

# Expression profiling of single cells using 3 prime end amplification (TPEA) PCR

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## ABSTRACT

**The ability to relate the physiological status of individual cells to the complement of genes they express is limited by current methodological approaches for performing these analyses. We report here the development of a robust and reproducible method for amplifying 3' sequences of mRNA derived from single cells and demonstrate that the amplified cDNA, derived from individual human lymphoblastoma cells, can be used for the expression profiling of up to 40 different genes per cell. In addition, we show that 3 prime end amplification (TPEA) PCR can be used to enable the detection of both high and low abundance mRNA species in samples harvested from live neurons in rat brain slices. This procedure will facilitate the study of complex tissue function at the cellular level.**

## INTRODUCTION

DNA sequence generated by genome and expressed sequence tag (EST) sequencing projects will provide the basis to further our understanding of the control and mode of action of individual gene products. In this respect, analysis and comparison of when, where and to what degree genes are expressed, commonly known as expression profiling, is certain to play an essential role in the functional characterization of newly identified genes (1,2).

Many tissues, such as the immune and nervous systems, are composed of highly heterogeneous cell populations. A key factor in understanding their physiology, and the role of specific gene products expressed within them, will be the ability to examine gene usage in the context of this cellular diversity. In the past, methods such as northern blotting and nuclease protection assays were employed to study gene expression. More recently, a number of powerful new technologies have been developed for assessing simultaneously the expression of large numbers of genes (3,4). All these techniques, however, require relatively large amounts of RNA and currently lack the sensitivity to

analyse specimens derived from small populations of cells or indeed from an individual cell.

At present, methodologies for the analysis of gene expression within single cells are limiting. Whilst *in situ* hybridization provides detailed information on a gene's cellular expression pattern in intact tissue, be it whole-mounts or tissue sections, the technique is relatively laborious to perform and is incapable of analysing more than a very small number of transcripts in a single preparation. In recent years, the polymerase chain reaction (PCR) has been used successfully to investigate gene expression in cytoplasmic samples derived from single cells (5). The nested-primer approach has good sensitivity, but the analysis is restricted to a small number of closely related genes from specific gene families (6). Other techniques capable of detecting the expression of unrelated genes in a single cell include T7 RNA polymerase amplification of mRNA (7,8), and PCR after prior homopolymeric tailing of the first strand cDNA (9–11). However, neither of these approaches have been demonstrated to be capable of analysing more than a small number of genes and are also not widely used. The former is technically difficult, whilst the latter may be biased against long transcripts and often requires subsequent cloning of the amplified products. There exists therefore a great need for improved methodologies that can quickly, easily and sensitively analyse gene expression at the cellular level.

Here we report the development of an approach, called 3 prime end amplification (TPEA) PCR, for the analysis of gene expression in small samples or single cells, which overcomes many of the technical limitations of existing methodologies. In this method, the 3' region of mRNA is amplified arbitrarily by PCR using a novel combination of primers. The amplified cDNA, which represents the most diverse region of gene sequence, can then be analysed by a second round of PCR using gene-specific primers. We demonstrate that it is possible to analyse the expression of up to 40 genes (20 in duplicate) in single human lymphoblastoma cells. We also show that this method is ideally suited to the analysis of genes expressed at low levels in small populations of cells, by demonstrating the expression of the adenosine A<sub>2a</sub> receptor in cholinergic neurons of the rat striatum.

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## MATERIALS AND METHODS

### Lymphoblastoma cell sorting

An Epstein Barr virus transformed lymphoblastoid cell line (HRC575, ECACC, Porton Down, UK) was maintained in log phase growth in RPMI 1640 medium supplemented with 16% foetal calf serum, 2 mM L-glutamine and penicillin–streptomycin (100 U/ml and 100 mg/ml, respectively). Cells at  $\sim 10^6$ /ml were stained with the bisbenzimidazole dye Hoechst 33342 (Sigma, Poole, UK) at 1  $\mu$ g/ml for 30 min at 37°C. Cells were sorted by using the Autoclone attachment of a Coulter Elite ESP flow cytometer, 300 mW of all lines UV from a Coherent 306 laser and by using single drop and complete abort sorting settings. Time of flight, forward and right angle scatter and Hoechst fluorescence peak and area measurements were used to ensure the sorting of single cells. The accuracy of sorting (both spatial and numerical) was tested by sorting single fluorescent beads (DNA Check, Coulter Corp.) into 96-well plates and viewing the plates on a fluorescence microscope.

