

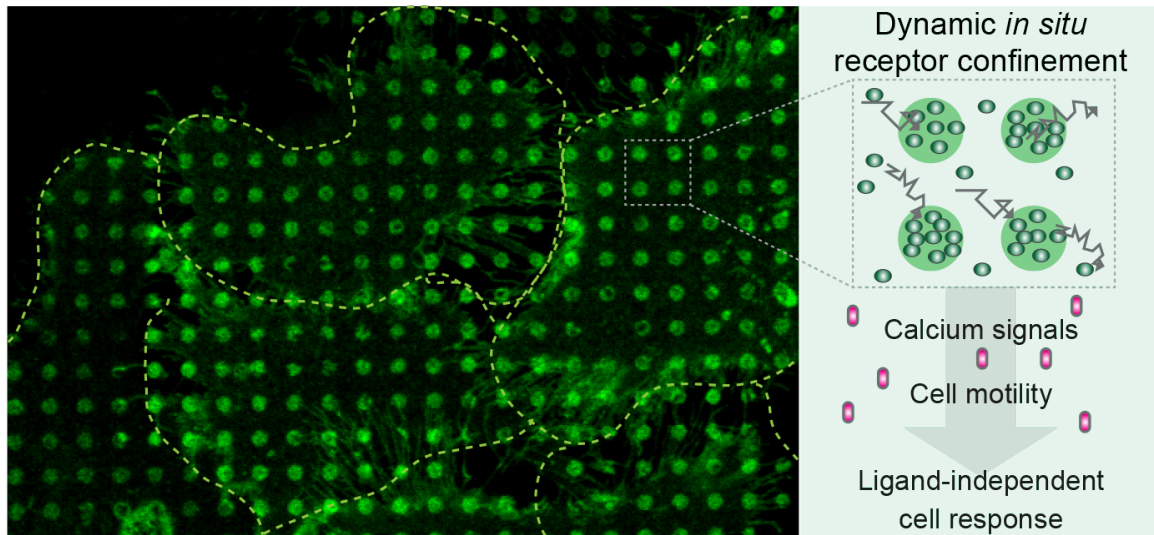
## 1 **Dynamic *in situ* confinement triggers ligand-free neuropeptide receptor** 2 **signaling**

3 M. Florencia Sánchez<sup>1</sup>, Marina S. Dietz<sup>2</sup>, Ulrike Müller<sup>3</sup>, Julian Weghuber<sup>3,4</sup>,  
4 Karl Gatterdam<sup>1</sup>, Ralph Wieneke<sup>1</sup>, Mike Heilemann<sup>2</sup>, Peter Lanzerstorfer<sup>3</sup>, Robert Tampé<sup>1,\*</sup>

5  
6 <sup>1</sup>Institute of Biochemistry, Biocenter, Goethe University Frankfurt, Max-von-Laue-Str. 9, 60438  
7 Frankfurt am Main, Germany; <sup>2</sup>Institute of Physical and Theoretical Chemistry, Goethe University  
8 Frankfurt, Max-von-Laue-Str. 7, 60438 Frankfurt am Main, Germany; <sup>3</sup>School of Engineering and  
9 Environmental Sciences, University of Applied Sciences Upper Austria, Wels, Austria; <sup>4</sup>FFoQSI -  
10 Austrian Competence Centre for Feed and Food Quality, Safety & Innovation, FFoQSI GmbH,  
11 Technopark 1D, 3430 Tulln, Austria

12  
13 \*To whom correspondence may be addressed Email: [tampe@em.uni-frankfurt.de](mailto:tampe@em.uni-frankfurt.de)

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15



17 **Abstract**

18 Membrane receptors are central to cell-cell communication. Receptor clustering at the plasma  
19 membrane modulates physiological responses, and mesoscale receptor organization is critical  
20 for downstream signaling. Spatially restricted cluster formation of the neuropeptide Y<sub>2</sub>  
21 hormone receptor (Y<sub>2</sub>R) was observed *in vivo*; however, the relevance of this confinement is  
22 not fully understood. Here, we controlled Y<sub>2</sub>R clustering *in situ* by a chelator nanotool. Due to  
23 the multivalent interaction, we observed a dynamic exchange in the microscale confined  
24 regions. Fast Y<sub>2</sub>R enrichment in clustered areas triggered a ligand-independent downstream  
25 signaling determined by an increase in cytosolic calcium, cell spreading, and migration. We  
26 revealed that the cell response to ligand-induced activation was amplified when cells were  
27 pre-clustered by the nanotool. Ligand-independent signaling by clustering differed from ligand-  
28 induced activation in the binding of arrestin-3 as downstream effector, which was recruited to  
29 the confined regions only in the presence of the ligand. This approach enables *in situ*  
30 clustering of membrane receptors and raises the possibility to explore different modalities of  
31 receptor activation.

32

33 **Keywords:** Chemical biology / chemical pharmacology / G protein-coupled receptors /  
34 membrane organization / membrane proteins / receptor clustering

## 35 Introduction

36 Cells translate stimuli into biochemical signals through membrane receptors controlling  
37 multiple aspects of cell behavior, including migration (Kupperman *et al.*, 2000; Stallaert *et al.*,  
38 2018), differentiation (Li & Rudensky, 2016; Luther & Cyster, 2001), apoptosis (Scott *et al.*,  
39 2009), as well as infectious diseases and cancer (Boncompain *et al.*, 2019; Haqshenas &  
40 Doerig, 2019; Kawai & Akira, 2005; Pasquale, 2010; Pike *et al.*, 2018; Sebestyen *et al.*, 2020;  
41 Tsukiyama *et al.*, 2020). Receptors form dynamic assemblies or clusters that modulate  
42 downstream signaling and the final physiological response. Upon activation, these receptors  
43 undergo transitions from freely diffusing monomers to less mobile nanoclusters and further to  
44 higher-order oligomers (Ojosnegros *et al.*, 2017; Su *et al.*, 2016). In signal transduction, the  
45 mechanisms for receptor cluster formation and cluster behavior have become physiologically  
46 relevant topics. However, the role of mesoscale (hundreds of nanometers) receptor  
47 organization in signal transduction remains unsolved, mainly because techniques to trigger  
48 receptor clustering *in situ* and monitor this assembly process in real-time are largely limited.

49 Nano- and microlithographic approaches have provided cell-compatible scaffolds to  
50 investigate confined ligand-receptor interactions. Various techniques, ranging from  
51 photolithography (Chen *et al.*, 2021; Scheideler *et al.*, 2020; Traub *et al.*, 2016) to electron-  
52 beam lithography (Cai *et al.*, 2018; Nassereddine *et al.*, 2021) and microcontact printing ( $\mu$ CP)  
53 (Lindner *et al.*, 2018; Sánchez *et al.*, 2018), have yielded information on how topology and  
54 mobility of the stimulus regulate cellular outcomes. Recently, optogenetics and optochemistry  
55 have provided the possibility of targeting receptor oligomerization with high spatiotemporal  
56 control (Bardhan & Deiters, 2019; Goglia & Toettcher, 2019; Taslimi *et al.*, 2014). However,  
57 approaches that can be easily adapted to a variety of receptors or experimental setups and  
58 that offer the ability to analyze large cell populations simultaneously are rare.

59 Heterotrimeric guanine nucleotide-binding protein (G protein)-coupled receptors (GPCR)  
60 are key cell surface proteins that regulate a plethora of cellular responses to external stimuli  
61 (Hilger *et al.*, 2018; Venkatakrisnan *et al.*, 2013; Wootten *et al.*, 2018). The Y<sub>2</sub> receptor (Y<sub>2</sub>R)  
62 is one of the four human neuropeptide Y (NPY) receptor subtypes, which belong to the  
63 rhodopsin-like (class A) GPCR superfamily (Parker & Balasubramaniam, 2008; Tang *et al.*,

64 2022). Y<sub>2</sub>R is linked to many important physiological processes, such as fear extinction  
65 (Méndez-Couz *et al.*, 2021), regulation of food intake (Huang *et al.*, 2014), and obesity  
66 (Lafferty *et al.*, 2021). Y<sub>2</sub>R activation by neuropeptide Y (NPY) has been shown to promote  
67 cell migration and proliferation (Ekstrand *et al.*, 2003; Movafagh *et al.*, 2006). It has been  
68 recently demonstrated that Y<sub>2</sub>Rs respond to light-guided microscale clustering at spatially  
69 defined locations (Sánchez *et al.*, 2021). Y<sub>2</sub>Rs are activated independently of canonical  
70 ligands, evoking elevated cytosolic calcium, a change in cell spreading behavior, and a  
71 localized migratory pattern.

72 Here, we established a versatile approach for *in situ* receptor clustering using a multivalent  
73 chelator nanotool (*tris* N-nitrilotriacetic acid, *tris*NNTA), which displays high affinity for histidine  
74 (His)-tagged proteins. The nanometer size of the tool in combination with pre-structured  
75 matrices enabled receptor clustering with high spatiotemporal resolution. The lateral  
76 organization of Y<sub>2</sub>Rs in living cells was controlled within minutes in a non-invasive and ligand-  
77 independent manner. Microscale receptor clusters with a high degree of homogeneity in size  
78 and density were generated at the plasma membrane. Analysis of the receptor mobility  
79 revealed a dynamic assembly with fast exchange of the receptors within the confined areas in  
80 contrast to the static clustering induced by an anti-His-tag antibody on the same matrix.  
81 Nanotool-induced receptor clustering triggered ligand-independent activation of signal  
82 transduction, as evidenced by an increase in cytosolic calcium and cell motility, effects also  
83 observed in ligand-induced receptor activation. Moreover, we demonstrated an amplification  
84 of the signal upon ligand-induced activation in cells pre-clustered with the nanotool. As  
85 additional downstream event, we uncovered high arrestin-3 (Arr3) co-recruitment to the  
86 patterned areas only in the presence of the canonical ligand, suggesting an Arr3-independent  
87 desensitization mechanism for the ligand-independent response. Compared to standard  
88 micropatterning techniques, this generic approach advances ligand-free receptor signaling  
89 studies, with the advantage that large cell populations can be imaged simultaneously, and no  
90 expensive equipment is required for implementation. The versatile nanotool can be adapted  
91 to a variety of systems and receptors through minimal modifications.



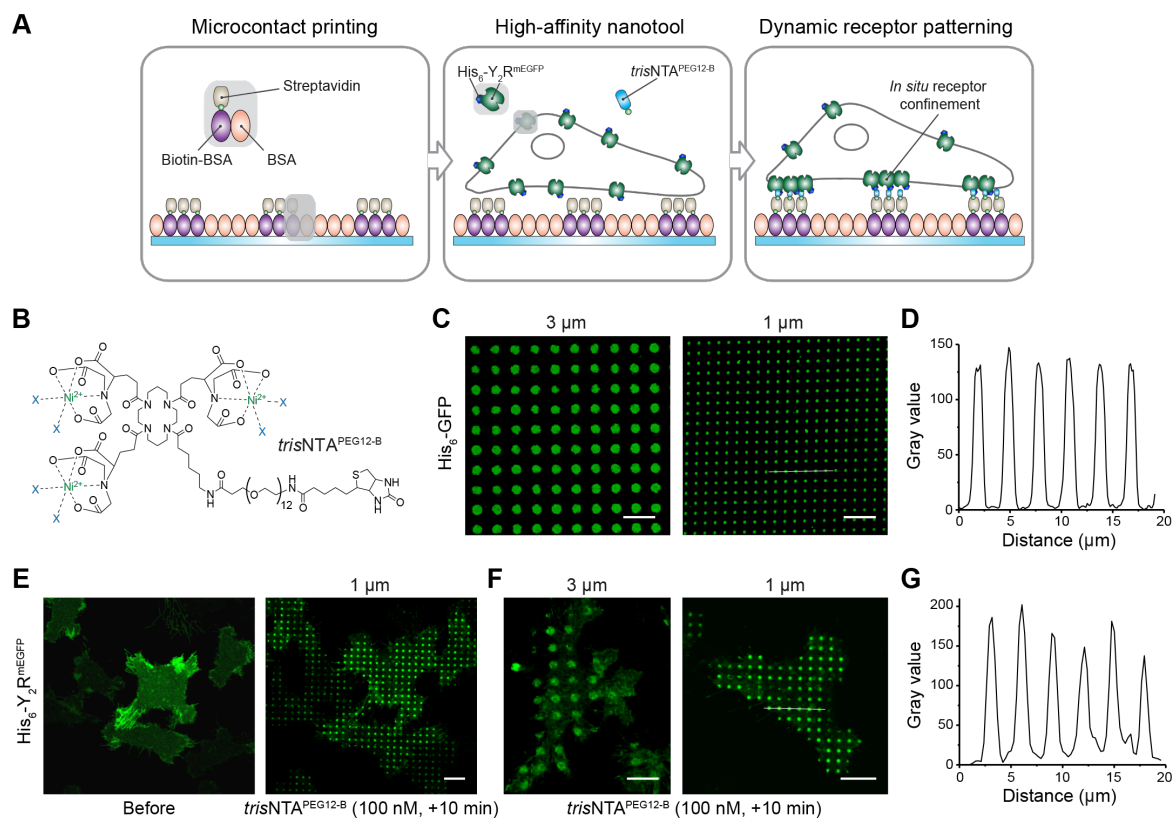
## 92 Results

### 93 *In situ* receptor clustering by a multivalent nanotool

94 We developed a system to induce dynamic receptor assembly *in situ* based on a multivalent  
95 chelator *tris*NTA nanotool (**Figure 1A**), which is equipped with a biotin moiety (*tris*NTA<sup>PEG12-B</sup>)  
96 (**Figure 1B**). *tris*NTA<sup>PEG12-B</sup> displays a high affinity for His<sub>6</sub>-tagged proteins ( $K_D \approx 1\text{-}10$  nM),  
97 resulting in a site-specific but reversible interaction with minimal steric constraints (Gatterdam  
98 *et al.*, 2018). Microcontact printing is a widely used method to investigate protein-protein  
99 interactions in living cells (Ruiz & Chen, 2007; Torres *et al.*, 2008). However, reproducible  
100 patterned substrates with a generic structure over extensive millimeter dimensions which allow  
101 simultaneous analyses of large cell populations are difficult to produce. We used a large-area  
102 perfluoropolyether (PFPE) elastomeric stamps inked with bovine serum albumin (BSA) to print  
103 96-well size glass (Hager *et al.*, 2021; Lanzerstorfer *et al.*, 2020). Wells within these plates  
104 containing a BSA-structured matrix were functionalized with biotinylated BSA (biotin-BSA) and  
105 streptavidin (SA) (**Figure 1A**). Subsequent functionalization with the nanotool and His<sub>6</sub>-tagged  
106 fluorescent proteins resulted in well-resolved protein patterns that were analyzed by confocal  
107 laser scanning microscopy (CLSM). The results confirmed the specificity of the nickel-loaded  
108 *tris*NTA chelator to capture His<sub>6</sub>-tagged proteins in defined regions of 1  $\mu\text{m}$  or 3  $\mu\text{m}$  diameter  
109 (**Figure 1C, D**).

