Journal of Molecular Histology 35: 397–402, 2004
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Investigation of different transcript quantitation tools for high-throughput mapping of brain gene expression using voxelation

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Received 21 November 2003 and in revised form 27 December 2003

Summary

Voxelation is a new approach for genome scale acquisition of brain gene expression patterns. The method employs high-throughput analysis of spatially registered voxels (cubes) to create multiple volumetric images of brain gene expression, similar to those obtained from biomedical imaging systems. The spatial resolution of voxelation depends on voxel size, with smaller voxels giving higher resolution. An important question is the applicability of different transcript profiling tools for the various levels of resolution that can be employed. Here, we describe the use of three methods to analyze voxel transcript abundance: real-time PCR, microarray analysis and linear amplification coupled with microarrays. We show statistically significant concordance between real-time PCR and microarray analysis for the myelin basic protein gene in human brain specimens at differing levels of spatial resolution. In addition, we also demonstrate the feasibility of using linear amplification coupled with microarray analysis to create voxelation maps from the mouse brain at high resolution, 1 μl. These data indicate the suitability of a number of transcript profiling tools for various levels of spatial resolution in voxelation.

Introduction

A comprehensive knowledge of all gene expression patterns will be an essential basis for understanding how the genome constructs the mammalian brain. One recently developed approach to facilitate this goal, voxelation, employs high-throughput transcript profiling of spatially registered cubes or voxels (Singh & Smith 2003). The gene expression information from each voxel allows the construction of multiple volumetric maps of gene expression for the brain analogous to the images reconstructed in biomedical imaging systems such as CT, PET and MRI.

Voxelation has been employed to analyze gene expression patterns in a number of different settings. One study analyzed coronal hemisections of the human brain at the level of the hippocampus from both normal individuals and individuals with Alzheimer's disease (Brown *et al.* 2002a). In this investigation, the coronal hemisections were divided in voxels of about 1 cm^3 and each voxel analyzed using a 2000 gene microarray. In another study, 40 voxel images of the mouse brain were analyzed using 9000 gene microarrays, an average volumetric resolution of 7.5 μl. (Brown *et al.* 2002c) Brains were studied from both normal mice and mice in which a model of Parkinson's disease was induced by toxic doses of methamphetamine.

The resolution of the gene expression maps obtained form voxelation depends on the size of the voxels, with smaller voxels resulting in higher resolution. As voxel size is decreased, dedicated instrumentation is necessary to permit semiautomated harvesting of the large numbers of voxels. Two devices consisting of 2D arrays of cutting blades have been created for high-resolution voxelation maps of the human and rodent brain (Singh *et al.* 2003). The human device results in voxels of 3.3 mm, a volumetric resolution of 87 μl for 8 mm sections, while the rodent device results in voxels of 1 mm, a volumetric resolution of 1 μl for 1 mm sections.

A variety of considerations are relevant in choosing an appropriate transcript quantitation tool for voxelation. Foremost among these are throughput and sensitivity. Microarrays have very high-throughput but only moderate sensitivity (Hegde *et al.* 2000). In contrast, real-time PCR has very high sensitivity but only moderate throughput (Gibson *et al.* 1996, Heid *et al.*

1996, Freeman *et al.* 1999, Medhurst *et al.* 2000). Use of linear amplification combined with microarray analysis should allow both high sensitivity and very high-throughput (Kacharmina *et al.* 1999).

A relevant question is the degree of concordance between data obtained using microarray and real-time PCR at different levels of resolution. An additional question is whether linear amplification combined with microarray analysis is sufficiently sensitive to analyze the small voxels, 1 μl, obtained from high resolution voxelation of the rodent brain. Here, we show good concordance between gene expression patterns obtained using voxelation and real-time PCR or microarrays at high and low spatial resolution. In addition, linear amplification and microarray analysis is shown to be a feasible tool for the high-throughput construction of 1 μl voxel gene expression maps of the rodent brain.

