Amino Acids (2005) 28: 279–290
DOI 10.1007/s00726-005-0177-x

Amino Acids
Printed in Austria

Expression profiling using human tissues in combination with RNA amplification and microarray analysis: assessment of Langerhans cell histiocytosis

Minireview Article

K. L. McClain[1], **Y.-H. Cai**[1], **J. Hicks**[2], **L. E. Peterson**[3], **X.-T. Yan**[1], **S. Che**[4,5], and **S. D. Ginsberg**[4,5,6]

[1] Texas Children's Cancer Center/Hematology Service, Baylor College of Medicine, Houston, Texas, U.S.A.
[2] Pediatric Pathology, Baylor College of Medicine, Houston, Texas, U.S.A.
[3] Department of Medicine, Baylor College of Medicine, Houston, Texas, U.S.A.
[4] Center for Dementia Research, Nathan Kline Institute, Orangeburg, New York, U.S.A.
[5] Department of Psychiatry, New York University School of Medicine, Orangeburg, New York, U.S.A.
[6] Department of Physiology & Neuroscience, New York University School of Medicine, Orangeburg, New York, U.S.A.

Received January 11, 2005
Accepted January 12, 2005
Published online March 30, 2005;

Summary. Advances in molecular genetics have led to sequencing of the human genome, and expression data is becoming available for many diverse tissues throughout the body, allowing for exciting hypothesis testing of critical concepts such as development, differentiation, homeostasis, and ultimately, disease pathogenesis. At present, an optimal methodology to assess gene expression is to evaluate single cells, either identified physiologically in living preparations, or by immunocytochemical or histochemical procedures in fixed cells *in vitro* or *in vivo*. Unfortunately, the quantity of RNA harvested from a single cell is not sufficient for standard RNA extraction methods. Therefore, exponential polymerase-chain reaction (PCR) based analyses, and linear RNA amplification including amplified antisense (aRNA) RNA amplification and a newly developed terminal continuation (TC) RNA amplification methodology have been used in combination with microdissection procedures such as laser capture microdissection (LCM) to enable the use of microarray platforms within individual populations of cells obtained from a variety of human tissue sources such as biopsy-derived samples {including Langerhans cell histiocytosis (LCH)} as well as postmortem brain samples for high throughput expression profiling and related downstream genetic analyses.

Keywords: cDNA microarray – Postmortem human brain – Cancer genomics – Translational neuroscience – Molecular fingerprint

Introduction

Contemporary molecular-based techniques have enabled the study of both genomic DNA and RNA populations from fresh, fixed, and paraffin-embedded tissues (Lehmann et al., 2000; Lewis et al., 2001; Van Deerlin et al., 2000; Van Deerlin et al., 2002). For example, upon identification of novel disease-related genes, retrospective mutation analysis in genomic DNA samples from archived tissues is tenable. Moreover, expression profiling of mRNA provides a real-time evaluation of gene expression. One mitigating factor is that the organs are complex structures with heterogeneous cellular populations. Each distinct cell type is likely to have a signature ''molecular fingerprint'' under normal and pathological conditions. For example, there is little data on comparative expression of cytokines in normal Langerhans cells (LC) versus those in Langerhans cell histiocytosis (LCH) and no data is available on the differences in cytokine expression in patients with 'low risk' versus 'high risk' disease. Thus, the pattern of mRNA expression in single cells or a population of cells may be more informative than regional expression patterns. Specifically, the molecular underpinnings that determine whether a given cellular population is vulnerable to degeneration or aberrant proliferation, i.e., ''selective vulnerability'', may be elucidated by single cell mRNA analyses (Galvin and Ginsberg, 2004; Ginsberg et al., 2004).

Advent of high throughput gene expression profiling methods, including cDNA (Brown and Botstein, 1999; Eisen and Brown, 1999) and oligonucleotide (Lockhart and Barlow, 2001; Lockhart et al., 1996) array technology, enables relative quantitative assessment of multiple (e.g.,

