Enabling Coupled Quantitative Genomics and Proteomics Analyses from Rat Spinal Cord Samples

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Translational research is progressing toward combined genomics and proteomics analyses of small and precious samples. In our analyses of spinal cord material, we systematically evaluated disruption and extraction techniques to determine an optimum process for the coupled analysis of RNA and protein from a single 5-mm segment of tissue. Analyses of these distinct molecular species were performed using microarrays and high resolution two-dimensional gels, respectively. Comparison of standard homogenization with automated frozen disruption (AFD) identified negligible differences in the relative abundance of genes (44) with all genes identified by either process. Analysis on either the Affymetrix or Applied Biosystems Inc. gene array platforms provided good correlations between the extraction techniques. In contrast, the AFD technique enabled identification of more unique proteins from spinal cord tissue than did standard homogenization. Furthermore use of an optimized CHAPS/urea extraction provided better protein recovery, as shown by quantitative two-dimensional gel analyses, than did solvent precipitation during TRIzol-based RNA extraction. Thus, AFD of tissue samples followed by protein and RNA isolation from separate aliquots of the frozen powdered sample is the most effective route to ensure full, quantitative analyses of both molecular entities. Molecular & Cellular Proteomics 6:1574–1588, 2007.

Expression analysis is a critical component of studies investigating biological mechanisms, including clinically relevant processes underlying disease, injury, and repair. Historically analyses of protein expression have often relied on inference from the characterization of mRNA changes. Molecular analysis of RNA has rapidly evolved from classical techniques such as the Northern blot to current large scale, high throughput microarray analyses (1–4). Proteomics offers an alternate means of expression analysis (5–9). As a direct rather than surrogate measure of protein expression, proteomics has evolved, like genomics, toward the capacity for routine, reliable, large scale discovery science. Both analytical paths have their own advantages and disadvantages and their own implications concerning data analysis and interpretation. Clearly quantitative, effectively coupled analyses would provide the most robust, critical, “systems-level” evaluation of a given sample set.

Coupled genomics and proteomics analyses of biological samples should thus satisfy both analytical pathways and, even more importantly, offer the most rigorous and comprehensive molecular assessments possible (10). A practical consideration in such coupled studies is whether the same sample can be used for both analyses (the “ideal” situation) or whether the nature of one analysis or the other necessitates the parallel generation of a separate sample. This consideration is even more critical for researchers investigating molecular processes in exceedingly small, valuable, and/or difficult to obtain biological material. Ideally the same sample could then be used in both analyses not only in the interest of practicality but also reproducibility and accountability between methods. To achieve this, the sample must somehow be randomized (“homogenized” in terms of handling tissues) such that each analysis is carried out on a representative portion of the original. The question then becomes whether the randomized sample must be partitioned and prepared independently for each application or if one extraction pathway is suitable for both RNA and protein analyses.

There are benefits and disadvantages in utilizing a single methodology for sample preparation in coupled analyses. Using one protocol for both RNA and protein extraction offers the benefit of requiring less starting material while still tracking RNA and protein expression profiles from the same single sample. However, it begs the question of whether a single protocol provides the breadth, depth, and resolution of analyses that may have been obtained had distinct procedures, optimized for RNA and/or protein extraction, been used. Can a single preparation method yield the same result as separate,
specialized procedures? All too commonly the use of a single methodology for coupled analysis is regarded as a compromise. Realistically it will be unacceptable to most researchers to reduce quality or certainty in one analysis despite optimizing the other.

Methods of extraction and preparation of either RNA or protein from biological samples have been thoroughly explored and characterized (1, 11). However, reports on concurrent RNA and protein extraction from small, valuable biological samples are relatively few, and none have critically evaluated the detailed, quantitative analyses of both types of molecule (10, 12, 13). In one study coupling genomics and proteomics analysis, the central proteomics analysis described differential enrichment in high and low molecular weight proteins concurrent with RNA extraction (10). In this case the focus was on cataloguing only a select portion of the proteome rather than the global analysis that must be undertaken if these methods are to effectively assist in delineating underlying molecular mechanisms. In a very recent study, the proteomics analysis was limited to a qualitative assessment of the sample by two-dimensional gel electrophoresis (2DE), and no fully comparative, quantitative proteomics analyses were carried out (13).

Nonetheless the rationale for extraction of both RNA and protein from the same sample is clear if one wishes to determine cause-and-effect relationships; specifically our interest is the effect of injury on gene and protein expression in the spinal cord. Thus, the focus here is on rat spinal cord samples, and our long term aim is to better understand the native complexity of this tissue as well as the diversity of molecular alterations that occur after injury to better assess treatment of spinal cord injury with neuroprotective agents administered postinjury. The goal of our initial investigation was to identify optimized techniques that would provide a full, rather than selective, means of coupled, quantitative genomics and proteomics analyses on the same tissue sample.

An avenue worthy of exploration is the use of RNA stabilization compounds. Many RNA species are extremely labile with half-lives of only hours or minutes in aqueous solution generally due to the activity of endogenous or exogenous (contaminating) RNase. Thus methods of stabilizing RNA are critical if global analyses representative of the native biological complexity are to be achieved. Generally RNA stabilization reagents operate by impeding RNase activity. In this regard, reagents such as TRIzol (Invitrogen) can be considered RNA-stabilizing agents as they act by nonspecifically denaturing proteins and thus disrupting enzyme (including RNase) activity. However, because the efficacy of TRIzol as a preparation technique was directly assessed in this study (see below), we also elected to test an independent method of RNA stabilization: RNAlater, a tissue storage reagent known to stabilize and protect RNA, is also commercially available (Ambion, Foster City, CA). Advantages of this approach include immediate and long term stability of the sample (eliminating the need to immediately freeze the sample) and its cryoprotectant properties that improve the longevity of the sample through multiple freeze-thaw cycles; these properties naturally appeal to laboratories collaborating at a distance. In addition, RNAlater is also attractive because it is relatively safe, non-toxic, and inexpensive.

The so-called TRI reagents are another attractive technology given our goals (10, 13, 14). These compounds are commercially available and marketed as a means of simultaneously extracting RNA/DNA and protein from a single biological sample, yielding products that the manufacturer claims are appropriate for both genomics and proteomics analyses, respectively. These solutions consist minimally of a monophasic solution of phenol and urea and are often combined directly with the biological sample during homogenization. The solution arrests RNase activity by rapid and thorough denaturation of proteins, thus preserving low abundance or labile mRNAs. TRI reagents, such as TRizol, are used predominantly in situations where RNA/DNA extractions and some form of genomics analysis are the primary goals (10, 13, 14). Few groups routinely use a TRI reagent when proteomics analysis is the primary goal (15), and no global characterization of the quality of TRizol-extracted proteomes has been described (10, 12, 13). Indeed it is generally recognized that specific protein losses occur with any kind of organic-based protein denaturation protocol, immediately calling into question the suitability of these extracts for truly quantitative, global proteomics analyses. Nonetheless many groups continue to routinely extract protein from biological samples using somewhat comparable organic precipitation protocols, and some select advantages to this practice are suggested (16, 17).

