Biological activities investigated by single cell analysis at nanoscales

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**BIOLOGICAL ACTIVITIES INVESTIGATED BY SINGLE
CELL ANALYSIS AT NANOSCALES**

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Summary

Single cell analysis is required to decipher a myriad of cellular processes; however, it is very challenging to develop a single cell analysis method with high sensitivity, excellent selectivity and high spatiotemporal resolution. This PhD research project focuses on the innovation of a unique optical and electrical single cell sensing platform to understand the physico-chemical fundamentals involved in the detection schemes and to investigate physiological processes in single cells for cell biology studies as well as disease diagnosis.

An optical fiber based nanobiosensor has been first constructed to detect the over-expression of a general cancer biomarker, telomerases in single cancer cells. The antibody-immobilized nanoprobe inserted into MCF-7 cell nucleus shows significantly higher average $(F-F_0)/F_0$ ratio than that of the negative control human mesenchymal stem cell (hMSC) nucleus. The average $(F-F_0)/F_0$ ratio in the MCF-7 cytoplasm is significantly smaller than that in the nucleus to clearly verify the nuclear localization of telomerase. The nanobiosensor may provide a potential method for cancer detection, and also demonstrate a universal approach to detect other low expression proteins in a single living cell. To study tumor metabolism at single cell level, a unique nanoscale optical fiber lactate sensor has been developed to successfully distinguish the higher extracellular lactate concentrations of cancer cells from that of normal cells, which supports Warburg hypothesis. Furthermore, lactate efflux inhibition profiles after exposure to a monocarboxylate transporter (MCT) inhibitor are different for HeLa and MCF-7 cancer cells demonstrating the nanosensor's potential to evaluate the effect of metabolic agents on cancer metabolism and survival.

A bifunctional electro-optical nanoprobe with integrated nanoring electrode and optical nanotip has also been designed to simultaneously detect both electrical and optical signals in real-time with high spatial resolution. Concurrent measurements of the oxidant generation and the intracellular antioxidant levels in single cells correlate the stronger oxidant generation with an altered initial antioxidant response in the cancer cells suggesting that cell malignancy is associated with the strength of oxidative stress. This method is promising in studying the dynamics of physiological processes, and also provides an opportunity to unravel the interplay of various signalling pathways.

Furthermore, we have investigated the subcellular location-dependent biochemical responses of a potent anticancer drug, β -lapachone (β -lap) by a reduced graphene oxide (rGO) functionalized optical nanoprobe that can deliver and simultaneously monitor the drug effect at nanoscales. For the first time, distinct oxidative responses and calcium alterations in three selected subcellular domains are discovered and clearly pinpoint that the perinuclear region as the subcellular site for β -lap to have the best anticancer efficacy. The results provide not only scientific insights of subcellular drug-cell interaction unobtainable from conventional methods but also valuable knowledge for rational design of subcellular targeted delivery.

This research project develops a nanobiosensor technology to analyze single cells with high spatiotemporal resolutions for revealing critical information on cell signalling and biomarker expression. The success of this project not only results in a sensitive single cell detection platform, but also provides new scientific insights in cellular processes to aid in disease diagnosis.

Chapter 1 Introduction

1.1 Background

The cell is the fundamental unit of life, and cellular heterogeneities exist among individual cells in the same population. Cells may differ in their chemical compositions, proliferation states or the dynamic responses to external stimuli. For example, tumor cells are often composed of mixtures of both clonally transformed cancer cells and other cell types. Conventional biological assays analyze millions of cells and only report the population averaged results. In contrast, single cell analysis can reveal the differences between individual cells to determine the actual distribution of cellular characteristics or responses, providing deep insights into the functions of cellular machinery.¹ In this way, cells can be classified with high resolution and rare cells may be identified. The high sensitivity of single cell analytical techniques is especially important to analyze cell types with limited number of passages such as zygotes, stem cells or primary cells.² Therefore, single cell analysis is crucial to unravel a myriad of cellular processes such as carcinogenesis, apoptosis and neurodegeneration to enhance the understanding of disease progression.¹⁻³

Since a cell functions as a system rather than a collection of sub-cellular organelles, molecular events should be real-time monitored in an intact living cell. The common pretreatment steps such as lysis and fixation can denature the cellular components to create artifacts.⁴ For instance, lysis disrupts the plasma membrane to release cellular components from intracellular compartments causing many orders of magnitude change in

cellular component concentration. Since the invasive preparation steps may terminate many reactions and disturb the physiological state of a cell,⁵ current researches are aiming to achieve a real-time analysis of single living cells with minimal invasiveness.

Despite the great outlook and overwhelming interests, single cell analysis with high sensitivity, specificity and spatiotemporal resolution is a challenging task due to the ultra-small sizes of a mammalian cell ($\sim 10\ \mu\text{m}$ in diameter) and its subcellular compartments, trace amounts of chemicals (e.g. 40 pg of DNA) and fast chemical reactions (in the order of μs) in each living cell.³ Through the years, a plethora of technologies such as flow cytometry, capillary electrophoresis, electrochemistry, and fluorescence microscopy has been developed to analyze single cells. Although these single cell technologies have exerted great impact on biomedical research, they have various limitations. For example, flow cytometry can only measure cell properties at specific time intervals but is not able to monitor in real-time; capillary electrophoresis is rather destructive; while electrochemical methods employing microelectrodes are usually limited to monitor electroactive species released to the extracellular environment. More importantly, the spatial resolutions of these methods have not yet reached a nanoscale level at which the molecular interactions take place. Therefore, developing a versatile biosensor to detect critical changes associated with various physiological processes in single living cells with high spatiotemporal resolution still present great challenges.

1.2 Motivations

Understanding of cell biology requires synchronous monitoring of multiple cellular processes in single living cells with high spatiotemporal resolutions. Unfortunately, most single cell analysis methods are still performed with fixed cells or are limited to monitor extracellular events such as exocytosis at a whole cell level. Therefore, this PhD research project is motivated to develop a sensitive single cell technique, capable of subcellular resolution multi-component analysis or localized intervention with minimal invasiveness.

Alterations in the expression levels of molecular markers, such as genes or proteins are particularly useful to characterize cancer progression.⁶ Current approaches like Western Blot and microarray require cell lysates from thousands of cells to determine the population averaged expression level.⁷ These methods cannot determine protein localization, which is essential to understand the mechanism of a critical disease. If homogenates from highly heterogeneous biopsy samples are analyzed, the information of the cell types expressing a target molecule is usually unavailable, thus more likely yielding false positive or negative results. In addition, preparation steps such as lysis, fixation and permeabilization may distort the native protein conformation to affect detection accuracy. Therefore, it is advantageous and necessary to measure the expression level of a biomarker in a living single cell.⁸

Studying cellular metabolism at high spatial resolution is also highly important. Currently, metabolism are mainly investigated using macro-sized probes that require large amount of samples ($>10^5$ cells) and only report the population averaged values. Since even genetically identical cells show large variations in molecular contents, single cell

metabolic analysis is preferred.⁹ Furthermore, a technique to spatially map metabolite distribution of a single cell is still under developed. Therefore, there is a great need to design a non-invasive and flexible system for spatially resolved metabolite monitoring of a single cell.

Complicated cellular activities such as DNA repair, oxidative activity and transcriptional regulation are usually controlled by multiple intricately interconnected signaling pathways.^{10, 11} Traditional work in biology has focused on studying individual components in a signaling pathway. Full unraveling of the complex cellular processes and their regulations demands simultaneous detections of multiple parameters correlated in time and space, which is still lacking. It is challenging to combine two or more detection modalities to synchronously monitor multiple cellular processes in real-time at a single cell level. In addition, precise delivery of exogenous molecules into specific subcellular compartments is vital to modulate a cell's signalling activity for probing the intracellular biochemical interactions that govern the cell's behavior. This represents another largely unexplored field that awaits urgent development.

1.3 Objectives

The overall objectives of this project are to establish a novel optical and electrical sensing platform for single cell analysis; to design and optimize single cell detection schemes by understanding the physico-chemical fundamentals such as optics theories, surface sciences, bio-conjugation principles and electrochemistry involved; to explore scientific insights into various cellular physiological processes at a single cell level; and to provide a potential method for disease diagnosis and treatment evaluation. This project focuses on developing nanosensors to investigate the cancer biomarker over-expression, the concentration profiles of a metabolite, the real-time fluctuations of biochemicals in single cells and the cellular responses to subcellular drug delivery.

An important task of this project is to construct an optical fiber nanobiosensor for detecting endogenous telomerases in a single living cell and subsequently distinguishing the over-expression of telomerase in cancer cells as compared to normal cells. The subcellular location of telomerase over-expression is also investigated to provide a potential method for cancer detection.

Single cell analysis for tumor cell metabolism is important since it promises to provide deep insights for carcinogenesis by revealing cell-to-cell variations. It is essential to develop an enzyme based optical fiber nanosensor with high spatial resolution, low background interference, and a physiologically relevant dynamic range to spatially map the extracellular lactate concentration. The lactate concentration profiles of cancer cells and normal cells can be compared to noninvasively study the fundamental metabolic

processes at a single cell level. Furthermore, the effect of a MCT inhibitor on HeLa and MCF-7 cells is investigated to evaluate the anti-cancer effect of various metabolic agents.

To understand the interplay of various cellular processes, a bifunctional electro-optical nanoprobe is designed to study the dynamic cellular oxidative activity at a single cell level by simultaneously detecting electrical and optical signals. The oxidative responses and intracellular antioxidant levels are synchronously measured to unveil the correlation between cell malignancy and the strength of oxidative stress. Furthermore, the nanoprobe is capable of localized detections in different membrane regions which can help to determine the activated enzyme targeting location.

Cell function control at a subcellular level is also a critical aspect in single cell analysis. An optical fiber nanoprobe is functionalized with rGO for subcellular delivery of an anticancer drug and simultaneous monitoring of the drug-specific biochemical responses. The loading and release of anticancer drugs is first evaluated and cellular responses such ROS generation and rise in Ca^{2+} level are subsequently monitored in different subcellular locations. Through studying the drug-cell interactions at subcellular domains, we aim to investigate subcellular location-dependent biochemical responses of bioactive molecules thus providing scientific insights in subcellular targeted pharmacodynamics.

1.4 Organization

Chapter 1 introduces the background and states the motivations and objectives of this PhD research project. Chapter 2 reviews the recent advances in the development of high resolution single cell analysis techniques, highlights the important biological findings, summarizes the advantages and limitations of existing techniques, and explains the principles of the optical fiber nanosensor. Chapter 3 describes the materials and experimental approaches used in this study. Following this, chapter 4 reports a sensitive optical fiber nanosensor combined with *in vitro* ELISA to detect telomerase over-expression in single living cancer cell. Chapter 5 demonstrates the applicability of a lactate dehydrogenase based optical fiber nanosensor to measure extracellular lactate concentrations of three cell lines and derives the inhibition profiles of lactate efflux upon CHC application. In chapter 6, an electro-optical nanoprobe based real-time monitoring of multiple cellular activities at selected membrane regions is presented for the first time. Chapter 7 then reports the development of a reduced graphene oxide (rGO) functionalized optical fiber nanoprobe for subcellular delivery of a potent anticancer drug, β -lapachone (β -lap) and simultaneous monitoring of the location-dependent biochemical responses in a single cell. Finally, in chapter 8, a general conclusion of the project and several promising future research directions are provided.

Chapter 2 Literature Review[†]

2.1 Overview

The fundamental human life processes such as signal transduction, intracellular trafficking, protein degradation and DNA repair often occur at a nanometer scale in subcellular compartments. It is critical to analyze single living cells at a high spatiotemporal resolution for observing previously indiscernible nanoscale architectures of cell organelles,¹² determining precise spatial organizations of cellular components, revealing spatial heterogeneities of chemical compositions,¹³ uncovering nanodomains for biochemical reactions¹⁴ and profiling cell-to-cell variations at a subcellular level.^{12, 15} Such information not only is critical to answer fundamental biological questions such as carcinogenesis and apoptosis, but also offers unique avenues for medical diagnosis and disease treatment. Currently, a great interest has been fueled up in developing analytical methods to observe, manipulate and explore single cells with nanoscale resolution. However, it is exceptionally challenging to achieve a high spatial resolution for observing the nanoscale structural details¹⁶ and a high temporal resolution for monitoring fast cellular dynamics.¹⁷ High sensitivity and specificity are also very important to detect minute amounts of biochemicals in individual organelle with high signal-to-noise (S/N) ratio,¹² while a minimally invasive approach is necessary to retain a cell in its native state.¹⁸

[†] Reproduced in part from X T Zheng and C M Li*, Single cell analysis at nanoscales. Chem Soc Rev, DOI: 10.1039/C1CS15265C.

With an excellent spatial resolution around 0.1-10 nm, electron microscopy (EM) is powerful to unravel structures and distributions of various nanometric cellular organelles such as vesicles, lipid rafts and etc. The main limitation of EM is that it only allows static, snapshot observations of fixed cells, and thus cannot be extended to live cell imaging.¹² On the other hand, optical microscopy is well suited to study dynamic cellular processes for understanding cell functions.¹⁹ Unfortunately, due to the diffraction-limited resolution in most microscopy techniques, the cellular organelles are too small to be observed in details. Recently, a number of “super-resolution microscopy” including stimulated emission depletion (STED) microscopy, stochastic optical reconstruction microscopy (STORM), and photoactivated localization microscopy (PALM) have successfully broken the diffraction limit to examine the unresolved cellular structures.¹⁶ Despite the power of the super-resolution microscope techniques to provide high resolution images, they offer little chemical information about the subcellular compartments. To understand the localization and function of cellular machineries, a powerful set of tools must be developed to analyze biochemical contents of the nanometric organelles.

In the past decade, the advancement of nanotechnology has brought a plethora of nanotools suitable for interrogating single cells at nanoscales for both observations of fine structures and determinations of biochemical compositions. The nanoprobe are elegantly designed to probe single cells in a minimally invasive manner and collect high resolution optical, electrical or force information from subcellular locations. They may be precisely positioned by a manipulator for point measurements or controlled by a scanning probe microscope for surface mapping. Different techniques to garner high resolution optical, electrical or force signals from single cells are discussed in the following sections.

2.2 Optical Techniques

The detections of localized optical signals (fluorescence, Raman and etc.) could greatly enhance our knowledge of cellular structures and their biological functions.

2.2.1 Near-field Scanning Optical Microscopy (NSOM) for Nanoscale Membrane Mapping

In conventional far-field microscopy, the diffraction limit determines that the spatial resolution is about several hundred nanometers for visible light excitation. NSOM represents the first optical microscopy technique that has broken the diffraction limit.²⁰ This is achieved by scanning a subwavelength-sized probe close (< 10 nm) to a cell in order to illuminate and/or detect in the near-field. The separation between the probe and the cell is independently controlled to generate a topographic image.¹³ Among different NSOM configurations, the aperture-type NSOM, which is incorporated into an inverted microscope for near-field excitation and far-field detection, is most widely applied in cellular studies (**Figure 2.1A**).¹³ In this type of NSOM, light coupled into a nanoprobe emanates from the aperture as evanescent waves, which exponentially attenuate away from the probe tip; thus exciting only molecules in close proximity to the tip. The resolution no longer depends on the excitation wavelength but is essentially determined by the aperture size, typically in the range of 50 to 100 nm.¹³ As illustrated in **Figure 2.1B**, the resolution of NSOM (85 nm) is three times smaller than that of confocal microscopy (270 nm).²¹ The resolution may be further increased by delicately designing probe geometry or structure. A tip-on-aperture nanoantenna (**Figure 2.1C**) has been developed to confine and enhance electromagnetic fields in a localized region < 30 nm

near the antenna apex. As a result, the nanoantenna can image individual antibodies with an unprecedented resolution of 26 ± 4 nm (**Figure 2.1D**).²² It is also suggested that careful tuning of the antenna geometry could improve the resolution to below 10 nm, opening a door for truly nanoscale cell imaging.²²

The exponentially decaying characteristic of the excitation field makes NSOM highly sensitive to surface features and therefore ideal for cell membrane investigations. NSOM has been frequently utilized to study the distribution,²³ organization²⁴ or co-localization²⁵ of membrane components such as proteins and lipids on fixed cells, thus disclosing a great wealth of details on membrane compartmentalization.^{13, 20} In one recent contribution, Zhong et al. have applied quantum dots instead of fluorescently labeled antibodies for immune-labeling of T-cell receptors to obtain highly reproducible NSOM images.²⁶ Another successful example employs single-molecule NSOM to understand the spatial-functional relationship between integrin LFA-1 and raft components (GPI-APs).²¹ The shortest distances from the (x,y) coordinate of LFA-1 to the (x,y) coordinate of the nearest GPI-AP can be measured from NSOM images to calculate the distribution of the nearest inter-domain distances. It has been discovered that ~50% of GPI-AP nanodomains reside close (< 200 nm) to LFA-1 nanoclusters at a resting state, but upon ligand binding to LFA-1, GPI-AP and LFA-1 form large supramolecules for efficient integrin-mediated cell adhesion.²¹ This method allows both quantitative nanoscale imaging and multicolor detection with equal resolution and position accuracy for all colors.

Figure 2.1 (A) Schematics showing the setup for a NSOM coupled with confocal microscope. The inset shows a representative NSOM probe (Scale bar=100 nm). (B) Composed confocal (Right) and NSOM (Left) image indicates the increased spatial resolution of NSOM (85 nm) compared to confocal microscopy (270 nm). Reprinted with permission from ref. 21. (C) The concept of probe-based optical antennas for imaging (D) fluorescently labeled antibodies. Top inset shows the intensity profile and Gaussian fits (red line). Bottom inset shows the SEM of a typical optical antenna. Reprinted with permission from ref. 22. Copyright 2010 Wiley.

