Using DSP, a reversible cross-linker, to fix tissue sections for immunostaining, microdissection and expression profiling

Charlie C. Xiang, Eva Mezey¹, Mei Chen, Sharon Key¹, Li Ma and Michael J. Brownstein*

Laboratory of Genetics, National Institute of Mental Health and ¹Basic Neuroscience Program, National Institute of Neurological Disorders and Stroke, NIH, 36 Convent Drive, Bethesda, MD 20892, USA

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ABSTRACT
Mammalian organs are typically comprised of several cell populations. Some (e.g. brain) are very heterogeneous, and this cellular complexity makes it difficult, if not impossible, to interpret expression profiles obtained with microarrays. Instruments, such as those manufactured by Leica or Arcturus, that permit laser capture microdissection of specific cells or cell groups from tissues were developed to solve this problem. To take full advantage of these instruments, however, one must be able to recognize cell populations of interest and, after they are harvested, to extract intact, unmodified RNA from them. Here we describe a novel, fast and simple method to fix and immunostain tissue sections that permits this to be done.

INTRODUCTION
The standard method used to prepare tissues for laser-assisted microdissections involves cutting frozen sections, dipping them in a precipitating fixative such as ethanol, and staining them lightly with hematoxylin or cresyl violet (1–5). As long as the cell-specific antibodies employed bind the cells or cell groups from tissues were developed to solve this problem. To take full advantage of these instruments, however, one must be able to recognize cell populations of interest and, after they are harvested, to extract intact, unmodified RNA from them. Here we describe a novel, fast and simple method to fix and immunostain tissue sections that permits this to be done.

MATERIALS AND METHODS
Comparison of DSP to other fixatives using cultured cells
Mouse fibroblast NIH 3T3 cells were seeded on single-well chamber slides (Nalge Nunc, Rochester, NY). The cells were grown to ~85% confluency, washed once with 1× PBS buffer, and then fixed in one of three different ways: (i) 4% formaldehyde (diluted from a 37% stock solution from Mallinckrodt, Hazelwood, MO with PBS) for 5 min. (ii) DSP (Pierce, Rockford, IL) at a final concentration of 1 mg/ml for 5 min. 50× stock solutions of DSP in 100% anhydrous DMSO (Sigma-Aldrich, St Louis, MO) were prepared and stored at –80°C. The stock solution was diluted to working concentration with 1× PBS immediately before use. To prevent the DSP from precipitating when the DMSO stock is added to the PBS, vortex the latter gently and add the stock solution dropwise. (iii) Ethanol fixation was performed according to the protocol of Kamme et al. (5) with minor modifications: 100% ethanol for 1 min, 95% ethanol for 10 s, 70% ethanol for 10 s, 50% ethanol for 10 s. Following tissue fixation, the slides were washed twice for 10 s each time with 1× PBS before staining. The cells were stained with an anti-beta actin antibody from Biogenex (San Ramon, CA) as follows: rabbit

*To whom correspondence should be addressed at Laboratory of Genetics, NIMH/NHGRI, NIH, Building 36, Room 3D06, 36 Convent Drive, Bethesda, MD 20892-4094, USA. Tel: +301 496 5351; Fax: +301 435 5465; Email: brownstm@mail.nih.gov
anti-beta actin (1:50) primary antibody for 10 min; two 10 s washes with 1× PB; Alex fluor 594 goat anti-rabbit IgG secondary antibody from Molecular Probes (Eugene, OR) (1:500) for 10 min; two 10 s washes with 1× PBS. The slides were left on the bench to air dry, and the stained cells were examined and photographed with a Leica DM RX microscope (Wetzlar, Germany). The stained cells were then scraped into 1.5 ml Eppendorf tubes, and were forced to the bottom of the tubes by brief centrifugation at 13,000 g. One hundred microliters of 1× PBS with RNasin (200 U/ml; Promega, Madison, WI) was added to re-suspend the cells. Two and a half microliters of 1 M DDT was added to the tube containing the DSP-fixed cells, and these were incubated at 37°C for 30 min. The cells were pelleted by centrifugation at 13,000 r.p.m. for 30 s and the supernatant was removed. Total RNA was extracted from this sample and from those fixed with formalin or ethanol with 100 μl of Trizol (Invitrogen, Carlsbad, CA) according to manufacturer’s instructions. The quality and quantity of RNA obtained was assessed using an Agilent Bio-analyzer (Agilent, Palo Alto, CA).

