Laser microdissection of immunolabeled astrocytes allows quantification of astrocytic gene expression

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Abstract

Astrocytes represent the major glial cell population within the central nervous system. In order to elucidate the function of astrocytes under physiological conditions and during the course of neurological disease, astrocytic gene expression profiling is necessary. However, since astrocytes form an intimately connected network with neurons and other cell types in the brain, gene expression analysis of astrocytes with a sufficient degree of cellular specificity is difficult. Here we are presenting a rapid and, thus, RNA preserving immunostaining protocol for the detection of astrocytes in rodent brain. This protocol can readily be combined with laser microdissection (Leica AS LMD platform) and quantitative RT-PCR (qPCR). Employing this method, we studied changes in glial fibrillary acidic protein (GFAP) expression in astrocytes of mouse entorhinal cortex following entorhinal cortex lesion. Using laser microdissection, astrocytes (n = 60) were collected in the tissue surrounding the lesion, the entorhinal cortex contralateral to the lesion, and in unlesioned control animals. Changes in GFAP mRNA were quantified using qPCR. GFAP mRNA levels were 82-fold higher in astrocytes of lesioned animals at the site of the lesion compared to GFAP mRNA levels in entorhinal cortex astrocytes of control mice. GFAP mRNA levels were only slightly elevated at the contralateral side (lesioned animals). This optimized protocol for immunolabeling and laser microdissection of astrocytes followed by qPCR allows quantification of astrocytic gene expression levels with a high degree of cellular specificity. It may similarly be employed in different settings where other cell types need to be identified and microdissected for gene expression profiling.

Keywords: Glia; Glial fibrillary acidic protein; Entorhinal cortex lesion; Gene profiling; Brain injury; Quantitative RT-PCR

1. Introduction

Astrocytes represent the major glial cell population within the central nervous system. They are widely appreciated as key players in maintaining optimal extracellular conditions for neuronal function (Hatton, 2002; Haydon, 2001). Furthermore, they are important for the formation and maintenance of the blood brain barrier (Prat et al., 2001), act as immunocompetent cells (Dong and Benveniste, 2001), and respond to different forms of brain injury (Eddleston and Mucke, 1993; Hatton et al., 1991; Ridet et al., 1997). Nonetheless, the complex functions of astrocytes are not yet fully understood. To further elucidate astrocyte functions in the CNS, astrocytic gene expression profiles under physiological conditions and during the course of neurological disease may be required.

Laser microdissection (LMD) is a new research tool allowing the rapid isolation of selected cell populations from complex tissues such as the brain. Combining this technique with qPCR facilitates gene expression analysis of defined tissue domains. Recently, we reported an optimized protocol for layer-specific gene expression profiling in the hippocampus (Burbach et al., 2003). However, principal cells of the hippocampus form densely packed and easily recognizable cell layers (Amaral and Witter, 1989; Stanfield and Cowan, 1979) and can, therefore, be readily microdissected as fairly homogenous neuronal cell populations (Burbach et al., 2003). In contrast, the harvesting of glial cell populations is far more challenging. Glial cells, such as astrocytes, are intermingled with other cell types and do not form distinguishable cell layers. Thus, single glial cells have to be identified, collected, and pooled in order to perform gene expression profiling with a sufficient degree of cellular specificity.
Unfortunately, standard histochemical stains are inadequate for distinguishing astrocytes from other cell types and are, thus, not useful for precise astrocyte microdissection. Only immunostaining protocols for astrocyte specific marker proteins such as glial fibrillary acidic protein (GFAP) offer the possibility of unequivocal astrocyte identification. To ensure reliable tissue transfer as well as preservation of intact RNA, however, the immunostaining procedure has to be adjusted. We have recently generated a protocol which can be used for the identification and microdissection of astrocytes in rodent brain (Burbach et al., 2004). This protocol, which yields high quality mRNA for astrocytic gene profiling is reported in detail here.

