Which method to estimate Kd and Ki in fluorescence polarization?

Methodology and Examples

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Outline

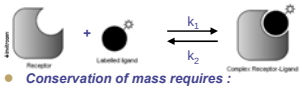
- *Context*
  - Saturation experiment
  - Competition experiment
- *Fluorescence Polarization*
  - Advantages
  - Principle
- *Estimation of Kd in fluorescence polarization*
  - Comparison of models
  - Examples & Simulations
- *Estimation of Ki in fluorescence polarization*
  - Comparison of models
  - Examples & Simulations
- *Conclusion*

Context: Ligand Binding Assay

- *A binding assay is used to measures the amount of binding or affinity between two molecules. There are numerous types of ligand binding assays.*
- *Widely applied in life science*
- *Examples*
  - Targeting protein-protein interactions
    - Protein–protein interaction (PPI) can contribute to many diseases, including cancer, and plays a key role in maintaining the malignant phenotype in tumor cells. Selective, small-molecule modulation of PPIs is therefore an area of interest to pharmaceutical science.
- *Two basic steps: saturation and competition experiments*

Two basic types of ligand binding experiment
1 – Saturation experiment

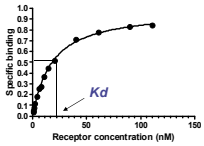
In saturation binding assays, a labelled ligand, [L], binds to a receptor, [R]*



$[R] + [L^*] \underset{k_2}{\overset{k_1}{\rightleftharpoons}} [RL^*]$

- *Conservation of mass requires :*
  $[L^*_T] = [L^*] + [RL^*]$ and $[R_T] = [R] + [RL^*]$
  with L^*_T, total concentration of labelled ligand
  R_T, total concentration of receptor
- *At equilibrium:* $k1\,[L^*]\,[R] = k2\,[RL^*]$

$$Kd = \frac{k2}{k1} = \frac{[L^*]\,[R]}{[L^*R]}$$



- *The equilibrium dissociation constant (Kd) provides a measure of affinity between the receptor [R] and the labelled ligand [L*]*

Two basic types of ligand binding experiment
2 – Competition experiment

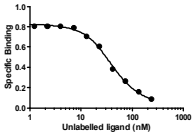
In competitive binding assays, an unlabelled ligand (competitor), [L], binds to a receptor [R] in competition with a labelled ligand, [L].*

$[R] + [L^*] \rightleftharpoons [RL^*]$

$+$

$[L]$

$[RL]$



- *Conservation of mass requires :*
  $[L^*_T] = [L^*] + [RL^*]$ $[L_T] = [L] + [RL]$
  $[R_T] = [R] + [RL^*] + [RL]$

with $[L^*_T]$ & $[R_T]$, total concentration of labelled ligand and receptor
$[L_T]$, total concentration of unlabelled ligand

- *At equilibrium:* $Ki = \frac{[L][R]}{[LR]}$
- *When the Kd of the labelled ligand [L*] is known, the equilibrium inhibition constant (Ki) provides an indirect measure of affinity between receptor [R] and unlabelled ligand [L]*

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Why use Fluorescence Polarization?

- *Previously, traditional radioligand binding experiments were widely used for saturation and competition experiments*
- *Fluorescence Polarization (FP) is one alternative to the traditional binding radioligand experiments, in particular for small molecules*
  - Application areas of FP:
    - Protein/Protein interactions
    - Enzyme/Substrate
    - Antigen/Antibodies
    - …
- *Fluorescence Polarization (FP) offers numerous advantages over the more conventional methods:*
  - Can be used in HTS
  - Faster and highly reproducible results (low variabilities intra/inter experiment)
  - No separation of bound and free ligand required
  - No radioactive materials

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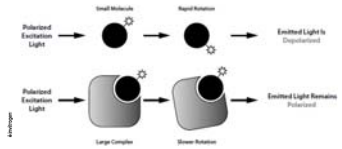
FP principle and signal expression

- *Fluorescence Polarization (FP) assays are based on measuring the polarization (P) of light caused by changes in molecular size.*
- *The rotational speed of a molecule is decreased once it is bound to a receptor.*



- *Polarized emission can be measured by polarization and anisotropy, r.*

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Relationship between anisotropy (r) and F_{bound}

