

DNA-SWCNT Biosensors allow real-time monitoring of therapeutic responses in Pancreatic Ductal Adenocarcinoma

Santanu Bhattacharya^{1,2}, Xun Gong³, Enfeng Wang¹, Shamit K. Dutta¹, Joseph R. Caplette², Manki Son³, Freddy T. Nguyen³, Michael S. Strano³, Debabrata Mukhopadhyay^{1,2*}

¹Department of Biochemistry and Molecular Biology, Mayo Clinic Collage of Medicine and Science, Jacksonville, FL 32224, USA.

²Department of Physiology and Biomedical Engineering, Mayo Clinic Collage of Medicine and Science, Jacksonville, FL 32224, USA.

³Department of Chemical Engineering, Massachusetts Institute of Technology, Cambridge, MA 02139, USA.

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Corresponding Author: *Debabrata Mukhopadhyay, Ph.D.
Griffin 411, Mayo Clinic Collage of Medicine and Science,
4500 San Pablo Road S,
Jacksonville, FL-32224,
Tel: (904) 953-6177,
Fax: (904) 953-0277,
E-mail: mukhopadhyay.debabrata@mayo.edu

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Abstract:

Pancreatic ductal adenocarcinoma (PDAC) is a highly desmoplastic cancer with limited treatment options. There is an urgent need for tools that monitor therapeutic responses in real-time. Drugs such as gemcitabine (GEM) and irinotecan elicit their therapeutic effect in cancer cells by producing hydrogen peroxide (H_2O_2). In this study, specific DNA- wrapped single-walled carbon nanotubes (SWCNTs), which precisely monitor H_2O_2 , were used to determine the therapeutic response of PDAC cells in vitro and tumors in vivo. Drug therapeutic efficacy was evaluated in vitro by monitoring differences of H_2O_2 in situ using reversible alteration of Raman G-bands from the nanotubes. Implantation of the DNA-SWCNT probe inside the PDAC tumor resulted in ~50% reduction of Raman G-band intensity when treated with GEM versus the pre-treated tumor; the Raman G-band intensity reversed to its pretreatment level upon treatment withdrawal. In summary, we demonstrate using highly specific and sensitive DNA-SWCNT nanosensors that dynamic alteration of a key analyte can be used to evaluate the effectiveness of chemotherapeutics both in vitro and in vivo.

Significance:

A novel biosensor is used to detect intratumoral hydrogen peroxide allowing real-time monitoring of responses to chemotherapeutic drugs.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is the third leading cause of cancer related death in the United States, with a 5-year survival rate of less than 8 %(1). PDACs are often diagnosed at a late stage and are unresectable with desmoplastic tumor microenvironments (TME) and show chemo-resistance (2-5). Gemcitabine (GEM) with Abraxane (6) or FOLFIRINOX (a combination of leucovorin, 5-fluorouracil, irinotecan, and oxaliplatin) (7) are the only first line treatments for PDACs. One major clinical challenge in the chemotherapy is the lack of methods available to monitor therapeutic efficacy in real-time. Clinicians are limited by waiting for drugs produce significant effects such that the measurable tumor size or metabolism changes can be monitored *via* expensive imaging techniques such as positron emission tomography-magnetic resonance imaging (8). In reality, the complex nature of the genetic makeup of the disease often determines the tumor's resistance to chemotherapy, and the entire duration of treatment will be lost due to lack of an available *in situ* monitoring system. Hence, the assessment of real-time therapeutic response for better clinical decision to treat cancer patients, in particular those with PDAC, is an unmet clinical need.

Integration of nanotechnology in medicine has successfully demonstrated unprecedented achievements in developing novel therapeutics and diagnostics. As biosensors, single-walled carbon nanotubes (SWCNTs) hold great promise for detecting key biological analytes, including nitric oxide (NO), glucose, hydrogen peroxide (H₂O₂) and several others (9-16), compared to other nanostructures, such as solid-state nanopores (17) and nanochannels (18). DNA-SWCNT conjugates are able to demonstrate DNA sequence-dependent molecular recognition. For example, (AT)15-wrapped SWCNTs perform as specific NO sensors (14,16) and (GT)15-wrapped SWCNT as H₂O₂ sensors in the biological system (10,12). SWCNTs exhibit

characteristic Raman and photoluminescence (PL) properties in the near-infrared (NIR) spectral region; these properties make them suitable candidates for bio-imaging, as most of the biological sample has minimal optical scattering and absorption in NIR range (700 nm-2500 nm) (19). Earlier, Heller et al. and Jin et al. (10,12) demonstrated that the single-stranded DNA, (GT)₁₅, wrapped around the SWCNT surface is highly selective to measure the presence of H₂O₂ with single molecule sensitivity and high spatial and temporal resolution from cells. These SWCNTs wrapped with (GT)₁₅ exhibit quenching of fluorescence upon exposure to H₂O₂ (12). Additionally, SWCNT's Raman signals have been utilized for tissue labeling (20,21) as well as sensor applications (22,23).

In cellular and preclinical experiments with small animals, several reports suggest that drugs, such as vitamin C (or ascorbate) (24,25) and GEM (24,25), induce production of H₂O₂ (26) within cancer cells during their cytotoxic activity. It is also evident that other routinely used anticancer therapeutics such as paclitaxel, cisplatin, arsenic trioxide, etoposide, and doxorubicin, likewise increase production of intracellular H₂O₂ in their cytotoxic activity (27-34). Hence, our hypothesis was to develop a minimally invasive, real-time, *in vivo* sensor-imaging platform to directly assess the status of the post-treatment tumor and its microenvironment, which can serve as indicators of treatment efficacy. We predict that if GEM is effective, there will be a corresponding production of H₂O₂ in the TME that suggests an effective response from drugs. Thus, if the designed sensor system can monitor the difference in H₂O₂ production in response to GEM treatment, we can correlate drug response to the tumor cell's cytotoxicity in real-time.

Herein, we report on the SWCNT nanosensors ability to specifically monitor H₂O₂ to evaluate chemotherapeutic response both *in vitro* and *in vivo*. In summary, the DNA-SWCNT hybrids display reversible alternation in Raman and PL properties with dynamic status of

endogenous and/or exogenous H₂O₂. Production of intracellular H₂O₂ is correlated with cellular cytotoxicity induced by chemotherapeutics, such as GEM and irinotecan. We observe both Raman and PL signals of SWCNTs in response to the alteration of H₂O₂ *in vitro*. To demonstrate the application of this biosensor as a real-time monitoring platform, we designed a longitudinal study in PDAC murine model, where this probe successfully recorded the release of H₂O₂ in response to GEM treatment. We also monitored that the Raman signals of SWCNTs reverse back to the initial pretreatment levels upon withdrawal of treatment, indicating inhibition of exogenous H₂O₂ production. Together these observations SWCNTs may be considered as a clinical tool to predict therapeutic outcome.

