Comprehensive qPCR profiling of gene expression in single neuronal cells

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A major challenge in neuronal stem cell biology lies in characterization of lineage-specific reprogrammed human neuronal cells, a process that necessitates the use of an assay sensitive to the single-cell level. Single-cell gene profiling can provide definitive evidence regarding the conversion of one cell type into another at a high level of resolution. The protocol we describe uses Fluidigm Biomark dynamic arrays for high-throughput expression profiling from single neuronal cells, assaying up to 96 independent samples with up to 96 quantitative PCR (qPCR) probes (equivalent to 9,216 reactions) in a single experiment, which can be completed within 2–3 d. The protocol enables simple and cost-effective profiling of several hundred transcripts from a single cell, and it could have numerous utilities.

INTRODUCTION

The human central nervous system (CNS) contains 10–100 billion neurons and glia, which mediate cognitive functions and regulate behavior. CNS neurons have been segregated into hundreds of different subtypes depending on their location, connectivity, neurotransmitter identity, passive and active electrophysiological properties and molecular markers. Dysfunction of specific neuronal subtypes—and perhaps of glia—underlies neuro-psychiatric disorders such as Parkinson's and Alzheimer's diseases and autism spectrum disorders. The autologous *de novo* generation of neurons useful for transplantation-based therapy of CNS disorders has been a central aim of regenerative medicine and, thanks to recent advances in stem cell biology, it is fast becoming a reality. This development has the capacity to revolutionize the understanding and treatment of CNS diseases.

Similarly to diverse neuronal types in the brain, neurons formed through cellular reprogramming are heterogeneous in the acquisition of lineage specificity. Thus, a major challenge in the neuronal stem cell field is the characterization of lineage-specific reprogrammed human neuronal cells, a process that necessitates the use of an assay sensitive to the single-cell level[1]. Traditional immunocytochemical analysis is limited both by the availability of specific antibodies and the limited number of proteins that can be assayed per cell. In contrast, single-cell gene profiling can provide definitive evidence regarding the identity of generated neurons, such as their expression of specific lineage markers and neurotransmitter identities. Together with functional analysis, the molecular characterization of single cells is crucial for characterizing the conversion of cells from various sources into neurons. Although detection of small numbers of transcripts from single cells has been performed for over 20 years[2–13], key limitations of these analyses have included the ability to comprehensively profile the expression of multiple genes from a single cell as well as the low throughput of comparisons between cells. Furthermore, whole-genome single-cell gene profiling[14,15] is still in its infancy and is prohibitively expensive for the analysis of multiple cells.

A number of applications that enable high-throughput qPCR (more than 384 reactions in an experiment) are in advanced development, such as emulsion-based digital PCR technologies (e.g., Quantalife's Droplet Digital; Raindance Technologies' Rainstorm), which could potentially enable absolute quantification of the number of transcripts in a sample, or microfluidic platforms for parallel analysis of multiple inputs[16] (e.g., Stokes Bio's high-throughput diagnostic system). In addition, direct quantification of target transcripts can be performed by multiplexed profiling (e.g., Nanostring's nCounter). To our knowledge, the only platform currently commercially available for comprehensive analysis of the limiting material found in single cells is Fluidigm's Biomark high-throughput qPCR chip. Therefore, this protocol is tailored to use of the Fluidigm Biomark system for comprehensive qPCR profiling of single cells. This system uses a pressure-regulated microfluidic circuit in order to perform mixing of nanoliter volumes of samples and probes within individual chambers on the microfluidic chip. After loading and mixing is complete, thermal cycling is performed, coupled to the imaging of the chip at the end of each cycle[17].

