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Region-Specific In Situ Hybridization-Guided Laser-Capture Microdissection on Postmortem Human Brain Tissue Coupled with Gene Expression Quantification

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Abstract

This chapter describes the procedure of in situ hybridization-guided laser-capture microdissection performed on postmortem human brain tissue. This procedure permits the precise collection of brain tissue within anatomically defined brain nuclei that is enriched with mRNA. The chapter emphasizes the specific handling of postmortem tissue and preservation of RNA integrity to ensure high-quality gene profiling. Downstream procedures including mRNA amplification, gene profiling using high-density microarray chips, and confirmation with quantitative real-time polymerase chain reaction (qPCR) are described. PCR primer design and cDNA quantification required for qPCR are delineated.

Keywords

In situ hybridization; Postmortem; Microdissection; Laser capture; Gene expression; PCR; Microarray; Brain; Nucleus

1. Introduction

Since the introduction of chip-based microarrays, gene expression profiling of human postmortem tissue has risen in popularity in the search for genetic underpinnings of many neuropsychiatric illnesses. However, studying the human brain is inherently difficult due to its heterogeneity and size. Different brain regions exhibit high levels of structural and functional differences: therefore, valid gene expression studies must possess high levels of anatomical precision. In addition, manner of death as well as postmortem delay can negatively impact mRNA integrity significantly altering gene expression measurements. In fact, these factors can alter gene expression profiles to such an extent so as to overwhelm the impact of disease on such profiles.

For successful gene profiling, it is essential to limit the extracted tissue just to the anatomical boundaries of the area of interest and to obtain mRNA of sufficient quality that is compatible with downstream gene expression assays. Laser-capture microdissection (LCM) permits precise acquisition of discrete cell populations, such as samples from entire brain nuclei. We developed radioactive in situ hybridization (ISH) labeling of nuclei-specific markers as a guide for LCM (Fig. 1), which yields sufficient quality mRNA. In this chapter,

we describe in detail the processing of human postmortem brain tissue using ISH-guided LCM for subsequent gene expression profiling using microarray and quantitative real-time polymerase chain reaction (qPCR). Application of this method has been recently validated (1, 2).

1.1. RNA Quality

RNA integrity is the single most important factor for successful gene expression profiling in postmortem tissue. There are three parameters that contribute significantly to RNA quality: postmortem interval, brain pH, and the agonal state of the decedent in the final hours before death (3–5). The postmortem interval, which is defined as the time between death and postautopsy preservation of the brain, should not exceed 36 h. Brain tissue pH can be assessed with a calibrated pH meter and should be verified in multiple samples from different brain regions. The cut-off for acceptable pH is 6.6. Brain pH and agonal state are correlated parameters. Individuals who suffered from a prolonged agonal state, which includes respiratory arrest, multiorgan failure, or coma, tended to have lower brain pH values; whereas those with brief deaths, caused by accidents, cardiac events, or asphyxia, most often present a normal brain pH. Human brain cells principally depend on continuous supply of nutrition and oxygen for their energy metabolism. It is known that human brain cells are vulnerable when oxygen supply is reduced. Under such prolonged hypoxic conditions, ordinary transcriptional machinery is reprogrammed to divert the cellular resources to stress response, apoptosis, and inflammation control. Especially, anaerobic metabolic pathways, such as lactic acid cycle, are activated, resulting in an enhanced production of acid equivalents, and contribute to intra- and intercellular acidosis (6, 7). Therefore, the agonal state of the individual included in gene expression profiling studies should be assessed through determination of cause of death and detailed reconstruction of the final hours before death.

