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Fluorescence-Enabled Electrochemical Microscopy with Dihydroresorufin as a Fluorogenic Indicator

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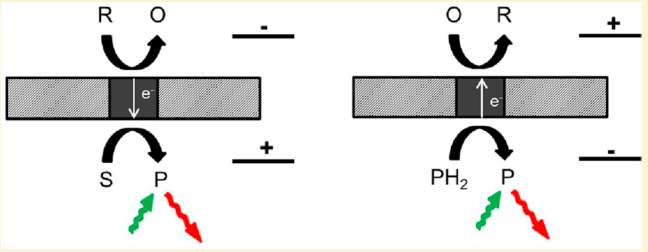
Supporting Information

ABSTRACT: Recently, we introduced a new electrochemical imaging technique called fluorescence-enabled electrochemical microscopy (FFEM). The central idea of FEEM is that a closed bipolar electrode is utilized to electrically couple a redox reaction of interest to a complementary fluorogenic reaction converting an electrochemical signal into a fluorescent signal. This simple strategy enables one to use fluorescence microscopy to observe conventional electrochemical processes on very large electrochemical arrays. The initial demonstration of FEEM focused on the use of a specific fluorogenic indicator, resazurin, which is reduced to generate highly fluorescent resorufin. The use of resazurin has enabled the study of analyte oxidation reactions, such as the oxidation of dopamine and H_2O_2. In this report, we extend the capability of FEEM to the study of cathodic reactions using a new fluorogenic indicator, dihydroresorufin. Dihydroresorufin is a nonfluorescent molecule, which can be electrochemically oxidized to generate resorufin. The use of dihydroresorufin has enabled us to study a series of reducible analyte species including $Fe(CN)_6^{3-}$ and $Ru(NH_3)_6^{3+}$. Here we demonstrate the correlation between the simultaneously recorded fluorescence intensity of resorufin and electrochemical oxidation current during potential sweep experiments. FEEM is used to quantitatively detect the reduction of ferricyanide down to a concentration of approximately 100 μM on a 25 μm ultramicroelectrode. We also demonstrate that dihydroresorufin, as a fluorogenic indicator, gives an improved temporal response and significantly decreases diffusional broadening of the signal in FEEM as compared to resazurin.



Electrochemical fluorogenic and fluoroquenching reactions have been of great interest for several applications. Fluorescent-voltammetry single-molecule spectroscopy (F-V/SMS) and, later, single-molecule spectroelectrochemistry (SMS-EC) were developed by Bard, Barbara and co-workers. These techniques were used to monitor single, reversible electron-transfer events within nanoparticles of organic polymer dyes.[1−3] Chen and co-workers also demonstrated the use of fluorogenic redox reactions to study site-specific electrocatalytic activity of single-walled carbon nanotubes.[4] A key in achieving single-molecule level sensitivity in these experiments is the use of fluorescence to significantly amplify the electrochemical signal.

Recently, we introduced the technique of fluorescence-enabled electrochemical microscopy (FEEM), which involves the use of a fluorogenic redox reaction to report the progress of a conventional, nonfluorogenic redox reaction of interest.[5] The key concept of FEEM includes the use of a closed bipolar electrode (BPE) to electrically couple two separate redox reactions and the use of fluorescence microscopy to simultaneously visualize electrochemical signals on parallel electrodes. A closed BPE consists of a conductor embedded in an insulator substrate that completely separates two solution compartments (Figure SI-1, Supporting Information). A potential can be applied across two driving electrodes to couple faradaic reactions at each pole of the BPE, enabling control of the system without any direct electrical connection to the BPE. Bipolar electrodes, especially those found in an open microfluidic platform, have been extensively studied by Crooks[6−8] and others[9−12] in the last two decades due to their increasing importance in analytical chemistry.

A key fluorogenic reaction used in FEEM is the reduction of resazurin (**S**), which generates a highly fluorescent product, resorufin (**P**). As a cathodic indicator process, this reaction can be used to study electrochemical oxidation processes of various analytes (**R**) through a closed BPE or its array. This same reaction has been the core of numerous studies by the Chen group to investigate (electro)catalytic activity at single nanoparticles.[4,13−16] A unique feature of FEEM is the use of highly sensitive fluorescence microscopy to optically monitor nonfluorogenic redox reactions of interest. Importantly, FEEM has enabled us to simultaneously monitor electrochemical kinetics on large arrays containing thousands or more ultramicroelectrodes.

Scheme 1a shows the two-electron, two-proton reduction of resazurin to form the highly fluorescent product resorufin.[17] In our previous work, we limited our discussion to the study of analyte oxidation reactions (**R** → **O**) coupled to this fluorogenic reduction process as shown in Scheme 1b. Since

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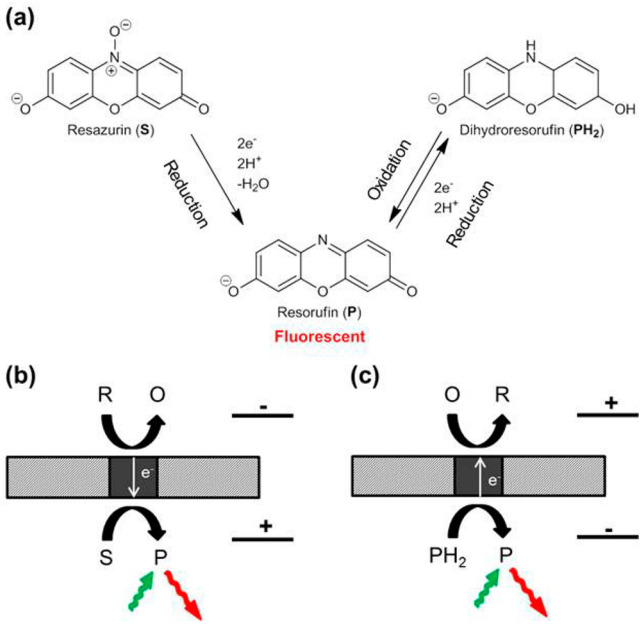
Scheme 1. (a) Reaction Scheme for the Fluorogenic Production of Resorufin from Either Resazurin by Reduction or from Dihydroresorufin by Oxidation and (b and c) Illustrations of FEEM for the Detection of an Oxidizable and a Reducible Species, Respectively[a]