Materials and Methods

Materials

For our study, we used raw HiPco™ SWCNT (batch HR29-039; NanoIntegris Inc), containing a distribution of small diameter SWCNTs, Dulbecco's Modified Eagle Medium (DMEM; Corning Incorporated); fetal bovine serum (FBS; Gibco™); penicillin-streptomycin (Gibco™); phosphate buffered saline (PBS; Life technologies); GEM (Sagent Pharmaceuticals, Inc.); irinotecan (LC laboratories); CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega Corporation); 6-carboxy-2',7'-dichlorodihydrofluorescein diacetate (Catalog number: C2938; from Invitrogen™); and 0.9% NaCl (AddiPak™).

Synthesis of DNA-SWCNT hybrid

(GT)15-SSDNA (IDT) and SWCNT were mixed in a 1 mg to 1 mg weight ratio in a 1 mL volume of 0.1 M NaCl. The mixture was chilled on ice and probe-tip sonicated (Qsonica Q125) with a 1/4 in. tip at 40% amplitude for 30 minutes. The dispersion was then centrifuged twice for 90 minutes at 16100 g to remove large particulates, undispersed SWCNTs, and other residual

impurities. The absorption spectra of SWCNT dispersions were collected (Cary 5000, Agilent Technologies) and concentrations approximated using the absorbance at 632 nm with extinction coefficient of $0.036 \text{ mg (L*cm)}^{-1}$ (35).

Cell culture

PANC1 (item number # CRL-1469 and batch number 62278038; ATCC) was cultured following a standard method, as previously described (36). Briefly, PANC1 was grown in DMEM supplemented with 10% FBS and 1% penicillin–streptomycin. Cells were thoroughly washed with PBS before any treatment mentioned in the study. For treatment, SWCNT, GEM, and irinotecan were dissolved in supplemented cultured media.

Cell viability assay

For cell viability assay, approximately, 5×10^3 cells were plated in 96 well plates and treated with GEM, and irinotecan. After 24 and 48 hours of treatment, cells were washed thoroughly with PBS three times. As per the manufacturer's protocol, cells were incubated with 100 μL media containing 20 μL One Solution reagents at 37°C for 30 minutes, and absorbance at 492 nm was measured using SpectraMax i3x (Molecular Devices, LLC.).

***In vivo* tumor growth**

All animal studies were performed as per procedures approved by the Mayo Clinic Institutional Animal Care and Use Committee. Orthotopic pancreatic cancer xenograft was developed in 6- to 8- week old female SCID mice that were obtained from the National Cancer Institute and housed in the institutional animal facilities. SCID mice were anesthetized with ketamine/xylazine prior to any surgical procedures. Effective anesthesia was tested by squeezing the rear paw or pinching the tail to test for reactions. Approximately 1×10^6 PANC-1 cells suspended in 50 μL PBS were injected orthotopically into the pancreases of the mice. Tumors were allowed to grow for three

weeks. Once tumor size was approximately 1 cm, tumor bearing mice were prepared for the longitudinal therapeutic study. Prior to imaging, surgery was performed on each mouse to access the tumor. After anesthesia, the region of interest was sterilized with an iodine solution and a transverse incision was made with scissors at the site of previous surgery to access the pancreatic tumor for imaging. After imaging, the peritoneal membrane and outer incision were sutured as separate layers with surgical absorbable suture, Vicryl 4-0 (undyed, braided).

SWCNT Characterization

NS3 NanoSpectralyzer (Applied NanoFluorescence, LLC) was used for all cuvette based spectroscopy. We used an InGaAs (Renishaw plc) detector for our study. A 532 nm laser and 671 nm laser were used for collection of PL and Raman signals, respectively, from SWCNTs. We have used inVia™ confocal Raman microscope (Renishaw plc), which is also equipped to record PL spectra and Raman spectra. For live cells and live animal imaging, we have used a 785 nm laser with output power 300 mW and CCD detector (Renishaw plc). For *in vitro* imaging we have used 40 × water immersion lens from Olympus and 1200 l mm⁻¹ gratings (Renishaw plc), and for *in vivo* imaging we used 5× air objectives from Leica and 600 l mm⁻¹ gratings (Renishaw plc). The absorption spectra of (GT)15 wrapped SWCNTs in the presence or absence of H₂O₂, fluorescence excitation-emission, and Raman spectra are presented in **Figures S1, S2, S3, and S4** respectively.

SWCNT size distribution was measured via NanoSight LM10 (Malvern Panalytical Ltd). Particles were tracked using forward scattered 405 nm laser light, a 10× objective lens and a CMOS camera. Experiments were performed at 293 K; 30 videos of 30 seconds each were taken and processed with a recently developed Bayesian algorithm to estimate the hydrodynamic

radius distribution of the particle population using Matlab and NanoSight NTA 3.2 software (Figure S5).

Transmission Electron Microscope (TEM)

Following treatment, PANC1 cells were fixed in EM fixative (4 % paraformaldehyde with 1% glutaraldehyde in phosphate buffered saline, pH 7.2), and placed into 2% low melting agar. Cells were then stained with 1% osmium tetroxide and 2% uranyl acetate, dehydrated through an ethanol series, and embedded into Spurr resin. Following a 24 hour polymerization at 60°C, 0.1 μm ultrathin sections were post-stained with lead citrate. Micrographs were acquired using a JEOL1400 Transmission Electron Microscope (Peabody, MA) equipped with a Gatan Orius camera (Gatan, Inc. Pleasanton, CA), operating at 80kV.

Statistical analysis

The data is represented as mean $\pm$ standard deviation (SD). All *in vitro* experiments were conducted independently in triplicate. Comparisons between treatment groups were done by Student t test. Statistical analysis was conducted with either Microsoft Excel or Origin labs. Statistical significance was accepted when $P < 0.05$.

Results

Raman and PL Response of DNA-SWCNT Hybrid Subjected to H_2O_2

In this study, we introduced a wide range of concentration from 5 μM to 200 μM of H_2O_2 to SWCNT suspension ($10\mu\text{g mL}^{-1}$), transferring the mixture in a quartz cuvette with path length of 1 cm (see supplemental materials for (GT)15–SWCNT absorbance, fluorescence, Raman, and size distribution characterization; S1-S5). The PL and Raman response of these nanotubes were collected by an NS3 NanoSpectralyzer spectrometer. As illustrated in **Figure 1a**, PL intensity decreased monotonically with the increasing concentration of H_2O_2 , as previously reported

(10,12). The PL quenching gradually slowed until 200 μM of H_2O_2 reaching a total response of 26% (**Figure 1b**). We also monitored similar modulation of the resonance Raman vibrational spectra of SWCNTs as illustrated in **Figure 1c** and **1d**.

