ACS SENSORS

Letter

Cite This: ACS Sens. XXXX, XXX, XXX−XXX

pubs.acs.org/acssensors

Epigenetic Process Monitoring in Live Cultures with Peptide Biosensors

Nur P. Damayanti,[†] Kevin Buno,[§] Sherry L. Voytik Harbin,[§] and Joseph M. K. Irudayaraj*[,‡]

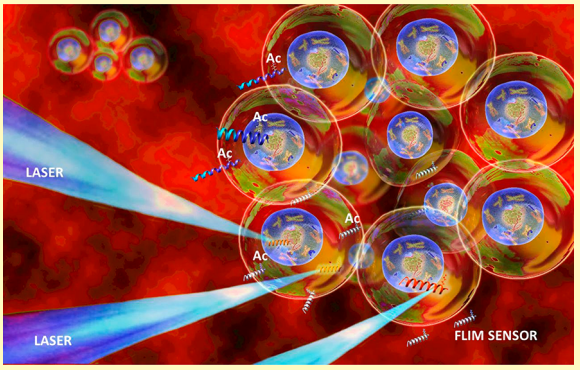
[†]Department of Agricultural and Biological Engineering, College of Engineering, Purdue University, West Lafayette, Indiana 47907, United States

[‡]Department of Bioengineering, Cancer Center at Illinois, University of Illinois at Urbana−Champaign, Biomedical Research Center, Carle Foundation Hospital, Urbana, Illinois 61801, United States

[§]Weldon School of Biomedical Engineering, Collage of Engineering, Purdue University, West Lafayette, Indiana 47907, United States

Supporting Information

ABSTRACT: Acetyltransferase is a member of the transferase group responsible for transferring an acetyl group from acetyl-CoA to amino group of a histone lysine residue. Past efforts on histone acetylation monitoring involved biochemical analysis that do not provide spatiotemporal information in a dynamic format. We propose a novel approach to monitor acetyltransferase acetylation in live single cells using time correlated single photon counting fluorescence lifetime imaging (TCSPC-FLIM) with peptide biosensors. Utilizing 2D and 3D cultures we show that the peptide sensor has a specific response to acetyltransferase enzyme activity in a fluorescence lifetime dependent manner ($P < 0.001$). Our FLIM biosensor concept enables real-time longitudinal measurement of acetylation activity with high spatial and temporal resolution in live single cells to monitor cell function or evaluate drug effects to treat cancer or neurological diseases.



KEYWORDS: *acetylation, FLIM, real-time detection, peptide sensor, live cultures*

Acetylation is post-translational modification pertinent for cellular function involving a class of acetyltransferase enzyme. P300/CBP associated factor (PCAF) is a tightly regulated acetyltransferase enzyme involved in diverse cellular processes such as transcriptional control, cell growth, and differentiation.[1] This ubiquitously expressed enzyme plays a key role in multiple cellular processes, owing to its cellular localization characteristics.[2,3] Distinct spatial and temporal profiles of PCAF results in different acetylation activity[4] which ultimately corresponds to a diverse set of cellular responses. Several methods[5−7] have reported monitoring of PCAF or other acetyl transferase protein activity for mechanistic evaluation. However, classical techniques to detect acetyl transferase activity, more specifically, PCAF, are generally limited to in vitro biochemical assays or immunofluorescence methods in fixed cells. Such methods do not provide dynamic information on protein acetylation. Some approaches have used genetically encoded biosensor or fluorescence resonance energy transfer (FRET) based biosensors[8,9] to monitor real-time acetylation. However, these approaches have limitations, because they require a uniform and stable transfection of genetically encoded sensors or depend on a FRET assay. More importantly, genetically encoded sensors will be challenging to implement in primary cells. Single molecule techniques have been used for characterizing epigenetic events;[10−12] however, few studies report on the dynamics of the process itself. In this work, we propose a novel approach with peptide biosensors[13] and fluorescence lifetime imaging (FLIM) to monitor acetylation in real-time. Fluorescence lifetime reporters can serve as ideal probes owing to its environmentally sensitive nanosecond decay changes. Furthermore, FLIM is not confounded by photobleaching or fluctuations in the excitation source and, hence, is a robust method for longitudinal observation. FLIM was applied to detect acetylation dependent fluorescence lifetime shifts (1−2 ns) in living 2D and 3D cultures. Detailed methods and instrumentations used in our work are provided in the Supporting Information (SI). We derive our motivation from our previous work with phosphorylation sensors.[13−15] The design of the sensor was based on the substrate of PCAF. PCAF primarily acetylates lysine 14 of histone 3;[14] therefore, we hypothesized that putative histone 3 peptide containing lysine 14 will be recognized by PCAF as its target substrate. Consequently, a