The NSOM imaging of live cell membranes is very challenging due to the mechanical interaction between nanoprobe and membrane. Korchev et al. have combined scanning ion conductance microscopy (SICM) and NSOM to simultaneously obtain optical and topographic images of living cardiomyocytes.²⁷ The probe-sample distance is controlled

by ionic currents rather than interaction forces for better reliability, thus achieving noncontact imaging to preclude any mechanical damages. This work proves the feasibility of NSOM for high resolution live cell imaging. A more recent development is the fluorescence correlation spectroscopy (FCS) coupled NSOM to investigate nanoscale molecular dynamics on intact living membranes.²⁸ The significant reduction in illumination area is the key to uncover lipid diffusion heterogeneities associated with nanoscale membrane compartmentalization. This NSOM-FCS platform provides a unique tool to characterize various diffusion controlled membrane processes and investigate events in membrane proximal cytosolic regions.

2.2.2 Optical Fiber based Nanosensor for Localized Chemical Analysis

Due to the slow scanning speed of NSOM, dynamic cellular processes have not been monitored at nanoscale resolution. Furthermore, its low penetration depth confines the detection at the top membrane. As many interesting biological phenomena happen in the cell interior, intracellular dynamic analysis of biochemicals is critical to understand cellular processes.¹⁶ A newly developed optical fiber based nanosensor is sufficiently small to penetrate into a cell for minimally invasive analysis (**Figure 2.2A-C**). In fiber-optics, light propagates in the higher refractive index fiber core via total internal reflection if the incident angle is larger than the critical angle. Some energy penetrates into the less dense medium as evanescent waves, which is a near-field standing wave that exponentially decays with distance from the boundary. For optical fiber nanosensor, an evanescent field is created at the nanotip with its penetration depth (d_p) defined by **Equation 2.1**.²⁹

$$d_p = \frac{\lambda}{2\pi(n_1^2 \sin^2 \theta - n_2^2)^{\frac{1}{2}}}$$

Where λ is the excitation wavelength, n_1 and n_2 are the refractive indexes of the fiber core and cladding, θ is the angle of incidence. Applying **Equation 2.1**, the penetration depths for visible excitation are calculated to be in the range of 200 – 300 nm. After functionalizing the nanotip with target-specific molecules such as fluorescent dyes, antibodies or peptides, important biochemicals and cellular events including intracellular pH,³⁰ cytotoxic compounds,¹⁸ and apoptosis³¹ have been reliably detected inside single living cells.

In the milestone work by Tan et al., nanofabrication of optical fiber tips has been combined with near-field photo-polymerization to achieve a thousand-fold miniaturization of sensor size, a million-fold sample reduction and a hundred-fold shorter response time.^{30, 32} An optical fiber pH sensor with internal calibration has been inserted into single rat embryo for accurate and non-destructive intra-embryonic pH determination. The nanosensor exhibits both low detection limit (less than 3000 hydrogen molecules) and good stability.³⁰ The sodium concentration variations in single mouse oocyte upon externally stimulated channel opening and closure, has been successfully monitored with an optical fiber sodium sensor later.³³

Figure 2.2 Photographs of (A) a 105 μm and (B) a submicrometre optical fiber pH sensor. Reprinted with permission from ref. 30. Copyright 1992 the American Association for the Advancement of Science. (C) Photograph of an antibody-based nanoprobe used to measure benzopyrenetetrol (BPT) in a single cell. Reprinted with permission from ref. 18. Copyright 2000 Nature Publishing Group.

Nanobiosensors have been immobilized with antibodies to detect fluorescent carcinogenic compounds including benzopyrene tetrol (BPT) and benzo[a]pyrene (BaP) directly in single MCF-7 cells. The intracellular concentrations can be easily quantified by *in vitro* calibrations.^{18, 34, 35} Two types of nanobiosensors have been designed to selectively monitor two critical proteins in the apoptotic pathway, namely, cytochrome c and caspase-9. The release of cytochrome c from the mitochondria, an early event in the apoptosis signal cascade, has been measured by a nanobiosensor inserted inside a single cell.³⁶ However, this two-step process does not allow real-time monitoring of the apoptosis process. A more elegant detection scheme is then designed to real-time monitor caspase-9 activation, a marker of apoptosis.³¹ Caspase-9 activity in single living MCF-7 cells has been assessed with a nanobiosensor immobilized with AMC labeled caspase-9 substrates. When apoptosis is induced, the activated caspase recognizes and cleaves the peptide substrates to release fluorescent AMCs. By comparing the AMC fluorescence

signal from cells with activated caspases and those with inactive caspases, caspase activity within a single living MCF-7 cell is accurately determined.³¹

2.2.3 Surfaced enhanced Raman Spectroscopy (SERS) Active Nanoprobes

Figure 2.3 (A) Scanning electron micrographs (SEM) of the SERS-active nanopipette. Inset shows the nanopipette tip covered with gold nanoparticles. (B) SERS spectra from the cell nucleus (upper spectrum) and cytoplasm (middle spectrum) obtained with the SERS-active nanopipette. The bottom spectrum (black line) was collected from the nanopipette tip before insertion. Reprinted with permission from ref. 37. Copyright 2009 American Chemical Society. (C) SEM micrograph of a gold-decorated CNT (D) Scheme depicting a SERS-enabled endoscope probing the intracellular environment. Reprinted with permission from ref. 38. Copyright 2011 Wiley.

In complement with fluorescence detection, SERS is a promising technique to study molecular phenomenon inside living cells. SERS can provide structure specific vibrational

spectra of analytes in close proximity to the probe surface down to a single molecule level.³⁹ The high sensitivity, comparable or even better than that of fluorescence-based techniques, is contributed from the strong enhancement of SERS. Apart from this, SERS is attractive because of its label-free nature and potentials for multiplexed detections. Up to date, intracellular SERS has been only realized by introducing gold or silver nanoparticles via endocytosis or nano-injection; however, control of intracellular distribution is the main limitation that interferes with accurate signal analysis.³⁹

A SERS substrate with fixed spatial arrangement of nanoparticles is then proposed to overcome the limitation. Gogotsi's group has applied a gold nanoparticles coated glass capillary with a 100 – 500 nm tip (**Figure 2.3A**) for *in situ* SERS analysis of single living cells.³⁷ The probe is precisely inserted into the nucleus or the cytoplasm, and the localized SERS signals recorded from these two compartments are clearly different (**Figure 2.3B**). An intracellular SERS probe can measure time-resolved SERS spectra to disclose cellular response to KCl stimulation.³⁷ A SERS-active fiber-optic nanoprobe with a 6 nm-thick silver layer is also functionalized with pH sensitive *para*-mercaptobenzoic acid to determine intracellular pH in living human cells.⁴⁰ Since both glass capillaries and optical fibers based nanoprobe have relatively large sizes, rigid structures and conical shapes, they often cause cellular damages.^{37, 41} To reduce probe-induced cellular perturbations, Niu et al. have employed a 20 nm gold nanoparticle-decorated carbon nanotube (CNT, 200 nm in diameter, 5 – 20 μ m in length) instead for intracellular SERS analysis (**Figure 2.3C & D**). The cylinder-shaped CNTs displace hundreds or even thousands times less cellular contents than the conical-shaped probes with a similar tip size to greatly minimize the cellular damage.³⁸ Maintaining cell health during probe interrogation is essential to

accurately measure cellular responses under physiological conditions. The high sensitivity of this SERS endoscope has been proven by detection of glycine down to 1 pM.³⁸ Furthermore, the molecular compositions of various cell organelles could be determined by precise placement of the endoscope. Although the variability of the SERS spectra still poses a significant challenge for analyte identification in the complex intracellular environment,³⁹ its multiplexing ability enables it to act as a nanodiagnostic tool to distinguish normal and diseased cells.

2.3 Electrical Methods

The applications of electrical sensors based on field effect transistors, microelectrodes, or patch clamps can be used to investigate various cellular processes or biomolecules such as cell secretion, ion transport, signaling messengers and functional proteins in single cells.⁴²

2.3.1 Nanowire-based Field effect transistors (FETs) for Cellular Electrophysiology

Many cell types are electrogenic and bioelectrical signals they produced underlie many critical cellular functions such as neuronal communication, muscle contraction and hormone secretion.⁴³ Patch clamp, the state of the art electrophysiological technique, is capable of resolving single ion channel activities. One major drawback is that micropipettes utilized in patch clamp measurements are generally too large to form good interfaces with small cells or fine cellular structures and are also difficult to multiplex.

Lieber and co-workers have employed “bottom up” fabricated silicon nanowire (SiNW) FETs to interface with living neurons and detect their bioelectricity⁴⁴. The

oriented p- and/or n-type SiNWs (~ 20 nm in diameter) are assembled into an FET array and further patterned with polylysine to direct and define neuron growth on the device. Multiple neurite-NW junctions can be made for the same neuron to monitor action potential propagations at a level of at least 50 “artificial synapses” per neuron (**Figure 2.4A**). Notably, the active junction area is only about 0.01 to $0.02 \mu\text{m}^2$, similar to natural synapses.⁴⁴ By parallel recording of spike propagations at multiple points, the propagation delays of spikes in neurites are clearly resolved. The signal propagation rates obtained from the slopes of latency time versus distance plots are 0.16 m/s and 0.43 m/s for dendrites and axons, respectively (**Figure 2.4B**). Histograms of propagation speeds collected from different neurons are then fitted to give Gaussian distributions of 0.15 ± 0.04 m/s for dendrites and 0.46 ± 0.06 m/s for axons respectively (**Figure 2.4C**).⁴² In addition, these nanoscale junctions can also stimulate and/or inhibit neuronal signal propagation. This work opens a new field using nanowire-based devices to interrogate subcellular structures and simultaneously detect bioelectricity with high spatiotemporal resolution.

As an alternative to SiNWs, single walled carbon nanotubes (SWCNTs) can interface with cells at higher spatial resolution because the diameter of SWCNT is only around 1 nm, comparable to the size of single protein. Antibody-coated SWCNTs have been incorporated in an electrolyte-gated transistor to interrogate single macrophage cells and study phagocytosis in real-time.⁴⁵ Another possible option is to use a single layer graphene sheet. Since two-dimensional graphene sheets are usually several microns in size, which are comparable to that of a single cell, the measured signal should represent an average value collected from the entire cell rather than spatially resolved information.⁴⁶

On the contrary, graphene nanoribbons with nanometric widths are expected to be more appropriate for realizing subcellular electrical measurements.

Figure 2.4 (A) Optical image of a cortex neuron lying on a nanowire array. (B) Plot showing latency time versus distance and (C) Histogram of propagation speed for axons (blue) and dendrites (red), respectively. Reprinted with permission from ref. 44. Copyright 2006 the American Association for the Advancement of Science. (D) SEM micrograph showing the CMOS compatible silicon nanowire array; the inset is a TEM image illustrating the rectangular cross-section of a nanowire. (E) Nanowire recordings on individual cardiomyocytes. The upper trace (I) represents the blank (without cell). The lower trace (II) is the recording

from a nanowire that was covered by a contracting myocyte. (F) A typical episode of the nanowire response from trace (II) is shown on the left. For comparison, an intracellular action potential recorded using a nanopipette is depicted on the right. Reprinted with permission from ref. 47. Copyright 2009 Wiley. (G) SEM image of a 3D nanoFET device. The yellow arrow and pink star mark the nanoscale FET and SU-8, respectively. (H) Electrical recording from beating cardiomyocytes: (I) extracellular recording, (II) transition from extracellular to intracellular recordings, and (III) steady-state intracellular recording. Reprinted with permission from ref. 48. Copyright 2010 the American Association for the Advancement of Science.

Assembly and integration of pre-synthesized nanowires are nontrivial and often give very low yield. Instead, a complementary metal oxide semiconductor (CMOS)-compatible “top-down” method has been chosen to mass produce chips with well-aligned SiNW arrays to detect bioelectrical signals in cardiomyocytes or smooth muscle cells (**Figure 2.4D-F**),⁴⁷ demonstrating a feasible method to fabricate inexpensive SiNW chips for high-throughput drug screening.

Until very recently, nearly all related works focus on planar FET devices, which could only conduct extracellular detections. A nanoscale FET (nanoFET) device has been successfully integrated at the tip of an acute-angle kinked SiNW to record intracellular potentials at high spatiotemporal resolutions in a beating cardiomyocyte (**Figure 2.4G & H**).⁴⁸ Overall, these nanowire-based FET devices are powerful platforms for electrophysiological measurements with unique advantages such as improved resolution, enhanced throughput and potential multiplexing capability.

2.3.2 Nanoelectrode-based Electrochemical Detections to Probe Cellular Chemistry in Nanodomains

Over the past two decades, ultramicroelectrodes have been widely developed for various applications^{49, 50} and further expanded to probe cellular chemistry, especially in real-time monitoring of exocytosis or ROS generation.⁴² However, the sizes of microelectrodes are close to that of mammalian cells, which preclude both accesses to cell interiors and interfaces with fine subcellular structures such as synapses.

Electrochemical detection in nanoscopic cellular domains requires fabrication of nanoscale electrodes. In fact, the reduction of electrode size to a nanoscale level can decrease Ohmic drop distortion, lower double-layer capacitance and enhance mass transfer rate to achieve ultrafast measurements.^{42, 51} A nanoelectrode not only offers high spatial resolution but also possesses excellent S/N ratio for sensitive detections.⁵¹ A carbon fiber nanoelectrode (~ 100 nm in diameter) has been applied for amperometric monitoring of dopamine release from PC12 cells with single vesicle resolution (**Fig. 5A**).⁵² Current spikes are only detected in ~ 30% of monitored membrane locations indicating that dopamine molecules are released at specific fusion sites rather than over the entire cell surface. It is discovered that multiple vesicles can release at the same site and the sequential release at the hotspot plays the major role in exocytosis.⁵² This nanoelectrode-based amperometric detection unravels the exocytotic mechanism insight at a nanoscale resolution.

Figure 2.5 (A) Photograph of actual nanoelectrode-cell arrangement; Inset shows current trace of sequential dopamine release from multiple vesicles at the same release site. Reprinted with permission from ref. 52. Copyright 2005 American Chemical Society. (B) Sequential SECM images of human gastric carcinoma (BGC) cell obtained with a 400-nm-radius Pt tip at every 7.5 min from (a) 0 to (t) 142.5 min in PBS solution. Reprinted with permission from ref. 53. Copyright 2010 Royal Society of Chemistry.

A further improvement is the application of nanoelectrodes in scanning electrochemical microscope (SECM) for spatially resolved electrochemical mapping of human breast cells.⁵⁴ In SECM, a nanoelectrode scans above a cell surface to measure the faradaic currents associated with oxidation or reduction of the electroactive compounds or freely diffusing ions in buffers.⁵⁴ As the nanoelectrode radius is ~ 1000 times smaller than that of a cell, it easily penetrates a cell without any apparent damage to the membrane. This technique has been successfully exploited to measure the trans-membrane charge transport, evaluate the membrane potential and probe subcellular redox properties at a spatial resolution of ~ 200 nm.⁵⁴ In most SECM studies, toxic exogenous mediators are used and could compromise the long term cell viability. To overcome the cytotoxicity problem, oxygen is chosen as an endogenous indicator to monitor nanoscale height changes of a single cell in physiological environment for viability evaluations (**Fig. 5B**).⁵³

With proper probe functionalization and integration, these nanoelectrodes cannot only be useful in fundamental cellular studies but also have potential in healthcare applications, especially the point-of-care devices.

2.3.3 Scanning Ion Conductance Microscopy (SICM) for High Resolution Surface Analysis

Figure 2.6 (A) Schematic diagram of the high-resolution SICM experimental setup (I: ion-current amplifier; II: scanner control). The inset shows an SEM image of the tip of a typical nanopipette. Reprinted with permission from ref. 55. Copyright 2006 Wiley. (B) A SICM probe scans the cell surface of an A6 cell to reveal microvillar structures. Reprinted with permission from ref. 56. Copyright 2003 PNAS. (C, D) Three-dimensional topographical images showing the vertically protruding mechanosensitive stereocilia of auditory hair cells as measured by HPICM in (C) colorscale or (D) grayscale; the arrow indicates a true cilium. Reprinted with permission from ref. 57. Copyright 2009 Nature Publishing Group.

SICM is a special scanning probe microscopy (SPM) that has been successfully adapted to image living cells at a nanoscale resolution. In SICM, a glass nanopipette is used to scan sample surface and the nanopipette-sample distance is feedback controlled by ion current flowing through the nanopipette without any physical contact (**Figure 2.6A**).⁵⁵ The resolution of SICM is determined by the inner radius of the nanopipette, commonly in the order of a few nanometers. The high resolution has been demonstrated by using a 13 nm-diameter quartz nanopipette to image single proteins on the plasma membrane of living cells.⁵⁵ SICM is particularly suitable for dynamic investigation of membrane structures such as microvilli on epithelial A6 cells (**Figure 2.6B**),⁵⁶ T-tubules and Z-grooves on cardiomyocytes.⁵⁸ For instance, high-resolution time-lapse SICM imaging reveals that microvilli undergo a life cycle of formation, steady state and retraction. By plotting the heights of individual microvillus against time, the heights are found to increase nonlinearly during their formation but decrease linearly at 1.2 nm/s during retraction.⁵⁶

The drawback of the conventional raster scanning SICM is prone to have probe-sample collision, and it is limited to image relatively flat surfaces. A hopping mode SICM (HPICM) overcomes the restraint for noncontact nanoscale imaging, even on the most convoluted surface structures.⁵⁷ In this mode, the nanopipette approaches a surface to measure heights only at selected imaging points where the current has a fixed percentage (e.g. 1%) of the reference current. It is rapidly withdrawn to a safe distance before moving on to the next imaging point. This HPICM technique can visualize a complex three-dimensional (3D) structure of mechanosensitive stereocilia on auditory hair cells as shown in **Figure 2.6C & D**.⁵⁷ A similar concept called standing approach (STA) mode has been

reported to image convoluted surfaces on living cell membrane, in which a field-programmable gate array (FPGA) is employed to enhance feedback regulation while speeding up the imaging process.⁵⁹ The ability to image membrane structures at nanometric resolution can be married to other functional imaging techniques to investigate structure-function relationship on a living cell membrane.

