Laser capture microdissection of single neurons with morphological visualization using fluorescent proteins fused to transmembrane proteins

https://doi.org/10.1523/ENEURO.0275-20.2021

Cite as: eNeuro 2021; 10.1523/ENEURO.0275-20.2021
Received: 23 June 2020
Revised: 12 July 2021
Accepted: 15 July 2021

This Early Release article has been peer-reviewed and accepted, but has not been through the composition and copyediting processes. The final version may differ slightly in style or formatting and will contain links to any extended data.

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Title
Laser capture microdissection of single neurons with morphological visualization using fluorescent proteins fused to transmembrane proteins

Abbreviated Title
Stable fluorescent labeling for microdissection

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CCC and AT designed research, analyzed data, and wrote paper. CCC and HTC performed research.

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Number of Figures: 7
Number of Tables: 0
Number of Multimedia: 0
Number of words for Abstract: 250
Number of words for Significance Statement: 110
Number of words for Introduction: 472
Number of words for Discussion: 1372

Acknowledgements
We thank Ms. Miaomiao Han for her technical assistance.

Conflict of Interest
Authors report no conflict of interest.

Funding sources
Abstract

Gene expression analysis in individual neuronal types helps understand brain function. Genetic methods expressing fluorescent proteins are widely used to label specific neuronal populations. However, because cell type specificity of genetic labeling is often limited, it is advantageous to combine genetic labeling with additional methods to select specific cell/neuronal types. Laser capture microdissection is one of such techniques with which one can select a specific cell/neuronal population based on morphological observation. However, a major issue is disappearance of fluorescence signals during tissue processing required for high quality sample preparation. Here, we developed a simple, novel method in which fluorescence signals are preserved. We use genetic labeling with fluorescence proteins fused to transmembrane proteins, which shows highly stable fluorescence retention and allows for the selection of fluorescent neurons/cells based on morphology. Using this method in mice, we laser-captured neuronal somata and successfully isolated RNA. We determined that ~100 cells are sufficient to obtain a sample required for downstream applications such as quantitative PCR. Capability to specifically micro-dissect targeted neurons was demonstrated by ~10-fold increase in mRNA for fluorescent proteins in visually identified neurons expressing the fluorescent proteins compared to neighboring cells not expressing it. We applied this method to validate virus-mediated single-cell knockout, which showed up to 95% reduction in knocked-out gene RNA compared with wild-type neurons. This method using fluorescent proteins fused to transmembrane proteins provides a new, simple solution to perform gene expression analysis in sparsely labelled neuronal/cellular populations, which is especially advantageous when genetic labelling has limited specificity.
Significance statement

Genetic labeling of specific cell types with reporter fluorescent proteins is widely used for gene expression analysis. However, because the specificity of genetic labeling is often limited, an additional method is required to collect samples from a specific cell type. Here, we developed a novel method to allow for selection of a cell/neuronal type of interest based on morphology before performing sample collection using laser capture microdissection. Stable fluorescent signals from fluorescent proteins fused to transmembrane proteins allows us to morphologically select and laser-capture hundreds of single neurons. This method would be applicable to genomic and transcriptomic analysis of different cell/neuronal types and could facilitate our understanding of brain functions.
Gene expression analysis of specific neuronal types has been successfully performed using genetic labeling with reporter fluorescent proteins and fluorescence activated cell sorting (FACS) (Xia et al., 2017, Daigle et al., 2018). However, one technical limitation is that genetic labeling often marks multiple neuronal types and/or other cell types (van Praag et al., 2002, Balthasar et al., 2004, Lagace et al., 2007, Harris et al., 2014) whereas FACS cannot separate such unwanted, labelled cell types. In addition, enzyme-mediated cell dissociation procedures could affect viability and gene expression (Hussain et al., 2018, Ho et al., 2018, Reichard et al., 2018). An alternative method that allows for separating cell/neuronal types without tissue dissociation steps would be useful as a complementary method.

Laser capture microdissection is a technique that dissects a small piece of tissue or a single cell by laser and collect them with gravity or assistance of laser. Instruments for laser capture microdissection are often equipped with a fluorescence light source (Emmert-Buck et al., 1996, Bonner et al., 1996, Fend et al., 2000). Therefore, one can observe the morphology of genetically labeled fluorescent cells and select a cell type of interest to capture. However, in practice, this is not a simple task. Commonly for laser capture microdissection, sections are prepared from freshly frozen tissue, rather than fixed tissue, to acquire high quality RNA. In such sections, fluorescent proteins diffuse out from cells and their fluorescence signals disappear during tissue processing procedures (Singh et al., 2019). Although an optimized protocol can improve the issue to some extent, instability of fluorescence signals makes it difficult to use fluorescence as a guide to distinguish cell types for laser capture microdissection. More stable fluorescence labeling method would be valuable for solving this technical limitation. In this line, studies (Rossner et al., 2006, Chatzi et al., 2019) demonstrated that nuclear targeted fluorescent proteins preserves their signals. However, because only nuclei, but not processes/neurites, is visualized, its application to morphological identification of cell types is limited.

