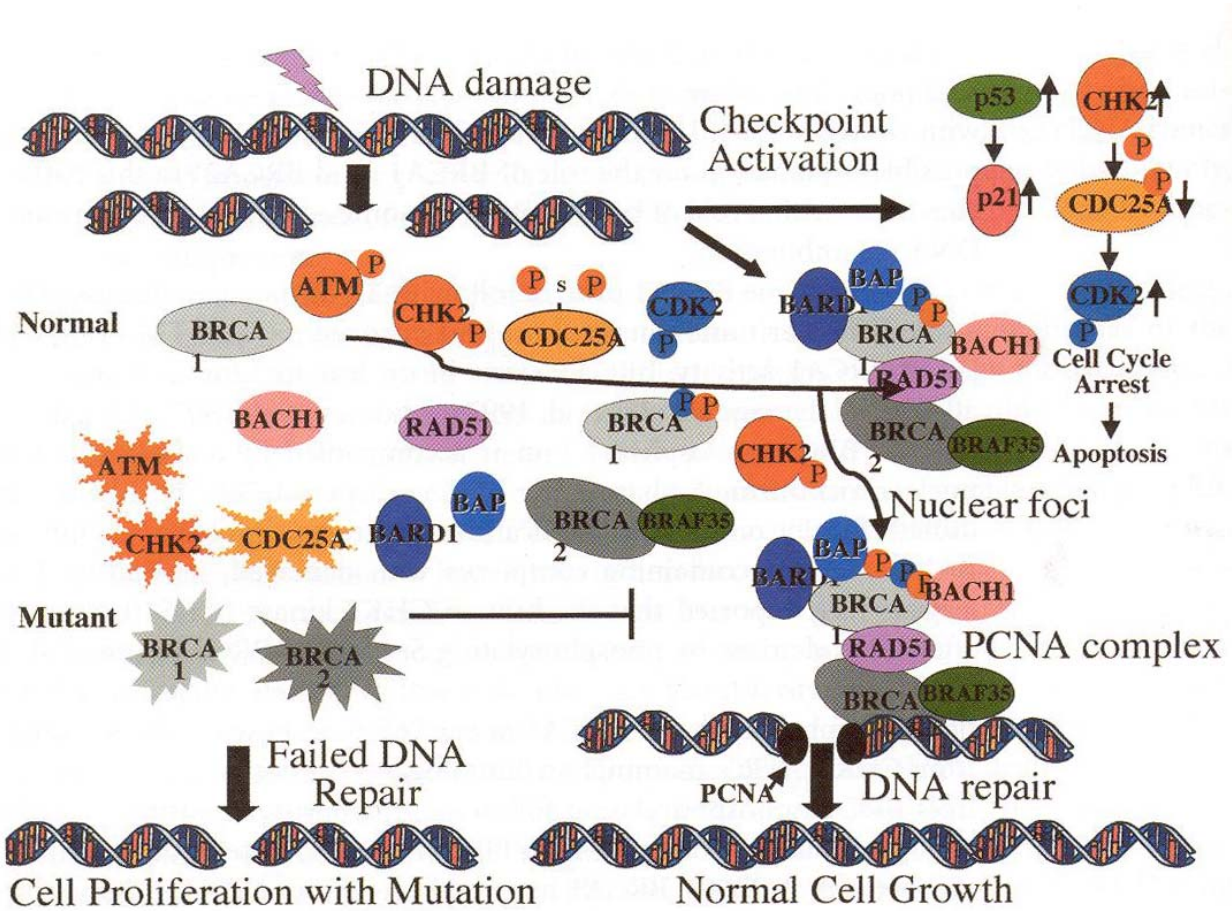
Protein-Ligand Interactions Are Responsible for Signal Transduction

Types of Interactions:

1. Protein-Protein
2. Protein-DNA (RNA)
3. Protein-small molecule



Dynamic Protein-Ligand Interaction

Dynamic interaction between Protein (T, target) and Ligand (L) assumes equilibrium

$$L + T \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} L \bullet T$$

The equilibrium binding constant is defined as: $$K_b = \frac{[L \bullet T]_{eq}}{[L]_{eq}[T]_{eq}} = \frac{k_{on}}{k_{off}}$$

The equilibrium dissociation constant is defined as: $$K_d = \frac{[L]_{eq}[T]_{eq}}{[L \bullet T]_{eq}} = \frac{k_{off}}{k_{on}}$$

where $[L \bullet T]_{eq}$, $[T]_{eq}$ and $[L]_{eq}$ are equilibrium concentrations of the complex, free protein and free ligand respectively.

It is obvious that $$\boldsymbol{K_b = K_d^{-1}}$$

K_b (K_d) can be found in equilibrium experiments by measuring somehow one of the following ratios:

$$\frac{[T]_{eq}}{[L \bullet T]_{eq}} \quad \text{or} \quad \frac{[L]_{eq}}{[L \bullet T]_{eq}}$$

at different $[T]_0$ or $[L]_0$ as we demonstrated with fluorescence anisotropy

Equilibrium Constant Measurements

Methods for equilibrium constant measurements:

1. Fluorescence
- Fluorescence anisotropy (limitation – small fluorophore)
- Fluorescence (absorbance) spectrum shift due to the change of microenvironment

2. Capillary Electrophoresis
- Capillary affinity electrophoresis (CAE)
- Non-Equilibrium Capillary Electrophoresis of Equilibrium Mixtures (NECEEM)

3. Molecular Electro-optics

4. Surface plasmon resonance

OFF and ON Rate Constants: k_{off} and k_{on}

To understand the DYNAMICS of interactions (how fast the complex forms and how fast it decays we need to know the rate constants of complex decay and formation (OFF and ONN constants): k_{off} and k_{on}.

The OFF constant may be measured by:

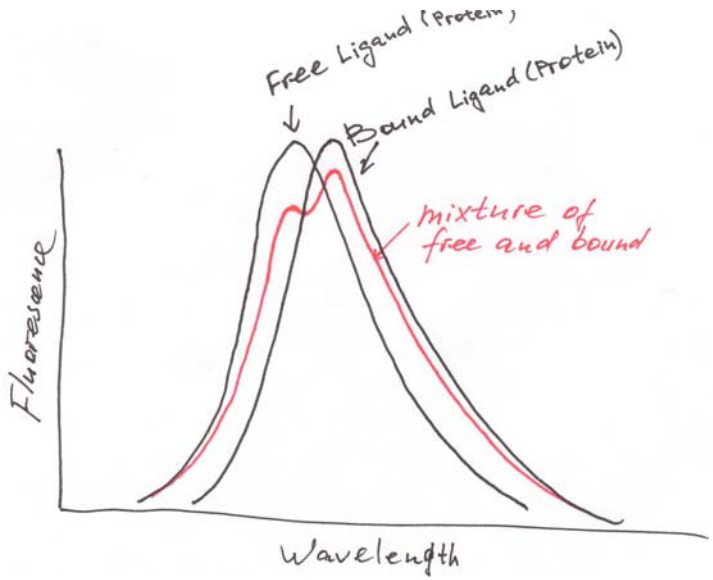
- NECEEM
- Surface plasmon resonance

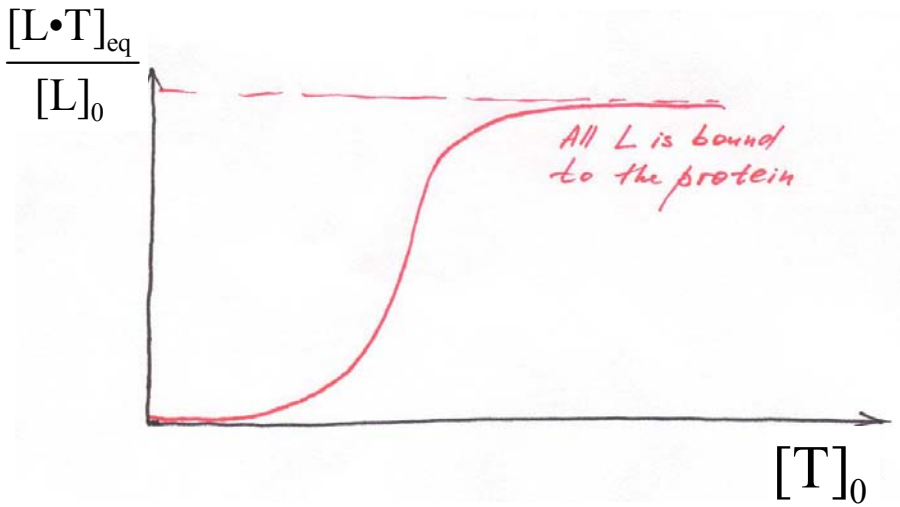
The ON constant can be determined by

1. Stopped-flow spectroscopic measurements
2. Sweeping Capillary Electrophoresis (SweepCE)

Fluorescence (absorbance) spectrum shift due to the change of microenvironment

Assume that L changes its fluorescence spectrum upon binding to T





Fluorescence intensity at a fixed wavelength λ is an extensive (additive) function:

$$I_\lambda^{tot} = I_\lambda^{L} \frac{[L]_{eq}}{[L]_0} + I_\lambda^{L \bullet T} \frac{[L \bullet T]_{eq}}{[L]_0}$$

