Structural and biophysical methods for biological macromolecules in solution

19 – 26 June 2016 | Suwon, Korea

Characterization of Protein Interactions by ITC, SPR and BLI

EMBO Global Exchange Lecture Course

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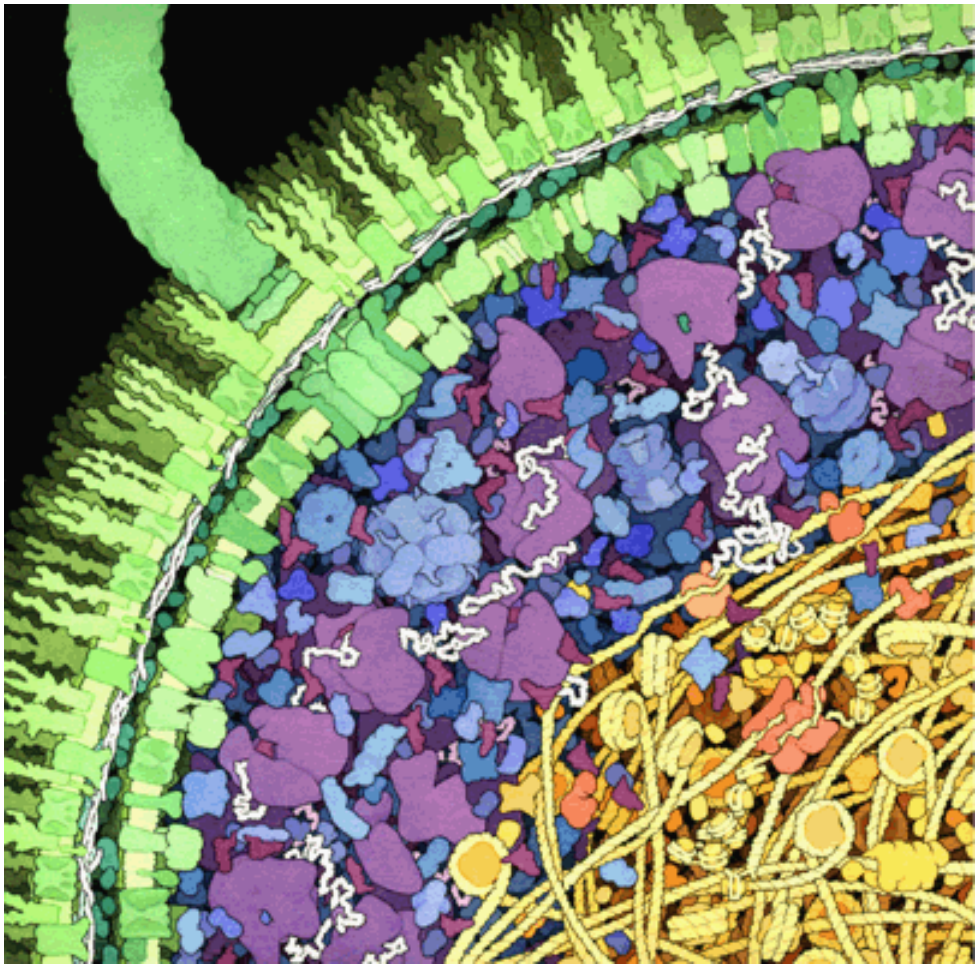
Outline

- Protein interactions: why bother?
- Calorimetry
- Optical methods: SPR and BLI
- Real-life example: hybrid approach

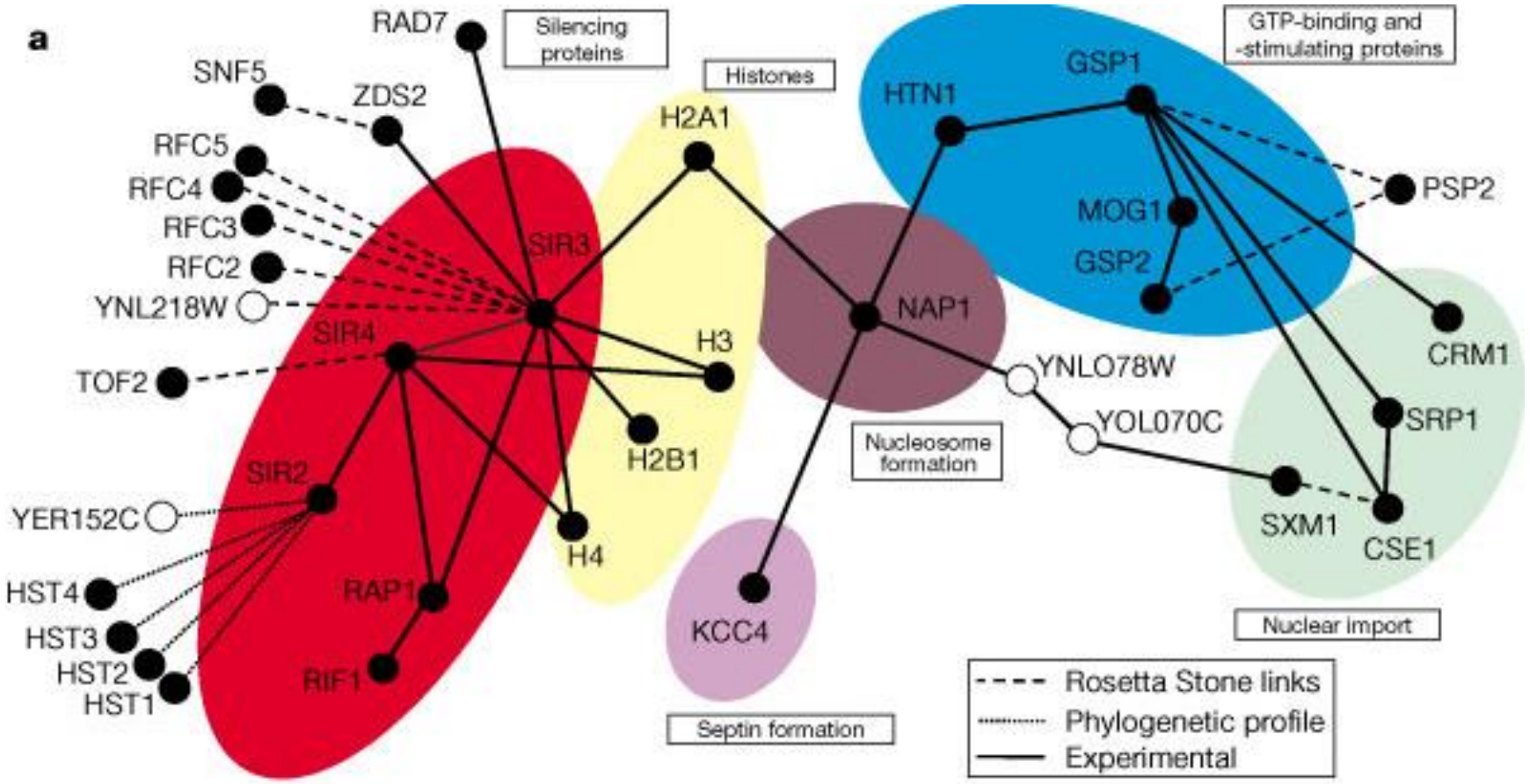
Protein interactions – why bother?

Protein interactions control the lives of cells



Escherichia coli drawn to molecular scale by David Goodsell

Protein interaction network



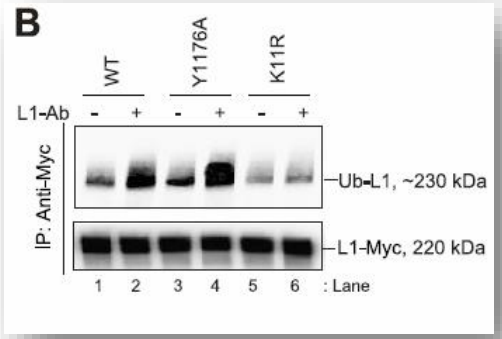
[*Nature* (2000)]

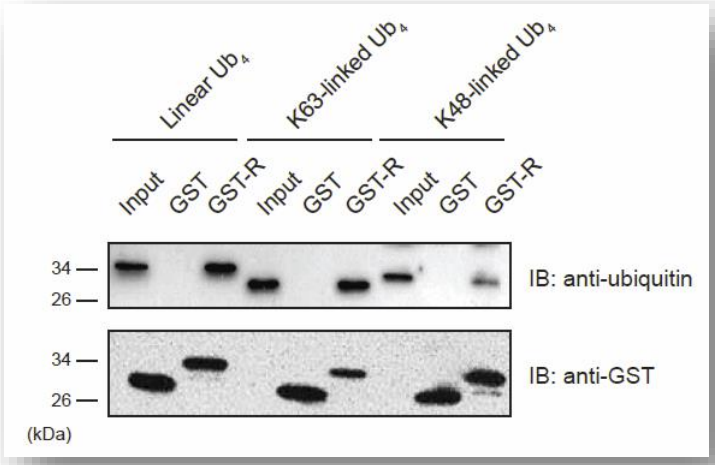
Protein interaction types

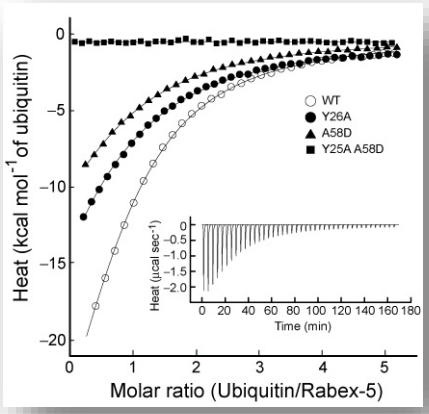
- Homologous interactions:
  - The same proteins
  - Oligomers
  - Coiled-coil
  - Amyloids

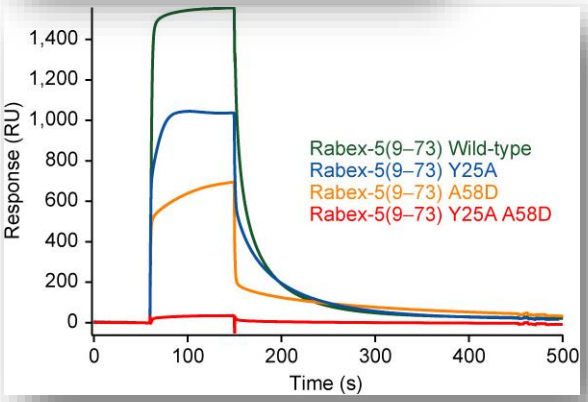
- Heterologous interactions:
  - Different proteins
  - Enzyme – inhibitors
  - Antibody – antigen
  - Protein complexes

Protein interactions: qualitative vs. quantitative





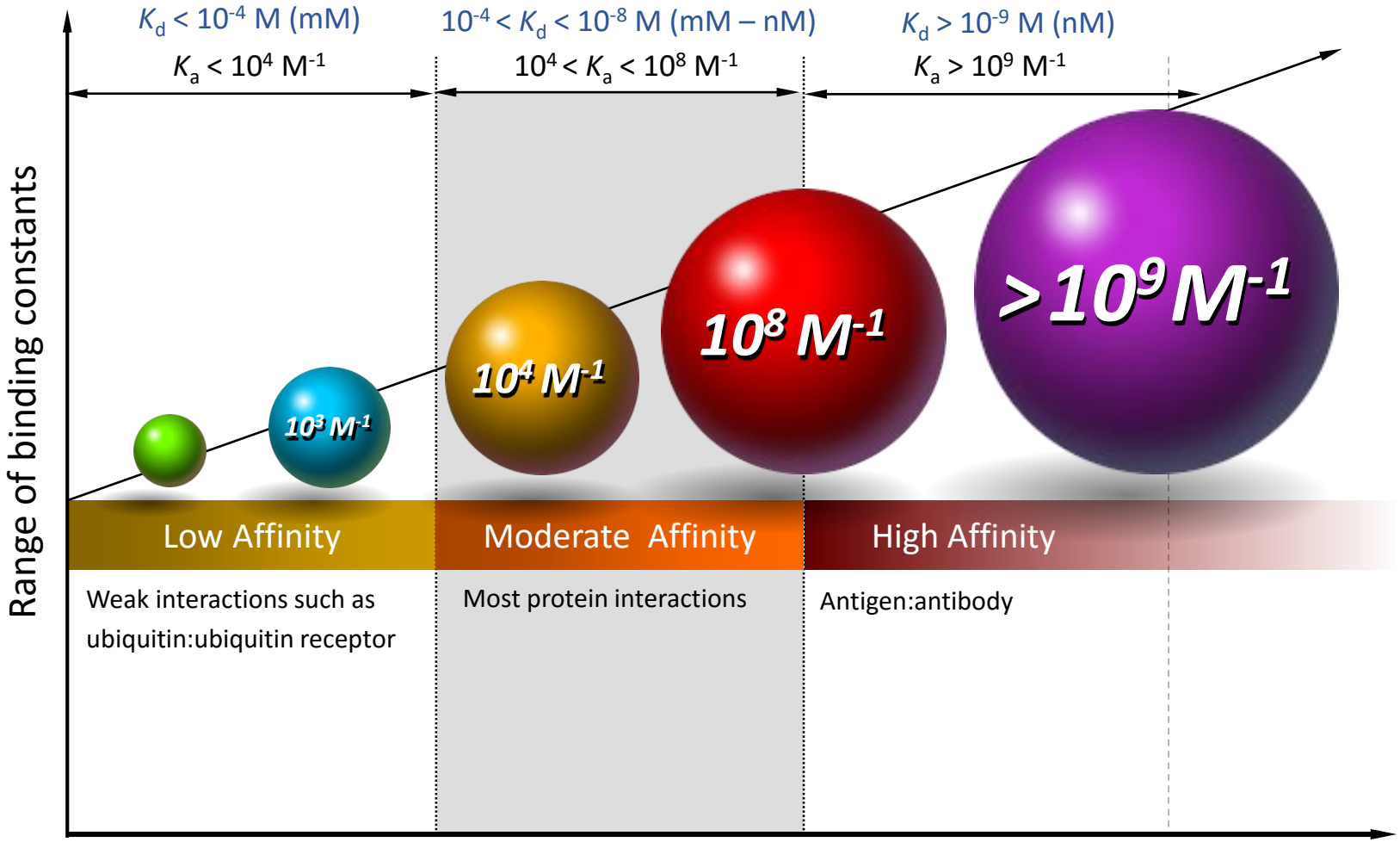




Immunoprecipitation (IP)
Pulldown
Qualitative or semi-quantitative

ITC, SPR, BLI
Fluorescence anisotropy
Quantitative

Protein interactions: binding affinity range



Dissociation constant: K_d

$$P + L \overset{K_a}{\leftrightarrow} PL$$

$$PL \overset{K_d}{\leftrightarrow} P + L$$

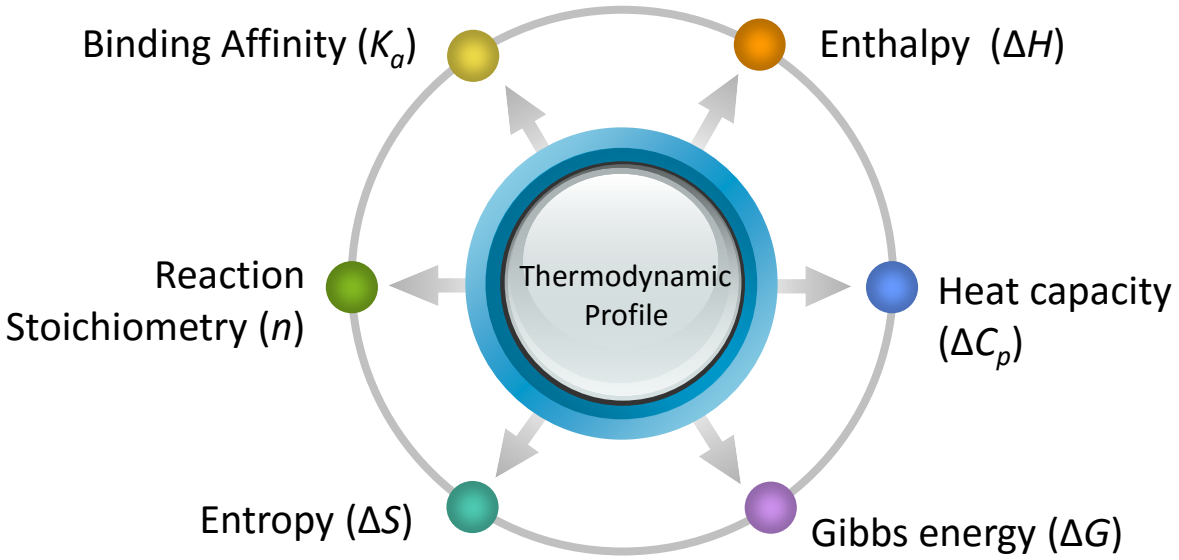
$$K_a = \frac{[PL]}{[P][L]} = \frac{k_{on}}{k_{off}}$$ M^{-1}

$$K_d = \frac{[P][L]}{[PL]} = \frac{k_{off}}{k_{on}}$$ M

$$K_a = \frac{1}{K_d}$$

Isothermal Titration Calorimetry

Isothermal titration calorimetry (ITC): Measuring heat



- *Calor* (Latin, *heat*) + *metry* (Greek, *measure*)
- Direct measurement of heat *q* either released or absorbed in molecular binding during gradual titration
- Label-free measurement
- Microcalorimeters: as low as 100 µl