In the course of an experiment for some other purpose, we happened to find that the fluorescence signals from transmembrane proteins fused with fluorescent proteins was extremely stable and kept visualizing dendritic morphology in air-dried, non-fixed brain sections. We later found that similar preservation of fluorescence signals from
transmembrane proteins fused with fluorescent proteins has been previously described in the context of flow cytometry of primary human fibroblasts (Kalejta et al., 1997). This observation made us reason that genetic labeling with fluorescent proteins fused to transmembrane proteins allows us to identify a cell type of interest based on their morphology and selectively capture them using laser capture microdissection. In this study, we tested this possibility and validated that the use of labeling with fluorescent proteins fused to transmembrane proteins allows us to select neurons over non-neuronal cells and perform downstream gene expression analyses.

**Materials and methods**

A flow chart of the methodology is shown in Figure 1. We freshly froze brains after retrieving them from the skull. We sectioned the brains, mounted sections on the glass slides and dehydrated/fixed them with ethanol. We distinguished neurons from glial cells based on their dendritic morphology under a fluorescence microscope, and then harvested the cell bodies of the selected neurons with laser capture microdissection. A total of 100-300 microdissected neurons were pooled for RNA extraction. As examples of downstream gene expression analyses, we performed quantitative PCR (qPCR) analysis after cDNA amplification.

**Mice**

We used 6- to 11-week-old C57BL/6 transgenic mice of either sex, housed in a 12 hours dark/light cycle with *ad libitum* access to water and food. Transgenic lines used were floxed NR1 mice (B6.129S4-Grin1tm2Stl/J) (Tsein et al., 1996) and Pomc-ChR2 mice, which were produced by crossing two transgenic lines, STOCK Tg(Pomc1-cre)16Lowl/J (Balthasar et al., 2004) (back-crossed with C57BL/6 mice) and B6.Cg-Gt(Rosa)26Sortm32(CAG-COP4*H134R/EYFP)Hze/J (Madisen et al., 2012). All three transgenic lines were originally purchased from Jackson laboratory (USA) and bred in our local facilities. All animal procedures were performed according to the approval from the IACUC at Nanyang Technological University.

**Preparation of brain sections and tissue processing**

Mice were deeply anesthetized with isoflurane. Mice were decapitated and their brains were dissected out from the skull within 2 min. The brains were transferred
onto an embedding mold and frozen in embedding medium (O.C.T. compound, Sakura Finetek, Japan) with isopentane on dry ice, which was completed within additional 1-3 min. Brains were stored in -80°C until cryo-sectioning. Immediately before cryo-sectioning, brains were kept in -20°C for at least 30 min. 10-12 µm-thick sections were produced using a cryostat microtome at -20°C. Brain sections were mounted on glass slides and air-dried for 5 min. Sections were dehydrated/fixed in increasing concentration of ethanol, 75%, 95%, and then 100%, for 30 sec each, followed by xylene for 1 min. We performed these procedures immediately before fluorescence visualization and laser capture microdissection.

Fluorescence visualization and laser capture microdissection
We used Zeiss PALM MicroBeam system (Zeiss, Germany) equipped with a fluorescence microscope. Each fluorescently-labeled cell was selected under the microscope with a LD Plan-Neofluar objective lens (40x/0.6NA), and laser-captured into an AdhesiveCap 500 opaque (Zeiss, Germany). A sample of up to ~100 cells were collected in a single AdhesiveCap and lysed with cell lysis buffer. When the collection took more than one hour, we lysed cells to minimize sample degradation and continued collection in a new Adhesivecap.

RNA extraction
After cell lysis in the Adhesivecaps, we extracted RNA using Ambion RNA isolation kit (Thermo Fisher Scientific, USA) according to the manufacturer’s protocol. When more than one Adhesivecaps were used for sample collection, cell lysates from multiple Adhesivecaps were pooled and passed through a single column. RNA concentration was measured using Quant-iT RiboGreen (Thermo Fisher Scientific, USA).