110 The ability to control the organization of membrane receptors *in situ* is important for  
111 dissecting the spatial complexity of cell signaling and the extracellular environment. With this  
112 aim, we established a monoclonal human cervical cancer HeLa cell line expressing low  
113 amounts of Y<sub>2</sub>R (~300,000 receptors/cell) utilizing a tetracycline-inducible (T-Rex) expression  
114 system (Sánchez *et al.*, 2021). Y<sub>2</sub>R displayed an N-terminal His<sub>6</sub>-tag to the extracellular space  
115 and a cytosolic C-terminal monomeric Enhanced Green Fluorescent Protein (mEGFP) (His<sub>6</sub>-  
116 Y<sub>2</sub>R<sup>mEGFP</sup>, in brief Y<sub>2</sub>R). It is important to mention that these modifications do not affect receptor  
117 activity, selectivity, or ligand binding as previously shown (Sánchez *et al.*, 2021). It has been  
118 further demonstrated that Y<sub>2</sub>R does not require the N terminus for ligand binding (Lindner *et*  
119 *al.*, 2009). Y<sub>2</sub>R-positive cells properly adhered to 1  $\mu\text{m}$  and 3  $\mu\text{m}$  SA-functionalized matrices  
120 and showed a homogeneous receptor distribution at the basal plasma membrane (**Figure 1E**).

121 Addition of  $\text{trisNTA}^{\text{PEG12-B}}$  (100 nM final) triggered receptor assembly. Within five minutes, all  
 122 cells showed receptor patterns at the plasma membrane comparable in size and density  
 123 (Figure 1E, F, Figure 1–figure supplement 1). Importantly, recruitment of soluble His<sub>6</sub>-  
 124 tagged GFP proteins as well as Y<sub>2</sub>R<sub>s</sub> to 1 μm pre-structured spots led to analogous intensity  
 125 profiles, reflecting that similar densities were obtained in both cases (Figure 1D, G).



126  
 127 **Figure 1.** *In situ* ligand-free receptor confinement. (A) Rational of the experimental design for ligand-  
 128 free receptor clustering. Matrices pre-structured with BSA are stepwise functionalized with biotin-BSA  
 129 and SA. Upon addition of the multivalent nanotool  $\text{trisNTA}^{\text{PEG12-B}}$ , His<sub>6</sub>-tagged receptors in HeLa cells  
 130 are captured to the pre-structured regions via multivalent His-tag/*trisNTA* interaction. (B) Chemical  
 131 structure of the  $\text{trisNTA}^{\text{PEG12-B}}$ . (C) Variable size protein patterns generated by further functionalization  
 132 of SA-matrices with the nanotool followed by incubation with His<sub>6</sub>-GFP (0.1 μM, 20 min). Images were  
 133 acquired by confocal laser scanning microscopy (CLSM). (D) Intensity profile of the 1 μm pattern (white  
 134 line in (C)) reflects high specificity of the interaction. (E) Large-scale cell patterning in living cells  
 135 occurred 10 min after incubation with the nanotool ( $\text{trisNTA}^{\text{PEG12-B}}$  100 nM final, 10 min). Y<sub>2</sub>R-  
 136 expressing cells were allowed to adhere to the functionalized matrix for 3 h and immediately imaged by  
 137 CLSM in live-cell imaging solution (LCIS) at 37 °C. (F) Customized Y<sub>2</sub>R assembly on 3 μm and 1 μm  
 138 SA-pre-structured matrices. (G) Intensity profile of the 1 μm pattern (white line in (F)) showed an  
 139 intensity comparable to a soluble His<sub>6</sub>-tagged protein. Scale bars: 10 μm.

140 In contrast, cells expressing Y<sub>2</sub>R<sub>s</sub> without the His<sub>6</sub>-tag (Y<sub>2</sub>R<sup>mEGFP</sup>) showed no receptor  
141 clustering after addition of *tris*NTA<sup>PEG12-B</sup> (**Figure 1–figure supplement 2**), demonstrating the  
142 specificity of the His<sub>6</sub>-tag/*tris*NTA interaction. Remarkably, ten minutes after receptor  
143 clustering by the multivalent nanotool, the Y<sub>2</sub>R enrichment resulted in an integrated receptor  
144 density equivalent to that of cells cultured on matrices functionalized with anti-His<sub>6</sub> antibodies.  
145 However, a 10-fold higher antibody concentration (1 μM final) was required compared to the  
146 multivalent nanotool, demonstrating its efficacy in capturing His<sub>6</sub>-tagged Y<sub>2</sub> receptors  
147 (**Figure 1–figure supplement 3**). The nanotool-induced 3 μm clusters presented a 9-fold  
148 increase in integrated density compared to 1 μm arrays, consistent with the increase in pattern  
149 area. Overall, our approach enabled versatile *in situ* receptor clustering with high specificity.

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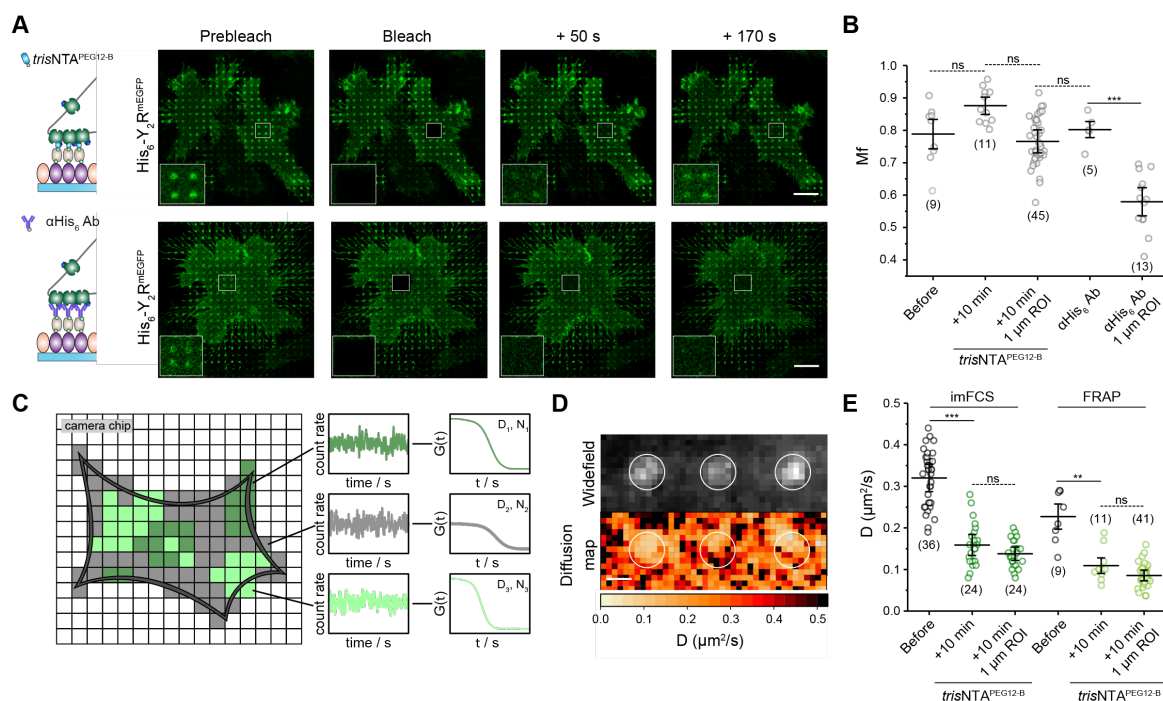
### 151 **Receptor diffusion and dynamic exchange in the confined regions**

152 GPCR signaling results from dynamic interactions among receptors, G proteins, and the  
153 complex surrounding membrane environment, which confers flexibility and versatility on this  
154 fundamental biological process. To characterize receptor clustering induced by the chelator  
155 nanotool, we first examined whether Y<sub>2</sub>R clustering affects lipid diffusion and distribution by  
156 labeling the membrane with the lipid-like dye CellMask. We observed a homogeneous staining  
157 of the plasma membrane, demonstrating that receptor confinement does not affect lipid  
158 distribution (**Figure 2–figure supplement 1**). To determine lateral diffusion coefficients (*D*),  
159 we performed fluorescence recovery after photobleaching (FRAP). *In situ* receptor clustering  
160 was triggered on Y<sub>2</sub>R-expressing cells cultured on SA-matrices by incubation with  
161 *tris*NTA<sup>PEG12-B</sup> (100 nM, +10 min), followed by membrane labeling with the lipid-like dye. In a  
162 subsequent step, square-shaped regions of interest (ROIs) covering four 1 μm-sized spots  
163 were photobleached. Fluorescence recovery was analyzed by a FRAP simulation approach  
164 that enabled calculation of diffusion coefficients independent of bleaching geometry  
165 (Blumenthal *et al.*, 2015). The lateral diffusion coefficient of lipids obtained by FRAP showed  
166 an average value of  $D_{\text{lipid}} = 0.66 \pm 0.10 \mu\text{m}^2/\text{s}$ , which is in agreement with literature values for  
167 free Brownian lipid diffusion at the plasma membrane (Schwille *et al.*, 1999; Wawrezynieck *et*  
168 *al.*, 2005).

169 To evaluate the Y<sub>2</sub>R mobility in the ligand-free induced clusters, we determined the lateral  
170 diffusion of Y<sub>2</sub> receptor in cells cultured on SA-matrices before and after receptor clustering  
171 by *tris*NTA<sup>PEG12-B</sup> (100 nM, 10 min). A square-shaped ROI covering four 1 μm-sized spots was  
172 photobleached. A significant decrease in the lateral diffusion of the Y<sub>2</sub>R was observed at the  
173 basal membrane of cells after receptor confinement by *tris*NTA<sup>PEG12-B</sup> ( $D_{\text{before}} =$   
174  $0.25 \pm 0.08 \mu\text{m}^2/\text{s}$  versus  $D_{\text{after}} = 0.10 \pm 0.03 \mu\text{m}^2/\text{s}$ ) (**Figure 2A, E**). Surprisingly, the receptor  
175 intensity showed a high recovery within ~3 min after photobleaching (**Figure 2A, Figure 2–**  
176 **figure supplement 2, Video 1**). Notably, no significant difference in receptor mobile fraction  
177 ( $M_f$ ) before and after addition of the nanotool was observed ( $M_f = 0.80 \pm 0.04$ ) (**Figure 2B**). In  
178 comparison, FRAP analyses of cells cultured on matrices functionalized with anti-His<sub>6</sub>  
179 antibodies presented a drastic decrease in receptor diffusion and mobile fraction at the  
180 clustered spots ( $M_{f,\text{anti-His6 Ab}} = 0.56 \pm 0.08$ ) (**Figure 2A, B, Figure 2–figure supplement 3,**  
181 **Video 2**). Despite the high affinity and kinetically stable binding ( $k_{\text{off}} = 0.18 \text{ h}^{-1}$ ) (Gatterdam *et*  
182 *al.*, 2018), the His-tag/*tris*NTA system relies on molecular multivalency, which enables  
183 competition of binding sites with histidine or other receptors, thus making the process of  
184 receptor assembly reversible. We rationalized that free receptors diffuse into the clustered  
185 spots and exchange with photobleached receptors at multivalent binding sites, leading to a  
186 dynamic confinement. Our results indicate that a high proportion of receptors is exchanged in  
187 and out of micrometer-sized clusters, an effect that likely depends on cluster size, with larger  
188 clusters showing less recovery (Sánchez *et al.*, 2021).

189 We also investigated the lateral receptor mobility with a higher spatiotemporal resolution  
190 using imaging fluorescence correlation spectroscopy (imFCS). FCS is used to study the  
191 diffusion of membrane proteins in living cells with single-molecule sensitivity (**Figure 2C**).  
192 These multiplexed FCS measurements are realized by analyzing many pixels simultaneously  
193 using a widefield setup (Harwardt *et al.*, 2018; Kannan *et al.*, 2006). Regions of interests  
194 (ROIs) on Y<sub>2</sub>R-expressing cells cultured on SA-matrices were analyzed before and after  
195 receptor clustering by *tris*NTA<sup>PEG12-B</sup>. Enrichment of Y<sub>2</sub>R at the basal membrane was observed  
196 with total internal reflection fluorescence (TIRF) microscopy (**Figure 2D**). Consistent with the  
197 FRAP measurements, the Y<sub>2</sub>R diffusion coefficient decreased upon cluster formation ( $D_{\text{before}}$

198 =  $0.32 \pm 0.06 \mu\text{m}^2/\text{s}$  and  $D_{\text{after}} = 0.16 \pm 0.05 \mu\text{m}^2/\text{s}$ ). The receptor diffusion coefficient  
 199 measured before clustering was comparable to membrane proteins of similar size (Lippincott-  
 200 Schwartz *et al.*, 2001), demonstrating that the microstructured confinement does not affect  
 201 receptor mobility.



202

203 **Figure 2.** Decrease of receptor mobility in confined regions. (A) FRAP analyses upon Y<sub>2</sub>R clustering  
 204 induced either by the nanotool *in situ* or by an anti-His<sub>6</sub> antibody (αHis<sub>6</sub> Ab). Y<sub>2</sub>R-expressing cells  
 205 were allowed to adhere to SA- or - αHis<sub>6</sub> Ab matrices for 3 h and immediately imaged by CLSM in live-cell  
 206 imaging solution (LCIS) at 37 °C. The trisNtA<sup>PEG12-B</sup> nanotool was added to a final concentration of  
 207 100 nM. Insets represent the bleached ROIs. Fast recovery of the clusters can be detected for the case  
 208 of the multivalent nanotool. (B) Quantification of the receptor mobile fraction for cell patterning by the  
 209 trisNtA<sup>PEG12-B</sup> and anti-His<sub>6</sub> antibody demonstrated unchanged receptor mobile fraction for the nanotool,  
 210 suggesting a high receptor exchange. The mean ± SD is shown. 9 cells before, 11 cells after  
 211 trisNtA<sup>PEG12-B</sup> addition (45x 1 μm ROIs), and 5 cells on anti-His<sub>6</sub> antibody matrices (13x 1 μm ROIs)  
 212 were analyzed. \*\*\*p ≤ 0.001 for Tukey test. (C) imFCS correlates fluorescence intensity fluctuations in  
 213 single camera pixels with a large degree of statistics, providing accurate diffusion coefficients with high  
 214 spatial and temporal resolution. (D) Widefield image of a ROI at the plasma membrane of a living cell  
 215 upon addition of the nanotool analyzed by imFCS (left). The analyses of numerous pixels  
 216 simultaneously provide two-dimensional diffusion data that draw a picture of the mobility of membrane  
 217 receptors and reveal local differences in the diffusion (right). (E) Both techniques demonstrated a  
 218 decrease in the lateral diffusion of the receptor at the plasma membrane after addition of the chelator  
 219 nanotool. Analysis of 1 μm clusters within the entire ROI led to a further decrease in the lateral diffusion  
 220 coefficient. For imFCS analyses, two-sample t-tests (α = 0.05) were applied to compare the diffusion  
 221 coefficients for the different conditions. The mean ± SD is shown. 36 and 24 cells for the conditions  
 222 before and after addition of trisNtA<sup>PEG12-B</sup> were analyzed. For FRAP, the mean ± SD is shown. 9 cells  
 223 before, 11 cells after trisNtA<sup>PEG12-B</sup> addition (41x 1 μm ROIs) were analyzed. \*\*\*p ≤ 0.001 for Tukey test.  
 224 Scale bar: 10 μm (A), 1 μm (D).

