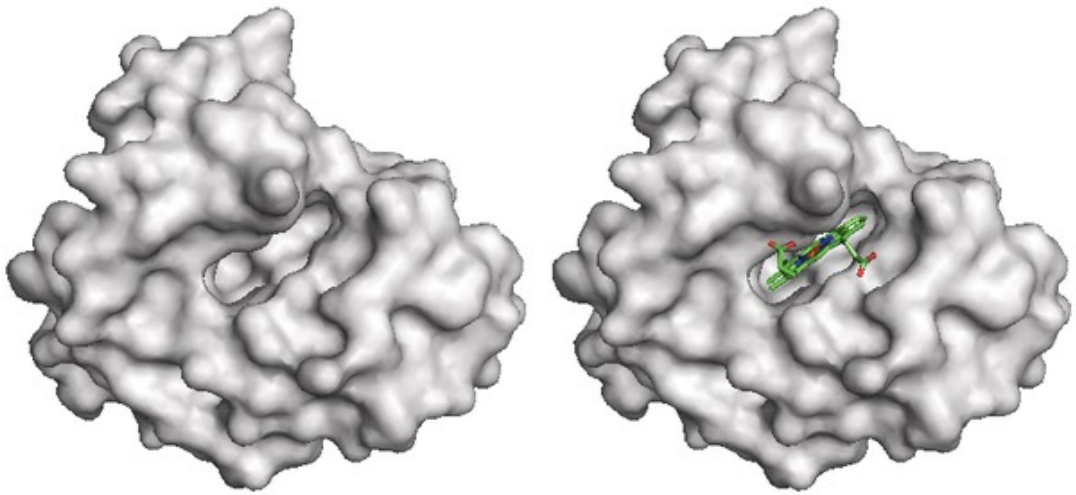


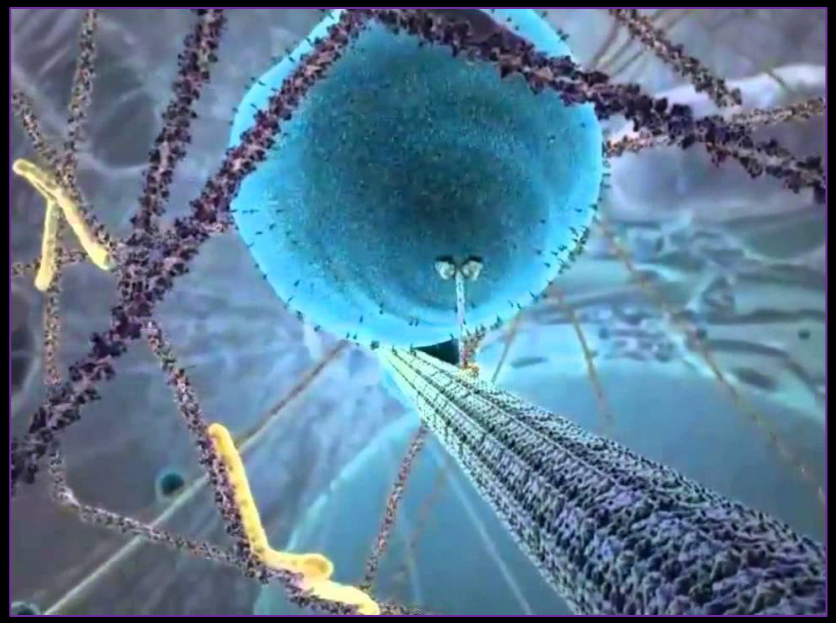
L4 – Quantitative Evaluation of Binding Interactions

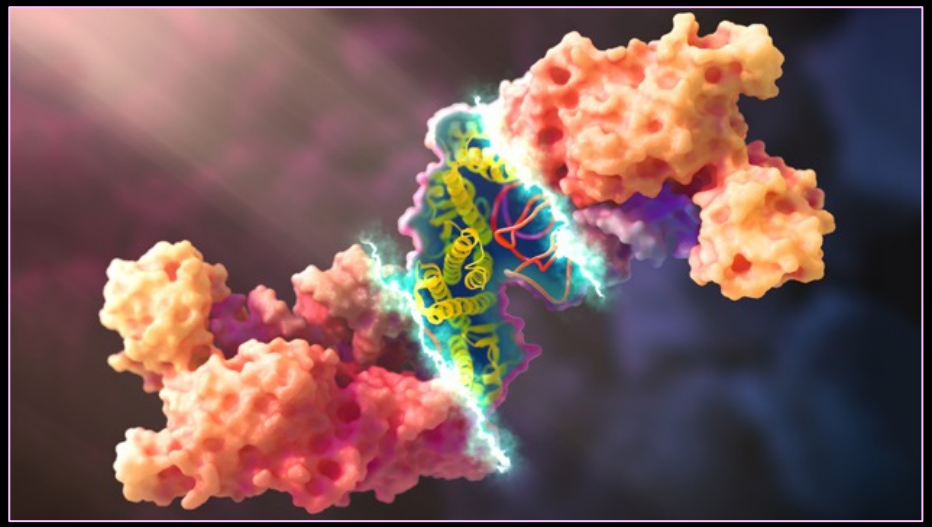
February 23, 2023

Molecular recognition is ubiquitous in biology

proteins, lipids, sugars, nucleic acids, metabolites, antibodies

The Inner Life of the Cell – Drs. Viel and Lue, Harvard





https://www.youtube.com/watch?v=FzcTgrxMzZk

8 minute video – watch it while you are running an experiment

Basic language of binding interactions

from 20.110

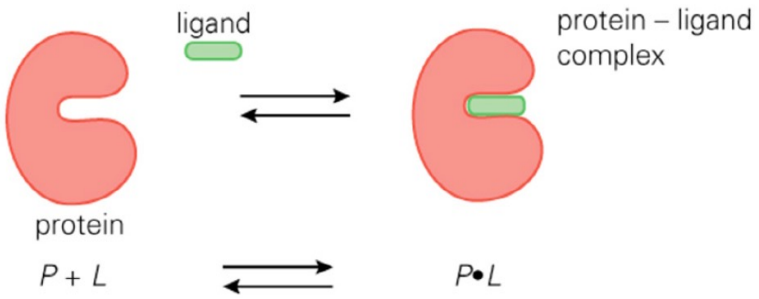
Affinity: strength of the interaction, measured by the corresponding decrease in free energy upon binding

Specificity: relative strength of interaction for a 'cognate' and 'non-cognate' receptor-ligand complex

There are two basic types of non-covalent interactions: simple binding and allosteric

Some binding interactions are 'simple' equilibria – each encounter is independent

simple interaction



Adapted from Kuriyan, The Molecules of Life, Chapter 12, Molecular Recognition

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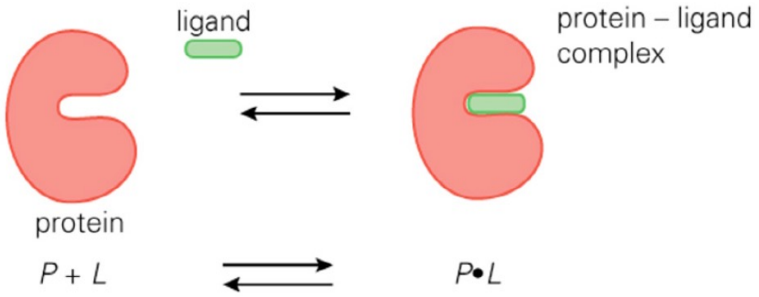
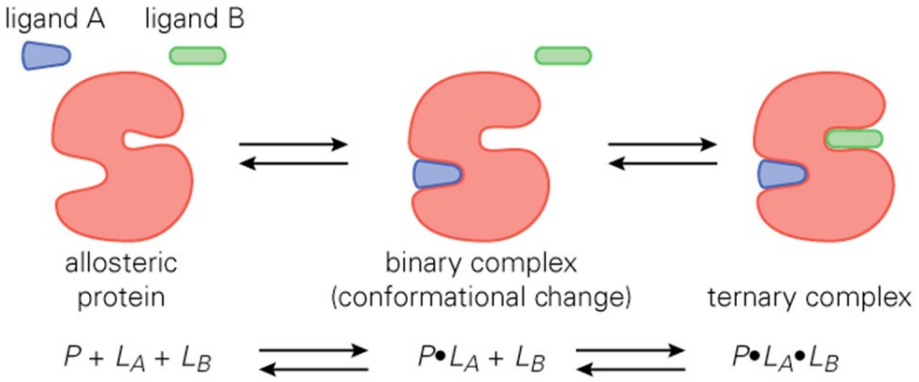


Others are more complex, involving allostery, where one ligand binding event alters the affinity for another ligand

allosteric interaction



Adapted from Kuriyan, The Molecules of Life, Chapter 12, Molecular Recognition

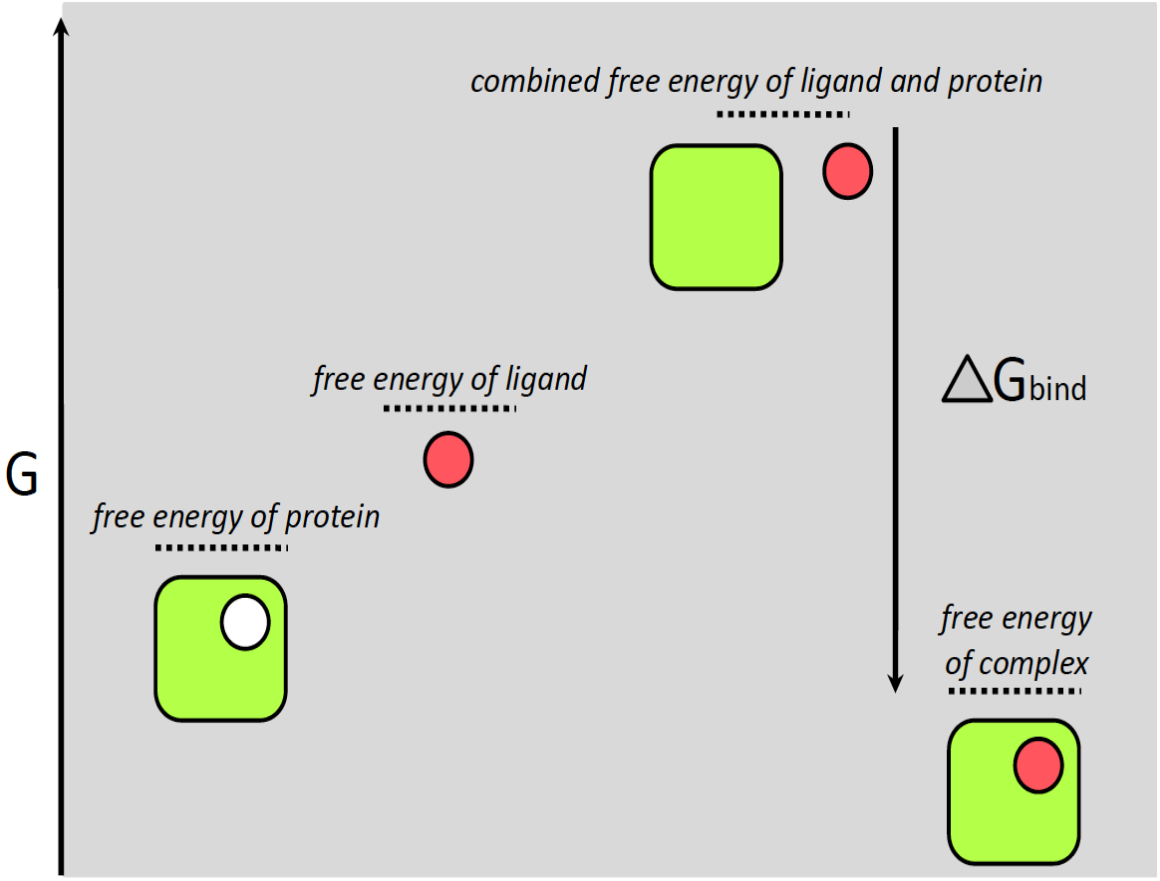
Thermodynamic analyses provide insight into molecular interactions

As you learned in 20.110, we can think about the following binding-related terms thermodynamically:

- affinity and specificity
- contribution of entropy and enthalpy
- dependence on temperature
- contributions of chemical groups on the ligand and/or the receptor

This information can in turn be used to understand a system and to alter the system (e.g. drug design)

Relationship of ligand binding free energy to association constants



From 20.110:

