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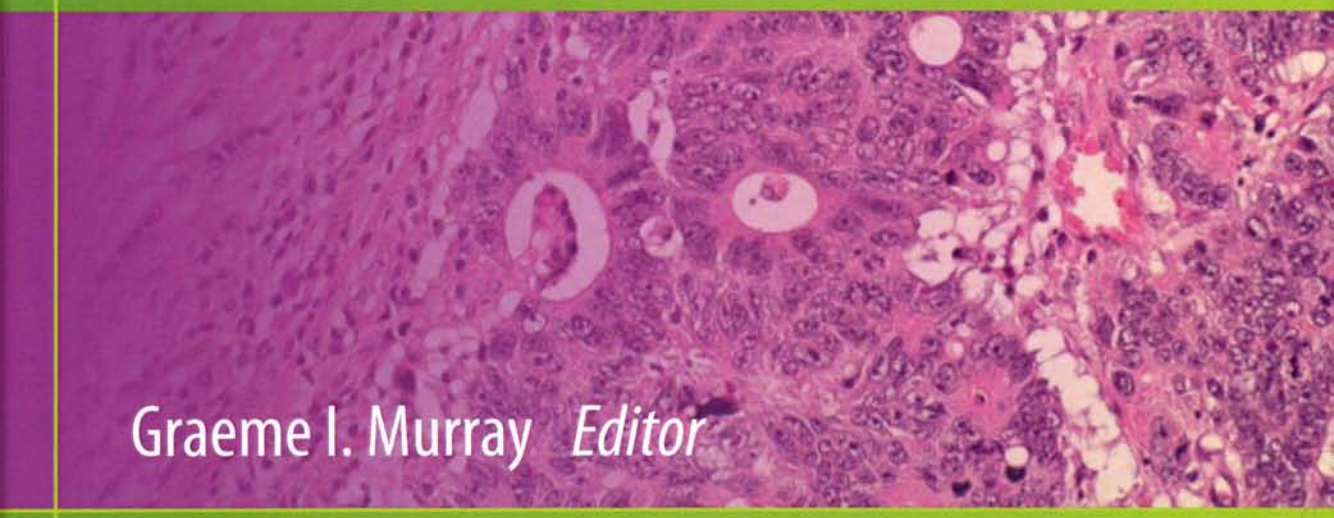

Graeme I. Murray *Editor*

Laser Capture Microdissection

Methods and Protocols

Second Edition

Humana Press

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Edited by

Graeme I. Murray

Department of Pathology, University of Aberdeen, Aberdeen, UK

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Editor
Graeme I. Murray, MB ChB, PhD, DSc, FRCPath
Department of Pathology
University of Aberdeen
Aberdeen, UK
g.i.murray@abdn.ac.uk

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Chapter 30

UV-Laser Microdissection and mRNA Expression Analysis of Individual Neurons from Postmortem Parkinson's Disease Brains

Jan Gründemann, Falk Schlaudraff, and Birgit Liss

Abstract

Cell specificity of gene expression analysis is essential to avoid tissue sample related artifacts, in particular when the relative number of target cells present in the compared tissues varies dramatically, e.g., when comparing dopamine neurons in midbrain tissues from control subjects with those from Parkinson's disease (PD) cases . Here, we describe a detailed protocol that combines contact-free UV-laser microdissection and quantitative PCR of reverse-transcribed RNA of individual neurons from postmortem human midbrain tissue from PD patients and unaffected controls. Among expression changes in a variety of dopamine neuron marker, maintenance, and cell-metabolism genes, we found that α-synuclein mRNA levels were significantly elevated in individual neuromelanin-positive dopamine midbrain neurons from PD brains when compared to those from matched controls.

