Journal of Neuroscience Methods 174 (2008) 219–226

Contents lists available at ScienceDirect

Journal of Neuroscience Methods

journal homepage: www.elsevier.com/locate/jneumeth

Validation of immuno-laser capture microdissection coupled with quantitative RT-PCR to probe blood–brain barrier gene expression *in situ*

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ARTICLE INFO

Article history:
Received 22 April 2008
Received in revised form 8 July 2008
Accepted 15 July 2008

Keywords:
Laser capture microdissection
Immuno-LCM
Microvessels
Heterogeneity
Blood–brain barrier

ABSTRACT

Laser capture microdissection (LCM) holds great potential for analyzing gene expression profiles *in situ*. Most recently, this laboratory employed a novel immunostain-based LCM protocol (immuno-LCM) to selectively retrieve brain microvascular endothelial cells (BMEC) from intimately associated perivascular cells. However, before this protocol can be confidently coupled to downstream analytical platforms, it must be demonstrated that any variability associated with it is minimal, so as not to obscure data interpretation. As various factors could contribute to variability, this study focused on determining whether technical inconsistency and/or biological diversity of sample populations, played such a role. Specifically, two separate immuno-LCM-derived BMEC samples derived from adjacent tissue sections of a single mouse (to detect only technical variability), and from analogous tissue sections of three different mice (to detect technical and biological variability) were compared for their relative expression of 16 genes, using quantitative-RT-PCR (qRT-PCR). Both significant linear and rank-order correlations were observed between different sections from the same animal, underscoring lack of technical variability in this LCM application. Furthermore, a three-dimensional scatter plot of gene expression profiles from the three animals was linear, and ANOVA showed absence of statistically significant differences between any of the animals, confirming lack of biological variability. These findings argue that immuno-LCM coupled to qRT-PCR affords a reproducible means to assay gene expression *in situ*.

1. Introduction

Ever since its introduction by Liotta and colleagues (Emmert-Buck et al., 1996), laser capture microdissection (LCM) has offered a novel and distinctly advantageous approach to analyzing gene and protein expression of enriched cell populations and extracellular components *in situ*. And when coupled to any one of several downstream expression platforms, the vast potential of LCM for unparalleled, in-depth molecular analysis of normal and pathogenic processes of the central nervous system (CNS) has been realized by several laboratories (Kamme et al., 2003; Kinnecom and Pachter, 2005; Churchill et al., 2006; Gozal et al., 2006; Lombardino et al., 2006; Demuth et al., 2007; Hellmich et al., 2007; Pen et al., 2007; Fernandez-Medarde et al., 2007; Novikova et al., 2008; Lu et al., 2007). This prospect holds for the two general classes of LCM currently available: infrared (IR) capture systems, and ultraviolet (UV) cutting systems. With the ability to obtain cellular and extracellular material from their *in situ* locales in a relatively quick and nondestructive manner, LCM averts caveats typically associated with protracted cellular and biochemical isolation procedures, such as considerable degradation and/or modification of nucleic acids, proteins and other molecules. Plus, by recovering cells from their physiologic environments, LCM avoids troublesome complications linked to tissue culture artefacts—such as phenotypic drift.

Notwithstanding the significant advantages of LCM, this technology has its own potential obstacles to overcome, necessitating that it be validated for the particular application at hand. For example, in cases where the objective is to analyze very closely apposed cell populations, it need be demonstrated that LCM results in precise cellular extrication with little or no cross-contamination. This critical issue was recently highlighted in a report by this laboratory (Kinnecom and Pachter, 2005), which described both coupling an IR-based LCM protocol to a novel double-immunolabeling technique (immuno-LCM), and performing extensive tissue dehydration by xylenes. Such an approach created particular conditions by which brain microvascular endothelial cells (BMEC) and tightly juxtaposed adventitial/perivascular cells could be resolved from each other and each population selectively captured—without any detectable cross-contamination as judged by RT-PCR. Thus, within

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doi:10.1016/j.jneumeth.2008.07.009

certain limits, high precision in retrieving designated cell populations by LCM can be achieved.

Aside from purity, another critical issue concerns reproducibility of the LCM procedure *per se*. As it is not routine to replicate experiments when coupling LCM to complex and expensive platforms such as microarray analysis (Paul et al., 2008) or proteomic evaluation (Mustafa et al., 2008), intrinsic variability in the LCM approach must be demonstrably minimal. If not, significant doubt would be cast on LCM's utility as an analytical tool to discern 'legitimate' differences in experimental samples. And while several reports have meticulously evaluated potential sources of variability associated with certain downstream applications coupled to LCM, e.g. RNA amplification and microarray analysis (King et al., 2005; Febbo et al., 2006; Caretti et al., 2008; Luzzi et al., 2003), much less attention has been focused on particular aspects of variability inherent with carrying out the actual dissection and extraction of tissue elements.

