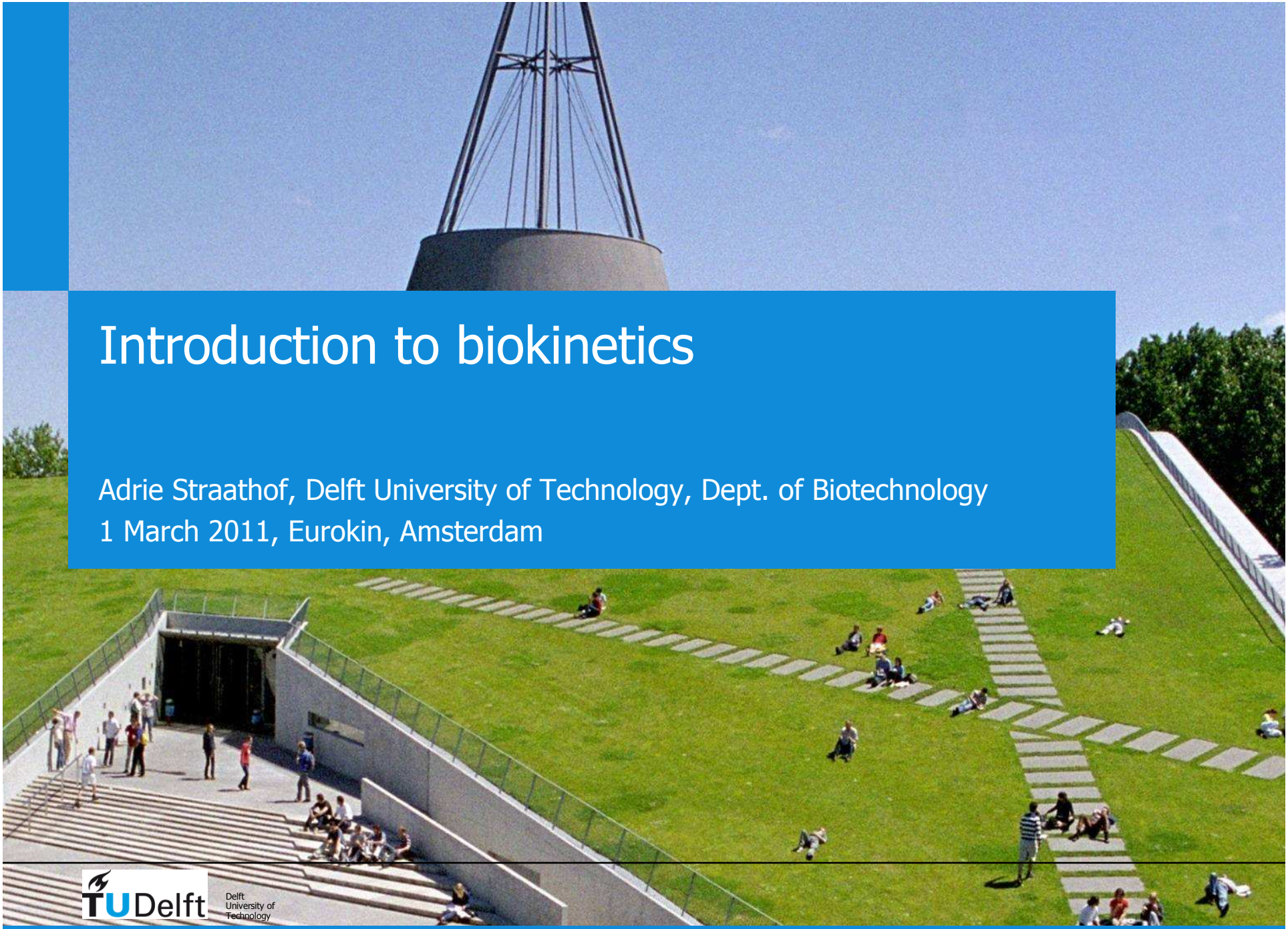

Introduction to biokinetics

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1 March 2011, Eurokin, Amsterdam

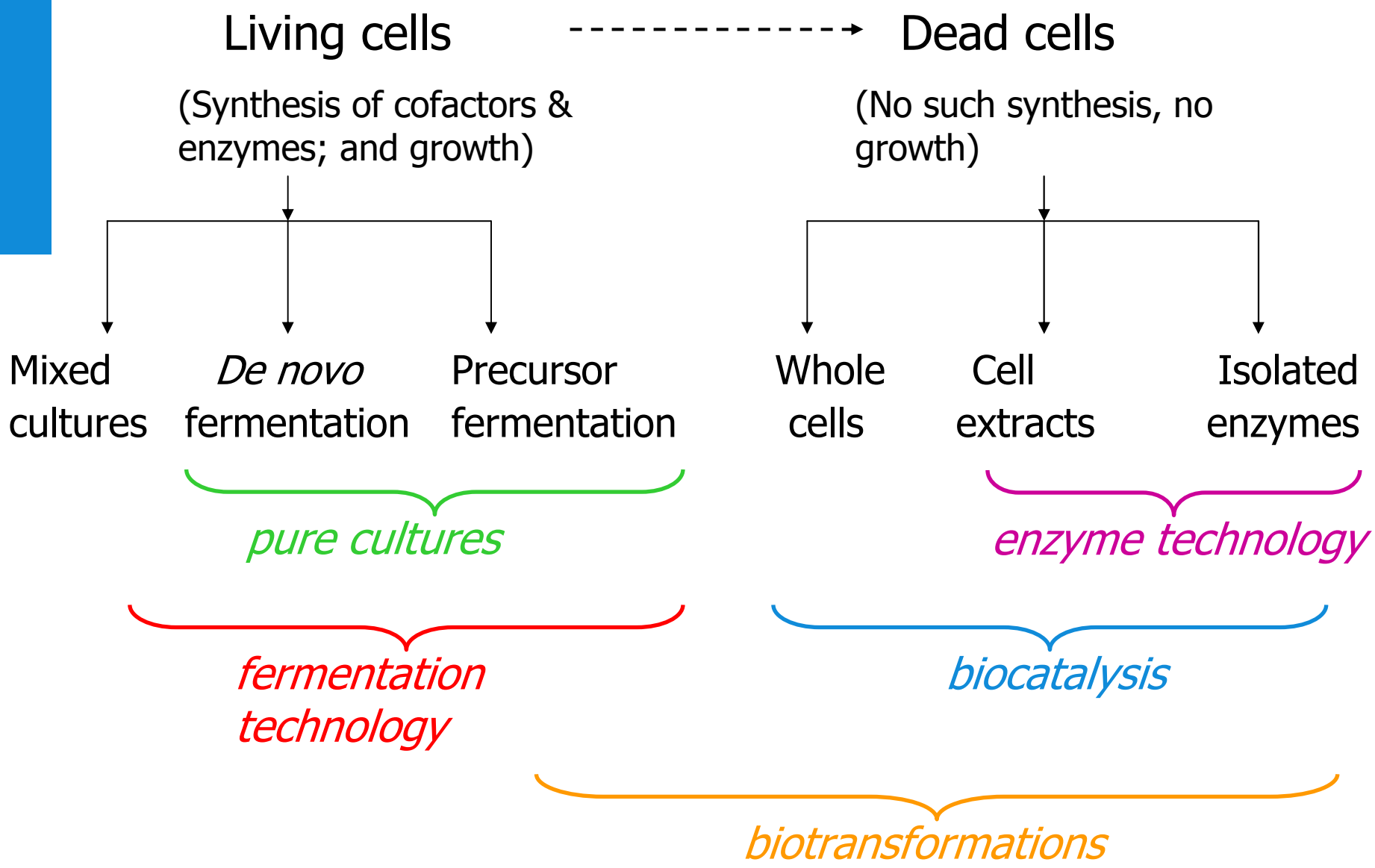
TU Delft
Delft University of Technology

Challenge the future

Research groups at Department of Biotechnology TU Delft

- Analytical Biotechnology
- Biocatalysis and Organic Chemistry
- Bioprocess Technology
- Bioseparation Technology
- Environmental Biotechnology
- Enzymology
- Industrial Microbiology
- Complex Fluids Theory
- Biotechnology and Society

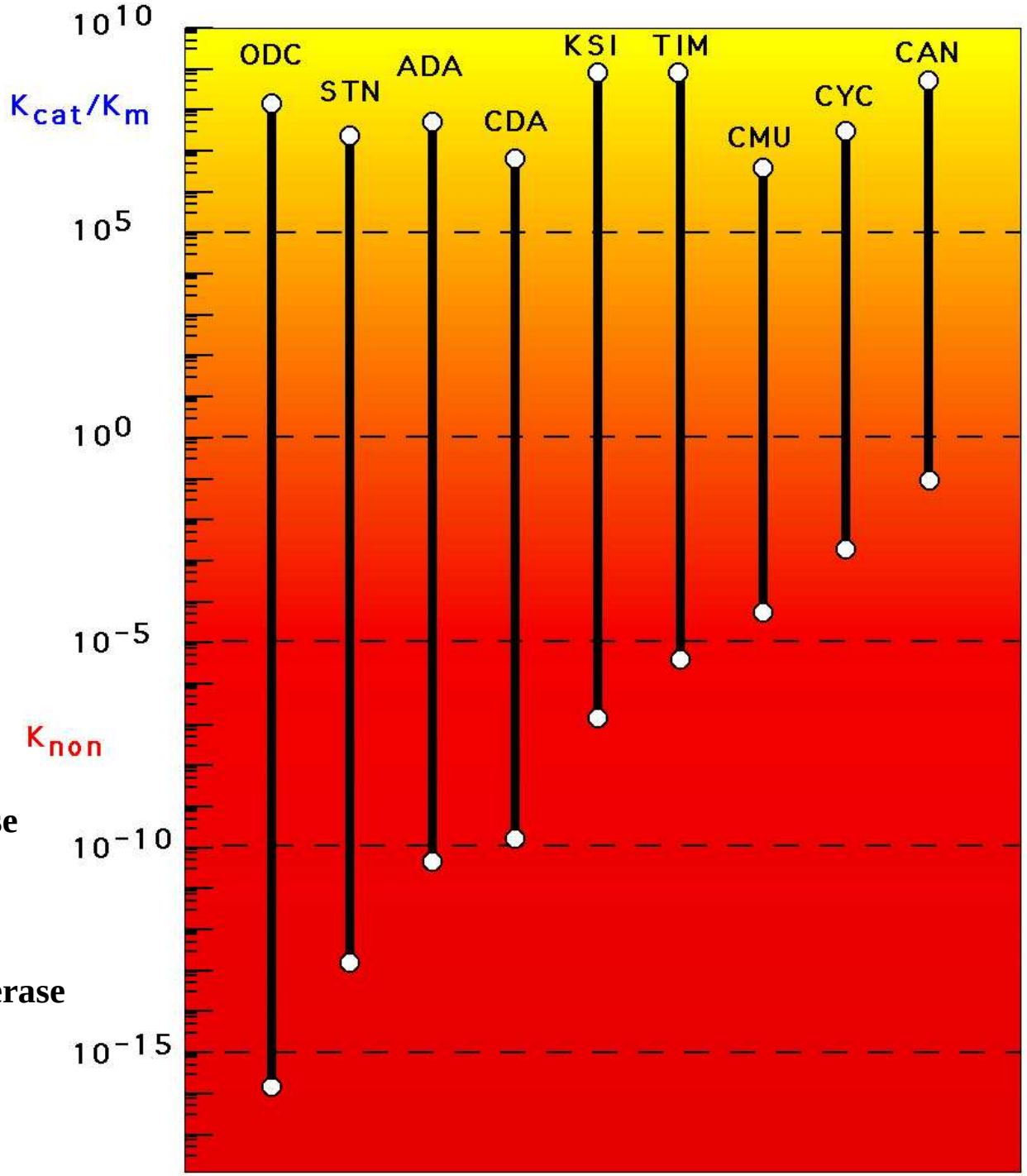
biokinetics is key expertise


Living cells
Dead cells
(Synthesis of cofactors & enzymes; and growth)
(No such synthesis, no growth)
Mixed cultures
De novo fermentation
Precursor fermentation
Whole cells
Cell extracts
Isolated enzymes
pure cultures
enzyme technology
fermentation technology
biocatalysis
biotransformations

Principles governing kinetics

- Enzymes: catalysis+transport
- Pure microbial cultures: also growth+regulation+evolution
- Mixed cultures: also growth+regulation+evolution +ecology

Rate enhancement by enzyme catalysis



ODC	**OMP decarboxylase**
STN	**Staphylococcal nuclease**
ADA	**Adenosine deaminase**
CDA	**Cytidine deaminase**
KSI	**Ketosteroid isomerase**
TIM	**Triosephosphate isomerase**
CMU	**Chorismate mutase**
CYC	**Cyclophilin**
CAN	**Carbonic anhydrase**

Rate equations

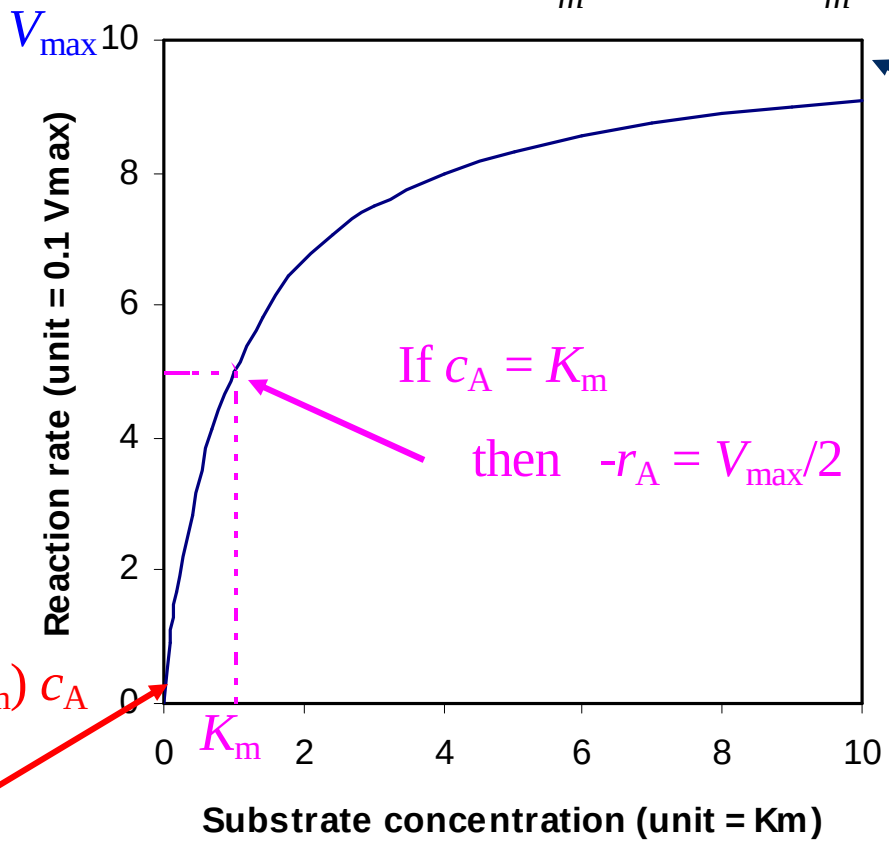
- $-r_A = f(p, T, c_A, c_B, c_P, c_{Enzyme}, c_{H^+}, c_{solvent}, \ldots)$
- $-r_{Enzyme} = f(p, T, c_A, c_B, c_P, c_{Enzyme}, c_{H^+}, c_{solvent}, \ldots)$

- Stoichiometry: $-r_A = -r_B = r_P$

- Rates are often studied at fixed p, T, pH; in water.

