1999

Fast methods for analysis of neurotransmitters from single cells and monitoring their releases in central nervous system by capillary electrophoresis, fluorescence microscopy and luminescence imaging

Ziqiang Wang
Iowa State University

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Fast methods for analysis of neurotransmitters from single cells and monitoring their releases in central nervous system by capillary electrophoresis, fluorescence microscopy and luminescence imaging

by

Ziqiang Wang

A dissertation submitted to the graduate faculty

in partial fulfillment of the requirement for the degree of

DOCTOR OF PHILOSOPHY

Major: Analytical Chemistry

Major Professor: Edward S. Yeung

Iowa State University

Ames, Iowa

1999

UMI Number: 9950127

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Major Professor

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For the Major Program

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For the Graduate College

DEDICATION

This dissertation is dedicated to my lovely wife: Yuan, Wenfei, for her encouragement and continuous support to my pursuit of educational advancement

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ABSTRACT

Fast methods for separation and detection of important neurotransmitters and the releases in central nervous system (CNS) were developed. Enzyme based immunoassay combined with capillary electrophoresis was used to analyze the contents of amino acid neurotransmitter from single neuron cells. The release of amino acid neurotransmitter from neuron cultures was monitored by laser induced fluorescence imaging method. The release and signal transduction of adenosine triphosphate (ATP) in CNS was studied with sensitive luminescence imaging method.

A new dual-enzyme on-column reaction method combined with capillary electrophoresis has been developed for determining the glutamate content in single cells. Detection was based on monitoring the laser-induced fluorescence of the reaction product NADH, and the measured fluorescence intensity was related to the concentration of glutamate in each cell. The detection limit of glutamate is down to 10^{-8} M level, which is 1 order of magnitude lower than previous reported detection limit based on similar detection methods. The mass detection limit of a few attomoles is far superior to that of any other reports. Selectivity for glutamate is excellent over most of amino acids. The glutamate content in single human erythrocyte and baby rat brain neurons were determined with this method and the results agreed well with literature values.

A noninvasive detection scheme based on above glutamate enzymatic assay combined with microscopy was developed to measure the glutamate release in CNS. The detection limit with CCD imaging is down to μM level of glutamate concentration with reasonable response (~30 s). The *in vitro* monitoring of glutamate release from cultured

neuron cells demonstrated excellent spatial and temporal resolutions that is superior to prevalent electrophysiological or chromatographic methods.

A novel luciferase-luciferin chemiluminescence assay method was developed to monitor the ATP release from living biological cell samples. The assay has linear response over 3 orders of magnitude for any set concentrations of enzyme and co-factors, the linear coefficient is over 99.9%. The detection limit of ATP is down to 10^{-8} M concentration levels at milliseconds exposure time scale with intensified charge coupled device (ICCD), which is superior to several seconds to tens of second of collection time with luminometer. The *in vitro* experiment of ATP release from astrocyte cultures was successfully developed, the ATP release upon stimulation from rat brain astrocyte cultures was recorded first time with ICCD imaging techniques in real time scale. The data shows that stimulation triggered an extracellular ATP wave, which reveals the communications among cells along the signaling pathway. Furthermore, the intracellular calcium wave propagation underneath this extracellular ATP wave was successfully simultaneously imaged. The data shows that the ATP extracellular signaling process is a dominant pathway for evoked calcium wave propagation in glial cells, but ATP release itself is not calcium-dependent.

CHAPTER 1

GENERAL INTRODUCTION

Dissertation Organization

This dissertation begins with a general introduction that consists of reviews of recent progress of glutamate analysis and ATP analysis in central nervous system with 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 in separate chapters. References for each paper and manuscripts are at the end of the chapter in which they are cited. The last chapter (chapter 5) presents general conclusions.

Glutamate in Central Nervous System

Overview

Five amino acids have been considered to serve neurotransmitter functions in central nervous system (CNS). Among these five compounds, glutamate and aspartate are considered to play excitatory function and γ-aminobutyric acid (GABA) , glycine and taurine are believed to work as inhibitory neurotransmitters(1).

Glutamate is considered as a major excitatory neurotransmitter compared to aspartate (1). The possibility of glutamate serving as an active excitatory neurotransmitter in CNS was first demonstrated in 1950s(2-4). It is found that glutamate could stimulate the depolarizing impact in spinal cord at single neuron level (3,4). In the meantime, scientist still could not accept the implication of discovery of glutamate as a neurotransmitter because of the high

concentration level and wide distribution of glutamate in CNS. Glutamate is found existing in cortex around 80um /g protein (5,6) and it in synaptic vesicles was reported as high as 60mM /g protein (7,8). The identified neurotransmitters at that time, acetylcoline and catcholamines, both exist in very restrict regions in CNS and at low concentrations.

Nevertheless, evidence was established to support the fact that glutamate is an excitatory neurotransmitter in the peripheral and CNS in the late 1970s and 1980s(9-11). In 1965, it is first reported (12) that glutamate is released from the cortex of cats with the surface superfusion approach. It is also found that the release of glutamate was accompanied by the release of aspartate, glycine and taurine after electrical stimulation. In 1976, it is observed that the release of glutamate from the visual cortex of rats by electrical or high K^+ stimulation was Ca^{2+} dependent (13).

However, there was no evidence that the sites of glutamate release were in the neural circuits of cortex at that time. Later in 1985, it is discovered that the ipsilateral cortico-cortical association fiber release glutamate as a transmitter (14). Around that time several areas were also found to release glutamate, such as the collosal projection fibers and the neostriatum in a moving rat (15,16). This led to the discovery of the spatial resolution of glutamate release.

There are five classes of synaptic receptors that have been identified for glutamate. Each of which is named for a selective agonist: N-methyl-D-aspartate (NMDA), Kainate (KA), AMPA, ACPD and AP4 (17-20).

Figure 1 illustrates the proposed metabolic cycle of glutamate in CNS. In glial cells glutamine is generated from either intake of released glutamate from neurons or from glutamate generated from the citric acid cycle (TCA) in the presence of glutamine

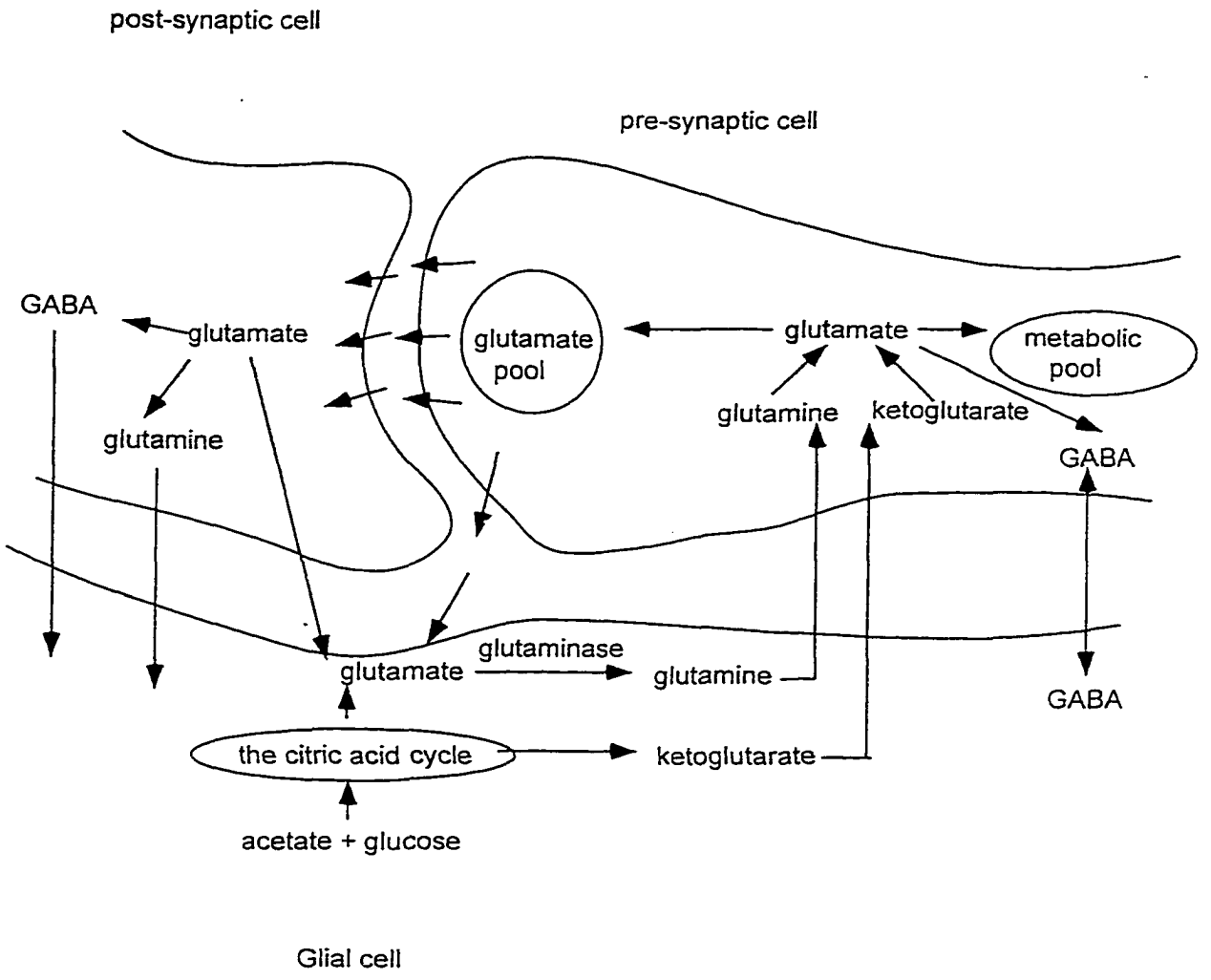


Figure 1: Glutamate in CNS

synthetase. Glutamine is then discharged, along with another component from TCA, α-ketoglutarate, from the glial cells into extracellular space and enters the nerve terminal of presynaptic neurons by a low affinity system or diffusion. At there, the glutamate is converted from glutamine by glutaminase and from α-ketoglutarate by glutamic dehydrogenase (GDH). The glutamate then has three pathways: the first is to move to transmission pool ready for synaptic transduction cycle; the second is functioning as the precursor of GABA, and the rest goes to metabolic pool. The released glutamate from presynaptic cell into extracellular space will be absorbed by postsynaptic cell to complete the signal transduction process and converted to GABA or glutamine there, and transfer to glial cells along with the rest excess amount of glutamate left in cleft and the cycle continues to next round.

Current approaches for glutamate analysis in CNS

The glutamate transmitter system has captured the attention of a wide range of investigators because it not only mediates standard fast excitatory synaptic transmission, via ionotropic receptors, but also participates in more complex neuronal process, such as development, learning and memory, and even neuropathology. Glutamate 's capacity to contribute to these processes is a product of the wide variety of intercellular signals that this transmitter can generate and thus interact with other neurons and glial cells through receptor-mediation systems. On the other hand, overstimulation of these signaling process by glutamate could also cause the neuronal damage associated with a wide variety of neurological insults and diseases, including ischemia, anoxia, stroke, hypoglycemia,

epilepsy, Huntington's disease, amyotrophic lateral sclerosis, lathyrisms and Alzheimer's disease.

Based upon the characteristics of CNS it is imperative for any successful analysis method to have following capabilities: (1) High selectivity. The CNS is a complicate network system formed by billions of various types of cells. With too many active processes undergoing at the same time the method ought to distinguish analyte molecules from thousands of possible interference factors; (2) High sensitivity. The concentrations of neurotransmitters presenting both in intracellular and extracellular fluids are usually very low, typically around μM level some of them even down to nM to perform normal neurotransmitter functions; (3) Fast response. The dynamic signal transduction process for neurotransmitters could happen and complete in few milliseconds. Under this magnitude any method that can not response fast enough would not be able to reveal the real behaviors of neurotransmitters in CNS; (4) Miniaturization. The dimension of neurons and glial cells in CNS is only at micron meter size and the distance between the presynaptic and postsynaptic cells is at nanometer range, so in order to obtain representative data of neurotransmitter at good spatial resolution for such extremely heterogeneous system, the sampling ability of the method should be able to fit such dimensions.

The current methods of glutamate measurement as neurotransmitter in CNS usually consist of multiple steps including sample collection followed by subsequent analysis with various techniques.

Sample collection

Sample collection includes evoking glutamate release and collection.

The types of samples include tissue slices, isolated bulk cells/ synaptosomes and intact tissue/cell cultures.

The techniques used to evoke glutamate release include ionophores, electrical stimulation and high concentrations of K^+. The first method applies certain compounds to sample cell membrane to open the voltage-sensitive ion channels such as sodium, potassium and calcium channels, and the intracellular concentrations of such ions thus increase and cause cell membrane depolarized and as the subsequence the glutamate ion is released with other ionic species through ion channels of the cell membrane. The side effect is that such compounds may cause cell damage after depolarization (21,22).

Applying electrical pulses to the neuron membrane is another technique to evoke glutamate release. When the electrical current passes the external bulk solution medium, a small fraction of it would pass across the cell membrane and thus some voltage dependent channels open and cause the depolarization of the cell (23).

The last method to evoke glutamate release is to raise the external solution K^+ concentration. This method is the most popular one among three techniques because of the ease for use. The high K^+ concentration required for stimulation ranges from 10 to 80mM while the normal concentration is about 2-4 mM. Upon such increase of K^+ concentration the cell membrane calcium channel will open and the intracellular calcium concentration will increase, and this triggers the exocytosis process of cell membrane and the content of glutamate pools in synapse area will be released. So this synaptic release of glutamate due to high K^+ concentration is calcium dependent (24).

The following collection of released glutamate is generally done by sample perfusions. Two types of techniques are usually used and based on their collection mode they could be categorized as "static" and "dynamic" modes. In first mode the sample tissue is incubated in the neurophysiological medium like Ringer's solution and is stimulated by evoke methods previous described, then the solution containing the released glutamate is collected and analyzed (25). This method can be used for "*in situ*" type experiments. Contrarily to this method, the second technique is aimed to collect glutamate released on a continuous basis. In this method small fractions of samples are continuously collected through specific devices during all the stimulation period, and by this way the dynamics of cell releasing glutamate can be studied. The most widely use of device in this technique is microdialysis tubing. A microdialysis tubing probe is inserted into sampling region, such as places right close to specific area of tissue slices, or cleft areas between different brain parts. The carrier medium is flown through the probe sensing tip and back, the glutamate in sensing region outside the tip will be collected into the medium based on different concentration inside and outside the tube. By using different types of dialysis tubing, species with high molecular weight can be excluded. In addition, this probe is flexible and will not damage the tissue and can be applied directly to the location of interest. This method can be used to "in vivo" type experiments (26).

Analytical methods

There are several analytical techniques available to determine the glutamate from CNS samples. These techniques include high performance liquid chromatography (HPLC), gas chromatography –mass spectrometry (GC/MS), isotope assays and enzymatic assays.

HPLC Method

HPLC is the most common technique for amino acid determinations. Numerous methods have been investigated for free amino acids determinations which mainly apply either pre-column or post –column derivatization method with ultraviolet, fluorescence or electrochemical detection schemes (27-32). Among these methods, separation on a reverse-phase column followed by fluorescence or electrochemical detection provides the most sensitive and easiest methodology.

The o-phthaldialdehyde (OPA) derivatization reaction is commonly used in pre-column derivatization method and with fluorescence or electrochemical detection. The OPA reagent reacts with amino acids rapidly to generate a highly fluorescent compound, thio-substituted isoindoles (33). The mobile phase includes methanol+buffer system such as acetate, phosphate at acidic pH and with a gradient system too reduce the retention time and stabilize the derivatives. The retention time for amino acid neurotransmitters is around 2-12min with this method. The detection limit is at about subpicomole range.

The HPLC system with fluorescence or electrochemical detection offers good detection limits and easy operation for amino acid measurements. The separation is fast and good resolution is obtained with gradient system. In addition, most of the amino acids can be separated and measured at the same time. The only disadvantage is that with gradient system it's time consuming to re-equilibrate the column between the runs.

Gas Chromatography- Mass Spectrometry (GC/MS)

GC/MS is the powerful tool for amino acid analysis. With derivatization procedure on GC column the detection limit down to 10 attomoles could be achieved (34). Because fragmentation occurred during MS step the pre-purification of sample is necessary to exclude interference ions to guarantee the maximum sensitivity.

Isotope assays

This method involves treating the amino acids with a radiolabled compound. The resulting radiolabled amino acids are detected by various assays (35). Radioreceptor assays are commonly used for amino acid neurotransmitters. In this procedure, the radiolabled components are incubated with the tissue that contains the neurotransmitter receptor of interest and the ligand binding to specific recognition sites is examined. Another assay is based on the double-isotope dansyl microassay (36). Amino acids sample is added to a mixture of ^{14}C-labeled amino acids, and the compound is dansylated by [^{3}H] –dansyl chloride. The dansylated amino acids are then separated by thin layer chromatography and the ratio between [^{3}H] and [^{14}C] for each dansyl amino acid is measured. The ratio shows a linear relationship to the unlabelled amino acid in the original sample.

This technique is reliable except the considerable manipulation of sample with isotope reagents.

Enzymatic Assays

This method is based on using an enzyme that is selective to the specific analyte and measuring the concentration of assay products, which can be related back to analyte concentration, by various detection methods. The enzymes that are selective to glutamate include glutamate dehydrogenase, glutamate oxidase, and glutamate decarboxylase. The common procedure used today is to add the enzyme and required cosubstrates to the sample and observed the signal generated from the reaction products by means of fluorescence, luminescence or spectrophotometry.

Various methods for measuring glutamate based on enzymatic assay have been developed (37-45). With the utilization of glutamate dehydrogenase, coupled with

cosubstrate NAD^+, the product of the reaction, NADH, can be monitored either by fluorescence or by absorption. The generated signal of NADH then can be calculated back to the measured glutamate concentration. For assay utilizing glutamate oxidase, the reaction includes consumption of oxygen and generation of ammonia and hydrogen peroxide. The measurements thus are completed by the detection of either the depletion rate of oxygen in reaction medium or the generation rate of ammonia and peroxide. Several oxygen and ammonia gas sensors were successfully built with this principle and applied to neurochemical analysis. In addition, the peroxide generated in the reaction can be further coupled to other peroxidase-based enzyme assays and the final products can be monitored by fluorescence and chemiluminescence.

Glutamate decarboxylase catalyzes the reaction of glutamate in the presence of water to generate γ-aminobutyric acid and carbon dioxide. The detection is accomplished either by monitoring the generation rate of carbon dioxide with gas sensor or by coupling to a pH sensitive dye, because the accumulation of carbon dioxide in reaction solution can cause the pH changes in a certain range, thus the signal from the dye can be measured spectrophotometrically.

The enzymatic assay based methods are fast and sensitive. They usually have a wide range of linear response to measured glutamate. The inherent high selectivity eliminated time-consuming sample pretreatment step aimed to exclude interference factors, and thereby offers simple, convenient and reliable approach for amino acid determination. The only disadvantage is that the enzymes used are usually expensive, so the conventional analysis format of addition of enzyme mixture to sample solution makes it impossible for the enzymes to be reused and is not cost-effective. This hinders the wide application of this

method in neurochemical analysis. The compromise approach prevalent today is to immobilize the enzyme onto a solid support in the form of a sensor, then the enzyme can be more stable and used repeatedly. Although this approach comes along with a certain degree of sacrifice of enzyme sensitivity from the immobilization procedure due to denaturalization of enzyme, it is proved that this biosensor format offers advantages on stability, ease of manipulation, signal reproducibility, and of course, the cost effectiveness.

Single cell analysis

With the development of microcolumn liquid chromatography and, especially in recent years, the fast progress of capillary electrophoresis techniques, single cell analysis technique has gained great interests and considerable efforts have been taken for the developments and advances (46-53).

The interests and development of single cell analysis technique come from two sources: first, all the conventional analysis tools described above are all bulk-average based methods, which means the signal is taken from a bulk of biological cell samples and finally the results are calculated back to single cell level. While this is acceptable and also is rather accurate for general biological sample analysis like blood analysis because the individual variances from the samples fall into a tolerant range and not causing big deviation from bulk average results, it is not true for neurochemical analysis. The CNS is a very complicated system with hundreds of different active molecules involved in numerous processes in different functional parts of the brain, it is also very heterogeneous among the individual cells in the same functional part, even in the different part of the same cell body. It is thus important to understand the variance for individual cells and their effect on physiological functions. The result from bulk average method will not able to tell the real dynamics and

mechanisms of the functions and pathways. It is also important for clinical diagnostic purpose, because the single cell analysis make it possible to find the first few abnormal cells at their early stage. Otherwise the majority of normal cell samples masks them out if analyzed by bulk average method.

Second, the development of chromatographic microcolumn technique, especially the progress in capillary electrophoresis (CE), make it practically possible to advance the single cell analysis. This is because: 1) the small dimension of capillary format, in addition to the ease of manipulation of CE, makes sampling of single cell without difficult. The inner diameter of capillary is usually range only from 10 –50 μM, which is at the same magnitude as that of cells; 2) the extremely high separation efficiency ($10^5 - 10^6$ theoretical plates) expedites the separation of complex constituents from intracellular species when cell lysed or released; 3) the capillary itself can be used as a reaction chamber after sampling, and so various on-column derivatization procedures or treatments to cell can be processed to investigate the cell functions and release kinetics of interest under to designed reaction conditions.

Several kinds of neuronal cells were studied by single cell analysis technique for the determination of neurotransmitters.

Dopamine, serotonin, tyrosine and tryptophan were identified and quantified from three different neurons, D2, E4 and F1, from the land snail Helix aspersa by open tubular liquid chromatography (OTLC) with voltammetric detection (47). The detection limit is at femtomole level, which is more sensitive than conventional methods. The advantage of this method comes from the inheritance of OTLC: high resolving power from HPLC column, in addition to the high sensitivity of electrochemical detector.

Catecholamines (norepinephrine and epinephrine) in single bovine adrenomedullary cells were determined quantitatively by C-18 reversed-phase microcolumn liquid chromatography (48). With electrochemical detector, the detection limit is down to tens of attomol. Through analysis of over 20 individual cell samples, the variance of catecholamines among these cells were demonstrated and their physiological implications were discussed. More progress was achieved on exploration of capillary electrophoresis (CE) technique. The common procedure is that single cell is injected into front end of capillary, which the sample volume is only sub-nanoliter with a 10-20μM i.d. capillary, and either on-column derivatization right on-site followed by separation or post-column derivatization after separation is carried. The detection is completed by various methods such as electrochemical, laser induced fluorescence and immunoassay detection (54-56). Compared to the pre-column derivatization commonly adopted in microcolumn liquid chromatography (50), the on-column and post-column derivatization is more used for detection of molecules that cannot fluoresce naturally in CE. It is because of the ease of manipulation advantage from CE format to carry derivatization on column, and more important, it limits the sample dilution factor by the size of the capillary, thus improve the sensitivity, and solves the multiple labeling problems associated with pre-column derivatization.

Hemoglobin and carbonic anhydrase in individual erythrocytes have been separated by CE and quantitated by using laser induced native fluorescence (LINF) detection with a UV line from an Ar ion laser (57).

The activities of several lactate dehydrogenase isoenzymes have been determined in individual human erythrocytes by monitoring the enzyme-catalyzed production of NADH using laser induced fluorescence (LIF) detection after separation of isoenzymes (58).

Exocytotic release of serotonin, insulin and catecholamine from single mast cells, pancreatic β-cells and adrenal chromaffin cells were quantitatively monitored by CE-LINF (59-61). An on-column derivatization procedure with LIF detection method by CE was developed and the analysis of individual rat pheochromocytoma (PC12) cell was demonstrated. Dopamine and 5 amino acids were detected and determined quantitatively (62).

The development of these CE-based methods demonstrates that CE is well suited for analysis of the wide variety of molecules found in cells, and is specially a power tool for solving heterogeneity complexity in neurochemical analysis.

