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Increased Detection of RNA Species in Histological Tissues Using a Two-Temperature Fixation Protocol

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Abstract

A number of diagnostic and research assays rely on accurately measuring the levels of ribonucleic acids (RNAs) in tissue. The best way to obtain high quality intact RNA from tissues is to use samples that are fresh. The standard for treating tissues used in pathology workflow is to treat with 10% neutral buffered formalin, alcohols, xylenes and waxes but produces lower quality material for detection of RNA. One barrier to analyzing RNA species in formalin-fixed paraffin embedded tissues (FFPE) is the use of variable times that institutions fix tissue samples which causes variable preservation and assay results. Another barrier is how long samples sit at room temperature (RT) before fixing, so called cold ischemia time. We report here on increased detection of RNA species from our rapid two-temperature formalin fixation protocol (cold+warm) which standardizes tissue collection and reduces cold ischemic time as variables in RNA ISH analysis. Human tonsils, mouse xenograft tumors and cell culture samples were used as model systems in fixation time course experiments to determine sensitivity of RNA preservation to the level of fixation. To extend these results colon, breast and lung carcinoma clinical patient samples were also examined. The amount of detectable β-actin RNA or miRNAs increased with increasing fixation times in model systems. In clinical tissues, the amount of detectable RNA species dramatically increased (20-40 fold) in tissues fixed with the cold +warm method. A dramatic reduction (20-50 fold) in RNA levels was observed when the tissues had longer cold ischemia times with standard room temperature fixation.

Keywords: RNA; RNA ISH; MicroRNA; Formalin; Tissue Fixation

Introduction

A number of diagnostic and research assays rely on accurately measuring the levels of RNA species in tissues [1-15]. The best way to obtain high quality intact RNA from tissues is to use samples that are fresh [16-22] or to use only alcohols to "fix" samples [23-25]. However, these options are not the standard way of treating tissues used in standard pathology workflow. Tissue is typically fixed in formalin, chemically processed and embedded into paraffin (wax) blocks. In these routine tissue samples the preservation of nucleic acids, especially RNA species, is a topic of growing importance in tissue diagnostics.

One barrier to analyzing RNA species in formalin-fixed paraffin embedded (FFPE) tissues is the lack of standard fixation times and tissue thickness which leads to variable penetration of the fixative from the edge of the tissue to the middle. Since an over whelming number of histology tests, IHC and ISH, rely on detecting epitopes from fully crosslinked and epitope retrieved tissues, these under-fixed samples often exhibit signal gradients (edge to middle) and errors in routine histological assays [9,26-33]. For example, phosphorylated protein kinase B (pAKT) expression in samples fixed for only 1-4 hours has been documented to exhibit a large spatial gradient from edge to middle [34]. Longer fixation protocols, greater than 8 hours, typically have fewer signal gradients and IHC and ISH signals are stronger in intensity. Unfortunately the duration of fixation of clinical samples is variable, often rushed, and leads to variable results for RNA *in situ* hybridization (ISH) assays.

Another barrier is how long the samples sit fresh at room temperature (RT) before being placed into fixative, the so called cold ischemia time. During cold ischemia, RNases are active and cause degradation of important RNA species. For high quality tissue samples, it will be imperative to develop and standardize rapid protocols for tissue fixation.

We previously reported on a novel, rapid two-temperature formalin fixation strategy that preserves key features of tissue such as morphology, as well as improving preservation of hard to detect markers [35]. In Calu3 mouse xenografts, human tonsil and cancerous tissues; significantly more phosphorylated-AKT protein was preserved in tissues with cold+warm fixation in comparison to standard room temperature procedures. To extend the capabilities of this rapid protocol, we investigated if this technology aided in the preservation of long and short RNA species in mouse xenografts, a cell culture system and human normal and carcinoma tissues. We report here on the increased preservation of RNA species from our rapid two-temperature formalin fixation protocol and the importance of cold ischemic time as a variable in RNA ISH analysis.

