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## Encapsulated enzymes with integrated fluorescence-control of enzymatic activity

### Supporting Information

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#### 1. Motivation

In the main manuscript the fluorescent pH indicator dye SNARF-1-dextran was used for to monitor the enzymatic reaction  $\text{OAA} + \text{NADH} + \text{H}^+ \rightleftharpoons \text{L-malic acid} + \text{NAD}^+$  (OAA = oxaloacetic acid, NADH = nicotinamide adenine dinucleotide) of malate dehydrogenase. In this supplementary information we demonstrate that the same reaction can also be followed with thymol blue, a non-fluorescent pH indicator. Thus the reaction can be followed by UV/vis absorption instead of fluorescence measurements. This way thymol blue has been used to complement the fluorescence measurements with SNARF-1. In addition thymol blue has different pK<sub>a</sub> values than SNARF-1-dextran, thus allowing measurements at different pH

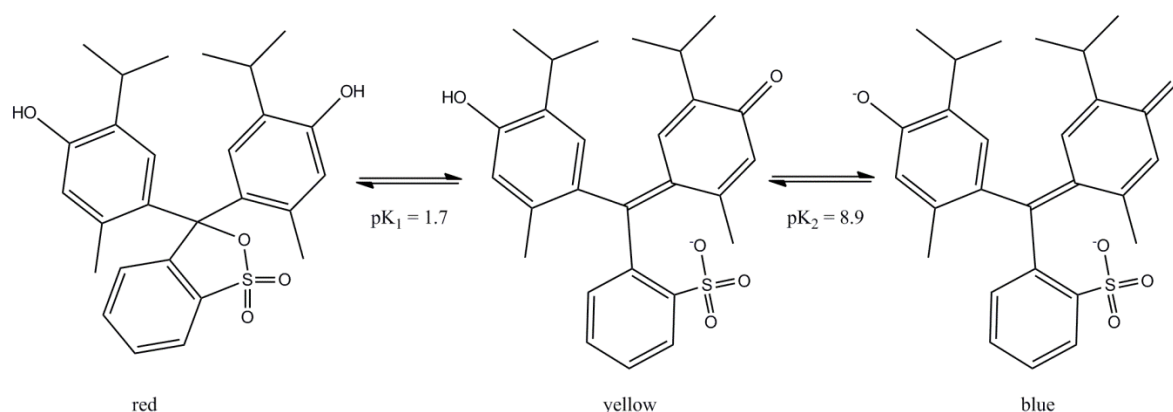
values. Furthermore, by using absorption measurements as detection technique, next to changes in pH as indicated by thymol blue absorption also changes in the concentrations of substrate and product of the enzymatic reaction can be followed by measuring their respective absorption spectra.

## 2. Chemicals

For all experiments, a fresh solution of 0.05 wt% of thymol blue (Riedel de Haën, Seelze, Germany, #32728) in ethanol (0.4 mg/mL, Roth, Karlsruhe, Germany, #5054.2) was prepared. Oxaloacetic acid (OAA, #4032.2), L-malic acid (#8684.1),  $\beta$ -nicotinamide adenine dinucleotide disodium salt (NADH, #AE12.1), and  $\beta$ -nicotinamide adenine dinucleotide (NAD<sup>+</sup>, #AE11.1) were obtained from Roth. Ultrapure water (ddH<sub>2</sub>O) with a resistance greater than 18.2 M $\Omega$  cm<sup>-1</sup> was used for all experiments. Borate buffer was prepared from buffer concentrates (Sigma-Aldrich, USA, #38747).

