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Comparison of Snap Freezing versus Ethanol Fixation for Gene Expression Profiling of Tissue Specimens

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Frozen tissue specimens are the gold standard for molecular analysis. However, snap freezing presents several challenges regarding collection and storage of tissue, and preservation of histological detail. We evaluate an alternative preservation method, ethanol fixation followed by paraffin embedding, by analyzing expression profiles of microdissected cells on Affymetrix oligonucleotide arrays of three matched benign prostatic hyperplasia (BPH) and tumor samples processed with each preservation method. Frozen samples generated an average present call of 26% of the probe sets, compared to 4.5% in ethanol-paraffin samples. Eighty-eight percent of the probe sets called present in the ethanol-paraffin samples were also present in the frozen specimens. Comparing ethanol-paraffin BPH to tumor, 52 probe sets showed a two-fold differential expression or higher in at least two cases, 23 of which were also differentially expressed in at least one frozen case. Despite a significant drop in the number of transcripts detectable, the data suggests that the obtainable information in ethanol-fixed samples may be useful for molecular profiling where frozen tissue is not available. However, ethanol fixation and paraffin embedding of tissue specimens is not optimal for high-throughput mRNA expression analysis. Improved methods for transcript profiling of archival samples, and/or tissue processing are still required. ***(J Mol Diagn 2004, 6:371–377)***

Formalin fixation and paraffin embedding is the standard tissue processing method used in histopathology laboratories. This protocol allows for permanent preservation of the tissues, easy storage, and optimal histological quality. Unfortunately, formalin fixation severely compromises analysis of biomolecules, in particular mRNA and proteins. We have recently demonstrated the utility of an alternative fixation method, 70% ethanol followed by paraffin embedding.[1] The advantages of formalin-fixed samples are maintained, including excellent preservation of visual details, while allowing for the recovery and analysis of biomolecules.[1–3] For example, RT-PCR for specific genes can be performed on RNA from ethanol-paraffin tissues, including small samples such as microdissected cells.[1] However, an in-depth evaluation of the global integrity of mRNA in ethanol-paraffin samples has not been performed.

In the present study we used oligonucleotide arrays to compare mRNA quality in ethanol-paraffin and frozen tissues, using ethanol-fixed prostate specimens as a test tissue. A number of molecular profiling studies of human prostate carcinoma have been previously published using grossly dissected tissue specimens.[4–8] More recent work[9,10] has shown the feasibility of gene expression profiling studies using pure populations of cells obtained with laser capture microdissection (LCM).[11] We modified established protocols[12–14] for analyzing mRNA from microdissected frozen tissues and applied this protocol to ethanol-paraffin tissue. The direct comparison of frozen and ethanol-paraffin tissues provides insight into the advantages and disadvantages of each tissue preservation method.

Materials and Methods

Tissue Preservation and Laser Capture Microdissection

Human prostate tissue specimens fixed with ethanol and embedded in paraffin were obtained from patients undergoing radical prostatectomy for clinically localized prostate carcinoma on an Institutional Review Board (IRB) approved protocol at the National Institutes of Health or the National Naval Medical Center. After surgery, prostatectomy specimens were placed immediately on ice. The

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margins were inked and the specimens transversely sectioned into 3- to 5-mm thick sections, each of which was fixed in 70% ethanol overnight at 4°C. Ethanol fixation and paraffin embedding followed previously established protocols.[1] Specimens obtained from patients on an IRB-approved protocol at the Catholic University in Santiago, Chile, were snap-frozen in OCT and stored at −80°C. Three frozen cases (Fr1, Fr2, and Fr3) and three ethanol-fixed, paraffin-embedded cases (EP1, EP2, and EP3) were selected for microdissection to obtain matched benign prostatic hyperplasia (BPH) and prostate carcinoma cells.

Frozen Tissue

Frozen tissue blocks were re-cut into 6-μm thick sections and kept at −80°C. When used for study, one re-cut slide at a time was removed from −80°C and immediately stained. Briefly, the tissue was rehydrated by dipping the slides sequentially in 100%, 95%, 70% ethanol. The tissue was then placed in Mayer's hematoxylin (Sigma-Aldrich, St. Louis, MO) for 20 seconds, followed by deionized water and bluing solution (Sigma-Aldrich) for 15 seconds each. The tissue was then stained with eosin (Sigma-Aldrich) for 5 seconds and dehydrated using increasing concentrations of ethanol. Finally, the tissue was completely dehydrated by dipping in xylenes for 2 minutes.