Materials and Methods

Tissue collection and fixation

For experiments involving human tumors Figures 1 and 2: Human colorectal (N=23), breast (N=10) and lung (N=10) carcinoma patient samples were collected in an OR setting with low cold ischemia times by Indivumed GmBH (Hamburg, Germany) to better preserve labile biomolecules under the company's IRB protocol. De-identified tissues were collected, fixed and processed by Indivumed GmBH scientists

and the wax blocks provided to Roche tissue diagnostics for microtomy and staining. Fixation consisted of immersion in 10% neutral buffered formalin (10% saturated aqueous formaldehyde from Fisher Scientific, Houston, TX, buffered to pH 6.8–7.2 with 100 mM phosphate buffer) for varying times at temperatures ranging from 4-45°C. Room temperature ranged from 20–25°C. The portion of the tumor which was allowed by the attending pathologist was split into 3 4mm thick sections and each fixed differently. One piece was wrapped in saline soaked gauze and kept at RT for 1 hour ("1 hour cold ischemia") before 24 hr fixation at RT. This was to test the effect of the longer guideline-prescribed cold ischemia interval of one hour. The remaining two pieces were used to test the effect of shorter (less than 17 minutes) cold ischemia.

One piece was placed directly into RT formalin for 24 hours ("24 hr"). A third piece was placed directly into 4°C formalin for 2 hours followed by 2 hours in 45°C formalin ("cold+warm")[35] to test if the process could be sped up to increase work efficiency in the hospital. Fixation was carried out in 100– 500 mL covered beakers in a refrigerator for 4°C treatment, or in a standard chemical fume hood for higher temperatures. Cold ischemia times for the "24" and "cold +warm" samples averaged 10 ± 3 minutes (range 6-17 min) (Figures 1 and 2).

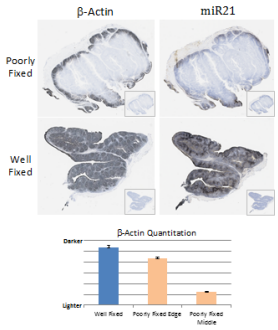

Figure 1: β-Actin and miR21 Staining on Poorly and well Fixed Tonsil. Whole human tonsil organs were soaked in 4 CO 10% neutral buffered formalin for 4 hours and then soaked in 45 CO 10% NBF for 1 hour (Top panels). Organs were sliced to 4 mm thickness and further processed overnight into wax blocks (Poorly fixed example). For well-fixed samples, 4mm thick human tonsils were fixed with the cold+warm method and processed into wax blocks. 4 um sections were stained with either a probe to β-Actin (left panels) or to miR21 (right panels). Insets at bottom right of pictures show staining of same tissues with a scrambled probe. Graph at bottom shows semi-quantitation of the silver signals from the well-fixed samples (blue bar) and from the edge and middle of the poorly fixed (Orange bars) samples.

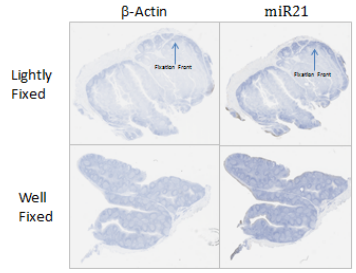

Figure 2: Anti-Sense Probe Controls. Human tonsil tissues were probed with an anti-sense probe to either β-actin or miR21. Stained tissue slides were scanned and images represent 2X.

For experiments involving human tonsil organs (Figures 3-5): Human tonsil organs deemed medical waste and de-identified were obtained on wet ice from a local medical center unfixed with the following procedure to ensure high quality tissues. Whole tonsil organs from surgeries were immediately placed into biohazard clear plastic bags, sealed and placed into a refrigerator. Periods of cold ischemia were unknown other than confirmation the associated surgery took place the day of delivery. Tonsil organs from those days' surgeries did not undergo any histologic or pathologic procedures, including grossing, and were delivered intact, unfixed and fresh.

