Split single-cell RT-PCR analysis of Purkinje cells

Shigeyuki Esumi[1,2], Ryosuke Kaneko[1], Yoshimi Kawamura[1] & Takeshi Yagi[1,3]

[1]KOKORO-Biology Group, Laboratories for Integrated Biology, Graduate School of Frontier Biosciences, Osaka University 1-3 Yamadaoka, Suita, Osaka 565-0871, Japan. [2]Department of Morphological Neural Science, Graduate School of Medicine, Kumamoto University, Kumamoto 860-8556, Japan. [3]CREST of Japan Science and Technology Corporation. Correspondence should be addressed to Y.T. (yagi@fbs.osaka-u.ac.jp)

Published online 7 December 2006; doi:10.1038/nprot.2006.343

This protocol details a method for analyzing the expression of multiple genes from a single Purkinje neuron, including the determination of whether the gene expression is monoallelic or biallelic. The protocol describes how to extract a single, living Purkinje cell for reverse transcription, divide the cDNAs into three equal samples and subject those to triplicate amplification of multiple targets by two rounds of PCR (first a multiplex PCR then a gene-specific nested PCR) and finally discriminate the allelic expression of the transcript by direct sequencing of the PCR products. In optimal conditions, this method permits the analysis of the expression of 18 genes in a single Purkinje cell. This protocol can be completed in 5–6 d.

INTRODUCTION

The brain consists of a huge number of neurons whose diverse characteristics and identities contribute to the generation of complex neuronal networks. Understanding the ultimate complexity of the brain relies on understanding the molecular mechanisms that specify the identity and gene-expression palette of single neurons. *In situ* hybridization and immunohistochemistry techniques are available for investigating the identity and diverse gene-expression patterns of single neurons; however, these methods have limitations, such as the difficulty of confirming a probe's specificity and the inability to analyze multiple target genes expressed by the same cell. Therefore, new methods that overcome these limitations are needed.

Recently, RT-PCR of mRNA from single neurons has been used to analyze their identity and diversity. Studies employing this method have typically used only a single PCR tube to amplify the genetic material[1,2]. This one-tube single-cell RT-PCR method has an important limitation, however. As the amount of mRNA contained in a single cell is very small, erroneous conclusions can be reached because of contamination between samples, and it is difficult to verify the absence of contamination. Furthermore, although one-tube single-cell RT-PCR has sometimes been used to analyze allelically regulated genes in a single cell, it has the potential to incorrectly indicate that expression is monoallelic. A well-organized work by Rhoades *et al.* overcame the latter limitation by aliquotting the first-strand cDNA sample (i.e., the reverse-transcribed product) from a single T cell into multiple tubes, which were then used for the amplification of two targets by PCR[3]. We call this method "split single-cell RT-PCR," which expands the analysis to multiple targets that are expressed in a Purkinje cell. The aliquotting of cDNA is mainly used to control for "jackpotting" during PCR, whereby one species is amplified by the same primers in the PCR and suppresses the other species, leading to underestimation of the presence or abundance of the other species. Thus, although using three aliquots still does not rule out the possibility of cross-contamination with a neighboring cell that occurred during reverse transcription, we have realistically diminished the possibility of the cross-contamination by careful handling of samples, reagents and equipment, and by gentle pipetting of the samples throughout the procedures. Our method consists of isolating a single living Purkinje cell, subjecting its RNA to reverse transcription, aliquotting it into three tubes and performing triplicate PCR amplification, and lastly determining the allelic expression by DNA sequencing (**Fig. 1**).

We have used this method to analyze the allelic expression of the *protocadherin* (*Pcdh*) genes and obtained good results[4,5]. The *Pcdh* genes are members of the cadherin superfamily, and are candidates for determining neuronal identity and diversity[6,7] because they are diverse and highly expressed in the vertebrate brain[8,9]. The *Pcdh* genes include *Pcdh-α* (also known as *CNR* (*cadherin-related neuronal receptor*)), *Pcdh-β* and *Pcdh-γ* clusters[6–9]. Their diverse isoforms are generated by differential splicing[6–9]. That is, the *Pcdh-α* and *Pcdh-γ* clusters contain variable regions (tandemly arrayed diverse variable exons) and constant regions (common, cis-spliced exons). We analyzed the gene regulation of the *Pcdh-α* and *Pcdh-γ* clusters using this split single-cell RT-PCR method[4,5]. Our observation revealed that the gene regulation of *Pcdh-α* and *Pcdh-γ* clusters involved both random yet combinatorial monoallelic expression and uniform biallelic expression within single Purkinje cells[4,5,10]. In addition, oligomerization between Pcdh-α and Pcdh-γ proteins led

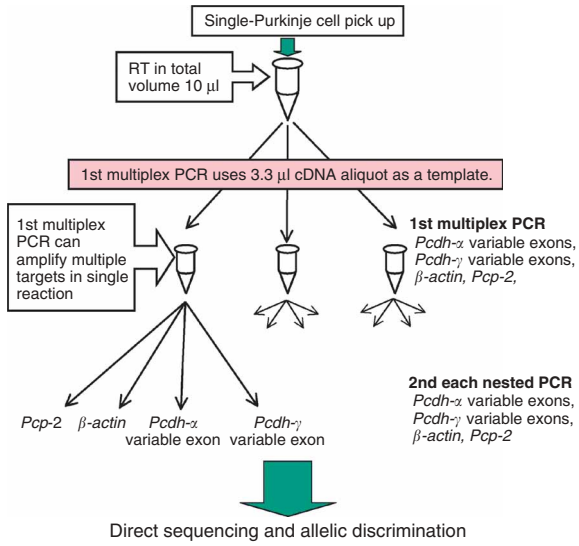

Figure 1 | Flow diagram of split single-cell RT-PCR analysis of a Purkinje cell.

to diverse combinational diversity on the cell surface of individual cells[11]. Here we describe our protocols for analyzing the expression patterns of the *Pcdh-α* and *Pcdh-γ* genes in single Purkinje cells.