Recently, we successfully converted human skin fibroblasts into functional neurons and addressed the challenge of identifying the subtype specificity of the newly converted neurons by using comprehensive qPCR-based single-cell gene profiling[18]. The protocol we developed uses microfluidic qPCR chips (Fluidigm Biomark), which enable high-throughput qPCR-based parallel analysis of multiple genes from a single-cell sample. The system is compatible with commercially available Taqman probes, as well as experimenter-designed primers in conjunction with DNA-binding dyes such as EvaGreen. Evagreen-based qPCR is substantially more cost-effective and maintains the capacity for enhanced flexibility and quality assurance. The method we describe is based on aspiration of single cells into a fine glass pipette followed by target-specific amplification using a mix of primers to the sequences of interest. Fluidigm BioMark dynamic arrays (48.48 or 96.96) are then used for high-throughput qPCR on a large number of independent samples (up to 48/96) across a large number of qPCR probes (up to 48/96), equivalent to 2,304 or 9,216 independent reactions on a single chip. Using this system, we explored the variation in neuronal gene expression patterns of individual induced neuronal (iN) cells[18].
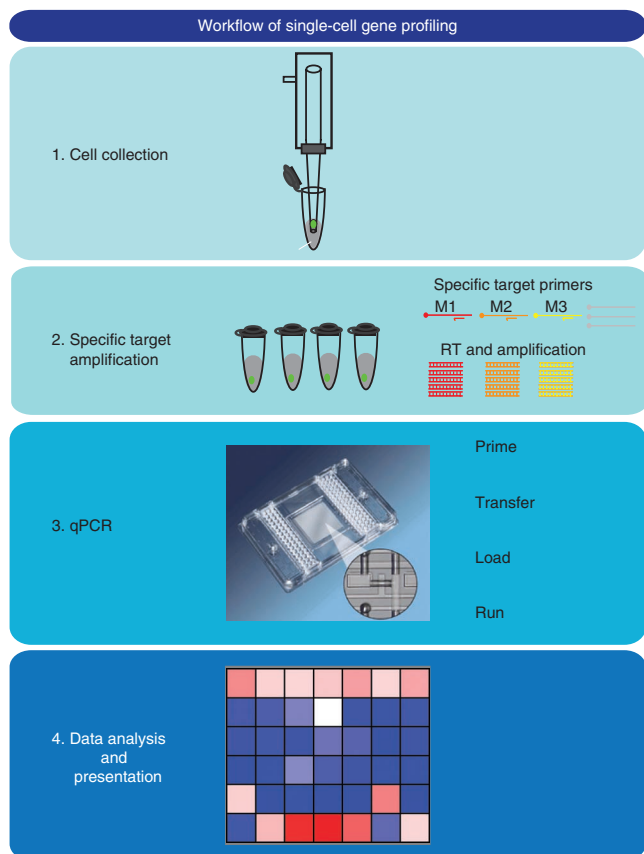
Figure 1 | A schematic workflow for single-cell gene profiling. The procedure for single-cell gene profiling consists of four major steps: (1) Collecting cells (for a detailed explanation, see **Fig. 2**). (2) Specific target amplifications using a mix of gene-specific primer pairs. M1–M3 represent genes of interest, gray mRNAs represent other genes. Target RNAs are subject to reverse transcription (RT) and amplification. (3) qPCR is performed on the Fluidigm Biomark system. Samples are loaded on one side of the array, whereas probes are loaded on the opposing side, with mixing occurring only in the appropriate wells, thereby allowing high-throughput parallel analysis of multiple samples across multiple probes. (4) Data analysis and presentation. At this point, quality assurance is performed to ensure the specificity and reliability of the results, followed by data analysis and presentation (potentially in the form of a heat map).

fibroblasts[19], as well as for addressing the heterogeneity among human induced pluripotent stem cells[20].

Applications of the method

The protocol we describe here, using microfluidic qPCR for single-cell gene profiling, enables the investigation of up to several hundred transcripts from single neuronal cells (**Fig. 1**). Although the version of the protocol described below is specific to cells in culture, further developments of this protocol could be applied to the profiling of single cells collected directly from tissue samples[10] or after cell sorting[3]. For example, in the context of investigating neuronal function in brain slice preparations after standard whole-cell patch-clamp electrophysiology, cytosolic material containing cellular mRNA can be aspirated into the patch pipette (**Fig. 2**). Single-cell gene profiling, using the protocol described here, will enable correlation of gene profiling with cellular function. This approach has been used in the past for correlating gene expression with cellular function, using traditional PCR[3,6,21,22], but it has been limited by the number of transcripts profiled from a single neuron. As neurons within the brain are very heterogeneous, both in their

A protocol describing the derivation of iN cells is currently in preparation. Similar approaches have also been used recently for the investigation of the variability in the acquisition of neuronal phenotypes after miRNA-induced conversion of human

Figure 2 | Micromanipulator-assisted manual collection of single neurons. Neurons are highly polarized cells, characterized by lengthy axonal projections and dendritic arbors. These morphological features cause the collecting of single neurons to be more challenging than that of other cell types, such as lymphocytes. Our chosen methodology uses the equipment available in a standard electrophysiology laboratory for manual selection and aspiration of target neuronal cells in culture. Thus, standard patch-clamp setups equipped with a fluorescent microscope and manipulator are used for the targeted collection of the soma of a single neuron, thereby minimizing the solution volume and the potential for contamination by other cell types. Furthermore, this setup potentially enables the analysis of the morphological and electrophysiological features of the cells parallel to their transcriptional profiling. (**a**) A schematic illustration of the method for collecting single cells using a patch pipette. Initially, a patch pipette with a tip opening of 2–3 μm (~0.5 MΩ resistance) is fixed to the pipette holder, which is attached through plastic tubing to a 3-ml syringe. Slight positive pressure (pres.) is applied through the syringe while inserting the pipette into the medium on the dish containing the target neuronal cells. Target cells are identified by their GFP expression and neuronal morphology, as assessed by differential interference contrast (DIC) imaging. The positive pressure driven through the pipette while it is lowered into the solution reduces the influx of liquid into the pipette because of capillary action. Under visual direction through the microscope, a micromanipulator is used to position the pipette close to the cell body. Once the tip touches the cell, the cell will normally adhere to the tip; at this point the pressure is relieved, and slight suction (suc.) applied, enabling gradual aspiration of the cell into the pipette tip. The