Received: September 29, 2018
Accepted: February 4, 2019
Published: February 4, 2019

ACS Publications © XXXX American Chemical Society

DOI: 10.1021/acssensors.8b01134
ACS Sens. XXXX, XXX, XXX−XXX

ACS Sens.
Downloaded from pubs.acs.org by UNIV DE BARCELONA on 02/09/19. For personal use only.

PCAF biosensor (HATS) was designed based on histone 3 sequences along with amino acids adjacent to lysine 14 (Figure 1a).

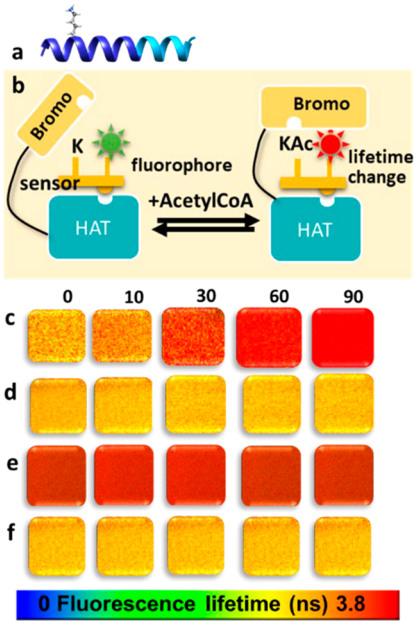


Figure 1. Schematic of proof of concept. (a) Peptide sensor: putative histone 3 (H3) (blue) within a DNA histone complex is a substrate for major acetyltransferase enzyme, and lysine 14 of H3 is a site for PCAF acetylation (K14) and a modified Tat sequence was added to facilitate sensor penetration into cells to enable live cell imaging (light blue). (b) Working principle of the peptide biosensor: the sensor binds to the histone acetyltransferase (HAT) target. Upon acetylation, phospholysine will bind to the PCAF bromodomain, resulting in an increased fluorescence lifetime of the reporter. (c–f) Fluorescence lifetime of HATS is acetylation-dependent. Monitoring PCAF acetylation in solution. General reaction condition is as follows: PCAF enzyme (Cayman Chemical, USA, cat. 0009115) was diluted 25 times in 50 μL of PCAF buffer (Cayman Chemical, USA, cat. 10009330), 20 μM acetyl-CoA and 10 μM peptide solution were added into the enzyme, and the mixture was incubated at 37 °C with (c) HATS, (d) HATS in the absence of acetyl-CoA, (e) acetylated HATS, and (f) acetylated HATS in the absence of PCAF enzyme. (c) After 90 min of incubation with HATS, fluorescence lifetime increased from 3.3 to 4.1 ns. (d) Same condition as in (c) but in the absence of the acetyl group donor, acetyl-CoA, showing constant fluorescence lifetime of HATS (3.3 ns). (e) Acetylated HATS in the same condition as in (c) showing constant fluorescence lifetime but higher fluorescence lifetime (4 ns) than nonacetylated HATS (3.3 ns). (f) Acetylated HATS in the same condition as in (e) but in the absence of PCAF enzyme showing the same lifetime with (c) nonacetylated HATS (3.3 ns) and remains constant during 90 min of observation.

