Enzymatic Oxygen Scavenging for Photostability Without pH Drop in Single-Molecule Experiments - Supporting Information

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Materials and Methods

Glucose oxidase (GOx), Type VII, from *Aspergillus niger*, pyranose oxidase (P2Ox) from *Coriolus sp.*, expressed in *E. coli*, protocatechuate-dioxygenase (PCD) from *Pseudomonas sp.* and catalase from bovine liver were available commercially from Sigma-Aldrich. A component binding to single-stranded DNA was found in the P2Ox from Sigma, hence, we additionally tested a P2Ox prepared from *Trametes multicolor* overexpressed in *Escherichia coli* with a C-terminal His-tag, and purified by a single metal-affinity chromatography step, which yielded a homogenous preparation of the enzyme [12]. The alternative P2Ox from *Trametes multicolor* did not show any ssDNA binding activity. Sigma-Aldrich enzymes were purified using an ssDNA-cellulose column and did not show any ssDNA binding activity after purification with a similar oxygen scavenging capability.

Oxygen concentrations in presence of the enzymes and their substrates were measured in an unstirred solution of Tris:HCl buffer (pH 8, 10 mM) with i) a Clark-type electrode (WTW CellOx 325, detection limit = 0.01 mg/L O_2) and ii) an O_2 Optode (OxySense Gen III 325i, detection limit = 15 ppb O_2). For POC and GOC, 7.5 U/mL of the oxidase, 1 kU/mL of catalase and 50 mM glucose was used. For PCD, 7.5 U/mL PCD and 50 mM PCA was used.

Single-molecule experiment buffers were composed of 20 mM Tris:HCl pH8, 50 mM NaCl and 40 mM glucose in a saturated aged Trolox-solution (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) at pH 8. When using PCD, its substrate PCA was used at 24 mM concentration instead of glucose. The solution was kept overnight to produce trolox-quinone to minimize fluorophore blinking [19]. The enzymes were dissolved in 10 mM Tris:HCl buffer at pH 8 with 50 mM NaCl.

PCD was used in a concentration of 175 nM or 3.7 U/mL in the imaging buffer (stock 12.5 mg/ml or 37.5 U/ml, from a batch with 3 U/mg). With GOx, 192 U/mL were typically used in the imaging buffer and for P2Ox, 3 U/mL of pyranose oxidase were used. Catalase, if needed, was added to a final concentration of 90 U/mL. To ensure that these mismatching activities of the enzymes did not influence the measurement, a comparison of POC and GOC at 3 U/mL was also performed, which did not yield any different behavior (after a 5 min waiting period to ensure equilibrium kinetics) in oxygen scavenging. Therefore, POC and PCD achieve anaerobic conditions similar to GOC despite their distinctly lower activity.

Biotinylated DNA double strands labeled with the cyanine dyes Cy3 or Cy5, respectively, were immobilized via neutravidin-biotin interaction, on a surface passivated with polyethylene glycol (PEG) of which 0.8 % (w/w) were labeled with biotin. The biotin-labeled binding sites, together with added neutravidin, then allowed the attachment of the biotinylated, fluorescently labeled DNA-molecules, as shown in Figure S1. The flow cell consisted of channels separated by double-sided tape between a cover slip and a drilled quartz objective slide and typically had a volume of about 40 µL.

All DNA oligos were purchased from Integrated DNA Technologies (and site-specifically labeled with NHS-Cy3 or NHS-Cy5 obtained from GE Healthcare (PA13101 and PA15101, respectively; Pittsburg, PA). \iAmMC6T\ denotes the amine-modified thymine with a C6 spacer used for site-specific labeling. \5BiosG\ denotes the 5' modification with biotin.

Name	Sequence (5' to 3')
AT-strand 1	(T30)\iAmMC6T\AAT TAT ATT TAA ATT TAA ATA TTA ATT AAT ATA TTA ATA T
AT-strand 2	\5BiosG\ATA TTA ATA TAT TAA TTA ATA TTT AAA TTT AAA TAT AAT \iAmMC6T\(T30)