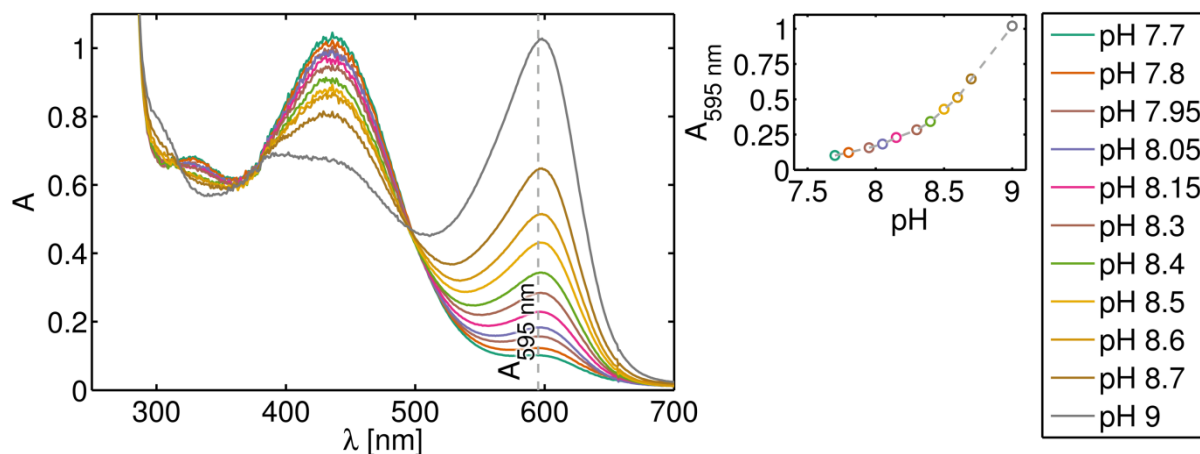
## 3. Absorption spectra of the pH indicator thymol blue

Thymol blue is a pH indicator with two transitions with pK<sub>a</sub> = 1.7 and pK<sub>a</sub> = 8.9, cf. Figure S1. During the first transition, the dye changes from red to yellow and during the second transition from yellow to blue. The change from yellow to blue can be measured with UV/vis absorption spectroscopy at 595 nm.



**Figure S1.** Structural transitions of thymol blue which causes different absorption of light depending on the pH. The first transition changes the color from red to yellow and the second transition changes the color from yellow to blue. The second transition can be quantified by the absorbance at 595 nm.

The calibration curves are shown in Figure S2. The absorbance at 595 nm increases strongly from pH 7.7 to 9.0. The graph is the first part of a sigmoidal curve which is expected for a pH indicator. Additionally, the presence of NAD<sup>+</sup> does not interfere with the UV/vis spectrum of thymol blue.



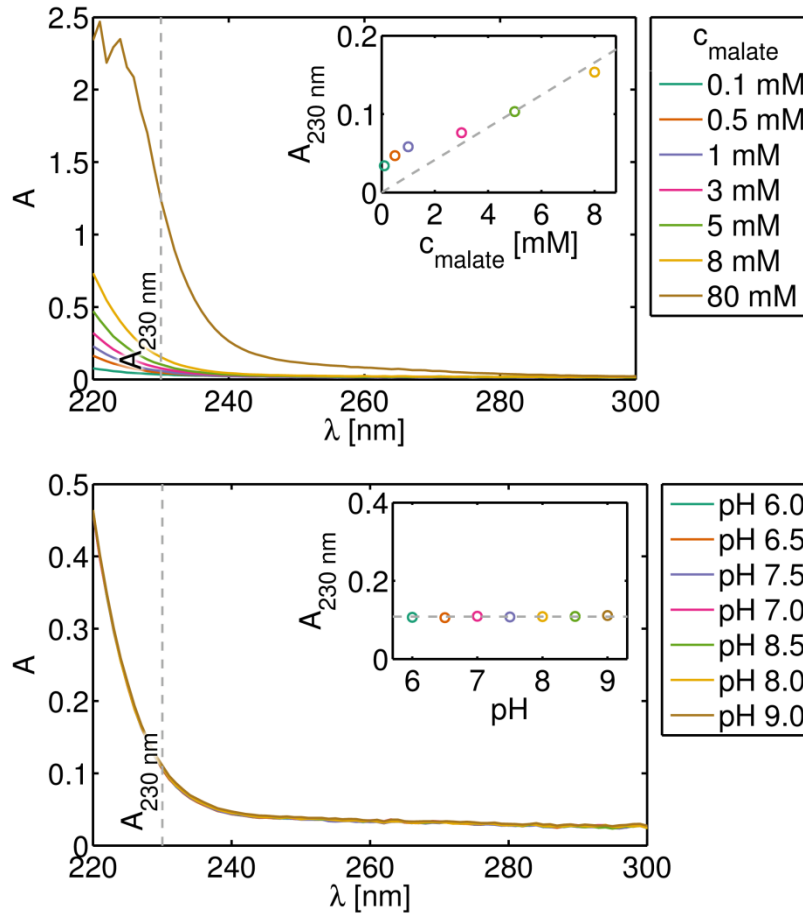
**Figure S2.** The absorption spectrum  $A(\lambda)$  of thymol blue versus different pH values. The UV/vis absorption spectrum of thymol blue shows two peaks in the visible in the pH region of 8-9. The peak  $A_{595\text{ nm}}$  at 595 nm strongly increases with increasing pH and can be used for pH measurements.

