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Expression microdissection isolation of enriched cell populations from archival brain tissue

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HIGHLIGHTS

- Expression microdissection (xMD) reduces issues of laser capture microdissection.
- xMD reduces procedure length compared to LCM.
- xMD can isolate immunopositive central nervous system cells (CNS) from FFPE tissue.
- RNA integrity number suggests RNA is of sufficient quality for further analysis.
- CNS cells isolated by xMD show enriched populations, confirmed by RT-PCR analysis

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ABSTRACT

Background: Laser capture microdissection (LCM) is an established technique for the procurement of enriched cell populations that can undergo further downstream analysis, although it does have limitations. Expression microdissection (xMD) is a new technique that begins to address these pitfalls, such as operator dependence and contamination.
New method: xMD utilises immunohistochemistry in conjunction with a chromogen to isolate specific cell types by extending the fundamental principles of LCM to create an operator-independent method for the procurement of specific CNS cell types.
Results: We report how xMD enables the isolation of specific cell populations, namely neurones and astrocytes, from rat formalin fixed-paraffin embedded (FFPE) tissue. Subsequent reverse transcriptase-polymerase chain reaction (RT-PCR) analysis confirms the enrichment of these specific populations. RIN values after xMD indicate samples are sufficient to carry out further analysis.
Comparison with existing method: xMD offers a rapid method of isolating specific CNS cell types without the need for identification by an operator, reducing the amount of unintentional contamination caused by operator error, whilst also significantly reducing the time required by the current basic LCM technique.
Conclusions: xMD is a superior method for the procurement of enriched cell populations from post-mortem tissue, which can be utilised to create transcriptome profiles, aiding our understanding of the contribution of these cells to a range of neurological diseases. xMD also addresses the issues associated with LCM, such as reliance on an operator to identify target cells, which can cause contamination, as well as addressing the time consuming nature of LCM.

1. Introduction

It has long been recognised that in order to unravel the underlying pathologies of central nervous system (CNS) disorders, it is necessary to identify the contributions of individual cell types to the overall disease process. We previously reported an immuno-LCM technique that combines rapid immunostaining with standard laser capture microdissection (LCM) to enable the isolation of an enriched population of cells from frozen post-mortem tissue (Waller et al., 2012). Using this approach, we have carried out a

Abbreviations: CNS, central nervous system; LCM, laser capture microdissection; EVA, ethylene vinyl acetate; DAB, 3′3-diaminobenzidine; xMD, expression microdissection; FFPE, formalin fixed paraffin embedded; RT-PCR, reverse transcriptase polymerase chain reaction; ABC, avidin/biotinylated complex; DEPC, diethylpyrocarbonate; TBS, Tris-buffered saline; UV, ultra violet; RIN, RNA integrity number.

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number of studies to enrich for populations of specific CNS individual cell types to compare the gene expression profile of disease and non-disease cells, including oligodendrocytes (Asi et al., 2014), astrocytes (Garwood et al., 2015) and neurones (Highley et al., 2014; Simpson et al., 2015). This approach has been useful in identifying disease-relevant gene expression related changes which can then be investigated further, but the protocols are somewhat lengthy and it can, on occasion, be difficult to confidently identify certain cells.

The development of the expression microdissection (xMD) technique (Tangrea et al., 2004), later refined (Hanson et al., 2011), offers a new approach to streamline and simplify the isolation of specific cell populations, removing the need for a microscope or cell identification by the user, making it an operator independent process. Similar to the LCM preparation process, for xMD, tissue sections undergo Immunohistochemistry for a cell-type-specific antigen. Tissue sections are then covered with an ethylene vinyl acetate (EVA) polymer film and the whole tissue is irradiated with a low energy infra-red laser. When the laser is fired over an immunopositive cell, the EVA film melts, causing a thermoplastic bond to occur between the EVA film and the immunopositive cell, adhering the cell to the EVA film. The film can then be removed from the tissue, taking with it only the immunopositive cells that have formed a thermoplastic bond with the EVA polymer, after which they can then be placed in extraction buffer for further downstream analysis (Fig. 1).