pipette is then retracted, and while positioning it in a pre-prepared PCR tube with 5 μl of reaction buffer the syringe is used to apply positive pressure to expel the cell into the solution in the tube. (**b**) Fluorescent and DIC image of the collection of an iN cell as described.
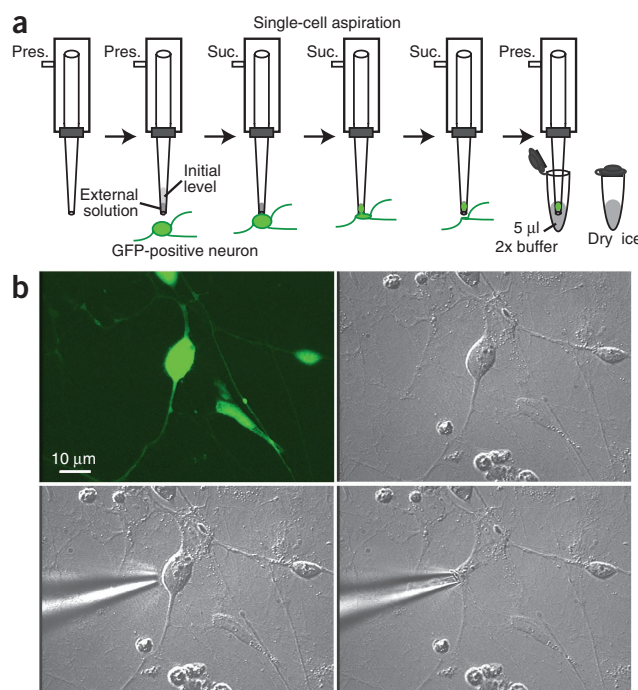
Box 1 | Choosing good primers

Excellent PCR primers and simple quality assurance measures are crucial to establishing the validity of single-cell qPCR analysis. Optimal PCR primers should provide specific and efficient amplification of the target, minimizing potential contamination[33]. Our preference is for simple nonspecific detection of qPCR products using fluorescent dyes such as EvaGreen. The principle of the reaction is that the binding of the fluorophore to double-stranded DNA (dsDNA) causes it to emit a strong fluorescent signal. The result is an increase in fluorescent intensity, which is directly proportional to the increase in dsDNA. This type of detection system is the simplest and most economical choice for qPCR, but it is not selective because of the potential for nonspecific amplification. Therefore, careful planning of qPCR primers, followed by vigilant quality assurance, is crucial for the acquisition of valid data. Notably, the use of fluorescent dyes to detect dsDNA enables simple quality assurance owing to the possibility of measuring the efficiency of the primer pair (by performing dilution analysis of a reference sample; **Fig. 3a,b**), as well as the specificity of the amplification (by addressing the melting curve of the amplicon; **Fig. 3c**). Choosing primers: optimal primers should be 16–30 bases long and have a melting temperature (T_m) of 56–68 °C (optimally 60–64 °C). The T_m of primers within a primer pair should be within 1 °C of each other. Product size should ideally be between 80 and 150 bp. If comparisons are to be made between products, the products should be of similar length. Nucleotide repeats should be avoided as they promote mispriming.

An optimal primer pair for qPCR of mRNA transcripts will be one in which at least one of the primers covers an exon-exon junction, or in which the amplicon contains an intron (intron-spanning), so as to avoid amplification of genomic DNA contaminants. A point often ignored is that of alternative splicing of the target sequence. It is important to note that when planning primers, attention should be given to alternative splice isoforms, and extra care should be taken when choosing primers, noting the splice variants that will be identified and those that will be missed.

In our experience, reliable resources for calculation of primers are software based on Primer3 (ref. 34; such as Roche Probefinder (http://qpcr.probefinder.com/organism.jsp) and NCBI primer-blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/)). Public repositories for qPCR primers are becoming increasingly prevalent, with popular ones being rtprimerdb (http://www.rtprimerdb.org/) and primerbank (http://pga.mgh.harvard.edu/primerbank/). Detailed, step-by-step descriptions of how to choose primers have been described previously[35].

