Machine learning–based inverse design for electrochemically controlled microscopic gradients of O2 and H2O2

Yi Chen,1 Jingyu Wang1,1, Benjamin B. Hoar4,5, Shengtao Lu2, and Chong Liu2,2

A fundamental understanding of extracellular microenvironments of O2 and reactive oxygen species (ROS) such as H2O2, ubiquitous in microbiology, demands high-throughput methods of mimicking, controlling, and perturbing gradients of O2 and H2O2 at microscopic scale with high spatiotemporal precision. However, there is a paucity of high-throughput strategies of microenvironment design, and it remains challenging to achieve O2 and H2O2 heterogeneities with microbiologically desirable spatiotemporal resolutions. Here, we report the inverse design, based on machine learning (ML), of electrochemically generated microscopic O2 and H2O2 profiles relevant for microbiology. Microwire arrays with suitably designed electrochemical catalysts enable the independent control of O2 and H2O2 profiles with spatial resolution of \( \sim 10^5 \) μm and temporal resolution of \( \sim 10^3 \) s. Neural networks aided by data augmentation inversely design the experimental conditions needed for targeted O2 and H2O2 microenvironments while being two orders of magnitude faster than experimental explorations. Interfacing ML-based inverse design with electrochemically controlled concentration heterogeneity creates a viable fast-response platform toward better understanding the extracellular space with desirable spatiotemporal control.

Ubiquitous spatiotemporal heterogeneity of natural environments fosters the diverse and fascinating biology that our world embraces, and motivates researchers to mimic natural environments with high spatiotemporal resolution (1–5). Given their close relevance in biochemical metabolisms, dioxygen (O2) and hydrogen peroxide (H2O2) as a surrogate of reactive oxygen species (ROS) are two ubiquitous biologically relevant species in extracellular medium (1, 6). Their extracellular spatial and temporal distributions, particularly at the microscopic scale ranging from 1 μm to 100 μm (7–11), are critical for signal transduction, protein expression, biochemical redox balance, and regulation for cellular metabolism with extensive ecological, environmental, and biomedical implications (Fig. 1A) (1, 3, 8–13). A programmable creation of the spatiotemporal concentration profiles of O2 and H2O2 offers the freedom to mimic, control, and perturb the microenvironments of O2 and H2O2 and hence advance our understanding in microbiology.

Despite recent progress (14–18), there remain major technical challenges, particularly in the achievable spatiotemporal resolution and high-throughput design of concentration profiles to suit a plethora of scenarios in microbiology. Approaches based on microfluidics and hydrogels have been able to achieve concentration gradients of O2 and H2O2 through the provision of either O2/H2O2 source (14, 19–21), O2/H2O2 scavenging agents (15, 22, 23), or a combination of both (24) across liquid-impermeable barriers such as agar layers or polymeric thin films (25, 26). Yet such approaches, dependent on passive mass transport and diffusion across more than \( 10^5 \) μm, are inherently incapable of achieving spatial features of less than 100 μm and temporal resolution smaller than \( 10^3 \) s, the prerequisites to investigate microbiology at cluster or single-cell levels (10–12). Moreover, the large variations of extracellular O2 and H2O2 gradients in different microbial systems demand an inverse design strategy, which, with minimal expenditure, quickly programs a desired concentration profile catering to a specific biological scenario (2–5). The current lack of inverse design protocol impedes the adoption of controllable extracellular heterogeneity to mimic and investigate microbial systems that are of environmental, biomedical, and sustainability-related significance.

We envision that the integration of electrochemically generated concentration gradients with inverse design based on machine learning (ML) will address the aforementioned challenges (Fig. 1B). Electrochemistry offers a venue for transducing electric signals into microscopic concentration profiles within \( \sim 10^5 \) μm to \( \sim 10^2 \) μm away from electrodes’ surface, following the specific electrode reaction kinetics and the mass transport governing equations in the liquid phase (27). Proper designs of electrodes’
microscopic spatial arrangement and electrochemical kinetics lead to concentration gradients that are spatiotemporally programmable by time-dependent electric signals of varying voltages (28). Such benefits of electrochemically generated concentration gradients lead us to employ electrochemistry as a tool to spatiotemporally control the concentration profiles in the extracellular medium. In one example, we found that wire arrays electrochemically active toward O2 reduction create anoxic microenvironment about 20 μm away from the aerobic external bulk environments, modulate the size and extent of O2 depletion in the anoxic microenvironment by the wire array’s morphology and applied electrochemical potential (Eapp), and hence enable O2-sensitive rhizobial N2 fixation in ambient air powered by renewable electricity (29). Moreover, while not reported before as far as we know, electrochemically generated concentration heterogeneity is commensurate with ML-based inverse design (30, 31), thanks to the mathematically well-defined electrochemical processes that can be numerically simulated (32, 33). We recently reported neural networks, trained by numerically simulated data, that explore the influence of electrode geometry on electrochemical N2 fixation and achieve optimized morphologies of wire array electrodes untenable without such an ML-based strategy (34). An inverse design for the electrochemically generated gradients will quickly program desirable microenvironments of O2 and ROS with high spatiotemporal resolutions, thanks to the well-reported electrochemical transformation related to O2 and H2O2 with high electrochemical selectivity (35, 36).

In this work, we report an inverse design based on neural networks for independent electrochemical creation of O2 and ROS microscopic gradients that are relevant, and mimic their extracellular heterogeneities in microbial systems. We hypothesize that careful design of electrocatalysis of O2 reduction reaction (ORR) can either facilitate four-electron ORR on Pt electrocatalyst for a controllable O2 spatiotemporal profile or promote two-electron ORR on Au electrocatalyst for a programmable generation of H2O2 gradient without significantly perturbing the O2 one, thanks to their concentration differences in biological mediums (∼10–1 M to ∼10 M for H2O2 and ∼10 M to ∼10 M for O2) (2, 7–11). Electrochemically active microwire array electrodes as exemplary model systems (Fig. 1C) are experimentally shown to achieve tunable heterogeneities of O2 and H2O2 independently, with spatial resolution of ∼1 μm and temporal resolution of ∼10 s, and are suitable as a platform for independently perturbing biologically relevant O2 and H2O2 profiles in microbial systems. We further established and experimentally validated two neural networks that inversely design the wire array electrodes’ morphologies toward targeted microenvironments of O2 and H2O2, respectively, which is at least one order of magnitude faster than trial-and-error numerical simulation and two orders of magnitude faster than experimental explorations. The demonstrated inverse design of electrochemically generated controlled gradients not only demonstrates a full electrochemical control of concentration profiles in an electrode’s proximity but also establishes an approach of spatiotemporally mimicking and perturbing extracellular space guided by artificial intelligence.

