Eph-ephrin signaling modulated by polymerization and condensation of receptors

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Abstract

Eph receptor signaling plays key roles in vertebrate tissue boundary formation, axonal pathfinding and stem cell regeneration by steering cells to positions defined by its ligand ephrin. Some of the key events in Eph-ephrin signaling are understood: ephrin binding triggers the clustering of the Eph receptor, fostering trans-phosphorylation and signal transduction into the cell. However, a quantitative and mechanistic understanding of how the signal is processed by the recipient cell into precise and proportional responses is largely lacking. Studying Eph activation kinetics requires spatiotemporal data on the number and distribution of receptor oligomers, which is beyond the quantitative power offered by prevalent imaging methods. Here we describe an enhanced fluorescence fluctuation imaging analysis, which employs statistical resampling to measure the Eph receptor aggregation distribution within each pixel of an image. By performing this analysis over time courses extending tens of minutes, the information-rich 4-dimensional space (*x, y, oligomerization, time*) results were coupled to straightforward biophysical models of protein aggregation. This analysis reveals that Eph clustering can be explained by the combined contribution of polymerization of receptors into clusters, followed by their condensation into far larger aggregates. The modeling reveals that these two competing oligomerization mechanisms play distinct roles: polymerization mediates the activation of the receptor by assembling monomers into 6- to 8-mer oligomers; condensation of the pre-assembled oligomers into large clusters containing hundreds of monomers, dampens the signaling. We propose that the polymerization-condensation dynamics creates mechanistic explanation for how cells properly respond to variable ligand concentrations and gradients.

Significance Statement.

Cell communication is a precisely orchestrated mechanism in which cell receptors translate extracellular cues into intracellular signals. The Eph receptors act as a model guidance system steering cells to defined positions by their ligand ephrin. However, we still lack a mechanistic understanding of how membrane receptors can read a wide range of ligand concentrations and gradients and integrate them into coherent cellular responses. Here we reveal the evolution of Eph aggregation upon ephrin stimulation with unprecedented resolution by extending current imaging methods. The results fit biophysical models of protein aggregation. In these models, two protein oligomerization modes, polymerization and condensation, correlate with the “on/off” switching of the receptor activation, providing a precise, proportional and dynamic response to variable ephrin inputs.

Introduction

Cells constantly respond to other cells and their environments through receptor-ligand interactions, leading to fundamental cell decisions such as patterning or division (1). Ligand stimulation often induces receptor oligomerization, fostering trans-activation (e.g. via phosphorylation) and transducing extracellular cues into intracellular signals (2, 3). Eph tyrosine kinase receptors represent a paradigmatic family of cell-cell communication molecules interacting with their ligand ephrin on the surface of neighboring cell membranes. Eph-ephrin signaling plays a central role in development, for example, during the establishment of vertebrate tissue boundaries (e.g. hindbrain cell segregation and somitogenesis) (4-6). Ephrin cues are also presented in the form of concentration gradients, apparently guiding axonal patterning in retinotectal mapping or stem cell migration in the developing intestines (4, 7-9). Despite ample

evidence for the precision of this signaling system in controlling cell patterning and positioning, the mechanism(s) by which different ephrin concentrations are interpreted by the Eph receptor into proportional responses is largely unknown.

The current model for Eph activation/clustering posits that the presentation of an ephrin dimer nucleates an Eph dimer, activating the receptor by the resulting trans-phosphorylation (10-13). Activated receptors then propagate the signal horizontally by recruiting neighboring monomers into large-scale clusters, which leads to the endocytosis of the aggregate and termination of the signal (12, 14-17). Receptor aggregation therefore has been interpreted as an "amplifier" which operates on the ligand signal and increases the receptor sensitivity for low ligand concentrations (3, 18). However, it is unclear how such simple signaling scheme, lacking an adaptation mechanism beyond endocytosis, offer the cell the ability to sense and transduce changes in ligand concentrations or gradients of ligands (13, 19).

Here, we combine quantitative imaging and biophysical modeling to a model for the oligomerization and activation dynamics of the Eph receptor. Measuring the dynamic evolution of aggregates on living cells exceeds the capabilities of conventional imaging approaches; this requires molecular-level sensitivity over the area of an entire cell, and temporal scales ranging from the msec-sec times over which receptor dynamics take place, to the tens of minutes over which cell responses manifest. We meet these challenges by using a fluorescence fluctuation analysis of the short term variations in the intensities of each pixel in an image, based on the powerful Number and Brightness (N&B) approach (20-23) (Fig. 1). N&B analysis has been implemented to study the aggregation of transcription factors (24), focal adhesion proteins (25), or membrane-tethered proteins (26-28), during short acquisition times. Conventional N&B analysis yields the median concentration (Number) and molecular aggregation state (Brightness)

of labeled proteins (Fig. 1c, gray bar) (20, 22, 23) . Our modeling showed that the median oligomerization (i-mer) state over a narrow time window of analysis was not adequate to test between different models of receptor activation. Here we define and deploy enhanced Number and Brightness (eN&B), which uses a more powerful statistical approach to reveal the distribution, rather than the mean, of Eph aggregation. Our eN&B analysis reveals a polymerization-condensation process mediating signal amplification and adaptation to the receptor signal.