### Reverse transcription and cDNA amplification

Lymphoblastoma cells were sorted into 96-well plates containing 7  $\mu$ l of freshly prepared lysis buffer [50 mM Tris–HCl (pH 8.3), 75 mM KCl, 3 mM MgCl<sub>2</sub>, 5 mM NP-40 (Sigma) and 1.5 U of RNase inhibitor (Pharmacia, Milton Keynes, UK)]. This buffer leaves the nucleus intact (11). After 5 min on ice, nuclei were removed by centrifugation (8000 g, 5 min at 4°C), the supernatant aspirated and the RNA reverse transcribed in a reaction volume of 10  $\mu$ l containing; 1  $\times$  first-strand buffer, 200 U M-MLV reverse transcriptase (Gibco BRL, Paisley, UK), 0.5 ng reverse transcription primer for 60 min at 37°C. The RT primer was composed of an anchored oligo(dT) primer with a specific 5' heel sequence: CTC TCA AGG ATC TTA CCG CTT TTT TTT TTT TTT (A,G,C).

Second-strand cDNA synthesis was initiated by incubation of the first-strand cDNA with 1 ng of a primer consisting of (5' to 3') a 20 base sequence selected due to its absence from the mammalian databases, a stretch of five random nucleotides and a defined pentameric sequence (CTG CAT CTA TCT AAT GCT CCNN NNN CGAGA where N represents C, G, T or A) for 15 min at 50°C under amplification conditions described below. Although this primer will undoubtedly prime second-strand DNA synthesis at many sites on the first strand cDNA, the subsequent PCR between the heel sequence of the oligo(dT) primer and the arbitrary primer closest to the 5' end, ensures amplification of cDNA sequences complementary to the 3' ends of the polyA tail. After allowing the second-strand primer to anneal, primer extension was performed at 72°C for 10 min using AmpliTaq DNA polymerase (0.35 U, Applied Biosystems, Warrington, UK) in PCR buffer containing 67 mM Tris–HCl (pH 8.3), 4.5 mM MgCl<sub>2</sub> and 0.5 mM dNTPs. Subsequently, 0.4 ng of 3' heel primer (CTC TCA AGG ATC TTA CCG C) was added and the reaction subjected to 10 cycles of 92°C for 2.5 min, 60°C for 1.5 min and 72°C extension for 1 min, followed by a final 10 min extension. A further 125 ng of second-strand primer and 50 ng of 3' heel primer were then added in 10  $\mu$ l of PCR reaction mix. After 15 cycles (as before), a further 10  $\mu$ l of PCR reaction mix containing 125 ng second-strand primer and 50 ng of 3' heel primer were added to the reaction and subjected to another 15 rounds of PCR.

The final reaction mixture was then diluted to 200  $\mu$ l with 10 mM Tris–0.1 mM EDTA (pH 8.1). Five microlitre samples were used for subsequent gene-specific PCR assays.

### Gene-specific PCR

Samples (5  $\mu$ l) of amplified cDNA were subjected to hot-lid PCR carried out in 1  $\times$  PCR buffer (3.5 mM MgCl<sub>2</sub>, pH 8.8) containing 12.5% sucrose, 0.1 mM cresol red, 12 mM  $\beta$ -mercaptoethanol, 0.5 mM dNTPs (Pharmacia), 0.6 U AmpliTaq DNA polymerase (Applied Biosystems), and primers at 100 ng/reaction. Amplifications were carried out on PTC-225 thermal cyclers (Tetrad, MJ Research, MA, US). Following an initial 2 min denaturing step (92°C), each PCR cycle consisted of 30 s denaturing (92°C), 90 s annealing (55°C) and 60 s elongation (72°C). After the final cycle the reaction was held for 10 min at 72°C. The PCR products were then separated on a 2.5% agarose gel, stained with ethidium bromide and photographed. All gene-specific primers are listed in Table 1.