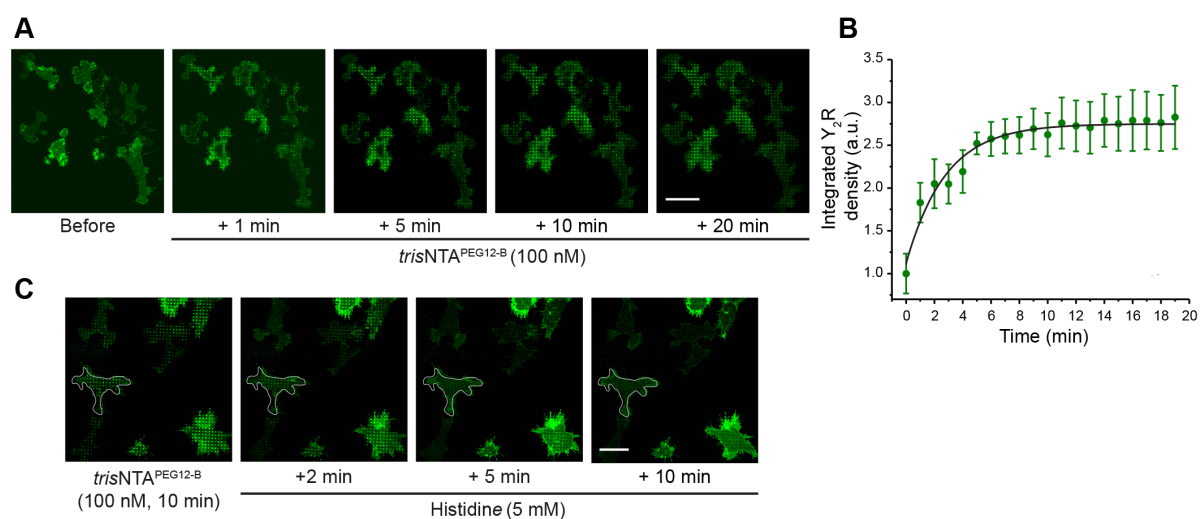
225 In contrast to FRAP, imFCS provides a two-dimensional diffusion map, which enables the  
226 determination of local differences in the lateral diffusion coefficient of membrane receptors  
227 with high precision. Quantitative analysis of the 1  $\mu\text{m}$  cluster spots in the acquired ROIs  
228 resulted in a lateral diffusion coefficient of  $D_{\text{spots}} = 0.14 \pm 0.03 \mu\text{m}^2/\text{s}$  (**Figure 2E**). Taking into  
229 consideration that imFCS detects mobile particles only, we determined a similar decrease in  
230 lateral diffusion in the patterned regions for cells cultured on matrices functionalized with anti-  
231 His<sub>6</sub> antibodies (**Figure 2-figure supplement 3**). Taken together, we unravel that in  
232 microscale clusters, associations between His<sub>6</sub>-tagged Y<sub>2</sub>R<sub>s</sub> and multivalent *tris*NTA<sup>PEG12-B</sup>  
233 resulted in a decreased lateral diffusion but dynamic receptor exchange with unchanged  
234 mobile fraction, which is similar to the behavior described for ligand-activated receptor  
235 clustering (Chavez-Abiega *et al.*, 2019).

236

### 237 **Ligand-independent receptor clustering triggers fast signaling**

238 To mimic a scenario in which receptors can cluster at the mesoscale and still reflect  
239 physiologically relevant dimensions for clustering at cell-cell interfaces (Guo *et al.*, 2008), Y<sub>2</sub>R-  
240 expressing cells were cultured on microstructured matrices with a diameter of 1  $\mu\text{m}$ , the  
241 smallest pattern we can produce and analyze with high accuracy. After addition of the  
242 multivalent nanotool, the receptor redistribution was tracked by CLSM at 37 °C. Receptor  
243 clustering occurred in the first minutes and increased within 10 min until an equilibrium was  
244 reached, resulting in a 2.5-fold increase in receptor density compared to the initial state  
245 (**Figure 3A, Video 3**). The kinetic profile of Y<sub>2</sub>R recruitment to the 1  $\mu\text{m}$  spots followed a  
246 pseudo-first-order assembly rate of  $0.35 \pm 0.05 \text{ min}^{-1}$  (**Figure 3A, B**). Considering the average  
247 cell area of  $1,420 \pm 50 \mu\text{m}^2$  ( $n = 66$  cells) and the enrichment factor (2.5-fold), we estimated a  
248 receptor density of  $\sim 500$  receptors/ $\mu\text{m}^2$  in the patterned regions ( $\sim 400$  receptors per 1  $\mu\text{m}$   
249 circular spot), a value comparable to other receptor studies utilizing fluorescence correlation  
250 spectroscopy (Bag *et al.*, 2015; Chen *et al.*, 2009). Addition of histidine to patterned cells  
251 resulted in rapid and complete disassembly of the receptor clusters, demonstrating the  
252 reversibility of the systems, a key advantage of the approach to investigate receptor dynamics  
253 (**Figure 3C**).





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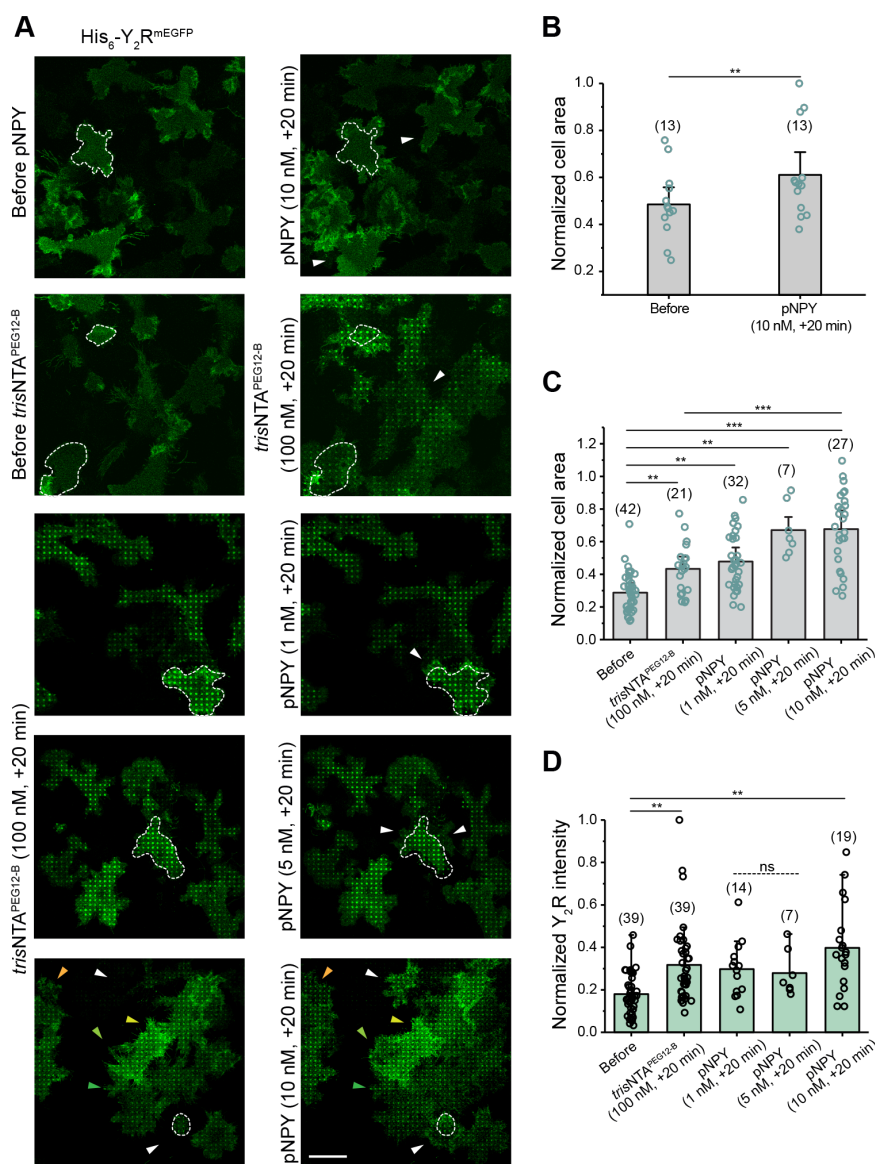
255 **Figure 3.** *In situ* receptor clustering with high spatiotemporal resolution. (A) Time-lapse imaging of Y<sub>2</sub>R  
256 assembly. Y<sub>2</sub>R-expressing HeLa cells were allowed to adhere to pre-structured SA-matrices for 3 h and  
257 were visualized by CLSM in LCIS at 37 °C. Time-lapse images were recorded for 20 min immediately  
258 after addition of *trisNTA*<sup>PEG12-B</sup> (100 nM). Scale bar: 20 μm. (B) Receptor-integrated density in the  
259 patterned regions increased mono-exponentially, leading to an assembly rate of  $0.35 \pm 0.05 \text{ min}^{-1}$  and  
260  $\tau_{1/2} = 3 \text{ min}$ . (50-200x 1 μm ROIs per experiment were analyzed from a total of 30 cells from three  
261 different experiments, 10 cells per experiment). (C) Reversal of the interaction and disassembly of the  
262 clusters is demonstrated upon addition of histidine. Y<sub>2</sub>R-expressing cells were allowed to adhere to the  
263 SA-matrices for 3 h, and then receptor confinement was induced by addition of *trisNTA*<sup>PEG12-B</sup> (100 nM).  
264 Subsequently, cells were incubated with histidine (5 mM) for 2 to 10 min followed by washing. Scale  
265 bar: 10 μm.

266

267 Y<sub>2</sub>R activation by its natural ligand NPY promotes cell migration and proliferation (Ekstrand  
268 *et al.*, 2003; Movafagh *et al.*, 2006). In cells cultured on SA matrices, a 17% increase in cell  
269 area was detected after addition of the agonist porcine neuropeptide Y (pNPY,  $K_D = 5.2 \pm$   
270  $2.0 \text{ nM}$ ) (**Figure 4A, B**). When clustering was induced by the nanotool, we also observed a  
271 fast change in cell spreading and motility and a 20% increase in the total cell area concomitant  
272 to receptor assembly (**Figure 4A, C**). This analogous effect indicates a ligand-independent  
273 response to receptor clustering. We did not observe change in cell motility upon addition of  
274 the *trisNTA*<sup>PEG12-B</sup> in cells cultured on matrices without SA. Furthermore, cells expressing  
275 Y<sub>2</sub>R<sup>mEGFP</sup> (lacking a His<sub>6</sub>-tag) on SA-matrices showed no significant change in cell spreading  
276 upon addition of the nanotool, demonstrating the specificity of the response. (**Figure 4-figure**  
277 **supplement 1**). To investigate the relevance of clustering for Y<sub>2</sub>R activation and the cell  
278 motility response, we evaluated the increase in cell area upon ligand-induced activation in  
279 cells that were non- and pre-clustered by the nanotool. We revealed that nanotool-induced  
280 clustering amplified the motility effect induced by the pNPY ligand. In pre-clustered cells,



281 stimulation with pNPY (10 nM) led to a 2-fold amplification and a 40% increase in cell area  
 282 compared to the initial state (**Figure 4A, C**). A dose-dependent increase in cell area  
 283 (**Figure 4A, C**) and cluster intensity (**Figure 4A, D**) was observed for *trisNTA*<sup>PEG12-B</sup>-pre-  
 284 clustered cells. Overall, these results indicate a critical function of the receptor clusters, an  
 285 amplification of the signal in pre-patterned cells, or, from the other point of view, a sensitization  
 286 of the receptor to lower concentrations of the natural ligand NPY.



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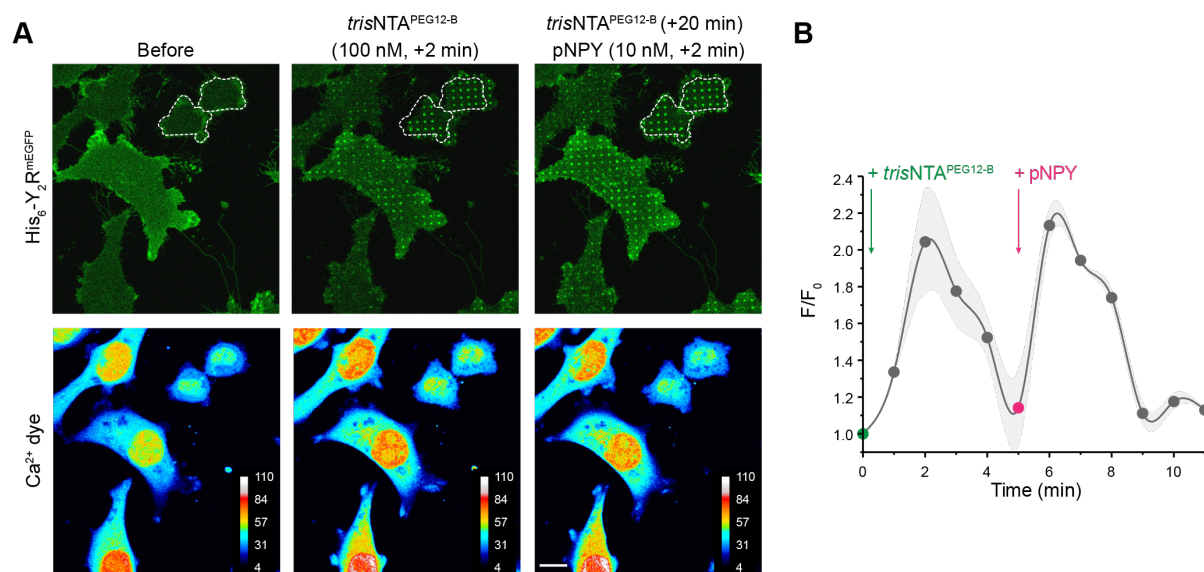
288 **Figure 4.** Receptor clustering amplifies the cell response induced by ligand activation. (A) Confocal  
 289 microscopy images of cells expressing Y<sub>2</sub>R exposed to different conditions. Y<sub>2</sub>R-expressing HeLa cells  
 290 were allowed to adhere to pre-structured SA-matrices for 3 h and visualized by CLSM in LCIS at 37 °C.  
 291 Cells were visualized and imaged for 20 min after addition of *trisNTA*<sup>PEG12-B</sup> or pNPY or both, first  
 292 *trisNTA*<sup>PEG12-B</sup> and subsequently pNPY (20 min incubation time, each). Scale bar: 20 μm. (B) Cell area  
 293 analysis before and 20 min after addition of pNPY (10 nM) showed a 20% area increase, confirming an  
 294 effect of ligand activation on cell motility. Values for cell area were normalized with respect to the highest  
 295 value. The mean ± SD (13 cells) is shown. \*\*p< 0.01 for Tukey test. (C) Cell area analysis before and  
 296 20 min after addition of *trisNTA*<sup>PEG12-B</sup> (100 nM) and subsequent addition of pNPY (1, 5, and 10 nM, one

297 well for each concentration) showed a dose-dependent area increase, demonstrating an amplification  
298 effect of receptor clustering in combination with pNPY. Values for cell area were normalized with respect  
299 to the highest value. The mean  $\pm$  SD (42 cells before, 21 cells after *trisNTA*<sup>PEG12-B</sup> and 14, 7, 19 for  
300 pNPY 1, 5, and 10 nM respectively) is shown. \*\* $p \leq 0.01$  and \*\*\* $p \leq 0.001$  for Tukey test. (D)  
301 Quantification of receptor intensity in the nanotool-induced patterned regions showed a significant  
302 increase in pattern intensity after addition of pNPY (10 nM), the concentration that had the largest effect  
303 on cell motility. The mean  $\pm$  SD is shown (19 to 39 cells and 50-220x 1  $\mu$ m ROI, were analyzed). \*\*\* $p \leq$   
304 0.001 for Tukey test.