#### 4. Role of substrates and cofactors NADH/NAD<sup>+</sup>

The substrates OAA and L-malic acid are part of the citrate cycle. Depending on the direction of the reaction the role between substrates and products changes. In case of the forward reaction  $\text{L-malic acid} + \text{NAD}^+ \rightarrow \text{OAA} + \text{NADH} + \text{H}^+$ , L-malic acid is the substrate and  $\text{NAD}^+$  is the cofactor. In case of the backward reaction  $\text{L-malic acid} + \text{NAD}^+ \leftarrow \text{OAA} + \text{NADH} + \text{H}^+$ , OAA is the substrate and NADH the cofactor. The cofactors NADH and  $\text{NAD}^+$  are essential for the metabolism of living organisms. While  $\text{NAD}^+$  absorbs UV light only at 260 nm, its reduced counterpart NADH also absorbs at 340 nm. This physical property allows monitoring of their interchange by the enzymatic reaction performed in our experiments. Calibration curves for both,  $\text{NAD}^+$  and NADH for the absorbance with respect to concentration in solution were made, as presented in the following. For measuring the absorption spectra of substrates and cofactors, fresh solutions in borate buffer (pH=8.0, constant pH) were prepared. The stock solutions were diluted using buffer solutions to vary the pH or the concentration.

#### 5. Absorption spectra of L-malic acid

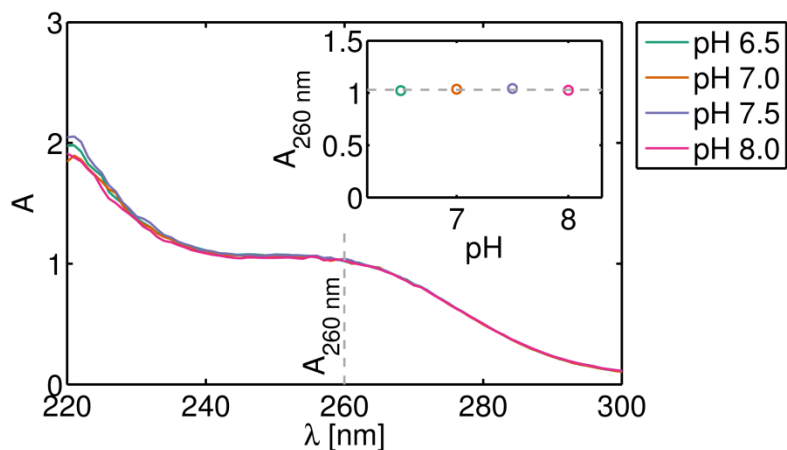
L-malic acid shows negligible absorption at low concentrations and only small absorption below 240 nm at high concentrations, cf. Figure S3. The spectrum does not change when the pH is changed. Therefore, no interference with the detection of the enzymatic reaction will occur.