Note that, although we used split single-cell RT-PCR to analyze the *Pcdh-α* and *Pcdh-γ* genes, this method is applicable to the study of other genes. We detected monoallelic and biallelic expression of the *Igf2r* gene in single Purkinje cells[12] (unpublished data). Furthermore, although this protocol describes methods for analyzing Purkinje cells from the P21 mouse, we obtained similar but not identical expression profiles from the Purkinje cells of P4, P7 and P11 mice (unpublished data). The developmental age of mice used to isolate Purkinje cells can be selected depending on the experimental purpose (our purpose was to analyze mature Purkinje cells[13], thus we collected Purkinje cells from a P21 mouse). In addition, other neuronal or non-neuronal cells besides Purkinje cells could be used in these protocols. Although our procedure to isolate a Purkinje cell is based on their unusually large size, the isolation procedure can be expanded to other cells. The identification and isolation of other neuronal cells is easily accomplished by using the same enzymatic dissociation solution and equipment from GFP knock-in or transgenic mice that show GFP-fluorescence in a specific population of cells[14–16] (unpublished data). Indeed, split single-cell RT-PCR has been used to analyze the allelic regulation of *interleukin-2* and *Pax-5* in single T cells[3] and the expression of multiple *Dscam* isoforms in *Drosophila* neurons[17]. Thus, the use of split single-cell RT-PCR is not limited to a single set of genes or a given cell type. However, especially in the case of some mRNAs that are not very abundant in a given cell, the method may not always reliably detect RNA species after dividing the cDNA from a single cell into three aliquots. Indeed, only 17% (59/352) of single cells from cerebral cortex of P1 mice gave two or three samples of *Pcdh-α* PCR product in triplicate PCR reactions (unpublished data). Cells from cerebral cortex of P1 mice are smaller than Purkinje cells, suggesting that less *Pcdh-α* mRNA was present. Furthermore, *Pcdh-α* genes are expressed at much lower levels in cerebral cortex than in Purkinje cells; it was estimated by quantitative RT-PCR that the amount of *Pcdh-α4* mRNA is less than 1% of that of *β-actin* mRNA in cerebral cortex cells (unpublished data). Taken together, the method provides a simple and reliable means for revealing gene expression profiles, including allele-specific expression, in single cells of many sorts.

MATERIALS

REAGENTS

- Animals: F1-hybrid mouse at postnatal day 21 ! CAUTION All animal experiments must comply with national regulations.
- Thermoscript reverse transcriptase (Invitrogen, cat. no. 12236-022). This enzyme was the most suitable for reverse transcription in our pilot study.
- LA-Taq PCR polymerase (TaKaRa, cat. no. RR002). This enzyme was the most effective for PCR in our pilot study.
- RNasin (Promega, cat no. N2111)
- Primers ▲ CRITICAL To avoid cross contamination, dissolve in DNase/RNase-free water carefully. To rule out the amplification of genomic DNA/ design primer pairs on different exons for the first and second PCR.
- DNase/RNase-free water (Invitrogen, cat. no. 10977-015)
- BigDye Terminator v3.1 (ABI) and reagents for DNA sequencing
- Polyethylene glycol (PEG) 6000 (Nacalai Tesque)
- Papain (Worthington, cat. no. 3120)
- DL-cysteine HCl (Sigma)
- DNase I (Sigma, cat. no. DN-25)
- BSA (Sigma)
- Glucose (Nacalai Tesque)
- Dulbecco's modified Eagle's medium (DMEM) (Sigma)
- 1:1 mixture of DMEM and Ham's F12 medium (Sigma)
- Horse serum (Invitrogen)

EQUIPMENT

- UV sterilization cabinet (Scie-Plas). ▲ CRITICAL To avoid cross-contamination, use this cabinet throughout the procedure, and UV-irradiate all pipettes, tips and tubes in this cabinet before use.
- Filtered tips (aerosol-resistant tips) ▲ CRITICAL To minimize the risk of cross-contamination, use filtered tips throughout the procedure and use them only in the UV sterilization cabinet. Also wear clean and new powder-free gloves after thawing a frozen single-cell sample.
- Sequence comparison computer software ▲ CRITICAL We use *SeqScape* software (ABI) for this purpose. The *SeqScape* and similar software on the market assist identification of monoallelic versus biallelic expression.
- Micromanipulator (Narishige) Although some groups use a micropipette attached to a mouth tube under a dissection microscope for collecting single cells[2], we obtained consistently good results using a micromanipulator. This was perhaps due to the small volume of medium incorporated into the reverse transcript reaction mix, which may interfere with the reaction.
- Microinjector (Narishige)
- ABI 3100 or ABI3730 DNA sequencer (ABI)
- Cell strainer (100-μm pore size) (Becton Dickinson, cat. no. 352360). ▲ CRITICAL A mature Purkinje cell is about 30–60 μm in diameter. Do not use a cell strainer with a 40-μm pore size.
- Glass micropipette ▲ CRITICAL Fine-tipped glass micropipettes must be made from borosilicate glass (O.D. 1.0 mm, I.D. 0.75 mm, length 10 cm, Sutter Instrument, cat. no. B100-75-10) using a micropipette puller (Sutter Instrument, model P-97/IVF) with the following settings: P = 500, HEAT = 640, PULL = 0, VEL = 20, TIME = 250.
- 0.2 ml thin-wall PCR tubes and 96-well PCR plates ▲ CRITICAL To minimize the risk of cross-contamination, use the PCR tubes and plates only in the UV sterilization cabinet. Also pipette gently throughout the procedure.

