Advanced Cell Biology and Biotechnology

ACBB 2021/22

…the lecture of December 15th is about to begin…

CELL-CELL COMMUNICATION

- methods to study cell-cell communication:
  - chemotaxis & chemokinesis
  - attraction & repulsion
  - substrate preference
  - bidirectional signalling

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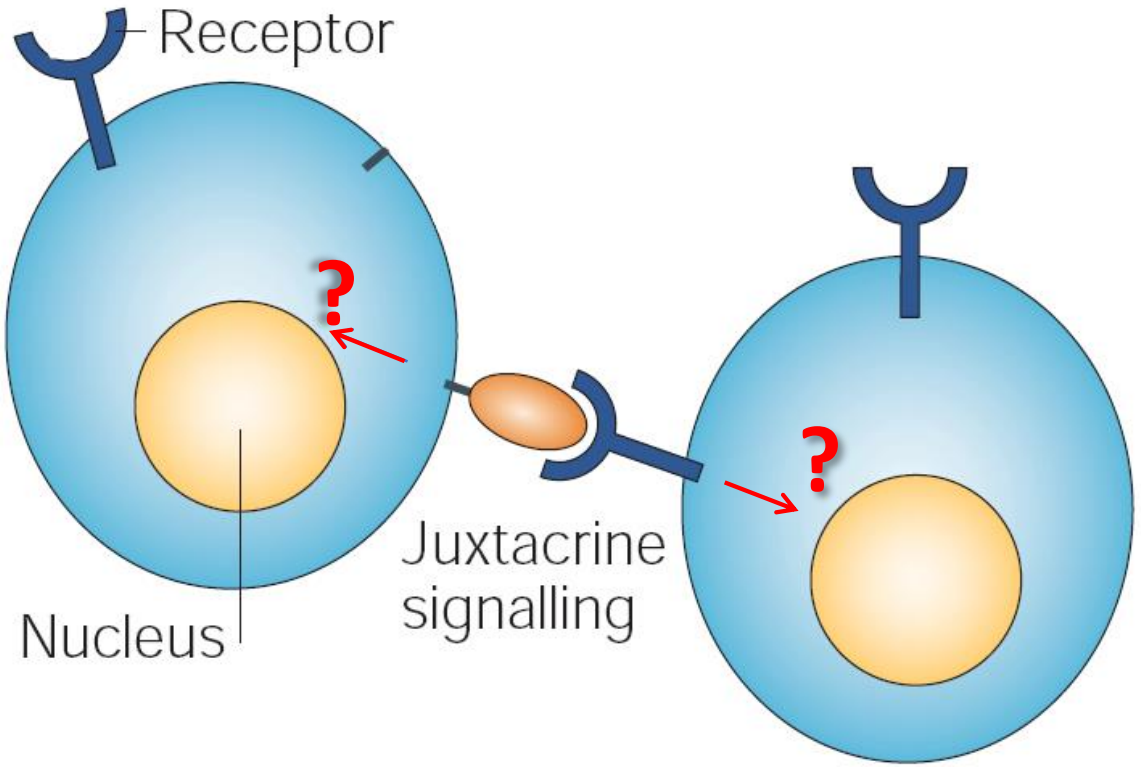
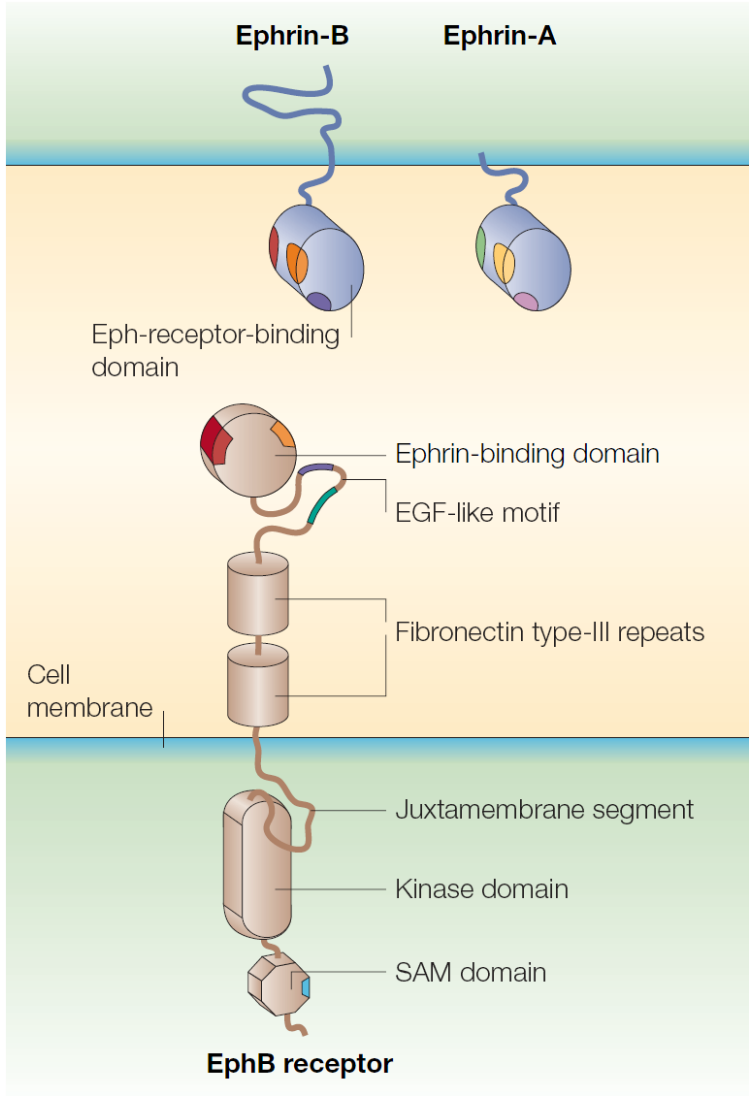
Cell-cell comunication:
how to study bidirectional signalling



Eph-ephrin as a model to study cell-cell communication



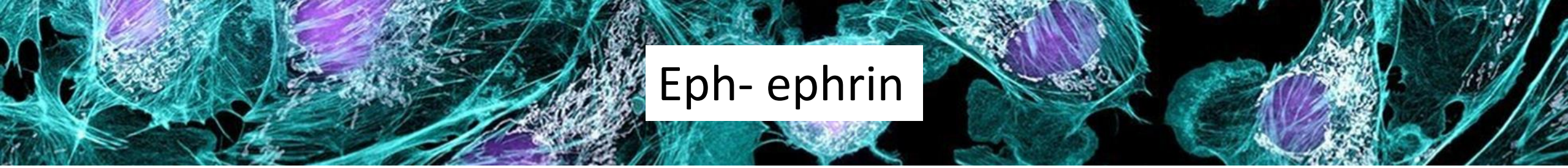
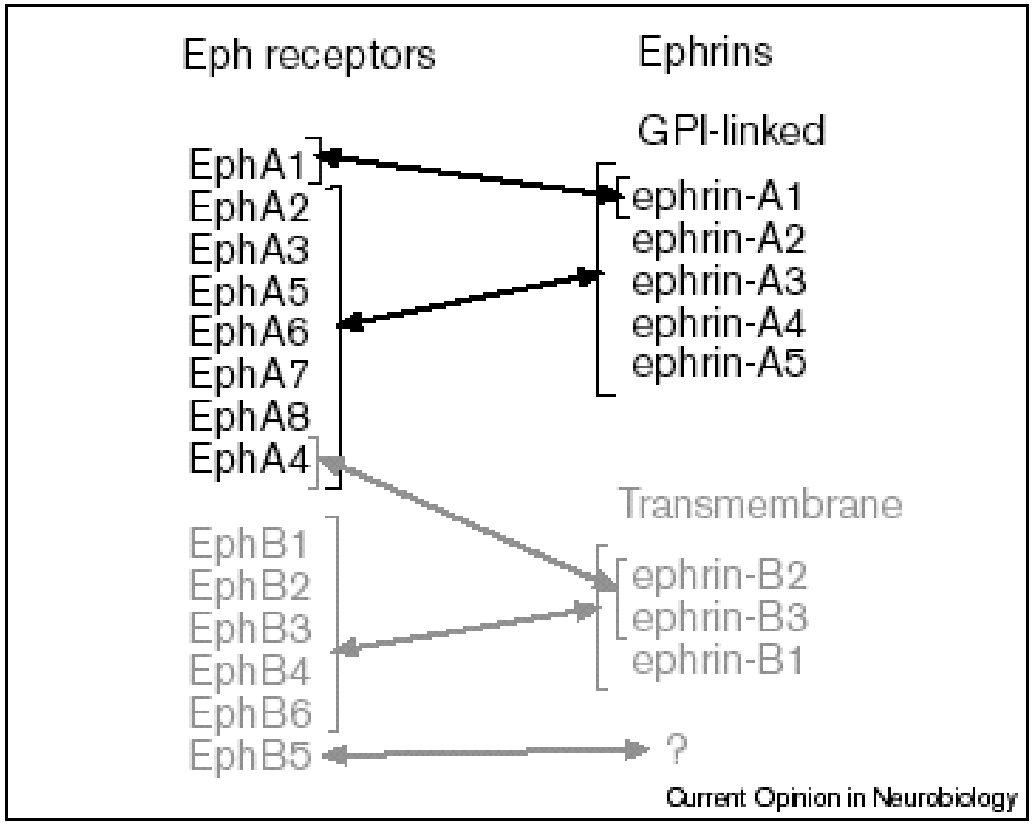


Table 1. Directional Guidance cues involved in CNS neuronal migration in vivo and in vitro

Ligands	Receptors	Defects in CNS neuronal migration in vivo	Neuronal migration in vitro
Slits	Robo	—	1. Slit repels postnatal SVZa cells[37] 2. Slit repels prenatal SVZ cells of GE[43]
Netrins	DCC	1. Abnormal pontine nuclei in DCC and netrin-1 mutants[46]	1. Netrin-1 attracts pontine nuclei[11]
	Unc-5h	2. Abnormal cerebellar development in unc-5h3[64]*	2. Netrin-1 repels postnatal cerebellar granule cells and prenatal SVZ cells[48,49] 3. Anti-DCC antibody blocks directed migration of postnatal SVZa cells[47]
Semaphorins	Neuropilin Plexin	1. Abnormal GABAergic interneurons in the striatum in neuropilin-2 mutants[50]	—
Ephrins	Eph	—	1. Disruption of Eph-B/Ephrin-B system affects the migration of postnatal SVZa cells[51]

*Unc-5h3/RCM mutant mice showed abnormal development of cerebellum. However, it is still unclear that the defect is primarily caused by migration abnormality or other reasons.

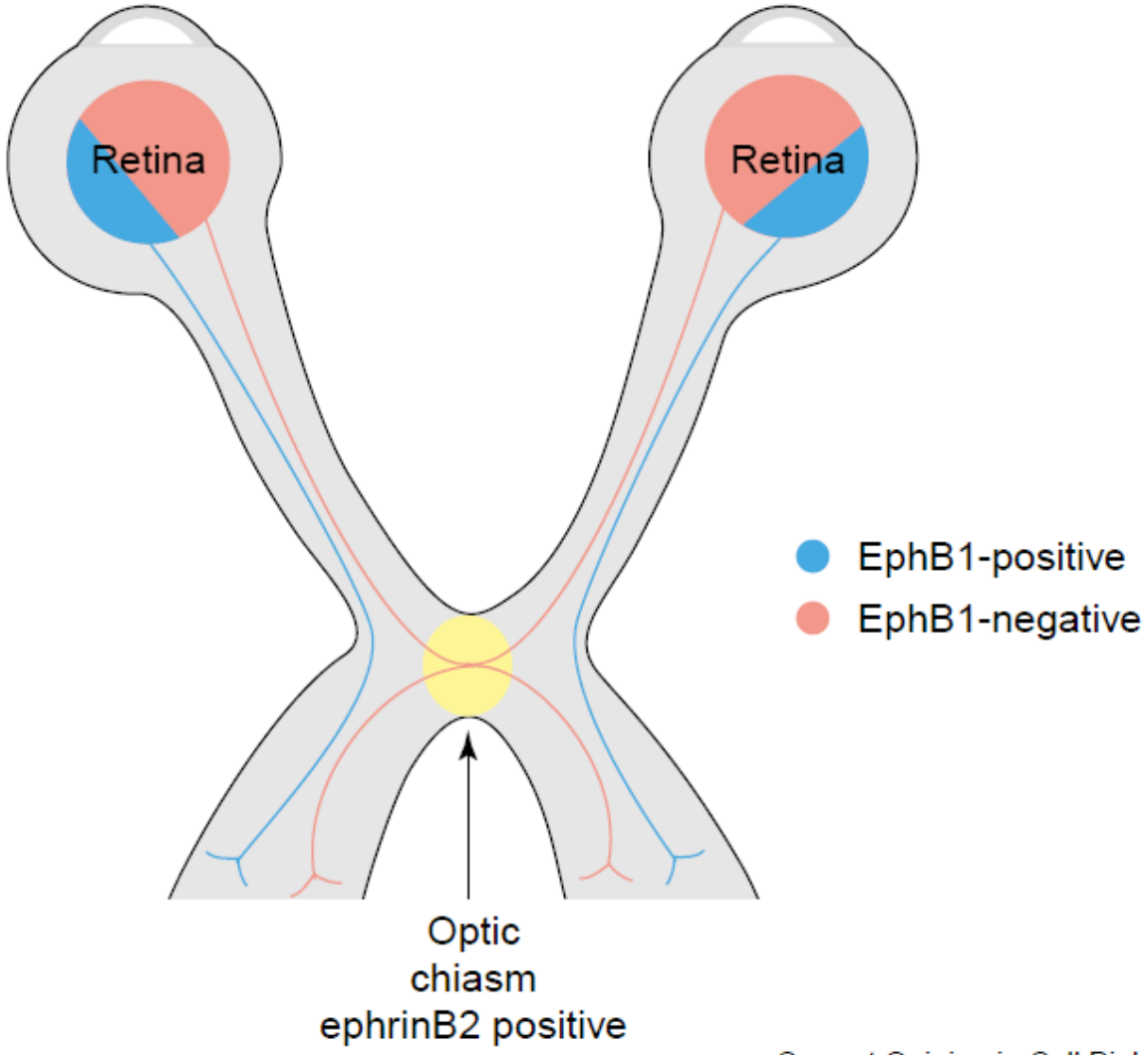
Eph receptors and ephrin ligands



Binding specificities of Eph receptors and ephrins. Eph receptors and ephrins fall largely into two binding specificity classes, with the exception of EphA4, which interacts with ephrin-A and some ephrin-B proteins. Differences exist, however, in the relative affinity of a receptor for different ephrins that may be functionally important. Additional ephrins probably exist, because EphB5 does not bind to any known ephrin.

*glycosylphosphatidyl-inositol membrane anchored

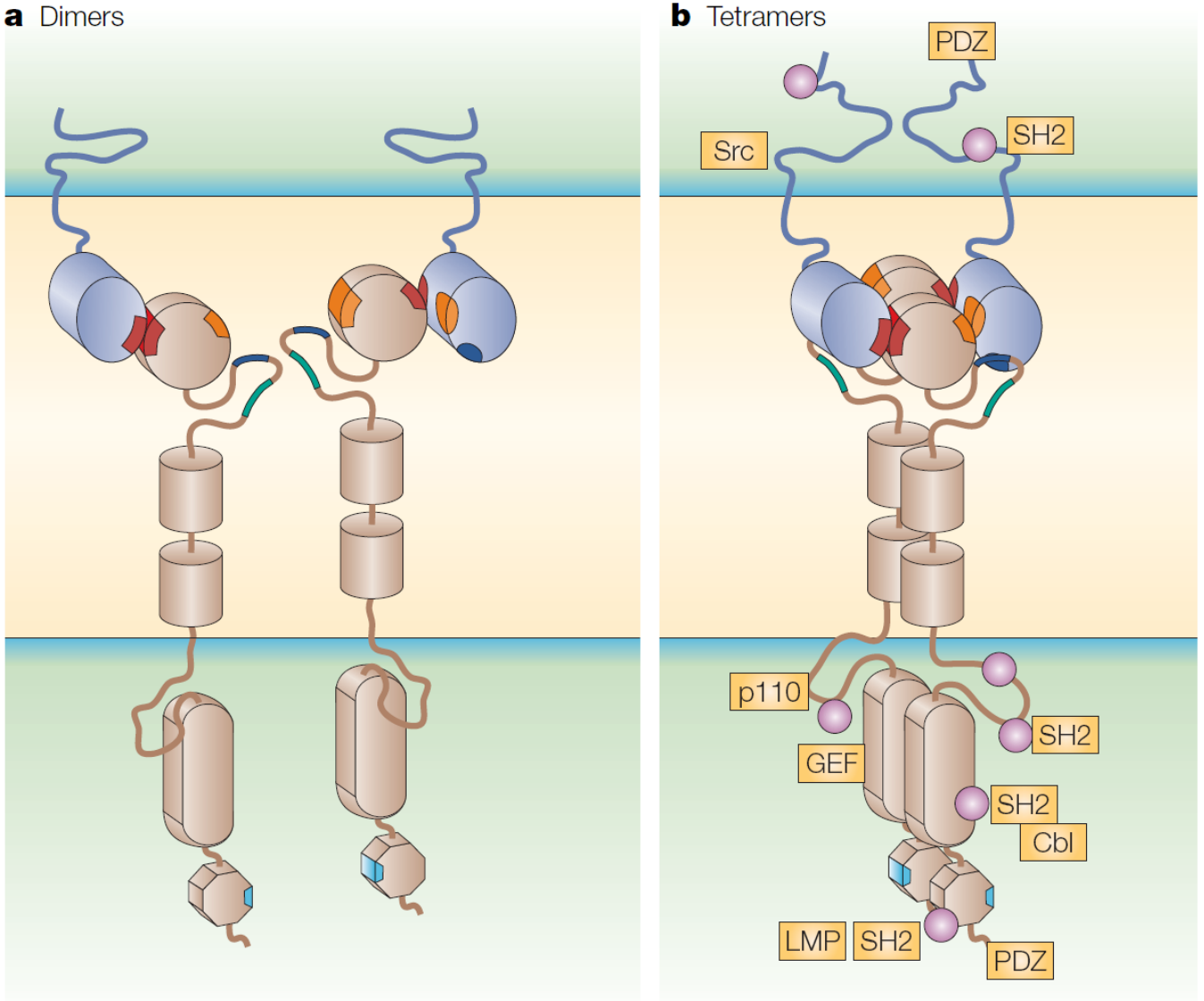
Midline guidance in the visual system



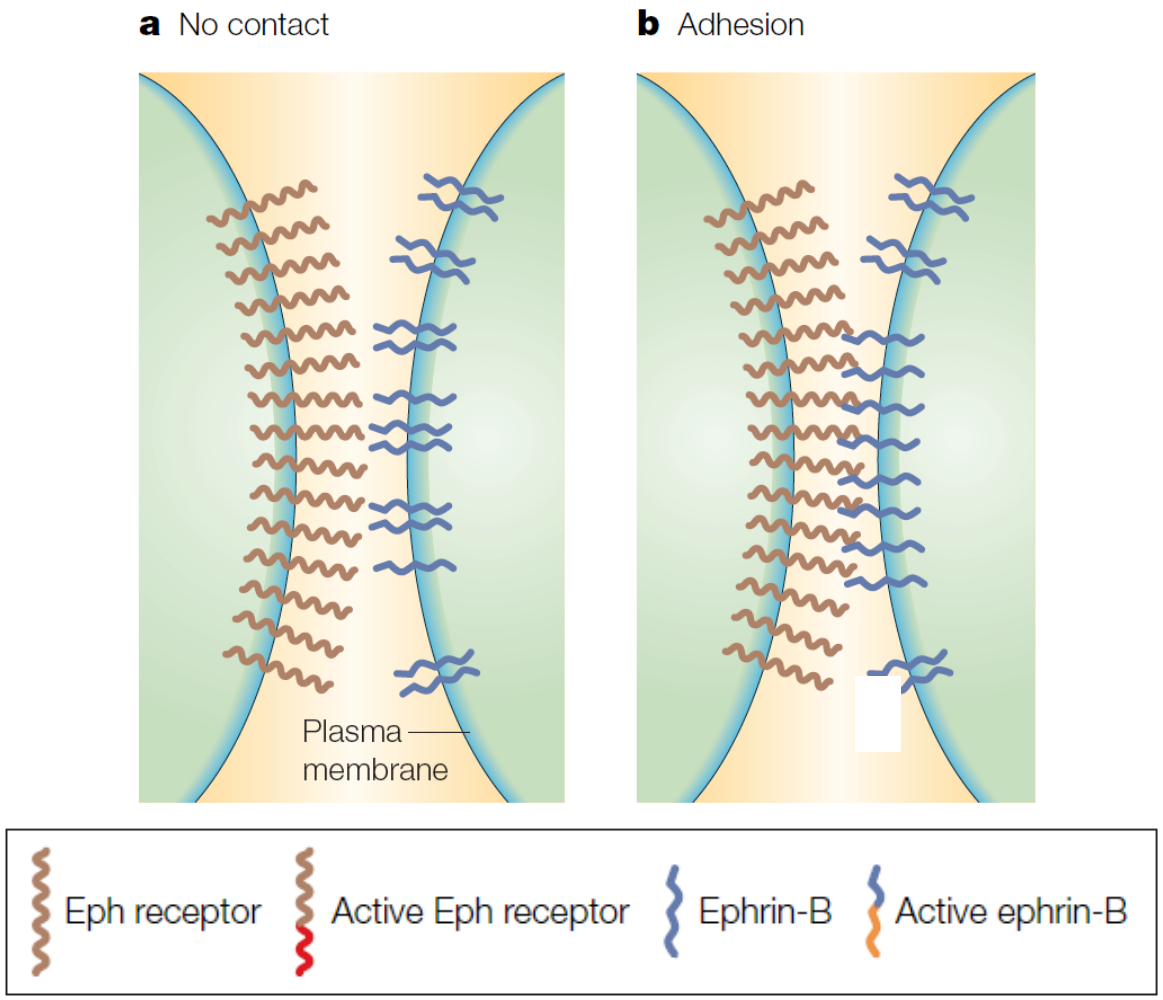
Current Opinion in Cell Biology

In animals with binocular vision, most retinal axons (**red**) cross to the contralateral side of the brain, while a smaller subset of retinal axons (**blue**) project to the ipsilateral side. Retinal axons expressing **EphB1** are repelled from the optic chiasm by **ephrinB2** and directed to an ipsilateral pathway. Contralaterally projecting axons do not express EphB receptors and therefore are not repelled by ephrinB2.

