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3 **Single-molecule imaging reveals distinct effects of ligands on**
4 **CCR5 dynamics depending on its dimerization status**

5 Fanny Momboisse¹, Giacomo Nardi², Philippe Colin³, Melany Hery¹, Nelia Cordeiro¹,
6 Olivier Schwartz¹, Nathalie Sauvonnet⁵, Fernando Arenzana-Seisdedos⁴, Thibault
7 Lagache^{2*}, Bernard Lagane³, Jean-Christophe Olivo-Marin², Anne BreLOT^{1*}

8

9 ¹ : Institut Pasteur, Université de Paris, CNRS UMR3569, Virus and Immunity Unit, F-
10 75015 Paris, France.

11 ² : Institut Pasteur, Université de Paris, CNRS UMR 3691, BioImage Analysis Unit, F-
12 75015 Paris, France.

13 ³ : Infinity, Université de Toulouse, CNRS, INSERM, UPS, 31024 Toulouse Cedex 03,
14 France.

15 ⁴ : Institut Pasteur, Université de Paris, INSERM U1108, Viral Pathogenesis Unit, F-
16 75015 Paris, France.

17 ⁵ : Institut Pasteur, Université de Paris, Group Intracellular Trafficking and Tissue
18 Homeostasis, F-75015 Paris, France.

19

20 *To whom correspondence may be addressed:

21 **Email:** anne.brelot@pasteur.fr; thibault.lagache@pasteur.fr

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24

25 **Abstract**

26 G protein-coupled receptors (GPCR) are present at the cell surface in different
27 conformational and oligomeric states. However, how these states impact GPCRs
28 biological function and therapeutic targeting remains incompletely known. Here, we
29 investigated this issue in living cells for the CC chemokine receptor 5 (CCR5), a major
30 receptor in inflammation and the principal entry co-receptor for Human
31 Immunodeficiency Viruses (HIV-1). We used TIRF microscopy and an original statistical
32 method to track and classify the motion of different receptors subpopulations. We
33 showed a diversity of ligand-free forms of CCR5 at the cell surface constituted of various
34 oligomeric states and exhibiting transient Brownian and restricted motions. These forms
35 were stabilized differently by distinct ligands. In particular, agonist stimulation restricted
36 the mobility of CCR5 and led to its clustering, a feature depending on β -arrestin, while
37 inverse agonist stimulation exhibited the opposite effect. These results suggest a link
38 between receptor activation and immobilization. Applied to HIV-1 envelope glycoproteins
39 gp120, our quantitative analysis revealed agonist-like properties of gp120s. Distinct
40 gp120s influenced CCR5 dynamics differently, suggesting that they stabilize different
41 CCR5 conformations. Then, using a dimerization-compromized mutant, we showed that
42 dimerization (i) impacts CCR5 precoupling to G proteins, (ii) is a pre-requisite for the
43 immobilization and clustering of receptors upon activation, and (iii) regulates receptor
44 endocytosis, thereby impacting the fate of activated receptors. This study demonstrates
45 that tracking the dynamic behavior of a GPCR is an efficient way to link GPCR
46 conformations to their functions, therefore improving the development of drugs targeting
47 specific receptor conformations.

48 **Introduction**

49 G protein-coupled receptors (GPCRs), also known as 7TM (seven transmembrane
50 helical) receptors, represent the largest group of cell surface receptors in humans that
51 transduce chemical signals from the extracellular matrix into the cell. They constitute one
52 of the primary drug target classes (Pierce et al., 2002).

53 GPCRs exist in different subpopulations at the cell surface, in part due to differential
54 post-translational modifications (Patwardhan et al., 2021; Scurci et al., 2021) and
55 arrangements of receptor loops and transmembrane domains (Deupi & Kobilka, 2010).
56 Receptor activation and G protein coupling indeed involves a series of conformational
57 changes from an inactive to an active state (Ahn et al., 2021). Coupling to different G
58 proteins or to other protein transducers (e.g. arrestins), as well as receptor
59 oligomerization expand the diversity of conformational states (Seyedabadi et al., 2019;
60 Sleno & Hebert, 2018). Molecular dynamics along with biophysical and structural studies
61 brought to light this variety of GPCR arrangements and showed how binding of different
62 ligands can stabilize or select different receptor conformations, which can in turn activate
63 different signaling pathways (Ahn et al., 2021). This concept of “functional selectivity” (or
64 “biased agonism”) opens the possibility to develop therapies specifically targeting a
65 selected receptor conformation, thereby increasing the effectiveness of drugs and
66 reducing their adverse effects (Seyedabadi et al., 2019).

67 The nature and proportion of the different forms of GPCRs vary depending on their
68 environment. This is likely to regulate the functional properties of the receptors (Colin et
69 al., 2018; Patwardhan et al., 2021). Few studies, however, confirmed this diversity of
70 receptors in living cells and investigated its regulation in time and space (Calebiro et al.,
71 2013; Gormal et al., 2020; Kasai et al., 2018; Martinez-Munoz et al., 2018; Sungkaworn
72 et al., 2017; Veya et al., 2015). In this study, we tracked the chemokine receptor CCR5

73 at the single molecule level to access its dynamic behavior at the plasma membrane and
74 identify the stoichiometry and the functional properties of the various receptor forms.

75 CCR5 is a class A GPCR expressed on the surface of hematopoietic and non-
76 hematopoietic cells. It is a key player in the trafficking of lymphocytes and
77 monocytes/macrophages and has been implicated in the pathophysiology of multiple
78 diseases, including viral infections and complex disorders with an inflammatory
79 component (Brelot & Chakrabarti, 2018; Flanagan, 2014; Vangelista & Vento, 2017). In
80 addition, the CCL5/CCR5 axis represents a major marker of tumor development
81 (Aldinucci et al., 2020). CCR5 binds several chemokines, including CCL3, CCL4, and
82 CCL5. Binding of chemokines results in conformational change of the receptor, which
83 then activates intracellular signaling pathways and leads to cell migration (Flanagan,
84 2014). CCR5 also binds the envelope glycoprotein of HIV-1, then acting as the major
85 HIV-1 entry co-receptor (Alkhatib et al., 1996; Brelot & Chakrabarti, 2018). One CCR5
86 allosteric ligand, maraviroc (MVC), is part of the anti-HIV-1 therapeutic arsenal (Dorr et
87 al., 2005), although emergence of MVC-resistant variants has been identified in some
88 patients (Tilton et al., 2010).

89 We and others showed the existence of various CCR5 populations present at the cell
90 surface (Abrol et al., 2014; Berro et al., 2011; Colin et al., 2013; Colin et al., 2018; Fox et
91 al., 2015; Jacquemard et al., 2021; Jin et al., 2014; Jin et al., 2018; Scurci et al., 2021).
92 Computational analysis predicts that CCR5 can adopt an ensemble of low-energy
93 conformations, each of which being differentially favored by distinct ligands and receptor
94 mutations (Abrol et al., 2014). CCR5 conformations display distinct antigenic properties,
95 which vary depending on cell types (Colin et al., 2018; Fox et al., 2015). The multiple
96 conformations interact differently with distinct ligands (agonist, antagonist, HIV-1
97 envelope glycoprotein) and differ in their biological properties, HIV co-receptor functions,

98 and abilities to serve as therapeutic targets (Abrol et al., 2014; Colin et al., 2013; Colin et
99 al., 2018; Jacquemard et al., 2021; Jin et al., 2014; Jin et al., 2018; Scurci et al., 2021).
100 In particular, coupling to G proteins distinguishes CCR5 populations that are differently
101 engaged by chemokines and HIV-1 envelope. This explains why HIV-1 escapes
102 inhibition by chemokines (Colin et al., 2013). In this context, the improved capacity of
103 chemokine analogs to inhibit HIV infection, as compared to native chemokines, is related
104 to their ability to target a large amount of CCR5 conformations (Jin et al., 2014).

105 Like other receptors of this class, CCR5 forms homo- and hetero-dimers with other
106 receptors, which contribute to the diversity of conformational states (Jin et al., 2018;
107 Sohy et al., 2009). We identified three homo-dimeric organizations of CCR5 involving
108 residues of transmembrane domain 5 (TM5) (Jin et al., 2018). Two dimeric states
109 corresponded to unliganded receptors, whereas binding of the inverse agonist MVC
110 stabilized a third state (Jin et al., 2018). CCR5 dimerization occurs in the endoplasmic
111 reticulum, thereby regulating the receptor targeting to the cell surface (Jin et al., 2018).
112 CCR5 dimerization also modulates ligand binding and HIV-1 entry into cells (Colin et al.,
113 2018). MVC stabilizes CCR5 homodimerization, illustrating that CCR5 dimerization can
114 be modulated by ligands (Jin et al., 2018), a feature shared with other chemokine
115 receptors (Isbilir et al., 2020). Allosteric interaction within CCR2/CCR5 heterodimers is
116 reported as well as cross-inhibition by specific antagonists (Sohy et al., 2009). This
117 suggests that dimerization impacts therapeutic targeting.

118 To characterize the diversity of CCR5 subpopulations at the cell surface and to
119 investigate the impact of CCR5 dynamics on its function, we tracked CCR5 fluorescent
120 particles by total internal reflection fluorescence (TIRF) microscopy (Calebiro et al.,
121 2013) and quantitatively classify their motion over time using an original statistical
122 method. We described CCR5 mobility patterns both at the basal state and after ligand

123 binding (using two agonists, the inverse agonist MVC, and HIV-1 envelope
124 glycoproteins) and under conditions that modulate CCR5 / G protein coupling, β -arrestin
125 binding, and dimerization. This study provides novel insights into the organization of a
126 GPCR at the cell surface and the mechanisms regulating its signaling and fate after
127 activation.