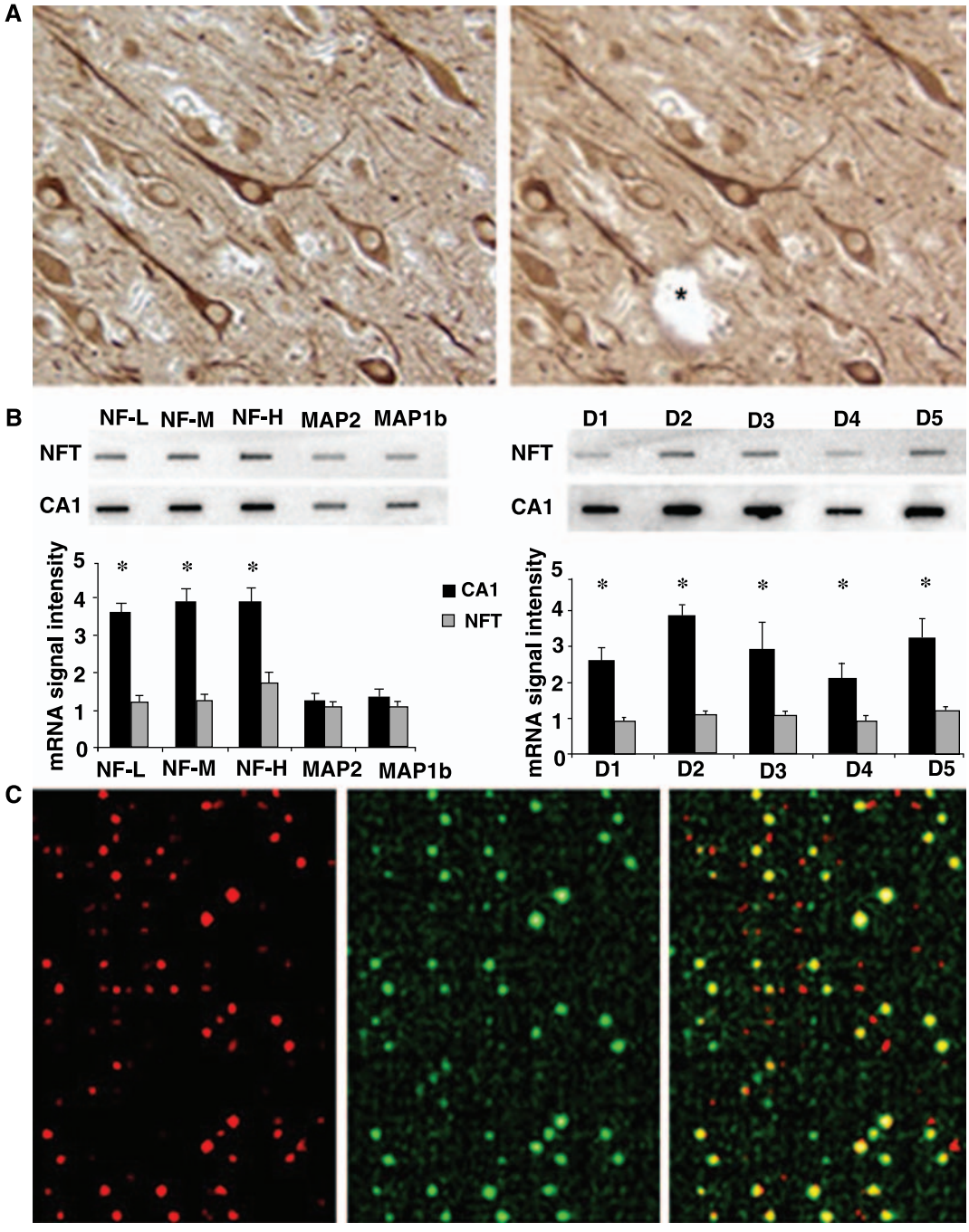

Fig. 1. Microaspiration and cDNA array analysis of single neurons. **A** Microaspiration of a neurofilament-immunoreactive CA1 pyramidal neuron obtained postmortem from the hippocampus of a normal control subject. The left hand panel illustrates the neuron before aspiration and the right hand panel (asterisk) shows the tissue section following microaspiration. **B** Representative histograms and custom-designed cDNA array analysis. Individual CA1 pyramidal neurons from normal control subjects (*CA1*) were compared to neurofibrillary tangle (*NFT*) bearing CA1 neurons from Alzheimer's disease (AD) brains. The left hand panel depicts down regulation of the three neurofilament subunits {low (*NF-L*), medium (*NF-M*), and high (*NF-H*)} in AD NFTs (asterisk denotes $p < 0.01$) with no significant regulation of the microtubule-associated proteins MAP2 and MAP1b. The right hand panel illustrates down regulation of the dopamine receptor genes D1–D5 in AD NFTs versus normal CA1 neurons. **C** A portion of a representative microarray is shown, illustrating a wide range of hybridization intensities using aRNA probes generated from NFT-bearing neurons (first panel; pseudocolored red), normal CA1 neurons (second panel; pseudocolored green), and an overlay of NFT-bearing and normal CA1 neuron hybridizations (third panel). In the latter panel, yellow indicates similar hybridization intensities for NFT-bearing neurons and normal CA1 neurons, green indicates a down regulation in NFT-bearing neurons relative to normal CA1 neurons, and red denotes an up regulation of an EST within diseased versus normal CA1 neurons

dozens to thousands) genes simultaneously from one tissue sample. Expression profiling is performed by accessing cells/tissues of choice via microaspiration or related technologies including LCM, extracting RNAs from sample tissues, amplifying RNA if necessary, labeling RNA/amplified RNA probes, and hybridizing labeled RNA to array platforms (Fig. 1). Quantification of hybridization signal intensity is performed to assess the relative signal intensity of each feature on the array platform. Gene expression is evaluated using univariate statistics, multivariate statistics, and informatics software, enabling high throughput coordinate analyses.

Gene expression profiling using fresh and fixed tissues

Assessment of cellular populations in optimally prepared, fresh frozen biopsy and fixed postmortem human tissues is desirable due to the abundance of specific tissues (e.g., brain samples and tumor samples) that are archived within individual laboratories and tissue banks. A variety of cells and tissues can be used to extract mRNA for gene profiling experiments. When employing mRNA as a starting material, one cannot over-emphasize the importance of the preservation of RNA integrity. RNA species are particularly sensitive to degradation by ribonuclease (RNAse). RNAses are found in virtually every cell type, and retain their activity over a broad pH range (Blumberg, 1987; Farrell Jr, 1998). Thus, RNAse-free precautions are essential for all microdissection-based studies. All biological samples require prompt handling, either through rapid RNA extraction, flash freezing, or fixation to minimize degradation. At present, no consensus protocol exists for the fixation and/or extraction of tissues obtained from biopsy or from postmortem human tissue. Several laboratories have evaluated the effects of different fixation protocols on RNA quality, ease of tissue microdissection, and success of cDNA array analysis (Goldsworthy et al., 1999; Grotzer et al., 2000; Klimecki et al., 1994; Su et al., 2004). Many variables, including antemortem characteristics, duration of fixation, and length of storage are relevant parameters that should be taken into consideration prior to the initiation of a study (Florell et al., 2001; To et al., 1998; Van Deerlin et al., 2000; Van Deerlin et al., 2002). A critical parameter lies in assessing RNA quality and quantity prior to performing expression profiling studies. A useful histochemical technique that lends itself to RNA assessment in tissue sections adjacent to those employed for expression profiling is acridine orange (AO) histofluorescence (Sarnat et al., 1986; Topaloglu and Sarnat, 1989). AO is a fluorescent dye that intercalates selectively into nucleic acids, and has been used to detect RNA and DNA in brain tissues and cancer-related paradigms (Ginsberg et al., 1997; Ginsberg et al., 1998; Pinto et al., 1990; Sarnat et al., 1986). Notably, individual RNA species (e.g., rRNA, tRNA, and mRNA) cannot be delineated by AO histofluorescence. Rather, this method provides a straightforward diagnostic test that can be performed on adjacent tissue sections to ensure the likelihood that an individual case has abundant RNA prior to performing expensive microdissection and microarray studies. A more definitive examination of RNA quality can be obtained via bioanalysis (e.g., 2100 Bioanalyzer, Agilent Technologies), which employs capillary gel electrophoretic methodologies to detect RNA quality and abundance (Che and Ginsberg, 2004a; Ginsberg and Che, 2004; Gottwald et al., 2001). Bioanalysis enables visualization of results in an electropherogram and/or digital gel format, and provides a means of RNA assessment at relatively high sensitivity. Investigators can also evaluate DNA and protein quality and abundance using bioanalysis platforms (Freeman and Hemby, 2004; Vasilyeva et al., 2004).

Accession of single cells from tissue sections

Microdissection of individual cells is performed to enable downstream gene expression profiling using cDNA microarrays or by PCR-based technologies. Provided that procedures are performed on well-preserved tissue sections and RNase-free conditions are employed, both immunocytochemical and histochemical procedures can be utilized to identify specific cell(s) of interest. Several different methodologies have been used to aspirate individual neurons or groups of cells including single cell microaspiration and laser capture microdissection (LCM). Microaspiration entails visualizing an individual cell (or cells) using an inverted microscope connected to a micromanipulator and a vacuum source. Microaspiration results in accurate dissection of cells of interest with minimal disruption of the surrounding tissue (Ginsberg et al., 1999; Ginsberg et al., 2000) (Fig. 1). LCM is a strategy for acquiring histochemically and/or immunocytochemically labeled cells from *in vivo* and *in vitro* sources (Dolter and Braman, 2001; Ehrig et al., 2001; Goldsworthy et al., 1999). LCM has become a widely used and reproducible technique that was developed originally at the NIH (Bonner et al., 1997; Emmert-Buck et al., 1996). There are two principal means of LCM, positive extraction and negative extraction. Positive extraction (a method used by the PixCell IIe from Arcturus) entails using a laser source

directly on the cell(s) of interest to adhere them to a matrix that will be lifted from the surrounding tissue. Negative extraction (or non-contact laser extraction) procedures employ a laser source to cut around the area of interest within a tissue section, and the microdissected material is catapulted into a microfuge tube (a method utilized by the P.A.L.M. system, P.A.L.M. Microlaser Technologies). In this manner, single cells as well as dozens to hundreds of cells can be acquired in a relatively short time. Moreover, RNA, DNA, and protein extraction methods can be performed on microdissected cells (Fend et al., 1999; Suarez-Quian et al., 1999; Craven et al., 2002; Mouledous et al., 2003; Simone et al., 2000).

RNA amplification

In order to generate a significant amount of RNA sufficient to perform microarray analysis and related downstream genetic analyses, an RNA amplification technique is often required. PCR-based amplification methods are not optimal, as exponential amplification can skew the original quantitative relationships between genes from an initial population (Kacharmina et al., 1999). Linear RNA amplification is another strategy that has been used successfully to generate enough input RNA for robust hybridization signal. One method of linear amplification termed amplified antisense RNA (aRNA) amplification, was developed by Eberwine and colleagues, and involves a T7 RNA polymerase based amplification procedure that enables quantitation of the relative abundance of gene expression levels from identified single cells and populations (Eberwine et al., 2001; Eberwine et al., 1992; Kacharmina et al., 1999). aRNA amplification entails the hybridization of an oligonucleotide primer consisting of thymidine triphosphates (TTPs) and a T7 RNA polymerase promoter sequence {oligo d(T)T7)} to mRNAs and conversion to an mRNA-cDNA hybrid by reverse transcriptase (RT) (Tecott et al., 1988; VanGelder et al., 1990). Upon conversion of the mRNA-cDNA hybrid to double stranded cDNA, a functional T7 RNA polymerase promoter is formed. aRNA synthesis occurs with the addition of T7 RNA polymerase and nucleotide triphosphates (Fig. 2). Each round of aRNA results in an approximate thousand fold amplification from the original amount of each polyadenylated {poly(A)+} mRNA in the sample (Eberwine et al., 2001; Eberwine et al., 1992). Two rounds of aRNA are typically necessary to generate sufficient quantities of aRNA for subsequent downstream analyses. Although aRNA is a laborious and difficult procedure, we have generated successful results obtained from microaspirated cells from biopsy-derived tumor specimens and postmortem human brain tissues utilizing a wide variety of cDNA array platforms (Ginsberg et al., 1999; Ginsberg et al., 2000; Hemby et al., 2003).