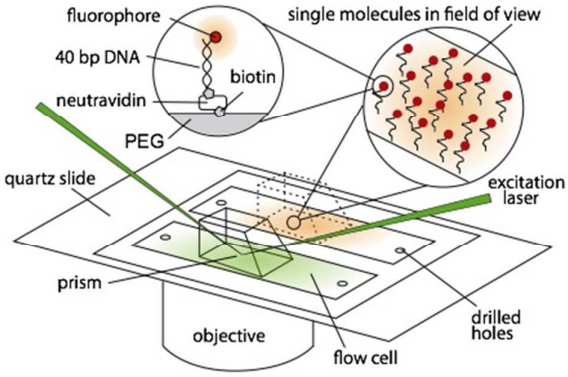


Fig. S1: Overview of the experimental setup (same as Figure 2a of the manuscript). The excitation laser is coupled to a Total-Internal-Reflection-Fluorescence (TIRF) excitation spot via a quartz prism placed on top of a drilled quartz slide. To excite the other flow cell, the sample slide can be moved relative to prism and objective (indicated by dashed prism outline), maintaining focal and excitation parameters. The molecular fluorescence is collected through an inverted microscope objective. The individual molecules labeled with fluorophores are immobilized in the flow cell through biotin-neutravidin interaction on a layer with biotinylated polyethylene glycol (PEG).

The single-molecule traces were recorded with a setup as shown in Fig. S1. An inverted microscope from Nikon was modified to include a Pellin-Broca prism for fluorescence excitation by total-internal reflection (TIR). A Coherent Sapphire laser at 532 nm wavelength was used to excite Cy3, while a Coherent Obis at 640 nm was used for Cy5. Most experiments were carried out in a channel arrangement as in Fig. S1, with one channel loaded with Cy3-labeled DNA molecules and the other channel loaded with Cy5-labeled DNA. Different enzymes and buffers were supplied to the experiment through the drilled holes shown in Fig. S1, allowing to keep the spot size and beam coupling and therefore excitation parameters constant for a given experiment.

The fluorescence was collected using an Andor electron-multiplying CCD-camera (iXon 897-D) and measurement software written in LabView. Post-processing code written in Matlab extracted single molecule intensity traces from the data as shown in Fig. S2a. These single molecule traces could then be averaged to produce the exponential decay curves as in Fig. S2b.

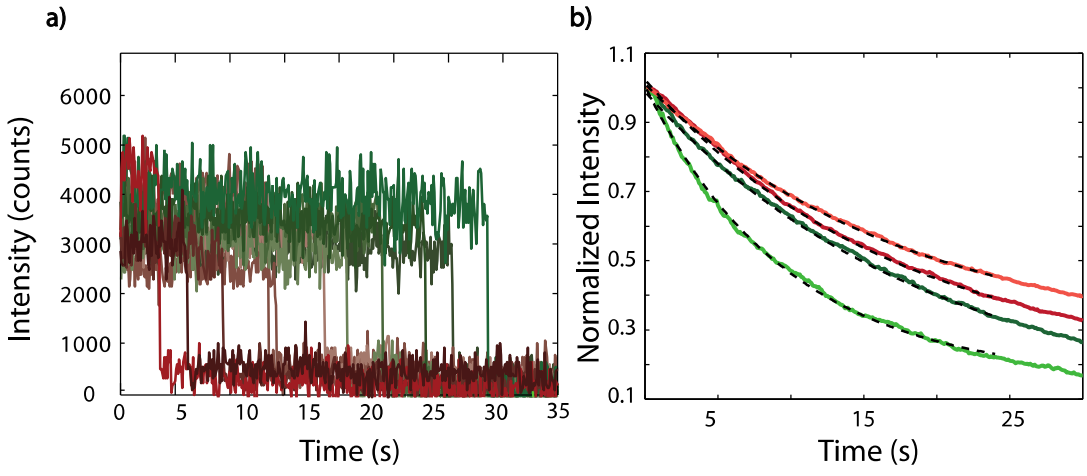


Fig. S2: a) Typical single molecule intensity traces of Cy3 fluorophores over time, with various bleaching times. b) Normalized decay curves obtained at pH 8 for Cy3 (green) and Cy5 (red) and POC (darker) vs. GOC (lighter color).

Figure S2b) shows four example decays from 1097 (POC, Cy3, dark green), 1340 (GOC, Cy3, light green), 2262 (POC, Cy5, dark red) and 1751 (GOC, Cy5, light red) molecules and the corresponding exponential nonlinear least-squares fit. Figure S2a depicts an example of individual molecule traces, which are added to obtain decay functions as in panel S2b. For each molecule, bleaching occurs after a given time and several hundred molecules make up a single scan for a given enzymatic condition. Blinking was rarely observed at our time resolution indicating that the Trolox solution successfully quenched triplet states.