In order to demonstrate the usefulness of this methodology, we have studied changes in GFAP mRNA expression levels in identified astrocytes following unilateral entorhinal cortex lesion ECL (for review see Deller and Frotscher, 1997; Deller et al., 2001; Steward, 1976). Injury to the adult CNS, such as ECL, results in a rapid response from resident astrocytes at the site of the lesion (Hatten et al., 1991; Ridet et al., 1997). This so called reactive astrogliosis is associated with an upregulation of both mRNA and protein of the major astrocytic intermediate filament molecule GFAP (Amaducci et al., 1981; Eng and Lee, 1995; Mathewson and Berry, 1985). In the present report, we have microdissected astrocytes in the mouse entorhinal cortex following lesion and have quantified GFAP mRNA levels of the lesion-associated astrocyte population with a very high degree of cellular specificity.

2. Materials and methods

2.1. Animals and surgical procedures

Male C57Bl/6 mice (6 month) were obtained from Charles River Laboratories (Sulzfeld, Germany). Since GFAP mRNA levels fluctuate across the estrous cycle and differing hormone levels may complicate result interpretation (Kohama et al., 1995) only male mice were used for this study. Unilateral ECLs were performed in five animals; five unlesioned animals served as controls. All surgical procedures were performed under deep anaesthesia with medazolam (0.1 mg/kg body weight i.p.), fentanyl (0.5 mg/kg body weight i.p.) and droperidol (16.5 mg/kg body weight i.p.) and were started only after animals failed to respond to light and touch stimuli. Complete unilateral ECLs were performed as described (Del Turco et al., 2003). In brief, after exposing the skull and estimating lateral coordinates for lesion placement a small hole was drilled above the transverse sinus. A wire-knife (David Kopf Instruments, Tujunga, CA, USA) was angled 10° backwards and placed immediately anterior to the sinus (+0.1 mm; L: 2.5 mm). The knife was lowered 4.5 mm, extended 3 mm and removed from the brain. All animal procedures were in agreement with the German law on the use of laboratory animals.

2.2. Tissue preparation

Mice were sacrificed using an overdose injection of pentobarbital (300 mg/kg body weight i.p.) 48 h after surgery. At this particular time point GFAP mRNA levels in lesioned brain are maximal (Horumb et al., 1990). The brain was removed, embedded in tissue freezing medium (Leica, Bensheim, Germany) and immediately flash-frozen in 2-methyl-butane at −40 °C for short term storage at −70 °C or instant sectioning. Serial horizontal cryostat sections (thickness: 6 μm) were cut and mounted on autoclaved polytetrafluoroethylene (PET) foil stretched on a metal frame (Leica). Sections were then fixed in ice-cold acetone for 2 min, dried on a heater at 40 °C for 10 min and subjected to GFAP immunostaining. Acetone fixation was chosen after several standard fixation solutions were tested for influence on morphological quality and background fluorescence as well as adhesion of the tissue to the PET foil.

2.3. GFAP immunostaining

First, sections were blocked for 3 min with 10% normal horse serum in PBS in DEPC water containing 0.3% Triton X-100. Then, a polyclonal GFAP-antibody solution (DAKO, Hamburg, Germany, 1:50 in 10% BSA in PBS containing 0.1% Triton X-100) was directly applied onto the mounted section, incubated at 40 °C for 5 min in a wet chamber and rinsed three times 15 s in PBS. After incubation with an Alexa568-labeled goat anti-rabbit antibody (MoBiTec, Göttingen, Germany, 1:25) for 2 min at room temperature, sections were again rinsed three times 15 s in PBS, and once in DEPC water. The sections were then dried at 40 °C for 10 min and immediately subjected to LMD. Several dilutions and proportions of primary to secondary antibody concentrations as well as various durations of different incubation steps were tested in serial experimental settings with the aforementioned details representing optimal conditions (fastest staining with adequate morphology).