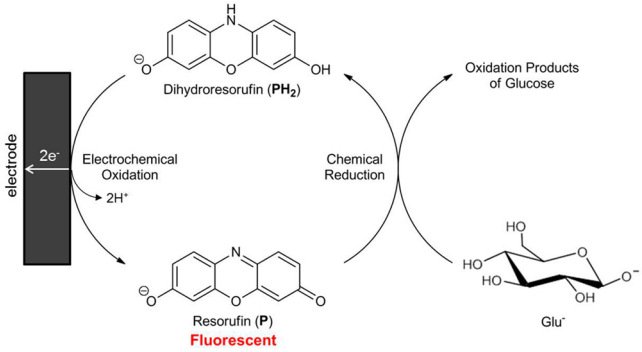


[a]In panel b, the oxidation of an analyte (**R**) at the anodic pole of a BPE is coupled to the fluorogenic reduction of resazurin (**S**) at the cathodic pole. In panel c, the reduction of an analyte (**O**) at the cathodic pole of a BPE is coupled to the fluorogenic oxidation of dihydroresorufin ($\mathbf{PH_2}$) at the anodic pole.

the development of FEEM, we have been interested in finding a suitable fluorogenic oxidation reaction to compliment the reduction of resazurin previously used with the technique. We sought a fluorophore with high extinction coefficient and quantum yield similar to resorufin in order to achieve sufficient signal amplification. Resorufin has an extinction coefficient, ε (570 nm), of approximately 57 000 M^{-1} cm^{-1} and a quantum efficiency, φ, of ~0.97,[13] making it an ideal fluorophore for FEEM. Fortuitously, resorufin can also be generated from the reversible electrochemical oxidation of the nonfluorescent dihydroresorufin ($\mathbf{PH_2}$). By starting with a solution of dihydroresorufin instead, we can extend the use of FEEM to the electrochemical detection and study of analyte reduction reactions ($\mathbf{O} \rightarrow \mathbf{R}$) (Scheme 1c).

Dihydroresorufin can be obtained from the chemical reduction of resorufin in a basic solution containing glucose, as shown in Scheme 2. This solution is often used in a chemistry classroom demonstration[18] commonly known as the "Vanishing Valentine Experiment" and is similar in concept to the more popular "Blue Bottle Experiment",[19,20] which uses methylene blue in place of resorufin. In the classroom demonstration, a pink solution of resorufin reacts with a deprotonated glucose molecule (Glu^-) to become the colorless dihydroresorufin over time. Oxygen enters the solution upon shaking, oxidizing dihydroresorufin and restoring the pink color and fluorescent properties. Alternatively, the chemically reduced species dihydroresorufin can undergo a heterogeneous electrochemical oxidation localized at the electrode surface. Here, we demonstrate the use of dihydroresorufin for the FEEM-based detection of reducible redox species such as

Scheme 2. Reaction Scheme Showing the Fluorophore-Consuming Chemical Reduction of Resorufin and the Fluorogenic Production of Resorufin through Electrochemical Oxidation[a]



[a]For further details regarding the deprotonation and subsequent chemical oxidation of glucose, readers are referred to reference 20.

ferricyanide and ruthenium(III) hexamine. Our results have shown three distinct advantages of using dihydroresorufin as an indicator molecule for FEEM as compared to our previous scheme. First, the presence of a strong reducing environment shifts the chemical equilibrium to dihydroresorufin, leading to a greatly suppressed fluorescence background. Second, the fluorescent product, once generated on the electrode surface, is quickly reduced back to the nonfluorescent substrate molecule, dihydroresorufin, significantly lowering the accumulation of fluorescent species around the electrode. Therefore, diffusion becomes less significant in our current work compared to our previous study. Third, unlike resazurin reduction, the fluorescent signal detected at any given time represents the rate at which dihydroresorufin is oxidized at the electrode. As such, one can monitor the electrochemical signal by directly monitoring the total fluorescence. Together, these two fluorogenic pathways shown in Scheme 1a provide a more complete fluorescent voltammetry tool kit with the ability to optically monitor the progress of any electrochemical reaction.

EXPERIMENTAL SECTION

Reagents, Chemicals, and Dihydroresorufin Solution Preparation. Resazurin sodium salt (Aldrich, dye content ~75%), sodium hydroxide (J.T. Baker, 98.5%), glucose (Sigma, 99.5%), potassium ferricyanide ($K_3Fe(CN)_6$, Sigma-Aldrich, 99%), potassium chloride (J.T. Baker, 99.8%), ascorbic acid (Sigma, 98%), and hexaammineruthenium(III) chloride ($Ru(NH_3)_6Cl_3$, Aldrich, 98%) were all used without further purification. Deionized water (>18 MΩ·cm) was obtained through a Barnstead Nanopure water purification system and used for all aqueous solutions. A dihydroresorufin solution containing glucose and 0.5 M NaOH was prepared fresh daily. The dihydroresorufin concentration was 100 μM and the glucose concentration was 67 mM except where noted. Ascorbic acid solutions were prepared analogously, substituting ascorbic acid for glucose and 0.5 M KCl for NaOH.