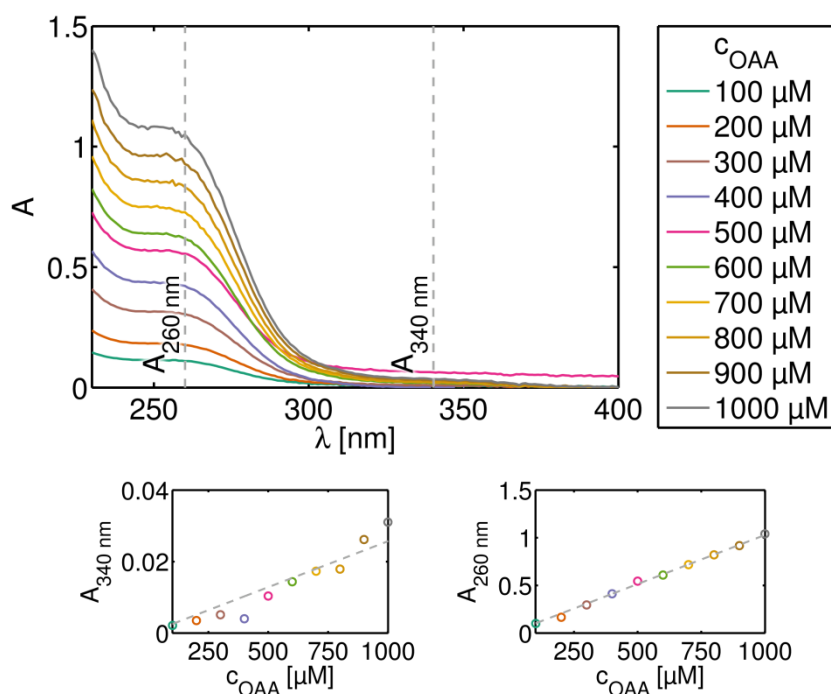
**Figure S3.** Absorption spectra  $A(\lambda)$  of L-malic acid depending on pH and concentration of L-malic acid  $c_{\text{malate}}$ . The absorption  $A_{230 \text{ nm}}$  at 230 nm is plotted versus pH and  $c_{\text{malate}}$ .

## 6. Absorption spectra of oxaloacetic acid (OAA)

The absorption spectrum of OAA has a shoulder between 240 nm and 260 nm and at high concentrations ( $\geq 5$  mM) a shoulder between 330 nm and 380 nm, *cf.* Figure S4 and Figure S5. The absorption increases linearly with the concentration and is independent of the pH. This means that there is interference between the absorption of OAA and  $\text{NAD}^+$  at 260 nm. However, only negligible absorption for  $\lambda > 300$  nm can be seen. Hence, the detection of NADH and the dye thymol blue is not influenced by OAA.



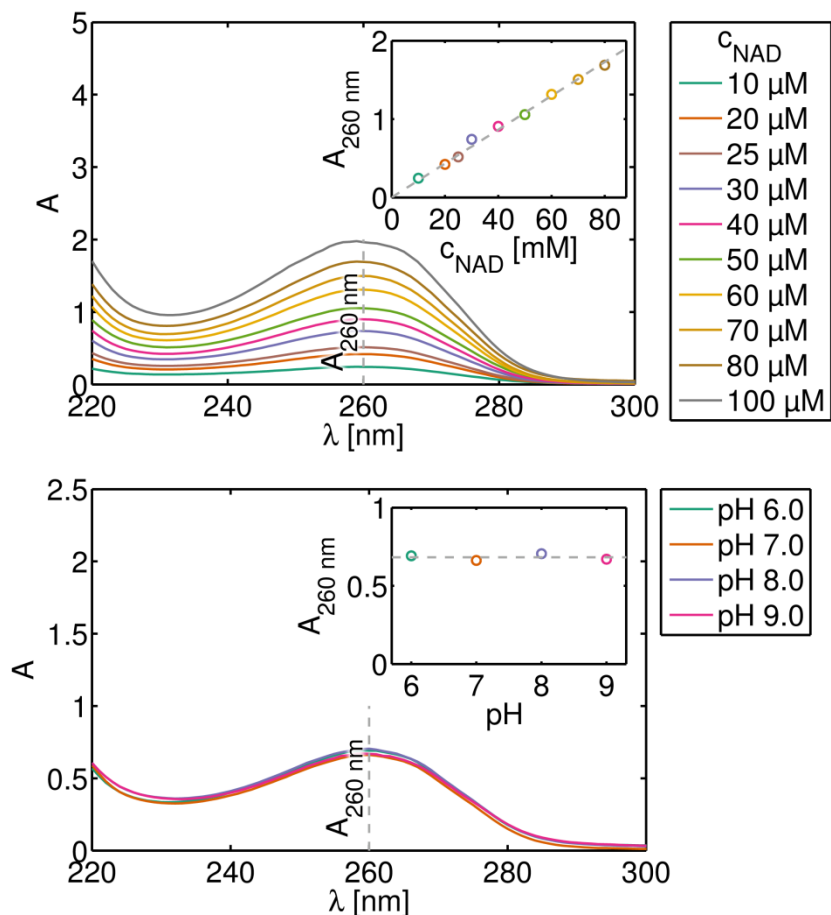
**Figure S4.** Absorption spectra  $A(\lambda)$  of oxaloacetic acid (OAA) depending on the pH. The absorption at 260 nm ( $A_{260\text{ nm}}$ ) is plotted versus the pH.