Michaelis-Menten equation

$$-r_A = \frac{V_{max}\, c_A}{K_m + c_A} = \frac{V_{max}\,\frac{c_A}{K_m}}{1+\frac{c_A}{K_m}} = \frac{k_{cat}\,\frac{c_A}{K_m}}{1+\frac{c_A}{K_m}}\, c_{Etot}$$



If $c_A \rightarrow \infty$

then $-r_A \rightarrow V_{max}$

(0^{th} order range)

If $c_A = K_m$

then $-r_A = V_{max}/2$

If $c_A \rightarrow 0$

then $-r_A \rightarrow (V_{max}/K_m)\, c_A$

(1^{st} order range)

Stoichiometry

most enzymatic reactions include up to 2 substrates and 2 products

1. Oxidoreductases	bi-bi, ter-bi (or other)
2. Transferases	bi-bi
3. Hydrolases	bi-bi
4. Lyases	uni-bi (usually)
5. Isomerases	uni-uni
6. Ligases	ter-ter (or more)

Difficult example (E.C. 6.3.1.14):

$2\ ATP + NH_3 + CO_2 + H_2O \rightarrow 2\ ADP + P_i$ + carbamoyl complex

http://www.expasy.org/enzyme/

Michaelis-Menten: A ==> P

• Glucose isomerase	A <==> P
• glucoamylase	A + H_2O <==> P + Q
• fumarase	A + H_2O <==> P
• aspartase	A + B <==> P
• aspartate decarboxylase	A ==> P + CO_2
• thermolysin	A + B <==> P + H_2O
• penicillin acylase	A + H_2O <==> P + Q

B, P and Q will influence the rate

Laboratory conditions

- $c_A << c_{water}$
- $c_A >> c_P, c_Q$

==> (pseudo) Michaelis-Menten suited

Industrial conditions

- c_A = initially as high as possible
- $c_A << c_P, c_Q$ at the end of the reaction

==> Michaelis-Menten equation unsuited

Simplest reaction scheme:

$$E + A \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EA \xrightarrow{k_2} E + P$$

- 4 species involved: E, A, EA and P
- 2 conservation equations; in batch with constant volume:

$$c_{Etot} = c_E + c_{EA}$$
$$c_{A0} = c_A + c_P + c_{EA}$$

- 4 - 2 = 2 macroscopic balances needed; in batch with constant volume:

$$-dc_A/dt = k_1 c_E c_A - k_{-1} c_{EA}$$
$$-dc_E/dt = k_1 c_E c_A - k_{-1} c_{EA} + k_2 c_{EA}$$

- Can hardly be solved analytically without any assumption

Simpifying assumptions

1. $c_A >> c_{Etot}$

2a. $k_{-1} >> k_2$ pseudo-equilibrium assumption

(EA is almost at equilibrium with E and A)

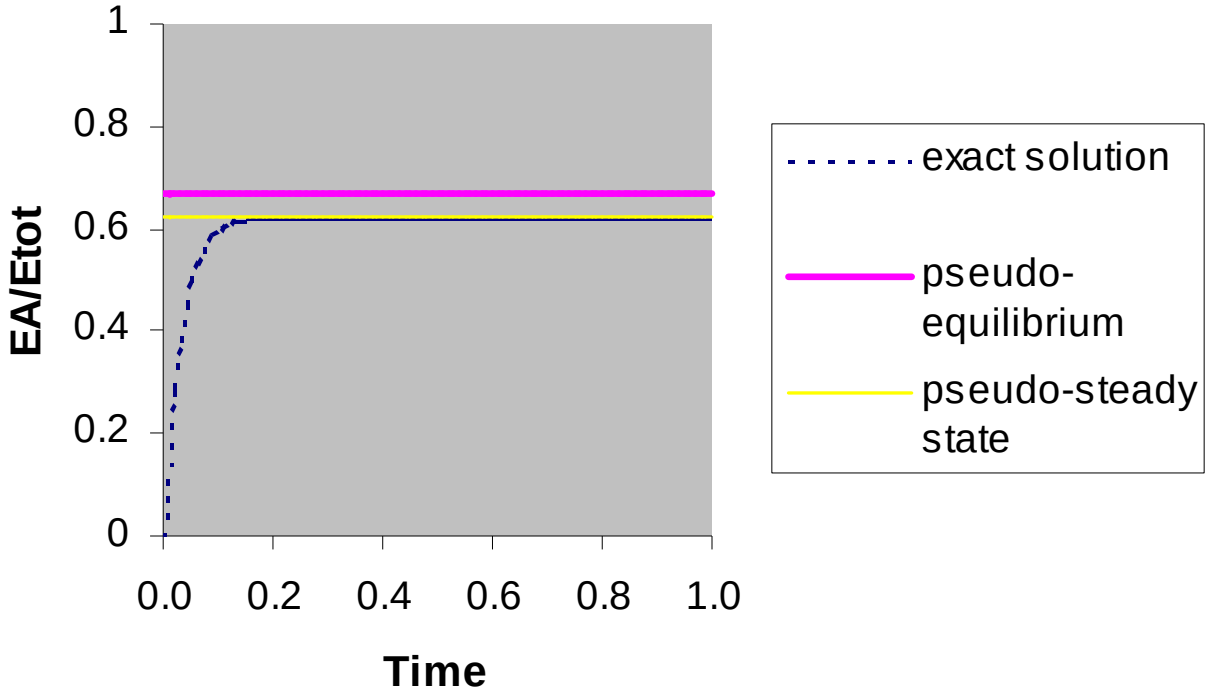
2b. $dc_E/dt = 0$ pseudo-steady state assumption

(the change in concentration of E is much smaller than the change in concentration of A)

Result for 2a and also for 2b: Michaelis-Menten type of equation

Comparison of approaches 1

(pre-steady state situation; no product formation yet)



$c_{A0} = 1$

$c_{Etot} = 0.001$

$k_1 = 20$

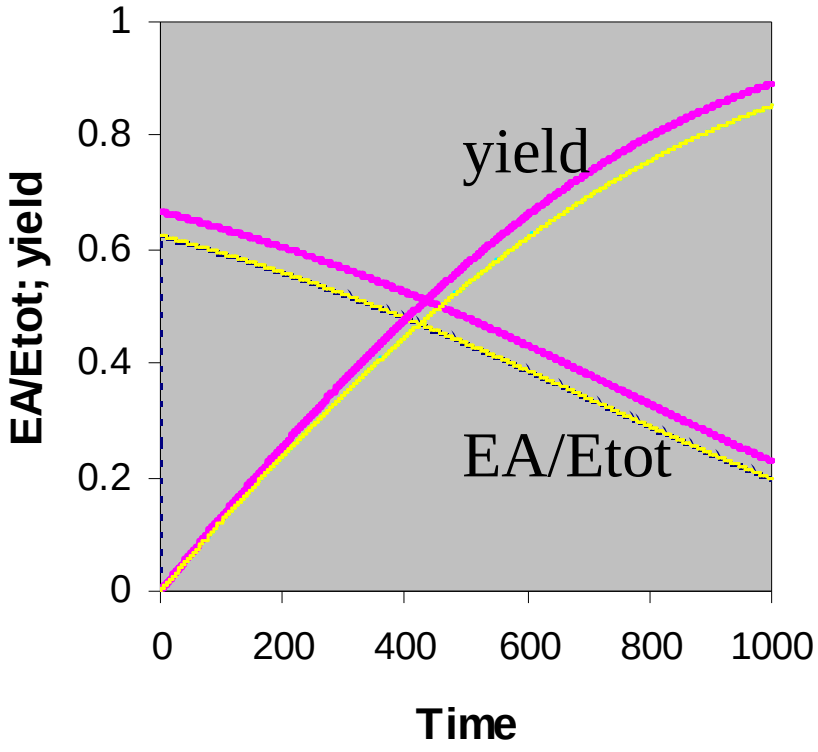
$k_{-1} = 10$

$k_2 = 2$

(arbitrary units)

Comparison of approaches 2

(steady-state situation; product formation)



$c_{A0} = 1$

$c_{Etot} = 0.001$

$k_1 = 20$

$k_{-1} = 10$

$k_2 = 2$

(arbitrary units)

PROCEDURES FOR DERIVATION OF RATE EQUATIONS

What is the rate equation for the following mechanism?

$$\mathrm{E} + \mathrm{A} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{EA} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \mathrm{EP} \xrightarrow{k_3} \mathrm{E} + \mathrm{P}$$

1. Write down the conservation balance for the *n* enzyme species involved (1 balance equation).

$$c_{Etot} = c_E + c_{EA} + c_{EP}$$

2. Use the pseudo-steady state approach for each but one of the enzyme species

$$\mathrm{d}c_{EA}/\mathrm{d}t = k_1 c_E c_A + k_{-2} c_{EP} - (k_{-1} + k_2) c_{EA} = 0$$

$$\mathrm{d}c_{EP}/\mathrm{d}t = k_2 c_{EA} - (k_{-2} + k_3) c_{EP} = 0$$

3. Sum the rates of substrate formation and conversion

$$-r_A = k_3 c_{EP}$$

4. Solve the set of $n + 1$ equations

Symbolic solution with Mathcad

Given

$$cEtot = cE + cEA + cEP$$

$$k1 \cdot cE \cdot cA + k\_2 \cdot cEP - (k\_1 + k2) \cdot cEA = 0$$

$$k2 \cdot cEA - (k\_2 + k3) \cdot cEP = 0$$

$$-rA = k3 \cdot cEP$$

$$\text{Find}(rA, cE, cEA, cEP) \rightarrow \begin{bmatrix} -k3 \cdot cEtot \cdot k2 \cdot k1 \cdot \dfrac{cA}{(k\_1 \cdot k\_2 + k\_1 \cdot k3 + k2 \cdot k3 + k2 \cdot k1 \cdot cA + k\_2 \cdot k1 \cdot cA + k3 \cdot k1 \cdot cA)} \\ cEtot \cdot \dfrac{(k\_1 \cdot k\_2 + k\_1 \cdot k3 + k2 \cdot k3)}{(k\_1 \cdot k\_2 + k\_1 \cdot k3 + k2 \cdot k3 + k2 \cdot k1 \cdot cA + k\_2 \cdot k1 \cdot cA + k3 \cdot k1 \cdot cA)} \\ (k\_2 + k3) \cdot cA \cdot k1 \cdot \dfrac{cEtot}{(k\_1 \cdot k\_2 + k\_1 \cdot k3 + k2 \cdot k3 + k2 \cdot k1 \cdot cA + k\_2 \cdot k1 \cdot cA + k3 \cdot k1 \cdot cA)} \\ cEtot \cdot k2 \cdot k1 \cdot \dfrac{cA}{(k\_1 \cdot k\_2 + k\_1 \cdot k3 + k2 \cdot k3 + k2 \cdot k1 \cdot cA + k\_2 \cdot k1 \cdot cA + k3 \cdot k1 \cdot cA)} \end{bmatrix}$$

5. Simplify the rate equation by replacing the groups of rate constants (k_1, k_2, etc that appear in the solution by kinetic parameters (K_m, V_{max}, etc.) according Cleland (1963).

$$V_{max} = \lim_{A\to\infty}(-r_A) = \frac{k_2 k_3}{(k_2 + k_{-2} + k_3)} c_{Etot}$$

so $$k_{cat} = \frac{V_{max}}{c_{Etot}} = \frac{k_2 k_3}{(k_2 + k_{-2} + k_3)}$$

$$\frac{V_{max}}{2} = \lim_{A\to Km}(-r_A)$$

so $$K_{mA} = \frac{k_{-1}(k_{-2} + k_3) + k_2 k_3}{k_1(k_2 + k_{-2} + k_3)}$$

$$-r_A = \frac{\dfrac{k_1 k_2 k_3}{k_{-1}(k_{-2} + k_3) + k_2 k_3} c_A c_{Etot}}{1 + \dfrac{k_1(k_2 + k_{-2} + k_3)}{k_{-1}(k_{-2} + k_3) + k_2 k_3} c_A}$$

5 microscopic rate constants

$$-r_A = \frac{V_{\max} c_A}{1 + \dfrac{c_A}{K_m}}$$

2 macroscopic (steady-state) kinetic parameters

Compare the two models that have been derived:

$$E + A \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EA \xrightarrow{k_2} E + P$$