ITC theory: Thermodynamics

- Scenario: a ligand (L) binds to a protein (P) at temperature T

$$P + L \overset{K_a}{\leftrightarrow} PL$$

- Release of absorption of heat due to binding

$$q = \Delta H^0(T) n_{PL} = \Delta H^0(T) V[PL]$$

- $\Delta H^0(T)$ and K_a (therefore K_d) can be determined by titration

$$q = \Delta H^0(T) V[P_T] \left(\frac{K_a[L]}{1 + K_a[L]} \right)$$

ITC theory: Thermodynamics

- Scenario: a ligand (*L*) binds to a protein (*P*) at temperature *T*

$$P + L \overset{K_a}{\leftrightarrow} PL$$

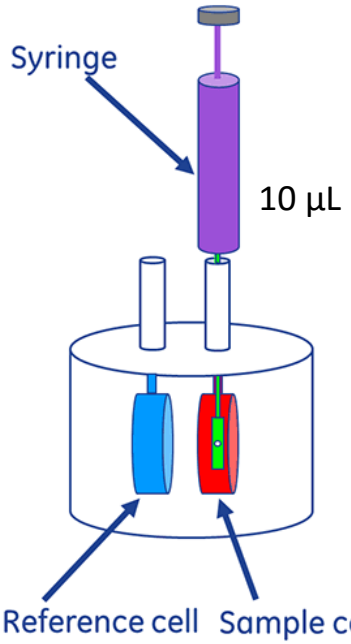
- Once you determine $\Delta H^0(T)$ and K_a (therefore K_d), ΔG^0 and ΔS^0 can be calculated.

$$\Delta G^0(T) = -RTlnK_a$$

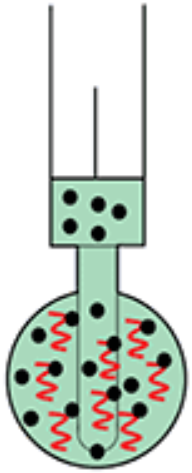
$$\Delta G^0(T) = \Delta H^0(T) - T\Delta S^0(T)$$

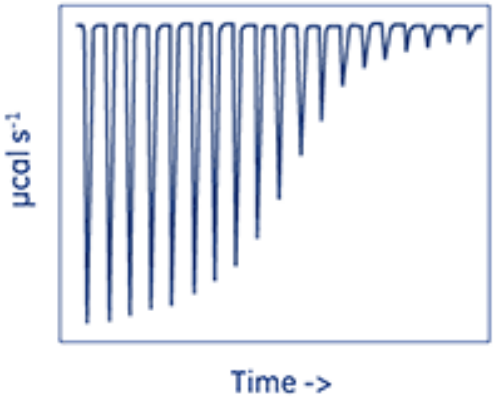
Representative instruments

ITC: Instrument components



1.4 (or 0.2) mL

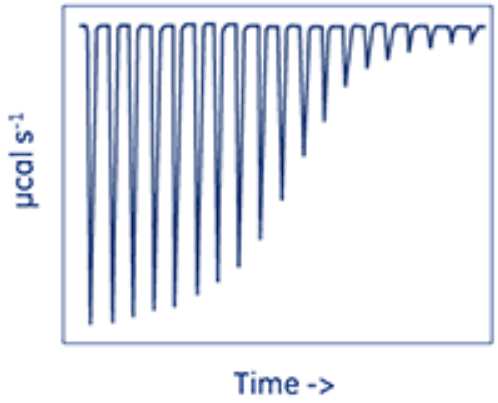


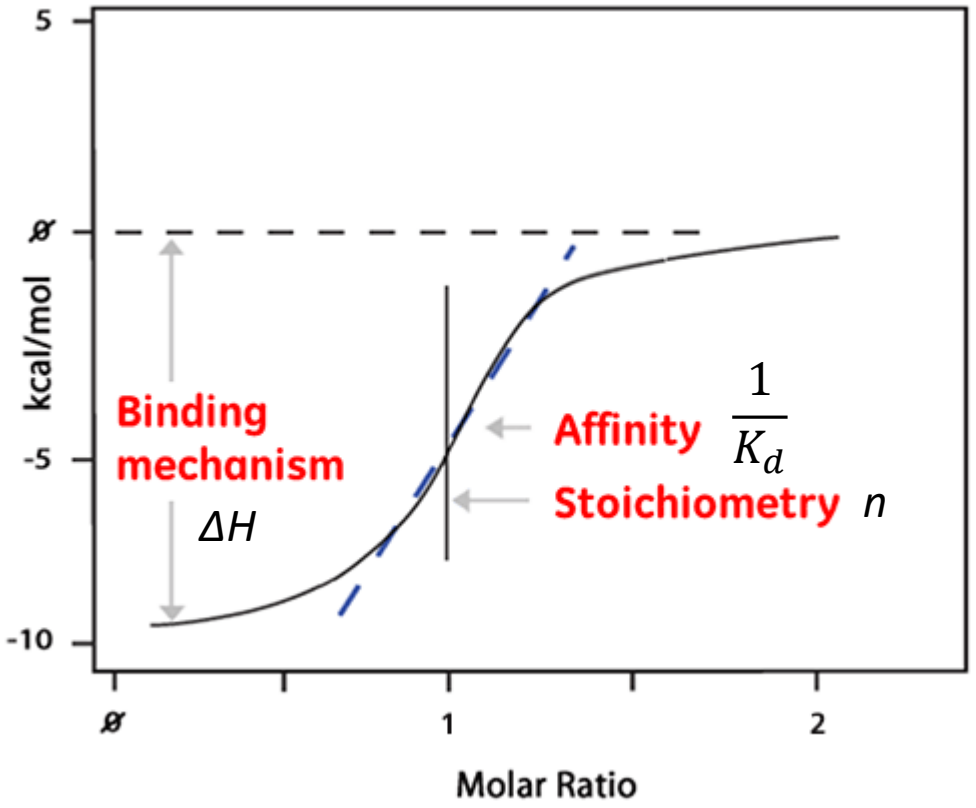


[www.Malvern.com]

- Exothermic reaction
- The sample cell becomes warmer than the reference cell.
- Binding causes a downward peak in the signal.
- Heat released is calculated by integration under each peak.

ITC: Data analysis

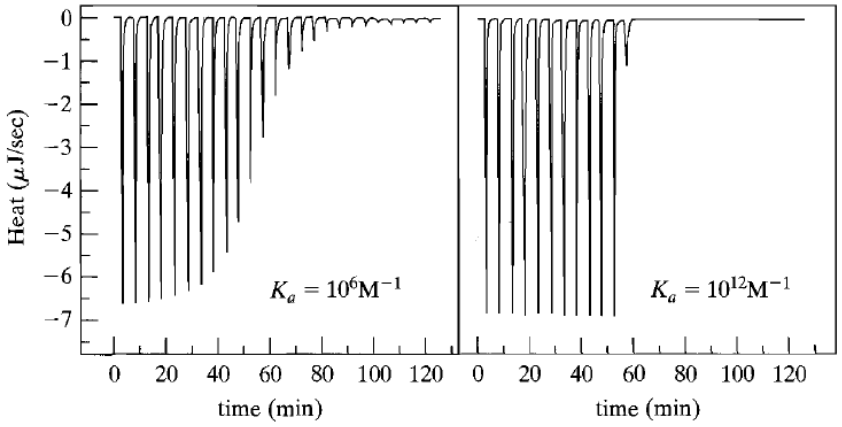




[www.Malvern.com]

ITC: Limitations and competitive binding techniques

Limits



[van Holde, *Principles of Physical Biochemistry*, 2nd Ed. (2006)]

Can't measure tight interactions

K_a by direct measurement:

10^2 M^{-1} - 10^9 M^{-1}

K_d (dissociation constant) = $1/K_a$

Work-around

(1) Weak ligand binds to protein

(2) Strong ligand displaces weak ligand:protein complex

$$K_{app} = \frac{K_{strong}}{1 + K_{weak}[L_{weak}]}$$

Can measure tight interactions

K_a by competitive technique:

10^9 M^{-1} - 10^{12} M^{-1}

Protein:protein interaction

nature
structural &
molecular biology

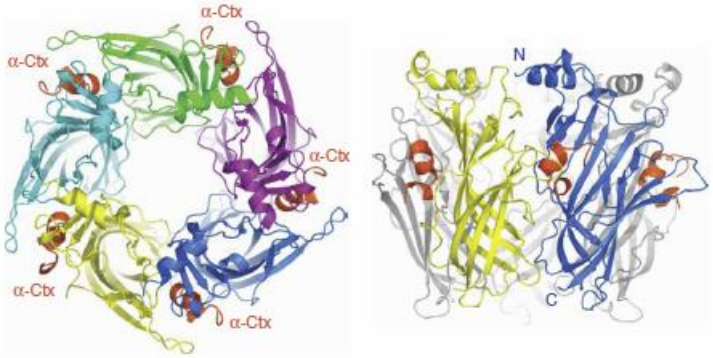
Crystal structure of nicotinic acetylcholine receptor homolog AChBP in complex with an α-conotoxin PnIA variant

Patrick H N Celie[1,5], Igor E Kasheverov[2,5], Dmitry Y Mordvintsev[2], Ronald C Hogg[3], Pim van Nierop[4], René van Elk[4], Sarah E van Rossum-Fikkert[1], Maxim N Zhmak[2], Daniel Bertrand[3], Victor Tsetlin[2], Titia K Sixma[1] & August B Smit[4]

Supplementary Table 1 Thermodynamic parameters

Protein	Ligand	K_d (nM)	N(mol/mol)	H (kcal/mol)	- T S (kcal/mol)
Ls-AChBP	PnIA[A10L,D14K]	27.5 ± 23.6	3.6 ± 0.1	- 1.44 ± 0.06	-8.78 ± 0.16
Ls-AChBP	PnIA[A10L]	85.0 ± 32.8	5.7 ± 0.3	- 1.13 ± 0.11	-8.46 ± 0.36
Ac-AChBP	PnIA[A10L,D14K]	32.6 ± 8.5	4.1 ± 0.3	- 5.64 ± 0.19	-4.47 ± 0.19
Ac-AChBP	PnIA[A10L]	36.7 ± 16.6	5.3 ± 0.2	- 3.91 ± 0.07	-6.19 ± 0.27

K_d is dissociation constant in nM, N is number of binding sites in each pentamer, H is heat change in kcal/mol, S is entropy change in kcal/mol/deg. Data are the mean values from two independent experiments.



Protein:DNA interaction

The EMBO Journal (2006) 25, 4503–4512 | © 2006 European Molecular Biology Organization | All Rights Reserved 0261-4189/06
www.embojournal.org

THE EMBO JOURNAL

Solution structure of the nonmethyl-CpG-binding CXXC domain of the leukaemia-associated MLL histone methyltransferase

Mark D Allen[1,4], Charles G Grummitt[1,4], Christine Hilcenko[2,4], Sandra Young Min[2,4], Louise M Tonkin[2], Christopher M Johnson[1], Stefan M Freund[1], Mark Bycroft[1] and Alan J Warren[2,3,*]

[1]Centre for Protein Engineering, Cambridge, UK, [2]MRC Laboratory of Molecular Biology, Cambridge, UK and [3]Department of Haematology, University of Cambridge, Cambridge, UK

Mixed-lineage leukemia: A type of childhood leukemia in which a piece of chromosome 11 has been translocated (broken off and attached itself to another chromosome). Children with this type of leukemia have a particularly poor prognosis (outlook). They do not respond at all well to the standard therapies for ALL (acute lymphoblastic or lymphocytic leukemia) and often suffer from early relapse after chemotherapy.

On both the clinical and laboratory levels, chromosome 11 childhood leukemia appears therefore to be a distinctive disease and not a subset of ALL. Armstrong and coworkers (Nature, Jan 2002) named it "mixed-lineage leukemia."

[*MedicineNet.com*]

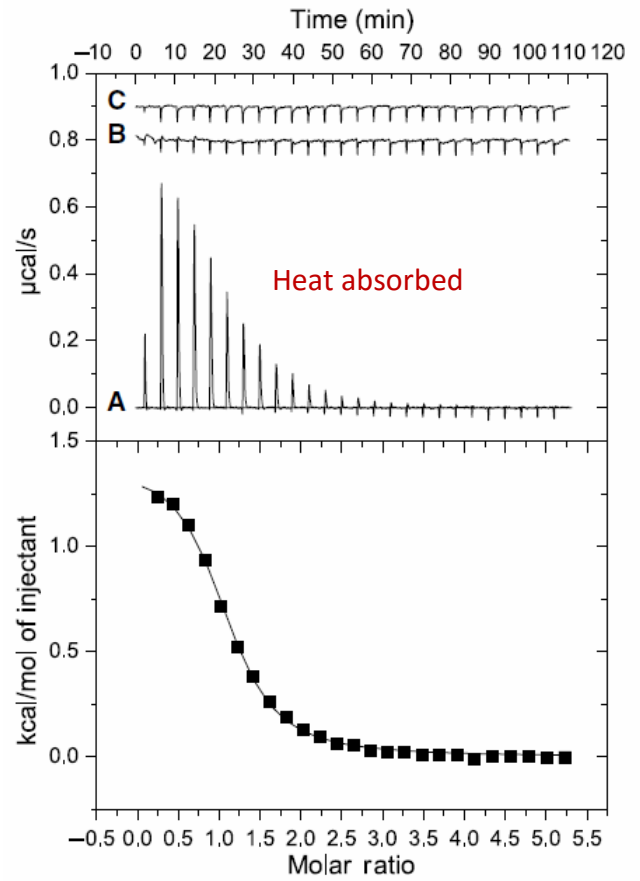


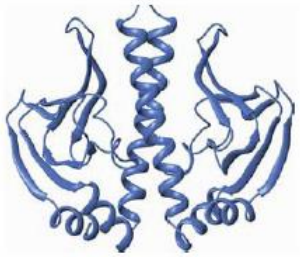
Figure 4 ITC analysis of DNA binding by the MLL CXXC domain. Typical ITC data are shown for the endothermic binding of the CXXC domain to a 12-mer CpG-containing DNA oligonucleotide at 22°C in 20 mM MES, pH 6.5, 250 mM NaCl, 5 mM β-mercaptoethanol. Upper panel: (**A**) CXXC domain (1.3 mM) into the calorimetric cell (1.4 ml) containing CpG 12-mer DNA (49 µM). (**B**) CXXC domain (970 µM) into ITC buffer. (**C**) ITC buffer into CpG 12-mer DNA (49 µM). Lower Panel: Integrated heat pulses, normalised per mole of injectant, giving a differential binding curve that is adequately described by a one-site binding model.