**Figure S5.** Absorption spectra  $A(\lambda)$  of oxaloacetic acid (OAA) depending on the concentration  $c_{\text{OAA}}$ . The absorption at 260 nm ( $A_{260\text{ nm}}$ ) and at 340 nm ( $A_{340\text{ nm}}$ ) is plotted versus the pH.

## 7. Absorption spectra of $\text{NAD}^+$

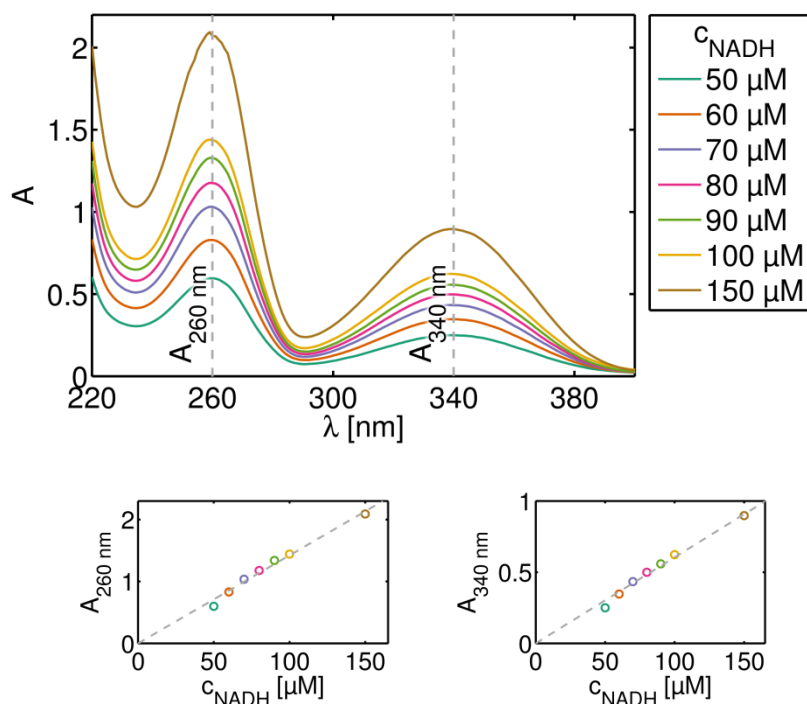
The absorption spectra of  $\text{NAD}^+$  shows an absorption band at 260 nm with an extinction coefficient of  $16,900\text{ M}^{-1}\text{ cm}^{-1}$ , cf. Figure S6. The absorption is even detectable at sub-millimolar concentrations. The absorption at 260 nm increases linearly with concentration between 0 and 100  $\mu\text{M}$  and is independent of the pH.



**Figure S6.** Absorption spectra  $A(\lambda)$  of  $\text{NAD}^+$  upon varying the pH and the  $\text{NAD}^+$  concentration ( $c_{\text{NAD}}$ ). The absorption at 260 nm ( $A_{260 \text{ nm}}$ ) is plotted versus  $c_{\text{NAD}}$  and the pH.

