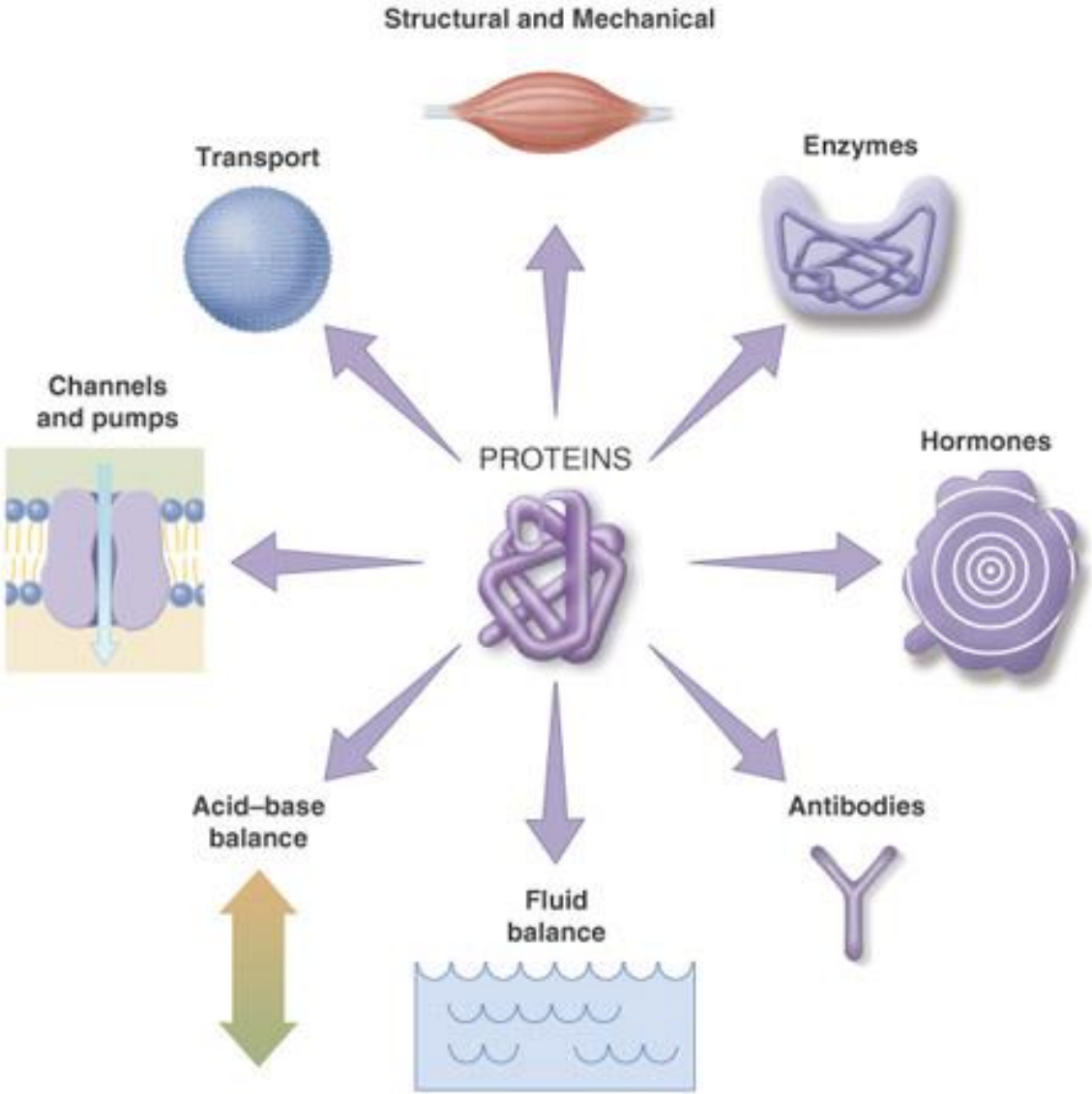
Proteine ricombinanti per studi strutturali.
studio delle interazioni intermolecolari

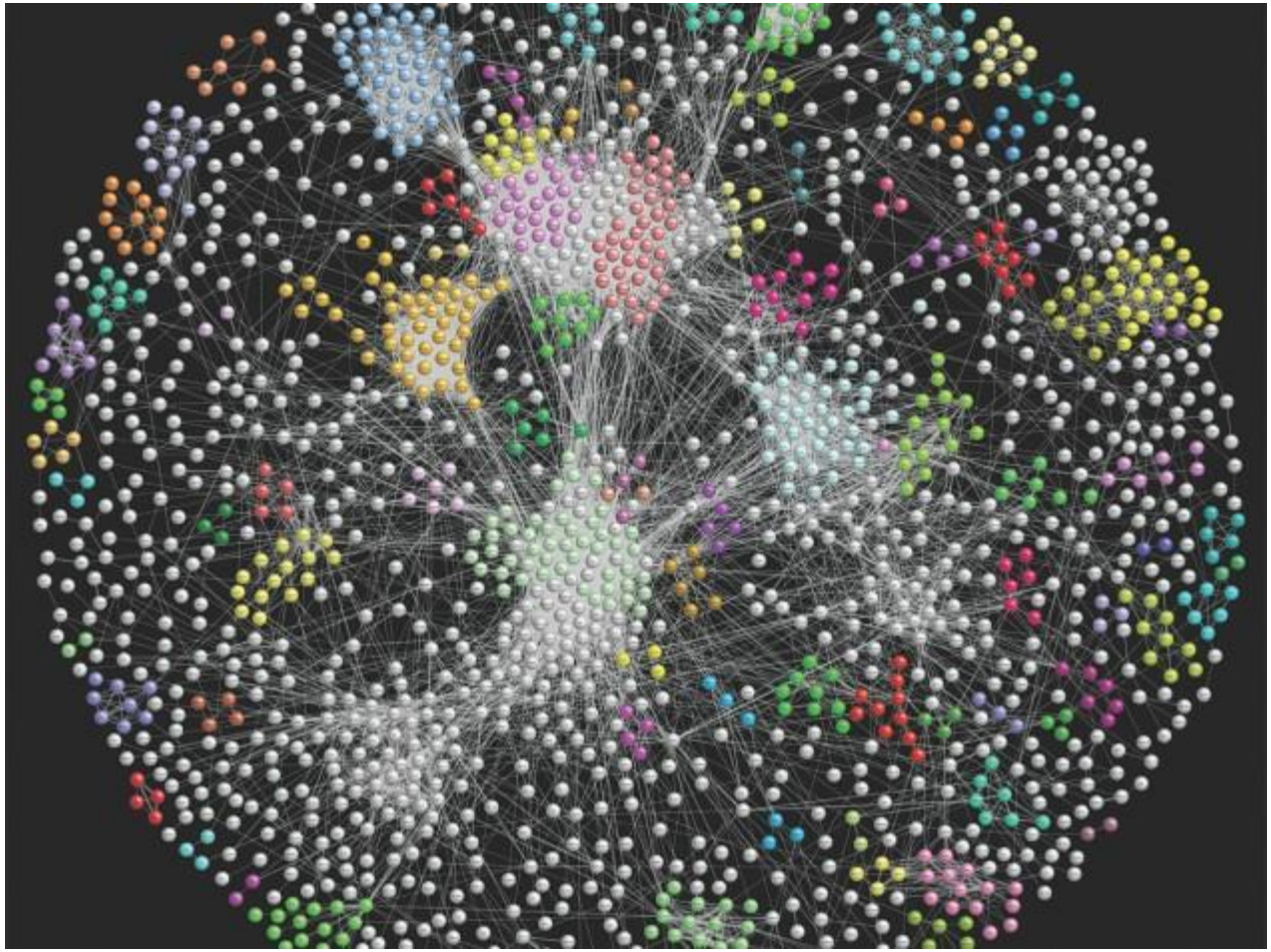
Studio dell'interazione proteina/proteina e proteina/ligando :

Interazione Proteina / Proteina (PPI) e Proteina / Ligando (PLI)



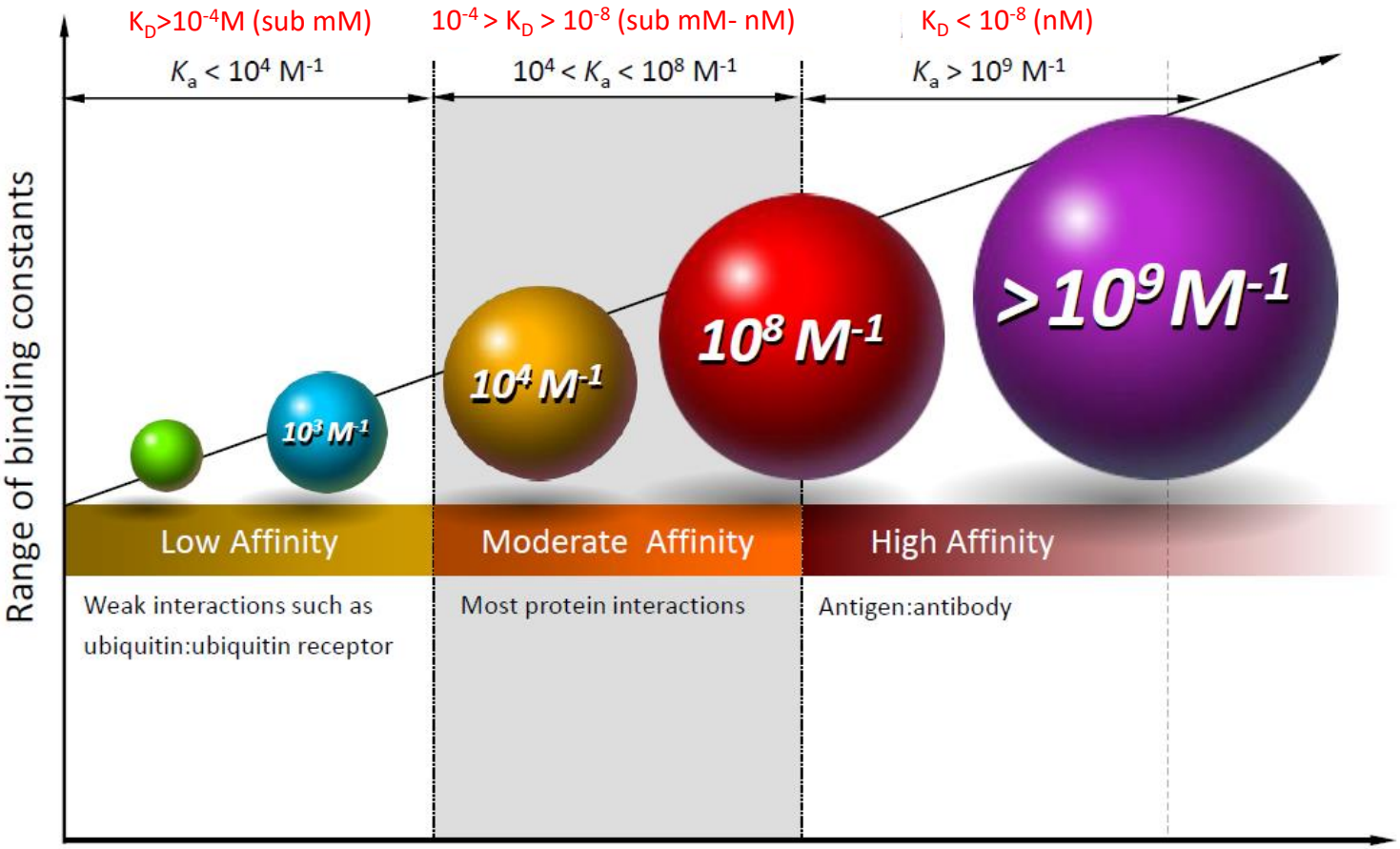
Studio dell’interazione proteina/proteina e proteina/ligando :

PPI network



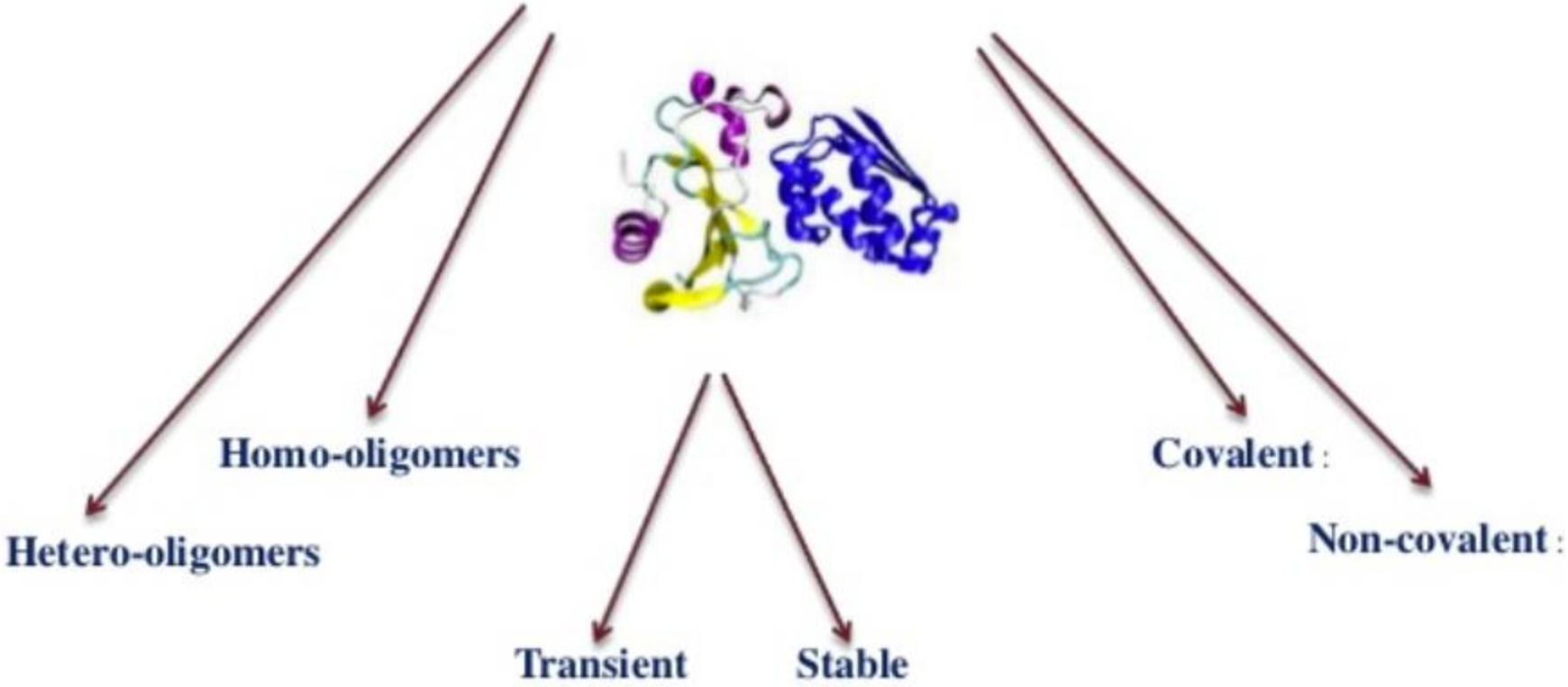
Studio dell'interazione proteina/proteina e proteina/ligando :

PPI affinity ranges



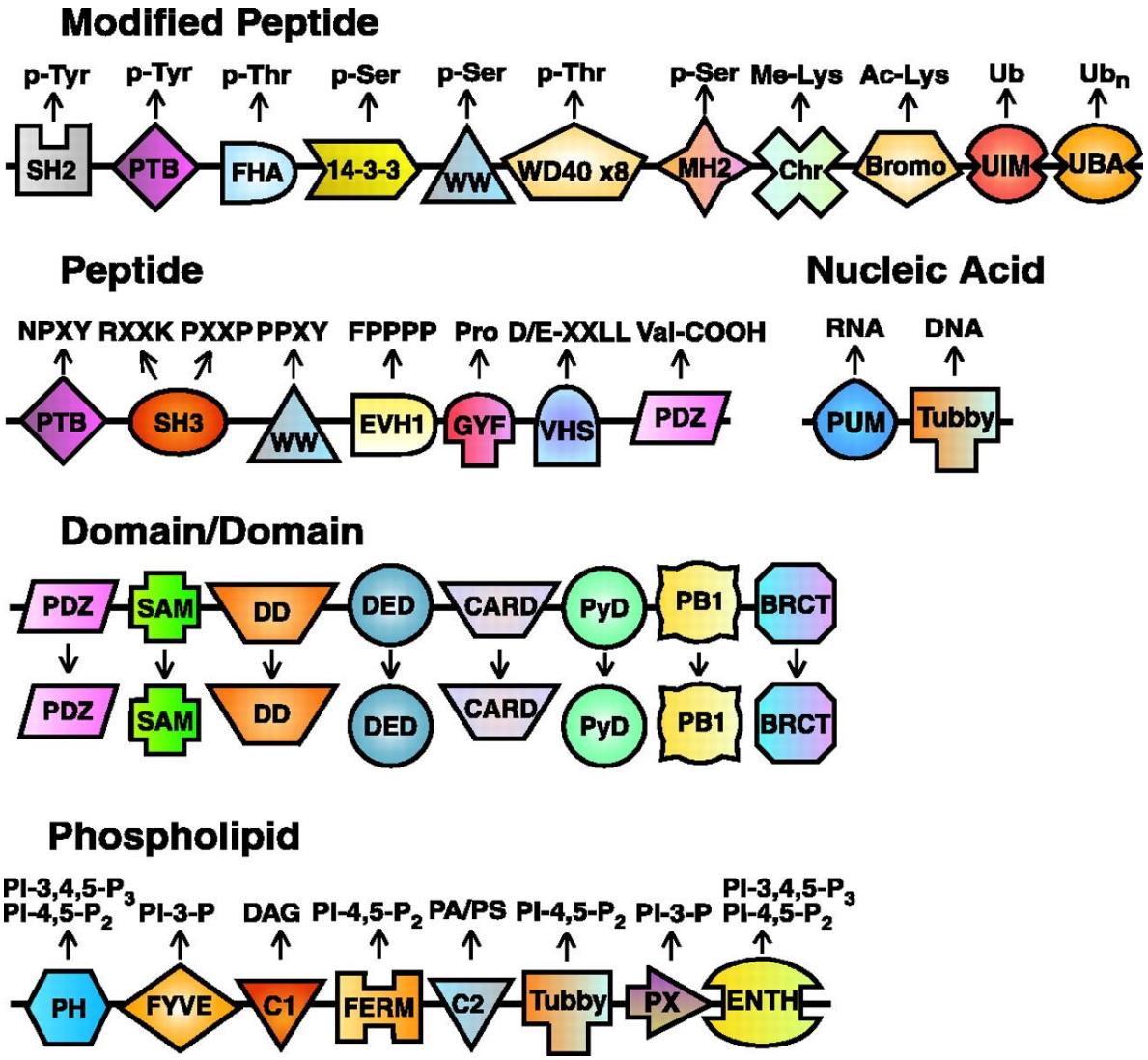
Studio dell’interazione proteina/proteina e proteina/ligando :

