Lecture #8

Enzyme measurements

Aims:

- Understand the basic principles and application-related significance of enzymes
- Get introduced into enzyme screens via fluorescence-activated droplet sorting
- Become familiar with your practical task

Lectures (CO 121)	Date & Topic	Details	Practical (location as color coded on next slide)
1	13.09 General Intro	Get to know teachers, TAs, students and aims of the course	17.09 Measure temperature using thermistor (using M&A explorer) TL
2	20.09 Lecture LabVIEW TL Group formation (A-F, 3 students, each)	Some first basic steps in LabVIEW programming	24.09 Brief intro into LabVIEW thermistor program (input and output) TL
3	27.09 Case study FACS, similarities and differences to droplet microfluidics Selection of case study topics	1.) Property to measure? 2.) Device? 3.) Working principle? 4.) Alternatives?	01.10 Preparation of bioinstrument case study
4	04.10 No course, preparation for case study		08.10 No course
5	11.10 Groups A-B presenting case study		15.10 Tour through LBMM workstation labs, intro into Nature Protocols (Groups A-B)
6	18.10 Lecture optics Homework: Students to prepare one laser/PMT blueprint FP	Mirrors, filters, microscope setup, lenses, etc.	22.10 Holidays
	25.10 Holidays, submit your blueprint by email		29.10 .10 Build workstation optics 1
7	01.11 Lecture electronics	FPGA, PMTs, amplifier, function generator	05.11 Build workstation 1 optics 2, laser alignment; build workstation electronics
8	08.11 Intro into enzyme concentration measurement experiment (kinetics, etc.) + task FP	Enzymes, kinetics, practical task	12.11 -
9	15.11 -	Software similar to Thermistor program, pdf on installation	19.11 Intro to droplet analysis software (LabVIEW) TL Build workstation software: Add output LED (mimicking sorting trigger) into analysis software
10	22.11 Fundamentals of microfluidics and microfluidic chips	Flow at the microscale, microfluidic chips (manufacturing), droplet microfluidic modules	26.11 Run microfluidic experiments, e.g. determine concentration of MMP in droplets
11	29.11 Prepare presentation		3.12 Sorting Demo on LBMM workstation1 (Groups A-B)
12	06.12 Prepare presentation		10.12
13	13.12 Groups B-A presenting results 13.12 Submit report (all!)		**17.12 – TUESDAY! - Individual Q & A sessions (10min, Groups A-B)**

Green shading: Single seminar/practical with all 18 students
Red shading: Individual seminar/practical with only 6 students required (= 3 sequential 90min slots, 4.5h in total)

Report & presentation

13.12.2024: presentations

13.12.2024: submit reports

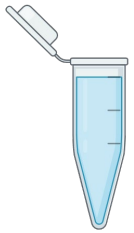
17.12.2024: individual Q&A

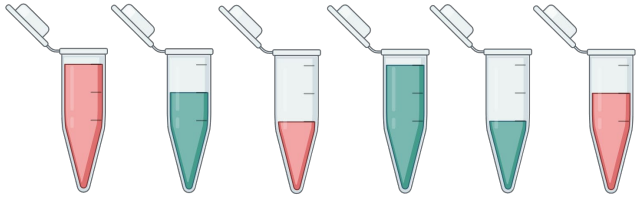
Final report: 8 – 10 pages

- introduction
- explain your design
- construction steps
- experimental analysis

Your practical task

Uncharacterized sample





Target: **Substrate**

Value: **Concentration**

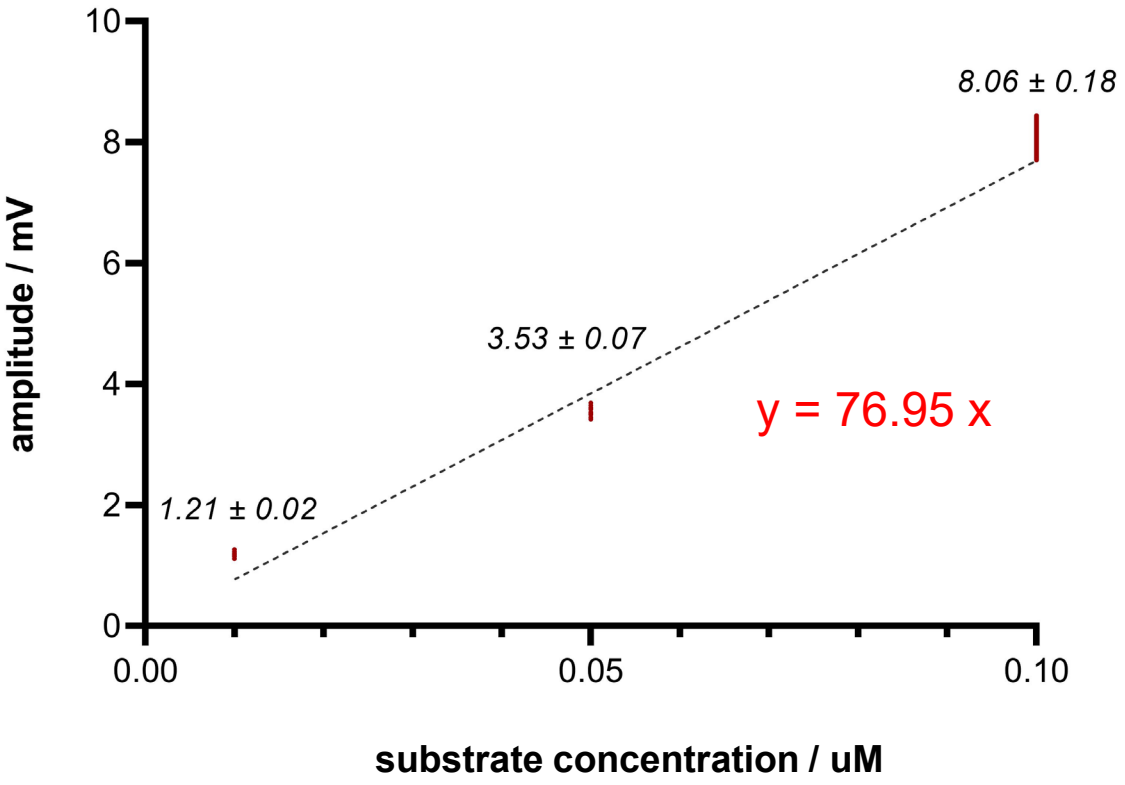
How…? Bioinstrument!

‘What can you measure with your setup?’

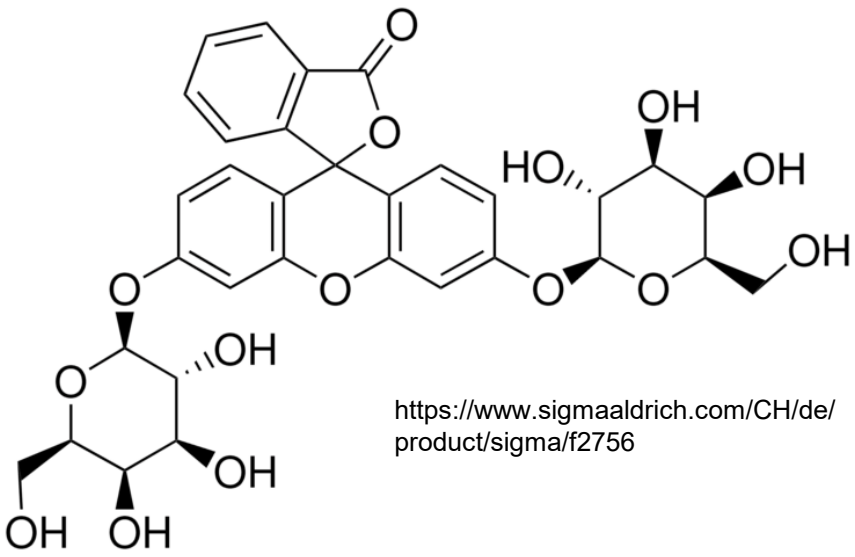
‘What kind of values do you get?’

Some help…

Calibration curve



Substrate conversion



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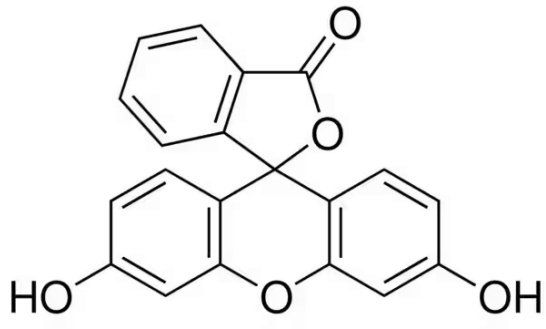

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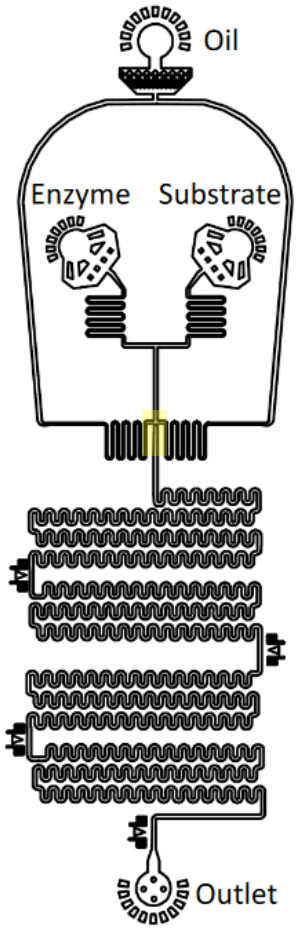
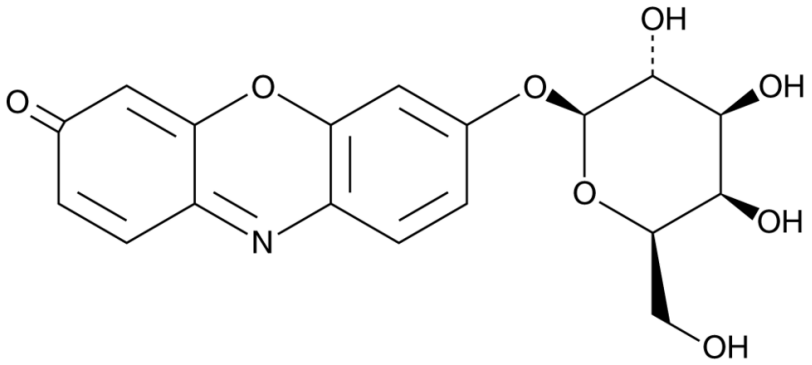


fluorescein di-β-D-galactopyranoside (FDG)

fluorescein

cleavage in presence of an <u>enzyme</u>

Substrate conversion



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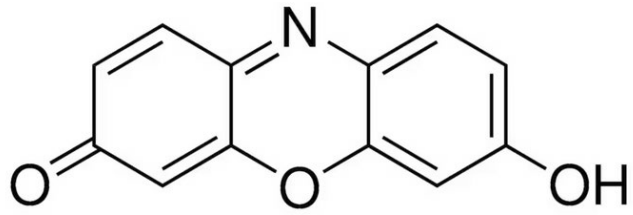

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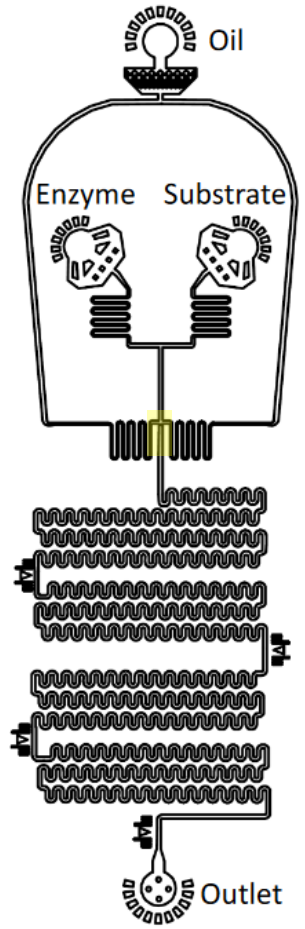


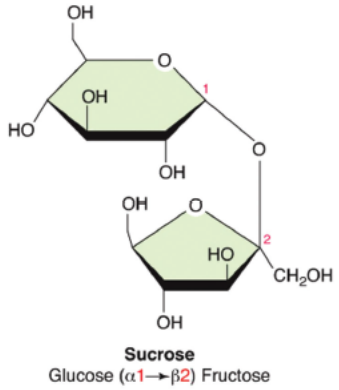
resorufin di-β-D-galactopyranoside (RDG)

resorufin

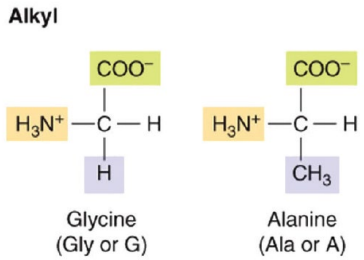
cleavage in presence of an enzyme

What are enzymes?