To facilitate live cell delivery, a TAT peptide sequence (light blue) was added to the putative histone 3 (Figure 1b, dark blue). Additionally, for visualization, a fluorophore, 5-FAM, was conjugated to lysine amino acids +4 of the acetylation site (Figure 1b, SI Figure 1a,b). The working principle of the proposed HATS is as follows: HATS will be recognized by PCAF, but the binding of sensor to PCAF itself will not change the fluorescence lifetime of HATS. However, upon acetylation of the lysine 5 amino acid residue in the HATS (Figure 1b, HAT box),[16] the acetylated lysine binds to the bromodomain of PCAF. This binding event alters the solvatochromic environment of the sensor reporter, resulting in a change in its fluorescence lifetime (Figure 1b). To demonstrate that the fluorescence lifetime of HATS changes in a PCAF acetylation-dependent manner, in vitro acetylation experiments were performed with HATS as a substrate and its fluorescence lifetime in solution was noted every 5 min for a 90 min period. FLIM images (Figure 1c) demonstrated a gradual increase in the average lifetime of the fluorophore in the HATS peptide biosensor in PBS exposed to the PCAF enzyme and acetyl-CoA suspended in PCAF buffer at 37 °C. The fluorescence lifetime decay curve was then fitted with a multiexponential model[17] to obtain an average lifetime at each time point. Our result shows that, in solution, the fluorescence lifetime of HATS depends on PCAF acetylation, increasing from 3.3 to 4.1 ns (Figure 1c). We also noted that there was no change in fluorescence lifetime (3.3 ns) (Figure 1d) in the presence of PCAF enzyme when acetyl-CoA was absent during this 90 min period, suggesting that there was no change in lifetime in the absence of acetylation. Using the same experimental conditions as in Figure 1c, when HATS was replaced by acetylated HATS (positive control) (SI Figure 1c,d), a higher fluorescence lifetime (4.05 ns compared to 3.3 ns) was noted in the presence of PCAF enzyme. However, upon acetylation, the lifetime of acetylated HATS remained the same, indicating the change is due an acetylation event. A lower fluorescence lifetime of acetylated HATS (3.3 ns) was noted in the absence of PCAF enzyme (Figure 1f). Collectively, our observations suggest that the fluorescence lifetime of HATS depend on the acetylation activity of PCAF enzyme and not merely to the binding of HATS to PCAF. After testing our concept in solution, we tested the performance of our sensor in live 2D cultures. One of the major challenges in genetically encoded or nanoparticle-based sensors is the uniformity of uptake when different cell lines[18] are used. Here, we tested the efficacy of PCAF sensor uptake in three different cell lines (SI Figure 2). Experiments indicated that the sensor was rapidly internalized by the cells tested within 20 min. To validate subcellular localization, the cells were stained for immunofluorescence and visualized with confocal microscopy (SI Figure 2). To evaluate acetylation and PCAF acetyl transferase dependence of the fluorescence lifetime shifts of HATS, positive and negative controls were used (Figure 2). For positive control, we treated LNCaP cells with MS-275, a histone deacetylation inhibitor which has been reported to result in the accumulation of acetylated histone 3.[19] We also tested our sensor by incorporating a small molecule inhibitor for PCAF acetyl transferase activity, anacardic acid (AA),[20] as negative control. The real-time performance of HATS was evaluated by stimulating cells with the activator MS-275 (Figure 2a–c) or PCAF inhibitor AA (Figure 2d–f) over a 25 min observation period. AA inhibition of PCAF acetylation was validated by measuring the acetylated status of the enzyme substrate, histone 3 lysine 14, with Western blot (SI Figure 3).