- *In FP, there is no necessity for separation of bound and free ligand*
- *The fraction of bound to free, F_{bound}, is directly deduced from the anisotropy, r, using the equation:*

$$F_{bound} = \frac{binding\ \_site\ \_occupied}{total\ \_ligand} = \frac{[RL^*]}{[L^*_T]} = \frac{r - r_{free}}{(r_{bound} - r)Q + (r - r_{free})}$$

Where r_{free} is the anisotropy of the free labelled ligand,
r_{bound} is the anisotropy of the ligand-protein complex at saturation and
Q is the ratio of fluorescence intensities of bound versus free ligand

- *The binding of a ligand to a receptor is expressed by the fraction of bound receptors F_{bound}*

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Estimation of Kd in saturation experiment in FP

- *F_{bound} = (binding sites occupied) / (total ligand) = $[RL^*] / [L^*_T]$*
- *F_{bound} allows to estimate the affinity a ligand has to a receptor (Kd):*

$$[R] + [L^*] \rightleftharpoons [RL]$$

Kd = $[R][L^*]/[RL^*]$

***Case 1:** Specific case*

$[R_T] >> [L^*_T]$ → $[R] \approx [R_T]$
- *Assumption of no receptor depletion*

$$F_{bound} = \frac{[RL^*]}{[L^*_T]} = \frac{[R_T]}{K_d + [R_T]}$$

***Case 2 :** General case*

$[R_T]$ not >> $[L^*_T]$ → $[R] = [R_T] - [RL^*]$

$$F_{bound} = \frac{[RL^*]}{[L^*_T]} = \frac{a - \sqrt{a^2 - 4[R_T][L^*_T]}}{2[L^*_T]}$$

$$with \quad a = (K_d + [R_T] + [L^*_T])$$

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Examples of estimations of Kd in FP

- *In FP experimental assay, when can we assume the assumption of no receptor depletion?*
- *Example 1:*
  - $[L^*_T]$=1.5 nM
  - $[R_T]$ is between 0.002 nM to 257.2 nM
- *Example 2:*
  - $[L^*_T]$=10 nM
  - $[R_T]$ is between 2.5 to 1280 nM

Examples	Kd [95%CI] (nM) Case 1: [R] = $[R_T]$ No receptor depletion	Kd [95%CI] (nM) Case 2: [R] = $[RL^*] - [R_T]$
Example 1	1.417 [1.230 ; 1.604]	0.702 [0.607 ; 0.798]
Example 2	7.336 [5.774,8.898]	3.230 [1.880,4.581]

- *In those examples, there is a factor around 2 between both methods to estimate the equilibrium dissociation constant Kd.*

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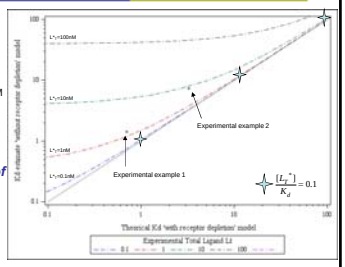
Simulations for Kd estimation

- ***Design***
  - **The FP were generated using receptor depletion hypothesis model (case 2) with the following factors:**
    - $[R_T]$ concentrations: 2.10^{-4} to 1.10^{4} nM
    - $[L^*_T]$ concentrations: [0.1 1 10 100] nM
    - Theoritical Kd from 0.1nM to 100nM
- ***Conclusion: providing $[L^*_T] < 10\%$ of Kd, we can assume that $[R] \approx [R_T]$***



Outline

Estimation of Ki in competition experiment

- ***The affinity of the competitor, unlabelled ligand [L], for the receptor [R] can be indirectly determined by measuring its ability to compete with, and thus inhibit, the binding of a labelled ligand [L*] to its receptor***

$$[R] + [L^*] \rightleftharpoons [RL^*]$$
$$[R] + [L] \rightleftharpoons [RL]$$
$$[RL] + [L^*] \rightleftharpoons [RL^*] + [L]$$

- ***The affinity of the unlabelled ligand for the receptor can be obtained from different methods:***
  - **Estimation of constant of inhibition (Ki)**
    - The Cheng-Prusoff equation
    - The *Nikolovska-Coleska & all* equation
    - …
  - **Direct estimation of the affinity of the competitor (Kd2)**
    - The Wang exact mathematical expression

Methods based on equation

- ***Shared assumptions***
  - **A single class of receptor binding sites**
  - **Assay is at equilibrium**
- ***Cheng-Prusoff equation***
  - **[R] ≈ $[R_T]$**
  - **$[L^*] \approx [L^*_T]$**

$$K_i = \frac{IC_{50}}{(\frac{[L^*]}{K_d} + 1)}$$

Where $[L^*]$ = *concentration of labelled ligand*; K_d = *dissociation constant of labelled ligand*