Testing primers: in order to ensure the quality of the primers, control reactions should be performed in which the specificity and efficiency of the primers is addressed. In these reactions, an abundant reference RNA is used (in the case of human iN cells, we used a commercial source of human brain RNA). A comprehensive titration of this RNA is prepared (e.g., eight fourfold dilutions from a concentration of ~1 µg µl⁻¹). This dilution curve should be subject to identical treatment to the samples (target-specific preamplification, ExoSAP-IT, and so on), preferably in duplicate parallel reactions, and analyzed in the same experiment as the single-cell samples. Because of the high-throughput nature of the Fluidigm Biomark PCR chips, it is preferable to include the control reactions in the first experiment in which they are introduced, so that a direct comparison of the results from the control reactions and the experimental cells may be performed. An additional control reaction important to include is the no-template control, which controls for contamination within the solutions used for the reaction.

Primer efficiency is a crucial aspect of qPCR analysis of limiting samples, as reduced efficiency would result in nonlinear amplification of the targets, which would render the results uninterpretable. Efficiency is calculated by plotting the results of the titration of the reference RNA (C_t versus log[dilution]; **Fig. 3b**) within the range of linear amplification. An optimally efficient PCR reaction will result in doubling of the quantity of the target RNA in each amplification cycle. Therefore, efficiency is defined as $100 \times (10^{(-1/slope)} / 2)$, such that the curve of the optimal slope is −3.3 ($10^{(-1/3.3)}=2$).

The specificity of the amplicon is addressed by visually inspecting the melting curve of the amplification product (**Fig. 3c**). Specific amplification will be demonstrated by a single prominent peak, common to all samples, whereas off-target amplification should be clearly evident (**Fig. 3c**). It should be noted that aside from sequencing the amplicons, there is no way of definitively ensuring that no off-target amplification occurred. Performing such analysis will entail setting up a parallel assay in a traditional PCR tube (as the amplified targets are not accessible from within the Fluidigm Biomark chip). However, a more practical (albeit less definitive) approach for gaining additional confidence in the specificity of the reaction may be to analyze a particular gene of interest with multiple primer pairs.

Other options for PCR primers include fluorescent resonance energy transfer (FRET)-based hydrolysis probes (such as Taqman), in which an additional probe (i.e., beyond the PCR primer pair) is included in the reaction, thus improving the specificity of the reaction. These options are currently substantially more expensive than the EvaGreen-based approach, and another limitation is that vendors often do not provide the sequences of the PCR primers.

function and in their incorporation into independent neuronal circuits, an assay enabling comprehensive single-cell profiling could be applied to achieving a higher resolution of experimentation. For example, experience-dependent plasticity relies on *de novo* gene transcription to encode memories[23], but only neuronal subcircuits are involved in encoding the experience[24]. More so, neurons encoding opposing functions may sit adjacent to each other within the tissue, and when assaying gene expression within a brain region[25] this cell- and circuit-specific information is lost. Comprehensive

single-cell gene profiling will enable the association of specific transcripts with specific cells, increasing the resolution of investigation. In addition, many neuronal genes, including ion channels and cell adhesion molecules, have complex splicing patterns[26,27]. Potentially, this could lead to new insight into mechanisms underlying CNS pathologies.

Obviously, applications of this technique can be extended to any context in which comprehensive information on gene expression at the single-cell level is of interest. This is true for all branches of stem
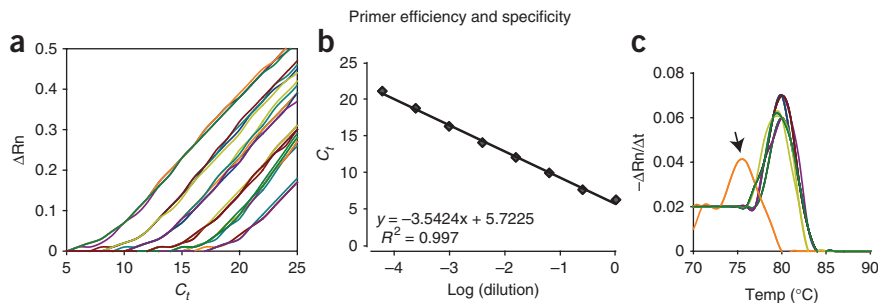
npg

Figure 3 | Assessment of primer efficiency and specificity. (**a**) An example of amplification curves using Fluidigm IFC arrays. Six sequential dilutions (fourfold) of total human brain RNA are shown, probed for expression of vGLUT2. (**b**) The efficiency of the primer pair for vGLUT2 was assessed by plotting the cycle threshold value (C_t) at each concentration against the logarithm of the fold dilution of the sample. The slope of a linear-regression trendline is indicative of primer efficiency, as defined in the text. (**c**) An example of melting curve analysis, showing the specific amplification of the target gene in the majority of the single-cell samples tested, as well as a nonspecific amplification observed in a single sample (indicated by an arrow).

cell investigation, as well as for diverse fields such as development, cancer and microbiology.

