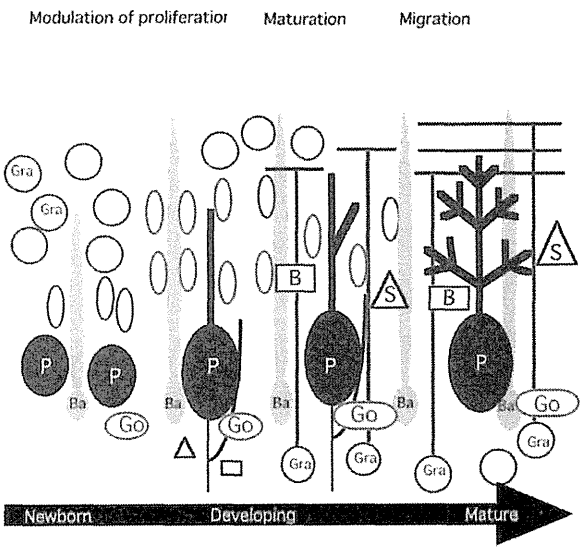

Fig. 1　Diagram of cerebellar development. In newborn cerebellum (1 to 3 days after birth), granule cell progenitors (Gra) proliferate in the EGL while immature Purkinje cells (P) form the PL with Golgi cells (Go) and some Bergmann glia (Ba). On developing, 5 to 7 days after birth, Gra-cells elongate their axon and migrate inside, and P-cells spread their dendrites and connect to other neurons during 2 weeks. B, Basket cell, S, satellite cell

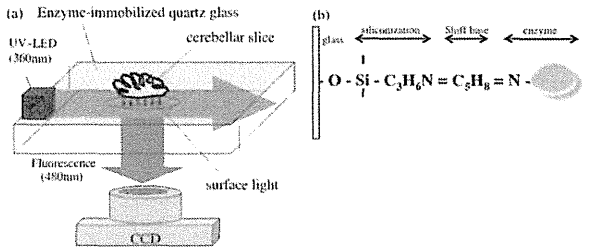

Fig. 2　Enzyme-linked photo assay device with the surface UV excitation lightning and covalent immobilization of enzymes (a). Transmitter molecules released from the sample are degenerated by the enzyme and generated NAD(P)H is excited by UV-LED generating narrow band 360 nm UV light. The cooled CCD catches the fluorescence. (b) N-terminal of enzymes is bound on the siliconized quartz glass surface.

3.　酵素光学法による伝達物質可視化デバイスの開発

そこで我々は，伝達物質 GABA やグルタミン酸の放出分布と時間変化を可視化するために新規光学デバイスを開発した（Fig. 2）．

本デバイスは，①酸化還元酵素を用いた伝達物質放出の蛍光観察，②UV-LED 光源を用い，ガラス導波路表面からの励起によって自家蛍光と組織障害を軽減，③シラン化剤による酵素のガラス表面固定化，を特徴とする[5]．薄切した小脳組織を十分な酸素供給の後ガラス基板上に載せ，組織と基板の界面に放出された GABA およびグルタミン酸は，GABA 分解酵素およびグルタミン酸脱水素酵素の酵素反応により化学量論的に還元型ニコチンアミドアデニンジヌクレオチド（リン酸）（NAD(P)H）を生成する．これを 360 nm で励起し 480 nm の蛍光を発生させ，放出分子の分布を観察する．基質選択性の高い酵素反応を用いるため，放出される伝達物質に特異的に反応して蛍光を発する（特徴①）一方，NAD(P)H は細胞内に普遍的に存在する分子であるため，励起光により細胞内から自家蛍光が発生する．そこで励起光源にガラス導波路を用い，表面からごく浅い範囲に励起光を照射することとした（特徴②）．さらにシラン化剤により酵素をガラス表面に固定化する（特徴③）ことで，簡易なバイオデバイスを構成した．

4.　発達期小脳で放出される GABA とその由来

発生過程の小脳皮質からの GABA 放出を観察したところ，出生直後の外顆粒層からの GABA 放出が観察された．この層には GABA 作動性神経細胞が殆ど分布しておらず，非神経性の GABA 放出であることが示唆された．外顆粒層からの GABA 放出は生後 1 週間程度続き，バスケット細胞・星状細胞が分布して神経性の GABA 放出が始まると消失することが観察された[6]（Fig. 3 a–d）．

外顆粒層では GABA の持続性電流も観察された．これらの GABA は顆粒細胞分裂を促進すると考えられた[7]．

小胞性 GABA トランスポーター（VGAT）遺伝子改変動物の観察[8]から，発達期の小脳皮質でグリア細胞の一種であるアストロサイトが GABA の放出と制御を行っていることが示唆された．さらに小脳以外の脳発生過程でも，伝達物質観察デバイスを用いることで，グリア細胞からの GABA 放出が脳発生に寄与することが観察された[9]．そこで小脳皮質から単離した

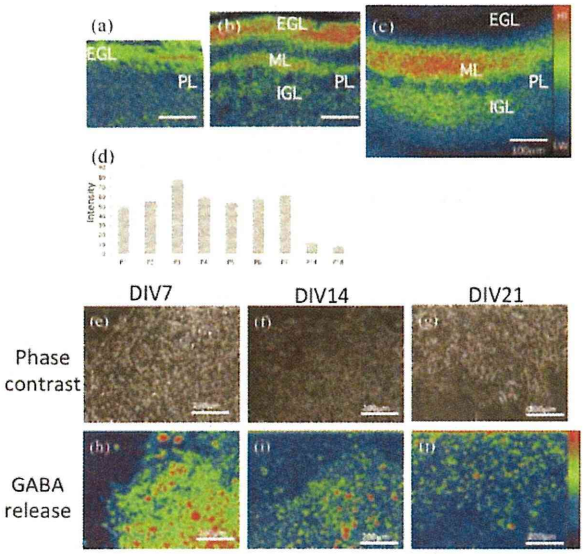

Fig. 3 GABA release from developing cerebellar cortex; (a) postnatal day 1 (P1), (b) P3, (c) P8. (d) shows temporal change of GABA release. (e) to (f) show GABA release from cultured cerebellar astrocytes. DIV: day *in vitro*.

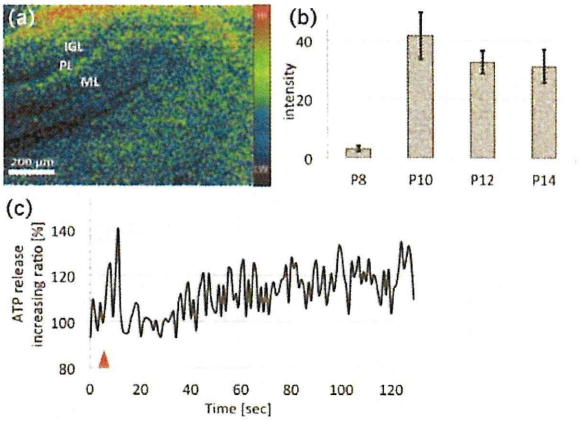

Fig. 4 (a) shows released ATP distribution in P10 cerebellar cortex. ATP release was suddenly increased from P10. (c) shows the time course of ATP release to 100 μM glutamate application.

培養アストロサイトを観察したところ，培養初期の細胞からGABA放出が観察された（Fig. 3 e–f）．これらはGABA合成酵素（GAD）を発現しており，発達期の小脳アストロサイトは神経細胞非存在でGABAを合成し放出していることが示唆された[10]．GABAの放出は培養日齢とともに減少し，同時にGAD，VGATの発現も急激に減少した．

5. アストロサイトが発生の場を作る

アストロサイトは従来神経細胞をサポートする非神経細胞ととらえられてきたが，近年神経伝達物質の取り込みおよび再放出によって，神経回路の「雰囲気」を作り，情報処理に直接作用することが知られてきた．特に発生期の脳組織では，アストロサイトが未分化の神経細胞の機能を代替することが知られている．我々は発生初期の小脳外顆粒層で，GABAが未分化の顆粒細胞からグルタミン酸放出を誘発することを観察している．未分化の培養顆粒細胞が細胞内リン酸化によってグルタミン酸を放出することは知られていたが，生体内ではアストロサイト由来のGABAによって顆粒細胞の増殖刺激とグルタミン酸放出に至る分化誘導が引き起こされることが示唆された．

では神経細胞間のシナプス形成や樹上突起発達に対し，アストロサイトはどのような寄与を示すのだろうか．アストロサイトの代表的な伝達物質ATPは痛覚や炎症の情報分子として知られており，発達期の小脳では，プルキンエ細胞と顆粒細胞のシナプス形成と，プルキンエ細胞樹上突起の発達に関与することが知られている[11]．ATPは通常ルシフェリン–ルシフェラーゼ発光反応（L-L反応）によって測定されるが，この反応は高感度だが光量が少なく，ATP放出の空間分布・時間変化をCCDで測定することが困難だった．

