3009 GPCR Learning objectives

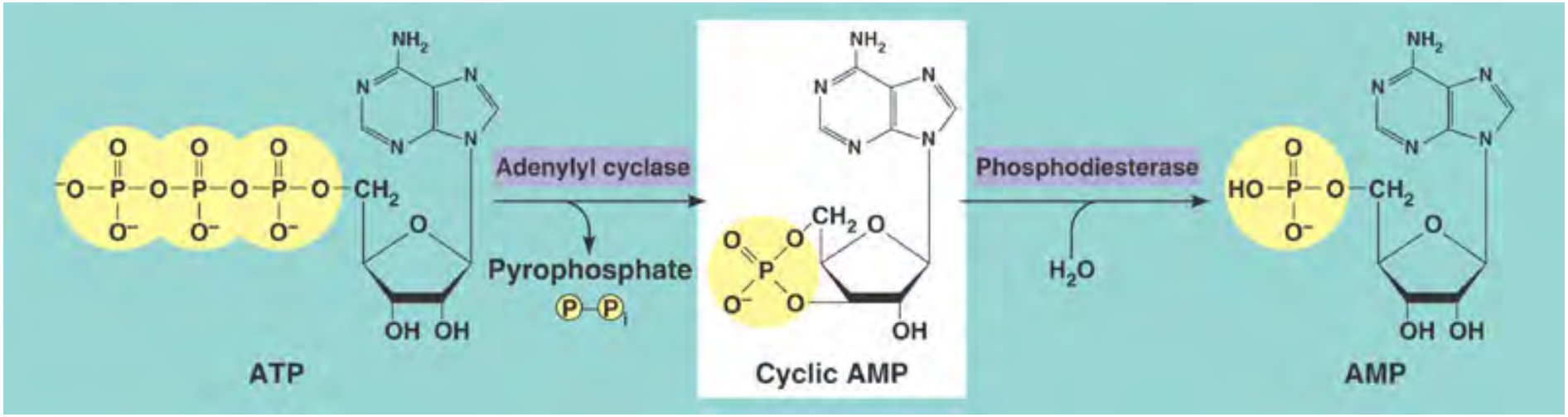
- Describe the G protein cycle
- Explain how cAMP acts as a second messenger
- Understand the basics of GPCR structure
- Explain the main accessory proteins associated with GPCR signalling
- Concepts of receptor dimerization and crosstalk
- *Explain fluorescence and its use in microscopy*
- *Describe the use of GFP to label proteins*

What do you need to be a 2nd messenger??

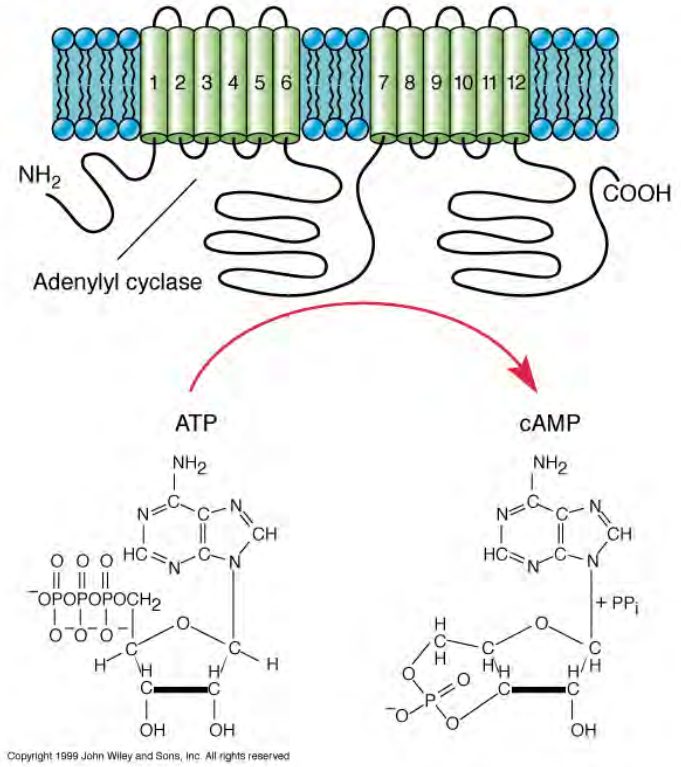
- Low amounts in resting state
- Regulated synthesis
- Regulated destruction
- Act through other proteins

* Cyclic AMP

* Calcium



Adenylyl cyclase turns ATP into cyclic AMP

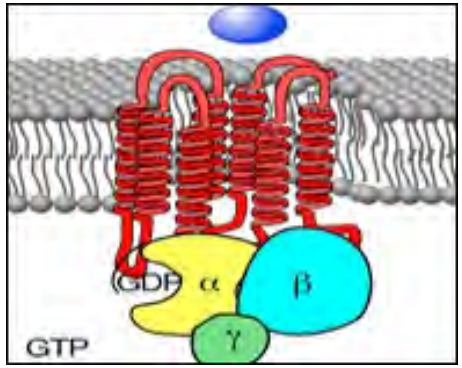


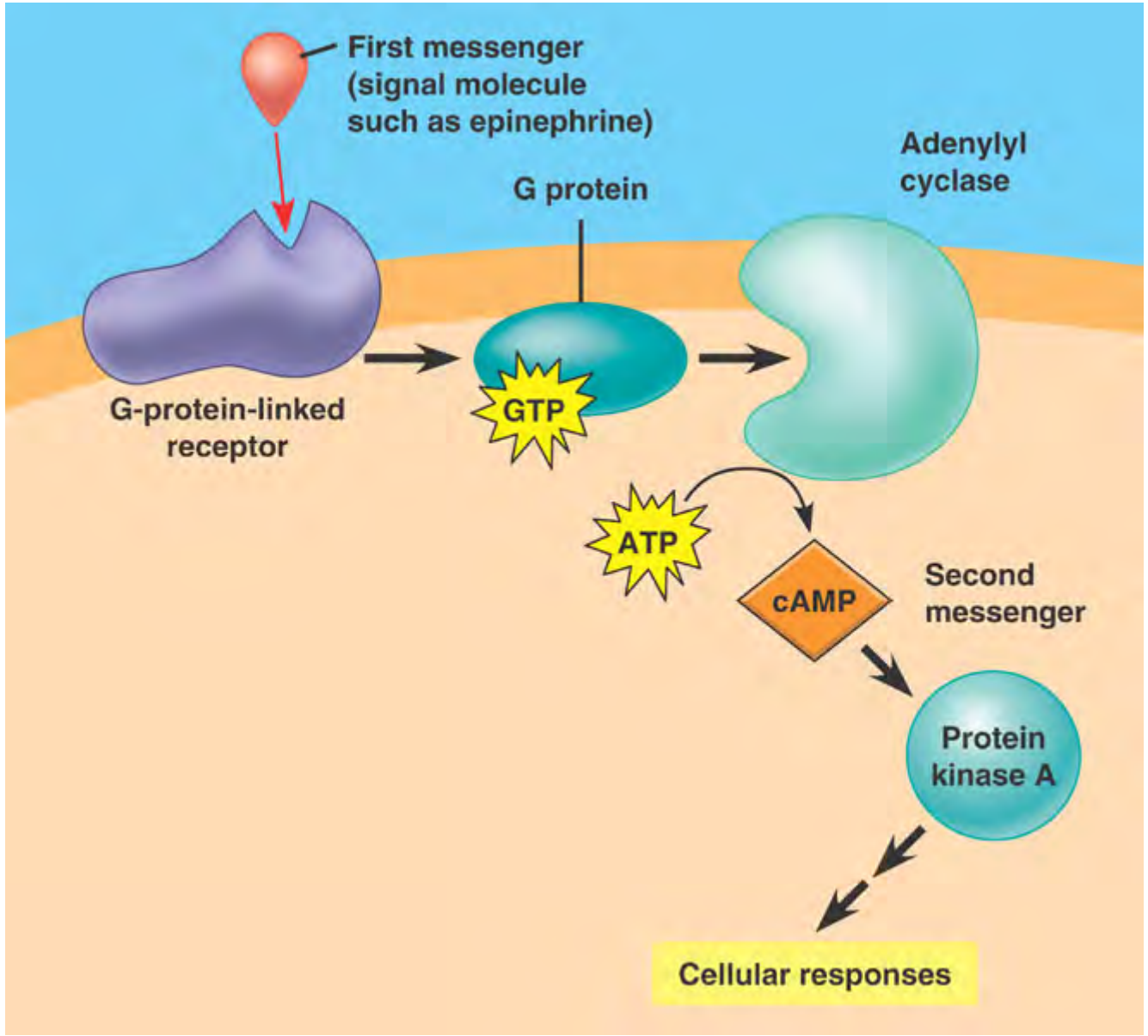
What regulates [cAMP]??

- ***Adenylyl Cyclase*** (AC) makes it from ATP…
- LOTS of ATP (~1 mM in cytoplasm)
- cAMP then activates ***KINASES***
- What regulates AC??

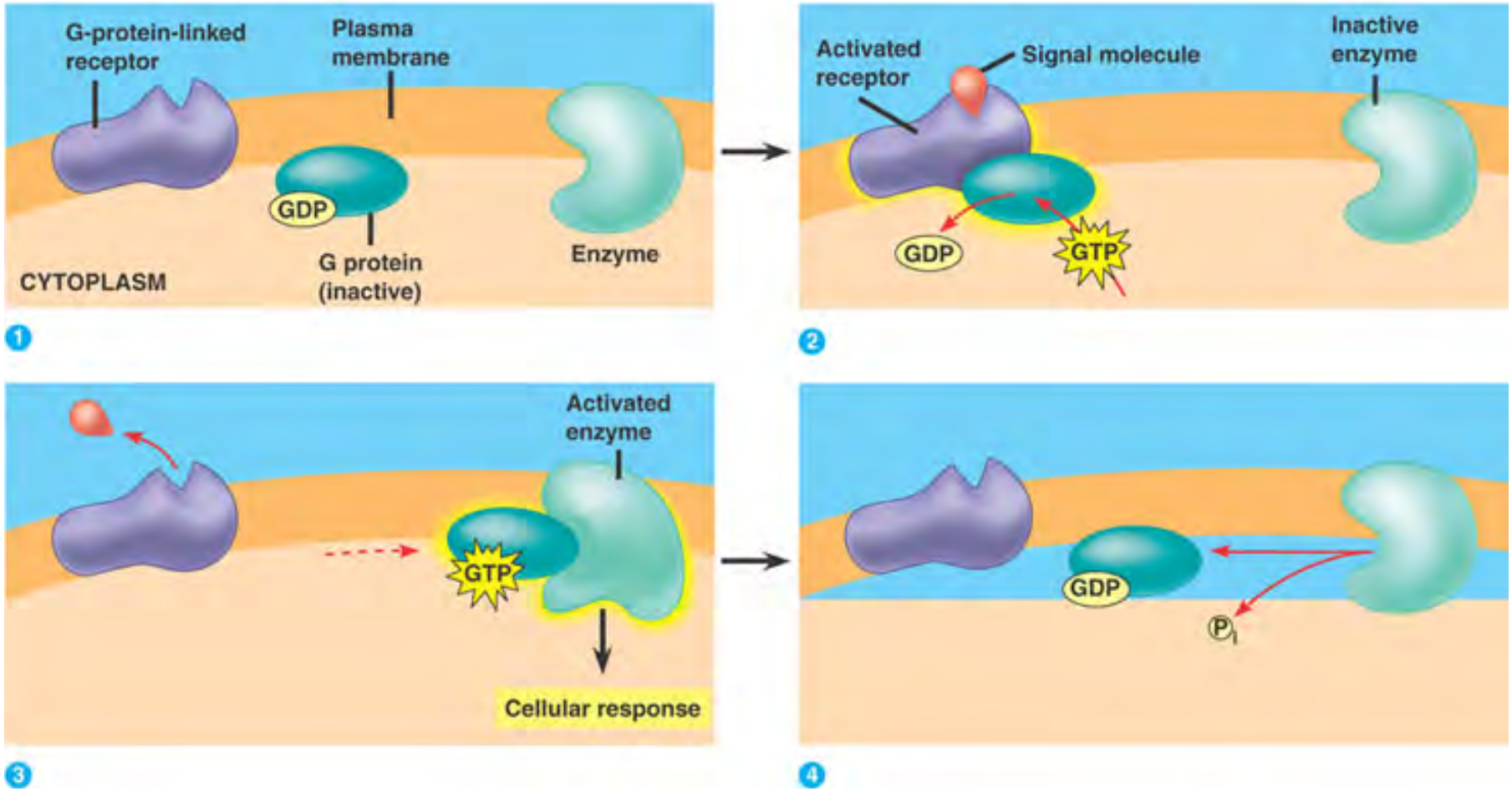
*G protein*s and G protein coupled *receptors (GPCR)*

- ***G proteins*** bind ***GTP*** (same as ATP but guanosine instead of adenosine
- G protein-coupled receptor activated
- G protein mobilised
- G protein activates effector (Adenylate Cyclase)
- cAMP produced and cascade is commenced!!




First messenger
(signal molecule
such as epinephrine)
G protein
Adenylyl
cyclase
G-protein-linked
receptor
GTP
ATP
cAMP
Second
messenger
Protein
kinase A
Cellular responses

A cycle – easy !


G-protein-linked receptor
Plasma membrane
GDP
G protein (inactive)
Enzyme
CYTOPLASM
1
Activated receptor
Signal molecule
Inactive enzyme
GDP
GTP
2
Activated enzyme
GTP
Cellular response
3
GDP
Pi
4

2012 Chemistry NP

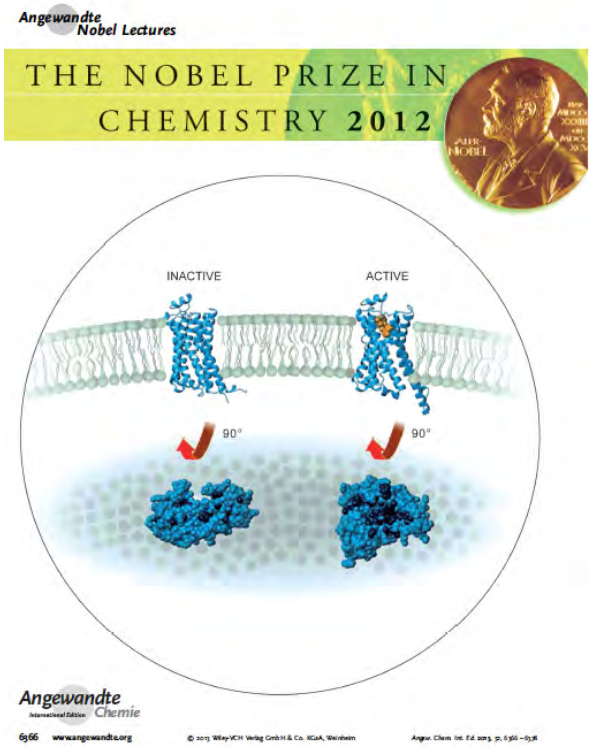
G-Protein-Coupled Receptors

DOI: 10.1002/anie.201301924

A Brief History of G-Protein Coupled Receptors (Nobel Lecture)**

*Robert J. Lefkowitz**

β_2 adrenergic receptor · G-protein-coupled receptors · Nobel lecture · protein structures · signaling



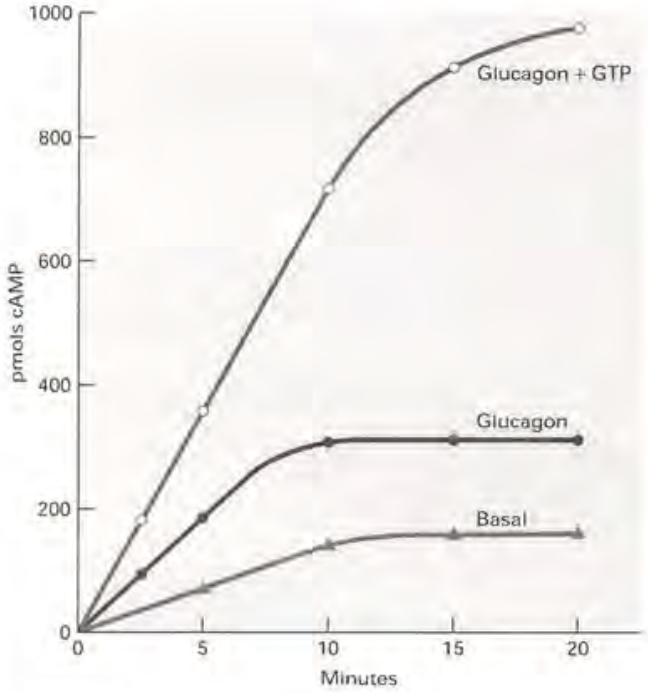
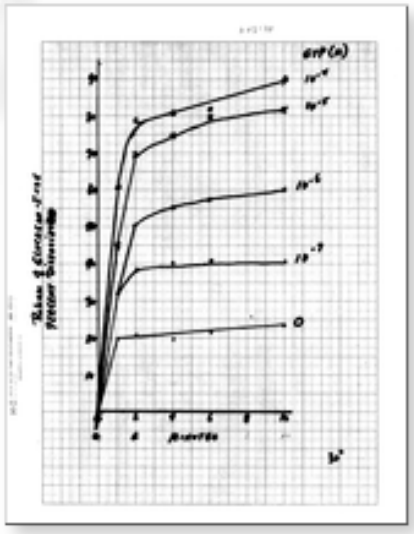


Figure 20.1
Effect of GTP on glucagon-stimulated cAMP production from AMP-PNP by purified rat liver membranes. In the absence of GTP, glucagon stimulates cAMP formation about twofold over the basal level in the absence of added hormone. When GTP also is added, cAMP production increases another fivefold. [Adapted from M. Rodbell et al., 1971, *J. Biol. Chem.* 246:1877.]