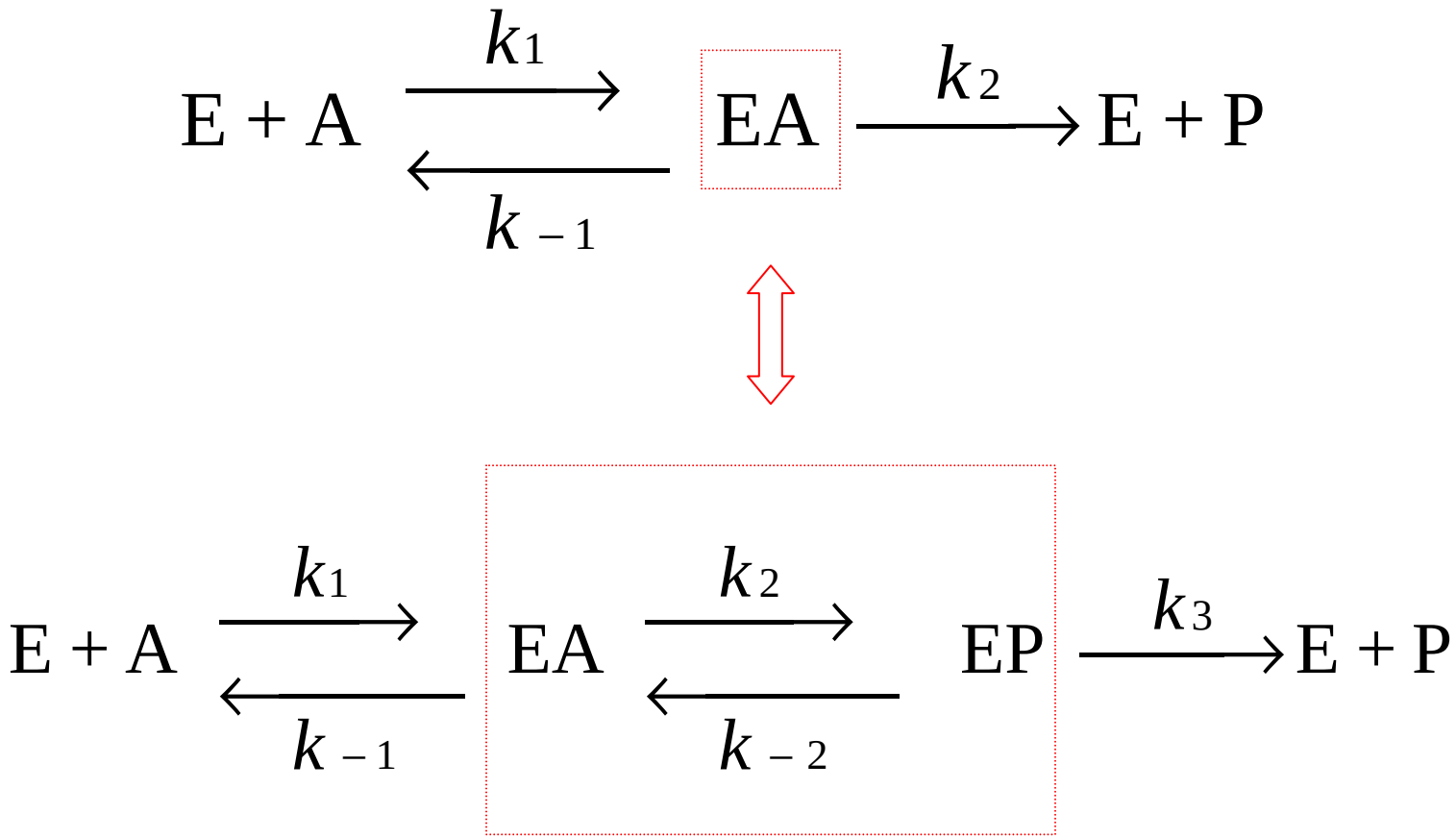
$$\Rightarrow \quad -r_A = \frac{\frac{k_1 k_2}{k_{-1} + k_2} c_A c_{Etot}}{1 + \frac{k_1}{k_{-1} + k_2} c_A} = \frac{\frac{V_{max}}{K_m} c_A}{1 + \frac{c_A}{K_m}}$$

$$E + A \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EA \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EP \xrightarrow{k_3} E + P$$

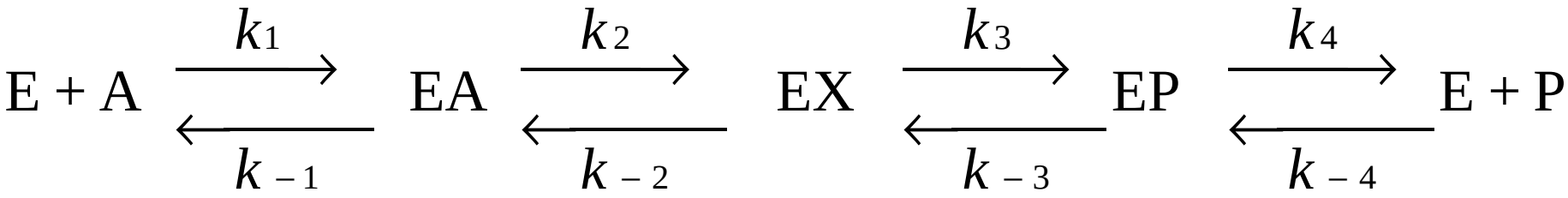
$$\Rightarrow \quad -r_A = \frac{\frac{k_1 k_2 k_3}{k_{-1}(k_{-2} + k_3) + k_2 k_3} c_A c_{Etot}}{1 + \frac{k_1(k_2 + k_{-2} + k_3)}{k_{-1}(k_{-2} + k_3) + k_2 k_3} c_A} = \frac{\frac{V_{max}}{K_m} c_A}{1 + \frac{c_A}{K_m}}$$

Identical model equations result

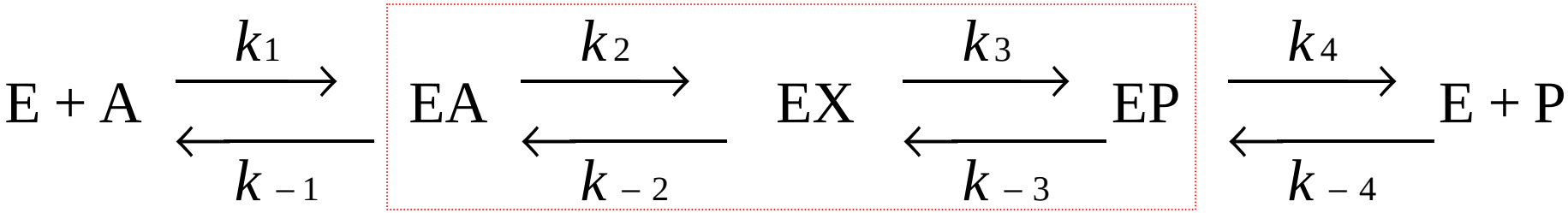
Isomerization steps can be lumped in one enzyme state:

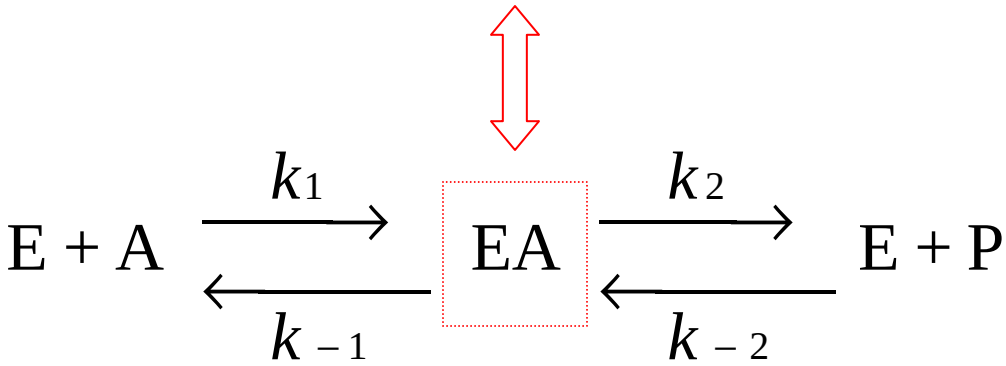


Consider the following mechanism:



What is the simplest mechanism that will give the same rate equation?





UNI-BI REACTIONS

compulsory order

$$E \underset{k_{-1}}{\overset{k_1 \cdot c_A}{\rightleftharpoons}} \{EA \Leftrightarrow EPQ\} \underset{k_{-2} \cdot c_P}{\overset{k_2}{\rightleftharpoons}} EQ \underset{k_{-3} \cdot c_Q}{\overset{k_3}{\rightleftharpoons}} E$$

$$-r_A = \frac{\frac{V_1}{K_{mA}}\left(c_A - \frac{c_P c_Q}{K_{eq}}\right)}{1 + \frac{c_A}{K_{mA}} + \frac{K_{mQ}}{K_{iQ}}\frac{c_P}{K_{mP}} + \frac{c_Q}{K_{iQ}} + \frac{c_P}{K_{mP}}\frac{c_Q}{K_{iQ}} + \frac{c_A}{K_{mA}}\frac{c_P}{K_{iP}}}$$

Example:

Mandelonitrile lyase

A = mandelonitrile

P = HCN

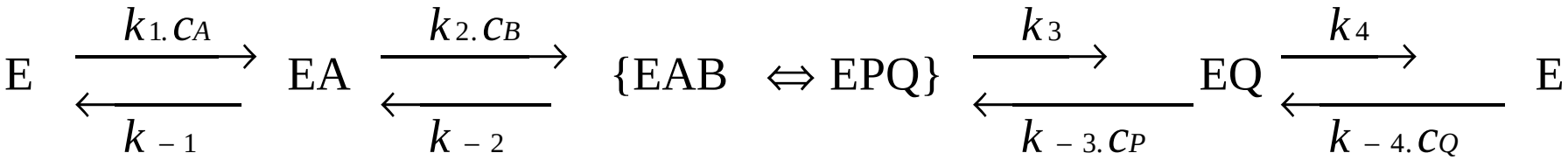
Q = benzaldehyde

random order

$$E \Leftrightarrow \{EA \Leftrightarrow EPQ\} \begin{matrix} \Leftrightarrow EQ \Leftrightarrow \\ \\ \Leftrightarrow EP \Leftrightarrow \end{matrix} E$$

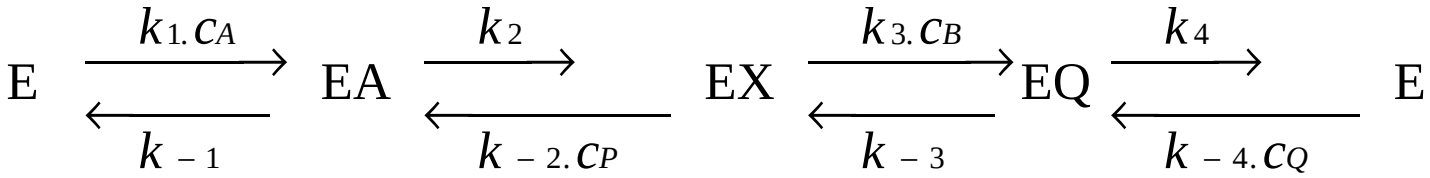
BI-BI REACTIONS

ordered ternary complex mechanism



Example: lactate dehydrogenase (A = NAD^+)

ping-pong mechanism



Example: penicillin amidase (A = penicillin)

***Table 1**. Pseudo-steady state parameters for some kinetic models (Cleland, 1963; Cornish-Bowden, 1995). Bi-uni definitions follow from uni-bi by reversal of A and Q, B and P, and + and -*

Par	Uni-uni	Uni-bi ordered	Bi-bi ordered ternary-complex	Bi-bi ping-pong
V_1	k_2c_{Etot}	$k_2k_3c_{Etot}/(k_2+k_3)$	$k_3k_4c_{Etot}/(k_3+k_4)$	$k_2k_4c_{Etot}/(k_2+k_4)$
V_{-1}	$k_{-1}c_{Etot}$	$k_{-1}c_{Etot}$	$k_{-1}k_{-2}c_{Etot}/(k_{-1}+k_{-2})$	$k_{-1}k_{-3}c_{Etot}/(k_{-1}+k_{-3})$
K_{mA}	$(k_{-1}+k_2)/k_1$	$(k_{-1}+k_2)k_3/[k_1(k_2+k_3)]$	$k_3k_4/[k_1(k_3+k_4)]$	$(k_{-1}+k_2)k_4/[k_1(k_2+k_4)]$
K_{mB}			$(k_{-2}+k_3)k_4/[k_2(k_3+k_4)]$	$k_2(k_{-3}+k_4)/[(k_2+k_4)k_3]$
K_{mP}	$(k_{-1}+k_2)/k_{-2}$	$(k_{-1}+k_2)/k_{-2}$	$k_{-1}(k_{-2}+k_3)/[(k_{-1}+k_{-2})k_{-3}]$	$(k_{-1}+k_2)k_{-3}/[(k_{-1}+k_{-3})k_{-2}]$
K_{mQ}		k_{-1}/k_{-3}	$k_{-1}k_{-2}/[(k_{-1}+k_{-2})k_{-4})]$	$k_{-1}(k_{-3}+k_4)/[(k_{-1}+k_{-3})k_{-4}]$
K_{iA}		k_{-1}/k_1	k_{-1}/k_1	k_{-1}/k_1
K_{iB}			$(k_{-1}+k_{-2})/k_2$	k_{-3}/k_3
K_{iP}		$(k_2+k_3)/k_{-2}$	$(k_3+k_4)/k_{-3}$	k_2/k_{-2}
K_{iQ}		k_3/k_{-3}	k_4/k_{-4}	k_4/k_{-4}
K_{eq}	$k_1k_2/(k_{-1}k_{-2})$	$k_1k_2k_3/(k_{-1}k_{-2}k_{-3})$	$k_1k_2k_3k_4/(k_{-1}k_{-2}k_{-3}k_{-4})$	$k_1k_2k_3k_4/(k_{-1}k_{-2}k_{-3}k_{-4})$

ordered ternary complex mechanism

$$-r_A = \frac{\frac{V_1}{K_{iA} K_{mB}} (c_A c_B - c_P c_Q / K_{eq})}{(1 + \frac{c_A}{K_{iA}} + \frac{K_{mA}}{K_{iA}} \frac{c_B}{K_{mB}} + \frac{c_A}{K_{iA}} \frac{c_B}{K_{mB}} + \frac{K_{mQ}}{K_{iQ}} \frac{c_P}{K_{mP}} + \frac{c_Q}{K_{iQ}} + \frac{c_P}{K_{mP}} \frac{c_Q}{K_{iQ}} + \frac{K_{mQ}}{K_{iQ}} \frac{c_A}{K_{iA}} \frac{c_P}{K_{mP}} + \frac{K_{mA}}{K_{iA}} \frac{c_B}{K_{mB}} \frac{c_Q}{K_{iQ}} + \frac{c_A}{K_{iA}} \frac{c_B}{K_{mB}} \frac{c_P}{K_{iP}} + \frac{c_B}{K_{iB}} \frac{c_P}{K_{mP}} \frac{c_Q}{K_{iQ}})}$$