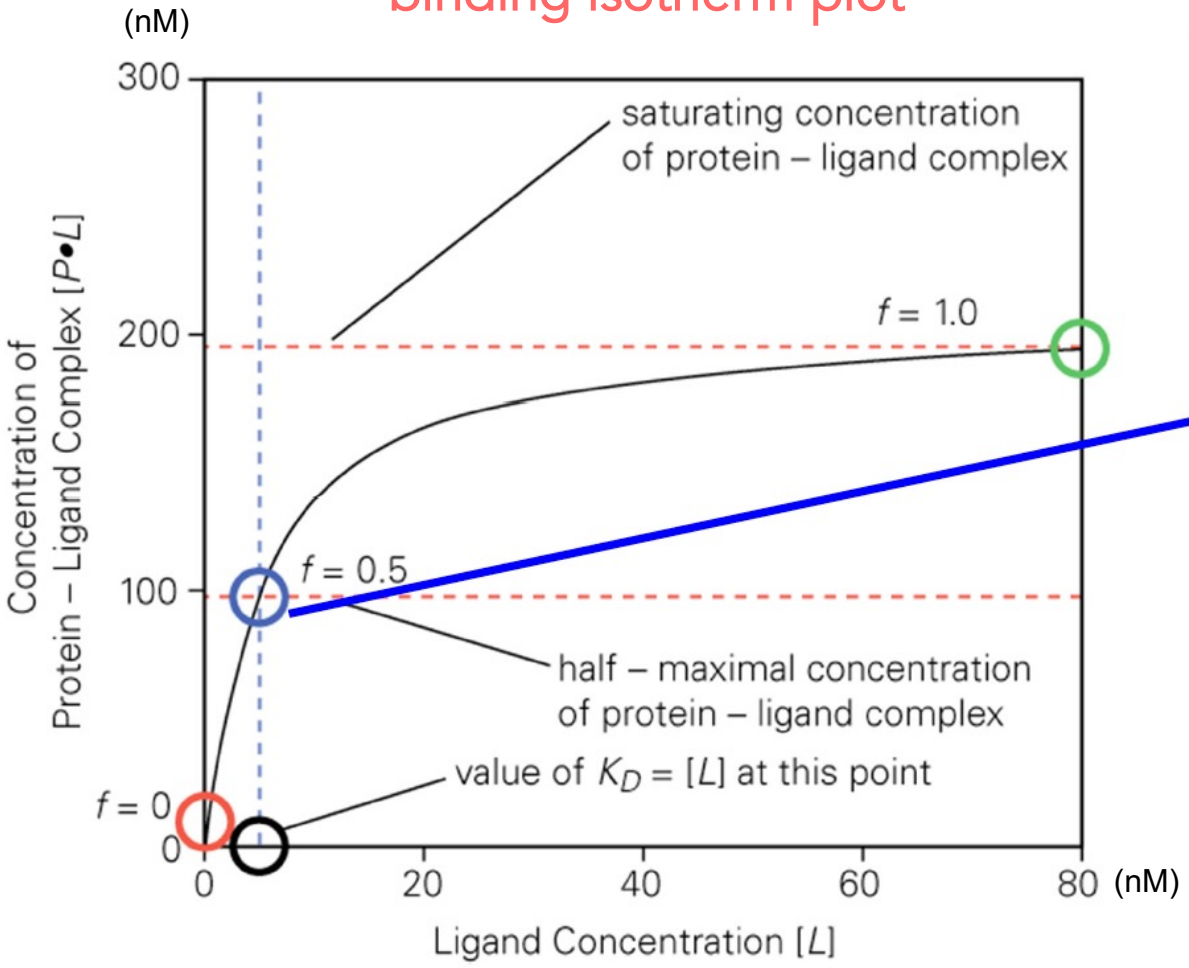
$$\Delta G^{\circ}_{bind} = -RT \ln K_A$$

$$K_D \underset{\text{(dissociation constant)}}{} = \frac{[P][L]}{[P \cdot L]} = \frac{1}{K_A}$$

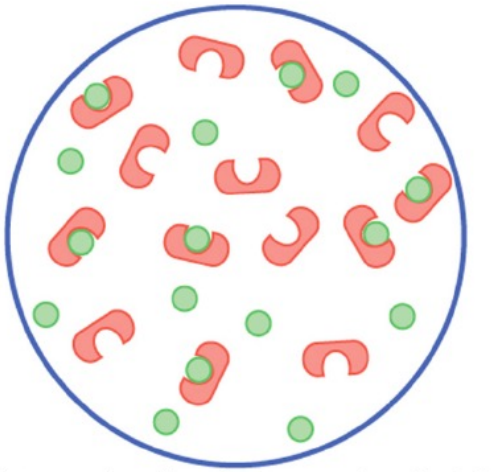
$$\Delta G^{\circ}_{bind} = +RT \ln K_D$$

Binding isotherms are half maximal at [L] = K_D

'binding isotherm plot'



$R \bullet L \leftrightarrows R + L$

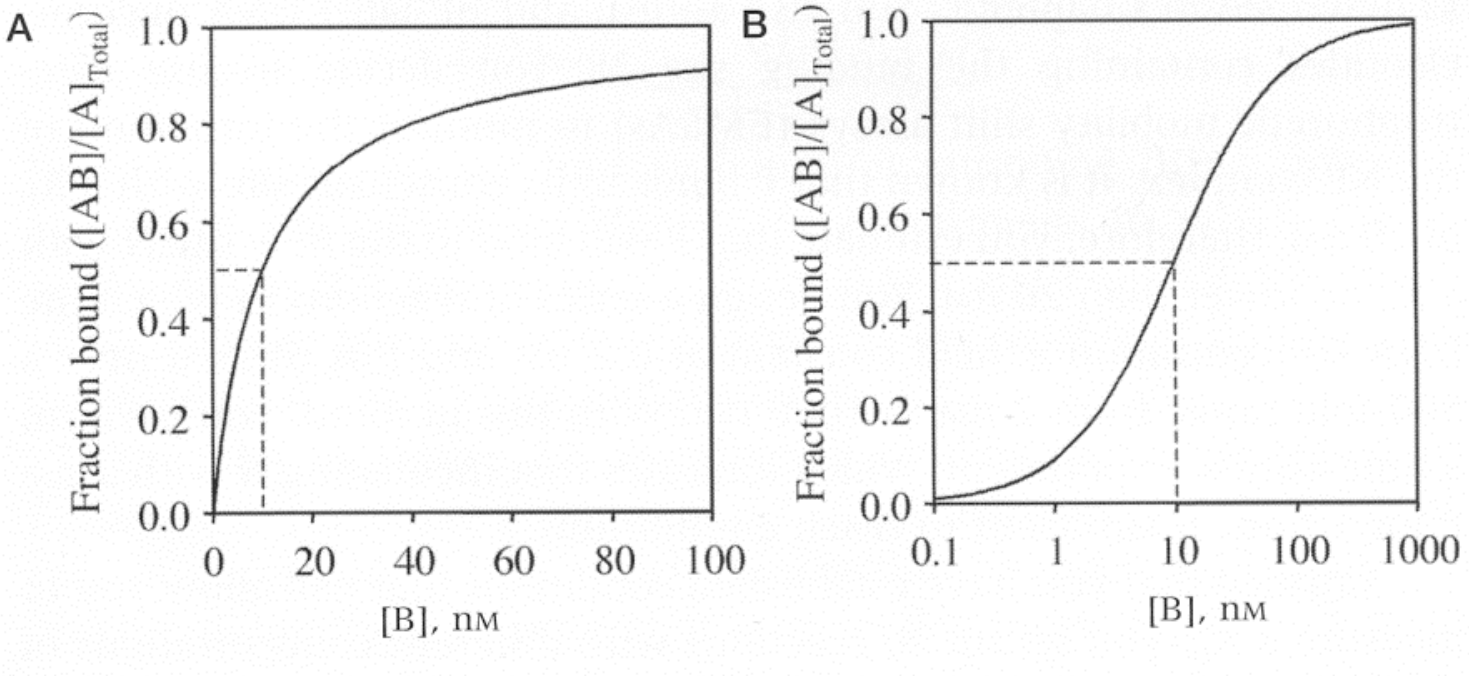


intermediate ligand concentration: $f = 0.5$
K_D = [L] when $f = 0.5$

steady-state equilibrium analysis

Logarithmic vs. Linear display of data

historic convention *current convention*



as a corollary, choose your concentrations wisely:

1, 3, 10, 30, 100, 300 nM

vs.

50, 100, 150, 200, 250, 300 nM

A range of affinities enable biology

Type of Interaction	K_D (molar)	ΔG^0_{bind} (at 300K) kcal/mol
Enzyme:ATP	~1×10^{-3} to ~1×10^{-6} (millimolar to micromolar)	**-4 to -8 kcal/mol**
signaling protein binding to a target	~1×10^{-6} (micromolar)	**-8 kcal/mol**
Sequence-specific recognition of DNA by a transcription factor	~1×10^{-9} (nanomolar)	**-12 kcal/mol**
small molecule inhibitors of proteins (drugs)	~1×10^{-9} to ~1×10^{-12} (nanomolar to picomolar)	**-12 to -17 kcal/mol**
biotin binding to avidin protein (strongest known non-covalent interaction)	~1×10^{-15} (femtomolar)	**-21 kcal/mol**