Detection of Chemotherapeutics Induce H_2O_2 Using DNA-SWCNT Hybrid

GEM (2',2' -difluoro-2' -deoxycytidine; dFdC) is a deoxycytidine analog known to be phosphorylated in the cytoplasm to produce GEM triphosphate within the cells. This metabolite then inhibits DNA synthesis (37), leading to cellular stress and H_2O_2 production (38), followed by induction of apoptosis and cell death (39). To evaluate if the production of H_2O_2 could be monitored by SWCNT, we used PANC1, a pancreatic cancer cell line. SWCNT ($10\ \mu\text{g mL}^{-1}$) was pre-incubated overnight in PANC1 cells, which were further treated with $10\ \mu\text{M}$ GEM for 12, 24, and 48 hours. Cells were thoroughly washed with PBS and harvested at each time point, followed by sonication, then PL measurement was conducted via NanoSpectralyzer (**Figure 2a**).

For comparison, we also used commercially available 6-carboxy-2',7'-dichlorodihydrofluorescein diacetate (carboxy-H2DCFDA) to monitor intracellular H_2O_2 upon GEM treatment. PANC1 cells were cultured in 96-well plates, then treated with $10\ \mu\text{M}$ GEM for 12, 24 and 48 hours. At each time point, cells were washed three times with PBS and incubated with $10\ \mu\text{M}$ carboxy-H2DCFDA. After one hour, green fluorescence was recorded using SpectraMax i3X plate reader from each well of the plate to measure the H_2O_2 . Both carboxy-H2DCFDA and SWCNT biosensor showed a similar trend in monitoring H_2O_2 production with respect to the different time points of GEM treatment, but the relative sensitivity for the SWCNT sensors was superior to that of the carboxy-H2DCFDA (**Figure 2a**). The relative sensitivity was determined using the following equation (1):

$$\Delta PL = 1 - \frac{I}{I_0} \quad (1)$$

I is the PL intensity from GEM-treated cells at any time point and I_0 is the PL intensity from cells without treatment. We also performed *in vitro* cell viability assay to measure the fate of the cells after exposure of 10 μ M GEM at 24- and 48- hour time points using the same 96-well experimental set up used for carboxy-H2DCFDA. Percentage viability was calculated using the following equation (2):

$$Viability\% = 100 \times \frac{Abs_{Treated} - Abs_{Blank}}{Abs_{Untreated} - Abs_{Blank}} \quad (2)$$

Abs represents the absorption. Results presented in **Figure 2b** clearly demonstrate that with time, cell viability significantly diminished. Hence, we found a direct correlation of cell viability with H_2O_2 production due to GEM treatment as previously reported (26).

Live Cell Raman Microscopy Provide Spatial Distribution of Chemotherapy-Induced H_2O_2

We further employed live cell imaging to understand the spatial distribution of H_2O_2 production. PANC1 was cultured in a quartz bottom 50 mm Petri dish and then incubated with the SWCNT biosensor at a final concentration of 10 μ g mL^{-1} in cell culture media overnight. After thorough washing with PBS three times, the cells were treated with two different concentrations of GEM, 5 μ M and 10 μ M, for 72 hours. Finally, the cells were washed and prepared for live cell imaging using a Renishaw inVia confocal microscope. Before each measurement with the instrument, Raman signal was calibrated with a silicon substrate. Using line scan with 15 mW laser power and 1second exposure time, Raman spectra collected from individual points of a line arbitrarily drawn over the cells; each point was separated by 1 μ m and the line consisted of 53 such points over the live cells. These points were overlaid on the bright field image of the corresponding cell as illustrated in **Figure 3a**. The inset of **Figure 3a** shows Raman spectrum collected from one such point and confirms the presence of SWCNT within the cell. Raman G-band intensity at

1590 cm^{-1} corresponding to each data point was then overlaid on the white light image. From the spectral signatures, we can specifically monitor spatial distribution of SWCNTs within the cells. Laser power was optimized prior to the experiment to prevent any detrimental effect to the live cells. Furthermore, these spectra were collected using confocal mode to minimize the influence of Raman signals from surrounding SWCNTs. The intracellular spatial variation of the Raman spectra is presented in **Figure 3b**. In the selected NIR excitation wavelength (785 nm), no significant auto fluorescence interference from cellular components was detected. **Figure 3c and 3d** display the Raman signals from cells treated with 5 μM and 10 μM GEM for 72 hours, respectively. As evident from the morphology of the cells, GEM was effectively toxic (**Figure 3c and 3d**). The vertical color bar in **Figures 3a, 3c, and 3d** indicates the range of intensity corresponding to the characteristic Raman peak G-band of SWCNTs in untreated and treated cells respectively and the significant reduction in maximum intensity range correlates to the Raman signal decrease due the presence of intracellular H_2O_2 . For treated cells, with increasing concentration of GEM, more attenuation of Raman signal was observed inside the PANC1 cells as summarized in **Figure 3e and 3f**. **Figure 3e** represents the spatial distribution of Raman signals and Figure 3f displays a bar graph to present variation in maximum intensity subject to GEM exposure. These data support current literature, where it has been shown that the higher concentration of GEM treatment results in increased H_2O_2 production (38), and hence, we found more attenuation in Raman signals of SWCNTs. Cell viability assay also confirmed the toxicity from GEM at 5 and 10 μM concentrations after 72 hours of exposure (**Figure 3g**). It is evident from **Figures 3a, 3c, and 3d** that there are spatial variations of H_2O_2 production inside the cells that display a varying amount of Raman signal modulation in SWCNTs. Gemcitabine triphosphate, not the GEM itself, binds to the DNA inside the cells (40). To illustrate that the

GEM itself is not contributing to SWCNT's PL quenching by binding with (GT)₁₅ present on the surface of SWCNTs, we incubated GEM with SWCNT biosensors for 72 hours. PL spectra from these samples were collected by NanoSpectralyzer as presented in **Figure 3h**. No variation was observed in PL intensity of signature peaks corresponding to SWCNTs in the presence of GEM, suggesting that GEM itself has no non-specific contribution to the PL response. We did not observed any significant alteration in PL spectra collected from SWCNTs incubated in PANC1 cells up to 72 hours (**Figure S6**).

Furthermore, we have incorporated another chemotherapeutic drug, irinotecan, which showed similar results (**Figure 4**). After overnight pre-incubation with SWCNT, PANC1 cells were exposed to 120 μ M of irinotecan for 72 hours. Cells were thoroughly washed with PBS before imaging. In **Figure 4a**, SWCNT displays a similar Raman response to that in **Figure 3a**. After 120 μ M irinotecan treatment, around 50% Raman signal change was monitored (as displayed in **Figure 4b** by the color scale) due to the production of intracellular H₂O₂ from treatment. These observations were very consistent throughout the cell culture dish for both Gem and irinotecan treatment (**Figure S7**). **Figure 4c** demonstrates the spatial distribution of Raman G-band signals over the line scan. Cell viability assay confirms that irinotecan at 120 μ M concentration delivers significant cytotoxicity to PANC1 cells after 72 hours of exposure (**Figure 4d**). **Figure 4e** summarizes the effect on spatial distribution of Raman G- band signals from 120 μ M irinotecan treatment which directly correlates with the level of intracellular H₂O₂. **Figure 4f** demonstrates the quantitative analysis of the results presented in Figure S7. To verify endocytosis of SWCNTs we have performed TEM analysis as shown in **Figure S8**.