The normalized fluorophore lifetimes from the manuscript are all given in Table S1. To estimate the number of photons obtained per molecule and unit time, the EMCCD-signal can be correlated with a photon number from the counts obtained at a given gain value. Additional estimates are needed to assess the number of photons collected by the microscope (spatial angle of observation) and lost in the optics between microscope and camera. Over 20 seconds, our camera collects (in normal conditions) approximately 40 000 photons from a single Cy3 fluorophore at 30 mW excitation power, or 2 000 photons/s. A rough estimate for Cy3 therefore results in 170 000 photons per Cy3-molecule for a lifetime of 20 s (in good agreement with literature values), assuming a camera quantum efficiency of 90%, 70% of the Cy3 emission spectrum collected by our setup, and 90% of the signal transmitted, after 1.5π have been collected by our water-immersion objective.

Table S1 gives numeric values of the bar values given in Figure 2, while Table S2 gives the number of molecules and fields of view of different experiments.

Table S1: Characteristic lifetimes for different fluorophores and different enzymes.

	1/e lifetime (s) ± standard error (s)					
pH value	Cy3 GOC	Cy3 POC	Cy3 PCD	Cy5 GOC	Cy5 POC	Cy5 PCD
7	17.6 ±2.4	15.0 ±0.9	40.6 ±6.8	30.5 ±4.4	45.8 ±4.7	55.9 ±4.4
7.5	16.6 ±5.1	22.2 ±3.2	31.4 ±4.7	42.0 ±5.4	36.7 ±5.3	54.9 ±7.5
8	18.6 ±2.0	19.6 ±2.2	22.3 ±3.7	32.8 ±2.5	35.7 ±10.6	51.7 ±4.3

Table S2: Molecule numbers and field of view for different experimental conditions.

	Number of molecules (number of fields of view)					
pH value	Cy3 GOC	Cy3 POC	Cy3 PCD	Cy5 GOC	Cy5 POC	Cy5 PCD
7	3154 (6)	2812 (6)	2483 (5)	5568 (8)	4911 (7)	4244 (6)
7.5	3555 (7)	4065 (8)	2732 (5)	5263 (8)	4853 (8)	5430 (8)
8	3734 (7)	4028 (8)	3736 (7)	5043 (10)	3648 (7)	5081 (8)
	Streptavidin measurements					
	Cy3 GOC	Cy3 POC	Cy3 PCD	AlexaGOC	AlexaPOC	AlexaPCD
7.5	5292 (14)	4185 (10)	4443 (13)	4367 (7)	6305 (9)	8491 (14)
	Blinking measurements: Cy3 / Cy5 molecule numbers					
Enzyme	1 min	5 min	30 min	120 min		
GOC	654 / 516	473 / 465	585 / 509	562 / 420		
POC	526 / 498	618 / 504	615 / 547	570 / 594		
PCD	521 / 440	425 / 425	460 / 476	501 / 455		

Molecular blinking evaluation

Long-term (two-hour) studies of molecule lifetimes were performed in the same flow chamber with prism and focal spot left untouched for comparison between different enzymes, as in previous bleaching experiments (see Figure S1).
To assess blinking, we evaluated the per-frame fluorescence intensity for single-molecule fluorescence intensity traces. Checking at given times after flush in, as in Figure 3, panels c) to f), a molecule was counted with a frame as "off", if the molecular fluorescence intensity in that frame, I_{off}, was below 35% of the range between the maximum and minimum intensity of the entire trace, I_{max} and I_{min}, respectively, added to I_{min}. The limit was therefore

$$I_{off} < 0.35 \times (I_{max} - I_{min}) + I_{min} < I_{on}$$

This limit is indicated as dashed-line in panels c) to f) in Figure 3. To select for single fluorophores and exclude double-fluorophore-traces or imperfections in the PEG-surface, traces had to show a clear single bleaching step to be counted. Thus, molecules that lived through the entirety of the scan were excluded for lacking distinction from scattering dirt or aggregates. Stepwise bleaching or apparent steps/switching in fluorescence intensity resulting from two fluorophores in one spot also led to exclusion of a trace as two molecules might also interact in their blinking kinetics. Counting the number of frames in "off"-state, n_{off}, versus the frames in "on"-state, n_{on}, before bleaching, integrating over all selected molecules in the scan and dividing to obtain the ratio $R_{off/on} = \sum n_{off} \div \sum n_{on}$, led to the curves plotted in Fig. 3 a) and b) at given times. Repeating the analysis for different single-molecule movies recorded at the same time yielded a difference in $R_{off/on}$ of approximately 1 % (for POC and GOC, after 120 min). While many blinking events were most likely faster than the 30 millisecond temporal resolution available through our EMCCD camera, the low intensity frames of a given trace represent intervals of low average intensity and therefore high blinking or large fractions of molecular "off"-time.