Processing brain tissue for LCM, especially at room temperature, accelerates the RNA degradation. All efforts must be taken to minimize processing time at room temperature and to limit the activity of RNases. For example, we do not recommend serial processing to laser capture more than one brain region per section. Our data indicate that the second dissected area is of lower RNA quality than the first nucleus within the same section. Because of the continued decline of RNA integrity, RNA quality needs to be assessed while processing. We recommend checking RNA quality after RNA tissue extraction before proceeding with amplification. Resulting electropherograms (Fig. 2) can be assessed in different ways: 28S/18S peak ratio, RNA integrity number (RIN), and area under the curve (AUC) assessments, which are described by Schoor et al (8). Postprocessing the gene expression data from the microarray chip deliver data that highly correlate with RNA quality: MAS5 present call (Fig. 3); 3'/5' GAPDH ratio; and slopes of RNA digestion plots (3). In Table 1, we list those measures for three nuclei processed as described in this chapter. Figure 3 displays correlation analyses between different RNA integrity measures and resulting gene expression microarray detectability. The results demonstrate that RIN and Schoor ratio are significantly correlated with microarray present call numbers; whereas 18S/28S ratio is not.

2. Materials

2.1. Brain Preparation, Sectioning, and Dehydration Solutions

1. Cryostat (Leica, Wetzlar, Germany).
2. Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA).
3. Stainless steel slide holding racks (for 20 slides; 75 mm long).
4. Diethylpyrocarbonate (DEPC).
5. 100% ethanol.
6. Xylene.
7. Fine-tooth saw.
8. pH meter (Corning, Cypress, CA).

2.2. LCM, RNA Isolation, and RNA Extraction

1. Autopix or Arcturus XT LCM machine (Molecular Devices, Sunnyvale, CA).
2. PicoPure RNA isolation system (Molecular Devices).
3. CapSure LCM macro caps (Molecular Devices).
4. RNase-free DNase Set (Qiagen Valencia, CA).

2.3. RNA Quality Assessment

1. Bioanalyzer 2100 (Agilent, Santa Clara, CA).
2. RNA PicoGreen LabChip Assay (Agilent).

2.4. RNA Amplification

1. Arcturus RiboAmp Plus 1.5 round kit (Molecular Devices).
2. RNeasy mini clean-up kit (Qiagen).

2.5. Biotinylation and Hybridization for Microarray Chips

1. BioArray High Yield RNA Transcription Labeling Kit (T7) (Enzo, Farmingdale NY).
2. NanoDrop 2000 (Thermo Scientific, Wilmington, DE).
3. Affymetrix (Affymetrix, Santa Clara, CA) or Illumina (Illumina, San Diego, CA) GeneChip microarray system.

2.6. Polymerase Chain Reaction

1. Quant-IT PicoGreen dsDNA assay (Invitrogen, Carlsbad, CA).
2. CytoFluor microplate reader (Applied Biosystems, Foster City, CA).
3. iQ SYBR Green Supermix (Bio-Rad, Hercules, CA).

4. iCycler system (Bio-Rad) with iQ Real-Time PCR Detection System (Bio-Rad).
5. 96-Well 0.2-ml thin-wall PCR plates (Bio-Rad).
6. Optical-quality sealing tape (Bio-Rad).

3. Methods

3.1. Tissue Preparation and In Situ Hybridization-Guided LCM

1. Frozen blocks of human brain tissue (0.75–1 cm thick) are placed on dry ice and dissected using a fine-tooth saw to generate tissue samples for subsequent cryostat sectioning. Such tissue blocks should be approximately 4 × 3 cm in size and stored immediately at –80°C.
2. Prepared tissue blocks are cut on a cryostat into 10-mm thick sections at –20°C and immediately thaw mounted onto a glass slides (one section per slide), and then stored at –80°C (see Note 1).
3. A section (adjacent to the one to be processed for LCM) needs to be treated to mark the area of interest. Several techniques can be applied (9, 10). We use digitized autoradiogram images from sections that underwent ³⁵S-radiolabeled ISH to mark RNA of interest. Details on this method with regard to processing postmortem sections can be found here (11). The ISH processed section can then be stained, using a Luxol Fast Blue method to reveal anatomical and histological landmarks. Histostained images complement the ones with the specific mRNA marker and are used as an orientation guide enhancing the accuracy of the microdissection (Fig. 1). It is not advisable to stain the section to be processed for LCM because it compromises RNA integrity (12).
4. Dehydration of slide mounted section as preparation for LCM (see Note 2).
 - (a) Remove from –80°C storage and leave at RT, 30 s
 - (b) Place in 75% ethanol, 30 s
 - (c) Place in 75% ethanol, 30 s
 - (d) Place in 95% ethanol, 30 s
 - (e) Place in 100% ethanol, 30 s
 - (f) Place in 100% ethanol, 30 s
 - (g) Place in xylene, 5 min
 - (h) Place in xylene, 5 min
 - (i) Air dry under hood, 20 min