Key words: Laser microdissection, Single cell, Real-time quantitative PCR, RNA integrity number, Reverse transcription, Postmortem, Human tissue, Synuclein, Parkinson's disease, Dopamine

1. Introduction

Gene expression analysis via quantitative PCR of reverse-transcribed RNA (RT-qPCR) has become a routine technique. Contact free UV-laser microdissection (UV-LMD) enables RT-qPCR-based mRNA analysis of specific tissue regions, homogeneous cell pools, and even individual cells (1, 2). Besides a variety of methodological considerations that could bias RT-qPCR results in general (3, 4), cellular specificity of gene expression analysis is a desired goal to prevent tissue-sample-generated artifacts, in particular, when the relative number of

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target cells present in a given tissue varies dramatically – e.g., between control and disease states in the context of neurodegenerative diseases. High cellular heterogeneity, selective neuron loss, and disease-related changes in nonneuronal cells will contribute to an altered composition of the diseased brain tissue compared to that in controls. Importantly, this will confound any conclusions about specific gene expression changes in the cell type of interest.

For example, one of the key pathological hallmarks of Parkinson's disease (PD) and its animal models is the loss of dopamine-containing (DA) midbrain neurons, in particular within the *substantia nigra* (SN) *pars compacta*. In PD, typical clinical motor symptoms manifest not until approximately 75 % of these DA midbrain neurons – the most prominent cell type within the SN – are lost (5, 6). This massive loss of SN DA neurons will confound mRNA expression analysis of PD midbrain tissue when compared to controls. In addition, gene expression analysis at the level of PD midbrain tissue will be furthermore distorted by altered numbers and functional states of nonneuronal cells such as microglia, astrocytes, and local T-cells, which are known to occur in PD (7). All these factors might explain the large number of different and even contrary findings of tissue-based gene expression studies in PD brains, e.g., for α-synuclein (reviewed in ref. 8) – a gene that can cause familiar forms of PD when mutated (PARK1) or duplicated/triplicated (PARK4) (9). Cell-specific quantification of gene expression with single cell resolution overcomes these tissue-related limitations of gene expression data from pathological tissues and controls, since it enables the unbiased detection of cell-specific transcriptional dysregulation.

Here, we describe a step-by-step protocol for UV-laser microdissection of individual neurons from frozen postmortem human midbrain tissue and subsequent reliable RT-qPCR gene expression analysis of individual cells or small cell pools (1, 10). Cell lysis and cDNA synthesis are performed in the same reaction tube without a distinct RNA isolation step to avoid RNA loss, contamination, and handling errors. We specifically focus on postmortem gene expression analysis of neuromelanin-positive (NM+) DA midbrain neurons from the SN of PD patients and respective controls and describe the robust detection of significantly higher mRNA levels of α-synuclein in NM+ DA neurons from PD brains compared to controls (1). These results suggest that transcriptional dysregulation of the α-synuclein gene and elevated α-synuclein levels not only cause rare familiar forms of PD (PARK4) (11–13) but also might additionally contribute to the risk and pathology of sporadic PD as suggested by recent genome-wide association and related expression quantitative trait loci (eQTL) studies (14–16).

2. Materials

2.1. Handling, Fixation, and Staining of Human Brain Tissue

1. To prevent contamination, human brain tissue specimens are stored in heat-sterilized tinfoil and RNase-ExitusPlus (AppliChem, Darmstadt, Germany)-treated parafilm-sealed boxes at −80 °C.
2. Microtome blades (Leica, Nussloch, Germany, Type 819) washed for 30 s in 70 % RNase-free ethanol and whipped with RNase-ExitusPlus and isopropanol (Sigma-Aldrich, St Louis, USA) (see Note 1).
3. Ethanol dilutions (2× 75 %, 95 %, 100 % absolute, and 100 % anhydrous) are freshly prepared on each experimental day and stored in 50 ml Falcon tubes at room temperature. One tube of 75 % ethanol is kept at −20 °C. 75 % and 95 % ethanol dilutions are prepared from ethanol absolute puriss. p.a. (Sigma-Aldrich) in RNase free water (5Prime, Hamburg, Germany). Ethanol anhydrous stock is stored with molecular sieve (Merck, Darmstadt, Germany, pore size: 0.3 nm, 25 g/l).
4. 1 % cresyl violet acetate staining dye (Sigma) is diluted in 100 % ethanol absolute puriss. p.a., stored in a tinfoil-covered and parafilm-sealed Falcon tube and incubated at least overnight before use.
5. Drying box with silica gel with moisture indicator (Merck).