In the current study, formalin-fixed paraffin embedded (FFPE) post-mortem tissue was used to highlight how this resource can be used in conjunction with the expression microdissection technique to acquire enriched central nervous system (CNS) cell populations, namely neurones and astrocytes. For the purpose of this proof of principle study the neurone marker NeuN and a widely used astrocyte marker GFAP were the antibodies of choice. Histopathologically well-characterised archival FFPE tissue is currently an underutilised resource that has the potential to unravel underlying mechanisms contributing to disease pathogenesis. To date, two studies have been conducted utilising this method as a means to isolate specific cell types (Grover et al., 2006; Hanson et al., 2006). However, neither of these studies isolated CNS cells, making the method described here novel for these specific cell types.

This paper describes the detailed methodology of xMD which can be used to isolate enriched populations of specific cell types from FFPE tissue, and confirms this enrichment by RT-PCR. Future studies can potentially use this technique in well characterised archived FFPE cohorts to investigate the contribution of specific cell types to the pathogenesis of a range of neurological diseases.

2. Materials and methods

2.1. Rat brain tissue

Formalin fixed paraffin embedded (FFPE) post mortem rat brain tissue blocks were obtained from 6 Sprague Dawley adult rats. Cell isolation was carried out from the hippocampal region.

2.2. Immunohistochemistry

5 μm sections were collected onto sterile charged slides. Sections were dewaxed in xylene and rehydrated to DEPC-water in a graded series of alcohols (100%, 100%, 95%, 70% EtOH for 5 min each). Immunohistochemistry was used following the standard avidin/biotinylated enzyme complex (ABC) staining method (Vector Laboratories, UK). All work was carried out at room temperature (RT) under RNase-free conditions. Sections were blocked in 1.5% relevant normal serum for 30 min, then incubated in the appropriate specific antibody (glial fibrillary acidic protein [Dako catalogue number Z0334] 1:500; NeuN [Chemicon catalogue number MAB377] 1:100) diluted in blocking serum for 1 h before washing with Tris-buffered saline (TBS) for 5 min. Sections were then incubated with 0.5% biotinylated secondary antibody for 30 min, washed with TBS for 5 min, and then incubated with 2% horse-radish peroxidise conjugated ABC for 30 min and washed with TBS for 5 min. Antibody staining was visualised with 3,3′-diaminobenzidine (DAB) as chromogen (Vector Laboratories, UK) for 3 min. Sections were rinsed in DEPC-water and dehydrated in graded alcohols (70%, 95%, 100%, 100% for 15 s each) then cleared in xylene for 5 min. Sections were left to air dry in an air flow hood for approximately one hour prior to baking. Each antibody was optimised in order to ensure a specific pattern of immunoreactivity was achieved with minimal background staining. The optimisation process included the appropriate negative controls with primary antibody exclusion and an isotype control to ensure there was no non-specific reactivity.

2.3. Film and slide preparation for microdissection

CoTran 19% ethylene-vinyl acetate (EVA) film (3M, cat. No. 3M CoTran 9715) was sterilised in an ultraviolet (UV) air flow hood for 20 min. Using a sterile scalpel and forceps, the film was positioned over the slide so that all the tissue section was covered, and the film was cut to fit the slide, leaving a 2–3 mm border to prevent the film catching on the LCM system. After placing the slides in a pre-warmed oven for 10 min at 60 °C, they were taken out and pressure was applied using a roller to increase the film-section contact area. Slides were then placed back in the oven for a further 10 min.

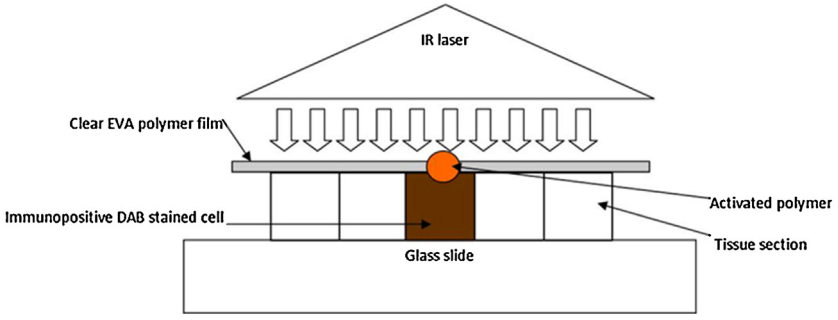

Fig. 1. Schematic representation of xMD. Immunopositive cells with the bound DAB chromogen are covered with a clear EVA polymer. The entire tissue is then irradiated with the IR laser of a LCM system. The EVA polymer melts at the site of the immunopositive cell due to the increased temperature caused by the chromagen DAB. This then bonds the EVA polymer film to the target cells.