2.3.4 Nanopipette-based Patch Clamp Measurements for Analyzing Ion Channel Activity at Subcellular Structures

The glass micropipettes used in conventional patch clamp measurements often suffer from poor resolution, and are not able to map ion channel distributions over cell surfaces. SICM allows both ultrafine positioning of the nanoprobe and high resolution imaging of the membrane. Since a SICM probe can be used as a patch pipette, a scanning patch clamp technique has been developed to facilitate single channel recording at submicron-sized cellular structures. Ion channel signals from the top of epithelial microvilli and openings of cardiomyocyte T-tubules are recorded at a nanometer precision.⁶⁰ The same technique has been utilized later to study the spatial distribution of maxi-anion channels in rat cardiomyocytes.⁶¹ It is found that maxi-anion channels are concentrated at the openings of T-tubules and along Z-lines in cardiomyocytes. In addition, the spatial distribution of ATP-releasing sites also matches with that of the maxi-anion channels to verify the ATP-conductive nature of the maxi-anion channels.⁶¹ Very recently, Schrlau et al. have employed carbon nanopipettes (CNPs), which integrate carbon nanopipes into the tips of pulled glass capillaries, for electrophysiological measurements in mouse hippocampal cells. The resting membrane potential and transient membrane response to

pharmacological agents have been measured.⁶² CNPs are advantageous because of their less breakage and clog thus facilitating lengthy measurements. They may eventually replace the glass nanopipettes if they could be reproduced at low cost.

2.4 Nanoscale Functional Analysis of a Living Cell Using Atomic Force Microscope (AFM)

AFM with excellent spatial control in an order of several nanometers has emerged as a powerful tool for high resolution cellular analysis to depict the detailed membrane morphologies, mechanical properties, receptor-ligand interactions.¹⁷ The spatial organization of peptidoglycan, a major wall constituent of bacterial cells has been probed by AFM. Topographic images reveal 25-nm-wide periodic ridges of peptidoglycan run parallel to the short cell axis on a mutant cell surface.⁶³ As AFM is able to measure forces ranging from piconewtons to nanonewtons, it is very suitable to examine mechanical properties of single cells. A DNA-coated AFM cantilever measures the cell adhesion force to be ~ 1000 pN, corresponding to roughly 20-25 linkages of 20 bp DNA duplex.⁶⁴ AFM has also been utilized to explore different mechanical properties between normal and cancerous human cervical epithelial cells.⁶⁵ As presented in **Figure 2.7A**, a clear difference in force curves can be obtained after processing the raw single cell force data with a brush-on-soft-surface model.⁶⁶ Normal cells exhibit one-slope force dependence and the force between the spherical AFM probe of radius R and the brush can be expressed by **Equation 2.2**^{65, 66}

$$F_{steric} \approx 50k_B T R N^{\frac{3}{2}} \exp\left(-\frac{2\pi h}{L}\right) L$$

where L , N and T are the brush length, the grafting density and the medium temperature, respectively while $0.1 < h/L < 0.8$. As for the two-slope force dependence of cancerous cells, the probe-cell force is estimated by **Equation 2.3**⁶⁵

$$F_{steric} \approx 50k_B T R \times \left[N_1^{3/2} \exp\left(-\frac{2\pi}{L_1} h\right) L_1 + N_2^{3/2} \exp\left(-\frac{2\pi}{L_2} h\right) L_2 \right]$$

where the indexes 1 and 2 of N and L correspond to the first and second brushes, respectively. It is not applicable if the distance h is between the lengths L_1 and L_2 , or greater than the larger L .

Figure 2.7 (A) Forces between the AFM probe and cell brush; the slower force decay at large distances in cancerous cells is highlighted by the circle. (B) Distribution histograms of brush lengths and (C) Schematic of cells with brushes drawn to scale for normal (left) and cancerous (right) cells. Reprinted with permission from ref. 65. Copyright 2009 Nature publishing group.

The fitting results indicate that normal cells have single-length brushes of $\sim 2.4 \mu\text{m}$ with a grafting density of ~ 300 units per μm^2 , whereas cancer cells have brushes with two

characteristic lengths of 0.45 and 2.6 μm with grafting densities of 640 and 180 units per μm^2 (**Figure 7B & C**), demonstrating the potential of AFM for cancer detection.⁶⁵ Efforts have been devoted to increase the scan speed of AFM for real-time visualization of dynamic conformational changes. A high-speed AFM has been used to obtain high-resolution movies of a light-driven proton pump, bacteriorhodopsin under realistic conditions. A cytoplasmic portion of each bacteriorhodopsin monomer is observed to contact with adjacent trimers upon illumination.⁶⁷ We anticipate that with further advances in high-speed AFM techniques, even molecular processes could be directly observed in the near future.

2.5 Combined Multi-machine Detections for Correlative Investigations of Cellular Processes

It is well recognized that intricately interconnected signaling pathways control complicated cellular activities. Therefore, synchronous detections of multiple parameters are required to fully unravel the interplays of cellular processes.⁶⁸ SICM is combined with laser confocal microscopy to simultaneously measure nanometric motions and local calcium concentration changes of cardiomyocytes.⁶⁹ An extension of this method called scanning surface confocal microscopy (SSCM) can concurrently record both topographic and fluorescence images of cell membranes at a nanoscale resolution.⁷⁰ SSCM retains the high resolution of SICM to image individual fluorescent particles on fixed or live cells. The interactions between single virus-like particles (VLPs) and cell membranes are recorded by SSCM to reveal the sinking of single particles into membrane invaginations prior to cell entry (**Figure 2.8A**).⁷⁰

Later on, more efforts are devoted into the developments of other hybrid systems including SECM/OM⁷¹ and SICM/SECM.⁷² The SECM/OM system is capable of simultaneously capturing electrochemical, fluorescent and topographic images from single gene-transfected HeLa cells.⁷¹ The co-expression of secreted alkaline phosphatases outside the cell and green fluorescent protein (GFP) inside the cell are concomitantly detected. Unfortunately, the probe with radius around 800 nm is slightly large and compromises the resolution for both electrochemical and optical imaging. About the same time, a SECM/SICM system with ionic current feedback control has imaged both topography and spatial distribution of electrochemical species.⁷² A nanopipette/nanoring electrode probe (aperture radius = 220 nm; outer ring radius = 550 nm) is employed to achieve electrochemical measurements at submicrometer resolution (**Figure 2.8C**). This system is able to acquire cellular topography in parallel with electrochemical mapping. For instance, the permeation property of electroactive species through cell membranes can be mapped.⁷²

Another smart design is a nanoneedle transistor-based sensor for selective detection of calcium ions in a living cell via both conductance and fluorescence signals.⁷³ Here, a SWNT-FET is fabricated at the end of a nanopipette and functionalized with Fluo4-AM fluorescent dyes. The selective bindings of Ca^{2+} ions onto the dye molecules alter the charge states, resulting in changes of source-drain current and fluorescence intensity.⁷³ Such a detection format can sense other intracellular molecules at a high resolution.

A powerful platform that combines SECM, NSOM and AFM has also been reported.⁷⁴ A specially designed probe (**Figure 2.8B**) is applied to reveal submicrometer-order structures and characteristic chemical sites of a neurite in three modes with

nanoscale resolutions. The bent probe has a tip with an 100 nm aperture surrounded by a 250 nm gold ring nanoelectrode and an outermost insulating polymer layer. The resolutions are 240 nm, 380 nm and 390 nm for AFM, SECM and NSOM modes, respectively.⁷⁴ This method has great potential to study various dynamic processes at nanoscale cellular structures.

Figure 2.8 (A) Overlay of topographic and fluorescence images of Cy3-labeled VLPs adsorbed to the surface of COS 7 cells simultaneously obtained by SSCM. Reprinted with permission from ref. 70. Copyright 2002 PNAS. (B) Conceptual diagram of the tip used for combined SECM/NSOM/AFM. Reprinted with permission from ref. 74. Copyright 2007 Wiley. (C) SEM micrograph of a SECM/SICM probe. Reprinted with permission from ref. 72. Copyright 2010 American Chemical Society. (D) Schematic of the multifunctional nanotube endoscope. Inset shows an SEM image of an as-assembled endoscope with 100 nm CNT tip. Reprinted with permission from ref. 41. Copyright 2011 Nature publishing group.

Very recently, a multifunctional CNT-based endoscope has been developed for cell interrogation, fluid transport and optical/electrochemical diagnostics at a single organelle level.⁴¹ The endoscope comprises of a MWCNT (200 nm in diameter, 50-60 μm long) assembled at the tip of a glass micropipette (**Figure 2.8D**) to achieve a spatial resolution of ~ 100 nm and is non-disruptive to cellular organelles. It is excellent in injecting attolitre volumes of fluid into a cell thus permitting localized stimulation or signal intervention. The conductive CNT is actually a nanoelectrode to record picoampere signals from cells. The coating of CNT with gold nanoparticles further enables intracellular SERS detections.⁴¹ Overall, this endoscope is a multifunctional device that promises breakthroughs in nanomedicine.

2.6 Nanoscale Operation of a Living Cell

Visualization, manipulation and interrogation of biological processes at subcellular level are the ultimate goals of biological research.⁷⁵ Micropipettes are traditionally used for cellular manipulations such as nucleus replacement and intracellular delivery. Unfortunately, they are very disruptive to cells and too large to access subcellular compartments. Since most fundamental biological processes occur at nanoscales, newly developed nanoneedles are very suitable for minimally invasive operation in a single living cell.⁷⁵

FIB has been applied to etch a pyramidal AFM tips into a high aspect ratio nanoneedle (200-300 nm diameter and 6-8 μm long) for successful nuclear insertion without apparent cellular damage.⁷⁶ Force distance curves obtained using AFM indicate that an indentation of only 1-2 μm precedes the penetration of a nanoneedle, whereas a

pyramidal AFM tip causes severe cell disruption (**Fig. 9A & B**).⁷⁶ The polylysine functionalized nanoneedles have positively charged surfaces to load negatively charged plasmids via electrostatic interactions. The nanoneedle-based gene delivery to mesenchymal stem cells achieves 70% transfection efficiency, considerably higher than other methods.⁷⁷

The FIB technique is limited because the etched tips are around several hundred nanometers. A further reduction of the tip size is required for better biocompatibility. One feasible way is to attach a 10-20 nm diameter multiwalled carbon nanotube (MWCNT) to an AFM tip for intracellular delivery of protein coated quantum dots. The quantum dots are attached to the MWCNT surface via disulphide based linkers that are specifically cleaved within the cell after 15-30 min incubation.⁷⁸ The effect of nanoneedle size on cell penetration is then investigated in detail. The smaller diameter nanoneedle significantly reduces the indentation depth to around 100-200 nm. Besides, the required penetration force is only 100-200 pN, ten times lower than FIB etched nanoneedles, resulting in very minimal cell deformation.⁷⁹

Besides AFM manipulation, nanoneedles can be coupled to the widely available micromanipulators for intracellular delivery and the positioning precision is dependent on the micromanipulator precision. Yum et al. have presented a nanoscale mechanochemical method to deliver quantum dots into living cells.⁸⁰ A pre-synthesized boron nitride nanotube is affixed to a macroscopic needle and then sputtered with a gold layer for facile surface functionalization. The as-fabricated nanoneedles are able to deliver a small number of quantum dots specifically to either the cytoplasm or the nucleus and the diffusion of single quantum dot (**Fig. 9C**) is also tracked to study its intracellular diffusion

dynamics.⁸⁰

Figure 2.9 Nanoscale operation of a living cell. (A, B) Force-distance curves during approach (blue arrow) and retraction (red arrow) from a melanocyte using a normal pyramidal AFM tip (A) and the nanoneedle (B), respectively. (C) Tracking of a QD inside living cells. A series of fluorescence images shows the trajectory (the green line) of a tracked QD. Reprinted with permission from ref. 76 and 80. Copyright American Chemical Society.

A wider range of substances can be continuously delivered by gently contacting a hollow AFM cantilevers with a nanoscale opening on a cell membrane.⁸¹ Recently, the temporal control has been improved by electrochemically de-conjugating protein coated quantum dots to achieve triggered release of cargos within tens of seconds.⁸² The ideal situation would be to combine targeted intervention with various sensing strategies at a high spatiotemporal resolution to control and subsequently uncover the details of biological processes.

2.7 Perspectives

In summary, the advanced nanotechnology has brought an excellent set of nanotools such as NSOM, optical fiber nanosensors, nanowire-based FETs, SICM and AFM, to capture optical, electrical or force signals from single living cells with high spatiotemporal resolutions for profound investigations of cellular processes at nanoscales. The key for these new techniques to succeed is the tailored nanometric detection elements utilized, which are comparable in size to subcellular targets. The newly developed technologies have offered many exciting opportunities to reveal 3D nanostructure of cellular organelles, spatial organizations of critical biomolecules, and biochemical reactions taking place in nanodomains.

Although these nanoprobe-based techniques hold enormous promises to uncover unprecedented details in important cellular processes, several critical issues should be addressed to exploit their full potentials. First, almost all the methods discussed so far have an inherent low throughput limitation because each nanoprobe can only interrogate one cell at a time. Fastidious measurements are therefore required to verify statistical significance of any new observations. Unveiling of cellular heterogeneities at a nanoscale level also requires high throughput. Several ways including automatic manipulation, integration with microfluidic platforms or detection in an array format are considered feasible to overcome this difficulty. Second, the resolutions achieved up to date are mostly larger than 100 nm. As biological processes are ultimately controlled by molecular interactions, the future trend is to improve the spatial resolution to tens of nanometers or even a few nanometers, i.e. a molecular level. This improvement is dependent on probe

size reduction, which on the other hand may compromise signal intensity. Therefore, we need to consider the tradeoffs between signal strength and resolution. Third, the temporal resolution represents another critical aspect. Dynamic analysis of biological processes requires techniques with fast detection capabilities. Current high resolution methods take substantially long duration (in the order of minutes) for signal collection or analysis, thus, precluding the monitoring of some rapid cellular processes (in the order of microseconds). A high-speed AFM technique is recently reported⁶⁷ which sheds light on the development of high spatiotemporal resolution techniques. Fourth, there are very few examples that integrate multiple detection modalities, thus hinting that great technical challenges are involved. The parallel multi-parametric analysis of biological pathways is of exceptional importance to study cell signaling and regulation. Advancements in this direction allow us to observe and manipulate cellular structures at nanoscale resolutions while simultaneously elucidate their sophisticated functions. Additionally, functionalization and/or surface modification of a nanoprobe to improve the specificity and sensitivity is also very challenging in our future research.

It is believed that the abovementioned challenges could be solved in the near future and we envision that with the appearance of established protocols and commercialized equipments, high resolution techniques will become readily available to the general scientific community, especially the biologists. The widespread application of high resolution techniques can certainly fuel up the next wave of biological research in single cell analysis, albeit at a nanometer scale.

Chapter 3 Experimental Approaches

3.1 Materials and Equipment

3.1.1 Chemicals and Reagents

Poly-L-lysine hydrochloride (15,000-30,000 Da), (3-aminopropyl)triethoxysilane (APTES), BiotinTag™ Micro Biotinylation Kit, sodium L-lactate, β -nicotinamide adenine dinucleotide hydrate (NAD^+), L-lactic dehydrogenase from porcine heart (LDH), α -cyano-4-hydroxycinnamic acid (α -CHC), phorbol 12-myristate 13-acetate (PMA), phenylarsine oxide (PAO), S-Nitroso-N-acetyl-DL-penicillamine (SNAP), 2', 7' - Dichlorofluorescein diacetate (DCF-DA), dopamine hydrochloride, catalase, liquefied phenol, 2-allylphenol, butyl cellosolve, methanol and β -lapachone (β -lap) were purchased from Sigma (St.Louis, MO). 50% (v/v) glutaldehyde in water was obtained from Merck (Hohenbrunn, Germany). Affinity purified polyclonal rabbit anti-telomerase was purchased from Rockland Immunochemicals (Gilbertsville, PA). Alkaline phosphatase stabilizer and wash solution were obtained from Kirkegaard & Perry Laboratories (Gaithersburg, MD). Streptavidin-alkaline phosphatase conjugate, 9-H-(1,3-dichloro-9,9-dimethylacridin-2-one-7-yl) phosphate, diammonium salt (DDAO phosphate), CellTracker™ Green CMFDA, MitoTracker™ Red, Fluo-4 AM and Rhodamine-phalloidin were purchased from Invitrogen (Carlsbad, CA). The deionized water (18.2 M Ω •cm) was obtained from a Millipore Milli-Q water purification system. All other reagents were of analytical grade.

3.1.2 Single Cell Analysis Platform

Figure 3.1 Schematics of the single cell analysis platform that is capable of simultaneously detecting both optical and electrical signals. Reproduced from ref. 83.

The fluorescence detection system (**Figure 3.1**) was assembled as reported previously.⁸⁴ The 610 nm light from the monochromator was focused into the nanoprobe coupled at the distal end of the delivery fiber. The nanoprobe was tied to the nanomanipulator on the Olympus IX71 inverted microscope stage to allow fine movements. Fluorescence emission was collected by the objective and passed through a long pass filter to a photomultiplier tube (PMT). Finally, PMT output signal were acquired by the data acquisition card. For simultaneous electrochemical measurements, both cyclic voltammetry and amperometry were performed using an Autolab electrochemical station (PGSTAT 30, Eco chemie). The reference electrode was Ag/AgCl electrode and the counter electrode was a Pt wire. All electrochemical measurements were performed inside a Faraday cage.