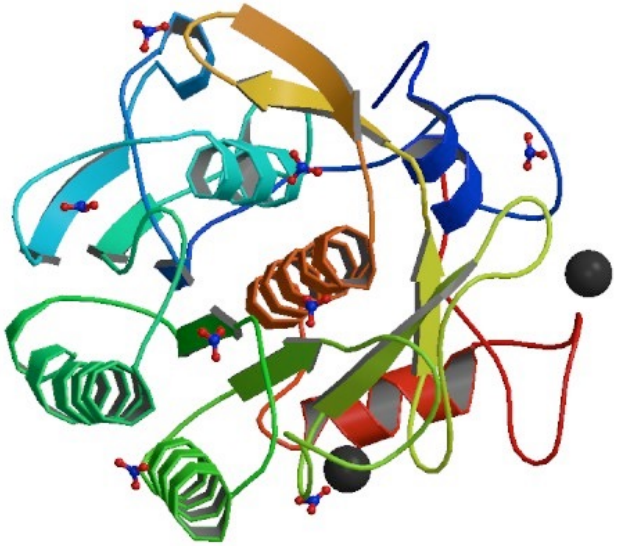
higher K_D value
weaker interaction

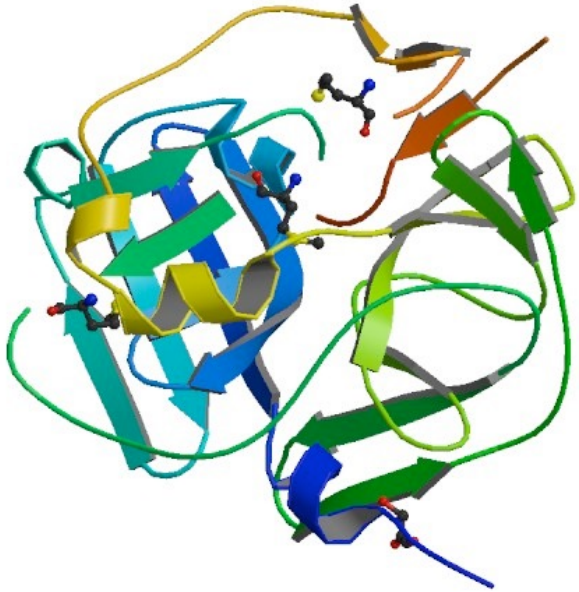
lower K_D value
stronger interaction

Adapted from Kuriyan, The Molecules of Life, Chapter 12, Molecular Recognition

Specificity in molecular recognition

discrimination among targets





Proteinase K

low specificity

Aliphatic/X
Aromatic/X

Lab Use - DNA/RNA preps

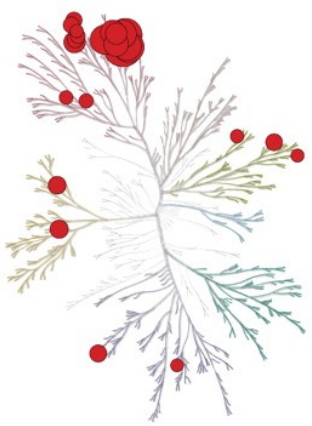
HRV 3C Protease

high specificity

Leu-Glu-Val-Leu-Phe-Gln/Gly-Pro

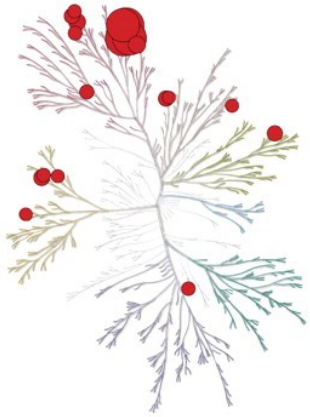
Lab Use – cleaving fusion proteins

Specificity in molecular recognition – kinase drugs



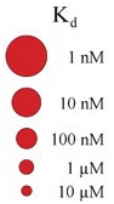
AC220

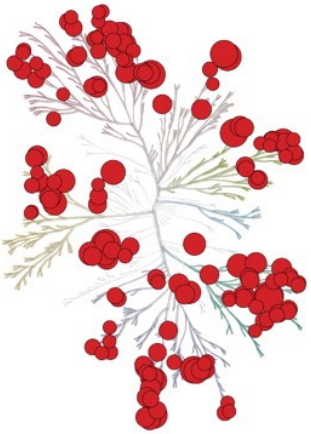
CEP-701



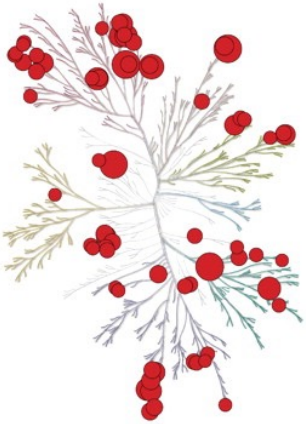
MLN-518

binding constants

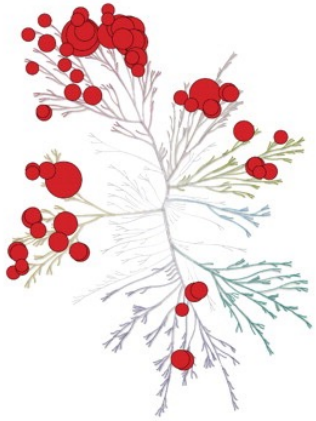




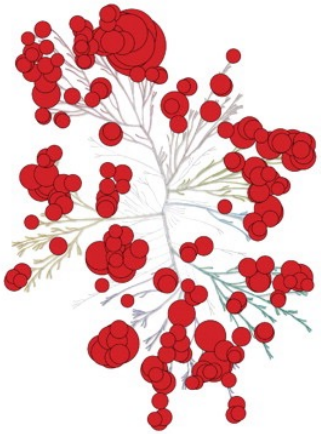
PKC-412



CGP-52421



Sorafenib

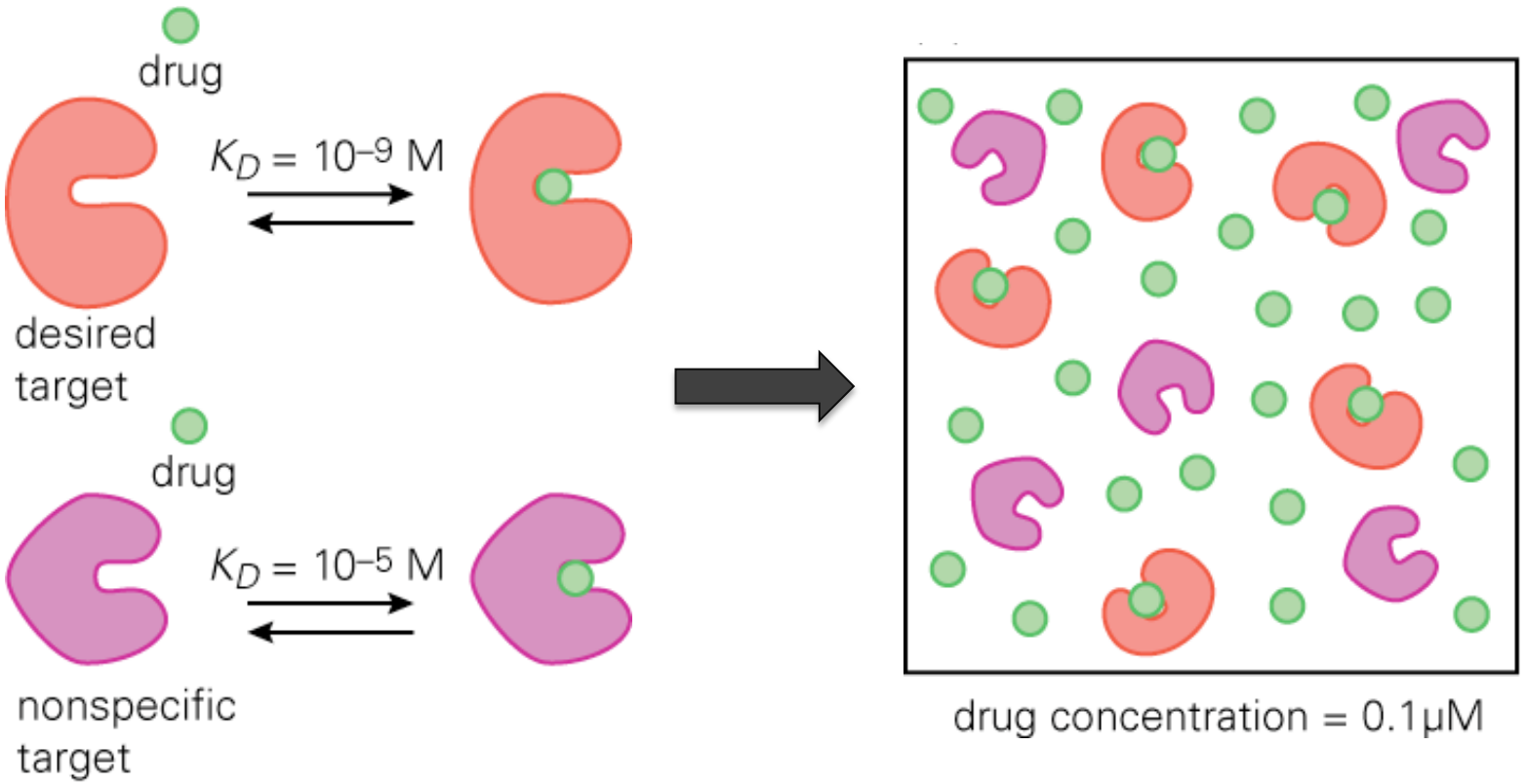


Sunitinib

Adapted from Zarrinkar et al, Blood (2009), 114: 2984-2992

Specificity in drug binding – fractional saturation

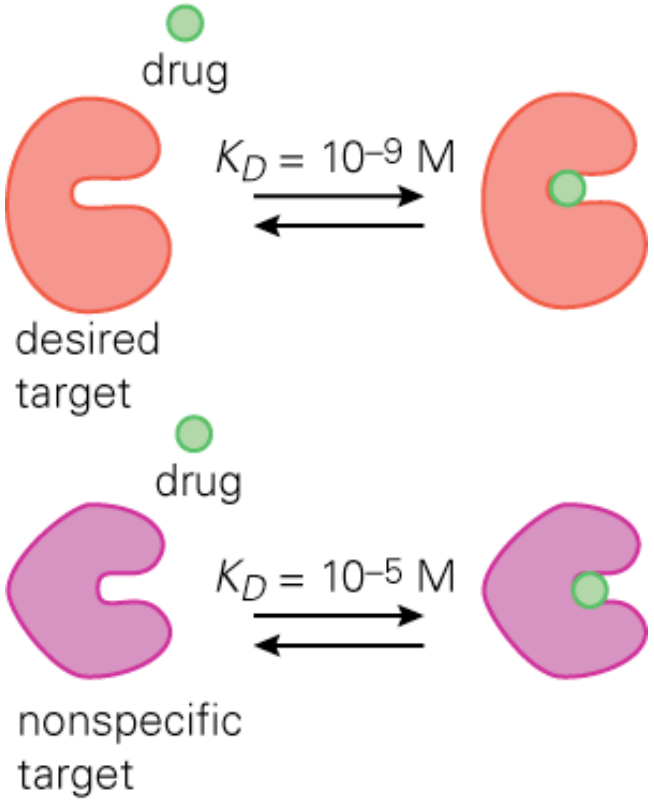
deliver the drug at a concentration below the K_D for non-cognate target



Adapted from Kuriyan, The Molecules of Life, Chapter 12, Molecular Recognition

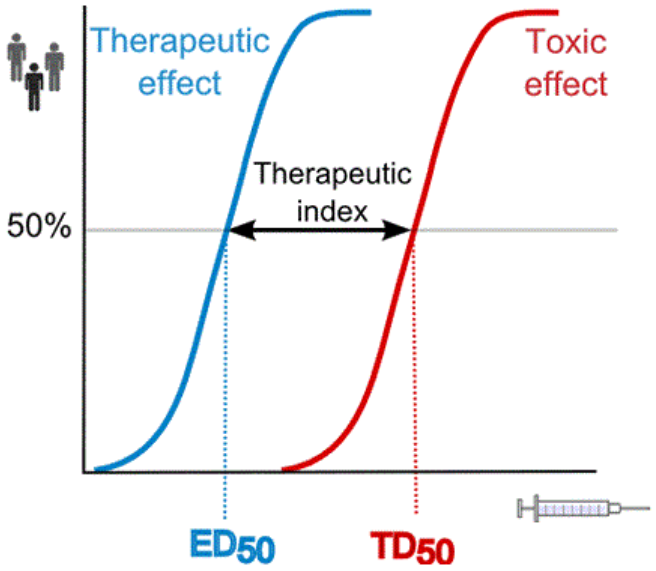
Specificity in drug binding – fractional saturation

deliver the drug at a concentration below the TD_{50} in patients



impact therapeutic effects?

'Therapeutic Window'



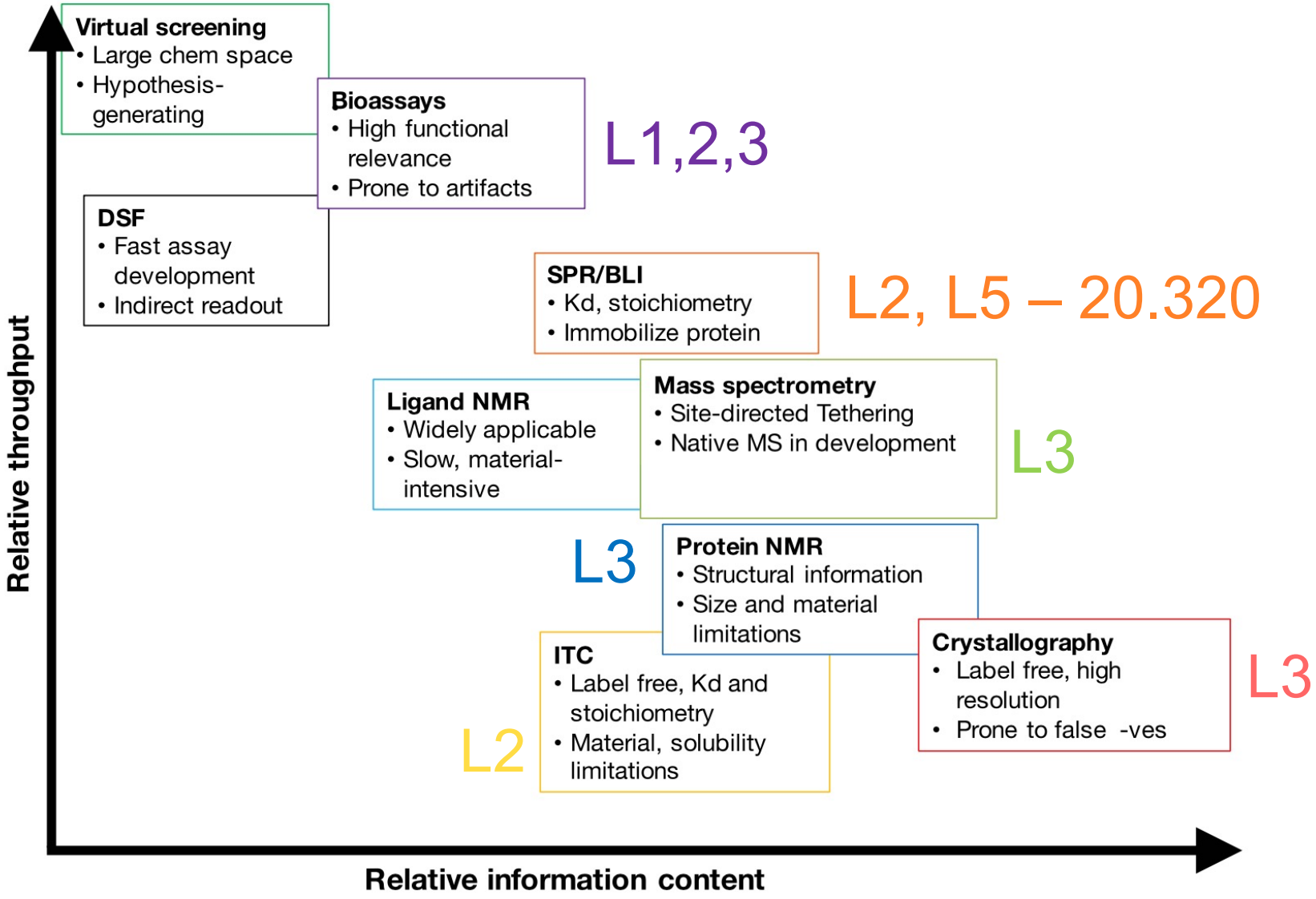
ED_{50} = effective in 50% patients

TD_{50} = toxic in 50% patients

Adapted from Kuriyan, The Molecules of Life, Chapter 12, Molecular Recognition

But how do we go about measuring these K_D values in a laboratory setting?