Suppose $c_P = 0$

$$-r_A = \frac{\frac{V_1}{K_{iA} K_{mB}} c_A c_B}{1 + \frac{c_A}{K_{iA}} + \frac{K_{mA}}{K_{iA}} \frac{c_B}{K_{mB}} + \frac{c_A}{K_{iA}} \frac{c_B}{K_{mB}} + \frac{c_Q}{K_{iQ}} + \frac{K_{mA}}{K_{iA}} \frac{c_B}{K_{mB}} \frac{c_Q}{K_{iQ}}}$$

Also suppose $c_B \rightarrow \infty$

$$-r_A = \frac{\frac{V_1}{K_{iA} K_{mB}} c_A c_B}{\frac{K_{mA}}{K_{iA}} \frac{c_B}{K_{mB}} + \frac{c_A}{K_{iA}} \frac{c_B}{K_{mB}} + \frac{K_{mA}}{K_{iA}} \frac{c_B}{K_{mB}} \frac{c_Q}{K_{iQ}}} = \frac{V_1 c_A}{K_{mA} + c_A + K_{mA} \frac{c_Q}{K_{iQ}}}$$

<u>competitive inhibition</u>

$$-r_A = \frac{V_{\max} c_A / K_m}{1 + c_A / K_m + c_I / K_i}$$

→ Inhibition type follows from the mechanism (and vice versa)

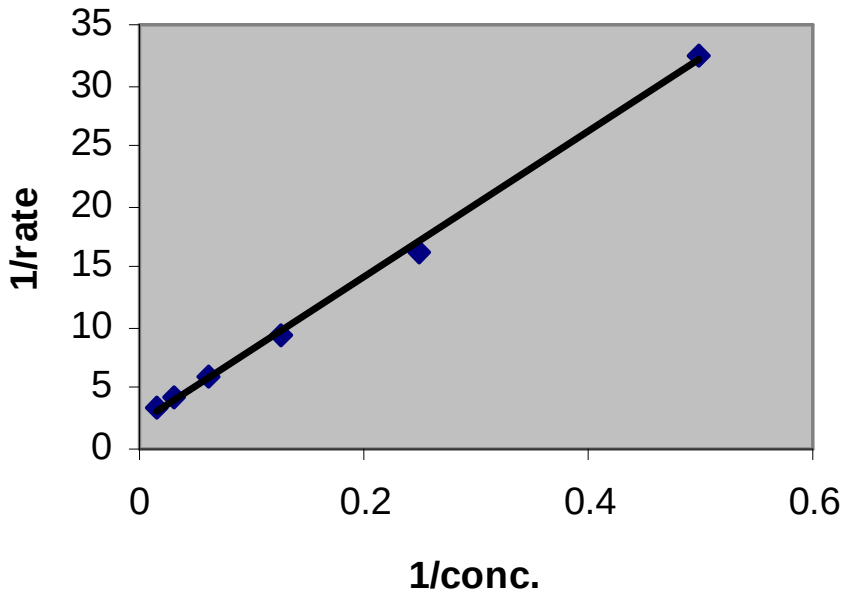
→ Mechanistic information can directly be used for process simulation

Estimation of steady-state kinetic parameters

- Simply measuring substrate consumption/product formation
- Popular on-line analyses: UV / fluorescence / pH stat
- Initial rate analysis or Progress curve analysis

Initial rate analysis

Lineweaver-Burk plot



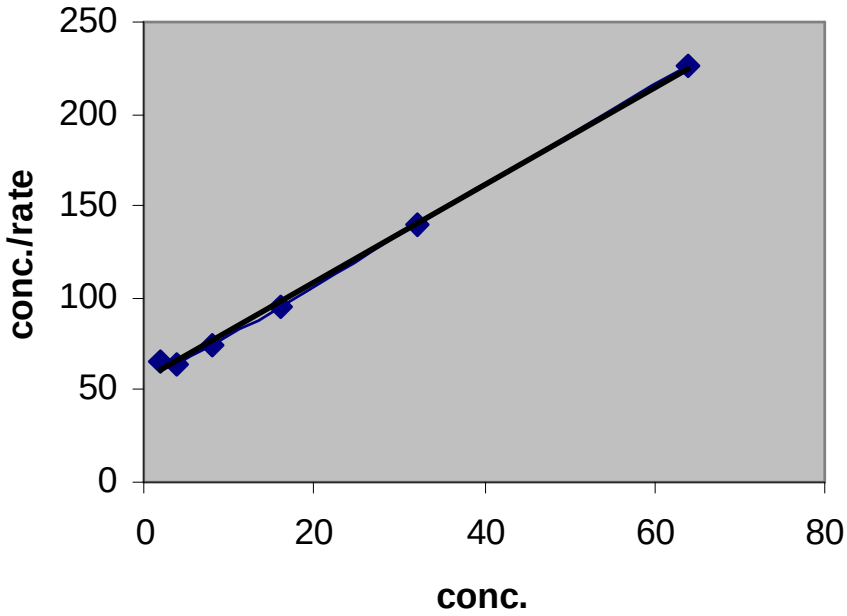
$$\frac{1}{r} = \frac{1}{V_{\max}} + \frac{K_m}{V_{\max}} \frac{1}{c_A}$$

$$V_{\max} = \frac{1}{\text{intercept}}$$

$$K_m = \text{slope} \cdot V_{\max}$$

Hanes plot

(more even error distribution)

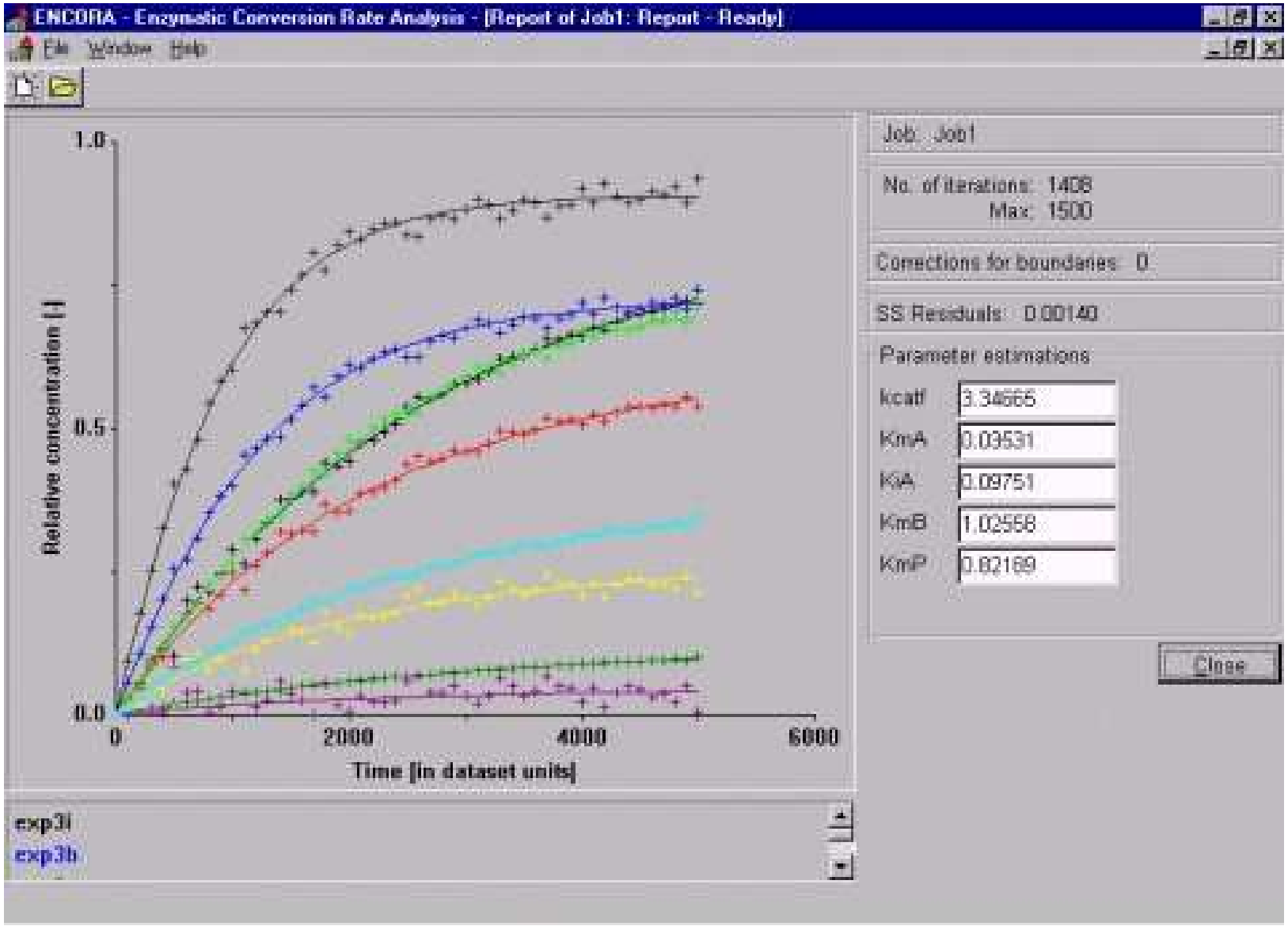


$$\frac{c_A}{r} = \frac{c_A}{V_{\max}} + \frac{K_m}{V_{\max}}$$

$$V_{\max} = \frac{1}{\text{slope}}$$

$$K_m = \text{intercept} \cdot V_{\max}$$

Progress curve analysis

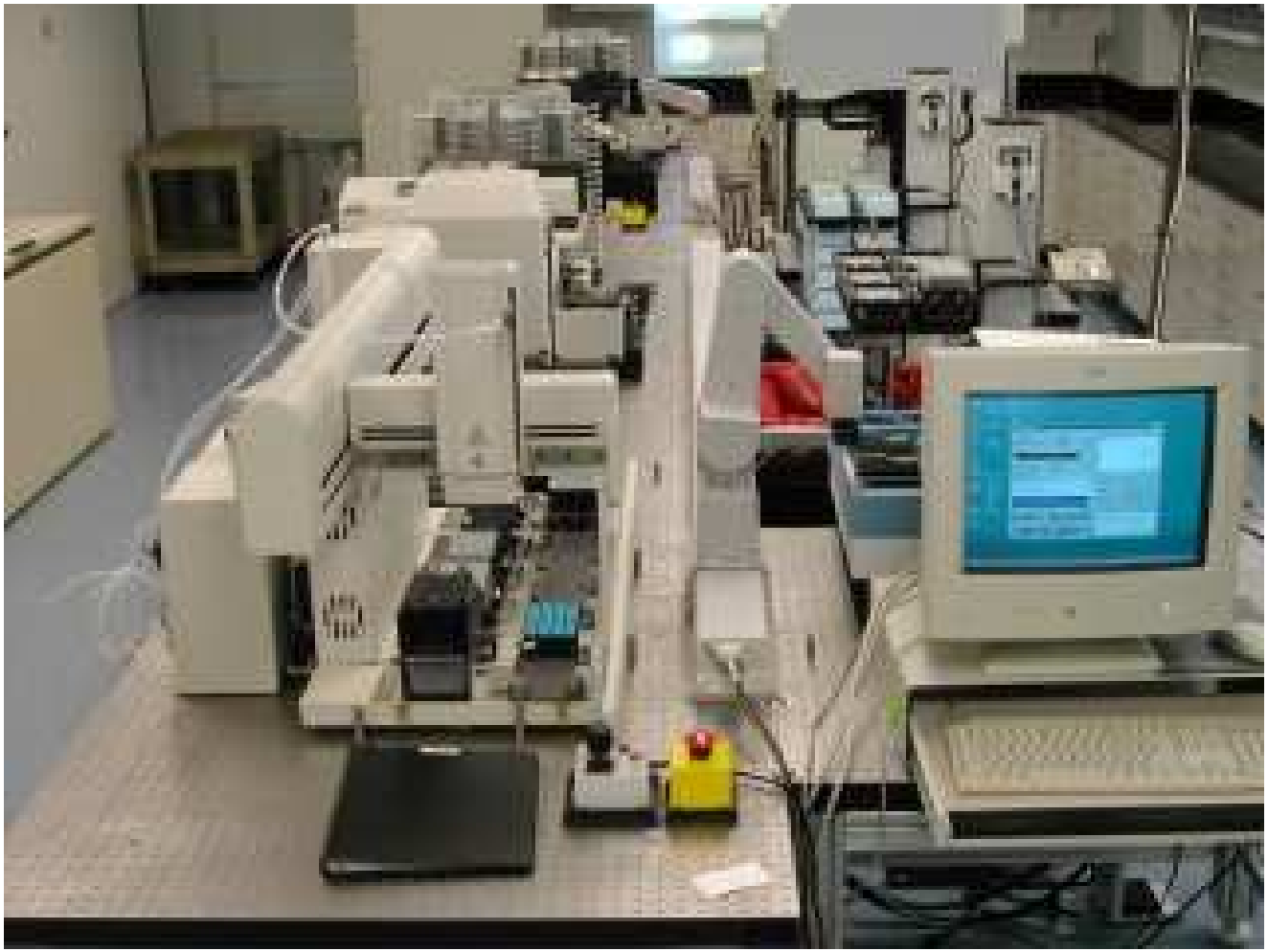


http://www.tnw.tudelft.nl/live/pagina.jsp?id=ca553f24-64c7-4d5e-a344-ce30830894ab&lang=en

