in two or more cells (in one of the three repetitions, where a repetition
422 relates to a different transfection) in each cell-activation state (stimulated or resting). We used
423 Diatrack⁵³ for fitting the point spread functions (PSFs) to a Gaussian with a 1.75 pixel width
424 (1 pixel \approx 0.097 nm) and then to track the particles by setting the search radius to 10 pixels.

425 The data was later analyzed by a custom MATLAB (Mathworks) adaptation of the trajectory
426 analysis part of a previously published multi-target tracing (MTT) code³². Immobile particles
427 (RMSD < 2 pixels) and trajectories shorter than 15 frames were excluded from analysis.
428 Stages of confined and free diffusion were detected according to equation 1, with $D_{\text{free}} = 2.15$
429 $\mu\text{m}^2/\text{sec}$ (Fig. S2b, bottom), $W = 4$ and t_w was the sum of the exposure time and the CCD
430 reading time (~19.7 ms). To detect time spent in confinement, each sequence was segmented
431 to non-overlapping windows of 5 frames and in each block of 5 frames, the ratio of
432 confined:total particles was calculated. Each value of one 5 frames-window is a count in the
433 histogram. All data processing and statistical analyses were performed in MATLAB.

434 **Statistical Tests**

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

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450 **Supplementary Materials**

451 **Fig.S1** Relationship between Lck diffusion coefficient and confinement

452 **Fig.S2** Diffusion coefficients histograms of wtLck, Lck10, LckY505F, LckY394F,
453 LckK273R and LckK273R, Y505F in stimulated and resting Jurkat cells

454 **Fig.S3** Illustration of confinement ratio analysis

455 **Fig.S4** Comparison of confinement analysis result

456 **Fig.S5** Comparison of confinement analysis result of wtLck-PAmCherry, LckK273R-
457 PAmCherry and LckK273R, Y505F-PAmCherry in stimulated and resting cells

458 **Movie S1**

459 **Movie S2**

460 **Movie S3**

461 **Movie S4**

462 **Movie S5**

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606 wrote manuscript. EP established analysis and helped write the manuscript. ZY was
607 responsible for the generation of Lck constructs. DJN and JG aided in writing and drafting of

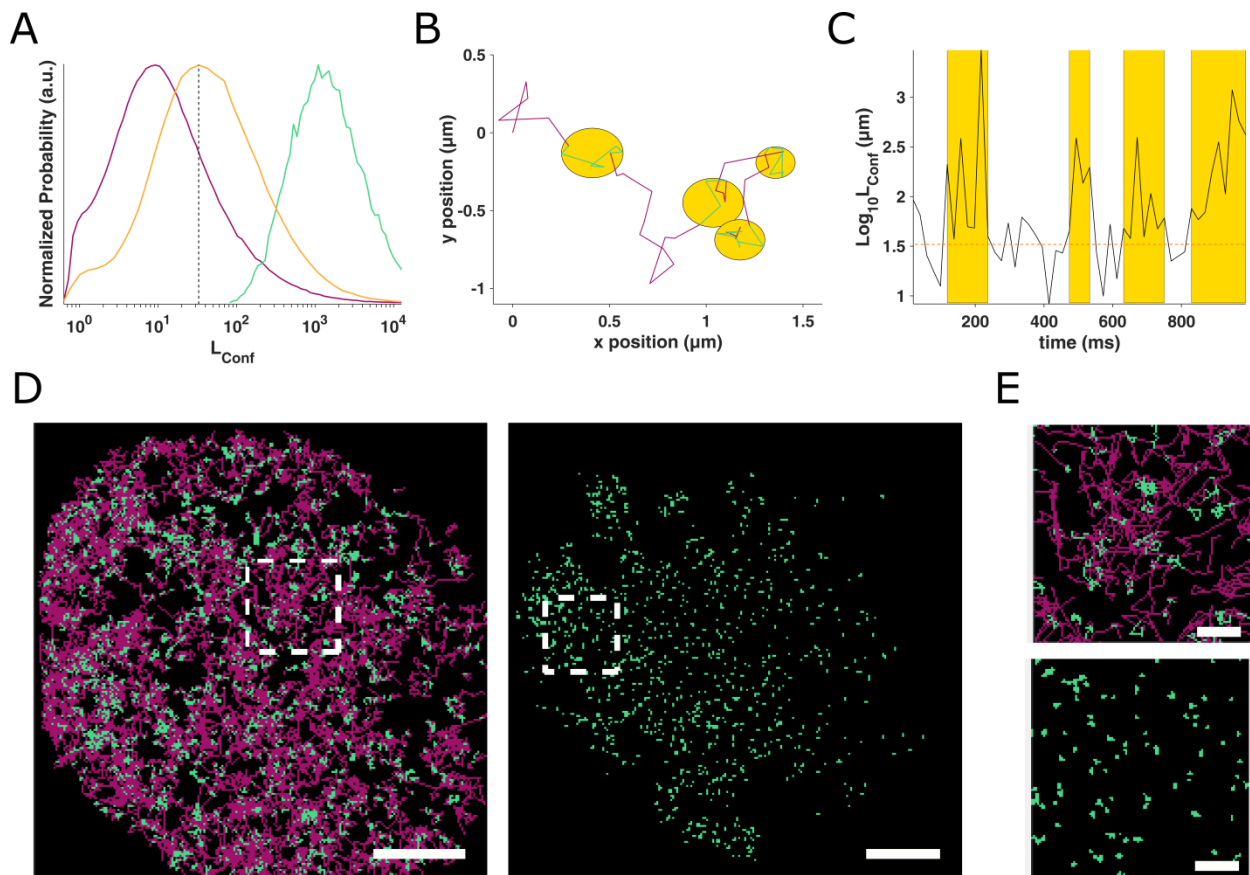
608 the manuscript. JR provided guidance with experiments. KG designed the project,