305  
306 As calcium signals are widely known to regulate cell motility, we monitored local calcium  
307 dynamics utilizing a far-red cell-permeable calcium-sensitive dye. By dual-color imaging,  
308 receptor assembly and the cytosolic calcium concentration were simultaneously recorded in  
309 living cells over the matrices. Upon addition of *trisNTA*<sup>PEG12-B</sup>, receptor recruitment led to a 2-  
310 fold increase in cytosolic calcium concentration with a rapid rise within two minutes  
311 (**Figure 5A, B**). A second peak in the cytosolic  $Ca^{2+}$  signals was detected upon subsequent  
312 addition of pNPY (10 nM). Contrary, no calcium signal was measured in cells over control  
313 matrices without SA (**Figure 5–figure supplement 1**). To confirm an enhancement of the  
314 response to ligand-induced activation in the presence of nanotool-induced receptor clusters,  
315 calcium signals were monitored in non- or pre-clustered cells (**Figure 5–figure supplement**  
316 **2**). After receptor clustering, we observed a 1.6-fold increase in cytosolic calcium signal upon  
317 pNPY stimulation compared to the initial state. In contrast, a 1.2-fold increase was detected  
318 for cells in the presence of the pNPY only.

319 Overall, our results show analogous calcium signaling for ligand-free *versus* ligand-induced  
320 systems and an amplification of the signal for ligand-induced activation in pre-clustered cells.  
321  $Y_2R$  has been found in a conformational equilibrium between inactive and active states in the  
322 absence of the ligand and forms high-affinity active complexes with G proteins (Ziffert *et al.*,  
323 2020). By ligand-free receptor clustering, the high local receptor density may increase the  
324 residence time of G proteins in vicinity and recruit further downstream effectors, which could  
325 boost the probability of activation and subsequent signaling. Based on the formation of the  
326 high affinity  $Y_2/G$  protein complexes and the short time regime (1-5 min) in which changes in  
327  $Ca^{2+}$  concentration and cell motility are observed, it is likely that the ligand-independent  
328 activation mechanism involves the G protein pathway. G protein signaling leads to the release  
329 of  $G\beta\gamma$  and activation of phospholipase C-beta that cleaves phosphatidylinositol 4,5-

330 bisphosphate into diacylglycerol and phosphatidylinositol (3,4,5)-trisphosphate (PIP<sub>3</sub>). PIP<sub>3</sub>  
331 opens intracellular calcium stores through PIP<sub>3</sub> receptors, leading to local activation of  
332 cytoskeletal proteins and causing the observed cell motility response.



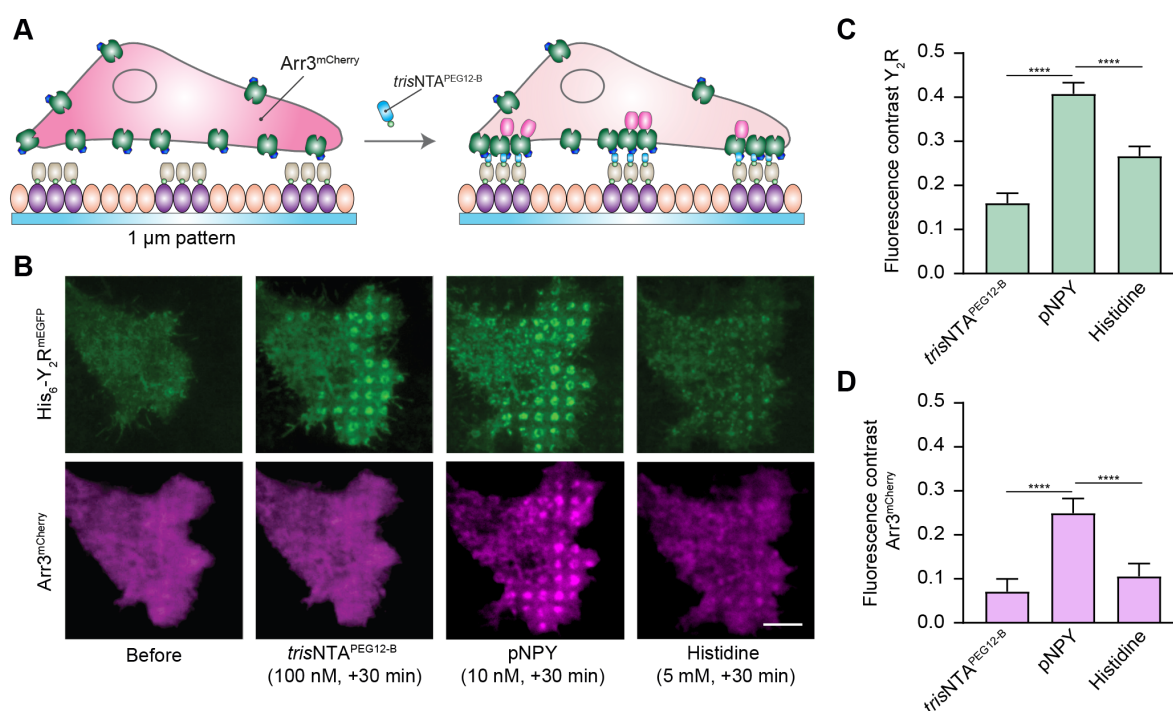
333  
334 **Figure 5.** Ligand-free receptor confinement provokes calcium signaling. (A) Representative confocal  
335 fluorescence images of the Y<sub>2</sub>R (upper panel) and color-coded images of the Ca<sup>2+</sup> dye (lower panel).  
336 Y<sub>2</sub>R-expressing cells on SA-pre-structured matrices were incubated with BioTracker 609 Red Ca<sup>2+</sup> AM  
337 dye (3 μM) for 30 min. After rinsing, cells were immediately imaged by CLSM in LCIS at 37 °C. Addition  
338 of trisNTPeG12-B showed a 2-fold increase in cytosolic calcium. Scale bar: 10 μm. (B) Analysis of the  
339 mean gray value for Ca<sup>2+</sup> signal before (F<sub>0</sub>) and upon addition of trisNTPeG12-B (F) versus time. Time-  
340 lapse images were recorded with 45 s interval before and after addition of trisNTPeG12-B (100 nM), and  
341 subsequent addition of pNPY (10 nM) after 5 min of nanotool addition (5 slices z-stack per time-point).  
342 ROIs covering the complete cell area were considered. The mean ± SD (10 cells) is shown.

343

#### 344 Ligand-free vs ligand-induced receptor activation differs in arrestin recruitment

345 We finally explored the impact of receptor clustering on downstream signaling by monitoring  
346 arrestin-3 recruitment. GPCR desensitization involves a complex series of events, e.g.  
347 receptor phosphorylation, arrestin-mediated internalization, receptor recycling, and lysosomal  
348 degradation (Ziffert *et al.*, 2020). Short-term desensitization occurs within minutes and is  
349 primarily associated with arrestin preventing G protein interaction with the GPCR. Arrestins  
350 bind to activated, phosphorylated GPCRs and block receptor-G protein interaction by steric  
351 hindrance at the receptor-coupling interface, while serving as adaptors for key components of  
352 the endocytic machinery and numerous signaling proteins (Hilger *et al.*, 2018; Wang *et al.*,  
353 2020). In the presence of high concentrations of the canonical ligand, an Arr3-dependent  
354 internalization, subsequent endosomal sorting, and recycling of Y<sub>2</sub>R to the cell membrane

355 were observed (Walther *et al.*, 2010; Wanka *et al.*, 2018). However, recent studies  
 356 demonstrated a strong and persistent activation of the  $G_{\alpha i}$ -pathway upon  $Y_2R$  activation, which  
 357 depletes the intracellular G protein repertoire before Arr3 binding can terminate signaling  
 358 (Ziffert *et al.*, 2020). To assess whether ligand-free clustering leads to Arr3 recruitment, we  
 359 transfected cells stably expressing the  $Y_2R$  with Arr3<sup>mCherry</sup> (in brief Arr3) and monitored Arr3  
 360 recruitment in real-time by total internal reflection fluorescence (TIRF) microscopy (**Figure 6A**).



361 **Figure 6.** Arrestin-3 recruitment upon ligand-induced receptor activation. (A) Schematic representation  
 362 of the experimental set-up. Cells co-expressing  $Y_2R$  and Arr3 were allowed to adhere to SA-pre-  
 363 structured matrices for 3 h and visualized by total internal reflection fluorescence (TIRF) microscopy in  
 364 LCIS at 37 °C. (B) Representative TIRF images of cells before and upon addition of trisNTA<sup>PEG12-B</sup>  
 365 (100 nM, 30 min) and subsequent incubation with pNPY (10 nM) and histidine (5 mM) in LCIS for 30 min  
 366 at 37 °C. All concentrations mentioned are final concentrations in the wells. Scale bar: 5 μm. (C)  
 367 Quantification of the fluorescence contrast in the patterned regions for  $Y_2R$  confirmed receptor  
 368 enrichment upon addition of trisNTA<sup>PEG12-B</sup>, (2-fold with respect to the basal signal before, 100 nM,  
 369 30 min), which further increased 4-fold upon addition of pNPY (10 nM, 30 min). Histidine addition led to  
 370 a decrease in the signal (1.7-fold decrease compared to pNPY, 5 mM, 30 min). Data were normalized  
 371 with respect to the fluorescence intensity before clustering and it is expressed as the means ± SEM (60  
 372 cells for each condition were analyzed). Tukey's multiple comparison test was applied (\*\*\*p ≤ 0.001).  
 373 (D) Fluorescence contrast analysis demonstrated no significant recruitment of Arr3 upon trisNTA<sup>PEG12-B</sup>  
 374 (1.4-fold with respect to the basal signal before, 100 nM, 30 min). Addition of pNPY increased the Arr3  
 375 signal (3.6-fold, 10 nM, 30 min), confirming co-patterning of the downstream signaling molecules.  
 376 Subsequent addition of histidine led to a decrease in the signal (2.3-fold, 5 mM, 30 min). Data was  
 377 normalized with respect to the fluorescence intensity before clustering and it is expressed as the means  
 378 ± SEM (60 cells for each condition were analyzed). Tukey's multiple comparison test was applied (\*\*\*p ≤  
 379 0.001).  
 380  
 381

382 In agreement with our results shown above, image analysis at an equilibrium state (30 min  
 383 after addition of the nanotool) showed a subsequent increase in  $Y_2R$  density in the clustered

384 regions upon addition of pNPY (**Figure 6B, C**). Surprisingly, upon microscale receptor  
385 confinement by *tris*NTA<sup>PEG12-B</sup>, we did not observe a significant increase in Arr3 recruitment by  
386 intensity-contrast analysis of the patterned spots, whereas a significant Arr3 recruitment was  
387 detected upon addition of the agonist pNPY (**Figure 6B, D**). Reversibility by specific  
388 competition with histidine showed that half of the intensity in the patterned regions was  
389 dissipated of the Y<sub>2</sub>R/Arr3 assemblies (**Figure 6B, D**). These results suggest that not all  
390 receptors within the cluster regions are associated with the nanotool upon addition of the  
391 ligand, supporting the observation of increased receptor density in the presence of the pNPY.  
392 Patterning of Arr3 was also detected in cells on an anti-His<sub>6</sub> antibody matrix within the first  
393 minutes after addition of pNPY (**Figure 6–figure supplement 1**). In this case, we did not  
394 observe a significant change in receptor density upon addition of the pNPY, indicating that the  
395 high degree of immobilization and large size of the antibody might restrict the transient  
396 enrichment of active receptors into the clustered regions. Specific clusters termed GPCR hot  
397 spots (40-300 nm) have been visualized at the plasma membrane of living cells (Calebiro &  
398 Jobin, 2019; Chavez-Abiega *et al.*, 2019; Hilger *et al.*, 2018; Sungkaworn *et al.*, 2017). These  
399 hot spots represent regions that preferentially engage signaling, and that are enriched in both  
400 receptors and G proteins. We hypothesize that the induced microscale clusters trigger the  
401 formation of hot spots, which provide an ideal environment for recruitment of more active  
402 receptors and thus amplification of the signal. By increasing the local effective receptor  
403 concentration, this organization may amplify both the speed and efficiency of receptor-  
404 G protein coupling while enabling local signal transduction. In summary, our results show a  
405 difference between Arr3 recruitment in the ligand-free mode compared to the ligand-activated  
406 state. These observations indirectly confirm a high-affinity interaction between the Y<sub>2</sub>R and  
407 G $\alpha_i$  and suggest active recruitment of G proteins that delay Arr3 recruitment and impair  
408 termination of G protein signaling (Ziffert *et al.*, 2020). Likewise, the increased recruitment of  
409 receptors observed after addition of the pNPY ligand may be directly related to the dynamic  
410 nature of the confined regions.



## 411 **Discussion**

412 We developed a versatile approach to cluster receptors *in situ* with minimal steric hindrance  
413 and disturbance. The transient association between the multivalent nanotool and the receptors  
414 revealed the generation of a dynamic platform for cell signaling. The dynamic exchange of  
415 molecules within induced Y<sub>2</sub>R microscale clusters may contribute to the formation of hot spots  
416 and final downstream signaling. This feature, as well as the broad applicability and the lower  
417 concentration required compared to established systems with immobilized ligands or  
418 antibodies, highlight the advantages of this versatile approach. Ligand-independent receptor  
419 activation by confinement was unraveled by cytosolic calcium increase and changes in cell  
420 spreading and motility, a response analogous to ligand-induced receptor activation.  
421 Furthermore, we demonstrated an amplification of the signal upon ligand-induced activation  
422 in cells pre-clustered with the nanotool. Subsequent addition of the neuropeptide ligand led to  
423 an enhancement of the calcium signal compared to ligand-induced activation without  
424 clustering. Interestingly, we demonstrated an increase in the receptor intensity in the clustered  
425 areas concomitant with ligand addition. We also uncovered a difference in downstream  
426 signaling for the ligand-free *versus* ligand-activated receptors as evidenced by co-recruitment  
427 of Arr3 to the clustered spots only occurring in the presence of the neuropeptide ligand. This  
428 finding is consistent with previous results demonstrating an Arr3-dependent internalization,  
429 subsequent endosomal sorting, and receptor recycling to the cell membrane in the presence  
430 of high concentrations of NPY (Walther *et al.*, 2010; Wanka *et al.*, 2018). We hypothesize that  
431 high-affinity Y<sub>2</sub>R/Gα<sub>i</sub> interactions drive the initial cell response, cytosolic calcium increase, and  
432 cell motility. High local receptor density in the spots increases the residence time of proximate  
433 Gα<sub>i</sub> proteins and recruits further downstream effectors, which boost the probability of  
434 activation (Sánchez *et al.*, 2021). Further, Y<sub>2</sub>R/Gα<sub>i</sub> interactions lead to persistent activation of  
435 the Gα<sub>i</sub> pathway, which depletes the intracellular Gα<sub>i</sub> protein repertoire before Arr3 binding  
436 can terminate signaling (Ziffert *et al.*, 2020). The time frame of imaging after addition of the  
437 nanotool (30 min) suggests a long-lasting Gα<sub>i</sub> protein activation and favors the hypothesis of  
438 a mechanism for Y<sub>2</sub>R activation and desensitization that is limited to the cell membrane and  
439 partially independent of Arr3 recruitment (Ziffert *et al.*, 2020).

