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Comparative Molecular Physiological Genomics

Heterologous Probing of cDNA Arrays

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Summary

The use of DNA microarrays has gained wider acceptance as a standard tool for molecular biology studies over the past decade. In particular, biomedical studies embraced this technology as soon as arrays were produced for the common laboratory species. Slower to develop, however, has been the use of microarray screening with non-standard animal models, even though these species present fascinating physiological phenomena for study. The very high cost and huge amount of work involved in developing and producing a DNA array or microarray for a new species is prohibitive for most researchers working in comparative biology. The alternative is to explore the use of heterologous array hybridization, screening for stress-induced gene expression in one species using an array developed for another species. This chapter provides a comprehensive review of the current literature on heterologous DNA array hybridization and explores the factors that must be taken into account when performing heterologous microarray analysis on nonstandard species. Changes in methodology (e.g. hybridization conditions, stringency of washing) to optimize the percent cross reaction, the potential for false positives and false negatives to occur, and techniques for downstream analysis and confirmation of array data are all discussed. Examples of cross-hybridization using human microarrays are discussed using phylogenetically diverse species ranging from ground squirrels to frogs to snails. As with any new technology, the willingness to grasp cross-species analysis has been slow but the future looks bright for heterologous DNA hybridization and microarray analysis now that the initial hurdles have been overcome.

Key Words: Comparative genomics; cross-species DNA array hybridization; mammalian hibernation; semiquantitative PCR.

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1. Introduction

The vast majority of research in biochemistry and molecular biology is centered on a very low number of model species; human, mouse and rat are the main mammalian models whereas fruit flies (*Drosophila melanogaster*) and nematodes (*Caenorhabditis elegans*) are primary invertebrate models. However, the genetic information programmed within numerous other species offers researchers unique insights into the molecular mechanisms that underlie organismal responses to numerous stresses, conditions and diseases. For example, study of appropriate animal model systems is the only way to determine how cells and organisms have evolved to endure environmental stresses including, but not limited to, oxygen deprivation, extremes of hot or cold temperature, freezing, and high or low salinity. Regulating these biological processes requires a concerted effort put forth by multiple signaling pathways eventually targeting specific genes to activate or repress expression in order to maintain sustained cellular function during stress. The development of DNA array screening technology has given researchers an immensely powerful new tool with which to study cellular responses to stress. Array screening can take a snapshot of the total gene expression patterns within a cell at any given time point providing insights into the responses of individual genes as well as an integrated view of the responses of functional units (e.g., metabolic pathways, signal transduction cascades, etc.).

Since the advent in the 1970s of the Southern blot that used DNA bound to nitrocellulose or nylon membranes, researchers have searched for ways to immobilize DNA on smaller and smaller scales and platforms. cDNA arrays and microarrays are the latest nucleic acid immobilization tool for molecular biologists. Since their introduction their physical size has decreased dramatically at the same time as the information they possess has greatly increased. The field has grown immensely since the introduction of DNA arrays in the mid-1990s ***(1–5)***, with researchers applying DNA array screening to nearly all areas of biology and medicine. Their initial use was in studying the mRNA expression profiles, or transcriptomes, of cells, tissues or organisms to gain insight into the changes in gene expression between two or more metabolic states, however, recent advances made using microarrays include identifying and genotyping numerous pathogens within clinical samples, also called metagenomics ***(6–8)***.

There are two primary methods for producing DNA arrays. These are largely the same today as they were a decade ago except that the cost to produce arrays has dropped significantly and the arrays themselves have become higher density and more complete, often covering entire genomes. The two methods are:

1. Photolithography; the synthesis of specific oligonucleotides on a support medium such as glass. Developed by Affymetrix, this method invokes light-directed oligonucleotide synthesis directly onto a glass slide or support ***(3)***.

2. Spotting cDNAs onto a glass or nylon support. The method, originally developed by Patrick Brown's lab at Stanford University, involves amplification and purification of cDNAs that are then spotted onto the support ***(1)***. Advances in robotics now allow production of high density cDNA arrays and microarrays.

Comparative molecular biology and physiology, particularly areas dealing with non-traditional model organisms, has only begun to grasp the power of DNA array technology, largely due to a lack of experimental platforms with which to proceed. Prevailing thought in the field has been that a homologous cDNA platform must be used in order to generate useful data. However, this is not the case, especially considering that large mixed populations of bacterial and viral pathogens can now be genotyped and identified on a single array ***(6–8)***. If this is the case, then heterologous cDNA array screening between closely related organisms should not pose a problem. The production of a DNA array for each and every new species is expensive and cost-prohibitive for the amount of information that is produced so studies have been slow to expand beyond the traditional model species. Recently, however, arrays have become available for more and more model species, representing widely differing groups of organisms and, thereby, broadening the range of studies for which homologous array screening can be used. For example, Affymetrix currently has platforms for a number of nonmammalian animals (the frog *Xenopus laevis*, the fruit fly *D. melanogaster*, the nematode *C. elegans*, zebrafish *Danio rerio*), plants (*Arabidopsis thaliana,* barley, grapes, maize, soybeans, tomato), and prokaryotes (*Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*). Thus, there is a large existing platform of pre-fabricated arrays that many comparative researchers can exploit for cross-species heterologous analysis.

The diversity of experimental animal models in use by researchers around the world is huge. Currently, it is simply not possible to construct and screen homologous DNA arrays for each and every species of interest at a cost that is reasonable for most researchers. Instead, approaches aimed at exploiting existing microarray technology (using microarrays fabricated for model organisms or other closely related organisms) need to be embraced. Heterologous probing, screening cDNA from one organism using an array produced from another species, has significant potential as a gene discovery tool. Although cross-species hybridization will never be 100%, with appropriate optimization of conditions, heterologous hybridization can allow the analysis of expression responses by thousands of genes. Thus, heterologous DNA hybridization is not only feasible and valid on scientific principles, but provides comparative biologists with a means to study the diversity of gene regulation without the prohibitive costs of having to create a microarray platform for each and every species under scientific study. To understand

the range of opportunities provided by this approach, we must first look at similarities at the genetic level between nonmodel organisms and the model organisms for which array platforms have already been fabricated and then decide whether or not such an undertaking is feasible.

The success of cross-species array hybridization depends largely on the degree of identity between genes from nontraditional model species and homologous genes in model organisms. Detailed analysis of orthologous human and rodent gene sequences has shown that, on average, protein coding regions share 85% nucleic acid identity, 5′ untranslated regions are approx 70% identical, and 3′ untranslated regions are approx 71% identical ***(9,10)***. When this analysis was extended to compare mammalian (human and mouse) to nematode (*C. elegans*) genes, 44% of human/rodent orthologs were found to have nematode counterparts that had a mean approx. 50% identity at the nucleotide level ***(11)***. This strong identity between orthologous gene sequences across the animal kingdom give us a good basis for predicting that heterologous cDNA array probing will succeed.

Our own studies have cloned and sequenced a variety of specific genes from hibernating small mammals including ground squirrels (*Spermophilus tridecemlineatus*.) and bats (*Myotis lucifugus*) and the results have shown that the degree of identity between gene orthologs of humans and hibernators is virtually the same as that noted above for humans and rodents. For example, analysis of the open reading frame sequences of the heart isoform of fatty acid binding protein (*h-fabp*) from *S. tridecemlineatus*, *M. lucifugus,* human, mouse and rat showed 91% nucleotide identity between the five species ***(12–14)***. Notably, fatty acid binding proteins are of key importance to hibernation as they facilitate the intracellular transport of fatty acids from the plasma membrane to the mitochondria, lipids being the primary fuel used during hibernation. Similar results have been obtained for the full or partial cDNA sequences of several other genes cloned from hibernators including *atpase6/8*, *coxII*, *hif1*α, *mlc2(v)*, *nd2*, *pag*, *pgc-1*α and *ppar*-γ ***(12,14–19)***. Other researchers have also shown a high degree of nucleotide identity among genes from hibernating mammals compared with their human, mouse or rat counterparts ***(20,21)***.