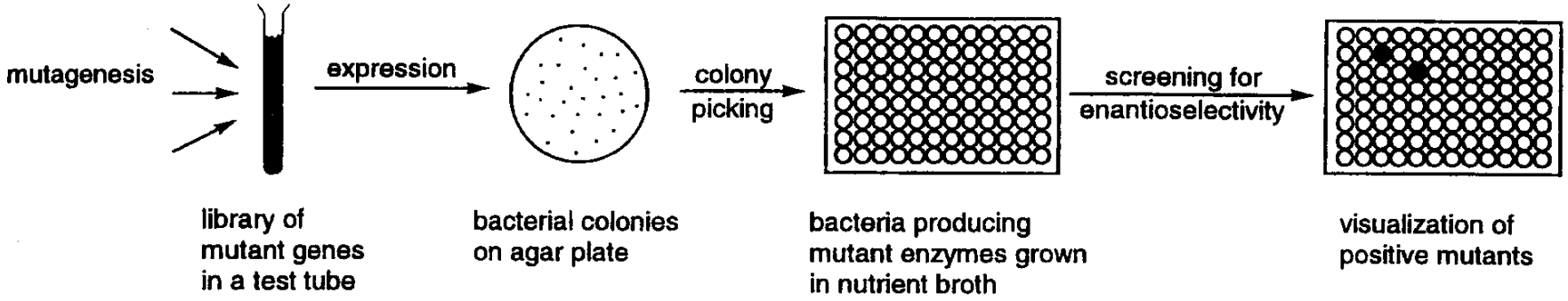
Comparision of methods

Method	Initial rate	Progression curve
# of samples	>5 up to 5-10% conversion	>20 up to full conversion
Analytical requirement	Higher sensitivity, also off-line	Lower sensitivity, only on-line
# of experiments	5 for k_{cat} and K_m; +20 per K_i	3 for k_{cat} and K_m; +3 per K_i
Product concentration	$c_P \approx c_{P0}$; $c_Q \approx c_{Q0}$	According to full rate equation
Product degradation	No	Yes
Enzyme degradation	No	Yes
Regression method	Linear possible (Lineweaver-Burk, Hanes)	Non-linear (Sophisticated software)
Calculations	Easy	Difficult
Results	Key parameters	All parameters
Application area	Basic kinetic studies	Process development

High-throughput methods



Screening for more active/selective/stable enzymes

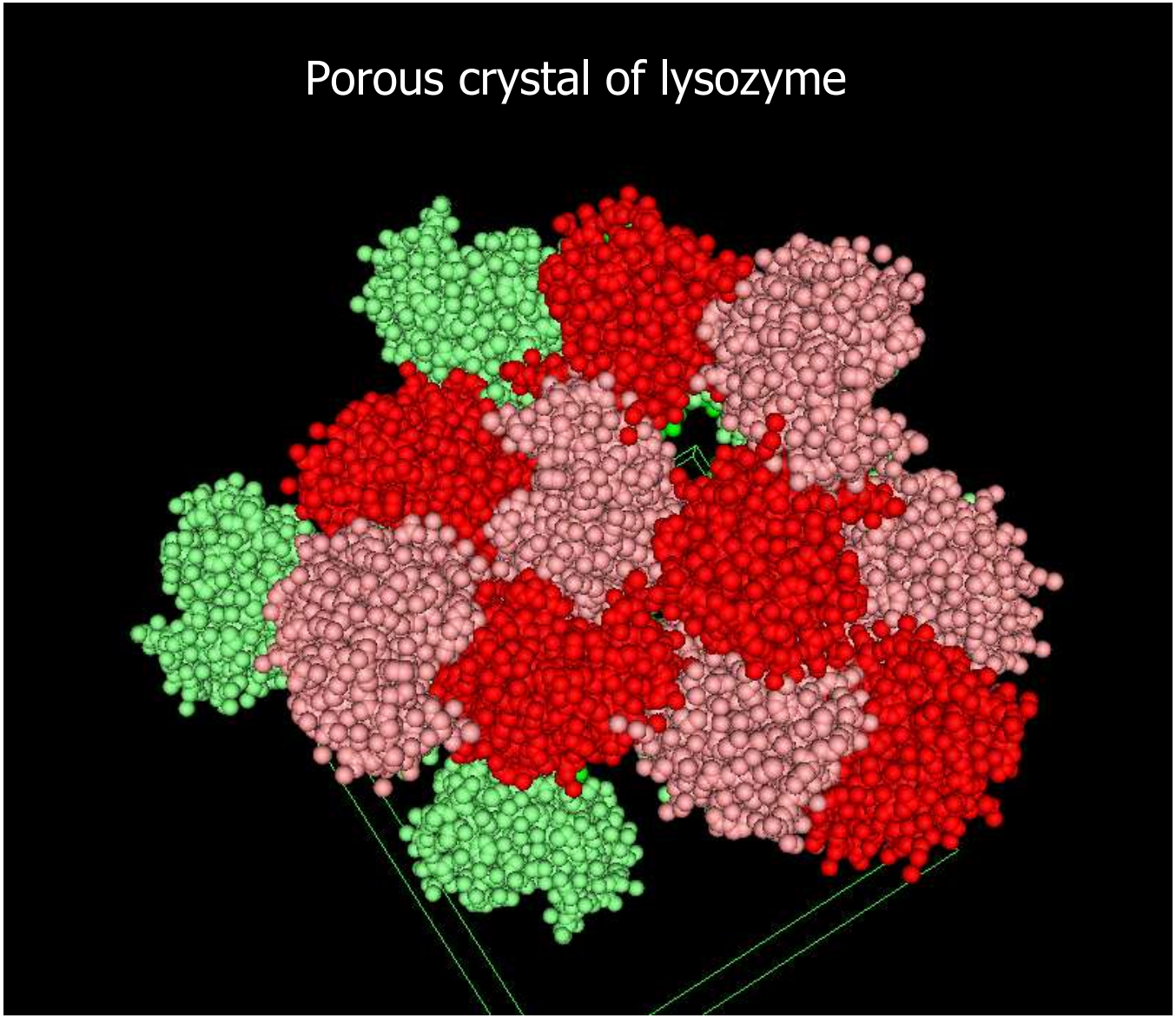


- Generation of improved enzymes has become highly sophisticated

- Kinetic assays are the usual bottleneck

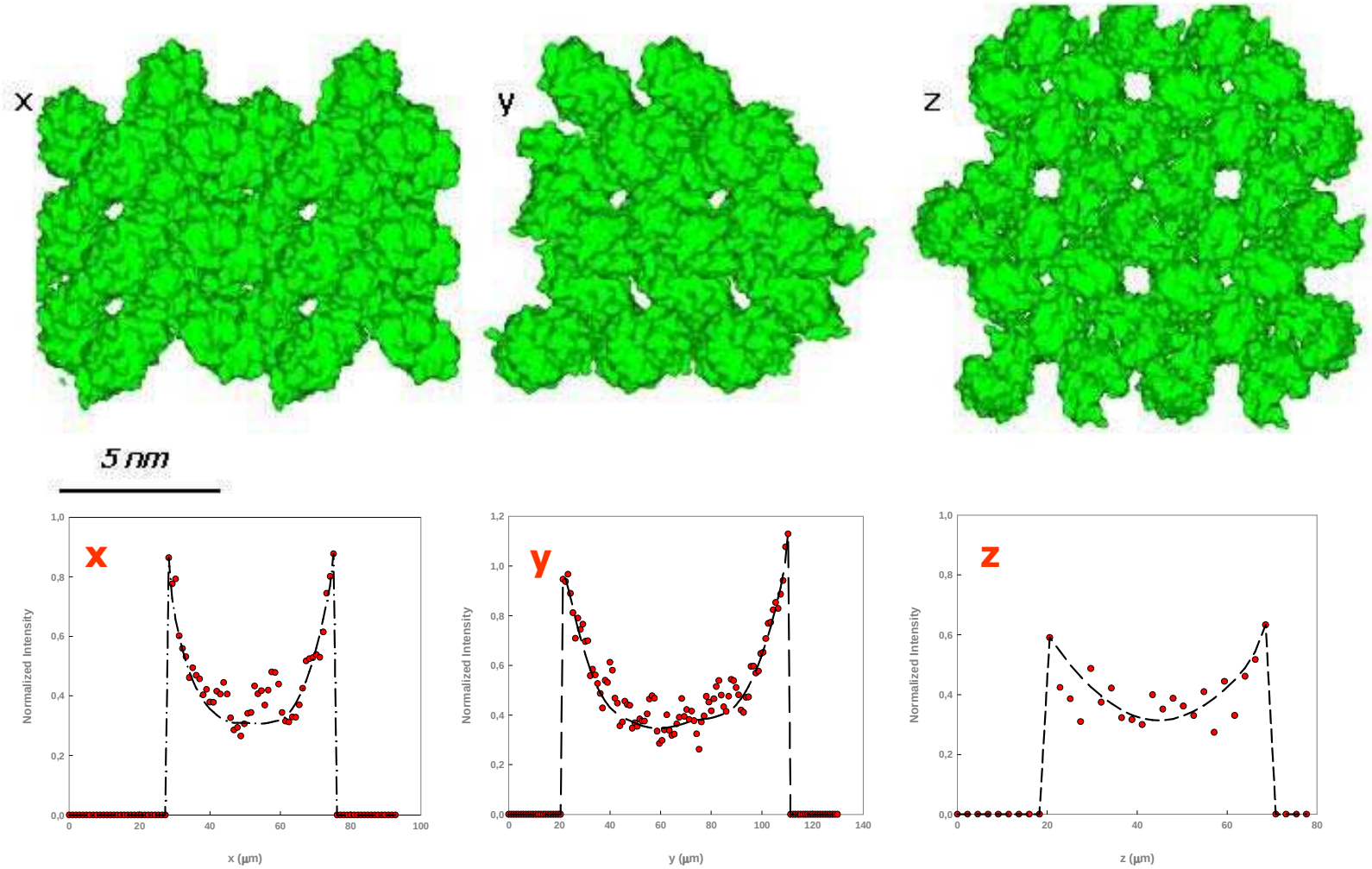
Immobilized enzymes

- Usually measurement of dissolved substrate/product; reaction-diffusion model → intrinsic kinetic parameters
- In some cases on-line measurement of adsorbed solute concentration (see next sheets)



Picture made with software from http://pymol.sourceforge.net/

Diffusion visualization by 3-D laser scanning confocal microscopy



$D_{xx} = 53\ 10^{-15}$ $D_{yy} = 79\ 10^{-15}$ $D_{zz} = 310\ 10^{-15}$ m^2/s

Cvetkovic et al., J. Am. Chem. Soc. 123 (2005) 875-879

Other issues in enzyme kinetics

- Variable temperature or pH; inactivating enzyme
- Parallel or consecutive reactions catalyzed by the same enzyme (depolymerizations; kinetic resolutions)
- Parallel or consecutive reactions catalyzed by other enzymes (lignocellulose hydrolysis)

How to get the microscopic constants?

