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# Single Enzyme Electroanalysis

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## Abstract

Traditional studies of enzymatic activity rely on the combined kinetics of millions of enzyme molecules to produce a product, an experimental approach that may wash out heterogeneities that exist between individual enzymes. Evaluating these properties on an enzyme-by-enzyme basis represents an unambiguous means of elucidating heterogeneities; however, the quantification of enzymatic activity at the single-enzyme level is fundamentally limited by the maximum catalytic rate, $k_{cat}$, inherent to a given enzyme. For electrochemical methods measuring current, single enzymes must turn over greater than $10^7$ molecules per second to produce a measurable signal on the order of $10^{-12}$ A. Enzymes with this capability are extremely rare in nature, with typical $k_{cat}$ values for biologically relevant enzymes falling between 1 and 10000 $s^{-1}$. Thus, clever amplification strategies are necessary to electrochemically detect the vast majority of enzymes. This review details the progress toward the electroanalytical detection and evaluation of single enzyme kinetics largely focused on the nanoimpact method, a chronoamperometric detection strategy that monitors the change in the current-time profile associated with stochastic collisions of freely diffusing entities (*e.g.,* enzymes) onto a microelectrode or nanoelectrode surface. We discuss the experimental setups and methods developed in the last decade toward the quantification of single molecule enzymatic rates. Special emphasis is given to the limitations of measurement science in the observation of single enzyme activity and feasible methods of signal amplification with reasonable bandwidth.

## Introduction

The ultimate sensitivity in electroanalysis may be regarded as the specific detection of a single entity (*e.g.*, molecule, nanoparticle, enzyme, etc.).[1, 2] Pursuing this level of sensitivity is essential to progress our understanding of nature, as the study of single entities permits the observation of phenomena that may be washed out in ensemble measurements over many

entities. For example, it was not until the fluorescence of single proteins were quantified one-at-a-time that we realized protein fluorescence is not continuous and instead exhibits an on/off blinking phenomenon.[3-5]

Indeed, optical techniques, including spectroscopy (*e.g.,* fluorescence[6-13] and Förster resonance energy transfer[14]) and microscopy *(e.g.,* scanning optical microscopy[15]), towards the study of single enzymes have already elucidated interesting conformational and catalytic properties unobserved in bulk systems. Detection strategies using probe techniques, such as scanning tunneling microscopy (STM) and atomic force spectroscopy (AFM)[16, 17] have also emerged as powerful tools to investigate the mechanical properties of enzymes, down to the single molecule level.  Importantly, electrochemistry is uniquely suited to directly investigate mechanisms involving electron transfer, which is at the heart of many of these biocatalytic processes.  Electrochemical detection offers a rather inexpensive, simple, and label-free detection scheme that uses simple instrumentation to measure the transfer of electrons. As such, probe techniques have been coupled with electrochemical detection in methods including electrochemical scanning tunneling microscopy (EC-STM) and conducting probe atomic force microscopy (CP-AFM), where they can be used to investigate the electronic mechanisms of a single enzyme bound to the probe tip.[18] Nanopore[19] and field effect transistor methods[20, 21] have also been employed for the label-free, electrical detection of single biomolecules. These sensing strategies require the difficult fabrication of a very small tip or pore to achieve single molecule detection.

Here, we focus on the electrochemical detection of single enzymes by the nanoimpact approach. Throughout this review, "nanoimpact" refers to the chronoamperometric detection of enzymes freely diffusing in a substrate-containing solution, where detection occurs at a micro/nanoelectrode after a stochastic collision event of an individual enzyme onto the electrode surface. The detection is marked by a transient or permanent (steady-state) change to the current-time profile. This method is simple to employ and high throughput, providing replicate measurements in the same system, where the frequency of the individual measurements can be easily tuned by modulating the concentration of freely diffusing entities. However, based on the expected catalytic current and instrumental limitations, it does not seem feasible to detect these events, let alone extract kinetic information from the resultant current-time transients (*vide infra*), without employing an amplification strategy.[1, 22] We discuss in detail the surprising experimental results and suggest future directions for these analyses.