Use solution for a maximum of five times, change container after two sets of 20 slides have been processed.
5. The sections can now be processed for LCM. Depending on the manufacturer, these steps may vary. We use the AutoPix instrument equipped with an inverted

microscope system using 4, 10, and 20 $\times$ objectives. Regional microdissections are performed under 4 $\times$ objective and with CapSure LCM macro caps (Arcturus). Next to the monitor with the microscope picture of the LCM image, have a second monitor (or printout) with the stained adjacent image on display, which will guide you when selecting the desired area (see Note 3). LCM settings used for human brain sections are: 50–75 mW power; 1,500–3,500 ms duration; and 200–250 mV intensity (see Note 4). When capture is completed, view captured cells on cap and the corresponding absence of cells on the tissue. Acquisition of image from the filled cap and processed section serves as a valuable quality control tool. Isolation is then initiated.

3.2. RNA Extraction

1. Place each cap into 0.5-ml tubes provided by the Arcturus PicoPure isolation kit.
2. Add 50 μ l of extraction buffer to each tube.
3. Invert the tube and incubate at 42°C for 30 min while gently shaking.
4. Centrifuge at 800 $\times g$ for 2 min, collect cell extract and remove LCM cap (see Note 5).

3.3. RNA Isolation

1. Preconditioning of RNA purification column.
 - (a) Add 250 μ l conditioning buffer.
 - (b) Incubate column for 5 min at RT.
 - (c) Centrifuge in provided collection tube at 16,000 $\times g$ for 1 min.
2. Add 50 μ l of 70% ethanol to RNA cell extract from step 3.2 and mix well by pipetting.
3. Pipette cell extract-ethanol mix ($\approx$ 100 μ l) onto preconditioned purification columns.
4. Centrifuge at 100 $\times g$ for 2 min, followed by centrifugation at 16,000 $\times g$ for 30 s.
5. Add 100 μ l of wash buffer W1 and centrifuge at 8,000 $\times g$ for 1 min.
6. Perform DNase treatment (see Note 6).
7. Add 100 μ l wash buffer W2 and centrifuge at 8,000 $\times g$ for 1 min.
8. Add another 100 μ l wash buffer W2 and centrifuge at 16,000 $\times g$ for 2 min. If residual buffer remains on the column, centrifuge again at 16,000 $\times g$ for 1 min.
9. Transfer purification column to provided 0.5-ml tube.
10. Add 20 μ l elution buffer onto membrane of purification column and incubate for 1 min at RT.
11. Centrifuge column at 1,000 $\times g$ for 1 min followed by 16,000 $\times g$ for 1 min.

12. Immediately process RNA or store at -80°C .

3.4. Agilent RNA Pico Assay and Assessment of RNA Quality

RNA integrity is crucial for the success of any gene expression profiling method. Postmortem samples will always have less than perfect quality. To know whether RNA quality is sufficient for reliable and reproducible results is imperative and there are many different methods to assess RNA integrity, such as 28S/18S peak ratio, RIN, and 3'/5' ratio of GAPDH expression. All these methods can tell a perfect from an imperfect sample. However, there is a gray zone at which a small amount of RNA degradation products are present, which will ultimately impact all three aforementioned measures. A recently published method (8), which we favor, indicates whether a moderately degraded RNA sample, such as from postmortem brain tissue, still produces reliable gene expression results. Next to RNA quality control, the RNA Pico assay has another purpose. It determines the amount of RNA in the sample, which is needed prior to RNA amplification reactions.