**Results**

**Wire Array Electrodes for Electrochemical Generation of O2 and H2O2 Gradients.** We applied a microwire electrode array loaded with selective ORR electrocatalysts to establish customizable O2 or H2O2 gradients (Fig. 1C). Si-based microwire arrays in a square lattice were constructed through photolithography and reactive ion etching in a five-step fabrication process (see Materials and Methods). After thermal annealing to generate an electrically insulating oxide layer, indium-doped tin oxide (ITO)
of 500 nm was deposited via sputtering near conformally onto the wire array, followed by the deposition of about 7 nm of Pt and Au for the generation of O₂ and H₂O₂ gradients via selective O₂RR, respectively. Here the deposition of the electrically conducted ITO layer ensures a uniform distribution of the applied electrochemical potential (E_{appl}). We employed Pt as the selective electrocatalysts of four-electron O₂RR and Au for two-electron O₂RR, and employed Au for Au-loaded wire array electrode to the reaction of O₂ reduction up to 50% at 0.5 V vs. RHE. Thanks to its selectivity of H₂O₂ generation for wire array (SI Appendix, Fig. S4) displayed a selectivity of H₂O₂ generation from O₂ reduction up to 50% at 0.5 V vs. RHE. Thanks to the reaction–diffusion model in the electrolyte and the electrochemical boundary conditions imposed by the microwire morphology, particularly a strictly O₂-free local environment in aerated PBS solution, linear scan voltammograms (20 mV/s) of the deposited Pt-coated wire array electrode under confocal microscopy, assisted by the corresponding calibration curves (SI Appendix, Figs. S9–S12; see Materials and Methods). When k = (15, 4, 50) for the wire array (Fig. 2B and C), the three-dimensional I_p mapping was recorded in a time sequence when the Pt-coated wire array was initially under an open-circuit condition (t < 15 s), subject to an electrochemical potential (E_{appl} = 0.5 V vs. RHE) from t = 15 and 45 s, and reverted back to the open-circuit condition when t > 45 s (see Materials and Methods). The side views of the three-dimensional I_p mapping were displayed when E_{appl} was initially absent (t = 0 s), E_{appl} = 0.5 V vs. RHE (t = 16 s), and E_{appl} was absent again at t = 48 s (“t = 0 sec,” “t = 16 sec,” and “t = 48 sec,” respectively, in Fig. 2B). The intensity of I_p was noticeably stronger within the wire array when E_{appl} = 0.5 V vs. RHE in comparison to the I_p values under the open-circuit conditions before and after the presence of E_{appl}. As I_p is inversely proportional to the local values of [O₂], this observation qualitatively suggested a decrease of [O₂], and hence an O₂ gradient covering the wire array region with microscopic resolution under a reductive electrochemical potential.

The averaged [O₂] values ([O₂]avg) at different distances above the bottom of the wire array z = 5, 50, and 100 μm were quantified and are displayed as a function of time t in Fig. 2C. While a negligible change of [O₂]avg was recorded at z = 100 μm (black in Fig. 2C), which was quite far away from the wire array, at z = 5 and 50 μm (blue and red, respectively, in Fig. 2C), significant changes of [O₂]avg up to a complete anoxic condition were observed, concurrent with the temporal presence of E_{appl}. Such data suggest that the established O₂ gradient can be temporally switched by electrochemical potentials faster than the temporal resolution of the confocal microscopy under the tested conditions (~2.7 s). The electrochemically established O₂ gradients for wire array k = (30, 3, 50) were similarly quantified at E_{appl} = 0.4, 0.5, and 0.6 V vs. RHE as a function of the distance above the bottom of the wire array (z) (black, red, and blue, respectively, in Fig. 2D). The steepness of the generated O₂ gradient increased at lower E_{appl} values under which the electrochemical activities of O₂ consumption on Pt were more pronounced thanks to the increased magnitude of reductive overpotential (SI Appendix, Fig. S4). Such an E_{appl}-dependent O₂ gradient showed that electrochemical input was capable of spatially controlling and yielding a desirable O₂ gradient for potential biological applications given biologically relevant [O₂] values and the spatial resolutions detected here (2, 46, 47). The electrochemically driven O₂ gradients were also quantified at E_{appl} = 0.5 V vs. RHE for k = (15, 4, 50), (30, 3, 50), and (30, 3, 50) (black, red, and blue, respectively, in Fig. 2E). Noticeably different O₂ gradients were observed, suggesting the capability of the wire array morphology to yield a specific O₂ gradient. Particularly, a strictly O₂-free local environment in aerated medium was established for k = (15, 4, 50). Such a customizable O₂ gradient will be of interests for the study of communal interactions among microorganisms of varying degrees of O₂ demands that are prevalent in nature (2).