Fluorescence Microscopy. All fluorescence microscopy experiments were conducted on an Olympus IX70 inverted microscope equipped with an IX-FLA inverted reflected light fluorescence observation attachment. The excitation source consisted of a Thorlabs M530L2 Collimated LED powered by a DC2100 LED Driver and filtered using a cube with a HQ535/50 excitation filter, a Q565lp dichroic mirror and a HQ610/75

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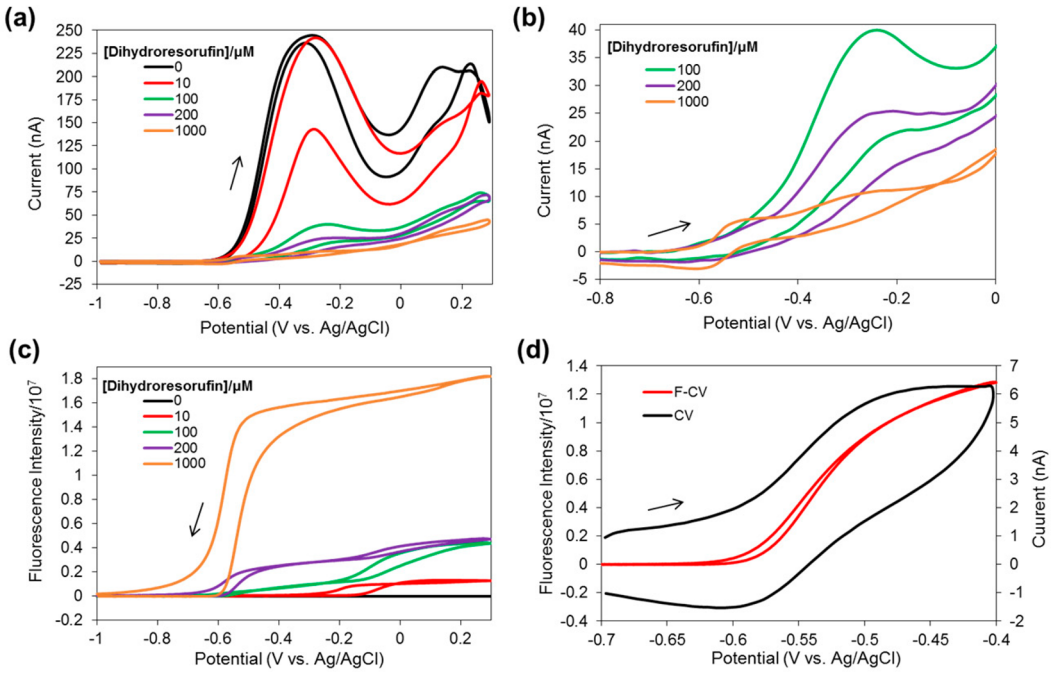

Figure 1. Electrochemical and fluorescence CVs obtained using a 25 μm Au electrode at various concentrations of dihydroresorufin in 67 mM glucose and 0.5 M NaOH. (a) Electrochemical CVs for a scan from −1 to +0.3 V. (b) Zoom-in of panel a for dihydroresorufin concentrations of 100, 200, and 1000 μM. Only the potential range from −0.8 to 0 V is shown. (c) Fluorescence CVs corresponding to the electrochemical CVs shown in panel a. (d) Overlaid plot of the simultaneously recorded fluorescence (red trace) and electrochemical (black trace) CVs of 1000 μM dihydroresorufin for a scan from −0.7 to −0.4 V at 10 mV/s.

emission filter. The excitation and emission wavelengths were chosen based off of a fluorescence spectrum of resorufin reported by the Chen group.[13] An Andor iXon+ EMCCD camera cooled to −80 °C and Andor SOLIS software was used to record and process all videos and images. Video was recorded at a frame rate of 33.887 Hz for all experiments using a preamplifier gain setting of 5.1.

Cyclic and Linear-Sweep Voltammetry. A Chem-Clamp voltmeter/amperometer (Dagan) interfaced to a Dell computer through a PCI-6251 data acquisition board (National Instruments) via a BNC-2090 analog breakout box (National Instruments) was used for all experiments. LabView 8.5 (National Instruments) was used for voltage function generation as well as acquisition of the current−voltage data. A scan rate of 200 mV/s was used for all potential sweep experiments unless noted otherwise. All reported potentials are referenced to Ag/AgCl.

RESULTS AND DISCUSSION

Fluorogenic, Reversible Oxidation of Dihydroresorufin. To better utilize dihydroresorufin in FEEM experiments, an understanding of its basic electrochemical behavior was needed. We used a conventional two-electrode (nonbipolar) cell to study the fluorogenic oxidation of dihydroresorufin. It was found that the chemically reduced dihydroresorufin solution provides a unique advantage over the resazurin solution previously used in FEEM experiments. Previously, we[5] and others[1,21] have demonstrated the proportionality between the time derivative of the fluorescence intensity and the electrochemical current for fluorogenic and fluoroquenching electrochemical reactions. In the resazurin/phosphate buffer solution, electrogenerated resorufin remains in solution and continues to fluoresce throughout the experiment. However, in the dihydroresorufin solution used here, the presence of glucose at a sufficiently high concentration (i.e., 67 mM, or 670 times greater than that of the dihydroresorufin) in a basic media assures that resorufin, when generated at the anodic pole, can be rapidly consumed from the solution, restoring dihydroresorufin and effectively eliminating fluorescence accumulation. It is therefore the fluorescence intensity itself and not the time derivative that is proportional to the electrochemical current.

Figure 1 shows the cyclic voltammograms (CVs) of a 25 μm diameter Au electrode in a conventional two-electrode setup along with the corresponding fluorescence cyclic voltammograms (F-CVs) for potential sweep experiments of various concentrations of dihydroresorufin in 67 mM glucose and 0.5 M NaOH. As can be seen in the CVs (Figure 1a,b), an oxidation wave with an onset potential of −520 mV is present, which corresponds to the oxidation of glucose.[22] As expected, there is no fluorescence signal when no dihydroresorufin is present (Figure 1c, black trace). When dihydroresorufin is added to solution, the electrochemical and fluorescence signals become coupled in an interesting manner. Predictably, increasing the concentration of dihydroresorufin increases the overall fluorescence signal, as more of the fluorogenic redox species is present in solution and available for fluorogenic oxidation. However, somewhat surprisingly, this results in a significantly diminished electrochemical current signal. As the first step of glucose oxidation involves its adsorption onto the electrode,[23,24] this pattern indicates that dihydroresorufin blocks this oxidation step, resulting in a decreased electrochemical signal as the dihydroresorufin concentration increases. It is also known that the second wave of glucose oxidation produces more weakly adsorbed products.[23,24] This results in a less-blocked surface, which we propose is the cause of the second wave of fluorescence intensity at −100 mV for