PPI tipi



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

PPI : basi



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, lactamase, galactosidase)
- FRET/BRET/BiFc

Experimental (*In vitro*)

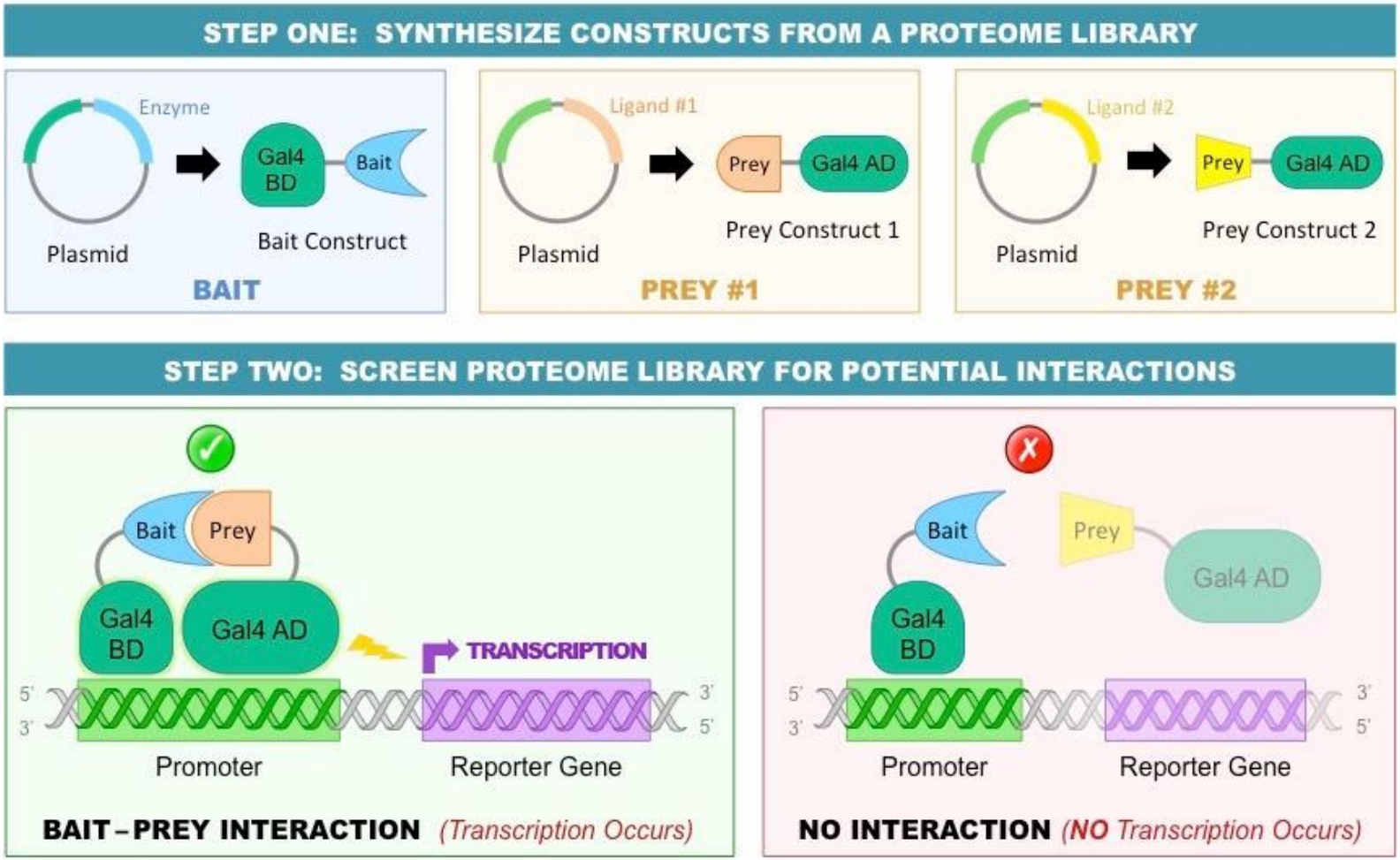
- Co-IP
- Pull-down
- SPR
- Phage Display
- HD-exchange MS/Protein Painting

Computational (*In silico*)

- BIND Biomolecular Interaction Network Database
- DIP Database of Interacting Proteins
- MINT Molecular INTeraction Database
- IntAct Molecular Interaction Database

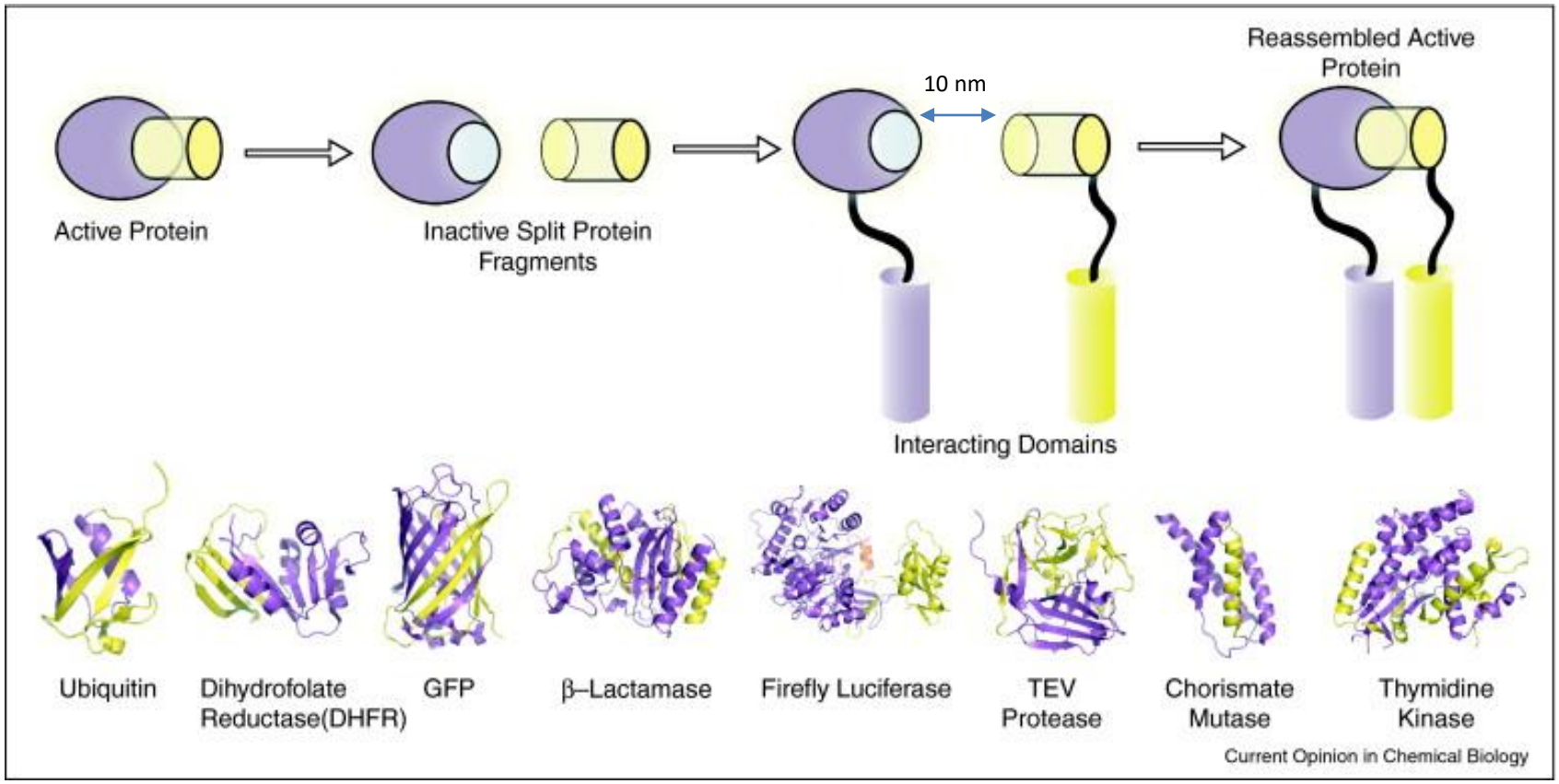
Studio dell'interazione proteina/proteina e proteina/ligando :

PPI : Two Hybrid system



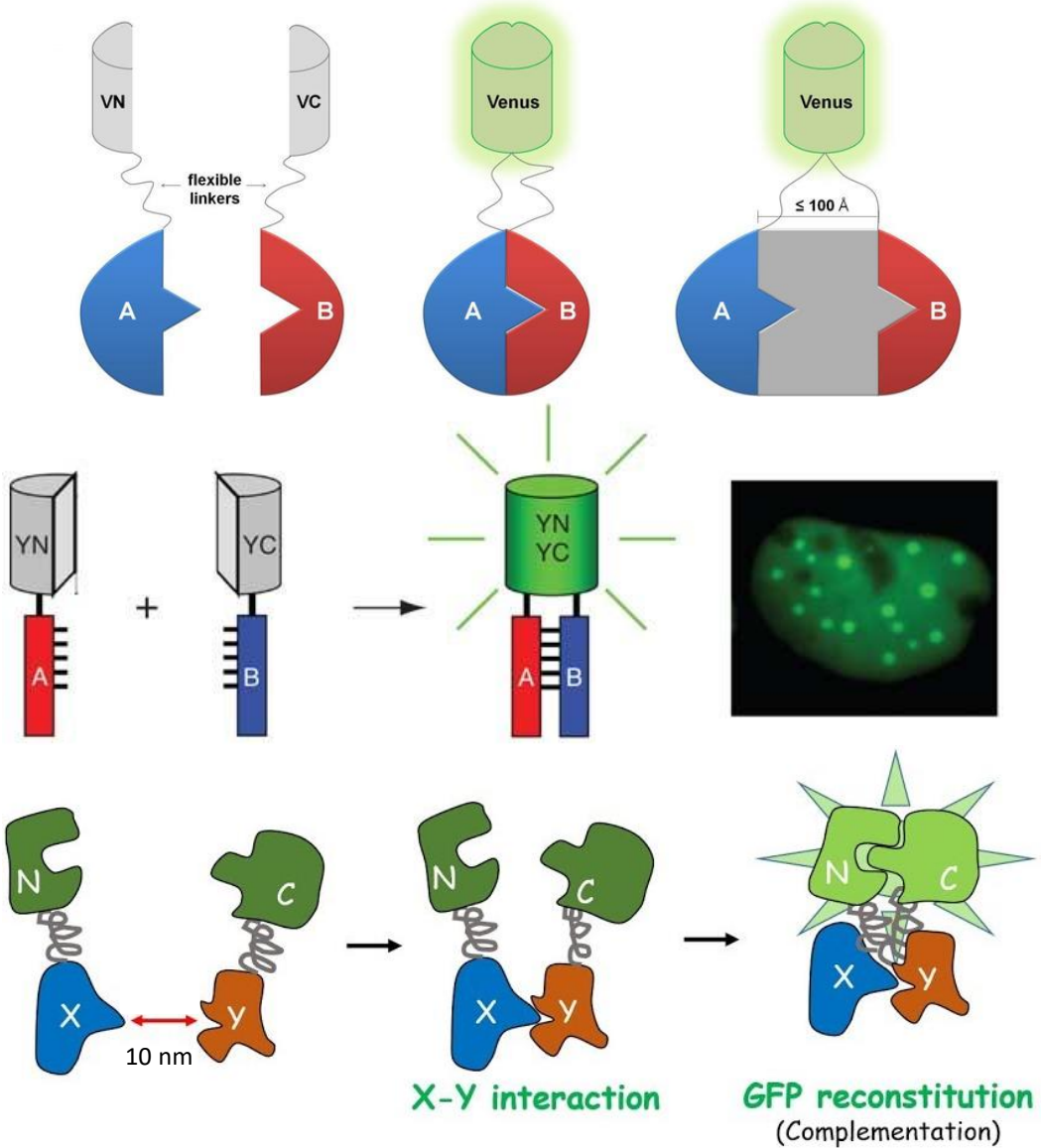
Studio dell'interazione proteina/proteina e proteina/ligando :

PPI : Protein-fragment complementation assay



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

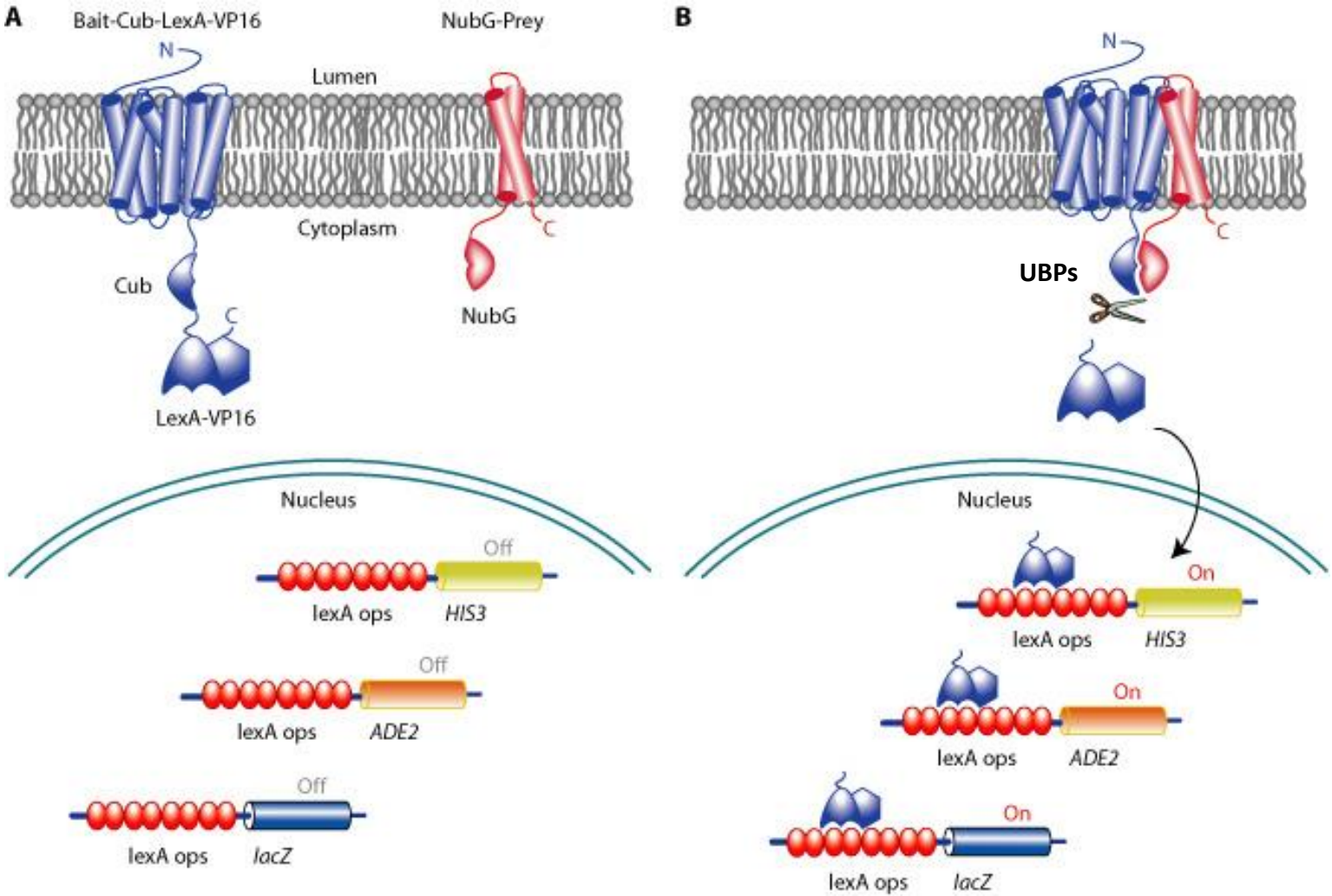
PPI: BiFc (Bimolecular Fluorescence Complementation)