Electrochemical Generation and Control of O₂ Concentration Profiles. The Pt-deposited microwire array electrode is capable of spatiotemporally controlling the electrochemically generated O₂ gradient. Thanks to its triple–triple quenching with 3O₂ (43), the phosphorescence emission of Tris(1,10-phenanthroline) ruthenium(II), Ru(phen)_3^2+, from the intersystem crossing after optical excitation between ~350 nm and ~500 nm (44), was utilized to spatiotemporally probe the local concentration of O₂ ([O₂]) (Fig. 2A). The constructed microwire array electrode was housed in a homemade fluidic device (SI Appendix, Fig. S7) under a confocal microscope with 470-nm optical excitation (see Materials and Methods). Under a constant flow of aerated PBS solution with 0.15 mM Ru(phen)_3^2+ ([O₂] = 0.246 mM saturated with ambient air (45)), the phosphorescence emission intensity (I_p, ~610 nm to ~650 nm), inversely proportional to the value of [O₂], was collected, and a calibration curve was established for the quantification of local [O₂] values (see Materials and Methods and SI Appendix, Fig. S8). When k = (15, 4, 50) for the wire array (Fig. 2 B and C), the three-dimensional I_p mapping was recorded in a time sequence when the Pt-coated wire array was initially under an open-circuit condition (t < 15 s), subject to an electrochemical potential (E_{appl} = 0.5 V vs. RHE) from t = 15 and 45 s, and reverted back to the open-circuit condition when t > 45 s (see Materials and Methods). The side views of the three-dimensional I_p mapping were displayed when E_{appl} was initially absent (t = 0 s), E_{appl} = 0.5 V vs. RHE (t ≈ 16 s), and E_{appl} was absent again at t = 48 s (“t = 0 sec,” “t = 16 sec,” and “t = 48 sec,” respectively, in Fig. 2B). The intensity of I_p was noticeably stronger within the wire array when E_{appl} = 0.5 V vs. RHE in comparison to the I_p values under the open-circuit conditions before and after the presence of E_{appl}. As I_p is inversely proportional to the local values of [O₂], this observation qualitatively suggested a decrease of [O₂], and hence an O₂ gradient covering the wire array region with microscopic resolution under a reductive electrochemical potential.

The averaged [O₂] values ([O₂]avg) at different distances above the bottom of the wire array z = 5, 50, and 100 μm were quantified and are displayed as a function of time t in Fig. 2C. While a negligible change of [O₂]avg was recorded at z = 100 μm (black in Fig. 2C), which was quite far away from the wire array, at z = 5 and 50 μm (blue and red, respectively, in Fig. 2C), significant changes of [O₂]avg up to a complete anoxic condition were observed, concurrent with the temporal presence of E_{appl}. Such data suggest that the established O₂ gradient can be temporally switched by electrochemical potentials faster than the temporal resolution of the confocal microscopy under the tested conditions (~2.7 s). The electrochemically established O₂ gradients for wire array k = (30, 3, 50) were similarly quantified at E_{appl} = 0.4, 0.5, and 0.6 V vs. RHE as a function of the distance above the bottom of the wire array (z) (black, red, and blue, respectively, in Fig. 2D). The steepness of the generated O₂ gradient increased at lower E_{appl} values under which the electrochemical activities of O₂ consumption on Pt were more pronounced thanks to the increased magnitude of reductive overpotential (SI Appendix, Fig. S4). Such an E_{appl}-dependent O₂ gradient showed that electrochemical input was capable of spatially controlling and yielding a desirable O₂ gradient for potential biological applications given biologically relevant [O₂] values and the spatial resolutions detected here (2, 46, 47). The electrochemically driven O₂ gradients were also quantified at E_{appl} = 0.5 V vs. RHE for k = (15, 4, 50), (30, 3, 50), and (30, 3, 50) (black, red, and blue, respectively, in Fig. 2E). Noticeably different O₂ gradients were observed, suggesting the capability of the wire array morphology to yield a specific O₂ gradient. Particularly, a strictly O₂-free local environment in aerated medium was established for k = (15, 4, 50). Such a customizable O₂ gradient will be of interests for the study of communal interactions among microorganisms of varying degrees of O₂ demands that are prevalent in nature (2).
“t = 22 sec,” and “t = 52 sec,” respectively, in Fig. 3B). While the absence of $E_{\text{appl}}$ correlates with the absence of fluorescent emission from resorufin (“t = 0 sec” and “t = 52 sec” in Fig. 3B), the presence of $E_{\text{appl}} = 0.5$ V vs. RHE (“t = 22 sec” in Fig. 3B) yielded significant fluorescent emission near the wire array that suggested electrochemically generated $\text{H}_2\text{O}_2$. Meanwhile, concurrently monitoring of $[\text{O}_2]$ suggests that the local $[\text{O}_2]$ are not significantly perturbed (SI Appendix, Fig. S13A), due to the relatively lower current density of ORR on the Au-based electrocatalysts under similar $E_{\text{appl}}$ values (SI Appendix, Figs. S4 and S5). This suggests that the electrochemically controlled $\text{H}_2\text{O}_2$ is nearly independent of aeration of the liquid medium, thanks to the catalytically selective generation of $\text{H}_2\text{O}_2$ and the low value of observed $\text{H}_2\text{O}_2$ (at most, up to 30 $\mu$M) relevant for biological studies (8, 48, 50) in comparison to the air-saturated $\text{O}_2$ solubility in water (246 $\mu$M) (45).

The average change of $[\text{H}_2\text{O}_2]$ values ([$\text{H}_2\text{O}_2$]$_{\text{avg}}$) at different distances above the bottom of the wire array $z = 5, 50,$ and $90\mu$m were quantified and displayed as a function of time $t$ in Fig. 3C. At all $z$ values, the time-dependent generation of $[\text{H}_2\text{O}_2]_{\text{avg}}$ measured from $I_p$ was well correlated with the presence of $E_{\text{appl}}$. A more gradual yet still relatively fast transition of the measured $I_p$ (~10 s), and hence $[\text{H}_2\text{O}_2]_{\text{avg}}$, was observed, which was presumably due to the limited temporal resolution of the fluorogenic reaction that was needed to track local $[\text{H}_2\text{O}_2]$ (51). Nonetheless, such data indicated the capability of temporally controlling the formation of $\text{H}_2\text{O}_2$ electrochemically, which can be handy as a perturbation to study the microbial response toward $\text{H}_2\text{O}_2$-based ROS (52). We also determined the electrochemically induced $\text{H}_2\text{O}_2$ gradient for wire array $k = (15, 4, 50)$ at $E_{\text{appl}} = 0.45, 0.5,$ and 0.55 V vs. RHE (black, red, and blue, respectively, in Fig. 3D). Significant different local accumulations of $\text{H}_2\text{O}_2$ up to 30 $\mu$M for different $E_{\text{appl}}$ values were observed despite the 50-mV change of $E_{\text{appl}}$. Such an observation suggests that the generated $\text{H}_2\text{O}_2$ gradient is highly sensitive and subsequently tunable by electrochemical driving forces. In addition, the morphology of wire array electrodes impacts the generated $\text{H}_2\text{O}_2$ gradient. The characterized $\text{H}_2\text{O}_2$ gradients for $k = (15, 4, 50), (30, 3, 50),$ and $(15, 4, 20)$ (black, red, and blue, respectively, in Fig. 3E) were noticeably different at the same $E_{\text{appl}} = 0.5$ V vs. RHE. The achievable range of $\text{H}_2\text{O}_2$ gradients at the microscopic level is commensurate with biologically observed ROS microenvironments (8), heralding the utility of the electrochemically generated $\text{H}_2\text{O}_2$ gradients in microbial studies.

