Deciphering the effect of microbead size distribution on the kinetics of heterogeneous biocatalysts through single-particle analysis based on fluorescence microscopy

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Table S1. Immobilization parameters. Co-immobilization of His-BsADH with NADH on AG-Co^{2+} and PEI coating

	Yield (%)	Load	Expressed activity(IU x g^{-1})	Immobilized Specific activity(IU x mg^{-1})	Relative Expressed Activity (%)
His-BsADH	41±1	16,3[a]	21±4	1.28	82±16
NADH	76	7.6[b]			

a: $mg_{protein}$ x $g_{carrier}^{-1}$; b: μmol_{NADH} x $g_{carrier}^{-1}$. Relative expressed activity means the percentages of the specific activity of the soluble retained after the immobilization.

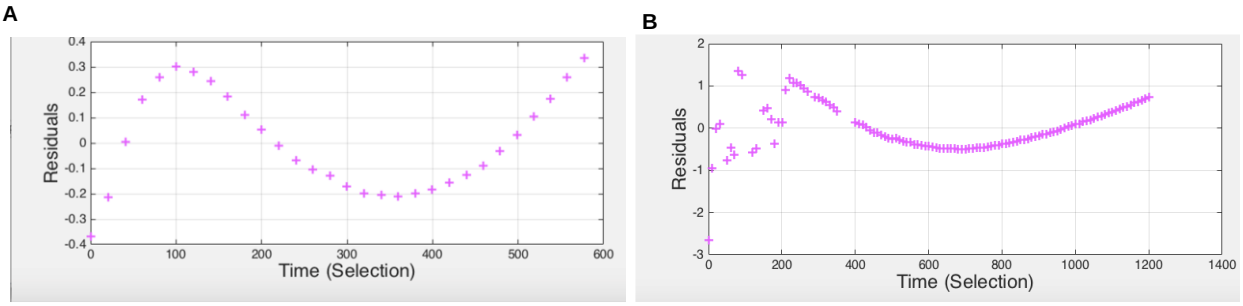

Figure S1. Residual plots of data estimated in Figure 2A(A) and 2B (B).

