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Technical Note

Method for single-cell microarray analysis and application to gene-expression profiling of GABAergic neuron progenitors

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Abstract

The mammalian central nervous system is populated with various types of neurons and glia. To investigate the functions and development of individual cells requires gene-expression analysis at the single-cell level. Here, we developed a microarray-based method for the gene-expression profiling of single cells and tested it for GABAergic neuron progenitors. Single GABAergic neuron progenitors were collected from the neocortex of GAD67-GFP knock-in mice by dissociation followed by the aspiration of GFP-positive cells. Complementary DNA from the single cells was amplified by a method in which Super SMART PCR and T7 RNA polymerase amplification were combined at a optimized condition. The cRNA was subjected to microarray hybridization and analysis, which yielded reliable and reproducible results.
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Keywords: GABA neuron; GFP; Microarray; Profiling; Single cell

1. Introduction

The mammalian central nervous system consists of various types of neurons and glia. The variation is derived from differences in gene expression from cell to cell. For instance, GABAergic neurons in the neocortex are classified into many subtypes by characteristic subtype markers as well as by their morphological and electrophysiological properties. To study the process of subtype differentiation, some subtype markers, such as calretinin and somatostatin, are quite useful, because they can be detected by immunohistochemistry from an early embryonic stage. However, the immunoreactivity of another marker, parvalbumin, does not appear in the mouse neocortex until after birth. In addition, immunohistochemistry only allows us to examine a few different markers co-localized in the same cell. RT-PCR may detect a greater number of expressed genes in a single cell, but it can still detect fewer than 10. A more detailed study of developing neural cells requires a more efficient method for detecting the gene expression in single cells. Therefore, we sought to develop a method for microarray analysis at the single-cell level.

To perform microarray analyses of single cells, a technique for amplifying the mRNA signals from a single cell is required. Pioneering studies of single-cell microarray analysis used a universal PCR amplification procedure (Iscove et al., 2002; Tietjen et al., 2003) or two rounds of T7 amplification (Kamme et al., 2003). These experiments led to the successful cloning of unique genes from the nasal epithelium and from hippocampal neurons. However, it was not clear whether the universal PCR amplification or two rounds of T7 amplification accurately expanded low-copy-number mRNA. Moreover, these previous methods required skill in molecular biology techniques and a profound knowledge of enzymology. Thus, here we report a simplified method for single-cell microarray analysis. We also

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tested our method by applying it to single GABAergic neuron progenitor cells.

2. Materials and methods

2.1. Experimental animals

Mice were housed and treated in strict accordance with the Rules for Animal Care and Use for Research and Education of Kumamoto University. The generation and characteristics of the GAD67 (GFP knock-in) knock-out mice have been described previously (Tamamaki et al., 2003). All the embryos used in the present study were obtained by time-mating C57BL/6 wild-type mice with heterozygous GAD67-GFP knock-in mice. The morning after the mating was defined as embryonic day 0 (E0). The day of birth was E19 (postnatal day 0: P0).

2.2. Recovery of single cells

Dissociated cells were prepared from a piece of tissue containing the intermediate zone (IZ), subventricular zone (SVZ), and VZ layers of the GAD67-GFP knock-in mouse neocortex at E18-P0 (Fig. 1A). The tissue was dissected and subjected to 0.5% trypsin and 0.05% DNaseI (Sigma) digestion at 37 °C for 5 min. The digested tissue was then dissociated into single cells by mild pipetting. To remove tissue debris, the dissociated cells were filtered through a 40-μm cell strainer (Falcon, USA). The cells were diluted to an appropriate concentration with medium (10% fetal bovine serum in DMEM; Invitrogen, USA) and pooled in a 6-cm plastic dish. The dissociated cells were observed under a fluorescence microscope (CKX41, Olympus, Japan), and GFP-positive cells were identified in photographs taken with a digital CCD camera (VB-6010, Keyence, Japan) (Fig. 1B and C). Single GFP-positive cells were aspirated by a Quixell Automated Cell Selection and Transfer System (Stoelting, USA) attached to the microscope. Each single cell was then ejected into a 5-μl droplet of DNase- and RNase-free water containing 0.5 μl (40 U/μl) of RNase inhibitor (Ambion, USA) under light microscopic observation.

2.3. Single-cell RT-PCR analysis

The solution containing an ejected cell was transferred to a PCR tube, and 6 μl of DNase- and RNase-free water, 1 μl of oligo(dT) primer (0.5 μg/μl) (Invitrogen, USA), and 1 μl of mixed dNTPs (10 mM each) (Invitrogen, USA), were added. The mixture was heated to 65 °C for 10 min and then cooled on ice for 1 min. Single-strand cDNA was synthesized by adding 4 μl of 5× first-strand buffer, 2 μl of dithiothreitol (DTT, 0.1 M), 0.5 μl of RNase inhibitor (40 U/μl), and 0.5 μl of Superscript III reverse transcriptase (200 U/μl; Invitrogen, USA). The mixture was incubated at 50 °C for 60 min. The mixture was heated to 70 °C for 15 min to terminate the reaction, and then cooled. Fragments of the genes of interest were amplified by nested PCR using the cDNA from a single GFP-positive cell as the template. The first PCR was performed using 2 μl of the cDNA solution, 5 μl of 10× PCR buffer, 5 μl of 2 mM dNTP mix, 0.4 μl of each primer (50 μM) (described below), 1 μl of HotStar Taq polymerase (Qiagen, USA), and 37.2 μl of water. The PCR profile was 10 min at 95 °C; 40 cycles of 30 s at 95 °C, 30 s at 57 °C, 1 min at 72 °C; 10 min at 72 °C. A second nested PCR was performed using 0.1 μl of the first PCR product, 2.5 μl of 10× PCR buffer, 2.5 μl of 2 mM dNTP mix, 0.2 μl of each primer (50 μM) (described below), 0.25 μl of Blend Taq polymerase (Toyobo, Japan), and 19.45 μl of water. The reaction profile was 2 min at 94 °C; 35 cycles of 20 s at 94 °C, 20 s at 57 °C, 30 s at 72 °C; 7 min at 72 °C. The second-round PCR product was separated by electrophoresis in a 1.0% agarose gel and stained with ethidium bromide. The PCR primers were designed based on the GenBank sequence for each target gene.

We detected β-actin and GAD67 mRNA by single-cell RT-PCR as a housekeeping gene and a GABAergic-neuron marker, respectively. The upper and lower primers for β-actin and GAD67 were located in different exons to rule out

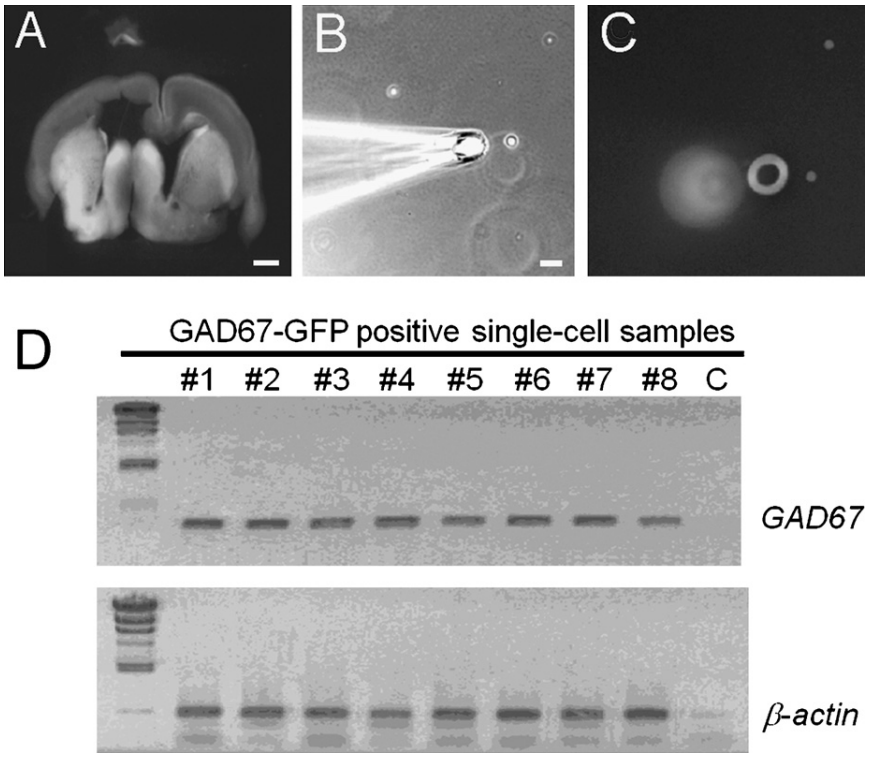

Fig. 1. Expression of GAD67 mRNA in GFP-positive cells revealed by single-cell RT-PCR. (A) Fluorescence image of a dissected piece of brain tissue containing the IZ, SVZ, and VZ obtained from a GAD67-GFP knock-in mouse neocortex at P0, (B) dissociated cells in a bright field image, (C) GFP-positive single cells in a fluorescent image and (D) PCR products of β-actin and GAD67 obtained by single-cell RT-PCR. The second-round PCR products are shown. β-actin and GAD67 mRNA were detected in all samples (53/53). Calibration bars in (A) and (B) are 1 mm and 10 μm, respectively. Bar in (B) applies to (C).