Results

Enhancement of N&B

Eph receptor aggregation takes place in response to ligand interaction, and as a result, a variety of oligomeric species must co-exist on the cell surface. Standard Number and Brightness (20) can interpret the fluctuations in an image to reflect the mean concentration and oligomerization in each pixel, but cannot offer insights into the full variety of oligomerization states (Brightness) that can co-exist in the same pixel. To answer this challenge, we created an enhanced N&B (eN&B) analysis, which employs a statistical resampling method (SI Materials and Methods, SI Text, Fig. S1) and can obtain the histogram of the Eph receptor aggregation (i-mer distribution) within each pixel of an image (Fig. 1c, cyan bars). The distribution of aggregation states for all the pixels in an image are determined from a rapid series of images acquired over a few seconds. As Eph receptor aggregation requires minutes, we bridge this temporal gap by extending the eN&B analysis over time[27, 28] (Fig. 1d), measuring the oligomerization dynamics of proteins in each pixel for the full time-course of each cell's response. The information-rich 4-dimensional

space (*x, y,oligomer distribution, time*) offered by eN&B can be related to Eph receptor activation by closely coupling analysis with mathematical modeling (Fig. 1f, g).

Aggregation dynamics using micro-printed ephrin presentation

Receptor aggregation was studied in transgenic cells (HEK293T) stably expressing the fusion protein EphB2_mRuby; alternatively, we used the Kinase Deficient (KD) construct KD_ EphB2_mRuby (Materials and Methods, Fig. S2), which can be used to study the role of endocytosis, since it cannot activate the endocytic pathway and is not internalized by the cell upon stimulation by ephrinB1 (19, 29). The ephrin-Fc protein was micro-contact printed on functionalized glass to present the ligand to the cells in a localized yet homogenous manner (see SI Materials and Methods); control experiments used functionalized glass printed with Fc alone or coated with poly-L-lysine (PLL).

Cells were imaged using Total Internal Reflection Fluorescence (TIRF) microscopy, which yields high signal-to-noise images of the cells' membranes as they interacted with the ligand-functionalized surfaces enabling N&B analysis on membrane proteins (23, 26-28, 30). This approach offered the needed pixel size and temporal resolution required for eN&B analysis. Fig. S1 shows the fluorescence intensity fluctuations for two representative pixels, one from an ephrinB1-stimulated cell (red trace), and another from a control cell (black trace). Over the 60 min data collection period, the fluctuations in fluorescence intensity increased in size and decreased in frequency in the ligand stimulated cell; such fluctuations yield the Brightness (B) in N&B analysis, and can be converted to mean oligomer sizes (i-mer) by multiplying the

149 brightness value of the unstimulated monomer ($B_{monomer}$=1.17, relative $B_{background}$=1.00, SI
150 Materials and Methods, Fig. S3).

151 Simple observation of the mean fluorescence intensity cannot distinguish stimulated and
152 unstimulated cells over 60 minutes (Fig. 2a). The eN&B analysis, instead, reveals large
153 differences by exploring oligomers distributions over time for each pixel. This multi-dimensional
154 dataset, however, cannot be directly represented as an image. To intuitively visualize this
155 information, we color code the image based on the average oligomer size, scaling from monomer
156 to 40-mer. This dimensionally-reduced representation reveals a striking difference between the
157 dramatic EphB2 clustering on the cells presented with ephrinB1 (Fig. 2a) and the near static
158 oligomerization level of cells presented only with PLL or Fc (Fig. S4).

159 The oligomerization state averaged across all pixels for ephrinB1 stimulated cells after 60
160 minutes (i-mer=28.2±0.9) was significantly higher ($p<0.01$) than the oligomerization in control
161 cells (i-mer= 3.7±0.2 for PLL coating; i-mer=2.9±0.2 for micro-printed Fc protein) (Fig. 2b).
162 The KD-EphB2 mutant showed intermediate levels of aggregation when presented with
163 ephrinB1 (i-mer=12.0 ±0.5). Such averaged results indicate that receptor clustering was strong
164 and specific to cells presented with ephrinB1. Ephrin stimulation did not have any impact on
165 GFP oligomerization in cells co-expressing membrane-tethered GFP and the Eph receptor (see
166 Fig. S4h), indicating that Brightness increase derives from specific EphB2 receptor
167 oligomerization rather than spurious phenomena such as membrane ruffling or cell adhesion
168 variability (27, 28, 30). We have also performed an automated tracking of the top 10% brightest
169 aggregates from several cells stimulated with micro-printed ligand on one of the sequence of 200
170 frames. The results (Fig. S4i) reveal high mobility of the clusters formed, suggesting that
171 receptor mobility is not compromised by micro-printing ligand delivery, probably due to the non-

172 covalent adsorption of ephrin to the surface. Internalization of Eph clusters occurred normally as
173 well after micro-printed ligand delivery (Fig. S4j).

174 The eN&B analysis over time revealed an orderly progression of Eph receptor aggregation after
175 stimulation with ephrinB1 over the 60 minutes of observation (i-mer plot; Fig. 2c). Initially,
176 low-order species dominate (monomer-pentamer), then decay rapidly (within the first 30
177 minutes). Each i-mer species increases in abundance in turn over the few minutes after its initial
178 appearance; thereafter, each one decreases as higher i-mers form. Extended observation (75
179 min) did not reveal an upper limit to the i-mers being formed (Fig. S4). The relatively fast
180 depletion of the monomers revealed by eN&B analysis in the presence of progressive EphB2
181 clustering indicates that higher order EphB2 oligomers cannot be assembled predominantly by
182 the recruitment of monomers; instead, it seems that oligomer growth must involve the
183 recruitment of smaller oligomers into larger complexes (31).

