Supporting Information - A Bayesian approach to understanding artificial enzymatic networks

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Materials and Instrumentation

Materials

All chemicals and reagents were used as received from commercial suppliers without any further treatment unless stated otherwise.

Enzymes

The enzymes immobilized on PEBs were purchased from Merck Sigma-Aldrich. Specifically:

- Lyophilized Trypsin (Tr) from bovine pancreas (product no. T1426)
- Lyophilized Glucose Dehydrogenase (GDH) from Pseudomonas sp. (product no. 19359)
- Lyophilized Hexokinase, type F-300 (HK) from Saccharomyces cerevisiae (product no. H4502)
- Lyophilized Glucose-6-phosphate dehydrogenase (G6P-DH) from Leuconostoc menesteroides (product no. G8529)

Trypsin reactions was conducted in a 200 mM Tris buffer, pH 7.8, with 20 mM of CaCl2. GDH, HK and G6P-DH reactions were conducted in a 200 mM Tris buffer, pH 7.8, with 10 mM of MgCl2.

Substrates

The substrates Cbz-Arg-AMC (R-AMC, CAS: 3701-04-6, New catnr: 4002540.0050) and Suc-Ala-Ala-Ala-AMC (AAA-AMC, CAS 73617-90-0, New catnr: 4006305.0050) were purchased from BioConnect B.V. and were dissolved as 150 mM stock in anhydrous DMSO and kept frozen at -20°C.

The substrates employed for all metabolic enzymatic reactions were: Glucose (anhydrous), glucose-6-phosphate hydrate , ATP disodium salt, ADP monosodium salt, NAD+ , NADH disodium salt, NADP+disodium salt and NADPH tetrasodium salt.

Instrumentation & Quantification protocols

Flow setup
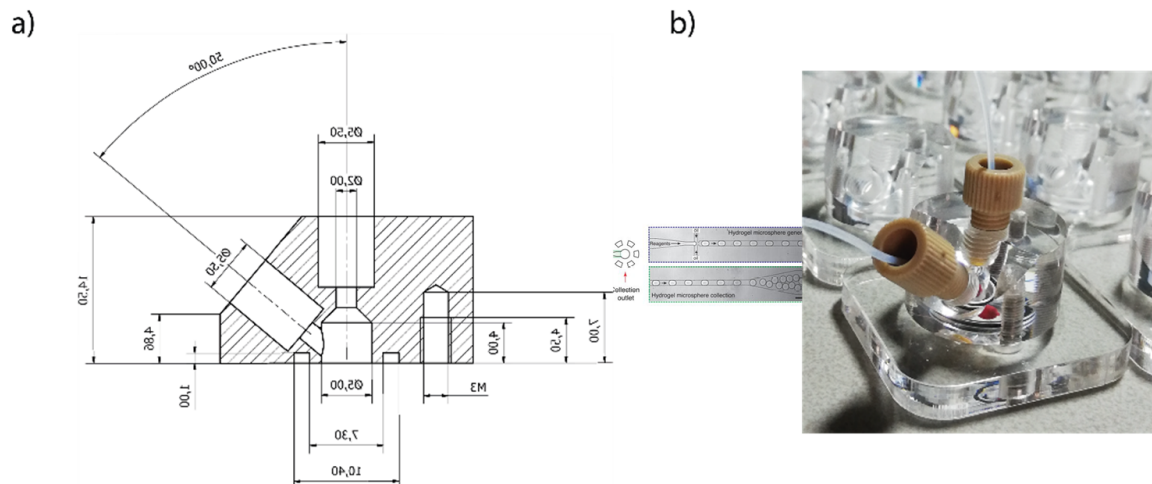
Figure 1: a) Schematic overview of the CSTR. b) Photograph of the CSTR

A custom made CSTR (see Figure 1 for the design schematic) was charged with PEBs (in a ratio of 1:31 mg beads:injection volume). The openings of the reactor were sealed with Whatman Nuclepore polycarbonate membranes (5 μm poresize) to prevent outflow of PEBs To subject the CSTR to various flow conditions we used Cetoni Low-Pressure High-Precision Syringe Pumps neMESYS 290N and gastight Hamilton syringes,