Quantitative polymerase chain reaction (qPCR)
Reverse transcription and cDNA amplification was performed using cDNA SMARTer™ PCR cDNA Synthesis Kit (Takara Bio, Japan) or GeneChip™ WT Pico Kit (Affymetrix, USA). qPCR was performed with iTaq Universal SYBR Green Supermix (Bio-rad, USA) and StepOnePlus™ Real-Time PCR System (Applied Biosystems, USA), using the following gene specific primers: NR1 forward, TGTCCTGGGACTGACTACCC, NR1 reverse, CTGACCAGCAGGATGA, YFP
forward, TATATCATGGCCGACAAGCA, YFP reserve,
TGTTCTGCTGGTAGTGGTCG, DCX Forward, GAGTGCGCTACATTTATACCATTG
DCX reverse, TGACATTCTGTGTACTCAACCT, CaMKIIα Forward,
AGGATGAAGACACCAAAGTGC, CaMKIIα Reverse,
GGTTCAAAGGCTGTATTCC, β-actin forward, AGCCATGTACGTAGCCATCC, β-
actin reverse, CTCTAGCTGTGGTGATGAA. Each sample was amplified in
triple. For Figure 4D, we used mouse liver total DNA that was included in cDNA
SMARTer™ PCR cDNA Synthesis Kit (Takara Bio, Japan).

RNA integrity assay
The quality of RNA samples that we isolated was evaluated by a service provided by
Novogeneait Genomics Singapore Pte LTD, using Agilent RNA 6000 Pico kit and
Agilent 2100 Bioanalyzer (Agilent, USA). The quality of RNA was determined as the
RNA Integrity number (RIN) (Schroeder et al., 2006).

Preparation of viral vectors
We prepared the viral vectors as previously described (Tashiro et al., 2015a). We
used Moloney murine leukemia virus-based retroviral vectors driving the expression
of eNpHR3.0-YFP, mCherry, GFP-F, ChR2(SFFO) or bicistronically eNpHR3.0-YFP
and Cre under the control of the CAG promoter (Zhao et al., 2006) and an adeno-
associated viral vector expressing mCherry-TRPV2 under the control of the CaMKIIα
promoter (Kitanishi et al., 2015).

Stereotaxic injections
Mice were bilaterally injected with retroviral vectors (1.5 µL, titer: 1x 10^7-1 x 10^6
colony forming unit/mL) into the dentate gyrus (Coordinate, AP: 2 mm from the
bregma, ML: 1.5 mm from the bregma; DV: 2.3 mm from skull surface). Preparation
and injection procedures were previously described (Tashiro et al., 2015a). For co-
injection, a mixture of virus solution was prepared with 1:1 ratio in volume.

Perfusion and fixation
For the purpose of visualizing fluorescence expression in neurons (in Figure 2 and
7C), brain sections were prepared from brains that were perfusion-fixed with 4%
paraformaldehyde. The procedure was previously described (Tashiro et al., 2015b).
Brain sectioning was performed in the same way as described in section 175 “preparation of brain tissue”. Sections were mounted on glass slides with coverslips and anti-fade mounting medium containing PVA-DABCO.

Freeze-thaw cycle with HEK 293 cell culture with or without cryoprotectant

We seeded 1X10^5 of HEK293 cells in wells in a 12-well plate. Next day, we transfected the cells with one of the plasmids listed below, with a calcium phosphate method with the following mixture: 500 ng plasmid DNA in 640 µL sterile milliQ water, 36 µL 2M CaCl₂, with 100 µL 2x HEBS (50 mM HEPES, 280 mM NaCl, 10 mM KCl, 1.5 mM Na₂HPO₄, 12 mM D-glucose, pH 7.05). The plasmid DNAs were retroviral transfer plasmids, RV-CAG-GFP, RV-CAG-mCherry, RV-CAG-eNpHR3.0-YFP, RV-CAG-ChR2-mCherry and RV-CAG-ChR2(SSFP)-YFP.

24 h after transfection, we removed culture media from the wells and washed twice with phosphate-buffered saline (PBS). For cells with cryoprotectant, we applied 500 µL 10% DMSO/90% fetal bovine serum on top of the cells before a freeze-thaw cycle. For cells without cryoprotectant, we air-dried and froze/thawed cells without cryoprotectant. We froze the cells by keeping the 12-well plate in a -80°C freezer for 30 min. We then thawed them at room temperature and performed imaging immediately.

Results

Fluorescence signals from fluorescent proteins fused to transmembrane proteins are preserved after tissue processing

In preliminary experiments, we tried to laser-capture single neurons labelled with regular fluorescent proteins, such as the green fluorescent proteins (GFP), which is soluble in the cytoplasm. After tissue processing procedures, fluorescence signals disappeared in sections prepared from freshly frozen brains and our attempt failed. However, in the course of an experiment for some other purpose, we happened to find that fluorescence signals from fluorescent proteins fused to transmembrane proteins is extremely stable even in air-dried, non-fixed brain sections prepared from freshly frozen brains. Under the same conditions, the fluorescence signals of regular fluorescent proteins diffuse and dissipate quickly in the order of seconds to minutes. This preliminary finding led us to test whether labeling with fluorescent proteins fused
to transmembrane proteins is preserved after the standard tissue processing procedure for laser capture microdissection.