184 **Polymerization-condensation model**

185 A mathematical model was used to interpret the rich information about oligomerization
186 dynamics contained in the multiple eN&B distributions, and to validate the hypothesis that
187 coalescence of oligomers contribute to aggregate growth beyond the point of monomer
188 depletion. We built our model based on the Lumry-Eyring biophysical theory on protein
189 aggregation (32-34) assuming that two oligomerization mechanisms foster receptor aggregation,
190 namely polymerization by accretion of monomers, and condensation by coalescence of
191 oligomers into larger aggregates. The rich eN&B data allowed us to explore the parameter space
192 of the polymerization-condensation model to study the relative impacts of polymerization and
193 condensation in controlling oligomer formation and the strength of the ensuing signal (see SI

194 Text). The best fit model shows that EphB2 receptor oligomer growth is not a monotonic
195 process, but instead results from the combined action of polymerization and condensation, which
196 are mechanistically uncoupled but whose contribution overlaps in time (Fig. 2d). Two growth
197 phases take place: a first phase in which free monomeric receptors form dimers by ephrin
198 induction (nucleation) and incrementally higher oligomers independent of additional ligand
199 binding (polymerization). The second growth phase involves both the accretion of any free
200 monomers and the coalescence (condensation) of two aggregates to form a larger one.

201 The polymerization-condensation model predicts an initial phase in which the addition of
202 monomers predominates (Fig. 2d) until observable monomer concentration falls to below 1%.
203 Condensation then becomes more important with a contribution of monomers being mainly
204 recruited from the unobservable part of the membrane. The excellent agreement between the
205 model prediction and the eN&B data supports the hypothesis of a dual oligomerization mode
206 (polymerization and condensation) contributing to receptor aggregation. In agreement, note that
207 the variance in the EphB2 oligomer sizes increases around minute 30 (Fig. 2e), when monomer
208 concentration is very low, as predicted if the oligomers grew by a condensation of previously
209 formed oligomers (32) . Processes in which oligomers grow only by adding monomers should
210 reveal a variance $\left(\sigma_{\mu}^{2}\right)$ that grows slowly with the mean aggregate size (μ)(see SI Text). The
211 stimulation of EphB2_KD cells with ephrinB1 (Fig. 3) showed impaired receptor aggregation, in
212 terms of a lower degree of oligomerization (measured by brightness maps) and slower
213 aggregation dynamics, as compared to the functional receptor. These results suggest a role of the
214 tyrosine kinase domain of the Eph receptor in the formation of high order clusters, possibly by
215 harboring specific interfaces needed for condensation of oligomers (35).

216

Aggregation dynamics using ephrin in solution

To show that the approach is valid for other means of ephrin presentation, we imaged cells that were stimulated by the addition of soluble pre-clustered ephrinB1. The soluble ligand, unlike the micro-printed ephrin, has no restriction in mobility, allowing us to test the impact of ligand mobility, a feature shown to impact Eph receptor response (31, 36). Also, the soluble ligand bathes the entire surface of the cell, thus minimizing the effects of any unobservable receptor monomer populations, which can move from any unstimulated surfaces of the cells to the imaged micro-printed surface and thereby obscure the impact of condensation (Fig. 4). eN&B revealed a qualitative difference in the temporal sequence of EphB2 oligomerization dynamics upon exposure to soluble ligand as compared to micro-printed ephrin. Oligomerization maps show aggregation as spatially heterogeneous, and a smaller number of larger clusters appeared (Fig. 4a). The average oligomerization of EphB2 cells stimulated with ephrinB1 for 60 minutes (Fig. 4b, imer= 21.7±0.9) was significantly higher ($p<0.01$) than cells stimulated with Fc (imer= 6.5±0.4) or cells expressing KD EphB2 (imer= 10.6±0.9). Despite the fact that the ligand concentration for the micro-printed and soluble case are not directly comparable, the i-mer plot of Eph oligomerization using soluble ephrin (Fig. 4c) reveals a fast decay of the smaller oligomers and the sequential appearance of larger oligomers is less pronounced than in the micro-printed case. Our model predicts a rapid depletion of the free monomer in the first minutes after ligand presentation to the entire cell surface (Fig. 4d). This reduction of available monomer and absence of a 'hidden' reservoir shifts the clustering dynamics towards a strong dominance of condensation, which is reflected by the large separation between the curve of the variance of the cluster sizes $\left(\sigma_{\mu}^{2}\right)$ and their average (μ) when using soluble ligand (Fig. 4e). While most

condensation events occur at the subpixel scale, some larger scale events are observable with simple confocal microscopy (Fig. 4f, movies S5-7)

Receptor phosphorylation

A dose-response curve was performed stimulating the cells with a 100-fold range of soluble ephrinB1concentrations and measuring the Eph receptor response by western blot densitometry of phosphorylated EphB2. The results revealed a uniform phosphorylation kinetics for all concentrations tested. (Fig. 5a, Fig. S5). The amount of phosphorylated receptor rapidly increased for 15 minutes, then slowed to an asymptote at around 30 minutes post-stimulation. The receptor response however was proportional to the ligand dose, the final amount of activation (phosphorylation) increased with larger concentrations of ephrinB1. Notably, these kinetics indicate that receptor activation and signaling primarily occur when low-order oligomers predominate (Fig. 4c, d), implying that the condensation phase dominates after receptor activation. Moreover, the broad phosphorylation range of EphB2 (Fig. 5e) was confirmed for oligomerization measurements as well. Stimulating EphB2 with increasing ephrinB1 concentrations induced larger dynamic responses in aggregation as reported by eN&B analysis (Fig. 5b). These results suggest a direct link between oligomerization dynamics and receptor activation.

