Detection of single-molecule HO signalling from epidermal growth factor receptor using fluorescent single-walled carbon nanotubes

The MIT Faculty has made this article openly available. Please share how this access benefits you. Your story matters.
Detection of single-molecule H$_2$O$_2$ signaling from epidermal growth factor receptor using fluorescent single-walled carbon nanotubes

Hong Jin$^1$, Daniel A. Heller$^1$, Marie Kalbacova$^2$, Jong-Ho Kim$^1$, Jingqing Zhang$^1$, Ardemis A. Boghossian$^1$ Narendra Maheshri$^1$ and Michael S. Strano$^1$,*

$^1$ Department of Chemical Engineering, Massachusetts Institute of Technology, Building 66-566, 77 Massachusetts Ave, Cambridge, MA 02139-4307

$^2$ Institute of Inherited Metabolic Disorders, 1st Faculty of Medicine, Charles University in Prague, Czech Republic

*CORRESPONDING AUTHOR FOOTNOTE

phone: (617) 324-4323 or (781) 330-7205  fax: (617) 258-8224

Email: strano@mit.edu
Abstract

An emerging concept in cell signaling is the natural role of reactive oxygen species (ROS), such as hydrogen peroxide (H$_2$O$_2$), as beneficial messengers in redox signaling pathways. Despite growing evidence, the nature of H$_2$O$_2$ signaling is confounded by the difficulty in tracking it in living systems both spatially and temporally at low concentrations. We demonstrate a platform for selectively measuring the H$_2$O$_2$ efflux from living cells at the single-molecule level. An array of fluorescent single-walled carbon nanotubes is capable of recording the discrete, stochastic quenching events that occur as H$_2$O$_2$ molecules are emitted from individual A431 human epidermal carcinoma cells in response to epidermal growth factor (EGF). We show mathematically that such arrays have the unique property of distinguishing between molecules originating locally on the cell membrane from other contributions. We find that EGF induces 0.2 nmol H$_2$O$_2$ over a period of 50 min. This platform promises a new approach to understand ROS signaling in biological cells.
Historically, H2O2 has been thought to have only a deleterious role in cell biology as a toxic metabolic waste product, or as part of the immune respiratory burst in response to microbial invasion. New findings suggest that it is a messenger in normal signaling pathways: H2O2 production has been observed in response to stimulation with various growth factors, cytokines and other signaling molecules across many cell types, and is known to activate specific downstream targets. The understanding of this emerging role for H2O2, and other reactive oxygen species (ROS), is hampered by their low concentration and short lifetime, and this has inspired innovative detection probes. However, such probes for H2O2 cannot map the entire transient signaling response over its duration with spatial resolution.

Our laboratory has pioneered the use of single-walled carbon nanotubes (SWNT) as fluorescent optical sensors for analytical detection from within living cells and tissues. Recent measurements by others and our own laboratory have extended the detection limit down to the single-molecule level by analyzing the stochastic quenching of exitons as molecules adsorb to the SWNT surface. In this work, we develop an array of such single-molecule sensors selective for H2O2 and show that we can image the molecular flux emanating from single living cells in real time with spatial precision, a demonstration that is impossible with any known sensor technology to date. These arrays can image the signaling flux of living A431 human epidermoid carcinoma cells, and resolve several questions about H2O2 generation upon growth factor stimulation, including the membrane activity compared to the other contributions, its spatial distribution, and through inhibition experiments, the chemical mechanism of the signal response. We show generally that such arrays can mathematically distinguish between
molecules generated at the array interface (near-field) from those contributions from a randomized component (far-field), an important property for cellular analysis.

A431 cells over-express the epidermal growth factor receptor (EGFR), which is one of four transmembrane growth factor receptor proteins, including EGFR (HER-1 or c-erbB-1), HER2, HER3 and HER4. EGFR is a 170-kDa glycoprotein with an extracellular receptor domain, a transmembrane domain and an intracellular domain. Its expression is approximately $10^6$ receptors per cell while only $4 \times 10^4$ to $10^5$ receptors per non-cancerous cell. The extracellular domain of EGFR can be divided into four subdomains: I, II, III and IV, with I and III participating in binding (Fig. 1a). Epidermal growth factor (EGF) stimulates cell growth, proliferation and differentiation upon binding to EGFR. It is a single polypeptide chain of 53 amino acid residues, the structure of which is held together in three dimensions by three disulfide bonds in cysteine (Cys) (Fig. 1a).

Upon activation by EGF, the receptor EGFR undergoes dimerization at the cell membrane and it has been shown that an $H_2O_2$ signal is generated in response. The chemical origin of this $H_2O_2$ signal and its relationship to the remaining cascade is largely unknown.