そこで，ATP要求性の酸化還元酵素反応であるグリセルアルデヒド3-リン酸脱水素酵素（GADPH）を用い，100 μMのグルタミン酸刺激によって放出されるATPを，酵素反応で化学量論的に発生するNADHによって可視化することを試みた．

生後8日の小脳組織ではほとんどATP放出が見られないが，生後10日の小脳では急激なATP放出の増加が見られた（Fig. 4c）．ATP放出はグルタミン酸刺激から約50 sec後にゆっくりと上昇し，従来のL-L反応の結果と一致していた．各種のグルタミン酸受容体アゴニストで刺激したところ，刺激される受容体サブタイプによってATP放出部位が異なることが見られ，アストロサイトが直接グルタミン酸刺激を受けるだけでなく，神経細胞の刺激を増幅・制御してATPを放出していることが示唆された[12]．

アストロサイトからの小脳発生に関わる情報分子をFig. 5に示す．出生直後の小脳ではGABA作動性神経細胞が未発達であるため，アストロサイトは神経細

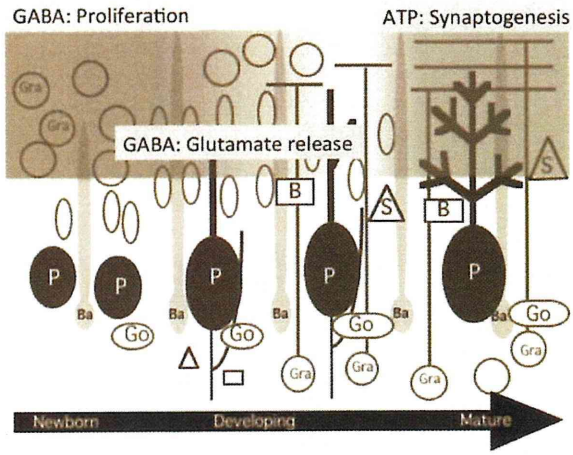

Fig. 5　Schematic diagram of glial coordination to cerebellar development. Released GABA would modulate granule cell proliferation and neuronal development, and release ATP would accelerate synaptogenesis between Purkinje cell and granule cells.

胞の代替としてGABAを放出し，顆粒細胞の増殖を促していると考えられる．さらに分化が進むと，顆粒細胞からグルタミン酸の放出を促し，同時にアストロサイトからのGABA放出は急激に消失する．代わりにグルタミン酸刺激によりATPを放出し，プルキンエ細胞と顆粒細胞のシナプス形成を誘導すると考えられる．

神経回路は，記憶することに見られるようにきわめて可塑性の高い組織である．これまで神経回路の秩序形成は，神経相互間の自己組織化から遺伝子発現による決定論的な形成まで様々の仮説で説明され，データの蓄積がなされてきた．そのなかでアストロサイトは，神経細胞が遊走する足場として，あるいは伝達物質やイオン環境の調整役として，「重要だがぼんやりした存在」と捉えられることが多かった．しかし脳皮質内で大きく広がり，細胞間の結合によって広い範囲の物質環境を制御するアストロサイトは，神経回路の発達過程で積極的に神経細胞を刺激し，構造的な秩序を作り出している．神経細胞との相互作用によりアストロサイト自身も分化し，機能を変化させながら，神経回路の機能発現にも関わっていることが示唆される．

6. 情報が見えることの意味

自分が知りたい情報を得る手段があるとは限らない．そういう情報を見たいがために開発してきた我々のデバイスは，小脳発生研究の枠を超えて，現在パーキンソン病の研究やアルツハイマー症，網膜変性の基礎研究に，あるいは糖尿病の予防効果の研究へと利用が広がっている．情報分子の可視化技術によって，神経細胞の生死を左右する脳の中のリズムを見ることが可能になり，神経回路の新しいルールが顕らかになることを期待している．

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参　考　文　献

1) Yamada, M., Terao, M., Terashima, T., Fujiyama, T., Kawaguchi, Y., Nabeshima, Y., Hoshino, M. (2007): Origin of climbing fiber neurons and their developmental dependence on *Ptf1a*, J. Neurosci., Vol.27, pp.10924–10934
2) Millen, K.J., Gleeson, J.G. (2008): Cerebellar development and disease, Curr. Opin. Neurobiol., Vol.18, No.1, pp.12–19
3) Ito, M. (1984): The Cerebellum and Neural Control. New York: Raven Press
4) Varju, P., Katarova, Z., Madarasz, E., Szabo, G. (2001): GABA signalling during development: new data and old questions, Cell Tissue Res., Vol.305, No.2, pp.239–246
5) Morishima, T., Uematsu, M., Furukawa, T., Yanagawa, Y., Fukuda, A., Yoshida, S. (2010): GABA imaging in brain slices using enzyme-linked photo analysis, Neuroscience Research, Vol.67, pp.347–353
6) Okochi, K., Hirano, S., Maruya, K., Morishima, T., Takayama, C., Fukuda, A., Yoshida, S. (2008): Ectopic GABA release in developing Cerebellar cortex is regulated by GABA transporters and glutamate, Neuroscience Research, Vol.61 (suppl.1), S114
7) Morishima, T., Kumada, T., Takayama, C., Yanagawa, Y., Yoshida, S., Fukuda, A. (2009): Temporal release of GABA from Bergmann glia accelerates granule cell precursors (GCPs) proliferation during rat cerebellar cortex development, IUPS2009
8) Uematsu, M., Hirai, Y., Karube, F., Ebihara, S., Kato, M., Abe, K., Obata, K., Yoshida, S.,

Hirabayashi, M., Yanagawa, Y., Kawaguchi, Y. (2008): Quantitative chemical composition of cortical GABAergic neurons revealed in transgenic Venus-expressing rats, Cerebral Cortex, Vol.18, pp.315–330

9) Wang, T., Kumada, T., Morishima, T., Kaneko, T., Yanagawa, Y., Yoshida, S., Fukuda, A. (2012): Gathering of GABAergic neurons rendering focal ambient GABA gradient and downregulation of KCC2 may underlie the microgyrus formation in mouse model, Cerebral Cortex, in press

10) Kurimoto, Y., Yonezawa, Y., Kobayashi, K., Hozumi, N., Takayama, C., Fukuda, A., Yoshida, S. (2011): GABA conditioning regulated by the transporters in cultured cerebellar glial cells, AP-IRC 2011, p.91

11) Casel, D., Brockhaus, J., Deitmer, J.W. (2005): Enhancement of spontaneous synaptic activity in rat Purkinje neurones by ATP during development, J. Physiol., Vol.568 (Pt. 1), pp.111–122

12) Muramoto, H., Sekino, Y., Hozumi, N., Fukuda, A., Yoshida, S. (2012): Developing transition of ATP release to glutamate stimulation in rat cerebellar slices using a new ATP imaging system, 42nd Neuroscience meeting, New Orleans

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Improvements in Enzyme-Linked Photoassay Systems for Spatiotemporal Observation of Neurotransmitter Release

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Neurotransmitters and neuronal releasing molecules are not only the regulators of neuronal function but also the indicators of neuronal conditions. Glutamate and γ-amino butyric acid (GABA) play important roles in cerebellar differentiation and function. In the mature cortex, they are released from synapses and taken up by transporter molecules. We have developed enzyme-linked photoassay systems for glutamate, GABA, and adenosine triphosphate (ATP), and reported their release in the developing cerebellar cortex. Our systems showed slow transmitter release in the immature cerebellum, whereas it was hard to detect the fast synaptic release from mature neurons, because there were some limitations in time resolution and data depth derived from a charge-coupled device (CCD), and the enzyme-linked photodevice was sometimes unstable. In this study, we report the dynamic observation of neurotransmitter release in the developing cerebellar slices using improved photodevices and a high-speed 16-bit CCD. With this new system, the rapid measurement of transmitter release in a young-adult cerebellar cortex is possible. We suggest that these photoassay systems are useful for observing synaptic release in several diseases.