Establishing Neural Networks for an Inverse Design Strategy.

We seek to establish computational models that can inversely predict the values of $E_{\text{appl}}$ and $k = (P, D, L)$ of the Pt- and Au-loaded wire array electrodes for targeted corresponding $\text{O}_2$ and $\text{H}_2\text{O}_2$ gradients ([O$_2$]$_k$ and [H$_2$O$_2$]$_k$), respectively. Such an inverse design strategy for $\text{O}_2$ and $\text{H}_2\text{O}_2$ microenvironments is proposed to be much more time efficient in comparison with the classical trial-and-error approach (Fig. 4), and will find plentiful applications given the high variabilities of biological applications in both spatial and temporal domains (2, 3, 8). Critical inside such computational models are neural networks, trained with sufficient amounts of...
Controlling H₂O₂ heterogeneity

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Data, which correlate \( \{E_{\text{appl}}, k = (P, D, L)\} \) with the \([O_2](z)\) and \([H_2O_2](z)\) distributions. In such a regard, we employed numerical simulations based on finite element methods (FEM) (32) to augment the available data (Fig. 4B). FEM-based electrochemical simulations have been widely used in the understanding and design of electrochemical applications, with satisfactory accuracies (32, 33, 53–55). We established electrochemical microkinetic models that include the mass transport of redox species and the electrochemical reduction of O₂ for the Pt and Au electrocatalysts (see Materials and Methods). FEM-based numerical simulations were conducted with COMSOL Multiphysics (version 5.3) for the O₂ and H₂O₂ gradients near the Pt- and Au-loaded wire array electrodes, respectively. Experimental \([O_2]_{\text{avg}}\) and \([H_2O_2]_{\text{avg}}\) values were compared with simulation results at different heights above the base of the wire array (z), as shown in the exemplary case when consistent results of O₂ and H₂O₂ gradients were observed for \(k = (30, 3, 50)\) and \(E_{\text{appl}} = 0.5\) V vs. RHE (SI Appendix; Fig. S13 B and C). The mean-squared errors (MSEs) between FEM-based simulations and experimental results are \(9.81 \times 10^{-4}\) mM² and \(4.84 \times 10^{-6}\) mM² for O₂ and H₂O₂ gradients, respectively (see Materials and Methods). Such a consistency of results between experimental characterization and FEM-based simulations motivates us to use the augmented data from FEM-based simulations to establish neural networks to inversely predict O₂ and H₂O₂ gradients.

The established neuron networks display good accuracy for the \([O_2](z)\) and \([H_2O_2](z)\) distributions near the wire array electrodes loaded with Pt and Au electrocatalysts. We previously constructed multilayer perception neuron networks (MLPNNs) that expand the explorable parameter space of wire array electrode morphologies in electrocatalytic reduction of N₂ (34). Here, we constructed MLPNNs that predict \([O_2](z)\) and \([H_2O_2](z)\) based on inputs of \(\{E_{\text{appl}}, k = (P, D, L)\}\), which were trained based on 10,000 data points augmented from the FEM-based simulations (see Materials and Methods). As the model-training process proceeds with an increasing number of epochs, monotonic decreases of the average MSE (AMSE) between the training and predicted data points for the datasets of both validation and training (red and black dots, respectively) were observed in Fig. 4 C and D for the O₂ and H₂O₂ gradients near Pt- and Au-loaded wire array electrodes, respectively. The fact that the values of AMSEs against the validation datasets were similar to the ones from the training datasets in Fig. 4 C and D indicates that there was no overfitting in the ML process (56). In the end, near-unity coefficients of determination \(R^2 \to 1\) were observed for both MLPNNs (MLPNN 1 that predicts O₂ gradient and MLPNN 2 that predicts H₂O₂ gradient) (Fig. 4 E and F, respectively). The values of AMSEs from the MLPNNs reach \(1.74 \times 10^{-4}\) mM² and \(1.81 \times 10^{-6}\) mM² for the prediction of \([O_2](z)\) and \([H_2O_2](z)\) based on inputs of \(\{E_{\text{appl}}, k = (P, D, L)\}\), respectively. Such small values of AMSEs suggest good accuracy of the developed neural networks for the inverse design of desirable O₂ and H₂O₂ microenvironments.

**Exemplary Inverse Design of O₂ and H₂O₂ Microenvironments Near Wire Array Electrodes.** Exemplary inverse design processes with direct biological relevance were experimentally tested, with
good predictabilities for the establishment of desirable O₂ and H₂O₂ microenvironments. In microbiology and microbial ecology, it is desirable to establish well-defined microenvironments whose sizes are ~20 μm to ~100 μm in order to mimic natural heterogenous distribution of biologically relevant extracellular species such as nutrients and other microbial resources (57). Within such length scales, establishing microoxic niche (i.e., O₂ ≈ 100 μM) in the midst of an oxic external environment (~10 d) and experimental characterization alone at one specified region (see Materials and Methods). It is intriguing to note that there existed multiple different wire array morphologies to yield the same desirable O₂ and H₂O₂ gradients, which may not be straightforward, intuitively. We estimated that the determination of O₂/H₂O₂ gradients for one parameter coordinate in the space of {Eappl, k = (P, D, L)} will take ~4 s for the MLPNN-based method, ~90 s from FEM-based simulations, and ~20 mins for the confocal characterization alone at one specific Eappl for a single wire array morphology, notwithstanding any time spent in any preceding protocols (see Materials and Methods). Therefore, a comprehensive exploration of the parameter space {Eappl, k = (P, D, L)} with more than 10⁴ trials as shown above is only possible with the use of MLPNN-based inverse design, because only the MLPNN is capable of screening 10,000 parameter combinations within a reasonable amount of time in practice (~12 h) in comparison to the ones based on FEM (~250 h, i.e., ~10 d) and experimental characterization (at least 3,000 work-hours without considering any practical concerns) (Fig. 4).