Martin Rodbell, Nobel Prize 1994

GTP is required for the ligand-induced stimulation of adenylate cyclase.

Overall: need, 1) a receptor, 2) a transducer (G-protein) and 3) an amplifier (adenylate cyclase) that generates large amounts of a second messenger.



The Nobel Prize in Physiology or Medicine 1994
Alfred G. Gilman, Martin Rodbell

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The Nobel Prize in Physiology or Medicine 1994

Alfred G. Gilman
Prize share: 1/2

Martin Rodbell
Prize share: 1/2

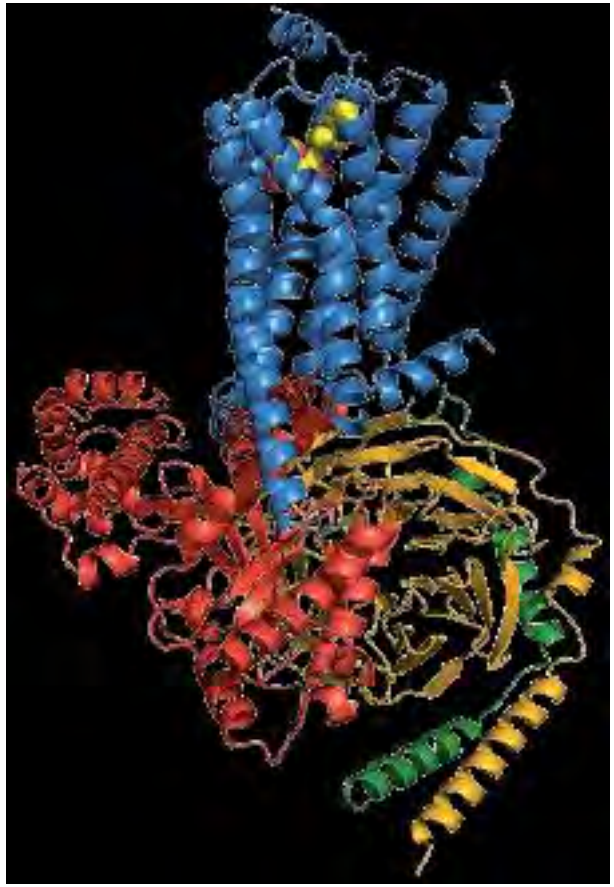
The Nobel Prize in Physiology or Medicine 1994 was awarded jointly to Alfred G. Gilman and Martin Rodbell *"for their discovery of G-proteins and the role of these proteins in signal transduction in cells"*

scraps of its likely genetic sequence. Kobilka then displayed "the first of many flashes of technological innovation", says Lefkowitz: he decided to construct a library of mammalian genomic sequences and screen it with the scraps of sequences they had. This would pull out longer clones that could be pieced together to reveal the full sequence.

The plan worked. And when the team stitched together the receptor sequence[6], it held a surprise: several strings of amino acids that are typically found in cell membranes showed that the receptor snaked through the membrane seven times. It was just like rhodopsin, the light-detecting receptor in the retina that was also known to activate a G protein. At the time, there was no reason to think that these receptors were going to look the same — especially when one was turned on by light, and the other by a hormone.

"It was a real eureka moment," recalls Lefkowitz. At the time, about 30 proteins were known to turn on G proteins. "We realized, oh my god, they're all going to look like this. It's a whole family!"

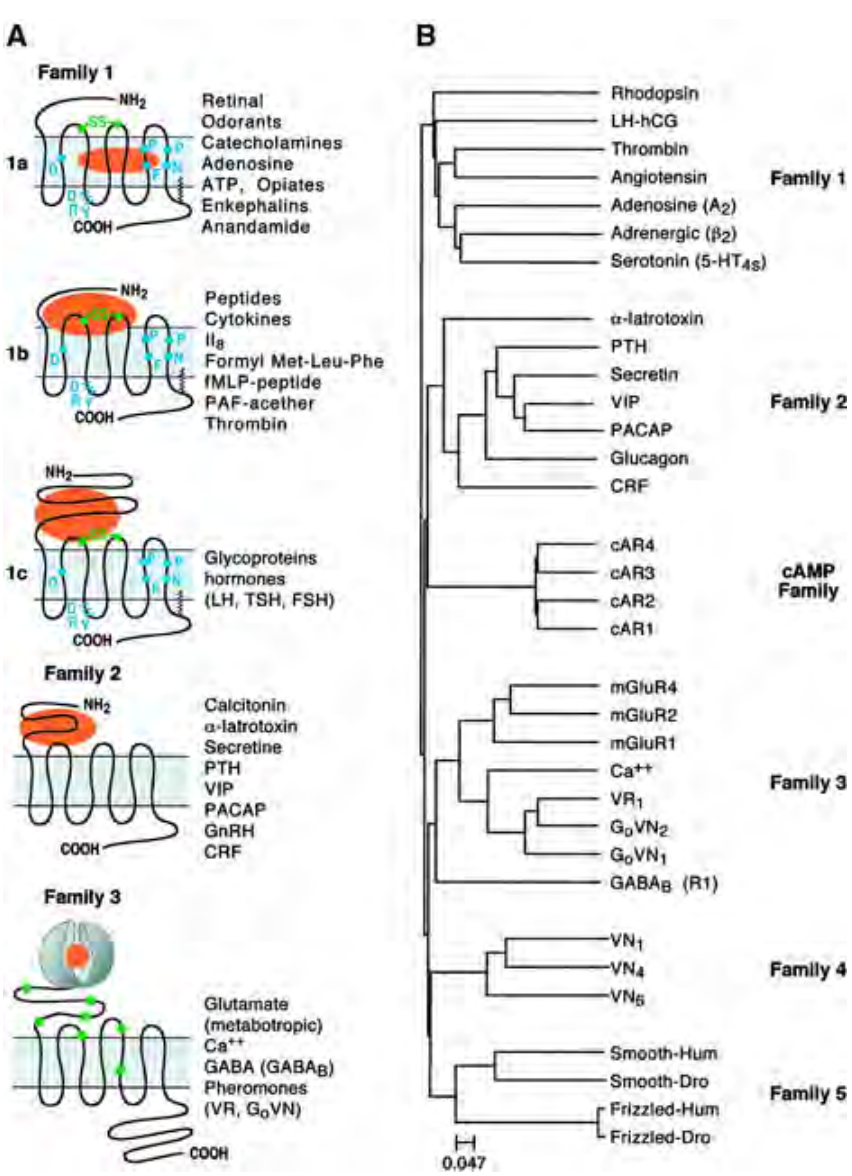
That family became known as seven-transmembrane receptors, or GPCRs — and is now known to have nearly 800 members in humans. Kobilka describes the watershed

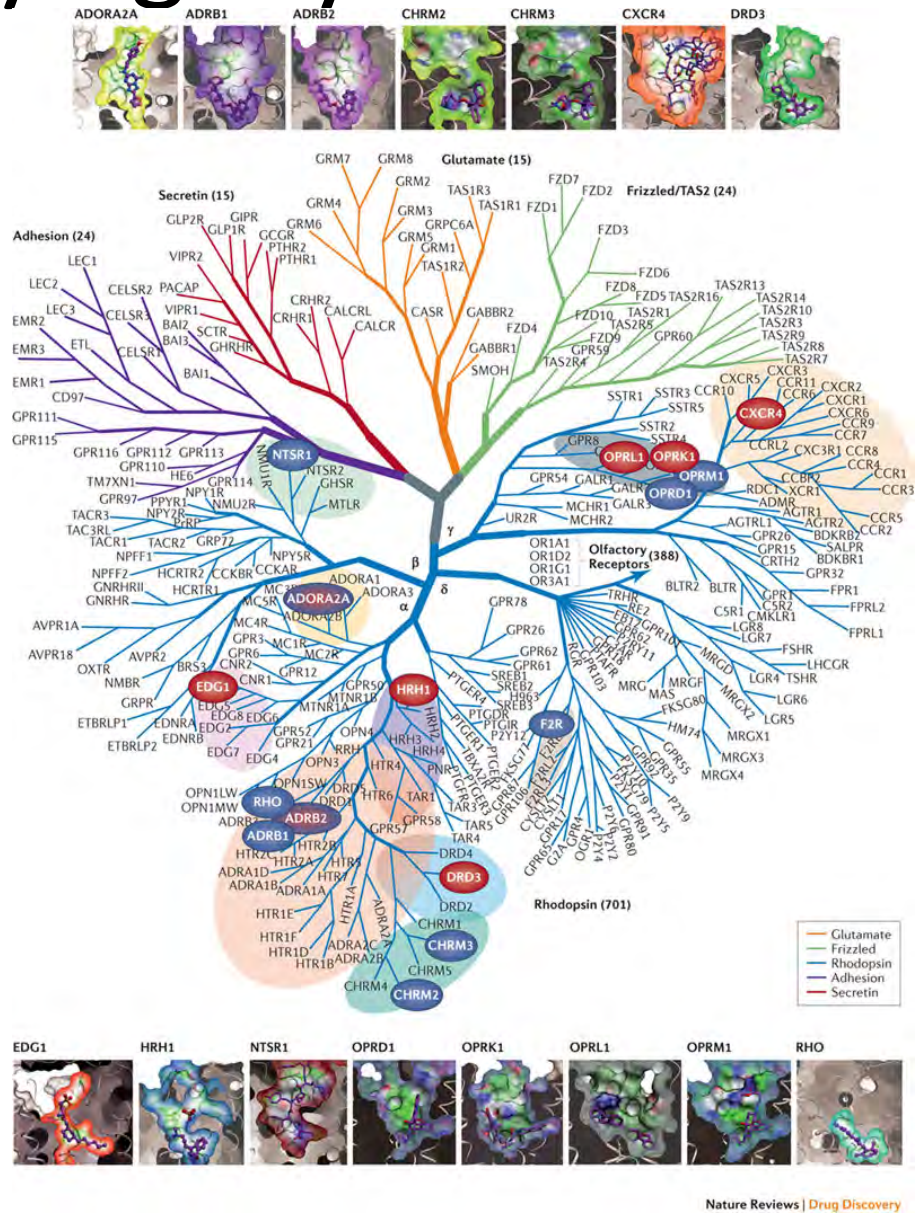


Big pharma likes GPCRz

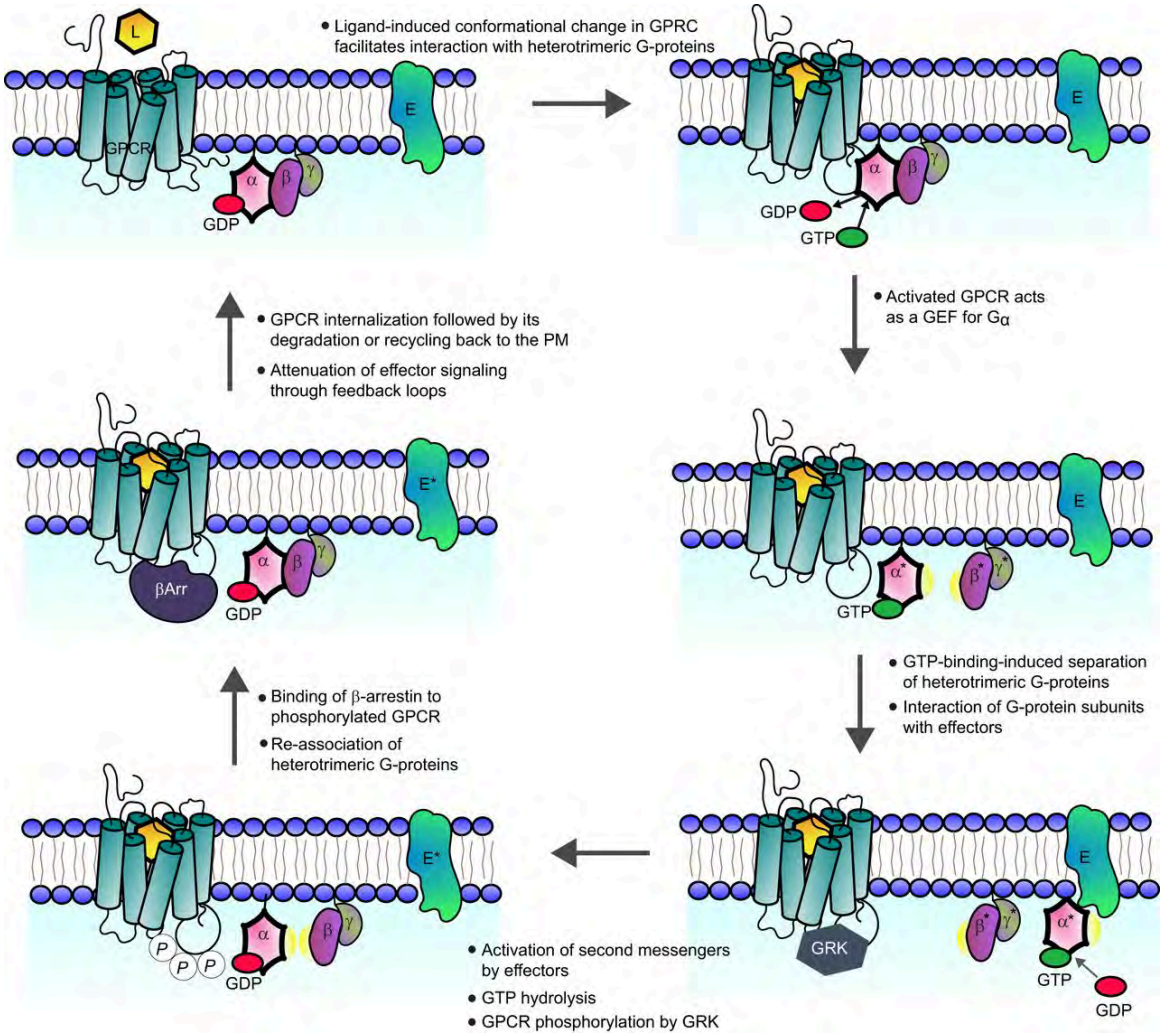
- 6 classes based on sequence homology and functional similarities. Class A (rhodopsin-like receptors),
- Class B (the secretin receptor family),
- Class C (metabotropic glutamate receptors),
- Class D (fungal mating pheromone receptors),
- Class E (cyclic AMP receptors) and
- Class F (Frizzled and Smoothened receptors).
- physiological events such as olfaction, taste, cognition, the regulation of blood pressure, immune response, behaviour and mood
- ***Currently, ~30–40% of marketed drugs target GPCRs, and these drugs include β-blockers, angiotensin receptor blockers, opioid agonists and histamine receptor blockers***
- Lots of disease mutations!

GPCR Phylogeny

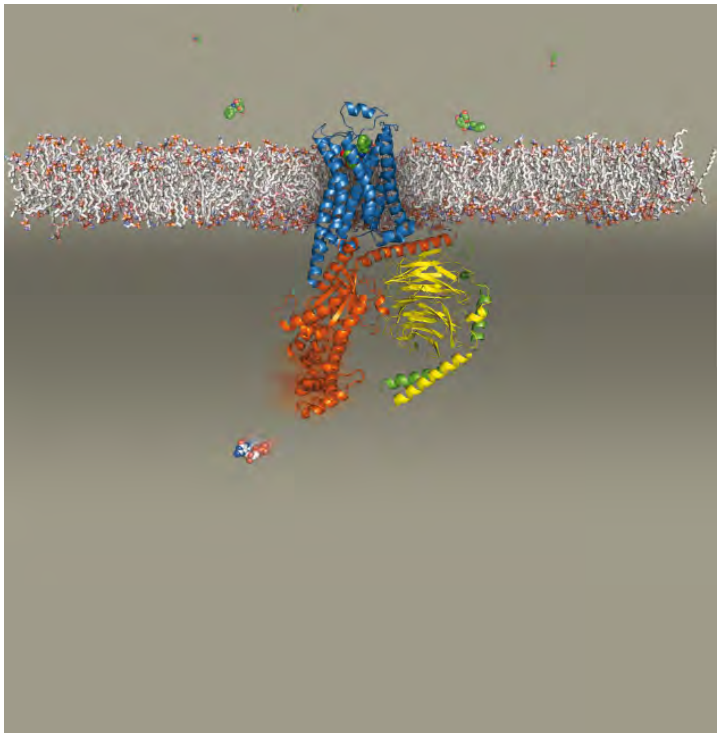


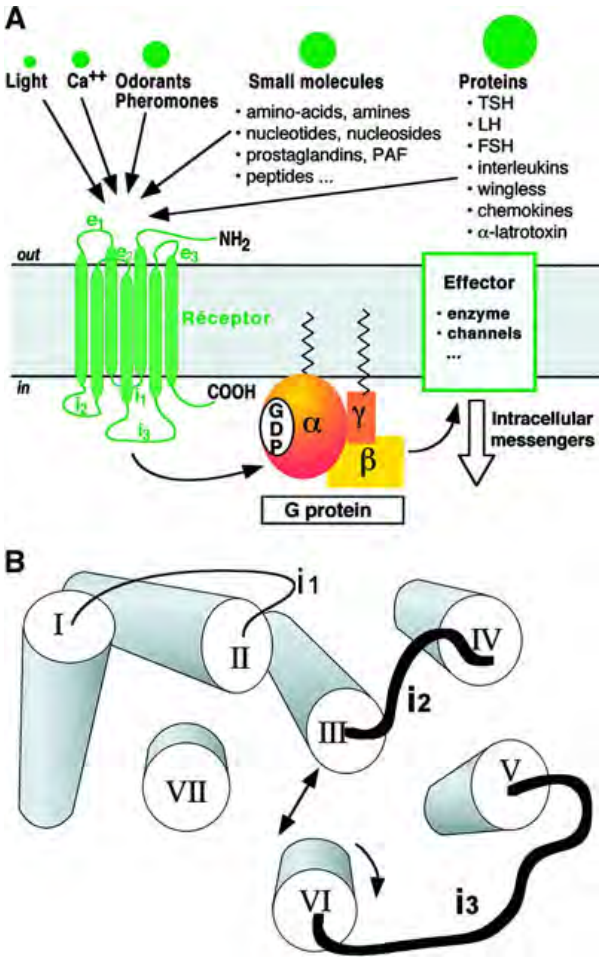


The GPCR cycle.