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dihydroresorufin concentrations from 10 to 200 μM. As seen in the CV for 1000 μM dihydroresorufin (Figure 1a,b, orange trace), a new oxidation wave arises with an onset of −600 mV. This coincides nearly exactly with the onset of the fluorescence signal (−580 mV), indicating that this wave is from the electrochemical oxidation of dihydroresorufin. This can be seen more clearly in Figure 1d, which shows the simultaneously recorded CV and F-CV obtained for a solution of 1000 μM dihydroresorufin overlaid. The fluorescence signal directly correlates to the electrochemical current signal, indicating that this reaction can be used as a fluorogenic indicator for FEEM. The CV gives a steady-state current of approximately 3.8 nA. The theoretical steady-state current, i_{ss}, can be calculated from the following equation:[25]

$$i_{ss} = 4nFC^*Dr \quad (1)$$

where n is the number of electrons transferred per molecule, F is Faraday's constant (96 485 C/mol), D is the diffusion coefficient, C^* is the bulk concentration of redox species, and r is the electrode radius. Using a diffusion coefficient of 4.8 × 10^{-6} cm^2/s,[26] the expected steady-state current for 1000 μM dihydroresorufin at a 25 μm diameter microelectrode is 4.6 nA. The experimental value corresponds nicely to this theoretical value when considering that glucose is also adsorbed onto the electrode surface and thus blocking some of the electroactive area from dihydroresorufin oxidation.

Studying variations in the oxidation of dihydroresorufin on a Au electrode as compared to a carbon fiber electrode (CFE) also provides insight into the coupled electrochemical and fluorescent properties of this system. Figure 2a shows the overlaid CV and F-CV for a solution of 100 μM dihydroresorufin, 67 mM glucose, and 0.5 M NaOH obtained with a 25 μm diameter Au working electrode for a potential sweep from −1 to +0.3 V. During the forward scan, the fluorescence onset potential is at −540 mV and a second higher intensity wave appears at approximately −100 mV. In addition to the potential-dependent blocking effect of adsorbed glucose and its oxidation products, we propose there is at least one other factor leading to the general shape of this curve. At electrode potentials between −540 and −100 mV, the fluorescence intensity is limited somewhat by the relatively high concentration of glucose in solution adjacent to the electrode. At potentials above −100 mV, glucose is electrochemically oxidized to a greater degree, as can be seen in the CV in Figure 2a, depleting the concentration of this species in the vicinity of the electrode surface. This process, in addition to the ongoing oxidation of dihydroresorufin, results in a second, higher intensity fluorescence wave. It is thought that both of these factors contribute to some degree to the presence of two distinct waves as well as the slightly higher fluorescence signal on the return scan. The three still-images presented in Figure 2b were taken from a video recorded during the F-CV experiment and show the burst of fluorescence at the 25 μm Au electrode as the potential is swept to oxidize dihydroresorufin. At +200 mV, the electrode spot size is noticeably larger as well due to the depletion of glucose and the therefore slower chemical reduction process. The fluorescence intensity (and the concentration of resorufin) at or near to the electrode in Figure 2b and subsequent figures is likely controlled by several processes including oxidation of dihydroresorufin, oxidation of glucose, the chemical reduction of resorufin by glucose, and various diffusion processes.

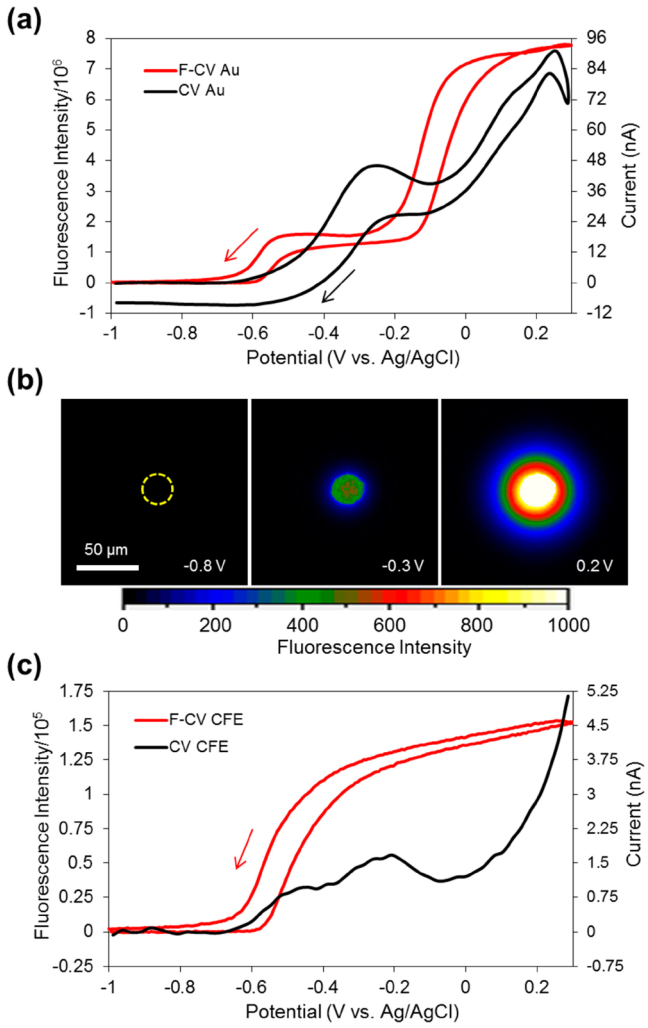

Figure 2. (a and c) Comparison of a conventional cyclic voltammogram (CV) with a corresponding, simultaneously recorded fluorescence cyclic voltammogram (F-CV) for (a) a 25 μm diameter Au disk electrode and (c) a 10 μm diameter CFE, direct connect (not BPE) in a solution containing 100 μM dihydroresorufin, 67 mM glucose, and 0.5 M NaOH. (b) Fluorescence still images captured from a video recorded during the potential sweep experiment shown in panel a at select potentials. The dashed yellow ring in the left panel of panel b indicates the actual size and position of the electrode. Note the different fluorescence intensity and current scales in panels a and c. Only the forward scan of the CV is shown in panel c.