Materials and methods

Voxelation of the human brain

The device for voxelation of the human brain consisted of steel razor blades embedded in Plexiglas (Liu & Smith 2003, Singh *et al.* 2003). The instrument resulted in a 425-voxel template, consisting of 25 voxels in length and 17 voxels in width, each voxel being a square of side 3.3 mm. The device was used to voxelate manually a coronal hemisection from a normal human brain. The donor was a 38 years old male who had died of a cocaine overdose. The postmortem interval was 9 h. The hemisection was 8 mm thick and was from the left side at the level of the hippocampus, corresponding to section 15 of the University of Maryland Brain and Tissue Bank protocol, method 2 (U Maryland Brain and Tissue Bank). The specimen was thawed from frozen and maintained in a firm condition on ice. The hemisection was then divided into three approximately equal parts, superior, middle and inferior, and each part voxelated. The specimens were digitally photographed before and after voxelation to allow identification of anatomical landmarks visible in an unstained section. Identification of the landmarks employed a human brain atlas (DeArmond *et al.* 1989). After voxelation, the voxels were extracted using a scalpel blade and total RNA extracted. The scheme resulted in 669 voxels for the whole hemisection.

RNA extraction and real-time PCR

RNA extraction and real-time PCR was performed as described (Liu & Smith 2003, Singh *et al.* 2003). Total RNA was extracted by placing the voxels in TRIzol Reagent (Gibco BRL, Gaithersburg, MD) and homogenized by passaging several times through a $26_{5/8}$G needle. Subsequent steps for RNA isolation were performed following the manufacturer's instructions. The average amount of total RNA from the human voxels was 28.8 ± 1.2 μg. Real-time reverse transcription (RT) PCR was performed using TaqMan technology on an ABI Prism 7900HT Sequence Detection system (Applied Biosystems, Foster City, CA) (Gibson *et al.* 1996, Heid *et al.* 1996, Freeman *et al.* 1999, Brown *et al.* 2002a, c, Leil *et al.* 2002). TaqMan One-Step RT-PCR mix was used for the RT and PCRS, following the manufacturer's instructions. Reverse transcription used 50 ng of total RNA. The primers used for amplification of the myelin basic protein gene (MBP) gene were: CCAGAGGCACGGATCCAA and CCCTGTC ACCGCCAAAGA. The TaqMan probe was FAM-CATCCTTGACTCCATCGGGCGCT-TA MRA, with FAM as the reporter dye and TAMRA as the quencher. The amplification primers were at 900 nm and the probe at 200 nm. A passive reference dye (ROX) provided an internal standard for normalization of FAM fluorescence, correcting for fluctuations due to volume changes. For relative quantitation, a standard curve was constructed for the primer and probe set, using total brain RNA. A ribosomal RNA control primer and probe set (Applied Biosystems) was used for normalization purposes. The possibility of genomic DNA contamination was excluded by the use of no reverse transcriptase controls in combination with the ribosomal primers. Normalization of data from different PCR runs was employed as described. (Brown *et al.* 2002a, c, Singh *et al.* 2003).

Images

Images were constructed using MatLab algorithms on a personal computer (Liu & Smith 2003). The algorithms converted gene expression levels into pseudocolor and also smoothed the images over voxels. The expression images were placed onto available human brain atlas sections (Virtual Hospital: The Human Brain). To allow registration, warping was accomplished using our own implementation of the thin-plate splines warping method (Bookstein 1989, Brown *et al.* 2002b). The warping employed approximately equidistant fiducial points on the digital photographs of the actual brain sections and the atlas sections. Affine transformation brought corresponding fiducials as close as possible using least Euclidean distances. Additional transformation used the thin-plate spline technique, which permitted non-linear grid deformations. The algorithms employed for the images are available on our web site (Smith Lab Web Site).

Microarray analysis

Both the unamplified control channel and the amplified channel employed total normal C57BL/6J mouse

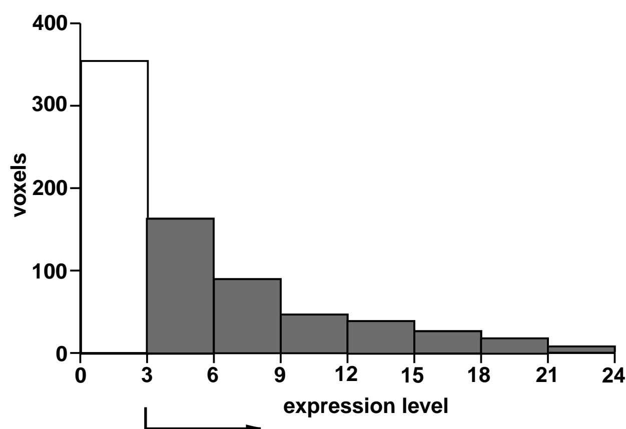

Figure 1. Distribution of MBP expression levels in the voxels of the human brain hemisection. The top 46% of voxels is indicated by the horizontal arrow (shaded bars).

