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Direct Methods for Dynamic Monitoring of Secretions from Single Cells by Capillary Electrophoresis and Microscopy with Laser-induced Native Fluorescence Detection

by

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TABLE OF CONTENTS

ABSTRACT.....	vi
GENERAL INTRODUCTION.....	1
Dissertation Organization	1
Single-Cell Chemical Analysis.....	1
Separation-Based Methods	2
Electrochemical Methods Using Microelectrodes	6
Fluorescence Imaging Microscopy	9
References.....	11
CHAPTER 1. MONITORING SINGLE-CELL PHARMACOKINETICS BY CAPILLARY ELECTROPHORESIS AND LASER-INDUCED NATIVE FLUORESCENCE	18
ABSTRACT.....	18
INTRODUCTION	18
EXPERIMENTAL.....	20
CE Apparatus.....	20
Reagents.....	21
Methods.....	21
RESULTS AND DISCUSSION.....	25
CONCLUSIONS.....	28
ACKNOWLEDGMENTS	30
REFERENCES	30
CHAPTER 2. ON-COLUMN MONITORING OF SECRETION OF CATECHOLAMINES FROM SINGLE BOVINE ADRENAL CHROMAFFIN CELLS BY CAPILLARY ELECTROPHORESIS.....	33
ABSTRACT.....	33
INTRODUCTION	34
EXPERIMENTAL.....	35
CE Apparatus.....	35
Reagents.....	36
Cell Isolation.....	37
CE Methods	38
RESULTS AND DISCUSSION.....	39
Separation of CA in Physiological Buffer and Treatment of Capillary	39

Quantitation of Release from Single Cells in Static Experiments	42
Peak Broadening in Static Experiments.....	49
Dynamic Release Experiments	52
CONCLUSIONS.....	55
ACKNOWLEDGMENTS	57
REFERENCES	58

CHAPTER 3. DIRECT VISUALIZATION OF SECRETION OF SINGLE BOVINE ADRENAL CHROMAFFIN CELLS BY LASER-INDUCED NATIVE FLUORESCENCE IMAGING MICROSCOPY.....62

ABSTRACT.....	62
INTRODUCTION	63
EXPERIMENTAL SECTION	64
Instrumentation and Imaging	64
Cell Isolation and Reagents	66
RESULTS AND DISCUSSION.....	66
Selectivity of Native Fluorescence Microscopy	66
Catecholamine Secretion Dynamics	67
Effect of UV laser on the cells.....	79
CONCLUSIONS.....	86
ACKNOWLEDGMENTS	86
REFERENCES	87

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CHAPTER 4. SPATIAL CHARACTERIZATION OF SEROTONIN RELEASE FROM SINGLE LEECH RETZIUS NEURONS BY LASER-INDUCED NATIVE FLUORESCENCE MICROSCOPY.....89

ABSTRACT.....	89
INTRODUCTION	90
EXPERIMENTAL	91
Cell Isolation and Culture	91
Imaging	92
Capillary Electrophoresis.....	94
Reagents.....	94
RESULTS AND DISCUSSION.....	95
LINF Images of Retzius Cells.....	95
Spatial Characteristics of Serotonin Release	98
CONCLUSIONS.....	103
ACKNOWLEDGMENTS	110
REFERENCES	110

*Preprint - Removed for
separate
processing*

GENERAL SUMMARY	114
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ACKNOWLEDGMENTS	115
-----------------------	-----

ABSTRACT

Microscale separation and detection methods for real-time monitoring of dynamic cellular processes (e.g., secretion) by capillary electrophoresis (CE) and microscopic imaging were developed. Ultraviolet laser-induced native fluorescence (LINF) provides simple, sensitive and direct detection of neurotransmitters and proteins without any derivatization.

An on-column CE-LINF protocol for quantification of the release from single cell was demonstrated. Quantitative measurements of both the amount of insulin released from and the amount remaining in the cell (β TC3) were achieved simultaneously.