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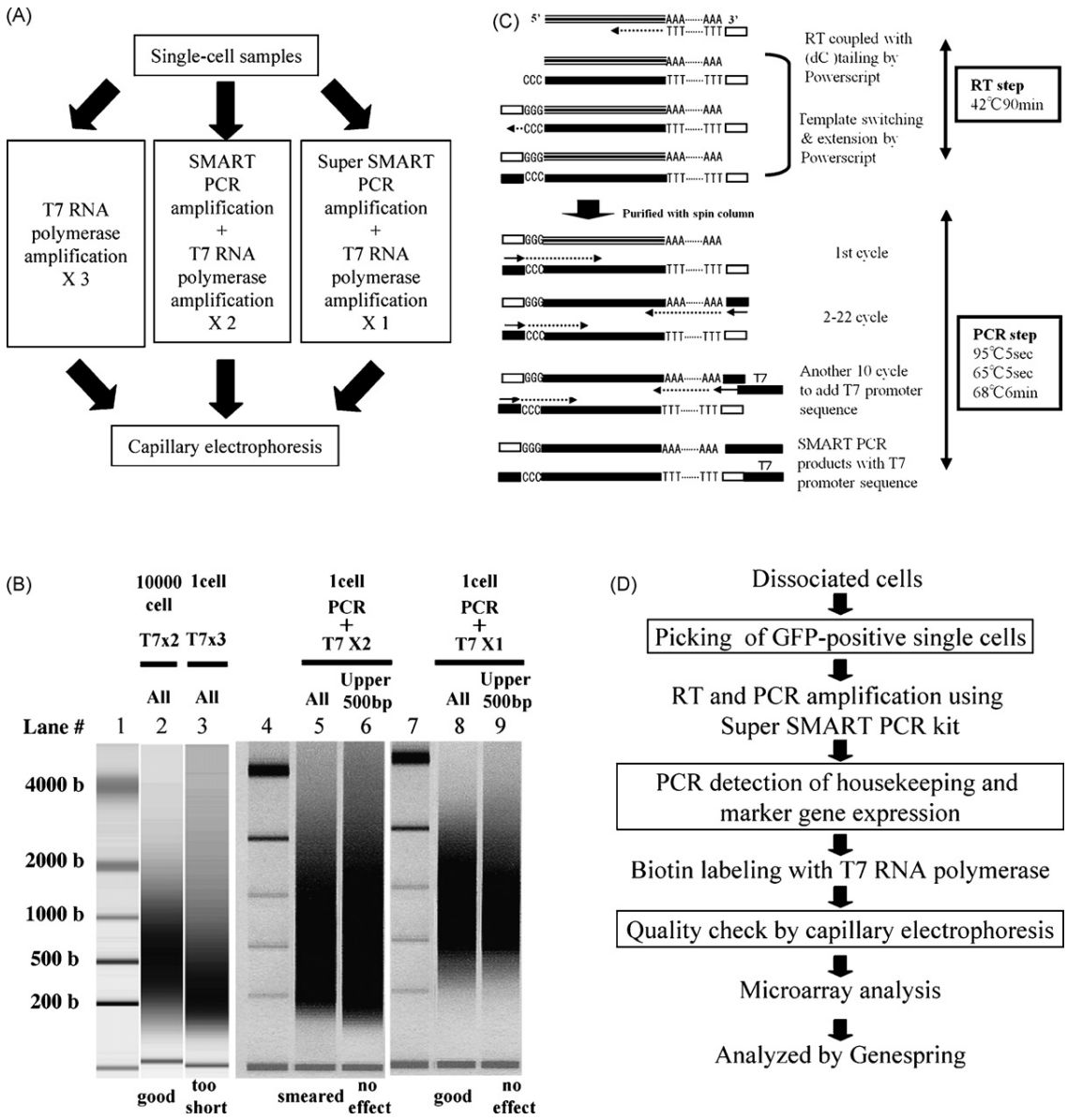

Fig. 2. Experimental design for single-cell microarray analysis. (A) Schematic diagram showing three different procedures for single-cell RNA amplification. In the first trial, single-cell samples were expanded with three rounds of T7 amplification. In the second, single-cell samples were processed with SMART mRNA amplification kit followed by PCR and two rounds of T7 RNA amplification. In the third, single-cell samples were processed with Super-SMART PCR amplification and one round of T7 RNA amplification, (B) capillary-electropherograms of cRNA produced by the three different procedures. Lanes 1, 4, and 7 are molecular-size markers (RNA 6000 nano-Marker; Ambion, USA). Control cRNA was produced from the total RNA obtained from 10^4 GFP-positive cells by an authentic procedure (two rounds of T7 amplification) and separated by electrophoresis (lane 2). Three rounds of T7 amplification yielded a sufficient amount of cRNA from a single cell for microarray analysis but reduced the length of the cRNAs drastically (compare to the control product) (lane 3). The procedure using SMART mRNA amplification kit followed by PCR and two rounds of T7 amplification yielded a sufficient amount of cRNA from a single cell for microarray analysis, with lengths ranging from 200 to 4000 bases (lane 5). However, the peak of cRNA length was broad, and much was in the short range. Although we tried to reduce the amount of short cRNA by gel-separation of the PCR product, there was no improvement in the final product. The procedure using Super-SMART PCR amplification and one round of T7 amplification yielded a sufficient amount of cRNA from a single cell for microarray analysis, with lengths ranging from 500 to 2000 bases (lane 8). This cRNA length was good for microarray analysis, and there was no need to include the step of gel-separation of the PCR product (lane 9). The comment under each lane refers to the cRNA length, (C) scheme of the cDNA amplification procedure using the Super-SMART PCR cDNA synthesis kit. Triple lines indicate mRNA, black lines indicate DNA, white boxes indicate primers, T7 indicates the promoter sequence for T7 RNA polymerase promoter, arrows indicate universal primer, and arrows with a broken line indicate DNA synthesis and (D) flowchart for single-cell microarray analysis. Boxes indicate the check points for the experimental products.

the amplification of genomic DNA. To check for contamination from the bathing solution of the dissociated cells, a droplet of bathing solution was processed similarly for every eight samples. The primers for β-actin were 5′-GCCAACCGTGAAAAGATGAC-3′ and 5′-GCACTGTGTTGGCATAGAGG-3′ in the first PCR, and 5′-GGCTGTGCTGTCCCTGTATG-3′ and 5′-CAAGAAGGAAGGCTGGAAAA-3′ in the second nested PCR. The expected product sizes were 556 and 391 bp, respectively (NM_007393). The primers for GAD67 were 5′-GCACCCGTGTTTGTTCTCAT-3′ and 5′-TGTGAGGGTTCCAGGTGACT-3′ in the first PCR, 5′-TCCCAGAAGTGAAGACAAAAA-3′ and 5′-TCTATGCCGCTGAGTTTGTG-3′ in the second nested

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PCR. The expected product sizes were 559 and 380 bp, respectively (GenBank accession NM_008077).

2.4. Fluorescence-activated cell sorting and RNA purification

GAD67-GFP-positive cells were obtained as described above and sorted by FACS Vantage SE (BD, USA). The sorted cells (8.6×10^5 GFP-cells) were suspended in Trizol (Invitrogen, USA). The RNA was denatured immediately, and the total RNA (1 μg) was purified according to the manufacturer's protocol (Invitrogen).

