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

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Abstract

Membrane receptors are central to cell-cell communication. Receptor clustering at the plasma membrane modulates physiological responses, and microscale receptor organization is critical for downstream signaling. Spatially restricted cluster formation of the neuropeptide Y₂ hormone receptor (Y₂R) was observed *in vivo*; however, the relevance of this confinement is not fully understood. Here, we controlled Y₂R clustering *in situ* by a multivalent chelator nanotool in prestructured matrices. Fast Y₂R enrichment in microscale arrays triggered a ligand-independent downstream signaling determined by an increase in cytosolic calcium, cell spreading and migration. We reveal that ligand-independent signaling by confinement differs from ligand-induced activation in the recruitment of arrestin-3 as downstream effector, which was recruited to confined regions only in presence of the ligand. The employed multivalent nanotool facilitated a dynamic receptor enrichment with high exchange in the confined regions, comparable to microscale condensates. This concept enables *in situ* organization of membrane receptors and the exploration of ligand-independent receptor signaling.

Keywords: G protein-coupled receptors / membrane organization / membrane proteins / phase segregation / receptor clustering

Introduction

Cells translate stimuli into biochemical signals through membrane receptors controlling multiple aspects of cell behavior, including migration (Kupperman *et al.*, 2000; Stallaert *et al.*, 2018), differentiation (Li & Rudensky, 2016; Luther & Cyster, 2001), apoptosis (Scott *et al.*, 2009), as well as infectious diseases and cancer (Boncompain *et al.*, 2019; Haqshenas & Doerig, 2019; Kawai & Akira, 2005; Pasquale, 2010; Pike *et al.*, 2018; Sebestyen *et al.*, 2020; Tsukiyama *et al.*, 2020). Receptors form dynamic assemblies, condensates, or clusters that modulate downstream signaling and thus the final physiological response. Upon activation, these receptors undergo transitions from freely diffusing monomers to less mobile nanoclusters and further to higher-order oligomers (Ojosnegros *et al.*, 2017; Su *et al.*, 2016). Recently, liquid-liquid phase separation (LLPS) has been considered as a general mechanism for higher-order receptor cluster formation. In these dynamic, liquid-like condensates driven by weak multivalent interactions, the likelihood of receptor activation increases as neighboring molecules can act together engaging ligands (Case *et al.*, 2019; Lyon *et al.*, 2021). In signal transduction, the mechanisms for receptor cluster formation and cluster behavior have become physiologically relevant topics. However, the role of mesoscale (hundreds of nanometers) receptor organization in signal transduction remains unsolved, mainly because concepts to trigger and analyze receptor clustering *in situ* are largely limited.

Nano- and microlithographic approaches have provided cell-compatible scaffolds to investigate confined ligand-receptor interactions (Manz & Groves, 2010; Nair *et al.*, 2011; Torres *et al.*, 2008). Various techniques, ranging from photolithography to polymer lift-off and microcontact printing (μ CP), have yielded information on how topology and mobility of the stimulus regulate cell responses (Bernard *et al.*, 2000; Doh & Irvine, 2006; Mossman *et al.*, 2005; Sánchez *et al.*, 2018; Traub *et al.*, 2016). So far, these methods utilize receptor ligands or antibodies to induce a rather static cluster formation. However, fast dynamic signaling at the plasma membrane requires tools that enable receptor clustering in a fast, specific, and *in situ* intervention of receptor networks.

Heterotrimeric guanine nucleotide-binding protein (G protein)-coupled receptors (GPCR) are key cell surface proteins that regulate a plethora of cellular responses to external stimuli (Rosenbaum *et al.*, 2009; Venkatakrishnan *et al.*, 2013; Wootten *et al.*, 2018). The Y₂ receptor (Y₂R) is one of the four human neuropeptide Y (NPY) receptor subtypes, which belong to the rhodopsin-like (class A) GPCR superfamily (Parker & Balasubramaniam, 2008; Tang *et al.*, 2021). Y₂R is linked to many important physiological processes, such as fear extinction (Méndez-Couz *et al.*, 2021), regulation of food intake (Huang *et al.*, 2014), and obesity (Lafferty *et al.*, 2021). Y₂R activation by neuropeptide Y (NPY) has been shown to promote cell migration and proliferation (Ekstrand *et al.*, 2003; Movafagh *et al.*, 2006). Recently, we demonstrated that Y₂Rs respond to light-guided microscale clustering at spatially defined locations (Sánchez *et al.*, 2021). Y₂Rs are activated independently of the presence of the canonical ligand, evoking an increase in cytosolic calcium, a change in cell spreading behavior, and a localized migratory pattern.

Here, we established a versatile concept for *in situ* receptor clustering using a multivalent chelator nanotool (*tris* N-nitrilotriacetic acid, *tris*NTA), which displays high affinity for histidine (His)-tagged proteins. The small size of the chelator tool in combination with a prestructured platform enabled high-content receptor clustering with high spatiotemporal resolution. The lateral organization of Y₂Rs in living cells was controlled within minutes in a non-invasive (“traceless”) and ligand-free manner. Microscale receptor condensates with a high degree of homogeneity in size and density were generated in the plasma membrane. Receptor clustering triggered ligand-independent activation of signal transduction, as evidenced by an increase in cytosolic calcium and cell motility. By evaluating arrestin-3 (Arr3) recruitment as a downstream signaling event, we uncovered high Arr3 co-recruitment to the patterned areas only in the presence of the canonical ligand, suggesting an Arr3-independent desensitization mechanism for the ligand-independent response. Strikingly, analysis of the receptor mobility revealed a dynamic receptor assembly with high exchange within the confined areas, showing similarities to liquid-like condensates. The versatility of this generic approach may advance studies of ligand-free receptor signaling by dynamic confinement.

Results

High-content receptor clustering in living cells

We developed a system to induce dynamic receptor assembly *in situ* based on a multivalent chelator *tris*NTA nanotool (Fig 1A), which is equipped with a biotin moiety (*tris*NTA^{PEG12-B}) (Fig 1B). *tris*NTA^{PEG12-B} displays a high affinity for His₆-tagged proteins ($K_D \approx 10$ nM), resulting in a site-specific but reversible interaction (Gatterdam *et al.*, 2018). Microcontact printing is a widely used method to investigate protein-protein interactions in living cells (Bernard *et al.*, 2000; Torres *et al.*, 2008). However, patterned substrates with a generic structure allowing high-content analyses of cells are difficult to produce. We used a large-area perfluoropolyether (PFPE) elastomeric stamps inked with bovine serum albumin (BSA) to print 96-well size glass (Hager *et al.*, 2021; Lanzerstorfer *et al.*, 2020). Wells within these plates containing a BSA-structured matrix were functionalized with biotinylated BSA (biotin-BSA) and streptavidin (SA) (Fig 1A). Subsequent functionalization with the nanotool and His₆-tagged fluorescent proteins, resulted in well-resolved protein patterns that were analyzed by confocal laser scanning microscopy (CLSM). This confirmed the specificity of the nickel-loaded *tris*NTA chelator to capture His₆-tagged proteins in defined regions of 1 μ m or 3 μ m diameter (Fig 1C, D).