brain RNA. The control channel was labeled with Cy3, while the amplified channel was labeled with Cy5. Linear amplification was performed using the MessageAmp aRNA Kit (Ambion Inc., TX) starting with 20 ng RNA. The samples were labeled using amino-allyl chemistry via the CyScribe cDNA Post Labeling Kit (Amersham Biosciences). After labeling, the samples were purified using Microcon filters (Millipore). The labeled RNA samples were co-hybridized to a 9000 gene microarray, as described (Hegde *et al.* 2000 Brown *et al.* 2002c). For hybridization, both the unamplified control channel and the amplified channel employed 1.8 μg RNA. Signal to noise ratios were 2.5-fold above background for each gene in both Cy3 and Cy5 channels.

Results

Expression pattern of the MBP gene in the human brain obtained using high-resolution voxelation

The human voxelation device was employed to analyze a coronal section of the human brain containing hippocampus, thalamus and striatum (Singh *et al.* 2003). The section was divided into three roughly equal parts and each part divided into voxels of 3.3 mm × 3.3 mm (surface) × 8 mm (depth) (87 μl) using the voxelation device. Total RNA was extracted from each of the voxels and real-time PCR was employed to analyze the expression pattern of the myelin basic protein (MBP) gene. This gene is involved in creation of the myelin sheath and would be expected to be strongly expressed in the white matter. The distribution of expression levels of the MBP gene throughout the voxels as judged using real-time PCR is shown in Figure 1. The anatomical location of voxels in the top 46% of voxels as judged from MBP expression levels are show in Figure 2. As expected, the voxels that most strongly express the MBP gene are mostly concentrated in the white matter. The expression pattern of the MBP gene using a pseudocolor presentation in which the expression levels were smoothed between voxels is shown in Figure 3. Again, this image suggested good agreement between the expression pattern of the MBP gene deduced from voxelation and the location of the white matter. In order to assess the reproducibility of the high-resolution voxelation for the MBP gene, the real-time PCR were repeated (Figure 4). Good reproducibility was found. There were a few voxels close to the bottom of the chart indicating greatly reduced expression in experiment 2, possibly related to experimental error, e.g. in pipetting.

