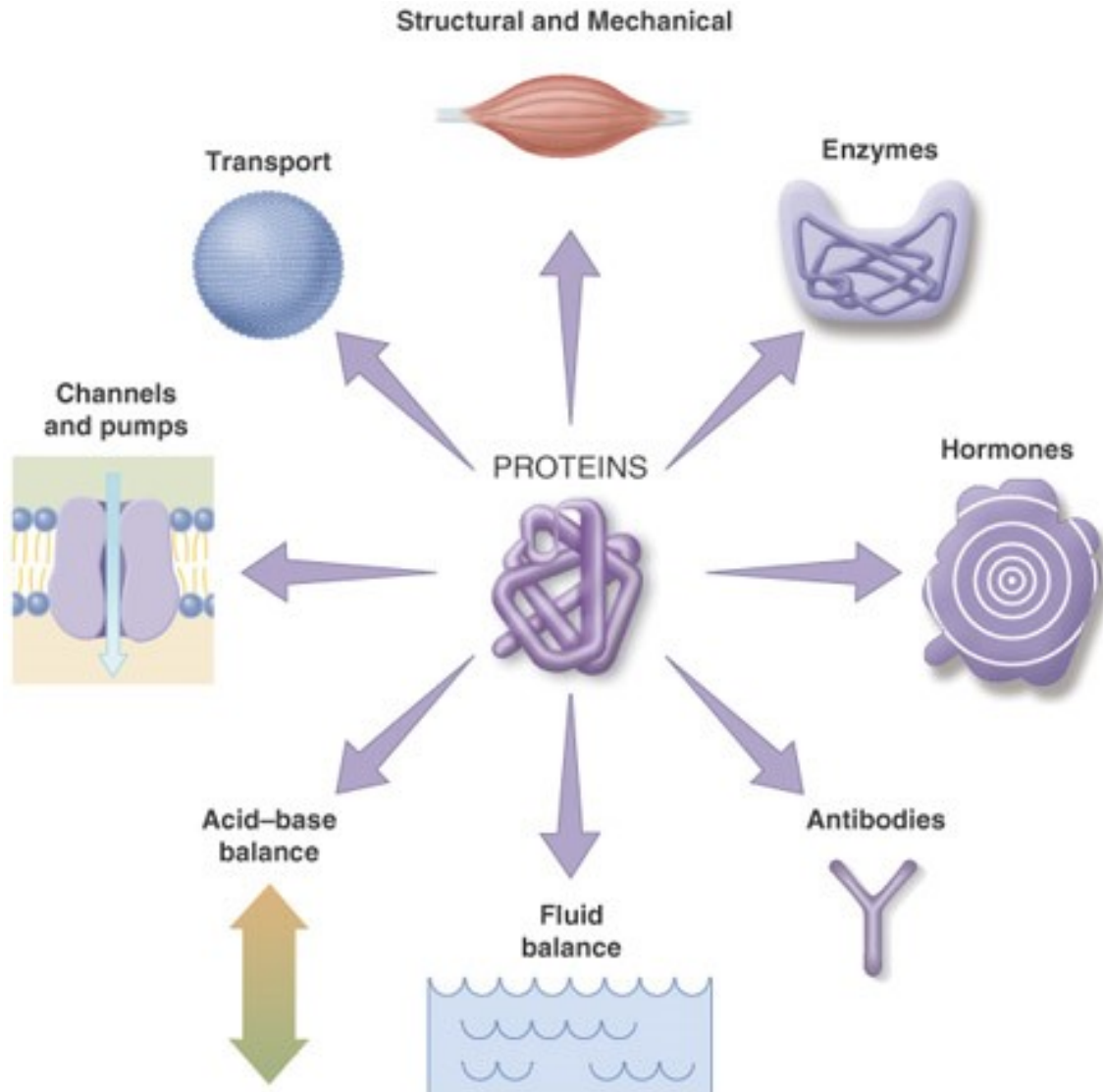


Proteine ricombinanti per studi  
strutturali.

studio dell'interazione  
proteina/proteina e  
proteina/ligando

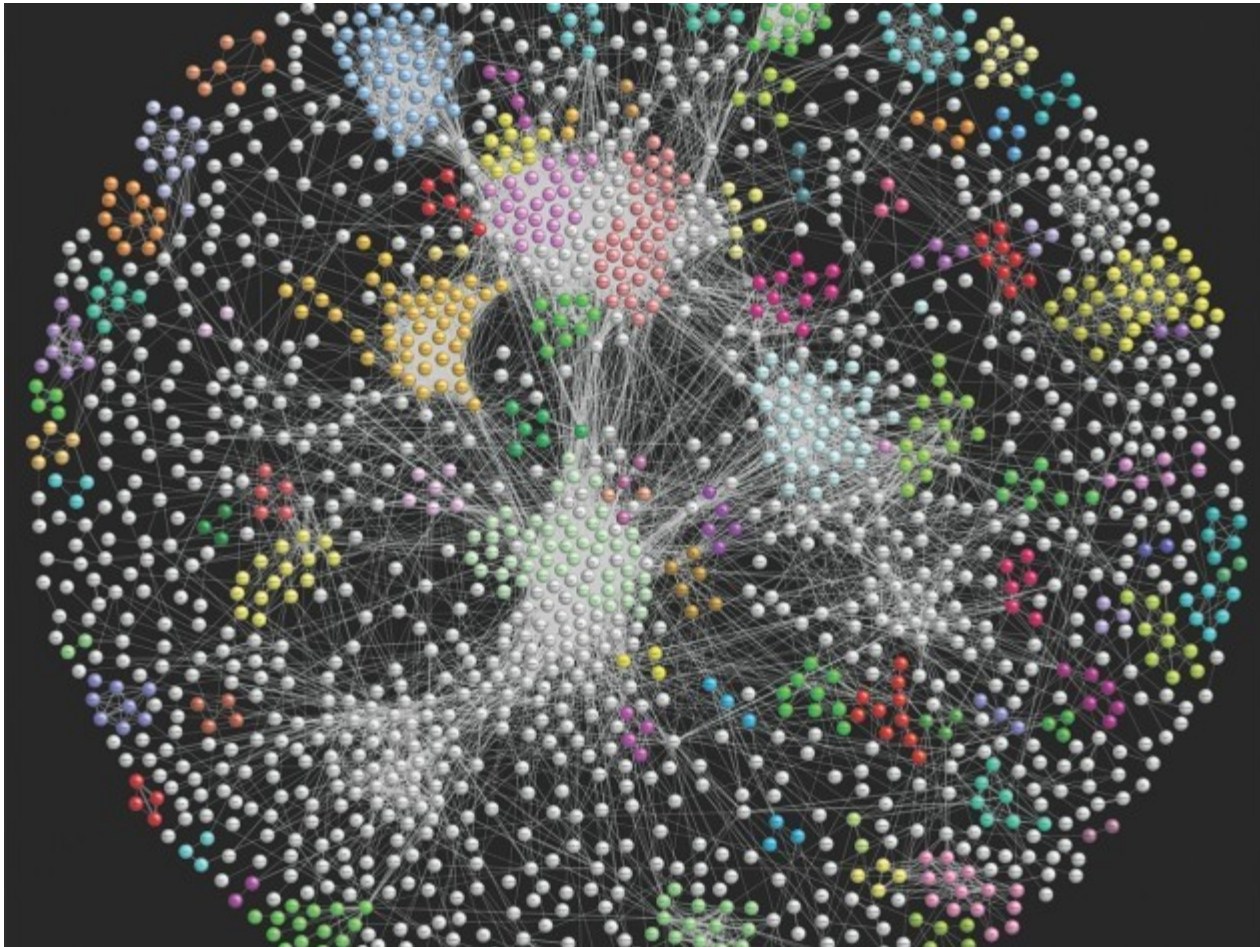
# Studio dell'interazione proteina/proteina e proteina/ligando :

## Interazione Proteina/ proteina (PPI)



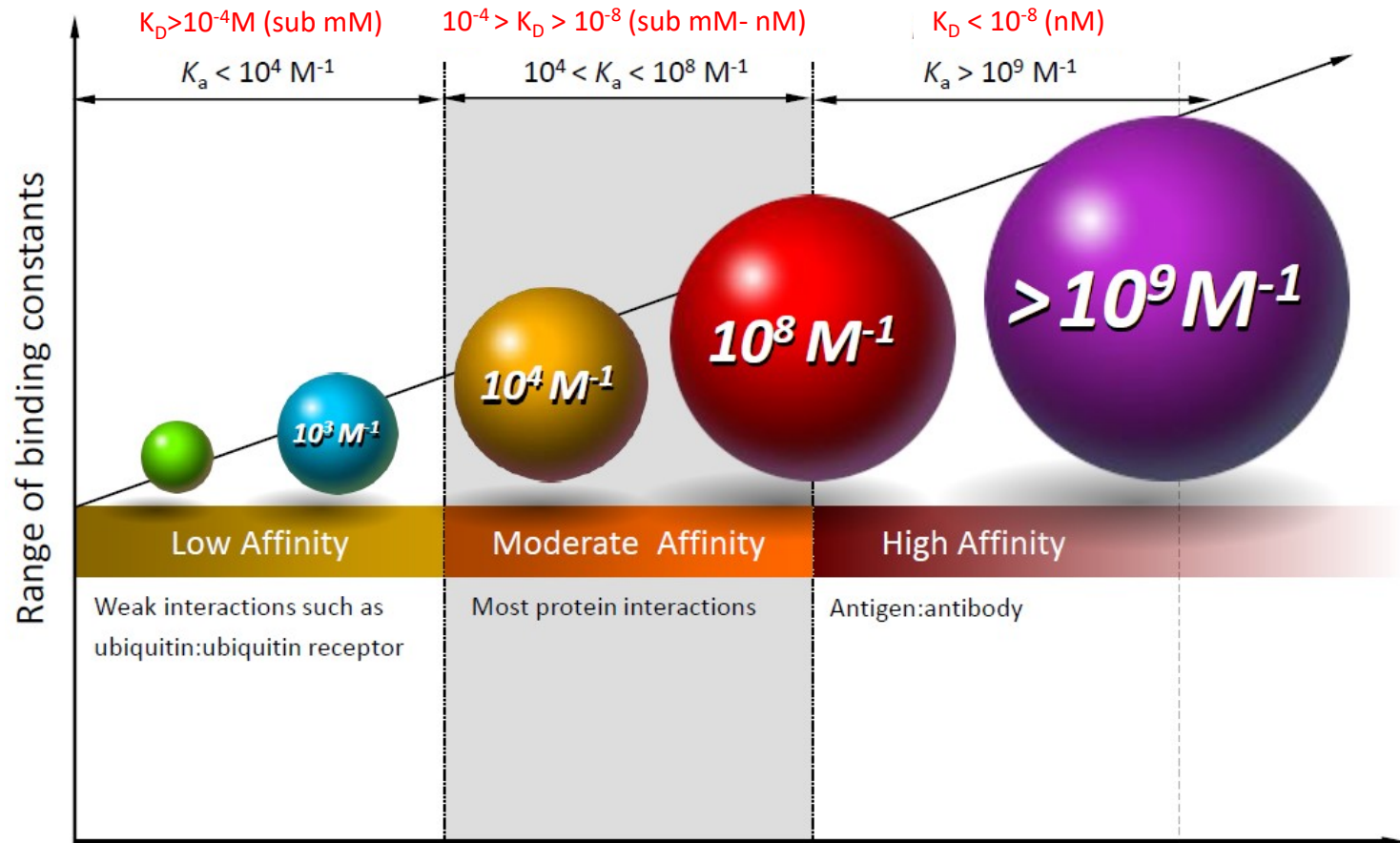
# Studio dell'interazione proteina/proteina e proteina/ligando :

## PPI network



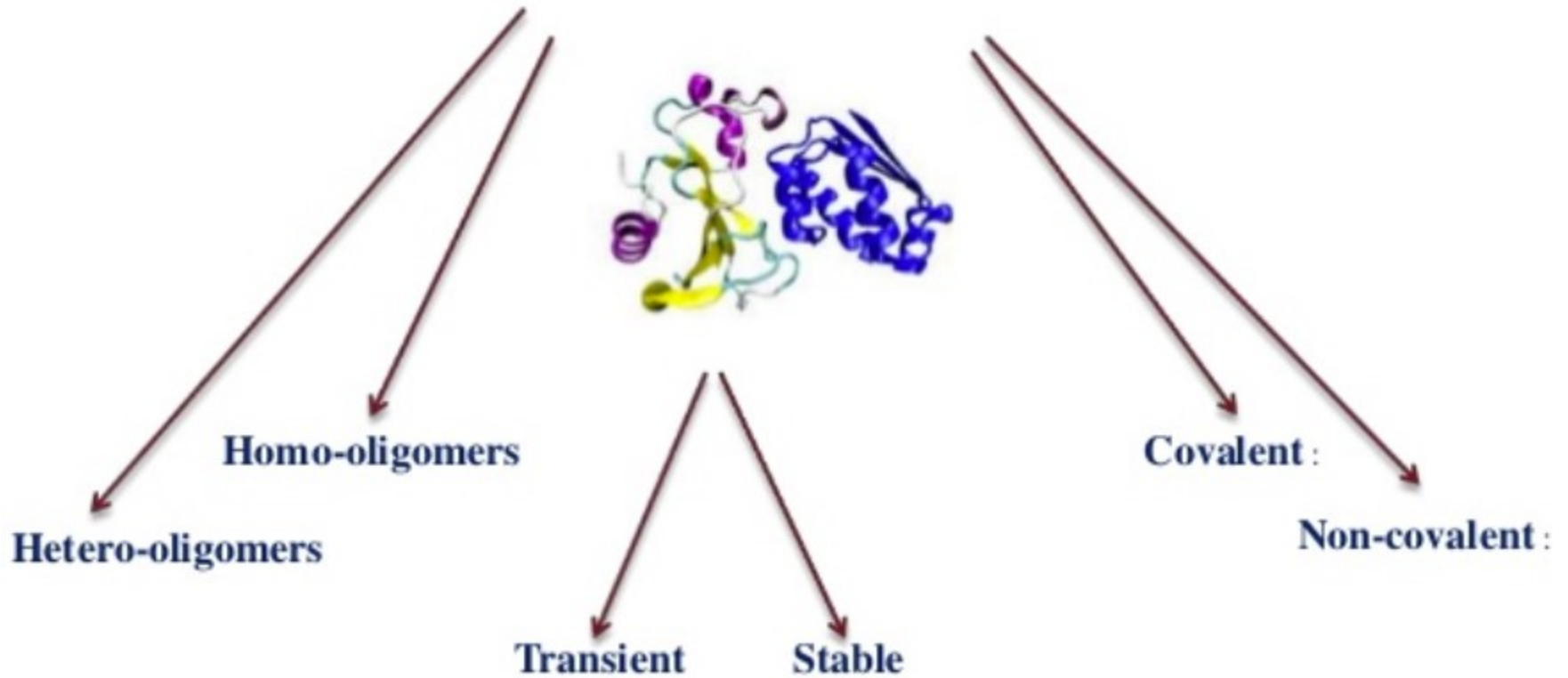
# Studio dell'interazione proteina/proteina e proteina/ligando :

## PPI affinity ranges



# Studio dell'interazione proteina/proteina e proteina/ligando :

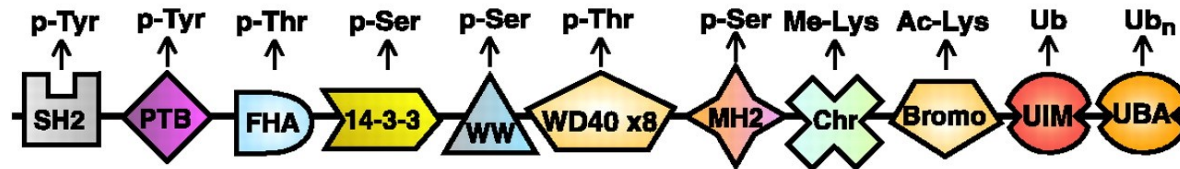
## PPI tipi



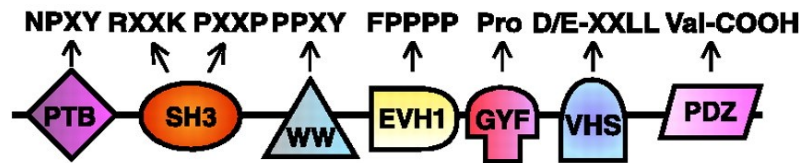
# Studio dell'interazione proteina/proteina e proteina/ligando :

## PPI : basi

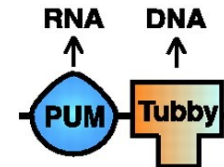
### Modified Peptide



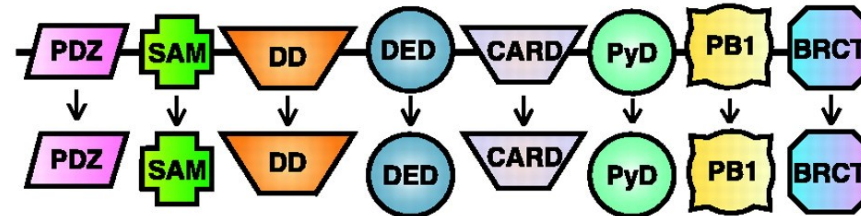
### Peptide



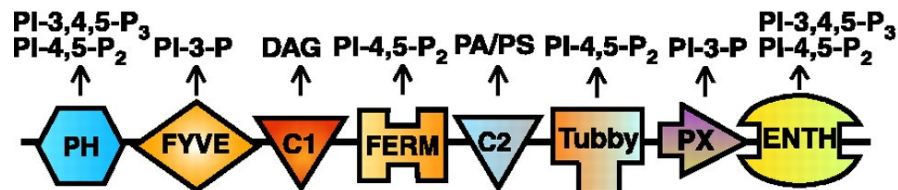
### Nucleic Acid



### Domain/Domain



### Phospholipid



**PPI : metodi di identificazione/mapping**

# Studio dell'interazione proteina/proteina e proteina/ligando :

## PPI : metodi di identificazione/mapping

### Experimental *(In vivo)*

- Yeast two-hybrid system
- PCA (split ub, GFP, lactamase, galactosidase)
- FRET/BRET/BiFc