REAGENT SETUP

Mice To distinguish whether mRNA was from the paternal or maternal allele, or both, use the F1 hybrid offspring of the mouse strain of interest. In our case, the offspring are obtained by intercrossing mice of the laboratory strain C57BL/6 (B6) (Purchased from Charles River) with the Japanese wild mouse strain JF1 or MSM (obtained from the National Institute for Genetics; Mishima, Shizuoka, Japan). The genomic DNA of these wild mouse strains contains many polymorphisms in comparison with the B6 DNA sequence, so in the F1 hybrids, the parental origin of many alleles can be readily identified. To analyze genes in the mature Purkinje cell, we mainly use mice at postnatal day 21.

Enzymatic dissociation solution 90 units papain, 0.002% DL-cysteine HCl, 0.05% DNase I, 0.1% BSA and 0.05% glucose (**Table 1**). To more rapidly dissolve the papain, warm it at 37 °C for 5 min. Except for the papain and DL-cysteine HCl, this solution is made from the following stock solutions: 1% DNase I, 1% BSA and 1% glucose. The stock solutions can be stored at –20 °C for several months. ▲ CRITICAL Prepare just before use and filter through a 0.22-μm syringe-driven filter unit.

Resuspension medium 5% FBS (Invitrogen), 5% horse serum (Invitrogen) and 90% 1:1 mixture of DMEM and Ham's F12 medium (Sigma). Filter through a 0.22-μm syringe-driven filter unit. DMEM can also be used as the resuspension medium.

PROCEDURE

Preparation of single-cell suspension ● TIMING 1.5 h

1| Prepare single Purkinje cells as follows (**Fig. 2**): kill a P21 F1 mouse by cervical dislocation and remove the cerebellum using clean surgery tools. Place the cerebellum on an ice-cold glass dish.

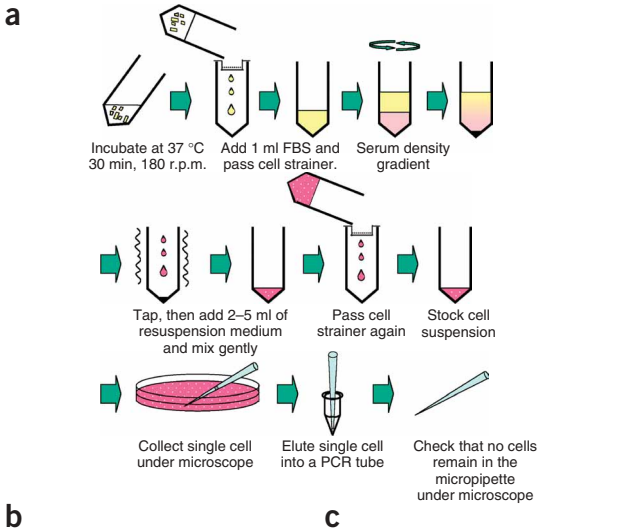

Figure 2 | Method for collecting a single Purkinje cell from a P21 F1 mouse. (**a**) Small pieces of cerebellum are incubated in enzymatic dissociation solution at 37 °C for 30 min with vigorous shaking. The undissociated tissues and debris are removed by passing the sample through a cell strainer and then a serum density gradient. The pellet is resuspended with ice-cold resuspension medium. The single Purkinje cell is collected with a glass micropipette under an inverted microscope equipped with a micromanipulator and microinjector. After collecting a single Purkinje cell, the holder, which still retains a glass micropipette involving the cell is detached from the micromanipulator, settled on a PCR tube by hand, and the cell and a small amount of medium is eluted into a PCR tube containing DNase/RNase-free water by microinjector. After elution of the cell, it is important to check that no remnants of the cell are left in the glass micropipette. Photographic images show the system to collect a single Purkinje cell (**b**) and the procedure to elute the single cell into a PCR tube (**c**).

2| Cut the cerebellum into 8–10 pieces using a clean knife.

3| Immerse the pieces of cerebellum into 10 ml of enzymatic dissociation solution (pre-warmed to 37 °C) for 30 min at 37 °C with vigorous shaking (180 r.p.m.).
▲ **CRITICAL STEP** If after 30 min the solution is not sufficiently turbid to indicate dissociation, you can extend the incubation time up to 40 min. If the tissues are aggregated, add more DNase I and/or shake more vigorously.
? TROUBLESHOOTING

4| Add 1 ml of FBS to stop protease digestion.

5| Pass the suspension through a cell strainer (100-μm pore size).

6| Remove debris by centrifuging the sample through a serum density gradient. In detail, layer the flow-through from the cell strainer into 10 ml of FBS (previously filtered through a 0.22-μm filter and pre-warmed to 37 °C), and centrifuge at 300*g* for 8 min. Remove the supernatant by aspiration and tap the tube to disaggregate the pellet.

7| Resuspend the pellet very gently by adding the appropriate volume (usually 2–5 ml) of ice-cold resuspension medium. To remove the debris completely, pass the sample once more through a cell strainer (100-μm pore size). This suspension will be the stock cell suspension. It should be stored on ice while collecting Purkinje cells.

Collecting single Purkinje cells ● TIMING ~2 h

8| Pour an appropriate volume of stock cell suspension (1/20 ~ 1/10 of the total volume) into a FBS pre-coated 6-cm plastic dish containing 3 ml of ice-cold resuspension medium. The Purkinje cells can be easily distinguished from other cells by their size. Mature Purkinje cells have a diameter of about 30–60 μm, while granule and other cerebellar cells have diameters of about 10–20 μm (**Fig. 3a**).