Fluorescence microscopic methods

Fluorescence microscopy is a powerful tool in that it allows spatial and temporal visualization of fluorescent material in microscope specimens. Because of this capability, this new technology has been used in neuroscience to address fundamental and important physiological functions and dynamics. It allows user to look inside the neuronal cells and brain tissue slices with exceptional detail. It provides the user with acquisition of spatial information and temporal alterations of objects that are either intrinsically fluorescent or which have been coupled to extrinsic fluorescent molecules. The combination of the specificity inherent in current fluorescence techniques with the sensitivity of the fluorescence imaging system has led to the ability to detect very small amounts of material with very high sensitivity and precision.

Various applications have been developed in neurochemical analysis by using fluorescence imaging techniques. To name a few, immunofluorescence, which can be used for the identification, localization and visualization of cell and tissue component molecules

based on the fact that is possible to produce highly specific antibodies against individual cell constituents (antigens) to label these antibodies with fluorescent molecules and the visualize these fluorescent antibodies after they have been applied to microscopic specimens; Fluorescence in situ hybridization (FISH), which is similar to immunofluorescence except that FISH allows the direct visualization and localization of DNA and RNA sequence on chromosomes in cells and in tissues; Digitized video microscopy, in which the intact cells and tissues' physiological functions are manipulated and measured by video microscopy so that the multiple cellular activities in sample specimen are recorded in same time and in real time scale. In general, the kinetic process in living cells, genetic conformation of DNA and RNA inside cell chromosomes and 2-D and 3-D characteristic cell image reconstruction are all been intensively explored with fluorescence microscopy techniques.

Many important biological macromolecular constituents and free ions such as H^+ and Ca^{2+} In signal transduction process have been studied with fluorescence microscopy. Fluorescent labeling probes for target molecules or native fluorescence method have been developed and representative data for molecule distribution, dynamics of processes such as endocytosis and excytosis have been presented.

Calcium imaging in neuronal cells is a well-established common method to monitor the distribution and dynamics of calcium ion movement in cells. With the calcium sensitive dyes fura-2 and fluo-3, the concentration changes of calcium ion in different kinds of neurons and glial cells (63-67) with respect to various pathways in which calcium ion is involved have been successfully exploited and great progress has been achieved on the understandings of nature of activities in brain.

Serotonin is another important neurotransmitter involved in many endocytosis and exocytosis processes in CNS. Because serotonin possess native fluorescence, the laser induced native fluorescence (LINF) method has been developed with the advantages of specificity, low background and high sensitivity. The uptake of serotonin by single astrocytes was first monitored by LINF microscopy with simultaneous temporal and spatial resolution (68). The exocytotic releases of serotonin from astrocytes, mast cells were also studied with LINF microscopy (69-70). The highly localized phenomenon of granule and vesicle release dynamics is much more revealed with the help of inherent advantages of simultaneous high spatial and temporal resolution of fluorescence microscopy.

For glutamate behavior in signal transduction, it is of no doubt that the real time imaging of glutamate movement and concentration change will greatly enhance the understanding of many important neuronal activities. Though some efforts has been made such as simulation study of real time release dynamics of glutamate between pre- and post-synaptic cells (71), no real imaging data has been reported yet. The difficulties lie in the fact of fast dynamics that the whole process of synaptic glutamate release may be finished in few milliseconds at a extremely small dimension (the cleft of between pre- and post-synaptic cells is usually only several hundred nanometer at distance), and so far there is no effective derivatization method established (the glutamate has no fluorophore group on it) to detect such fast, transient process.

Despite these factors, the interest on glutamate keeps strong for reasons described previously and new progress will be made in the future with the development of higher sensitive imaging techniques, specific assay probes and new derivatization methods.

ATP in Central Nervous System

Overview

Adenosine-3"-triphosphate (ATP) is best known as the most important energy source in cellular metabolism. Almost all living cells contain ATP and it plays a central role in the energy status of the cell because it serves as the energy source for all kinds of metabolic changes and cellular functioning (72,73). In addition, ATP is also found to have regulation ability for intracellular enzyme activity (74,75).

Analytical approaches for ATP measurement

Several analytical techniques have been developed for the determination of ATP in biological samples.

Electrochemical based methods: cyclic voltammetry and chronoamperometry are used to develop the ATP measurement method based on competitive receptor binding with hexacyanoferrate (II) ion, with which the ATP-receptor binding is otherwise nonelectroactive (76). Theoretical study shows the sub-millimolar concentration of ATP could be detected. Also, Sinusoidal Voltammetry method has been successfully developed to directly measure the ATP concentration, and the detection limit of submicro molar level can be reached with this technique (77).

Spectroscopic based methods: it is reported (78) that the rapid Fourier transform infrared (FTIR) spectroscopy and time-resolved single wavelength infrared spectroscopy (IR) have been used to follow the photochemical release of ATP molecule. The temporal resolution can be reached around millisecond-to-second scale. Though biological application of these IR techniques has not been developed yet.

Luminescence based method: the most widely used technique in biological science so far for the determination of ATP content from samples is based on firefly luciferase-luciferin bioluminescence assay technique (79-87). In this assay, the enzyme extract from firefly *photinus pyralis*, the luciferase, is coupled to co-substrates luciferin (D-form), and with the existence of magnesium ion, catalyzes the ATP molecule to AMP and pyrophosphate. Accompanied with the result products of the reaction is the light emittance with wavelength around 560nm, as depicted in following equations:

$$E + LH2 + ATP \rightarrow E\text{-}LH_2\text{-}AMP + PP$$

$$O_2 \downarrow \text{——} \text{light (560nm)}$$

$$E + L + ATP \rightarrow E\text{-}L\text{-}AMP + PP$$

E: luciferase; LH_2: D-luciferin; PP: pyrophosphate; L: oxyluciferin.

Oxygen is required for the light emitting step.

This reaction is fast, specific and sensitive. After only few hundred milliseconds, or even shorter in practical situation upon enzyme coupling to ATP molecule, light output can be observed; The specificity of the enzyme is excellent: only ATP can trigger the light emission reaction, many analogues of ATP such as ADP, AMP, UDP and other purine nucleotides have no effect on enzyme, so the interference factor can be controlled to minimal content; the reaction is also very sensitive: with the right combination of enzyme and substrate concentrations and proper instrument conditions, the total amount down to attomoles of ATP from extracts of cell samples can be detected.

The typical light output curve from this reaction is shown in figure 2. Through carefully study of this light emission it is found that the flash shape like peak height at the beginning can be used to quantitatively determine the correspondent amount of ATP in the reaction. The linear relationship is excellent (over 99.9%) and the dynamic response ranges over 4 to 5 magnitudes and can start from down to 10^{-18} mole level, even with commercial assay kit without any modification.

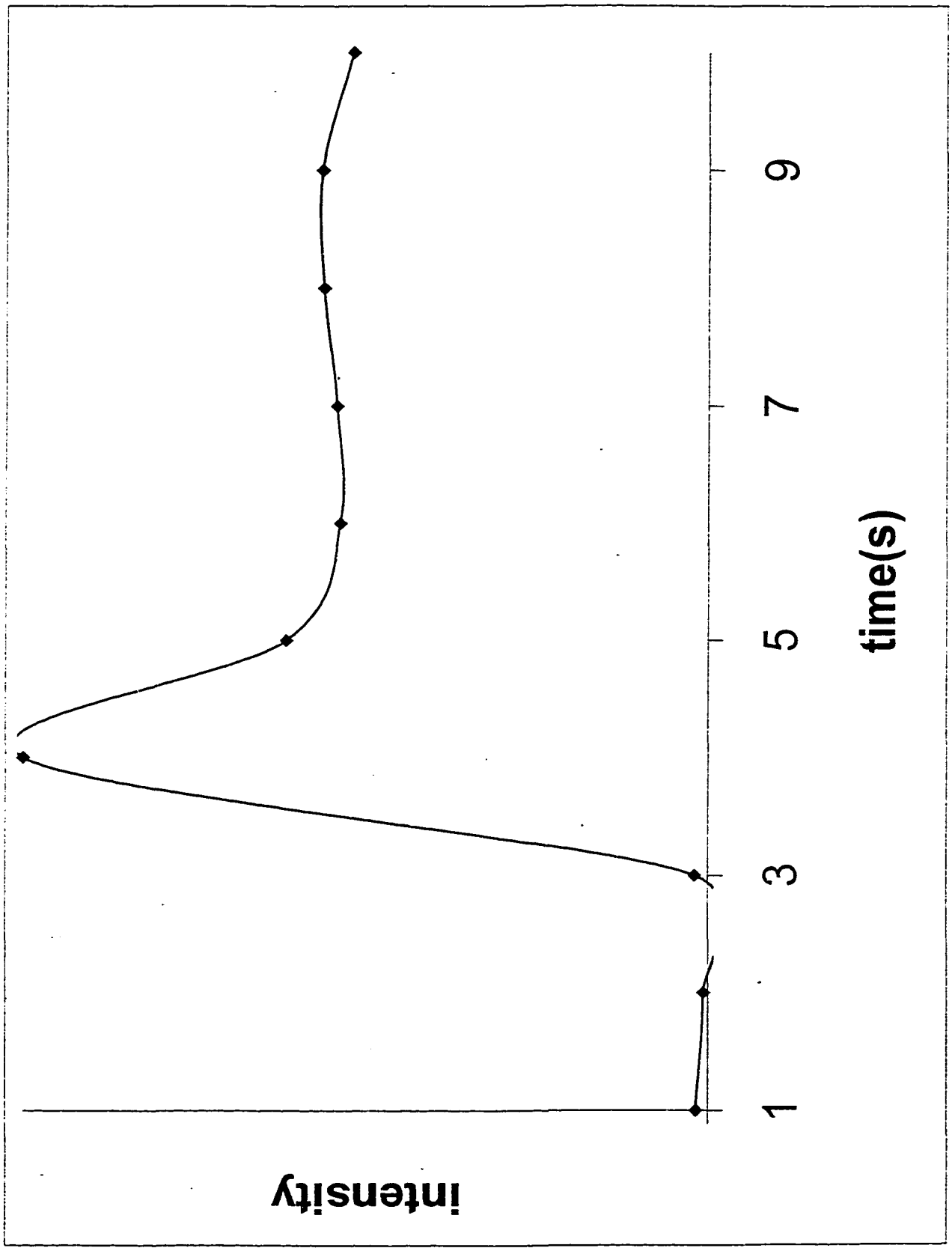
Various applications based on this assay in biological study have been reported (88-95). The determinations of ATP content in different types of cells and tissues were used in clinical diagnostics, such as bacteria testing and antibiotic assay; it is also used in routine microbiology area, food industry, pharmaceuticals, environmental monitoring, even space technology.

ATP as neuromessenger in CNS

In 1970s, ATP is first found to have an effect on neurotransmission in some CNS systems (96), though there was skepticism for ATP as a neurotransmitter or neuromodulator then. In late 20 years, the critic evidences for ATP as a neurotransmitter or neuromodulator have been found and the role of ATP in CNS is confirmed and established (97-101).

It is therefore a very interesting and important subject of study of ATP as neurotransmitter in CNS at the cellular and subcellular level. The release of ATP from micro-dissected organ of Corti has been measured with bioluminescent assay (102). The extracellular concentration of ATP in hippocampal slices from seizure-prone mice has been estimated (103). Co-releases of ATP with some other well-known neurotransmitters such as norepinephrine and acetylcholine were discovered and studied by electrophysiological

Figure 2: Light emission response to ATP from luciferase-luciferin enzyme reaction.


intensity
1
3
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7
9
time(s)

methods (104-108). The groups of ATP-specific receptors have been identified and classificated (109).

While advances are making everyday, the image of ATP function and metabolic cycle in CNS is far to be completely constructed yet.

References

1. Christopher, J. *Surv. Ophthalmol.* **1985**, *29(5)*, 355-360.
2. Hayashi, T. *Keio J. Med.*, **1954**, 302, 183-188.
3. Curtis, D.; Eccles, R. *J. Physiol. (London)* **1958**, *141*, 435-450.
4. Curtis, D.; Philis, J.; Watkins, J. *Nature* **1959**, *183*, 611-613.
5. Banay-Schwartz, M.; Lajtha, A.; Palkovitz, M. *Neurochem. Res.* **1986**, *373*, 252-257.
6. Palkovitz, M.; Lang, T.; Patthy, A.; Elekes, I. *Brain Res.* **1986**, *373*, 252-258.
7. Burgar, P.; Mehl, E.; Cameron, P.; Maycox, P.; Baumert, M.; Lottspeich, F.; De Cammili, P.; Jahn, R. *Neuron* **1989**, *3*, 715-719.
8. Naito, S.; Ueda, T. *J. Neurochem.* **1985**, *44*, 99-105.
9. Fagg, G.; Foster, A. *Neuroscience* **1983**, *9*, 701-706.
10. Fonnum, F. *J. Neurochem.* **1984**, *42*, 1-11.
11. Roberts, P.; Storm-Mathisen,J.; Johnston, G. *Glutamate: Transmitter in the Central Nervous System* John Wiley and Sons, New York, **1981**.
12. Jasper, H.; Khan, R.; Elliott, K. *Science* **1965**, *147*, 1448-1450.
13. Clark, R.; Collins, G. *J. Physiol. (London)* **1976**, *263*, 383-388.
14. Hicks, T.; Guedes, R.; Veals, W.; Veenhuizen, *J. Exp. Brain Res.* **1985**, *58*, 421-423.
15. Druce, D.; Peterson, D.; De Belleronche, J.; Bradford, H. *Brain Res.* **1982**, *247*, 303-306.
16. Peinado, J.; Iribar, M.; Myers, R. *Neurochem. Res.* **1987**, *12*, 629-633.

17. Mayer, M.; Westbrook, G. *Prog. Neurobiol.* **1987**, *28*, 197-200.

18. Tadaharu, T. *Neurosci. Res.* **1990**, *9*, 79-82.

19. Bradford, H. *Chemical Neurobiology: An Introduction to Neurochemistry* W. H. Freeman and Co., New York, **1986**.

20. Fagg, G. *TINS,* **1985,** May.

21. Walz, W. in Boulton, A; Baker, G.(*eds.*) *Neuromethods 3: Amino Acids,* Humana Press, Clefton, NJ, **1985.**

22. Nicholls, D.; Attwell, D. *TiPS,* **1990,** 11, 462-468.

23. Greenshaw, A. in Boulton, A; Baker, G.(*eds.*) *Neuromethods 3: Amino Acids,* Humana Press, Clefton, NJ, **1985.**

24. Lipton, P. in Boulton, A; Baker, G.(*eds.*) *Neuromethods 3: Amino Acids,* Humana Press, Clefton, NJ, **1985.**

25. Parpura, V.; Liu, F. Brethorst, S.; Jeftinija, K.; Jeftinija, S.; Haydon, P. *FEBS Letters* **1995**, *360*, 266-270.

26. Ungerstedt, U. in Marsden (*eds.*) *Measurement of Neurotransmitter Release in Vivo,* John and Sons, New York, **1984.**

27. Cosnier, S.; Innocent, C.; Allien, L.; Poitry, S.; Tsacopoulos, M. *Anal. Chem.* **1997**, *69*, 968-971.

28. Boutelle, M.; Fellows, L.; Cook, C. *Anal. Chem.* **1992**, *64*, 1790-1794.

29. Lindroth, P.; Mopper, K. *Anal. Chem.* **1979**, *51*, 1667-1674.

30. Zecca, L.; Zambotti, F.; Zonta, N.; Mantegazza, *J., Chromatogr.* **1982**, *233*, 307-312.

31. Roger, K.; Philibert, A.; Allen, A.; Molitor, J.; Wilson, E.; Dutton, G. *J. Neurosci. Methods*. **1987**, *22*, 173-179.

32. Caudill, W.; Honck, G.; Wightman, R. *J. Chromatogr.* **1982**, *227*, 331-339.

33. Durkin, T.; Anderson, G. *J. Chromatogr.*, **1988**, 428, 9-13.

34. Wood, P.; Cheney, D. in Boulton, A; Baker, G.(*eds.*) *Neuromethods 3: Amino Acids,* Humana Press, Clefton, NJ, **1985.**

35. Ferkany, J. in Boulton, A; Baker, G.(*eds.*) *Neuromethods 3: Amino Acids,* Humana Press, Clefton, NJ, **1985.**

36. Butterworth, R. in Boulton, A; Baker, G.(*eds.*) *Neuromethods 3: Amino Acids,* Humana Press, Clefton, NJ, **1985.**

37. Nicholls, D. *J. Neurochem.* **1989**, *52*, 331-341.

38. Fosse, M.; Kolstad, J.; Fonnum, F. *J. Neurochem.* **1986**, *47*, 340-347.

39. Dremel, B.; Schmidt, R.; Wolfbeis, O. *Anal. Chim. Acta,* **1991**, 248, 351-358.

40. Kar, S.; Arnold, M. *Anal. Chem.* **1992**, *64*, 2438-2443.

41. Opdjcke, W.; Park, S.; Meyerhoff, M. *Anal. Chim. Acta*, **1983**, 155, 11-20.

42. Wolfbeis, O.; Weis, L.; Leiner, M.; *Anal. Chem.*, **1988**, 60, 2028-2035.

43. Villarata, R.; Cunningham, D.; Guilbault, G. *Talanta*, **1991**, 38, 49-60.

44. Yao, T.; Kobayashi, N.; Wasa, T. *Anal. Chim. Acta*, **1990**, 231, 121-135.

45. Diaz, G.; El-Issa, L.; Arnold, M.; Miller, R. *J. Neurosci. Meth.*, **1988**, 23, 63-67.

46. Kennedy, R. T.; St. Claire III, R. L.; White, J. G.; Jorgenson, J. W. *Mikrochim. Acta,* **1987**, 11,37-45.

47. Kennedy, R. T.; Jorgenson, J. W. *Anal. Chem.* **1989**, 61, 436-441.

48. Cooper, B. R.; Jankowski, J. A.; Leszczyszyn, D. J.; Wightman, R. M.; Jorgenson, J. W. *Anal. Chem.* **1992**, 64, 691-694.

49. Wallingford, R. A.; Ewing, A. G. *Anal. Chem.* **1988**, 60,1972-1975.

50. Kennedy, R. T.; Gates, III. D.; Cooper, B. R.; Nickerson, B.; Jorgenson, J. M. *Science*, **1989**,246,57-63.

51. Olefirowicz, T. M.; Ewing, A. G. *Anal. Chem.* **1990**, 62, 1872-1876.

52. Olefirowicz, T. M.; Ewing, A. G. *Chimia,* **1991**, 45, 106-108.

53. Hogan, B. L.; Yeung, E. S. *Anal. Chem.* **1992**, 66, 2841-2845.

54. Ewing, A. G.; Mesaros, J. M.; Gavin, P. F. *Anal. Chem.* **1994.** 66, 527A-537A.

55. Yeung, E. S. Optical Detectors for Capillary Electrophoresis, in *Advances in Chromatography,* Vol. 35, Brown, P. R. and Grushka, E. Eds., Marcel Dekker, New York, **1995**, pp.20-31.

56. Regnier, F. E.; Patterson, D. H.; Harmon, B. J. *Trends Anal. Chem.* **1995,** *14,* 177-181.

57. Lee, T. T.;Yeung, E. S. *Anal. Chem.*, **1992**, 64, 3045-3051.

58. Xue, Q.; Yeung, E. S. *Anal. Chem.* **1994,** 66, 1175-1178.

59. Lillard, S. J.; Yeung, E. S.; McCloskey, M. A. *Anal. Chem.* **1996**, 68, 2879-2904.

60. Tong, W.; Yeung, E. S. *J. Chromatogr. B* **1997**,689,321-325.

61. Tong, W.; Yeung, E. S. *J. Neurosci. Meth.* **1998,** 82(2), 151-158.

62. Gilman, S. D.; Ewing, A. G. *Anal. Chem.* **1995,** 34, 58-64.

63. Williams, D. A.; Fay, F. S. *J. Biophys.* **1985**, 47, 131A-139A.

64. Murphy, T.; Baraban, J.; Wier, W.; Blatter, L. *Science* **1994**, 263: 529-532.

65. Cornell-Bell, A.; Finkbeiner, S.; Cooper, M.; Smith, S. *Science* **1990**, 247: 470-473.

66. Pol, A.; Wuarin, J.; Dudek, F. *Science* **1990**, 250; 1276-1278.

67. Jovin, D.; Nicoud, M.; Kaufman, S.; Jovin, T. *Science* **1985**, 230: 247-256.

68. Tan, W.; Parpura, V.; Haydon, P. G.; Yeung, E. S. *Anal. Chem.* **1995,** 67, 2575-2579.

69. Lillard, S.J.; Yeung, E. S. *J. Neurosci. Meth.*, **1997**, 75, 103-109.

70. Tan, W.; Haydon, P. G.; Yeung, E. S. *Appl. Spectroscopy.* **1997**, 51, 1139-1133.

71. Dmitri, A.R.; Dimitri, M. K. *J. Neurosci.*, **1998,** 18(9), 3158-3170.

72. Bridger, W.; Henderson, J. *Cell ATP,* Wiley, New York, **1983**

73. Alberts, B.; Bray, D.; Lewis, J.; Raff, M.; Roberts, K.; Watson, J. *Molecular Biology of the Cell.* Garland Publishing, New York, 1983, 43-91, 483-549.

74. Lundin, A.; Styrelius, I. *Clin. Chim. Acta.* **1978,** 87, 199-209.

75. Lundin, A.; Karnell, U.; Baltscheffsky, M. *Acta. Chem. Scand.* **1979**, B33, 608-609.

76. Bianchi, A.; Domenech, A.; Garcia-Espana, E. *Anal. Chem.* **1993,** 65, 3137-3142.

77. Singhal, P.; Kuhr, W. G. *Anal. Chem.* **1997,** 69, 3552-3557.

78. Barth, A.; Hauser, K.; Mantele, W. *J. Am. Chem. Soc.* **1995**, 117, 10311-10316.

79. Stanley, P.; McCarthy, B.; Smither, R. *ATP Luminescence*, Blackwell Scientific Publications, Oxford, **1989.**

80. Imbeault, N.; Paquet, M.; Cote, R. *J. Water Qual. Res. Canada.* **1998**, 33,403-415.

81. Abraham, E.; Okunieff, P.; Scala, S.; Vos, P.; Oosterveld, M.; Chen, A. Shrivastav, B. *Science,* **1997,** 275, 1324-1326.

82. Bitler, B.; McElroy, W. *Arch. Biochem, Biophys.* **1957,** 72, 358-368.

83. Denburg, J.; Reiko, L.; McElroy, W. *Arch. Biochem, Biophys.* **1969,** 134, 381-394.

84. Rhodes, W.; McElroy, W. *J. Biol. Chem.* **1958,** 233, 1528-3157.

85. Schifman, R.; Wieden, M.; Broker, J.; Chery, M.; Delduca, M.; Norgard, K.; Palen, C.; Reis, N.; Swanson, J.; White, J. *J. Clin. Microbiol.* **1984,** 20, 644-648.