Several different strategies have been employed by independent laboratories to evaluate and improve linear RNA amplification efficiency (Dafforn et al., 2004; Iscove et al., 2002; Klur et al., 2004; Wang et al., 2000). We have developed a new linear RNA amplification procedure that utilizes a method of terminal continuation (TC). TC RNA consists of synthesizing first strand cDNA complementary to the RNA template, subsequently generating second strand

Fig. 2. aRNA amplification scheme. An oligo-d(T)T7 primer is hybridized to polyA+ mRNAs and a double stranded mRNA-cDNA hybrid is formed by reverse transcription. The double stranded mRNA-cDNA hybrid is then converted into double stranded cDNA. Following the removal of tertiary structures and drop dialyzing the double stranded cDNA against RNase-free water, the first round of aRNA synthesis occurs using T7 RNA polymerase and NTPs. A second round of aRNA amplification begins by annealing random hexamers to the newly formed aRNA, and synthesizing a cDNA strand. The oligo(dT)-T7 primer is then reintroduced and a double stranded cDNA template is formed. aRNA probes are then generated with fluorescent, biotin, or radiolabeled second round aRNA products

Fig. 3. Overview of the TC RNA amplification method. **A** A TC primer (containing a bacteriophage promoter sequence for sense orientation) and a poly d(T) primer are added to the mRNA population to be amplified (green rippled line). First strand (blue line) synthesis occurs as an mRNA-cDNA hybrid is formed after reverse transcription and terminal continuation of the oligonucleotide primers. Following RNase H digestion to remove the original mRNA template strand, second strand (red line) synthesis is performed using *Taq* polymerase. The resultant double stranded product is utilized as template for *in vitro* transcription, yielding high fidelity, linear RNA amplification of sense orientation (green rippled lines). **B** Schematic similar to A, illustrating the TC RNA amplification procedure amplifying RNA in the antisense orientation (yellow rippled lines)

Fig. 4. Custom-designed cDNA array analysis of microdissected LC and tissue dissection of LCH from bone, spleen, and liver. **A** Representative hybridization signal intensity from microdissected LC and LHC. Upper panel is from normal skin LC and the lower panel from a spleen highly infiltrated with LHC in a patient with 'high risk' LCH. Duplicate samples of M-CSF, TGF-beta receptor, and IL-1α (arrows) show prominent expression of these genes in both the normal LC and diseased LCH tissue. **B** Custom-designed cDNA array analysis from amplified RNA obtained using whole tissue (spleen and liver) specimens. Intense hybridization signal intensity was observed for TNFα, RANK, RANKL, TNFRp55, TGFβR, NFκB, IL-13, and IL-13R1. **C** Dendogram derived from densitometric analysis of custom-designed cDNA arrays. The dendogram summarizes data from **A** and **B** as well as arrays for hybridization of aRNA from bone lesions of two different patients (data not shown). Columns 1 (liver) 2 (bone), and 5 (spleen) are data from analysis of whole tissue RNA from those sources. Columns 3 and 4 (LH spleen and LH skin) represent data from microdissected LHC of LCH spleen and LC of normal skin. The red color represents gene expressed at the highest level, white-intermediate, and blue-lowest relative expression