3.2 Methodology

3.2.1 Cell Cultures

Human mesenchymal stem cells (hMSCs) were cultured using MesenPRO RSTTM Basal Medium (Gibco) supplemented with MesenPRO RSTTM Growth Supplement (Gibco) and 2 mM of L-glutamine. Human cervical cancer HeLa cells were cultured using RPMI1640 medium (Hyclone) supplemented with 10% heat inactivated FBS and 50 U/mL penicillin/streptomycin (Gibco). DMEM/F12 medium (Gibco) mixed with 10% heat inactivated FBS and 0.3 mg/mL G418 sulphate were used for human fetal osteoblast (hFOB) cell culture. Human mammary epithelial MCF10A cell line was cultured in Clonetics® MEGM® Mammary Epithelial Cell Growth Medium (Lonza, Walkersville, MD). Human breast cancer MCF7 cells were grown in Minimum Eagle's Medium (MEM) (PAA Laboratories, Pasching, Austria) supplemented with 10% heat inactivated fetal bovine serum (FBS) (PAA Laboratories), 1 mM sodium pyruvate (PAA Laboratories) Gibco, Grand Island, NY) and 50 U mL⁻¹ penicillin/streptomycin (Gibco). HER2 over-expressing MCF7 (MCF7/HER2) cells were cultured with Dulbecco's Modified Eagle's Medium (DMEM) (PAA Laboratories) supplemented with 10% heat inactivated FBS, 0.5 mg/mL G418 (PAA Laboratories) and 50 U mL⁻¹ penicillin/streptomycin (Gibco). The cells were grown in a humidified incubator at 37 °C (hFOB cells were cultured at 33.5 °C) with 5.0% CO₂. Cells were seeded at a density of 1×10^4 cells cm⁻² onto poly-L-lysine (0.1 mg mL⁻¹) coated coverslips for cell attachment.

3.2.2 Nanoprobe Fabrication

Optical fiber nanoprobe were fabricated from multimode fibers with 400 μm diameter core (Oz optics, Canada) by heating and pulling method using a laser-based micropipette pulling device (Model P2000, Sutter Instruments, Novato, CA). The nanoprobe fabrication was conducted in two stages and the programmable parameters used are presented in **Table 3.1**. Different strategies can be applied to coat the sidewalls of the nanoprobe as illustrated below.

Table 3.1 The programmable parameters used in nanoprobe fabrication

Stage	Heat	Filament	Velocity	Delay	Pull
1	850	2	165	110	125
2	980	1	110	100	200

3.2.2.1 Fabrication of silver-coated nanoprobe

The lateral wall of pulled nanotip was coated with a layer of silver via silver mirror reaction as reported previously.⁸⁴ This approach could effectively coat silver on the lateral wall of the nanoprobe for preventing light leakage, while leaving the aperture free for optical detection.

3.2.2.2 Fabrication of aluminum-coated nanoprobe

The lateral wall of pulled nanotip was sputter-coated with 100 nm aluminum. The distal end of the nanotip was left free for transmitting light by careful adjustment of the fiber placement angle in the sputter chamber. The aluminum coating is stable for subsequent fabrication procedures.

3.2.2.3 Fabrication of electro-optical nanoprobe

The lateral wall of pulled nanotip was sputter-coated with 100 nm gold. For electrical contact of a gold-coated optical fiber nanotip, the sputtered gold layer was connected to a copper wire with silver epoxy (Chemtronics, Kennesaw, GA) and cured at 80 °C for 15 min. The nanoelectrodes were insulated via copolymerization of 2-allylphenol (90 mM) and phenol (60 mM) at 4.0 V vs. a platinum counter/reference electrode as reported.⁸⁵ The gold nanoring was exposed by milling with FIB.

Chapter 4 Single Living Cell Detection of Telomerase Over-expression for Cancer Detection by an Optical Fiber Nanobiosensor[‡]

4.1 Introduction

Single cell analysis is of importance since cells may seem identical but are heterogeneous in their composition.^{1, 2, 86, 87} Diversity in a large population of cells is a critical parameter in understanding a myriad of cellular processes. For example, the heterogeneous characteristics of many tumors may be enhanced in the course of carcinogenesis.² Therefore, monitoring carcinogenic transformations at a single cell level may lead to discovery of biomarkers for cancer diagnosis, which is crucial for effective treatment and significant reduction of mortality. Alterations in the expression levels of molecular markers, such as genes or proteins are particularly useful in characterizing cancer development.⁶

Telomerase, a ribonucleoprotein enzyme that catalyzes telomere maintenance, is required in cellular immortalization.⁸⁸ Its expression is up-regulated in 85-90% of human tumours but generally repressed in normal somatic cells. Thus, it is regarded as a general cancer biomarker.⁸⁹⁻⁹¹ Telomerase detection has great potential for applications in cancer diagnosis and evaluating anti-cancer therapeutics.⁹² A variety of techniques have been

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developed to analyze telomerase activity and the most popular telomerase assay is the telomeric repeat amplification protocol (TRAP).⁸⁸ Recently, an array of alternative approaches has been developed to detect telomerase using optical,⁹³⁻⁹⁶ electrochemical,⁹⁷⁻⁹⁹ surface plasmon resonance¹⁰⁰ and magneto-mechanical sensors.¹⁰¹ However, these telomerase detection techniques require cell lysates from thousands of cells or fixed cells⁷ for analysis, and only the population averaged results can be obtained. They also cannot determine the protein localization, which is essential to understand the mechanism of an infectious disease. If homogenates from highly heterogeneous tumor biopsy samples are analyzed for telomerase, the information of the cell types expressing telomerases is usually unavailable, thus more likely yielding false positive or negative results when cell types are mixed. In addition, the preparation steps such as lysis, fixation and permeabilization may also distort the native protein conformation thus affecting detection accuracy. Therefore, a living single cell analysis becomes even more advantageous⁸ and necessary for accurate telomerase detection. Up to date, green fluorescent protein (GFP) CD-tagging of proteins at their endogenous chromosomal locations is the only reported approach to detect proteins in single living cells, but it is not applicable for low abundance protein such as telomerase since it can only detect proteins expressed at relatively high levels ($\sim 10^4$ /cell).¹⁰² Furthermore, CD-tagging technique is rather complicated and time-consuming as the transfected cells need to be sorted and screened for correctly tagged clones.

Due to the extremely small size of a single cell and ultra trace amount of a cellular component, it still remains great challenges for a single living cell analysis with high sensitivity and good selectivity. Previously, optical fiber nanosensors have been

developed to monitor intracellular pH,³⁰ cytotoxic compounds,^{18, 34} and apoptosis.^{31, 36} In this work, we report an optical fiber nanobiosensor for the first time to detect endogenous telomerase in its native environment, the nucleus of single living cells. After the nanoprobe captured telomerases from the nucleus, enzyme-linked immunosorbent assay (ELISA) was conducted on the nanotip *in vitro* for significant signal amplification, which is another key factor for the telomerase detection at a single cell level as telomerase is usually present in low copy numbers¹⁰³ in individual cells.

4.2 Preparation and Measurement

4.2.1 Immobilization of Anti-telomerases

The silver-free surfaces of the nanotip ends were silanized by treatment with 5% (v/v) aminopropyltriethoxysilane (APTES) in ethanol at 60 °C for 3 h. The silanized nanoprobe were washed with ethanol and dried in vacuum at 120 °C for 2 h. After drying, the nanoprobe were activated by immersing in 5% (v/v) glutaldehyde at 4 °C for 12 h followed by rinsing with deionized water. Finally, the activated nanoprobe were incubated in 0.1 mg/mL anti-telomerase solution for 24 h at 4 °C. The as-fabricated nanoprobe were rinsed with phosphate buffered saline (PBS) and blocked in 1% BSA/PBS overnight at 4 °C.

4.2.2 Sandwich ELISA Immunoassay

For detection in the nucleus, the nanoprobe was carefully manipulated to penetrate both plasma membrane and nuclear membrane to be incubated in the nucleus for 30 min.

Telomerase in the nucleus shows a diffuse nucleoplasmic distribution.^{104, 105} To improve the collection efficiency of the nanoprobe, the nanotip was moved to several different locations in the nucleus within the 30 min incubation duration to ensure successful capture of telomerases. For detection in the cytoplasm, the nanoprobe was moved past the plasma membrane and extended a short distance into the cytoplasm without affecting the integrity of the nuclear envelope. The nanoprobe was then taken out of the cell and rinsed with PBS. As monitored under optical microscope, the cells being probed remain viable within the 30 min incubation time. We believe that this mechanical intrusion does not trigger nuclear protein synthesis or degradation within the 30 min incubation time. However, they start to show signs of dying after the nanoprobe's withdrawal and most of them are dead after 1 h. The nanoprobe was incubated with 0.1 mg/mL biotin-labeled anti-telomerase for 1 h. Following a rinse step with 0.5% Tween 20 in PBS, the nanoprobe was incubated with 0.2 mg/mL streptavidin-alkaline phosphatase conjugates for 1 h. Finally, the nanoprobe was washed in alkaline phosphatase wash solution and mounted. DDAO-phosphate solution (0.05 mM) was added to the chamber for fluorescence measurement.

4.3 Single Cell Telomerase Detection Principle

Figure 4.1 Single cell telomerase detection by optical fiber nanobiosensor

A typical eukaryotic somatic cell expresses about 10,000 different proteins averaging 600 copies each,¹ while telomerase expression in a single HEK-293 cell is estimated to be 20 to 50 molecules.¹⁰³ It is thus very challenging to detect telomerase at such a low level in a single cell. In this work, several strategies are employed to achieve sensitive detection of telomerases from a single living cell as illustrated in **Figure 4.1**.

Figure 4.2 Field emission-scanning electron microscope (FE-SEM) micrographs of (a) bare nanotip with diameter around 100 nm and (b) silver-coated nanotip with coating thickness around 100 nm. Inset shows the silver-free surface of nanotip end. Scale bar indicates 50 nm.

The optical fiber nanoprobe has a nanotip with diameter around 100 nm and the sidewall is coated with a thin layer of silver (100-200 nm) to prevent light leakage along the taper length for efficient light transmission to the nanotip (**Figure 4.2**). Overall, the tip diameter is around 500 nm which is much smaller than the size of a cell. In addition, the nanotip has a small taper angle ($< 10^\circ$); thus it is sufficiently small and sharp to penetrate into the nucleus of a single living cancer cell to capture endogenous telomerases directly via antigen-antibody interactions. Here, the silver-free surface area of the tip end is estimated to be in the order of 10^4 nm^2 and the size of telomerase is measured to be around 10 nm from the X ray diffraction of telomerase protein crystals.¹⁰⁶ Assuming 50% nanotip surface coverage, the nanotip would harvest ~ 50 telomerase molecules and consequently tens of alkaline phosphatases. It is rather difficult to detect this low number of enzymes because of the high background caused by light scattering or fluorescent impurities. However, this is successfully overcome by working with an ultras small

detection volume, an approach that has achieved the sensitivity for single molecule detection.¹⁰⁷⁻¹⁰⁹ Typically, when monochromatic light beam is coupled into the nanotip, a subwavelength light source is created since the light beam cannot penetrate the nanotip and the light energy is then transmitted as evanescent wave with exponential decay from the boundary. This evanescent field has extremely low penetration depth (~300 nm) to effectively reduce the detection volume allowing molecules only in close proximity to the nanotip to be excited,¹¹⁰ while significantly eliminating interference from other parts of the 1 mL detection chamber for high signal to noise (S/N) ratio. In this work, signal amplification is also achieved with cleaving DDAO-phosphates by alkaline phosphatases to produce a large amount of DDAOs with fluorescence at 610 nm excitation. The fluorescent signal caused by non-specific adsorption of alkaline phosphatases on the nanoprobe surface is effectively reduced by overnight blocking in bovine serum albumin (BSA).

4.4 Optical Detection of Telomerase in Single Cells

Figure 4.3 Typical fluorescence change obtained upon addition of DDAO-phosphates to nanobiosensors in the detection chamber for four different experimental groups

Fluorescence intensity was monitored in real-time upon addition of the enzyme substrate, DDAO-phosphate. Rather than reporting absolute fluorescence intensities, $(F-F_0)/F_0$ ratio was introduced to reduce the interferences caused by different probes, where F_0 and F are detected fluorescence intensities upon addition of DDAO-phosphates at 0 s and 1800 s, respectively. As depicted in **Figure 4.3**, the $(F-F_0)/F_0$ ratio detected by the nanosensor inserted in MCF-7 nucleus significantly increases within 250 s after DDAO-phosphate addition and continues to increase for up to 1800s. The increase rate of fluorescence presented here is comparable to that of conventional ELISA performed in

microplates. This is conceivable since the reaction kinetics depends on the concentration of the enzyme rather than its absolute number. Although only several tens of telomerases can be captured, leading to also low copies of alkaline phosphatases to be bound, these enzymes are restricted to the nanotip surface in the attoliter detection volume defined by the evanescent field, giving rise to high concentration of enzymes. In fact, an earlier report on the reaction kinetics of single enzyme molecules in femtoliter vials also demonstrates a similar pattern.¹¹¹

In our experiment, nucleus of hMSC cell was used as the negative control since it is reported that hMSC shows low telomerase activity by TRAP assay and its telomerase transcription is repressed.^{112, 113} As compared to MCF-7 nucleus, the fluorescence for nanoprobe incubated in hMSC nucleus only increases slightly for the first 250s and reaches saturation thereafter. Meanwhile, telomerase expression in MCF-7 cytoplasm was also probed and no significant fluorescence increase was observed. This result is in good accordance with literature reports, where fusion protein of GFP and telomerase (GFP-hTERT) expression in telomerase negative cells,¹⁰⁴ immunostaining^{114, 115} and western blot¹¹⁶ of cancer cells have verified that telomerases are mainly localized in the nucleus. Both the MCF-7 cytoplasm and the hMSC nucleus show similar fluorescence profiles as the blank (nanoprobes incubated in cell-free medium), suggesting the extremely low expression levels of telomerases in these cases.

4.5 Telomerase Expressions in Different Cell Lines

Figure 4.4 Optical detection of telomerase. (A) Single cells results (B) Population-averaged results (n = 20 for MCF-7 nucleus, n = 12 for MCF-7 cytoplasm, n = 10 for hMSC nucleus and n = 13 for blank). Error bars represent standard errors of mean (s.e.m). t-test: *p<0.05, **p<0.01, ***p<0.001

Telomerase detection results of individual cells are presented in **Figure 4.4A** as cell-to-cell variations are critical for understanding fundamental biological questions. Large variation in $(F-F_0)/F_0$ ratios between MCF-7 cell nucleus is observed and it is expected that the dominant source of variation comes from the large cell-to-cell variability in protein expression.^{102, 117, 118} This large global difference between cells arises from the difference in cell size, cell cycle state as well as the stochasticity in protein production.¹¹⁸ The statistical significance of the differing expression levels of telomerases in MCF-7 cancer cell nucleus is then verified by performing t-test (**Figure 4.4B**). The average ratio for MCF-7 cancer cell nucleus is significantly larger than that of the negative control (hMSC nucleus) (p<0.001), confirming the positive response of cancer cells and demonstrating that successful detection of telomerases in a single living cell by the optical nanobiosensor. Cytoplasmic detection gives significantly smaller average ratio as

compared to the nuclear detection for MCF-7 cells ($p < 0.05$). The extremely low expression of telomerases in normal stem cells or cytoplasms is not distinguishable by the nanoprobe ($p > 0.05$). Nevertheless, the higher expression level of telomerase in the nucleus of MCF-7 cancer cell¹⁰⁵ can be clearly determined, thus the as-fabricated nanobiosensor is very useful in distinguishing over-expression of telomerases from normal expression levels in individual cells for cancer detection.

4.6 Conclusions

In summary, detection of telomerase over-expression in a single living cell is demonstrated by an optical nanoprobe inserted in a living MCF-7 cancer cell nucleus, showing significantly higher $(F-F_0)/F_0$ fluorescent response than that in the negative control hMSC nucleus. The unique design of the nanoprobe, *in situ* sampling in a living single cell nucleus and followed *in vitro* sandwich ELISA in this method play critical roles in the sensitive detection. Due to the importance of telomerase in cellular proliferation, senescence and carcinogenic transformation, the as-fabricated optical nanoprobe renders an approach to investigate telomerase regulation in various cell types at a single cell level. Particularly, the successful detection of telomerase over-expression in a single living cell for the first time may provide a potential method for cancer detection, and also demonstrates a universal approach that can be used to detect other low expression proteins in a single living cell.

Chapter 5 Optical Detection of Single Cell Lactate Release for Cancer Metabolic Analysis[§]

5.1 Introduction

Cancer cells acquire unique metabolic phenotypes during carcinogenesis; therefore, study of tumor metabolism has great potential for a multitude of applications in oncology, including early cancer diagnosis, disease prognosis, treatment efficiency evaluation and therapeutic development.¹¹⁹ Metabolic study at a single cell level is exceptionally advantageous since it offers the opportunity to reveal cellular heterogeneity and deconvolute complicated metabolic interactions, leading to the deciphering of various metabolic pathways.¹²⁰ Thus, a biosensing platform capable of distinguishing the metabolism of a cancer cell from that of a normal cell at single cell level is particularly important for understanding the molecular basis of carcinogenesis and for early cancer diagnosis and prognosis as well.

A metabolism change that is universal to invasive cancers is the up-regulation of glycolysis, whereby glucose is persistently converted into lactate even when sufficiently oxygenated. The increase in glucose uptake and lactate production, uniquely observed in tumor cells, is known as aerobic glycolysis or ‘Warburg effect’.¹²¹⁻¹²⁴ Particularly, lactate, the end product of glycolysis, needs to be exported from cells to the microenvironment to maintain intracellular homeostasis, which leads to lowering of the extracellular pH and

[§] Adapted with permission from X. T. Zheng, H. B. Yang, C. M. Li*: Optical Detection of Single Cell Lactate Release for Cancer Metabolic Analysis. *Anal Chem*, 82 (12), 5082–5087; Copyright 2010 American Chemical Society.

consequently creating a hostile environment for neighboring healthy cells.^{123, 125} Since a correlation between increased intratumoral lactate concentration and the increased incidence of metastasis has been established in many cancer types, lactate is considered as an important metabolite marker for malignant cancer.¹²¹ Thus, in this work selecting lactate as the detection target to compare metabolisms of different cell lines indeed has fundamental and practical significance.