Ethanol-Fixed, Paraffin-Embedded Tissue

Preserved tissue blocks were re-cut into 6-μm thick sections and kept at room temperature. When used for study, one re-cut slide at a time was first dewaxed, rehydrated, and then stained for hematoxylin and eosin (H&E) using the protocol described above. To remove the paraffin wax, slides were dipped in two consecutive baths of xylenes for 5 minutes each. After H&E staining, all slides were immediately used for LCM with the PixCell II LCM system (Arcturus, Mountain View, CA).

RNA Isolation and Measurement

Before LCM, the tissue was scraped from sample slides of each case and the RNA isolated using the RNeasy Mini Kit (Qiagen, Valencia, CA). RNA quality and quantity were assessed using the Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA).

After LCM, total RNA was isolated using the PicoPure RNA Isolation Kit (Arcturus) following the manufacturer's instructions, which included a DNase step. Isolated total RNA was then measured with the Bioanalyzer 2100.

RNA Amplification

RNA was amplified by modifying a previously established protocol that combines the RiboAmp (Arcturus) and Affymetrix (Affymetrix, Inc., Santa Clara, CA) systems.[12] The resulting product is biotin-labeled, antisense cRNA that can be used for oligonucleotide microarrays. Ten nanograms of total RNA were used as the starting material for all samples. The RiboAmp HS Kit (Arcturus) was used to perform two rounds of linear amplification and then, using 500 nanograms of input antisense RNA (aRNA) from round two, double-stranded cDNA for a third round of amplification was synthesized. During the final cDNA synthesis, double the amounts of reagents were used due to the kit's design for small samples.

The cDNA was then used for *in vitro* transcription (IVT) with the BioArray RNA Transcript Labeling Kit according to the manufacturer's protocol (Enzo Life Sciences, Farmingdale, NY). In total, each sample underwent three rounds of amplification to ensure abundant material for hybridization. After a 5-hour incubation at 37°C, the biotinylated-cRNA was quantified by UV absorbance. Ten μg of each biotinylated sample were then fragmented according to the recommended protocol (Affymetrix, Inc.) followed by hybridization to the gene chip.

Microarray Hybridization and Scanning

Samples were hybridized to Human Genome U133A GeneChip arrays (Affymetrix, Inc.) for 16 hours. Microarrays were washed and stained using the manufacturer's standard "EuKGE-WS2v4" protocol. This step involves antibody-mediated signal amplification. The chips were then scanned using the Affymetrix GeneChip Scanner 3000.

Data Analysis

Microarray Analysis Suite 5.0 (MAS 5.0) (Affymetrix, Inc.) was used to process the images. The data for each microarray were initially normalized by scaling all signals to a target intensity of 500. Expression analysis was performed using the defaults parameter settings. Present calls required a P value of $P < 0.05$ and marginal calls required $P < 0.065$ for all probe sets. Probe sets with $P > 0.065$ were marked absent. Each array was analyzed based on the present, marginal, or absent call for each probe set. A probe set is the group of probes for a single transcript. In the U133A chip, most probe sets relate to a known gene, but some relate to hypothetical proteins or ESTs. In addition, some genes have multiple corresponding probe sets. This chip contains approximately 23,000 probe sets.

A report including hybridization controls, housekeeping gene information, raw signal averages, and present call was then generated. The data were then uploaded to the National Cancer Institute's mAdb microarray website (http://nciarray.nci.nih.gov) for further analysis.

Using the mAdb web site, both absolute expression analyses and comparison analyses were performed. For absolute expression analyses, unfiltered data were subjected to average linkage hierarchical clustering of the arrays using a classical Pearson correlation.[15] For comparison analysis, determinations of gene expression increases or decreases required a $P < 0.002$. All calls of marginal increase, no change, or marginal decrease were excluded from further analysis, as were probe sets called marginal or absent in either one or both arrays. In comparing BPH to tumor, BPH was always used as the