Whole tonsils were immediately sectioned into 4 mm thick samples, routine histologic size, and placed into 10% NBF at RT or 4°C. Samples were removed from the RT container at various times as indicated up to 24 hrs to make a time course of fixation. Alternatively for cold +warm fixation, samples were soaked in 4°C NBF for 2 hours, followed by 2 hours in 45°C NBF for 2 hours (cold+warm). For experiments on whole tonsils, the entire tonsil was placed directly into 4°C or room temperature NBF for the indicated times (Figures 3 and 4).

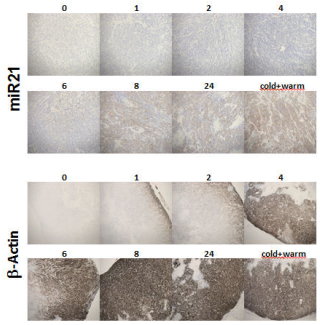

Figure 3: Fixation Time Course Dependence of miR21 and β-actin. MCF7 mouse xenografts tumors were fixed for 0-24 hours or with the cold+warm protocol as labeled. Tissues were probed with a probe to either miR21 (top pictures) or β-actin (bottom pictures) and developed with DAB (brown).

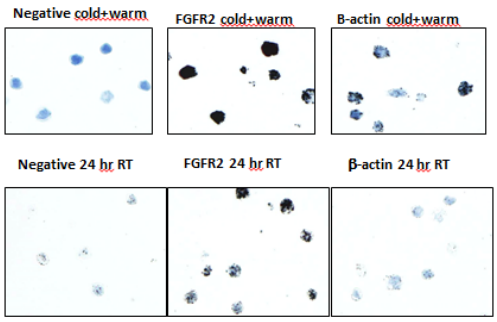

Figure 4: Staining of mRNA in Cell Culture Model. SNU-16 cells, which are highly amplified for FGFR2 expression, were fixed either with the cold+warm (top) or 24 hr (bottom) fixation methods. A multi-block containing both samples was made and stained with a scrambled negative control (left), FGFR2 probe sequence (center), or β-actin (right).

For experiments involving mouse xenografts (Figures 5): An MCF7 cell line (ATCC HTB-22) was obtained from The American Type Culture Collection (Manasas, Virginia) and xenograft tumors were provided by the Assay Development Support Service (ADSS) at Ventana Medical Systems, Inc. (Tucson, AZ). Whole MCF7 mouse xenograft tumors were immediately sectioned into 4mm thick samples, routine histologic size, and placed into 10% NBF at RT or 4°C. Samples were removed from the RT container at various times as indicated up to 24 hrs to make a time course of fixation.

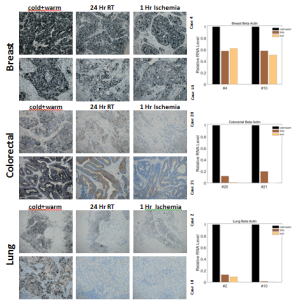

Figure 5: β-actin staining on diseased tissue samples. Representative samples from 10 breast carcinomas, 23 colorectal carcinomas and 10 lung carcinomas. Three samples from each patient case was fixed according to figure labels (cold+warm, 24 Hr RT or 1 Hr Ischemia +24 Hr RT). Amount of silver was assessed by digital means (see Methods) and expressed as relative RNA level (graphs). Black: cold +warm, Brown: 24 Hr RT Orange: (Isch) 1 Hr purposeful Ischemia with 24 Hr RT fixation.

Alternatively for cold+warm fixation, samples were soaked in 4°C NBF for 2 hours, followed by 2 hours in 45°C NBF for 2 hours (cold +warm), a procedure worked out in a previous publication that correlates tissue fixation with acceptable pathologic scoring (Figure 5) [35].