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

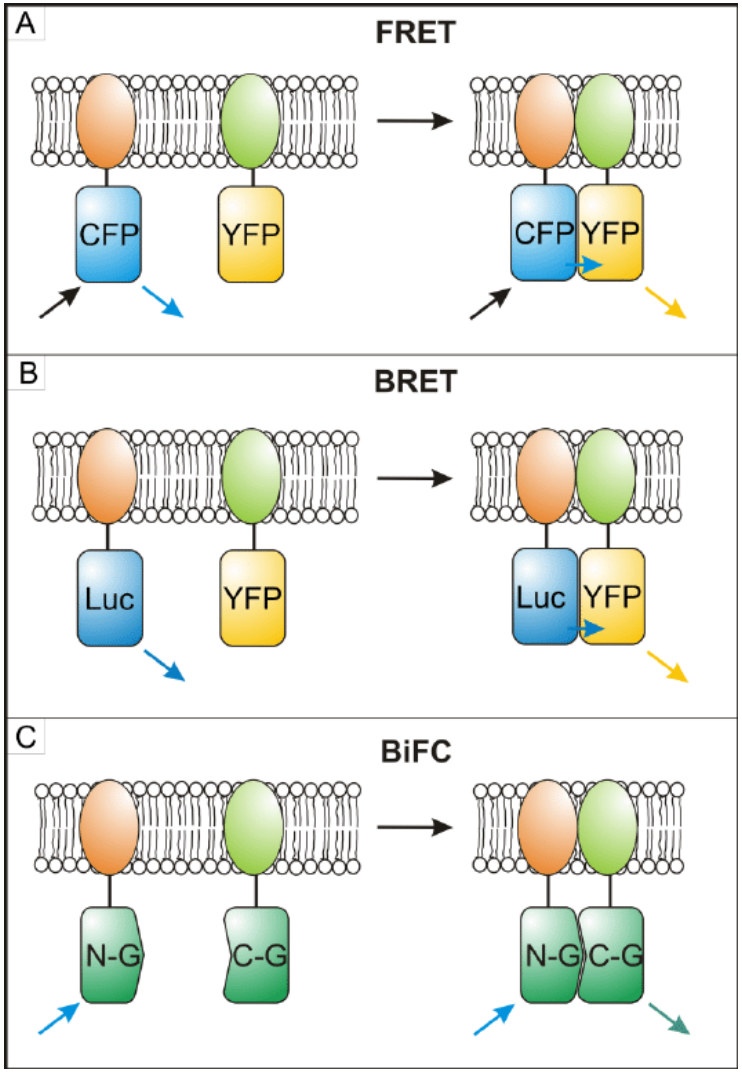
PPI : Two Hybrid system

Per complessi binari a livello di membrana



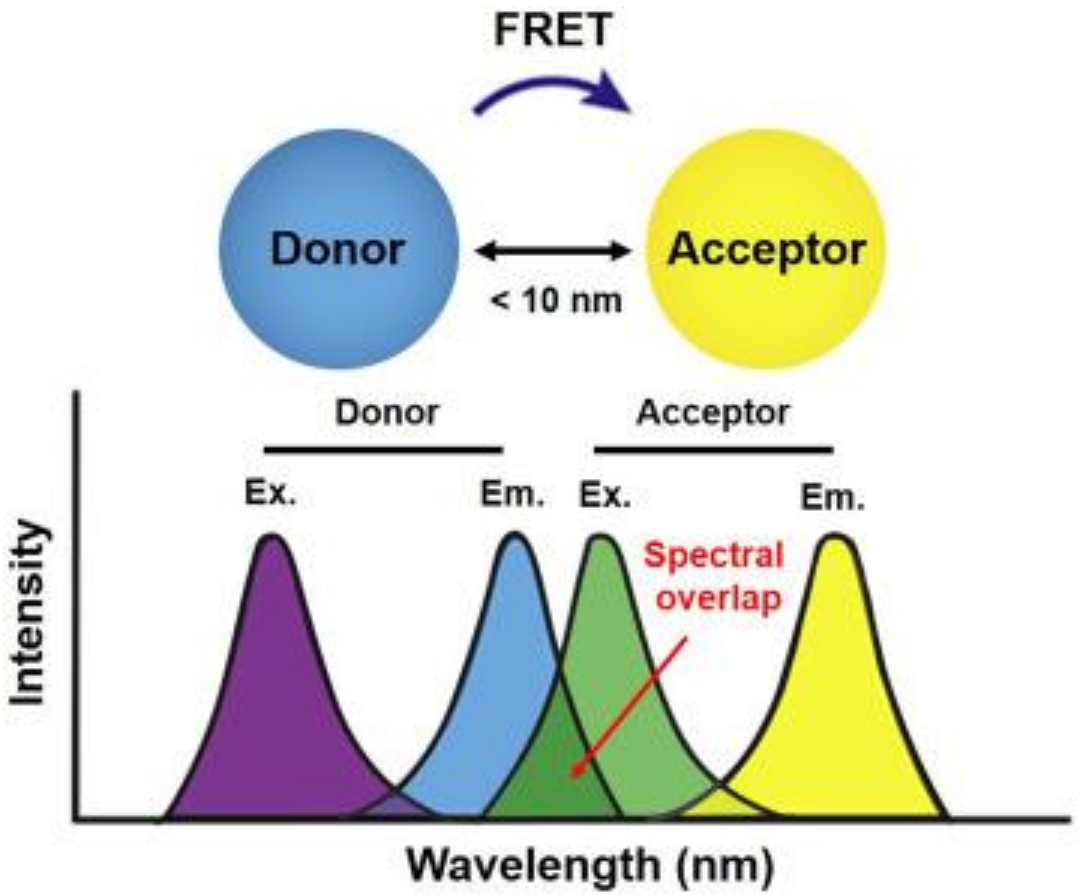
Studio dell'interazione proteina/proteina e proteina/ligando :

PPI : Fluorescence based methods



Studio dell’interazione proteina/proteina e proteina/ligando :

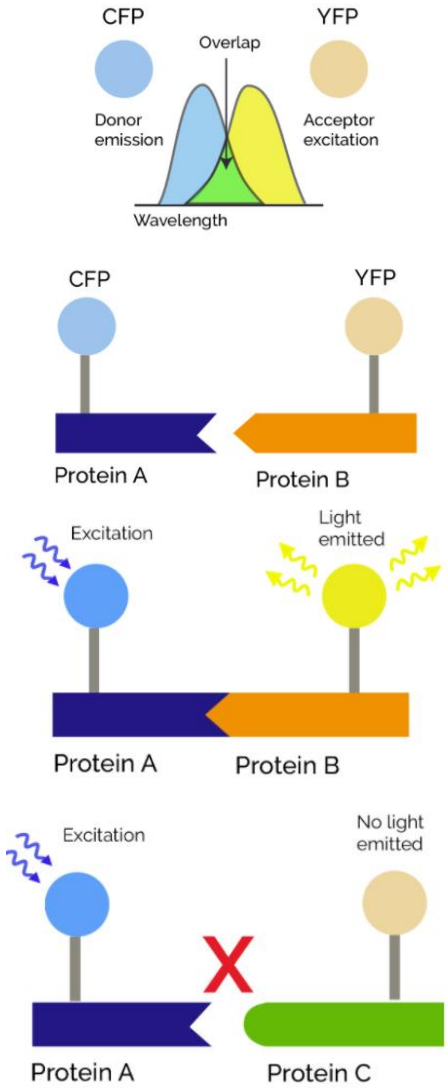
PPI: FRET (Förster Resonance Energy Transfer)



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:

Espresse come proteine di fusione con proteine fluorescenti

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

Coniugate chimicamente

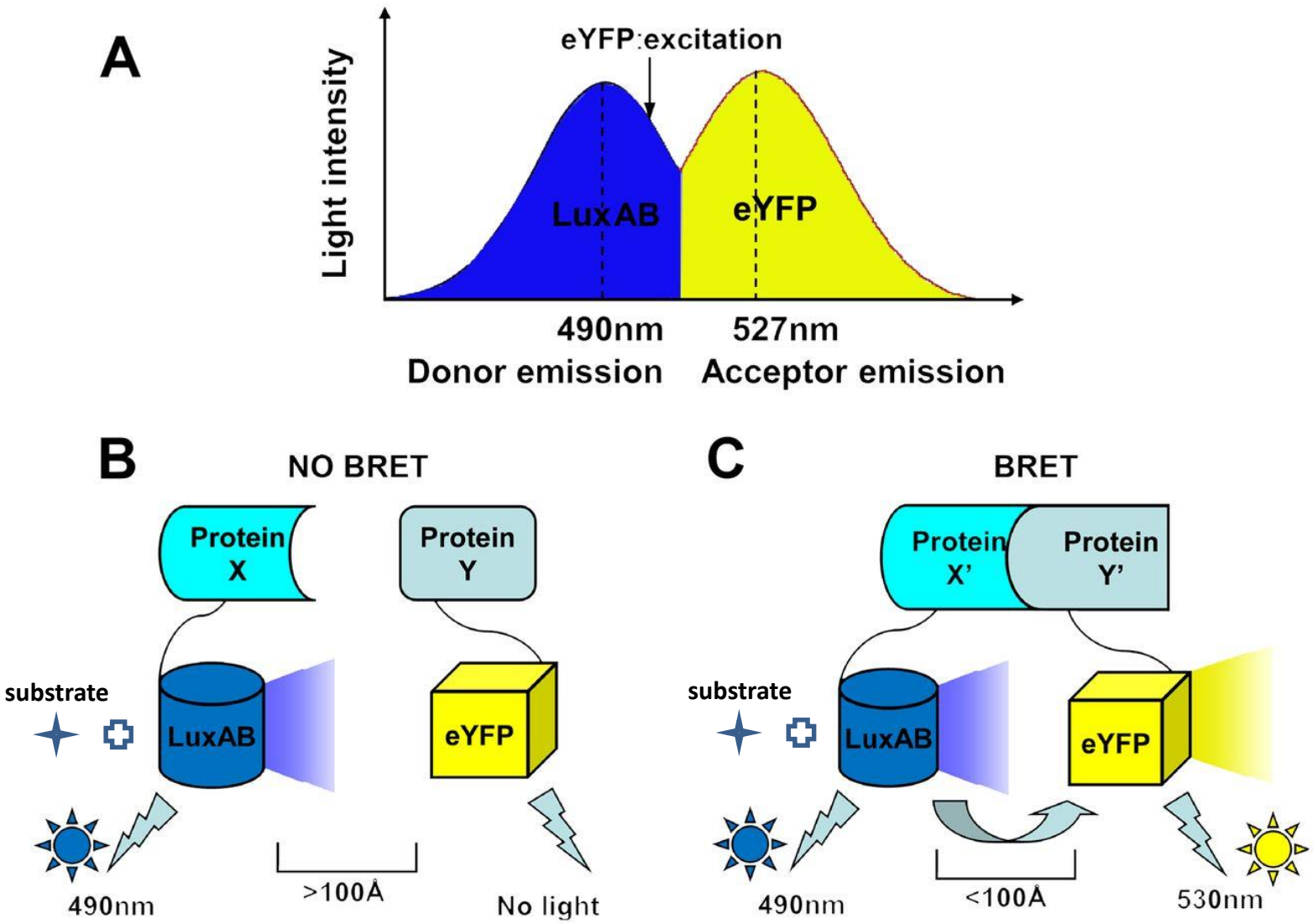
- Cy3-Cy5
- Alexa488-Alexa555
- Alexa488-Cy3
- Alexa594-Alexa647
- FITC-TRITC
- Terbium (III)-Fluorescein
- DiSBAC4(3)-CC2-DMPE (voltage sensitive)

In vivo: nell'ambiente fisiologico (batteri, funghi e cellule di mammifero)

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

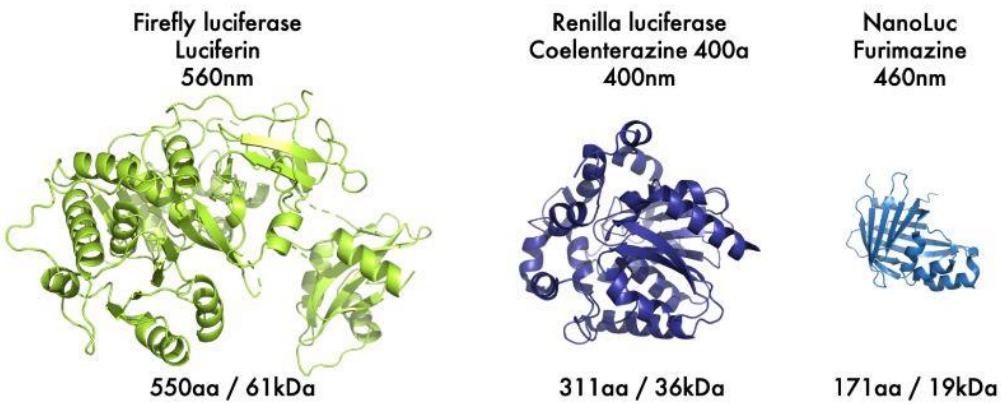
Studio dell'interazione proteina/proteina e proteina/ligando :

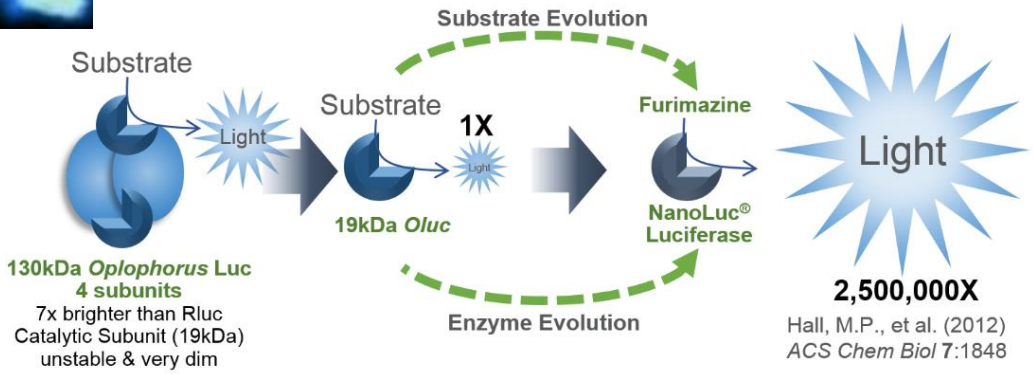
PPI: BRET (Bioluminescence Resonance Energy Transfer)



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

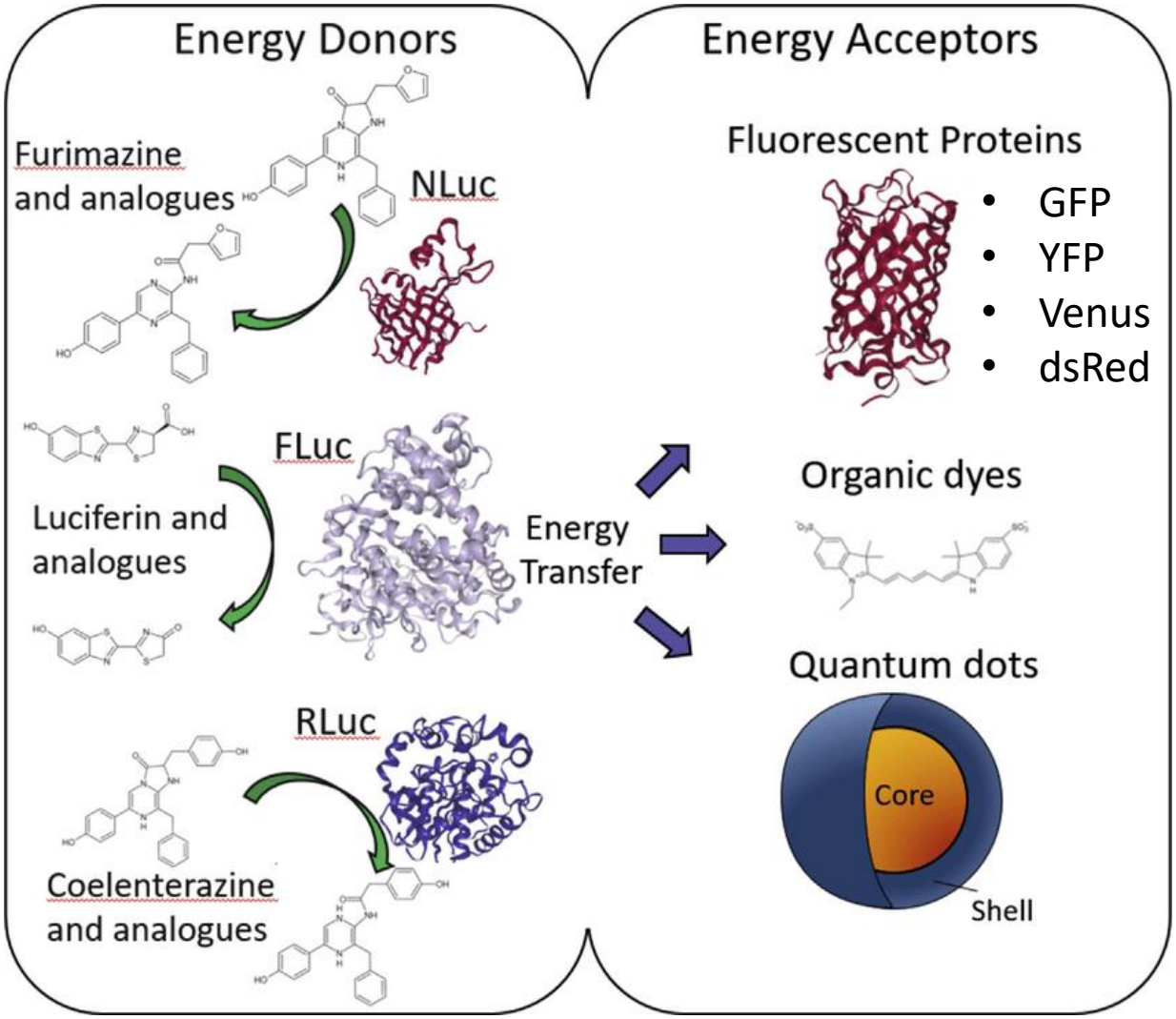
PPI: BRET (Bioluminescence Resonance Energy Transfer)





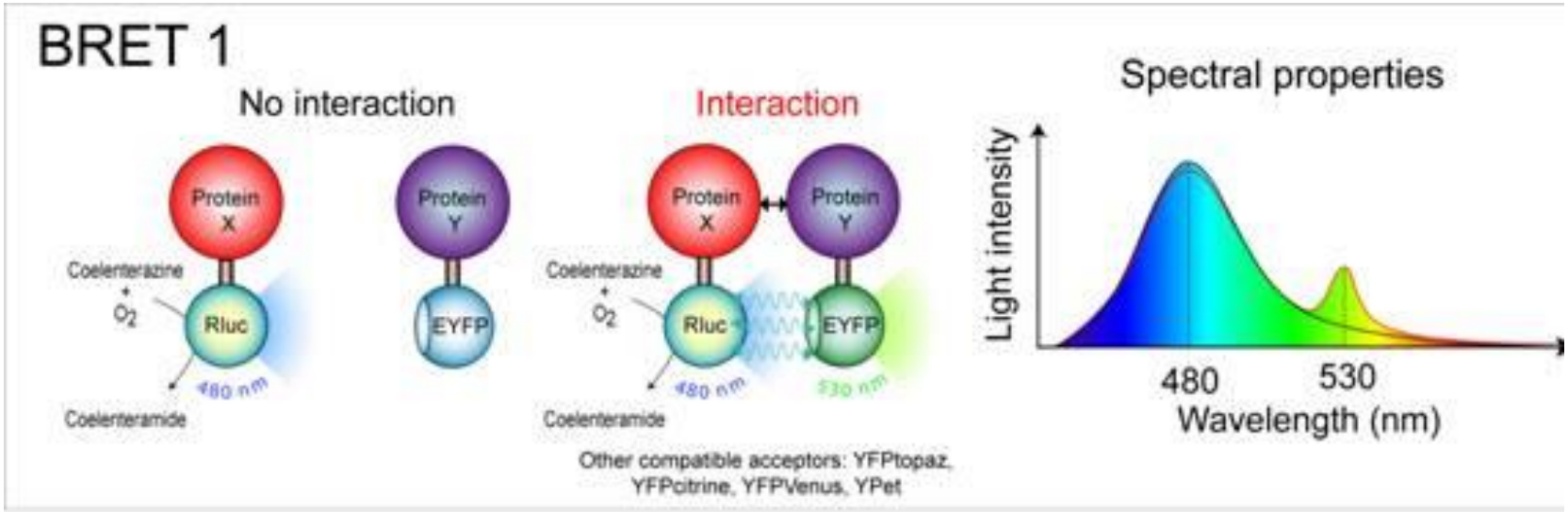
Studio dell'interazione proteina/proteina e proteina/ligando :