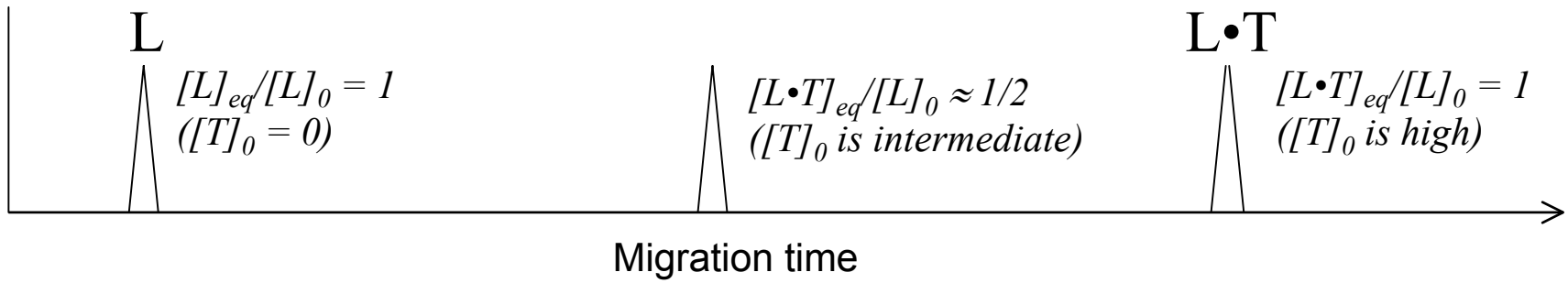
Absolute fluorescence intensities of pure L and L•T

Capillary affinity electrophoresis (CAE)

Assume that:

1. Ligand is detected (by absorbance or fluorescence).
2. Free Protein (T) and Ligand (L) have different migration times
3. L • T will have intermediate migration time

Add protein to the run buffer at different concentrations
Observe migration time shift as a function of $[T]_0$. The following are the results of three different experiments:



Mobility is an additive function:

$$\mu = \mu_L \frac{[L]_{eq}}{[L]_0} + \mu_{L\bullet T} \frac{[L \bullet T]_{eq}}{[L]_0}, \qquad \mu = \frac{v}{E} = \frac{L}{tE}$$

$$\frac{1}{t} = \frac{1}{t_F} \frac{[F]_{eq}}{[F]_0} + \frac{1}{t_{P\bullet F}} \frac{[P \bullet F]_{eq}}{[F]_0}$$

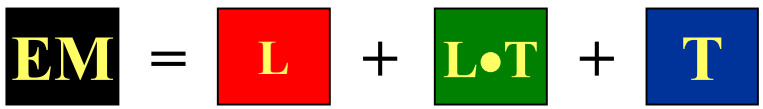
Assignment: Derive formulas for ACE-based determination of K_d using fluorescence anisotropy as a prototype

Non-equilibrium capillary electrophoresis of equilibrium mixtures (NECEEM)

Assume that **Protein (T) and Ligand (L) have different migration times**

Incubate L and T to reach the equilibrium.

The equilibrium mixture contains three components: L, T, and L•T.

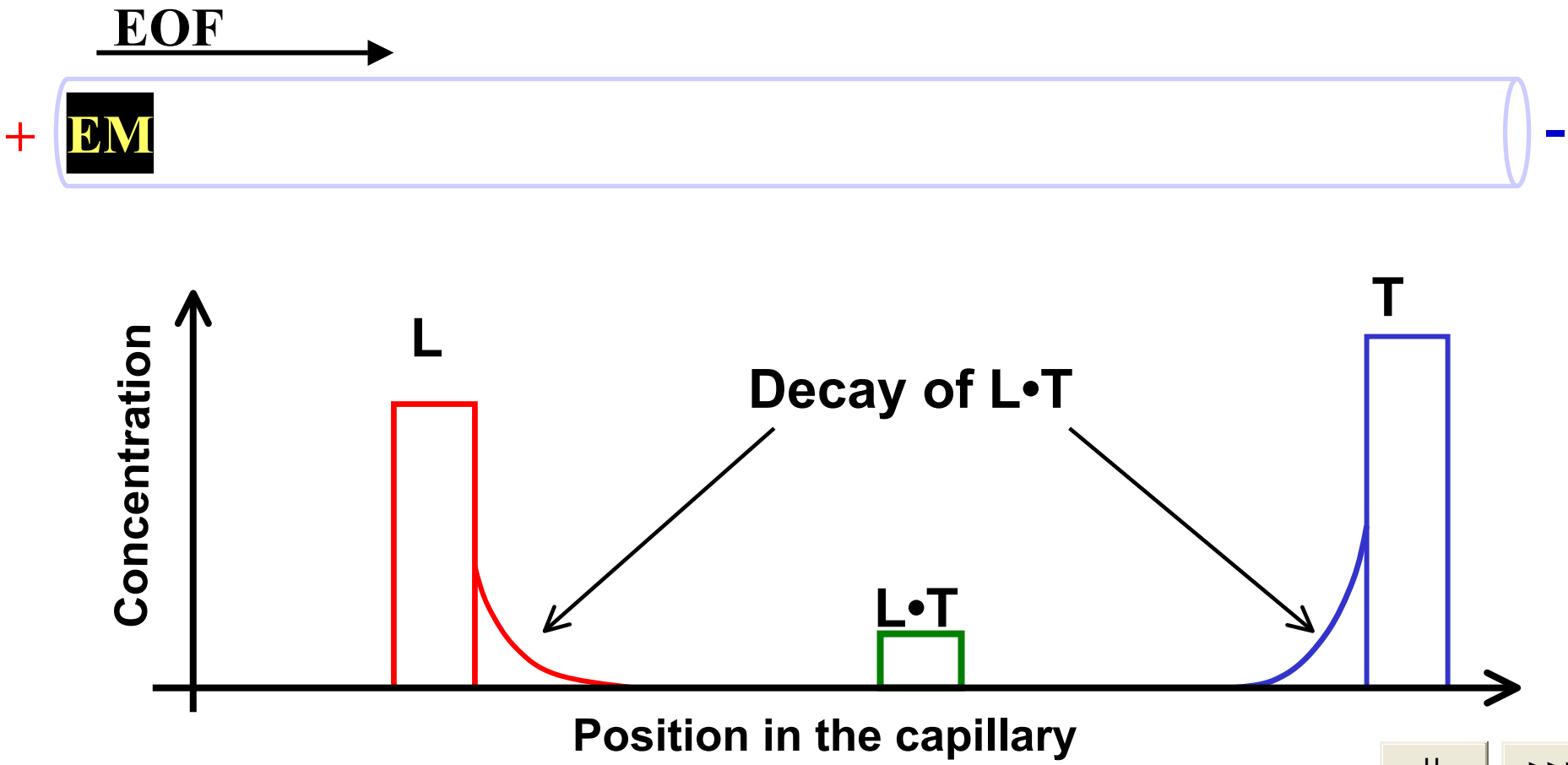


Separate them by electrophoresis.

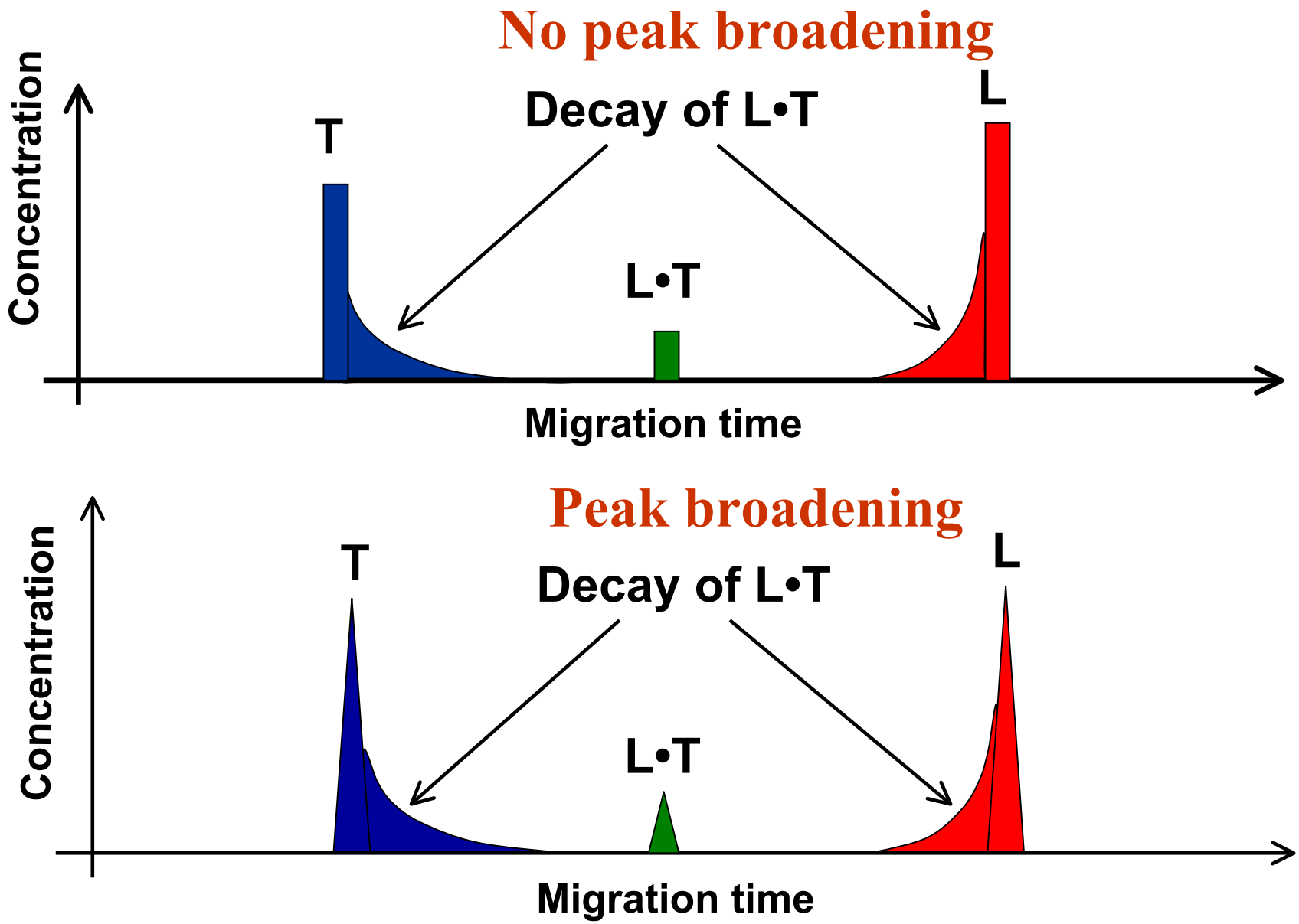
Electrophoretic zones in NECEEM

- Equilibrium fractions of L and T migrate as single zones
- Equilibrium fraction of L•T decays and generates smears

