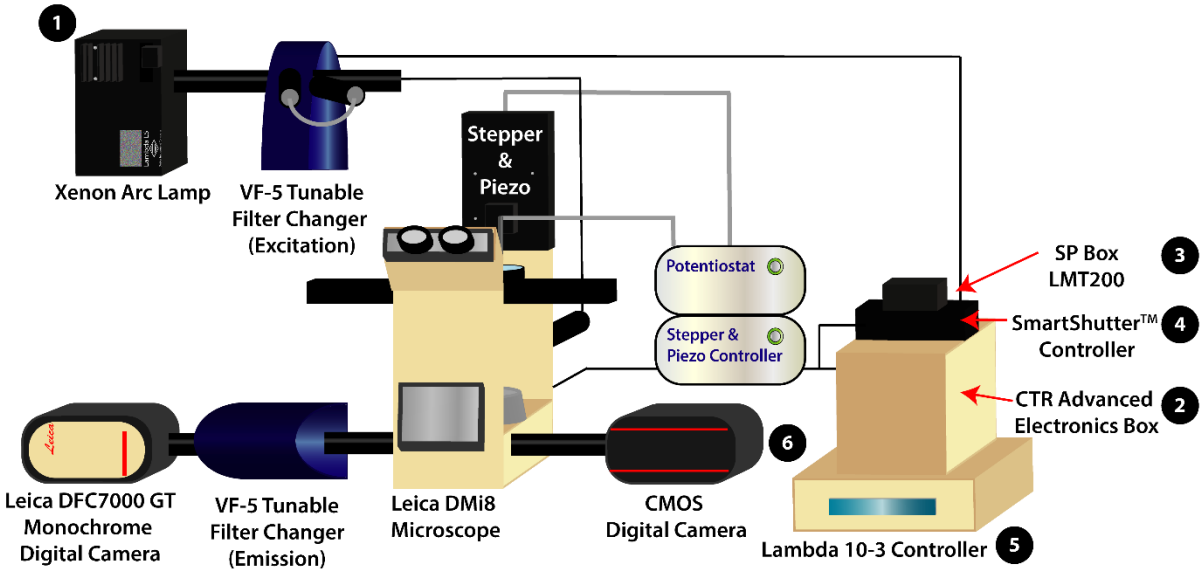
Electronic Supporting Information

# Investigating the Cytotoxic Redox Mechanism of PFOS within Hep G2 by Hyperspectral Assisted-Scanning Electrochemical Microscopy

Sondrica Goines[a] and Jeffrey E. Dick[a, b]*

[a]Department of Chemistry, The University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, USA
[b]Lineberger Comprehensive Cancer Center, The University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, USA
*To whom all correspondence should be addressed: jedick@email.unc.edu

Table of Contents




**Scheme S1.** Schematic representation of the variable fluorescence bandpass hyperspectral imaging platform coupled with a 920D bipotentiostat for hyperspectral assisted-scanning electrochemical microscopy. Equipment is numbered based on the power on mechanism.

**Table S1. Available VersaChrome® Tunable Filters**

| Location | Product # | Description |
|---|---|---|
| VF-5 Tunable Filter Changer (Excitation, SA or S1) | O573380 | OPT, VF,380/16 NM BANDPASS FILTER |
| | O573440 | OPT, VF,440/16 NM BANDPASS FILTER |
| | O573490 | OPT, VF,490/15 NM BANDPASS FILTER |
| | O573550 | OPT, VF,550/15 NM BANDPASS FILTER |
| | O573620 | OPT, VF,620/14 NM BANDPASS FILTER |
| VF-5 Tunable Filter Changer (Emission, SB or S2) | O573440 | OPT, VF,440/16 NM BANDPASS FILTER |
| | O573490 | OPT, VF,490/15 NM BANDPASS FILTER |
| | O573550 | OPT, VF,550/15 NM BANDPASS FILTER |
| | O573620 | OPT, VF,620/14 NM BANDPASS FILTER |
| | O573700 | OPT, VF,700/13 NM BANDPASS FILTER |

## Detailed Experimental

### Hyperspectral assisted-scanning electrochemical microscopy of Hep G2

A cryovial containing 1 mL Hep G2 P26 cells in freezing media (*i.e.,* 95% full growth media/5% DMSO) was removed from the -80 °C freezer, then thawed in a water bath set to 37 °C. Next, the cryovial contents were counted using an automated Corning Cell Counter and the associated CytoSMART application. The counter was used to generate an image, which was used to approximate the number of cells ($1.62 \times 10^6$ cells $mL^{-1}$). Based on this approximation, $3.13 \times 10^5$ cells were delivered to a 3.5 cm-diameter tissue culture dish in a 193 µL aliquot, then combined with 2 mL full growth media. The resulting Hep G2 P27 cells were placed in the incubator set to 37 °C and 5% $CO_2$. At 30 to 40% confluence (*i.e.,* low to medium density), spent media was removed from the dish and cells were rinsed with 1 mL DPBS. Following the rinse, 2 mL full growth media was placed in the dish.