Gene cloning from other nonmodel species also supports the suggestion that the sequences of many genes are highly conserved across a broad range of vertebrate and invertebrate species. We have recently sequenced a number of genes from the wood frog, *Rana sylvatica*, a species that survives the freezing of its body fluids over the winter months. The genes for fibrinogen α and fibrinogen γ are up-regulated in the liver of freezing frogs and sequencing of the partial cDNA for wood frog fibrinogen γ showed that it was 67 % identical with human fibrinogen γ over the same region ***(22)***. The wood frog *aat* gene encoding the ATP/ADP translocase showed 70% nucleic acid

identity compared with the human gene ***(23)*** and other cloned genes from wood frogs showed remarkably high identity with their mammalian counterparts including the inorganic phosphate carrier, *pic*, and the acidic ribosomal protein, *P0* ***(24,25)***. Mitochondrial genes cloned from the turtle, *Chrysemys picta marginata*, also displayed high homology to the corresponding mammalian genes ***(26)***. The genes for ribosomal protein L26 and ferritin, cloned from the marine snail, *Littorina littorea* ***(27–29)*** provide even more evidence that genetic similarity between non-model and model species can be utilized for heterologous cDNA array screening. Lastly, a muscle-LI*M* gene cloned from the cold hardy gall insect, *Epiblema scudderiana*, shows gene homology of 60% over the entire mRNA and 75% identity within the open reading frame (ORF) compared to that of the appropriate model species, in this case *D. melanogaster* ***(30)***. Thus, our data on gene homologies strongly suggest that comparative DNA array hybridization will work. Actual array screening results from our lab and others documents this, as discussed below.

Given the demonstrated high degree of nucleotide identity in comparisons of cloned hibernator genes with other mammals, the capacity to utilize cross-species array hybridization as a means of gene discovery in hibernation has never been better. The same applies to many other heterologous pairings. Thus, cross-species array hybridization has become a rational approach to studying nontraditional model animals as a means of deciphering the complex modifications in the transcriptome under different stresses. In addition to our studies, many experiments have been performed using other species as well, harnessing the high-throughput capabilities of cDNA arrays. To this end, we have carried out gene expression studies on animal species using heterologous probing with either Clontech ATLAS™rat cDNA arrays and human 19K cDNA arrays (University Health Network, Toronto). These studies have been highly effective for identifying: a) previously unrecognized target genes that participate in environmental stress tolerance, and b) tracing the cellular signaling pathways that are active in stress response. Our first studies used ATLAS™nylon arrays containing rat cDNAs to assess changes in gene expression during mammalian hibernation in the ground squirrel, *S. tridecemlineatus*. Cross-hybridization was very high between the two rodent species and the results showed, among others, a clear up-regulation of *a-fabp* (the adipose isoform of fatty acid binding protein) in brown adipose tissue ***(14)*** and a striking 50% suppression of genes encoding numerous ribosomal proteins, including *S12*, *L21,* and *L36a*, in skeletal muscle during hibernation ***(31)***. Subsequent work from our lab has shown that mammalian (bat or ground squirrel) hybridizations to cDNA arrays can give up to 85–90% hybridization ***(31)***, lower vertebrates (wood frogs) on human arrays will give 60–80% hybridization ***(32)*** and the marine snail *Littorina littorea* will hybridize to 18% of genes on 19K human

arrays ***(33)*** using low stringency hybridizations. Such cDNA array studies with the heart of wood frogs have identified a number of genes that are putatively up-regulated in the organ during freezing. Interestingly, three genes designated as up-regulated on the cDNA microarray experiments were previously identified as freeze-responsive genes by other means, specifically the ATP/ADP translocase, glucose transporters and glucose-6-phosphate dehydrogenase ***(22,34,35)***. Other labs have also made good use of mammalian cross species hybridizations proving that this method of comparative analysis is sound ***(14,31,36–44)***. Indeed, the study by Rinaudo and Gerin ***(40)*** showed that heterologous screening of a 4000 human gene microarray with woodchuck liver cDNA resulted in no false positives and only 29 false negatives (0.7%); a false positive was defined as a gene that shows putative up- or down-regulation on the array but no differential expression on downstream analysis whereas a false negative was a gene that does not cross react on the array. The false negatives appeared to occur because of high genetic distance between humans and woodchucks for these particular genes. The most promising aspect of this work was the complete lack of false positives, suggesting that rigorous heterologous microarray analysis yields real useable data. Analyzing hybridization patterns of woodchuck liver samples in comparison to human liver samples on nylon filter arrays, it was found that human liver samples hybridized to 20–60% of array probes (depending on temperature and salt concentrations during washing), whereas woodchuck liver samples hybridized to nearly the same degree, showing 18–53% hybridization to array probes under identical conditions ***(40)***.

Another study used human cDNA arrays containing 4400 genes to study UV-induced melanoma in the opossum, *Monodelphis domestica*. This heterologous probing worked very well and the results showed that 79 genes were up-regulated by UV-treatment whereas 28 were down-regulated ***(43)***. It was noted that the majority of published gene sequences for this evolutionarily distant marsupial mammal showed 70–80% identity with the corresponding human genes.

A study on a porcine model of vascular remodeling analyzed gene expression on a human ATLAS cDNA platform ***(45)***. The resulting analysis and downstream characterization found differential expression of *col1a1* and *col3a1*. More recently, studies on pig mRNA expression have been performed on human nylon DNA arrays reproducibly detecting the expression of 4324 porcine genes ***(36)***. Investigators have also begun to use high-density microarrays to investigate the possibility of using heterologously probed cDNAs produced from porcine samples. Initial results using human Affymetrix high density oligonucleotide arrays are promising ***(41)*** as are those using porcine samples on human UniGEM microarrays ***(46)***.

Studies analyzing gene probes from cattle, pig, and dog on human and mouse Affymetrix high-density oligonucleotide arrays showed that successful hybridization can be performed with a high degree of statistical significance ***(47)***. By slightly lowering the sensitivity of array hybridizations and washings, mainly through altering the salt concentration in washing buffers, hybridization patterns were detected for 2972 transcripts from cattle heart and liver on human U133A GeneChips covering 18,000 human transcripts, with a correlation coefficient of 0.792 across all 2972 genes when compared to expression profiles of human heart and liver ***(47)***. In another study, the response of bovine macrophages to *Escherichia coli* OH157:H7 LPS treatment was investigated on human UniGE*M* microarrays developed by Incyte ***(44)*** as a model for the effects of pathogen invasion. These researchers found that nearly 80% of genes located on the array produced a sufficient and detectable hybridization signal for analysis, 5644 hybridizations out of 7075 total targets. Of genes randomly selected for downstream analysis, 90% gave results in agreement with the microarray data and homology between cow and human genes ranged from 76% to 96%, with a mean of 86%, once again suggesting that the high degree of homology between humans and other mammals provides a strong basis for the use of cDNA arrays in comparative studies.

What cross-species DNA array analysis has told us is that different species display a remarkable degree of conservation at the gene level that is evidenced by a high degree of cross-hybridization on DNA arrays. Thus, from studies to date with cross-hybridization between two mammalian species, we can expect up to 85–90% cross-reactivity of genes found on any set of microarrays ***(31)*** or by limiting probe hybridizations via increased stringency during washing ***(40)***, we can limit and focus our attention on downstream targets that are likely to be significantly up- or down-regulated genes eliminating the possibility of pursuing false positives. Indeed, the use of whole genome or near whole genome microarrays in a cross-hybridization analysis can actually allow a broader coverage of the transcriptome than if researchers were to create arrays from their own cDNA libraries at either high or low density. Since the focus of many researchers is to use the arrays to identify genes that would be good subjects for downstream analysis, researchers are better served by using existing microarray platforms to do their initial screening and direct their major research efforts into downstream analysis of gene regulation and protein function.