Fig. 4. The development of inverse design for electrochemically generated O₂ and H₂O₂ gradients. (A) Comparison between the conventional protocol and our inverse design approach for the development of suitable experimental conditions, represented as {Eappl, k} in order to achieve desirable spatiotemporal distributions of O₂ and H₂O₂ concentrations ([O₂](r,t) and [H₂O₂](r,t), respectively). MLPNN 1 & 2, multiple-layer perceptron neural networks for O₂ and H₂O₂ gradients, respectively. (B) Protocols of data augmentation for the establishment of MLPNN: Eappl, Pt/Au and Eappl, Au, the exchange current densities of four-electron and two-electron ORRs on Pt and/or Au electrocatalysts, respectively. (C and D) The AMSE in the training (blue) and validation (pink) datasets at different epochs for the gradients of O₂ (MLPNN 1 in C) and H₂O₂ (MLPNN 2 in D). (E and F) Comparisons between the MLPNN-predicted values ([O₂]predict and [H₂O₂]predict) and training values ([O₂]train and [H₂O₂]train) for the local average concentrations of O₂ (E) and H₂O₂ (F), respectively. R², coefficient of determination.
We also conducted a spot check for the predicted $O_2$ and $H_2O_2$ microenvironments by experimental validations. A Pt-based wire array electrode with $k = (46, 6, 20)$ was picked from Fig. 5C as a desirable geometry, experimentally prepared (Fig. 1E), and experimentally tested for the established $O_2$ gradient at $E_{appl} = 0.5 V$ vs. RHE. Satisfactory consistency with MSE $= 5.63 \times 10^{-4} \text{ mM}^2$ was achieved between the experimental and targeted values of $[O_2](z)$ (dots and line, respectively, in Fig. 5E). Similarly, an Au-based wire array electrode with $k = (17, 3, 30)$ was picked from Fig. 5D, experimentally prepared (Fig. 1E), and experimentally tested for the $H_2O_2$ gradient at $E_{appl} = 0.45 V$ vs. RHE. We also observed satisfactory consistency with MSE $= 7.22 \times 10^{-6} \text{ mM}^2$ between the experimental and targeted values of $[H_2O_2](z)$ (dots and line, respectively, in Fig. 5F). While we were unable to experimentally exhaust all of the predicted parameter space for the desirable micro-environments of $O_2$ and $H_2O_2$, our experimental validations offer convincing evidence for the validity of the developed MLPNN-based inverse design for future microbiology-related research.

Discussion

In summary, we presented a ML-based inverse design strategy for $O_2$ and $H_2O_2$ concentration profiles with the use of electrochemical catalysis of ORR. We demonstrated a proof-of-concept closed-loop protocol for inversely designing $O_2$ and $H_2O_2$ gradients with properly designed microwire electrodes in PBS solution, the go-to culturing medium in microbiology. By achieving concentration differences and spatial resolutions that are relevant to microbial microenvironments, the demonstrated $O_2$ and $H_2O_2$ gradients are applicable for studies in microbiology. While the reported research focuses on one specific form of electrochemical boundary conditions, namely, microwire array, the reported inverse design procedures are generally applicable for any electrochemical systems that can be parameterized and analyzed by neural networks. As mass transport and concentration profiles in an electrode’s proximity are important components in electrochemistry, this work demonstrates the power of ML-based inverse design in electrochemistry. Moreover, our results will lead to a general platform that inversely designs suitable electrochemical systems for any targeted environments of $O_2$ and $H_2O_2$ in microbiology. Future research will focus on the platform’s application of in vivo microbial communities and fundamental insights that can be fetched thanks to our system’s unique capabilities. Moreover, since electrochemistry is capable of modulating any redox active species such as extracellular Fe(II)/(III) species (60), as well as other extracellular metabolites sensitive to oxidative stress, such as pyocyanin (61–63), our inverse design approach based on electrochemistry is capable of controlling the other microenvironments beyond $O_2$ and ROS and is generally applicable in the study of ubiquitous microenvironments in extracellular medium.

Materials and Methods

Chemical and Materials. ITO-coated glass slides (06499-AB) were purchased from SPI Supplies (~30 $\Omega$ to ~60 $\Omega$, 22 x 40 mm). Silver (Ag) paste...
(16040-30) was purchased from Ted Pella. Si wafers (p-type, boron-doped, <100>, ~1 Ω-cm~1 to ~10 Ω-cm~1) were purchased from University Wafer, Inc. Platinum (Pt) wires (CHI 115) and glassy carbon electrodes (CHI 104, diameter = 3 mm) were purchased from CH Instruments. Unless specially mentioned, all chemicals and materials were purchased from Sigma-Aldrich or WWR.