Methods to find or evaluate binding interactions



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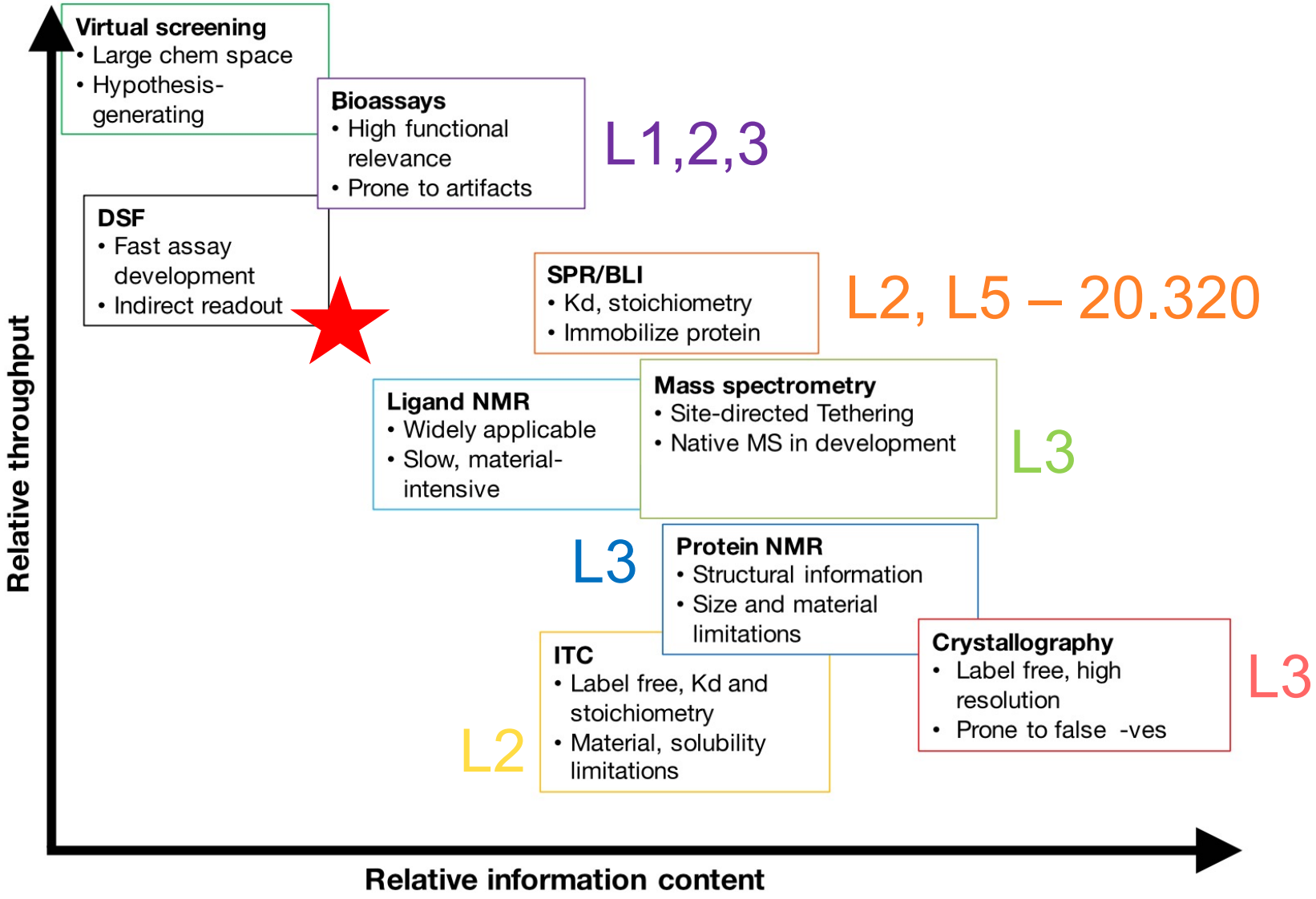
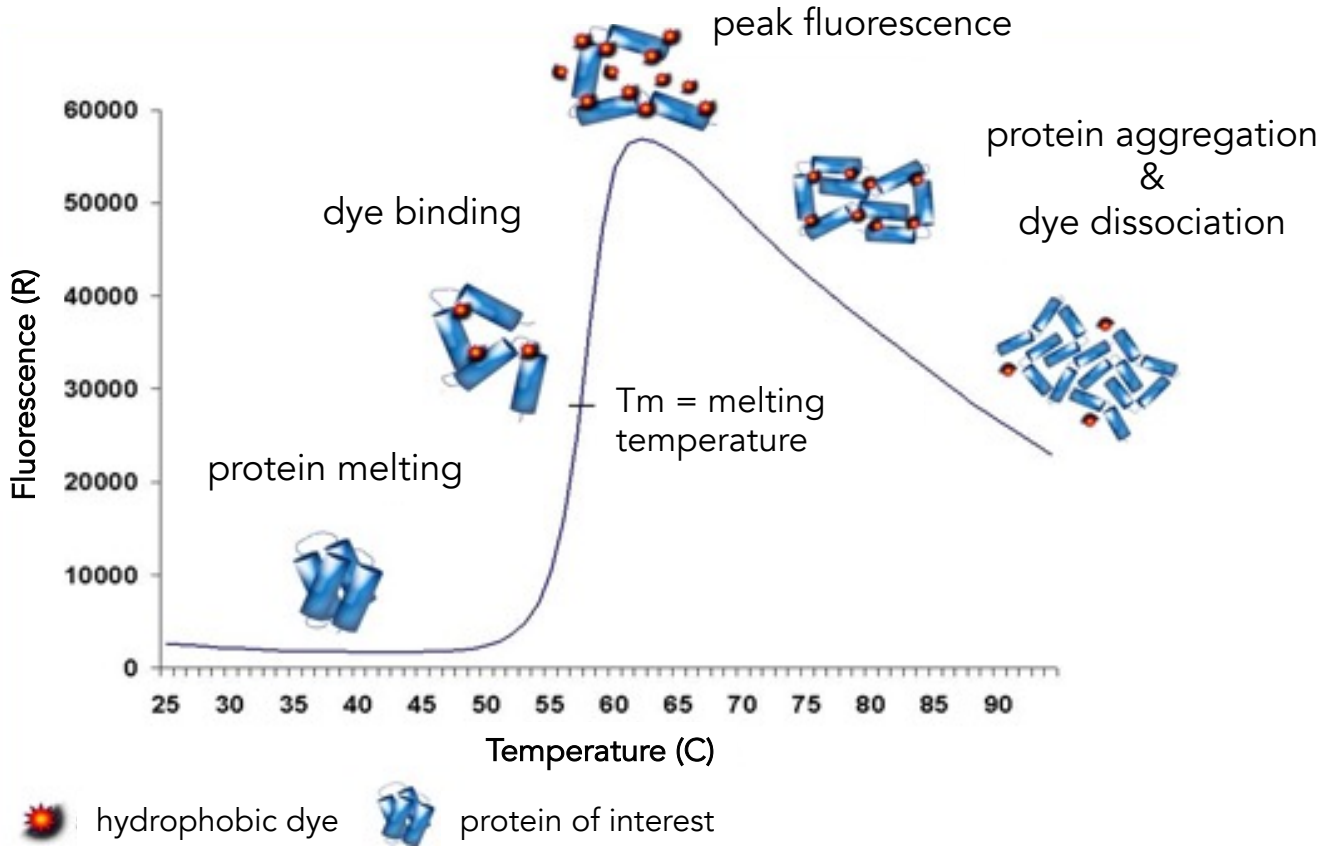
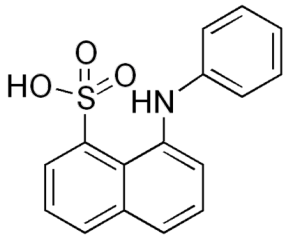


Measuring a thermal melt profile for a protein



Dyes used to detect protein unfolding



ANS

8-anilinonapthalene-1-sulfonic acid

(1965)

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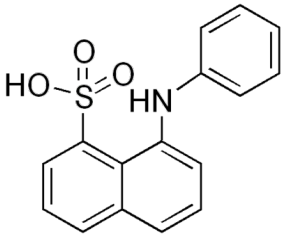

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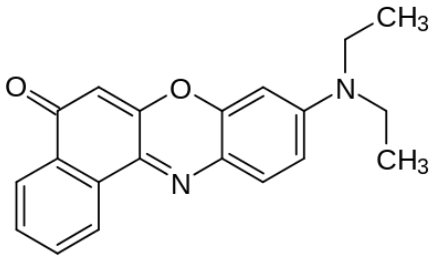


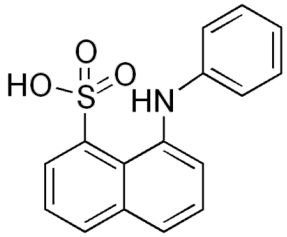
Nile Red

9-diethylamino-5-benzo[a]phenoxazinone
(1985)

solvatochromic

Nile Red under visible and
UV light in different solvents

Dyes used to detect protein unfolding



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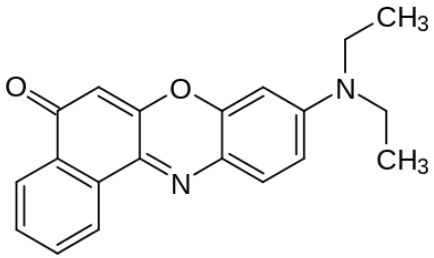

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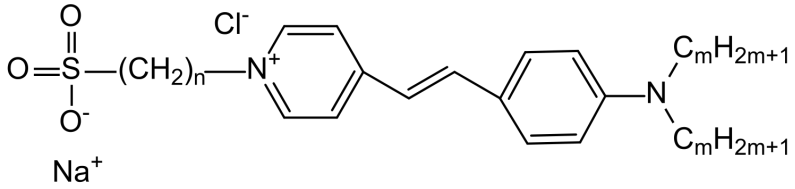
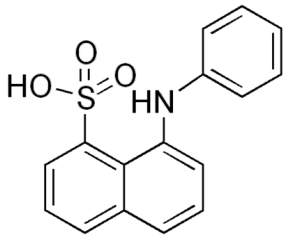


SYPRO® Orange

Most common dye for DSF/TS
(2004)

binds nonspecifically to hydrophobic surfaces;
water quenches fluorescence

Dyes used to detect protein unfolding



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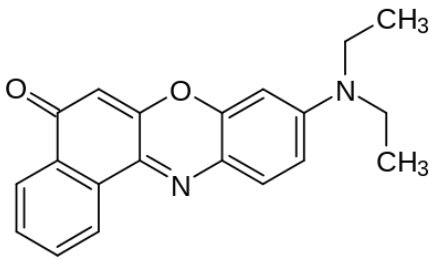

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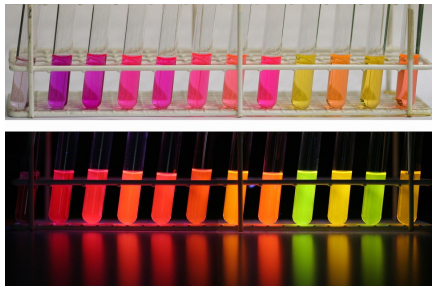
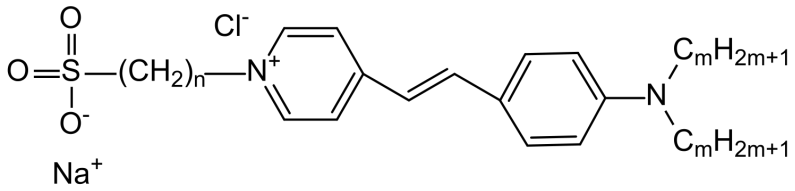


solvatochromic

Nile Red under visible and UV light in different solvents



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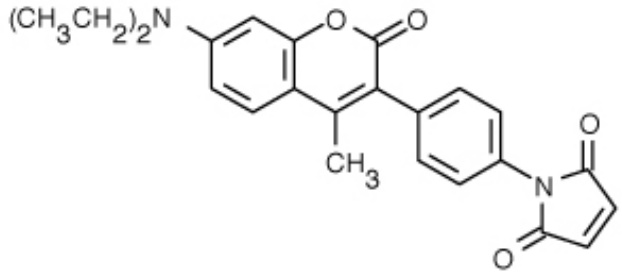
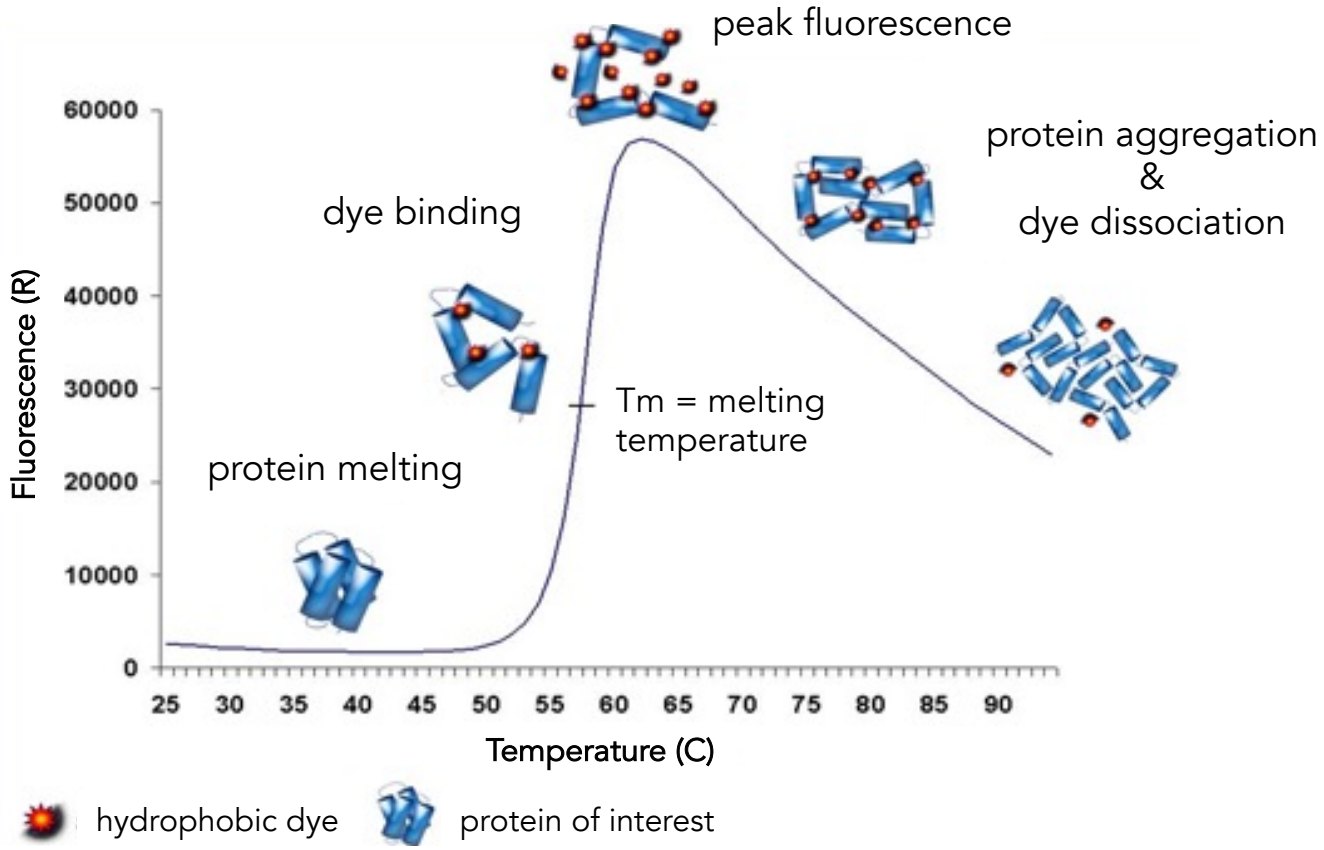


