Label-Free *in Situ* pH Monitoring in a Single Living Cell Using an Optical Nanoprobe

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Abstract

Intracellular pH plays critical roles in cell and tissue functions during processes such as metabolism, proliferation, apoptosis, ion transportation, endocytosis, muscle contraction and so on. It is thus an important biomarker that can readily be used to monitor the physiological status of a cell. Thus, disrupted intracellular pH may serve as an early indicator of cell dysfunction and deterioration. Various methods have been developed to detect cellular pH, such as pH-sensitive labeling reagents with fluorescent or Raman signals. However, excessive cellular uptake of these reagents will not only disrupt cell viability but also compromise effective long-term monitoring. Here, we present a novel fiber-optic fluorescent nanoprobe with a high spatial resolution for label-free, subcellular pH sensing. The probe has a fast response time (~20 seconds) with minimum invasiveness and excellent pH resolution (0.02 pH units) within a biologically relevant pH environment ranging from 6.17 to 8.11. Its applicability was demonstrated on cultured A549 lung cancer cells, and its efficacy was further testified in two typical cytotoxic cases using carbonylcyanide 3-chlorophenyl hydrazine, titanium dioxide, and nanoparticles. The probe can readily detect the pH variations among cells under toxin/nanoparticles administration, enabling...
direct monitoring of the early onset of physiological or pathological events with high spatiotemporal resolution. This platform has excellent promise as a minimum invasive diagnostic tool for pH-related cellular mechanism studies, such as inflammation, cytotoxicity, drug resistance, carcinogenesis, stem cell differentiation and so on.

Graphical Abstract

Keywords

optical nanoprobe; intracellular pH; single-cell label-free subcellular sensing; high spatiotemporal resolution; minimum invasiveness

INTRODUCTION

Eukaryotic cell establishes and stringently maintains distinct transmembrane pH levels in a dynamic way (Casey, Grinstein, & Orlowski, 2010). These molecular-level protonation-deprotonation events are crucial for many metabolic and physiological processes (Whitten & Hilser, 2005). For instance, the cross-membrane proton-motive force ($\psi_{\text{H}^+}$) takes part in the cellular energy generation and conversion processes via ATP production through inner membrane H\(^+\)-ATP synthase ($F_1F_0$-ATPase) (Casey et al., 2010). The endocytosis and phagocytosis are also controlled by cellular pH, where a gradual luminal acidification process takes place from high pH endocytic vesicles to low pH lysosomes. Such luminal acidification is also in charge of routine membrane trafficking between endocytic sub-compartments (Weisz, 2003). A large majority of cellular behaviors, including proliferation (Pouyssegur, Franchi, L’allemain, & Paris, 1985), migration (Denker & Barber, 2002; Meima, Mackley, & Barber, 2007), apoptosis (Schelling & Jawdeh, 2008), as well as tissue scale events, such as muscle contraction, are being influenced or initiated by cellular pH. Where in the latter case, extracellular protons act as neurotransmitters on muscle cells at the proton-gated cation channels (Beg, Ernstrom, Nix, Davis, & Jorgensen, 2008).

On the other hand, disrupted homeostasis of cytosolic pH usually associates with the occurrence or progression of cellular dysfunction, or pathophysiologic status. For instance, ischemia-induced hypoxia in tissues and organs results in cellular level anaerobic metabolism and the production of an excessive number of protons with accumulated lactate
and pyruvate, which in turn induces hyperactive Na\(^{+}\)-H\(^{+}\) exchanger 1 (NHE1), followed by Na\(^{+}\) overload and Ca\(^{2+}\) influx. These events then induce a cascade of deleterious phenomena, such as altered membrane excitability/contractility, generation of toxic free-radicals, cellular hypertrophy, apoptosis and necrosis (Casey et al., 2010; Obara, Szeliga, & Albrecht, 2008; Vaughan-Jones, Spitzer, & Swietach, 2009). An extreme case of pH disruption is cancerous cells, whose pH appearance is highly twisted to optimize their progression through tissues with normal pHs (Swietach, Vaughan-Jones, Harris, & Hulikova, 2014). Extracellular acidification of cancerous cells supports their migration and protects them against alkaline drugs such as doxorubicin (Gerweck, Kozin, & Stocks, 1999; Raghunand et al., 1999). This prominent role of pH in cancer progression attracts increasing interest in developing new therapeutic innovations (Swietach et al., 2014; Webb, Chimenti, Jacobson, & Barber, 2011). Attempts to target pH-handling proteins using low molecular weight inhibitors or monoclonal antibodies have been developed recently (Fais, De Milito, You, & Qin, 2007; Le Floch et al., 2011; Neri & Supuran, 2011). To this end, we learn that the early-stage occurrence or disruption of cellular acidification may well serve as an upstream hallmark for the prediction of late-stage cellular catastrophe.