Bidirectional communication

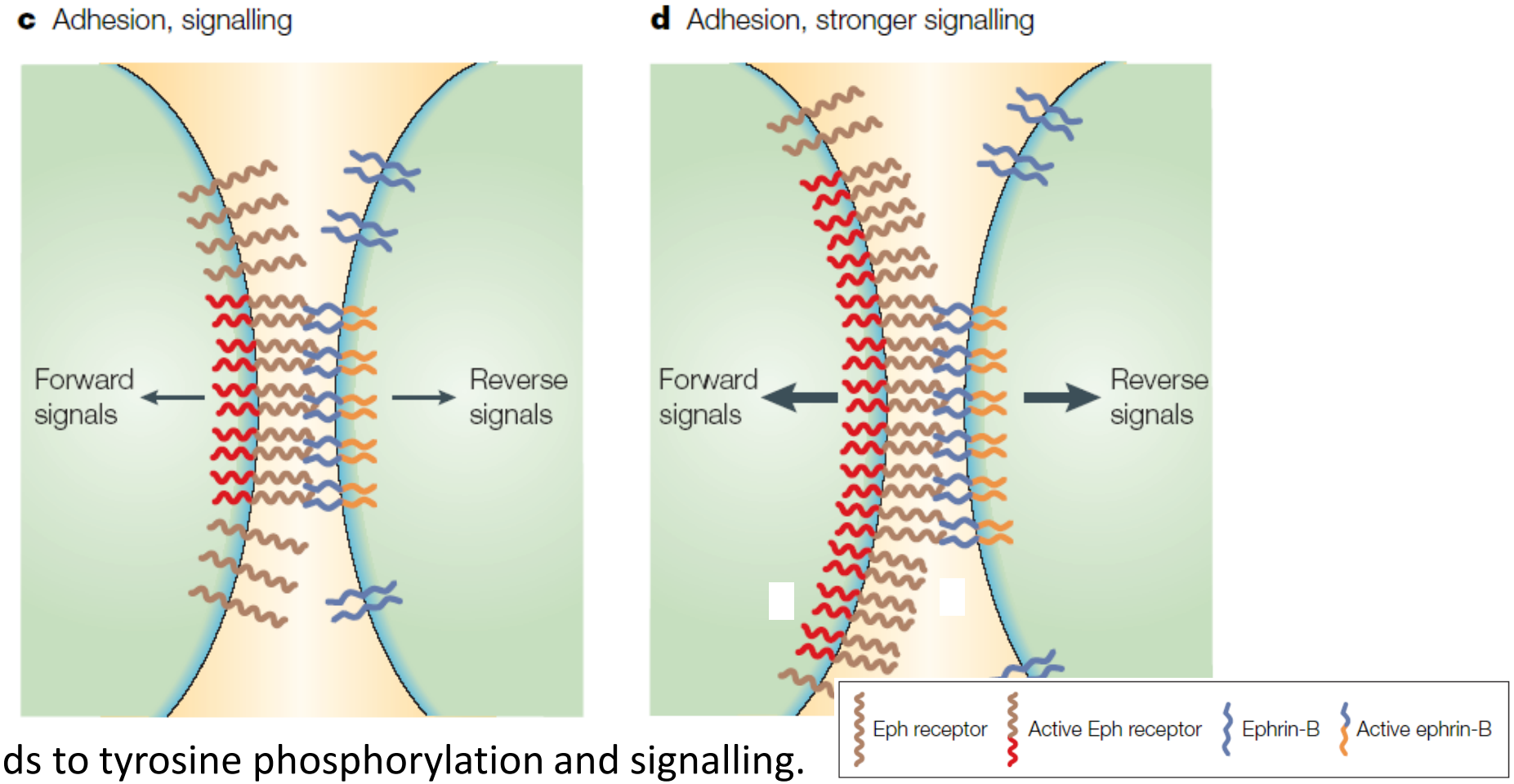


Steps in cell-contact-dependent Eph bidirectional signalling



a,**b -** Eph receptors and ephrins on opposed cell surfaces mediate cell adhesion on cell contact.

Steps in cell-contact-dependent Eph bidirectional signalling



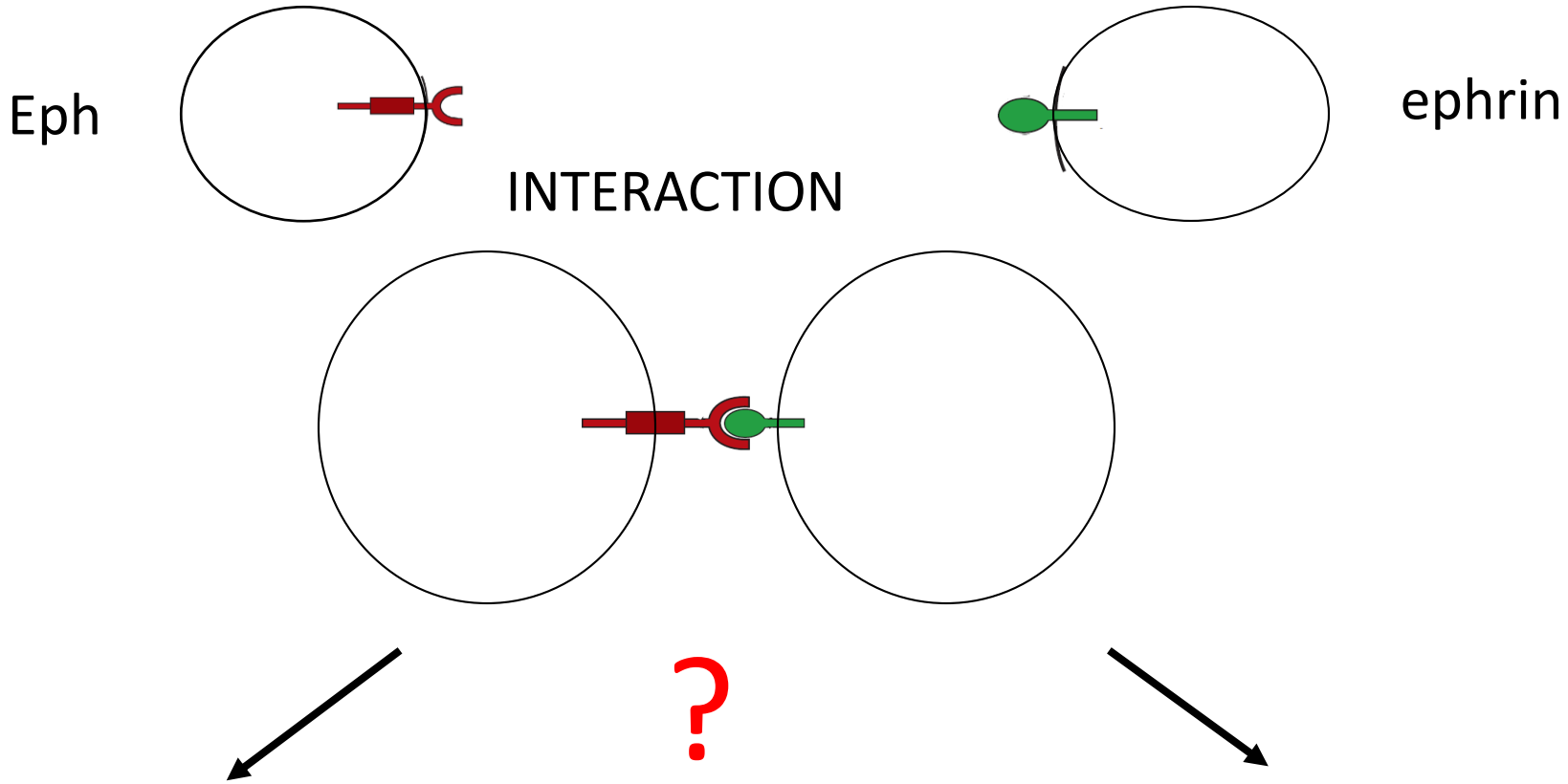
c - tetramerization leads to tyrosine phosphorylation and signalling.

d - the tetrameric complexes can further grow into larger clusters that, in the Eph receptor-expressing cells, can extend beyond the region of contact through homophilic interactions between Eph receptors.

The degree of clustering might regulate signal intensity and the nature of the signals.

Paradoxes of Eph signalling

- **Paradox 1.** The interaction between Eph receptors and ephrins requires cell–cell contact and mediates strong cell adhesion, but often the ensuing signals induce the separation of the two cells.



How can two cells separate after ligand-receptor strong interaction?

How can two cells separate after ligand-receptor strong interaction?

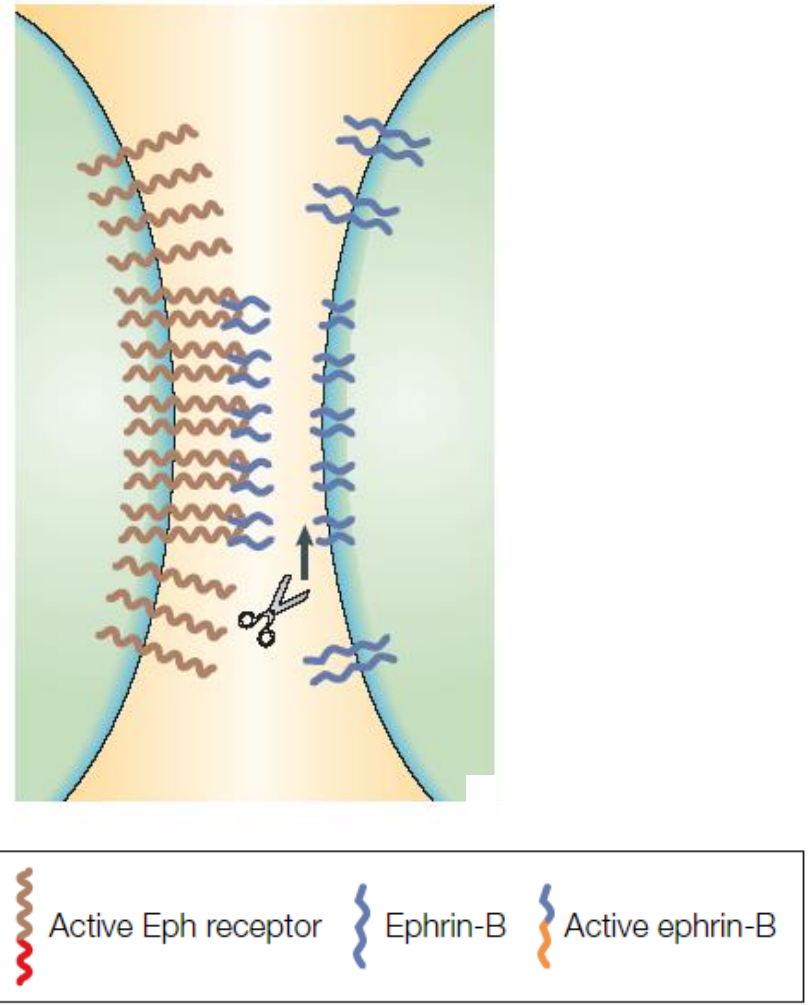
• many axon guidance molecules, including ephrins, netrins, semaphorins and slits, elicit repulsive responses when bound to their receptors; some of these factors are diffusible and growth cones respond to concentration gradients, whereas others, including the ephrins, are membrane-bound and repulsion happens after cell–cell contact

• interactions between repellent guidance cues and their receptors are high affinity, contrasting with the rapid process of contact-mediated repulsion

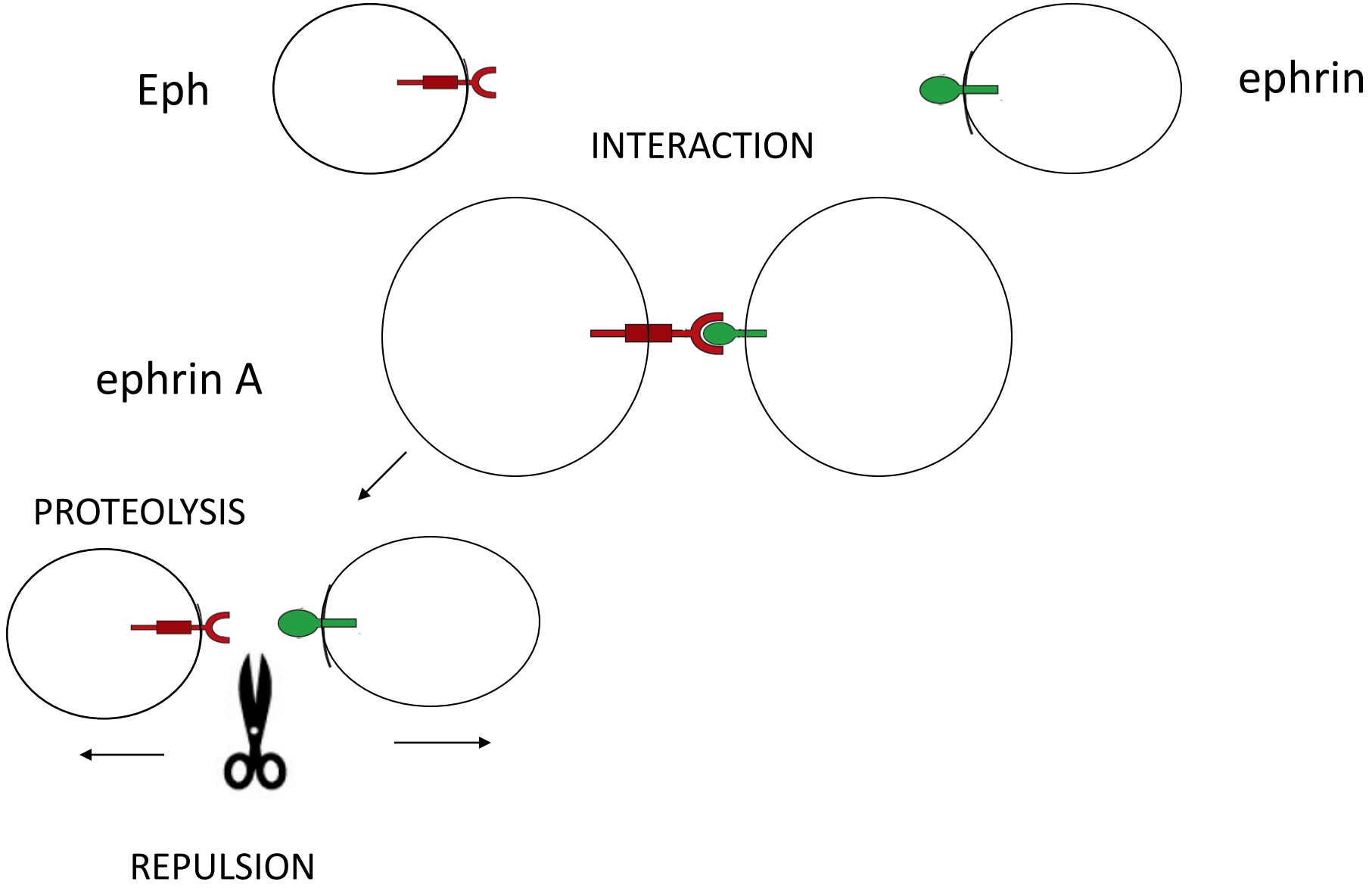
• this results in a paradox: although the formation of a complex between ligand and receptor is an adhesive event, it results in detachment and retraction of cells and their cellular processes

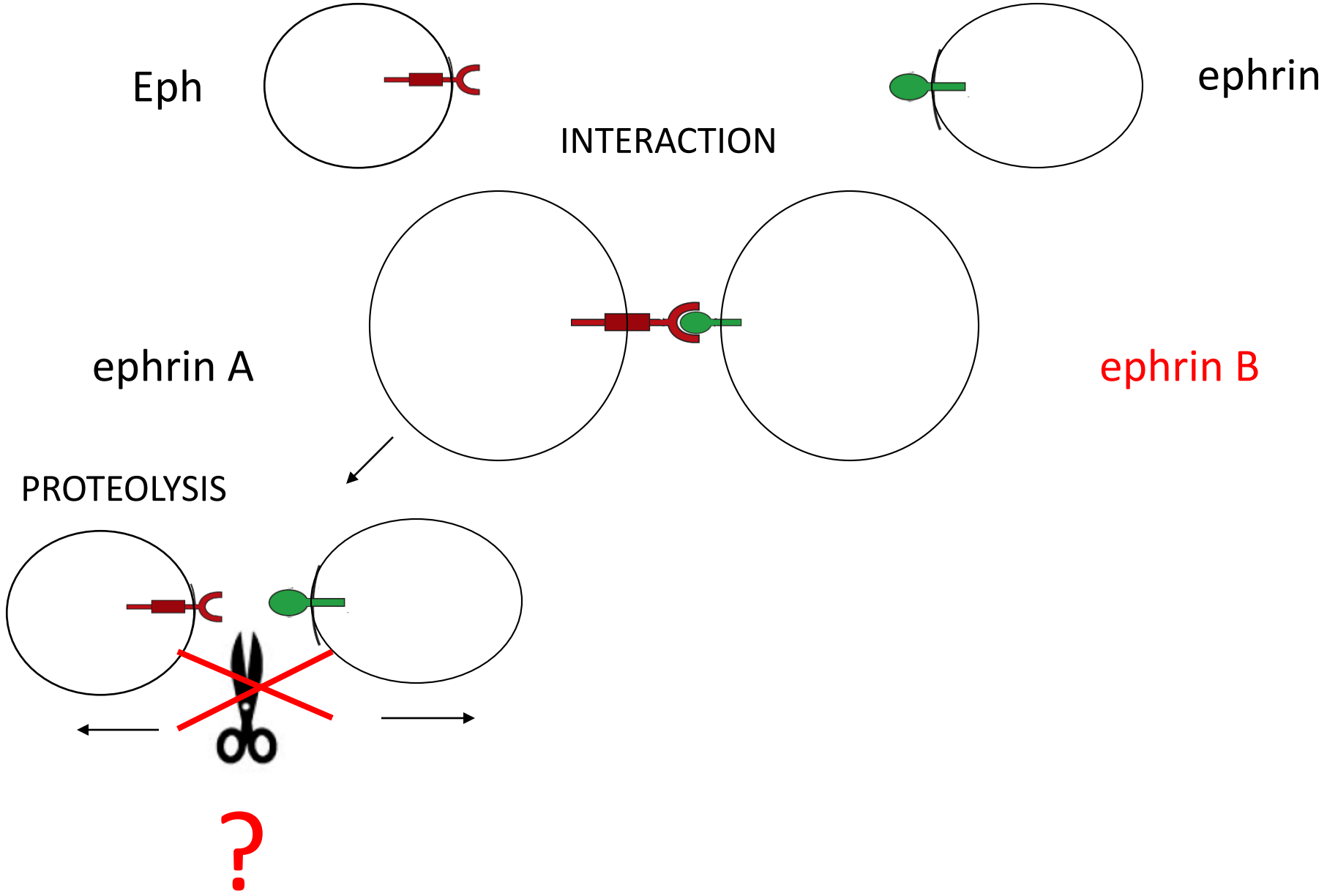
→ one mechanism that may remove ligand–receptor complexes from the cell surface is **PROTEOLYTIC CLEAVAGE**