Table 1. Microdissection, RNA Yield, and Array Performance

Sample	LCM Shots	Total RNA	Round 1 aRNA	Round 2 aRNA	Round 3 template	Round 3 cRNA	Present call (%)	GAPDH 3'/5'	β-Actin 3'/5'
Fr 1 BPH	1496	10 ng	1.2 ng	16.6 μg	500 ng aRNA	21.3 μg	15.9	3.1	6.0
Fr 1 Tu	2006	10 ng	1.9 ng	25.4 μg	500 ng aRNA	16.6 μg	12.7	2.3	4.7
Fr 2 BPH	1692	10 ng	9.5 ng	35.1 μg	500 ng aRNA	21.7 μg	31.6	4.6	4.4
Fr 2 Tu	1049	10 ng	5.8 ng	37.9 μg	500 ng aRNA	18.3 μg	31.1	8.2	7.3
Fr 3 BPH	2616	10 ng	13.1 ng	51.8 μg	500 ng aRNA	10.4 μg	28.6	7.4	49.9
Fr 3 Tu	2679	10 ng	19.2 ng	54.9 μg	500 ng aRNA	13.9 μg	36.2	5.9	9.5
Fr Average	1923	10 ng	8.5 ng	36.9 μg	500 ng aRNA	17.0 μg	26.0	5.3	13.6
E–P 1 BPH	10827	10 ng	3.2 ng	1.5 μg	500 ng aRNA	47.2 μg	3.7	1.7	10.3
E–P 1 Tu	19032	10 ng	2.3 ng	7.4 μg	500 ng aRNA	36.1 μg	3.0	1.2	0.5
E–P 2 BPH	16441	10 ng	2.2 ng	8.2 μg	500 ng aRNA	40.6 μg	7.5	3.2	3.9
E–P 2 Tu	9256	10 ng	17.6 ng	56.3 μg	500 ng aRNA	32.7 μg	4.2	0.4	0.8
E–P 3 BPH	10023	10 ng	73.0 ng	71.6 μg	500 ng aRNA	34.1 μg	2.5	0.4	0.6
E–P 3 Tu	7771	10 ng	78.5 ng	39.7 μg	500 ng aRNA	35.5 μg	6.1	1.5	2.1
E–P Average	12225	10 ng	29.5 ng	30.8 μg	500 ng aRNA	37.7 μg	4.5	1.4	3.0

baseline array, with tumor as the experimental array. Only intra-patient comparisons were done. This minimized any transcript expression differences that could be due to unrelated patient or tissue handling factors. Ethanol-paraffin samples were not compared directly to frozen samples due to the extreme differences in present call.

Results

Microdissection, RNA Amplification, and Microarray Hybridization

The 12 samples were evaluated following each step of the protocol and the data are presented in Table 1. To collect 10 ng of total RNA, the frozen samples required an average of 1923 LCM shots, while the ethanol-paraffin samples required an average of 12,225 shots. In addition to more material being needed to obtain an equivalent amount of RNA, the ethanol-paraffin samples also generated RNA of poorer quality than that from the frozen samples (Figure 1). RNA from all of the ethanol-paraffin samples was fragmented, appearing only as a smear, the largest fragments of which reached to 1 kb with an average fragment size of approximately 300 bases. Although the RNA from most of the frozen samples was of excellent quality, with sharp ribosomal RNA bands approaching a 2:1 ratio of 28S and 18S RNA, there was some variation, with both the BPH and tumor from the Fr1 specimen being of lower quality. The first round of amplification using the Riboamp HS Kit generated approximately a 50-fold amplification of the mRNA in the original 10 ng total RNA from each sample, while the second round generated approximately a 1000-fold amplification of the first round aRNA. The third round, using a combination of the Riboamp HS Kit for cDNA synthesis followed by the ENZO Kit for IVT, generated RNA amplifications of approximately 20- to 100-fold, with the higher apparent yields from the ethanol-paraffin samples (an average of 37.7 mg versus 17.0 mg from the frozen samples). Fol-

