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Single-Cell Analysis of Gene Expression in the Nervous System

Measurements at the Edge of Chaos

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Abstract

The characteristic functions of tissues and organs result from the integrated activity of individual cells. Nowhere is this more evident than in the nervous system, where the activities of single neurons communicating via electrical and chemical signals mediate complex functions, such as learning and memory. The past decade has seen an explosion in the identification of genes encoding proteins, such as voltage-gated channels and neurotransmitter receptors, responsible for neuronal excitability. These studies have highlighted the fact that even within a neuroanatomically defined region, the coexistence of multiple cell types makes it difficult, if not impossible, to correlate patterns of gene expression with function. The recent development of techniques sensitive enough to study gene expression at the single-cell level promises to break this bottleneck to our further understanding. Using examples taken from our own laboratories and the work of others, we review these techniques, their application, and discuss some of the difficulties associated with the interpretation of the data.

Index Entries: Expression profiling; single-cell RT-PCR; multiplex RT-PCR; gene expression; patch-clamp.

Introduction

Throughout the history of neuroscience, each step forward in our understanding of nervous tissue function has come through the application of new techniques whose resolution, in some manner of speaking, exceeded that of its predecessor. Whereas in the 19th century Ramon y Cajal (1909) was able to classify neurons based on their location and dendritic morphology, modern immunohistochemical techniques have established relationships between neurons and their cellular components that previously could only have been guessed at. Similarly, understanding of the ionic basis of the action potential in the early

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1950s was first made possible by the development of voltage-clamp technology (Hodgkin and Huxley, 1952). However, analysis of the biophysical properties of voltage-gated channels that underlie the action potential was at a relative impasse until the development of the "patch-clamp" in the late 1970s (Sakman and Neher, 1983).

The properties that distinguish neurons from other cells and from each other reflect specific differences in patterns of gene expression. One goal of modern neurobiology is to identify these differences and the mechanisms that trigger, coordinate, and maintain them. Using conventional molecular biology techniques, including the construction and screening of cDNA libraries, the polymerase chain reaction (PCR) to identify specific genes, together with northern blot, *in situ* hybridization, and related techniques to study their pattern of expression, this challenge has been met with great success. For example, we now know the anatomical localization of genes encoding receptor subunits for the primary chemical neurotransmitters in the brain. However, these studies have limited resolution and generally present us only with a picture of gene expression that represents an average across a population of cells; differences in gene expression that might distinguish different cell types or even individual variance between cells are often lost to these techniques. Here we discuss the use of new analytical tools, whose sensitivity permits analysis of the small quantities of mRNA present within a single-cell (<1 pg; Alberts et al., 1989), that provide exciting opportunities to understand how gene expression is coordinated in individual cells.

Expression Profiling

The use of patch-clamp techniques to study neuronal excitability has provided a natural bridge in developing methods to study gene expression in single-cells. In patch-clamp analysis, a high-resistance seal is formed between a glass recording pipet and the cell membrane. Subsequently, the membrane beneath the mouth of the pipet can be broken, establishing a low-resistance pathway between the interior of the cell and the recording pipet for analysis of whole-cell currents. This also provides a conduit through which the cell's cytoplasm can be harvested. Eberwine and his colleagues (Van Gelder et al., 1990; Eberwine et al., 1992), have taken advantage of this arrangement to develop a technique known as expression profiling that permits analysis of mRNAs present in a single-cell. In a typical experiment, a patch-clamp pipet is used to form a high-resistance seal with the surface membrane of a neuron and the whole-cell recording configuration established. In addition to the recording saline, the internal solution in the pipet contains reagents necessary to prime first-strand cDNA synthesis from poly-A-containing RNA, including a modified oligo-dT primer that contains the T7 RNA polymerase promoter sequence at its 5'-end. Because of diffusion of reagents during the course of the electrophysiological recording, first-strand cDNA synthesis is begun in the cell. Using mild suction, the contents of the cell are aspirated into the pipet and first-strand synthesis followed by conversion to double-stranded cDNA is completed in a microfuge tube, creating a pool of cDNA representing the mRNAs present in the cell. Using T7 RNA polymerase, it is then possible to generate a highly amplified mixed cRNA probe that can be used in a reverse northern to screen a panel of double-stranded cDNAs for specific genes.