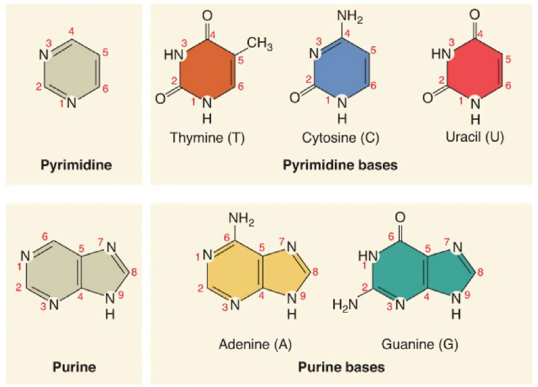
Sugars



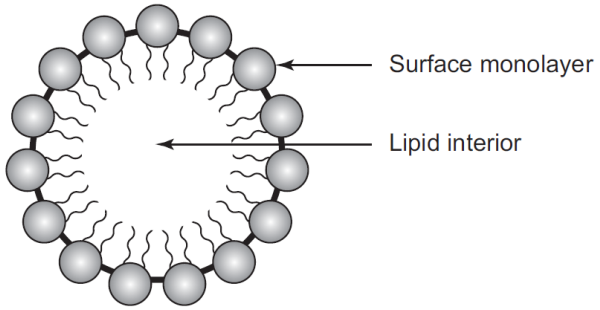
Amino acids

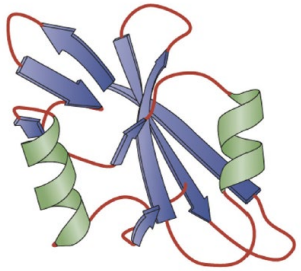


Nucleotides

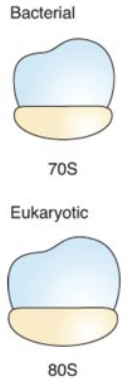


Fatty acids





Enzymes

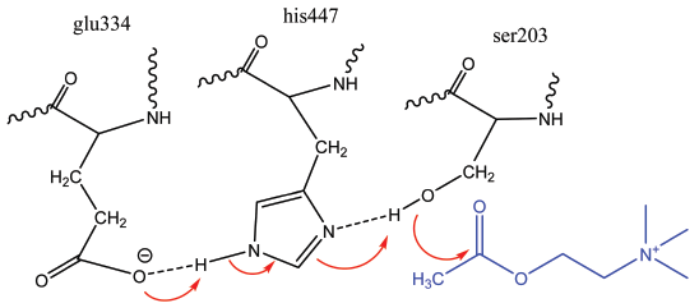


Ribozymes

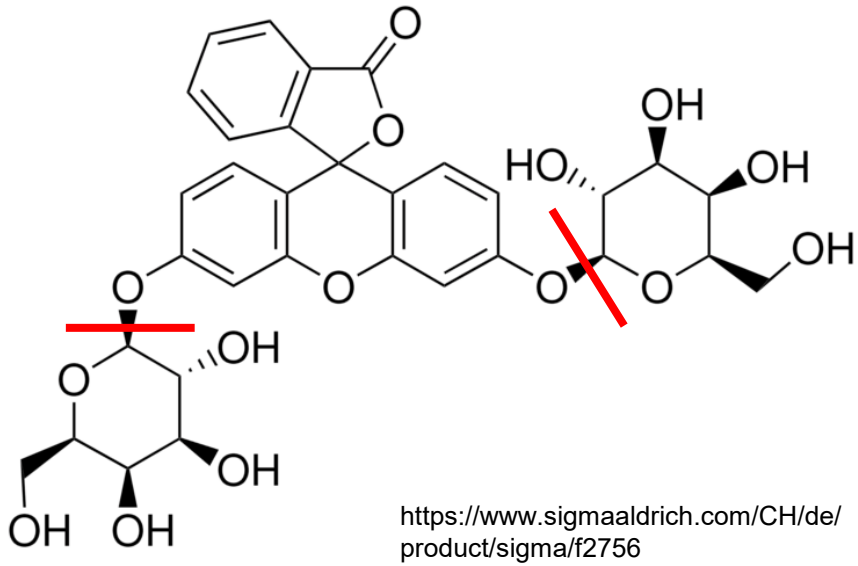
Ochs, Raymond S., *Biochemistry*, 2022

What are enzymes?

- catalysis occurs in the active site
- enzymes act specifically



Ochs, Raymond S., *Biochemistry*, 2022



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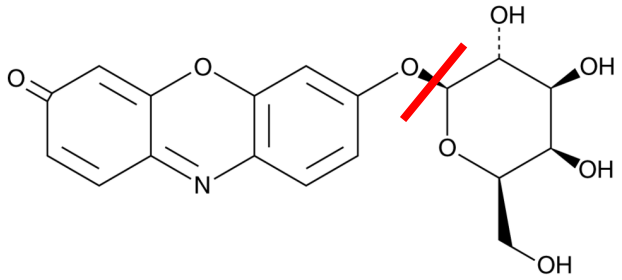


https://www.caymanchem.com/product/28015

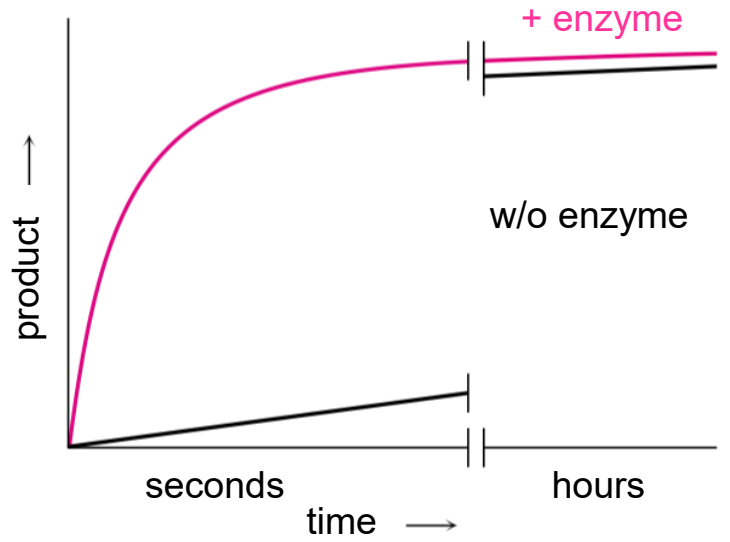
What are enzymes?

= biological catalysts

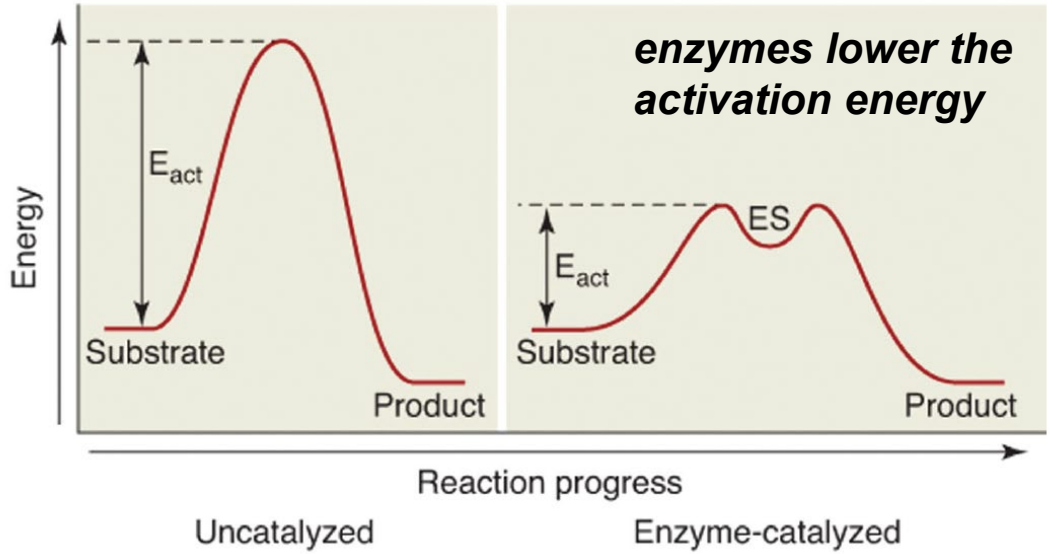
= increasing the reaction rate

= not affecting the thermodynamic equilibrium

$\Delta G^0 = R\,T\,\ln(K_{eq})$



adapted from: Berg, Jeremy M., Tymoczko, John L., Gatto jr., Gregory J., *Stryer Biochemie*, 2017



Ochs, Raymond S., *Biochemistry*, 2022

Why are enzymes of interest?

