European School On Nanosciences & Nanotechnologies

Advanced biophysics for micro-system design

Learning from nature the future of nanobiotechnology

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KTH VETENSKAP OCH KONST

Structure of course

Part I – Biological systems
Labeling and Interactions

1 • Cellular conditions

2 • Individual

3 • Labeling proteins

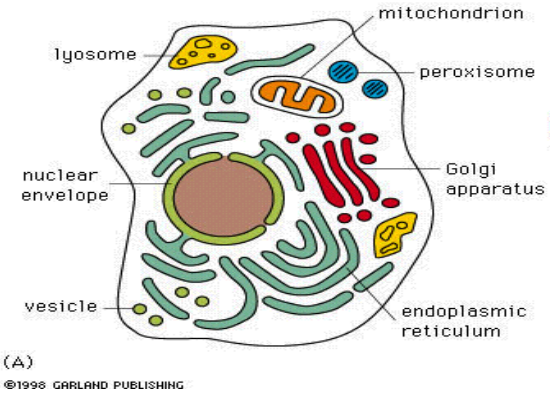
4 • Molecular interactions

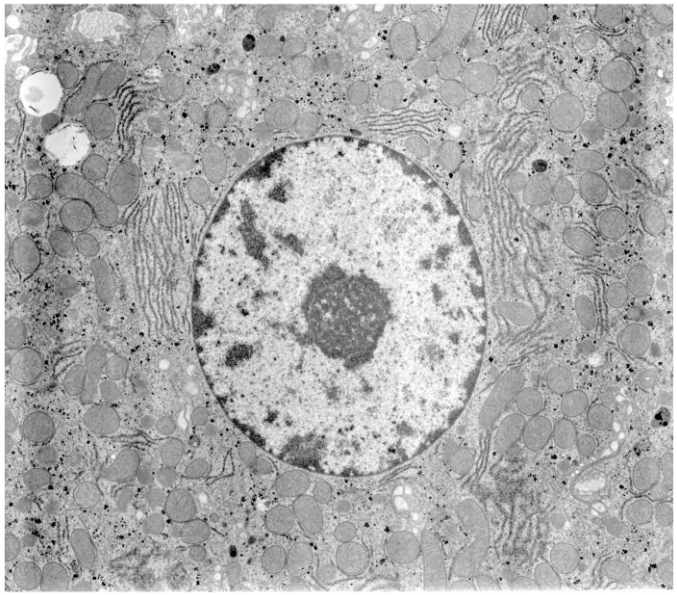
5 • Molecular sensors and machines

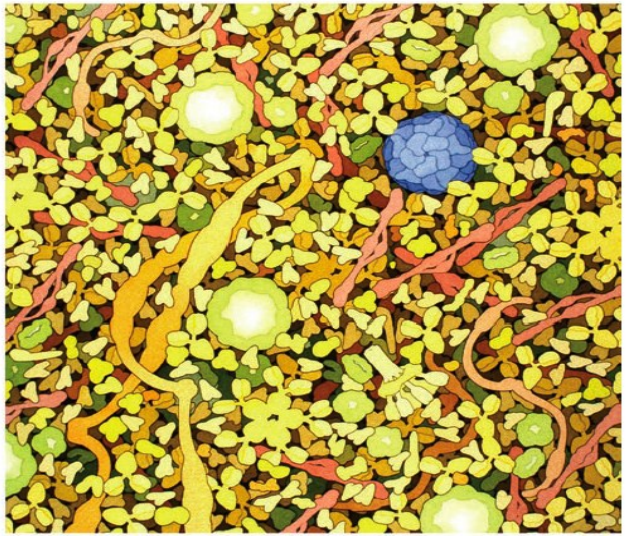
Part II - Molecular characterization
Methods and results

- Fluorescence and Single molecule detection
- Tracking of objects
- Super-resolution methods
- Single molecule experiments
- Single or few molecule devices

1 • Cells - Very complicated conditions







Goodsell, Nat Chem Biol 3 (2007) p.681

- Highly heterogeneous - compartments with very different contents
- Transport and separation
- Highly crowded
- Many different components – e.g. ~5’000 different gene products

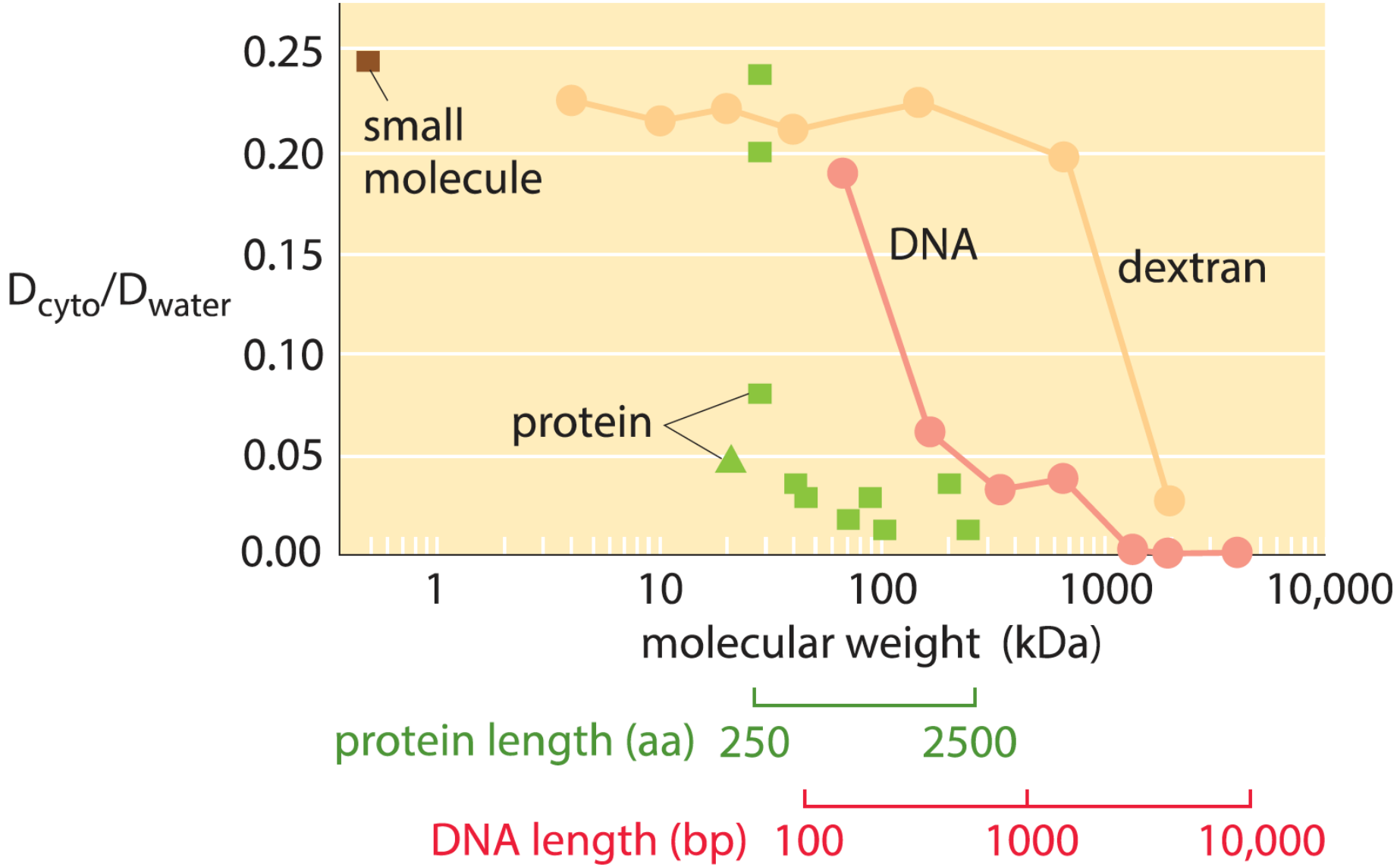
=> Very far from an ideal system in physico-chemical terms

1 • Crowding & Size-dependent hinderance

versus

1 • Effects of Crowding: Size-dependent hinderance

Diffusion in the cytosol vs in water



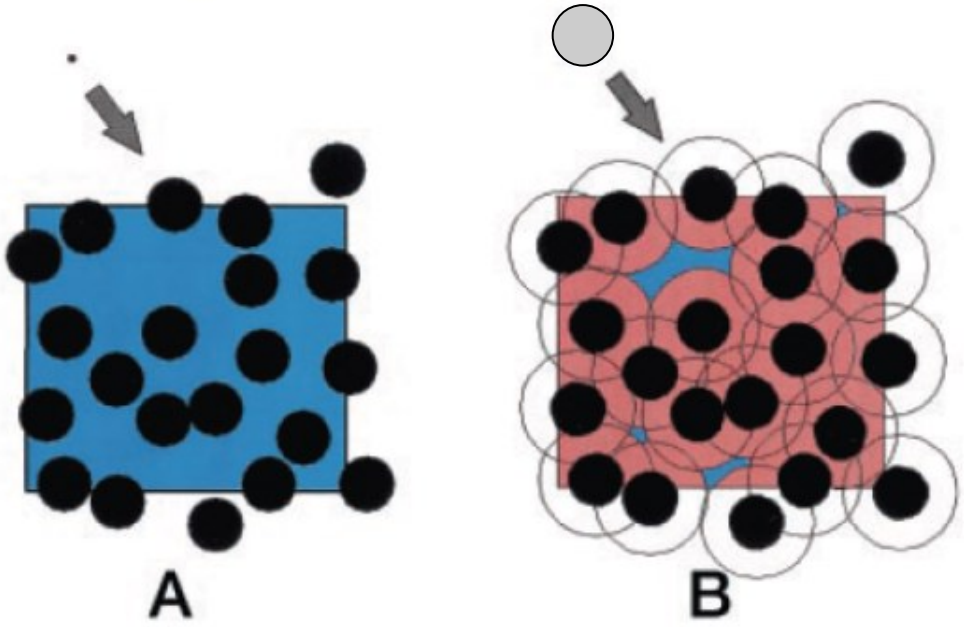
http://book.bionumbers.org/ see also Lalwarczk,Bioinformatics,2012

1 • Effects of Crowding: Excluded volume & confinement

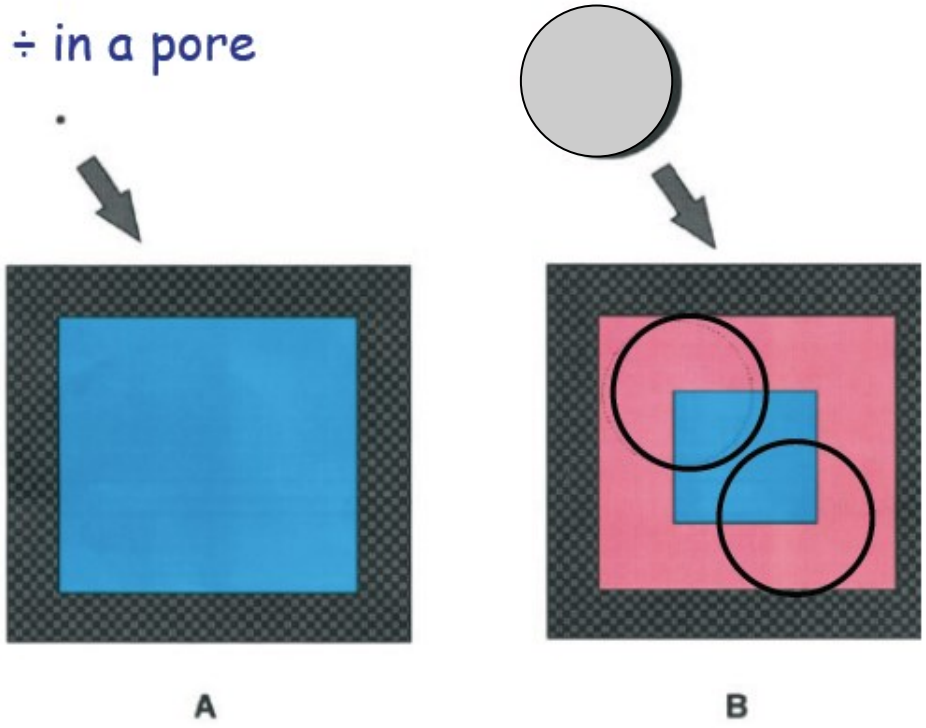
Size matters !!