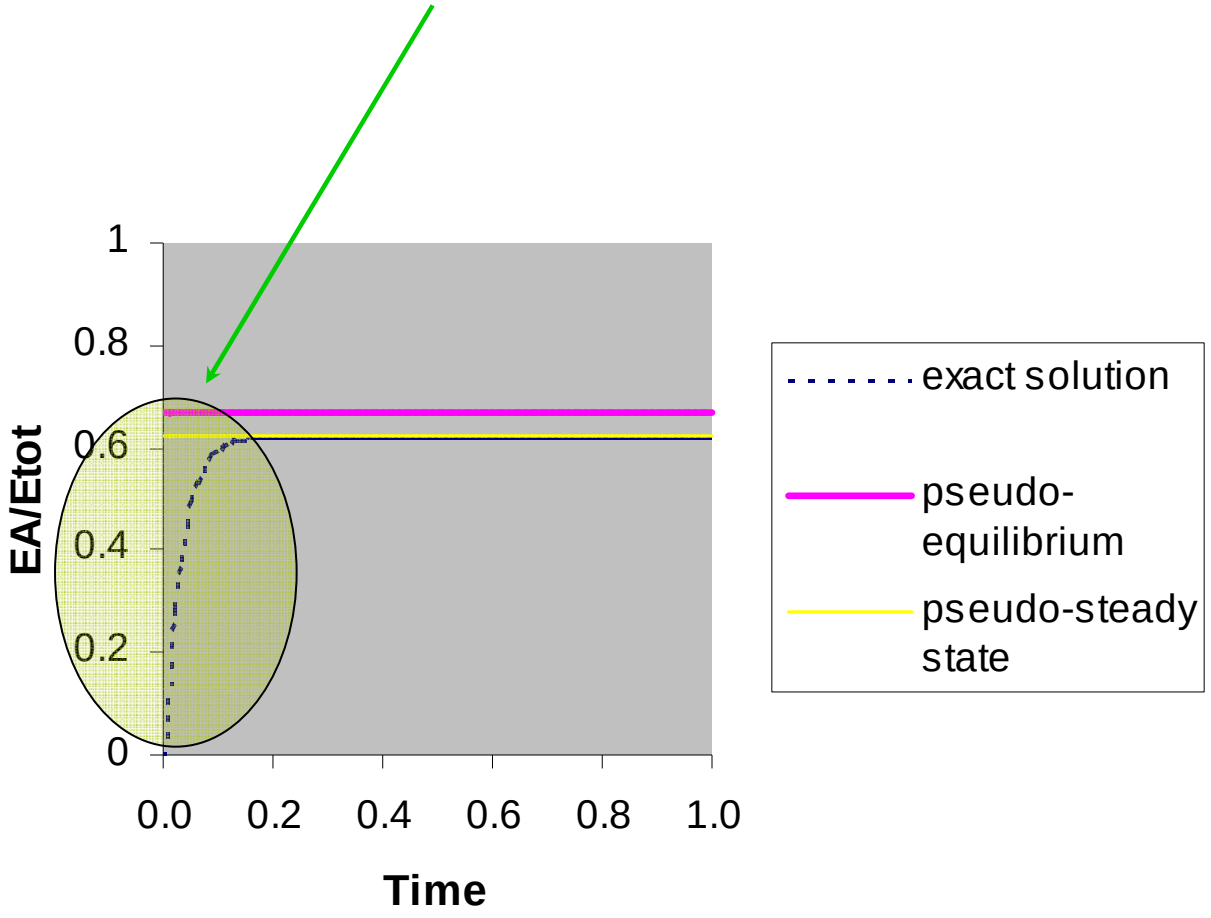
$$-r_A = \frac{\frac{k_1 k_2 k_3}{k_{-1}(k_{-2} + k_3) + k_2 k_3} c_A c_{Etot}}{1 + \frac{k_1(k_2 + k_{-2} + k_3)}{k_{-1}(k_{-2} + k_3) + k_2 k_3} c_A}$$

5 microscopic rate constants

$$-r_A = \frac{V_{\max} c_A}{1 + \frac{c_A}{K_m}}$$

2 macroscopic (steady-state) kinetic parameters

Pre-steady-state kinetics



$c_{A0} = 1$

$c_{Etot} = 0.001$

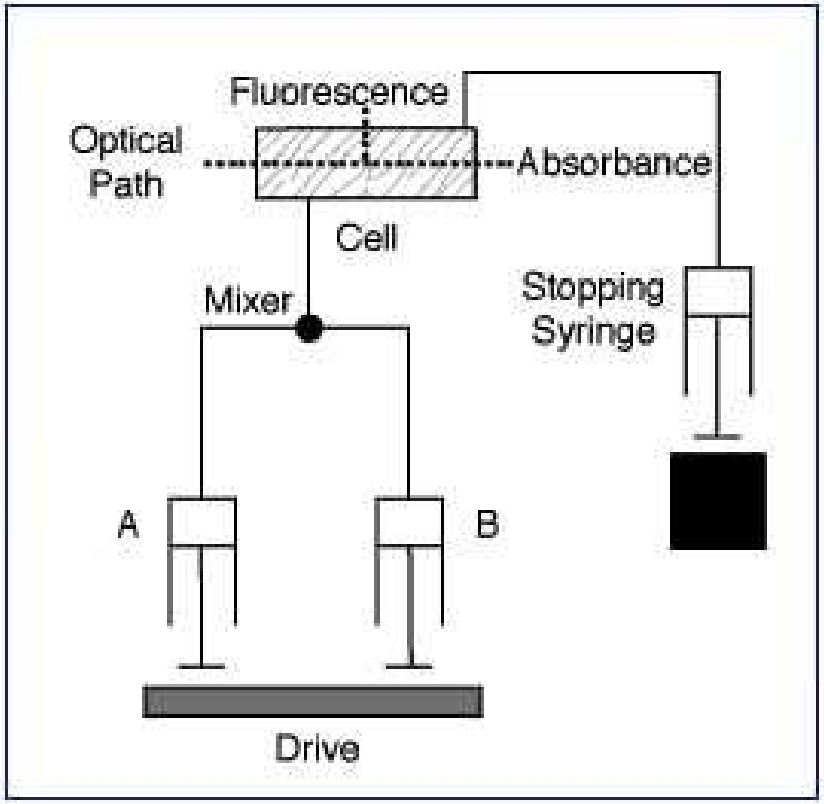
$k_1 = 20$

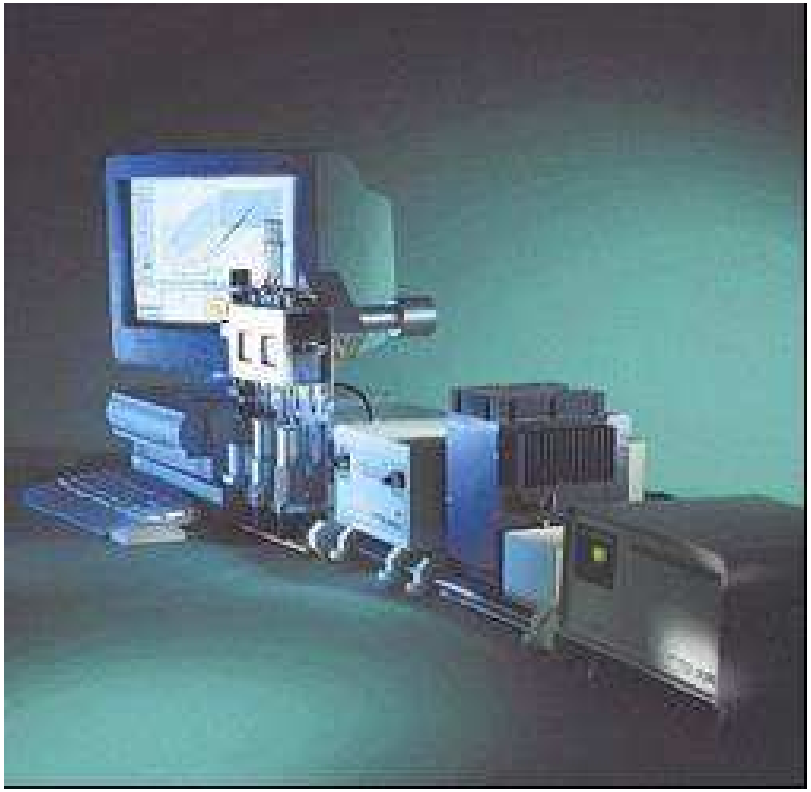
$k_{-1} = 10$

$k_2 = 2$

(arbitrary units)

Stopped flow equipment





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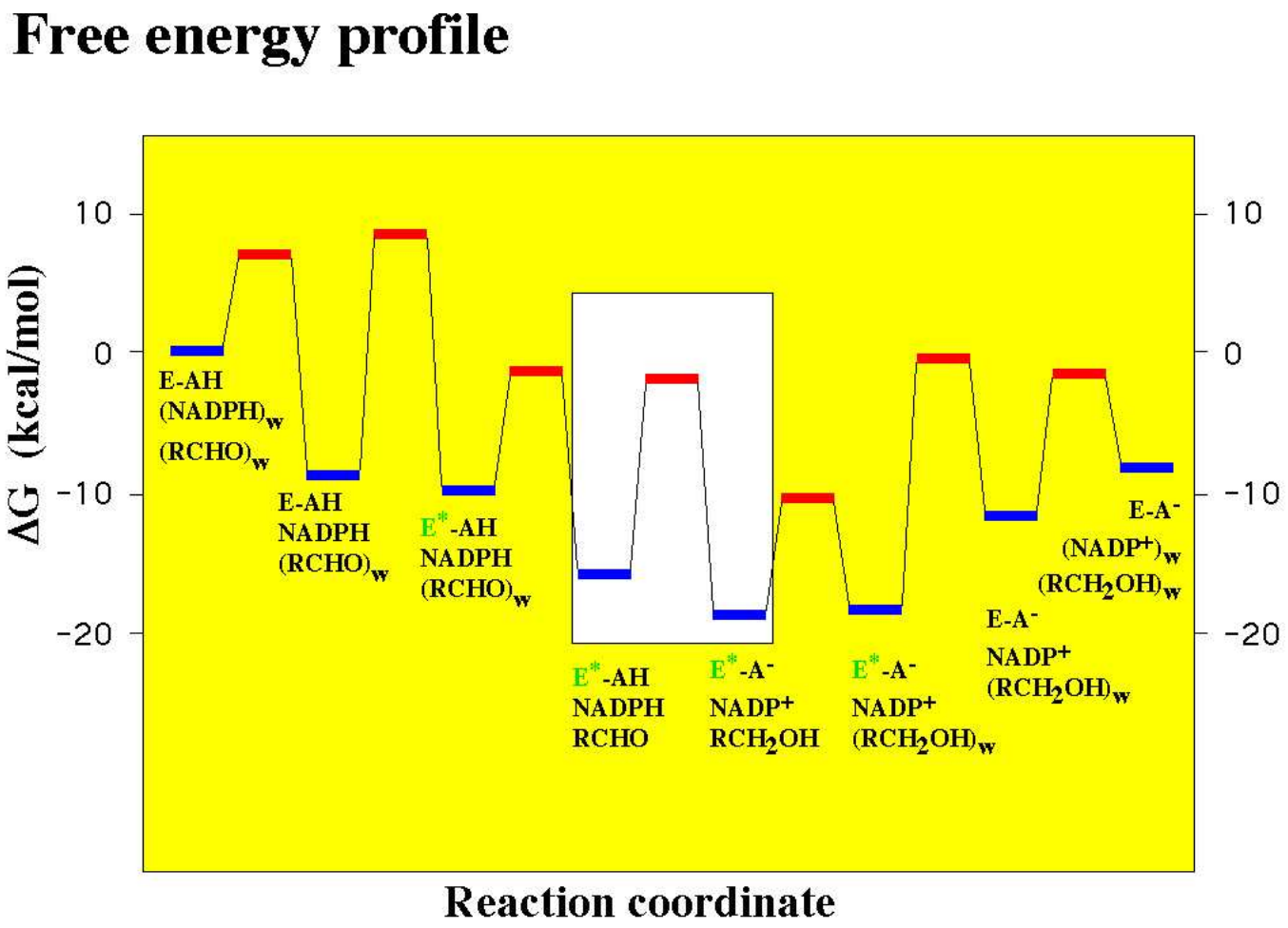
Applied Photophysics, Inc.