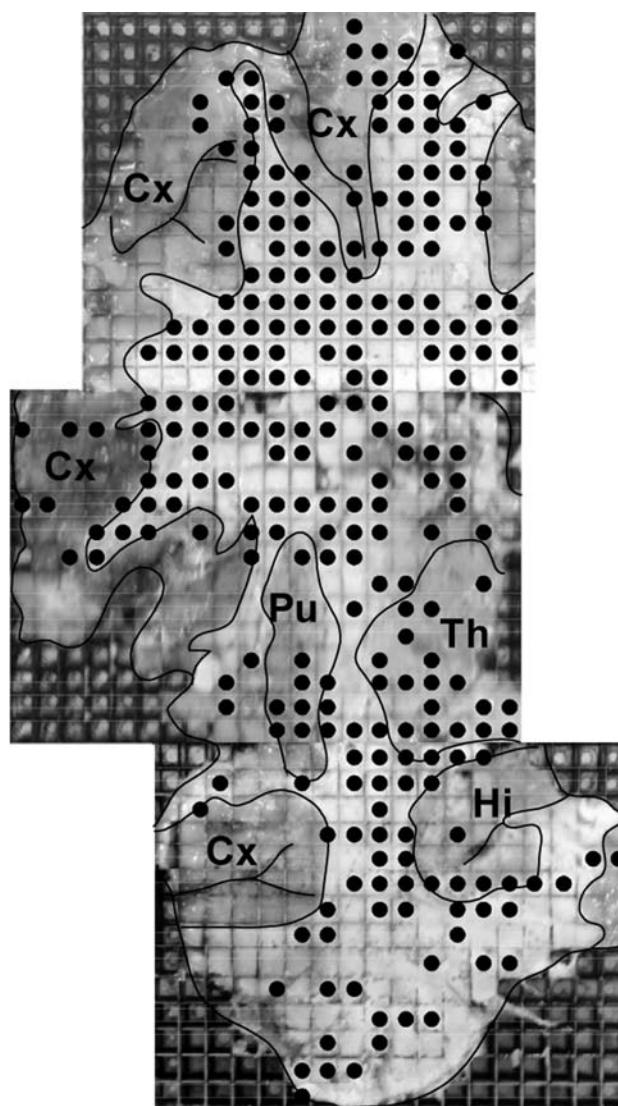

Figure 2. Human brain coronal hemisection. The hemisection was divided into three approximately equal sections, superior, middle and inferior, and then voxelated as shown. Those voxels in the top 46% of all voxels with regards to expression of the MBP gene are indicated by the filled in circles. Pu – putamen, Cx – cortex, Hi – hippocampus, Th – thalamus.

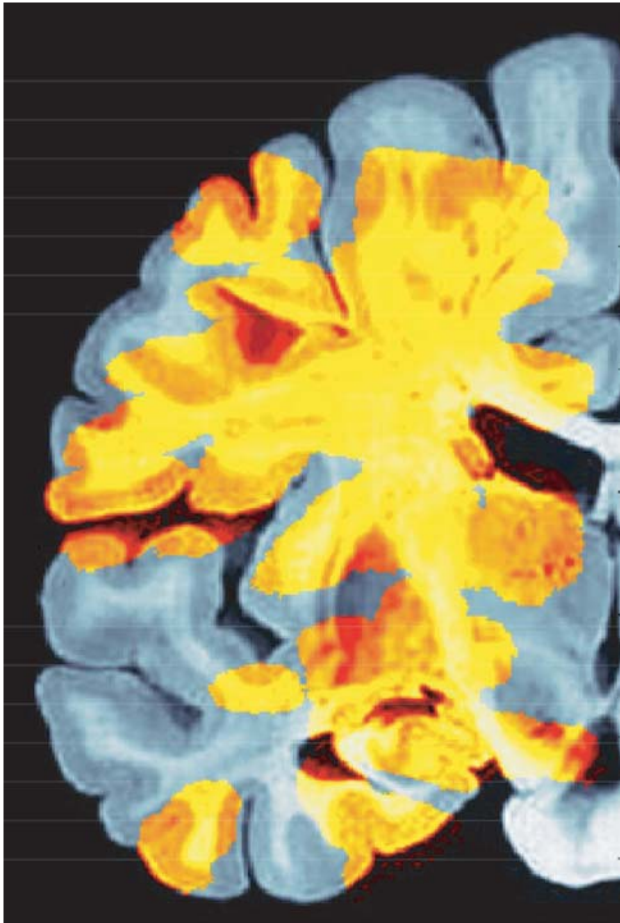

Figure 3. Pseudocolor image of MBP gene expression. Red – high, yellow – intermediate, blue – low.

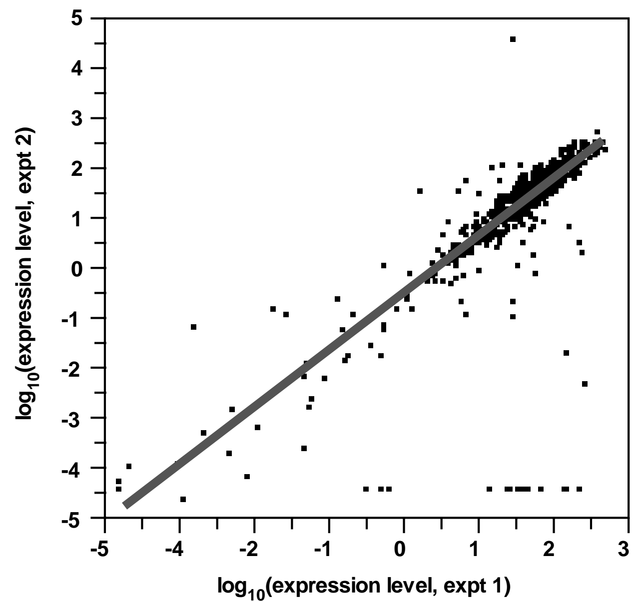

Figure 4. Replicability of high resolution MBP gene expression data deduced from real-time PCR, $r = 0.74$, $p < 0.0001$.