Though effective ways to detect biosystems at quasi- or real single-cell levels have long been pursued (as summarized in Supplementary Table 1), including considerable efforts that focused on pH measurement, such as those methods based on: absorbance (De Meyer, Hemelsoet, Van Speybroeck, & De Clerck, 2014; Han & Burgess, 2009), fluorescence (Sijie Chen et al., 2013; Han & Burgess, 2009; Orte, Alvarez-Pez, & Ruedas-Rama, 2013; Shirmanova et al., 2015), nuclear magnetic resonance (NMR) (Soto, Zhu, Evelhoch, & Ackerman, 1996; Yu, Cui, Bourke, & Mason, 2012), surface-enhanced Raman spectroscopy (SERS) (Fazio et al., 2016; Kneipp, Kneipp, Wittig, & Kneipp, 2010; Pallaoro, Braun, Reich, & Moskovits, 2010), Förster Resonance Energy Transfer (FRET) (Dennis, Rhee, Sotto, Dublin, & Bao, 2012; Suzuki, Husimi, Komatsu, Suzuki, & Douglas, 2008), and modified quantum dots (Dennis et al., 2012; Medintz et al., 2010). However, most of these methods require intracellular labeling to enable in situ visualization, which always involving cytosolic uptake or accumulation of excessive amount of dye molecules, radioactive indicators (Soto et al., 1996; Yu et al., 2012), gold or silver substrates for SERS (Fazio et al., 2016; Kneipp et al., 2010; Pallaoro et al., 2010), or quantum dots (Dennis et al., 2012; Suzuki et al., 2008). These indicators are exogenous, mostly toxic (Alford et al., 2009; Boisselier & Astruc, 2009; Derfus, Chan, & Bhatia, 2004; Han, Han, & Tung, 2013; Hauck, Anderson, Fischer, Newbigging, & Chan, 2010; Levard, Hotze, Lowry, & Brown Jr, 2012), modified as membrane-impermeable once entered the cell, thus hard to be expelled or digested by cells (Alford et al., 2009; Boisselier & Astruc, 2009; Derfus et al., 2004; Han et al., 2013; Hauck et al., 2010; Levard et al., 2012). The resulted long-term co-existence of these materials with intracellular organelles may alter the original intra-/extra-cellular conditions, induce cell deterioration, and finally impair the capability of these materials as effective pH indicators.

Recently, we have innovated a basic version 1-in-6 fiber optic single cell pH probe, and preliminarily showed its capability in detecting different cellular pHs between single live cells (Yang, Wang, Chen, et al., 2015). In the present work, a newly developed fiber-optic fluorescent nanoprobe based on our previous inventions (Sisi Chen, Yang, Xiao, Shi, & Ma,
2017; Cheng et al., 2014; Lan et al., 2014; Yang, Wang, Chen, et al., 2015; Yang, Wang, Lan, et al., 2015; Yang et al., 2014) is developed. This novel probe allows the measuring of intracellular pH with a high spatiotemporal resolution with, up till now, the lowest invasiveness. The 8-hydroxypyrene-1, 3, 6-trisulfonate (HPTS) pH dye with high photostability was adopted to embed into an organically modified silicate (OrMoSil) nanolayer network on top of the sensing tip to support pH indication. The probe’s functionality was validated by measuring individual human lung cancer A549 cells. The probe’s applicability was then demonstrated via cell monitoring firstly under normal and then toxic conditions (titanium dioxide (TiO$_2$) nanoparticles (NPs) and carbonylcyanide 3-chlorophenyl hydrazine (CCCP)). The developed nanoprobe may thus establish a potential method to detect or predict early-stage cell deterioration observed in many cytotoxic and pathological phenomena.