Protein:cofactor interaction

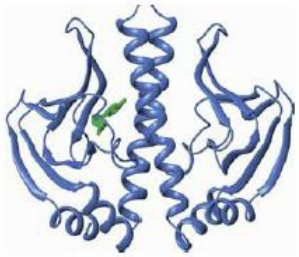
nature
structural &
molecular biology

Dynamically driven protein allostery

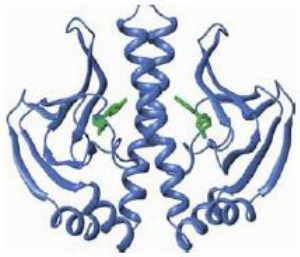
Nataliya Popovych[1], Shangjin Sun[1], Richard H Ebright[2] & Charalampos G Kalodimos[1]



Apo-CAP^N



cAMP$_1$–CAP^N



cAMP$_2$–CAP^N

CAP: catabolite activator protein (dimer)
cAMP: cyclic AMP

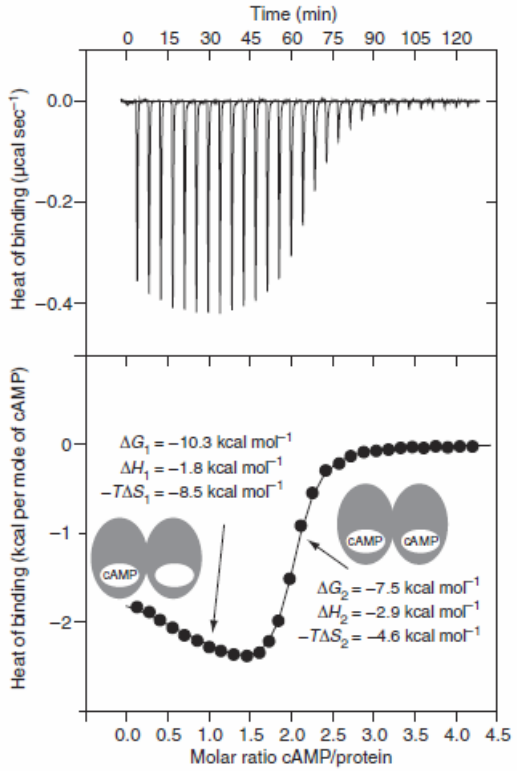
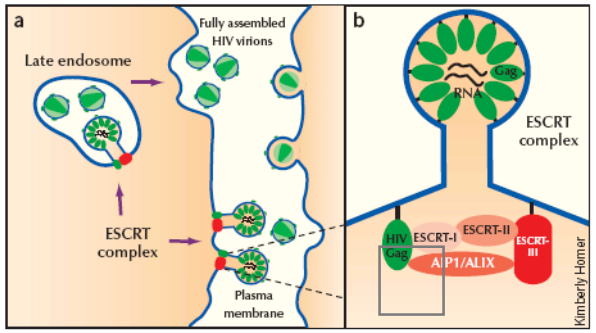
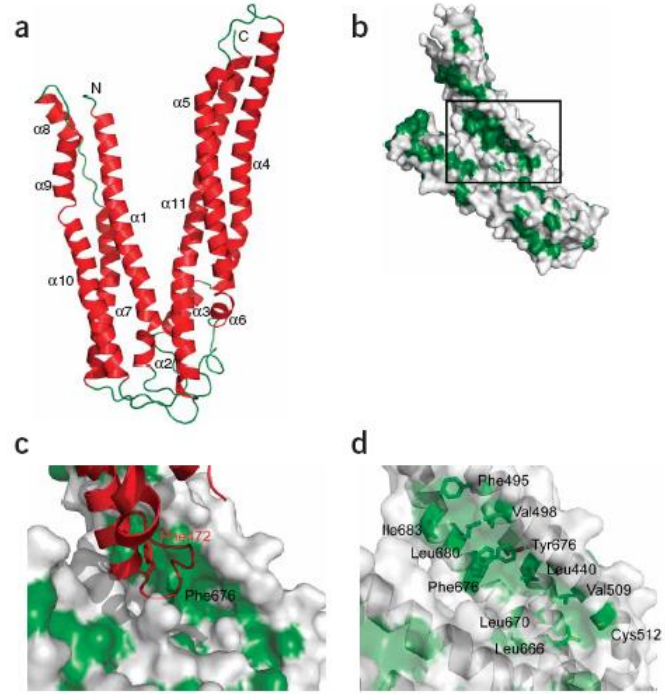
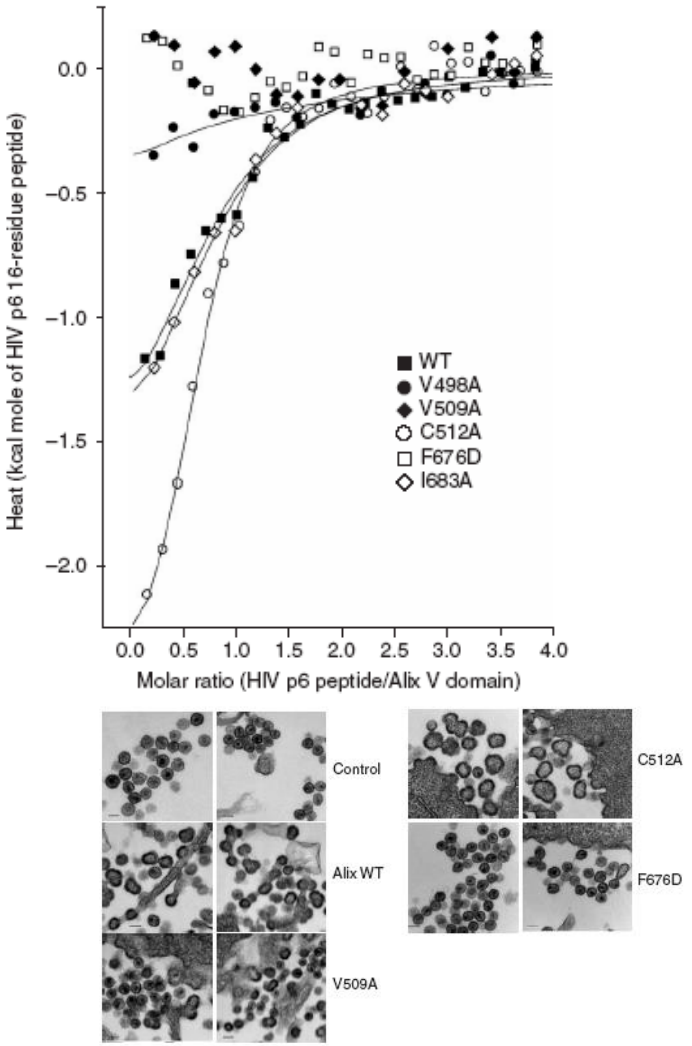


Figure 7 Energetics of cooperative sequential binding of cAMP to CAP^N. ITC traces (top) and binding isotherm (bottom) of the calorimetric titration of cAMP into CAP^N. Solid line in bottom chart represents the fit to a sequential binding site model. $K_d = 0.04$ µM and 4 µM for the first and second cAMP binding steps, respectively.

Protein:protein interaction – HIV Gag p6:Human Alix

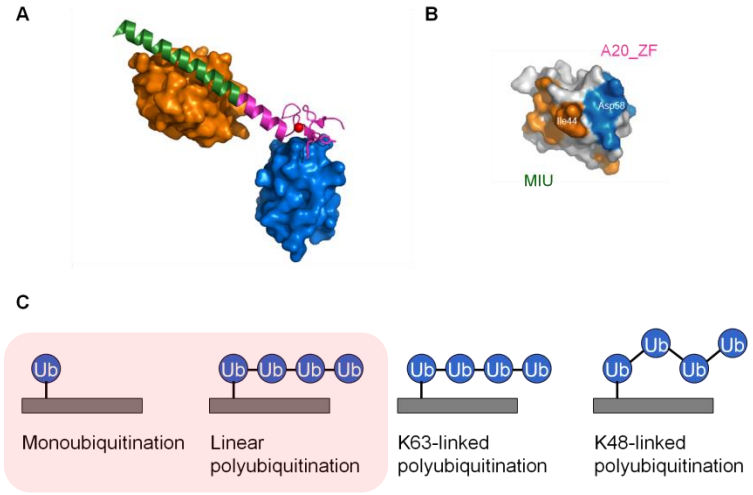






[Sangho Lee et al. *Nat. Struct. Mol. Biol.* (2007)]

Protein:protein interaction – Rabex-5:Polyubiquitin



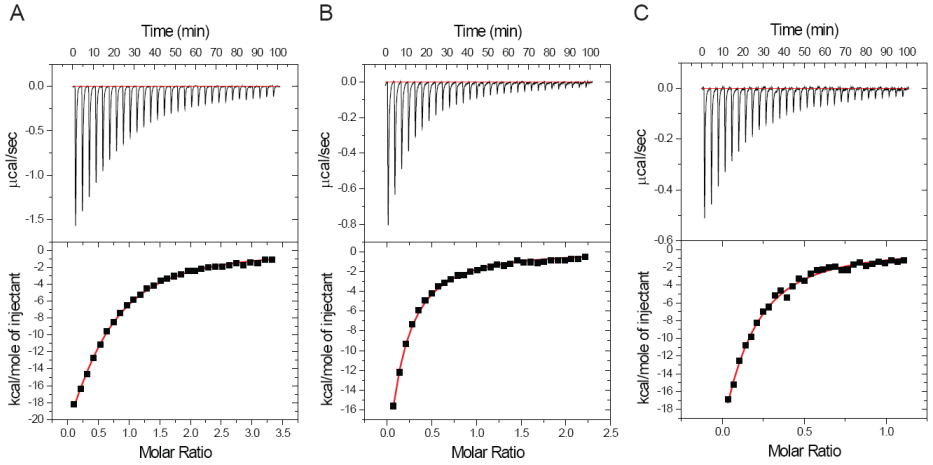


Table 1. Binding affinities of Rabex-5(9-73) mutants to linear tetraubiquitin

Rabex-5	Ubiquitin	K_d (μM) [a]
Rabex-5(9-73)Wild-type	Ub	18 ± 11
	Linear-Ub4	8 ± 8 [b]
Rabex-5(9-73)A58D	Ub	41 ± 20
	Linear-Ub4	6 ± 1
Rabex-5(9-73)Y25A/Y26A	Ub	21 ± 7
	Linear-Ub4	10 ± 2

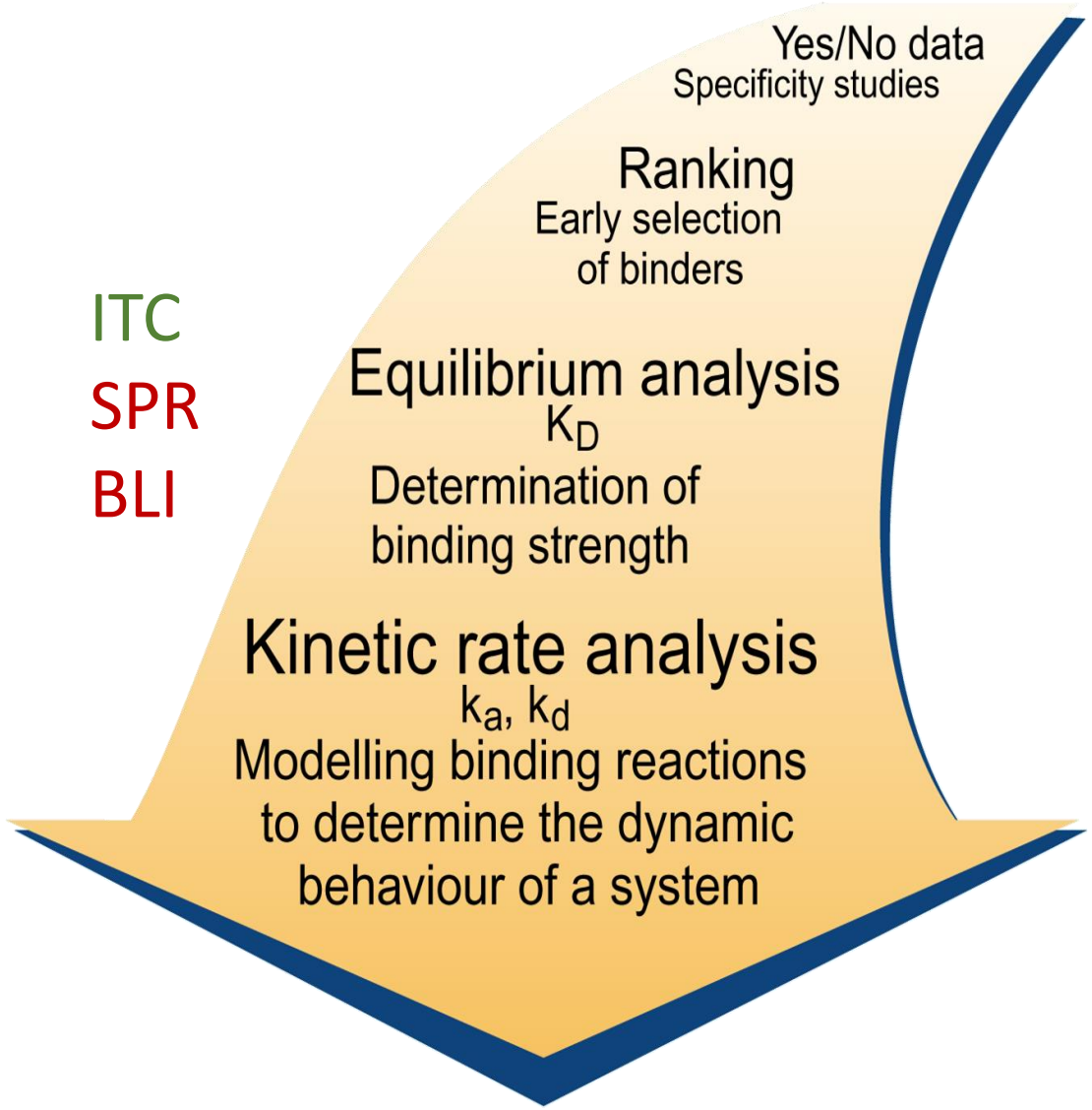
[a] Apparent dissociation constants based on a single-site model.

[b] Values calculated from experiments in duplicates.

[Donghyuk Shin, Sangho Lee et al. (2012) *Biochem. Biophys. Res. Commun.*]

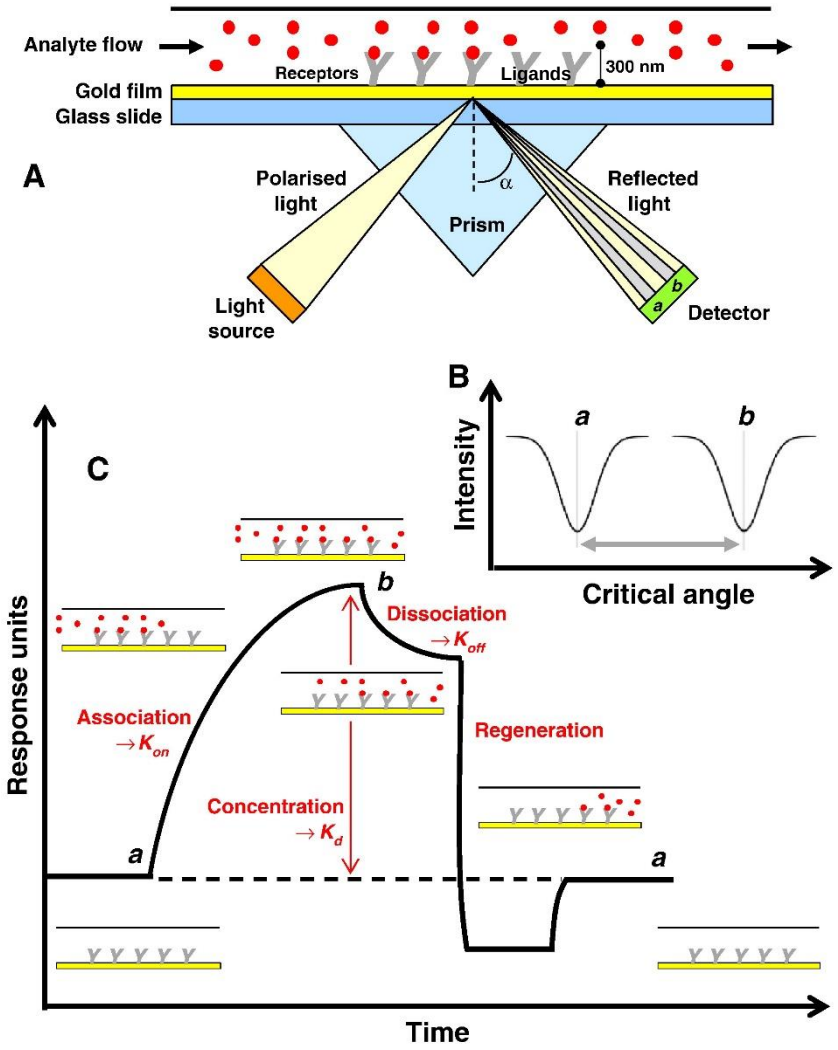
Surface plasmon resonance

Surface plasmon resonance (SPR): Assay objectives



[BiaCore]