2.4. Astrocyte LMD, RNA isolation and reverse transcription

PET foil metal frames were mounted on a Leica AS LMD system (Leica) with the section facing downwards. Using a 63 or 150× objective, cutting intensity; aperture and velocity were adjusted as follows: 63× objective, aperture 5, intensity 45, speed 1, offset 45; 150× objective, aperture 1, intensity 35, speed 1, offset 57. Then, the pulsed UV laser beam was carefully directed along the borders of single astrocytes. For each serial section of the operated animals, astrocytes from the lesion site and a corresponding area at the contralateral non-lesioned side were collected separately. To avoid a biased selection of intensely immunofluorescent astrocytes, all immunolabeled and lesion-associated cells within a single visual field were collected indiscriminately regarding their staining intensity. The cells cut (n
Table 1
qPCR assay for GFAP detection

<table>
<thead>
<tr>
<th>Target</th>
<th>Designation</th>
<th>Primer or probe sequence (5′ → 3′)</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFAP</td>
<td>GFAPF</td>
<td>ACC GCA TCA CCA TTC CTG TAC</td>
<td>85</td>
</tr>
<tr>
<td></td>
<td>GFAPR</td>
<td>TGG CCT TCT GAC ACG GAT TT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GFAP probe</td>
<td>TCC AGA TCC GAG AAA CCA GGC TGG A</td>
<td></td>
</tr>
<tr>
<td>18S rRNA</td>
<td>18SF</td>
<td>CGG CTA CCA CAT CCA AGG AA</td>
<td>181</td>
</tr>
<tr>
<td></td>
<td>18SR</td>
<td>GCT GGA ATT ACC GCG GCT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>18S probe</td>
<td>TGC TGG CAC CAG ACT TGC CCT C</td>
<td></td>
</tr>
</tbody>
</table>

= 60) were then transferred by gravity alone into a micro-
 centrifuge tube cap placed directly underneath the section.
The tube cap was filled with a guanidine isothiocyanate
(GITC)-containing buffer (Buffer RLT, RNeasy Mini Kit,
Qiagen, Hiden, Germany) to ensure isolation of intact
RNA. Cell collection was verified by inspecting the tube
cap. Microcentrifuge tubes were immediately transferred
on ice after tissue collection followed by three freeze-thaw
cycles in a dry ice/ethanol bath. Total RNA was isolated
using the RNeasy Micro Kit (Qiagen) according to the

![Fig. 1. Astrogliosis following unilateral entorhinal cortex lesion. Mice
were sacrificed 48 h after unilateral entorhinal cortex (EC) lesion. Brain
was flash-frozen, cut and immunostained with a GFAP antibody. Whereas
multiple GFAP-labeled astrocytes (arrows) were observed surrounding the
lesion site (asterisks) in the ipsilateral EC (a, ipsi), fewer GFAP-labeled
astrocytes were found in the unlesioned contralateral side (b, contra).
Insets demonstrate selected single astrocytes at higher magnification. Scale
bars: 50 μm; inset: 5 μm.](image1)

![Fig. 2. Microdissection of astrocytes. GFAP-immunostained sections were
mounted on a Leica AS LMD system. Single GFAP-positive astrocytes
were identified in mouse EC (a). After adjustment of cutting intensity,
aperture, and speed, astrocytes were carefully microdissected under direct
microscopic visualization. A snapshot during the process of microdissec-
tion is depicted in (b), the respective area after microdissection in (c).
Minor edge artifacts as a direct consequence of drying immunofluores-
cent sections do not interfere with the recognition of astrocyte contours
(closed dashed line). Scale bars: 5 μm.](image2)
manufacturer’s recommendations with an elution volume of 12 µl and the addition of poly-A carrier RNA to the lysate. DNA digestion was not required since primers and probe were intron-spanning (Table 1). Integrity of the isolated RNA was determined using the Agilent RNA 6000 Pico Lab Chip (Agilent Technologies, Waldbronn, Germany). RNA was reverse transcribed using TaqMan Reverse Transcription Reagents (Applied Biosystems) and random primers.