86. Molin, O.; Nilsson, L.; Ansehn, S. *J. Clin. Microbiol.* **1983,** 18, 521-525.

87. Deluca, M.; McElroy, W.(eds.) *Bioluminescence and Chemiluminescence.* Academic Press, New York, **1981.**

88. Dadoo, R.; Seto, A.; Colon, L.; Zare, R. *Anal. Chem.* **1994,** 66, 303-306.

89. Beckers, B.; lang, H.; Schimke, D.; Lammers, A. *Euro. J. Clin. Microbiol.* **1985,** 4, 556-561.

90. Bussey, D.; Tsuji, K. *applied & Environ. Microbiol.* **1986**, 51, 349-355.

91. Daigneault, R.; Larouche, A. Thibault, G. *Clin. Chem.* **1979**, 25, 1639-1643.

92. Hamilton, R. Holm-Hansen, O. *Lumnology and Oceanography.* **1967,** 12, 319-324.

93. Macleod, N.; Chappelle, E.; Crawford, A. *Nature,* **1969,** 223, 267-268.

94. Levin, G. *Adv. Applied Microbiol.* **1968,** 10, 55-71.

95. Olsson, T.; Sandsted, K.; Holmberg, O. Thore, A. *Biotech. Applied Biochem.* **1986,** 8, 361-369.

96. Bobbin, R.; Thompson, M. *Ann. Otol. Rhinol. Laryngol.* **1978**, 87, 185-190.

97. Mockett, B.; Housley, G.; Thorne, P. *J. Neurosci.* **1994**, 14, 6992-7007.

98. Ashmore, J.; Ohmori, H. *J. Physiol.(Lond)* **1990**, 428, 109-131.

99. Lin, X.; Hume, R. Nuttall, A. *J. Neurophysiol.* **1993**, 70, 1593-1605.

100. Dulon, D.; Mollard, P.; Aran, J. *Neuroreport.* **1991,** 2, 69-72.

101. Yamasita, T.; Amano, H.; Kumazawa, T. *ORL J. Otorhinolaryngol. Relat. Spec.* **1993**, 55, 201-204.

102. Wangemann, P. *Audit. Neurosci.* **1996**, 2, 187-192.

103. Wieraszko, A.; Goldsmith, G.; Seyfried, T. *Brain Res.* **1989**, 485, 244-250.

104. Silinsky, E.; Redman, R. *J. Physiol.(Lond)* **1996,** 492, 815-822.

105. Redman, R.; Silinsky, E. *J. Physiol.(Lond)* **1994,** 477, 117-127.

106. Morel, N.; Meunier, F. *J. Neurochem.* **1981**, 36, 1766-1773.

107. Sawynok, J.; Downie, J.; Reid, A.; Cahill. C.; White, T. *Brain Res.* **1993**, 610, 32-38.

108. Cena, V.; Rojas, E. *Biochim. Biophys. Acta* **1990**, 1023, 213-222.

109. Burnstock, G.; Kennedy, C. *Gen. Pharmacol.* **1985,** 16, 433-440.

CHAPTER 2

DUAL-ENZYME ASSAY OF GLUTAMATE IN SINGLE CELLS BASED ON CAPILLARY ELECTROPHORESIS

A paper published in the Journal of Chromatography B: Biomedical Applications[1]

Ziqiang Wang and Edward S. Yeung

ABSTRACT

A new dual-enzyme on-column reaction method combined with capillary electrophoresis has been developed for determining the glutamate content in single cells. Glutamate dehydrogenase and glutamic pyruvic transaminase were used to catalyze the glutamate reaction. Detection was based on monitoring the laser-induced fluorescence of the reaction product NADH, and the measured fluorescence intensity was related to the concentration of glutamate in each cell. Glutamate dehydrogenase catalyzed the formation of NADH, and glutamic pyruvic transaminase drives the glutamate dehydrogenase reaction by

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removing a reaction product and regenerating glutamate. The detection limit of glutamate is down to 10^{-8} M level, which is 1 order of magnitude lower than previous reported detection limit based on similar detection methods. The mass detection limit of a few attomoles is far superior to that of any other reports. Selectivity for glutamate is excellent over most of amino acids. The glutamate content in single human erythrocyte and baby rat brain neurons were determined with this method and the results agreed well with literature values.

Keywords: Glutamate; Enzymes

INTRODUCTION

Biochemical analysis and research has developed to a stage where cellular and subcellular measurement techniques become possible. The capability for analysis at the single-cell or even single-molecule levels could provide insights into cellular and subcellular chemical processes without the necessity of averaging over large cell populations. Considerable efforts have been given to the development of analytical methods for glutamate due to its active role in human sensory system, in liver function, and most importantly, in the central nervous system(CNS). Glutamate functions as a primary excitatory neurotransmitter in CNS. The transport and communication of glutamate with other active species in CNS was proposed to be an important part of brain and CNS signaling process(1,2).

HPLC(3-8), GC-MS(9,10), isotope assay(11, 12), immunocytochemical method (13,14) and enzymatic assays (15-17)have all been applied to glutamate measurement with good sensitivity. The detection limits reported with these techniques have reached submicromolar concentration level or subfemtomole amount in biological samples. Despite the extensive published works, few dealt directly with glutamate function at the cellular or subcellular level due to the limitations of the analytical techniques used. However, the importance of such techniquess should be emphasized, because CNS is a complicated biological system extremely heterogeneous in both physical and chemical properties in different sections of the brain, among neighboring cells, and even different regions of a single cell.

Capillary electrophoresis(CE) offers several important advantages for the analysis of biological samples. The ability of CE to sample extremely small volumes (subnanoliter), its high separation efficiency, and the existence of extremely sensitive detection schemes make CE compatible with single cell and subcellular analysis relevant to neurochemical studies. Various methods and applications have been reported in recent years for single cell analysis by using CE (18-31). The components typically present in single cells are at the femtomole to zeptomole levels, which makes quantitative detection of these compounds extremely challenging.

Laser-induced fluorescence(LIF) detection, electrochemical detection and immunoassay have all been used to obtain sufficiently low mass detection limits for quantitative determination in individual cells (18, 20, 32). In this article we describe a dual-

enzyme reaction method combined with CE-LIF for determining the glutamate content in single cells. The enzymes used are glutamate dehydrogenase (GDH) and glutamic pyruvic transaminase (GPT). The reactions scheme is as follows:

$$\text{glutamate} + NAD^+ \rightarrow \alpha\text{-ketoglutarate} + NH_4^+ + NADH$$

(GDH catalyzed)

$$\alpha\text{-ketoglutarate} + \text{alanine} \rightarrow \text{pyruvate} + \text{glutamate}$$

(GPT catalyzed)

GPT drives the GDH reaction in the desired direction by removing a reaction product and regenerating glutamate. The detection is achieved by monitoring the fluorescence of the product NADH, and the measured fluorescence intensity is related to the concentration of glutamate. Single human erythrocytes and single rat neurons can be studied in this way.

EXPERIMENTAL

Reagents and Chemicals

L-glutamic acid(monosodium salt), L-alanine,β-NAD^+, NADH,Tris-phosphate, L-glutamate dehydrogenase (GDH, 36 units per mg protein, from bovine liver), glutamic pyruvic transaminase(GPT, 80 units per mg protein, from porcine heart), all amino acids used in the interference study, sodium chloride and sodium phosphate(monobasic) were purchased from Sigma (St. Louis, MO, USA). All experiments were carried in the 10 mM Tris-phosphate buffered solution with pH adjusted to around 7.

Enzyme solutions were prepared by diluting enzyme extracts with buffer solution to the 10^{-8} M level. All solutions were prepared daily to prevent possible contamination and changes due to bacteria growth.

Capillary Electrophoresis Instrumentation

Capillary electrophoresis experiments were carried out using an in-laboratory system (18,20). 20 kV was applied to a 20 μ I.D., 360 μ O.D. fused-silica capillary tube(Polymicro Technologies, Inc., Phoenix, AZ, USA) from a power supply(0-40kv, EH series; Glassman High Voltage, Inc., Whitehouse Station, NJ, USA) for separation of the enzyme reactions products. The total capillary length is 64 cm and effective separation length is 45 cm. The LIF detection system consisted of an argon ion laser(Model 2045, Spectra-Physics, Mountain View, CA, USA) with wavelength at 305 nm. The detection window was made by removing a 3 to 5 mm section of polyimide coating on the capillary. A 1 cm focal length quartz lens(Melles Griot Corp., Irvine, CA, USA) was used to focus the laser beam into the detection region. The fluorescence signal emitted from NADH was collected by a 20X microscope objective(Edmund Scientific Co., Barrington, NJ, USA). A 456 nm interference filter was placed in front of the photomultiplier tube(PMT, Model IP28, Hamamatsu Corp., Bridgewater, NJ, USA) to exclude scattered light. The output signal from the PMT was acquired by ChromPerfect software(Justice Innovations, Palo Alto, CA, USA) at 5 Hz and stored in IBM PC for subsequent data analysis.

Human Red Blood Cells

Human red blood cells were obtained from a normal adult male and sampled fresh each time before the experiment. The cells were washed according to following procedure (23). After centrifugation, the supernatant serum was siphoned off and a 135 mM NaCl and 20 mM sodium phosphate solution at pH 7.4(PBS) with a volume equivalent to 4-6 times the volume of red blood cells was added. The mixture was then shaken gently and centrifuged again (at 200g for 3 min), followed by removal of the supernatant. This siphoning-mixing-centrifuging cycle was repeated at least 6 times before use. For easy manipulation at the cell injection stage, further dilution might be necessary if the red blood cell number density was found to be too high.

Rat Neuron Cells

Hippocampi were removed from 18 day old rat embryos and washed with Earl's balanced salt solution (EBSS) for 5 min. before incubation in 0.25% trypsin solution for 15 min. The tissue was then washed for 5 min. prior to stopping the enzyme action with trypsin inhibitor. The tissue was washed again in EBSS for 5 min. before a 1-min. incubation in serum containing-medium. Medium was removed and fresh medium was added to the tissue at a rate of 1 ml per 6 hippocampi. The hippocampi were then dissociated by gentle trituration with a 5-ml glass serological pipette until no visible clumps remained.

The dissociated cells were prepared for freezing by adding enough dry sterile dimethyl sulfoxide (DMSO) to produce a 9% solution and mixed thoroughly. This cell

solution was divided into 250-μl aliquots in freezing vials and place at 40°C for 15 min. The vials were then positioned in a freezing box which allows gradual freezing and then placed in a -80°C freezer.

Before experiment, the sample vial was taken out of the freezer and thawed in 40°C water bath. 2.0 ml PBS solution was added to the vial and then centrifuged gently (200 g) for 10 min. The supernatant solution was then poured off carefully, and 2.0 ml of fresh PBS solution was added to the neuron cells and shaken thoroughly before use.

Introduction of Cell and On-column Reaction

Cell injection into the capillary was done under a microscope. The single cell injection protocol was reported earlier (20).A drop of cell suspension was placed on a plastic slide and was examined under the microscope with a magnification of 100X. The first several millimeter coating of the capillary coating at the injection end was removed, and the tip of the capillary was immersed into the droplet on the slide so that the opening of the tubing could be clearly seen. The buffer vial with the other end of capillary immersed into it was sealed by a air-tight septum with a 20 ml syringe attached. By manually controlling the syringe the cell would be drawn into capillary, which can be confirmed with the microscope. As soon as a cell was introduced into the capillary it was allowed to settle down and adhere to the inner wall of capillary. Then the capillary was immersed into enzymes+cosubstrates solution to electrokinetically inject the reactants. The injection time was set at 10 s and the voltage is 20 kV. The cell was lysed in seconds due to the osmotic

shock, and the contents were released for reaction with the enzyme solution. After this injection was completed the capillary was placed back into the standard running buffer. The power supply remained off to provide a 15 min incubation period prior to separation and detection.

RESULTS AND DISCUSSIONS

Characteristics of Enzymatic Essay and Optimization

In dual-enzyme assay scheme used in this study, the glutamate dehydrogenase(GDH) catalyzes the formation of NADH from NAD^+ during the oxidation of glutamate. This reaction has been used as standard assay method for glutamate determination from biological samples (33, 34) and has been shown to have good selectivity for neurochemical measurements of glutamate (35, 36), with a larger linear response range and a low detection limit. The only draw-back is that this reaction alone is thermodynamically unfavorable for the production of NADH. The equilibrium constant with NADH as the reaction product is only approximately 10^{-14} (37). The thermodynamically favored products of GDH catalysis are glutamate and NAD^+. Because of this, in practical applications, the GDH reaction is often coupled with second enzyme reaction which consumes one of the products of GDH reaction , driving the reaction in the desired direction and improving the response. Glutamic pyruvic transaminase(GPT) is one of enzymes that is often adapted for this purpose(15, 38, 39).

Several parameters were studied to optimize the reactions, including concentrations of co-substrates NAD^+ and alanine, concentrations of enzymes, pH and buffer species. For a 6.25 μM glutamate solution, maximum response was approached for a NAD^+/glutamate ratio 500 and above. A similar dependence was also found for alanine; the ratio of alanine/glutamate for maximum response is about 2000. The concentration level of glutamate in the capillary for cell studies would be at 10^{-7} M due to dilution. Therefore, in all subsequent experiments, the concentrations of NAD^+ and alanine were kept at $5x10^{-5}$ M and $2x10^{-4}$ M, respectively. For enzyme concentrations, it was found that when the concentration levels was increased from 10^{-8} M to 10^{-7} M, the signals did not increase significantly; in some cases they even decreased. So, both GDH and GPT concentrations were kept at 10^{-8} M for all experiments. We found that NADH fluorescence signal was more stable and GPT retained higher activity in Tris buffer, while GDH was more stable in phosphate buffer. As a result, Tris-phosphate was chosen as a compromise. pH of all solutions, including the CE running buffer, was kept around 7.4. This is because it approximates physiological conditions of living cells, and because it is close to the pH for maximum enzymes activities.

It is important to control the incubation time in order to provide reliable quantitation. Fortunately, reaction does not occur until the cell lyses, and 15 min is a long period tha can be reliably controlled to ±1%. For a constant incubation time, standard solutions can be used to establish the calibration curve.

Interference Study

All 20 amino acids which may exist in cells were studied for possible interferences. The result are shown in Table 1. It can be seen that this method has very high specificity; only glutamine and lysine give about 1-2% signal response compared with the same concentration of glutamate. A possible reason for glutamine signal is the partial hydrolysis of glutamine, which produces glutamate. The reason for the presence of a small lysine signal is unknown.

Glutamate Determination of Single Red Blood Cell

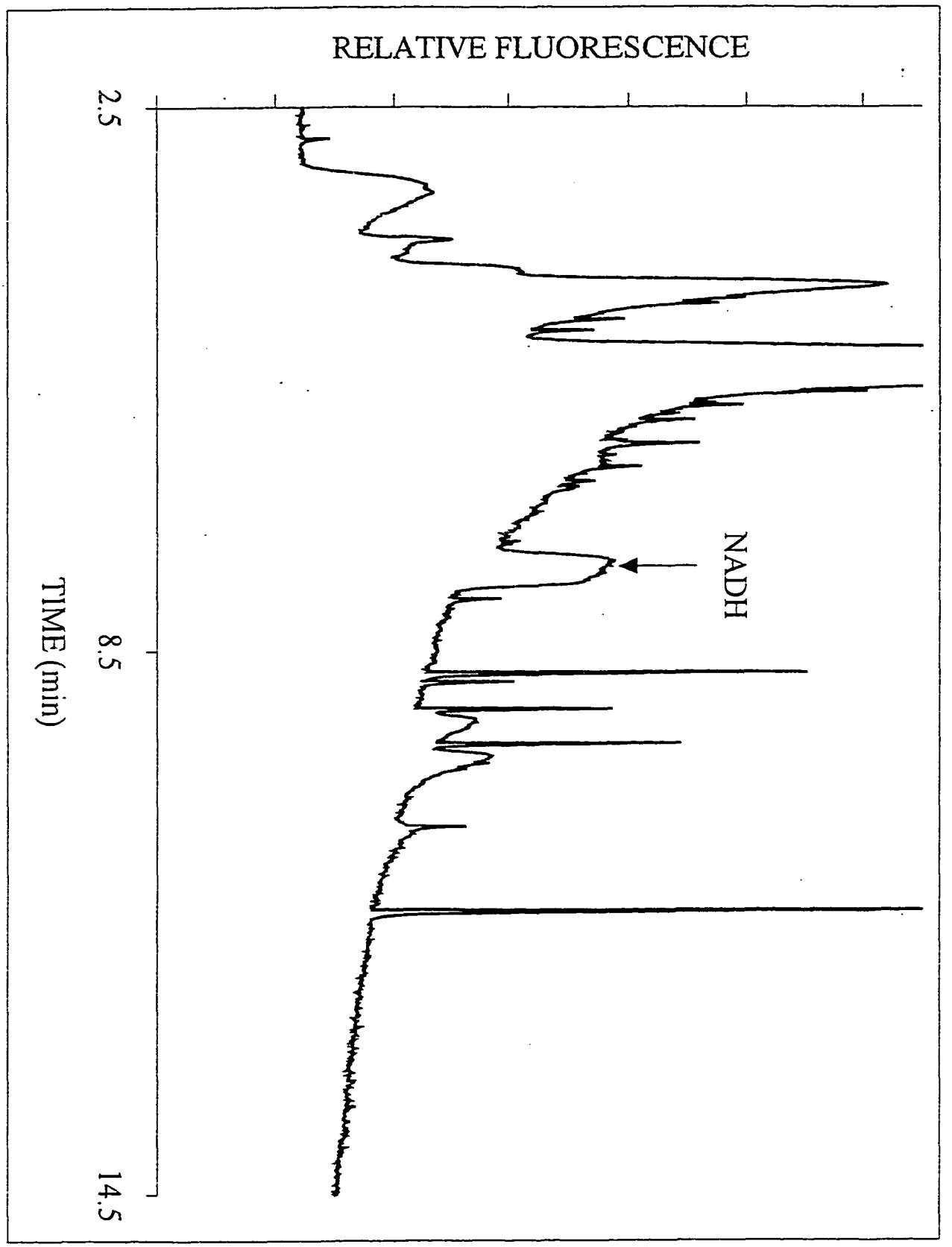
The electropherogram for glutamate determination in a single human erythrocyte is shown in Figure 1. The step-like NADH peak shape at 7.5min is due to the continuous formation of NADH when injected enzyme solution plug moved along the capillary. The integrated area of the step is proportional to the amount of glutamate. The broad peaks from 3min to 5.5min are from enzymes, and was confirmed by control experiments — injecting only the enzyme solution without glutamate. Enzymes are proteins which usually possess some native fluorescence. The detection limit is about 10^{-8} M (S/N=3) or attomole level for NADH. This means the detection limit for glutamate is also in the same range. The baseline returns to the original level at the end of each run, which indicates that the enzymes and cell contents are not absorbed to the capillary walls. A standard series of enzyme assays with glutamate concentration from $5x10^{-8}$ M to $1x10^{-5}$ M (r^2= 0.991) is used to calibrate the amount of NADH produced from single red blood cells and in turn to calculate the amount

Table 1: Interference factors for glutamate determination.

Interference factors

compound	%response	compound	%response
L-glutamate	100	L-alanine	0
L-arginine	0	L-asparagine	0
L-aspartate	0	L-cysteine	0
L-glutamine	~2	L-glycine	0
hydroxy L-proline	0	L-histidine	0
L-isoleucine	0	L-leucine	0
L-lysine	<1	L-methionine	0
L-phenylalanine	0	L-proline	0
L-serine	0	L-threonine	0
L-tryptophan	0	L-tyrosine	0
L-valine	0		

Figure 1: Electropherogram of the enzymatic assay of a single red blood cell.


RELATIVE FLUORESCENCE
2.5
8.5
14.5
TIME (min)
NADH

of glutamate in the cells. The results of analyses of 19 single red blood cells are shown in Figure 2. According to these 19 runs, the amount of glutamate in single red blood cell is range from 3.0×10^{-17} mol to 2.8×10^{-16} mol, or a concentration range from 0.33mM to 3.1mM. These values reflect real differences among single cells, since the precisioin for repeated measurements of standard solutions is much higher. Compared to literature values (40, 41), which are based on bulk average, this result here are in good agreement.

Glutamate Determination in Single Rat Neuron Cell

The electropherogram resulting from enzyme assay of a single rat neuron is shown in Figure 3. The results of analyses of 22 single neurons are shown in Figure 4. According to these results, the amount of glutamate in single neuron is about 2.0×10^{-18}mol to 3.3×10^{-16}mol. or concentration levels from 3.8μM to 0.64 mM (assuming the diameter of neuron is 10 μm). Again, large cell-to-cell variations are evident.

No previous reported value of glutamate amount in single rat neuron was found to compare with our results. All data found in the literature about glutamate in rat brain neurons have the unit (μg(or pg)/wet weight of tissue), which is not comparable here. However, it is reported (42) that the average concentration level of glutamate in rat central neural system is about 1 mmol/kg wet weight of tissue, with the highest concentration exists in astrocytes (about 10 mmol/kg wet weight), and the lowest value in extracellular fluid (about 1-3 μmol/kg wet weight). The concentration gradient of glutamate across the plasma membrane is about a thousand fold. Also, from several publications (43-45) which studied the CNS glutamate uptake system it has been suggested that the glutamate uptake rate for

Figure 2: Glutamate amounts in 19 individual human erythrocytes.

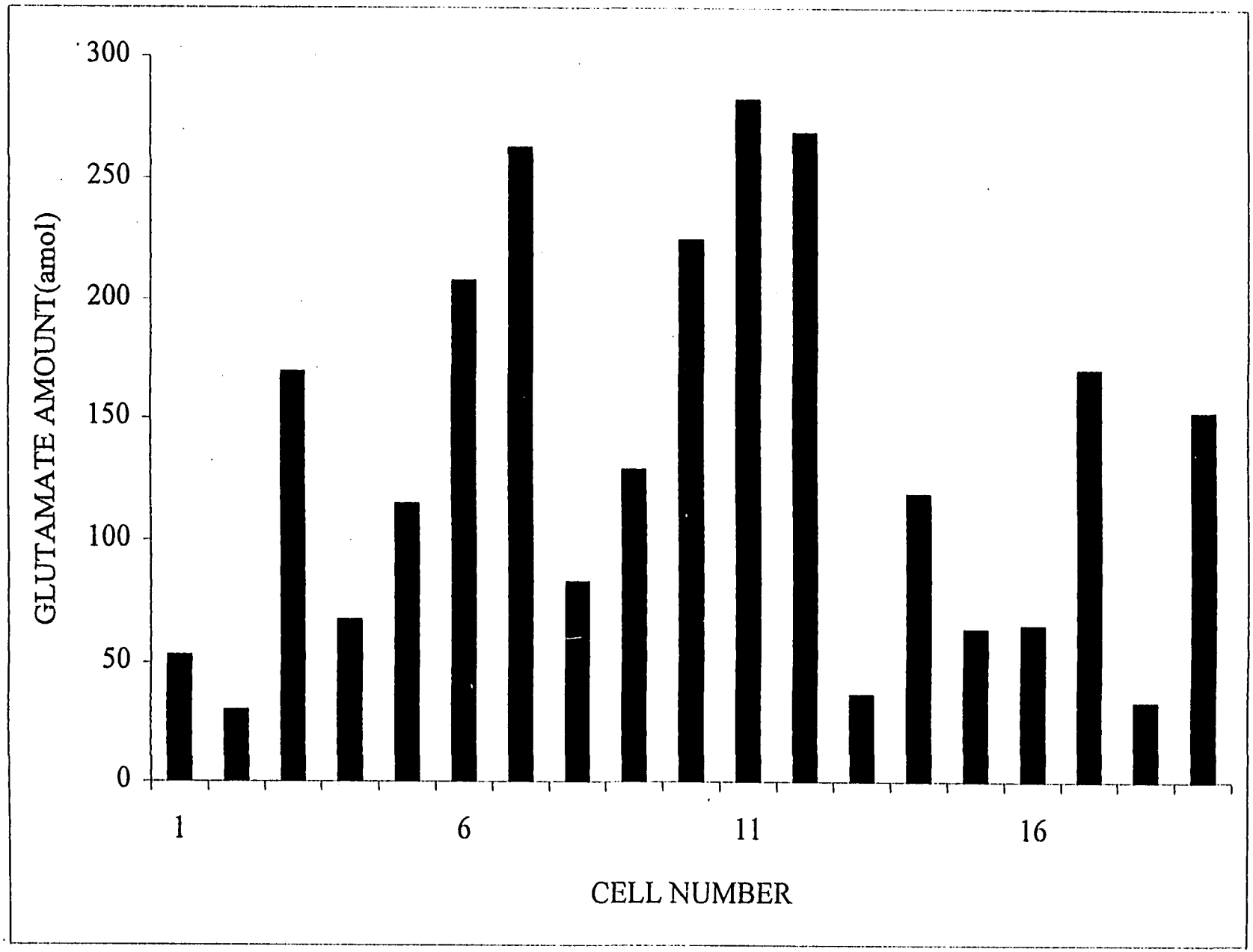

300
250
200
150
100
50
0
GLUTAMATE AMOUNT(amol)
1
6
11
16
CELL NUMBER

Figure 3: Electropherogram of the enzymatic assay of a single rat neuron.