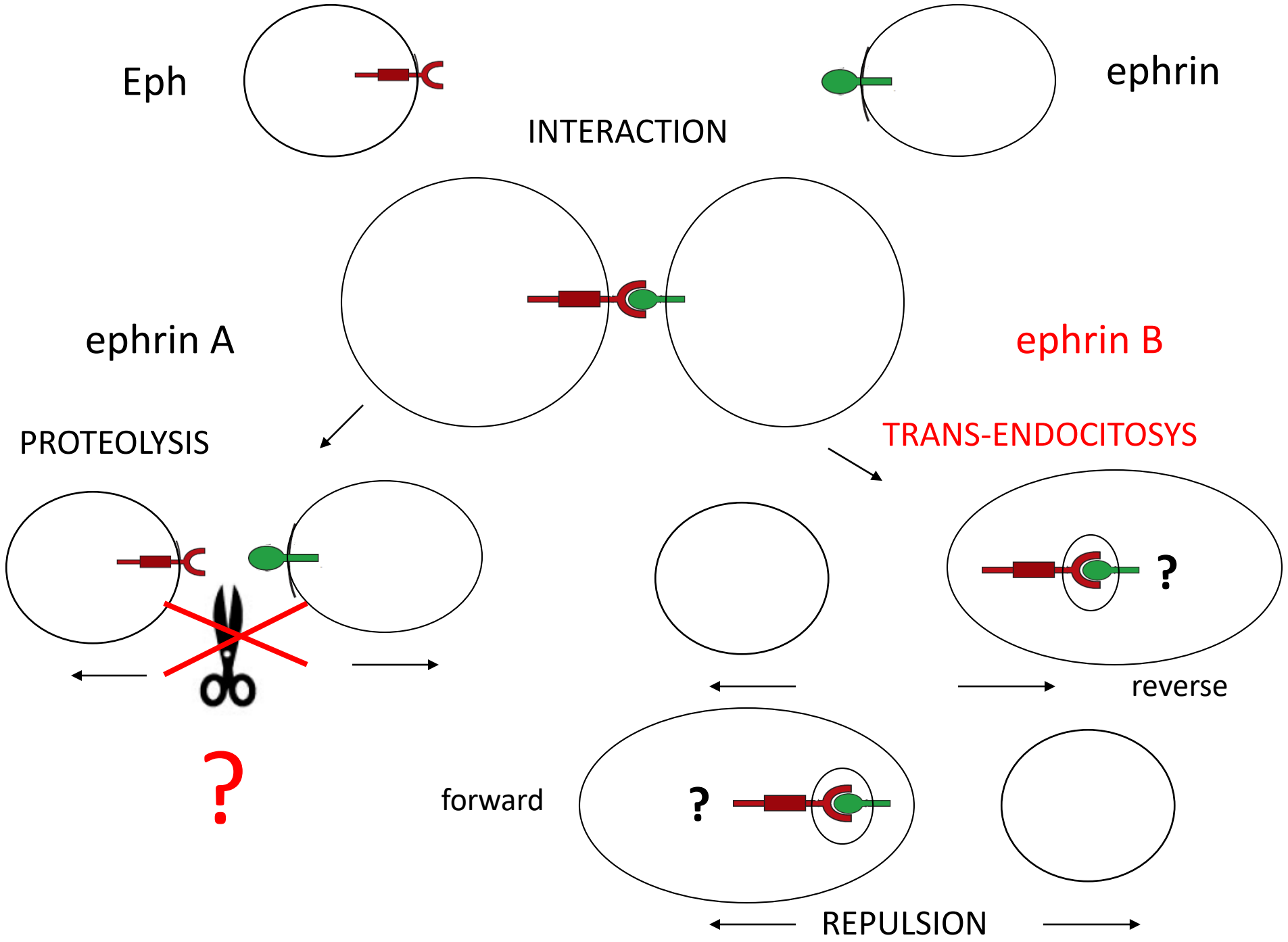
Mechanisms of Eph signal attenuation and termination

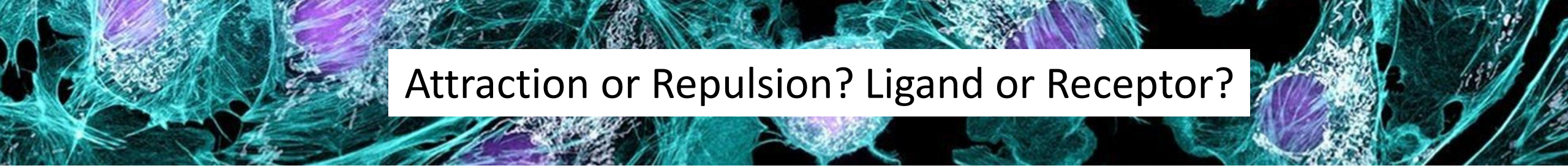


Cleavage of the ephrin by a protease allows cell separation following Eph–ephrin engagement


Eph
ephrin
INTERACTION
ephrin A
PROTEOLYSIS
REPULSION


Eph
ephrin
INTERACTION
ephrin A
ephrin B
PROTEOLYSIS
?


Eph
ephrin
INTERACTION
ephrin A
ephrin B
PROTEOLYSIS
TRANS-ENDOCITOSYS
?
?
reverse
forward
?
REPULSION



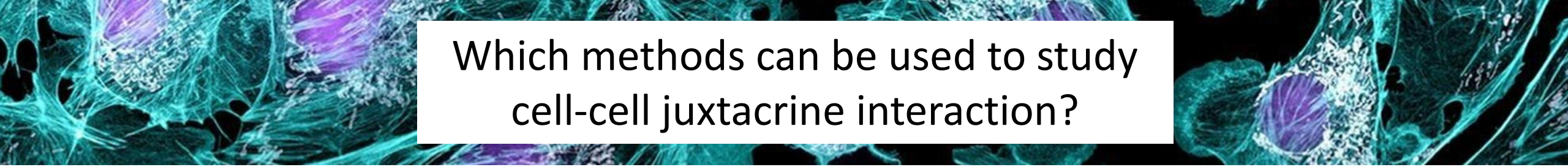
Attraction or Repulsion? Ligand or Receptor?

Repulsion by ephrin A ligands requires **CLEAVAGE**

- growth cone contact
- ectodomain shedding
- collapse and withdrawal

Repulsion by ephrin B ligands requires **TRANS-ENDOCYTOSIS** of ephrinB/EphB complexes

- growth cone contact
- trans-endocytosis
- collapse and withdrawal



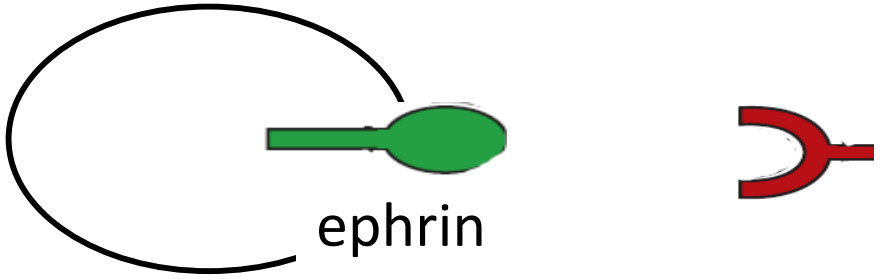
Which methods can be used to study cell-cell juxtacrine interaction?

- cells expressing the receptor stimulated with the soluble ligand
- cells expressing the ligand stimulated with the soluble receptor

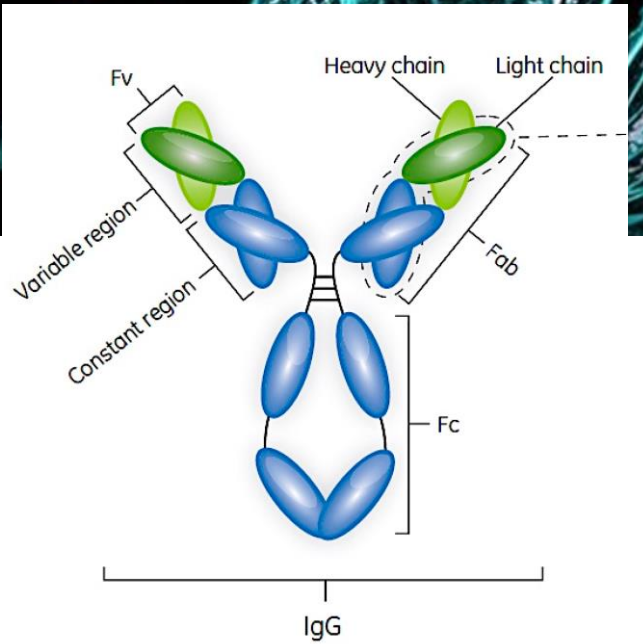
Signal transduction is usually studied by stimulating the target cells with a recombinant soluble ligand or with a recombinant soluble receptor

- cells expressing transmembrane receptor stimulated with soluble ligand

- cells expressing transmembrane ligand stimulated with soluble receptor



Fc fusion proteins



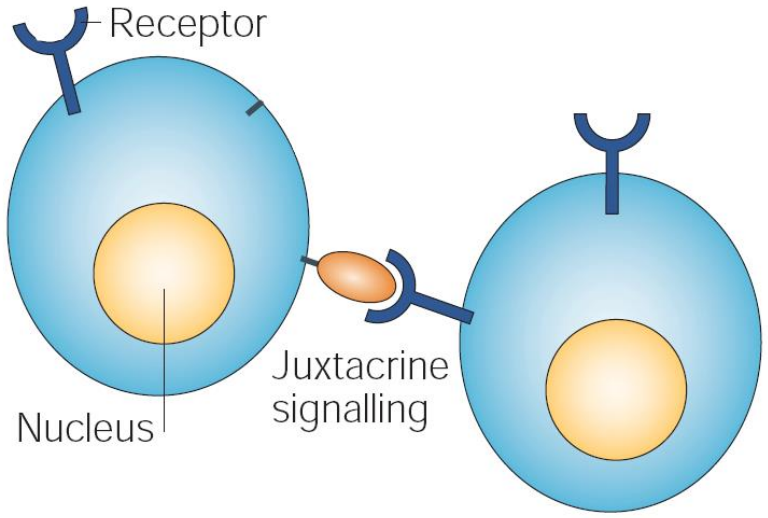
IgG-Fc tag is the constant region of **immunoglobulin heavy-chain**. It is fused to the C-terminus of a protein and it resembles a chimeric antibody. **Fc-fusion protein** is also called Fc chimeric protein.

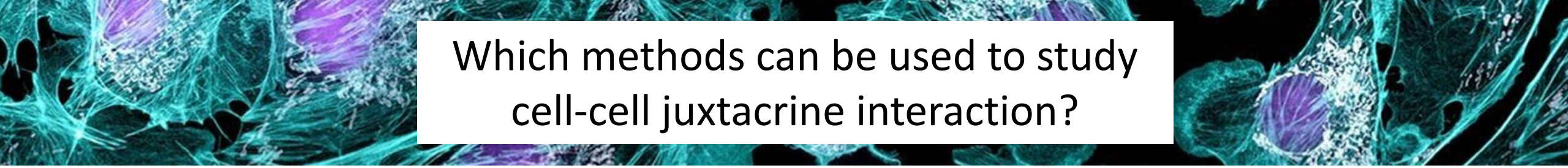
Adding an IgG-Fc tag to a protein allows **rapid and simple detection** by ELISA kit, but also assists **affinity purification** of the Fc-tagged protein by **protein A*** affinity purification resins. Adding an IgG-Fc tag to a protein often increases protein expression yield.

Fc-tagged proteins behave as dimers because IgG antibody heavy chain naturally forms dimers through the cysteine residuals. Fc region can be of any IgG antibodies from multiple species, depending on the application.

***Protein A** is a 42 kDa surface protein originally found in the cell wall of the bacteria *Staphylococcus aureus*. It is used in biochemical research because of its ability to bind immunoglobulins.

Sometime this approach is deeply different from the physiological process of intercellular communication, in part because the proteins that act as ligands for certain receptors are themselves anchored to the cell membrane



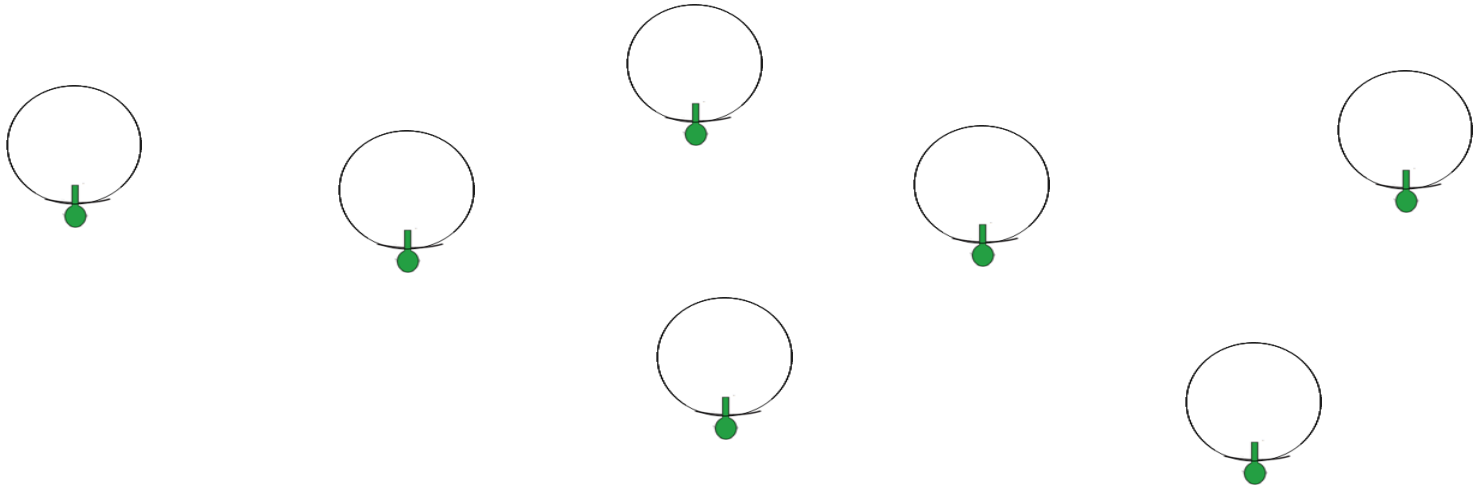


Which methods can be used to study cell-cell juxtacrine interaction?

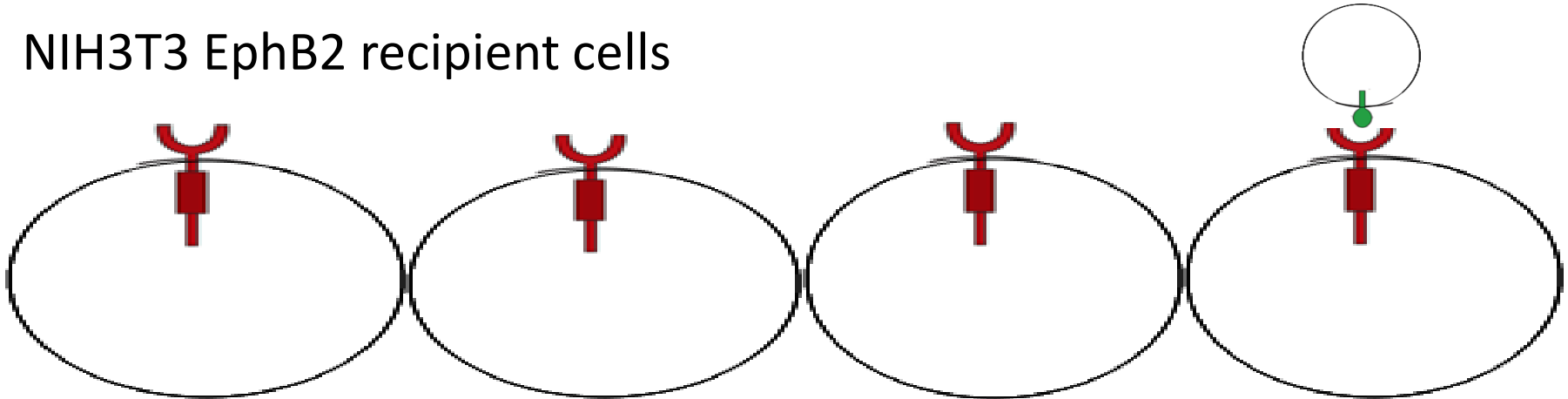
- cells expressing the receptor stimulated with the ligand
- cells expressing the ligand stimulated with the receptor
- cells expressing the receptor stimulated with cells expressing the ligand
- cells expressing the ligand stimulated with cells expressing the receptor

Stimulation of “recipient” cells expressing the receptor with cells expressing the ligand

NIH3T3 ephrin stimulator cells

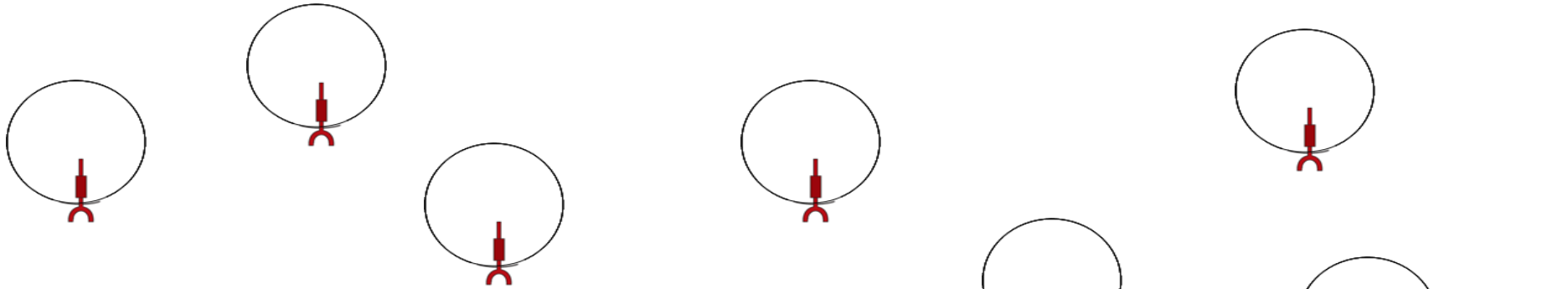


NIH3T3 EphB2 recipient cells

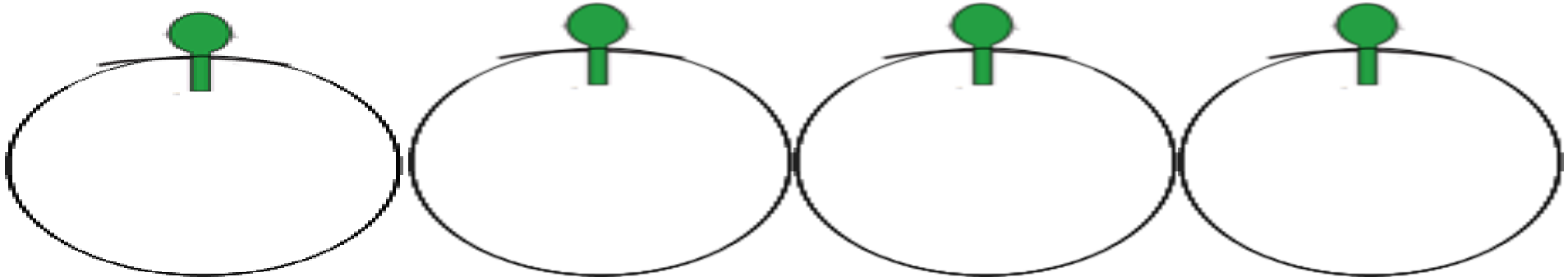


Stimulation of “recipient” cells expressing the ligand with cells expressing the receptor

NIH3T3 EphB2 stimulator cells



NIH3T3 ephrinB1 recipient cells

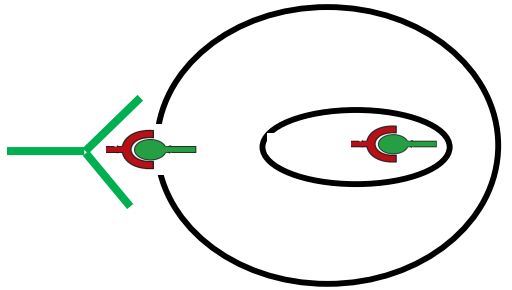


A sparse monolayer of ‘recipient cells’, is first cultured on glass cover slips. Next, ‘stimulator cells’ are taken in suspension by a *mild* treatment and added onto the recipient cells. After 10 min, all cells are fixed and stained.

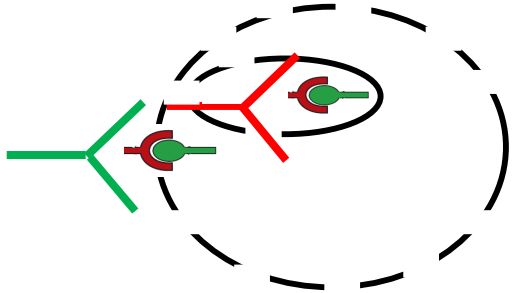
How can you distinguish between proteins on the external surface and intracellular proteins?

A sparse monolayer of ‘recipient cells’, is first cultured on glass cover slips. Next, ‘stimulator cells’ are taken in suspension by a *mild* treatment and added onto the recipient cells. After 10 min, all cells are fixed and stained.

Cells are fixed <u>in the absence of detergents</u> and immunolabelled for **ephrin on the cell surface**.