Figure 2 illustrates the instability of cytoplasmic fluorescent proteins and stability of fluorescent proteins fused to transmembrane proteins in sections from freshly frozen brains, which we processed by the standard protocol of tissue processing used for laser capture microdissection. We injected a mixture of two retroviral vectors into the dentate gyrus of adult mice (Figure 2A). One of them was for expressing yellow fluorescent proteins (YFP) fused to transmembrane proteins (enhanced halorhodopsin, eNpHR3.0) while the other was for expressing mCherry, which is cytosolic fluorescence proteins. Some of the mice were perfusion-fixed with 4% paraformaldehyde, while the brains of the other mice were freshly frozen without fixation. All brains were then subjected to the standard tissue processing procedure. As expected, paraformaldehyde-fixed brain sections preserved both YFP and mCherry signals (Figure 2B, upper panel). On the other hand, in sections from freshly frozen brains, YFP signals were preserved while mCherry signals were completely lost (Figure 2B, lower panel). Thus, fluorescent proteins fused to transmembrane proteins, but not cytosolic fluorescent proteins, preserve the fluorescence signals after the standard tissue processing procedure for laser capture microdissection. Importantly, dendritic morphology of YFP+ neurons was preserved and clearly visualized in the sections from freshly frozen brains (Figure 2B).

In addition to enhanced halorhodopsin, we tested fluorescent proteins fused to another type of transmembrane proteins, channelrhodopsin-2 (ChR2). We prepared brain sections from 1) POMC-ChR2-YFP mice expressing ChR2 fused to YFP in a subset of granule cells in the dentate gyrus and 2) adult mice that were injected with retroviral vector expressing a stable step function opsin (SSFO) mutant of ChR2 fused to YFP (Figure 2A) into the dentate gyrus. Then we performed tissue processing. As shown in Figure 2C and D, fluorescence signals in neurons were preserved in freshly frozen sections.

ChR2 and eNpHR3.0 are structurally similar proteins. To test whether fusion to a different type of transmembrane proteins also makes fluorescence signals preserved, we prepared brain sections from mice injected with an adeno-associated viral vector
expressing transient receptor potential cation channel V2 (TRPV2) (Figure 2A) into the dentate gyrus. As shown in Figure 2E, fluorescence signals were again preserved in freshly frozen sections.

To test whether membrane-targeted fluorescent proteins with a farnesylation signal are preserved after tissue processing, we injected a retroviral vector expressing green fluorescent proteins fused with a farnesylation signal (GFP-F) (Moriyoshi et al., 1996, Jiang et al., 1998) (Figure 2A) into the dentate gyrus of adult mice. In cells, a farnesyl group is added to GFP-F and facilitates the attachment to cellular membrane. Although sections from brains fixed with paraformaldehyde preserved clear fluorescence labeling of neurons (Figure 2F, upper panel), no fluorescence signal was detected in the sections from freshly frozen brains (Figure 2F, lower panel). Thus, membrane-targeted fluorescence proteins do not preserve its fluorescence signals through the standard tissue processing procedure.

We found that the loss of fluorescence signals from regular fluorescent proteins also occurs in HEK 293 cells after freezing and thawing (Figure 3A). However, when we added cryoprotectant (10% DMSO/90% fetal bovine serum), fluorescent signals were retained. This is presumably because cryoprotectant prevented the freeze-thaw cycle from rupturing the plasma membrane. In contrast, fluorescence signals from fluorescent proteins fused to transmembrane proteins were preserved after a freeze-thaw cycle without cryoprotectant (Figure 3B). The results suggest that the breakage of plasma membrane caused by freezing causes the leakage of regular cytoplasmic fluorescent proteins but still keeps fluorescent proteins fused to transmembrane proteins in HEK293 cells, which is likely same in brain sections.

Laser capture microdissection of morphologically identified neurons

Next, we performed laser capture microdissection of single neurons labelled with fluorescent proteins fused to transmembrane proteins. We injected a retroviral vector expressing eNpHR3.0-YFP into adult mice (Figure 4A) and, after 14 days, harvested their brains and prepared brain sections using the standard tissue processing protocol. When injected into the dentate gyrus of adult mice, the type of retroviral vectors we used in this study is known to mainly transduce in newly-generated neurons in the dentate gyrus (Figure 2B). However, a modest number of non-
neuronal cells are also infected to express transgenes. Under a fluorescence microscope, we were able to distinguish neurons from non-neuronal cells based on their morphology (Figure 4B). The soma of labeled neurons were quickly identified and laser-captured before being photo-bleached by excitation light. Figure 4C shows fluorescence images from sections before and after laser capture microdissection, which demonstrates the specific removal of the fluorescence signals in the soma of single neurons while maintaining surrounding tissue intact.

