

MIT OpenCourseWare
<http://ocw.mit.edu>

5.36 Biochemistry Laboratory
Spring 2009

For information about citing these materials or our Terms of Use, visit: <http://ocw.mit.edu/terms>.



A



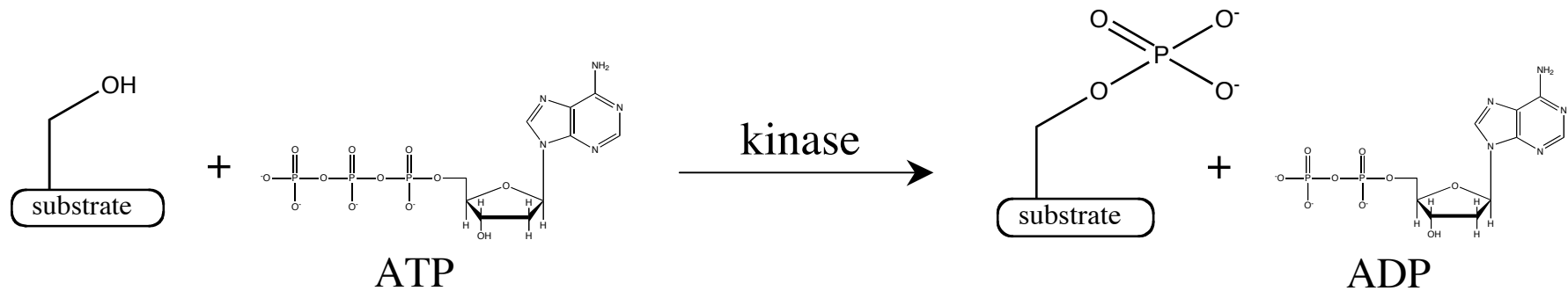
B

Which is a kinase domain? A, B, or both?

Detecting Kinase Activity in vitro and in vivo

- I. In vitro activity assays (for purified kinases)
 - A. ^{32}P incorporation assay
 - B. Gel analysis with a phospho-specific antibody
 - C. Kinase coupled-activity assay (lab sessions 13 and 14)
- II. Fluorescent peptide-based probes
 - A. Environment-sensitive fluorescent probes
 - B. Chelation-enhanced fluorescent probes
- III. In-vivo protein probes (FRET)
- IV. Laboratory report guidelines

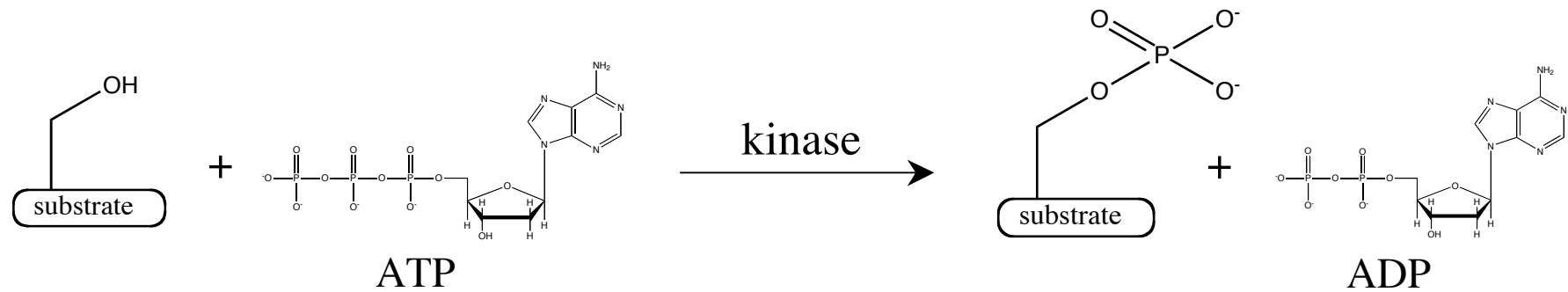
Detecting protein kinase activity in vitro and in vivo



Why are scientists interested in detecting kinase activity?

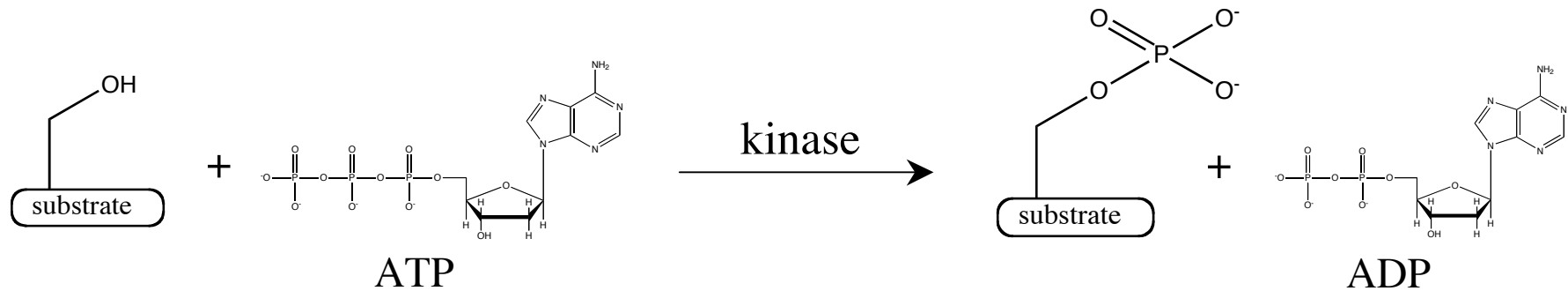
- Enables monitoring of kinase activity and inhibition
- Used for screening of **drug candidates**
- Facilitates the unraveling of complex cell-signalling pathways implicated in processes ranging from cell cycle regulation to cellular **migration** (including tumor metastasis).

Detecting protein kinase activity in vitro and in vivo



Enzyme activity assays detect either product formation or starting material consumption.

Detecting protein kinase activity in vitro and in vivo

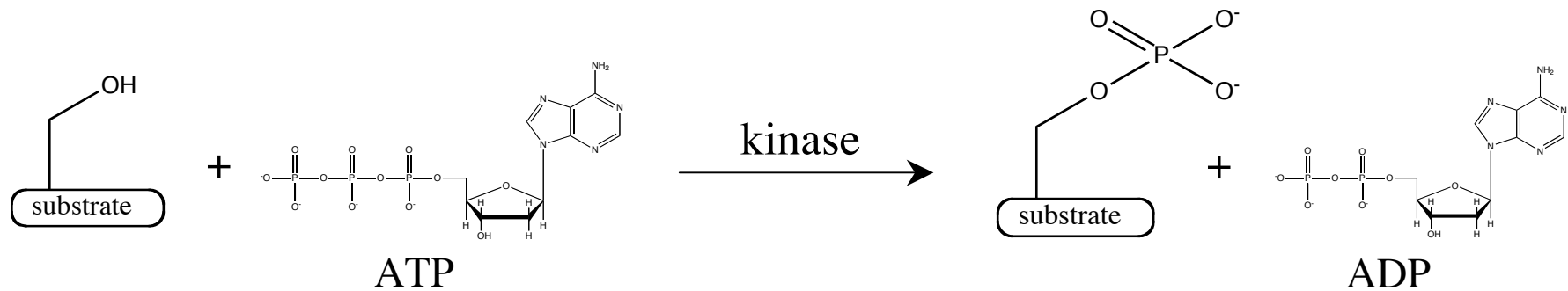


Enzyme activity assays detect either product formation or starting material consumption.

For kinases this means

- Detection of phosphorylated **substrate** (product) *or*
- Detection of **ADP** (product) formation or **ATP** (starting material) consumption

Detecting protein kinase activity in vitro and in vivo



*However, phosphorylation is spectroscopically **silent**.*

Neither the formation of phosphorylated product nor the conversion of ATP to ADP can be monitored *directly* using spectroscopy.