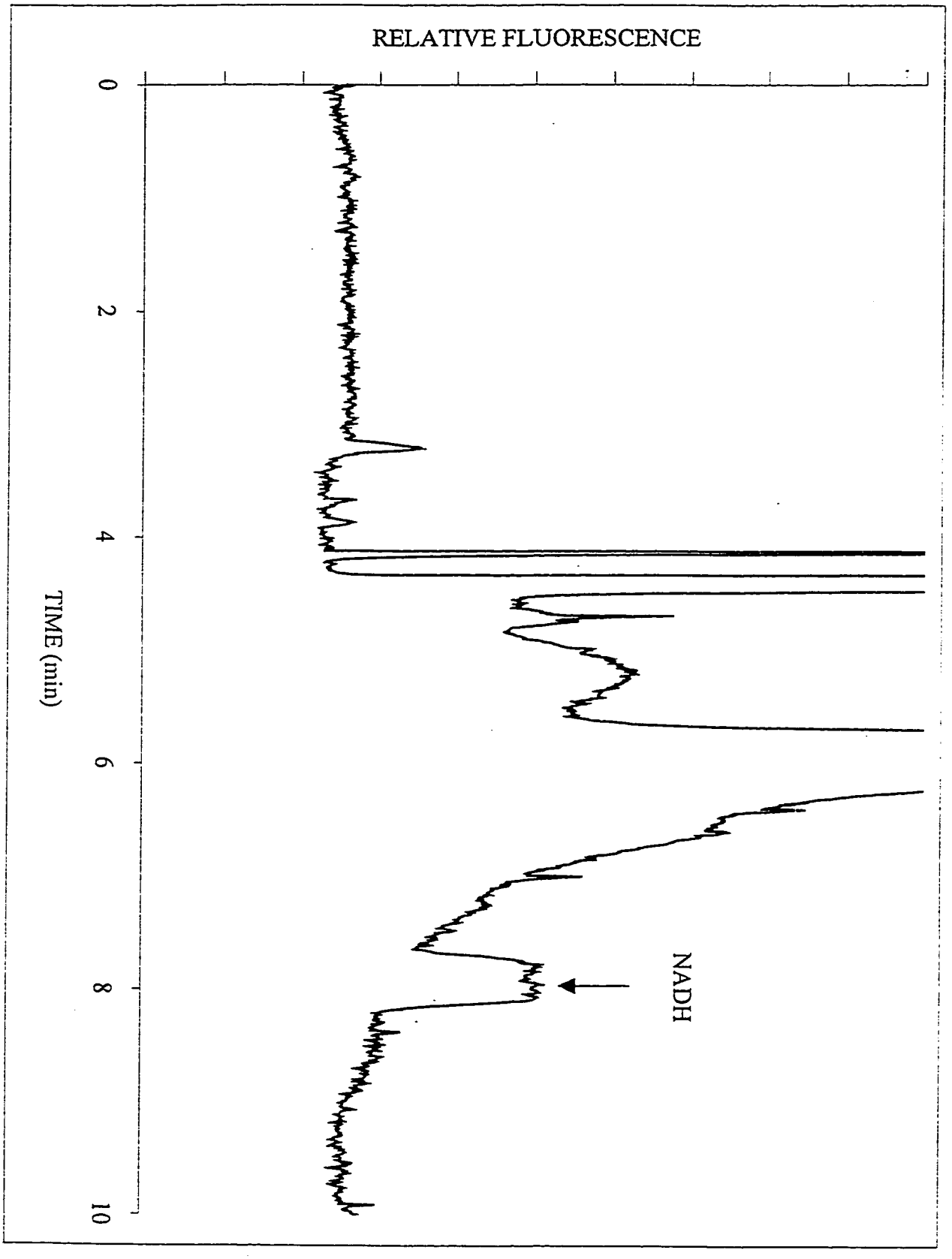
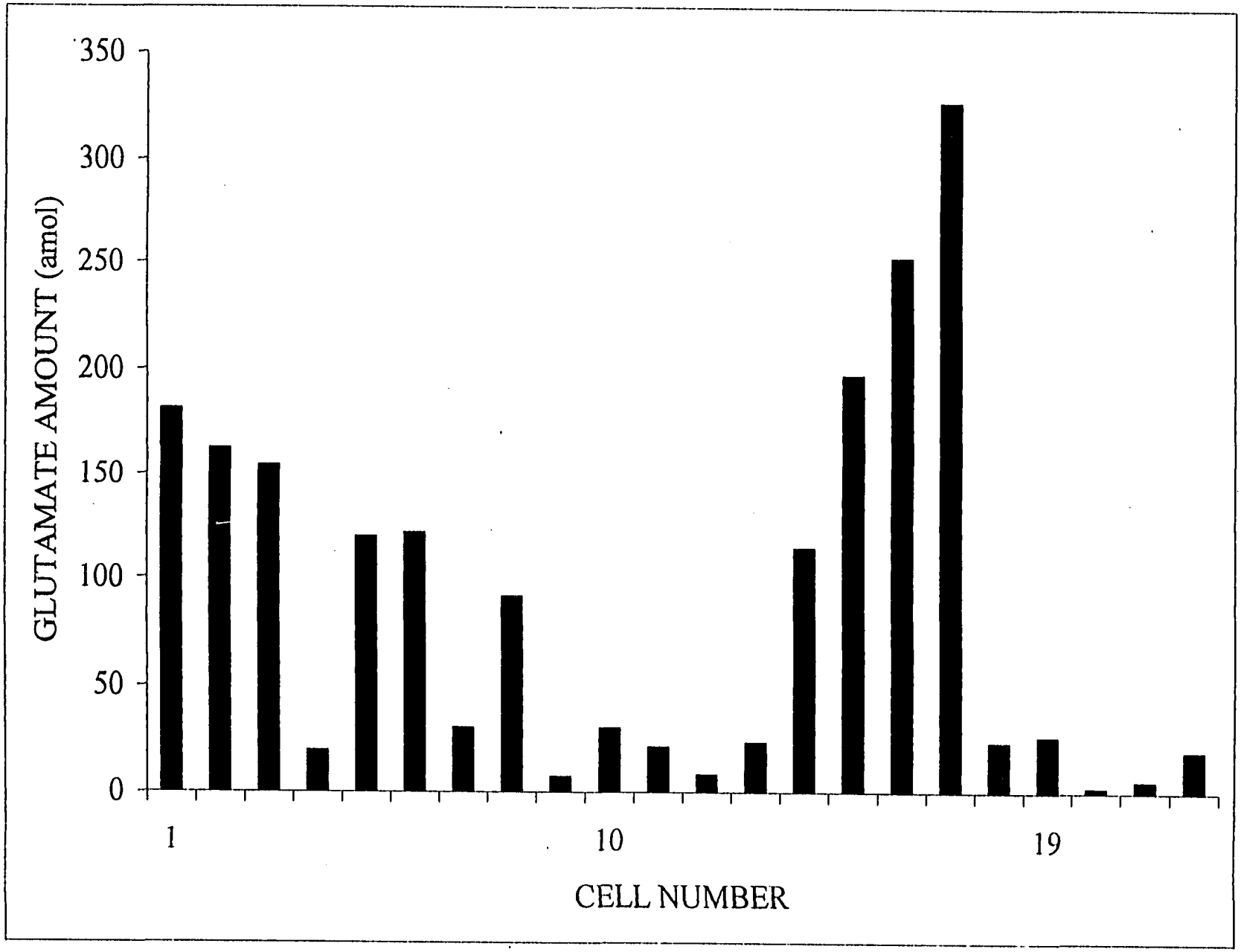

RELATIVE FLUORESCENCE
0
2
4
6
8
10
TIME (min)
NADH

Figure 4: Glutamate amounts in 22 individual rat neurons.


350
300
250
200
150
100
50
0
GLUTAMATE AMOUNT (amol)
1
10
19
CELL NUMBER

neurons is about 10 attomole/min per cell in vivo. Therefore, these data suggest that our results have lies in a reasonable range.

ACKNOWLEDGMENTS

The authors thank Philip Haydon for providing the rat neuron cells. The Ames Laboratory is operated for the U.S. Department of Energy by Iowa State University under Contract W-7405-Eng-82. This work was supportedd by the Director of Energy Research, Office of Basic Energy Sciences, Division of Chemical Sciences.

REFERENCES

1. Fonnum, F. (1984) *J. Neurochem.* **42**, 1-11.
2. Parpura, V., basarsky, T.A., Liu, F., jeftinija, K., jeftinija, S. and Haydon, P.G. (1994) *Nature*, **369**, 744-747
3. Lindroth, P. and Mopper, K. (1979) *Anal. Chem.* **51**, 1667-1674
4. Lee, P.L. (1974) *Biochem. Med.* **10**, 107-121
5. Zecca, L., Zambotti, F., Zonta, N. and Mantegazza(1982) *J. Chromatogr.* **233**, 307-312
6. Roger, K.L., Philibert, R.A., Allen, A.J., Molitor, ., Wilson, E.J. and Dutton, G.R. (1987) *J. Neurosci Methods.* **22**, 173-179
7. Caudill, W.L., Honck, G.P. and Wightman, R.M. (1982) *J. Chromatogr.* **227**, 331-339
8. Graham, L.T. and Aprison, M.H. (1966) *Anal. Biochem.* **15**, 487-497
9. Wolfensberger, M. and Amsler, U. (1982) *J. Neurochem.* **38**, 451-456

10. Wood, P.L. and Cheney, D.L. (1985) in *Neuromethods 3: Amino Acids*(Boulton, A.A. and Baker, G.B. eds.) pp. 51-89, Humana Press, Clifton, N.J.

11. Ferkany, J.W. (1985) in *Neuromethods 3: Amino Acids*(Boulton, A.A. and Baker, G.B. eds.) pp. 117-154, Humana Press, Clifton, N.J.

12. Butterworth, R.F.(1985) in *Neuromethods 3: Amino Acids*(Boulton, A.A. and Baker, G.B. eds.) pp. 81-96, Humana Press, Clifton, N.J.

13. Wu, J.Y. and Lin, C.T.(1985) in *Neuromethods 3: Amino Acids*(Boulton, A.A. and Baker, G.B. eds.) pp. 155-178, Humana Press, Clifton, N.J.

14. Nakane, P.L. and Pierce, G.B. Jr. (1967) *J. Cell. Biol.* **33**, 307-318

15. Wang, A. and Arnold, M.A. (1992) *Anal. Chem.* **64**, 1051-1055

16. Nicholls, D.G. (1989) *J. Neurochem.* **52**, 331-341

17. Fosse, M., Kolstad, J. and Fonnum, F. (1986) *J. Neurochem.* **47**, 340-347

18. Xue, Q. and Yeung, E.S. (1994) *Anal.Chem.* **66**, 1175-1178

19. Yeung, E.S., Wang, P., Li, W. and Giese, R.W. (1992) *J. Chromatogr.* **608**, 73-77

20. Hogan, B.L. and Yeung, E.S. (1992) *Anal. Chem.* **64**, 2841-2845

21. Xue, Q. and Yeung, E.S. (1994) *J. Chromatogr.* **661**, 287-295

22. Lee, T.T. and Yeung, E.S. (1992) *J. Chromatogr.* **595**, 319-325

23. Lee, T.T. and Yeung, E.S.(1992) *Anal. Chem.* **64**, 3045-3051

24. Wallingford, R.A. and Ewing, A.G.(1988) *Anal. Chem.* **60**, 1972-1975

25. Ewing, A.G. (1993) *J. Neurosci. methods.* **48**, 215-224

26. Kristensen, H.K., Lau, Y.Y. and Ewing, A.G. (1994) *J. Neurosci. Methods.* **51**, 188-189

27. Kennedy, R.T., St. Claire, R.L., White, J.G. and Jorgenson, J.W. (1987) *Mikrochim. Acta.* **2**, 37-45

28. Kennedy, R.T., Oates, M.D., Cooper, B.R., Nickerson, B. and Jorgenson, J.W. (1989) *Scciene*(Washington, D.C.) **246**, 57-63

29. Cooper, B.R., Jankowski, J.A., Leszczyszyn, D.J., Wightman, M.R. and Jorgenson, J.W.(1992) *Anal. Chem.* **64**, 691-694

30. Chien, J.B., Wallingford, R.A. and Ewing, A.G. (1990) *J. Neurochem.* **54**, 633-638

31. Olefirowicz, T.M. and Ewing, A.G. (1990) *J. Neurosci. methods.* **34**, 11-15

32. Rosenzweig, Z. and Yeung, E.S., *Anal. Chem.*, (1994), **66**, 1771-1776

33. Fisher, H.F. (1985) *Methods in Enzymology.* **113**, 16-27

34. Olson, I.A., and Anfinsen, C.B.(1955) *J. Biol. Chem.* **202**, 841-845

35. Ayoub, G.S. and Copenhagen, D.R. (1991) *J. Neurosci. Methods.* **37**, 7-14

36. Nicholls, D.G. (1989) *J. Neurochem.* **52**, 331-341

37. Subramanian, S. (1978) *Biophys. Chem.* **7**, 375-378

38. Saier, M.H. and Jenkins, W.T. (1967) *J. Biol, Chem.* **242**, 91-93

39. Ernster, L. (1985) *Methods in Enzymology.* **113**, 309-312

40. Pennell, R.B. (1964)in *The Red Blood Cell*(Bishop, C. and Surgenor, D.M. eds.) pp. 29-70, Academic Press, New York, NY

41. Mcmenamy, R.H., Lund, C.C., Neville, G.J. and Wallach, D.F. (1960) *J. Clin. Invest.* **39**, 1675-1705

42. Otterson, O.P., Zhang, N. and Walberg, F. (1992) *Neurosci.* **46**, 513-534

43. Danbolt, N.C. (1994) *Prog. in Neurobiology.* **44**, 377-396

44. Schonsbon, A., Fosmarr, H. and Hertz, L. (1975)*J. Neurochem.* **25**, 909-911

45. Gordon, R.D. and Balâzs, R. (1983) *J. Neurochem.* **40**, 1090-1099

CHAPTER 3

FLUORESCENCE IMAGING OF GLUTAMATE RELEASE IN NEURONS

A paper submitted to the Journal of Neuroscience Methods

Ziqiang Wang and Edward S. Yeung

ABSTRACT

A noninvasive detection scheme based on glutamate dehydrogenase (GDH) enzymatic assay combined with microscopy was developed to measure the glutamate release in central nervous system (CNS). The enzyme reactions is very specific and sensitive. The detection limit with CCD imaging is down to μM level of glutamate concentration with reasonable response (~30 s). The standard glutamate test shows a linear response over 3 orders of magnitude, from μM to 0.1mM range. The *in vitro* monitoring of glutamate release from cultured neuron cells demonstrated excellent spatial and temporal resolutions that is superior to prevalent electrophysiological or chromatographic methods.

Keywords: imaging; glutamate; microscopy; neurons

INTRODUCTION

Extensive efforts have been undertaken towards the development of analytical methods for study of glutamate in the central nervous system since late 1950s, after it was identified as the major excitatory neurotransmitter in CNS (Curtis et al. (1958); Curtis et al. (1959); Rostas et al. (1986); Palkovitz et al.(1986); Christopher(1985); Burgar et al. (1989); Naito and Ueda (1985); Fagg and Foster (1983); Fonnum (1984); Roberts et al. (1981); Jasper et al. (1965); Clark and Collins (1976); Hicks et al. (1985); Druce et al. (1982); Peinado et al. (1987); Mayer and Westbrook (1987); Tsumoto (1990); Bradford (1986)). Various techniques including electrochemical analysis (Kar and Arnold (1992); Cosnier et al. (1997); Boutelle et al. (1992)), chromatography (Lindroth and Mopper (1979); Zecca et al. (1982); Roger et al. (1987); Caudill et al. (1982); Graham and Aprison (1966); Wolfensberger and Amsler (1982)) and enzymatic assay (Nicholls (1989); Fosse et al. (1986); Kuhr and Cosford (1996)) have been used for glutamate analysis in CNS. Currently, the microelectrode-based electrophysiological detection (Clements et al. (1992); Otis et al. (1997); Greengard et al. (1991)) and chromatographic method such as HPLC (Boutelle et al. (1992); Lindroth and Mopper (1979); Parpura et al. (1995)) and capillary electrophoresis (CE) (Kuhr and Cosford (1996); Orwar et al. (1996); Lada et al. (1997); Zhou et al. (1995)) combined with sampling techniques such as microdialysis are the most widely used techniques. While progress has been made by these methods, there is one common disadvantage that these methods can only probe a specific locality at a time and cannot obtain comprehensive spatial and temporal information simultaneously.

In recent years fluorescence imaging has been widely employed in neurochemical analysis. It has a number of advantages for the study of neuronal cells and other biological cells: (1) a particular molecule species or cellular constituent can be selectively visualized in the presence of a large excess of other species in a heterogeneous environment; (2) lower concentration detection limit can be achieved because of the inherent associated with fluorescence; (3) low excitation intensities can be so that long-term observation can be realized while the viability of specimen is preserved; and (4) excellent spatial resolution can be obtained with light microscope so subcellular compartments can be identified. With good sensitivity, temporal and spatial resolution, the flux of ions and molecules and the distribution and dynamics of intracellular species can be measured in real time with specific fluorescent probes (Murphy et al. (1994); Cornell-Bell et al. (1990); Pol et al. (1990); Jovin et al. (1985)), substrates (Miesenbock and Rothman (1997)) or with native fluorescence (Tan et al. (1997); Lillard and Yeung (1997)).

Though impressive progress has been achieved with fluorescence imaging in neurochemical analysis, so far it is still difficult to measure glutamate directly in CNS. This is due to the nature of the molecule, which possesses few functional groups and no fluorophore that can be used for detection. To adopt some kind of chemical labeling usually requires multiple steps such as sampling, separation, derivatization and detection. The treatment procedure maybe fatal to living cells process and unwanted interference associated with the derivatization process may need to be eliminated in order to obtain good detection. This makes the *in vivo* or *in vitro* monitoring of glutamate activities in real time almost impossible in the small volumes typical of individual cells in mammalian CNS.

Enzymatic assay is an interesting alternative to chemical derivatization. Enzyme reaction normally is very specific and occurs naturally in cellular environments. The detected product molecule usually has distinctive characteristics from other cosubstrates and analytes so there is no need for separation. The detection efficiency is also enhanced by avoiding possible sample degradation and dilution during the sampling and separation steps.

We have successfully developed a fluorescence imaging method for directly monitoring change in glutamate concentration in CNS based on glutamate dehydrogenase (GDH) enzymatic assay. The glutamate is oxidized to a-ketoglutarate and the cosubstrate NAD^+ is reduced to the highly fluorescent NADH. The detection scheme has linear response to glutamate concentration over 3 orders of magnitude. The *in vitro* monitoring of glutamate release from cultured rat brain neuron cells is demonstrated. Satisfactory spatial and temporal resolution has been achieved simultaneously. This represents the first real-time biological imaging study based on enzymatic assay.

EXPERIMENTAL SECTION

Materials

Sodium chloride, potassium chloride, magnesium chloride hexahydrate, calcium chloride dihydrate, D-glucose, Tris-hydrochloride, L-glutamic acid (monosodium salt), L-alanine, glutamate dehydrogenase (36 units per mg protein, from bovine liver), glutamic pyruvic transaminase (GPT, 80 units per mg protein, from porcine heart),β-NAD^+, NADH were from Sigma (St. Louis, MO). All chemicals were used as received. All solutions were prepared fresh with purified and deionized water on a daily basis.

Buffers

All experiments were carried in physiological buffer with following recipe: 135 mM sodium chloride, 5mM potassium chloride, 7mM magnesium chloride, 2mM calcium chloride, 6mM D-glucose, 10mM Tris. The final pH is adjusted to 7.35. In the cell stimulation solution the potassium ion concentration was elevated to 80mM and sodium ion concentration was decreased accordingly to maintain the same ionic strength. All experiments were done at room temperature.

Cell Culture

Low-density embryonic hippocampal cultures were prepared using a modification method of Goslin and Banker (1991). Briefly: hippocampi dissected from E18 rat fetuses (6 per ml) were dissociated by trypsinization [0.25% trypsin in Earl's balanced salt solution (EBSS) for 15 minutes followed by trituration in modified minimal essential medium (MMEM) (Eagle's minimum essential medium, 2mM glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin and 100 mg/ml streptomycin) supplemented with 10% fetal bovine serum. The cell solution was diluted to 1:10 in MMEM and plated onto 12 mm round cover slips coated with poly-L-lysine (1mg/ml in borate buffer, pH 8.4) and placed at 37°C in a humidified 5% CO_2/ 95% air atmosphere for 1 h to allow cell attachment. The media was then replaced with 1 ml of serum substituted MEM. After 6-10 days growth in incubator the cell is ready to use. Before the experiment begins everyday the culture dish is washed with

fresh pH 7.35 buffer solutions described above to eliminate potential bacterial growth and other interference factors.

Fluorescence Imaging System

The imaging system consists of an ultraviolet argon-ion laser (Model 2045, Spectra-Physics, Mountain View, CA) with multiple wavelength (of which the 305nm is isolated and used as the excitation source), an inverted light microscope (Nikon Diaphot 300, Fryer, Edina, MN) and a frame transfer CCD system (Model 1024x512FT, Princeton Instruments, Trenton, NJ) attached to the camera mount of microscope to record fluorescence. An electronic shutter (Uniblitz T132, Vincent Associates, Rochester, NY) was placed in the light path to block the laser beam when CCD was not taking data to avoid possible bleaching of NADH, as depicted in Figure 1. The flow cell was made from a piece of Plexiglas slide by drilling a hole with a diameter 8mm and depth 1.8mm, the volume is about 90 μl. There was one tunnel on each side of the cell used as solution inlet and outlet respectively. A microsyringe was inserted to the inlet tunnel for the injection of glutamate solution in the calibration experiments and for the enzyme assay mixture in neuron cell experiments. The laser power at the focus was controlled to less than 1mW to avoid cell damages due to sensitive nature of neuron cells.

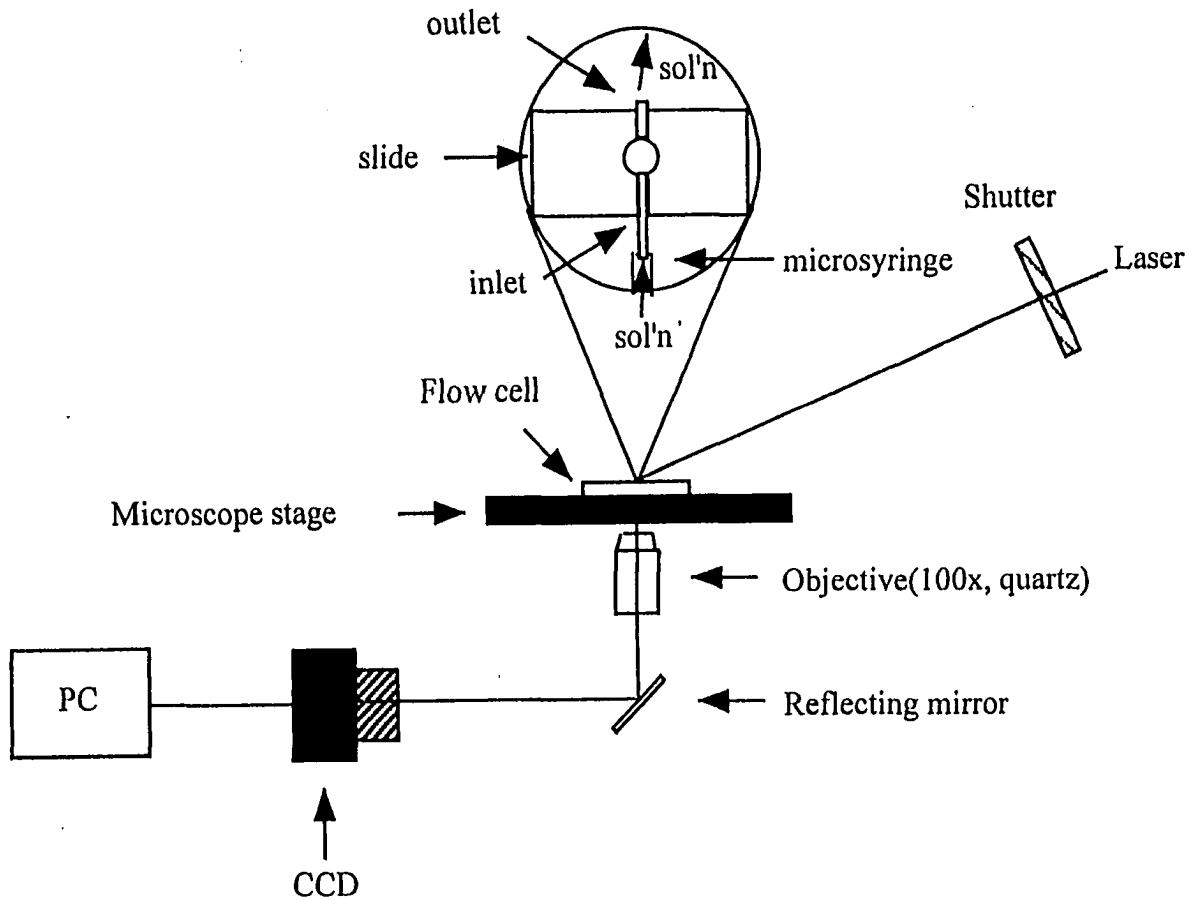


Figure 1: Instrumental setup for glutamate imaging.