1. Introduction

Neurotransmitter molecules released from neurons are not only the regulators of neuronal transduction but also the indicators of neuronal conditions.[1–3] Glutamate and γ-aminobutyric acid (GABA) are known as typical transmitters in the brain's cortex, and they play important roles as stimulators and suppressors, respectively. Lack of balance in the release of glutamate and GABA may lead to autism, epilepsy, or Parkinson's disease.[4]

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To observe spatiotemporal neurotransmitter release in the cerebellar cortex, we have recently developed an enzyme-linked photoassay system, which is a device with an immobilized enzyme on a quartz glass surface. Using this system, we observed glutamate or GABA release in developing cerebellar slices using either new or authorized methods.[5] Enzyme-linked photoassay is sensitive and selective, and it can discriminate the substrates from their pharmacological analogues. Our system can detect transmitter release in the cerebral cortex,[6] hippocampus, retina, and cultured cells,[7] and made it possible to detect the release of adenosine triphosphate (ATP),[8] glucose, sucrose, and fructose. On the other hand, enzymes tend to denature and separate from the quartz. For the detection of transmitter release in mature neuronal circuits, increasing the sensitivity and stability of the device is required.

In this paper, we propose new immobilizing methods and discuss the optimization of the enzyme-linked photoassay.

2. Materials and Methods

2.1 *Substrate and enzyme reaction*

Imaging neurotransmitter release was monitored for the reaction in which oxidoreductases generate reduced nicotinamide adenine dinucleotide (NAD^+) or diphosphonucleotide ($NADP^+$). For glutamate, GABA, or adenosine triphosphate (ATP) imaging, we used glutamate dehydrogenase, GABA disassembly enzyme [GABase, Fig. 1(a)] or glyceraldehyde 3-phosphate dehydrogenase, respectively.[9–11] The NADH or NADPH, the reductants of NAD^+ or $NADP^+$, respectively, which is generated stoichiometrically, emits 480 nm fluorescence after excitation at 340–365 nm.

2.2 *Surface photoexcitation*

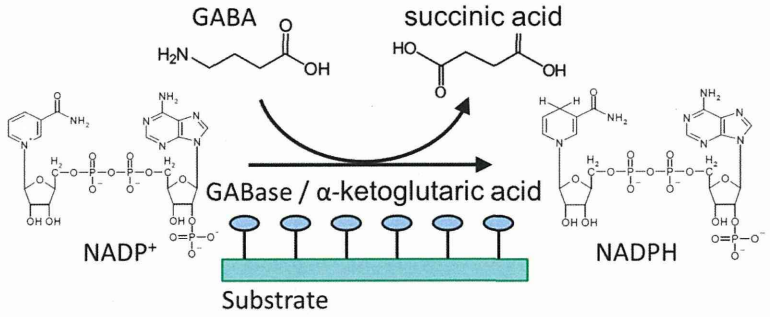
For UV excitation, a quartz glass plate illuminated with an ultraviolet light-emitting diode (UV-LED, Nichia, Tokushima, Japan) was used. Leaking UV light onto the glass surface excited fluorescent NADH or NADPH [Fig. 1(b)].

2.3 *Imaging apparatus*

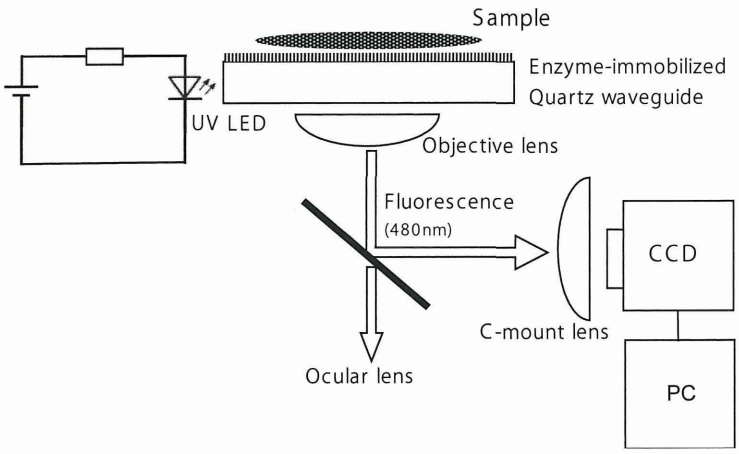
All fluorescence images through the inverted microscope (IX73, Olympus Co., Ltd., Tokyo, Japan) were observed by a cooled charge-coupled device (CCD) (ORCA-ER CCD) or a high-speed complimentary metal-oxide semiconductor (CMOS) (ORCA-Flash 4.0) camera, supplied by Hamamatsu Photonics Co., Ltd., Hamamatsu, Japan. Imaging data were analyzed by iVision software (BD Biosciences, San Jose, CA, USA).

2.4 *Enzyme immobilization and sample preparation*

Enzymes were typically covalently immobilized on the quartz glass surface using a silane coupling agent and a crosslinking agent, 3-aminopropyltriethoxy silane (3-APTS) and glutaraldehyde, respectively [Fig. 2(a)].[12] These surface modifications determine both the stability of the enzyme reaction and the distance between the sample and the glass surface.



(a)



(b)

Fig. 1. (Color online) (a) Released neurotransmitters are oxidized by the oxidoreductase, and NAD(P)H is formed stoichiometrically. In GABA oxidization, released GABA is converted to succinic acid with NADPH formation by GABase. (b) Schematic diagram of the enzyme-linked photoassay system. The oxidoreductases are immobilized on the quartz glass surface, and the excitation light radiating from UV-LED passes through the quartz waveguide. Fluorescent images are obtained by CCD or CMOS, and analyzed using a computer system.

In some cases, glass surfaces were treated with either aromatic crosslinkers, 1,4-phenylene diisothiocyanate (1,4-DIC), or 1,3-phenylene diisothiocyanate [1,3-DIC, Fig. 2(b)], and glutaraldehyde (GA).[13] Others were treated with a phosphonic acid, 11-aminoundecylphosphonic acid [11-AUPA, Fig. 2(c)], as a replacement for 3-APTS.[14]

Cerebellar acute slices were treated from postnatal day 3 (P3) to P15 in rats, sliced sagittally to a thickness of 400 μm with a rotor slicer (Dohan EM, Kyoto, Japan),

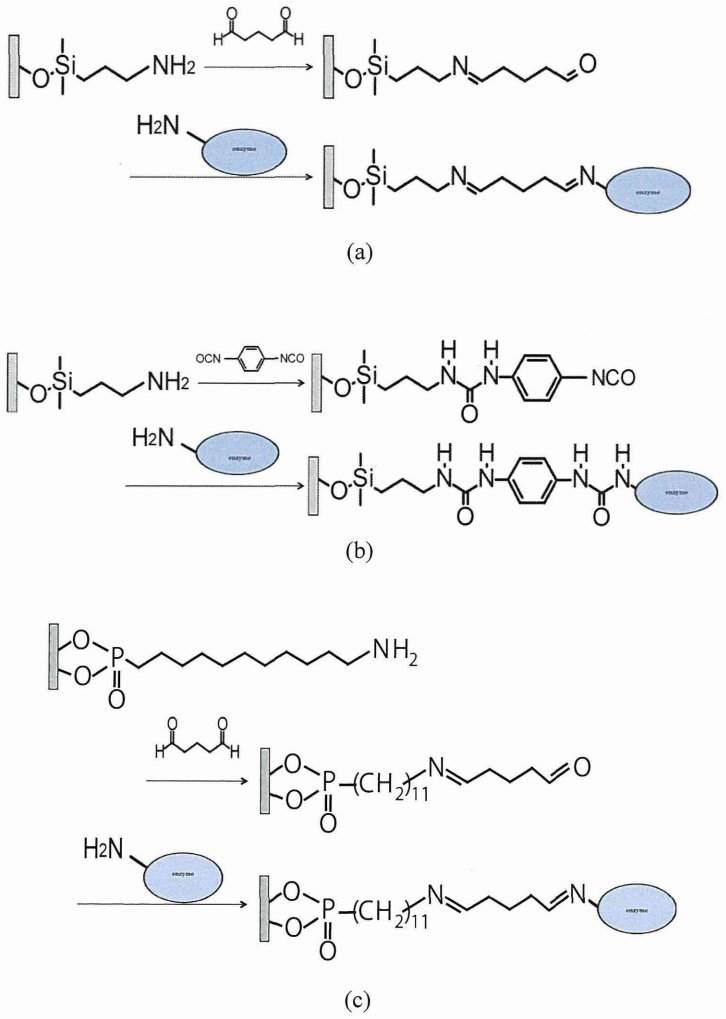


Fig. 2. (Color online) (a) Method of enzyme immobilization using 3-APTS and glutaraldehyde. Surface treatment and crosslinking between waveguide quartz and enzyme. (b) Crosslinking using 1,3-DIC. (c) Surface treatment with 11-AUPA.

and incubated in oxygen-aerated PBS for 45 min. All experimental procedures were approved by the committee for the use of animals at Toyohashi University of Technology and by the guidelines of the Ministry of Education, Culture, Sports, Science and Technology, Japan.