Surface plasmon resonance (SPR): Theory

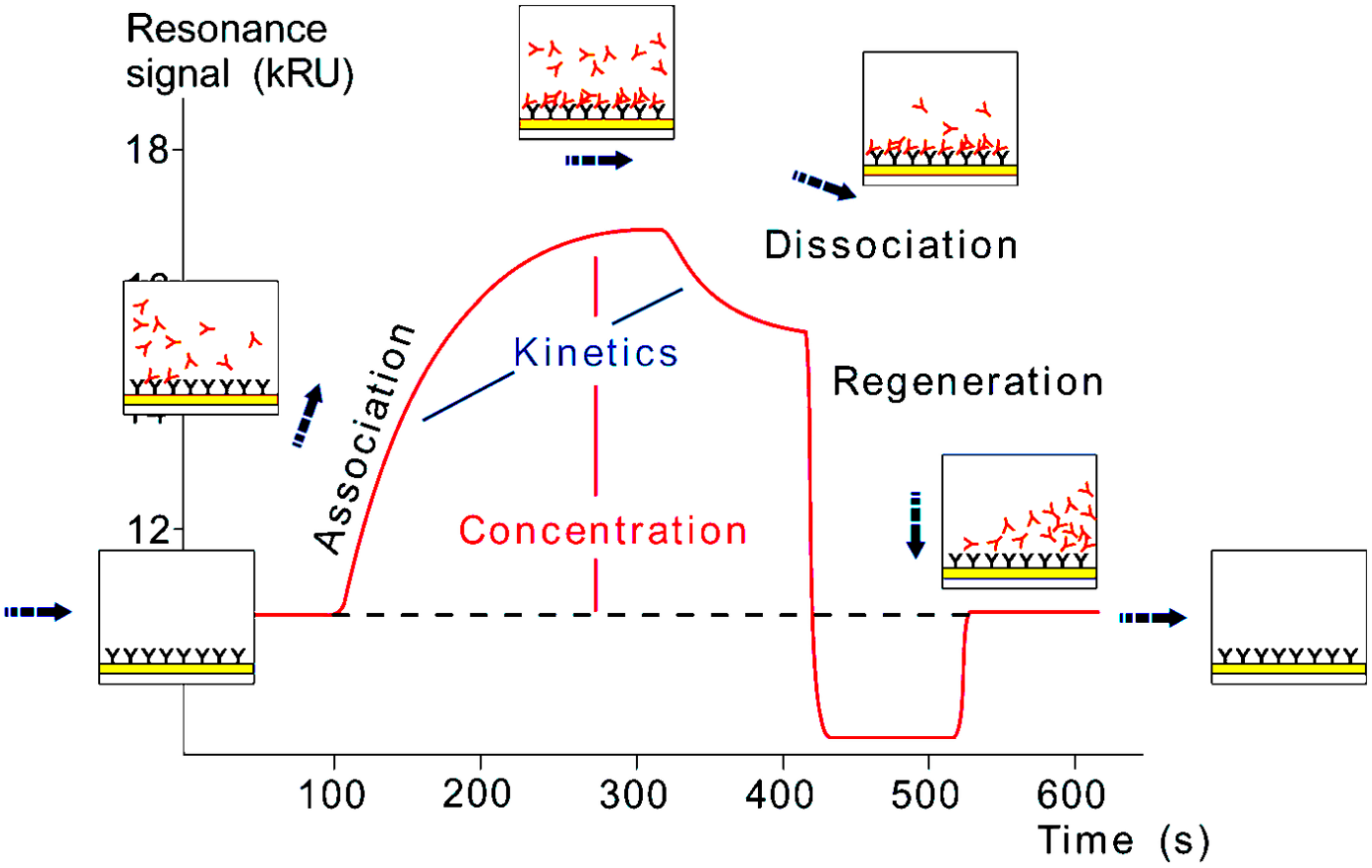


- To measure the refractive index near to a sensor surface
- Polarised light is directed through a prism to the under surface of the gold film where surface plasmons are generated at a critical angle of the incident light.
- This absorption of light is seen as a decrease in intensity of the reflected light. Resonance or response units (RU) are used to describe the increase in the signal, where 1 RU is equal to a critical angle shift of 10^{-4} deg or 10^{-12} g mm^{-2}.
- When a steady-state is achieved (all binding sites occupied), the maximum RU is determined (*n*: No. of binding sites in Ligand)

$$RU_{max} = nRU_L\left(\frac{MW_A}{MW_L}\right)$$

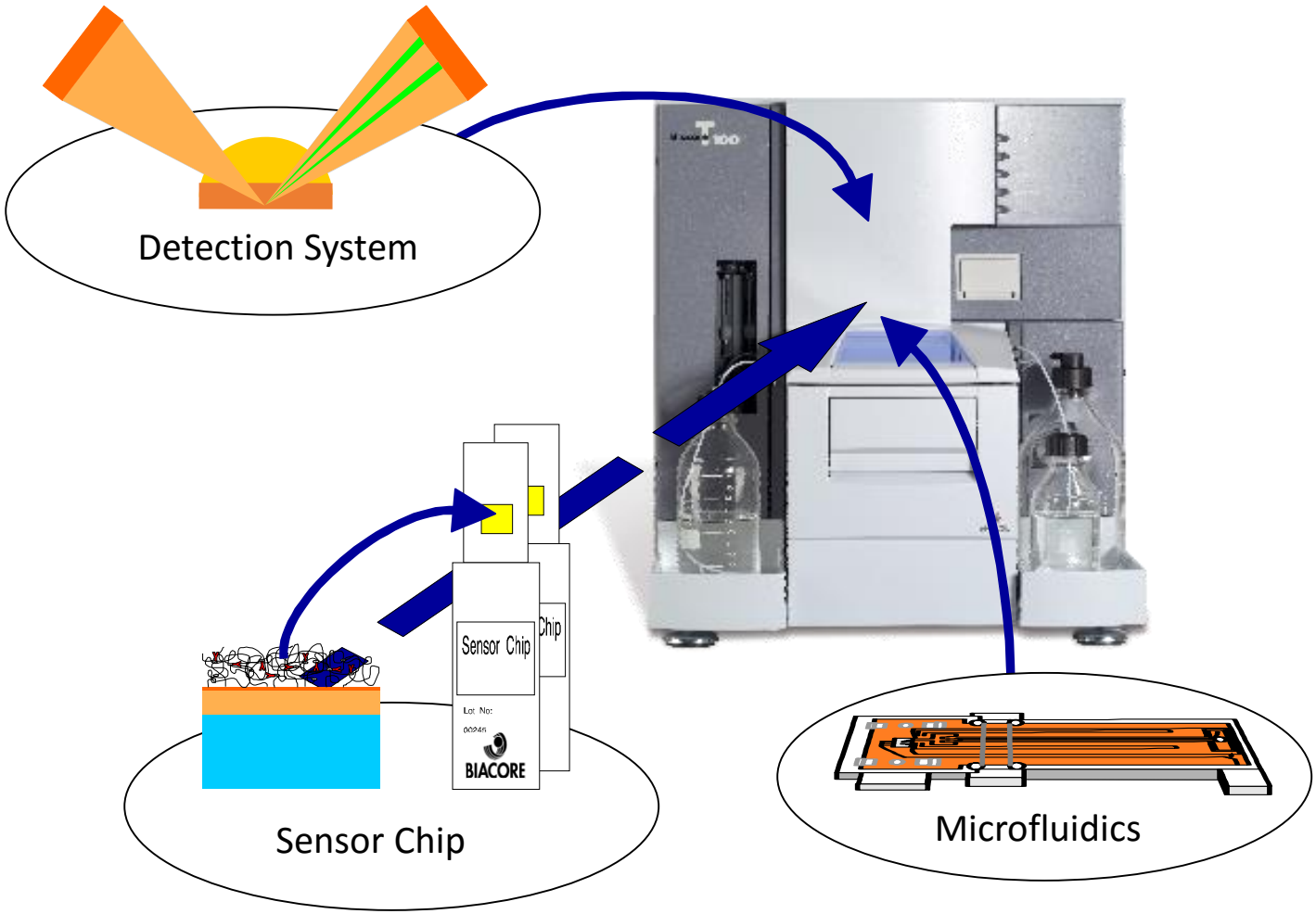
[Patching, *Biochim. Biophys. Acta* (2014)]

Surface plasmon resonance (SPR): Sensorgram



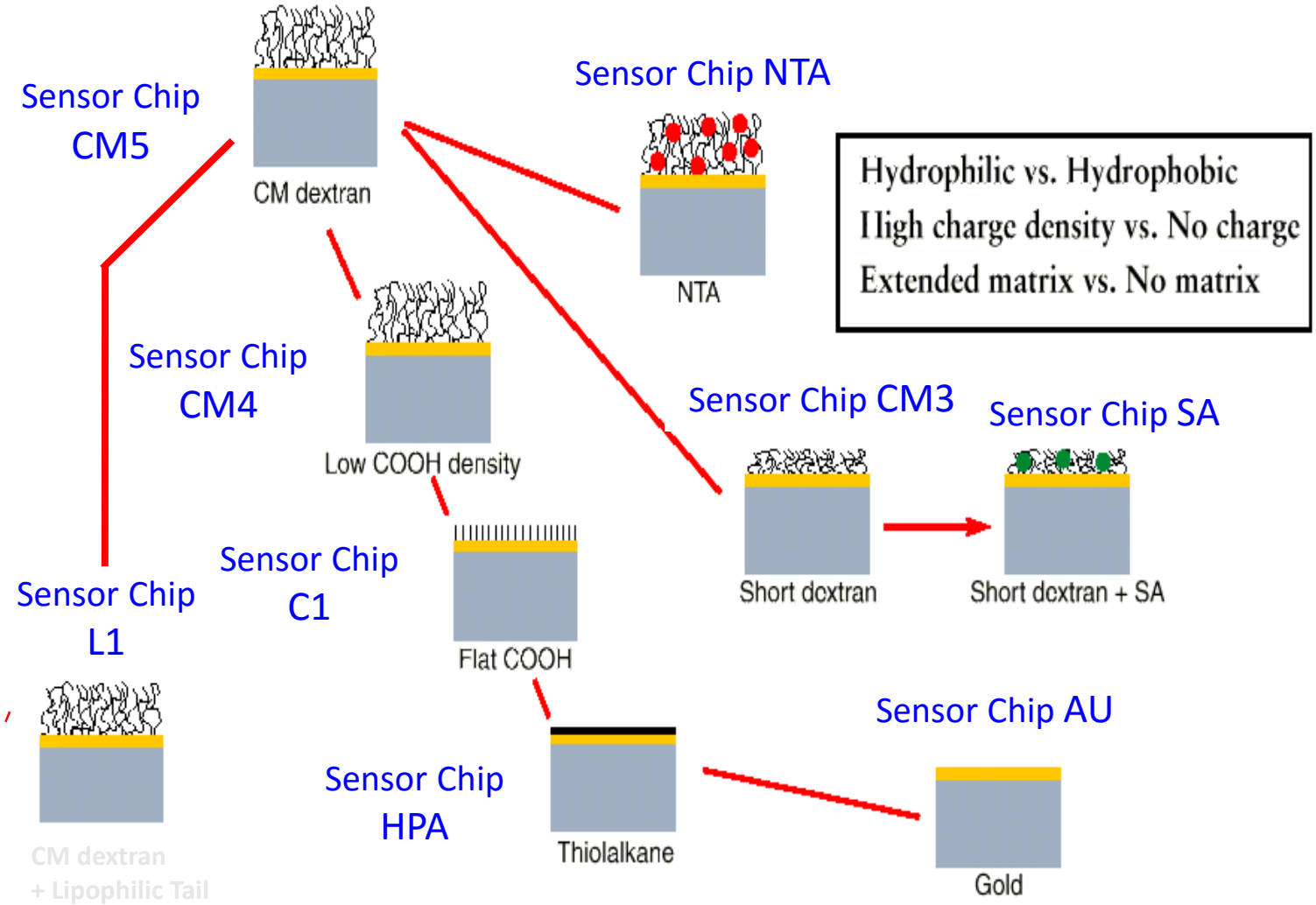
[BiaCore]

Surface plasmon resonance (SPR): Components



[BiaCore]

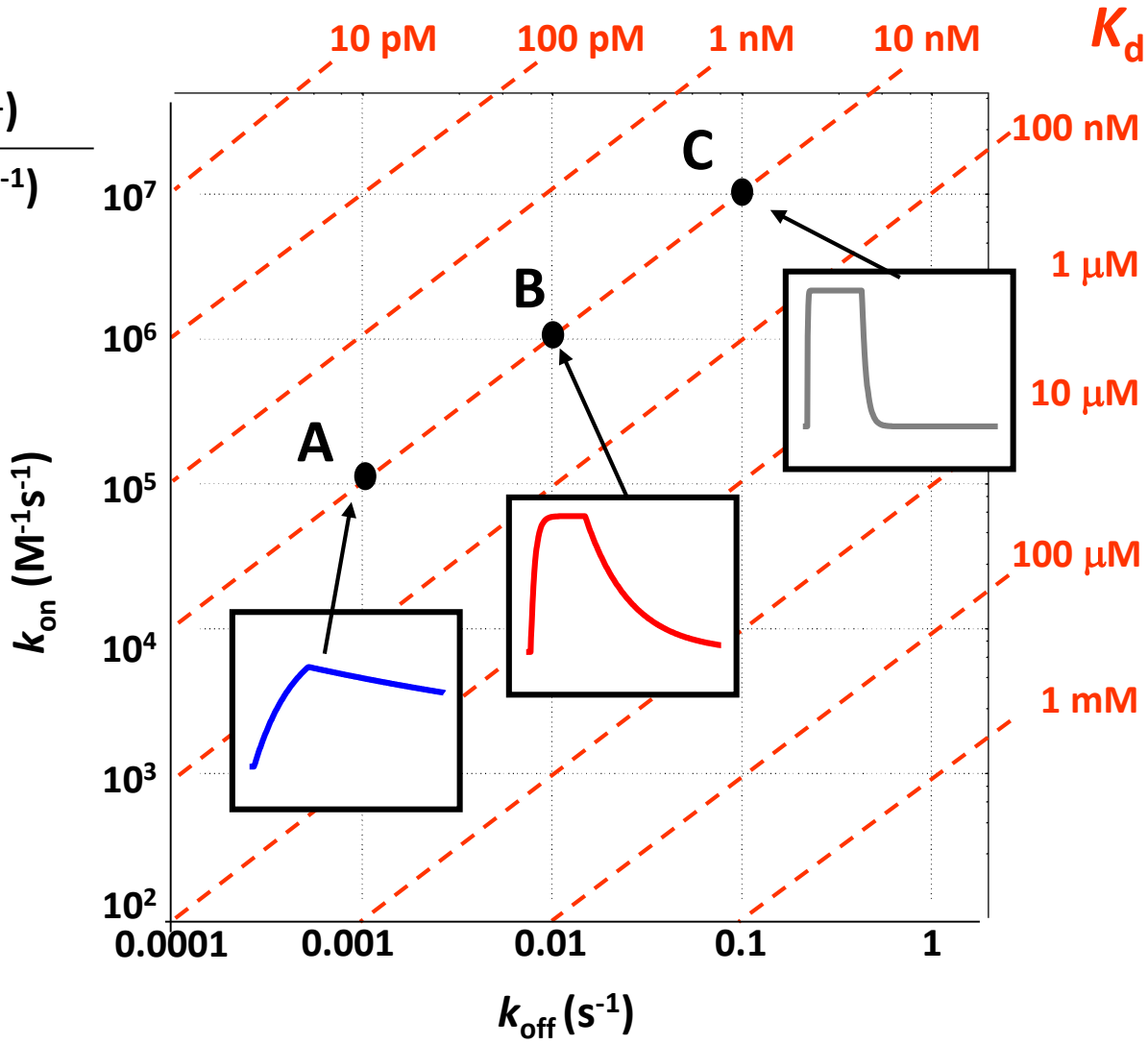
Surface plasmon resonance (SPR): Sensor chips



[BiaCore]

Kinetic analysis: Why important?

$$K_d = \frac{k_{off}\,(s^{-1})}{k_{on}\,(M^{-1}s^{-1})}$$



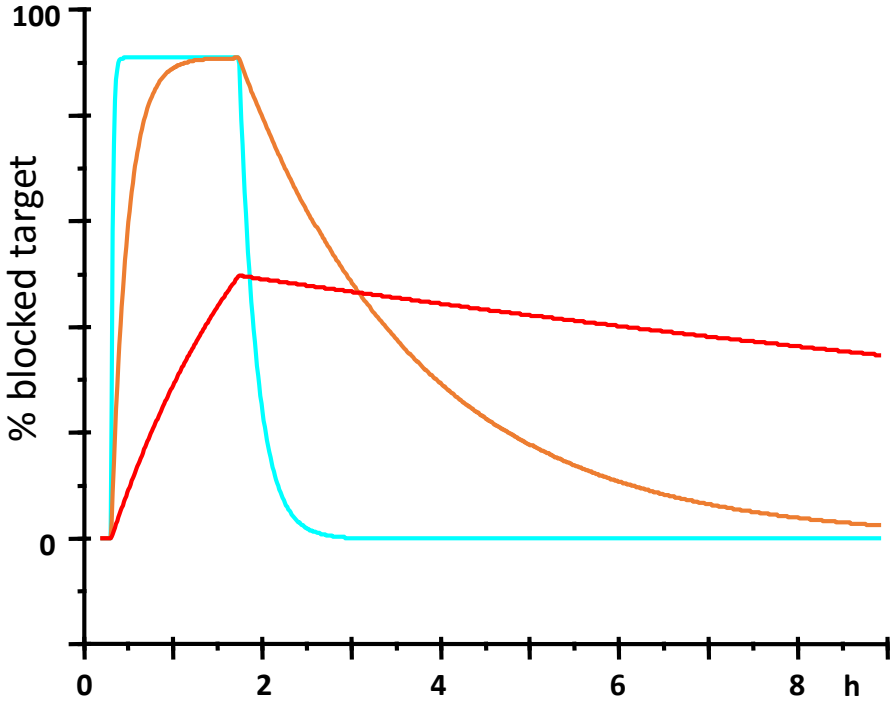
[BiaCore]

Kinetic analysis: Same affinity, different kinetics

Compare sensorgrams for three different interactions

- Same 1 nM affinity (K_d)
- Different kinetics

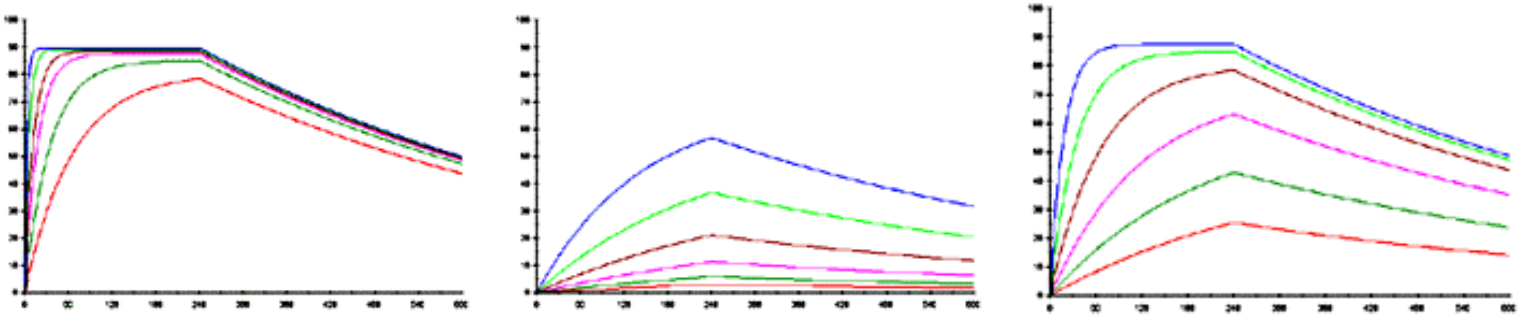
$$K_d = \frac{k_d}{k_a}$$



[BiaCore]

Things to consider: Analyte concentration

- Run analyses over a wide range of analyte concentrations, ideally 100-fold or more: The range should span 10x below the K_d to 10x above the K_d.
- Accurate analyte concentration is critical!
- Include a zero-concentration sample in the analyses.