The ability to control the organization of membrane receptors in real time is important for dissecting the spatial complexity of cell signaling and the extracellular environment. To this end, we utilized a tetracycline-inducible (T-Rex) expression system to establish a monoclonal human cervical cancer HeLa cell line expressing low amounts of the Y₂R (~300,000 receptors/cell) (Sánchez *et al.*, 2021). Y₂Rs displayed an N-terminal His₆-tag to the extracellular space and a cytosolic, C-terminal monomeric Enhanced Green Fluorescent Protein (mEGFP) (His₆-Y₂R^{mEGFP}, in brief Y₂R). It is worth mentioning that these modifications do not affect receptor activity, selectivity, or ligand binding as demonstrated (Sánchez *et al.*, 2021). In addition, studies determined that Y₂R does not need the N-terminus for ligand binding (Lindner *et al.*, 2009). Y₂R-positive cells properly adhered to 1 μ m and 3 μ m SA-functionalized matrices and showed a homogeneous receptor distribution at the basal plasma

membrane (Fig 1E). Addition of *trisNTA*^{PEG12-B} (100 nM final) triggered receptor assembly. Within five minutes, all cells showed receptor patterns at the plasma membrane comparable in size and density (Fig 1E, F, Fig EV1). Importantly, recruitment of soluble His₆-tagged GFP proteins as well as Y₂Rs to 1 μm prestructured spots led to analogous intensity profiles, reflecting that similar densities were obtained in both cases (Fig 1D, G). In contrast, cells expressing Y₂Rs lacking the His₆-tag (Y₂R^{mEGFP}) showed no receptor clustering after addition of *trisNTA*^{PEG12-B} (Fig EV2), demonstrating the specificity of the His₆-tag/*trisNTA* interaction in living cells. Remarkably, ten minutes after receptor clustering by the multivalent nanotool, the Y₂R enrichment resulted in an integrated receptor density equivalent to that of cells cultured on matrices functionalized with anti-His₆ antibodies. However, a tenfold higher antibody concentration (1 μM) was required compared to the multivalent nanotool, demonstrating its efficacy in capturing His₆-tagged membrane receptors (Fig EV3). The 3 μm diameter nanotool-induced receptor clusters presented a ninefold increase in integrated density compared to 1 μm arrays, consistent with the increase in pattern area (Fig EV3). Overall, our approach enabled versatile *in situ* receptor organization with high specificity.

Ligand-independent receptor clustering triggers fast signaling

To follow receptor clustering *in situ*, Y₂R-expressing cells were cultured on microstructured matrices with a diameter of 1 μm, the smallest pattern we can produce and analyze with high accuracy. After addition of the multivalent nanotool, the receptor redistribution was tracked by confocal microscopy. Receptor clustering occurred in the first minutes and increased steadily within 10 min, resulting in a twofold increase in receptor density compared to the initial state (Fig 2A, Movie 1). Considering the average cell area of $1,420 \pm 50 \mu\text{m}^2$ (n = 66 cells) and the enrichment factor (twofold), we estimated a receptor density of ~400 receptors/μm² in the patterned regions (~300 receptors per 1 μm spot), a value comparable to other receptor studies utilizing Fluorescence Correlation Spectroscopy methods (Bag *et al.*, 2015; Chen *et al.*, 2009). The kinetic profile for Y₂R recruitment to the 1 μm spots followed a pseudo-first-order assembly rate of $0.20 \pm 0.03 \text{ min}^{-1}$ (Fig 2A, C). Despite of the high affinity and kinetically

stable binding ($k_{\text{off}} = 0.18 \text{ h}^{-1}$) (Gatterdam *et al.*, 2018), the His-tag/*tris*NTA system relies on molecular multivalency, which enables competition of the binding sites with histidine, thus making the process of receptor assembly reversible. Addition of histidine to patterned cells led to the disassembly of the receptor clusters in a rapid and complete manner, demonstrating a key advantage of the approach to investigate receptor dynamics (Fig 2B).

Upon receptor clustering by the multivalent interaction nanotool, we observed a fast change in cell spreading and motility. Analysis of cell spreading revealed a 20% increase in the total cell area concomitant to receptor assembly (Fig 2A, D, Fig 3A), indicating ligand-independent receptor activation upon clustering. Cells cultured on matrices in the absence of SA as well as cells expressing Y_2R^{mEGFP} (lacking a His₆-tag) on SA-matrices showed no significant change in cell spreading upon addition of the *tris*NTA^{PEG12-B} (Fig EV4). As calcium signals are widely known to regulate cell motility, we monitored local calcium dynamics utilizing a far-red cell-permeable Ca^{2+} -sensitive dye. By dual-color imaging, receptor assembly and the cytosolic calcium concentration were simultaneously recorded in living cells over the matrices. Upon addition of *tris*NTA^{PEG12-B}, receptor recruitment led to a twofold increase in cytosolic calcium concentration with a rapid rise within two minutes (Fig 3B, C), contrary to control matrices without SA (Fig EV5). This indicates that calcium signals are associated with the motility change. To correlate ligand-free *versus* canonical receptor activation, the agonist porcine neuropeptide Y (pNPY, $K_D = 5.2 \pm 2.0 \text{ nM}$) was added to pre-patterned cells. An increase in cytosolic Ca^{2+} was detected, which reached a plateau after one minute, followed by a slow decay of the signal over a period of four minutes (Fig 3B, C). Strikingly, more receptors were recruited to the patterned areas after activation via the agonist, as reflected by the increase in integrated density in the patterned regions (Fig 3D). These results indicate that ligand-activated Y_2Rs diffuse into areas where increased receptor density is present and preferentially localized there. Recently, specific clusters termed GPCR hot spots (40-300 nm) were visualized at the plasma membrane of living cells (Calebiro & Jobin, 2019; Chavez-Abiega *et al.*, 2019; Hilger *et al.*, 2018; Sungkaworn *et al.*, 2017). These hot spots represent regions where signaling preferentially occurs and where both receptors and G proteins are

enriched. We hypothesize that the induced microscale clusters may also trigger the formation of hot spots providing the ideal environment for recruitment of more active receptors and thus amplification of the signal. By increasing the local effective receptor concentration, this complex organization may increase both the speed and efficiency of receptor-G protein coupling while enabling local signaling transduction.

Downstream signaling by ligand-free receptor clustering

We next explored the impact of receptor clustering on downstream signaling. GPCR desensitization involves a complex series of events, e.g. receptor phosphorylation, arrestin-mediated internalization, receptor recycling, and lysosomal degradation (Ziffert et al, 2020). Short-term desensitization occurs within minutes and is primarily associated with arrestin preventing G protein interaction with the GPCR. Arrestins bind to activated, phosphorylated GPCRs and block receptor-G protein interaction by steric hindrance at the receptor-coupling interface, while serving as adaptors for key components of the endocytic machinery and numerous signaling proteins (Hilger et al, 2018, Wang et al, 2020). In the presence of high concentrations of the canonical ligand, an Arr3-dependent internalization, subsequent endosomal sorting, and recycling of Y₂R to the cell membrane were observed (Walther *et al.*, 2010; Wanka *et al.*, 2018). However, recent studies demonstrated a strong and persistent activation of the G_{ai}-pathway upon Y₂R activation, which depletes the intracellular G protein repertoire before Arr3 binding can terminate signaling (Ziffert et al, 2020). To assess whether ligand-free clustering leads to Arr3 recruitment, we transfected cells stably expressing the Y₂R with Arr3^{mCherry} (in brief Arr3) and monitored Arr3 recruitment in real-time by total internal reflection fluorescence (TIRF) microscopy (Fig 4A). Upon microscale receptor confinement by *tris*NTA^{PEG12-B}, we did not observe a significant increase in Arr3 recruitment by intensity-contrast analysis of the patterned spots, whereas a significant Arr3 recruitment was detected upon addition of the agonist pNPY (Fig 4B, C). Again, an increase in Y₂R density in the clustered areas was detected upon addition of pNPY (Fig 4B, D). In a subsequent step, a partial dissipation of the Y₂R/Arr3 assemblies by specific competition with histidine was