For experiments involving cell culture (Figure 6): Cells grown in culture were obtained from Ventana Medical Systems Inc. CDx Pharma Services group. Cells were trypsinized and pelleted at 250 xg for 5 minutes. Pelleted cells were fixed in two different ways. For standard 24 hour fixation cells were vortexed and incubated for 24 hours in formalin at 4°C. For cold+warm fixation cells were vortexed, incubated at 4°C in formalin for 2 hours, pelleted at 250 x g for 5 minutes, cold formalin was removed and replaced with warm (45°C) 10% neutral buffered formalin, vortexed, and incubated at 45°C for 2 hours.

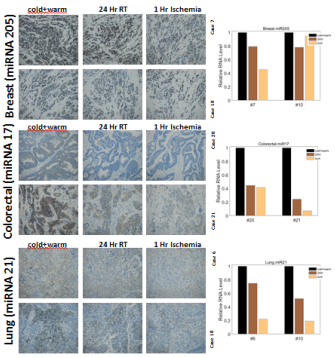

Figure 6: Fixation Dependence of miRNA in Diseased Tissue. Representative samples from 10 breast carcinomas, 23 colorectal carcinomas and 10 lung carcinomas. Three samples from each patient case was fixed according to figure labels (cold+warm, 24 Hr RT or 1 Hr Ischemia + 24 Hr RT). Amount of silver was assessed by digital means (see Methods) and expressed as relative RNA level (graphs). Black: cold+warm, Brown: 24 Hr RT Orange: (Isch) 1 Hr purposeful Ischemia with 24 Hr RT fixation.

Media was removed and replaced with cold (4°C) 10% neutral buffered formalin (SIGMA HT501128). After the formalin fixation was complete cells were pelleted at 250 xg for 5 minutes, formalin was removed, and cold (4°C) 70% ethanol was added. To embed the cells into a form capable of being sectioned, cells were centrifuged at 250 xg for 5 minutes. 70% ethanol was removed, and the cells transferred to 2ml microcentrifuge tubes (BIO PLAS, catalog #4204). Cells were centrifuged at 250 xg for 5 min and remaining ethanol removed.

Cells were mixed with hot (70°C) HistoGel (Thermo, Catalog # HG 4000-012) by aspiration, and incubated on ice for 10 minutes. Histogel embedded cells were then placed in a cassette in cold 70% ethanol and embedded in paraffin. cold+warm fixed cells and standard 24 hour fixed cells were embedded in the same paraffin block (Figure 6).

Tissue processing of all tissue samples: All samples after fixation were further processed on an automated Leica tissue processor with cycle times as follows: 70% ethanol for 2 hours, 95% ethanol for 3 hours, 100% ethanol for 3 hours, xylene for 3 hours, and paraffin for 3 hours. Ethanol solvent processor steps were kept at RT under ambient pressure, xylene steps were kept at 42◦C and the paraffin step was held at 62°C under vacuum (Figure 5).

Design and synthesis of probes: Probes for each mRNA target were selected from their respective reference sequence in GenBank. First, potential repetitive sequences were removed by conducting the repeatmasker; then overlapping 80 nucleotide long fragments were generated and screened by GC content filter (40-60%), devoid of continuous runs of nucleotides of 5 As, 4 Cs, 4 Gs, or 5 Ts for synthesis

accuracy; calculated melting temperature ranges from 70-90°C. 80-mers that pass these filters are subject to search against the human RNA library with BLASTN and results were inspected for specificity and accuracy.