**Tissue fixation with DSP: dissection of supraoptic nucleus (SON) and optic chiasm (CHI) samples**

Male 250–300 g Sprague–Dawley rats (Taconic, Germantown, NY) were housed 2–3 per cage at 21–23°C, and fed rat chow ad libitum. The lights in the animal room were on from 6 a.m. to 6 p.m. The rats were killed by decapitation at 1 p.m., and their brains were immediately removed and frozen on dry ice. All procedures were carried out in accordance with NIH guidelines governing the care and use of animals, and in the context of an approved protocol. The brains were sectioned at 12 μm intervals with a Leica CM3000 microtome/cryostat (Leica, Bannockburn, IL) at −18°C, and thaw-mounted (37°C for 1–2 min) onto membrane-coated glass slides (Leica Microsystems Inc. glass foil PEN slides). The sections can be stained immediately, but were typically stored in a −80°C freezer until they were processed. To study RNA recovery and quality, sections were fixed with DSP, formalin or ethanol as above, and the fixed sections were scraped off the slides into Eppendorf tubes. Some slides with DSP-fixed sections were stained with anti-oxytocin–neurophysin antibody (see below) and scraped into Eppendorf tubes. This allowed us to compare the quality of RNA from unfixed, DSP-fixed and DSP-fixed/stained samples. The RNA was extracted and analyzed as described above.

Samples of SON and CHI were cut from rat brain sections with a laser using a Leica AS LMD microdissection system (Wetzlar, Germany). Sections were fixed with DSP for 5 min, rinsed for 30 s in DEPC water, and incubated for 15 min at room temperature in a 1:50 dilution of anti-oxytocin–neurophysin antibody PS 36 (10) provided by H. Gainer (NINDS, Bethesda, MD). They were then incubated for 15 min in a 1:100 dilution of biotinylated anti-mouse IgG (Vector Laboratories, Burlingame, CA) and subsequently placed in a 1:60 dilution of ABC (Avidin/biotinylated enzyme) complex from Vector Laboratories for 10 min. Finally, they were exposed to Vector Red alkaline phosphatase substrate (20 μl of Reagents 1, 2 and 3 in 1 ml of 100 mM Tris–HCl, pH 8.3; Vector Laboratories) for 20 min. The oxytocin-positive neurons were stained red. Note that the times for each of the incubations above were decreased from those recommended by the manufacturer to minimize RNA degradation. To facilitate this, the concentrations of the antibodies and the ABC reagent were increased 5–10 times over those normally used. The slides were briefly washed with 1× PBS buffer between incubations and RNasin (200 U/ml; Promega, Madison, WI) was added to all of the solutions used. (It is worth mentioning that the horseradish peroxidase method was used to visualize cells too. No RNA was recovered from tissue stained this way. Perhaps, it was degraded by the hydrogen peroxide used in the staining reaction.)

To improve the sensitivity of the immunostaining reactions, the DakoCytomation (Carpinteria, CA) EnVision antibody/alkaline phosphatase complex was tested. Unlike the anti-mouse IgG and ABC complex used above, the DakoCytomation reagent is a conjugate of ~20 anti-mouse or rabbit antibodies plus 100 alkaline phosphatase molecules. Brain sections were incubated for 15 min in 1:100 dilutions of antibodies directed against oxytocin–neurophysin (PS36), vasopressin–neurophysin (PS 41), somatostatin (product number YC7 60132A, BD Biosciences, San Jose, CA), tyrosine hydroxylase (product number 22941, ImmunoStar, Hudson, WI), or NeuN (product number MAB377, Chemicon, Temecula, CA). Following a quick water rinse, they were placed in the DakoCytomation antibody/enzyme complex for 20 min, rinsed in water and added to DakoCytomation coloring solution for 1–5 min. (The above solutions contained 200 U/ml of RNasin. Some solutions, including the antibody/enzyme complex, were found to have RNase activity, which was inhibited by this reagent.) All of the antibodies tested stained appropriate populations of neurons in the sections studied. In spite of this, it should be emphasized that some antibodies will undoubtedly not stain cells well enough to permit microdissections to be done, and the optimal antibody and substrate concentrations, exposure times, etc. for any given staining may not be defined above. These will have to be determined empirically.