Recent progress in the creation of specific fluorescent probes for $H_2O_2$ has enabled unambiguous confirmation of signal generation in A431 in response to EGF, and has also identified a similar mechanism in neuronal cell signaling for the first time. The platform developed in this work differs in its single-molecule detection limit, and infinite photoemission lifetime that allows continuous detection over the time course of the entire
response. The array of single-molecule sensors can map the signal response spatially and in real time, and is able to discriminate generation at the membrane from sources originating from the cell interior and far field. Such mapping is demonstrated before and after EGF stimulation of A431 cells and NIH-3T3 murine fibroblast cells in real time, where we show that this data informs the chemical mechanism of the signaling cascade.

**A sensitive, selective platform for single-molecule H₂O₂ detection in vitro**

Two properties of the SWNT array make it ideally suited for imaging H₂O₂ fluxes from living cells. The first is H₂O₂ binds with a forward (77.8 M⁻¹s⁻¹) and reverse (0.0006 s⁻¹) rate constant¹⁰ that enables both sensitive detection and reversibility to the exclusion of other reactive species with long lifetimes. In previous work¹⁰, we demonstrated how Hidden Markov Modeling can be used to determine forward and reverse rate constants from single-molecule adsorbates on SWNT. Figure 1c is the application of the technique to several species of interest in this work. Except for nitric oxide (NO), H₂O₂ has the largest forward rate constant of all species considered, meaning that its capture probability is highest. Protons (H⁺) can be detected with a forward rate of 8.1 M⁻¹s⁻¹, but their reverse rate constant is high (0.0011 s⁻¹) such that, at physiological pH (7.4) their contribution is negligible. Likewise, interfering nitrites and nitrates have small rate constants and there is no contribution from components of cell media. Nitric oxide has a high forward binding rate of 80.0 M⁻¹s⁻¹, however its reverse rate is almost immeasurable under these conditions. Therefore, it is easily distinguished as irreversible sensor deactivation. Since no such events were observed in this work, NO can be excluded. In addition, our sensor appears inert to singlet oxygen (¹O₂) and superoxide (O₂⁻) (Fig. 1c).
The second property is the sensor immobilization within a thin film, 2 nm in roughness (Fig. S1) with open porosity (pore size ~ 30 nm). Only the most stable species emanating from the cell are detected. For example, \(^1\)O\(_2\), O\(^2\)\(^-\) and OH\(^-\) have lifetimes of 4 µs\(^1\), 1 µs and 1 ns respectively\(^2\). These species are not likely to diffuse substantially into the film, even at high concentrations\(^3\). The selectivity combined with the diffusion barrier results in an array of sensors specifically designed to H\(_2\)O\(_2\) in real time. This does not limit the approach to H\(_2\)O\(_2\) exclusively. Our recent work shows that by varying the chemistry of the encapsulating matrix\(^4\), or by utilizing multiple orthogonal optical responses (multimodality)\(^5\), it may be possible to selectively detect any analyte emanating from the cell in this manner as future work will underscore.

The detection limit for this class of single-molecule sensor is theoretically one molecule, but the penalty is in an extended observation time (Fig. S2). For instance, an array of SWNT can detect a concentration of 1 µM (10 µM) provided an acquisition time of 14 min (3 min).

**Detecting and spatially mapping single-molecule H\(_2\)O\(_2\) cellular efflux**

When no cells are plated onto the collagen-SWNT array, the result is a photoluminescence intensity of constant root mean square value. A Hidden Markov algorithm finds no quenched states outside of the noise floor as expected. SWNT sensors near or underneath plated A431 cells show discrete quenching transitions of the type observed previously\(^6\). Stepwise quenching and dequenching reactions are clearly
observable (Fig. 1e), compared to the control experiment (Fig. 1d). Because the collagen-SWNT array has such high selectivity towards H$_2$O$_2$, we assign this flux of single-molecules as H$_2$O$_2$ originating from cellular metabolic activity and also non-specific receptor-ligand binding. To further support this assignment, we use manganese oxide (MnO$_2$) to selectively catalyze the decomposition of H$_2$O$_2$ around the A431 cells both with and without EGF stimulation, to show that the quenching reverses significantly (Fig. S3) as the H$_2$O$_2$ is catalytically depleted.

A Hidden Markov algorithm was applied to each SWNT signal in the array, yielding the spatial and temporal detection of single-molecules emitted from the cell in real time. The typical observation time was 3000s and Figure 2a-d describes the spatial distribution of single-molecule detection frequencies for both live (Fig. 2a, b) and fixed (Fig. 2c, d) A431 cells after the addition of EGF (500ng/mL) at t = 0 using a Matlab program written in-house. Each sensor was binned according to its number of quenching transitions within the 3000s observation window Fig 2a-e. The control array, exposed to 10 µM H$_2$O$_2$ in the absence of cells, demonstrates a spatially random distribution of transition frequencies (Fig. 2e). However, when A431 cells are present, the frequency distribution possesses a sharp mode invariably confined to the region immediately under the cell. The behavior is seen for both live and fixed cells. The locations of these “hot spots” do not remain invariant over the course of the 3000 s experiment, but shift to alternate regions. The EGFR receptor lifetime is approximately 30 min, long enough to prevent spatial averaging of the membrane signal. Example fluorescence traces of nanotube right under the cell (green star, pink circle, Fig. 2a) and removed from the cell (dark blue star,
pink circle, Fig. 2a) are shown in Fig. 2f and g. It is clear that the dominant contribution of the H$_2$O$_2$ flux comes from the A431 cells, and the data suggest that at any given time it is concentrated at specific locations on the array, unlike the case of a uniformly exposed control.