A variety of companies now produce commercial microarrays and some of them produce arrays for multiple species; **Table 1** lists a few of these major microarray producers that make microarrays at a reasonable cost. By no means is this list intended to be complete as new companies and institutes produce arrays at a rapid pace. As described above and in the discussions that

Table 1
Companies Producing DNA Arrays. Companies and institutes offering a wide variety of DNA array platforms that can be exploited for comparative purposes

Company	High Density	Probe
Affymetrix	Yes	Cy3, Cy5
Amersham	Yes	Cy3, Cy5
Clontech	Yes and No	Cy3, Cy5 and ^{32}P
Ontario Cancer Institute	Yes	Cy3, Cy5
Superarray Bioscience	No	^{32}P

follow, we show that a number of different cDNA arrays have been used in comparative research with a high degree of success. Before considering the use of DNA arrays for comparative molecular biology, researchers must first identify the likelihood that the cDNA produced from their model species will cross-react with a particular array and generate sufficient hybridization signals to produce meaningful data.

Successful cross-species microarray analysis does not just apply to mammalian genes and models, but broadly across the animal kingdom. For example, researchers using rainbow trout, *Oncorhynchus mykiss*, as a model for zinc exposure in the environment, assessed gene expression by probing a gill array containing 18,432 cDNA clones made from the puffer fish, *Fugu rubripes*. Genes involved in energy production, protein synthesis and the inflammatory response were up-regulated in gills after 6 days of exposure to zinc ***(37)***.

2. Materials

All chemicals used are of molecular biology grade or their equivalent and of the highest purity. All plastic and glassware, including bottles and pipette tips, are autoclaved and gloves must be worn at all times during operations involving nucleic acid manipulation. cDNA ATLAS arrays are purchased from Clontech. Human 19K cDNA arrays are purchased from the Ontario Cancer Institute.

2.1. Total RNA Isolation

1. Diethyl pyrocarbonate (DEPC) (Sigma-Aldrich, St. Louis, MO) is added to water at a concentration of 0.1% (v/v), stirred overnight (>12 h), autoclaved. Tips, tubes and other plastic or glassware may be purchased as certified RNase-free or treated by stirring overnight in DEPC-treated water to destroy any RNases present. DEPC-treated water and RNase-free plastic or glassware are used to make up all solutions in this section and to dissolve the final RNA samples.

2. Trizol reagent (Invitrogen, Carlsbad, CA).
3. Chloroform (Fisher Scientific, Fairlawn, NJ).
4. Isopropanol (Fisher Scientific).
5. 70% Ethanol. Add 30 mL of DEPC treated water to 70 mL of 100% ethanol (Pharmco, Brookfield, CT).

2.2. Denaturing RNA Gel Electrophoresis

1. Stock 10× MOPS buffer: 200 m*M* 3-(*N*-morpholino) propanesulfonic acid (MOPS), 50 m*M* sodium acetate, 10 m*M* EDTA, pH 7.
2. 1% (w/v) agarose formaldehyde denaturing gel: Melt 3 g of agarose in 217 mL of ddH_2O containing ethidium bromide (1 μg/mL) in a sterile flask. Place solution in an incubator set to 55°C. Into a separate sterile flask, add 30 mL of MOPS 10× buffer and 53 mL of formaldehyde 37% (v/v) and place this solution at 55°C. Once both solutions have equilibrated to 55°C, combine the contents of both flasks together in a fumehood and gently swirl without introducing bubbles, and pour into large gel tray to desired thickness.
3. RNA sample buffer: 1× MOPS buffer, 2.2 *M* formaldehyde, 50% (v/v) formamide.
4. RNA Loading buffer 6× stock: 1× MOPS buffer, 50% (v/v) formamide, 40% (v/v) glycerol. Add a few flakes of bromophenol blue and xylene cyanol as tracking dyes.

2.3. mRNA Isolation

1. Oligotex poly$(A)^+$ mRNA isolation kits (Qiagen). Note: the three buffers listed below come with the kit.
2. Oligotex binding buffer (OBB): 20 m*M* Tris-HCl, pH 7.5, 1 *M* NaCl, 2 m*M* EDTA, 0.2% w.v sodium dodecyl sulfate (SDS; Sigma-Aldrich).
3. Oligotex wash buffer (OWB): 10 m*M* Tris-HCl, pH 7.5, 150 m*M* NaCl, 1m*M* EDTA
4. Oligotex elution buffer (OEB): 5 m*M* Tris-HCl, pH 7.5.

2.4. cDNA Probe Synthesis

1. 1 μg of mRNA sample.
2. Polymerase chain reaction (PCR) Thermalcycler (e.g., Bio-Rad iCycler [Bio-Rad], PTC-100 [MJ Research]).
3. Oligo-5′-dT_{20}N-3′ (Bio S&T, Montreal, QC).
4. Random primer (100 m*M* $d(N)_6$; New England Biolabs).
5. CDS Primer mix (Clontech).
6. [α-^{32}P]dATP (3000 Ci/mol; GE Healthcare).
7. dNTP mix 1 (dCTP/dTTP/dGTP; 2.5 m*M* each).
8. 20 m*M* dNTP mix 2 (6.67 m*M* each of dATP, dGTP, dTTP).
9. 2 m*M* dCTP.
10. Cy3-dCTP, Cy5-dCTP (GE Healthcare).

11. Dithiothreitol (DTT) (Sigma-Aldrich). Make up a 0.1 *M* stock with sterile ddH_2O.
12. Superscript II RNase H reverse transcriptase (200 U/μL) (Invitrogen).
13. RNasin (20 U/μL; Promega).
14. 0.5 *M* EDTA (Sigma-Aldrich).
15. 10 *M* NaOH (Sigma-Aldrich).
16. 5 *M* acetic acid (Sigma-Aldrich).
17. Isopropanol (Fisher Scientific).
18. 70% Ethanol. Prepare as described in **Subheading 5.1.**
19. TE buffer : 10 m*M* Tris-base, pH 8.0, 1 m*M* EDTA.

2.5. DNA Array Hybridization and Washing

1. Church's buffer: 0.25 $M Na_2HPO_4$, 0.25 $M NaH_2PO_4$, pH 7.5, 7% SDS w/v.
2. 20× SSC: 3.0 *M* NaCl, 0.3 *M* sodium citrate (Sigma-Aldrich).
3. 20% SDS w/v.
4. Yeast tRNA (10 mg/mL) (Invitrogen).
5. Calf thymus DNA (10 mg/mL) (Sigma).
6. DIG Easy Hybe Solution (Roche).

2.6. DNA Array Analysis

1. X-ray film or phoshorimaging plate (for ATLAS^{TM}cDNA arrays).
2. Microarray reader (for Human 19K cDNA arrays). A number of companies (AlphaInnotech, Affymetrix, VersArray Chip Reader) have array readers available for purchase but some companies or services (and many core facilities at research institutions) will scan and read arrays for a fee, which is considerably more cost effective that purchasing your own array reader.
3. Downloadable analysis software (e.g., Scanalyze; http://rana.lbl.gov/).