***In Vivo* Raman Imaging and Assessment of Real-Time Chemotherapeutic Outcome of Pancreatic Cancer in a Longitudinal Mice Model**

We also present a longitudinal study of H₂O₂ sensing in an *in vivo* animal model by monitoring dynamic status of H₂O₂ inside the tumor of a live animal through (GT)15-wrapped SWCNT following GEM treatment. Briefly, every mouse underwent three survival surgeries during the study and was imaged for three times. Orthotopic pancreatic cancer xenograft was developed at first survival surgery. After tumor size reached approximately 1 cm, the second survival surgery was executed to implant SWCNT sensors in the tumor. A small well was made in the tumor using a puncher, then 20 μ L of SWCNT probe was delivered in the well and secured in the tumor using biological glue. The dry spot from the biological glue was later used to locate the sensor implantation site. Using a Renishaw inVia confocal microscope, we first assessed the optical slice with maximum intensity profile using a 785 nm laser at 30 mW power (1second exposure), and Raman spectra was collected using 600 1 mm⁻¹ grating and Renishaw CCD detector. Selection of NIR laser allowed us deep tissue imaging in our study. Nominal laser power was used to avoid tissue damage. We performed a depth profile, where Raman spectra were collected from different tumor depths, surface to 2000 μ m deep, with 200 μ m interval in longitudinal direction (**Figure 5**). This depth analysis confirmed that SWCNTs located at approximately 200 μ m below the surface produced maximum Raman signals. Then, we performed an area scan surrounding the implanted site at a depth where maximum Raman signals of SWCNTs were acquired ($\sim$ 200 μ m) in all mice. The separation between points in the area map was 20 μ m in both lateral directions. To maintain mice hydration, all mice received sterile 0.9% NaCl prior to live imaging and lubricant eye drops from Refresh Plus (Allergan, Inc.) during imaging. These mice were maintained under mild anesthesia using a continuous flow of 1% to 2% isoflurane and placed on heating stage set to 37°C. After imaging, the peritoneal membrane and outer incision were sutured as separate layers with surgical absorbable suture, Vicryl 4-0. The G-band peaks at

1590 cm^{-1} of SWCNTs were monitored in these spectra. A typical map is presented in **Figure 6a** using intensity profile at 1590 cm^{-1} . It has been previously described that cancer cells produce H_2O_2 at a basal level (41). Hence the recorded Raman signal from implanted SWCNTs (**Figure 6a**) was considered the basal level of H_2O_2 in the TME.

One day after the second survival surgery, each mouse was treated with a 40 mg Kg^{-1} dose of GEM for three days (42). In agreement with previous studies from our laboratory and others, GEM at this dose, was effective enough and started killing the tumor cells *in vivo*, hence predicting elevation of H_2O_2 level in the TME (43). Hence, we performed the third survival surgery to assess whether the nanosensors were able to detect the change of H_2O_2 production in TME after GEM treatment in live mice. The surgery was done on the microscope stage and anesthesia was maintained with a continuous flow of isoflurane. For imaging, the same experimental parameters were used as mentioned earlier for live mouse imaging. The biological glue used previously for securing SWCNTs within the tumor was spotted, and the scanning area was determined. The optical plane for area scan was again determined by performing a depth scan in each mouse to obtain maximum Raman signals from SWCNTs. The characteristic G-band of SWCNTs was noticed in the Raman spectra obtained from the area scan from the live animal, though the intensity of these peaks was significantly reduced, as evident from the color map scale in **Figure 6b**. This Raman signal change can be attributed to the production of H_2O_2 at an elevated level in tumor microenvironment due to GEM treatment.

The plasma half-life for GEM is approximately 0.28 hour in mice (44). To examine whether withdrawal of GEM treatment could rescue the H_2O_2 in the TME to its pre-treatment levels, we kept each mice alive for two more weeks without any further treatment. Mice were then sacrificed and tumor mass was scanned at the same area specified by the biological glue. We

performed another depth scan to optimize the optical plane with maximum Raman signals from SWCNTs and performed area scan thereafter. The Raman signal in each mouse was found to be almost similar to their corresponding pre-treatment level, as presented by the color map scale in **Figure 6c**. The reduced spatial distribution in **Figure 6c** may be due to the orientation of the mouse during imaging. Pancreatic cancer is characterized by its highly desmoplastic TME but minimal diffusion SWCNTs cannot be ignored. We performed an area scan over the tumor cross section and mapped for Raman signals of SWCNTs, confirmed the localization and integrity of SWCNTs in the implanted site (**Figure S9**). In **Figure 6d**, we presented a comparative analysis of maximum Raman G-band intensities obtained from the above mentioned longitudinal animal study on three mice. Data corresponding to each individual mouse is displayed by separate colors. It is evident that the signal significantly decreased with GEM treatment in mice compared to both pre- and post- treatment levels. These observations also confirmed that GEM therapy can induce the production of H_2O_2 , and upon withdrawal of treatment, the elevated level of H_2O_2 diminished.

Discussion

Chemotherapeutics that show significant cytotoxicity to cancer cells by several key mechanisms, often produce oxidative stress in the cell (27-31,34). Hence, generation of H_2O_2 in these circumstances can be evaluated as a key parameter to estimate the merit of any chemotherapeutics in chemoresistance pancreatic cancer. So far, several fluorophore-based assay systems have been explored to measure the endogenously expressed H_2O_2 (45). Among them, the most commonly used H_2O_2 indicator is carboxy-H2DCFDA, which provide green-fluorescent upon exposure to reactive oxygen species (ROS) in cells (46). But their application in animal study is very limited due to signal attenuation and autofluorescence of live tissue. To overcome

these limitations a significant effort has been made to develop an H_2O_2 sensor with NIR absorption and fluorescence as tissues are optically transparent at the NIR spectral window. One such successful nanoplatform is SWCNT, which performs as a highly selective and sensitive H_2O_2 sensor when wrapped with (GT)15 (10,12).