### Extraction of neuronal contents

Coronal slices (300  $\mu$ m) from 14–28 day-old male Sprague–Dawley rats containing the striatum were viewed with a Zeiss Axioskop microscope (Carl Zeiss Ltd, Welwyn Garden City, UK) fitted with a  $\times 64$  water-immersion objective lens together with gradient contrast optics (Luigs and Neumann, Ratingen, Germany). Light in the infrared range ( $>740$  nm) was used in conjunction with a contrast-enhancing Newvicon camera (Hamamatsu, Hamamatsu City, Japan) to resolve individual neurones within slices (27). The cytoplasm from large cells ( $>30$   $\mu$ m in one dimension) was gently aspirated under visual control into a patch-clamp recording electrode until at least 40% of the somatic cytoplasm had been collected. The electrode was then withdrawn from the cell to form an outside-out patch which prevented contamination when the electrode was subsequently withdrawn from the slice. The contents of the electrode were forced into a microtube and reverse transcribed, subjected to 3' cDNA amplification, and 2.5% of the product used in each gene specific PCR reaction.

## RESULTS

### TPEA-PCR

Assay development was performed on lymphoblastoma cells in the G<sub>0</sub>/G<sub>1</sub> phase of the cell cycle. Groups of 100, 10 and single cells were flow sorted into wells containing lysis buffer and the mRNA reverse transcribed. A proportion of the sorted cells then underwent 3' end amplification, as described in Materials and Methods (Fig. 1). Gene-specific PCR assays for eight 'housekeeping' genes were carried out on lymphoblastoma cDNA, before and after cDNA amplification. Following reverse transcription only, the expression of each of the housekeeping genes could be detected when cDNA generated from between one and 100 cells was used in each PCR assay (Fig. 2). It was not possible, however, to detect the expression of any of these genes when cDNA equivalent to less than one cell was assayed. Following TPEA-PCR, however, the expression of all eight of the genes was detectable even when amplified cDNA generated from the equivalent of as little as 5% of one cell was present in the PCR assay (Fig. 2).