**MATERIALS AND METHODS**

1. **Chemicals**

Fetal bovine serum (FBS) was purchased from American Type Culture Collection (ATCC) (Manassas, VA). Ham’s F-12K medium with added L-glutamine was purchased from Fisher Scientific (Pittsburgh, PA). Trypsin–EDTA (ethylenediaminetetraacetic acid) (0.25%), and 0.1 M phosphate-buffered saline (PBS) were purchased from Life Technology Co. (Carlsbad, CA). Ultra-pure water was generated with a Milli-Q system (Millipore, Bedford, MA). The cetyltrimethylammonium bromide (CTAB), ethyltriethoxysilane (ETEOS), (3-glycidoxypropyl) trimethoxysilane (GPTMS), mitochondria inner membrane potential depolarization reagent CCCP, TiO$_2$ NPs at 99.0% purity, pH-sensitive dye HPTS, and mitochondrial potential indicator dye 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) (Perelman et al., 2012) were purchased from Sigma-Aldrich (Saint Louis, MO).

2. **Single-cell pH micro-probe fabrication and validation**

A novel optical microprobe with a hexagonal 1-in-6 fiber configuration (Figure 1 and 2, Figure S1) was fabricated as described previously with minor modifications. (Sisi Chen et al., 2017; Yang, Wang, Chen, et al., 2015; Yang, Wang, Lan, et al., 2015) Briefly, single-mode optical fiber (SMF-28, Corning Inc., NY) was used and the probe was fabricated by using a home-built coaxial-twisting and gravitational-stretching system (Figure S1) (Yang, Wang, Chen, et al., 2015). Once the 1-in-6 fiber taper stem was fabricated, a uniform gold (Au)/palladium (Pd) layer with an approximate thickness of 350 – 450 nm was sputter-coated, to optically isolate the probe tip from environmental influences. Then focused ion beam (FIBs, Helios Nanolab 600, FEI) milling was applied to remove the very tip (< 5 μm) Au/Pd layer. Immediately the tip was subjected to an organic-modified silicate (OrMoSil) sol-gel coating to introduce the embedded pH-sensing HPTS dye (ex. 488nm/em. 525 nm). The featured small sensing tip and the designed 1-in-6 fiber configuration ensured a high spatial resolution detection and highly efficient emission-collection. All fabricated probes were validated through measuring a series of standard pH buffer solutions with known pH (Figure 2a), which have been pre-confirmed by an Accumet AB15 Plus pH meter (Thermo Fisher Scientific Inc., MA).
3. **Cell culture and NPs dosing**

The human bronchoalveolar carcinoma-derived A549 cell line was purchased from ATCC (CCL-185) (Manassas, VA) and used as an in vitro cytotoxicity model in this study. This cell line has been widely used in particulate matter-related pulmonary toxicity studies (Oberdörster, Oberdörster, & Oberdörster, 2005; Oberdörster, 2000; Ramakrishna & Ghosh, 2003) and was used in our previous work to demonstrate irradiation-enhanced ZnO NP cytotoxicity (Yang, Lin, Burton, Park, & Ma, 2016; Yang & Ma, 2014). Cells were maintained in Ham’s F-12K medium supplemented with 10% FBS, 100 units per mL penicillin, and 100 μg mL$^{-1}$ streptomycin and grown at 37 °C in a 5% carbon dioxide (CO$_2$) humidified environment. In each test, cells were seeded and allowed to attach for 18 hours (overnight) before microprobe testing or NPs exposure. A cell density of $1 \times 10^5$ per milliliter was used. Cells without NPs exposure were used as the control in each experiment. All NPs suspensions were freshly made each time with designed dosages in serum-free F-12K culture medium and subjected to 15 minutes ultrasonication. Pre-seeded cells will be washed by PBS three times and co-cultured with NPs suspensions. Cells used as control will also be washed by PBS and continuous cultured using a serum-free F-12K medium. The use of serum-free medium constantly in NP-dosing experiments was to avoid nanocytotoxicity controversy that may be caused by the formation of protein corona outside NPs. (Mortensen et al., 2013)