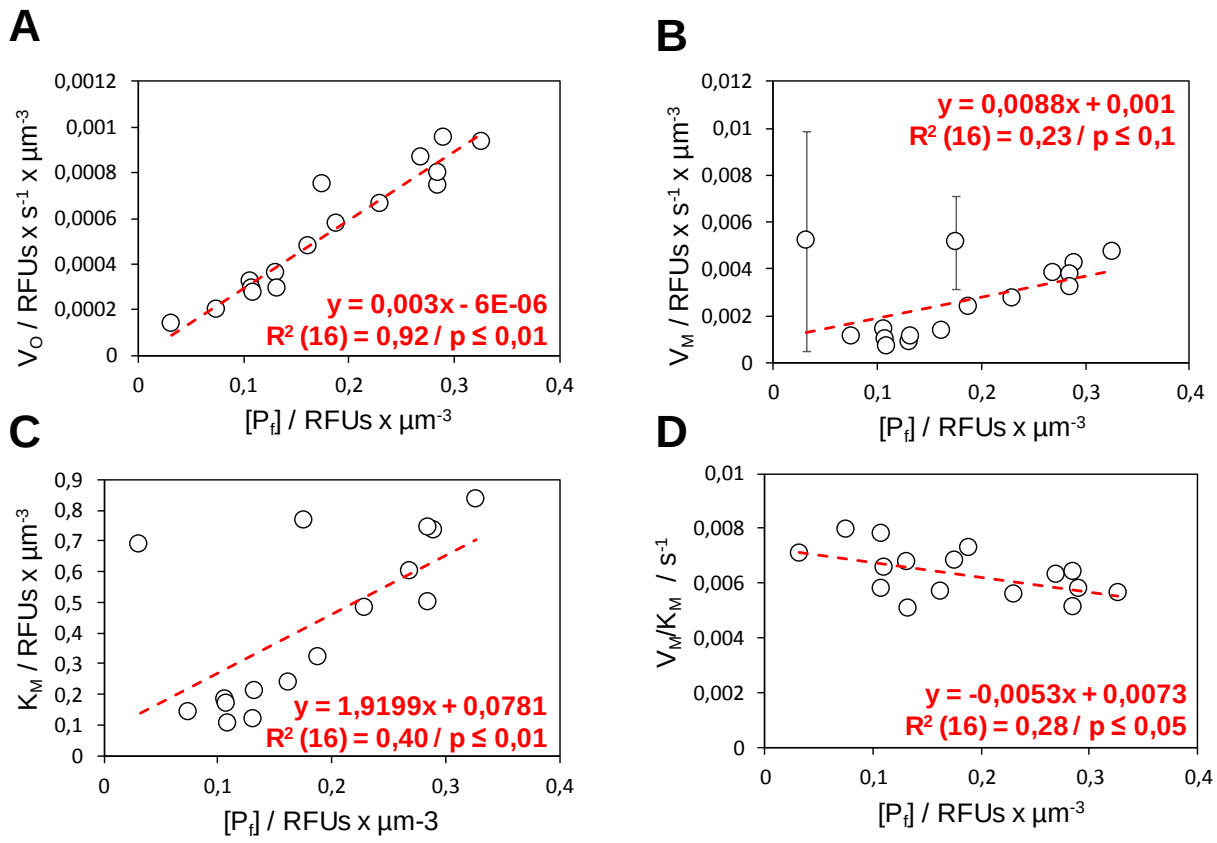

Figure S2. Effect of the formed product [P_f] on the kinetic parameters (see in bold) of the immobilized His-BsADH and NADH using acetone as substrate. For single particle analysis, apparent V_0 **(A)**, V_M **(B)**, K_M **(C)**andV_M/K_M **(D)** were determined and plotted against [P_f]. Dashed red line represents the linear correlation between each kinetic parameter and the intraparticle cofactor concentration. Linear regression equations are shown within each graph. The multiple correlation coefficient R^2, considering the number of samples between parenthesis, and the *p* were calculated with an ANOVA statistical analysis and shown in blue.

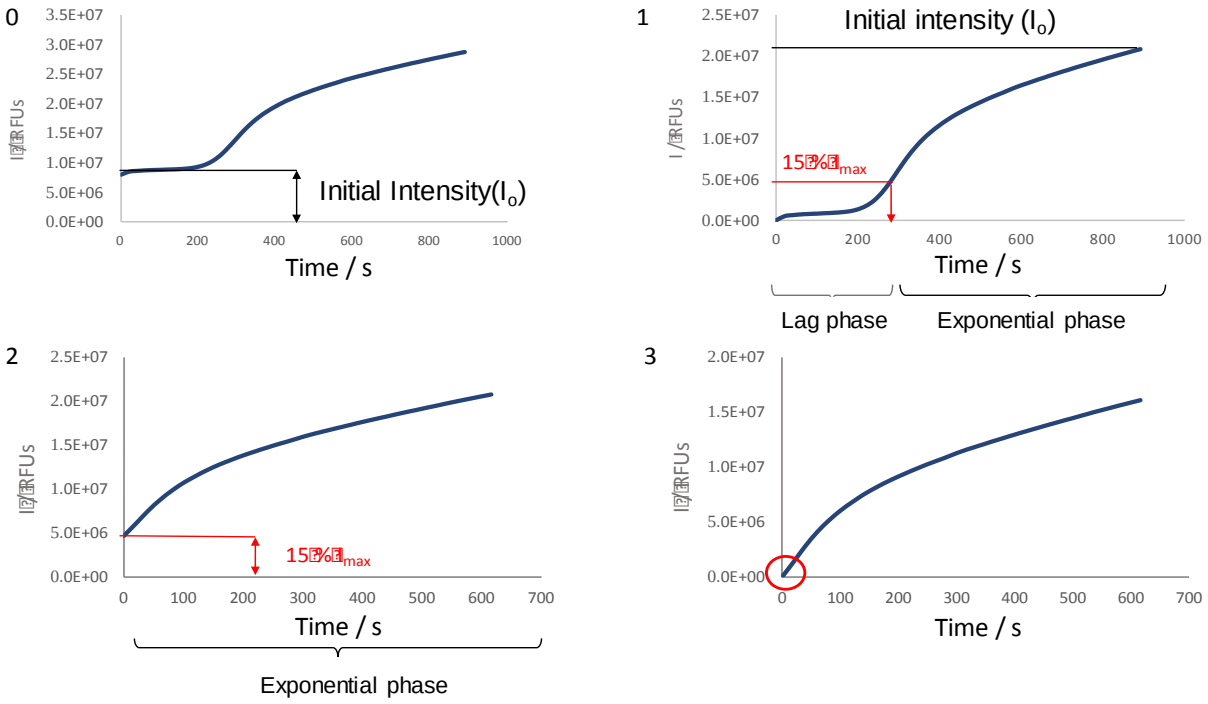

Figure S3. Example of lag-phase removal for one single-particle experiment where the fluorescence intensity increases along the time by the action of the formate dehydrogenase co-immobilized with NAD+ (reduction reaction of NAD+ to NADH) using sodium formate as substrate. 1) Substraction of the background (intensity at time 0) from all data points. 2) Selecting the data point where the intensity is 15% of the maximum intensity of the experiment (time 600 s) as the time 0 data point. 3) Substracting the value of the 15% maximum intensity to the data set to have a data point at time 0 with a value of 0.