EM = L + L•T + T

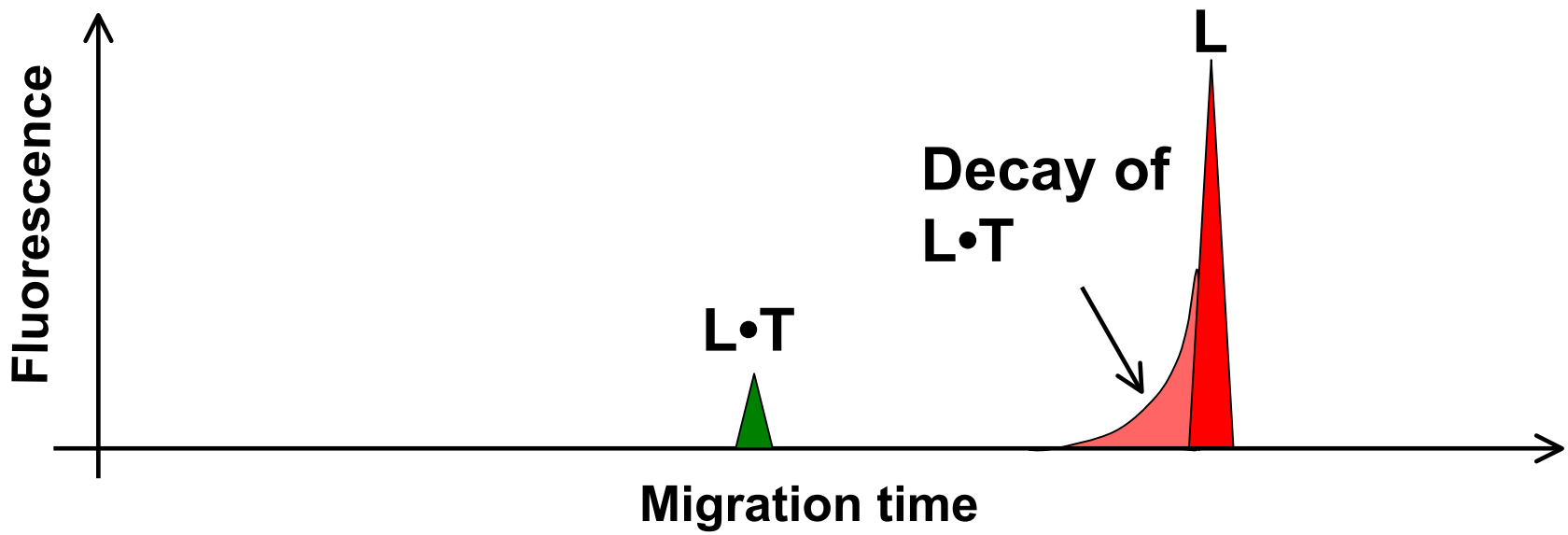


NECEEM Electropherograms



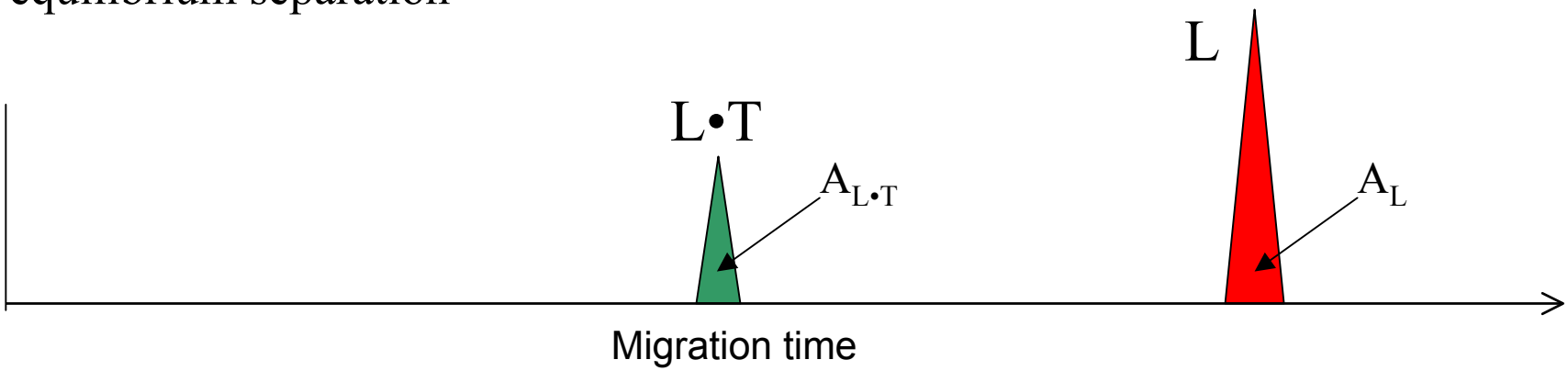
NECEEM Electropherograms – Fluorescence Detection of One Component

L has a fluorescent tag
T does not have it



Determination of K_d with NECEEM

Case 1: Complex is stable enough (does not decay during the separation). Then two peeks will be observed in a single experiment. NECEEM is reduced to equilibrium separation



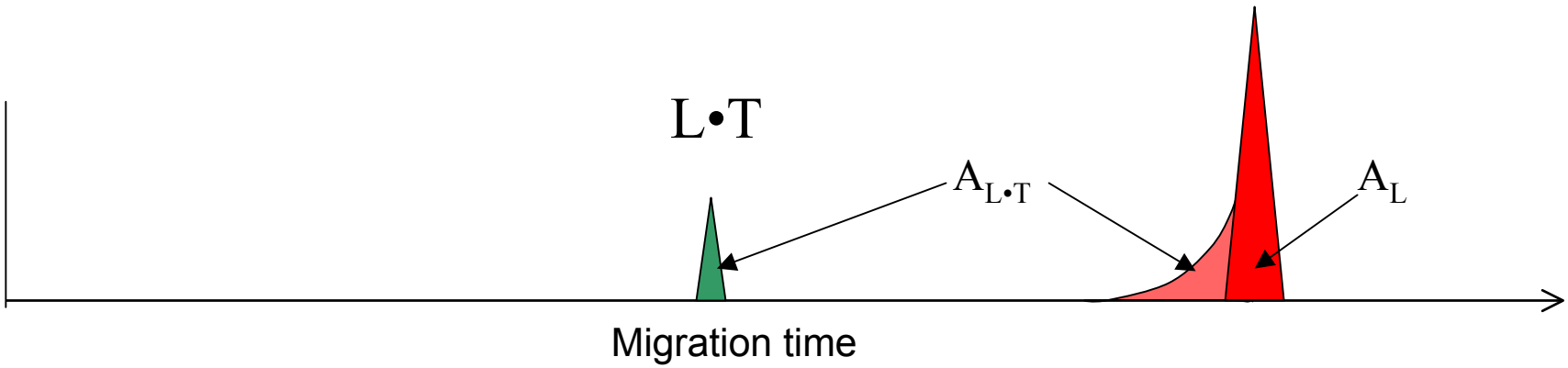
Peak areas correspond to the equilibrium concentrations of L and L • T:

$A_L \sim [L]_{eq}$, $A_{L•P} \sim [L•T]_{eq}$,

Thus, $$K_d = \frac{[T]_0(1+R)-[L]_0}{(1+1/R)} \quad \text{where } R = \frac{[L]_{eq}}{[L•T]_{eq}} = \frac{A_L}{A_{L•T}}, \quad 0 < R < 1$$

Determination of K_d with NECEEM contd.

Case 2: Complex is NOT stable enough (does decay during the separation). Then the L•T zone will be decaying during separation. The red area adjacent to peak L is produced by decay of L•T.