Compare a small (A) and a large (B) molecule for accessible to centre of mass (blue) and excluded (pink) volume with in a solution containing large solutes ●

÷ in bulk solution



÷ in a pore



Minton, JBC, 2001

1 • Effects of Crowding: Effective concentrations

Molar concentrations [***A***] are multiplied by an *activity coefficient* α which depends on

i) total solute concentration and *ii)* molecular size of ***A***

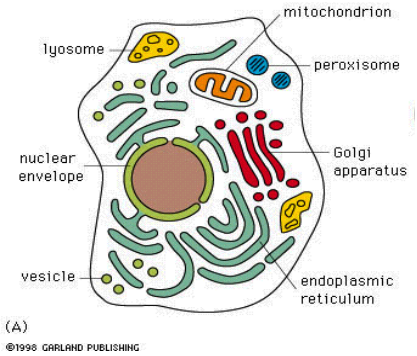
1 • Effects of Crowding: Chemical equilibria

Imagine an oligomerization reaction: $nA \rightleftharpoons A_n$

characterized by a dissociation constant $K_d = \frac{[A]^n}{[A_n]}$

2 • Quantities in biology

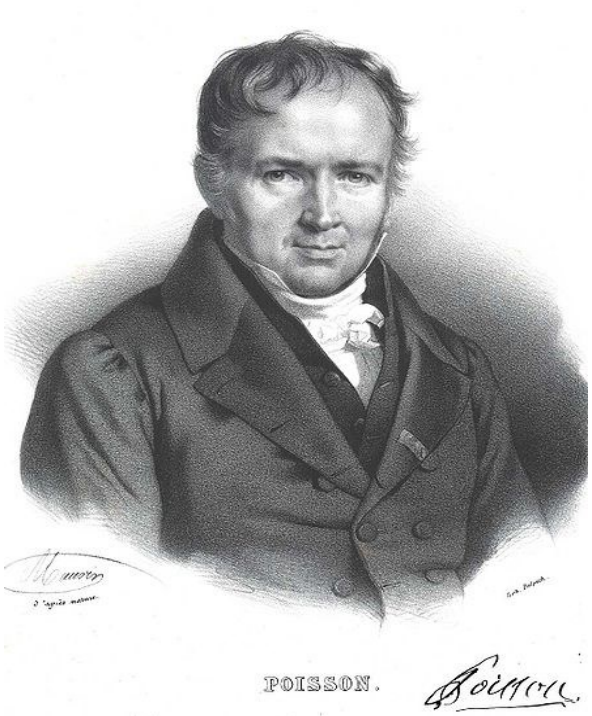
	Macroscopic world		Cellular world
Length	1 decimeter		1 μm
Volume	1 liter		10^{-15} liter



- Consequence of scale on the ***number of molecules*** present

		Macroscopic	Cellular
Salts	100 mM	10^{23}	10^{8}
Synaptic transmitter	1 mM	10^{21}	10^{6}
Hormone	1 nM	10^{15}	1

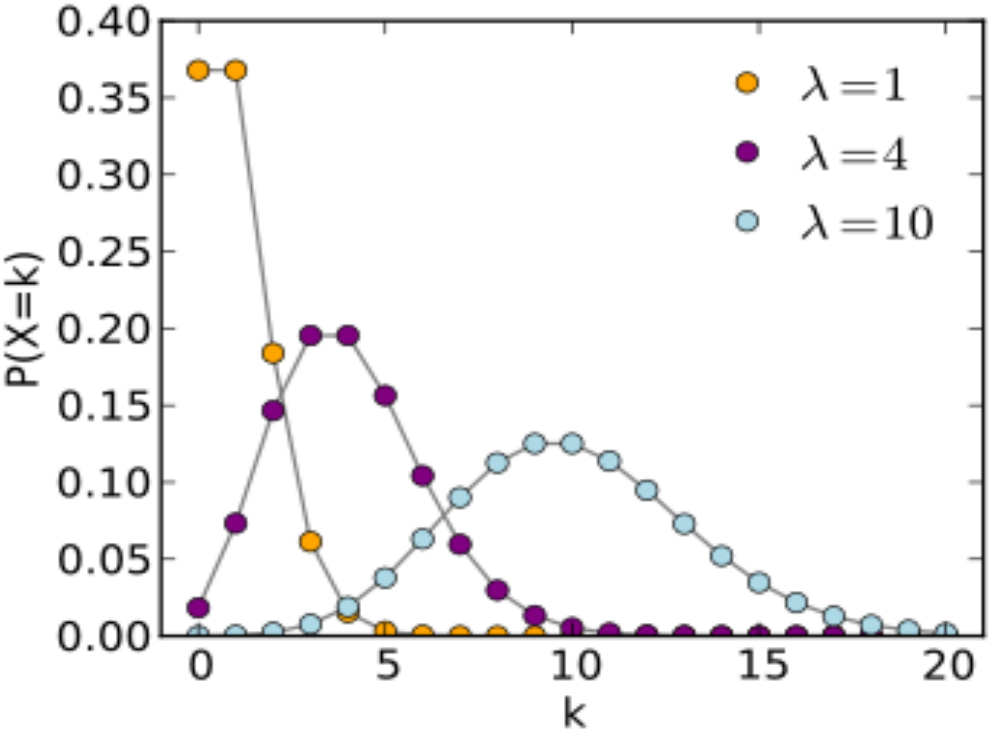
2 • Poisson distribution: To be or not to be



Siméon Denis Poisson
1781 - 1840

“when the average is small,
the change to observe nothing is not null !”

In fact, there is a distribution of the average ***λ*** with different probabilities ***p*** to observe value ***k*** *:*



$$p(k) = P(X = k) = e^{-\lambda}\frac{\lambda^k}{k!}$$

=> the lower the average ***λ***, the longer one has to observe to be sure !

2 • Small numbers reveal individual molecular characters

Two examples :

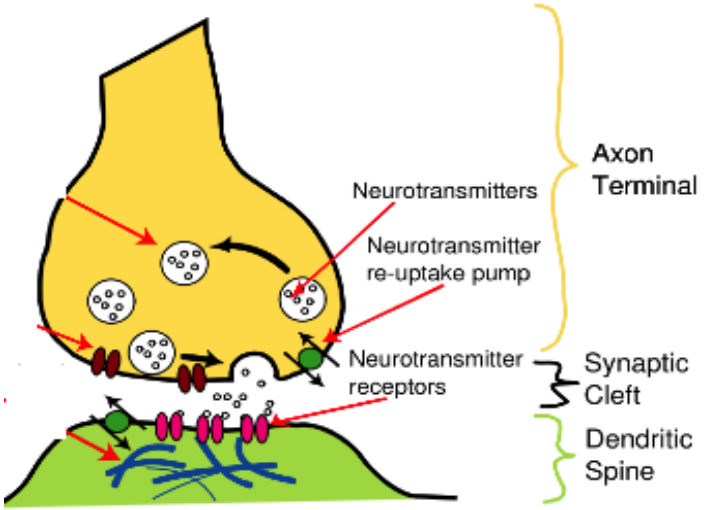
i • Detection of molecular properties of ***N*** molecules

signal $\propto$ ***N*** *standard deviation* $\propto \sqrt{\boldsymbol{N}}$

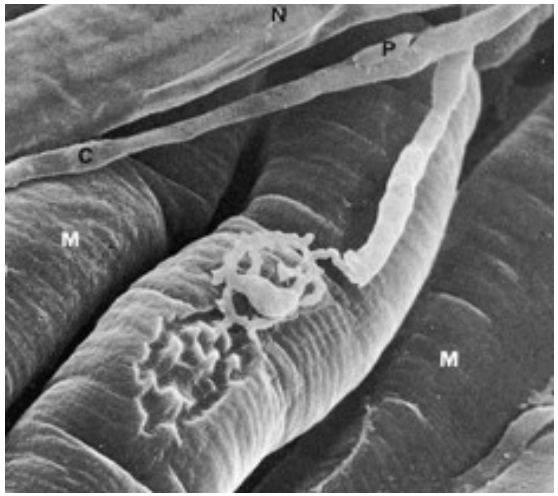
2 • Small vs big numbers

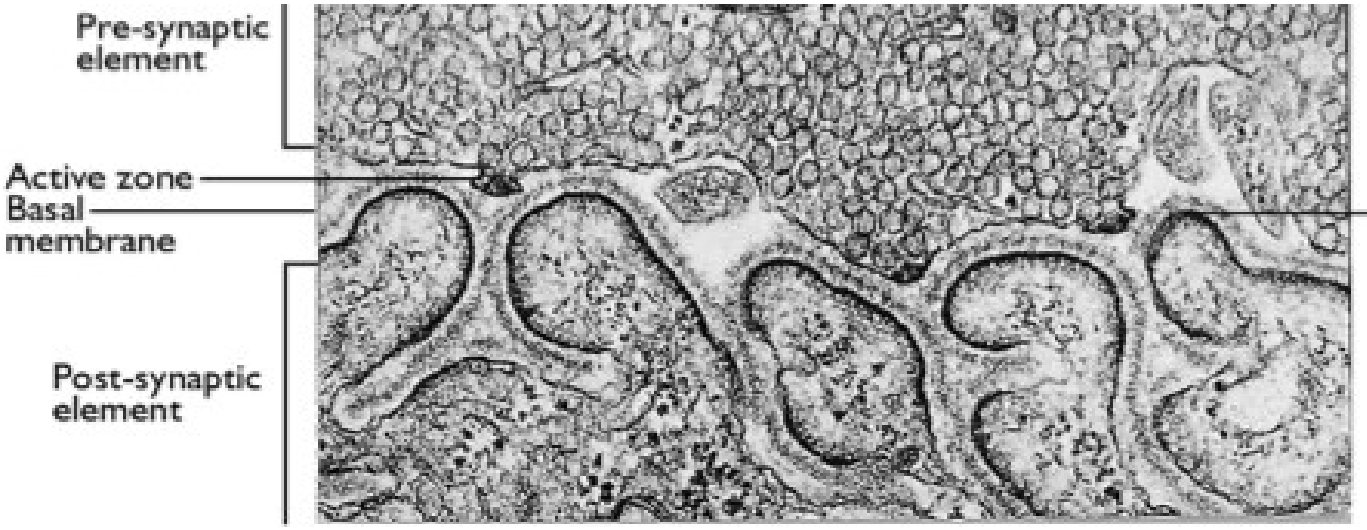
Synapses as example :