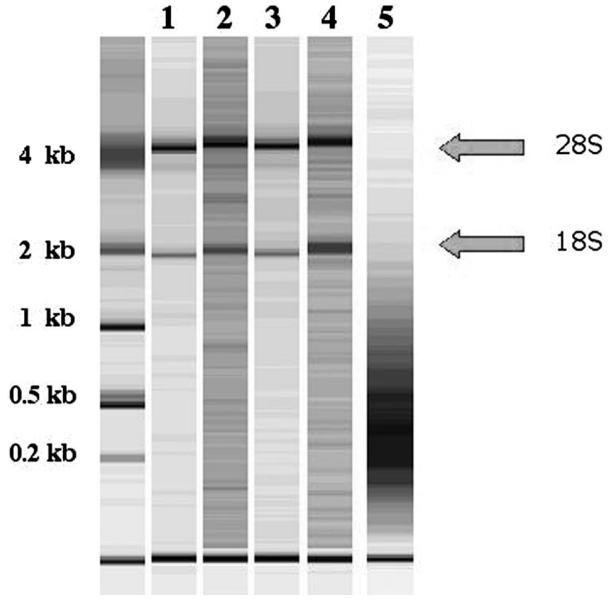

Figure 1. Total RNA after microdissection, before amplification. **Lanes 1** to **4**, frozen samples. **Lane 5**, ethanol-paraffin sample.

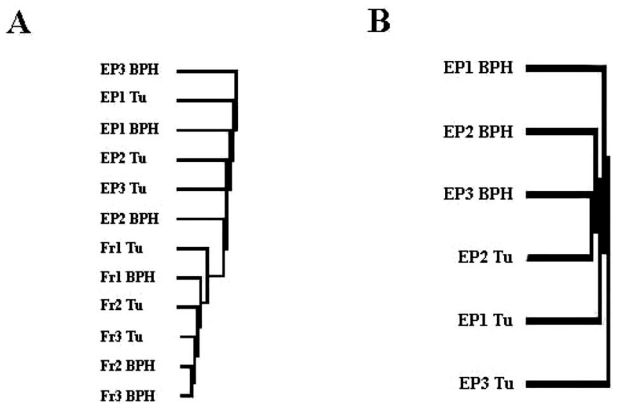

Figure 2. Hierarchical clustering of arrays using all probe sets. **A:** Hierarchical clustering of all arrays. **B:** Hierarchical clustering of ethanol-paraffin samples. E-P, ethanol-paraffin; Fr, frozen; BPH, benign prostatic hyperplasia; Tu: tumor.

lowing microarray hybridization, the 3′/5′ ratios for the housekeeping genes *GAPDH* and *β-actin* were lower in the ethanol-paraffin samples than the frozen samples, although the overall present calls for all probe sets was higher for the frozen samples. Using high quality RNA, the number of probe sets called present typically ranges between 30 and 55% of the total available on the U133A chip, depending on the source and processing of the sample. In our experience, prostate cell lines have provided a 48 to 50% present call, whereas frozen prostate tissue, whether or not the RNA was amplified, provided a 25 to 30% present call. The average present call for these samples was 26.1% for the frozen tissues and 4.5% for the ethanol-paraffin specimens.

Absolute Expression Analysis

On initial examination of the data, hierarchical clustering including every probe set clearly differentiated frozen from ethanol-paraffin samples (Figure 2A), which was the expected result since it has been well established that RNA quality greatly influences gene expression profiling efforts and RNA quality is compromised in non-frozen samples. To determine the overall relationship of the ethanol-paraffin arrays, these were separately clustered, again including all probe sets. The BPH and tumor samples appeared on separate sides in the cluster (Figure 2B).

To determine whether the probe sets called present were skewed toward highly abundant transcripts as a result of the preservation method, the normalized signal of probe sets called present for each array was compared to the normalized signal of all probe sets on the array (regardless of present call) (Table 2). Comparing

Table 2. Relative Signal of Probe Sets Called Present on Normalized Arrays

Sample	Average signal of probe sets called present	Average signal of all probe sets on array	Fold increase
Cell lines	1334	704	1.9
Frozen	2840	843	3.4
Ethanol-paraffin	10790	889	12.1

Table 3. Number of Probe Sets Called Present in Frozen and Ethanol-Paraffin Samples

Array	Probe sets present in at least 1 of 3 arrays	Probe sets present in all 3 arrays	% of probe sets consistent across arrays
Fr BPH	8402	2666	31.7
Fr Tu	9131	2245	24.6
E–P BPH	2465	82	4.2
E–P Tu	2331	114	6.0

the signal of present probe sets to the overall probe set signal accounts for variable scaling factors between arrays. Two arrays from unamplified prostate cell line RNA were included to provide a baseline for comparison. The average signal of present probe sets was 1.9-, 3.4-, and 12.1-fold increased over the entire array signal for the cell lines, frozen samples, and ethanol-paraffin samples, respectively.