TABLE 6.1 Classification of Enzymes by the First Category of the Enzyme Commission (EC)

EC Type	*Category*	*Example*
1	Oxidoreductase	Lactate Dehydrogenase (Chapter 9)
2	Transferase	Hexokinase (Chapter 9)
3	Hydrolase	Sucrase (this chapter)
4	Lyase	Aldolase (Chapter 9)
5	Isomerase	Glucose Phosphate Isomerase (Chapter 9)
6	Ligase	Pyruvate Carboxylase (Chapter 13)
7	Translocase	Cytochrome c Oxidase (chapter 10)

Ochs, Raymond S., *Biochemistry*, 2022

Enzymes = fundamental necessities for life

- Research application
- Industrial application
- Medical application

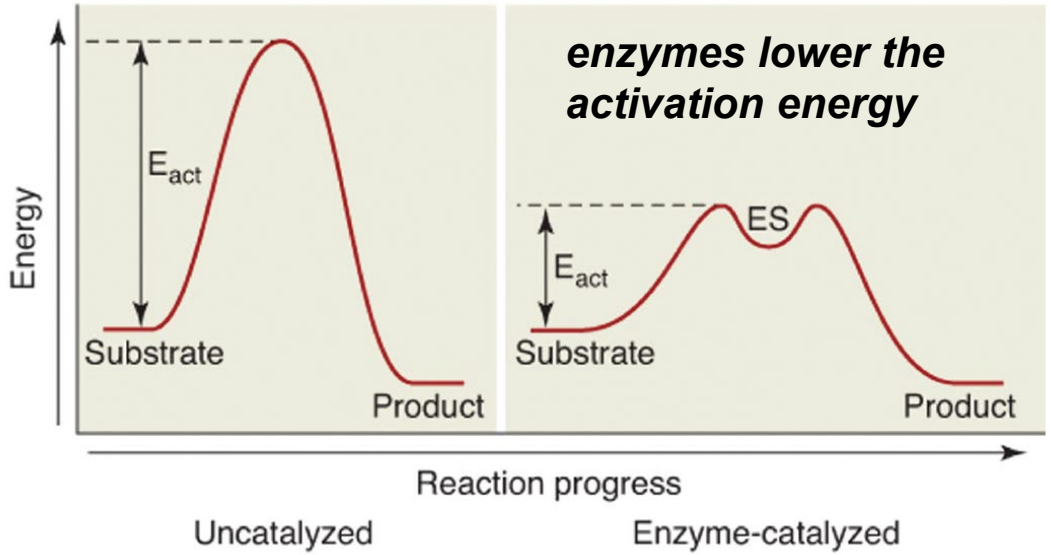
… reactivity, specificity, kinetics

Bioinstrument: **measures product formation** or **substrate conversion**

How do enzymes work?

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_{cat}} E + P$$

Ochs, Raymond S., *Biochemistry*, 2022

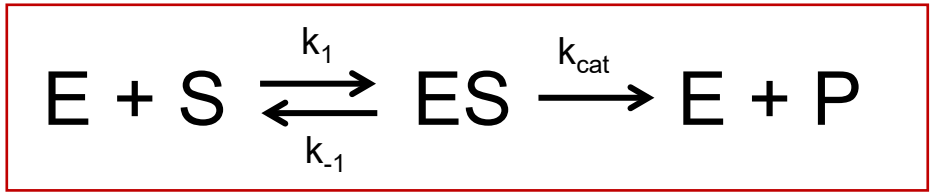


(1) Steady state: rate of ES formation = rate of ES destruction

(2) Enzyme concentration: $[E]_{total} = [E] + [ES]$

(3) Initial velocity: $v = k_{cat}[ES]$

Michaelis-Menten equation



(1) rate of ES formation = rate of ES destruction

$$k_1 [E] [S] = k_{-1} [ES] + k_{cat} [ES]$$

$$\frac{[E]\ [S]}{[ES]} = \frac{k_{cat} + k_{-1}}{k_1}$$

$$\frac{[E]\ [S]}{[ES]} = \mathbf{K_m}$$

(2) $$\frac{([E]_{total} - [ES])\ [S]}{[ES]} = \mathbf{K_m}$$

$[E]_{total} = [E] + [ES]$

$$[ES] = \frac{[E]_{total}\,[S]}{\mathbf{K_m} + [S]}$$

(3) $$v = \frac{k_{cat}\,[E]_{total}\,[S]}{\mathbf{K_m} + [S]}$$

$v = k_{cat} [ES]$

Michaelis-Menten equation

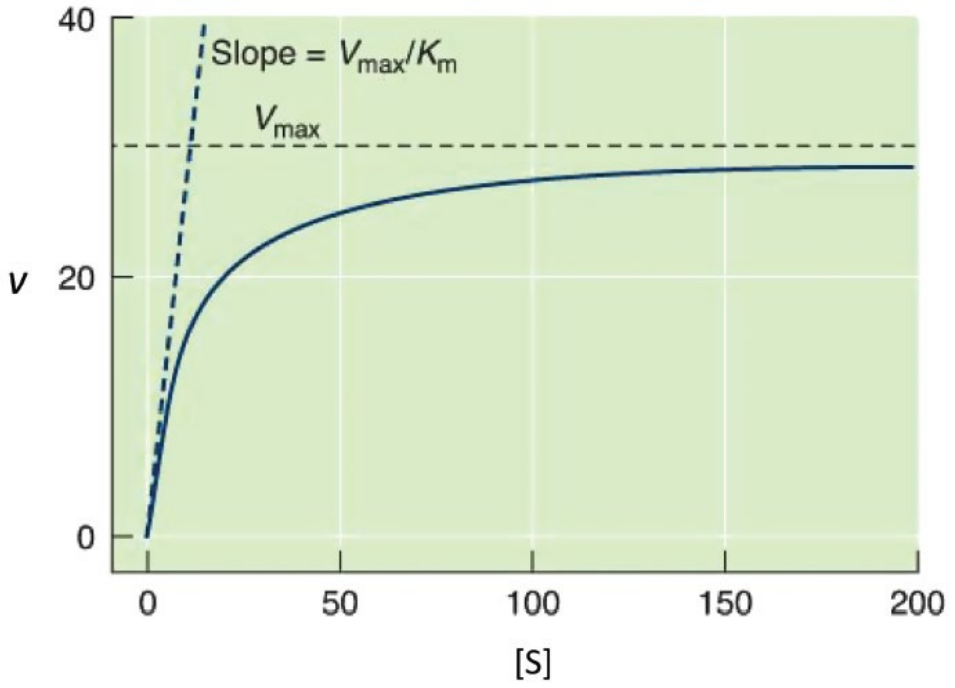
$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_{cat}} E + P$$

$$v = \frac{k_{cat}[E]_{total}[S]}{\mathbf{K_m} + [S]}$$

(4) $v_{max} = k_{cat}[E]_{total}$

$$v = \frac{v_{max}[S]}{\mathbf{K_m} + [S]}$$

Ochs, Raymond S., *Biochemistry*, 2022



Michaelis-Menten equation

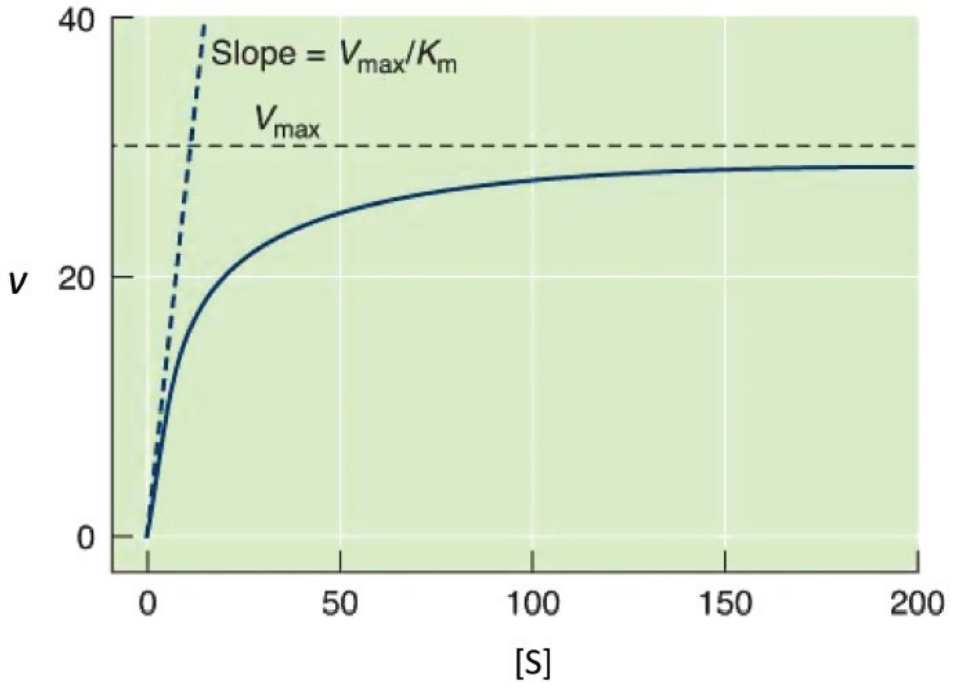
$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_{cat}} E + P$$

$$v = \frac{v_{max}[S]}{K_m + [S]}$$

case 1: [S] >> K_m $\quad v = v_{max}$

case 2: [S] << K_m $\quad v = \frac{v_{max}[S]}{K_m}$

Ochs, Raymond S., *Biochemistry*, 2022



Michaelis-Menten equation: K_m

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_{cat}} E + P$$

K_m / mol/L

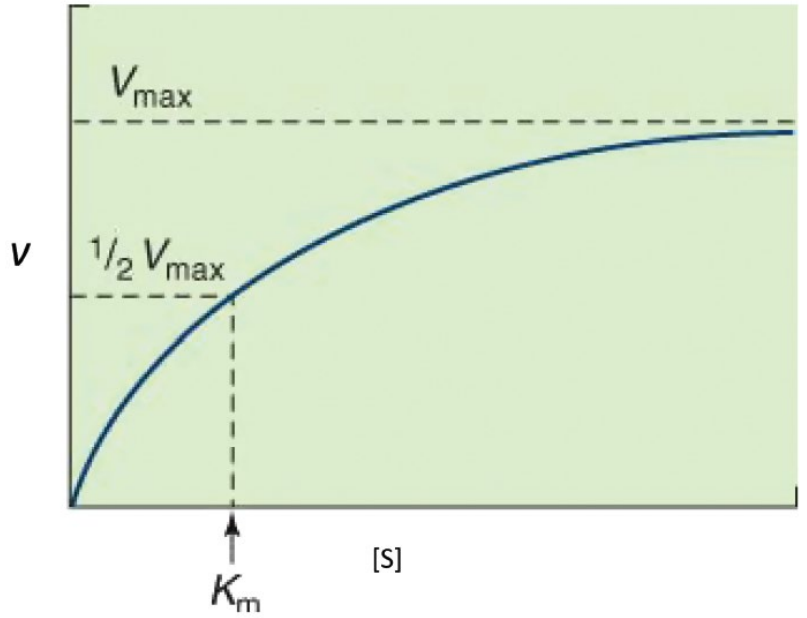
$$v = \frac{v_{max}[S]}{K_m + [S]}$$

$$v = \frac{1}{2}v_{max} = \frac{v_{max}[S]}{K_m + [S]}$$

$$\frac{1}{2} = \frac{[S]}{K_m + [S]}$$

$$K_m = [S]$$

Ochs, Raymond S., *Biochemistry*, 2022



K_m

1.) comparing different substrates ('specificity')
2.) estimating intracellular concentration ?!