CPM

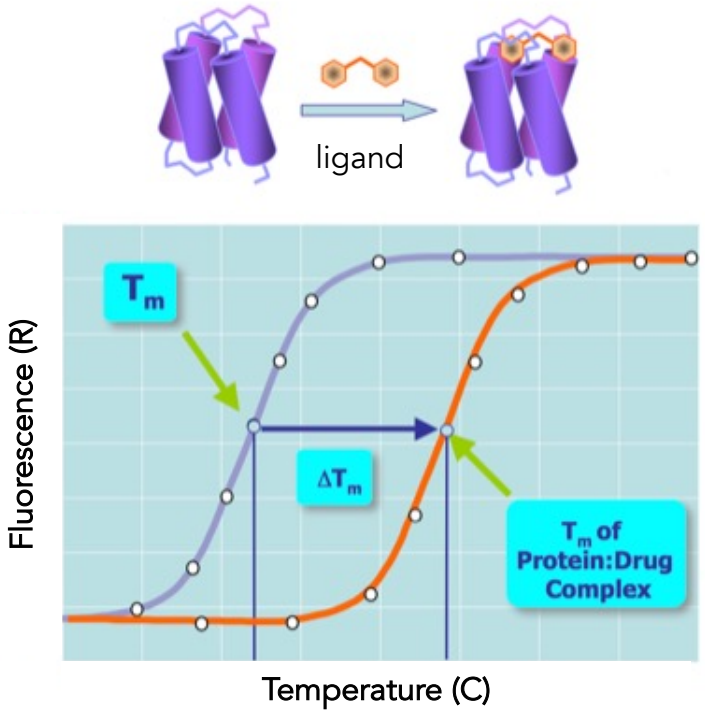
N-[4-(7-diethylamino-4-methyl-3-coumarinyl)phenyl]maleimide
(2008)

only fluoresces after reacting with Cys residues

What happens when you add a small molecule?

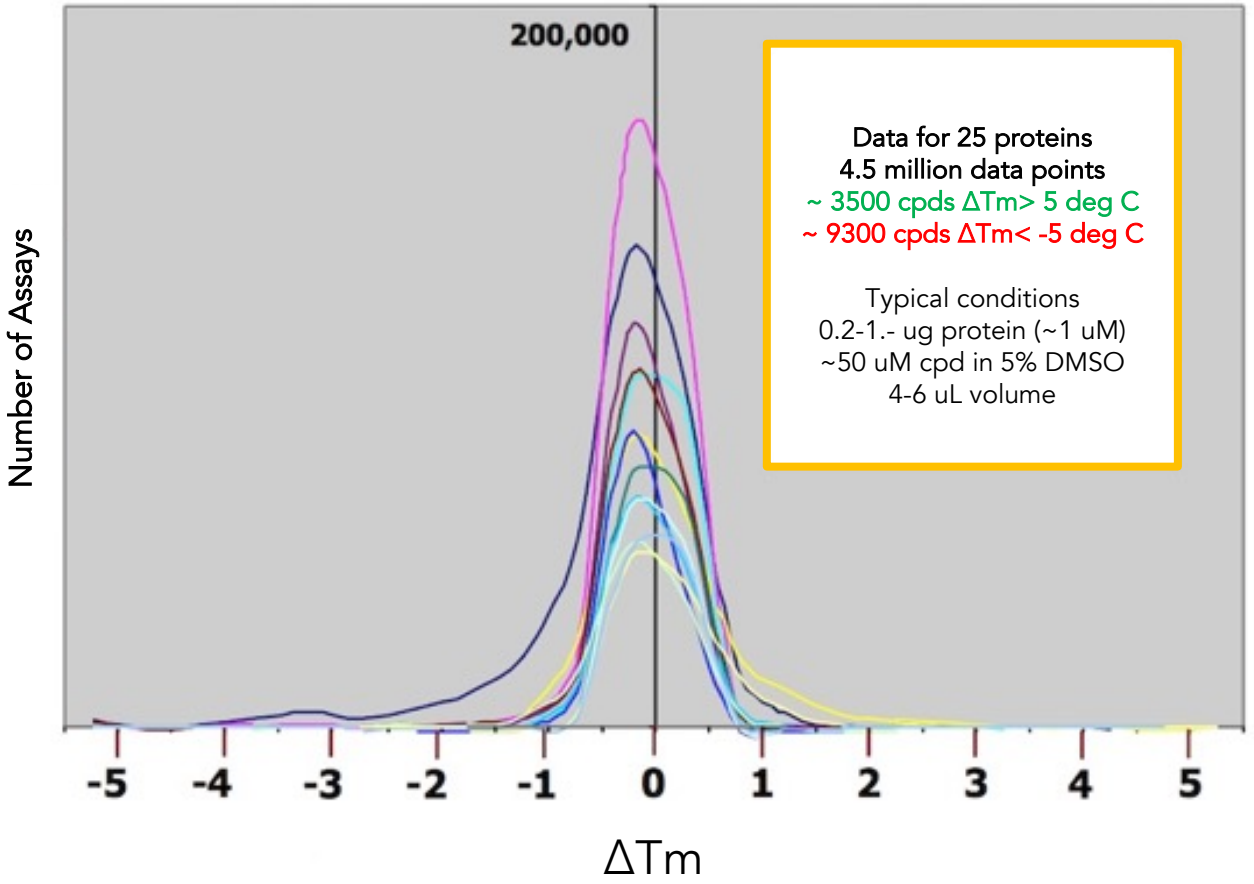


Thermal shift assays with small molecules



Adapted from Dr. Salemme at thermofluor.org

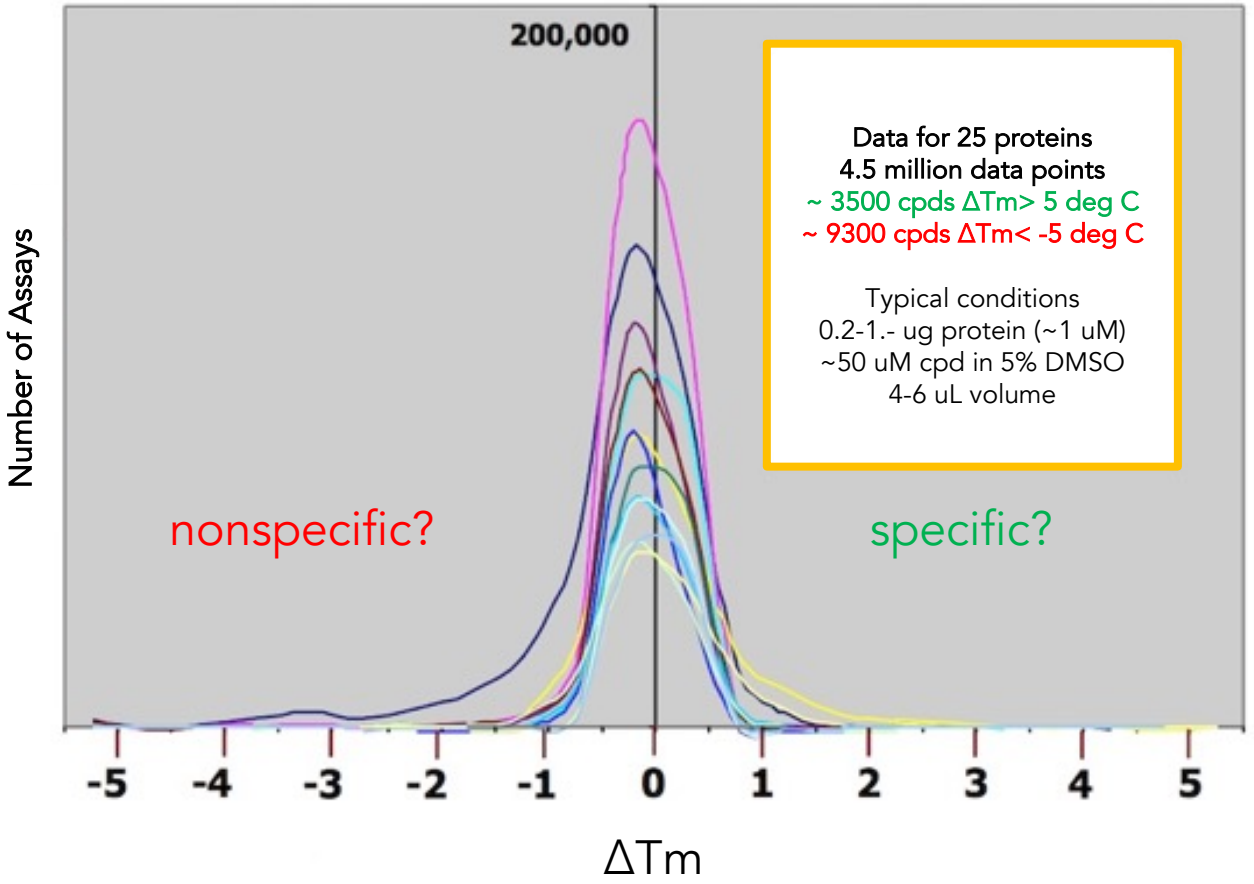
Real thermal shift screens with small molecules



preferential ligand binding to unfolded states?

Adapted from Dr. Salemme at thermofluor.org

Real thermal shift screens with small molecules

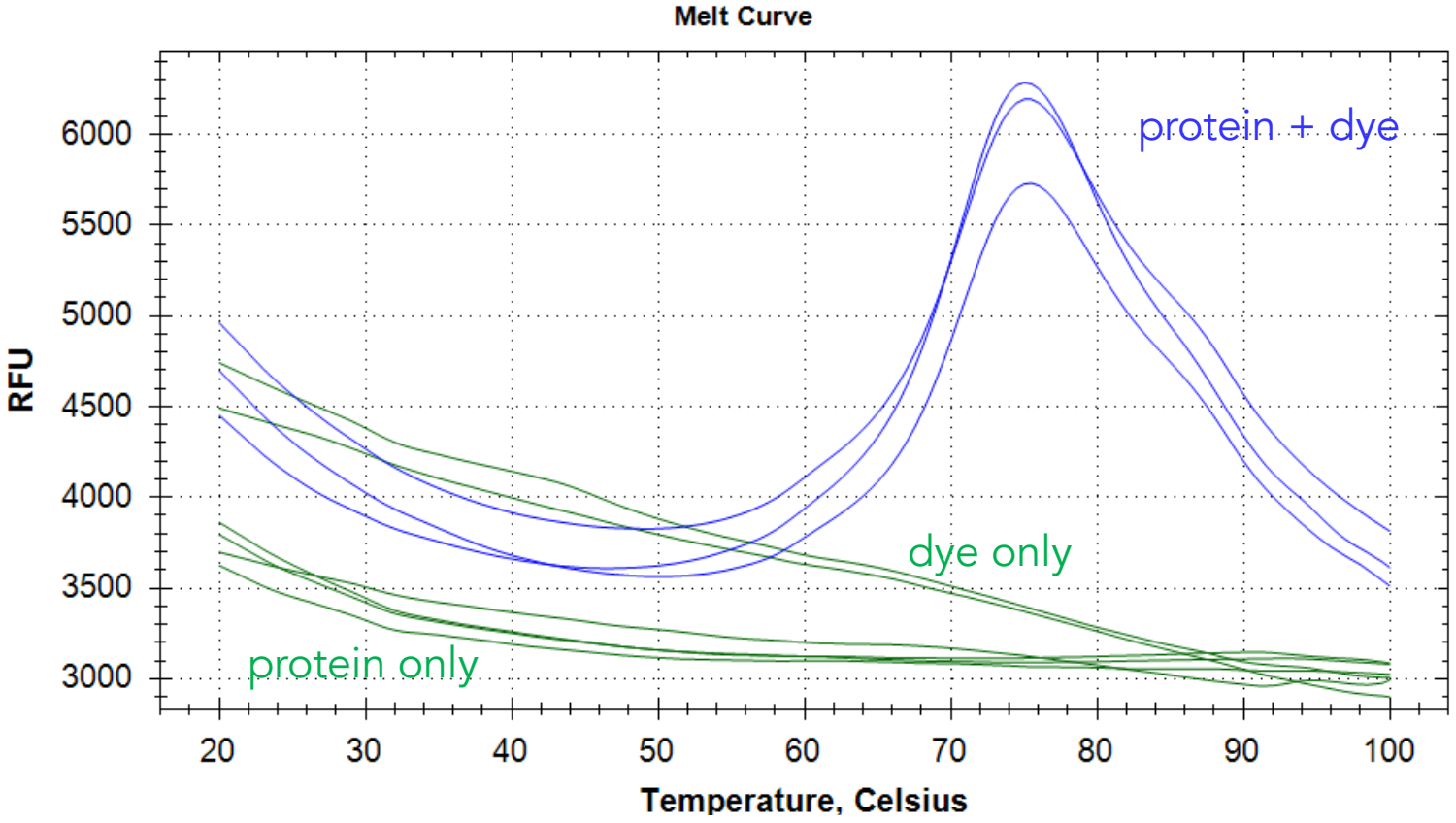


preferential ligand binding to unfolded states?

