Gene Expression Analysis by Multiplex Single-Cell RT-PCR

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► To cite this version:

Ludovic Tricoire, Bruno Cauli, Bertrand Lambolez. Gene Expression Analysis by Multiplex Single-Cell RT-PCR. Glutamate Receptors, pp.139-154, 2019, 10.1007/978-1-4939-9077-1_10 . hal-03044302

HAL Id: hal-03044302
https://hal.sorbonne-universite.fr/hal-03044302v1

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Metadata of the chapter that will be visualized online

Chapter Title	Gene Expression Analysis by Multiplex Single-Cell RT-PCR	
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Abstract

Brain circuit assemblies comprise different cellular subpopulations that exhibit morphological, electrophysiological, and molecular diversity. Here we describe a protocol which, combined with whole-cell patch-clamp recording and morphological reconstruction, allows the transcriptomic analysis of the recorded cell. This protocol provides recipes on how to detect simultaneously the expression of 24 genes/markers at the single-cell level using polymerase chain reaction (PCR),

how to design gene-specific probes, and how to validate them. This technique provides multiplexed expression data that cannot be easily obtained by other approaches such as immunological co-labeling.

Keywords (separated by '-') mRNA - cDNA - Cell type - Transcriptome - Electrophysiology

Chapter 10 1

Gene Expression Analysis by Multiplex Single-Cell RT-PCR 2

Ludovic Tricoire, Bruno Cauli, and Bertrand Lambolez 3 AU1

Abstract 4

Brain circuit assemblies comprise different cellular subpopulations that exhibit morphological, electrophysiological, and molecular diversity. Here we describe a protocol which, combined with whole-cell patch-clamp recording and morphological reconstruction, allows the transcriptomic analysis of the recorded cell. This protocol provides recipes on how to detect simultaneously the expression of 24 genes/markers at the single-cell level using polymerase chain reaction (PCR), how to design gene-specific probes, and how to validate them. This technique provides multiplexed expression data that cannot be easily obtained by other approaches such as immunological co-labeling. 5–11

Key words mRNA, cDNA, Cell type, Transcriptome, Electrophysiology 12

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1 Introduction 13

Figure 1 outlines the general protocol for multiplex single-cell PCR (scPCR). After recording of a cell in the whole-cell configuration of the patch-clamp technique, cell content is collected into the electrode and expelled into a test tube. Reagents are then added to perform first-strand complementary DNA (cDNA) synthesis from harvested mRNAs. Following overnight incubation of the reverse transcription (RT) reaction, a PCR is set in the same tube to amplify multiple cDNAs of interest by mixing all different primer pairs. Hence, the procedure is fast and simple because one tube corresponds to one cell and few manipulations are required. The purpose of the first PCR round is to increase the numbers of cDNA copies to ensure a reliable aliquoting for subsequent re-amplification. Indeed, primer dimers and nonspecific primer-amplicon interactions substantially decrease multiplex PCR amplification efficiency. The first PCR products are re-amplified through a second gene-specific PCR to achieve consistent detection by agarose gel electrophoresis. Besides flexibility and robustness, a major advantage of scPCR resides in its speed and ease of application, which allows rapid feedback between functional and molecular analyses. These 14–32

Corinna Burger and Margaret Jo Velardo (eds.), *Glutamate Receptors: Methods and Protocols*, Methods in Molecular Biology, vol. 1941, https://doi.org/10.1007/978-1-4939-9077-1_10, © Springer Science+Business Media, LLC, part of Springer Nature 2019