Secretion of catecholamines (norepinephrine (NE) and epinephrine (E)) from individual bovine adrenal chromaffin cells was determined using the on-column CE-LINF. No apparent relationship was found between the ratio of NE/E released and the original ratio of NE/E in the cell. Temporal information about the release was traced out with a dynamic stimulating method by pumping secretagogue continuously passing the cell with the aid of electroosmotic flow and measuring the peak broadening of the released catecholamines in the electropherogram.

Direct visualization of the secretion process of individual bovine adrenal chromaffin cells was achieved by LINF imaging microscopy with high temporal and spatial resolution. Large variations were found among cells in the amount of catecholamines secreted and the rate of secretion. Local heterogeneity in the rate of secretion was also found.

The secretion of serotonin from individual leech Retzius neurons was directly characterized by LINF microscopy with high spatial resolution. The results from images of co-cultured pressure sensory (P) (non-serotonergic) and Retzius (serotonergic) cells, as well as capillary electrophoresis, suggested that the major native fluorescence excited by the laser was derived from intracellular serotonin. A much more substantial release of serotonin was

found at the axon stump left during cell isolation, a preferred region for synapse formation.

The release of serotonin was also characterized to be calcium dependent.

GENERAL INTRODUCTION

Dissertation Organization

This dissertation begins with a general introduction of recent progress in the area of single-cell chemical analysis with a list of cited references. The following chapters are arranged in such a way that published papers or manuscripts submitted or in preparation to be submitted are each presented as separate chapters. These chapters are followed by a general conclusion. References for each paper and manuscript are at the end of the chapter in which they are cited.

Single-Cell Chemical Analysis

In recent years, single-cell chemical analysis has received considerable interest. Because of the ultrasmall sample volume and the concomitantly low absolute amount of analyte involved, chemical analysis of single cells is challenging for analytical chemist. Since some of the early work in late 80's and early 90's [1-5], a number of review articles [6-13] have been published summarizing the separation based and voltammetric or amperometric microelectrode based methods for single-cell chemical analysis.

There are several reasons that single-cell analysis is considered important. First, because of the high heterogeneity among individual cells, it is important to understand the variability between cells, from chemical composition to physiological response. This is then related to their overall biological functionality. Second, cells, especially endocrine and neurological cells, also exist in an extremely heterogeneous environment: the cells we are interested in usually coexist with other cells and therefore are exposed to a mixture of hormones and neurotransmitters released from neighboring cells. Single-cell analysis has the inherent advantage that it provides a controlled environment precluding the influence of other

cells. This is extremely important for the understanding of the dynamic cellular processes such as secretion, uptake and metabolism. Third, single-cell analysis is also potentially useful as a powerful early diagnostic tool. It is well known that if cells are analyzed individually, the chance of finding abnormal cells or the cells that carry certain biological markers for certain diseases is substantially greater. This a big advantage over conventional bulk analysis where a large population of cells is used. The effect of the lower number of abnormal cells present at the early stages of diseases are usually masked by the majority healthy cells.

Although membrane capacitance measurements with the patch-clamp technique [14] provides a fast means to measure the extent of exocytosis by measuring the change of surface area caused by fusion of secretory vesicles with cell membrane, it is a physical or indirect method and therefore will not be discussed further here. Separation-based methods (microcolumn liquid chromatography and capillary electrophoresis), electrochemical methods using microelectrodes (amperometry and fast scan voltammetry) and microscopic imaging methods are among the most common methods used for single-cell chemical analysis. In the remainder of this introduction, the recent advances and future prospects in these areas are discussed.

Separation-Based Methods

Microcolumn separation methods, especially capillary electrophoresis (CE) are well suited for single-cell analysis because: 1) the small dimension of the capillary column is compatible with the small "sample" volume of a single cell and dilution by the running buffer is avoided; 2) the extremely high separation efficiency (10^5 - 10^6 theoretical plates) and fastness allows for the separation of complex constituents from inside a single cell in a short time scale; 3) the capillary column itself can be used as the site of single-cell manipulation,

such as derivatization of cellular components or physiological stimulation (secretion or uptake), prior to detection.