9| Under an inverted microscope equipped with a micromanipulator and microinjector, use a glass micropipette to pick up individual Purkinje cells (**Fig. 3b**) and place each Purkinje cell in a thin-walled 200-μl PCR tube with 6 μl DNase/RNase-free water.
▲ **CRITICAL STEP** Check that no cells are remaining in the glass micropipette after elution of each cell into a PCR tube.
? TROUBLESHOOTING

10| Freeze each Purkinje cell–containing tube immediately in liquid nitrogen.

11| After picking up Purkinje cells for 20 to 30 min, replace the cell suspension in the 6-cm dish with fresh cell suspension from the cold stock cell suspension.
▲ **CRITICAL STEP** Collect the Purkinje cells for no longer than 2 h. Taking longer to isolate the cells can negatively affect the PCR amplification step, resulting in poor amplification efficiency or cross-contamination.
? TROUBLESHOOTING Use one glass micropipette per Purkinje cell to avoid cross-contamination. To verify that there is no contamination, collect some of the solution bathing the dissociated cells after every seventh Purkinje cell collected, and use

it as a cell-free control in the remainder of the steps. If this cell-free sample gives any PCR amplification, do not use the results obtained from the cells that were isolated in the same batch as the cell-free sample.

■ **PAUSE POINT** The frozen tubes containing the single-cell samples can be stored at –80 °C for 1–2 months.

Reverse transcription with gene-specific primers ● TIMING 2 h

12| Thaw the single-cell samples on ice and centrifuge at 200*g* at 4 °C for 1 min.

13| To each thawed sample tube containing a single Purkinje cell, add 1 µl of gene-specific primer mix (which contains 2.5-µM gene-specific primers) and 1 µl of 10-mM dNTP mix. Keep samples at 4 °C while adding reagents.

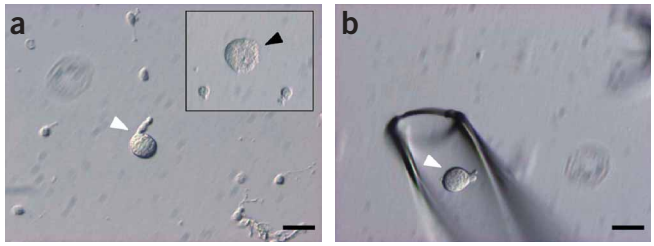

Figure 3 | Collecting a single living Purkinje cell with a glass micropipette. (**a**) Photomicrograph taken of cells under the inverted microscope. The cell suspension contains living Purkinje cells (white arrowhead) and many non-Purkinje cells (not marked). Inset shows a dead Purkinje cell (black arrowhead). (**b**) A living Purkinje cell (white arrowhead) is being picked up with a glass micropipette. Scale bars, 50 µm.

14| Heat the mixture at 65 °C for 5 min and then place it on ice immediately for at least 2 min.

15| Add 2 µl of 5× cDNA synthesis buffer (Invitrogen, supplied with Thermoscript), 0.5 µl of 0.1-M DTT (Invitrogen, supplied with Thermoscript), 0.5 µl of RNasin and 0.5 µl (7.5 unit) of Thermoscript reverse transcriptase per reaction. Mix gently and incubate at 55 °C for 60 min. Stop the reaction by heating the sample to 85 °C for 5 min.

■ **PAUSE POINT** The cDNA samples can be stored at 4 °C for several days and at –20 °C for several months.

Split single-cell RT-PCR: multiplex PCR followed by gene-specific nested PCR ● TIMING 10 h

16| Aliquot the cDNAs derived from each Purkinje cell into three PCR tubes (i.e., as 3.3-µl aliquots).

17| Perform the first, multiplex PCR using all three tubes containing the cDNA from a single cell. To each 3.3-µl aliquot, add 5 µl of 10× *LA-PCR* buffer (Mg^{2+}-free), 3.9 µl of 25-mM $MgCl_2$, 4 µl of each 2.5-mM dNTP mix, 0.6 µl of each 10-µM primer, 0.5 µl of *LA-Taq* polymerase and water up to 50 µl. Use the following PCR conditions: 1 cycle of 2 min, 94 °C; 30 cycles of 15 s, 95 °C; 10 min, 65 °C; 1 cycle of 10 min, 72 °C.

18| Perform the second round of PCR (gene-specific nested PCR) using 0.5 µl of the first PCR product as the template, 2 µl of 10× *LA-PCR* buffer (Mg^{2+}-free), 2 µl of 25-mM $MgCl_2$, 1.6 µl of each 2.5-mM dNTP, 0.3 µl of each 10-µM primer, 0.2 µl of *LA-Taq* polymerase and water up to 20 µl. Use the following PCR conditions: 1 cycle of 5 min, 95 °C; 35 cycles of 30 s, 95 °C; 30 s, 60 °C; 1 min, 72 °C.

▲ **CRITICAL STEP** The primer sequences we used are listed in ref. 5. Primers for RT-PCR should be positioned on different exons to avoid amplification from genomic DNA.

? TROUBLESHOOTING

■ **PAUSE POINT** RT-PCR products can be stored at 4 °C for several days and at –20 °C for several months.

Agarose gel electrophoresis ● TIMING 30 min

19| Load 5 µl of the second-round PCR product into the well of a 1.5% agarose gel. Run at 100 V for 15 min. After electrophoresis, stain the gel with ethidium bromide solution (~20 min) and visualize the bands under UV light.

Purification of PCR products by PEG precipitation and DNA sequencing ● TIMING 5 h or more, depending on sequencer used

20| Add 15 µl of 20% PEG6000 to 15 µl of PCR product that showed a detectable amount of PCR product in the agarose gel, then mix vigorously using a vortex mixer.