Then, cells are <u>permeabilized</u> with detergents and stained for **total ephrin** using a different primary antibody. Staining that appears exclusively after permeabilization represents the **intracellular pool of ephrin**



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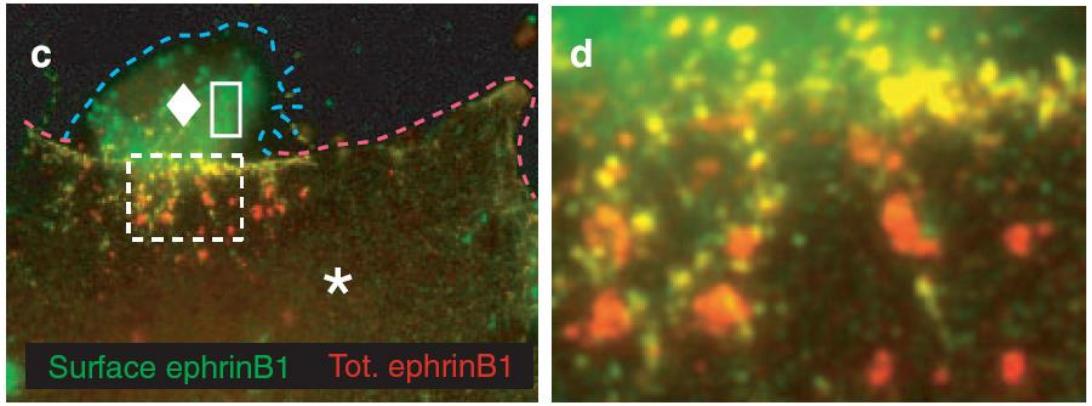
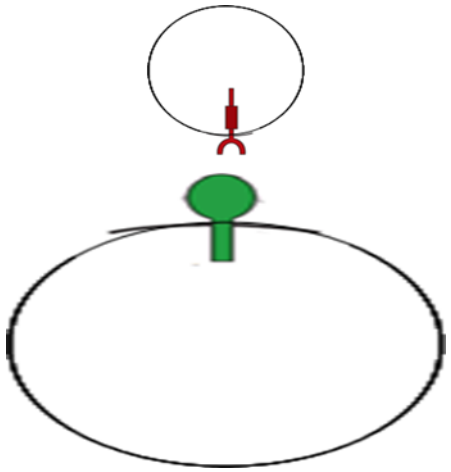
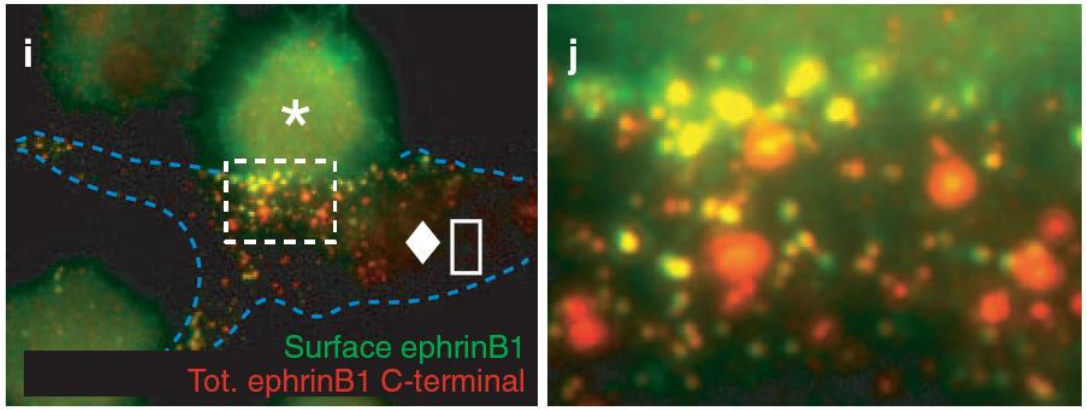
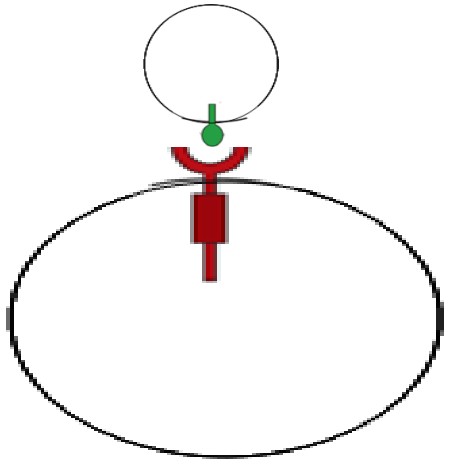




If they use 3T3 EphB2 (♦)stimulator cells with 3T3 ephrinB1 (∗) recipient cells, they observe rapid and localized co-clustering of ephrinB1 and EphB2 at the site of cell–cell contact. These clusters were partially endocytosed and the direction of internalization was in a *reverse* manner, that is, into the recipient 3T3 ephrinB1 cells

A sparse monolayer of ‘recipient cells’, is first cultured on glass cover slips. Next, ‘stimulator cells’ are taken in suspension by a mild treatment and added onto the recipient cells. After 10 min, all cells are fixed and stained.





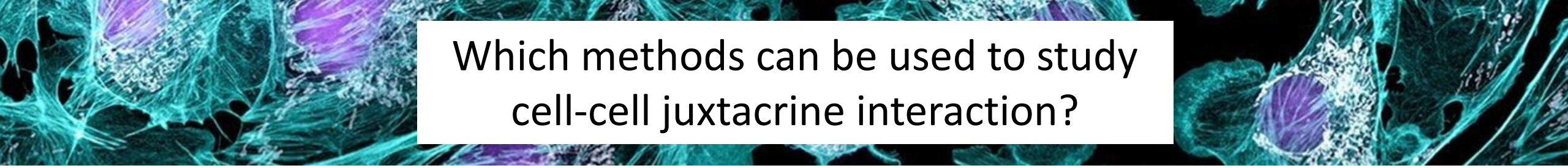
Next, they did the reverse experiment and used 3T3 ephrinB1(⁎) as stimulator cells and 3T3 EphB2 (♦) as recipient cells. EphrinB1 was internalized in a *forward* manner by 3T3 EphB2 cells

- this experiment involved the stimulation with cells in suspension
- endocytosis was predominant in the pre-plated recipient cells

Why?

- it is possible that the recipient cells have an advantage in their organization of the endocytic and membrane trafficking machinery over the freshly seeded stimulator cells as the endocytic machinery might be linked to the actin cytoskeleton

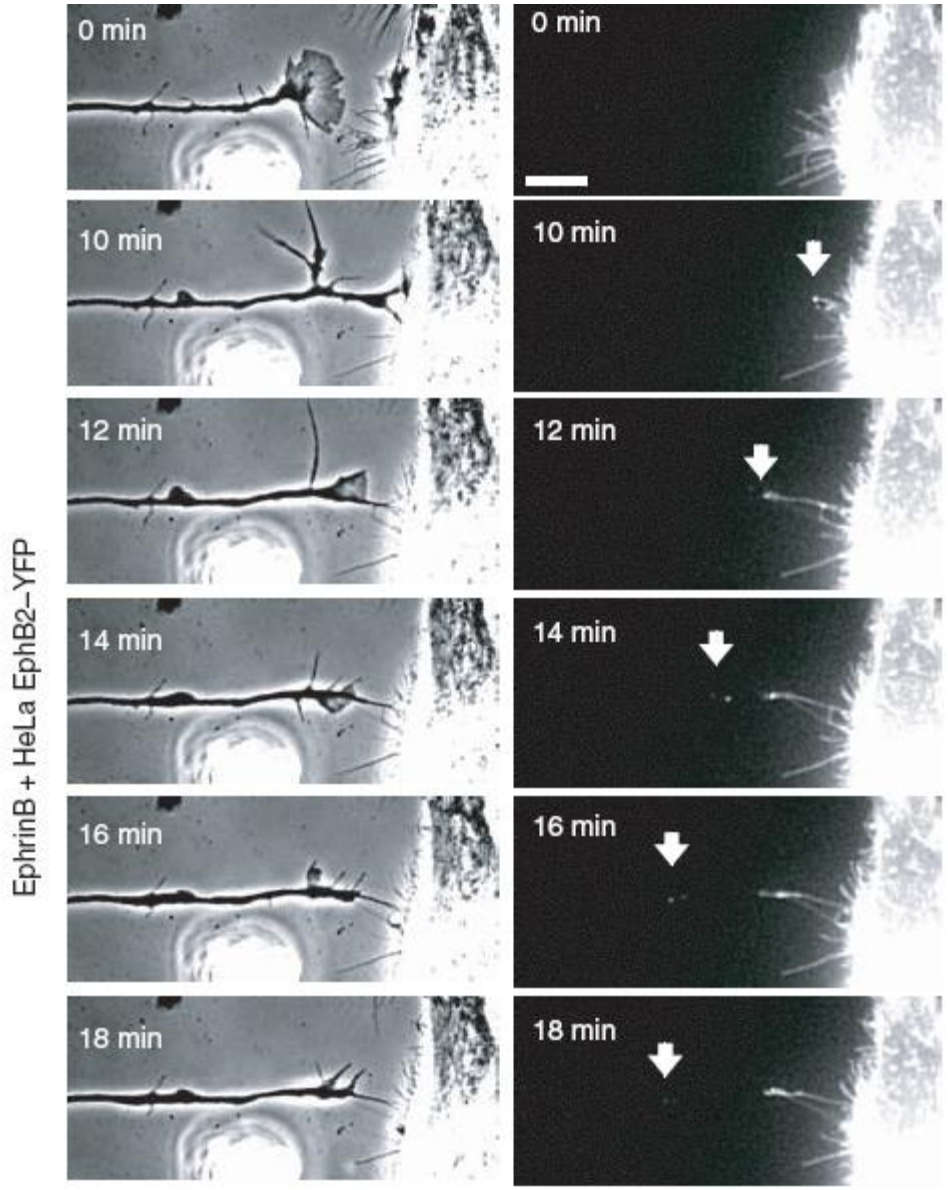
- how can you study cell-cell interactions with both cells adhering on the cell culture dish?



Which methods can be used to study cell-cell juxtacrine interaction?

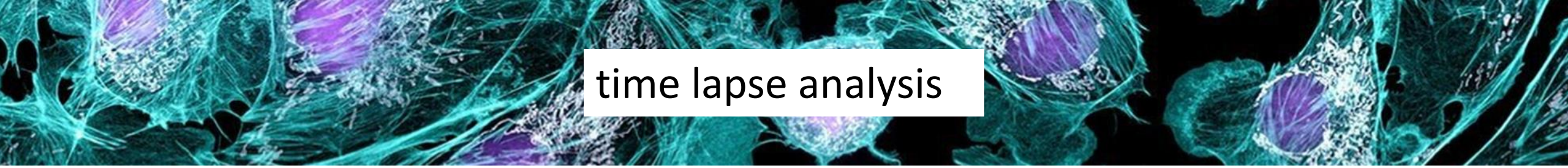
- cells expressing the receptor stimulated with the ligand
- cells expressing the ligand stimulated with the receptor
- cells expressing the receptor stimulated with cells expressing the ligand
- cells expressing the ligand stimulated with cells expressing the receptor
- time lapse analysis of the interactions between cells expressing the receptor with cells expressing the ligand

EphrinB1 and EphB2 uptake and transport by primary neurons



Forebrain neurons from E14.5 mouse embryos were co-cultured with HeLa cells transiently expressing **EphB2–YFP**. Growth cones were imaged by time-lapse microscopy at 1 frame per min. The images show a neuronal growth cone before contact with a HeLa cell and collapse of the growth cone within 10 min after contact. At the time of collapse, a fluorescent cluster of EphB2 forms at the tip of a single protrusion of the HeLa cell (arrow at 10 min). The growth cone partially retracts and pulls a protrusion out. Two EphB2 clusters are retrogradely transported into the neurite (arrows).

These results demonstrate that the full-length EphB2 receptor is taken up by the neuron, probably owing to ephrinB reverse endocytosis in the growth cones.

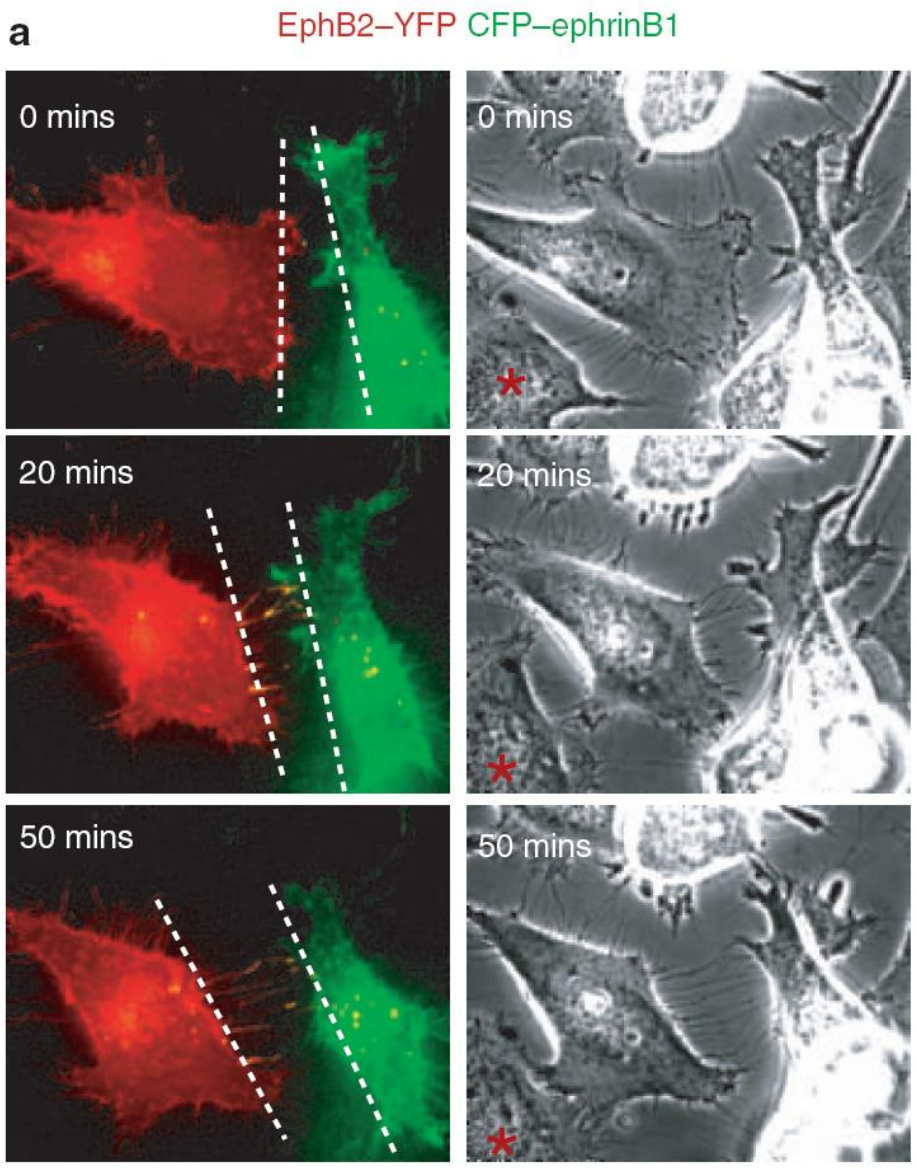


time lapse analysis

• to determine whether bi-directional endocytosis affects repulsive cell migration, an *in vitro* assay was developed in which cells expressing fluorescently tagged EphB2 receptor (**EphB2–YFP**) were co-cultured with cells expressing fluorescently tagged ephrinB1 (**CFP–ephrinB1**)

• HeLa cells were chosen because they express low levels of endogenous ephrinB and EphB proteins and high levels of transfected proteins; they are also very motile, which makes them ideal for fluorescence time-lapse imaging.

Bi-directional trans-endocytosis regulates repulsion response and cell detachment



HeLa cells were transiently transfected with full-length **EphB2–YFP** and full-length **CFP–ephrinB1** and then cocultured before time-lapse imaging.

Left, selected fluorescence images with **EphB2–YFP** in **red** and **CFP–ephrinB1** in **green**.

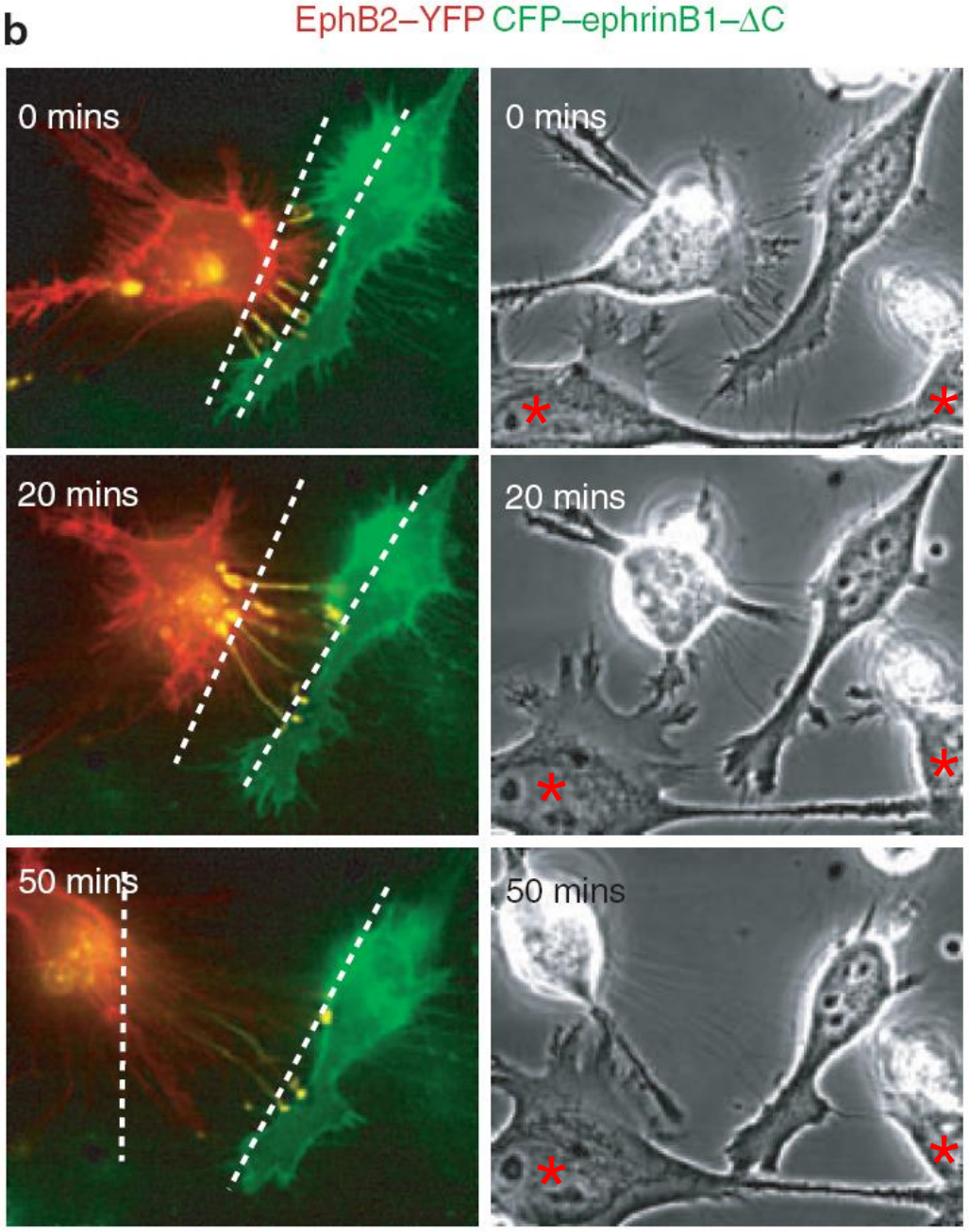
Right, phase contrast images.

Intense clustering of EphB2 and ephrinB1 is seen in **yellow** at the contact site between the two cells at 20 min, the **EphB2–YFP** cell retracts a lamellipodium from the **ephrinB1 cell** (indicated by the distance between the two stippled lines).

- in almost all observed cases, when a ruffling lamellipodium of an **EphB2–YFP** cell collides with an **CFP–ephrinB1** cell, strong co-clustering of receptor with ligand occurs within 1 min and the initial clusters always appear in filopodia-like protrusions.

- during the retraction of **EphB2–YFP** positive lamellipodia, receptor–ligand complexes endocytose bi-directionally

- contacts of **EphB2–YFP-** or **CFP–ephrinB1-**transfected cells with untransfected cells in the same culture do not result in clustering nor cell retraction (asterisks in the figure)



HeLa cells were transiently transfected with full length **EphB2–YFP** and C-terminally truncated **CFP–ephrinB1-ΔC** then cocultured before time-lapse imaging.

Left, selected fluorescence images with **EphB2–YFP** in **red** and **CFP–ephrinB1- ΔC** in **green.**

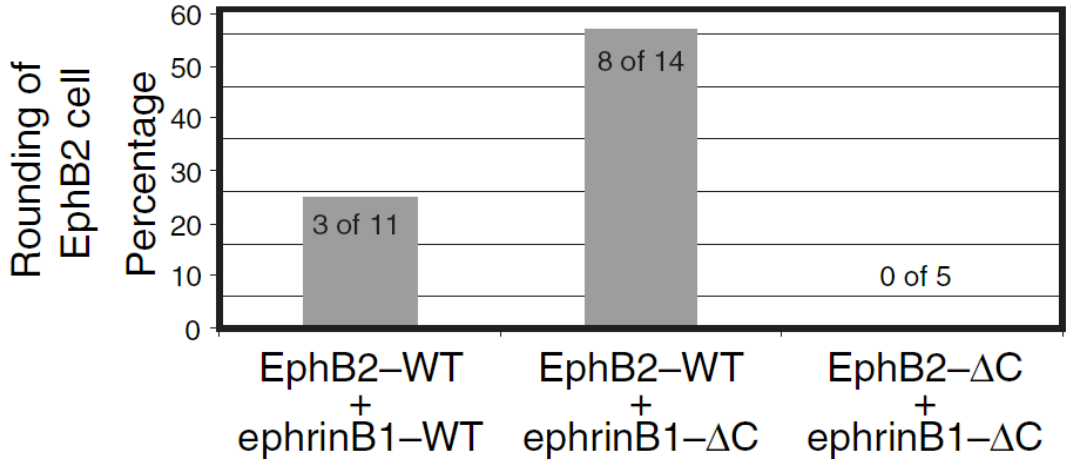
Right, phase contrast images.

EphB2–YFP clusters (in **yellow**) are uni-directionally endocytosed into the **EphB2–YFP** expressing cell. Strong repulsion and rounding of **EphB2–YFP** expressing cell is observed.