2.2. UV-LMD

1. A contact-free LMD microscope is needed. This protocol was successfully tested with both the Zeiss (Munich, Germany) PALM UV-LMD setup and the Leica UC-LMD6000 and 7000 setups. Heat sterilization (180 °C, 2 h) of all LMD microscope parts that are in contact with the tissue slides (i.e., slide holder) or reaction tubes (i.e., cap or tube holder) prevents RNase contamination (see Note 1).
2. PEN-membrane slides (2.0 µm, MicroDissect, Herborn, Germany) for mounting of tissue sections and laser microdissection, treated with UV-C light for 15 min.
3. RNase-free thin-walled 0.5-ml PCR reaction tubes with flat cap for cell collection and combined cell lysis and cDNA synthesis, UV-C treated for 45 mins.

2.3. Preparation of Cap-Mix for Combined Cell Lysis and cDNA Synthesis

1. Cell lysis and cDNA synthesis are performed in the same buffer (*Cap-Mix*), containing 0.5 % NP-40 (Roche, Basel, Switzerland, light-sensitive 10 % stock stored in aliquots in dark at +4 °C; see Note 2), 5 U SUPERase-In (Ambion, Austin TX, USA, stored in aliquots at −20 °C), 0.5 mM dNTPs (GE Healthcare, Freiburg, Germany, stored as 20 mM stock at −20 °C), 5 µM random hexamer primer (Roche,

stored as 1 mM stock aliquots at −20 °C), 500 ng poly-inosine (Sigma-Aldrich, stored as 1 µg/µl stock at −20 °C), 2 mM Tris–HCl pH 7.4 (Sigma-Aldrich, 100 mM stock stored at −20 °C), 10 mM DTT (Invitrogen, Carlsbad, USA, stored as 100 mM stock at −20 °C) in 1× first-strand buffer (Invitrogen, 5× stock: 250 mM Tris–HCl, 375 mM KCl, 15 mM $MgCl_2$, pH 8.3, stored in aliquots at −20 °C) at a final volume of 4.7 µl per reaction.

2. Cap-Mix sufficient for the number of samples that are collected (plus positive and negative controls) is freshly prepared on each experimental day and stored on ice in a light-protected, RNase-free 0.5-ml single sealed reaction tube (biopure, Eppendorf, Hamburg, Germany). All components are carefully added, mixed by finger flipping, and quickly centrifuged. SUPERase-In (Ambion, Austin, USA) is added directly from −20 °C to the reaction mix. Poly-inosine, NP40, and SUPERase-In are viscous and special care has to be taken during pipetting to avoid air bubbles. If bubbles are formed, the mix is centrifuged briefly until all bubbles disappear (see Note 3).
3. 100 U SuperScript II Reverse Transcriptase (Invitrogen, stored in aliquots at −20 °C) is added to each reaction *after* lysis (see subheading 3.3). The enzyme aliquots are stored in a benchtop freezer (Techne, Stone, UK) at −20 °C during the experiment to avoid "freeze–thaw cycles".