**Table 1.** Primer sequences used for gene-specific assays

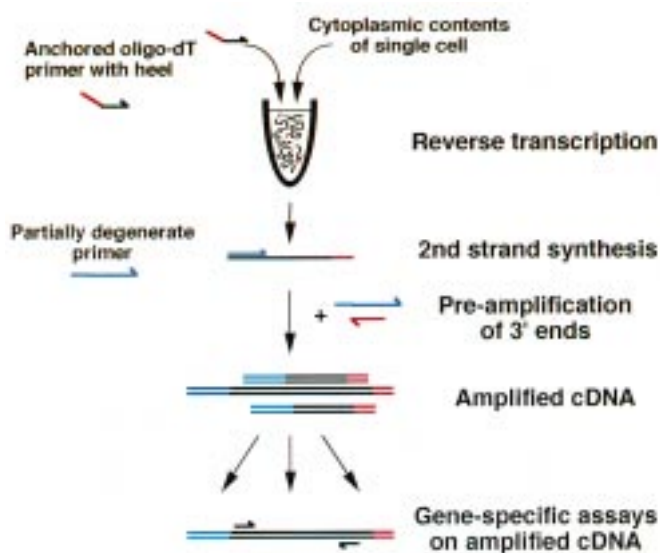
Human primers				
Gene	Symbol	Acc. No.	Forward	Reverse
Riboprotein L5	RPL5	U14966	GAACAGCGTAACTCCAGACATG	CCGCTCCTGAGCTCTGAG
Riboprotein L21	RPL21	U14967	GTGCGTATTGAGCACATAAAGC	TTCCAGCAGCTCAGGCTC
Riboprotein L27a	RPL27a	U14968	GACAAATTGTGGACTTTGGTCA	ATGTGCTTCAAGCCACCAG
Riboprotein L28	RPL28	U14969	ATCAACAAGAATGCTCGCG	TGACTTTATTGCTCTGGGGG
Riboprotein S5	RPS5	U14970	GTGAACCCAGGCCATCTGG	CCAAAGGGCAGACAGGTTTA
Riboprotein S9	RPS9	U14971	CATTGTCCGCCTGGATTG	TGTTTATTTGGCAGGAAAACG
Riboprotein S10	RPS10	U14972	GGAGATTGTGCTGCCAC	AAACTGGAATTCGGTTGCTG
Riboprotein S29	RPS29	U14973	ATTCGGCCAGGGTTCTC	TTTTTCATTGAGTAGATGCCCC
Housekeeping protein Q27	HSKPQ27	M81806	GGAACCTCCTCTGGGAACCT	GGAGGTCAAGTCAAGCTCCA
Glyceraldehyde-3-phosphate dehydrogenase	G-3-PDH	M33197	CGACCACCTTTGTCAAGCTCA	AGGGGTCTACATGGCAACTG
Cytoplasmic beta-actin	ACTB	AB004047	CGTGGACATCCGTAAGACC	ACATCTGCTGGAAGGTGGAC
JUND	JUND	X56681	CTCGGGGAACAAACGTTG	ACAACAGGAATGTGGACTCG
Elongation factor 1	EF1	M82882	CCAAGAAAGCAACTTGAGCC	TGCAGAAGACATTTTTTCTTG
Cell division cycle 25B	CDC25B	M81934	AGTGCCTTGCATACCCAAAC	CCCCCATCTTGAGATTGAGA
CD19	CD19	X13312	CCAACCTCTGGAGCAATGTT	GGAATACAAAGGGGACTGGA
CD79A	CD79A	L32754	CTTCTGGGGGCTTCTTAGT	GTTAGGAGGTGGGGCAGTTT 3
Immunoglobulin M	IGM	X17115	GTCCCCGGAGAAGTATGTGA	GACTTGTCCACGGTCTCTC
CD2	CD2	M16445	TCTTGAACCTCAGCCATGTG	GGCTGCTTAGTGAGACCC
Sucrase-isomaltase	SI	X63597	CAGGGTCTCTGTTTTGGGA	GGAAGCGAATTTTTATTCCG
INTRON	-	[see ref 18]	GTGGATATAGTTGAGTCC	CACTGTTGACCACTCCAT
Rat Primers				
Gene	Symbol	Acc. No.	Forward	Reverse
Beta-actin	<i>Actb</i>	V01217	CATCCATGCCCTGAGTCC	ACACCTCAAACCACTCCAG
Alpha-tubulin	<i>Tuba</i>	V01226	CACTGGTACGTGGGTGAGG	TTTGACATGATACAGGGACTGC
Cyclophilin	<i>Cyca</i>	M19533	ACTGCCAAGACTGAGTGGCT	AATGGTTTGTAGGGTAAATGC
Ribosomal protein L18	<i>Rpl18</i>	M20156	AGGTGTACCAGACATTTGGC	TTTTGTAGCTCGGCTGG
Choline acetyltransferase	<i>Chat</i>	[see ref 28]	TACTAAGCTCTGTTCCATCCC	ACCCAGTTGCTTCCAAAC
Glutamic acid decarboxylase	<i>Gad67</i>	X57573	ATCTTGCTTCAGTAGCCTTTGC	TGCTTCAAACACATTTGTGGG
Neurokinin receptor 1	<i>Nk1</i>	J05097	CTGGAAGAGAGGAGCCTTGTG	CTGAGACGGAAGGAACAGC
Neurokinin receptor 2	<i>Nk2</i>	M31838	TTCTGCAGTGAGGAGCTGG	TTGGCTTTCAGAGGGCAC
Neurokinin receptor 3	<i>Nk3</i>	J05189	TTTGTGACCGCAGAAGAGC	TTGTGTGTGGGAAGTCAAGTCA
Adenosine receptor 2a	<i>Adora2a</i>	M91214	TCTGACCAACAAAGCTGGC	TGGAAGGAAAGGCAGTAGTCA