Cells were imaged the following day. A stock solution of 4.31 mM DCF-DA was prepared by combining 2.10 mg DCF-DA and 1 mL DMSO in a 1.5 mL microcentrifuge tube. For fluorescence imaging, 23 µL of the stock solution was diluted to a 10 mL solution of 10 µM DCF-DA with DPBS. For electrochemical imaging, 2.05 mg $FcCH_2OH$ was weighted out in a 20 mL scintillation vial and diluted to a solution of 0.948 mM $FcCH_2OH$ in 10 mL DPBS. Next, the $FcCH_2OH$ solution was vortexed and placed in a sonicator for 15 minutes to fully dissolve the solid. To analyze the intracellular ROS content, the cells were rinsed with 1 mL DPBS, then incubated in 1 mL 10 µM DCF-DA in DPBS for 10 minutes at room temperature while covered with foil. After the DCF-DA incubation, the solution was removed and the cells were rinsed with 1 mL DPBS. Next, the $FcCH_2OH$ stock was diluted to 0.5 mM $FcCH_2OH$ in the dish using DPBS.

The dish was placed on the microscope stage and three electrodes were placed in solution: a salt bridge to an external Ag/AgCl (1 M KCl) reference electrode in DPBS, a glassy carbon rod counter electrode ($r$ = 1.5 mm), and a Pt microelectrode SECM tip ($r$ = 5 µm) using a 3D printed holder attached to the piezoelectric positioner of the 920D bipotentiostat. Cells were brought into focus using a 20× objective lens and the SECM tip was positioned within the acquisition area. To place the SECM tip above cells for imaging, first, the tip was biased at +0.5 V vs. Ag/AgCl (*i.e.,* sufficiently positive to oxidize $FcCH_2OH$ to ferrocenium methanol, $Fc^+CH_2OH$), then used to approach the insulating dish in the $z$ direction until negative feedback was observed (*i.e.,* $i_T < i_{T,\infty}$). Here, the tip was moved towards the dish vertically 588 µm to be theoretically 3 µm from the insulating dish (**Figure S1**); next, the tip was retracted 588 µm and used to approach to the cell surface. The resulting approach curves were used to approximate the distance from the cells during electrochemical imaging (**Figure S1**).

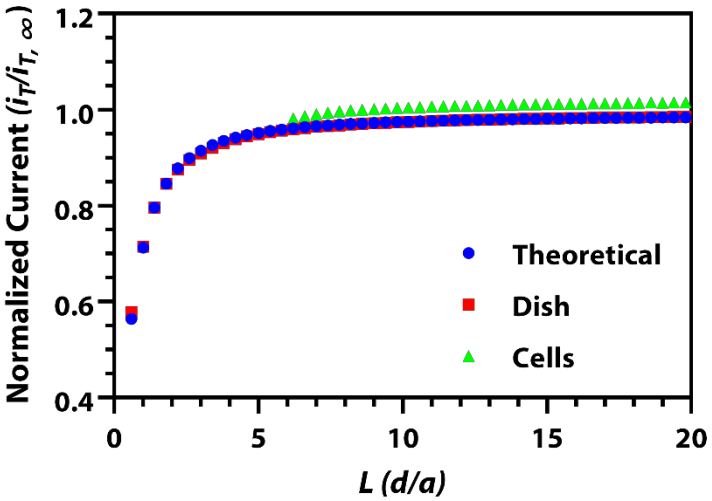



**Figure S1.** Normalized approach curves obtained using a Pt microelectrode SECM tip ($r$ = 5 µm) biased at +0.5 V vs. Ag/AgCl (1 M KCl) within 0.5 mM $FcCH_2OH$ in DPBS (1X, pH 7.4) above Hep G2 P27 cells in a 3.5 cm-diameter tissue culture dish. A glassy carbon rod counter electrode ($r$ = 1.5 mm) was used.

Prior to electrochemical imaging, a fluorescence image of DCF, a redox reporter for ROS formed from DCF-DA, was obtained after setting the hyperspectral system to an excitation of 465 nm and an emission of 535 nm. The hyperspectral system was then used to obtain hyperspectral data from the emission of 500 nm to 650 nm at an excitation of 465 nm. After moving the tip up 1 µm with the piezoelectric positioner, the SECM tip was used to scan the imaged area in the *xy* plane at +0.5 V vs. Ag/AgCl. The resulting correlated data is shown in **Figure S2** below.