### Experimental *(In vitro)*

- Co-IP
- Pull-down
- SPR
- Phage Display
- HD-exchange MS/Protein Painting

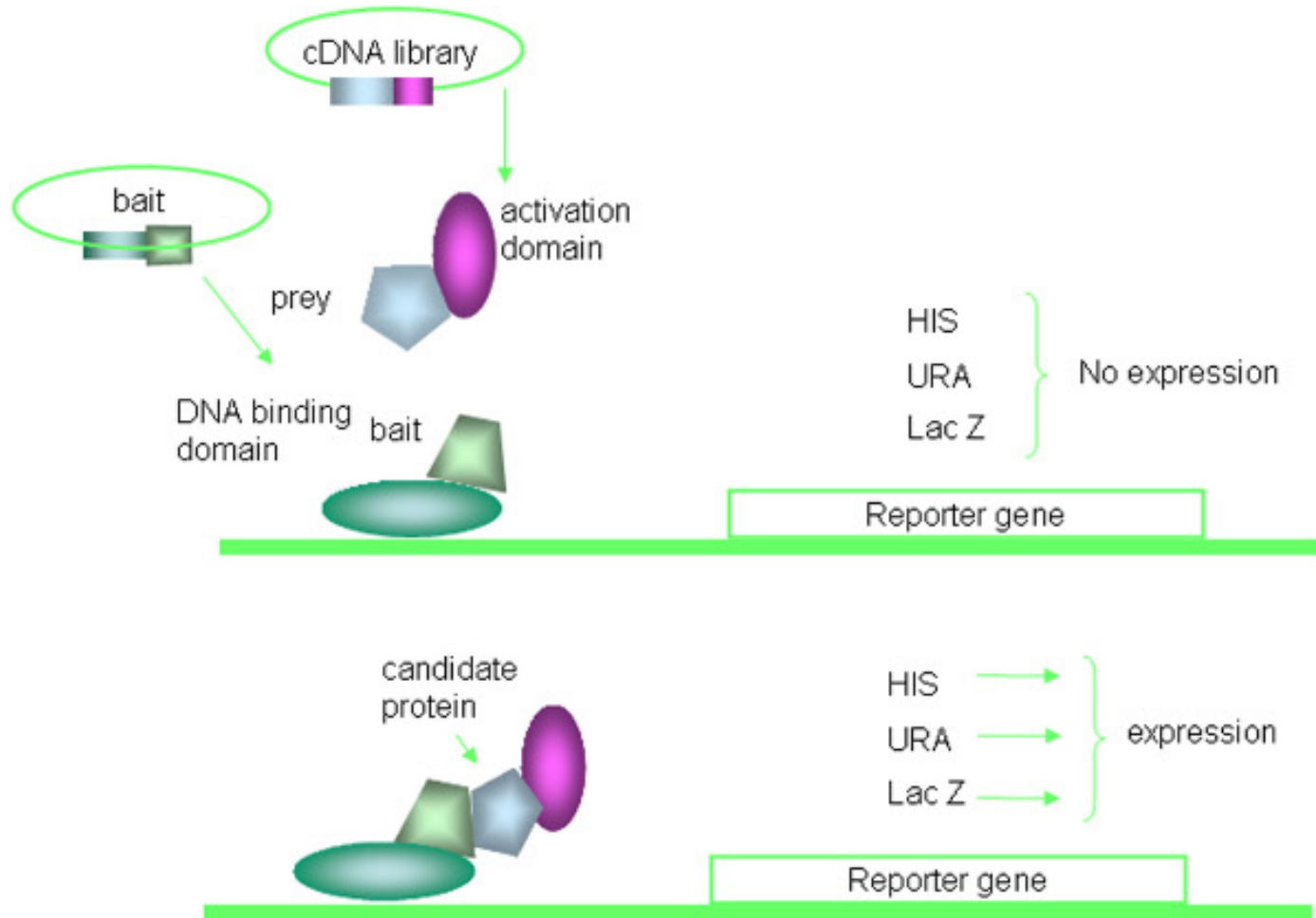
### Computational *(In silico)*

- BIND [Biomolecular Interaction Network Database](#)
- DIP [Database of Interacting Proteins](#)
- MINT [Molecular INTERaction Database](#)
- IntAct [Molecular Interaction Database](#)



# Studio dell'interazione proteina/proteina e proteina/ligando :

## PPI : Two Hybrid system



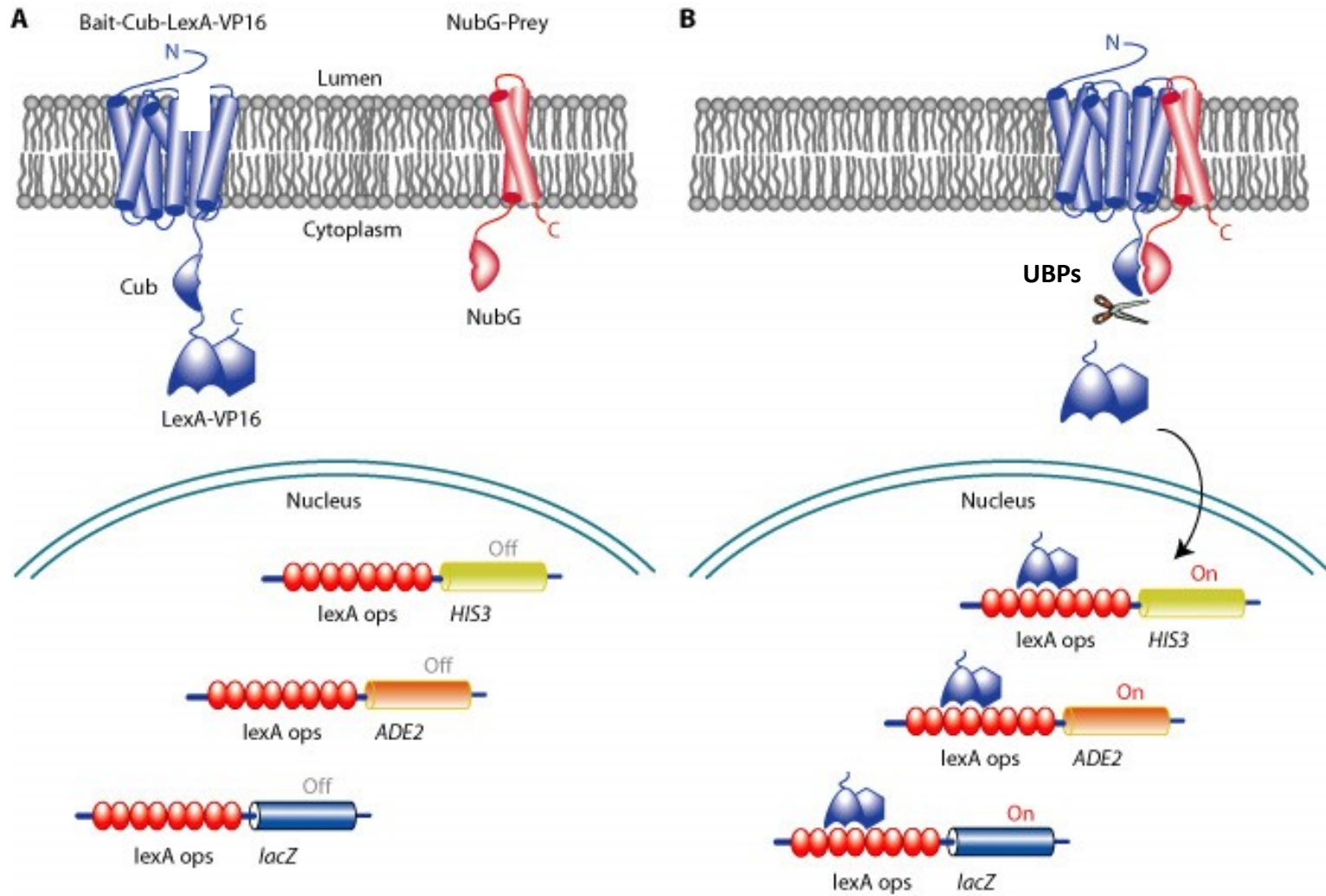
# Studio dell'interazione proteina/proteina e proteina/ligando :

## PPI : Two Hybrid system

Per complessi binari a livello di membrana

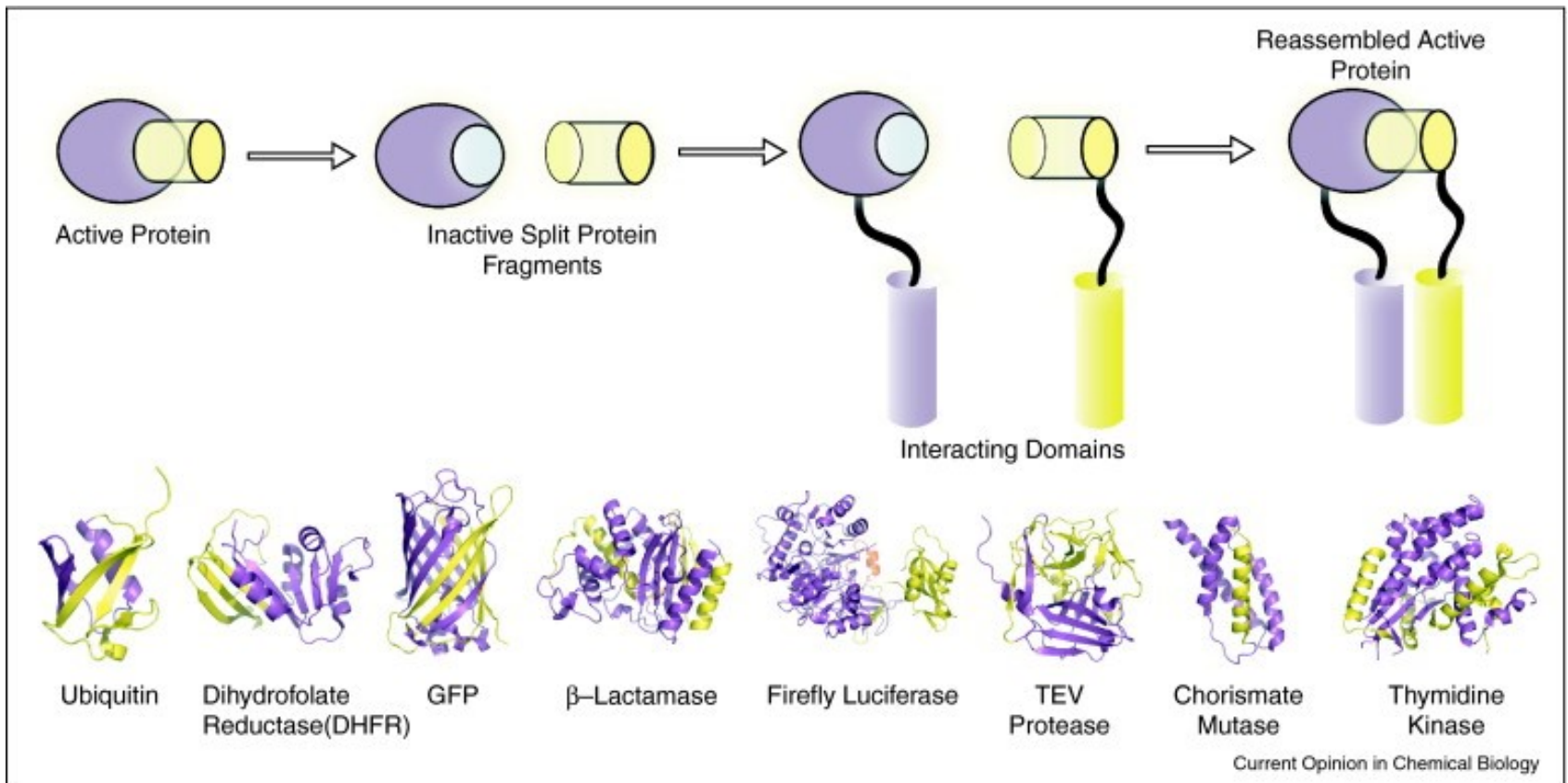


Ubiquitin



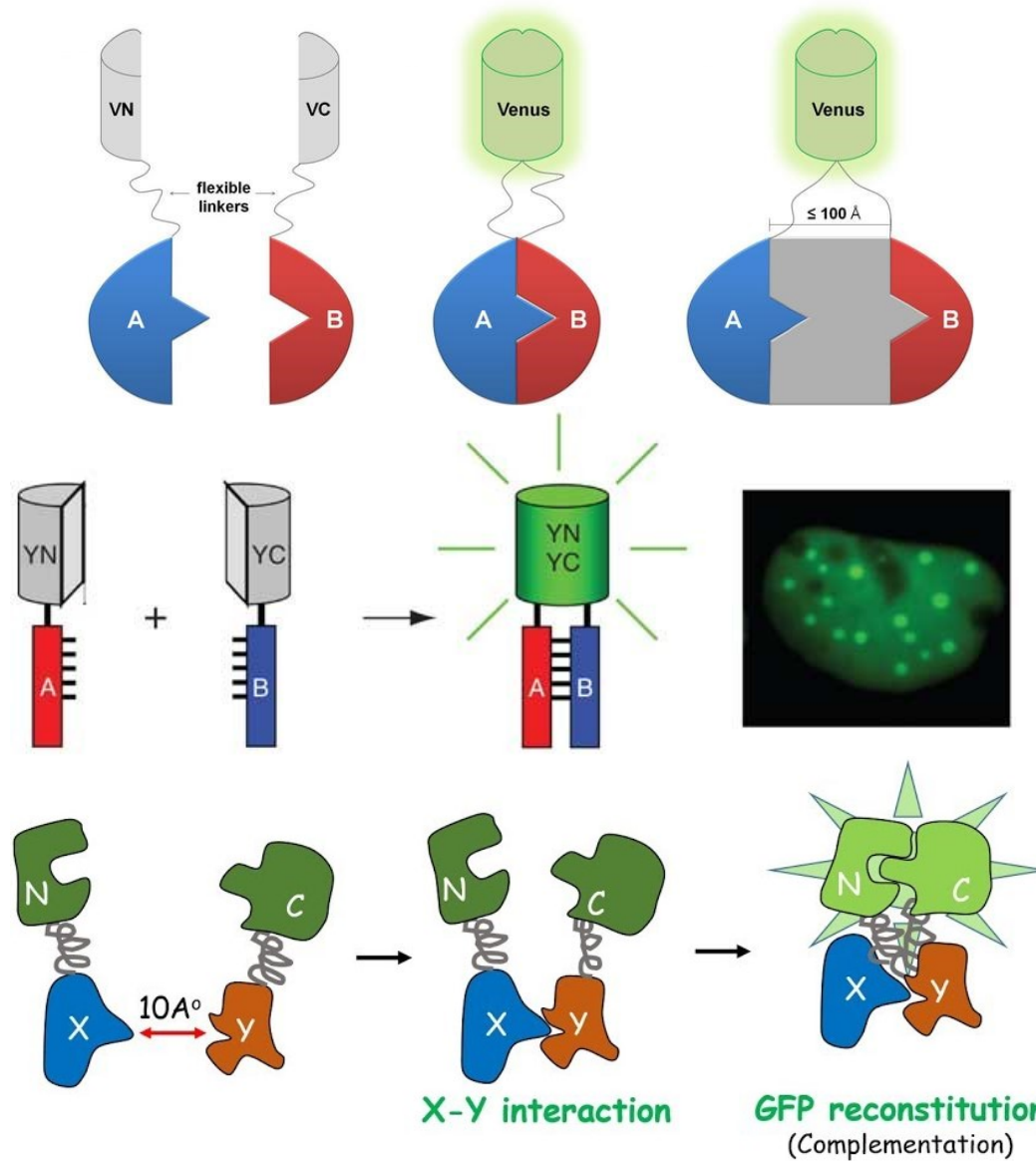
# Studio dell'interazione proteina/proteina e proteina/ligando :

## PPI : Protein-fragment complementation assay



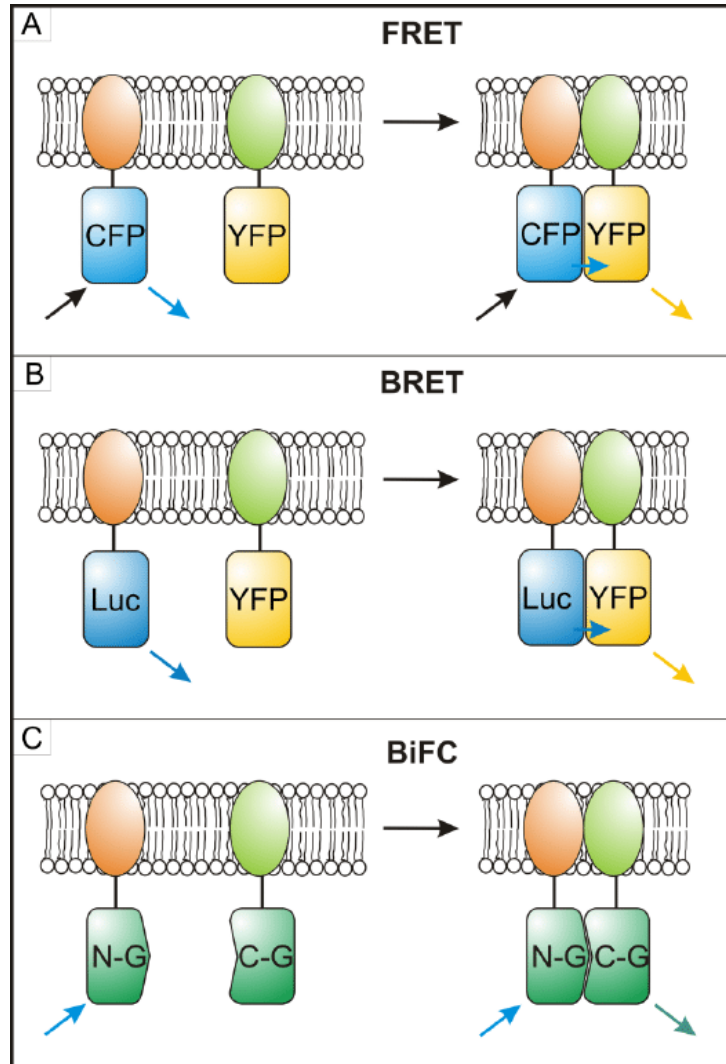
# Studio dell'interazione proteina/proteina e proteina/ligando :