128 **Results**

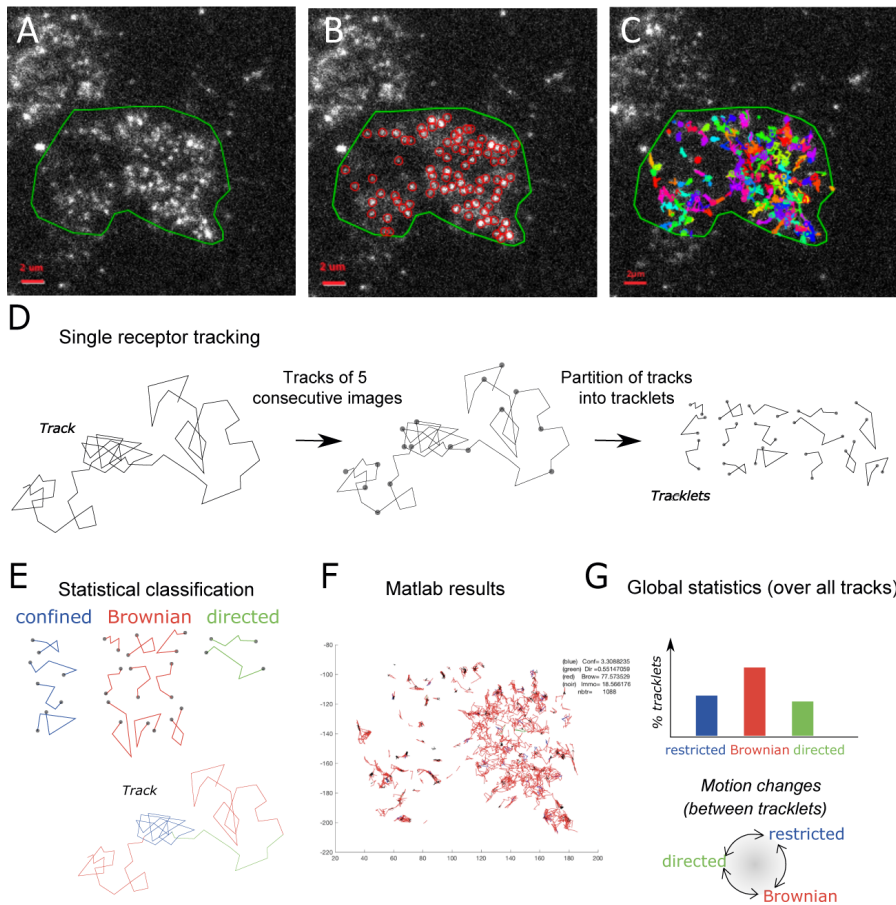
129 **Statistical classification of receptor trajectories at the cell membrane**

130 We generated a HEK 293 cell line stably expressing a low density of eGFP-CCR5 at the
131 cell surface (< 0.5 particles/ μm^2), which is critical for single particle tracking on the
132 surface of living cells (Calebiro et al., 2013). We choose HEK 293 cells because they do
133 not express CCR5. Fusion of eGFP to the N-terminus of CCR5 does not alter cell
134 surface expression of the receptor or its intracellular trafficking (Boncompain et al.,
135 2019).

136 To study the dynamics of CCR5 as a single particle at the plasma membrane of living
137 cells, we used TIRF microscopy, which restricts the observation to the first 200 nm from
138 the coverslip. The acquisitions were carried out at 37 °C with a frequency of 30 Hz (1
139 image / 33 ms). From the movies obtained, we tracked the motion of the particles over
140 time using the *Spot tracking* plugin of the ICY software (Chenouard et al., 2013; de
141 Chaumont et al., 2012) (**Figure 1A-C, Videos 1, 2, 3**, see Material and Methods).

142 The method generally used to evaluate the dynamics of a particle is based on Mean
143 Square Displacement (MSD) analysis (Qian et al., 1991). However, MSD is a global
144 analysis of particle trajectory that does not handle possible changes in particle motion. In
145 particular, it indicates whether the observed motion is standard Brownian motion and
146 computes the related diffusion coefficient of the trajectory, but it cannot characterize

147 more complex stochastic motions as the frequency of motion changes. In addition, the
148 MSD analysis does not provide a statistical significance of classified motion. To robustly
149 characterize the complex stochastic motions of single receptors at the cell membrane,
150 we used an alternative statistical method. We first partitioned single receptor trajectories
151 into small tracklets (with $N = 5$ consecutive detections each) to mitigate the risk of
152 tracking errors over long trajectories, and to detect potential motion changes between
153 tracklets within each single receptor trajectory (**Figure 1D**). We first evaluated immobile
154 objects and then used a robust statistical method to classify tracklet motion (see Material
155 and Methods and **Figure 1E-F**). Briefly, for each tracklet X , we computed the statistics
156 $S(X, N)$ introduced in (Briane et al., 2018) that evaluate the ratio between the maximal
157 distance reached by the tracklet particle from the initial point and the motion standard
158 deviation. We then used the statistics $S(X, N)$ to classify each tracklet into one of the
159 three following motion categories: *confined*, *Brownian*, or *directed* stochastic motion. For
160 this, we computed $S(X, N)$ for each tracklet and compared it to the quantiles $(q_\alpha, q_{1-\alpha})$,
161 which are statistical reference values of Brownian motion at level α and $(1 - \alpha)$.
162 Quantiles of $S(X, N)$ only depend on N and α (Briane et al., 2018), and can be evaluated
163 independently of the characteristics of experimental trajectories. Finally, tracklets X were
164 classified according to the associated stochastic motion: confined (if $S(X, N) < q(\alpha)$),
165 Brownian (if $q(\alpha) \leq S(X, N) < q(1 - \alpha)$), and directed motion (if $q(1 - \alpha) \leq S(X, N)$)
166 (**Figure 1-figure supplement 1**). We used this statistical classification procedure, with
167 $\alpha = 0.05$, to characterize the dynamics of CCR5 particles at the cell membrane.



168

169 **Figure 1.** Single particle detection of eGFP-CCR5 using TIRF microscopy and analysis with the
 170 statistical method. (A) Distribution of eGFP-CCR5 stably expressed in HEK 293 cells. Imaging
 171 was acquired at 30 Hz. The region of interest defined by the green line is used for A-C and F.
 172 Analysis of movies was performed using the ICY software and (B) the *Spot detection* and (C) the
 173 *Spot tracking* pluggins. Scale bare 2 μm . (D) Single receptor tracks were partitionned into
 174 tracklets of 5 images each. (E) Analysis of tracks with the statistical method: tracklets were
 175 classified into confined, Brownian, and directed motion. (F) Results obtained from Matlab. (G)
 176 Pooled tracklets classification provided a global estimate of receptor dynamics and the number of
 177 motion changes along the track (transition rates). (Restricted motions: immobile and confined
 178 motions).

179 **Video 1.** TIRF movie of a cell stably expressing eGFP-CCR5-WT acquired at 30 Hz. The region
 180 of interest was defined by the green line.

181 **Video 2.** TIRF movie of the same cell as in video 1 analyzed using the Icy software. Red circles
 182 correspond to the detection of bright spots using the *Spot detection* pluggin.

183 **Video 3.** TIRF movie of the same cell as in video 1 and 2 analyzed using the Icy software and the
 184 *Spot tracking* pluggin. Colored lines correspond to the tracked spots.

185 **Figure supplement 1.** Validation of the statistical method using simulated trajectories.

186 **CCR5 particles have different motions at the plasma membrane**

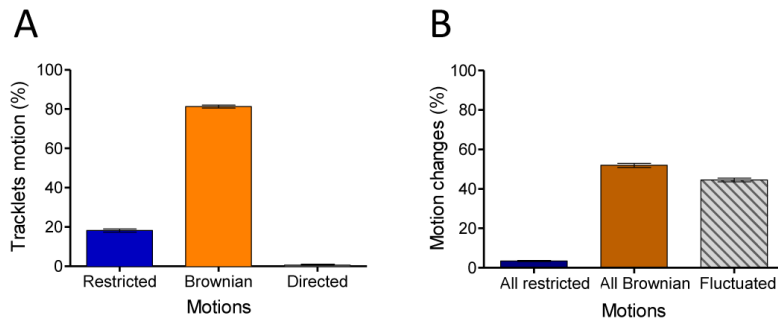
187 We investigated CCR5 mobility in the basal state using the statistical method described
188 above (**Figure 1**). The result of the classification of all the pooled tracklets provided a
189 global estimate of the receptor dynamics, while the number of motion changes along the
190 same trajectory gave us an estimate of the overall stability of the motion (**Figure 1G**).

191 In the basal state, the eGFP-CCR5 particles distributed homogeneously over the entire
192 membrane surface (**Figure 1A, Videos 1, 2, 3**). However, the motions of eGFP-CCR5
193 particles were heterogeneous (**Figure 2A**). Eighty percent of the pooled CCR5 tracklets
194 were mobile with Brownian motion, while 20 % were classified as restricted motion (i.e.
195 immobile and confined) (**Figure 2A**). We observed almost no directed trajectories (< 0.5
196 %). Around fifty percent of particles (52 %) exhibited Brownian motion over the entire
197 length of the path (**Figure 2B**). The other half fluctuated between Brownian and
198 restricted motion (**Figure 2B**). This high degree of fluctuation between motions within
199 one trajectory suggested the existence of transient conformations of CCR5 at the
200 plasma membrane.

201 Together, these analyses revealed heterogeneity of CCR5 motion at the basal state
202 consistent with the diversity of CCR5 forms described previously by other methods
203 (Abrol et al., 2014; Colin et al., 2013; Fox et al., 2015; Jin et al., 2018; Scurci et al.,
204 2021).

205

206



207

208 **Figure 2.** In the basal state, CCR5 exhibits different motions at the plasma membrane. (A)
209 Distribution of tracklets motion: restricted, Brownian, or directed (mean \pm SEM, n= 28 305 tracks
210 from 19 cells, 3 independent experiments). (B) Distribution of tracklets motion changes along
211 tracks (mean \pm SEM, n= 48 237 tracks from 45 cells, 7 experiments).