Probe	Sequence
asACTB-1	MVVVVMCGGGAGCCACACGCAGCUCAMUUGUAGAAGGUGUGGUGCCAMGAUUUUCUCCAUGUCGUCCCMAGUUGGUGACGAUGCCGUGCMVVVVMT
asACTB-2	MVVVVMACGAUGCCAGUGGUACGGCCMAGAGGCGUACAGGGAUAGCAMCAGCCUGGAUAGCAACGUACMAUGGCUGGGGUGUUGAAGGUMVVVVMT
asACTB-3	MVVVVMGAAGCAGCCGUGGCCAUCUCMUUGCUCGAAGUCCAGGGCGAMCGUAGCACAGCUUCUCCUUAMAUGUCACGCACGAUUUCCCGMVVVVMT
sns18S	MVVVVMCUGAAACUUAAAGGAAUUGAMCGGAAGGGCACCACCAGGAGMUGGAGCCUGCGGCUUAAUUUMGACUCAACACGGGAAACCUCMVVVVMT
ExR22	MVVVVMGUGUAACACGUCUAUACGCCCAMVVVVMT
asmiR-21	MVVVVMUCAACAUCAGUCUGAUAAGCUAMVVVVMT
asmiR-21_TL	MVVVVMGUUGCCAUGAGAUUCAACAGUCMVVVVMT
asmiR-21_S	MVVVVMCAGCCAUGGAGAUGUCACGAUGMVVVVMT
asmiR-17	MVVVVMCUACCUGCACUGUAAGCACUUUGMVVVVMT
asmiR-205	MVVVVMCAGACUCCGGUGGAAUGAAGGAMVVVVMT
asmiR200c	MVVVVMUCCAUCAUUACCCGGCAGUAUUAMVVVVMT
asmiR-25	MVVVVMUCAGACCGAGACAAGUGCAAUGMVVVVMT
M	abasic amine
V	deoxynucleotide A

Table 1: Probe names and sequences.

Each 80-mer probe (Table 1) incorporates 7 abasic amines during the synthesis. Five amines were placed every 20 nucleotide on an 80-mer. To increase the amine load on the probes, two "tails" of four deoxynucleotide A were added to both 5' and 3' ends, and two additional amines were added at the end of "tails". MicroRNA probes, which are usually 20-24 nucleotide long, incorporate four amines, with two at the 5' and 3' ends and two additional by using two "tails" as in 80-mer probes. Labeling of amine probes with Digoxigenin NHS-ester (Sigma-Aldarich, St. Louis, MO) was performed according to the manufacturer's instructions, and labeled probes were purified with the Oligo Clean & Concentrator kit (Zymo Research, Irvine, CA).

Probes were synthesized on a MerMade 192 oligonucleotide synthesizer (BioAutomation, Irving, TX), 2'-o-methyl RNA phosphoramidites were purchased from Glen Research (Sterling, Virginia), Amine phosphoramidite was synthesized in house. All other reagents for synthesis were purchased from BioAutomation. Synthesis protocol was as recommended by the vendors.

RNA *in situ* hybridization and tyramide-chromogen detection

All FFPE tissue sections were mounted on positively charged glass microscope slides and stained using the Ventana Medical Systems, Inc. Discovery XT automated stainer. Slides were de-paraffinized and antigen retrieved using CC1 reagent and protease 3 (cat. 760-2020, Ventana Medical Systems Inc, Roche Group, Tucson, AZ). Following retrieval, the 2' O-methyl RNA oligomer probe(s) labeled with digoxigenin were diluted in DISCOVERY RiboHybe (cat. 760-104, Ventana Medical Systems Inc, Roche Group) and dispensed onto the slide, denatured at 80°C for 8 min, and hybridized at 75°C for 2 hrs. After hybridization, slides were rinsed with reaction buffer (cat. 950-300, Ventana Medical Systems Inc, Roche Group) three times at 65°C to prevent non-specific hybridized probe binding followed by inactivation of endogenous tissue peroxidase activity using PO inhibitor (cat. 760-4143, Ventana Medical Systems Inc, Roche Group). Silver chromogen detection of each bound probe was accomplished in a two tier amplification method. The first tier applies an anti-digoxigenin antibody (cat.11333062910, Roche) conjugated to horseradish peroxidase (HRP) and incubated for 32 minutes at 37°C, followed by a HRP catalyzed hapten amplification using a tyramide-DIG conjugate and TSA-H2O2 reagent (cat. 760-4141, Ventana Medical Systems Inc, Roche Group). The second tier applies another application of the anti-digoxigenin HRP antibody for 32 minutes at 37°C which catalyzes the deposition of an insoluble silver chromogen (ultraView SISH Detection Kit cat. 780-001 Ventana Medical Systems Inc, Roche Group). The slides were counterstained with hematoxylin (cat. 790-2208 Ventana Medical Systems Inc, Roche Group) for 4 minutes at 37°C and then counterstained with bluing reagent (cat. 760-2037 Ventana Medical Systems Inc, Roche Group) for 4 minutes to visualize tissue morphology. Slides were then dehydrated using gradient alcohols, xylene and cover slipped. Sense strand probes were used as negative controls to determine background resulting from any non-specific probe interactions.