Expression profiling has been applied successfully in a number of different cell types in the mammalian CNS, including acutely dissociated hippocampal neurons (Eberwine et al., 1992), CA1 neurons in hippocampal slices (Mackler et al., 1992; Mackler and Eberwine, 1993), cultured hippocampal neurons (Miyashiro et al., 1994), cultured cerebellar Purkinje cells (Van Gelder et al., 1990) and acutely isolated striatonigral neurons (Surmeier et al., 1992). This technique has been used to establish correlations between the electrophysiological properties and expres-

sion levels of multiple mRNAs within individual cells (Mackler et al., 1992). These and other studies have also shown that considerable molecular variance may exist between individual neurons of a given cell type (Eberwine et al., 1992; Mackler et al., 1992; Mackler and Eberwine, 1993). In addition, this technique has provided insight into the subcellular distribution of mRNAs (Miyashiro et al., 1994).

Whereas the majority of these studies are qualitative, by normalizing the amount of probe bound to each target cDNA to an internal standard, expression profiling also lends itself to quantitative measures of gene expression. Using a neurofilament cDNA standard, Mackler and colleagues (Mackler et al., 1992) identified several genes whose expression was altered in single hippocampal CA1 neurons during synaptic potentiation induced by tetanic stimulation of Schaffer collaterals. Many of the mRNA species that exhibited altered levels of expression might have been anticipated from the results of previous studies. For example, as predicted by the finding that CamKII antagonists block development of LTP (Malinow et al., 1989), CamKII mRNA levels increased in single CA1 pyramidal neurons during synaptic potentiation (Mackler et al., 1992). However, some data obtained by expression profiling are not consistent with findings obtained using other techniques. In particular, levels of BDNF mRNA assayed by *in situ* hybridization have been shown to increase in CA1 pyramidal cells following induction of LTP (Patterson et al., 1992), but apparently decrease when assayed at the single-cell level (Mackler et al., 1992). Even more surprising is the observation that glutamic acid decarboxylase, the rate-limiting enzyme responsible for the synthesis of the inhibitory neurotransmitter GABA can be expressed by neurons that are known to be glutamatergic (Eberwine et al., 1995). Differences inherent in these techniques may underlie the apparent inconsistencies.

As with all single-cell analyses, the high sensitivity and limited amounts of material used in expression profiling make selecting the appropriate controls critical. Generally, a target cDNA for a highly expressed gene, such as β-actin or neurofilament protein, serves as a positive control and a sample of the plasmid into which the target sequences have been cloned is included as a negative control. Hybridization of the amplified mRNA probe is performed at high stringency and the identity of the sequences hybridizing to the target cDNAs is generally assumed. In one case, where expression profiling revealed the unexpected coexpression of three different dopamine receptor mRNAs in single striatonigral neurons, their presence was confirmed by subsequent RT-PCR analysis of amplified RNA prepared from single-cells (Surmeier et al., 1992). More recently a technique has been developed to examine directly the amplified mRNA sequences that hybridize to target cDNAs. Using a ligation-mediated PCR strategy to clone and sequence hybridizing amplified mRNAs, Pekhletski et al. (1996) demonstrated that reverse northerns can be subject to a high frequency of false positives. Hybridization of amplified mRNAs derived from noncomplementary genes can be mediated by a variety of sequences, including short repeated structures, that are represented in many genes. It is important, therefore, that experimental design takes this possibility into account, by sequencing the hybridizing species directly (Pekhletski et al., 1996), by looking for a consistent pattern of hybridization to two or more cDNA targets representing nonoverlapping regions of the gene of interest, or through confirmation of the presence of particular messages in single-cells by RT-PCR.