212

213 **Multiple ligands impact CCR5 mobility differently**

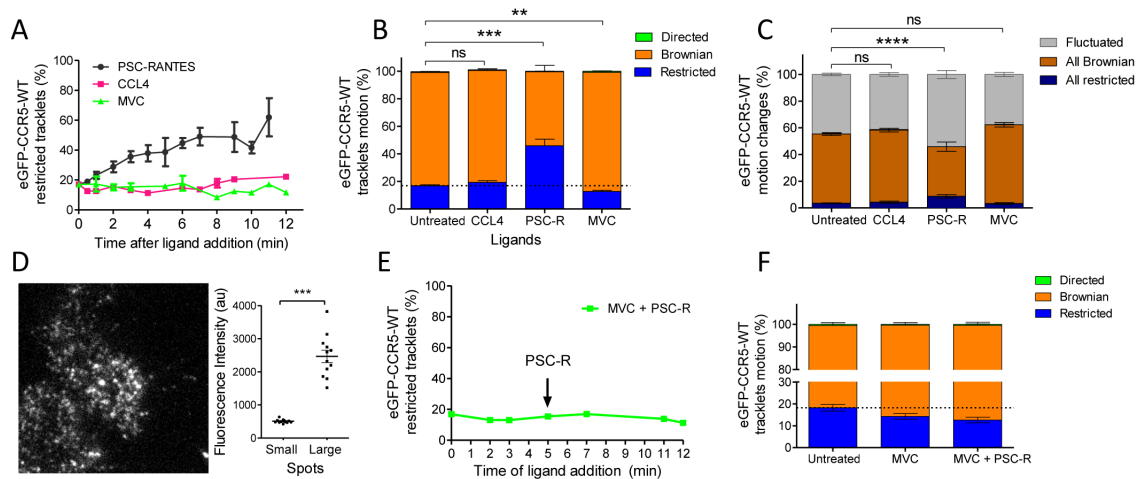
214 Since ligands modulate the conformation of CCR5 (Colin et al., 2018; Jacquemard et al.,
215 2021; Jin et al., 2018), we investigated the impact of ligand binding on its spatiotemporal
216 dynamic properties. We evaluated the effect of saturating concentration of ligands (two
217 agonists with different efficacies and the inverse agonist MVC, i.e. a ligand with a
218 negative efficacy) on CCR5 trajectories at the plasma membrane over time. We first
219 incubated the cells in the presence of the native chemokine CCL4 at a saturating
220 concentration (> 100 nM, $k_d = 0.4$ nM) (Colin et al., 2013) for the indicated time. The
221 mobility of the receptor was then assessed immediately after addition of the ligand in a
222 window of 1 to 12 min (**Figure 3A**). CCL4 triggered no significant change in CCR5
223 mobility after 10 min of stimulation (**Figure 3B**). However, a longer time of CCL4
224 stimulation (> 12 min) increased the percentage of restricted CCR5 tracklets, indicating
225 localized immobility of a small fraction of receptors (**Figure 3-figure supplement 1**). We
226 also noted the formation of large and immobile spots after 12 min of stimulation (**Video**
227 **4**).

10

228 We compared the effect of CCL4 with that of an agonist targeting a greater proportion of
229 receptor conformations and displaying a greater agonist efficacy, PSC-RANTES (Escola
230 et al., 2010; Jin et al., 2014). We incubated the cells in the presence of a saturating
231 concentration of PSC-RANTES (20 nM, $K_i = 1.9$ nM) (Colin et al., 2013) and evaluated
232 the motion of the receptors under the same conditions. PSC-RANTES triggered a
233 progressive increase in the number of tracklets classified as restricted motion over time
234 (**Figure 3A**). Ten minutes after stimulation with PSC-RANTES, about 50 % of eGFP-
235 CCR5 tracklets were in a restricted state (46 %) against 17 % under basal conditions
236 (**Figure 3B**). Consequently, the fraction of all Brownian trajectories decreased, while the
237 fraction of fluctuated and all restricted trajectories increased (**Figure 3C**).
238 Simultaneously, we observed the formation of large immobile spots (5 to 10 per cell) in
239 PSC-RANTES-treated cells (**Figure 3D, left**). These large spots had a long lifespan (50
240 to 100 frames) (**Video 5**). The quantification of the fluorescence intensity of the spots
241 from the frame 1 of live-imaging movies showed that the large spots had, on average,
242 intensity 4 times higher than the other spots, indicating a clustering of at least 4
243 receptors per large spot (**Figure 3D, right**). These results revealed a change in CCR5
244 mobility upon activation towards receptor immobilization and clustering, supporting
245 receptors trapping in nanodomains.

246 Unlike agonists, the inverse agonist MVC (10 μ M, $K_d = 1$ nM) did not restrict receptor
247 mobility (**Figure 3A, B, C**). On the contrary, the fraction of restricted eGFP-CCR5
248 tracklets at the surface of MVC-treated cells showed a slight decrease compared to
249 untreated cells (**Figure 3B**). We verified the specificity of PSC-RANTES-induced CCR5
250 immobility by treating cells with MVC before PSC-RANTES stimulation. MVC treatment
251 impaired PSC-RANTES-induced receptor immobilization (**Figure 3E-F**), indicating that
252 CCR5 immobilization depended on PSC-RANTES binding to CCR5.

253 These results showed that distinct ligands differently stabilize CCR5 in living cells, in
 254 accordance with our previous results (Colin et al., 2013; Colin et al., 2018; Jin et al.,
 255 2014; Jin et al., 2018). Interestingly, the amount of receptors immobilized correlates with
 256 the efficacy of ligands (PSC-RANTES > CCL4 > MVC), suggesting a link between
 257 receptor activation and immobilization.
 258



259

260 **Figure 3.** Different ligands, agonists and inverse agonist, impact CCR5 mobility differently. eGFP-
 261 CCR5-WT expressing cells were treated or not with a saturating concentration of agonists (CCL4,
 262 200 nM or PSC-RANTES, 20 nM) or inverse agonist (maraviroc, 10 μ M) and single particle
 263 tracking analysis was performed. (A) Percentage of restricted tracklets after treatment over time
 264 (n= tracks from 10, 4, and 3 cells for PSC-RANTES, CCL4, and MVC conditions respectively, at
 265 least 3 independent experiments). (B) Distribution of tracklets motion after 10 min of treatment
 266 (mean \pm SEM, n= 40 564, 15 421, 11 213, 9 828 tracks for each condition from 38, 14, 12, 9 cells
 267 respectively, at least 3 independent experiments). Unpaired t test on restricted motions only: ns,
 268 nonsignificant; $**P \leq 0.005$; $***P \leq 0.0001$. (C) Distribution of tracklets motion changes along
 269 tracks after 10 min of treatment (mean \pm SEM, n= 48 237, 8 954, 16 668, 9 828 tracks from 45, 9,
 270 17, 9 cells for each condition, at least 3 experiments). Unpaired t test on all restricted motions
 271 only: ns, nonsignificant; $****P \leq 0.0001$. (D) (Left) Single particle detection of eGFP-CCR5-WT
 272 after 3 min of stimulation with PSC-RANTES (20 nM) from frame 1 of live-imaging movie (one
 273 representative image). (Right) Mean of the sum of fluorescence intensity under large immobile
 274 spots and small mobile spots after 3 to 10 min of stimulation (mean \pm SEM, n= at least 40 spots
 275 from 12 cells, 3 experiments). (E) Percentage of restricted tracklets after successive stimulation

276 with maraviroc (10 μ M, 5 min) and PSC-RANTES (20 nM, 5 to 12 min) (one representative
277 experiment). (F) Distribution of tracklets motions after successive stimulation with maraviroc (10
278 μ M, during 5 min) and PSC-RANTES (20 nM, during 6 min) (mean \pm SEM, n= 14 467, 3 601, 2
279 075 tracks from 14, 2, 2 cells respectively, 1 experiment).

280 **Figure supplement 1.** Effect of CCL4 on CCR5 mobility.

281 **Video 4.** TIRF movie acquired at 30 Hz of a cell stably expressing eGFP-CCR5-WT and treated
282 by CCL4 (100 nM) for 14 min.

283 **Video 5.** TIRF movie acquired at 30 Hz of cells stably expressing eGFP-CCR5-WT and treated
284 by PSC-RANTES (20 nM) for 3 min.

285

286 **Gi coupling and β -arrestin association influence CCR5 motion differently under basal state** 287 **and stimulated conditions**

288 To further address the above hypothesis, we sought to determine whether the mobility of
289 CCR5 is influenced by its coupling to Gi protein, which stabilizes the receptor in an
290 activated state. We analyzed the pool of restricted CCR5 tracklets in the presence of
291 pertussis toxin (PTX), which uncouples the receptor from Gi proteins (**Figure 4A**).

292 In the basal state, the fraction of restricted eGFP-CCR5 tracklets from cells pre-treated
293 with PTX decreased compared to untreated cells (**Figure 4A**). Under this condition, PTX
294 also inhibited chemotaxis, a process that depends on CCR5 coupling to Gi proteins
295 (**Figure 4-figure supplement 1**). These results thus suggested that a small subset of
296 CCR5 is in a Gi protein-bound form in its basal state, which may contribute to the
297 transient restriction of the motion of CCR5 at the cell surface.