2.4. Quantitative Real-Time PCR

1. 2× QuantiTect Probe PCR Master Mix (Qiagen, Hilden, Germany).
2. 20x TaqMan Primer-Probe Assay (e.g., Applied Biosystems, Warrington, UK, α-synuclein assay number: HS00240906_m1, detects α-synuclein splice variants 112 and 140) (see Note 4).
3. cDNA for generation of standard curves to assess assay performance, e.g., SN cDNA (e.g., serial dilutions from 30 to 0.03 ng cDNA, derived from human SN tissue total RNA, Ambion, 1 mg/ml).
4. A real-time quantitative PCR (qPCR) instrument, e.g., the GeneAmp 7900HT real-time qPCR system (Applied Biosystems) used here, or a comparable instrument.
5. Suitable qPCR MicroAmp 96-well reaction plates and optical adhesive covers (e.g., both Applied Biosystems).

3. Methods

To guarantee successful UV-LMD and subsequent gene expression analysis of small cell pools and individual cells, it is essential to work in a strictly RNase-free regime. For details on RNase-free

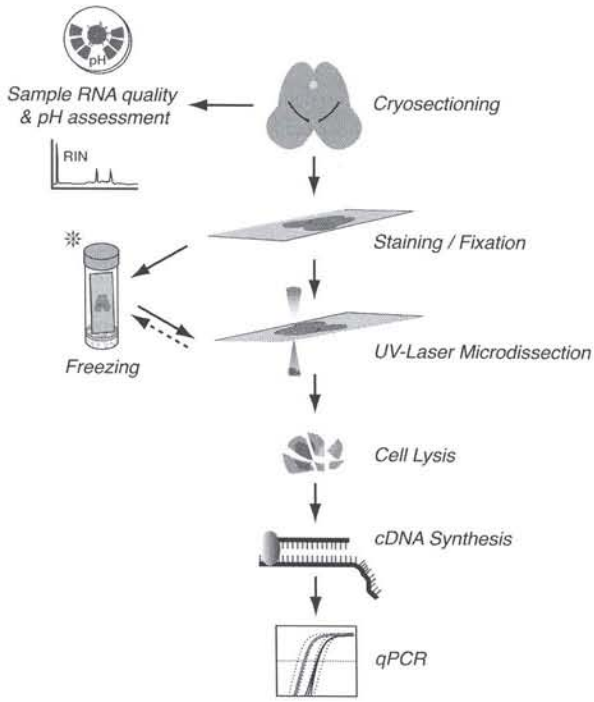

Fig. 1. Flowchart illustrating the experimental procedure for UV-laser microdissection and gene expression analysis of individual human dopaminergic neurons from postmortem midbrains. For details, please see text. *RIN* RNA integrity number.

working conditions, see Note 1. The protocol described below was used to quantify mRNA levels in NM + DA SN pars compacta neurons from human postmortem midbrain tissue blocks, provided by the German Brain Bank. However, it is applicable to various postmortem tissues from different species and can be principally adapted to many other tissues of interest. An overview of the experimental procedures is illustrated in Fig. 1.

3.1. Storage, Cryosectioning, and Staining of Human Brain Tissue

1. On the experimental day, human midbrain tissue is transferred (on dry ice) from −80 °C to the quick-freeze panel of a pre-cooled cryostat (−35 °C) and glued with tissue freezing medium (Leica) on a specimen holder (see Note 5). After an equilibration period of 20 min at −35 °C, the cryostat is set to the optimal cutting temperature (depends on the processed tissue, for our specimens −19 °C) and further equilibrated for 45 min before 12 µm horizontal midbrain sections including the SN are cut. Chippings from the trimming procedure are collected for RNA quality and tissue pH analysis (see Fig. 1 and Note 6).
2. The brain sections are mounted on UV-C treated PEN-membrane slides and allowed to thaw briefly (see Note 7). Once thawed, the slide is transferred to a 50 ml Falcon tube with 75 % ethanol at −20 °C and fixed for 2 min.

3. The slide is removed with a sterile forceps and 0.5 ml 1 % cresyl violet is applied directly on the slide using a sterile filter syringe (0.1 µm), incubated for 1 min, then dipped briefly in 75, 95, and 100 % ethanol absolute and finally incubated for 1 min in 100 % ethanol anhydrous.
4. The fixed and stained slides, each containing several brain sections, are stored in a drying chamber containing silica gel for at least 45 min before UV-LMD.
5. Alternatively, after drying (min. 45 min) the slides can be stored at −80 °C in storage jars (containing silica gel) and used later (see Fig. 1 and Note 8).