° Synaptic contact between neurons
only few active receptors present



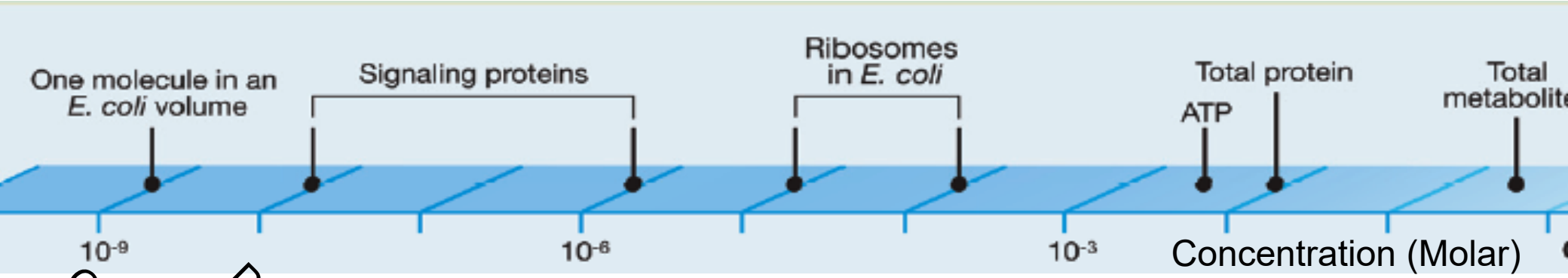
° Neuro-muscular junction
thousands of receptors





2 • The “n” in biology

Escherichia coli:
Volume ~10^{-15} L



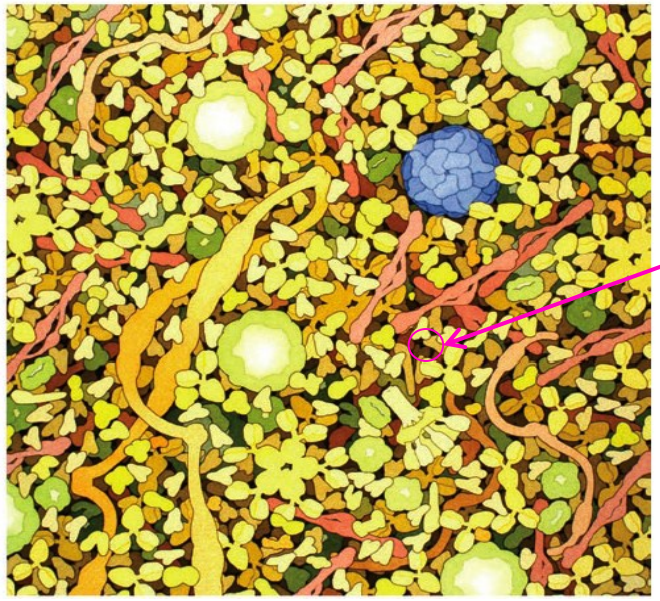
Chromosome

DNA Polymeras

Several important molecules are rare, almost alone
=> these molecules are decisive or deciding !!

SNAP shot Key numbers in Biology (2010) Cell 141
S.Xie (2006) Science Vol 311

3 • Finding a needle in a haystack



protein of interest

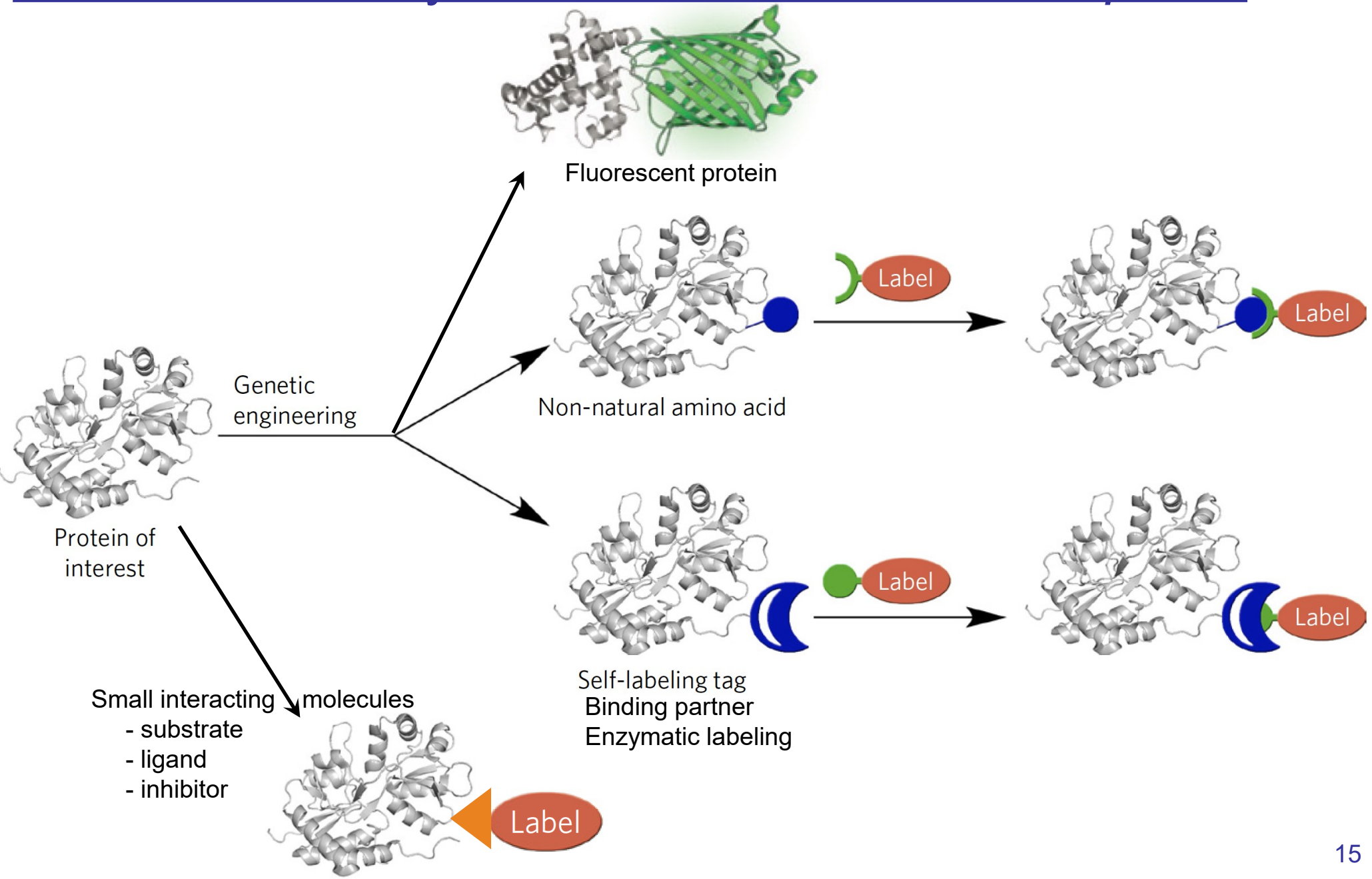
Advantages of fluorescence methods

- Non-invasive => *in vivo*
- Sensitive => down to a single molecule
- On line => direct info from ns to days
- High spatial & temporal resolution
- High information content

Focus:
Bio-orthogonal labelling of proteins

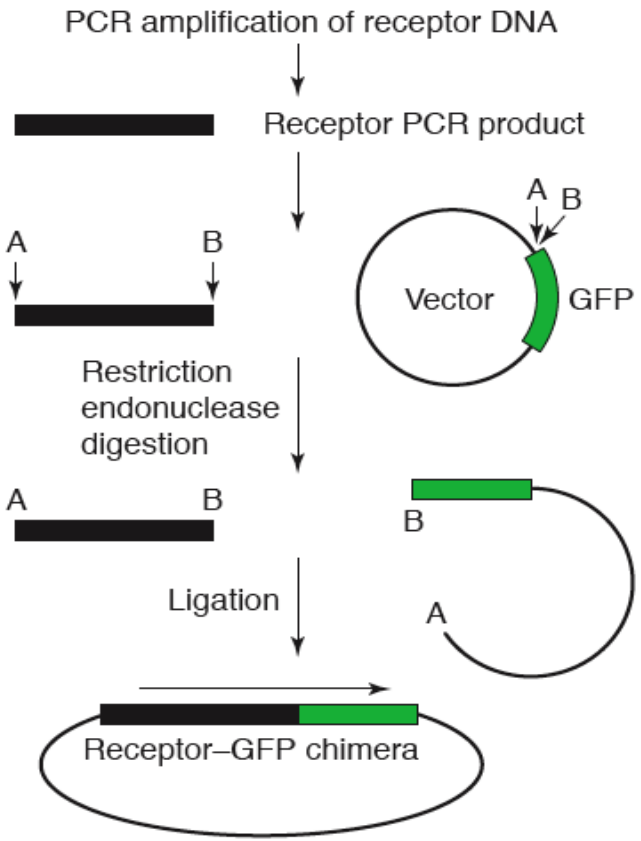
Fluorescent labelling is needed to discriminate between molecules of interest and the rest

3 • How to label your **P**rotein - **o**f - **I**nterest "*poi*" ?