Within 25 min of MS-275 stimulation, HATS exhibited increased fluorescence lifetime (Figure 2a,b). A signal-response event was observed in MS-275 treated cells: PCAF activity was noted in the nucleus following MS-275-induced signal activation. Our observation is consistent with previous findings which state that increased cytoplasmic PCAF acetylation will increase the cells' nuclear export.[7] The opposite response was observed for HATS within 25 min of treatment with AA (SI Figure 2d,e). Quantitative analysis (Figure 2c) confirmed that HATS in LNCaP cells treated with MS-275 (orange line) not

DOI: 10.1021/acssensors.8b01134
ACS Sens. XXXX, XXX, XXX–XXX

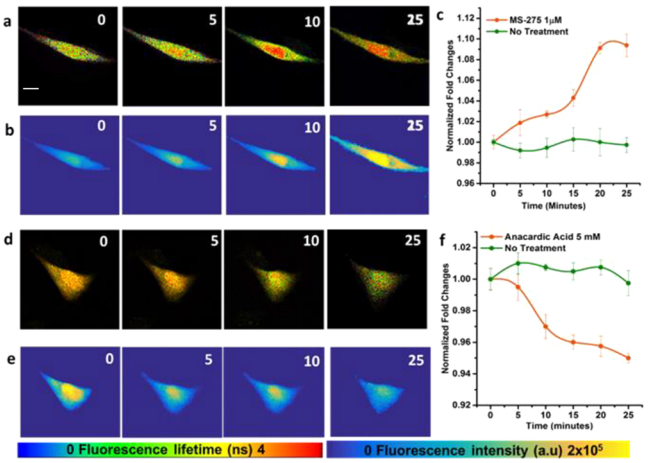


Figure 2. Fluorescence lifetime of HATS depends on PCAF acetylation. (a,b) 25 min time course images show a higher (a) average florescence lifetime and (b) intensity of HATS in LNCaP cells treated with histone deacetylase inhibitor, MS-275 (5 μM), at 37 °C. Changes were most prominent in the nucleus. (c) Quantitative analysis of (a) FLIM time course show increasing average florescence lifetime, especially in cell nucleus compared to control (2.45 to 2.9 ns) from t = 0 to t = 25 min. (d,e) 25 min time course images show a lower (d) average florescence lifetime and (e) intensity of HATS in LNCaP cells treated with the PCAF inhibitor, AA (0.5 μM). (f) Quantitative analysis of (e) FLIM time course of LNCap cells treated with AA show a gradual decrease of fluorescence lifetime (3.1 to 2.9 ns) from t = 0 to t = 25 min. Observations are representative of three technical and three independent biological replicates. Scale bar = 20 μm.

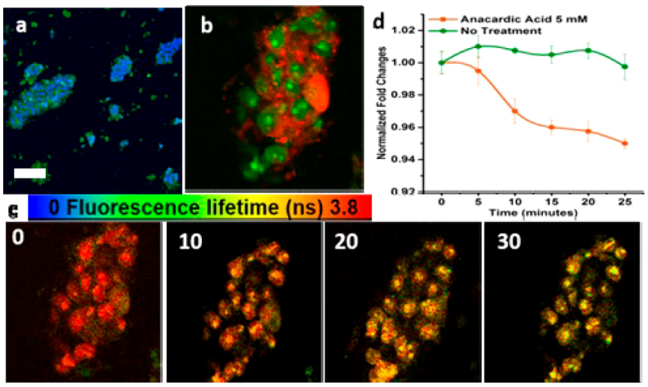


Figure 3. PCAF acetylation in 3D LNCaP cells monitored with fluorescence lifetime. (a) Confocal image of HATS (green) was uniformly uptaken by live LNCaP in collagen 3D culture. (b) Confocal image of live LNCaP 3D culture stained with Mitotracker red (red) and HATS (green) shows that HATS did not affect cell health. (c) FLIM image of HATS acetylation in 3D cultures over 25 min upon treatment with AA 5 μM. (d) Quantitative analysis of (c) anacardic acid (orange) and control of untreated cells (green) demonstrates no apparent decrease in control cells (green) and gradual decrease of HATS fluorescence lifetime in treated cells (orange) during the course of 25 min. Scale bar = 20 μm.

only exhibited a higher average fluorescence lifetime than that of control, but also experienced detectable changes in the initial average fluorescence lifetime. On the other hand, the control did not show any noticeable change in average lifetime during the observation period (green line). Data is representative of three technical and three independent biological replicates. In negative control experiments, quantitative analysis also validated the real-time response of HATS upon PCAF inhibition.