The probe sets called present on each of the 12 arrays were quantified and compared regarding preservation method and tissue type. The data are presented in Table 3. The number of probe sets called present in any one of the three ethanol-paraffin BPH or tumor arrays was approximately 25 to 30% of those in the frozen arrays. When comparing probe sets called present in all three BPH or tumor arrays for each preservation method, the number of probe sets called present in the ethanol-paraffin group was only approximately 5% of the number for the frozen group. However, of the 82 probe sets called present in all of the ethanol-paraffin BPH arrays, 72 (87.8%) were also in the frozen BPH arrays. Of the 114 probe sets present in all of the ethanol-paraffin tumor arrays, 100 probe sets (87.7%) were also in the frozen tumor arrays. A total of 159 different probe sets were present in the ethanol-paraffin BPH or tumor arrays. Of these, 37 probe sets representing 32 genes were common to BPH and tumor (Table 4), yielding a 23.3% overlap.

Comparison Expression Analysis

To compare the gene expression of BPH cells to tumor, individual comparisons (ie, each intra-patient comparison) were grouped and only probe sets showing a significant ($P < 0.002$) and twofold or greater change were selected. With these criteria, 19 probe sets consistently differentiated all BPH from tumor in all of the frozen samples. When any two of the three possible BPH arrays were compared to tumor, 297 differentially expressed probe sets were identified.

The same guidelines as above were applied to the comparison of ethanol-paraffin BPH to tumor. No probe set differentiated BPH from tumor when requiring a twofold or greater change in all array comparisons in ethanol-paraffin samples. When only two of the three possible BPH arrays were compared to tumor, 52 probe sets were identified. Of these 52 sets, 23 probe sets representing 22 genes were also differentially expressed in at least one of the three frozen comparisons (Table 5).

Table 4. Probe Sets Present in Both Frozen and Ethanol-Paraffin BPH and Tumor

Gene	Description
HNRPL	heterogeneous nuclear ribonucleoprotein L
SEMA3F	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3F
KIAA0397	KIAA0397 gene product
PHF1	PHD finger protein 1
TRAP95	thyroid hormone receptor-associated protein, 95-kD subunit
GGA1	golgi associated, gamma adaptin ear containing, ARF binding protein 1
CGI-40	CGI-40 protein
LOC222070	hypothetical protein LOC222070
CRYM	crystallin, mu
SFN	Stratifin
FAU	Finkel-Biskis-Reilly murine sarcoma virus (FBR-MuSV); ribosomal protein S30
RPL34	ribosomal protein L34
RPS10	ribosomal protein S10
PSMB2	proteasome (prosome, macropain) subunit, beta type, 2
RPL37A	ribosomal protein L37a
RHEB2	Ras homolog enriched in brain 2
SNRPN	small nuclear ribonucleoprotein polypeptide N
LAMP1	lysosomal-associated membrane protein 1
TAT	tyrosine aminotransferase
MKRN4	makorin, ring finger protein, 4
RPL39	ribosomal protein L39
RPS28	ribosomal protein S28
RPS6	ribosomal protein S6
LRRFIP1	leucine rich repeat (in FLII) interacting protein 1
RPL13	ribosomal protein L13
CHRNE	cholinergic receptor, nicotinic, epsilon polypeptide
EEF1D	eukaryotic translation elongation factor 1 delta (guanine nucleotide exchange protein)
KIAA1827	KIAA1827 protein
	Homo sapiens Alu repeat (LNX1) mRNA sequence
GLTSCR2	glioma tumor suppressor candidate region gene 2
MLPH	Melanophilin
KPNB2	karyopherin (importin) beta 2

Discussion

Development of tissue fixation and embedding procedures are needed that allow for the preservation of biomolecules for analysis without the disadvantages presented by snap freezing. Ethanol fixation offers high quality histology, easy storage of blocks, as well as the possibility of recovering biomolecules for analysis. We have been able to generate proteomic data using microdissected cells from ethanol-paraffin samples.[3,16] How-

Table 5. Two-fold Changes between BPH and Tumor Identified Both in Frozen and Ethanol-Paraffin Samples