Peak areas still correspond to the equilibrium concentrations of L and L•T :

$A_L \sim [L]_{eq}$, $A_{L•P} \sim [L•T]_{eq}$,

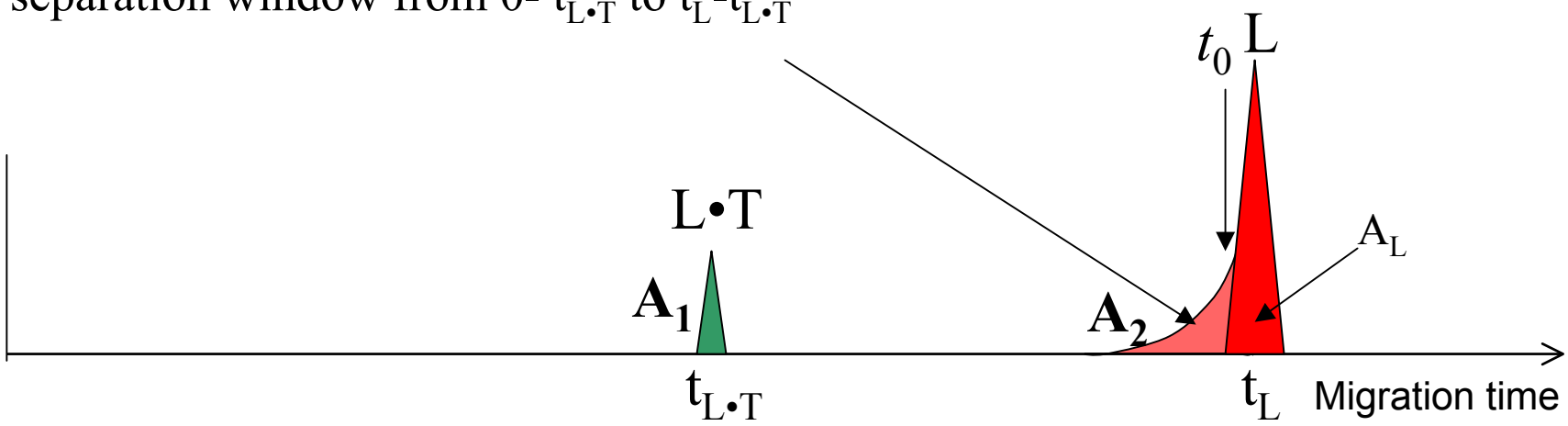
However, $A_{L•T}$ will now consist of two parts, the remnants of peak L•T and signal produced by the decay of L•T. It does not change, however, the formula for K_d calculation:

$$K_d = \frac{[T]_0(1+R)-[L]_0}{(1+1/R)} \quad \text{where } R = \frac{[L]_{eq}}{[L•T]_{eq}} = \frac{A_L}{A_{L•T}}, \quad 0 < R < 1$$

Determination of k_{off} constant by NECEEM

Case 2: Complex is NOT stable (it decays during the separation). The forward reaction does not proceed since free T and L are separated by electrophoresis. So that the only reaction going is: $L \bullet T \xrightarrow{k_{off}} L + T$

Exponential decay, $I = I_0 \exp\{k_{off} a(t-t_0)\}$, where $a = t_{L\bullet T}/(t_L - t_{L\bullet T})$ is a constant needed to compensate for apparent change of the separation window from 0- $t_{L\bullet T}$ to t_L-$t_{L\bullet T}$



Thus, k_{off} can be found by fitting the exponential decay line.

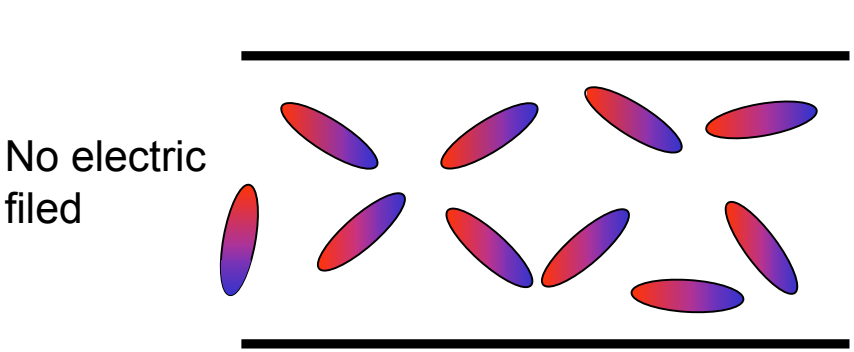
Alternatively: $$k_{off} = \ln\left(\frac{A_1 + A_2}{A_1}\right) / t_{L\bullet T}$$

Molecular Electro-Optics

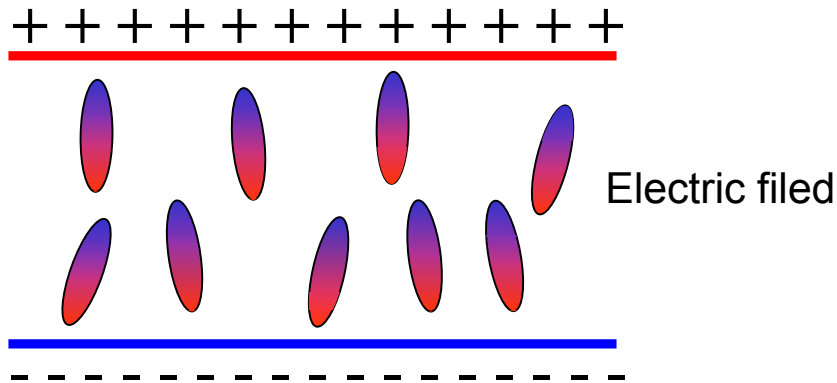
Physical Bases: The method is based on a change of protein alignment in an electric filed upon binding to a ligand. The change can be registered optically, ……

Implementation: Short pulses of electric field are applied to a solution of protein. Since all proteins are dipoles, the electric filed induces the alignment of electrical dipoles in the field. This molecular alignment is recorded by measurements of:

- Absorbance of polarized light by protein (electric dichroism)
- Anisotropy of the refractive index (electric birefringence)
- Fluorescence anisotropy
- Light scattering
- Etc.



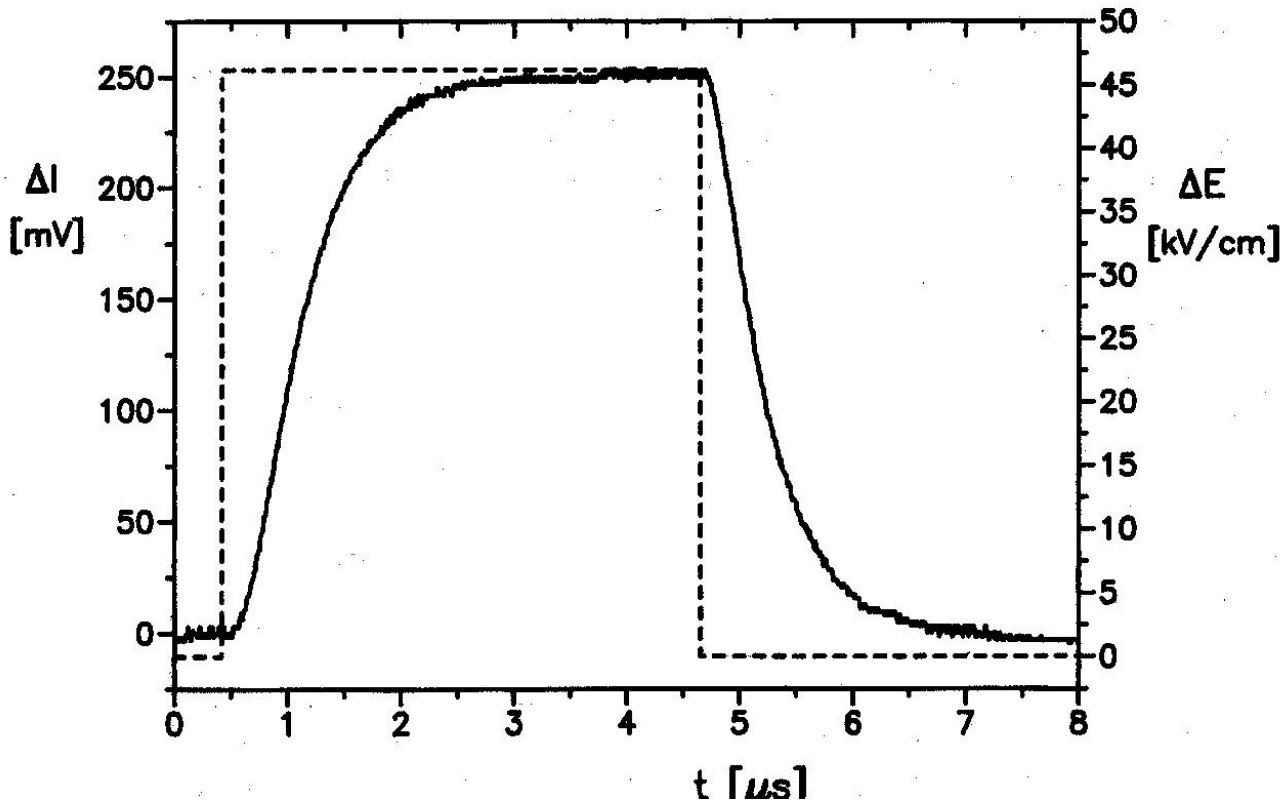
In the absence of an external filed the distribution of protein molecules is random



In the presence of external filed protein molecules are partially oriented in the direction of the electric filed