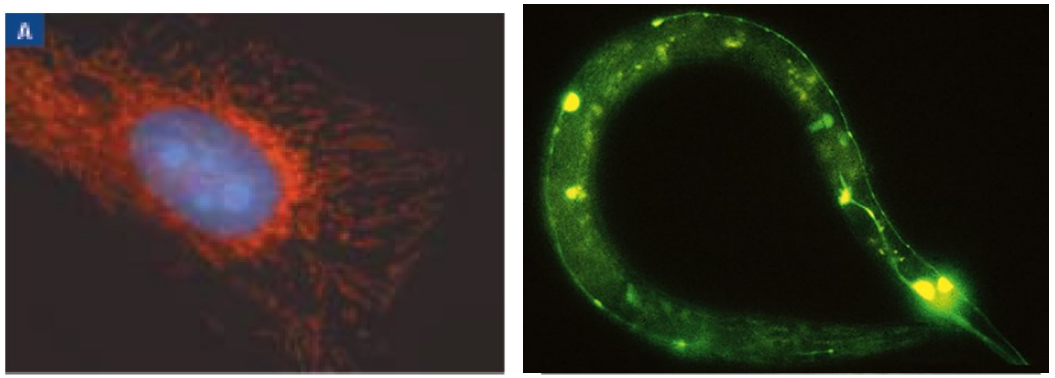


3 • Fluorescent proteins as label

- Fusion to protein of interest
  N- or C- terminal, internal

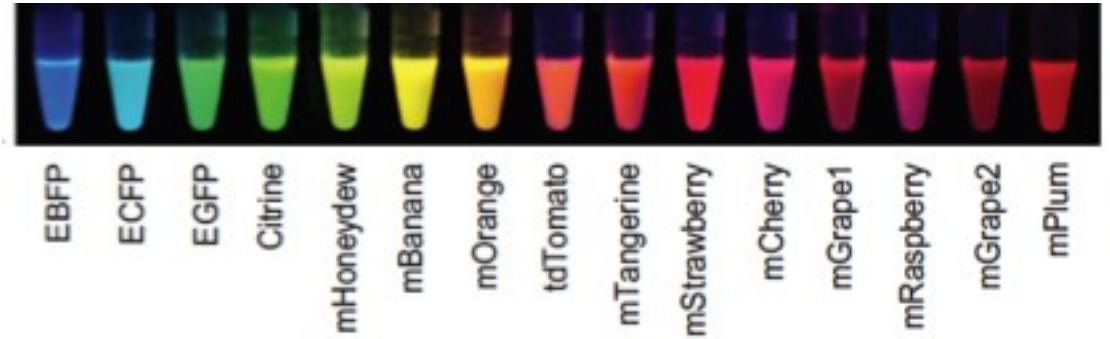


- Within single cells or living organisms



R. Y. Tsien

- Parallel labelling of several proteins with different fluorescent proteins

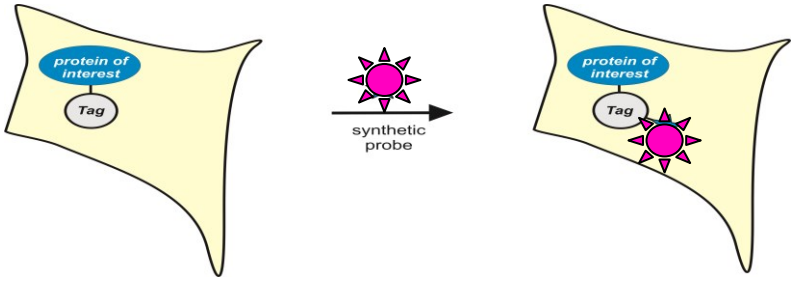


Which one to use ? => fpbase.org

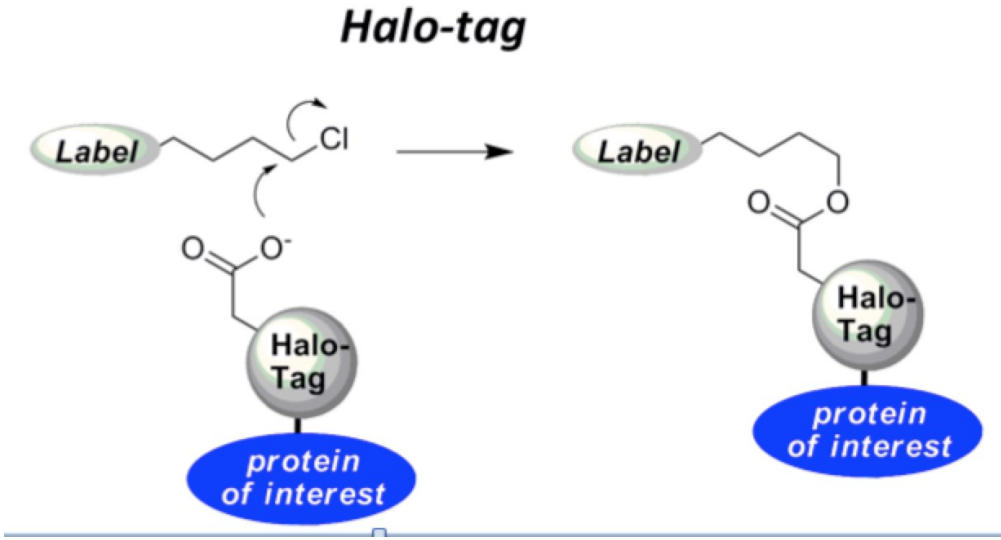
Where to get ? => addgene
The nonprofit plasmid repository

T. Kogure, Nat Biotech 24 (2006) 577

3 • Self-labelling fusion tags



- Fuse tag to protein-of-interest
- Tag = a “suicide” enzyme
- Providing proteins with properties that cannot be genetically encoded
- Using a single tag for different applications
- Many different probes can react with the same tag
- Popular examples are ***Halo-tag*** or ***Snap-tag***



- **Halo-tag**
  - based on a haloalkane dehalogenase
  - provide very fast labeling

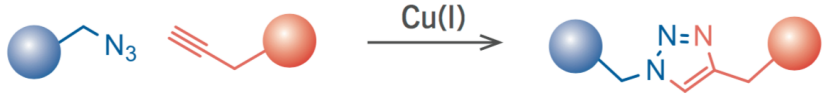
Promega

3 • Non-natural amino acids

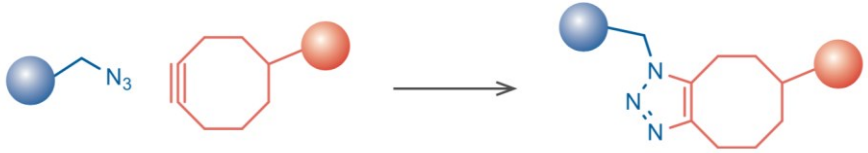
Introduce amino acid analogues with handles for modification by “click” chemistry

Click-ing molecules together :

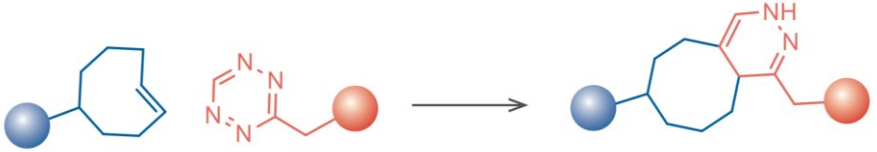
÷ The original Cu^{+}-catalyzed



÷ Cu-free strain-promoted



÷ Inverse Diels-Alder reaction

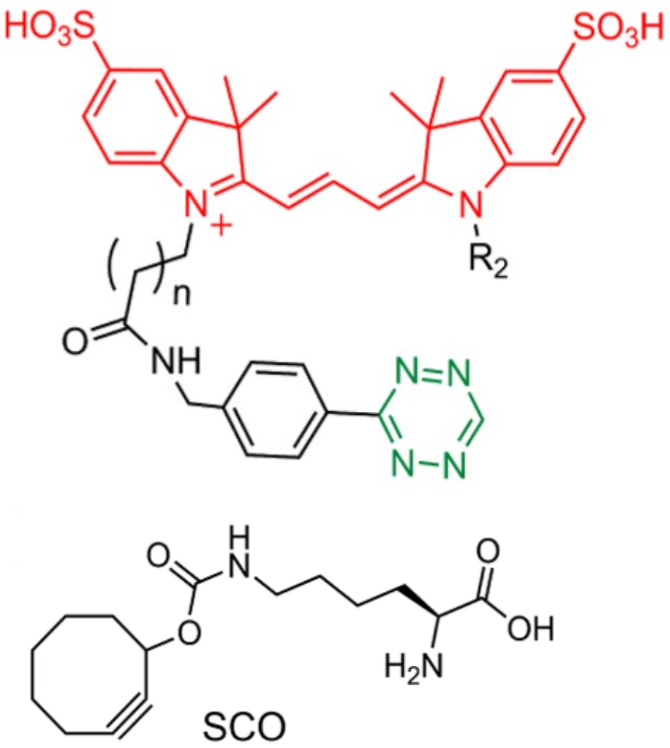


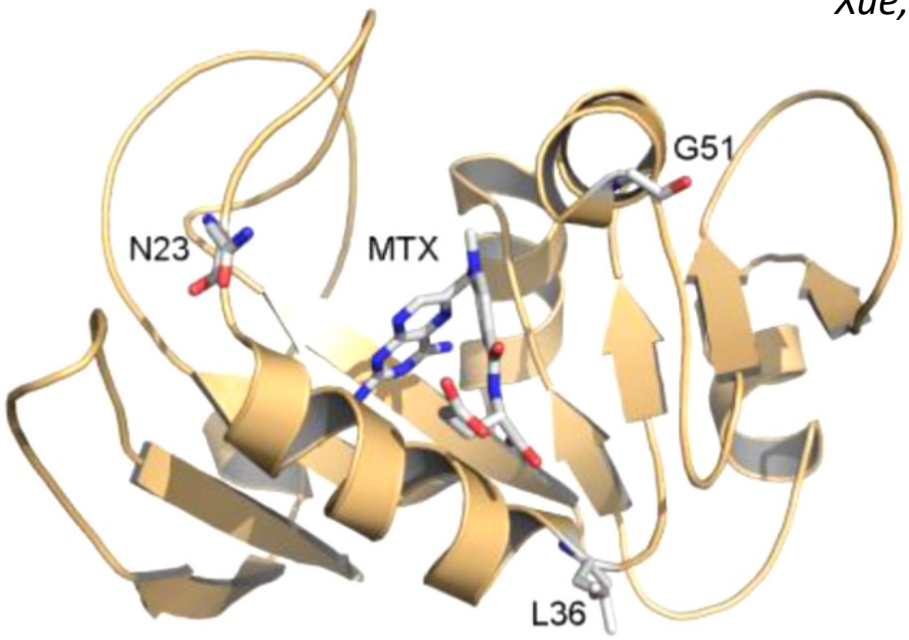
Click Chemistry Tools
Bioconjugate Technology Company

3 • Non-natural amino acids

An example of a “click-pair

Xue, JACS 2016

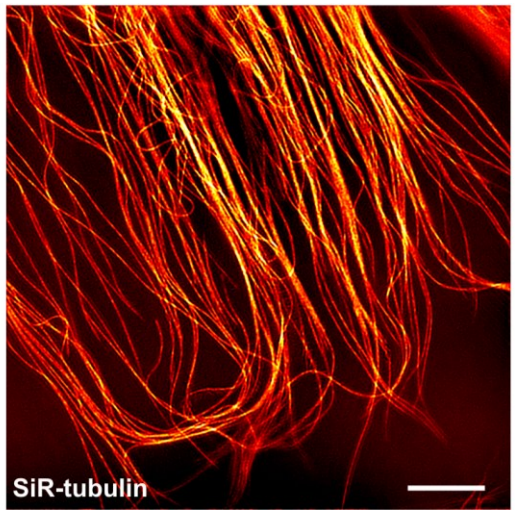


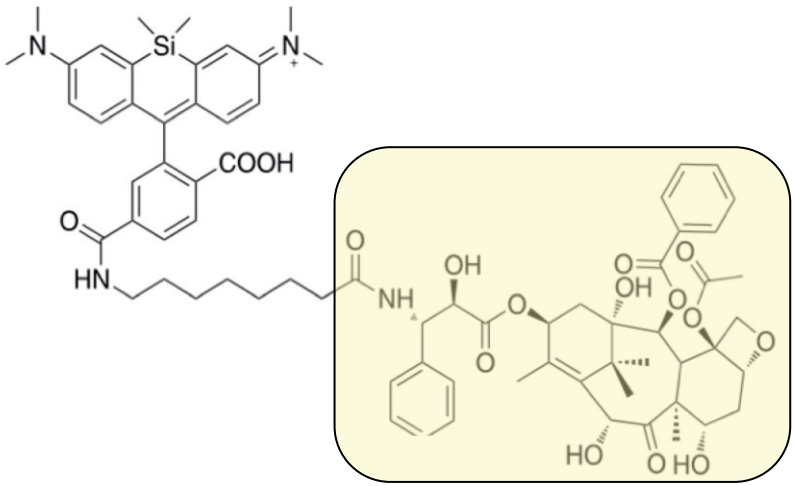


The **combination** of **non-natural amino acids** and **click chemistry** yields a **high spatial precision** for the introduction of the fluorophore