- ***Nikolovska-Coleska & al. equation***
  - **[R] = $[R_T]$ - [RL]**
  - **$[L^*] = [L^*_T]$ - [RL]**

$$K_i = \frac{[L_{50}]}{(\frac{[L^*_{50}] + [R_0]}{K_d} + 1)}$$

Where

$[R_0]$ = *Initial concentration of receptor*
$[L]$ = *Concentration of unlabelled ligand*
$[R_{eq}] = (-(K_{d1} + [L^*_t] - [R_t]) + ((K_{d1} + [L^*_t] - [R_t])^2 + 4[R_t]K_{d1})^{0.5})/2$
$[RL_{eq}] = [R_t] - [R_{eq}]$
$[RL^*_{eq}] = [RL^*_{tot}]/2$
$[L^*_{eq}] = [L^*_t] - [RL^*_{eq}]$
$[L_{50}] = IC_{50} - [R_t] + [RL^*_{eq}](1 + K_{d1}/[L^*_{eq}])$

Wang model: An exact analytical treatment of competitive binding

- ***Wang has described an exact expression of competitive binding:***

$$R + L^* \xleftrightarrow{K_{d1}} RL^* \qquad R + L \xleftrightarrow{K_{d2}} RL$$

$$K_{d1} = \frac{[R]\cdot[L^*]}{[RL^*]}(Eq.1) \qquad K_{d2} = \frac{[R]\cdot[L]}{[RL]}(Eq.2)$$

Conservation of mass requires:

$$[R_T] = [RL^*] + [RL] + [R]$$
$$[L^*_T] = [RL^*] + [L^*]$$
$$[L_T] = [RL] + [L] \quad (Eq.3)$$

- ***After substitution and rearrangement, this leads to the following equation:***

$$F_{bound} = \frac{[RL^*]}{[L^*_T]} = \frac{2\cdot\sqrt{(a^2-3b)}\cdot\cos\frac{\theta}{3} - a}{3K_{d1} + 2\cdot\sqrt{(a^2-3b)}\cdot\cos\frac{\theta}{3} - a}$$

Where

$$a = K_{d1} + K_{d2} + [L_T] + [L^*_T] - [R_T]$$
$$b = K_{d2}\cdot([L^*_T] - [R_T]) + K_{d1}\cdot([L_T] - [R_T]) + K_{d1}\cdot K_{d2}$$
$$c = -K_{d1}\cdot K_{d2}\cdot[R_T]$$

- ***$[R_T]$ and $[L_T]$ are the experimental constants***
- ***Kd1 must have been previously obtained from a direct binding experiment***

Comparison of Ki

- ***Example 1:***
  - **$[L^*_T]$ = 1.5 nM and $[R_T]$ = 6 nM**
  - **$[L_T]$ is between 0.51 nM to 10000 nM**
  - **Kd = 1.08 nM and IC50 = 22.3 [17.5 ; 28.6] nM**
- ***Example 2:***
  - **$[L^*_T]$ = 10.0 nM and $[R_T]$ = 20.0 nM**
  - **$[L_T]$ is between 0.67 to 238 nM**
  - **Kd = 3.23 nM and IC50 = 68.1 [40.1 ; 115.3] nM**

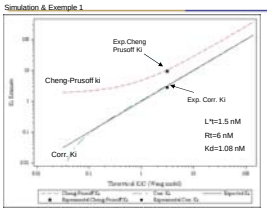
Experiment	*Ki Cheng Prusoff*	*Corrected Ki Nikolovska-Coleska & al.*	*Kd2 Wang model*
Example 1	9.35	2.81	3.14 [2.395 ; 3.888]
Example 2	16.62	7.33	5.34 [3.896 ; 6.778]

Simulations of competition experiment (1)