3.4.1. Agilent RNA Pico Assay—Preparing ladder

1. Thaw and spin ladder to bottom of the tube. Heat to denature for 2 min at 70°C .
2. Immediately cool the tube on ice.
3. Add 90 μl of RNase-free water and mix thoroughly.
4. Prepare aliquots in RNase-free tubes with the required amount for a typical daily use and store them at -70°C .
5. Before use, thaw ladder aliquots and keep them on ice (avoid extensive warming upon thawing process).

Preparing gel matrix

6. Remove reagents from 4°C storage and bring them slowly to RT.
7. Add 550 μl of gel matrix to a spin column and centrifuge at $1,500 \times g$ for 10 min.
8. The filtered gel can be used for up to 1 month when stored at 4°C .

Preparing gel-dye mix and electrodes

9. Place 65 μl of the filtered gel matrix (at room temperature) in a 0.5-ml tube.
10. Add 1 μl of pico dye, vortex, and then centrifuge at $13,000 \times g$ for 10 min.
11. Gel-dye mix is light sensitive and good to use for 24 h. One chip requires about 30 μl of gel-dye mix.
12. Clean electrodes by adding 400 μl of DEPC water to a clear cleaning chip, which is put into the bioanalyzer for 5 min. Open bioanalyzer lid and air dry for at least 2 min. Repeat this cleaning procedure once.

3.4.2. Loading the PicoChip

1. Place samples into 70°C water bath for 2 min. Do not heat ladder.

2. Open a new PicoChip and place in loading station.
3. Load 9 μ l of gel-dye mix into a well marked with a “white G in black circle.”
4. Close top of loading station and depress plunger until it is below retaining notch.
5. Wait 30 s before releasing notch carefully and pull plunger slowly back to the 1 μ l mark. Open loading station slowly.
6. Load 9 μ l of gel-dye mix into two remaining wells marked with a “G.”
7. Load 9 μ l of pico conditioning solution into a well marked with “CS.”
8. Load 5 μ l of pico marker into the remaining 12 wells which includes “ladder.”
9. Load 1 μ l of pico ladder in the well marked “ladder.” Then load 1 μ l of each sample into the sample wells (see Note 7).

3.4.3. Sample Run on PicoChip

1. The chip is placed securely in the supplied IKA vortexer and vortex for 1 min.
2. Place the chip in the bioanalyzer and close the lid (see Note 8).
3. Start bioanalyzer program (Agilent 2100 Expert software) and select Eukaryote total RNA pico, name samples, and then start the sample run (see Note 9).
4. Save the data and export them to an Excel spreadsheet format.
5. Clean electrodes with distilled water and let them air dry.

3.5. RNA Quality Assessment (8)

1. In Excel, determine the beginning and ending of elution time for the 18S and 28S peak (see Note 10).
2. Calculate the AUC from the appearance of the first peak of the sample to the beginning of 18S peak.
3. Calculate the AUC of the 28S peak and the total sample AUC that ranges from the appearance of the first peak to the last. The latter is usually the 28S peak.

According to the algorithm determined by Schoor et al., a sample with the following parameters delivers valid and reproducible gene expression results:

1. Less than 65% of the total signal is contained in the pre-18S area.
2. The 28S peak accounts for more than 4% of the total signal. Figure 2 shows a representative electropherogram.