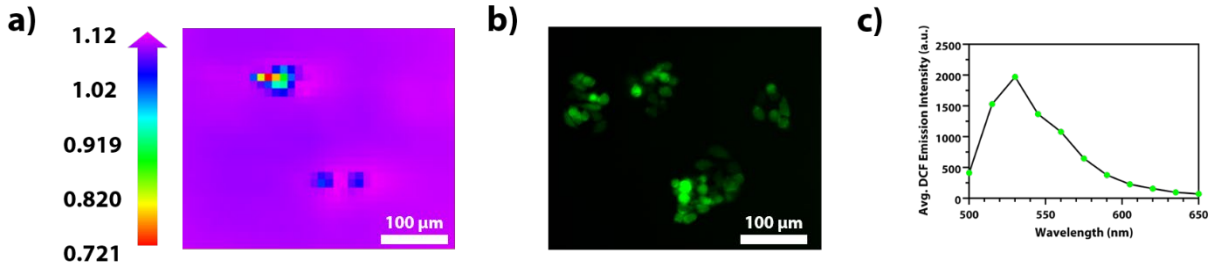



**Figure S2.** Correlated **(a)** electrochemical image, **(b)** fluorescence image, and **(c)** spectrum of Hep G2 P27 cells in 0.5 mM $FcCH_2OH$ in DPBS (1X, pH 7.4). Hep G2 cells loaded with DCF-DA prior to imaging. Electrochemical image obtained using a Pt microelectrode SECM tip ($r$ = 5 µm) at +0.5 V vs. Ag/AgCl (1 M KCl) and a glassy carbon rod counter electrode ($r$ = 1.5 mm); image normalized by $i_{T,\infty}$ when $d \geq 100$ µm. Fluorescence image of ROS indicator, DCF, obtained at $\lambda_{ex}/\lambda_{em}$ 465/535 nm. Hyperspectral images used to produce **(c)** obtained at $\lambda_{ex}$ 465 nm. The fluorescence image was false colored for visual representation.

**Figure S2** demonstrates our ability to capture correlated electrochemical, optical, and hyperspectral images of Hep G2 cells loaded with DCF-DA to express DCF fluorescence upon

reacting with intracellular ROS (*i.e.,* DCF is the fluorescence reporter for intracellular ROS[1, 2]). All images were captured in the presence of $FcCH_2OH$ – a well behaved, one-electron transfer, cell permeable redox mediator[3]. The electrochemical image (**Figure S2a**) has been normalized by the limiting current (*i.e.,* $i_{T,\infty}$ when $d >> 2a$, where $d$ is the distance from the insulating dish and $a$ is the radius of the Pt microelectrode SECM tip); electrochemical images were normalized to remove ambiguity associated with variations in the electrochemical signal due to the tip-to-substrate distance. It is important to note that electrochemical images were obtained with a pixel size of 10 μm × 10 μm based on the diameter of the Pt microelectrode SECM tip (*i.e.,* diameter = 10 μm) to avoid oversampling. Areas of increased electrochemical signal were positioned above Hep G2 cells based on the corresponding fluorescence image shown in **Figure S2b**. The correlated spectrum shown in **Figure S2c** is the spectral representation of a two-dimensional stack of fluorescence images (*i.e.,* a λ stack of images) captured at an excitation of 465 nm; the images were captured at wavelengths from 500 nm to 650 nm with 15 nm increments between each image. This spectrum is representative of a hyperspectral image, in which a spectrum can be reported for each individual pixel within the λ stack. Within **Figure S2c**, DCF is excited using an excitation of 465 nm. Later, an excitation of 495 nm is used to capture an emission $\lambda_{max}$ at 535 nm to reduce photobleaching during imaging.

**Hyperspectral assisted-scanning electrochemical microscopy of Hep G2 following exposure to PFOS: reactive oxygen species content analysis**

Initially, Hep G2 P19 cells were cultured in a 10 cm-diameter tissue culture dish (VWR International, LLC) using full growth media and maintained in an incubator set to 37 °C and 5% $CO_2$. At 50% confluence (*i.e.,* medium density), spent media was removed from the dish and the cells were rinsed with 5 mL DPBS. Next, cells were suspended in a 2:1 mixture of DPBS to TrypLE Express (*i.e.,* 4 mL DPBS to 2 mL TrypLE Express). The dish was placed in the incubator for 7 minutes to promote cell suspension, then 9 mL full growth media was added to the suspension to halt trypsinization. The suspension was centrifuged at 1000 rpm for 5 minutes at room temperature. After removing the supernatant, the cells were resuspended in 1 mL full growth media and counted using an automated Corning Cell Counter and the associated CytoSMART application. Three images were generated with the counter and used to average the number of suspended cells ($6.77 \times 10^6 \pm 1.01 \times 10^6$ cells $mL^{-1}$, $n = 3$). Aliquots of 185 µL (approximately $1.25 \times 10^6$ cells) of the cell suspension were added to five 3.5 cm-diameter tissue culture dishes with 1.815 mL full growth media. The resulting Hep G2 P20 cells were placed in the incubator.