3. Results

3.1 *Spatiotemporal observation of glutamate release*

Figure 3(a) shows an illustration of rat cerebellar development. In the developing cerebellum, neuronal arrangement and circuit formation progress after birth. Granule cells, small input neurons, proliferate and migrate down from the external granular layer (EGL) to the internal granular layer (IGL). Purkinje cells, major output neurons, develop their dendrites and associate neuronal connections between granule cells and other interneurons. The layer of Purkinje cell somas is identified to be the Purkinje layer (PL). A neuronal circuit layer forms the molecular layer (ML).[15,16] To understand the roles of neurotransmitters in the cerebellar development, we have developed a new visualizing device and, with it, we have observed spatiotemporal molecular dynamics.

Using the enzyme-linked photoassay system, we have observed many kinds of transmitter release in several developmental stages and organs. Our system has visualized both spontaneous and responsive transmitter release processes with 0.5 s time resolution. Figures 3(b)–3(k) show the transitions in glutamate release in response to 100 μM GABA application in developing cerebellar slices.[17] Glutamate was released in both the EGL and the IGL, whereas the PL was indicated by a negative line. In the developing cerebellum, the granule cells that distributed in the EGL and IGL are the only neurons that release glutamate, so both layers showed fluorescence activities. Glutamate release in P3 cerebellar slices appeared in both layers slowly but continuously, whereas it started rapidly in the lower EGL and then spread to the IGL within a short time in P7 celebellar slices.[18] Granule cells in the P3 cerebellum did not develop sufficiently to react to GABA stimulation nor release the transmitter actively, but they still proliferated. On the other hand, the granule cells in the P7 cerebellum developed sufficiently to react to GABA stimulation, so they released glutamate rapidly.

Although spatiotemporal observation could give us dynamic information about neuronal reaction, our system needs to be improved in terms of stability, sensitivity and time resolution for us to observe fast synaptic transmissions. The targets of our improvements were the (1) sensing CCD, (2) excitation waveguide, and (3) manner of enzyme immobilization shown in Fig. 2.

3.2 *Effects of new crosslinkers and surface treatment*

Two types of glass devices with either aromatic crosslinkers, 1,3-DIC or 1,4-DIC, and GA were examined to observe spontaneous GABA release with 500 ms time resolution using ORCA ER CCD. The device formed using 1,3-DIC and GA gave images with a better contrast of GABA release than the GA crosslinked device in the P10 cerebellar slice [Fig. 4(a)], whereas it showed no difference in the P6 cerebellar slice. The 1,4-DIC crosslinked device yielded no good images.

The aromatic crosslinkers make the glass surface hydrophobic. Because mature brain tissues become hydrophobic as the myelin structure develops, 1,3-DIC crosslinking should increase the affinity of the enzyme for the tissues.

The binding between the glass and the acceptor molecules has been weak, because the silane coupling agents tend to undergo hydrolysis under biological conditions. The

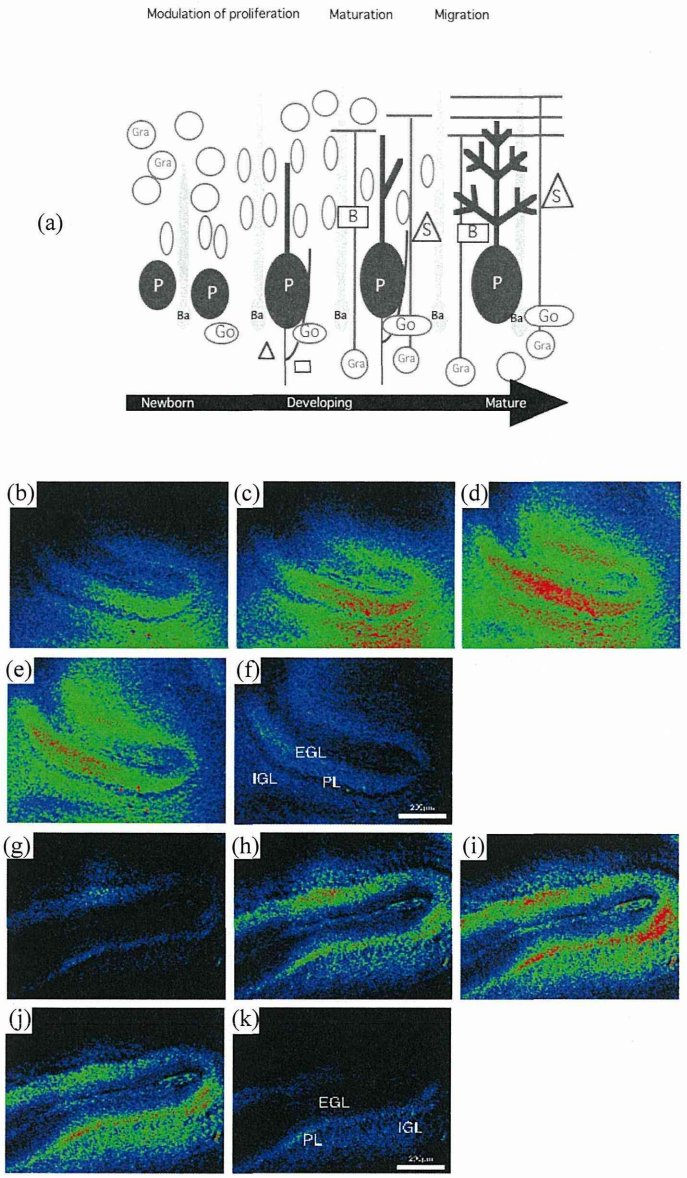


Fig. 3. (a) Diagram of cerebellar development. In the newborn cerebellum (1 to 3 days after birth), granule cell progenitors (Gra) proliferate in the EGL, while immature Purkinje cells (P) form the PL with Golgi cells (Go) and some Bergmann glia (Ba). During the developement, 5 to 7 days after birth, Gra-cells elongate their axon and migrate inside, and P-cells spread their dendrites and connect to other neurons within two weeks. B denotes basket cells, and S, satellite cells. Evoked glutamate wave with GABA application in developing cerebellar cortex. (b)–(f): 2.0, 4.5, 7.0, 12.5, and 23.5 s after stimulation in P3 cerebellar cortex, respectively. (g)–(k): 0.5, 2.5, 4.0, 11.0, and 16.0 s after stimulation in P7 cerebellar cortex, respectively.

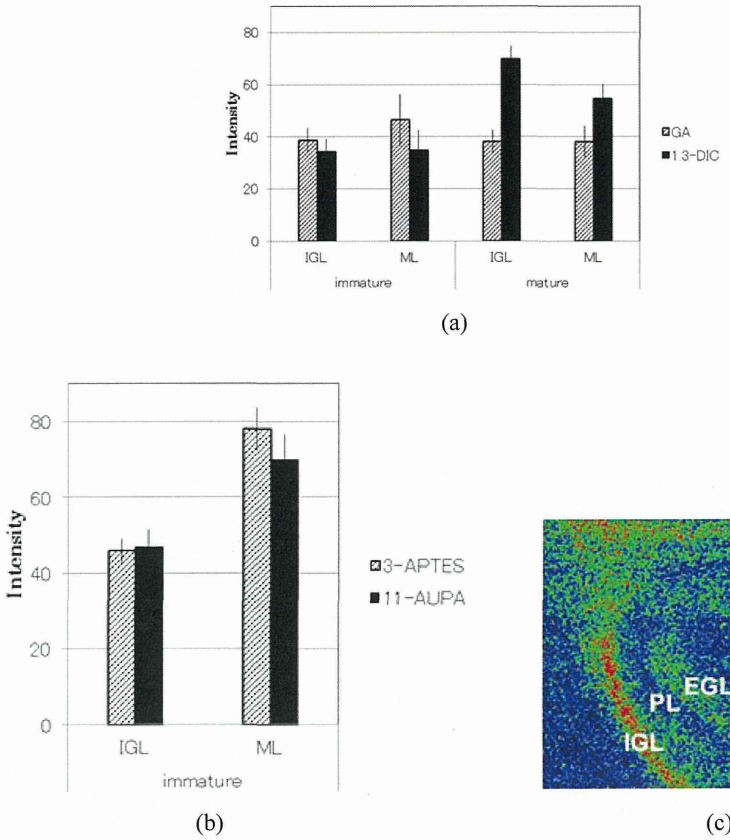


Fig. 4. (Color online) (a) New crosslinker, 1,3-DIC, gave us better contrast images than GA in mature cerebellar observation, while it made little difference from GA in immature organ. (b) The 11-AUPA-GA treatment showed the same result as the APTS-GA treatment in the immature organ. (c) Spontaneous GABA release image in P12 cerebellar slice using 1,3-DIC crosslinking glass device.

surface treatment by 11-AUPA, as a replacement for 3-APTS, was expected to inhibit hydrolysis, but it had low affinity for the glass. We constituted a new glass device with 11-AUPA-coupling enzymes and examined its sensitivity and stability. Figure 4(b) shows that the new device performed with the same sensitivity and stability as the device with APTS.