Upon AA treatment, HATS not only exhibited a lower average fluorescence lifetime compared to control, but also a detectable decrease from its initial fluorescence lifetime (Figure 2f). After testing HATS in live 2D cultures, the next step is to translate our study to a more physiologically relevant system in 3D cultures of LNCaP cells. In our tissue-like 3D culture, the collagen matrix model (Figure 3, SI Figure 4) of LNCaP cells formed an acinar structure morphology in 5 days, rather than its spindle-like morphology in 2D cultures (SI Figure 4e), consistent with our previous studies with 3D prostate cancer models.[21,22] To test the performance of the sensor in 3D cultures, HATS was tested in three different cells lines, LNCaP, RWPE, and PC3.

Within 30 min of incubation with HATS, the living cells in the 3D tumor structures had internalized the sensor (SI Figure 4g–i). Biocompatibility is an important factor in live cell studies.[23] To evaluate biocompatibility, ROS levels were assessed upon internalization of HATS. The difference was not statistically significant (data not shown). Our result indicates that HATS (20 μM) is not cytotoxic and does not increase the production of ROS, which can lead to cellular DNA damage and apoptosis.[24] We further assessed the cytotoxicity of HATS in 3D cultured cells by testing its mitochondrial health with Mitotracker red-FM.[25] No change in fluorescence intensity of MitoTracker-FM was noted (Figure 3b), indicating that the HATS did not disrupt mitochondria health. After testing the biocompatibility of HATS in 3D cells, PCAF acetylation was monitored as in 2D cells. Consistent with our results in 2D cell cultures, after treatment with AA, the signal of HATS decreased (Figure 3c). Quantitative analysis of FLIM images (Figure 3d) further indicated that the lifetime of HATS (orange) decreased after treatment with AA, while the nontreated control (green) remained the same.

We have successfully developed a peptide biosensor based FLIM strategy to monitor in real-time acetyltransferase enzyme activity in vitro as well as in 2D and 3D cultures at the single cell level. Unlike other techniques which involve a genetically expressed sensor, our strategy is relatively easier to implement in most cells including primary cells or embryonic cells that are notoriously difficult for transfection. Our FLIM detection is also less prone to photobleaching compared to fluorescence intensity based methods because the laser intensity used in our studies is only 4 μW. Our approach provides dynamic information with single cell resolution allowing the interrogation of key acetylation events in a live cell context to explore epigenetic regulation and drug effect in cancer or other diseases. The methods developed can be translated to study acetylation in a multiplex format by expanding the number of acetyltransferase sensors with appropriate fluorophore reporters with lifetime in a different dynamic range.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssensors.8b01134.

Peptide characterization, immunofluoroescence staining, Western blot, detailed methods, and instrumentation (PDF)

DOI: 10.1021/acssensors.8b01134
ACS Sens. XXXX, XXX, XXX−XXX

AUTHOR INFORMATION

Corresponding Author

*E-mail: jirudaya@illinois.edu.

ORCID

Joseph M. K. Irudayaraj: 0000-0002-0630-1520

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

The authors acknowledge the grant from W. M. Keck Foundation to J.I. J.I. gratefully acknowledges funding in part from the Purdue University Center for Cancer Research, NIH Grant P30CA023168 and the Indiana CTSI Grant 358 UL1TR001108 from NIH.