SNARF-1 Measurements

Chamber design for SNARF-1 experiments

Experiments tracking the pH evolution via the SNARF-1 fluorescent probe were performed in a chamber closely resembling the single-molecular experimental flow chambers. The arrangement is depicted in Figure S3. The slide was placed inside the fluorescence spectrometer as is shown, in the path of the excitation light and adjusted manually to emit toward the emission grating. Due to an angular dependence of the excitation-emission-pathways, each placement of the slide required individual calibration with prepared buffers. After flushing in by suction through the tubing depicted in the lower part of Figure S3, spectra were acquired over 120 minutes.. The channel volume was between 10 and 20 µL and the reservoir-flow channel contact hole diameter was 0.75 mm.

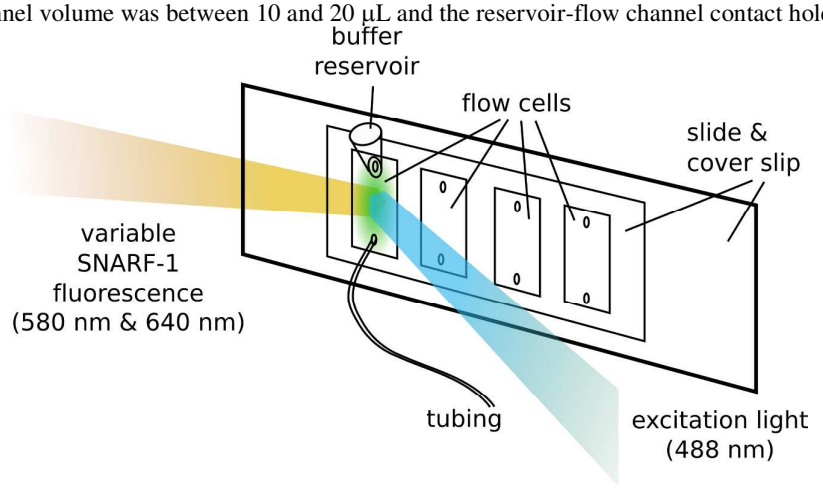


Fig. S3: Chamber arrangement for SNARF-1 experiments in Perkin-Elmer fluorescence spectrometer (not shown). The cuvette of conventional bulk experiments is replaced by our flow chamber arrangement resembling the single-molecule flow chambers (see Figure 2) to emulate the oxygen influx dynamics of these chambers.

Calibration of SNARF-1 and fitting of pH value

SNARF-1 fluorescence spectra were recorded on a Perkin Elmer LS55 fluorescence spectrometer with an excitation at 488 nm (10 nm slit) and the emission between 520 nm and 750 nm (15 nm slit). The flow cell was placed in the spectrometer as described in [14]. The test solution was mixed outside the fluorescence spectrometer and rapidly (<30 s) injected after application of the oxygen scavenging enzyme.

We obtained the pH values of a given solution including the SNARF-1 sensor by starting from

$$pH = pK_a + c\ log\left(\frac{R_{A/B} - R_{min}}{R_{max} - R_{A/B}}\right) + log\left(\frac{I_{acid}}{I_{base}}\right)$$