609 interpreted the data and wrote the manuscript.

610 **Competing interests:** The authors declare no competing interests.

611

612 **Figures:**



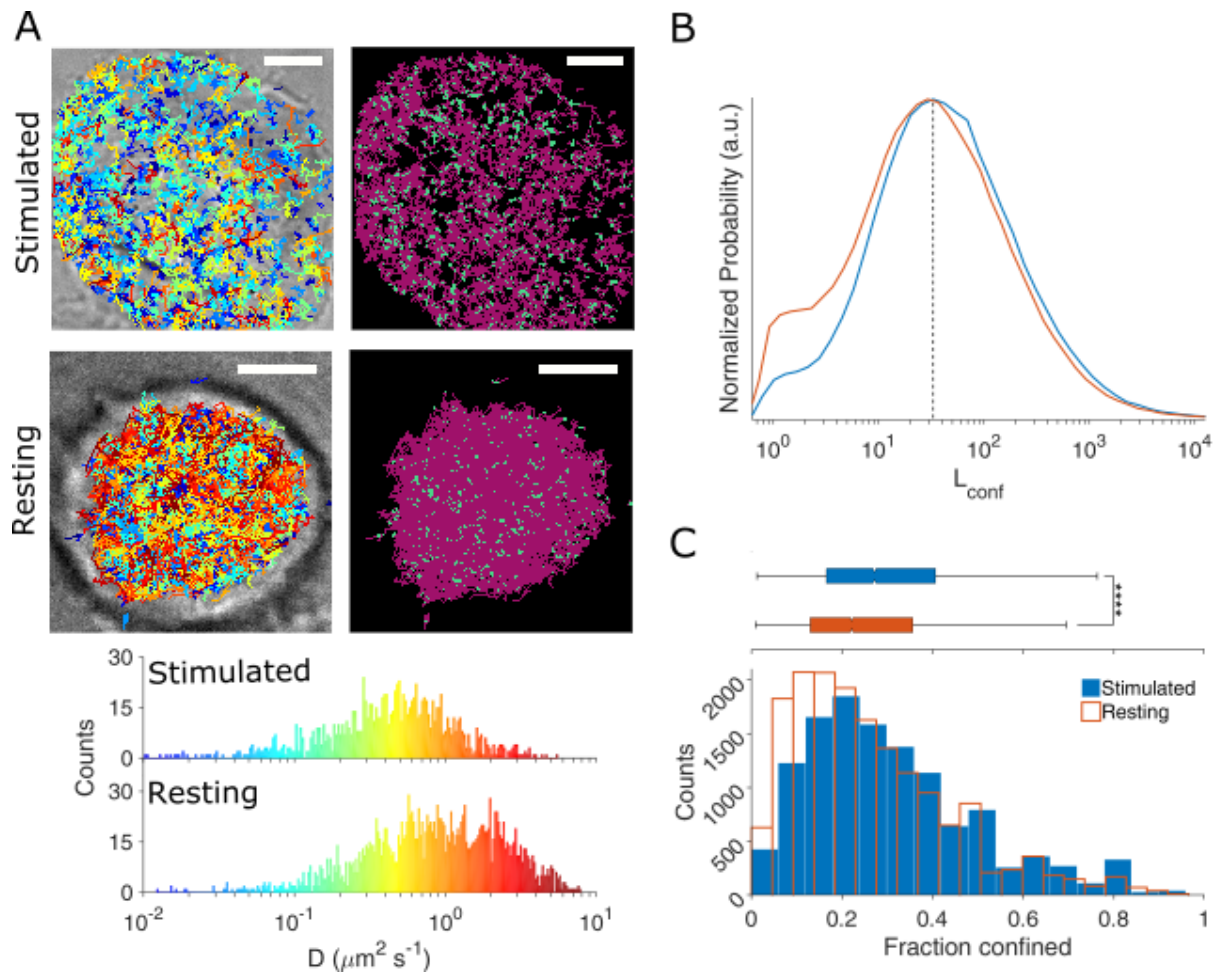
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614 **Fig.1 wtLck switches between free and confined states.** (A) L_{Conf} acquired for Lck10-
615 PAmCherry in resting Jurkat cells (purple), wtLck-PAmCherry in stimulated Jurkat cells
616 (orange) and wtLck-PAmCherry in fixed cells (cyan), normalized to peak value. The dashed
617 vertical line marks the threshold where a particle was to be considered confined, i.e., if it had
618 three or more consecutive steps with an L_{Conf} value greater than that threshold. (B) An
619 experimental trajectory decomposed to free (magenta) and confined (cyan) states, with the
620 confinements highlighted in yellow circles. (C) Time evolution of L_{Conf} values for the
621 trajectory in (B) with the threshold marked with an orange dashed line and the confined
622 periods with a yellow shade. (D) Trajectory decomposition maps of wtLck-PAmCherry in a
623 stimulated live cells (left) and fixed Jurkat cells (right) Free periods are colored magenta,