We extended the polymerization-condensation model to predict EphB2 phosphorylation based on eN&B oligomerization data (SI Text). To do so we assume that tyrosine phosphorylation is mediated only by polymerization (the binding of free monomers to pre-existing phosphorylated receptors) and not by condensation (14-17, 19). The model prediction shows good agreement with the monomer concentration calculated by eN&B (Fig. 5c) and also confirms the asymptotic

kinetics reported by the Western blot measurements. Half-maximal or full (asymptotic) tyrosine kinase activation occur when 5-mers (maximum value after 15min stimulation: $TK_{50\%}$=5.2±3.1,) or 8-mers (maximum value after 30min stimulation: TK_{full}=8.5±3.6), respectively, are the dominant species in the oligomer population. The later appearance of oligomers of 40-mers and beyond indicates that activation is decoupled from this high-order clustering.

Performing the simulations with and without condensation contributing to the dynamics offer ample evidence of the importance of condensation in the activation (Fig. 5d). For an example where 40-mer was the largest oligomer allowed to assemble before truncation (SI Text and Fig. S5), removing condensation from the system delayed the time to reach the maximum signal as well as increasing the signal amplitude. These results suggest that with condensation, the time required to form large oligomers can be reduced (Fig. 6b), contributing to the signal adaptation and serves as mechanism for dynamic range control.

Discussion

The regulation of receptor dynamics is critical for the fidelity information flow in cell-cell communication (1). Previous studies have suggested that, in the absence of modulation, unlimited receptor clustering would amplify any given ligand input to the same maximum level of activation (3, 18, 37). While highly sensitive, such transduction dynamics would seem to be unnecessarily slow since complete activation must await the assembly of large-scale clusters. Uncontrolled receptor clustering would also blunt the dynamic response of the receptor to integrate the information encoded in ligand gradients, as active signaling involves a winner-takes-all formation of high-order aggregates (38). In order to obtain a combination of sensitivity

and range of response the cell must control the degree to which the Eph receptor activity induced by the ligand can propagate towards free neighbor monomers.

We tackle the question on how receptor clustering dynamics can be regulated by using the eN&B analysis. This powerful tool allowed us to time-resolve the evolution of a wide spectrum of EphB2 species during ephrin-induced oligomerization, overcoming the previous limitation of measuring only the weighted averages of species (19, 32, 33). The fine-grained results enable fitting rich oligomerization data into standard biophysics models. The eN&B method offers unique space and time resolution and could be implemented to study different receptor and cellular responses, such as neuronal differentiation or immune response, induced by space-structured ligands (44, 45).

The eN&B data can be largely explained by a Lumry-Eyring biophysics process of protein aggregation, in which polymerization and condensation run in parallel and combine with each other (32-34). Activation takes place during nucleation and polymerization of monomers in the immediate 15-30 minutes following ephrin stimulation, reaching the maximum activation when pentamers-to-octamers predominate (Fig. 5c and 6). Similar timing has been reported for other RTKs (39). Moreover, previous studies using artificial dimerization of the Eph receptor suggested that complete activation can be reached without the assembly of large-scale clusters, in agreement with our measurements (19). After reaching maximal activation, our results show that aggregation of receptors is mainly driven by condensation of oligomers.

Our model also provides an explanation on how condensation can contribute to the receptor dynamic response to a broad range of ephrin concentrations (Fig. 5b-d). The polymerization-condensation model suggests that coalescence of oligomers into larger aggregates reduces the

overall recruitment of free monomers, by accelerating the formation of large-scale (slow-diffusing) clusters and subsequent induction of endocytosis and signal termination (Fig. 5d and 6b)(29). Hence condensation can adapt signal propagation by dampening the lateral recruitment of free receptors, thus creating a fast and transitory response to the ligand (40, 41). This amplification-adaptation strategy provides a simple mechanistic explanation on how receptor clustering combines the sensitivity and the dynamic range needed for the cell to respond the range of ephrin concentrations and gradients found in animal tissues (42, 43). Salaita et al. demonstrated that high-order oligomerization plays a central role in cytoskeleton remodeling and cell invasiveness (31). We think our complementary models suggest a dual role of large Eph clusters as space-concentrators of the signal (local cytoskeleton remodeling) and signal terminators (endocytosis induction).