3.3 *Observation using high-speed CMOS camera*

The fluorescence intensity of NADH is very low and is only a few thousands of the intensity of typical artificial fluorescence. To collect data with sufficient time resolution, a highly sensitive and rapid data transferring camera is required. The time resolution

shown in Fig. 3 is 0.5 s for the 12-bit ORCA CCD, which is too low to detect the synaptic transmitter reaction.

A 16-bit CMOS camera, Flash 4.0, could detect weak light and transfer data in less than a microsecond. Using this camera, transient glutamate release could be detected with a 20 ms time resolution (Fig. 5). In developing the P7 cerebellum, glutamate release was increased in the EGL by applying a glutamate receptor-stimulating agent, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA). Even in the premature P14 cerebellum, the increment in the rate of glutamate release was observed. It was not strong and noiseless, but the AMPA stimulation-induced glutamate release was observed in the ML and IGL where the glutamatergic neurons are distributed.

4. Discussion

The detection of neurotransmitter release gives us important information about developmental conditions and diseases. Parkinson's disease, a degenerative disorder of the central nervous system, is caused by the alteration of the release of neurotransmitters. The detection of the spatial or temporal alteration of the release would require early diagnosis and treatment of Parkinson's disease. In immature or lesioned neuronal organs, transmitters are released and taken up slowly, so the time resolution required is from 0.5 to 1 s. In young-adult stages, the release speed becomes higher than that in the immature stage within 20 ms.

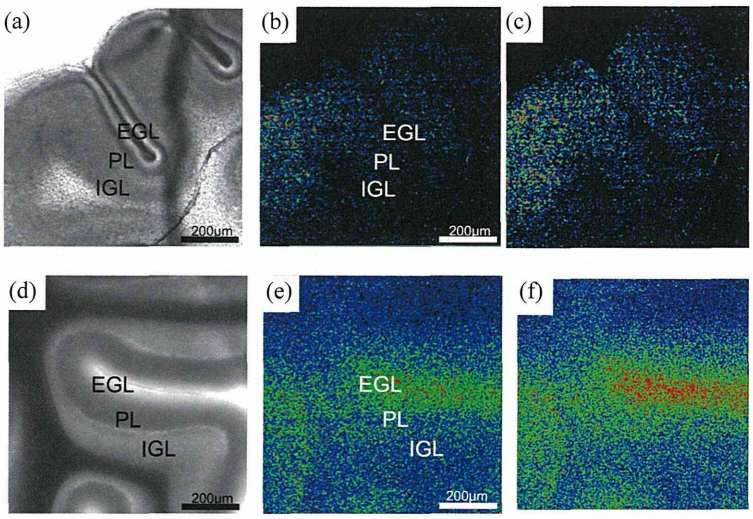


Fig. 5 (Color online) Evoked glutamate release images to AMPA stimulation for 20 ms time resolution using Flash 4.0 CMOS system. (a)–(c) P7 cerebellar slice; (d)–(f) P14 cerebellar slice. (a) and (d) Phase contrast light images. (b) and (e) Fluorescence images before stimulation and (c) and (f) just after AMPA stimulation.

Enzyme-linked assays were applied previously for chemical detection because of their specificity. In spatial observations, however, their fluorescence intensity is too weak to detect. Our enzyme-linked photodevice was developed to detect spatiotemporal neurotransmitter release, and it was improved to observe rapid synaptic release. New crosslinkers could contribute to a more sensitive detection, and the phosphonic surface treatment would expand the range of applications. In order to detect a high-speed transmitter release, both the light accumulation system for weak fluorescence and the close contact between the specimen and the enzyme are required. At present, our photodetection system detects several ms releases from neuronal synapses in the presence of noise, and in the future, it could give us more noiseless observations using an optimal image processing system.

5. Conclusions

The newly developed enzyme-linked photoassay is useful for the visualization of neurotransmitter release in brain slices. In the immature cerebellum, the granule cells release glutamate slowly or rapidly at their stage of neuronal development and synaptogenesis.

Using a fast new system, the rapid measurement of transmitter release in a young-adult cerebellar cortex became possible. Crosslinkers and other device techniques are required for stable observations. We suggest that the photoassay systems have advantages for the observation of synaptic release in several diseases.

Acknowledgements

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References

1 J. Moran and M. Rivera-Gaxiola: J. Neurosci. Res. **33** (1992) 239.
2 P. Varju, Z. Katarova, E. Madarasz and G. Szabo: Cell Tissue Res. **305** (2001) 239.
3 D. Casel, J. Brockhaus and J. W. Deitmer: J. Physiol. **568** (2005) 111.
4 M. Ochi, S. Shiozaki and H. Kase: Neuroscience **127** (2004) 223.
5 T. Morishima, M. Uematsu, T. Furukawa, Y. Yanagawa, A. Fukuda and S. Yoshida: Neurosci. Res. **67** (2010) 347.
6 T. Wang, T. Kumada, T. Morishima, S. Iwata, T. Kaneko, Y. Yanagawa, S. Yoshida and A. Fukuda: Cereb Cortex **24** (2014) 1088.
7 S. Yoshida and N. Hozumi: JNNS **20** (2013) 14.
8 H. Muramoto, Y. Sekino, N. Hozumi, A. Fukuda and S. Yoshida: 42th Neuroscience Meeting (2012) (CD-ROM).
9 B. Innocenti, V. Parpura and P. G. Haydon: J. Neurosci. **20** (2000) 1800.
10 M. Israel and B. Lesbats: J. Neurochem. **67** (1996) 2624.
11 S. Yoshida: Japanese Patent No. 4480952 (2010).
12 L. Blasi, L. Longo, PP. Pompa, L. Manna, G. Ciccarella, G. Vasapollo, R. Cingolani, R. Rinaldi, A. Rizzello, R. Acierno, C. Storelli and M. Maffia: Biosens. Bioelectron. **21** (2005) 30.

13 N. Aissaoui, J. Landoulsi, L. Bergaoui, S. Boujday and J. F. Lambert: Enzyme Microb. Technol. **52** (2013) 336.
14 M. Bariana, M. S. Aw, M. Kurkuri and D. Losic: Int. J. Pharm. **443** (2013) 230.
15 M. Yamada, M. Terao, T. Terashima, T. Fujiyama, Y. Kawaguchi, Y. Nabeshima and M. Hoshino: J. Neurosci. **27** (2007) 10924.
16 M. Ito: The Cerebellum and Neural Control (Raven Press, New York, 1984) Chap. 1.
17 M. L. Fiszman and A. Schousboe: J. Neurosci. Res. **76** (2004) 435.
18 K. Okochi, S. Hirano, K. Maruya, T. Morishima, C. Takayama, A. Fukuda and S. Yoshida: Neurosci. Res. **61** (2008) S114.

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Paper

Visualization of Spatially Distributed Bioactive Molecules using Enzyme-Linked Photo Assay

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In this paper, we propose a new simple device for visualizing bioactive molecules with a fine spatial resolution by using a membrane in which a specific enzyme is immobilized. The layer produces fluorescence after association with a specific substance. The layer, on which a biological tissue is to be mounted, is deposited on a quartz substrate that is used as a light guide to introduce UV light to the layer. Substance release is observed by a CCD camera from the opposite side of the substrate. In order to shorten the experiment time, we had automated the optical device. The paper also describes the reduction of background fluorescence by means of image processing technique. Images were acquired by employing two UV-LEDs with slightly different angle. Image processing was performed to separate background and target fluorescence by means of independent component analysis. Finally the release of GABA(γ-aminobutyric acid) and glutamate from specific layers in rat cerebellum was successfully observed. It is expected that, using this method, both real-time transmitter release and its response to medicine can be observed.

Keywords : bioactive molecules, enzyme-linked photo assay, independent component analysis

1. Introduction

Light guide is composed of a dielectric material that can enclose the light propagation. In addition to being applied to communication, it is useful for sensing as well. In chemical sensing the surface of the light guide has to be coated with some specific chemical that may change its optical property depending on chemical reactions. Such a function can be applied to chemical imaging, if the light guide has a flat surface. This study proposes an application of two-dimensional light guide, of which surface is chemically modified, to biochemical imaging.