624 whereas confined periods are colored cyan. Scale bar = 5 μm . **(E)** 5 μm by 5 μm zoomed-in

625 regions of interest in **(D)** (top – live, bottom - fixed). Scale bar = 1 μm .

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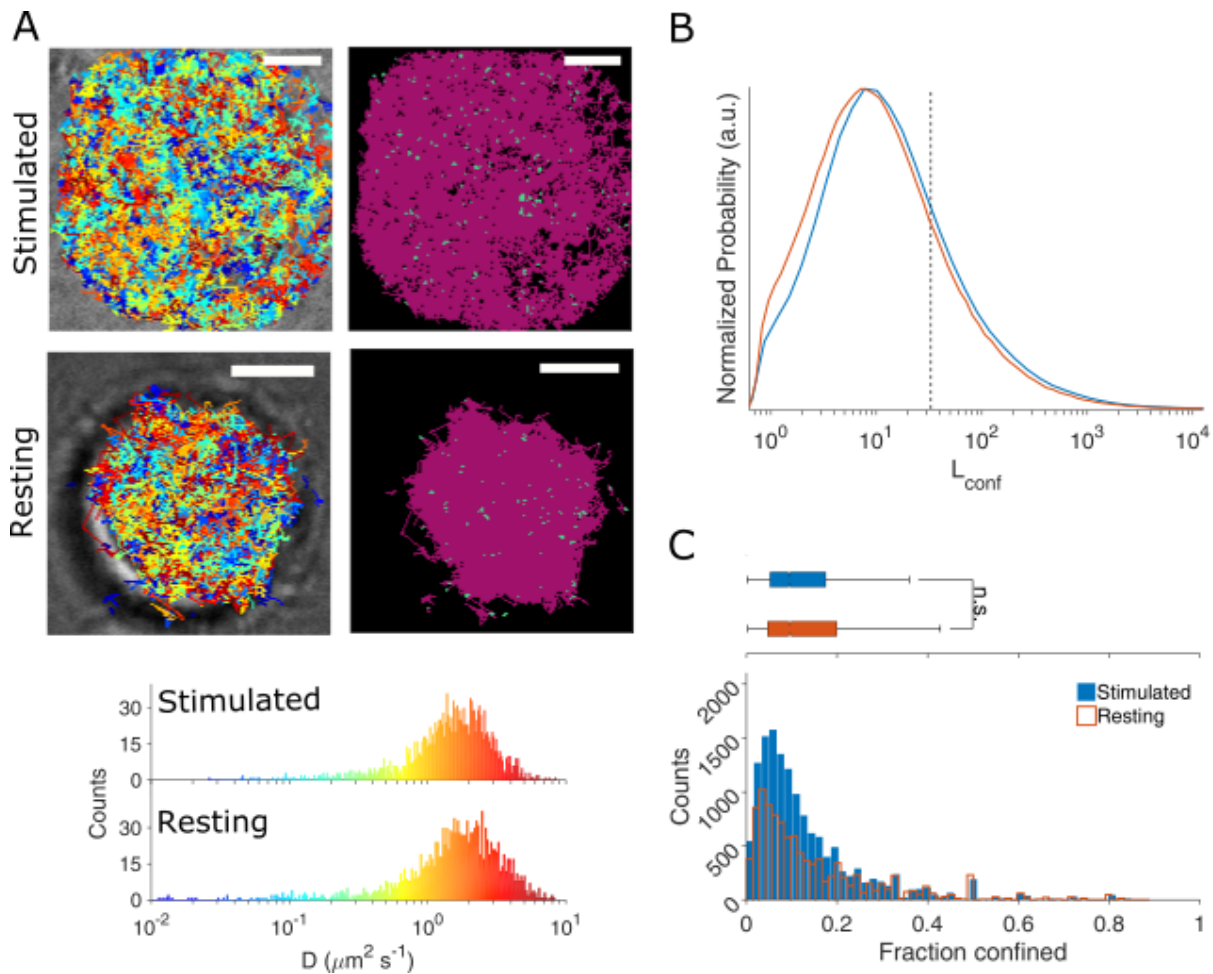
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629 **Fig.2 wtLck-PAMCherry is more confined in stimulated cells.** (A) Representative
 630 stimulated and resting Jurkat E6-1 cells expressing wtLck-PAMCherry. The left panels show
 631 bright field images of the cells with detected trajectories overlaid,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

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643 **Fig. 3 Lck10-PAmCherry demonstrates free-diffusion in resting and stimulated cells.**

644 (A) Representative stimulated and resting Jurkat E6-1 cells expressing Lck10-PAmCherry.

645 The left panels show bright field images of the cells with detected trajectories overlaid, color-

646 coded according to their initial diffusion. The right panels show the free (magenta) and

647 confined (cyan) modes of diffusion. Scale bar = 5 μm . Bottom: diffusions histogram

648 corresponding to the cells above, sharing mutual color-coding. (B) L_{Conf} histograms for