**Real-time quantitative analysis from EGF stimulation across two cell lines**

First, we analyze the total dynamic count rate of each single cell in response to EGF stimulation to measure the duration of the induced efflux. The quenching rate was calculated in real time for EGF stimulation (500ng/mL EGF was added at t=0) on live and fixed 3T3 (Fig. 3a) and A431 cells (Fig. 3b). Compared to the no cell control and unstimulated cell data, the quenching rate of both A431 and 3T3 cells were increased by EGF stimulation. A431 cells with a higher EGFR density have a much higher quenching rate than 3T3 cells. As can be seen from Fig. 3a-b, the behaviors of single A431 cells after EGF stimulation are similar: the quenching rate increased rapidly right after stimulation. However, the time point of maximal response ranges from 600s to 1800s after stimulation. There is no significant difference between live and fixed A431 cells.

Removal of EGF decreases the quenching (Fig. S4). Compared to ensemble measurements on thousands of cells, our platform allows real-time quantification on single isolated cells for the first time. To confirm that the above results correlated with overexpression of EGFR, we then compared the EGFR density in 3T3 cells and A431 cells. Both cell lines were immunostained using rabbit polyclonal to EGFR as the primary antibody and Alexa Fluor 568 donkey anti-rabbit IgG as the secondary antibody (Methods). Confocal images (Fig. 3c, d) confirm that A431 cells express substantially
more EGFR than 3T3 cells (Fig. S5). From a calculation on the immunostaining images of 100 cells, the EGFR density of A431 cells is approximately 10 times that of 3T3 cells, consistent with literature values\textsuperscript{14,15}.

**Far-field component subtraction and near-field generation from the membrane**

Rank ordering the sensor responses from lowest to highest capture rate constructs the cumulative distribution. Let $x$ be the number of sensors having a response less than $y$, so that $\frac{x(y)}{n}$ is then the probability of finding a sensor with a number of counts less than $y$.

For the case of equal capture probabilities, the rank ordered response is a modified Gamma distribution (Fig. S6a-b):

$$x = \frac{n\int_{0}^{y} e^{-t}t^{a-1}dt}{\int_{0}^{\infty} e^{-t}t^{a-1}dt} = \frac{n\Upsilon(a, y)}{\Gamma(a)} = nP(a, y)$$

(Eq. 1)

Where $\Upsilon$, $\Gamma$ and $P$ represents the lower incomplete, ordinary and regularized Gamma functions respectively with $a$ as mean value of $y$.

A kinetic Monte Carlo simulation of $10^4$ H$_2$O$_2$ molecules randomly binned into a sensor array consists of 300 sensors ($n=300$) is well-described by Eq. 1 after rank ordering\textsuperscript{23} (Fig. S6a-b). Note that there are no fit parameters in this simulation.

An array of sensors capable of detecting discrete, single-molecules has the following unique property: it is possible to distinguish between those near-field component
generated at the interface and those comprising a far-field component with no memory of origination.

On top of the far-field component described by Eq. 1, molecules generated near the array surface (i.e. at the cell membrane surface) are easily distinguished. The algorithm for extracting this interfacial generation at the interface simply accounts for non-binomial contributions to the frequency distribution. The local response, $y_{\text{local}}(x)$, is:

$$y_{\text{Local}}(x) = y(x) - P^{-1}(a, \frac{x}{n})$$

(Eq. 2)

The mean value $a$ can be found by computing the slope of the experimental data in the $x \to 0$ limit:

$$\frac{\partial y}{\partial x} = \frac{\Gamma(a)}{ny^{a-1}e^{-y}}$$

(from $\frac{\partial \Gamma(a,y)}{\partial y} = -y^{a-1}e^{-y}$)

It can be shown from Monte Carlo simulation that membrane generation near the array interface of sufficient activity always biases the rank ordered response to the highest activity sensors (Fig. 4a-b, Fig. S6e-i). As a result, a small number of data points at $x \to 0$ are enough to extract the far-field component (Eq. 1) from any experimental curve with the membrane generation recovered from Eq. 2. A practical sensor array constructed from SWNT embedded in a collagen matrix as described above has a distribution of sensor capture probabilities as each sensor varies slightly in length and orientation. A beta-distribution is a generic, empirical function that can describe this variation. It has the advantage that the far-field component then becomes a cumulative-beta-binomial distribution (Fig. S6c-d), and an analogous deconvolution can be derived (Fig. 4c-d).

