Single-cell molecular biology

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The need for single-cell mRNA analysis is evident given the vast cellular heterogeneity of the CNS and the inability of conventional methodologies such as northern blotting or RNAse protection to distinguish individual cellular contributions to mRNA abundance differences. The history of single-cell molecular biology is relatively new when compared to many areas of neuroscience investigation. The first single-cell analysis involved the characterization of mitochondrial DNA[1]. Single-cell DNA analysis (particularly genomic DNA) is important and may be informative in the analysis of genetics of cell clonality, genetic anticipation and single-cell DNA polymorphisms. However, the more important area of analysis for most neuroscientists is mRNA expression analysis. It is the relative abundances of mRNAs and their regulation that give rise to a cell's identity, potential for synaptic responsiveness and ability to undergo cellular plasticity.

In situ hybridization (ISH) was the first example of single-cell mRNA analysis. This procedure allowed the detection and precise localization of mRNAs in cells and subcellular domains. Initially, ISH lacked sensitivity and could not simultaneously detect more than a handful of mRNAs on the same tissue section. (There are not enough distinguishable probes to permit more than 5–10 different mRNAs to be detected simultaneously on a single tissue section.) Although the sensitivity issue has largely been overcome with various signal enhancement strategies, the latter difficulty still remains.

Rather than attempting to increase the number of reporter molecules, it was apparent that amplification of the endogenous mRNA population could provide a means for obviating both problems with ISH. Isolation of mRNA from individual cells can be performed blindly from randomly selected cells, from electrophysiologically characterized cells or from cells expressing particular markers such as green fluorescent protein (GFP). The RNA is isolated using a patch pipette to penetrate the cell membrane. The cellular contents are then aspirated into the pipette and transferred to a microcentrifuge tube. The amplified antisense RNA (aRNA) procedure[2] is a linear nucleic acid amplification procedure in which the mRNA complement of a cell is converted into cDNA using an oligo-dT primer that contains a T7 RNA polymerase promoter site. After the cDNA is double-stranded, T7 RNA polymerase is used to transcribe RNA copies. Because of the positioning of the T7 RNA polymerase promoter site, these RNA copies are antisense. The procedure produces more than 10^6-fold amplification when optimally done. The first paper devoted to analysis of the mRNA complement from an individual cell[3] used a single, cultured primary hippocampal neuron from which the mRNA complement was isolated using a patch pipette (**Fig. 1**, left).

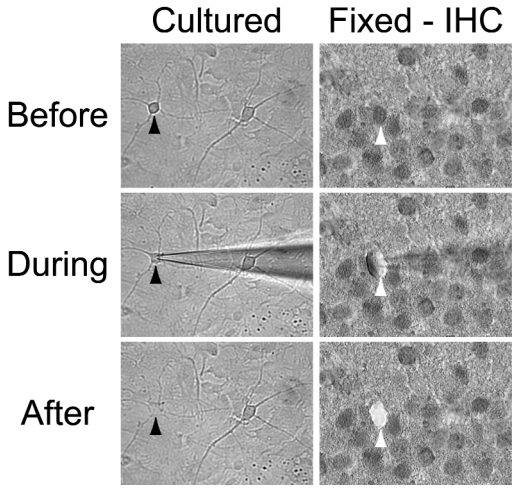

Fig. 1. Harvesting of mRNA from single live and fixed cells. Left panels are from single live neurons; right panels illustrate the harvesting from fixed immunostained neurons. Photo courtesy of Janet Estee Kacharmina and Paolo Marciano.

The polymerase chain reaction (PCR) can also be used to amplify mRNAs from single cells. Two groups[4,5] independently showed that the mRNAs encoding individual members of the ionotropic glutamate receptor family are present in neurons at differing abundances. Others have used these methods to correlate electrophysiological responsiveness of neurons with the presence of particular receptor and channel mRNAs within the same cell[6].