2.7. Confirmation of Results: Semiquantitative Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

1. DNAman software (Lynnon Biosoft).
2. Primer Designer software (Scientific and Educational Software).
3. Bio-Rad iCycler (Bio-Rad) or other PCR thermalcycler with gradient capabilities.
4. 50× TAE buffer: 242 g of Tris-base, pH 8.5, 57.1 mL of glacial acetic acid, 37.2 g of EDTA, 1 L of ddH_2O.
5. 1% TAE agarose gel: 1× TAE buffer, 1% agarose (w/v), ethidium bromide (1 μg/mL) in 100 mL of water.
6. DNA loading dye: 0.25% w/v bromophenol blue, 0.25% w/v xylene cyanol FF, 50% v/v glycerol.
7. DNA ladder (Invitrogen). A ladder should be chosen based on the size of the expected PCR products (ranging from 100 bp up to several kilobases).

3. Methods

Before beginning any microarray experiment, it is essential to have proper controls and time points so that the data obtained is biologically meaningful. For considerations on choosing proper controls *see* **Notes 1** and **2**.

3.1. Total RNA Isolation

1. Poly(A)$^+$mRNA isolation kits can be used to harvest poly(A)$^+$ mRNA from frozen tissue samples; however, our experience with one-step isolations is that the yield is generally low when using kits. Instead, we recommend using the traditional method of total RNA isolation, followed by checking to ensure that the RNA is of good quality, and then proceeding with mRNA isolation and array hybridization.
2. Homogenize control and experimental samples in Trizol reagent (1 ml per 100 mg of frozen tissue) and then add chloroform immediately (0.2 mL/mL of Trizol). Invert samples rapidly and repeatedly (15 s) and incubate at room temperature (5 min). Centrifuge at maximum speed for 15 min at 4°C.
3. Remove the aqueous (top) layer and transfer to a sterile RNase-free Eppendorf tube. Add an equal volume of isopropanol to precipitate the RNA. Incubate at room temperature for 10 min.
4. Pellet the RNA by centrifuging the samples at maximum speed in a microcentrifuge for 10 min at 4°C. Carefully, aspirate the supernatant and wash the pellet with 70% ethanol (250 µL). Recentrifuge the RNA at maximum speed for 5 min. Aspirate the ethanol and air-dry the pellet. Be careful not to overdry the pellet, as it will be difficult to resuspend. Dissolve the RNA in DEPC-treated water in a ratio of approx 10 µL of water per 10 µg of pellet and store at –20°C (if it will be processed within a week) or at –80°C (long-term storage).
5. Determine RNA concentration and purity spectrophotometrically by measuring absorbance at 260 and 280 nm. The $A_{260}:A_{280}$ ratio should fall between 1.6 and 2.0 for RNA of good purity. Proceed to analyze the quality via RNA gel electrophoresis. Calculate how much total RNA is required for 10–20 µg using the standard of 40 µg of RNA gives an A_{260} reading of 1.00.

3.2. Denaturing RNA Gel Electrophoresis

1. Prepare a 1% agarose formaldehyde denaturing gel and submerse it in enough 1× MOPS buffer to cover the wells. Prerun the gel for 15 min (while RNA samples are being prepared).
2. Aliquot appropriate volumes of total RNA, containing between 10 and 20 µg, into labeled tubes on ice and dilute up to 15 µL with DEPC treated water. Add 15 µL of RNA sample buffer into each tube and 6 µL of 6× RNA loading buffer.
3. Incubate samples at 55°C for 10 min and place immediately on ice. Add the appropriate volume of RNA loading buffer to each tube to give a final 1× concentration of loading buffer in each sample.
4. Mix the RNA samples gently and briefly centrifuge tubes to collect the entire sample at the bottom of each Eppendorf tube.

5. Load the entire contents of each tube into the wells of the gel and record sample order for reference.
6. Perform gel electrophoresis at 100 V until the loading dye front reaches the end of the gel. Place the gel on plastic wrap and visualize the RNA using a UV light. The 28S and 18S ribosomal RNA (rRNA) bands are used as an indicator of RNA quality and should be found in a ratio of approx 2:1. This step ensures that you have good quality total RNA before isolating mRNA for DNA array hybridization. Although total RNA can be used for probe synthesis, isolating mRNA is recommended. If RNA is not of good quality the ratio of the 28S rRNA and 18S rRNA bands will be much less than 2:1 or there may be smearing of the sample. (*see* **Note 3** for considerations on maximal amount of RNA to be used for microarray analysis.)

3.3. mRNA Isolation Using Oligotex Mini Kit (Qiagen)

1. Heat Oligotex suspension to 37°C in a water bath, mix by vortex-mixing, and then place at room temperature.
2. Set a heating block to 70°C and heat the OEB.
3. Begin with 0.25–0.5 mg of good quality total RNA although this may need to be modified subsequently for selected tissues or animals if the mRNA yield is not sufficient.
4. Pipet total RNA into an RNase-free 1.5-mL Eppendorf tube and adjust the volume to 500 μL with RNase-free water.
5. Add 500 μL of OBB and 30 μL of Oligotex suspension.
6. Incubate the samples for 3 min at 70°C in a heating block to disrupt secondary structure.
7. Remove samples from the heating block and place at room temperature for 10 min to allow the oligo dT_{30} on the Oligotex particles to hybridize to poly$(A)^+$ tails of mRNA.
8. Pellet the Oligotex–mRNA complex by centrifugation at maximum speed in a microcentrifuge for 2 min and then carefully remove the supernatant.
9. Resuspend the Oligotex–mRNA pellet in 400 μL of OWB by pipetting up and down, pipet the suspension onto a spin column (provided with the kit) and place the column in an RNase-free 1.5-mL Eppendorf tube. Centrifuge for 1 min at maximum speed.
10. Transfer the spin column to a fresh RNase-free 1.5-mL Eppendorf tube and apply 400 μL of OWB to the column, centrifuge at maximum speed, and discard the flow through.
11. Transfer the spin column to a new RNase-free 1.5-mL Eppendorf tube and apply 20 μL of hot (70°C) OEB onto the column, pipet up and down 3–4 times to resuspend the Oligotex–mRNA resin, and centrifuge for 1 min at maximum speed and save the eluant which contains purified mRNA.
12. Repeat step 11 for maximal mRNA yield and pool the eluants from the two centrifugations. (*see* **Note 4** as further mRNA treatment may be inadvisable at this point.)