The slides were quickly dried with compressed gas (AccuDuster III, CleanTex, Nanuet, NY) at room temperature before the microdissections were performed. SON samples (8–10) were dissected from the 6 sections on each slide using a Leica AS LMD at the following settings: aperture of 9, intensity of 42 and speed of 5. After the SON samples were removed, we harvested 10 pieces of optic chiasm from the same sections. These were roughly equal in size to the SONs.

**Tissue fixation with DSP: dissection of vasopressin- and oxytocin-containing magnocellular neurons**

An Arcturus PixCell II LCM instrument (Mountain View, CA) was used to dissect magnocellular neurons from the SON. Eight micron thick rat brain sections containing the SON were cut, thaw mounted onto regular glass slides and stained with one of two primary antibodies, PS 36, a mouse anti-oxytocin (OT)–neurophysin antibody, or PS 41, a mouse anti-vasopressin (VP)–neurophysin antibody (10). Both antibodies were provided by H. Gainer (NINDS, Bethesda, MD). The DSP fixation and immunostaining procedures used were the same as those described earlier. Our goal was to obtain 1–10 ng of total RNA per sample. Based on the amount of RNA that can be extracted from a whole SON that has not...
undergone immunostaining (100 ng; N. Mutsuga, personal communication) and the number of cells in an SON (3000–4000; M. Palkovits, personal communication) we estimated that each magnocellular neuron has 20–35 pg of potentially extractable total RNA. (This is about half as much RNA as an estimate based on cell volume. If the amount of RNA in a cell is linearly related to its volume, magnocellular neurons should have about 10 times as much as smaller, 10 μm diameter neurons, which are thought to contain ~10 pg per cell.) Thus, 200–400 neurons were collected from the 6 sections on each slide. Note that the sections were 8 μm thick, and the diameter of magnocellular neurons is ~20 μm. Therefore, only part of each neuron was harvested: 31 μg and 70 μg of RNA were produced from the OT and VP neurons, respectively, in two consecutive amplification reactions. Since the literature in the Arcturus RiboAmp HS kit indicates that two rounds of amplification should yield 20 μg of product per ng of template, we estimate that ~8 pg of RNA were obtained from each (partial) cell harvested. This is consistent with our expectations.

**RNA extraction from microdissected samples and RNA template amplification**

A PicoPure RNA Isolation kit (Arcturus, Mountain View, CA) was used to extract RNA from the SONs, CHIs and vasopressin- and oxytocin-containing magnocellular neurons. The SONs and CHIs were collected in two separate empty 0.2 ml PCR tube caps, and 10 μl of PicoPure XB buffer with DTT (25 mM) were added to the caps after the samples had been dissected. Then microfuge tubes were pressed onto the caps.

Vasopressin- and oxytocin-containing magnocellular neurons were isolated on CapSure HS LCM Caps from Arcturus. Immediately afterwards, 10 μl of XB buffer with 25 mM DTT were added to the cells and 0.5 ml Eppendorf tube was pressed onto the collection cap.

The capped tubes containing SON, CHI or magnocellular neurons were left inverted, placed in 15 ml plastic tubes, and incubated in a 42°C water bath for 30 min. Next, the tubes were turned right side up, placed in a microfuge, and centrifuged briefly at 13 000 g to force the extracts into the bottoms of the tubes. RNA was then isolated with an Arcturus PicoPure RNA Isolation kit, and two rounds of RNA amplification were performed using an Arcturus RiboAmp HS RNA Amplification kit according the manufacturer’s instructions.

**Microarray studies**

Total RNAs from cultured mouse NIH 3T3 cells and rat brain sections, and amplified RNAs from microdissected rat brain regions (SON and CHI) or magnocellular neurons were analyzed with mouse DNA microarrays having 11 136 elements. The cDNAs were provide by Bento Soares; for information about the microarrays, please visit our website http://intramural.nimh.nih.gov/research/log/index2.html. Since the brain samples came from rats, experiments were executed in advance to determine the feasibility of using mouse arrays to study rat samples: 94% of the elements that gave significant signals when probes made from mouse hypothalamus were hybridized to mouse cDNA arrays also gave significant signals when probes made from rat hypothalamus were used (data not shown). Elements representing the mRNAs that encode the rat vasopressin- and oxytocin-precurors were printed on the arrays because of their particular importance in the present study.