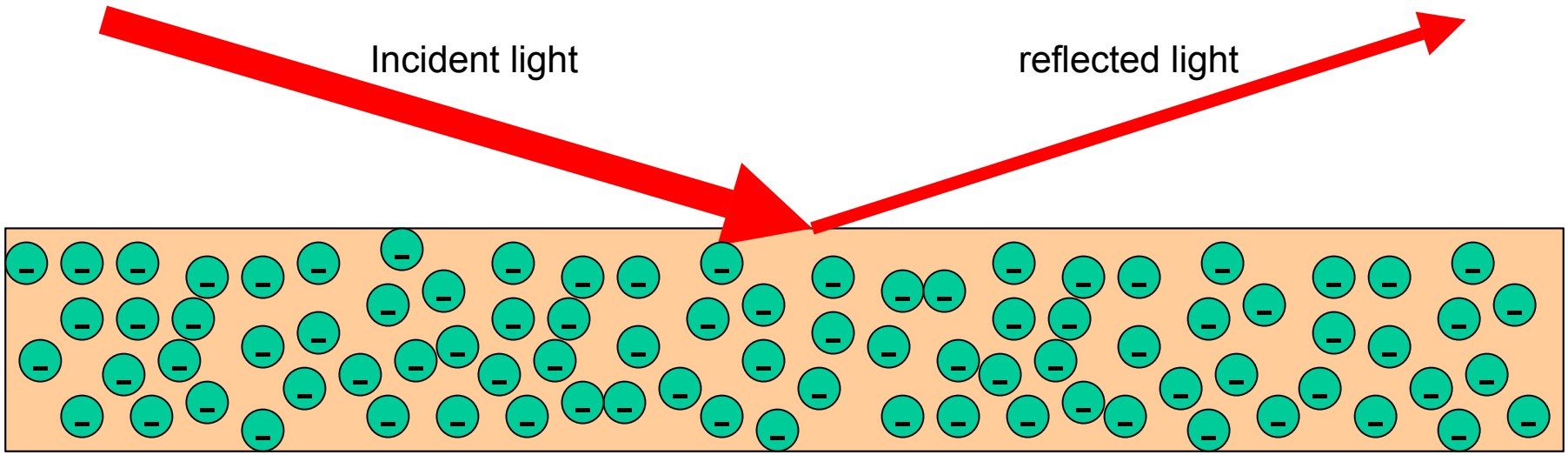
Molecular Electro-Optics contd.

When the field is turned off the molecules relax back to the random orientation. The rate of alignment and the rate of relaxation are equal to each other and depend on the size and shape of the molecule and the viscosity of the solution. This rate changes upon binding of the protein to the ligand



Surface Plasmon Resonance (SPR)

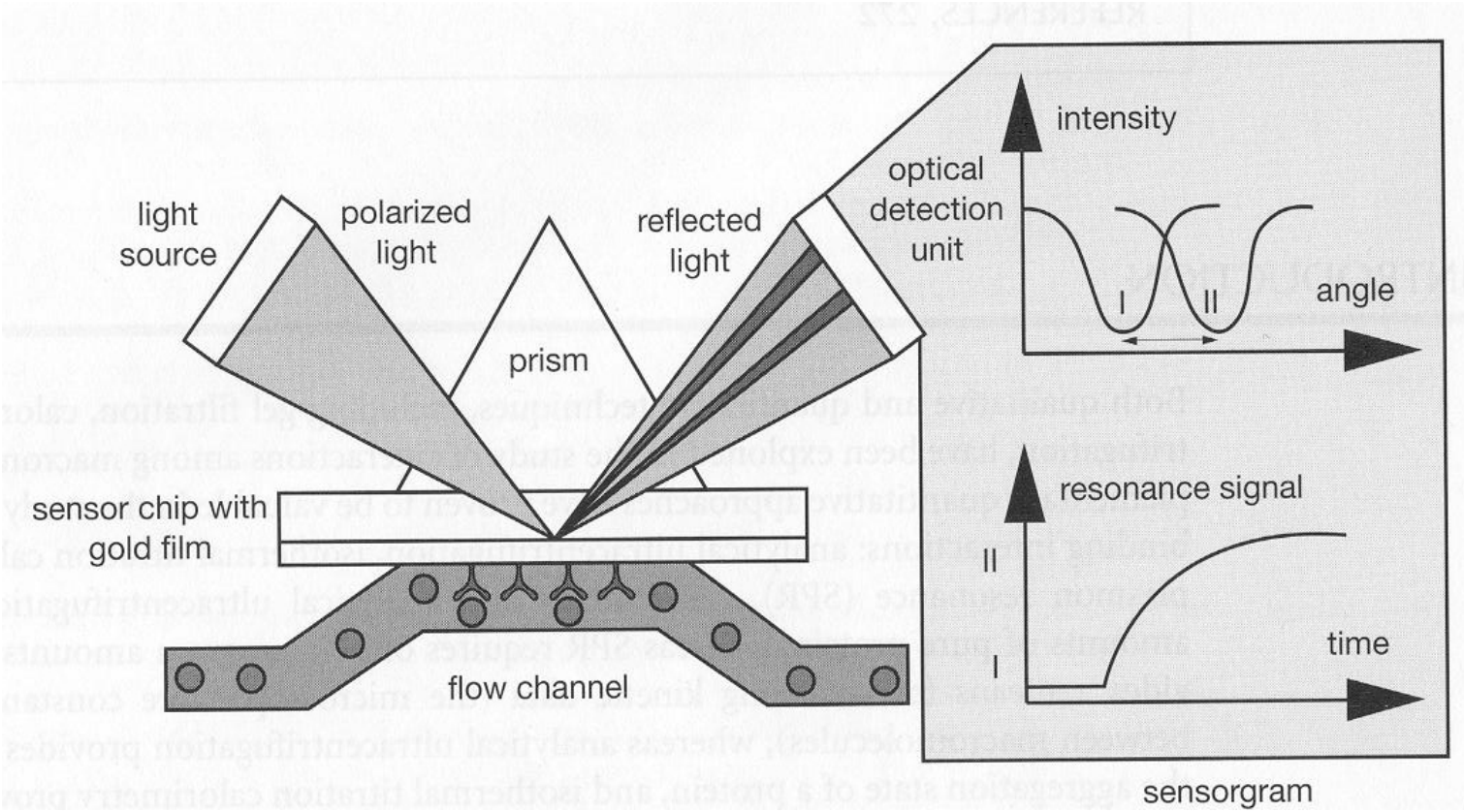
- SPR is an resonance between light and a cloud of free electrons in a thin layer of gold (electromagnetic filed of light penetrates inside the gold to small depth). Resonance results in dissipation of light energy and thus decreasing intensity of light reflected from the gold surface.
- There is a certain angle between incident light and surface at which the intensity of reflected light is minimum (energy absorbance or SPR is maximum)
- Resonance depends on the refractive index of the media on the back side of the gold mirror. Refractive index is sensitive to the change of surface chemistry.



Refractive index of the media on this side of the mirror changes when surface bound ligand interacts with the protein. The change in the refractive index leads to the change in SPR.

Practical Aspects of SPR

- What is detected in SPR? The change in the angle at which the intensity of reflected light is minimum (SPR is maximum)
- The ligand is immobilized on the gold surface
- The protein solution flows over the surface
- When the protein binds to the ligand the angle of minimum reflected light changes. The change is proportional to the mass of protein bound to the surface.



Ligand Immobilization in SPR

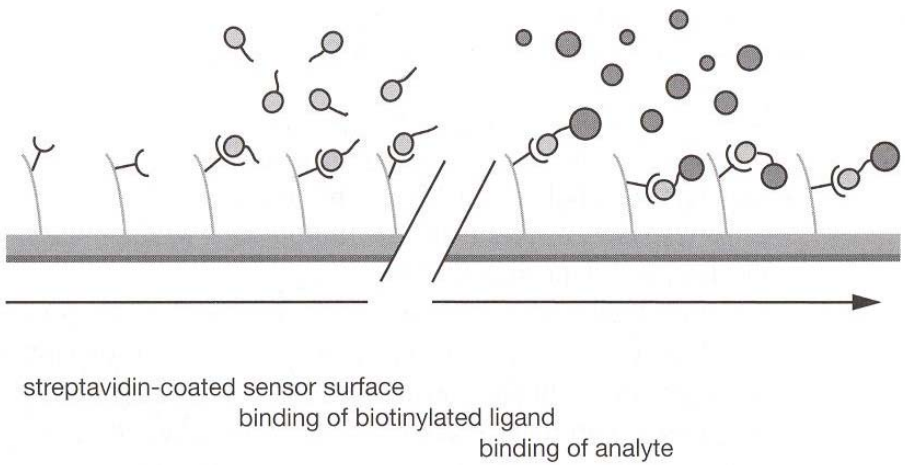
Ligand immobilization without disruption of its activity is the most challenging part of experimentation in SPR.

Direct immobilization of ligand to the surface has a number of drawbacks:

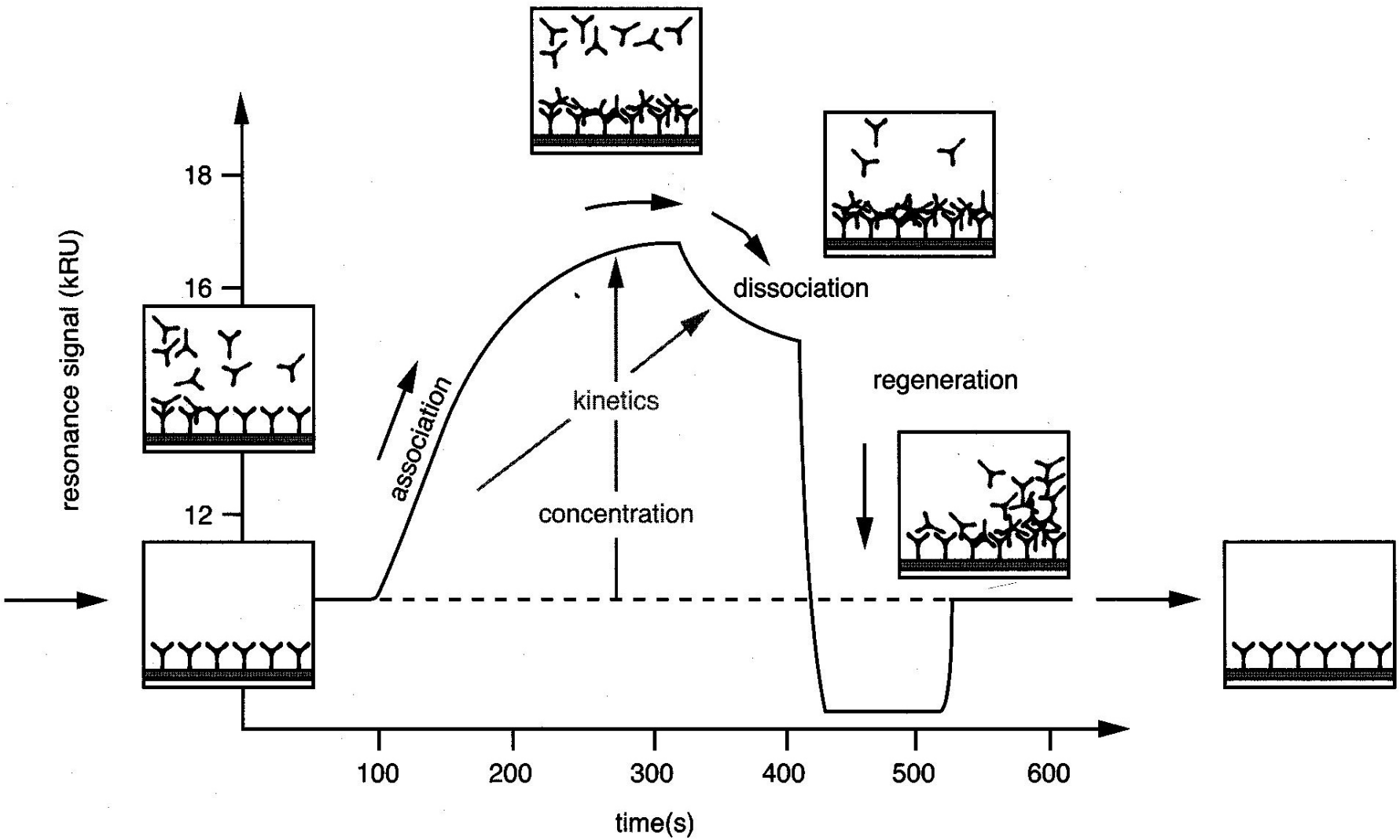
- Provides only heterogeneous immobilization due to multiple reactive groups on ligands (proteins)
- Often disrupts binding to the protein
- Require pure ligand since the impurities will bind to the surface as well

Indirect immobilization of ligand to the surface through another molecule:

- Ligand is seldom inactivated
- The ligand does not have to be pure; it can be captured from a "crude" sample (e.g. by using antibodies)
- Immobilized molecules can have homogeneous orientation

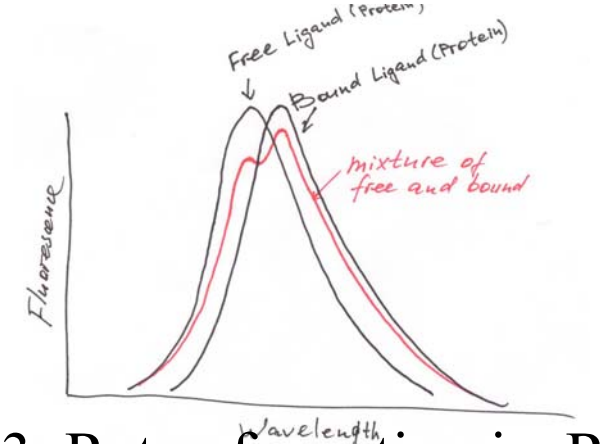


Sensogram in SPR



Stopped-Flow Spectroscopy

Is based on spectral changes of one of the components (e.g. L) upon binding to another one

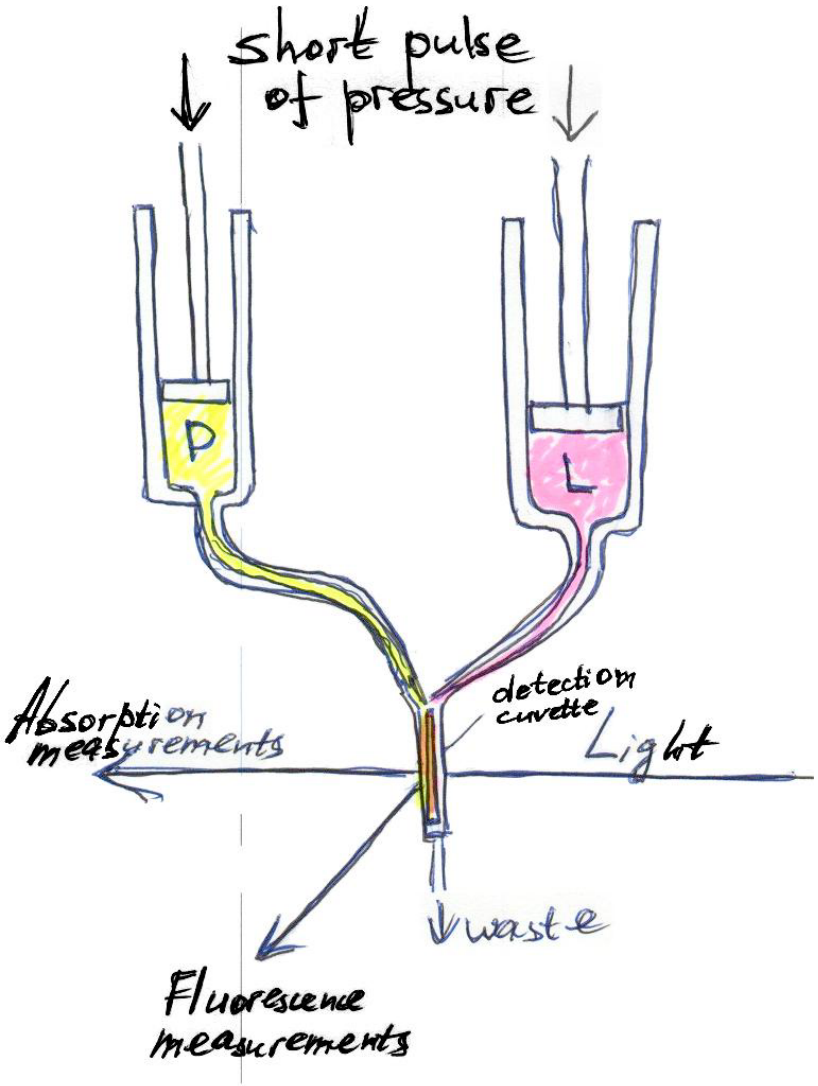


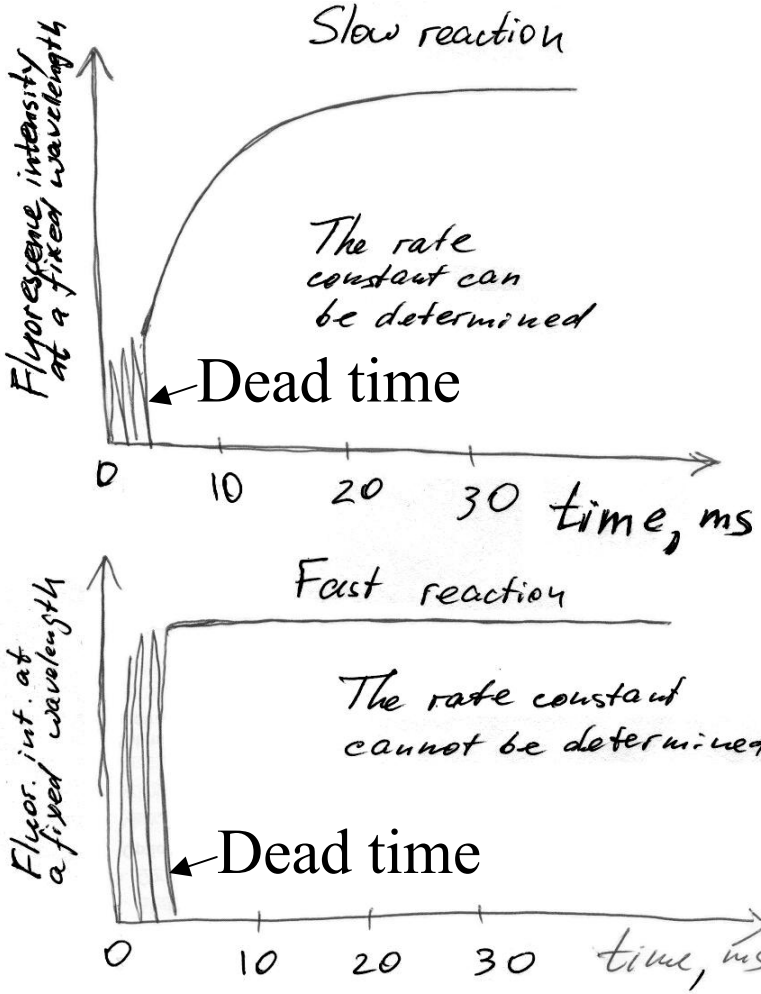
1. Reacting components are mixed fast:
2. In the initial stages the reverse reaction is negligible

$$L + T \xrightarrow{k_{on}} L{\bullet}T$$

3. Rate of reaction is: Rate = k_{on} [L][T]
4. The simplest approach to find k_{on} is based on the assumption of the pseudo-first order reaction. For this, the "invisible" component (e.g. T) is taken in excess to the "visible" component (e.g. L). It is assumed that T does not change during the reaction: $[T] \approx [T]_0$. Therefore, rate of reaction $\approx (k_{on}[T]_0)$ [L]. The apparent rate constant, k_{app} ($= k_{on}[T]_0$), is found from fitting the exponential line. The rate constant is then calculated as $k_{on} = k_{app}/[T]_0$