4. **Single-cell pH measuring**

The A549 cell line was cultured and exposed to NPs as described above but in an environmentally controlled chamber that mounted on an Olympus IX51 inverted fluorescent microscope to enable long-term cell observation, manipulation, and measurement. When conducting single-cell probing, appropriate number of cultured cells (10 – 30 cells) were trypsinized and gently washed and picked up using the cell holding probe through applying −1 to −5 mbar negative pressure (as demonstrated in Scheme 1b) followed by intracellular insertion of the pH microprobe at a 180° angle opposite to the holding probe. The probe was kept inside the cytoplasm for approximately 10 – 15 seconds for pH equilibration. Then excitation laser was turned on for 3 – 5 seconds to acquire stable signal accumulation, followed by gentle retracting of the probe out of cell membrane. Spectrometer collected data and time points will then be analyzed and plotted.

5. **Statistical Analysis**

All cell population-based experiments were run in triplicate for statistical validation purposes. However, single-cell probing was not limited under this category due to the intrinsically high heterogeneity between cells. When appropriate, experimental data was normalized against the corresponding control group and has been expressed as the mean ± standard deviation (SD). Statistical analyses were performed using Prism 5 (Graph-Pad Software, CA) including a one-way analysis of variance test (ANOVA) followed by a post hoc Tukey test to determine the statistical significance. Differences were established to have statistical significance at $p < 0.05$ (*).
RESULTS AND DISCUSSION

Scheme 1c shows the architectural view of the nanoprobe design. The exposed inner structure had five hierarchies that were composed of: 1) the centrally positioned fiber for delivery of the excitation laser light, 2) the peripheral six fibers for collection of the fluorescence signals, 3) the Au/Pd sputtered shielding layer for optical isolation along the fiber to avoid ambient light interference, 4) the exposed tip region by trimming off the Au/Pd coating for pH sensing layer coating, and 5) the outer-most tip-only OrMoSil-HPTS coating for pH sensing.

The fabricated nanoprobe will then be used for single-cell pH detection while the cells are alive in the culture medium, as illustrated in Scheme 1a. The sensing principle is briefly described here: firstly, a 488 nm laser beam is free-space-coupled into the central fiber core using a focusing lens and a programmable beam shutter. Secondly, when the diameter of the fiber bundle becomes small enough due to the tapering of the tip structure, the excitation light propagates inside the central fiber core will be evanescently coupled into the surrounding fibers and thus, excites the OrMoSil-HPTS coating at the very tip. The fluorescence signal is then collected by the surrounding six fibers and routed back to the optical spectrum analyzer. The 1-in-6 probe design can effectively separate the fluorescence signals from the excitation signal so that the interference of the excitation light will be significantly minimized. A zoom-in schematic (Scheme 1b) illustrates the insertion of the probe into the cytoplasmic region of a single cell for intracellular pH measurement. A tapered hollow glass tube with the other end connected to a controllable vacuum pump was used to pick up and hold a single cell in position. Under an inverted microscope, the single-cell insertion is thus able to be conducted with the combination of several translational stages. For adherent cells, the nanoprobe will still be applicable for its small size and ease of control.

To fabricate the nanoprobe, briefly, a tapered hexagonal 1-in-6 fiber structure (Figure 1a) was firstly created using a home-built coaxial-twisting and gravitational-stretching system as detailed elsewhere (Yang, Wang, Chen, et al., 2015) (Figure S1). The pre-alignment of the fibers and the balancing of the inner tensile strength between fibers are very important in achieving a rigid taper with the excellent optical performance.