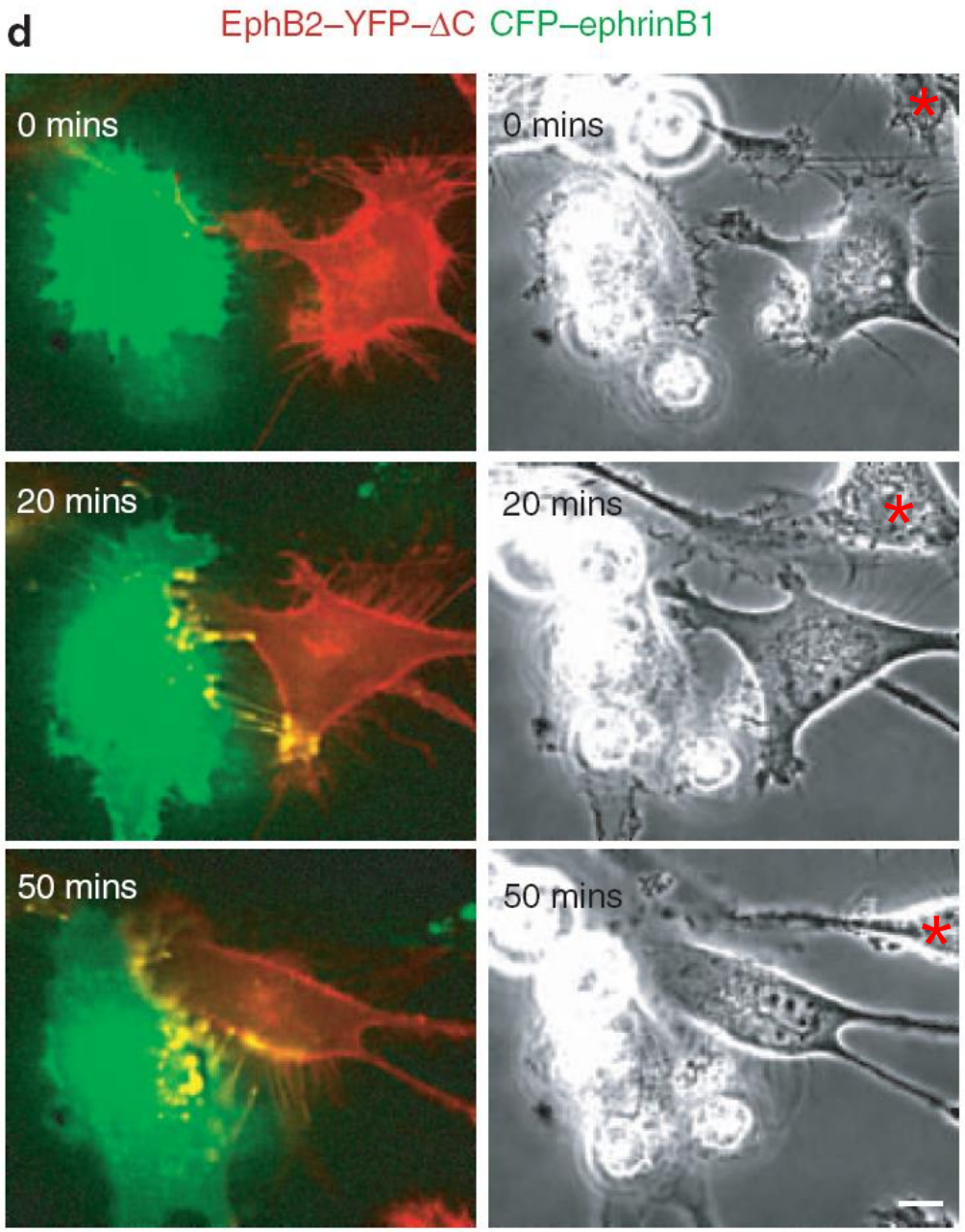
• when ephrinB1 endocytosis was blocked by a C-terminal truncation (**CFP–ephrinB1-ΔC**), markedly different cell behaviour was observed

• rapid co-clustering with **EphB2–YFP** occurs after contact, but these clusters remain in part localized to the surface of the ligand expressing cell, where they grow to much larger complexes

• the **EphB2–YFP** cell engulfs the clusters vigorously, retracts strongly, and in most cases even rounds up, a behaviour rarely observed with wild-type ephrinB1



→ therefore, a mutation that blocks ephrinB1 endocytosis results in a stronger EphB2 cell retraction response



HeLa cells were transiently transfected with C-terminally truncated **EphB2–YFP- ΔC** and full length **CFP–ephrinB1** then cocultured before time-lapse imaging.

Left, selected fluorescence images with **EphB2–YFP- ΔC** in **red** and **CFP–ephrinB1** in **green.**

Right, phase contrast images.

EphB2–YFP clusters (in **yellow**) are strongly uni-directionally endocytosed into the **CFP–ephrinB1** expressing cell.

Otherwise normal cell behaviour similar to un-transfected cells is observed.

How do cells react to unidirectional ephrinB reverse signalling?

- as expected, **CFP–ephrinB1** cells strongly endocytose receptor–ligand clusters, whereas **EphB2–YFP- ΔC** cells fail to endocytose these complexes

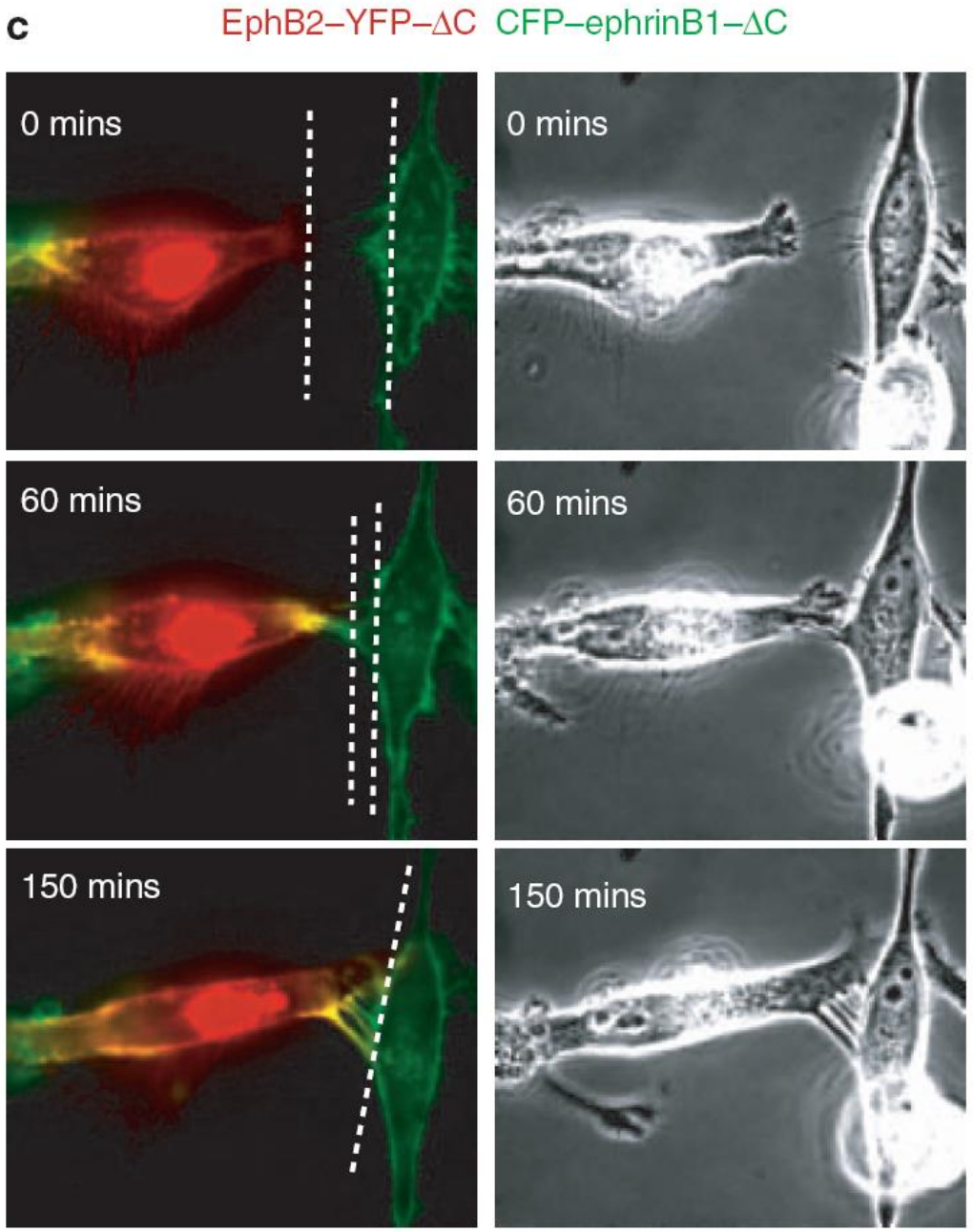
- however, the cells **neither retract nor adhere** to each other

- cell behaviour is indistinguishable from non-transfected cells

→ **ephrinB1 reverse trans-endocytosis is sufficient to terminate adhesion and to cause cell detachment**

****

What happens when cells are transfected with C-terminally truncated **EphB2–YFP- ΔC** and **CFP–ephrinB1-ΔC** **?**



HeLa cells were transiently transfected with C-terminally truncated **EphB2–YFP- ΔC** and **CFP–ephrinB1-ΔC** then co-cultured before time-lapse imaging.

Left, selected fluorescence images with **EphB2–YFP- ΔC** in **red** and **CFP–ephrinB1- ΔC** in **green.**

Right, phase contrast images.

Cells strongly adhere to each other forming large fascicles filled with **EphB2–YFP- ΔC** and **CFP–ephrinB1-ΔC** **yellow** complexes

• when both ephrinB1 and EphB2 are truncated at the C-terminal (**EphB2–YFP- ΔC** and **CFP–ephrinB1-ΔC**), the cells strongly adhere to each other and large receptor-and ligand-bearing fascicles are formed at the contact zone

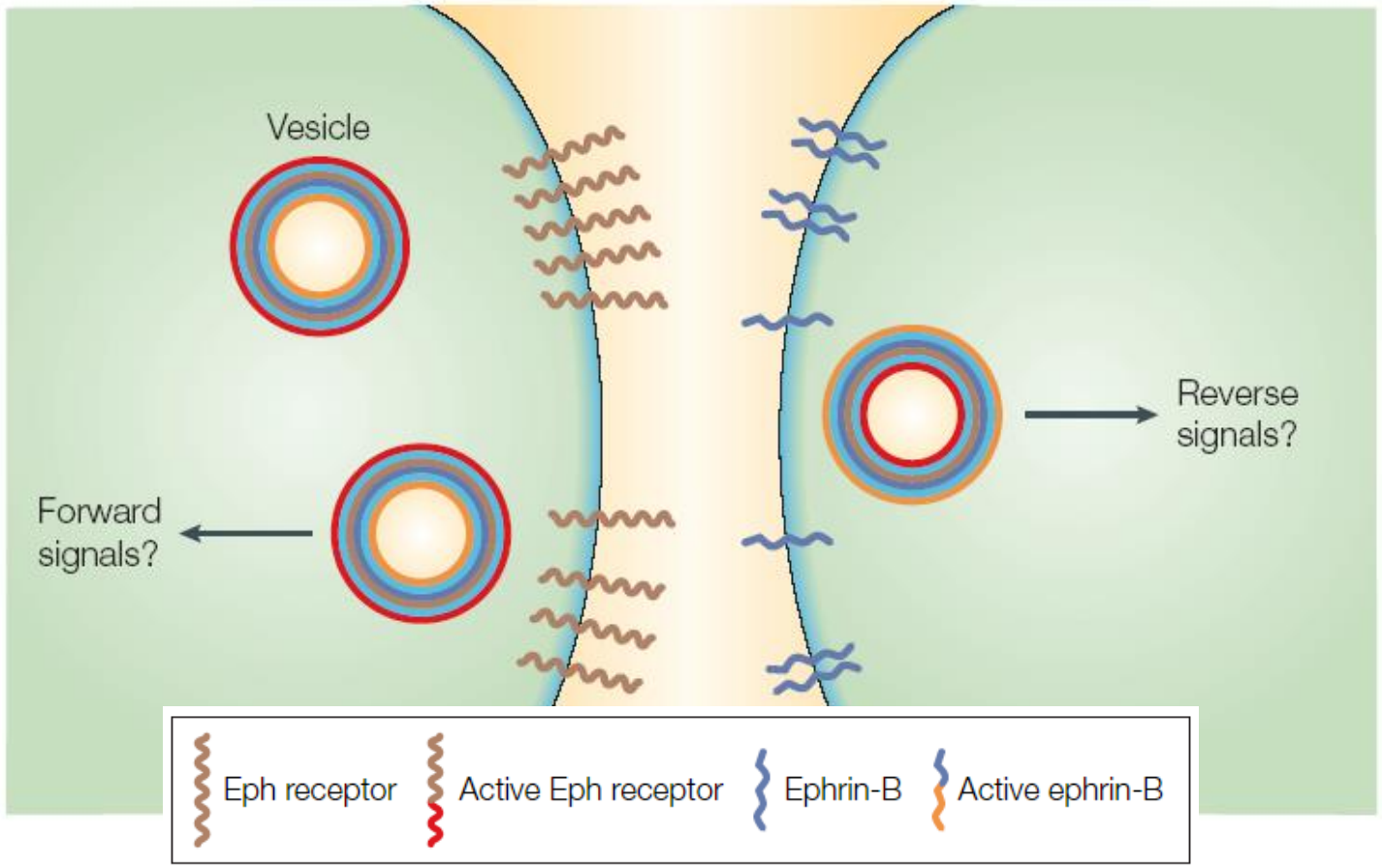
→ **ephrinB and EphB proteins can function as adhesion molecules if endocytosis and other signalling events are blocked.**

Conclusions

- EphB2 forward signalling induces forward endocytosis of EphB2–ephrinB1 complexes **and** a lamellipodial retraction response
- ephrinB1 reverse signalling only mediates reverse endocytosis
- in the absence of reverse endocytosis a gain-of-function phenotype is observed: enhancement of repulsion by EphB receptor forward signalling
- ephrinB–EphB complexes endocytosis occurs in a bi-directional fashion involving full-length proteins: one of the interaction partners is trans-cytosed from one cell to its neighbour

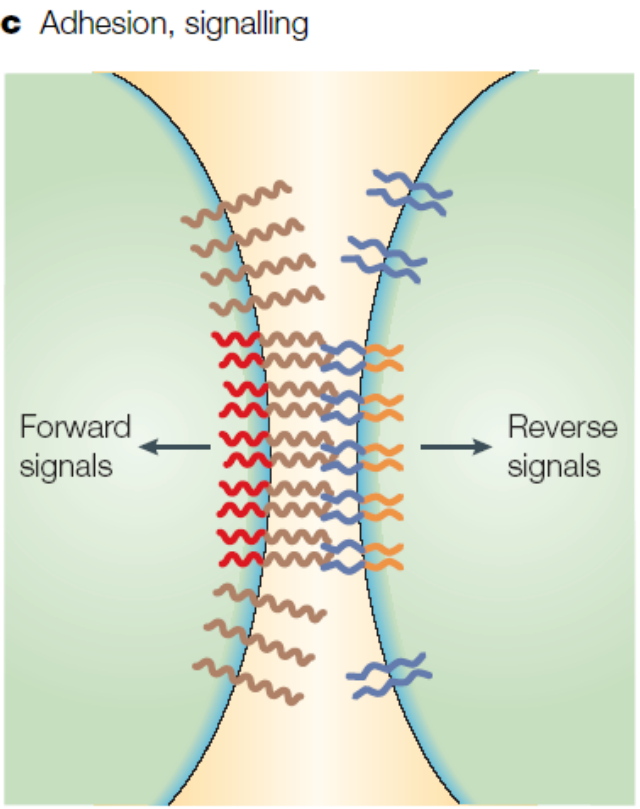
- **the relative contribution of reverse *versus* forward endocytosis may largely depend on cellular context**
- the underlying mechanism of EphB2 endocytosis may resemble phagocytosis

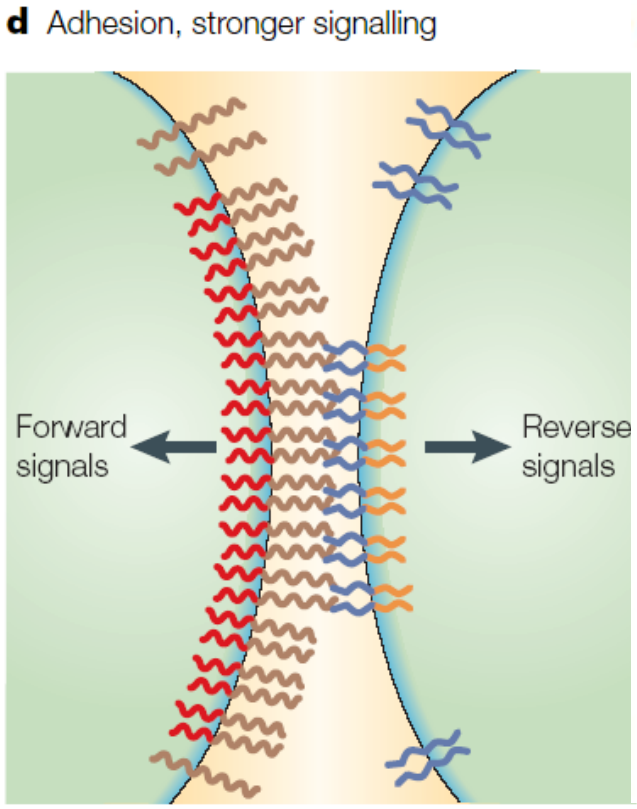
Mechanisms of Eph signal attenuation and termination

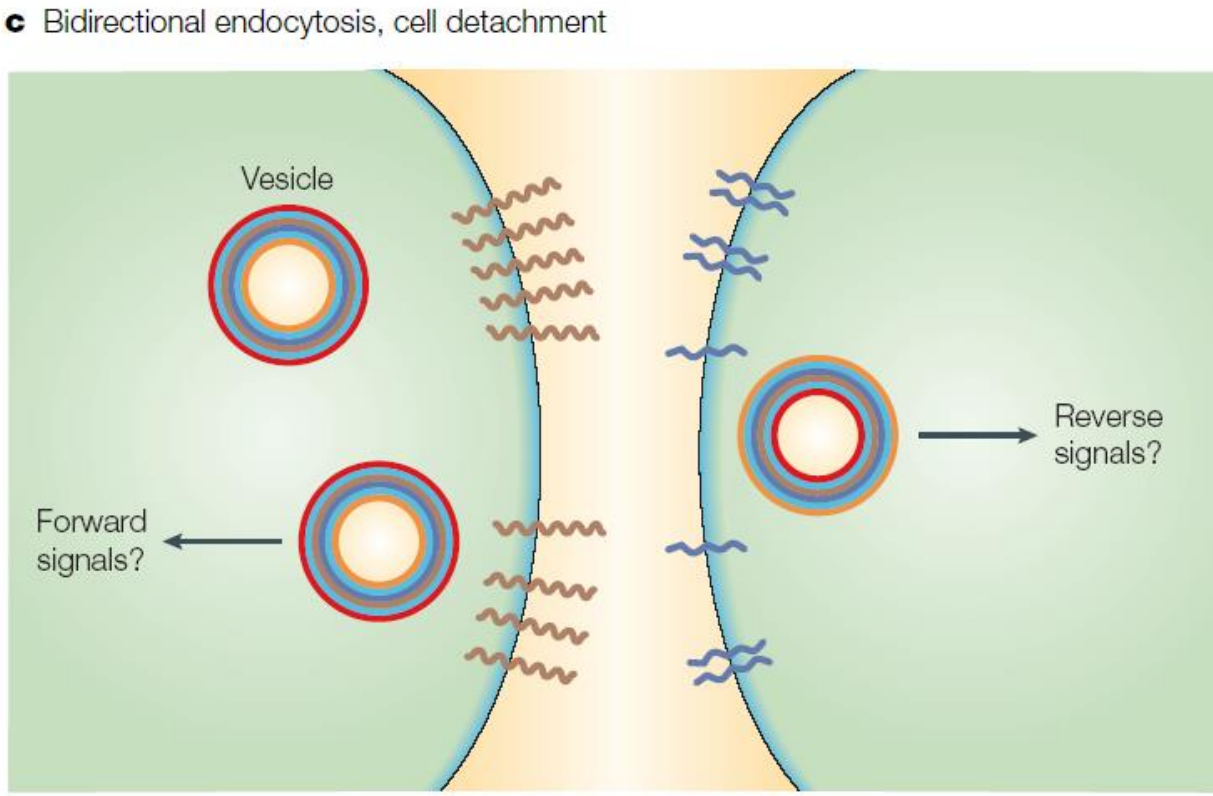


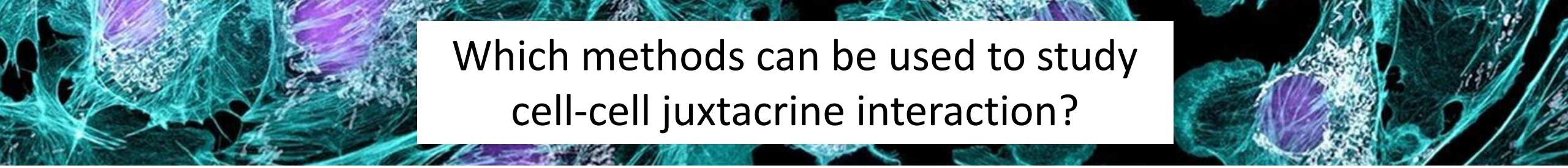
Internalization of Eph-receptor–ephrin complexes together with their surrounding plasma membranes, which can occur into the receptor- and the ligand-expressing cell, allows disengagement of the two cells and gives rise to internalized double-membrane vesicles.