Figure 2: a) Overview of the experimental setup, with left the fraction collection, lower center the CSTR on a stirring plate, and lower right th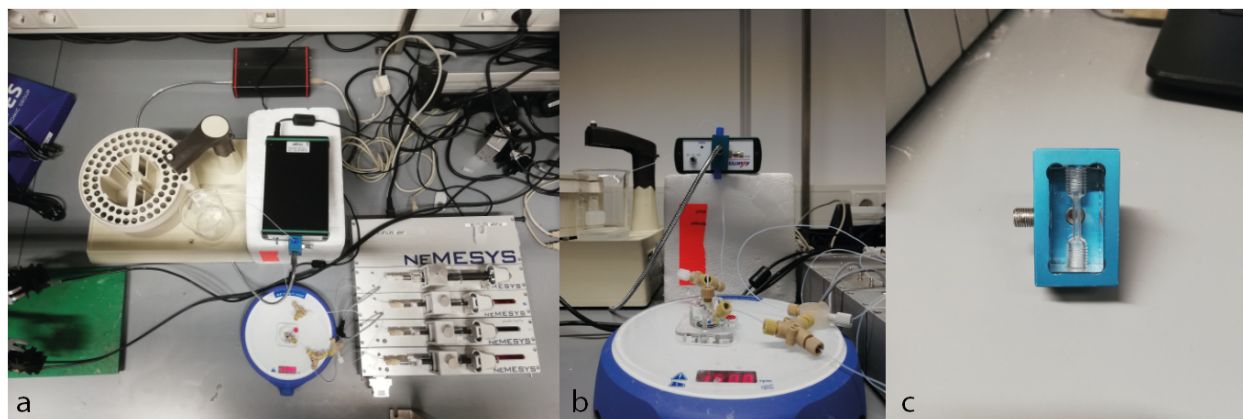e neMESYS pump system. b) Close-up of the CSTR connected to input and output tubing. c) Close-up of the flow cuvette used for online absorbance detection, provided by LabM8

with all flowrates programmed using the Cetoni neMESYS software. For all methods of offline detection, the outflow of the CSTR was connected to a BioRad 2210 Fraction collector, collecting for 7.5 (absorbance) or 10 minutes (HPLC) per fraction. This experimental setup is shown in Figure 2a-b

Spectroscopy

Offline fluorescence   Collected fractions were pipetted onto a microplate (Greiner Bio One, transparent, polysterene, 96 flat bottom chiney wells) at 60 μL per well. Fluorescence measurements for enzyme activity determination were performed with a Tecan Spark 10M plate reader. The fluorescence intensity of wells containing 30-200 μL of the reaction mixture was monitored for 1-4 min (shaking 3s/orbital mode/amplitude 4mm) at 23 °C using top or bottom reading mode, at $\lambda_{ex}/\lambda_{e}m = 380\text{nm}/460\text{nm}$ for 7-amino-4-methylcoumarin (AMC) based substrates.

Offline absorbance   Collected fractions were pipetted onto a microplate (Greiner Bio One, transparent, polysterene, 96 flat bottom chiney wells) at 60 μL per well. The absorbance of each sample at 340 nm was measured in a TECAN SPARK M10 platereader. Using a calibration curve, the absorbance data was converted to NADH concentration.

Online absorbance   Absorbance in flow experiments was continuously measured at the reactor's output with a custom made flow cell kindly provided to us by LabM8 (shown in Figure 2c), connected to an AvaLight 355 nm LED lamp. Absorbance at 380 nm was detected using an AvaSpec-2048 with 1,005 ms integration time and averaging at 200 times per recorded datapoint. Using a calibration curve, the absorbance data was converted to NADH concentration.

High-performance liquid chromatography

High-performance Liquid Chromatography (HPLC) was performed using Shimadzu NexeraX3/Prominence system under a 0.9 mL/min flow at 45 °C with a Shimadzu WAX-1 column. For the Shimadzu system, an injection volume was subjected to a 25 min gradient program was used starting from 20 mM of potassium phosphate at pH 7.0

- 1 min – 20 mM at pH 7.0
- 16 min – 480 mM at pH 6.8
- 19 min – 480 mM at pH 6.8
- 22 min – 20 mM at pH 7.0
- 25 min – 20mM at pH 7.0

Retention time of ADP is at 8.8 minutes, ATP at 15.5 minutes.

G6P-DH assay

Collected fractions were pipetted onto a microplate (Greiner Bio One, transparent, polysterene, 96 flat bottom chiney wells) at 60 μL per well. To these wells was added an 20 μL of 5 mM of NADP+ and 1 μL of 500 u/mL of G6P-DH. The absorbance of each sample at 340 nm was continuously measured in a TECAN SPARK M10 platereader. Using a calibration curve, the absorbance data was converted to NADPH concentration. We assumed this NADPH concentration equivalent to G6P concentration.