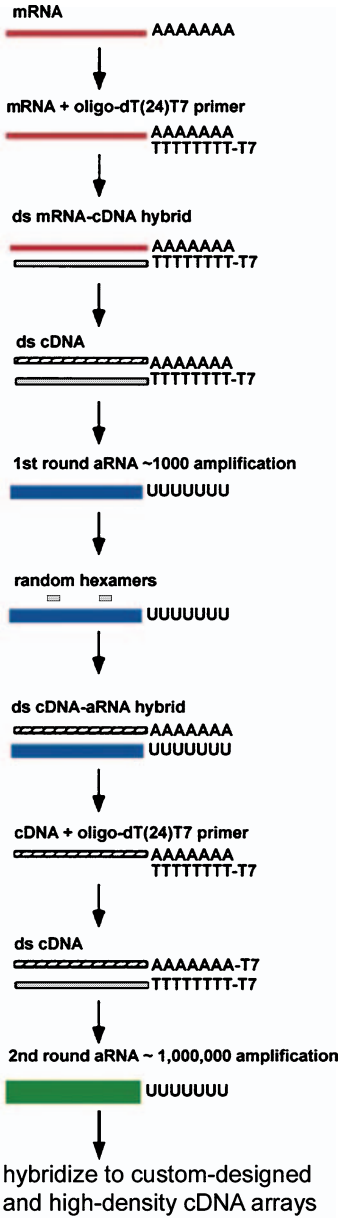

Fig. 2

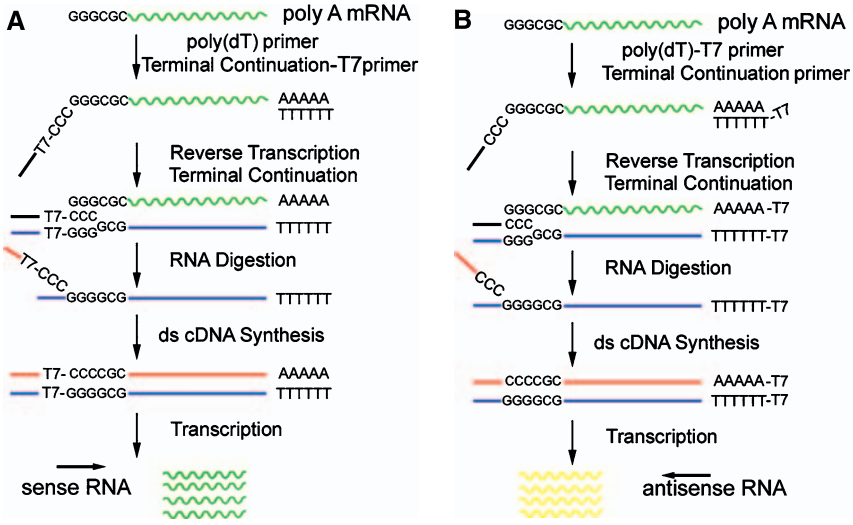

Fig. 3

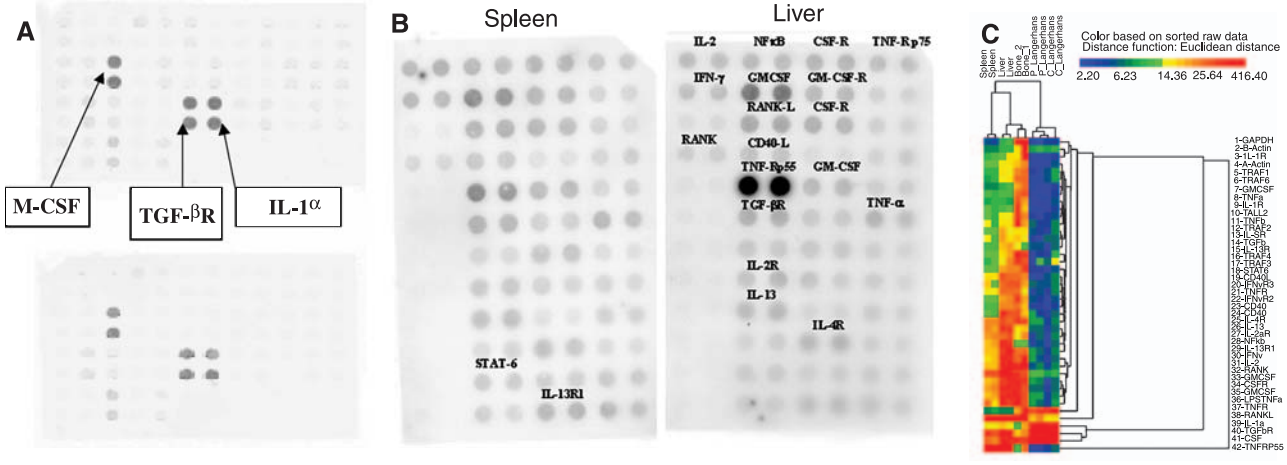

Fig. 4

cDNA complementary to the first strand cDNA, and finally *in vitro* transcription using the double stranded cDNA as template (Che and Ginsberg, 2004a; Che and Ginsberg, 2004b) (Fig. 3). By providing a known sequence at the 3′ region of first strand cDNA and a primer complementary to it, hairpin loops will not form. Second strand cDNA synthesis can be performed with robust DNA polymerases, such as *Taq*, and the TC reaction is highly efficient. One round of amplification is sufficient for downstream genetic analyses (Che and Ginsberg, 2004a; Che and Ginsberg, 2004b). Furthermore, TC RNA transcription can be driven using a promoter sequence attached to either the 3′ or 5′ oligonucleotide primers. Therefore, transcript orientation can be in an antisense orientation (similar to conventional aRNA methods) when the bacteriophage promoter sequence is placed on the poly d(T) primer or in a sense orientation when the promoter sequence is attached to the TC primer, depending upon the design of the experimental paradigm (Fig. 3). Following TC RNA amplification, a large proportion of genes can be assessed quantitatively as evidenced by bioanalysis and cDNA microarray analysis in human postmortem brain tissues (Che and Ginsberg, 2004a; Ginsberg and Che, 2002; Ginsberg and Che, 2004; Ginsberg and Che, 2005). Importantly, increased sensitivity appears greatest for genes with relatively low abundance. Moreover, background hybridization is significantly attenuated when using TC RNA amplification (Ginsberg and Che, 2002; Ginsberg and Che, 2004; Mufson et al., 2002).

Microarray analysis

Once input sources of RNA are isolated and/or amplified, fluorescent or radiolabeled probes can be generated for subsequent hybridization to microarray platforms. Synthesis of cDNA microarrays entails adhering cDNAs or expressed sequence-tagged cDNAs (ESTs) to solid supports such as glass slides, plastic slides, or nylon membranes (Brown and Botstein, 1999; Eisen and Brown, 1999). A parallel technology uses photolithography to adhere oligonucleotides to array media (Lockhart and Barlow, 2001; Lockhart et al., 1996). Arrays are imaged using a laser scanner for fluorescently labeled probes and a phosphor imager for radioactively labeled probes. Gene expression data collected using single cells and/or homogeneous populations via RNA amplification and cDNA array analysis does not allow absolute quantitation of mRNA levels, but generates an expression profile of the relative changes in mRNA levels (Eberwine et al., 2001; Ginsberg et al., 2004; Hemby et al., 2002). Differential expression greater than approximately two-fold is accepted conventionally as relevant for further examination (Galvin and Ginsberg, 2004; Hemby et al., 2003; Kotlyar et al., 2002). Computational analysis is critical for optimal use of microarrays due to the enormous volume of data that is generated from a single probe (Aittokallio et al., 2003). Additionally, access to relational databases is desirable, especially when evaluating hundreds of oligonucleotides and ESTs that may/may not be linked to genes (and subsequent proteins) of known function.

Practical application of microarray analysis: comparison of LC and LCH

There is little data on comparative expression of cytokines in normal Langerhans cells (LC) versus those in Langerhans cell histiocytosis (LCH) and no data on the differences in cytokine expression in patients with ‘low risk’ versus ‘high risk’ disease. As an example of the power of microarray analysis within this paradigm, we compared cytokine expression profiles of microdissected LC from normal and LCH tissue to that of whole tissue cytokine expression using a custom-designed array platform.

LCH, known previously as histiocytosis X, is a reactive, proliferative disease characterized by the accumulation of morphologically normal LCs along with lymphocytes, eosinophils, and macrophages to form infiltrates typical for the disease (McClain et al., 1994). The incidence of LCH is approximately 5 cases per million children (Carstensen and Ornvold, 1993). Only one-third as many adults may be afflicted (Islinger et al., 2000). LCH includes a wide range of clinical presentations from single system involvement such as the skin or bone lesions (‘low risk’ patients) to diffuse involvement of spleen, liver, lungs, bone marrow (‘high risk’ patients), and even the central nervous system. The course of the disease is unpredictable, varying from rapid progression and death to repeated recurrences with chronic sequelae, or to spontaneous regression and resolution (Minkov et al., 2002).

The etiology of LCH is unknown although most investigators consider LCH a form of immunologic dysfunction. There are no cytogenetic or immunophenotypic differences in the cells to further characterize patients as to possible outcome of therapy, although one study using comparative genomic hybridization found gains and losses in several chromosomes (Murakami et al., 2002). Several cytokines have been identified with higher expression in LC, lymphocytes, or macrophages in LCH lesions including IL-1α, interferon-γ, M-CSF, GM-CSF, TNF-α, leukemia inhibitory factor, interleukins-2, -4, -5, and CD40/CD40L (Arenzana-Seisdedos et al., 1986; Egeler

Table 1. LCH-associated genes

TNF family	TNF-α	TNF-β	TNF-R1	TNF-R2	TNFR-3	TRAF1
	TRAF2	TRAF3	TRAF4	TRAF5	TRAF6	
	CD40	CD40L	RANK	RANKL	NFκB	STAT6
Cytokines	GM-CSF	GM-CSF-R	INF-γ	INF-γ-R1	INF-γ-R2	
Interleukins	IL-1α	IL-1α-R	IL-2α	IL-2α-R	IL-4	IL-4R
	IL-5-R	IL-10	IL-10R	IL-13	IL-13-R1	IL-13-R2
Other	TGFβ	TGFβ-R	α-actin	β-actin	GDPH	

TNF, tumor necrosis factor; *TNF-R*, tumor necrosis factor receptor, *TRAF*, tumor necrosis factor receptor associated factor; *TRAIL*, TNF-related apoptosis inducing ligand; *RANK*, receptor-activator of NF-kappa B; *RANKL*, receptor-activator of NF-kappa B ligand; *NFκB*, nuclear factor-kappa B; *STAT*, signal transducer and activator of transcription; *GM-CSF*, granulocyte-macrophage colony stimulating factor; INF-γ, interferon gamma; *INF-γ-R*, interferon gamma receptor; IL, interleukin; *IL-R*, interleukin receptor; *TGFβ*, transforming growth factor beta; *TGFβ-R*: transforming growth factor beta receptor; *GDPH*, glyceraldehyde-3-phosphate dehydrogenase

et al., 1999; Yokota et al., 1996) (see Table 1 for gene abbreviations). Differences in the relative expression of cytokines from the various cell types in the LCH lesions have been defined by immunocytochemistry, suggesting that the interaction of various cellular components could be very important in the pathophysiology of LCH. For example, LC in LCH have been proposed to be immature dendritic cells as judged by lack expression of Class II antigens on the cell surface and absence of CD83 and CD86 (Geissmann et al., 2001) and that IL-10 produced by macrophages in the lesion were one cause of the maturational arrest. The relative numbers of IL-10 producing macrophages in different types of LCH lesions correlated with clinical outcome in that the lowest numbers were found in skin and bone lesions that often heal spontaneously. Another possible cause of maturational arrest is the presence of transforming growth factor-beta (TGF-β) that is abundant in LCH lesions (Geissmann et al., 1999).

It appears that in LCH normal LC physiology is disrupted because of imbalances in the cytokine milieu. We have begun to study the expression of several potential LCH-related genes plus others relevant to the growth and function of dendritic cells to establish a method for studying biologic differences between normal LC and those in LCH lesions as well as correlates to different clinical responses between the subgroups of LCH patients.