The rank ordered sensor responses of SWNT-collagen arrays exposed to constant,
uniform concentrations of $\text{H}_2\text{O}_2$ from 10 to 100 $\mu\text{M}$ are described by beta-binomial distributions with parameters $\alpha = 1.2 \pm 0.15$ and $\beta = 3.0 \pm 0.12$ (Fig. S7a-d). These parameters were used to correct the measured responses for the variation of capture sensitivities of each SWNT.

Membrane activities on single live, fixed A431 cells and live 3T3 cells before and after the EGF stimulation over 3000s were extracted from the above algorithm (Fig. 4e-g, Fig. S8). Before EGF stimulation, the local activity is negligible. After EGF stimulation, the membrane generation observed for both live and fixed A431 cells, however not for live 3T3 cells. The increased activities after EGF stimulation in the unit of number of quenching transitions per sensor are summarized in Table 1. For A431 cells after simulation, the local $\text{H}_2\text{O}_2$ concentration is determined through calibration to be 2$\mu\text{M}$ using control experiments in the cell-free system (Fig. S7e). The local generation rate from each membrane source is then 0.04 nmol $\text{H}_2\text{O}_2$/min after correcting for diffusion from Eq. S3.

**A consistent $\text{H}_2\text{O}_2$ signal generation mechanism**

The spontaneous or catalytic breakdown of superoxide anions is considered to be the source of $\text{H}_2\text{O}_2$ in many biological pathways, not only for immune cells, but also in a variety of eukaryotic cells. Superoxide anions can be produced by the partial reduction of oxygen by cytochrome c oxidase in mitochondria or by membrane-associated NAD(P)H oxidase. Extensive literature has shown that EGF stimulated $\text{H}_2\text{O}_2$ generation originates from NAD(P)H oxidase instead of mitochondria for various nonphagocytes,
including A431 cells \textsuperscript{6,24,25}. Growth factors like EGF induce the formation of a complex on NAD(P)H oxidase to promote the electron transfer from NAD(P)H to molecular oxygen\textsuperscript{25}. Fixation of A431 cells using 4\% paraformaldehyde were designed to remove the influence of the mitochondria\textsuperscript{26} in our experiments, as has been used in the literature to kill the cells without affecting the binding abilities of EGFR\textsuperscript{22}. The fact that we see no quantitative difference between live and fixed A431 cells in their EGF inducible membrane generation (Table 1) is consistent with the all existing literature where mitochondria do not affect the EGF-induced H\textsubscript{2}O\textsubscript{2} generation. In addition, our array platform of single-molecule sensors allows for the signaling flux from the cell to be differentiated from a diffuse far-field component for the first time, and can therefore inform the discussion of the nature of the H\textsubscript{2}O\textsubscript{2} signal. Our analysis above confirms that the H\textsubscript{2}O\textsubscript{2} that increases in response to EGF binding is generated at the membrane and not in the cell interior (Fig. 4e-g). Recently, DeYulia and co-workers demonstrated that the H\textsubscript{2}O\textsubscript{2} production is EGFR-ligand-dependent in A431 cells\textsuperscript{22}, where the inhibition of EGFR phosphorylation did not affect the H\textsubscript{2}O\textsubscript{2} generation. At this point, both the signaling network post-NAD(P)H oxidation, and how the activation of NAD(P)H oxidase connects with the EGFR-ligand-dependent generation, are unclear in the literature. We further performed inhibition experiment using NAD(P)H oxidase inhibitors (Fig. S9a-b) and EGFR inhibitor (Fig. S9c), and we found that consistent with our speculation, NAD(P)H oxidase inhibitors prevent H\textsubscript{2}O\textsubscript{2} from forming while EGFR inhibitor has no effect on the H\textsubscript{2}O\textsubscript{2} produced.
It is also not clear what catalytic portion of EGFR may be responsible for membrane
H$_2$O$_2$ generation in the work by DeYulia and co-workers$^{22}$. Tryptophan (Trp) is proposed
to be responsible for the conversion of singlet oxygen to H$_2$O$_2$ in antibodies$^{27}$. This
antibody-mediated process is triggered upon binding of singlet oxygen to conserved
binding sites within the antibody fold$^{28}$, where the antibody serves as the catalyst,
stabilizing the intermediate (H$_2$O$_3$) and directing its conversion to H$_2$O$_2$. Trp is present in
both EGF$^{15}$ (Trp 49, Trp 50) and EGFR$^{29}$ (Trp 140, 176, 453, 492) (Fig. 1a). If lacking
these Trp residues, EGFR does not bind ligand with high affinity$^{29}$. It is possible that
EGFR, upon binding with EGF, allows greater access to sites on the receptor itself that
catalyze the conversion of singlet oxygen to H$_2$O$_2$. To explore this, 1 mM sodium azide
(NaN$_3$), a scavenger of singlet oxygen$^{30}$, was added to fixed A431 cells with and without
the presence of EGF and the single-molecule efflux of H$_2$O$_2$ was again recorded.
Compared to the un-stimulated control (green curve, Fig. 4h), NaN$_3$ greatly diminished
both the near and far-field portions of the H$_2$O$_2$ response to EGF (black and red curve,
Fig. 4h). Further, we observed an increase in H$_2$O$_2$ after we exchanged water with D$_2$O
(the lifetime of singlet oxygen in D$_2$O is 67 $\mu$s$^{31}$, a factor of 16 times greater than that in
water) for the EGF stimulation experiment on fixed A431 cell (purple curve, Fig. 4h),
compared to the parallel experiment conducted with water (blue curve, Fig. 4h). In
previous studies, the level of H$_2$O$_2$ before and after EGF stimulation is not affected when
shutting down the mitochondria$^{24, 25}$. The fact that we were able to observe an obvious
decrease in H$_2$O$_2$ level even below the basal level when adding NaN$_3$ before and after
EGF stimulation, and an increase in H$_2$O$_2$ level when extending the lifetime of singlet
oxygen, supports a complex pathway involving singlet oxygen.
One possible signaling network that may explain this more complex response starts from superoxide anion, which is produced from the reduction of molecular oxygen by NAD(P)H oxidase in A431 cells (Fig. 4k) \(^ {24,28} \).