Software

Python scripts and Jupyter notebooks were used to create the Bayesian models and perform inference and predictive sampling. Example notebooks with explanation (`example_simple.ipynb` and `example_complex.ipynb`), and notebooks used for creating the figures in the publication (`fig_*.ipynb`) can be found on the accompanying github repository. This repository also includes version information for all software dependencies. PDF reproductions of the example notebooks are also included in this supporting information.

To determine likelihoods of partially observed enzyme networks, a custom Theano operator was written to calculate likelihoods from steady-state conditions. This SteadyStateOperator can be found in the BayERN Python package, alongside documentation, and is included in the dependencies of the manuscript github repository.

Production and characterisation of polyacrylamide-enzyme beads

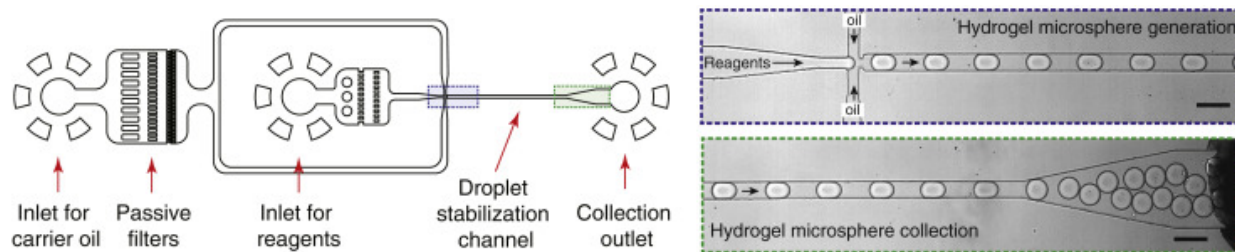## Microfluidic devices fabrication for beads production

Figure 3: Schematic of the microfluidic device for droplets production (*adapted from[1]*)

Microfluidic devices, designed according to the scheme shown in Figure 3[1], were produced in silicon wafers by photo and soft lithography, with different orifice sizes (20-80μm) at the T-junction, for droplet formation. After that, the wafers were used for the production of polydimethylsiloxane (PDMS) devices. The PDMS replicas were separated from the wafer, and inlets and outlets of 1mm inner diameter were punched. After the replica was bonded by oxygen plasma treatment to a glass slide, channels were coated with 2% 1H,1H,2H,2H-Perfluoro-octlytriethoxysilane to make them hydrophobic[2].

Method 1: Enzyme-first functionalization

This method was used for the synthesis of PEBs containing Trypsin.

Synthesis 6-acrylaminohexanoic acid succinate (AAH-Suc) linker

N-6-acryloyl amido hexanoic acid (30 mg, 0.172 mmol, 1 eq) and N-Hydroxy succinimide (20mg, 0.172 mmol, 1eq) were added in a large glass vial and brought under N2 atmosphere. Subsequently, they were dissolved in 1 mL of anhydrous DMF and cooled down to 0°C. Then, N, N'-dicyclohexylcarbodiimide (DCC, 38mg, 0.172mmol, 1eq) was added, and the vial was sealed and left to stir at 0°C for one hour. Subsequently, the reaction was brought to 4°C and stirred for 16h. The reaction mixture was assumed quantitative and immediately used for enzyme functionalisation without further analysis[3].

Enzyme functionalization

The desired enzyme (1 eq) was added to a falcon tube containing 0.1 M NaHCO3 (4mL), followed by addition of AAH-Suc (7.5 eq) in DMF. The mixture was left to stir at 21 °C for 1 h, after which the reaction mixture was dialysed and lyophilised.

General Enzyme Immobilization During Polymerization (EIDP) method

An emulsion of monodisperse water-in-oil droplets was produced by using the microfluidic device described above with a 20 μm-wide T-junction, following an adapted procedure from Rivello et al.[3] The reagents phase used for hydrogel formation was composed by 9.7% (w/v) acrylamide, 0.4% (w/v) bisacrylamide, 1.5% (w/v) 2,2'Azobis(2-methylpropionamidine) dihydrochloride (AAPH) and 0-8% (v/v) of a solution of functionalized enzyme (100 μM). The oil phase was composed of fluorinated fluid HFE-7500 (3M) and 1.5% (v/v) Pico-Surf™ 1. The flow rates used to produce monodisperse beads of 50μm average diameter were 600$\mu L/h$ (Qw) for the reagents phase and 900$\mu L/h$ for the oil phase (Qo). The emulsion of droplets created in the microfluidic device was collected in an Eppendorf with 100 uL mineral oil to avoid evaporation and breaking the emulsion and polymerised for 10 minutes under UV light. After polymerisation, beads were washed three times with 20% (v/v) 1H,1H,2H,2H perfluorooctanoic (PFO) in HFE-7500 oil to break the emulsion. The obtained beads were then washed three times with 1% (v/v) Span 80 in heptane, three times with 0.1% (v/v) Triton X-100 in miliQ and three times with Milli-Q. After every washing step, the beads were