Figure 2c shows the results of an experiment similar to that in Figure 2a except the working electrode was replaced with a 10 μm diameter carbon fiber electrode (CFE). The intensity of both the electrochemical CV and the F-CV were significantly lower than that of the Au electrode, likely due to relatively lower electrocatalytic activity of carbon, but the initial fluorescence onset potential was still at approximately −540 mV. Glucose oxidation occurs at a much lower rate for carbon as compared to Au,[27] therefore leaving the concentration of glucose relatively unperturbed prior to the chemical reaction with resorufin. We propose this is why only one wave is observed in the CFE F-CV, indicating that the extent of the glucose side reactions (i.e., adsorption and electrochemical oxidation) on a CFE is much smaller as compared to on a Au electrode.

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To more completely understand the dynamics of the fluorogenic electrochemical oxidation of dihydroresorufin and subsequent chemical reduction of the product resorufin by glucose, the effect of altering the glucose concentration was studied. Figure 3 presents F-CVs from potential sweep

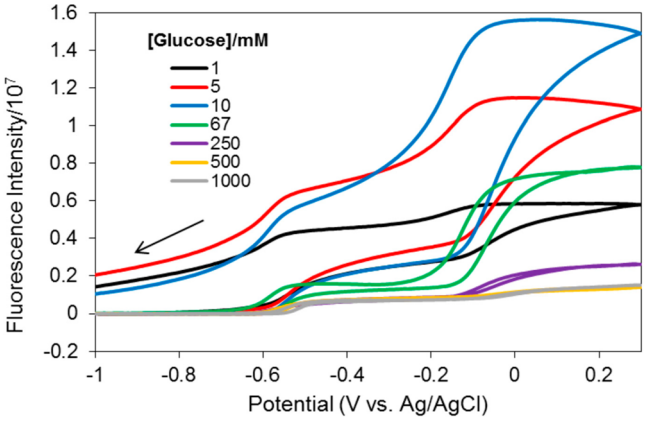

Figure 3. F-CVs obtained with a 25 μm diameter Au disk electrode in solutions with a total resazurin/resorufin/dihydroresorufin concentration of 100 μM and varying concentrations of glucose in 0.5 M NaOH. The potential was swept from −1 to +0.3 V at 200 mV/s.

experiments conducted using a conventional two-electrode setup in which the concentration of glucose was varied from 1 to 1000 mM. A 25 μm Au working electrode was used, and the total concentration of resazurin/resorufin/dihydroresorufin was 100 μM for all solutions. The fluorescence intensity was found to increase as the glucose concentration in solution was increased from 1 to 10 mM. This can be explained by the increasing concentration of dihydroresorufin in solution. Altering the concentration of glucose will affect the equilibrium between these three species, with higher concentrations of glucose favoring the fully reduced species, dihydroresorufin. This can be observed visually (Figure SI-2, Supporting Information), as the solution color changes from blue (resazurin) to pink (resorufin) to clear (dihydroresorufin) as the glucose concentration is increased. A higher concentration of dihydroresorufin enables the fluorogenic oxidation to proceed at a higher rate, resulting in an increased fluorescence signal.

Interestingly, further increasing the glucose concentration to 67 mM and beyond results in a clear decrease in both the recorded fluorescence intensity and the hysteresis of the F-CV. We propose two factors contribute to this decrease in intensity. First, the amount of surface-adsorbed glucose on the electrode likely increases with solution concentration. The adsorbed glucose physically blocks dihydroresorufin oxidation, resulting in a lower rate of reaction and hence a lower fluorescence intensity. Second, at higher solution concentrations, more glucose will be present in solution adjacent to the electrode surface. This glucose can quench fluorescence by chemically reducing resorufin back to the nonfluorescent dihydroresorufin. This quenching effect becomes greater as the glucose concentration increases, thus decreasing overall fluorescence intensity. The quenching effect also explains the change in hysteresis of the fluorescence voltammograms. At low concentrations of glucose (i.e., 1−10 mM), resorufin produced at the electrode via dihydroresorufin oxidation has a much longer lifetime in solution than at higher glucose concentrations, as each resorufin molecule is less likely to encounter glucose and undergo reduction back to dihydroresorufin. This time delay between resorufin production via dihydroresorufin oxidation at the electrode and resorufin consumption via reduction by glucose becomes less as the glucose concentration increases, thereby decreasing the hysteresis in the F-CV. The use of ascorbic acid as an alternative reducing agent was also briefly investigated, with the results presented in Figure SI-3 (Supporting Information). The same overall trends were observed, but the fluorescence signal was more than 2 orders of magnitude lower with ascorbic acid. We suspect this is due to solution instability, as a precipitate began forming shortly after preparing the solutions.

Use of Dihydroresorufin in FEEM to Study Analyte Reduction. With an understanding of the electrochemical and fluorescence properties of the dihydroresorufin oxidation reaction, we could then use it in a bipolar FEEM setup to study analyte reduction reactions. We used a simple bipolar setup (diagram shown in Figure SI-4, Supporting Information) to demonstrate the electrochemical coupling of the fluorogenic oxidation of dihydroresorufin to the reduction of ferricyanide ($Fe(CN)_6^{3-}$). Two 25 μm diameter Au disk electrodes were connected in series to form a closed BPE as described previously.[28,29] The cathodic pole was placed in 250 μM ferricyanide with 1 M KCl supporting electrolyte and the anodic pole was placed in a solution of 100 μM dihydroresorufin with 67 mM glucose and 0.5 M NaOH and positioned on a microscope for observation. The dihydroresorufin solution was illuminated to excite resorufin fluorescence, and a triangular waveform from +1.2 to +0.2 V was applied to two Ag/AgCl driving electrodes to reduce ferricyanide at the cathodic pole. This reduction is coupled to the oxidation of dihydroresorufin at the anodic pole, resulting in the production of the fluorescent product, resorufin.