Experimental Establishment of Testing Platform. Modified from a previously published protocol (29), the experimental testing platform as shown in SI Appendix, Fig. S7 consists of a fluidic cell with a three-electrode system, in order to electrochemically generate desirable gradients of O2 and H2O2. As the working electrode in the setup, microwire array electrodes were fabricated similarly as previously described (29). The microwire arrays were fabricated by photolithography with the use of the deep reactive-ion etching process (DRIE). After treatment in hexamethyldisilazane vapor for 10 min, precleaned Si wafers were coated with photoresist (MicroChemicals, AZ5214E, 3000 rpm spin coating) by soft baking (100 °C for 75 s), exposed in the hard contact mode by a contact aligner (Carl Suss MA6), hard baked (120 °C, 5 min), and developed to generate the periodic patterns for the array (mixture of MicroChemicals AZ400K water, 1:4 volume ratio). After creation by DRIE (Unaxis Versaline FDSE III), microwire arrays of desirable lengths were annealed under ambient air at 1,050 °C for 9.5 h to yield the isolating oxide layer, coated by 500-nm ITO through reactive sputtering (Denton Discovery 550 sputtering system), and finally deposited with a 7-nm layer of Pt or Au by an Anatech Hummer 6.2 sputtering system. The structure of the wire array was examined by SEM (ZEISS Supra 40VP SEM), and the element distribution was examined by SEM (JEOL JSM-6700F) equipped with EDS (Ametek). Patterned ITO-coated glass slides were used as the counter electrodes in the established testing platform, after the selective removal of ITO by 6 M HCl in undesirable areas on the glass slides. Ag paint as the pseudoreference electrode was applied on select areas of the ITO-coated slides so as to cover a 5 mm × 5 mm square with a layer of silver, serving as the reference electrode, while Pt was deposited on other ITO-coated areas for the creation of a counter electrode in the setup. An optically transparent fluidic cell of 250-μm height was constructed by assembling the microwire array electrodes with the prefabricated ITO glass slides, while a Gamma Interface 1010B potentiostat was used to enforce the electrochemical driving force. The setup was mounted on an inverted laser confocal microscope (Leica SP8 SMD) with sufficient working distance (680 μm), and a syringe pump was used to maintain a fixed liquid flow rate. As we particularly ensure the accuracy of applied electrochemical potentials, cyclic voltammetry in a standard Zobell’s solution (3.3 mM K2Fe(CN)6, 3.3 mM K4Fe(CN)6, and 0.1 M KCl, 0.43 V vs. standard hydrogen electrode) was employed to calibrate the electrochemical potential of an Ag-based pseudoreference electrode as shown in SI Appendix, Fig. S14 (64, 65). The Ag-based pseudoreference electrode was calibrated as 0.75 V vs. RHE in PBS solution at pH 7.4.

Electrochemical Characterization of the Deposited Electrocataysts. When the deposited Pt electrocatalysts have been characterized in our prior report (29), experiments were conducted to analyze the electrocatalytic activities of ORR for the coated Au electrocatalysts. An experiment of a rotating ring-disk electrode (Pine Research, Inc., AFE61R1PT) was conducted in PBS solution using a setup with a Pt wire counter electrode, Ag/AgCl (1 M KCl) reference electrode and a modulated speed rotator (Pine Research, Inc., AFMSRCE). While a Pt ring electrode was kept at 1.9 V vs. RHE, linear scan voltammograms (20 mV/s) were recorded between 0.1 and 1.1 V vs. RHE with different rotating speeds (100, 225, 400, 625, 900, 1,225, 1,600 rpm, and 2,025 rpm) in electrolytes saturated with O2 and N2, respectively. The measurements in N2-saturated solution were used as the signal background.

Quantification and Calibration of O2 Concentration Profiles. Aerated PBS solution consisting of 0.2 U/mL HRP and 120 μM Amplex Red (1× working solution) was prepared in the dark and fed into the assembled testing platform at a flow rate of 0.8 mL/min. The fluorescent intensity mapping under confocal microscopy was conducted in a 1-min time sequence. During the 1-min confocal microscopy measurement, programmed 30-s electrolysis was performed with a particular potential on the working electrode from t = 20 s to t = 50 s. The excitation wavelength was set as 470 nm, and we gathered emission intensity from 590 nm to 650 nm as fluorescence emission intensity I. The fluorescence emission intensity distribution was further translated into the concentration profiles, based on the linear relationship between O2 concentration and the inverse of I (noted as I−1) that was experimentally determined. Ru(phen)3Cl2-containing PBS solutions of different O2 concentrations, ranging from [O2] = 25 μM to 375 μM, were prepared by bubbling a N2/O2 mixture of tunable ratio through the solution, and were pumped into the assembled fluidic device (0.8 mL/min) for calibration.

Quantification and Calibration of H2O2 Concentration Profiles. Aerated PBS solution consisting of 0.2 U/mL HRP and 120 μM Amplex Red (1× working solution) was prepared in the dark and fed into the assembled testing platform at a flow rate of 0.8 mL/min. The fluorescent intensity mapping under confocal microscopy was conducted in a 1-min time sequence. During the 1-min confocal microscopy measurement, programmed 30-s electrolysis with a particular potential on the working electrode from t = 20 s to t = 50 s. The excitation wavelength was set as 470 nm, and we gathered emission intensity from 590 nm to 650 nm as fluorescence emission intensity I. The fluorescence emission intensity distribution was further translated into the concentration profiles, based on the corresponding calibration curves. In the experiments of calibrating H2O2 concentrations, darkly prepared PBS solution consisting of 0.4 U/mL HRP and 240 μM Amplex Red was combined with PBS solutions of different H2O2 concentrations, ranging from 5 μM to 60 μM, and was pumped into the assembled devices for the measurement of confocal microscopy. We found that the H2O2-induced fluorescence intensity I is also dependent on the specific morphologies of the wire array (K) and the distance away from the bottom of the wire array (z) (SI Appendix, Fig. S15), owing to the scattering and, possibly, optical absorption of the wire array electrodes (66–68). Therefore, individual calibration curves were determined for every z location in wire arrays with all possible k values (SI Appendix, Figs. S9–S12). Specific I correction was made to compensate the difference between the calibration experiment and gradient optical detection due to practical restrictions (SI Appendix, Supplementary Text and Fig. S16).

FEM-Based Numerical Simulations for O2 Gradient and H2O2 Gradients. FEM simulation of both O2 gradient and H2O2 gradient was achieved in “electroanalysis” module in COMSOL Multiphysics (Version 5.3).

Geometry description. The shape of wire was represented by a column with diameter D and length L. We located each wire in the center of a cuboid of P × P × 200 μm, and the difference between the cuboid and column was geometrically defined for the electrolyte. For each point in the electrolyte, if its distance from the top of the wire not smaller than the diffusion distance, dp, we considered that it belonged to the bulk electrolyte in which [O2] = 246 μM, independent of time. The boundary surface was defined as the area of which the distance to the top of the wire is dp. On the boundary surface, the [O2] was the same as that in the bulk. A periodic boundary condition was applied to describe the wire array. The value of dp was set as 20 μm for O2 gradient simulation on Pt-loaded wire array electrodes (29). For O2 gradient and H2O2 gradient simulation on Au-loaded wire array electrodes, dp was measured as 50 μm (SI Appendix, Fig. S17).