2.4. LCM cap preparation

The film layer of a CapSure Macro LCM cap (Life technologies or Applied Biosystems Arcturus Engineering, Mountain View, CA, USA) was peeled off using sterile forceps and discarded, and the cap placed back into the tray. Although the cells are not collected onto the LCM cap, it is still needed to focus the laser when carrying out the xMD process.

2.5. Expression microdissection

Expression microdissection was performed using a Veritas LCM system (Applied Biosystems). The following parameters were used: 50 μm spot size, 50 ms laser pulse duration and 70 mW laser power, set to continuously fire.

2.6. RNA extraction

After microdissection, a sterile scalpel was used to cut the film surrounding the target area and carefully removed from the slide before being placed in a sterile 0.2 ml Eppendorf tube. Total RNA was extracted from microdissected cells using a modified protocol that utilised the Recoverall Total Nucleic Acid Kit for FFPE (Life Technologies, CA, USA) and RNAqueous Micro kit (Life Technologies, CA, USA). Approximately 200 cells were collected. RNA extraction was conducted with the following protocol. Protease digestion, and nucleic acid isolation were carried out as in the manufacturer's protocol for Recoverall Total Nucleic Acid Kit for FFPE kit (step C1 through to step D2). Briefly, 100 μl digestion buffer and 4 μl protease was added to the Eppendorf containing the film. Samples were then mixed by swirling followed by incubation at 50 °C for 15 min, then 80 °C for 15 min. 120 μl isolation additive and 275 μl 100% ethanol were then added to the mixture and mixed by pipetting. Samples were then transferred to cartridge filters included in the RNAqueous Micro kit for wash steps, elution and DNase treatment, carried out as in the manufacturer's protocol (step B5 through to step C4). All the sample was loaded onto the cartridge assembly and centrifuged for 1 min at 10,000 × *g*. 180 μl wash buffer 1 was added to the cartridge assembly and centrifuged for 1 min at 10,000 × *g*. This was followed by two washes with wash buffer 2/3, each of which was centrifuged for 30 s at 10,000 × *g*. The cartridge assembly was then centrifuged for 1 min at 10,000 × *g* to remove any residual fluid. 10 μl elution solution, preheated to 95 °C was added to the cartridge assembly and incubated for 5 min at RT, followed by centrifugation for 1 min at 10,000 × *g*. This step was repeated to produce a final total elution volume of 20 μl. The sample then underwent DNase treatment. 2 μl 10× DNase I buffer and 1 μl DNase I was added and mixed by gentle pipetting. Samples were then incubated for 20 min at 37 °C. 2 μl DNase inactivation reagent was added and incubated for 2 min at RT. Samples were then centrifuged for 90 s at 16,000 × *g*.

A NanoDrop 1000 spectrophotometer (Thermoscientific, UK) was used to determine the quantity of the RNA and 2100 Bioanalyzer, RNA 6000 Pico LabChip (Agilent, Palo Alto, CA, USA) was used to determine RNA quality.

2.7. Reverse transcriptase polymerase chain reaction (RT-PCR)

Reverse transcriptase polymerase chain reaction (RT-PCR) is a standard and reliable method of assessing the cell phenotype specific genes from the RNA extracted from LCM isolated cells (Vincent et al., 2002), as well as xMD isolated cells (Hanson et al., 2006), making this a viable method of validating the isolation of an enriched, specific cell population, as well as indicating contamination levels of non-specific cells.

Table 1
Primer sequence.