3.6. RNA Amplification

For human tissue, the Agilent Pico Chip assay indicates a range from 1,000 to 10,000 pg/ μ l of RNA depending on LCM sample size and area. Most microarray experiments require nearly 1 mg RNA sample for detection. Amplification of the RNA is therefore required. RNA amplification is not necessary if only RT-PCR processing is desired. For amplification, we used isovolumetric sample amounts of RNA. For amplification reactions,

we employed the RiboAmp Plus 1.5 round kit and the BioArray High Yield RNA Transcription Labeling Kit (T7). The end product of the RiboAmp Plus 1.5 round protocol is amplified cDNA, which can be directly used for RT-PCRs (see Note 11). When intending to perform microarray experiments, only a portion of cDNA is retained for PCR; whereas the remainder is used with the BioArray High Yield RNA kit to produce biotin-labeled amplified RNA for microarray experiments.

1. Round 1 – first strand cDNA synthesis (see Note 12).
2. Round 1 – second strand cDNA synthesis.
3. Round 1 – cDNA purification (see Note 13).
4. Round 1 – In vitro transcription.
5. Round 1 – aRNA Purification.
6. Round 2 – first strand cDNA synthesis.
7. Round 2 – second strand cDNA synthesis.
8. Round 2 – cDNA purification (see Note 14).

3.7. Biotinylation for Microarray Experiments

1. We followed the instructions from Enzo BioArray High Yield RNA Transcript Labeling Kit. This kit uses two different biotinlabeled nucleotides (Biotin-CTP and Biotin-UTP), which enables more uniform and efficient labeling. Specifically, we use only 7 μ l of the Round 2 cDNA in the in vitro transcription (IVT) reaction and store the remainder of the sample at -80°C for RT-PCR. The reaction runs for 16 h and can stay at 4°C for a short length of time before purification.

The biotinylated RNA needs to be cleaned up. We use the Qiagen RNeasy Kit for this step.

2. Add 40 μ l of RNase-free water to each sample and then add 280 μ l of buffer RLT (do not add β -mercaptoethanol to the RLT buffer). Mix by pipetting up and down.
3. Add 340 μ l of 100% ethanol and mix again by pipetting up and down.
4. Load the entire volume onto a supplied spin column placed in a 2-ml collection tube. Close the lids and centrifuge at $8,000 \times g$ for 15 s. Discard flow-through.
5. Add 700 μ l buffer RW1 to the spin column. Close the lids and centrifuge at $8,000 \times g$ for 15 s. Discard flow-through.
6. Add 500 μ l buffer RPE to the spin column. Close the lids and centrifuge at $8,000 \times g$ for 15 s. Discard flow-through. Add another 500 μ l of buffer RPE and centrifuge at $8,000 \times g$ for 2 min.
7. Place the spin column in a new 1.5-ml collection tube. Add 30 μ l RNase-free water directly onto the column. Wait 2 min and then centrifuge at $8,000 \times g$ for 1 min to elute cleaned up RNA, which still needs to be quantified prior to array chip hybridization (see Note 15). The samples should be stored at -80°C .

3.8. Gene Chip Microarrays

1. After the biotin-labeled cRNA is prepared and purified, it can be used on Gene Chips. For the Affymetrix Expression system, the cRNA is fragmented and added to a hybridization cocktail containing probe array controls.
2. It is then hybridized to the Gene Chip during a 16 h incubation.
3. Immediately following hybridization, the array is washed and stained with a streptavidin phycoerythrin conjugate using an automated protocol on the GeneAtlas™ Fluidics Station, followed by scanning on the GeneAtlas™ Scanner. For Illumina Bead Chips, the cRNA is added directly to the hybridization cocktail and applied to the chip which is hybridized for 16–20 h at 58°C.
4. The chip is then washed and incubated with streptavidin-Cy3. The chip is again washed, dried, and scanned with an Illumina Bead Chip scanner.
5. The data is analyzed with BeadStudio software.

3.9. Quant-iT PicoGreen Assay for PCR Input Determination

Because we do not want to rely on reference housekeeping genes to determine relative abundance of gene expression, we utilized the Quant-iT PicoGreen dsDNA assay to determine nucleic acid concentration in our sample. This assay offers great sensitivity at relatively low cDNA concentrations (see Note 16).