Figure 4 shows five background-corrected still-images of the anodic pole taken from a video recorded over the potential

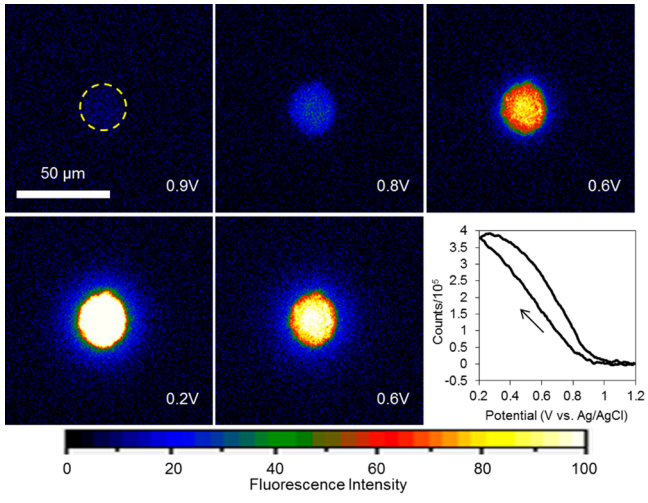

Figure 4. Series of fluorescence still-images captured from a video of the FEEM detection of 250 μM ferricyanide at a 25 μm diameter Au disk electrode using a solution of 100 μM dihydroresorufin, 67 mM glucose, and 0.5 M NaOH. Potential was cycled from +1.2 to +0.2 V at a scan rate of 200 mV/s as applied to two Ag/AgCl driving electrodes. The bottom-right panel shows fluorescence intensity at the electrode as a function of electrode potential. The dashed yellow ring in the top-left panel indicates the actual position and size of the electrode.

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sweep experiment. The last panel of the figure displays the voltage-dependent total fluorescence intensity recorded at the anodic pole. As can be seen, the onset of the fluorescent burst occurs at approximately 830 mV. This onset potential can be understood from an analysis of the onset potentials for the reduction of ferricyanide (310 mV vs Ag/AgCl in a two-electrode cell, Figure SI-5, Supporting Information) and that for the oxidation of dihydroresorufin (−540 mV vs Ag/AgCl in a two-electrode cell, Figure 2a). The onset potential of the coupled ferricyanide reduction/dihydroresorufin oxidation here corresponds roughly to the difference in the two onset potentials,[30] agreeing within 20 mV. As the potential is swept in the negative direction, ferricyanide is reduced at an increasing rate at the cathodic pole, resulting in an increasing rate of dihydroresorufin oxidation at the anodic pole and thus greater fluorescence intensity. As the potential is swept back in the positive direction, ferricyanide reduction slows down, resulting in a decrease in fluorescence intensity until fluorescence is no longer observed. The fluorescence intensity is also slightly higher on the return scan. This can be visualized by comparing the top-right (+0.6 V forward scan) and bottom-middle (+0.6 V return scan) panels of Figure 4. We propose this is due to the depletion of glucose in solution adjacent to the electrode surface on the forward scan, which lessens the quenching effect and results in a slightly higher fluorescent signal on the return scan. We also used dihydroresorufin as an indicator molecule to report the reduction of $Ru(NH_3)_6^{3+}$. Figure SI-6 (Supporting Information) shows a series of fluorescence images recorded during the electrochemical reduction of 250 μM $Ru(NH_3)_6^{3+}$ in a separate FEEM experiment. Importantly, the onset potential of the coupled reaction, as determined by the fluorescent signal, agreed to within 10 mV of the predicted onset potential. These results demonstrate that dihydroresorufin can be used in FEEM for the study of reducible redox species.

We then investigated the use of dihydroresorufin for the quantitative detection of a reducible analyte using FEEM. Again, two 25 μm Au electrodes were connected in series to produce a single BPE. The anodic pole was placed in a solution of 100 μM dihydroresorufin, 67 mM glucose, and 0.5 M NaOH and positioned on a microscope for observation, and the cathodic pole was placed in a solution containing ferricyanide with 1 M KCl supporting electrolyte. The potential was swept from +1.2 to +0.2 V at 200 mV/s. Presented in Figure 5a is a series of fluorescence intensity signals recorded over the course of the voltage scans with analyte concentrations from 25 to 750 μM. The concentration dependence of the fluorescence intensity at the switching potential is shown in Figure 5b. The concentration range over which the data is linear is from 50 to 400 μM ferricyanide. Above 400 μM ferricyanide, the curve begins to plateau as the oxidation of dihydroresorufin at the anodic pole of the BPE becomes limiting. At ferricyanide concentrations of 50 μM and below, the fluorescence response becomes insensitive to the concentration of ferricyanide and is approximately constant, as oxygen reduction, rather than ferricyanide reduction, is now primarily coupled to the oxidation of dihydroresorufin. Deoxygenating these low concentration solutions results in no observable fluorescence signal, however, indicating that the current from ferricyanide reduction in these solutions is below the threshold current necessary for fluorescence generation (Figure SI-7, Supporting Information). This can be understood by considering the electrochemical and chemical processes in the dihydroresorufin