3 • Small interacting molecules

- A cell-permeable microtubule-binding conjugate of a taxol-analogue and Silicon Rhodamine



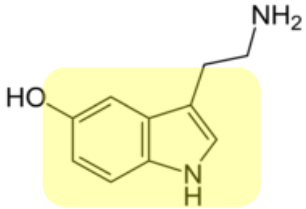


SPIROCHROME
Probes for Bioimaging

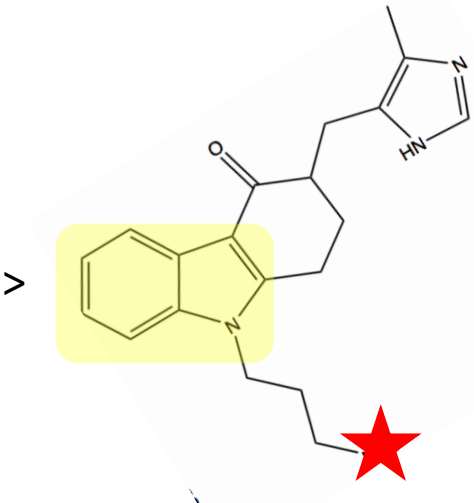
Nature Methods 2014

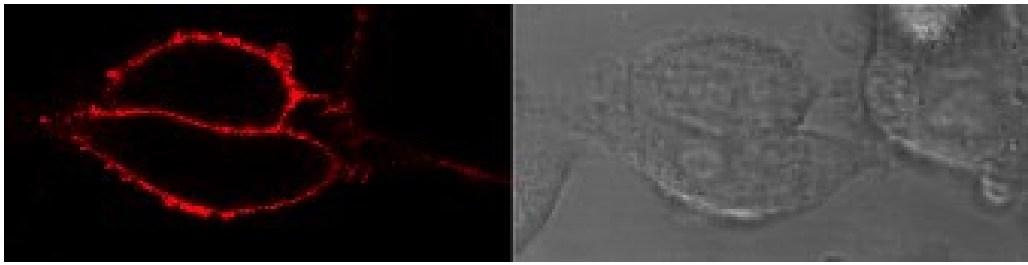
- A labeled serotonin-analogue of interacting with the serotonin receptor in the cell membrane

serotonin



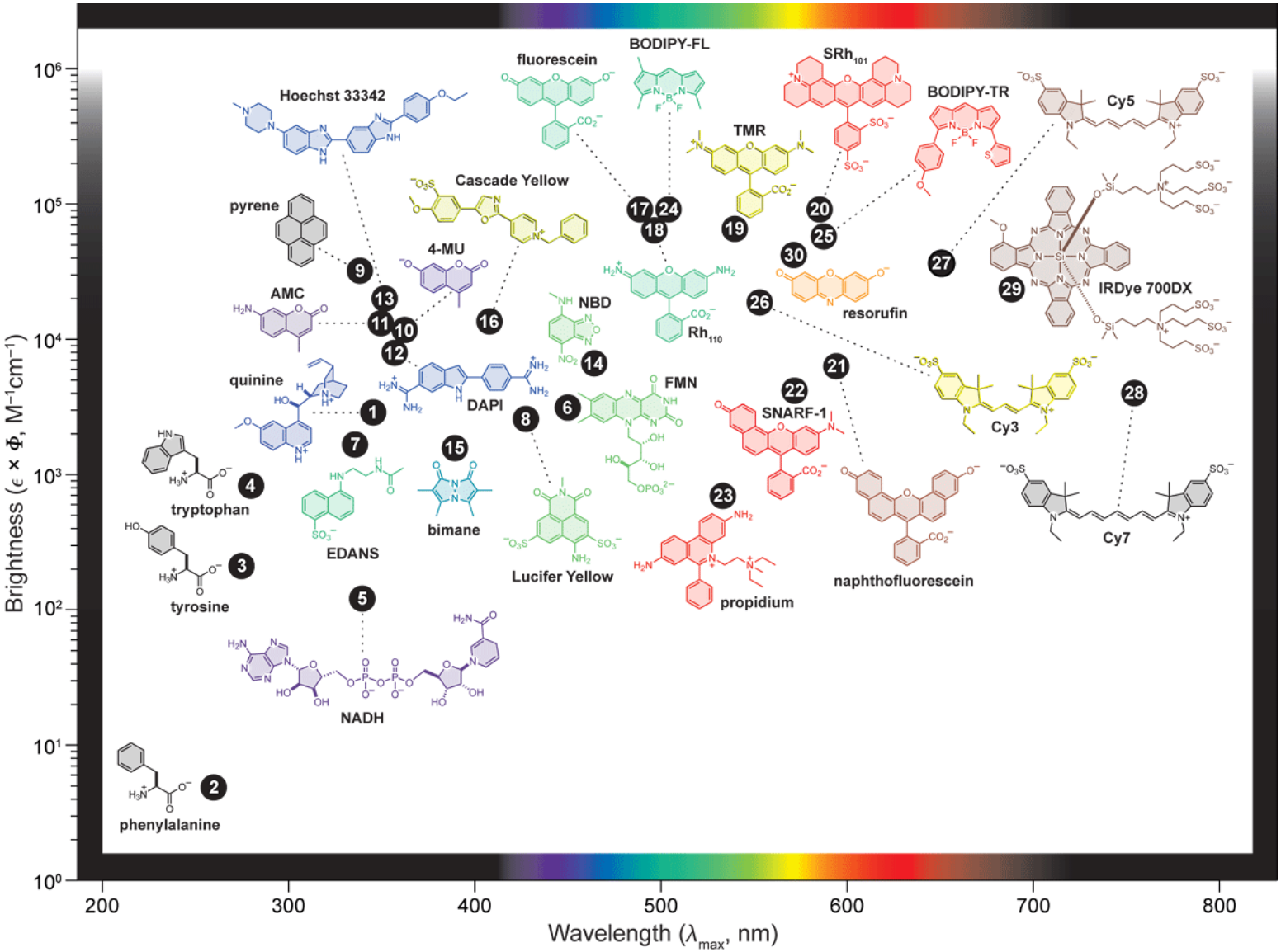
=>





Wohland, Biochemistry 1999

3 • Labelling with organic dyes - Which one ?



Many available dyes :

Alexa's Invitrogen

Cy dyes GE

Atto's Atto-tec

CF's Biotium

SiR's Spirochrome

Abbrerior

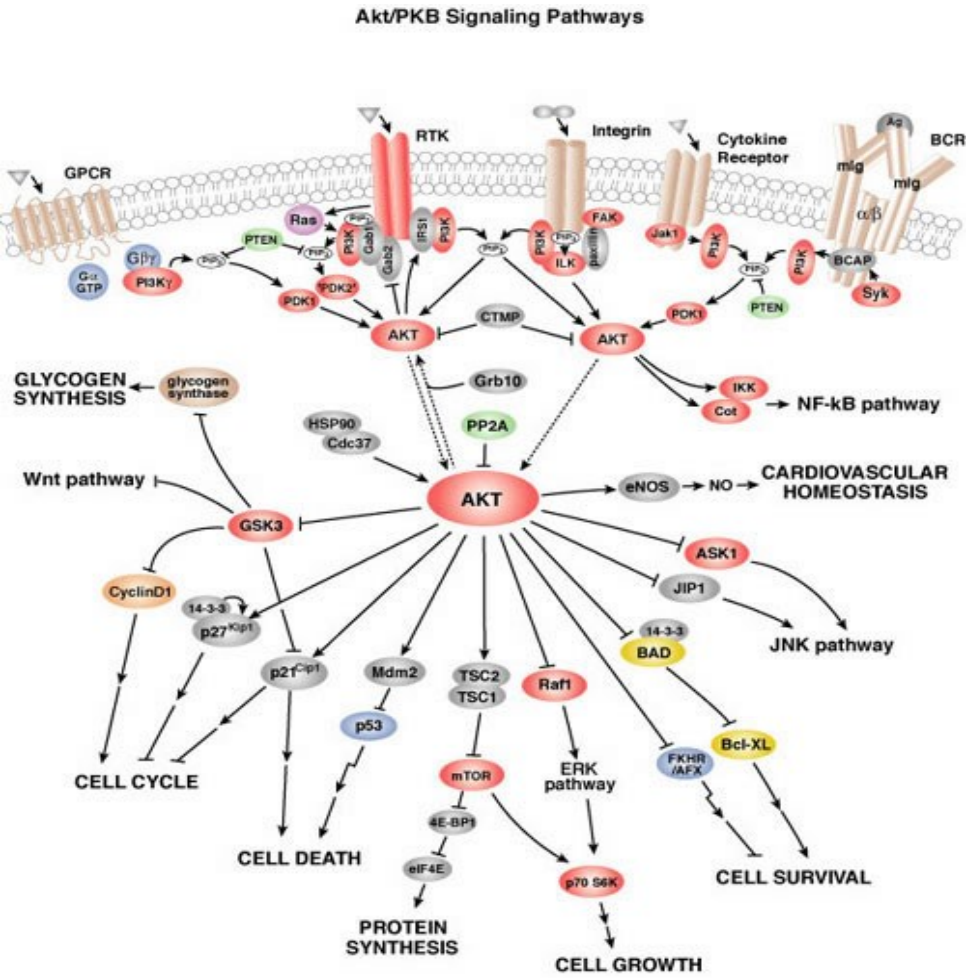
.....