Total RNA (5 μg) or 10–30 μg of amplified RNA were used to prepare Cy3- or Cy5-labeled probes. The synthesis of DNA in the probe labeling reaction was driven by amine-modified random primers (11). After the hybridization and washing steps, the arrays were scanned with an Axon GenePix 4000A (Union City, CA) at 10 μm resolution. PMT voltage settings were varied to obtain maximum signal intensities with >1% probe saturation. TIFF images were captured and analyzed with IPlab (Scanalytics, Fairfax, VA) and ArraySuite (NHGRI, Bethesda, MD) software. Calibrated ratios of signals from the two probes applied to each array were obtained by a normalization method based on ratio statistics (12). To determine the reliability of individual ratio measurements, quality scores (Q) ranging from 0 to 1 were assigned to each ratio, and elements with Q of 0 were removed from the data sets (12). In the studies of unfixed and fixed/stained cultured cells, samples were compared by generating and analyzing scatter plots. In the studies of tissue samples (SON versus CHI and oxytocin versus vasopressin producing neurons, both the raw signal intensities and the calibrated ratios of signals from specific elements were examined.

**RESULTS**

To develop and test our methods, we initially worked with cultured mouse NIH 3T3 cells. As shown in Figure 1A–C, these cells could be stained with an anti-actin antibody regardless of the method of fixation used. It is worth noting, however, that following ethanol fixation the staining was weaker and less uniform than it was following DSP or formalin fixation.

Ethanol fixation was even less satisfactory when an antibody directed against the oxytocin–neurophysin was used to stain hypothalamic magnocellular neurons in sections prepared from frozen rat brains. While DSP and formalin fixation anchored the antigen into the tissue and permitted it to be visualized in cells, ethanol did not (Figure 1D–F).

Oxytocin–neurophysin levels are moderately high in magnocellular neurons, and we had no trouble detecting this marker or vasopressin–neurophysin with a method based on the avidin–biotin complex (ABC) and alkaline phosphatase. While we could visualize the neurophysins with these staining reagents, it was difficult (thought not impossible) to detect less abundant antigens. Consequently, we replaced the ABC and alkaline phosphatase in our staining reactions with a secondary antibody/alkaline phosphatase complex (see Materials and Methods). This allowed us to decrease the reaction times in some cases, and to detect all of the cell-specific antigens that we have looked at to date. Sections stained with the antibody/alkaline phosphatase complex are shown in Figure 2. The images are not as nice as they would be if the sections had been coverslipped, but they reflect what one sees when laser dissections are performed.

The three fixation methods tested affected our ability to extract RNA from processed tissues differently. It was difficult, if not impossible, to extract RNA from formalin-fixed tissues, and even though it was simple to prepare RNA from
ethanol-fixed samples, it appeared degraded (Figure 3). Some researchers have reported this previously (13,14), but others have said that intact RNA could be extracted following ethanol fixation (15). Given the latter, we cannot explain our repeated failure to generate intact RNA from ethanol-fixed samples whether we stained them or not.

DSP-treated NIH 3T3 cells readily release RNA which looks similar to that extracted from unfixed samples, even following immunostaining of the cells (Figure 3). The 28S/18S ratios from unfixed, DSP-fixed and fixed/stained cells ranged from 2.0 to 2.15, ratios typical of intact RNA. On the other hand, RNA obtained from unfixed brain sections was slightly degraded (28S/18S = 1.65) in the example shown, and RNAs from fixed and fixed/stained sections (28S/18S = 1.36 and 1.34, respectively) were somewhat less intact than RNA from unprocessed ones. As noted
below, this was not always the case. The quality of RNA from fixed/stained sections was sometimes as good as that in unfixed, control sections, and in any case, we have shown previously that partially degraded RNA gives reliable expression profiles with the amplification and labeling methods used in the present study (16).