SWCNTs have been well characterized for their intrinsic NIR fluorescence and Raman spectroscopy. Raman signal of SWCNT is largely observable due to the phenomena of resonance Raman scattering (47,48), where the incident light is near the frequency of the electronic transition of a specific SWCNT chirality, augmenting Raman emissions. SWCNT absorbance decreases with H_2O_2 (**Figure S2**), which should result in a corresponding decrease in Raman signal. Raman signature of SWCNT is sensitive to the local environment and has been used for sensor application (23). Since H_2O_2 has this well documented effect on the strength of the optical absorption transition, and the resonant Raman peak intensities necessarily couple to these transitions, there is a mechanism for detecting H_2O_2 using Raman. This mechanism is the same as what has been previously observed for H^+ (49). It is important to note from excitation-emission maps (**Figure S3**) that there exist a distribution of SWCNT chiralities, as well as a distribution of SWCNT lengths in these sensor solutions. The calibration here relates to the mean population response to H_2O_2 and is applied as such in subsequent sections. It is this modulation of PL and Raman signals, explored extensively in the biological environment (50), that is now applied to the case of chemotherapeutic response.

We have noticed that, both PL and Raman signals attenuate monotonically with increasing concentrations of H_2O_2 (**Figure 1**). To monitor influence of chemotherapeutics in production of H_2O_2 , we treated PANC1 cells with both GEM and irinotecan at concentrations that significantly deliver cytotoxicity to PANC1 cells. As shown in **Figure 2**, SWCNTs display

higher sensitivity over carboxy-H₂DCFDA in detection of intracellular H₂O₂. Furthermore, study of Raman signal attenuation from SWCNTs illustrates spatial distribution of H₂O₂ production inside the cells treated with GEM and irinotecan (**Figure 3 and 4**).

To understand the dynamic influence of therapeutics by monitoring resulted variation in H₂O₂ production, we designed a longitudinal study on PANC1 orthotopic xenograft model in mice. (GT)15-wrapped SWCNTs were implanted in PDAC tumor during a critical survival surgery. The Raman signals were immediately recorded from tissue-embedded SWCNTs and utilized as reference for further analysis. To identify the optical plane of maximum signals, Raman signals were collected from different depths of tissue (tumor surface was set as origin) (**Figure 5**). This precautionary exercise was repeated for further measurements. A significant reduction in Raman intensity from SWCNTs was monitored in GEM-treated mice, confirming the elevated level of intratumor H₂O₂ production. Upon recovery from the influence of GEM treatment, H₂O₂ levels returned to normal and Raman signals reached to the reference level (**Figure 6**).

To the best of our knowledge, there is no report of using a SWCNT-based biosensor, or perhaps any other sensor system, to longitudinally evaluate the chemotherapeutic outcome of any drug in a live animal tumor model, in real-time. This assessment of dynamic changes of H₂O₂ in real-time and also in situ, can be employed further to evaluate efficacy of other drugs in different tumor types.

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Figure Captions

Figure 1. Detection of H_2O_2 using DNA-SWCNT hybrid: a) Photoluminescence spectra obtained from SWCNT ($10 \mu\text{g mL}^{-1}$) after incubation with H_2O_2 concentrations from 0 – $200\mu\text{M}$. b) Percentage of PL quenching has been plotted with concentration of H_2O_2 . c) Raman spectra collected from SWCNT after incubation of H_2O_2 concentrations from 0 – $200\mu\text{M}$. d) Raman intensity corresponding to G-band has been plotted with concentration of H_2O_2 .

Figure 2. Detection of H_2O_2 in gemcitabine-treated cancer cell and cytotoxicity study: a) Endogenous expression of H_2O_2 measured using PL signals corresponding to carboxy-H2DCFDA and SWCNT biosensor. b) Cell viability assay of PANC1 after exposure to $10\mu\text{M}$ GEM treatment at three different time points.

Figure 3. Monitoring spatial distribution of H_2O_2 with varying concentrations of GEM: a) PANC1 treated with SWCNT. Raman spectra collected from each point in the dotted line. The color map in the dotted line is based on intensity at 1590 cm^{-1} . One such spectrum is shown in the inset and the corresponding point in the line scan is identified by the arrow sign. Color bar displays the range of Raman signals recorded in individual image. b) Spectra collected from each point has been plotted in a 3D graph, where x-axis represents the wavenumber (cm^{-1}), y-axis represents the coordinate of the line, and z-axis represents the intensity in arbitrary unit. c) A representative picture of the line scan on cells after treatment with $5\mu\text{M}$ GEM. Color bar displays the range of Raman signals recorded in each individual image. d) A representative picture of line scan on cells after treatment with $10\mu\text{M}$ GEM. Color bar displays the range of Raman signals recorded in each individual image. e) 3D representation of intensity profile from Figures a, c, and d, demonstrating effective decrease of Raman signals with increasing dose of GEM *in vitro*. The data set has been presented with offset in x axis to demonstrate their intensity profile without

overlapping. f) Bar graph represents alteration of maximum intensity of Raman signals with increasing concentration of GEM. ***** represents p value <0.0001. g) Cell viability assay of PANC1 after exposure of 5 μ M and 10 μ M GEM treatment for 72 hours. h) PL spectra obtained from SWCNT incubation with (red line) or without (black line) GEM. Both spectra overlap showing no effective influence of GEM on PL spectra of SWCNT.

Figure 4. Detection of H₂O₂ and their spatial distribution in irinotecan-treated cancer cell: PANC1 cells incubated with 10 μ g mL⁻¹ SWCNT overnight followed by irinotecan treatment for 72 hours. a) Cell without irinotecan treatment and b) cell with 120 μ M irinotecan treatment. Color bar displays the range of Raman signals recorded in individual image. c) Spectra collected from each point have been plotted in a 3D graph where x-axis represents the wavenumber (cm⁻¹), y-axis represents the coordinate of the line, and z-axis represents the intensity in arbitrary unit. d) Cell viability assay of PANC1 after incubation with 120 μ M irinotecan for 72 hours. e) 3D representation of intensity profile from Figures a, and b, demonstrating decrease of Raman signals with irinotecan treatment *in vitro*. The data set has been presented with offset in x-axis to demonstrate their intensity profile without overlapping. f) Maximum Raman intensity presented in bar graph display the effect of irinotecan treatment on H₂O₂ production. ***** represents p <0.0001.

Figure 5. Depth profile of Raman G-band intensity: The tumor was scanned in longitudinal direction to find the depth corresponding to maximum Raman signals.

Figure 6. Longitudinal live animal imaging: Tumor site of same mice imaged a) before initiation of treatment, b) after three treatments of GEM, and c) two weeks after withdrawal of treatment. The red color map was done based on the Raman signals from SWCNT at 1590 cm⁻¹

wavenumber. d) The maximum level of Raman signals from SWCNT corresponding to these three distinct treatment conditions is displayed for all three mice. * and ** indicate $p < 0.05$ and < 0.01 , respectively.