440 Multiscale analyses of the interactions between receptor clusters, G proteins, the lipid  
441 environment and actin-myosin assemblies are critical to confirm cluster behavior and  
442 dynamics. *In vitro* reconstitution systems utilizing lipid bilayers have proven useful to  
443 investigate receptor signaling (Huang *et al.*, 2021). Comparing fluid-patterned lipid bilayers  
444 with our established platform together with advanced quantitative fluorescence microscopy  
445 techniques such as fluorescence resonance energy transfer (FRET) and single-molecule  
446 localization microscopy will help us to decode cluster behavior and decipher the complete  
447 ligand-independent signaling pathway. In summary, the developed nanotool and matrices  
448 allow the investigation of ligand-independent receptor activation *in situ*, facilitating the  
449 investigation of early key processes in cell signaling.



## 450 **Materials and Methods**

451 **Synthesis of *tris*NTA<sup>PEG12-B</sup>**: Cyclam-Lys-*tris*NTA (Gatterdam *et al.*, 2018) (5.0 mg, 4.8  $\mu$ mol),  
452 Biotin-PEG<sub>12</sub>-NHS (23.0 mg, 24.0  $\mu$ mol) and DIPEA (12.2  $\mu$ L, 72.0  $\mu$ mol) were dissolved in  
453 0.5 ml dry DMF and stirred for 2 h at RT. After reaction, the volatile components were removed  
454 by lyophilization. Raw product was purified by reverse-phase (RP)-HPLC (mobile phase A:  
455 H<sub>2</sub>O + 0.1% TFA, B: CAN + 0.1% TFA; gradient 5% to 80% B in 20 min; MZ-PerfectSil, 300  
456 ODS, 5  $\mu$ m, 250 x 10 mm, flow 4 ml/min). A biotin moiety was integrated into the nanotool for  
457 immobilization to SA in the pre-structured matrices. The PEG<sub>12</sub> linker between the biotin and  
458 the *tris*NTA unit increased the flexibility of the molecule. The identity of *tris*NTA<sup>PEG12-B</sup> was  
459 confirmed by liquid chromatography-coupled mass spectrometry (LC-MS, Waters BioAccord  
460 System). Datasets were recorded with an ACQUITY UPLC I-Class Plus chromatography  
461 system and ACQUITY RDa Detector, which was set to a cone voltage of 25 V, capillary voltage  
462 of 1.2 kV and a desolvation temperature of 500 °C operating in positive ionization mode. For  
463 reverse-phase separation, an ACQUITY UPLC Peptide BEH C18 column (300 Å, 1.7  $\mu$ m,  
464 2.1 mm x 100 mm) was used (**Figure 1–figure supplement 4**). *tris*NTA<sup>PEG12-B</sup> was dissolved  
465 in HBS buffer (20 mM HEPES-NaOH pH 7.5, 150 mM NaCl) and incubated with 10-fold  
466 excess of NiCl<sub>2</sub>. After 30 min incubation at 4 °C, the excess of Ni(II) was separated by a size  
467 exclusion chromatography gravity column (PD MidiTrap G-10).

468

469 **Microcontact printing**. Large-area microcontact printing was performed as described  
470 previously (Lanzerstorfer *et al.*, 2020) with modifications. In short, a field of a large-area PFPE  
471 elastomeric stamp (1  $\mu$ m grid size), obtained by the EV-Group (St. Florian am Inn, Upper  
472 Austria, Austria), was cut out, and washed by flushing with ethanol (100%) and distilled water.  
473 After drying with nitrogen, the stamp was incubated in 50 ml BSA solution (1 mg/ml, Sigma-  
474 Aldrich) for 30 min followed by washing the stamp with phosphate-buffered saline (PBS) and  
475 distilled water. After drying with nitrogen, the stamp was placed with homogeneous pressure  
476 onto a clean epoxy-coated glass substrate (Schott Nexterion Slide E) and incubated overnight  
477 at 4 °C. The next day, the stamp was stripped from the glass with a forceps, and the

478 microstructured glass was bonded to a 96-well plastic casting using an adhesive tape (3M)  
479 and closed with an appropriate lid.

480

481 **Functionalization of the pre-structured matrices.** BSA-pre-structured wells were incubated  
482 with biotin-BSA (0.1 mg/ml, Thermo Fisher Scientific, Waltham, MA, USA) and SA (1  $\mu$ M,  
483 Sigma-Aldrich, Munich, Germany) in PBS, each for 1 h at RT. Incubated wells were washed  
484 thoroughly with PBS after each step to remove unbound biotin-BSA and SA. For binding of  
485 soluble His-tagged proteins, wells were incubated with *tris*NTA<sup>PEG12-B</sup> (0.5  $\mu$ M) in HBS buffer  
486 for 1 h at RT. For nickel-loading, the pre-structured matrices were sequentially incubated with  
487 imidazole (1 M, 2 min), EDTA (100 mM, 2 min), and NiCl<sub>2</sub> (10 mM, 5 min). Wells were carefully  
488 washed after each step. Finally, HBS buffer containing EDTA (50  $\mu$ M) was used to remove  
489 the free, non-complexed nickel ions. His<sub>6</sub>-GFP (100 nM) previously expressed and purified  
490 was added to the wells and incubated for 30 min at RT. Experiments were performed in  
491 biological replicas (N=5).

492

493 **Cell culture.** HeLa Flp-In<sup>TM</sup> T-Rex<sup>TM</sup> Y<sub>2</sub>R cells (His<sub>6</sub>-Y<sub>2</sub>R<sup>mEGFP</sup> or Y<sub>2</sub>R<sup>mEGFP</sup>) were generated  
494 and cultured at 37 °C, 5% CO<sub>2</sub>, and 95% humidity (Sánchez *et al.*, 2021). For culturing the  
495 stable cell line, high glucose Dulbecco's modified Eagle's medium (DMEM) (Gibco/Thermo  
496 Fisher Scientific) was supplemented with 10% tetracycline-free fetal calf serum (FCS,  
497 Bio&Sell), blasticidin S HCl (1  $\mu$ g/ml, Thermo Fisher Scientific), and hygromycin B (50  $\mu$ g/ml,  
498 Thermo Fisher Scientific). To induce receptor expression the cell medium was replaced with  
499 fresh medium containing tetracycline (0.1  $\mu$ g/ml, Fluka) 18 h before imaging. The same  
500 concentration of tetracycline resulting in an efficient plasma membrane targeting was used for  
501 all the experiments. The cells were regularly tested for mycoplasma contamination.

502

503 **Receptor confinement in real-time by *tris*NTA<sup>PEG12-B</sup>.** Cells expressing Y<sub>2</sub>R (His<sub>6</sub>-Y<sub>2</sub>R<sup>mEGFP</sup>  
504 or Y<sub>2</sub>R<sup>mEGFP</sup>) were trypsinized and allowed to adhere to SA pre-structured matrices for 3 h or  
505 overnight. 15-18 h prior to the experiment, the cell medium was replaced with fresh medium  
506 containing tetracycline (0.1  $\mu$ g/ml) to induce receptor expression. The cells were visualized by

507 CLSM in live-cell imaging solution (LCIS, Thermo Fisher Scientific) at 37 °C. Cells were  
508 subsequently incubated with nickel-loaded *tris*NTA<sup>PEG12-B</sup> (final concentration 100 nM) in LCIS  
509 for 10-15 min at 37 °C. Excess of unbound *tris*NTA<sup>PEG12-B</sup> was removed by washing with LCIS.  
510 For reversibility experiments, micropatterned cells were incubated with histidine (5 mM) in  
511 LCIS for 2 to 10 min followed by washing with LCIS. Experiments were performed in biological  
512 replicas (N=4).

513

514 **Receptor confinement on antibody-micropatterned matrices.** Wells pre-structured with  
515 BSA were subsequently incubated with biotin-BSA (0.1 mg/ml), SA (1 μM), and a biotinylated  
516 anti-His<sub>6</sub> antibody (1 μM) (ab106261, Abcam) in PBS for 1 h at RT. Wells were washed  
517 thoroughly with PBS to remove unbound antibody. Cells expressing Y<sub>2</sub>R were trypsinized and  
518 seeded onto the antibody patterns. After 3 h, cells were visualized by CLSM in LCIS at 37 °C.  
519 Experiments were performed in biological replicas (N=5).

520

521 **Time-lapse calcium imaging.** 18 h after seeding the cells onto pre-structured SA-matrices,  
522 cells were incubated with BioTracker 609 Red Ca<sup>2+</sup> AM dye (3 μM, Merck Millipore) in fresh  
523 medium for 30 min. The cell-membrane permeable dye is de-esterified by cellular esterases  
524 and remains trapped in the cytosol. After incubation with the Ca<sup>2+</sup> dye, cells were rinsed three  
525 times with PBS and imaged by CLSM in LCIS at 37 °C. For investigation of Ca<sup>2+</sup> signal, time-  
526 lapse images were taken (5 slices z-stacks, 45-s interval) before and after addition of  
527 *tris*NTA<sup>PEG12-B</sup>. Fluorescence intensity ( $\lambda_{ex/em}$  590/609 nm) of the dye changes depending on  
528 the intracellular Ca<sup>2+</sup> concentration. Maximum intensity projections of single channels were  
529 analyzed. The ImageJ ROI tool was used to define the areas of the image to be analyzed. We  
530 consider a ROI covering the complete cell contour. Mean gray values (F) were background  
531 subtracted and normalized to the fluorescence in cells before F<sub>0</sub>. Experiments were performed  
532 in biological replicas (N=3).

533

534 **Plasma membrane staining.** Live-cell membrane staining was performed directly after  
535 receptor assembly in living cells grown on pre-structured matrices. CellMask™ deep red

536 plasma membrane stain (Thermo Fisher Scientific) was used according to manufacturer's  
537 instruction. 1  $\mu$ l of the stock solution (1000x dilution) was dissolved in 1 ml of warm LCIS (final  
538 concentration 5  $\mu$ g/ml) and subsequently added to the cells, incubated for 5 min at 37 °C, and  
539 washed with LCIS before visualization. Experiments were performed in biological replicas  
540 (N=3).

541

542 **Confocal laser scanning microscopy.** Images were recorded by using a CLSM Zeiss LSM  
543 880 (Carl Zeiss) equipped with a Plan-Apochromat 63x/1.4 Oil DIC M-27 objective. Sequential  
544 settings for dual-color imaging were used. Excitation wavelengths for the different  
545 fluorophores: 488 nm (argon laser) for mEGFP; 594 nm for the Ca<sup>2+</sup> dye; 633 nm (helium-  
546 neon laser) for the plasma membrane dye. Signals were detected after appropriate filtering on  
547 a photomultiplier. Intensities of channels were adjusted over the whole image for better  
548 visualization of overlap and exported by Zen blue (version 2.3 lite, Zeiss). Detector  
549 amplification, laser power, and pinhole were kept constant for all studies.

550

551 **Image analysis.** Fluorescence images were processed with Zen blue, ImageJ, and Fiji  
552 software (Schindelin *et al.*, 2012; Schneider *et al.*, 2012). All images were background  
553 subtracted. Integrated density, mean gray value and cell area were obtained with ImageJ.  
554 Data were plotted with OriginPro.

555

556 **Fluorescence recovery after photobleaching.** FRAP experiments were conducted at the  
557 CLSM Zeiss LSM 880 using 63 x/1.4 Oil DIC objective. Rectangular-shaped regions (6-10  $\mu$ m  
558 radius) were bleached within 10 s with high laser intensities. Fluorescence recovery was  
559 monitored by repetitively imaging an area containing the photobleached region at 0.1 frame/s  
560 for ~150 s. For the analysis, a simulation approach that allows computation of diffusion  
561 coefficients regardless of bleaching geometry used in the FRAP series was applied  
562 (Blumenthal *et al.*, 2015). The method is based on fitting a computer-simulated recovery to  
563 actual recovery data of a FRAP series. The algorithm accepts a multiple-frame TIFF file,  
564 representing the experiment as input, and simulates the diffusion of the fluorescent probes

565 (2D random walk) starting with the first post-bleach frame of the actual data. Once the  
566 simulated recovery is finished, the algorithm fits the simulated data to the real one and extracts  
567 the diffusion coefficient. The algorithm iteratively creates a series of simulated images, where  
568 each frame corresponds to a single iteration. The intensity values are extracted from the (user  
569 indicated) bleached area of the simulated frames, thus determining the general shape of the  
570 recovery curve. The “time” axis at this stage is in arbitrary units (iterations). To extract the  
571 diffusion coefficient, the simulated recovery curve needs to be fitted to the real recovery curve,  
572 by appropriately stretching the “time” axis. The time between frames in the actual data set is  
573 obviously known, thus once overlapping optimally the simulated curve with the real one, the  
574 duration of one iteration, in real-time units, is determined. The diffusion coefficient of the  
575 simulated series is then calculated according to eq. 1, where  $D_s$  is the simulation-extracted  
576 diffusion coefficient,  $l$  is the step of a molecule in each iteration of the simulation,  
577 corresponding to one pixel in the image (the pixel size is calibrated previously, by imaging a  
578 known calibration sample), and  $t_i$  is the time interval between steps (determined as explained).

$$D_s = \frac{l^2}{4t_i} \quad (1)$$

579 The simulation proceeds until a plateau is reached (equilibration of the fluorescence intensity  
580 in the bleached area). The number of data points in the simulated recovery is typically different  
581 (larger) than the number of experimental points. In addition, the real experimental data may  
582 not have been acquired until equilibration of fluorescence. To determine  $t_i$ , the algorithm  
583 scans a range of possible values for the total duration represented by the simulation and  
584 calculates a value  $X^2$  for the goodness-of-fit between the simulated data and the real FRAP  
585 data. Total simulation duration is selected as the one that produces the minimal  $X^2$ .  
586 Experiments were performed in biological replicas (N=3).