Too high concentration

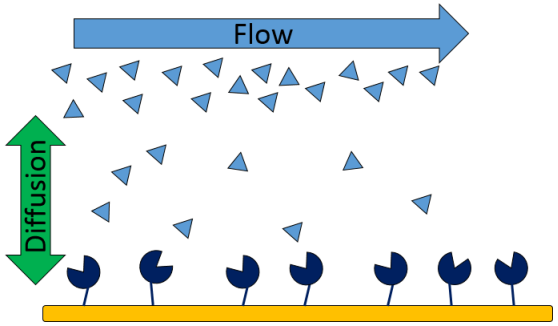
Too low concentration

Optimized

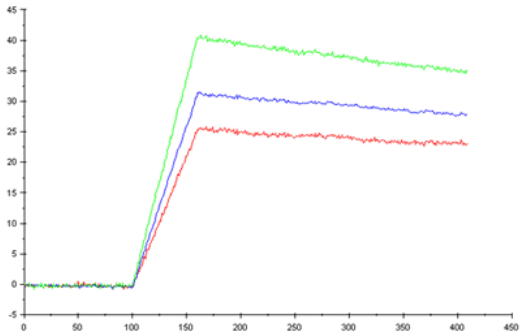
[BiaCore]

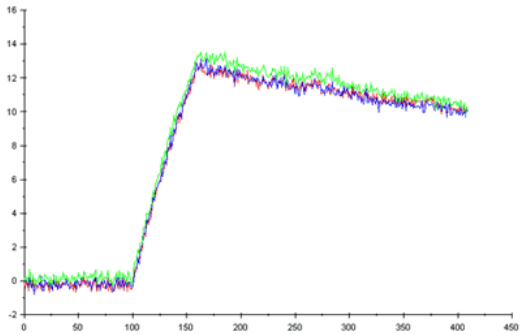
Things to consider: Mass transfer

- If the diffusion rate is slower than the association rate, mass transfer effects can be observed
- Low RU_L reduces analyte consumption in “no-flow zone”
- Apparent rate constants are smaller when mass transport limited binding occurs (inaccurate kinetic data)
- Work-arounds: higher flow rates, lowest ligand density



At different flow rates





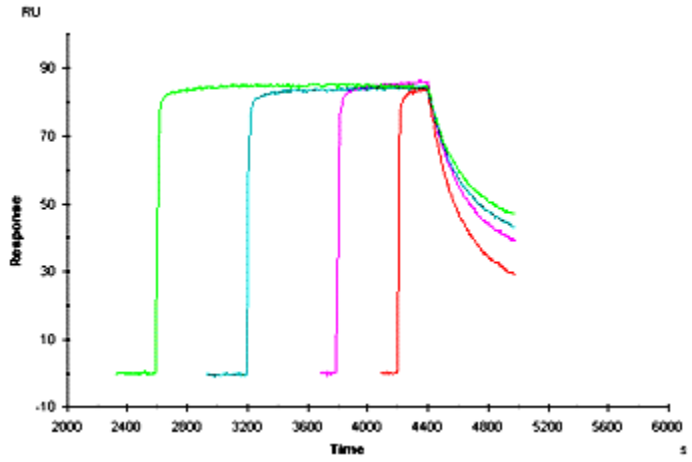
Mass transfer limitation

No limitation

[BiaCore]

Things to consider: Conformational changes

- Conformational changes during interaction may cause kinetic parameters to change
- Inject analyte at a fixed concentration
- Vary contact times
- Overlay the sensorgrams



Do relative dissociation rates change?
If so, a conformational change is occurring.

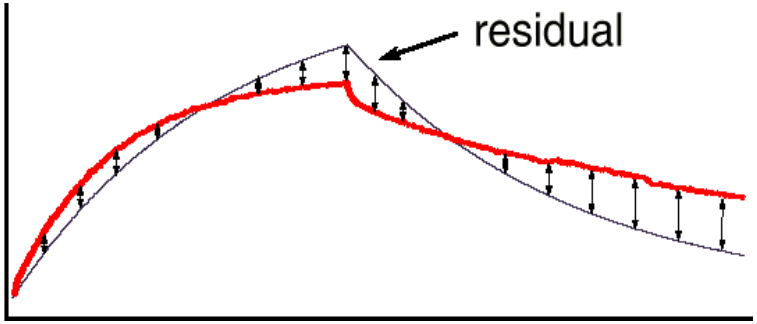
Confirm with other techniques.

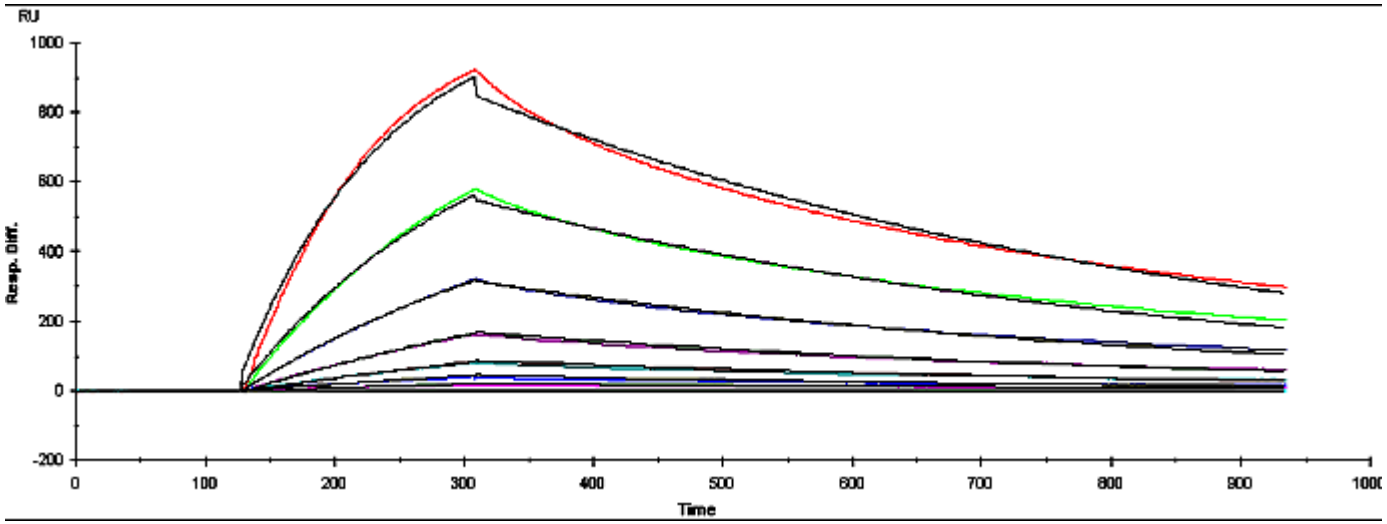
[BiaCore]

Data analysis: Curve fitting in kinetic analysis

k_{on}, k_{off}, and RU_{max} are calculated by global curve fitting

$$A + B \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} AB$$





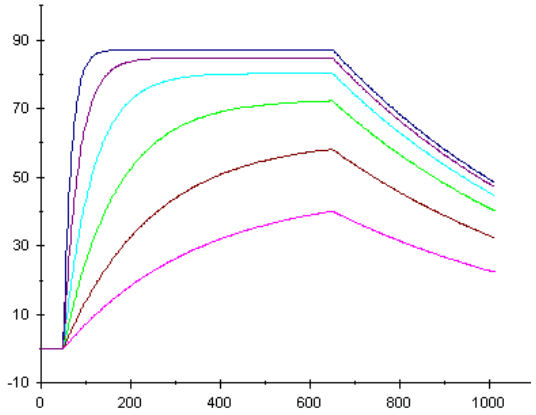
[BiaCore]

Data analysis: Steady-state affinity determination

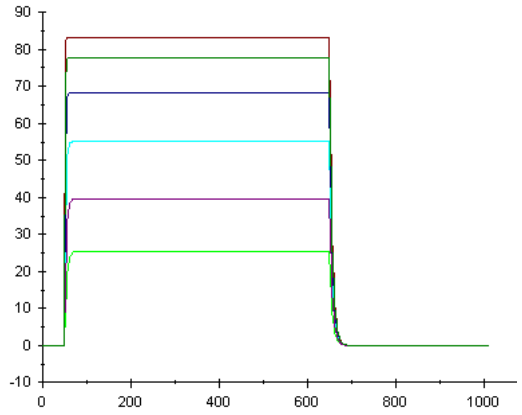
- Kinetic determinations give an independent value

$$K_a = \frac{k_{on}}{k_{off}} \qquad K_d = \frac{k_{off}}{k_{on}}$$

- Steady-state response levels give one value for affinity constants
- Steady-state can be used for fast interactions where kinetics are not available



Kinetics and affinity

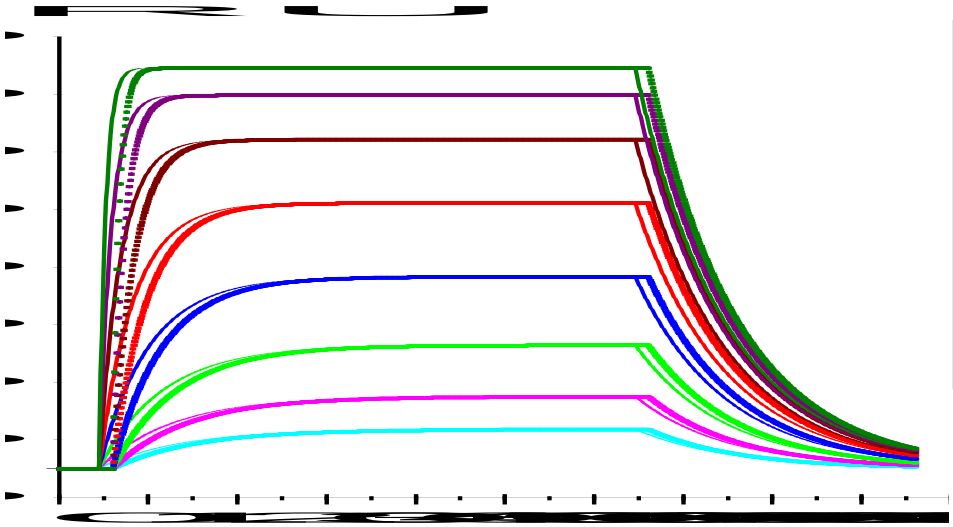


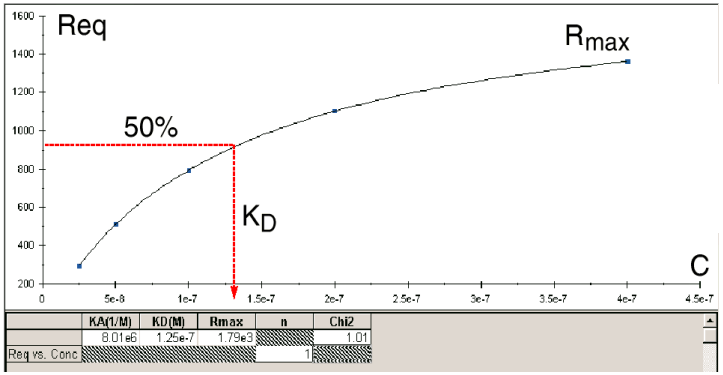
Affinity only

[BiaCore]

Data analysis: Steady-state affinity determination

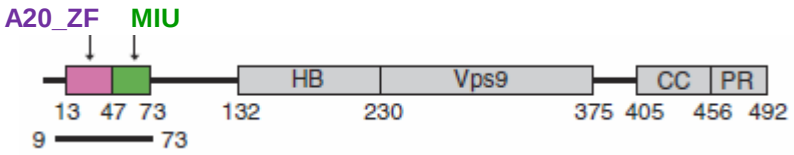
- **Response at equilibrium can be plotted against the concentration to determine the affinity**
  - Response should be at or close to equilibrium at all concentrations for a reliable measurement



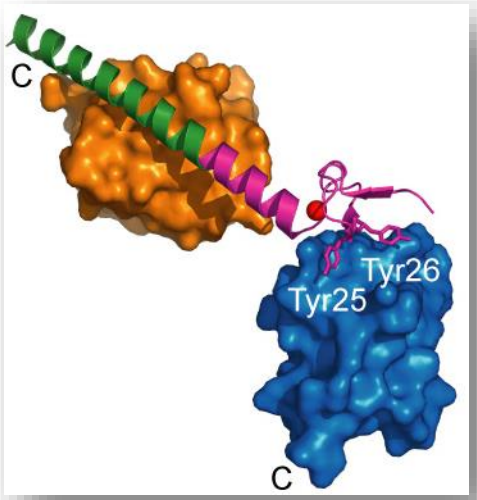


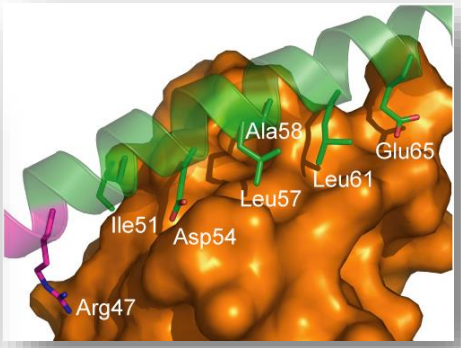
[BiaCore]

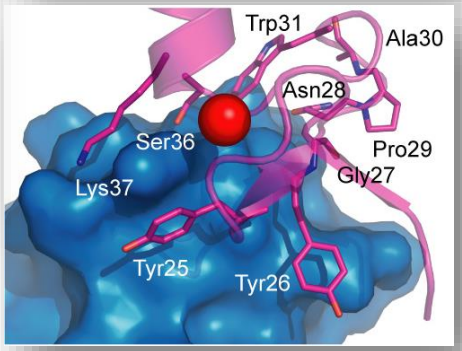
Qualitative and quantitative interaction analysis: Rabex-5 and ubiquitin

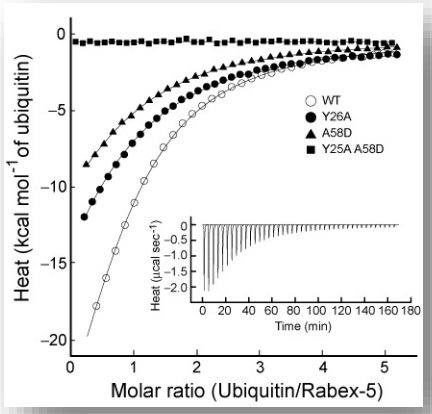


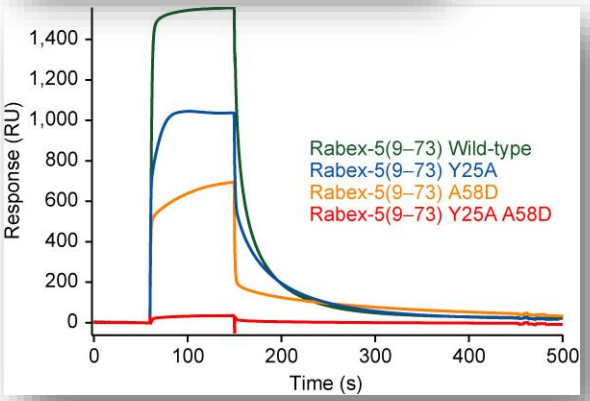
- Rabex-5: guanine exchange factor (GEF) for Rab5 in intracellular trafficking
- Two ubiquitin binding domains: A20_ZF, MIU