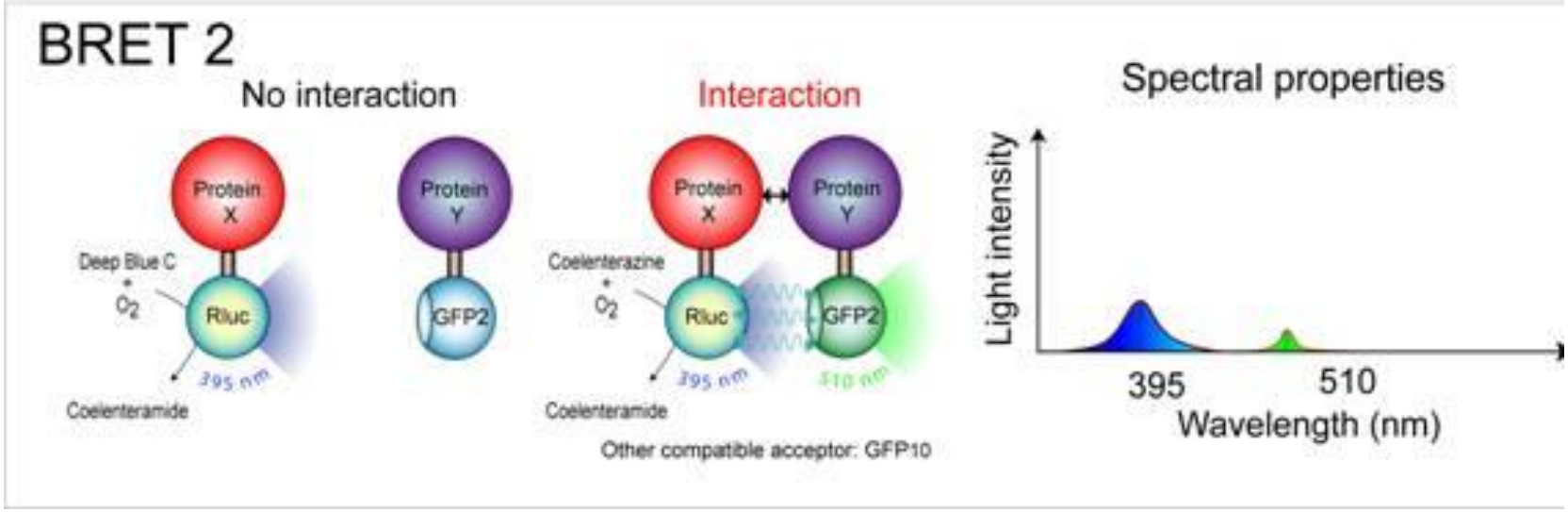
PPI: BRET (Bioluminescence Resonance Energy Transfer)



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

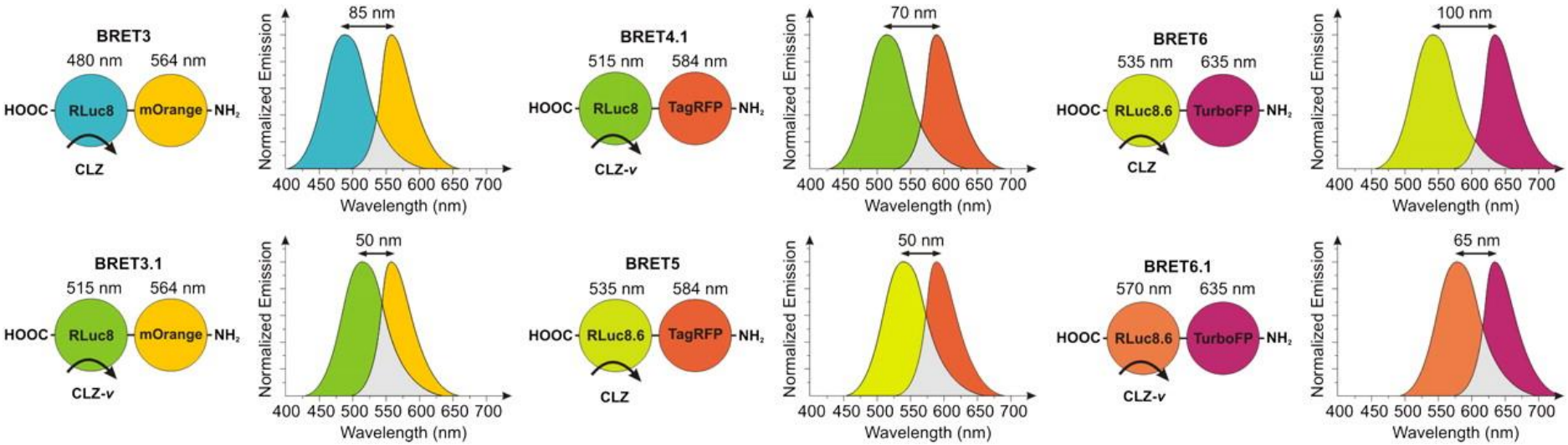
PPI: BRET (Bioluminescence Resonance Energy Transfer)



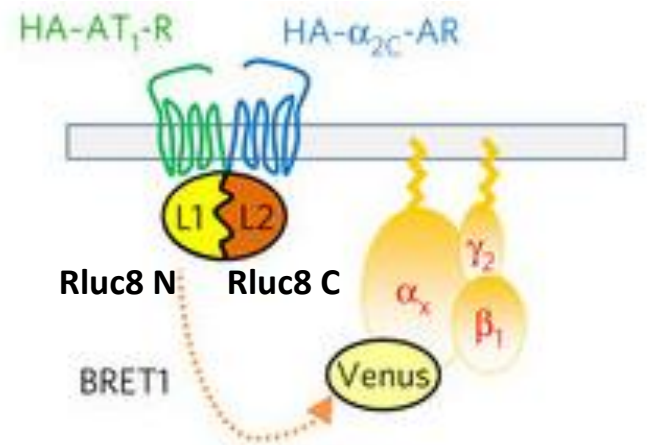


Studio dell'interazione proteina/proteina e proteina/ligando :

PPI: BRET (Bioluminescence Resonance Energy Transfer)

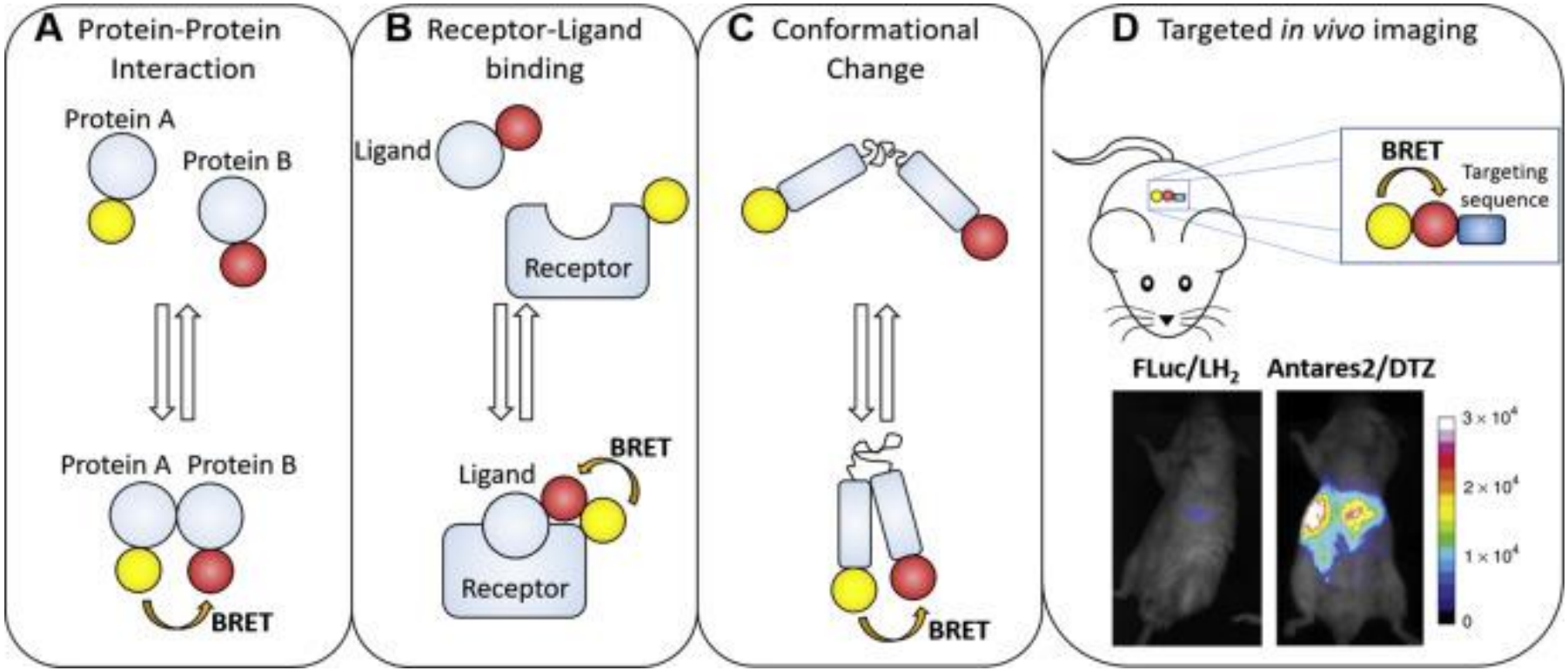


CODA-RET (BRET+PCA): complessi ternari



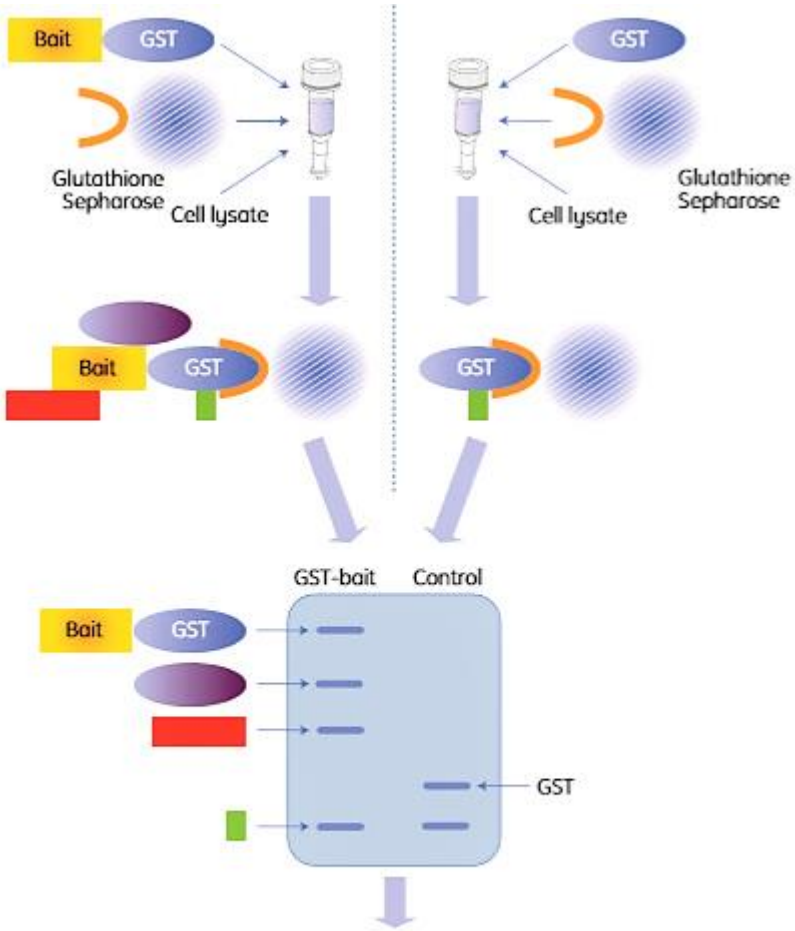
Studio dell'interazione proteina/proteina e proteina/ligando :

PPI: BRET (Bioluminescence Resonance Energy Transfer)

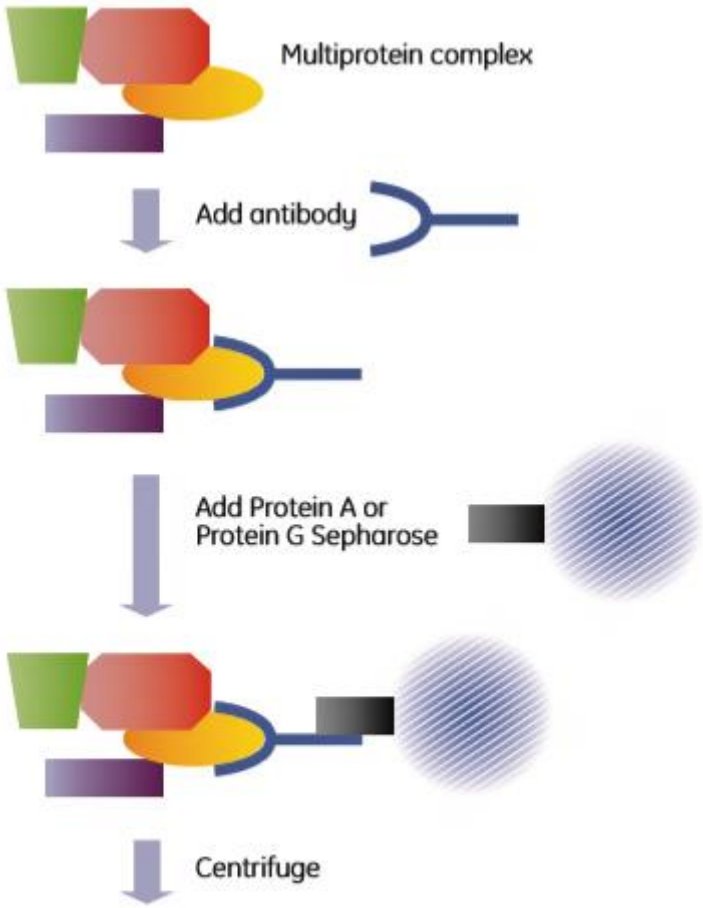


Studio dell'interazione proteina/proteina e proteina/ligando :

PPI : Pull-down e CoIP



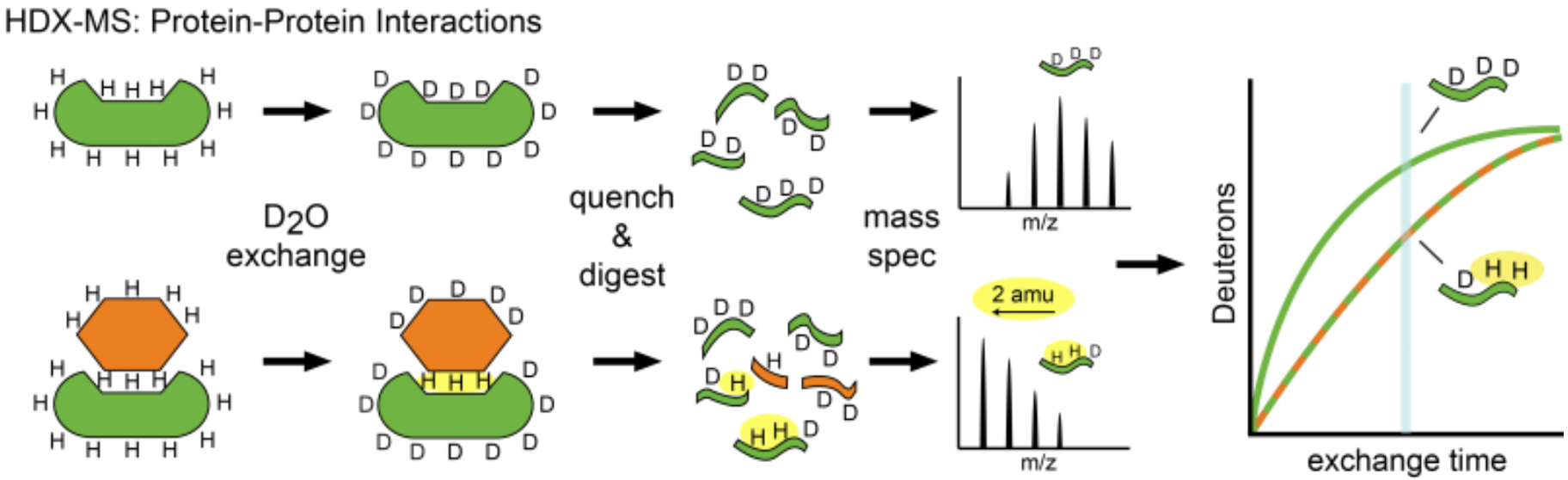
Excision of bands corresponding to purple and red proteins, followed by trypsin digestion, analysis by mass spectrometry, and protein identification through data searches.



Elution and separation with SDS-PAGE followed by trypsin digestion, analysis by mass spectrometry and protein identification through database searches.