A third possible technology of immediate interest in proteomics but historically also in mRNA, lipid, and carbohydrate analyses is so-called automated frozen disruption (AFD) (18). Essentially AFD is a method of thoroughly powdering tissue samples in a deep frozen state, akin to what is commonly done with a liquid nitrogen-chilled mortar and pestle, but offers a mechanized variation that is much more thorough, reproducible, and appropriate for even smaller samples (18–22). The advantage of mechanical disruption in a deep frozen state is clear: at this temperature enzyme activity in the biological sample is essentially nonexistent; certainly it is extremely reduced from that at physiological temperature or even on ice. Moreover, nonspecific degradation of proteins seems not to occur during AFD because once thawed enzyme activity is restored (23). It was recently demonstrated that AFD is the most appropriate available tissue disruption strategy for
proteomics analyses (by 2DE), enabling the efficient handling of even very small and/or very hard, dense tissue samples (18, 20, 22). It has additionally been reported that AFD is the optimal tissue disruption strategy in terms of total RNA extraction especially so from denser or otherwise difficult to homogenize materials (20, 22). However, a detailed analysis of AFD in terms of its suitability for coupled protein and RNA analyses and for RNA analyses by microarrays has not been reported.

Finally another potential analytical path is also of critical interest for proteomics analyses: sample prefractionation has been demonstrated to be of immediate and substantial benefit for gel-based proteomics. Briefly sample prefractionation strategies separate proteomes into physically (e.g. size, density, and cellular localization), chemically (e.g. hydrophobicity, charge properties, and specific proteins), or otherwise defined fractions prior to resolving proteins in each of these separate fractions (24). Chief among the advantages of this approach is improved resolution and detection of low abundance proteins: many proteins that fall below detection sensitivity in separations of the total proteome are effectively concentrated by virtue of the sample prefractionation strategy and are thus detectable in an optimized separation of the defined fraction. However, prefractionation strategies are not without their own complications. The approach necessarily introduces more steps into the analytical process, increasing the potential for error; even with inhibitors present, the additional handling will nonetheless enhance the potential for nonspecific proteolysis and/or artifactual modifications. Moreover, a proteomics analysis involving prefractionation inherently requires a larger amount of starting material than one without; because no prefractionation process is 100% efficient, the additional handling compounds losses, and thus enough starting material must be present so that analyses can be carried out on each individual fraction. Thus, sample prefractionation becomes a challenging prospect when small, rare, and/or expensive tissue samples are to be analyzed as was the case in the present study. A prefractionation strategy separating tissue proteomes into physically defined total membrane and soluble protein components has been thoroughly characterized as practical, rapid, and effective (8, 18, 25), but its viability for the analysis of such scarce amounts of starting material, further limited by the constraints of coupled genomics and proteomics analyses, has not yet been explored.

Thus we systematically tested these available methods, and combinations thereof, with the goal of optimizing quantitative, coupled genomics and proteomics molecular analyses from small tissue samples of rat spinal cord (see Fig. 1). It is hoped that an optimized methodology will lay the groundwork for more detailed, systems-level analyses of the native spinal cord (and other tissues) and ultimately for coupled analyses of spinal cord injury and recovery processes.

**RNA and Protein Analyses of a Single Tissue Sample**

**EXPERIMENTAL PROCEDURES**

**Experimental Animals**—Male Sprague-Dawley rats (University of British Columbia Animal Facility, Vancouver, British Columbia, Canada) weighing 260–300 g were used. All animal procedures were performed in accordance with the guidelines of the Canadian Council for Animal Care and approved by the institution’s animal care committee.

**Surgery**—For the spinal cord injury sham procedure, rats were anesthetized with an intraperitoneal injection of ketamine hydrochloride (72 mg/kg) and xylazine hydrochloride (9 mg/kg), and all surgery was carried out under sterile conditions. Once anesthetized, the spinal cord was exposed, and the lamina of T9 was removed with fine rongeurs. Subsequently the rat was held for 10 min by clamping the dorsal process of T8 and T10 with Allis clamps (equivalent to the time needed to perform a contusion injury). No impact was given to these sham control animals. The skin incision was subsequently closed using wound clips. Postoperative care included keeping the animals on a thermostatically regulated heated pad until completely awake, the administration of 6 ml of Ringer’s solution subcutaneously, expressing the bladder three times daily, checking for signs of infection, and monitoring postoperative weight loss.

**Spinal Cord Segment Collection**—Spinal cord segments were collected 24 h postsurgery. To minimize degradation of spinal cord samples, anesthetized rats were perfused intracardially with 100 ml of cold (4 °C) PBS, which lowered body temperature −10 °C within 1 min. The rats were then placed on ice, and a laminectomy of the entire thoracic and lumbar spinal cord was performed. The isolated spinal cord kept at 4 °C while a 5-mm segment (the “injury site” was the epicenter) was excised. The excised segments were placed in precooled (4 °C) sterile RNase/DNase-free microcentrifuge tubes and immediately flash frozen in liquid N2. All cord segments were stored at −70 °C until further processing. To evaluate the suitability of RNAlater for coupled analyses, some samples (where indicated) were treated with the protective agent at three points during sample collection. (i) The animals were perfused with 25 ml of cold (4 °C) RNAlater (Ambion) immediately following standard PBS perfusion, (ii) the spinal cord was bathed in RNAlater immediately after it was exposed during surgery and again following the laminectomy, and (iii) excised spinal cord segments were soaked in 0.3 ml of fresh RNAlater at 4 °C overnight before the bath was removed and the tissue was stored at −70 °C.

**Sample Preparation Conditions**—Three combinations of existing preparative techniques were tested (Fig. 1): Condition 1, standard homogenization (SH): in the presence of TRizol the tissue was macerated in a microcentrifuge tube using a fitted pestle; Condition 2, AFD followed by TRizol extraction (AFD-T) of the frozen pulverized tissue; and Condition 3, AFD followed by direct CHAPS/urea extraction/solubilization (AFD-D) of the frozen pulverized tissue.