## 8. Absorption spectra of NADH

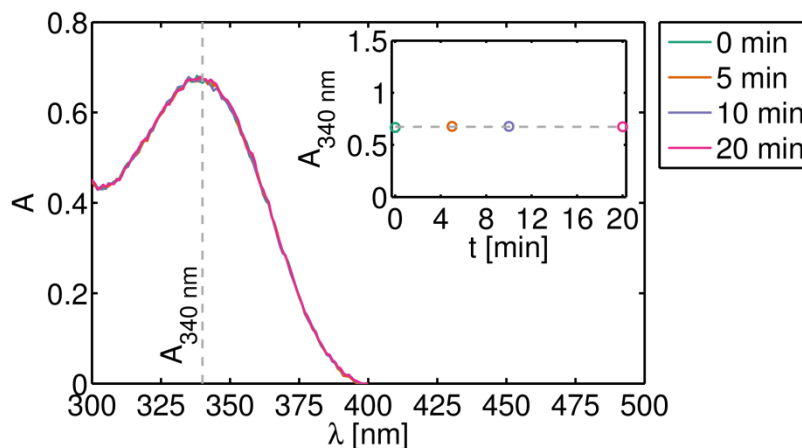
The absorption spectra of NADH show absorption bands at 260 nm and 340 nm, *cf.* Figure S7. The extinction coefficients are  $14,400 \text{ M}^{-1} \text{ cm}^{-1}$  and  $6,220 \text{ M}^{-1} \text{ cm}^{-1}$ , respectively. The absorption at 340 nm is a unique feature regarding the reagents used in our experiments, which allows tracking the generation or the loss of NADH during the reaction by measuring time-resolved absorption at 340 nm. There is furthermore no absorption at higher wavelengths. The ratio between the absorption at 260 nm and 340 is constant (*ca.* 2.4). This result is close to the literature value of 2.31.<sup>1</sup>



**Figure S7.** Absorption spectra  $A(\lambda)$  of NADH upon varying the concentration  $c_{\text{NADH}}$ . The absorption at 260 nm ( $A_{260 \text{ nm}}$ ) and at 340 nm ( $A_{340 \text{ nm}}$ ) is plotted versus  $c_{\text{NADH}}$ .

### 9. Absorption spectra of a mix of OAA and NADH (without enzyme)

This control experiment was conducted in order to check if a reaction between the substrate and the cofactor occurs without the enzyme. To test this, the absorption spectra of a solution containing 500  $\mu\text{M}$  of NADH and 10 mM OAA, adjusted to pH 7, were recorded over time. The absorption of NADH at 340 nm was used to determine possible consumption of NADH. As shown in Figure S8, no change in the absorption can be seen which indicates that there is no reaction between the two reactants over time. Even after 20 minutes, no change occurred which leads to the conclusion that there is no or only a very slow reaction without the enzyme.



**Figure S8.** Absorption spectra  $A(\lambda)$  of a mixture of NADH and OAA over time  $t$ .  $A_{340 \text{ nm}}$  is the absorbance at 340 nm.

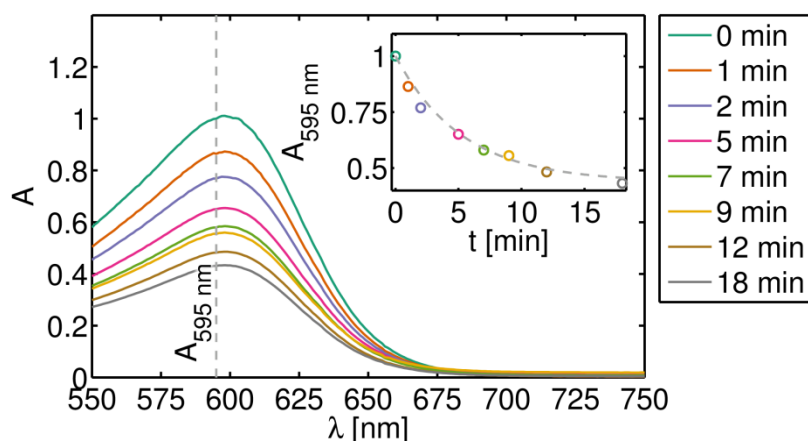
### 10. Following the forward reaction by absorption measurements

The reaction in the forward direction ( $\text{L-malic acid} + \text{NAD}^+ \rightarrow \text{OAA} + \text{NADH} + \text{H}^+$ ) was used to verify if the reaction is in principle capable to create a change in pH. According to the reaction equation upon enzymatic processing of L-malic acid protons should be released and thus pH should go down. For this, we used in a first step the pH indicator thymol blue and monitored the change at 595 nm with UV/vis spectroscopy.