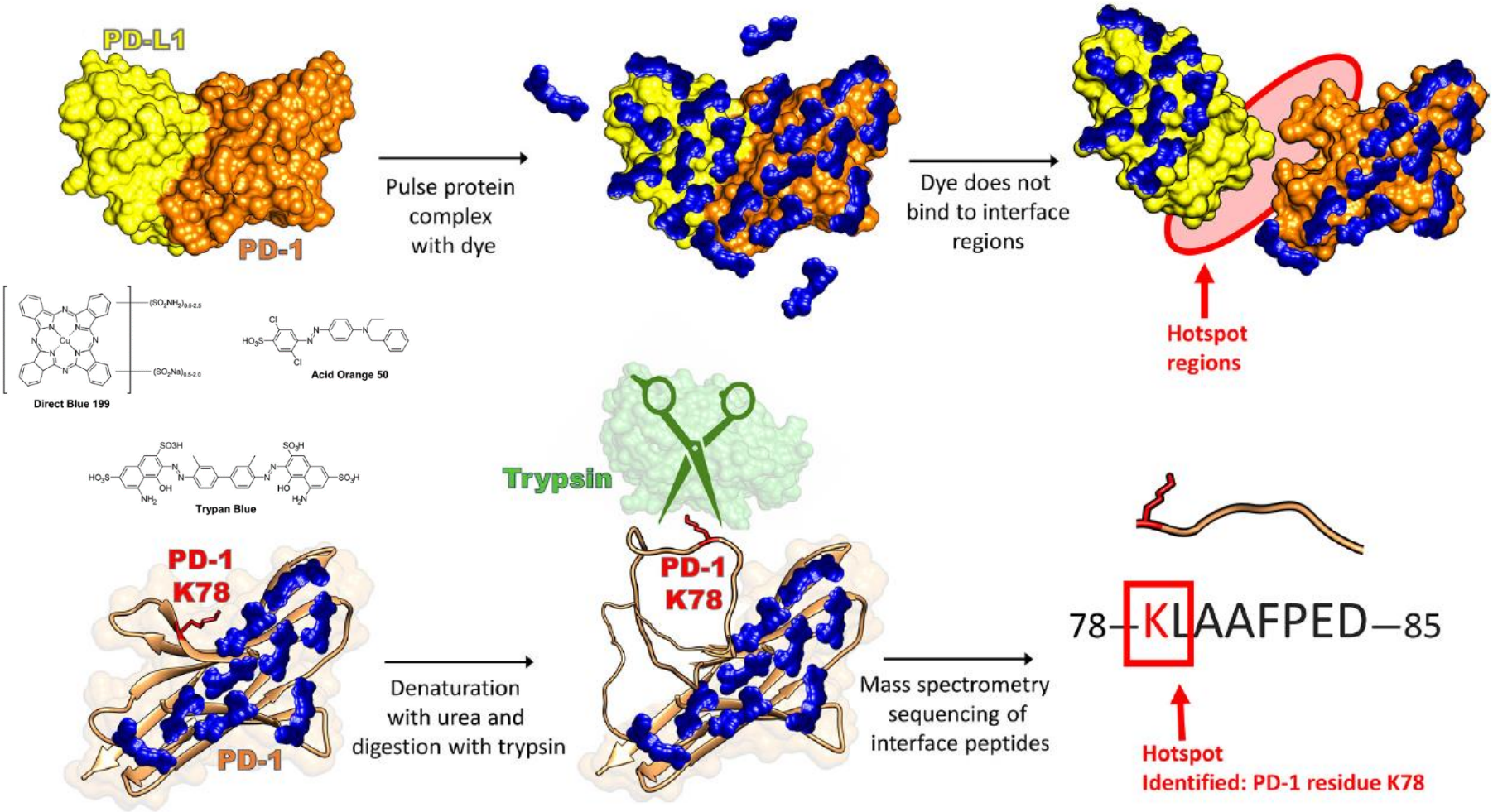
Studio dell'interazione proteina/proteina e proteina/ligando :

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



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

PPI&PLI: Protein Painting

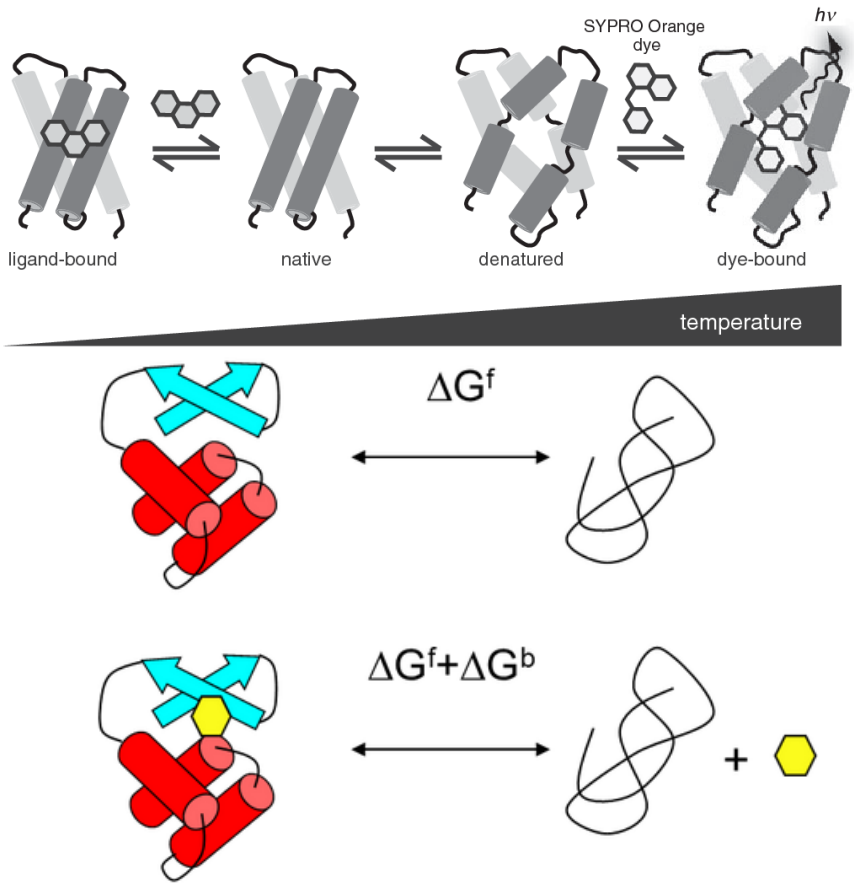


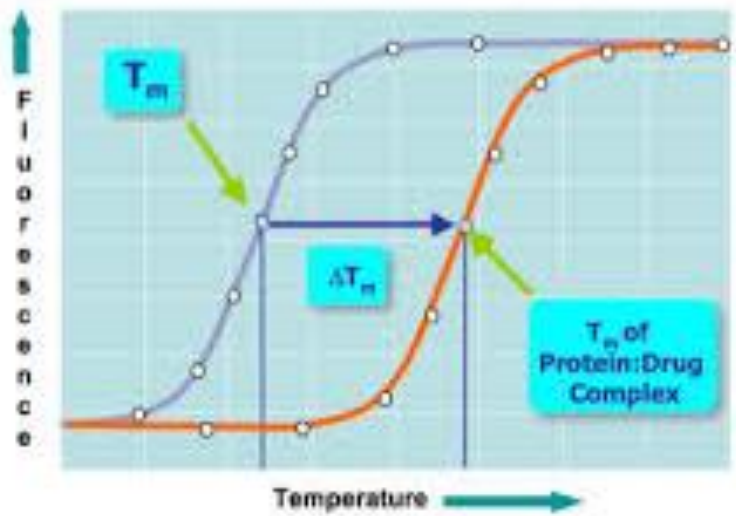
Interazioni intermolecolari:
metodi di caratterizzazione quantitativa

DSF

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

DSF: Applicazioni
1) PLI: Screening di ligandi



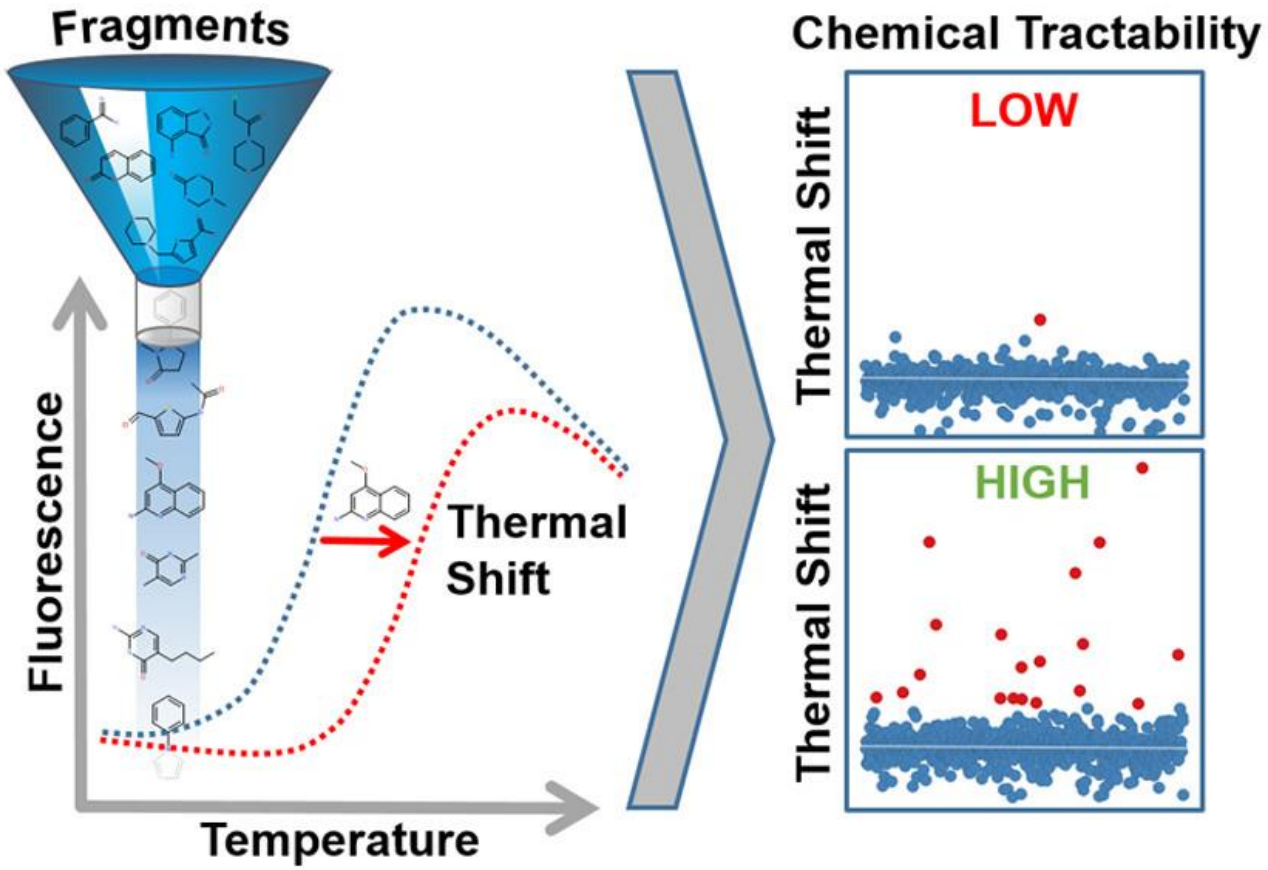


Correlazione stabilizzazione/affinità dell'interazione proteina/ligando: $IC_{50} < 1\mu M \rightarrow \Delta T_M > 4°C$

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

DSF: Applicazioni
2) PLI: HIT PROFILING

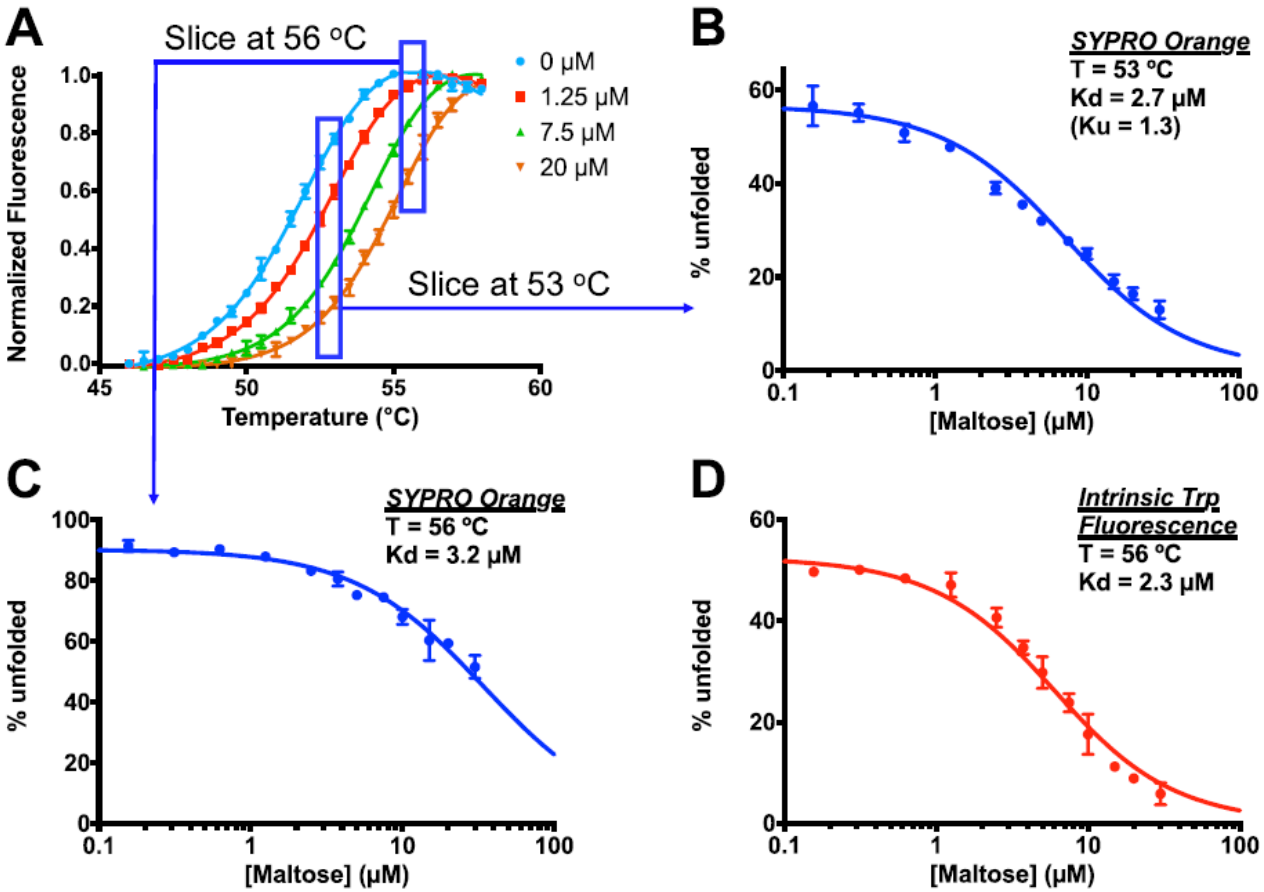
Confrontando i valori Tm
→ ranking della forza di interazione dei ligandi/stima dell' energia di legame



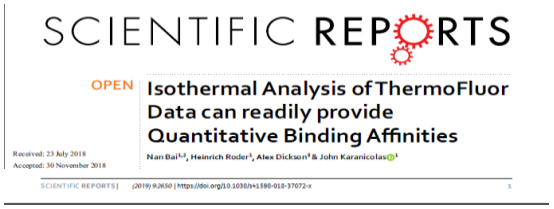
Studio dell'interazione proteina/proteina e proteina/ligando :

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

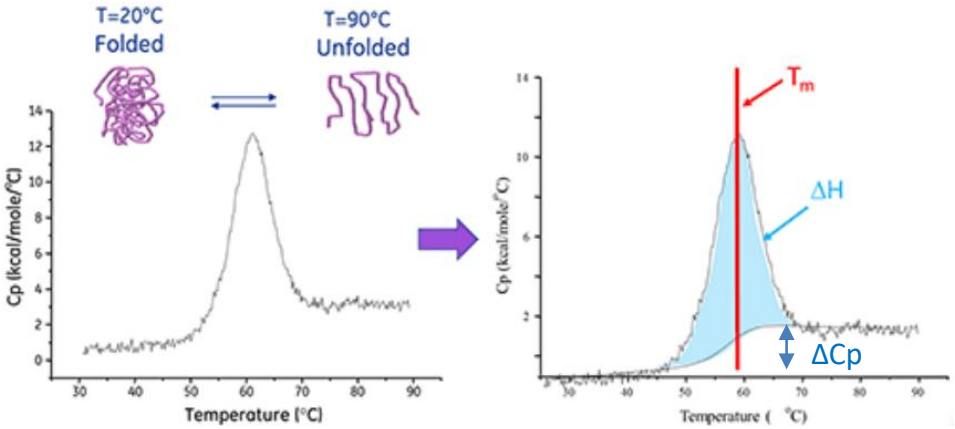
Nan Bai[1,2], Heinrich Roder[1], Alex Dickson[3] & John Karanicolas[1]

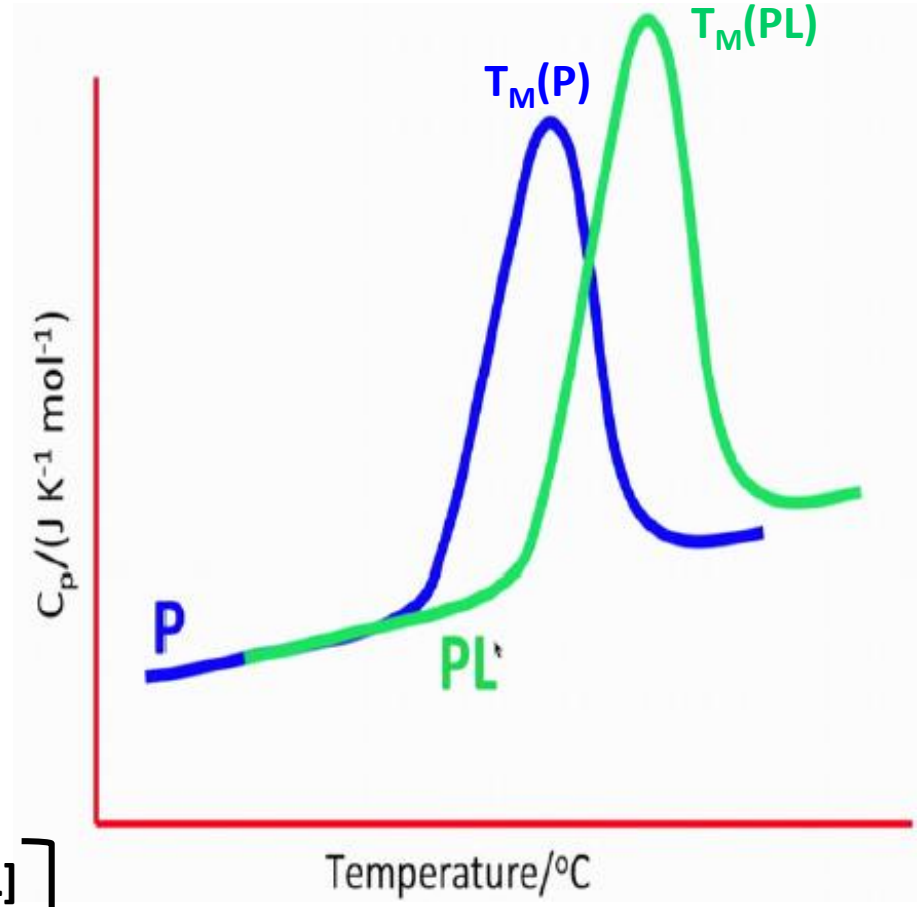
SCIENTIFIC REPORTS | (2019) 9:2650 | https://doi.org/10.1038/s41598-018-37072-x 1