**Standard Homogenization**—TRizol extraction of total RNA and subsequent extraction of protein was carried out essentially according to the manufacturer’s specifications (Invitrogen) with minor modifications (SH, Condition 1). To a 5.0-mm spinal cord segment (3-mm diameter), 1 ml of TRizol was added, and the sample was homogenized with a disposable pestle for 3 × 15 s using continuous rotary motion supplied by a small electric motor (Pellet Pestle Cordless Motor, Kontes, Vineland, NJ). Following incubation for 5 min at room temperature on a rotator, 0.2 ml of chloroform was added, and the tubes were shaken for 15 s and then incubated for 3 min while rotating at room temperature. This mixture was then centrifuged at 12,000 × g for 15 min at 4 °C. The aqueous phase was transferred to a new tube, and the lower organic phase (Fraction 1) was saved for protein extraction. Isopropanol (0.5 ml) was added to the aqueous phase, and the sample was mixed by inversion and then incubated for 10 min at room temperature prior to centrifugation at 12,000 × g for
10 min at room temperature. The supernatant (Fraction 2) was removed to a new tube and kept for protein extraction. The RNA pellet was washed with 1 ml of 75% ethanol, centrifuged at 7,500 × g for 5 min at room temperature, and air-dried in sterile conditions for 5-10 min. The RNA pellets were dissolved in 20 μl of RNase-free water and stored at −80 °C.

Protein was isolated from the two protein-containing fractions by isopropanol precipitation. First DNA was removed from the organic phase of Fraction 1 by the addition of 0.3 ml of 100% ethanol. The sample was mixed by inversion, incubated for 3 min at room temperature, and centrifuged at 2,000 × g for 5 min. The phenol/ethanol phase was removed and combined with the supernatant from the RNA precipitation stage (Fraction 2). To this mixture, 2.5 ml of isopropanol was added, the sample was mixed and incubated for 10 min, and the precipitated protein was pelleted at 12,000 × g for 10 min. The supernatant was removed, and the pellet was washed three times using 1 ml of 0.3 M guanidine HCl with 20-min incubations. After centrifugation at 7,500 × g for 10 min the sample was vacuum dried and stored at −80 °C.

Automated Frozen Disruption—Spinal cord segments were mechanically disrupted in the deep frozen state using a Mikro-Dismembrator (B. Braun Melsungen AG, Melsungen, Germany) (18). Briefly a self-sealing Teflon chamber containing a stainless steel ball was prechilled in liquid N2. The deep frozen sample was loaded, and a vibrational frequency of 40 Hz was applied for 60 s. The resulting pulverized cord sample was divided equally: one aliquot of frozen powdered tissue for RNA and protein extraction using TRIzol (AFD-T, Condition 2) and the other for direct extraction of the protein using a CHAPS/urea extraction (AFD-D, Condition 3). In each case the sample remained deep frozen until the addition of the required reagent: either TRIzol or a CHAPS/urea solution (see below), respectively. In this case, the TRIzol extraction was performed as described above except 0.5 ml of TRIzol was used, and all other subsequent reagent additions were halved.

CHAPS/Urea Extraction—Powdered frozen tissue homogenates were thawed directly in 2DE solubilization buffer containing 8 M urea, 2 M thiourea, 4% CHAPS, and a broad spectrum protease inhibitor mixture (25, 26). Precipitated and vacuum-dried protein isolates from TRIzol extracts were solubilized in the same manner. The sample was vortexed vigorously for 20 min at room temperature and then centrifuged at 12,000 × g at 4 °C for 10 min to pellet and remove any detergent-insoluble material. Although no visible pellet was observed, the supernatant was isolated into a fresh tube for subsequent analysis.

Sample Prefractionation—Sample prefractionation was carried out essentially as described by Butt and Coorsen (25). Briefly the powdered frozen tissue homogenates were thawed directly into 2DE solubilization buffer containing 8 M urea, 2 M thiourea, 4% CHAPS, and a broad spectrum protease inhibitor mixture (25, 26). Precipitated and vacuum-dried protein isolates from TRIzol extracts were solubilized in the same manner. The sample was vortexed vigorously for 20 min at room temperature and then centrifuged at 12,000 × g at 4 °C for 10 min to pellet and remove any detergent-insoluble material. Although no visible pellet was ever observed, the supernatant was isolated into a fresh tube for subsequent analysis.

RNA Analyses: Microarrays—RNA quality was initially assessed using spectrophotometric readings and separation on 1% denaturing formaldehyde-agarose gels and subsequently confirmed by analysis on an Agilent 2100 Bioanalyzer. At the British Columbia Genome Sciences Centre, individual RNA samples (3 μg each) were processed on the Affymetrix rat 230 2.0 chip using the manufacturer’s recommended protocols. For processing on the Applied Biosystems Inc. (ABI) rat arrays, 200 ng of RNA was used, and sample processing was performed by the Winship Cancer Institute DNA Microarray Core Facility (Emory University School of Medicine, Atlanta, GA) using the manufacturer’s recommended protocols. Triplicate samples were analyzed on individual arrays unless noted otherwise.

Protein Analyses: 2D Gel Electrophoresis—Total solubilized protein was quantified using the EZ-Q protein quantitation assay (Molecular Probes, Eugene, OR). Thus, sample normalization was achieved by the handling of consistent amounts of total protein for every sample. Uniformly, 100 μg of protein in solubilization buffer was combined 1:1 with an ampholyte buffer containing 8 M urea, 2 M thiourea, 4% CHAPS, protease inhibitor mixture, 1% broad range carrier ampholytes (pH 3–10), and 0.2% each of five narrow range ampholytes (pH 2.5–4.0, 3.5–5.0, 5.0–7.0, 7.0–9.0, and 8.0–9.5) such that the final concentration of ampholytes was 1% (w/v). Samples were sequentially reduced and alkylated with (i) 2.6 mM tributylphosphine and 45 mM dithiothreitol and (ii) 230 mM acrylamide, respectively, for 1 h each at 25 °C. Sample loading was by passive hydration onto 7-cm (miniformat) linear pH = 3–10 IPG strips (Bio-Rad) for 10 h at 25 °C.

Isoelectric focusing was carried out at 17 °C using the Bio-Rad Protean IEF cell. A constant current of 50 μA/gel was applied during the ramping phase: 250 V maximum for 15 min (desalting) and linear ramping to 4,000 V maximum over 2 h. Electrode wicks were changed periodically to ensure the effective removal of trace salts and enable smooth ramping. Once attained, a constant voltage of 4,000 V was applied for 37,500 V-h. IEF run data were captured in real time with a personal computer using an RS232 serial connection protocol.