observed (Fig 4B, C). Interestingly, half of the intensity in the patterned regions was dissipated in the presence of the pNPY, indicating that not all receptors within the cluster areas are associated with the nanotool upon addition of the ligand. In addition, patterning of Arr3 was also detected in cells on an anti-His₆ antibody matrix within the first minutes after addition of pNPY (Fig EV6). In this case, we did not observe a significant change in receptor density upon addition of the pNPY, indicating that the high degree of immobilization and large size of the antibody might restrict the transient enrichment of active receptors into the clustered regions. Overall, our results reflect a difference between Arr3 recruitment in the ligand-free mode compared to ligand-activated state. These observations indirectly confirm a high-affinity interaction between the Y₂R and Gα_i (Ziffert et al, 2020) and suggest an active recruitment of G proteins that delay Arr3 recruitment and impair termination of G protein signaling.

Receptor diffusion and dynamic exchange in confined regions

GPCR signaling results from dynamic interactions among receptors, G proteins, and the complex surrounding membrane environment, which confers flexibility and versatility on this fundamental biological process. GPCR hot spots are highly heterogeneous in terms of diffusion of receptors and G proteins, which rapidly switch among phases of rapid and slow diffusion (Calebiro & Jobin, 2019; Sungkaworn *et al.*, 2017). First, we examined whether Y₂R clustering affects lipid diffusion and distribution by labeling the membrane with the lipid-like dye CellMask. We observed a homogeneous staining of the plasma membrane, demonstrating that receptor confinement does not affect membrane organization (Fig EV7). To determine diffusion coefficients (*D*), we performed fluorescence recovery after photobleaching (FRAP) technique. *In situ* receptor clustering was triggered on Y₂R-expressing cells cultured on SA-matrices by addition of *tris*NTA^{PEG12-B} (100 nM, 10 min). In a subsequent step, the membrane was labeled and square-shaped regions of interest (ROIs) covering four 1 μm-sized spots were photobleached. Fluorescence recovery was analyzed by a FRAP simulation approach that enabled calculation of diffusion coefficients independent of bleaching geometry (Blumenthal *et al.*, 2015). The diffusion coefficient of lipids obtained by FRAP

showed an average value of $D_{\text{lipid}} = 0.66 \pm 0.10 \mu\text{m}^2/\text{s}$ in agreement with literature values for free Brownian lipid diffusion at the plasma membrane (Schwille *et al.*, 1999; Wawrezinieck *et al.*, 2005).

To evaluate the Y₂R mobility in the ligand-free induced clusters, we determined the receptor lateral diffusion of Y₂R-expressing cells cultured on SA-matrices before and after addition of trisNTA^{PEG12-B} (100 nM, 10 min). A square-shaped ROI covering four 1 μm -sized spots and recovery was photobleached. A significant decrease in the lateral diffusion of the Y₂R was observed at the basal membrane of cells after receptor confinement by trisNTA^{PEG12-B} ($D_{\text{before}} = 0.25 \pm 0.08 \mu\text{m}^2/\text{s}$ versus $D_{\text{after}} = 0.10 \pm 0.03 \mu\text{m}^2/\text{s}$) (Fig 5A, E). Surprisingly, the receptor density showed a high recovery within ~3 min after photobleaching (Fig 5A, Fig EV8, Movie 2). Notably, no significant difference in receptor mobile fraction (M_f) before and after addition of the nanotool was observed ($M_f = 0.80 \pm 0.04$) (Fig 5B). Importantly, FRAP analyses of cells cultured on matrices functionalized with anti-His₆ antibodies presented a significant decrease in the receptor diffusion as well as in the M_f at the clustered spots ($M_{f,\text{anti-His6 Ab}} = 0.56 \pm 0.08$) (Fig 5B, Fig EV9, Movie 3). In contrast to monovalent antibody immobilization, receptor clustering by the multivalent interaction nanotool is dynamic, allowing fast exchange of His₆-tagged receptors or competition by histidine. We rationalized that free receptors can diffuse into the clustered spots and exchange with photobleached receptors at multivalent binding sites, leading to a dynamic confinement. Our results point out that a high receptor exchange is possible even in micrometer-sized clusters, an effect that likely depends on cluster size, with larger clusters showing less recovery (Sánchez *et al.*, 2021).

Finally, we investigated the lateral receptor mobility with a higher spatiotemporal resolution using imaging fluorescence correlation spectroscopy (imFCS). FCS is used to study the diffusion of membrane proteins in living cells with single-molecule sensitivity (Fig 5C). These multiplexed FCS measurements are realized by analyzing many pixels simultaneously using a widefield setup (Harwardt *et al.*, 2018, Kannan *et al.*, 2006). Regions of interests (ROIs) on Y₂R-expressing cells cultured on SA-matrices were analyzed before and after addition of trisNTA^{PEG12-B}. Enrichment of Y₂R at the basal membrane was observed with total internal

reflection fluorescence (TIRF) microscopy (Fig 5D). Consistent with the FRAP measurements, a decrease in the Y₂R diffusion coefficient was determined upon cluster formation ($D_{\text{before}} = 0.32 \pm 0.06 \mu\text{m}^2/\text{s}$ and $D_{\text{after}} = 0.16 \pm 0.05 \mu\text{m}^2/\text{s}$). The receptor diffusion coefficient measured before clustering is comparable to similar membrane proteins (Lippincott-Schwartz et al, 2001), demonstrating that the microstructured confinement does not affect the mobile fraction of receptors. In contrast to FRAP, imFCS provides a two-dimensional diffusion map, which enables the determination of local differences in the lateral diffusion coefficient of membrane receptors with high precision. Quantitative analysis of the 1 μm cluster spots in the acquired ROIs resulted in a lateral diffusion coefficient of $D_{\text{spots}} = 0.14 \pm 0.03 \mu\text{m}^2/\text{s}$ (Fig 5E). Taking into consideration that imFCS detects only mobile particles, we determined a similar decrease in lateral diffusion in the patterned regions (Fig EV9) for cells cultured on matrices functionalized with anti-His₆ antibodies. Taken together, we unravel that in microscale clusters, associations between His₆-tagged Y₂Rs and multivalent *tris*NTA^{PEG12-B} resulted in decreased diffusion rate but dynamic receptor exchange with unchanged mobile fraction, which is similar to the behavior described upon ligand-activated receptor clustering. This plasticity of the confined receptors likely has a relevant impact on the efficiency of hot spot formation and GPCR signaling.