DSC

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

DSC: applicazioni
PLI: Caratterizzazione quantitativa





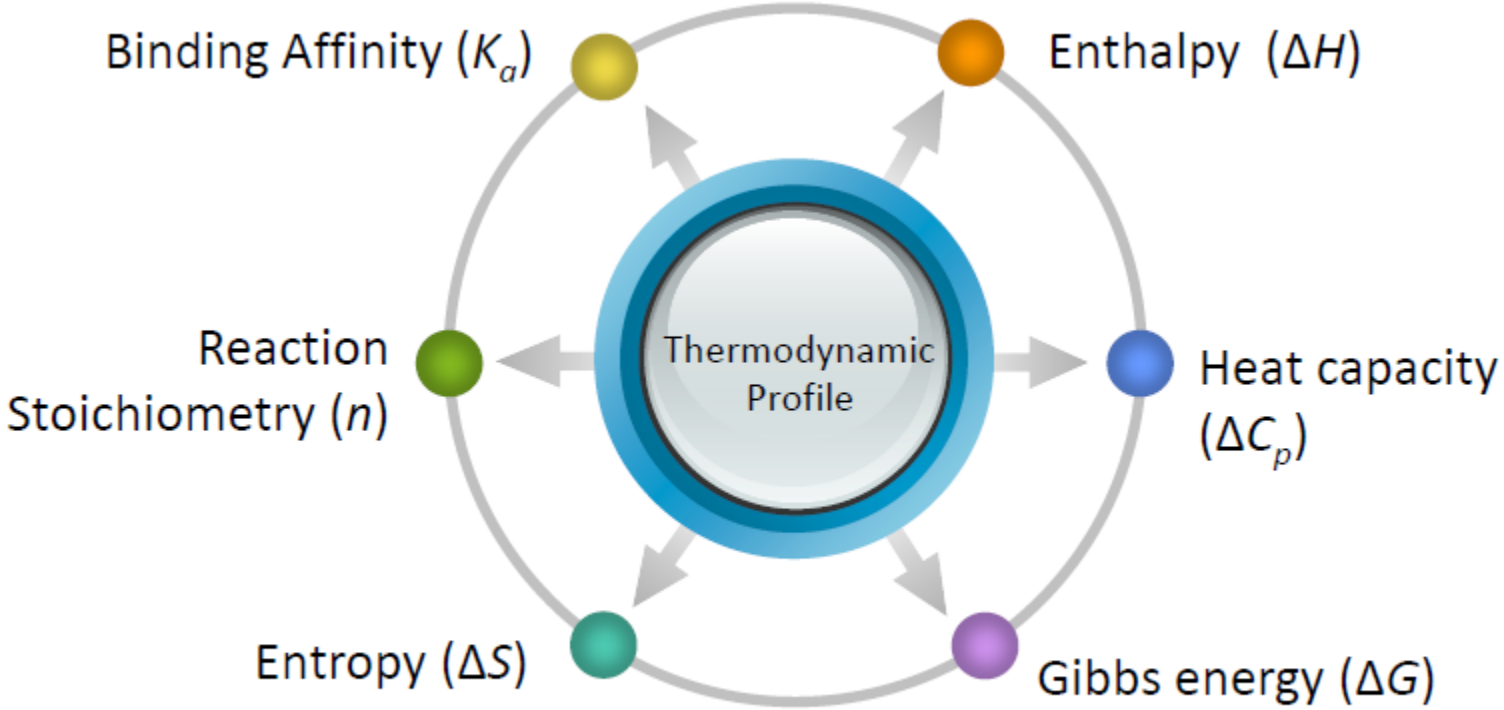
Per l'interazione **P + L ↔ PL**

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

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

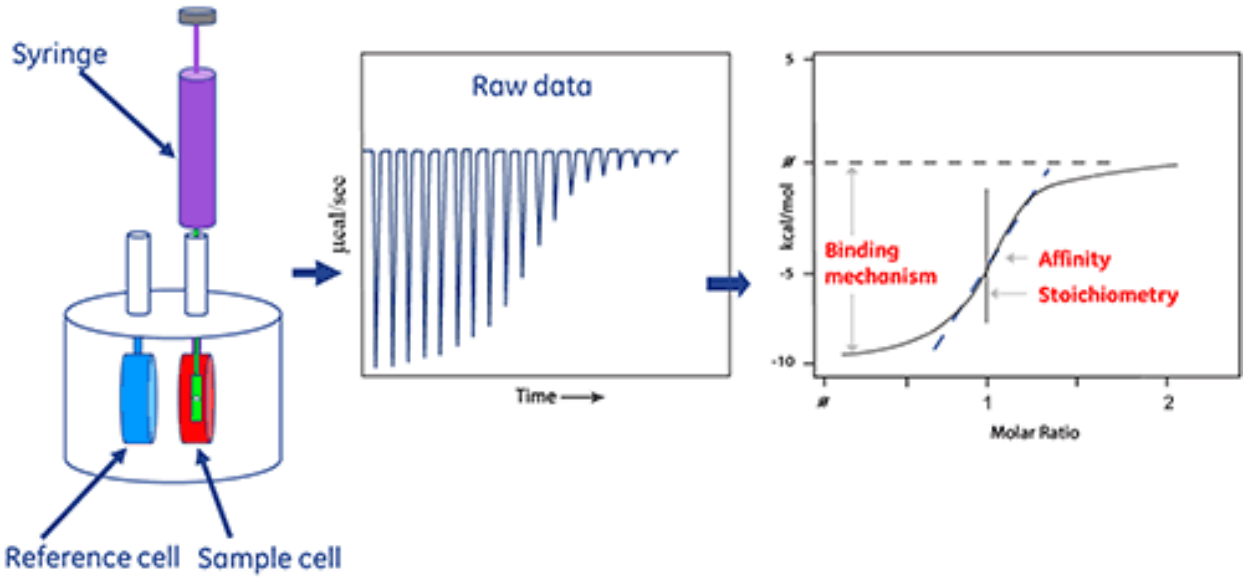
A **pressione costante**,

P + L ↔ PL

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

ΔH variazione di entalpia molare
V_0 volume della cella calorimetrica

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



A saturazione **[PL] / $[P]_T$ ≈ 1**

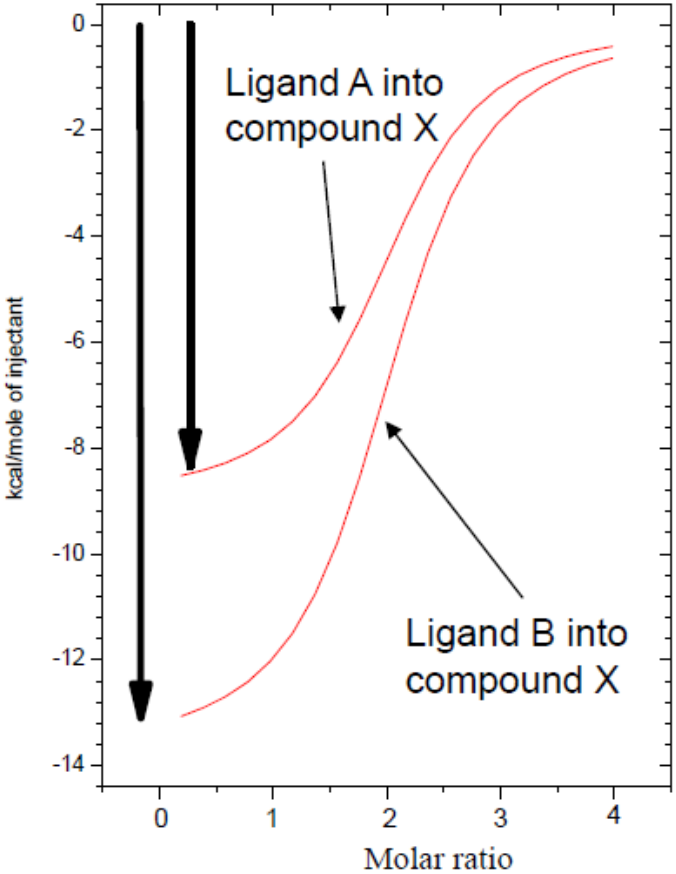
$$\Delta H = \frac{Q_T}{V_0 \, [P]_T}$$

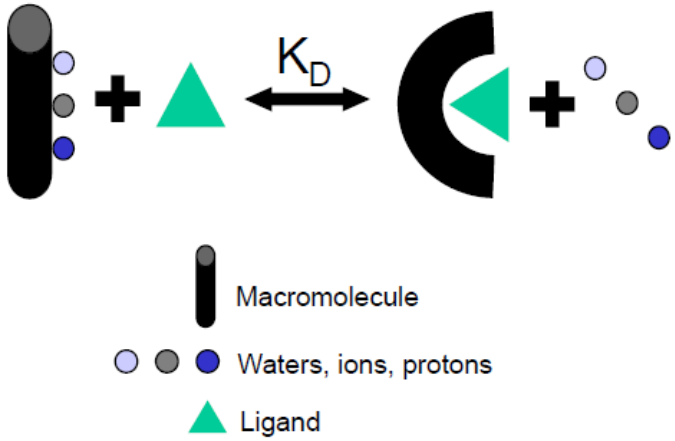
Studio dell'interazione proteina/proteina e proteina/ligando :

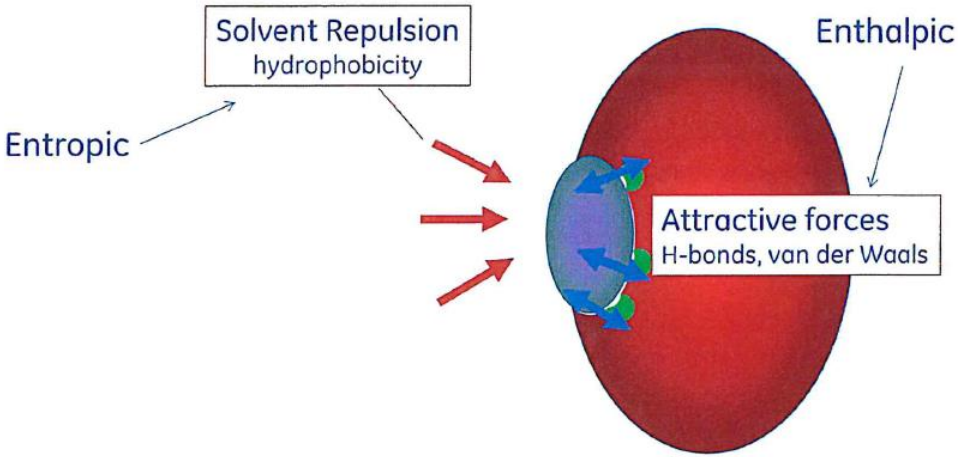
ITC: basi

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

→ **diversi meccanismi di legame**

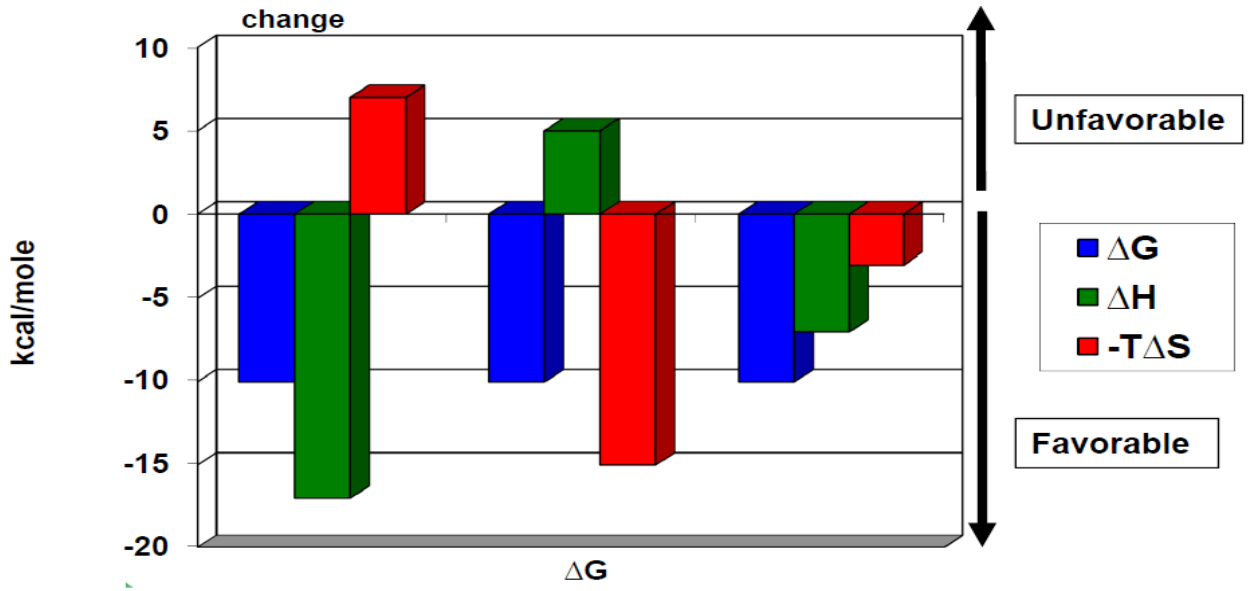






Studio dell'interazione proteina/proteina e proteina/ligando :

ITC: basi



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

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

ITC: basi

CASI ESTREMI DI PLI:

A. ΔH driven (ligandi polari):

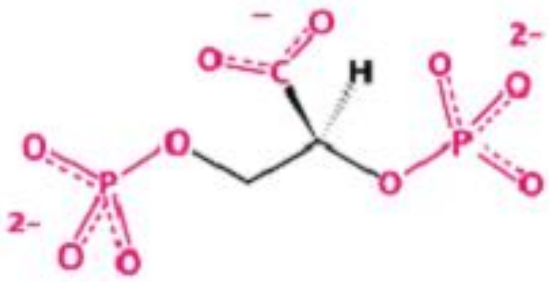
con grande grado di flessibilità
con distanze ottimali per legami H
molto specifici

AFFINITA' BASSE

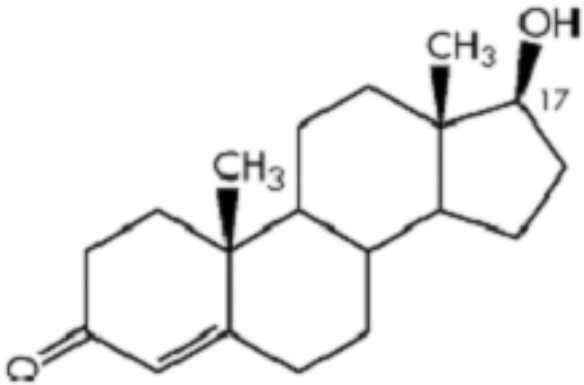
B. -TΔS driven (ligandi idrofobici):

spesso molto grandi
meno specifici

AFFINITA' ALTE



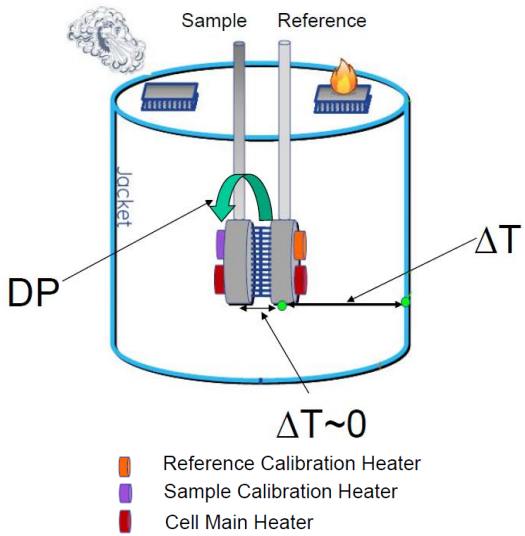
2,3-Bisphosphoglycerate
(2,3-BPG)

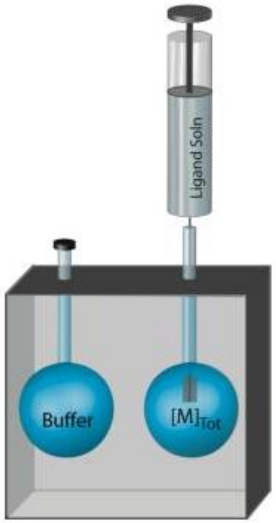


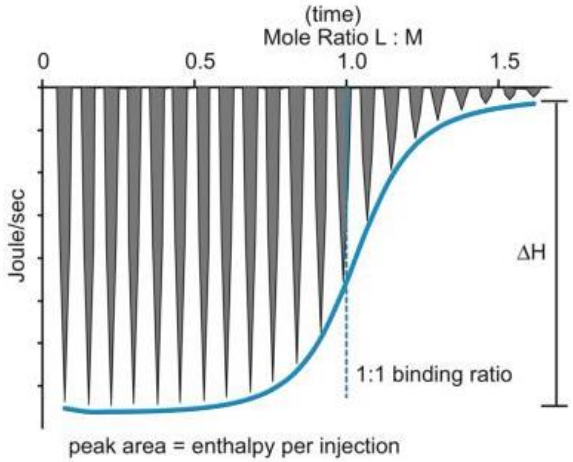
Testosterone

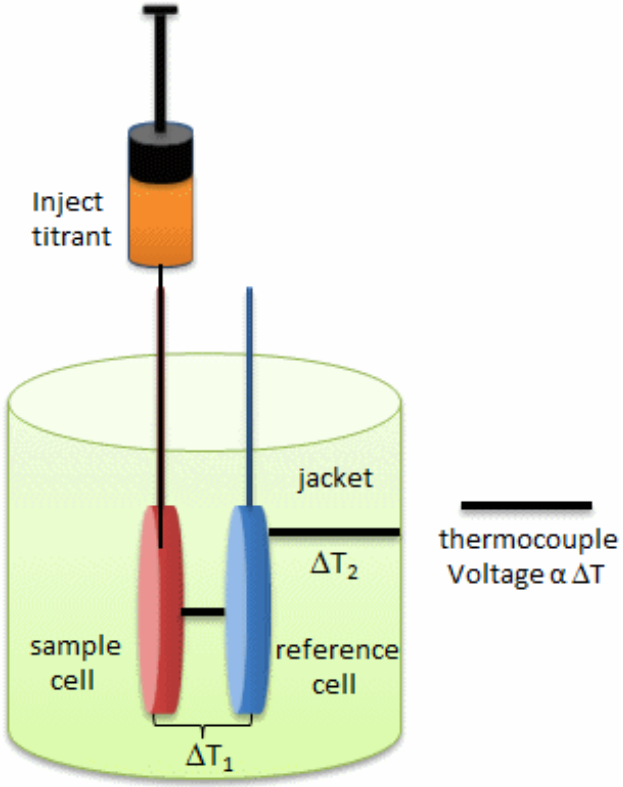
Studio dell'interazione proteina/proteina e proteina/ligando :