## PPI: BiFc (Bimolecular Fluorescence Complementation)



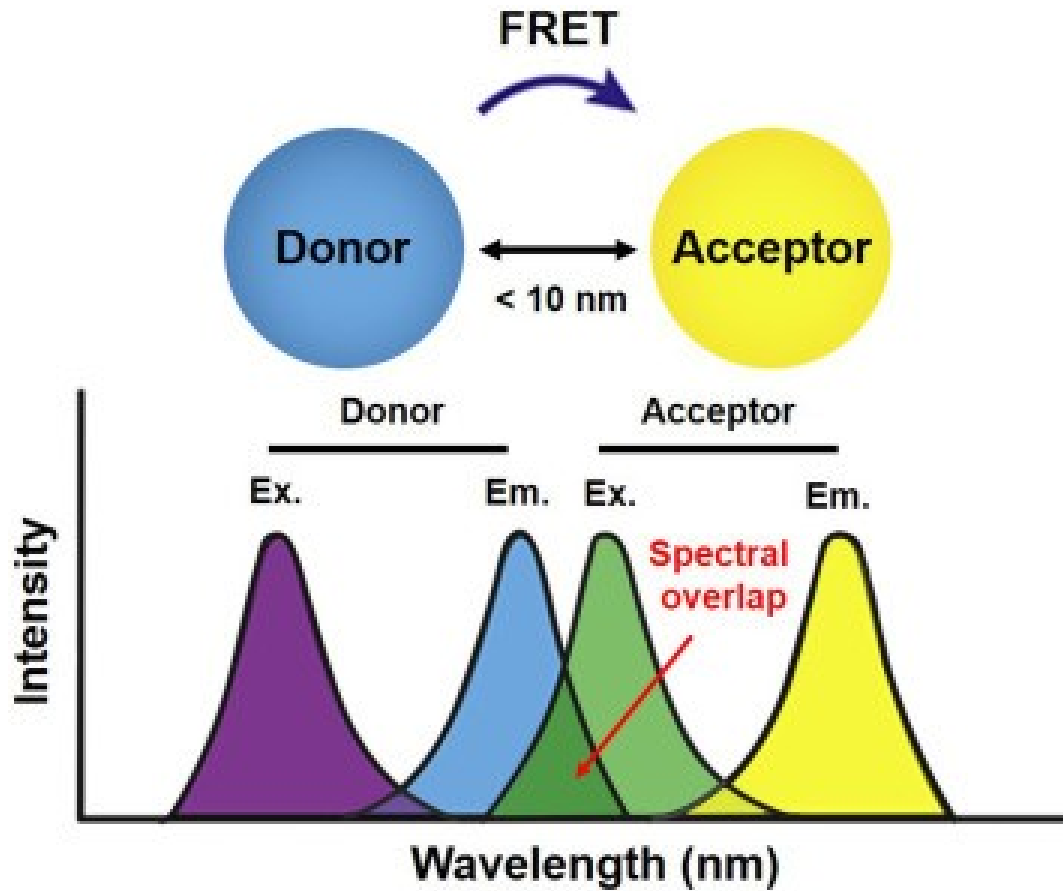
# Studio dell'interazione proteina/proteina e proteina/ligando :

## PPI : Fluorescence based methods



# Studio dell'interazione proteina/proteina e proteina/ligando :

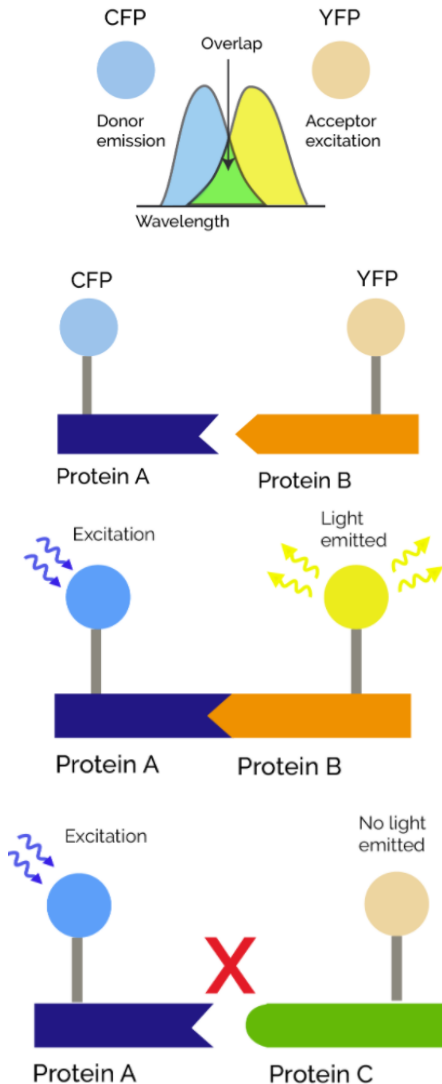
## PPI: FRET (Förster Resonance Energy Transfer)



consente di determinare la vicinanza/orientamento di due fluorofori

# Studio dell'interazione proteina/proteina e proteina/ligando :

## PPI: FRET (Förster Resonance Energy Transfer)



Coppie FRET di fluorofori per marcare in modo specifico biomolecole:

Esprese come proteine di fusione con proteine fluorescenti

- BFP-GFP
- CFP-dsRED
- CFP-YFP

Coniugate chimicamente

- Cy3-Cy5
- Alexa488-Alexa555
- Alexa488-Cy3
- Alexa594-Alexa647
- FITC-TRITC
- Terbium (III)-Fluorescein
- DiSBAC4(3)-CC2-DMPE (a voltage sensitive FRET pair)

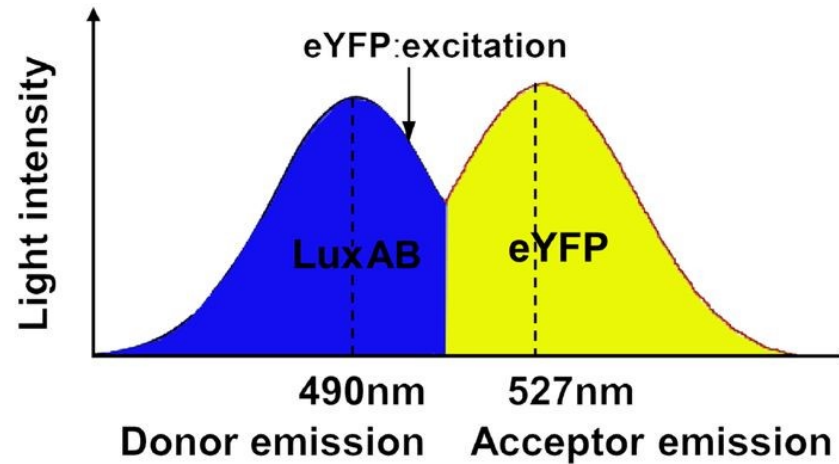
**In vivo: all'interno dell'ambiente fisiologico (batteri, funghi e cellule di mammifero)**

**In vitro consente di calcolare la distanza/orientamento di due fluorofori di studiare cambiamenti conformazionali**

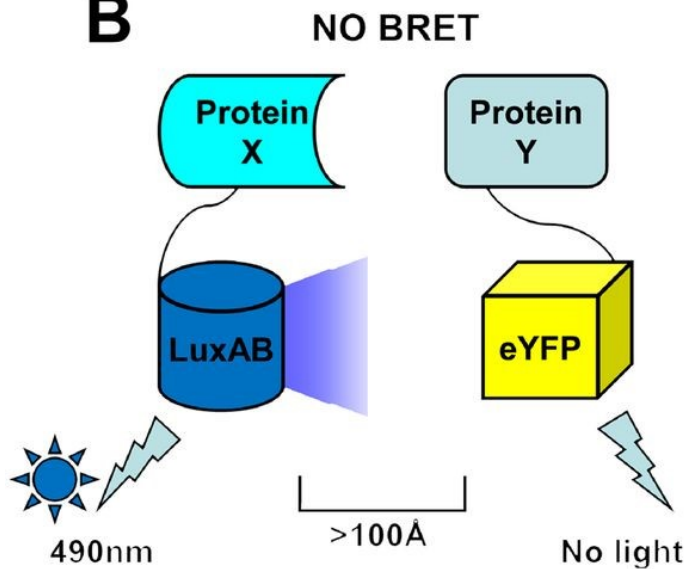
# Studio dell'interazione proteina/proteina e proteina/ligando :

## PPI: BRET (Bioluminescence Resonance Energy Transfer)

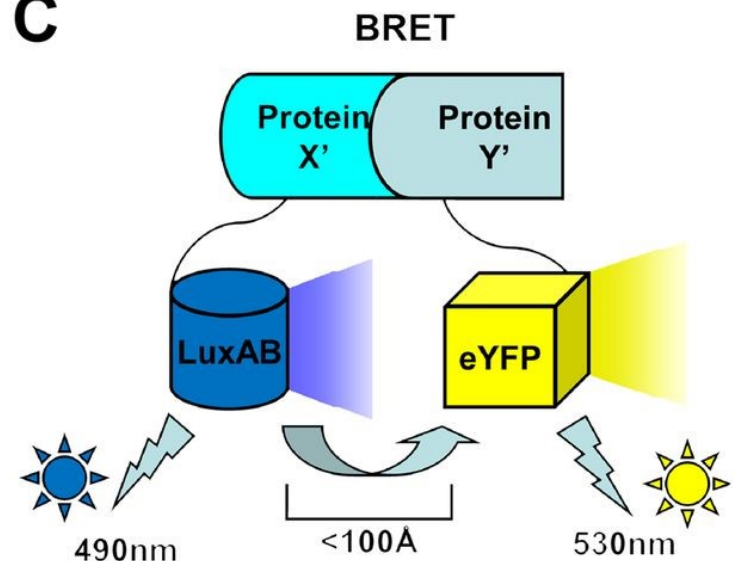
**A**



**B**



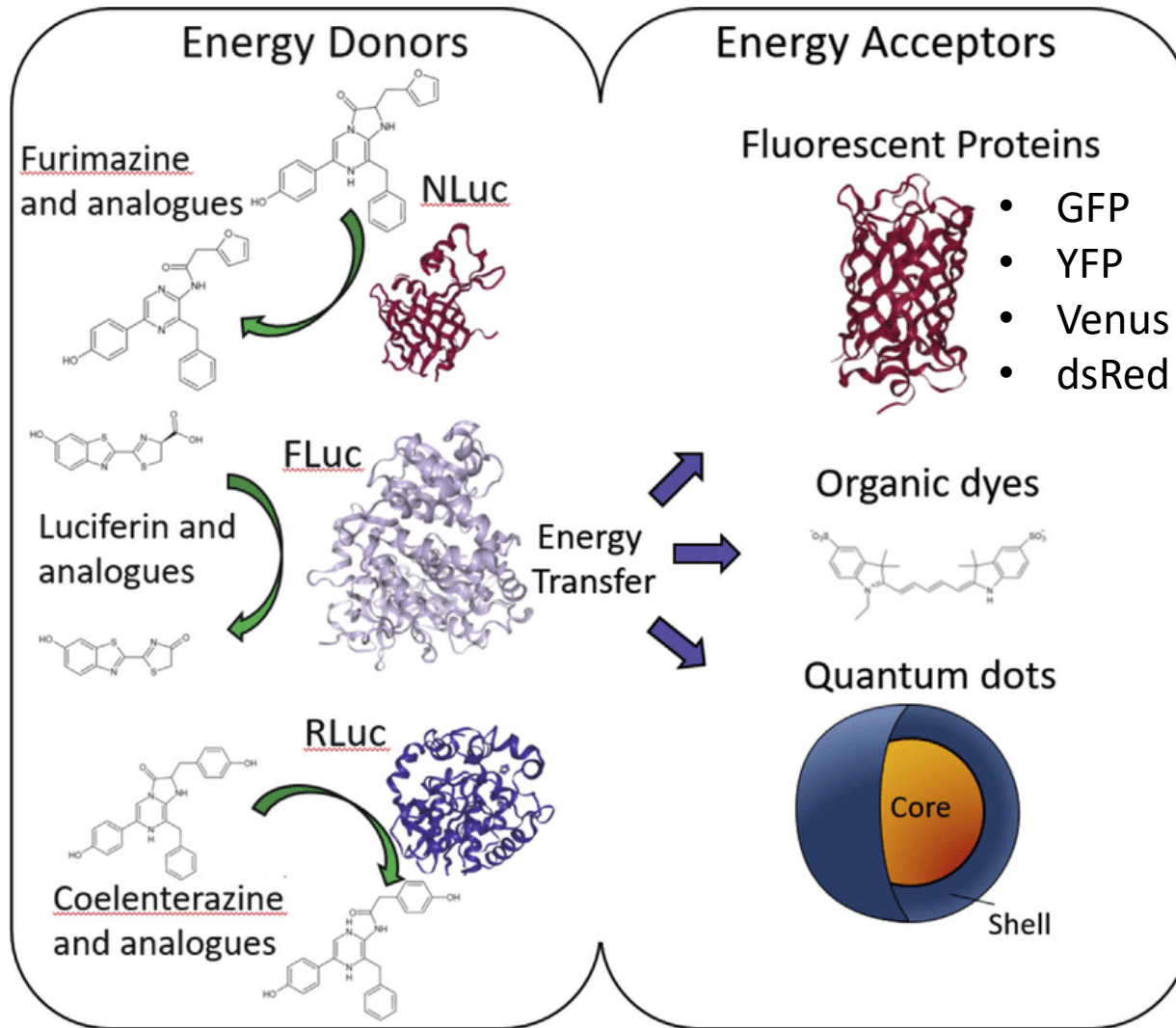
**C**





# Studio dell'interazione proteina/proteina e proteina/ligando :

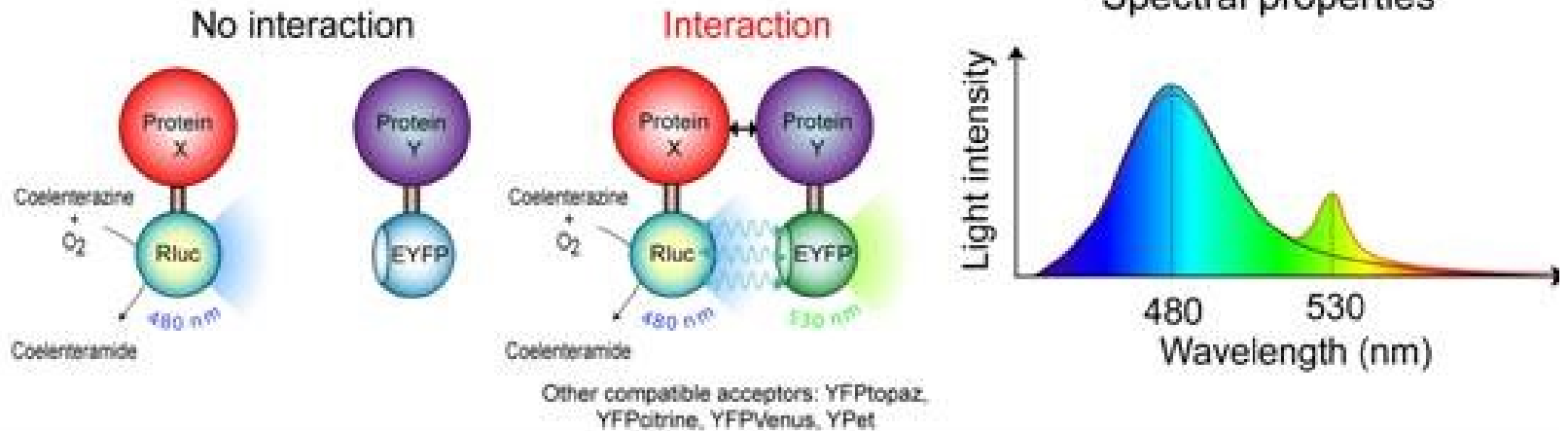
## PPI: BRET (Bioluminescence Resonance Energy Transfer)



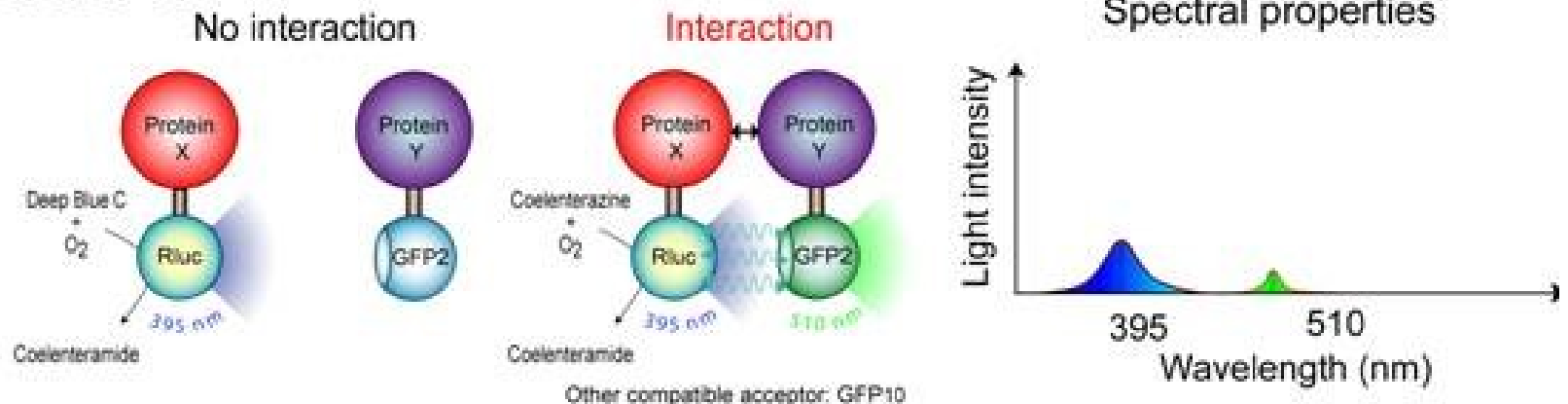
# Studio dell'interazione proteina/proteina e proteina/ligando :