2.5. Super SMART cDNA amplification

The total RNA from a single cell was reverse-transcribed into cDNA using a Super SMART PCR cDNA Synthesis Kit (Takara Clontech, USA). A single cell was ejected into a 5-μl droplet of DNase- and RNase-free water containing 0.5 μl of RNase inhibitor (40 unit/μl) (Ambion, USA). To this was added 45 μl of DNase- and RNase-free water, 7 μl of 12 μM 3′SMART CDS primer IIA, and 7 μl of 12 μM template switching SMART IIA oligonucleotide. The sequence of the 3′SMART CDS Primer IIA was 5′-AAGCAGTGGTATCAACGCAGAG-TACT$_{(30)}$VN-3′ (V = G, C, or A) and that of the SMART IIA Oligonucleotide was 5′-AAGCAGTGGTATCAACGCAGAGTACGCGGG-3′. The mixture was heated to 65 °C for 3 min and then incubated at 42 °C for 2 min. The following reagents were then added: 20 μl of 5× first-strand buffer, 2 μl of DTT (100 mM), 10 μl of dNTP (10 mM), 4.5 μl of RNase Inhibitor (20 U/μl), and 5 μl of Powerscript Reverse Transcriptase (Takara-Clontech, USA). The cDNA synthesizing reaction was performed at 42 °C for 90 min, and terminated by adding 2 μl of EDTA (0.5 M). To purify the cDNA obtained from a single cell, we used NucleoSpin Extract II (Macherey-Nagel, USA) according to the manufacturer's instructions. We then recovered the cDNA in 85 μl of DNase- and RNase-free water. The whole cDNA derived from a single cell was used as a template for the subsequent PCR amplification. Ten microliters of 10× Advantage2 PCR buffer, 2 μl of dNTP (10 mM), 2 μl of 12 μM Primer IIA, and 2 μl of 50× Advantage2 Polymerase Mix (Takara-Clontech, USA) were added to the PCR solution. The sequence of Primer IIA was 5′-AAGCAGTGGTATCAACGCAGAGT-3′. The reaction profile was 1 min at 95 °C; 22 cycles of 5 s at 95 °C, 5 s at 65 °C, 6 min at 68 °C. We then added 2 μl of 12 μM modified Primer IIA and 2 μl of 50× Advantage2 Polymerase Mix again, and performed another thermal cycling for 1 min at 95 °C; 10 cycles of 5 s at 95 °C, 5 s at 65 °C, 6 min at 68 °C; 6 min at 68 °C. The sequence of modified Primer IIA with the T7 promoter was: 5′-GGCCAGTGAATTGTAATACGACTCACTATAGGGAGGCGGAAG-CAGTGGTATCAACGCAGAGTACTTT-3′.

A portion of the final PCR products from the single cells (1 μl) was used as a template for PCR to detect β-actin, βIII-tubulin, and GAD67 cDNA and to assess the quality of the final PCR product. The conditions for nested PCR were the same as for the single-cell RT-PCR. The primers for β-actin were the same as those used in the single-cell RT-PCR. The primers for βIII-tubulin were 5′-AGTGCGGCAACCAGATAGGG-3′ and 5′-TGCGGAAGCAGATGTCGTAG-3′ in the first PCR, and 5′-TCTGGCGCCTTTGGACACCT-3′ and 5′-GCGTTGTAGGGCTCCACCAC-3′ in the second nested PCR. The primers were set to bind within 600 bp from the poly-A site but in different exons. The expected product sizes were 609 and 323 bp, respectively (GenBank accession number NM_023279). The primers for GAD67 were 5′-GGGGAAATAGTAG-CAGTCTT-3′ and 5′-GCTAAGCGAGTCACAGAGAT-3′ in the first PCR, and 5′-ATTGCCATTGTCTATCTTTG-3′ and 5′-AAGCAAGATTTCCATAACAT-3′ in the second nested PCR. The primers were set to bind within 600 bp from the poly-A site within the same exon. The expected product sizes were 556 and 340 bp (GenBank accession number NM_008077).

2.6. Microarray hybridization and analysis

The final PCR product was purified by NucleoSpin Extract II (Macherey-Nagel, USA). The purified extract and a set of control markers (*E. coli* labeling markers, Affymetrix, USA) were subjected to one cycle of the T7 RNA polymerase reaction to produce biotinylated cRNA (Affymetrix, USA) (Fig. 2A). We then assessed the length of the biotinylated cRNA by capillary electrophoresis (RNA 6000 nano-labo, Agilent, USA) (Fig. 2B). The cRNA was subsequently fragmented under alkaline conditions and hybridized to a GeneChip Mouse Genome MOE430A array (22,626 genes on one chip; Affymetrix, USA), according to the manufacturer's protocol. The microarray image data were processed with a GeneChip Scanner 3000 (Affymetrix, USA) to generate CEL data. The CEL data were then subjected to analysis with the GeneChip Operating Software (GCOS; Affymetrix, USA). We compared data from each chip using GCOS in the default setting ("user defined" and "scale factor: 1" in "expression analysis setting"). Absolute calls (present, absent, and marginal) were calculated with GCOS in the default setting. Further data analysis was carried out using the GeneSpring software (Agilent, USA). Raw signal data were imported from GCOS in the default setting. We performed per-chip normalization (normalized to the 50th percentile). More information about the protocol for the single-cell microarray analysis is available at: http://srv02.medic.kumamoto-u.ac.jp/dept/morneuro/microarray.html.

2.7. Quantitative real-ltime PCR analysis

Quantitative real-time PCR analysis was carried out using Power SYBR-Green PCR master mix (ABI, USA) according to the manufacturer's protocols. Final Super SMART PCR product from a single cell (0.25 μl), 10 μl of 2× Power SYBR Green PCR master mix, 0.4 μl of each primer (10 μM) (described below), and 8.95 μl of water were added to a PCR tube. The PCR profile was: 10 min at 95 °C; 50 cycles of 15 s at 95 °C, 1 min at 60 °C using ABI7500 real-time PCR system. At the end of the program a dissociation curve analysis was done. Control solutions were made by diluting pCRII cloning plasmid (Invitrogen, USA). Data were analyzed by SDS7500 software (ABI, USA). Primers for reelin were 5′-CAACACCAGCAAAGTATGATG-3′ and 5′-TTTAAG-CATGGGCTTTTAGC-3′. The expected product size was 97 bp (NM_011261). Primers for β-actin were 5′-GACAGGATGCAGAAGGA-3′ and 5′-TGATCCACATCTGCTGGAA-3′. The expected product size was 142 bp (NM_007393). Primers for GAD67 were 5′-ACCTAAGG-CAATGTTTCTGA-3′ and 5′-TCTTCTTGCACACCCTAAAT-3′. The expected product size was 102 bp (NM_008077).

2.8. Immunocytochemistry

Dissociated cells were prepared as described in the above. The dissociated cells were spun for 3 min at 1000 × *g*, and the pellet was resuspended in DMEM (Invitrogen, USA). Resuspended cells were spread onto MAS-coated slides (Matsunami, Japan), air dried, fixed in 4% paraformaldehyde in phosphate buffer salin (pH 7.4), and then processed for double-immunofluorescence staining of GFP, ARX, DLX2 and Reelin. The primary antibodies used were as follows: rabbit anti-GFP (1:500; Tamamaki et al., 2003) and rat anti-GFP (1:500; Nacalai, Japan), rabbit anti-ARX (1:1000; a gift from Dr. Kitamura), mouse anti-reelin (1:2000; Calbiochem, USA), guinea-pig anti-Dlx2 (1:3000; a gift from Dr. Yoshikawa). Secondary antibodies were Goat anti-rabbit IgG conjugated with Alexa 488 (1:500; Molecular Probes, USA), Goat anti-rat IgG conjugated with Alexa 488(1:500; Molecular Probes, USA), donkey anti-gunia-pig IgG conjugated with Cy3 (1:200; Jackson Immunoresearch, USA), donkey anti-mouse IgG biotinylated (1:500; Chemicon, USA), donkey anti-rabbit IgG conjugated with Alexa 594(1:500; Molecular Probes, USA). Tertiary solutions were Alexa594-conjugated streptoavidin (1:500; Molecular Probes, USA).