270 mg L-malic acid and 3 mg  $\text{NAD}^+$  were dissolved in 9 mL ddH<sub>2</sub>O water. 1 mL thymol blue solution (0.05 wt%) was added and the solution thoroughly mixed. The pH was then adjusted with NaOH to 9.3. The colour of the solution was blue. To start the reaction, 5  $\mu\text{L}$  of diluted malate dehydrogenase enzyme (1/8 dilution of the stock solution) were injected into the reaction solution while moderately stirring.

At different time points, a UV/vis absorption spectrum was taken. Within a few minutes, a significant change of the absorbance at 595 nm occurred. Caused by the reaction, the absorbance at 595 nm increased quickly, indicating, that the pH of the solution was lowering due to release of  $\text{H}^+$  ions. In addition to monitoring the UV/vis spectra, the change was visible by eye as the solution quickly turned green and then slowly changed its colour into the yellowish. This behaviour was visible only when all the reactants needed for the catalysis were present in solution. Very importantly, this experiment shows that significant amounts of hydrogen ions are released due to the reaction and that this change is easily detectable by a common pH indicator. At the end of the reaction the pH had fallen to 8.5. Time dependent decrease in pH can be seen in Figure S9.

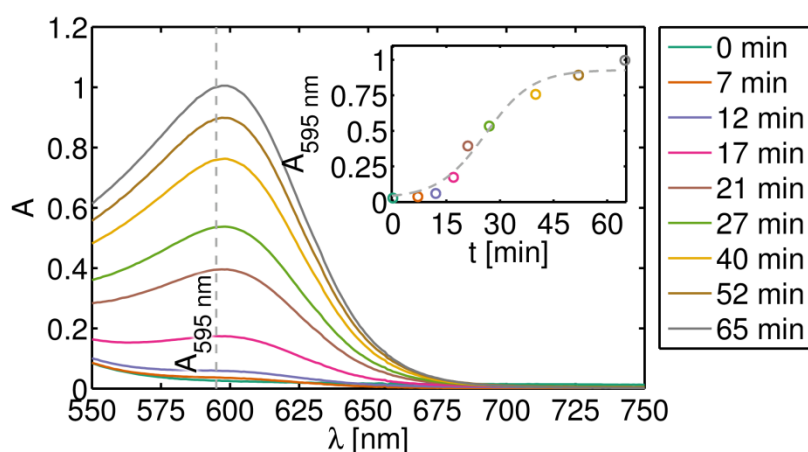




**Figure S9.** UV-Vis absorption spectra  $A(\lambda)$  of thymol blue following the reaction of the free enzyme,  $L$ -malic acid +  $NAD^+ \rightarrow OAA + NADH + H^+$ . The lowering of pH can be seen in the continuous reduction of the absorption  $A_{595\text{ nm}}$ , cf. Figure S2.

### 11. Following the backward reaction by absorption measurements

The backward reaction  $L$ -malic acid +  $NAD^+ \leftarrow OAA + NADH + H^+$  was experimentally investigated using the complementary substrate, co-substrate and enzyme, as have been used for observing the forward reaction. As previously, the substrates and the dye were dissolved and the pH adjusted to 6.8 before 1  $\mu$ L of MDH was added. Afterwards UV/vis absorption spectra were taken at different points in time. It can be seen that there is a significant increase of absorption at 595 nm within the first 20 minutes which indicates that the pH of the solution becomes more basic which is expected from the reaction. Note, that the direction of the reaction is given by the initial pH, which was set to 6.8 in case of the backward reaction and to 9.3 in case of the forward reaction.



**Figure S10.** UV/vis absorption spectra  $A(\lambda)$  of thymol blue under the influence of the backward reaction of the free enzyme,  $L$ -malic acid +  $NAD^+ \leftarrow OAA + NADH + H^+$ . The increment of pH can be seen in the continuous reduction of the absorption  $A_{595\text{ nm}}$ , cf. Figure S2.

## 12. References

1. J. M. Siegel, G. A. Montgomery and R. M. Bock, *Arch. Biochem. Biophys.*, 1959, **82**, 288-299.