At 75% to 80% confluence (*i.e.,* medium to high density), spent media was removed from three dishes of the Hep G2 P20 cells and the cells were rinsed with 1 mL DPBS. Next, 2 mL solutions of 0, 50, and 100 µM PFOS-supplemented full growth media were added to one of the three dishes providing one sample per PFOS concentration. Each dish was labeled based on the concentration of PFOS added, then placed in the incubator for 16 hours overnight.

The next day, a stock solution of 2.16 mM DCF-DA was prepared by combining 1.05 mg DCF-DA and 1 mL DMSO in a 1.5 mL microcentrifuge tube. For fluorescence imaging, 70 µL of the stock solution was diluted to a 15 mL solution of 10 µM DCF-DA with DPBS. For electrochemical imaging, 2.21 mg $FcCH_2OH$ was weighted out in a 20 mL scintillation vial and diluted to a solution of 1 mM $FcCH_2OH$ with DPBS. Next, the $FcCH_2OH$ solution was vortexed and placed in a sonicator for 15 minutes to fully dissolve the solid.

After incubating the cells for 16 hours overnight, spent PFOS-supplemented full growth media was removed from the dishes. To analyze the intracellular ROS content, the cells were rinsed with 1 mL DPBS, then incubated in 1 mL 10 µM DCF-DA in DPBS. The dishes were covered with foil and placed on a rotator plate set to 10 rpm for 10 minutes at room temperature. After the DCF-DA incubation, the solution was removed and the cells were rinsed with 1 mL DPBS. Next, 2 mL full growth media was added to each dish and the dishes were placed in the incubator for later use.

The 1 mM $FcCH_2OH$ solution was then diluted to a 2 mL solution of 0.5 mM $FcCH_2OH$ in DPBS in an empty 3.5 cm-diameter tissue culture dish. The dish was placed on the microscope stage, then three-electrode system used previously was placed in solution. After connecting each electrode to the proper electrode leads of the bipotentiostat, the Pt microelectrode SECM tip was biased at +0.5 V vs. Ag/AgCl (*i.e.,* sufficiently positive to oxidize $FcCH_2OH$) and used to approach the insulating tissue culture dish in the *z* direction. After obtaining a negative feedback response, the SECM tip was used to scan the dish surface in the *xy* plane at +0.5 V vs. Ag/AgCl to electrochemically image the tilt of the stage (**Figure S3**).