649 Lck10-PAmCherry in resting (orange) and stimulated (blue) cells. (C) Histograms of the

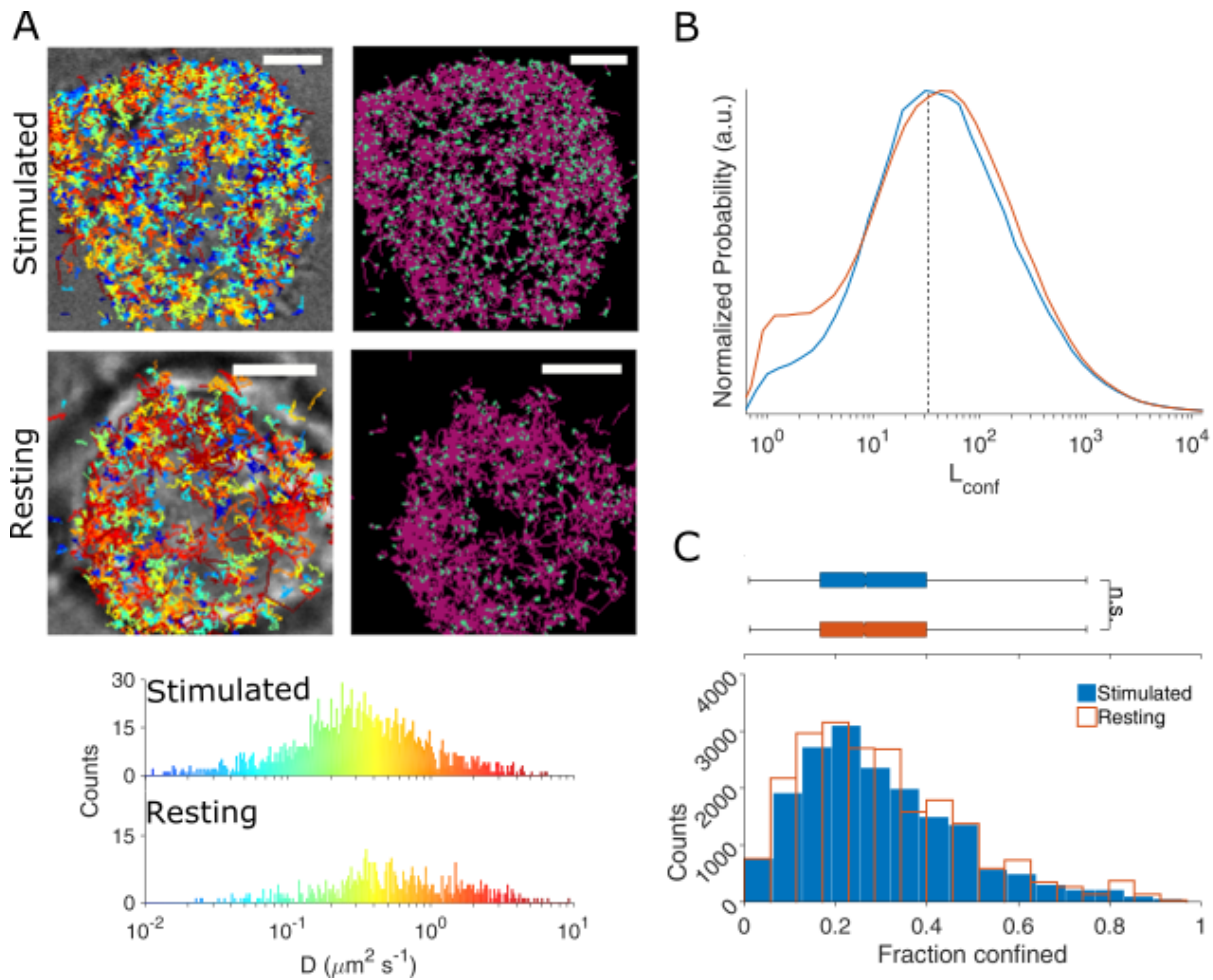
650 fraction of confined Lck10-PAmCherry molecules obtained for 19 stimulated (blue) and 15

651 resting (orange) Jurkat cells. Box plot shows the median. Notch 95% confidence interval, box

652 edges first and third quartile, lines Tukey's fences, n.s. $p > 0.01$.

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657 **Fig. 4** Lck^{Y505F}-PAmCherry is equally confined in stimulated and resting cells. (A)

658 Representative stimulated and resting Jurkat E6-1 cells expressing Lck^{Y505F}-PAmCherry. The

659 left panels show bright field images of the cells with detected trajectories overlaid, color-

660 coded according to their initial diffusion. The right panels show the free (magenta) and

661 confined (cyan) modes of diffusion. Scale bar = 5 μm. Bottom: diffusions histogram

662 corresponding to the cells above, sharing mutual color-coding. (B) L_{Conf} histograms for