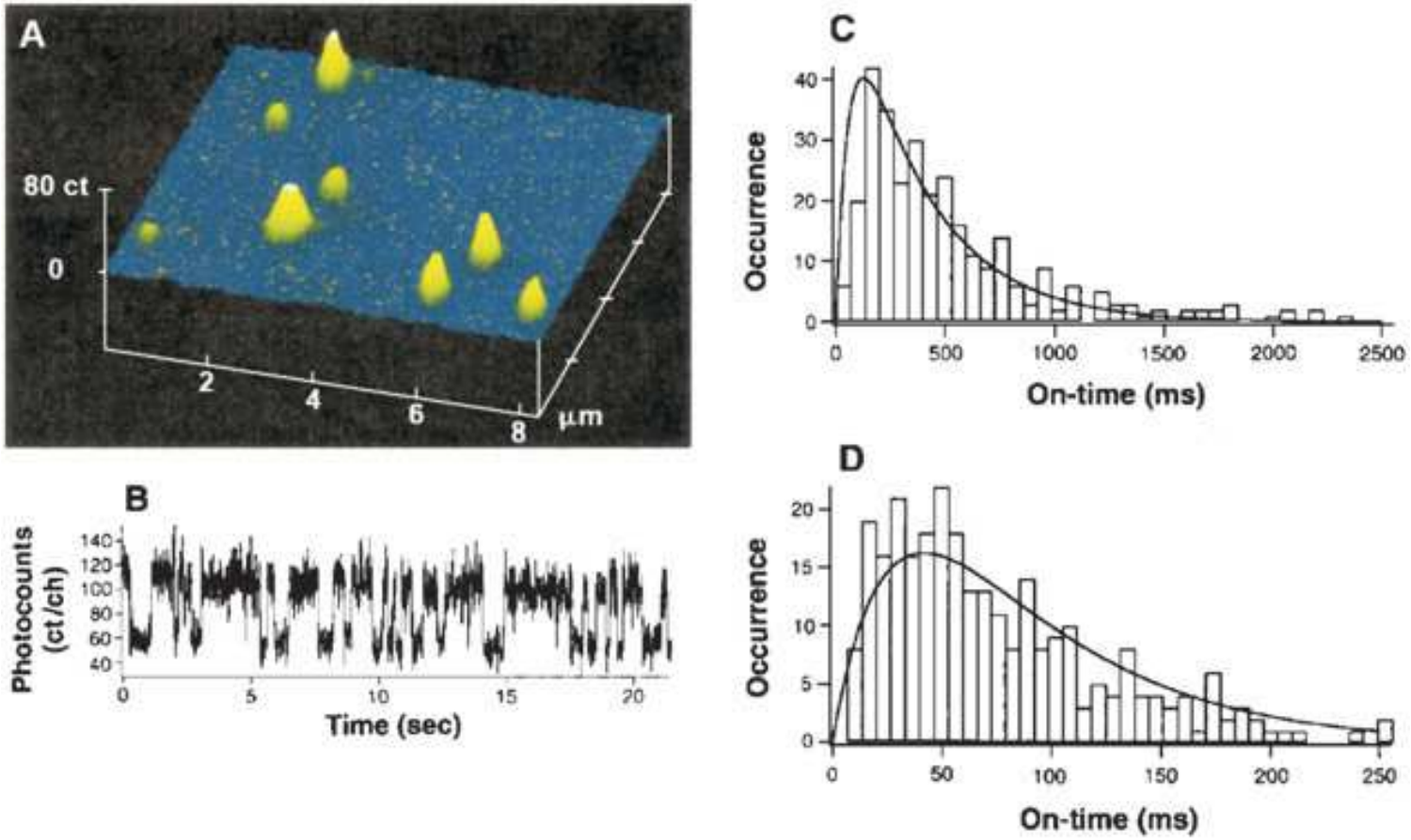
(Human) Aldose reductase



Varnai, P. and Warshel, A. (2000) *J. Am. Chem. Soc.*, 122, 3849-3860

Single enzyme kinetics

Because different enzyme molecules have different conformations



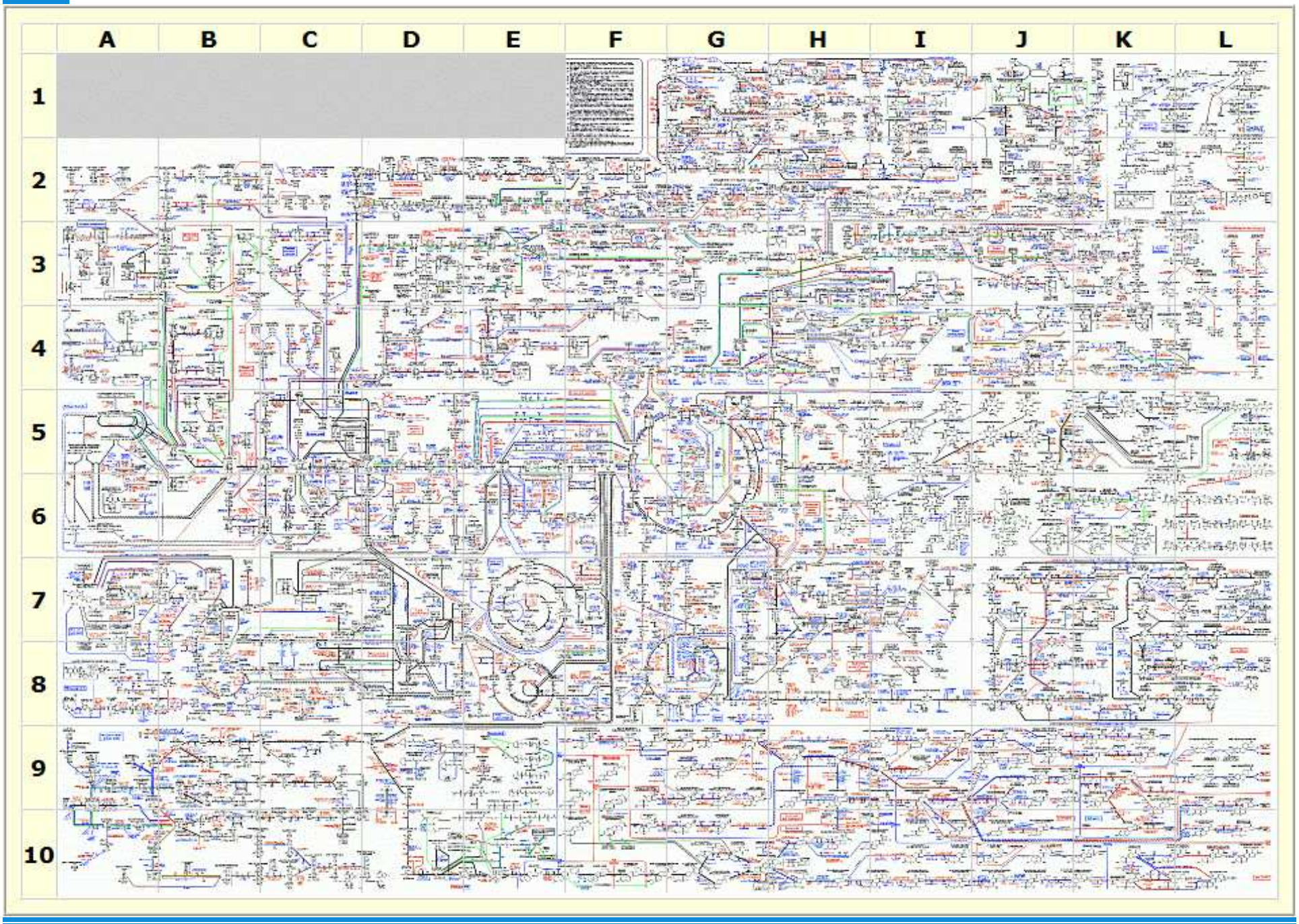
Kinetics of microbial cells

- Thousands of regulated reactions (from DNA level to metabolite level)
- Regulation leads to continuous fast changes of rates → chemostats (CSTRs) to obtain reproducible steady states
- Conventionally 5-10 overall rates externally measured (C-source, N-source, O_2, CO_2, H^+, product, cell mass, …)

→ All reactions lumped in 2-3 overall reactions (~6 kinetic parameters)

→ Unsatisfactory understanding, so empirical genetic engineering

NEW: Systems biology approach: measure and model *all* rates


A
B
C
D
E
F
G
H
I
J
K
L
1
2
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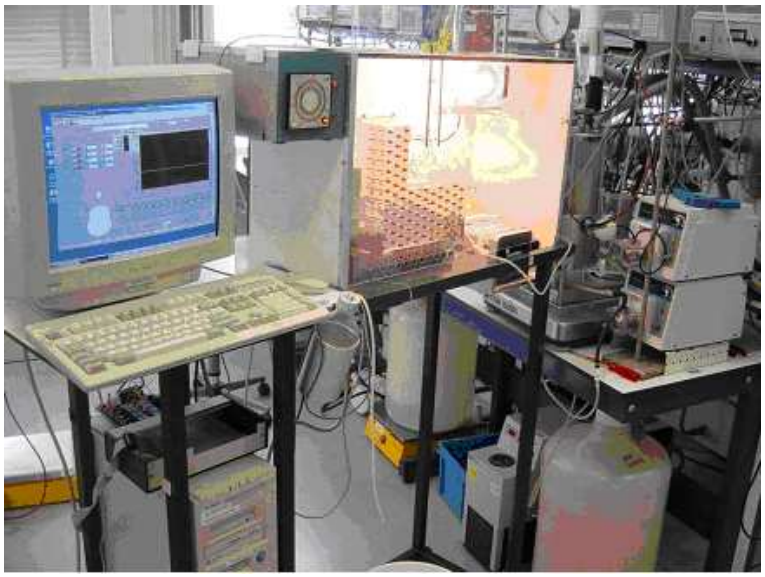
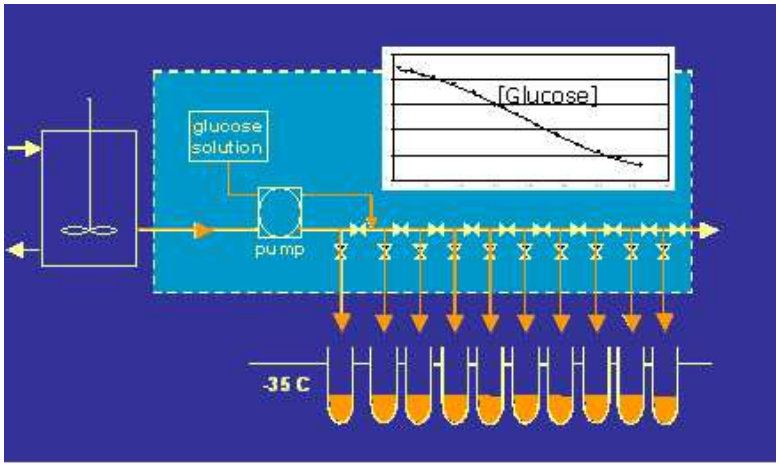
5
6
7
8
9
10

BioScope

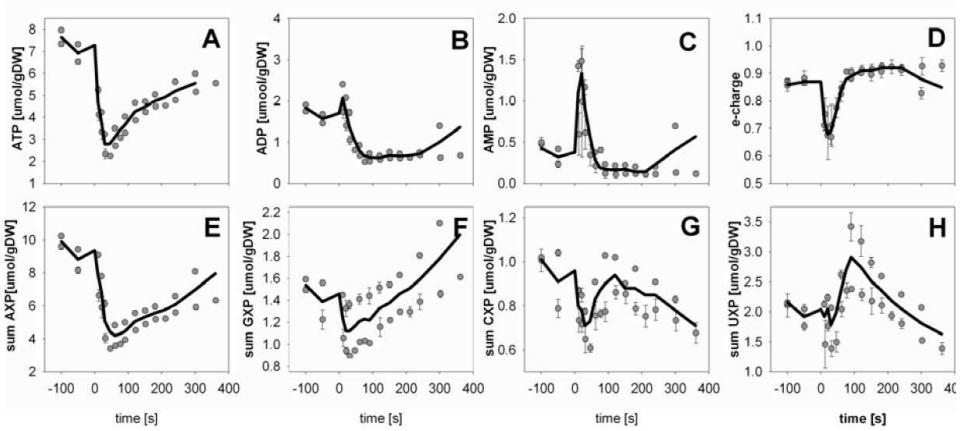
- 0-500 s stimulus-response of cells growing in a steady-state chemostat
- Rapid sampling, quenching, metabolite extraction, isotope dilution LC/MS for ~200 intracellular metabolites.
- Genome wide transcript levels are measured with Affymetrix chips
- Protein level measurements: in progress
- Dynamic simulations to model the metabolic rates (using phemenological enzyme kinetics)