## PPI: BRET (Bioluminescence Resonance Energy Transfer)

### BRET 1



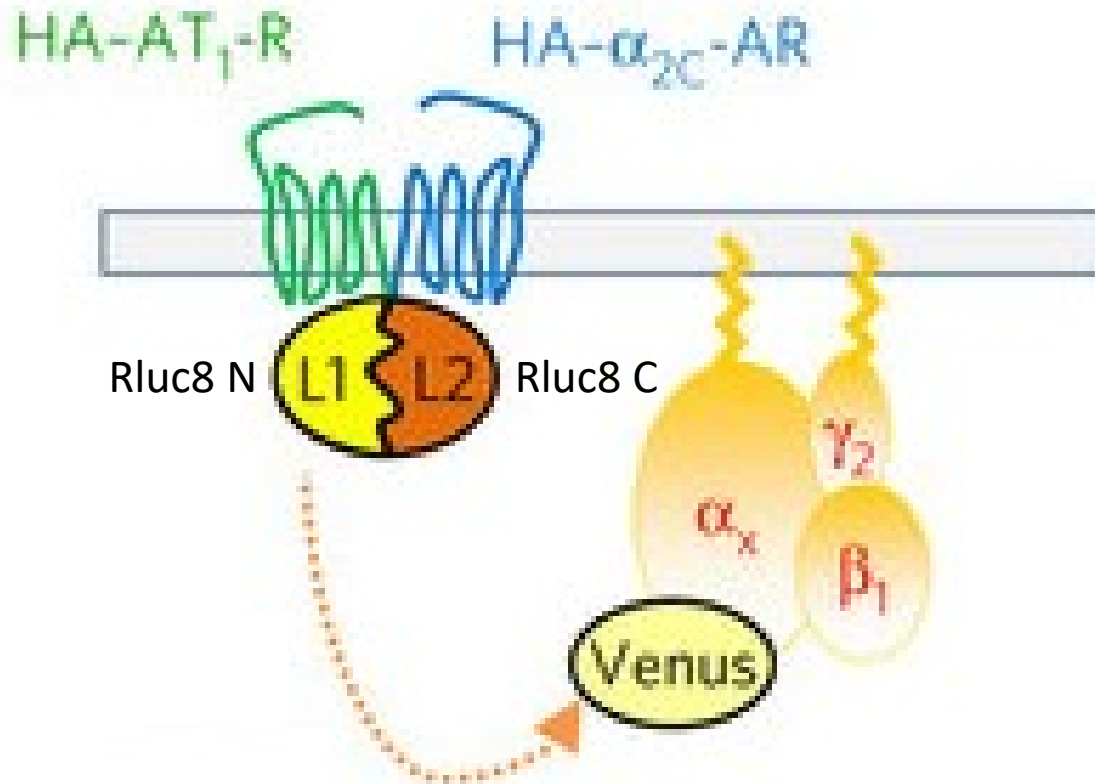
### BRET 2



# Studio dell'interazione proteina/proteina e proteina/ligando :

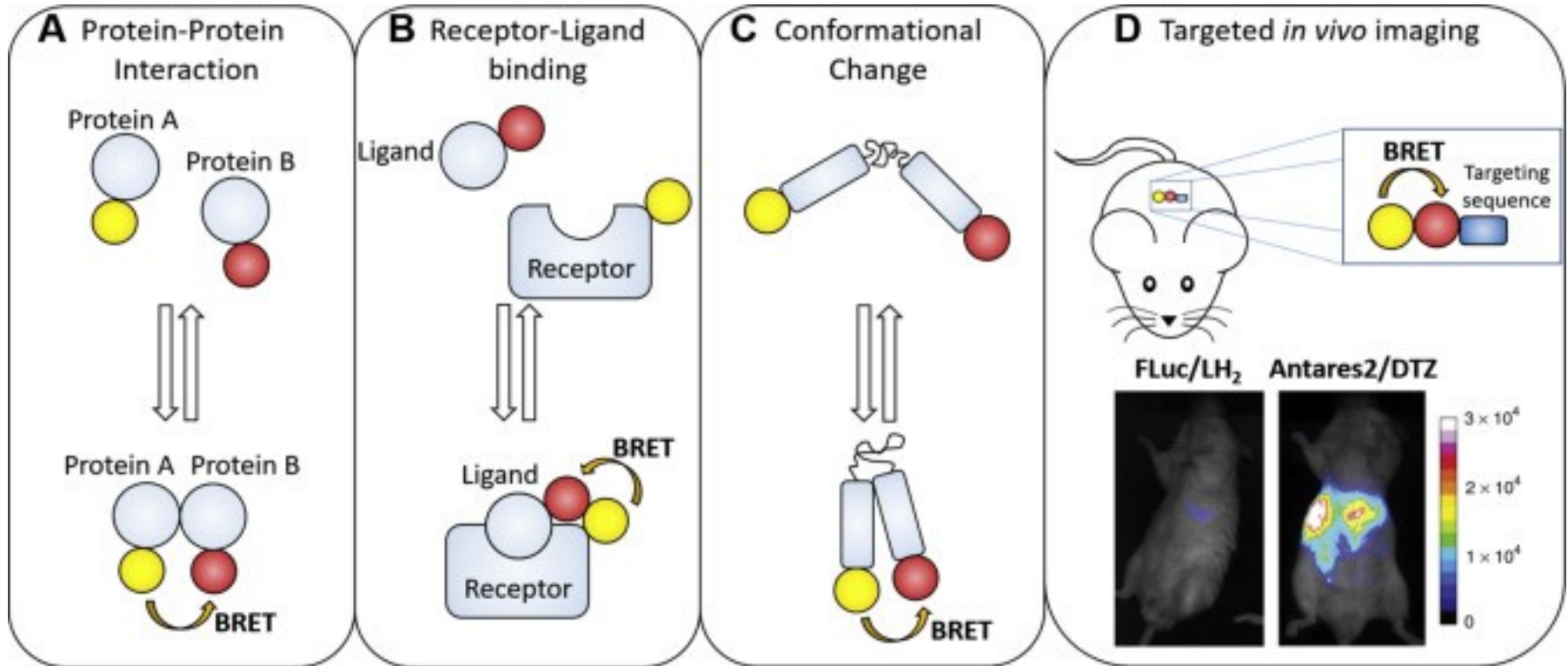
PPI: BRET (Bioluminescence Resonance Energy Transfer)

CODA-RET (BRET+PCA): complessi ternari



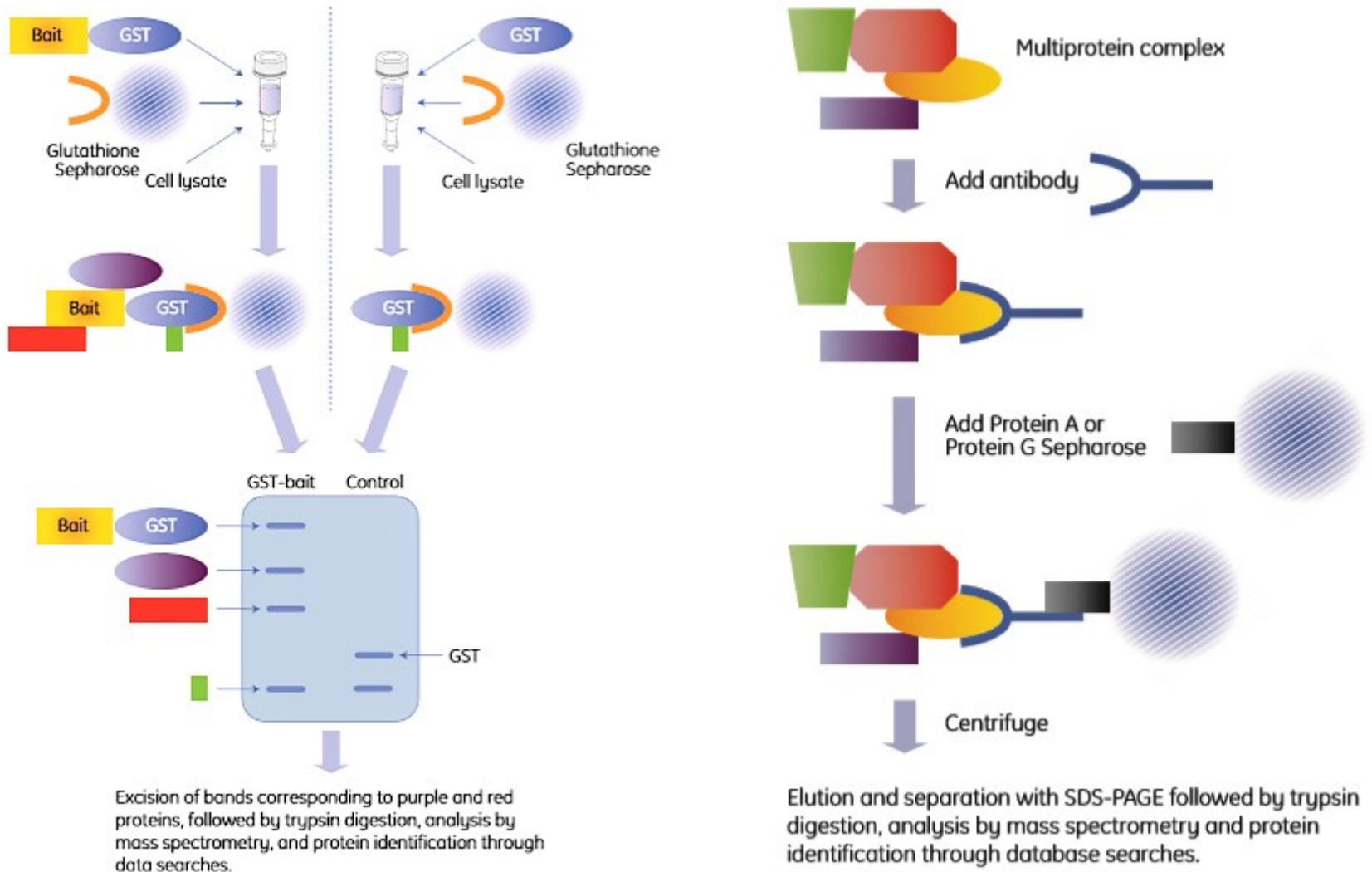
# Studio dell'interazione proteina/proteina e proteina/ligando :

## PPI: BRET (Bioluminescence Resonance Energy Transfer)



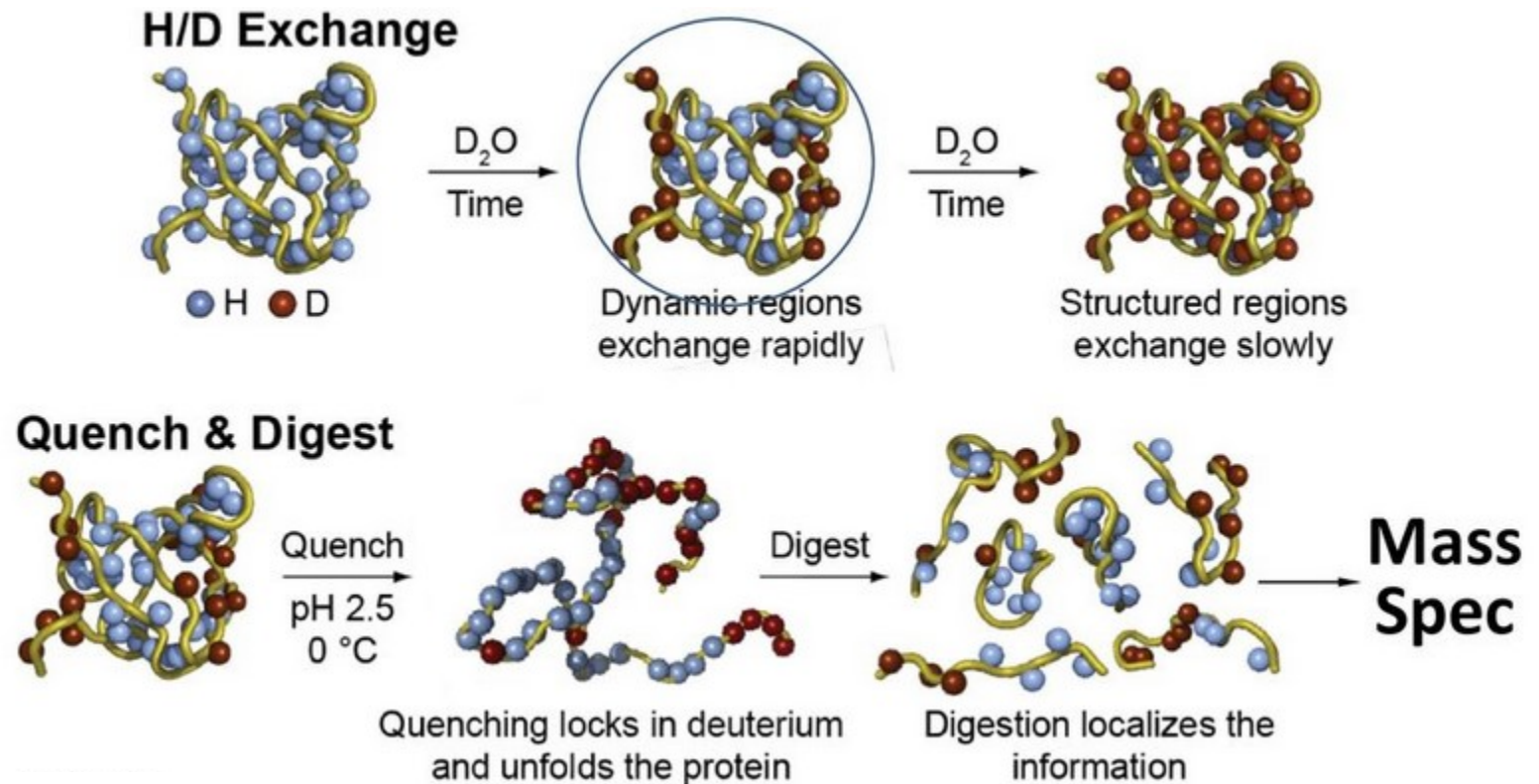
# Studio dell'interazione proteina/proteina e proteina/ligando :

## PPI : Pull-down e CoIP



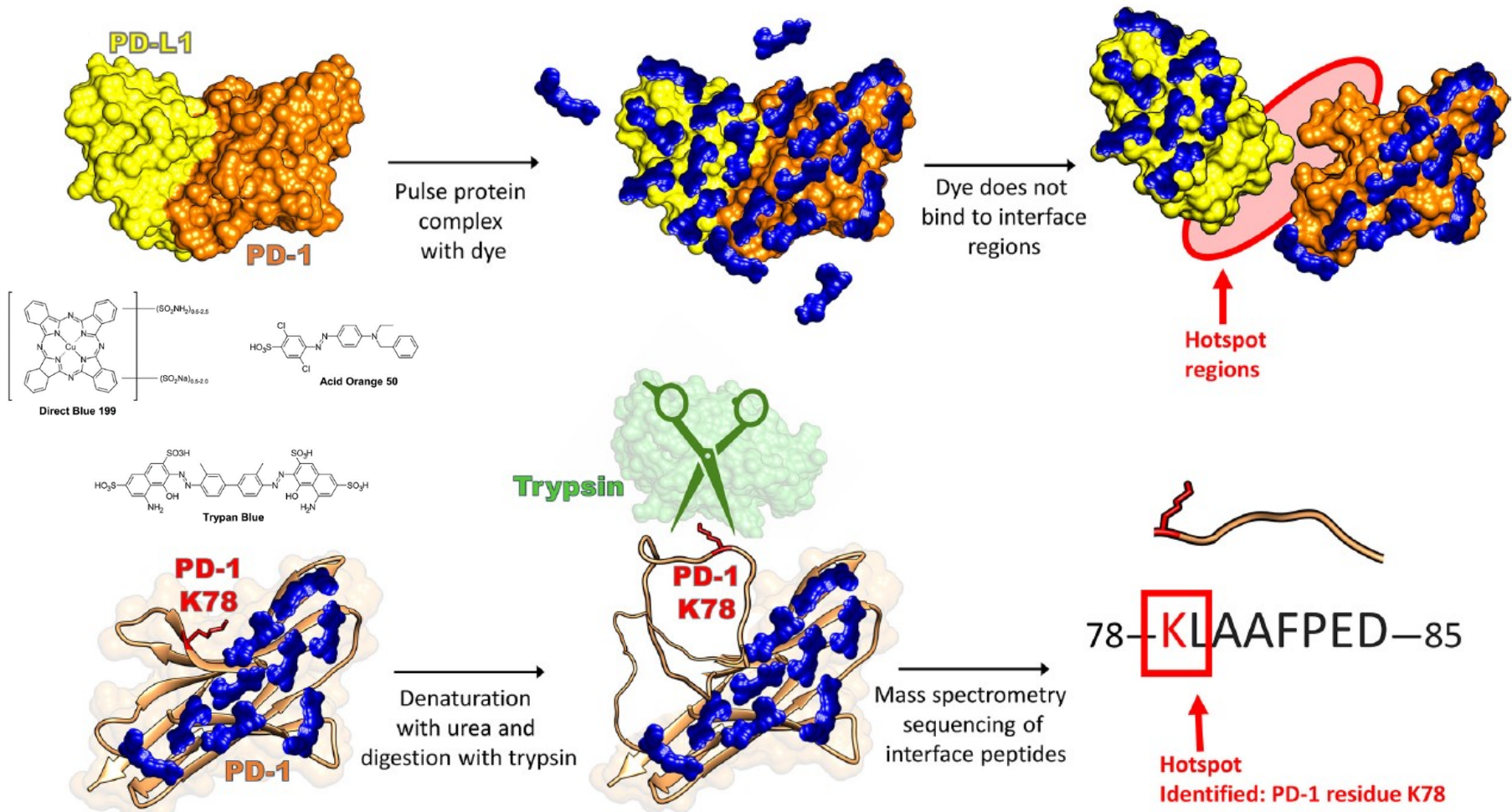
# Studio dell'interazione proteina/proteina e proteina/ligando :