Glutamate Standard Experiment

The flow cell (fixed on the microscope stage) was first filled with enzyme assay mixture but without glutamate. The reaction was then initiated by the injection of glutamate from the microsyringe attached to the side of flow cell. A series of glutamate standard solution with concentrations ranging from 10^{-6} M to 3X 10^{-4} M were injected to the flow cell. The NADH fluorescence generated was recorded by the CCD in sequential frames and sent to computer for storage and data analysis.

In vitro Neuron Culture Glutamate Release Monitoring

The neuron culture cover slip was carefully attached to the lower surface of flow-cell chamber with cultured cell side facing up. The flow chamber was placed on the microscope stage, filled with normal buffer to keep cells alive. The enzyme mixture was also present in this solution. Both sides of slide were then sealed with vacuum grease to form a closed chamber and to prevent leaking. Stimulation of cell to cause glutamate release was via injection of high-potassium buffer from the microsyringe. The NADH fluorescence signal generated due to reaction of glutamate with enzymes and cosubstrates was recorded by the CCD in sequential frames and stored in the computer.

RESULTS AND DISCUSSION

Enzyme Reaction Characteristics and Optimized Conditions

In this study we used a dual-enzyme reaction theme as that reported earlier (Wang and Yeung (1997)) for analysis of glutamate in single cells by capillary electrophoresis:

$$\text{Glutamate} + NAD^+ \rightarrow \alpha\text{-ketoglutarate} + NH_4^+ + NADH \quad (1)$$

(GDH catalyzed)

$$\alpha\text{-ketoglutarate} + \text{alanine} \rightarrow \text{pyruvate} + \text{glutamate} \quad (2)$$

(GPT catalyzed)

Besides enhancing the specificity of the reaction, the introduction of the second enzyme, glutamic pyruvate transaminase, greatly enhances the reaction efficiency by taking up α-ketoglutarate to drive the reaction toward NADH generation. The reaction rate becomes much faster compared to conventional single GDH-enzyme reaction as reported in literature (Fisher (1985); Olson and Anfinsen(1955)). Consistent with what we found in the CE experiments, it was necessary to have a large excess amount of cosubstrates NAD^+ and L-alanine to accelerate the reaction to assure that the CCD signal is linear with initial glutamate concentration. Under our experiment conditions, we were able to obtain satisfactory NADH fluorescence image over the course of 1 min after injection for initial glutamate concentrations down to 1 μM and for CCD exposure times at 500 ms per frame.

Glutamate Calibration

The response curve for glutamate is shown in Figure 2. The intensities are the average result from a 100x100-pixel area. The enzyme reaction provides useful response over 3 orders of magnitude from 3 μM to 100μM, the 1μM glutamate signal was merely

above the background noise and approached the detection limit. Signals of concentration higher than 100 μM would reach a plateau, that is, the signal becomes saturated. The optimized conditions were similar to those on CE experiments. This shows that the flow cell characteristics (geometry, material, volume) did not have any adverse effects on the reaction.

Figure 3 shows the fluorescence intensity increase vs. time after standard glutamate is added to the flow cell. It can be seen that under optimized conditions NADH signal begins to accumulate right after the injection of glutamate and continue until around 60 s, when it reaches a relatively stable plateau. Therefore, in all standard test, we took the average (5 frames) intensity increase from around 60 s after injection as the response for every concentration of glutamate used in the standard curve.

In vitro Glutamate Release Monitoring

Figure 4 and Figure 5 show the typical (n=20 experiments) fluorescence images of NADH generated from the glutamate enzyme reaction. All fluorescence images are the result of subtraction of final frame (1 min after stimulation) from initial frame (before stimulation) after background correction but with no other modifications. By this flat-fielding method all contributing interference signals such as fluorescence from background matrix, cell membranes and cell nuclei, basal release of glutamate and stray light were cancelled out.

Figure 2: Glutamate response curve. Each data point is the averaged result over 4 repetitive runs. The 0.3 mM data point is not shown here.

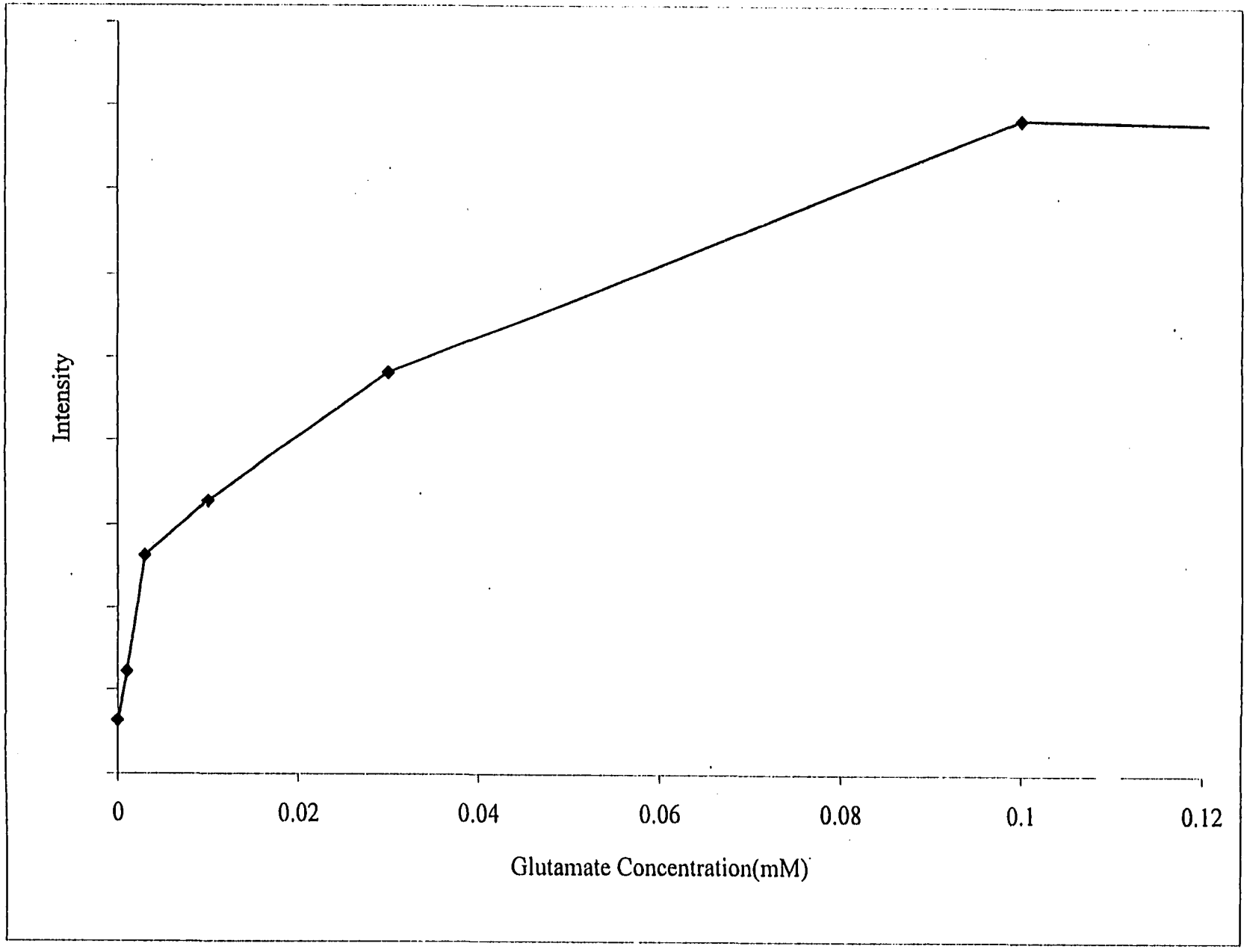

Intensity
0
0.02
0.04
0.06
0.08
0.1
0.12
Glutamate Concentration(mM)

Figure 3: Temporal response of glutamate enzymatic assay. The concentration of glutamate injected into the flow cell is 10^{-4} M.

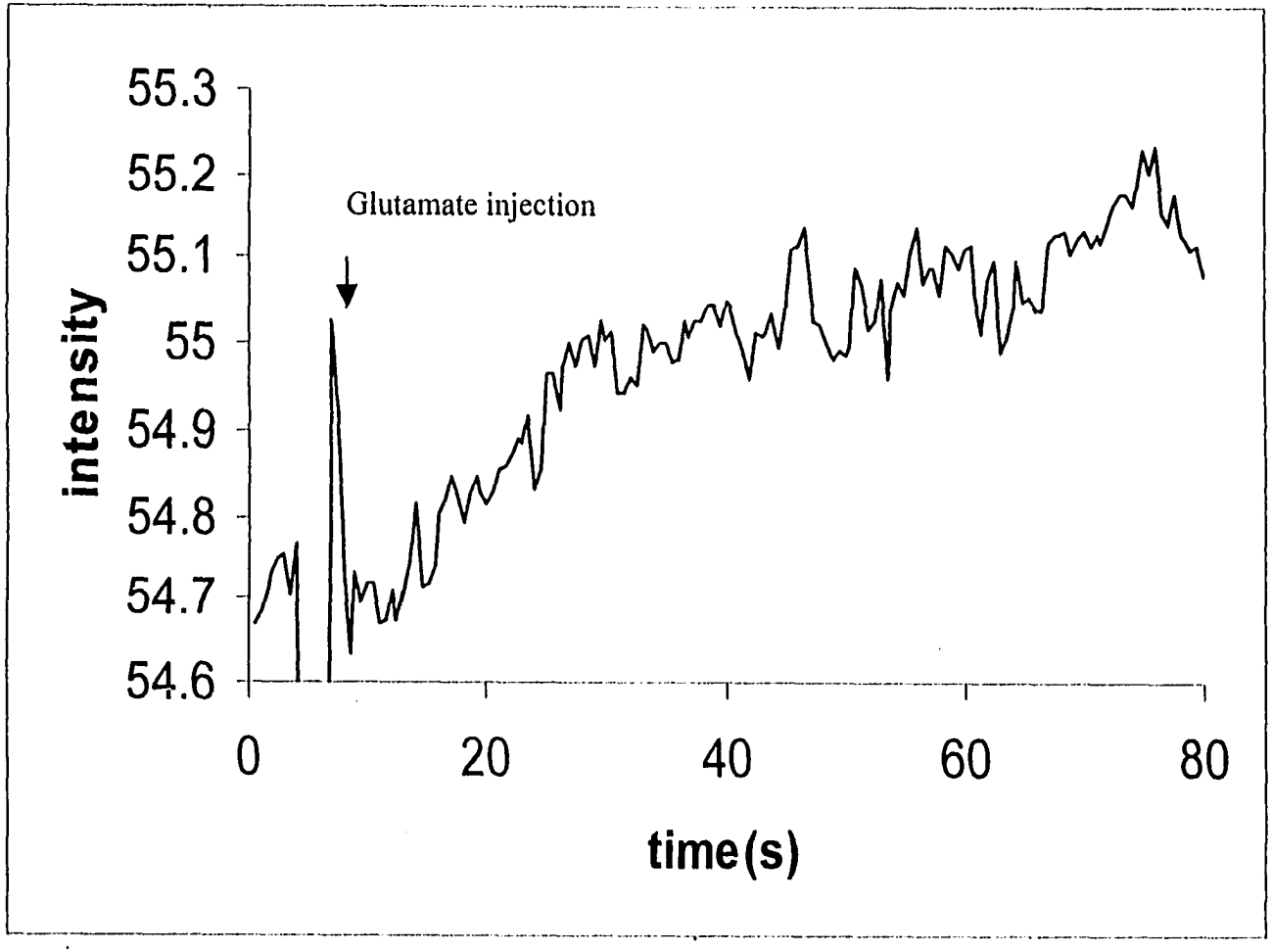
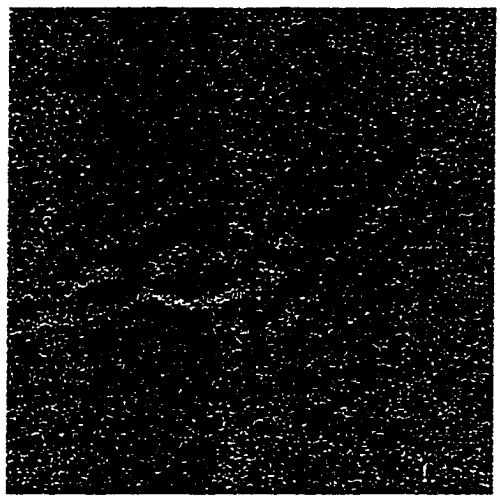

55.3
55.2
55.1
55
54.9
54.8
54.7
54.6
intensity
Glutamate injection
0
20
40
60
80
time(s)

Figure 4: Glutamate release from a single neuron in relatively isolated environment. A: optical image of the neuron and B: corresponding fluorescence image. The exposure time is set at 500 ms per frame.

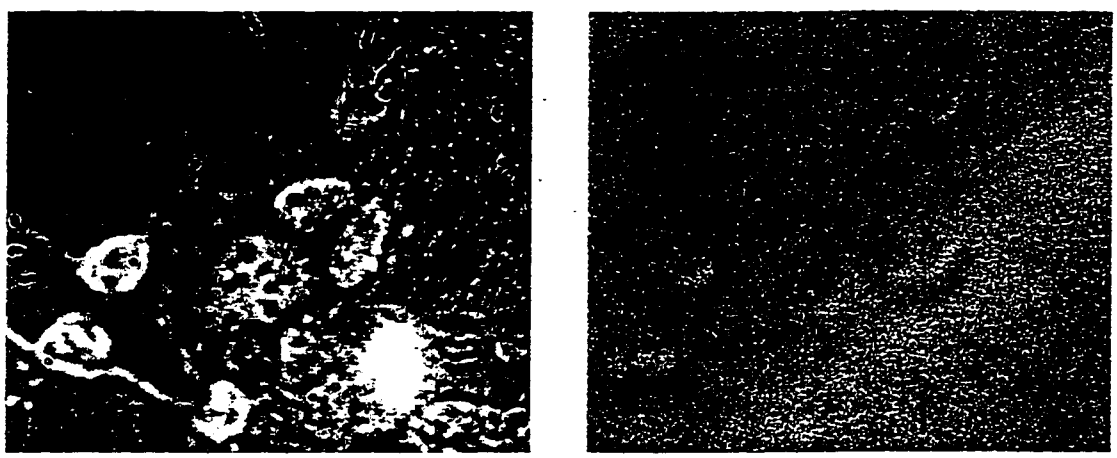
Frames of fluorescence image were taken sequentially during and after stimulation. The image shown was the average of 5 consecutive frames.

A



B

Figure 5: Multiple neurons in densely cultured area. A: optical image of multiple neurons and B: corresponding fluorescence image. The conditions were the same as in Figure 4.



A B

The NADH generated is the result of enzyme reaction from released glutamate from neurons due to high-K^+ stimulation because control experiments at normal potassium concentrations gave no detectable signal under the same conditions (data not shown).

From these images it can be seen that although glutamate released is released all over the cell body area, only some parts of the cell, mostly along the edge of the cell, has strong release and are thus represented by bright area of NADH fluorescence. It is believed (Siegel et al. (1989)) that in CNS the structure of neuronal network consists of dendrite and axon fibers extended from neuron bodies to neighboring neurons and glial cells, near and far away, covering each other and forming signaling patch pairs. At the end under the butons from the fibers there are nerve terminals which form pre- and post-synaptic terminals. Neurotransmitters are released from pre-synaptic terminals, upon stimulation, into the cleavage space between the synapse pair and move to post-synaptic terminals to activate specific receptor groups to complete the signal transduction process. Though glutamate is spread all over the neuronal cell compartments (Rostas et al. (1986); Palkovitz et al.(1986)), the average concentration is relative low. Only glutamate released from synapses due to stimulation, in the form of vesicles (and granules) expelled mainly through exocytosis (due to high-K^+ stimulation in our case), would have temporarily localized or higher (Christopher(1985); Burgar et al. (1989)) concentrations. This high concentration of glutamate would also quickly decrease to the low level of extracellular concentration due to dilution through diffusion and absorption by post-synaptic terminals to complete signal transmission and to protect cells from exocytotoxity (Clements et al. (1992); Choi (1992); Leutwyler (1997)). This fast phenomenon is the major reason that makes the direct

monitoring glutamate release in CNS difficult. However, this behavior favors observation of the signal derived from the first quanta of release on stimulation with elevated potassium concentration. This is because as long as the glutamate is released rapidly, only the localized high concentration of glutamate would be captured by transient reaction with the enzyme mixture to generate enough NADH for detection. For somatic release from cell bodies, the diluted glutamate would not able to produce large signals and would only form background in the image.

In principle, the enzymatic assay should be linear with respect to initial glutamate concentration over some magnitudes under optimized conditions. However, in this study the quantification of released glutamate was not pursued. In order to not irritate the cells, we limited the cosubstrates concentrations of NAD^+ and L-alanine to only 2.5mM and 10mM respectively. As stated above, although the average concentration level over the neurons may be around μM, the localized glutamate concentration in the released vesicles via exocytosis by stimulation may be as high as mM. With this in mind the enzyme reaction occurring at the release sites on the cell surface may not be linear in any case due to a decreased ratio of cosubstrate/analyte. Moreover, with a depleted supply of cosubstrates after initial release, it will take some time for NAD^+ and L-alanine from neighboring saline regions to travel to reactive enzyme site for replenishment. During this period, the observed signal will be time dependent.

CONCLUSION

We have successfully developed a fluorescence imaging method for following glutamate released from neuron cultures. The method is sensitive and specific. Since no pretreatment with fluorescent dyes is needed, the native cell characteristics and functions are preserved. The *in vitro* experiment in neuron cultures demonstrates that the detection scheme could simultaneously provide satisfactory spatial and temporal resolution. The approach is complementary to electrophysiological and chromatographic methods, it promises to become a useful for signal transduction study and neurochemical analysis. To the best of our knowledge there are no previously published reports on glutamate imaging or on enzyme-based imaging. We believe that with more advanced lasers and more sensitive CCD systems in the future, glutamate monitoring in CNS will become even more sensitive and the spatial and temporal resolution will become even higher.

ACKNOWLEDGEMENT

The authors thank Philip Haydon for providing the rat neuron cells and for many helpful discussions. The Ames Laboratory is operated for the U.S. Department of Energy by Iowa State University under Contract No. W-7405-Eng-82. This work was supported by the Director of Energy Research, Office of Basic Energy Sciences, Division of Chemical Sciences and by the National Institutes of Health.

REFERENCES

Banker G, Goslin K (eds.).: Culturing Nerve Cells 1991, MIT Press, Cambridge, MA, 251-281.

Boutelle M, Fellows L, Cook C. Enzyme packed bed system for the on-line measurement of glucose, glutamate and lactate in brain microdialysis. Anal. Chem. 1992; *64*: 1790-4.

Bradford H. Chemical Neurobiology: An Introduction to Neurochemistry W. H. Freeman and Co., New York, 1986

Burgar PM, Mehl E, Cameron PL, Maycox PR, Baumert M, Lottspeich F, De Cammili PD, Jahn R. Synaptic vesicles immunoisolated from rat cerebral cortex contain high levels of glutamate. Neuron 1989; 3: 715-20.

Caudill W, Honck G, Wightman RM. Determination of γ-aminobutyric acid by liquid chromatography with electrochemical detection. J. Chromatogr.1982; 227: 331-9.

Choi, D. Bench to bedside: the glutamate connection. Science 1992; 258: 241-3.

Christopher JP. Retinal neurotransmission. Surv. Ophthalmol. 1985; 29(5): 355-65.

Clark R, Collins G. An analysis of the influence of membrane potential and metabolic poisoning with azide on the sodium pump in skeletal muscle. J. Physiol. (London) 1976; 263: 383-403.

Clements J, Lester R, Tong G, Jahr C, Westbrook G. The time course of glutamate in the synaptic cleft. Science 1992; 258:1498-1.

Cornell-Bell A, Finkbeiner S, Cooper M, Smith S. Glutamate induces calcium waves in cultured astrocytes: long-range glial signaling. Science 1990; 247: 470-3.

Cosnier S, Innocent C, Allien L, Poitry S, Tsacopoulos M. An electrochemical method for making enzyme microsensors. Anal. Chem. 1997; 69: 968-71.

Curtis DR, Eccles RM. The excitation of renshaw cells by pharmacological agents applied electrophoretically.J. Physiol. (London) 1958; 141:435-45

Curtis DR, Philis JW, Watkins JC.. Chemical excitation of spinal neurons. Nature 1959; 183: 611-2.

Druce D, Peterson D, De Belleronche J, Bradford HF. Differential amino acid neurotransmitter release in rat neostriatum following lesioning of the cortico-striatal pathway. Brain Res. 1982; 247: 303-7.

Fagg GE, Foster AC. Amino acid neurotransmitters and their pathways in the mammalian central nervous system. Neuroscience 1983; 9: 701-19.

Fisher H. L-glutamate dehydrogenase from bovine liver. Methods Enzymol. 1985; 113: 16-27.

Fonnum F. Glutamate: a neurotransmitter in mammalian brain. J. Neurochem. 1984, 42, 1-11.

Fosse M, Kolstad J, Fonnum F. A bioluminescence method for the measurement of L-glutamate. J. Neurochem. 1986; 47: 340-9.

Graham, LT, Aprison MH. Fluorometric determination of aspartate, glutamate and γ-aminobutyrate in nerve tissue using enzyme methods. Anal. Biochem. 1966; 15: 487-97.

Greengard P, Jen J, Nairn A, Stevens C. Enhancement of the glutamate response by cAMP-dependent protein kinase in hippocampal neurons. Science 1991; 253: 1135-8.

Hicks TP, Guedes RW, Veale, WL, Veenhuizen J. Aspartate and glutamate as synaptic transmitters of parallel visual cortical pathways. Exp. Brain Res. 1985; 58: 421-5.

Jasper HH, Khan RT, Elliott KA. Amino acids released from the cerebral cortex in relation to its state of activation. Science 1965; 147: 1448-9.

Jovin D, Nicoud M, Kaufman S, Jovin T. Fluorescence digital imaging microscopy in cell biology. Science 1985; 230: 247-56.

Kar S, Arnold M. Fiber-optic ammonia sensor for measuring synaptic glutamate and extracellular ammonia. Anal. Chem. 1992; 64: 2438-3.