Single-Cell RT-PCR

RT-PCR provides a second highly sensitive method that can be used to analyze mRNA species present in cell cytoplasm harvested with a whole-cell recording electrode. Once the cytoplasm has been harvested, first-strand cDNA synthesis is generally performed using

random hexanucleotides (Lambolez et al., 1992; Smith and O'Dowd, 1994), although sequence-specific antisense (Gurantz et al., 1996) and oligo-dT (Tong et al., 1994) primers have also proven effective. Subsequently, target sequences are amplified by PCR and the reaction products analyzed by gel electrophoresis. The feasibility of this approach was first demonstrated by Lambolez and colleagues (1992), who examined the AMPA receptor expression profiles of cerebellar Purkinje neurons and granule cells. Early studies restricted their analysis to only one gene or closely related members of a gene family. However, multiplex single-cell RT-PCR, in which several primer pairs are combined in a single PCR reaction, has extended the utility of this technique to the analysis of multiple-target mRNAs (Ruano et al., 1995; Kato et al., 1996). Together with its high specificity and relative simplicity compared to expression profiling, single-cell RT-PCR is a good first choice for most investigators interested in studying gene expression at the single-cell level. In the remainder of this article, therefore, we will discuss some of the technical considerations that should be taken into account when designing a single-cell RT-PCR experiment, as well as some of the issues that relate to interpretation of the data.

Primer Selection

Primer selection for single-cell RT-PCR should be guided by the same parameters used in primer selection for standard RT-PCR. In general, primers should be approx 20 nucleotides long, and have a GC content of about 50%. Complementarity between primers, particularly at their 3'-ends, should be avoided. Most commercial DNA analysis programs (e.g., MacVector, Eastman Kodak; Lasergene, DNAStar) contain modules that automate primer selection and optimize for these various parameters. To ensure a high level of amplification efficiency, sequences to be amplified should be kept short, preferably in the range of 100–300 bp. Overall, our experience has been that primers that work well with total RNA (i.e., produce a strong band on an ethidium bromide-stained gel when amplifying cDNA equivalent to 50–100 ng of total RNA), also work well in single-cell applications. Similarly, when selecting primers for the first round of multiplex PCR, complementarity between primers should be avoided and primer pairs selected whose optimal annealing temperatures (Rychlik et al., 1990) are as closely matched as is practical.

Patch-Clamp Electrophysiology

Although the electrophysiological setup is similar to that used for conventional whole-cell recording, precautions should be taken to exclude RNase contamination. Glass used to make recording pipets should be handled with gloves and baked overnight to reduce residual RNase activity (Sambrook et al., 1989). Glass pipets should be pulled in small batches as needed during the course of the experiment, again to reduce the possibility of inadvertent contamination with RNase. In making up internal solutions used in the recording pipet, care must be taken to ensure that they too are free of RNase activity. Although many investigators use DEPC-treated water (Sambrook et al., 1989) to make up the internal solution, we have only found it necessary to make solutions with ultrafiltered autoclaved distilled H_2O in sterile plasticware. Whereas single-cell RT-PCR appears to be relatively tolerant of variations in the composition of the internal solution, one study has reported that CsCl may inhibit reverse transcriptase (Sucher and Deitcher, 1995). However, in the majority of studies in which the glutamate receptors have been examined the internal recording solution has contained CsCl (Lambolez et al., 1992; Audinat et al., 1994; Bochet et al., 1994; Ruano et al., 1995). Although we have not made a systematic study, internal solutions 5–15 mosM lower than the external bathing solution

appear optimal for stable recording and ease with which the contents of the cell can be harvested.

Harvesting Cytoplasm of Single Cells

As described above, the technique of harvesting the cytoplasm from individual cells was first utilized by Eberwine and colleagues (Van Gelder et al., 1990; Eberwine et al., 1992). The successful application of this technique using slices of mammalian brain demonstrates that high cell density does not preclude its use (Mackler et al., 1992). However, aspiration of the contents of a single cell, without contamination arising from adjacent cells, relies on maintaining the integrity of the seal during the harvesting procedure and therefore requires that seal resistance be monitored throughout the harvesting procedure. In situations in which individual cells can be clearly visualized, for example, in acutely dissociated preparations or low-density cell culture, glass pipets can be used to harvest the contents of single cells without making physiological recordings. A recent study reports the use of both recording and nonrecording electrodes in the same experiment; electrophysiological measurements were first made from an outside-out patch pulled from the cell followed by the use of a second, larger electrode to harvest the cell's contents (Geiger et al., 1995).