Materials and Methods

Lentiviral constructs

mRuby was first excised from a pCDNA3.1 construct (46) using *BamHI* and *BsrGI* sites and cloned into pCDNA3_EphB2_GFP construct, a generous gift from R.Klein lab (29). Lentiviral constructs driving the expression of EhpB2_mRuby receptor were generated by cloning a PCR amplified cassette containing wild-type or mutated EhpB2_mRuby between BamHII and SalI sites of pLenti CMV Puro (Thermo Fisher. The kinase deficient (KD) receptor was first generated in the pCDNA3_EphB2_mRuby expression vector by amplification of the whole vector containing the wild-type EhpB2_mRuby construct with specific 5' pospho-primers designed to generate an A to G point mutation (KD-EhpB2_mRuby) in the EhpB2_mRuby receptor:

KD-FW_℗-ATGACCCCAGGCATGAGGATCTATATAGATCCT

KD_RV_℗-AGGATCTATATAGATCCTCATGCCTGGGGTCAT

Then wild-type or mutated EhpB2mRuby were amplified from pCDNA3_EhpB2_mRuby vector with the following primers:

FW_CGCGGGCCCGGGATCCGCCACCATGAACTTTATCCCAGTCGA

RV_ GAGGTTGATTGTCGACTCAAACCTCTACAGACTGG

PCR products were cloned into pLenti CMV Puro by using In-Fusion® HD Cloning Kit (Clontech).

Production of lentiviruses

HEK293T cells (standard cell line in the field(16), Thermo Fisher) were grown on gelatin coated plates and transfected with pLenti.*CMV*:EphB2_mRuby using Lipofectamine 2000 along with the ViraPower Lentiviral Packaging Mix (Thermo Fisher) according to the manufacturer's protocol. Supernatants were collected 48 and 72 hrs after transfection, pulled together, filtered at 0.45 µm and ultracentrifuged at 50,000×g for 2 hr at 4°C to obtain virus concentration.

Lentiviral transduction and cell lines.

$1x10^6$ HEK293T cells were infected in suspension and then plated in 10 cm plate. After two passages the cells were infected a second time following the same protocol. After two additional passages the cells were trypsinized and mRuby positive cells were selected by cell sorting. Two lines were generated: HEK293T:EphB2_mRuby, HEK293T:KD-EphB2_mRuby. All cells lines were routinely tested for mycoplasma by real-time PCR.

The plasmid pCS2-eGFP-CtermHras encodes for a GFP targeted to the membrane by the fusion to the C terminal domain of HRas.

Western Blot

Protein extracts, separated by SDS-PAGE and transferred onto PVDF membranes, were probed with antibodies against Anti-phosphoY594-Eph receptor B1/B2 (ab61791, 1:500, Abcam) or actin (A1978, 1:5000, Sigma) or Anti-EphB2 (AF467, 1:2000, R&D) (Fig. S5). Proteins of interest were detected with anti-rabbit IgG antibody (NA934, 1: 10000, GE Healthcare, Uppsala, Sweden) or anti-mouse IgG antibody (NA931, 1: 5000, GE Healthcare, Uppsala, Sweden) or anti-goat IgG antibody (P0160, 1: 2000, Dako) and visualized with the Amersham ECL Western blotting detection reagents (RPN2209, GE Healthcare, Uppsala, Sweden), according to the provided protocol.

Ethics Statement

The experiments presented in this study were conducted following protocols approved by the Institutional Review Board of the leading institution (CMR[B]).

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Author Contributions

S.O. and J.J.O. performed experiments. S.O. and F.C. analyzed the results. D.R. and S.O. designed mathematical model. F.C. and C.C. designed analysis algorithms. V.H. and E.M. designed the micro-printing protocol. C.T., A.S. and S.O. performed western blots and cell cultures. S.M. performed FCS analysis. M.L., E.M, A.R and S.E.F. contributed to the experimental design. S.O., F.C., D.R. and S.E.F. wrote the manuscript.

Competing interests

The authors declare no competing financial interests.

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Figure legends

Fig. 1. Experimental pipeline overview. (A) Ephrin-B1 is micro-contact printed on glass bottom imaging cell culture dishes. Cells are seeded and prepared for Total Internal Reflection Fluorescence (TIRF) imaging. (B) Short scale time imaging (seconds) is performed to capture fluorescence fluctuations, basis of N&B analysis (N, number, B, Brightness). (C) Statistical enhancement of N&B expands oligomerization information for each pixel from a median value (N&B, gray) to a distribution (eN&B, cyan). (D) Photobleaching compensation(47, 48) (see SI Materials and Methods) allows long scale time imaging (minutes) providing a distribution of oligomers for each time point in each pixel. (E) Oligomerization map distribution for a Region of

Interest (ROI) in a cell over long scale imaging (minutes). Color coding (Jet colormap) represents oligomerization level; each color is color-mapped to black to represent the relative percentage of molecules at a specific oligomer state. Vertical direction represents progression over time. (F) Mathematical modeling is coupled to the eN&B analysis. (G) Model interprets the information rich content obtained via eN&B to access biological mechanisms information.