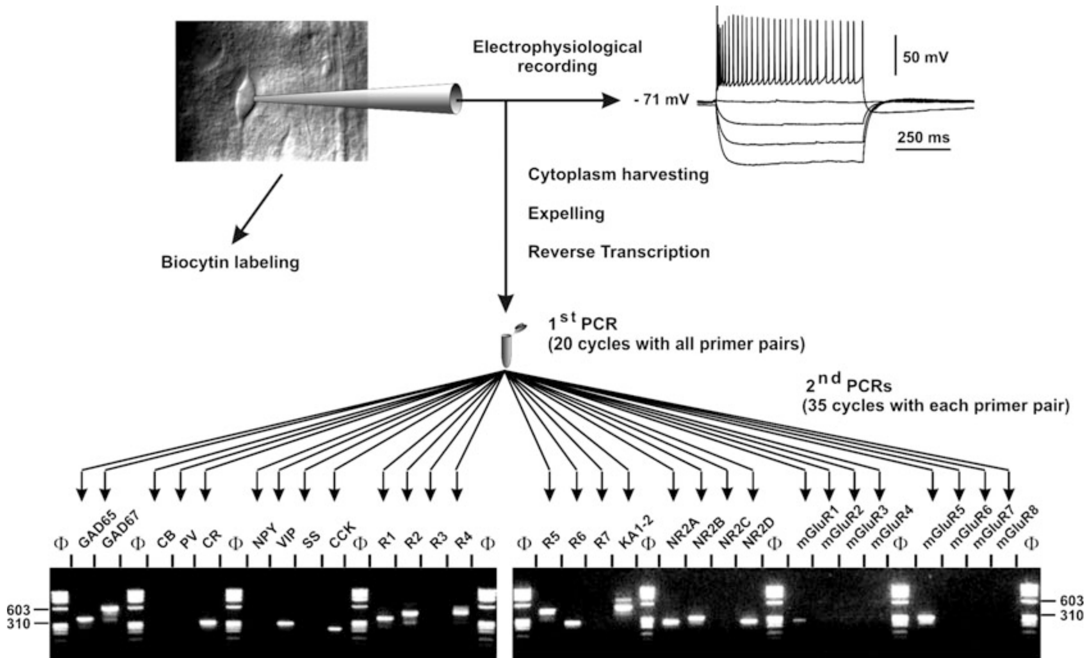

Fig. 1 Gene expression profiling by multiplex scPCR. In the first amplification step, all cDNAs of interest are co-amplified by mixing their specific PCR primers together. PCR products are then individually re-amplified using their specific PCR primers and analyzed by agarose gel electrophoresis. Note that expression of 16 genes was detected in this cortical GABAergic neuron among the 31 genes probed, helping to determine cell-type-specific expression profiles (*see* [21] for the list of genes analyzed in this cell). During the recording, biocytin present in the intracellular solution diffuses in the neuron and allows post hoc morphological reconstruction

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properties prove essential to establishing correlations between molecular and functional properties at the single-cell level, beyond the large cellular diversity of brain tissues. In parallel with the recording, biocytin included in the intracellular recording solution diffuses throughout and fills the patched cell allowing performing morphological analyses.

1.1 Correlating mRNA Expression and Functional Properties of Single Cells

The scPCR technique was initially designed to establish correlates between the functional properties of native glutamate receptor channels of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) subtype and their mRNA expression [1]. AMPA receptors are multimeric assemblies of four different subunits GluA1–4 and mediate fast excitatory synaptic transmission in the central nervous system (CNS). Further diversity is generated by alternative splicing and mRNA editing. The initial scPCR report established the reliability and selectivity of this technique by showing that single cerebellar Purkinje neurons expressed a relatively constant set of AMPA receptor subunit mRNAs, which differed from that found in cerebellar granule cells [1].

The functional relevance of scPCR analyses was next assessed by demonstrating that the calcium permeability of AMPA receptor is determined by transcriptional control of the expression level of the GluA2 subunit. This was established by showing that the abundance of the GluA2 mRNA relative to that of other subunits correlates with the channel properties of native AMPA receptors in different cell types [2, 3]. Besides its scientific importance, the Jonas et al. [3] study was the first to report scPCR from acute brain slices, which preserve the cytoarchitecture and connectivity of brain tissues.

1.2 Quantitative Analyses by scPCR

Studies of AMPA receptors relied on relative quantification between AMPA receptor subunit mRNAs and their splice and editing variants expressed by single neurons. This was made possible by the fact that a single primer pair co-amplified the four subunit cDNAs and that their original mRNA ratios were maintained throughout the whole RT-PCR process [1, 4]. This relative quantification was performed by using subunit-specific restriction enzymes following amplification with radiolabeled primers [5] or fluorescently labeled primers [6, 7]. This approach has been recently used to quantify the expression ratio of GluD1 and GluD2, the two members of the delta family of glutamate receptors [7].