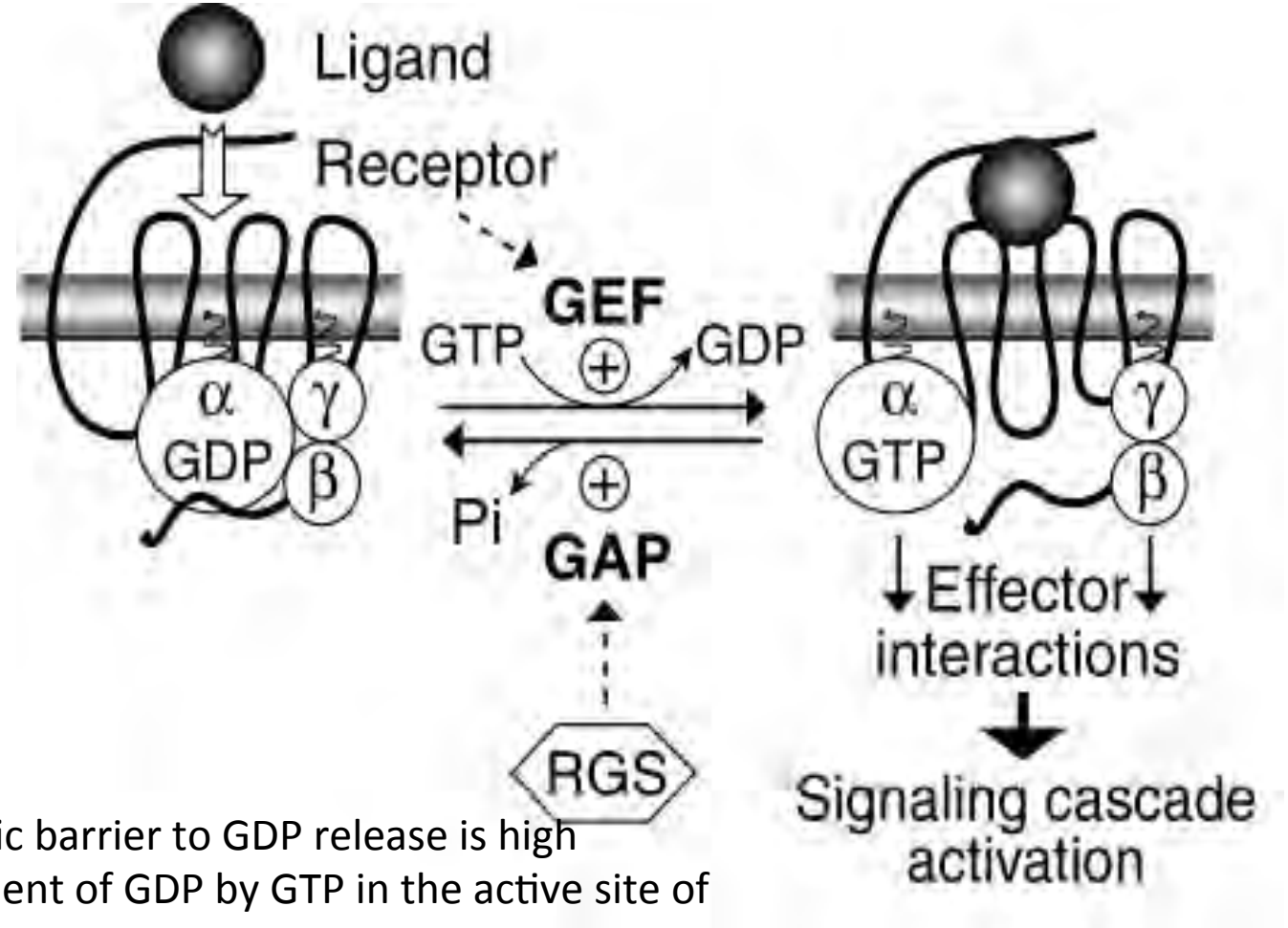
Caitlin D. Hanlon, and Deborah J. Andrew J Cell Sci 2015;128:3533-3542





The EMBO Journal (1999) **18,** 1723–1729

GEF and GAP

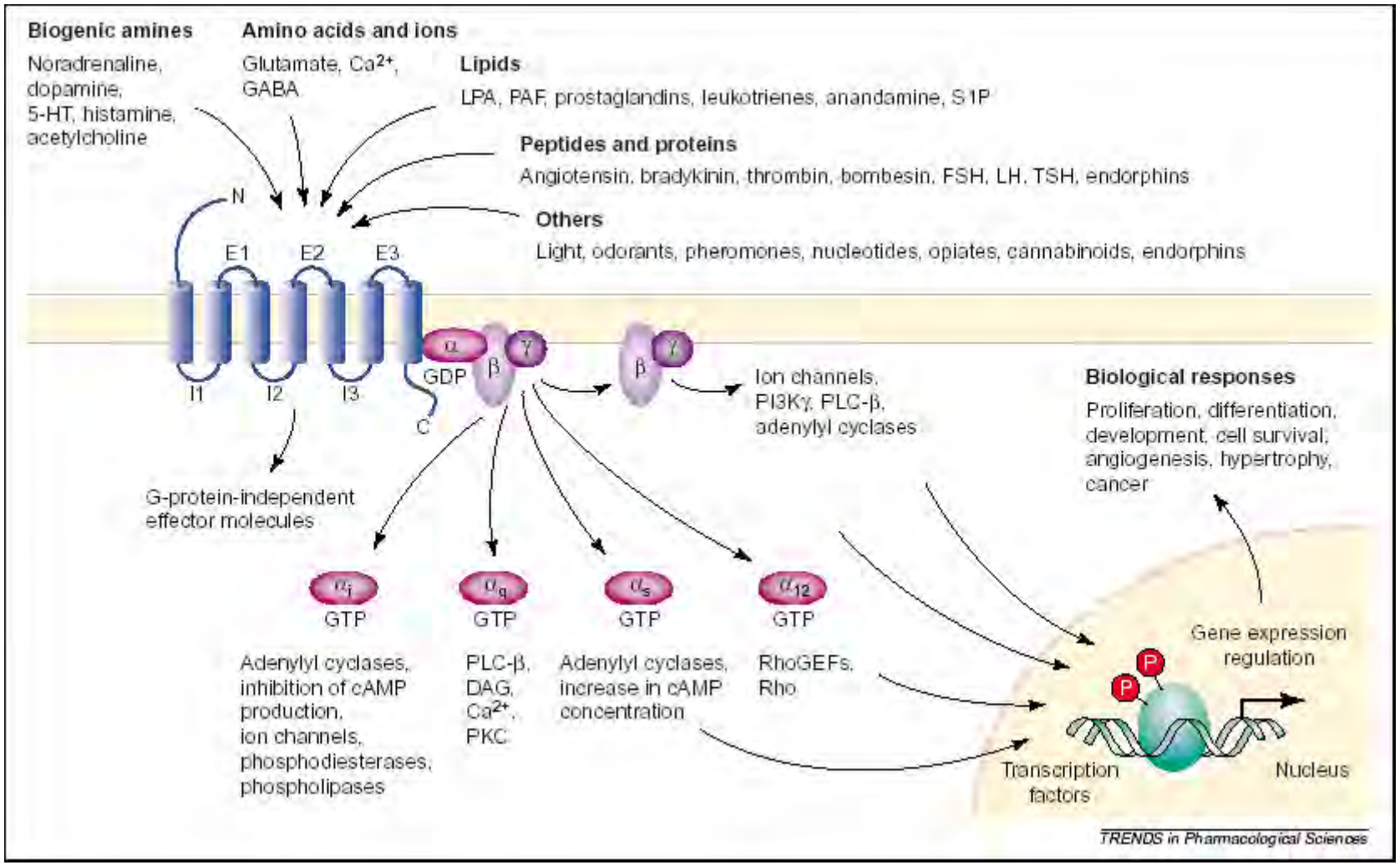


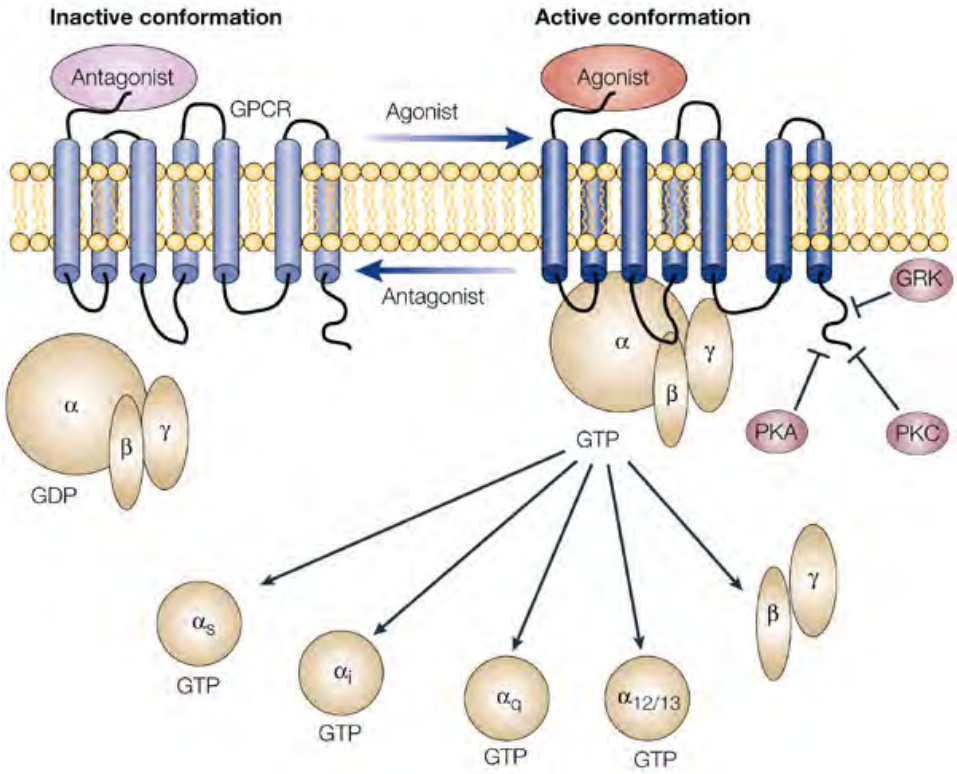
the kinetic barrier to GDP release is high replacement of GDP by GTP in the active site of a G protein is the turn-on signal that requires the assistance of a guanine nucleotide exchange factor, or **GEF.**

GAP = GTPase activating protein
RGS = regulator of G protein signalling

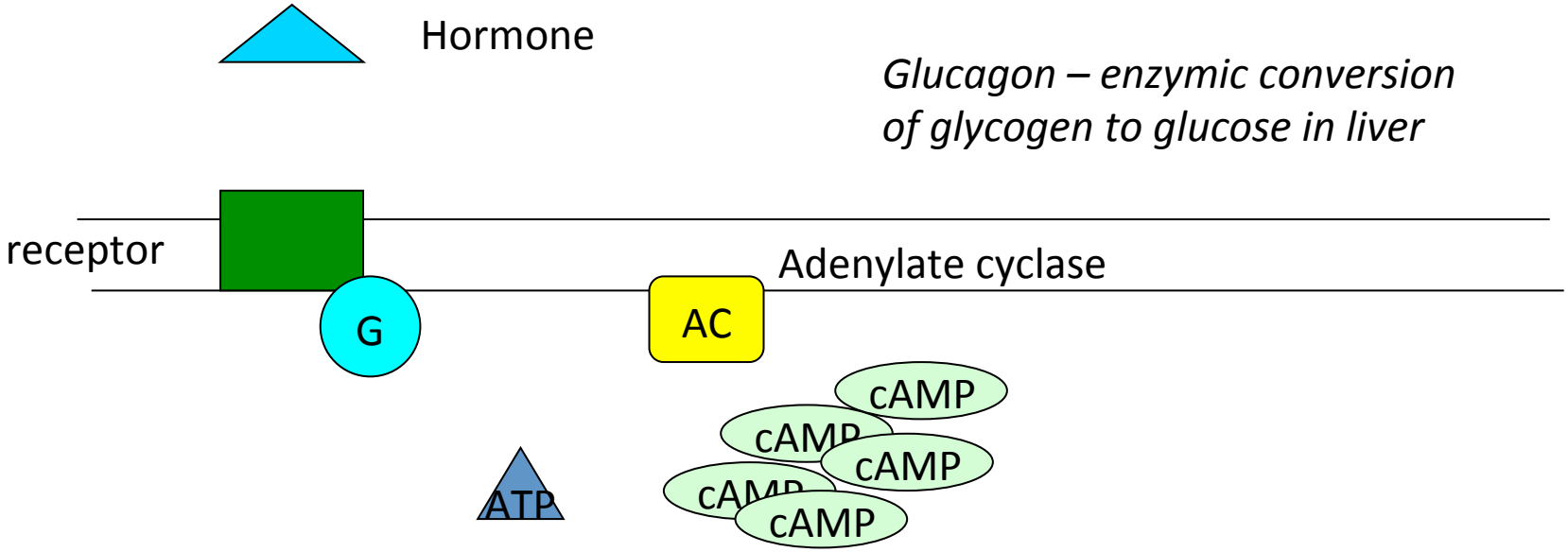
- **Hollinger et al. 2000** *Pharmacological Reviews*