Materials and methods

Sample acquisition

Patient specimens were obtained from the Texas Children's Hospital Pathology department. Tissue accession and accrual procedures were in accordance with IRB standards at the Texas Children's Hospital, Baylor College of Medicine, Nathan Kline Institute, and NYU School of Medicine. Samples were studied from three different patients and a foreskin sample from a normal child who had a circumcision. All patients had been diagnosed with LCH according to guidelines of the Histiocyte Society (McClain et al., 1994). LC were identified using an antibody directed against CD1a. Two male patients, ages 6 and 9 years, had only single bone lesions that were biopsied. These children are off therapy for more than one year and have no evidence of recurrent disease. The spleen sample was obtained from a two year old girl with LCH involving spleen, liver, skin, bone, and bone marrow. Her spleen was removed because of respiratory compromise. She ultimately died and liver tissue was obtained at autopsy. Samples were fixed in 4% paraformaldehyde/phosphate buffered saline (PBS) overnight at 4°C and paraffin embedded.

Tissue preparation

Six micron-thick sections of skin or LCH tissue specimens were utilized in concert with anti-CD1a (Immunotech, Marseille, France), anti-TNFα, or anti-IL-13 (R&D Systems, Minneapolis, MN). Primary antibodies were incubated on the sections for 20 minutes. Sections were processed with secondary antibodies (DakoCytomation, Carpinteria, CA) and developed with 0.3% hydrogen peroxide in PBS with 0.1% sodium azide and 0.05% diaminobenzidine in PBS buffer for 10 minutes as described previously (Ginsberg et al., 2000). LC was microaspirated using LCM instrumentation (PixCell IIe, Arcturus, Mountain View, CA). Three hundred CD1a+ LC were captured per reaction. Regional isolation of LCH tissue was performed using approximately 1 mm^3 pieces of tissue. RNA was isolated using RNA STAT-60 (Tel-Test, Friendswood, TX) followed by phenol:chloroform and ethanol extraction.

RNA amplification

An oligonucleotide primer consisting of 24 TTPs is coupled to a T7 RNA polymerase promoter sequence {oligo-dT(24)T7} was hybridized to the cellular poly A+ mRNA (Eberwine et al., 2001; Ginsberg et al., 1999; Ginsberg et al., 2000). cDNA is synthesized with avian myeloblastosis virus reverse transcriptase (AMVRT, 0.5 U/μl, Seikagaku America, Falmouth, MA) in Tris-HCl buffer (pH 8.3) containing 6 mM $MgCl_2$, 120 mM KCl, 7 mM dithiothreitol, 250 uM dNTPs, and 0.12 U/ul RNAsin. The aRNA is amplified from the double stranded cDNA template with T7 RNA polymerase (Epicentre Technologies, Madison, WI). The first-round aRNA synthesis yields approximately a 1000-fold amplification of the original starting material (Eberwine et al., 2001; Ginsberg et al., 1999; Ginsberg et al., 2000). After the second round of amplification the aRNA represents >1 million-fold amplification (1000-fold from each round of amplification) (Eberwine et al., 2001; Kacharmina et al., 1999). Initially the substrate RNA from the 300 LC is in the range of several picograms by spectrophotometric analysis. After the two amplification steps we estimate

the aRNA would be approximately 0.4 μg. Hybridization probes were synthesized by *in vitro* transcription using ^{32}P incorporation in 40 mM Tris (pH 7.5), 7 mM $MgCl_2$, 10 mM NaCl, 2 mM spermidine, 5 mM of DTT, 0.5 mM of ATP, GTP, and UTP, 10 μM of cold CTP, 20 U of RNase inhibitor, 1000 U T7 RNA polymerase (Epicentre), and 40 μCi of ^{32}P-UTP (GE Amersham, Piscataway, NJ). The reaction was performed at 37°C for 4 hours.

Hybridization to custom-designed arrays

Custom-designed cDNA arrays were synthesized using 500 base pair segments of genes listed in Table 1. All the plasmids were obtained from the Baylor College of Medicine DNA microarray core facility. Five micrograms of each insert was arrayed in duplicate onto nylon membranes (Hybond, GE Amersham). This represents a several thousand-fold excess of target over probe for hybridized sequences (Che and Ginsberg, 2004b; Ginsberg and Che, 2005). Prehybridization (12 hours) and hybridization (48 hours) was performed in 50% formamide, 6X SSPE, 5X Denhardt's solution, 0.1% sodium dodecyl sulfate 200 μg/ml denatured salmon sperm DNA, and 1 mM sodium pyrophosphate at 42°C. The arrays were washed in sequential solutions of 2X, 1X, and 0.5X SSC containing 0.1% sodium dodecyl sulfate at 42°C (Ginsberg and Che, 2002; Ginsberg et al., 1999; Ginsberg et al., 2000). Hybridization intensity of labeled products was detected by phosphor imaging. Data presented in this report represents analysis of duplicate custom-designed arrays for each tissue sample and the arrays were used only once.

Statistical analysis

Hierarchical cluster analysis was performed on gene expression levels with the CLUSFAVOR program (Peterson, 2002). Briefly, the specimens (columns) were clustered using expression levels standardized with row (gene) averages and standard deviations. The distance function used for agglomeration was one minus correlation (i.e., $1 - r_{jk}$) where r_{jk} is the correlation in standardized expression between specimen j and specimen k. For each pair of specimens (j, k) out of the total sample of p specimens, the minimum jackknife correlation $min\{r(j,k)^{(1)}, r(j,k)^{(2)}, \ldots, r(j,k)^{(n)}\}$ was used, where $r(i,j)^{(i)}(i = 1, 2, \ldots, n)$ represents correlation when gene i is dropped from the calculation, and n is the total number of genes (Heyer et al., 1999). Clustering was then performed on the genes, with standardization based on column (specimen) averages and standard deviations. T-tests for significant differences in mean expression of genes in the array groups were performed by the Welch test. Significance was set at the $p < 0.05$ level.

Results

A surprising similarity of genes was expressed in normal and diseased LC, with highest expression seen for GM-CSF, TGFβ-R, and IL-1α (Fig. 4). The majority of candidate cytokine genes showed no statistically different expression in the LC obtained from normal versus diseased tissue. Several genes (CD40L, interferon-γ, IL-1R, TGFβ, and TNFα) were more highly expressed in the normal LC. These observations are further illustrated by cluster analysis and presented in a dendrogram (Fig. 4). Results indicate that LC and LCH samples have very low expression of a majority of the genes except for TNFR (p75), RANKL, IL-1α, TGFβ-R and GM-CSF. There is minimal, if any, difference between the two sources of isolated LC for expression of these genes. In contrast, there is a more generalized increased expression of the cytokine panel from whole tissue RNA in bone, liver, or spleen tissue, most likely reflecting expression from lymphocytes, macrophages, and/or eosinophils.

Given the relative similarity of gene expression in LC from normal or diseased tissue it was of interest to compare the expression profiles from whole tissues (e.g., bone, liver, and spleen) (Fig. 4). Table 2 summarizes the genes expressed at statistically different levels in 'high risk' tissues (e.g., liver and spleen) versus 'low risk' LCH lesion (e.g., bone). Both the liver and spleen had higher relative levels

Table 2. Comparison of expression profiles obtained from bone, spleen, and liver LCH lesions. Cytokine gene expressed at the higher relative levels in bone are in italics, those expressed more prominently in spleen or liver are in bold type