## PPI&PLI: Hydrogen/Deuterium Exchange (DX-MS)



# Studio dell'interazione proteina/proteina e proteina/ligando :

## PPI&PLI: Protein Painting



**PPI&PPL : metodi di caratterizzazione quantitativa**

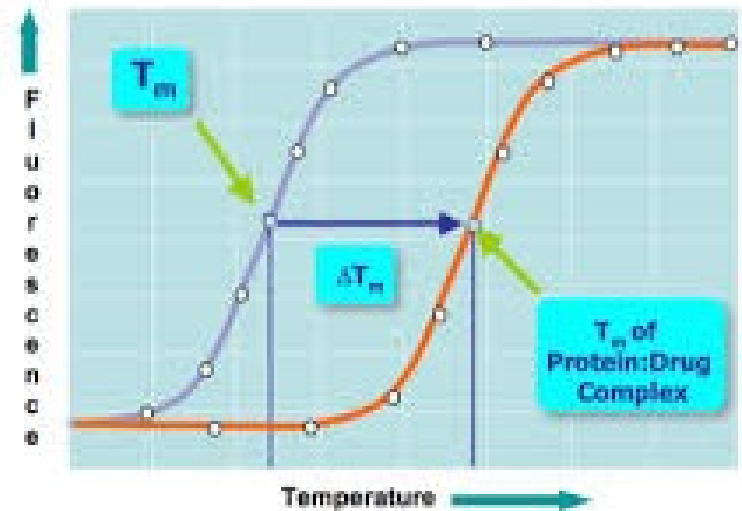
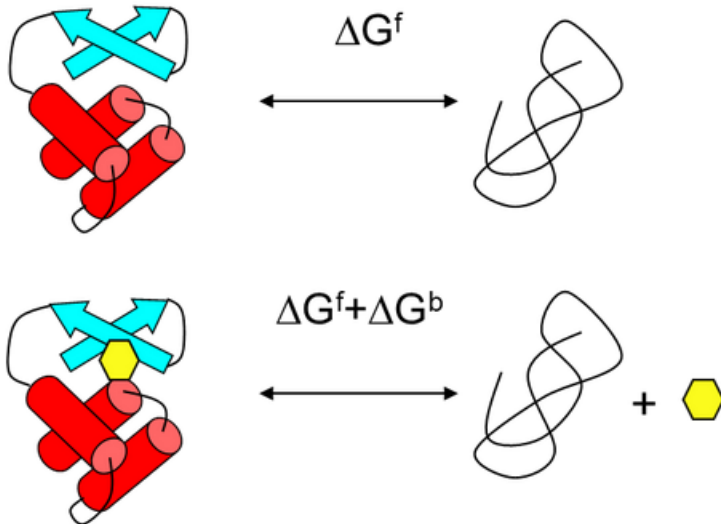
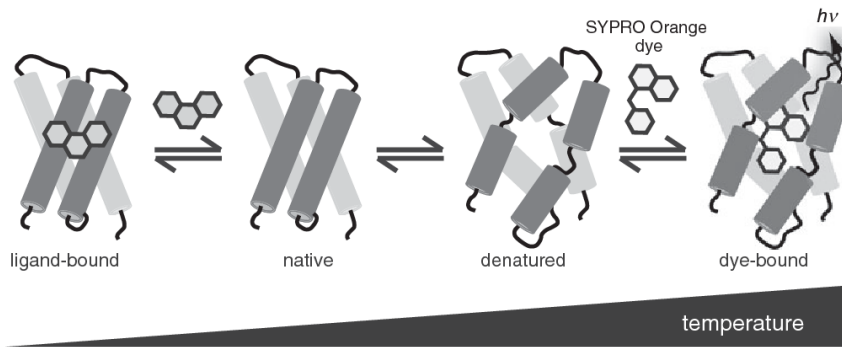


DSF

# Studio dell'interazione proteina/proteina e proteina/ligando :

## DSF: Applicazioni

### 1) PLI: Screening di ligandi



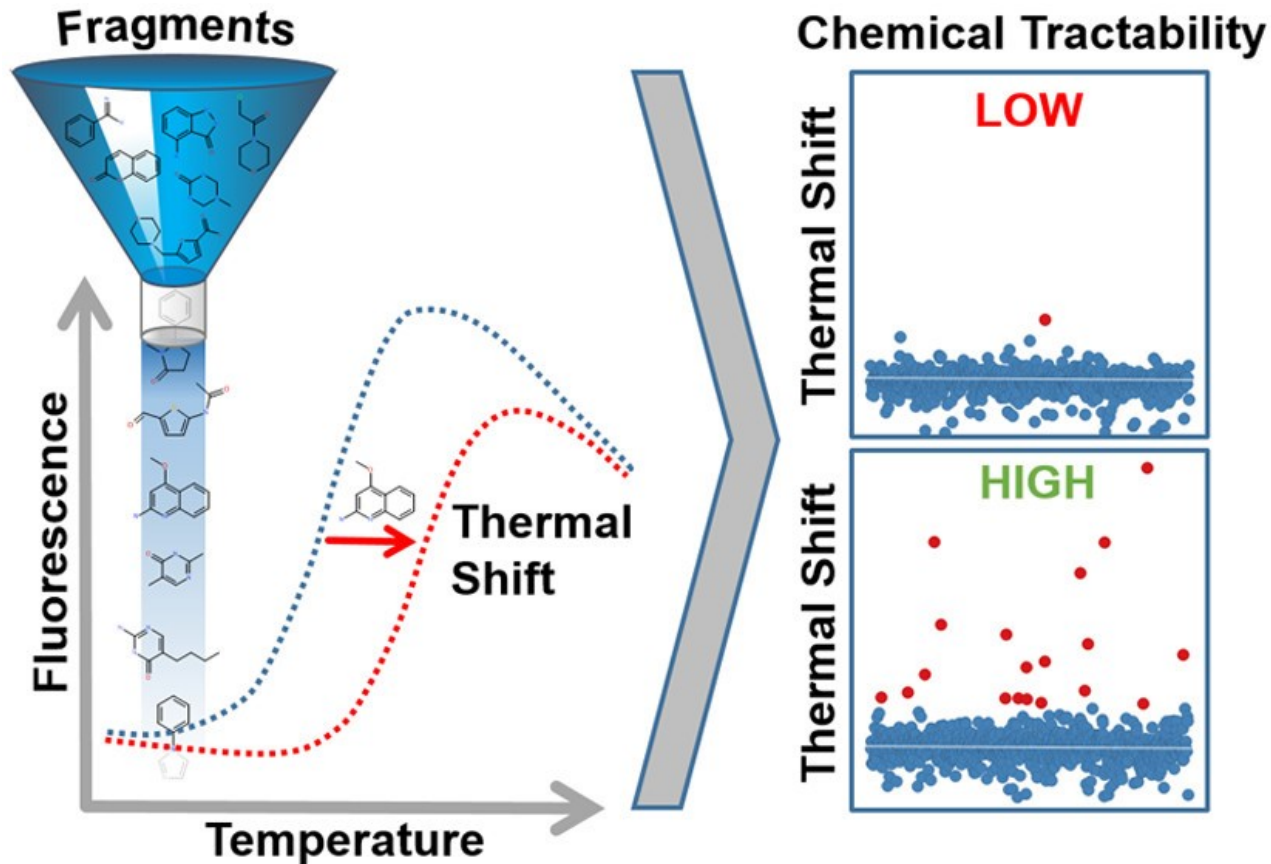
Correlazione stabilizzazione/affinità dell'interazione proteina/ligando:  $IC_{50} < 1\mu M \rightarrow \Delta T_M > 4^\circ C$

# Studio dell'interazione proteina/proteina e proteina/ligando :

## DSF: Applicazioni

### 2) PLI: HIT PROFILING

Il ranking della forza di interazione del ligando e la stima dell'energia di legame possono essere ottenuti confrontando i valori  $T_m$  di proteina apo e dei complessi proteina-ligando.

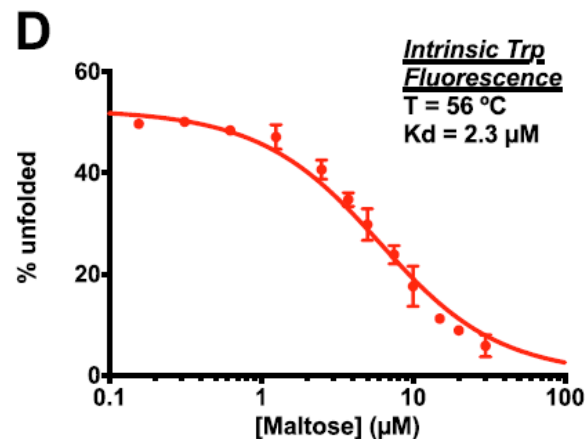
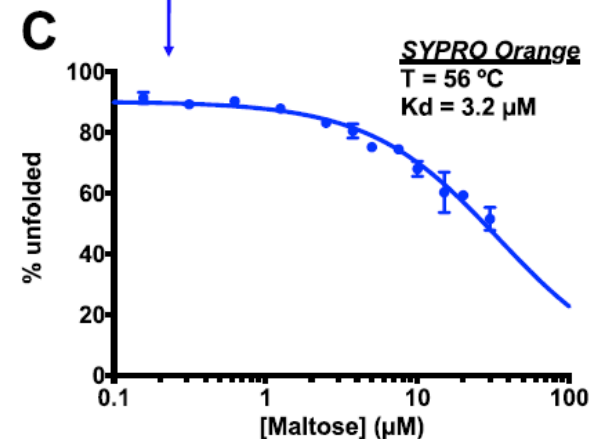
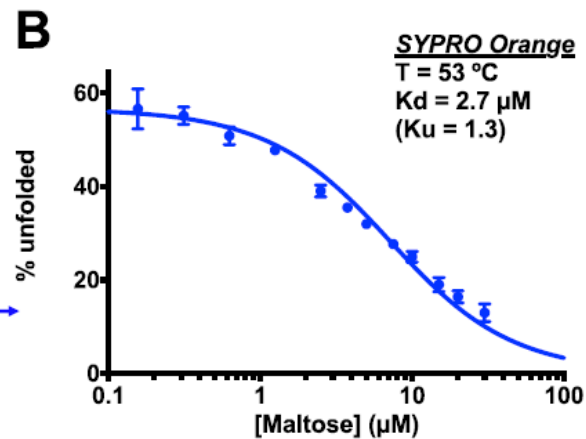
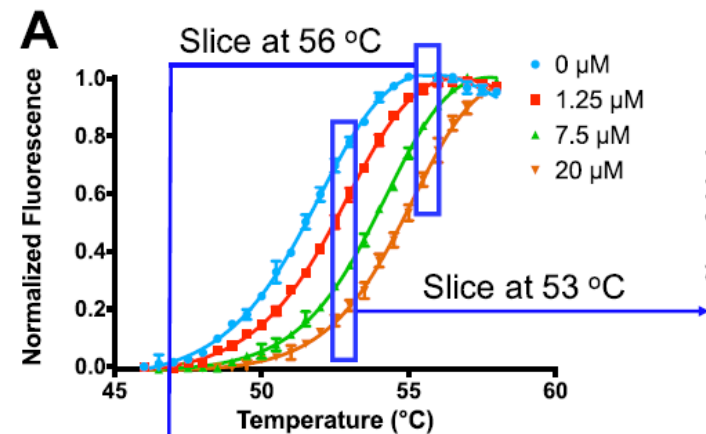


# Studio dell'interazione proteina/proteina e proteina/ligando :

## DSF: Applicazioni

### 3) PLI: Caratterizzazione quantitativa

A T costante: due di equilibri accoppiati (folding / unfolding della proteina e legame / dissociazione del ligando).



Approccio "isotermico": dalle curve di melting a diverse [ligando] → frazione di proteina denaturata a una data T →  $K_D$ .

SCIENTIFIC REPORTS

OPEN Isothermal Analysis of ThermoFluor Data can readily provide Quantitative Binding Affinities

Received: 23 July 2018  
Accepted: 30 November 2018

Nan Bai<sup>1\*</sup>, Heinrich Roder<sup>1</sup>, Alex Dickson<sup>1</sup> & John Karanicolas<sup>1,2</sup>

SCIENTIFIC REPORTS | (2019) 9:2650 | https://doi.org/10.1038/s41598-019-37072-4

1

DSC

# Studio dell'interazione proteina/proteina e proteina/ligando :

DSC: applicazioni

PLI: Caratterizzazione quantitativa

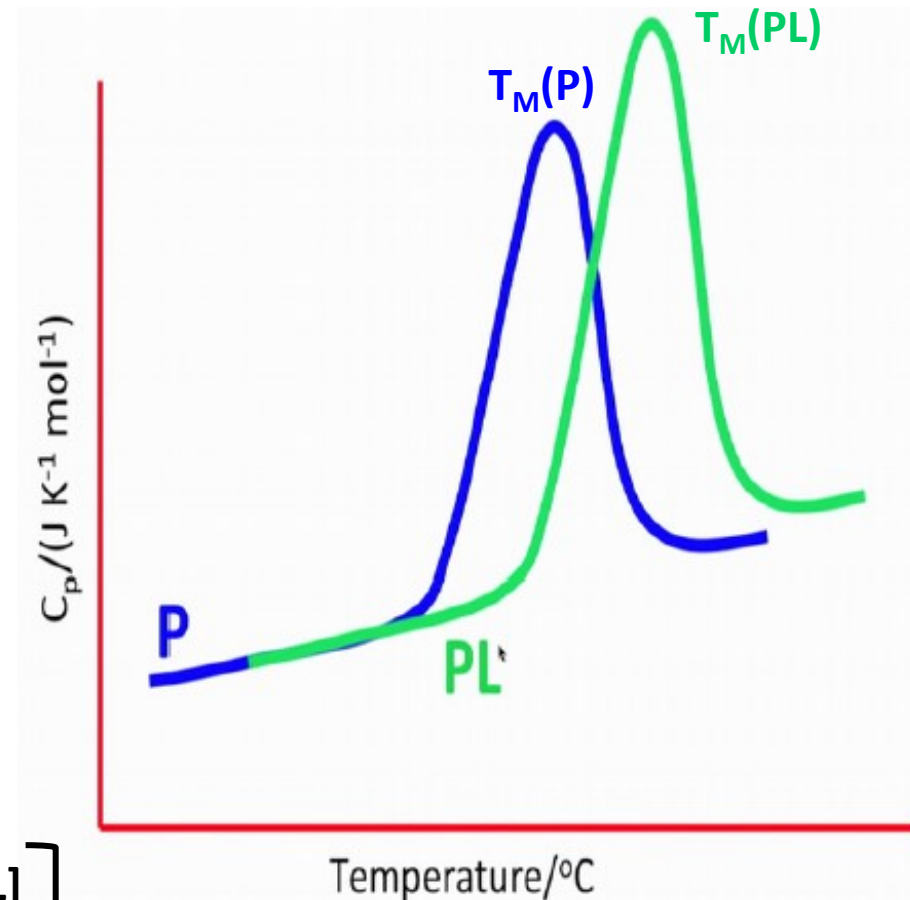
L'aumento della  $T_M$



Shift del termogramma verso destra consente di stimare l'affinità di legame:

Per l'interazione  $P + L \leftrightarrow PL$

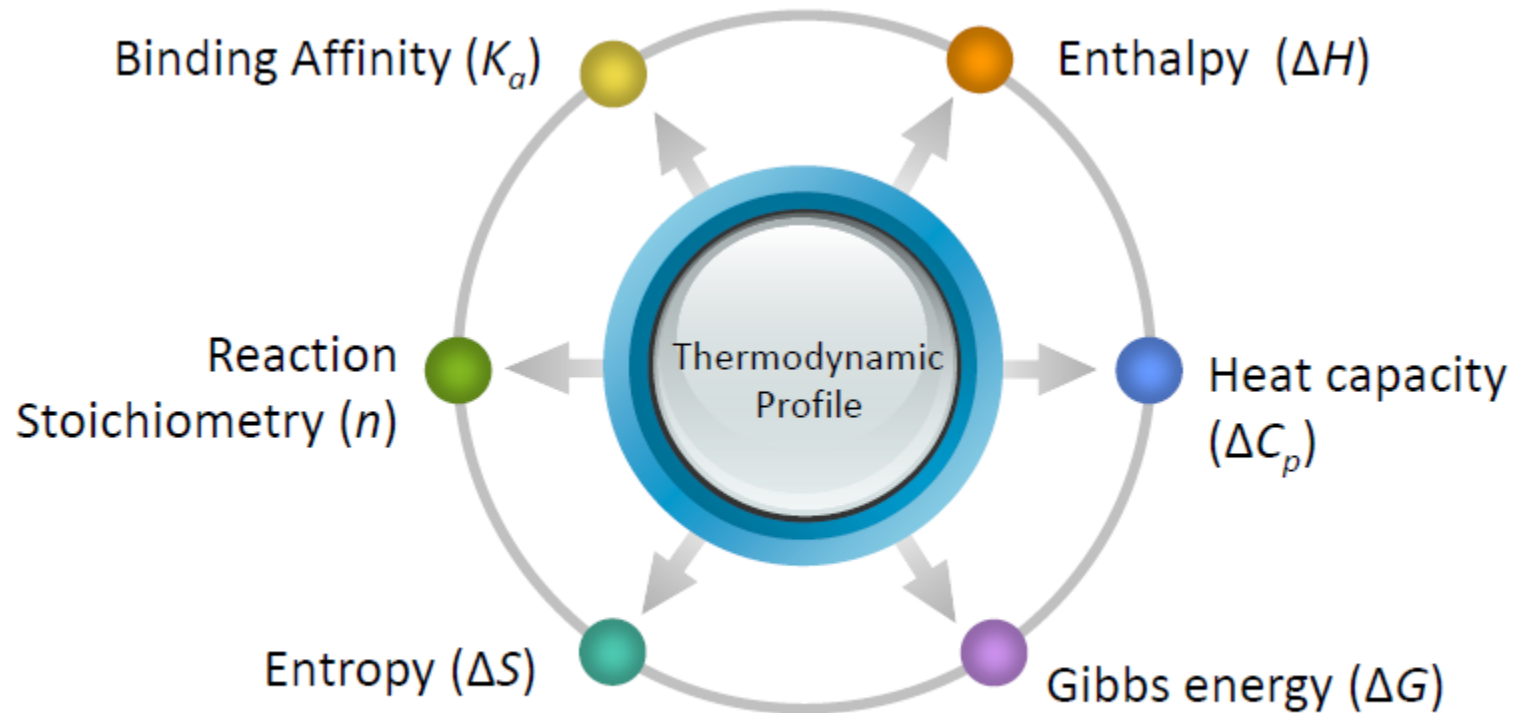
$$\frac{\Delta T_M}{T_M(PL)} = \frac{T_M(PL) - T_M(P)}{T_M(PL)} = \frac{R T_M(P)}{\Delta H(P)} \ln \left[ 1 + \frac{[L]}{K_D} \right]$$