The 1-in-6 fiber taper was then sputter-coated with a uniform Au/Pd layer with approximately 250 – 300 nm in thickness (Figure 1b). This relatively thick coating optically isolates the nanoprobe from the environment along the taper. The Au/Pd coating at the tip of the taper was then removed using the focused ion beams (FIBs, Helios Nanolab 600, FEI) to expose the last 5 μm shaft (Figure 1c). Then, a full-wave simulation using COMSOL was conducted to predict the behavior of the photons at the probe tip (Figure S2, a – d). The simulation results indicate that the excitation light that was originally propagating in the central fiber core, evanescently coupled into the surrounding fibers, as the tapered tip size became smaller (Figure S2, f – i). Thus, it becomes important to assure the fluorescence excitation region is small enough so that the probe will be accountable for the intracellular detection. Our simulation showed that a tip region with a length of about 15 μm to the end is already unable to limit the excitation laser within the central fiber. This result affirmed our
design of the shielding Au/Pd layer to limit the laser-fluorophore contacting-length less than 5 μm at the tip, to realize true intracellular sensing. Then, the dispersive X-ray spectroscopic (EDS) mapping of the FIBs modified taper tip indicated that the sputtered metal layer has been successfully removed and the inner glass core is completely exposed (Figure 1d and Figure S3). Immediate sol-gel dip-coating, aging, and curing procedures were then performed to bond the pH-sensitive OrMoSil-HPTS layer onto the exposed silica surface of the tip (Figure S1h). Figure 1e shows the microscopic fluorescent image of a finalized pH nanoprobe under FITC channel. The pH-sensitive region is well confined at the tip of the probe with a dimension of less than 1μm in diameter and 5 μm in length. This small active region ensures the high spatial resolution needed for intracellular measurement. The use of a 1-in-6 structure could also ensure the high efficiency of the excitation and collection of the weak fluorescence signals, thus maximizing the signal-to-noise ratio.

The pH-sensing ability is achieved by the pH-sensitive HPTS dye (Kermis, Kostov, & Rao, 2003; Lee, Kumar, & Tripathy, 2000; Schulman et al., 1995) that hosted in the OrMoSil coating processed by a hybrid sol-gel method (Figure 1f). The HPTS dye was mixed (1:2 in molar ratio) with CTAB to form a HPTS-CTAB ion pair (IP) complex, and then further mixed with the prepared ETEOS and GPTMS based sols. After curing for 48 hours, a complete dye entrapment within the nano-structured aerogel networks was achieved. The nanostructure of the thin OrMoSil film was tailored to ensure complete encapsulation of the dye-containing ion-pairs without leaching. The HPTS dye has good photo-stability at a pK_a of ~7.30, and its conjugation with CTAB through ion-pairing strengthens the dye immobilization through the formed hydrophobic hairpin. This design avoids unwanted molecular resonance and bathochromic shift, which may occur in the formation of a covalent bond. The use of the partially polar GPTMS and an ETEOS precursor provides a hydrophilic outer matrix in order to promote proton permeability and to support mechanical stability and the inner hydrophobic backbone, respectively (Figure 1f) (Wencel, MacCraith, & McDonagh, 2009). No apparent dye leaking was observed during the protonation and deprotonation process (Figure 1g). The molar ratio of the reactants and catalysts in sol preparation was adjusted to optimize dye loading efficiency and to maintain a balance between the sensing ability and the film stability (Wencel et al., 2009). Once excited by the 488 nm photons, the coating emits ~510 nm fluorescence with its peak intensity varying depending on the surrounding pH.

The fabricated nanoprobe was characterized through SEM/EDS scanning and validated by pH measurements under physiologically relevant conditions. Figure 1h shows the cross-section view of the probe tip prepared by cutting at the position of about 15 μm away from the tip end using FIBs, where seven fibers are well aligned in the 1-in-6 configuration. Figures 1i and 1j are the zoom-in views of the porous nanonetworks of the OrMoSil-HPTS coating. The depth of the coating is estimated to be about 450 nm (Figure 1j) as shown in the yellow square boxes in Figure 1h. EDS scanning showed an increased level of carbon (C), with lower levels of the underneath SiO_2, demonstrating a uniform deposition of the OrMoSil-HPTS layer onto the probe surface.

The pH nanoprobe was calibrated by measuring a series of standard buffer solutions. Figure 2a shows the raw fluorescence spectra of the probe. The fluorescence emission intensity
decreased as the pH values increased. An iterated curve-fitting (OriginLab) was conducted to fine-tune the result (Figure S4j). The emission spectral peak areas (Figure 2b, inset: peak area covered between 492 and 640 nm in the spectrum plot) under individual pH testing conditions were used to correlate with the respectively applied pHs. A good linear correlation ($R^2 = 0.9895$) was observed (Figure 2b) within the biologically relevant pH range from 6.17 to 8.11. The sensitivity of the nanoprobe was then defined as the change in the fluorescent peak area per pH unit ($\Delta A_{peak}/U_{pH}$). Our results showed an average of $\Delta A_{peak}/U_{pH} = 14,867$ a.u. within a biologically relevant pH that ranged from 6.17 to 8.11. Thus, the pH resolution was calculated based on the previously established method (Yang, Wang, Chen, et al., 2015) with an average value of 0.06 pH unit. Due to a relatively higher sensitivity near the neutral pH region (~pH7.0 – pH 7.3) (Yang, Wang, Chen, et al., 2015), an optimal pH resolution within that range can reach up to ~0.02 pH unit.