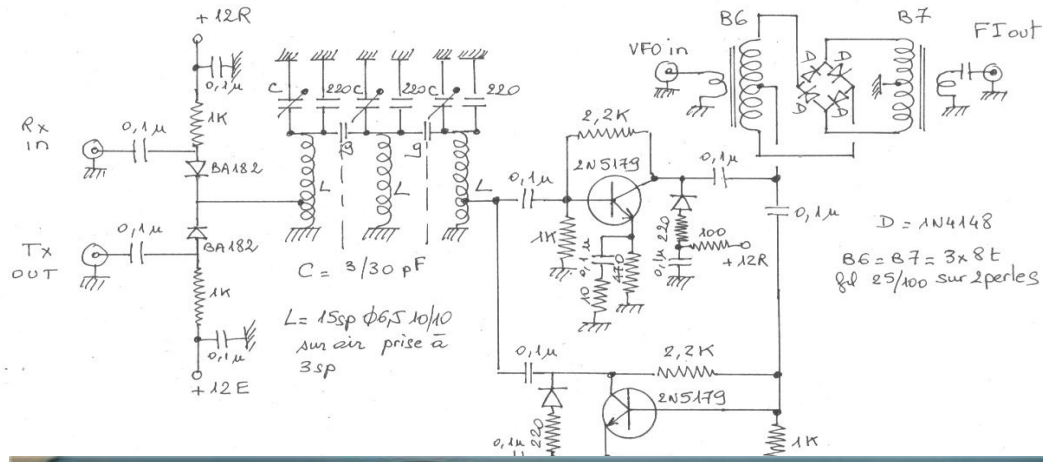
Criteria : Brightness, stability, non-specific interactions & cell-permeability

http://www.hhmi.org/research/tailoring-fluorescent-molecules-biological-applications

4 • Molecular interactions - Biological networks

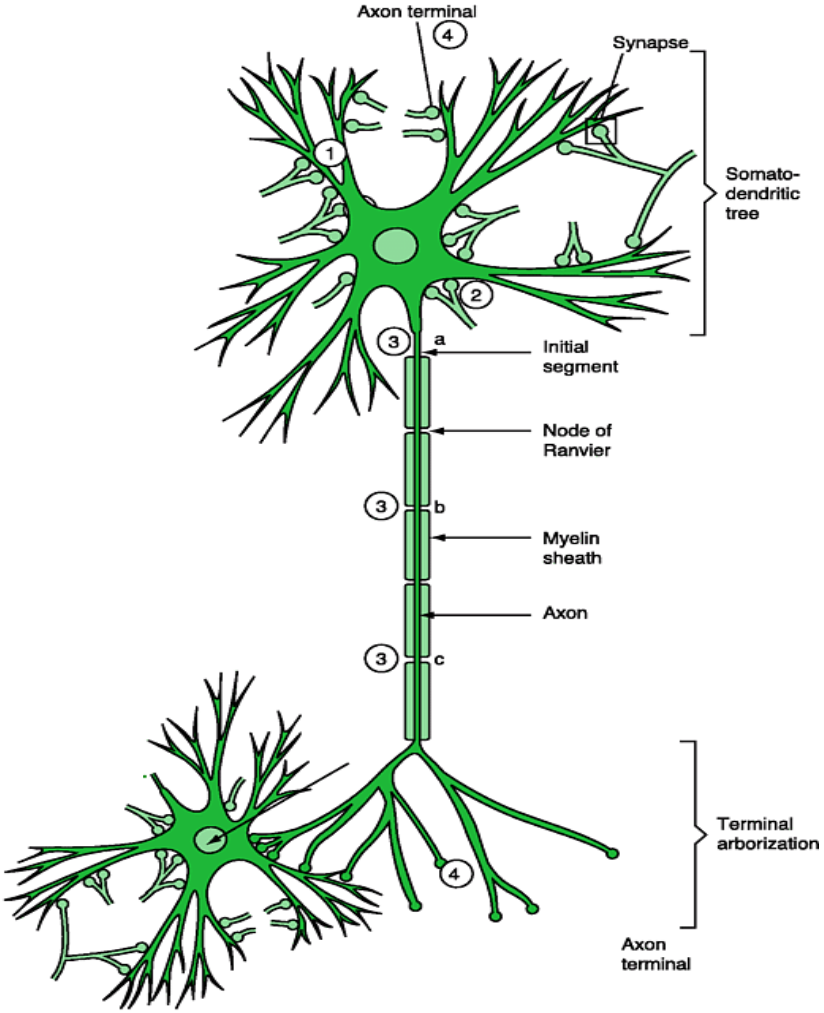
- The cell is extremely complex : many intertwined processes of signaling and metabolism :
  - multi-dimensional networks
  - a strong spatial-temporal organization





4 • Biological networks – Reversible molecular interactions

- Interacting neurons

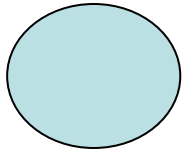


Hammond "Cell & Mol Neurobiology"
Kim, Nat Rev Neurosci 5 (2004) 772

4 • Proteins - Modular assembly of functional domains

- Proteins must be multi-functional
  - specific interaction
  - regulation of interaction
  - effector

- Proteins are in general not monolithic globules



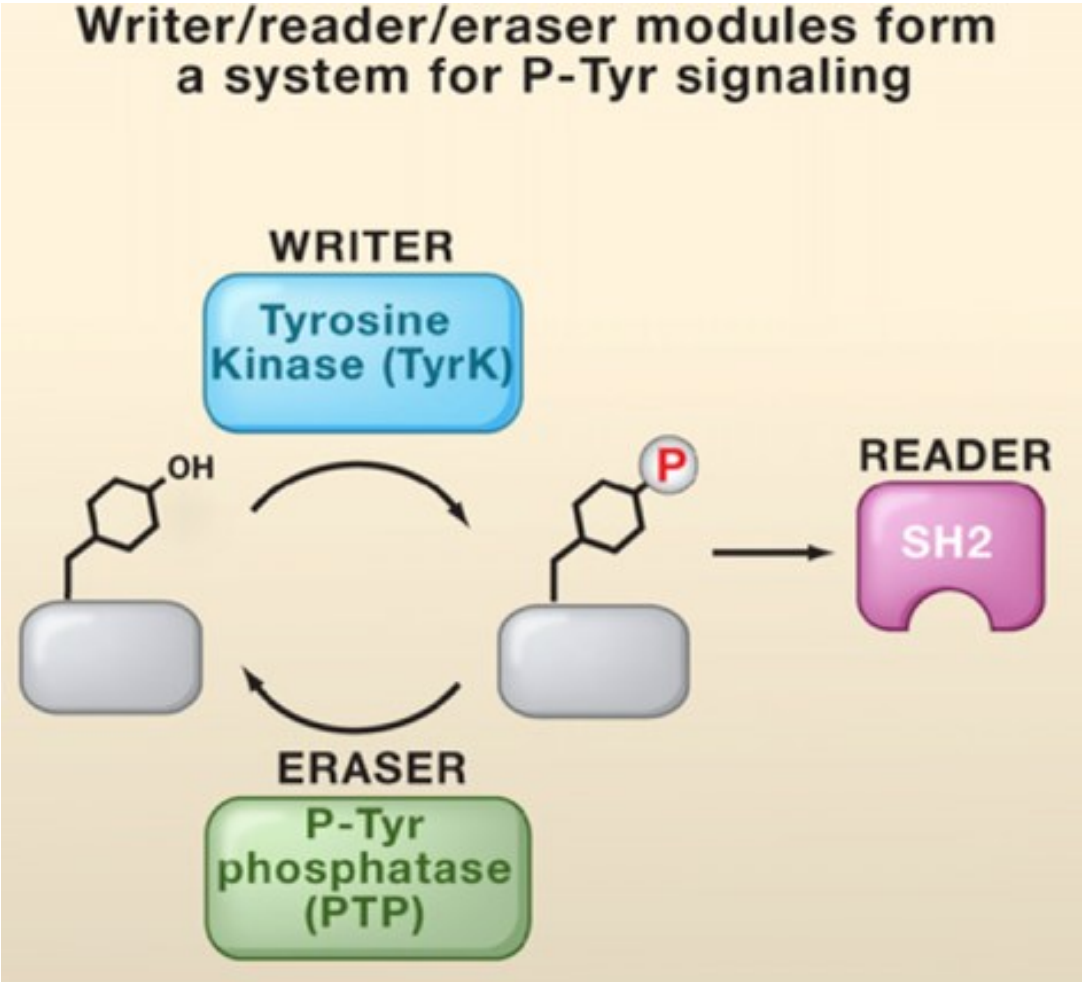
but rather have a beads-on-a-string multi-domain structures, where ***each domain has a specific function***

"Modular Protein Domains" Cesareni e.a. (Eds) 2005 Wiley

4 • Phospho-tyrosine signalling

Growth factor-activated receptors in plasma membrane

=> Active receptors phosphorylate themselves and other proteins



Writers:
- Receptor Tyr-Kinases :
  activity is ligand-dependent

Erasers:
- p-tyrosine phosphatases :
  activity is generally constitutive

Readers :
- proteins with SH2-domains that bind P-Tyr

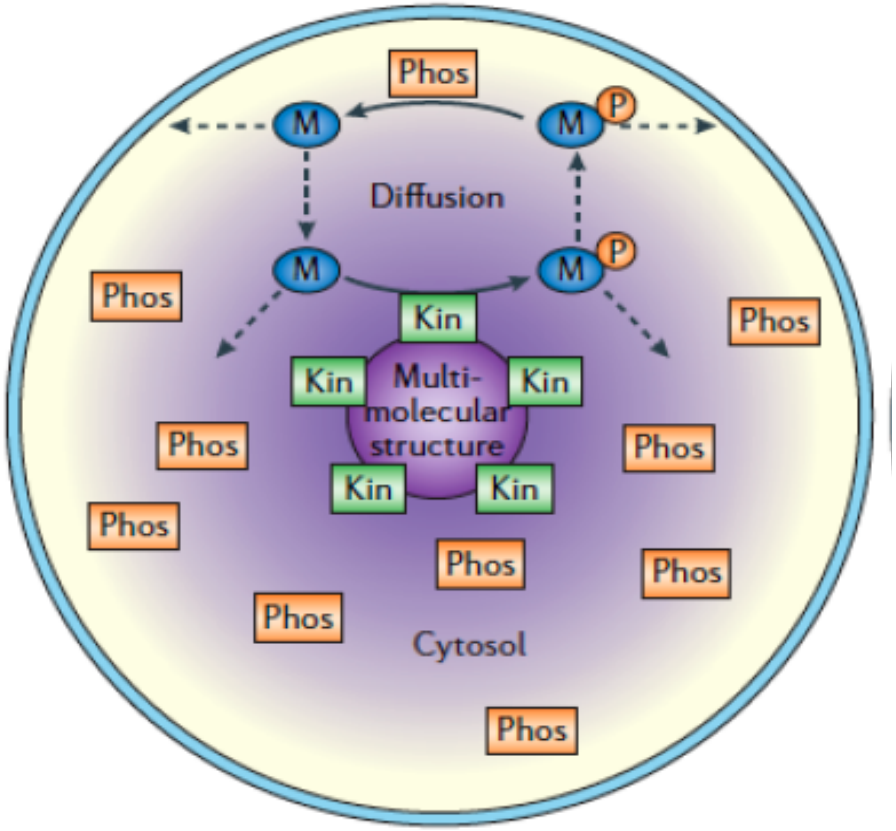
Lin (2010) Cell 142, 661

4 • Space & time in signalling: A thought experiment

E.g. for the phosphorylation state of protein M (purple hue indicate [M-Pi].