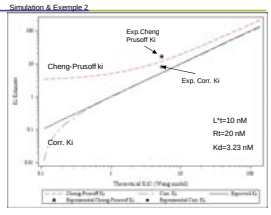
- ***Design***
  - **FP were generated using the Wang model under 2 experimental conditions with the following factors:**
    - $[L_T]$ is between 0.1 nM to 10000 nM
    - Theoretical Kd2 of competitor from 0.1nM to 10nM
  - 1st simulation (Example 1):
    - $[L^*_T]$ = 1.5 nM $[R_T]$ = 6 nM and Kd=1.08 nM
  - 2nd simulation (Example 2):
    - $[L^*_T]$ = 10 nM $[R_T]$ = 20 nM and Kd=3.23 nM
  - **EC50, Cheng-Prusoff Ki and Coor. Ki (Nikolovska-Coleska & al.) were estimated.**

Simulations of competition experiment (2)

Simulation & Exemple 1



Simulation & Exemple 2



- ***In these experimental conditions:***
  - **the Cheng-Prusoff Ki was over estimated**
  - **The Corr. Ki was:**
    - **always lower than the Cheng-Prusoff Ki**
    - **very close to the affinity of the competitor simulated with the Wang model (Kd2)**

Conclusion

- ***In FP, classical binding models need to be adapted to the Fbound***
- ***Which method to estimate Kd in FP?***
  - **The classical model, [R] ≈ [R_T], is not well adapted: over estimation of Kd**
  - **The general model, taken into account the receptor depletion [R] = [R_T] – [RL*], must be used in FP experiments**
- ***Which method to estimate Ki in FP?***
  - **Ki calculated with Cheng-Prusoff biaised the affinity of a competitor for a receptor**
  - **The Nikolovska-Coleska equation (corr. Ki) and the exact analytical treatment of competitive binding (Wang model) are equivalent methods:**
    - Advantage of Wang model : 95% confidence interval is estimated
    - Advantage of Corr. Ki : equation for uncompetitive inhibition is available
    - Both methods: difficult to generalize in particular cases as dimeric models

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References

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- Xinyi HUANG. "Fluorescence polarization competition assay: the range of resolvable inhibitor potency is limited by the affinity of the fluorescent ligand.", *J Biomol Screen*, 2003, 8, 34-38.

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Back-up slides

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Estimation of Kd in saturation experiment in FP

- F_{bound} = (binding sites occupied) / (total ligand) = [RL*] / [L^*_T]
- F_{bound} allows to estimate the affinity a ligand has to a receptor (Kd):

$$[R] + [L^*] \rightleftharpoons [RL]$$

$$Kd = [R]\,[L^*]/\,[RL^*]$$

Case 1: Specific case

$[R_T] >> [L^*_T] \rightarrow [R] \approx [R_T]$

- Assumption of no receptor depletion

$$F_{bound} = \frac{[RL^*]}{[L^*_T]} = \frac{[R_T]}{K_d + [R_T]}$$

Case 2 : General case

$[R_T]$ not $>> [L^*_T] \rightarrow [R] = [R_T] - [RL^*]$

$$F_{bound} = \frac{[RL^*]}{[L^*_T]} = \frac{a - \sqrt{a^2 - 4[R_T][L^*_T]}}{2[L^*_T]}$$

$$with \quad a = (K_d + [R_T] + [L^*_T])$$

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Estimation of Kd in saturation experiment in FP

- **Case 1: Specific case**
  - Assumption of no receptor depletion

$$K_d = \frac{[L^*][R]}{[RL]} \qquad [R] = [R_T] \qquad [L^*] = [L_T^*] - [RL^*]$$

$$K_d = \frac{([L_T^*] - [RL])([R_T]}{[RL]}$$

$$K_d + [R_T] = \frac{([L_T^*] - [RL])([R_T]}{[RL]} + [R_T] \longrightarrow K_d + [R_T] = \frac{([L_T^*] - [RL])([R_T]}{[RL]} + \frac{[RL][R_T]}{[RL]}$$

$$K_d + [R_T] = \frac{[L_T^*][R_T] - [RL][R_T] + [RL][R_T]}{[RL]}$$

$$K_d + [R_T] = \frac{[L_T^*][R_T]}{[RL]}$$

$$F_{bound} = \frac{[RL^*]}{[L^*_T]} = \frac{[R_T]}{K_d + [R_T]}$$

Estimation of Kd in saturation experiment in FP

- **Case 2: General case**
  - Assumption of receptor depletion

$$K_d = \frac{[L^*][R]}{[RL]} \qquad [R] = [R_T] - [RL^*] \qquad [L^*] = [L_T^*] - [RL^*]$$

$$K_d = \frac{([L_T^*] - [RL^*])([R_T] - [RL^*])}{[RL^*]} \longrightarrow K_d[RL^*] = ([L_T^*] - [RL^*])([R_T] - [RL^*])$$