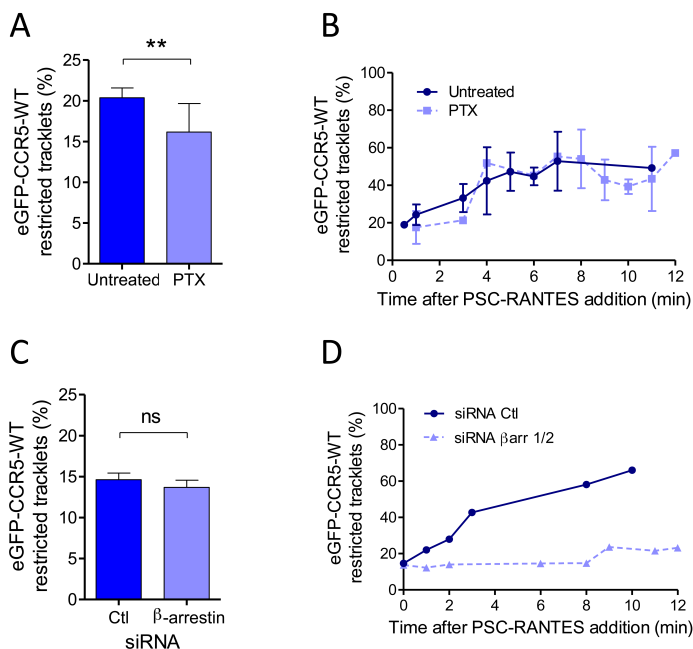
298 After stimulation, receptor immobilization could be due to the recruitment of receptors in
299 hub areas where the receptor meets the activation machinery and in particular the G
300 protein (Sungkaworn et al., 2017). To evaluate the role of Gi coupling on receptor
301 immobilization after PSC-RANTES stimulation, we analyzed tracks of TIRF movies of
302 PSC-RANTES-stimulated cells pretreated or not with PTX. In this condition, the fraction
303 of restricted tracklets increased over time after stimulation in the same proportion

304 regardless of PTX treatment (**Figure 4B**). This suggested that Gi coupling was not
305 involved in PSC-RANTES dependent immobilization of CCR5 after several minutes of
306 stimulation. This result is actually consistent with our previous study showing high affinity
307 interaction of PSC-RANTES with Gi protein uncoupled CCR5 (Colin et al., 2013).

308 After stimulation by PSC-RANTES, CCR5 follows a clathrin-dependent endocytosis
309 pathway, involving β -arrestins, which bridge the receptor to AP2 and clathrin (Delhaye et
310 al., 2007; Jin et al., 2014). We previously showed that silencing β -arrestin 1 and β -
311 arrestin 2 endogeneous expressions with siRNA decreased CCR5 internalization after
312 PSC-RANTES stimulation (Jin et al., 2014). Silencing β -arrestins in eGFP-CCR5 cells
313 with siRNA did not impact eGFP-CCR5 motion in the basal state (**Figure 4C**) but
314 inhibited PSC-RANTES-induced eGFP-CCR5 immobilization and clustering (**Figure 4D**).

315 These experiments indicated that β -arrestins contributed to CCR5 immobilization after
316 stimulation.

317 Together, these results pointed to the existence of a fraction of CCR5 in a transient pre-
318 assembled signaling complex in the basal state, which is consistent with previous
319 studies showing CCR5 constitutive activity (Garcia-Perez et al., 2011; Lagane et al.,
320 2005). They also suggested that the fate of CCR5 several minutes after activation is
321 independent of Gi coupling but dependent on β -arrestin recruitment, in accordance with
322 receptor desensitization and uncoupling after activation (Flanagan, 2014).



323

324 **Figure 4.** Gi coupling and β -arrestins association restrict CCR5 mobility at basal state or after
325 PSC-RANTES stimulation. (A) Percentage of restricted tracklets in eGFP-CCR5-WT expressing
326 HEK 293 cells pre-treated or not with 100 ng/ml of PTX for 3 h (mean \pm SEM, n= 8 614 and 11
327 377 tracks for each condition, 12 and 15 cells respectively, 3 independent experiments). Unpaired
328 t test: p value 0.0083**. (B) Percentage of restricted tracklets over time of eGFP-CCR5-WT
329 expressed on PSC-RANTES (20 nM) treated cells after incubation or not with PTX (100 ng/ml)
330 (mean \pm SD, n= 3 independent experiments). (C) Proportion of restricted tracklets in eGFP-
331 CCR5-WT expressing cells transfected with siRNA β arr1/2 (mean \pm SD, n= 6 754 and 8 854
332 tracks for each condition, from 7 and 8 cells respectively). Unpaired t test: p value 0.46, ns. (D)
333 Percentage of restricted tracklets over time of eGFP-CCR5-WT expressed on PSC-RANTES (20
334 nM) treated cells after siRNA β arr 1/2 transfection (n= 1 representative experiment).

335 **Figure supplement 1.** Effect of PTX treatment on chemokine-mediated chemotaxis.

336

337 **Immobilization of CCR5 after stimulation depends on its oligomeric state**

338 We previously showed by energy transfer experiments that a point mutation of CCR5 in
339 TM5 (L196K) leads to a receptor, which has a reduced dimerization capacity compared
340 to CCR5-WT (Jin et al., 2018). To study the role of CCR5 dimerization on its mobility, we

341 generated HEK 293 cells stably expressing eGFP-CCR5-L196K in the same proportion
342 to the clone expressing eGFP-CCR5-WT.

343 We studied the molecular composition of both eGFP-CCR5-L196K and eGFP-CCR5-WT
344 in these cells by analyzing the fluorescence intensity of eGFP per spot from the frame 1
345 of live-imaging movies. In a previous study, we calibrated the fluorescence intensity of
346 eGFP while spotted on glass coverslip (Salavessa et al., 2021). We showed that most of
347 eGFP spots bleached in a single step, suggesting that eGFP corresponds to 1 molecule,
348 with an average fluorescence intensity of 300-500 au (Salavessa et al., 2021). In eGFP-
349 CCR5 expressing cells, the fluorescence intensities were distributed in Gaussians, which
350 we classified with the Akaike information criterion (AIC, see Material and Methods)
351 (Akaike, 1974). We observed three types of Gaussians with double or triple mean
352 intensities (300, 600, 900 au), which likely correspond to spots comprising 1, 2, or 3
353 receptors (**Figure 5A**). This reflected the existence of a heterogeneous distribution of
354 receptors. In this classification, the WT receptor distributed in 50 % monomers, 40 %
355 dimers, and 10 % oligomers (trimers or more) at the plasma membrane, while eGFP-
356 CCR5-L196K was mostly in a monomeric form (75 % monomers, 25 % dimers) (**Figure**
357 **5B**). These results revealed that eGFP-CCR5-L196K was mostly monomeric at the
358 surface of living cells and that the fusion of eGFP to CCR5 did not alter the effect of
359 L196K mutation on CCR5 dimerization.

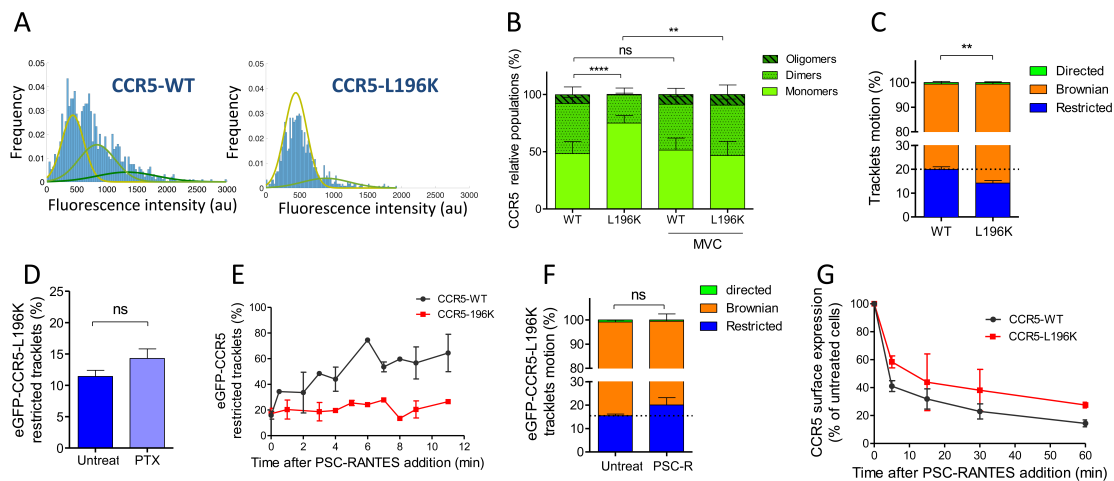
360 In the presence of MVC, both eGFP-CCR5-WT and eGFP-CCR5-L196K distribution
361 exhibited 50 % monomers, 40 % dimers, and 10 % oligomers (**Figure 5B**). The change
362 of eGFP-CCR5-L196K stoichiometry distribution in the presence of MVC confirmed our
363 previous results showing that MVC stabilized CCR5 in a novel dimeric form, which was
364 not disrupted by the introduction of a lysine in TM5 (Jin et al., 2018).

365 To investigate the impact of CCR5 dimerization on its mobility, we compared the motion
366 of eGFP-CCR5-L196K to eGFP-CCR5-WT at the cell surface. As for eGFP-CCR5-WT,
367 eGFP-CCR5-L196K tracklets were predominantly classified as Brownian tracklets
368 motion (85 % of the tracklet motions are Brownian), suggesting that monomeric and
369 dimeric forms of CCR5 exhibited similar behavior at the surface of cells in the basal state
370 (**Figure 5C**). However, we observed a decrease in the proportion of restricted tracklets
371 for eGFP-CCR5-L196K compared to eGFP-CCR5-WT (**Figure 5C**). These data
372 suggested that dimerization contributed to the stability of CCR5 molecules at the cell
373 surface, as previously proposed (Calebiro et al., 2013).

374 To test whether eGFP-CCR5-L196K coupling to Gi protein accounts in its restriction as
375 shown for eGFP-CCR5-WT, we pre-treated cells with PTX. Contrary to eGFP-CCR5-
376 WT, PTX treatment did not alter the proportion of the eGFP-CCR5-L196K restricted
377 tracklets pool (**Figure 5D**), suggesting that most of eGFP-CCR5-L196K were not
378 precoupled to the Gi protein at the basal state or that G protein precoupling induces
379 differential effects on the dynamics of both receptors. Supporting the first hypothesis,
380 previous biochemical and energy transfer experiments on a distinct GPCR showed that
381 there could be a link between dimerization and Gi coupling at basal state (Maurice et al.,
382 2010).

383 To investigate whether dimerization affected CCR5 mobility after stimulation, we
384 analyzed single-molecule movies of eGFP-CCR5-L196K cells after PSC-RANTES
385 treatment (**Figure 5E-F**). Contrary to eGFP-CCR5-WT massive immobilization and
386 clustering upon PSC-RANTES treatment (**Figure 3A-B**), eGFP-CCR5-L196K was only
387 slightly immobilized after 10 minutes of treatment (**Figure 5E-F**), while large immobile
388 spots were not detected (**Video 6**). This result indicated that CCR5 immobilization and
389 clustering after stimulation depend on CCR5 dimerization.