Discussion

We developed a versatile concept to cluster receptors *in situ* with minimal steric hindrance and disturbance. Ligand-independent receptor activation by confinement was unraveled by cytosolic calcium increase and changes in cell spreading and motility. Addition of the canonical ligand led to a similar calcium increase and interestingly, an amplification of the signal was observed in the clustered areas concomitant with an increased receptor density. Surprisingly, we revealed a difference in triggering of downstream signaling for the ligand-free *versus* ligand-activated receptors as evidenced by co-recruitment of Arr3 to the clustered spots only occurring in the presence of the canonical ligand. These findings are consistent with previous results demonstrating an Arr3-dependent internalization, subsequent endosomal sorting, and

receptor recycling to the cell membrane in the presence of high concentrations of NPY (Walther *et al.*, 2010; Wanka *et al.*, 2018). We hypothesize that high-affinity $Y_2R/G\alpha_i$ interactions drive the initial cell response, cytosolic calcium increase, and cell motility. High local receptor density in the spots increases the residence time of proximate $G\alpha_i$ proteins and recruits further downstream effectors, which boost the probability of activation (Sánchez *et al.*, 2021). Further, $Y_2R/G\alpha_i$ interactions lead to persistent activation of the $G\alpha_i$ pathway, which depletes the intracellular $G\alpha_i$ protein repertoire before Arr3 binding can terminate signaling (Ziffert *et al.*, 2020). The time frame of imaging after addition of the nanotool (30 min) suggests a long-lasting $G\alpha_i$ protein activation and favors the hypothesis of a mechanism for Y_2R activation and desensitization that is limited to the cell membrane and partially independent of Arr3 recruitment (Ziffert *et al.*, 2020).

Many transmembrane receptors form dynamic assemblies that promote downstream signal transduction in cells. Recently, dynamic liquid-like microscale clusters were identified as critical modulators of cell signaling (Case *et al.*, 2019; Lyon *et al.*, 2021). In our system, the transient association between the nanotool and the receptors revealed the generation of a dynamic platform for cell signaling, which may have characteristics akin liquid-like clusters generated by LLPS. The dynamic exchange of molecules within induced Y_2R microscale clusters may contribute to hot spots formation and final downstream signaling. This characteristic together with the lower concentration required compared to established systems with immobilized ligands or antibodies, highlight the relevance of this versatile approach along with the developed nanotool. Multiscale analyses of the multivalent interactions between receptor condensates, G proteins, the lipid environment and actin–myosin assemblies are critical to confirm the cluster behavior and dynamics. *In vitro* reconstitution systems utilizing lipid bilayers have proven phase-segregation in two dimensions (Banjade & Rosen, 2014; Ditlev *et al.*, 2019). The comparison of a mobile system based on model membranes and our established platform, together with advanced quantitative fluorescence microscopy techniques such as fluorescence resonance energy transfer (FRET) and single-molecule localization microscopy will help us to decode condensates behavior and ligand-independent

311 signaling. In summary, the developed tool and matrices allow the investigation of ligand-
312 independent receptor activation *in situ* facilitating the investigation of early key processes in
313 cell signaling.

Materials and Methods

Synthesis of *trisNTA*^{PEG12-B}: Cyclam-Lys-*trisNTA* (Gatterdam *et al.*, 2018) (5.0 mg, 4.8 μ mol), Biotin-PEG₁₂-NHS (23.0 mg, 24.0 μ mol) and DIPEA (12.2 μ L, 72.0 μ mol) were dissolved in 0.5 ml dry DMF and stirred for 2 h at RT. After reaction, the volatile components were removed by lyophilization. Raw product was purified by semi-preparative RP-HPLC (mobile phase A: H₂O + 0.1% TFA, B: CAN + 0.1% TFA; gradient 5% to 80% B in 20 min; MZ-PerfectSil, 300 ODS, 5 μ m, 250 x 10 mm, flow 4 ml/min). A biotin moiety was integrated into the nanotool for immobilization to SA in the prestructured matrices. The PEG₁₂ linker between the biotin and the *trisNTA* unit increased the flexibility of the molecule. *trisNTA*^{PEG12-B} was confirmed by liquid chromatography-mass spectrometry (LC-MS, Waters BioAccord System). Dataset was recorded with an ACQUITY UPLC I-Class Plus chromatography system and ACQUITY RDa Detector, which was set to a cone voltage of 25 V, capillary voltage of 1.2 kV and a desolvation temperature of 500 °C operating in positive ionization mode. For reverse-phase separation, an ACQUITY UPLC Peptide BEH C18 column (300 Å, 1.7 μ m, 2.1 mm x 100 mm) was used (Fig EV10). The *trisNTA* chelator compounds were dissolved in HBS buffer (20 mM HEPES-NaOH pH 7.5, 150 mM NaCl) and incubated with tenfold excess of NiCl₂. After 30 min incubation at 4 °C, the excess of Ni(II) was separated by ultra-centrifugal filters (Amicon Ultra-0.5 ml; 3 kDa and 10 kDa MWCO).

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

at 4 °C. The next day, the stamp was stripped from the glass with a forceps, and the microstructured glass was bonded to a 96-well plastic casting using an adhesive tape (3M) and closed with an appropriate lid.

Functionalization of the prestructured matrices. BSA-prestructured wells were incubated with biotin-BSA (0.1 mg/ml, Thermo Fisher Scientific) and SA (1 μM, Sigma-Aldrich) in PBS, each for 1 h at RT. Incubated wells were washed thoroughly with PBS after each step, to remove unbound biotin-BSA and SA. For binding of soluble His-tagged proteins, wells were incubated with *tris*NTA^{PEG12-B} (0.5 μM) in HBS buffer for 1 h at RT. For nickel-loading, the prestructured matrices were sequentially incubated with imidazole (1 M, 2 min), EDTA (100 mM, 2 min), and NiCl₂ (10 mM, 5 min). Wells were carefully washed after each step. Finally, HBS buffer containing EDTA (50 μM) was used to remove the free, non-complexed nickel ions. His₆-GFP (100 nM) previously expressed and purified was added to the wells and incubated for 30 min at RT. Experiments were performed in biological replicas (N=5).

Cell culture. HeLa Flp-InTM T-RexTM Y₂R cells (His₆-Y₂R^{mEGFP} or Y₂R^{mEGFP}) were generated as previously described (Sánchez *et al.*, 2021) and cultured at 37 °C, 5% CO₂, and 95% humidity. For culturing the stable cell line, high glucose Dulbecco's modified Eagle's medium (DMEM) (Gibco/Thermo Fisher Scientific) was supplemented with 10% tetracycline-free fetal calf serum (FCS, Bio&Sell), blasticidin S HCl (1 μg/ml, Thermo Fisher Scientific) and hygromycin B (50 μg/ml, Thermo Fisher Scientific). To induce receptor expression, the cell medium was replaced with fresh medium containing tetracycline (0.1 μg/ml, Fluka) 18 h before imaging. The same concentration of tetracycline resulting in an efficient plasma membrane targeting was used for all the experiments. The cells were regularly tested for mycoplasma contamination.

Receptor confinement in *real-time* by *trisNTA*^{PEG12-B}. Cells expressing Y₂R (His₆-Y₂R^{mEGFP} or Y₂R^{mEGFP}) were trypsinized and allowed to adhere to SA prestructured matrices for 3 h or overnight. 15-18 h prior to the experiment, the cell medium was replaced with fresh medium containing tetracycline (0.1 µg/ml) to induce receptor expression. The cells were visualized by CLSM in live-cell imaging solution (LCIS, Thermo Fisher Scientific) at 37 °C. Cells were subsequently incubated with nickel-loaded *trisNTA*^{PEG12-B} (100 nM) in LCIS for 10-15 min at 37 °C. Excess of unbound *trisNTA*^{PEG12-B} was removed by washing with LCIS. For reversibility experiments, micropatterned cells were incubated with histidine (5 mM) in LCIS for 2 to 10 min followed by washing with LCIS. Experiments were performed in biological replicas (N=4).