3.2. UV-Laser Microdissection of Individual Neurons from Human Brain Samples

1. All work spaces are cleaned to ensure RNAse-free working conditions (see Note 1).
2. The slides with tissue specimens are placed on the sterile slide holder and transferred to the UV-LMD microscope. Tissue quality and staining are inspected under low and high magnifications, and only sections that allow clear identification of individual cells are used for the experiment. Optimal laser settings need to be adjusted for each individual slide/section.
3. After the brain region of interest is found (in our case SN), an UV-C treated thin-walled PCR reaction tube is placed in the cap holder and transferred to the microscope. The reaction tube cap is inspected with the *cap-control* function to exclude rarely occurring contaminations with dust particles. Individual cells are cut and harvested into the cap of the reaction tube. It is recommended to visually control that all laser microdissected cells are successfully harvested (*cap-control* function, see Fig. 2).
4. If the cap-control is positive, the cap holder is removed and 4.7 µl Cap-Mix is added to the cap immediately. Any direct contact between the cap and the pipette tip must be avoided.

3.3. Lysis and cDNA Synthesis of Individual Laser Microdissected Neurons from Human Brain Samples

1. The reaction tube is carefully removed from the cap holder and the tube is closed upside down to ensure that the Cap-Mix remains in the cap.
2. The reaction tube is placed upside down on the cap in a preheated (72 °C) thermoblock and incubated for 2 min for cell lysis (see Note 9).
3. Afterwards, the tube is transferred onto an ice-cold metal block, again upside-down, and allowed to cool for 1 min.
4. The Cap-Mix is then spun down at 12,900 rpm in a benchtop centrifuge for 1 min at room temperature.
5. 0.3 µl SuperScript II is added directly to the Cap-Mix at the bottom of the tube after spinning.