In a simple approximation, the current from a single enzyme's catalytic turnover rate ($k_{cat}$) can be described by:

$$i_{cat} = k_{cat}qnsN \qquad \textit{Equation 1}$$

where $q$ is the charge of an electron, $n$ is the number of electrons, $s$ is the number of active sites on an enzyme, and $N$ is the number of enzymes. A common enzyme used in bioanalytical devices is glucose oxidase, often reported to have a $k_{cat}$ around 500 s$^{-1}$ [23, 24], two active sites, and oxidizes glucose in a two-electron process. From the above equation, the catalytic current expected from a reaction involving a single enzyme is less than 1 fA. With common

electrochemical methods, changes in current can be detected in the picoampere regime, which would require the studied enzyme to have a turnover of on the order $10^7$ s$^{-1}$ to produce a detectable current, suggesting that almost all enzymes are undetectable without significant technological advances or creative amplification methods. Under a picoampere, shot noise sets the limit of quantitation.[25] In 2008, Lemay and colleagues demonstrated the abilities of state-of-the-art instrumentation by quantifying the minimum observable enzymatic current for a hydrogenase system. In this report, a catalytic current of 22 fA was obtained on a lithographically fabricated gold nanoelectrode (70 nm x 70 nm) with adsorbed enzymes by protein film voltammetry (PFV) in an oxygen-free environment. Based on previously reported $k_{cat}$ values (9000 – 1500 s$^{-1}$)[26] for the hydrogenase, and the observed current, the authors estimated to have measured a signal arising from 8 - 46 enzymes.[27] Since this time, five reports of single enzyme catalysis *via* electrochemical detection have emerged in the literature, all of which report catalytic activities several orders of magnitude higher than previously reported bulk experiments suggest (*i.e.*, superactivity). These reports are summarized in Table 1 and described below in two major categories: Nanoimpact and Confined-volume Entrapment.

**Nanoimpact of the single enzyme**

Though stochastic electrochemical experiments date back to the early 1990s[28-31], the technique was popularized by Lemay's 2004 landmark report describing the amperometric detection of individual latex microspheres at an ultramicroelectrode (UME).[32] Synonemously known as nanoparticle impact, or nanoimpact, this method exploits the small dimensions of ultramicroelectrodes and nanoelectrodes to isolate the signal resulting from the collision of a single entity, supporting the modern field of single entitiy electrochemistry.[1] These flexible and robust experiments provide insight into properties of colliding entities based on current transients generated by the blocking of a heterogeneous electrochemical reaction (Figure 1A, stepwise decrease in current), *via* eletrocatalytic amplification where electron transfer is mediated by the colliding entity (Figure 1B, stepwise or blip/spike increase in current), or the introduction of a redox species contained within a finite nanoreactor such as a water nanodroplet (Figure 1C, blip/spike in current).[2]
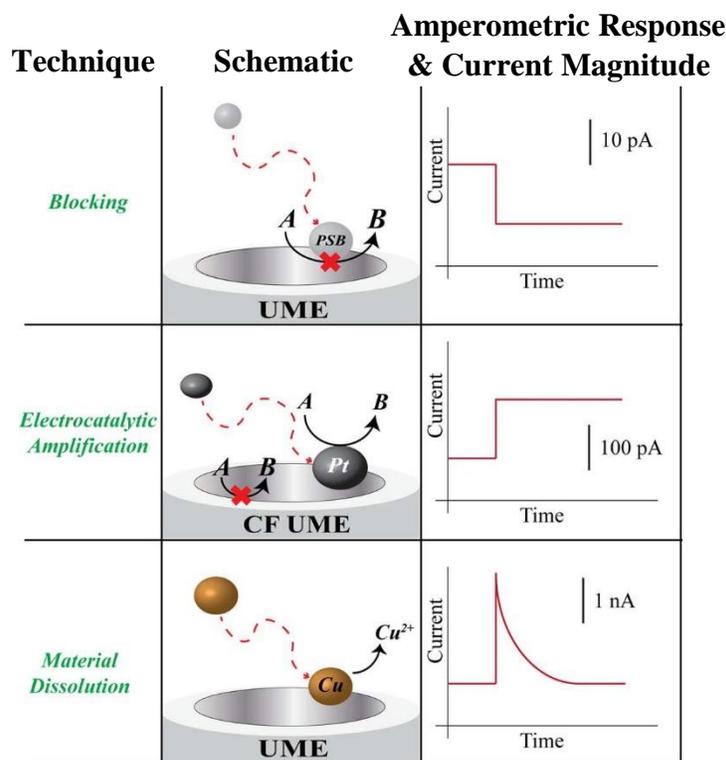
**Fig. 1.** Schematic representation of typical nanoimpact techniques for single entity detection, including: insulating nanoparticle blocking, nanoparticle electrocatalysis, and nanoparticle electrolysis. Adapted with permission from S.; Dick, J. E., Review—Electrochemistry's Potential to Reach the Ultimate Sensitivity in Measurement Science. *Journal of The Electrochemical Society* 2019, *167* (3), 037505. Copyright (2019) American Chemical Society.