Because of its association with electrophysiology, the primary use of this technique to date has been in the nervous system. However, it is not limited to use in nervous tissue but can be applied to any tissue, in which one can access single-cells with a harvesting pipet and the cell's contents can be aspirated without potential contamination from nearby cells. For example, single-cell RT-PCR has been used to examine mRNA expression in cardiac myocytes (Krown et al., 1995) and cells in the immune system (Trumper et al., 1993; Pearson-White et al., 1995). There are also a wide range of different cell sizes in which this technique has been successfully employed. At the small end of the spectrum are nonneuronal cells, acutely dissociated from E14 ciliary ganglion (Smith and O'Dowd, 1994), and multiplex RT-PCR in cells from E12–E16 chick hindbrain (Kato et al., 1996) where the capacitance measurements can be as small as 2 pF, corresponding to cell diameters of approx 8 μm.

Modifications to increase harvesting efficiency and/or recovery of intact mRNA from the pipet include the use of sialinized pipets and addition of carrier tRNA to the internal solution. Although these modifications may be helpful, we have not found them to be necessary in any of the systems in which we have worked, and in the interest of keeping the system as simple as possible, we would not normally include them.

First-Strand cDNA Synthesis and PCR Amplification

Oligo-dT, specific, and random primed cDNA have proven to be effective templates in single-cell PCR reactions. Random primed templates have a greater general utility and may even be preferred for multiplex PCR applications. In our own laboratories, reagents for the first-strand cDNA synthesis, except reverse transcriptase, are premixed and 7.5-μL aliquots placed into microfuge tubes and stored on ice ready to receive the cell extracts (Smith and O'Dowd, 1994; O'Dowd et al., 1995). Two microliters of the internal solution containing the cell extract are expelled from the electrode tip onto the side wall of the microfuge tube and rapidly mixed with the first-strand cDNA premix by brief centrifugation and again stored on ice. At the end of the recording session, first-strand cDNA synthesis is initiated by addition of 0.5 μL (100 U) of MMLV reverse transcriptase and the reaction allowed to proceed for 1 h at 37°C. The reaction is stopped and DNA/RNA hybrids denatured by heating the mixture for 5 min at 95°C, followed by cooling on ice prior to being added to the PCR reaction mixture.

Reagents for PCR are assembled as a premix and aliquoted into PCR reaction tubes on ice. Following addition of cDNA, the tubes are placed in a thermal cycler for amplification. Cycle parameters that are found to work well for a particular primer set in conventional RT-PCR are a good starting point for amplification of single-cell templates. The number of cycles required, however, depends on the application. For some templates, a single round of 35–40 cycles is sufficient (Lambolez et al., 1992; O'Dowd et al., 1995), others may require two rounds of amplification using a nested primer set (Lambolez et al., 1992; Smith and O'Dowd et al., 1994). At the end of the PCR reaction, aliquots of the reaction mixture are analyzed by gel electrophoresis. Several methods of detection have been employed, including ethidium staining (Lambolez et al., 1992), Southern blotting (Lamolez et al., 1992) and direct autoradiography of the amplified bands through incorporation of a radiolabeled primer in the PCR reaction (Smith and O'Dowd, 1994; O'Dowd et al., 1995).