Solution preparation

1. TE buffer: 20× dilution with sterile, distilled RNase-free water.
2. PicoGreen reagent: 200× dilution with 1× TE.
3. DNA standard: Two-step dilution (1:50, then 1:400) with 1× TE.

Sample processing

1. 1 µl cDNA sample.
2. 100 µl 1× TE buffer.
3. 100 µl PicoGreen reagent.

Standard curve – low range

1× TE buffer (µl)	50 ng/ml DNA standard (µl)	PicoGreen reagent (µl)	Final DNA conc.
0	1,000	1,000	25 ng/ml
900	100	1,000	2.5 ng/ml
990	10	1,000	250 pg/ml
999	1	1,000	25 pg/ml
1,000	0	1,000	0

Light-protected incubation of all samples is recommended for 2–5 min. Thereafter, samples (run in triplicates) and standard solutions are assayed in final volume of 200 µl in a 96-well microplate and processed on a CytoFluor microplate reader. Samples are excited at 485 nm

and measured with a 535 nm emission filter for 0.1 s. Sample concentrations were determined with the standard curve.

If cDNA from amplified material is used, we recommend a two-step dilution to obtain a final concentration of 50 pg/μl, which is the input concentration for subsequent qPCR.

3.10. Polymerase Chain Reaction

1. Genomic DNA and mRNA sequences are downloaded from NCBI Entrez Gene at <http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene>. PCR primer pairs are designed to anneal within 500 bp of the 3' end to generate a single amplicon that is shared by all of the known splice variants, between 75 and 150 bp in size using Primer3 software (13). Secondary structure is minimized for each amplicon using DNA Mfold (14) (<http://www.bioinfo.rpi.edu/applications/mfold/>), and primers are then designed against regions lacking predicted secondary structure.
2. Amplification reactions are carried out in 96-well 0.2-ml Thin-Wall PCR plates.
3. The total volume for each real-time PCR is 20 μl – which included 5 μl of cDNA, forward and reverse primers at a final concentration of 450 nM, and 10 μl of iQ SYBR Green Supermix, containing 50 U/ml iTaq/DNA polymerase, 6 mM MgCl₂, 0.4 mM of each dNTP, and 20 nM fluorescein.
4. All amplifications and fluorescence quantifications are performed in real-time on a Bio-Rad iCycler using a SYBR-488 detection protocol, at a peak excitation wavelength of 490 nm and peak emission wavelength of 530 nm.
5. To minimize amplification of nonspecific products, a hot-start PCR protocol is employed as follows: denature at 95°C for 30 s, followed by ten cycles of denaturing at 95°C for 15 s annealing at 65–60°C for 15 s, extension at 72°C for 15 s. Annealing temperature is decreased at each step of the cycle by 0.5°C from a maximum of 65°C to the final temperature of 60°C. This is followed by 35 cycles of denaturing at 95°C for 15 s, annealing at 60°C for 15 s, extension at 72°C for 15 s. Following each extension step, primer dimers are denatured by raising the temperature to 83°C for 30 s, and then fluorescence is quantified. This cycling was followed by a final extension step at 72°C for 5 min.
6. Following amplification, PCR products are denatured by sequential increases in temperature from 72°C to 95°C in 0.5°C increments. At each step, the temperature was held constant for 10 s, and fluorescence was quantified. Presence of specific amplification products was confirmed by the single peak on the melting curve, plotted as the negative derivative of fluorescence as a function of temperature.

4. Notes

1. Ideally, the cryostat is dedicated to sectioning of human specimens only to prevent contamination with human pathogens. It needs to be thoroughly cleaned before and after use with 70% ethanol. Always wear protective gloves.