3.4. cDNA Probe Synthesis

1. Preheat thermal cycler to 70°C and add 1 μg (at least 0.5 μg/μL) of each mRNA sample (control and experimental) into separate 0.5 mL (or 0.2 mL depending on block size of thermal cycler) PCR tubes. To each tube, add 100 ng of Oligo-5′-dT_{20}N-3′ and 100 ng of random primers (200 ng total) to ensure labeling of the full mRNA pool.
2. Clonetch ATLAS™kits recommend using 1 μL of the CDS primer mix included with their kits, which includes sequence specific primers for the genes on their arrays. Although the CDS primer mix works relatively well with mRNA samples from hibernating mammal species, we have found that by replacing the CDS primer mix with 100 ng of Oligo-5′-dT_{20}N-3′ and 100 ng random primers (200 ng total), we get similar results for highly expressed genes and a better representation of all genes in samples. This is because the CDS primer mix contains species-specific primers that may fail to label genes that have sequence differences within the region covered by the CDS primer sequence. For screening nonmammalian species that are more distant on the phylogenetic tree, the use of Oligo-5′-dT_{20}N-3′ and random primers is absolutely required.
2. Dilute the reaction to 3 μL total by adding the necessary amount of DEPC-water and incubate for 2 min on the thermal cycler before reducing the temperature to 50°C for 2 min to allow for sufficient hybridization of the primers to mRNA.
3. To make the ATLAS™master mix for Clontech ATLAS™arrays, add to a 0.5-mL Eppendorf tube per reaction: 2 μL of 5× reaction buffer (included with Superscript II, Invitrogen), 1 μL of dNTP mix 1 (2.5 m*M* each of dTTP, dCTP, and dGTP; Invitrogen), 0.5 μL of 100 m*M* DTT, and 3.5 μL of [α-^{32}P]dATP (3000 Ci/mol; GE Healthcare). Alternatively, other radiolabeled nucleotides can be used provided that they are not included in the dNTP mix (i.e., [α-^{32}P]dGTP can be included with a dNTP mix consisting of dATP, dTTP, and dCTP). To create a master mix for synthesizing fluorescently labeled cDNAs (*see* **Note 5** for other considerations when using fluorescently labeled dNTPs), add per reaction: 8 μL of 5× reaction buffer, 3 μL of 20 m*M* dNTP mix 2 (6.67 m*M* each of dATP, dGTP, dTTP), 1 μL of 2 m*M* dCTP, 1 μL of 1 m*M* Cy3 or Cy5 dCTP (use one to label the control sample and the other to label experimental sample), 4 μL of 0.1 *M* DTT and 20 μL of water to bring the reaction up to 37 μL for each reaction. Keep master mixes on ice.
4. Finish preparing the master mix by adding Superscript II reverse transcriptase (RT) to the master mix tubes. ATLAS™array kits include an MMLV-RT enzyme that we have found to contain little very little activity from time to time. We recommend replacing the 1 μL of MMLV-RT with 1 μL of Superscript II RT per reaction is thus. The addition of 1 μL of Rnasin (Promega) per reaction is also advisable to prevent RNA degradation. Mix by pipetting up and down several times.
5. After the 2 min of incubation of mRNA at 50°C, add 8 μL of the ATLAS™master mix for synthesis of radiolabeled cDNAs or 37 μL of fluorescent master mix for synthesis of fluorescent cDNAs to each reaction and incubate at 42°C. For radiolabeled probe, the reaction should be incubated for at least 25 min. For

fluorescently labeled probe, reaction times are significantly longer (at least 2–3 h) due to the poor incorporation of the Cyanine dyes. The reactions are stopped by adding 1 μL of 0.5 *M* EDTA. Once this is done, the stopped reactions can be stored overnight at –20°C.

6. Probe cleanup varies depending on whether fluorescent probes or radiolabeled probes are made. Radiolabeled probes are cleaned up using the spin columns included with the Clontech ATLAS™kits to remove unincorporated nucleotides. Each individually prepared radiolabeled cDNA probe is then hybridized to its own individual array. Fluorescent probes are purified first by RNA hydrolysis: add 2 μL of 10 *M* NaOH and incubate at 65°C for 20 min, then neutralize the reaction with 4 μL of 5 *M* acetic acid. The labeled cDNA is then precipitated using 100 μL of isopropanol on ice for 30 min followed by centrifugation and washing with 70% ethanol. The labeled Cy3 and Cy5 labeled cDNA samples are then dried, each diluted in 5 μL of water or TE (10 m*M* Tris-base, pH 8.0, 1 m*M* EDTA) buffer, and then the two samples are combined into a cDNA pool before hybridization to an array.

3.5. DNA Array Hybridization and Washing

3.5.1. Hybridization and washing of Clontech ATLAS™ Arrays (See **Note 6**)

1. The suggested hybridization temperature for the arrays is 68°C for homologous hybridization. However, for heterologous hybridization, we find that the temperature should be lower. For mammalian hibernator samples, hybridizing at 68°C can generate a signal but better hybridization occurred at 55°C and we ultimately found that overnight hybridization at 44°C in Church's buffer generated the best hybridization result. For heterologous hybridization with nonmammalian species, reducing the temperature to 40°C gives the best hybridization signal with very little background.
2. After hybridization, the washing steps need to be modified and monitored to ensure that no cross-hybridization signal is lost with heterologous systems. Washes are started at 5× SSC (diluted from the 20× stock), 1% SDS, followed by washes at 2× SSC, 1% SDS, then 1× SSC, 0.5% SDS, and finally 0.5 × SSC, 0.5% SDS. After each wash step, ATLAS™arrays should be checked for hybridization signal using a Geiger counter. When a wash results in the signal dropping to 500–1000 cpm, washing should be stopped and ATLAS™arrays exposed to X-ray film or phosphorimager plates. After developing the film or reading the phosphorimager plate, the two films or two images (control vs. experimental) can be overlaid to identify differentially expressed targets first by visual inspection. To obtain a more quantitative result, each image generated from a phosphorimager or from scanned ×-ray film should be converted into .tiff files to be compatible with image analysis software.

3. ATLAS™ arrays can be reused at least three times. Arrays are stripped by boiling in 10% SDS for 10 min and washing with 2× SSC to remove all the SDS. Then the arrays are wrapped in cellophane and stored at –20°C until reuse.

3.5.2. Hybridization and Washing of Human 19 K microarrays (adapted from the Ontario Cancer Institute protocol, www.microarray.ca/, See also Note 6)

1. Prepare the hybridization solution by taking 100 µL of DIG Easy Hyb solution per hybridization and adding 5 µL of yeast tRNA (10 mg/mL) (Invitrogen) and 5 µL of calf thymus DNA (10 mg/mL) (Sigma) to reduce nonspecific binding. The mixture is heated to 65°C for 2 min and cooled to room temperature.
2. Add 80 µL of the prepared hybridization solution to a pooled pair of Cy3 and Cy5 dye-labeled cDNA samples and again heat to 65°C for 2 min followed by cooling the mixture to room temperature.
3. When using the 19K human microarrays, the genes are arrayed over two slides, so care must be taken when adding the prepared probe to the slides. One slide will lie on top of the other with the array sides facing inwards. Carefully apply the probe mixture in hybridization solution slowly and evenly along one of the edges ensuring that there are no bubbles present.
4. Place the extra hybridization solution inside a hybridization chamber (a sealable slide chamber placed horizontally in a 37°C incubator) to ensure that humidity levels are maintained so the hybridization reaction does not dry out. Place the slides inside the chamber and incubate overnight at 37°C. No modifications in hybridization temperature are needed.
5. After hybridization, wash slides in 2× SSC to remove hybridization buffer and perform subsequent washes by placing the microarray slides in a slide rack and washing in pre-warmed (50°C) 2× SSC, 0.1% SDS for 10 minutes followed by a wash in prewarmed (50°C) 1× SSC, 0.1% SDS. Finally, dip slides in 1× SSC, followed by a brief wash in isopropanol and centrifuge at 500 × g to remove any unbound fluorescent cDNAs. The microarrays can then be scanned at two wavelengths to quantify the different fluors. Two image files are generated and each is analyzed for fluorescent intensities. In cases where evolutionary distance is a concern for generating a good hybridization signal, it is advisable to wash much less stringently. For example, in studies utilizing cDNAs that are only 60–80% identical, it is advisable to lower the washing temperature to 45°C and only perform the 2× SSC wash. In our experience and in the experience of others ***(40)***, the salt concentration of the wash buffer has the greatest effect on removing probe from the arrays.

3.6. Array Analysis

Analyzing cDNA arrays has become easier with time. Our analysis has primarily been done using the Scanalyze program developed by Michael Eisen, which is available free of charge to academic researchers (http://rana.lbl.gov/),

coupled with visual inspection of target spots on the arrays themselves. Scanalyze allows users to input two array images, usually one image generated from scanning Cy3 hybridized targets and one image generated from Cy5 targets. Further information can be found at http://rana.lbl.gov/manuals/ScanAlyzeDoc.pdf. Other DNA array analysis programs abound; for further information *see* **Note 7**.