2.5. Quantitative reverse transcription-PCR
cDNAs were subjected to PCR utilizing the ABI Prism 7000 Sequence Detection System (Applied Biosystems) and TaqMan Universal PCR Master Mix (Applied Biosystems). GFAP primers and an intron-spanning probe bridging the border between Exon 7 and Exon 8 of the GFAP gene were selected using Primer Express software (Applied Biosystems, Table 1). For normalization of Ct-values an endogenous control, eukaryotic 18S ribosomal RNA gene were selected using Primer Express software (Applied Biosystems). GFAP primers and an intron-spanning probe bridging the border between Exon 7 and Exon 8 of the GFAP gene were selected using Primer Express software (Applied Biosystems). Primers were labeled to an endogenous control, eukaryotic 18S ribosomal RNA gene were selected using Primer Express software (Applied Biosystems). Primers were labeled to an endogenous control, eukaryotic 18S ribosomal RNA for 2 min, 1 cycle of 95°C for 15 s and 60°C for 1 min. Direct detection of the PCR product was enabled by the 5′ → 3′ exonuclease activity of Taq polymerase thereby terminating the close proximity of the reporter dye to the quencher dye by reporter fluorochrome cleavage leading to an increase of reporter dye fluorescence equivalent to the increase of amplified PCR product. Using standard curves of serial control cDNA dilutions a relative quantitation of target cDNA expressed in 2^-x-fold differences was performed. All quantitations were normalized to an endogenous control (18S rRNA) to account for variability in the initial concentration and quality of total RNA and in the conversion efficiency of the reverse transcription reaction. Statistical analysis was performed using SPSS for Windows (SPSS, München, Germany). The non-parametric Kruskal–Wallis test was used to compare differences among the groups investigated, pair-wise comparison using the Mann–Whitney U-test. Differences were considered statistically significant if the P-value was ≤ 0.05.

3. Results
3.1. Microdissection of GFAP-immunostained astrocytes and isolation of high quality RNA for qPCR analysis
Using the rapid immunostaining protocol outlined in the previous paragraph, individual GFAP-labeled astrocytes could readily be detected on entorhinal cortex sections mounted on PET foils (Figs. 1 and 2). Acetone fixation assured adhesion of the section to the PET foil during the staining procedure and, thus, convenient tissue handling. Optimized settings during microdissection ensured straightforward collection of astrocytes (Fig. 2). Capillary electrophoresis using the RNA 6000 Pico Lab Chip demonstrated distinct ribosomal RNA peaks and no substantial shift of RNA size distribution to small size fragments indicating high RNA quality (Fig. 3). By choosing primers residing in two different exons and an intron-spanning probe, specific detection of GFAP mRNA but not genomic DNA was assured. Data reproducibility was excellent if samples were run in triplicates.

3.2. Upregulation of GFAP mRNA levels in astrocytes surrounding the site of the lesion following ECL
Astrocytes located close to the margin of the ECL were microdissected. After RNA extraction GFAP mRNA levels were measured by qPCR. Relative GFAP mRNA quantification in astrocytes of lesioned animals demonstrated a 82-fold increase at the site of the lesion compared to entorhinal cortex astrocytes in unlesioned control animals (Fig. 3, Burbach et al., 2004). Astrocytes located in the contralateral non-lesioned entorhinal cortex, which is partially denervated following ECL, showed slightly elevated GFAP mRNA levels. Non-parametric testing (Kruskal–Wallis) followed by pair-wise comparison using the Mann–Whitney U-test revealed significant differences to control GFAP mRNA levels for both, lesion site and contralateral EC (P ≤ 0.05).

4. Methodological considerations
4.1. Preliminary remarks
Standard precautions for the LMD/qPCR procedure have been reviewed in detail by Fink et al. (Fink and Bohle, 2002). Furthermore, general recommendations during combined LMD/qPCR as well as platform-specific measures for using the Leica AS LMD system have been reported recently (Burbach et al., 2003). In this methodological report, we will, therefore, focus on technical aspects concerning the rapid immunofluorescent detection and microdissection of astrocytes.