The products appear **identical** to the starting materials due to identical absorption features and extinction coefficients.

Kinase assays: in buffer, in cell lysate, in vivo

Depending on your application, you should consider whether you need an assay that can be performed only with purified enzyme, in a cell lysate mixture, or in vivo.

Some other considerations may include a protocol that is:

- Easy and safe to use
- **General** versus specific
- **Continuous** (versus an end-point assay)
- A turn-on versus a turn-off assay (some researchers prefer the generation of a positive signal rather than a decrease in signal)
- Amenable to high-throughput applications
- Sensitive to low levels of kinase activity

Detecting Kinase Activity in vitro and in vivo

I. In vitro activity assays (for purified kinases)

- A. ^{32}P incorporation assay
- B. Gel analysis with a phospho-specific antibody
- C. Kinase coupled-activity assay (lab sessions 13 and 14)

II. Fluorescent peptide-based probes

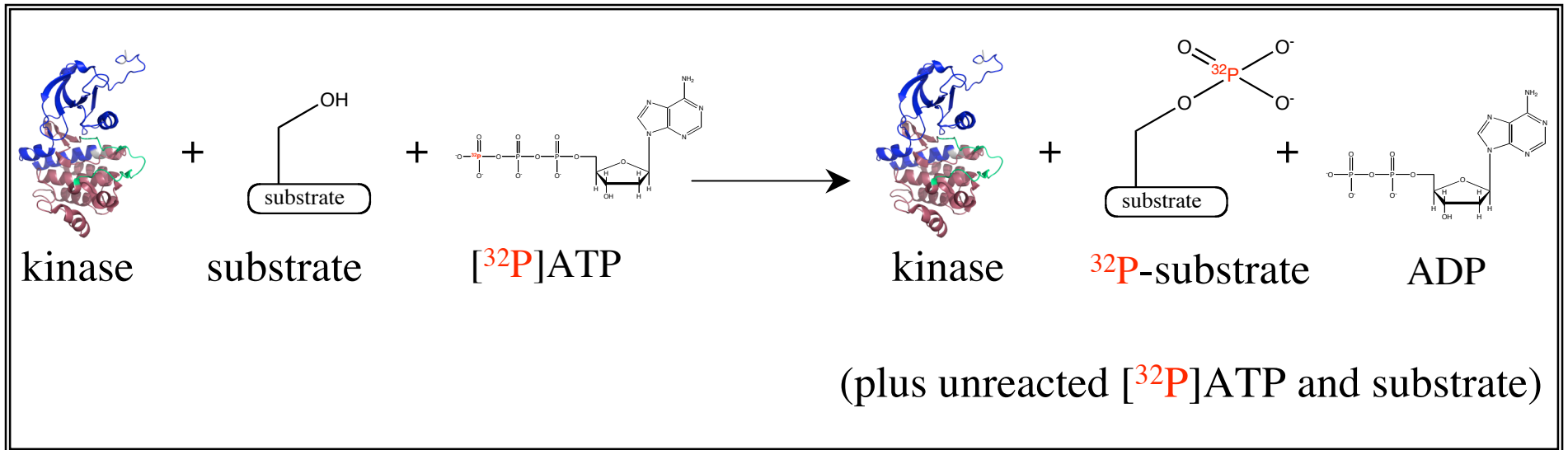
- A. Environment-sensitive fluorescent probes
- B. Chelation-enhanced fluorescent probes

III. In-vivo protein probes (FRET)

IV. Laboratory report guidelines

^{32}P incorporation assay

Detection of a radiolabeled phosphorylated substrate

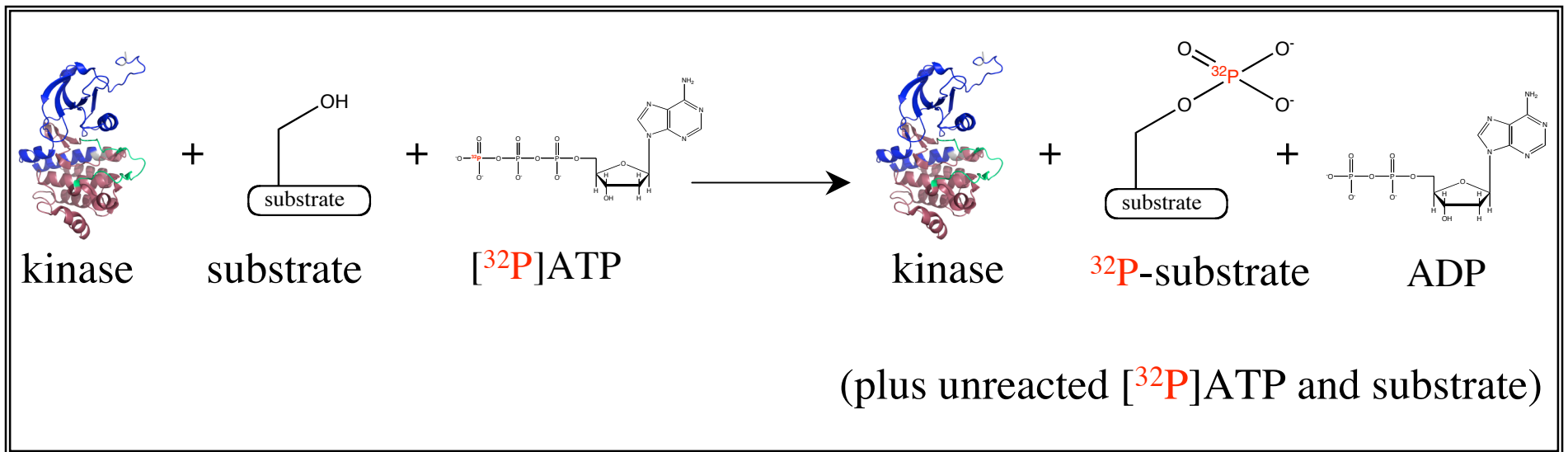


The kinase of interest is incubated with a substrate peptide or protein, radiolabeled $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and MgCl .

Following *separation* of the unreacted $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, the incorporation of ^{32}P into the substrate is quantified on a scintillation counter.

³²P incorporation assay

Detection of a radiolabeled phosphorylated substrate

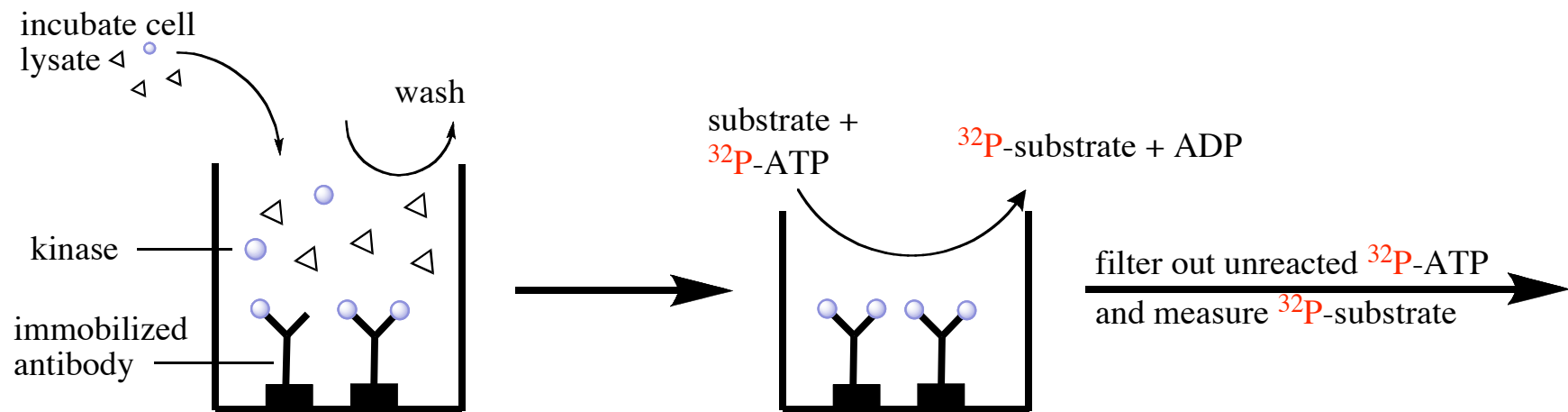


The requirement for residual ATP separation is the major drawback of this method. Additional assay considerations (both negative and positive):

- generates **radioactive** waste
- non-continuous, meaning that it is necessary to take time points if you need kinetic data.
- General and sensitive. The assay works for **any** kinase.