The issue of comparing mRNA expression levels between different cells has been further addressed using real-time PCR following patch-clamp harvesting of the cell's content and RT reaction as described above [8, 9]. It is noteworthy that in the procedure described by Tricoire et al. [9], the protocol of the first PCR is modified to obtain a linear pre-amplification of the target cDNA. The second PCR step is replaced by a real-time PCR reaction based on TaqMan chemistry (Applied Biosystems). In a single well, the gene-specific amplification is performed at the same time as the amplification as a reference gene allowing normalization. The amplifications of the two genes in a single well are followed on two separate fluorescence channels. Absolute quantification of AMPA receptor subunit mRNAs expressed by single neurons was performed by introducing just prior to the RT an internal standard consisting in a full-length GluA2 RNA bearing a point mutation [4]. This allowed determining the number of AMPA receptor mRNA copies harvested by patch-clamp, estimating the proportion of AMPA receptor mRNA harvested from single neuron and their respective distribution in somatic versus neuritic compartments.

1.3 Molecular and Functional Phenotyping of Neuronal Types

Initially, scPCR was developed to quantify expression of a homologous gene, but then it was extended to the detection of multiple unrelated genes in single cells (as outlined in Fig. 1), aimed at establishing detailed electrophysiological, gene expression, and morphological phenotypes of single neurons [10–12]. This

approach was further used to correlate pharmacological response to the expression of cognate receptors [13–16]. This approach has significantly contributed to establishing the distinctive molecular, functional, and morphological phenotypes of the diverse neuronal types in the cerebral cortex and in the hippocampus [9–11]. Finally, these multivariate phenotypes have been used to classify cortical and hippocampal neuronal types based on unsupervised multiparametric clustering analyses, hence establishing their statistical significance on a large array of features [9–11, 17, 18].

Recently, studies have reported the development of RNA sequencing on single neurons after whole-cell patch-clamp recording [19, 20]. The entire somatic compartment is aspirated into the recording pipette. After reverse transcription of RNA and cDNA amplification, a sequencing library is prepared. Although this approach appears quite extensive, it is difficult to implement in a classical molecular biology lab and requires high capacity of sequencing and expensive equipment. The protocol described further is easier to implement and provides results very quickly and does not require complex analysis of the data. We routinely use this protocol to probe expression of 30 or more different genes from single neurons (e.g., [21]). In this chapter, the protocol is given for the simultaneous analysis of 24 genes per cell and is based on a 96-well format.

2 Materials

2.1 Reagents (See Note 1)

1. RNase-free H_2O.
2. RNase-free dithiothreitol (DTT).
3. Hexamer random primers.
4. Deoxynucleotide (dNTP) solution set.
5. Oligonucleotide primers for PCR. Desalted grade (resuspended at 100 μM).
6. Recombinant RNase inhibitor (40 units/μl).
7. Reverse transcriptase (RT; 200 units/μl).
8. Taq DNA polymerase (5 U/μl).
9. Agarose.
10. 0.5× Tris-borate-EDTA buffer (TBE).
11. DNA ladder.

2.2 Equipment

1. Glass pipettes.
2. Powder-free gloves.
3. 96-well PCR plate suitable for your thermal cycler.
4. Adhesive and aluminum seals suitable for the 96-well PCR plate.

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5. Filter tips. Do not stand autoclave. 142
6. 1.5 ml, 2 ml, and 5 ml plastic tubes. 143
7. Homemade expeller. It is made by assembling an old pipette holder with a 10–20 ml syringe. This piece of equipment is used to expel the intracellular solution containing the cytoplasmic harvest into a clean tube. 144–147
8. 0.5 ml thin wall PCR tubes. The tubes have to match the size of the wells of the PCR machine used. Given the high efficiency required in each step of the reaction, this point has to be stressed. 148–151
9. Programmable thermal cycler. 152
10. Electrophoresis system suitable for loading with multichannel pipette. 153–155

2.3 Equipment Setup

1. Glass pipettes have to be heated to 150–200 °C for 2 h to destroy RNase. RNase cannot be inactivated by autoclaving. 156–157
2. A dedicated workbench space must be set up to prepare the first and second PCR reaction. The plate containing the second PCR reaction should never be brought back on this bench to avoid contamination by PCR DNA. Reverse transcription reaction for primer validation must be set up on a different workbench to avoid contamination by mRNA and cDNA. 158–164