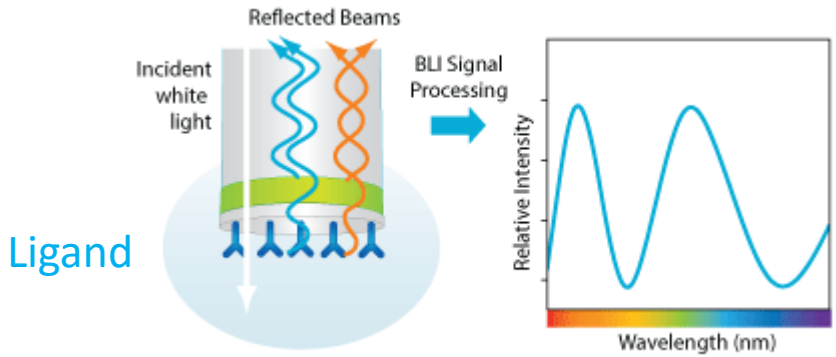




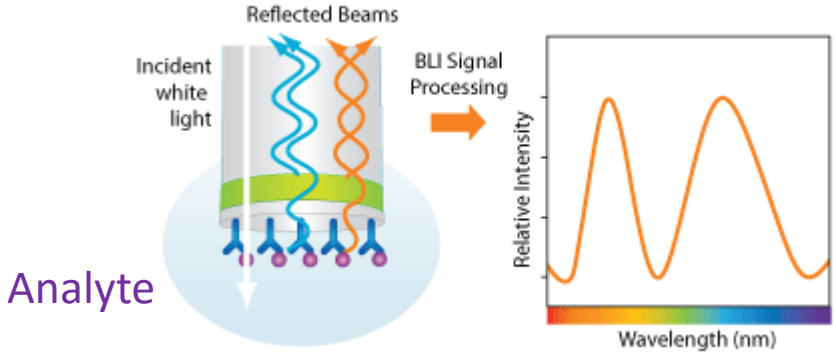
[Sangho Lee et al. (2006) *Nat. Struct. Mol. Biol.*]

Biolayer interferometry

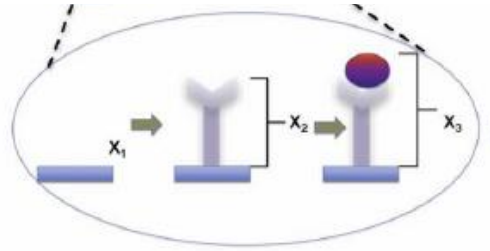
Biolayer interferometry (BLI): Theory



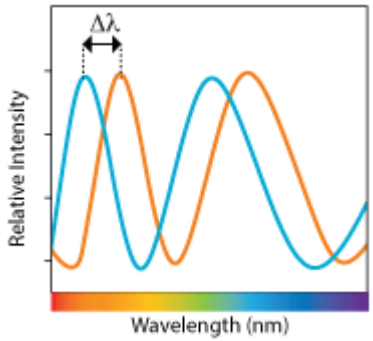
Ligand



Analyte



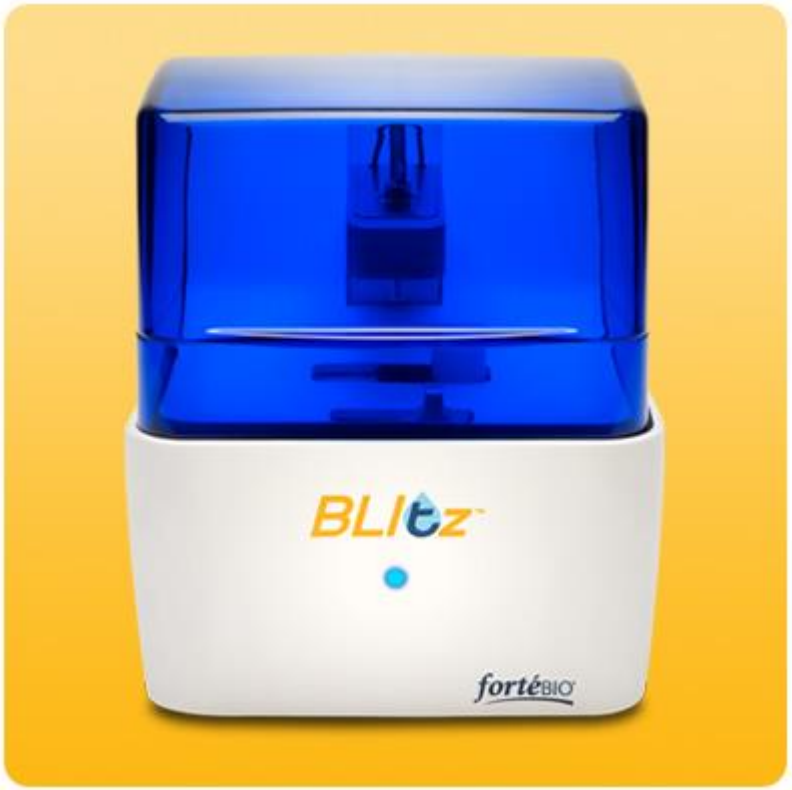
igand:Analyte



Optical thickness change at the sensor tip due to binding causes wavelength shift $\Delta\lambda$

[ForteBio; Citartan et al. *Analyst* (2013)]

BLI: Experimental platforms

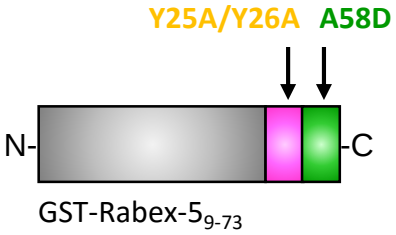


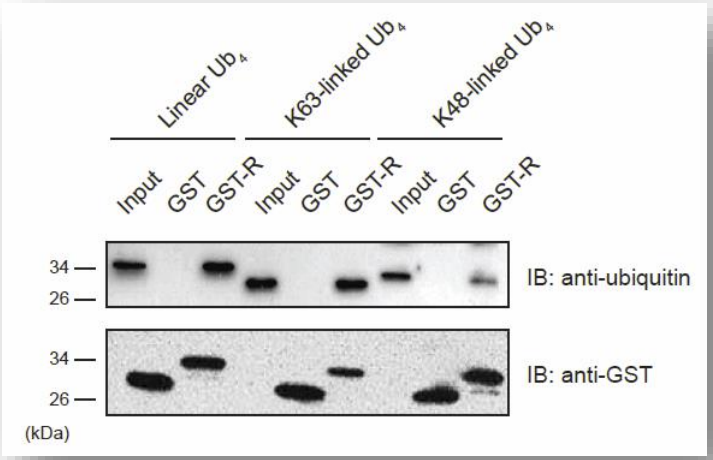
[ForteBio]

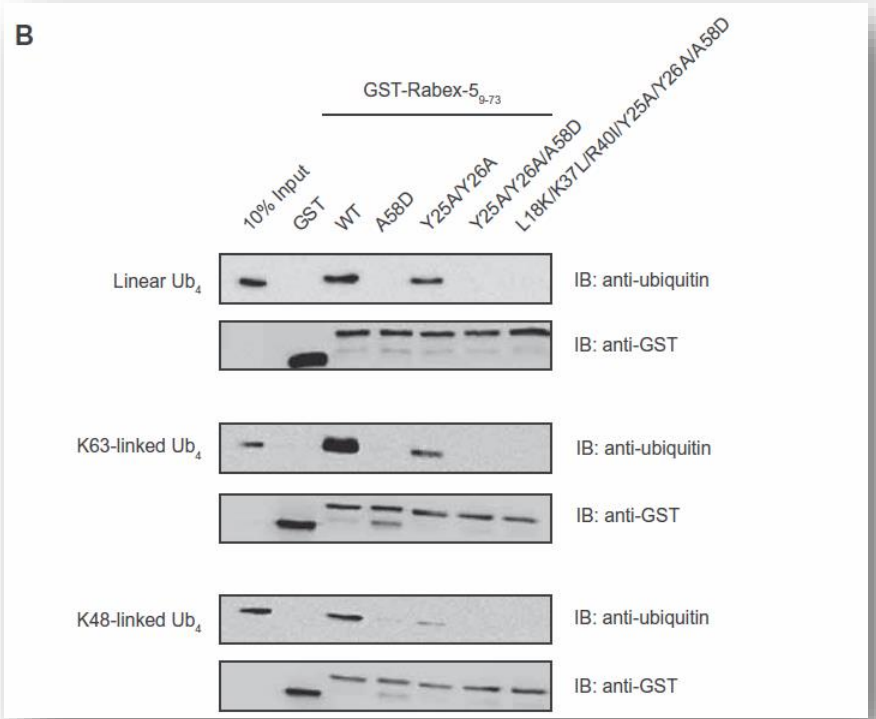
BLI: Practical considerations

- pH Scouting is done for optimal ligand immobilization on a sensor.
- Molecular weight of the analyte matters.
- Choice of data analysis method (kinetic or steady state) depends on the nature of protein interactions.

BLI example system: Rabex-5 and polyubiquitin

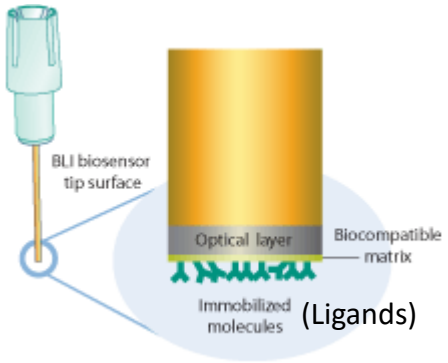


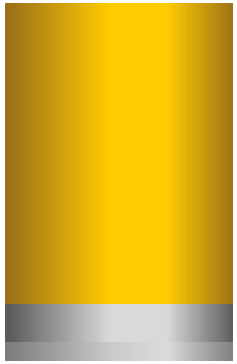




[Donghyuk Shin, Sei Young Lee, Shuo Ren, Soyoun Kim, Yoshikatsu Aikawa, and Sangho Lee (2012) *Biochem. Biophys. Res. Commun.*]

Experimental design





Analytes

GST-Rabex-$5_{9\text{-}73}$ N- -C

GST

Rabex-$5_{9\text{-}73}$

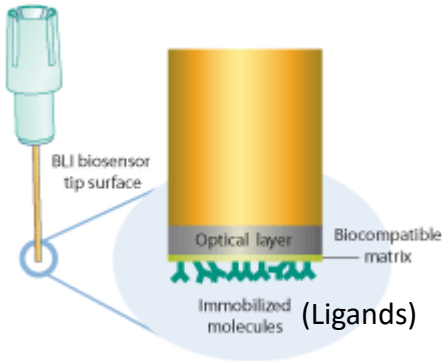
Ligands

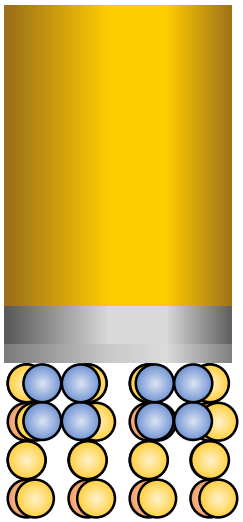
Linear Ub_4

K63-linked Ub_4

K48-linked Ub_4

Experimental design





Analytes

GST-Rabex-$5_{9\text{-}73}$ N- -C

GST

Rabex-$5_{9\text{-}73}$

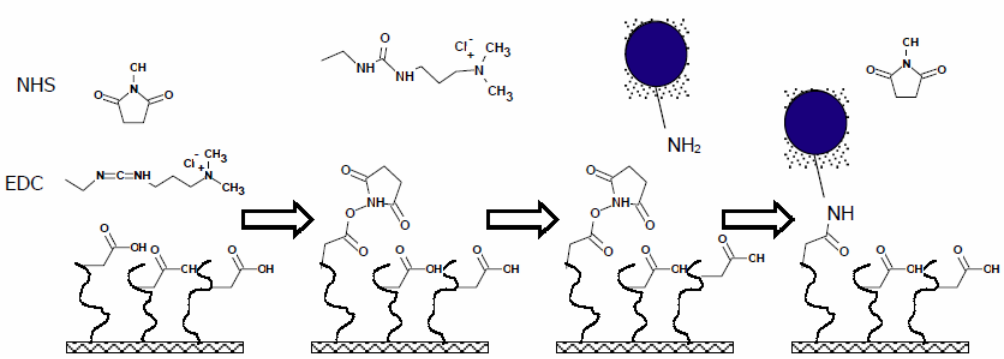
Ligands

Linear Ub_4

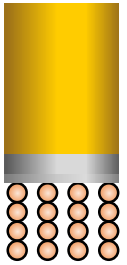
K63-linked Ub_4

K48-linked Ub_4

Ligand immobilization: pH Scouting



[Thermo.com]



0.25 µM Linear Ub_4

3. **Ligand preparation** - Dilute the ligand to be immobilized in 100 mM MES at the appropriate pH. Follow the guidelines below for selecting the appropriate pH of MES buffer.

   c. If the pI of the protein is unknown it may be necessary to test the immobilization of the ligand at all three pHs provided (pH 4.0, 5.0, 6.0).

fortéBIO

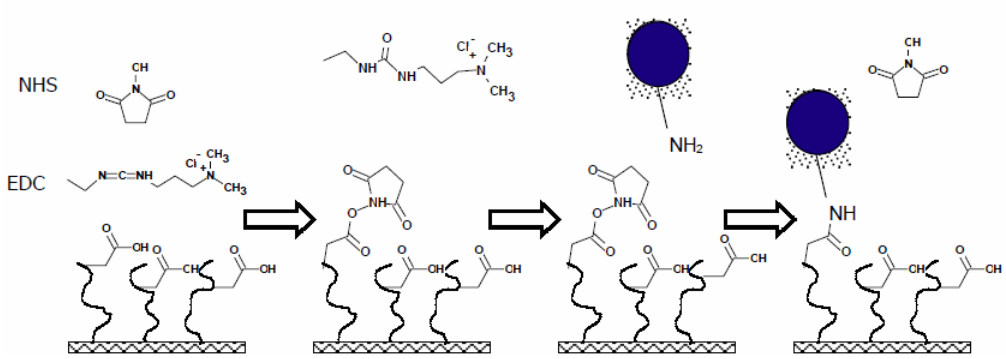
Amine Coupling Reagent Kit

Reagent Kit for use with Amine Reactive Biosensors
Product Code: 18-5017

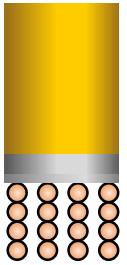
Table 1: Buffer formulation for creation of 10 mL of 100 mM MES at the pH indicated.

Target pH	Volume of 100 mM MES pH 4.0 (mL)	Volume of 100 mM MES pH 5.0 (mL)	Volume of 100 mM MES pH 6.0 (mL)
4.1	9.45	0.55	----
4.2	9.2	0.8	----
4.3	8.7	1.3	----
4.4	8.1	1.9	----
4.5	7.3	2.7	----
4.6	5.4	4.6	----
4.7	4.9	5.1	----
4.8	4.0	6.0	----
4.9	3.3	6.7	----
5.1	----	9.4	0.6
5.2	----	8.9	1.1
5.3	----	8.2	1.8
5.4	----	7.5	2.5
5.5	----	6.2	3.8
5.6	----	5.1	4.9
5.7	----	3.9	6.1
5.8	----	2.6	7.4
5.9	----	1.2	8.8

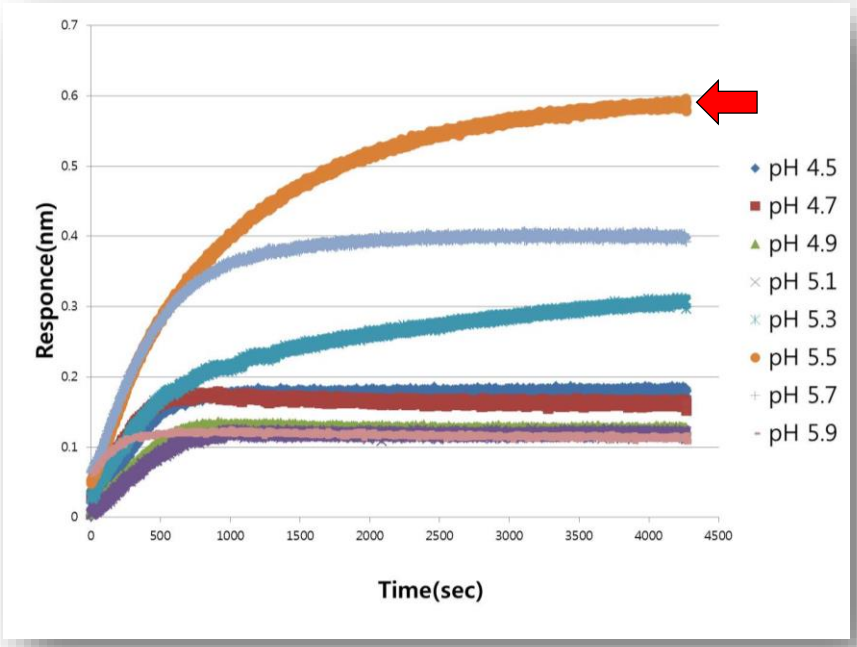
Ligand immobilization: pH Scouting

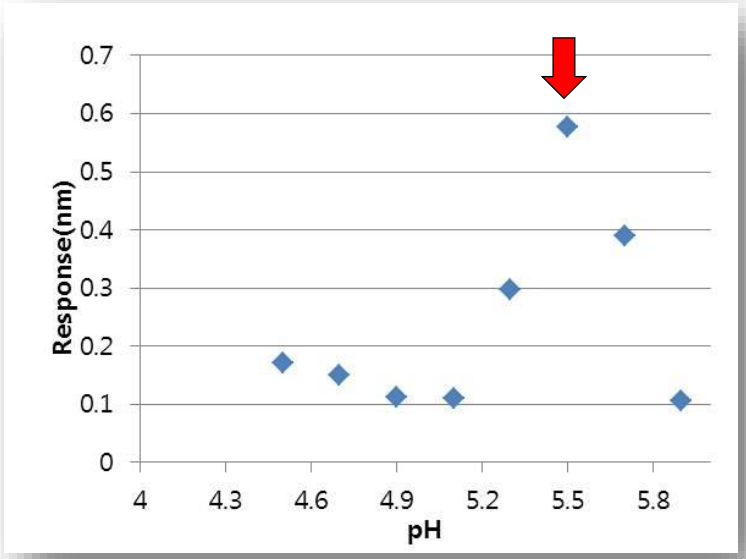


[Thermo.com]