ITC: setup



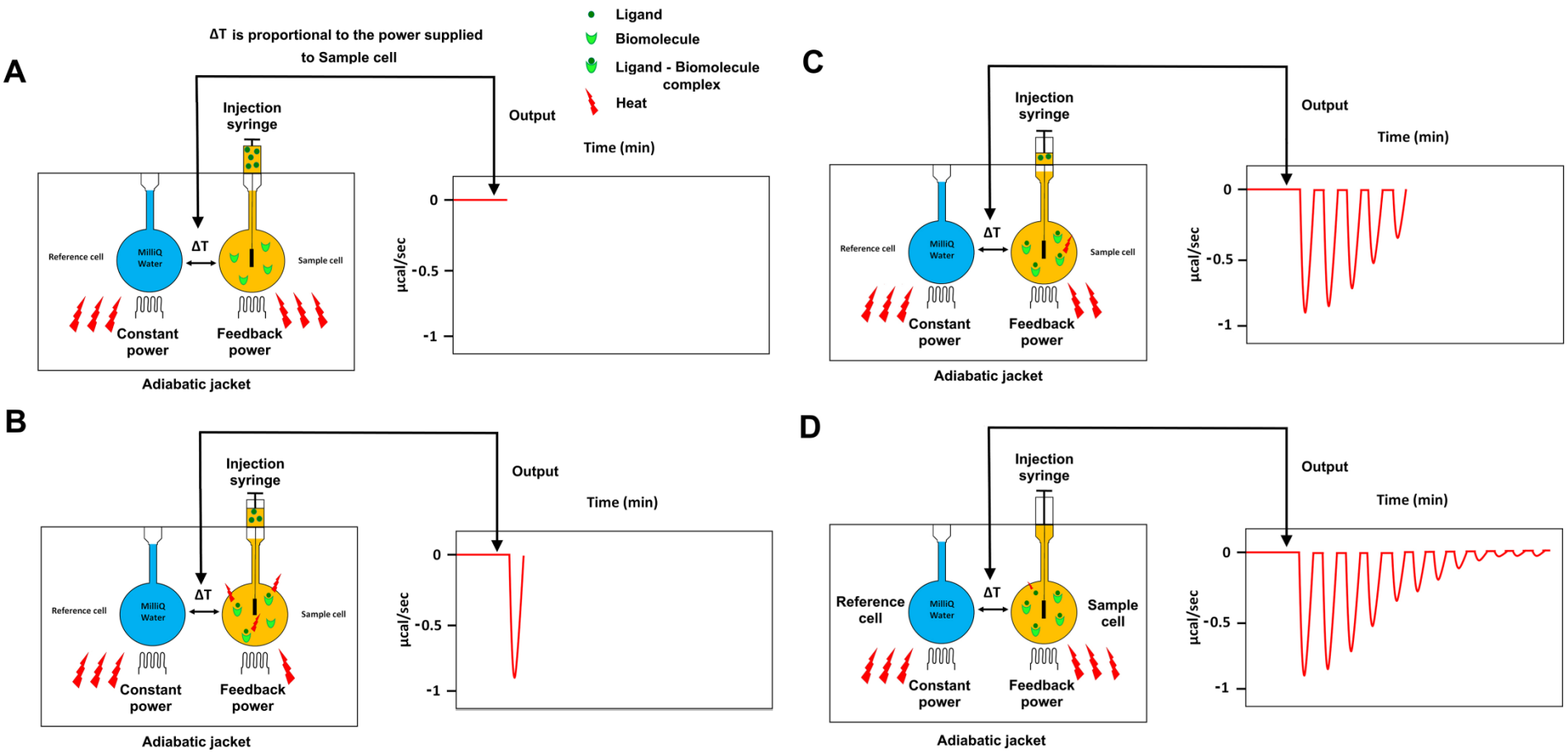




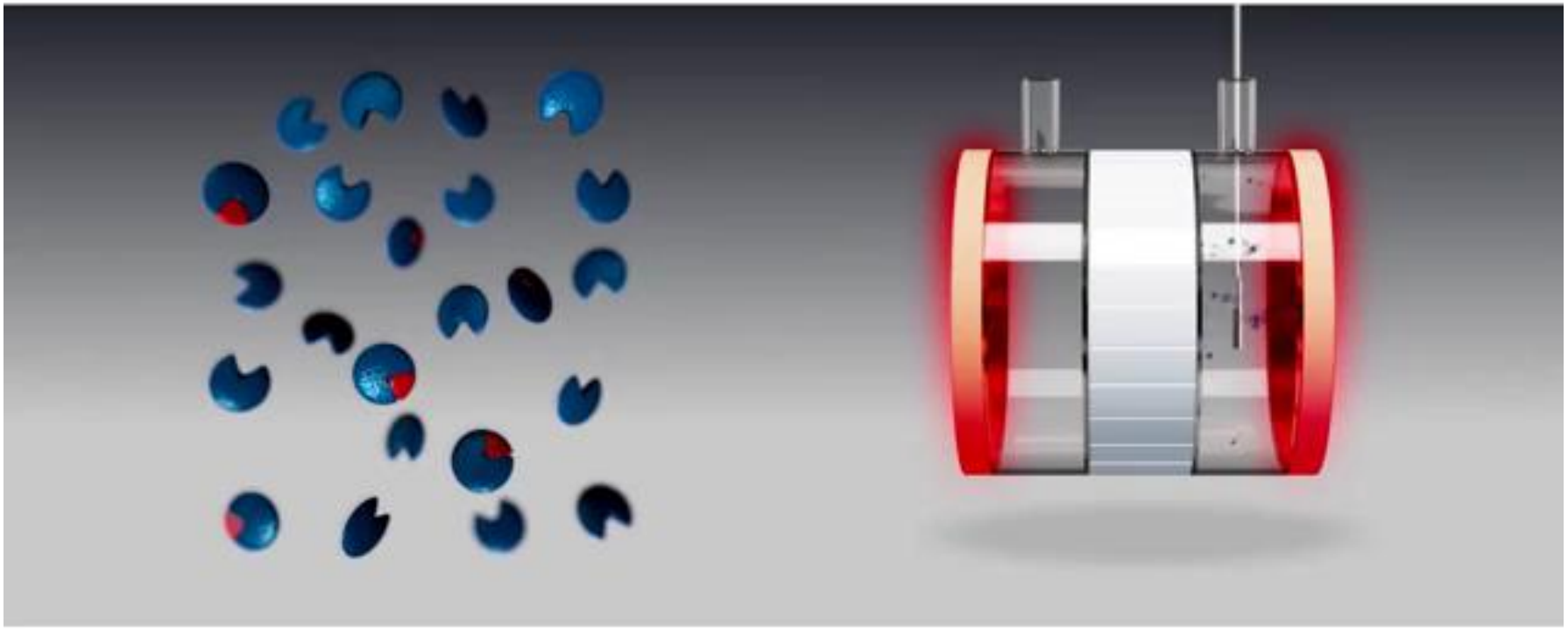


Studio dell'interazione proteina/proteina e proteina/ligando :

ITC: setup



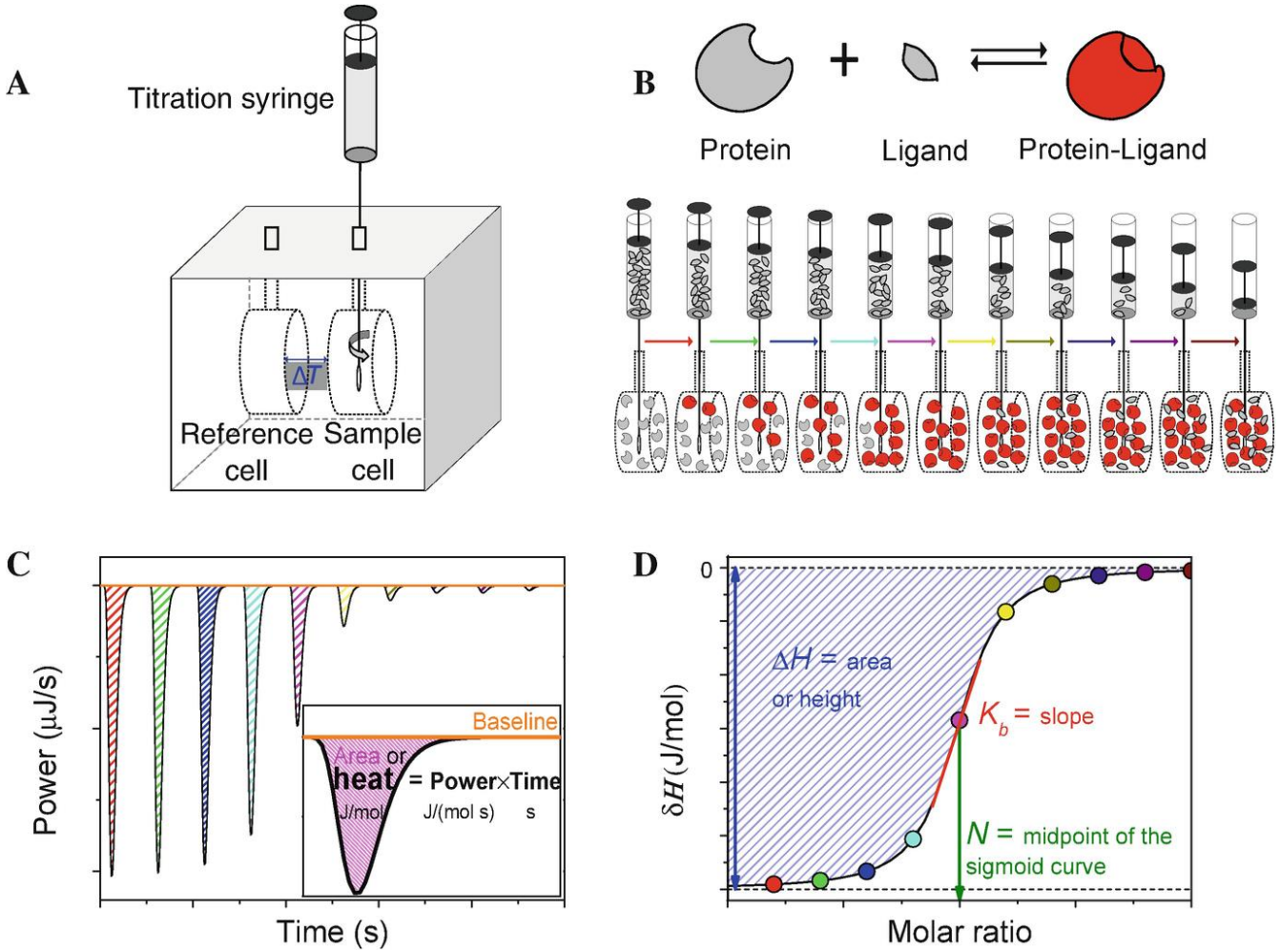
Studio dell’interazione proteina/proteina e proteina/ligando :



Malvern

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_{200}

MicroCal PEAQ™ ITC

MicroCal Auto-iTC_{200}

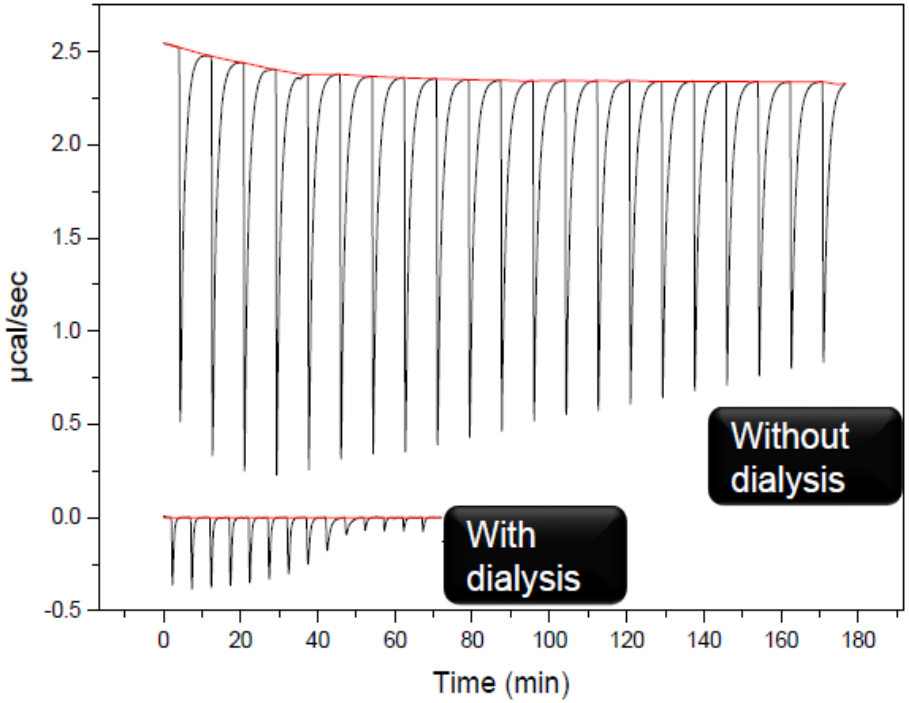
MicroCal PEAQ ITC Automated

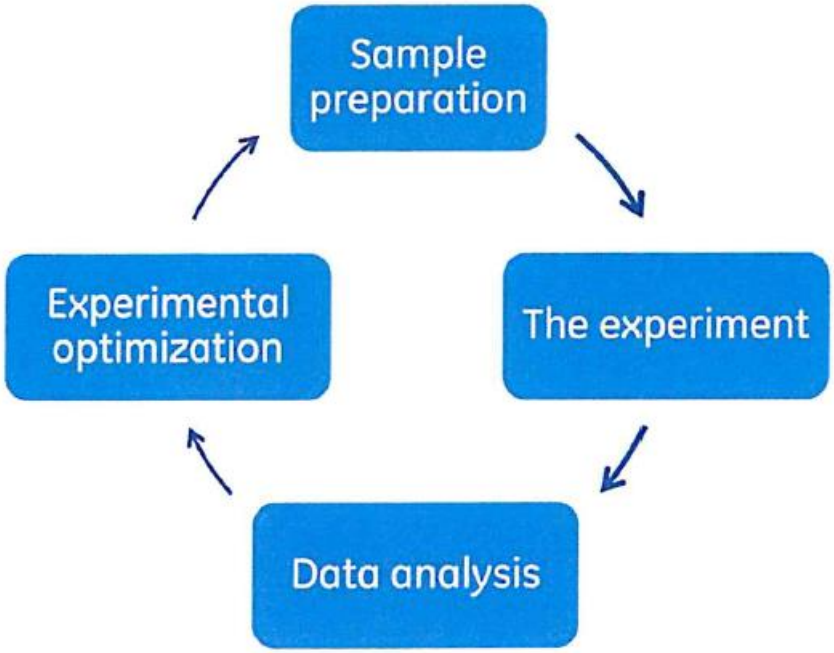
Studio dell'interazione proteina/proteina e proteina/ligando :

ITC: condizioni sperimentali

preparazione del campione:

1. Buffer matching





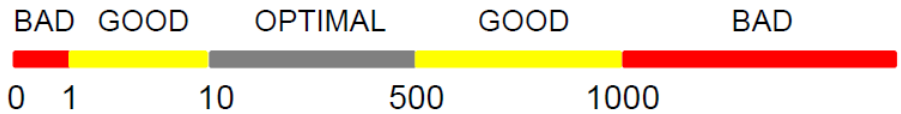
2. Misura della []

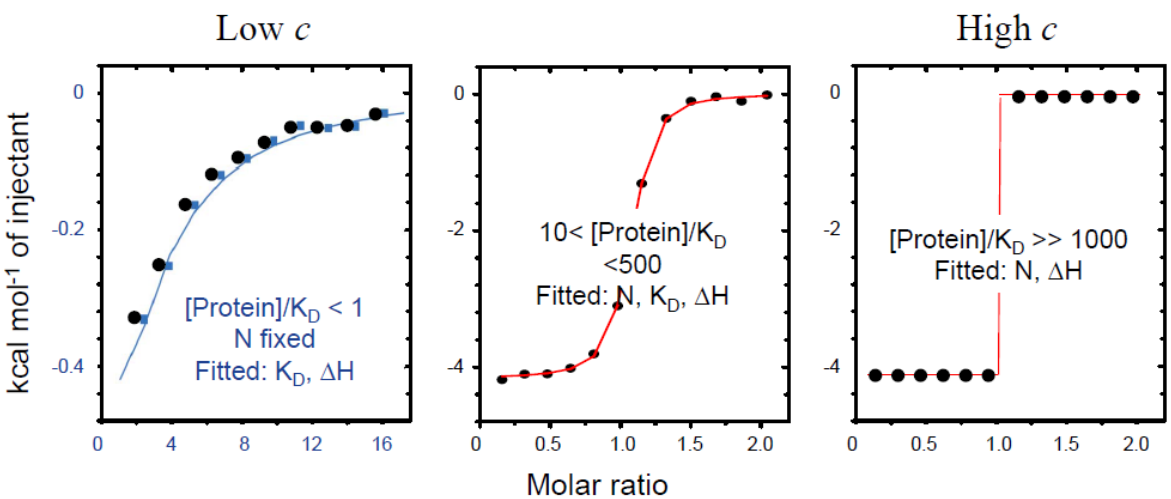
Studio dell'interazione proteina/proteina e proteina/ligando :