Following IEF, IPG strips were equilibrated according to standard protocols (8, 18, 25). Briefly equilibration solution consisted of 6 M urea, 20% glycerol, 2% SDS, and 375 mM Tris-HCl (pH 8.8); the solution was supplemented with 2% dithiothreitol for the first wash, and this was then replaced with equilibration solution containing 2.5% acrylamide for the second wash; each equilibration wash was carried out at room temperature for 10 min.

Two alternate second dimension separations were used. First standard minigels (5.5 cm) were used for initial separations. For greater resolving power, large format (20 cm) gels were used in later experiments. All gels consisted of a 12.5% T, 1.6% C acrylamide for the second wash; each equilibration wash was carried out at room temperature for 10 min.

Although stacking gels were used, two alternate second dimension separations were used. First standard minigels (5.5 cm) were used for initial separations. For greater resolving power, large format (20 cm) gels were used in later experiments. All gels consisted of a 12.5% T, 1.6% C acrylamide resolving gel with a 5% T, 1.6% C stacking gel. Both separating and stacking gels were buffered with 375 mM Tris-HCl (pH 8.8) (25, 27). Electrophoresis was carried out at 4 °C at a constant current of 16 mA/gel during migration through the stacking gel; once stacking was complete, the separation was continued at 24 mA/gel (with a maximum potential of 300 V) to completion.

Slight modifications to existing SYPRO Ruby staining protocols for minigels were incorporated to achieve similar performance with large gels. Briefly gels were fixed by immersion in 7% acetic acid and 10% methanol for 1 h with gentle rocking. The solution was discarded and replaced, and fixation was continued for 12 h. Gels were washed with distilled water a total of four times for 30 min each before staining with SYPRO Ruby for 12 h overnight. Gels were briefly destained in fixative solution for 15 min and washed with distilled water a total of four times for 30 min each. SYPRO Ruby was excited in the visible spectrum (480 nm) and a digital image was captured (620 nm) using the ProXPRESSION Proteomic Imaging System (Genomic Solutions, Boston, MA). At this point all gel images were normalized by making...
small adjustments to the exposure time to achieve a common maximum fluorescence signal intensity.

**Digital Image Analysis**—Automated analysis of digital images was carried out using the average analysis mode of Progenesis Work station version 2005 software (NonLinear Dynamics, NewCastle, UK). Briefly all 2D gel separations were carried out in triplicate, and relative “average gels” were created from these replicates for mathematical/statistical comparison. For inclusion, observed protein spot differences between average gels must be both significant (t test, \( p < 0.05 \), \( n = 3 \)) and 100% reproducible across the relevant data set. For convenience, only differences 2.5-fold or greater were labeled on gel images, but all differences meeting inclusion criteria are reported. By establishing rigid inclusion criteria, the most significant differences between resolved proteomes are highlighted, and the inclusion of artifactual differences is minimized.

**RESULTS**

To most effectively enable fully coupled, quantitative genomics (RNA/microarray) and proteomics (2DE) analyses, we systematically evaluated widely used tissue handling, homogenization, and extraction techniques (Fig. 1). Our immediate goals were detailed, global (i.e., systems-level) genomics and proteomics analyses of (5-mm) rat spinal cord samples.

**RNA Analysis**—Approximately 10–15 μg of RNA was recovered from each 5-mm segment of spinal cord using the SH extraction procedure (Fig. 2A). RNA analysis indicated \( A_{260/280} \) ratios of 1.75–1.85 with RNA integrity numbers between 8.6 and 9.3 (Agilent 2100 Bioanalyzer). RNA profiles yielded rRNA (28 S/18 S) ratios of 1.1–1.3 with a noted amount of small (50–200-nucleotide) RNAs. The presence of degraded RNA having less than 50 nucleotides was not seen in any of our samples. From samples processed by AFD, half of the resulting powder (the equivalent of 2.5 mm of tissue) was processed by TRIzol extraction for RNA and protein analyses (AFD-T), whereas the other half was processed directly for protein analysis (AFD-D). Both the yield and the quality of RNA recovered from the AFD-T samples were equivalent to that of the RNA derived from the SH samples (Fig. 3). RNA recovered from direct CHAPS/urea extraction of AFD-prepared material (AFD-D) yielded RNA quantities of less than 3 μg/segment (Fig. 2); therefore this method was not pursued further, and only direct TRIzol extracts were compared in microarray analyses.
RNA samples were subsequently analyzed using both the Affymetrix rat 230 2.0 microarrays and the ABI rat GeneChip platforms. The two platforms, Affymetrix and ABI, both enable efficient and reproducible detection of differential gene expression. However, due to the limited availability of RNA, the ABI GeneChips were run using 200 ng of total RNA (low RNA protocol) compared with the 3 μg of RNA used on the Affymetrix arrays (standard RNA protocol). Hence we expected the ABI platform to have more variability and thus less ability to detect differences. Comparisons of log intensities between AFD-T and SH protocols using both the Affymetrix and ABI chips are shown (Fig. 3). The correlation coefficient for the ABI data is 0.988 (Fig. 3A), and the correlation coefficient for the Affymetrix data is 0.995 (Fig. 3B). The chip-to-chip correlation of log intensity within each platform was high (0.975–0.966 for ABI and 0.987–0.986 for Affymetrix but low between platforms (0.650–0.668)). Nonetheless overall both platforms indicated a good correlation between the two disruption techniques tested: SH and AFD-T.

A set of 12,484 common genes were identified by matching gene identities from annotation files of the ABI and Affymetrix array results, again showing that both array systems identified a high degree of similarity between the tissue disruption techniques that were compared. A comparison of the distribution of log ratios of the two disruption methods indicated that ABI results had a wider range of log ratio distribution compared with the Affymetrix results (data not shown).

In each platform, a modified t test was used to identify alterations in RNA abundance differences between the two extraction methods (Fig. 4). Using a 2-fold change and a p value of 0.01 as cutoff criteria, 63 genes were identified as significantly up-regulated between the disruption methods (SH relative to AFD-T) on the Affymetrix arrays. Using the same criteria on the ABI platform, 104 genes showed increased abundance and 43 genes showed reduced abundance in SH relative to AFD-T. After a Benjamini and Hochberg correction of multiple testing for false discovery rate (28), the Affymetrix arrays had 44 genes showing increased abundance in SH relative to AFD-T, whereas the ABI arrays identified none. Nonetheless both array types identified the same set of common genes from both tissue preparation strategies.