ITC

# Studio dell'interazione proteina/proteina e proteina/ligando :

## Isothermal Titration Calorimetry (ITC)





# Studio dell'interazione proteina/proteina e proteina/ligando :

## Isothermal Titration Calorimetry (ITC): basi

Per l'interazione a **pressione costante**,



$$Q_T = V_0 \Delta H [PL]$$

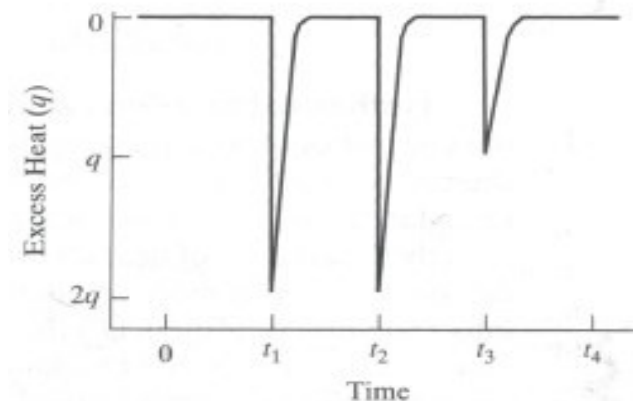
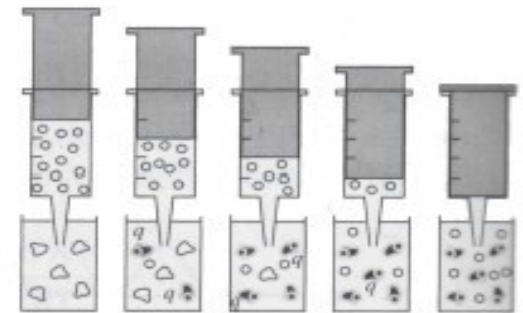
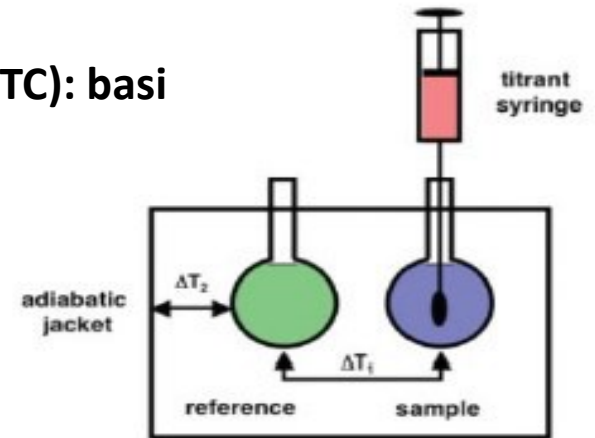
$\Delta H$  variazione di entalpia molare

$V_0$  volume della cella calorimetrica

$$\frac{[PL]}{[P]_T} = \frac{Q_T}{V_0 \Delta H [P]_T}$$

Si ottiene  $\Delta H$  se

- Saturazione completa o  $[PL] / [P]_T \approx 1$
- Sottrazione adeguata del background
- $[P]_T$  accurata

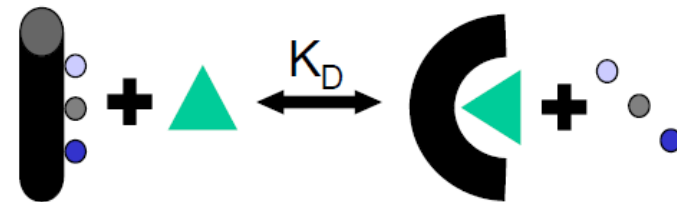
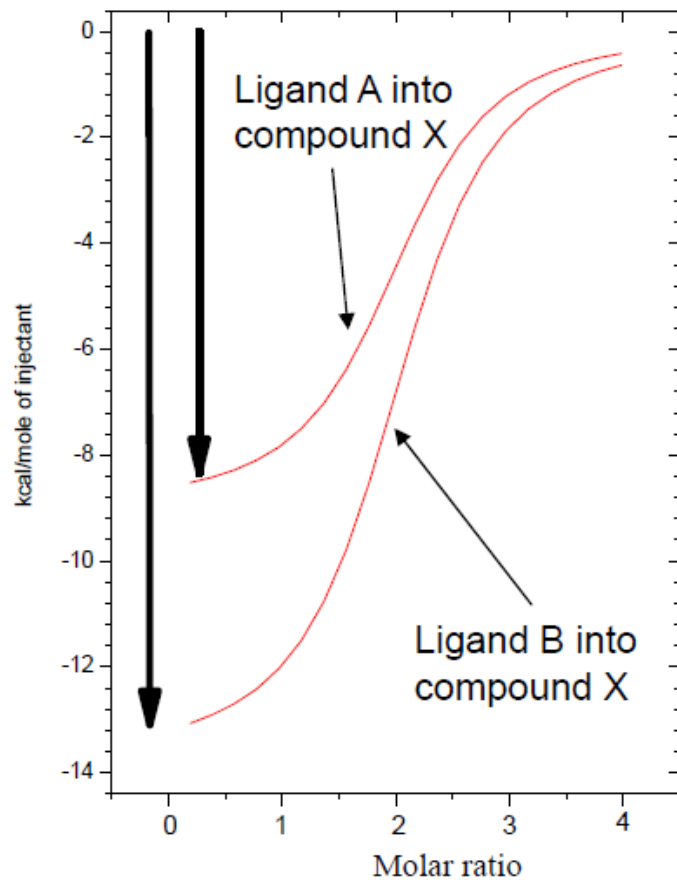


# Studio dell'interazione proteina/proteina e proteina/ligando :

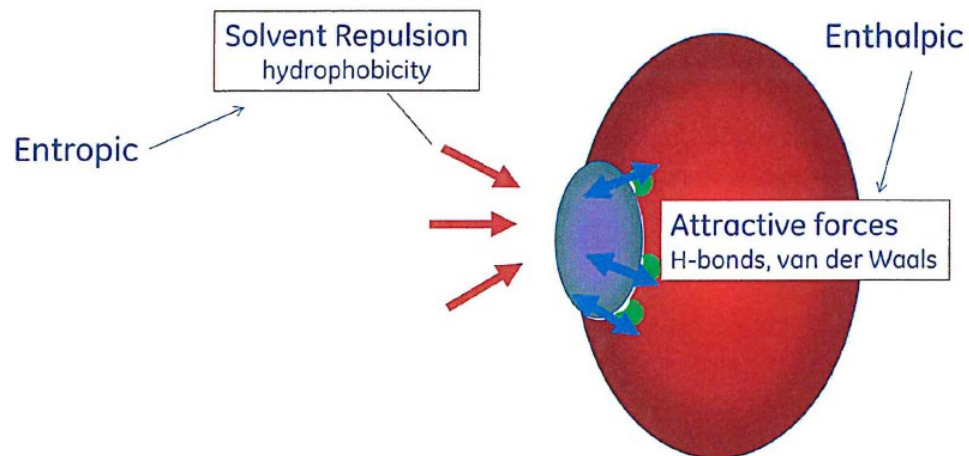
## ITC: basi

$$\Delta G = RT \ln K_D = \Delta H - T \Delta S$$

→ diversi meccanismi di legame

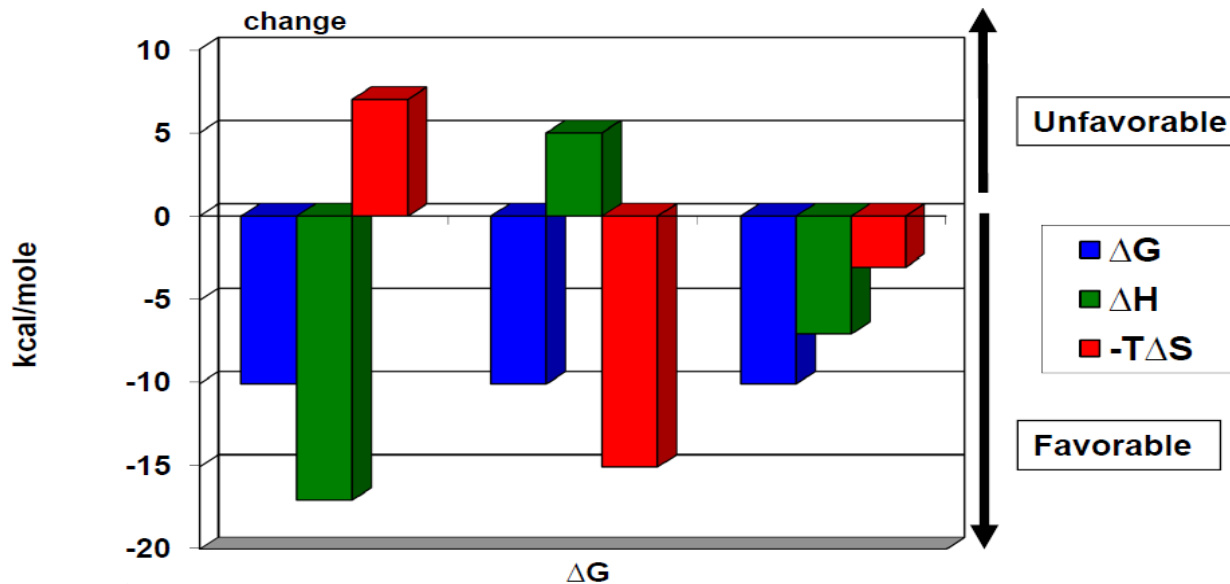


Macromolecule  
Waters, ions, protons  
Ligand



# Studio dell'interazione proteina/proteina e proteina/ligando :

## ITC: basi



$\Delta H$	$-T\Delta S$	$\Delta G = \Delta H - T\Delta S$
-	-	Processo favorito da entrambi e spontaneo a tutte le T
-	+	Processo favorito da entalpia ma sfavorito da entropia, spontaneo a $T < \Delta S / \Delta H$
+	-	Processo sfavorito da entalpia ma favorito da entropia, spontaneo a $T > \Delta S / \Delta H$
+	+	Processo sfavorito da entrambi e mai spontaneo

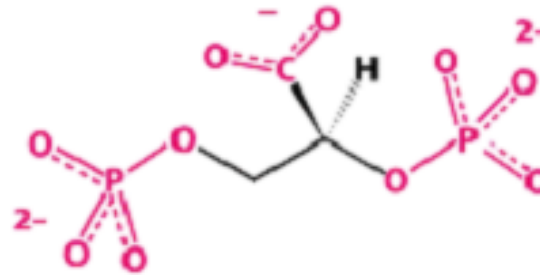
# Studio dell'interazione proteina/proteina e proteina/ligando :

## ITC: basi

CASI ESTREMI DI PLI:

### A. $\Delta H$ driven (ligandi polari):

con grande grado di flessibilità  
con distanze ottimali per legami H  
molto specifici



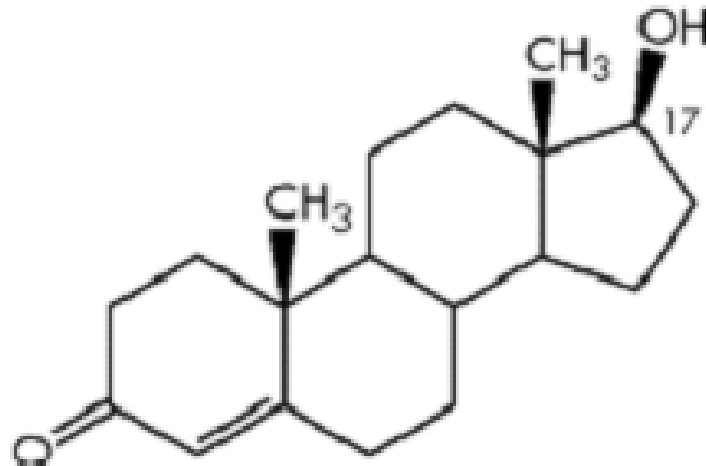
**2,3-Bisphosphoglycerate**  
**(2,3-BPG)**