587

588 **imFCS analysis.** imFCS measurements were performed as described earlier (Harwardt *et al.*,  
589 2018; Harwardt *et al.*, 2017). A home-built widefield setup with total internal reflection  
590 fluorescence (TIRF) illumination was used for imFCS analysis. The experimental setup was  
591 equipped with a 488 nm diode laser (100 mW, Obis, Coherent, USA). The excitation light

592 passes through an acousto-optical tunable filter (AA Opto-Electronic, Orsay, France) and a  
593 telescope consisting of two achromatic lenses (Thorlabs, USA) with  $f = -40$  mm and 750 mm.  
594 A third achromatic lens ( $f = 400$  mm, Thorlabs) directed the excitation light to the TIRF mirror  
595 and had its focus on the back focal plane of the objective. The TIRF mirror was placed on a  
596 motorized translation stage (25 mm, #MTS25/M-Z8, Thorlabs) controlled by a motion  
597 controller (K-Cube Brushed DC Servo Motor Controller, #KDC101, Thorlabs) to switch  
598 between widefield and TIRF illumination. The light entered an Eclipse Ti microscope (Nikon,  
599 Japan) was reflected by a dichroic mirror (TIRF-Quad filter set 405/488/561/640 consisting of  
600 a QuadLine Laser Clean-up ZET405/488/561/640x, QuadLine dichroic zt405/488/561/640rpc,  
601 QuadLine rejection band ZET405/488/561/640 TIRF, all AHF Analysentechnik AG, Tübingen,  
602 Germany), and was directed onto the sample by an oil-immersion TIRF objective (UapoN  
603 100xOTIRF, 1.49 Oil, Olympus, Japan). A nosepiece stage (IX2-NPS, Olympus) was used for  
604 z-plane adjustment and drift minimization. Emission light was collected by the same objective  
605 and passed the dichroic mirror. In the detection path a TwinCam (Acal Bfi, Germany) with a  
606 BrightLine HC 525/45 bandpass filter (AHF Analysentechnik AG) was implemented, and the  
607 signal was detected by a scientific complementary metal-oxide semiconductor (sCMOS)  
608 camera (Zyla 4.2, Andor, Belfast, UK). Data were collected using the open-source software  
609  $\mu$ Manager (Edelstein *et al.*, 2010). For data acquisition the following settings were applied:  
610  $24 \text{ W/cm}^2$  laser intensity, a bit depth of 16 bit, pixel readout rate of 540 MHz, frame time 4 ms,  
611 4x4 binning, and 4,000 frames per film. For each film, a 40x25 pixel (or 40x20 pixel) region of  
612 interest (ROI) was chosen and the measurement was performed with TIRF illumination to  
613 observe membrane diffusion of  $Y_2R$ . In total 36 untreated cells, 24 cells with *trisNTA*<sup>PEG12-B</sup>  
614 immobilized receptors, and 26 cells with anti-His<sub>6</sub> antibody immobilized receptors were  
615 measured. Each condition contains data from at least three independent measurement days  
616 (N=3).

617

618 **imFCS data analysis.** Analysis of imFCS films was performed using the imFCS plugin  
619 (version 1.52) (Sankaran *et al.*, 2010) for Fiji (Schindelin *et al.*, 2012). The following correlation  
620 settings were chosen: emission wavelength = 515 nm, NA = 1.49, correlator scheme P = 16

621 and  $Q = 8$ , lateral PSF = 0.8, binning = 1, pixel size = 5.75  $\mu\text{m}$ , magnification = 25 for 4x4  
622 binning, and linear segment bleach correction with linear segments of 500 frames. Diffusion  
623 coefficients were obtained for each pixel by fitting the correlation curves according to the  
624 literature (Sankaran *et al.*, 2010). To compare the overall diffusion coefficients with those of  
625 the patterned regions, ROIs were placed around patterned regions and analyzed separately.  
626 For further analysis, the pixelwise diffusion coefficients for all measurements were imported  
627 into OriginPro 2019 (OriginLab Corporation, Northampton, USA). For box plots of diffusion  
628 coefficients, median diffusion coefficients were determined for each cell. Mean diffusion  
629 coefficients per condition were obtained by averaging over the median diffusion coefficients  
630 per measurement and calculating the standard error of the mean. Two-sample t-tests ( $\alpha =$   
631 0.05) were applied to compare the diffusion coefficients for the different conditions. All  
632 datasets were tested for normality using the Kolmogorov-Smirnov test ( $\alpha = 0.05$ ). Significance  
633 was assigned as follows:  $p > 0.05$  no significant difference between populations (n.s.),  $p <$   
634 0.05 significant difference (\*),  $p < 0.01$  significant difference (\*\*), and  $p < 0.001$  significant  
635 difference (\*\*\*). Two-dimensional maps of diffusion coefficients were generated also in  
636 OriginPro. Diffusion coefficients were color-coded from light yellow to dark red in the range of  
637 0 to 0.5  $\mu\text{m}^2/\text{s}$ . Pixels that yielded correlation curves with diffusion coefficients higher than  
638 0.5  $\mu\text{m}^2/\text{s}$  are presented in black. Pixels that yielded correlation curves which could not be  
639 fitted by the imFCS plugin in Fiji are shown in light grey. To generate frequency distribution  
640 plots, diffusion coefficients were log-transformed and binned in the interval between -5.3 and  
641 1.0 with a bin size of 0.1 for each cell. Logarithmic diffusion coefficients were re-transformed,  
642 frequency counts were averaged over all cells per condition, and normalized. Frequency  
643 counts were plotted logarithmically against diffusion coefficients. Errors bars represent  
644 standard errors of the mean.

645

646 **Arr3 recruitment upon receptor confinement.** Microstructured surfaces were functionalized  
647 with biotin-BSA and SA or SA and anti-His<sub>6</sub> antibody as described before. For transient co-  
648 transfection with Arr3<sup>mCherry</sup>, cells were sub-cultured the day before and then transfected with  
649 the Arr3<sup>mCherry</sup> plasmid using the TurboFect<sup>TM</sup> transfection reagent (Thermo Fisher Scientific),



650 according to the manufacturer's instructions and induced with Tetracycline (0.1 µg/ml) 18 h  
651 before microscopy. Cells co-expressing His<sub>6</sub>-Y<sub>2</sub>R<sup>mGFP</sup> and Arr3 were seeded onto the  
652 microstructured matrices and visualized by total internal reflection fluorescence (TIRF)  
653 microscopy in LCIS at 37 °C after 3 to 4 h to ensure a homogeneous cell membrane adhesion,  
654 which is a prerequisite for quantitative TIRF microscopy. For antibody experiments, cells  
655 grown on pre-structured matrices were incubated with pNPY (10 nM, Tocris) in LCIS for  
656 30 min at 37 °C. For *tris*NTA<sup>PEG12-B</sup> experiments, cells grown on SA-matrices were  
657 subsequently incubated with nickel-loaded *tris*NTA<sup>PEG12-B</sup> (100 nM final) and pNPY (10 nM  
658 final) in LCIS for 30 min at 37 °C. For reversibility, cells were incubated with histidine (5 mM)  
659 in LCIS for 30 min. Experiments were performed in biological replicas (N=2).

660

661 **Arr3 imaging by TIRF microscopy.** The detection system was set up on an epi-fluorescence  
662 microscope (Nikon Eclipse Ti2). For selective fluorescence excitation of mGFP and mCherry,  
663 a multi-laser engine (Toptica Photonics, Munich, Germany) was used at 488 and 561 nm,  
664 respectively. The samples were illuminated in total internal reflection (TIR) configuration  
665 (Nikon Ti-LAPP) using a 60x oil immersion objective (NA = 1.49, APON 60XO TIRF). After  
666 appropriate filtering using standard filter sets, the fluorescence was imaged onto a sCMOS  
667 camera (Zyla 4.2, Andor, Northern Ireland). The samples were mounted on an x-y-stage  
668 (CMR-STG-MHIX2-motorized table, Märzhäuser, Germany), and scanning was supported by  
669 a laser-guided automated Perfect Focus System (Nikon PFS).

670

671 **Contrast quantification and statistical analyses.** Contrast analysis was performed as  
672 described previously (Lanzerstorfer *et al.*, 2014; Lanzerstorfer *et al.*, 2020; Schütz *et al.*, 2017).  
673 Initial imaging recording was supported by the Nikon NIS Elements software. Images were  
674 exported as TIFF frames and fluorescence contrast analysis was performed using the Spotty  
675 framework (Borgmann *et al.*, 2012). The fluorescence contrast  $\langle c \rangle$  was calculated as  $\langle c \rangle =$   
676  $(F^+ - F^-)/(F^+ - F_{bg})$ , where  $F^+$  denotes the intensity of the inner pixels of the pattern.  $F^-$  shows  
677 the intensity of the surrounding pixels of the micropattern, and  $F_{bg}$  the intensity of the global  
678 background. Data are expressed as the means  $\pm$  SEM. Comparisons of more than two

679 different groups were performed using one-way ANOVA, which was followed by Tukey's  
680 multiple comparisons test in GraphPad Prism software (version 9.1.2).

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685 (Goethe University Frankfurt) for the generation of the Y<sub>2</sub>R<sup>mEGFP</sup> constructs with and without  
686 His<sub>6</sub>-tag. We thank Dr. Cornelius Krasel (Philipps University of Marburg) for the Arr3<sup>mCherry</sup>  
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694

695 **Author contributions**

696 M.F.S. performed the cell-based assays and imaging experiments. M.S.D. carried out the  
697 imFCS experiments and analyzed the data together with M.H.. U.M., P.L., and J.W. prepared  
698 the pre-structured surfaces, performed the Arr3 recruitment assays and the intensity-contrast  
699 analysis. K.G. synthesized and characterized the chelator compound. M.F.S., R.W., and R.T.  
700 wrote the manuscript with contributions from all authors. R.T. conceived the study.

701

702 **Competing interest.** The authors declare no competing interest.

703

704 **Data availability.** Data and movies are available in the supplementary materials.

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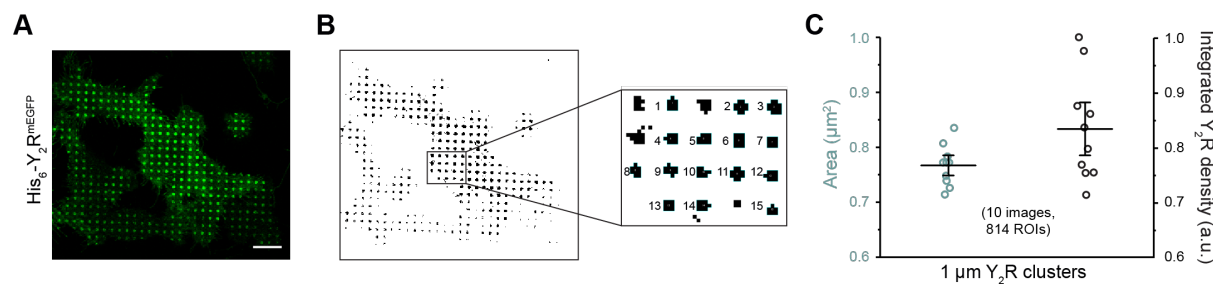


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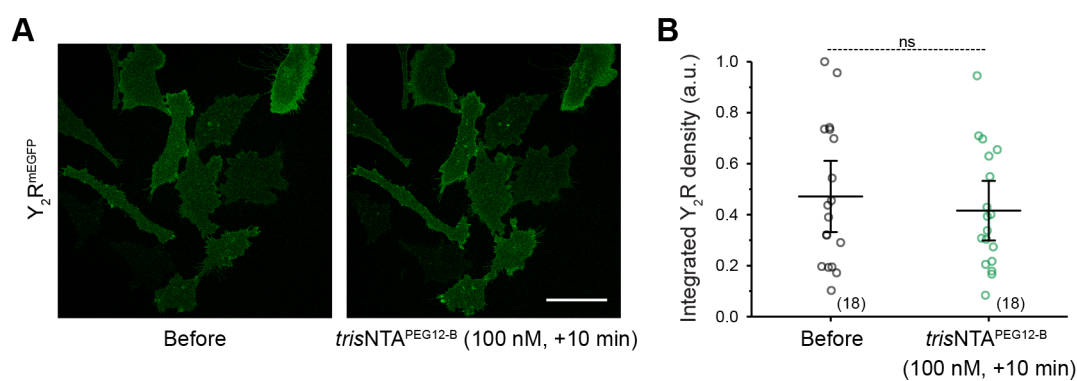
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- 887

888 **Supplemental figures**



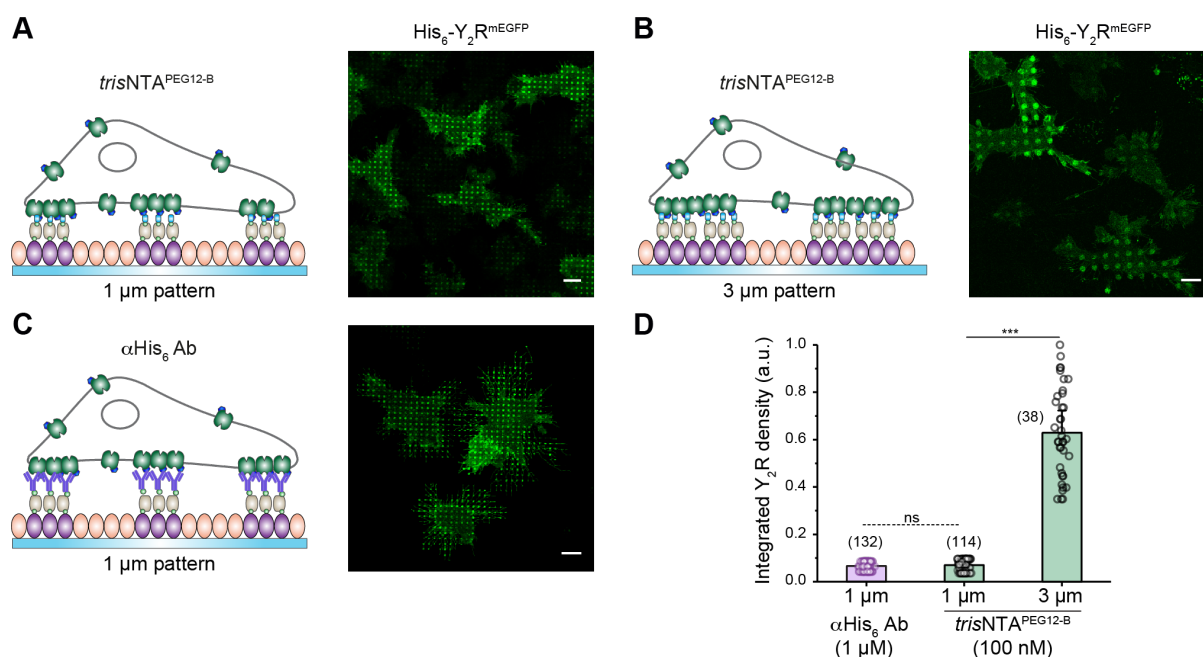
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890 **Figure 1–figure supplement 1.** Receptor confinement with high reliability. (A)  
891 Representative confocal image of a cell patterned by *trisNTA*<sup>PEG12-B</sup>. (B) Automatic cluster  
892 analysis performed by ImageJ requires a “binary”, black and white, image. A threshold range  
893 is set to select the objects of interest apart from the background. All pixels in the image whose  
894 values lie under the threshold are converted to white and all pixels with values above the  
895 threshold are converted to black. Further selection of the clusters according to area and  
896 roundness enable a large-scale analysis. (C) Change in cluster area and integrated density of  
897 the receptor within different 96-well plates, different months and cell stocks reflected a reliable  
898 and reproducible approach. The average area in the clustered regions ( $0.77 \pm 0.03 \mu\text{m}^2$ ) and  
899 integrated density of ten images in five different experiments (841x ROIs of  $1 \mu\text{m}$  in total) is  
900 shown. Scale bar:  $10 \mu\text{m}$ .