21| Keep solution at –80 °C for 30–60 min and then centrifuge it at 4 °C at 15,000*g* for at least 20 min. Remove supernatant.

22| Add 100 µl of 70% EtOH and centrifuge it at 4 °C at 15,000*g* for 15 min. Remove supernatant.

23| Dissolve pellet in 10 µl of DNase/RNase-free water.

24| Check the concentration of the purified PCR products using agarose gel electrophoresis.

25| Sequence the DNA following the laboratory's preferred protocol. We use a BigDye DNA sequencing Kit (ver 3.1, ABI) and an ABI Prism 3100 or ABI Prism 3730 Genetic Analyzer.

▲ **CRITICAL STEP** Use a 96-well plate and multi-channel pipette to handle many samples at one time.

■ **PAUSE POINT** Purified RT-PCR products can be stored at 4 °C for several days and at –20°C for several months.

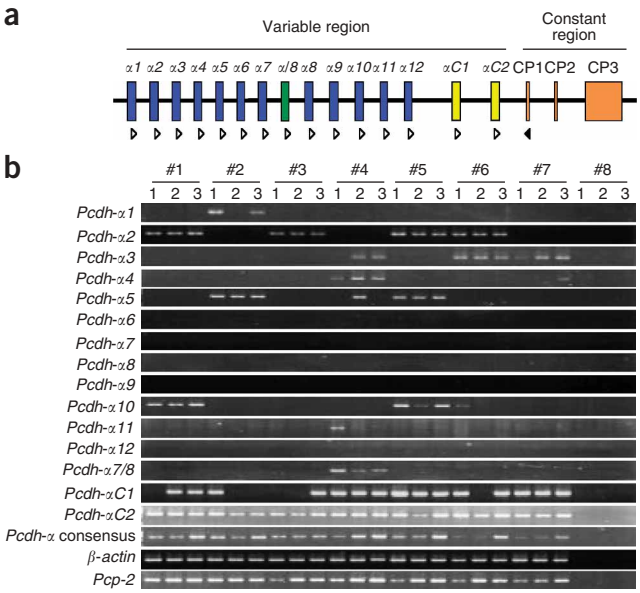

Figure 4 | Split single-cell RT-PCR analysis of *Pcdh-α1–12, C1, C2, β-actin* and *Pcp-2* in individual Purkinje cells. (**a**) Structure of the *Pcdh-α* gene cluster. The positions of gene-specific nested forward primers (white arrowheads) and a consensus nested reverse primer (black arrowhead) for *Pcdh-α* genes are indicated. (**b**) Results of the second-round PCR. Purkinje cells were collected from the P21 F1 progeny of a cross between the B6 and JF1 strains. Individual Purkinje cells are labeled as #1–#7. Sample #8 is a cell-free negative control that gave no bands, indicating there was no cross-contamination. Each reverse-transcribed sample was divided into three aliquots and then subjected to PCR, followed by agarose gel electrophoresis. The PCR product from the three independent tubes is labeled at the top as 1, 2 or 3.

Allele discrimination ● TIMING 1 h

26| To discriminate monoallelic from biallelic expression, analyze the sequencing data using sequence-comparison software.

▲ CRITICAL STEP To determine allelic expression patterns, use only data in which all three tubes yielded PCR amplification. Theoretically, if only one or two aliquots showed amplification, the expression pattern could be incorrectly identified as monoallelic.

? TROUBLESHOOTING

● TIMING

Steps 1–7; Preparation of single cell suspension: 1.5 h
Steps 8–11; Collecting single Purkinje cells: ~2 h
Steps 12–15; Reverse transcription: 2 h
Steps 16–18; Split single-cell RT-PCR: 10 h
Step 19; Agarose gel electrophoresis: 30 min
Steps 20–25; Purification of PCR products by PEG precipitation and DNA sequencing: 5 h or more, depending on sequencer used.
Step 26; Allele discrimination: 1 h

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Problem	Possible reason	Solution
Incomplete dissociation of cerebellum (Step 3)	Insufficient enzymatic dissociation	Extend enzymatic dissociation period to 40 min
		Mix vigorously at 180 r.p.m. with inclination of tube
	Low DNase I activity	Increase DNase I concentration to 0.15%
No viable Purkinje cells (Step 9)	Pipetting was too vigorous	Pipette very gently after dissociation
	Too long an enzymatic dissociation period	Shorten the enzymatic dissociation period to less than 40 min
Loss of Purkinje cells during pick-up (Step 9)	Attachment of cell to the inside of glass micropipette	Pre-coat glass micropipette with FBS
No and/or low efficiency of PCR amplification (Step 18)	Degradation of mRNA	Store single cell samples at –80 °C and use within 1–2 months
	Failure to pick up the cell	Visually check both for the presence of the cell in the glass micropipette after picking up cell and for the absence of the cell in the glass micropipette after eluting into PCR tube, under the microscope
	DNA polymerase activity is low	Use *LA-Taq* PCR polymerase in first multiplex PCR
	Unsuitable combination of primers in first multiplex PCR	Carefully determine the primer set and PCR conditions
	Picked cell is dead	Shorten the pick-up time to 2 h. Be sure to select live Purkinje cells, by examining their morphology (see **Fig. 3a**)

TABLE 1 | Troubleshooting table (continued).