Staining and quantitation of silver signals

Each slide was imaged on a microscope (Nikon Eclipse 80i) with a 20X objective (Nikon Plan Apo) and images were collected at a field of view that was representative of the specimen. Collected images were dynamically compensated for the illumination pattern of the microscope using a top hat algorithm to produce an optically flat image. RGB images were then converted to intensity images (i.e., grayscale) using the total luminosity which calculates grayscale values based on a weighted sum of the red, green, and blue channels (For example see Figure 7).

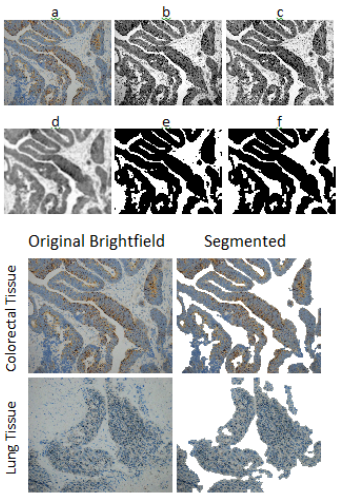

Figure 7: Description of image segmentation algorithm. a) Original brightfield image. b) Luminance of original displayed in greyscale. c) Luminance image after illumination compensation. d) Low-pass filtered image. e) Binomial thresholded image. f) Final segmentation after removing small disjoint objects and filling small holes in contiguous tissue sections. Lower Panels indicate two different tissue samples before (bottom left) and after (bottom right) segmentation.

The greyscale image was smoothed with a low-pass filter. Next, regions of tissue where identified by using Otsu's binomial separation method to calculate an optimal threshold to distinguish background from tissue. Small isolated sections of tissue were removed and small holes within sections of the tissue were filled in to produce a contiguous mask representing adjoining sections of the tissue. Large regions of the images that were negative for tissue of interest (e.g. blank regions, stroma) were excluded from the image analysis so that the size of the tissue did not bias the results. Finally, black pixels positive for silver stain were identified against the lighter background using a linear binary threshold on the intensity image. The total number of positive pixels and the total number of pixels contained within the tissue were used to calculate the percentage of the tissue that expressed silver stain. In Figures 1 and 2 the percentage of tissue staining was normalized to the value for the cold+warm fixation to account for the large absolute differences in the amount of staining between the different types of tissue.

Results

To show the sensitivity of RNA ISH assays to fixation time, we compared a partially fixed whole tonsil (2 hours RT formalin) to one that was well fixed (2 hr cold + 2 hr warm fixation) (Figure 1). Sections from these whole tonsils were probed with either a sequence to the β-actin mRNA or with a sequence to a shorter RNA, miR21 (See Methods). Tissue samples were subjected to a silver stain procedure on a Ventana Medical Systems Inc. Discovery XT automated slide stainer to reveal the location of probe binding. Poorly fixed tonsil exhibited extreme edge staining forming a dark silver ring structure. Staining quickly disappeared towards the center of these poorly fixed tissues indicating that robust probe binding only occurs in areas of sufficient fixative penetration and crosslinking. This result is consistent with ring-like darker hematoxylin staining on negative control (sense probes) (picture insets and Figure 2). In contrast, the well-fixed tonsil had uniform and strong silver signals throughout the entire tissue sample, as well as consistent hematoxylin staining across the negative control (sense probes). Both probe types (mRNA or miRNA) showed the same pattern indicating that RNA ISH assays are in general sensitive to the amount of crosslinking during fixation.