Finite mixing time leads to the phenomenon of "dead time"





Limitation of Stopped flow spectroscopy

The spectral change may be insignificant

⇓

To detect the small change large concentrations of L and T must be used

⇓

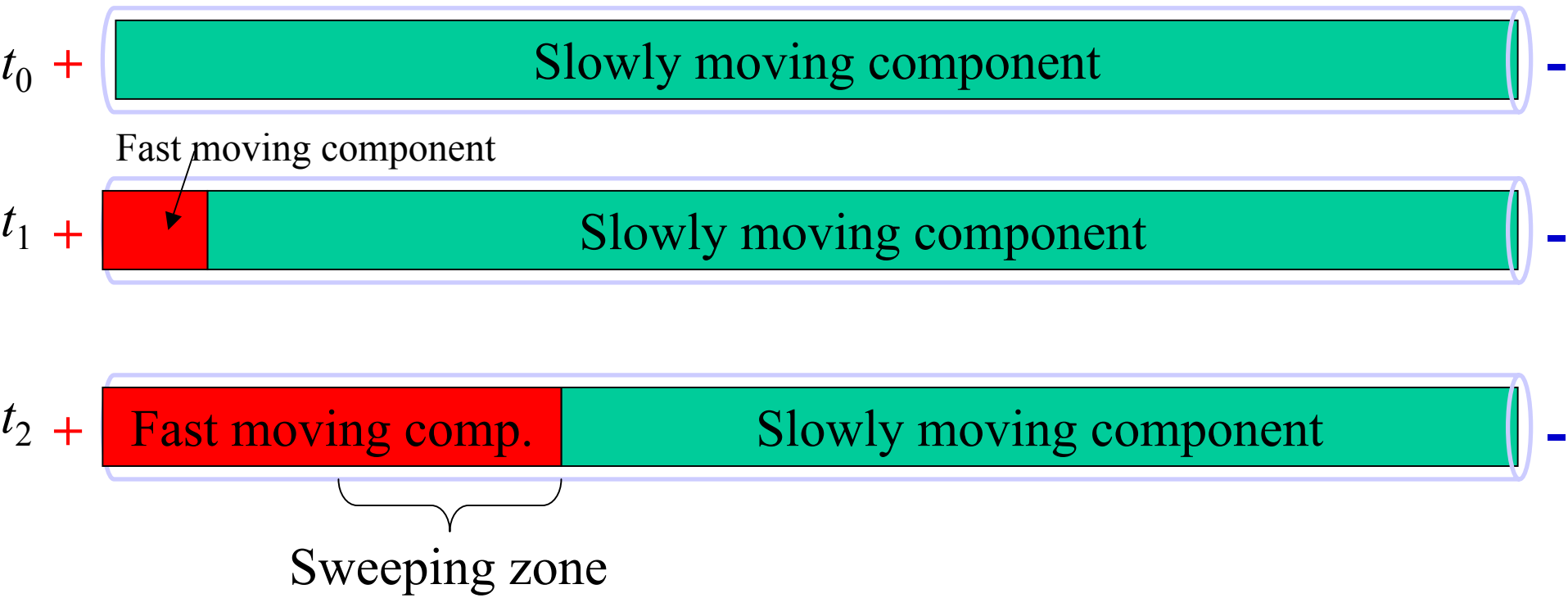
Reaction rates become very fast at large concentrations so that the reaction reaches equilibrium during the mixing period

⇓

As a result large k_{on} cannot be often measured

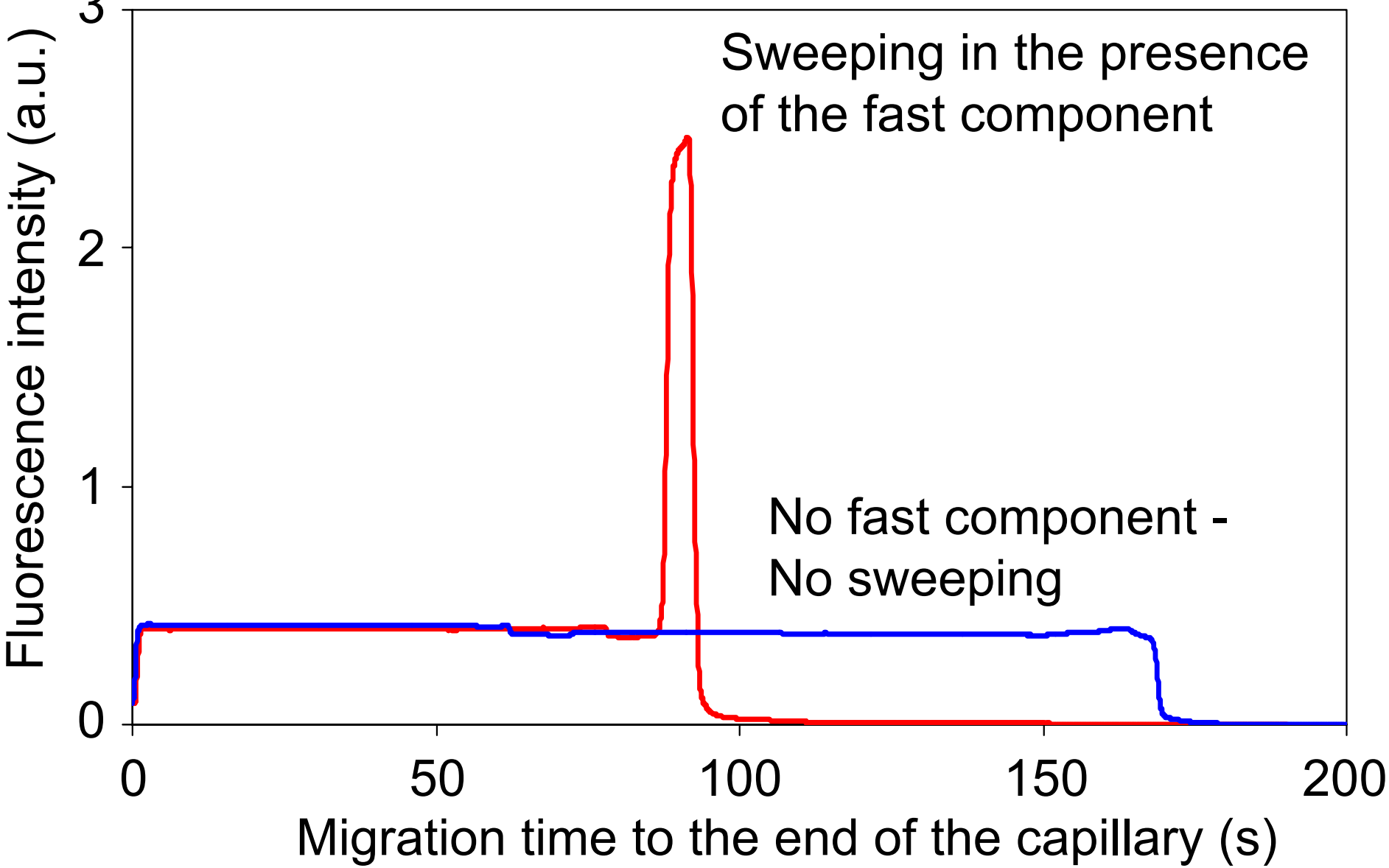
Sweeping Capillary Electrophoresis (SweepCE)

- SweepCE is the only non-stopped flow technique for measuring k_{on} of fast reactions
- Is based on sweeping of a slowly-moving component by a fast-moving component



SweepCE Electropherogram

Slow-moving component is fluorescent



Mass transfer equations to describe SweepCE

Reaction: L + T $\rightarrow$ L•T

$$\frac{\partial T(t,x)}{\partial t} + v_T \frac{\partial T(t,x)}{\partial x} = -k_{\text{on}} L(t,x) T(t,x)$$

$$\frac{\partial L(t,x)}{\partial t} + v_L \frac{\partial L(t,x)}{\partial x} = -k_{\text{on}} L(t,x) T(t,x)$$

$$\frac{\partial L\bullet T(t,x)}{\partial t} + v_{L\bullet T} \frac{\partial L\bullet T(t,x)}{\partial x} = k_{\text{on}} L(t,x) T(t,x)$$