Conceivably, major sources of variability associated with execution of the LCM process can be grouped into two categories: (1) technical variability; and (2) biological variability. Technical variability would cover inconsistencies such as capturing variable amounts of contaminating tissue, and differences in tissue 'lifting efficiency' (i.e., the degree to which the targeted cells can be lifted away from the surrounding tissue). Also included in the technical category would be variations in processing the retrieved samples post-LCM (e.g., extracting/isolating the molecules to be analyzed). Contamination, as stated above, can be managed by labeling both the cell type(s) targeted for LCM and those cell populations one wishes to avoid (Kinnecom and Pachter, 2005); for BMEC, astrocyte foot processes are the most numerous cellular elements likely to be a source of contamination. Multiple labeling, however, particularly if involves deposition of layers of antibody and chromogenic precipitate, runs the risk of inconsistently thwarting tissue lifting efficiency, as well as frustrating the recovery of nucleic acid and/or protein (Gutstein and Morris, 2007). It further need be appreciated that the extensive dehydration necessary to support lifting efficiency and highly pure brain microvascular retrieval (Kinnecom and Pachter, 2005) may, in turn, variably affect immunostaining. Such intricate possibilities must therefore be definitively ruled out. To date, variability stemming from these technical facets of LCM has not received formal attention.

Biological variability, on the other hand, would derive from possible natural heterogeneity from specimen to specimen within a given population, and/or regional variability within a single specimen. This matter is particularly acute when employing LCM to assess blood vessels, the endothelium of which exhibits profound phenotypic diversity at the segmental and regional levels (Ge et al., 2005; Aird, 2007). Without forehand knowledge that the process of acquiring and processing molecular samples by a particular LCM approach does not itself result in significant *within-group* variability – technical and/or biological – any observed effects between different experimental groups would necessarily be suspect.

An issue interconnecting the predicaments of technical and biological variability is 'pooling' LCM samples from different sections—either from the same subject or different subject. Carried out in order to overcome low nucleic acid or protein content (Williams et al., 2008; Wang et al., 2005; Marcaccini et al., 2008), pooling is potentially a cause of high level within-group variability that could obscure real treatment; i.e., *between-group*, effects. Pooling is apt to be most necessary when targeted cells are small and/or thin, as with endothelial cells (Kinnecom and Pachter, 2005), or few in number/section. Additionally, use of thin sections to minimize potential contaminating cell types that lie above or below the plane of the cell of interest (Kamme et al., 2003), but which would greatly reduce RNA/protein yield, could further obligate pooling. However, the legitimacy of pooling necessitates forehand knowledge that such independent LCM retrievals performed under a 'specific' set of conditions do not, by themselves, contribute to variability.

Given all these considerations, we sought to answer the following question: Is our double-label, immuno-LCM procedure to obtain a select population of microvascular endothelial cells (Kinnecom and Pachter, 2005) sufficiently reproducible, and without statistically significant variability, so as to enable future efforts at comparative, global profiling of functionally distinct microvascular beds in the central nervous system (CNS)? Quantitative real-time-PCR (qRT-PCR) was used for the readout of gene expression, as this is considered the "benchmark technology" (Bustin et al., 2005) and "gold standard" (Klur et al., 2004; Wang et al., 2006) for detection and comparison of RNA levels. Specifically, we concentrated on investigating the variability associated with using different brain tissue sections from the same mouse or from different mice, as this incorporated potential aspects of both technical and biological variability that might be unique to our LCM application. Inasmuch as BMEC form the preeminent cellular component of the blood–brain barrier (BBB) (Ge et al., 2005; Hawkins and Egleton, 2008), validation of this integrated approach would strongly encourage its use in investigating gene regulatory events underlying BBB function. To this end, we examined LCM-acquired BMEC from the inbred C57 BL/6 mouse strain for relative expression of 16 genes associated with various aspects of BBB function. This strain was chosen as it exhibits minimal brain vascular heterogeneity from animal to animal (Ward et al., 1990), and capillaries were exclusively examined as they constitute the vast majority of the brain microvascular tributaries (among arterioles, capillaries and venules). Moreover, by studying a delimited population of cortical capillaries only, variability due to regional and segmental heterogeneity, respectively, was avoided (Ge et al., 2005). This design thus allowed for technical variability specifically associated with the immuno-LCM process to be analyzed.

Expression levels of the 16 BBB-related genes – spanning a wide quantitative range – showed a significant linear correlation when comparable tissue samples were pooled from adjacent brain sections of the same mouse. They further maintained the same rank order and showed no statistically significant quantitative differences from sample to sample. Lastly, this lack of variability was additionally observed when comparing analogous samples obtained from different mice. These results strongly support use of this immuno-LCM approach for quantitative comparative analysis of gene expression along the brain microvascular endothelium.

2. Methods

2.1. Animals

Male C57 BL/6 mice, 8–10 weeks of age and obtained from Charles River Laboratories, Inc. (Wilmington, MA), were used in order to minimize microvascular heterogeneity due to confounding variables such as genetic variability, sex, and age. Animals were euthanized by CO_2 inhalation, in accordance with measures stipulated by the Animal Care and Use Guidelines of the University of Connecticut Health Center (Animal Welfare Assurance # A3471-01).

2.2. Tissue preparation and sectioning

Immediately following euthanasia, the entire brain was removed, snap-frozen in liquid N_2, and stored at −80 °C. Frozen