2.4 Reagent Setup for Reverse Transcription

1. 20× DTT. A 1 M solution (154 mg in 1 ml H_2O) is prepared in water and filter sterilized. Working solution (20×) is 0.2 M in H_2O stored as 50 μl aliquots in 500 μl tubes. 165–167
2. 5× RT mix. Stock solution of random hexamer primers is dissolved in TE pH 7 at 3 μg/μl (770 μM). Each dNTP is supplied as a 100 mM solution. A working RT mix solution (5×) of random primers and dNTPs is prepared. For 300 μl: 168–171 AU2

   This working mix is stored as 21 μl aliquots in 500 μl tubes at −80 °C. 172–173
3. Reverse transcriptase and RNase inhibitor must be aliquoted (5 μl). 174–175
4. All these reagents must be stored at −80 °C until the day of use and then kept at −20 °C during the day. 176–178

2.5 Reagent Setup for PCR

2.5.1 First PCR Primer Mix

1. Make a 50× dNTP (2.5 mM each dATP, dCTP, dGTP, dTTP). 179

   20 μl of each 100 mM dNTP. 180

   720 μl RNase-free H_2O. 181

   Dispense in 100 μl aliquots. 182
2. Make PCR primer mix. All primers designed for the first PCR must be mixed to reach a final concentration of 1 pmol/μl. In a 5 ml tube, add: 183–185

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832 µl H_2O. 186

16 µl each primer at 100 µM (48 primers = 24 genes). 187

Dispense in 64 µl aliquots, i.e., one aliquot for three cells (20 µl per PCR, i.e., 20 pmoles of each primer). 188 189

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2.5.2 Second PCR Primer Mix

1. Prepare one primer mix for each gene to be analyzed. Primer concentration in this mix is 2 pmoles/µl. 191 192
   From primers stocks at 100 µM: 193

   18 µl primer sense. 194

   18 µl primer antisense. 195

   864 µl H_2O. 196
2. Transfer primer mixes in a 96-well plate. The plate consists of 8 rows (labeled from A to H) and 12 columns (numbered from 1 to 12). The 24 primer mixes must be deposited in 3 columns of 8 wells. For gene #1, dispense 55 µl of the primer mix in wells labeled A1, A4, A7, and A10. 197 198 199 200 201
3. Repeat **step 2** with three plates. 202
4. For gene #2, repeat **steps 2** and **3** by dispensing the corresponding primer mix in wells B1, B4, B7, and B10. 203 204
5. Repeat **step 4** for other genes to be analyzed. The four plates should be completely filled with 55 µl of primer mix in each well. Cover the plates with plastic seal and store them at −20 °C. 205 206 207 208

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2.6 Contamination Prevention

Contamination is of course one main concern when using PCR. In this instance contamination by either RNase or DNA or RNA molecules must be carefully avoided. Although the absence of contamination for a given PCR reaction has to be routinely tested (*see* below and in the example section), we have found that the observance of a few rules efficiently prevents contamination. RNase contamination does not seem to be a major problem, and using standard procedures (*see* below), contamination, either from reagents, solutions, or labware, can be avoided. At the beginning of the day, the silverware of the recording electrode needs to be rechlorinated with bleach to inactivate possible RNase contamination of the wire. 210 211 212 213 214 215 216 217 218 219 220 221

Contamination by laboratory plasmids is the major problem given the enormous amount of molecules produced (for instance, 1 µl of a 1 mg/ml solution of a 6 kb plasmid contains 1011 copies of the insert). Our advice is therefore not to set up any of the reactions for single-cell RT-PCR in a room used to manipulate plasmids containing the cDNA to be detected. Even contamination of non-disposable labware by washing is a concern in this case. The same advice is given for in vitro transcripts, although the problem is less critical. 222 223 224 225 226 227 228 229 230

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As a rule we have a set of pipettes especially reserved for single-cell RT-PCR and related preliminary tests. With these pipettes we never manipulate natural RNA or cDNA solutions containing more than 1 ng/μl. We never use these pipettes for plasmids or in vitro transcript solutions containing more than 10^3 molecules/μl. In addition, these pipettes are never used either for solutions containing PCR-amplified fragments. Although we worked for 1 year without aerosol-blocking tips and avoided contamination, we now routinely use them.

All solutions are kept as aliquots, rather than in stock.

Keep in mind that pH electrodes can be a source of contamination.

We always wear gloves.

Harvesting of mRNA from cells surrounding the target cell or released from dying cells is yet another possible source of contamination. Thus it is important to have good quality slices and to include, in the set of gene, one gene that is only express in surrounding cells (*see* **Note 2**).