AFFINITA' BASSE

### B. $-\Delta S$ driven (ligandi idrofobici):

spesso molto grandi  
meno specifici

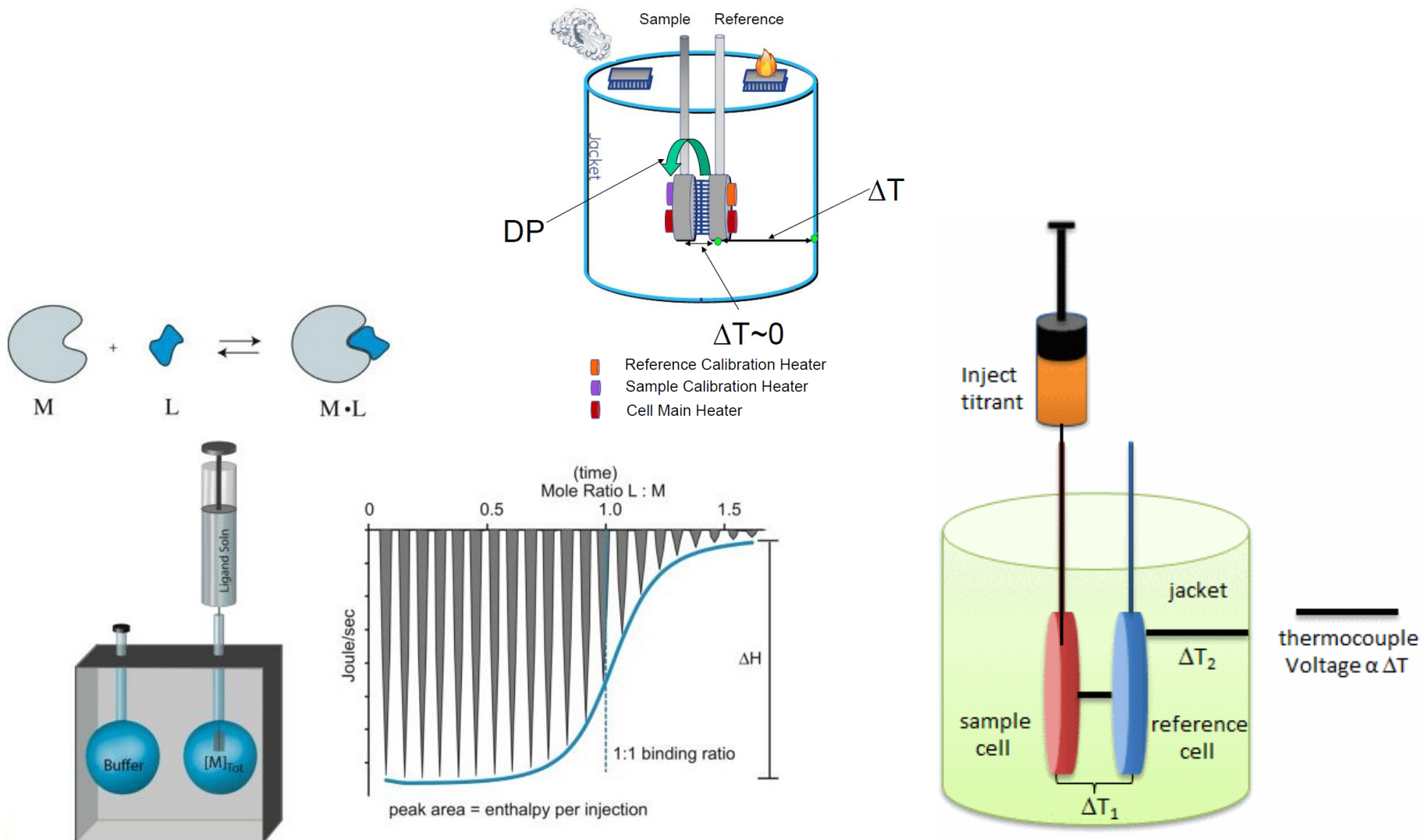
AFFINITA' ALTE



**Testosterone**

# Studio dell'interazione proteina/proteina e proteina/ligando :

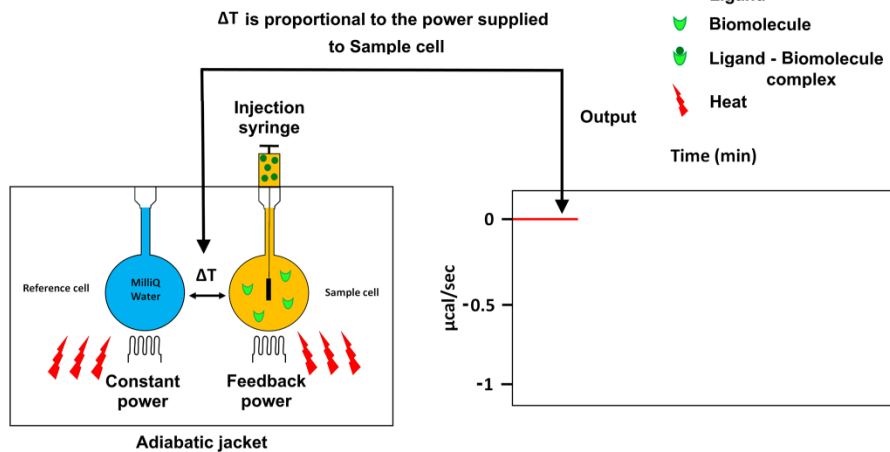
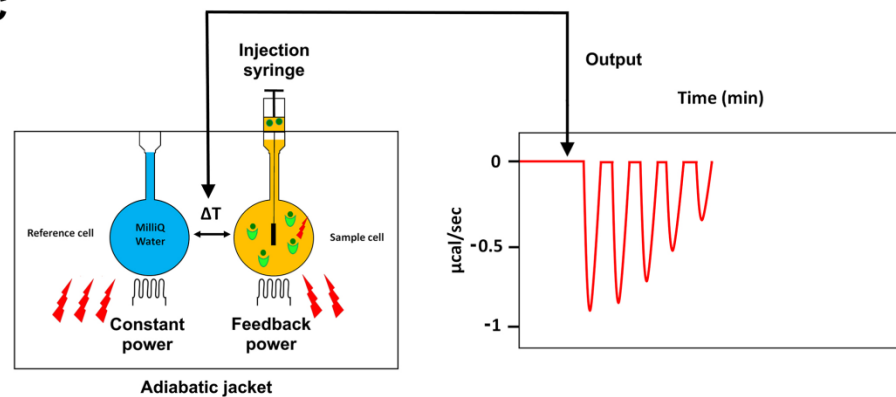
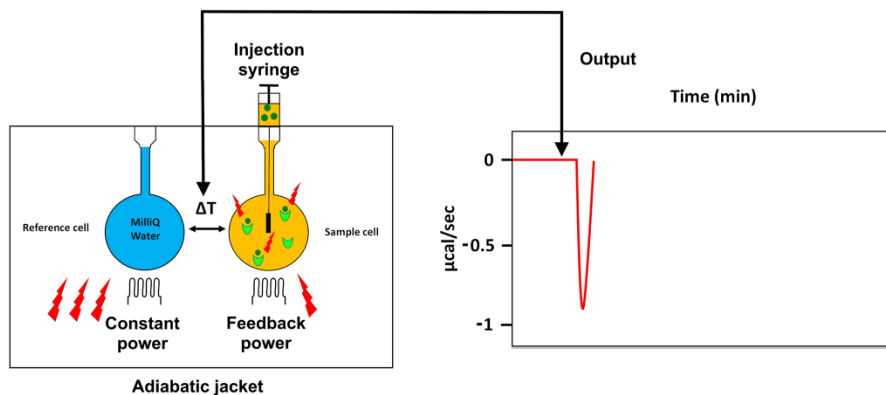
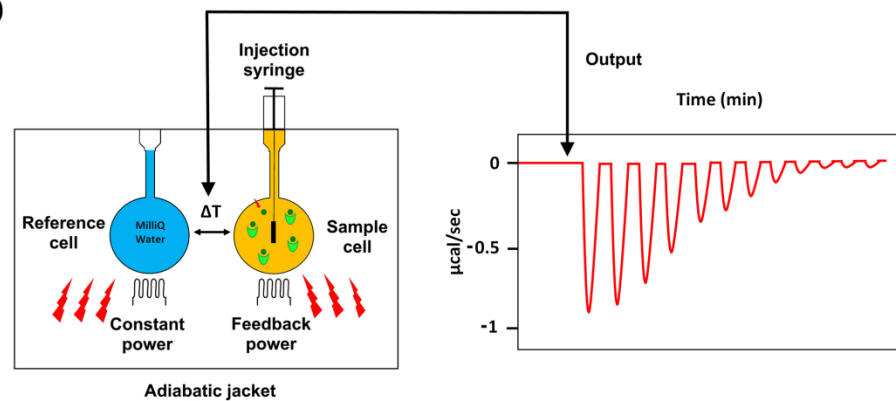
## ITC: setup



# Studio dell'interazione proteina/proteina e proteina/ligando :

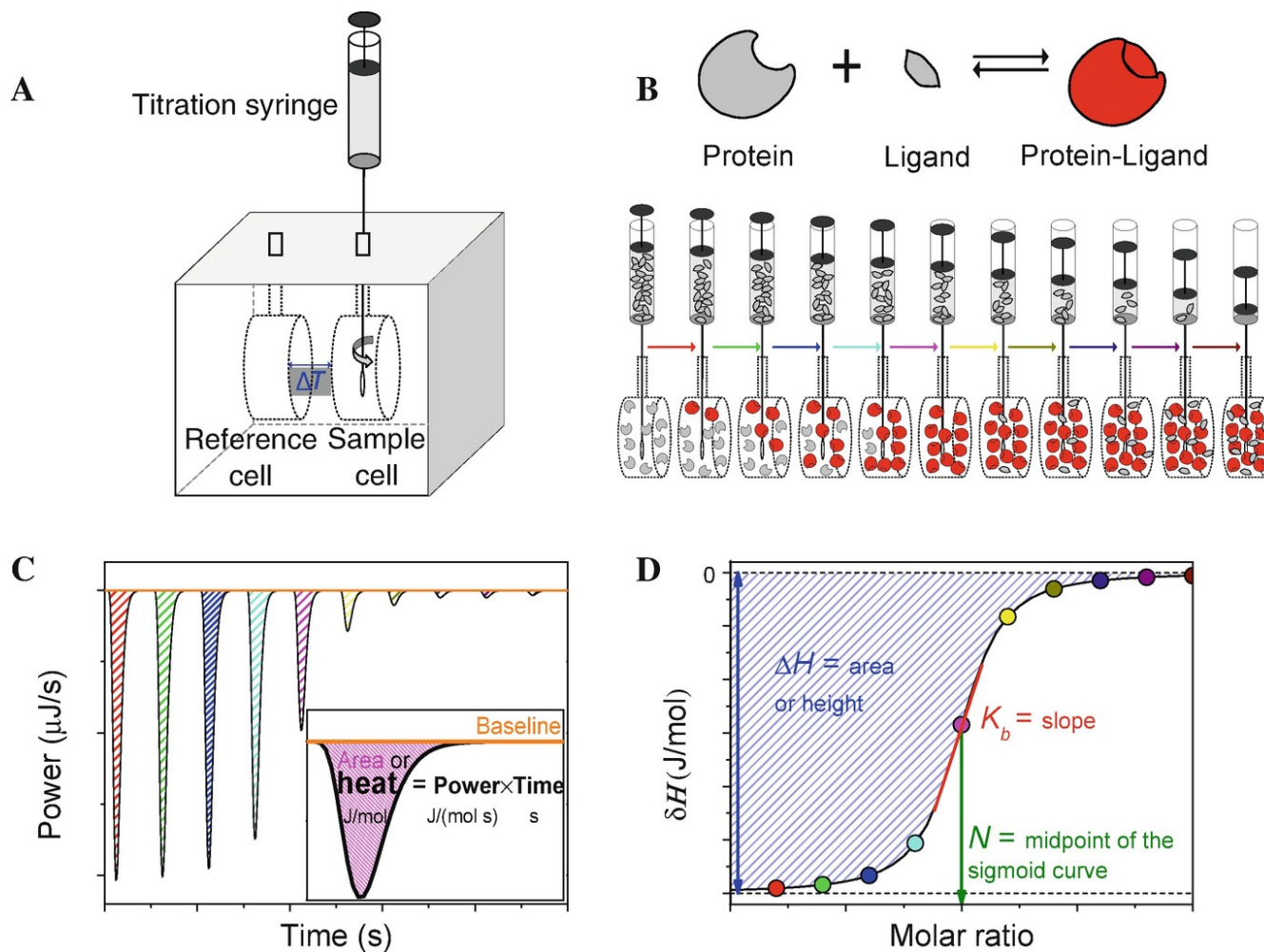
## ITC: setup

- Ligand
- Biomolecule
- Ligand - Biomolecule complex
- 🔥 Heat

**A****C****B****D**

# Studio dell'interazione proteina/proteina e proteina/ligando :

## ITC: setup



## ISOTERMA DI LEGAME

# Studio dell'interazione proteina/proteina e proteina/ligando :

## ITC: strumentazione

MicroCal VP-ITC



MicroCal iTC<sub>200</sub>



MicroCal PEAQ™ ITC



MicroCal Auto-iTC<sub>200</sub>



MicroCal PEAQ ITC Automated



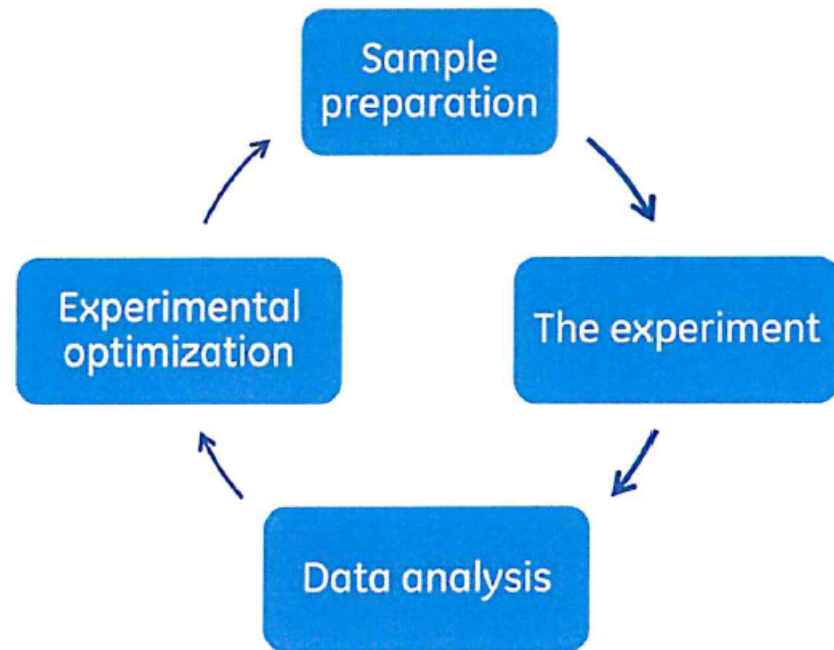
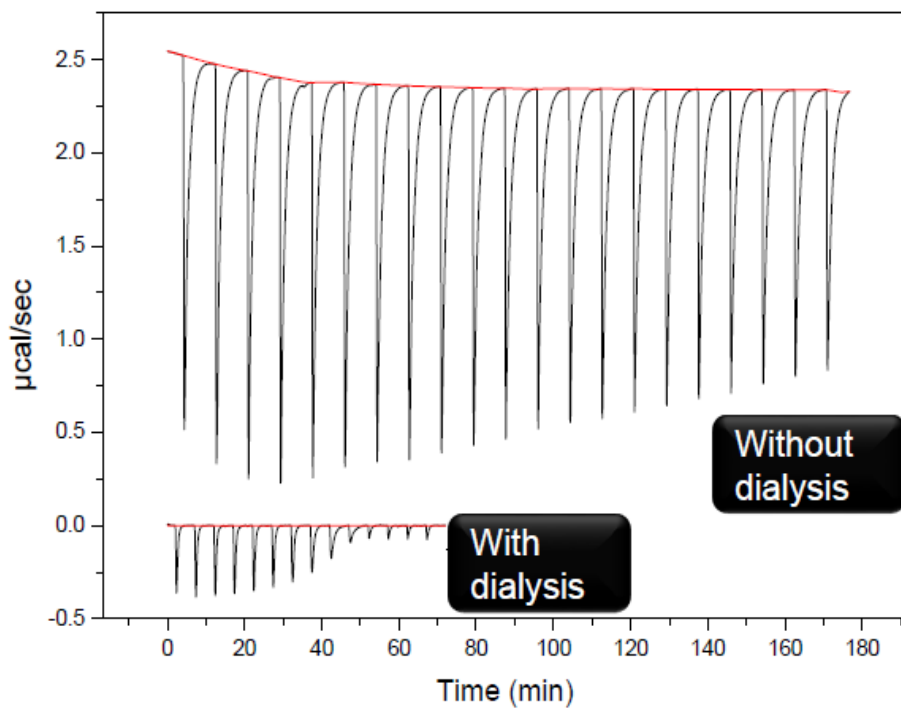


# Studio dell'interazione proteina/proteina e proteina/ligando :

## ITC: condizioni sperimentali

### preparazione del campione

1. I tamponi della cella e della siringa devono essere identici



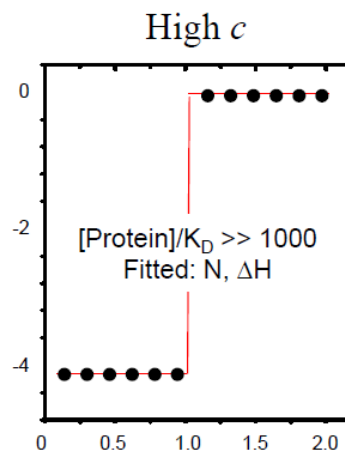
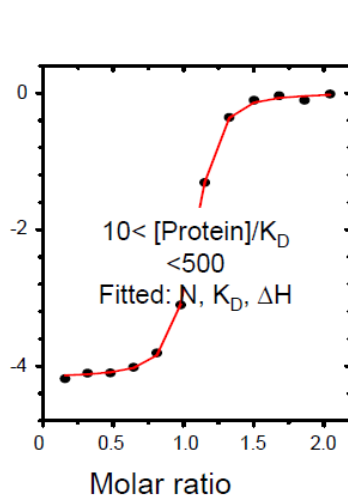
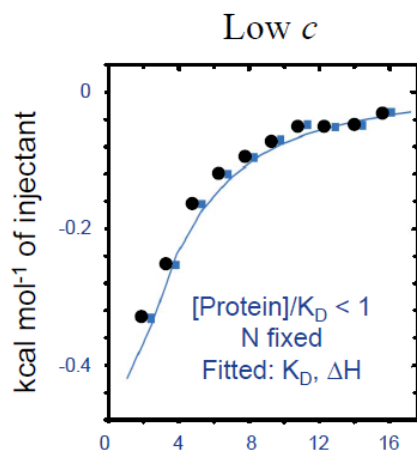
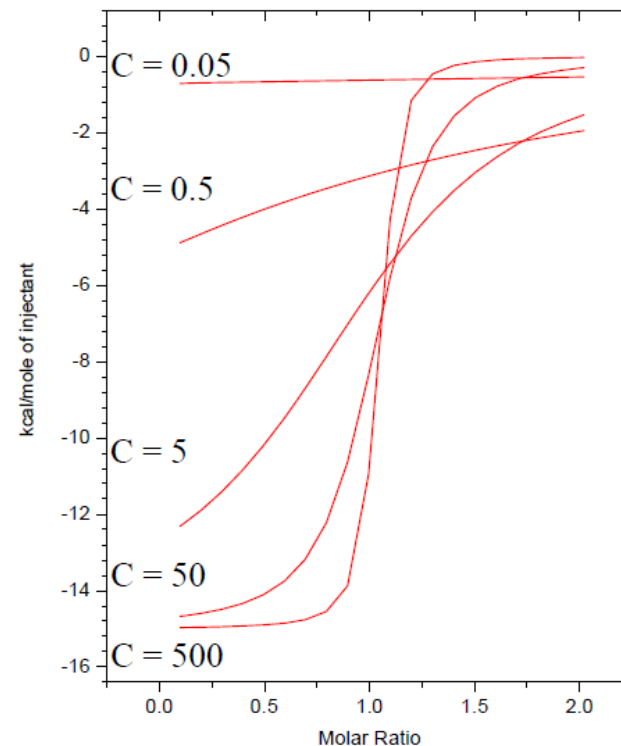
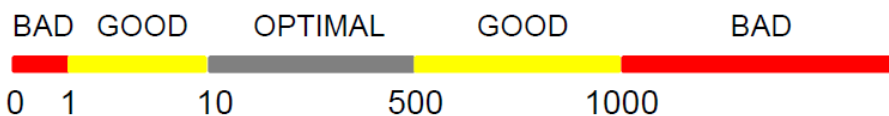
2. Misura della []

# Studio dell'interazione proteina/proteina e proteina/ligando :

## ITC: condizioni sperimentali

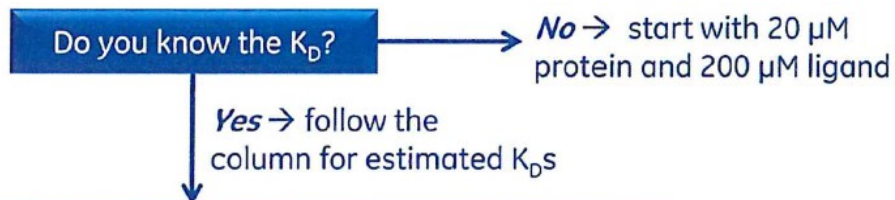
[P] nel  $\mu\text{M}$

$$C = [P]/K_D$$



# Studio dell'interazione proteina/proteina e proteina/ligando :

## ITC: condizioni sperimentali

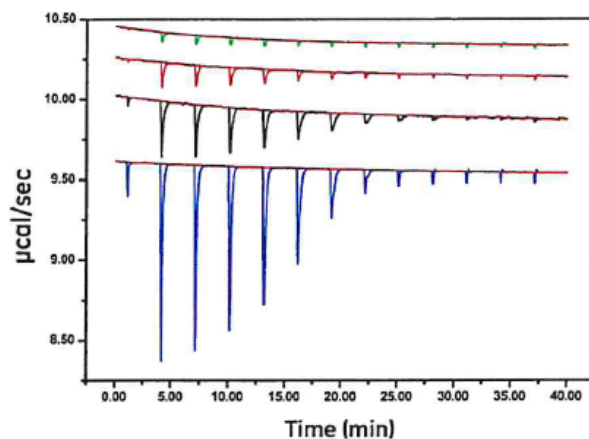


Estimated $K_D$ $\mu\text{M}$	[Protein] $\mu\text{M}$	[Ligand] $\mu\text{M}$	[Protein]/ $K_D = C$
<0.5	10	100	>20
0.5-2	20	200	10-40
2-10	50	500	5-25
10-100	30	40* $K_D$	0.3-3
>100	30	20* $K_D$	<0.3