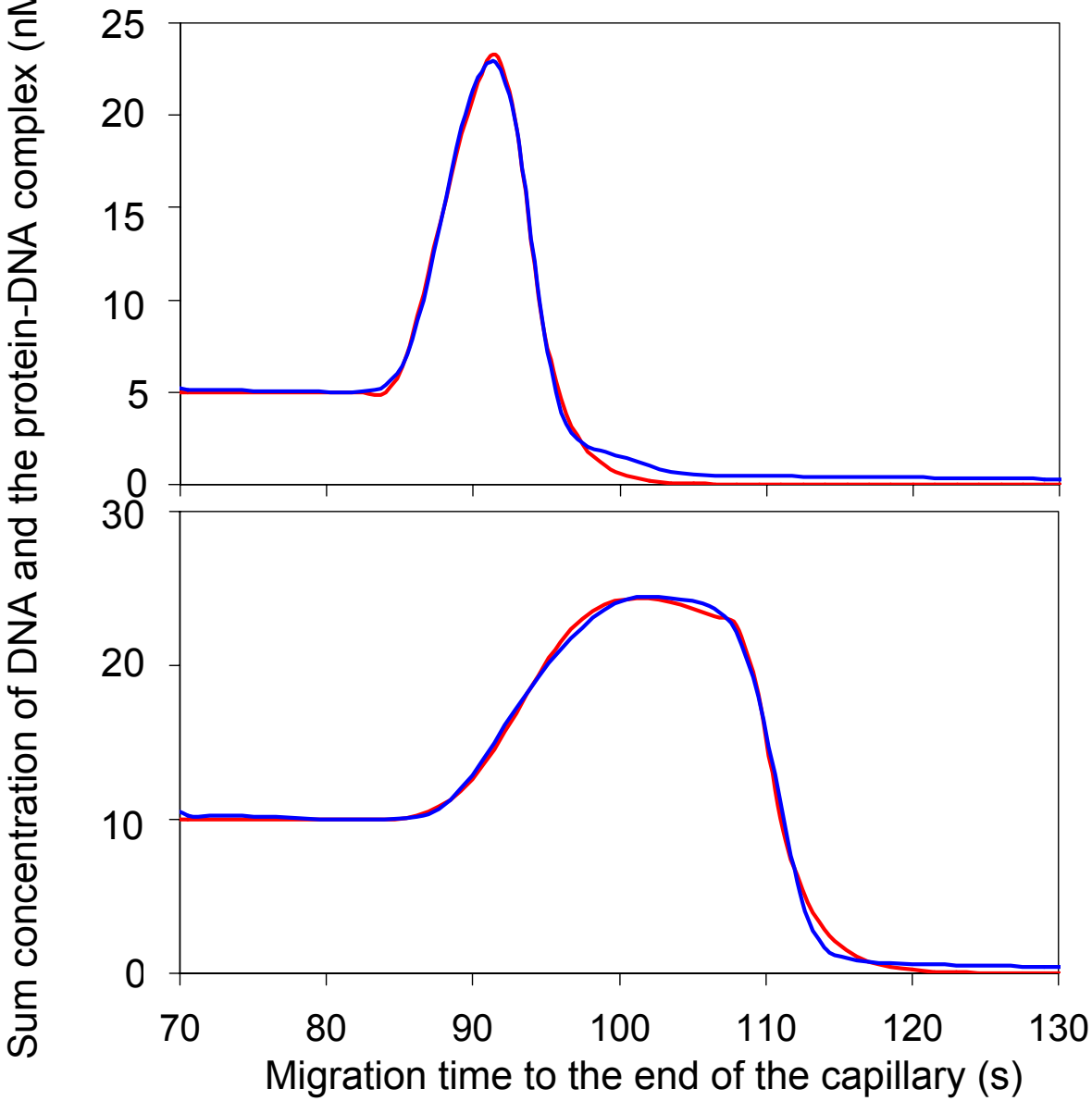
Analytical solution for SweepCE

$$T(t,x) = T_0 \frac{\exp\left(k_{\mathrm{on}} T_0 \dfrac{x - v_{\mathrm{T}} t}{v_{\mathrm{T}} - v_{\mathrm{L}}} \right) \theta(-x + v_{\mathrm{T}} t)}{\left[\left\{ \exp\left(-k_{\mathrm{on}} T_0 \dfrac{x - v_{\mathrm{T}} t}{v_{\mathrm{T}} - v_{\mathrm{L}}} \right) - 1 \right\} \theta(-x + v_{\mathrm{T}} t) + \right.}$$
$$\left. + \left\{ \exp\left(k_{\mathrm{on}} L_0 \dfrac{x - v_{\mathrm{L}} t}{v_{\mathrm{T}} - v_{\mathrm{L}}} \right) - 1 \right\} \theta(x - v_{\mathrm{L}} t) + 1 \right]$$

$$L(t,x) = L_0 \frac{\exp\left(k_{\mathrm{on}} L_0 \dfrac{x - v_{\mathrm{L}} t}{v_{\mathrm{T}} - v_{\mathrm{L}}} \right) \theta(x - v_{\mathrm{L}} t)}{\left[\left\{ \exp\left(-k_{\mathrm{on}} T_0 \dfrac{x - v_{\mathrm{T}} t}{v_{\mathrm{T}} - v_{\mathrm{L}}} \right) - 1 \right\} \theta(-x + v_{\mathrm{T}} t) + \right.}$$
$$\left. + \left\{ \exp\left(k_{\mathrm{on}} L_0 \dfrac{x - v_{\mathrm{L}} t}{v_{\mathrm{T}} - v_{\mathrm{L}}} \right) - 1 \right\} \theta(x - v_{\mathrm{L}} t) + 1 \right]$$

$$L \bullet T(t,x) = k_{\mathrm{on}} \int_0^t L(t - \tau, x - \tau v_{\mathrm{L} \bullet \mathrm{T}}) T(t - \tau, x - \tau v_{\mathrm{L} \bullet \mathrm{T}}) d\tau$$

Finding k_{on} by fitting experimental SweepCE electropherograms



[T] = 40 nM
[L] = 5 nM
$k_{on} = 3.9 \times 10^6\ M^{-1}s^{-1}$

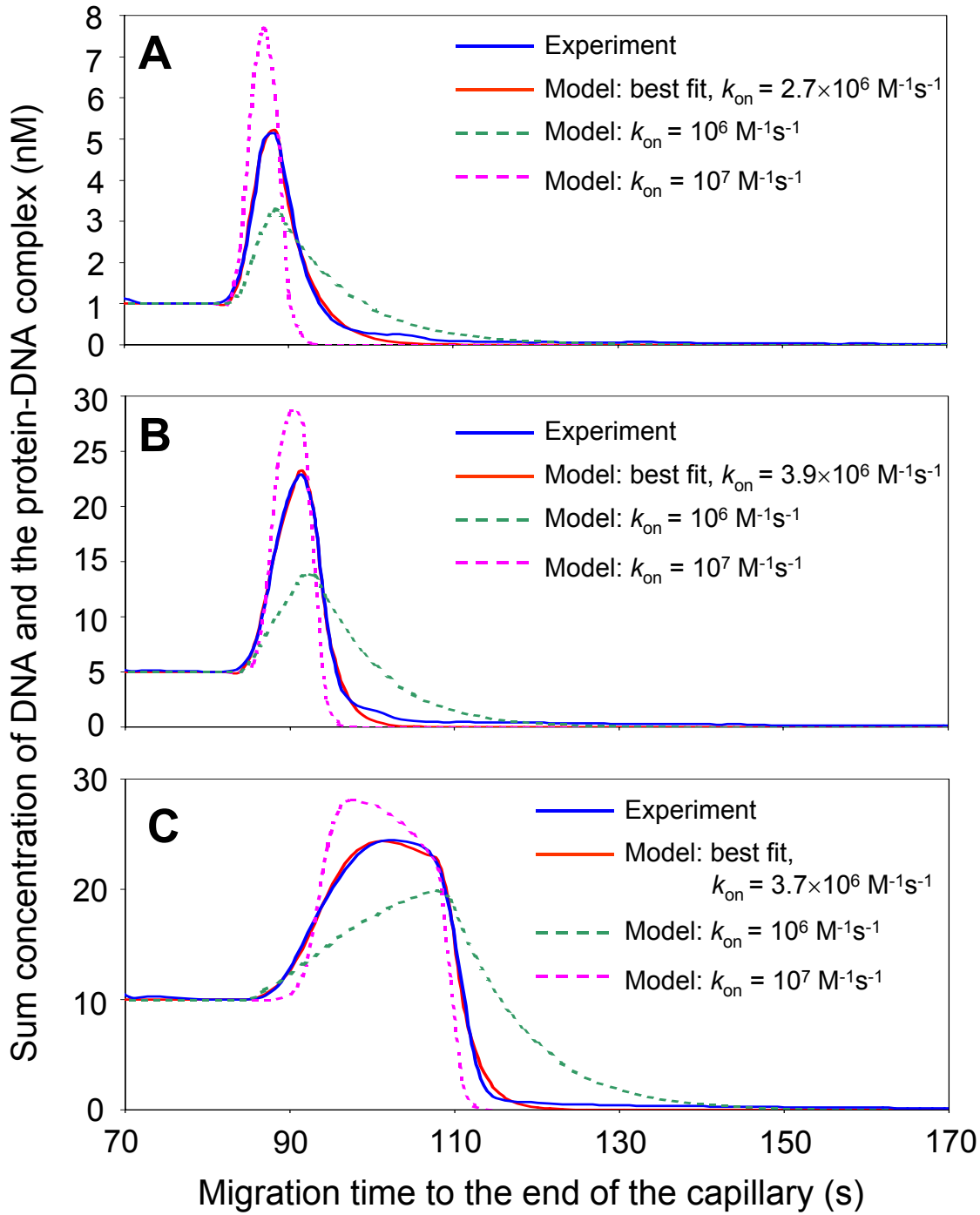
[T] = 40 nM
[L] = 10 nM
$k_{on} = 3.7 \times 10^6\ M^{-1}s^{-1}$

Accuracy of SweepCE

A: [T] = 40 nM
[L] = 1 nM

B: [T] = 40 nM
[L] = 5 nM

C: [T] = 40 nM
[L] = 10 nM



Advantages of SweepCE over Stopped-flow Spectroscopy

Does not require spectral changes

⇓

Can employ low concentration of a "visible" component

⇓

Can be used for measuring very high k_{on}

Can also be used for measuring k_{off} if numerical modeling is used to fit the experimental data (under development)