### Reproducibility and specificity of 3' amplification of cDNA from single cells

The reproducibility of this technique was assessed when the expression of 20 genes was examined in duplicate on single lymphoblastoma cells. Duplicate PCR assays revealed almost identical expression profiles both between assays on a single cell and between different cells (Fig. 3). The expression of 11 housekeeping genes was reproducible in all of these single cell expression profiles, demonstrating consistent amplification of cDNA from the transcripts of these genes. However four genes (JUND, EF1, CDC25B and CD19) were not found to be expressed in every cell tested. Two gene-specific assays, sucrase-isomaltase and CD2, were included as negative controls as they were not expected to be expressed in these cells since their mRNAs have only previously been observed in the gastrointestinal tract (12) and in populations of T-cells (13), respectively. Another set of primers, designed to amplify an intronic sequence from a gene found in the Xq 2.5 region (R.Brooksbank and A.Coffey, personal communication), were used to detect genomic contamination. All three sets of primers have been demonstrated to provide a sensitive assay for their cDNA and genomic DNA targets, respectively (data not shown). Since these assays were negative in all the cells examined ( $n = 20$ ), and expression of any gene was not detected without prior reverse transcription, false positives arising from amplification of genes not expressed in these cells (SI and CD2) or genomic contamination, do not appear to complicate interpretation of results.

### Analysis of complex cell systems: expression profiling of neurons

Having shown that TPEA-PCR permits expression profiling of single cells, we wished to apply it to the analysis of a complex cell system *in vivo*. Striatal cholinergic interneurons are readily identifiable in rat brain slices due to their large size (>30  $\mu\text{m}$  diameter) when compared to surrounding cell types, which are predominately medium spiny neurons (<15  $\mu\text{m}$  diameter). After electrophysical characterisation of the cells (14), cytoplasmic samples were taken using a patch pipette, reverse transcribed and the cDNA amplified. The expression of a variety of genes of interest was then investigated (two representative expression profiles of cholinergic interneurons are shown in Fig. 4). The expression of four known housekeeping genes was demonstrated, confirming the integrity of sample collection and of the RNA. In addition, the expression of choline acetyltransferase (the acetylcholine synthesising enzyme) was observed in each, thus unequivocally confirming the cholinergic phenotype of the sampled neurons. In order to control against the possibility of contamination by the surrounding population of medium spiny neurons, which are known to express the adenosine  $A_{2a}$  receptor (15), we assayed the samples for the presence of the mRNA of glutamic acid decarboxylase (*Gad67*), which is highly expressed in medium spiny, but not cholinergic neurons. The *Gad67* primers amplify from medium spiny neurons (data not shown), and the absence of *Gad67* expression in cholinergic interneurons demonstrates that samples had not been contaminated. All neurons tested for the expression of the neurokinin (NK) receptors



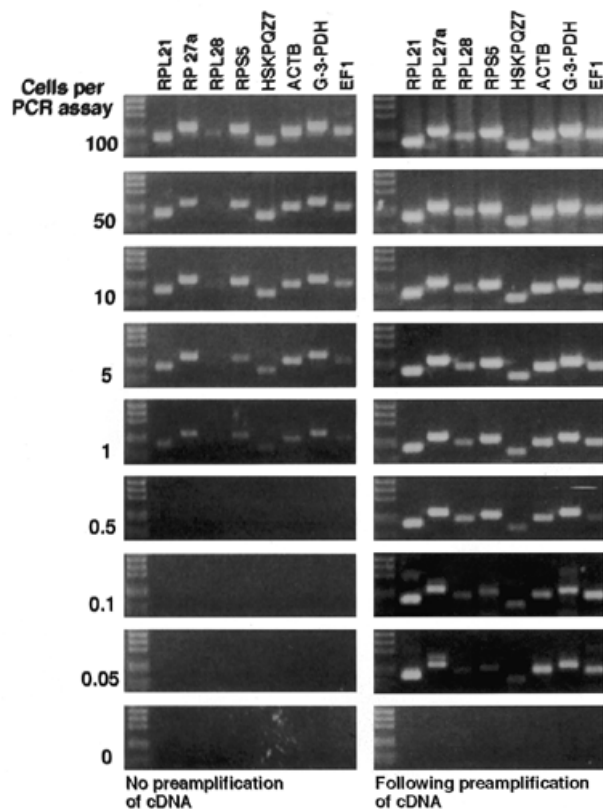
**Figure 1.** Outline of TPEA-PCR. Details of the protocol are given in Materials and Methods, as are the sequences of the anchored oligo(dT) primer and the partially degenerate second strand primer.

were negative for NK2 and NK3 receptor mRNA, but positive for that of the NK1 receptor. 27% (7 out of 26) of the cholinergic interneurons tested expressed the adenosine A<sub>2a</sub> receptor.