Problem	Possible reason	Solution
No amplification of *Pcp-2* (Step 18)	Non-Purkinje cell was selected	Take care to correctly identify the Purkinje cells
Some PCR amplification in control cell-free sample (Step 18)	Cross contamination derived from a different sample	Be sure to use filter-attached pipette tips and a UV sterilization cabinet
	Contaminated reagent	Use a new reagent and use filter-attached pipette tips
	Leaking of mRNA and/or DNA into bathing solution	Shorten the pick-up period to 2 h or less
Low yield of DNA after PEG precipitation (Step 24)	Different MW PEG was used	Use PEG6000
	Low efficiency of second-round PCR amplification	Increase PCR cycles to 40 and increase the second-round PCR volume to 50 μl
	Short incubation at −80 °C	Keep at −80 °C for at least 1 h after adding 20% PEG6000 to PCR products
High background noise in sequence data (Step 26)	Insufficient purification of PCR products	Confirm PEG precipitation. Use an accurate amount of PCR product as the template in the sequence reaction
	Nonspecific PCR byproducts were amplified in second-round PCR	Check PCR conditions and the primer sequences used in second-round PCR
No polymorphism (Step 26)	PCR-amplified region is too short	Extend the PCR-amplified region
	No polymorphism in the gene of interest in the mouse strain	Use another mouse strain. For information, check the National Institute for Genetics (http://www.shigen.nig.ac.jp/mouse/strain/index_e.php)
No sequence data in the direct sequence (Step 26)	Wrong sequence primers were used	Use second-round PCR primers as the sequence primers
	Non-specific PCR byproducts were amplified in second-round PCR	Check the PCR conditions and the primer sequences used in the second-round PCR
Difficulty in discriminating monoallelic *versus* biallelic expression (Step 26)	Low peak and high background-to-noise ratio	Check the PCR conditions and primer sequences used in the second-round PCR
		Use *SeqScape* software in the direct sequence analysis
		Read sequence from both sides using forward and reverse primers
	Settings for the *Seqscape* software are not optimal	Adjust *SeqScape* software settings

ANTICIPATED RESULTS

We devised split single-cell RT-PCR to analyze the allelic expression patterns of multiple genes within a single neuron. The critical points of this protocol are (i) collecting a single living Purkinje cell, (ii) splitting the cDNA from each cell into three aliquots, (iii) using multiplex PCR as the first PCR and (iv) direct sequencing of the PCR products.

Collecting a single living Purkinje cell

Picking up a single living Purkinje cell with a glass micropipette, without contamination by other cells, is one of the most important steps in this protocol. It is accomplished by careful observation under the microscope and retrospective confirmation using PCR of the Purkinje cell marker gene *Pcp-2* (ref. 18). A living Purkinje cell has a large soma and a smooth surface (**Fig. 3a**, white arrowhead), unlike the small soma of other types of living cells or the rough surface of a dead cell (**Fig. 3a** inset, black arrow head). Every living Purkinje cell derived from the P21 mouse cerebellum shows intense PCR amplification of *Pcp-2* (**Fig. 4**). Usually, we can collect 16–28 living Purkinje cells along with 2–4 cell-free control samples within 2 h. After 2 h, even when the cell suspension has been stored on ice, most Purkinje cells appear to be dead (**Fig. 3a** inset, black arrowhead). Although it takes a total of three and a half hours from killing the mouse to finishing collecting single cells, it seems that the preparation time of 3.5 h does not affect gene expression properties, since there was no detectable difference between Purkinje

cells collected after a short preparation time versus those collected over a long preparation time. Also, the results obtained by our split single-cell RT-PCR are consistent with the results obtained by *in situ* hybridization.

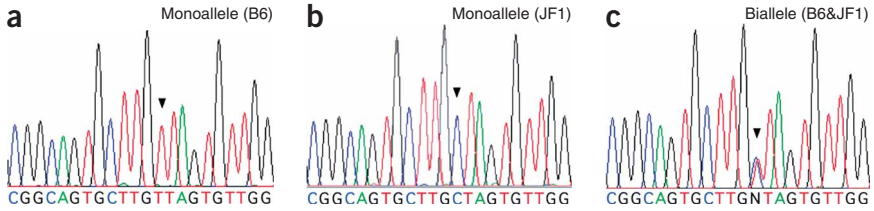

Figure 5 | Direct sequence analysis of the second PCR product. Representative electropherograms of monoallelic expression from the maternal (B6) (**a**) or paternal (JF1) (**b**) allele and the biallelic expression (**c**) of *Pcdh-α7* are shown. The black arrowheads indicate the positions of polymorphisms between the B6 and JF1 strains. The polymorphisms permit determination of the source of each allele.

Splitting the cDNA into three aliquots

A recent report indicates that single-cell RT-PCR analysis performed in one tube can incorrectly indicate monoallelic expression, especially for transcripts of low copy number[3]. In theory, running the procedure with the cDNA sample split into three aliquots drops the risk of misidentifying biallelic gene expression as monoallelic to only 25% of cells analyzed[3]. Therefore, we use three separate cDNA aliquots from each cell as the template for the first PCR. Aliquotting the cDNA into three tubes will prevent a researcher from making an erroneous conclusion based on PCR reactions that result from jackpotting, in which one species amplified by the same primers in the PCR somehow overpowers the reaction and effectively suppresses the other. We have provided results from an actual experiment in **Figure 4** as an example of what might be expected. In general, all three aliquots show uniform and intense PCR amplification of *Pcp-2* and *β-actin* (**Fig. 4**). However, the expression profiles of the *Pcdh* genes in individual Purkinje cells are complicated and differ from cell to cell. For example, cell #5 shows the amplification of *Pcdh-α2*, *α5*, *α10*, *αC1* and *αC2*, whereas cell #7 shows *Pcdh-α3*, *αC1* and *αC2*

TABLE 2 | Primer mixtures for reverse transcription and multiplex PCR.