3. Results

3.1. Single-cell RT-PCR

In the GAD67-GFP knock-in mouse neocortex at E18-P0, GFP-positive cells were present in the subventricular zone (SVZ) and the intermediate zone (IZ), as well as in the cortical plate (CP) (Fig. 1A). We examined the expression of GABA synthetase, GAD67, a marker for GABAergic neurons, in the GFP-positive cells by single-cell RT-PCR (Fig. 1). Dissociated cells were obtained from the deeper half of the neocortex

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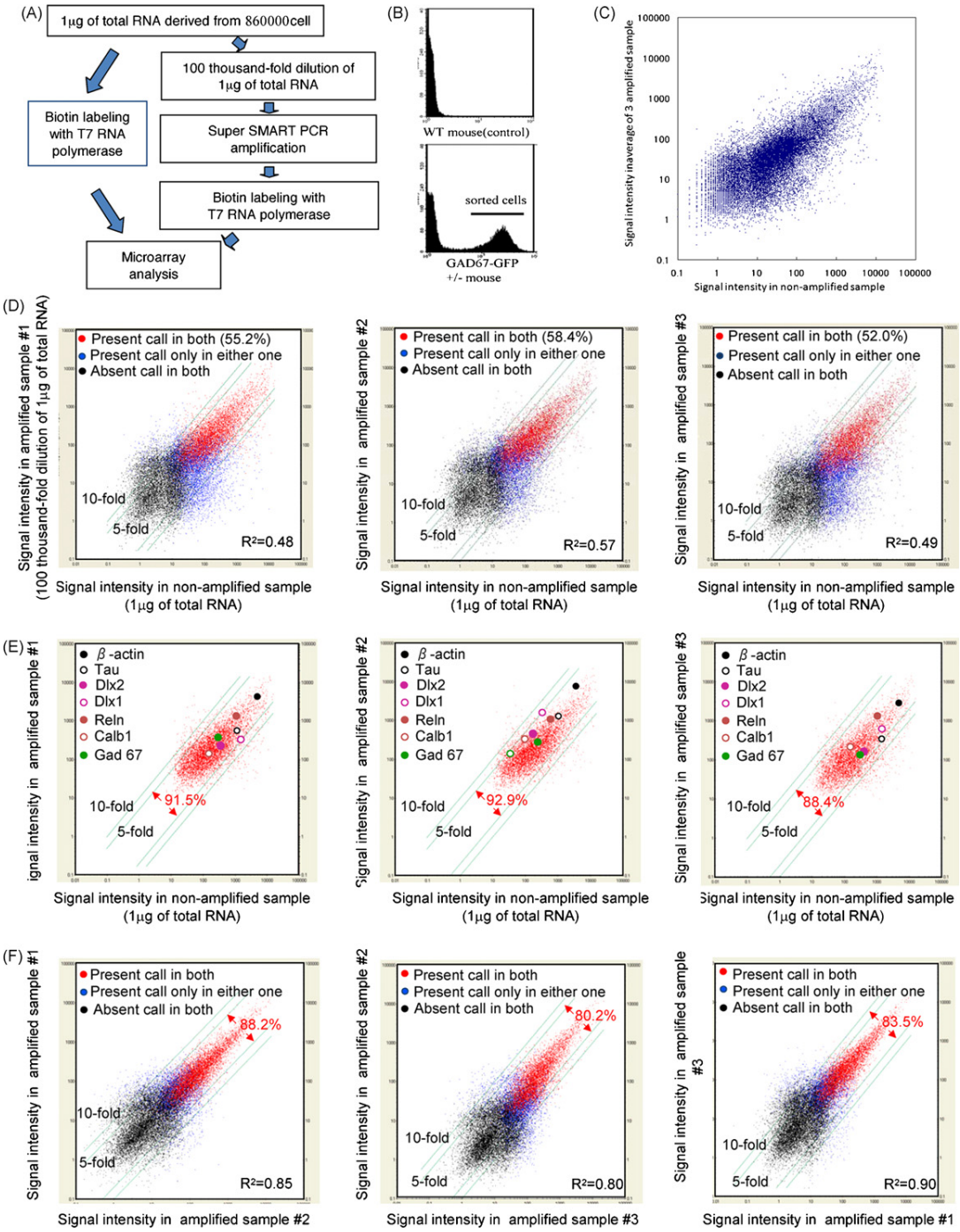

Fig. 3. Examination of cDNA amplification. (A) Experimental design for comparing non-amplified and amplified samples. The final products from the two types of preparation were hybridized to microarrays. The gene-expression profile of the original sample was then compared with that of samples prepared after dilution and amplification, (B) histograms of the numbers of cells obtained from the brain tissue of wild-type and GAD67-GFP knock-in heterozygote mice at E18 as a function of fluorescence intensity. Black bar indicates the collection gate for GAD67-GFP-positive cells. One microgram of total RNA was obtained from 8.6×10^5 GFP-positive cells, (C) scatter plots of signal intensity obtained from a non-amplified sample and those obtained from average of three diluted and amplified samples, (D) scatter plots of signal intensity obtained from a non-amplified sample and from three diluted and amplified samples, respectively. The 5-fold and 10-fold difference lines are indicated by green lines. Red points indicate genes having a present call on both microarrays. Blue points indicate genes having an absent call on one of the microarrays. Black points indicate genes having an absent call on both of the microarrays, (E) scatter plots of the intensity of genes that had a present call on both the microarray for the non-amplified sample and for each diluted-and-amplified sample. The signal data for several genes (β-actin, Tau, Dlx2, Dlx1, Reelin, Calbindin, and GAD67) were overlaid on the scatter plots and (F) scatter plots of signal intensity obtained from two of the three diluted-and-amplified samples, respectively. The 5-fold and 10-fold differences are represented by green lines. Red points indicate the genes having present calls on both of the microarrays. Blue points indicate the genes having an absent call on one of the microarrays. Black points indicate genes having absent calls on both of the microarrays. The number between two red arrows indicates the percentage of genes with a present call that were plotted between the 5.0-fold difference lines.

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(Fig. 1A) and pooled in a plastic dish. A single GFP-positive cell was identified by its green fluorescence, aspirated, and ejected into a water droplet under bright field microscopy (Fig. 1B and C). The water droplet was transferred to a PCR tube and processed for RT-PCR. Total RNA from the single cell was reverse-transcribed into cDNA from the poly-A site using oligo-dT as a primer. Then, several genes of interest were amplified by PCR using the cDNA raised from a single cell as the template. We detected GAD67 expression in all of the β-actin-positive samples (53/53).

3.2. Development of a single-cell microarray analysis method

The single-cell RT-PCR described above is useful for examining the expression of up to five, and at best 10, genes. For a more detailed analysis of the gene expression in a single GAD67-GFP-positive cell, we sought to develop a single-cell microarray-based method. This required amplifying the cDNA raised from a single cell. First, we examined the advantages and disadvantages of several methods for amplifying mRNA. T7 RNA polymerase amplification (T7 amplification) and PCR amplification are commonly used methods. T7 amplification can increase the starting amount of mRNA up to 1000-fold in one round of reaction. It is also possible to perform two or three rounds of T7 amplification. During T7 amplification, the relative amount of each mRNA contained in the starting solution (the proportion of mRNA) is believed to be maintained rather well. However, each round of T7 amplification seems to reduce the length of the mRNA. On the other hand, PCR can amplify cDNA while maintaining its length, although preservation of the proportion of mRNA is less certain. A gene-chip expression array consists of 11 matched and mismatched pairs of 25-mer oligonucleotide probes chosen from 600 bases upstream from the poly-A for a single gene (Affymetrix, MOE430A). One microarray contains the sequences for 22,626 genes. At least 20 μg of biotinylated cRNA is needed for hybridization with one microarray. Thus, we needed to raise 20 μg of biotinylated cRNA from a single cell in the range of 600 bases upstream from the poly-A without disturbing the proportion of the messages.