v_{max} / mol/sec

maximum enzyme velocity

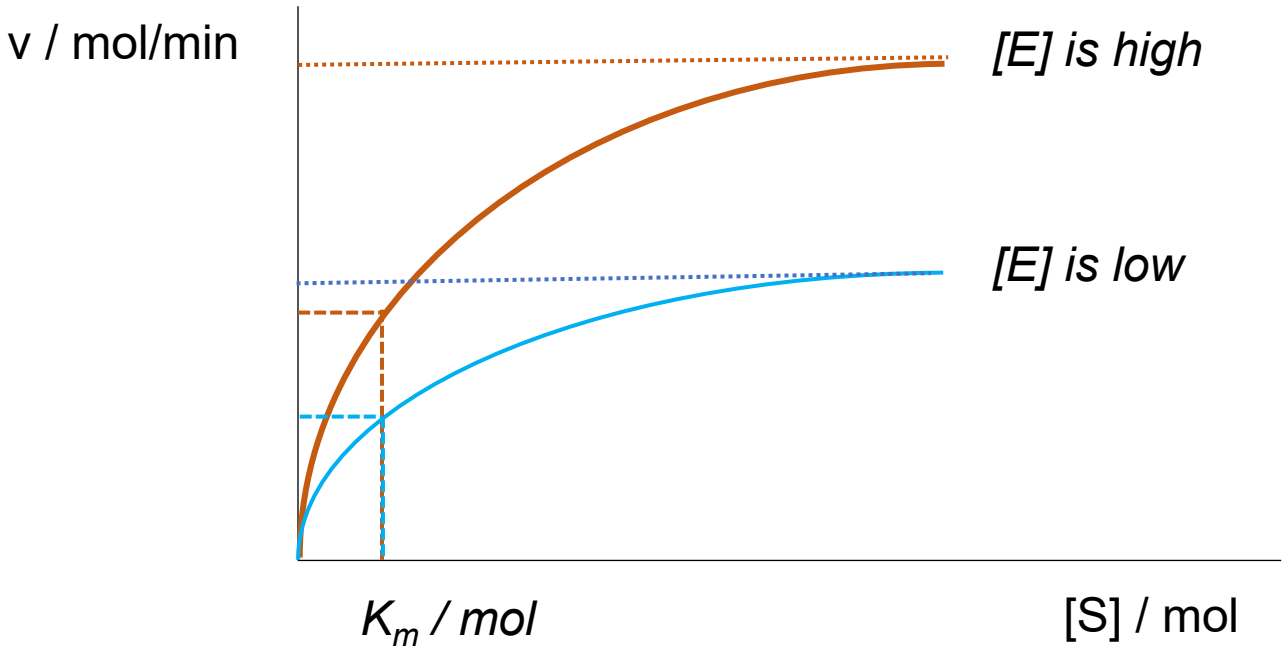
Enzyme kinetics

exemplary calculation:

> substrate in excess, enzyme is limiting

'How should the curve look like?'

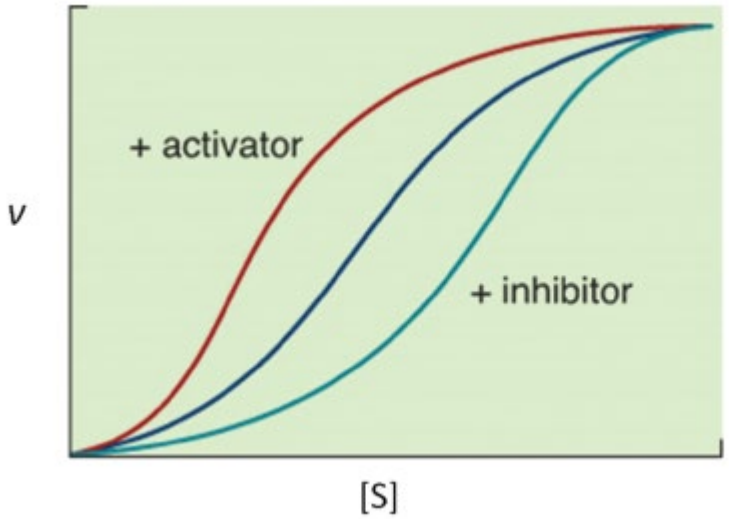
'Does the K_m change?'



Allosteric enzymes & cooperativity

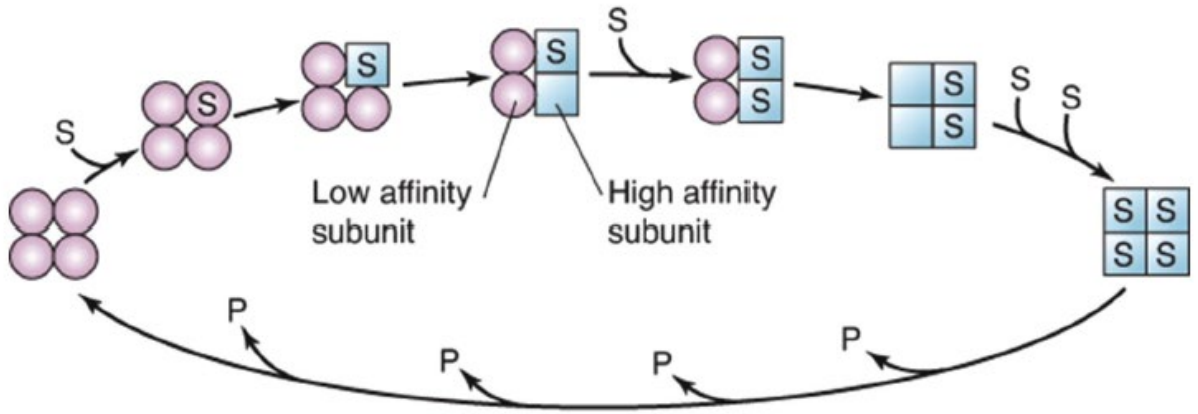
= enzymes with <u>modifiers</u> binding to <u>another place than the active site</u> (= ‘effectors’) (‘heterotropic effect’)

Ochs, Raymond S., *Biochemistry*, 2022

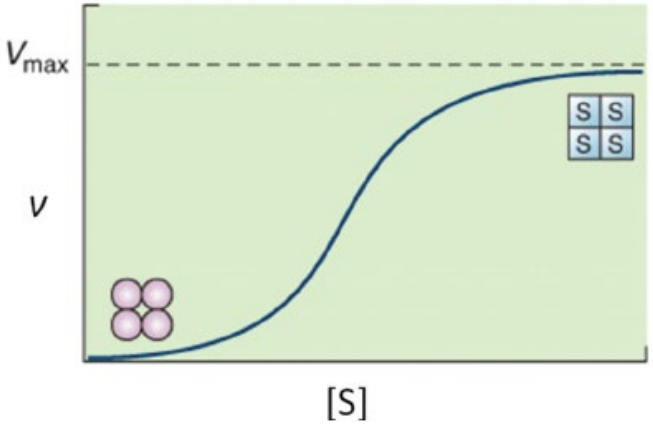


= <u>regulation</u> by metabolites other than the physiological ligand

Ochs, Raymond S., *Biochemistry*, 2022



= often composed of multiple subunits
= often showing **cooperative behavior** (here: ‘homotropic effect’)



‘How would you design an inhibitor?’

Enzyme assays

(1) monitoring formed product

(2) monitoring consumed substrate

> what is preferred > *why?*

Table 2

Sensitivity of selected detection methods assuming standard instrumentation and time required for signal acquisition (e.g. in a droplet sorter). The inverse of the integration time indicates the upper limit of possible throughput for a given detection method, irrespective of limitations imposed by droplet dynamics

Detection Method	Sensitivity	Integration time
Fluorescence Intensity	High	µs
Fluorescence Anisotropy		µs
Fluorescence Lifetime	nM	s
Resonance Energy Transfer		µs
Fluorescence Microscopy		µs
Chemiluminescence		ms
Electrochemistry		ms
Surface EnhancedRamanScattering	µM	ms
Light Scattering		µs
Absorbance		µs
Brightfield Microscopy	mM	ms
	Low	µs (MHz), ms (kHz), s (Hz)

Mair et al., 2017 (doi: 10.1016/j.cbpa.2017.02.018)

Table 12.1 Detection methods used in enzyme assays

Technique	Detection of	Enzyme assay for
Optical measurements		
UV spectroscopy	NADH, A_{340nm}	Alcohol dehydrogenase; lactate dehydrogenase; malate dehydrogenase
Visible spectroscopy	*p*-Nitrophenol, A_{405nm}	Alkaline phosphatase
Polarimetry	Optical rotation, [α]	Invertase
Turbidimetry (Nephelometry)	Attenuation of incident light (intensity of scattered light)	Lysozyme
Fluorimetry	Fluorescein; ↓at 470 nm and ↑at 510 nm	Cholinesterase; acylase; chymotrypsin
Luminometry	Luciferin; ↑at 562 nm	Luciferase
Electrochemical measurements		
pH meter/pH-stat	$[H^+]$ change	Lipase; cholinesterase; urease; glucose oxidase
	Carbon dioxide	Carbonic anhydrase
Potentiometry	Fe^{2+}/Fe^{3+}	Oxidase reactions (cytochromes)
Amperometry	O_2	Oxygenases; glucose oxidase
Manometric measurements		
Warburg manometer	O_2 consumed, CO_2 released	Respiratory enzymes; decarboxylases
Radiotracer measurements		
Scintillation counter	β-Emission	Dehydrogenases (^3H); glutamate decarboxylase (^{14}C); protein synthesis (^{35}S); kinases; enzymes of nucleic acid metabolism (^{32}P)

Punekar, N.S., *Enzymes: Catalysis, Kinetics and Mechanisms*, 2018

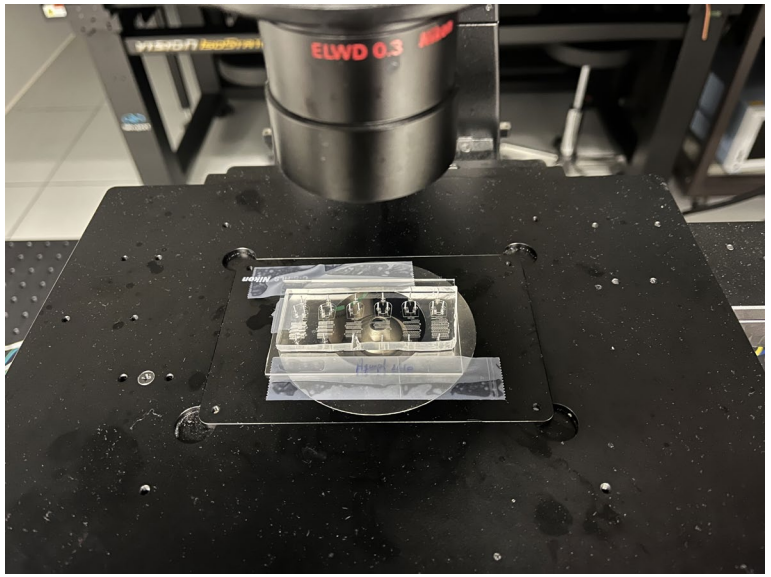
Enzyme assays

Fluorometric assays

= more sensitive vs. absorption-based methods

…however, providing a ***relative value***

'Which factors could vary between experiments?'