2. Postmortem human brain samples, by virtue of their harvesting methodology, have less than perfect RNA quality. Therefore, it is important to prevent further RNA degradation during processing of each tissue sample. Aqueous solutions can contain RNases, which need to be deactivated. We use the following protocol to produce RNase-free water.
 - (a) Add 1 ml of 0.1% diethylpyrocarbonate (DEPC) to 1,000 ml distilled water
 - (b) Mix well and let set at room temperature overnight
 - (c) Autoclave solution
 - (d) Let cool to room temperature prior to useUse DEPC-treated water for making ethanol dilutions. Moisture or other contaminants on the brain section, such as unevaporated xylene, contaminated ethanol solutions, fingerprints, or frost hinder the ability to perform a satisfactory LCM.
3. Next to shape, use anatomical landmarks such a tissue border, fiber tracts, and holes from blood vessels as alignment guides for your area of interest. Use roughly the same magnification as on the monitors. Newer LCM software may also permit to overlay the stained image from the adjacent section with your target section.
4. Before starting a series of collection, test fire the laser on a piece of tissue outside the area of interest. The laser focus is verified by observing black, melted rings of plastic. When slides are changed, readjustment of focus may be necessary. Keep the caps free from any contamination. Adjustments to the laser power and duration may be needed during the tissue collection process from multiple slides. In general, if the plastic does not melt increasing the power might help and if cells are not detached laser duration should be increased. Steadily increasing power settings over time to achieve cell lifting indicate that the sections are absorbing moisture.
5. It is advisable to extract only and store the sample at -80°C until all the samples for one study are captured. From RNA isolation step, all samples of one study should be processed together to minimize variability.
6. DNase treatment is crucial step to remove genomic DNA. We use RNase-Free DNase Set from Qiagen.
 - (a) For one sample: Add 5 μl DNase I Stock solution to 35 μl buffer RDD. Mix gently. According to the number of samples to be processed, calculate the needed total amount of this mix and make one large batch.
 - (b) Pipette 40 μl DNase incubation mix into the purification column membrane. Incubate at RT for 15 min.
 - (c) Pipette 40 μl PicoPure RNA Kit Wash Buffer 1 (WB1) into the purification column membrane and centrifuge at $8,000 \times g$ for 15 s.
 - (d) Proceed with RNA isolation.

7. Perform steps 6–9 relatively fast with no breaks in between. Use the loaded chip within 5 min.
8. It is important that no bubbles are introduced during this step. Close and open the lid slowly several times to ensure the absence of any air bubble. If a message about a bad chip appears right at the beginning of the run, there are probably bubbles in a well. Lid then needs to be opened and closed three times before restarting the assay. If this does not resolve the problem, the chip may need to be rerun.
9. If after a rerun of the chip, the results are still not satisfactory, the pico head may need to be cleaned. Electrodes are gently scrubbed with a soft toothbrush, then sonicated for 10 min in RNase-free water. Change water and repeat procedure once with water and then twice with 100% ethanol. Dry pico head at 37°C overnight before using again.
10. To calculate AUC, use the numerical approximation method called the trapezoidal rule. Divide the desired AUC into very small segmental trapezoids and add their areas together. The smallest possible time increment between two signal data points equals the height of one trapezoid. Make sure to subtract the height from the baseline first before calculating the AUC.
11. The kits contain only a slight volume coverage of components. Be careful with pipetting. Use a fresh pipette tip for each addition to each sample. This will provide a consistency between samples. You can stop at any of the points indicated in the protocol and store the samples as described. It is possible to complete all of Round 1 in one day and store the samples at –80°C overnight.
12. The kit allows you to use up to 11 µl of RNA from the LCM sample. The amount you use will depend on the concentration of the RNA determined from the Pico Chip (Subheading 3.4, step 1). For the RiboAmp Plus kit, between 10 and 40 ng of starting material is needed. All samples will have different concentrations and it is possible to adjust the volume you use so that all samples have the same starting concentration. Alternatively, one can use the same volume for all samples as long as the concentrations fall within the range indicated by the kit.
13. Be careful when adding the DNA binding buffer to your sample tube. The PCR tube will be almost full and mixing will be difficult. Be careful when centrifuging your tubes in the final step. It is possible to break the lids off if the tubes are not placed in the centrifuge correctly. Protocol directions should be strictly followed.
14. Round 2 will take less than 3 h. If you intend to start the final IVT reaction during the same day, store the samples on ice or complete Round 2 just before you begin the IVT reaction which will incubate overnight.
15. The RNA samples can be quantified with a NanoDrop 2000 UV-Vis photospectrophotometer. We use 1 µl of sample on the NanoDrop. Concentrations usually range from 500 to 2,000 ng/µl. Chip input amounts vary depending on manufacturer: While the Illumina 8 sample chip requires only 750 ng of human aRNA, the HG-U133 Plus 2.0 from Affymetrix array requires 15 mg aRNA.