Considerations for choice of methodology

This method is applicable in situations where parallel analysis of a number of different cellular markers is necessary to characterize the variation among single cells in a population. Given the limitations of the throughput and availability of reagents for techniques based on immunohistochemistry and *in situ* hybridization, single-cell qPCR array is our method of choice. Additional protocols describing single-cell PCR have been described elsewhere[4,28–31].

Comparison with other methods

Expression profiling of single cells can be performed either at the level of RNA or protein. Obviously, it is more informative to assay protein levels within a cell, as a gap may exist between RNA levels and protein levels. Nevertheless, the techniques for parallel analysis of multiple proteins within a single cell, such as immunohistochemistry-based techniques, are extremely limited both by the availability of good antibodies and by the availability of fluorophores enabling multiplexed analysis of a single sample. Recent advances allow parallel analysis of up to 100 different proteins in a single immune cell[32]. Although this suggests exciting prospects for the future, the wide implementation of the technique may not be trivial.

A number of methods exist for analysis of RNA levels within single cells. One approach is based on *in situ* hybridization, which

offers high sensitivity and the potential to probe different splicing variant of a specific gene. However, the throughput of the readout is very limited in that only one or two genes can be probed in a single cell. At the other end of the spectrum, single-cell deep sequencing[14,15] is a recent development that enables comprehensive profiling of transcripts from a single cell. However, the technology is still in its infancy and is costly and labor intensive, rendering it impractical for standard lab use at this stage.

In contrast, the use of microfluidic-based qPCR chips, using regular qPCR primers (**Box 1** and **Fig. 3**) with a DNA-binding dye, is highly cost effective, and it enables the parallel analysis of a large number of samples across many probes with relatively low effort. For the collection of cells, although it is laborious, we use manual aspiration of single cells, as this allows the unambiguous collection of specific cells (on the basis of their morphology) to be performed in a minimal volume, while minimally disrupting the complex morphology of the neuronal branches (in contrast to the enzymatic treatment necessary for fluorescence-aided cell sorting).

Current limitations of the protocol

- In its present form, the throughput of the protocol is limited by the laborious collection of individual neurons.
- The success of the experiment depends on the quality of the primers (**Box 1** and **Fig. 3**). Once a primer pair has been confirmed to be adequate in terms of specificity, it provides a tool that can be used repeatedly with confidence.
- Because of the minute quantities of starting material, certain transcripts may fall below the detection limit.
- It is limited to effectively processing 200–300 probes per cell and is not applicable for whole-genome expression analysis.
- The quantitative nature of the results is not yet clear. It is likely that it can be used for qualitative analysis, but further work is necessary to evaluate whether it is truly quantitative (because of biological variability in terms of cell size, processing of single cells for preparation of RNA, and so on).
- mRNA transcripts do not necessarily correlate with protein expression; there may be a discrepancy between the two.

MATERIALS

REAGENTS

- BioMark 48.48 or 96.96 Dynamic Array (Fluidigm, cat. nos. BMK-M-48.48; BMK-M-96.96)
- Gene expression 48.48 or 96.96 Dynamic Array sample & loading reagent kit (Fluidigm, cat. nos. 85000800 and 85000802)
- CellsDirect reaction mix (Applied Biosystems, cat. no. 11753-100)
- TaqMan gene expression master mix (Applied Biosystems, cat. no. 4369016)

- DNA-binding dye sample loading reagent (Fluidigm, cat. no. 100-0388)
- EvaGreen DNA-binding dye (Biotium, cat. no. 31000)
- Assay loading reagent (Fluidigm, cat. no. 85000736)
- Low-EDTA (0.1-mM EDTA) TE buffer (Teknova, cat. no. T0227)
- ExoSAP-IT (Affymetrix USB, cat. no. 78201)
- Sodium chloride (NaCl; Sigma-Aldrich, cat. no. S3014)
- Potassium chloride (KCl; Merck, cat. no. 104938)
- Calcium chloride, dihydrate ($CaCl_2$, $2H_2O$; Merck, cat. no. 208291)