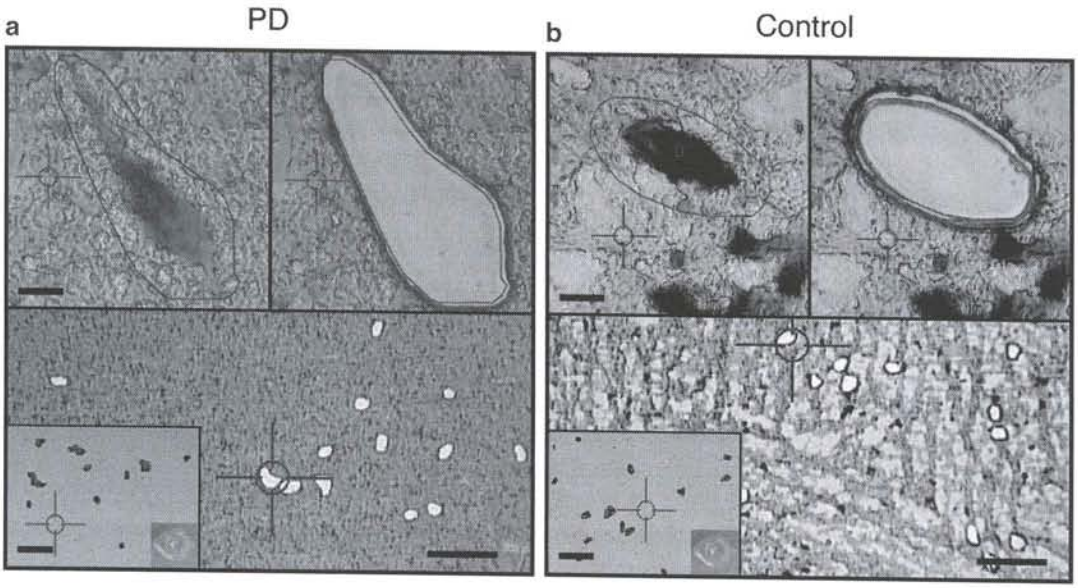

Fig. 2. UV-laser microdissection (UV-LMD) of individual neuromelanin-positive (NM+) dopamine midbrain neurons of human postmortem Parkinson's disease (PD) (**a**) and control (**b**) tissue sections. *Upper row*: individual neurons before (*left*) and after UV-LMD (*right*). *Scale bars*: 25 µm. *Lower row*: overview of the horizontal midbrain section containing substantia nigra after UV-LMD of 15 individual NM + neurons. *Scale bars*: 250 µm. *Inserts*: inspection of the reaction tube cap for cell collection. *Scale bars*: 250 µm.

6. The tube is transferred to a preheated (38 °C) thermomixer (350 rpm for 10 s every 10 min) and cDNA synthesis is carried out for at least 2 h or (recommended) overnight. For overnight incubation, all samples are spun down briefly and transferred to a preheated thermobox for final overnight cDNA synthesis (38 °C). After cDNA synthesis, samples are stored at −20 °C until further processing.
7. For each set of experiments, suitable positive (e.g., ~2 ng purified midbrain tissue RNA) and negative controls (no LMD-harvested cell in cap) are processed in parallel.

3.4. Quantitative Real-Time PCR of UV-LMD cDNA Samples and Data Analysis

1. The following procedures are all carried out in a UV-C-treated sterile workbench.
2. Each single cell cDNA sample is diluted 1:11 by adding 50 µl molecular-biology-grade water to the tubes with cDNA in 5 µl Cap-Mix. The diluted cDNA is vortexed and spun down. The tubes are stored in ice-cold metal blocks to ensure constant cooling. Alternatively, single cell samples are purified via ethanol precipitation (see Note 10) (17).
3. A master mix for quantitative real-time PCR in 20 µl reactions is prepared by mixing 10 µl 2× QuantiTect Probe PCR Master Mix, 1 µl 20× primer/probe mix (for gene of interest, e.g., α-synuclein), and 4 µl water (molecular-biology grade) for

each UV-LMD sample/PCR-reaction (volumes are multiplied by the number of samples +1).

4. 5 µl of UV-LMD sample derived cDNA (diluted or purified; for higher concentrations, see Note 10) is added to the bottom of a MicroAmp 96-well reaction plate. 15 µl of master mix is added to each cDNA sample and the plate is sealed with an optical adhesive cover. After spinning for 2 min (1,000 × *g*, at 4 °C), the plate is transferred to a RT-qPCR system (we use the 7900HT, Applied Biosystems) and the qPCR reaction is run using the appropriate cycling conditions (e.g., specific for our TaqMan assays: 2 min at 50 °C, 15 min at 95 °C and subsequently 50 cycles, 15 min at 94 °C and 1 min at 60 °C each, see Note 11).
5. A serial dilution of a cDNA standard is run in parallel with each experiment (e.g., 300-0.3 ng SN tissue-derived cDNA in serial-dilution steps of 10), which is used as a PCR-positive control, as well as to generate standard curves to assess assay performance and sensitivity and to calculate the cDNA concentration of the UV-LMD samples.
6. For data analysis, the detection threshold is set in the exponential phase of the qPCR amplification plot (relative fluorescence plotted against PCR cycle number, see Fig. 3). To quantify the expression of a certain gene via qPCR for a set of