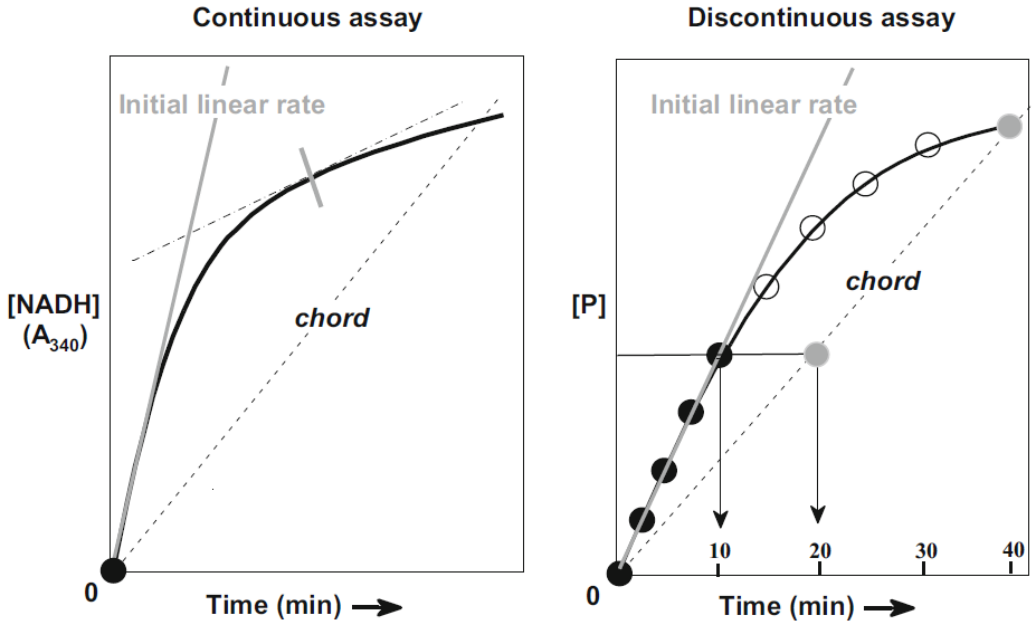


Enzyme assays

> direct assay vs. indirect assay (usually, coupled-enzyme assays)

'Difficulties with indirect assays?'

> continuous assay vs. discontinuous/end-point assay



Punekar, N.S., *Enzymes: Catalysis, Kinetics and Mechanisms*, 2018

Enzyme assays

> loss of linearity… *why?*

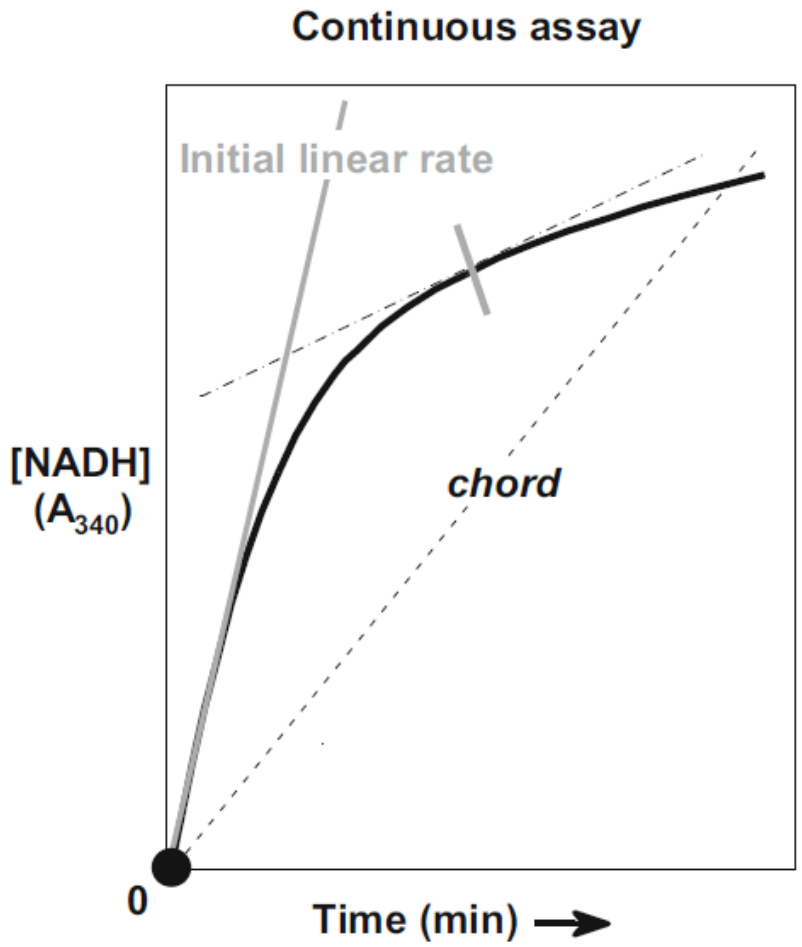
(1) continuous substrate consumption

(2) more product: increased backward reaction

(3) formed product may inhibit the enzyme

(4) product formation could change the pH

(5) instability of enzyme or substrate



Punekar, N.S., *Enzymes: Catalysis, Kinetics and Mechanisms*, 2018

Enzyme assays

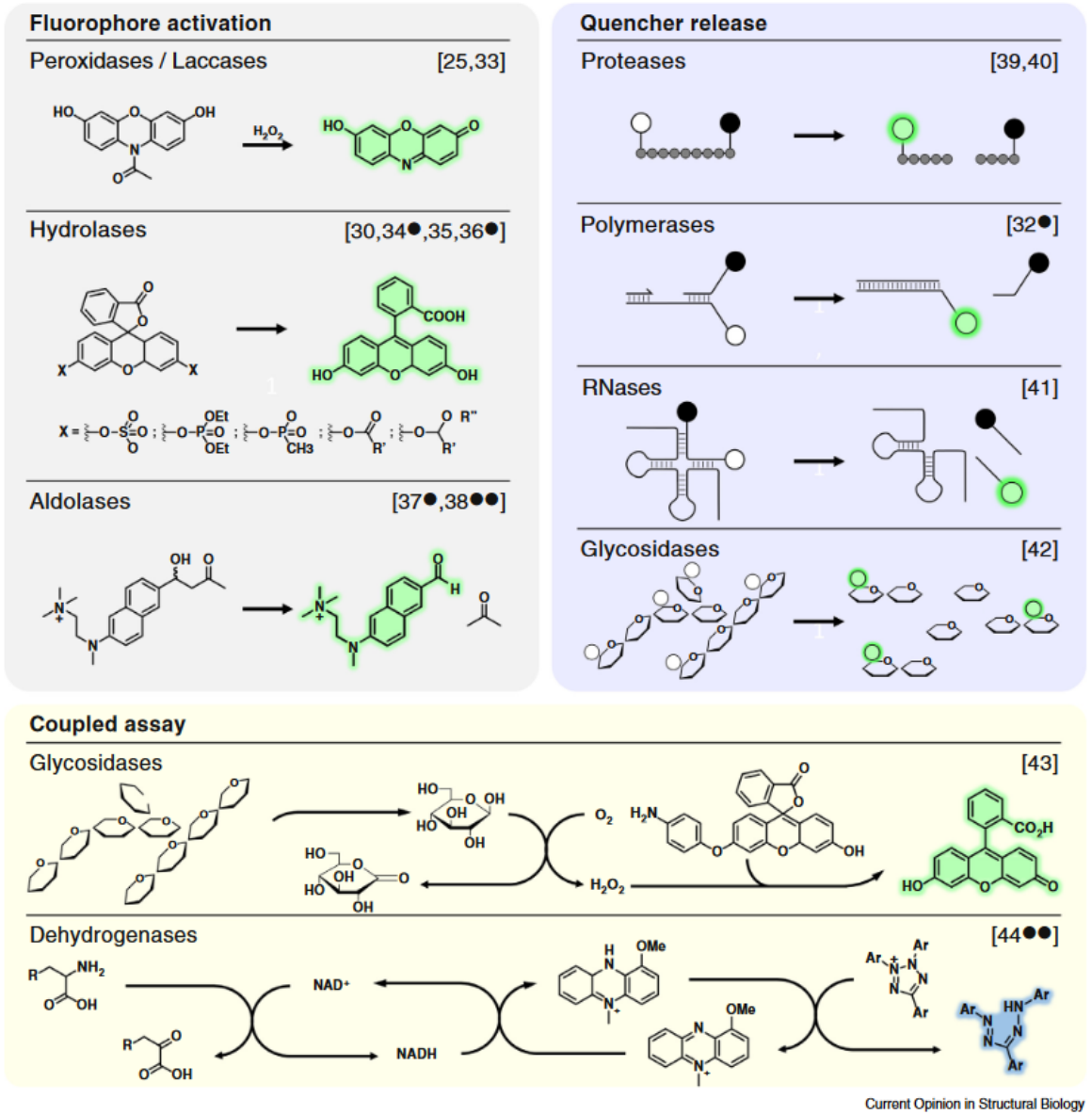
Good practices

> apply concentrated stock solutions (*why?*)

> controls: enzyme minus & substrate minus

> consider: pH, ionic strength and temperature

Enzyme applications

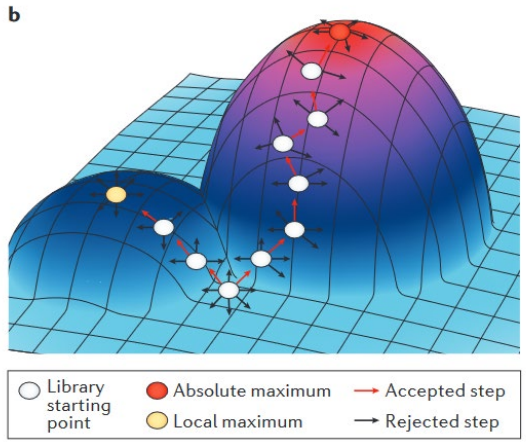


Bunzel et al., 2018
(doi: 10.1016/j.sbi.2017.12.010)

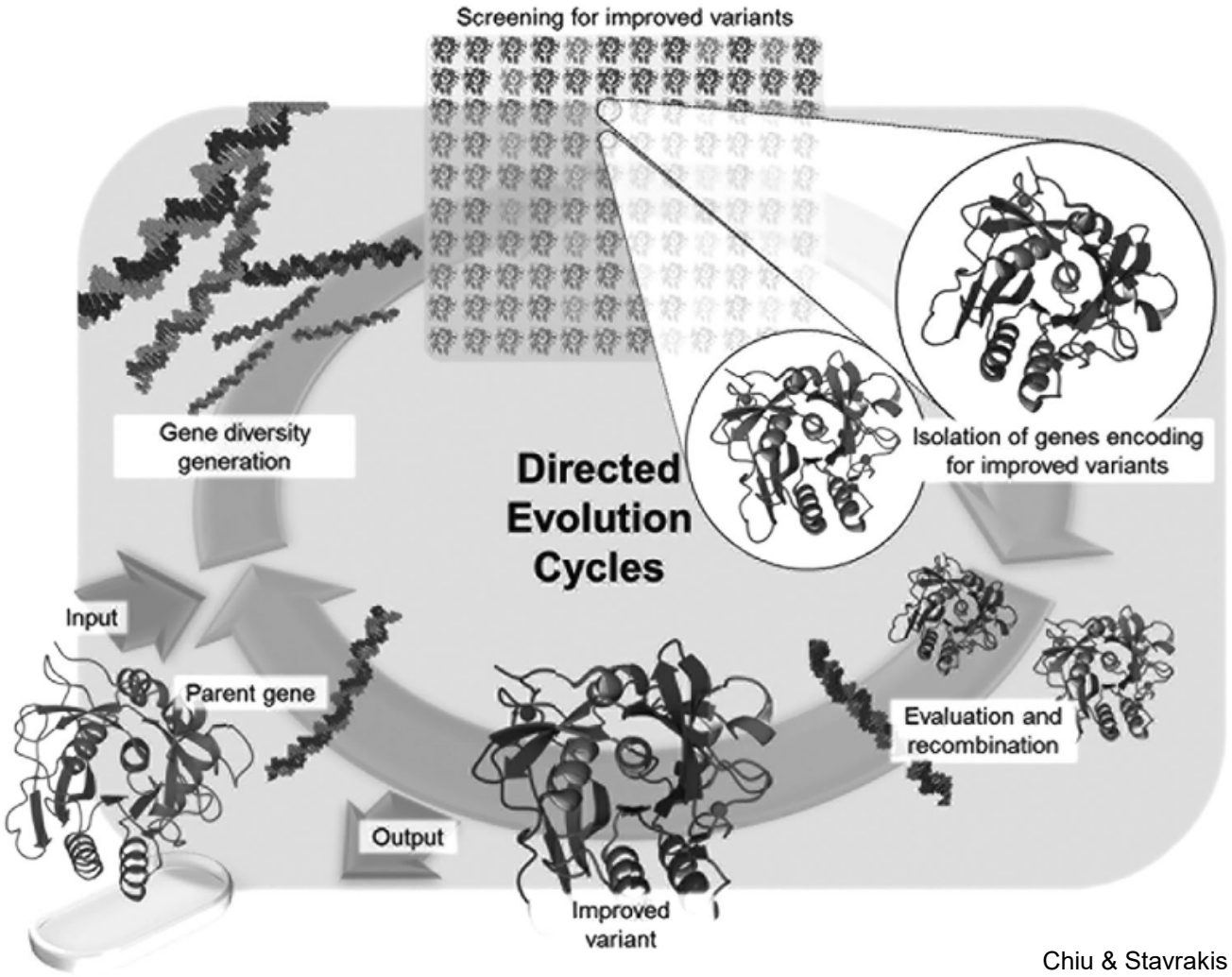
Enzyme applications

Directed evolution

- new enzyme variants
- desired activity
- *e.g.*, digesting unnatural substrates