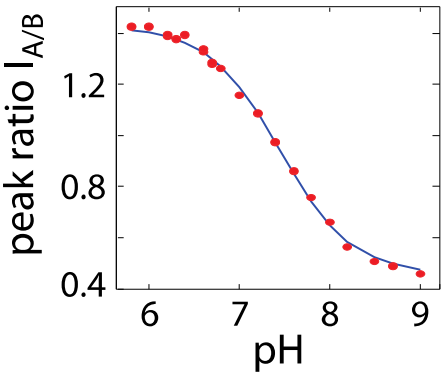
as given by Whitaker *et al.* [15]. We treat c and $log\left(\frac{I_{acid}}{I_{base}}\right)$ as fit parameters, and calibrate the sensor using a set of buffer solutions at defined pH values ranging from pH 6 to pH 9. From our set of calibration buffers we can then deduce a function $R_{A/B}$ dependent on the pH value as

$$R_{A/B} = \frac{R_{max} \exp\left(\frac{pH - pK_a - log\left(\frac{I_{acid}}{I_{base}}\right)}{c}\right) + R_{min}}{\left(1 + \exp\left[\frac{pH - pK_a - log\left(\frac{I_{acid}}{I_{base}}\right)}{c}\right]\right)}$$

as done by Shi *et al.* [14], using a pK_a=7.62 for SNARF-1. We use nonlinear least-squares fitting as implemented in MATLAB.

The calibration curve obtained by our set of defined pH buffers is shown in Figure S4. The flat edges at the lower pH values indicate the limits of the SNARF sensor sensitivity which we ran into using GOC and PCD at starting pH values of 7.5 and 7, respectively.

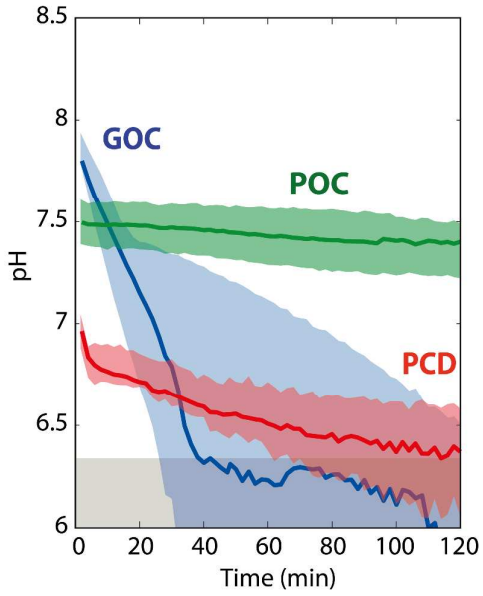
Fig. S4: Calibration curve of SNARF-1 peak ratios from a set of buffers with defined pH values, using the equations above.



Interval of Confidence for the SNARF-1 pH measurements

To assess the precision of our SNARF-1 experiments, we performed the same experiment 3 times (PCD and POC) and 4 times (GOC). Figure S5 depicts the resulting curves for PCD with a starting pH of 7 (red), POC with a starting pH of 7.5 (green) and GOC starting at pH 8 (blue). The lines are the average pH evolution with the transparent colored shades indicating the respective maximum and minimum measured values over the 3 experiments. The spread is largest for GOC (blue), which we attribute to different oxygen mass transport kinetics between experiments. This spread illustrate the lack of control on pH conditions when GOC is used. The gray area indicates again the limits of the pH sensitivity of SNARF-1, where measurements did not yield reliable differences in emission peak intensity ratios anymore.

Fig. S5: Intervals of confidence for SNARF-measurements. In general, GOC shows the strongest decline in pH, whereas POC remains mostly constant. PCD is depicted to drop from starting pH 7 (just outside the PCA buffering range) to about 6.5 and below, which also marks the limits of the experiment.



Enzyme performance dependence on glucose concentration or PCA presence

Bleaching lifetime experiments with PCD were performed in presence of glucose to keep buffer conditions as comparable as possible. As glucose is not required for PCD function, we tested its influence on PCD in an independent set of experiments. No clear evidence of hampered function due to glucose could be found, nor could a clear indication of reduced intensity or lifetime be discerned in our experiments (see Fig. S6).