390 Because CCR5-WT immobilization involved β -arrestins (**Figure 4D**), an explanation for
 391 the lack of PSC-RANTES induced eGFP-CCR5-L196K immobilization is that eGFP-
 392 CCR5-L196K fails to recruit β -arrestins and therefore, is not desensitized and/or
 393 internalized after stimulation. To test this hypothesis, we evaluated PSC-RANTES-
 394 induced internalization of the dimerization-compromised mutant compared to the WT
 395 receptor in feeding experiments using FLAG-SNAP-CCR5 expressing cells (Delhaye et
 396 al., 2007; Jin et al., 2018). A saturating concentration of PSC-RANTES decreased cell
 397 surface expression of both receptors, but not in the same proportion (**Figure 5G**),
 398 suggesting that CCR5 dimerization impacted its internalization process. However, while
 399 eGFP-CCR5-L196K immobilization was drastically impaired, its internalization was not
 400 fully abrogated. These results supported that dimerization is a pre-requisite to the
 401 immobilization of the receptor, but was not essential for receptor internalization. This
 402 ruled out a necessary step of massive receptor immobilization before internalization and
 403 revealed that different mechanisms may contribute to CCR5 internalization depending
 404 on the receptor conformation.



405

406 **Figure 5.** Dimerization through TM5 alters CCR5 mobility and trafficking. (A) Distribution of the
 407 fluorescence intensity of spots detected at the surface of HEK 293 cells expressing eGFP-CCR5-

408 WT or eGFP-CCR5-L196K. One representative experiment out of 6 (n= 943 spots from 6 cells
409 and 1 207 spots from 8 cells for each condition); (B) Quantification of the fluorescent populations
410 depending on the mean of the gaussian at the surface of cells treated or not with MVC (10 μ M)
411 (mean \pm SD, nWT = 5 171 spots from 47 cells, 9 experiments; nL196K = 3 144 spots from 30
412 cells, 5 experiments; nWT-MVC = 3 055 spots from 25 cells, 4 experiments; nL196K-MVC = 1
413 776 spots from 16 cells, 3 experiments). Unpaired t test on monomers: p value $**P \leq 0.005$; $****P$
414 ≤ 0.0001 ; ns $P \geq 0.05$; (C) Distribution of pooled tracklets motion of eGFP-CCR5-WT and eGFP-
415 CCR5-L196K (mean \pm SEM, n = 11 321 tracks from 10 cells and 10 460 tracks from 12 cells in
416 each condition; 2 independent experiments). Unpaired t test on the restricted tracklets: p value
417 0.0015**. (D) Percentage of restricted tracklets in eGFP-CCR5-L196K cells pre-treated or not
418 with 100 ng/ml of PTX for 3 h (mean \pm SEM, n= 7 cells). Unpaired t test: p value 0.15, ns. (E)
419 Percentage of restricted tracklets over time of PSC-RANTES induced eGFP-CCR5-WT or eGFP-
420 CCR5-L196K expressing cells (mean \pm SD of 3 independent experiments). (F) Distribution of
421 tracklets motion after 10 min of PSC-RANTES stimulation (20 nM) (mean \pm SEM, n= 11 218
422 tracks from 10 cells and 5 433 tracks from 4 cells for untreated and PSC-RANTES treated cells
423 respectively, 2 independent experiments). Unpaired t test: p value 0.055, ns. (G) CCR5
424 internalization. Cell surface expression of FLAG-SNAP-CCR5-WT or FLAG-SNAP-CCR5-L196K
425 was monitored by flow cytometry in stable HEK 293 cell clones after stimulation with a saturating
426 concentration of PSC-RANTES (20 nM) for the indicated time. The percentage of total bound
427 anti-FLAG antibody was calculated from the mean fluorescence intensity relative to untreated
428 cells (mean \pm SD from two independent experiments).

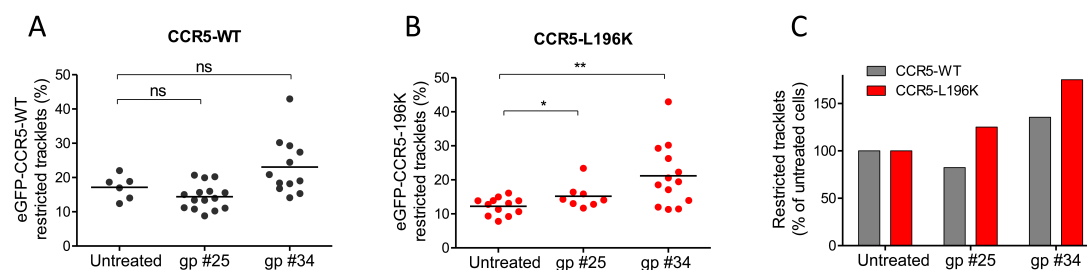
429 **Video 6.** TIRF movie acquired at 30 Hz of a cell stably expressing eGFP-CCR5-L196K and
430 treated by PSC-RANTES (20 nM) for 2 min.

431

432 **Distinct HIV-1 envelope glycoproteins gp120 differently influenced CCR5 dynamics**

433 Pharmacological studies suggested that distinct CCR5 conformations at the cell surface
434 differentially engaged distinct HIV-1 envelope glycoproteins gp120 (Colin et al., 2018).
435 Since we showed here that CCR5 mobility and ligand engagement are intrinsically
436 linked, we used our mobility classification method to characterize the effect of different
437 HIV-1 gp120s on CCR5 mobility and tested in living cells whether different gp120s
438 engaged different conformational states of CCR5.

439 We tested the effect of two soluble gp120s, gp #25 and gp #34, described to induce
440 distinct conformational rearrangements in CCR5 (Jacquemard et al., 2021), and to have
441 different binding capacities to the receptor and fusogenic efficacies (Colin et al., 2018).
442 Twenty min of gp120s exposure slightly modulated the mobility of eGFP-CCR5-WT,
443 although this trend was not statistically significant (**Figure 6A, C**). However, and in
444 contrast to what we observed using chemokines as ligands, the HIV-1 gp120s
445 immobilized eGFP-CCR5-L196K, with gp #34 having the highest effect (**Figure 6B, C**).
446 This suggested (i) that gp120s stabilized CCR5 conformations, which were different from
447 those stabilized by chemokines, and (ii) that different envelopes also stabilized
448 differently CCR5 conformations, in accordance with our previous result (Colin et al.,
449 2013; Colin et al., 2018).
450



451

452 **Figure 6.** HIV-1 gp120s binding restricts CCR5 mobility. Soluble gp120s were incubated 30 min
453 at RT in the presence of soluble CD4 (ratio sCD4/gp120 >5) to allow their binding to CCR5. Then,
454 gp120-sCD4 complexes were added to live eGFP-CCR5-WT or eGFP-CCR5-L196K expressing
455 cells during at least 20 min before single particle analysis. The proportion of restricted tracklets
456 after gp #25 and gp #34 treatment (100 nM) (in complex with sCD4) on eGFP-CCR5-WT (A, C)
457 or eGFP-CCR5-L196K (B, C) expressing cells was represented (n=3 independent experiments).
458 Unpaired t test: ** $P \leq 0.005$; *** $P \leq 0.0001$; ns $P \geq 0.05$.

459

460 **Discussion**

461

462 In this study, we developed a statistical method to classify the motion of fluorescent
463 particles at the cell surface. We applied this method to track eGFP-CCR5 under different
464 stimuli and different conformations. We showed that the receptor fluctuates between
465 Brownian and restricted motions at the cell surface, depending on (1) precoupling to Gi
466 proteins at the basal state; (2) the type of ligand bound to the receptor, and in particular
467 its efficacy on receptor activation and interaction with β -arrestins; and (3) receptor
468 dimerization. Indeed, CCR5 mobility changes following agonist stimulation were
469 dependent on β -arrestins recruitment and receptor dimerization, but were independent of
470 receptor interaction with Gi proteins. This study demonstrated that coupling single
471 molecule tracking to a statistical classification of trajectories is a powerful approach to
472 characterize the dynamic behaviors of functionally different receptor populations at the
473 plasma membrane.

474 **Diversity of ligand-free forms of CCR5 at the cell surface.**

475 Quantitative analysis of the motion of CCR5 particles and their numbering within the
476 fluorescent spots present at the cell membrane of HEK 293 cells revealed in the basal
477 state (i) two classes of receptor trajectories, Brownian (80 %) and restricted (20 %)
478 (**Figure 2**) and (ii) different oligomeric states (**Figure 5**): monomers (50 %), dimers (40
479 %), and oligomers (more than three receptors) (10 %). These features shared with other
480 GPCRs (Gormal et al., 2020; Martinez-Munoz et al., 2018; Sungkaworn et al., 2017;
481 Tabor et al., 2016; Veya et al., 2015), established the existence of multiple CCR5 forms
482 at the cell membrane.

483 In addition, our statistical method highlighted a fluctuation between Brownian and
484 restricted states during the same trajectory, suggesting the existence of transient
485 populations of receptors (**Figure 2B**). The change in mobility between periods of

486 confinement separated by free diffusion could be attributed to the molecular organization
487 of the receptor oscillating between different oligomeric forms at the cell surface
488 (monomers, dimers, oligomers), as proposed for CCR5 (Jin et al., 2018) or other
489 receptors (Isbilir et al., 2020; Kasai et al., 2018; Martinez-Munoz et al., 2018; Tabor et
490 al., 2016). In agreement with this, we observed differences in mobility between
491 monomers and dimers of CCR5 (**Figure 5C**). Change in mobility could also be linked to
492 a transient coupling to G proteins, leading to a transient immobility of the receptor in the
493 basal state. This hypothesis is supported by our data in the presence of PTX (**Figure**
494 **4A**) or in the presence of the inverse agonist MVC (**Figure 3A, B**), which both uncouple
495 the receptor from G proteins and decreased the proportion of immobile receptors. These
496 data are consistent with dual-color TIRF-M analysis of adrenergic receptor and G
497 protein, showing that an active receptor-G protein complex is formed in a confined
498 region of the plasma membrane at the basal state and lasts around 1 second
499 (Sungkaworn et al., 2017). However, they contrast with a study on mGluR3 showing
500 higher mobility of the receptor when complexed with G protein (Yanagawa et al., 2018).
501 This suggested that dynamics of distinct GPCRs can be differently impacted by coupling
502 to G proteins. Regarding β -arrestin association, we showed using siRNA that CCR5 was
503 not precoupled to β -arrestins in its basal state (**Figure 4C**). This result suggests that
504 CCR5 conformations, which bind to G proteins are not recognized by β -arrestins. This is
505 consistent with the idea that the conformations of receptors interacting with G proteins
506 and β -arrestins are different (Lagane et al., 2005).