The nanoimpact blocking technqiue has been extended to bio-analysis, where a wide range of biological species (*i.e.*, "soft" nanoparticles) have been studied, including DNA[31], RNA[33], viruses[34], bacteria[35], and enzymes.[36] This method allows for simple, *in situ*, direct detection of single entities in solution. Using the blocking technique, the concentration and size of single enzyme molecules may be quantified after colliding with ultramicroelectrodes, but these experiments give no information on single enzyme reactivity.[36]

Electrocatalytic amplification is the most relevant method for the study of single enzyme kinetics. Of the five reports regarding electrochemical measurements of single enzyme activity, four used the nanoimpact method in this mode. While the following discussion indicates that interesting behavior is observed with the nanoimpact method, further experimentation and independent validation must be performed to understand whether or not the authors are elucidating reactivity if single enzymes.

In 2016, Sekretaryova et al. reported the first single enzyme electrochemical measurement.[37] In their experiments, freely diffusing laccase enzymes collided with a gold ultramicroelectrode surface and adsorbed, allowing electrons to be relayed to the enzyme *via* direct electron transfer and enabling the enzymatic reduction of oxygen to water in a four electron process. They obtained amperometric *i-t* traces where collisions of single enzyme molecules were detected as current spikes on the order of picoamperes (Fig.2a). The height of the resultant current spikes (Fig. 2b-c) was used to determine individul $k_{cat}$ values for each colliding enzyme, where the average $k_{cat}$ was calculated to be $3.8 \times 10^5$ s$^{-1}$ (Fig. 2d).

Importantly, one might expect that the arrival of an enzyme to the electrode surface would produce a stepwise increase in the observed current due to the continuous turnover of substrate (oxygen) to product (water) by enzyme catalysis. However, Sekretaryova et al. observed and analyzed current spikes, which they hypothesized did not attain steady-state due to rapid enzyme denaturation at the electrode surface, effectively shutting off the reaction. It should be noted that this system lacks selectivity, and the resultant blip-type current events offer little information as to the mechanisms of electron transfer detected at the electrode surface. Here, the current events are attributed to the direct electron transfer of electrons from the electrode surface to a single laccase, where this claim is supported by previous reports of direct catalysis of oxygen reduction on various gold surfaces[38, 39], and the experimental application of a potential +0.210 V versus NHE, a potential that drives no faradaic current in the absence of the enzyme and is insufficient to oxidize the expected enzymatic product ($H_2O$). However, correlated experiments are absolutely necessary moving forward to validate these claims.
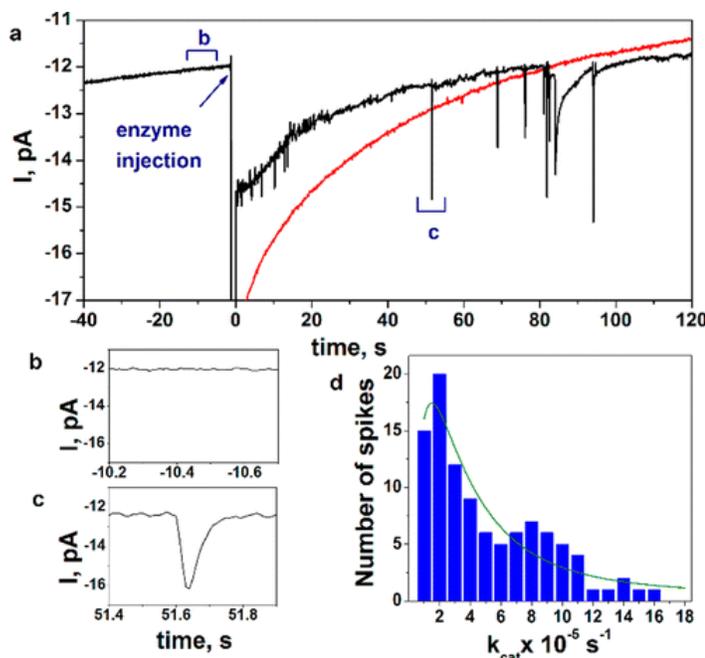


**Fig. 2.** (a). Amperometric $i$–$t$ curves in the presence of 0.1 U mL$^{-1}$ enzyme solution in a deoxygenated solution (red) and in an oxygen containing solution (black). The AuUME was biased at +0.210 V vs NHE over the entire experimental time. (b and c) Magnified $i$–$t$ curves, of the parts indicated in a, showing the background and a clear spike-shaped response, respectively. (d) Distribution of turnover rates of the enzyme molecules, calculated from the collision experiment using the peak height value, fitted by log-normal statistics. The mean value is $(3.8 \pm 1.1) \times 10^5$ s$^{-1}$. Experimental conditions were: pH 5.0 (0.1 M acetate buffer), $T = 20$ °C. Reprinted with permission from Sekretaryova, A. N.; Vagin, M. Y.; Turner, A. P. F.; Eriksson, M., Electrocatalytic Currents from Single Enzyme Molecules. *Journal of the American Chemical Society* 2016, *138* (8), 2504-2507. Copyright 2016 American Chemical Society.