1. Open tiff files corresponding to Cy3 and Cy5 scanned images of 19K cDNA arrays in Channel 1 and Channel 2, respectively.
2. Once the images are loaded, click on the redraw button and adjust the gain and normalization of each image such that they have the same brightness and intensity upon visual inspection.
3. Grid the images such that each of the 19K cDNA "spots" is outlined by a Scanalyze generated circle. For each new batch of arrays, a new grid must be created. Click on the *New Grid* button on the *Grid control* form and select between 1 and 32 grids.
4. Enter the number of columns and rows per grid, column and row spacing and column and row height.
5. Because array printing is sometimes not entirely perfect, the grid may or may not fit the array exactly. In this case, the directional buttons in Scanalyze can be used to adjust the array grid up, down, left or right as well as stretching the grid in the same direction. When the array grid is close to being perfectly overlaid on each image, Scanalyze can perform fine tuning of the grid by pressing the refine button. If selected spots are misaligned, they can be further manipulated by selecting the "spot" option and using the directional buttons to align spots individually.
6. Once the grid has been made and fits the array, hit the save data button. Scanalyze will calculate the output information for each spot on the array and provide it in a tab delimited format that can be opened in Microsoft Excel.
7. By far the quickest and easiest type of analysis is to determine the hybridization ratio of intensities generated Channel 1: Channel 2 (e.g., control vs. hibernating). This will give a general indication of the ratio of gene levels between one state and another. Because the data is in Microsoft Excel, it can then be sorted based on highest to lowest (or lowest to highest) ratios by clicking on the Data tab and then selecting Sort. The genes corresponding spots on the arrays that show the greatest up- or down-regulation are then identified and downstream analysis is performed (*see* **Note 8**).

3.7. Confirmation of Results: Semiquantitative PCR (See Note 9)

1. For each gene of interest, obtain sequences for the homologous gene from other animals by downloading gene sequences from National Center for Biotechnology Information (www.ncb.nlm.nih.gov).
2. Select nucleotide from the drop down menu and enter the gene interest either by name or abbreviation.
3. Once one gene sequence is obtained, it is often easier to do a BLAST search (www.ncbi.nlm.nih.gov/BLAST/) to obtain the sequence of the gene from other species. Download the sequence of the gene from multiple sources. For example,

for studies of ground squirrel genes, other rodent and/or mammalian sequences (e.g., mouse, rat, human) might be chosen. For example: with hibernator genes, we typically use *Homo sapiens*, *Mus musculus* and *Rattus norvegicus* as a starting point for determining homologous regions. For animals of greater evolutionary distance, it is helpful to have sequences that are more diverse and/or from species that are more closely related phylogenetically to the species of interest. For example, if analyzing a turtle gene, a choice of frog, chicken and rat sequences might be more appropriate for initial analysis. Three alternate sequences is often sufficient, but sometimes with a well-studied gene, more species can be added to the analysis.

4. Open each sequence (*H. sapiens*, *M. musculus,* and *R. norvegicus*) in DNAman. Select Edit, Select All from the drop down menu and enter each sequence (Sequence, load channel) into its own channel. Once sequences are loaded, select Sequence, Multiple Alignment, Add From Channel and enter in all the sequences. Select the full alignment button and hit OK. A comprehensive gene alignment will be displayed with regions of homology identified by dark shading.
5. Once regions of high homology are found, Primer Designer software (Scientific and Educational Software) is used to generate putative primer sequences. Primer sequences showing no mismatched bases within the last 10 bases at the 3′ end can be used to generate primers. In some cases where sequence variability is high, a degenerate PCR primer is designed instead and used to obtain a cDNA sequence from the organism of interest. With this newly obtained sequence, species-specific primers can then be designed for use in expression analysis. The species-specific primer can also be used in the technique of rapid amplification of cDNA ends (RACE-PCR) in order to obtain the full sequence of the open reading frame of the gene of interest ***(12)***.
6. The protocol for creating first strand cDNA is identical to that used in DNA array probe synthesis section (**Subheading 3.4.;** *see also* **Note 10**) with the exception that no labeled nucleotides are added. The master mix contains 2 μL of 5× reaction buffer (Invitrogen), 0.5 μl 100 m*M* DTT, and 1 μl 10 m*M* dNTPs (2.5 m*M* each dATP, dTTP, dCTP, dGTP).
7. To optimize PCR conditions for a new sequence from a new animal, we routinely set up a temperature gradient on a gradient enabled thermal cycler (Bio-Rad iCycler) in a range of 50–70°C. The heating blocks are usually set up to run a gradient across eight samples so the temperature increments are 2.5°C. Set up a master mix on ice for each 50 μl reaction containing 5 μL of 10× reaction buffer, 2.5 μL of 50 m*M* $MgCl_2$, 1 μl 10 m*M* dNTPs, 1 μl 0.5 μ*M* PCR primers, 1 μL of template, 0.25 μL of *Taq* DNA polymerase (Invitrogen) and 39.25 μL of water.
8. The PCR protocol generally used is one initial denaturation step at 95°C for 2 min, and then 35 cycles of 95°C for 45 s, annealing (50°C to 70°C for 45 s), and extension at 72°C. The extension time at 72°C depends on the size of the product being amplified. Taq polymerase is a highly active so the general rule of 1 min per kilobase of DNA being amplified is more than sufficient but the time can be scaled back as required for shorter amplifications. After the 35 cycles are performed, a final 72°C extension step is carried out for 10 min and then the reaction is set on hold at 4°C or placed at 4°C in a refrigerator.

9. Run all of the reactions on a 1% TAE agarose gel, stain with ethidium bromide and visualize under UV light and take a picture for a photographic record. Identify the PCR annealing temperature (and/or extension time) that gave the highest amount of amplification and use these conditions for subsequent work.
10. Sequence the PCR product to confirm that it is indeed the gene of interest.
11. Once the annealing conditions are worked out and the PCR product has been confirmed as being the gene of interest, the control and experimental samples can be compared. Prepare serial dilutions of the first-strand cDNA for both control and experimental samples (i.e., 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}) for the gene of interest. Prepare an identical set of serial dilutions to be used to amplify a control gene. Commercial primers for genes such as α-tubulin, β-actin, or glyceraldehyde-3-phosphate dehydrogenase (more commonly referred to as GAPHD on commercial websites) may work for species with high sequence identity but for animals further separated through evolution, designing your own primers for control genes from highly identical regions is advisable.
12. PCR is performed on each dilution in the control and experimental samples. When complete, the products are electrophoresed on a 1% TAE agarose gel, which is stained with ethidium bromide and visualized under UV. Band intensities in the different lanes are measured using imaging software (Imagequant). Bands from one or more of the lower dilutions will likely be saturating in intensity so choose a band that is subsaturating but gives sufficient signal as the one to be quantified. This procedure ensures that the band chosen for quantification has an intensity that falls within the linear range of the imaging software used. Band intensity of the gene of interest in each lane can be normalized against the intensity of the corresponding control gene band to normalize for any unequal loading.

3.8. Outlooks: Comparability of Microarray Data, Comparative Genomics, and Hibernation

One of the early concerns with DNA array analysis was the lack of useful public domains to house the wealth of information that was produced ***(48–50)***. The initial hope was to create unique public databases that would allow researchers free access to microarray data in order facilitate more rapid discovery in areas that may be seemingly unrelated. For example, a researcher studying a particular gene, would be able to look at various microarray profiles and determine where and when the gene is up- or down-regulated and formulate a hypothesis about it's regulation in relation to other genes. The introduction at the NIH of the gene expression omnibus (GEO) (http://www.ncbi.nlm.nih.gov/geo/) has allowed researchers to do just this ***(51,52)***. Other microarray databases also exist. The Stanford University Microarray Database (http://genome-www5.stanford.edu/) lists published data, references and the organisms from which the data were obtained and ArrayExpress (http://www.ebi.ac.uk/arrayexpress/), a part of the European Bioinformatics

Institute ***(53–56)***, performs similarly to the NIH GEO database containing data from over 12,000 hybridizations covering at least 35 species. Currently, GEO remains the largest and fully open database that allows scientists free access to data acquired by high-throughput technology, including those relating to mRNA expression, genomic DNA analysis, serial analysis of gene expression (SAGE), mass spectroscopy, and proteomics. While these databases are useful, particularly for researchers working on model species, they have only just begun to be utilized by researchers undertaking comparative studies.