Controls

Owing to its high sensitivity, single-cell RT-PCR is vulnerable to low levels of contamination that result in false positives. It is critically important, therefore, to have controls in place to detect these should they occur. Care should be taken throughout the preparation and handling of reagents and samples not to introduce contaminating DNA or RNA that might serve as a template. Thus, gloves, aerosol-resistant pipetters, and a draft-free environment are minimal precautions that should be taken to guard against contamination. UV irradiation of the electrophysiology setup and covering the computer keyboard with plastic film (Saran wrap) have proven necessary when working with primers that have high homology to human genes (O'Dowd and Smith, unpublished observations). Contaminated reagents may be detected using a blank control in which water is substituted for cell cytoplasm during the first-strand cDNA synthesis. Equally important, however, is to exclude experiments in which samples may have become contaminated during harvesting of the cell cytoplasm. Such contamination may come from a variety of sources, including mRNA released into the bathing medium during the recording session or contaminants present on the electrode wire or electrode holder. Such contamination can be detected by a media control in which bathing medium in the vicinity of a cell is sampled using a standard recording pipet and processed as a sham cell. In a typical experiment, we generally include one media control for every five cells, and only analyze data from experiments in which both water and media controls are negative (Smith and O'Dowd, 1994; O'Dowd et al., 1995).

Since the nucleus is frequently harvested with the contents of the cell, the possibility that false positives result from amplification of genomic DNA needs to be considered. Cerebellar granule cells do not normally express the GluR5 glutamate receptor subunit (Bettler et al., 1990). Based on their inability to amplify GluR5 sequences from granule cell cytoplasm samples that include the nucleus, Johansen et al. (1995) concluded that genomic sequences are not amplified by single-cell RT-PCR. Similarly, as expected from *in situ* hybridization studies (Wisden and Seeburg, 1993), mRNAs encoding GluR 5, KA-1, and KA-2 subunits can be detected in single Purkinje cells, but sequences for GluR-6 and GluR-7 cannot (Ruano et al., 1995). The ability of genomic alleles to act as templates, however, may depend on whether a given gene is normally transcribed by the cell (which may impact on its accessibility to RNA polymerase II [Kornberg and Lorch, 1995]) and the number of PCR cycles used in the amplification step. In a recent study that examines Kv channel gene expression in *Xenopus* neurons during development, reverse transcription was followed by two sequential 40 cycle PCR reactions (Gurantz et al., 1996). Since the PCR fragments amplified from mRNA did not span an intron, pretreatment with DNase was required to prevent amplification from genomic DNA.

When aspiration of the nucleus was visibly ascertained and both DNase pretreatment and reverse transcription were omitted, amplification of the PCR fragments from Kv genes occurred in every cell tested. A second study has also shown that genomic β-actin sequences can be amplified from single human embryonic kidney cells (Tong et al., 1994). Thus, unless the primers being used flank an intron or other preventive measures, such as DNase I pretreatment (Tong et al., 1994) are undertaken, it cannot be assumed that an amplified band derives from mRNA, a consideration that becomes particularly important when analyzing expression of an intronless gene.

Quantitative Single-Cell Analysis

Whereas amplified mRNA and RT-PCR based techniques make quantitative estimates of the level of mRNA in a single-cell possible, inability to measure the efficiency of the harvesting step has meant that quantitative measures of gene expression using single-cell techniques have been restricted to measures of relative abundance in which mRNA levels are normalized to an internal standard. Such measures, however, often assume a pattern of expression of the reference gene based on data collected by other techniques and may not be valid at the single-cell level. Therefore, the selection of the reference gene and possible impact on the interpretation of the data should be carefully considered. Additional complications owing to differences in amplification efficiency can also arise when comparing the abundance of two or more genes to a reference gene. Such differences however, can be eliminated or minimized by using a competitive PCR strategy (Gilliland et al., 1990a,b) where a single primer pair can be employed to amplify transcripts of homologous genes or alternatively spliced mRNAs. For example, using primers complementary to conserved sequences in the AMPA receptor subunits GluR1–GluR4, followed by restriction mapping and Southern blot analysis to identify the amplified products, it was shown that the patterns of expression for these subunits in cerebellar Purkinje cells and granule cells are cell specific (Lambolez et al., 1992). Subsequently, a modification of this strategy was adopted to examine the relative abundance of the GluR1–4 AMPA receptor subunit mRNAs expressed in hippocampal and cortical neurons (Geiger et al., 1995). The same polyspecific primer pair was used to coamplify all of the GluR1–4 cDNAs. In the second round of amplification, the primers were extended to include restriction sites to facilitate high-efficiency cloning of the products amplified from each cell. By screening the resulting single-cell plasmid libraries with oligonucleotide probes specific for each of the GluR subtypes, differences in AMPA receptor-mediated Ca^{2+} permeability observed between different classes of neurons were shown to correlate with expression of GluR-2 and GluR-4 subunits (Geiger et al., 1995).