Successful cDNA preparation and qPCR analysis
We pooled 150 and 300 laser-captured, single neuronal somata and extracted RNA. From these neuronal somata, we acquired 350 and 900 pg of RNA samples, respectively. These yields of RNA are sufficient for reverse-transcription and cDNA amplification using GeneChip™ WT Pico Kit, which requires a minimum of 100-pg sample input. We used 270 pg RNA as sample input and acquired yield of 77.8 µg cRNA and 19.1 µg single strand cDNA. qPCR amplification of the cDNA with a housekeeping gene β-actin showed an amplicon at the expected molecular size on agarose gel electrophoresis (Figure 4D). This result shows that the method for tissue processing and microdissection yields sufficient quality of RNA for downstream qPCR analysis. According to the manufacturer’s instruction, cDNA yield we acquired is also sufficient for a potential application to perform microarray analysis [e.g. Affymetrix Whole-Transcriptome array (Affymetrix, USA)]. Using another sample of cDNA prepared in the same way, we confirmed that the successful laser capture collection of neurons by the amplification of the CaMKIIα (for excitatory neurons) and doublecortin (DCX for newborn neurons) (Figure 4D).

Evaluation of RNA quality
Our method uses conventional freezing, dehydration, laser capture and RNA isolation procedures. There is no extra step to potentially reduce RNA quality, and therefore we expect that we can achieve the isolation of RNA in good quality, which is a requirement for successful qPCR amplification described above. To confirm this expectation, we laser-captured individual neurons in the granule layer one by one and pooled 5000 cells in each sample. Then we evaluated RNA integrity using Agilent RNA 6000 Pico Kit and 2100 Bioanalyzer (Schroeder et al., 2006). As described above, the yield of RNA collected from 100-300 cells was sufficient for
successful downstream qPCR analysis but does not meet the minimum sample requirement for the RNA quality test. Therefore, we used larger samples by pooling 5000 cells for this analysis.

As shown in the electrophoresis images and electropherogram in Figure 5, two distinct bands of 18S and 28S ribosomal RNA were detected. RNA integrity numbers for two samples reached 7.5 and 7.2 (Schroeder et al., 2006). These values indicate good quality of RNA, which is typically obtained by laser capture methods and sufficient for downstream qPCR and transcriptomic applications (Butler et al., 2016; Ong et al., 2020). Thus, we validated that our hydration, laser capture and RNA isolation method produce good quality RNA.

**Specificity of microdissection**

The specificity for microdissection of single neuronal somata can be an issue since a targeted cell and its neighboring cells are often in close proximity. In addition, typical thickness of brain sections (10-15 µm) used for laser capture microdissection allows multiple neuronal soma overlap in z-direction. Therefore, a certain degree of contamination during the isolation is unavoidable. To evaluate specificity of microdissection, we examined YFP gene expression in visually identified YFP+ neurons and YFP− cells. For this experiment, we used POMC-ChR2-YFP mice that express ChR2-YFP in ~10% of granule cells in the dentate gyrus. YFP+ granule cells and neighboring YFP− cells were individually captured (Figure 6A). The identity of YFP− cells was not confirmed but most of them are likely granule cells because we have selected them from the granule cell layer. Approximately 300 cells were pooled for each sample of YFP+ and YFP− neurons. Collected RNA was reverse-transcribed and amplified into cDNA with GeneChip™ WT Pico Kit. The amplification yield of 107 (YFP+) and 102 (YFP−) µg of cRNA and 16.9 (YFP+) and 16.6 (YFP−) µg of ss-cDNA. From the amplified cDNA, the amount of YFP mRNA was quantified by qPCR. The result indicates as low as 10% of YFP mRNA in YFP− cells compared with that in neighboring YFP+ granule cells (Figure 6B), indicating high specificity in microdissection of single neuronal somata.

**Application of the technique to validate retrovirus-mediated single cell gene knockout**
Virus-mediated expression of Cre recombinase has been used to achieve sparse, single-cell gene knockout (Tashiro et al., 2006a, b). Validation of such sparse gene knockout is not straightforward because unaffected, wild-type cells are intermixed. We applied our method of laser-capture microdissection with fluorescent proteins fused to transmembrane proteins, to validate that such virus-mediated single-cell gene knockout affects the expression of a target gene.