We tried three different combinations of PCR amplification and T7 amplification (Fig. 2A). In the first trial, cDNA from a single cell was subjected to three rounds of T7 amplification. In the second trial, the cDNA was processed with a SMART mRNA amplification kit and amplified by 20-cycle PCR followed by two rounds of T7 RNA polymerase reaction. In the third trial, the cDNA was processed with a Super-SMART kit and amplified by 22-cycle PCR followed by one round of T7 RNA polymerase reaction (Fig. 2A). We then compared the products from the three different procedures by capillary electrophoresis (Fig. 2B). In the first trial, the total amount of biotinylated cRNA reached 20 μg, the amount sufficient for hybridization with one microarray. However, the length of the cRNA was drastically reduced during the three rounds of T7 amplification (Fig. 2B, lane 3; compare the product to that made from 10 ng of total RNA by two rounds of T7 amplification; Fig. 2B, lane 2). In the second trial, the combination of processing using a SMART mRNA amplification kit, amplification by 20-cycle PCR, and only one round of T7 amplification did not yield enough cRNA for hybridization with one microarray. Although the combination of processing using a SMART mRNA amplification kit, amplification by 20-cycle PCR, and two rounds of T7 amplification yielded 100 μg of cRNA, the length of the cRNA was much reduced and ranged from 200 to 4000 bases (Fig. 2B, lane 5). We tried to eliminate the short cDNA product by adding a gel electrophoresis step between the PCR and the two rounds of T7 amplification, as suggested in another report (Kurimoto et al., 2006, 2007). However, we found no improvement in our case (Fig. 2B, lane 6). In the third trial, processing using a Super-SMART kit, amplification by 22-cycle PCR, and one round of T7 amplification yielded enough cRNA for hybridization with 1–4 microarrays from a single cell. In addition, the length of the products ranged from 500 to 2000 bases (Fig. 2B, lane 8). Eliminating the short cDNA product before the T7 amplification did not result in any change in the final product (Fig. 2B, lane 9). The major difference between the second and third trials was primer sites made for PCR amplification. SMART mRNA amplification kit added T7 promoter sequence to one end of cDNA, while super-SMART kit did not. However, T7 promoter sequence did not often work for PCR amplification as shown below. Thus, we chose the third combination of amplification procedures for the following study. In the process using the Super-SMART kit (Fig. 2C), mRNA is reverse-transcribed from poly-A with a proper PCR primer (3′SMART CDS primer IIA). An oligonucleotide (SMART IIA oligonucleotide) containing a stretch of $G_{(3)}$ nucleotides is included in the reaction solution. Using the $C_{(3)}$-hang at the end of the cDNA as a cohesive end, reverse transcriptase copies the SMART IIA oligonucleotide sequence to the end of the cDNA (Fig. 2C). After this step, we amplified the whole cDNA with 22 cycles of PCR using Primer IIA as a universal primer. Then, the T7 promoter sequence was added to the amplified cDNA, and another 10 PCR cycles were performed. We subcloned the PCR products into a TA-cloning vector (Invitrogen, USA), sequenced 16 clones, and found the T7 promoter sequence in 7 of the 16 clones (43.3%). This indicated that the additional 10 cycles of PCR did not amplify the cDNA very much. The final PCR product was examined by performing another PCR for housekeeping gene and specific genes, such as β-actin, βIII-tubulin, and GAD67 (Fig. 2D). This examination step is important not to waist many microarrays with unsuitable probes. Fig. 2D summarizes the procedure for single-cell microarray analysis. Only the samples that passed three checkpoints (boxes in the flowchart) were used for microarray analysis.

Next, we examined whether the procedures used for mRNA amplification worked as we expected (Fig. 3). We obtained 1 μg of total RNA from 8.6×10^5 GFP-positive cells and used it for microarray analysis (Fig. 3A and B). The 1 μg of total RNA was reverse-transcribed from poly-A using a poly-T primer with the T7 promoter sequence, double-stranded, and

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biotinylated with T7 RNA polymerase (one-cycle target labeling kit; Affymetrix, USA). A portion of the total RNA was diluted 10^5-fold, and 10 pg of total RNA, which is the amount expected from 0.75 HeLa cells, according to the manufacturer's instructions, was processed with the Super SMART kit and amplified by PCR and one round of the T7 RNA polymerase reaction ($n = 3$).

We hybridized the final products to the microarray and compared the gene-expression profiles of the sample raised from 1 μg of total RNA and the three samples raised from the diluted and amplified RNA. We plotted all signal intensities obtained from a non-amplified sample and those from average of three diluted and amplified samples (Fig. 3C). The vast majority (79.9%; 18082/22626) of these plotted points were within the 5-fold difference. Forty-eight percent of all the genes (10942/22626) had a "present" call in the sample raised from 8.6×10^5 GAD67-GFP positive cells. This means that 48% of the genes represented on the microarray were expressed in the 8.6×10^5 cells with high probability. However, 30.9 ± 1.5% of all the genes (7026/22626, 7390/22626, 6545/22626) in the samples that were 10^5-fold diluted and amplified had a present call, and 26.7 ± 1.3% of all the genes (6049/22626, 6398/22626, 6546/22626) had a present call on the microarrays from both types of samples. Therefore, the mRNAs for 55.2 ± 2.6% (6049/10942; 6397/10942, 5693/10942, red spots in Fig. 3D) of the genes having a present call in the sample raised from undiluted total-RNA, were amplified properly after 10^5-fold dilution, while 42.4 ± 2.8% (4646/10942; 4267/10942, 5020/10942, blue spots in Fig. 3D) of those mRNAs were not amplified. The remaining 2.3 ± 0.18% (247/10942; 278/10942, 229/10942, data not shown) of the genes had a marginal call. The reduction in present calls might be due to PCR error in the amplification procedure. Plots of the signal intensities for the genes that had a present call on both arrays (red spots in Fig. 3D) were aligned on a 45° line ($R^2 = 0.51 \pm 0.04$). Moreover, the vast majority (90.9 ± 2.3%; 5540/6049, 5945/6397, 5033/5693) of these plotted points were within the 5-fold difference lines (Fig. 3E). Since the signal intensity of a spot on a microarray usually correlates with the number of copies of mRNA, we concluded that the Super-SMART amplification does not distort the proportion of mRNA during amplification very much. To demonstrate the quality of the correlation, we

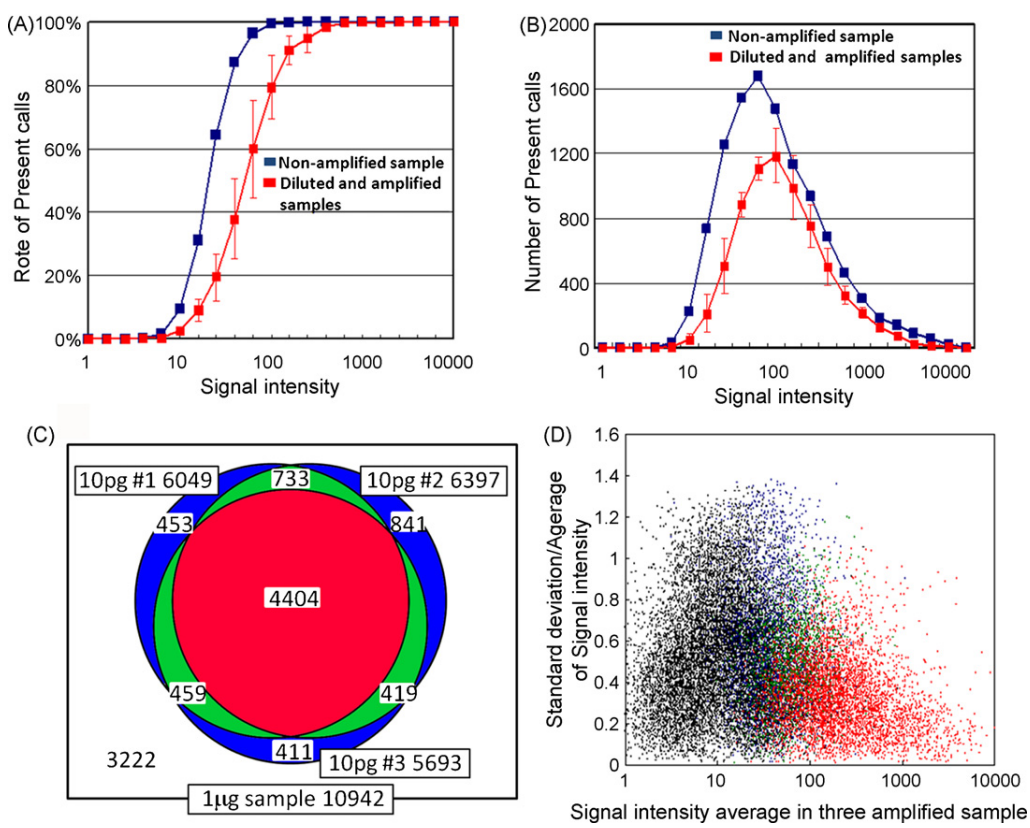

Fig. 4. (A) Rate of present calls for detected genes as a function of signal intensity observed in the nonamplified (blue) sample and amplified samples (red), (B) number of present calls as a function of signal intensity observed in the nonamplified (blue) sample and amplified samples (red), (C) number of present calls for detected genes of three amplified samples and nonamplified sample indicated in a Venn diagram. Red area indicates the number of genes having present calls on three of the three amplified samples. Green area indicates the number of genes having present calls on two of the three amplified samples. Blue area indicates the number of genes having a present call on one of the three diluted-and-amplified samples and (D) ratio of the deviation to the average signal for each gene. Vertical axis indicates the value of (standard deviation)/(average signal intensity of a gene in the three amplified samples). Horizontal axis indicates the average signal intensity of each gene in the three amplified samples. Red points indicate the genes having present calls on three of the three amplified samples. Green points indicate the genes having present calls on two of the three amplified samples. Blue points indicate the genes having a present call on one of the three diluted-and-amplified samples. Black points indicate the genes having Absent calls on all the amplified samples.