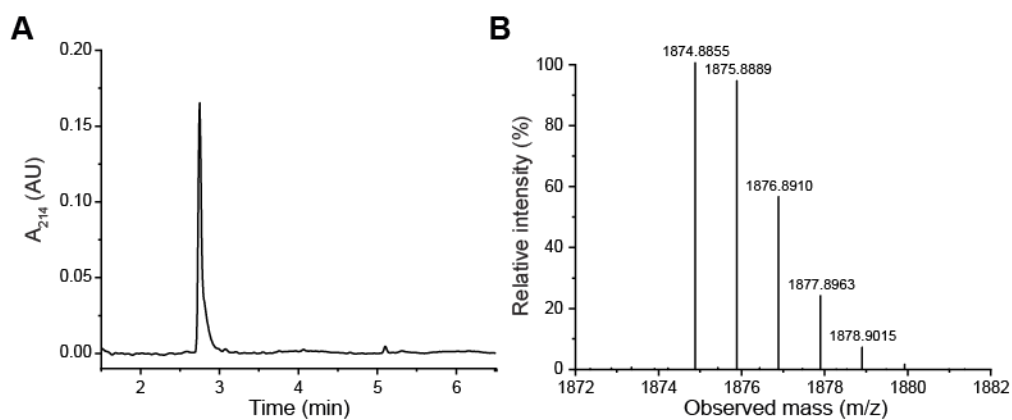
901

902 **Figure 1-figure supplement 2.** Y<sub>2</sub> receptors lacking a His<sub>6</sub>-tag do not cluster in confined  
903 areas. (A) Representative confocal images of cells expressing Y<sub>2</sub> receptors without N-terminal  
904 His<sub>6</sub>-tag over SA-pre-structured matrices before and after addition of the nanotool. Within the  
905 timeframe of imaging, there was neither a pattern formation nor a change in the integrated  
906 receptor density. (B) Quantification of the integrated Y<sub>2</sub>R density before and after addition of  
907 trisNTA<sup>PEG12-B</sup>. The mean ± SD (18 cells) is shown. \*\*p ≤ 0.01 for Tukey test. Scale bar: 50 μm.



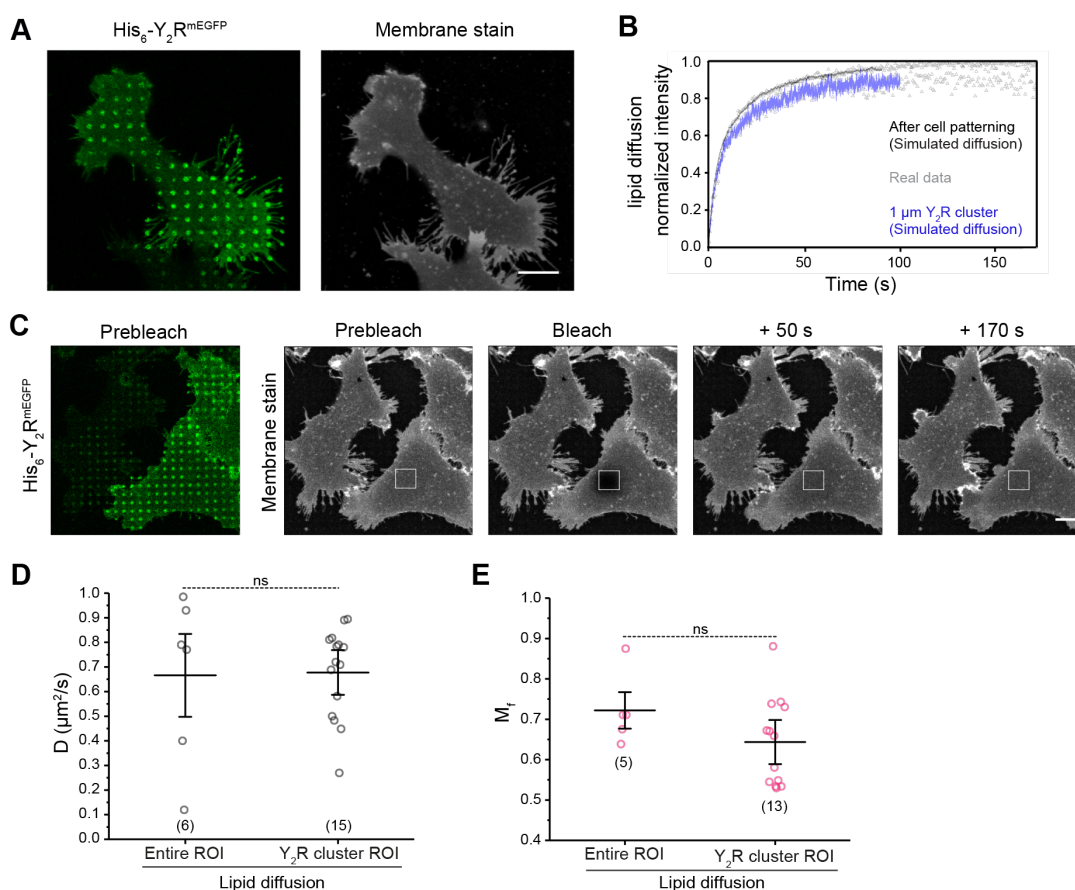
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909 **Figure 1–figure supplement 3.** Receptor density correlates with the pre-  
 910 structured regions. (A, B) BSA-pre-structured matrices, 1 μm (A) or 3 μm (B), were stepwise  
 911 functionalized with biotin-BSA and SA. Y<sub>2</sub>R-expressing HeLa cells were allowed to adhere to  
 912 the functionalized matrix for 3 h and immediately imaged by CLSM in live-cell imaging solution  
 913 (LCIS) at 37 °C. Incubation with *trisNTA*<sup>PEG12-B</sup> (100 nM final) led to *in situ* receptor assembly.  
 914 (C) 1 μm BSA-pre-structured matrices were stepwise functionalized with biotin-BSA, SA, and  
 915 a biotinylated anti-His<sub>6</sub> antibody. Y<sub>2</sub>R-expressing cells were allowed to adhere to the  
 916 functionalized matrix for 3 h and immediately imaged by CLSM in LCIS at 37 °C. (D)  
 917 Quantification of the receptor-integrated density. *In situ* receptor confinement by *trisNTA*<sup>PEG12-</sup>  
 918 <sup>B</sup> resulted in a receptor density that is comparable to cells in contact with pre-structured  
 919 antibodies. For the 3 μm patterns, receptor density correlated with pattern area. The mean ±  
 920 SD (38 to 132x 1 μm ROIs) is shown. \*\*\*p ≤ 0.001 for Tukey test. Scale bars: 10 μm.



921

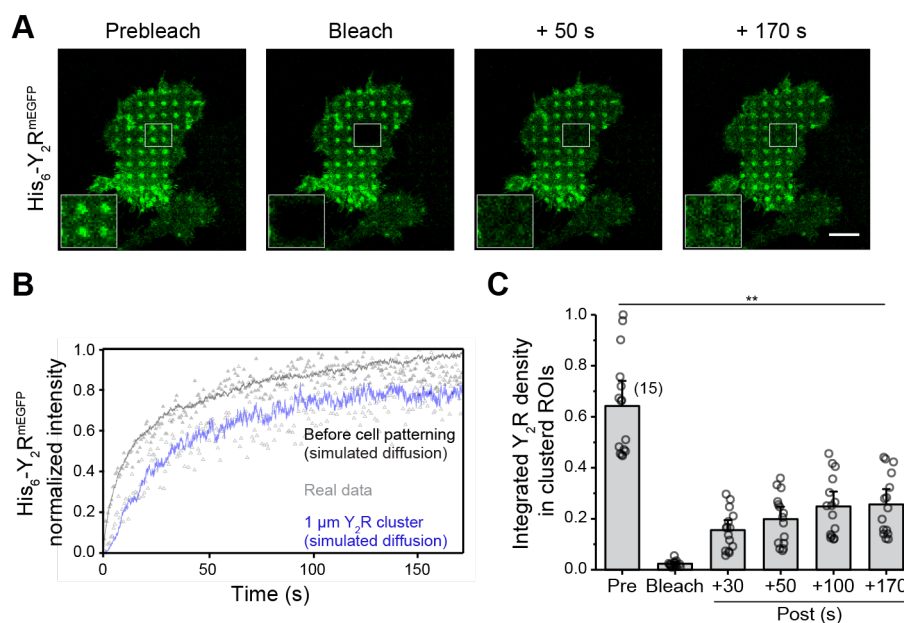
922 **Figure 1–figure supplement 4.** Multivalent nanotool  $\text{trisNTA}^{\text{PEG12-B}}$  analyzed by LC-MS. (A)  
923  $\text{trisNTA}^{\text{PEG12-B}}$  chromatogram reflecting the purity of the synthesized nanotool. (B) LC-MS of  
924  $\text{trisNTA}^{\text{PEG12-B}}$ , yielding the experimental mass ( $M_{\text{exp.}}$ ) of 1874.85 Da ( $M_{\text{theor.}} = 1873.90$  Da).



925

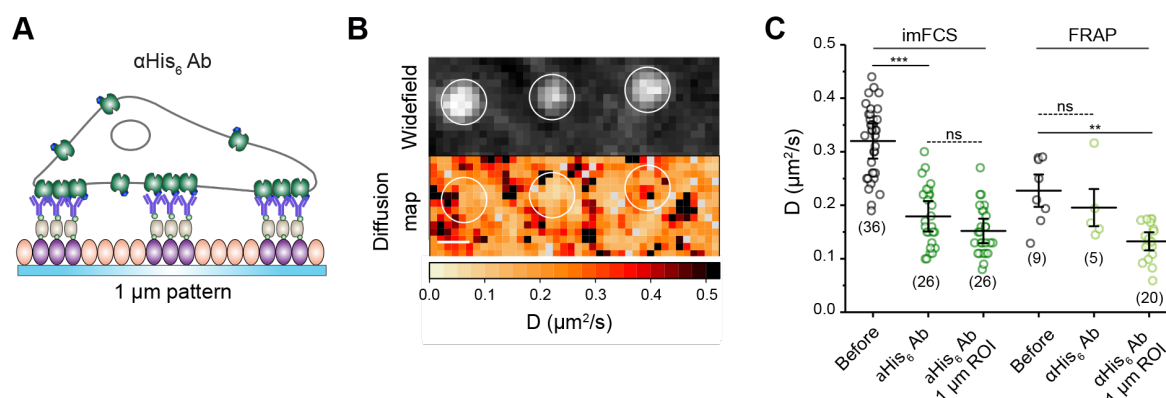
926 **Figure 2-figure supplement 1.** Lipid localization and dynamics after receptor confinement.  
 927 (A) Confocal microscopy images of the live-cell plasma membrane staining, which was  
 928 performed 15 min after Y<sub>2</sub>R assembly in living cells. 5 μg/ml CellMask staining solution was  
 929 incubated for 5 min at 37 °C and washed with LCIS before visualization. Lipid distribution is  
 930 not affected by receptor confinement as shown by the homogeneous staining of the membrane.  
 931 (B, C) FRAP recovery curve (B) and time-lapse (C) for the lipid dye demonstrated a rapid  
 932 recovery for the lipids. Diffusion was measured in the entire rectangular ROI or at the Y<sub>2</sub>R  
 933 cluster spots (region selected based on the receptor channel image). An image of the receptor  
 934 channel confirmed the presence of the pattern. (D) The analysis did not show any differences  
 935 in lipid diffusion coefficients for the entire rectangular ROI or at the 1 μm clustered regions  
 936 ( $D_{\text{entire ROI}} = 0.66 \pm 0.10 \mu\text{m}^2/\text{s}$  and  $D_{\text{spots}} = 0.67 \pm 0.17 \mu\text{m}^2/\text{s}$ ). The mean  $\pm$  SD (6 cells, 15x  
 937 1 μm ROIs) is shown. \*\* $p \leq 0.01$  for Tukey test. (E) Quantification of the mobile fraction ( $M_f$ )  
 938 for FRAP measurements of the lipid dye reflected no significant difference. The mean  $\pm$  SD (5  
 939 cells, 14x 1 μm ROIs) is shown. \*\* $p \leq 0.01$  for Tukey test. Scale bars: 10 μm.





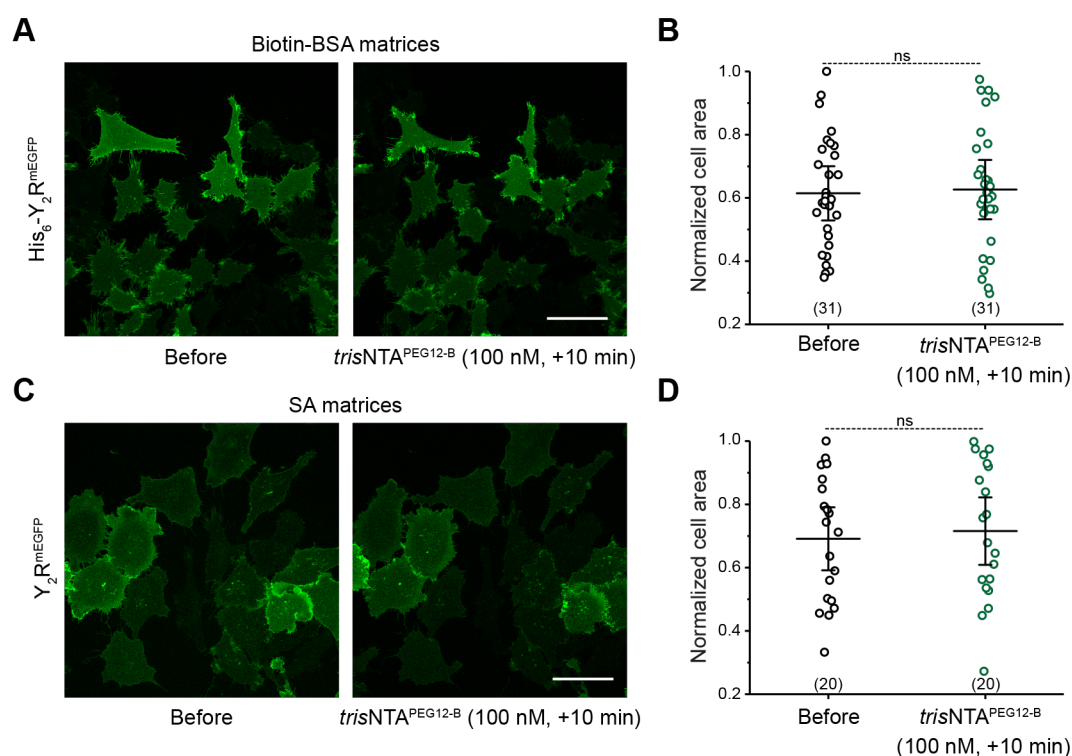
940

941 **Figure 2-figure supplement 2.** Dynamic receptor exchange in confined clusters. (A)  
942 Representative confocal images of FRAP measurements for  $\text{Y}_2\text{R}$ -expressing cells on SA-pre-  
943 structured matrices 10 min after addition of the nanotool. (B) FRAP recovery curves reflecting  
944 the entire bleached area or an analysis performed only in the clustered 1  $\mu\text{m}$  regions. The  
945 analysis is based on a simulation approach which fits a computer-simulated recovery to actual  
946 recovery data of a FRAP series and determines the diffusion coefficient regardless of  
947 bleaching geometry. (C) Quantification of the receptor density in the confined regions showed  
948 50% recovery indicating a high exchange rate. The mean  $\pm$  SD (6 cells, 15x 1  $\mu\text{m}$  ROIs  
949 analyzed) is shown. \*\* $p \leq 0.01$  for Tukey test. Scale bar: 10  $\mu\text{m}$ .