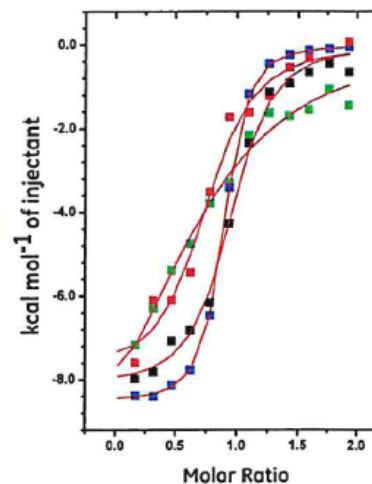
[Furosemide] = 10 \* [BCA II]

[BCA II], C

$K_D \sim 500 \text{ nM}$


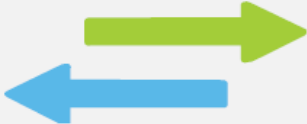
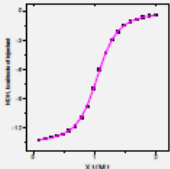



5  $\mu\text{M}$ , C = 10  
 10  $\mu\text{M}$ , C = 20  
 20  $\mu\text{M}$ , C = 40  
 50  $\mu\text{M}$ , C = 100



# Studio dell'interazione proteina/proteina e proteina/ligando :

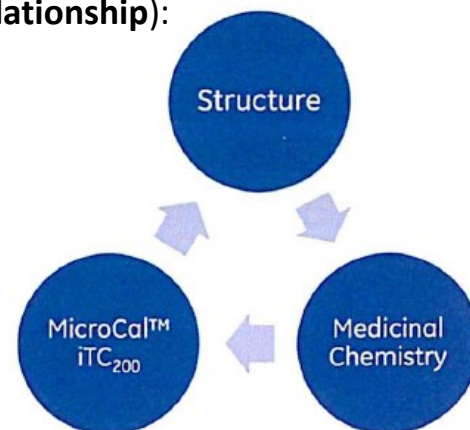
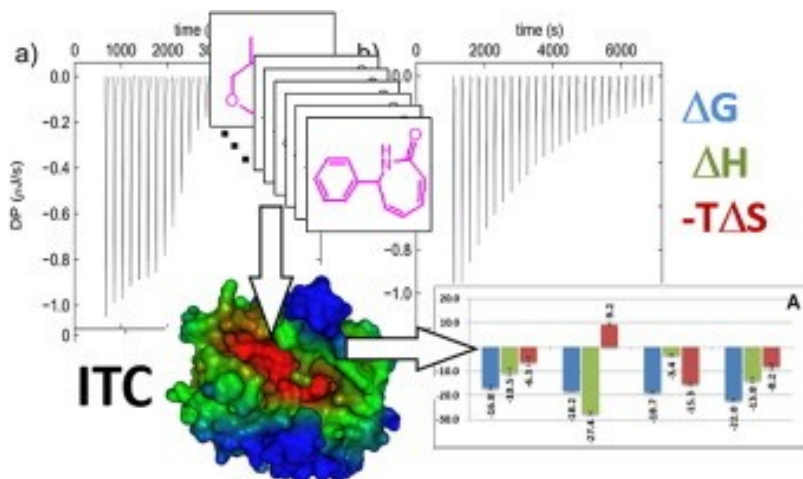
## ITC: vantaggi

Label-free	Broad dynamic range	Information rich	Ease-of-use
<ul style="list-style-type: none"><li>• Direct measurement of heat change (ITC)</li><li>• Direct measurement of melting transition temperature to predict thermal stability (DSC)</li></ul> 	<ul style="list-style-type: none"><li>• Native molecules in solution (biological relevance)</li><li>• Very sensitive to accomodate range of affinities</li></ul> 	<ul style="list-style-type: none"><li>• All binding parameters (affinity, stoichiometry, enthalpy and entropy) in a single ITC experiment</li></ul> 	<ul style="list-style-type: none"><li>• No labeling or immobilization necessary</li><li>• No assay development</li><li>• Wide range of solvent/buffer conditions</li></ul> 

# Studio dell'interazione proteina/proteina e proteina/ligando :

## ITC & Drug Design

- termodinamica per guidare l'ottimizzazione del farmaco (**SAR: Structure–activity relationship**):



Due approcci: ottimizzare

$\Delta H$

$\Delta S$

Ma

perdita di specificità  
impossibilità di ottenere  $K_A$  alte  
polimorfismi associati a drug-resisten

ITC guida nella scelta del tipo di modifiche  
determina variazioni nel tipo di legame  
associate a polimorfismi

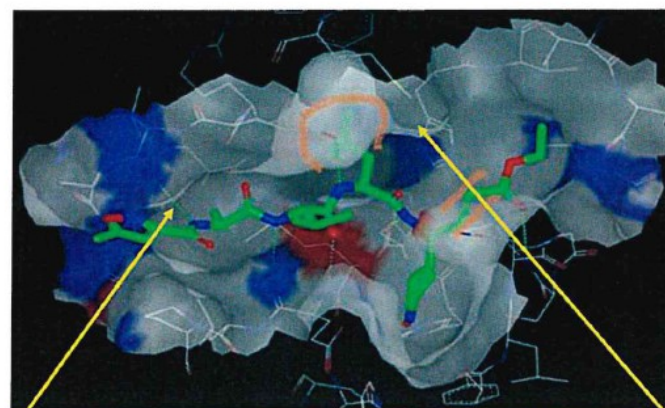


Image by permission of Professor Dr. Knut Baumann Institut fuer Pharmazetische Chemie, Technische Universitaet Braunschweig, Germany

$\Delta H$ - indicates H Bonding and van der Waals interactions

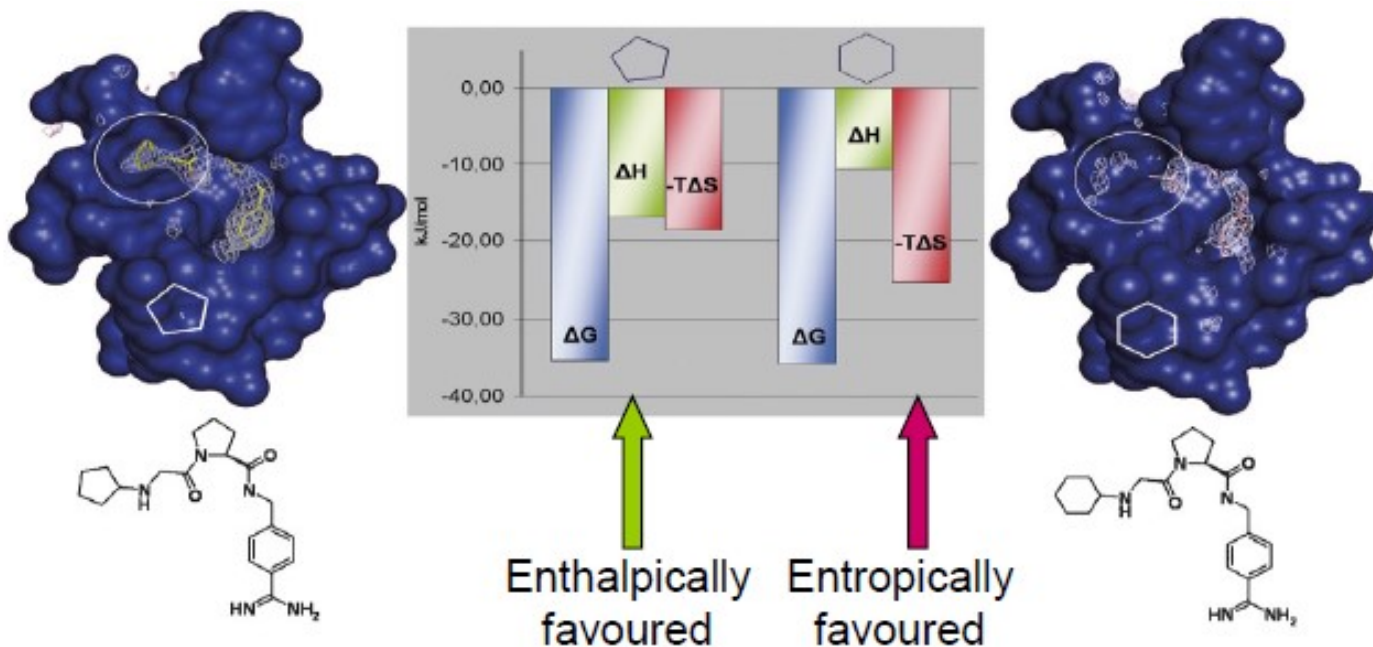
$T\Delta S$ - indicates hydrophobic interactions and conformational

# Studio dell'interazione proteina/proteina e proteina/ligando :

## ITC:

Applicazioni nella scoperta e lo sviluppo di nuovi farmaci:

Inibitori della trombina:



Strutture cristalline di due inibitori della trombina strettamente correlati con un gruppo ciclopentile o cicloesile



Drug Discovery Today

Volume 11, Issues 13–14, July 2006, Pages 580–594



Review

Foundation

Virtual ligand screening: strategies, perspectives and limitations

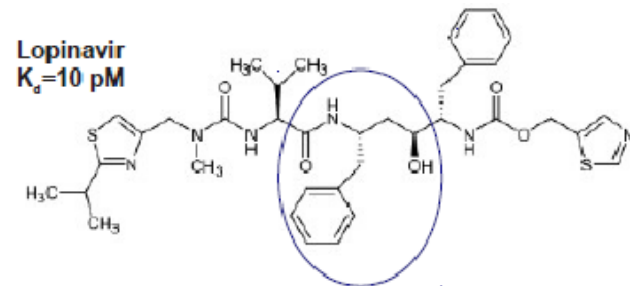
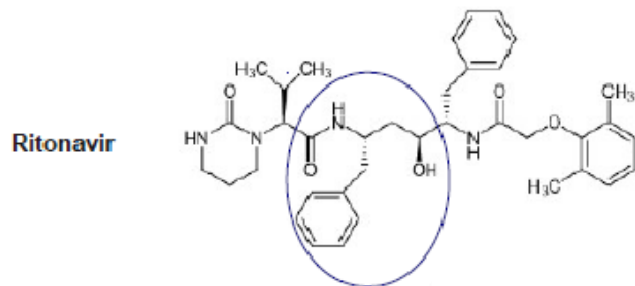
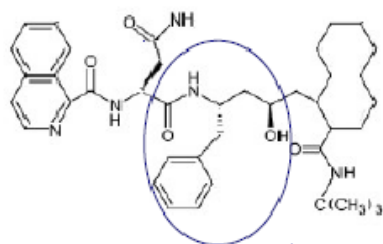
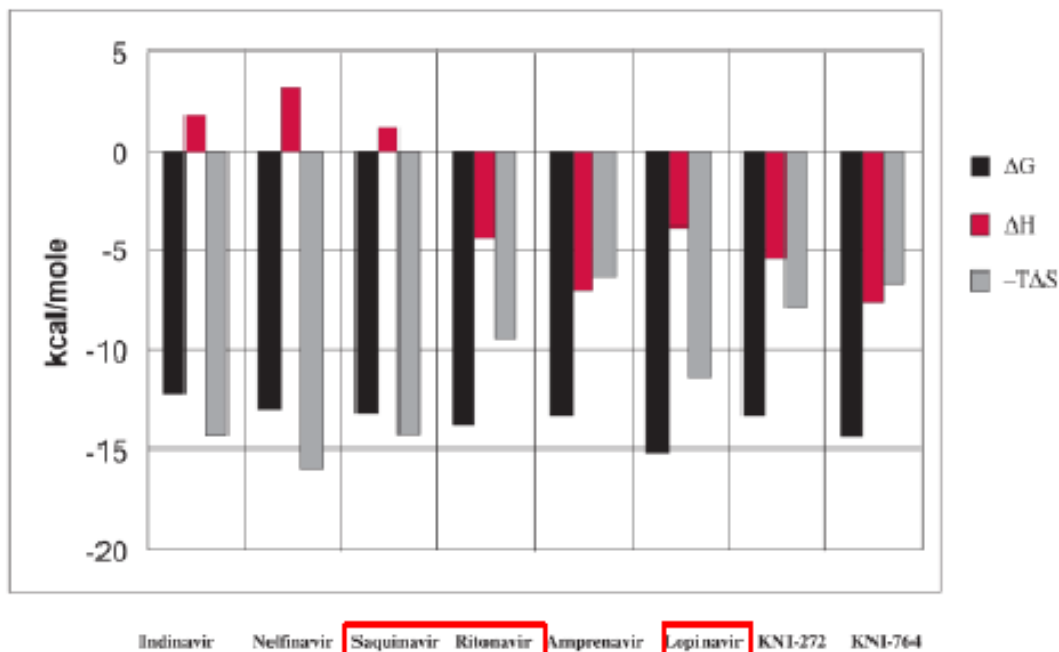
Gerhard Klebe

# Studio dell'interazione proteina/proteina e proteina/ligando :

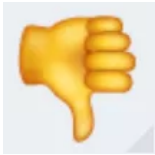
## ITC:

Applicazioni nella scoperta e lo sviluppo di nuovi farmaci:

Inibitori della proteasi di HIV-1

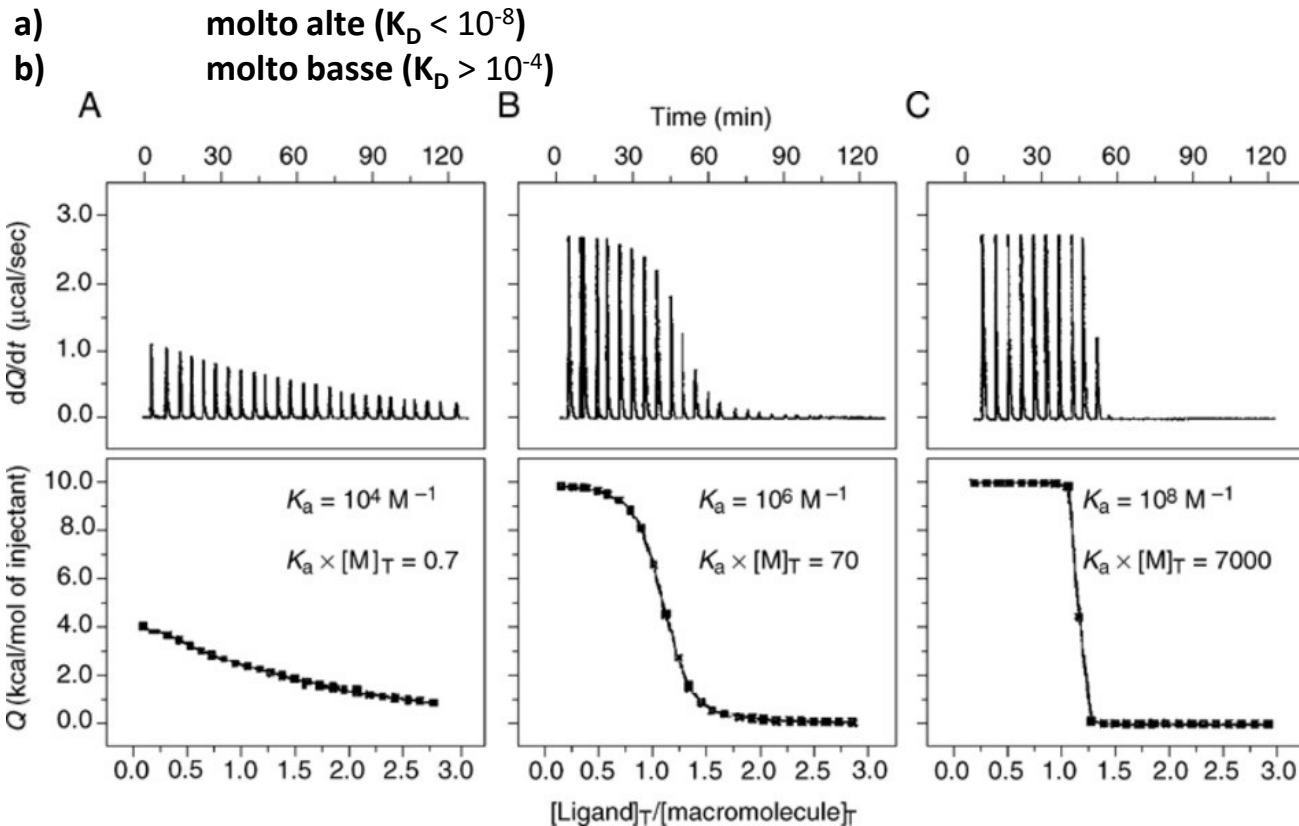


# Studio dell'interazione proteina/proteina e proteina/ligando :



## ITC:

- Misura il calore globale
- solo per PPI/PLI con calore misurabile
- Elevato consumo di campione
- Non può misurare direttamente **affinità**





# Studio dell'interazione proteina/proteina e proteina/ligando :

ITC:

Metodi di DISPACEMENT:

Prerequisito: **competitore noto:**

$$K_{app} = K_A / (1 + K_{comp} [comp])$$

a) DEBOLE (W)  
Misurabili  $K_D$  fino a  $10^{-12}$

b) FORTE (S)  
Misurabili  $K_D$  fino a  $10^{-3}$