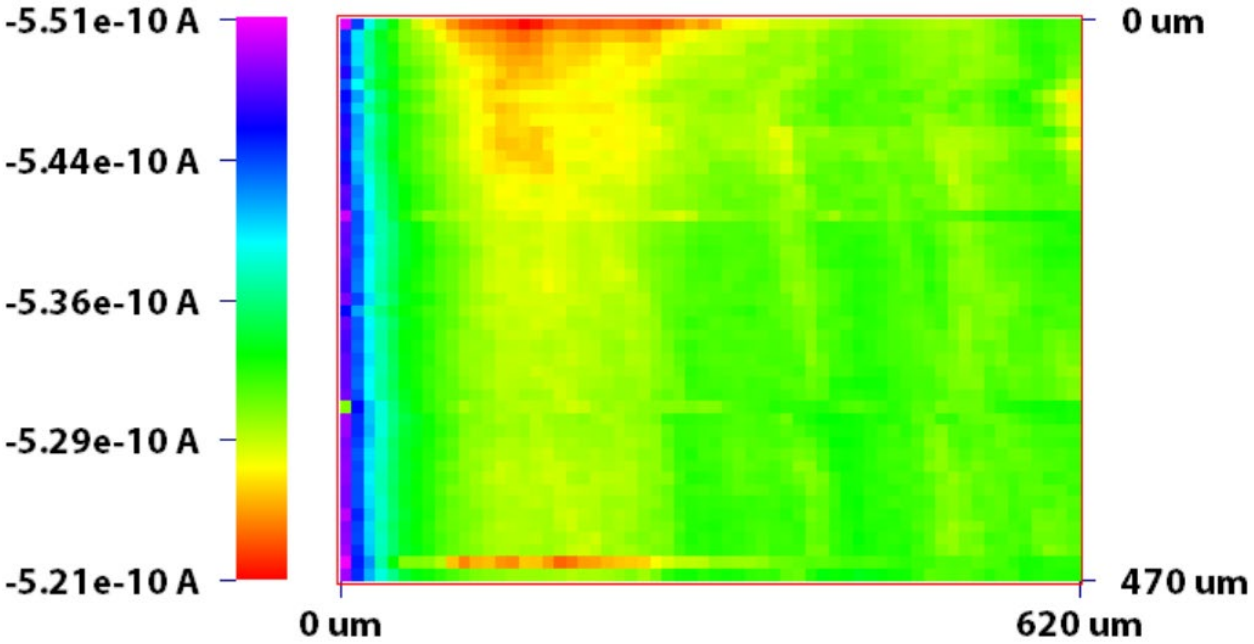



**Figure S3.** Polarographic electrochemical image of a 3.5 cm-diameter tissue culture dish containing 0.5 mM $FcCH_2OH$ in DPBS (1X, pH 7.4). Image obtained with a Pt microelectrode SECM tip ($r$ = 5 μm) at +0.5 V vs. Ag/AgCl (1 M KCl) and a glassy carbon rod counter electrode ($r$ = 1.5 mm).

After confirming insignificant tilt (*i.e.,* $\Delta i \leq 30$ pA), the samples exposed to PFOS-supplemented full growth media were imaged. To image the Hep G2 P20 cells exposed to 100 μM PFOS-supplemented full growth media for 16 hours, spent media was removed from the sample and the cells were rinsed two times with approximately 1 mL DPBS before the 1 mM $FcCH_2OH$ stock solution was diluted to 0.5 mM $FcCH_2OH$ with DPBS directly in the sample dish. Next, the dish was placed on the microscope stage, and the three-electrode system previously used to image the tilt was placed in solution. Cells were brought into focus in bright field using a 20× objective lens. After connecting electrodes and the 920D bipotentiostat electrode leads, the cells were approached with the SECM tip at +0.5 V vs. Ag/AgCl until feedback (**Figure S4**) and cell movement was observed.

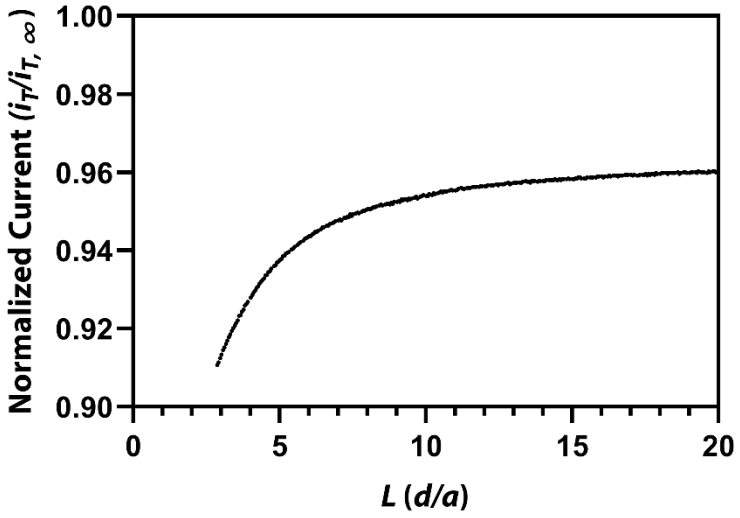



**Figure S4.** Approach curve to 100 µM PFOS treated Hep G2 P20 cells in 0.5 mM $FcCH_2OH$ in DPBS (1X, pH 7.4) obtained using a Pt microelectrode SECM tip ($r$ = 5 µm) biased at +0.5 V vs. Ag/AgCl (1 M KCl) in a 3.5 cm-diameter tissue culture dish. A glassy carbon rod counter electrode ($r$ = 1.5 mm) was used.

It is important to note that it is not uncommon to observe negative feedback with cell movement, rather than positive feedback, if cells are pushed away from the electrode tip as the tip approaches the insulating dish. After obtaining a feedback response and observing cell movement, the Pt microelectrode tip was moved upward 10 µm until no cell movement was observed. Next, a fluorescence image of DCF, indicative of ROS content within cells, was obtained after setting the hyperspectral system to an excitation of 495 nm and an emission of 535 nm. The hyperspectral system was then used to obtain a lambda scan from the emission of 535 nm to 550 nm with a step size of 1 nm at an excitation of 495 nm (**Figure S5i**). A corresponding electrochemical image was obtained with the SECM tip at +0.5 V vs. Ag/AgCl. This series of imaging steps was repeated with samples exposed to 50 µM and 0 µM PFOS-supplemented full growth media. All electrochemical, optical, and spectral data shown in **Figure S5**.