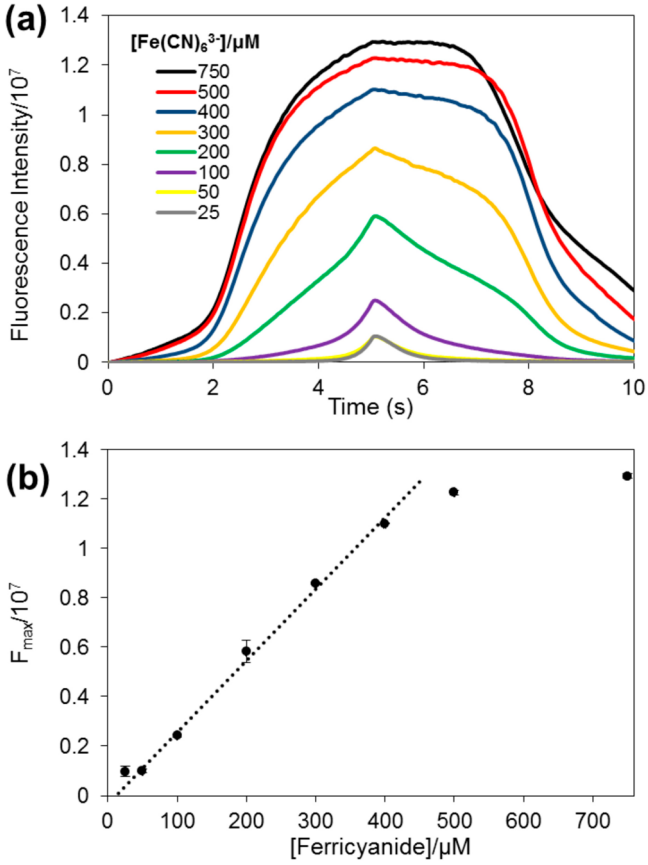

Figure 5. FEEM-based detection of various concentrations of ferricyanide at a 25 μm diameter Au disk BPE using a solution containing 100 μM dihydroresorufin, 67 mM glucose, and 0.5 M NaOH. Potential was cycled from +1.2 to +0.2 V at a scan rate of 200 mV/s as applied to two Ag/AgCl driving electrodes.

solution. There are three competing processes at the anodic pole: the electrochemical oxidation of dihydroresorufin to generate resorufin, the electrochemical oxidation of glucose, and the chemical reduction of resorufin back to dihydroresorufin. When the concentration of ferricyanide is below 50 μM, the electrochemical oxidations of dihydroresorufin and glucose are both relatively slow, resulting in a very small rate of resorufin generation. As such, in the presence of a large concentration of glucose, resorufin is quickly reduced back to dihydroresorufin. Admittedly, the range over which a linear concentration response is observed is very narrow and the lower limit of detection leaves much to be desired, limiting the use of this system as a quantitative technique to readout analyte concentrations. However, it is anticipated that modifying the electrode to selectively oxidize dihydroresorufin or using a nonfouling reducing agent that is electrochemically inactive in the desired potential range will lower the limit of detection.

Improved Spatial and Temporal Resolution of FEEM using Dihydroresorufin. In spite of the relatively high limit of detection of FEEM using dihydroresorufin, we found that this system gives rise to several significant benefits. As already demonstrated, the electrochemical current signal can be reported by directly monitoring the total fluorescence signal rather than the time derivative of this signal as with our previous report. More importantly, FEEM using dihydroresorufin as the fluorogenic reporter appears to give enhanced spatial and temporal resolution than FEEM using resazurin as the

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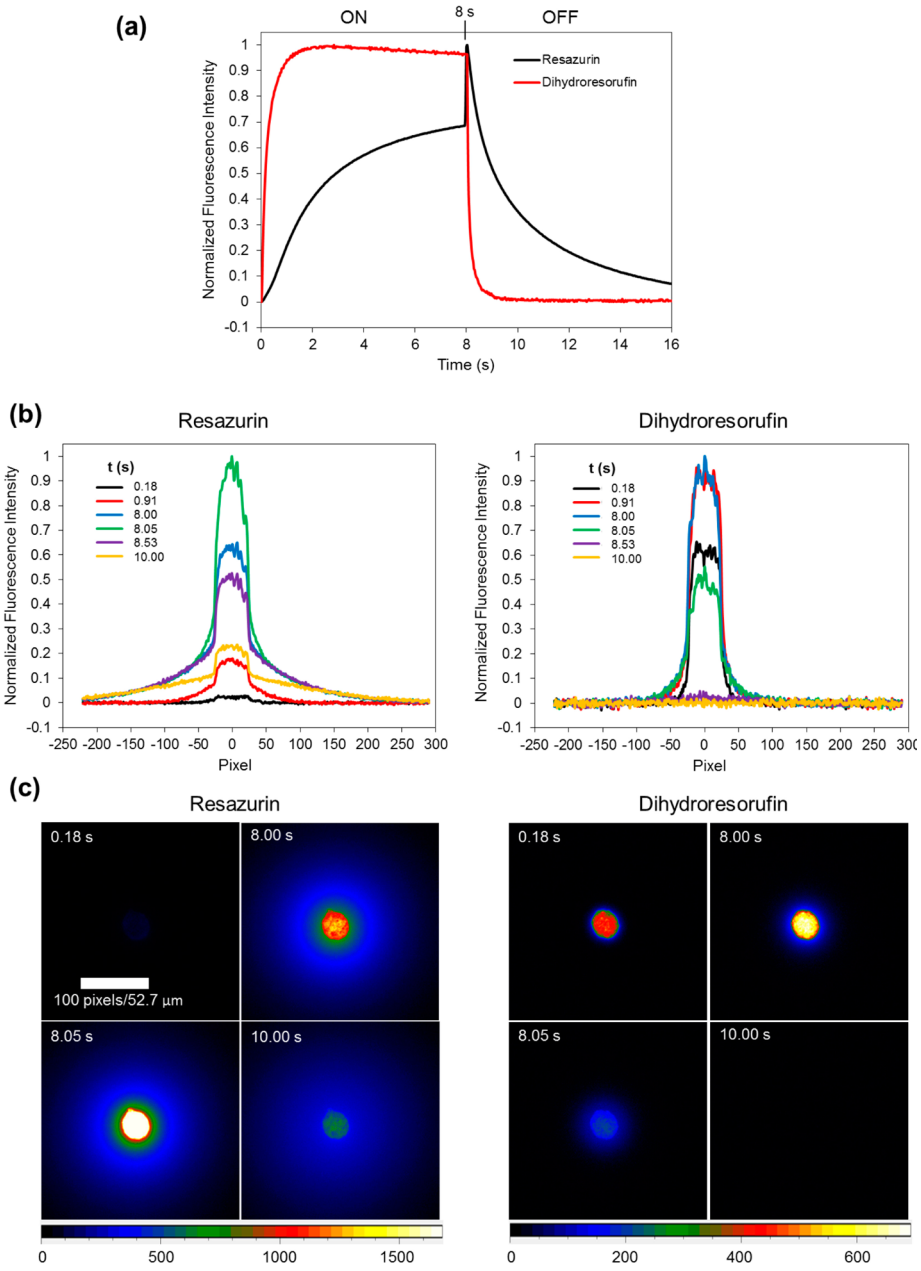