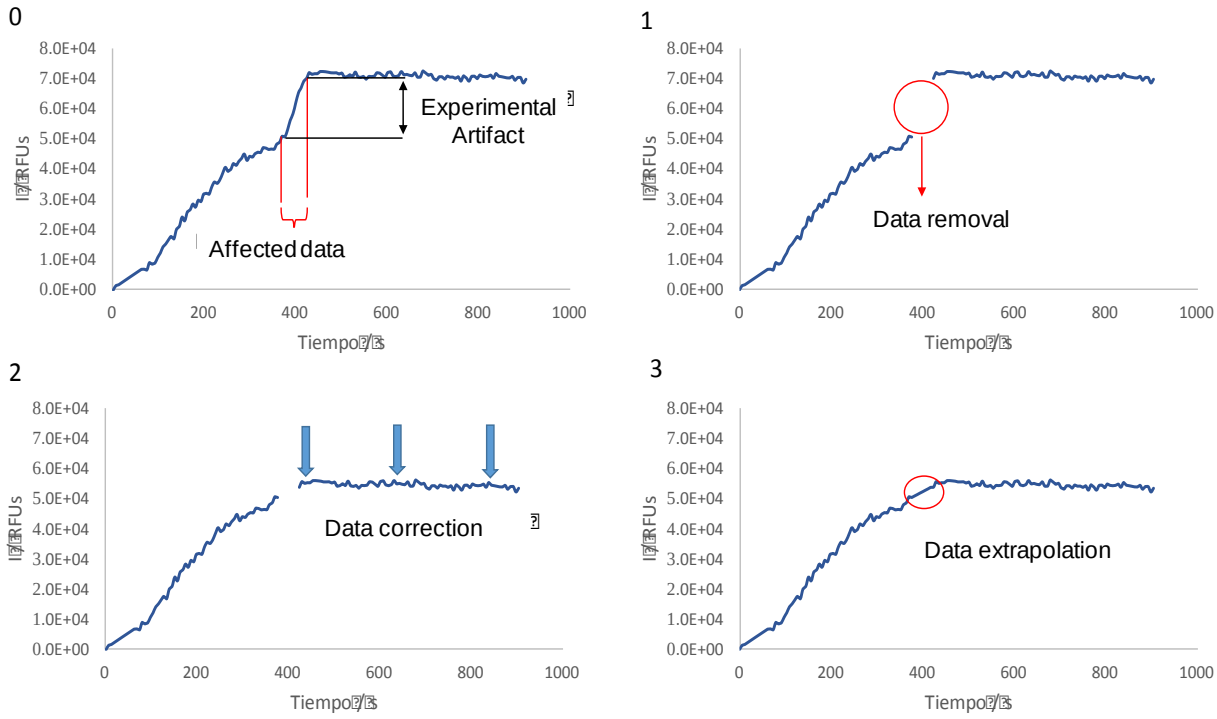

Figure S4. Removal of outlier points from one single-particle experiment where using the inverted time-course. The experiment was carried out by the action of the alcohol dehydrogenase co-immobilized with NADH (oxidation reaction of NADH to NAD+) using acetone as substrate. The inverted intensity refers to the production of NAD+ as result of the enzymatic reduction. 1) removal of the outlier values, 2) correction of the data after the artifact if needed and 3) extrapolation of the reliable data point after eliminating the outlier ones.

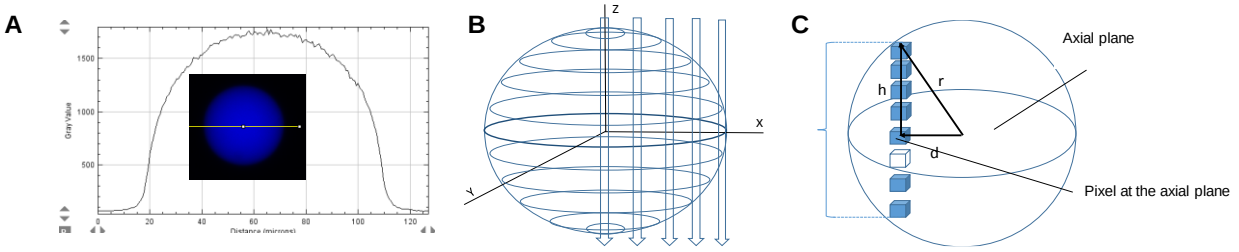

Figure S5. Graphical explanation of how to calculate the volumetric fluorescence intensity. Specific intensity of each pixel depends on their distance to the bead center. (A) Plot profile of the time 0 before triggering the reaction for one bead imaged with the epiflorescence microscope. The fluorescence intensity should be uniformly distributed across the bead volume. (B) Fluorescence accumulation depending on the coordinates. (C) Scheme to calculate the intrinsic pixel volume