Gene	Description
Up-regulated:	
	Homo sapiens, clone IMAGE:4429946, mRNA
SAT	spermidine/spermine N1-acetyltransferase
GADD45B	growth arrest and DNA-damage-inducible, beta
	Human clone 137308 mRNA, partial cds.
Down-regulated:	
NEFH	neurofilament, heavy polypeptide 200kDa
LARS2	leucyl-tRNA synthetase, mitochondrial
PPARD	peroxisome proliferative activated receptor, delta
MSF	MLL septin-like fusion
LOC283445	hypothetical protein LOC283445
CGI-40	CGI-40 protein
FBLN1	fibulin 1
HOMER3	homer homolog 3 (Drosophila)
	Homo sapiens mRNA; cDNA DKFZp566G0746 (from clone DKFZp566G0746)
SFN	stratifin
NDUFB8	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 8, 19kDa
MYH11	myosin, heavy polypeptide 11, smooth muscle
TCIRG1	T-cell, immune regulator 1, ATPase, H+ transporting, lysosomal V0 protein a isoform 3
NCOR2	nuclear receptor co-repressor 2
ARPC2	actin related protein 2/3 complex, subunit 2, 34kDa
MYL6	myosin, light polypeptide 6, alkali, smooth muscle and non-muscle
GLTSCR2	glioma tumor suppressor candidate region gene 2
FLJ13993	hypothetical protein FLJ13993

ever, we have not assessed the global integrity of mRNA using a high-throughput approach such as microarray analysis.

In the present study, we evaluated mRNA quality in ethanol-fixed, paraffin-embedded prostate tissues by comparing oligonucleotide array data generated from these samples to those from frozen tissues. Cells were laser capture microdissected from tissue sections, and the mRNA was amplified three times before expression array analysis. The starting mRNA amount for amplification was normalized in all cases (10 ng), as well as the total amount of amplified cRNA hybridized on the chips (10 μg). Three rounds of linear amplification have previously been shown to decrease the average distribution size of aRNA without affecting reproducibility.[14] Overall, the ethanol-paraffin samples required significantly more time and effort for microdissection to procure 10 ng of total RNA. This could prove to be a limiting factor in the utility of this preservation technique.

Surprisingly, the 3′/5′ ratios for the housekeeping genes *GAPDH* and *β-actin* were lower in the ethanol-paraffin samples than the frozen samples. The 3′/5′ ratio is a useful measure of the degree to which an entire RNA transcript was amplified. Since amplification travels from the 3′ toward the 5′ end, one expects the 3′ probe signal to be greater than the 5′ probe signal, as transcription may stop before an entire sequence is transcribed. While manufacturer recommendations are for the 3′/5′ ratio to be less than 3.0 in non-amplified material, amplified RNA yields significantly higher values.[12] Although many of our ratios are less than 3.0, we do not believe this reflects full transcript amplification. As initial RNA transcript lengths become smaller and smaller due to fragmentation that results from isolating RNA from ethanol-paraffin tissue, even fewer of the 3′ probes are amplified, and thus the ratio falls. Therefore, the low values for the housekeeping gene ratios seen among the ethanol-paraffin samples may signify loss of 3′ probes in addition to the 5′ probes. That more 5′ probes for *β-actin* were called absent than for *GAPDH* is likely due to *β-actin's* longer transcript length: 1.79 kb versus 1.28 kb for *GAPDH*.

The ethanol-paraffin samples had a significantly lower percent present call than frozen samples, 4.5% versus 26%. First we assessed whether these probe sets correspond to a subset of higher abundance genes. If the full range of transcript abundance were represented in each array, then one would expect the ratio of the signal for present probe sets to the overall array signal to be the same for all samples, regardless of percent present call, since each array was independently scaled to a target signal of 500. The probe sets present in ethanol-paraffin arrays had a much higher relative signal than the frozen and cell line arrays. Therefore, we can conclude that on average the more abundant transcripts from ethanol-paraffin tissue hybridized to the arrays. Affymetrix platform has multiple controls in place to prevent or at least easily identify potential array-based discrepancies, and none were observed. We believe there is a threshold effect by which only the most abundant transcripts in ethanol-paraffin tissue can be amplified. We think that the primary cause for artifactual absent calls in the Affymetrix experiments is that the transcripts have been shortened predominantly during the tissue processing, beyond the site where they would be detected by the probe set. Previously, we analyzed arrays with non-amplified material obtained from scrapes of ethanol-fixed, paraffin-embedded normal prostate and lymph node tissues (unpublished data). Several micrograms of RNA were procured. The RNA showed degradation (no ribosomal bands) already before the amplification. cDNA arrays from the NCI array facility were used for the analysis. Even though several lymphocytic-related transcripts were up-regulated in the lymph node, and epithelial or smooth muscle-related transcripts up-regulated in the prostate sample, a significantly smaller proportion of genes than expected were detected differentially expressed between the two tissues types.