Neurotransmitter molecules released from neurons are not only regulators of neuronal transduction but also indicators of neuronal conditions. Glutamate and γ-aminobutyric acid (GABA) are known as typical transmitters in brain cortex that play important roles as stimulator and suppresser, respectively. Lack of balance in the release of glutamate and GABA may lead to autism, epilepsy or Parkinson's disease[1][2].

In order to observe the spatio-temporal release in cerebellar cortex, we have newly proposed the enzyme-linked photo assay system, which is realized even using normal CCD camera, and observed GABA release in developing cerebellar slice using either new or authorized methods[3].

In this paper, we propose a new simple device for this purpose by using a reactive layer in which a specific enzyme is immobilized, and produces fluorescence after association with a specific substance released from mounted slice. This layer is bound a quartz substrate that is used as a light guide for UV light excitation. Fluorescence derived from a substance is observed by a CCD camera from the opposite side of the substrate.

The paper describes the reduction of background fluorescence by means of image processing technique. Finally it will be shown that the release of transmitters from specific layers in rat cerebellum was successfully observed.

2. Specimen Preparation and Photo Excitation System

Imaging of neurotransmitter release was monitored the reaction of oxidoreductases generating reduced nicotinamide adenine dinucleotide (NAD^+) or diphosphonucleotide ($NADP^+$). For glutamate and GABA, we used glutamate dehydrogenase and GABA disassembly enzyme (GABase), respectively.

Enzymes were covalently immobilized on the quartz glass substrate using a silane coupling agent and a crosslink agent. The substrate was as thick as 1 mm. Stoichiometrically generated NADH or NADPH emits 480 nm fluorescence after excitation at 340-365 nm.

Existence of glutamate and GABA lead to fluorescence when co-existing with specific enzyme and co-enzyme. A glass substrate on which specific enzyme is coated is in contact with the biological specimen. A chamber space is created around the specimen. The space is filled with buffer liquid and co-enzyme. On the glass substrate therefore, the specimen is in contact with both enzyme and co-enzyme.

Consequently glutamate or GABA, that is released from the tissue spontaneously by stimulation, makes an oxidation-reduction reaction on the substrate. Although both glutamate and GABA do not produce fluorescence by themselves, NAD(P)H that is created as the result of the above chemical reaction makes fluorescence. As the ratio of glutamate or GABA and NAD(P)H is 1:1, the

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fluorescence can be correlated to the amount of released glutamate or GABA.

In the experiment, rat cerebellum was sliced sagittally at 400 μm thick and incubated in oxygen-aerated HEPES-Na^+ buffer for 40 min. The slice was placed on the quartz glass substrate with both $NADP^+$ and α-ketoglutarate. Figure 1 shows the schematic diagram of the observation system including the device. The enzyme was immobilized covalently on the glass as shown in Fig. 2. Figure 3 shows chemical reactions taking place on the substrate. $NADP^+$ (nicotinamide adenine dinucleotide phosphate) changes into NADPH (reduced nicotinamide adenine dinucleotide phosphate) just as glutamate and GABA degeneration. Synthesized NADPH was illuminated by 360 nm surface UV-LED, and emitted the 480 nm fluorescent light observed by cooled CCD (ORCA ER, Hamamatsu Photonics). The quartz substrate can be recognized as a light guide to illuminate the surface of the substrate.

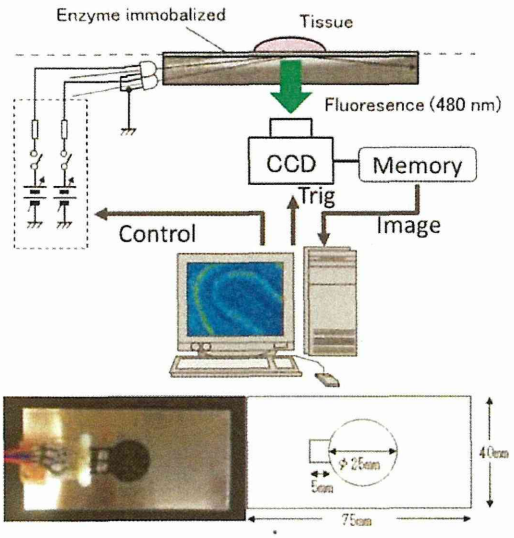


Fig. 1. Schematic diagram of the observation system including the device and its outlook

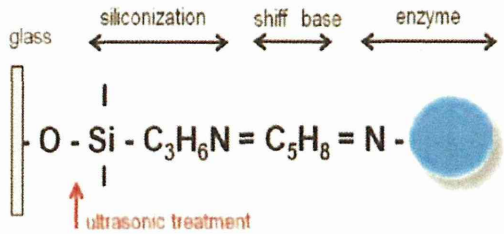


Fig. 2. Immobilized enzyme

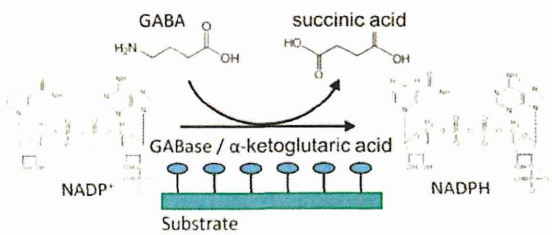


Fig. 3. Chemical reaction on the substrate

3. Image Processing

The fluorescent light detected by the CCD camera is divided into target light and background light. As significant intensity of background light is detected, it is assumed that fluorescence is excited by the light that is refracted on the interface between the substrate and tissue system including the layer. The light, being generated by LEDs and propagates though the substrate, can be decomposed into plane waves with different angles of propagation. Each plane wave transfers across the enzyme layer and comes into the tissue. We assume that both target and background light were predominantly excited by normal light. As the background light significantly damage the quality of the image, it should be reduced as much as possible. Making use of the evanescent light may be a solution, however, it may make the system complicated, and the target light may be not as significant as this case. Therefore we tried to reduce the background by means of a simple image processing.

Assuming that the light is a plane wave and scatter can be neglected, wave propagation and detected fluorescence can be illustrated as Fig. 4. In the figure, fluorescence, attributed to the layer where the enzyme is fixed, is represented as I_0. This is

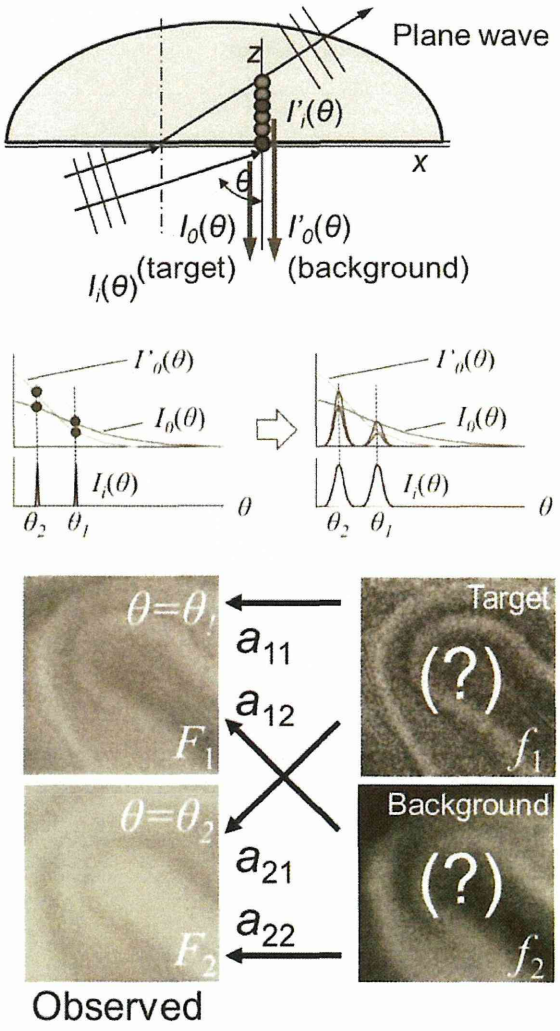


Fig. 4. Fluorescence detected by CCD camera

defined as to be the target. The fluorescence attributed to the tissue is represented as I'_0. This is defined as to be the background. Both I_0 and I'_0 depends on the incident angle θ. The thickness of the quartz plate, which is used as a light guide, is as thick as 1 mm. As it is much thicker than the diameter of normal optical fiber it is relatively easy to introduce two kinds of lights of which angles of center axes are significantly different. In addition, in practice, they depend differently on the incident angle. As the result, the proportion (I_0/I'_0) is not the same along θ. This is true even if the incident angle has distributed.