507 **Different ligands recognize/stabilize different sets of CCR5.**

508 We showed that CCR5 mobility is influenced differently according to the ligand it binds.
509 Chemokine-induced activation of eGFP-CCR5-WT decreased mobility and leads to
510 clustering (**Figure 3B, D**), effects not observed with the inverse agonist MVC and

511 abolished by MVC (**Figure 3A, B and Figure 3E, F**). This result reinforces the link
512 between GPCR mobility and ligand binding proposed for GPCRs of different classes
513 (Gormal et al., 2020; Moller et al., 2020; Veya et al., 2015; Yanagawa et al., 2018).

514 We also showed that two agonists targeting different amount of receptors (CCL4 and
515 PSC-RANTES) (**Figure 3**) restricted receptor motion in a different proportion, leading to
516 speculate a relationship between mobility restriction and quantity of targeted receptor,
517 the greater the number of receptors targeted, the more receptors immobilized and
518 trapped. Although other possibilities should be considered, such as the dynamic nature
519 of the ligand-bound conformations, we proposed that characterizing ligands by their
520 impact on receptor motion opens a new way to classify biased ligands.

521 Applied to viral envelope glycoproteins, our single-particle approach revealed that HIV-1
522 gp120s displayed an agonist-like influence on CCR5 mobility, albeit to different extent
523 according to the nature of the gp120 (**Figure 6**). This feature contrasts with the cryo-EM
524 structure of the CD4-gp120-CCR5 complex, showing that CCR5 adopts inactive
525 conformation (Shaik et al., 2019). However, it is in line with gp120s-induced CCR5
526 signaling (Brelot & Chakrabarti, 2018; Flanagan, 2014) and with recent MD simulations
527 showing that gp120 binding reorients characteristic microswitches involved in GPCR
528 activation (Jacquemard et al., 2021). The fact that the fraction of immobilized receptors
529 varied between gp120s could reflect that they do not bind to the same CCR5
530 conformations, as previously shown (Colin et al., 2018; Jacquemard et al., 2021), and
531 suggests that these gp120s behave themselves as biased agonists. These features of
532 gp120s will help understand the determinants of HIV-1 tropism.

533

534 **Single particle tracking analysis revealed that dimerization regulates the fate of activated**
535 **CCR5.**

536 Our results suggest that receptor dimerization may regulate precoupling of CCR5 to Gi
537 proteins. Indeed, the mobility of the dimerization-compromized mutant eGFP-CCR5-
538 L196K was not affected by PTX treatment, in contrast to the WT receptor (**Figure 4A**
539 **and Figure 5D**). This suggests that most eGFP-CCR5-L196K receptors that reside
540 preferentially as monomers are not coupled to Gi proteins in the basal state, in
541 agreement with previous conclusion on CXCR4 (Isbilir et al., 2020). Alternatively, but not
542 exclusively, CCR5-L196K dimers might also be impaired in their ability to be precoupled
543 to Gi proteins, contrary to WT receptor dimers.

544 Our analysis suggests that dimerization is a pre-requisite to receptor immobilization and
545 clustering upon activation by chemokine agonists. Indeed, unlike eGFP-CCR5-WT,
546 eGFP-CCR5-L196K receptors are only marginally immobilized in the presence of PSC-
547 RANTES (**Figure 5E**). This result is not due to impaired binding of the chemokine,
548 because we controlled that PSC-RANTES induced efficient endocytosis of the mutant
549 receptor (**Figure 5G**). Receptor immobility and clustering were independent of Gi protein
550 coupling, as exemplified by unaffected CCR5 mobility after 10 min of agonist stimulation
551 in PTX pre-treated cells (**Figure 4B**), but most likely related to uncoupled and
552 desensitized form of CCR5 that accumulate in CCS (clathrin-coated structures), as
553 proposed (Grove et al., 2014; Yanagawa et al., 2018). This hypothesis was strengthened
554 with the essential role of β -arrestins in activated receptor immobility and clustering
555 (**Figure 4D**). We cannot rule out that activated receptor clustering may in addition
556 correspond to an accumulation of receptor in early endosome for a second phase of
557 activation (Irannejad et al., 2013).

558 In line with this, we showed that dimerization regulates endocytosis (**Figure 5G**). The
559 lack of immobilization of the dimerization-compromised mutant leads to a suboptimal
560 internalization of the receptor. This could be due to the impact of dimerization on the
561 route of endocytosis (dependent or independent of CCS) or on the association of β -
562 arrestins with the receptor, which stability regulates the fate of the receptor (Bonsch et
563 al., 2015). This later hypothesis is in accordance with studies showing an impact of
564 dimerization on β -arrestins recruitment (Fillion et al., 2019). Differential effects of gp120
565 on immobilization of CCR5 WT and L196K (**Figure 6**), compared to chemokines (**Figure**
566 **5**), could also be explained by differences in recruitment of β -arrestins, linked to
567 differences in the stabilized conformations of receptors.

568 Finally, our study suggested that CCR5 can be activated whether monomeric or dimeric.
569 We showed that eGFP-CCR5-L196K, while mostly monomeric in its basal state (**Figure**
570 **5B**), is still internalized (**Figure 5G**), suggesting that monomers can be activated, which
571 is consistent with studies reporting that GPCR monomers can be active enough on their
572 own to be functional (Whorton et al., 2007).

573 In summary, our single-particle tracking analysis established that a diversity of CCR5
574 forms exists at the surface of living cells and that distinct ligands stabilize different
575 receptors. This approach also revealed that receptor dimerization is involved in Gi
576 protein-coupling in the basal state, and in the immobilization of receptors after activation,
577 pointing out that receptor conformation regulates GPCRs signaling and fate after
578 activation. In addition, our work suggested that the different receptor conformations likely
579 engaged different ways of regulation, expanding GPCRs functions.

580

581 **Materials and Methods**

582

583 **Cell culture and reagents**

584 The HEK 293 cells stably expressing SNAP-FLAG-CCR5-WT and SNAP-FLAG-L196K
585 and the A3.01 human T cell line stably expressing CCR5 (A3.01-R5) were previously
586 described (Colin et al., 2013; Jin et al., 2018). These cell lines were maintained in
587 Dubelcco's modified Eagle medium (DMEM) (Thermo Fisher Scientific) or RPMI 1640
588 medium supplemented with 10 % Fetal Bovine Serum (FBS, GE Healthcare) and 100
589 µg/ml penicillin/streptomycin (Life technologies).

590 The CCR5 inverse agonist maraviroc (MVC) was obtained from the National Institutes of
591 Health. The native chemokine CCL4 was chemically synthesized by F. Baleux (Institut
592 Pasteur, Paris, France). The chemokine analog PSC-RANTES (N- α -(n-nonanoyl)-des-
593 Ser(1)-[L-thiopropyl(2), L cyclohexylglycyl(3)] RANTES(4-68)) was obtained through the
594 Center for Aids reagents, National Institute for Biological Standards and Control (NIBSC,
595 UK). The primary antibodies used are the anti-GFP (Roche), the anti-CCR5 2D7 mAb
596 (BD-Biosciences); the anti-FLAG monoclonal antibodies M1 or M2 (Sigma-Aldrich).
597 Secondary antibodies used were a phycoerythrin (PE)-conjugated anti-mouse antibody
598 (BD Biosciences). The toxin from Bordetella pertussis (PTX) used at a 100 ng/ml
599 concentration were from Sigma. The β arr1/2 siRNA (5'-ACCUGCGCCUCCGCUAUG-
600 3') and a scrambled siRNA (control, 5'-UGGUUUACAUGUCGACUAA-3') (Dharmacon)
601 were transfected by RNAimax (Invitrogen) according to the instructions of the
602 manufacturer, as described (Jin et al., 2014). To select siRNA positive cells, cells were
603 co-transfected with a plasmid coding the fluorescent protein mcherry (gift of F. Perez,
604 Institut Curie). Soluble, monomeric HIV-1 glycoprotein gp120 was produced using a
605 semliki forest virus (SFV) system as described (Benureau et al., 2016; Colin et al.,
606 2018). The sequence coding for gp120 #25 and gp120 #34 were from PBMCs of

607 patients collected early after seroconversion or in the AIDS stage of infection,
608 respectively (Colin et al., 2018). Recombinant soluble CD4 (sCD4), produced in S2 cell
609 lines, was purified on a strep-Tactin column using the One-STrEP-tag fused to the CD4
610 C-tail as a bait (production and purification of recombinant proteins technological
611 platform, C2RT, Institut Pasteur).

612 [Generation of cell lines](#)

613 The eGFP-CCR5 plasmid was a gift of F. Perez (Institut Curie, Paris, France)
614 (Boncompain et al., 2019). The mutant eGFP-CCR5-L196K (substitution of L196 with a
615 lysine) was generated by site-directed mutagenesis using the QuickChange II
616 Mutagenesis kit (Agilent Technologies) according to the manufacturer's instruction. This
617 mutant was verified by sequencing (Eurofins). HEK 293 cells stably expressing eGFP-
618 CCR5-WT and HEK 293 cells stably expressing eGFP-CCR5-L196K were generated by
619 calcium phosphate transfection and cultured for several weeks in 1 mg/ml G418
620 (Geneticin, Invitrogen). Cell Clones were screened and sorted by flow cytometry (Attune
621 NxT flow cytometer, Thermo Fisher) using an anti-GFP monoclonal antibody.