Besides, four biologically relevant interferences, ionic strength, temperature, glucose and protein (bovine serum albumin, BSA) concentrations, were examined to evaluate the probe’s applicability under realistic cellular matrix conditions (Figure 2, d – g). The ionic strength and temperature variations were found to correlate slightly with the probe measurement results within a physiologically relevant range. The ionic strength variation of 0.06 M caused a maximum difference of $\rho_{ionic} = 0.0873$ pH unit. The temperature variation of 3 °C produced a maximum difference of $\rho_{temp} = 0.0629$ pH unit (Figure 2, d and e) (Mouat & Manchester, 1998). The glucose and BSA interferences on the probe were too small to observe (Figure 2f, 2g). These test data demonstrated the reliability of the probe for pH sensing in a realistic physiological niche environment. In addition, a potential photobleaching effect was examined by casting a laser beam onto a glass coverslip coated with the same aerogel layer and evaluated using time-lapse microscopic imaging (Figure S5). Gradual but slow fluorescence total intensity attenuation was observed, which resulted in a ~10% decrease of the original level after 900 seconds, and ~20% after 1,500 seconds. Such photostability may thus be supportive of the realistic applications without concerns of fast photobleaching.

Next, the developed nanoprobe was tested for sensing intracellular pH by using the human lung carcinoma A549 cells (Figure 3). Figure 3a shows an exemplary microscopic image of probe insertion into an alive cell. Mild insertions were carefully conducted into the peripheral cytoplasmic areas to reduce adverse effects on the cell (Figure 3a). The dark-field images show the DAPI stained cell nucleus (blue) and the cell boundaries (yellow dash line), while the inset shows the inserted probe tip inside the cell with a tiny lightening spot under the FITC channel (Figure 3b). The probe tip only sensed a 3 – 5 μm depth region in the cytosolic part, indicating a high spatial resolution of the probe for intracellular measurement. It took 10 – 15 seconds in dark to equilibrate the probe within the cell, then the laser was turned on and the signal was recorded immediately, followed by the shutting down of the laser. As such, the probe’s intracellular positioning and sensing process can be well-controlled, and a high spatiotemporal sensing resolution was achieved at a couple of micrometers depth beneath the cell membrane within 20 seconds of total measurement time. Figure S6 and video clip 1 show more details of the cell capturing, inserting, sensing, and releasing processes.
Cell insertion, though, as an invasive method, has drawn major concerns regarding cell sustainability and viability. In our case, only mild and shallow insertion and quick detection were performed to enable the minimum level of invasiveness. The small size tip will typically create a membrane entrance with a diameter less than 1 μm, thus no noticeable cytoplasmic membrane abruption was observed after probe retreating. Figure S6g shows the detected intracellular pH values among 15 randomly selected A549 single cells. The measured pHs mostly fell into the range of pH 6.8 – pH 7.6, which agreed with the generally reported intracellular values. However, individual cell heterogeneity does exist. Our probe recorded two extraordinary low (6.34) and high (8.10) cellular pH levels, though all cells were cultured under identical conditions. The unprecedentedly revealing of such divergence provides significant pieces of evidence for future single-cell studies such as stem and precursor cell differentiation and/or carcinogenesis.