3 Methods

3.1 Design of Gene-Specific Primers

Before starting the design of the primer, you need to collect as much information regarding the structure of your gene of interest such as the presence of introns and whether splice variants have been described in the literature. During the harvesting procedure, it is possible to harvest some genomic DNA. If your gene is intronless, it is impossible to distinguish amplification from genomic DNA or from mRNA-derived cDNA. Therefore we recommend designing primers on two different exons. You have to make sure of the sequence of your coding sequence and that it is the most updated. As much as possible, use the sequences labeled "reference sequence" in Genbank. You can get this information on the page of your gene of interest in Genbank (http://www.ncbi.nlm.nih.gov/gene/). This page also indicates the genomic structure of your gene. Usually in reference sequence, the beginning and end of exons are given along with the sequence.

The requirement for designing primers for single-cell RT-PCR is the same as for a basic PCR. The melting temperature should be around 60 °C; the %GC should not exceed 60%. They should not form secondary structure such as hairpin, and the two primers should not hybridize to each other. Any program for primer design takes into account these parameters. An example of such program is Primer3 (http://primer3.ut.ee/). The size of the PCR product is typically in the range 200–500 bp.

Primers should be specific for your gene of interest and should not significantly recognize other gene sequence. To check the specificity of the designed primers, you can run a nucleotide blast

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search (NCBI website) of your sequence against the nucleotide collection of Genbank using the blastn program. Alternatively, you can use the primer-BLAST program of NCBI that designs in a single step the sequence of the primers using primer3 and run the BLAST against reference sequence database. 277 278 279 280 281

When possible, it is better if the primers for the second PCR are different from those of the first PCR. The use of internal (nested) primers during the second PCR round enhances amplification specificity and minimizes the carry-over of primer dimers from the first PCR, improving amplification efficiency and specificity. It increases the confidence in the identity of the PCR product and further guarantees that you are not amplifying another gene that shares some homology with your gene of interest. Depending on the gene, it is complicated to design two different sets of primers. You can use a common primer for the two PCRs. In the worst-case scenario, the primers can be the same for the two PCRs. 282 283 284 285 286 287 288 289 290 291 292 293

In case your gene is intronless, the approach for the design is the same, but you have to include in your PCR a control reaction intended for the detection of genomic DNA. We routinely probe the presence of the intron of somatostatin gene [15]. After the second PCR, if we obtain a positive result for this intron, we discard the results of intronless genes obtained from the same cell harvesting. 294 295 296 297 298 299 300

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3.2 Validation on Total RNA

1. Prepare mRNA from tissue known to express your genes of interest. We routinely prepare total RNAs using Trizol according to manufacturer instructions. 302 303 304
2. Perform reverse transcription in 20 μl total volume using reagent provided with the enzyme: 305 306

   1 μg of RNA. 307

   4 μl 5× RT mix. 308

   4 μl 5× first-strand buffer. 309

   l RNase-free H_2O. 310 AU3
3. Warm at 65 °C for 5 min and then cool down on ice for 1 min. 311
4. Add: 312

   2 μl 0.1 M DTT. 313

   1 μl RNase inhibitor. 314

   1 μl reverse transcriptase. 315
5. Incubate overnight at 37 °C. 316
6. Dilute cDNA to a final concentration of 1 ng/μl. 317
7. Prepare one PCR reaction per primer pair to test: 318

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cDNA @ 1 ng/μl	1 μl
50× dNTP mix	2 μl
10× Taq polymerase buffer	10 μl
Taq polymerase (5 U/μl)	0.5 μl
RNase-free H_2O	To 80 μl

8. Incubate for 1 min at 95 °C in the PCR instrument, and then add 20 μl of primer mix containing forward and reverse primers in RNase-free H_2O (primer final concentration: 2 pmol/μl).
9. Run PCR program:

One time:	95 °C/3 min
40 times:	95 °C/30 s
	60 °C/30 s
	72 °C/35 s
One time:	72 °C/10 min
Then cool down at 15 °C	