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overlaid the signal data of several genes that are often expressed in GABAergic neurons and their progenitors on the scatter plots (Fig. 3E). We also examined the signal deviation among the three different gene-expression profiles obtained by amplifying the same diluted total RNA in three different tubes, and confirmed that our method had good reproducibility (Fig. 3F). The reciprocal scatter plots of the three profiles showed good correlation, and 84.0 ± 3.3% of the intensities of genes that had a present call were plotted between the 5.0-fold difference line ($R^2 = 0.84 \pm 0.04$).

Next, we plotted the rate of present calls for detected genes (present call/detected genes at each signal intensity) as a function of signal intensity (Fig. 4A). This analysis showed that more than 80% of the genes with a present call in the amplified sample had signal intensities less than 100. We also plotted the number of present calls in non-amplified and amplified samples as a function of signal intensity (Fig. 4B). These figures show that the genes switching from present call to absent call had low signal intensities. The reduction in present calls of low signal intensity would be due to the failure of PCR amplification from a small number of templates. A better amplification method for single-cell microarray analysis would decrease the difference between the blue and red lines in Fig. 4B. Further, we showed the number of present calls of the three amplified samples and nonamplified sample in a Venn diagram (Fig. 4C). In the diagram, 4404 genes had present calls in all the three amplified samples, 1611 genes in two of the three amplified samples, 1705 genes in one of the three amplified samples. In fact 7720/10942 (70.5%) genes had present calls in either one of the three amplified samples. In other words, majority (73.0 ± 3.4%; 4404/6049, 4404/6397, 4404/5693) of genes that had present calls in either one of the three amplified samples had present calls in all of the three amplified samples. We also plotted the ratio of the standard deviation in signal intensity to the average intensity of the signals in the three amplified samples for all the genes on the microarray (Fig. 4D). The genes with higher signal intensities had smaller deviations.

To examine the background level in this method, we aspirated the bathing solution of a dissociated-cell pool, and processed it for reverse-transcription, Super-SMART amplification, and T7 amplification. The sample raised from the bathing solution was then hybridized with a microarray. Although 2.6% (577/22626) of all the genes in the bathing solution had a present call, the average signal intensity of the spots with a present call was 26.3, and the average for all the spots was 8.2. These averages were about 5.3% of the average intensities in the microarray hybridized with the 10^5-fold diluted and amplified sample. Since the statistical algorithm in the GCOS software often generates false present calls in the analysis of microarray data at low signal intensities, present calls based on spots with signals less than 5.3% of the intensity of the other data should be ignored.

3.3. Single-cell microarray analyses of GAD67-GFP-positive cells

Next, we used the method for single-cell microarray analysis to examine the gene-expression profiles of GAD67-GFP-positive cells obtained from the mouse neocortex at P0 (Fig. 1). We prepared 56 Super-SMART PCR amplified sample, and detected β-actin in 53 samples (53/56; 94.6%), and detected β-actin and βIII-tubulin in 42 samples (42/56; 75.0%), and detected β-actin, βIII-tubulin and GAD67 in 18 samples (18/56; 32.1%) using less than 1/100 of the Super-SMART PCR product obtained from single cells as a PCR template. Although there were no introns between the pairs of PCR primers for GAD67 in this case, contamination with genomic DNA could be ruled out, because the GAD67 sequence was detected at a high rate (Fig. 5A). Eight biotinylated-cRNA samples obtained from single cells were hybridized with microarrays and analyzed. We found 1277 (#3) to 3147 (#5) present calls in each sample, respectively. First of all, we made a scatter plot of data obtained from the GFP-positive cell #5 and the bathing solution of a dissociated-cell pool (background control sample) (Fig. 5B). The scatter plot revealed that all the data of control samples corresponded to the data with absent calls in the sample of cell #5 and fallen in the left lower corner of the plot, which implies that the present calls in the background control sample is nonsense. The scatter plot in Fig. 5C shows the difference and similarity between cells #4 and #5 (Fig. 5C). The red spots indicate genes common to the two cells (5.3%, 1203/

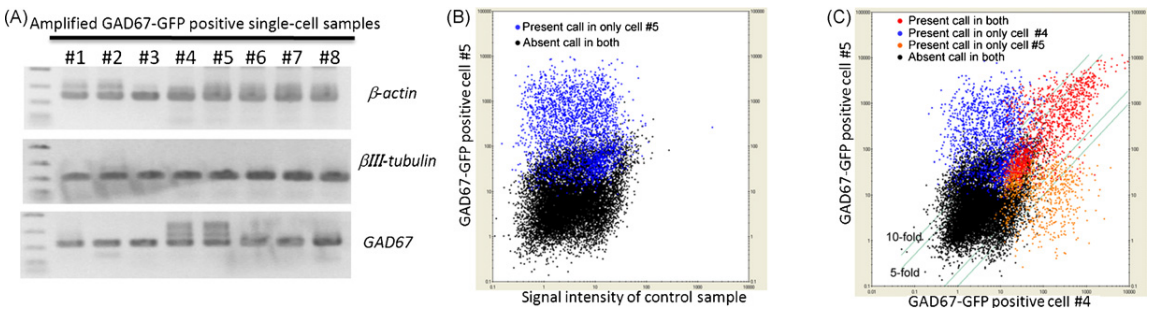

Fig. 5. Single-cell microarray analysis of GABAergic neuron progenitors. (A) Detection of β-actin, βIII-tubulin and GAD67 in Super-SMART PCR product, (B) scatter plots of data obtained from GFP-positive cell #5 and the bathing solution of a dissociated-cell pool (background control sample) and (C) scatter plots of data obtained from two different GFP-positive cells. The scatter plot reveals the difference and similarity in the gene expression of the two GFP-positive-cells. The 5-fold and 10-fold differences are represented by green lines. The majority of genes with a present call in both of the cells (75.8%) were confined between the 5-fold difference lines.

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22626), and may represent house-keeping genes or genes for common functions of GABAergic neurons. The blue and orange spots indicate the genes unique to cell #4 (4.2%, 954/22626) or to cell #5 (8.2%, 1868/22626), respectively. Black spots indicate genes not detected in either cell (80.0%, 18091/22626). As we suggested above, the black spots include absent calls that changed from present calls due to a failure of PCR amplification. However, the blue and orange spots might represent genes unique to either cell, especially those appearing with high signal intensity.

We further analyzed the gene-expression profiles of eight single cells with special reference to genes that are reported to be characteristic of GABAergic neurons and pyramidal neurons in the adult mouse neocortex (Table 1 and Sugino et al., 2006). Fig. 6A shows the signal intensity of genes characteristic of GABAergic neurons in eight cells by pseudo-colors (from green to red; by Genespring software, Agilent, USA). The eight cells showed unique gene-expression profiles, respectively. GAD67 had a present call in cells #4, #5, and #8, while all of the eight cells were