centrifugated 30 seconds at 5000 rcf, and the supernatant was removed by pipetting. The resulting beads were freeze-dried and re-dissolved in miliQ at a concentration of 0.0322mg/μL. Bead size was obtained after freeze-drying by imaging with a light microscope with a 40x objective lens. The average bead size was 50 μm.

Method 2:

This method was used for the synthesis of PEBs containing GDH, HK, and G6PDH.

Empty bead production method

The microfluidics device described above was used to produce gel beads with a 20 μm wide T-junction, following an adapted procedure from[2]. The gel solution phase consisted of 9.6% (w/v) acrylamide, 0.4% (w/v) N,N'-methylenebisacrylamide, 0.5% (w/v) acrylic acid and 1.5% (w/v) 2,2'Azobis(2-methylpropionamidine) dihydrochloride. The oil phase contained 1.5% (v/v) Pico-Surf™ 1 in fluorinated fluid HFE-7500 (3M). The flow rates for gel phase and oil phase were 600 $\mu L/h$ and 900 $\mu L/h$, respectively. The outflow emulsion was collected in the tube which was filled with 100 μL mineral oil. Afterwards beads were polymerised using UV lamp for 10 minutes at 70% gain. After polymerisation the lowest layer containing fluorocarbon phase was carefully removed with a P200 pipette. The remaining beads were washed 3 times with 20% (v/v) 1H,1H,2H,2H-Perfluoro-1-octanol in HFE-7500 (3M), then 3 times with 1% (v/v) Span 80 in hexane, 3 times with 0.1% (v/v) Triton X-100 in Milli-Q and finally 3 times with Milli-Q. Every washing step was finalised with mixing the tube using vortex, centrifuging at 5000 x g for 3 min and removing the layer which was not containing beads. Furthermore, beads were flash frozen using nitrogen and freeze dried overnight. After re-wetting beads, their size was determined using light microscope with 40x magnitude objective. The average size was 50 μm in diameter.

General Enzyme Immobilisation after Polymerisation (EIAP) Method

Empty acrylamide beads were re-dissolved in Milli-Q at a concentration of 0.0322 mg/μL. 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (100 mM) and N-Hydroxysuccinimide (100 mM) were added to the reaction mixture. The total volume of the activation solution was 5-fold of the beads volume. The reaction mixture tube was put on the roller bank for 30 min. After this, beads were centrifuged at 5000 x g for 3 min. The supernatant was carefully removed using P200 pipette. Beads were washed 3 times by adding Milli-Q, mixing using vortex, centrifuging and removing the supernatant. Speficif to each batch of PEBs, a certain amount of enzyme was added to the beads (see Table 1). The tube was put on the roller bank for 2 h coupling step. Sequentially, beads were washed 8 times by adding Milli-Q, centrifuging and removing the supernatant. Finally, beads were flash frozen using nitrogen and freeze dried overnight.

Overview of PEBs

Table 1: Overview of PEB batches

Enzyme	Batch number	Enzyme concentration added	Volume per mg of beads
GDH	1	0.54 kUnit/mL	100 μL/mg
	2	2 kUnit/mL	100 μL/mg
	3	1 kUnit/mL	100 μL/mg
HK	1	1 kUnit/mL	100 μL/mg
G6P-DH	1	1 kUnit/mL	100 μL/mg

Overview of experiments

Processed datafiles from these experiments can be found on the accompanying github repository in the `data` folder as csv-files. The files are directly used during analysis performed in the Jupyter notebooks.

In the tables below, the volume refers to the volume of PEBs suspension injected into the CSTR, where the PEBs suspension itself is created by suspending 1 mg of dry PEBs in 31 μL buffer, or other quantities in the same ratio (1:31). The batch refers to the batch of PEBs, which can contain different effective enzyme concentation. See Table 1 for an overview of all PEB batches.