It is clear from that cross-species microarray analysis allows comparative researchers an opportunity to further research output immensely. Two areas that cross-species array screening does not address are ***(1)*** the case of genes displaying low homology between the array and the target organism, and ***(2)*** the occurrence of novel genes that are specific to a particular organism and, therefore, not represented on commercial arrays. However, arrays are being produced for more and more species all the time so, to some extent, both issues may dissipate with time as arrays become available for species that are phylogenetically closer to the species of interest. In the hibernation field, for example, the laboratory of Matt Andrews ***(57)*** has recently produced a DNA array with over 4000 cDNAs derived from a *S. tridecemlineatus* cDNA library and used this array to analyze the heart transcriptome in hibernation. With 4000 genes arrayed, a high percentage of the genome remains unrepresented and, hence, at the present time, heterologous probing still gives a broader result; e.g., we achieved 85–90% hybridization by ground squirrel cDNA with commercial 19,000 gene human arrays. However, the species-specific array offers the potential opportunity to find novel genes that occur only in hibernators (i.e., not found in the human genome) and, hence, the species-specific array could make a unique contribution toward the complete genetic analysis of the hibernation phenotype.

Because data from array screening must be followed up with rigorous downstream analysis, regardless of which array platform is used, the advantage for comparative biologists clearly currently lies with the use of cross-species cDNA array hybridizations. This is particularly true in the cases where the species of interest displays remarkable gene identity to a model species. Because the genomes of a number of non-traditional model species are currently being sequenced around the world, including *S. tridecemlineatus,* which is slated for full genome sequencing by the Human Genome Research project (http://www.genome.gov/), the field of cross-species microarray analysis should bloom in the coming years. Annotation and analysis of genes and gene structures from many species will shed more light into precise gene identity and homology, further confirming the usefulness of microarrays for future cross-species work by comparative biologists.

4. Notes

1. A critical part of any array study is establishing guidelines and using proper controls. As in all scientific endeavors, the choice of the appropriate control situation is the key to being able to properly interpret the gene expression changes that occur in the experimental situation. This seems especially important in array screening studies because these are analyzing mRNA pools and mRNA typically has a very short half-life in cells so for these studies it is best to have control and experimental samples that are as closely matched in time and in preexperimental state as possible. For example, a current controversy in the hibernation field illustrates this. We want to know how torpor is regulated and what genes need to be up-regulated to help an animal enter torpor and/or stabilize metabolism for long term survival the torpid state. Hence, we choose control and experimental animals that are as closely matched as possible: in this case, controls are euthermic animals at 37°C body temperature that have not entered torpor in the 5°C cold room versus animals in torpor in the same cold room with body temperature near ambient. This shows us the pool of gene expression changes that define the difference between active and torpid states. By contrast, some other groups advocate comparisons of summer active animals with winter torpid ones ***(58)***. This could show seasonal differences in the pools of mRNA in organs but is inappropriate for investigating the control of torpor because there are too many other differences between summer and winter animals including environmental conditions (e.g., photoperiod, thermoperiod), physiological states (e.g., actively feeding or not; active above ground vs. sleeping in burrows), and reproductive states that make it impossible to "dissect out" the gene expression changes that are torpor-specific. Hence, summer active animals are, at best, an extremely poor biological control and, at worst, a time point that is erroneous and detrimental to hibernation research as a whole. Indeed, the experimental protocol that we use (euthermic vs. torpid winter animals) for our gene screening has shown that a wide variety of genes are specifically up-regulated when animals enter torpor; these appear to perform essential biological functions in the torpid state. We also find extensive organ-specific activation of stress-induced signal transduction pathways in torpid versus euthermic animals including different classes of mitogen-activated protein kinases ***(17,59)*** which shows that organs maintain substantial metabolic activity during torpor. This actually goes against some previous "conventional wisdom" in hibernation research, which had the notion that most biological processes were turned down or off during torpor.
2. It is worth noting that our stress marker screening using Kinexus Kinetworks™ phospho-protein screens (**Table 2**) also revealed that selected proteins show an altered phosphorylation state during torpor in liver of *S. tridecemlineatus* further supporting the idea that hibernators do maintain metabolic activity during torpor. The results included an unchanged phosphorylation status of $p38^{MAPK}$ (Thr^{180}/Tyr^{182}), elevated phosphorylation of JUN (Ser^{73}), decreased phosphorylation of AKT at Ser^{473} but not Thr^{308}. This data are in agreement with previous studies in *S. richardsonii* that showed that during torpor $p38^{MAPK}$activity was

Table 2
Phospho-protein screen for *S. tridecemlineatus* liver

Protein Name	Abbreviation	Epitope	Euthermic	Torpid
N-methyl-D-aspartate glutamate receptor subunit 1 (112)	NR1	S896	133	—
Adducin α (121)	Adducin a	S724	1000	894
Adducin γ (80)	Adducin g	S662	485	404
Oncogene SRC (49)	SRC	Y529	347	834
Signal transducer and activator of transcription 5 (94)	STAT5	Y694	616	—
p38 α MAP kinase (38)	p38 MAPK	T180/Y182	205	136
Protein kinase C α (S657)	PKCa	S657	1658	3710
Oncogene SRC (49)	SRC	Y418	714	1413
Protein kinase C α/β (T368)	PKCa/b	T638/641	1128	1356
MAP kinase kinase 6 (MEK6) (36)	MEK6	S207/T211	188	240
MAP kinase kinase 3 (MEK3) (33)	MEK3	S207/T211	295	321
S6 kinase p70 (80)	p70 S6K	T389	688	905
Protein kinase C ε	PKCe	S719	618	921
Signal transducer and activator of transcription 3 (83)	STAT3	S727	1255	2315
Oncogene JUN (40)	JUN	S73	—	426
Oncogene Raf 1 (69)	RAF1	S259	656	411
Oncogene Raf 1 (63)	RAF1	S259	986	943
Protein kinase C δ	PKCd	T505	407	947
Protein kinase B-α (Akt1) (T308)	PKBa	T308	341	417
Protein kinase B-α (Akt1) (S473)	PKBa	S473	1193	547
Glycogen synthase kinase 3αa (45)	GSK3a	S21	172	54
dsRNA dependent protein kinase (68)	PKR	T451	243	653
Glycogen synthase kinase 3α (45)	GSK3a	Y279	437	286
Glycogen synthase kinase 3β (40)	GSK3b	Y216	—	317

Extracts were prepared from liver of euthermic and hibernating ground squirrels and assessed using the Kinexus Kinetworks™screens for phosphoprotein status to identify activation or suppression of signaling proteins during hibernation. The epitope to which the antibody is derived is given and relative expression levels of each protein in the euthermic and torpid states are given in arbitrary units.

unaltered, while JNK activity, the kinase that phosphorylates JUN, was highly elevated ***(59)*** and studies in *S. tridecemlineatus* that showed that Ser^{473} but not Thr^{308} was decreased during hibernation and torpor ***(60)***. Hence, data from array screening agrees well with data gathered by more traditional assays.