As the result, the captured fluorescence with different angle of optical axis is composed of target and background fluorescence with different mixture ratios. This can be represented as:

$$\begin{pmatrix} F_1 \\ F_2 \end{pmatrix} = \begin{pmatrix} a_{11} & a_{12} \\ a_{21} & a_{22} \end{pmatrix} \begin{pmatrix} f_1 \\ f_2 \end{pmatrix} \quad \cdots\cdots (1)$$

where $F_1(x,y)$ and $F_2(x,y)$ are captured fluorescence image, $f_1(x,y)$ and $f_2(x,y)$ are spatial distributions of fluorescence as the target and background, a_{11}, a_{12}, a_{21}, a_{22} are constants. Although the image acquisition is sequential, ICA is performed by assuming that two images, $F_1(x,y)$ and $F_2(x,y)$ are acquired with a negligible time lag. Reproduced images $f'_1(x,y)$ and $f'_2(x,y)$ are calculated from F_1 and F_2. As the result of periodical acquisitions of F_1 and F_2, time dependent images of f'_1 and f'_2 are calculated. Eq. (1) can also be described using a matrix expression as:

$$\mathbf{F} = \mathbf{A} \cdot \mathbf{f} \quad \cdots\cdots (2)$$

The target and background fluorescence distribution can be calculated by applying $\mathbf{A}^{-1}$ to $\mathbf{F}$. In practice, only contrast of the image would be enough to recognize the distribution. In such a case $\mathbf{A}^{-1}$ can be represented as:

$$\begin{pmatrix} 1 & \alpha \\ \beta & 1 \end{pmatrix} \quad \cdots\cdots (3)$$

After capturing two images $\mathbf{F}_1$ and $\mathbf{F}_2$ by changing the angle of optical axis, the target and background images can be separated by finding appropriate numbers for α and β. α and β can be tuned manually by monitoring the quality of reproduced image, however, the theory of independent component analysis (ICA) may be powerful for solving such a problem[4].

Stochastic distribution of pixel intensity in images f'_1 and f'_2 are represented as $p(y_{1i})$ and $p(y_{2j})$, where y_{1i} and y_{2j} represent the intensity.

$$\left.\begin{aligned} p(y_1) &\equiv \{p(y_{11}), \cdots, p(y_{1i}), \cdots p(y_{1n})\} \\ p(y_2) &\equiv \{p(y_{21}), \cdots, p(y_{2j}), \cdots p(y_{2n})\} \end{aligned}\right\} \quad \cdots\cdots (4)$$

$p(y_{1i}, y_{2j})$ represents the probability that the intensity of a pixel in image f'_1 is y_{1i} and that of the corresponding point in image f'_2 is y_{2j}. In other words $p(y_1)$ and $p(y_2)$ are probabilities that cases y_1 and y_2 take place, respectively, and $p(y_1, y_2)$ is the probability that cases y_1 and y_2 takes place simultaneously. Variables y_1 and y_2 are considered to be independent when

$$p(y_1, y_2) = p(y_1)p(y_2) \quad \cdots\cdots (5)$$

is established. Kullback-Leibler(K-L) parameter is often employed to indicate the independency of variables:

$$KL \equiv \sum_{i,j} p(y_{1i}, y_{2j}) \log \frac{p(y_{1i}, y_{2j})}{p(y_{1i})p(y_{2j})} \quad \cdots\cdots (6)$$

The K-L parameter is zero when two sets of variables y_1 and y_2 are completely independent together. In practice, α and β in Eq. (3), which determine the probabilities $p(y_1)$, $p(y_2)$ and $p(y_1, y_2)$, can be tuned so that the K-L parameter indicates the minimum.

The process of ICA is illustrated in Fig. 5. The equation described in the form of matrix indicates that two images, F_1 and F_2, derive from linear combination of unknown original images f_1 and f_2. If an appropriate inverse matrix can be found then the original images can be reproduced. However as the matrix to describe the linear combination is unknown as well, ICA algorithm is applied to find the most appropriate matrix (as the inverse matrix). In the

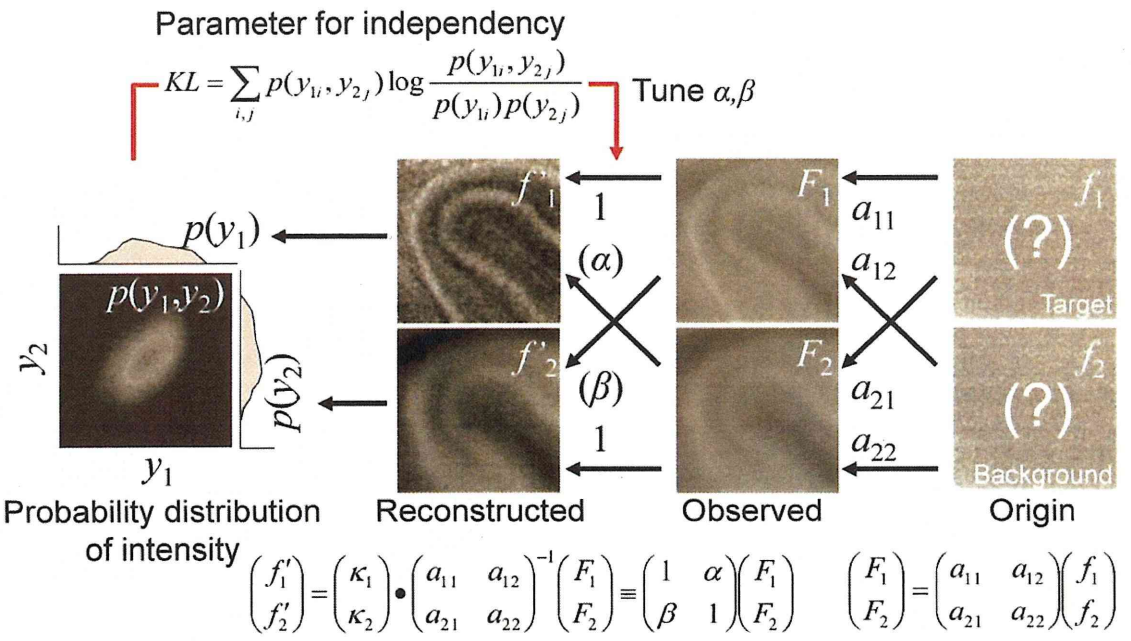


Fig. 5. Illustration for image processing based on independent component analysis

ICA process K-L parameter is calculated in order to evaluate the probabilistic independency of images f'_1 and f'_2. It can be considered that in the reproduction algorithm the core process is the calculation of the K-L parameter. In this preliminary study K-L parameter is successively calculated by manually changing the inverse matrix, and images are assumed to be reproduced when the K-L parameter indicates the minimum.

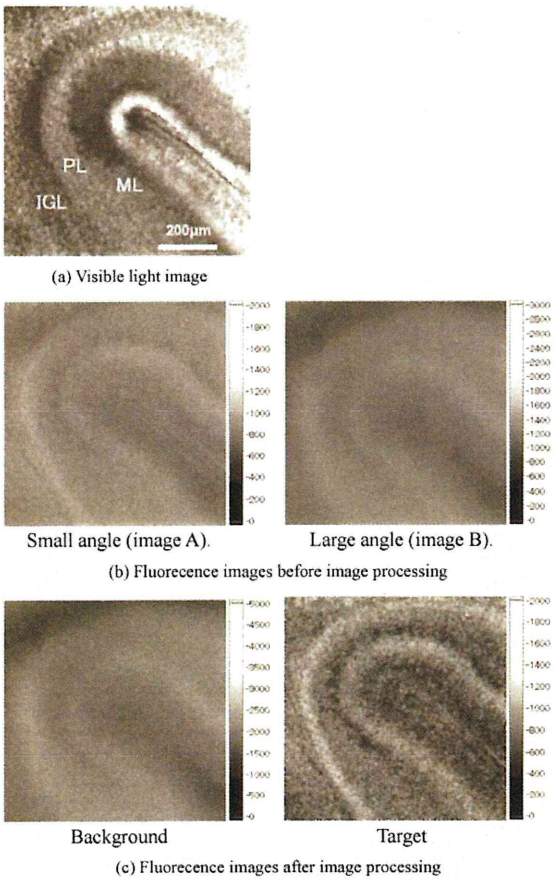
4. Results and Discussion

4.1 Image Processing using the ICA Figure 6 (a) shows visible light image of the cerebellum with postnatal 21 days. In developing cerebellum, granule cells, small input neurons, proliferate and migrate down from the external granular layer (EGL) to the internal granular layer (IGL). As the development proceeds, EGL turns into molecular layer (ML) whereas IGL remains. Purkinje cells, big output neurons, develop their dendrites and associate neuronal connections between granule cells and other interneurons. Neuronal circuit layer forms the ML. As the cerebellum shown in Fig. 6 (a) is mature, ML, PL, IGL are clearly visible. Note that ML is on the outer side of the cerebellum, and a wrinkle surrounded by the ML is seen in Fig. 6 (a).