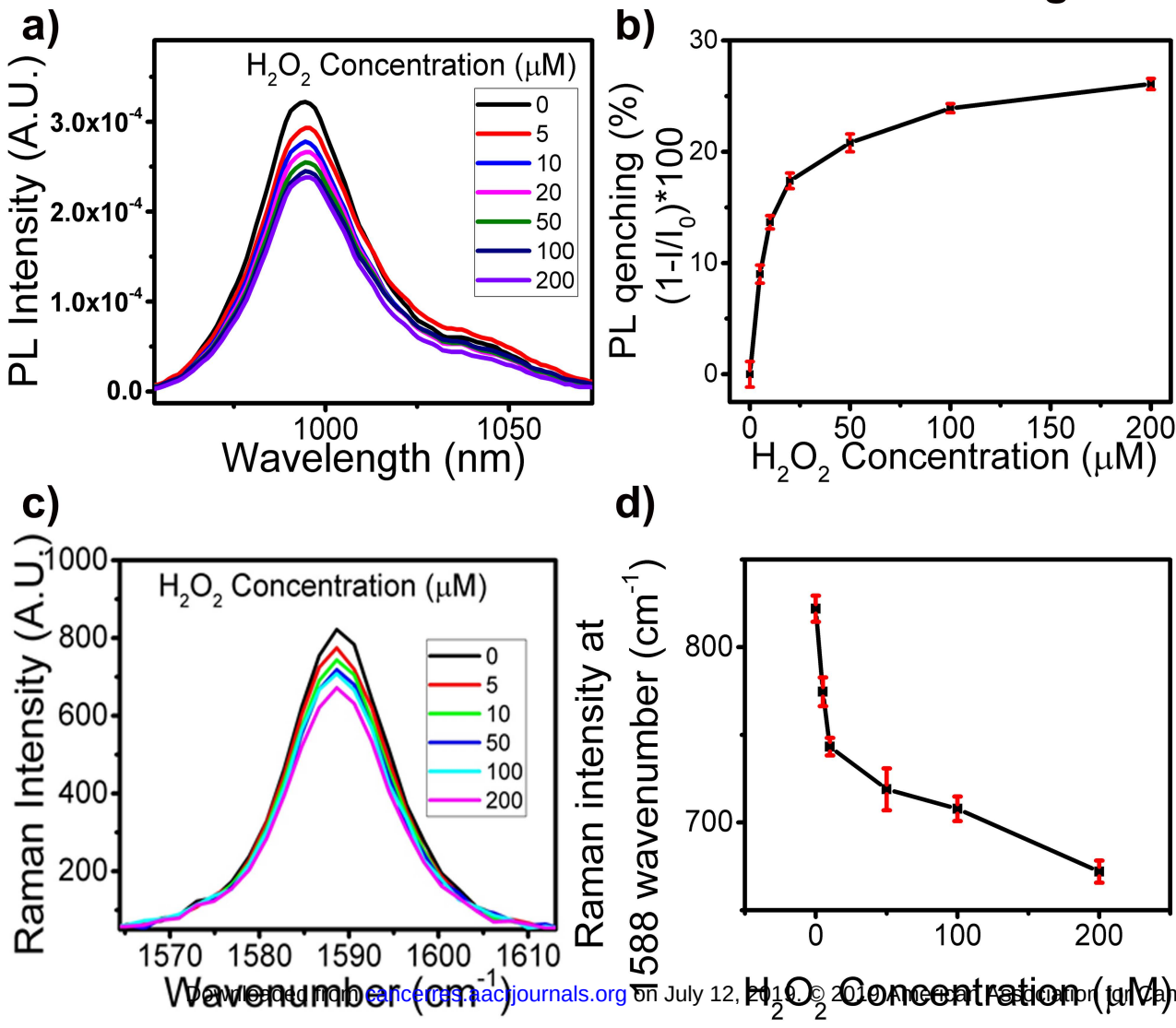


Figure 2

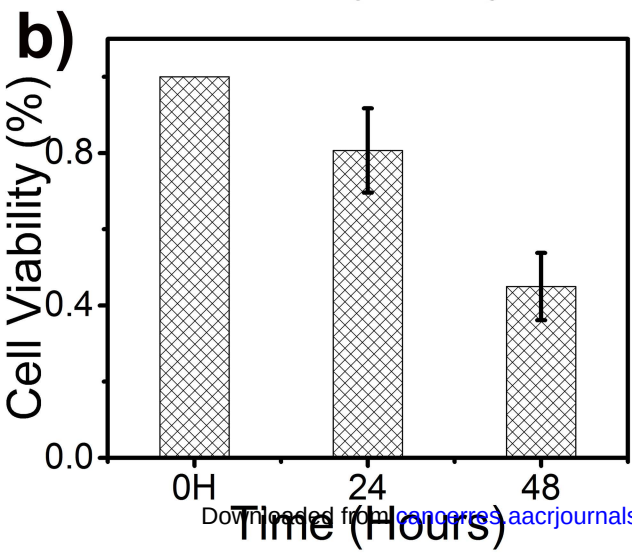
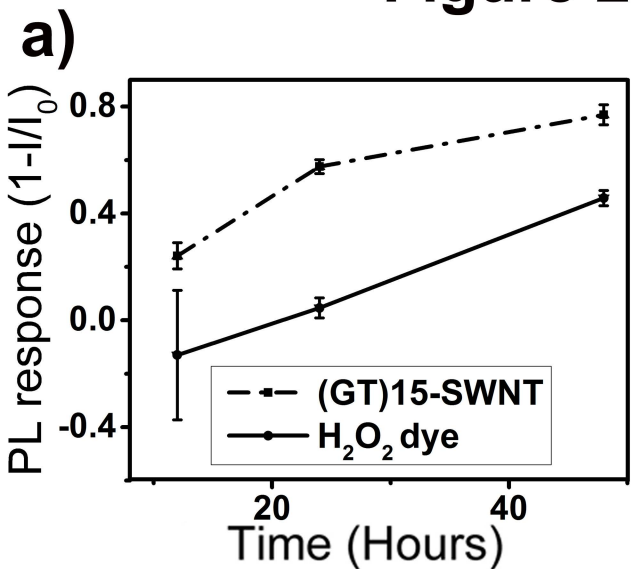