Gene	Average		T-test Bone vs.	Average	T-test Bone vs.
	Bone	Spleen	spleen t	Liver	liver t
α-Actin	17.57	10.96	2.75	14.02	1.06
β-Actin	25.83	6.41	1.72	8.66	1.42
CD40	19.31	11.12	2.29	28.01	−1.64
CD40L	*19.53*	*14.54*	*4.00*	22.16	−1.25
M-CSF	32.26	21.20	2.39	34.88	−0.47
M-CSFR	29.07	18.36	2.62	33.38	−0.84
GAPDH	47.14	5.39	4.64	9.30	3.85
GM-CSF	*30.44*	*16.98*	*6.85*	31.95	−0.49
IFNγ	33.13	15.13	1.55	32.15	0.08
IFNγR2	22.86	12.61	2.21	18.10	0.86
IFNγR3	20.64	14.23	2.04	23.36	−0.75
IL-13	**15.16**	**18.76**	**−3.14**	**27.17**	**−4.64**
IL-13R	11.33	9.96	0.64	16.90	−1.85
IL-13R1	*21.24*	*15.76*	*8.03*	**33.70**	**−3.10**
IL-1α	14.10	13.12	0.25	15.00	−0.18
IL-1R	*21.92*	*8.27*	*3.51*	8.21	2.90
IL-2	*42.33*	*15.43*	*4.58*	36.09	0.72
IL-2αR	16.86	18.18	−0.44	22.92	−1.50
IL-4R	24.88	19.55	2.07	23.60	0.34
IL-5R	*14.85*	*12.07*	*9.42*	15.36	−0.19
LPSTNFα	36.28	17.78	1.46	31.03	0.40
NFκB	**12.65**	**16.68**	**−19.83**	**31.75**	**−9.63**
RANK	26.73	24.96	0.52	41.42	−2.56
RANKL	**37.69**	**62.81**	**−12.22**	**78.73**	**−13.31**
STAT6	23.06	11.87	2.33	18.65	0.79
TALL2	21.78	10.06	2.10	10.68	1.81
TGFβ	14.86	11.97	1.58	15.91	−0.52
TGFβR	**17.91**	**25.79**	**−4.03**	**40.21**	**−5.09**
TNFα	*21.99*	*9.26*	*6.51*	*10.13*	*4.45*
TNFβ	23.10	10.34	2.66	13.66	1.83
TNFRP75	24.79	7.38	2.45	6.53	2.30
TNFRP55	**15.20**	**407.64**	**−36.48**	**73.88**	**−5.15**
TRAF1	*20.11*	*8.71*	*14.64*	*13.70*	*3.30*
TRAF2	25.58	11.74	1.72	15.00	1.26
TRAF3	*25.73*	*9.72*	*8.10*	*16.22*	*2.65*
TRAF4	33.14	10.90	3.13	16.28	2.10
TRAF6	*21.16*	*7.98*	*21.19*	*14.21*	*4.71*

of several genes with largest differences found in TNFR p55, RANKL, TGFβ-R, NFκB, and IL-13. Liver tissue also had higher levels of the IL-13 receptor-1. Bone lesions and isolated LC had minimal expression of NFκB, making this the most conspicuous up-regulated gene in the 'high risk' patients (Table 2). In contrast the relatively lower expression of TNFR p75 as well as TALL2, IL-1-R, and GM-CSF were evident in the spleen and liver tissue RNA. Bone lesions had statistically higher levels of the TNFR p75, GM-CSF, IL-1-R, IL-2, IL-4, IL-13R, several TRAFs, CD40/CD40L, interferon-γ, interferon-γ-R, GM-CSF, GM-CSF-R, and STAT6 as compared to the liver and spleen tissues (Table 2).

Discussion

LC and LCH expression profiling

Preliminary expression profiling using microdissected samples from LC and LCH tissues demonstrated no significant difference in the relative hybridization signal of genes expressed at the highest levels (GM-CSF, TGF-αR, and IL-1α) of microdissected LC. Cytokine expression in whole tissue specimens showed significantly higher levels of IL-13, NFκB, TNFR p55, RANK-L, CD40-L, and TGF-βR in liver and spleen tissues from 'high risk' patients. Statistically significant differences in the gene profile from lesions of patients with only bone involvement versus organs of patients with multisystem involvement were found including CD40/CD40L, GM-CSF, GM-CSF-R, IL-13-R1, IL-2, IL5R, TNFα, TGFβ, and TRAF1, and TRAF6. We hypothesize that cells other than LC, including lymphocytes, monocytes, and/or eosinophils are responsible for the pathophysiology of LCH.

To date, a direct comparison of expression profiles in normal LC versus those in LC from LCH patients has been lacking. In order to develop hypotheses on the pathophysiology of LCH, we have sought to develop a more comprehensive panel of relevant cytokine genes and use this to explore gene expression in isolated LC as well as tissue biopsies. These data sets are preliminary due to the initial small number of patients and types of lesions examined. However, this preliminary analysis illustrates the power of expression profiling in microdissected tissues using RNA amplification methodologies coupled with custom-designed cDNA array analysis. Specifically, there is a remarkable similarity in the gene expression profile of dissected LC from normal skin or diseased spleen. One of the three highly expressed genes was GM-CSF, which is an important cytokine for differentiation of CD1a+ dendritic cells from monocytes *in vitro* when incubated with IL-4 (Palucka et al., 1998). These cells express the GM-CSF-R, which under stimulation with IL-10, could induce macrophage development (Rieser et al., 1998). Given the plasticity of dendritic cells and macrophage cells, it is not surprising that many LCH patients are affected by a hemophagocytic syndrome in their bone marrow that markedly complicates their treatment (Favara et al., 2002). It will be necessary to extend these studies using more patients to confirm the differences found and further define genes with prognostic potential. These data illustrate the pleiotropic effects of cytokines likely occurring as a result of T cell activation and pro-inflammatory responses. Specific expression profiles of T cells in LCH are currently under investigation. In addition, the relatively high level expression of several members of the TNFα family of genes as well as cytokines and their receptors in LCH suggest these groups of genes should be more extensively investigated as central to the pathophysiology of the disease.

Expression profiling using microdissected cell samples

One of the most important aspects of single cell or single population gene expression analysis is that different cell types can be discriminated based upon their molecular fingerprint. For example, populations of neurons that express proteins selectively such as cholinergic basal forebrain neurons (Ginsberg et al., 2004; Mufson et al., 2002) or midbrain dopaminergic nigral neurons (Fasulo and Hemby, 2003; Tang et al., 2003) as well as LC and LCH (see above) are amenable to single cell RNA amplification and subsequent microarray analysis. Moreover, cells that lack a distinct or selective signature phenotype can be analyzed using a variety of Nissl and immunohistochemical stains (Ginsberg and Che, 2004) for downstream genetic applications. Discrimination of adjacent cell types is quite important because this enables the differentiation of the desired cells from the surrounding elements. Thus, a goal is to assess pathological changes in gene expression within vulnerable populations while avoiding potential contamination from spared cell types.

Conclusions

As our ability to refine expression profiling paradigms increases, the development of pharmacotherapeutic agents that are more selective, or potentially specific, for individual cell types become tenable. The next level of understanding of cellular and molecular mechanisms underlying normative

function and pathological conditions lies in the ability to combine these aforementioned technologies with appropriate models to recapitulate the structure and connectivity of these systems *in vivo* and *in vitro*. Complex biological processes are not likely to be governed solely by the action of a single isolated gene. Rather, coordinate interactions of a multiplicity of genes may regulate normative function. When these gene programs or mosaics undergo increased or decreased expression during the lifespan, they may contribute to the mechanisms underlying disease pathogenesis. Single cell and population cell profiling techniques coupled with microarray platforms have the potential to quantify simultaneous expression levels of numerous genes and proteins in a given cell, thereby allowing for previously unobserved gene interactions, and ultimately protein interactions, to become more evident. Study of fresh and archival human tissue and the development of transgenic animal models that recapitulate pertinent pathologic aspects of human disease (e.g., neurodegenerative disorders and hematological/oncological disorders) will help answer questions regarding the mechanisms leading to disease pathogenesis. Critical to the success of these paradigms will be the development of relational databases and repositories for single-cell data, similar to platform sharing access that is available currently for microarray users.

Acknowledgements

We thank Dr. Karolina Palucka for helpful discussions and critical comments on the manuscript. We thank Ms. Faith Brzostoski, Ms. Irina Elarova, and Mr. Marc D. Ruben for expert technical assistance. Support for this project comes from the Histiocytosis Association of America (KDM), NINDS (NS43939; SDG), NIA (AG10668, AG14449; SDG), NCI (CA94520; SDG), and Alzheimer's Association (SDG). We also express our appreciation to the families of the patients studied here who made this research possible.