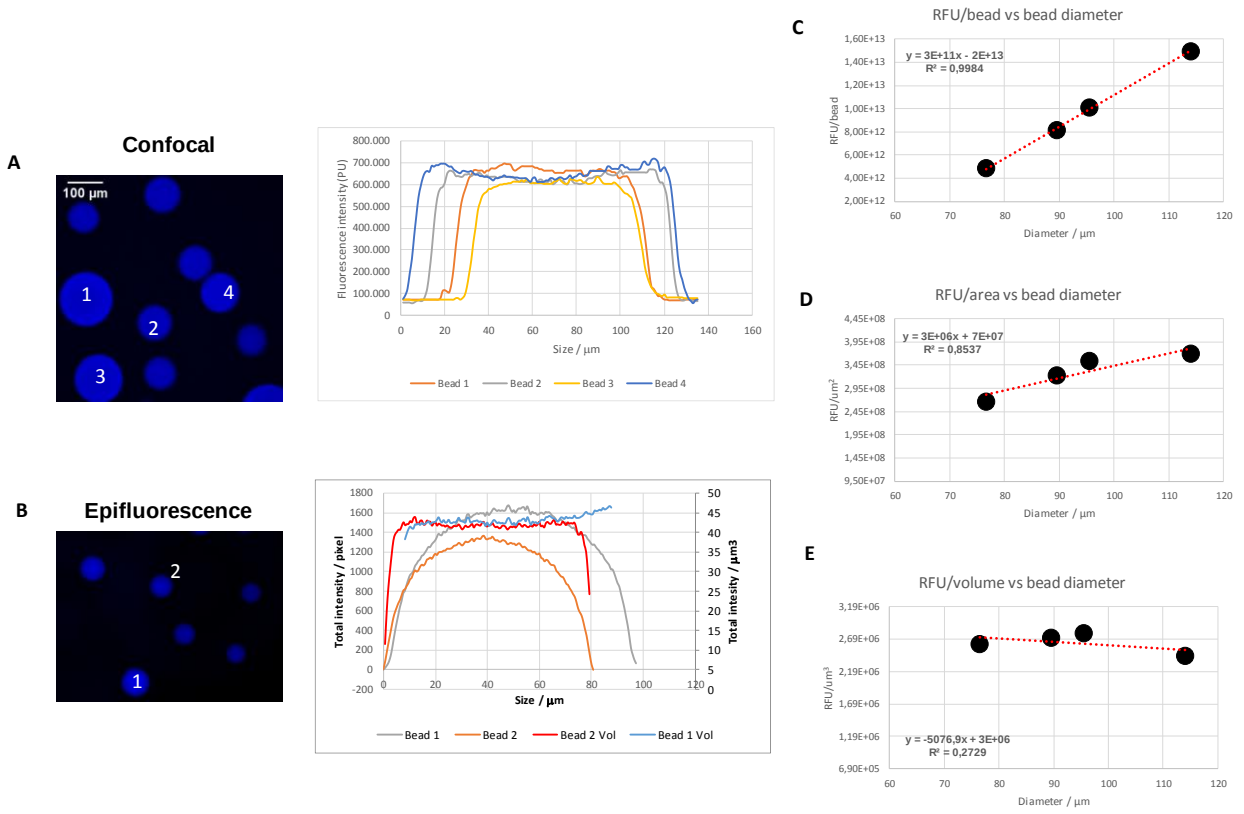

Figure S6. Cofactor distribution across the surface of agarose microbeads functionalized with PEI. **(A)** Confocal laser scanning microscopy image *left:* Plot profile analysis of different beads with different diameters. **(B)** Epifluorescence microscopy image *left:* Plot profile analysis of different beads with different diameters (grey and orange) and the volumetric profile (red and blue) according to the equations 4 and 5 (see Figure S5 C). Correlation between the total intensity per bead **(C),** the total intensity per area **(D)** and the total intensity per volume **(E)** and the particle diameter. The data to make these plots resulted from the analysis of four beads from the epifluorescence microscopy image presented in (B).