Through the years, many types of lactate sensors, inclusive of optical¹²⁶⁻¹³¹ and electrochemical¹³²⁻¹³⁶ probes have been fabricated to detect lactate with micromolar detection limit and fast response time. However, most of these methods are based on macro-sized probes which require large amount of samples ($>10^5$ cells) and usually only report the population averaged values. Since even genetically identical cells show large variations in molecular content and such heterogeneity is crucial to understand the cellular processes, single cell analysis is preferred.⁹ Due to the extremely small size of a single cell, the fast speed of biochemical reactions and the trace amount of released chemicals, single cell analysis can only be achieved with a highly sensitive and responsive probe which has comparable or smaller size to a single cell. In recent years, microelectrodes integrated in a microfluidic system has been used to determine the intracellular lactate concentration of a single cell by permeabilizing single myocyte isolated in a microchamber.^{137, 138} Although simultaneous monitoring of Ca^{2+} transients, extracellular pH and cell contraction has been achieved on this microfluidic platform,¹³⁸ this approach requires the target cell to be lysed prior to detection, possibly introducing variations of the original target. Scanning electrochemical microscopy (SECM) has been applied to record the lactate production profile of single cancer cells,¹³⁹ but this method is still technically

difficult as the microprobe employed easily damages the cell during scanning. Another approach is to assay the lactate content of the spent medium for each embryo on an automated microfluidic platform.¹⁴⁰ Unfortunately, embryo isolation and nanoliter medium transfer steps are proven to be tedious and labor-intensive. Therefore, great challenges remain in developing a non-invasive and flexible system for single cell lactate monitoring.

Here, an enzyme based optical fiber nanosensor is developed to detect extracellular lactate concentration at a single cell level. The nanoscale tip is critical in the present study to achieve high spatial resolution, reduce background interferences and avoid cellular damage by the UV excitation light. In addition, the selectivity of the nanosensor is ensured by lactate dehydrogenase, which is covalently immobilized on the nanotip. The as-fabricated optical nanosensor is successful in determining the extracellular lactate concentrations for single cells. Moreover, this nanosensor can also be applied to monitor the effect of metabolic agents on lactate efflux, which opens opportunities to evaluate anti-cancer effects of metabolic agents.

5.2 Preparation and Measurement

5.2.1 Immobilization of Lactate Dehydrogenases (LDH)

The aluminum-free silica surfaces of the nanotip ends were silanized and activated as reported previously.¹⁴¹ Briefly, the silica nanotip ends were silanized by treatment with 5% (v/v) APTES in ethanol at 60 °C for 3 h. After vacuum drying at 120 °C for 2 h, the

nanotip ends were activated by immersing in 5% (v/v) glutaldehyde at 4 °C for 12 h. Next, the activated nanotip ends were incubated in 1.6 mg/mL LDH (Sigma) for 24 h at 4 °C. Finally, the nanotip ends were carefully rinsed three times with PBS and kept in PBS at 4 °C if not in use. This strategy can effectively immobilize LDHs on the aluminum-free silica surfaces.

5.2.2 *In vitro* Calibration

The nanoprobe was first incubated in 1 mL culture medium with 2 mM NAD⁺ to record the steady state background fluorescence (F_0). A known volume of 5 mM standard lactate solution was then added and the resulted steady state fluorescence (F) was measured. Further addition of the standard solution to increase the measured target concentration was repeated and the steady state fluorescence was measured up to 3 mM lactate for the calibration curve.

5.2.3 Lactate Detection for Single Cells

Prior to the single cell detection, the cells were rinsed and NAD⁺ was added to a final concentration of 2 mM in culture medium (phenol red, pyruvate and lactate free). Lactate detection was performed with isolated single cells that were at least 300 μm apart to eliminate interferences from each other. With a 3-axis nanomanipulator system, the nanoprobe was positioned accurately with a deviation less than $\pm 0.5 \mu\text{m}$. To detect the extracellular concentrations of lactate secreted from different cells, the nanoprobe was slowly and precisely located on the cell plasma membrane, whereby the released lactate under catalysis of LDH produced fluorescent NADH and the fluorescence was excited

and collected by the fluorescence detection system assembled as reported in our previous work.^{84, 141}

5.2.4 Effect of MCT Inhibitor on Lactate Release

Before α -CHC treatment, the extracellular lactate concentration was first determined. The same coverslip of cells was then incubated with α -CHC from 0.2 – 0.8 mM for 15 min and after rinsing, lactate concentration was determined again to construct a dose response curve.

5.2.5 Effect of Extracellular NAD⁺ on Cellular Lactate Production

The lactate concentration in the cell supernatant was determined enzymatically by monitoring NADH absorbance. Cells at a concentration of 1×10^6 /mL were incubated in DMEM with 2 mM NAD⁺ for 5 min (the typical experiment duration). Control cells were incubated in NAD⁺ free DMEM. After 5 min incubation, samples of the cell supernatants were drawn and mixed with NAD⁺ (5 mM) in Trizma buffer (pH 8.5). Initial absorbance was recorded. Lactate dehydrogenases (200 U) were then added to the reaction mixture. The absorbance value was recorded again after 10 min. The difference in absorbance was used to calculate the lactate concentrations using a pre-determined lactate calibration curve.

5.3 Single Cell Lactate Detection Principle

Figure 5.1 Detection of extracellular lactate by an optical fiber based nanobiosensor. The nanobiosensor is parked on the plasma membrane of a single cell. The evanescent field from the nanotip illuminates a spatially confined region. The immobilized lactate dehydrogenases catalyze the conversion of lactate into pyruvate and the fluorescence of the byproduct NADH can be monitored by the fluorescence measurement system to determine the lactate concentration. The inset at the top right corner of the figure shows the nanoscale excitation light spot and the inset at the bottom left corner shows the FESEM image of the aluminium-coated nanotip.

Lactate detection relies on the enzymatic action of immobilized LDH, which converts lactate into pyruvate while reducing the cofactor NAD^+ to NADH. When excited at 360 nm, the enzymatic product, NADH, emits fluorescence maximally at 460 nm, which can be detected by our fluorescence detection system. Since the amount of NADH generated is proportional to the lactate concentration, the lactate concentration can be readily determined by monitoring the NADH fluorescence. In the present work, the optical fiber

nanosensor with immobilized LDH is applied for extracellular lactate detection at a single cell level. When the nanoprobe is parked in close vicinity to cell membrane, lactate secreted from the cell is immediately catalyzed by the LDH immobilized on nanotip and NADH fluorescence can be monitored in real time. As shown in the optical micrograph of **Figure 5.1**, the nanotip size is ~100 times smaller than the cell. Field emission-scanning electron microscopy (FESEM) confirms that the diameters of the pulled tips are around 50 nm and aluminum coating increases the diameters to around 250 nm (inset of **Figure 5.1**). This subwavelength aperture at the nanotip allows the formation of a nanoscale light source which is crucial to achieve a high spatial resolution while suppressing the background fluorescence.^{31, 142} By precise positioning of the nanoprobe, the lactate release profiles of individual cells can be mapped to investigate the spatial heterogeneity in extracellular lactate concentrations and further be applied for intracellular analysis. The evanescent field at the nanotip has extremely low penetration depth (~ 300 nm) resulting in an attoliter excitation volume, which is at least 3 orders smaller than that of a microprobe due to significantly reduced free-beam propagation often caused by a micro-aperture, thus effectively suppressing the background.^{31, 142} In particular, the excitation of intracellular NADH is minimized, thus the background from the native NADH becomes negligible. The high signal to noise ratio (S/N) of the nanoprobe was demonstrated experimentally as shown in **Figure 5.2**, in which the background (unmodified probe) is negligible in comparison to the lactate nanosensor. Furthermore, since UV excitation light is harmful to cells, the utilization of a nanoprobe significantly reduces the cell exposure, thus minimizing the damage of the target cell.

Figure 5.2 Comparison between fluorescence signals obtained by the unmodified nanoprobe and the lactate sensitive nanoprobe when parked next to a single cell (n = 3)

5.4 Effect of NAD^+ Concentration on Sensor Response

Since the cofactor NAD^+ is involved in LDH enzymatic reaction, the effect of its concentration on the lactate nanosensor response was first investigated. The lactate nanosensor response in the presence of 4 mM lactate was monitored at various NAD^+ concentrations. As shown in **Figure 5.3**, the fluorescence signal increases with increasing NAD^+ concentration until 2 mM at which a plateau value achieves. This optimal NAD^+ concentration is consistent with that in the literature.¹⁴³ It is also expected that the reaction rate becomes independent of the NAD^+ concentration that is well above the reported K_m value (0.1 mM).¹²⁶ Therefore, the NAD^+ concentration used in all subsequent experiments was 2 mM.

Figure 5.3 Effect of cofactor NAD⁺ concentration on the normalized sensor response in the presence of 4 mM lactate

5.5 *In vitro* Calibration

After optimizing NAD⁺ concentration, each used nanosensor was calibrated *in vitro* for lactate concentrations up to 3 mM prior to the single cell detection. Since NAD⁺ and lactate are non-fluorescent, the absolute fluorescence increase is due to the enzymatically generated NADH. A steady state NADH concentration is established when the rate of enzymatic production is counterbalanced by diffusion of NADH away from the excited region. The background subtracted fluorescence intensity $F - F_0$ is then plotted against lactate concentration and a typical calibration curve is displayed in **Figure 5.4a**. It can be observed that an increase in lactate concentration results in an increase in the fluorescence. The linear response range is from 0.06 mM to 1 mM ($R^2 = 0.996$), which

covers the physiological range of the lactate released from a single cell.¹⁴⁴⁻¹⁴⁶ When the lactate concentration increases to 2 mM, a plateau response occurs, demonstrating a typical enzyme-catalyzed reaction kinetics behavior.^{49, 147} A linear Lineweaver-Burk plot ($R^2 = 0.998$) is also obtained as in **Figure 5.4b**, which further verifies that the enzymes follow the Michaelis-Menten kinetics. Meanwhile, the detection limit is calculated to be *ca.* 20 μM . The real-time response of the nanosensor (inset of **Figure 5.4a**) upon addition of 1 mM lactate shows that the response time is about 1 s and a low background with a signal to background ratio of ~ 80 . The fast response time is possibly attributed to the small sample volume (1 μL) which allows rapidly achieving a uniform concentration by convection transport and greatly enhanced 3-D mass transport rate at the nanotip. Each nanoprobe was calibrated before its use and the measured $F-F_0$ values have a standard deviation of $\sim 7\%$. These results verify that the as-fabricated nanoprobe is suitable for the single cell lactate detection.

Figure 5.4 (a) Calibration curve for lactate detection using the lactate dehydrogenase based optical fiber nanosensor. The inset is the measured real time response of the nanosensor upon addition of 1 mM lactate indicated by the arrow. (b) Lineweaver-Burk plot for the corresponding data in (a).

5.6 Single Cell Lactate Detection

The fabricated nanoprobees were applied to detect the extracellularly released lactate concentration around a single cell. The target cells grown to adhere on the coverslips were rinsed three times to remove previously secreted lactate and fresh medium was added before each measurement. Lactate secreted from the individual isolated cell creates a lactate concentration gradient around a single cell. To determine this concentration gradient, the nanoprobe was first parked 200 μm away from a single HeLa cell and it was manipulated to gradually approach the cell. The fluorescence signal was observed to increase as the nanoprobe approaches the cell membrane. The lactate concentrations at different positions are shown in **Figure 5.5**. The result indicates that the highest lactate concentration exists on the cell membrane. It quickly drops to 40% of the maximum at 25 μm distance away from the cell and becomes even lower at 50 μm away. When the nanoprobe is placed further at 100 or 200 μm from a single HeLa cell, the lactate concentration detected is negligible. The experimental diffusion profile of the released lactate (**Figure 5.5**) is then compared with the theoretical diffusion model expressed as follows:^{148, 149}

$$C_x = C_b + \frac{(C_s - C_b)R}{R + x} \quad (\text{Equation 5.1})$$

Where R is the cell radius, x represents the distance from the cell and C_b and C_s are the lactate concentrations in the bulk solution and at the single cell surface, respectively. Assuming $C_b = 0 \text{ mM}$, $C_s = 0.6 \text{ mM}$ and $R = 10 \text{ }\mu\text{m}$, the simulated lactate response as a function of distance from the cell surface is plotted in **Figure 5.5**, indicating that the

experimental result fits well to the diffusion model. Furthermore, this concentration profile is also consistent with the lactate production profile from a single cancer cell as determined by SECM.¹³⁹ Overall, the result demonstrates that the nanoprobe is capable of determining the extracellular lactate concentration of single cells. It is critical to precisely position the nanoprobe on the cell membrane for accurate lactate detection, which was also emphasized by Wightman et al. in their pioneering studies on single cell exocytosis.¹⁵⁰⁻¹⁵²

Figure 5.5 Measured (data points with standard deviation bars) and simulated (solid line) diffusion profiles of the released lactase. The inset illustrates the hemispherical lactate diffusion zone around a single cell adhered on a glass coverslip.

5.7 Extracellular Concentration Difference between Cancer Cell and Normal Cell

As shown in **Figure 5.6a**, the measured extracellular lactate concentrations adjacent to individual cells vary and this spread is most probably due to cell-to-cell variability in metabolism. Statistical analysis of single cell lactate detection results for three different cell lines is presented in **Figure 5.6b**, showing that the extracellular lactate concentrations for the two human cancer cell lines are significantly higher than the normal hFOB cells (t-test, $p < 0.05$). This result is as predicted by the Warburg effect which generalizes that cancer cells switch on aerobic glycolysis in the course of carcinogenesis leading to higher lactate production and efflux.¹²¹⁻¹²⁴ Besides, HeLa cells of the cervical cancer origin release 70% more lactate than MCF-7 cells, a non-metastatic breast cancer cell line ($p < 0.01$). This difference is quite conceivable as these two cell types are of different tissue origins and are at different stages of cancer development which results in the variation in lactate production rates of these two cell types. Furthermore, this phenomenon may also be explained by the differential expressions of monocarboxylate transporters (MCTs), membrane proteins on the plasma membranes responsible for lactate flux of these two cancer cell lines. HeLa cells express both MCT1 and MCT4,^{153, 154} whereas MCF-7 cells mainly express MCT1.¹⁵⁵ MCT4 is found to be responsible for lactate efflux in highly glycolytic cells;¹²⁵ thus, its exclusive expression in HeLa cell may also account for the higher level of extracellular lactate detected as compared to MCF-7 cells.

Figure 5.6 Cancerous cells exhibit higher extracellular lactate concentrations in comparison to normal cells. (a) Single cell results and (b) statistical analysis of single cell results for HeLa cells (n = 8), MCF-7 cells (n = 7) and hFOB cells (n = 7). Error bars represent standard errors; ** and * indicate $p < 0.01$ and $p < 0.05$ respectively.

5.8 Effect of α -CHC on Lactate Release from Cancer Cells

To demonstrate the capability of the nanoprobe in monitoring metabolic flux inhibition at a single cell level, lactate release after exposure to α -CHC, a MCT inhibitor was investigated. We examined two cancer cell lines including MCF-7 and HeLa cells to construct inhibition profiles from statistical analysis of the single cell responses. As presented in **Figure 5.7**, the extracellular lactate concentrations are lowered for both cell lines after exposure to 0.2 mM α -CHC for 15 min. As α -CHC concentration increases from 0.2 to 0.8 mM, the extracellular lactate concentrations are further reduced. This concentration range is selected because K_i for α -CHC in tumor cells has been estimated to be 0.5 mM.¹²³ In general, the dose-response curves for both cell lines show similar decreasing trends, which apparently verify that α -CHCs can inhibit MCT transporters and consequently block the lactate efflux. However, α -CHC is 3 times more effective in inhibiting lactate efflux from MCF-7 cells in comparison to HeLa cells as revealed by the steeper slope in the inhibition profile for MCF-7 cells. The differential expressions of MCT isotypes may help to explain this disparity in dose responses. Unlike MCF-7 cells which express MCT1 predominantly, HeLa cells express both MCT1 and MCT4 substantially as mentioned previously. Furthermore, the affinity of α -CHC for MCT4 is approximately 5 times lower;^{156, 157} thus, the less effective inhibition of MCT4 leads to the higher level of extracellular lactate for HeLa cells than that of MCF-7 cells after α -CHC application. Inhibition of the lactate efflux by the extracellularly bound α -CHC can cause lactate accumulation in the cytosol and subsequently lowers intracellular pH, which eventually disturbs the cell homeostasis. It is anticipated that prolonged α -CHC application also inhibits pyruvate reduction and prevents the cycling of NAD^+ , thus,

causing metabolic crisis in cancer cells.¹²³ Lactate efflux inhibition profile may provide important indications on the anticancer effect of various metabolic agents. Recently, it is proposed that α -CHC can be used as an anticancer agent to prohibit cancer cell proliferation.¹²³ In our experiment, it was observed that the cell growth is greatly inhibited for both MCF-7 and HeLa cells when α -CHC final concentration in culture medium is greater than 2 mM. Thus, monitoring the lactate flux inhibition at a single cell level provides great potential in evaluating the anti-cancer effect of various metabolic agents.

Figure 5.7 Change in extracellular lactate concentrations of MCF-7 (n = 6) and HeLa cells (n = 4) in response to increasing concentrations of α -CHC, a MCT transporter inhibitor expected to blocks lactate efflux and consequently lowers the extracellular lactate concentrations. Error bars represent standard errors; ** indicates $p < 0.01$.

Since NAD^+ is an important molecule for energy metabolism,¹⁵⁸ it is necessary to understand whether the extracellular addition of NAD^+ possibly affects the cellular

metabolism. It is known that the immediate effects of extracellularly applied NAD^+ are usually exerted through the catalysis of an enzyme, CD38.^{159, 160} Since this enzyme is not expressed in the three target cell lines used,¹⁶¹⁻¹⁶³ the metabolic outcomes are unlikely to be altered significantly in our experiments. Further experiments have been conducted to determine the lactate concentrations from the cell supernatant of cell populations (**Figure 5.8**), also indicating that the extracellular lactate concentrations are not significantly altered after 5 min incubation in cell medium with 2 mM NAD^+ . To fully understand the potential effects of the extracellular NAD^+ on cells and allow this technique to be universally applied to all cell types, we intend to integrate the cofactor-free lactate detection schemes^{130, 131} with our nanoprobe for investigation.

Figure 5.8 The extracellular application of 2 mM NAD^+ does not affect the lactate productions of the cell populations ($n = 3$). The error bars represent the standard deviations. Student's t test was performed to confirm that lactate production of the NAD^+ treated cells is not significantly different from the control cells.