\[
NAD(P)H + 2O_2 \xrightarrow{\text{NAD(P)H oxidase}} NAD(P)^+ + H^+ + 2O_2^{•-} \quad \text{(Reaction. 1)}
\]

EGF was found to increase the production of superoxide anion in A431 cells while an inhibitor of NAD(P)H oxidase halts this mechanism in a manner that was also mitochondria independent\(^ {24} \). This observation, together with the previous known EGF-EGFR induced NAD(P)H oxidase activation mechanism for EGF stimulated A431 cells from numerous literature\(^ {6,24,25} \), and our inhibition experiment results (Fig. S9a-b), supports Reaction 1. Superoxide anion can then be decomposed by superoxide dismutase (SOD). It has been found that SOD is a master regulator of growth factor signaling and the inhibition on SOD1, which is an abundant copper/zinc enzyme found in the cytoplasm, increases the steady-state levels of superoxide and decreases the levels of H\(_2\)O\(_2\) in A431 cells\(^ {32} \). These reactions are rapid and occur with a diffusion limited reaction rate.

\[
2O_2^{•-} + 2H^+ \xrightarrow{\text{SOD}} O_2 + H_2O_2 \quad \text{(Reaction. 2)}
\]

\[
2O_2^{•-} + 2H_2O \xrightarrow{\text{SOD}} O_2 + H_2O_2 + 2OH^- \quad \text{(Reaction. 3)}
\]

Transition metal ions such as iron or copper can catalyze the reduction of H\(_2\)O\(_2\) by superoxide anion\(^ {35-37} \).

\[
O_2^{•-} + H_2O_2 \xrightarrow{\text{transition metal ions}} O_2 + OH^- + OH^- \quad \text{(Reaction. 4)}
\]
Singlet oxygen can decay to the ground state oxygen. It has been shown that the decay of singlet oxygen is determined by its interactions with water in the cell and not by interactions with other cell constituents with a decay rate constant of $3 \times 10^5 \text{ s}^{-1}$.

\[ ^1\text{O}_2 \xrightarrow{\text{water}} \text{O}_2 \]  
(Reaction. 5)

Ferrous also reacts with $\text{H}_2\text{O}_2$ known as the Fenton reaction.

\[ \text{Fe}^{2+} + \text{H}_2\text{O}_2 \longrightarrow \text{Fe}^{3+} + \cdot\text{OH} + \text{OH}^- \]  
(Reaction. 6)

\[ \text{Fe}^{3+} + \text{O}_2^- \longrightarrow \text{Fe}^{2+} + \text{O}_2 \]  
(Reaction. 7)

Upon EGF stimulation, singlet oxygen is converted into $\text{H}_2\text{O}_2$ catalyzed by EGF-EGFR.

\[ 2\text{H}_2\text{O} + \text{EGF–EGFR} \xrightarrow{\text{EGF–EGFR}} 2\text{H}_2\text{O}_2 + \text{O}_2 \]  
(Reaction. 8)

From solving the proposed pathway numerically (with the initial values for in vitro ROS obtained from the literature: superoxide, ~1nM, $\text{H}_2\text{O}_2$, 1µM, singlet oxygen, ~1nM) assuming a well-mixed condition and using the methods in previous work, the concentration of superoxide and $\text{H}_2\text{O}_2$ increases with the addition of EGF (Fig. 4i), consistent with the experimental observations by us and others. The addition of $\text{NaN}_3$ causes decrease of $\text{H}_2\text{O}_2$ (Fig. 4j), even below the initial value, consistent with our observations in Fig. 4h. While the pathway that we proposed here is compelling, future work is necessary in order to conclusively rule out alternate mechanisms.