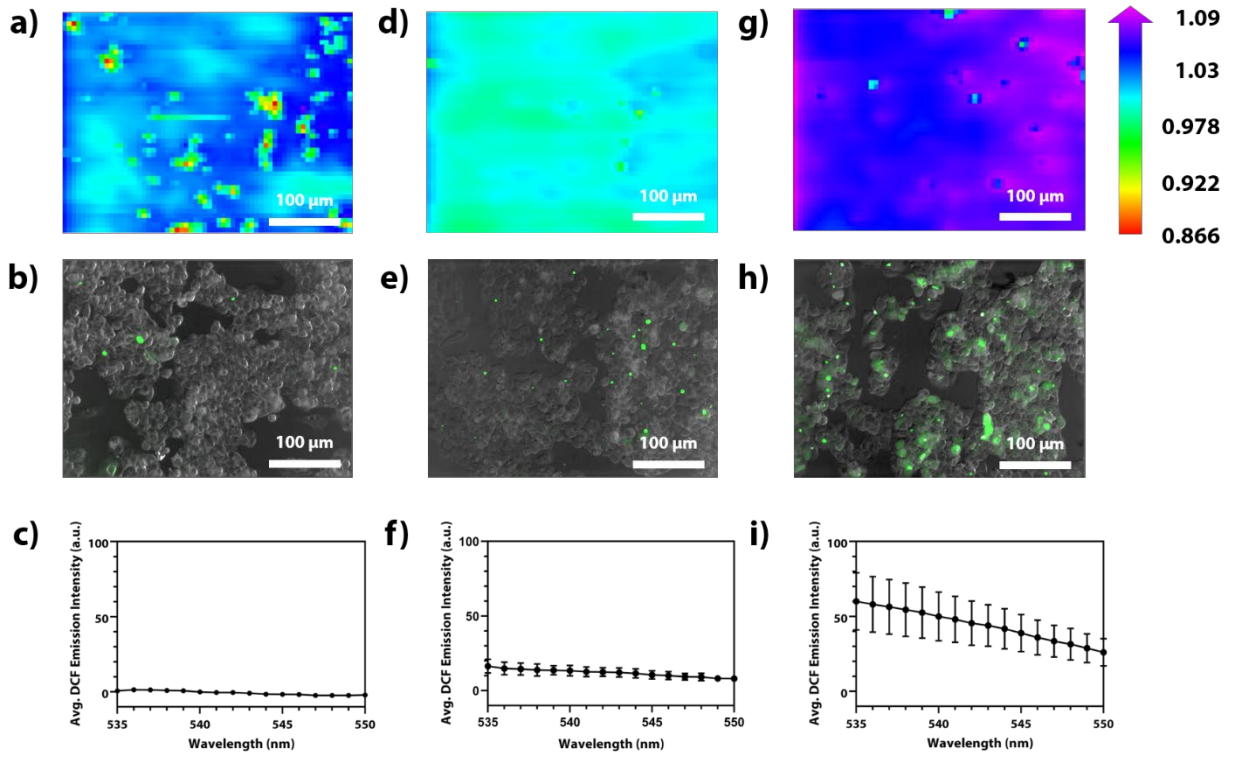



**Figure S5.** Correlated electrochemical, optical, and spectral data for Hep G2 P20 cells exposed to 0 (a-c), 50 (d-f), and 100 (g-i) µM PFOS-supplemented media for 16 hours at 37 °C and 5% $CO_2$. Images obtained in 0.5 mM $FcCH_2OH$ in DPBS (1X, pH 7.4). Electrochemical images (**a**, **d**, and **g**) obtained using a Pt microelectrode SECM tip ($r$ = 5 µm) at +0.5 V vs. Ag/AgCl (1 M KCl) and a glassy carbon rod counter electrode ($r$ = 1.5 mm); images normalized by $i_{T,\infty}$ when $d \geq 100$ µm. Fluorescence images (**b**, **e**, and **h**) of ROS indicator, DCF, obtained at $\lambda_{ex}/\lambda_{em}$ 495/535 nm. Hyperspectral images used to produce (**c**, **f**, and **i**) obtained at $\lambda_{ex}$ 495 nm. The fluorescence images were false colored for visual representation.

**Cell viability following PFOS exposure**

Hep G2 P22 cells were cultured in full growth media in a 10 cm-diameter tissue culture dish. At >65% confluence, spent media was removed from the dish and cells were rinsed with 5 mL DPBS. After rinsing the cells, a mixture of 2:1 DPBS to TrypLE Express was added to the dish (*i.e.,* 4 mL DPBS to 2 mL TrypLE Express). The dish was placed in the incubator set to 37 °C and 5% $CO_2$ to facilitate suspension of cells. After 6 minutes, 9 mL of full growth media was added to the dish to halt trypsinization and the suspension was centrifuged at 1000 rpm at room temperature for 5 minutes. After removing the supernatant from the cell pellet, cells were resuspended in 1 mL full growth media and counted using the automated Corning Cell Counter. With $5.73 \times 10^6$ cells $mL^{-1}$, aliquots of 109 µL (*i.e.,* $6.25 \times 10^5$ cells per aliquot) were delivered to nine 3.5 cm-diameter tissue culture dishes. The following day, 2 mL solutions of 0, 50, and 100 µM PFOS-supplemented full growth media were added to three of the dishes to prepare three samples per PFOS concentration. Next, the samples were incubated overnight for 16 hours.