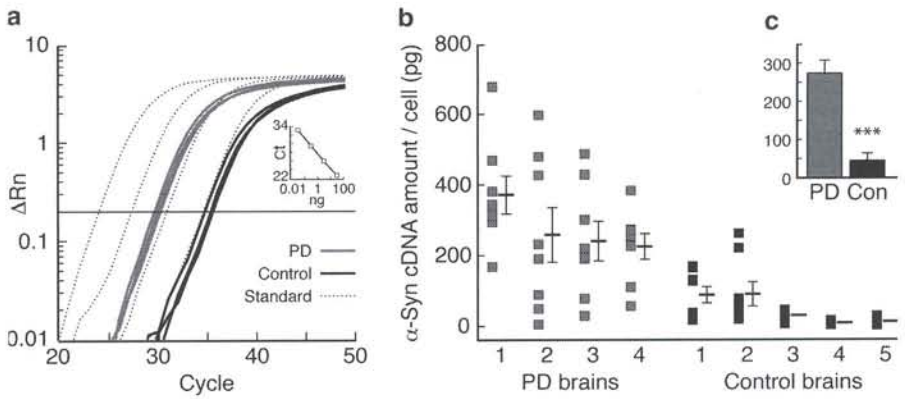

Fig. 3. Real-time PCR (qPCR) quantification of α-synuclein cDNA derived from individual laser microdissected neuromelanin-positive (NM+) substantia nigra (SN) neurons of human midbrain from postmortem control (Con) and Parkinson's disease (PD) cases. (**a**) α-synuclein qPCR amplification plot showing the change in relative fluorescence (ΔRN) per qPCR cycle for three pools of 15 dopamine neurons each, from control (*grey*) and PD (*black*) brains, as well as the qPCR amplification of standard curve cDNA (*dashed lines*, 30-0.03 ng cDNA, from left to right, generated from human SN tissue-derived mRNA (Ambion)). *Insert:* plot of threshold cycle (C_t) value vs cDNA amount (ng) for the tested standard curves. (**b**) Calculated α-synuclein cDNA amount per single cell (pg-equivalents of standard cDNA derived from SN-tissue per cell) for all analyzed UV-LMD collected dopamine neuron pools for 4 PD (*grey boxes*) and 5 control (*black boxes*) brains. Each box represents an individual UV-LMD sample. *Horizontal bars* represent average α-synuclein cDNA amount ± standard error of the mean per single cell for each individual brain (standard curve slope: −3.40, $Y_{intercept}$: 30.0). (**c**) Average α-synuclein cDNA amount per single NM+ dopaminergic midbrain neuron for all analyzed PD (274 ± 33 pg, $n=4$) and control brains (46 ± 18 pg, $n=5$, $P<0.001$).

samples, the same threshold value is used for all tested samples and standards. *Threshold cycle* (C_t) values of each sample as well as slope and Y-intercept of the standard curve are read out directly using an appropriate sequence detection software (e.g., SDS2.3, Applied Biosystems).

7. The average cDNA amount per cell in relation to the utilized standard is calculated according to the following formula:

$$\text{cDNA amount per cell} = \frac{S^{\left[\left(Ct - Y_{\text{intercept}}\right)/\text{slope}\right]}}{\text{No}_{\text{cells}} \times \text{cDNA fraction}}.$$

S corresponds to the serial dilution factor of the standard curve (e.g., 10 for serial dilution in steps of 10), No_{cells} refers to the number of harvested neurons per sample and cDNA fraction to the fraction of the UV-LMD cDNA sample used as template in the real-time PCR reaction, e.g., 5/55. The unit magnitude corresponds to the respective standard utilized, which defines the unit at the $Y_{\text{intercept}}$ (e.g., pg-equivalents of standard cDNA, derived from SN-tissue/cell, see Fig. 3 and Note 12).