Adapted from Dr. Salemme at thermofluor.org

Real results from thermal shift studies

assay development

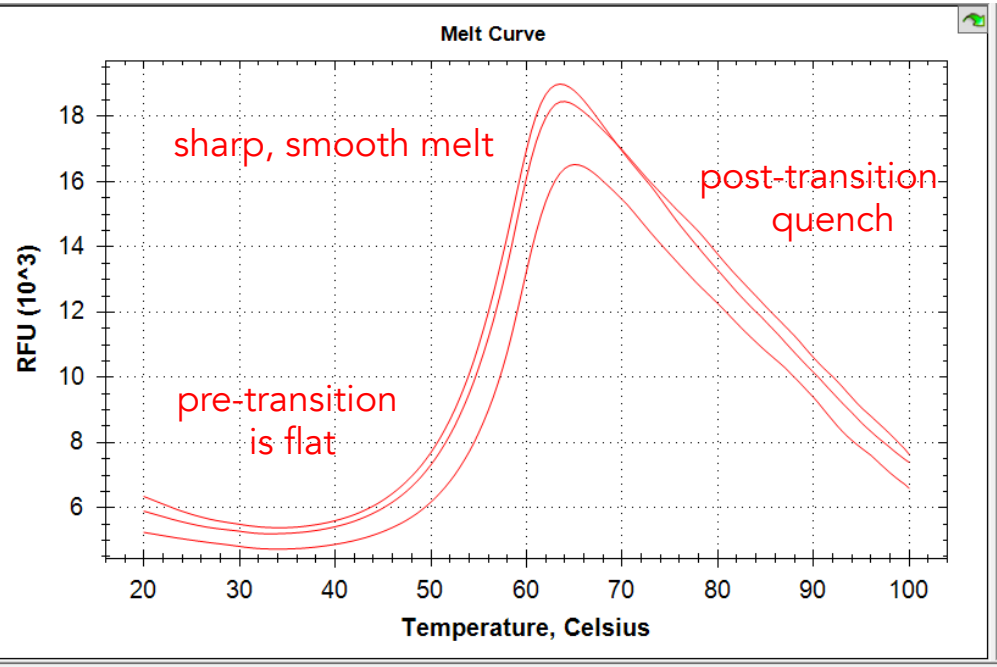


consider optimizing buffer conditions – pH, cofactors

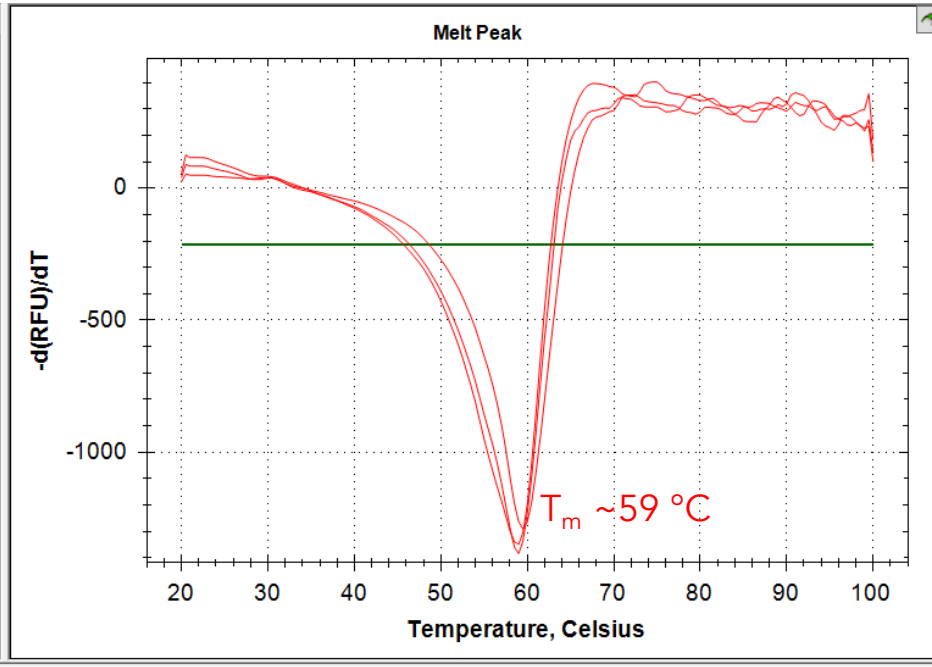
Adapted from Collaborative Crystallisation Centre

Real results with thermal shift assays

three replicates for a single experiment



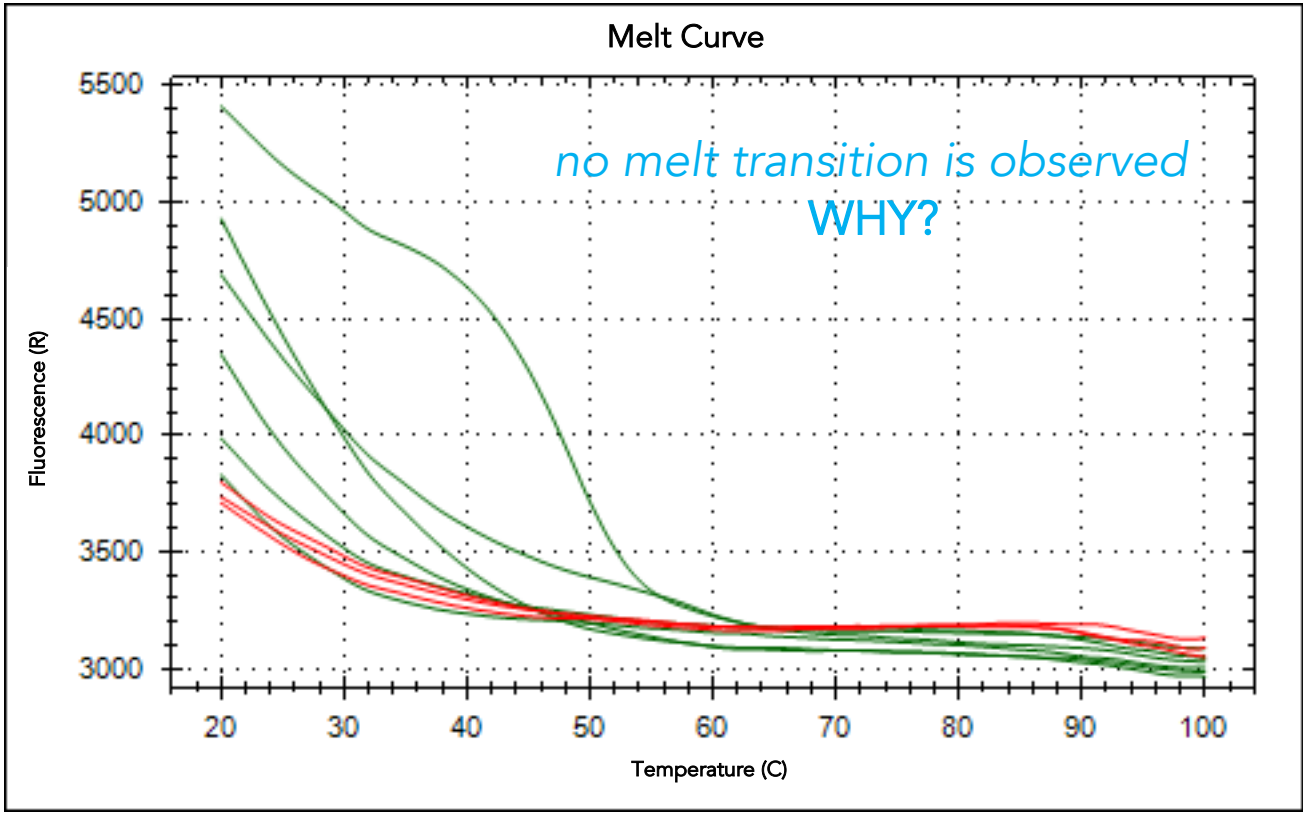
raw fluorescence thermal curves



first derivative representation

Adapted from Collaborative Crystallisation Centre

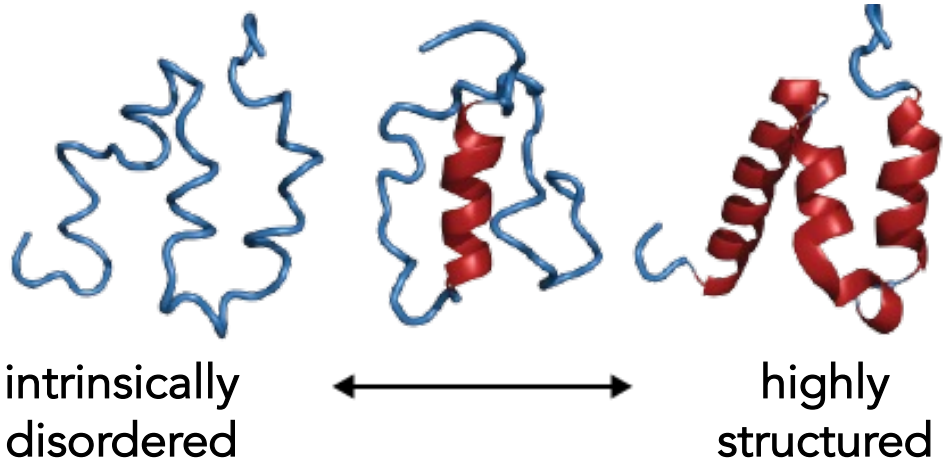
Real results with thermal shift assays



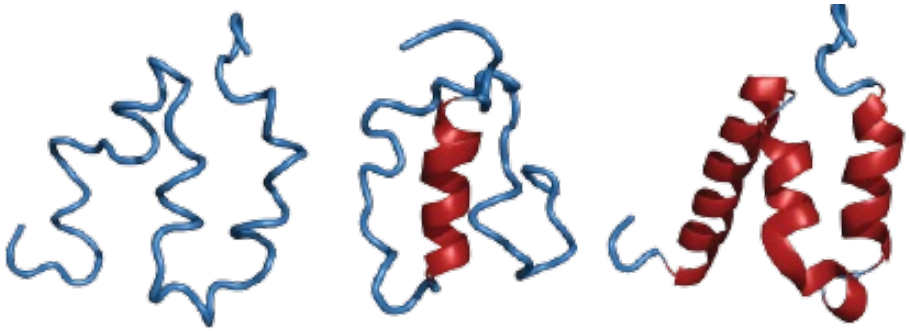
raw fluorescence thermal curves

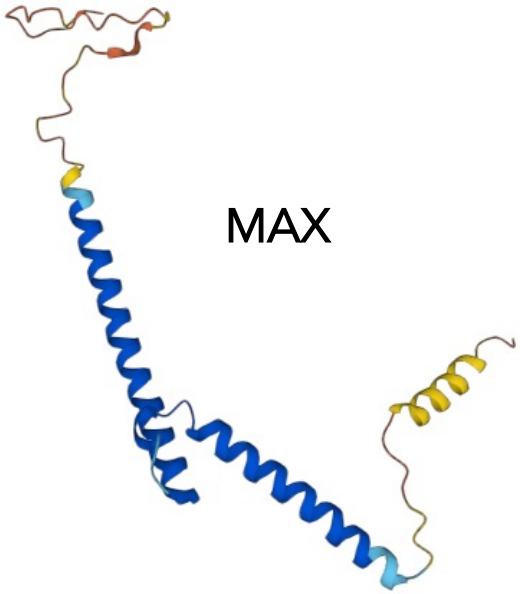
Adapted from Collaborative Crystallisation Centre

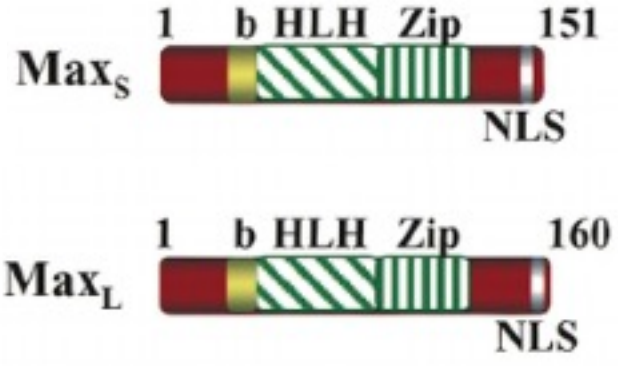
Protein disorder continuum



Protein disorder continuum

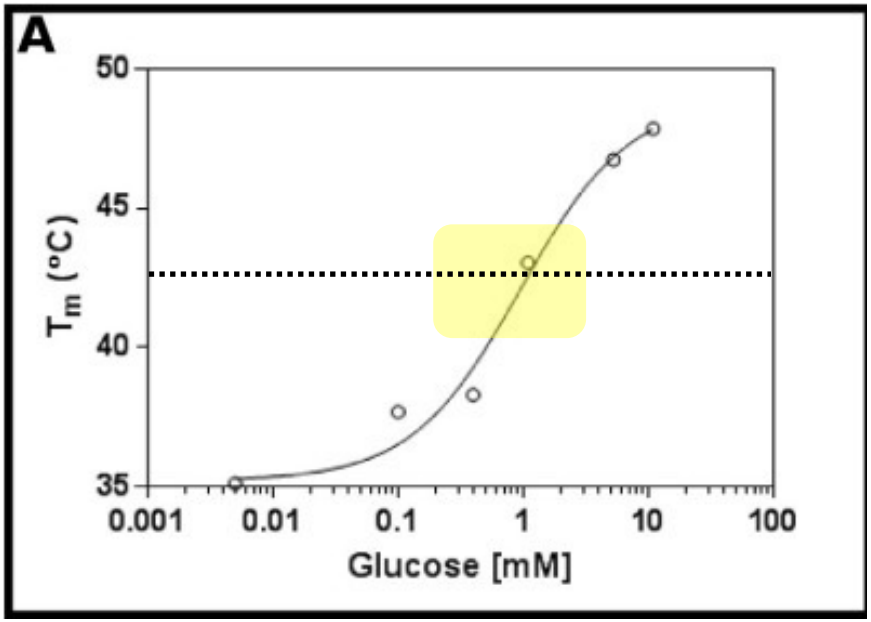






Determining apparent dissociation constants

hexokinase (receptor) and glucose (ligand)