- the phosphatase **Phos** is homogeneously distributed in the cytosol, but
- the kinase is bound to

a central structure or

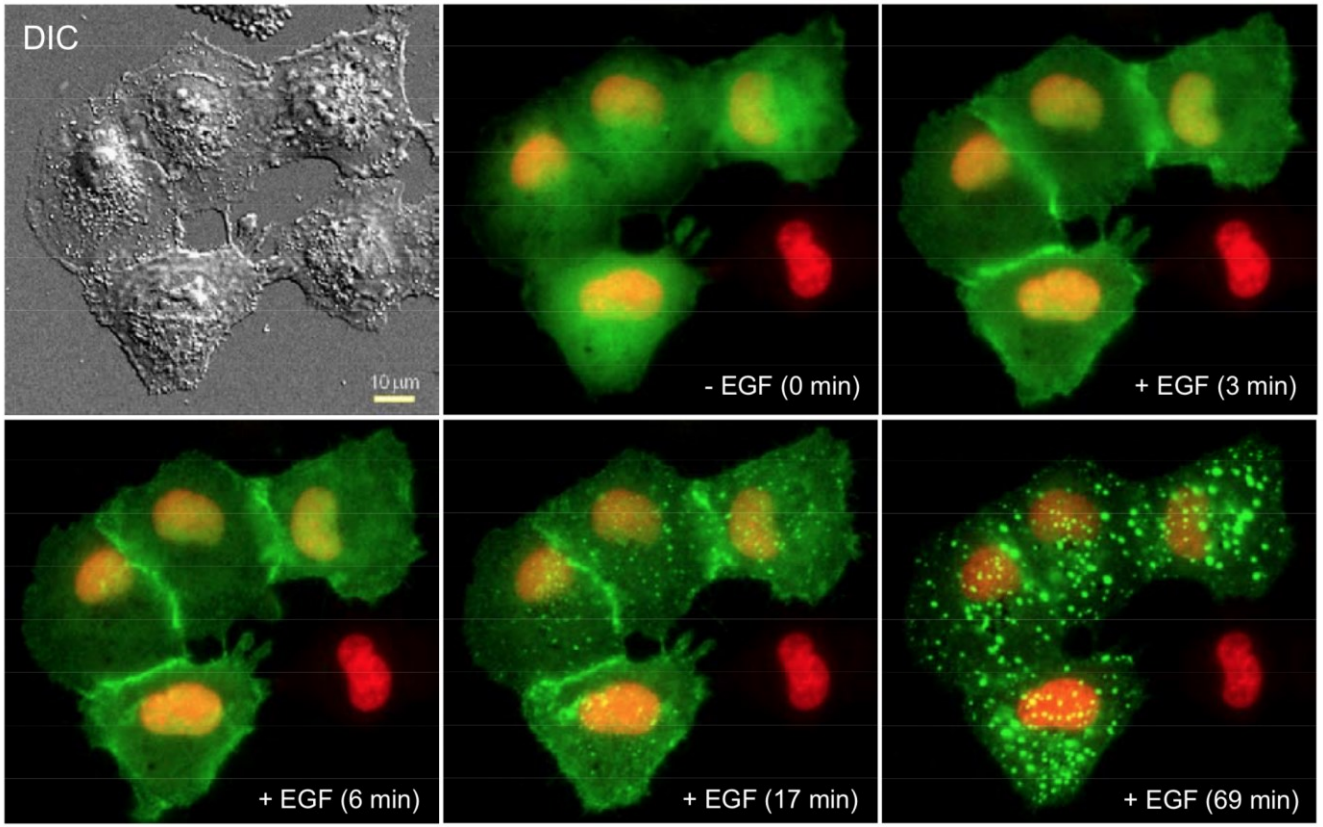


Kholodenko, Nat Rev Mol Cell Biol 2006

=> Localized activity creates concentration gradients

4 • Imaging tyrosine phosphorylation *in vivo*

- A fusion of and SH2 domain and GFP is expressed in mammalian cells
- At t = 0, the epidermal growth factor (EGF)is added



SH2-GFP translocates to plasma membrane binding to the phosphorylated EGF-receptor

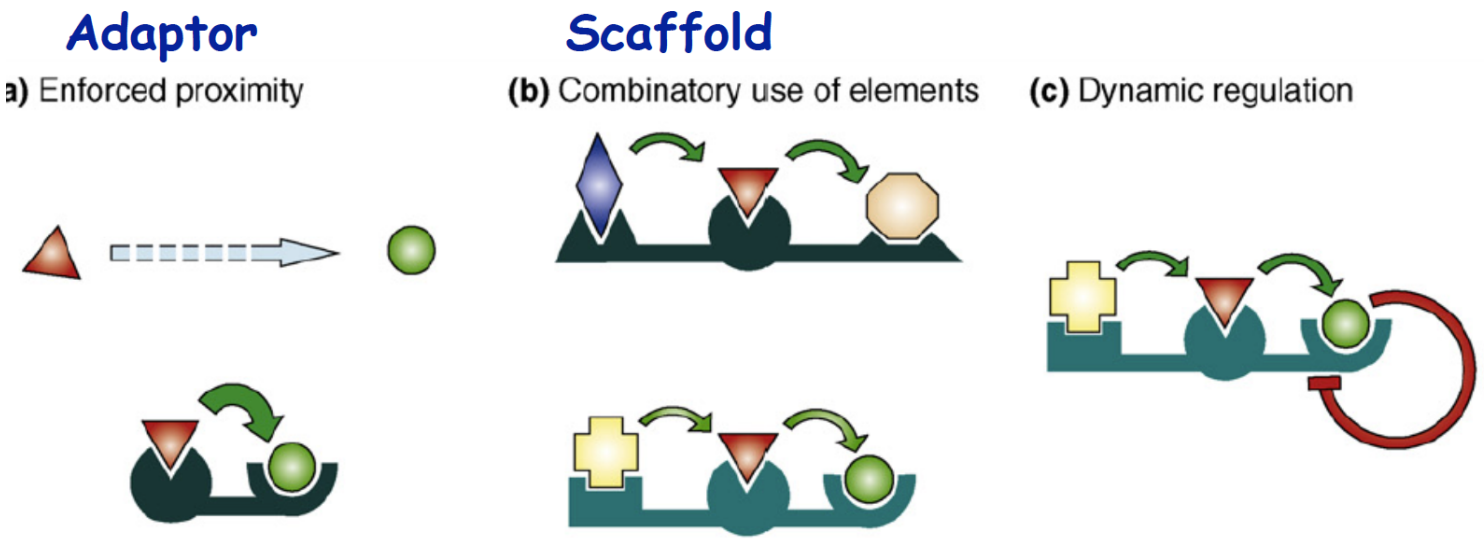
Finally, the EGF-receptor is internalized.

NB : Nucleus is red

=> Hormone-induced phosphorylation and re-localisation of SH2 domain and receptor

4 • Scaffolds facilitate molecular encounters

Adaptors & scaffolds: allow certain proteins to get together in a specific orientation and order at a specific site in the cell

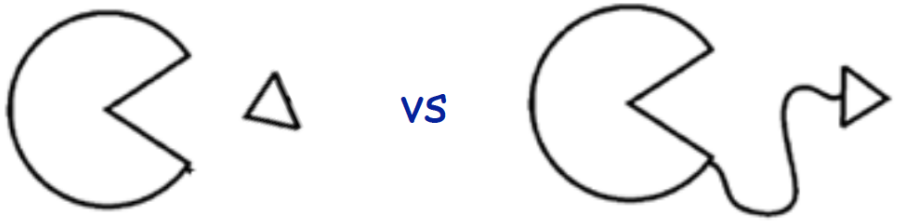


Zeke TiCB 2009

4 • Effective molarity

A protein with a binding site for triangles and with an "internal" ligand ◁.
Q: how does this internal ligand compete with an external one ◀ ?

Or, what is the effective concentration of ◁ ?

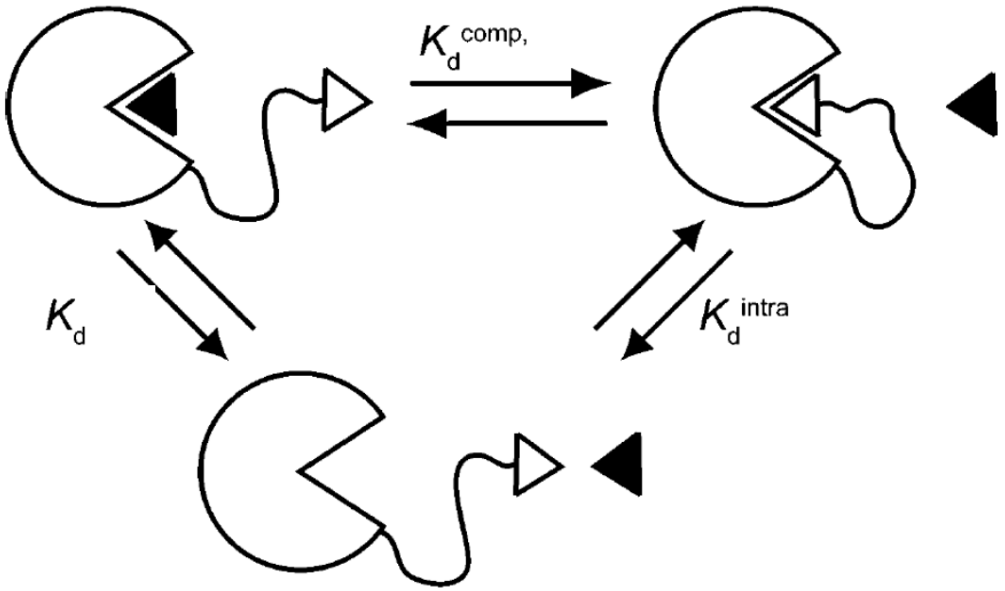


G. Whitesides **J. AM. CHEM. SOC. 2007**, *129*, 1312−1320

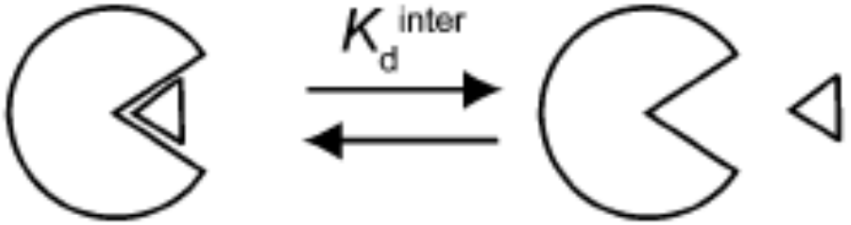
4 • Effective molarity

A protein with a binding site for triangles and with an "internal" ligand ◁.
Q: how does this internal ligand compete with an external one ◀ ?

Or, what is the effective concentration M_{eff} of ◁ ?



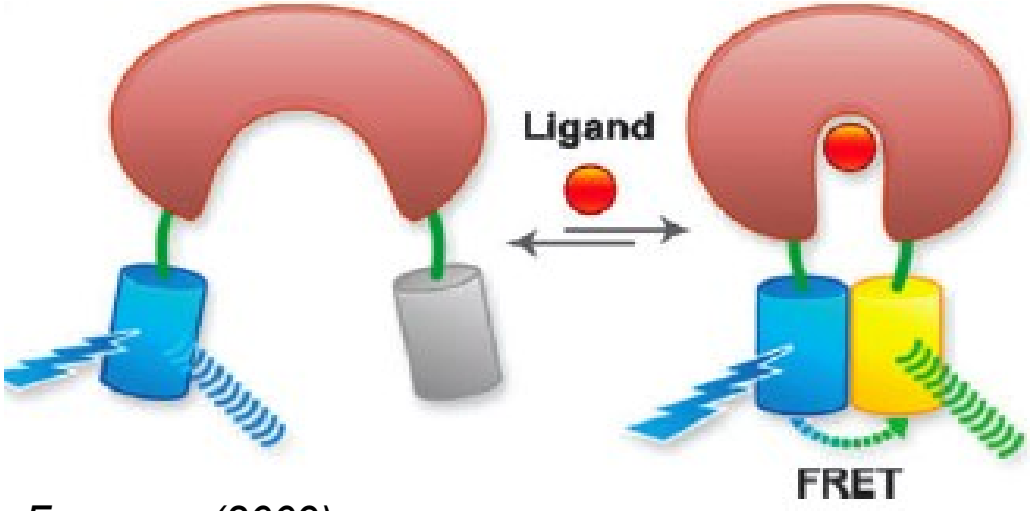
$$M_{\text{eff}} = K_{\text{d}}^{\text{inter}} / K_{\text{d}}^{\text{intra}}$$



• Effective molarity is concentration independent !!