Gene	Primer sequence	Product size
Gfap	F: CTG ATT CAG CCC AGA GGG TTA R: TGA AGA GCA GGG AGC ATA AAG C	82bp
Cd68	F: CAC AGT GGA CAT TCT CAG CG R: AGA GCA GGT CCA GGT GAA TTG	123bp
Olig2	F: ACC TGG GGG CTT GAC AAA AG R: GCA TCG CGA TTT CGT TGA T	136bp
Tub3	F:GGA GGA GGG GGA GAT GTA TGA A R:CCA GCT GCG AGC AAC TTC AC	77bp

Key: F, forward; R, reverse.

cDNA was synthesised using the Superscript III reverse transcriptase kit (Life Technologies, CA, USA) with random primers, as described in the manufacturer's protocol. Gene-specific PCR primers were either designed in house or obtained from commercially available sources. PCR was performed using 50 ng cDNA, 2× ReddyMix PCR master-mix (Thermoscientific, UK) with optimised concentrations of forward and reverse primers (Table 1), in a total volume of 20 μl. A denaturation step at 95 °C for 10 min was followed by amplification of the products: 30 cycles at 95 °C for 15 s, 60 °C for 60 s, then 72 °C for 15 min. PCR products were visualised on a 3% agarose gel stained with ethidium bromide.

3. Results

3.1. Expression microdissection enables the enriched isolation of specific cell-types from FFPE tissue

Standard immunohistochemistry enabled the identification of the target cell type. $NeuN^+$ neurones displayed large round immunopositive nuclei, while $GFAP^+$ astrocytes displayed stellate morphology with processes extending out of the cell body (Fig. 2). NeuN+ neurones and GFAP+ astrocytes were isolated using expression microdissection on a Veritas laser-capture microdissection system. The laser irradiated the entire tissue, only activating the EVA polymer film over the immunopositive cells that bound the DAB chromogen, due to the transient increase in temperature, fusing the film with the underlying cells. When the film was lifted off, the unwanted (unlabelled) cells were left behind (Fig. 3).

3.2. RNA quality

Following RNA isolation from the xMD isolated specific immunoreactive cells, the RIN value was assessed (Fig. 4). In the representative examples shown, the RIN value decreased by 0.3.

3.3. PCR of cell phenotype specific transcripts confirms enrichment of specific cell populations using xMD

Expression microdissection is suitable for the procurement and isolation of neurones and astrocytes, resulting in enriched target cell samples. RNA isolated from $NeuN^+$ cells had high levels of Tubulin 3 transcripts with no transcripts for *Gfap*, *Cd68* or *Olig2*, confirming that extracted RNA represents a highly-enriched neuronal population (Fig. 5). This was also true for $GFAP^+$ cells, which had high levels of *Gfap* transcripts with lower levels of Tubulin 3 and no detectable *Cd68* and *Olig2* transcripts (Fig. 5).

4. Discussion

The isolation of enriched cell populations from post-mortem material is key to identifying the role(s) these cells play in neurological diseases. To date, the majority of studies have examined disease-associated changes in gene expression in whole tissue

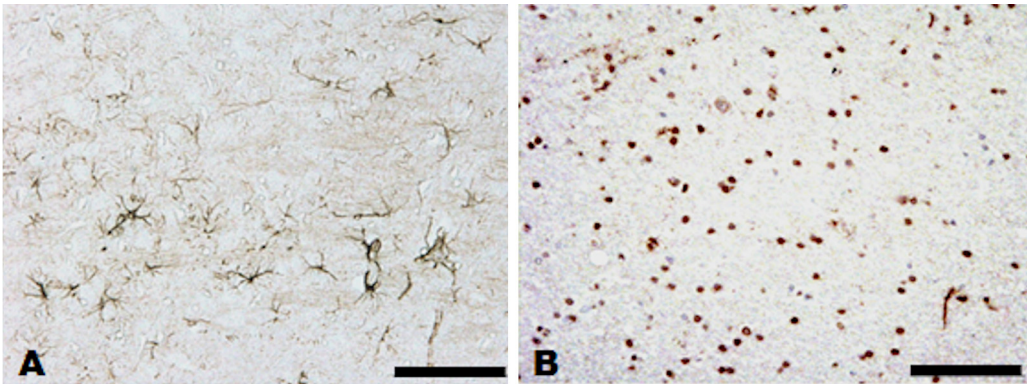

Fig. 2. Immunohistochemical identification of astrocytes and neurones. (A) Glial fibrillary acidic protein (GFAP) was used to identify astrocytes and (B) NeuN was used to identify neurons. Scale bar represents 50 μm.