Receptor confinement on antibody-micropatterned matrices. Wells prestructured with BSA were subsequently incubated with biotin-BSA (0.1 mg/ml), SA (1 µM), and a biotinylated anti-His₆ antibody (1 µM) (ab106261, Abcam) in PBS for 1 h at RT. Wells were washed thoroughly with PBS to remove unbound antibody. Cells expressing Y₂R were trypsinized and seeded onto the antibody patterns. After 3 h, cells were visualized by CLSM in LCIS at 37 °C. Experiments were performed in biological replicas (N=5).

Time-lapse calcium imaging. 18 h after seeding the cells onto prestructured SA-matrices, cells were incubated with BioTracker 609 Red Ca²⁺ AM dye (3 µM, Merck Millipore) in fresh medium for 30 min. The cell-membrane permeable dye is de-esterified by cellular esterases and remains trapped in the cytosol. After incubation with the Ca²⁺ dye, cells were rinsed three times with PBS and imaged by CLSM in LCIS at 37 °C. For investigation of Ca²⁺ signal, time-lapse images were taken (5 slices z-stacks, 45-s interval) before and after addition of *trisNTA*^{PEG12-B}. Fluorescence intensity (($\lambda_{ex/em}$ 590/609 nm) of the dye changes depending on the intracellular Ca²⁺ concentration. Maximum intensity projections of single channels were analyzed. The ImageJ ROI tool was used to define the areas of the image to be analyzed. We consider a ROI covering the complete cell contour. Mean gray value (F) were background

subtracted and normalized to the fluorescence in cells before F_0 . Experiments were performed in biological replicas (N=3).

Plasma membrane staining. Live-cell membrane staining was performed directly after receptor assembly in living cells grown on prestructured matrices. CellMask™ deep red plasma membrane stain (Thermo Fisher Scientific) was used according to manufacturer's instruction. 1 µl of the stock solution (1000x dilution) was dissolved in 1 ml of warm LCIS (final concentration 5 µg/ml) and subsequently added to the cells, incubated for 5 min at 37 °C, and washed with LCIS before visualization. Experiments were performed in biological replicas (N=3).

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

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

Fluorescence recovery after photobleaching. FRAP experiments were conducted at the CLSM Zeiss LSM 880 using 63 x/1.4 Oil DIC objective. Rectangular-shaped regions (6-10 μm radius) were bleached within 10 s with high laser intensities. Fluorescence recovery was monitored by repetitively imaging an area containing the photobleached region at 0.1 frame/s for ~ 150 s. For the analysis, a simulation approach that allows computation of diffusion coefficients regardless of bleaching geometry used in the FRAP series was applied (Blumenthal *et al.*, 2015). The method is based on fitting a computer-simulated recovery to actual recovery data of a FRAP series. The algorithm accepts a multiple-frame TIFF file, representing the experiment as input, and simulates the (pure) diffusion of the fluorescent probes (2D random walk) starting with the first post-bleach frame of the actual data. Once the simulated recovery is finished, the algorithm fits the simulated data to the real one and extracts the diffusion coefficient. The algorithm iteratively creates a series of simulated images, where each frame corresponds to a single iteration. The intensity values are extracted from the (user indicated) bleached area of the simulated frames, thus determining the general shape of the recovery curve. The “time” axis at this stage is in arbitrary units (iterations). To extract the diffusion coefficient, the simulated recovery curve needs to be fitted to the real recovery curve, by appropriately stretching the “time” axis. The time between frames in the actual data set is obviously known, thus once overlapping optimally the simulated curve with the real one, the duration of one iteration, in *real-time* units, is determined. The diffusion coefficient of the simulated series is then calculated according to eq. 1, where D_s is the simulation-extracted diffusion coefficient, l is the step of a molecule in each

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

iteration of the simulation, corresponding to one pixel in the image (the pixel size is calibrated previously, by imaging a known calibration sample), and t_i is the time interval between steps (determined as explained). Technically, the simulation proceeds until a plateau is reached (equilibration of the fluorescence intensity in the bleached area). The number of data points in the simulated recovery is typically different (larger) than the number of experimental points. In

addition, the real experimental data may not have been acquired until equilibration of fluorescence. To determine t_i , the algorithm scans a range of possible values for the total duration represented by the simulation and calculates a value X^2 for the goodness-of-fit between the simulated data and the real FRAP data. Total simulation duration is selected as the one that produces the minimal X^2 . Experiments were performed in biological replicas (N=3).

imFCS measurements. imFCS measurements were performed as described earlier (Harwardt *et al.*, 2018; Harwardt *et al.*, 2017). A home-built widefield setup with total internal reflection fluorescence (TIRF) illumination was used for imFCS analysis. The experimental setup was equipped with a 488 nm diode laser (100 mW, Obis, Coherent, USA). The excitation light passes through an acousto-optical tunable filter (AA Opto-Electronic, Orsay, France) and a telescope consisting of two achromatic lenses (Thorlabs, USA) with $f = -40$ mm and 750 mm. A third achromatic lens ($f = 400$ mm, Thorlabs) directed the excitation light to the TIRF mirror and had its focus on the back focal plane of the objective. The TIRF mirror was placed on a motorized translation stage (25 mm, #MTS25/M-Z8, Thorlabs) controlled by a motion controller (K-Cube Brushed DC Servo Motor Controller, # KDC101, Thorlabs) to switch between widefield and TIRF illumination. The light entered an Eclipse Ti microscope (Nikon, Japan), was reflected by a dichroic mirror (TIRF-Quad filter set 405/488/561/640 consisting of a QuadLine Laser Clean-up ZET405/488/561/640x, QuadLine dichroic zt405/488/561/640rpc, QuadLine rejection band ZET405/488/561/640 TIRF, all AHF Analysentechnik AG, Tübingen, Germany), and was directed by an oil-immersion TIRF objective (UapoN 100xOTIRF, 1.49 Oil, Olympus, Japan) onto the sample. A nosepiece stage (IX2-NPS, Olympus) was used for z plane adjustment and drift minimization. Emission light was collected by the same objective and passed the dichroic mirror. In the detection path a TwinCam (Acal Bfi, Germany) with a BrightLine HC 525/45 bandpass filter (AHF Analysentechnik AG) was implemented and the signal was detected by a scientific complementary metal-oxide semiconductor (sCMOS) camera (Zyla 4.2, Andor, Belfast, UK). Data were collected using the open-source software

μ Manager (Edelstein *et al.*, 2010). For data acquisition the following settings were applied: 24 W/cm² laser intensity, a bit depth of 16 bit, pixel readout rate of 540 MHz, frame time 4 ms, 4x4 binning, and 4,000 frames per film. For each film, a 40x25 pixel (or 40x20 pixel) region of interest (ROI) was chosen and the measurement was performed with TIRF illumination to observe membrane diffusion of Y₂R. In total 36 untreated cells, 24 cells with *trisNTA*^{PEG12-B} immobilized receptors, and 26 cells with anti-His₆ antibody immobilized receptors were measured. Each condition contains data from at least three independent measurement days (N=3).