Packer & Liu
(doi: 10.1038/nrg3927)

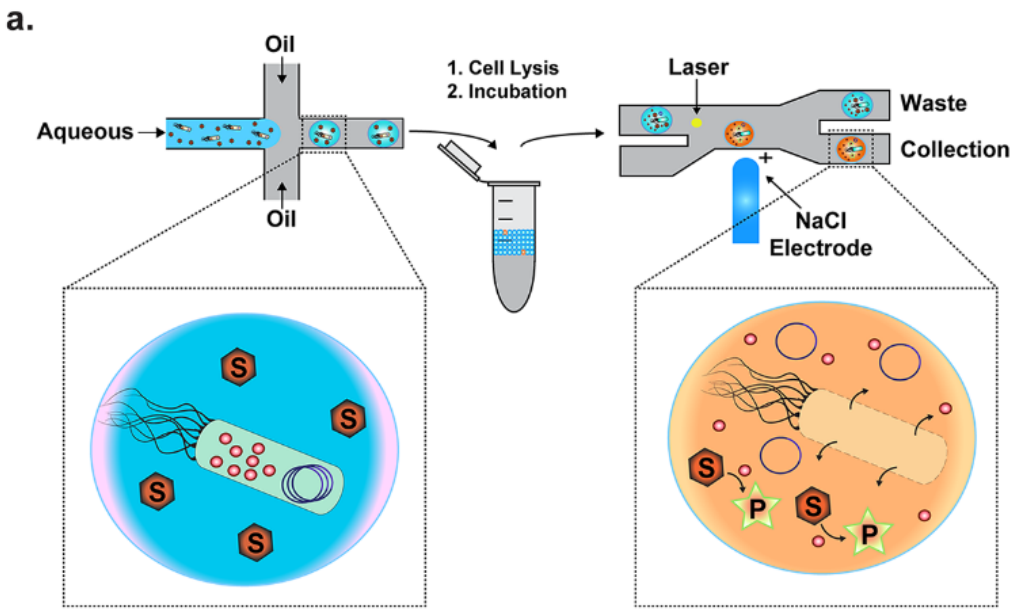


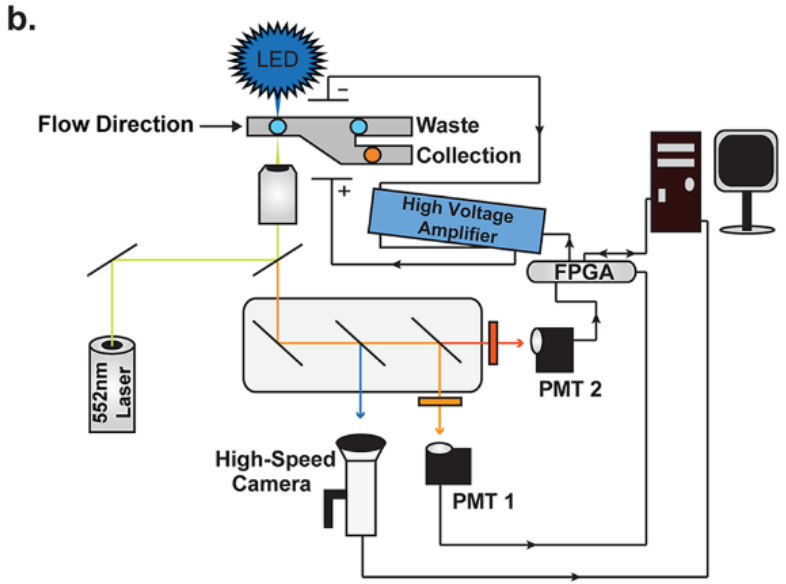
Chiu & Stavrakis
(doi: 10.1002/elps.201900222)

Enzyme applications

Directed evolution

- new enzyme variants
- desired activity
- *e.g.*, digesting unnatural substrates

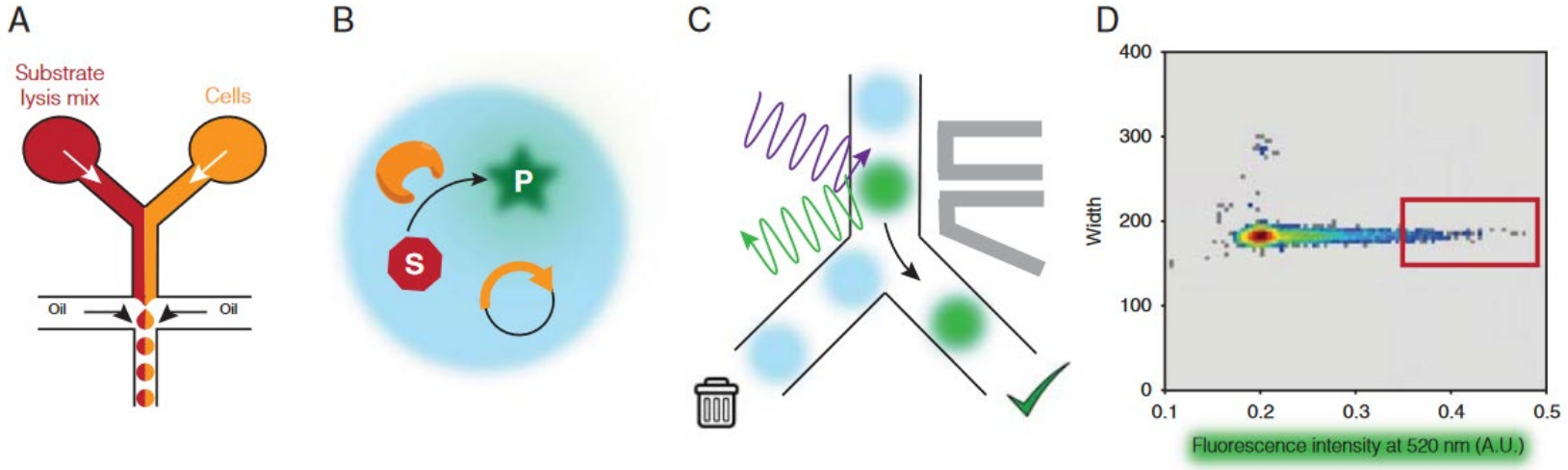


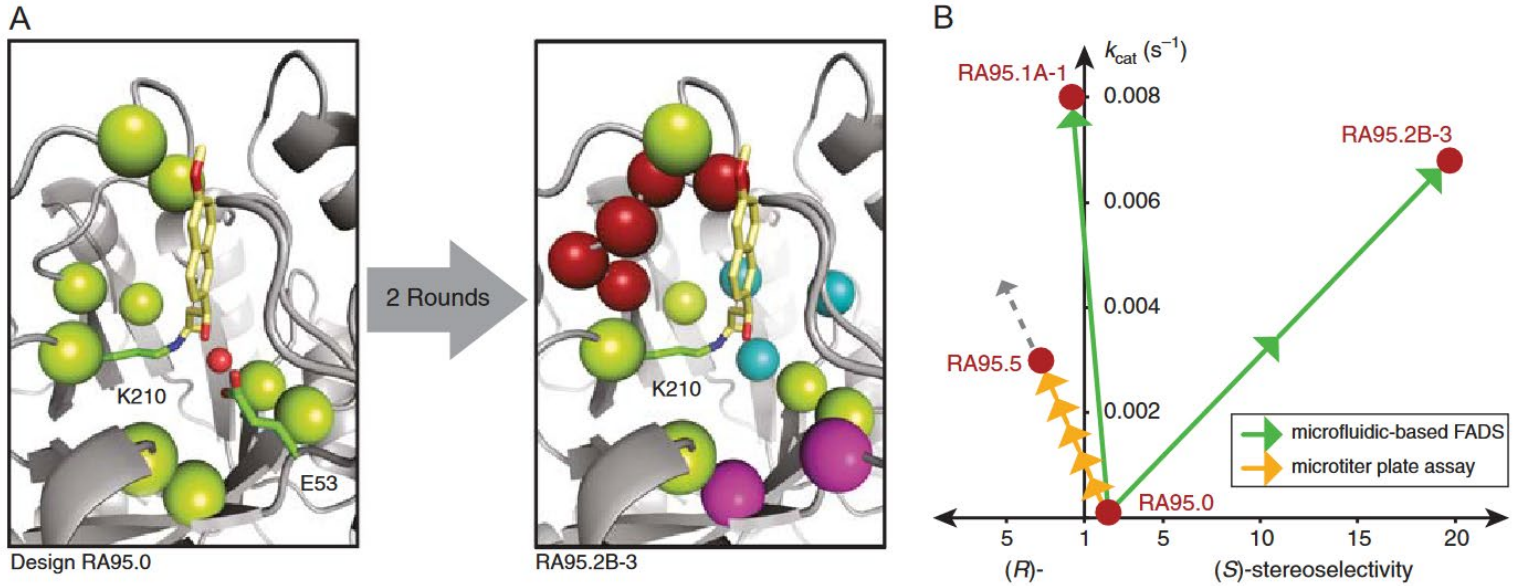


Vallejo et al., 2019
(doi: 10.1021/acssynbio.9b00103)