Note: ^{32}P incorporation (or other in-vitro) assays can be used for cell lysate if the kinase of interest is first purified from the cell lysate by [immunoprecipitation](#).

Immunoprecipitation is the use of an [antibody](#) to specifically bind a target protein and provide a handle for protein isolation.



modified from Asthagiri, A. R. et al. *Anal. Biochem.* **269**, 342-347 (1999)

Gel Analysis with a Phospho-specific Antibody

Western blot detection of a phosphorylated substrate

Ie. Detection of phosphorylated Crk (an Abl substrate) from cells with increasing concentrations of PDGF.

Image removed due to copyright restrictions.
See Fig. 3c in Ting, A. Y. et al. Genetically encoded fluorescent reporters of protein tyrosine kinase activities in living cells. *PNAS*. 98 (2001): 15003-15008.

PDGF= platelet derived growth factor
PDGF → PDGFR → Src → Abl

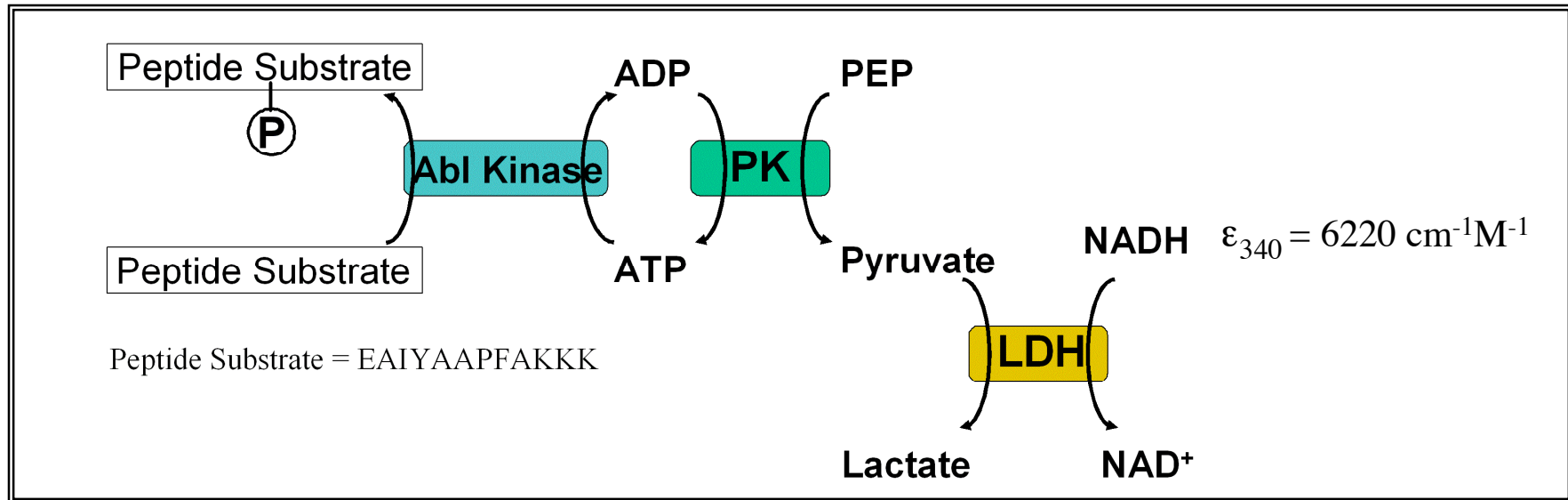
Ting A. Y. et.al. *PNAS* **98**,15003-15008 (2001)

Assay considerations (depending on your specific application)

- Non-continuous
- Often used qualitatively
- Highly **specific** and often used with crude cell lysate

Coupled Phosphorylation Assay (Session 13 and 14)

(Indirect) detection of ADP formation/ATP consumption



Through two enzymatic reactions, Abl kinase activity is coupled to the conversion of **NADH** to **NAD⁺**.

The specific activity of the kinase can be calculated based on the decrease in NADH absorbance at **340** nm over time.

Extinction coefficient (ϵ)

A molecule's extinction coefficient is a measure of the extent to which the molecule **absorbs** light at a given wavelength.

ϵ is typically reported for the wavelength of maximum absorbance (ϵ_{\max}) in units of $\text{cm}^{-1}\text{M}^{-1}$.