## DISCUSSION

Current methodologies for analysing gene expression in small samples of RNA, particularly from single cells, are severely limited in terms of the number and diversity of genes that it is possible to analyse, and the difficulty of experimental procedures. The methodology outlined here overcomes many of these limitations.

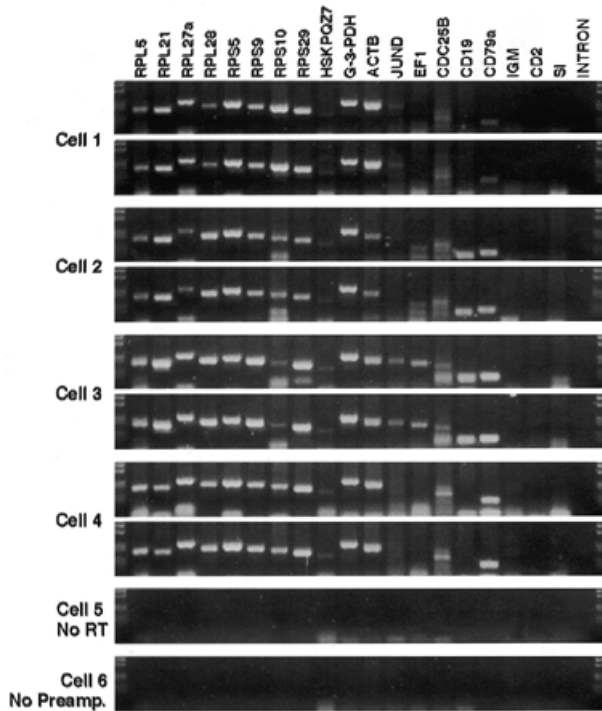
Sequence diversity between genes is at its greatest in the 3' untranslated region (16) and this region provides the most unique target for gene-specific assays; this is especially important when wishing to differentiate between closely related members of a gene family. Our aim in designing this procedure has therefore been to amplify preferentially this portion of the mRNA sequences. cDNA synthesis by reverse transcriptase is initiated by an anchored oligo-dT priming so that the 3' region of all genes is represented in the resulting single-stranded cDNA. A 5'-specific heel is incorporated into this primer for use in the subsequent amplification procedure. For second-strand synthesis, it was seen as desirable that ~1 kb from the 3' end of each gene could be selected and amplified by PCR. Assuming a completely random length of nucleotide sequence, it would be expected that a given five base sequence would appear every 1024 bases (4<sup>5</sup>), even though some nucleotide sequences are more common than others (17). A pentameric sequence was selected that would enable the primer to initiate second-strand synthesis in an arbitrary manner within 1 kb of the 3' end of the mRNA. A search of 30 gene sequences revealed that at least one copy of this five base sequence was present in this region of each gene. 5' to this, five bases of random sequence (N<sub>5</sub>) were incorporated in order to stabilize the interaction of the arbitrary pentameric sequence, which in turn was flanked by a specific heel sequence. A similar



**Figure 2.** Demonstration of cDNA amplification. cDNA was prepared from varying numbers of sorted lymphoblastoma cells, and analysed by RT-PCR for the expression of eight 'housekeeping' genes, before (left) and after (right) TPEA-PCR. After cDNA amplification, expression of all eight genes assessed could be detected after carrying out gene-specific PCR on only 5% of the amplified cDNA generated from a single cell. Without cDNA amplification, however, expression of none of the genes assayed could be detected at this level. Genes assayed: RPL21 (riboprotein L21), RP27a (riboprotein 27a), RPL28 (riboprotein L28), RPS5 (riboprotein S5), HSKPQZ7 (housekeeping protein), ACTB (Cytoplasmic beta-actin), G-3-PDH (Glyceraldehyde-3-phosphate dehydrogenase) and EF1 (Elongation factor 1).

primer design strategy has been demonstrated to be useful for DOP-PCR primers (18). After a single round of second-strand synthesis, each cDNA contains a specific priming site 5' and 3' to the region of interest, thus allowing amplification of the intervening sequence. At present, the exact representation of each mRNA species in the amplified cDNA relative to the starting material is unclear. As far as we have been able to ascertain, however, the majority of the mRNA species represented in the first-strand cDNA pool before amplification, as detected by conventional RT-PCR, were also detectable after amplification.