Proteomics Analysis—In early experiments, the rat spinal
cord tissue was prefractionated into gross membrane and soluble protein fractions as described by Coorssen and co-workers (8, 18, 25, 29) to determine the feasibility of this approach for downstream analyses (Fig. 1). A total protein assay indicated that the amount of membrane protein exceeded the amount of soluble protein by a factor of ~12-fold in these 5-mm spinal cord segments. Moreover, the amount of soluble protein available from a single cord was only ~1/4 of that minimally required for a single, high resolution miniformat 2D gel. Thus, to carry out gel-based proteomics analysis of the soluble protein fractions, four to five spinal cord segments would minimally need to be pooled to carry out a single separation. As this was impractical with the current sample set and initial experimental design, we eliminated sample prefractionation from this coupled analysis of rat spinal cord tissue. Thus all proteomics analyses were carried out on total protein samples.

RNAlater was initially used as a means of controlling possible RNA degradation (Fig. 1), which was never seen in any samples; however, perfusion/bathing of the spinal cord sample with RNAlater dramatically affected the subsequent resolution of proteins by 2DE as seen even by simple visual inspection (Fig. 5, A and B). The consequence of having RNAlater in the sample can be divided into four major effects: (i) substantially retarded voltage ramping during the IEF separation (slow separation; Fig. 5C), (ii) horizontal and vertical streaking (poor first and second dimension resolution); (iii) pattern distortion (changes in protein spot position and large tracts of “missing” proteins; Fig. 5, A and B), and (iv) a high degree of sample-to-sample variability in the extent of the different effects noted in (ii) and (iii) above (data not shown).

To more firmly establish the source of the observed problem, RNAlater was spiked directly into protein isolates from spinal cords previously unexposed to the additive immediately prior to electrophoretic separation. The rate of voltage ramping during IEF was impeded, in a dose-dependent manner, by this direct addition of RNAlater (Fig. 5C). The pattern distortions characteristic of RNAlater perfusion were also apparent in separations from samples spiked with the additive postextraction (Fig. 5, D–G). The severity of the horizontal streaking and pattern distortion were correlated with both the difficulty of voltage ramping during IEF and the concentration of RNAlater added. Although at low concentrations of RNAlater, proteomic maps retain some degree of similarity to controls (e.g. samples spiked with 0.5% RNAlater exhibit ~74% total spot correlation with untreated parallel controls derived from the same sample; Fig. 5, D and E), separations carried out in the presence of higher doses of the additive became progressively dissimilar to the control to the point that the quality of the separation was so poor as to render it effectively useless for comparative analytical purposes. The perfusion with and storage of samples in RNAlater during spinal cord collection was omitted from all subsequent analyses.

Thus two potential analytical routes were eliminated, and the focus became assessing the identified methods of tissue homogenization and RNA/protein extraction (summarized in Fig. 1). SH was coupled exclusively with TRIzol extraction, which is a widely accepted standard protocol for RNA extractions. No attempt to extract protein directly from SH preparations was made because the superiority of AFD relative to

![Fig. 4. Comparison of differentially expressed genes between ABI (A) and Affymetrix (B) gene arrays.](image-url)
such classical homogenization methods has been exhaustively demonstrated for proteomics analyses (18). However, neither the suitability of AFD for RNA extractions of the spinal cord material nor the potential of TRIzol extractions to “rescue” proteins from classically homogenized samples has been investigated. Thus AFD was coupled with TRIzol extraction (AFD-T) for comparisons with SH. Finally suitability of TRIzol extractions for broad scale comparative proteomics analyses has not been rigorously explored. Thus the spinal cord segments were additionally homogenized by AFD and directly solubilized (AFD-D) using 2DE solubilization buffer as is the standard protocol for optimal gel-based proteomics analyses (18, 25).

Protein yields differed significantly between these three preparative methods (Fig. 2). AFD-D yielded the most protein per cord sample, AFD-T yielded slightly less, and SH yielded the least total protein, although in all cases the material available was sufficient for replicate gels (Fig. 2). At the level of 2D gel electrophoresis and automated protein spot detection, total numbers of proteins detected in the gels generated from the three different handling methods were statistically indistinguishable (Fig. 6A). On average a total of 1,573 ± 38 spinal cord proteins were resolved and detected (n = 9). However, for comparative purposes we eliminated spots for which detection was variable between gel replicates to strictly limit any potentially spurious data. Thus, proteins that were detected

Fig. 5. Effect of RNAlater on 2DE performance. Shown is miniformat 2DE of rat spinal cord segments following perfusion and washing without (A) and with (B) RNAlater during surgery and after excision. To independently assess the effect of RNAlater on 2DE, spinal cord segments previously not exposed to the additive were spiked immediately prior to 2DE. C, voltage ramping during isoelectric focusing (first dimension) with respect to the concentration of RNAlater. The severity of characteristic gel distortion and poor resolution resulting from RNAlater contamination is correlated with the concentration of the contaminant: D, control; E, 0.5% RNAlater; F, 1% RNAlater; G, 2% RNAlater.
with less than 100% reproducibility within a given sample set \((n = 3)\) were eliminated. Subtle but significant differences in the number of 100% reproducible spots between the three preparation conditions were observed: the fewest reproducibly resolved and detected proteins were in the SH preparation, and the greatest number were in the AFD-D preparation (Fig. 6B). These data suggest that there must be proteins that are undetectable in some preparations relative to the others.

Thorough automated differential image analysis was carried out to annotate the specific protein spots underlying these differences between preparation methods. To further limit potential artifacts, only significant quantitative differences in those proteins reproducibly detected in all cases \((t\text{-test}, p < 0.05, n = 3)\) were reported. In comparisons between SH and AFD-T preparations, the objective was to identify the optimal homogenization strategy for TRizol-extracted protein samples. Despite overt visual similarity, a total of 58 proteins were significantly different between SH and AFD-T preparations (Fig. 7). Of these, two were resolved and detected only in the SH preparation (absent from AFD-T), whereas 12 were resolved and detected only in the AFD-D preparation (absent from SH; Fig. 7). The remaining 44 proteins were detected in both preparations but differed significantly in relative abundance (Fig. 7); of these, six proteins showed a greater than 2.5-fold change (Fig. 7). All quantitative differences are summarized in Supplemental Table 1.
In comparisons between AFD-T and AFD-D preparations, the objective was to determine the suitability of TRIzol extraction for broad scale comparative proteomics analyses. Although the two preparations produced visually similar protein spot patterns in 2DE separations, automated image analysis indicated extensive quantitative differences between the two (Fig. 8). A total of 241 proteins met our inclusion criteria in comparisons of AFD-T with AFD-D, representing fully 1/4 of the 100% reproducibly detected proteome (Fig. 8). Of these, 22 proteins were resolved and detected only in the AFD-T preparation (absent from AFD-D), whereas a total of 54 proteins were resolved and detected only in the AFD-D preparation (absent from AFD-T; Fig. 8, A and D, and Supplemental Table 2). The remaining 165 quantitative differences between AFD-T and AFD-D were detected in both preparations but differed significantly in relative abundance (Supplemental Table 2); 56 of these alterations in protein abundance represented greater than 2.5-fold differences (Fig. 8, B and C).