An interesting issue refers to which of the steps, fixation or embedding, is affecting the integrity of the RNA. Two recent studies have shown that the use of different fixatives has a significant effect on RNA. Kim et al[17] showed that methacarn, a combination of methanol, chloroform, and acetic acid was the optimal fixative. RNA was assessed by microcapillary electrophoresis using Agilent Labchips, as well as RT-PCRs. Vincek et al[18] show that both histomorphology and macromolecules can be preserved when a new universal molecular fixative (UMFIX, Sakura Finetek USA, Inc., Torrance, CA) is used. Quality and quantity of DNA, RNA and proteins were assessed. According to these data, the fixation method is an important factor in the preservation of RNA. In a previous study on the effect of fixatives on the preservation, we also assessed the use of alternative embedding procedures, such as low-temperature melting polyester wax.[1] Higher and more intense RNA smears were seen when embedding was performed with low-temperature melting polyester waxes (Figure 6A from reference[1]). This data suggests that in addition to fixation, the conventional paraffin-embedding step, used in most of the laboratories, also significantly influences RNA stability.

Another key question was whether the probe sets called present in ethanol-paraffin samples correspond in fact to true gene expression rather than to some form of non-specific hybridization. The ability of the unfiltered data to separate the three ethanol-paraffin BPH samples from the tumor samples suggests that the data correspond to biologically expressed genes. In addition, although a smaller number of probe sets were consistent between arrays in the ethanol-paraffin BPH and tumor groups than frozen samples, nearly 90% of these appeared in the corresponding frozen list, and only 23% of the probe sets overlapped between BPH and tumor in the ethanol-paraffin group. These data suggest that probe sets that appear consistently using ethanol-paraffin tissue are truly expressed in the sample and are not a result of non-specific hybridization ("sticky spots"). Though sticky spots are an inherent problem to cDNA arrays, the Affymetrix platform uses a perfect match and single mismatch algorithm to calculate present call, which minimizes this problem. Some overlap between the BPH and tumor samples should be expected considering that both are epithelial, replicating prostate cells. In fact, as ex-

pected, many of these probe sets are genes that encode ribosomal proteins.

Of the 52 probe sets with at least a twofold difference between all three of the ethanol-paraffin BPH and tumor samples, almost half of them also appeared in at least one of the three intra-patient frozen comparisons. These data provide further evidence that while fewer genes can be measured from ethanol-paraffin tissue, the measured changes are valid.

In summary, there are significant limitations of mRNA analysis of ethanol-paraffin tissues, such as increased dissection time and loss of measurable transcripts. However, the approach is able to consistently generate approximately 5% present calls per microdissection, equivalent to about 1000 genes per experiment. Thus, it is possible to identify differentially expressed genes that are expressed at relatively high levels in at least one of the biological samples under study. In a recent publication, Kabbarah et al[19] reported an ethanol fixation and paraffin-embedded protocol that allows for GeneChip analysis of 800 to 4400 cells microdissected from mouse tissue samples. The protocol involves 16 to 24 hours of ethanol fixation, is performed at 4°C, and is followed by conventional paraffin embedding or low-melting temperature paraffins. It is likely that additional degradation takes place when working with clinical samples due to the time of the surgical procedure, as well as the longer processing required, such as whole mount sectioning, margin labeling, etc.

There are still clear and significant improvements that are needed in tissue processing methods to facilitate gene expression profiling. A fixative that resulted in less RNA degradation would negate all of the current pitfalls of ethanol fixation. On the other hand, there is a great need to develop an amplification protocol that reliably amplifies all mRNA fragments, including small segments such as those recoverable from formalin-fixed, paraffin-embedded tissues. This would enable the exploration of the stockpiles of fixed tissues that have been archived worldwide.

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