Another PCR-based procedure, differential display (DD), has been used to analyze the entire mRNA complement of single cells. DD has been used to identify various mRNAs that are localized to the dendritic domain of neurons[7]. These studies have highlighted the previously unexpected diversity and extent of mRNA transported to dendrites. DD has also been used to identify and clone novel mRNAs including those encoding pheromone receptors from single sensory neurons of the rat vomeronasal organ[8]. Although quantitative assessment of mRNA populations is difficult with DD, mRNAs can be easily identified with single-cell DD.

One example of basic science information that can be gleaned from single-cell analysis is the extent of alternative mRNA splicing occurring in a single cell. The near completion of the sequencing of the human genome has provided a conundrum. How can 30,000 genes generate the vast complexity (50 to 60,000) of proteins that are made? In an individual cell there are an estimated 10,000 mRNAs expressed all at differing abundances, the population of which gives rise to 30,000 or more distinct proteins. Aside from post-translational modifications, one answer is that extensive alternative splicing occurs resulting in multiple mRNA and protein species from one gene. Single-cell PCR and/or aRNA amplification methodologies have been used to show which splice forms of various glutamate receptor mRNAs coexist within the same cells[5,9].

The application of these methodologies to the characterization of human diseases is an area of intense interest, and the only ready source of human disease tissue is fixed pathological tissue. mRNA isolated from fixed tissue is truncated (between 50–100 bases) and the yield is low. The aRNA procedure has been modified[10] for use in harvesting the *in situ*-transcribed cDNA[11] from fixed immunostained tissue sections, after which aRNA amplification is performed; cDNA is primed with oligo-dT containing a T7 RNA polymerase promoter site (**Fig. 1**, right). The aRNA probe generated from such tissue can be used to screen macro and microarrays[12]. This area of single-cell molecular analysis is in its infancy but promises to provide important insight into the molecular consequences of neuronal dysfunction in human disease.

One of the challenges of single-cell mRNA analysis procedures is data analysis. This is particularly true with regard to microarray analysis of single-cell

amplified products. Even with amplification of the mRNA complement of a single cell, the resulting amplified product is still relatively low—on the order of 50–100 ng—meaning that the lower fluorescent intensity measurements will need to be quantitated. This poses a problem for how data normalization should be accomplished. Challenges such as this await resolution.

1. Li, H. *et al. Nature* **335**, 414–417 (1988).
2. VanGelder, R. *et al. Proc. Natl. Acad. Sci. USA* **87**, 1663–1667 (1990).
3. Eberwine, J. *et al. Proc. Natl. Acad. Sci. USA* **89**, 3010–3014 (1992).
4. Lambolez, B., Audinat, E., Bochet, P., Crepel, F. & Rossier, J. *Neuron* **9**, 247–258 (1992).
5. Mackler, S. & Eberwine, J. *Mol. Pharmacol.* **44**, 308–315 (1993).
6. Mermelstein, P., Song, W., Tkatch, T., Yan, Z. & Surmeier, D. *J. Neurosci.* **18**, 6650–6661 (1998).
7. Miyashiro, K., Dichter, M. & Eberwine, J. *Proc. Natl. Acad. Sci. USA* **91**, 10800–10804 (1994).
8. Dulac, C. & Axel, R. *Cell* **83**, 195–206 (1995).
9. Jonas, P., Racca, C., Sakmann, B., Seeburg, P. & Monyer, H. *Neuron* **12**, 1281–1289 (1994).
10. Crino, P., Dichter, M., Trojanowski, J. & Eberwine, J. *Proc. Natl. Acad. Sci. USA* **93**, 14152–14157 (1996).
11. Tecott, L., Barchas, J. & Eberwine, J. *Science* **240**, 1661–1664 (1988).
12. Chow, N. *et al. Proc. Natl. Acad. Sci. USA* **95**, 9620–9625 (1998).