4.2. Morphology and immunostaining procedure
4.2.1. Astrocyte identification
Histochemical staining of whole brain sections allows the microdissection of densely packed cell layers in highly organized tissues like the hippocampus (Burbach et al., 2003). Astrocytes, however, do not form separately distinguishable cell layers and standard histochemical stains are, therefore, inadequate. Immunostaining for the astrocyte-specific marker protein GFAP in general follows a straightforward protocol. However, staining procedures for LMD and subsequent qPCR analysis are complicated by the risk of
Fig. 3. Astrocytic GFAP mRNA is upregulated at the lesion site following ECL. LMD was performed as indicated using the rapid immunostaining protocol described in this study. Microdissected astrocytes from operated animals at the site of the lesion, at the contralateral side and from control animals at corresponding tissue areas were collected. RNA was isolated and subjected to quality analysis on a Agilent 2100 bioanalyzer. Electropherograms demonstrated clear 18S and 28S rRNA peaks and no significant shift of RNA fragments to shorter migration times indicating high RNA quality (a).

Relative quantification of GFAP mRNA levels by qPCR demonstrated a 82-fold increase in astrocytes surrounding the site of the lesion compared to astrocytes from a corresponding area in control animals. GFAP mRNA levels in astrocytes from the contralateral entorhinal cortex of operated animals were slightly, however significantly, elevated. ∗P ≤ 0.05 is considered statistically significant.

RNA degradation that is greatest in aqueous environments (Burbach et al., 2003; Fink and Bohle, 2002). Therefore, routine immunostaining procedures that often require overnight antibody incubation are not suitable for qPCR studies and can only be applied for DNA or proteomic analysis (Hofbauer et al., 2003; Kaserer et al., 2000; Mouledous et al., 2003). Consequently, a rapid albeit highly specific immunostaining protocol had to be developed to limit the risk of RNA degradation. Protocols for the identification of selected cell populations in brain (Bi et al., 2002; Prosniaik et al., 2003) as well as in non-CNS tissues (Fend et al., 1999; Fink et al., 2000a,b; Lindeman et al., 2002; Murakami et al., 2000; Trogan et al., 2002) have already been generated. However, a detailed description of this methodology and its concurrent pitfalls has not been published yet. Furthermore, platform-specific application protocols are required since the different LMD platforms currently available (Emmert-Buck et al., 1996; Kolble, 2000; Schütze et al., 1998) call for different technical approaches. During the present study, we have developed a protocol which takes the technical specifications of the Leica AS LMD system as well as the need for very short incubation times into account.

4.2.2. Staining times

In order to minimize staining times, Fend et al. have successfully applied a three-step streptavidin biotin technique with pre-diluted primary antibodies (DAKO Quick staining kit, DAKO (Fend and Raffeld, 2000)). Unfortunately, this staining kit is no longer commercially available. Therefore, standard PAP, APPAP, streptavidin-biotin or immunofluorescence protocols have been adapted for the purpose of targeted microdissection and subsequent RNA analysis (Bi et al., 2002; Fink et al., 2000a,b; Lindeman et al., 2002; Prosniaik et al., 2003). For this study, we have chosen an immunofluorescent staining approach since immunofluorescence protocols, in general, can be shortened to a greater extent than other methods, and the RNA recovery rate is higher (Fink et al., 2000b).

4.2.3. Platform-specific adaptations

Use of the Leica AS LMD system requires sections placed directly on PEN (polyethylene) or PET-foil anchored to a slide or metal frame. We have shown previously that sections mounted on the thinner PET-foil revealed significantly better morphology if standard histochemical stains were applied (Burbach et al., 2003). Again, immunostaining on PET-foil used during this study demonstrated reliable morphological quality and lowest fluorescent background levels. Because the use of dried sections without coverslip is necessary for optimal tissue capture, autofluorescent edge artifacts may occur and confuse the investigator. If so, the careful application of a few 100% ethanol drops onto the tissue section on the demounted slide eliminates these artifacts without interfering with the subsequent microdissection procedure.