5.9 Conclusions

Single cell analysis for tumor cell metabolism is of great importance since it promises to provide deep insights for carcinogenesis by revealing cell-to-cell variations. This work presents an enzyme based optical fiber nanosensor with high spatial resolution, low background interference, and a dynamic range (0.06–1 mM) comparable to the physiological range of the single cell released lactate to well detect the extracellular lactate concentration associated with the tumor cell metabolism at a single cell level. The extracellular lactate detections for three cell lines demonstrate that the nanosensors are capable of distinguishing the higher extracellular lactate levels for cancer cell lines as compared to the normal cell line. Furthermore, the effect of the MCT inhibitor on HeLa and MCF-7 cells was also investigated, indicating that the different lactate efflux inhibition profiles corroborated with the difference in MCT isotype expressions. This work provides a powerful tool to noninvasively study the fundamental metabolic processes at a single cell level and could further be used in diagnosis of early development of cancer cells. Further work is underway to explore the spatial variation of lactate concentrations at a fixed distance from the single cell and to investigate the effect of aging on single cell lactate profile.

Chapter 6 Bifunctional Electro-optical Nanoprobe to Real-time Detect Local Biochemical Processes in Single Cells^{}**

6.1 Introduction

Complicated cellular activities such as DNA repair, oxidative activity, programmed cell death and transcriptional regulation are usually controlled by multiple intricately interconnected signaling pathways.^{10, 11} Traditional work in biology has focused on studies of individual components in a signaling pathway. Although these investigations have made significant progress in discovering various signaling components, fully unraveling of the complex cellular processes and their regulations demands simultaneous detections of multiple parameters correlated in time and space. As a critical cellular process, the maintenance of redox homeostasis depends on the interplay of reactive oxygen species (ROS) generation and elimination systems. The deregulation of such systems can lead to oxidative stress that inflicts biological damages and is implicated in various pathological conditions including cancer, cardiovascular, inflammatory and degenerative diseases.^{164, 165} Thus, a real-time quantitative detection method to simultaneously analyze the dynamics of ROS production and antioxidant system is of great importance to study the oxidative stress in living cells. Through the years, many methods including optical^{166, 167} and electrochemical methods¹⁶⁸⁻¹⁷¹ have been developed for the real-time monitoring of oxidative bursts in single living cells. However, these

^{**} Reproduced with permission from X. T. Zheng, W. H. Hu, H. Wang, H. Yang, W. Zhou and C. M. Li*, Bifunctional Electro-optical Nanoprobe to Real-time Detect Local Biochemical Processes in Single Cells. *Biosens Bioelectron*, 26, 4484-4490, 2011. Copyright Elsevier.

studies either focus on the measurement of ROS production alone, or perform unrelated asynchronous measurements of the ROS production and antioxidant contents, leading to difficulty in data interpretation.

To achieve real-time simultaneous detections of multiple biochemical processes, combinations of different techniques including electrical, fluorescence or surface plasmon resonance have been proposed.¹⁷²⁻¹⁷⁵ However, these combinations mainly rely on macro or micro-sized probes which are incompatible with localized detections. Previous studies have confirmed that spatial heterogeneities of biomolecule distributions and biochemical processes exist in single cells.¹⁷⁶ Thus, the ability to monitor local biochemical events is greatly needed for a more precise and in-depth analysis. Recently, combined scanning electrochemical microscopy (SECM)/optical microscopy (OM) utilizing a nanosized scanning probe has been reported to obtain static fluorescent and electrochemical images of PC12 neurites⁷⁴ and Gene-transfected HeLa cells⁷¹ with high spatial resolution, but its long acquisition time renders the hybrid system inapplicable for dynamic simultaneous detections of fast cellular activities such as oxidant generation and antioxidant change under oxidative stress. Furthermore, these unmodified probes do not guarantee the target specificity for the electrochemical measurements and usually only allow qualitative comparisons. In addition, mediators are frequently added extracellularly, which could affect the cell physiology to interfere with the measured results.

Figure 6.1 Schematics of the experimental setup for simultaneous detections. The nanoprobe is placed on the cell membrane for simultaneous optical and electrochemical detections of cellular activities. The excitation light from the nanoaperture illuminates the fluorescent dye loaded single cell and the emitted fluorescence is recorded by the fluorescence measurement system. Meanwhile, the electroactive species released from single cell is detected electrochemically by the gold nanoring electrode.

Herein, a unique electro-optical nanoprobe was fabricated from a gold-coated optical fiber nanotip via a focused ion beam (FIB) process to detect oxidative stress in a single cell, in which the nanoscale probe can investigate a local biological process while the

bifunctional electro-optical sensing scheme provides real-time detection with more information. As depicted in **Figure 6.1**, the optical fiber core of the nanoprobe efficiently delivers the excitation light to achieve high-resolution optical detection of intracellular activities, while the electrocatalyst modified gold nanoring surrounding the silica core as a nanoelectrode can simultaneously monitor a specific extracellular chemical event by quantitative electrochemical measurements. By precise positioning of the nanoprobe at selected locations on the cell membrane, distinct ROS generation and antioxidant levels are site-detected in cancer cells as compared to the normal ones. This is the first demonstration of electro-optical nanoprobe based real-time local dynamic monitoring of multiple cellular activities at a single cell level.

6.2 Preparation and Measurement

6.2.1 Cell Preparation

The cells adhered to a coverslip were washed with buffer solution and loaded with one of the following dyes: DCF-DA (20 μ M) or CMFDA (40 μ M) for 30 min at 37°C. After incubation, the stained cells were rinsed three times with buffer and transferred to the detection chamber for single cell measurements. The cells may be incubated with PAO (5 μ M) for 20 min at 37 °C prior to detection.

6.2.2 Electro-optical Nanoprobe Modification

The gold nanoring was exposed by milling with FIB. The nanoelectrode was further modified by the electrochemical deposition of Prussian Blue (PB) in a 2 mM $K_3[Fe(CN)_6]$

with 0.1 M KCl and 1 mM HCl.⁴⁶ The potential was cycled at 50 mV s⁻¹ between -0.2 and 0.6 V for 50 cycles. The PB modified nanoelectrode was subsequently rinsed with DI water and allowed to dry at room temperature.

6.2.3 Electrochemical Characterization of the Electro-optical Nanoprobe

Both cyclic voltammetry and amperometry were performed using an Autolab electrochemical station (PGSTAT 30, Eco chemie). The reference electrode was Ag/AgCl electrode and the counter electrode was a Pt wire. All electrochemical measurements were performed inside a Faraday cage. Cyclic voltammetry was performed in 10 mM K₃[Fe(CN)₆] to estimate the actual electrode size, while amperometry was performed at 0.0 V vs. Ag/AgCl to obtain a calibration curve for H₂O₂. The difference between the two steady state currents, before and after each H₂O₂ injection was defined as the nanoprobe's response and plotted against H₂O₂ concentration to establish the calibration curve. The low H₂O₂ concentration detection limit was defined by a signal/noise (S/N) ratio of 3. The electrodes were characterized before and after each measurement. Each electrode can be used to detect 3-5 cells without significant fouling.

6.2.4 Simultaneous Optical and Electrochemical Measurements in Single Cells

Single cell experiments were performed at room temperature (23 °C) on the stage of an inverted microscope (Olympus IX71) in a Faraday cage with all room lights switched off. The detection chamber containing adherent cells was washed three times with buffer and filled with fresh buffer immediately prior to measurements. The nanoprobe was precisely positioned with a nanomanipulator (0.5 µm precision) at fixed locations on the cell membrane of an isolated cell that was at least 300 µm away from other cells. The

ROS generation upon 10 μM PMA stimulation was detected in real-time by amperometry. At the same time, the intracellular fluorescence signal was also recorded by the fluorescence detection system assembled as reported.^{84, 141}

6.3 Characterization of the Electro-optical Nanoprobe

Figure 6.2 Characterization of the electro-optical nanoprobe. (a) FIB image of the electro-optical nanoprobe; (b) Cyclic voltammogram (CV) of a nanoprobe in 10 mM $\text{K}_3[\text{Fe}(\text{CN})_6]$ and 0.1 M KCl at a scan rate of 50 mVs^{-1} ; (c) CV of the unmodified and PB modified nanoprobe in 0.01 M PBS buffer (pH 7.4) at 50 mVs^{-1} ; (d) The calibration plot of steady state currents against H_2O_2 concentration. The inset shows the linear region.

The FIB image (**Figure 6.2a**) of a typical electro-optical nanoprobe clearly reveals an optical fiber aperture (*ca.* 100 nm in radius) surrounded by a ring of gold (*ca.* 100 nm thickness), and further protected by a layer of insulating polymers (*ca.* 100 nm thickness). The aperture, being smaller than the excitation wavelength, effectively confines the excitation light in the evanescent field with extremely low penetration depth (~ 300 nm), leading to highly localized excitation. Such design is crucial for data acquisition as it is able to achieve low background and high spatial resolution.¹⁷⁷ Furthermore, the reduced excitation intensity from the aperture could also minimize dye bleaching.

The typical voltammetric response of the nanoelectrode in 10 mM potassium ferricyanide ($\text{K}_3[\text{Fe}(\text{CN})_6]$) (**Figure 6.2b**) shows a sigmoidal curve with a diffusion-limited current of 0.65 nA. Although the nanoprobe appears to be a ring electrode, it can be approximated as a disk electrode because the ratio of the outer-to-inner radius is greater than 1.25.¹⁷⁸ Based on the diffusion-limited current equation for a disk electrode ($i = 4nFDcr$, where n is the number of electrons transferred per molecule, F is the Faraday constant, D is the diffusion coefficient, c is the concentration of electroactive species, and r is the radius),¹⁷⁸ the radius is calculated to be 220 nm, which is consistent with the radius measured from the FIB image (**Figure 6.2a**). Similar to the optical detection, a nanoelectrode senses only local electrochemical properties of a sub-micrometer sized domain, because the thickness of the diffusion layer is equal to a few nanoelectrode radii.⁵⁴ Another great advantage is that the small double layer capacitance of nanoelectrodes with high signal to noise (S/N) ratio enables submicrosecond monitoring of cellular activities,⁴² which is very critical to investigate a fast kinetic bioprocess in a

single cell. Moreover, the markedly enhanced fluxes at nanoelectrodes can further improve S/N ratio in electrochemical sensing.⁴²

Figure 6.3 (a) Typical amperometric responses of a PB-modified nanoelectrode to successive additions of H_2O_2 at 0.0 V vs. Ag/AgCl reference electrode in 0.01 M PBS buffer (pH 7.4). (b) Calibration curves for H_2O_2 obtained at unmodified and PB-modified nanoelectrodes at 0.0 V vs. Ag/AgCl reference electrode. (c) The selectivity of the PB-modified nanoelectrode against common interfering agents including ascorbic acid (AA), dopamine (DA) and nitric oxide (NO) at 0.0 V vs Ag/AgCl reference electrode.

ROS generated during oxidative stress usually encompass many species. To specifically monitor a particular ROS, the nanoelectrode needs further modification for good specificity. Here, the nanoelectrode is electrodeposited with an electrocatalyst Prussian Blue (PB) to selectively detect H_2O_2 , a valuable marker for oxidative stress. PB is also known as “artificial enzyme peroxidase” due to its high activity and selectivity in H_2O_2 reduction.¹⁷⁹ **Figure 6.2c** shows a characteristic redox pair at the formal potential of ~ 0.20 V corresponding to the reversible conversion of Prussian White to PB¹⁸⁰ for the modified nanoelectrode, while it is absent from the unmodified nanoelectrode, confirming the successful electrodeposition of PB on the nanoelectrode. Typical amperometric responses of a PB-modified nanoelectrode to successive concentration changes of H_2O_2 measured at 0.0 V vs. Ag/AgCl are shown in **Figure 6.3a**. The corresponding calibration curve is illustrated in **Figure 6.2d** where the highest catalytic current can be obtained for up to 850 μM H_2O_2 , and the inset shows the linear range of the calibration curve. It is thus evident from the graph that the amperometric response towards the reduction of H_2O_2 is linear up to 260 μM ($R^2 = 0.994$) with a measured sensitivity of $12.4 \text{ pA}\mu\text{M}^{-1}$, a detection limit of 0.387 μM , and a response time of ~ 0.5 s. However, no amperometric responses are observed at the unmodified nanoelectrode with the addition of H_2O_2 at the same potential (**Figure 6.3b**). This is due to the excellent electrocatalytic activity of PB towards H_2O_2 , which allows the H_2O_2 reduction at a low potential to achieve the maximum diffusion controlled current. Furthermore, at this low potential of 0.0 V, the common coexisting electroactive species such as ascorbic acid, dopamine and nitric oxide at a concentration of 40 μM produce no observable current responses (**Figure 6.3c**), verifying that the PB-modified nanoelectrode is highly selective for H_2O_2 detection. These results

indicate that the nanoprobe fulfils the requirements of in situ H_2O_2 detection at a single cell level.¹⁸¹

6.4 Simultaneous Optical and Electrochemical Detections of Oxidative Stress in a Single Living Cell

Figure 6.4 Fluorescence images of MCF10A (a, d & g) MCF7 cells (b, e & h) and MCF7/HER2 cells (c, f & i) stained for its intracellular redox state (a-c) and reduced thiol level (d-e) by DCF-DA (20 μM) and CMFDA (40 μM) for 30 min prior to PMA stimulation, respectively.

To investigate the relationship between oxidative stress and cancer progression, the electro-optical nanoprobe was applied to examine oxidative stress in three breast cell lines including a normal mammary epithelial cell line (MCF10A), a mammary adenocarcinoma cell line (MCF7) and a drug-resistant HER2-overexpressing MCF7 cell line (MCF7/HER2). The over-expression of HER2, an oncoprotein belonging to the human epidermal growth factor receptor (HER) family, has been correlated with chemoresistance

of breast cancer.¹⁸² Prior to simultaneous detections, the target cells were stained with specific fluorescent dyes such as DCF-DA or CMFDA for monitoring the intracellular redox state or intracellular reduced thiol level, respectively. The whole cell fluorescent images recorded by confocal laser scanning microscope (CLSM) confirm the successful staining of the cells (**Figure 6.4**). Fluorescent dyes are most suitable for assessing the general intracellular status, however they may not be specific to a single target and usually only give qualitative results; whereas an electrochemical sensor can easily quantify the target and real-time data can be further analyzed to obtain critical parameters about the biochemical process. As a demonstration, here we combine the fluorescence and electrochemical detections for a thorough investigation of the oxidative stress in single cancer cells.

Figure 6.5 Dynamics of H_2O_2 burst release to extracellular space (a-c) and simultaneous changes of intracellular redox state (d-f) or reduced thiol level (g-i) in MCF10A (a, d & g), MCF7 (b, e & h) and MCF7/HER2 (c, f & i) detected by an electro-optical nanoprobe. The current decay profiles simulated according to Equation (6.2) are represented by $\square$, $\circ$ and Δ for MCF10A, MCF7 and MCF7/HER2 cells, respectively.

During each detection, the nanoprobe is precisely positioned in the peripheral domain of the membrane to ensure fair comparison of the data obtained from three types of cells. **Figure 6.5** displays the typical real-time traces from the electrochemical detection of H_2O_2 generation dynamics and the simultaneous optical detection of intracellular changes in the three target cell lines upon phorbol 12-myristate 13-acetate (PMA) stimulation. PMA, a diacylglycerol analogue which activates protein kinase C (PKC), is applied to elicit oxidative responses in the target cells.^{183, 184} For single MCF7 cell, both the amperometric and DCF fluorescence signals (reflects the intracellular redox state) increase immediately following PMA injection (**Figure 6.5b and e**), indicating that the H_2O_2 release to extracellular environment synchronizes with the shift to a more oxidized intracellular state. In contrast, the same addition of PMA in the absence of cells does not produce any appreciable amperometric and fluorescence responses (data not shown), confirming that both amperometric and the fluorescence signals originate from the activation of single cell's oxidative activity by PMA. On the other hand, in the absence of PMA, both the signals show no significant change within the detection duration of 600 s for all three cell lines (**Figure 6.6**), proving that PMA stimulation is necessary for eliciting the oxidative stress. It is worth mentioning that the introduction of catalase, a selective H_2O_2 scavenger,¹⁸¹ into the cell medium completely suppresses the current (**Figure 6.7**), verifying that H_2O_2 is the ROS species detected, and the acquired amperometric response can be attributed to H_2O_2 .

Figure 6.6 Time courses of the amperometric signals (top), fluorescence signals representing the intracellular redox state (middle) and the reduced thiol level (bottom) of MCF10A, MCF7 and MCF7/HER2 cells as detected by an electro-optical nanoprobe in the absence of PMA stimulation.

Figure 6.7 The addition of catalase completely suppresses the amperometric signal thus the current can be ascribed to H_2O_2 reduction.

Upon the PMA injection, the amperometric signal from single MCF7 cell immediately rises to achieve a current peak of 80 pA after about 40 s (**Figure 6.5b**), indicating an increase in the local concentration of H₂O₂ in response to the PMA injection. Due to the cell-adhered location and the fast response of the nanoelectrode, we can estimate that the local concentration of H₂O₂ at the cell surface increases from 0 to 6.97 μ M in 40 s according to the calibration curve shown in **Figure 6.2d**. In this process, the released H₂O₂ diffuses into the extracellular bulk solution and the effective thickness (δ) of the diffusion layer at the time of peak current around the cell can be calculated based on

$$\delta = (\pi Dt)^{1/2} \quad \text{(Equation 6.1)}$$

where D is the diffusion coefficient of H₂O₂ and t is release time (from the onset of H₂O₂ release to that reaching peak current). Assuming a typical value of $10^{-5} \text{ cm}^2 \text{ s}^{-1}$ for D and a constant concentration gradient in the diffusion field,¹⁸⁵ we can calculate the amount of released H₂O₂ by the cell during the PMA stimulation, as listed in **Table 6.1**, which clearly illustrates that the cancer cells produce much larger quantities of H₂O₂ in a shorter release time upon oxidative stress as compared with the normal cells.