Later that same year, using a similar method, the Zhan group detected horseradish hydroperoxidase on modified gold nanoelectrodes.[40] Both papers report current spikes on the order of picoamperes, large diversity in current response, and $k_{cat}$ values several orders of magnitude higher than previously reported the the literature (Table 1).

We note that lacking in these early papers is a key control experiment that must be performed in nano-impact measurements: the frequency with which enzymes collide with the electrode should be studied as a function of enzyme concentration. This rather simple control experiment unambiguously validates that the signal observed is coming from the analyte being added. Additionally, we emphasize that for nanoimpact measurements that are transient in nature (*i.e.*, a large change in current followed by a return to baseline), measurement bandwidth (sampling frequency) is a critical consideration. For instance, the faster one samples, the higher a current 'spike' will be in the amperometric response. Thus, no meaningful knowledge can be gained from a current 'spike' magnitude without full consideration of the measurement sampling frequency.[41] Filters are also often very important, as they can drastically change the shape of the transient response.[42]

In response to the detection of single enzymes *via* collisions on ultramicroelectrodes, Compton's group presented a theoretical argument with computational analysis of the electrode response for a freely diffusing enzyme with an electroactive product.[41] They discuss the challenges and complications arising from the conversion of the analog measurement to the recorded current response, with special attention given to how filtering can affect the signal. As a model system they used catalase, an enzyme that decomposes hydrogen peroxide to oxygen. A carbon ultramicroelectrode was biased at -1.0 V vs. SCE, a potential sufficient to reduce oxygen. The experimental *i-t* amperograms show current spikes with heights on the order of $10^{-10}$ A. Using a literature $k_{cat}$ ($7.24 \times 10^5$ s$^{-1}$)[43,] and the simple estimation ($I_0 = k_{cat}e_0$) where the expected current ($I_0$) is proportional to the enzyme turnover number ($k_{cat}$) multiplied by the charge of an electron ($e_0 = 1.60 \times 10^{-19}$ C), the predicted current magnitude is $1.16 \times 10^{-13}$ A for a single catalase enzyme. While Compton's group concluded that the observed spikes cannot be attributed to single enzyme activity where they are detecting the products of the catalase reaction, they indicated that the experimental observation might be explained by a direct electron transfer mechanism. This paper gave rise to a lively debate, adding to the literature discussion about the possibility of electrochemical single enzyme detection.[22]

In the year following it's publication, Sekretaryova and Compton published several correspondances to this paper, providing additional arguments for consideration towards the discussion: can the nanoimpact method detect the activity of a single enzyme?

Sekretaryova *et al*. argued against Compton's comparision to a $k_{cat}$ determined in bulk studies, and the assumption used in their theoretical modeling, namely that the enzyme steadily transforms substrate into product.[44] Referencing literature that report conformational fluctuations and dynamics in enzymatic catalysis[7, 45], they assert that the catalytic constant ($k_{cat}$) obtained from single molecule experiments should be expected to be different than a catalytic constant determined in a bulk experiment. Additionally, Sekretaryova et al. discuss the effects of using an electrode biased at a significant overpotential to perform direct electron transfer to an adsorbed laccase enzyme, acting as the reductive enzymatic substrate. Where traditional ensemble experiments are obtained in equilbrium systems, in the laccase model, because of the influence of overpotential, the experimental conditions are likely not at equilibrium. Sekretaryova suggested that the use of overpotential in place of a diffusing chemical substrate shifts the enzymatic rate dependence to either the intramolecular electron transfer or the subsequent reaction with oxygen. In terms of the catalase model, Sekretaryova indicated uncertainties in the experimental conditions, such as the possible influence of direct electron

transfer, avalanche reductions of oxygen nanobubbles, and contaminents present in the commercial protein.

While Compton's group largely agreed with these points, assuming the detection is direct electron transfer, they clarified that their model considered only the mechansim of probing the catalase enzymatic product. They concluded that while the direct electrochemistry of enzyme collisions is a plausible explanation to the experimental observations, quantitative analysis against a suitable model for impact frequency, size, and shape along with more kinetic data are needed to support these nanoimpact claims.