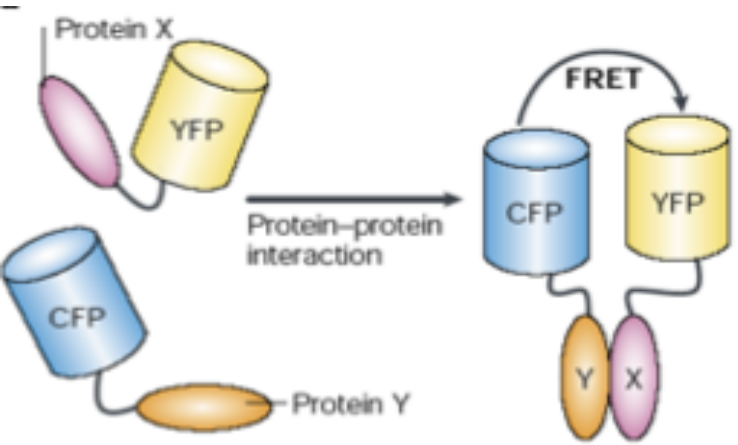
5 • Sensors based on fluorescent proteins and molecular interaction

° Analyte detection



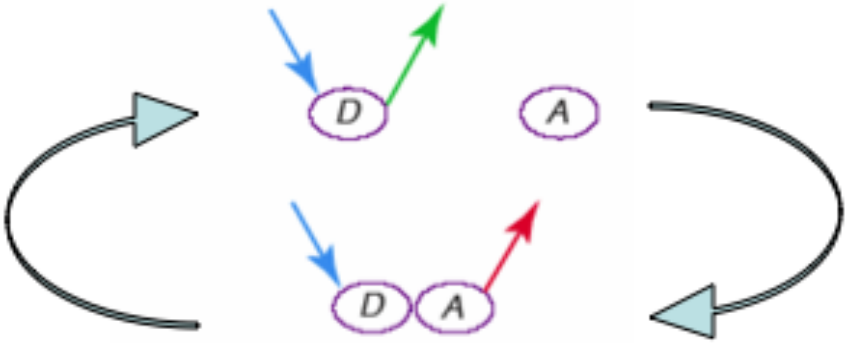
Frommer (2009)

° Molecular interactions



J. Zhang, Nat Rev MolCelBiol 3 (2002) 906

Föster resonance energy transfer (**FRET**):



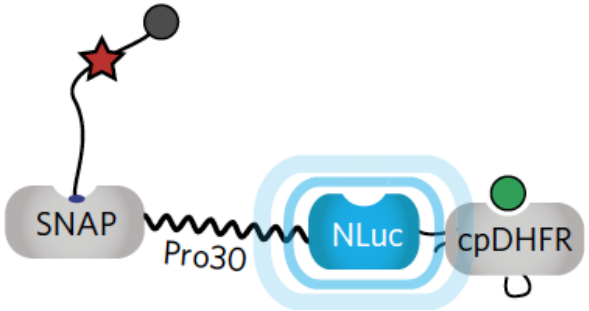
Transer of excitation energy from one fluorophore “Donor” to an “Acceptor”; possible only when Donor and Acceptor are within a few nm.

Up-to-date list:
codex.dpb.carnegiescience.edu/db/biosensor

5 • Snif-it's for drug monitoring : LUCID's

- **LUCID's** : **Luc**iferase-based **I**ndicators of **D**rugs

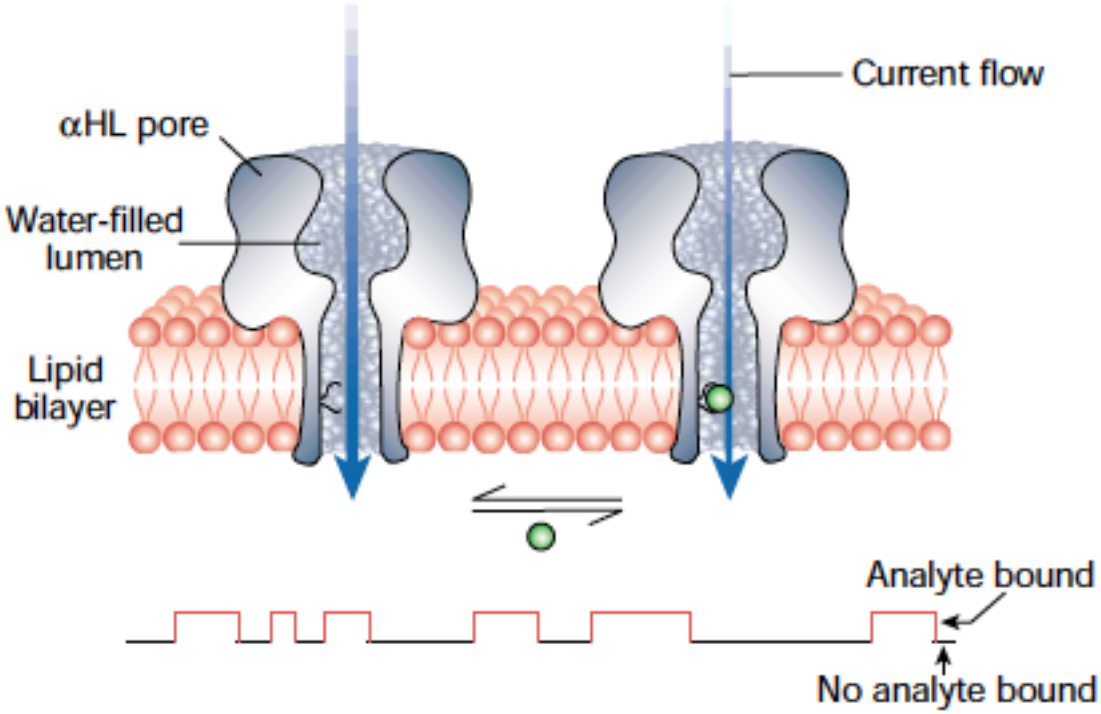
  e.g. for the anti-cancer drug methotrexate  with intern analog 

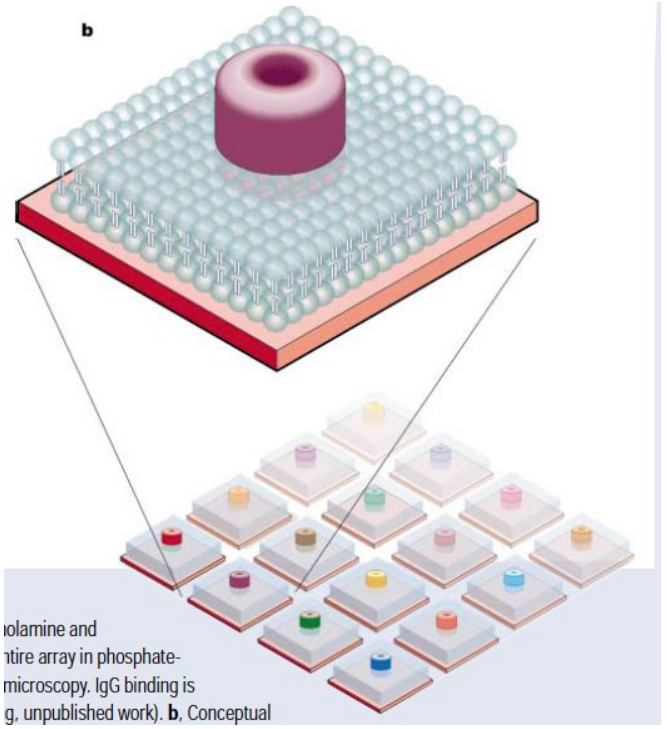


Griss (2014) Nat Biotech *Schena (2015) Nat Commun*

5 • Nanopore sequencing : From dream to reality

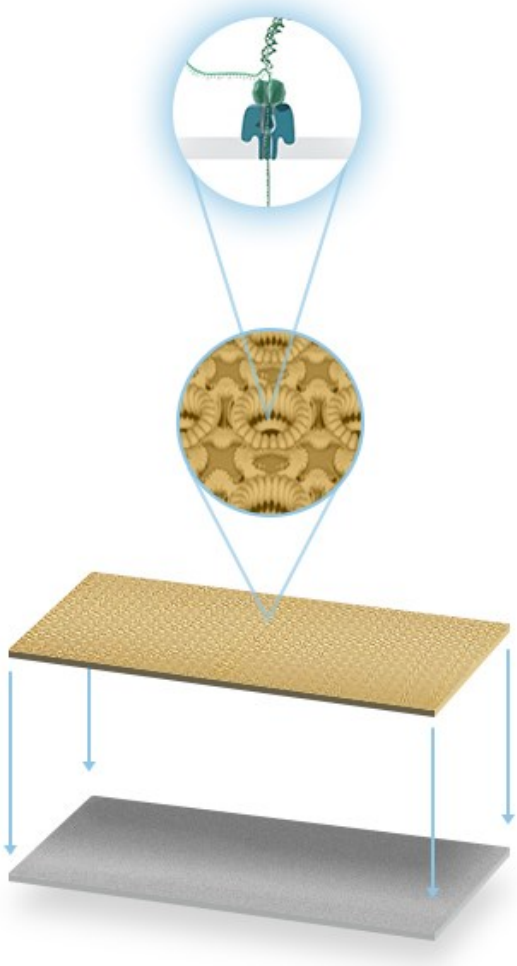
~2000 : The dream Hagan Bayley (Oxford)





5 • Nanopore sequencing : From dream to reality

Today's reality Minion from Oxforn Nanopore



Protein sequencing :https://www.biorxiv.org/content/10.1101/2021.07.13.452225v1

Reviews

- **Chemical labelling**

Schafer ea “Recent advances in Bioorthogonal reactions” Chimia 2019,73,308-312

Lang & Chin “Bioorthogonal Reactions for Label”
ACS Chem Biol (2014) 9, 16-20

Lang & Chin “Cellular Incorporation of Unnatural Amino Acids and Bioorthogonal Labeling of Proteins”
Chem Rev (2014) 114, 4764-4806

Resch et al “Quantum dots versus organic dyes as fluorescent labels”
Nature Methods (2008) 5, 763–775

- **Fluorescent proteins**

Wang et al “Fluorescence Proteins,Live-Cell Imaging, and Mechanobiology”
Annu. Rev. Biomed Eng. (2008) 10, 1-38.

Tsien, Campbelle.a.. “The Growing and Glowing Toolbox of Fluorescent and Photoactive Proteins”
doi.org/10.1016/j.tibs.2016.09.010

- **Fluorescent ligands**

Baindur & Triggle “Concepts and Progress in the Development and Utilization of Receptor-Specific Fluorescent ligands” *Med Res Rev* (1994) 14, 591-664

Current Opinion in Chemical Biology

Cell Biology by the Numbers http://book.bionumbers.org/

4 • Space & time in signalling: A cellular mechanism

E.g. regulation of cell division of yeast.

Observations:
- only big cells divide
- all components needed seem to be present continuously
- When nucelar Cdk1 is active => cell division
- Pom1 inhibits Cdk1 via a cascade