imFCS data analysis. Analysis of imFCS films was performed using the imFCS plugin (version 1.52) (Sankaran *et al.*, 2010) for Fiji (Schindelin *et al.*, 2012). The following correlation settings were chosen: emission wavelength = 515 nm, NA = 1.49, correlator scheme P = 16 and Q = 8, lateral PSF = 0.8, binning = 1, pixel size = 5.75 μ m, magnification = 25 for 4x4 binning, and linear segment bleach correction with linear segments of 500 frames. Diffusion coefficients were obtained for each pixel by fitting the correlation curves according to the literature (Sankaran *et al.*, 2010). To compare the overall diffusion coefficients with those of the patterned regions, ROIs were placed around patterned regions and analyzed separately. For further analysis, the pixelwise diffusion coefficients for all measurements were imported into OriginPro 2019 (OriginLab Corporation, Northampton, USA). For box plots of diffusion coefficients, median diffusion coefficients were determined for each cell. Mean diffusion coefficients per condition were obtained by averaging over the median diffusion coefficients per measurement and calculating the standard error of the mean. Two-sample t-tests (α = 0.05) were applied to compare the diffusion coefficients for the different conditions. All datasets were tested for normality using the Kolmogorov-Smirnov test (α = 0.05). Significance was assigned as follows: $p > 0.05$ no significant difference between populations (n.s.), $p < 0.05$ significant difference (*), $p < 0.01$ significant difference (**), and $p < 0.001$ significant difference (***). Two-dimensional maps of diffusion coefficients were generated also in OriginPro. Diffusion coefficients were color coded from light yellow to dark red in the range of

0 to 0.5 $\mu\text{m}^2/\text{s}$. Pixels that yielded correlation curves with diffusion coefficients higher than 0.5 $\mu\text{m}^2/\text{s}$ are presented in black. Pixels that yielded correlation curves which could not be fitted by the imFCS plugin in Fiji are shown in light grey. To generate frequency distribution plots, diffusion coefficients were log-transformed and binned in the interval between -5.3 and 1.0 with a bin size of 0.1 for each cell. Logarithmic diffusion coefficients were re-transformed, frequency counts were averaged over all cells per condition and normalized. Frequency counts were plotted logarithmically against diffusion coefficients. Errors bars represent standard errors of the mean.

Arr3 recruitment upon receptor confinement. Microstructured surfaces were functionalized with biotin-BSA and SA or SA and anti-His₆ antibody as described before. For transient co-transfection with Arr3^{mCherry}, cells were sub-cultured the day before and then transfected with the Arr3^{mCherry} plasmid using the TurboFectTM transfection reagent (Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturer's instructions and induced with Tetracycline (0.1 $\mu\text{g}/\text{ml}$) 18 h before microscopy. Cells co-expressing His₆-Y₂R^{mGFP} and Arr3 were seeded onto the microstructured matrices and visualized by total internal reflection fluorescence (TIRF) microscopy in LCIS at 37 °C after 3 to 4 h to ensure a homogeneous cell membrane adhesion, which is a prerequisite for quantitative TIRF microscopy. For antibody experiments, cells grown on prestructured matrices were incubated with pNPY (10 nM, Tocris) in LCIS for 30 min at 37 °C. For *trisNTA*^{PEG12-B} experiments, cells grown on SA-matrices were subsequently incubated with nickel-loaded *trisNTA*^{PEG12-B} (100 nM) and pNPY (10 nM) in LCIS for 30 min at 37 °C. For reversibility, cells were incubated with histidine (5 mM) in LCIS for 30 min. Experiments were performed in biological replicas (N=2).

Arr3 imaging by TIRF microscopy. The detection system was set up on an epi-fluorescence microscope (Nikon Eclipse Ti2). For selective fluorescence excitation of mGFP and mCherry, a multi-laser engine (Toptica Photonics, Munich, Germany) was used at 488 and 561 nm, respectively. The samples were illuminated in total internal reflection (TIR) configuration

(Nikon Ti-LAPP) using a 60x oil immersion objective (NA = 1.49, APON 60XO TIRF). After appropriate filtering using standard filter sets, the fluorescence was imaged onto a sCMOS camera (Zyla 4.2, Andor, Northern Ireland). The samples were mounted on an x-y-stage (CMR-STG-MHIX2-motorized table, Märzhäuser, Germany), and scanning was supported by a laser-guided automated Perfect Focus System (Nikon PFS).

Contrast Quantification and Statistical Analysis. Contrast analysis was performed as described previously (Lanzerstorfer *et al.*, 2014; Lanzerstorfer *et al.*, 2020; Schütz *et al.*, 2017). Initial imaging recording was supported by the Nikon NIS Elements software. Images were exported as TIFF frames and fluorescence contrast analysis was performed using the Spotty framework (Borgmann *et al.*, 2012). The fluorescence contrast $\langle c \rangle$ was calculated as $\langle c \rangle = (F^+ - F^-)/(F^+ - F_{bg})$, where F^+ denotes the intensity of the inner pixels of the pattern. F^- shows the intensity of the surrounding pixels of the micropattern, and F_{bg} the intensity of the global background. Data are expressed as the means $\pm$ SEM. Comparisons of more than two different groups were performed using one-way ANOVA, which was followed by Tukey's multiple comparisons test in GraphPad Prism software (version 9.1.2).

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Author contributions

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

Competing interest. The authors declare no competing interest.

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

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Figures

Figure 1. High-content ligand-free receptor confinement.

A Rational of the high-content ligand-free receptor clustering. Matrices prestructured with BSA are stepwise functionalized with biotin-BSA and SA. Upon addition of the multivalent nanotool trisNTA^{PEG12-B}, His₆-tagged receptors in HeLa cells are captured to the prestructured regions via the His-tag/trisNTA interaction.

B Chemical structure of the trisNTA^{PEG12-B}.

C Variable size protein patterns generated by further functionalization of SA-matrices with the nanotool followed by incubation with His₆-GFP (0.1 μM, 20 min). Images were acquired by confocal laser scanning microscopy.

D Intensity profile of the 1 μm pattern (white line in C) reflects high specificity of the interaction.

E Large-scale cell patterning in living cells occurred after addition of the trisNTA^{PEG12-B} (100 nM, 10 min).

F Customized Y₂R assembly on 3 μm and 1 μm SA-prestructured matrices.

752 G Intensity profile of the 1 μm pattern (white line in f) showed an intensity comparable to a
753 soluble His₆-tagged protein. Scale bars: 10 μm .

Figure 2. Ligand-independent response triggered by *in situ* receptor confinement.

A Time-lapse imaging of Y₂R assembly. Y₂R expressing HeLa cells were allowed to adhere to prestructured SA-matrices for 3 h and visualized by CLSM in LCIS at 37 °C. Time-lapse images were recorded for 10 min immediately after addition of *trisNTA*^{PEG12-B} (100 nM).

B Reversal of the interaction and disassembly of the clusters is demonstrated upon addition of histidine. Y₂R expressing cells were allowed to adhere to the SA-matrices for 3 h, and then receptor confinement was induced by addition of *trisNTA*^{PEG12-B} (100 nM). Subsequently, cells were incubated with histidine (5 mM) for 2 to 10 min followed by washing.

C Receptor integrated density in the patterned regions increased mono-exponentially, leading to an assembly rate of $0.20 \pm 0.03 \text{ min}^{-1}$ and $\tau_{1/2} = 5 \text{ min}$.

D Fast cell spreading upon cluster formation was observed. Cell area analysis before and 10 min after nanotool addition showed a 20% area increase, confirming an effect of the receptor clusters on cell response. Values for cell area were normalized with respect to the highest value. The mean $\pm$ SD (20 cells) is shown. ** $p \leq 0.01$ for Tukey test. Scale bars: 10 μm .