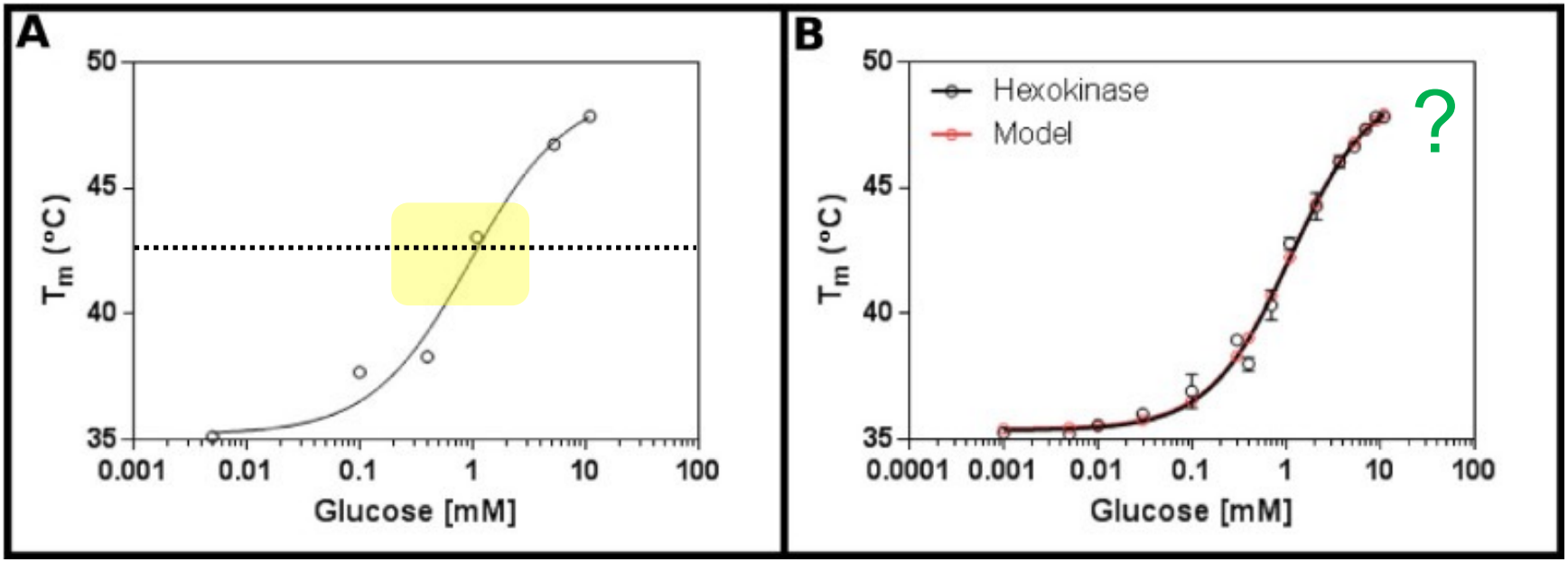
Experiment 1:

test a wide range of glucose concentrations

K_D is likely between 0.2 and 1.7 mM

Determining apparent dissociation constants

hexokinase (receptor) and glucose (ligand)



Experiment 1:

test a wide range of glucose concentrations

K_D is likely between 0.2 and 1.7 mM

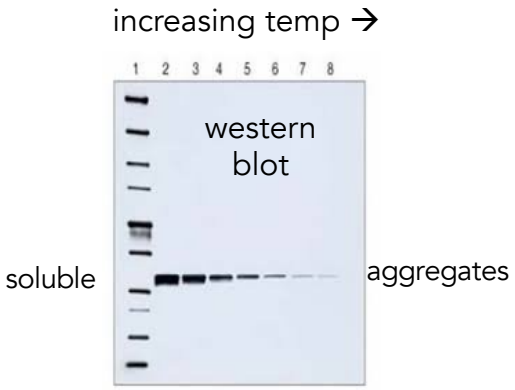
Experiment 2:

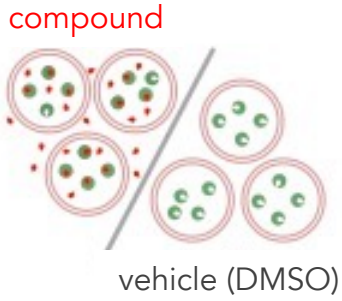
test 16 concentration of glucose
fit to single binding event model (red)

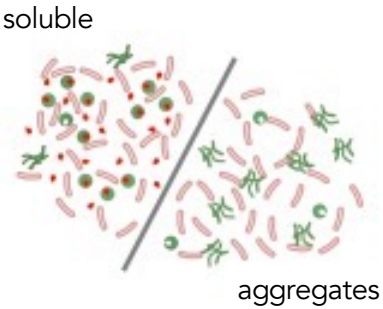
apparent K_D ~ 1.12 +/- 0.05 mM

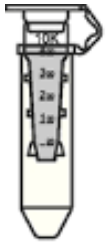
Target engagement in cells: cellular thermal shift assays (CETSA)

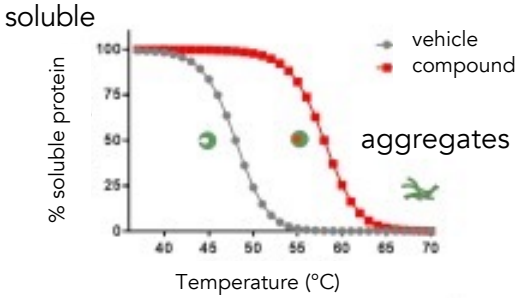
monitor levels of **soluble** proteins







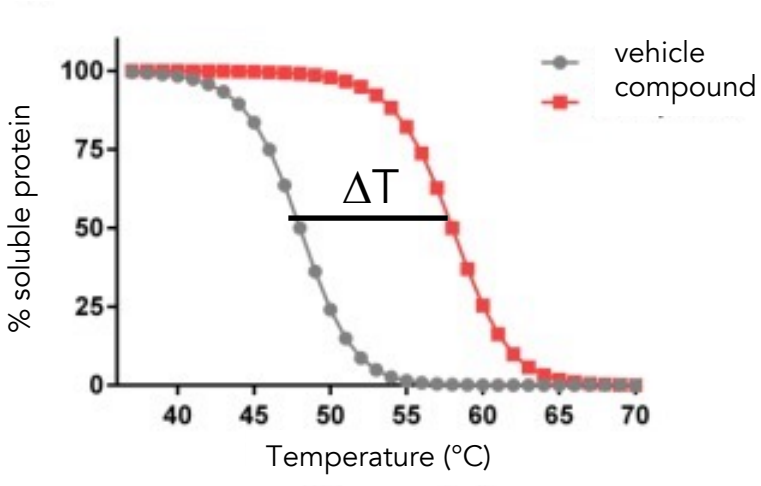




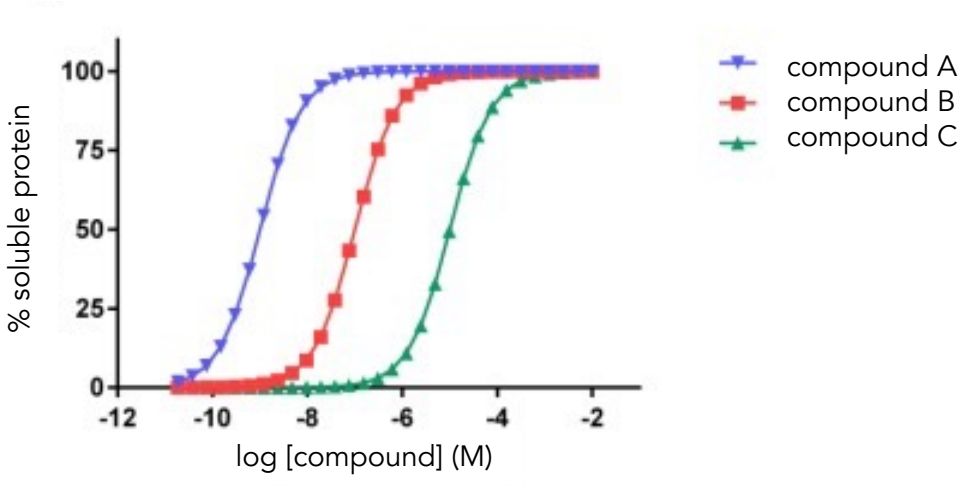
compound treatment in live cells → heating and cooling → lyse cells → separation of aggregates (optional) → detection