Figure 3

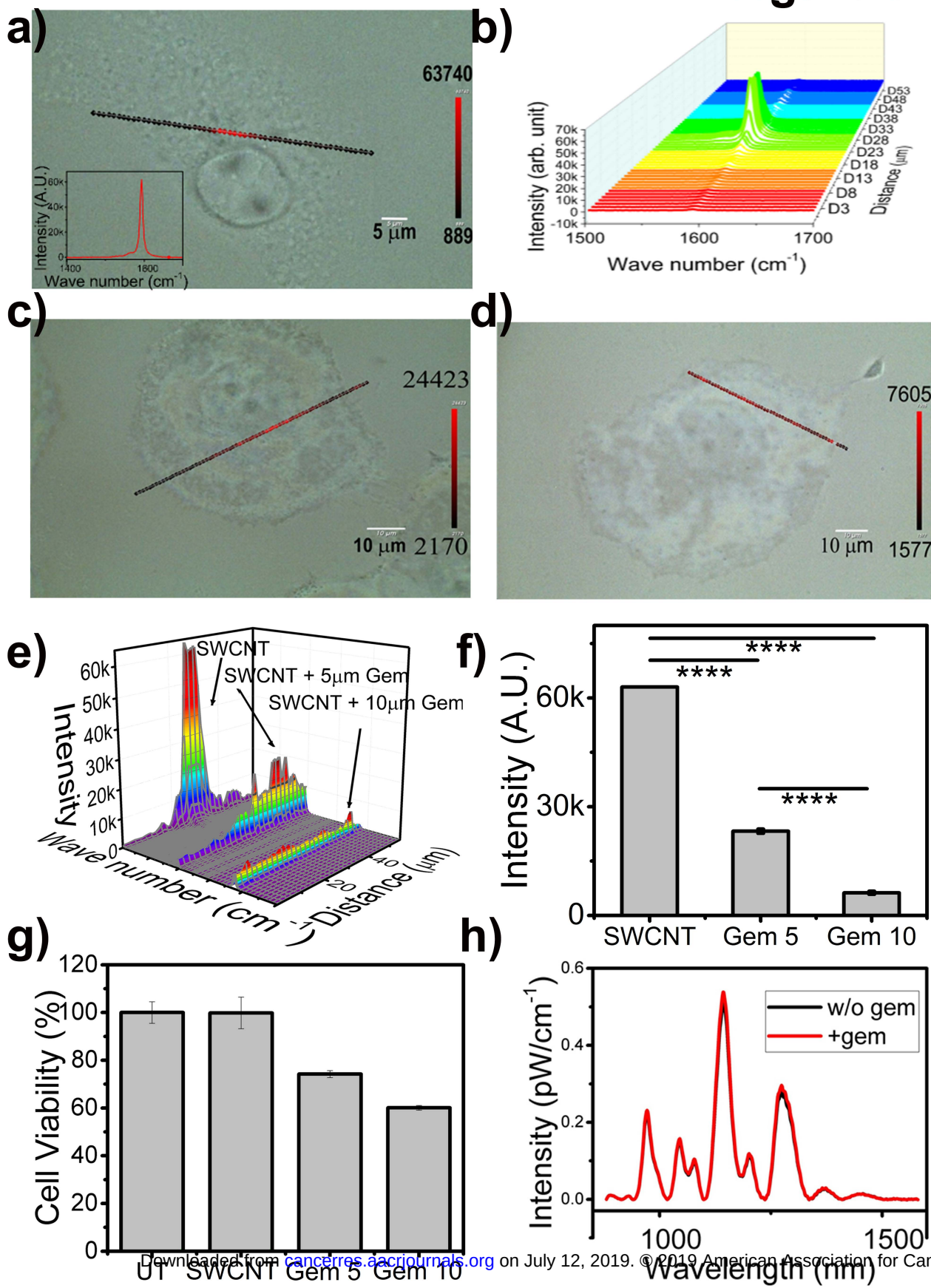


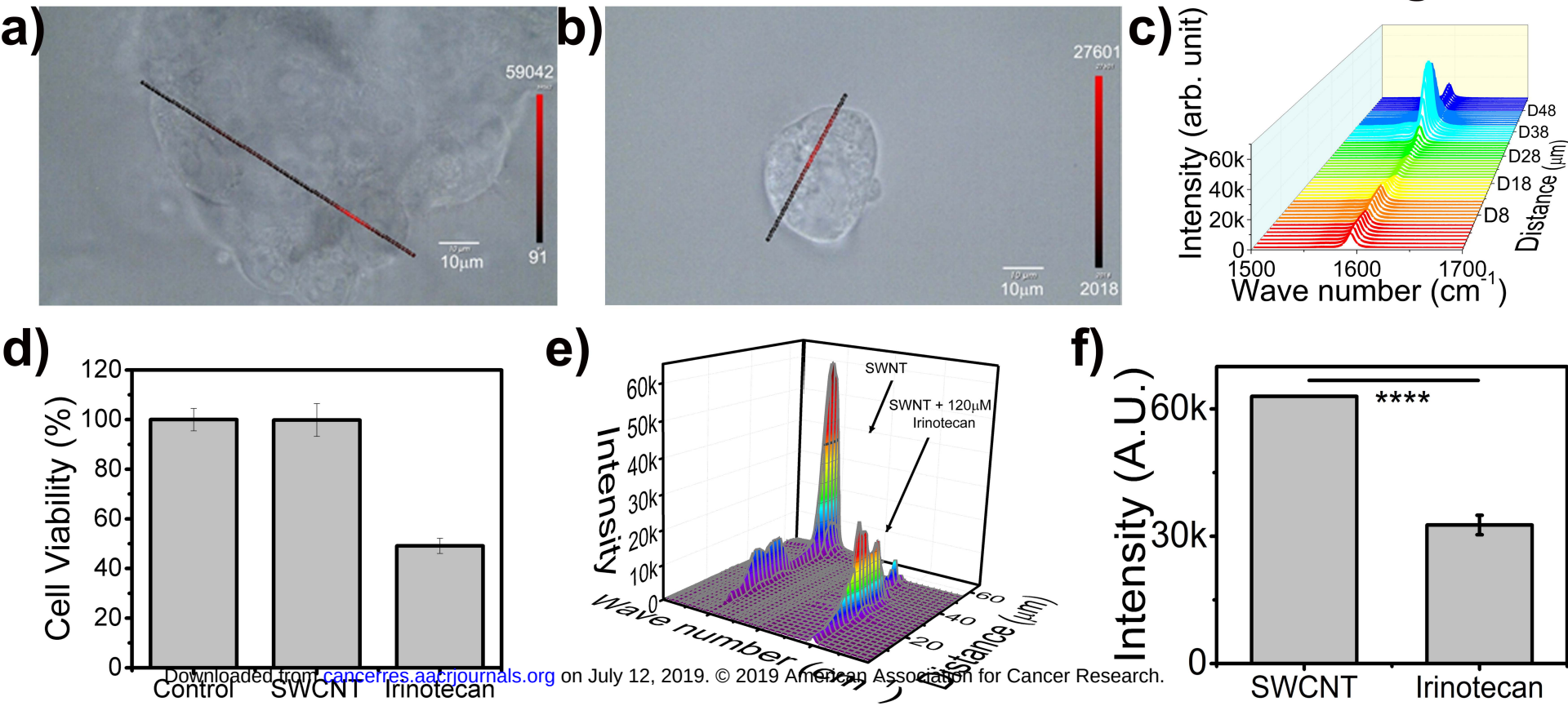
Figure 4

Figure 5

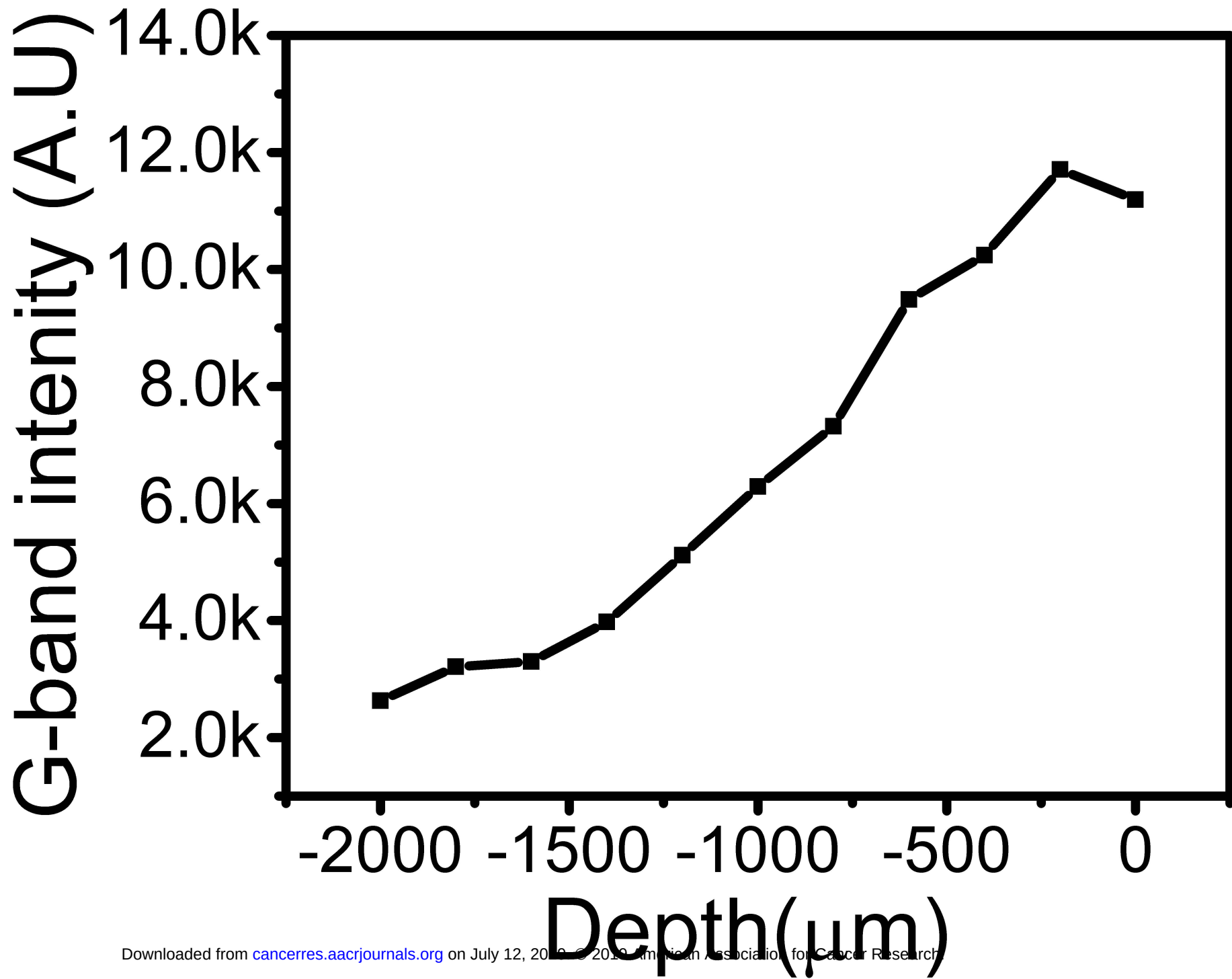
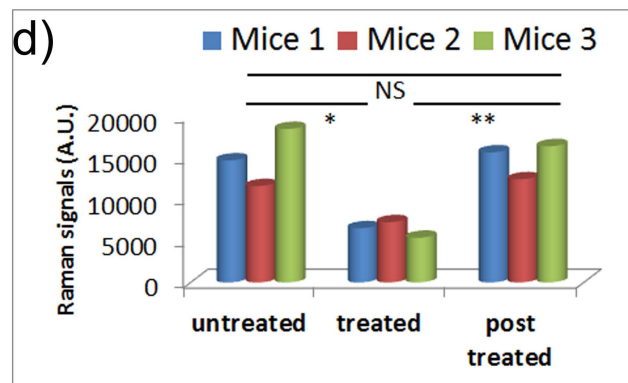
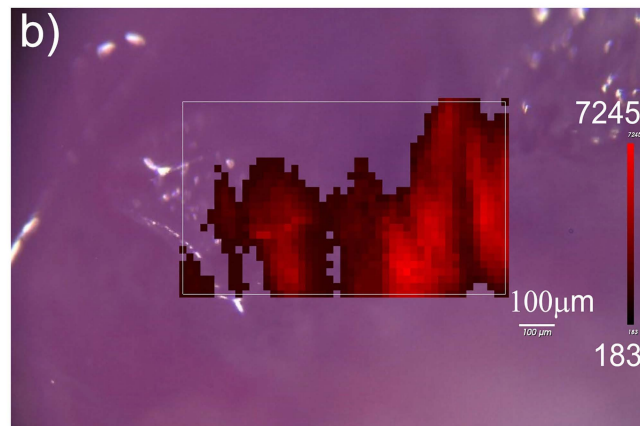
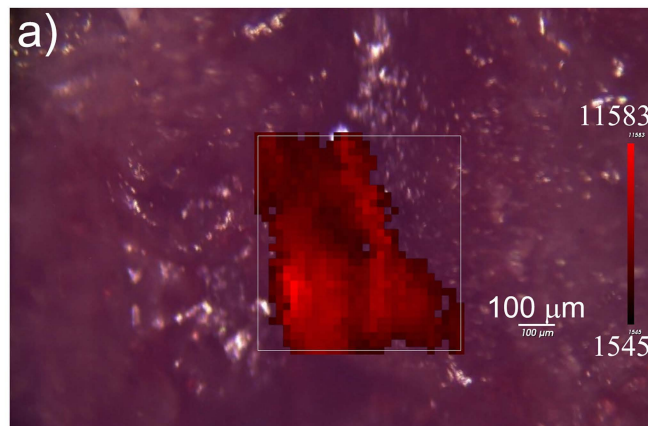


Figure 6



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DNA-SWCNT Biosensors allow real-time monitoring of therapeutic responses in Pancreatic Ductal Adenocarcinoma

Santanu Bhattacharya, Xun Gong, Enfeng Wang, et al.

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