3. cDNA probes for microarray analysis are labeled using either fluorescence or radioactivity. Fluorescence probes are used exclusively for high-density DNA microarrays whereas ^{32}P-labeled probes are generally used for macroarrays, such as Clontech ATLAS™arrays. In situations of heterologous probing, our experience has shown that outcomes can be highly successful when several conditions are met:

   a. There is a high percent identity of genes between the two species (the test species vs. the one used to make the array),
   b. Optimal amounts of starting material are available, and
   c. Slightly less stringent hybridization conditions are applied than would be used in homologous probing.

   From tissue samples, we first purify RNA using the Trizol (Invitrogen) method of RNA extraction followed by mRNA [or $poly(A)^+$] purification using Qiagen Oligotex purification kits according to manufacturer's protocols. Our work has also found that while hybridizations can be performed with limited starting material (0.25–0.5 μg mRNA or 1–2 μg of total RNA at concentrations of at least 0.5 μg/μL), significantly higher hybridization intensity is obtained using purified mRNA isolated using Qiagen Oligotex–mRNA purification kits according to the manufacturer's protocols. The maximum mRNA suggested by each array protocol is usually at least 1 μg of mRNA. cDNA probes for microarray analysis are then prepared from the mRNA using either fluorescence or radioactivity. Fluorescence probes are used exclusively for high density DNA microarrays whereas ^{32}P-labeled probes are generally used for macroarrays, such as Clontech ATLAS™arrays. In order to compare two samples (e.g., control vs. experimental) with ^{32}P-labeled probes, one set of radiolabeled cDNA is hybridized to one array and another probe set is hybridized to a duplicate array.

4. Once the mRNA is prepared, it is suggested by most protocols that DNase treatment be performed prior to array hybridization to rid samples of any contaminating genomic DNA. While it is advisable to rid the sample of any contaminations, we found that eliminating this step had essentially no effect on array hybridization. In fact, when we eliminate this step, we rarely see binding above background levels (areas of the arrays spotted with buffer and no DNA) to genomic DNA array spots found on arrays. Thus, in cases where the mRNA sample is extremely limited, we feel that the possibility of RNases being introduced through increased handling outweigh the risk of some a small fraction of genomic DNA contaminating the reaction.
5. For fluorescence labeling, cyanine dyes (Cy3 and Cy5 for short), which are extremely light sensitive, are the preferred choice due to their relative ease of hybridization to microarrays (e.g., these dyes show little steric hindrance compared

to other fluorescent tags). In this case, one sample is Cy3 labeled and the other is Cy5 labeled and then the two probes are hybridized on a single chip. Further, for microarray studies involving fluorescently labeled cDNA, we generally perform two sets of hybridizations, in the first set labeling the control sample with Cy3 dye and the experimental sample with Cy5 dye and in the second set reversing this to eliminate any bias in the labeling procedure. Although the absolute fluorescence generated from each individual hybridization may vary, similar expression ratios are typically found on reciprocally probed arrays.

6. For our studies using Clontech ATLAS™arrays, we first used the suggested conditions given in the protocols included with the array kits to prepare radiolabeled cDNA. For our hybridization to microarrays, we began by following guidelines set out by the Microarray Center of the Ontario Cancer Institute. Subsequently, we found that altering some of the protocols increased our ability to detect transcripts on the arrays during heterologous probing. In general, preparation of cDNAs incorporating ^{32}P and fluorescent tags are the same with the major exception being that fluorescent probes should be made in the dark or reduced light to minimize excitation and degradation of the fluorescent signal.
7. While our analysis using Scanalyze has proven sufficient for identifying target genes for further studies, other analysis programs may offer more to other researchers. Other freeware applications available to researchers include the HTML based programs known as "Bullfrog" for Affymetrix arrays and "Spot" for custom and other types of cDNA arrays ***(61)***. Also available is the Gene Expression Open Source System (GEOSS) formerly known as Gene X Va ***(62)***, which allows users to input hybridization ratios and prepares data for clustering by easily converting hybridization ratios into color intensities for visualization and producing Venn diagrams (if multiple experimental samples are used). **Table 3** lists companies and other researchers that offer analysis software. Open source platforms are generally made freely available to academic and nonprofit researchers, usually with registration and referencing of the program. Other programs available are licensed to researchers at a price but do not necessarily improve upon the large number of freeware applications available to date.
8. A 1.5-fold change in gene expression seems to be sufficient to document a statistically significant difference between control and experimental situations in most cases but may still miss some important clues. Another layer of analysis can be used in which groups of related genes are assessed for overall changes in pathway response to stress. For example, a study by Mootha et al. ***(53)*** found that when analyzing 22,000 genes from skeletal muscle of age-matched human males falling into three categories (normal glucose tolerance, impaired glucose tolerance, diabetic), no genes were differentially regulated according to prior standards of statistical analysis or more simply, on a gene by gene basis, there was little detectable expression difference between genes in the three groups. However, by enriching the genes into sets for a pathway-based analysis, they found that genes involved in oxidative phosphorylation were co-coordinately down-regulated in diabetics and this was traced to the action of the transcription factor PGC-1αthat

Table 3
Microarray analysis programs

Array Program	Developer	Open Source	Reference
ArrayPro	Media Cybernetic	No	
Bullfrog and Spot	Zapala et al.	Yes	***(61)***
F-Scan	Munson et al., NIH	Yes	***(64)***
GenePix	Axon Instruments	No	
GEOSS	Lee et al.	Yes	***(52)***
ImaGene	BioDiscovery	No	
MatArray	Wang et al., National Research Center for Juvenile Diabetes	Yes	***(65)***
P-scan	Munson, et al., NIH	Yes	***(64)***
Quantarray	Packard Bioscience	No	
Scanalyze	Michael Eisen	Yes	

showed a approx 20% decrease in expression in diabetics ***(63)***. The development the Gene Set Enrichment Analysis (GSEA) approach for clustering genes into ordered groups according to known signaling pathways aids in the identification of biochemical functions that are associated with a particular stress. Of course, this analysis requires prior knowledge of signaling pathways and their downstream effects on gene expression, which may or may not be available for comparative systems.

9. While the preferred method of downstream microarray analysis is generally stated to be quantitative RT-PCR (Q-PCR), in reality, we feel that Q-PCR is unnecessary for downstream analysis of data derived from microarray screening in comparative studies. This is because the key outcome being sought is the relative change in gene expression between control and experimental conditions (i.e., is a gene up- or down-regulated), not the quantitative amount of each mRNA transcript type in each sample. Thus, semiquantitative RT-PCR is more than sufficient. Further, the term "quantitative" applied to PCR is not only incorrect, but also misleading. For a result to be truly quantitative, controls must be performed at every step of the analysis, including controlling for % yields during RNA isolation through to spiking the isolated RNA with a control mRNA of known quality and quantity so that downstream analysis can be performed on the control to give a precise standard for all downstream applications. For confirmation of gene expression changes highlighted by heterologous screening, the two methods most often used are Northern blotting (including dot or slot blotting) or semiquantitative RT-PCR. In cases where a differentially regulated target gene has already been cloned and is available to researchers, Northern blotting or dot blotting would be the preferred method of downstream analysis. In most cases, however, comparative

biologists would choose semiquantitative RT-PCR which is highly effective for two reasons: (a) PCR is conducive to high-throughput analysis of gene expression, and (b) the technique also generates material for a partial sequence analysis which, after translation, allows researchers to assess changes to amino acid sequence and putative structure/function differences of the protein in the species of interest as compared with its homologues in Genbank.

10. When using RT-PCR for validation, it is important to amplify as much of the total transcript population as possible. Oligo-5′-dT_{20}N-3′ priming works for many small mRNAs (<2 kB) but larger mRNAs and mRNAs containing a lot of secondary structure require additional priming. Thus, it is wise to also include random primers for first strand cDNA synthesis.

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