To analyze cell viability after the incubation, spent PFOS-supplemented full growth media was removed from each sample. The cells were rinsed with 1 mL DPBS, then a 1.5 mL solution of 2:1 DPBS to TrypLE Express was added to each dish to suspend cells. After incubating the dishes for 5 minutes, 1 mL full growth media was added to each suspension to halt trypsinization. The suspensions were centrifuged as done previously. After removing the supernatant, cell pellets were resuspended in 1 mL full growth media. Prior to using the automated cell counter to analyze the viability of a suspension, 1 mL Trypan Blue was added to the suspension to stain dead/membrane damaged cells. The results of this experiment shown in **Figure S6** demonstrate that Hep G2 cells exposed to up to 100 µM PFOS for up to 16 hours remain intact and viable, with viability above >95%.

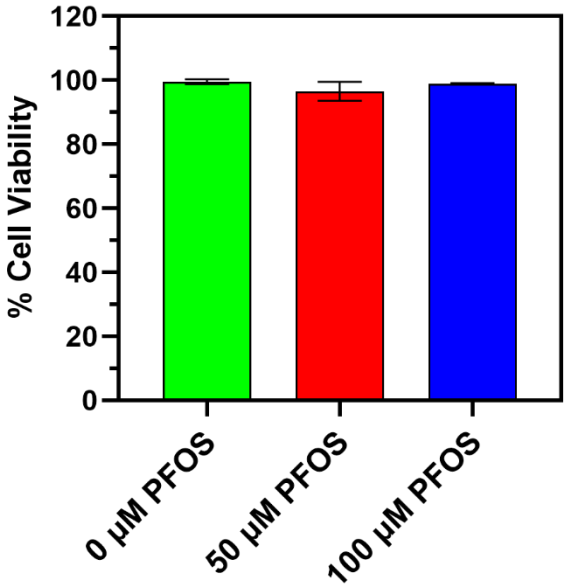



**Figure S6.** Cell viability of Hep G2 P23 cells exposed to 0, 50, and 100 µM PFOS-supplemented full growth media for 16 hours at 37 °C and 5% $CO_2$ (*N*=3).

**Ferrocenium methanol reduction via glutathione**

To provide evidence for possible ferrocenium methanol ($Fc^+CH_2OH$) reduction *via* GSH production at the cell membrane in the previous experimental method, the hypothesized redox mechanism was investigated in bulk solution using cyclic voltammetry. Initially, 122 mg GSH was weighed into a 20 mL scintillation vial, then 20 mL DPBS was added to the vial. Next, 798 mg $FcCH_2OH$ was weighed into an additional 20 mL scintillation vial, then 20 mL DPBS was added to the vial. Both vials were vortexed, then placed in the sonicator for 15 minutes to prepare 19.9 mM GSH and 1.85 mM $FcCH_2OH$ stock solutions. After ensuring the solids were fully dissolved, the $FcCH_2OH$ stock solution was diluted to 0.5 mM $FcCH_2OH$ with DPBS.

After purging the 0.5 mM $FcCH_2OH$ solution for 5 minutes with argon gas, an initial cyclic voltammogram was obtained with a three-electrode system similar to that used in the microscopy experiment: a Pt macroelectrode ($r$ = 1 mm), a Ag/AgCl (1 M KCl) reference electrode, and a glassy carbon rod counter electrode ($r$ = 1.5 mm). To obtain the voltammogram, the Pt macroelectrode was scanned at 0.2 V/s from 0 V to +0.5 V vs. Ag/AgCl then in the reverse direction for the oxidation of $FcCH_2OH$ and reduction of the produced $Fc^+CH_2OH$, respectively, in the polarographic convention. Next, 0.5 mM GSH in DPBS was prepared using the GSH stock solution previously made. After purging the 0.5 mM GSH solution for 5 minutes, a cyclic voltammogram was obtained using the three-electrode system used previously as well as an identical potential window. These controls are shown in **Figure S7** with an additional control for the GSH redox-indicative probe, monochlorobimane (MBCl).