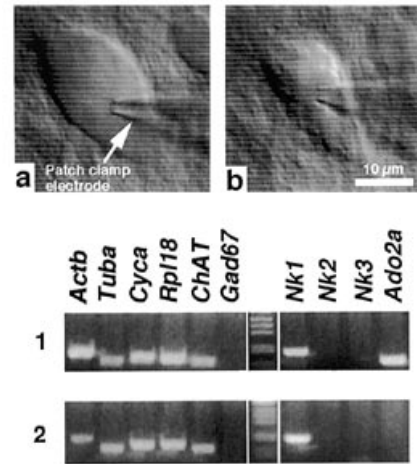
Problems that can be inherent in the detection of gene expression at the single cell level include the possibility of contamination by other cell types and the interpretation of negative results, which could be due to cell heterogeneity or an artefact of the detection system. In order to minimize these concerns, we chose to develop the system on sorted lymphoblastoma cells. These cells might be expected to be relatively homogeneous, so allowing an assessment of assay reproducibility by comparison of expression profiles between different cells and would also



**Figure 3.** Multiple gene expression analysis in single lymphoblastoma cells. Four cells (1–4) were lysed, reverse transcribed, the cDNA amplified and gene-specific PCR performed on the product. CD2, SI and intron primer pairs serve as negative controls to check for genomic contamination of the samples. Eleven of the other genes are expressed in all four cells in duplicate, while JUND, EF1, CDC25B and CD19 expression was not consistent between cells. Genes assayed: RPL5 (riboprotein L5), RPL21 (riboprotein L21), RPL27a (riboprotein 27a), RPL28 (riboprotein L28), RPS5 (riboprotein S5), RPS9 (riboprotein S9), RPS10 (riboprotein S10), RPS29 (riboprotein S29), HSKPQZ7 (Housekeeping protein), ACTB (Cytoplasmic beta-Actin), G-3-PDH (Glyceraldehyde-3-phosphate dehydrogenase), EF1 (Elongation factor 1), JUND (JUND), CDC25B (cell cycle factor CDC25b) and the cell surface antigens CD19, CD79a, CD2, IGM (immunoglobulin IgM), SI (the intestine-specific enzyme, sucrase-isomaltase).

avoid contamination by other cell types. The use of sorted cells also provides a ready source of single cell material.

Figure 3 clearly demonstrates the reproducibility of the gene-specific assays following amplification from single cells, with each of 11 housekeeping gene assays being detectable in duplicate reactions on each of four lymphoblastoma cells. There was some apparent variability in the expression of four genes (JUND, EF1, CDC25B and CD19) studied in these cells. It is unclear at this stage, however, whether this represents experimental variability, fluctuations in transcriptional activity within these cells or 'real' consistent differences in the expression of these genes in cells that we can only assume to be homogeneous. Such variability in gene expression has been encountered in other cell groups thought to be homogeneous (19). This analysis also demonstrates that the procedure does not lead to false positives due to either over amplification (as no signal was detected for genes SI and CD2 known not to be expressed in these cells) or genomic contamination (as demonstrated by the lack of signal from intronic primers or from the gene-specific primers when the cell contents were not reverse transcribed prior to amplification).



**Figure 4.** Demonstration of adenosine 2a receptor expression in striatal cholinergic interneurons. (a) An infrared video image of a rat striatal cholinergic interneuron during electrophysiological characterization and (b) after aspiration of cytoplasm. The expression of four housekeeping genes, the transmitter synthesising enzymes choline acetyltransferase (found only in cholinergic neurons) and glutamic acid decarboxylase (found in GABAergic, medium spiny neurons), three tachykinin (NK) receptors and the adenosine  $A_{2a}$  receptor was assessed in 26 striatal cholinergic neurons. Two representative neurons are shown; 1 expresses the  $A_{2a}$  receptor; 2 does not, while the expression of the other genes tested are the same in both.