Table 2: Overview of single-enzyme experiments

Code	flowrate ($\mu L/h$)	enzyme	volume (μL)	batch	inputs	obervables	observation techniques
SNCA14	750	GDH	10	1	G, NAD	NADH	offline abs.
SNCA15	750	GDH	10	1	G, NAD	NADH	offline abs.
SNCA17	750	HK	10	1	G, ATP	G6P	G6P assay
SNCA18	750	HK	1	1	G, ATP	G6P	G6P assay
SNKS03	750	HK	1	1	G, ATP	G6P	G6P assay
SNKS04	750	HK	1	1	G, ATP	G6P,ATP	G6P assay, HPLC
SNKS08	750	G6PDH	10	1	G6P, NAD	NADH	offline abs.
SNKS11	750	GDH	0.5	2	G, NAD	NADH	online abs.
SNKS12	750	GDH	0.5	2	G, NAD	NADH	online abs.
SNKS18	750	GDH	2.0	3	G, NAD	NADH	offline abs.
SNKS20	750	G6PDH	2.0	1	G6P, NAD	NADH	online abs.

Table 3: Overview of multi-enzyme experiments

Code	flowrate ($\mu L/h$)	enzymes	volumes (μL)	batches	inputs	obervables	observation techniques
SNKS06	750	GDH,HK	10.0, 1.0	1, 1	G, NAD, ATP	NADH, ADP	online abs., HPLC
SNNS002	750	GDH,HK	0.5, 1.0	2, 1	G, NAD, ATP	NADH	online abs.
SNNS003	750	GDH,HK	0.25, 1.5	2, 1	G, NAD, ATP	NADH	online abs.
SNNS004	750	GDH,HK	0.333, 1.33	2, 1	G, NAD, ATP	NADH	online abs.
SNNS005	750	GDH,HK	0.666, 0.666	2, 1	G, NAD, ATP	NADH	online abs.
SNNS006	750	GDH,HK	0.75, 0.5	2, 1	G, NAD, ATP	NADH	online abs.
SNNS007	750	GDH,HK	0.5, 1.0	2, 1	G, NAD, ATP	NADH	online abs.

Overview of computational methods

All computational studies were performed with Jupyter notebooks. Datasets were loaded in from csv-files with Pandas, and if relevant, concatenated together into larger objects.

The Bayesian model, including the determination of prior probabilities and likelihood function, was created using PyMC3. Generally, prior probabilities for Michaelis-Menten parameters were chosen as uniform distributions over a specified interval. These distributions were used as uninformative priors to ensure no subjective information would enter the model, while garanteeing correct sampling and estimation of parameters. Priors for the uncertainty estimations (denoted by `sigma` in the notebooks) were given an exponential distribution, which also acted as an uninformative distribution, while garanteeing correct sampling. In larger models, where multiple likelihoods were combined, hyperpriors were placed on the k_{cat} and σ priors to increase convergence of the sampling algorithm.

All sampling was peformed using the No-U-Turn Sampler (NUTS), which is an adaptive step-size Hamiltonian Monte Carlo sampler. When (automatically, or via a custom operator) the gradients of the likelihoods with respect to the kinetic parameters were given, this samples is much more efficient then a classical Metropolis Monte Carlo sampler, showing faster convergence and needing less samples for precise posterior estimations. Generally, sampling was performed using 4 or 8 independent chains on 4 or 8 cpu cores, all with 1000 tuning steps, and 1000 sampling steps, and a target step acceptance probability of 0.95. These values were found to yield good sampling results without becoming computatially inefficient.

The samples obtained from the posterior distribution were further analysed using standard statistical tools in Python, the Numeric Python package and the Scientific Python package (NumPy and Scipy). To ensure the accessibility and reproducibility of these results the datasets and Jupyter notebooks, used for the analysis and creation of figures found in the publication, are made available as additional Supporting Information, and directly on github at https://github.com/huckgroup/Bayesian-enzymatic-networks_manuscript_2022.