**DISCUSSION**

The enabling of concurrent genomics and proteomics analyses of the same sample requires validation of the preparative techniques used to obtain the analytes from the tissue sample. Our objective was to determine which techniques could be used to subsequently provide the best possible global molecular analysis of these tissues (and likely others) without sacrificing one analysis for the other. We found that AFD followed by partitioning of the powdered sample for separate, optimized RNA and protein extractions yields quantitative, coupled analyses of the necessary rigor.

*RNAlater Detrimentally Affects Proteomics Analyses*—To determine the practical applicability of RNAlater for coupled
Molecular & Cellular Proteomics 6.9

RNA and Protein Analyses of a Single Tissue Sample

genomics and proteomics analyses of rat spinal cord segments, spinal cords were perfused with the protective agent during surgery and additionally soaked in this solution after excision. However, RNAlater was a substantial impediment to effective IEF (Fig. 5C). Qualitatively samples exposed to RNAlater never yielded proteomic maps of the quality routinely achieved from samples not exposed to the additive regardless of whether RNAlater was introduced by perfusion/bath application prior to tissue homogenization (Fig. 5, A and B) or directly introduced to previously unexposed protein isolates immediately prior to gel electrophoresis (Fig. 5, D–G). Compared with unexposed controls, gels of all samples exposed to RNAlater displayed characteristically poor resolution that included substantial horizontal and vertical streaking, protein spots that were missing or altered in position, proteins that were smeared, and large zones in which no detectable proteins were resolved (Fig. 5). Both the severity of the reduction in IEF performance and the magnitude of pattern distortions in resulting 2DE gels were proportional to the concentration of RNAlater added, suggesting that the impediment to IEF is a likely cause of the observed effects on 2D gel patterns (Fig. 5).

All of the observed effects of RNAlater on 2DE are indicative of a major problem with the separation. These problems associated with RNAlater contamination of protein samples for 2DE are most generally characteristic of salt contamination, although the contaminant could be any ion in principle. This is the primary reason why salts, despite benefiting protein solubility, are typically strictly omitted from gel electrophoretic separations especially 2DE (30). The possibility that RNAlater contains some percentage of organic solvent, either to disrupt enzyme activity or to assist in the solubility of the active ingredients, also cannot be excluded. If this were the case, the observed impediment to successful 2DE via RNAlater contamination could be the result of partial denaturation/precipitation of the protein sample during electrophoresis.

Interestingly the distortions and poor resolution were observed in separations of RNAlater-exposed tissue samples regardless of the tissue preparation method used. Whether the sample was prepared by AFD-D (Fig. 5, A and B), AFD-T, or SH (data not shown), the effect was similar. If the offending contaminant is indeed a salt or organic small molecule, it would be expected to be washed away either during the TRIzol extraction itself or the exhaustive cleanup that was subsequently used prior to protein solubilization. Additionally in an attempt at postharvest cleanup, RNAlater-contaminated protein samples were dialyzed using centrifugal concentration devices with 3-kDa size exclusion filters to repeatedly concentrate protein and replace the aqueous buffer. However, even triplicate replacement of the aqueous buffer was insufficient to relieve the characteristic RNAlater effect (data not shown). If small ionic species are responsible for the detrimental effects of RNAlater contamination on 2DE, they must be exceedingly concentrated in the commercial product. Alternately the offending ingredients may be small peptides that partition both with proteins in the solvent precipitation/cleanup during TRIzol extraction and with proteins during size exclusion filtration. It should be noted that although severe contamination of the sample with RNAlater yields proteomic maps that are completely unrecognizable (Fig. 5G), mild contamination can yield a somewhat reasonable, albeit distorted, proteome. Ultimately, however, caution must be exercised: protein separations of RNAlater-contaminated samples are not consistent with rigorous global analyses of the underlying biological complexity. Thus we deem the data from RNAlater-contaminated spinal cord segments unsuitable for comparative proteomics analyses, and RNAlater application was eliminated as a potential means of protecting RNA for coupled genomics and proteomics analyses.

Membrane Versus Soluble Protein Abundance—Sample prefractionation has been extensively demonstrated to improve the resolution and detection of proteins (24). Here well characterized methods of separating the homogenized tissue into total membrane and soluble proteomes were tested for their applicability in the analysis of spinal cord segments (18). The concern was that with such a limited amount of available starting material (e.g. one 5-mm spinal cord segment per sample) there may simply be insufficient protein for complete analysis of both fractions. This was indeed the case, although for a somewhat unexpected reason: the amount of soluble protein, as defined by this prefractionation strategy, was insufficient for our proteomics analyses. This is the first instance, out of an extremely wide array of different tissue samples, in which we found disproportionately more membrane and associated proteins than soluble proteins. Separate fractions could conceivably be analyzed if several spinal cords were pooled, but even further tissue collection and pooling would be required to generate replicate gels. Thus, this particular prefractionation strategy is inappropriate for proteomics analysis of these spinal cord segments. Based on our goals, we were unwilling to accept the loss of quantitative data from one of these tissue fractions at the cost of optimizing the other. This unfortunate situation will persist until such time as in-gel detection sensitivity is improved to the extent that 2DE can be carried out with substantially less protein (31). Given the total protein yields, sufficient for several gels per spinal cord segment, other prefractionation strategies, such as charge fractionation of the protein isolates could be attempted (32). Alternately even larger gels with correspondingly higher total protein loads and greater resolving power could be applied in future analyses (25). Lastly in the absence of an effective prefractionation approach, postfractionation strategies could also be applied to further enhance protein resolution (25).