Diversity of GPCR signalling

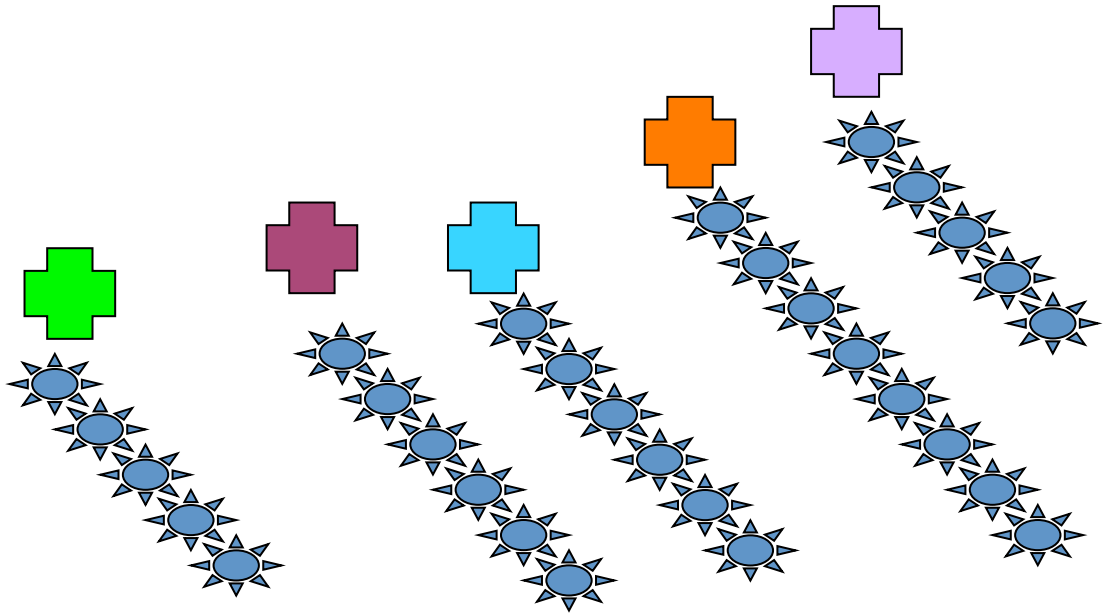


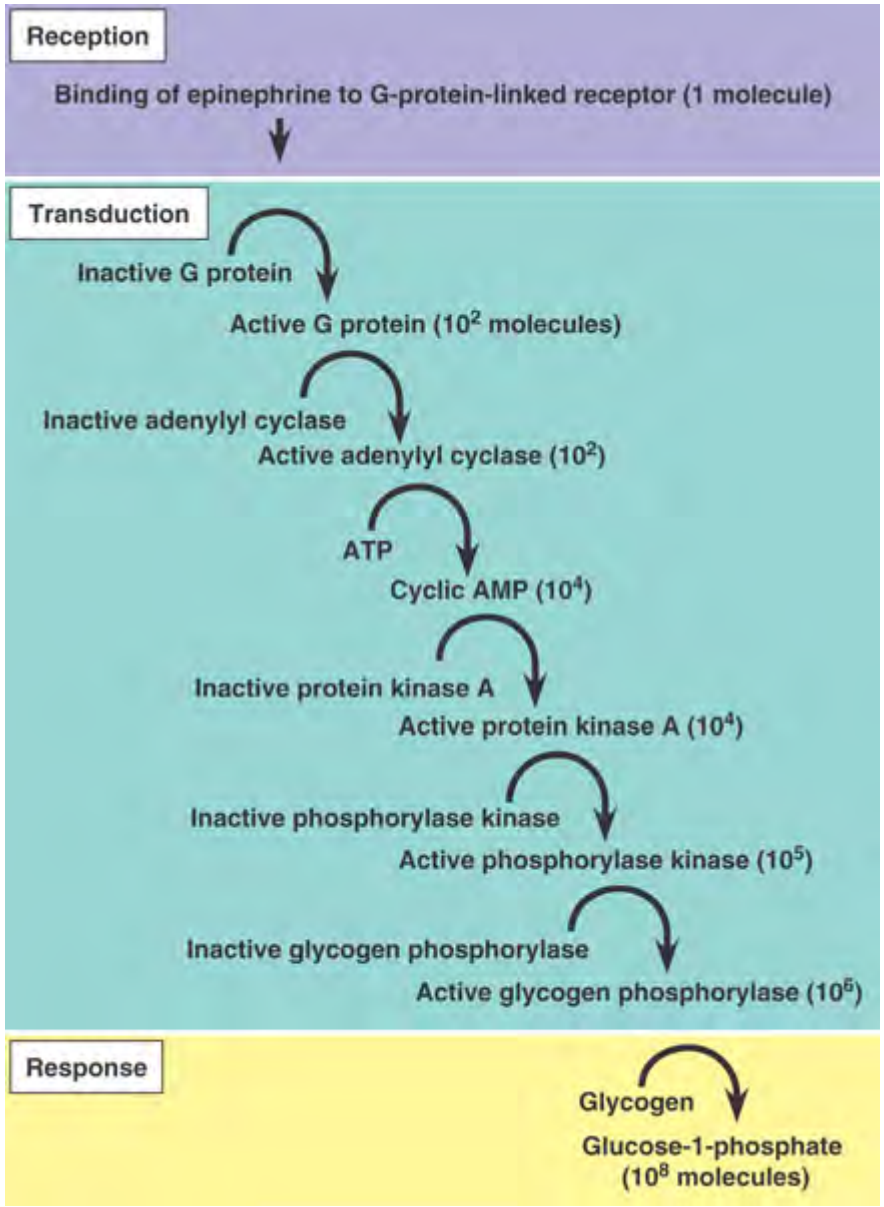


Family	α_s	α_i	α_q	$\alpha_{12/13}$	βγ
Members	$G\alpha_s$	$G\alpha_{i1/i2/i3}$	$G\alpha_q$, $G\alpha_{14}$	$G\alpha_{12}$	β (1–5)
	$G\alpha_{sXL}$	$G\alpha_o$, $G\alpha_t$	$G\alpha_{11}$, $G\alpha_{15/16}$	$G\alpha_{13}$	γ (1–8)
	$G\alpha_{olf}$	$G\alpha_z$, $G\alpha_{gust}$			
Effectors	Adenylyl cyclases	Adenylyl cyclases	PLCβ	Rho GEFs	Ion channels
	Increased cAMP	Inhibition of cAMP	DAG	Rho	PI3Kγ
	PKA	Ion channels	Ca^{2+}		PLCβ
		Phosphodiesterases	PKC		Adenylyl cyclases
		Phospholipases			



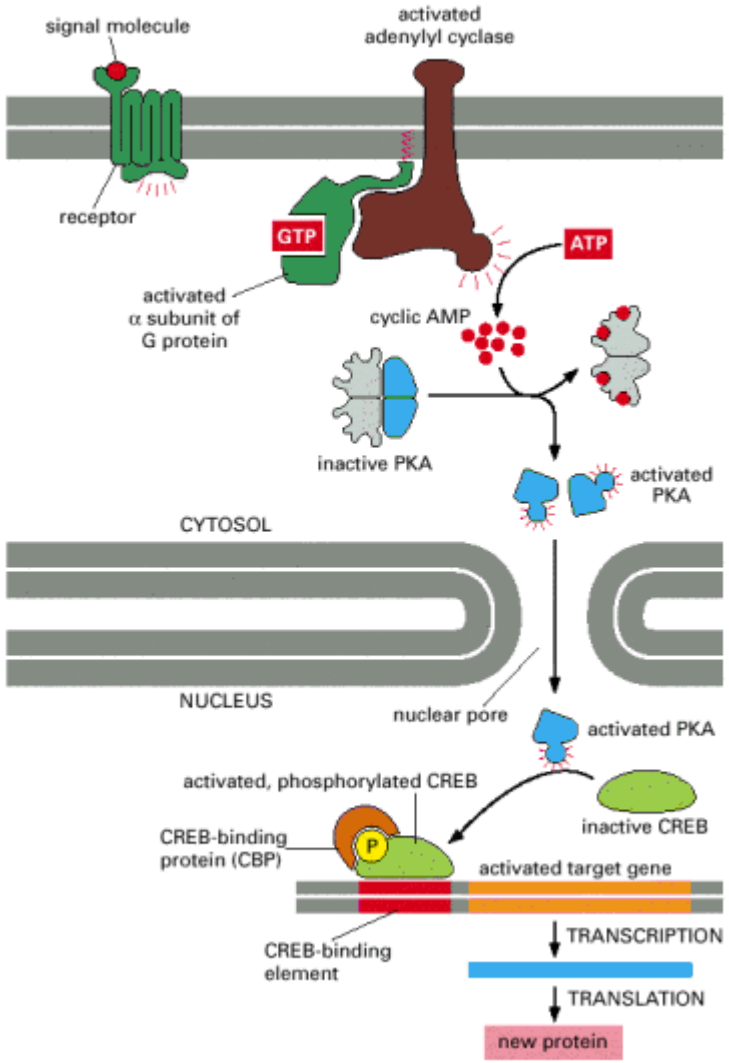
cAMP sensitive activators





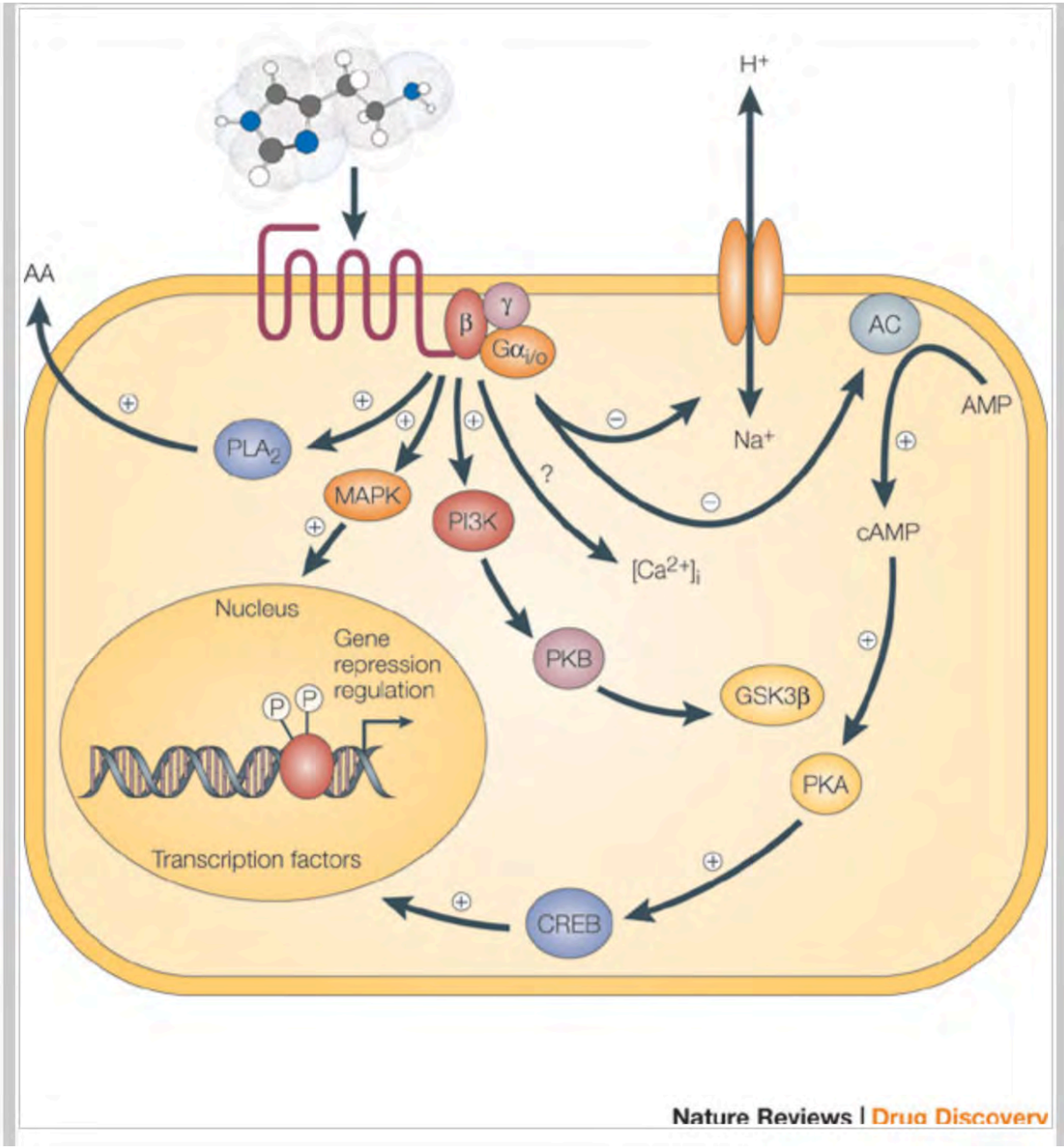
Amplification!!!!!

Activation of cAMP responses by Gs coupled GPCRs

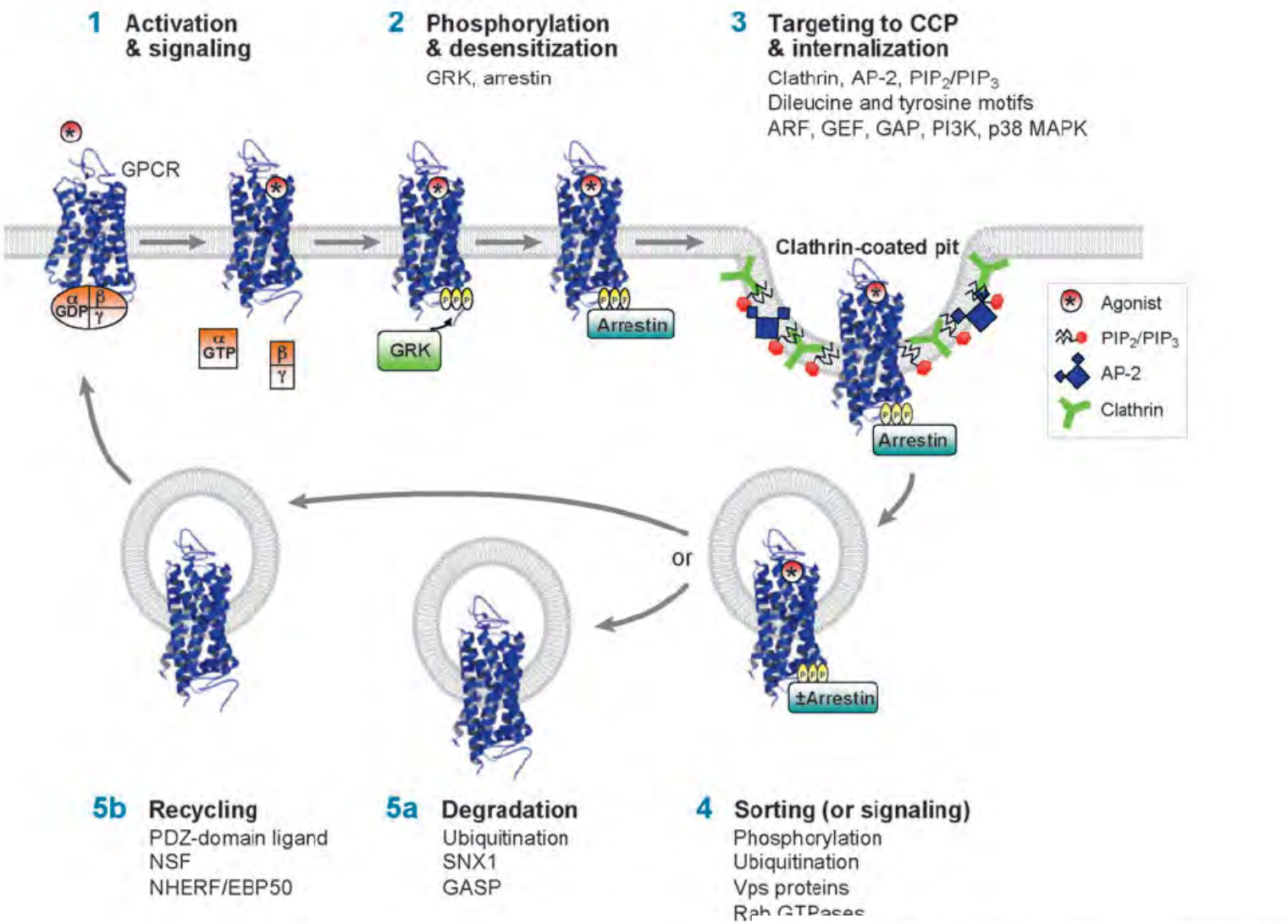


How gene transcription is activated by a rise in cyclic AMP concentration.

Molecular Biology of the Cell


H+
AA
β
γ
Gαi/o
AC
AMP
PLA2
Na+
MAPK
PI3K
?
cAMP
[Ca2+]i
Nucleus
Gene
repression
regulation
P
P
PKB
GSK3β
PKA
Transcription factors
CREB
Nature Reviews | Drug Discovery

What about switching off (desensitization) and beta-arrestins and GRKs



www.annualreviews.org • Receptor Trafficking

GRK = GPCR kinase

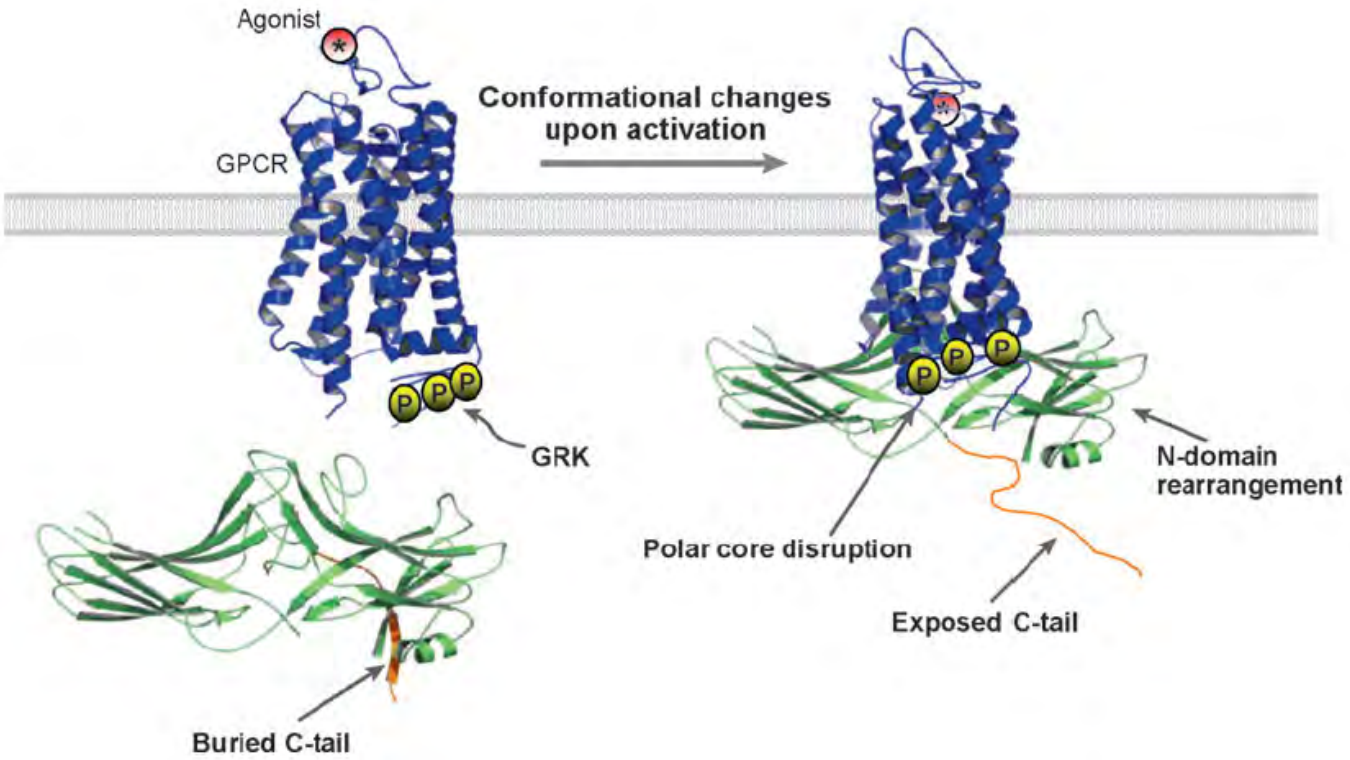


Figure 3

Model depicting conformational changes in arrestin that occur upon receptor binding. Some of the proposed conformational changes that occur upon receptor binding include disruption of the polar core and rearrangements of the N- and C-terminal domains. These changes enhance receptor binding and unmask binding sites for clathrin and β2-adaptin. Abbreviations used: GPCR, G protein–coupled receptor; GRK, G protein–coupled receptor kinase.