Transport properties. The diffusion of oxygen and hydrogen peroxide was simulated based on the following Eqs. 1 and 2. DO2 and DH2O2 are the diffusion coefficients of oxygen and hydrogen peroxide in aqueous solution, which were 2.2 × 10−9 m2/s and 1.5 × 10−9 m2/s, respectively.

\[
\frac{d[D_2]}{dt} = D_{O_2} \nabla^2 [O_2] \tag{1}
\]

\[
\frac{d[H_2O_2]}{dt} = D_{H_2O_2} \nabla^2 [H_2O_2]. \tag{2}
\]

Electroanalysis. The potential window of Epot is from 0.6 V vs. RHE to 0.2 V vs. RHE.

On the surface of Pt-loaded wire array electrodes, two-electron and four-electron ORR reactions (2e-ORR and 4e-ORR, respectively) took place at the same time as potential-dependent selectivity.
2e − ORR: O₂ + 2H⁺ + 2e⁻ → H₂O₂
4e − ORR: O₂ + 4H⁺ + 4e⁻ → 2H₂O.

On the surface of Pt-loaded wire array electrodes, the supply-consumption equilibrium was simulated as Eqs. 3 and 4 in the formalism of concentration-dependent Tafel kinetics (27),

\[ i_{SC} = i_{ORR} = -i_{o_{ regulates}} \left( \frac{[O_2]}{C_{O_2}} \right) \exp \left( \frac{-\alpha \tau_{RT}}{RT} \right) \]

\[ J_0 = \frac{i_{SC}}{4F} \text{.} \]

Here \( i_{SC} \) denotes the current density of 4e-ORR, \( i_{ORR} \) denotes the exchange current density of 4e-ORR, \( i_{o_{ regulates}} \) denotes the local current density of four-electron reduction of O₂ on the electrode surface, \( J_0 \) denotes the local flux of O₂ consumption from electrolyte, and \( \eta_{ORR} \) is the overpotential that is defined as the difference between \( E_{ORR} \) and the standard redox potential of O₂/H₂O, \( E^0_{O_2/H_2O} \) (1.23 V vs. RHE). The \( \alpha \) equals 0.5 as the transfer coefficient, \( F \) denotes the Faraday constant, \( R \) is the gas constant, \( T \) is the temperature, and \( \eta_{ORR} \) is the electron transfer number before the rate-determining step of 4e-ORR, of which the value is one (SI Appendix, Fig. S4). The local oxygen concentration is denoted as \([O_2]_{C_{O_2}}\), \( J_0 \) is the oxygen concentration in air-saturated water at \( T \).

The above equation follows the textbook equations that account for the mass transport and chemical stoichiometry at electrode interfaces (27). On Pt-loaded wire array electrodes, \( i_{o_{ regulates}} \) is found to be \( 3.0 \times 10^{-6} \) A/m² (SI Appendix, Fig. S4).

On the electrode surface of Au-loaded wire array electrodes, the supply-consumption equilibrium was simulated as Eqs. 5–7.

\[ i_{SC} = i_{ORR} = -i_{o_{ regulates}} \left( \frac{[O_2]}{C_{O_2}} \right) \exp \left( \frac{-\alpha \tau_{RT}}{RT} \right) \]

\[ i_{o_{ regulates}} = -i_{o_{2, Au}} \left( \frac{[O_2]}{C_{O_2}} \right) \exp \left( \frac{-\alpha \tau_{RT}}{RT} \right) \]

\[ J_0 = \frac{i_{SC}}{4F} + \frac{i_{ORR}}{2F} \]

\[ J_{0, Au} = -\frac{i_{ORR}}{2F} \text{.} \]

While most of the definitions of variables in the case of Au-loaded wire array electrodes with Eqs. 5–7 are the same as the Pt-loaded case in Eqs. 3 and 4, we noted that \( i_{ORR} \) instead stands for the local current density of both two-electron and four-electron reduction of O₂ on the electrode surface, \( i_{o_{2, Au}} \) the current density of 2e-ORR, and \( \eta_{ORR} \) is electron transfer number before the rate-determining step of 2e-ORR, of which the value is 0.7 (SI Appendix, Fig. S5). On Au-loaded wire array electrodes, the catalysis selectivity of Au toward 4e-ORR and 2e-ORR is dictated by the exchange current densities \( i_{o_{2, Au}} \) and \( i_{o_{2, Au}} \), respectively. The \( i_{o_{2, Au}} \) is 2.0 × 10⁻⁸ A/m², and \( i_{o_{2, Au}} \) is 8.0 × 10⁻⁹ A/m², based on literature and measurement (SI Appendix, Fig. S5) (69, 70).

Based on a comparison with experimental gradients, the AMSE of O₂ gradient simulation and H₂O₂ gradient simulation on Pt-loaded wire array electrodes and Au-loaded wire array electrodes is 9.81 × 10⁻⁴ m²/m² and 4.84 × 10⁻⁶ m²/m², respectively. The range of \( E_{SS, ORR} \) was set within the ORR potential window, from 0.6 V vs. RHE to 0.2 V vs. RHE. Besides, a three-dimensional block was defined in the space of \((P, D, L)\) as the range of morphology, from \( P = 1 \mu m \) to 100 \( \mu m \), \( L = 1 \mu m \) to 150 \( \mu m \), and \( D = 0.2 \mu m \) to 10 \( \mu m \). By using Simulink in Matlab, we were able to generate random \( \kappa \) value within the morphology space and calculate the corresponding gradient curves. To fulfill the ML functions, gradient profiles under 10,000 experimental conditions were included in the dataset for each developed multiple-layer perceptron neural network, respectively.

Model Selection and Training. The implementation of all ML code was done on a MacBook Pro with a 1.4 GHz quad-core Intel Core i5 processor and 8GB of RAM, with code specifically deployed using the JupyterLab Notebook, a Python-based programming platform widely used in data science and ML (34). In this paper, we selected MLPNNs as the ML model for inverse design. We use FEM-simulated gradients to develop the MLPNNs. Gradient data were imported from FEM results in the form of .csv files and combined into a library of data. Prior to the model development, a random 20% of the data were split from the whole dataset for later model validation. The rest of the data were split into training and validation data, of which the percentages were 65% and 15%, respectively. Multiple cycles of model training, each cycle termed as one epoch, were conducted in order to develop the targeted MLPNNs. In each epoch, MLPNNs will be trained from the training data, followed by a validation process in the validation dataset used to provide estimates of final model accuracy after each round of training. The total ML process will include 10 epochs of forward and backward propagations. The accuracies of the developed MLPNNs model as a function of epoch numbers are plotted in Fig. 4 C and D.