Kuhr W, Cosford R. Capillary biosensor for glutamate. Anal. Chem. 1996; 68: 2164-9.

Lada M, Vickroy T, Kennedy R. High temporal resolution monitoring of glutamate and aspartate in vivo using microdialysis on-line with capillary electrophoresis with laser-induced fluorescence detection. Anal. Chem. 1997; 69: 4560-5.

Leutwyler K. Stopping strokes. Scientific American 1997; April: 26-8.

Lillard SJ, Yeung ES. Temporal and spatial monitoring of exocytosis with native fluorescence imaging microscopy. J. Neurosci. Methods; 1997:*75*, 103-9.

Mayer M, Westbrook G. The physiology of excitatory amino acids in the vertebrate central nervous system. Prog. Neurobiol. 1987; 28: 197-276.

Miesenbock G, Rothman J. Pattrens of synaptic activity in neural networks recorded by light emission from synaptolucins. Proc. Natl. Acad. Sci. U.S.A. 1997; 34: 3402-7.

Murphy T, Baraban J, Wier W, Blatter L. Visualization of quantical synaptic transmission by dendritic calcium imaging. Science 1994; 263: 529-2.

Naito S, Ueda T. Characterization of glutamate uptake into synaptic vesicles. J. Neurochem. 1985; 44: 99-105.

Nicholls DG. Release of glutamate, aspartate and γ-aminobutyric acid from isolated nerve terminals. J. Neurochem. 1989; 52: 331-41.

Olson IA, Anfinsen CB. Kinetic and equilibrium studies on crystalline L-glutamate acid dehydrogenase. J. Biol. Chem. 1955; 20: 841-56.

Orwar O, Jardemark K, Jacobson I, Moscho A, Fishman H, Scheller R, Zare R. Patch-clamp detection of neurotransmitters in capillary electrophoresis. Science 1996; 272: 1779-2.

Otis T, Kavanaugh M, Jahr C. Postsynaptic glutamate transport at the climbing fiber-purkinje cell synapse. Science 1997; 277: 1515-8.

Palkovitz M, Lang T, Patthy A, Elekes I. Distribution and stress-induced increase of glutamate and aspartate levels in discrete brain nuclei of rats. Brain Res. 1986; 373: 252-8.

Parpura V, Liu F, Brethorst S, Jeftinija K, Jeftinija S, Haydon PG. α-Latrotoxin stimulates glutamate release from cortical astrocytes in cell culture. FEBS Letters 1995; 360: 266-0.

Peinado J, Iribar M, Myers R. Hemidecortication selectively alters release of glutamate in perfusates collected from cerebral cortex of unrestrained rats. Neurochem. Res. 1987; 12: 629-634.

Pol A, Wuarin J, Dudek F. Glutamate, the dominant excitatory transmitter in neuroendocrine regulation. Science 1990; 250; 1276-8.

Roberts P, Storm-Mathisen J, Johnston G. Glutamate: Transmitter in the Central Nervous System John Wiley and Sons, New York, 1981.

Roger KL, Philibert RA, Allen AJ, Molitor J, Wilson EJ, Dutton GR. HPLC analysis of putative amino acid neurotransmitters released from primary cerebellar cultures. J. Neurosci. Methods. 1987; 22: 173-9.

Rostas J, Brent V, Heath J, Neame R, Powis D, Weinberger R, Dunkley P. The subcellular distribution of a membrane-bound calmodulin-stimulated protein kinase. Neurochem. Res. 1986; 11(2): 253-68.

Siegel G, Agranoff B, Albers R, Molinoff P. (eds.) Basic Neurochemistry 1989, Raven Press, New York, 457-507.

Tsumoto T. Excitatory amino acid transmitters and their receptors in neural circuits of the cerebral neocortex. Neurosci. Res. 1990; 9: 79-102.

Tan W, Haydon PG, Yeung ES. Imaging neurotransmitter uptake and depletion in astrocytes. Appl. Spectroscopy. 1997; 51: 1139-3.

Wang Z, Yeung ES. Dual-enzyme assay of glutamate in single cells based on electrophoresis. J. Chromatogr. B 1997; 695: 59-65.

Wolfensberger M, Amsler U. Mass fragmentographic method for the determination of trace amounts of putative amino acids neurotransmitters and related compounds from brain perfusates collected in vivo. J. Neurochem. 1982; 38: 451-6.

Zecca L, Zambotti F, Zonta N, Mantegazza J. Determination of γ-aminobutyric acid in brain areas by high-performance liquid chromatography of dansyl derivatives with ultraviolet detection. J. Chromatogr. 1982; 233: 307-12.

Zhou S, Zuo H, Stobaugh J, Lunte C, Lunte S. Continuous in vivo monitoring of amino acid neurotransmitters by microdialysis sampling with on-line derivatization and capillary electrophoresis separation. Anal. Chem. 1995; 67: 594-9.

CHAPTER 4

MONITORING ATP RELEASE IN RAT ASTROCYTE CULTURES WITH LUMINESCENCE ASSAY-BASED IMAGING METHOD

A manuscript to be submitted to Analytical Chemistry

Ziqiang Wang, Philip Haydon, and Edward Yeung

ABSTRACT

A novel luciferase-luciferin chemiluminescence assay method was developed to monitor the adenosine-triphosphate (ATP) release from living biological cell samples. The assay has linear response over 3 orders of magnitude for any set concentrations of enzyme and co-factors, the linear coefficient is over 99.9%. The detection limit of ATP is down to 10^{-8} M concentration levels at milliseconds exposure time scale with intensified charge coupled device (ICCD), which is superior to several seconds to tens of second of collection time with luminometer. The *in vitro* experiment of ATP release from astrocyte cultures was successfully developed, the ATP release upon stimulation from rat brain astrocyte cultures was recorded first time with ICCD imaging techniques in real time scale. The data shows

that stimulation triggered an extracellular ATP wave, which reveals the communications among cells along the signaling pathway. Furthermore, the intracellular calcium wave propagation underneath this extracellular ATP wave was successfully simultaneously imaged. The data shows that the ATP extracellular signaling process is a dominant pathway for evoked calcium wave propagation in glial cells, but ATP release itself is not calcium-dependent.

INTRODUCTION

ATP is widely distributed in almost every type of biological cells. It is best known as the energy substrate for various cellular metabolic functions and regulator of enzymatic activities, including hydrogen production for electron transfer, biosynthesis, photosynthesis, mitosis, respiration, DNA replication, RNA synthesis, muscle contraction, membrane ion channel pump, hormonal effects and metabolic regulations[1,2]. In recent years, ATP in extracellular fluids in central nervous system (CNS) was found to have modulation effects on many neuronal activities, such as neurotransmission, epithelial secretion, endocrine/exocrine secretion, cardiovascular performance, and immune, inflammatory reaction, etc[3,4]. In several types of nervous system such as auditory organ and visionary retina, lines of support evidence[5,6] are establishing the role of neurotransmitter or neuromodulator for ATP. Besides, several reports very recently show that in brain glial cells ATP is a promising dominant neurotransmitter candidate for extracellular signal pathway[7-9]. Several kinds of technique were developed to measure the ATP contents in biological samples, including UV absorption[10-12], electrochemical detection[13,14] and luminescence assay[15-22].

UV absorption detection is a common technique for measuring ATP in biological fluids due to its wide availability and ease of operation. It was incorporated into various determination modes such as high performance liquid chromatography (HPLC). The detection limit with HPLC method is about 10^{-5} M concentration level that is suitable for routine measurement.

Electrochemical detection including chronoampemetry, cyclic voltammetry and sinusoidal voltammetry were also developed to measure purine-base nucleic acids including ATP with the improved sensitivity. The detection limit for ATP was in the order of 70-200 nM at electrode surface, the interference factors were also decreased.

The most widely developed technique to measure ATP in biological fluids and tissues is bio/chemiluminescence assay by using enzyme firefly luciferase. The reaction has fast response (~ milliseconds) and broad linear range for ATP concentrations. For any given set of enzyme/cosubstrate concentrations, the linear response range is over at least three orders of magnitude. This method is specific and highly sensitive. Only ATP would react with enzymes, no other adenosine containing nucleotides such as AMP or ADP would give signals. The sensitivity is extremely high because the background signal is theoretically zero. The detection limit is easily reached down to 10^{-10} M concentration level or 10^{-18} moles amount at normal conditions. Based on these advantages various applications have been developed for ATP measurement from many types of biological tissues and samples under different environments with this method[16, 21,23-26].

In the present work, we demonstrate the development of a microscopic imaging method with this chemiluminescence assay for the purpose of real time measurement of ATP releases from living biological cell samples. With the coupling of assay reaction to

intensified charge-coupled-device (ICCD) detection technique with single-photon sensitivity level[27, 28], the detection limit of ATP is able to reach 10^{-8} M concentration range within only milliseconds temporal resolution, which is superior compared to the collection time at least several seconds magnitude for measurement with luminometer. The linear response range is over 3 orders of magnitude of concentrations and the linear coefficient is over 99.9%. The excellent temporal resolution with low detection limit, in addition to the inherent spatial characteristics from imaging aspect, makes it an ideal tool to study ATP activities in living biological cell samples. The ATP releases from rat brain astrocyte cultures were studied with this method. For the first time the complete profile of ATP release to extracellular fluid from astrocytes was recorded at real time scale. The signaling patterns of ATP in extracellular pathway in rat glial cells were studied. Furthermore, with the development of simultaneously monitoring intracellular calcium change ($[Ca^{2+}]_i$) by using fluo-3, along with the measurement of ATP luminescence signal, we studied the dependencies between ATP and $[Ca^{2+}]_i$ in glial cells.

EXPERIMENTAL SECTION

Reagent and chemicals

Sodium chloride (NaCl), potassium chloride (KCl), magnesium chloride ($MgCl_2$), calcium chloride ($CaCl_2$), D-glucose, HEPES free acid, luciferase from firefly *photinus pyralis* (EC 1.13.12.7), D-luciferin, thapsigargin, suramin and ATP were purchased from Sigma Chemical Co. (St. Louis, MO). Fluo-3 and fluo-3 AM, BAPTA-AM were obtained from Molecular Probes (Eugene, OR). U-73122 and U-73343 were from Research

Biochemicals(Natick, MA). Dimethyl sulfoxide (DMSO) was from Fisher Scientific (Pittsburgh, PA). All reagents were used as received.

Buffers

All experiments were carried out in fresh saline prepared with purified and deionized water. The buffered saline contains NaCl (135mM), KCl (5mM) $MgCl_2$ (7mM), $CaCl_2$ (2mM), D-glucose (6mM) and HEPES (10mM). The final pH is adjusted to 7.35. All experiments were done at room temperature.

Cell culture

The enriched astrocyte cultures were prepared as follows[29]: Cortices of 1-4 day postnatal rat pups were dissected, dissociated and placed into culture flasks containing phenol-free modified minimal essential medium (MMEM) (Eagle's minimum essential medium, 2mM glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin and 100 mg/ml streptomycin) supplemented with 10% fetal bovine serum. These flasks were placed in a humidified 5% CO_2/ 95% air atmosphere for 8-10 days. The flasks were then rinsed twice with ice cold MMEM and the flasks tapped abruptly to dislodge any loose cells (neurons) and placed on an orbital shaker for 1.5 hours at 260PRM. The flasks were again rinsed twice with ice cold MMEM, tapped and returned to the shaker for 18 hours. The adherent cells were detached by trypsinization (0.1%). The detached cells were spun at 750RPM for 10 min and the supernatant discarded. The cells were resuspended in MMEM and plated into 12mm round coverslips. They were placed at 37°C in a humidified 5% CO_2/ 95% air atmosphere and allowed to grow to confluency (~4 days) and are ready for use. Before the experiment begins everyday the culture dish is washed with fresh saline described above to eliminate potential bacterial growth and other interference factors.

Instrumentation

The imaging system consists of an inverted light microscope (Nikon Diaphot 300, Fryer, Edina, MN) and a microplate coupled ICCD (EEV 576x384, Princeton Instrument, Trenton, NJ) attached to the camera mount of the microscope to record signals. The signal was collected by a 20X objective (N/A 0.75, Zeiss, Germany). The experiments were carried out in a flow chamber on the microscope stage. The flow chamber is made from a piece of Plexiglas slide by drilling a hole with a diameter of 8mm and a depth of 1.8mm, the volume is about 90 μl. For standard ATP calibration experiment, a microsyringe was inserted to chamber through a side tunnel for injection of ATP solution. For *in vitro* cell experiment a mechanical tip made from tungsten rod was used for stimulation to the cells underneath on coverslip in the flow chamber. The mechanical tip was etched to 6-8 μm diameter and controlled by micromanipulator, as depicted in Figure 1. Under both the standard calibration test and *in vitro* cell experiment the aliquot of luciferase enzyme mixture was first placed in chamber before experiment begins, the solution was kept still while taking data. For simultaneously monitoring calcium-Fluo-3 fluorescence signal with ATP chemiluminescence signal, a 488-nm argon-ion laser (Cyonics, San Jose, CA) was coupled to the system as excitation source and a 488nm notch filter (Oriel) was put in front of the ICCD to exclude scatter light. A customized mechanical shutter was placed in front of the laser, it was controlled by programmed digital I/O signal sent by ICCD controller so that the fluorescence signal was chopped with ATP signal. When ICCD was taking ATP frame, the shutter was closed to block the laser beam, so only chemiluminescence signal of ATP would come into the ICCD. The next frame the shutter is open, both chemiluminescence and laser induced fluorescence signal were taken together, but the fluorescence intensity was much

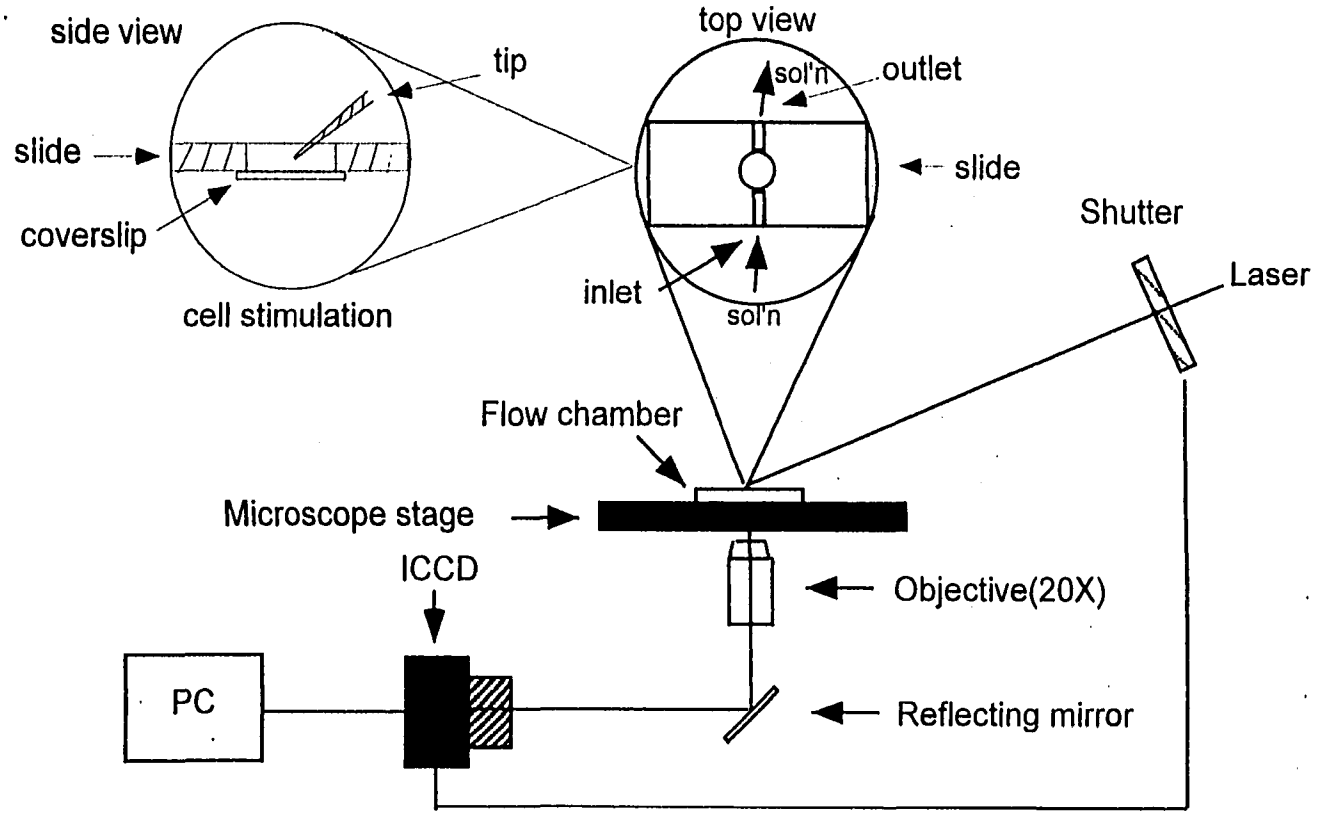


Figure 1: ATP luminescence imaging instrumentation

higher (over 100 times) than chemiluminescence so the latter could be neglected in this frame. Then this cycle of shutter programming was repeated and by this way the chopped ATP and calcium fluorescence frames were taken sequentially in experiments. In every experiment the ICCD was always to run first to take a few frames of background signals before ATP injection or cell stimulation.

Standard ATP calibration

Aliquots of standard ATP solution with concentration range from 10^{-8} M to 10^{-6} M were injected to flow chamber to react with luciferase-luciferin mixture solution. The generated light signal was recorded sequentially by ICCD at 1-second exposure time. The concentrations of enzyme mixture in chamber were 40μg/ml and 0.5mM for luciferase and D-luciferin, respectively. Each ATP concentration point was run in at least triplicate.

***In vitro* astrocyte cultures experiment**

The astrocyte culture coverslip was carefully attached to the lower surface of the flow chamber with the cultured cell side facing up. The flow chamber was placed on the microscope stage and filled with reaction reagents prepared in saline. Stimulation to the cells to cause ATP release was via gentle tapping to the cell membrane with the mechanical tip, as described elsewhere[30, 31]. The chopped ATP signal and calcium signal were respectively recorded sequentially with ICCD at approximate 2 second frame rate, with the exposure time 500 milliseconds and 50-100 milliseconds for ATP and calcium signals, respectively. The final concentrations were 100-200 μg/ml and 100-200 μM for luciferase and luciferin, respectively. The laser power at the focus was controlled to less than 1 mW to avoid possible cell damage.

Treatment of purinergic antagonist to astrocytes

Suramin was dissolved in saline and was add to chamber with final concentration 100μM for 5 min before starting cell stimulation and recording signal.

Monitoring intracellular calcium by fluo-3 fluorescence

Intracellular calcium concentration changes were monitored by using the fluorescent indicator Fluo-3[32]. The astrocyte cultures were loaded with fluo-3 AM (~10 μM) for 30-45 min at 37° C. the coverslip was then rinsed and kept in fresh saline for 30 min to let Fluo-3 de-esterify before use[9]. The loading solution was prepared by dissolving solid fluo-3 AM into dry DMSO, then diluted into saline to final solution. The calcium-Fluo-3 fluorescence induced by 488nm argon ion laser was recorded by ICCD.

Treatment of intracellular calcium chelators to astrocytes

The intracellular calcium chelator reagent 1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetate (BAPTA) AM (25 μM) was co-loaded with fluo-3 AM into astrocyte cultures for 45min, and coverslip was rinsed before experiment. The preparation of BAPTA AM solution is similar to that of fluo-3 AM. Also, another block reagent thapsigargin was dissolved in saline and cell coverslip was immersed into this solution for 30 min with thapsigargin concentration 1 μM and rinsed before experiment[31, 33].

Cell-free region experiment

The defined width (50-200 μm) cell-free lanes were made between regions of confluent astrocyte cultures by drawing a fine size mechanical tip through the coverslip, and allowed for cell recovery. At the time of experiment, the cell-free lanes were bordered by

healthy cells loaded with fluo-3 and displayed normal baseline fluorescence compared with controls.

Phospholipase-C (PL-C) inhibition experiment

The PL-C inhibitor aminosteroid 1-[6-[[17β-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1H-pyrrole-2,5-dione (U-73122) was dissolved in saline and was placed in flow chamber for at least 5 min before experiment with 10 μM final concentration. The solution was kept in chamber all the time during experiment with no rinse step. Similar experiment was also carried out in parallel with its inactive form U-73343 (10 μM)[34-36].

RESULTS AND DISCUSSION

Chemiluminescence assay for ATP measurement with firefly luciferase

The Chemiluminescence assay includes enzyme luciferase from firefly, D-luciferin, ATP and existence of magnesium ion. The reaction scheme is as follows:

$$E + LH_2 \xrightarrow{Mg} E\text{-}LH_2 + ATP \longrightarrow E\text{-}L\text{-}AMP + PP_i$$

$$E\text{-}L\text{-}AMP \xrightarrow{O2} E\text{-}L + AMP + h\nu\ (560nm)$$

E: luciferase LH_2: D-luciferin PP_i: Pyrophosphate

L: deoxy-luciferin AMP: adenosine-monophosphate

The D-luciferin combines luciferase to form enzyme-coenzyme E-LH_2 intermediate. When ATP exists in solution, this intermediate will quickly react with ATP to form E-L-AMP complex on the enzyme surface and release pyrophosphate; under normal aerated condition, the E-L-AMP complex will dissociate to E-L and AMP, accompanied with light generation around 560nm wavelength.

This reaction needs Mg^{2+} existence in solution[37], which was supplied from saline in our experiment. Without Mg^{2+} the reaction efficiency is greatly decreased (over 90 percent). The optimal pH is 7.8 for maximum enzyme activity[38]. At pH 7.35, which was our experiment condition, the enzyme activity was retained about 80 percent of maximum. It is reported[37] that few anions, SCN^-, I^-, NO_3^-, and Br^-, pose inhibition effects to the reaction. Because these anions were generally not presenting in our experiments, there was no worry for the interference.

Fig 2a shows the light intensity observed as a function of time from the assay. In milliseconds after reactants mixed there is at first a rapid rise in light intensity, then the signal will fall down fast to the second phase that is a relative slow decreasing curve. This is mainly due to the reaction kinetics hinders by the generation and accumulation of pyrophosphate from the reaction[39].

The peak height of the first flash is found to be linear to the ATP concentration if ATP is the limiting reagent and the linearity is over 99.9% from luminometer measurement[40, 41].