Final word to Bob…

Thus, it not only desensitizes G protein-mediated signaling but simultaneously serves as a signal transducing system in its own right. β-Arrestins act as adaptors or scaffolds which link the receptors to an ever growing number of intracellular molecules.[53,54] Some of the pathways which have been demonstrated over the past few years are shown here as are the resulting cellular physiological consequences. Most thoroughly studied have been the MAP kinase enzymes. β-Arrestins also mediate clathrin coated pit endocytosis by interacting with a growing list of elements of the endocytic machinery.[55] Thus, the β-arrestins mediate three types of function, desensitization, receptor internalization and signaling.

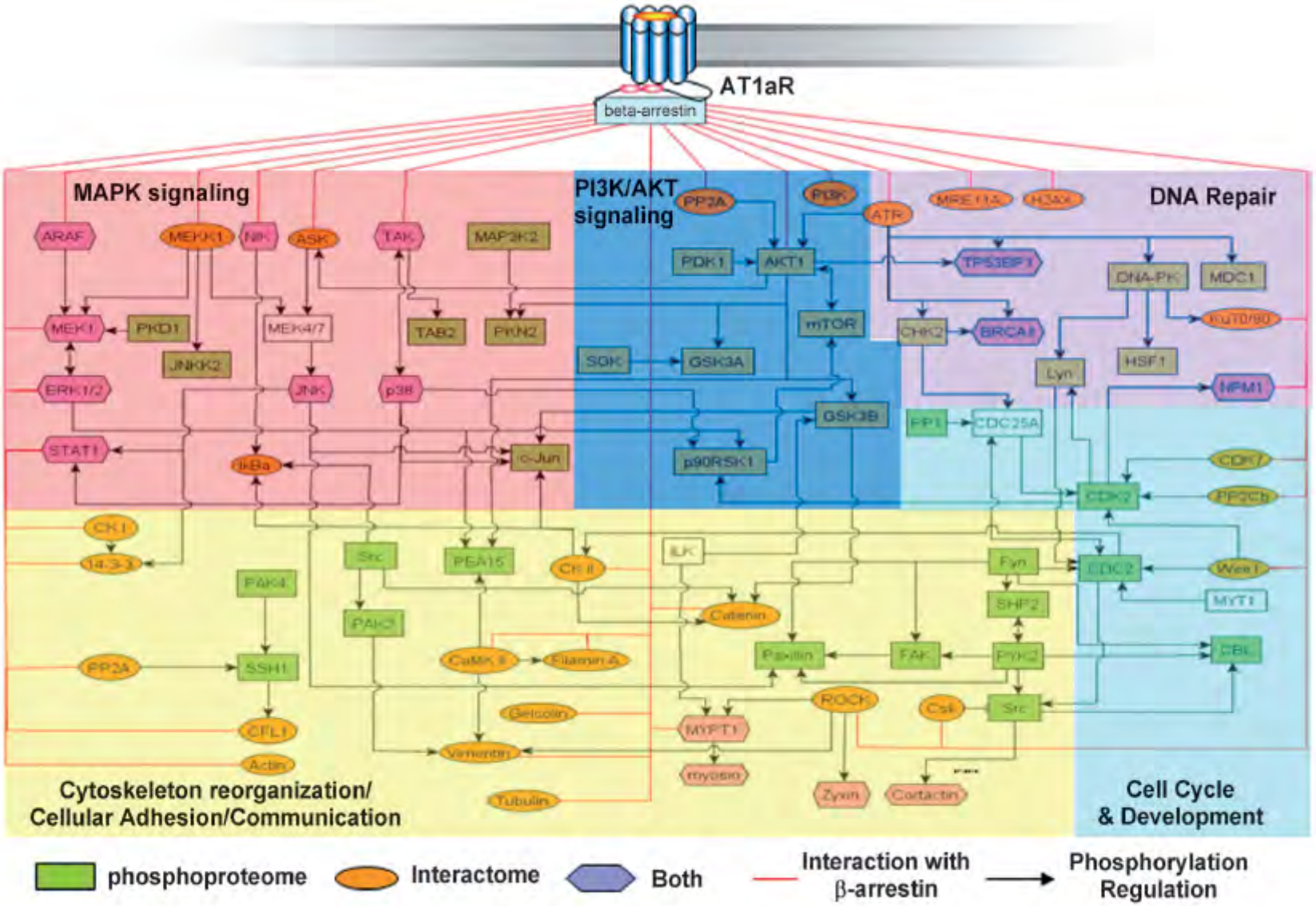


Figure 14. A β-arrestin-dependent kinase network downstream of the angiotensn AT_{1a} receptor reproduced with permission from Ref. [59].

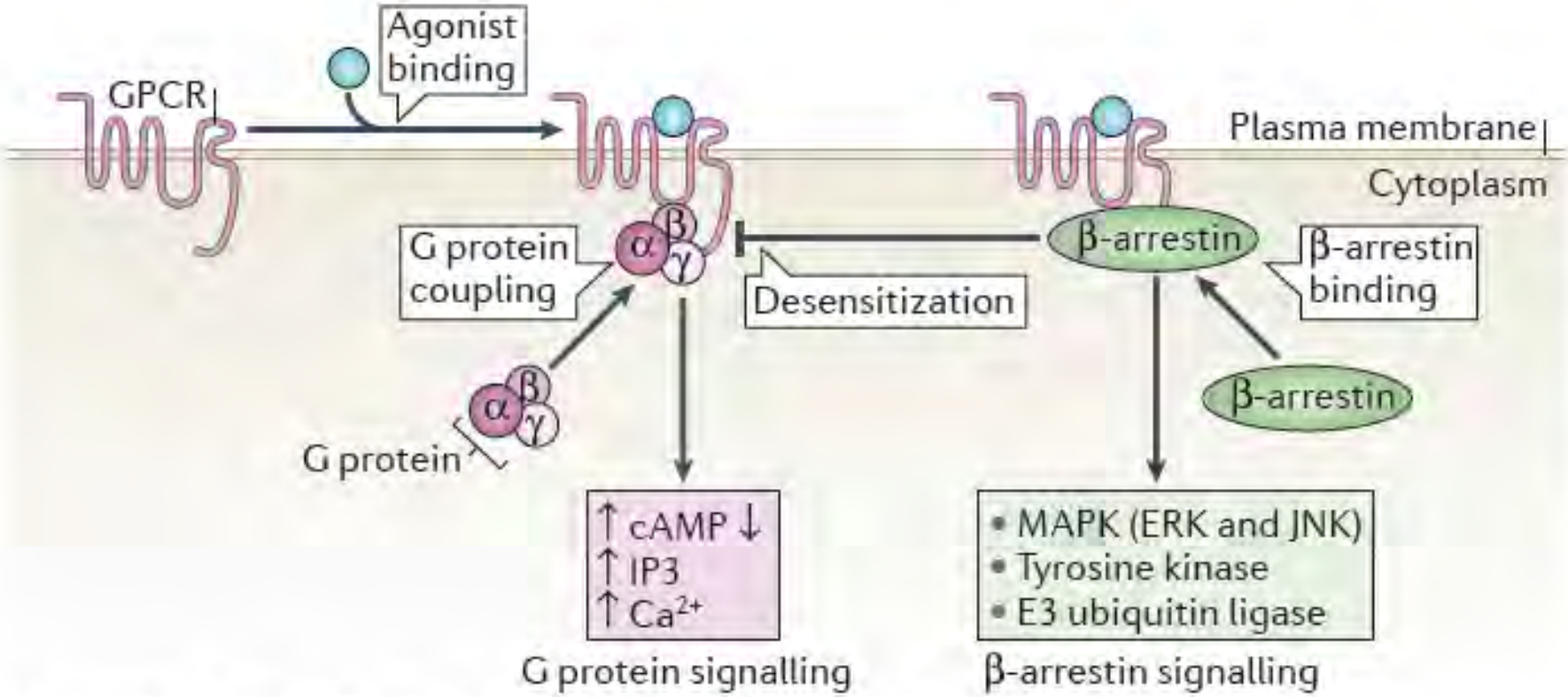


Figure 1 | **A simplified schematic of GPCR signalling.** Binding of an agonist (activating ligand) induces a conformational change in the G protein-coupled receptor (GPCR) to activate it. Activated receptors couple to heterotrimeric G proteins composed of Gα, Gβ and Gγ subunits. Subsequently, the heterotrimeric G proteins dissociate and G protein signalling mediates the generation of second messengers such as cyclic AMP, inositol triphosphate (IP3) and Ca^{2+}. Activated receptors are phosphorylated, primarily in the carboxyl terminus, by GPCR kinases. Phosphorylated receptors recruit β-arrestins, which are multifunctional adaptor proteins that block further G protein–GPCR coupling, potentially through a steric hindrance mechanism (referred to as desensitization).

Ghosh et al, Nat Rev: mol Cell biol 16:69 2015

Journal of Cell Science (2015) 128, 3533-3542 doi:10.1242/jcs.175158

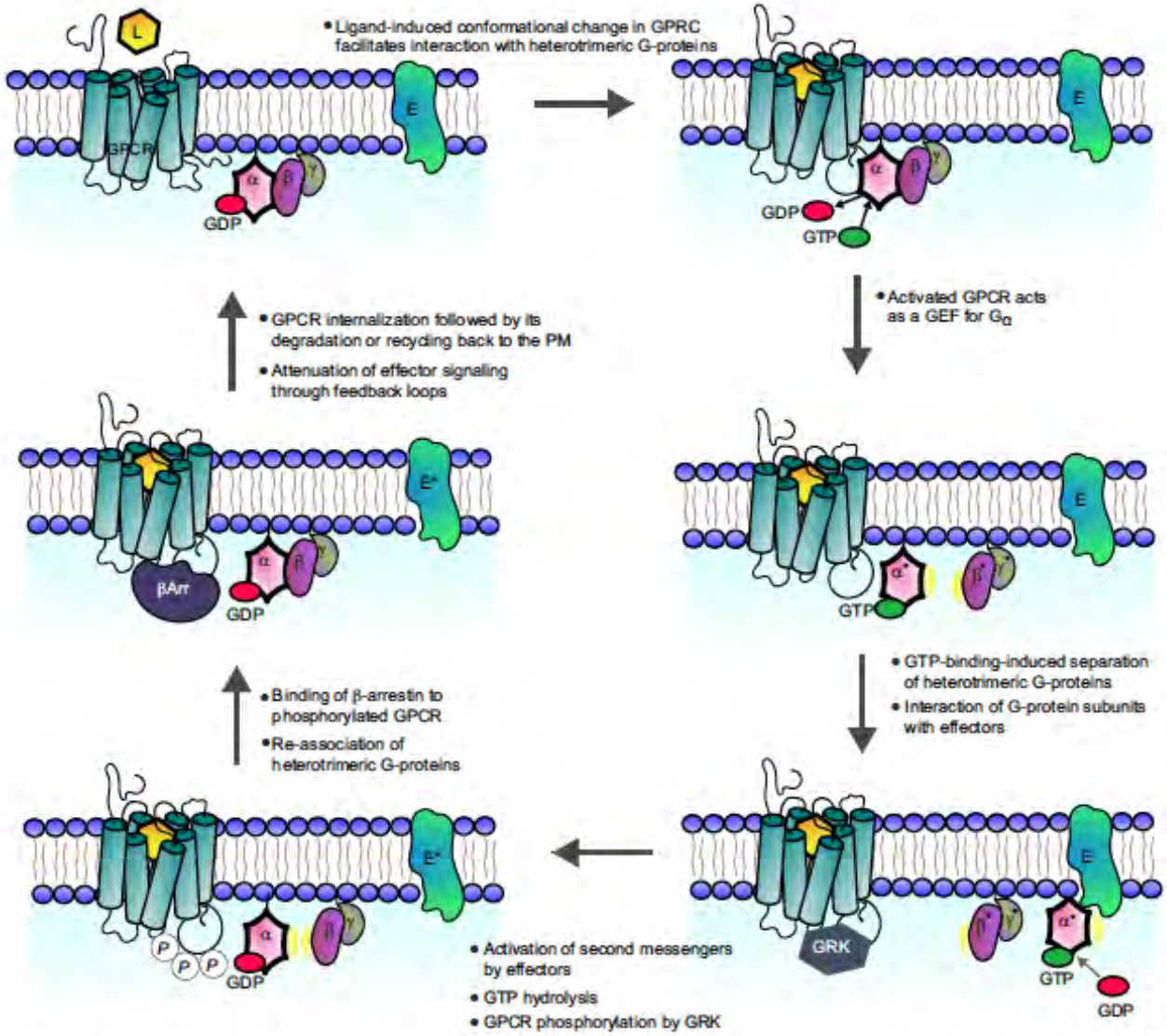


Fig. 1. The GPCR cycle. The GPCR cycle starts on the top left of the figure. In its basal state, a GPCR is free of ligand (L). Gα binds to GDP and associates with Gβγ. The heterotrimeric protein complex might associate with the receptor at this point, or remain free in the membrane as pictured. Upon ligand binding the GPCR becomes activated and undergoes a conformational change. The activated GPCR acts as a GEF for Gα. The resulting GTP-bound Gα separates from βγ, and the heterotrimeric proteins are active (*). Activated Gα can then interact with an effector (E), such as PLC or adenylate cyclase, which results in effector activation (*) and initiation of a second-messenger cascade. The GTP in Gα is then hydrolyzed to GDP through the activity of Gα and RGS proteins (not shown), leading to Gα inactivation and reassociation of the heterotrimeric protein complex. Independently, GRKs bind to and phosphorylate the GPCR. This stimulates its binding by β-arrestin (βArr), which promotes internalization of the receptor. The GPCR can then be recycled back to the cell surface without ligand, restarting the cycle.