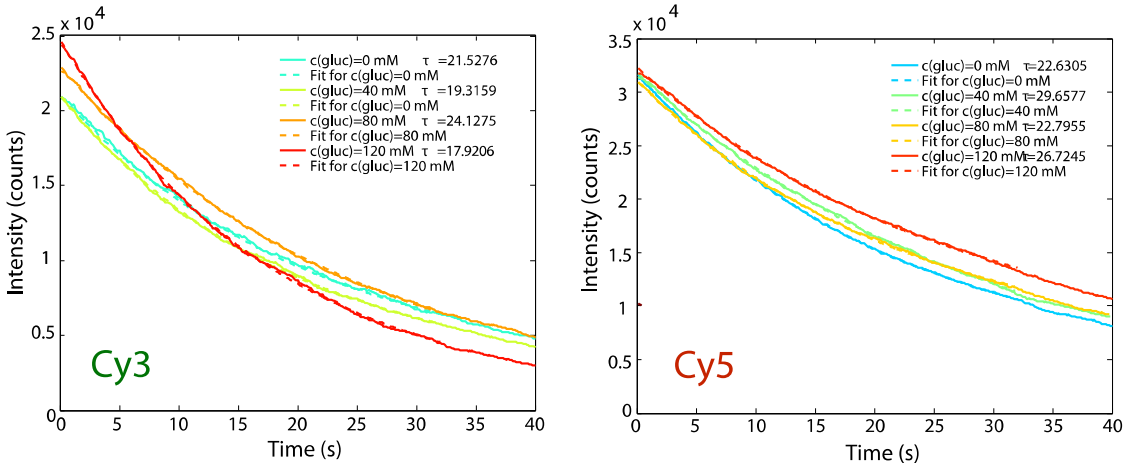


Fig. S6: Integrated single-molecule bleaching traces for Cy3 and Cy5 with PCD for varying glucose concentrations of 40mM, 80mM and 120mM and with corresponding characteristic lifetimes. No clear effect of glucose on PCD performance or overall brightness is apparent.

To test the influence of PCA on the fluorophore lifetimes, we performed comparative experiments with 24 mM PCA in the buffers of POC and GOC. These results are compared with PCD in a buffer containing 40 mM glucose, as shown in Fig. S6. Fig. S7 summarizes the PCA-buffer control experiment. The observed increase of the Cy3 lifetime with PCA in the buffers could not be reliably reproduced, but may, for GOC, be due to the additional buffering by PCA. All experiments were performed starting at pH 8.

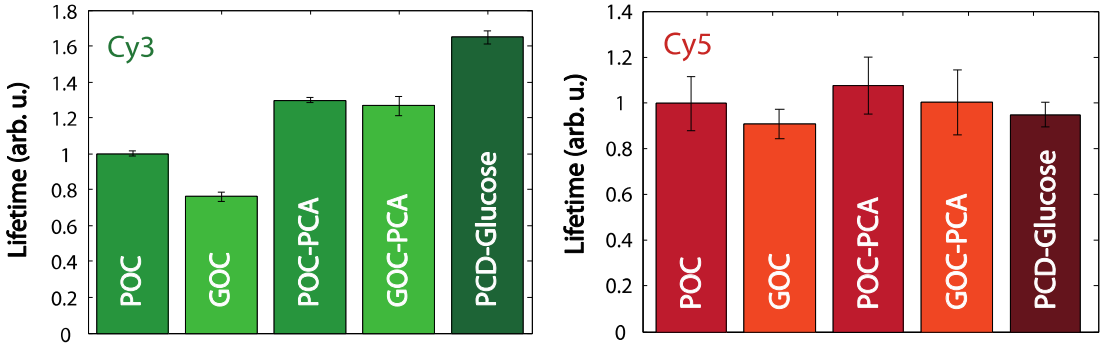


Fig. S7: Normalized lifetimes of Cy3 and Cy5 fluorophores for buffers containing no PCA (POC, GOC) and 24 mM PCA (POC-PCA, GOC-PCA, PCD-glucose). All buffers contained 40 mM glucose and had an initial pH 8.

Buffering capability of PCA

The online chemical computational tool SPARC [17] was used to calculate deprotonation curves over a changing pH value for PCA and included the same calculation with Tris as the compound for comparison (see Fig. S8). The gray area indicates the pH region in which many single-molecule experiments are performed.
While Shi *et al.*[14] did not observe a drop in pH value using PCD, we show this to be due to the buffering capacity of the PCA substrate. The pK_a values of PCA are 4.4, 8.7 and 10.7-13.00 [18]. Therefore, at pH 8 the drop in pH is limited because (i) PCA has a buffering effect at pH 8 (see pKa2) and (ii) the oxidation of one PCA to two carboxylic acid group result in less than two proton release since some of the phenolic hydroxyl groups are deprotonated (about 16% at pH 8 according to pK_a values). However both contributions are decreasing at lower pH values, therefore the effect on pH is more important for lower pH values.