**Conclusions**

In conclusion, an array of SWNT sensors has been used to image, for the first time, the incident flux of $\text{H}_2\text{O}_2$ molecules that stochastically absorb and quench the emission with
spatial and temporal resolution. Notably, arrays of this type can distinguish between molecules originating near an interface and those with no memory of origination, attributed as the far-field component. The signaling activity of EGFR on single A431 cells has been successfully measured using this sensor array. **We find that the EGF stimulation induces on average 2 nmol H₂O₂ over a period of 50 min in A431 cells.** Corresponding inhibition experiments suggest a mechanism whereby water oxidizes singlet oxygen at a catalytic site on the receptor itself, generating H₂O₂ in response to receptor binding. An EGFR-mediated H₂O₂ generation pathway that is consistent with all current and previous findings has been proposed and numerically tested for consistency.

**Acknowledgements**

M.S.S is grateful for a Beckman Young Investigator Award and a National Science Foundation (NSF) Career Award. This work was funded under the NSF Nanoscale Interdisciplinary Research Team on single-molecule detection in living cells using carbon nanotube optical probes. Part of this work was supported by the national grants Ministry of Education of the Czech Republic project No. MSM0021620806 and KAN Grant No. 400100701. We thank S. Tannenbaum, G. Wogan and L. Trudel and acknowledge a seed grant from the Center for Environmental Health Sciences at MIT. We also thank M. Balastik at Harvard Medical School for assistance with the confocal experiments, K. D. Wittrup, G. Stephanopoulos, J-H. Ahn, J-H Han at Chemical Engineering at MIT, S. Sheffield, Mathematics Department, MIT and Y. Li at University of Illinois Urbana Champaign for helpful discussions.
Author contributions
H.J. and M.S. conceived the experiments, derived the models and wrote the manuscript. H.J. performed the experiments and analyzed the data. D.H., M.K., J-H.K., J.Z and A.B. all assisted in the experiments. H.J. and M.S. co-wrote the paper with input from N.M.

Methods
Suspension of SWNT in Collagen
Single-walled HiPco carbon nanotubes (Rice University) were suspended in type 1 collagen (BD Biosciences) via 1min probe-tip sonication (1/4” tip, 40% amplitude). One mg of SWNT was used per mL of 3.41 mg/mL collagen stock in 0.02 N acetic acid for sonication. The mixture was centrifuged for 270min at 16300g and the pellet discarded, retaining the supernatant for future experiments.

Collagen-SWNT Thin Films
Collagen-SWNT was diluted with stock collagen (3.41 mg/mL) to make 272 mg/L SWNT concentration. This solution was diluted to 50 μg/mL collagen with 0.02 N acetic acid with a final concentration of SWNT of 8mg/L for imaging purposes (the concentration of SWNT is found by trial and error to achieve a desirable coverage of SWNT on the film) and pipetted onto glass bottom 35mm Petri dishes (MatTek Corp., P35G-1.5-14-C) in 500 μL aliquots to completely cover the glass region in the center of the dish. The collagen was dried at room temperature in a laminar flow hood. The dried film was rinsed well with PBS to remove the remaining acid. Everything was done under a sterilized environment.
Singlet Oxygen and Superoxide Generation

Rose bengal was used to generated singlet oxygen and superoxide in real time using a procedure described from a previous study. Briefly, 50 nM of rose Bengal was illuminated at 561 nm at 200mW for 30 min and the fluorescence of nanotubes upon this illumination was recorded in real time. It is reported that this procedure will generate both singlet oxygen and superoxide. MnO₂ was added to the solution to prevent any interference from H₂O₂.

Fluorescence Microscopy on Live and Fixed Cell

Human epidermoid carcinoma A431 cells and murine NIH-3T3 cells were cultured with Dulbecco’s Modified Eagle’s Medium (DMEM, ATCC) supplemented with 10% fetal bovine serum (FBS, Gemini Bio-Products) and 1% Pen-Strep Solution (10,000 U/mL Penicillin-G 10,000 µg/mL Streptomycin Penicillin-Streptomycin Solution, Gemini Bio-Products) at 37 ºC with 5% CO₂ on a collagen film in a glass bottom 35mm Petri dish (MatTek Corp., P35G-1.5-14-C) after serum starvation. Right before imaging, the cell medium was changed into Leibovitz’s L-15 medium, which buffers the pH in the atmosphere. The nanotubes in the collagen film beneath the cells are then imaged using a fluorescence microscope (Carl Zeiss, Axiovert 200), with a CCD camera (Carl Zeiss, ZxioCam MRm) and 2D InGaAs array (Princeton Instruments OMA 2D). Movies were acquired using the WinSpec data acquisition program (Princeton Instruments). The nanotubes were excited by a 658 nm laser (LDM-OPT-A6-13, Newport Corp) at 35mW. After a stable fluorescence intensity was observed (Fig. S1), 500 µL of each reagent was
added to reach the desirable final concentration. For experiment with fixed cells, A431 cells were washed with PBS, fixed in 4% paraformaldehyde (pH 7.4) for 10 min, washed 3 times and ready for imaging.