Fig. 2. eN&B analysis of EphB2 clustering using micro-printed ligand stimulation. (A) Time-lapse oligomerization map of HEK293T:EphB2_mRuby cells acquired with the TIRF microscope. The cells were seeded on plates coated with PLL with either no additional coating or 2μM ephrinB1. For every coating condition, the top panels show grey-scale snapshots of the cells after photobleaching compensation(47, 48) at indicated time points. The bottom rows depict the oligomerization maps of the same images. Every pixel in the cell represents the weighted average i-mer species color-coded according to the color scale bar. PLL and 2μM ephrinB1 experiments were replicated respectively 47 and 61 times, (see also movies S1-3). (B) Distribution of average and standard deviation oligomerization values for multiple cells (N_{PLL} = 10, N_{FC} = 8, $N_{EphrinB1}$ = 19, N_{KD} = 16) presented with the relevant ligand for 60 minutes. KD, kinase deficient mutant; PLL poly-L-lysine. (c) i-mer evolution plot (see SI Materials and Methods). (C) Evolution of the concentration of each aggregate (A_i) over time from the ephrinB1 stimulated cell in (A), normalized by the initial concentration of free receptor (R_0). i-mer values are color-coded according to the color scale bar. (D) Model fitting to experimental data (see SI Text section 3). The experimental data from (C) (dashed lines) was used to fit 12 selected species into the mathematical model (solid lines). Additional fittings can be found in the SI Text. (E)

Mean (μ) and covariance (σ^2) of the aggregate size for eN&B measurements and prediction of the polymerization-condensation model for micro-printed ephrinB1.

Fig. 3. EphB2 Kinase Deficient (KD) mutant oligomerization. (A) Time-lapse brightness map of HEK293T:EphB2_KD cells acquired with the TIRF microscope. The cells were seeded on plates functionalized with 2μM Fc protein, 2μM ephrinB1 or presented with 0.2μM Fc or 0.2μM ephrinB1 in solution. Every pixel in the cell depicts the weighted average i-mer aggregate color-coded according to the color scale bar. (B) Distribution of average and standard deviation brightness values for multiple HEK293T:EphB2_KD cells (N>5) that have been presented with the relevant ligand for 60 minutes. (C) i-mer evolution plot (see SI Materials and Methods). Evolution of the concentration of each aggregate (A_i) over time from the ephrinB1 stimulated cell in (A), normalized by the initial concentration of free receptor (R_0). i-mer values are color-coded according to the color scale bar.

Fig. 4. eN&B analysis of EphB2 clustering using soluble ligand stimulation. (A) Time-lapse oligomerization maps. HEK293T:EphB2_mRuby cells were stimulated with 0.2μM Fc or 0.2μM ephrinB1 in solution. The weighted average i-mer species is color-coded according to the color bar. Experiments for 0.2μM Fc and 0.2μM ephrinB1 were repeated respectively 22 and 40 times (see also movie S4). (B) Distribution of average and standard deviation oligomerization values for multiple cells ($N_{ephrinB1}$= 40, N_{Fc}= 22, N_{KD}= 32) that have been presented with the relevant ligand for 60 minutes. KD, kinase deficient mutant. (C) i-mer evolution plot (see SI Materials and Methods). Evolution of the concentration of each aggregate (A_i) over time from the cell in

(A) stimulated with ephrinB1 in solution, normalized by the initial concentration of free receptor (R_0). i-mer values are color-coded according to the color scale bar. $TK_{50\%}$ and TK_{full} indicate timepoints 15 and 30 mins. where 50% and the entire receptor population, respectively, is phosphorylated. (D) Mathematical model fitting of selected 12 species from (C) (see SI Text section 3). The dashed lines represent the experimental eN&B measurement, and the solid lines the model prediction. Additional fittings can be found in the SI Text. (E) Mean (μ) and covariance (σ^2) of the aggregate size for eN&B measurements and prediction of the polymerization-condensation model for ephrinB1 in solution. (F) Time-lapse, 3D confocal reconstruction of HEK293T cells transfected with EphB2-GFP after stimulation (time=h:min:sec) with soluble ephrinB1 (see also movies S5-7). The red circle highlights Eph receptor clusters merging into larger aggregates.

Fig. 5. EphB2 activation kinetics. (A) EphB2 dose-response phosphorylation curve of cells stimulated with different ephrinB1 concentrations measured by western blot densitometry. (B) EphB2 oligomerization range. Distribution of average and standard deviation oligomerization values for multiple cells ($N_{0.2\mu M}$= 29, $N_{0.64\mu M}$ = 37, $N_{2\mu M}$= 28) presented with the different ephrinB1 concentrations for 60 minutes. (C) Phosphorylation kinetics (blue line) from the cell in Fig. 4a, c, as predicted from the polymerization-condensation model (SI Text). Red circles indicate the total monomer concentration obtained from eN&B. Vertical lines highlight 15 and 30 minute timepoints. (D) The relative amount of receptors assembled in clusters (proportional to the receptor activation) was quantified for a truncation limit of N=40-mer (see SI Text, section 4.1) in the presence or absence of condensation.

Fig. 6. Schematic representation of the EphB2 polymerization-condensation model. (A) The model shows the nucleation of an EphB2 dimer upon interaction with ligand ephrinB1, which triggers the transactivation of the receptor. Lateral recruitment of receptors into low-order oligomers by polymerization (thin black arrows) leads to full activation. The coalescence of oligomers (condensation, thick black arrows) results in the formation of large-scale Eph aggregates, the recruitment of monomers slows down, and endocytosis leads to signal termination. (B) Condensation accelerates the formation of large aggregates. By introducing condensation, the same given size receptor cluster (i.e. 20-mer) can be assembled with less binding events compared to cluster growth by polymerization exclusively.