We used transgenic mice with floxed NR1 gene, in which expression of Cre recombinase results in the deletion of NR1 gene (also called GluN1) encoding a subunit of N-methyl-D-aspartate receptor (Tashiro et al., 2006a). We injected these mice with a bicistronic retroviral vector expressing YFP-fused enhanced halorhodopsin and Cre (RV-CAG-eNpHR3.0-YFP-IRES-Cre) to knock out the NR1 gene. For control, transgenic mice were injected with a retroviral vector expressing YFP-fused enhanced halorhodopsin only. The vector constructs were described in Figure 7A. Brain sections were prepared 11 days after virus injection (Figure 7B,C). For each sample, we captured >100 YFP+ neurons and pooled them for RNA extraction. We prepared four samples each for control and NR1 knockout. Extracted RNA was reverse-transcribed and amplified into cDNA with GeneChip™ WT Pico Kit. The amplification yielded 63, 101, 203 and 197 µg (Control) and 62, 102, 178 and 210 µg (NR1 knockout) of cRNA and 14.4, 15.2, 14.4 and 14.5 µg (control) and 14, 15.6, 14.6, and 14.5 µg (NR1 knockout) of ss-cDNA. qPCR analysis showed 95 ± 0.077% reduction of NR1 gene in the NR1 knockout neurons compared to the control (Figure 7D,E), validating the successful removal of NR1 gene in Cre-expressing granule cells.

Discussions

In this study, we demonstrated that genetic labelling with fluorescent proteins fused to transmembrane proteins allows for the selective collection of genetically-labelled fluorescent neurons using laser capture microdissection. Fluorescent proteins fused to transmembrane proteins preserve fluorescence signals in sections prepared from freshly frozen brains after the standard tissue processing procedure for laser capture microdissection. Stable fluorescence signals allowed us to distinguish neurons from non-neuronal cells and laser-capture hundreds of neurons easily. We successfully isolated RNA from the collected neuronal somata and determined that ~100 cells are...
sufficient to obtain samples required for downstream applications such as qPCR and DNA microarray analysis.

Compared with conventional protocols of laser capture microdissection, observation and selection of fluorescently labelled cells are the only additional step. This additional step can be easily performed as far as a genetic method is available to label a cell type of interest with fluorescent proteins fused to transmembrane proteins.

While this requirement may be a practical limitation for some applications, recent popularity in the use of optogenetic transmembrane proteins (Deisseroth et al., 2006, Pastrana et al., 2011, Pathak et al., 2013), which are often fused to fluorescent proteins, lowers a hurdle for this requirement. A plenty of transgenic and viral tools expressing channelrhodopsin or halorhodopsin are available commercially and/or from public depositories such as Addgene (http://www.addgene.org), which makes it relatively easy to express fluorescent proteins fused to transmembrane proteins in any neuronal (or other cell) types of interest.

We showed that fluorescent proteins fused to three different types of transmembrane proteins, ChR2, eNpHR and TRPV2, can preserve fluorescence signals. ChR2 and TRPV2 are ion channels formed by multiple subunits with multiple transmembrane regions. eNpHR is an ion pump but its structure has similarity to ChR2. Thus, it is important to note that we tested relatively similar types of transmembrane proteins; therefore it is not clear how much our result would be extended to other types of transmembrane proteins, for example those with a single transmembrane domain.

We do not know why fluorescent proteins fused to those three transmembrane proteins can preserve fluorescence signals, but not regular or membrane-targeted one does. However, it is known that freeze-thaw cycles damage the plasma membrane, and soluble proteins, such as haemoglobin, are released from cells (Lovelock, 1953). Regular cytoplasmic fluorescent proteins are likely to diffuse out from cell bodies in this way. Transmembrane proteins are anchored tightly with and may stay with the plasma membrane while the link of membrane-targeted fluorescent protein might not be strong enough to keep them associated with the membrane.
To validate the specificity of RNA collection from selected cells, we used transgenic mice expressing ChR2-YFP and laser captured YFP+ and YFP− cells separately. qPCR analysis showed 10-fold difference in the amount of YFP mRNA between YFP+ and YFP− cells, demonstrating the high specificity of RNA collection using this method, although a small amount of contamination from neighbouring cells may not be avoidable.

We applied this method to demonstrate the effect of gene removal by viral expression of Cre recombinase in floxed NR1 mice. A bicistronic viral vector expressing eNpHR-YFP and Cre was injected into the dentate gyrus of adult mice, and we laser-captured YFP+ cells. The amount of NR1 mRNA in these YFP+ cells was reduced by up to 95% compared with wild-type neurons. Thus, this experiment provides an example of successfully applying the technique to an actual experiment. This method would be useful for similar experimental needs for validating single cell genetic manipulations (Zong et al., 2005, Judkewitz et al., 2009, Luo et al., 2016, Kaneko, 2017, Nishiyama et al., 2017).