Anticipated results from CETSA assays

T_{agg} curve



ITDRF curves

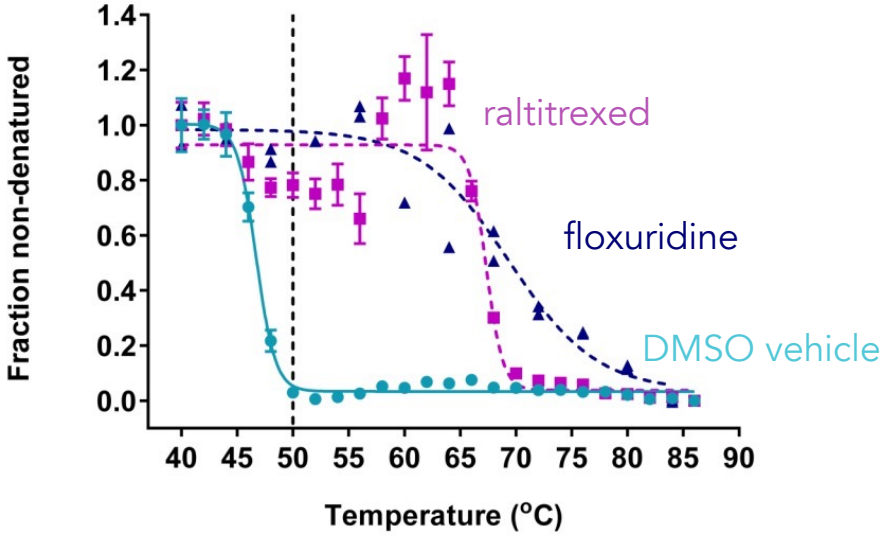


IsoThermal Dose Response Fingerprint
'apparent potencies' at single temp

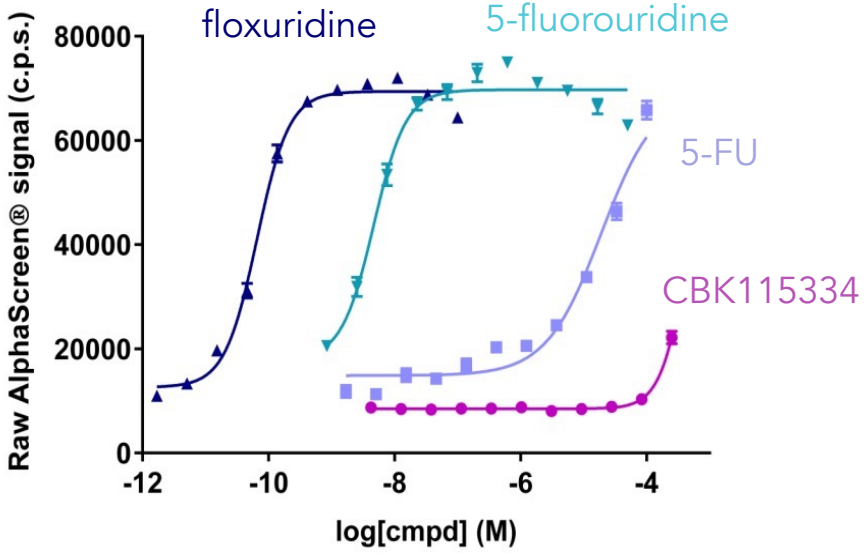
Real results from CETSA assays

thymidylate synthase drugs in K562 cells

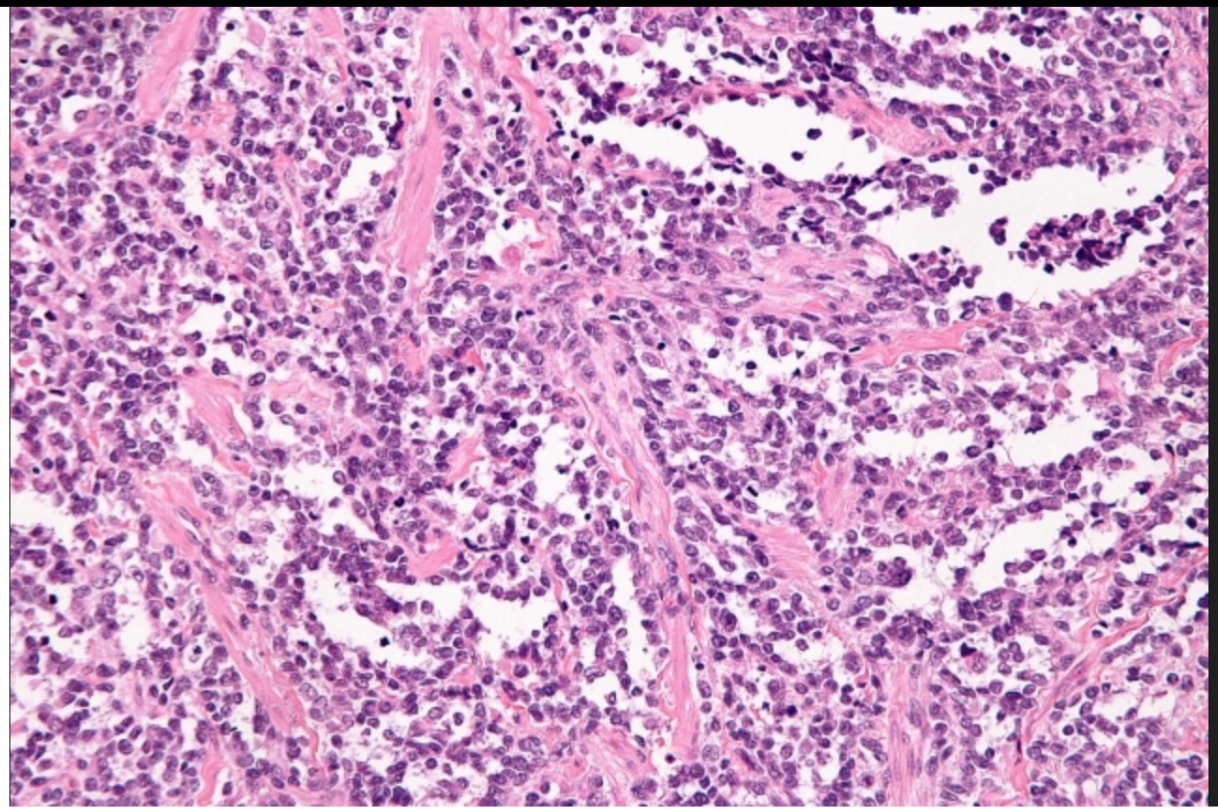
T_{agg} curve



ITDRF curves at 50 °C



quadruplicate data from one independent experiment



Alveolar rhabomyosarcoma, a soft tissue cancer

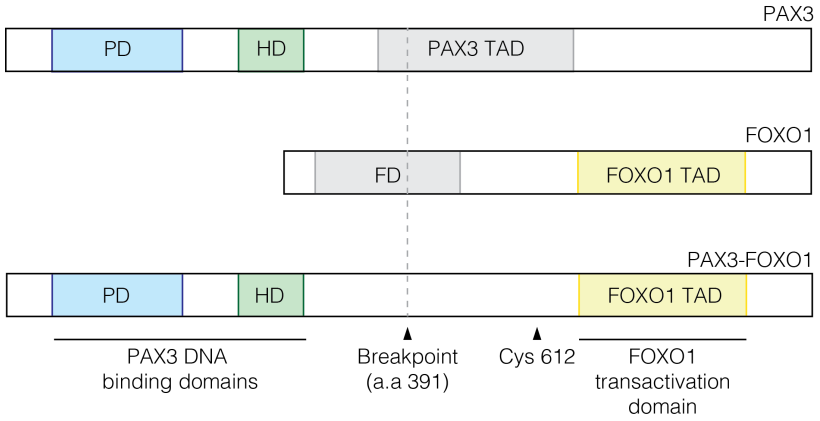
Image: Michael Bonert/Wikimedia Commons

Taking a moonshot at a rare childhood cancer

Team of researchers including MIT Professor Angela Koehler obtains $5.8 million grant to study fusion-positive alveolar rhabdomyosarcoma.

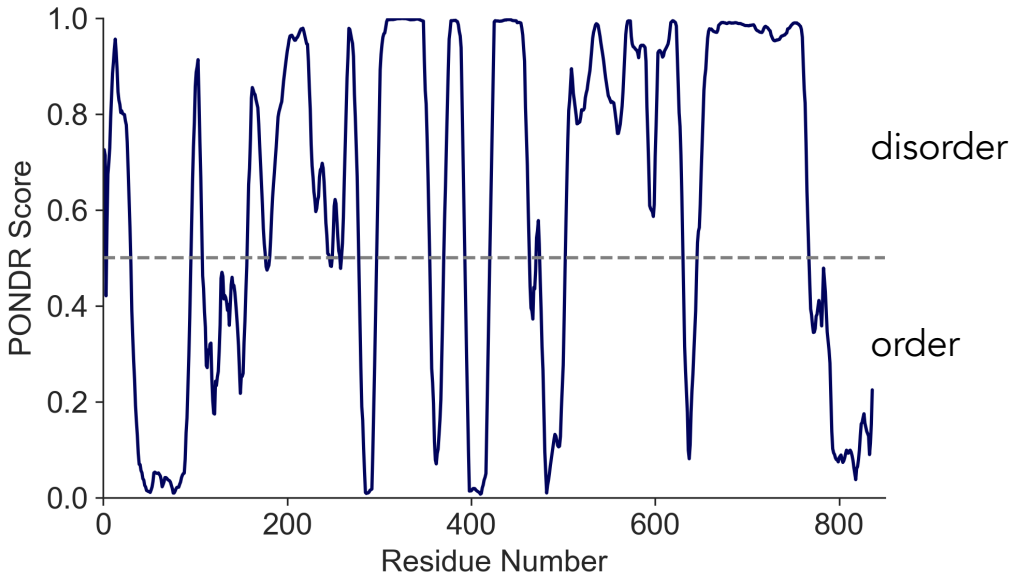
PAX3-FOXO1

pathognomic fusion in alveolar rhabdomyosarcoma



PONDR®

Predictor of Natural Disordered Regions

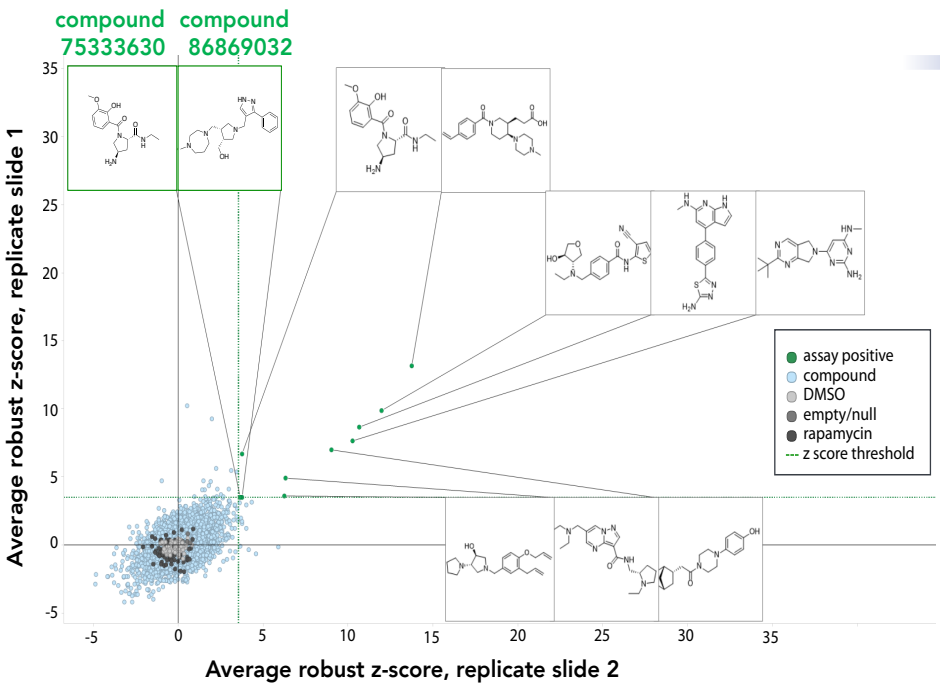


PAX3-FOXO1

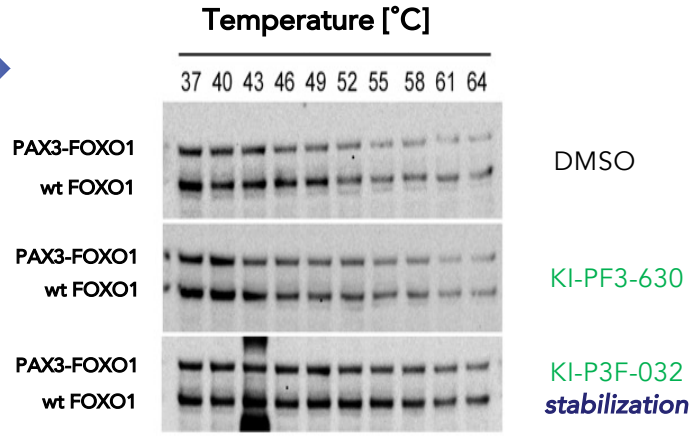
pathognomic fusion in alveolar rhabdomyosarcoma

Preliminary SMM screening data for PAX3-FOXO1 from **HEK293T cell lysates**

Pilot: ~10,000 small molecules

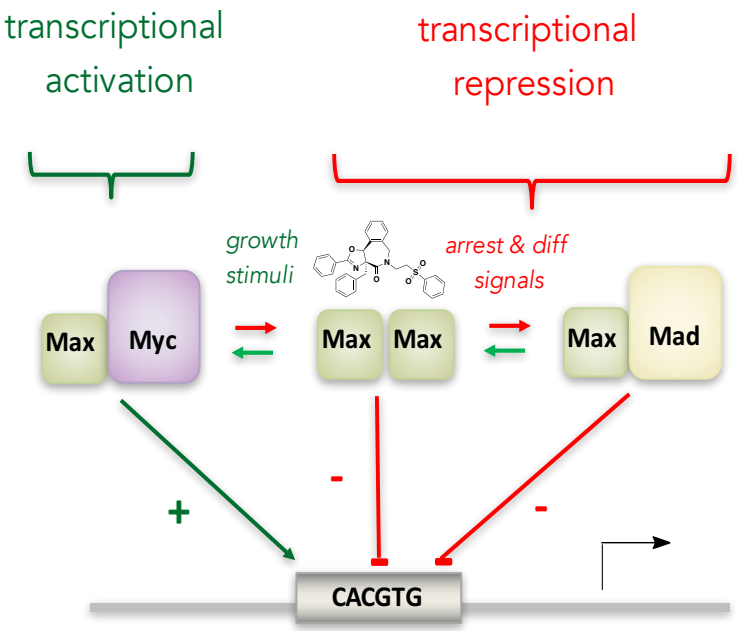


PAX3-FOXO1, FOXO1
CETSA

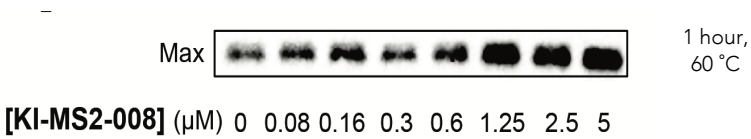


Shelby Doyle, Becky Leifer, Marco Wachtel, Beat Schaefer

CETSA for MAX Binder KI-MS2-008

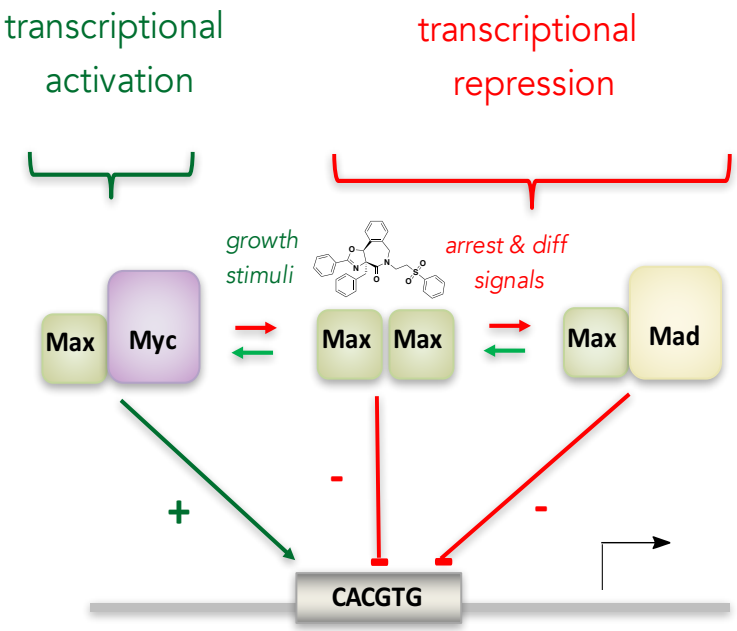


dose-dependent
cellular thermal shift assays (CETSA)
in live cells



Struntz et al., *Cell Chem Biol*, 26, 711-723 (2019)

CETSA for MAX Binder KI-MS2-008



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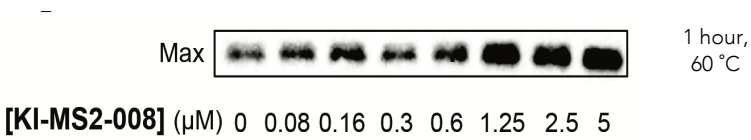

EXPERIMENT FOR FUTURE 20.109 STUDENTS EVALUATING YOUR MAX SMM HITS?

Struntz et al., *Cell Chem Biol*, 26, 711-723 (2019)

Upcoming Lectures

2/9/23	Lecture 1	Intro to chemical biology: small molecules, probes, and screens
2/14/23	Lecture 2	Small Molecule Microarray (SMM) technique
2/16/23	Lecture 3	Our protein target – MAX
2/21/23	No Lecture	
2/23/23	Lecture 4	Quantitative evaluation of protein-ligand interactions
2/28/23	Lecture 5	An SMM ligand discovery vignette for sonic hedgehog
3/2/23	Lecture 6	KB-0742: A Phase 2 clinical candidate discovered by SMMs
3/7/23	Lecture 7	Wrap up discussion for Mod 1 experiments and report