SI MATERIALS AND METHODS

Surface Coating and soluble ligand preparation

35 mm Glass bottom dishes (MatTek) were coated with poly-L-lysine (Sigma-Aldrich) solution at 0.05% (w/v) in PBS for 90 min and then rinsed with PBS and Milli-Q water. Flat Polydimethylsiloxane (PDMS) stamps (SYLGARD® 184 , Ellsworth Adhesives) were fabricated by mixing a 10:1 mass ratio of silicon elastomer base and curing agent. PDMS was degassed under vacuum, poured on flat Petri dishes and cured overnight at 60ºC. Stamps were cut in 12 mm round discs and cleaned with ethanol in an ultrasonic bath for 5 min. 2 μM recombinant mouse ephrinB-Fc Chimera or Recombinant Human IgG_1 Fc (R&D Systems Inc.) solution, hereafter referred as Fc, were conjugated with Goat Anti-Human IgG (Jackson ImmunoResearch 109-005-088) at a 2:5 molar ratio for 30 min under constant shaking. Thereafter, stamps were inked with ephrinB1-Fc solution for 45 min. Afterwards the stamps were thoroughly rinsed with PBS and Milli-Q water and air dried. Inked stamps were brought into conformal contact with previously poly-L-lysine coated surfaces for 10 min. Flat stamps were carefully removed and conjugated ephrinB1-Fc or Human IgG_1 Fc were transferred to the surface. After printing, surfaces were rinsed with PBS and Milli-Q water. Stimulations with the soluble ligand were carried out as commonly used in the field. A DMEM solution of 0.4μM of either ephrinB1 or Fc were incubated with Recombinant Human IgG_1 Fc (R&D Systems Inc.) at a 1:5 mass ratio, for 30 minutes under constant shaking. After warming up at 37ºC, 1 ml of the solution was added to the culture plate to reach a final 0.2μM concentration.

For every experiment, approximately 10^6 cells where freshly harvested from a culture plate and gently resuspended in DMEM without phenol red for immediate use. The cell suspension was then transferred into the functionalized plates containing either the micro-printed or soluble ligand, and spun down using a plate centrifuge at 1000rpm for 1 minute. When using the micro-printed plates, the clock was set to zero at the end of the centrifugation process. The samples were then quickly taken to the microscope for observation.

FCS and RICS measurements of EphB2 mobility

HEK293T cells were seeded into LabTek glass bottom chamber slides (Nalgene) and transiently co-transfected with a paGFP tagged EphB2 and membrane localizing mCherry (mem-mCherry) (Fig. S3). 24h following transfection cells were imaged using a Zeiss LSM 780 laser scanning confocal microscope and avalanche photodiodes of the Confocor 3 (Zeiss, Jena). A water ×63/1.4 NA objective (Zeiss, Jena) was used for imaging, photoactivation, paFCS and RICS. The paGFP was photoactivated with the 405 nm laser line for FCS and RICS acquisition following previously described protocols (46). FCS and RICS data were acquired using the ZEN Software FCS and RICS modules (Zeiss, Jena), respectively. For FCS acquisition, a point was selected along the membrane identified with the mem-mCherry marker and acquired for 25s with 4 repetitions, and analyzed through previously established paFCS protocols probing for anomalous and free diffusion (46). RICS data was performed by acquiring 100 consecutive frames with a 50 nm pixel size and pixel dwell time of 25 μs. Region-of-Interest analysis was performed by selecting small

regions along the membrane, and was fit to a single species. For both analyses the laser waist (ω_r) was calibrated as previously outlined (47).

Image acquisition

The diffusion rate of EphB2 was measured using standard single point FCS and Raster Image Correlation Spectroscopy (RICS) (47) and analyzed using ZEN (Zeiss, Jena, DE) and SimFCS (www.lfd.uci.edu) obtaining a value of 0.25+/- 0.08 $\mu m^2/s$.

We acquired the time series for N&B analysis using a commercial STORM microscope system from Nikon Instruments (NSTORM) equipped with an EMCCD camera (Andor iXon3 897) set to frame transfer mode and a 1.4 NA 100x objective and a 1.5x lens tube for additional magnification. The microscope was used in TIRF mode to illuminate only the portion of the cell membrane in direct contact with the glass surface. Cells were illuminated with 561nm light at low laser intensity (3 W/cm^2 power density) for 200 frames with 500 ms exposure time (1min 40sec total acquisition time). Every time-point acquisition was initiated 2 to 3 minutes after the termination of the previous one. Acquisition of every time point Exposure time was chosen so it fell in the linear range of the autocorrelation curve shown in Fig. S3. Waiting time between time points was 2.5 minutes. Camera calibration for N&B with dark was performed using SimFCS (www.lfd.uci.edu). Further processing was done with custom Matlab scripts (will be published elsewhere).

Cells used for monomer calibration were seeded for 24h on PLL. The value of Brightness retrieved for monomer was B(monomer)=1.17 with sigma = 0.08. The acquired data was detrended using boxcar filtering on each pixel. This detrending mode has been demonstrated to maintain fluctuations while improving the performance of N&B (43, 44). The values of aggregates were calculated as percentages from the Brightness histogram using the formula B(nmer) = 1+(n*(B(monomer)-1)) with variance measured from monomer calibration.

Cells undergoing apoptosis and out of the TIRF evanescent wave focal plane were excluded from analysis.