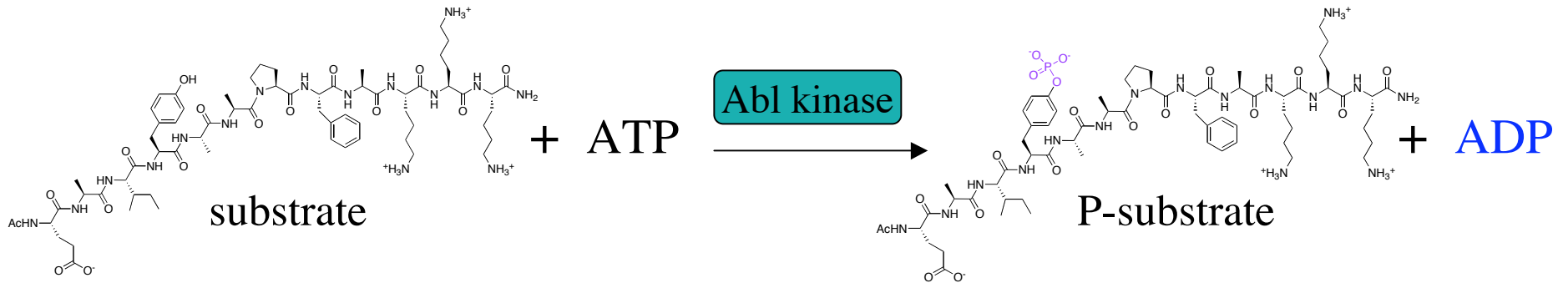
Beer's Law

$$\text{Abs} = \epsilon cl$$

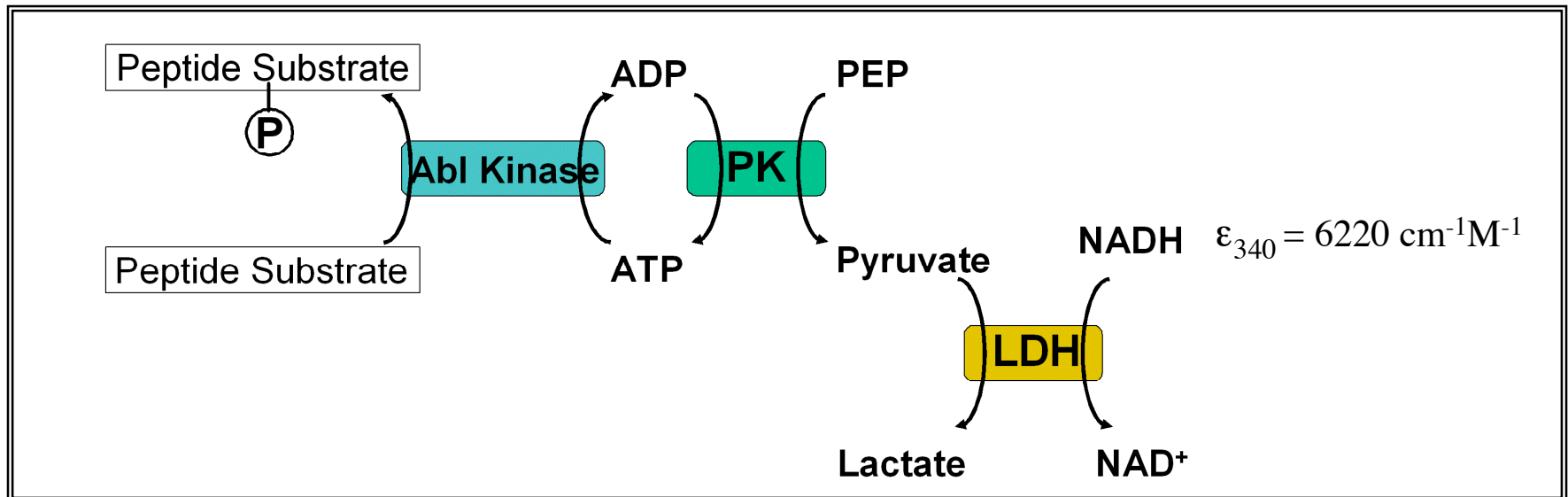
where c = concentration, and l = pathlength of the cuvette

Beer's law allows you to use ϵ to calculate the **concentration** of a sample in solution by measuring the Abs at a given wavelength.

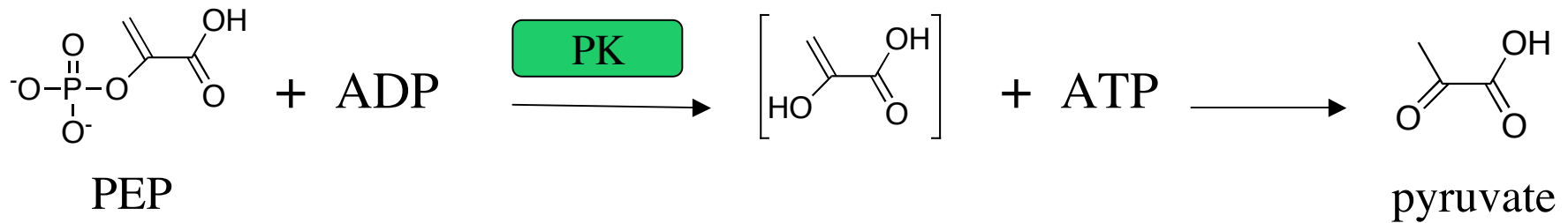
Coupled Phosphorylation Assay (Session 13 and 14)



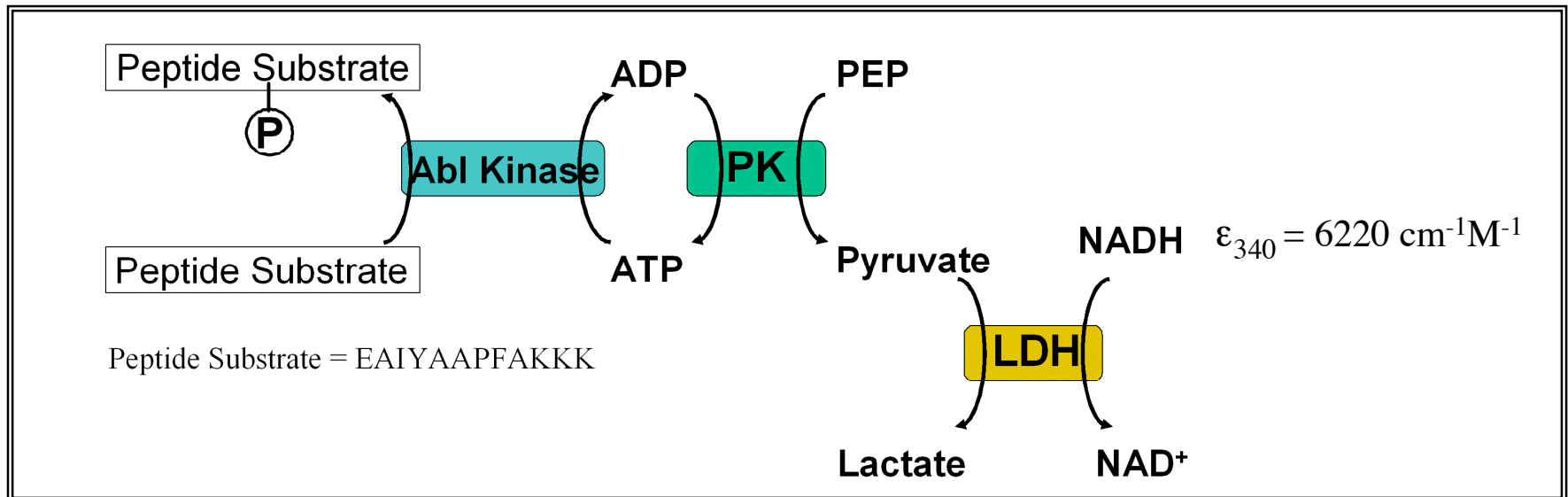
A protein kinase catalyzes the transfer of a phosphate group from ATP to a T, S, or Y on the peptide substrate. For our Abl assays, the substrate is EAIYAAPFAK⁺KKK.



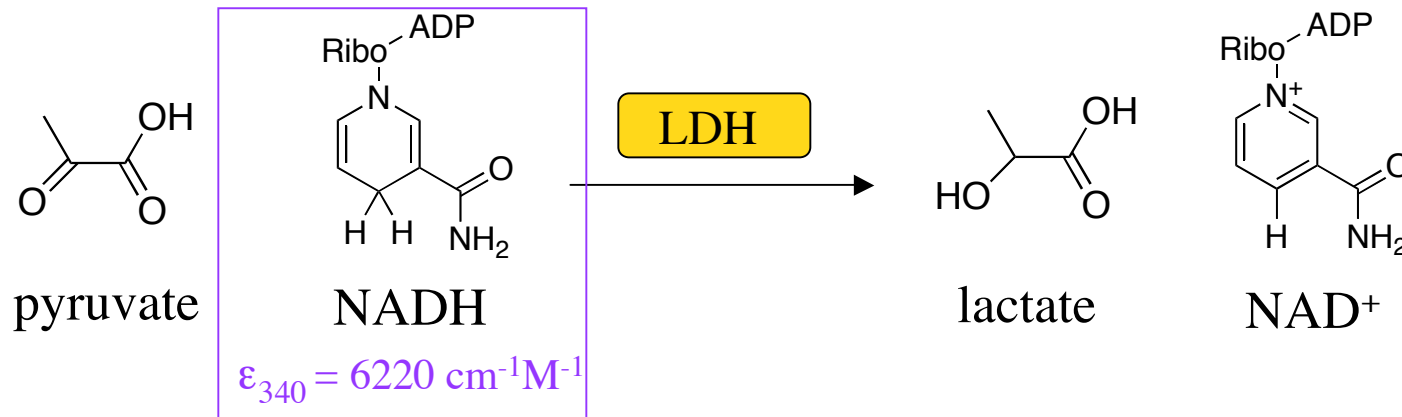
Coupled Phosphorylation Assay (Session 13 and 14)