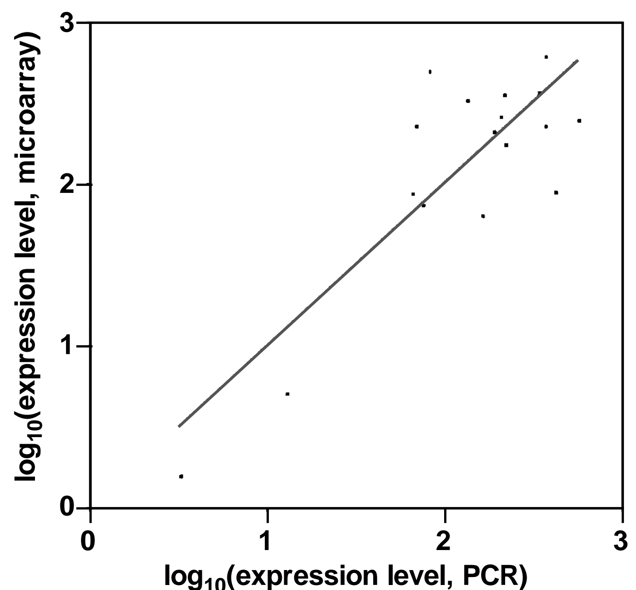

Figure 5. Scatterplot comparing MBP gene expression levels deduced from high resolution images and real-time PCR and low resolution images and microarray analysis. $r = 0.84$, $p < 0.0001$.

Concordance between high-resolution real-time PCR and low-resolution microarray voxelation images for the MBP gene

A relevant question for voxelation is the degree of agreement between gene expression patterns obtained from various transcript profiling technologies at different levels of resolution. A previous investigation used microarrays to examine gene expression patterns from coronal hemisections of the human brain at a spatial resolution of $\approx 1\ cm^3$ (Brown *et al.* 2002a). The MBP gene was present on the microarrays employed in this investigation, providing an opportunity to examine the concordance between the expression of the MBP gene deduced from the low resolution microarray studies with the high resolution voxelation studies reported here using real-time PCR.

In order to perform this comparison, gene expression profiles of the MBP gene obtained at high resolution were down-sampled (approximately 28–1) and averaged to give equal numbers of voxels for the high and low resolution data sets. A scatterplot showing the agreement between the two data sets is shown in Figure 5. A good level of agreement was obtained, suggesting that relatively robust agreement can be obtained between data obtained at various levels of resolution and analyzed using different transcript profiling tools.

Potential use of linear amplification and microarrays for high resolution voxelation of the rodent brain

A device for high resolution voxelation of the mouse and rat brain has been constructed (Singh *et al.* 2003).

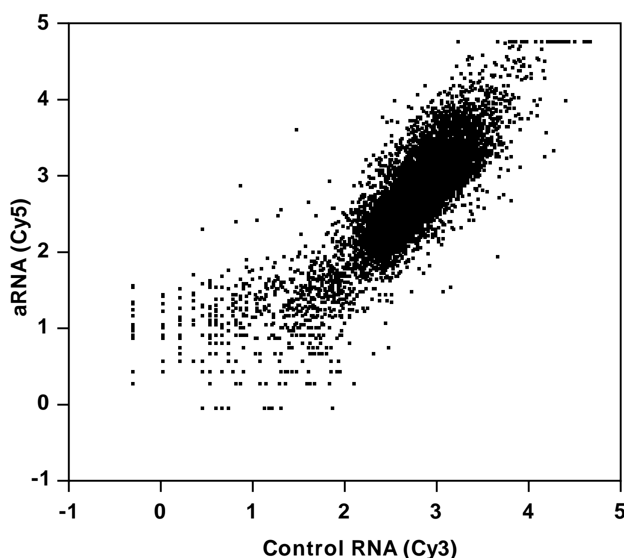

Figure 6. Scatterplot comparing microarray signals from unamplified RNA (Cy3) and linearly amplified RNA (aRNA, Cy5). $r = 0.8$, $p < 0.0001$.