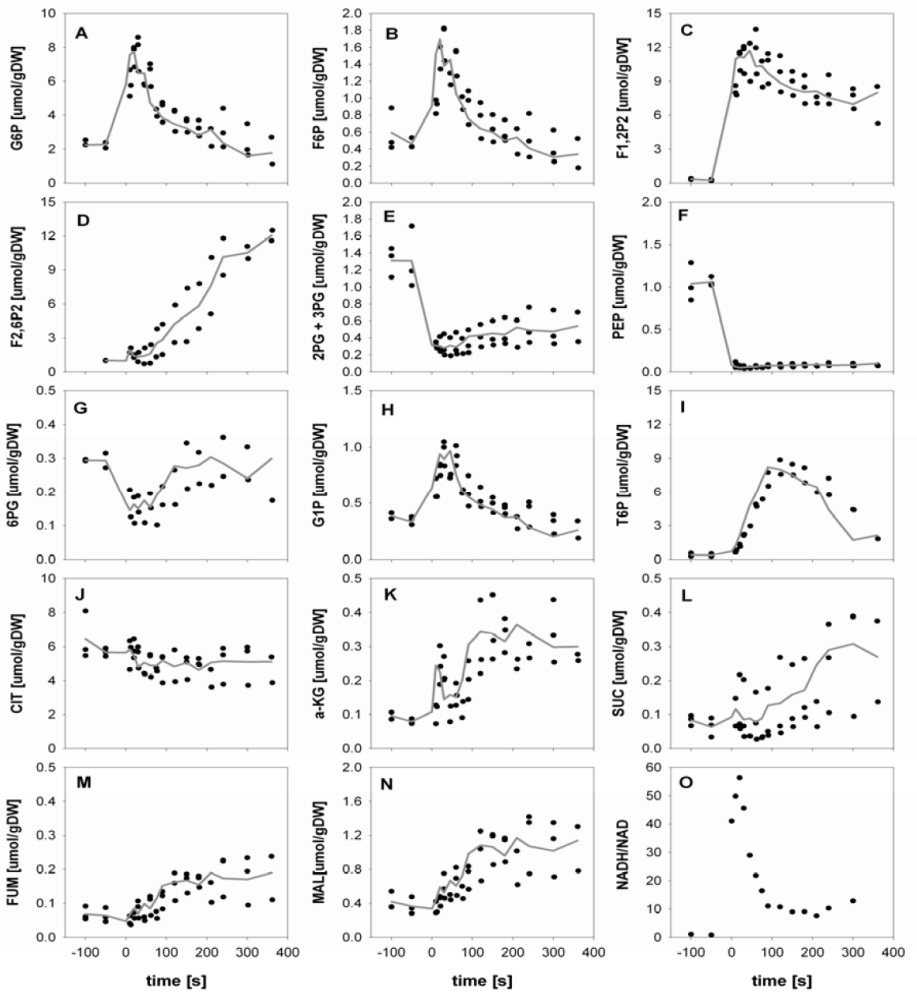
Heijnen group, Biotechnology, TU Delft





Example results 1



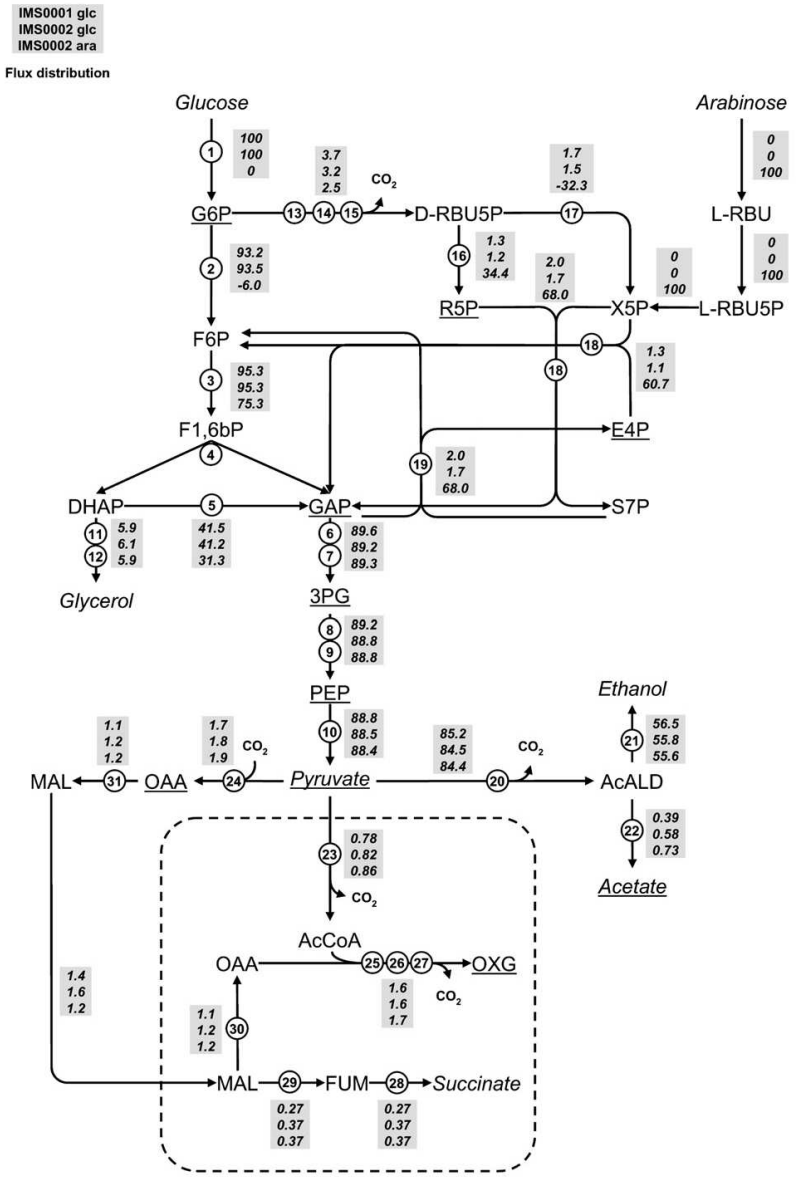


Example results 2

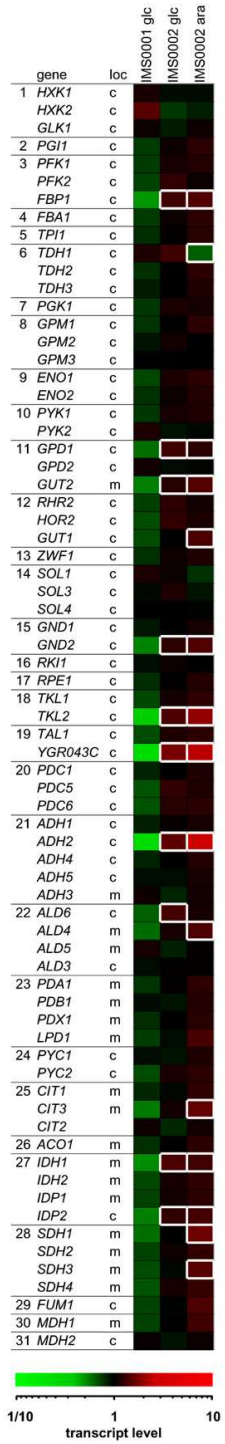
- Metabolic flow rates in ethanol fermentation
- Explains results of genetic engineering
- Provides new genetic engineering targets

Wisselink et al., Metabolic Eng. 2010

A

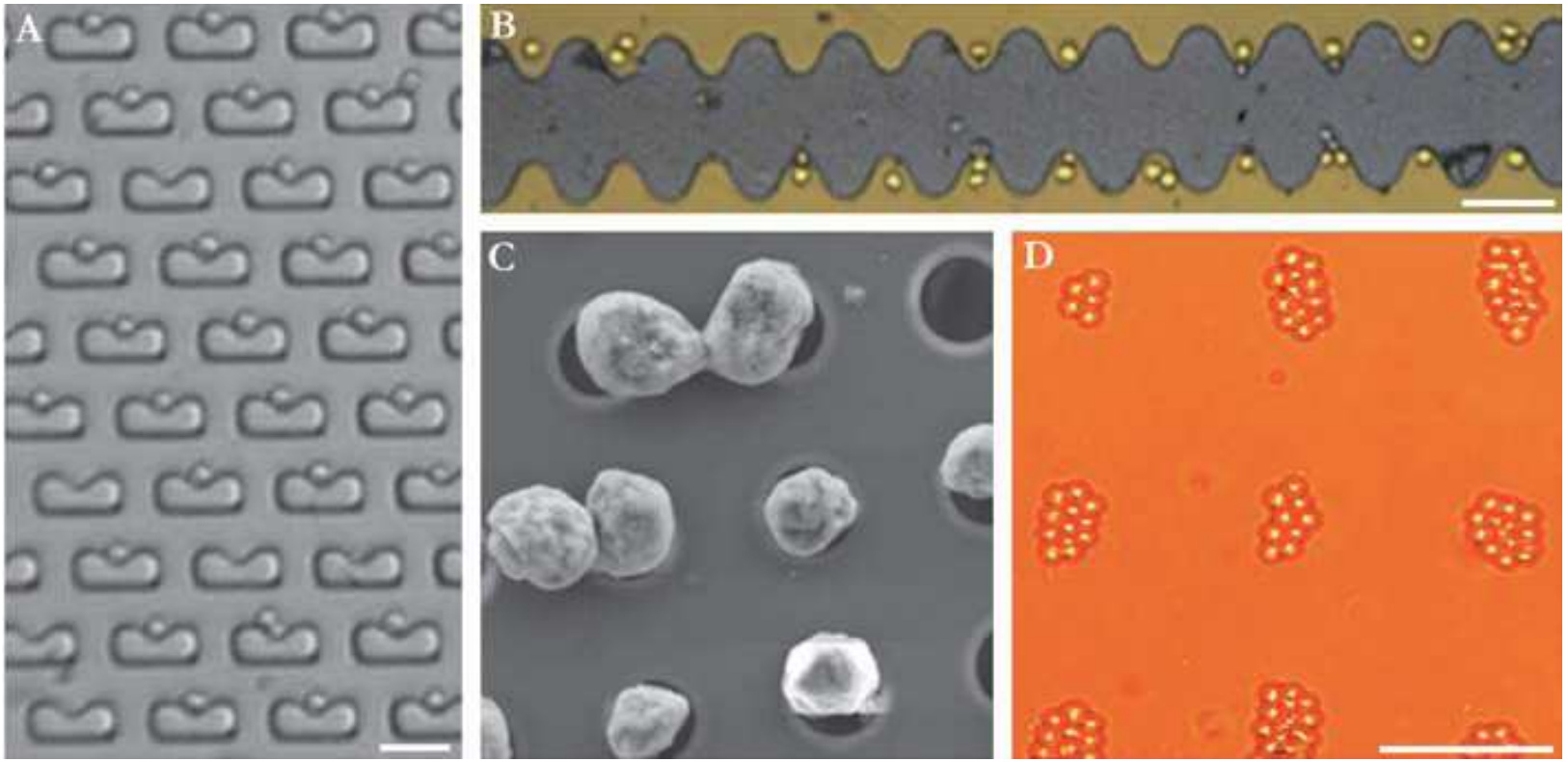


B



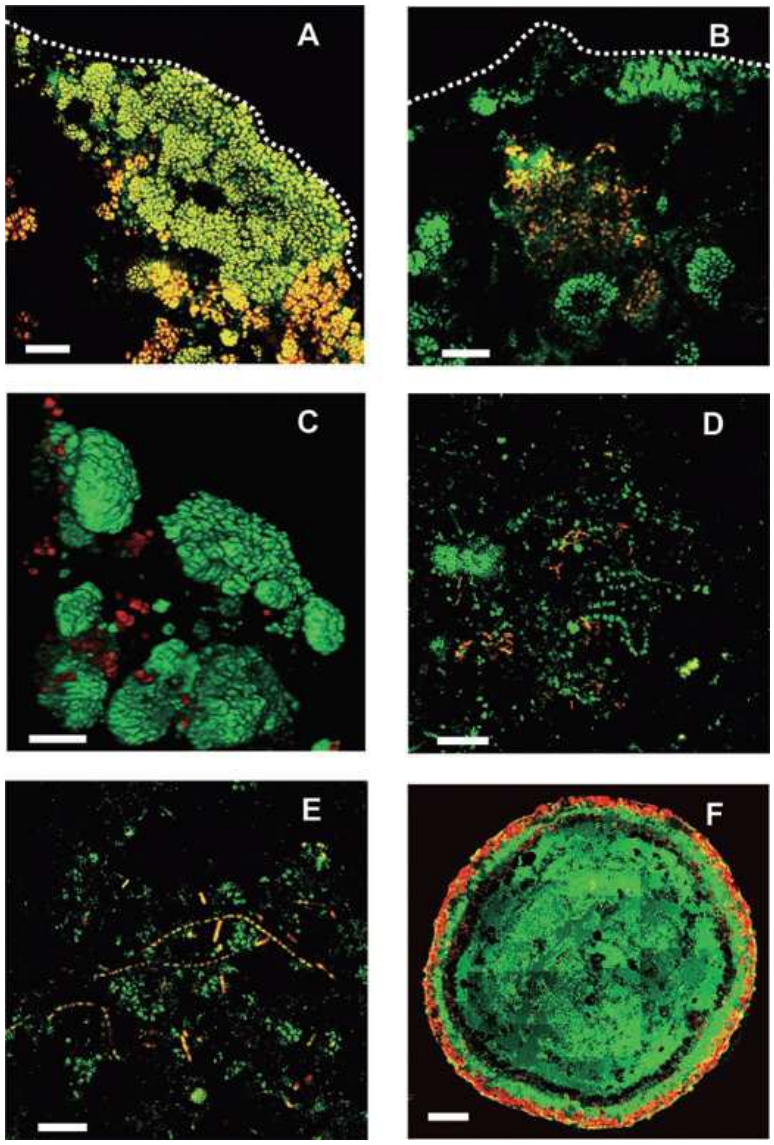
Single cell kinetics (Microfluidics)

Because different cells behave differently – especially temporally

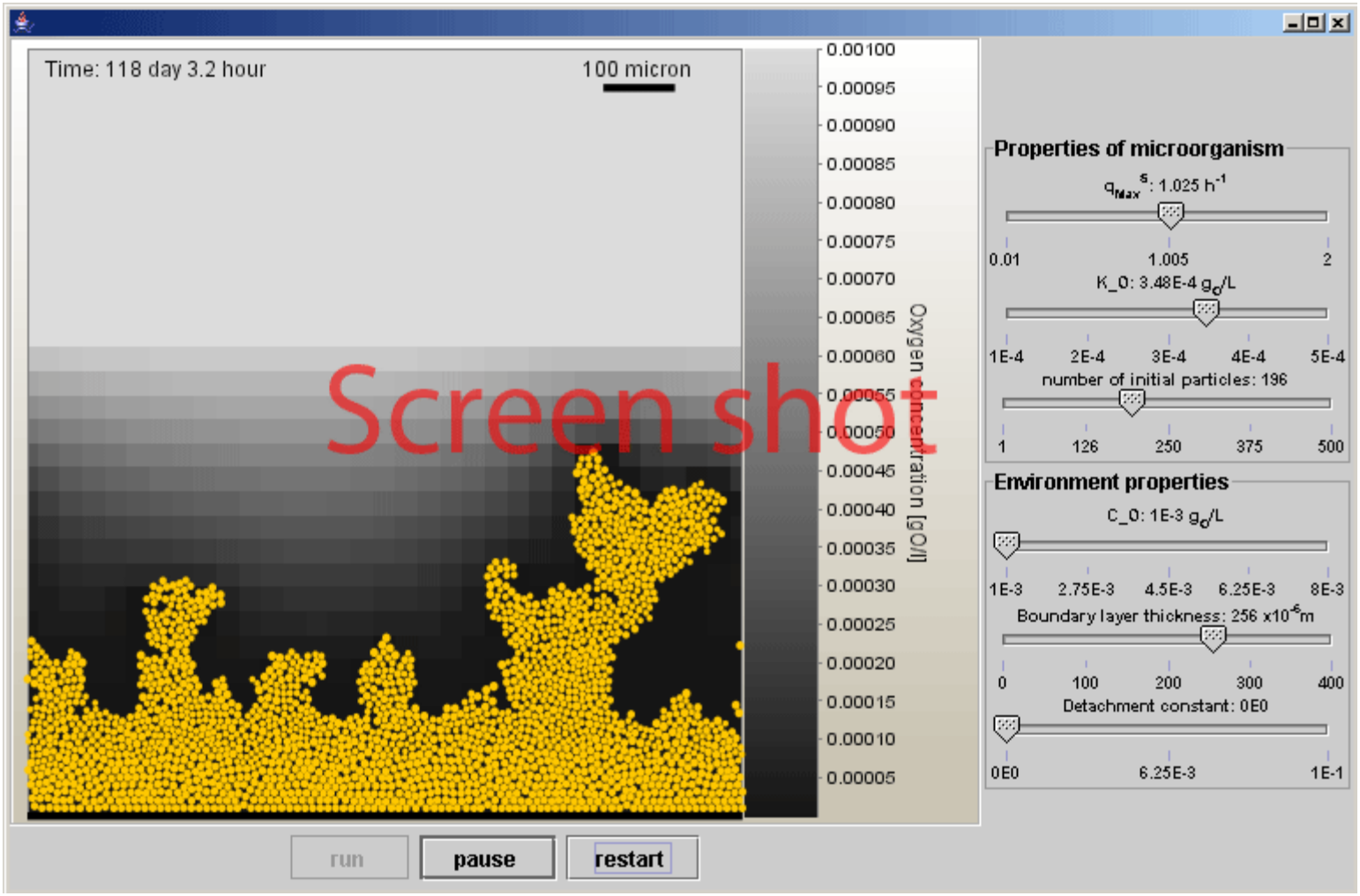


Mixed cultures

- Traditionally: Overall rates measured, black block model
- Fluorescent probes to differentiate populations of microorganisms
- Biofilms: reaction-diffusion



Monospecies 2D biofilm model



Conclusions

- Steady-state enzyme kinetics are mature; used in biochemistry just like in bioprocess development
- Pre-steady state and single enzyme methods are used for advanced enzymology
- High-throughput assays are bottlenecks in development of biocatalytic processes
- Monitoring individual cells is becoming important
- Numerous new methods to determine intracellular rates drive the field of Systems Biology