Assessing Tissue Homogenization Strategies for Coupled Genomics and Proteomics Analyses—There is substantial evidence, if limited literature, to suggest that AFD is the premiere tissue homogenization strategy currently available as a preparative method for general molecular analyses. AFD has
been described as optimal for the extraction of protein, RNA/DNA, carbohydrates, and lipids when directly compared with competing homogenization strategies (18, 19, 21, 22, 33). This is especially true of tough, fibrous, or otherwise difficult to homogenize biological material (18, 20, 22). From a proteomics standpoint, the benefits of AFD include (but are not limited to) enabling the detection of proteins that are undetectable by other classical homogenization strategies (18). This is partially the result of the greater thoroughness and completeness of the homogenization provided by AFD relative to competing strategies but also partly the result of maintaining a deep frozen state during homogenization, preventing degradation of the tissue sample (18). In some respects further comparison of SH with AFD is superfluous. However, in the previous study, it was observed that the magnitude of the benefits afforded by AFD varied from tissue to tissue, so the comparison was made here in part to confirm that AFD was also the better choice for spinal cord samples. Additionally, although AFD appears to be the most appropriate tissue disruption method available for proteomics analysis, in the interest of coupled genomics and proteomics analyses, the method must additionally satisfy the rigorous requirements of RNA microarray analysis. Lastly, although TRI reagents are not designed primarily for proteomics applications, AFD and more classical tissue homogenization strategies have not yet undergone rigorous comparison as preparative methods specifically for TRizol extractions. Indeed by some combination of selective extraction and concentration, it is possible that TRizol extraction might rescue proteins that were previously undetectable in manually (or similarly) homogenized samples (e.g. previously seen only in samples prepared by AFD) (18). In this regard, comparison of spinal cord segments prepared by SH or AFD-T was inevitable. Both homogenates were subjected to parallel TRizol extraction for RNA isolation with subsequent protein isolation and cleanup by solvent precipitation; standard microarray and 2DE analyses of the separate molecular fractions followed.

The two homogenization strategies were effectively interchangeable from the perspective of the genomics analysis. Both methods yielded RNA of equivalent quality and quantity. Moreover microarray analyses demonstrated that either preparative technique, SH or AFD-T, can be used essentially interchangeably because the number of genes identified and the intensity of fluorescence signal (proportional to the number of RNA molecules present) of the arrays were essentially identical after both preparative techniques (Figs. 3 and 4). The Affymetrix analysis showed a slight difference between the two disruption methods as a number of genes showed increased abundance in SH relative to AFD-T. Nonetheless the same set of common genes was identified in both arrays for both tissue homogenization techniques, and no specific mRNA was completely lost after one technique relative to the other. Thus in terms of RNA microarray analysis, the differences between disruption techniques were negligible, indicating that both protocols are viable alternatives for these RNA analyses.

There was a modest discrepancy between arrays in terms of the number of genes differing in abundance between homogenization strategies. Whereas analysis of Affymetrix data identified 44 differences, the ABI analysis identified none. It is likely that this discrepancy is the result of disparate RNA inputs; the Affymetrix arrays utilized a standard RNA input (3 μg), whereas the ABI arrays were analyzed with a minimal RNA input (200 ng). Nonetheless it was reassuring to see that both arrays identified the same genes, and from this perspective there is good concordance between the two platforms. Therefore, for RNA analyses, either tissue disruption technique followed by extraction of RNA with TRizol and subsequent analysis on either Affymetrix or ABI chips can be performed with a high degree of confidence.

However, AFD-T was marginally superior to SH from a proteomics standpoint. Consistent with a previous study, AFD-T yielded more solubilized total protein per unit of starting material than did SH (Fig. 2B). It has been demonstrated that AFD provides the most physically thorough and complete homogenate currently possible (18); it seems that by virtue of the greater surface area thus achieved, less of the sample is wasted (e.g. as poorly homogenized material removed by centrifugation), and more total protein is available for efficient extraction and solubilization. However, the difference in protein yield between AFD-T and the competing tissue homogenization strategy was smaller in this study than for any tissue tested previously (18). It seems that in terms of protein yield, SH (with TRizol extraction) is superior to manual homogenization alone (18). However, AFD is still superior in terms of total protein yield, which is an important consideration given a small, rare, or expensive sample from which multiple analyses are to be derived.

At the level of 2DE, both AFD-T and SH resulted in excellent, high quality, high resolution proteomic maps of the biological material (Fig. 7). In fact, 2DE patterns of spinal cord segments generated by these two preparation methods were nearly indistinguishable by eye. There was no immediately obvious detriment to SH as a homogenization method for proteomics applications (Fig. 7). However, consistent with a previous study (18), there were a large number of significant homogenization-dependent differences in specific proteins identified by automated quantitative image analysis (Fig. 7). A large number of these differences in the relative abundance of specific proteins were detected in both preparations (Fig. 7 and Supplemental Table 1). It seems that, by virtue of the greater exposed surface area achieved by AFD (see previous section), some proteins are rendered more accessible for extraction and solubilization following AFD (18). The relative abundance of such proteins would be expected to be elevated in AFD-T relative to SH. Conversely proteins that do not experience such enhanced extraction as a result of AFD would be expected to be reduced in relative abundance.
Although all of these proteins were detected in both SH and AFD preparations, and thus none of these data are truly “lost,” prior evidence indicates that AFD offers the greatest confidence that a truly representative and uniform homogenate has been prepared (18). The suggestion is that the substantial quantitative differences between SH and AFD are indicative of somewhat incomplete homogenization by SH and thus evidence that the resulting extract does not provide the most quantitative representation of the native biological complexity of the sample. Supporting this, a number of proteins were detected exclusively in the AFD-T homogenate and were absent from the SH preparation (Fig. 6). It should be considered that these proteins, detectable by one preparative method but not the other, may not represent proteins that are effectively either present or absent. Rather many “unique” proteins (e.g. detectable in one sample but not the other) may in fact be present in both preparations but differ in relative abundance between samples such that this difference bounds the threshold of minimal detection sensitivity as was demonstrated previously in central nervous system tissue (18).

Interestingly two proteins were detectable only in the SH preparation and were absent from AFD-T (Fig. 7). In a previous study, there were no proteins detected by manual homogenization that could not be detected by parallel AFD in samples from six different tissues that varied from mouse brain, liver, and cardiac muscle to spinach leaf and cricket leg (32). It seems that here SH was able to approach the performance of AFD by virtue of the subsequent TRIzol extraction: TRIzol extraction appears to improve the efficiency of classical homogenization methods (18), and some proteins missed by our previous approach (manual homogenization) can thus be rescued independently of AFD. Part of the benefit afforded by AFD results from arresting enzyme activity by carrying out the homogenization in a deep frozen state and maintaining this state until a denaturing agent is applied (18). During SH, strong denaturing agents (i.e. TRIzol) are included throughout, and although these reagents seem to introduce issues of their own (see below), they offer the benefit of an alternate means of broadly arresting enzyme activity (including that of proteinase) during tissue homogenization.

Ultimately the fact is that more proteins are lost during SH and “rescued” by using AFD than vice versa (Figs. 6 and 8). Coupled with the evidence that AFD provides greater protein yields from this small tissue sample, AFD seems to be the optimal choice for the proteomics and thus should be applied in future coupled genomics and proteomics analyses.