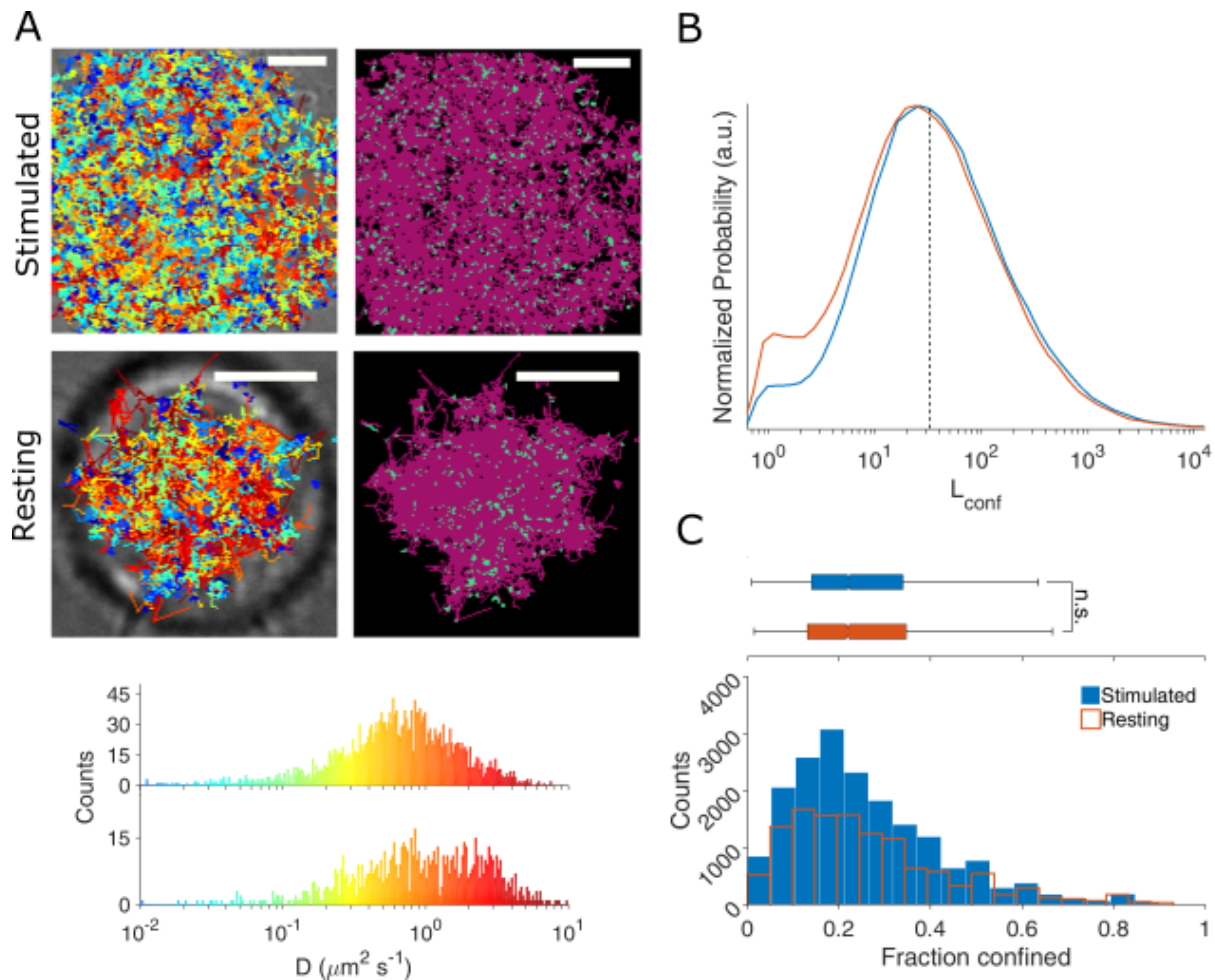
663 Lck^{Y505F}-PAmCherry in resting (orange) and stimulated (blue) cells. (C) Histograms of the

664 fraction of confined Lck^{Y505F}-PAmCherry molecules obtained for 14 stimulated (blue) and 18

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

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671 **Fig. 5** $\text{Lck}^{\text{Y394F}}$ -PAmCherry is equally confined in stimulated and resting cells. (A)

672 Representative stimulated and resting Jurkat E6-1 cells expressing $\text{Lck}^{\text{Y394F}}$ -PAmCherry. The

673 left panels show bright field images of the cells with detected trajectories overlaid, color-

674 coded according to their initial diffusion. The right panels show the free (magenta) and