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An impulse to the brain—using *in vivo* electroporation

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To understand the mechanisms and processes that underlie neural development, plasticity, physiology and function, it is essential to be able to monitor and manipulate the behavior of cells over time and to modify gene expression in viable cells, embryos and tissues. Recent progress in a number of technologies, including *in vivo* imaging (see article in this issue by Lichtman and Fraser), embryo culture and gene transfer by *in vivo* electroporation (EP), are now permitting us to approach important questions in a new way. Here we summarize some of the potential uses and advantages of combining *in vivo* EP with other methods for neuroscience research.

Efficient gene transfer by the EP technique has been widely used to introduce exogenous molecules into both prokaryotic and eukaryotic cells. Although the detailed mechanisms are unknown, transient pores generated by electric shocks at the cellular membrane allow charged macromolecules such as proteins, RNA and DNA to actively penetrate into cells by means of electrophoresis. The difficulty in applying this approach to living tissues or organisms had been that the electric pulses often damage cells and result in substantial cell death. A key breakthrough was the discovery that a rapid series of controlled square wave pulses, instead of averaged or bell-shaped exponential pulses, dramatically reduced the levels of cell death. In 1997, Muramatsu and colleagues first reported remarkable results using *in vivo* electroporation in developing chicken embryos[1]. This important and convenient technology is now routinely being used by chick embryologists and is also being applied to many other living tissues and organisms, including mammals[2–6]. Although much of the current use centers on early development, this method is equally applicable to adult tissues, organs or differentiated post-mitotic cell populations.

How does this new technology bring benefits to the field of neuroscience? *In vivo* EP has several advantages for investigating neural development, as it facilitates and complements both genetic and manipulative approaches in many experimental systems. The neural tube or brain vesicles are particularly easy to target, as a gene expression vector can be placed in the lumen, permitting the directed transfer of DNA, which carries a negative charge, to the side of the positive electrode (**Fig. 1a**)[2–4,6]. If an enhancer/promoter combination (for example, rous or cytomegalo virus) capable of directing expression in most cell types is used, it is routinely possible to obtain expression in 10–100% of transfected cells by optimizing conditions through variation of voltage and numbers of pulses (**Fig. 1a**). It is also possible to target specific cell types or restricted populations through the use of enhancers capable of mediating spatial, temporal or tissue-specific expression[2]. Furthermore, the EP technique provides an effective approach for mapping and identifying *cis*-regulatory elements[2,7]. However, there can be variability in expression with some electroporated regulatory regions, presumably because the electroporated DNA generally does not become integrated. This may be analogous to the differences seen in experiments using transient transfection versus stable cells lines or transgenes.

A further refinement of the EP approach arises from variations in types of electrodes and application of the voltage. Specific promoter/enhancer elements are not the only way to modulate gene expression within restricted tissues and/or groups of cells, as the targeting can also be directed by careful positioning of the electrodes to apply the electric field in a specific manner. By using electrodes of different size or type (from a fine point to a long wire or plate) or modifying the voltage, it is possible to control the relative size of the transfected area, which can vary from a single cell to entire tissues[4,5,8,9]. If the DNA can be injected in the proper position, the electrodes do not always need to be placed in the embryo itself, as long as a current is passed through the relevant tissues (**Fig. 1a** and **b**). This permits a wide spectrum of electrode orientations to enable gene transfer along different axes (anteroposterior, dorsoventral, mediolateral, left-right, proximodistal). By performing the operation at different or multiple stages in the embryo or adult tissues, one can control the timing of expression for exogenous genes *in vivo* to study many aspects of neural differentiation and patterning[9,10]. Multiple genes can simultaneously be examined by co-electroporation of mixed expression vectors[2,11] and blocking gene function or expression is also possible using dominant negative molecules[12]. The expression of fluorescent reporter genes can act as lineage tracers in grafting and fate-mapping experiments[6]. Furthermore, different combinations of these tools or approaches can be used with multiple rounds of electroporation on the same embryo, as viability or integrity of tissues and embryos is high using the EP technique. These analyses can be done in