We attempted to determine how efficient the recovery of RNA from fixed and stained brain sections was by scraping such sections off of slides and extracting RNA from them. DSP-fixed sections yielded about two-thirds as much RNA as unfixed sections did when the modified PicoPure extraction method described below or Trizol was used to isolate the product. Six sections, or 0.24 mg of tissue, were homogenized in 200 μl of lysis buffer or Trizol, and unfixed tissue gave about 4 μg of total RNA (data not shown). Placing the sections in phosphate-buffered saline (PBS) containing 200 U/ml of RNasin for brief periods (1–5 min) resulted in a significant loss of RNA; the RNA remaining in the samples following such incubations was only one-quarter to one-third of the amount in the unfixed control sections. After 20 min in PBS, the RNA recovery was no worse however. Thus, following DSP fixation there appear to be two pools of RNA. One of these is rapidly degraded or diffuses out of the sections when they are placed in aqueous solutions; the other seems to be retained. Incubating sections in PBS for 40 min or immunostaining them with either ABC/alkaline phosphatase or the antibody/alkaline phosphatase complex resulted in a further loss of RNA which was comparable in all cases; only 10–15% of the RNA in the original samples could be extracted following these procedures, but this RNA was typically as intact as the material obtained from unfixed tissue. Reducing the time required for the staining reactions, which seems feasible in...
some instances, could improve RNA recovery by a factor of two.

We employed RNA extracted from DSP-fixed, immuno-stained 3T3 cells and untreated cells to generate the scatter plots shown in Figure 4. Comparisons of probes made from 3T3 cell RNAs from unixed (self-on-self), fixed/stained (self-on-self), and unixed versus fixed/stained samples gave correlation coefficients of 0.97, 0.977 and 0.95 respectively. It is clear that there is more scatter when RNAs from unixed and fixed/stained cells are compared than in the self-on-self comparisons. Therefore, it is best to design experiments in which fixed tissues are compared to one another.

As a preliminary test of the general utility of our fixation and staining methods for microarray work, we stained rat brain sections with an anti-oxytocin–neurophysin antibody, and excised samples of the supraoptic nucleus (SON, Figure 5A–C) and adjacent optic chiasm (CHI) from the same sections using a Leica AS LMD (Laser Microdissection) microscope. We chose these brain regions because they are easy to recognize, well circumscribed and fairly homogenous. The SON is comprised of large neurons that, for the most part, synthesize either vasopressin or oxytocin. These cells send their axons through the median eminence to the posterior pituitary where they release their peptide products into the periphery. The CHI, on the other hand, is made up of myelinated axons. Most of the cells there are oligodendrocytes. RNAs extracted from the SON and CHI samples were subjected to two rounds of amplification, and 20 μg of each product were used for probe labeling. We examined elements on the array that represent genes known to be especially abundant in either the SON—vasopressin, oxytocin, dynorphin, neuronatin (17)—or in myelin/oligodendrocytes—myelin basic protein, proteolipid protein, 3′-cyclic nucleotide 3′-phosphodiesterase (18). The results of this analysis were consistent in several independent experiments. Data from two separate representative experiments are shown in Table 1; SON-specific genes were much more abundant in SON samples than in those from the CHI and vice versa.

A critical test of our method, of course, was to isolate two populations of neurons with known differences in gene expression and to determine whether these differences could be detected with arrays. For this purpose, we chose the vasopressin (VP)- and oxytocin (OT)-producing neurons in the SON. Based on the work of others (19) and our own experience (data not shown), we inferred that quantitative comparisons of two samples could not be undertaken with >1–10 ng

![Image](https://via.placeholder.com/150)

**Figure 5.** Microdissections of DSP-fixed hypothalamic sections. Immunostained oxytocinergic (A) and vasopressinergic (D) neurons in the supraoptic nucleus. Images B and E are photographs of the same sections shown in images A and D respectively, after microdissection of the entire SON (B) or 18 individual vasopressin-positive neurons (E). Images C and F show the dissected samples in the collection vessels. All of the photomicrographs were made with the microscopes used for the microdissections. The sections were not cover slipped. The stained cells are easier to distinguish when you examine the sections with the microscope than they are in the micrographs. The size scale in box C (for images A–C) is 80 μm long, and the scale in box F (for images D–F) is 100 μm long.