→ **TRANS-ENDOCYTOSIS** may provide an alternative mechanism for the removal of ligand–receptor complexes from the surface









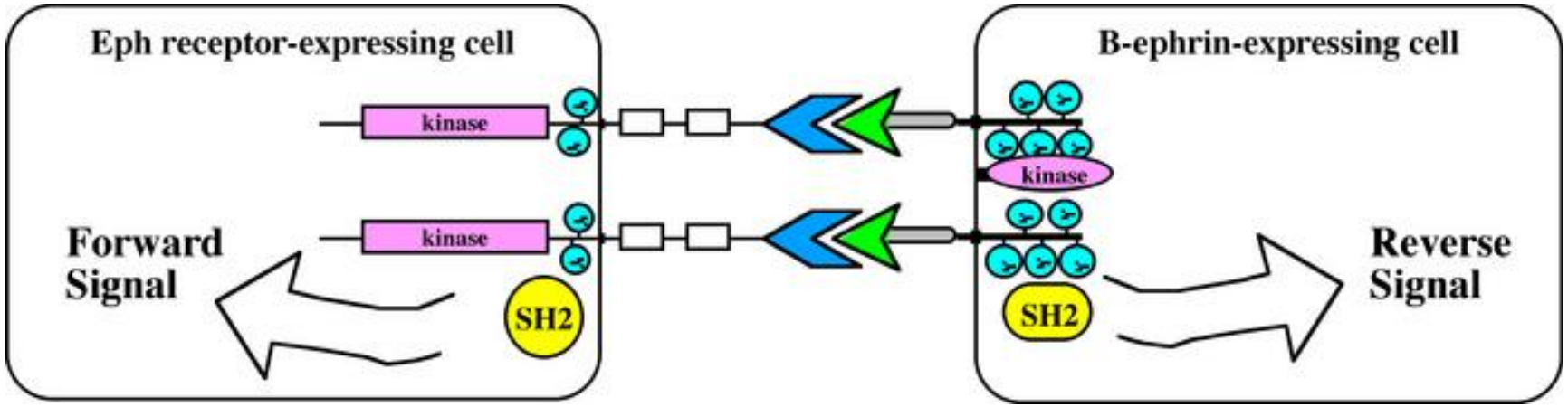
Which methods can be used to study cell-cell juxtacrine interaction?

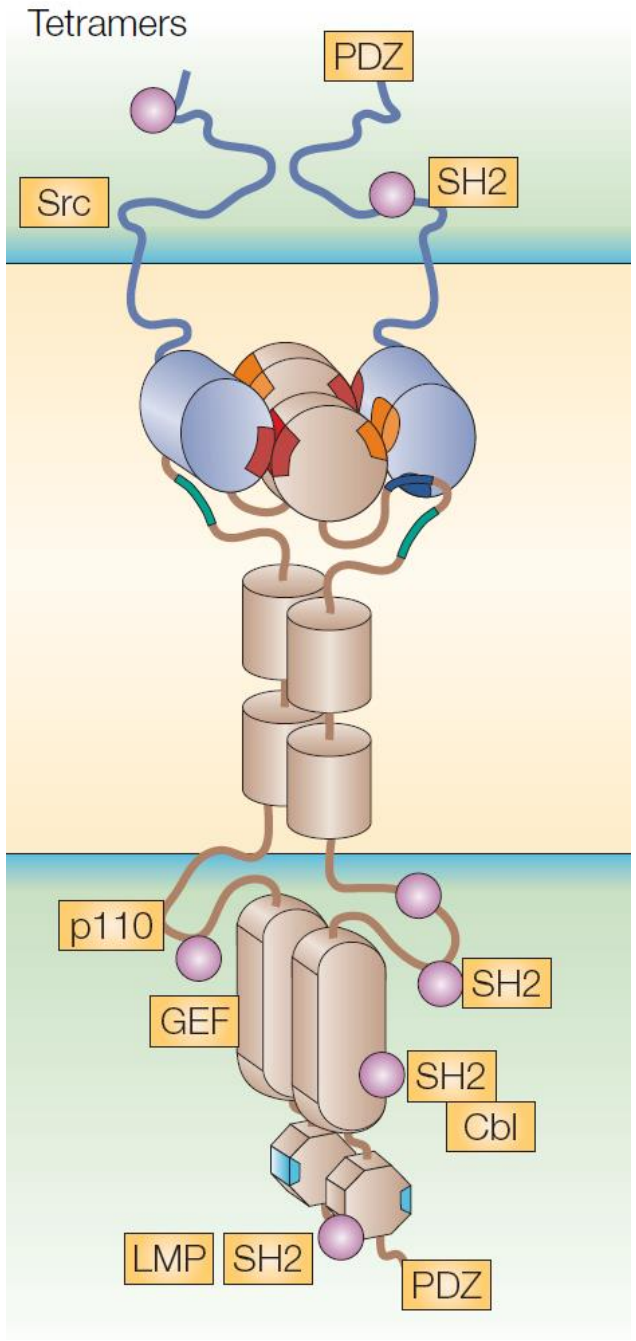
- cells expressing the receptor stimulated with the ligand
- cells expressing the ligand stimulated with the receptor
- cells expressing the receptor stimulated with cells expressing the ligand
- cells expressing the ligand stimulated with cells expressing the receptor
- time lapse analysis of the interactions between cells expressing the receptor with cells expressing the ligand
- quantitative bidirectional signalling

❁ direct interactions between transmembrane Eph receptor tyrosine kinases (EphRs) and their membrane bound ephrin ligands frequently lead to mutual **cell repulsion** and are important for **axon guidance** and **boundary formation** during tissue development

❁ clustering of B-type EphRs and ephrins at the surface of adjacent cells activates phosphotyrosine (pTyr) signaling in both the EphR- and ephrin-expressing cells, termed **forward** and **reverse** signaling, respectively

❁ systematic analysis of cell-specific networks in distinct populations of interacting cells is challenging primarily because the unique properties of each cell type are lost once co-cultured cells are processed for biochemical analysis, such as by immunoblotting





?

How can you quantitatively study the signal transduction pathways in two adjacent interacting cells?

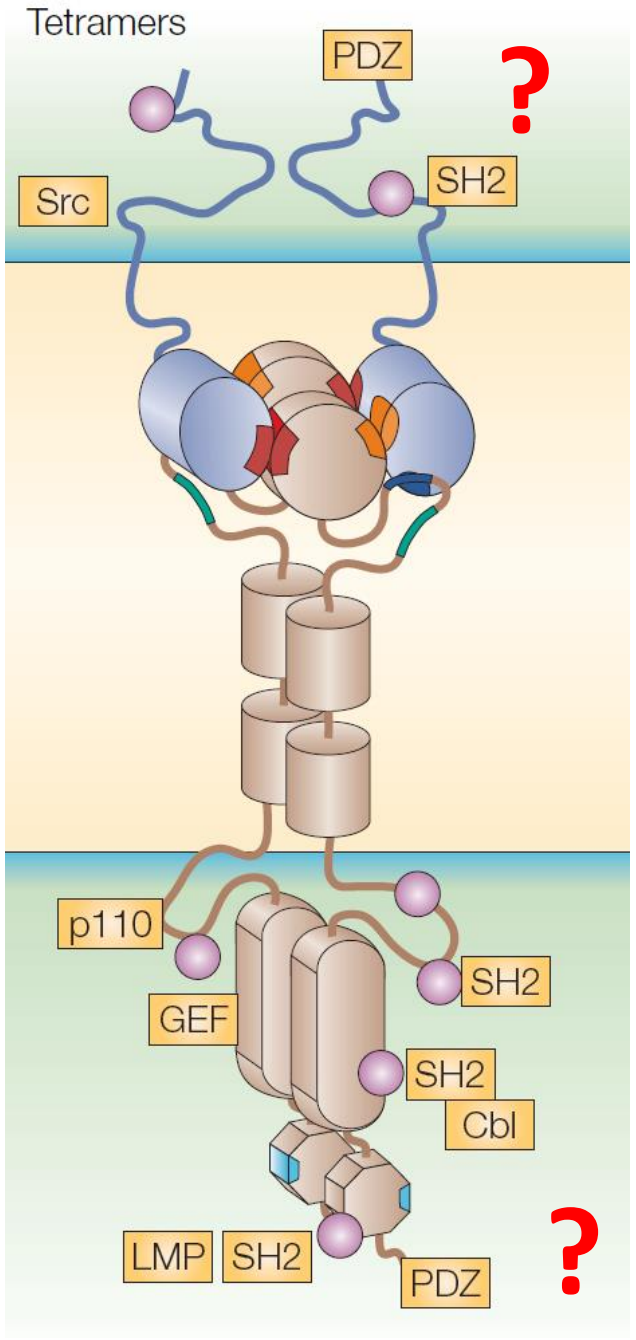
?

-> by quantitative bidirectional signalling analysis

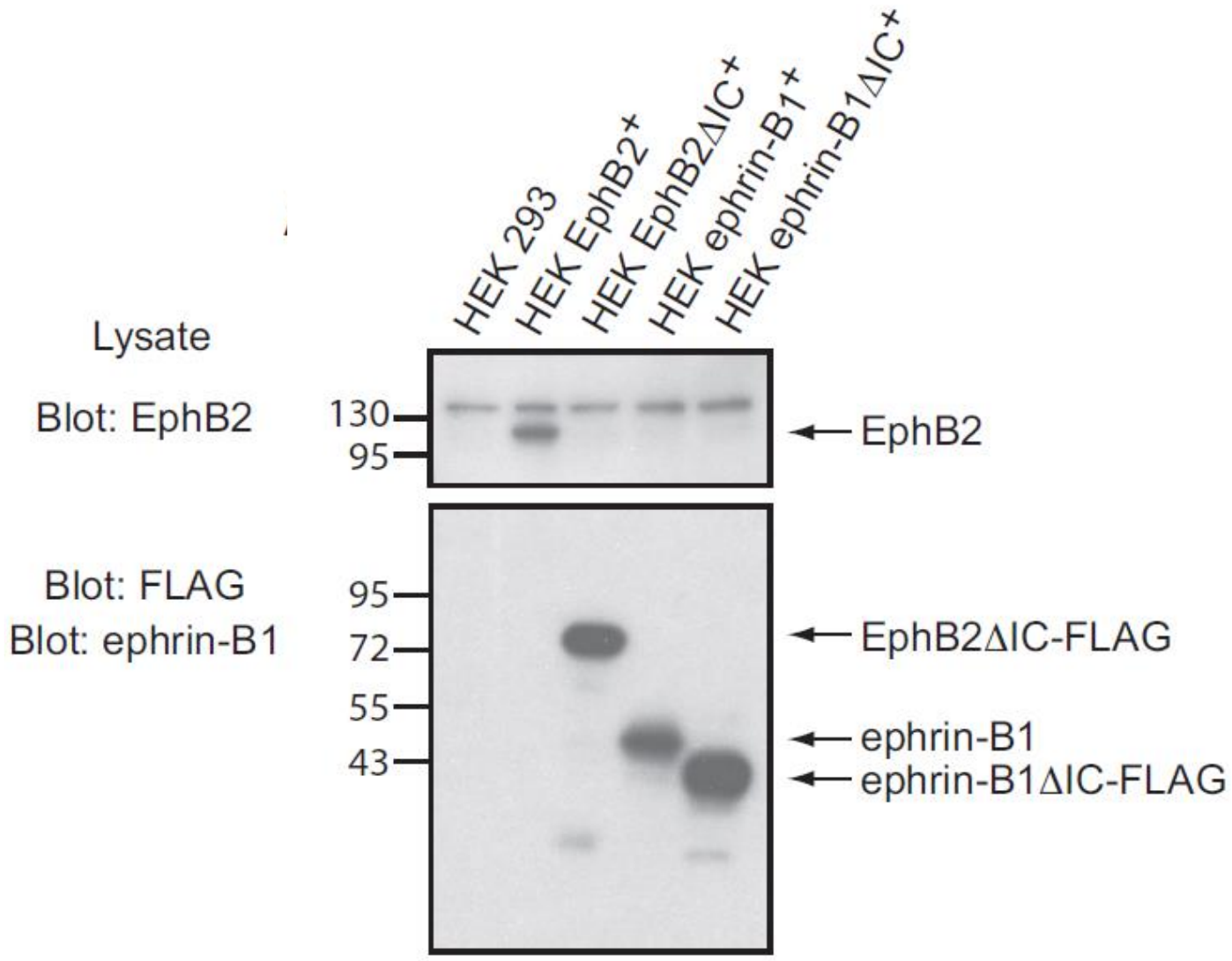
11 DECEMBER 2009 VOL 326 **SCIENCE** www.sciencemag.org

Cell-Specific Information Processing in Segregating Populations of Eph Receptor Ephrin–Expressing Cells

Claus Jørgensen,[1] Andrew Sherman,[1,2] Ginny I. Chen,[1,2] Adrian Pasculescu,[1] Alexei Poliakov,[3] Marilyn Hsiung,[1] Brett Larsen,[1] David G. Wilkinson,[3] Rune Linding,[4]* Tony Pawson[1,2]*



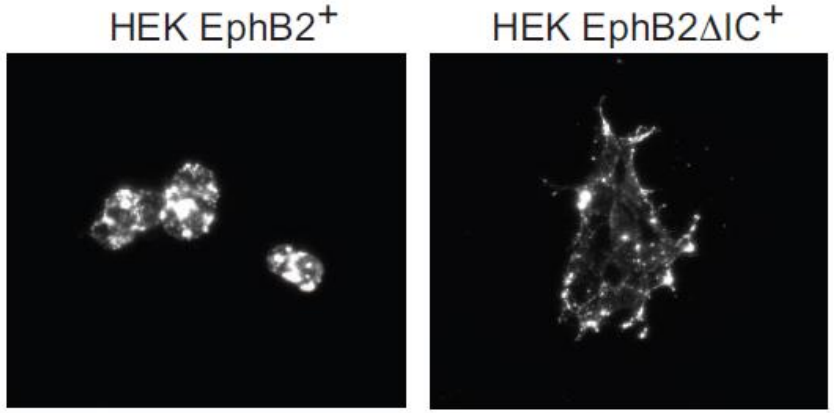
Quantitative analysis of Bidirectional Signaling (qBidS)



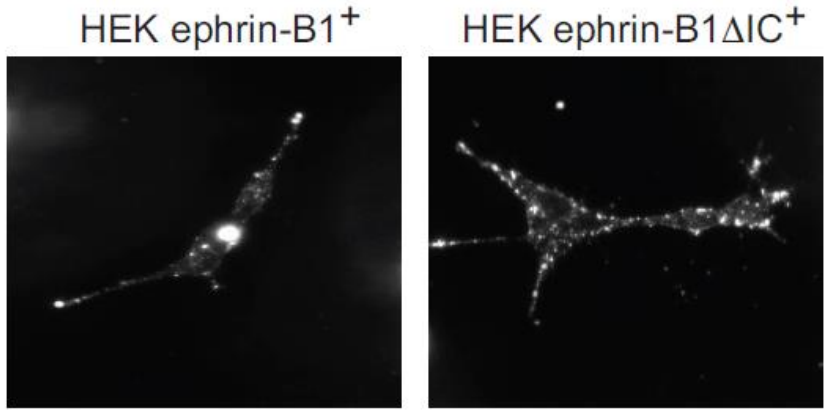
To study bidirectional EphR-ephrin signaling, they used the human embryonic kidney (HEK)293 cell line engineered to express either EphB2 (EphB2$^+$ cells) or ephrin-B1 (ephrin-B1$^+$ cells).

How can you verify if the protein is correctly expressed in the membrane?

Quantitative analysis of Bidirectional Signaling (qBidS)



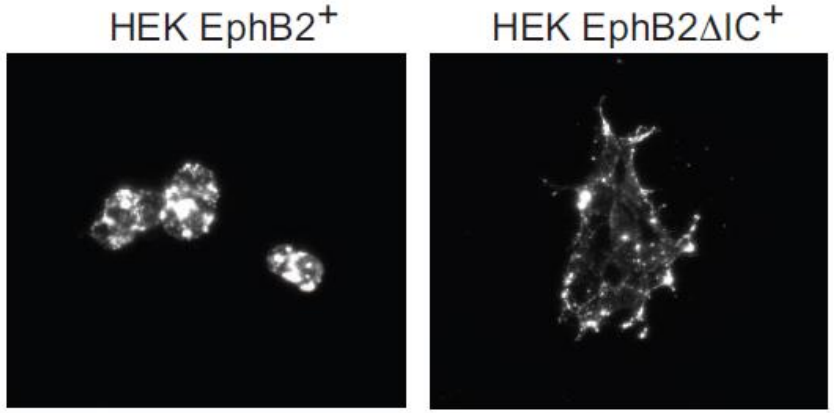
ephrin-B1-Fc alexa 488



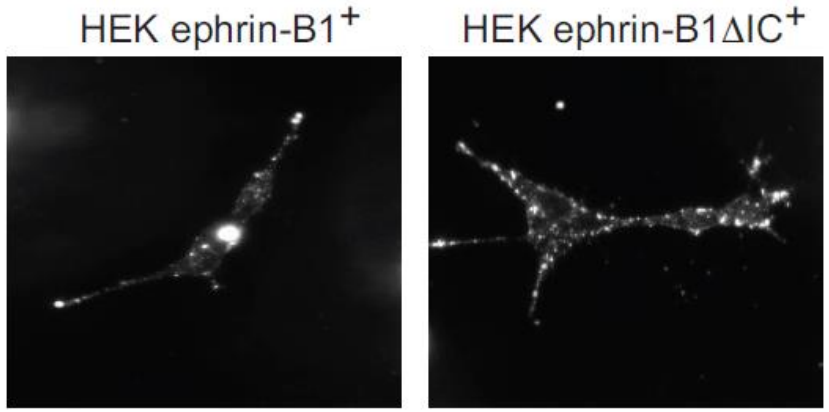
EphB2-Fc alexa 488

Relative surface levels of these proteins were tested using immunofluorescence (IF).
Ectodomain-Fc fusions of EphB2 or ephrin-B1 were labeled with Alexa-488 and used to mark surface exposed Eph receptor or ligand in live cells.
Analysis by IF revealed similar surface levels of wild type or mutant forms of EphB2 and ephrin-B1 in all stable cell lines used.

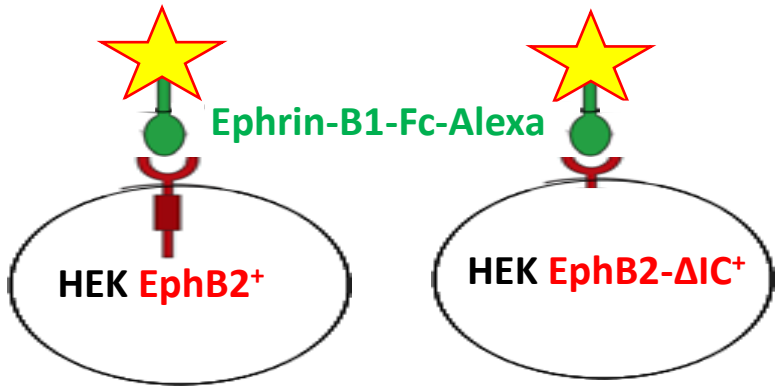
Quantitative analysis of Bidirectional Signaling (qBidS)