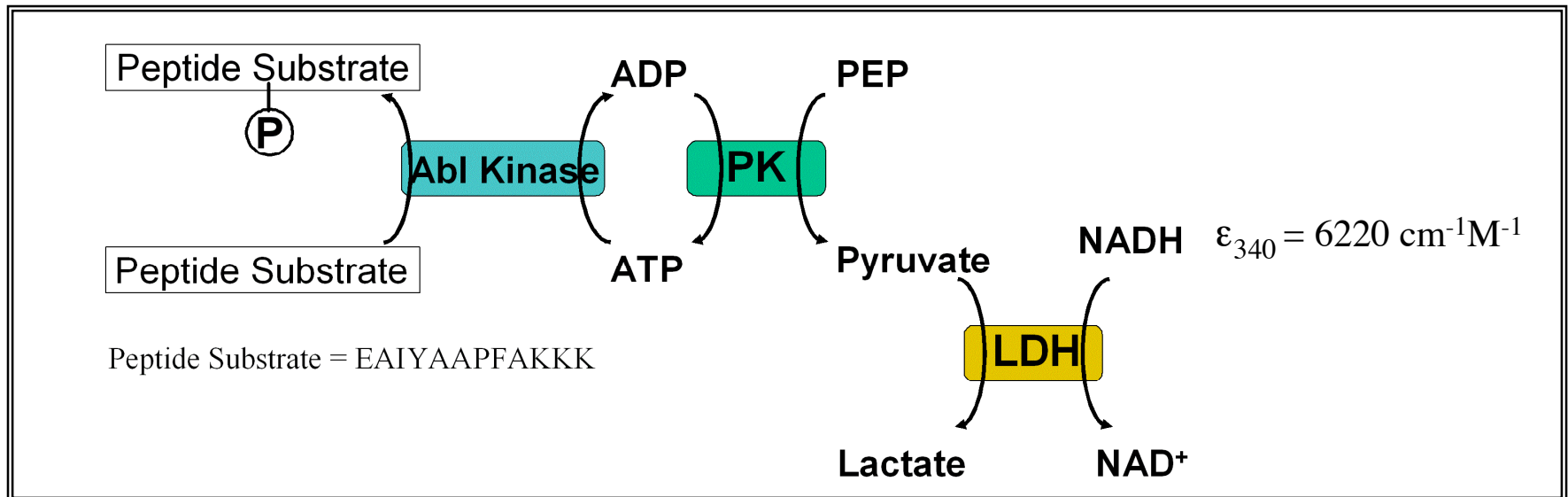
Pyruvate kinase (**PK**) transfers a phosphate group from phosphoenolpyruvate (PEP) to ADP to form **pyruvate** and ATP.



Coupled Phosphorylation Assay (Session 13 and 14)



Lactate dehydrogenase (LDH) catalyzes the reduction of pyruvate to **lactate** with concomitant oxidation of the coenzyme **NADH** to NAD⁺.



Coupled Phosphorylation Assay (Session 13 and 14)

You will monitor the **loss** of NADH absorption at **340** nm to quantify the **specific activity** of **wt** and **H396P** Abl kinase domain

- 1) in the absence of any inhibitors
- 2) with the Abl inhibitor Gleevec
- 3) with the Abl inhibitor Dasatinib

Specific activity in biochemistry is defined as the amount of product formed by an enzyme in a given amount of time.

Specific activity is often reported as:

units (U) per **mg** of enzyme,

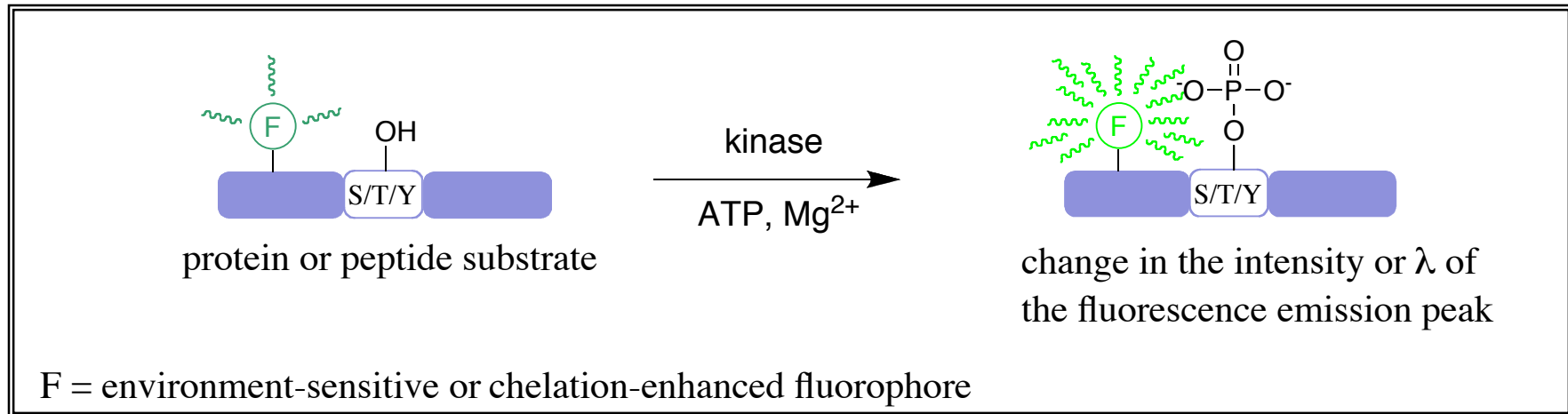
where 1 unit is equal to 1 μmol of product formed per minute.

Detecting Kinase Activity in vitro and in vivo

- I. In vitro activity assays (for purified kinases)
 - A. ^{32}P incorporation assay
 - B. Gel analysis with a phospho-specific antibody
 - C. Kinase coupled-activity assay (lab sessions 13 and 14)
- II. Fluorescent peptide-based probes
 - A. Environment-sensitive fluorescent probes
 - B. Chelation-enhanced fluorescent probes
- III. In-vivo protein probes (FRET)
- IV. Laboratory report guidelines

Fluorescent Peptide-based Probes

Detection of the phosphorylated substrate



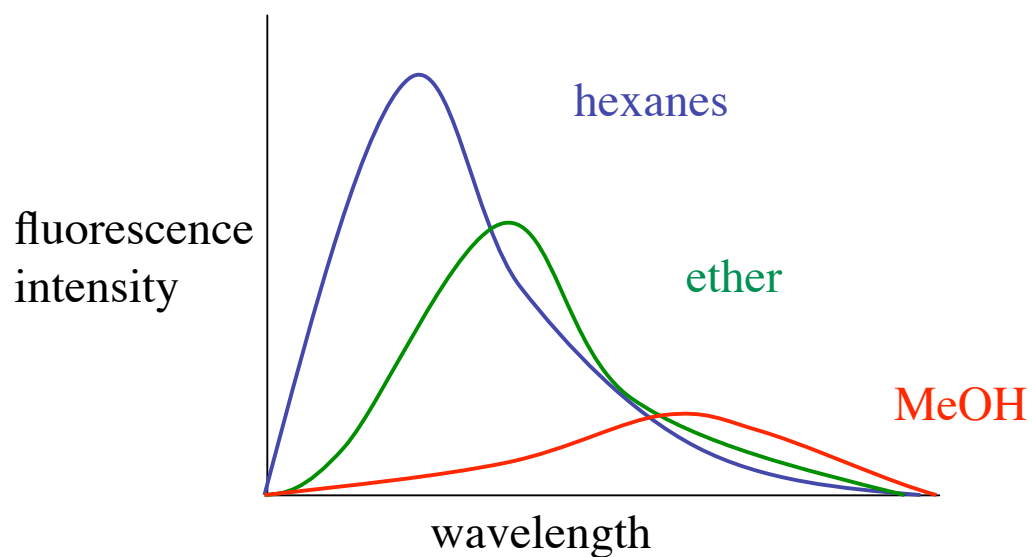
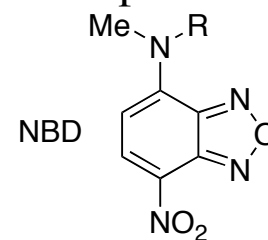
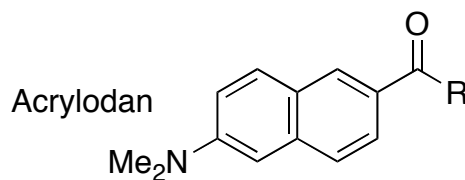
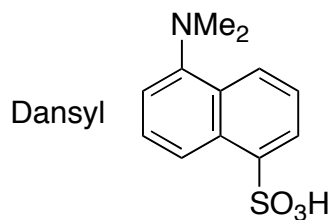
These probes comprise a substrate peptide that includes an amino acid recognition sequence for a specific kinase and an appropriately positioned **environment**-sensitive or **chelation**-enhanced fluorophore.