**Fluorescent Staining of Cells**

Cells were incubated with 4% PFA/PBS (USB Corporation) at 4 °C for 5 min, at room temperature for 10 min, then with 100% methanol (Sigma) at -20 °C for 10 min. The fixed cells were washed 3 times with PBS (Hyclone), permeabilize with 0.1% Triton X 100 (Sigma)/PBS for 20 min at room temperature followed by another washing with PBS. The cells were then incubated in 1% FBS/0.05% Tween (Sigma)-20/PBS for 20 min at room temperature, after which they were incubated with the primary antibody (rabbit polyclonal to EGFR, ABCAM Inc) in 1% FBS/0.05% Tween-20/PBS for 1h at room temperature. Washing and blocking were repeated. In the dark, the secondary antibody (Alexa Fluor 568 donkey anti-rabbit IgG, Invitrogen) was added in 1% FBS/0.05% Tween-20/PBS for 1h at room temperature (Alexa 1:500), after which 4',6-diamidino-2-phenylindole (DAPI, Sigma Aldrich Co.) was added with a final concentration of 1µg/L. The washing step was repeated. The sample was then mounted in Moviol (Shandon Immu-Mount, Thermo Fisher Scientific). The samples were then analyzed in Zeiss LSM 510 Meta confocal microscope using the same configuration and processed in LSM image Browser software from Zeiss.

**Atomic Force Microscope (AFM)**
MFP-3D (Asylum Research) was used for tapping mode atomic force microscopy (AFM) imaging. Samples were directly deposited on a 75 mm x 25 mm glass slide (VWR International) and imaged using rectangular silicon tips (Olympus AC240TS) with a nominal spring constant of 2 N/m. Both topographic and height images were recorded during AFM analysis. Height analysis was performed using Igor Pro software.

**Figure Legends**

**Figure 1** The nanotube sensing platform. (a) A431 cell was cultured on the collagen-SWNT film. Zoomed from red circle: domain I, III bind to EGF, generating H$_2$O$_2$. (b) A431 cell was cultured on the film where the SWNT sensors were excited by a 658 nm excitation laser at 1mW at the sample through Alpha Plan-Apo 100x/1.46 oil emersion objective. (c) Sensor binding rate and reverse binding rate for SWNT embedded in collagen film for various analytes: SWNT sensors are selectively sensitive for H$_2$O$_2$. (d) The control trace from the control experiment where no cell was present showed no steps. (e) The fluorescence trace from the SWNT in the red circle in (a) showed reversible, stepwise quenching (green trace), modeled by HMM (red).

**Figure 2** Spatially mapping the quenching transitions over single A431 cells. The quenching activity (in the unit of counts) over the 3000s observation window of each sensor was binned into sixteen categories represented by sixteen different color bars with red the highest quenching activity and black the lowest for live (a, b) and fixed (c, d) A431 cells. Control experiment where 10μM H$_2$O$_2$ was present in the absence of a cell (e). Phase contrast images without the overlap of quenching activities are shown on the left of
each panel. The fluorescence trace of the green star (f) and the dark blue star (g) highlighted by the pink circles.

**Figure 3** SWNT quenching is EGFR density dependent. The real-time quenching rate (#/sensor/s) for live 3T3 cells (a) and live/fixed A431 cells (b). The number of sensors under each single cell is 255, 200, 250, 150, 255, 200 respectively for cell 1, 2, 3, 4, unstimulated and control in (a); 160, 110, 126, 174, 140, 180, 180, 200 respectively for cell 1, 2, 3, 4, fixed cell 1, 2, unstimulated and control in (b). Representative confocal images for 3T3 cells (c) and A431 cells (d) with EGFR (red) labeled with rabbit polyclonal to EGFR and Alexa Fluor 568 donkey anti-rabbit IgG. The nuclei (blue) is labeled with 4',6-diamidino-2-phenylindole (DAPI).