0.25 µM Linear Ub_4

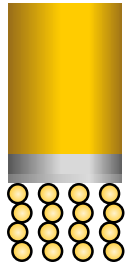




Analyte selection: Size matters

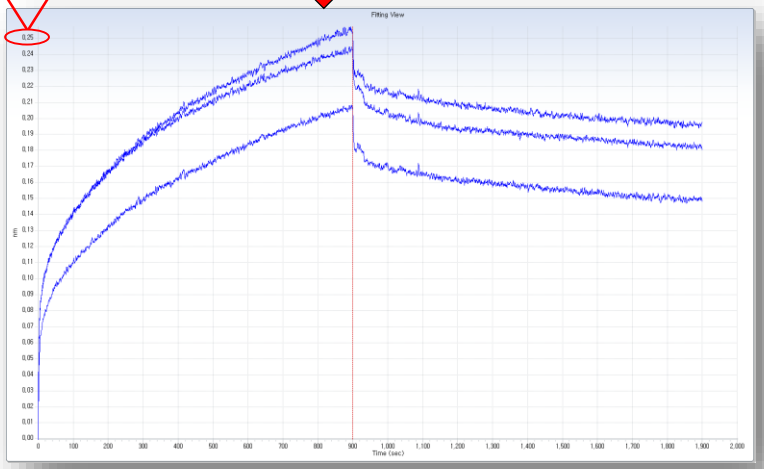
Rabex-5_{9-73} 

GST-Rabex-5_{9-73} N-  -C

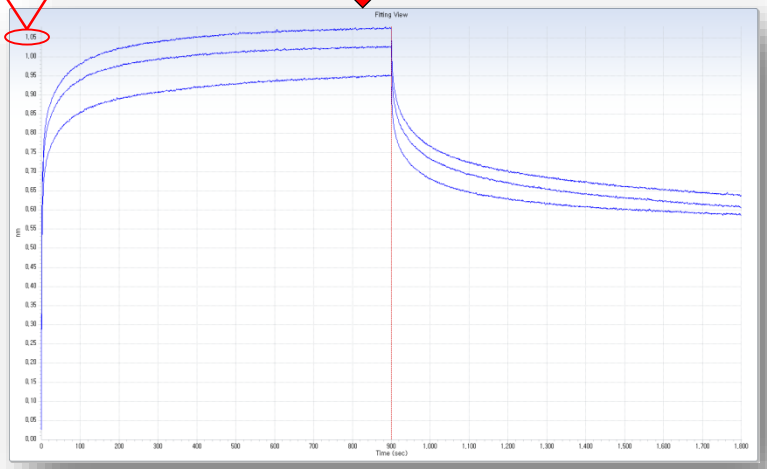


0.25 µM K63-linked Ub_4

0.25 **No** saturation

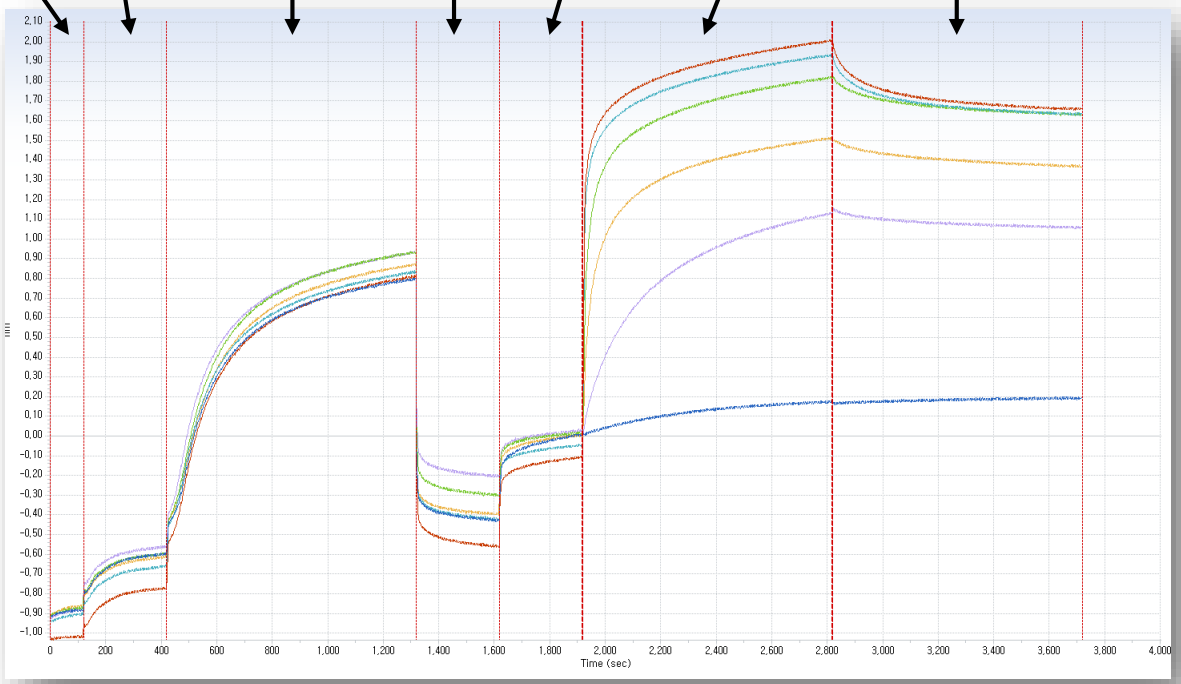


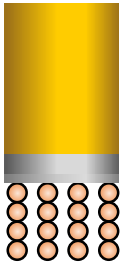
1.05 Saturation



Full sensorgram: Everything optimized

Baseline Activation **Loading** Quenching Baseline **Association** Dissociation





0.25 µM Linear Ub_4

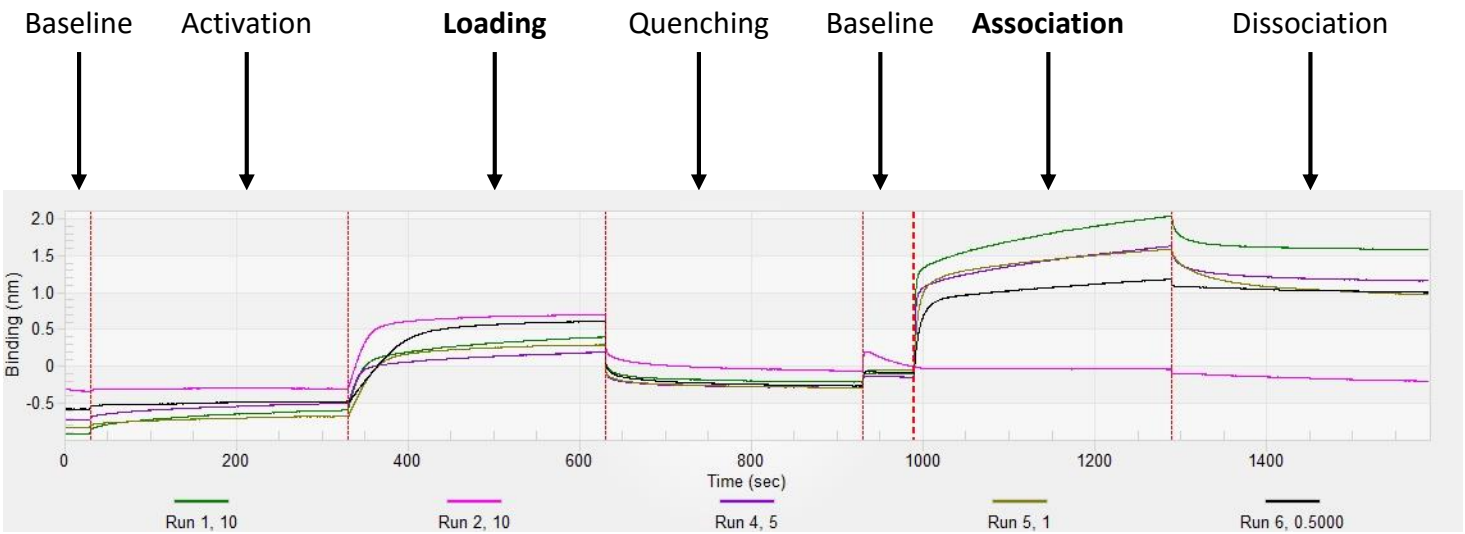
GST-Rabex-5_{9-73}

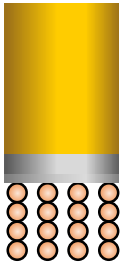
10 µM
5 µM
1 µM
0.5 µM
0.1 µM

GST

10 µM

Full sensorgram: Everything optimized





0.25 µM Linear Ub_4

GST-Rabex-5_{9-73}

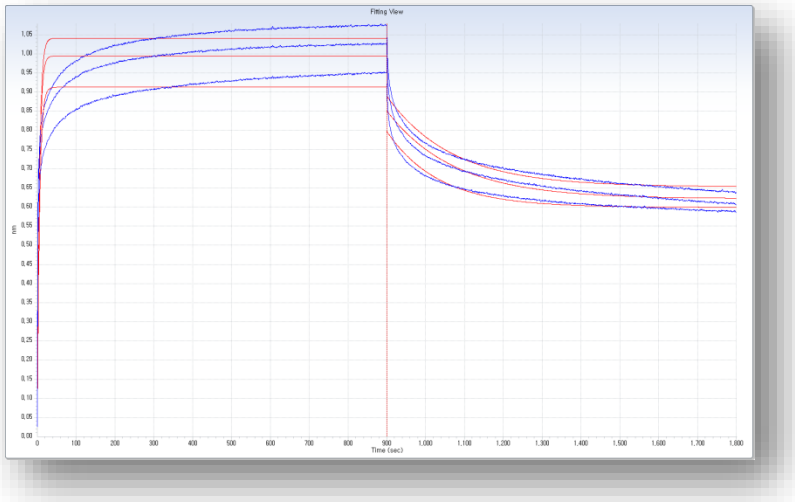
10 µM
5 µM
1 µM
0.5 µM

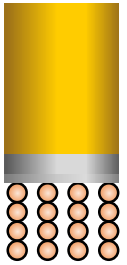
GST

10 µM

Data analysis: Kinetic *vs.* steady-state

Kinetic

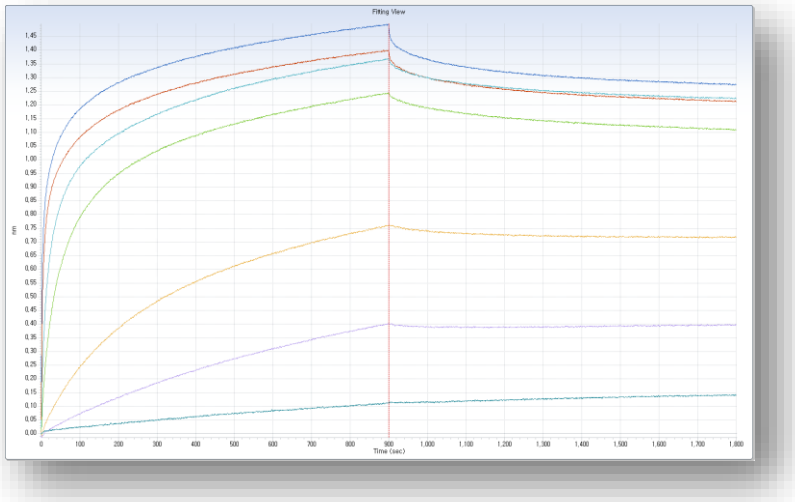


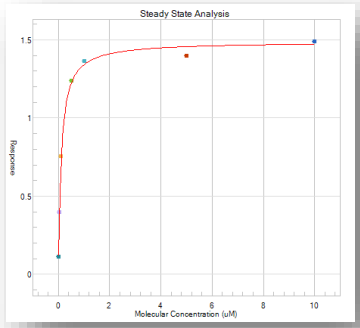


0.25 µM Linear Ub_4

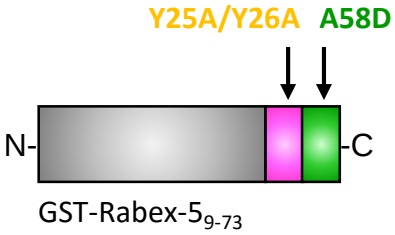
GST-Rabex-5_{9-73}

Steady-state





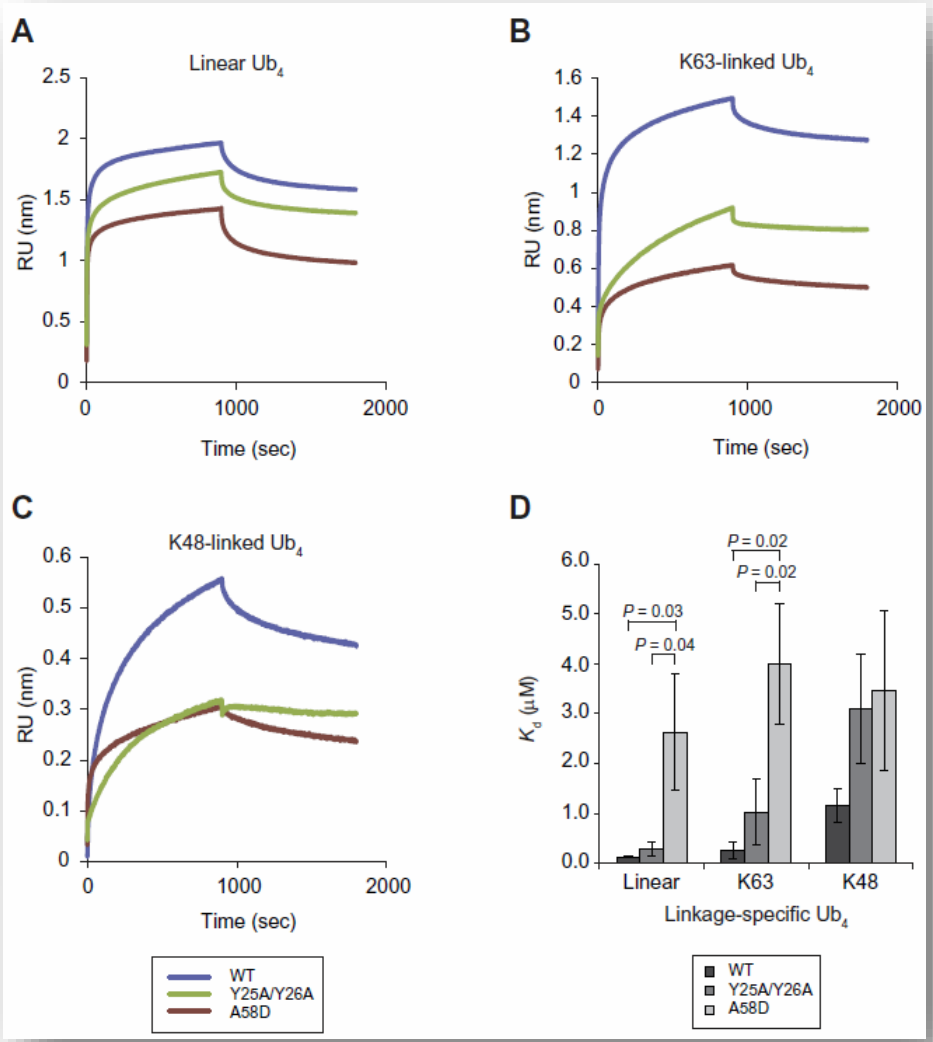
Qualitative and quantitative interaction analysis: Rabex-5 MIU domain and polyubiquitin



Binding affinities of Rabex-5_{9-73} to linkage-specific tetraubiquitin chains[a].

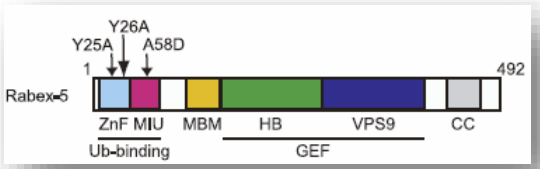
Rabex-5_{9-73} mutant	K_d (μM)[b]		
	Linear Ub_4	K63-linked Ub_4	K48-linked Ub_4
WT	0.13 ± 0.02	0.26 ± 0.17	1.2 ± 0.33
Y25A/Y26A	0.28 ± 0.14	1.0 ± 0.65	3.1 ± 1.1
A58D	2.6 ± 1.2	4.0 ± 1.2	3.5 ± 1.6

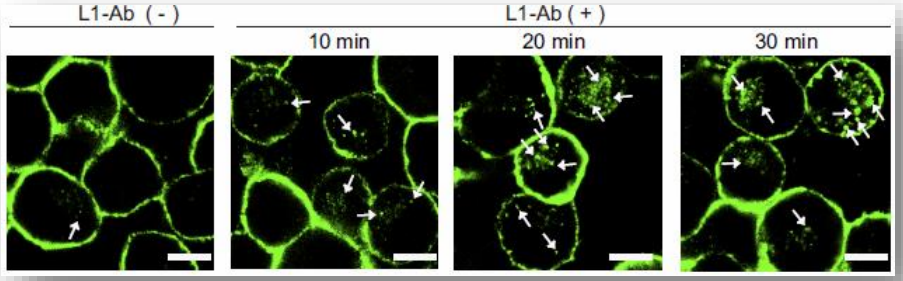
[a] Each linkage-specific Ub_4 chain was immobilized on a sensor and GST-Rabex-5_{9-73} mutants were added.
[b] K_d values were calculated based on triplicate data and limited to two significant figures.