Table 6.1 Important parameters obtained based on the chronoamperograms displayed in Figure 6.5

Cell line	Peak current (pA)	Peak concentration (μM)	Release time (s)	Diffusion layer thickness (μm)	Total amount of H ₂ O ₂ released (pmol mm ⁻²)
MCF10A	13.4	1.08	57.8	426	0.230
MCF7	86.4	6.97	41.4	361	1.23
MCF7/HER2	56.7	4.57	40.9	358	0.818

After attaining the peak current, the current gradually decreases because the released H₂O₂ continuously diffuses to the bulk solution, resulting in decrease of the local concentration. The spontaneous decomposition of H₂O₂ is slow so it is negligible within the measurement duration.¹⁸⁶ Assuming that at time $t = t_0$ all H₂O₂ molecules start to diffuse from the cell surface, the concentration profile in the diffusion field is a function of the position and the time according to¹⁸⁷

$$C(x, t) = \frac{N_0}{\sqrt{\pi Dt}} \exp\left(\frac{-x^2}{4Dt}\right) \quad \text{(Equation 6.2)}$$

where x represents the distance to the cell surface ($x = 0$ on the cell surface) and N_0 represents the amount of H₂O₂ released by the cell (mol mm⁻²). The simulated curves based on **Equation 6.2** are presented in **Figure 6.5a-c**, which fit well with the experimental curves. It can be noted from **Equation 6.2** that the concentration decreases

exponentially with the distance x . Therefore, to sensitively detect the species released by cell, the electrode should be positioned as close as possible to the cell and at $x = 0$ (i.e., on the cell surface) the strongest response could be obtained, which has been proved by previous work.¹⁶⁸ Larger x value can result in two major drawbacks: lower amperometric response is likely to be detected for a given release while the peak current temporally delays as compared to the cell release so that the amperometric response loses its real-time nature. Therefore, the application of such a nanoprobe designed in the present study that can be placed intimately at the cell surface is very critical for real-time detection and high sensitivity.

At the same time, the DCF fluorescence first increases by 20%, followed by a slower rise phase lasting 280 s before the signal starts to decay (**Figure 6.5e**). Both signals confirm the onset of oxidative stress in the MCF7 cell upon PMA stimulation. Simultaneous measurements offer the advantage to verify and compare the signals, thus eliminating false positive detections and ensuring an accurate evaluation of cellular activities. By successfully acquiring the intracellular and extracellular signals, another important feature of the bifunctional nanoprobe can thus be demonstrated for its ability to correlate two cellular events taking place in two separate compartments.

It is known that the cellular redox homeostasis is controlled by balancing ROS generation with their elimination through the ROS scavenging system.¹⁶⁴ As such, it is advantageous to simultaneously measure the ROS generation along with the antioxidant level in the same single living cell for correlation of their changes and evaluation of the cellular oxidative status under oxidative stress. Herein, concurrent measurements of the intracellular reduced thiol, a major antioxidant, and H_2O_2 generation are achieved by a

bifunctional nanoprobe positioned on a CMFDA labeled MCF7 cell. As observed from **Figure 6.5h**, PMA triggers a significant increase in the CMF fluorescence lasting around 300 s, after which the fluorescence starts to drop and eventually decreases below the initial level. In other words, in order to counteract the oxidative stress, the MCF7 cell raises its intracellular thiol level initially, which is depleted eventually.

Figure 6.8 Statistical comparison on the amplitude of (a) H_2O_2 burst detected amperometrically; (b) alteration in intracellular redox state and reduced thiol level via fluorescence monitoring upon PMA stimulation in single MCF10A, MCF7 and MCF7/HER2 cells ($n = 12$). Error bars represent standard errors. $p < 0.05$ for comparison between any two groups.

It is evident from **Figure 6.5 and 6.8** that the PMA induced oxidative responses differ significantly among the three cell lines, which may be correlated with the cell malignancy. The H_2O_2 generation is the highest in MCF7 cells, considerably less in MCF7/HER2 cells and the lowest in MCF10A cells from the statistical analysis of single cell data (**Figure 6.8a**). The higher levels of H_2O_2 released from the cancerous MCF7 and MCF7/HER2 cells as compared to the normal MCF10A cells indicate the higher oxidative stress in cancer. This observation agrees well with literature reports that ROS level is elevated in many types of cancer cells.¹⁶⁴ As shown in **Figure 6.8b**, the extents of the PMA-induced shift to a more oxidized cellular state also decreases in the order of the cancerous MCF7 cells, the MCF7/HER2 cells and the normal MCF10A cells, which is consistent with the trend of H_2O_2 release.

In response to a minimal oxidative stress, a MCF10A cell only requires a slight rise in the thiol level to counteract the stress. In contrast, the intracellular thiol levels in MCF7 and MCF7/HER2 cells display a trend opposite to the change in the redox state. PMA elicits a much larger initial increase in the thiol level in MCF7/HER2 cells, both larger in amplitude and longer in duration, in comparison to MCF7 cells. This observation implies that the former can better counteract the oxidative stress upon stimulation, which also explains the milder oxidative stress in MCF7/HER2 cells. Based on the above results, it is reasonable to hypothesize that the drug resistance characteristics of MCF7/HER2 cells may derive from their superior ability to maintain a redox balance upon drug stimulation.

Generally, distinct oxidative responses and antioxidant levels are detected in the three breast cell lines. Redox balance is maintained in normal MCF10A cells by delicately balancing the generation and elimination of ROS. Malignant transformation leads to the

ROS generation in cancerous MCF7 and MCF7/HER2 cells which can only be partially compensated by the induction of antioxidants, resulting in an overall oxidizing environment. The more intense oxidative stress and the altered level of antioxidant molecules in the cancerous cells indicate critical genetic changes during malignant transformation. Further increase in the antioxidant level in MCF7/HER2 cells may confer drug resistance by protecting the tumor from excessive oxidative stress. Simultaneous real-time analysis of ROS generation and antioxidant level helps to understand the redox changes associated with the malignant transformation, which is potentially useful in cancer diagnosis and prognosis.

6.5 NADPH Oxidase Inhibition Suppresses the Oxidative Response

Figure 6.9 Simultaneous real-time detections of H_2O_2 burst (a) and intracellular redox state (b) before (red) and after (blue) applications of PAO to the MCF10A (top), MCF7 (middle) and MCF7/HER2 (bottom) cells

In many biological pathways, H_2O_2 is derived from the spontaneous or catalytic breakdown of superoxide, which in turn is produced by the partial reduction of oxygen in mitochondria or by membrane-associated NAD(P)H oxidase (NOX).¹⁸⁸ To determine whether this enzyme complex is responsible for PMA-stimulated oxidative response in these breast cell lines, we performed inhibition experiments using a NOX inhibitor – phenylarsine oxide (PAO).¹⁸⁸ As shown in **Figure 6.9**, the application of PAO leads to complete diminishment of the H_2O_2 amperometric signal; while the increase in the DCF fluorescence can no longer be detected, indicating that no change is induced in the intracellular oxidation state. These observations evidence that the NOX activation is a major source of ROS in PMA-induced oxidative stress in these breast cell lines.

6.6 Subcellular Location-dependent Detection of Oxidative Response

Taking advantages from the submicrometer size of the nanoprobe, we further investigate the oxidative response of the PMA-stimulated MCF7 cells at specific locations. Simultaneous detections were performed at two selected membrane domains: the peripheral domain and the central domain lying right above the nucleus (Inset of **Figure 6.10a**). As shown in **Figure 6.10a and b**, both the H_2O_2 signal and the DCF fluorescence is about two times higher in the central domain than the peripheral domain, indicating that oxidative stress is spatially confined which may be caused by the specific targeting of the activated NOX enzymes to the central domain,¹⁸⁹ giving rise to the higher H_2O_2 production level as well as the more oxidized intracellular state. Such large contrast in the obtained data is confirmed by the statistical analysis (**Figure 6.10c and d**). It is

important to point out that detections in the peripheral domains at different positions on the cell circumference give consistent data with insignificant variations (data not shown), confirming that the oxidative response mainly differs along the radial direction, i.e., positions at different distances to the nucleus. This result not only demonstrates the advantage of the sub-micrometric size of the probes to distinguish specific activities of different membrane regions, but also discovers that the main target of oxidative stress is spatially confined in the central domain of a cell.

Figure 6.10 Localized detection of the oxidative response at different cellular locations. (a) Dynamics of H_2O_2 burst release to extracellular space, (b) simultaneous changes in intracellular redox state; statistical comparison on the amplitude of (c) H_2O_2 burst detected and (d) alteration in intracellular redox state in different membrane domains of MCF7 cells ($n = 12$). Error bars represent standard errors, * indicates $p < 0.05$ and ** indicates $p < 0.01$. The inset in (a) shows the top view of an adherent cell. The peripheral and central domains are indicated by the blue and red crosses, respectively. The current decay profiles simulated according to Equation (2) are represented by $\circ$ and $\square$ for peripheral and central domains, respectively.

6.7 Conclusions

We developed a bifunctional electro-optical nanoprobe to study the local dynamic cellular oxidative activity at a single cell level by simultaneously detecting electrical and optical signals. The optical fiber core allows monitoring the intracellular fluorescence change corresponding to the general redox state or reduced thiol level, while the nanoring electrode enables real-time, quantitative and sensitive amperometric detection of extracellular H_2O_2 generation at the same location. The simultaneous detection reveals distinctively higher oxidative responses and altered antioxidant levels in the cancer cells as compared to the normal ones, suggesting that the cell malignancy correlates with the strength of oxidative stress, and the higher antioxidant level may be the cause of drug resistance. Furthermore, the nanoscale probe enables localized simultaneous detections in different membrane regions to determine the activated enzyme targeting location. This work demonstrates that this uniquely structured nanoprobe with ultra-low detection volume is potentially applicable for universal monitoring biochemical reactions with significantly reduced background and enhanced S/N ratio, providing a unique opportunity to probe chemical dynamics in a nanoscopic environment for elucidating key biological processes.

Chapter 7 Anticancer Efficacy and Subcellular Site of Action Investigated by Real-time Monitoring of Cellular Responses to Localized Drug Delivery in Single Cells^{††}

7.1 Introduction

Subcellular targeted drug delivery has become extremely attractive to cure infectious diseases and cancers, because many drugs have their targets confined to specific subcellular compartments as the action sites.¹⁹⁰ Drugs need to be delivered to the appropriate subcellular sites of action for maximum therapeutic effect and minimal side effects,¹⁹⁰ but the sites of action are usually not predictable because many drugs, especially those derived from natural origins, may not have identified molecular targets yet, or may act on multiple targets associated with different organelles.^{176, 191} Since around half of the small molecule anticancer drugs discovered such as camptothecin, etoposide and paclitaxel are derived from natural origins like herbal plants,¹⁹² it is important to investigate drug-cell interactions at a nanoscale level to evaluate the medical effects and identify the subcellular site of action, which could provide scientific insights for designing subcellular targeted drug delivery strategies to realize full therapeutic potentials.^{190, 193}

Currently, medical effects of drugs are mainly studied by tissue-specific or cell-specific delivery.¹⁹⁴⁻¹⁹⁸ Unfortunately, this approach leads to either drug sequestration in endocytotic pathways or pervasive drug distribution throughout a cell,¹⁹⁰ and it is very

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difficult to accurately evaluate drug efficacy and side effects. Recently, subcellular targeting ligands have been attached to nanocarriers for delivery into desired intracellular organelles.¹⁹⁰ Nevertheless, the complicated intracellular environment still presents a significant barrier to efficient organelle targeting.^{190, 199, 200} Therefore, subcellular drug delivery and simultaneous detection of cellular responses remain a largely unmet challenge.

β -lapachone (β -lap), as one example of the naturally derived drugs, was first isolated from the bark of the Lapacho tree (genus *Tabebuia*). It is a potent antitumor agent that selectively kills cancer cells but is non-toxic to normal cells.^{201, 202} β -lap is able to trigger a series cellular responses including reactive oxygen species (ROS) generation, Ca^{2+} elevation, ATP depletion and poly(ADP-ribose) polymerase 1 (PARP-1) hyperactivation that eventually leads to cancer cell death.²⁰³ However, the detailed mechanism of action has not been elucidated and the subcellular site of action for the highest anticancer efficacy has not been determined.²⁰¹

In this study, we investigate the drug-target interaction at a subcellular resolution using a nanoscale rGO-functionalized optical fiber probe to efficiently load and deliver an anticancer drug, β -lapachone (β -lap) to subcellular domains while concurrently detect the cellular responses in real-time (**Figure 7.1a**). The results reveal the spatial heterogeneities of the cellular responses to subcellular β -lap delivery. Analysis of ROS and Ca^{2+} changes can identify the subcellular site of action for β -lap to have the best anticancer efficacy. Overall, this study provides valuable scientific information about the subcellular level drug-cell interaction that is not obtainable from conventional cell-specific delivery and evaluation methods.

Figure 7.1 Subcellular delivery of anticancer drugs into living cells. (a) Schematic illustrating the subcellular delivery of drug and simultaneous monitoring of cellular responses. (b) Field-emission scanning electron microscopy (FESEM) image of a rGO-functionalized optical fiber nanoprobe. (c) High magnification FESEM image showing the nanoscale rGO-functionalized tip (d) Procedure for assembling rGO on the nanoprobe surface via electrostatic interactions.

7.2 Preparation and Measurement

7.2.1 Cell Preparation

Prior to localized intracellular delivery, the MCF-7 cells were stained with DCF-DA (20 μM) or Fluo-4 AM (5 μM) and/or Mitotracker red (200 nM) for 30 min at 37 °C. The actin filaments were stained by incubating fixed and permeabilized cells with Rhodamine-phalloidin for 30 min.

7.2.2 Fabrication of rGO-functionalized Optical Fiber Nanoprobe

Optical fiber nanoprobe was pulled and silanized as reported.^{141, 177} Stable dispersion of rGO was prepared as reported.⁴⁶ The aminopropyl triethoxysilane (APTES) functionalized nanoprobe was then modified with rGO by incubating with rGO solution (0.05 mg mL⁻¹) for 1 hr. The morphology of the nanoprobe was examined by field-emission scanning electron microscope (FESEM; JEOL, JSM-6700F, 5 kV). Zeta potential measurements were performed using a Zetasizer 3000 (Malvern Instruments).

7.2.3 Localized Intracellular Drug Delivery by Optical Fiber Nanoprobe

A rGO modified nanoprobe was immersed in aqueous β -lap solution (0.1 mg mL⁻¹) at specified durations to load β -lap. With the help of a nanomanipulator (0.5 μ m precision), the nanoprobe was precisely inserted into specific intracellular locations in an isolated cell for localized β -lap delivery. The change in DCF or Fluo-4 fluorescence can be measured by the home-built fluorescence detection system to determine the drug action at intracellular targeted sites.

7.3 Characterization of rGO-functionalized Optical Fiber Nanoprobe

Optical fiber probes with $\sim$ 100 nm tips were tapered from micrometer multimode optical fibers by a heating and pulling method.^{68, 141, 177} Unlike the conventional conical glass nanopipette, the nanoprobe has a small taper angle, leading to a gradual increase in the tip diameter away from the tip end. The diameter only increases to $\sim$ 500 nm at 3 μ m from the tip end (a typical inserted depth), and the displaced volume upon the insertion is

~ 0.2 fL, 3 orders smaller than the volume of a single cell. Thus, the possible mechanical cellular damage upon penetration could be effectively minimized. The optical fiber nanoprobe could also detect optical and/or electrochemical signals from single living cells to monitor drug responses.^{68, 84, 141, 177}

Figure 7.2 (a) β -lapachone loading capacity of rGO as compared to SiO_2 (b) release percentage after 24-hr incubation in PBS or culture medium

The hydroxyl terminated silica surface of the optical fiber shows very weak interaction with aromatic drugs and thus has almost no ability of loading β -lap (**Figure 7.2a**). An effective surface modification is very essential to enhance drug-surface interaction for a high loading density. We have previously discovered that rGO can efficiently load a neural and hydrophobic anticancer drug, β -lap via favorable π - π interaction (**Figure 7.2a**), and the rGO-loaded β -lap release is negligible in PBS, but $\sim 14\%$ in cell culture medium after 1-day incubation (**Figure 7.2b**). Functionalizing the nanoprobe with rGO^{204} is anticipated to enhance drug-surface interactions not only to improve drug loading but also to stabilize the drug molecules before their delivery.

Figure 7.3 Time series of (a, c, e) bright-field images and (b, d, f) the corresponding CellTracker Green fluorescence images showing the shape and the viability of a targeted living cell (a) before the nanoprobe penetrates the cell membrane, (c) while the nanoprobe is inserted into the cell, and (e) after the nanoprobe is withdrawn from the cell.

In rGO functionalization, the hydroxyl-terminated silica surface of optical fiber nanoprobe was first modified with aminopropyltriethoxysilane (APTES) to make the surface positively charged. On the other hand, rGO nanosheets has a zeta potential of -45.6 ± 13.8 mV at a neutral pH and is negatively charged, which can be assembled on the modified optical fiber nanoprobe via electrostatic interactions as illustrated in **Figure 7.1d**. The wrinkle-like structures on the originally smooth optical fiber surface illustrates the successful rGO functionalization without noticeable increase in the tip diameter (**Figure 7.1b&c**), which could allow functionalized nanoprobe to access subcellular

compartments in a minimally invasive manner, while does not impede the light transmission at the nanoprobe for optical detection due to the high transparency of rGO²⁰⁵ immobilized. **Figure 7.3** shows that the cell is not deformed during the entire penetration-incubation-withdrawal process. The actin filament network is also not damaged after the nanoprobe penetration (**Figure 7.4a**), whereas a conical glass nanopipette causes severe cytoskeleton disruption indicated by large perforations (**Figure 7.4b**). Furthermore, we have found that the nanoprobe insertion does not induce any observable cytosolic Ca^{2+} elevation, thus confirming that it imparts negligible mechanical stress to the cell (**Figure 7.4c**).