Figure 6. Voltage pulsing experiment comparing the FEEM detection of ferricyanide using dihydroresorufin to the detection of ferrocyanide using resazurin at a 25 μm Au electrode. (a) Comparison of the normalized fluorescence intensity over time of the resazurin system and dihydroresorufin system. The voltage pulse was in the "on" state (a potential sufficient to drive the coupled reactions of interest) for 8 s and then switched to the "off" state for 8 s. (b) Linescans across the electrode over the course of the voltage pulse. (c) Fluorescence images of the electrode over the course of the voltage pulse. The maximum intensity on the color scale was set to the maximum pixel intensity recorded at the electrode at 8 s.

reporter. A simple potential step experiment, shown in Figure 6, demonstrates this point. In this experiment, two 25 μm diameter Au electrodes were connected to form a closed BPE. One pole was placed in a solution that was 250 μM in both ferricyanide and ferrocyanide with 1 M KCl as supporting electrolyte. The opposite pole was placed in either 100 μM dihydroresorufin, 67 mM glucose, and 0.5 M NaOH or 100 μM resazurin in 50 mM phosphate buffer. An 8 s pulse at a potential sufficient to drive the coupled redox reactions was applied ("on" state), followed by an 8 s period at which the potential was adjusted below the onset potential ("off" state). For the coupled dihydroresorufin oxidation/ferricyanide reduction, the "on" state was +0.3 V and the "off" state was +1.2 V. For the coupled resazurin reduction/ferrocyanide oxidation, the "on" state was +1.2 V and the "off" state was +0.3 V. Figure 6a shows the normalized fluorescence intensity recorded over the length of the pulse cycle for both coupled reactions. As seen, the fluorescence response of the two fluorogenic indicators is markedly different. The dihydroresorufin system reaches a steady-state fluorescence intensity within 2 s of the start of the "on" pulse, while the fluorescence intensity of the resazurin system increases throughout the "on" pulse, failing to reach a steady-state in 8 s. When the potential is switched to the "off" state, the fluorescence intensity of the dihydroresorufin system decays to its initial intensity within 1 s, while the intensity of the resazurin system shows an immediate

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spike, followed by a slow decay, failing to return to its initial intensity within 8 s.

The difference in the temporal response of the two systems can be explained by considering the differing mechanisms through which the electrogenerated fluorescence decays. In the dihydroresorufin system, electrogenerated resorufin is chemically reduced back to nonfluorescent dihydroresorufin. In the resazurin system, electrogenerated resorufin is only "consumed" by photobleaching or diffusion out of the field of view. The photobleaching and diffusion processes are much slower than the chemical reduction process, which accounts for a more sluggish fluorescence response from the resazurin system. We propose the large spike in fluorescence intensity observed in the resazurin system when the potential is switched to the "off" state is due to resazurin adsorbed onto the electrode surface. In the "on" state, these surface-bound molecules are fully reduced to dihydroresorufin, and upon switching the potential to the "off" state, they are immediately oxidized back to resorufin, resulting in the observed spike.

The effect of fluorophore diffusion can be readily seen in Figure 6b, which presents a series of line scans across the electrodes over the course of the voltage pulse. The fluorescent signal from the resazurin system broadens significantly over the course of the experiment due to diffusion of electrogenerated resorufin. By comparison, the signal from the dihydroresorufin system shows minimal broadening, as the electrogenerated resorufin is chemically reduced back to dihydroresorufin before it has time to diffuse a significant distance from the electrode surface. Figure 6c presents background corrected images of the electrodes over the course of the voltage pulse. The different temporal response and the effect of fluorophore diffusion can be easily observed in these images. At 0.18 s into the "on" state, a strong signal is observed in the dihydroresorufin system whereas the resazurin signal is weak. At 8 s, however, both systems show strong signals, but the signal from the resazurin system has significantly broadened due to diffusion. At 8.05 s (0.05 s into the "off" state), the resazurin system shows a large spike in intensity, while the signal from the dihydroresorufin system has decayed to about half its steady-state intensity. At 10 s, no signal is observed from the dihydroresorufin, while a strong, diffusion-broadened signal is still observed from the resazurin system. As can be seen, using dihydroresorufin as a fluorogenic indicator in FEEM gives an improved temporal response and lower diffusional signal broadening than resazurin. We expect these properties of improved spatial and temporal resolution in FEEM to be especially important in FEEM imaging applications where high-density arrays of thousands or more ultramicroelectrodes would be used to image dynamic electrochemical processes. These applications include screening of new electrocatalysts and imaging transient biological processes such as neuronal exocytosis. Work is currently underway to image dynamic diffusion layers of ultramicroelectrodes, which we expect to report on in due course.

CONCLUSION

In summary, we have demonstrated the ability to use fluorescence-enabled electrochemical microscopy for the analytical detection of reducible species using a solution containing dihydroresorufin that undergoes a fluorogenic electrochemical oxidation to form resorufin. This process, together with the previously described fluorogenic reduction of resazurin, provides for a more complete electrochemical fluorogenic toolkit and greatly increases the applicability of FEEM. The fluorescence intensity measured from the system described here was found to be directly proportional to the electrochemical current. The signal from this system is therefore easier to interpret than the signal from the fluorogenic reduction of resazurin where we previously showed the correlation of the time derivative of fluorescence intensity and the electrochemical current. Due to the presence of a chemical reducing agent that quickly consumes the electrogenerated fluorophore, this system was found to have an improved temporal response as well as lower signal broadening from diffusion than the resazurin system.

ASSOCIATED CONTENT

Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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