Compton's group published two developments toward a model that predicts the experimentally observed current spikes for the catalase system. They first presented a computational model that demonstrated that the nanoimpact method can detect freely diffusing enzymes if a small electrode is used, there is good bandwidth, and the enzyme has a high turnover rate, where these findings apply to enzymes operating at constant turnover. However, in this model, the experimental data still showed significant discrepancy in magnitude and duration of response.[46] The experimental $i$-$t$ amperograms show current spikes with heights on the order of $10^{-10}$ A and average width (at half spike) to be 0.0054 s (Fig. 3a-b). In direct comparison, the simulation of the same system indicated an average spike height three orders of magnitude smaller ($10^{-13}$ A) and an average half-spike width two orders of magnitude larger (Fig. 3c-d). It is noted that the recorded spike shape is, in part, determined by the sampling frequency and the potentiostat filter. As the expected current is on the order of $10^{-13}$ A, a signal too weak to be resolved from the background in a real experiment, they maintain the position that these current spikes cannot be attributed to single enzyme activity where they are detecting the enzymatic products.
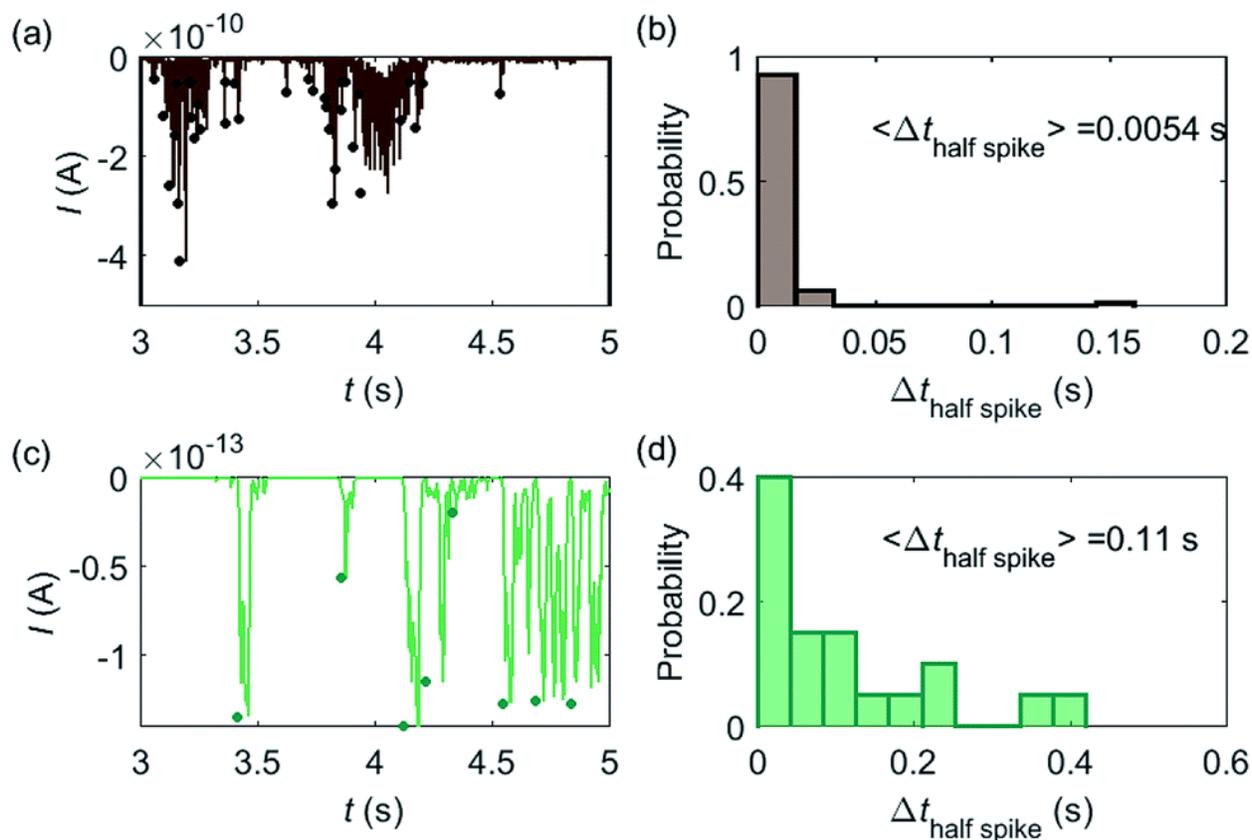
**Fig. 3.** (a) is an experimentally found chronoamperogram of 9 pM catalase in a 100 mM hydrogen peroxide solution at an applied potential of −1.0 V *versus* SCE, measured at a 5 μm radius microdisc electrode; (b) is the corresponding histogram of the half-spike width of the current spikes in (a); (c) and (d) are the simulated chronoamperograms (see text) referring to single catalase detection at a microdisc electrode and the corresponding histogram of the half-spike width. The total recording time is 50 s for both experiment and simulation. The simulation space is from 5 nm to 10 μm. Reproduced from Lin, C.; Kätelhön, E.; Sepunaru, L.; Compton, R. G., Understanding single enzyme activity via the nano-impact technique. *Chemical Science* 2017, *8* (9), 6423-6432 - Published by The Royal Society of Chemistry.