• Magnesium chloride, hexahydrate (MgCl$_2$, 6H$_2$O; AppliChem, cat. no. A3618)
• HEPES (Sigma-Aldrich, cat. no. H3375)
• D-(+)-Glucose (Sigma-Aldrich, cat. no. G8270)
• Clear adhesive tape (e.g., Scotch tape, 3M)

EQUIPMENT
▲ CRITICAL All equipment should be treated as potentially contaminated with RNase.
• Inverted tissue culture microscope with phase contrast and epifluorescence, equipped with ×10 and ×40 objectives (Axiovert 40 CFL, Zeiss; Olympus BX51W upright microscope (Olympus); ×4 objective (Olympus XL Fluoro ×4/340 objective, 0.28 NA, Olympus)
• TMC 65-560 anti-vibration table (Technical Manufacturing Corporation)
• Glass micropipettes (inner diameter 1.0 ± 0.05 mm; outer diameter 1.5 ± 0.05 mm; Garner Glass Company, cat. no. KG33)
• PC-10 glass microelectrode puller (Narishige)
• PCR tube strips (USA Scientific)
• Tubing
• Syringe (3 ml; BD Bioscience)
• Micromanipulator (Sutter, MPC-385)
• Primer design software (**Box 1**)

• Genepattern software package (available from the Broad Institute; http://genepattern.broadinstitute.org)
• Biomark system (Fluidigm; Fluidigm integrated fluidic circuit (IFC) controller, real-time PCR analysis software)

REAGENT SETUP
Extracellular solution Make up a bath solution containing 140 mM NaCl, 5 mM KCl, 2 mM CaCl$_2$, 2 mM MgCl$_2$, 10 mM HEPES and 10 mM glucose, pH 7.4. The final osmolarity of the solution should be 310 ± 10 mOsm.
▲ CRITICAL Store the solution at 4 °C for up to 1 week.
Primer and sample organization Owing to the relatively high-throughput nature of the protocol (analyzing up to 96 samples across 96 probes), the organization of the components is extremely important. Our preference is to use tubes of different colors for the samples and the probes, organize them consistently (e.g., strips of 8, organized on 96-well plates) and label them with a simple code (e.g., numbers for probes and letters for samples).
Glass pipette preparation Prepare glass pipettes using a PC-10 two-stage puller. Adjust the heating temperature of the two steps so that the tip of the glass pipette is close to a diameter of ~2 to 3 μm (~0.5 MΩ).

PROCEDURE
Cell aspiration ● TIMING 5 min per cell
1| Prepare PCR strip tubes with 5 μl of 2× CellsDirect reaction mix per tube (**Fig. 1**). Keep the tubes on ice.
▲ CRITICAL STEP Use RNase-free reagents and RNA protective technique throughout the protocol (clean surfaces of working area, wear gloves, avoid mouth pipetting, and use RNase-free filter tips and tubes).

2| Mount the pipette (with a tip opening between 2 and 3 μm) on a micromanipulator connected to tubing to enable transfer of pressure through the pipette.

3| Visually identify the isolated cell of choice under the microscope, use a micromanipulator to approach it with positive pressure (applied through a 3-ml syringe) to clear its surroundings and aspirate it into a patch pipette, ensuring minimal contamination by surrounding cells or cell debris (**Fig. 2**).
▲ CRITICAL STEP Use minimal pressure to aspirate the cell so as to raise a minimal amount of fluid into the pipette.

4| Retract the pipette and remove the holder with syringe from the setup (maintaining the connection to the syringe). Lower the pipette tip into the solution at the bottom of the PCR tube. Apply positive pressure to eject the cell into the solution while retracting the tip of the pipette out of the solution. Freeze the cell immediately on dry ice.
▲ CRITICAL STEP Use minimal pressure to eject the cell into the solution so as not to blow the solution out of the tube. Maintaining positive pressure while retracting the pipette is important for minimizing capillary action that could drive solution back into the tip of the pipette.
▲ CRITICAL STEP Each micropipette can be used only once for transferring each individual cell into reaction buffer.
▲ CRITICAL STEP It is essential to include a negative control sample for the qPCR analysis, in which no individual cell is picked; instead, transfer external solution equivalent to the carryover volume collected during the single-cell transfer into reaction buffer. The negative control will enable the detection of contamination in the subsequent steps of the procedure.
■ PAUSE POINT Cells can be stored for several months at −80 °C until processing.

Specific target amplification ● TIMING 6 h
5| Prepare primer pairs at a concentration of 20 μM for each primer. Mix the primers used for specific-target amplification (up to 100 primer pairs), bringing the final concentration of each primer to 200 nM (if you are using < 100 primer pairs, dilute accordingly with RNase-free water). Large stock solutions of this primer mix may be made and stored at −20 °C if the same primers are to be used continuously. Note that if more than 100 genes are to be assayed, higher stock concentrations of primer pairs should be made (above 20 μM).