Experiment	Genes of interest	Primers for RT	Primers for first multiplex PCR
#1	*Pcp-2*	Pcdha Constant R (1)	Pcdha Consensus F (5)
15 genes	*β-actin*	Pcp-2 R (3)	Pcdha Constant R (1)
	Pcdh-α1 to α12	b-actin R (4)	Pcp-2 F (13)
	Pcdh-α consensus		Pcp-2 R (4)
			b-actin F (14)
			b-actin R (4)
#2	*Pcp-2*	Pcp-2 R (3)	Pcp-2 F (13)
17 genes	*β-actin*	b-actin R (4)	Pcp-2 R (4)
	Pcdh-α1 to α12	Pcdha Constant R (1)	b-actin F (14)
	Pcdh-aC1		b-actin R (4)
	Pcdh-αC2		Pcdha Constant F (5)
	Pcdh-α consensus		Pcdha Consensus R (1)
			Pcdha C1F (6)
			Pcdha C2F (7)
#3	*Pcp-2*	Pcp-2 R (3)	Pcp-2 F (13)
6 genes	*Pcdh-αC1*	Pcdha Constant R (1)	Pcp-2 R (4)
	Pcdh-αC2	Pcdhg Constant R (2)	Pcdha Constant R (1)
	Pcdh-γC3		Pcdhg Constant R (2)
	Pcdh-γC4		Pcdha C1F (6)
	Pcdh-γC5		Pcdha C2F (7)
			Pcdhg C3F (10)
			Pcdhg C4F (11)
			Pcdhg C5F (12)
#4	*Pcp-2*	Pcp-2 R (3)	Pcp-2 F (13)
18 genes	*Pcdh-α1 to α12*	Pcdha Constant R (1)	Pcp-2 R (4)
	Pcdh-α consensus	Pcdhg Constant R (2)	Pcdha Constant F (5)
	Pcdh-γA7		Pcdha Consensus R (1)
	Pcdh-γA10		Pcdhg Constant R (2)
	Pcdh-γB1		Pcdhg A7A10 Consensus (8)
	Pcdh-γB7		Pcdhg B1B7 Consensus (9)

The primer mixtures listed are used for the reverse transcription step (primers for RT) and the first-round, multiplex PCR (primers for first multiplex PCR). The second-round PCR is performed using gene-specific nested primers. The primer sequences are shown in ref. 5. The numbers in parentheses are the same as in ref. 5.

TABLE 3 | Distribution of the B6 and JF1 alleles as revealed by split single-cell RT-PCR followed by DNA sequencing.

Cell	tube	*α1*	*α2*	*α3*	*α4*	*α5*	*α6*	*α7*	*α8*	*α9*	*α10*	*α11*	*α12*	*α7/8*	*αC1*	*αC2*	*Pcdh-α* consensus	*Pcp-2*	*β-actin*
	1		JF1								JF1					B6JF1	○	○	○
#1	2		JF1												B6JF1	B6JF1	○	○	○
	3		JF1								B6				JF1	B6JF1	○	○	○
	1	JF1				B6JF1						(α4)		(α4)	B6	B6JF1	○	○	○
#2	2					B6JF1								(α4)		B6JF1	○	○	○
	3	JF1				B6								(α4)		B6JF1	○	○	○
	1		B6JF1													B6JF1	○	○	○
#3	2		B6													B6JF1	○	○	○
	3		JF1												JF1	B6JF1	○	○	○
	1				JF1										B6JF1	B6JF1	○	○	○
#4	2			B6	JF1	JF1									B6JF1	B6JF1	○	○	○
	3			B6	JF1										B6	B6JF1	○	○	○
	1		JF1			B6					JF1				JF1	B6JF1	○	○	○
#5	2		JF1			B6					JF1				B6	B6JF1	○	○	○
	3		JF1			B6					JF1				B6	B6JF1	○	○	○
	1		B6	JF1							JF1				JF1	B6JF1	○	○	○
#6	2		B6	JF1												B6JF1	○	○	○
	3		B6	JF1											B6JF1	B6JF1	○	○	○
	1			B6											B6JF1	B6JF1	○	○	○
#7	2			JF1											B6JF1	B6JF1	○	○	○
	3			B6JF1											B6JF1	B6JF1	○	○	○

To determine whether the amplified PCR products were from the B6 or JF1 allele, we performed direct sequencing of all three tubes for each of seven Purkinje cells. This analysis could not distinguish between B6 and JF1 for *Pcdh-α6*. The samples that were not sequenced are indicated with an open circle.

expression, suggesting there is combinatorial gene regulation of the *Pcdh-α* gene cluster. Furthermore, some aliquots do not show PCR amplification for a particular gene that is present in the other aliquots (see **Fig. 4**, for example, *α1* in cell #2, *α3* in cell #4, *α5* in cell #4). We suppose that the nonequivalent amplification in three aliquots indicates a low amount of transcript.

Using multiplex PCR as the first PCR

Multiplex PCR permits the amplification of many targets of interest within one reaction by using multiple primer pairs. Our multiplex PCR can amplify a Purkinje cell marker (*Pcp-2*), a control house-keeping gene (*β-actin*) and other genes of interest (*Pcdh*). Note that careful determination of the PCR conditions and primer combinations are necessary to get reproducible results. We established four combinations of primer sets for multiplex PCR to investigate *Pcdh* expression: (i) 15 genes including 13 *Pcdh-α* isoforms, *Pcp-2* and *β-actin*; (ii) 17 genes including 13 *Pcdh-α* isoforms, two *C*-type *Pcdh-α* isoforms, *Pcp-2* and *β-actin*; (iii) six genes including two *C*-type *Pcdh-α* isoforms, three *C*-type *Pcdh-γ* isoforms and *Pcp-2* and (iv) 18 genes including 13 *Pcdh-α* isoforms, 4 *Pcdh-γ* isoforms and *Pcp-2* (see **Table 2** for details).