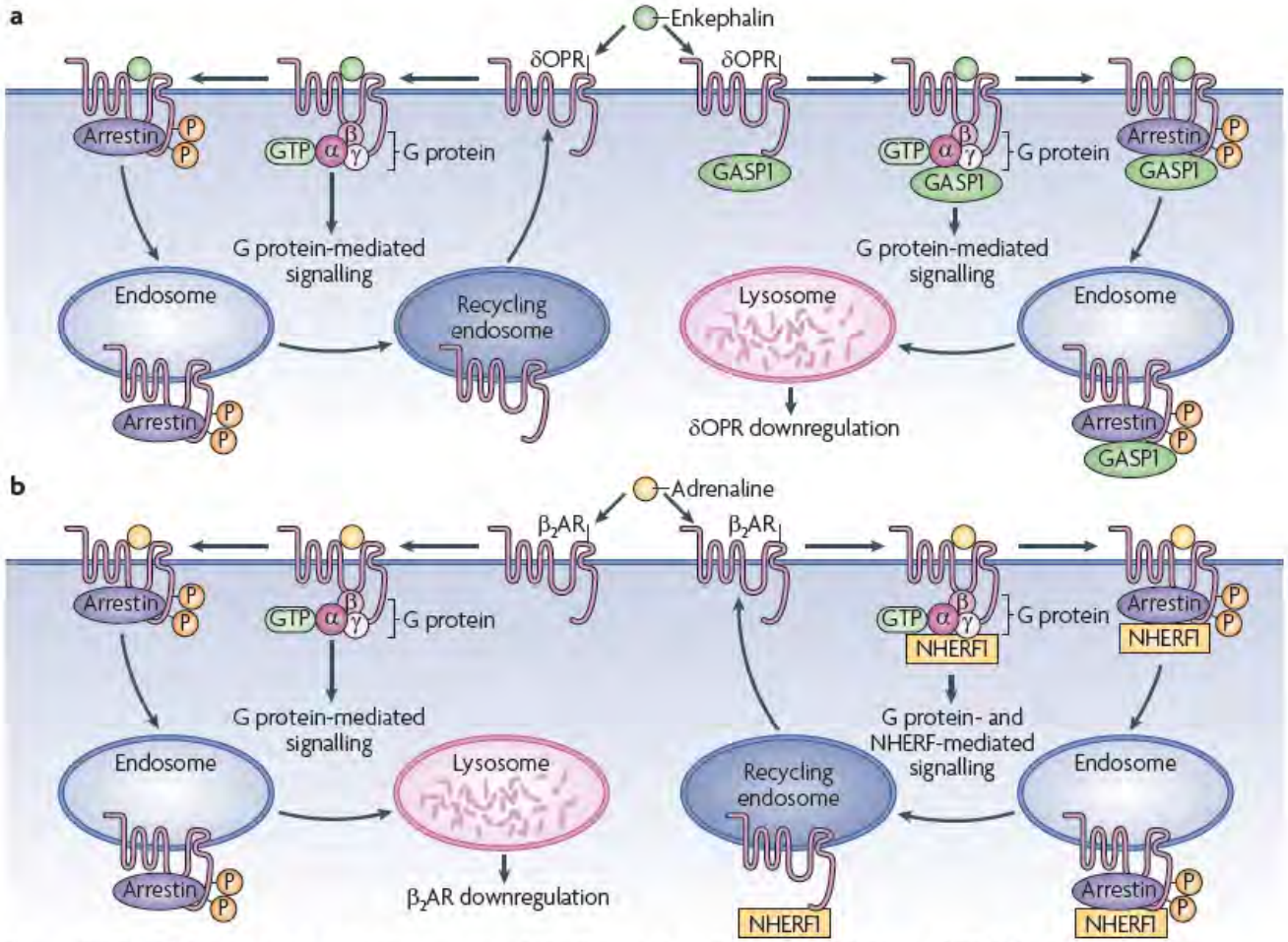
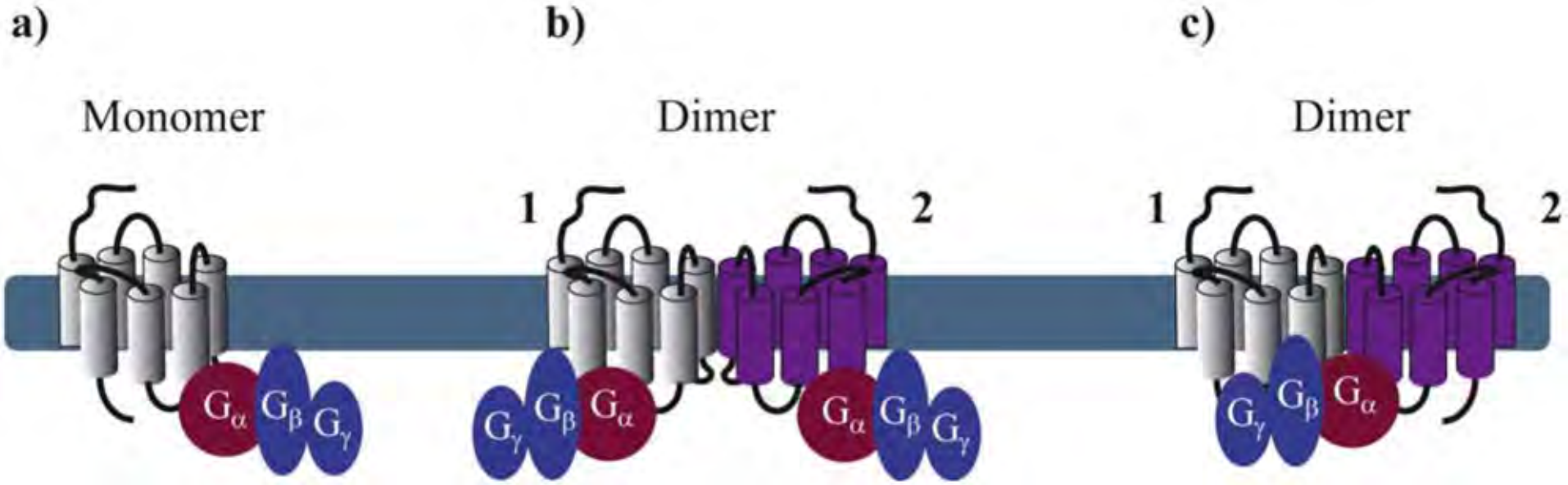


Figure 3 | **GPCR-interacting proteins can regulate the post-endocytic trafficking of GPCRs.** Following agonist-induced receptor endocytosis, some G protein-coupled receptors (GPCRs) are targeted for proteolytic and/or lysosomal degradation, whereas other GPCRs rapidly recycle back to the plasma membrane. **a** | The interaction between GPCR-associated sorting protein 1 (GASP1) and δ-type opioid receptor (δOPR) promotes the endocytic targeting of agonist-internalized δOPRs to lysosomes, where the receptors are degraded. However, in a distinct cellular compartment (or distinct cell type) that lacks GASP1, as shown on the left, δOPRs are rapidly recycled back to the plasma membrane. **b** | By contrast, the interaction between the GPCR-interacting protein Na^+–H^+ exchange regulatory factor 1 (NHERF1; also known as EBP50 and SLC9A3R1) and the β_2-adrenergic receptor (β_2AR) promotes the rapid recycling of receptors following agonist-promoted internalization. However, in a distinct cellular compartment (or distinct cell type) that lacks NHERF1, as shown on the left, β_2ARs are preferentially targeted to lysosomes for degradation.

GPCRs can homo- and heterodimerise



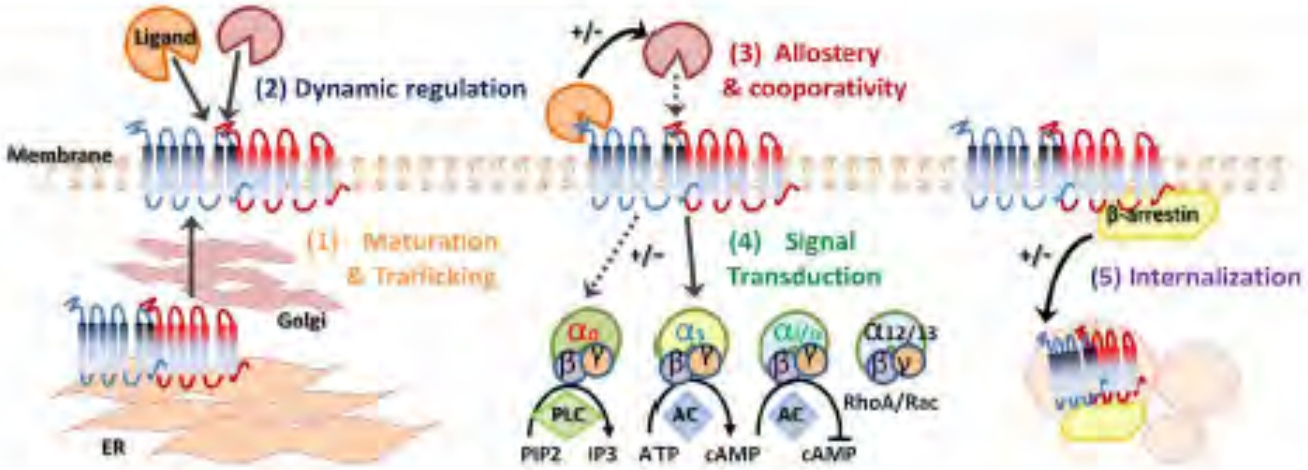
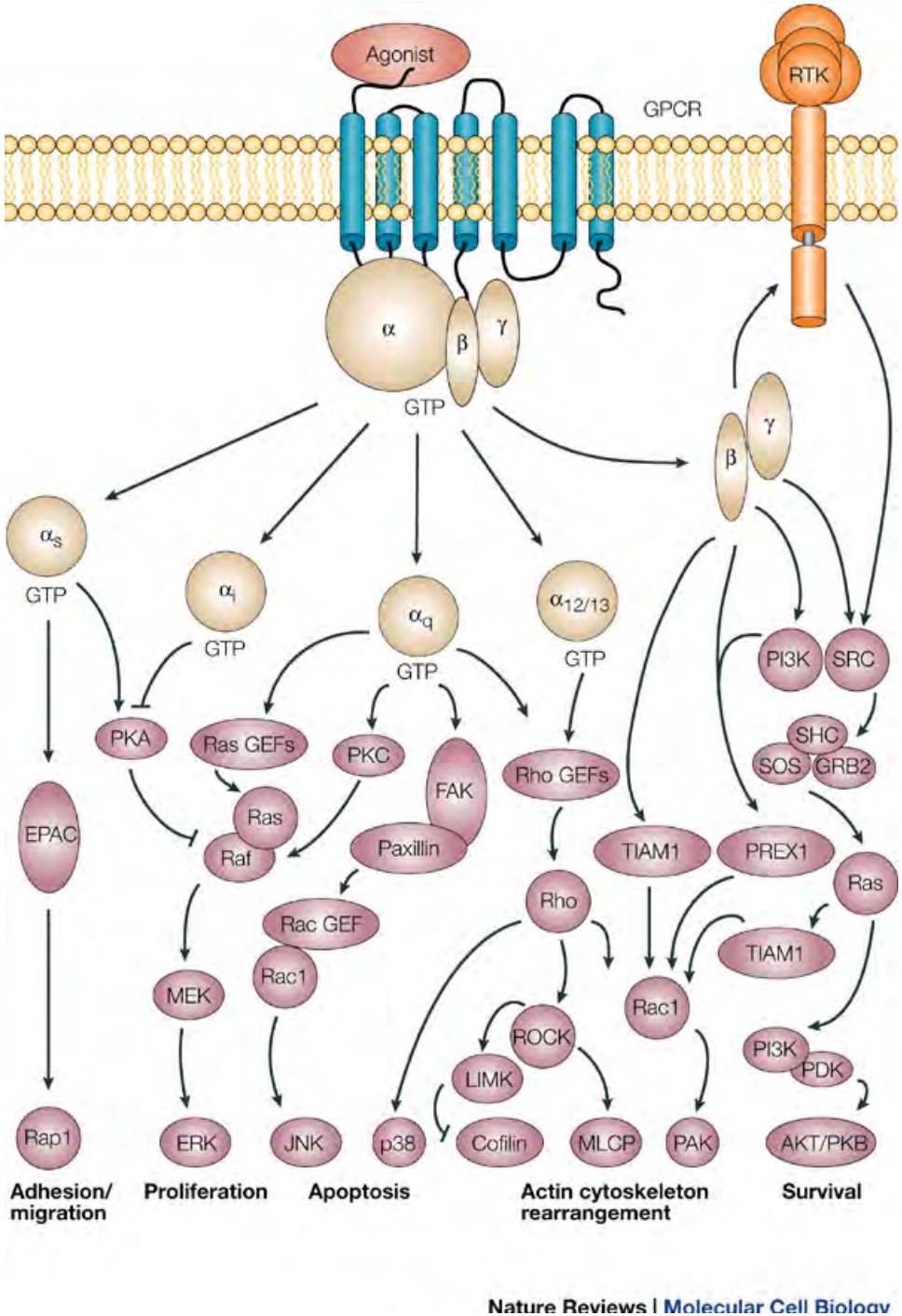


FIGURE 1 | Heterodimerization of G-protein coupled receptors. G-protein coupled receptor (GPCR) oligomerization has several biological functions and consequences. Receptor dimerization can play a role in receptor maturation and correct trafficking **(1)**. Specific ligand binding can dynamically regulate heterodimerization **(2)** and allostery can enhance or suppress downstream signaling **(3)**. In addition, GPCR heterodimerization may demonstrate preferential G protein coupling **(4)**. Finally, agonist-promoted GPCR endocytosis and co-internalization may lead to signal attenuation **(5)**. +/− indicates increase or decrease, respectively.



Plus Small GTPases - small monomeric GTPases that function Independently
Ras etc

How can we measure GPCRs?

- How can we detect the signalling pathways
- How can we detect GPCR interactions ?

- Fluorescence based techniques are one important answer…

What is fluorescence?

- ***Fluorescence* is the property of emitting electromagnetic radiation in the form of light as the result of (and only during) the absorption of light from another source.**

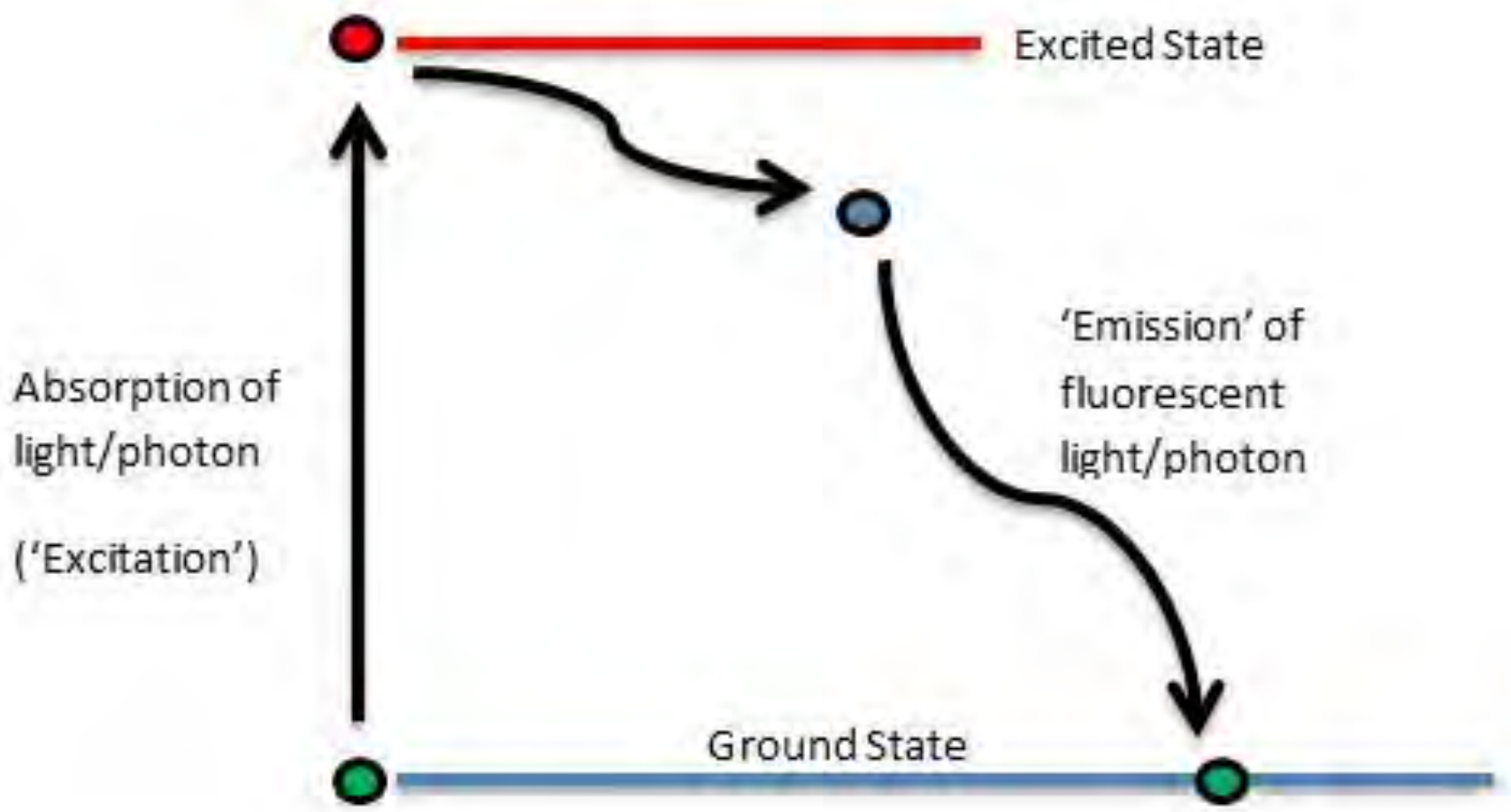
1. It is the result of the absorption of light.