Figure 3. Ligand-free receptor confinement triggers calcium signaling.

773 A Representative images of His₆-Y₂R^{mEGFP} expressing cells over SA-prestructured matrices
774 before and in the presence of *trisNTA*^{PEG12-B} demonstrated a significant change in cell area.
775 Scale bar: 20 μm upper panel, 10 μm lower panel.

776 B Representative fluorescence images of the Y₂R (upper panel) and color-coded images of
777 the Ca²⁺ dye (lower panel). Y₂R expressing cells on SA-prestructured matrices were
778 incubated with BioTracker 609 Red Ca²⁺ AM dye (3 μM) for 30 min. After rinsing, cells were
779 immediately imaged by CLSM in LCIS at 37 °C. Addition of the nanotool showed a twofold
780 increase in cytosolic calcium.

781 C Analysis of the mean gray value for Ca²⁺ signal before (F₀) and upon addition of
782 *trisNTA*^{PEG12-B} (F) *versus* time. Time-lapse images were recorded with 45 s interval before
783 and after addition of *trisNTA*^{PEG12-B} (100 nM) (5 slices z-stack per time-point). ROIs covering
784 the complete cell area were considered. The mean ± SD (10 cells) is shown. Scale bar:
785 10 μm.

786 D Quantification of receptor integrated density in the nanotool patterned regions showed an
787 increase in the density of the receptors within the clusters after addition of the pNPY. The
788 mean ± SD is shown (113 1 μm ROI were analyzed). ***p≤ 0.001 for Tukey test.

Figure 4. Selective arrestin-3 recruitment upon ligand-induced receptor activation.

A Schematic representation of the experimental set-up. Cells co-expressing Y₂R and Arr3 were allowed to adhere to SA-prestructured matrices for 3 h and visualized by total internal reflection fluorescence (TIRF) microscopy in LCIS at 37 °C.

B Representative TIRF images of cells before and upon addition of *trisNTA*^{PEG12-B} (100 nM, 30 min) and subsequent incubation with porcine neuropeptide pNPY (10 nM) and Histidine (5 mM) in LCIS for 30 min at 37 °C. Scale bar: 5 μm.

C Fluorescence contrast analysis demonstrated no significant recruitment of Arr3 upon *trisNTA*^{PEG12-B} (1.4-fold with respect to the basal signal before, 100 nM, 30 min). Addition of pNPY increased Arr3 signal (3.6-fold, 10 nM, 30 min) confirming co-patterning of the downstream signaling molecule. Subsequent addition of histidine led to a decrease in the signal (2.3-fold, 5 mM, 30 min). Data was normalized with respect to the fluorescence intensity before clustering and it is expressed as the means ± SEM (60 cells for each condition were analyzed). Tukey's multiple comparisons test was applied (***p ≤ 0.001).

D Quantification of the fluorescence contrast in the patterned regions for Y₂R confirmed receptor enrichment upon addition of *trisNTA*^{PEG12-B}, (2-fold with respect to the basal signal

806 before, 100 nM, 30 min) which further increased 4-fold upon addition of pNPY (10 nM, 30
 807 min). Histidine addition led to a decrease in the signal (1.7-fold decrease compared to
 808 pNPY, 5 mM, 30 min). Data was normalized with respect to the fluorescence intensity
 809 before clustering and it is expressed as the means $\pm$ SEM (60 cells for each condition were
 810 analyzed). Tukey's multiple comparisons test was applied (**p $\leq$ 0.001).

Figure 5. Decrease of receptor mobility in confined regions.

A Confocal images of FRAP measurements upon Y_2R clustering induced either by the nanotool *in situ* or by an anti- His_6 antibody. Insets represent the bleached ROIs. Fast recovery of the clusters can be detected for the case of the multivalent nanotool.

B Quantification of the receptor mobile fraction for cell patterning by the $trisNTA^{PEG12-B}$ and anti- His_6 antibody demonstrated a significant decrease in the receptor mobile fraction for the antibody matrices suggesting a lower receptor exchange. The mean $\pm$ SD is shown. 9 cells before, 11 cells after $trisNTA^{PEG12-B}$ (45x 1 μm ROIs) and 5 cells on anti- His_6 antibody matrices (13x 1 μm ROIs) were analyzed. *** $p \leq 0.001$ for Tukey test.

C imFCS correlates fluorescence intensity fluctuations in single camera pixels with a large degree of statistics, providing accurate diffusion coefficients with high spatial and temporal resolution.

D Widefield image of a ROI at the plasma membrane of a living cell upon addition of the nanotool analyzed by imFCS (left). The analyses of numerous pixels simultaneously provide two-dimensional diffusion data that draw a picture of the mobility of membrane receptors and reveal local differences in the diffusion (right).

E Both techniques demonstrated a decrease in the lateral diffusion of the receptor at the plasma membrane after addition of the nanotool. Analysis of 1 μm clusters within the entire ROI led to a further decrease in the lateral diffusion coefficient. For imFCS analyses, two-sample t-tests ($\alpha = 0.05$) were applied to compare the diffusion coefficients for the different conditions. The mean $\pm$ SD is shown. 36 and 24 cells for the conditions before and after addition of *trisNTA*^{PEG12-B} were analyzed. For FRAP, the mean $\pm$ SD is shown (9 cells before, 11 cells after *trisNTA*^{PEG12-B}, 41 1 μm ROIs were analyzed). *** $p \leq 0.001$ for Tukey test. Scale bar: 10 μm (A), 1 μm (D).

Expanded View Figures

Figure EV1. Receptor confinement with high reliability.

A Representative confocal image of a cell patterned by *trisNTA*^{PEG12-B} (100 nM).

B Automatic cluster analysis performed by ImageJ requires a “binary”, black and white, image. A threshold range is set to select the objects of interest apart from the background. All pixels in the image whose values lie under the threshold are converted to white and all pixels with values above the threshold are converted to black. Further selection of the clusters according to area and roundness enable a large-scale analysis.

C Change in cluster area and integrated density of the receptor within different 96-well plates, different months and cell stocks reflected a reliable and reproducible approach. The average area in the clustered regions ($0.77 \pm 0.03 \mu\text{m}^2$) and integrated density of ten images in five different experiments (841x ROIs of 1 μm in total) is shown. Scale bar: 10 μm.

Figure EV2. Y₂ receptors lacking a His₆-tag do not cluster in confined areas.

A Cells expressing Y₂ receptors without N-terminal His₆-tag over SA-prestructured matrices before and after addition of the nanotool. Within the timeframe of imaging, there was neither a pattern formation nor a change in the integrated receptor density.

B Quantification of the integrated Y₂R density before and after addition of *trisNTA*^{PEG12-B}. The mean ± SD (18 cells) is shown. **p ≤ 0.01 for Tukey test. Scale bar: 50 μm.

Figure EV3. Receptor density correlates with the area of the prestructured regions.

A,B BSA-prestructured matrices (1 μm, A or 3 μm, B) were stepwise functionalized with biotin-BSA and SA. Y₂R expressing HeLa cells were allowed to adhere to the functionalized matrix for 3 h and immediately imaged by CLSM in live-cell imaging solution (LCIS) at 37 °C. Addition of *trisNTA*^{PEG12-B} (100 nM) led to *in situ* receptor assembly.