The power of this technique lies in its potential to facilitate expression profiling of cells derived from complex cell populations, even when they form only a small proportion of the population as a whole, and in its ability to detect low abundance transcripts. We therefore investigated the expression of the neurokinin (NK) receptors and the adenosine  $A_{2a}$  receptor in single striatal cholinergic interneurons which constitute a small fraction of the total cellular mass of the striatum. Of the neurokinin receptors, the NK1 receptor is widely accepted as being expressed in these cells (20), so the expression of this gene was examined as an example of an mRNA species expressed at far lower levels than housekeeping genes. In contrast there is considerable controversy as to whether the  $A_{2a}$  receptor is expressed in cholinergic interneurons (15,21–23), suggesting that the corresponding mRNA species may be present at low levels as suggested by one *in situ* hybridisation study (24) or not present at all, as suggested by other studies (25). Expression of the  $A_{2a}$  receptor in these cells has important implications for the mechanism of action of adenosine and because of the potential of the  $A_{2a}$  receptor to act as a target for novel drugs for the amelioration of Parkinson's Disease (22). Using a patch pipette it was possible to harvest upwards of an estimated 40% of the cellular contents of these neurons (Fig. 4a). This was then subjected to the cDNA amplification procedure, followed by gene-specific PCR assays. Testing for the expression of four housekeeping genes and the marker enzymes choline acetyltransferase and *Gad67*, was included in order to demonstrate the quality of collection and amplification, and to corroborate the cell lineage, respectively. NK1 receptor mRNA was detected in all the cholinergic neurons tested, although the NK2 and NK3 receptors were not, confirming that Substance P exerts its effects on these cells via the NK1 receptor (26). Expression of the adenosine  $A_{2a}$  receptor mRNA was detected in 27% of the cholinergic neurons assayed (Fig. 4b), a percentage close to that

observed previously (24). It is not clear whether the apparent heterogeneity in expression of this receptor is due to differences in the temporal expression of this gene (i.e. that all of these cells possess A<sub>2a</sub> receptor protein, but only 27% actually express the gene at any one time), or to an absolute difference in gene expression within the striatal cholinergic interneuron population.

An important advantage of TPEA-PCR is the relative ease of the methodology. Other protocols can be time-consuming and complex, involving DNA purification and precipitation from one step to another. The current cDNA amplification technique, however, can be carried out in a single tube with a need for only limited manual intervention. This therefore makes it possible to amplify large numbers of samples relatively easily. The ability to then analyse the expression of many genes of unrelated sequence, both at high and low abundance, in samples taken from as little as a single cell, will potentially allow it to be used in high throughput screening systems.

We have demonstrated that this approach can be used to analyse gene expression in samples from as little as a single cell (Fig. 4) to that contained in 100 cells (Fig. 2). Amplification from a single cell currently provides enough material for ~40 gene-specific PCR reactions. Whilst this is already an improvement over existing protocols, it should theoretically be possible to improve the efficiency of the TPEA reaction to provide far higher yields of 3' cDNA product. This would then not only allow the number of gene-specific PCR reactions performed on each sample to be increased, but more importantly could allow the procedure to be linked to other analysis procedures. With improvements to the amplification regime and addition of fluorescent or radioactive nucleotide label to the reaction, it should be possible in the future to analyse the product using array based hybridisation technologies (4) which currently require relatively large amounts of RNA for a single assay. Such developments would potentially allow the expression profiling of hundreds or thousands of genes in samples derived from biopsies or single cells.

In summary, this report describes a rapid, robust and reproducible procedure, called TPEA-PCR, capable of amplifying 3' fragments of cDNA prior to analysis by other techniques. We believe that this will greatly facilitate the analysis of gene expression in samples obtained from small samples of tissue or single cells. In so doing, it will allow the utilisation of the wealth of new sequence data now available, to further our understanding of disease processes and the cellular physiology of complex tissues.

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