<table>
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<th>Clone title</th>
<th>CalR1</th>
<th>CalR2</th>
<th>S1-SON</th>
<th>S1-CHI</th>
<th>S2-SON</th>
<th>S2-CHI</th>
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<td>160.40</td>
<td>4019.60</td>
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<td>Vasopressin</td>
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<td>119.00</td>
<td>2834.60</td>
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<td>Dynorphin</td>
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<td>340.10</td>
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<td>16561.80</td>
<td>4586.00</td>
<td>37717.40</td>
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The supraoptic nucleus (SON, see Figure 3) and optic chiasm (CHI) were laser microdissected from DSP-fixed tissue slices stained with an anti-oxytocin–neurophysin antibody, and RNAs extracted from the samples were amplified and used for probe labeling. The SON (Cy5) and CHI (Cy3) probes from the two samples were combined and hybridized to a microarray. Raw signal intensities (S) from the SON and CHI samples, and the ratios of the calibrated signal intensities (CalR; SON/CHI) are shown. Genes that are known to be expressed in the SON gave high CalRs and genes known to be expressed in myelin (three among many observed to be different) gave low ones. Results from two independent experiments are shown.
of RNA template. Consequently, we used the Arcturus Laser Capture Microdissection technique to remove ~200 oxytocinergic neurons and 400 vasopressinergic neurons from sections stained with antibodies PS 36 (anti-oxytocin–neurophysin) or PS 41 (anti-vasopressin–neurophysin), respectively (see Figure 5D–F). Two rounds of RNA amplification yielded 31 μg of RNA from the OT sample and 70 μg of RNA from the VP one. To prepare probes, 30 μg of each were used, and these were mixed and hybridized to an 11 136-element array. The VP cell probe gave a VP signal (with background subtracted) that was much stronger than that seen with the probe from OT cells—4467 versus 586 (see Figure 6). OT cells, on the other hand, gave an OT signal that was much stronger than the one from VP cells—3918 versus 520.

**DISCUSSION**

We conclude that, unlike ethanol, DSP fixes soluble antigens and protects RNA in tissue sections—even ones that have been immunostained. Furthermore, we agree with others who have reported that is difficult to extract intact RNA from formalin-fixed tissue. Although such tissue is easy to stain, even brief emersion in a dilute (0.1%) formalin solution prevented us from isolating significant amounts of RNA (data not shown).

DSP-fixed, immunostained, laser-captured tissue samples yielded RNA that could be amplified efficiently, and the amplified material gave expression profiles resembling those seen with unfixed/unstained samples. Furthermore, genes that are differentially expressed in the SON versus chiasm or VP versus OT cells could be shown to have different expression levels in our array studies of DSP-fixed tissue samples. One might have expected the ratio of the VP signals from VP versus OT cells to be much larger than 7; the same thing is true of ratio of the OT signals from OT versus VP cells. When Xi et al. (20) used a quantitative RT–PCR method to measure VP and OT mRNAs in single dissociated magnocellular neurons harvested with glass micropipettes, they found average VP/OT mRNA ratios of 182 in VP cells, and average OT/VP mRNA ratios of 256 in OT cells. The six VP cells studied contained 0.232–1.368 amol of VP mRNA; the four OT cells examined had 0.136–1.211 amol of OT. One of the 11 cells isolated had similar levels of VP and OT—0.237 and 0.106 amol, respectively. If ‘ambivalent’ neurons of this sort make up about 10% of the magnocellular population, and if they contain about as much VP and OT as the cells that make one of these peptides preferentially, then one might expect the VP/OT ratio in VP cells and the OT/VP ratio in OT cells to be about 10, close to the number we obtained. On the other hand, it is possible that the ratios we found were lower than they should have been because we underestimated the background levels associated with DNA elements, or used too little RNA in our amplification reactions to give quantitative results. A greater number of cells may have to be dissected in future studies to overcome the latter problem. Finally, our dissections may not have been as clean as Xi’s. Small fragments of adjacent or overlapping neurons may have remained attached to cells obtained by means of the Arcturus method; and, worse yet, loose bits of debris on the surface of sections may have stuck to the cap onto which the cells were harvested. If small numbers of cells are collected, contamination by tiny tissue fragments could have a dramatic effect on the expression profiles observed. To prevent this, we scanned the caps that we used for ‘dirt’ and attempted to brush it away when we found any, but we may not have succeeded completely.

It is worth noting that the volume of lysis buffer used in the experiments designed to measure RNA recovery from sections was 50× greater than the volume of the tissue extracted. When individual magnocellular neurons were captured and pooled in subsequent experiments, the volume of lysis buffer used to extract them was 1000× greater than the volume of the cells. This should result in very efficient RNA isolation, but we have no way to determine this directly.

In spite of the caveats listed above, our results are quite encouraging, and what works for brain will probably work for most tissues. Whether our techniques can routinely be used for quantitative comparisons of samples remains to be seen, but we should surely be able to use them to characterize the genes expressed by specific cell populations with microarrays, and to obtain samples for quantitative PCR measurements.

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