4.2.4. Fluorophores

The choice of strong and stable fluorophores for labeling was critical since laser microdissection procedures may last up to 2 h (Burbach et al., 2003). During this time, bleaching should be minimized to ensure straightforward collection of several astrocytes in the same visual field. So far, most authors have used cyanine dyes or rhodamine and fluorescein-coupled first or secondary antibodies in their immunofluorescent staining protocols (Bi et al., 2002; Fink et al., 2000a,b; Murakami et al., 2000). The favored fluorochrome coupled to the primary antibody needed for a specific study, however, is often not available. We have, therefore, generated a rapid two-step immunostaining protocol and used...
an Alexa568-labeled secondary antibody. Alexa dyes and their conjugates are more fluorescent and photostable than the aforementioned fluorophores (Panchuk-Voloshina et al., 1999). For rapid immunostaining, a 20-fold increase of the polyclonal GFAP antibody concentration used for routine immunocytochemistry best facilitated the development of a positive signal within a minimal amount of time. This observation is in agreement with findings in other tissues or using other antibodies (Bi et al., 2002; Murakami et al., 2000).

The optimal concentration for the secondary antibody in this setting was 10-fold higher than the concentration used for routine immunocytochemistry. By applying these optimized antibody titters, the total exposure time to aqueous media could be reduced to 10 min with little effect on staining quality. Effects on RNA integrity were negligible (Section 4.3, Fig. 3). Although other authors have successfully used air drying for up to 30 min as a preparatory step before LMD/qPCR (Murakami et al., 2000; Prosniaik et al., 2003), we recommend drying the sections on a heater at 40°C for 10 min before and after the staining procedure. In our experience, this step significantly supports the minimization of RNA degradation. Dehydration steps involving xylene treatment were not applied since Alexa dye intensity is substantially affected by xylene exposure (Bi et al., 2002).

4.3. Laser microdissection

During the present study, astrocytes were carefully microdissected under direct microscopic visualization. Others have used immunostained sections only as a guide adjacent to the microdissection taking place in a histochimically stained serial section (Martinet et al., 2003). In our experience, however, the targeted and precise microdissection of single astrocytes is only possible if cells are visualized and dissected in the same section.

4.3.1. Settings

For maximal tissue recovery and optimal cutting efficiency, cutting intensity, aperture and velocity were adjusted in areas of no interest. The settings mentioned in the methods section assured that the dissected tissue was directly transferred into the microcentrifuge cup without further use of physical force. Although UV laser adjustment allows the precise dissection of irregularly shaped cells at high magnification (Koblje, 2000), the excision of very fine structures is limited. Since the adjusted laser beam diameter (63 and 150× objective) exceeds the diameter of fine astrocytic processes, we have confined the dissection to the mRNA-containing cell body including proximal astrocytic processes. This precise microdissection approach assured the collection of astrocytic tissue with a high degree of cellular specificity. For a specific isolation of astrocytes this method appears to be superior to collecting astrocytes with an infrared laser-based system, where significant carry-over of neighboring cell material occurs (Prosniaik et al., 2003).

4.4. Pooling of microdissected astrocytes

During the present study, 60 astrocytes were microdissected per probe and pooled for subsequent RNA isolation and qPCR analysis. Because the staining intensity of astrocytes may vary between control and lesioned animals and strongly immunostained astrocytes may preferentially be harvested by an investigator, all immunolabeled cells located within a single visual field were collected indiscriminately regarding their staining intensity. This random selection of astrocytes minimized a potential selection bias.

The cell-type specific gene expression analysis of pooled microdissected astrocytes is advantageous compared to the analysis of heterogeneous tissue homogenates or single cells. In the case of tissue homogenates, larger tissue blocks are used in which astrocytes as well as several other cell types are present. In these tissue samples, astrocytic gene expression changes may be below the level of detection because mRNA harvested from neurons or microglial cells may mask an effect. In contrast, in the case of single cell RT-PCR analysis, mRNA from only one cell is studied. This bears the risk of producing artifacts through preferentially amplifying non-desired templates or contamination traces (Fend and Raffeld, 2000). Furthermore, gene expression profiles of single cells might not represent gene expression of the respective cell population as a whole. Gene expression in the cell analyzed might be determined by locally restricted extrinsic or intrinsic events, e.g. the position of the cell in the cell cycle. Taken together, the pooling of microdissected cells, which increases both sample representation as well as detection sensitivity (Fend and Raffeld, 2000), may turn out to be the preferred methodological approach to study gene expression patterns of defined cell populations.