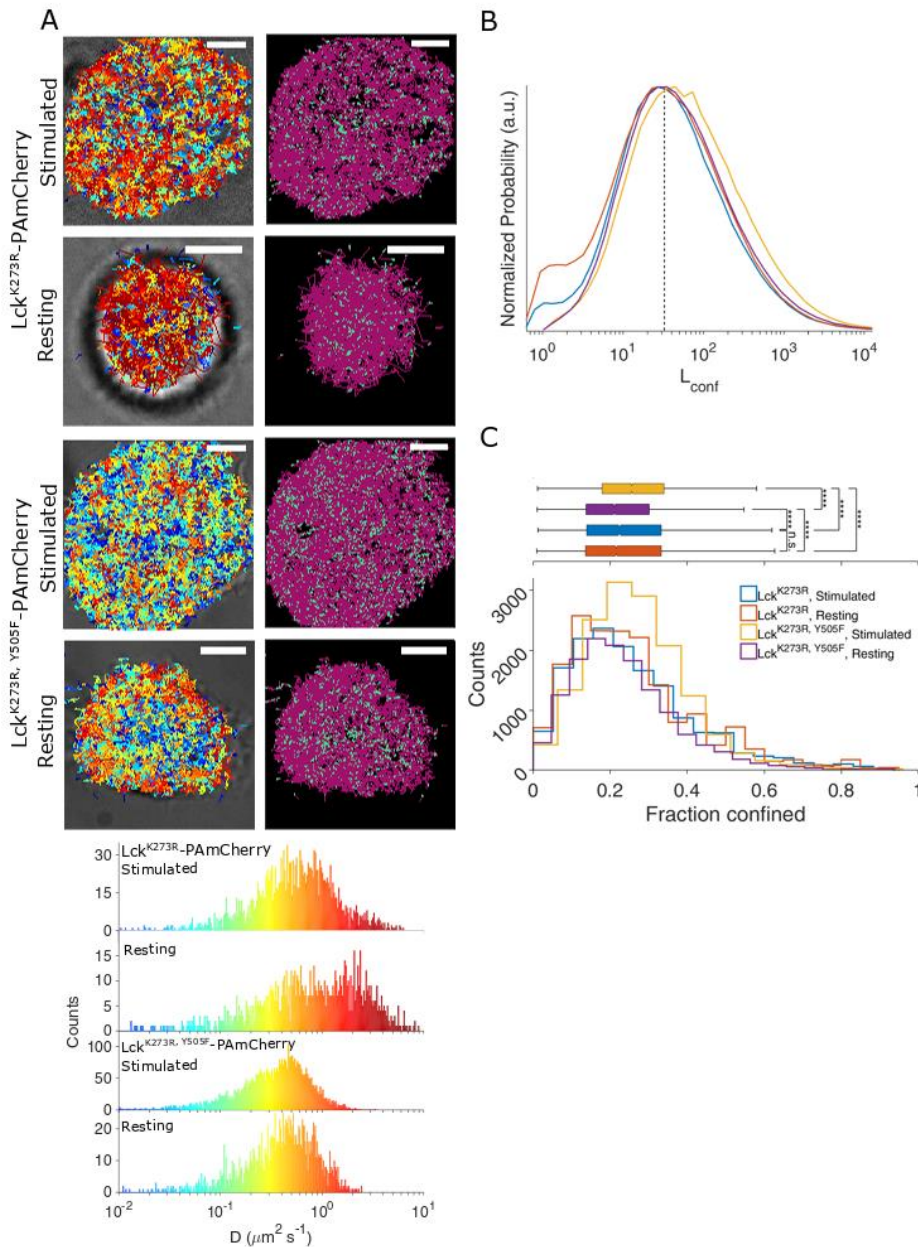
675 confined (cyan) modes of diffusion. Scale bar = 5 μm . Bottom: diffusions histogram

676 corresponding to the cells above, sharing mutual color-coding. (B) L_{Conf} histograms for

677 $\text{Lck}^{\text{Y394F}}$ -PAmCherry in resting (orange) and stimulated (blue) cells. (C) Histograms of the