10. Analyze your PCR product by agarose gel electrophoresis. You must verify you obtain a single band at the correct size. We routinely run all PCR reactions on 2% agarose gel in 0.5× TBE buffer next to a DNA molecular marker.
11. Mix and dilute the primers for the first PCR (48 different primers) at 1 pmol/μl in water. This mix can be aliquoted and stored at −20 °C.
12. Set up the first PCR reaction:

cDNA at 1 ng/μl	1 μl
dNTP at 2.5 mM each	2 μl
10× Taq polymerase buffer	10 μl
Taq polymerase (5 U/μl)	0.5 μl
RNase-free H_2O	To 80 μl

13. Incubate 1 min at 95 °C in the PCR machine, and then add 20 μl of first PCR primer mix prepared in Subheading 2.5.
14. Run PCR program:

One time:	95 °C/3 min
20 times:	95 °C/30 s
	60 °C/30 s

(continued)

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	72 °C/35 s
One time:	72 °C/10 min
Then cool down at 15 °C	

15. Set up the second PCR.

	For one gene	Mix for 24 genes (×25)
RNase-free H_2O	75.5 µl	1887.5 µl
10× Taq buffer	10 µl	250 µl
50× dNTP	2 µl	50 µl
First PCR reaction	2 µl	50 µl
Taq polymerase (5 U/µl)	0.5 µl	12.5 µl

16. Transfer 90 µl of the mix in 24 PCR tubes, one per gene to analyze.
17. Add 10 µl of gene-specific primer mix in each tube.
18. Run the PCR program:

One time:	95 °C/3 min
35 times:	95 °C/30 s
	60 °C/30 s
	72 °C/35 s
One time:	72 °C/10 min
Then cool down at 15 °C	

19. Analyze your PCR product by agarose gel electrophoresis. You must verify you obtain a single band of the expected size. In case one or several bands are absent, it indicates that some primers are incompatible, and a new set of primer pairs have to be designed.
20. It is important to perform negative control experiments from time to time to check the absence of contamination (*see* **Note 2**). This is primarily achieved by running the same protocol as described above using no RNA (H_2O control). You also have to perform the protocol described in Subheadings 3.4–3.6 using just intracellular recording solution. This step allows you to check the quality of the recording solution.

3.3 Patch-Clamp Harvesting of Single Cells

A key issue in single-cell molecular biology, especially in tissue slices, is to harvest selectively the cellular content without contamination from surrounding cells. The whole-cell configuration of the patch-clamp technique confers a tightly insulated physical and

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electrical access to the cell's cytoplasm. It thus provides ideal means of harvesting in complex tissue following electrophysiological characterization. Patch-clamp harvesting of a GABAergic neuron from a slice of rat cerebral cortex is illustrated in Fig. 2. 388 389 390 391

The very first step of the single-cell RT-PCR protocol starts with the electrophysiological recording of the neuron to be analyzed. The cell is first visually identified and chosen according to morphological criteria. The patch pipette filled with 8 μl intracellular solution (*see* **Note 3**) is then advanced toward the neuron with positive pressure in order to avoid contamination by the surrounding tissue. 392 393 394 395 396 397 398

Once the pipette reaches the neuron, a gigaohm seal is obtained which ensures a very tight contact between the cell membrane and the pipette avoiding the risk of contamination (cell-attached configuration). The patch membrane under the pipette is then broken by applying a brief negative pressure establishing a physical and electrical continuity between the cytoplasm and the pipette solution. At the end of the whole-cell recording, which 399 400 401 402 403 404 405