4.5. Total RNA isolation, reverse transcription and qPCR

4.5.1. RNA isolation

The choice of RNA extraction clearly depends on the amount of cells isolated during the laser microdissection procedure (Burbach et al., 2003; Fink and Bohle, 2002). If less than 100 cells were isolated, commercially available RNA extraction kits up to recently were unsuitable for use because elution volumes were higher than desirable. Therefore, some authors performed reverse transcription without prior RNA isolation (Fink and Bohle, 2002). However, in this case, transcription efficiency might be not optimal because of cellular debris and protein remnants. In the meantime, silica column-based RNA isolation technology was developed which allows the purification of RNA from very small amounts of tissue. For this particular study we applied the recently introduced RNeasy Micro Kit (Qiagen) and used an elution volume of 12 μl (end volume: 10 μl) ensuring highly concentrated RNA. Poly-A carrier RNA (4 ng/μl) was added to the lysate to enhance RNA recovery.
4.5.2. RNA quality
A major concern during staining and microdissection for subsequent gene expression analysis is the preservation of intact RNA. The greatest reduction of incubation times associated with minimal loss of RNA is obtained by a rapid immunofluorescent technique (Fink et al., 2000b), which was applied during this study. Standard control of RNA quality by denaturing gel electrophoresis and assessment of 28S and 18S rRNA band intensity (Margan et al., 2000) was not applicable since RNA amounts isolated from less than 100 cells are not sufficient for obtaining feasible data. Therefore, integrity of the isolated RNA was determined by capillary electrophoresis using the RNA 6000 Pico Lab Chip (Agilent Technologies, Waldbronn, Germany). Run on an Agilent 2100 bioanalyzer (Agilent Technologies), this microchip device is capable of rapidly sizing RNA fragments. Since total RNA was isolated, 18S and 28S ribosomal peaks were readily detected accounting for the integrity of the RNA isolated. During this study, only RNA with clearly distinguishable ribosomal peaks and without a major shift in RNA size distribution to small size fragments was used (Fig. 3).

4.5.3. Primer and probe design
Digestion of potential genomic DNA contamination was not necessary since primers and probe were designed to be intron-spanning (Table 1). After identifying appropriate mouse GFAP mRNA and gene sequences, both were aligned to detect intron-exon borders utilizing the mRNA-to-genomic alignment program Spidey (National Center for Biotechnology Information, Bethesda, MA, USA). Primer express software (Applied Biosystems) was used to identify GFAP primers in two different exons and an intron-spanning probe. Both primers and probe were checked for homologies with non-desired targets using the Basic local alignment search tool (BLAST, Altschul et al., 1990). During this study, primers in exons 7 and 8 and a probe bridging the exon-exon-border were picked. This primer/probe set ensured the specific detection of mouse GFAP mRNA without amplification of undesired RNA or DNA targets.

4.5.4. qPCR
The use of fluorochrome-labeled secondary antibodies during the staining procedure does not interfere with qPCR analysis (Fink et al., 2000a). Assessment of qPCR efficiency, use of internal control primer/probe sets and relative quantification of gene expression have already been described in detail previously (Burbach et al., 2003).

5. Conclusions
The present methodological report demonstrates that the combination of an optimized immunostaining-LMD protocol with qPCR is a highly convenient tool for astrocytic gene expression analysis. Although the protocol was developed for this specific application, it may also be used for the detection, microdissection and subsequent qPCR analysis of different cell types in brain or other tissues.

In addition, this report demonstrates the usefulness of the LMD/qPCR technique for studies focusing on changes in gene expression during the course of neurological disease. Relative changes in astrocytic gene expression following brain injury can reliably be detected as well as quantitated. Because these quantitative data represent changes in gene expression within cells rather than changes in gene expression within tissue volumes, they are independent of the specific experimental paradigm which is employed or the specific brain region which is being analyzed. Thus, this method allows a direct comparison of changes in astrocytic gene expression under different conditions of brain lesioning (Burbach et al., 2004).

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