This device uses a two dimensional array of cutting blades in combination with a stamping device to divide sections of the rodent brain into voxels of 1 mm, a volumetric resolution of 1 μl. The average amount of RNA obtained from these voxels (mean ± SEM) for the rat was 111.2 ± 10.3 ng and for the mouse 216.3 ± 16.2 ng. In order to assess the feasibility of microarray analysis using these small amounts of RNA, we performed a linear amplification experiment. In this experiment, we began with amounts of RNA from a whole mouse brain that were much less than that obtained from an individual 1 mm voxel, 20 ng. This amount of RNA was amplified to 1.8 μg and labeled with Cy5. In addition, 1.8 μg of unamplified total brain RNA was labeled with Cy3. These two RNA samples, sufficient for a strong signal, were then co-hybridized to a 9000 gene microarray. The results of the analysis are shown in Figure 6. There was good concordance between the amplified and unamplified samples, suggesting that linear amplification combined with microarray analysis should be a feasible approach to genome scale acquisition of gene expression patterns using voxelation of the rodent brain at high resolution.

Discussion

Various transcript profiling tools and levels of spatial resolution can be employed for the high-throughput acquisition of brain gene expression patterns using voxelation. Gene expression tools that have been used include real-time PCR and microarrays, although serial analysis of gene expression (SAGE) (Velculescu *et al.* 1995) and RNase protection (Brown *et al.* 2002b) could also be employed. Voxelation has been performed at a number of different spatial levels of resolution. For the human brain, studies have been performed at a volumetric resolutions of 1 cm^3 and 87 μl. For the mouse brain, studies have been performed at 7.5 and 1 μl.

A relevant issue for voxelation is the degree of concordance between gene expression patterns obtained using various transcript profiling tools at different levels of spatial resolution. In the present study, advantage was taken of two different data sets for the MBP gene. One data set was obtained using real-time PCR from a human brain hemisection at high volumetric resolution, 87 μl. The other data set was obtained using microarrays at low volumetric resolution, 1 cm^3. A significant degree of concordance was found for these two very different types of data set. This suggests good replicability between high-resolution real-time PCR and low resolution microarray voxelation gene expression images. In addition, these observations suggest that it is possible to obtain good image registration between various brain specimens at different levels of spatial resolution.

At very high levels of spatial resolution in the rodent brain, only limited amounts of RNA are recovered from each voxel. In order to perform voxelation at these high levels of spatial resolution on a genome-wide scale, it is necessary to evaluate the feasibility of using linear RNA amplification in combination with microarray analysis. In this study, it was shown that use of 20 ng of brain RNA in combination with linear RNA amplification and microarray analysis provided good agreement with expression profiles from unamplified RNA. Since 1 μl voxels provided 111.2 ng from the rat brain and 216.3 ng from the mouse brain (Singh *et al.* 2003), these results indicate that plenty of RNA is obtained from each voxel for linear RNA amplification and microarray analysis. Thus, genome wide analyses of gene expression patterns in the rodent brain at high spatial resolution should be feasible.

Voxelation is modality independent, providing the opportunity to analyze multiple molecular domains. For example, the proteome can be analyzed using high-throughput tools such as protein microarrays and mass spectrometry. Another recently developed approach, immuno-detection amplified by T7 polymerase (IDAT), uses RNA amplification coupled with antibody binding as a sensitive protein detection method (Zhang *et al.* 2001) and may be applicable to voxelation. As these and other technologies mature and become incorporated into voxelation, it will be relevant to assess the concordance between expression maps obtained using different technologies. Identification of similarities and differences between transcriptomic and proteomic expression maps are especially likely to provide interesting insights into the molecular basis of the brain.

Acknowledgements

Supported by the NIH (DA015802, DA05010), National Foundation for Functional Brain Imaging, NARSAD Young Investigator Award, Tobacco-Related Disease Research Program (11RT-0172), and Alzheimer's Association (IIRG-02-3609). The human brain specimen was obtained from the University of Maryland Brain and Tissue Bank under NIH contract N01-HD-1-3138.

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