The same competitive PCR strategy also works well for the analysis of alternatively spliced mRNAs. Agrin is an extracellular matrix protein that triggers differentiation of the postsynaptic apparatus of the neuromuscular junction. Agrin is encoded by a single gene, but diversity of gene expression is achieved by splicing of relatively small exons that have been shown to affect the activity of the protein (Bowe and Fallon, 1995). Using primers flanking one particular alternatively spliced locus, we (Smith and O'Dowd, 1994) demonstrated that agrin pre-mRNA splicing was cell-specific and that the relative abundance of different transcripts changed during development. The same techniques were also used to correlate changes in voltage-gated sodium currents with the patterns of alternatively spliced Na^+ channel mRNAs expressed in cultured *Drosophila* neurons (O'Dowd et al., 1995). In these two studies, differences in the size of the alternatively spliced mRNAs were small, and primers were selected to minimize the average size of the amplified fragments such that the overall amplification efficiency was high. Inclusion of a small amount of radiolabeled primer in the PCR

reaction greatly simplified the quantitative analysis by eliminating the need to correct for differences in the molecular weights of the products. Amplification efficiency is affected by such variables as template length and secondary structure, and it may be advisable to determine empirically how such differences affect the experimental outcome. However, the observation that following amplification of in vitro transcripts of the flip and flop AMPA receptor isoforms, the ratios present in the original mixtures are preserved suggests that amplified products from single-cells are likely to reflect the relative abundance of mRNAs in the cell extract (Lambolez et al., 1996).

Multiplex RT-PCR

A potential limitation of single-cell RT-PCR is the number of genes that can be simultaneously examined in a single-cell. Whereas expression profiling is limited only by the number of target sequences that can be hybridized to the probe, single-cell RT-PCR had, until recently, been restricted to the analysis of transcripts from homologous genes or generated through alternative splicing. This constraint has been removed with the demonstration that single-cell RT-multiplex PCR (RT-mPCR), in which multiple primer pairs are combined in a single PCR reaction, can be used to analyze the molecular composition of kainate receptors expressed in cultured hippocampal neurons (Ruano et al., 1995). High levels of KA-2 and Glu-R6 subunit mRNAs are present in the hippocampus (Wisden and Seeburg, 1993), and expression studies have demonstrated that GluR-6 and KA-2 subunits coassemble to generate glutamate receptors activated by kainate and AMPA (Herb et al., 1992). With the technique of single-cell RT-mPCR, Ruano and colleagues have demonstrated that the majority of cultured hippocampal neurons sensitive to kainate contain mRNA encoding the GluR-6 subunit, whereas GluR-5 and GluR-7 mRNAs were not detected (Ruano et al., 1995). These data suggest that kainate receptors in these cells are homoligomeric proteins.

We have used a modified single-cell RT-mPCR approach to examine expression of homeotic genes during early hindbrain development (Kato et al., 1996). First-strand randomly primed cDNA, synthesized from RNA isolated from single-cells acutely dissociated from embryonic stage 12–16 chick rhombomeres, was coamplified with oligonucleotide primers for three homeotic genes together with primers for β-actin as a positive control. Subsequently, aliquots of the first PCR were reamplified in separate PCR reactions using a second set of internal primers specific for each of the target sequences. This strategy of using nested primers and two rounds of amplification with the multiplex step in the first round prevents amplification of nonspecific products generated in the first round (perhaps owing to the use of nonoptimal annealing temperatures to accommodate the use of multiple primers) as might be possible in previous studies (Ruano et al., 1995). The results of these studies show heterogeneity in the expression pattern of hox genes and developmental changes in the pattern of coexpression of specific transcription factors in cells from single rhombomeres (Kato et al., 1996).