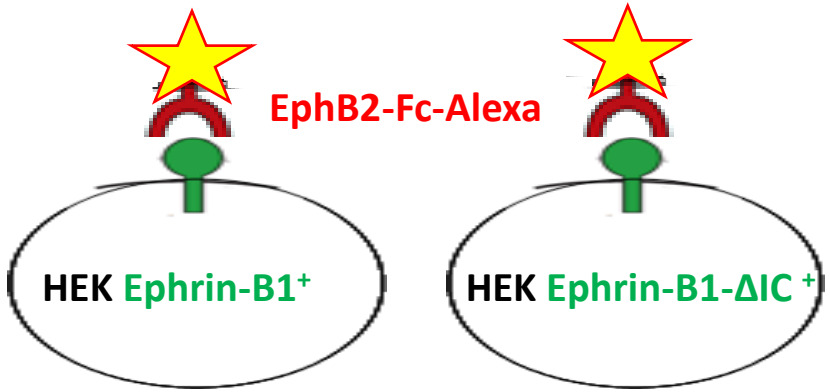


ephrin-B1-Fc alexa 488

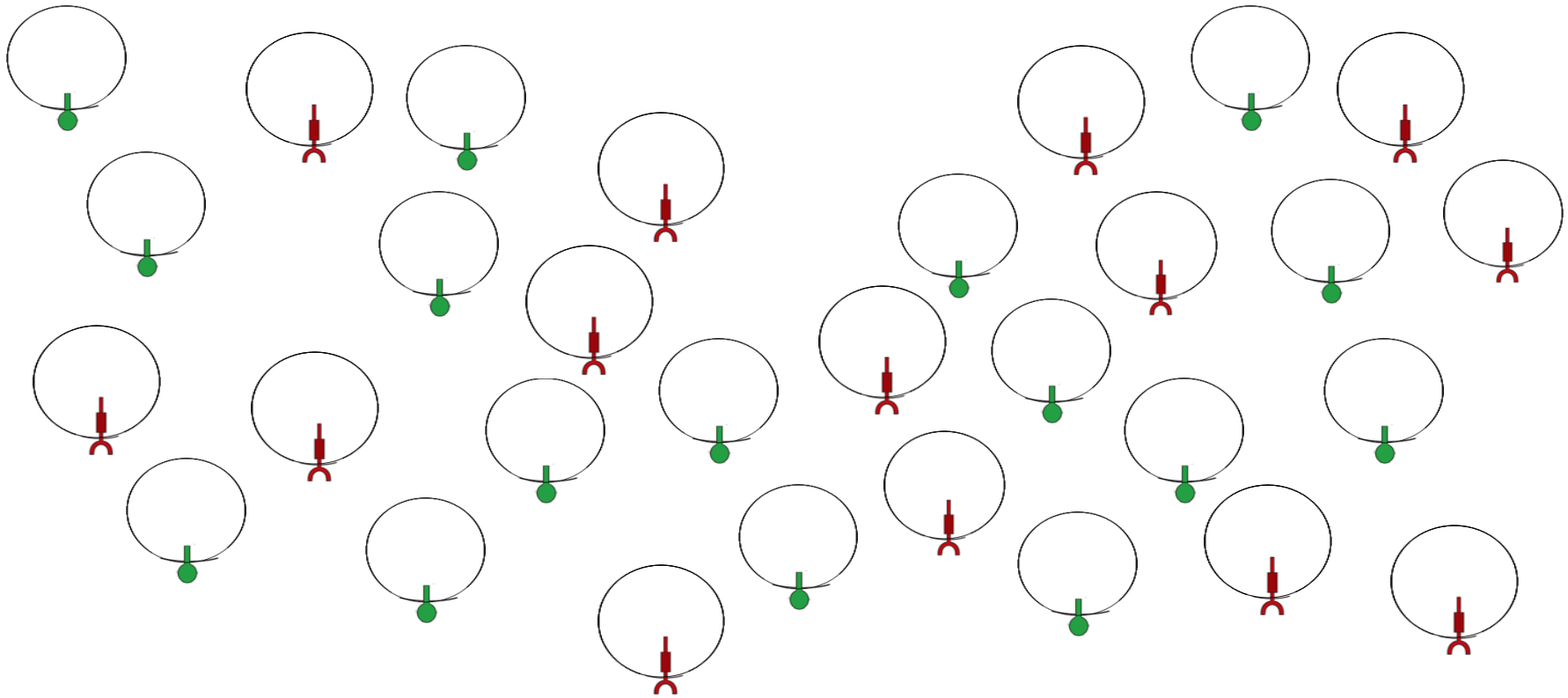


EphB2-Fc alexa 488



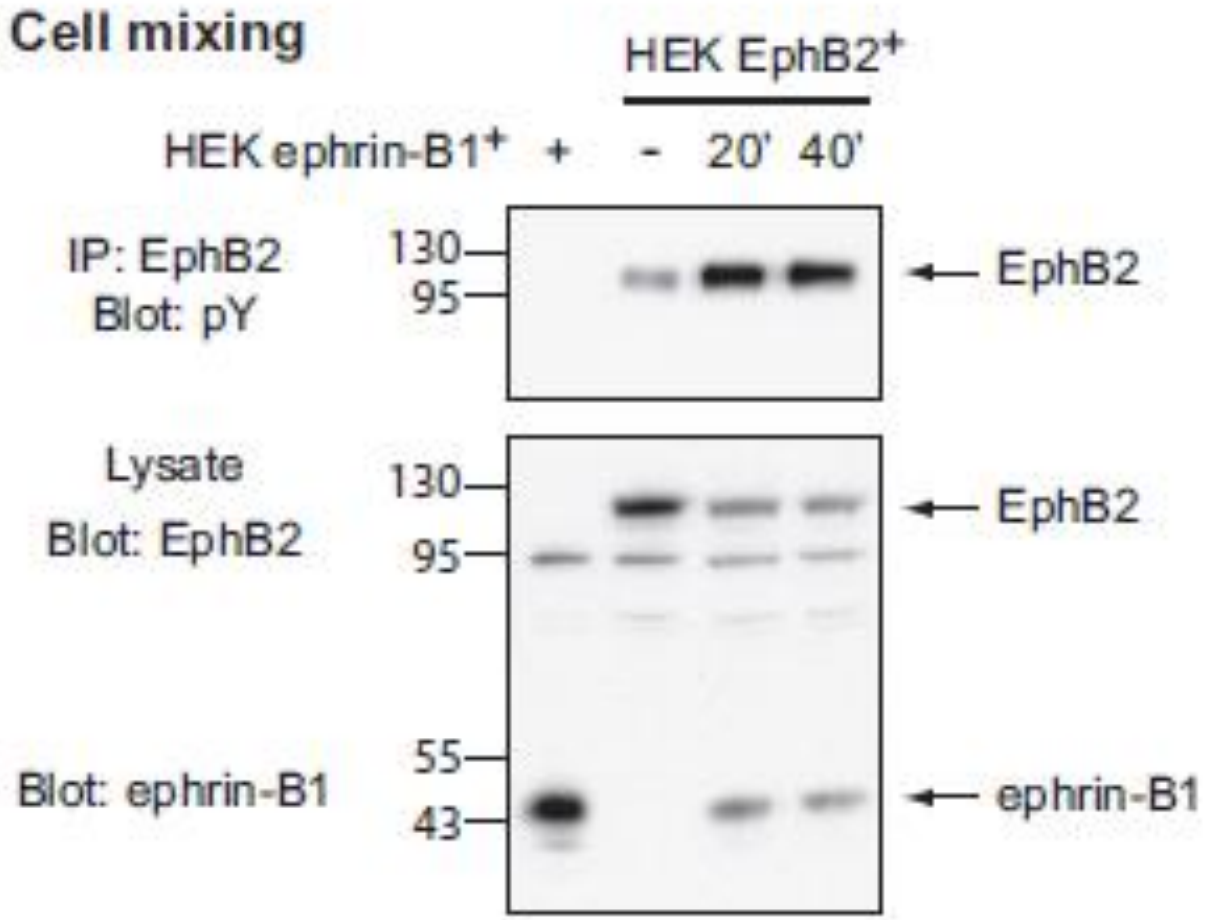


To verify the ability of the EphB2$^+$ and ephrin-B1$^+$ cells to induce a pTyr response, signaling was initiated by cell mixing.
EphB2$^+$ cells were mixed with **ephrin-B1$^+$ cells** for 20 or 40 minutes.

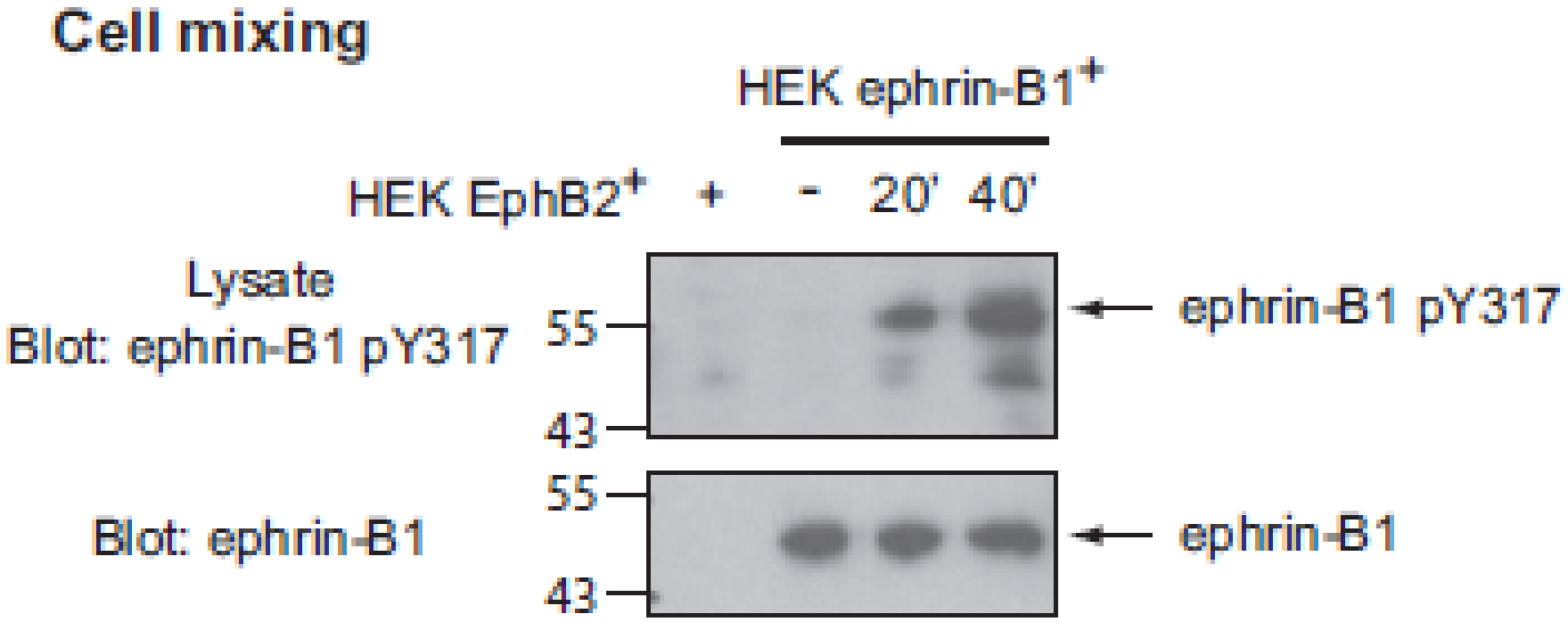


Ephrin -B1$^+$ stimulator cells + **EphB2$^+$ stimulator cells**

• EphB2^{+} cells were mixed with ephrin-B1^{+} cells for 20 or 40 minutes.
• Tyrosine phosphorylation of EphB2 was analyzed by immunoprecipitation of EphB2 followed by blotting with anti-pTyr antibody (pY).
• Equal loading of EphB2 or ephrin-B1 was assessed by blotting cell lysates with anti-EphB2 or anti-ephrin-B1 antibodies.

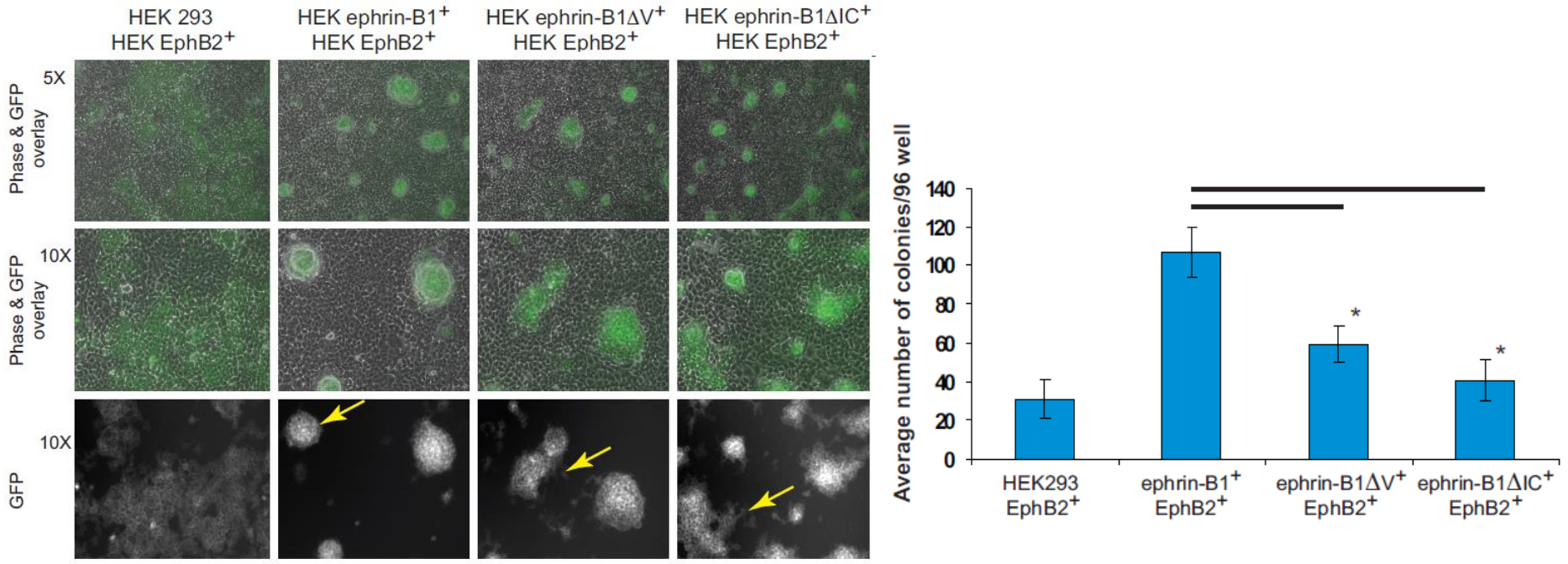


• Tyrosine phosphorylation of ephrin-B1 (Y317) was analyzed by immunoblotting with a phospho-specific antibody to this site.
• Equal loading of ephrin-B1 was assessed by blotting cell lysates with anti-ephrin-B1 antibodies.



There is extensive tyrosine phosphorylation of both ephrin-B1 and EphB2, demonstrating a functional tyrosine kinase response.

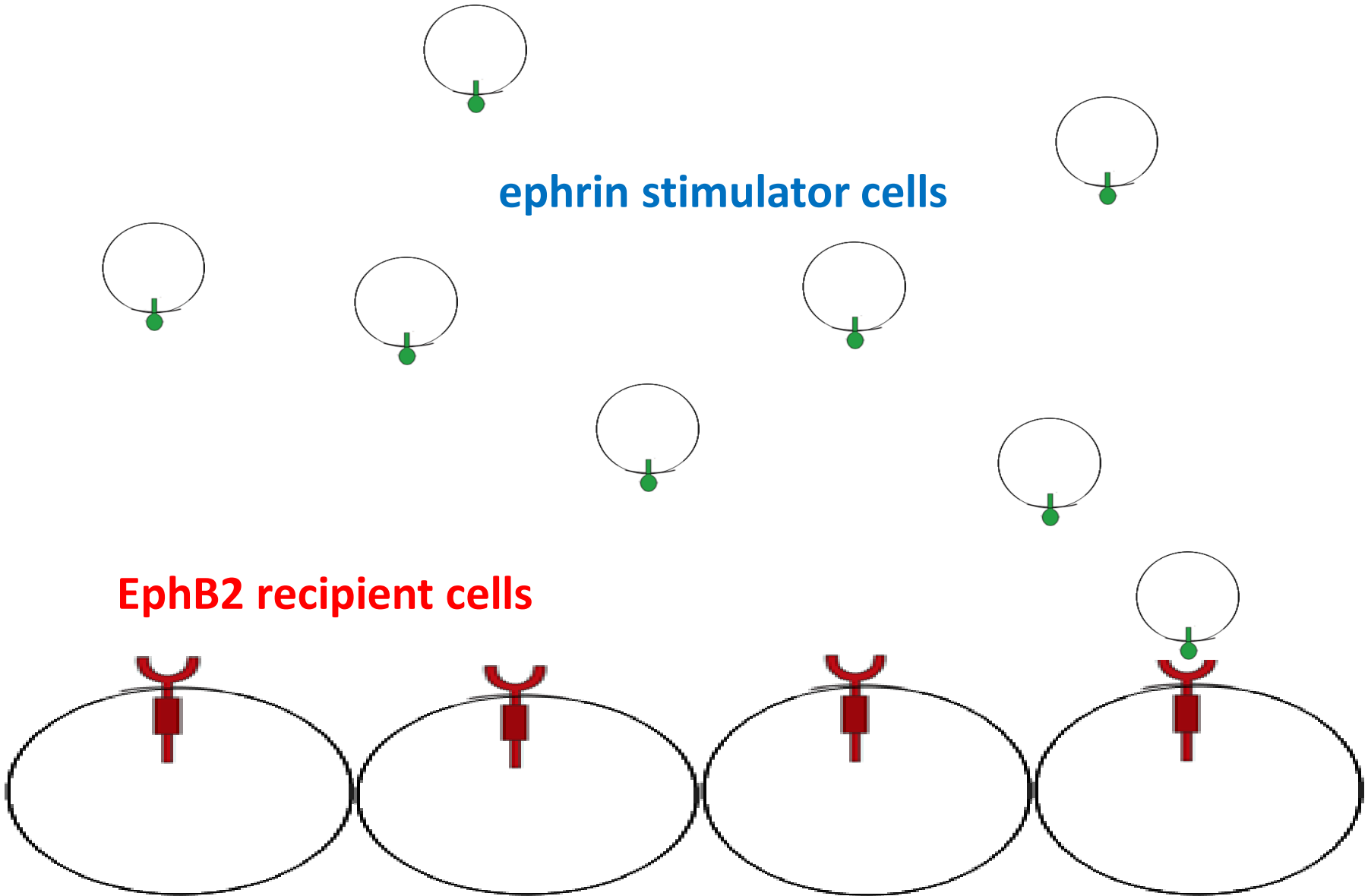
EphB2$^+$ cells, which coexpress **myristoylated GFP**, were mixed with ephrin-B1$^+$ cells. The ability of mixed populations of EphB2$^+$ and ephrin-B1$^+$ cells to sort and organize into distinct multi-cellular structures (colonies) was also confirmed, suggesting that all the relevant molecules required for this process are expressed within these cells.



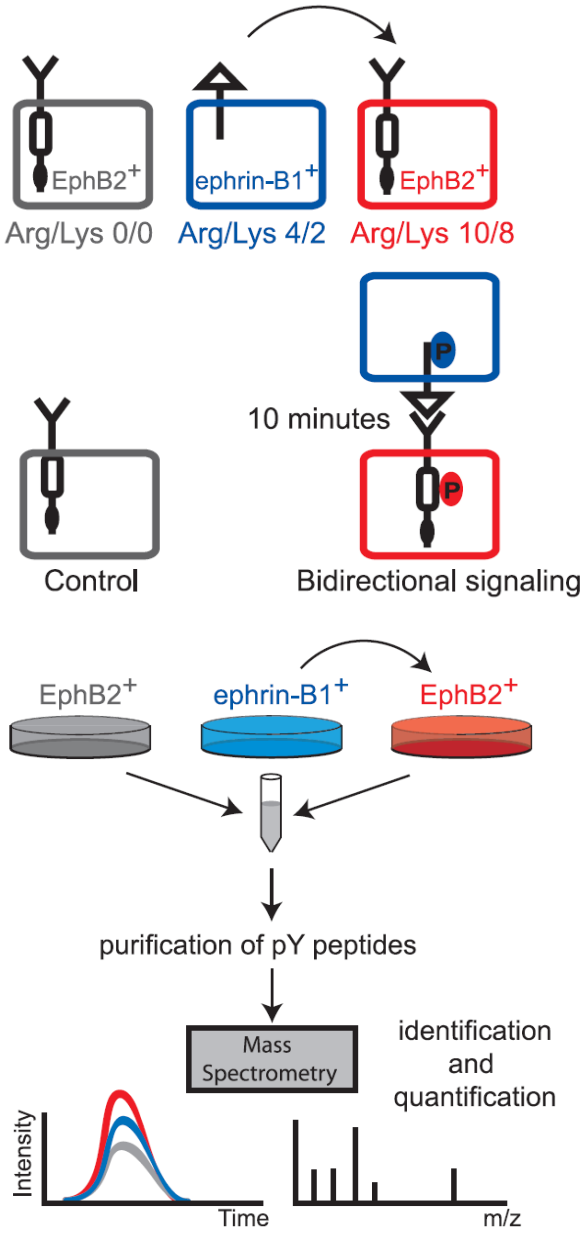
Myristoylation is a lipid anchor modification of eukaryotic and viral proteins targeting them to membrane locations, involving the **addition of a 14-carbon unsaturated fatty acid, myristic acid,** to the **N-terminal glycine** of a subset of proteins. The N-myristoyltransferase (NMT) recognizes the sequence motif of appropriate substrate proteins at the N terminus and attaches the lipid moiety to the N-terminal glycine residue.

Quantitative analysis of Bidirectional Signaling (qBidS) to better understand EphB2- and ephrin-B1-regulated cell sorting

Stimulation of adherent recipient cells (expressing transmembrane receptor) with cells in suspension, (expressing transmembrane ligand).



Quantitative analysis of Bidirectional Signaling (qBidS)



EphB2^{+} cells were labeled independently with

- **"light" ($C^{12}N^{14}$) arginine and lysine**

or

- **"heavy" ($C^{13}N^{15}$) arginine and lysine**

ephrin-B1^{+} cells were labeled with

- **"medium" ($C^{12}N^{15}$) arginine and lysine**

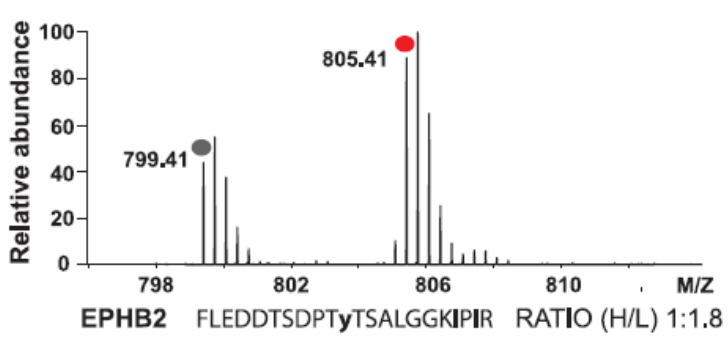
Bidirectional signaling was initiated, mixing **heavy labeled EphB2+ cells** and **medium labeled ephrin-B1+ cells**

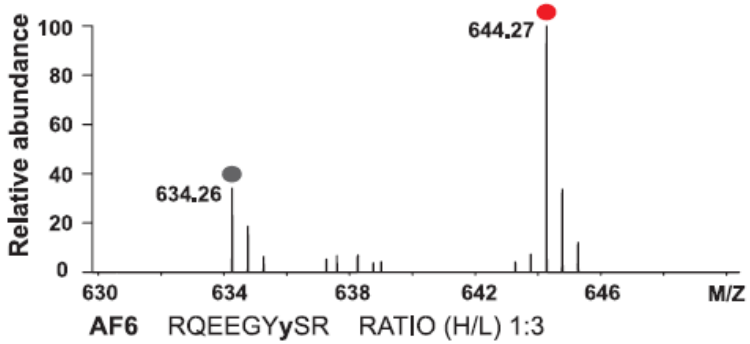
non-stimulated light-labeled EphB2+ cells served as a control.