Electrochemical detection provides sensitive detection for electroactive cellular species [9]. Many works on single-cell analysis were performed using amperometric detection after microcolumn LC and CE separation. A species is detected if it is oxidized at the potential of the electrode and results in a current response. The detection limit is as low as attomole to zeptomole level. Neurotransmitters were quantitatively analyzed in cytoplasmic injections from single neurons in pond snail *Planorbis corneus* and land snail *Helix aspersa* using microcolumn LC and CE with electrochemical detections [1, 2, 6, 15-17]. Catecholamines in single mammalian adrenal medullary cells as well as their secretion were quantified by microcolumn LC with electrochemical detection [18, 19]. Analysis of single neurons in *P. corneus* by CE with electrochemical detection was used to provide evidence supporting a two-compartment model for neurotransmitters in single nerve cells [20]. CE with scanning electrochemical detection was also used to identify the identity of the species by its voltammetric characteristics [16, 21]. Derivatization with naphthalene-2,3-dicarboxaldehyde (NDA) prior to analysis extends the scope of electrochemical detection to some non-electroactive species such as some amino acids with attomole detection limits [22].

Laser-induced fluorescence (LIF) is another well-established detection mechanism for single-cell analysis [23]. Many biomolecules including biogenic amines, some proteins, alkaloids, vitamins and steroids will fluoresce naturally when excited with suitable wavelengths [24]. Many proteins (such as hemoglobin, methemoglobin, carbonic anhydrase, hemoglobin variants and insulin, etc.) that have tryptophan and/or tyrosine residues can be quantified at the single cell level by CE using UV laser (usually 275 nm) excited native fluorescence detection [5, 25-28]. Cellular neurotransmitters (such as catecholamine, serotonin) and steroids were also detected using CE and laser-induced native fluorescence

(LINF) [29, 30]. Since LINF is a direct detection method, it avoids the problem of incomplete derivatization reactions or slow derivatization kinetics and therefore extremely useful for dynamic monitoring of single cell events such as secretion or uptake. Exocytotic release of serotonin, insulin and catecholamine from single mast cells, pancreatic β -cells and adrenal chromaffin cells were quantitatively monitored by CE-LINF [31-33]. Although the quantum efficiency of native fluorescence is usually low, by selecting the appropriate pH and avoiding band broadening plus the sensitivity of LIF, one can still achieve detection limits in the range of 10^{-10} M or 10^{-19} mole.

However, for the majority of biomolecules which cannot fluoresce naturally, derivatization with a fluorophore is often used either in pre-column, on-column or post-column mode with detection limits as low as 10^{-18} - 10^{-21} moles [34]. Pre-column derivatization of single cells with NDA was used to identify several amine containing analytes in individual snail neurons [2] and bovine adrenal medullary cells [35]. On-column derivatization uses the inlet end of the capillary as the reaction chamber. Thiols inside single human red blood cells were derivatized on-column with monobromobimane (mBBBr) transported through the cell membrane [5]. Individual pheochromocytoma (PC12) cells were analyzed by CE after on-column derivatization with NDA [36]. The advantage of on-column derivatization over pre-column derivatization is that the sample dilution is limited by the size of the capillary and the only source of dilution is the diffusion of the analytes along the capillary bore which is usually very small. Post-column derivatization is one of the ways to solve the multiple labeling problem associated with pre-column derivatization. Derivatization reagents (such as o-phthaldialdehyde (OPA), fluorescamine and NDA with 3-mercaptoethanol) that can react rapidly are preferred for post-column derivatization. Amino acids from homogenates of *Planorbis corneus* neurons and hemoglobin in a single human erythrocyte were analyzed after post-column derivatization with NDA [37]. OPA post-

column derivatization using a coaxially connected capillary for the determination of hemoglobin and carbonic anhydrase in single human erythrocytes was also demonstrated [38]. Peptides labeled with fluorescamine [39] and fluorescein isothiocyanate (FITC) [40] were analyzed in single neuron cells. Some of the problems associated with derivatization procedures include: extensive sample handling which can cause loss of analytes and/or contamination; dilution of reaction products which may cause diminished sensitivity; slow reaction kinetics which may cause incomplete reactions and lead to irreproducibility; and, multiple labeling of solutes with several functional groups [34]. A way to circumvent these problems is to use indirect fluorescence detection [41] for non-fluorescent ionic analytes. Intracellular K^+ , Na^+ [5, 42], lactate and pyruvate [43] in single human erythrocytes were all monitored by indirect fluorescence detection.