With the development of analytical microscopic imaging technique for biological study[28, 42-44], it would be an advantageous tool to combine this assay with imaging method to study the cell dynamics, especially in neurochemical analysis. The neurochemical activities

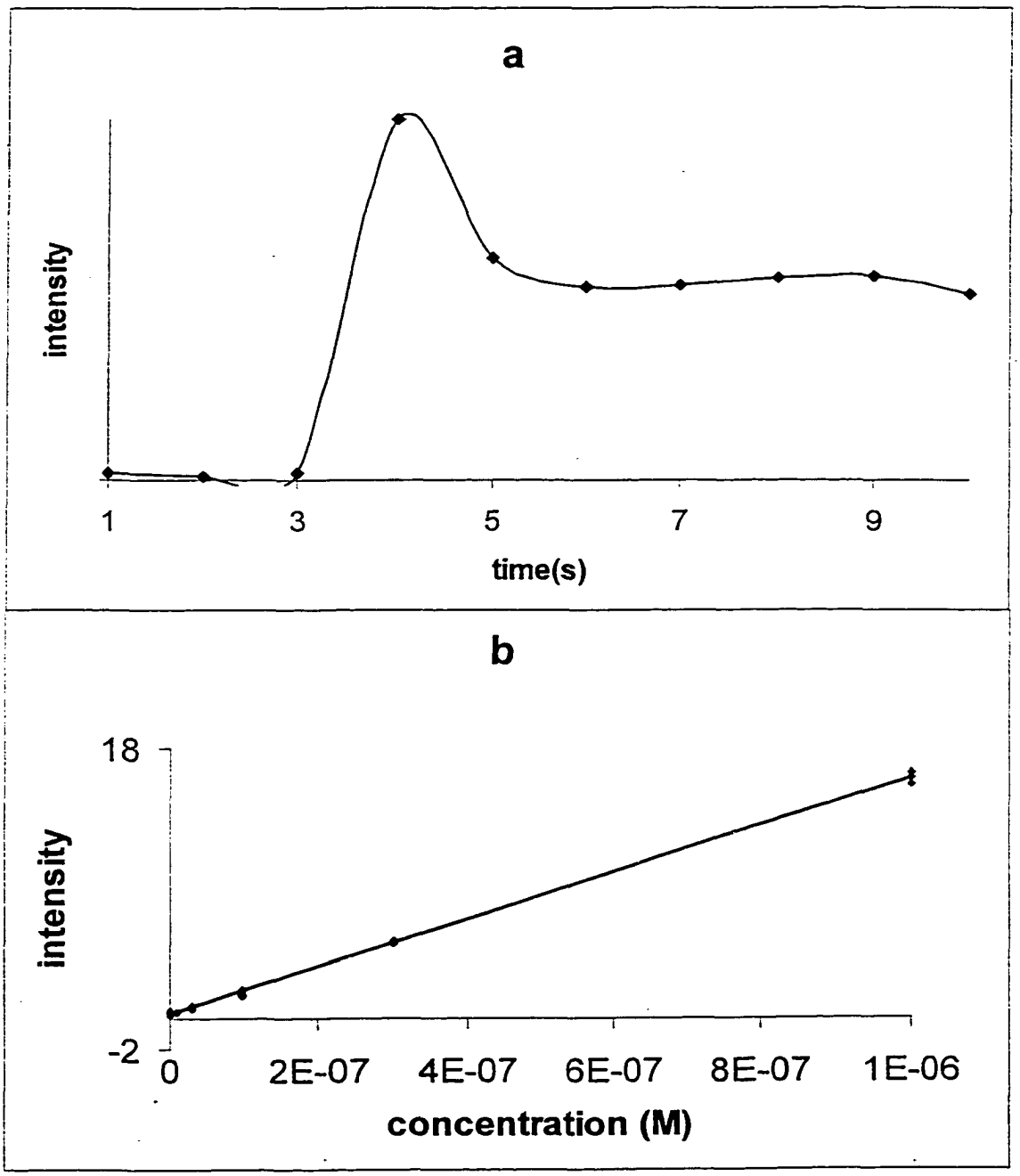


Figure 2: Time course of ATP reaction and standard calibration curve

a: time course. b: standard calibration curve

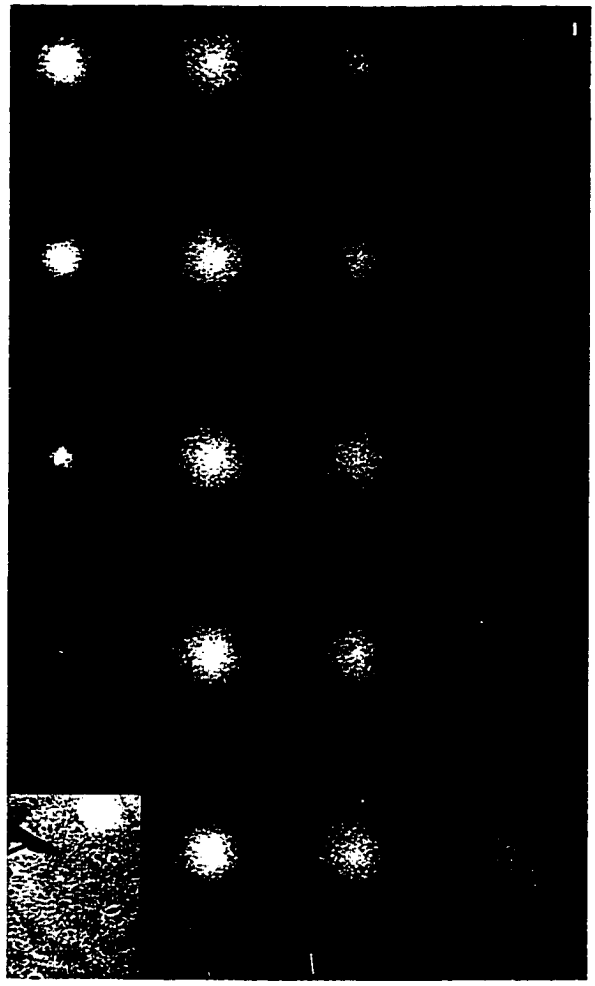
possess fast dynamics in an extremely heterogeneous environment. The luminometer-based bulk average sampling and analysis method is not suitable in order to reveal the real characteristics of the processes.

Fig 2b shows the standard assay calibration from our imaging setup. The linearity of this enzyme standard reaction is retained. The linear coefficient is 0.999. The detection limit is down to 10^{-8} M of ATP concentration with sub-second ICCD exposure at given enzyme concentrations. The dynamic range is over at least 3 orders of magnitude for any set of enzyme-coenzyme concentrations. The ATP concentration in extracellular fluids in central nervous system (CNS) is reported range from few hundred nM to mM[8, 45] which makes this chemiluminescence assay-based imaging method promising in neurochemical analysis.

Monitoring ATP releases from rat astrocyte cultures

Fig. 3 shows the signal of ATP released from astrocyte cultures with luciferase assay imaging method. It is clearly seen that upon stimulation, ATP was released from the cell stimulated into extracellular fluid and triggered the neighboring cells to release more ATP, which in turn triggered more further neighboring cells to form an extracellular ATP wave. The effective radius of stimulated ATP wave propagation was calculated at about 258 ± 50 μm $(n = 25)$. This confirms that the ATP wave consists the contribution of ATP from cells along the signaling pathway, and against the single point release model, in which the effective signal travel distance is 110 ± 30 μm[46, 47]. On the other hand, it is also necessary to point out that this wave was not propagating at the same magnitude, it was gradually decreased along the propagation. This supports the postulation[48, 49] that astrocytes express several subtypes of purinergic receptor through which ATP triggers the cell, and not all the

Figure 3: Temporally and spatially resolved ATP release patterns from astrocytes taken by an ICCD camera. The exposure time is 500 ms/frame. All frames were taken at 2 s intervals. The image sequence starts from upper left to right, then down one row, and so on. The first image corresponds to the bright-field image of the astrocyte culture, showing the microtip used for mechanical stimulation positioned over a glial cell, and the second corresponds to the frame at which the stimulation is applied. The solution in the perfusion chamber is kept still when images are taken during the experiment. Scale bar at lower right: 50 μm.



receptor types show identical response, in addition, cells show individual variations that means these subtypes of receptor are not evenly distributed on every cell. These factors result in the gradually decreasing pattern of ATP wave in astrocytes.

Synchronization of ATP wave and evoked intracellular calcium wave signals

Fig. 4 shows the simultaneous extracellular ATP wave and intracellular calcium wave ($[Ca^{2+}]_i$)underneath taken from programmed shutter experiments described in experimental section. Fig. 4b-c shows the time courses of both ATP signal and correspondent calcium signal from several individual cells in Fig. 4a. In Fig 4a, Cell 1 is right at the position of stimulation. At time 0 s only the calcium level in cell 1 increased. As the experiment progresses, cells 2 and 3, then cells 4 and 5, and finally cell 6 showed increases in calcium level synchronized with the arrival of the ATP wave. These data show that the increase of $[Ca^{2+}]_i$ synchronizes with the ATP wave propagation, both spatially and temporally. This implies that ATP is the messenger for the $[Ca^{2+}]_i$ wave in astrocytes.

Repetitive stimulation of astrocytes

To demonstrate that the mechanical stimulation does not cause artifact from possible cell breakage, we did repeat stimulations on same astrocytes. Fig. 5 shows that most cells response to stimulation with at least 2 times, after first stimulation the cell returned to normal conditions, which was confirmed from $[Ca^{2+}]_i$ fluorescence; and the signal responses were at a reduced magnitude at second stimulation. This indicates that the cells were intact during stimulation and showed genuine biological response. The fact that there was almost no or very little signal after third stimulation means overstimulation is reached.

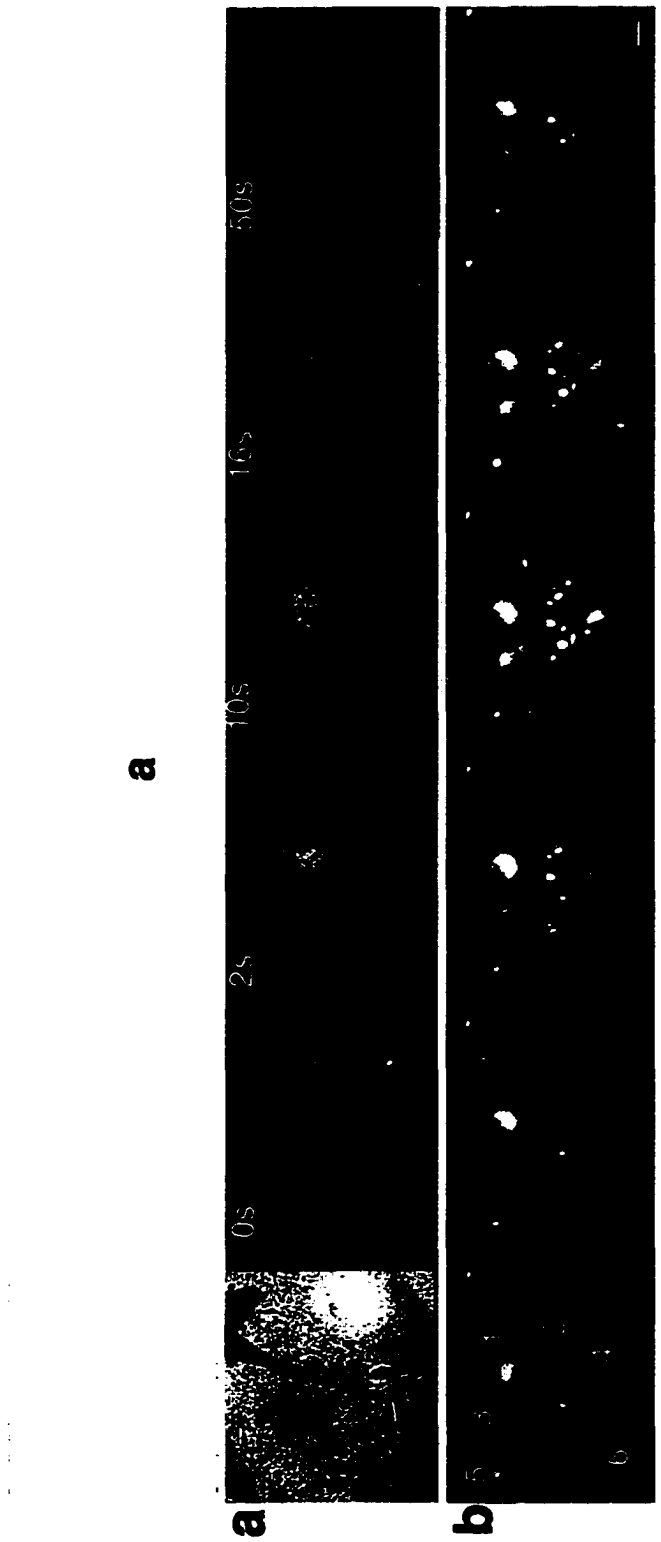
Figure 4: Synchronization of ATP release and $[Ca^{2+}]_i$ change from astrocyte cultures

a: images showing simultaneous release of ATP and calcium wave.

Calcium levels in astrocytes were monitored using Fluo-3. a: bright-field image showing the position of the stimulation tip followed by ATP images taken under the same conditions as in Fig. 3. Times shown in each image are relative to the time of stimulation, which is referred to as 0 s. b: corresponding calcium images taken at 0.5 s delay relative to each ATP image shown above it. Numbers 1-6 mark the location of several cells. Cell 1 is right at the position of stimulation. Scale bar at lower right: 50 μm.

b: time course of ATP releases of individual cells from a

c: correspondent calcium changes in cells from b show synchronization with ATP release


a
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2s
10s
16s
50s
a
b
b

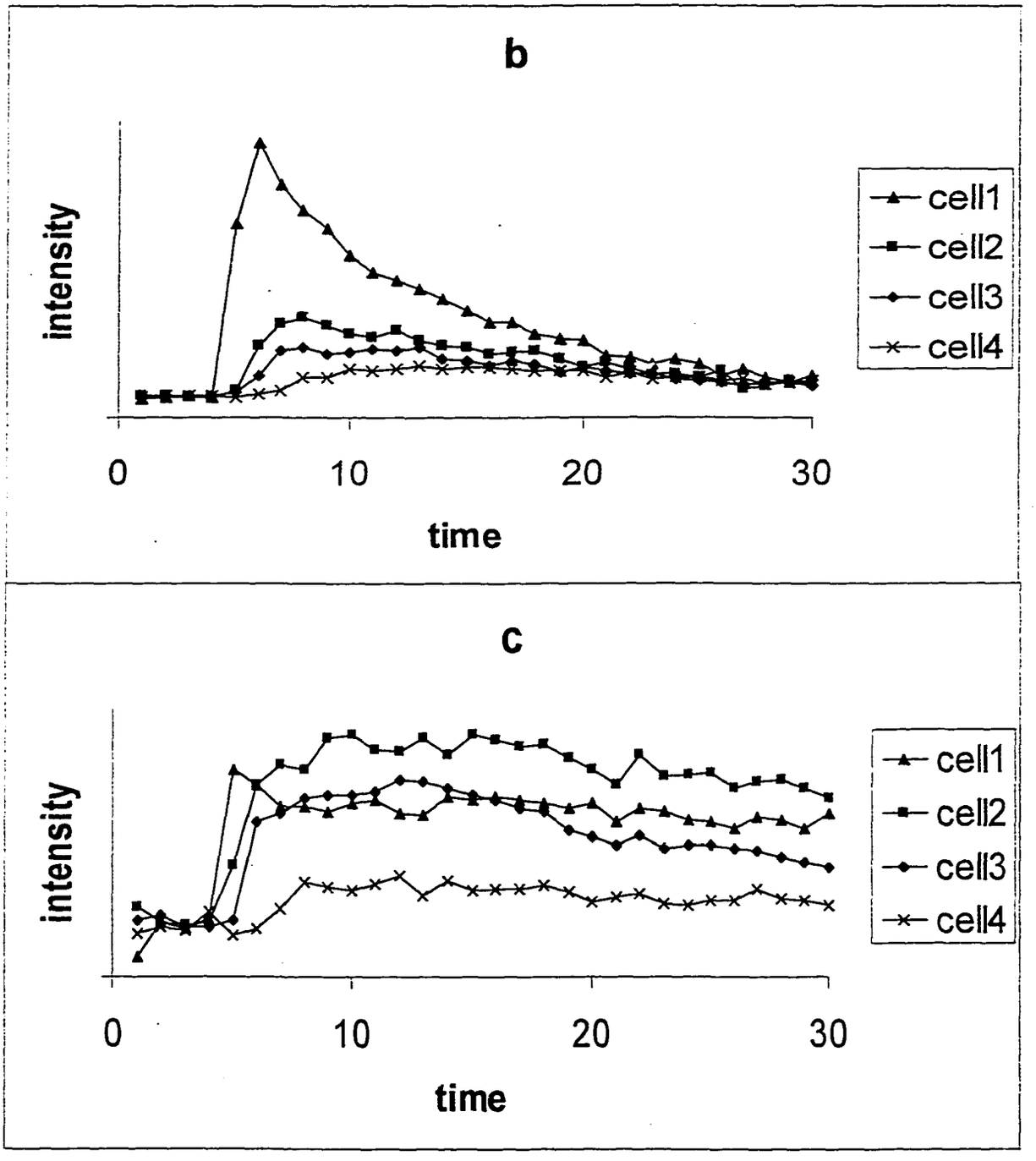
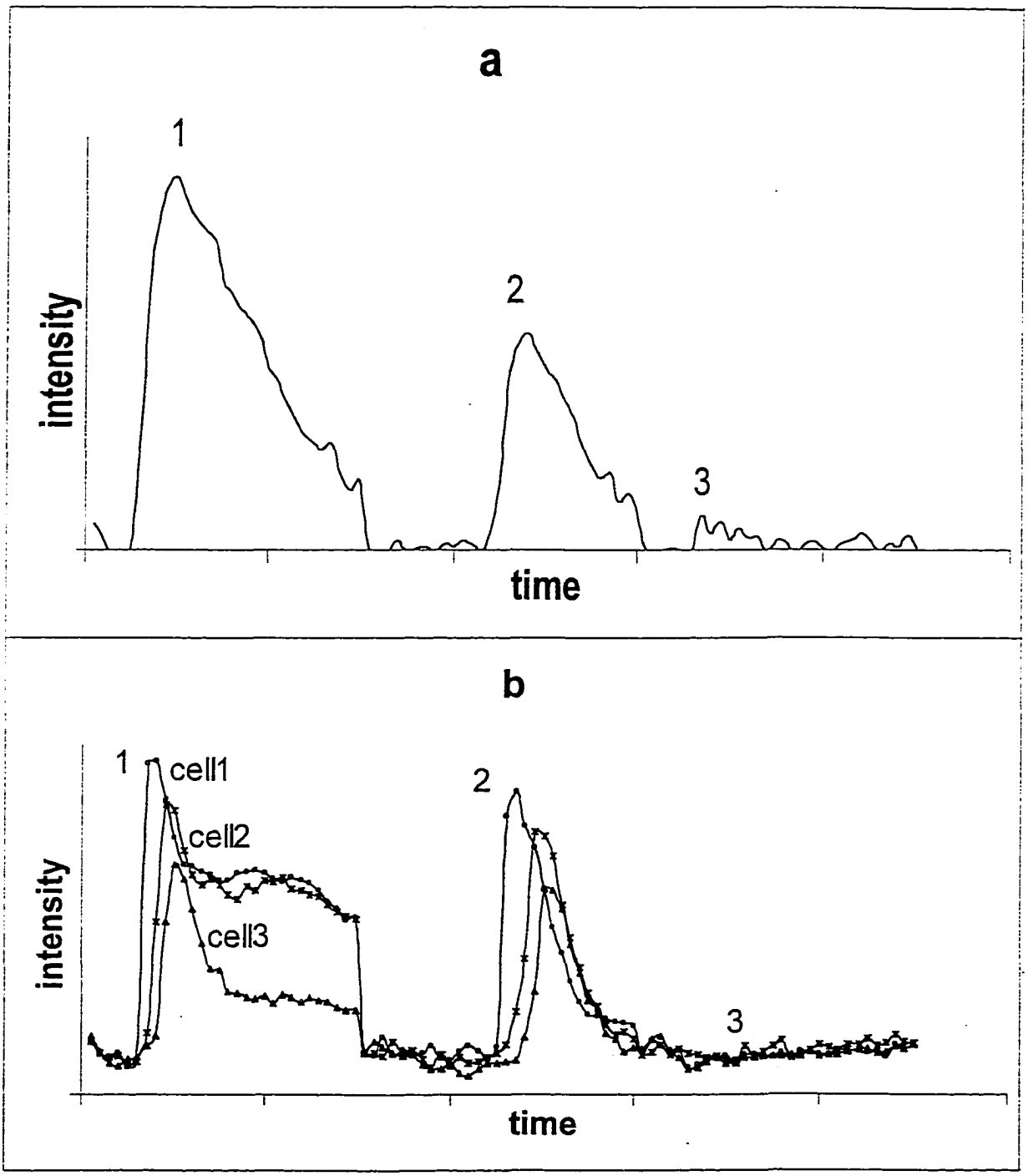


Figure 4 (continued)

Figure 5: repetitive simulation of astrocytes.

a: ATP releases, 1: first stimulation, 2: second stimulation to same cells after few minutes; and 3: third stimulation. Cell1-3 is according their distance to stimulation point. Cell 1 is closest and cell 3 is farthest, cell 2 is in middle.

b: Calcium changes in those cells during stimulations


a
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cell1
cell2
cell3
2
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intensity
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ATP signaling on physically isolated cells(cell-free region)

Observations[8, 9] suggested that ATP signals astrocytes through an extracellular pathway. We did experiment in which a cell-free lane was made as described in experimental section. Fig 6 shows the result that, the ATP released on one side of the lane was able to travel through the narrow lane (<120μm, n=8 of 11) area and triggered the cells on the other side, and in turn caused the increase of $[Ca^{2+}]_i$ in those cells. This is confirmed by the simultaneous monitoring of calcium fluorescence, as seen in Fig. 6b. Cell 1 is at the stimulation position. Cells 2 and 3 are on the same side of the gap as the stimulated cell, while cell 4 is on the opposite side of the gap. Cells on the same side of the gap show evoked calcium elevation first according to the order of their distances to the stimulation position. After a delay, [Ca2+]i elevation was observed from cells on the other side of the gap, synchronized to ATP crossing the gap. Because there was no physical connection between cells on two sides of the lane, the only way ATP molecules can reach another side is by diffuse in extracellular fluids to cross over. Our results support the ATP extracellular signaling pathway postulation in astrocytes.

Comparison of ATP release intensity and evoked $[Ca^{2+}]_I$ changes in individual cells of different distances

Fig. 7 shows the effects of ATP release intensity on $[Ca^{2+}]_I$ changes from individual cells according to their distance from stimulation point. It shows that while the magnitude of ATP released from cells gradually decreased over the distance, the $[Ca^{2+}]_I$ increase ratio kept steady for several relative close cells, and gradually decreased on further cells. This implies the possibility of a linear relationship between the $[Ca^{2+}]_I$ and ATP intensity, with the

Figure 6: ATP diffuses across cell free zone to trigger cells with no physical connection to stimulated cells.

a: bright-field image showing the stimulation position and a physically induced cell-free zone followed by images showing the ATP wave propagating to different distances at different times. The gap was in the middle part of the image and the width was about 70-120 μm. Times in each frame are relative to the stimulation at time equal to 0 s.

b: corresponding simultaneous calcium fluorescence images. Scale bar at lower right: 50 μm.