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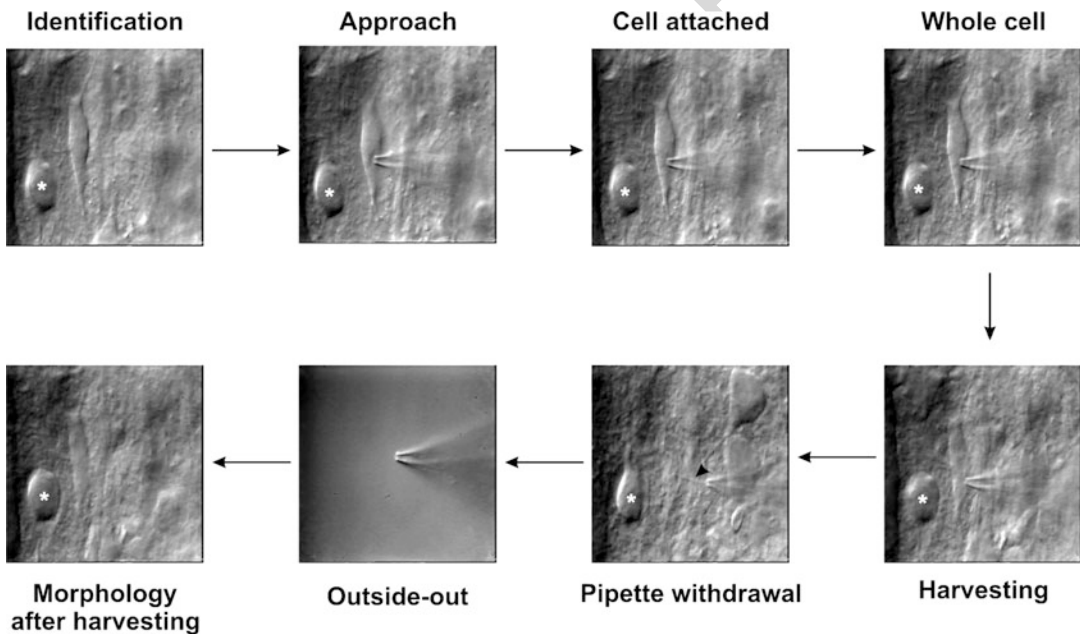

Fig. 2 Patch-clamp harvesting in brain slices. Once a cell is visually identified, the recording pipette is approached with positive pressure in order to avoid cellular debris contamination at the tip of the pipette. Note the dimple on the membrane of this neuron. Positive pressure is then interrupted in order to form a cell-attached configuration with a gigaohm tight seal, and subsequently brief suctions allow going into whole-cell configuration. At the end of the recording, the cytoplasm is harvested by applying a gentle negative pressure into the pipette, while the tight seal is maintained. Note the shrinkage of the cell body during the harvesting procedure. The recording pipette is then gently withdrawn to form an outside-out patch, which favors cell membrane closure for subsequent biocytin staining, and preservation of harvested material in the patch pipette

should not last more than 20 min for RNA preservation, the cell's content is aspirated into the recording pipette by applying negative pressure under electrophysiological and visual control in order to avoid contamination and to ensure a good cytoplasm collection. During the harvesting procedure, a careful (re)positioning of the pipette away from the nucleus (*see* **Note 4**), which can obstruct the pipette tip, is sometimes required. In slices, the harvesting procedure is stopped if the gigaohm seal is lost. After the cytoplasm collection, the pipette is gently withdrawn from the neuron to favor the closure of the cell membrane. Ideally, an outside-out configuration is then achieved which allows the preservation of the pipette's content and of the cell.

The electrophysiological control of this harvesting procedure offers several advantages. Firstly, only mRNA from the recorded neuron is harvested with very limited risk of contamination by the tissue surrounding the cell. Secondly, the harvesting procedure allows a good preservation of the collected mRNAs. Thirdly and inherently to the single-cell RT-PCR technique, the analyzed neuron can be electrophysiologically and pharmacologically characterized. Finally, when a diffusible tracer (e.g., biocytin) is added into the patch solution, a detailed morphology of the neuron can be obtained for further anatomical identification.

3.4 General Protocol for the Single-Cell RT Reaction

1. During the course of the electrophysiological experiments, the aliquots of intracellular solution, 5× RT mix, and 20× DTT are kept on ice mix and RNase inhibitor and reverse transcriptase kept at −20 °C. The patch pipette is filled with 8 μl patch intracellular solution. At the end of the recording, 2 μl of 5× RT mix and 0.5 μl of 20× DTT are pipetted into the RT-PCR tube (0.5 ml PCR tube).
2. After recording and aspirating the cell, the pipette's content is expelled (we usually collect 6.5 μl) in this tube. The tip of the pipette should be broken in order to facilitate the expelling in the PCR tube containing 2 μl of 5× RT mix and 0.5 μl 20× DTT. We then add 0.5 μl RNase inhibitor and 0.5 μl RT, and the tube is flicked and briefly centrifuged. The final volume should then be roughly 10 μl with final concentrations: 0.5 mM each dNTP, 5 μM random primers, 10 mM DTT, and 20 units of RNase inhibitor and 100 units of reverse transcriptase.
3. The RT reaction then proceeds overnight at 37 °C. After this incubation, the tube is kept at −80 °C until the PCR reaction.

The remainder of the aliquots of 5× RT mix and 20× DTT is discarded after each day of experiment.