REFERENCES

(1) Love, I.; Sekaric, P.; Shi, D.; Grossman, S.; Androphy, E. The histone acetyltransferase PCAF regulates p21 transcription through stress-induced acetylation of histone H3. *Cell Cycle* **2012**, *11* (13), 2458−2466.

(2) Sadoul, K.; Wang, J.; Diagouraga, B.; Khochbin, S. The Tale of Protein Lysine Acetylation in the Cytoplasm. *J. Biomed. Biotechnol.* **2011**, *2011*, 970382.

(3) Pickard, A.; Wong, P.-P.; McCance, D. J. Acetylation of Rb by PCAF is required for nuclear localization and keratinocyte differentiation. *J. Cell Sci.* **2010**, *123* (21), 3718−3726.

(4) Bosic, M. M.; Brasanac, D. C.; Stojkovic-Filipovic, J. M.; Zaletel, I. V.; Gardner, J. M.; Cirovic, S. L. Expression of p300 and p300/CBP associated factor (PCAF) in actinic keratosis and squamous cell carcinoma of the skin. *Exp. Mol. Pathol.* **2016**, *100* (3), 378−385.

(5) Spilianakis, C.; Papamatheakis, J.; Kretsovali, A. Acetylation by PCAF Enhances CIITA Nuclear Accumulationand Transactivation of Major Histocompatibility Complex Class II Genes. *Mol. Cell. Biol.* **2000**, *20* (22), 8489−8498.

(6) Blanco, J. C. G.; Minucci, S.; Lu, J.; Yang, X.-J.; Walker, K. K.; Chen, H.; Evans, R. M.; Nakatani, Y.; Ozato, K. The histone acetylase PCAF is a nuclear receptor coactivator. *Genes Dev.* **1998**, *12* (11), 1638−1651.

(7) Blanco-Garcia, N.; Asensio-Juan, E.; de la Cruz, X.; Martinez-Balbas, M. A. Autoacetylation regulates P/CAF nuclear localization. *J. Biol. Chem.* **2009**, *284* (3), 1343−52.

(8) Sasaki, K.; Ito, T.; Nishino, N.; Khochbin, S.; Yoshida, M. Real-time imaging of histone H4 hyperacetylation in living cells. *Proc. Natl. Acad. Sci. U. S. A.* **2009**, *106* (38), 16257−16262.

(9) Sasaki, K.; Yoshida, M. Genetically encoded FRET indicators for live-cell imaging of histone acetylation. *Methods Mol. Biol. (N. Y., NY, U. S.)* **2014**, *1071*, 151−61.

(10) Vidi, P.-A.; Chen, J.; Irudayaraj, J. M. K.; Watts, V. J. Adenosine A(2A) receptors assemble into higher-order oligomers at the plasma membrane. *FEBS Lett.* **2008**, *582* (29), 3985−3990.

(11) Chen, J.; Miller, A.; Kirchmaier, A. L.; Irudayaraj, J. M. K. Single-molecule tools elucidate H2A.Z nucleosome composition. *J. Cell Sci.* **2012**, *125* (12), 2954−2964.

(12) Chen, Y.; Damayanti, N. P.; Irudayaraj, J.; Dunn, K.; Zhou, F. C. Diversity of two forms of DNA methylation in the brain. *Front. Genet.* **2014**, *5*, 46.

(13) Damayanti, N.; Parker, L.; Irudayaraj, J. Fluorescence Lifetime Imaging of Biosensor Peptide Phosphorylation in Single Live Cells. *Angew. Chem., Int. Ed.* **2013**, *52* (14), 3931−3934.

(14) Damayanti, N. P.; Buno, K.; Cui, Y.; Voytik-Harbin, S. L.; Pili, R.; Freeman, J.; Irudayaraj, J. M. K. Real-Time Multiplex Kinase Phosphorylation Sensors in Living Cells. *ACS Sensors* **2017**, *2*, 1225.