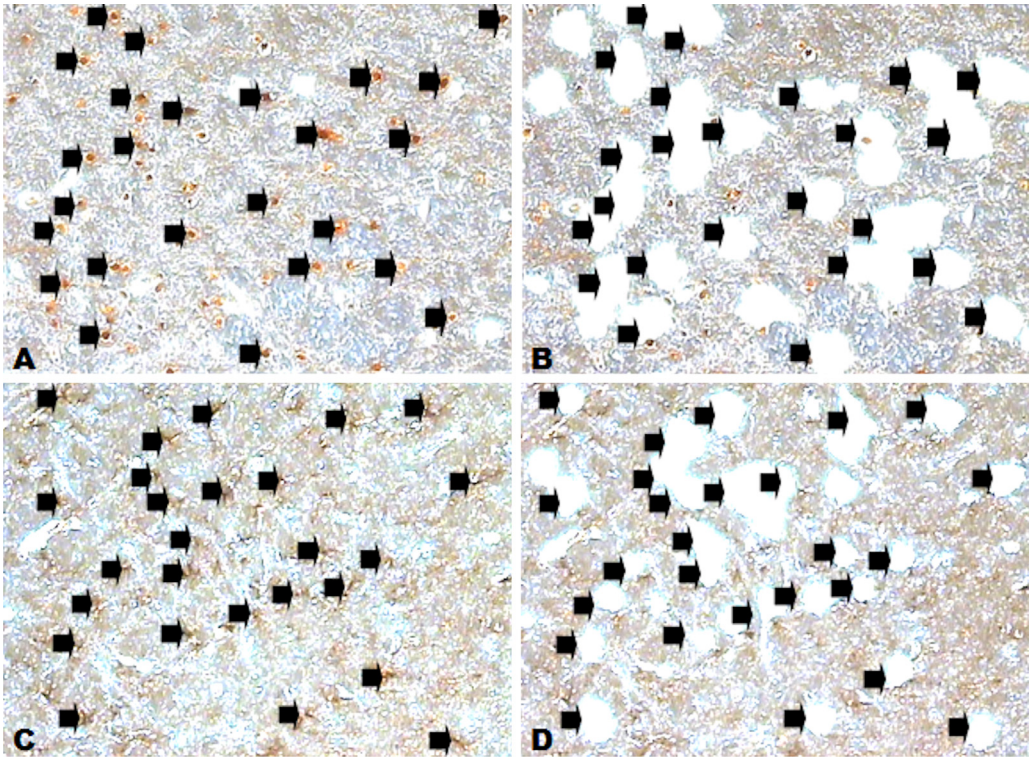

Fig. 3. Expression microdissection of (A,B) NeuN+ neurones and (C,D) GFAP+ astrocytes from FFPE post-mortem rat brain tissue. Immunopositive cells were isolated using expression microdissection on a Veritas laser-capture microdissection system. The laser irradiated the entire tissue, only activating the EVA polymer film on the immunopositive cells bound the DAB chromogen, due to the transient increase in temperature. The film fused with the underlying cells. The film was lifted off, leaving unwanted cells behind (B,D). The isolated cells were attached to the film, to then undergo RNA extraction.

extracts, which likely mask the specific contribution of individual cell populations (Bossers et al., 2009; Grunblatt et al., 2007; Wang et al., 2006). In the current study we demonstrate that enriched cell populations can be successfully isolated from CNS tissue by xMD. Whilst not being entirely homogeneous, the extract achieved using this approach is highly enriched for the cell type of interest, as confirmed by RT-PCR. While isolated neurones had no detectable levels of glial transcripts, low levels of neuronal transcripts were present in the xMD-isolated astrocytes, likely reflecting the target cells' proximity to axons. Therefore, xMD is suitable for the procurement and isolation of neurones and astrocytes from post-mortem FFPE tissue, resulting in enriched target cell samples. In addition to demonstrating the high recovery rate of the specific cell of interest, we also show that the RNA integrity of the xMD isolated cells is suitable for further downstream analysis, confirming previous xMD studies (Grover et al., 2006; Hanson et al., 2006).