As for fluorescence observation, three different images were acquired. Two were with different inclination of the excitation light source, and one was with no excitation light. Each of the two images with excitation light was subtracted with the image with no excitation light, in order to reduce the background light from the outside. These two images after the subtraction were defined as images A and B.

Figure 6 (b) shows these images for a rat cerebellum. Both images are very unclear, because of the background fluorescence. Figure 6 (c) shows the result of image processing. It is clearly shown in the image entitled as "target" that the fluorescence intensity is high in two layers, whereas that entitled as "background" is not clear. By morphological inspection these layers are recognized as ML and IGL. These layers are known that GABAergic neurons distribute in mature cerebellum. Studies using HPLC and electrophysiological method have shown that GABA is released from the postnatal cerebellar cortex even before synaptogenesis, and that GABA receptors act on the developing cerebellar Purkinje cells[4][5]. However, dynamic GABA release could not be observed unless the enzyme-linked photo assay is used. In addition, because cytoplasmic autofluorescence becomes noisy background light, it is useful that the image processing system extracted the image of GABA release from the autofluorescence-contained image. Using this method, both real-time transmitter release and its response to medicine can be observed.

4.2 Transition after Chemical Stimulation In relatively developed cerebellum, cells distributed in the ML and IGL are only the neurons of glutamate release, so that both layers showed fluorescent activities. Figure 7 indicates release distribution of glutamate in comparison with normal optical image illuminated with visible light. The fluorescent image, indicating glutamate release, is after the ICA processing. Figure 7 (c) indicates the regions of interest for analysis. Regions highlighted as ML and IGL have relatively strong intensity in fluorescence. They have a contrast to the region highlighted as PL. Release from white matter (WM), which is mostly composed of fatty materials, is much less significant.

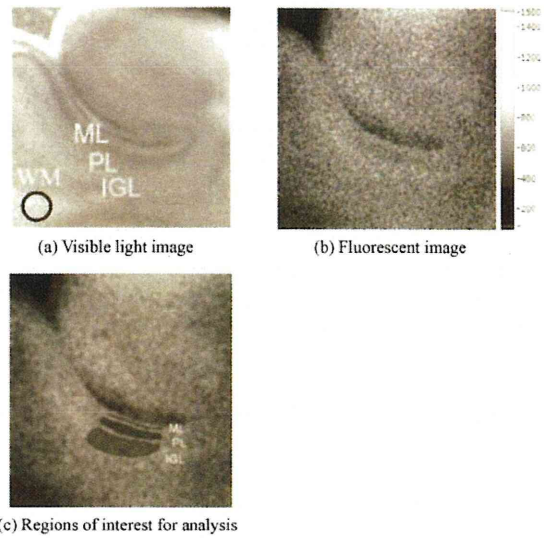


(a) Visible light image

(b) Fluorecence images before image processing

(c) Fluorecence images after image processing

Fig. 6. Cross sectional mages of cerebellar cortex: (a) Visible light image, (b) original fluorescent images with different angle of optical axes, and (c) fluorescent images after the image processing. Scales are indicated in arbitrary unit. Specimen: rat cerebellum (postnatal 21 days), target: GABA



(a) Visible light image

(b) Fluorescent image

(c) Regions of interest for analysis

Fig. 7. Cerebellum with postnatal 7 days observed with visible light and fluorescent light indicating glutamate release. 0.9 mm × 0.9 mm. Gray scale is arbitrary. ML: molecular layer, PL: Purkinje layer, IGL: internal granular layer, WM: white matter. Specimen: rat cerebellum (postnatal 7 days)

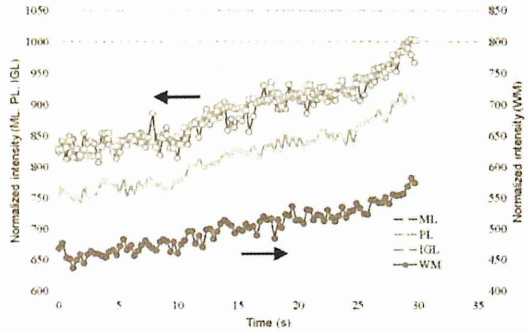


Fig. 8. Transition in fluorescence intensity in each layer (normalized by the intensity of ML 30 s after stimulation that is indicated as 1000). Specimen: rat cerebellum (postnatal 7 days), target: glutaminate

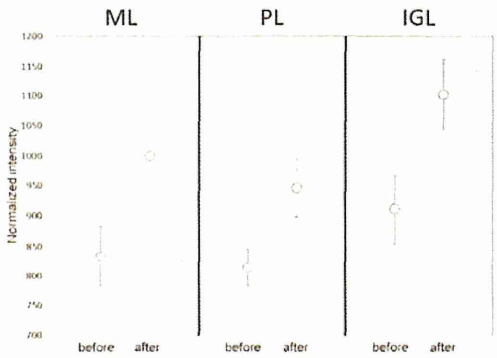


Fig. 9. Change in fluorescence intensity before and after AMPA stimulation (normalized by the intensity of ML 30 s after stimulation that is indicated as 1000). Specimen: rat cerebellum (postnatal 7 days), target: glutaminate

Our system can visualize both spontaneous and responsive transmitter release with about 0.2 s time resolution. Figure 8 shows the transition of glutamate release in response to 100 µmol/ℓ (S)-alpha-Amino-3-hydroxy- 5 methylisoxazole-4-propionic acid (AMPA) application in cerebellar slices. All values are normalized by the intensity of ML 30 s after stimulation that is indicated as 1000. Fluorescence, as indication glutamate release, was intense in both the IGL and ML, whereas the PL was indicated with lower intensity. As shown in Fig. 8, a clear increase in fluorescence was observed after stimulation. Transition in fluorescence was similar for ML and IGL, suggesting that these layers are activated. However PL, which was not expected to release glutamate, showed fluorescence as well although it was less intense than ML and IGL. As this specimen was taken from relatively young rat (postnatal 7 days), the cerebellar development was not totally completed, and the layers were not separated enough. It is hence considered that diffusion from ML and IGL to PL would take place, leading to an increase in fluorescence in this layer. The increase in fluorescence in WM suggests that glutamate might have been diffused into WM as well, although the absolute value was much lower than ML and IGL.

Figure 9 compares the fluorescence in each layer before and after stimulation. Four different specimens were used for the observation, in order to confirm reproducibility. It is clear that the AMPA stimulation brought a significant glutamate release from ML and IGL, although the increase is also seen with PL.

5. Conclusions

A new method for visualization of spatially distributed bioactive molecules using enzyme-linked photo assay has been proposed. It is based on fluorescent reaction assisted by an enzyme immobilized on the substrate, however, background fluorescence disturbs the observation. In order to reduce the background fluorescence, two images were acquired by changing the optical axis of UV illumination. Image processing based on independent component analysis made the target image clear. Observation of rat cerebellum was successfully performed and GABA and glutamate release from two specific layers was clearly indicated.

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References

(1) N. C. Danbolt : "Glutamate Uptake", *Neurobiology*, Vol.65, pp.1-105 (2001)
(2) N. Kasai, Y. Jimbo, O. Niwa, T. Matsue, and K. Torimitsu : "Rea-time Multisite Observation of Glutamate Release in Rat Hippocampal Slices", *Neurosdence Lett.*, Vol.304, pp.112-116 (2001)
(3) T. Morishima, M. Uematsu, T. Furukawa, Y. Yanagawa, A. Fukuda, and S. Yoshida : "GABA Imaging in Brain Slices Using Enzyme-linked Photo Analysis", *Neurosci. Res.*, Vol.67, pp.347-353 (2001)
(4) V. Calhoun, G. Pearlson, and T. Adali : "Independent Component Analysis to fMRI Data, A Generative Model for Validating Results", VLSI Signal Processing, Vol.37, pp.281-291 (2004)
(5) F. F. Trigo, M. Chat, and A. Marty : "Enhancement of GABA Release through Endogenous Activation of Axonal GABA(A) Receptors in Juvenile Cerebellum", *J. Neurosci.*, Vol.27, No.46, pp.12452-63 (2007)
(6) K. Obata : "Excitatory and Trophic Action of GABA and Related Substances in Newborn Mice and Organotypic Cerebellar Culture", *Dev Neurosci*, Vol.19, No.1, pp.117-119 (1997)

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