Table 1
Characteristic genes of GABAergic neurons and glutamatergic neurons

Gene symbol	Common gene name	Comment	Probe set ID	Accession number
2610042L04Rik	2610042L04Rik	Hypothetical protein (2610042L04Rik)	1438206_a_at	NM_025575
Actb	Beta-actin	Actin, beta, cytoplasmic	1419734_at	NM_007393
Adcyap1	PACAP	PACAP ligand precursor	1423427_at	NM_009625
Alox12b	Aloxe2, Alox12b	Arachidonate 12-lipoxygenase, pseudogene 2	1418266_at	NM_009659
Arx	Arx	Aristaless related homeobox gene	1450042_at	NM_007492
Calb1	CalbindinD	Calbindin-28K	1417504_at	NM_009788
Calb2	Calretinin	Calretinin	1451129_at	NM_007586
Cck	Cholecystokinin	Cholecystokinin	1419473_a_at	NM_031161
Cdh9	T1-cadherin, cadherin9	T1-cadherin	1427618_at	XM_907532
Chrna6	Chrna6	Nicotinic acetylcholine receptor subunit alpha6 (NICA6 gene)	1450426_at	NM_021369
Cnr1	Cannabinoid receptor 1	Cannabinoid receptor 1 (brain)	1419425_at	NM_007726
Col19a	Col19a	Procollagen, type XIX, alpha 1	1430676_at	NM_007733
Col5a1	Collagen a1(V)	Collagen a1(V), complete cds	1434479_at	AK157100
Cox6a	Cox6a	Cytochrome *c* oxidase subunit VIaH	1417607_at	NM_009943
Crym	Crym	Mu-crystallin (Crym)	1416776_at	NM_016669
Ctsc	Ctsc	Cathepsin C	1416382_at	NM_009982
Cxcl14	Chemokine ligand 14	Chemokine ligand 14	1418457_at	NM_019568
Dcn	Decortin, DSPG, mDCN	Decorin(PG40, PGII, PGS2SLRR1B)	1449368_at	NM_007833
Diap3	Dia2	Diaphanous-related formin (Dia2)	1422944_a_at	NM_019670
Dlx1	Dlx1	Distal-less homeobox 2	1449470_at	NM_010053
Dlx2	Dlx2	Distal-less homeobox 2	1448877_at	NM_010054
Dlx3	Dlx3	Distal-less homeobox 3	1450475_at	NM_010055
Dlx4	Dlx4(Dlx7)	Distal-less homeobox 4	1427641_at	BC006923
Dlx5	Dlx5	Distal-less homeobox 5	1449863_a_at	NM_010056
Dlx6	Dlx6	Distal-less homeobox 6	1452507_at	AF022078
Ece1	Ece1	Endothelin converting enzyme1	1422586_at	NM_021306
Gad1	Gad67	Glutamic acid decarboxylase 1 (67)	1416561_at	NM_008077
Gad2	Gad65	Glutamic acid decarboxylase 2 (65)	1421978_at	NM_008078
Gata3	Gata3	GATA-binding protein 3	1448886_at	NM_008091
Gna14	Gna14	Guanine nucleotide binding protein, alpha 14	1449848_at	NM_008137
Htr3a	Serotonin receptor 3a	Serotonin (5-HT) receptor 3A	1418268_at	NM_013561
Ifi203	Ifi203	Interferon activated gene 203	1448775_at	NM_001045481
Klk26	Klk1b26	Mouse kallikrein 1 related peptidase b 26	1419090_x_at	NM_010644
Krtap12-1	Krtap12-1	High cysteine keratin-associated protein 12.1	1450535_at	NM_010670
Lhx1	Lhx1	LIM homeo box protein 1	1421951_at	NM_008498
Ndrg1	CAP43, DRG1	*N*-myc downstream regulated gene1	1423413_at	NM_010884
Nek7	Nek7	Never in mitosis gene a (NIMA)-related expressed kinase 7	1416816_at	NM_021605
Neurod6	Nex, Math2	Neurogenic differentiation 6	1418047_at	NM_009717
Nfe2l3	Nrf3	Nuclear factor erythroid derived 2, like3	1417520_at	NM_010903
Nos1	nNOS	Nitric oxide synthase 1, neuronal	1422949_at	NM_008712
Npnt	Nctn, Npnt	Nephronectin	1452106_at	AB059656
Nppc	Nppc	Natriuretic peptide precursor type C	1422790_at	NM_010933
Npy	Neuropeptide Y	Neuropeptide Y	1419127_at	NM_023456
Pdlim1	Pdlim1(Clim1)	Carboxyl terminal LIM domain protein	1416554_at	NM_016861
Plxnd1	PlexinD1	PlexinD1	1451475_at	BC019530
Postn	Periostin	Periopstin, Osteoblast specific factor	1423606_at	NM_015784
Ptprc	Cd45, B220, Ly-5, T200	Protein tyrosine phosphatase, receptor type, C	1422124_a_at	NM_011210
Ptprm	Ptprm	Protein tyrosine phosphatase, receptor, type M	1422541_at	BC079621
Pvalb	Parvalbumin	Parvalbumin	1417653_at	NM_013645

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Table 1 (*Continued*)

Gene symbol	Common gene name	Comment	Probe set ID	Accession number
Reln	Reelin	Reelin	1449465_at	NM_011261
Sepp1	Sepp1	Plasma selenoprotein P (SELP) gene	1452141_a_at	NM_001042613
Sfrp2	Sfrp2	Secreted frizzled related protein sFRP-2	1448201_at	NM_009144
Slc6a1	Gat1, GABA transporter1	GABA transporter 1	1452142_at	NM_178703
Sln	Sacrolipin	Sacrolipin	1420884_at	NM_025540
Sox2	Sox2	SRY-box containing gene 2	1416967_at	BC057574
Sst	Somatostatin	Somatostatin	1417954_at	NM_009215
Tac1	Tachykinin 1	Tachykinin 1	1416783_at	NM_009311
Tac2	Tachylinin2	Preprotachykinin B,	1419411_at	NM_009312
Tal1	Tal1	T-cell acute lymphocytic leukemia 1	1449389_at	NM_011527
Tcap	Telethonin	Telethonin genomic sequence	1423145_a_at	NM_011540
Tcrb-V13	Tcrb-V13(BV13S1)	Mouse T-cell receptor germline beta-chain gene, V-region promoter	1427848_at	M87849
Tuba1	Tubulin, alpha1	Tubulin, alpha1	1418884_x_at	NM_011653
Thy1	Thy1	Mouse Thy-1.2 glycoprotein gene	1423135_at	NM_009382
Tyro3	Tyro3, Brt, Dtk, RSE, Sky.Tif	Tyro3 protein tyrosine kinase	1425249_a_at	NM_019392
Viaat	VGAT(slc32a1)	Vesicular GABA and glycine transporter	1456457_at	BC025013
Vip	Vasoactive intestinal peptide	Vasoactive intestinal peptide gene	1428664_at	NM_011702
Zfp312	Zfp312, Fez	Zinc finger protein 312	1418790_at	NM_080433

positive for GAD67 as revealed by PCR (Fig. 5A). In addition, some of the 8 cells seemed to be characterized by typical subtype markers even from perinatal stage. Reelin (Reln) had present calls in cells #5 and #6. Neuropeptide Y (Npy) had present calls in cells #5, #6 and #8. Although we also examined the expression levels of genes characteristic of glutamatergic neurons in the eight cells, almost all of them were very low and dark green in the pseudo-color scheme (Fig. 6B). Most house-keeping-genes had present calls in the eight cells (Fig. 6C).

Fig. 6. (A and B) Gene-expression profiles of eight single cells with special reference to genes that are reported to be characteristic of GABAergic neurons (A) and pyramidal neurons (B) in the adult mouse neocortex, respectively (Sugino et al., 2006). The normalized expression level is indicated by pseudo-colors (from green to red; by GeneSpring software) and (C) expression profiles of house-keeping-genes in 8 single cells.

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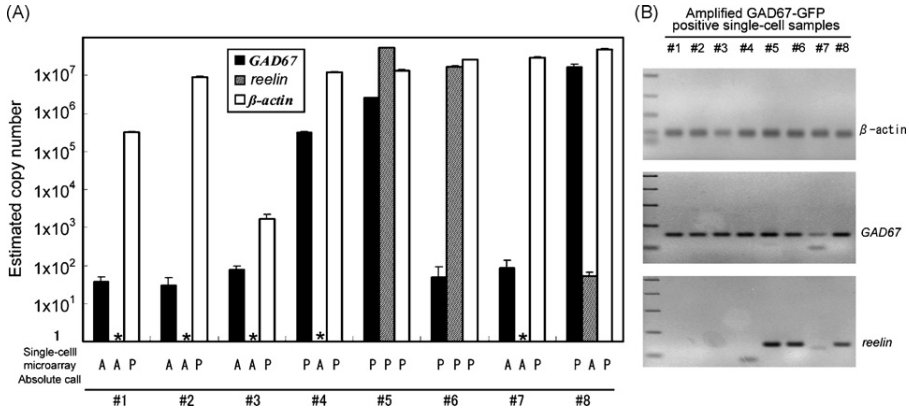

Fig. 7. (A) Copy numbers of cDNA detected in Supar SMART PCR products by quantitative real-time PCR analysis. The number of cDNA copies were contained 1 μl of the Super SMART PCR product. P and A along the *x*-axis indicate the gene that had present call or absent call in single-cell microarray analysis, respectively. The asterisk indicated the sample with no PCR product and (B) PCR products of β-actin, GAD67 and reelin obtained by the quantitative real-time PCR.