4. Notes

1. Ribonuclease contamination is a crucial concern for successful cDNA synthesis of single laser microdissected cells or small cell pools. The ubiquitous RNase A is a highly stable and active ribonuclease, which is present on human skin as well as in the specimens and can easily contaminate any lab environment. Thus, creating and maintaining an RNase-free work environment and RNase-free solutions is essential for successfully performing RT-qPCR analysis. Therefore, we strongly recommend to follow these guidelines:
   (a) Always wear gloves when handling chemicals and sections/samples containing RNA. Change gloves frequently especially after touching potential sources of RNase contamination such as doorknobs, pens, pencils, and human skin.
   (b) If available, always use certified RNase-free tubes, pipette tips, and chemicals *for all steps* involved in the experiments (e.g., ethanol/staining solutions and jars for preparation of tissue sections for UV-LMD). Keep chemicals, tubes, etc. tightly sealed.
   (c) Treat UV-LMD (membrane) slides for 15 min with UV-light (e.g., in a sterile hood).

(d) Heat-sterilize all metal objects (forceps, spatulas, LMD cap holder, LMD slide holder), glassware, and any other equipment that gets in contact with slides or reaction tubes during UV-LMD experiments at 220 °C overnight.

(e) Clean pipettes, benches, and all other equipment that cannot be heat-sterilized with RNase decontamination solutions, e.g., RNase-ExitusPlus (AppliChem) and/or RNaseZapWipes (Ambion).

2. In our hands, NP40 seems to be less effective over time, even when continuously stored at 4 °C in the dark. Thus, we use NP40 aliquots for about three months.
3. Low-retention filter tips should be used for all pipetting steps. To avoid any RNase or DNA contamination. We recommend preparing the Cap-Mix under a sterile fume hood.
4. We recommend using qPCR amplicon sizes below 80 bp when working with tissues of significantly reduced/different RNA qualities (as assessed for example via Agilent RNA integrity number (RIN) analysis), which is often the case for human postmortem brain or other human tissues (1).
5. To reuse the specimen for several experiments, the brains are fixed on cork disks with a tissue freezing medium. These cork disks can be frozen quickly with a drop of water on the specimen holder of the cryostat and easily be removed after the experiment and stored again at −80 °C.
6. Tissue chippings of the cryosectioning procedure are used to assess overall RNA quality and tissue pH of each specimen. Transfer chippings into a liquid nitrogen precooled Falcon tube with cold sterile forceps and store at −80 °C until further usage, e.g., RNA extraction and RIN evaluation, or pH analysis.
7. LMD PEN-membrane slides with a strong iridescence must not be used. These membranes are damaged.
8. PEN-membrane slides with tissue sections can be stored in 50 ml Falcon tubes at −80 °C and reused for later experiments. To ensure that the slides stay dry, silica gel is added to the Falcon tube (see Fig. 1). A small sieve is used to separate the silica gel from the slide and to prevent contamination. For reuse, the slides are removed from −80 °C and allowed to equilibrate at −20 °C (15 min), −4 °C (15 min), and finally at room temperature (15 min) before usage.
9. Our mild lysis protocol is optimized for single UV-laser microdissected cells or small pools of individual cells from ethanol-fixed tissue sections. Please note that it is neither suited for lysis of larger microdissected tissue samples nor

suited for lysis of single cells from PFA-fixed tissue sections, and it is not tested for lysis of individual plant cells.

10. If the undiluted cDNA reaction is used for quantitative real-time PCR analysis or microarray experiments, cDNA needs to be purified by direct in-tube precipitation to avoid qPCR detriment (17). In this case, employing a reaction tube that is suited for longer high-speed centrifugation (e.g., no thin-walled PCR tubes) is recommended to be used for harvesting of UV-LMD samples. If cDNA precipitation is not required, a maximum of 10 % of the cDNA reaction should be used for quantitative downstream analysis to avoid well-described inhibitory effects of cDNA synthesis reaction components (17).
11. Note that optimal qPCR conditions depend on the qPCR assay system, master mix, and the respective primer/probes utilized.
12. Relative quantification, i.e., normalization against a so-called "housekeeping gene", is not recommended for RT-qPCR analysis of single cells or small pools of individual cells (18). Thus, it is crucial to use chemicals from the same stocks and lots for all experiments of a study, since even small differences in enzyme efficiencies or reagent concentrations can introduce a strong bias.

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