C 1 μm BSA-prestructured matrices were stepwise functionalized with biotin-BSA, SA, and a biotinylated anti-His₆ antibody (αHis₆ Ab). Y₂R expressing cells were allowed to adhere to the functionalized matrix for 3 h and immediately imaged by CLSM in LCIS at 37 °C.

D Quantification of the receptor integrated density. *In situ* receptor confinement by *trisNTA*^{PEG12-B} resulted in a receptor density, which is comparable to cells in contact with prestructured antibodies. For the 3 μm patterns, receptor density correlated with pattern area. The mean ± SD (38 to 132x ROIs of 1 μm) is shown. ***p ≤ 0.001 for Tukey test. Scale bars: 10 μm.

Figure EV4. Changes in cell motility are exclusively triggered upon receptor clustering.

A,B Confocal images of cells expressing His₆-tagged Y₂R on matrices which do not contain SA but only biotin-BSA. Addition of the *trisNTA*^{PEG12-B} nanotool confirms no effect on cell spreading and motility as shown in the quantification of the cell area (B). The mean ± SD (31 cells) is shown. **p ≤ 0.01 for Tukey test.

C Confocal images of cells expressing Y₂ receptors lacking the His₆-tag on SA-matrices do not present significant changes in cells spreading upon addition of the nanotool.

D Quantification of the cell area before and after addition of *trisNTA*^{PEG12-B} (100 nM). Values for cell area were normalized with respect to the highest value. The mean ± SD (20 cells) is shown. **p ≤ 0.01 for Tukey test. Scale bar: 50 μm.

Figure EV5. Calcium signaling is a specific response upon clustering.

A Representative fluorescence images of the Y₂R (upper panel) and color-coded images of the Ca²⁺ dye (lower panel). Y₂R expressing cells over prestructured matrices in absence of streptavidin were incubated with BioTracker 609 Red Ca²⁺ AM dye (3 μM) for 30 min. After rinsing, cells were immediately imaged by CLSM in LCIS at 37 °C. Addition of *trisNTA*^{PEG12-B} showed neither clustering nor change in cytosolic calcium. Scale bar: 10 μm

B Analysis of the mean gray value for Ca²⁺ signal before (F₀) and upon addition of *trisNTA*^{PEG12-B} (F) versus time. Time-lapse images were recorded with 45 s interval before and after addition of *trisNTA*^{PEG12-B} (100 nM) (5 slices z-stack per time-point). ROIs covering the complete cell area were considered. The mean ± SD (10 cells) is shown.

Figure EV6. Arrestin-3 recruitment on antibody-confined regions.

A Schematic representation of the experimental set-up. Cells co-expressing Y₂R and Arr3 were allowed to adhere to anti-His₆ antibody prestructured matrices for 3 h and visualized by total internal reflection fluorescence (TIRF) microscopy in LCIS at 37 °C.

B Representative TIRF images of cells before and upon addition of pNPY (10 nM) in LCIS for 30 min at 37 °C. Scale bar: 10 µm.

C Quantification of the fluorescence contrast in the Y₂R-patterned regions showed no significant change in receptor intensity yet a recruitment of Arr3 upon addition of pNPY (twofold). Data are expressed as the means ± SEM (30 cells for each condition were analyzed). Tukey's multiple comparisons test was applied (**p ≤ 0.01).

Figure EV7. Lipid localization and dynamics after receptor confinement.

A Live-cell plasma membrane staining was performed 15 min after Y₂R assembly in living cells. 5 µg/ml CellMask staining solution was incubated for 5 min at 37 °C and washed with LCIS before visualization. Lipid distribution is not affected by receptor confinement as shown by the homogeneous staining of the membrane.

B Representative confocal images of FRAP measurements for the lipid dye. An image of the receptor channel confirmed the presence of the pattern.

C FRAP recovery for the lipid dye measured in the entire rectangular ROI or at the Y₂R cluster spots (region selected based on the receptor channel image) demonstrated a rapid recovery for the lipids.

D The analysis did not show any differences in lipid diffusion coefficients for the entire rectangular ROI or at the 1 µm clustered regions ($D_{\text{entire ROI}} = 0.66 \pm 0.10 \mu\text{m}^2/\text{s}$ and $D_{\text{spots}} = 0.67 \pm 0.17 \mu\text{m}^2/\text{s}$). The mean $\pm$ SD (6 cells, 15x 1 µm ROIs) is shown. ** $p \leq 0.01$ for Tukey test.

E Quantification of the mobile fraction (M_f) for FRAP measurements of the lipid dye reflected no significant difference. The mean $\pm$ SD (5 cells, 14x 1 µm ROIs) is shown. ** $p \leq 0.01$ for Tukey test. Scale bars: 10 µm.

Figure EV8. Dynamic receptor exchange in confined clusters.

A Representative confocal images of FRAP measurements for Y₂R expressing cells on SA-prestructured matrices 10 min after addition of the nanotool.

B FRAP recovery curves reflecting the entire bleached area or an analysis performed only in the clustered 1 μ m regions. The analysis is based on a simulation approach which fits a computer-simulated recovery to actual recovery data of a FRAP series and determines the diffusion coefficient regardless of bleaching geometry.

C Quantification of the receptor density in the confined regions showed 50% recovery indicating a high exchange rate. The mean $\pm$ SD (6 cells, 15x 1 μ m ROIs analyzed) is shown. ** $p \leq 0.01$ for Tukey test. Scale bar: 10 μ m.

Figure EV9. Receptor mobility on antibody structured matrices.

A Scheme representing the experimental set-up.

B Representative widefield image (left) of a ROI at the plasma membrane of a living cell grown on prestructured matrices with an anti-His₆ antibody (αHis₆ Ab) analyzed by imFCS and the derived two-dimensional diffusion map (right).

C Lateral diffusion of the receptor analyzed by FRAP and imFCS. Both techniques demonstrated a decrease in D at the plasma membrane ($D_{\text{before}} = 0.32 \pm 0.06 \mu\text{m}^2/\text{s}$ and $0.25 \pm 0.08 \mu\text{m}^2/\text{s}$; $D_{\text{anti-His}_6 \text{ Ab}} = 0.18 \pm 0.06$ and $0.19 \pm 0.06 \mu\text{m}^2/\text{s}$ for imFCS and FRAP respectively), concurring with the values obtained for the measurements upon addition of the nanotool. Analysis of clustered regions (1 μm) within the selected ROIs led to a further decrease in the diffusion 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, respectively). For imFCS measurements, two-sample t-tests ($\alpha = 0.05$) were applied to compare the diffusion coefficients for the different conditions (** $p \leq 0.001$). The mean $\pm$ SD is shown. 36 and 26 cells for the conditions before and after addition of anti-His₆ antibody were analyzed. For FRAP, the mean $\pm$ SD is shown. Here, 9 cells before, 5 cells after addition of anti-His₆ antibody, 20x ROIs were examined. ** $p \leq 0.01$ for Tukey test. Scale bar: 1 μm .

Figure EV10. Multivalent nanotool *trisNTA*^{PEG12-B} analyzed by LC-MS.

A *trisNTA*^{PEG12-B} chromatogram reflecting the purity of the synthesized nanotool.

B LC-MS of *trisNTA*^{PEG12-B}, yielding the experimental mass ($M_{\text{exp.}}$) of 1874.85 Da ($M_{\text{theor.}} = 1873.90$ Da).