Enzyme applications

Directed evolution



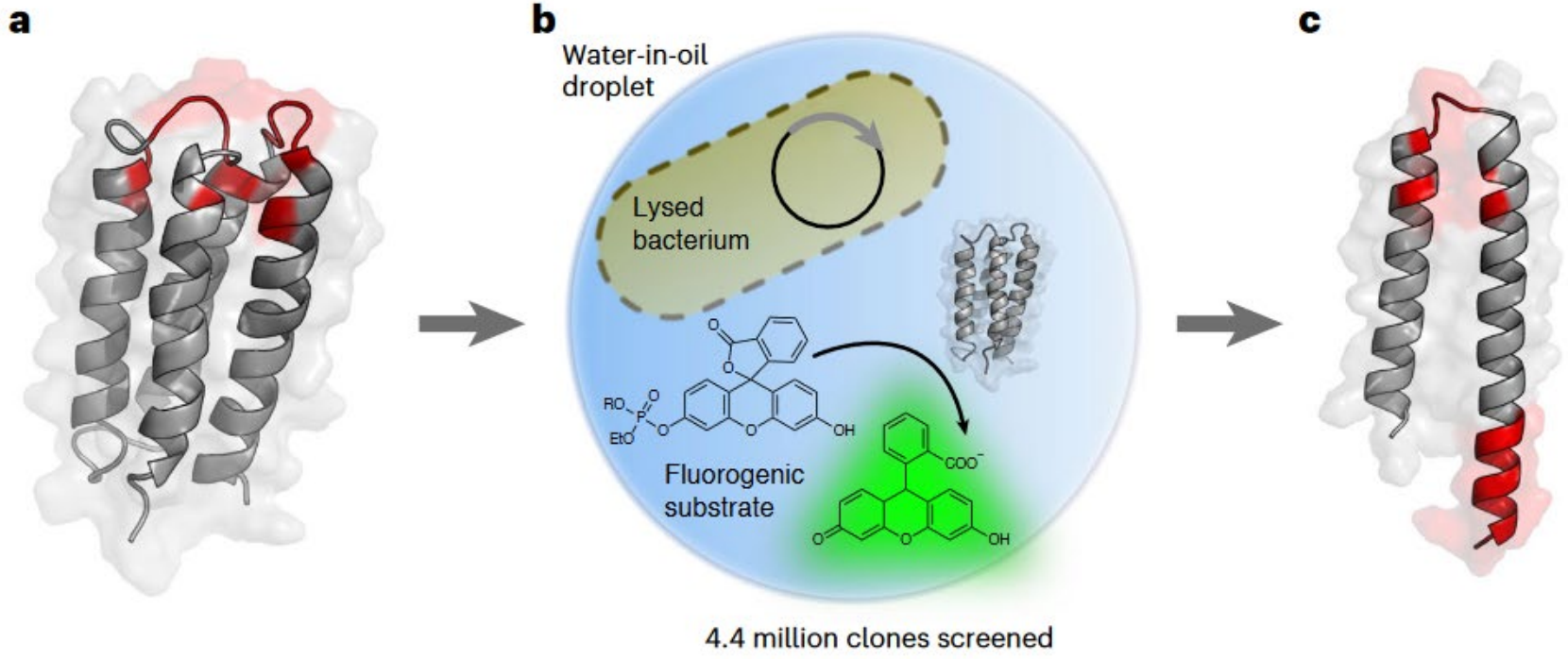


Obexer et al., 2016
(doi: 10.1093/protein/gzw032)

Enzyme applications

Directed evolution

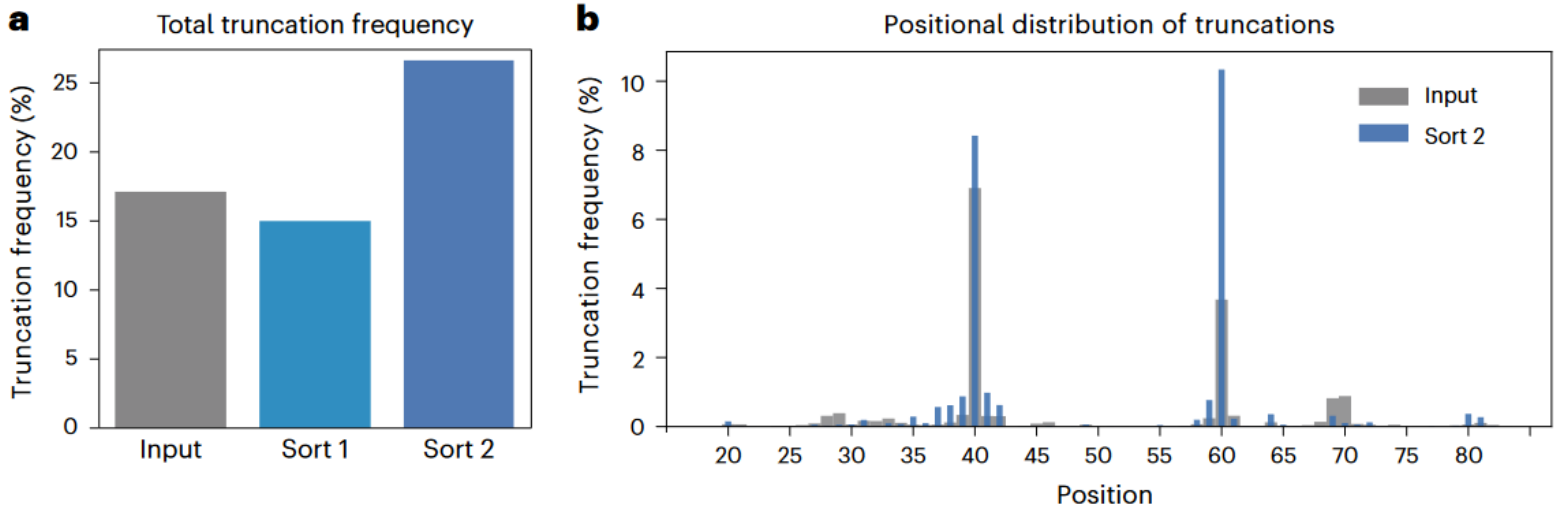
Selection of a promiscuous minimalist cAMP phosphodiesterase from a library of de novo designed proteins

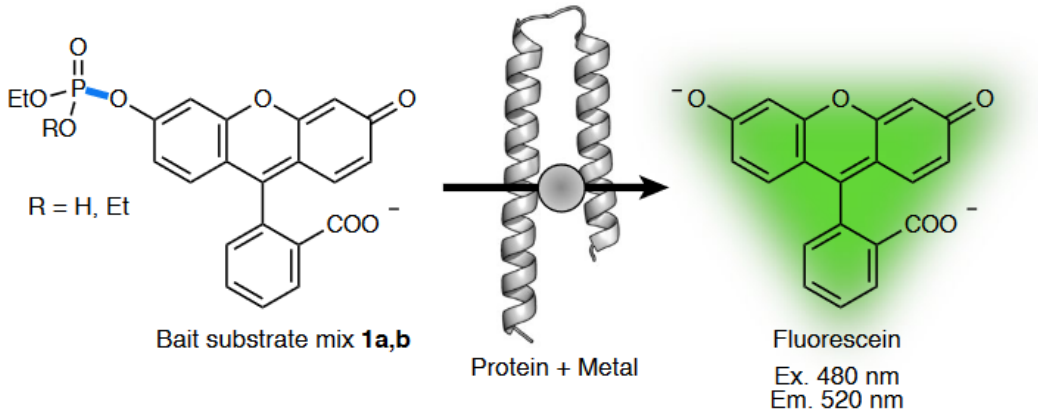


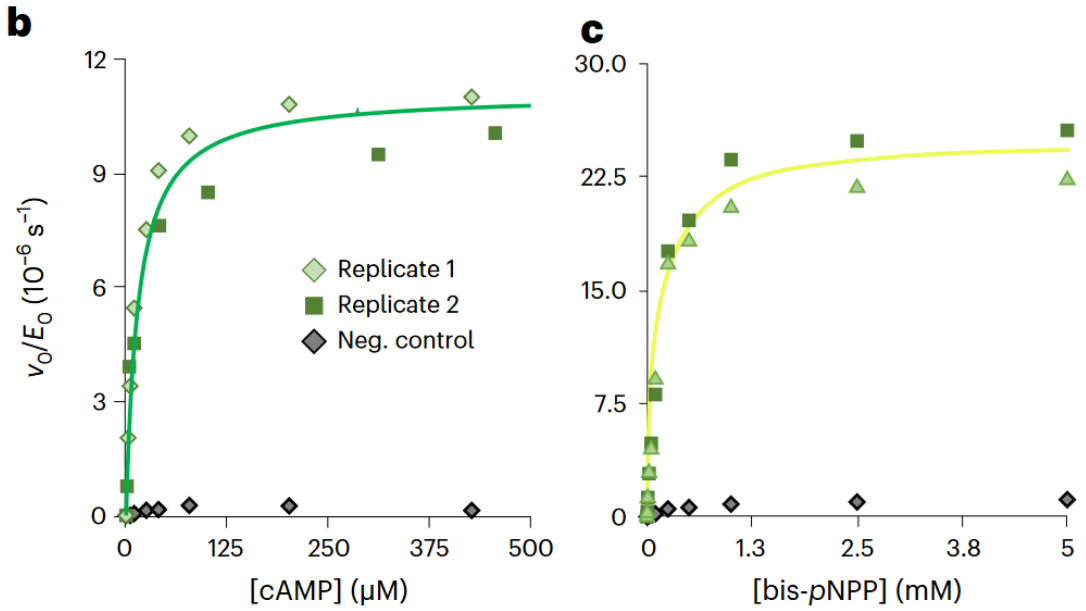
Schnettler et al., 2024
(doi: 10.1038/s41557-024-01490-4)