Direct sequencing of PCR products

Direct sequence analysis is a reliable method for detecting polymorphism(s)[19]. Representative electropherograms of monoallelic expression from the maternal (B6) or the paternal (JF1) allele and of biallelic expression are shown in **Figure 5,** and the allelic discrimination results obtained using the PCR products shown in **Figure 4** are summarized in **Table 3**. This method can discriminate monoallelic from biallelic expression, even if there is no restriction enzyme site within the amplified region. However, it is time-consuming to analyze many data sequences manually, and in some cases an indistinct signal, due to background noise, confounds the discrimination. To solve these problems, we use sequence comparison software (for example *SeqScape* software from ABI). The software facilitates the discrimination of monoallelic versus biallelic expression. For a Purkinje cell from a P21 mouse, *β-actin* (high expression level), *Pcdh-αC2* (low expression level) and *Pcdh-γC3* (low expression level) can be used as positive controls for biallelic expression, and *Pcdh-α1–12* and *Pcdh-γA7*, *-γA10*, *-γB1* and *-γB7* can be used as controls for monoallelic expression.

ACKNOWLEDGMENTS We thank H. Kato, M. Kawaguchi and K. Hirano for their assistance and discussion. This work was supported by a Grant-in-Aid for Scientific Research on Priority Areas (Molecular Brain Science) from the Ministry of Education, Culture, Sports, Science and Technology of Japan (14104025 to T.Y.), Grants-in-Aid from the Ministry of Education, Science, Sports, and Culture of Japan (17024034 to T.Y.) and CREST in the Japan Science and Technology Corporation (T.Y.).

COMPETING INTERESTS STATEMENT The authors declare that they have no competing financial interests.

Published online at http://www.natureprotocols.com

1. Chess, A., Simon, I., Cedar, H. & Axel, R. Allelic inactivation regulates olfactory receptor gene expression. *Cell* **78**, 823–834 (1994).
2. Shen, S.I., Gao, C. & Singer-Sam, J. Use of a reverse transcriptase-polymerase chain reaction assay to analyze allele-specific expression in individual hippocampal neurons. *Mol. Genet. Metab.* **63**, 96–102 (1998).
3. Rhoades, K.L. *et al.* Allele-specific expression patterns of *interleukin-2* and *Pax-5* revealed by a sensitive single-cell RT-PCR analysis. *Curr. Biol.* **10**, 789–792 (2000).
4. Esumi, S. *et al.* Monoallelic yet combinatorial expression of variable exons of the protocadherin-α gene cluster in single neurons. *Nat. Genet.* **37**, 171–176 (2005).
5. Kaneko, R. *et al.* Allelic gene regulation of *Pcdh-*α and *Pcdh-*γ clusters involving both monoallelic and biallelic expression in single Purkinje cells. *J. Biol. Chem.* **281**, 30551–30560 (2006).
6. Kohmura, N. *et al.* Diversity revealed by a novel family of cadherins expressed in neurons at a synaptic complex. *Neuron* **20**, 1137–1151 (1998).
7. Wu, Q. & Maniatis, T. A striking organization of a large family of human neural cadherin-like cell adhesion genes. *Cell* **97**, 779–790 (1999).
8. Yagi, T. & Takeichi, M. Cadherin superfamily genes: functions, genomic organization, and neurologic diversity. *Genes Dev.* **14**, 1169–1180 (2000).
9. Hirayama, T. & Yagi, T. The role and expression of the protocadherin-alpha clusters in the CNS. *Curr. Opin. Neurobiol.* **16**, 336–342 (2006).
10. Chess, A. Monoallelic expression of protocadherin genes. *Nat. Genet.* **37**, 120–121 (2006).
11. Murata, Y., Hamada, S., Morishita, H., Mutoh, T. & Yagi, T. Interaction with protocadherin-γ regulates the cell surface expression of protocadherin-α. *J. Biol. Chem.* **279**, 49508–49516 (2004).
12. Hu, J.F., Oruganti, H., Vu, T.H. & Hoffman, A.R. Tissue-specific imprinting of the mouse insulin-like growth factor II receptor gene correlates with differential allele-specific DNA methylation. *Mol. Endocrinol.* **12**, 220–232 (1998).
13. Hashimoto, K. & Kano, M. Postnatal development and synapse elimination of climbing fiber to Purkinje cell projection in the cerebellum. *Neurosci. Res.* **53**, 221–228 (2005).
14. Tamamaki, N. *et al.* Green fluorescent protein expression and colocalization with calretinin, parvalbumin, and somatostatin in the GAD67-GFP knock-in mouse. *J. Comp. Neurol.* **467**, 60–79 (2003).
15. Osada, T. *et al.* Developmental pluripotency of the nuclei of neurons in the cerebral cortex of juvenile mice. *J. Neurosci.* **25**, 8368–8374 (2005).
16. Wu, S.X. *et al.* Pyramidal neurons of upper cortical layers generated by NEX-positive progenitor cells in the subventricular zone. *Proc. Natl. Acad. Sci. USA* **102**, 17172–17177 (2005).
17. Neves, G., Zucker, J., Daly, M. & Chess, A. Stochastic yet biased expression of multiple *Dscam* splice variants by individual cells. *Nat. Genet.* **36**, 240–246 (2004).
18. Oberdick, J., Baader, S.L. & Schilling, K. From zebra stripes to postal zones: deciphering patterns of gene expression in the cerebellum. *Trends Neurosci.* **21**, 383–390 (1998).
19. Taguchi, Y., Koide, T., Shiroishi, T. & Yagi, T. Molecular evolution of cadherin-related neuronal receptor/protocadherin (*CNR/Pcdh*α) gene cluster in *Mus musculus* subspecies. *Mol. Biol. Evol.* **22**, 1433–1443 (2005).