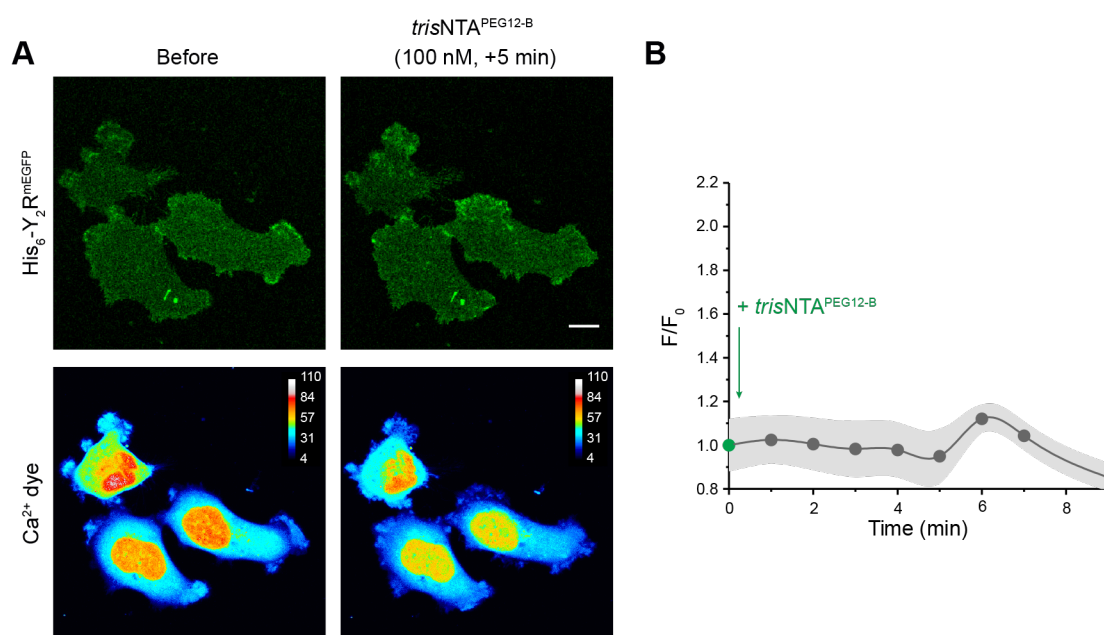
950

951 **Figure 2–figure supplement 3.** Receptor mobility on antibody structured matrices. (A)  
 952 Scheme representing the experimental set-up. (B) Representative widefield image (left) of a  
 953 ROI at the plasma membrane of a living cell over a pre-structured matrices with an anti-His<sub>6</sub>  
 954 antibody analyzed by imFCS and the derived two-dimensional diffusion map (right). (C) Lateral  
 955 diffusion of the receptor analyzed by FRAP and imFCS. Both techniques demonstrated a  
 956 decrease in  $D$  at the plasma membrane ( $D_{before} = 0.32 \pm 0.06 \mu\text{m}^2/\text{s}$  and  $0.25 \pm 0.08 \mu\text{m}^2/\text{s}$ ;  
 957  $D_{\text{anti-His}_6 \text{ Ab}} = 0.18 \pm 0.06$  and  $0.19 \pm 0.06 \mu\text{m}^2/\text{s}$  for imFCS and FRAP, respectively), concurring  
 958 with the values obtained for the measurements upon addition of the nanotool. Analysis of  
 959 clustered regions ( $1 \mu\text{m}$ ) within the selected ROIs led to a further decrease in the diffusion  
 960 coefficient ( $D_{\text{spots}} = 0.15 \pm 0.05 \mu\text{m}^2/\text{s}$  and  $0.13 \pm 0.03 \mu\text{m}^2/\text{s}$  for imFCS and FRAP,  
 961 respectively). For imFCS measurements, two-sample t-tests ( $\alpha = 0.05$ ) were applied to  
 962 compare the diffusion coefficients for the different conditions ( $***p \leq 0.001$ ). The mean  $\pm$  SD is  
 963 shown. 36 and 26 cells for the conditions before and after addition of anti-His<sub>6</sub> antibody were  
 964 analyzed. For FRAP, the mean  $\pm$  SD is shown. Here, 9 cells before, 5 cells after addition of  
 965 anti-His<sub>6</sub> antibody, 20x  $1 \mu\text{m}$  ROIs were examined.  $**p \leq 0.01$  for Tukey test. Scale bar:  $1 \mu\text{m}$ .



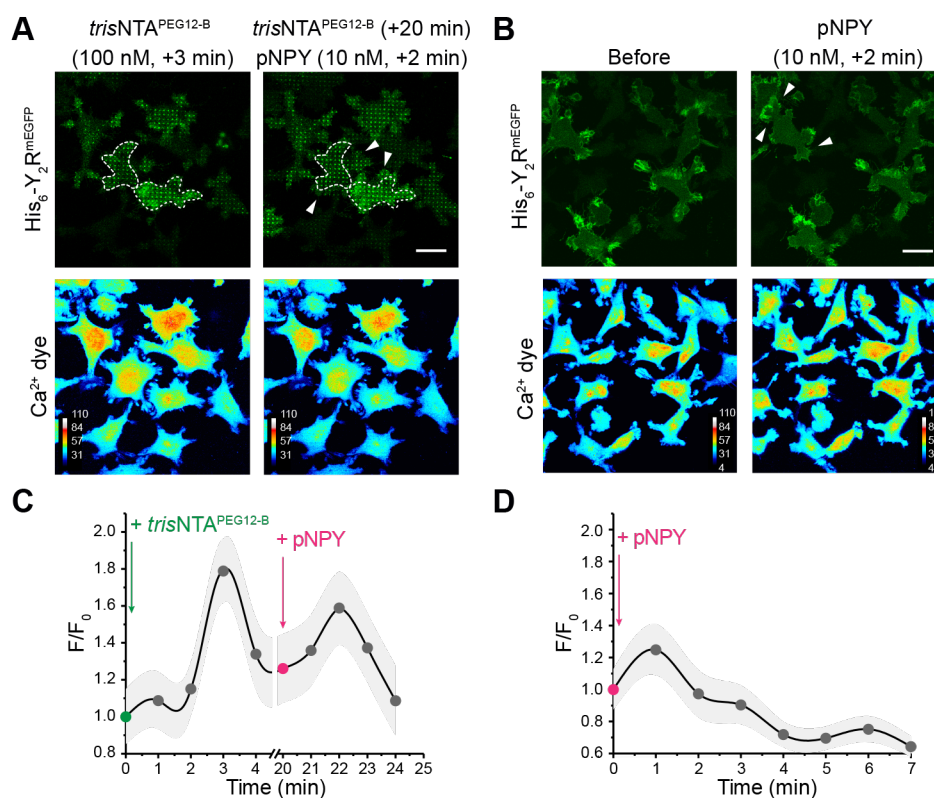
966

967 **Figure 4-figure supplement 1.** Changes in cell motility are exclusively triggered upon  
968 receptor clustering. (A, B) Confocal images of cells expressing His<sub>6</sub>-tagged Y<sub>2</sub>R on matrices  
969 which do not contain SA but biotin-BSA only. Addition of the trisNNTA<sup>PEG12-B</sup> nanotool confirmed  
970 no effect on cell spreading and motility as shown in the quantification of the cell area (B). The  
971 mean ± SD (31 cells) is shown. \*\*p ≤ 0.01 for Tukey test. (C) Confocal images of cells  
972 expressing Y<sub>2</sub> receptors lacking the His<sub>6</sub>-tag on SA-matrices do not present significant  
973 changes in cells spreading upon addition of the nanotool. (D) Quantification of the cell area  
974 before and after addition of trisNNTA<sup>PEG12-B</sup> (100 nM). Values for cell area were normalized with  
975 respect to the highest value. The mean ± SD (20 cells) is shown. \*\*p ≤ 0.01 for Tukey test.  
976 Scale bar: 50 μm.



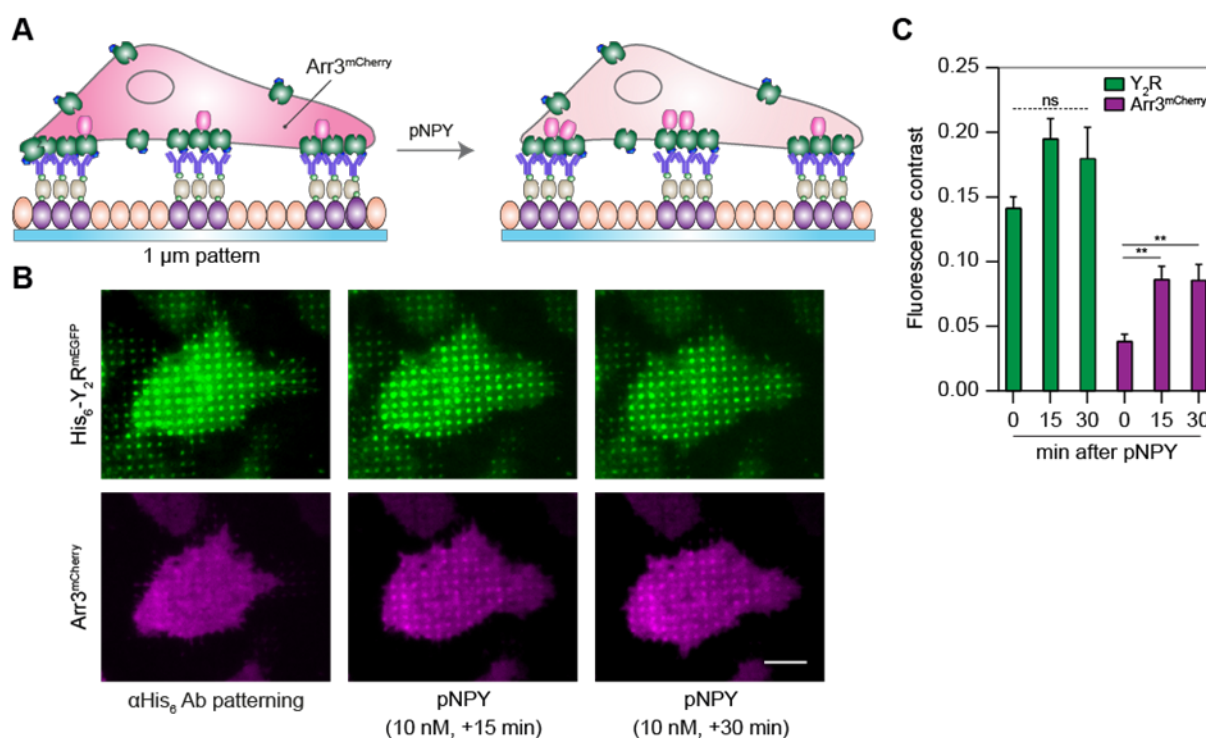
977

978 **Figure 5–figure supplement 1.** Calcium signaling is a specific response upon clustering. (A)  
979 Representative fluorescence images of the Y<sub>2</sub>R (upper panel) and color-coded images of the  
980 Ca<sup>2+</sup> dye (lower panel). Y<sub>2</sub>R-expressing cells over pre-structured matrices in the absence of  
981 streptavidin were incubated with BioTracker 609 Red Ca<sup>2+</sup> AM dye (3 μM) for 30 min. After  
982 rinsing, cells were immediately imaged by CLSM in LCIS at 37 °C. Addition of *trisNTA*<sup>PEG12-B</sup>  
983 showed neither clustering nor change in cytosolic calcium. Scale bar: 10 μm. (B) Analysis of  
984 the mean gray value for Ca<sup>2+</sup> signal before (F<sub>0</sub>) and upon (F) addition of *trisNTA*<sup>PEG12-B</sup> versus  
985 time. Time-lapse images were recorded with 45 s interval before and after addition of  
986 *trisNTA*<sup>PEG12-B</sup> (100 nM) (5 slices z-stack per time-point). ROIs covering the complete cell area  
987 were considered. The mean ± SD (10 cells) is shown.



988

989 **Figure 5–figure supplement 2.** Receptor clustering potentiates calcium signaling. (A, B)  
 990 Representative fluorescence images of the Y<sub>2</sub>R (upper panel) and color-coded images of the  
 991 Ca<sup>2+</sup> dye (lower panel). Y<sub>2</sub>R-expressing HeLa cells were allowed to adhere to pre-structured  
 992 SA-matrices for 3 h. Before visualization, cells were incubated with BioTracker 609 Red Ca<sup>2+</sup>  
 993 AM dye (3 μM) for 30 min. After rinsing, cells were visualized by CLSM in LCIS at 37 °C and  
 994 imaged before and after addition of only pNPY or before and after *trisNTA*<sup>PEG12-B</sup> and  
 995 subsequent addition of pNPY. Scale bar: 20 μm. (C, D) Analysis of the mean gray value for  
 996 Ca<sup>2+</sup> signal before (F<sub>0</sub>) and upon (F) addition of *trisNTA*<sup>PEG12-B</sup>/pNPY versus time. Time-lapse  
 997 images were recorded with 45 s interval before and after addition of *trisNTA*<sup>PEG12-B</sup>/pNPY (5  
 998 slices z-stack per time-point). ROIs covering the complete cell area were considered. The  
 999 mean ± SD (10 cells for each condition) is shown.



1000

1001 **Figure 6–figure supplement 1.** Arrestin-3 recruitment on antibody-confined regions. (A)  
1002 Schematic representation of the experimental set-up. Cells co-expressing Y<sub>2</sub>R and Arr3 were  
1003 allowed to adhere to anti-His<sub>6</sub> antibody pre-structured matrices for 3 h and visualized by total  
1004 internal reflection fluorescence (TIRF) microscopy in LCIS at 37 °C. (B) Representative TIRF  
1005 images of cells before and upon addition of pNPY (10 nM) in LCIS for 30 min at 37 °C. Scale  
1006 bar: 10  $\mu\text{m}$ . (C) Quantification of the fluorescence contrast in the Y<sub>2</sub>R-patterned regions  
1007 showed no significant change in receptor intensity yet a recruitment of Arr3 upon addition of  
1008 pNPY (2-fold). Data are expressed as the means  $\pm$  SEM (30 cells for each condition were  
1009 analyzed). Tukey's multiple comparison test was applied (\*\*p $\leq$  0.01).