CE-based enzyme assays are useful for the detection of sub-attomole levels of intracellular species because of the large amplification factors provided by enzymatic reactions [44]. The use of LIF detection further lowers the detection limit and is demonstrated useful for the analysis of LDH isoenzymes in single human erythrocytes [45]. The LIF enzyme assay detection limit for LDH-1 was down to 10^{-21} mol (800 molecules). The enzyme assay protocol was applicable to the study of LDH activity, a possible marker for leukemia, in lymphocytes [46]. A particle-counting immunoassay using antibody coated latex particles was miniaturized for capillary electrophoresis to analyze glucose-6-phosphate dehydrogenase (G6PDH) using laser scattering detection [47]. In fact, the activity and concentration of G6PDH from a single human erythrocytes was determined simultaneously with this method [48]. The detection limit of this method is 10^{-21} mol of G6PDH (600 molecules). A CE-based immunoassay with LIF detection was used to determine insulin content and secretion from a single islet of Langerhans [49], and an on-line competitive assay system was built to monitor the insulin secretion from a single islet [50].

Radiochemical detection methods involve culturing isolated cells in ^{35}S -methionine and then detecting the newly synthesized methionine-containing peptides which have incorporated the radiolabel [11]. Peptides in single *Aplysia* neurons, as well as their release, were measured with HPLC methods [51-55]. A postcolumn radionuclide detection method for CE was also developed [56] and applied to measure the neuropeptides in single buccal cells of *Aplysia californica* [57].

Optical detection usually provides relatively little information about the chemical identity of analytes (unless the migration times can be correlated with standards). Mass spectrometry (MS) can provide chemical analysis at the single cell level with the benefit of unsurpassed chemical specificity. Recent developments in CE, electrospray ionization (ESI) and Fourier transform ion cyclotron resonance (FTICR) MS have made direct analysis of cellular proteins possible [58]. In one early report, CE-ESI-FTICR was used to analyze both the α and the β chains of hemoglobin acquired from 10 human erythrocytes (corresponding to 4.5 fmol of hemoglobin) [58]. Later, selective ion accumulation techniques based on quadrupolar excitation (QE) was incorporated and the hemoglobin from a single human erythrocytes (≈ 450 amol) was characterized [59]. Using CE-ESI-FTMS with an ultralow flow rate ($\leq 4\text{ nL/min}$), a resolving power of about 60,000 for injection of 0.7 to 3 attomole of 8- to 29-kDalton proteins with errors of <1 Dalton was achieved [60]. Single human erythrocytes were also analyzed by microspot matrix-assisted laser desorption ionization (MALDI) MS even without separation [61].

Electrochemical Methods Using Microelectrodes

Amperometric and fast scan voltammetric methods using microelectrodes provide millisecond time resolution for fast cellular events like exocytosis as well as spatial resolution for measurements in intracellular compartments [62]. The earliest uses of

microelectrodes were for *in vivo* neurochemical measurements [62]. Since that time, many new developments have been made to follow the exocytosis at the single cell level in real time [6, 12, 63, 64]. Several neurotransmitters like catecholamine, serotonin, certain amino acids and peptides are electroactive and can be oxidized or reduced at reasonable potentials. The most widely used microelectrodes were made of carbon fiber constructed both in disk [63] or cylindrical [65] shape with the diameters close to that of a single cell (15-20 μ m).

Exocytosis from single bovine adrenal chromaffin cells was monitored in this way [66-68]. This method can discriminate the release of single secretory granules of catecholamine from single cells through a series of amperometrically measured current "spikes". Each "spike" observed on the current vs. time plot corresponded to catecholamine release from a single granule within the cell. Using Nafion-coated carbon fiber microelectrodes, this method was used to identify the released catecholamines as epinephrine (E) and norepinephrine (NE) [69]. Integration of the current observed for each spike provided an estimate of catecholamine content equaling 5 - 8 amol for each granule using Faraday's Law [66]. Catecholamine release from PC12 cells was also monitored with microelectrode amperometry at the zeptomole level [70]. Other electroactive species such as serotonin, histamine [71, 72], oxygen [73] and tryptophan- or tyrosine-containing peptides [74] were also monitored at the single cell level.