Enhanced Number and Brightness analysis

In this work we use the Number and Brightness (N&B) method (20) to measure the average number of molecules and brightness in each pixel of the fluorescent images acquired. N&B is a powerful tool that distinguishes pixels with different aggregation states by determining the mean intensity and variance of their relative fluorescence intensity fluctuations. The method has been successfully applied in both confocal (20, 22, 23) and EMCCD based systems (23) for measuring aggregation of proteins (22, 30, 48). In its most general form, the apparent Brightness, which represents the molecular aggregation level, is

calculated as the ratio of variance to average intensity while the apparent molecular Number is the ratio of total intensity over Brightness:

$$B = \frac{\sigma^2}{\langle k \rangle}$$

$$N = \frac{\langle k \rangle^2}{\sigma^2}$$

Step 1. Oligomerization enhancement. We acquire multiple time point datasets of the same specimen over approximately 60 minutes and apply N&B analysis to map the EphB2 receptor aggregation over time. However, the original N&B method has been used to give an averaged Brightness and Number for a given pixel over one contiguous dataset of F images acquired over one specific time range (tn) of the aggregation. In this work, we enhanced the resolution capability of the method by calculating within each pixel the distribution of aggregates and its dynamic over multiple time points (t1,t2,..tn). We name this the enhanced Number and Brightness (eN&B).

The enhancement is accomplished by analyzing the dataset with a circularly sliding window through the number F=200 of frames acquired in each time point. The analysis window was chosen to have length w=100 to provide a stack size with statistical confidence, 5 times larger than the minimum number suggested in the original N&B paper [17]. The analysis window was applied on the dataset with the same principle of circular buffers, in which we sub-sample the overall frames to build statistical distribution. The approach we chose uses the same size for this circular sliding window therefore ensuring same statistical weight to each frame and each Brightness calculated. Hence for each pixel (i,j) we obtain an array of F values of Brightness B. Each Brightness arise from a sliding window defined as follow:

$$B_s = \begin{cases} \left.\frac{.\sigma^2}{\langle k \rangle}\right|_n^{n+w} & if\ n < F - w \\ \left.\frac{.\sigma^2}{\langle k \rangle}\right|_n^{F} + \left.\frac{.\sigma^2}{\langle k \rangle}\right|_1^{w-(F-n)} & if\ n > F - w \end{cases}$$

where s goes from 1 to F. $\left.\frac{.\sigma^2}{\langle k \rangle}\right|_n^{n+w}$ is the B arising from the window of length w starting from position n and ending in n+w while $\left.\frac{.\sigma^2}{\langle k \rangle}\right|_n^{F} + \left.\frac{.\sigma^2}{\langle k \rangle}\right|_1^{w-(F-n)}$ joins frames from position n to last frame F and from position 1 until reaching the number w

Similarly we obtain corresponding F values of apparent Number N.

$$N_s = \begin{cases} \left.\frac{\langle k \rangle^2}{\sigma^2}\right|_n^{n+w} & if\ n < F - w \\ \left.\frac{\langle k \rangle^2}{\sigma^2}\right|_n^{F} + \left.\frac{\langle k \rangle^2}{\sigma^2}\right|_1^{w-(F-n)} & if\ n > F - w \end{cases}$$

As the sliding window maintains the time structure, this method can be considered as consecutive N&B measurement with time delay equals to the frame rate. After repeating the analysis for T time points we obtain a 5 dimensional hypercube of information with x, y pixel position, distribution of apparent Number and apparent Brightness in each pixel, and time.

Step 2. Time enhancement. The aggregation dynamics of EphB2 are captured, and analyzed using eN&B over multiple time point datasets to build a time evolution of the oligomerization. We enhanced the time resolution of eN&B by applying a set of detrending algorithms, which has been developed and optimized for reducing the effect of photobleaching on N&B while preserving the fluctuations(43, 44).

Oligomer calculation

The values of aggregates were calculated from the brightness distributions using the formula B(i-mer) = 1+(i*(B(monomer)-1)) with the spread measured from monomer calibration.

I-mer plots calculation

The time-evolution of oligomers is represented on i-mer plots. The parameters A_i and R_0 of experimental data were directly extracted from eN&B analysis. A_i is the relative concentration of each oligomer of size i (i-mer) which results from the sum of the concentrations of oligomers with same size, for all pixels in a cell. The relative concentration of every i-mer is described in the previous section. R_0 is the total concentration of receptors at the initial time-point calculated as $R_0 = R(t_0) + \sum_{i=2}^{N} i \cdot A_i\,(t_0)$. The theoretical calculation of A_i and R_0 is described the equation (19) from section 3 of the SI Text.

Automatic tracking of EphB2 clusters

Tracking was performed on one the dataset from cells stimulated with micro-printed ligand using Bitplane Imaris software and targeting the top 10% brightest aggregates. The sequence of 200 frames (100 sec) ensures statistical confidence with over 120.000 tracks performed.

Statistics

Welch's t test was calculated using MATLAB. P values for Figs. 2b and 4b $P_{Sol}= 4.11*10^{-6}$, $P_{mp}= 1.78*10^{-11}$. Combining all samples, with both soluble and micro-printed ligand presentation, negative controls and mutant cell lines, we analyzed 312 cells distributed over 36 experiments. The results of the analysis were robust and reproducible across experiments (see Fig. S4).

Confocal videos

3D confocal videos of large clusters condensation (movies S5-7) were acquired using ZEISS LSM 5 Exciter confocal microscope and rendered using Bitplane Imaris.

Data Availability

All data supporting the findings of this study are available from the corresponding authors on request.

Code Availability

All custom scripts are available from the corresponding authors on request.