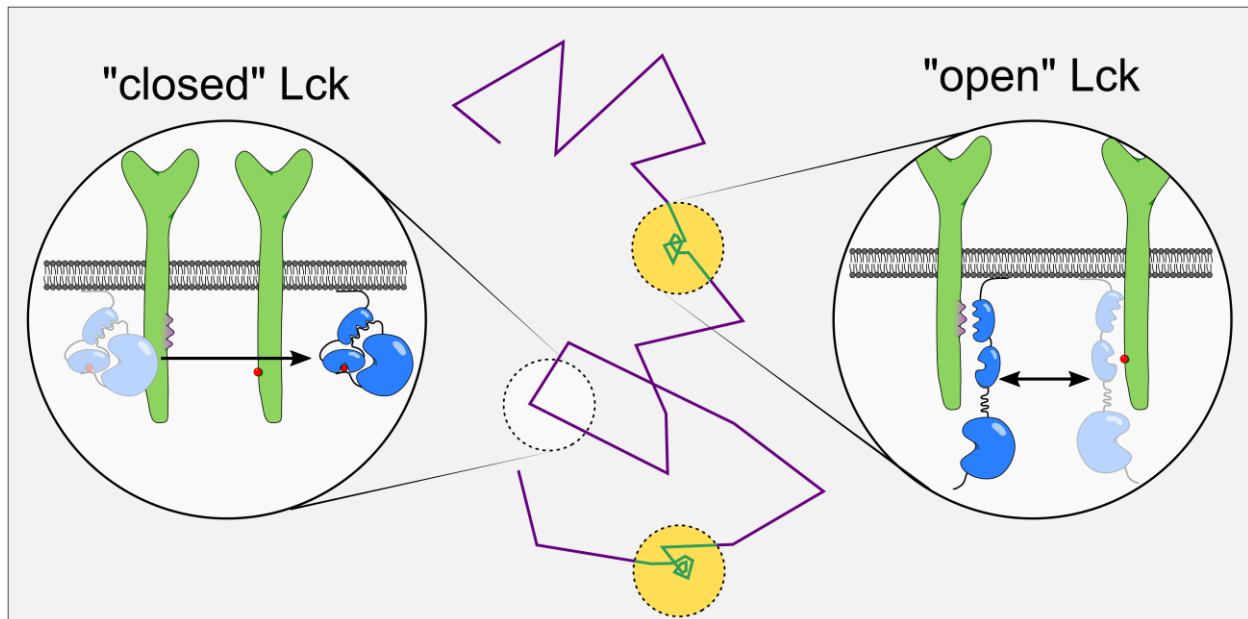
678 fraction of confined Lck^{Y394F}-PAmCherry molecules obtained for 16 stimulated (blue) and 14
679 resting (orange) Jurkat cells. Box plot shows the median. Notch 95% confidence interval, box
680 edges first and third quartile, lines Tukey's fences, n.s. $p>0.01$.

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 683 **Fig. 6 Confinement analyses for Lck^{K273R}-PAmCherry and Lck^{K273R, Y505F}-PAmCherry**
 684 **in stimulated and resting cells. (A)** Representative stimulated and resting Jurkat E6-1 cells
 685 expressing Lck^{K273R}-PAmCherry and Lck^{K273R, Y505F}-PAmCherry. The left panels show bright
 686 field images of the cells with detected trajectories overlaid, color-coded according to their
 687 initial diffusion. The right panels show the free (magenta) and confined (cyan) modes of
 688 diffusion. Scale bar = 5 μm. Bottom: diffusions histogram corresponding to the cells above,
 689 sharing mutual color-coding. **(B)** L_{Conf} histograms for Lck^{K273R}-PAmCherry and Lck^{K273R,}
 690 ^{Y505F}-PAmCherry in and stimulated cells. (orange, blue, purple and yellow, respectively). **(C)**

691 Histograms of the fraction of confined Lck^{K273R}-PAmCherry molecules obtained for 12
692 stimulated (blue) and 14 resting (orange) Jurkat cells and histograms of the fraction of
693 confined Lck^{K273R, Y505F}-PAmCherry obtained for 8 stimulated (yellow) and 8 resting (purple)
694 Jurkat cells. Box plot shows the median. Notch 95% confidence interval, box edges first and
695 third quartile, lines Tukey's fences, , **** p \leq 0.00001, n.s. p $>$ 0.01.
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699 **Fig. 7 Two-stage diffusion model of Lck that combines an efficient search strategy with**

700 **a high phosphorylation rates of substrates.** Lck (illustrated in blue) exists in two main

701 conformations: a closed conformation characterized by low catalytic activity and mediated by

702 intramolecular interactions; and an open conformation characterized by high catalytic activity

703 and free SH2 and SH3 domains. Our data propose that the closed conformation diffuses

704 unimpeded (purple line), whereas the open conformation interacts with other membrane

705 proteins (illustrated in green) via SH2 and SH3 domain mediated interactions and becomes

706 confined (yellow circles) through rapid rebinding (teal line). Free diffusion allows Lck to

707 scan large membrane areas while confinement in the open conformation enables high

708 substrate phosphorylation rates.

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