Figure 7.4 Effect of nanoprobe insertion on the cytoskeleton and the calcium response. The merged bright-field and fluorescent images of MCF7 cell actin cytoskeletons after insertion of (a) the as-fabricated 100 nm nanoprobe and (b) a conical glass nanopipette; (c) Typical intracellular Ca^{2+} response upon the insertion of (I) an as-fabricated 100 nm nanoprobe and (II) a conical glass nanopipette

7.4 Cellular Responses to the Localized β -lap Delivery into Specific Subcellular Domains

Figure 7.5 ROS generation profile observed when the β -lap is added into the extracellular solution to exert a global stimulation

There are both scientifically and technically great challenges to investigate the subcellular sites of action for drugs, particularly those of natural origins, by conventional techniques.^{176, 190} Here for the first time the drug-cell interactions are studied at the nanoscale to determine the subcellular site of action using an rGO-functionalized optical fiber nanoprobe to deliver β -lap into specific domains and concomitantly monitor the cellular responses. The main medicinal effect of β -lap is to induce dramatic reactive oxygen species (ROS) formation for extensive damage of DNA and finally killing cancer cells.²⁰¹ In comparison, we have detected the ROS generation following stimulation with 10 nmole of β -lap dissolved into the cell medium (**Figure 7.5**). After ~ 300 s, the intracellular ROS level increases slowly, and the level keeps rising even after 2400s. In

contrast, we monitored the ROS generations upon insertion of the β -lap loaded nanoprobes into three selected subcellular domains as illustrated in **Figure 7.6a**. The nuclear compartment is marked by a red cross, while the cytoplasmic compartment is divided into a perinuclear region (marked by the green cross) and a general cytosol (marked by the blue cross). The monitored real-time ROS generation profiles from the three subcellular locations are displayed in **Figure 7.6b-d**. In the cytoplasm, the ROS level increases rapidly after the first 100 s of latency to reach a plateau at ~ 600 s. The much shorter latency period and faster ROS generation rate as compared to global stimulation are possibly because the inserted nanoprobe bypasses the cell membrane barrier to directly deliver drugs to the subcellular target site. There is no significant difference between the perinuclear region and the cytosol, suggesting that the ROS generation is about the same throughout the entire cytoplasm, but differently, the ROS level constantly fluctuates even with a small magnitude in the nuclear compartment. Although the detailed mechanism is not completely clear, this phenomenon may be explained by the major cytosolic localization of the enzyme NAD(P)H: quinone oxidoreductase-1 (NQO1), which catalyzes β -lap in a futile manner to generate ROS²⁰⁶. In comparison, control experiments using β -lap free nanoprobes show negligible changes in the ROS levels, verifying that the distinct ROS generation patterns are due to the subcellularly delivered β -lap. The different ROS responses detected in cytoplasmic and nuclear compartments reveal the spatial heterogeneities in responses to drugs which may provide scientific insights to understand the anticancer mechanism.

Figure 7.6 Different ROS generation profiles observed when the β -lap is delivered to specific subcellular locations. (a) Merged fluorescent image for DCFDA (green) and Mitotracker red (red) double stained MCF7 cell; ROS responses detected in (b) the perinuclear region (green cross) (c) the cytosol (blue cross) or (d) the nucleus (red cross), respectively. Controls show negligible changes in fluorescence.

To pinpoint the subcellular region with the best anticancer effect, we further analyzed alterations in intracellular Ca^{2+} , an important mediator of cancer cell death,²⁰⁷ following precise β -lap delivery to the three subcellular locations. Although a rise in Ca^{2+} level is generally observed at all three locations, the signals differ in their magnitudes as well as the rates of increase (**Figure 7.7**). The nuclear delivery attains a lower magnitude for Ca^{2+} rise, which is only half of that for the cytoplasmic delivery. More importantly, we have discovered that while the peak magnitudes for the delivery to the perinuclear region and the cytosol are quite similar, perinuclear delivery obviously leads to a much faster rise in

the Ca^{2+} level. Collectively, the results suggest β -lap interacts well with molecular targets in this narrow perinuclear region for generating/releasing second messengers such as Ca^{2+} . The strongest and fastest Ca^{2+} response in the perinuclear region helps to identify β -lap's subcellular site of action, which can further guide the rational design of subcellular targeted delivery. The fact that both Ca^{2+} concentration and ROS level rise within minutes of localized delivery also indicates that these two events may actually take place concurrently rather than sequentially.

Figure 7.7 Change in intracellular calcium concentration upon β -lap delivery to specific subcellular locations

It is also worthy of a note that the fast ROS and Ca^{2+} responses observed (only minutes after insertion) implies accelerated dissociation of β -lap from rGO in the intracellular environment, which is likely due to the binding of β -lap molecules with abundant cellular proteins.²⁰⁸ This observation helps to explain the high potency of

various graphene-drug hybrids despite the negligible *in vitro* release. In addition, we measured the cell responses upon inserting nanoprobe with different amounts of loaded β -lap into the perinuclear region. The amount of drug loaded was adjusted by increasing the drug loading duration. It is found that the strength of ROS response correlates with the drug loading amount (**Figure 7.8**), thus allowing the study of drug dose response at subcellular resolutions.

Figure 7.8 Dose dependent ROS responses elicited by adjusting the duration of drug loading

7.5 Conclusions

In summary, we have investigated the drug-cell interaction at a subcellular level by using an rGO functionalized optical fiber nanoprobe. Distinct ROS generation, the main medical effector and rise in Ca^{2+} level, an important mediator of cancer cell death, observed at different subcellular locations pinpoint that the perinuclear region is the subcellular site of action. This work provides not only scientific insights of subcellular drug-cell interaction but also valuable knowledge for rational design of subcellular targeted delivery or spatially resolved signal intervention.

Chapter 8 General Conclusions and Directions for Future Research

8.1 General Conclusions

In this dissertation, the recent progress toward analyzing single cells at a nanoscale level, in particular the emerging techniques developed by nanotechnology are thoroughly reviewed. An optical and electrical single cell sensing platform has been developed to analyze various aspects of a single living cell in accordance with the project goals.

A primary accomplishment is the detection of telomerase over-expression by an optical fiber nanobiosensor inserted in a living MCF-7 cancer cell nucleus. The unique design of the nanoprobe, *in situ* sampling in a living single cell nucleus and *in vitro* sandwich ELISA in this method play critical roles in the sensitive detection. The nanotip inserted into MCF-7 cell nucleus provides significantly higher average $(F-F_0)/F_0$ ratio than that of human mesenchymal stem cell (hMSC) cell nucleus, demonstrating the successful detection of the telomerase over-expression in cancer cells as compared to normal cells. In addition, the detection in the MCF-7 cytoplasm shows much smaller average ratio than that in the nucleus to clearly verify the nuclear localization of telomerase. The successful detection of telomerase over-expression in a single living cell for the first time not only provides a potential method for cancer detection, but also demonstrates a universal approach to detect other low expression proteins in a single living cell.

Sensitive detection of extracellular lactate concentration at single cell level has also been achieved by a nanoscale optical fiber lactate sensor with lactate dehydrogenases immobilized on the nanotip. The nanoscale tip achieves both high spatial resolution and background interference minimization, meeting the requirements for single cell analysis. Dynamic range of this nanosensor is from 0.01 mM to 1 mM, comparable to the physiological range of single cell lactate release. The results indicate that single cancer cells such as HeLa and MCF-7 cells exhibit higher extracellular lactate concentrations than normal cells, which supports the Warburg hypothesis. In addition, a MCT inhibitor exerts different effects on the lactate effluxes of HeLa and MCF-7 cells, which corroborates with the difference in MCT isotype expressions. This work provides a powerful tool to noninvasively study the fundamental metabolic processes at a single cell level and could also be used in early cancer diagnosis.

A further development is the construction of a bifunctional electro-optical nanoprobe to study the local dynamic cellular oxidative activity at a single cell level. The optical fiber core can monitor the intracellular fluorescence changes corresponding to the general redox state or reduced thiol level, while the nanoring electrode enables real-time, quantitative and sensitive amperometric detection of extracellular H_2O_2 generation at the same location. The results reveal distinctively higher oxidative responses and altered antioxidant levels in the cancer cells as compared to the normal ones, suggesting that the cell malignancy correlates with the strength of oxidative stress, and the higher antioxidant level may be a cause of drug resistance. Furthermore, localized detections in different membrane regions uncovers the spatial heterogeneity in oxidative stress to indicate the targeting of activated NOX enzymes to a central domain. This uniquely structured

nanoprobe with ultra-low detection volume, significantly reduced background and enhanced S/N ratio provides a unique opportunity to probe chemical dynamics in a nanoscopic environment for elucidating key biological processes.

To address the challenge of manipulating cells at high resolutions, I have innovated an rGO functionalized optical fiber nanoprobe to subcellularly deliver a potent anticancer drug, β -lap at nanoscales and simultaneously monitor the drug-induced biochemical responses. Localized delivery at nanoscales is achieved by directly inserting an rGO-functionalized nanoprobe with loaded β -lap into different subcellular locations without compromising the cell viability. Distinct ROS generation, the main medical effect and rise in Ca^{2+} level, the important mediator of cancer cell death, observed at different subcellular locations pinpoint the perinuclear region as the subcellular site of action. Since rGO-based materials could efficiently load other drugs/bioactive molecules such as small molecule inhibitors, DNAs and enzymes, this multifunctional nanoprobe could be used as a universal approach to investigate subcellular location-dependent biochemical responses of bioactive molecules. This work provides not only scientific insights of subcellular drug-cell interaction but also valuable knowledge for rational design of subcellular targeted delivery or spatially resolved signal intervention.

In summary, this project develops a series of nanosensors to study fundamental biological activities such as biomarker expression, metabolism alteration, oxidative stress and drug-cell interaction at single cell level. This sensitive single cell detection platform not only provides new scientific insights in cellular processes but also presents a potential way to improve healthcare in terms of better disease diagnosis and treatment.

8.2 Directions for Future Research

This nanosensing platform holds enormous promises for uncovering unprecedented details in various important cellular processes at a single cell level. To exploit its full potential, several future research directions are proposed.

Since each nanoprobe can only interrogate one single cell at a time, repeated measurements are currently required to verify statistical significance of any new observations. The improvement of throughput to concurrently record at multiple cells/cellular sites is particularly important. Automatic manipulation, integration with microfluidic platforms and utilization of fiber-optic bundles/arrays are possible ways to solve this problem.

The spatial and temporal resolutions achieved so far are hundreds of nanometers and milliseconds, respectively. As biological processes are ultimately controlled by rapid molecular interactions, the future trend is to improve both the spatial and temporal resolutions. It is expected to improve the spatial resolution to a few nanometers, i.e. a molecular level. Meanwhile, the targeted temporal resolution should reach the order of microseconds.

The multi-parametric analysis of biological pathways is of exceptional importance to study cell signaling and regulation. We have demonstrated the combination of two detections modalities to simultaneously monitor two parameters. More sensing schemes need to be designed to detect a wider range of targets. Further developments to incorporate more than two detection modalities will allow us to decipher the complicated signaling network that determines a cell's fate.

So far, the power of this technology has been mainly demonstrated on analyzing adherent cells. It is also necessary to extend its application to non-adherent cells such as blood cells. A feasible way is to integrate this nanosensing platform with microwell arrays or microfluidic cell traps that can confine single suspended cells to defined locations.

Lastly, since this emerging technique has great potential for medical applications, clinical samples obtained from biopsy should be tested to establish a correlation between the detection result and the clinical outcome. In addition, a robust commercial system and standard protocols are necessary for broad clinical applications such as diagnosis and treatment evaluation.

List of Abbreviations

EM: electron microscopy

STED: stimulated emission depletion microscopy

STORM: stochastic optical reconstruction microscopy

PALM: photoactivated localization microscopy

NSOM: Near-field scanning optical microscopy (NSOM)

SICM: scanning ion conductance microscopy

FCS: fluorescence correlation spectroscopy

SERS: Surfaced enhanced raman spectroscopy

CNT: carbon nanotube

FET: Field effect transistors

SiNW: silicon nanowire

SWCNT: single walled carbon nanotubes

CMOS: complementary metal oxide semiconductor

SECM: scanning electrochemical microscope

SPM: scanning probe microscopy

HPICM: hopping mode scanning ion conductance microscopy

STA: standing approach

FPGA: field-programmable gate array

CNP: carbon nanopipette

AFM: atomic force microscope

SSCM: scanning surface confocal microscopy

GFP: green fluorescent protein

OM: optical microscopy

FIB: focused ion beam

MWCNT: multi-walled carbon nanotubes

ELISA: enzyme linked immunosorbent assay

hMSC: human mesenchymal stem cell

hFOB: human fetal osteoblast

MCF10A: mammary epithelial cell line

MCF7: mammary adenocarcinoma cell line

HER: human epidermal growth factor receptor

MCF7/HER2: HER2-overexpressing MCF7 cell line

APTES: 3-aminopropyl triethoxysilane

NADH: β -nicotinamide adenine dinucleotide hydrate

LDH: L-lactic dehydrogenase from porcine heart

α -CHC: α -cyano-4-hydroxycinnamic acid

PMA: phorbol 12-myristate 13-acetate

PAO: phenylarsine oxide

SNAP: S-Nitroso-N-acetyl-DL-penicillamine

DCF-DA: 2', 7' - Dichlorofluorescein diacetate

PMT: photomultiplier tube

MEM: Minimum Eagle's Medium

DMEM: Dulbecco's Modified Eagle's Medium

PBS: phosphate buffered saline

TRAP: telomeric repeat amplification protocol

S/N: signal to noise

BSA: bovine serum albumin

FESEM: Field emission-scanning electron microscopy

K_m : Michaelis constant

K_i : dissociation constant for inhibitor binding

MCT: monocarboxylate transporter

LDH: lactate dehydrogenases

ROS: reactive oxygen species

$K_3[Fe(CN)_6]$: potassium ferricyanide

PB: Prussian Blue

CLSM: confocal laser scanning microscope

PKC: protein kinase C

NOX: NAD(P)H oxidase

PAO: phenylarsine oxide

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Awards

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MOE-GLC scholarship, 2004

Research Interest

- Optical fiber based nanobiosensor for single cell detection
- Simultaneous optical and electrochemical detection of single cell signals
- Advanced nanomaterials for targeted drug delivery

Professional Skills

- Fabrication of optical fiber based nanoprobe and operation of single cell electro-optical detection system
- TCSPC single photon detection system
- Confocal laser scanning microscopy and total internal reflection microscopy
- Cell culture and basic biochemical assays
- Synthesis of nanomaterials and its characterization techniques such as SEM, UV-vis, PL, XRD, EDX

List of Publications

Journal Articles

1. **X. T. Zheng** and C. M. Li*, Single Cell Analysis at Nanoscales. *Chem Soc Rev*, DOI: 10.1039/C1CS15265C.
2. **X. T. Zheng** and C. M. Li*, Anticancer Efficacy and Subcellular Site of Action Investigated by Real-time Monitoring of Cellular Responses to Localized Drug Delivery in Single Cells. Under Review, **2012**.
3. **X. T. Zheng** and C. M. Li*, Restoring Basal Planes of Graphene Oxides for Highly Efficient Loading and Delivery of β -lapachone. *Mol Pharmaceutics*, DOI: 10.1021/mp2005356.
4. **X. T. Zheng**, W. H. Hu, H. Wang, H. Yang, W. Zhou and C. M. Li*, Bifunctional Electro-optical Nanoprobe to Real-time Detect Local Biochemical Processes in Single Cells. *Biosens Bioelectron*, 26, 4484-4490, **2011**.
5. **X. T. Zheng**, H. B. Yang, C. M. Li*: Optical Detection of Single Cell Lactate

- Release for Cancer Metabolic Analysis. *Anal Chem*, 82 (12), 5082–5087, **2010**.
6. **X. T. Zheng**, C. M. Li*: Single Living Cell Detection of Telomerase Over-expression for Cancer Detection by an Optical Fiber Nanobiosensor. *Biosens Bioelectron*, 25, 1548–1552, **2010**.
 7. C. X. Guo#, **X. T. Zheng**#, S. R. Ng, Y. Lai, Y. Lei and C. M. Li*: In-situ molecular detection of ischemic cells by enhanced protein direct electron transfer on a unique horseradish peroxidase-Au nanoparticles-polyaniline nanowires biofilm. *Chem Commun*, 47, 2652-2654, **2011** (# Equal contribution to this work)
 8. Y. Hu,* **X. T. Zheng**, J. S. Chen, M. Zhou, C. M. Li, X. W. Lou,* Silica-based Complex Nanorattles as Multifunctional Carrier for Anticancer Drug, *J Mater Chem*, 21, 8052-8056, **2011**.
 9. C. X. Guo, **X. T. Zheng**, Z. S. Lu, X. W. Lou, C. M. Li*: Biointerface by Cell Growth on Layered Graphene-Biomimetic Enzyme-Protein Nanostructure for in Situ Quantitative Molecular Detection. *Adv Mater*, 22, 5164-5167, **2010**.
 10. Z. S. Lu, Z. H. Zhu, **X. T. Zheng**, Y. Qiao, J. Guo and C. M. Li*: Biocompatible fluorescence-enhanced ZrO_2 -CdTe quantum dot nanocomposite for in vitro cell imaging. *Nanotechnology*, 22, **2011**.
 11. Z. S. Lu, Y. Qiao, **X. T. Zheng**, Mary B. Chan-Park, C.M. Li*: Effect of particle shape on phagocytosis of CdTe quantum dot-cystine composites. *Med Chem Comm*, 1, 84-86, **2010**.
 12. X. Q. Cui, C. M. Li*, H. F. Bao, **X. T. Zheng**, J. F. Zang, C. P. Ooi, J. Guo: Hyaluronan-assisted Photoreduction Synthesis of Silver Nanostructures: From Nanoparticle to Nanoplate. *J Phys Chem C*, 112(29), 10730-10734, **2008**.
 13. X. Q. Cui, C. M. Li*, H. F. Bao, **X. T. Zheng**, Z. S. Lu: In-Situ Fabrication of Silver Nanoarrays in Hyaluronan/PDDA Layer-by-Layer Assembled Structure. *J Colloid Interface Sci*, 327, 459–465, **2008**.

Book Chapter

14. **X. T. Zheng**, C. M. Li*, Optical Fiber Nanobiosensor for Single Living Cell Detections of Cancers, *Biosensors and Cancer*, World Scientific Publishing, **2011**.