16. The sensitivity of this assay is limited to 25 pg/μl cDNA. If no amplification step is used, one needs to collect more tissue (sections) to yield more RNA. The amounts provided in Table 1 can serve as orientation. Also consider that 50 pg/μl cDNA is needed for qPCR.

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Fig. 1.

(a) Pseudocolored ISH image of a coronal brainstem section with TH mRNA (*red in online version*) enriched in the LC and TPH2 mRNA (*green in online version*) enriched in the nuclei MR and DR. (b) Klüver–Barrera stain of adjacent section displaying corresponding anatomical landmarks such as fiber tracts and nuclei. (c) Adjacent section to (b) on which LCM was performed to selectively remove LC tissue. *Overlaid black dots* represent TH mRNA signal from Fig. 1a to illustrate the precision and selectivity of the laser capture. (d) Adjacent section to (c) on which LCM was performed to selectively remove MR and DR tissue. *Overlaid black dots* represent TPH mRNA signal from Fig. 1a to illustrate the precision and selectivity of the laser capture.

Fig. 2.
Representative electrophoretic trace of a postmortem RNA sample extracted with LCM. Pre-18S, 18S, and 28S peak areas are important denominators in the assessment of RNA integrity.

Fig. 3.

Correlation of RNA integrity measures obtained from Bioanalyzer electropherograms with present call detection rates from corresponding gene expression microarray chips ($N = 21-23$). Schoor ratio (pre-18S/28S) and RNA integrity number show significant ($p < 0.05$) correlations ($r^2 = 0.28$ and 0.33 , respectively), whereas 18S/28S peak ratio did not correlate ($p = 0.50$; $r^2 = 0.02$) with MAS5 present call rates (%).

Table 1

RNA quality assessments obtained from electrophoretic traces from extracted RNA and indirect RNA quality measures derived from gene expression microarray chip analysis ($N=10/\text{nucleus}$; except for DR: $N=9$)

	Isolated RNA conc. (ng/μl)	RNA integrity number (RIN)	Schoor et al. values			% present call rate MAS5CALLS algorithm	GAPDH 3'/5' ratio	RNA degradation slopes
			Pre-18S/total AUG	28S/total AUG	28S/18S peak ratio			
Locus coeruleus	6.6 ± 2.5	4.58 ± 0.51	47.4 ± 11.9	10.8 ± 2.6	0.64 ± 0.19	46.12 ± 2.62	1.51 ± 0.05	10.60 ± 0.14
Dorsal raphe	3.8 ± 1.6	5.76 ± 0.31	44.3 ± 7.2	11.2 ± 3.8	0.72 ± 0.11	49.12 ± 4.03	1.37 ± 0.03	10.85 ± 0.50
Median raphe	7.7 ± 1.4	4.51 ± 0.91	45.3 ± 7.5	9.4 ± 2.4	0.61 ± 0.15	38.30 ± 6.37	1.46 ± 0.03	9.51 ± 0.56

Note that all RNA quality measures are concentration independent and that MR values are generally worse than LC and MR which is probably due to longer exposure at room temperature during LCM procedure