Compton's final model, presented about 1.5 years after their initial report, incorporated fast-slow activity fluctuations to account for the observed higher $k_{cat}$ in single catalase studies. In the simulation, they use two major parameters to descibe the fluctuations, $P$ and $\Delta t_{switch}$, representing the probability of the enzyme being active and the minimum time spent in one state before the next possible swith, respectively. Chronoamperograms normalized by $-k_{cat}e_0$ illustrate the deviation of the single enzyme catalysis from their average prediction (Fig. 4a-b) and are compared to simulated fluctuations in enzyme catalysis (Fig. 4c-d). The experimental data show kinetic enhancements up to three orders of magnitude and the simulations suggest that dynamic fluctuations can lead to temporary enhancements of this magnitude. The group concluded that the catalytic ability of single enzymes can be temporarily much higher than expected by the time-averaged Michaelis-Menten model, and these differences can be observed with the nanoimpact method.[47]
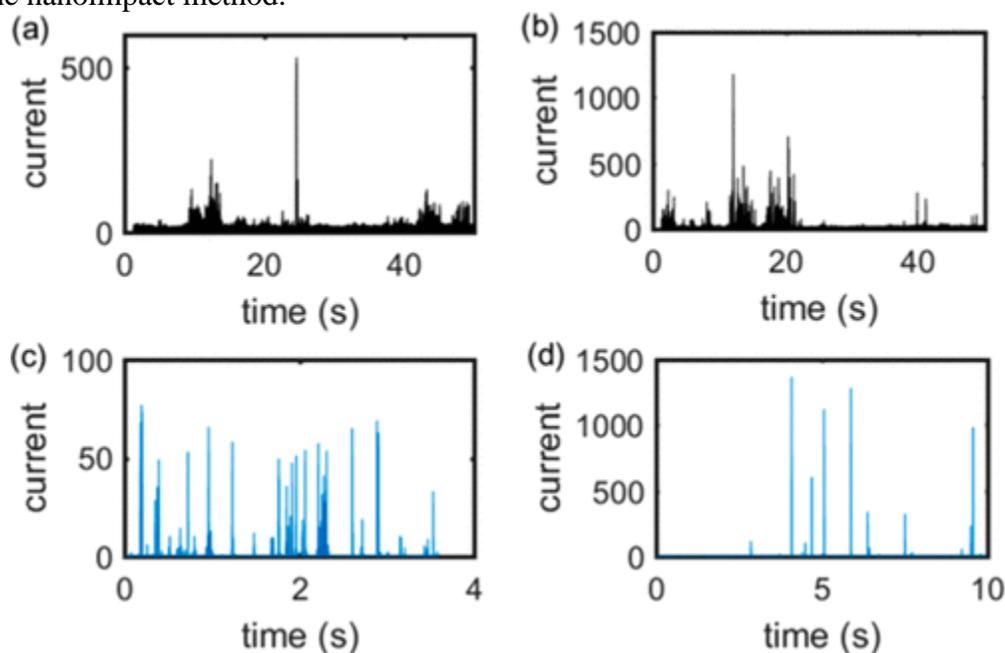


**Fig. 4.** (a,b) Experimental single catalase signals from two independent measurements. (c,d) Simulated single enzyme signals evaluated for two different fluctuation kinetics, where (c) $P = 0.01$, $\Delta t_{switch} = 1.0$ ms; (d) $P = 0.0005$, $\Delta t_{switch} = 0.1$ ms. In the simulation, the diffusion coefficient of O$_2$ is $D_{O2} = 1.96 \times 10^{-9}$ m$^2$ s$^{-1}$; the electrode radius $r_{el} = 5.0$ μm; $\langle k_{cat} \rangle = 7.4 \times 10^5$ s$^{-1}$. The current in both experiment and simulation is not the original current but normalized by $-\langle k_{cat} \rangle e_0$. Reprinted with permission from Lin, C.; Sepunaru, L.; Kätelhön, E.; Compton, R. G., Electrochemistry of Single Enzymes: Fluctuations of Catalase Activities. *The Journal of Physical Chemistry Letters* 2018, *9* (11), 2814-2817. Copyright 2018 American Chemical Society.