622 [Receptor cell surface expression levels and internalization measured by flow cytometry](#)

623 Flow cytometry was used to quantitate the internalization of FLAG-SNAP-CCR5-WT
624 compared to FLAG-SNAP-CCR5-L196K stably expressed in HEK 293 cells (Delhaye et
625 al., 2007; Jin et al., 2018). We measured the levels of cell surface CCR5 stained with the
626 anti-FLAG M2 antibody and with an anti-mouse coupled to phycoerythrin (PE) after
627 chemokine treatment or not. Cells were incubated with a saturable amount of M2 for 45
628 min to label receptors present at the plasma membrane, then incubated in the presence
629 (or not) of 20 nM PSC-RANTES for the indicated time at 37°C. Cells were chilled to 4°C
630 and stained with a PE conjugated anti-mouse IgG. Mean values were used to compute
631 the proportion of internalized receptors as indicated by a decrease of immune-reactive

632 surface with PSC-RANTES compared with untreated cells. Cells were analyzed with
633 Attune NxT flow cytometer (Thermo Fisher). At least 5,000 cells were analyzed per
634 experiment using Kaluza software. Background was subtracted using the fluorescence
635 intensity obtained on the parental HEK 293 cells.

636 Chemotaxis

637 CCR5 expressing A3.01 cells (A3.01-R5, 1.5×10^5), pre-treated or not with PTX (100
638 ng/ml) during 3 h, in prewarmed RPMI-1640 supplemented with 20 mM HEPES and 1%
639 serum, were added to the upper chambers of HTS-Transwell-96 Well Permeable
640 Supports with polycarbonate membrane of 5 μ m pore size (Corning). PSC-RANTES
641 (33.7 nM) or SDF-1 (control, 10 nM) was added to the lower chambers. Chemotaxis
642 proceeded for 4 h at 37 °C in humidified air with 5% CO₂. The number of cells migrating
643 across the polycarbonate membrane was assessed by flow cytometry with Attune NxT
644 flow cytometer (Thermo Fisher). Specific migration was calculated by subtracting
645 spontaneous migration from the number of cells that migrated toward the chemokine.

646 Live cell TIRF imaging

647 Round 25 mm No. 01 glass coverslips (Fisher Scientific) were pre-cleaned with 70%
648 ethanol followed by acetone, with three consecutive washes in ddH₂O. 1.15×10^5 cells
649 were plated onto pre-cleaned coverslips 72 h before imaging. Cells were imaged in TIRF
650 medium (25 mM HEPES, 135 mM NaCl, 5 mM KCl, 1.8 mM CaCl₂, 0.4 mM MgCl₂,
651 4.5 g/l glucose and 0.5% BSA, pH 7.4). Movies were acquired with an LSM 780 Elyra
652 PS.1 TIRF microscope (Zeiss) equipped with an EMCCD Andor Ixon 887 1K camera,
653 and using an alpha Pin Apo 100x/1.46 oil objective, a 488 nm (100 mW) HR solid laser
654 line, and a BP 495-575 + LP 750 filter to detect eGFP-CCR5. Image acquisition was
655 done at 1 frame / 33 msec (30 Hz) (100 to 200 frames), with 8 % (tracking) or 15 %
656 (stoichiometrie) laser power at 37°C. Approximately 5-10 cells were acquired per

657 condition, per experiment. All live-imaging movies were analyzed using the open-source
658 software Icy (Institut Pasteur).

659 [Track analysis protocol](#)

660 *Tracking receptors in TIRF imaging with Icy software*

661 To automatically detect eGFP-CCR5 tracks at the plasma membrane upon time, we
662 used the software Icy (<http://icy.bioimageanalysis.org>) and the plugin *Spot tracking*,
663 which reports their *xy* displacement and intensities, as previously described in Bertot *et al.*
664 *(Bertot et al., 2018)*. *Spot tracking* was set to detect spots with approximately 3 pixels,
665 and a threshold of 135. All other parameters were as default. Tracks were analyzed with
666 the *Track manager* plugin. All data was exported to *Excel* for further analysis.

667 Tracks containing more than 10 % of virtual detections and more than three successive
668 virtual detections were excluded from the track classification.

669 *Splitting tracks into tracklets*

670 We deal with trajectories that have very different lengths and we want to estimate motion
671 variations along the trajectory. Thus, we split all long tracks into several tracklets in order
672 to better classify local motions. According to Section 1, this is done by setting $N = 5$ and
673 considering only the tracks with length larger than 6. Then, the different successive
674 tracklets are defined by using the position between the $(5k)^{\text{th}}$ and $(5(k + 1))^{\text{th}}$ frame
675 with $k \geq 0$.

676 *Detecting immobile receptors*

677 To classify tracklets and identify distinct receptor dynamics, we first identified immobile
678 receptors. In time lapse imaging, a tracklet X is defined by the vector of its successive
679 positions at the different time frames $X = (X_0, \dots, X_{N-1})$, with N the length of the tracklet.

680 We considered that a receptor was immobile if

681

$$\max_{i \neq j=0, \dots, N-1} \|X_i - X_j\| < \sqrt{2} l$$

682 where l is the size of the object ($l=2$ pixels typically). In other words, the previous
683 criterion states that a tracklet is immobile if the maximal distance between two different
684 positions is at most equal to the length of the diagonal of the square of edge l .

685 *The 3 types of motion of mobile receptors*

686 To classify the other tracklets corresponding to mobile receptors, we used the statistical
687 method introduced in (Briane et al., 2018), which allows to distinguish three main types
688 of motions:

689 (i) **Brownian motion**: the object (receptor) evolves freely and its trajectory is denoted by
690 σB_t where σ is called the *diffusion coefficient*. The position of the object X_t at time t is
691 given by $X_t = X_0 + \sigma B_t$. Brownian increments σdB_t at each time are independent and
692 normally distributed.

693 (ii) **Directed motion**: the object is actively transported by a deterministic force, and its
694 motion can be modelled by the following stochastic differential equation:

$$dX_t = \mu dt + \sigma dB_t,$$

695 where μ is a 2D-vector called *drift* and represents the deterministic force, and σ is the
696 *diffusion coefficient* modelling the random Brownian motion.

697 (iii) **Confined motion**: the object is confined in a domain or evolves in an open but
698 crowded area. This kind of motion can be modeled by an Ornstein-Uhlenbeck process:

$$dX_t = -\lambda(X_t - \mu)dt + \sigma dB_t.$$

700

701 We refer to (Durrett, 2018) for more properties about Brownian motion and stochastic
702 calculus.

703 *Statistical classification of mobile tracklets*

704 The motion classification criterion defined in (Briane et al., 2018) essentially considers
705 the ratio between the maximal distance from the initial point and the length of the
706 tracklets. This can be evaluated by defining the following statistics

707

$$S(X, N) = \frac{\max_{i=0, \dots, N} |X_{t_i} - X_{t_0}|}{\left[\frac{1}{2} \sum_{i=1}^N |X_{t_i} - X_{t_{i-1}}|^2 \right]^{\frac{1}{2}}}$$

708

709 where $|\cdot|$ denotes the 2D-Euclidean norm. The classification is made by using the
710 quantiles of order α and $1-\alpha$ ($\alpha = 0.05$) of such a statistic for Brownian tracklets.

711 These quantiles, denoted by $q(\alpha)$ and $q(1-\alpha)$ respectively, depend on α and N , and can
712 be computed by Monte Carlo simulations (see (Briane et al., 2018)). This essentially
713 consists in simulating a high number of Brownian tracklets, computing their statistics
714 values and then evaluating the quantiles.

715 Then the tracklet motion is said to be confined if $S(X, N) < q(\alpha)$, directed if $S(X, N) > q(1-\alpha)$,
716 and Brownian otherwise. For $N=5$ and $\alpha = 0.05$, we obtained $q(\alpha) = 0.724$ and $q(1-$
717 $\alpha) = 2.464$.

718 *From local classification of tracklet motion to global analysis of receptors' tracks*

719 The above statistical classifier allows estimating the local motion of each receptor. In a
720 second time, we analyzed the difference of tracklet motions along the same longer
721 receptor track. In particular, we evaluated if a receptor changed its type of motion along
722 its trajectory.

723 Finally, our statistical framework for classifying tracklets motion provided a two-scales
724 picture of the receptors' dynamic behavior: the classification of tracklets provided a

725 global estimation of receptors' motion, while the identified changes of receptors' motion
726 along their full trajectories indicated the stability of each receptor's motion.

727 [Stoichiometry analysis](#)

728 Icy software was used to determine the intensity distribution of eGFP-spots. Spots were
729 detected using the Spot detector wavelet-based algorithm (Olivo-Marin, 2002), and then
730 converted to ROIs with 2 pixels radius. Data was exported to Excel. We observed a
731 multimodal distribution of eGFP spots' intensities, and we decided to use the AIC
732 criterion (Akaike information criterion) (Akaike, 1974) to uncover the number of modes in
733 intensity distribution. Each mode putatively corresponds to a number of molecules.
734 Therefore, statistical characterization of the multimodal distribution of eGFP spots'
735 intensity will help to classify each spot with respect to its mode and, therefore, to its
736 estimated number of molecules.

737 AIC analysis starts with the modeling of the empirical distribution $e(x)$ of eGFP
738 spots' intensities with a weighted sum of Gaussian laws,

$$e(x) = \sum_{i=1}^p \alpha_i N(\mu_i, \sigma_i)$$

739 where p is the number of Gaussian laws in the mixture, α_i the weight of each law and
740 (μ_i, σ_i) the corresponding mean and variance. For a fixed p , we first searched for the
741 optimal parameters $(\alpha_i^*, \mu_i^*, \sigma_i^*)$, for $i = 1..p$ that maximize the likelihood L of the model to
742 the data:

743

$$L_p(\alpha_1, \mu_1, \sigma_1, \dots, \alpha_p, \mu_p, \sigma_p) = \prod_{j=1}^n \left[\sum_{i=1}^p \frac{\alpha_i}{\sqrt{2\pi\sigma_i}} \exp\left(-\frac{(x_j - \mu_i)^2}{2\sigma_i}\right) \right]$$

744 where (x_1, x_2, \dots, x_n) are the observed eGFP intensities in the considered frame of the
745 time-lapse sequence.