$$K_d[RL^*] - ([L_T^*] - [RL^*])([R_T] - [RL^*]) = 0 \longrightarrow -[RL^*]^2 + (K_d + [R_T] + [L_T^*])[RL^*] - [R_T][L_T^*]) = 0$$

$$[RL^*] = \frac{(K_d + [R_T] + [L_T^*]) - \sqrt{(K_d + [R_T] + [L_T^*])^2 - 4[R_T][L_T^*]}}{2}$$

$$[RL^*] = \frac{(K_d + [R_T] + [L_T^*]) - \sqrt{(K_d + [R_T] + [L_T^*])^2 - 4[R_T][L_T^*]}}{2}$$

$$F_{bound} = \frac{[RL^*]}{[L_T^*]} = \frac{(K_d + [R_T] + [L_T^*]) - \sqrt{(K_d + [R_T] + [L_T^*])^2 - 4[R_T][L_T^*]}}{2[L_T^*]}$$

Wang model: An exact analytical treatment of competitive binding (1)

- **Wang has described an exact expression of competitive binding:**

$$R + L^* \xleftarrow{K_{d1}} RL^* \qquad R + L \xleftarrow{K_{d2}} RL$$

$$K_{d1} = \frac{[R]\cdot[L^*]}{[RL^*]} (Eq.1) \qquad K_{d2} = \frac{[R]\cdot[L]}{[RL]} (Eq.2)$$

Conservation of mass requires:

$$[R_T] = [RL^*] + [RL] + [R]$$
$$[L_T^*] = [RL^*] + [L^*]$$
$$[L_T] = [RL] + [L] \quad (Eq.3)$$

- **Expressing [RL*] and [RL] as function of total ligand (L^*_T) and total competitor concentrations (L_T) yield Eq.4 and 5**

$$\Rightarrow \quad RL^* = \frac{[R]\cdot[L_T^*]}{K_{d1} + [R]} \quad (Eq.4) \qquad RL = \frac{[R]\cdot[L_T]}{K_{d2} + [R]} \quad (Eq.5)$$

- **Substitution of Eq.4 and 5 in Eq. 3, yields Eq. 6, which after rearrangement corresponds to the cubic Eq. 7**

$$[R_T] = \frac{[R]\cdot[L_T^*]}{K_{d1} + [R]} + \frac{[R]\cdot[L_T]}{K_{d2} + [R]} + [R] \quad (Eq.6)$$

$$[R]^3 + a[R]^2 + b[R] + c = 0 \quad (Eq.7)$$

Where

$$a = K_{d1} + K_{d2} + [L_T] + [L_T^*] - [R_T]$$
$$b = K_{d2}\cdot([L_T^*] - [R_T]) + K_{d1}\cdot([L_T] - [R_T]) + K_{d1}\cdot K_{d2}$$
$$c = -K_{d1}\cdot K_{d2}\cdot[R_T]$$

Wang model: An exact analytical treatment of competitive binding (2)

- **The only meaningful solution of Eq.7 can be written as in Eq.8 and the expression of θ is given in Eq. 9.**

$$[R] = -\frac{a}{3} + \frac{2}{3}\cdot\sqrt{(a^2 - 3b)}\cdot\cos\frac{\theta}{3} \qquad where \qquad \theta = \arccos\left[\frac{-2a^3 + 9ab - 27c}{2\cdot\sqrt{(a^2 - 3b)^3}}\right] \quad (Eq.8)$$

$$\frac{[RL^*]}{[L_T^*]} = \frac{[R]}{K_{d1} + [R]} = \frac{2\cdot\sqrt{(a^2 - 3b)}\cdot\cos\frac{\theta}{3} - a}{3K_{d1} + 2\cdot\sqrt{(a^2 - 3b)}\cdot\cos\frac{\theta}{3} - a} \quad (Eq.9)$$

Where

$$a = K_{d1} + K_{d2} + [L_T] + [L_T^*] - [R_T]$$
$$b = K_{d2}\cdot([L_T^*] - [R_T]) + K_{d1}\cdot([L_T] - [R_T]) + K_{d1}\cdot K_{d2}$$
$$c = -K_{d1}\cdot K_{d2}\cdot[R_T]$$

- **$[R_T]$ and $[L_T]$ are the experimental constants**
- **Kd1 must have been previously obtained from a direct binding experiment**