FFPE CNS tissue blocks are widely used to immunohistochemically characterise the detailed cellular pathology associated with specific neurological diseases. Whilst classical neuropathology is a key element of many studies, we demonstrate that the quantity and quality of RNA collected from FFPE tissue utilising the xMD method is of sufficient quality to carry out further molecular analyses. We demonstrate cellular isolation from cortical regions, but it should be noted that other studies have used different tissues with higher cellular densities to obtain cells via xMD (Hanson et al., 2011; Tangrea et al., 2004). While it is inevitable that some RNA will be degraded during the fixation and processing of the tissue, we demonstrate that the RIN value of the isolated cells is comparable with cells isolated from frozen CNS tissue (Waller et al., 2012). In

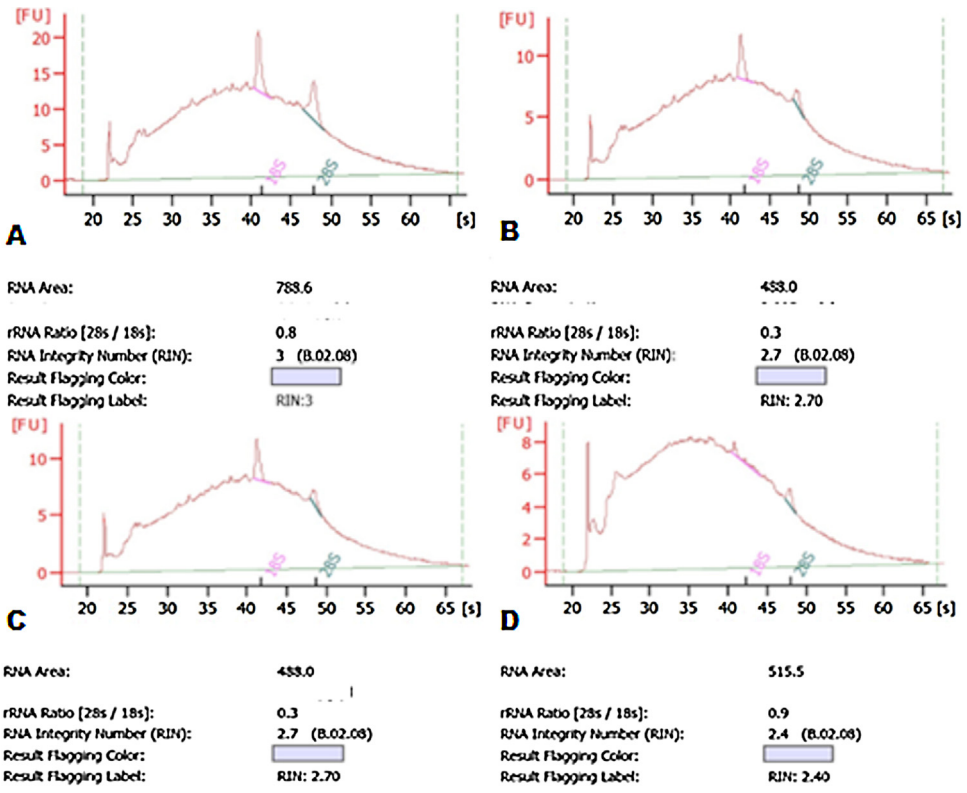

Fig. 4. Representative RNA integrity number (RIN) of samples before and after xMD. (A) Before (RIN 3.0) and (B) after (RIN 2.7) xMD isolation of NeuN$^+$ neurones. (C) Before (RIN 2.7) and (D) after (RIN 2.4) xMD isolation of GFAP$^+$ astrocytes.