References

Aittokallio T, Kurki M, Nevalainen O, Nikula T, West A, Lahesmaa R (2003) Computational strategies for analyzing data in gene expression microarray experiments. J Bioinform Comput Biol 1: 541–586

Arenzana-Seisdedos F, Barbey S, Virelizier JL, Kornprobst M, Nezelof C (1986) Histiocytosis X. Purified (T6+) cells from bone granuloma produce interleukin 1 and prostaglandin E2 in culture. J Clin Invest 77: 326–329

Blumberg DD (1987) Creating a ribonuclease-free environment. Methods Enzymol 152: 20–24

Bonner RF Emmert-Buck M, Cole K, Pohida T, Chuaqui R, Goldstein S, Liotta LA (1997) Laser capture microdissection: molecular analysis of tissue. Science 278: 1481–1483

Brown PO, Botstein D (1999) Exploring the new world of the genome with DNA microarrays. Nat Genet 21 [Suppl]: 33–37

Carstensen H, Ornvold K (1993) Langerhans-cell histiocytosis (histiocytosis X) in children. Ugeskr Laeger 155: 1779–1783

Che S, Ginsberg SD (2004a) Amplification of transcripts using terminal continuation. Lab Invest 84: 131–137

Che S, Ginsberg SD (2004b) RNA amplification methodologies. In: Progress in RNA research. Nova Science Publishing (in press)

Craven RA, Totty N, Harnden P, Selby PJ, Banks RE (2002) Laser capture microdissection and two-dimensional polyacrylamide gel electrophoresis: evaluation of tissue preparation and sample limitations. Am J Pathol 160: 815–822

Dafforn A, Chen P, Deng G, Herrler M, Iglehart D, Koritala S, Lato S, Pillarisetty S, Purohit R, Wang M, et al. (2004) Linear mRNA amplification from as little as 5 ng total RNA for global gene expression analysis. Biotechniques 37: 854–857

Dolter KE, Braman JC (2001) Small-sample total RNA purification: laser capture microdissection and cultured cell applications. Biotechniques 30: 1358–1361

Eberwine J, Kacharmina JE, Andrews C, Miyashiro K, McIntosh T, Becker K, Barrett T, Hinkle D, Dent G, Marciano P (2001) mRNA expression analysis of tissue sections and single cells. J Neurosci 21: 8310–8314

Eberwine J, Yeh H, Miyashiro K, Cao Y, Nair S, Finnell R, Zettel M, Coleman P (1992) Analysis of gene expression in single live neurons. Proc Natl Acad Sci USA 89: 3010–3014

Egeler RM, Favara BE, van Meurs M, Laman JD, Claassen E (1999) Differential *In situ* cytokine profiles of Langerhans-like cells and T cells in Langerhans cell histiocytosis: abundant expression of cytokines relevant to disease and treatment. Blood 94: 4195–4201

Ehrig T, Abdulkadir SA, Dintzis SM, Milbrandt J, Watson MA (2001) Quantitative amplification of genomic DNA from histological tissue sections after staining with nuclear dyes and laser capture microdissection. J Mol Diagn 3: 22–25

Eisen MB, Brown PO (1999) DNA arrays for analysis of gene expression. Methods Enzymol 303: 179–205

Emmert-Buck MR, Bonner RF, Smith PD, Chuaqui RF, Zhuang Z, Goldstein SR, Weiss RA, Liotta LA (1996) Laser capture microdissection. Science 274: 998–1001

Farrell RE Jr (1998) RNA Methodologies, 2nd edn. Academic Press, San Diego

Fasulo WH, Hemby SE (2003) Time-dependent changes in gene expression profiles of midbrain dopamine neurons following haloperidol administration. J Neurochem 87: 205–219

Favara BE, Jaffe R, Egeler RM (2002) Macrophage activation and hemophagocytic syndrome in Langerhans cell histiocytosis: report of 30 cases. Pediatr Dev Pathol 5: 130–140

Fend F, Emmert-Buck MR, Chuaqui R, Cole K, Lee J, Liotta LA, Raffeld M (1999) Immuno-LCM: laser capture microdissection of immunostained frozen sections for mRNA analysis. Am J Pathol 154: 61–66

Florell SR, Coffin CM, Holden JA, Zimmermann JW, Gerwels JW, Summers BK, Jones DA, Leachman SA (2001) Preservation of RNA for functional genomic studies: a multidisciplinary tumor bank protocol. Mod Pathol 14: 116–128

Freeman WM, Hemby SE (2004) Proteomics for protein expression profiling in neuroscience. Neurochem Res 29: 1065–1081

Galvin JE, Ginsberg SD (2004) Expression profiling and pharmacotherapeutic development in the central nervous system. Alzheimer Dis Assoc Disord 18: 264–269

Geissmann F, Revy P, Regnault A, Lepelletier Y, Dy M, Brousse N, Amigorena S, Hermine O, Durandy A (1999) TGF-beta 1 prevents the noncognate maturation of human dendritic Langerhans cells. J Immunol 162: 4567–4575

Geissmann F, Lepelletier Y, Fraitag S, Valladeau J, Bodemer C, Debre M, Leborgne M, Saeland S, Brousse N (2001) Differentiation of Langerhans cells in Langerhans cell histiocytosis. Blood 97: 1241–1248

Ginsberg SD, Che S (2002) RNA amplification in brain tissues. Neurochem Res 27: 981–992

Ginsberg SD, Che S (2004) Combined histochemical staining, RNA amplification, regional, and single cell analysis within the hippocampus. Lab Invest 84: 952–962

Ginsberg SD, Che S (2005) Expression profile analysis within the human hippocampus: comparison of CA1 and CA3 pyramidal neurons. J Comp Neurol (in press)

Ginsberg SD, Crino PB, Lee VM-Y, Eberwine JH, Trojanowski JQ (1997) Sequestration of RNA in Alzheimer's disease neurofibrillary tangles and senile plaques. Ann Neurol 41: 200–209

Ginsberg SD, Galvin JE, Chiu T-S, Lee VM-Y, Masliah E, Trojanowski JQ (1998) RNA sequestration to pathological lesions of neurodegenerative disorders. Acta Neuropathol 96: 487–494

Ginsberg SD, Crino PB, Hemby SE, Weingarten JA, Lee VM-Y, Eberwine JH, Trojanowski JQ (1999) Predominance of neuronal mRNAs in individual Alzheimer's disease senile plaques. Ann Neurol 45: 174–181

Ginsberg SD, Hemby SE, Lee VM-Y, Eberwine JH, Trojanowski JQ (2000) Expression profile of transcripts in Alzheimer's disease tangle-bearing CA1 neurons. Ann Neurol 48: 77–87

Ginsberg SD, Elarova I, Ruben M, Tan F, Counts SE, Eberwine JH, Trojanowski JQ, Hemby SE, Mufson EJ, Che S (2004) Single cell gene expression analysis: implications for neurodegenerative and neuropsychiatric disorders. Neurochem Res 29: 1054–1065

Goldsworthy SM, Stockton PS, Trempus CS, Foley JF, Maronpot RR (1999) Effects of fixation on RNA extraction and amplification from laser capture microdissected tissue. Mol Carcinog 25: 86–91

Gottwald E, Muller O, Polten A (2001) Semiquantitative reverse transcription-polymerase chain reaction with the Agilent 2100 Bioanalyzer. Electrophoresis 22: 4016–4022

Grotzer MA, Patti R, Geoerger B, Eggert A, Chou TT, Phillips PC (2000) Biological stability of RNA isolated from RNAlater-treated brain tumor and neuroblastoma xenografts. Med Pediatr Oncol 34: 438–442

Hemby SE, Ginsberg SD, Brunk B, Arnold SE, Trojanowski JQ, Eberwine JH (2002) Gene expression profile for schizophrenia: discrete neuron transcription patterns in the entorhinal cortex. Arch Gen Psychiat 59: 631–640