Our probe’s ability in high spatiotemporal resolution detection, with continuous monitoring capability, makes it a perfect tool to discover subtle changes within a single cell before the occurrence of mass incidents, such as the early onset of cell deterioration caused by toxic chemicals or materials. Here, CCCP and 40 nm TiO$_2$ NPs were used separately to create two typical cytotoxic cases. Firstly, mitochondria are known for their ability to control energy metabolism and they are crucial for many cellular events in response to the surrounding stimuli (Huss & Kelly, 2005). Interrupting the mitochondrial function using inhibitors, such as CCCP, will directly damage cellular energy consumption and may even cause cell death (Lin & Beal, 2006; Melser, Lavie, & Bénard, 2015; Salminen et al., 2015; Wallace, 2012). Here, a relatively mild dose (50 nM) of CCCP ($IC_{50} = 8 – 10 \mu M$) was applied to depolarize the mitochondrial inner membrane potential ($\Delta \psi_m$), and thus defunctionalize the organelles (Park, Kang, & Bae, 2015). Cell status was closely monitored using: 1) a $\Delta \psi_m$ sensitive dye J-aggregate-forming lipophilic cation (JC-1) (Reers et al., 1994; Smiley et al., 1991), and 2) our pH nanoprobe. At first glance, the fluorescent raw images of the JC-1-stained cells showed differences between control and CCCP-treated cell groups (Figure S7). However, after quantification based on the total fluorescent intensity ratio of Cy3/FITC (580nm/530nm) using ImageJ software (NIH), the latter group did not show significant $\Delta \psi_m$ decrease (Figure 3c, Figure S8) within the first 120 minutes. Comparatively, by accurately monitoring 10 individual cells (5 control and 5 CCCP-treated) by our nanoprobe, we found rapid cellular pH decrease only 40 minutes after CCCP-treatment, with an averaged intracellular pH decreasing of ~2.2 pH units (Figure 3d). Prolonged monitoring of the JC-1 stained group did not show apparent $\Delta \psi_m$ damage until 3 hours later. These results demonstrated an outstanding capability of our probe in detecting the early onset of cellular events. The recorded CCCP-related pH change is in agreement with previously reported stringent connection between intracellular pH and mitochondria (Khaled, Kim, Hofmeister, Muegge, & Durum, 1999; Lagadic-Gossmann, Huc, & Lecureur, 2004; Liu et al., 2000; Orij, Postmus, Ter Beek, Brul, & Smits, 2009), which suggests that the intracellular pH may serve as a specific early-stage indicator of mitochondrial dysfunction and cell deterioration.

The recent development of nanoparticles has drawn many concerns on the applications and regulations of such materials. Thus, we took a type of widely adopted TiO$_2$ NP as an example to testify the applicability of our probe in monitoring its cytotoxic effect, which may have long been underestimated. Again, single-cell pH was constantly monitored using
our probe for twelve hours with or without exposure to 100 μg/mL, 40 nm TiO$_2$ NPs (Figure 3e). The results showed an immediate tendency of decrease in the pH of NP-treated cells, while the control group remained relatively unchanged. Significant pH variation ($p < 0.05$) was detected between the NP-applied and control groups right after 2 hours of NP-exposure, which may require a longer time for other cell population-based methods to display such distinct change. These preliminary but crucial results made our probe a strong candidate for precise monitoring of early onset of cytotoxic events on an individual cell resolution, and a valuable technique for long-term cell status monitoring, with minimum invasiveness.

CONCLUSIONS

A novel fiber-optic pH nanoprobe was developed to measure pHs within a single alive cell. A featured “tip-modified” design allowed us to measure intracellular pH accurately with high spatial-resolution and minimum invasiveness, which has long been pursued in the single-cell research field. The probe has a pH sensitivity of about 0.06 pH unit in the range 6.17 – 8.11, making it feasible to track subtle cellular pH variations under a practical culturing environment. The probe also has a fast equilibrating and data acquisition processes, enabling a near real-time pH monitoring capability. The probe was primarily proven to be useful for prolonged intracellular sensing over 10 hours. In sum, this pH nanoprobe provides a novel way of stain-free, in situ, and high spatiotemporal resolution pH monitoring on a single living cell, which unprecedentedly enabled the investigation of early-stage physiological variations via subtle cytoplasmic pH fluctuation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- A novel fiber-optic fluorescent nanoprobe was developed for subcellular pH sensing
- High pH resolution (~0.02 pH unit) was achieved in cell-relevant pH environment
- High spatial resolution and label-free detection were also achieved with this probe
- Successfully revealed the heterogeneity of cultured A549 cells via pH measuring
- Revealed early cell deterioration that induced by toxic chemical and nanoparticles
**Figure 1. Fabrication and validation of the μ-pH probe.**