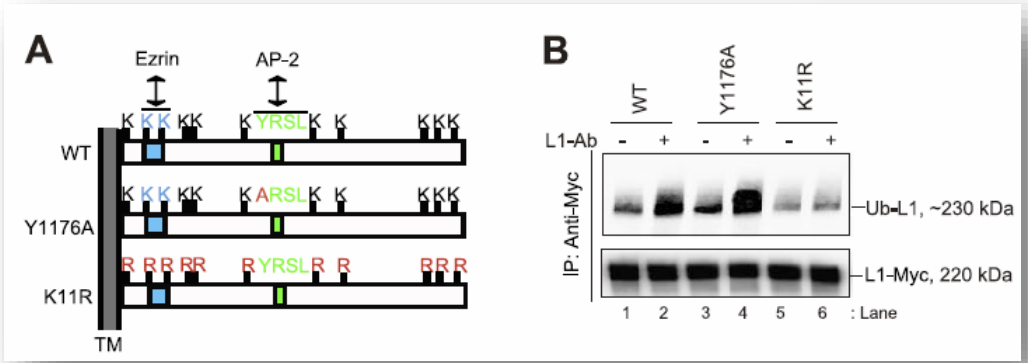


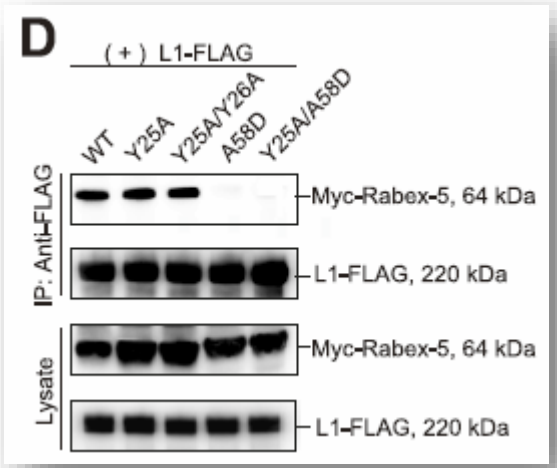
[Donghyuk Shin, Sei Young Lee, Shuo Ren, Soyoun Kim, Yoshikatsu Aikawa, and Sangho Lee (2012) *Biochem. Biophys. Res. Commun.*]

Qualitative and quantitative interaction analysis: Rabex-5 MIU domain and polyubiquitin





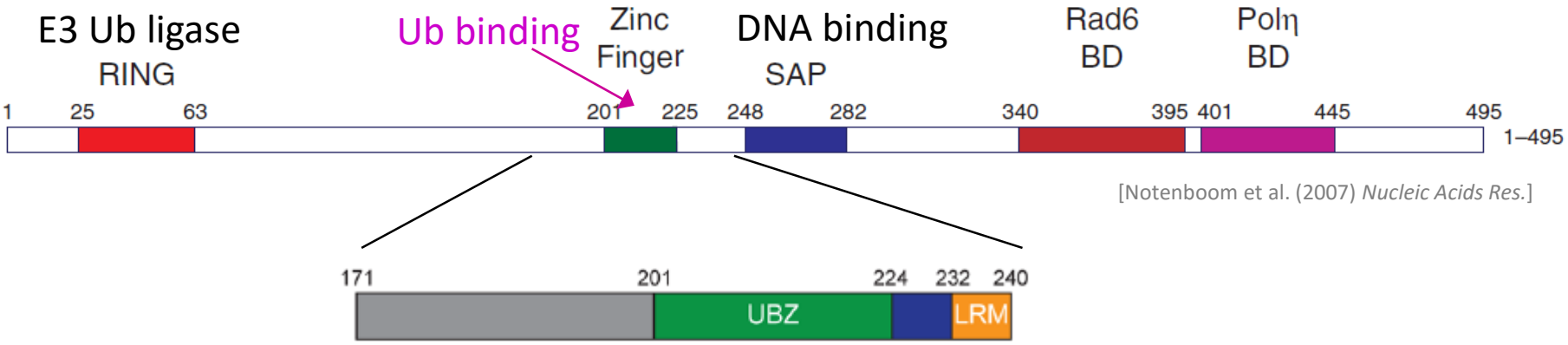




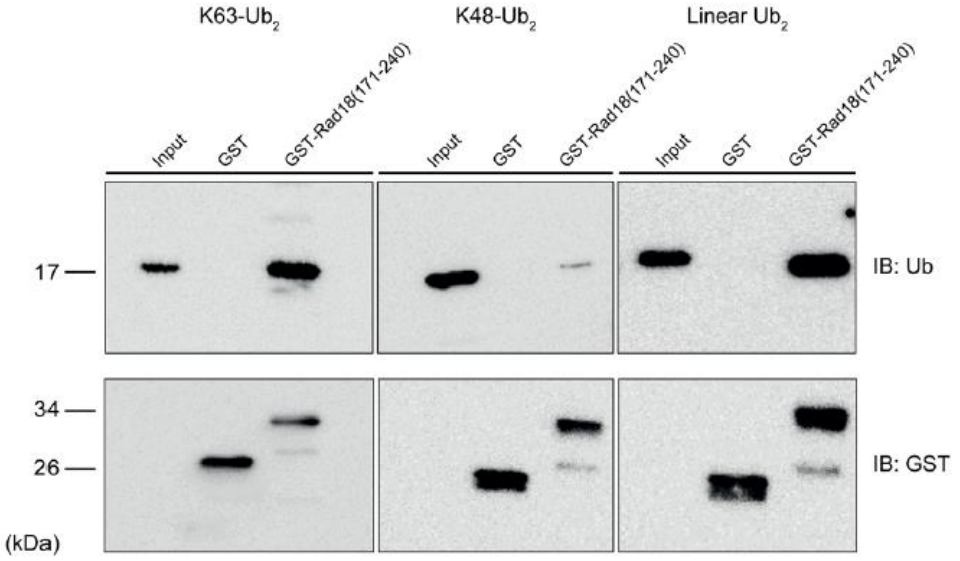
[Yoshikatsu Aikawa, Sangho Lee et al. (2012) *J. Biol. Chem.*]

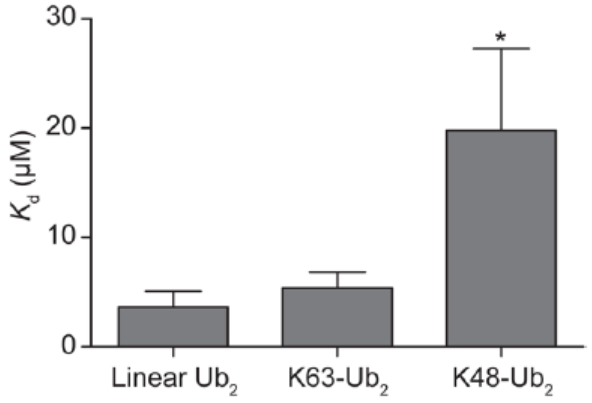
Real-life example: hybrid approach

Regions between UBZ and LRM of Rad18 Are Involved in Polyubiquitin Recognition



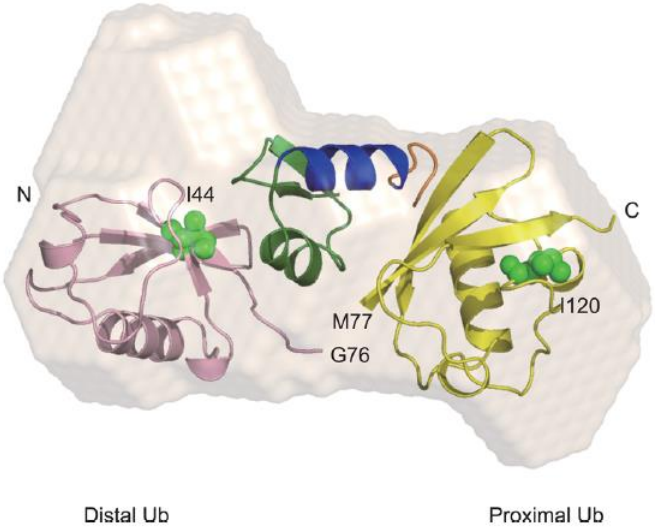
[Notenboom et al. (2007) *Nucleic Acids Res.*]

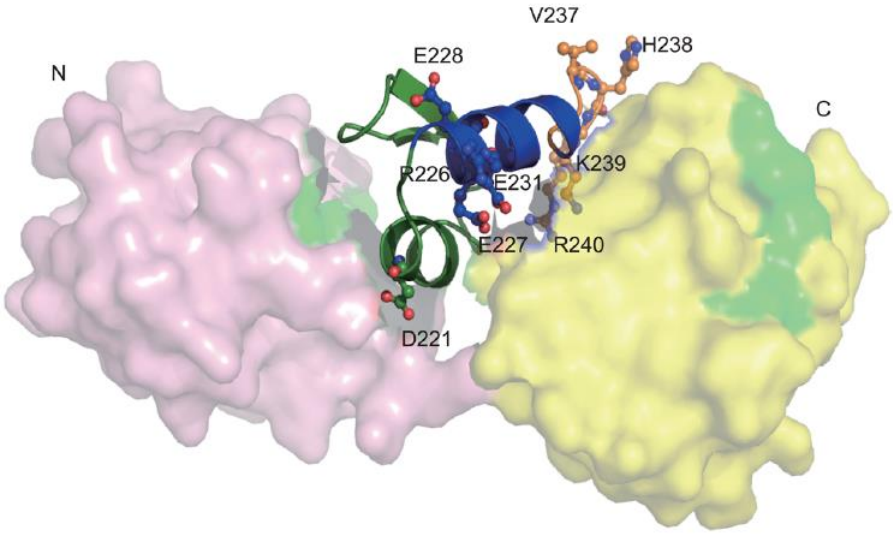


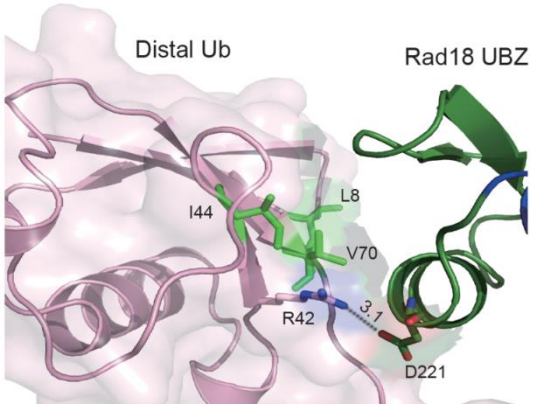


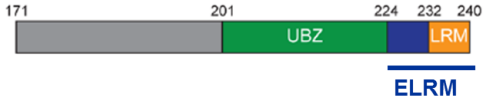
[Trung Thanh Thach, Namsoo Lee, Donghyuk Shin, Seungsu Han, Gyuhee Kim, Hongtae Kim, and Sangho Lee (2015) *Biochemistry*]

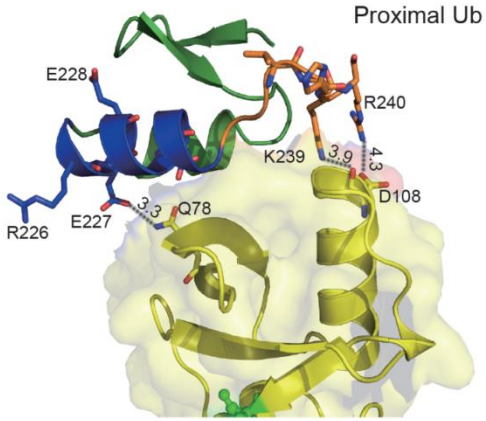
SAXS-based model for Rad18(201-240):Linear Ub_2





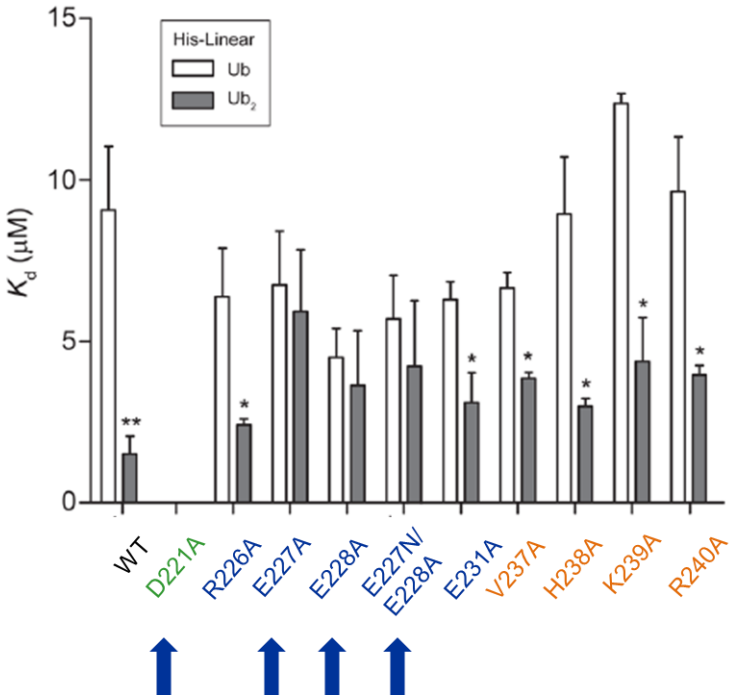


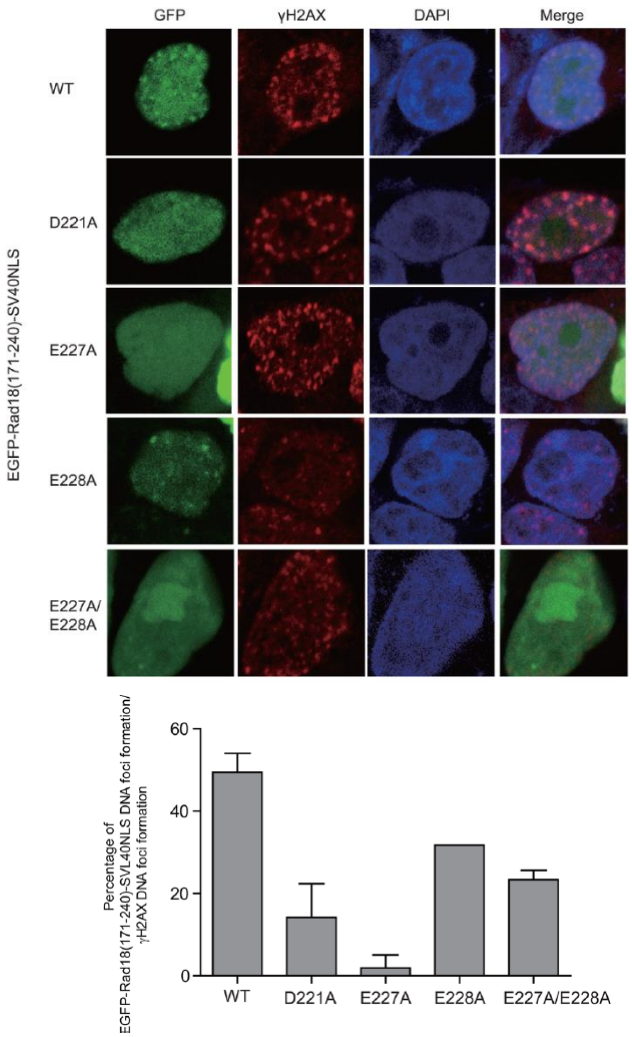




[Trung Thanh Thach, Namsoo Lee, Donghyuk Shin, Seungsu Han, Gyuhee Kim, Hongtae Kim, and Sangho Lee (2015) *Biochemistry*]

Validation of Rad18(201-240):Linear Ub_2 Interaction





[Trung Thanh Thach, Namsoo Lee, Donghyuk Shin, Seungsu Han, Gyuhee Kim, Hongtae Kim, and Sangho Lee (2015) *Biochemistry*]

Summary

	ITC	SPR, BLI
Affinity range (K_d)	nM to sub-mM (pM with competition)	nM to low mM
Pros	• Thermodynamic parameters (ΔG, ΔH, ΔS) • No immobilization	• Kinetic parameters (k_{on}, k_{off}) • "Dirty" samples possible • "Less" sample required High throughput
Cons	• "More" sample required • Lows to medium throughput	• Mass transfer limitation • Immobilization artifacts