Although voltammetric methods usually provide a relatively narrow electrode potential window to identify different electroactive species, such characterization ability is still useful for identification in single-cell analysis. Epinephrine and norepinephrine released from single cells was differentiated using background subtracted fast-scan cyclic voltammetry (CV) at 800V/s by scanning to sufficiently high voltage (> 1V) to observe a second oxidative wave for E [75]. Histamine and serotonin were discriminated similarly during the course of the forward and the reverse CV scan [71]. In addition, Nafion-coated microelectrodes were

often used to screen out the most common interferate ascorbate and DOPAC [76-78]. Poly pyrrole-coated electrodes were used to increase catecholamine sensitivity [79]. However, the additional layer decreases the temporal response. Also, high-pass filtering is also used to discriminate against ascorbic acid, DOPAC and acidic pH changes [80].

By using a smaller size microelectrodes, the intracellular compartments were individually and selectively measured. The penetration of electroactive drugs into neurons of the marine snail, *Aplysia californica* was monitored with intracellular voltammetry [81]. Carbon ring electrodes were used to monitor dopamine in the cytoplasm of single neurons of *Planorbis corneus* [82, 83].

Many biologically important species are not as easy to detect even though their oxidation or reduction is thermodynamically favored. Chemically-modified electrodes (CME) can solve some of these problems by using mediators modified at the electrode surface to shuffle electrons between analytes and the electrode surface [13]. Exocytotic release of insulin from single pancreatic β -cells was monitored using polynuclear ruthenium oxide modified microelectrodes [84, 85]. Enzyme-modified microelectrodes were used for monitoring the excitatory neurotransmitter glutamate [86] and glucose transients at the single cell level [87,88].

Amperometry using microelectrodes has found wide application in characterizing the exocytosis process. For example, neurotransmitter release from endocrine cells was speculated to be much slower than in the peripheral or the central nervous system. It was initially suggested that this could be due to diffusional broadening [89]. However, after analyzing the spike width and the pre-spike feature at different pH and temperature values, it was subsequently argued that the factors involved in the exocytotic processes, such as pH-dependent conformation and Ca^{2+} -dependent binding affinity of chromogranin A (a major protein in the vesicles), are not negligible [90-92]. However, the combination of

amperometry and capacitance measurements suggested that the persistence of secretion after depolarization is due to diffusion of Ca^{2+} between channels and release sites, implying that Ca^{2+} channels and secretory vesicles are not colocalized [93, 94]. Similar method with isolated mast cells and chromaffin cells also showed that when photolysis of caged Ca^{2+} was used to stimulate secretion there is a delay between increases in capacitance and the release of oxidizable transmitters. The increases in capacitance that are not resolved as steps cannot be readily interpreted as secretory events unless they are confirmed independently [95]. Careful analysis of amperometric spikes shows that about 70% are preceded by a small "foot" (the trickle of transmitters out of the early fusion pore). 20-50% of the foot signals exhibit rapid fluctuations that are interpreted as flickering of the fusion pore and those "stand-alone" foot signals may reflect transient fusions (vesicles do not collapse completely into the plasma membrane) [96]. Using smaller size microelectrodes (1 μm radius), some spatial resolution of exocytotic release sites was achieved and results showed that release sites are spatially localized on endocrine cells [97].

Fluorescence Imaging Microscopy

Microscopic imaging techniques can obtain spatial and temporal resolution of cellular processes simultaneously. In fact, the goal of research on and application of fluorescence microscopy is to *watch* in greater detail (i.e., improve spatial resolution); to *measure* more specifically, sensitively and quickly (i.e., improve spectroscopic information and temporal resolution); and to *manipulate* more precisely (i.e., improve control) simultaneously at the intact single cell level [98]. Fluorescence imaging as well as confocal fluorescence imaging microscopy have become conventional tools in biological Sciences [98, 99]. Usually, some fluorescent dyes are used as intracellular probes in fluorescence microscopy for certain intracellular species such as calcium and some other ions. Alternatively, chemiluminescence

microscopy is also used for some species [100]. However, similar problems as mentioned in derivatization detection of CE and LC also exist. Direct microscopic imaging will be addressed in the remainder of this introduction.