Upon phosphorylation of the substrate probe, the fluorophore's emission properties change, enabling detection.

Fluorescent Peptide-based Probes: *Environment-Sensitive Fluorophores*

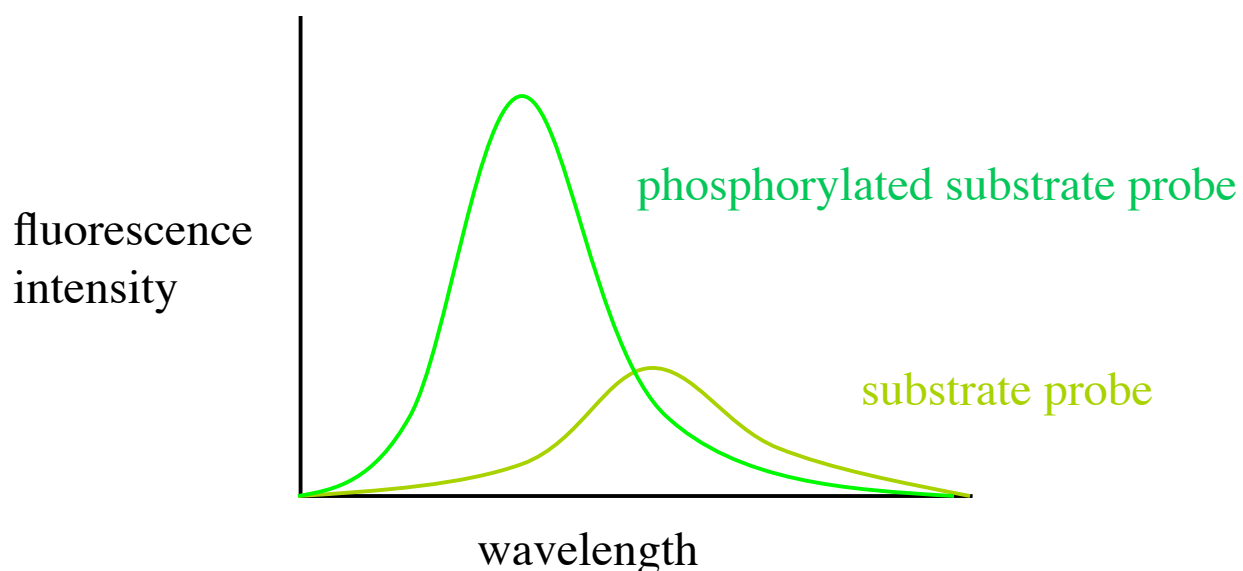
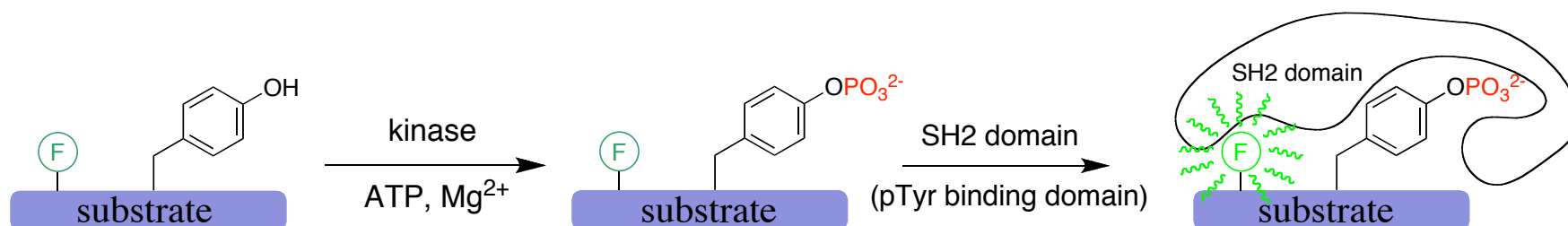
The signal intensity and maximum emission wavelength of environment-sensitive fluorophores (also referred to as solvatochromic dyes) are affected by the **polarity** of the fluorophore's immediate environment.

Examples: The fluorophores below are quenched and red-shifted in polar environments.



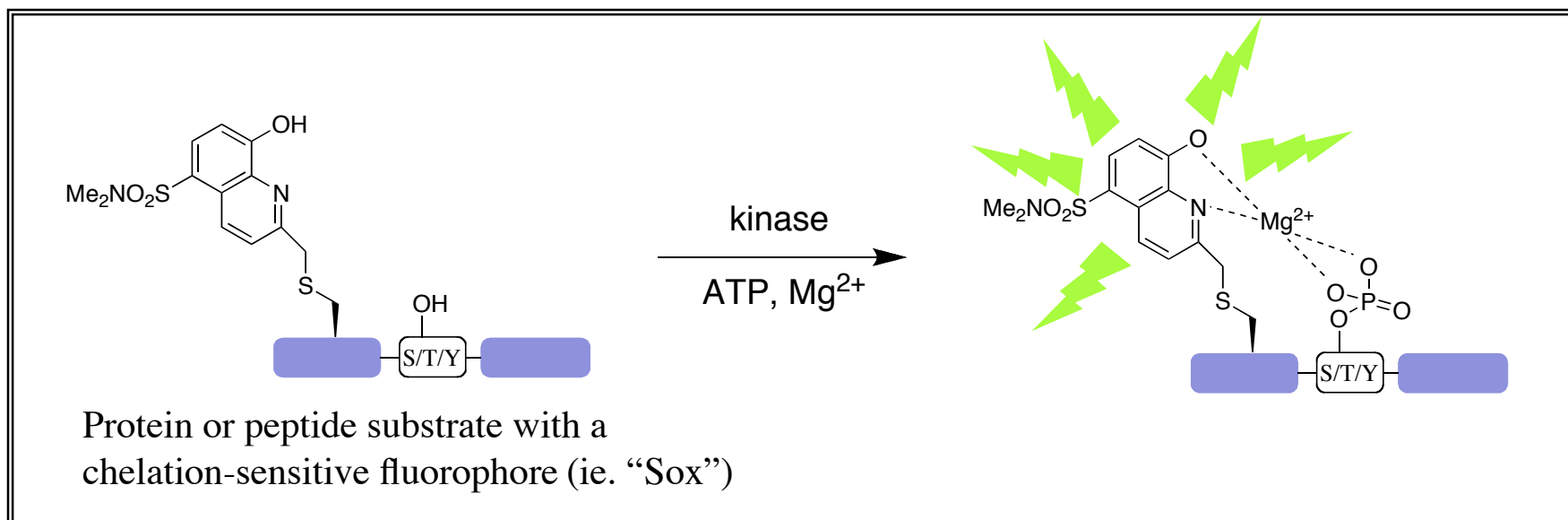
Fluorescent Peptide-based Probes: *Environment-Sensitive Fluorophores*

A phosphorylation-induced conformational change or intramolecular **binding** event that results in a change in the fluorophore's local environment can be detected by a change in fluorescence intensity.