Hemby SE, Trojanowski JQ, Ginsberg SD (2003) Neuron-specific age-related decreases in dopamine receptor subtype mRNAs. J Comp Neurol 456: 176–183

Heyer LJ, Kruglyak S, Yooseph S (1999) Exploring expression data: identification and analysis of coexpressed genes. Genome Res 9: 1106–1115

Iscove NN, Barbara M, Gu M, Gibson M, Modi C, Winegarden N (2002) Representation is faithfully preserved in global cDNA amplified exponentially from sub-picogram quantities of mRNA. Nat Biotechnol 20: 940–943

Islinger RB, Kuklo TR, Owens BD, Horan PJ, Choma TJ, Murphey MD, Temple HT (2000) Langerhans' cell histiocytosis in patients older than 21 years. Clin Orthop 379: 231–235

Kacharmina JE, Crino PB, Eberwine J (1999) Preparation of cDNA from single cells and subcellular regions. Methods Enzymol 303: 3–18

Klimecki WT, Futscher BW, Dalton WS (1994) Effects of ethanol and paraformaldehyde on RNA yield and quality. Biotechniques 16: 1021–1023

Klur S, Toy K, Williams MP, Certa U (2004) Evaluation of procedures for amplification of small-size samples for hybridization on microarrays. Genomics 83: 508–517

Kotlyar M, Fuhrman S, Ableson A, Somogyi R (2002) Spearman correlation identifies statistically significant gene expression clusters in spinal cord development and injury. Neurochem Res 27: 1133–1140

Lehmann U, Bock O, Glockner S, Kreipe H (2000) Quantitative molecular analysis of laser-microdissected paraffin-embedded human tissues. Pathobiology 68: 202–208

Lewis F, Maughan NJ, Smith V, Hillan K, Quirke P (2001) Unlocking the archive-gene expression in paraffin-embedded tissue. J Pathol 195: 66–71

Lockhart DJ, Barlow C (2001) Expressing what's on your mind: DNA arrays and the brain. Nat Rev Neurosci 2: 63–68

Lockhart DJ, Dong H, Byrne MC, Follettie MT, Gallo MV, Chee MS, Mittmann M, Wang C, Kobayashi M, Horton H, Brown EL (1996) Expression monitoring by hybridization to high-density oligonucleotide arrays. Nat Biotechnol 14: 1675–1680

McClain K, Hutter J, Cassady J (1994) Langerhan's cell histiocytosis. In: Cassady J (ed) Radiation therapy in pediatric oncology. Springer, New York, pp 337–350

Minkov M, Grois N, Heitger A, Potschger U, Westermeier T, Gadner H (2002) Response to initial treatment of multisystem Langerhans cell histiocytosis: an important prognostic indicator. Med Pediatr Oncol 39: 581–585

Mouledous L, Hunt S, Harcourt R, Harry JL, Williams KL, Gutstein HB (2003) Proteomic analysis of immunostained, laser-capture microdissected brain samples. Electrophoresis 24: 296–302

Mufson EJ, Counts SE, Ginsberg SD (2002) Single cell gene expression profiles of nucleus basalis cholinergic neurons in Alzheimer's disease. Neurochem Res 27: 1035–1048

Murakami I, Gogusev J, Fournet JC, Glorion C, Jaubert F (2002) Detection of molecular cytogenetic aberrations in Langerhans cell histiocytosis of bone. Hum Pathol 33: 555–560

Palucka KA, Taquet N, Sanchez-Chapuis F, Gluckman JC (1998) Dendritic cells as the terminal stage of monocyte differentiation. J Immunol 160: 4587–4595

Peterson LE (2002) CLUSFAVOR 5.0: hierarchical cluster and principal-component analysis of microarray-based transcriptional profiles. Genome Biol 3, SOFTWARE0002

Pinto A, Sarnat HB, Vogler C, Trevenen CL, Grant LH (1990) Acridine orange-RNA histofluorescence of sarcomas and small round cell tumors of childhood. Arch Pathol Lab Med 114: 585–588

Rieser C, Ramoner R, Bock G, Deo YM, Holtl L, Bartsch G, Thurnher M (1998) Human monocyte-derived dendritic cells produce macrophage colony-stimulating factor: enhancement of c-fms expression by interleukin-10. Eur J Immunol 28: 2283–2288

Sarnat HB, Curry B, Rewcastle NB, Trevenen CL (1986) Cytoplasmic RNA in nervous system tumors in children: a fluorochromic histochemical study using acridine orange. Can J Neurol Sci 13: 31–41

Simone NL, Remaley AT, Charboneau L, Petricoin EF 3rd, Glickman JW, Emmert-Buck MR, Fleisher TA, Liotta LA (2000) Sensitive immunoassay of tissue cell proteins procured by laser capture microdissection. Am J Pathol 156: 445–452

Su JM, Perlaky L, Li XN, Leung HC, Antalffy B, Armstrong D, Lau CC (2004) Comparison of ethanol versus formalin fixation on preservation of histology and RNA in laser capture microdissected brain tissues. Brain Pathol 14: 175–182

Suarez-Quian CA, Goldstein SR, Pohida T, Smith PD, Peterson JI, Wellner E, Ghany M, Bonner RF (1999) Laser capture microdissection of single cells from complex tissues. Biotechniques 26: 328–335

Tang WX, Fasulo WH, Mash DC, Hemby SE (2003) Molecular profiling of midbrain dopamine regions in cocaine overdose victims. J Neurochem 85: 911–924

Tecott LH, Barchas JD, Eberwine JH (1988) *In situ* transcription: specific synthesis of complementary DNA in fixed tissue sections. Science 240: 1661–1664

To MD, Done SJ, Redston M, Andrulis IL (1998) Analysis of mRNA from microdissected frozen tissue sections without RNA isolation. Am J Pathol 153: 47–51

Topaloglu H, Sarnat HB (1989) Acridine orange-RNA fluorescence maturing neurons in the perinatal rat brain. Anat Rec 224: 88–93

Van Deerlin VMD, Ginsberg SD, Lee VM-Y, Trojanowski JQ (2000) Fixed post mortem brain tissue for mRNA expression analysis in neurodegenerative diseases. In: Geschwind DH (ed) DNA microarrays: the new frontier in gene discovery and gene expression analysis. Society for Neuroscience Washington, DC, pp 118–128

Van Deerlin VMD, Ginsberg SD, Lee VM-Y, Trojanowski JQ (2002) The use of fixed human post mortem brain tissue to study mRNA expression in neurodegenerative diseases: applications of microdissection and mRNA amplification. In: Geschwind DH, Gregg JP (eds) Microarrays for the neurosciences: an essential guide. MIT Press Boston, pp 201–235

Van Gelder R, von Zastrow M, Yool A, Dement W, Barchas J, Eberwine J (1990) Amplified RNA (aRNA) synthesized from limited quantities of heterogeneous cDNA. Proc Natl Acad Sci USA 87: 1663–1667

Vasilyeva E, Woodard J, Taylor FR, Kretschmer M, Fajardo H, Lyubarskaya Y, Kobayashi K, Dingley A, Mhatre R (2004) Development of a chip-based capillary gel electrophoresis method for quantification of a half-antibody in immunoglobulin G4 samples. Electrophoresis 25: 3890–3896

Wang E, Miller LD, Ohnmacht GA, Liu ET, Marincola FM (2000) High-fidelity mRNA amplification for gene profiling. Nat Biotechnol 18: 457–459

Yokota K, Ariizumi K, Kitajima T, Bergstresser PR, Street NE, Takashima A (1996) Cytokine-mediated communication between dendritic epidermal T cells and Langerhans cells. *In vitro* studies using cell lines. J Immunol 157: 1529–1537

Authors' address: Stephen D. Ginsberg, Ph.D., Center for Dementia Research, Nathan Kline Institute, New York University School of Medicine, 140 Old Orangeburg Road, Orangeburg, NY 10962, U.S.A.,
Fax: 845-398-5422, E-mail: ginsberg@nki.rfmh.org