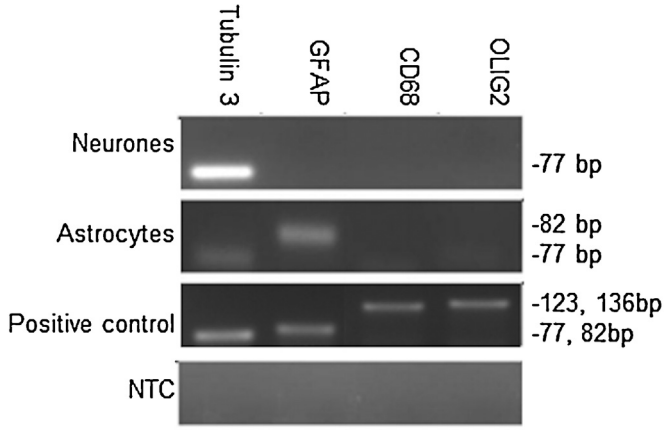

Fig. 5. RT-PCR of transcripts in cells isolated by expression microdissection. NeuN$^+$ neurones are associated with high levels of *Tubulin 3* transcripts (77bp). GFAP$^+$ astrocytes are associated with high levels of *Gfap* transcripts (82bp). *CD68* (123bp), *Olig2* (136bp). NTC: no template control; positive control: whole tissue extract.

theory, this method could also be applied to frozen tissue; however, a modified rapid immunostaining protocol, as previously described (Waller et al., 2012), is advised. Previous studies have also shown that samples with a wide range of RIN values (RIN 1–8.5) can be used in gene expression studies with successful outcomes (Kojima et al., 2014; Trabzuni et al., 2011).

In the current study we demonstrate that xMD is a robust approach for isolating an enriched population of specific CNS cell types, and suggest that with optimisation, this method could be extended and further applied to the isolation of any desired cell type provided a suitable cell-specific antibody is available to allow binding of a chromogen in order for the xMD process to be carried out. Whilst we performed this study on rat tissue, it could in theory, be used to isolate other CNS specific cell types from other species, including human and mouse. Human tissue has been shown to be suitable for use in conjunction with xMD (Grover et al., 2006; Hanson et al., 2006).

xMD, whilst still in its infancy, addresses many of the limitations associated with basic LCM techniques, such as the need for operator-based identification of the cell of interest based on morphology, which can result in the non-specific cell contamination of the enriched cell sample. The current study outlines the detailed methodology of how different CNS cell types can be microdissected without the need for visualisation by an operator, thereby reducing the chance of a cell being wrongly identified. It also prevents the isolation of a cell with similar morphology to the cell type of interest from being mistakenly picked, as only cells that are immunopositive adhere to the film when the tissue is irradiated. xMD also negates the need for a microscope or visualisation of the cell of interest, making the process operator-independent.

A further advantage of the xMD technique is its potential to address the low recovery rate associated with basic LCM, as larger numbers of the cell of interest are likely to adhere to the EVA polymer film than can be identified by the operator using LCM. LCM is also a highly time consuming process and this too is addressed by xMD, as the laser can be set to continuously fire without an operator being present during this process. Therefore, xMD can be employed to rapidly isolate enriched cell-specific populations from post-mortem material.

Further developments into xMD are still occurring (Hanson et al., 2011). These include the development of a handheld device that would further streamline the process, making it less time consuming for the operator due to the laser only having to fire over the tissue a few times in quick succession, meaning that this process in theory make take just a few minutes. A handheld device would

also make it more accessible to laboratories where LCM equipment is not readily available.

5. Conclusion

To summarise, this paper documents a detailed methodological approach for the isolation of specific cell types from FFPE tissue utilising expression microdissection as the method of isolation, whilst also addressing and overcoming the pitfalls of LCM to acquire enriched CNS cell populations. Whilst the use of FFPE tissue does have its limitations, it is an extremely valuable resource that is widely available and easy to store, making the RNA obtained from xMD-isolated specific, enriched cell populations from FFPE material a viable option for studying changes in the gene expression profile associated with the pathogenesis of a range of neurological diseases.

While xMD has some limitations, this technique allows enriched cell populations to be isolated from FFPE tissue, thereby enabling the role of these cells in neurological disease to be investigated. Utilising this approach, specifically by applying it to well characterised human tissue, would enable underlying pathogenic mechanisms to be elucidated and to identify potential targets for developing novel treatment strategies. In this respect, xMD is more relevant than other useful, but less reflective, approaches to understanding neurodegenerative disease, such as the use of cell lines and animal models (Jucker, 2010).

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