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3.5 First PCR-PCR Multiplex

1. Set up PCR reaction on ice. Amounts can be scaled depending of the number of cells to process.

   For one cell/RT reaction

RNase-free H_2O	59.5 µl
10× Taq buffer	10 µl
Taq polymerase (5 U/µl)	0.5 µl

2. Add 70 µl of the mix to each RT tube without touching the content. Change tip for each tube.
3. Incubate 1 min at 95 °C in the PCR machine, and then add 20 µl of multiplex primer mix to each tube, one by one. Change tip for each tube.
4. Run PCR program:

One time:	95 °C/3 min
21 times:	95 °C/30 s
	60 °C/30 s
	72 °C/35 s
One time:	72 °C/10 min
Then cool down at 15 °C	

3.6 Second PCR-PCR Simplex

1. Cool down the 96-well PCR plate on ice prior to setting up the PCR reaction (*see* **Note 5**).
2. For each cell, prepare the following mix in a 5 ml tube in the order:

	For one gene	Mix for 24 genes (×25)
RNase-free H_2O	75.5 µl	1887.5 µl
10× Taq buffer	10 µl	250 µl
dNTP 50× (2.5 mM each)	2 µl	50 µl
First PCR reaction	2 µl	50 µl
Taq polymerase (5 U/µl)	0.5 µl	12.5 µl

3. Vortex and then centrifuge for few seconds with a small workbench centrifuge.
4. Dispense 90 µl of the mix in the 96-well plate. One cell occupies three columns of eight wells. One plate is for four cells. Change tip for each cell.
5. Take the plate containing the primers simplex (nested primer) out of the fridge, and centrifuge them for 10 s. Using the multichannel pipette, add 10 µl of second PCR primer mix by

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following the organization of the second PCR mix plate prepared in Subheading 2.5.

6. Cover the plate with aluminum seal; transfer directly the plate from ice to the PCR machine. The block must have been pre-warmed at 94 °C.
7. Run PCR program:

One time:	95 °C/3 min
35 times:	95 °C/30 s
	60 °C/30 s
	72 °C/35 s
One time:	72 °C/10 min
Then cool down at 15 °C	

8. Analyze your PCR product by agarose gel electrophoresis. You must verify you obtain a single band of the correct size. For loading the agarose gel, use a multichannel pipette different from the one used for loading the second PCR primer mix.

4 Notes

1. Given the high sensitivity of the method and the high risk of contamination, we recommend using the following reagents which have been tested by us and others lab successfully: RNase-free dithiothreitol (Sigma), hexamer random primers (Roche or Life Technologies), deoxynucleotide solution set (New England Biolabs), recombinant RNAsin (Promega), reverse transcriptase SuperScript II (Life Technologies), and Taq DNA polymerase (Qiagen).
2. For each new primer pair or combination of primers, we test mRNA contamination from surrounding tissue by placing a patch-clamp pipette into the slice without establishing a seal. Following removal of the pipette, its content is then processed by RT-PCR. In our hands, this control has always yielded negative results. It must be noted, however, that a better negative control consists in scPCR of cells devoid of the mRNA of interest, from the same biological preparation.
3. Virtually any type of patch-clamp intracellular solution can be used, as long as it does not interfere with RT-PCR efficiency in cell-free assays. In our experience, intracellular solutions containing K+ or Cs + cations and Cl- and gluconate or methyl sulfate anions are equally suitable to scPCR.

4. When expression of intronless genes is analyzed, genomic DNA can be a source of false positives, and avoiding nucleus collection is here essential. We addressed this issue by including a control amplifying an intronic DNA sequence in the scPCR reaction and found that this control efficiently detected genomic DNA contamination [16].
5. In case of scaling down the whole procedure, instead of using 96-well format, you can use individual 0.2 or 0.5 ml PCR tubes.

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Query Refs.	Details Required	Author's response
AU1	Please check whether the author names and affiliation are presented correctly.	
AU2	Please check and confirm the presentation of list levels in throughout the chapter. dNTP (dATP, dCTP, dGTP, dTTP; final: 2.5 mM each) 7.5 l each dNTP Random primer (final: 25 M) 9.7 l dH_2O 260.3 l	
AU3	Please check whether the list "l RNase-free H_2O" is presented correctly here.	
AU4	Please check if edit to sentence starting "In our experience..." is okay.	

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