Assessing TRIzol Extraction as an Analytical Route to Coupled Genomics and Proteomics Analyses—Although AFD seems to be the optimal method of tissue homogenization for the resolution of proteomes by 2DE, the issue of posthomogenization handling remains to be resolved. Direct solubilization of the tissue without TRIzol extraction (AFD-D; standard practice for proteomics) was quickly eliminated as a potential analytical route for RNA analysis because the resulting RNA yields were untenably low (Fig. 2). Direct solubilization of the sample cannot be applied for coupled global analyses of both RNA and protein, and a TRIzol extraction is mandatory from the standpoint of RNA extraction and subsequent microarray analysis.

Thus, the objective became to determine whether protein isolated from the same TRIzol extract was satisfactory for quantitative proteomics analyses or whether the homogenate must be partitioned for separate, optimized extraction of RNA and protein. To address this we directly compared TRIzol extraction with direct solubilization of spinal cord homogenates prepared by AFD (AFD-T and AFD-D, respectively).

Qualitatively both preparation methods resulted in high quality, high resolution proteomic maps. Overall, gels from both extraction methods appeared highly similar, and there was no immediately obvious problem with either extraction protocol. It should be appreciated that qualitatively both protocols produced proteomic maps of the general quality expected for comparative analysis (Fig. 8). Quantitatively, however, there proved to be an extensive number of significant differences between TRIzol-extracted (AFD-T), and directly solubilized (AFD-D) spinal cord proteomes (Fig. 8 and Supplemental Table 2). The vast majority of these differences represent proteins that were detectable regardless of the method of protein extraction but that differed in relative abundance between the two techniques (Fig. 8, B and C, and Supplemental Table 2). However, there were also many proteins that were detectable only in the AFD-D preparation and were undetectable in TRIzol-extracted sample (Fig. 8D). A smaller number of proteins were detectable only in the TRIzol-extracted samples and could not be detected in the directly solubilized sample (Fig. 8A).

The data suggest that TRIzol extraction and solvent precipitation of the spinal cord sample results in selective extraction of proteins. Parallel selective losses may thus occur during the TRIzol extraction or during organic precipitation and cleanup, partitioning selectively with either RNA/DNA fractions during their isolation or with the subsequent solvent washes, respectively.

However, the observed extraction-dependent differences are extensive (241 significant differences in total; Supplemental Table 2). These quantitative differences represent fully ¼ of those proteins resolved and detected with 100% reproducibility within the sample sets (Figs. 6 and 8). Thus, selective extraction alone may be insufficient to explain the magnitude of this quantitative difference between preparation methods (Fig. 8 and Supplemental Table 2). One obvious potential cause for the observed difference between extraction methods is the possibility for contamination of the AFD-T sample with trace components of TRIzol: ionic detergents and solvents have well documented detrimental effects on IEF performance and 2DE (29, 34, 35). Despite rigorous solvent washing to remove components of TRIzol and vacuum drying of the sample to remove solvents, it may be that trace con-
tamination can never be completely eliminated, arguing strongly for the simpler method of directly solubilizing protein without the addition of other extraneous chemicals. This is likely the case even if alternative methods of proteome analysis are to be used. On the other hand, it may be that delipidation of the sample via TRIzol extraction and solvent precipitation alters the resolution of specific proteins. It has been extensively demonstrated that different lipids or lipid-like detergents improve the solubility of specific proteins, including membrane proteins, enhancing their resolution and detection in gel-based proteomics (29, 36, 37). Is it possible that enhanced solubility due to the presence of endogenous native lipids is responsible for part of the difference between extraction methods? This might prove to be a fruitful area for further investigation in terms of the quantitative analysis of membrane proteomes (8).

In contrast, it could also be argued that there are proteins in the TRIzol-extracted sample that are of greater abundance than in the directly solubilized sample, and thus there are several proteins that are only detected when a TRIzol extraction is carried out. This would imply that there is something to be gained from extraction using TRIzol. However, considering the parallel selective protein losses that are suffered, it is understandable that the relative abundance of some proteins appeared to be enhanced when a consistent amount of total protein was resolved by 2DE (Fig. 8). Thus it is perhaps not surprising that several proteins seemed to be more abundant in the TRIzol-extracted sample relative to the directly solubilized sample (Fig. 8).

Overall the evidence suggests that TRIzol extraction does not support the most comprehensive, representative, or quantitative analysis of the native protein complexity of the sample. At best, high quality proteomic maps can be prepared from protein isolated following a TRIzol extraction. The quality of TRIzol-extracted proteomes is unmistakable, and the general feeling that TRIzol extracts are completely unsuitable for proteomics analysis appears to be unfounded. Although there are some proteins that are only detected when a TRIzol extraction is performed, there is more to be gained than lost by direct solubilization of the sample; it appears direct solubilization of the sample offers the more quantitative option from a proteomics standpoint.

Concluding Remarks—Our objectives were to identify analytical paths enabling coupled genomics and proteomics analyses from limiting, single tissue samples; we sought to eliminate less than optimal possibilities and identify potential pitfalls to be avoided in coupled RNA/protein analyses of spinal cord injury in rats. In testing available technologies, we were able to systematically eliminate analytical paths that, despite showing great potential promise, proved less than optimal in practice. The result is quantitative confirmation of the optimal analytical path for coupled genomics and proteomics analyses from a single tissue sample using readily available technology. In this regard, use of the protective agent RNAlater was discarded due to its deleterious effect on 2DE performance. It was also determined that protein prefractionation was less than satisfactory for these spinal cord segments because the amount of soluble protein present was surprisingly low and insufficient for detailed, gel-based proteomic analyses. A TRIzol (or comparable) extraction is a demonstrable necessity for coupled analyses to ensure efficient isolation of RNA; in the absence of a TRIzol extraction, the RNA yield was unacceptably low. Interestingly as preparative tissue disruption methods for TRIzol extractions, SH and AFD produced comparable RNA isolates, which are essentially indistinguishable at the level of RNA microarray analysis. AFD, however, significantly improved both total protein yield (an important consideration for all small, rare, and expensive tissue samples) and the subsequent quantitative analysis of the spinal cord proteins. Thus, if a single homogenization strategy is to be applied to a single tissue sample for coupled analysis, AFD is the optimal choice. Finally although high quality, high resolution proteomes can unquestionably be resolved from the TRIzol extract, quantitative differences exist relative to proteomes resolved following direct solubilization of the protein. The suggestion is that the TRIzol extraction introduces a somewhat selective representation of the proteome. Thus, from the standpoint of enabling more quantitative and systems-level assessments, AFD followed by independent, optimized extractions of RNA and protein from separate aliquots of the powdered tissue homogenate is the method of choice for the most rigorous possible analyses.

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