3.4. Quantitative real-time PCR analysis

We hybridized eight microarrays with probes that were positive for β-actin, βIII-tubulin, and GAD67. However, GAD67 had present calls only in three cells. Thus we examined the number of copies of β-actin, GAD67, and reelin cDNA in the Super-SMART PCR product by quantitative real-time PCR (Fig. 7). As the results, we detected more than 1×10^5 copies (in 1 μl of the Super SMART PCR product) of GAD67 cDNA in the samples of cells #4, #5, and #8, and more than 50 copies in the other five samples. We also detected reelin cDNA in the samples of cells #4, #5, and #8, but not in the other samples. However, in the case that the number of cDNA copies remained less than 8×10^1, GAD67 and reelin genes had absent calls. More than 1×10^3 copies of β-actin were detected in all the eight samples, and β-actin had present call in all the cells. Fig. 7B shows PCR products obtained in the real time PCR analysis. There was no reelin PCR products in five samples.

3.5. Immunocytochemical detection of gene products

According to single-cell microarray analysis, Arx and Dlx2 genes had present calls only in five cells (Fig. 6), whereas the Arx- and Dlx2-expression has been reported in vast majority of GAD67-positive cells. .Thus we examined the proportion of ARX- and DLX2-positive cells to GFP positive cells by immunocytochemistry. Fig. 8 shows cells double-positive for GFP and ARX, GFP and DLX2, and GFP and Reelin (Fig. 8). Basically all GFP-positive cells were positive for ARX (98.0%; 147/150) and vast majority of GFP-positive cells were positive for DLX2 (95.8%; 204/213). The proportion of Reelin-positive cells to GFP positive cells was 5.2% (11/211).

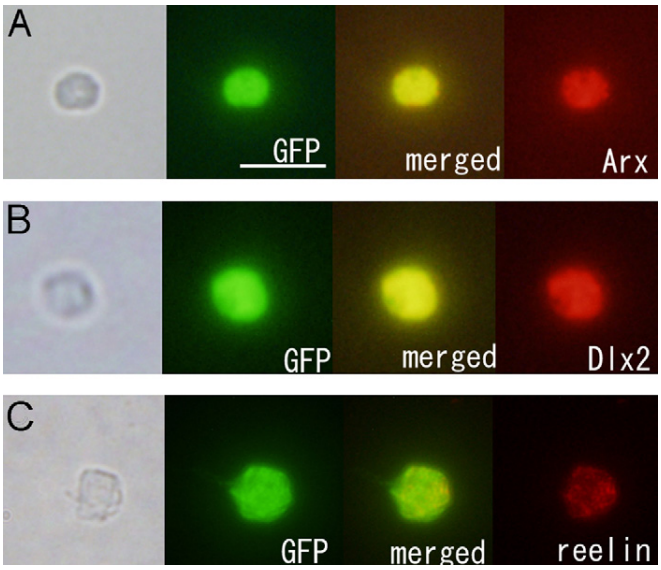

Fig. 8. Colocalization of GFP and ARX, GFP and DLX2, and GFP and Reelin in the IZ, SVZ, and VZ layers of the GAD67-GFP knock-in mouse neocortex at P0. GFP is in green in all the panels. (A) ARX is in red, (B) DLX2 is in red and (C) Reelin is in red. Scale bar in (A) is 10 μm.

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4. Discussion

In this study, we have established a method for single-cell microarray analysis that is based on several preexisting techniques and studies. As a result of extensive trial-and-error examinations, both our method and that of Kurimoto et al. (2006, 2007) converged and settled on almost identical protocols using PCR amplification and one round of T7 amplification. The only difference found in the two independent reports is in how the primer site is added at the 5′ end of the mRNA. We used a $G_{(3)}$-hang made by a reverse transcriptase and annealing of an oligonucleotide to the 5′ end of the mRNA followed by strand-switch extension of cDNA (Super-SMART PCR cDNA amplification kit), while Kurimoto et al. (2006, 2007) used a terminal poly-A made by terminal transferase to add a universal primer at the 5′ end of the sense DNA. We set the number of PCR cycles to 22 to yield enough cRNA for more than one microarray hybridization, while Kurimoto and his colleagues set the cycles to a similar number to reduce distortion in the proportion of the message from single cells. By comparing a sample raised from unamplified total RNA to samples raised from the total RNA 10^5-fold diluted and amplified, we found that the success rate of cDNA amplification using our procedure reached 55%.

Pioneering studies of single-cell microarray analysis used a universal PCR amplification (Iscove et al., 2002; Tietjen et al., 2003) or two rounds of T7 amplification (Kamme et al., 2003). However, it was not shown whether either of these amplification techniques accurately expanded the low-copy mRNA. In our experiments, neither two rounds of T7 amplification nor a process with SMART mRNA amplification kit followed by 20 cycle of PCR and one round of T7 amplification yielded sufficient amounts of cRNA to perform microarray hybridization from a single cell, although both methods yielded enough cRNA if a small number of cells were used as the starting sample. Lim et al. (2006) examined the genes, biological processes, and gene interactions for neocortical SVZ adult neurogenesis by microarray analysis of a small number of FACS-sorted cells after SMART-PCR amplification and one round of T7 amplification. In addition, Sugino et al. (2006) reported the gene-expression profiles of adult neocortical neurons by microarray analysis of a small number of sorted neurons after two rounds of T7 amplification. The efficiency of processes of SMART-mRNA amplification followed by PCR and T7 amplification was recently compared (Subkhankulova and Livesey, 2006). Gustincich et al. (2004) examined gene-expression profiles of single dopaminergic amacrine cells after processes of SMART-mRNA amplification, PCR, and two round of T7 amplification. Further, Jensen and Watt (2006) analyzed cultured human epidermal keratinocytes using same method. Recently, Trimarchi et al. (2007) examined developing retinal ganglion and amacrine cells by universal PCR-based single-cell microarray analysis.

In the experiment of single-cell RT-PCR, all the GFP-positive cells were positive for GAD67 (Fig. 1 and Wu et al., 2005). Although all eight cells used for the single-cell microarray analysis were also positive for GAD67, as revealed by an additional nested-PCR (Fig. 5A), the GAD67 gene had a present call only in cells #4, #5, and #8 in the single-cell microarray analysis (Fig. 6A). When 10 pg of the total RNA pooled from 8.6×10^5 GAD67-GFP-positive cells was amplified and subjected to microarray analysis (Fig. 3), the signals for the GAD67 gene had a present call. Therefore, we assume that the GAD67 mRNA was a difficult template to amplify with the primers we used in the single-cell microarray experiment. Moreover, the GAD67 gene was knocked out in the GAD67-GFP knock-in mouse and the gene expression was lower than that in wild mice (Tamamaki et al., 2003). In addition, it is well known that not all neocortical GABAergic neurons contain the same amount of GAD67 mRNA. GAD67 expression level was also altered depending on the subtype of GABAergic neurons. In the case that GAD67 cDNA was not amplified more than 1×10^5 copies in 1 μl of the Super SMART PCR products from the total RNA in a single cell, GAD67 may have absent call in the single-cell microarray analysis. Accepting these false negative (up to 45%), we would like to emphasize that we did not encounter any false positive in absolute calls obtained by the GCOS analysis.

It is common knowledge that not all templates can be amplified under the same PCR conditions. PCR amplification depends on the template sequence, such as its GC content, redundancy, hairpin-loop formation, and homology. Our method amplified 55% of a cDNA library prepared from 10 pg total RNA. Although the number of genes actually expressed in the cell might be twice that, this represents a tremendous improvement in single-cell analysis. Moreover, our amplification method preserved the proportion of signals in a single cell rather well, and more than 90% of the present calls were confined to a 5-fold range, which means that a change in gene-expression level of over one order of magnitude (10-fold) could be detected by this single-cell microarray method. In the future, the rate of successful amplification might be improved by adding another pair of primers (four primers total) during the reverse-transcription step and optimizing the PCR parameters for templates with two different GC contents.

Our findings indicate that single-cell microarray analysis can be useful for detecting the expression of thousands of genes in a cell, but that it is necessary to confirm the expression for critically important genes by single-cell RT-PCR. Single-cell RT-PCR and single-cell microarray analysis can thus complement each other to overcome the disadvantages of each and produce consistent data.

Acknowledgements

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