Unlike competitive PCR, primers used in RT-mPCR amplify unrelated target cDNAs. Nevertheless, competition for other reagents and amplification efficiencies of different templates may influence the sensitivity of mPCR and should be taken into account. The results of preliminary studies suggest that as the complexity of the mPCR reaction increases, the yields from some templates can fall below the level of detection (Kato, personal communication). One possible explanation for this observation is that amplification of primer dimers that form in high order RT-mPCR reactions, but not in lower-order reactions, outcompete template-specific amplification reactions. If this were the case, then one would expect to see amplified products lost in a pairwise fashion. Alternatively, the abundance and/or amplification efficiency of a given template may be

such that in the face of competition from other templates for limited amounts of dNTPs and polymerase, it is lost during the exponential amplification reaction. If the former is the case, changing the primers may help. For the latter scenario, increasing the amount of dNTPs and/or *Taq* polymerase in the first PCR may help. Either way, experiments should be performed to ensure that the frequency of expression of a particular gene in a given cell population does not differ significantly using RT-mPCR from that observed using the same primers alone.

How Closely Do the Observed Patterns of Expression Mirror Reality?

Single-cell analysis of gene expression has demonstrated that even within a given cell type, there is considerable heterogeneity of gene expression. Individual variation is likely to make an important contribution to the emergent properties of a population of cells. It is important, therefore, to have a sense of how reliable single-cell techniques are in informing us about the pattern of gene expression in a given cell.

In virtually all single-cell RT-PCR experiments, a certain population of cells exist for which no PCR product is obtained, raising the question of how to interpret these negative results. Lacking information concerning such variables as harvesting efficiency and integrity of the RNA collected for any given cell, the absence of a product cannot be interpreted as absence of template. For this reason, many studies have confined their analysis to cells for which the gene of interest was amplified. One strategy that has been employed to come to grips with this issue has been to define an internal criterion for success, generally amplification of a ubiquitously expressed high copy number control gene (Gurantz et al., 1996). However, two caveats need to be addressed when considering the validity of this approach. First, even though insufficient RNA may be harvested from a cell to produce a detectable product for the test gene, a high copy number criterion gene might still be within the range of detection. Second, differences in subcellular localization between the test and criterion genes may also influence the outcome of these types of experiments. For example, mRNAs encoding integral membrane proteins will be bound to the rough endoplasmic reticulum, and some transcripts, such as the CAM Kinase II mRNA, exhibit a dendritic pattern of spatial localization (Benson et al., 1992). Such differences are likely to impact on the efficiency with which a particular template can be harvested from a cell, and it may be inappropriate therefore to use a housekeeping gene translated in the cytoplasm as a criterion for amplification of genes with more heterogeneous patterns of dispersal within a cell. A second solution to the problem of how to interpret negatives is outlined in a study of gene expression during early pattern formation in the chick hindbrain (Kato et al., 1996). The frequency with which identical PCR products could be amplified from cDNA obtained from single-cells split between two independent PCR reactions, was examined. The number of cells in which the sister reactions were both positive (coherent positives), both negative (coherent negatives), or in which one reaction was positive and the other negative (incoherent) was determined. A low frequency of incoherent reactions suggested that the false negative rate was low. Based on the fraction of incoherent positive PCR reactions, the expected rate of false negatives can be estimated for comparison with the observed rate under standard reaction conditions. For the two genes tested in this fashion, sek1 and krox20, the rate of false negatives was <10% (Kato et al., 1996).