Enzyme applications

Directed evolution



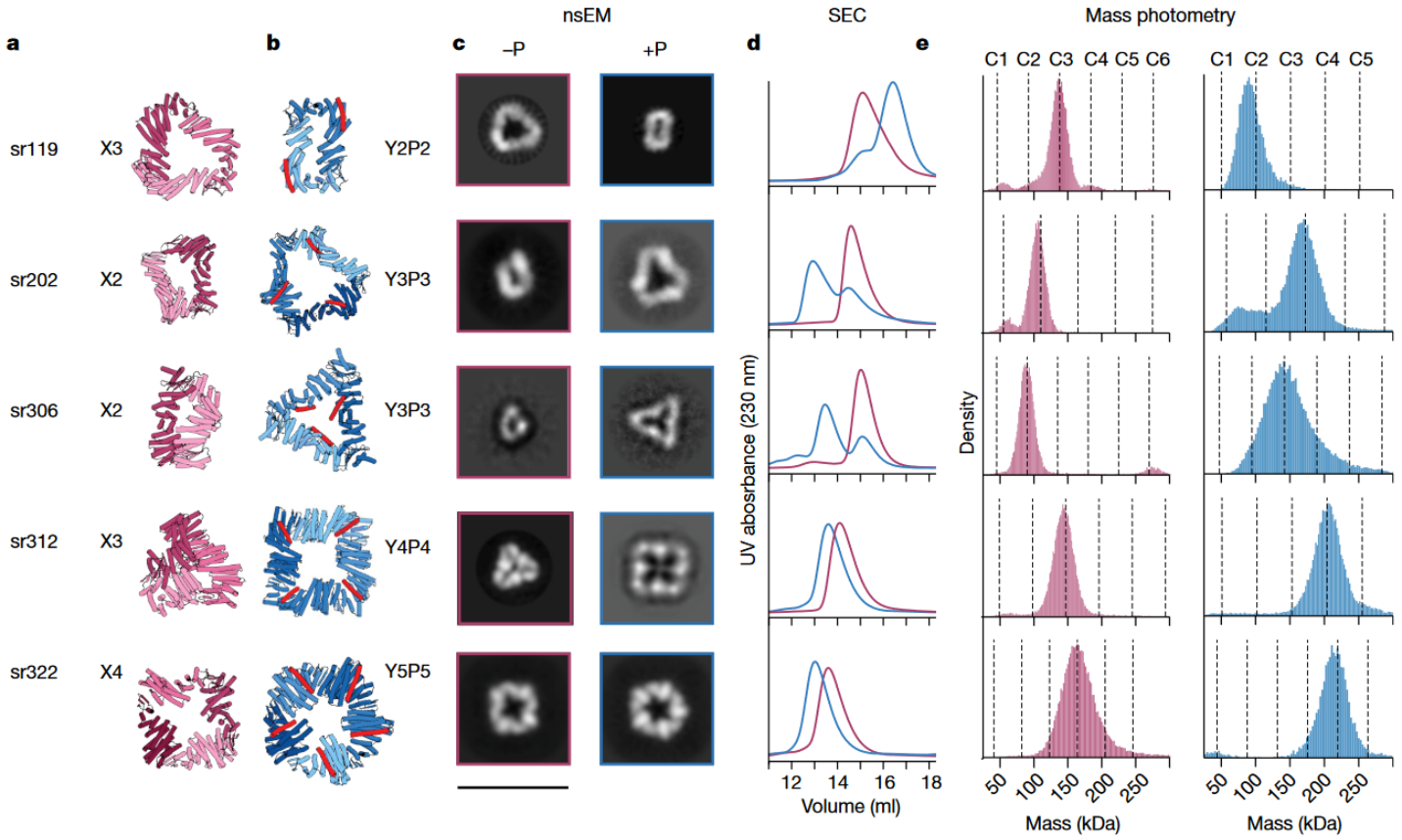


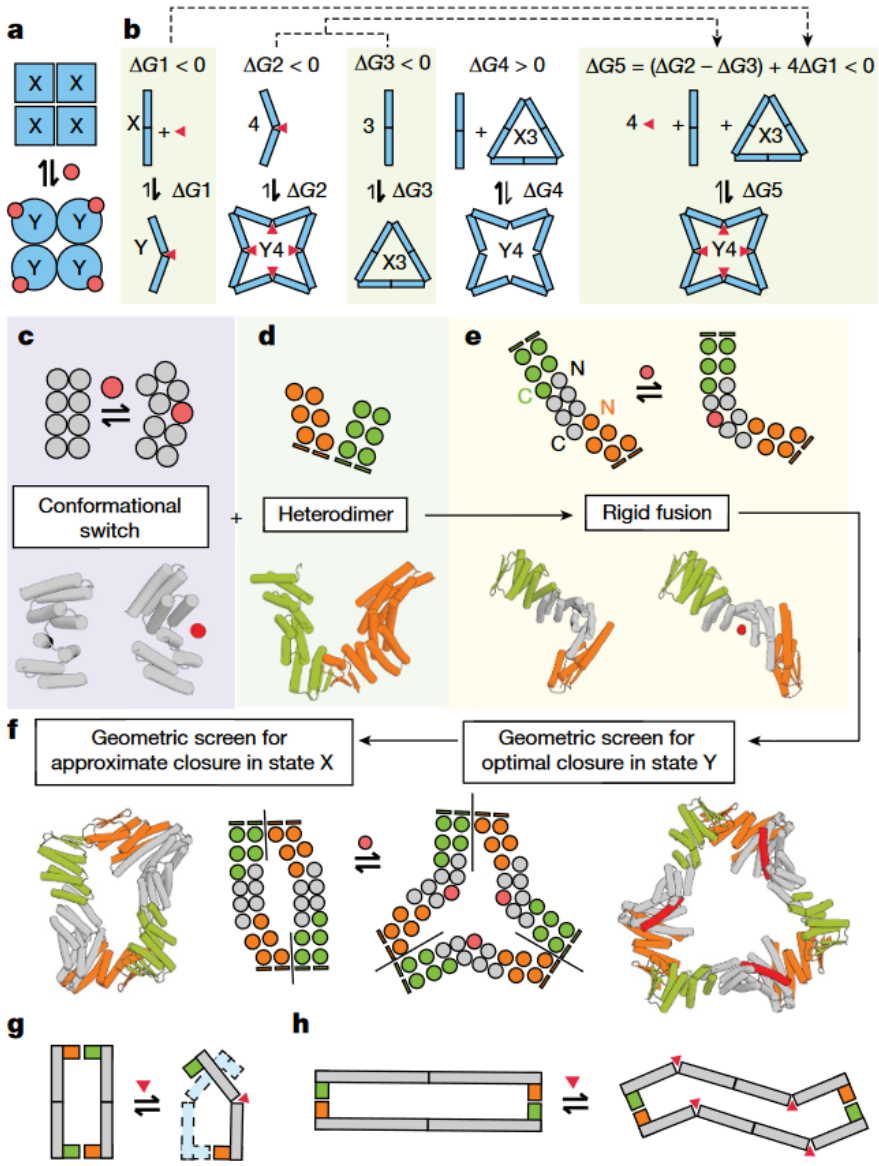


Schnettler et al., 2024
(doi: 10.1038/s41557-024-01490-4)

Current research

De novo design of allosterically switchable protein assemblies



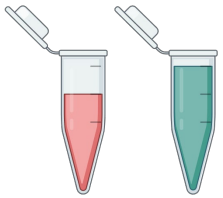


Pillai et al., 2024
(doi: 10.1038/s41586-024-07813-2)

Back to your practical task

2 groups and 2 different substrates

Beta-galactoside (EC 3.2.1.23)



Substrate: fluorescein di-β-D-galactopyranoside (FDG) (498 nm / 517 nm)

resorufin di-β-D-galactopyranoside (RDG) (571 nm / 585 nm)

'What is your substrate concentration?'

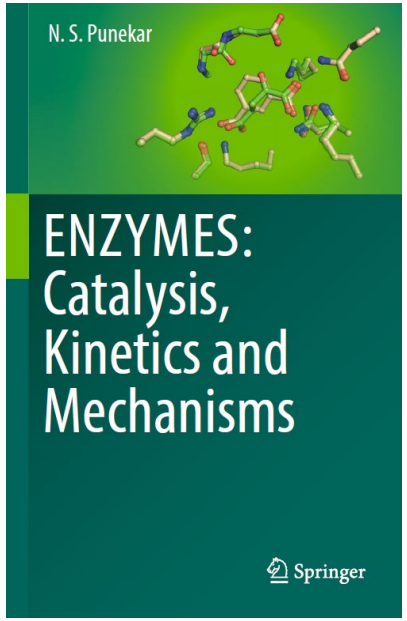
'What is the physiological role of your enzyme?'

'What are (potential) applications?'

Final report

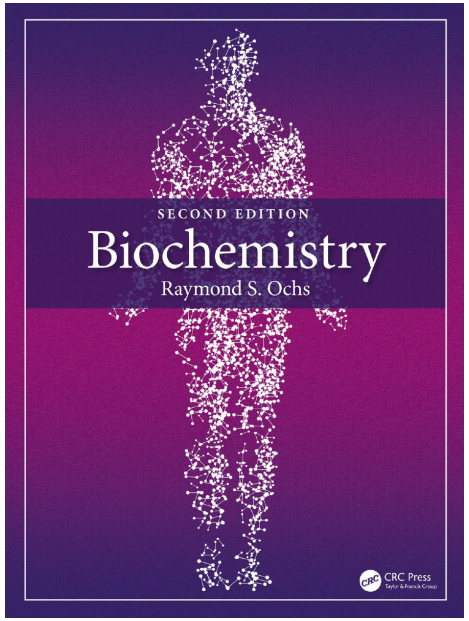
Graphical illustrations were made with **BioRender.com**

References and further literature



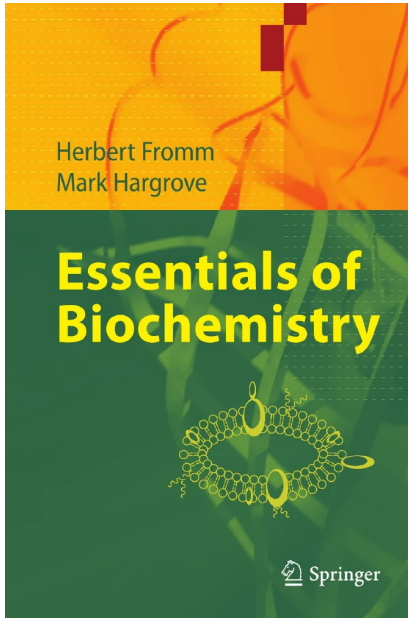
https://doi.org/10.1007/978-981-13-0785-0

Punekar, N.S., *Enzymes: Catalysis, Kinetics and Mechanisms*, Singapore, Springer Nature, 2018



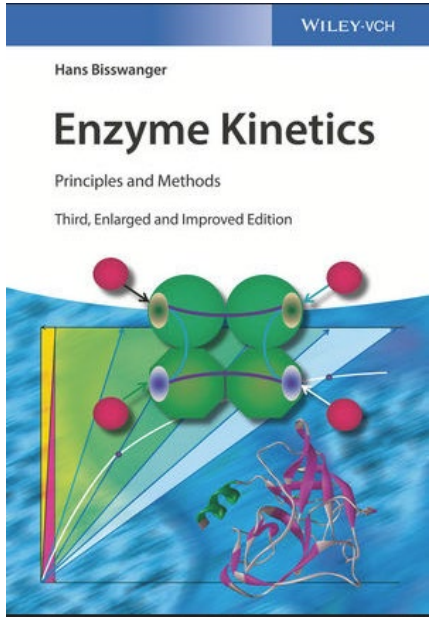
https://doi.org/10.1201/9781003029649

Ochs, Raymond S., *Biochemistry*, 2nd edition, Boca Raton, CRC Press, 2022



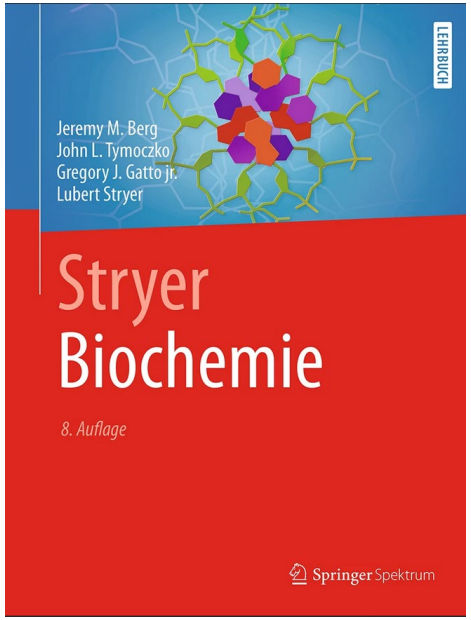
https://doi.org/10.1007/978-3-642-19624-9

Fromm, Herbert & Hargrove, Mark, *Essentials of Biochemistry*, Berlin, Heidelberg, Springer, 2012



https://doi.org/10.1002/9783527806461

Bisswanger, Hans, *Enzyme Kinetics: Principles and Methods*, 3rd edition, Weinheim, Wiley-VCH, 2017



https://doi.org/10.1007/978-3-662-54620-8

Berg, Jeremy M., Tymoczko, John L., Gatto jr., Gregory J., *Stryer Biochemie*, 8th edition, Berlin, Heidelberg, Springer Spektrum, 2017

Publications:

Mair et al., 2017 (doi: 10.1016/j.cbpa.2017.02.018)

Chiu & Stavrakis (doi: 10.1002/elps.201900222)

Vallejo et al., 2019 (doi: 10.1021/acssynbio.9b00103)

Obexer et al., 2016 (doi: 10.1093/protein/gzw032)

Bunzel et al., 2018 (doi: 10.1016/j.sbi.2017.12.010)

Packer & Liu, 2015 (doi: 10.1038/nrg3927)