Fluorescent Peptide-based Probes: *Chelation-Enhanced Fluorophores*

Chelation-Enhanced Fluorophores demonstrate dramatic fluorescence intensity increases upon metal chelation.



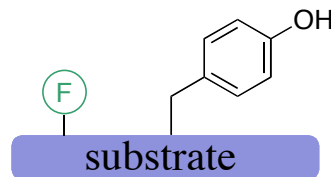
In CHEF-based kinase activity probes, the probes have low affinity for Mg^{2+} in the non-phosphorylated form. Once phosphorylated the binding affinity increases drastically, resulting in a 2 to 12 fold fluorescence enhancement.

Shults, M. D., Carrico-Moniz, D., and Imperiali, B. *Anal. Biochem.* **352**, 198-207 (2006)

Lukovic, E., Gonzalez-Vera, J. A., and Imperiali, B. *J. Am. Chem. Soc.* **130**, 12821-12827 (2008)

Advantages to peptide based probes

- Assays enable continuous readings (no work-up steps prior to detection) making them ideal for kinetic studies.
- Peptide probes are amenable to large scale **synthesis**.
- Compared to proteins, peptides are incredibly **robust** for handling and storage.
- Analogs to sense different kinases and explore different sensing techniques are readily accessible.
- Fluorescence change upon phosphorylation in many peptide-based sensors greatly exceeds 100%.



Disadvantage

- It is challenging to uniformly introduce peptide probes into cells for in-vivo studies. (Most peptides are not cell-permeable.)

Introducing Peptide probes into cells (current methods)

The major hurdle for using many of the peptide-based sensors in cell applications is introducing the sensor into the cell.

- [microinjection](#)
- Protein transduction domains (typically Arg-rich peptide sequences) that are fused to target peptides or small proteins and render them cell permeable.
- Introduction of a non-natural amino acid during protein expression (hijacking RNA translational machinery) for a genetically-encoded probe.

Detecting Kinase Activity in vitro and in vivo

I. Assays for purified kinases (in-vitro)

- A. ^{32}P incorporation assay
- B. Antibody visualization
- C. Kinase coupled-activity assay (lab sessions 13 and 14)

II. Fluorescent peptide-based probes

- A. Environment-sensitive fluorescent probes
- B. Chelation-enhanced fluorescent probes

III. In-vivo protein-based probes (FRET)

Protein Probes

Protein probes for sensing kinase activity typically employ fluorescence resonance energy transfer (**FRET**) to sense a conformational change induced by protein phosphorylation.

FRET

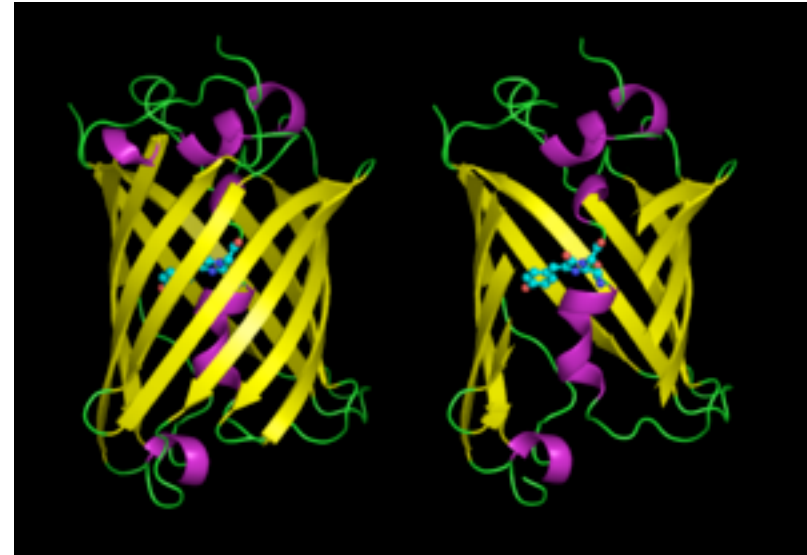
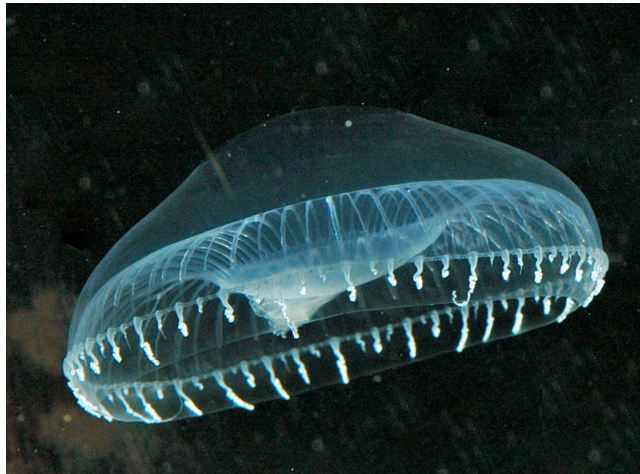
FRET is a distance-dependent interaction between a donor and an acceptor fluorophore and is used as a “molecular ruler”.

When the donor and acceptor fluorophores are in close proximity (10-100 Å), the acceptor fluorophore is excited by the donor emission and emits a signal*.

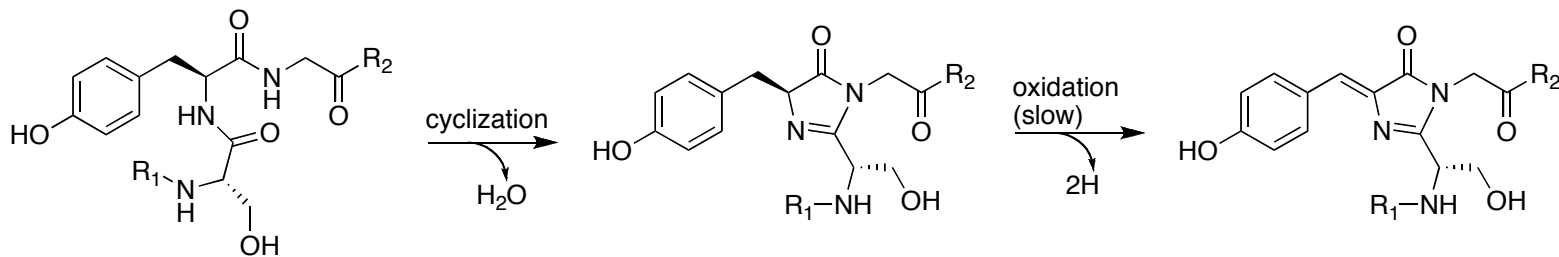


*In FRET, the emission spectrum of the donor must overlap with the absorption spectrum of the acceptor.

Green Fluorescent Protein (GFP) and related fluorescent proteins

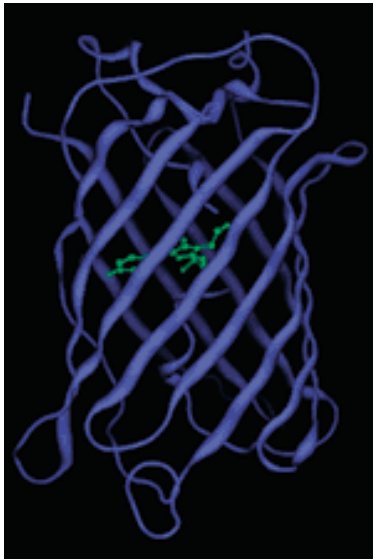


- **238** amino acid GFP first isolated from the Jellyfish *Aequorea Victoria*
- The chromophores of GFP and related fluorescent proteins arise from natural amino acids, so they can be expressed **in-vivo**.