746 This first step of the AIC analysis provides the calibrated parameters
747 $(\alpha_i^*, \mu_i^*, \sigma_i^*)_{i=1..p}$ when fitting a p -mixture model to data. Then, we computed the optimal
748 number of modes p^* that would describe the different populations of eGFP spots with
749 respect to their estimated number of molecules by minimizing the AIC:

$$AIC(p) = 2k_p - 2 \log(L_p^*)$$

750 where L_p^* is the maximized likelihood the p -mixture model, and $k_p = 3p - 1$ is the
751 number of free parameters of the p -mixture model.

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770

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772

773 **Author Contributions**

774 Fanny Momboisse, Conceptualization, Methodology, Validation, Formal analysis,
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776 Giacomo Nardi, Conceptualization, Methodology, Software, Validation, Formal analysis,
777 Writing - original draft preparation;

778 Philippe Colin, Validation, Formal analysis, Investigation;

779 Melany Hery, Validation;

780 Nelia Cordeiro, Validation;

781 Olivier Schwartz, Resources, Supervision, Funding acquisition;

782 Nathalie Sauvonnnet, Conceptualization, Formal analysis, Resources, Writing - original
783 draft preparation, Funding acquisition;

784 Fernando Arenzana-Seisdedos, Resources, Supervision, Funding acquisition;

785 Thibault Lagache, Conceptualization, Methodology, Software, Formal analysis, Data
786 curation, Writing - original draft preparation;

787 Bernard Lagane, Conceptualization, Formal analysis, Resources, Writing - original draft
788 preparation, Supervision, Funding acquisition;

789 Jean-Christophe Olivo-Marin, Resources, Supervision, Funding acquisition;

790 Anne BreLOT: Conceptualization, Methodology, Validation, Formal analysis, Investigation,
791 Resources, Data curation, Writing - original draft preparation, Visualization, Supervision,
792 Project administration, Funding acquisition.

793

794 **List of Figure Supplement**

795 Figure1-figure supplement 1

796 Figure3-figure supplement 1

797 Figure4-figure supplement 1

798

799 **Videos Title**

800 **Video 1.** TIRF movie of a cell stably expressing eGFP-CCR5-WT acquired at 30 Hz. The
801 region of interest was defined by the green line.

802 **Video 2.** TIRF movie of the same cell as in video 1 analyzed using the Icy software. Red
803 circles correspond to the detection of bright spots using the *Spot detection* plugin.

804 **Video 3.** TIRF movie of the same cell as in video 1 and 2 analyzed using the Icy
805 software and the *Spot tracking* plugin. Colored lines correspond to the tracked spots.

806 **Video 4.** TIRF movie acquired at 30 Hz of a cell stably expressing eGFP-CCR5-WT and
807 treated by CCL4 (100 nM) for 14 min.

808 **Video 5.** TIRF movie acquired at 30 Hz of cells stably expressing eGFP-CCR5-WT and
809 treated by PSC-RANTES (20 nM) for 3 min.

810 **Video 6.** TIRF movie acquired at 30 Hz of a cell stably expressing eGFP-CCR5-L196K
811 and treated by PSC-RANTES (20 nM) for 2 min.

812

813 **References**

814

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Figure supplement

Single-molecule imaging reveals distinct effects of ligands on CCR5 dynamics depending on its dimerization status.

Fanny Momboisse¹, Giacomo Nardi², Philippe Colin³, Melany Hery¹, Nelia Cordeiro¹, Olivier Schwartz¹, Nathalie Sauvonnnet⁵, Fernando Arenzana-Seisdedos⁴, Thibault Lagache^{2*}, Bernard Lagane³, Jean-Christophe Olivo-Marin², Anne BreLOT^{1*}

Corresponding author: Anne BreLOT and Thibault Lagache

Anne BreLOT: anne.brelot@pasteur.fr

Thibault Lagache: thibault.lagache@pasteur.fr

This PDF file includes:

Figure1-figure supplement 1
Figure3-figure supplement 1
Figure4-figure supplement 1
Legends for Videos 1 to 6

Other materials for this manuscript include the following:

Video 1
Video 2
Video 3
Video 4
Video 5
Video 6

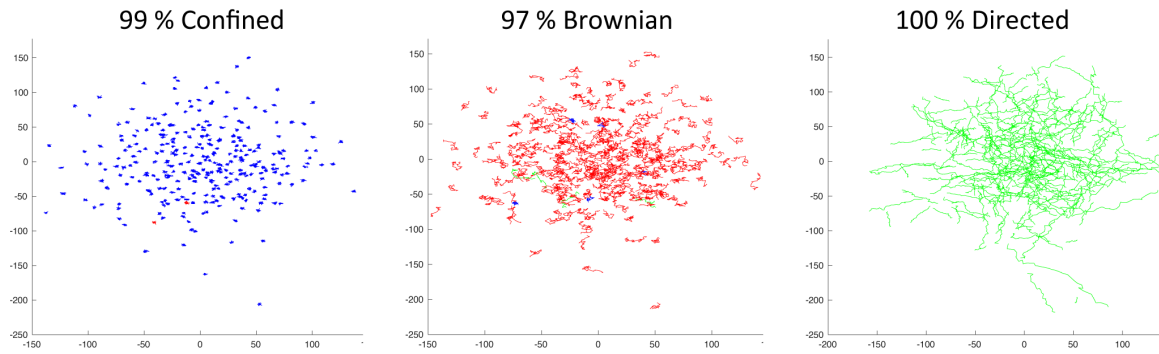


Figure 1-figure supplement 1. Validation of the statistical method using simulated trajectories. The three motions, confined, Brownian, and directed, were simulated by Monte Carlo experiments. For each type of motion, we simulated 300 tracks with length 11, with a Matlab program by using equations given in Materials and Methods. Trajectories were classified with the statistical method. The parameters used for the statistical classification were the following: $N=10$, $\alpha=0.05$, $q(\alpha)=0.725$, and $q(1-\alpha) = 2.626$. The percentage of detection of the different trajectories were indicated.

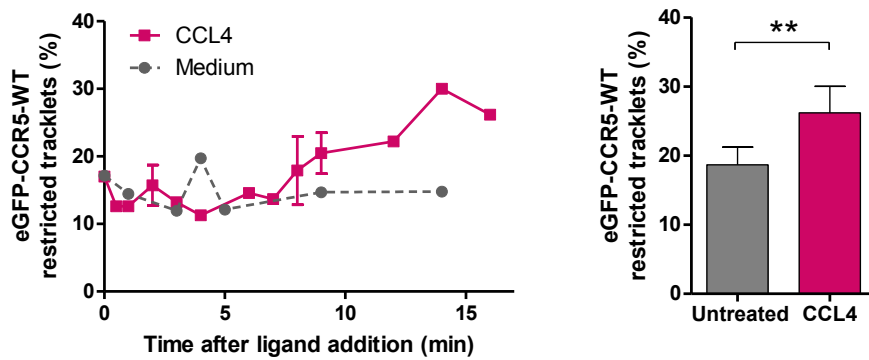


Figure 3-figure supplement 1: Effect of CCL4 on CCR5 mobility. eGFP-CCR5-WT expressing cells were treated or not with a saturating concentration of CCL4 (200 nM) and single particle tracking analysis was performed. Percentage of restricted tracklets after treatment over time (left) and after 12 to 16 min of treatment (right) (mean \pm SD, $n = 9\ 951$ and $4\ 320$ tracks for untreated and CCL4 conditions, from 6 and 3 cells respectively). Unpaired t test: p value 0.0088**.

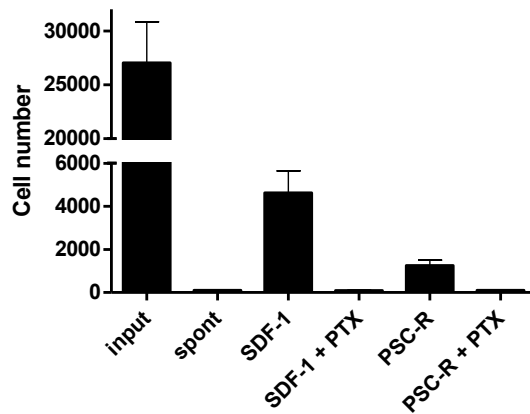


Figure 4-figure supplement 1. Effect of PTX treatment on chemokine-mediated chemotaxis. A3.01-R5 cells treated with 100 ng/ml PTX for 3 h were added to the upper chambers of HTS-transwell. Chemokines were to the lower chambers and chemotaxis were proceeded for 4 h. The number of cells migrating across the membrane was assessed by flow cytometry. PTX-treatment impaired SDF-1 (10 nM) and PSC-RANTES (33.7 nM)-mediated chemotaxis of A3.01-R5 cells. One representative experiment of 2 independent experiments (mean \pm SD of triplicates). *Spont*: Spontaneous migration (without chemokines in the lower chamber).

Videos legends

Video 1. TIRF movie of a cell stably expressing eGFP-CCR5-WT acquired at 30 Hz. The region of interest was defined by the green line.

Video 2. TIRF movie of the same cell as in video 1 analyzed using the Icy software. Red circles correspond to the detection of bright spots using the *Spot detection* pluggin.

Video 3. TIRF movie of the same cell as in video 1 and 2 analyzed using the Icy software and the *Spot tracking* pluggin. Colored lines correspond to the tracked spots.

Video 4. TIRF movie acquired at 30 Hz of a cell stably expressing eGFP-CCR5-WT and treated by CCL4 (100 nM) for 14 min.

Video 5. TIRF movie acquired at 30 Hz of cells stably expressing eGFP-CCR5-WT and treated by PSC-RANTES (20 nM) for 3 min.

Video 6. TIRF movie acquired at 30 Hz of a cell stably expressing eGFP-CCR5-L196K and treated by PSC-RANTES (20 nM) for 2 min.