In addition to populations of cells for which no PCR product is amplified, some single-cell RT-PCR analyses also reveal heterogeneity between cells for which PCR products are obtained. For example, whereas hippocampal neurons that respond to kainate express GluR6 subunit mRNA, a subset of these neurons also

contain mRNA encoding the GluR5 subunit (Ruano et al., 1995). Differences in the relative abundance of edited vs unedited variants of the GluR6 subunit were also observed in the same population of cells (Ruano et al., 1995). Similarly, retinal ganglion cells have been reported to display considerable variability in β-subunit expression for the $GABA_A$ receptor (Grigorenko and Hermes, 1994). Our own analysis of *para* sodium channel mRNA and agrin mRNA expression demonstrated qualitative and quantitative differences in the abundance of alternatively spliced mRNA isoforms between individual cells. Beginning with the hypothesis that stochastic variation associated with exponential amplification of low copy number sequences might account for these observations, we predicted that splitting the cDNA from a single-cell and amplifying it in two separate reactions would result in different profiles for each amplification. Splitting experiments, in which we examined agrin mRNA in chick ciliary ganglion neurons and sodium channel mRNA in *Drosophila* neurons, demonstrate that reproducible profiles can be obtained in separate PCR reactions from a single-cell (Smith and O'Dowd, 1994; O'Dowd et al., 1995). At least some of the heterogeneity in expression of alternatively spliced isoforms therefore reflects real differences in gene expression among these cells. However, instances in which sister PCR reactions resulted in different profiles were also observed (O'Dowd et al., 1995) consistent with the interpretation that some heterogeneity is inherent in the technique, a possibility that should be considered when analyzing the results of single-cell RT-PCR experiments.

Ensemble analysis of single channel electrophysiological records has been used to examine the role of specific channel types in generating whole-cell currents. By analogy, averaging the expression profile for a group of genes or alternatively spliced mRNAs obtained from single-cells produces an ensemble analysis of gene expression as a first step toward understanding the properties of the population. This is particularly important in instances in which there is heterogeneity in gene expression among single cells that, as discussed above, may reflect real variation or limitations of the technique. For example, whereas a strict correlation between sodium current expression and presence of a specific alternatively spliced *Drosophila para* mRNA encoding a voltage-gated Na^+ channel could not be seen in any one neuron, ensemble analysis clearly revealed a positive correlation between the presence of one particular isoform and levels of sodium current expression (O'Dowd et al., 1995). Analysis of the average relative abundance of GluR A/B/C/D and flip vs flop isoforms from a number of single-cells within any one class of cells was also useful in comparing the functional properties and subunit composition of native AMPA receptors in a variety of different cell types (Geiger et al., 1995). Ensemble analysis tests the hypothesis that all of the variation apparent at the cellular or organismal level can be accounted for by variation observed in single-cells. For example, the finding that the *para* sodium channel mRNA profile from whole-embryo RNA was similar to the average profile obtained from single neurons suggests that the population of cells sampled during the single-cell experiments was representative of the population in vivo. In addition, exon usage was not altered in the chosen culture conditions (O'Dowd et al., 1995). Similarly ensemble average data for some alternatively spliced agrin mRNAs, for example, the B11 transcript, was in good agreement with that obtained from RNA prepared from whole ganglia at the same developmental stage (Smith and O'Dowd, 1994). The observation that not all developmental changes in agrin pre-mRNA splicing could be accounted for in this manner suggests that other components are present in the ganglion that are missing from the single-cell analysis, sounding a note of caution in the interpretation of these data.

Conclusions

The enormous complexity of the nervous system represents a formidable challenge to designing experiments aimed at understand-

ing how changes in gene expression subserve fundamental processes that go on within it. Techniques that facilitate analysis of gene expression at the level of a single-cell hold great promise in this respect. Already these techniques have shed light on important questions, such as the subunit composition of oligomeric proteins in primary neurons, that could only have been guessed at from in vitro expression studies. As might have been anticipated, they also show that consistent patterns of gene expression exist within a single class of cells. Conversely, and perhaps of greatest interest, just as one sees differences in the heights of children in a class, single-cell analyses suggest that for any given gene, each cell within a class has a unique expression profile. Although this may simply reflect limitations on the ability of cells to regulate gene expression, it is also consistent with the possibility that individual cells are precisely tuned or preadapted such that the population as a whole is able to contend with a wide range of stimuli, producing the best response to a given stimulus. As techniques evolve that permit more rigorous quantitation of changes in gene expression in single-cells in response to experimental manipulation, it will be interesting to learn whether these complex patterns of expression that border the edge of chaos are, in fact, adaptive.

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