**Figure 4** Quantitative analysis based on the result from the SWNT sensor array. (a) Simulation of sensor response on $10^5$ H$_2$O$_2$ randomly falling onto 300 sensors (blue), with additional response to local generation (red). (b) Rank-ordered sensor response from (a). (c) Simulation of sensor response on $10^5$ H$_2$O$_2$ falling onto 300 sensors following beta distribution (blue), with additional response to local generation (red). (d) Rank-ordered sensor response from (c). After far-field component subtraction from the rank-ordered sensor response (dark, -EGF; green, +EGF), the local generation before (blue, star) and after (red, star) EGF stimulation for live (e), fixed (f) A431 cell and live 3T3 cell (g). (h) The real-time quenching rate for fixed A431, before (green) and after (blue) EGF stimulation. Sodium azide decreases the quenching, with (red) and without (black) EGF. Extending the singlet oxygen lifetime using D$_2$O increases the quenching (purple). (i) The concentration profiles on log-log scale for different species from solving Reaction
1-8. (j) The concentration profiles on log-log scale for different species considering the NaN₃ effect when solving the reaction network. (k) The scheme of the proposed pathway for H₂O₂ generation.

**Table 1** Number of quenching transitions per sensor from receptors alone calculated for live, fixed A431 cells and live 3T3 cells.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Number of quenching transitions per sensor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Live A431 cell #1</td>
<td>6.6</td>
</tr>
<tr>
<td>Live A431 cell #2</td>
<td>4.0</td>
</tr>
<tr>
<td>Live A431 cell #3</td>
<td>5.0</td>
</tr>
<tr>
<td>Live A431 cell #4</td>
<td>7.5</td>
</tr>
<tr>
<td>Fixed A431 cell #1</td>
<td>4.5</td>
</tr>
<tr>
<td>Fixed A431 cell #2</td>
<td>7.8</td>
</tr>
<tr>
<td>A431 cells average</td>
<td>6.0</td>
</tr>
<tr>
<td>Live 3T3 cell #1</td>
<td>1.0</td>
</tr>
<tr>
<td>Live 3T3 cell #2</td>
<td>1.1</td>
</tr>
<tr>
<td>Live 3T3 cell #3</td>
<td>1.1</td>
</tr>
<tr>
<td>Live 3T3 cell #4</td>
<td>1.0</td>
</tr>
<tr>
<td>3T3 cells average</td>
<td>1.1</td>
</tr>
</tbody>
</table>
References

20. Singlet oxygen, superoxide anion and hydroxyl radicals no longer interfere beyond a distance of 90, 45 and 1 nm away from the generating source respectively after twice their lifetimes, assuming that the diffusion coefficient of each is one tenth in our film than in water. Because SWNT in the film are an average of 200 nm away from the interface, it is unlikely for these short lived species to diffuse into the film.
23. All simulations in this study were done using Matlab and were repeated 100 times with the average reported unless specified otherwise.
**Figure 1**

(a) Intracellular domain of EGFR

(b) nIR image of SWNT sensors underneath A431

(c) A431 cell image from CCD camera

(d) Control: no cell

(e) From the red circle in Fig 1b

H$_2$O$_2$ extracellular domain (I, II, III, IV) bound

Intracellular domain of EGFR

SWNT sensors

Normalized Intensity

Normalized Intensity
Figure 2

(a) (b)

(c) (d)

(e)

(f) (g)
Figure 3 (a) EGF Stimulation on 3T3 cells

Figure 3 (b) EGF Stimulation on A431 cells

Figure 3 (c) 3T3

Figure 3 (d) A431
Figure 4

(a) (b) (c) (d) (e) (f) (g) (h) (i) (j) (k)

Figure 4: Graphs showing the number of quenching transitions over time for different conditions.

(e) (f) (g) (h) (i) (j) (k)

Figure 4: Graphs showing the number of quenching transitions over time for different conditions.

(e) (f) (g) (h) (i) (j) (k)

Figure 4: Graphs showing the number of quenching transitions over time for different conditions.

(e) (f) (g) (h) (i) (j) (k)

Figure 4: Graphs showing the number of quenching transitions over time for different conditions.

(e) (f) (g) (h) (i) (j) (k)

Figure 4: Graphs showing the number of quenching transitions over time for different conditions.

(e) (f) (g) (h) (i) (j) (k)

Figure 4: Graphs showing the number of quenching transitions over time for different conditions.

(e) (f) (g) (h) (i) (j) (k)

Figure 4: Graphs showing the number of quenching transitions over time for different conditions.

(e) (f) (g) (h) (i) (j) (k)

Figure 4: Graphs showing the number of quenching transitions over time for different conditions.

(e) (f) (g) (h) (i) (j) (k)

Figure 4: Graphs showing the number of quenching transitions over time for different conditions.

(e) (f) (g) (h) (i) (j) (k)

Figure 4: Graphs showing the number of quenching transitions over time for different conditions.

(e) (f) (g) (h) (i) (j) (k)

Figure 4: Graphs showing the number of quenching transitions over time for different conditions.

(e) (f) (g) (h) (i) (j) (k)

Figure 4: Graphs showing the number of quenching transitions over time for different conditions.