Ser 65 - Tyr 66 - Gly 67

Green Fluorescent Protein (GFP) and related fluorescent proteins



For (S65T) GFP:
Excitation: 488 nm
Emission: 507 nm

The denatured GFP protein is NOT fluorescent.
The fluorophore is **environment**-sensitive.

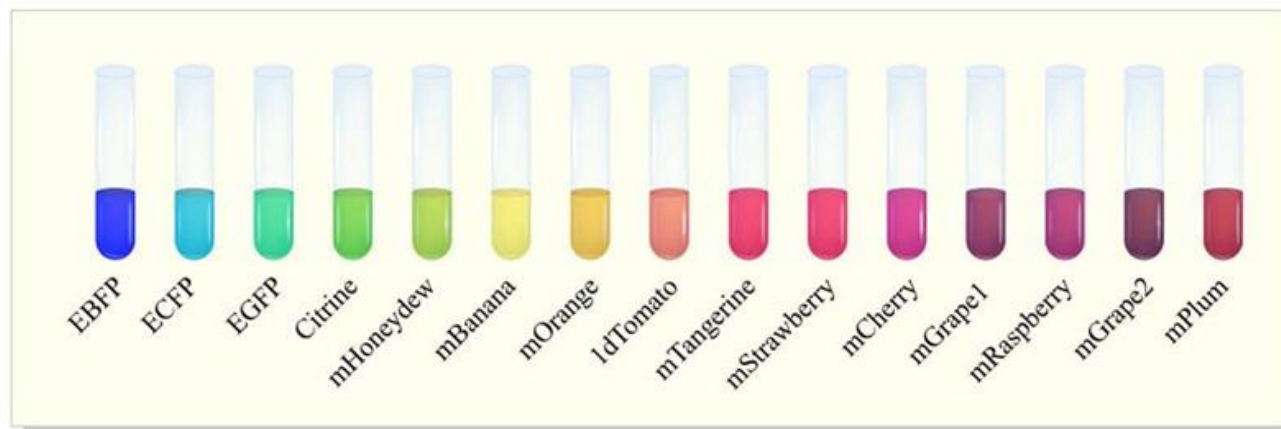


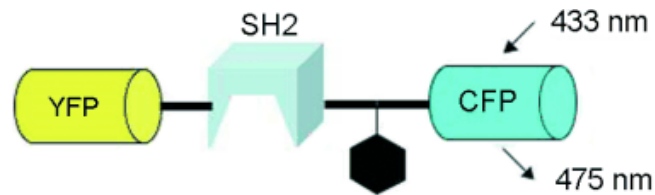
Image by MIT OpenCourseWare

Protein engineering has led to the development of a rainbow of other GFP analogs with a range of excitation and emission wavelengths. All are **expressible**, but also **large** (~230 amino acids).

FRET-based phosphorylation sensors:

Detection of a phosphorylation-induced **conformational** change

For example, below is a cartoon of a FRET-based sensor for protein tyrosine kinases (PTK) using the CFP/YFP FRET pair.

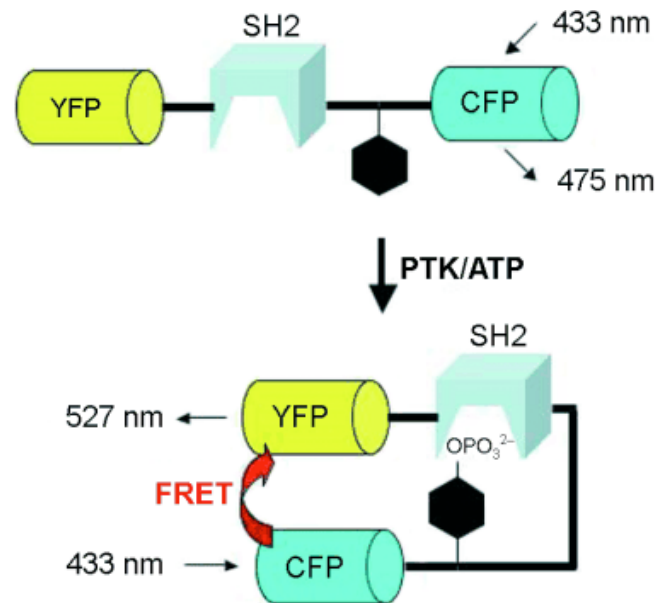


Lawrence, D. S. and Wang, Q. *ChemBioChem* **8**, 373-378 (2007)

FRET-based phosphorylation sensors:

Detection of a phosphorylation-induced **conformational** change

For example, below is a cartoon of a FRET-based sensor for protein tyrosine kinases (PTK) using the CFP/YFP FRET pair.



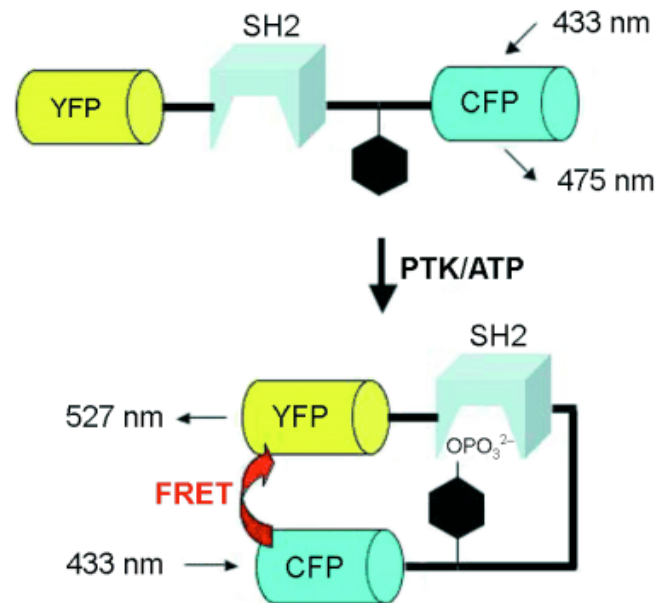
Lawrence, D. S. and Wang, Q. *ChemBioChem* **8**, 373-378 (2007)

The major advantage to FRET-based probes is that they can be expressed in cells.

FRET-based phosphorylation sensors:

Detection of a phosphorylation-induced **conformational** change

For example, below is a cartoon of a FRET-based sensor for protein tyrosine kinases (PTK) using the CFP/YFP FRET pair.



However, the large **size** of FRET fluorophore pairs is a concern, particularly in comparison to small-molecule fluorophores.

Also, most FRET-based probes demonstrate a fluorescence increase of less than **60%** following phosphorylation.

Example of a FRET sensor for Abl phosphorylation: The highest Abl activity is detected in PDGF-induced membrane ruffles

Images removed due to copyright restrictions.
See Fig. 3d and 3e in Ting, A. Y. et al. Genetically encoded fluorescent reporters of protein tyrosine kinase activities in living cells. *PNAS*. 98 (2001): 15003-15008.

PDGF= platelet derived growth factor
PDGF → PDGFR → Src → Abl

Laboratory Report Guidelines

Laboratory Report: **42 points**

Abstract: **5 points**

Introduction: **10 points**

Explanation of purpose/relevance: (5)

Demonstration of understanding: (5)

Results: **5 points**

Organization/Clarity: (5)

Discussion: **10 points**

Interpretation of data: (5)

Demonstration of understanding: (5)

Appendices: **12 points**

PyMol Structure Worksheet: (4)

Materials and Methods: (4)

Supplementary Info: (4)

Laboratory Work: **8 points**

Overall technique: (4)

Effort and attitude: (4)