(a) An actual 1-in-6 probe shaft imaged in the bright-field channel under an inverted microscope. The tip part of the probe circled in the red square is shown in (b) after a thick sputter coating of Au and Pd. (c) An exemplary probe tip after FIBs milling (cutting) of the first 5 μm sputter-coated Au/Pd layer to expose the underneath SiO$_2$ surface. (d) EDS mapping of the “tip-modified” area to show the exposed Si underneath after the sputter-coated Au was milled off by FIBs. A fully fabricated probe with OrMoSil-dye coating is then shown under an inverted microscope in (e) under FITC channel. (f) Sketch showing the ion pair (IP) formation of the HPTS-CTAB conjugates, as well as the basic formula of the hybrid OrMoSil sol-gel for probe dip-coating. (g) Schematic representation of the pH-sensing principle of the porous OrMoSil sensing layer with an entrapped HPTS-IP complex. (h) A SEM image of the cross-section of the probe (positioned 15 μm away from the tip-end) indicates a well-maintained hexagonal configuration until the very end of the probe. (i) A zoom-in view of the porous nanostructure of the OrMoSil-dye coated probe surface (the right yellow square box in h). The comparison of EDS elemental scanning between h and i illustrate a substantial distribution of carbon on the outer probe layer, demonstrating a successful aerogel deposition. (j) A zoom-in view of the left yellow square box in h shows an averaged ~450 nm thickness of the coated OrMoSil-dye sensing layer. Scale bar: b, 6 μm; c and d, 10 μm; e, 50 μm; h, 5 μm; i and j, 1 μm. Objects in f and g are not drawn to scale.
Figure 2.
(a) Detected fluorescent spectra by the probe using standardized gradient pH buffer solutions. (b) Linear correlation between standardized pHs and the signal peak-area covers a wavelength that ranged from 492 nm to 640 nm. The usability of the pH probe was further validated using different solutions or conditions: (d) ionic strength (sodium chloride), (e) temperature, (f) glucose concentration and (g) BSA concentration, adjusted under the same pH and physiological-relevant conditions.
Figure 3. Early-stage cell deterioration illustrations by in situ single cell pH sensing. One exemplary single A549 cell capturing and intracellular probing process is shown under bright-field (a) and fluorescent channels (b). Merged fluorescent images were taken under DAPI (cell nucleus) and FITC (HPTS dye fluorescence) channels. The inset zoom-in views of the probe tip lightening area demonstrate a tiny sensing spot within the cytoplasmic region. (c) Images were calculated, and 3-D plotted using ImageJ software based on the fluorescent intensity ratio of Cy3/FITC and were finally averaged and plotted vs. time. (d) Intracellular pH real-time measurement for five single A549 cells treated by the same
CCCP-toxication condition, as in c, and compared with non-treated cells. (e) Single-cell real-time monitoring of intracellular pH during cell exposure to 100 μg/mL, 40 nm TiO₂ NPs for 12 hours. Red lines are CCCP-treated cells while blue lines represent controls. Scale bars: a - b, 10 μm.
Scheme 1.
Schematics of system setup and single-cell pH nanoprobe design. (a) A schematic illustration of the single-cell pH probing system assisted with an inverted epifluorescence microscope. A continuous-wave Ar-ion laser was used and optically coupled into the central fiber core for excitation, and the surrounding six fibers were connected with a portable spectrometer (USB2000, Ocean Optics). Accurate single cell insertion was achieved by a home-built oil-pressured single-cell capturing device and a 3-D nanoprobe manipulation platform. (b) A schematic zoom-in view of a single cell probing device. A cell holding probe assists with single-cell capturing and probe insertion. (c) Schematic of the pH probe head structure and the sensing principle. The probe consisted of a bundle of highly tapered, seven hexagonally configured optical multimode fibers, using a coaxial-twisting and gravitational-stretching system. An Au/Pd shielding layer was deposited onto the peripheral six fibers with a tip trimming-off region (≤5 μm), which was finally covered by the OrMoSil-dye sensing layer. The excitation laser was introduced through the central fiber, and the peripheral six fibers only collected fluorescent signals at the non-shielded region.