The debate between the two groups sparked others to become engaged in the discussion. The Foord group also followed-up on Compton's 2016 notion that the impacts could not be

attributed to the detection of the catalase product but may be explained by direct electron transfer. While they used a very similar experiment to Compton's, they employed a custom boron-doped diamond ultramicroelectrode, as the substrate has been shown to interact with catalase and achieve low background currents.[48] The ultramicroelectrode was held at a potential insufficient to reduce hydrogen peroxide or oxygen (-0.2 V vs. Ag/AgCl) and current spikes with magnitudes of 10s of picoamperes were observed, suggesting that direct electron transfer was occurring between the enzyme and the ultramicroelectrode (Fig. 5a-c). The frequency of collisions was analyzed and determined to be consistent with steady-state, diffusion-controlled flux of particles to an ultramicroelectrode surface (Fig. 5d). Additionally, they observed an increase in spike frequency with the addition of the substrate (hydrogen peroxide), which was attributed to a >30% increase in enzyme diffusion based on correlated fluorescence spectroscopy measurements of fluorescently labeled catalase. The authors did not attempt a kinetic analysis using the observed spike height.
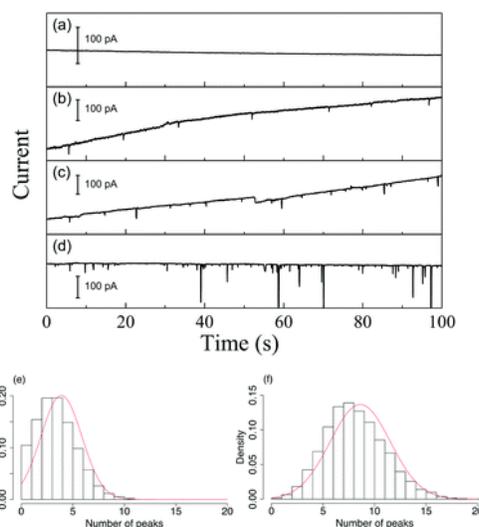


**Fig. 5.** (a–d) Typical *i–t* curve at BDD-UME at −0.2 V in a solution containing (a) 0.1 M PBS and (b) 5 pM catalase; (c) 10 pM catalase; and (d) 10 pM catalase mixed with 100 mM $H_2O_2$; (e and f) histogram of detected number of spikes shows the average number of spikes per 10 s, corresponding to a sample size of 1000 observations and 10000 replicates, randomly sampled over the duration of the experiment (2000 s). A fitted normal distribution yields an average spike count of 3.8 (standard error of the mean 0.02) for 10 pM catalase in (e) and 8.6 (standard error of the mean 0.02) for 10 pM with $H_2O_2$ in (f). Reproduced from Jiang, L.; Santiago, I.; Foord, J., Observation of nanoimpact events of catalase on diamond ultramicroelectrodes by direct electron transfer. *Chemical Communications* 2017, *53* (59), 8332-8335 with permission from The Royal Society of Chemistry.

## Confined-volume entrapment of single enzymes

While kinetic amplification due to nanoconfinement has not yet been addressed electrochemically, the method deserves discussion given electrochemistry's ability to rigorously quantify kinetics in nanoconfined volumes.[49, 50] These types of experiments might also provide insight into amplification observed from the nanoimpact method.

Trapping small numbers of enzymes is a fairly common method of isolation, dating back to the famous 1961 experiment where Rotman encapsulated single *β-d*-galactosidase enzymes in

microdroplets and related the confined fluorescence intensity to a Poisson distribution.[51] Confinement allows for the measurement of a single enzyme molecule moving freely in solution. To improve the reliability of the method, there has been a significant effort to increase the uniformity of the micro/nanoreactors, and modern measurements have since been used to observe heterogeneity on the single molecule level.[52-55] In 2016, the Zhang group claimed to detect single alkaline phosphatase enzymes using enzyme-induced metallization for signal amplification and digital analysis based on Poisson statistics.[56] The authors trapped enzymes in reactors on a microelectrode array where the reaction volume was controlled to include no more than one enzyme per microelectrode. They calculated a Michaelis Menten constant similar to the ensemble measurement. While they do not calculate the activity or $k_{cat}$, they qualitatively note that there is obvious activity variation among single enzymes (Fig. 6).