Exocytotic events were quantitatively counted by observing the cell under differential interference contrast (DIC) or Normarski optics with video-microscopy [101, 102]. However, these methods do not provide any chemical information. As mentioned above, LINF is a powerful method for direct fluorescence measurement of some biomolecules. In fact, autofluorescence from NAD(P)H (excitation: 360 nm; emission: 400-500 nm) was used as an intrinsic probe to study cellular metabolism by fluorescence microscopy in the late 50's [103]. The uptake of serotonin by single living astrocytes was first monitored by LINF imaging microscopy with simultaneous high temporal and spatial resolution [104]. Later, the exocytotic release of serotonin from single astrocytes [105], mast cells [106], as well as the release of catecholamine from single adrenal chromaffin cells [107] were also studied through LINF microscopy. Simultaneously achieved spatial and temporal resolution in LINF microscopy enables one to find the local heterogeneity in neurotransmitter release kinetics for different areas of neuroendocrine cells much more easily [105-107] as compared to amperometric methods using smaller sized microelectrodes [97].

Although UV-excited autofluorescence of NAD(P)H was developed into a non-invasive optical method to monitor cellular respiration [108], photobleaching of the autofluorescence as well as chromatic aberration between the UV excitation and the visible fluorescence in confocal microscopy remained serious problems. Two-photon excitation applied to laser scanning microscopy was demonstrated to solve some of these problems [109]. Recently, three-photon excitation was reported to excite short wavelength UV fluorescence of tryptophan and serotonin, and serotonin distribution in living rat basophilic leukemia (RBL-2H3) cells was measured [110]. This technique circumvents the limitation imposed by

photodamage, scattering and discriminates against the background encountered in other UV microscopies.

The spatial resolution of conventional optical techniques mentioned above is limited by diffraction limit ($\lambda/2$, where λ is the wavelength). The realization of better resolution by subwavelength light sources has led to the concept of near-field optics (NFO) and near-field scanning optical microscopy (NSOM) [111]. Some recent review articles summarized the development and application of this technique [112-114].

In conclusion, chemical analysis at the single-cell level is becoming a mature and conventional technique not only for analytical chemists, but also for biologists. While the development of more sensitive detection schemes for single-cell analysis is very important, better spatial and temporal resolution in direct dynamic monitoring of cellular processes is another equally exciting direction.

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GENERAL CONCLUSIONS

The high degree of heterogeneity of nervous and endocrine systems makes it important to study certain cellular processes at the single cell level. Laser-induced native fluorescence has proven to be a powerful direct detection method not only for measuring the intracellular components but also for monitoring the dynamic cellular processes. Capillary electrophoresis, with its compatibility with extremely small sample volumes and high separation efficiencies, has established itself as one of the most commonly used quantitative methods for single cell chemical analysis. Fluorescence microscopy features high sensitivity, high spatial and high temporal resolution making it an excellent choice for real-time monitoring of cellular processes.

This dissertation describes the advances in the direct analytical observation and measurement of pharmacokinetic responses (secretion under stimulation) at the single cell level using laser-induced native fluorescence. Capillary electrophoresis was used to quantify single or multiple neuropeptide and neurotransmitters released from individual cells. In addition to the quantitative information, temporal information was also observed. Laser-induced native fluorescence microscopic imaging was used to visualize the secretion process of individual cells directly with simultaneous high spatial and temporal resolution. These techniques can be easily extended to the study of non-fluorescent species with on-column or post-column derivatization. It is expected that interaction of small biological samples with their environments could also be studied using similar protocols to those developed in this dissertation. Drug metabolism at the cellular level may also be probed. With the development of similar imaging technologies, single molecules (e.g., HIV virus, antibody or a particular gene) may be eventually detected at single-cell level.

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