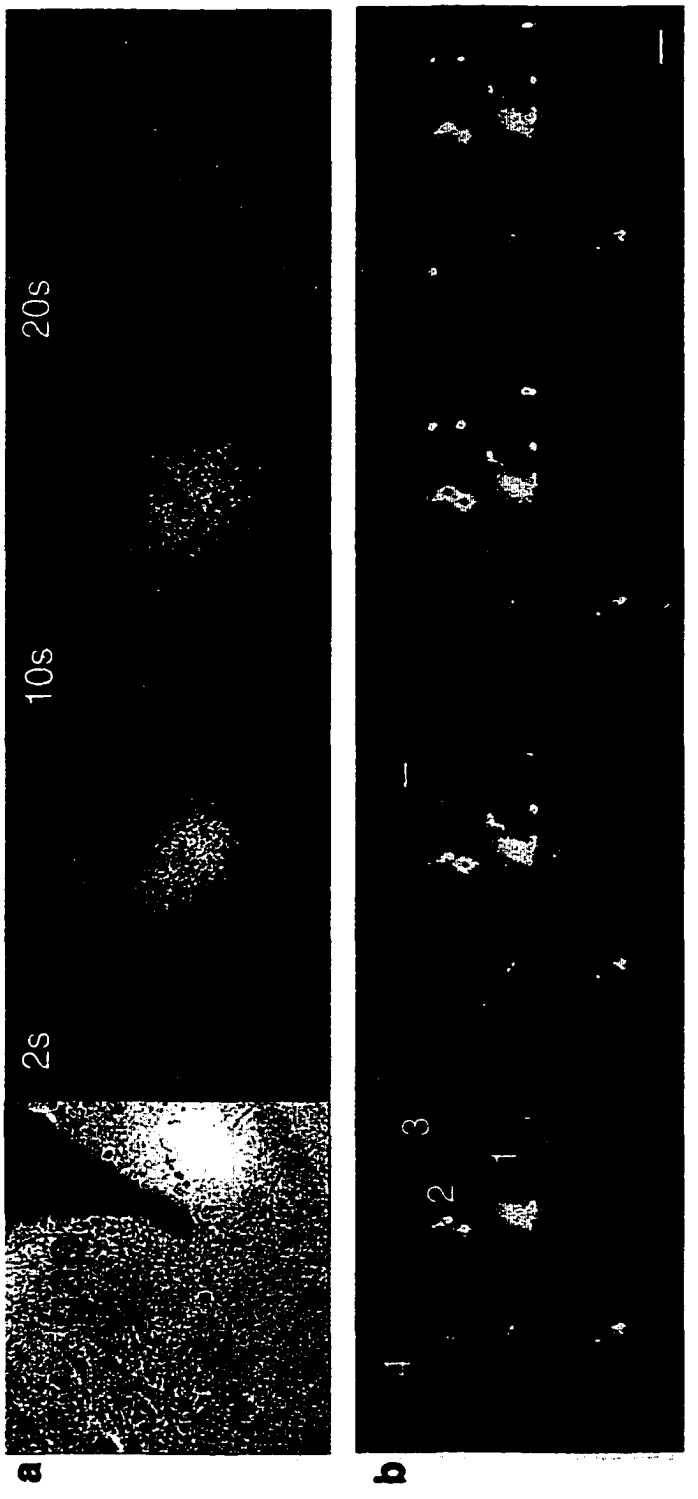
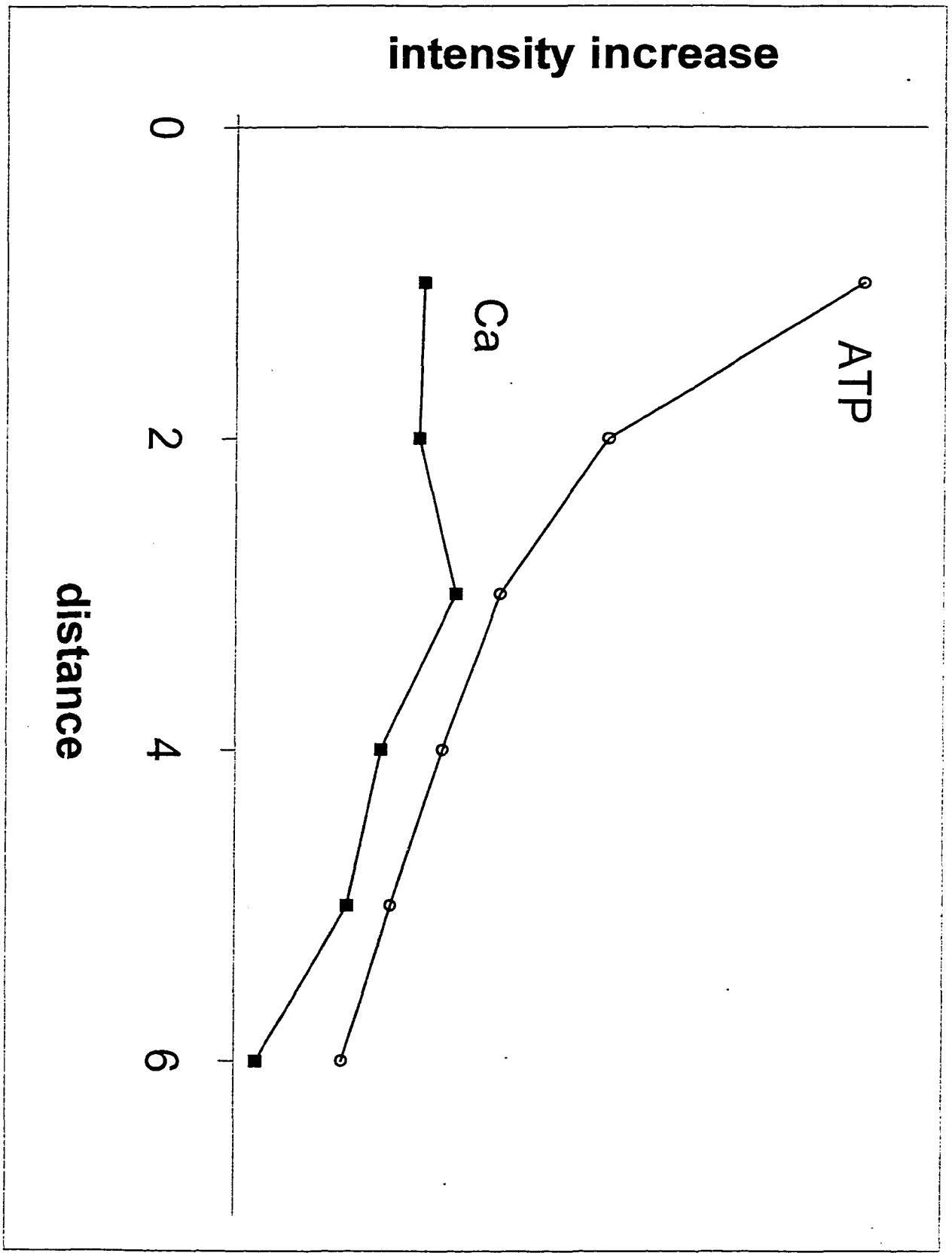

a
2s
10s
20s
b
1
2
3

Figure 7: correlation between released ATP intensity and $[Ca^{2+}]_i$ changes along the distance with regard to stimulation point (n=25 experiments)


intensity increase
0
2
4
6
distance
Ca
ATP

situation that there exists an upper threshold over which ATP intensity is strong enough to saturate the $[Ca^{2+}]_I$ response capability.

Dependency studies on ATP and $[Ca^{2+}]_i$ in astrocytes

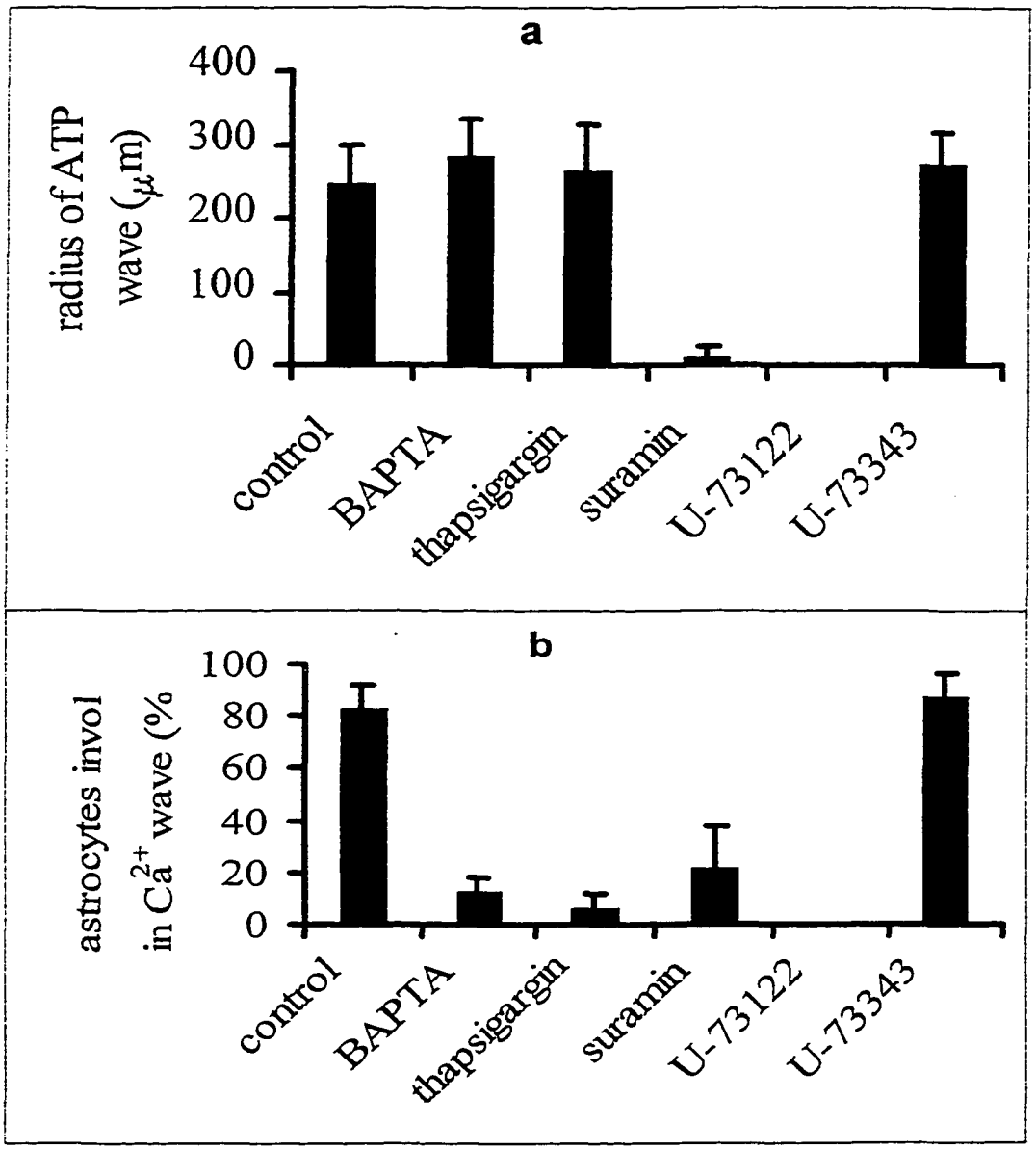
To investigate the interdependence between ATP and $[Ca^{2+}]_i$, we studied the effect of several factors on these astrocyte cultures, as shown in Fig. 8. The generic purinergic antagonist suramin is known to abolish the bioactivity of cellular ATP[3]. Treatment of suramin effectively blocks the chemiluminescence associated with ATP. There was none or only localized ATP release and the propagating ATP wave was absent (Fig. 8a). There was also no accompanying calcium wave (Fig. 8b). This result suggests that the ATP extracellular signaling system is a dominant pathway for glial calcium wave propagation. On the other hand, we also investigated the effect of 1,2-bis (2-aminophenoxy) ethane-*N,N,N',N'*-tetraacetate (BAPTA) and thapsigargin on the behavior of astrocytes. Both BAPTA and thapsigargin are known potent blockers of the elevation of cytosolic-free calcium from intracellular endoplasmic reticulum stores and have been shown to significantly reduce the calcium wave propagation[33, 50]. Under both situations we saw no calcium waves, as expected, but the ATP waves were still present in all cases with no signs of reduction in intensity or excursion distance (Fig. 8). These results suggest that $[Ca^{2+}]_i$ elevation is neither necessary to cause ATP release nor for the propagation of the ATP wave.

To further investigate the factors that affect ATP release, we studied the effect of U-73122 on astrocytes. U-73122 inhibits the activity of PL-C in cells, which catalyzes the rapid hydrolyzation of phosphatidylinositol 4,5-bisphosphate to diacylglycerol (DAG) and

Figure 8: Statistical results for dependency study of ATP and $[Ca^{2+}]_i$

a: ATP releases under different conditions

b: calcium changes in correspondent to same conditions in a


a
radius of ATP wave (μm)
400
300
200
100
0
control
BAPTA
thapsigargin
suramin
U-73122
U-73343
b
astrocytes invol in Ca2+ wave (%
100
80
60
40
20
0
control
BAPTA
thapsigargin
suramin
U-73122
U-73343

inositol 1,4,5-trisphosphate (1,4,5-IP_3). The accumulation of IP_3 in cells, in turn, will mobilize calcium from its intracellular stores to elevate $[Ca^{2+}]_i$.[34-36] The application of U-73122 to astrocytes totally blocked both calcium and ATP responses to stimulation such that neither ATP release nor $[Ca^{2+}]_i$ elevation was observed (n = 9 of 9). The application of a closely related inactive analog U-73343 to astrocytes resulted in no inhibition to either ATP or $[Ca^{2+}]_i$ (n = 9 of 9) such that both ATP and calcium waves were present to an extent similar to control experiments (Fig. 8). To ensure that there was no interference or loss of activity of luciferase on introduction of U-73122 (or U-73343), a small amount of standard ATP (100 μM) was injected into the cell chamber after the stimulation experiment. Immediately, a sharp increase in chemiluminescence was observed (data not shown). These results suggest that blocking the production of IP_3 does abolish the release of ATP on stimulation.

CONCLUSION

The chemiluminescence assay based imaging method for monitoring ATP dynamics and determination in biological samples was successfully developed. The assay shows broad range linear response to ATP concentrations and excellent low detection limit. The imaging method demonstrates excellent temporal and spatial resolutions. The ATP releases from rat brain astrocyte cultures were successfully monitored by this method. The release profile of ATP in astrocytes was recorded for the first time. With simultaneously monitoring intracellular calcium changes with ATP, we confirmed that in astrocytes ATP extracellular

signaling process is dominant pathway for intracellular calcium wave propagation, but ATP release itself is no calcium dependent.

ACKNOWLEDGEMENT

The Ames Laboratory is operated for the U.S. Department of Energy by Iowa State University under Contract W-7405-Eng-82. This work was supported by the Director of Energy Research, Office of Basic Energy Sciences, Division of Chemical Sciences and by the National Institutes of Health.

REFERENCE

1. Bridger, W.A.; Henderson, J.F. *Cell ATP.* John Wiley & Sons, New York, 1983.
2. De Robertis, E.D.P.; Saez, F.A.; De Robertis, E.M.F., Jr. *Cell and Molecular biology.* Saunders, Philadelphia, 1980.
3. Dubyak, G. R.; El Moatassim, C. *Am. J. Physiol.* **1993**, 265, C577-C606.
4. Bruns, R.F. *Ann. NY Acad. Sci.* **1990,** 603, 211-226.
5. Newman, E. A.; Zahs, K. R. *Science.* **1997, 275,** 844-847.
6. Wangemann, P. *Auditory Neurosci.* **1996, 2,** 187-192.
7. Cotrina, M. L.; Lin, J.H.C.; Alves-Rodrigues, A.; Liu, S.; Li, J.; Azmi-Ghadimi, H.; Kang, J.; Naus, C.C.G.; Nedergaard, M. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, 95, 15735-15740.
8. Guthrie, P. B.; Knappenberger, J.; Segal, M.; Bennett, M.V.L.; Charles, A.C.; Kater, S.B. *J. Neurosci.* **1999,** 19(2), 520-528.

9. Hassinger, T. D.; Guthrie, P.B.; Atkinson, P.B.; Bennett, M.V.L.; Kater, S.B. *Proc. Natl. Acad. Sci. U.S.A.* **1996,** 93, 13268-13273.

10. Fujitaki, J.M.; Nord, L.D.; Willis, R.C.; Robins, R.K. *J. Biochem. Biophys. Meth.* **1992,** 25(1), 1-10.

11. Au, J.L.; Su, M.H.; Wientjes, M.G. *Clin. Chem.* **1989,** 35(1), 48-51.

12. Karon, B.S.; Nissen, E.R.; Voss, J.; Thomas, D.D. *Anal. Biochem.* **1995,** 227(2), 328-333.

13. Singhal, P.; Kuhr, W. G. *Anal. Chem.* **1997,** 69, 3552-3557.

14. Bianchi, A.; Domenech, A.; Garcia-Espana, E. *Anal. Chem.* **1993,** 65, 3137-3142.

15. Stanley, P.; McCarthy, B.; Smither, R. *ATP Luminescence*, Blackwell Scientific Publications, Oxford, **1989.**

16. Abraham, E.; Okunieff, P.; Scala, S.; Vos, P.; Oosterveld, M.; Chen, A. Shrivastav, B. *Science,* **1997,** 275, 1324-1326.

17. Bitler, B.; McElroy, W. *Arch. Biochem, Biophys.* **1957,** 72, 358-368.

18. Denburg, J.; Reiko, L.; McElroy, W. *Arch. Biochem, Biophys.* **1969,** 134, 381-394.

19. Rhodes, W.; McElroy, W. *J. Biol. Chem.* **1958,** 233, 1528-3157.

20. Schifman, R.; Wieden, M.; Broker, J.; Chery, M.; Delduca, M.; Norgard, K.; Palen, C.; Reis, N.; Swanson, J.; White, J. *J. Clin. Microbiol.* **1984,** 20, 644-648.

21. Molin, O.; Nilsson, L.; Ansehn, S. *J. Clin. Microbiol.* **1983,** 18, 521-525.

22. Deluca, M.; McElroy, W.(eds.) *Bioluminescence and Chemiluminescence.* Academic Press, New York, **1981.**

23. Dadoo, R.; Seto, A.; Colon, L.; Zare, R. *Anal. Chem.* **1994,** 66, 303-306.

24. Leach, F.R. *J. Appl. Biochem.* **1981,** 3, 473-517.

25. Lin, S.; Cohen, H.P. *Anal. Biochem.* **1968,** 24, 531-540.

26. Imbeault, N.; Paquet, M.; Cote, R. *J. Water Qual. Res. Canada.* **1998**, 33,403-415.

27. Xu, X.; Yeung, E.S. *Science,* **1997,** 276, 1106-09.

28. Parpura, V.; Tong, W.; Yeung, E.S.; Haydon, P.G. *J. Neurosci. Methods.* **1998,** 82, 151-58.

29. Banker G.; Goslin K. (eds.): *Culturing Nerve Cells* **1991**, MIT Press, Cambridge, MA, 309-336.

30. Charles, A. C. *Dev. Neurosci.* **1994**, 16, 196-206.

31. Araque A.; Parpura, V.; Sanzgiri, R. P.; Haydon, P. G. *Eur. J. Neurosci.* **1998,** 10, 2129-2142.

32. Kao, J. P. Y.; Hartootunian, A. T.; Tsien, R. Y. *J. Biol. Chem.* **1989,** 14, 8179-8184.

33. Araque A.; Sanzgiri, R. P.; Parpura, V.; Haydon, P. G. *J. Neurosci.* **1998,** 18(17), 6822-6829.

34. Smallridge, R. C.; Kiang, J.G.; Gist, I. D.; Fein, H.G.; Galloway, R. J. *Endocrinology* **1992,** 131(4), 1883-1888.

35. Yule, D. I.; Williams, J. A. *J. Biol. Chem.* **1992,** 267, 13830-13835.

36. Yamada, M.; Yamada, M.; Richelson, E. *Eur. J. Pharmacol.* **1992,** 226, 187-188.

37. Denburg, J.; McElroy, W. *Arch. Biochem, Biophys.* **1970,** 141, 668-675.

38. Seliger, H. H.; McElroy, W. D. *Arch. Biochem. Biophys.* **1960, 88**, 136-141.

39. McElroy, W. D.; Seliger, H. H. in *Light and Life* (Eds. McElroy, W. D. and Glass, B.) John Hopkins Press, Baltimore, MD, **1961,** 219-257.

40. Stehler, B. L. in *Methods of Enzymatic Analysis* (Eds. Bergmeyer, H. U.). Academic Press, **1974,** 4, 2112-2113.

41. Leach, F. R.; Webster, J. J. *Methods in Enzymology.* **1986,** 133, 51-70.

42. Tan, W.; Haydon, P. G.; Yeung, E. S. *Appl. Spectrosc.* **1997,** 51, 1139-43.

43. Lillard, S. J.; Yeung, E. S. *J. Neurosci. Methods.* **1997,** 75, 103-109.

44. Tan, W.; Parpura, V.; Haydon, P. G.; Yeung, E. S. *Anal. Chem.* **1995,** 67, 2575-2579.

45. Silinsky, E. M.; Redman, R. S. *J. Physiol.* **1996,** 492, 815-822.

46. Crank, J. *The Mathematics of Diffusion* Oxford, Clarendon, **1975.**

47. Hazel, J. R.; Sidell, B. D. *Anal. Biochem.* **1987,** 166, 335-341 ().

48. Pearce, B.; Langley, D. *Brain Res.* **1994,** 660, 329-332.

49. Ho, C.; Hicks, J.; Salter, M. W. *Br. J. Pharmacol.* **1995,** 116, 2909-2918.

50. Wang, Z.; Tymianski, M.; Jones, O. T.; Nedergaad, M. *J. Neurosci.* **1997,** 17, 7359-7371.

CHAPTER 5

GENERAL CONCLUSIONS

A new dual-enzyme on-column reaction method combined with capillary electrophoresis has been developed for determining the glutamate content in single cells. Glutamate dehydrogenase and glutamic pyruvic transaminase were used to catalyze the glutamate reaction. Detection was based on monitoring the laser-induced fluorescence of the reaction product NADH, and the measured fluorescence intensity was related to the concentration of glutamate in each cell. Glutamate dehydrogenase catalyzed the formation of NADH, and glutamic pyruvic transaminase drives the glutamate dehydrogenase reaction by removing a reaction product and regenerating glutamate. The detection limit of glutamate is down to 10^{-8} M level, which is 1 order of magnitude lower than previous reported detection limit based on similar detection methods. The mass detection limit of a few attomoles is far superior to that of any other reports. Selectivity for glutamate is excellent over most of amino acids. The glutamate content in single human erythrocyte and baby rat brain neurons were determined with this method and the results agreed well with literature values.

Following the successfully developed enzymatic assay method as described above, we continued to develop a fluorescence imaging method for monitoring glutamate released from neuron cultures with this enzymatic assay. The method is sensitive and specific. Since

no pretreatment with fluorescent dyes is needed, the native cell characteristics and functions are preserved. The *in vitro* experiment in neuron cultures demonstrates that the detection scheme could simultaneously provide satisfactory spatial and temporal resolution. The approach is complementary to electrophysiological and chromatographic methods, it promises to become a useful for signal transduction study and neurochemical analysis. To the best of our knowledge there are no previously published reports on glutamate imaging or on enzyme-based imaging. We believe that with more advanced lasers and more sensitive CCD systems in the future, glutamate monitoring in CNS will become even more sensitive and the spatial and temporal resolution will become even higher.

A novel chemiluminescence assay based imaging method for monitoring ATP dynamics and determination in biological samples was successfully developed. The assay shows broad range linear response to ATP concentrations and excellent low detection limit. The imaging method demonstrates excellent temporal and spatial resolutions. The ATP releases from rat brain astrocyte cultures were successfully monitored by this method. The release profile of ATP in astrocytes was recorded for the first time. With simultaneously monitoring intracellular calcium changes with ATP, we confirmed that in astrocytes ATP extracellular signaling process is dominant pathway for intracellular calcium wave propagation, but ATP release itself is no calcium dependent.

ACKNOWLEDGEMENTS

I would like to express my thankfulness and gratefulness to everyone who make this dissertation possible.

First, I would like to thank my major advisor, Dr. Yeung, for his support and guidance for my Ph.D. research program. It is his excellence in the field, inspiration and insight to the research that guide me to an independent researcher, and his patience and encouragement lead me through all the difficulties and obstacles in these years. The precious training I got from his lab with so many broad inter-discipline research projects will benefit my whole career.

I would also like to thank my committee members, Professors Dennis Johnson, Marc Porter, Pat Thiel and Steven Rodermel, for their help on my project and time for my examinations.

I am grateful to Professor Philip Haydon at the Department of Zoology and Genetics, and to his lab members, for their cooperation on the project, discussion of biological aspects, and cell preparations.

I would like to thank all Yeung group members, both past and present. You were always there whenever I have questions, equipment to find, and cooperation on research schedule, etc.. During my years here I owe many thanks to Ho-Ming Pang, Wei Tong, Sheri Lillard, Michael Shortreed, just to name a few, for their friendship, support and cooperation.

Special thanks to Sze-Shun Wong from Professor Porter's group, for making the microtips for my cell experiments.

To the memory of my grandparents, who brought me up to where I am today. I am in huge debt to them, which I am not able to ever repay.

To my daughter, she stayed with me during my last project. Always refreshing and cheerfully, she brought me so much joy and that helped me to complete this dissertation successfully.

Finally, I would like to dedicate this dissertation to my wife, Wenfei Yuan (wini), for her encouragement to my educational pursuit, for her endurance of my negligence when I was busy on work, and for her strength to share all our pains and happiness in life.

This work was performed at Ames Laboratory under Contract no. W-7405-Eng-82 with the U.S. department of Energy. The Unite States government has assigned the DOE Report number IS-T 1891 to this thesis.