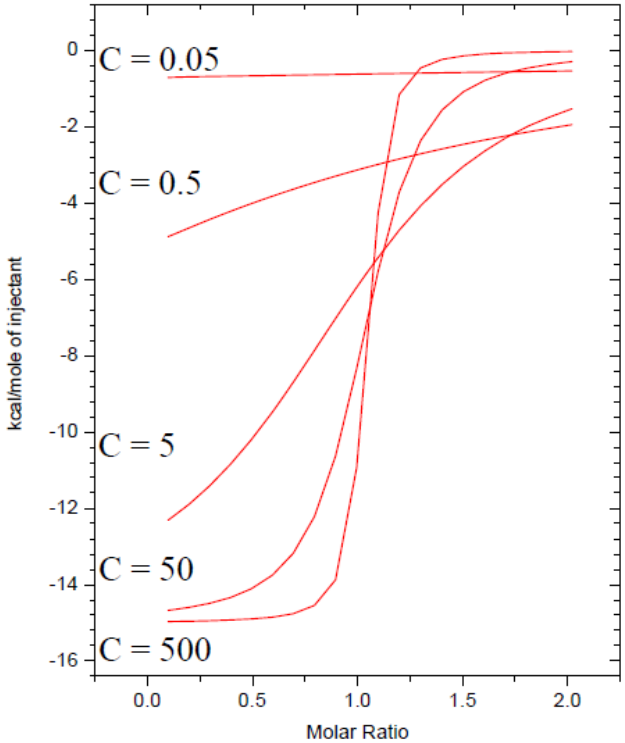
ITC: condizioni sperimentali

[P] nel µM

$C = [P]/K_D$







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

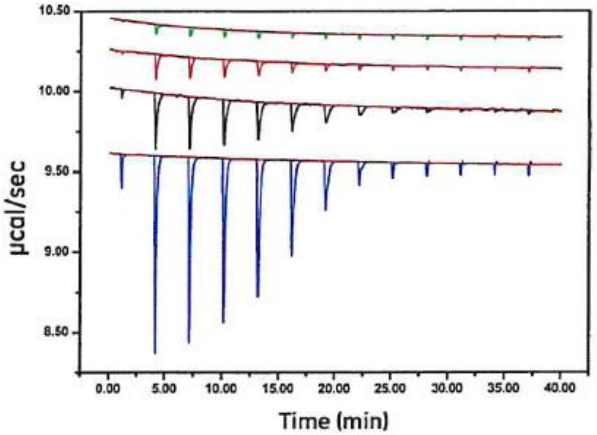
ITC: condizioni sperimentali

Do you know the K_D? → *No* → start with 20 μM protein and 200 μM ligand

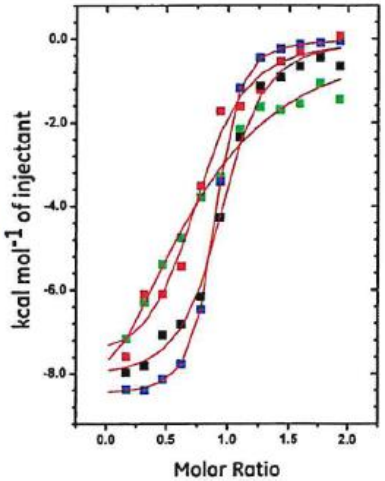
Yes → follow the column for estimated K_Ds

Estimated K_D μM	[Protein] μM	[Ligand] μM	[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 ~ 500 nM



5 μM, C = 10
10 μM, C = 20
20 μM, C = 40
50 μM, C = 100



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

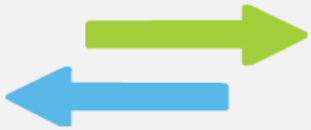
ITC: vantaggi

Label-free

- Direct measurement of heat change (ITC)
- Direct measurement of melting transition temperature to predict thermal stability (DSC)

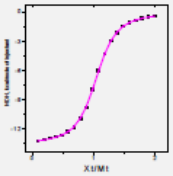
Broad dynamic range

- Native molecules in solution (biological relevance)
- Very sensitive to accomodate range of affinities



Information rich

- All binding parameters (affinity, stochiometry, enthalphy and entropy) in a single ITC experiment



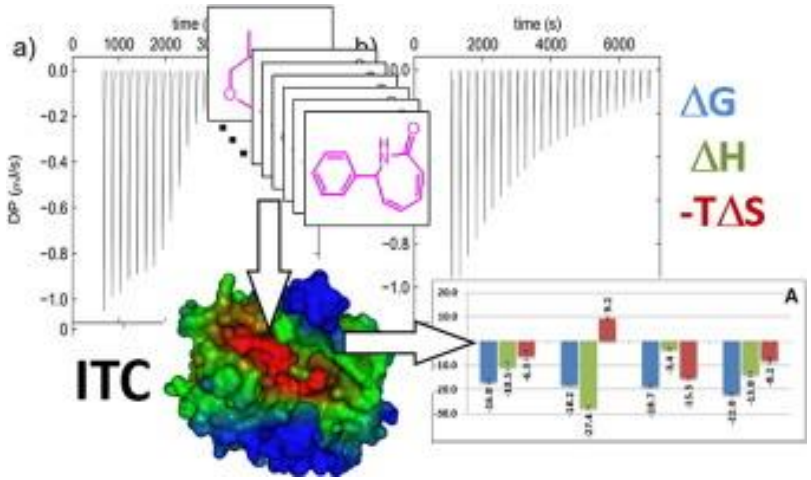
Ease-of-use

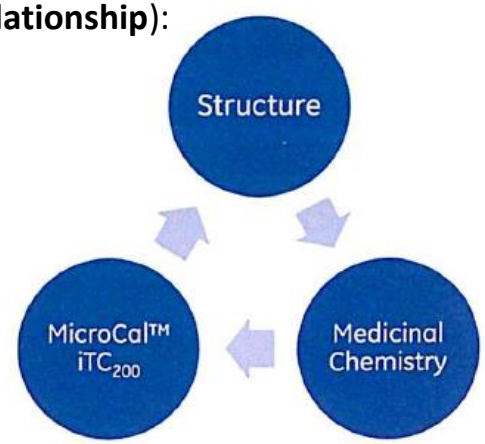
- No labeling or immobilzation necessary
- No assay development
- Wide range of solvent/buffer conditions

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

Δ H

Δ S

Ma perdita di specificità
impossibilità di ottenere $\mathbf{K_A}$ alte
polimorfismi associati a drug-resistence

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

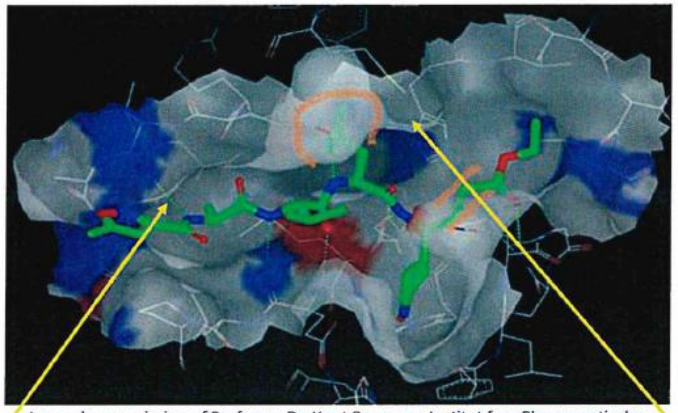


Image by permission of Professor Dr. Knut Baumann Institut fuer Pharmazetische Chemie, Technische Universitaet Braunschweig, Germany

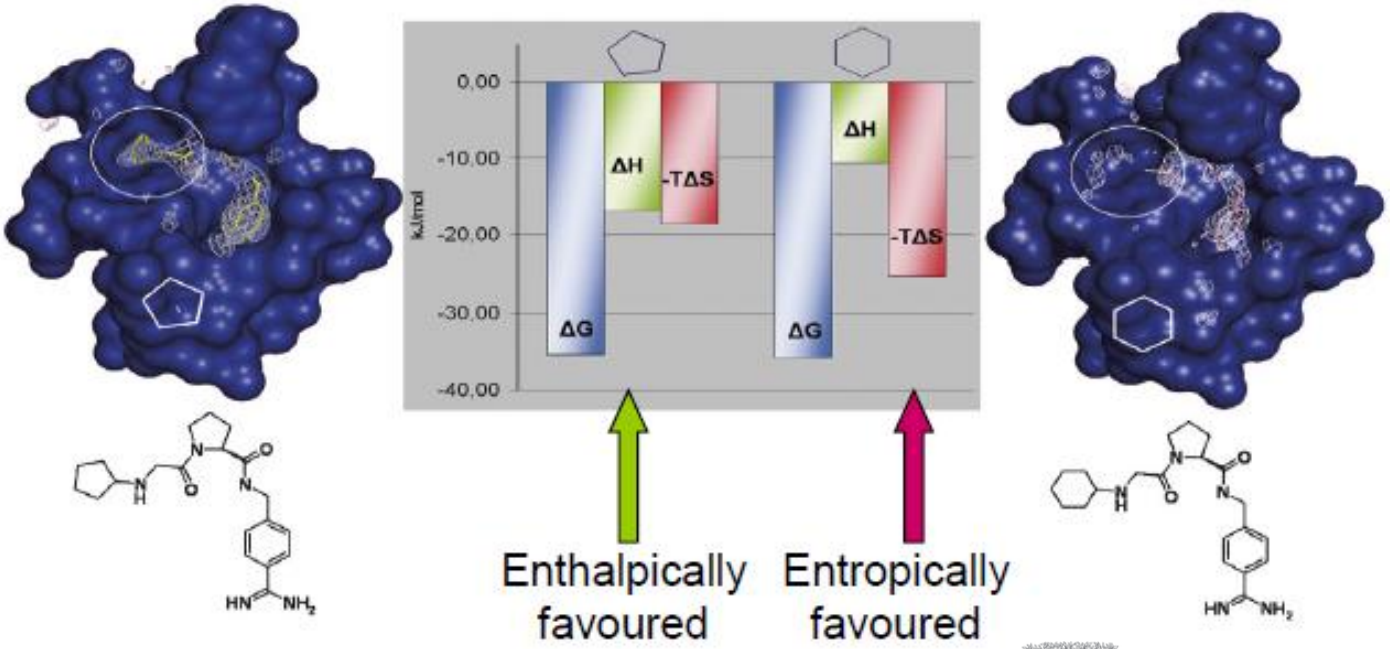
ΔH- indicates H Bonding and van der Waals interactions

TΔS- 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

ELSEVIER
Drug Discovery Today
Volume 11, Issues 13–14, July 2006, Pages 580-594

Review
Foundation

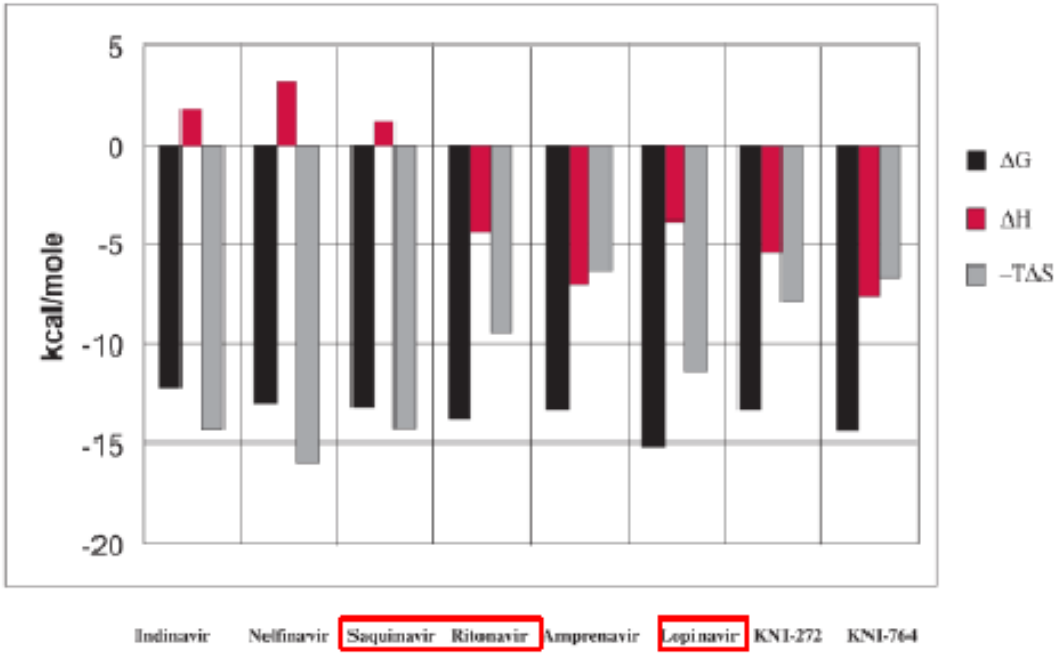
Virtual ligand screening: strategies, perspectives and limitations

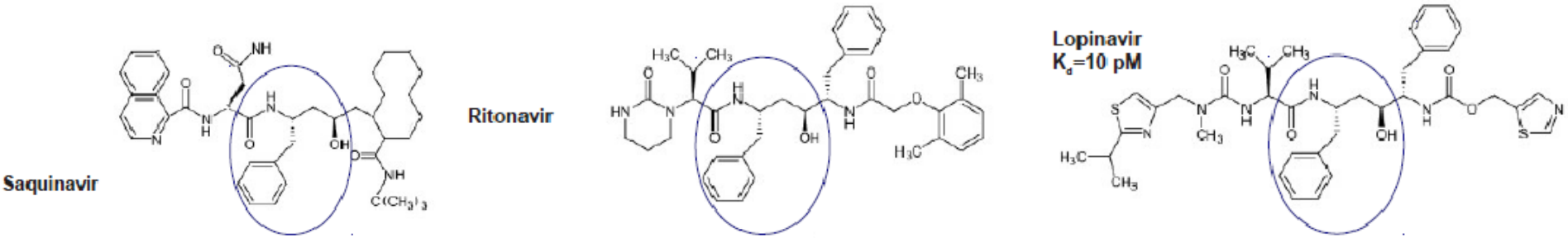
Gerhard Klebe

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

ITC:

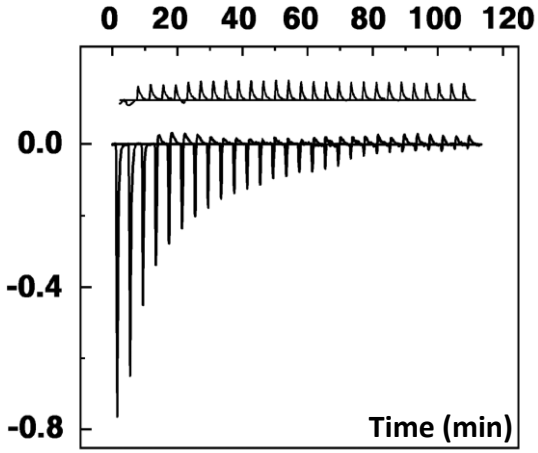
Applicazioni nella scoperta e lo sviluppo di nuovi farmaci:
Inibitori della proteasi di HIV-1



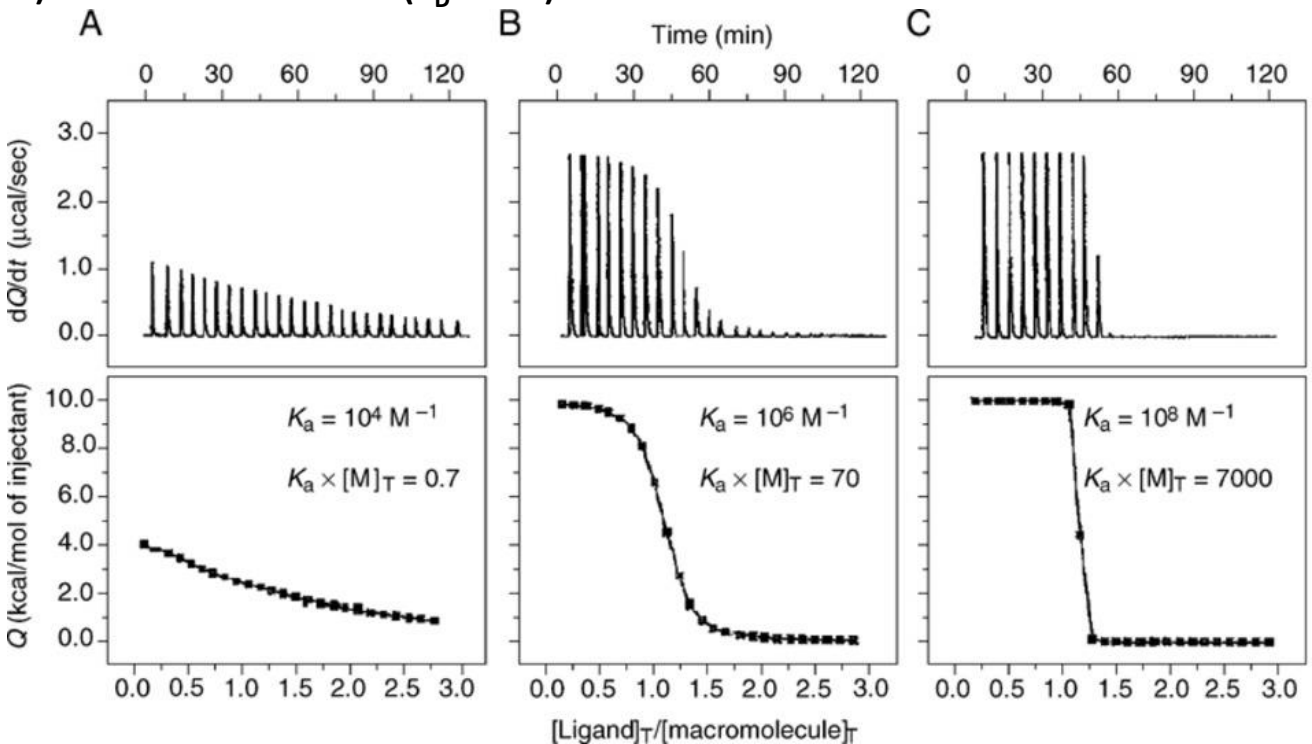


Studio dell'interazione proteina/proteina e proteina/ligando :

ITC:



- Misura il calore globale
- Solo per PPI/PLI con calore misurabile
- Elevato consumo di campione
- Non può misurare direttamente **affinità**
  - **a) molto alte ($K_D < 10^{-8}$)**
  - **b) molto basse ($K_D > 10^{-4}$)**



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}