6| Prepare the reaction mix as follows:

Component	Volume (µl; per sample)
SuperScript III/RT Platinum Taq mix	0.2
Primer mix (200 nM)	2.5
RNase-free H$_2$O	1.3

7| Thaw the cells from dry ice directly in a 50 °C bath for 15 s, and then place them on ice.

8| Add 4 µl per well of reaction mix and put it in a thermocycler, preprogrammed to run as follows:

Time	Temperature (°C)	Purpose
15 min	50	Reverse transcription
2 min	95	Denaturation
Cycle 18–20 times:		
15 s	95	Denaturation
4 min	60	Annealing and elongation
Indefinitely	4	End

9| Add 4 µl of ExoSAP-IT and return the reaction to a PCR machine preprogrammed to perform as follows:

Time (min)	Temperature (°C)	Purpose
15	37	Digest primers and dNTPs
15	80	Denature enzyme

■ **PAUSE POINT** cDNA can be stored indefinitely at −20 °C until further processing.

qPCR analysis of samples ● **TIMING** 5 h
10| Prepare the qPCR premix (according to the vendor's protocol, as described below):

Component	Volume (µl; per sample)
TaqMan gene expression master mix	2.5
DNA-binding dye sample loading reagent	0.25
EvaGreen DNA-binding dye	0.25

11| In prelabeled PCR strip tubes, prepare samples as follows. Then briefly vortex and spin down. Store samples on ice until loading.

Component	Volume (µl; per sample)
Reaction mix	3
ExoSAP-IT–treated samples	2

12| In a separate set of prelabeled PCR strip tubes, prepare assays as follows. Then briefly vortex-mix and spin down. Store tubes on ice until loading.

Component	Volume (μl; per sample)
Assay loading reagent	2.5
qPCR primer pairs (20 μM)	2.5

13| Prime the Fluidigm Biomark qPCR chip for loading the assays and samples by placing the chip in the IFC controller and activating the 'prime chip' program (~15 min).
▲ **CRITICAL STEP** The chip must be loaded within 1 h of priming so as to reduce the loss of pressure within the chip.

14| Pipette 5 μl of each assay and 5 μl of each sample into their respective inlets on the chip.
▲ **CRITICAL STEP** Use caution while pipetting the samples, as bubbles can be introduced. A fresh sterile tip can be used to remove or pop bubbles by partial aspiration of the bubble while pulling it out of the well. Care should be taken to avoid cross-contamination or loss of reaction volume.
? TROUBLESHOOTING

15| Load samples and assays into the chip by inserting the chip into the IFC controller and activating the 'load mix' program (~1 h).

16| Remove dust particles from the surface of the chip by applying adhesive tape to the surface, smoothing it onto the surface of the chip, and removing it carefully, ensuring that the surface of the chip is completely clean.
▲ **CRITICAL STEP** Do not blow dust particles away, as this could introduce contaminations. Only remove them with tape.

17| Load the chip onto the Biomark and initiate the qPCR reaction; include melting curve analysis. The program to be run is described below:

Time	Temperature (°C)	Purpose
2 min	50	Thermal mixing
30 min	70	Thermal mixing
10 min	25	Thermal mixing
2 min	50	Uracil-*N*-glycosylase activation
10 min	95	Hot start for Taq polymerase
Cycle 35 times:		
15 s	95	Denaturation
1 min	60	Annealing and elongation
Ramp	60–95	Melting curve

18| Initialize chip scanning. Allow the initial ROX (reference dye) scanning to proceed. Open ROX scan files in the appropriate software. Scans should demonstrate equivalent ROX loading in all wells. Dust particles will show up as streaks on top of the ROX signal and should be removed by repeating Step 16. If no problems appear, allow the software to proceed with the full qPCR reaction.
■ **PAUSE POINT** Data can be analyzed as follows at the time of your choice.
? TROUBLESHOOTING

Data analysis and visualization ● TIMING 4 h

19| Analyze data using Fluidigm's teal-time PCR analysis software. After the definition of cycle threshold values, the data may be viewed as a heat map with probes as columns and samples as rows, enabling an overview of the experiment and identification of potential problems in loading. Choosing a column allows the rapid visual inspection of the melting curves obtained from the single-cell samples in comparison with the reference reactions used for dilution curve analysis (**Box 1**). The full data can then be exported for further analysis in Microsoft Excel or other similar software.
? TROUBLESHOOTING

20| Visualize the data. Vendor software allows heat map visualization of results and exporting to spreadsheets for further analysis. Our preference is to visualize the raw data using the Heatmapviewer component of the Genepattern package available from the Broad Institute (http://genepattern.broadinstitute.org).