(15) Damayanti, N. P.; Buno, K.; Narayanan, N.; Voytik Harbin, S. L.; Deng, M.; Irudayaraj, J. M. K. Monitoring focal adhesion kinase phosphorylation dynamics in live cells. *Analyst* **2017**, *142* (15), 2713−2716.

(16) Zeng, L.; Zhou, M. M. Bromodomain: an acetyl-lysine binding domain. *FEBS Lett.* **2002**, *513* (1), 124−8.

(17) Damayanti, N.; Craig, A.; Irudayaraj, J. A hybrid FLIM-elastic net platform for label free profiling of breast cancer. *Analyst* **2013**, *138* (23), 7127−7134.

(18) González-Vera, A. J.; Morris, C. M. Fluorescent Reporters and Biosensors for Probing the Dynamic Behavior of Protein Kinases. *Proteomes* **2015**, *3* (4), 369.

(19) Rao-Bindal, K.; Koshkina, N. V.; Stewart, J.; Kleinerman, E. S. The Histone Deacetylase Inhibitor, MS-275 (Entinostat), Down-regulates c-FLIP, Sensitizes Osteosarcoma Cells to FasL, and Induces the Regression of Osteosarcoma Lung Metastases. *Curr. Cancer Drug Targets* **2013**, *13* (4), 411−422.

(20) Kusio-Kobialka, M.; Dudka-Ruszkowska, W.; Ghizzoni, M.; Dekker, F. J.; Piwocka. Inhibition of PCAF by anacardic acid derivative leads to apoptosis and breaks resistance to DNA damage in BCR-ABL-expressing cells. *Anti-Cancer Agents Med. Chem.* **2013**, *13* (5), 762−7.

(21) Harma, V.; Virtanen, J.; Makela, R.; Happonen, A.; Mpindi, J. P.; Knuuttila, M.; Kohonen, P.; Lotjonen, J.; Kallioniemi, O.; Nees, M. A comprehensive panel of three- dimensional models for studies of prostate cancer growth, invasion and drug responses. *PLoS One* **2010**, *5* (5), No. e10431.

(22) Gao, D.; Vela, I.; Sboner, A.; Iaquinta, P. J.; Karthaus, W. R.; Gopalan, A.; Dowling, C.; Wanjala, J. N.; Undvall, E.; Arora, V. K.; Wongvipat, J.; Kossai, M.; Ramazanoglu, S.; Barboza, L. P.; Di, W.; Cao, Z.; Zhang, Qi F.; Sirota, I.; Ran, L.; MacDonald, T. Y.; Beltran, H.; Mosquera, J.-M.; Touijer, K. A.; Scardino, P. T.; Laudone, V. P.; Curtis, K. R.; Rathkopf, D. E.; Morris, M. J.; Danila, D. C.; Slovin, S. F.; Solomon, S. B.; Eastham, J. A.; Chi, P.; Carver, B.; Rubin, M. A.; Scher, H. I.; Clevers, H.; Sawyers, C. L.; Chen, Y. Organoid Cultures Derived from Patients with Advanced Prostate Cancer. *Cell* **2014**, *159* (1), 176−187.

(23) Moussy, F.; Reichert, W. M. Biomaterials community examines biosensor biocompatibility. *Diabetes Technol. Ther.* **2000**, *2* (3), 473−7.

(24) Jena, N. R. DNA damage by reactive species: Mechanisms, mutation and repair. *J. Biosci.* **2012**, *37* (3), 503−17.

(25) Wirbisky, S. E.; Damayanti, N. P.; Mahapatra, C. T.; Sepúlveda, M. S.; Irudayaraj, J.; Freeman, J. L. Mitochondrial Dysfunction, Disruption of F-Actin Polymerization, and Transcriptomic Alterations in Zebrafish Larvae Exposed to Trichloroethylene. *Chem. Res. Toxicol.* **2016**, *29* (2), 169−179.

DOI: 10.1021/acssensors.8b01134
ACS Sens. XXXX, XXX, XXX−XXX