While there is only one report of confined volume single enzyme detection, there are several articles in the literature that developed nanofluidic devices in conjunction with amperometric detection of enzyme catalysis.[49, 50, 57] In 2014, Lemay's group immobilized tyrosinase enzymes in a nanofluidic device and amplified the current by redox cycling. While the authors believe single enzyme detection may be possible using this method, the limit of detection in these experiments was about 5000 enzymes.[58]
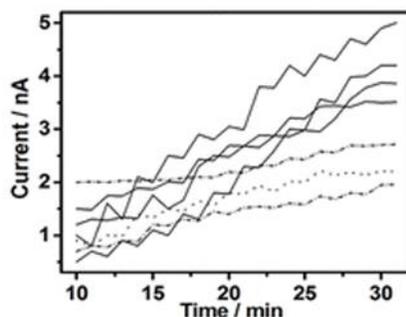


**Fig. 6.** Current-time traces of single ALD enzymes showing activity variation. Reprinted with permission from from Wu, Z.; Zhou, C.-H.; Pan, L.-J.; Zeng, T.; Zhu, L.; Pang, D.-W.; Zhang, Z.-L., Reliable Digital Single Molecule Electrochemistry for Ultrasensitive Alkaline Phosphatase Detection. *Analytical Chemistry* 2016, *88* (18), 9166-9172. Copyright 2018 American Chemical Society.

**Table 1**: Summary of Electrochemical Measurements of a Single Enzyme

| Group | Sekreyaryova[37] | Zhan[40] | Zhang[56] | Foord[48] | Compton[41, 46, 47] |
|---|---|---|---|---|---|
| Year | 2016 | 2016 | 2016 | 2017 | 2016-2018 |
| Enzyme | Laccase | Horseradish peroxidase | Alkaline phosphatase | Catalase | Catalase |

| Method of isolation | Nanoimpact | Nanoimpact | Confined volume | Nanoimpact | Nanoimapct |
|---|---|---|---|---|---|
| Electrode | Gold UME | Gold nanoelectrode | Gold UME | Boron-doped diamond UME | Carbon UME |
| Electroactive species | DET enzyme | DET enzyme | Silver | DET enzyme | Oxygen |
| Technique | Amperometry | Amperometry | Amperometry | Amperometry | Amperometry |
| Current magnitude | pA | pA | nA | 10-100 pA | 10-100 pA |
| Calculated $k_{cat}$ | $3.8 \times 10^5$ s$^{-1}$ | $6.2 \pm 1.9 \times 10^5$ s$^{-1}$ | Not reported | Not reported | $10^8$ s$^{-1}$ |
| Comparative bulk $k_{cat}$ | 500 s$^{-1}$ [59] | 1 s$^{-1}$ [60] | 83 s$^{-1}$ [61] | $7.24 \times 10^5$ s$^{-1}$ [43] | $7.24 \times 10^5$ s$^{-1}$ [43] |

### Conclusions and Outlook

This minireview highlights the experimental observations of single enzyme electroanalysis, mainly focused on the nanoimpact method. Moving forward, rigorous steps must be taken to validate experimental results. In particular: 1.) In the nanoimpact experiments, the frequency of collision must be studied as a function of enzyme concentration. A rigorous frequency analysis can also help indicate if practitioners are observing the current due to a single enzyme or a large aggregate. 2.) The steady-state limiting current, $i_{lim}$, to a sphere on a plane ($i_{lim} = 4\pi ln(2)nFDCa$, where $n$ is the number of electrons, $F$ is Faraday's Constant, $D$ is the diffusion coefficient of substrate, $C$ is substrate concentration, and $a$ is the electrode – or enzyme – radius) is not an accurate predictor of the current one should expect from a single catalytic enzyme. 3.) While experimentally difficult, correlated microscopy measurements are necessary to offer experimental validation while answering important mass transfer questions. 4.) One must recognize that half a century ago, the measurement of picoampere currents was considered a nearly insurmountable task. As science progresses forward, the ability to measure sub-femtoampere currents with reasonable bandwidth may elucidate single enzyme reactivity in a more robust way. While at present it is not clear how this will be achieved, perhaps room temperature single electron transistors will play a role.[62] 5.) Finally, just as electrocatalytic amplification allows insight into a single nanoparticles heterogeneous reactivity, clever amplification strategies are needed to observe single enzyme. Given the literature behind enhanced rates under nanoconfinement, aqueous nanodroplet nanoelectrochemistry may present interesting possibilities in single enzyme electroanalysis.

An undeniable reality of single enzyme nanoimpact experiments is that most groups have observed much higher enzyme activity compared to bulk enzymatic rate values. However, it is important to note that just as the absence of evidence is not evidence of absence, the presence of evidence is not the evidence of a single enzyme. Moving forward, groups must independently

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validate the observation of single enzyme turnover. Further experimentation is also necessary to rigorously understand the nature of the electrode surface and the environment in which the single enzyme is reacting.

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