2. It involves the emission of light.

3. An outside source of energy is required.

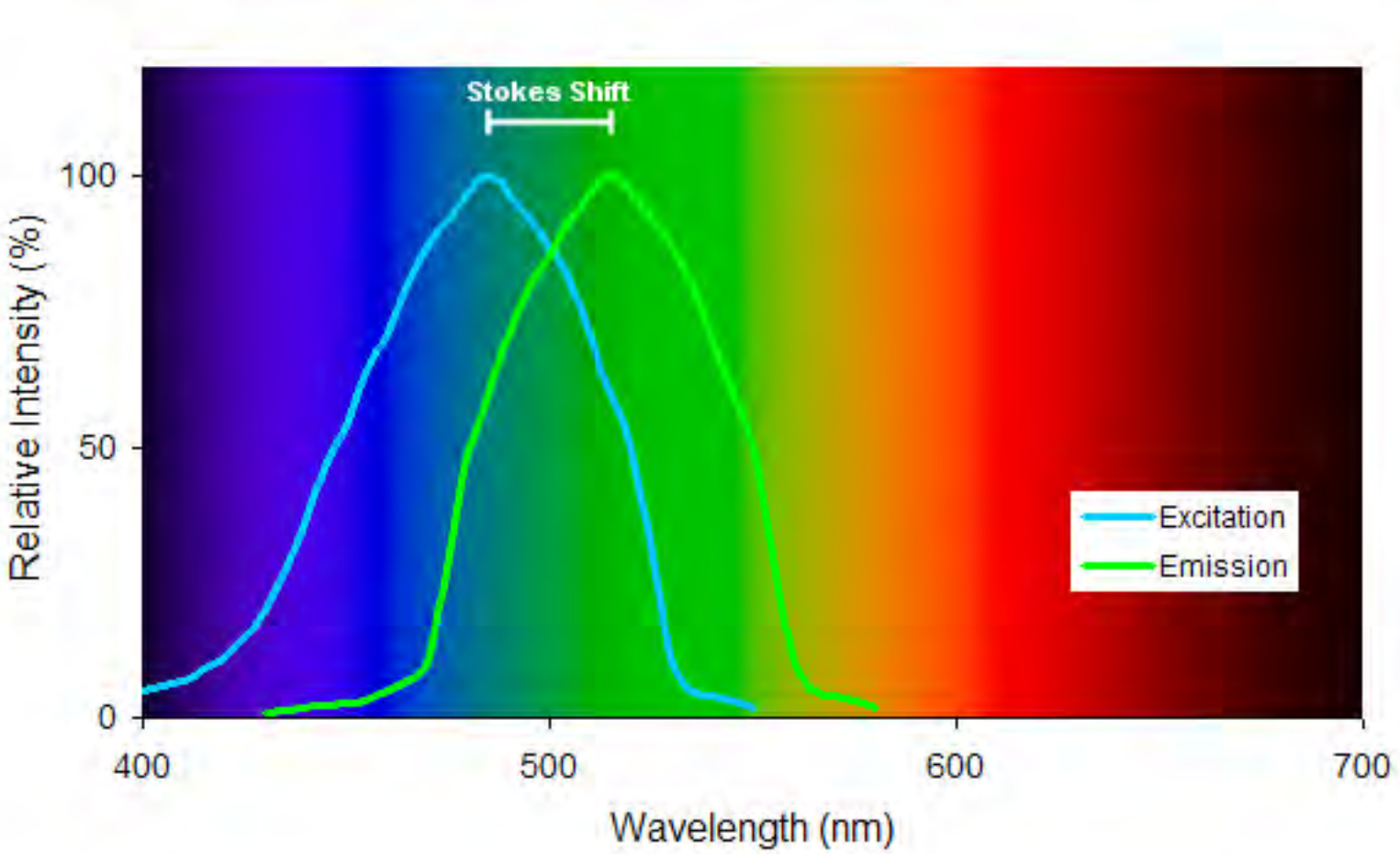
- A fluorophore emits light after exposure to a photon
- The light absorbed is by an electron of the fluorophore and this higher energy excites the fluorophore
- As the fluorophore returns to the “ground” state it emits a photon at a longer wavelength
- Excitation and emission wavelengths

Jablonski Diagram



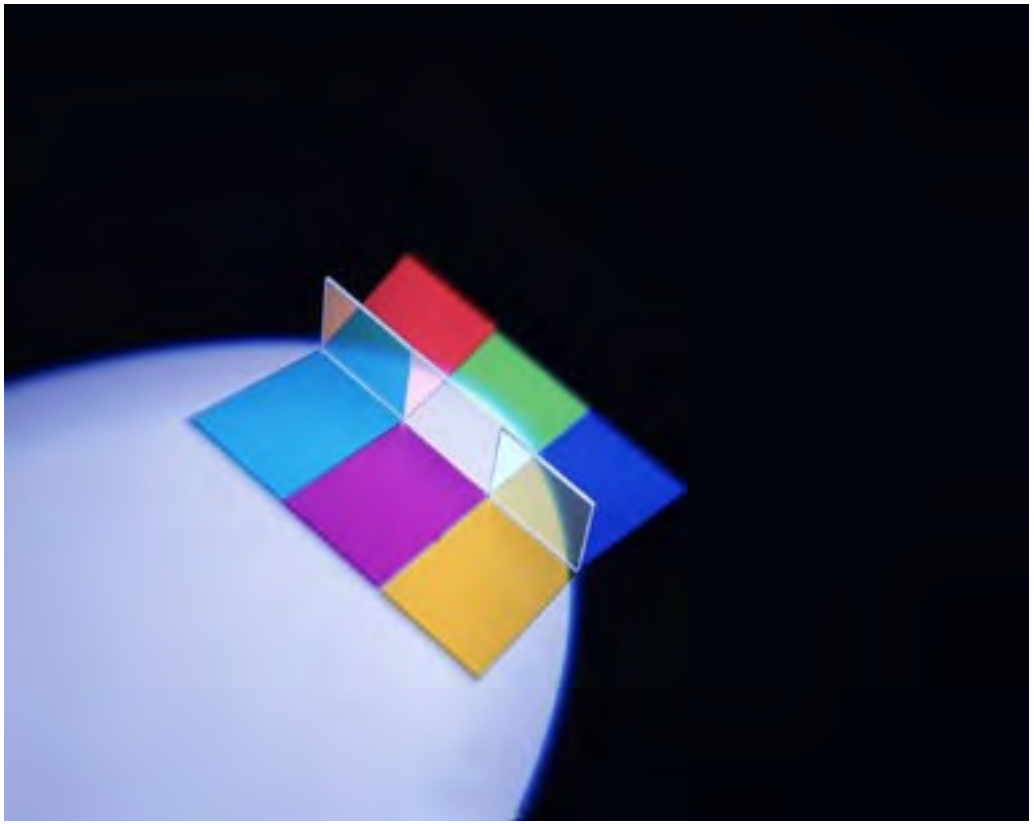
Stokes Shift

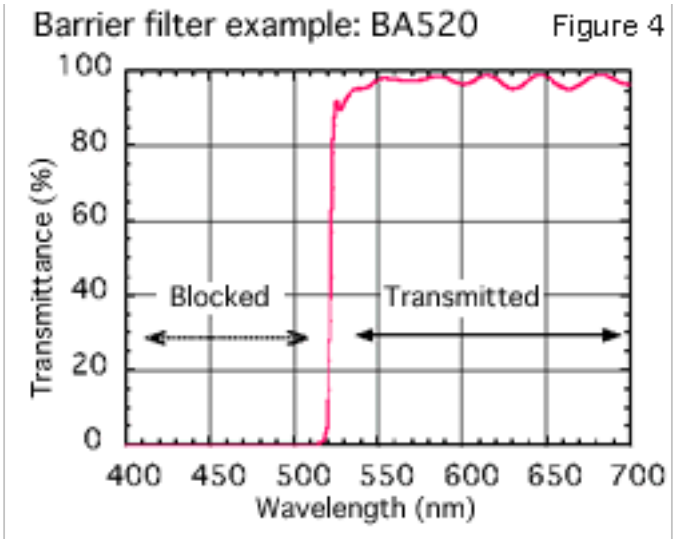
- Difference in nm between the peak excitation

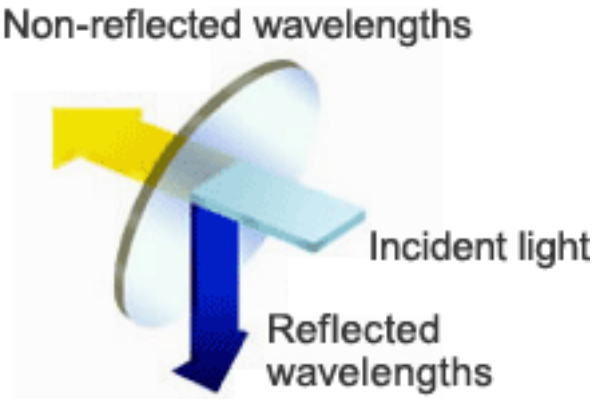


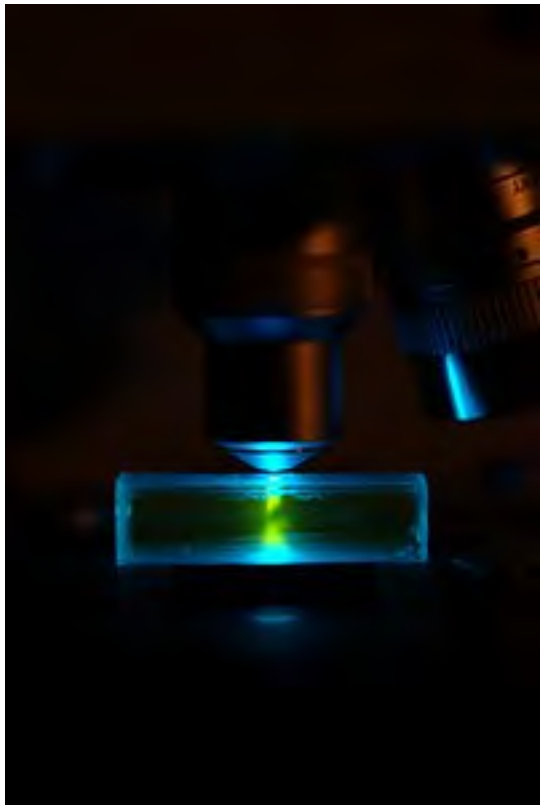
How to sort out light !

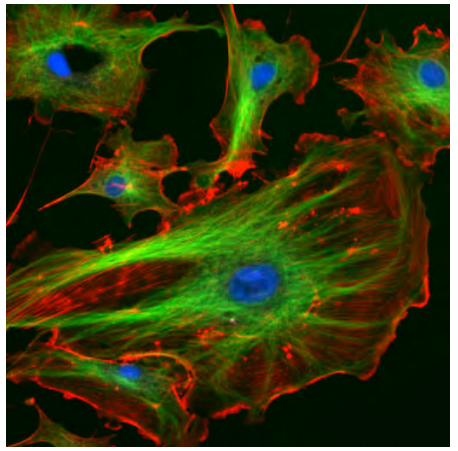
Beam splitters – dichroic mirrors – barrier filters

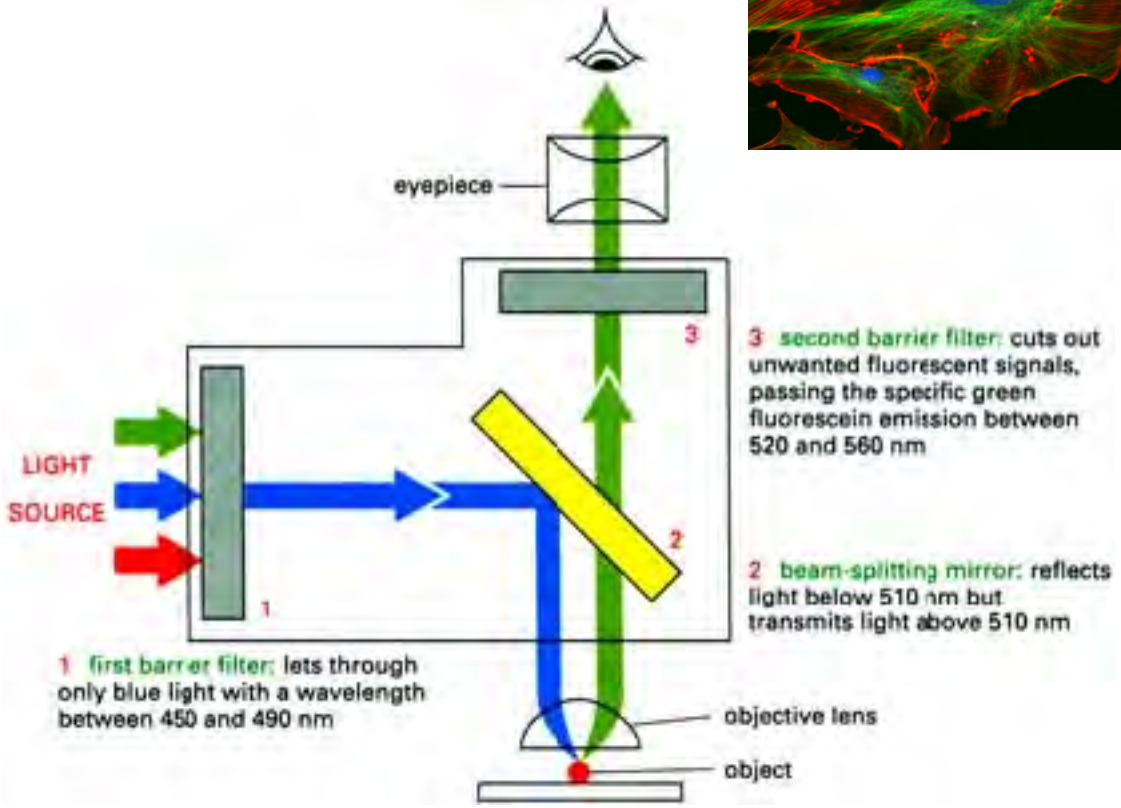


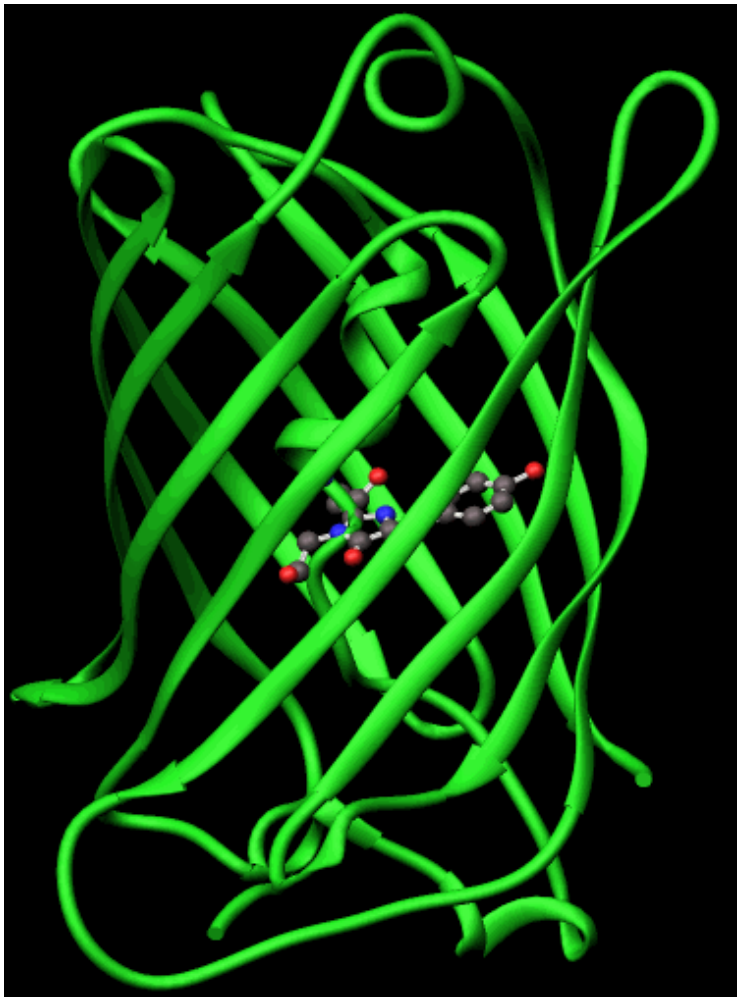


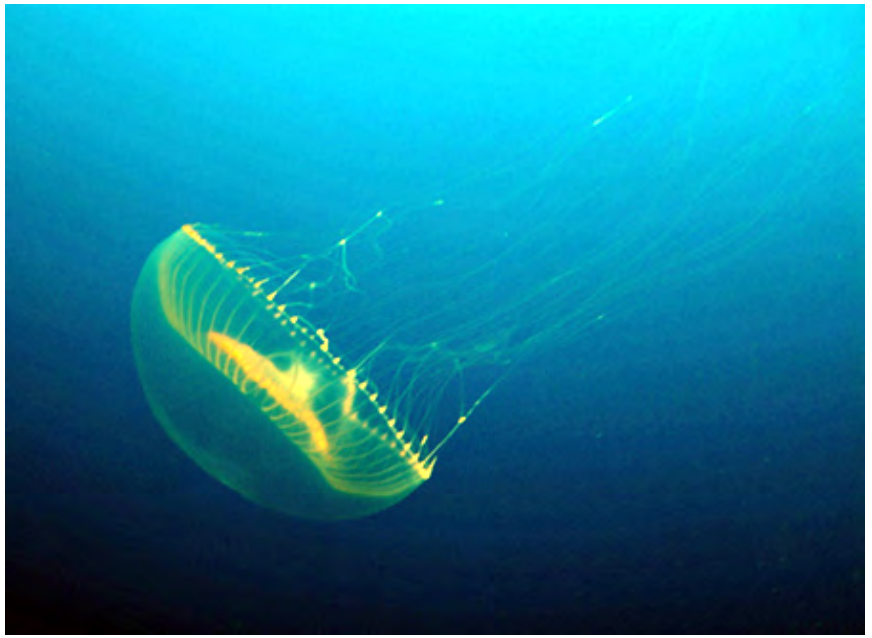


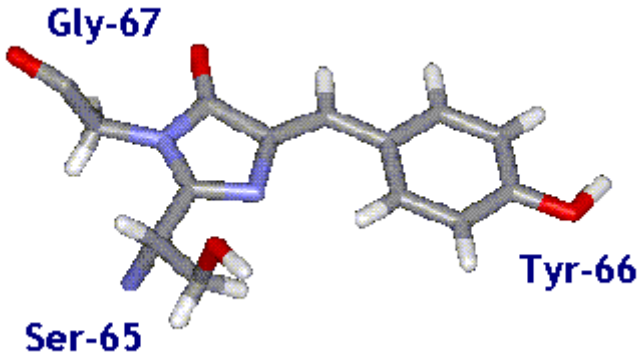





eyepiece
3
3 second barrier filter: cuts out unwanted fluorescent signals, passing the specific green fluorescein emission between 520 and 560 nm
LIGHT
SOURCE
2
2 beam-splitting mirror: reflects light below 510 nm but transmits light above 510 nm
1
1 first barrier filter: lets through only blue light with a wavelength between 450 and 490 nm
objective lens
object