Extended iterative combination of experiments

See Figure 4 for an extended version of the figure, showing posterior updating per iteration of new experiment.
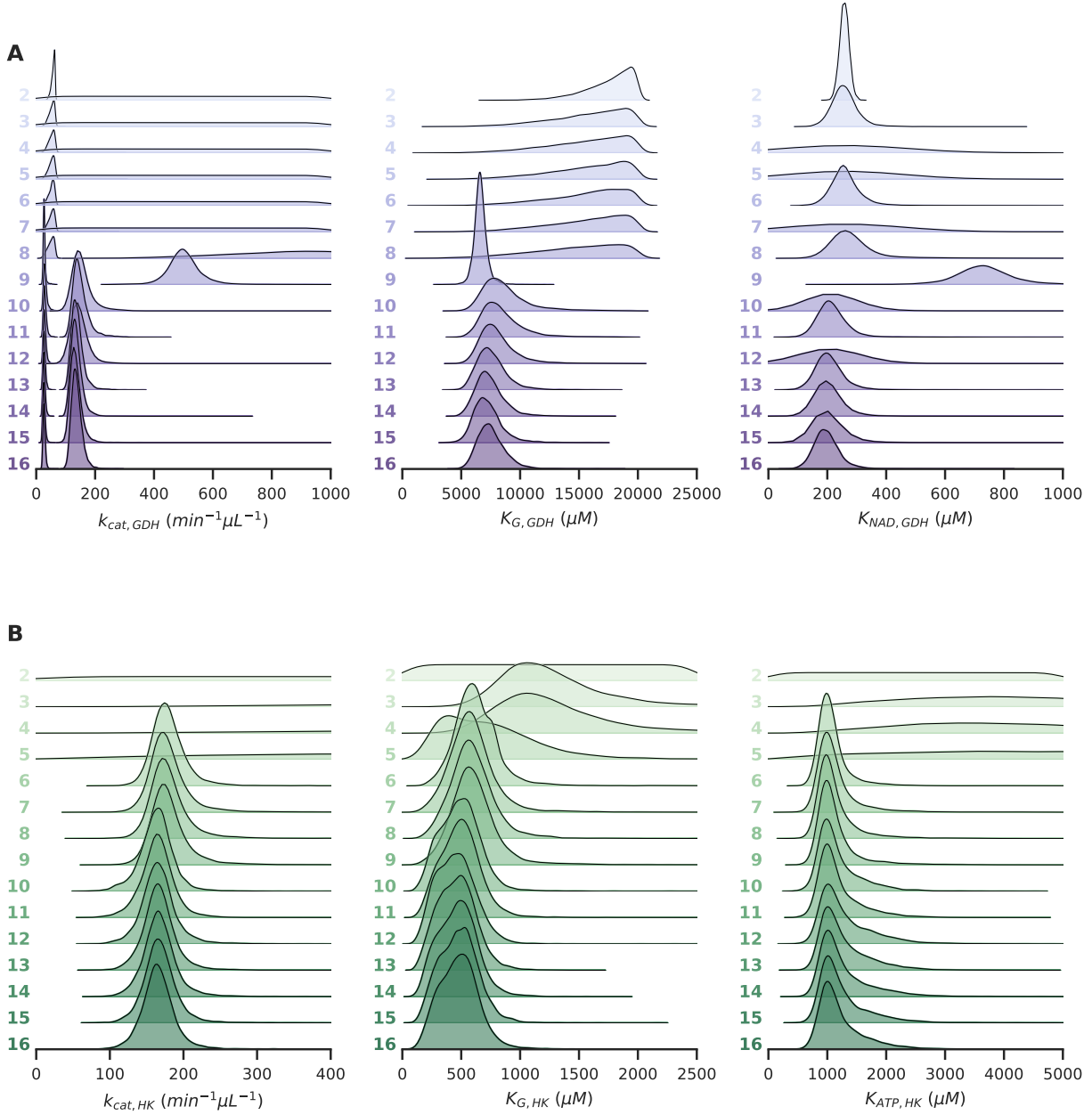
Figure 4: **Extended iterative posterior updating A,B** Posterior parameter estimates obtained from the model combining all three (GDH, HK, GDH+HK) observation likelihoods. For every parameter, the distributions are shown for 15 different datasets, with each following dataset containing an extra experiment, added in chronological order. Distributions are shifted and scaled to increase visibility. For the GDH k_{cat}, two estimates are obtained because PEBs with two different enzyme concentrations were used in different experiments.

References

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(2) Matula, K.; Rivello, F.; Huck, W. T. S. Single-Cell Analysis Using Droplet Microfluidics. *Advanced Biosystems* **2020**, *4* (1), 1900188. https://doi.org/10.1002/adbi.201900188.

(3) Semenov, S. N.; Markvoort, A. J.; Gevers, W. B. L.; Piruska, A.; Greef, T. F. A. de; Huck, W. T. S. Ultrasensitivity by Molecular Titration in Spatially Propagating Enzymatic Reactions. *Biophysical Journal* **2013**, *105* (4), 1057–1066. https://doi.org/10.1016/j.bpj.2013.07.002.