As described in the section above, the training dataset was a collection of concentration gradients under different \( E_{appl} \) and \( \kappa \) values. We used AMSE and SD, defined in more detail below, from Eqs. 8–11 to quantify the ability of ML models to correctly reveal the connection between gradient curves and the two impacting factors, \( E_{appl} \) and \( \kappa \). In model selection, we selected multiple-layer perceptron neural networks for gradient prediction due to the low AMSE and SD value gradient prediction (SI Appendix, Fig. S1B).

For both O₂ and H₂O₂ gradients, the predicted curve included 20 local concentration datum along the wire array from \( Z = 5 \mu m \) to 100 \( \mu m \) every 5 \( \mu m \). Z is defined as the distance from the bottom of the wire array. In O₂ gradient prediction, MSE and SD are defined as the following equations:

\[ MSEE = \frac{1}{20} \sum_{i=1}^{19} (\text{[O}_2\text{]_NN} - \text{[O}_2\text{]_real})^2 \]

\[ SDSE = \sqrt{\frac{1}{N} \sum_{n=1}^{N} (MSEE_n - \text{AMSE}_n)^2} \]

In Eqs. 8 and 9, \([O_2]_{real}\) stands for the predicted oxygen concentration from MLPNNs predicting the O₂ gradient on the Pt-loaded wire array. \([O_2]_{real}\) is the oxygen concentration in the simulation dataset. \( MSEE \) is defined based on the average square of concentration difference over the whole gradient profile. \( \text{AMSE}_N \) is the average MSE over the whole dataset. We calculated the SD over data under a wide range of experimental conditions to evaluate the overall precision of predictions from MLPNNs predicting the O₂ gradient on the Pt-loaded wire array.

MSE and SD in the H₂O₂ gradient were defined in a similar pattern. In Eqs. 10 and 11, \([H_2O_2]_{NN}\) stands for the predicted hydrogen peroxide concentration from MLPNNs predicting the H₂O₂ gradient on the Au-loaded wire array. \([H_2O_2]_{real}\) is the hydrogen peroxide concentration in the simulation dataset. \( MSEH \) is defined based on the average square of concentration difference over the whole gradient profile. \( \text{AMSE}_N \) is the average MSE over the whole dataset.

\[ MSEH = \frac{1}{20} \sum_{i=1}^{19} (\text{[H}_2\text{O}_2\text{]_NN} - \text{[H}_2\text{O}_2\text{]_real})^2 \]

\[ SDH = \sqrt{\frac{1}{N} \sum_{n=1}^{N} (MSEH_n - \text{AMSE}_n)^2} \]

Morphology Prediction for Desired O₂ Gradient and H₂O₂ Gradient. In morphology prediction for the desired O₂ gradient, we assigned \( E_{appl} = 0.5 \) V vs. RHE. Initially, MLPNNs predicting the O₂ gradient on the Pt-loaded wire array randomly selected one morphology in the morphology space and calculated the corresponding O₂ gradient. The similarity score between the calculated O₂ gradient and target O₂ gradient, \( S_O \), was quantified by Eq. 12. In the prediction process, the neural networks would find out the top 10,000 morphologies with the highest \( S_O \) values. In Eq. 12, \([O_2]_T\) is the local oxygen concentration in the target O₂ gradient, and \([O_2]_{NN}\) is the calculated local oxygen concentration. The similarity score \( S_O \) is the average relative error among a collection of different \( z \) values, \( C_D = [5, 10, 15, 25, 35, 45, 70] \), where the unit is micrometers.

\[ S_O = 1 - 100\% \times \frac{1}{7} \sum_{z} \left( \frac{[O_2]_{NN} - [O_2]_T}{[O_2]_T} \right) \]
morphologies with the highest $S_m$ values. In Eq. 13, $[H_2O_2]$ is the local hydrogen peroxide concentration in the target $H_2O_2$ gradient, and $[H_2O_2]_{min}$ is the calculated local hydrogen peroxide concentration. The similarity score, $S_m$, is the average relative error among a collection of different values, $C_i$, weighted to the number of micrometers. 

$$S_m = 1 - 100\% \cdot \sum_{x \in \mathbb{R}} \frac{[H_2O_2]_{min} - [H_2O_2]}{[H_2O_2]_{min}}$$

In the sliced mapping of the $H_2O_2$ similarity score, the similarity score at $L = 5$ μm was the average of from $L = 0$ μm to 10 μm. The similarity score at $L = 20$ μm was the average of from $L = 10$ μm to 30 μm. The similarity score at $L = 40$ μm was the average of from $L = 30$ μm to 50 μm. The similarity score at $L = 60$ μm was the average of from $L = 50$ μm to 70 μm. The similarity score at $L = 80$ μm was the average of from $L = 70$ μm to 90 μm. The similarity score at $L = 95$ μm was the average of from $L = 90$ μm to 100 μm.

Data Availability. All study data, except the code for the neural networks, are included in the article and/or SI Appendix. The code for the neural networks is available on B.B.H.’s GitHub account http://github.com/bbhoar/02_H2O2_ML_PNAS.

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Author affiliations: *Department of Chemistry and Biochemistry, University of California, Los Angeles, Los Angeles, CA 90095; and *California NanoSystems Institute, University of California, Los Angeles, Los Angeles, CA 90095.

Author contributions: C.L. supervised the project; Y.C. and S.L. fabricated the microelectrode systems; Y.C. and S.L. measured the concentration gradients with conical microscopy; J.W. characterized the electrochemical properties and conducted the FEM simulations in data augmentation; B.B.H. developed the neural network models and analyzed the computational results of inverse design; Y.C. and C.L. wrote the initial draft of the manuscript; and Y.C., J.W., B.B.H., S.L., and C.L. discussed the results and assisted during the manuscript preparation.
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