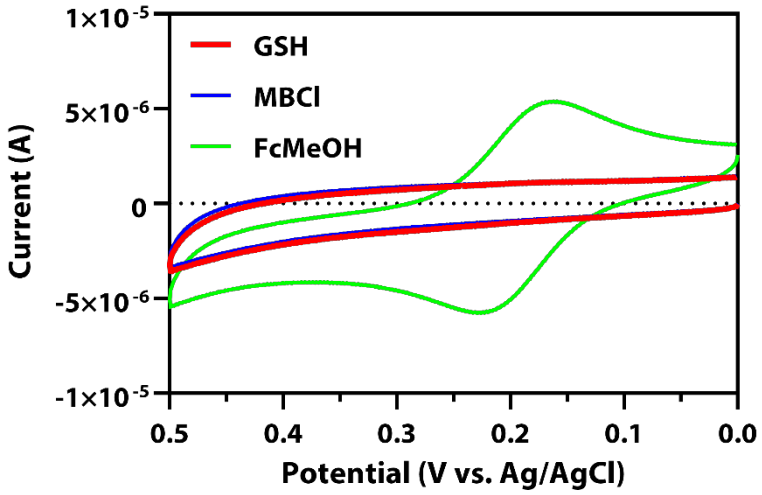



**Figure S7.** Overlay of cyclic voltammograms of 0.5 mM $FcCH_2OH$ (green), 0.5 mM GSH (red), and 0.5 mM MBCl (blue) in DPBS (1X, pH 7.4) at a CHI Pt macroelectrode ($r$ = 1 mm) vs. Ag/AgCl (1 M KCl) after purging each solution for 5 minutes. A glassy carbon rod ($r$ = 1.5 mm) was used as the counter electrode.

**Hyperspectral imaging of Hep G2 following exposure to PFOS: glutathione contribution analysis**

To provide evidence for the contribution of glutathione (GSH) in the redox mechanism associated with positive feedback at the surface of Hep G2 cells, cells were exposed to PFOS-supplemented full growth media, loaded with 10 µM DCF-DA in DPBS, then imaged in the presence of $FcCH_2OH$ and MBCl. MBCl is a non-fluorescent indicator for GSH; upon conjugation with GSH, the conjugate emits light at an excitation of 390 nm and an emission of 490 nm. An example fluorescence image and corresponding spectrum is shown in **Figure S8**.

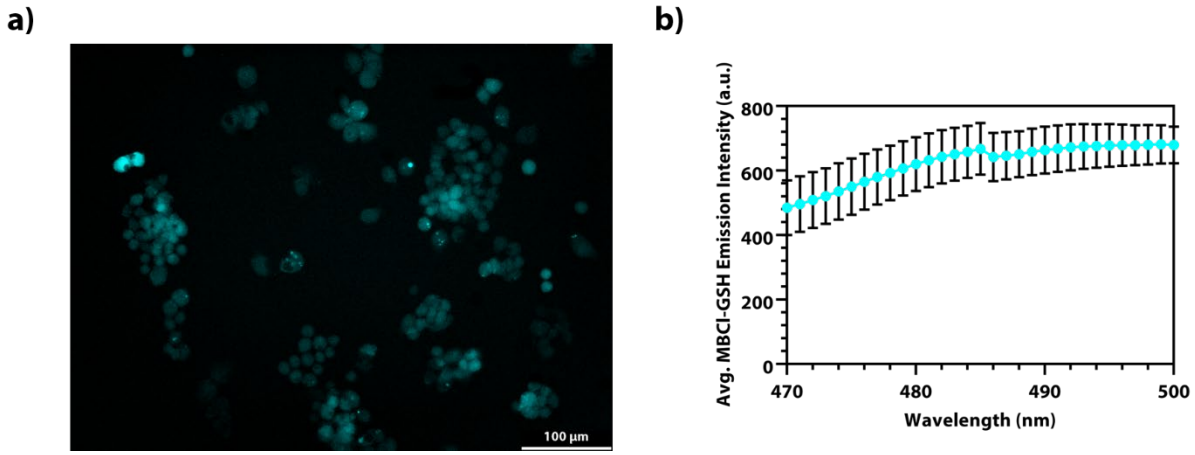



**Figure S8.** (**a**) Fluorescence image of Hep G2 P19 cells exposed to 100 µM PFOS-supplemented media for 16 hours, then loaded with 10 µM DCF-DA. Cells imaged in the presence of 0.5 mM $FcCH_2OH$ /20 µM MBCl in DPBS (1X, pH 7.4). Image of MBCl-GSH conjugate fluorescence at $\lambda_{ex}/\lambda_{em}$ 390 nm/490 nm. Image false colored for visual representation. (**b**) Corresponding emission spectrum at $\lambda_{ex}$ 390 nm. Technical average of three spectra of the same area of cells ($n$ = 3).

Mean emission intensities at 490 nm were averaged over three distinct regions of interest ($N$ = 3) within a dish to produce data presented in **Figure 4**.

## References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2. X. Z. Hu and D. C. Hu, *Archives of Toxicology*, 2009, **83**, 851-861.
3. S. Kuss, D. Polcari, M. Geissler, D. Brassard and J. Mauzeroll, *Proceedings of the National Academy of Sciences*, 2013, **110**, 9249.