One of a useful application of this method would be to examine gene expression in different neuronal types. This sort of gene expression study has been successfully performed using genetic labelling with regular cytoplasmic fluorescent proteins and fluorescent-based sorting. However, genetic labelling often marks multiple neuronal types and/or other cell types (van Praag et al., 2002, Balthasar et al., 2004, Lagace et al., 2007, Harris et al., 2014), which make it difficult to achieve RNA collection from a single neuronal type. For example, while the expression of parvalbumin is often used to genetically define a subtype of interneurons, it is well known that parvalbumin-expressing interneurons contain multiple neuronal types defined morphologically, including basket cells and axo-axonic cells (Hu et al., 2014, Nassar et al., 2015). In the conventional fluorescent-based sorting, these neuronal types cannot be separated. However, using our method, one should be able to separately laser-capture these two morphologically distinct neuronal types and analyse gene expression of each neuronal type. One caveat for this application is a requirement of relatively sparse labelling to allow for visualization of individual neurons’ dendrites. For example, in Pomc-ChR2-YFP mice that we used in this study, ~10% of granule cells express ChR2-YFP (Uemura et al., 2021) and we can clearly observe their 

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dendrites (Figure 2C). However, in another line of Pomc-ChR2-YFP that we used in a different study, nearly all granule cells express ChR2-YFP; the whole molecular layer of the dentate gyrus, which is occupied by granule cell dendrites, is filled with YFP fluorescence (Åmellem et al., 2021). In this case, we cannot visualize the dendritic morphology of individual neurons. Therefore, not all fluorescence labelling methods work for this approach, and one may need to select a method for relatively sparse labelling (Badea et al., 2009; Zong et al., 2005, Judkewitz et al., 2009, Luo et al., 2016, Kaneko, 2017, Nishiyama et al., 2017).

Some of morphological classification of neuronal types are largely subjective and may be difficult to reach agreement between individual researchers. Or even if they are visually clearly distinguishable, there is no common ground for judging whether they should be classified as separate neuronal types. We believe that our method could provide a solution for this issue by giving an independent rationale for such “subjective” classification criteria. For this purpose, one needs to apply a “subjective” morphological classification and perform gene expression analysis of those neuronal types using our method. If the two neuronal types have clear difference in gene expression, one can conclude that they are two different neuronal types. If they do not have difference in gene expression, one can conclude that they may not be different neuronal types. Furthermore, if some genes are expressed only in one of two neuronal types, one can use them as a marker for the neuronal type, which would make it easier to further confirm and utilize the classification in future studies.

When it is difficult to make clear morphological criteria, one can test multiple subjective criteria. When the criteria separate two neuronal types better, difference in gene expression would be clearer. Based on this idea, one determines the best version of morphological classification in terms of difference in gene expression. Our method should be helpful to provide such tentative classification of neuronal subtypes based on morphology with an independent basis/rationale in terms of gene expression.

Related to the importance of morphological visualization, fluorescent proteins fused to transmembrane has an additional advantage. Compared with cytosolic fluorescent proteins, fluorescent proteins fused to transmembrane fluorescence proteins distribute along cellular membrane and visualize small processes better than
cytosolic fluorescent proteins (Moriyoshi et al., 1996)(Figure 2B). Such clearer visualization of dendritic morphology facilitates the identification of neuronal types.

In addition to gene expression profiling, laser capture collection of selective neuronal population may be applied to different types of genomic analyses. Such applications include ChIP-seq to identify binding sites for DNA-binding proteins (Park, 2009), ATAC-seq to map chromatin accessibility (Buenrostro et al., 2013) and ChIA-PET, Hi-C and HiChip to examine tridimensional chromatin conformation (Fullwood and Ruan, 2009; van Berkum et al., 2010; Mumbach et al., 2016).

Our study demonstrated the stability of fluorescence signals of fluorescent proteins fused to transmembrane proteins, which allows for selectively collecting a cell type of interest with laser capture microdissection. We validated that RNA sample isolated with this method is suitable for the downstream gene expression analysis experiments such as qPCR. The isolation of single neurons is specific with minimum contamination from neighbouring cells. This methodology provides a novel alternative approach for gene expression analysis targeting a specific neuronal/cell type, which is especially advantageous when the specificity of genetic labelling is not sufficient and additional selection based on morphology is required.
Figure legends

Figure 1: Flow chart of methodology.