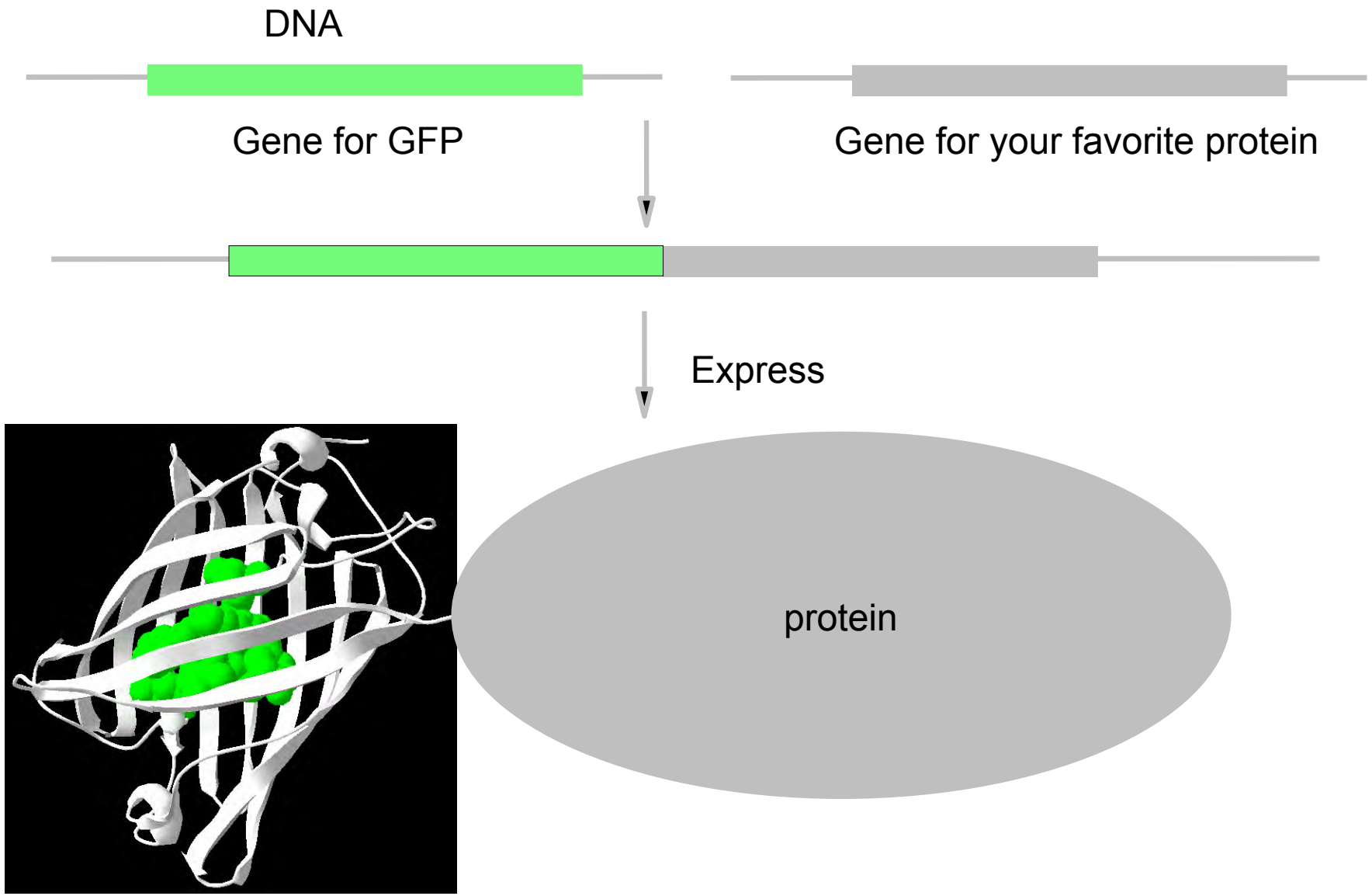


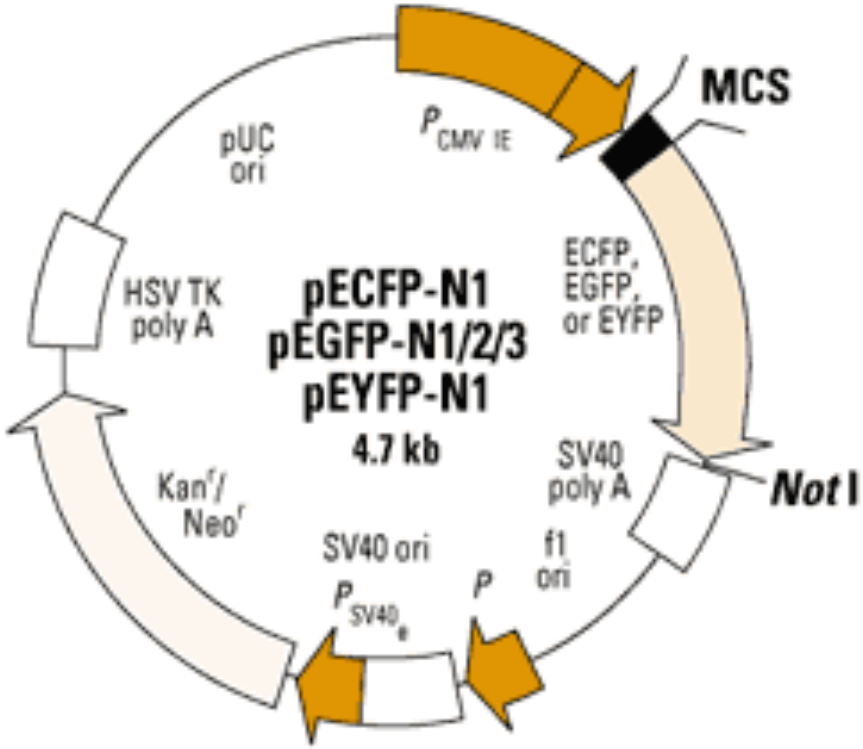


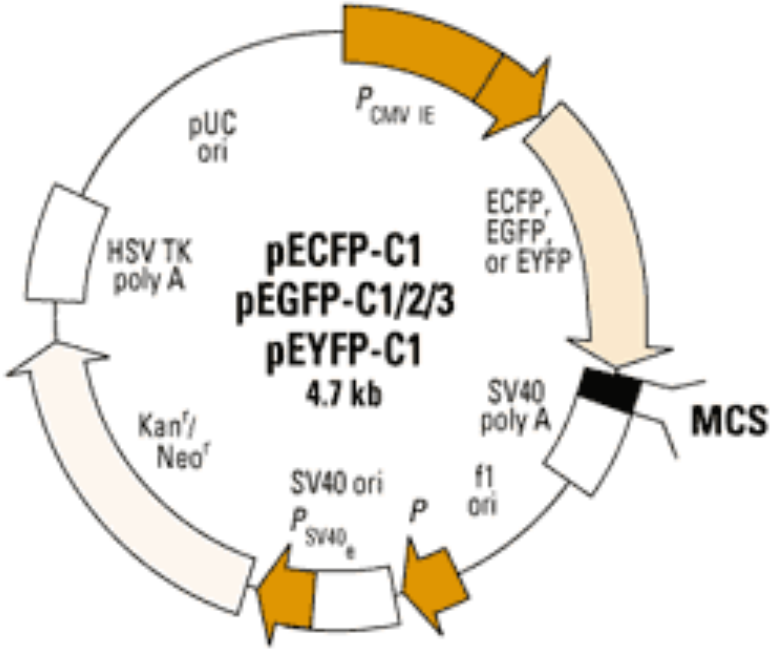


“the elegant assemblage is composed of 238 amino acids arranged in a stable birdcage-shaped structure with a small glowing strand of matter suspended within like an incandescent parakeet on a perch."

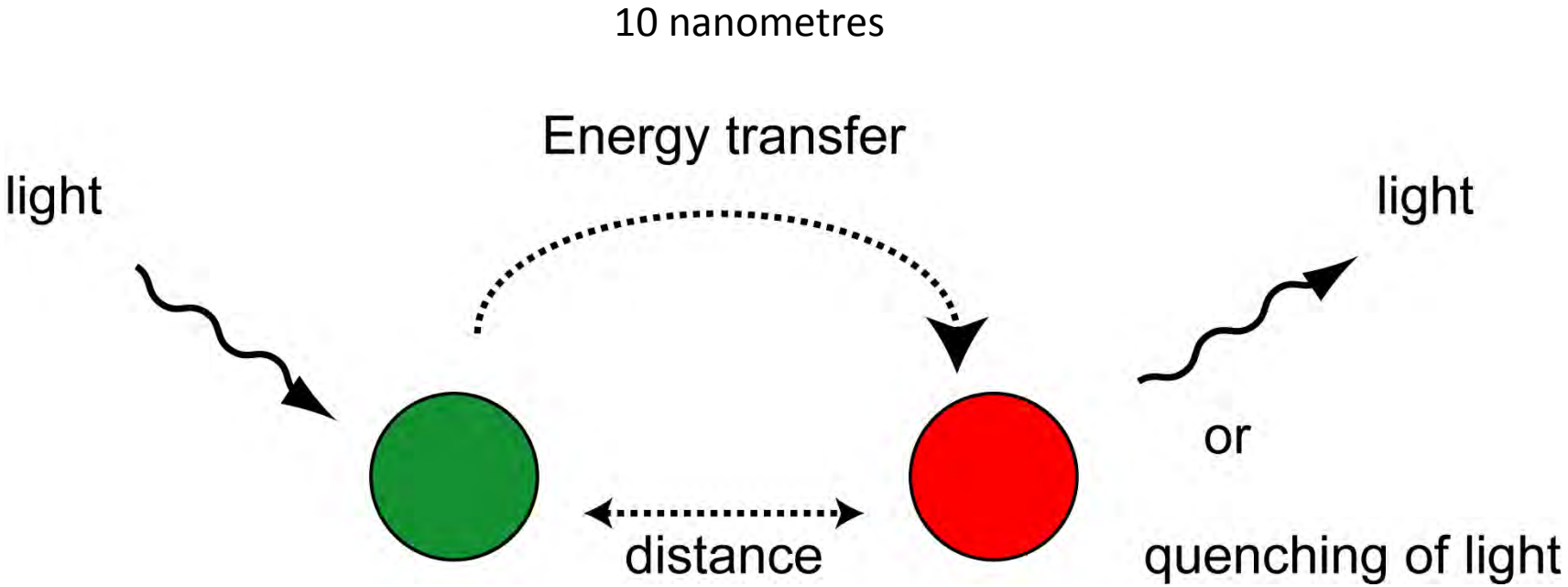
The biologist's method for fluorescent labeling of living cells:
attach a fluorescent protein



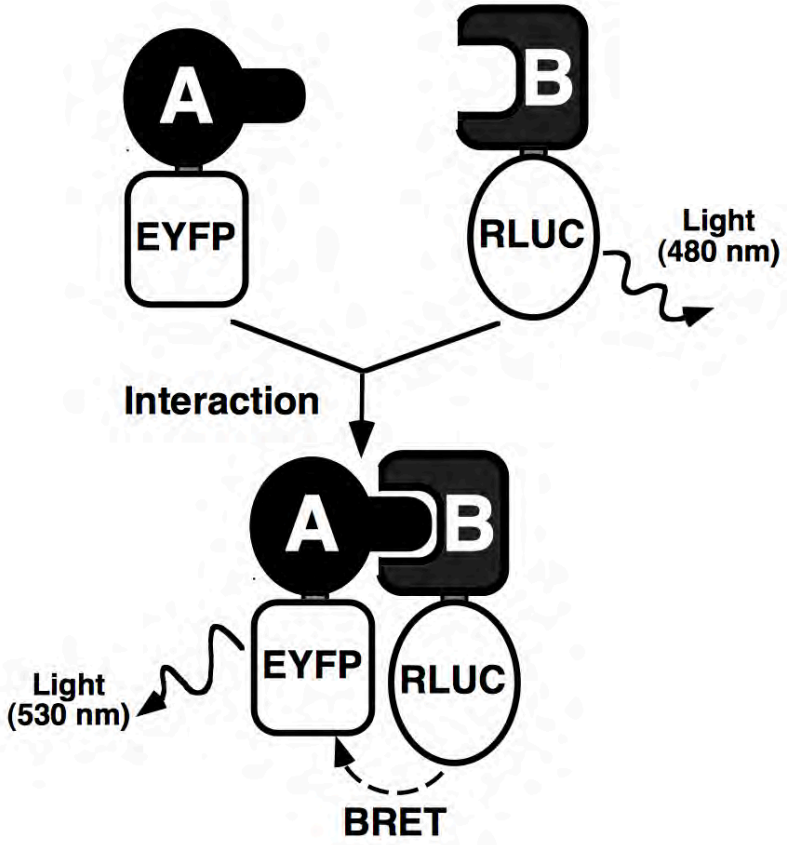

MCS
pUC ori
$P_{CMV\ IE}$
ECFP, EGFP, or EYFP
HSV TK poly A
pECFP-N1
pEGFP-N1/2/3
pEYFP-N1
4.7 kb
SV40 poly A
Not I
Kan^r/ Neo^r
SV40 ori
P_{SV40_e}
P
f1 ori

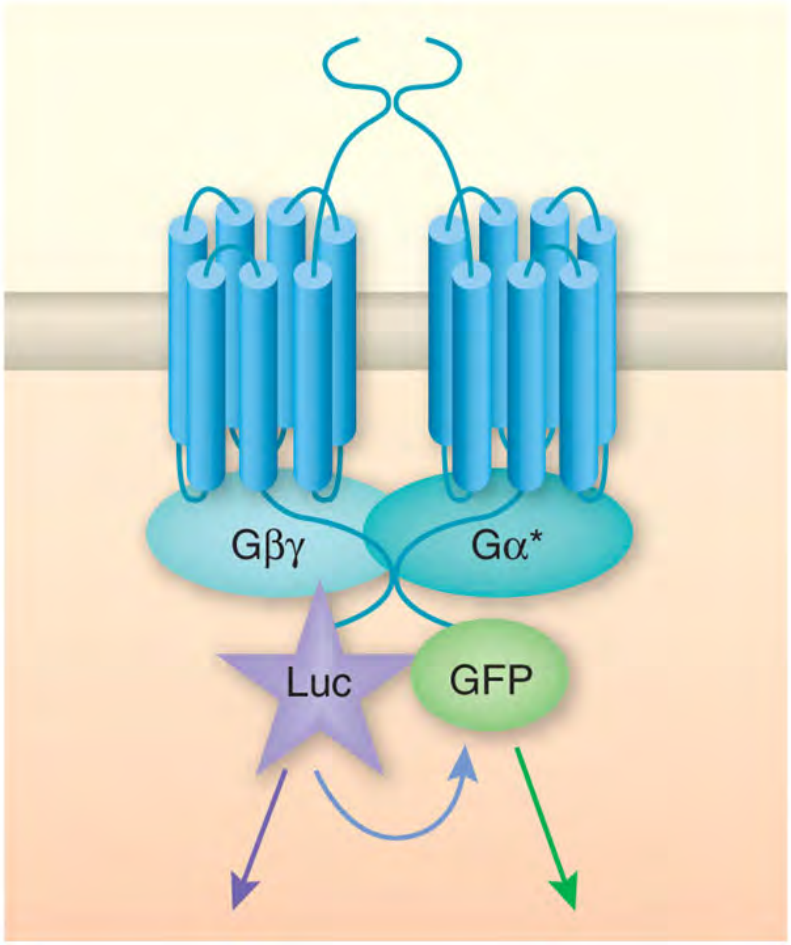

pUC ori
$P_{CMV\ IE}$
ECFP, EGFP, or EYFP
HSV TK poly A
pECFP-C1
pEGFP-C1/2/3
pEYFP-C1
4.7 kb
SV40 poly A
MCS
Kan^r/ Neo^r
SV40 ori
P_{SV40_e}
P
f1 ori

Bioluminsence resonance energy transfer BRET



Fluorescence resonance energy transfer FRET


A
B
EYFP
RLUC
Light
(480 nm)
Interaction
A
B
EYFP
RLUC
Light
(530 nm)
BRET


Gβγ
Gα*
Luc
GFP

Bimolecular fluroscence complementation assay - BiFC