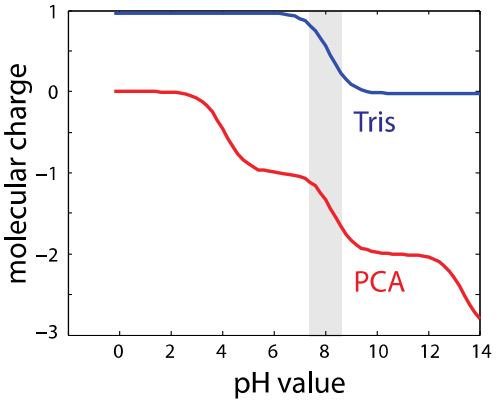


Fig. S8: Calculated molecular charge curves from SPARC [17] hinting toward a buffering capacity of PCA around pH 8.

Two-hour single-molecule lifetimes

Long-term stability of fluorophores in presence of POC, compared with GOC and PCD was measured over an interval of 120 minutes, at different times. The first measurement was performed after flushing the buffers into the experimental chamber and set as the 1-minute data point. Further experiments were performed after 3, 5, 15, 30, 60 and 120 minutes. The resulting average molecule lifetimes from exponential fitting are shown in Figure S9. Due to a change of focal parameters, for Cy5, POC is always compared to only one other enzyme in equal conditions, with PCD in Figure S9 b) and with GOC in c). All experiments were performed at a starting pH of 7.5, for Cy3 (see a) in Figure S9), PCD performs very well, while POC and GOC are comparable. For Cy5, in S9 b), a steep increase in lifetime from the first to the third minute indicates the initial removal of oxygen from the buffer mixing as seen in the pH drop curves. From 5 min after start of the experiment, the lifetime remains largely constant, albeit slightly better for PCD. Also comparing POC and GOC for Cy5 in Fig. S9 c), no clear difference between the two enzymes can be distinguished. We conclude that the molecular bleaching lifetime is largely unaffected over an experiment length of up to two hours. Effects of acidification are much more pronounced when looking at individual molecule blinking (see Fig. 3 a), b), e), and f) in the manuscript).

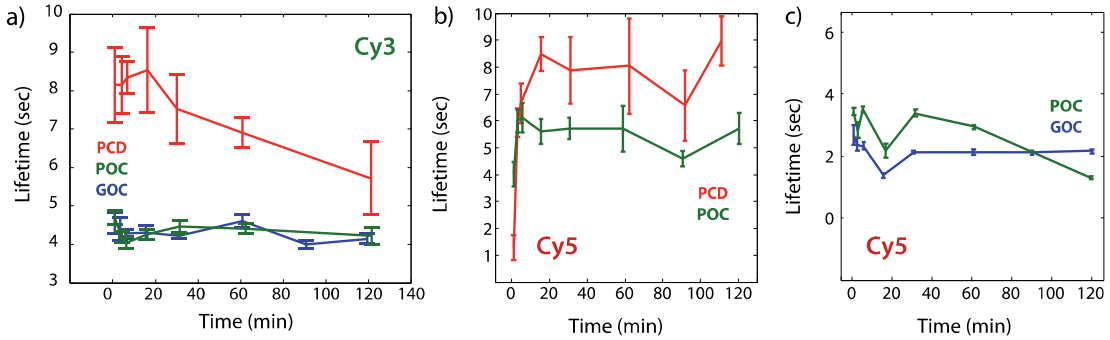


Fig. S9: Molecule bleaching lifetimes over 2 hours, all with starting pH 7.5, 10 mM Tris:HCl, 50 mM NaCl, saturated Trolox, for a) Cy3, b) Cy5, comparing PCD and POC, c) Cy5 comparing POC and GOC. The sharp rise of lifetime in the first two minutes in b) strongly hints at removal of initial oxygen from the buffer solution.

Test of enzyme compatibility with proteins (Streptavidin)

As many experiments involving single-molecule fluorescence work with proteins, we tested oxygen scavenging with POC, GOC and PCD with Streptavidin, labeled with Cy3 (from Sigma, catalog number S6402) and Alexa-647 (from Invitrogen, S-21374). No significant difference in molecule lifetime could be discerned (see Figure S9), suggesting that POC can indeed replace GOC or PCD in experiments with proteins as well as other biomolecules.

Fig. S10: Molecule lifetimes before bleaching for protein streptavidin labeled with Cy3 (green) and Alexa-647 (red). Cy3-lifetimes have been normalized with respect to the value obtained from PCD, Alexa-647-lifetimes have been normalized with respect to GOC.