Figure 2: The signals of fluorescent proteins fused to transmembrane proteins are preserved after the standard tissue processing procedure for laser capture microdissection. (A) Schematic structures of the viral vector genomes. (B-F) Images of fluorescently-labelled granular cells in the dentate gyrus of (B) mice injected with a mixture of two retroviral vectors expressing YFP-fused enhanced halorhodopsin, eNpHR3.0-YFP, or mCherry, (C) POMC-ChR2-YFP transgenic mice, and mice injected with (D) a retroviral vector expressing ChR2(SSFO), (E) an adeno-associated viral vector expressing TRPV2 and (F) a retroviral vector expressing a membrane targeted form of GFP, GFP-F. Brain sections were prepared from paraformaldehyde fixed (top) or freshly frozen brains and went through the standard tissue processing procedure. Note that fluorescence signals of eNpHR3.0-YFP (B), ChR2-YFP (C), ChR2(SSFO)-YFP (D) and mCherry-TRPV2 (E), but not mCherry (B) and GFP-F (F), were preserved in sections prepared from freshly frozen brains. Arrows and arrowheads in A indicate somata labelled by eNpHR3.0-YFP and mCherry. RV: retroviral vector, AAV: adeno-associated viral vector, LTR: long terminal repeat, CAG: the CAG promoter, WPRE: Woodchuck hepatitis virus post-transcriptional regulatory element, CaMKIIα: the CaMKIIα promoter, ITR: inverted terminal repeat. Scale bars: 100 µm.

Figure 3: Cryoprotectant prevents the loss of fluorescence signals from regular fluorescent proteins in HEK293 cell culture after a freeze-thaw cycle. A) Representative images showing fluorescence signals from regular, cytosolic fluorescent proteins (GFP and mCherry) after a freeze-thaw cycle with or without cryoprotectant. Fluorescence signals disappeared without cryoprotectant while cryoprotectant preserved fluorescence signals. B) Representative images showing fluorescence signals from fluorescent proteins fused to transmembrane proteins [eNpHR3.0-YFP, ChR2-mCherry, ChR2(SSFO)-YFP]. Fluorescence signals were preserved without cryoprotectant after a freeze-thaw cycle. Scale bar: 50 µm.

Figure 4: Laser capture microdissection of fluorescence labelled neurons.
(A) A schematic structure of the viral vector genome. (B) Images of eNpHR3.0-YFP-expressing neurons and non-neuronal cells in the dentate gyrus of mice which were injected with a retroviral vector expressing eNpHR3.0-YFP. (C) Fluorescence images of sections before and after laser capture microdissection. Red dotted ovals show the location of somata of fluorescently labelled neurons selected for laser capture microdissection. Blue dots with yellow labels indicates the centre of selected area used for laser capture. Scale bar: 20 µm. (D) An electrophoresis image showing PCR amplification end products for the DCX, CaMKIIα and β-actin genes. Note high expression of neuronal markers in fluorescently labelled neurons compared with a total liver RNA sample where DCX and CaMKIIα were not detected. M: Molecular marker, NTC: Non-template control.

Figure 5: RNA quality assay. (A) Electrophoresis images of RNA samples. Clear bands of 18S and 28S ribosomal RNAs are visible. Ladder: 0.2, 0.5, 1.0, 2.0, 4.0 and 6.0 kb. (B) Electropherogram showing the distinct peaks of 18S and 28S ribosomal RNAs. FU: Fluorescence Unit, Pink and thick green solid lines: baseline for 18S and 20S peaks, respectively, Thin green solid line: overall baseline.

Figure 6: Evaluation of specific RNA collection from neurons targeted by laser capture microdissection. (A) Images of brain sections sparsely containing YFP+ neurons in the dentate gyrus. The brain sections were prepared from Pomc-ChR2-YFP mice. YFP+ neurons (top) and their neighboring, YFP− cells (bottom) were visually selected for microdissection (red dotted ovals). Scale bar: 20 µm. (B) Relative amount of YFP RNA in YFP+ neurons and their neighboring YFP− cells, quantified by qPCR analysis.

Figure 7: Validation of retrovirus-mediated, single-cell gene knockout in adult-born neurons in the dentate gyrus. (A) Schematic structures of the viral vector genomes. (B) Experimental time line. (C) Images of the dentate gyrus of floxed NR1 mice which was injected with a bicistronic retroviral vector expressing eNpHR3.0-YFP and Cre. Scale bar: 50 µm. (D) qPCR amplification end products in gel electrophoresis. M: Molecular marker, KO: NR1 Knockout neurons with Cre expression, Control: control neurons without Cre expression, NTC: Non-template control.
control. (E) qPCR quantification of NR1 RNA in Cre-expressing (NR1 KO) and control (Con) adult-born neurons. Note ~95% reduction in Cre-expressing neurons.


Ramirez JM, Jones AR, Svoboda K, Han X, Turner EE, Zeng H (2012) A toolbox of