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

TABLE 1 | Troubleshooting table.

| Step	Problem	Possible reasons	Solution
14	Bubbles forming in sample/assay inlets	The chip does not maintain pressure	Make sure to load the chip within 1 h of priming
	Many bubbles in sample/assay wells	Bubbles are introduced during pipetting	Pipette more carefully
18	ROX passive reference is uneven	Problem in loading the chip	Make sure to load the chip within 1 h of priming and ensure no bubbles are formed while loading chip
19	Multiple peaks for a single primer pair in the melting curves	Primers are not specific to the target	Optimize new primers against the target
		Primers are contaminated	Redilute primers to make a fresh primer pair
	Multiple peaks in multiple primer pair melting curves	ExoSAP-IT did not fully digest primers	Ensure proper mixing before incubation for the ExoSAP-IT reaction; if the problem persists, repeat reaction with new ExoSAP-IT reagent
	No amplification detected for a single primer pair	Primers are not optimal	Redesign primer pair
	No amplification detected for a single sample	Cell was lost during selection	Repeat the cell selection
		Bubbles in sample wells	Repeat the experiment, paying attention to remove or pop bubbles by partial aspiration of the bubble using sterile pipette tips
	Results appear contaminated (e.g., neurons appear to be contaminated by cells from another source)	Additional material was aspirated, contaminating the reaction	Use caution in collecting samples
	Amplification in no-template control	Solutions may be contaminated	Study the melting curves carefully to determine the extent of contamination; you may need to rerun the reaction with new reagents
	No reaction detected in any sample	Samples were not loaded properly on the chip	View passive dye reference (ROX), to address the loading of the sample onto the chip; if it is defective, use a new chip
		Reverse transcription or PCR are defective	Repeat with new reagents
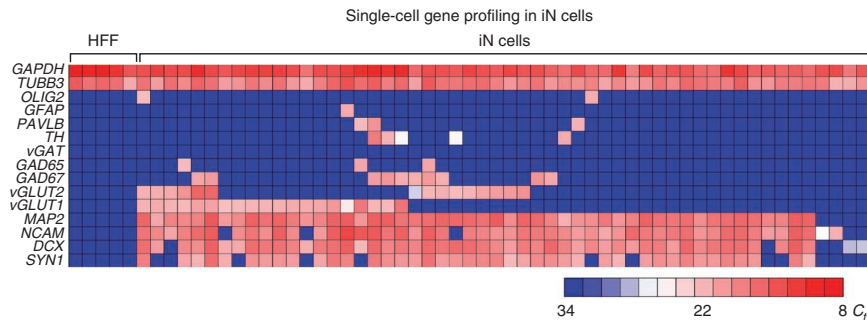
Figure 4 | Single-cell gene expression profiling using Fluidigm dynamic arrays. Rows represent the evaluated genes and columns represent individual cells. Heat map (blue to red) represents the threshold C_t values as indicated. HFF, human fetal fibroblast. From Pang *et al.*[18].

● **TIMING**
Steps 1–4, Single-cell aspiration (day 1): 5 min per cell and 6 h for ~50 cells
Steps 5–9, Specific target amplification (day 2): 6 h
Steps 10–18, qPCR analysis of samples (day 2): 5 h
Steps 19 and 20, Data analysis and visualization (day 3): 4 h

ANTICIPATED RESULTS
The Biomark system is equipped with user-friendly qPCR analysis software, in which results may be presented either as a heat map or spreadsheet, and all the key parameters of the reaction such as the threshold cycles and melting temperature are listed. Because of the high-throughput nature of the assay, we routinely include a number of internal controls, such as *ACTB*, *GAPDH* and *MAP2*, as well as neuron-specific reference genes, such as *TUBB3*, *NCAM1* and *SYN1*, with the expectation that essentially the whole population of neuronal cells assayed will express these reference genes. The signal obtained from the internal controls enables quality assurance for multiple steps in the process, from the aspiration of single cells, through reverse transcription, specific target amplification and qPCR. Furthermore, a no-template control should be routinely included in order to identify potential sources of contamination. Identification of cellular transformation to a specific neuronal subtype is made possible by the use of neuron subtype–specific probes (**Fig. 4**). If the protocol is followed carefully, a success rate of close to 100% (as evaluated on the basis of the amplification of reference genes in the single cells) is expected. Although we have routinely performed two or three replicates per reaction (either loading the samples or the probes in replicates) and have essentially always observed consistent results, we recommend performing the assay in duplicate unless there are experimental limitations.

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