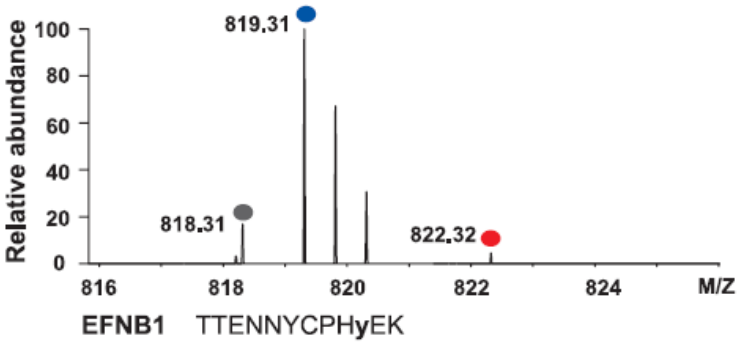
Mixed populations of cells were harvested after 10 min and combined with nonstimulated control cells.

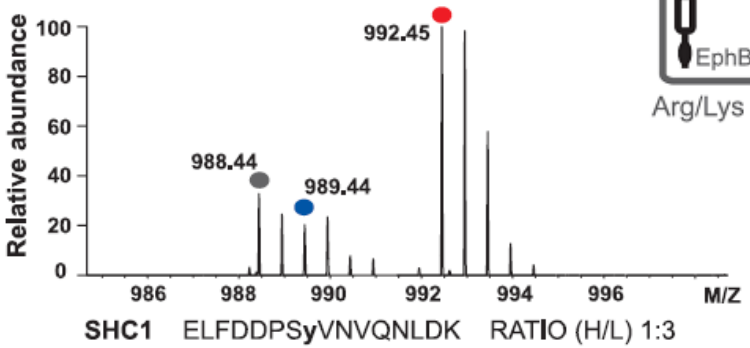
Cell lysates were digested with trypsin, and tyrosine-phosphorylated peptides were isolated and analyzed with liquid chromatography-mass spectrometry (LC-MS).

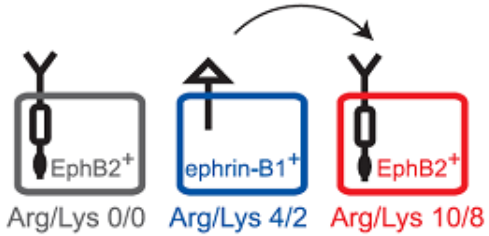
Peptides from mixed-cell populations were differentiated and quantified via their distinct isotopic labels





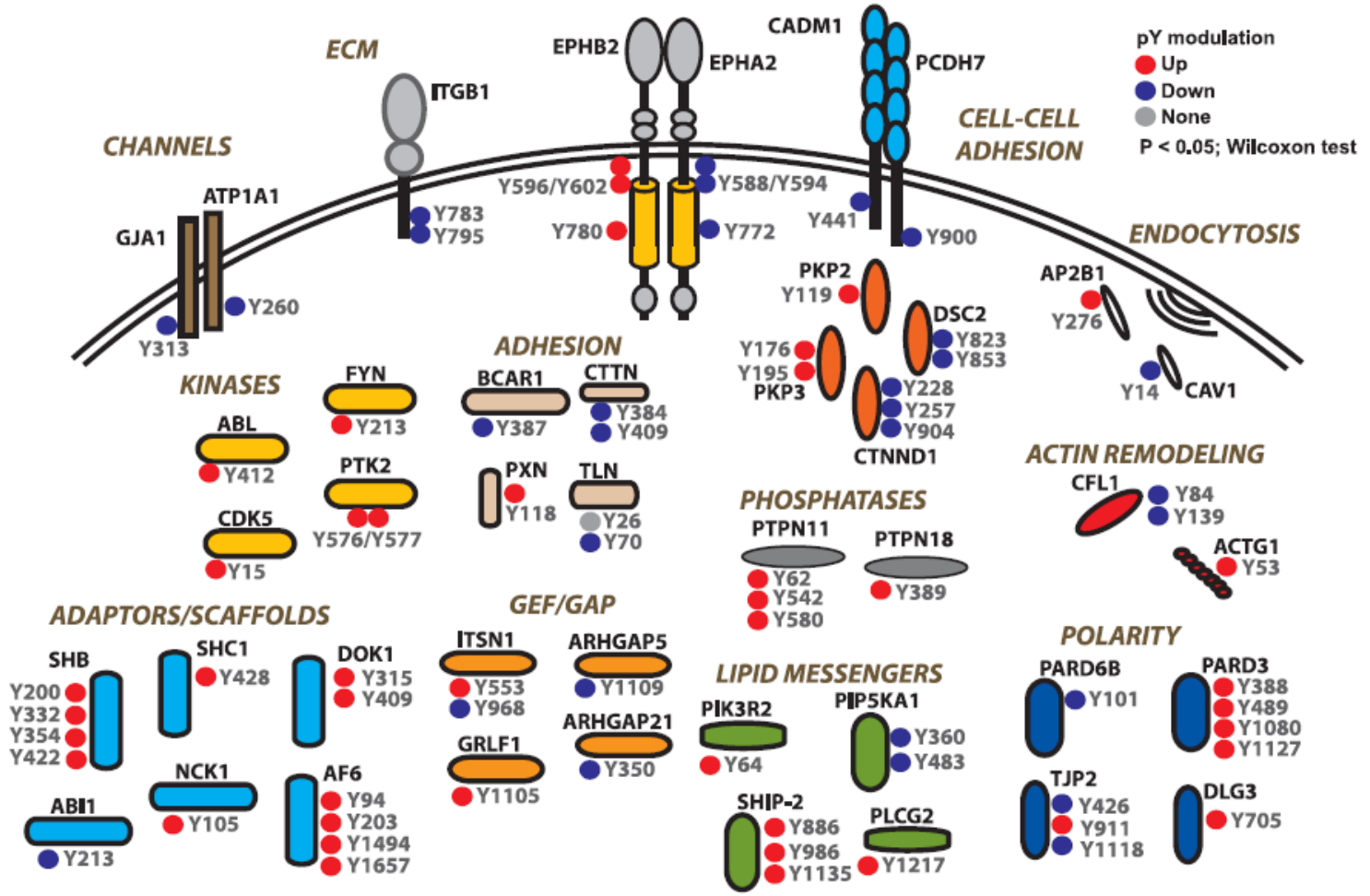






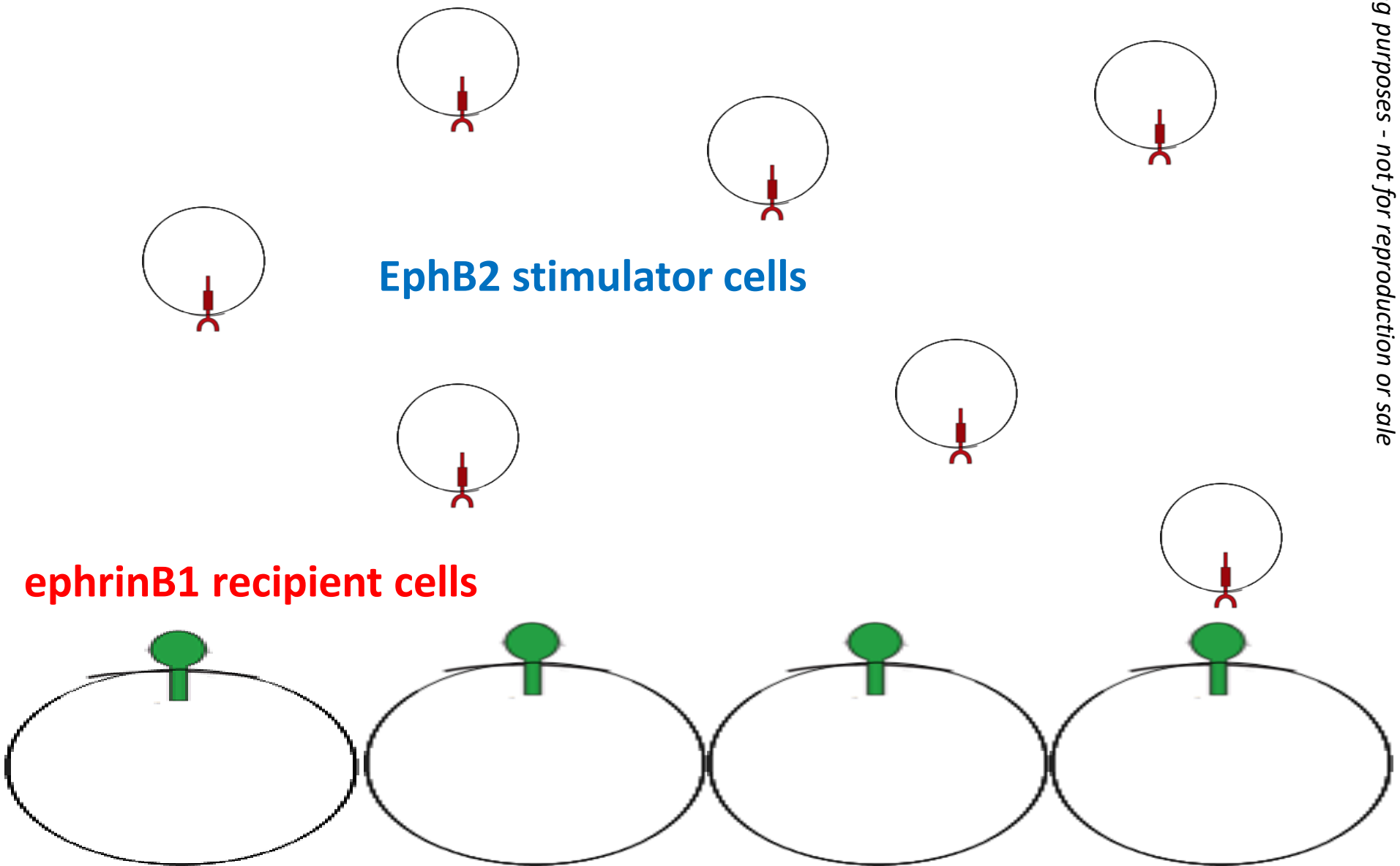
In **EphB2⁺ cells** mixed with **ephrin-B1⁺** cells, phosphorylation of EPHB2 was increased by 80%, as determined from the ratio of **heavy-** to **light**-labeled peptides.
Tyrosine phosphorylation of ephrin-B1 was only observed with a **medium** label, indicating that this peptide originates specifically from the **ephrin-B1⁺ cells**.
The previously described EphB2 targets AF6 and SHC1 display threefold increased levels of phosphorylation in the **heavy-labeled EphB2⁺ cell** population.

Overview of selected proteins in EphB2^{+} cells modulated by tyrosine phosphorylation after mixing with ephrin-B1+ cells.

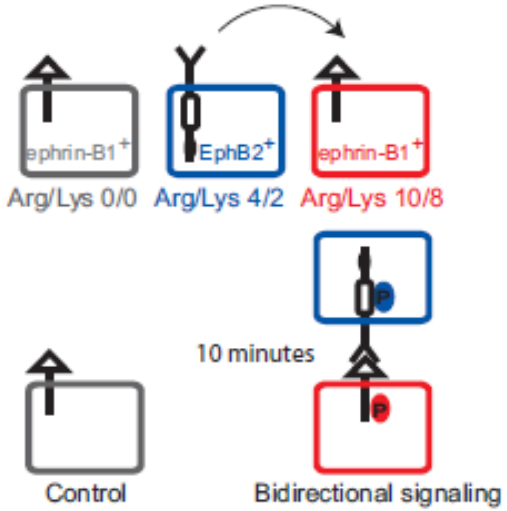


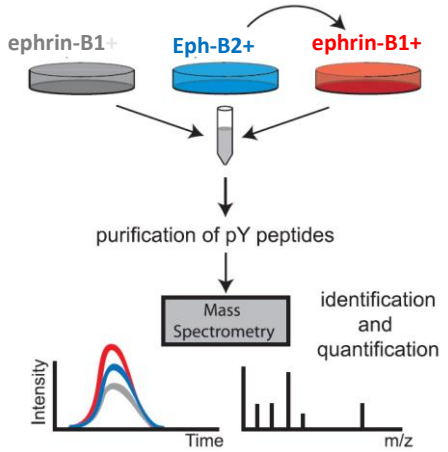
The modulation of pTyr sites is indicated as **significantly increased (red),** **not modulated (gray),** or **decreased (blue).** Molecules that are involved in a wide variety of cellular functions are modulated by tyrosine phosphorylation, indicating that phospho-regulation of numerous cellular processes may be important for cell sorting.

Stimulation of adherent recipient cells (expressing transmembrane ligand) with cells in suspension (expressing transmembrane receptor)



Quantitative analysis of Bidirectional Signaling (qBidS)





ephrin-B1^{+} cells were labeled independently with

-**"light" ($C^{12}N^{14}$) arginine and lysine** or

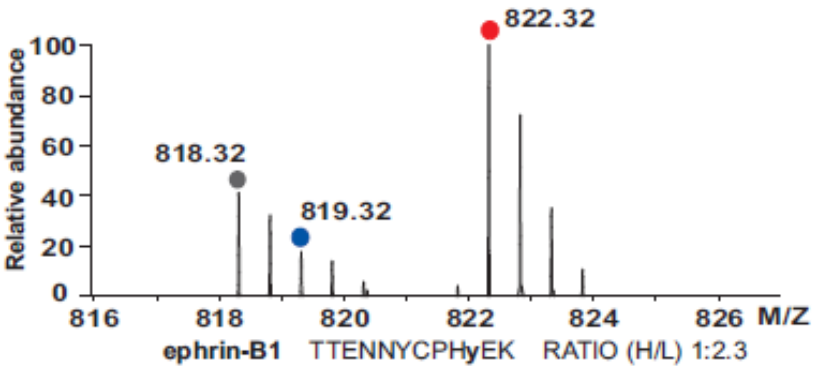
-**"heavy" ($C^{13}N^{15}$) arginine and lysine**

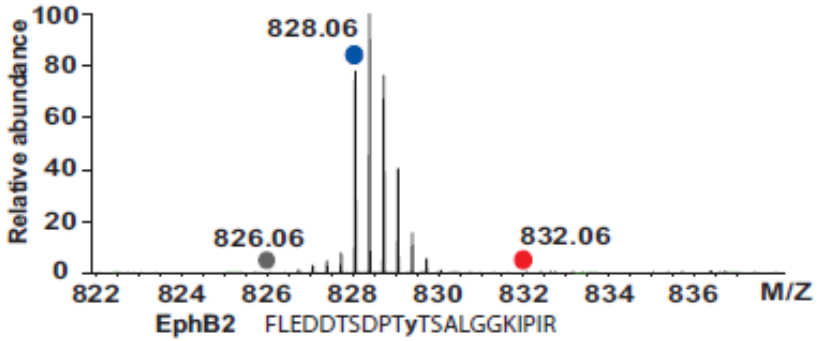
Eph-B2^{+} cells were labeled with

-**"medium" ($C^{12}N^{15}$) arginine and lysine**

Bidirectional signaling was initiated, mixing **heavy labeled ephrin-B1+ cells** and **medium labeled Eph-B2+ cells**;

non-stimulated light-labeled ephrin-B1+ cells served as a control.





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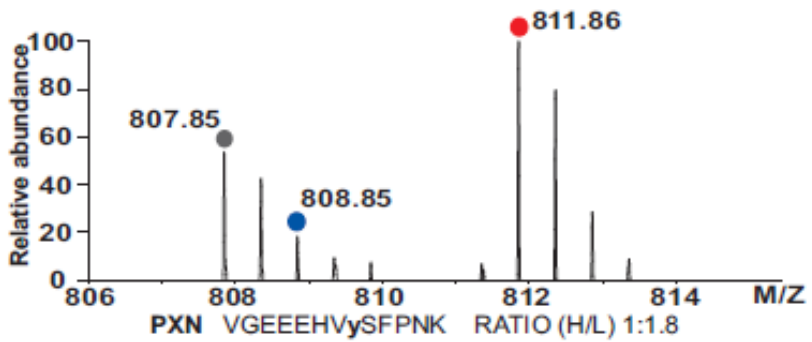
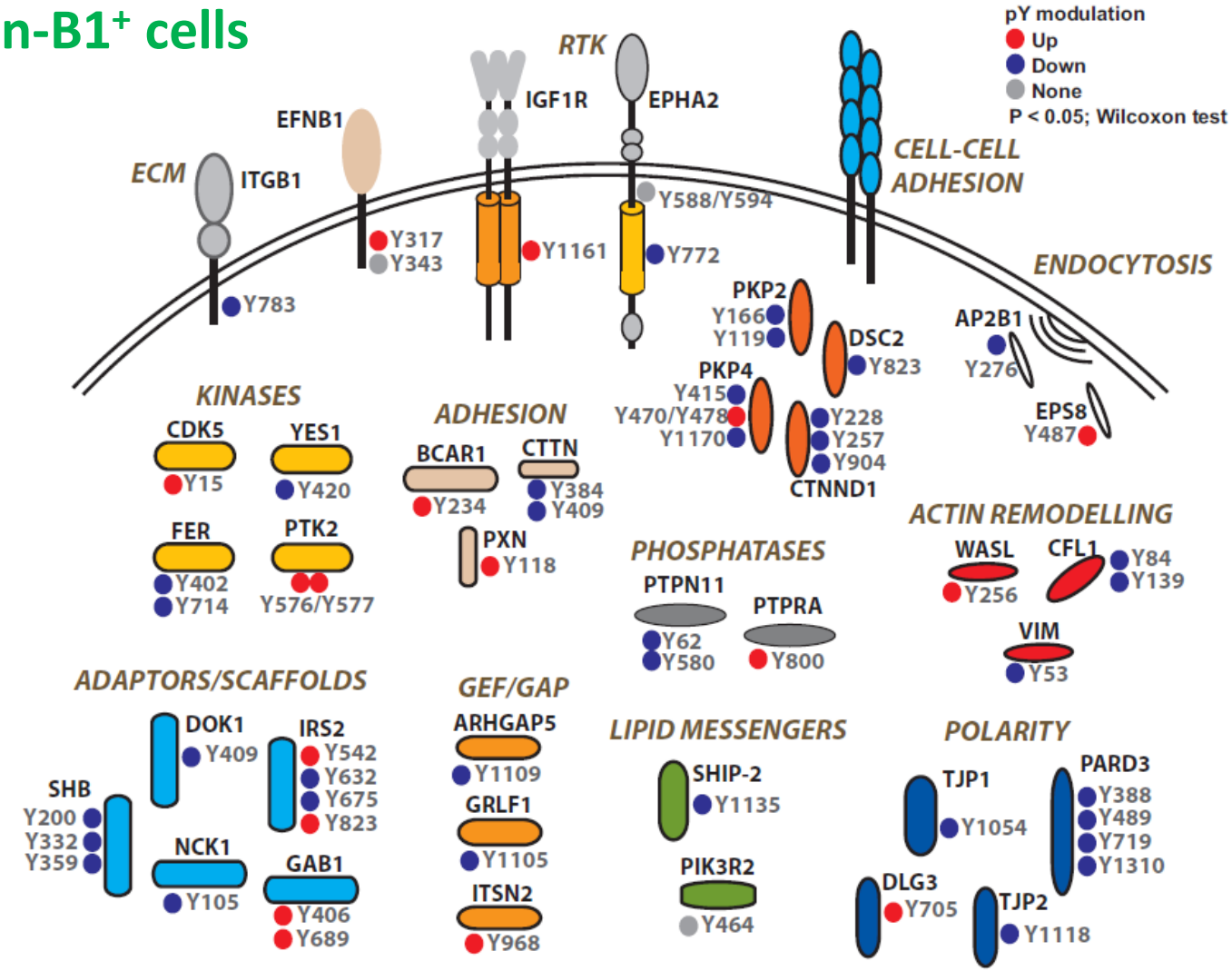


Overview of selected tyrosine phosphorylation sites regulated in ephrin-B1^{+} cells



The positions of identified tyrosine phosphorylation sites are shown and are color-coded to display whether their phosphorylation was **significantly increased (red), decreased (blue)** or **not modulated (grey).** Proteins involved in a variety of cellular functions were modulated in ephrin-B1^{+} cells following contact with EphB2^{+} cells.

Bidirectional signaling between **EphB2-** and **ephrin-B1**-expressing cells is **asymmetric**.

Analysis of EphB2-ephrin-B1 bidirectional signaling identified:

- 557 distinct tyrosine phosphorylation sites in **EphB2$^+$ cells** of which **276** were significantly regulated in **185** proteins.

- 353 distinct tyrosine phosphorylation sites in **ephrin-B1$^+$** of which **166** were significantly regulated in **119** proteins.

→ Analysis of the global changes in tyrosine phosphorylation induced by contact between **EphB2$^+$** and **ephrinB1$^+$** cells identified a total of

- **442** tyrosine phosphorylation sites on **304** target proteins