Since there is not yet an accepted method, qualitative or quantitative, of scoring silver signals, we developed a segmentation-based algorithm to score samples in this study. This was tested on the tissue samples in Figure 1 and confirms the large gradient between the edge and middle of poorly fixed tissues (Figure 1 graph).

To determine if there are benefits of using the rapid two-temperature fixation protocol (cold+warm), we studied in house model systems where the time of fixation and/or cold ischemia (time to fixation) conditions could be carefully manipulated. A mouse xenograft (MCF7) tumor model and human tonsil samples were fixed for increasing times (0-24 hours) or subjected to the cold+warm method (Figure 3). Samples were incubated with probes to the β-actin mRNA sequence (tonsil) or miR21 (MCF7) as described and detected as in Figure 1. At short (incomplete) fixation times, a clear gradient of staining was observed from the periphery to center of the samples (Figure 3, miR21 and β-actin 1-6 hours). The gradient was not as evident on samples that were fixed for longer than 6hours(Figure 3 miR21 and β-actin 8-24 hours). As expected, the number of positively staining cells and the signal strength greatly increased as the length of fixation time increased. More importantly, samples fixed with the cold +warm protocol (4 hours total fixation) had similar levels of preservation as 24 hour RT samples (compare cold+warm with 24 hours).

We also wanted to determine if placing cells directly from culture into 4°C 10% NBF would show the same results as the tissue models as a way to suggest a mechanism for this increased preservation. One possible mechanism is the rapid decrease in temperature limits the activity of RNAses in the cell that might degrade the molecules during RT fixation methods. Another consideration is the time taken for the formalin to penetrate the tissue; this delay is eliminated in the cell culture model as these are single cells. We fixed SNU-16 cells with both the cold+warm and standard 24 hour RT protocols (see materials and methods) and compared the level of FGFR2 messages (Figure 4). The level of background was significantly reduced with the cold+warm protocol when a non-homologous sequence was used (compare left panels, negative mRNA ISH). More importantly, the level of two specific mRNA messages, FGFR2 (middle) and β-actin (right), was significantly higher in the cold+warm compared to the 24 HR RT protocol. This suggests that dropping the temperature in the initial collection is a key component for biomolecule preservation.

We next wanted to determine if the cold+warm fixation protocol preserves more RNA in clinical samples that could be used for routine histological assays. We probed a set of breast, lung and colorectal carcinoma patient samples collected with cold ischemia times under 15 minutes but with different fixation protocols (see Methods). Each patient sample was split into three pieces and fixed in a conventional manner (24 hr of RT 10% NBF) or fixed after a purposeful 1 hr of additional cold ischemia (1 hr ischemia) or fixed with the cold+warm protocol. The amount of β-actin mRNA and various miRNAs that were subsequently detected were greatly dependent on the fixation protocol in these clinical samples (Figures 5 and 6). In all patient samples tested the cold+warm fixation protocol resulted in greater RNA signals than

conventional fixation and usually much greater than when additional cold ischemia was present. In breast samples cold+warm fixation resulted in nearly twice as much β-actin expression compared to both 24 hr and 1 hour ischemic fixation. Additionally, colorectal and lung tissue samples fixed with cold+warm fixation exhibited a 5 to 10 fold increase in β-actin staining over the other two fixation protocols. For miRNAs, cold+warm fixation samples repeatedly expressed 20-100% more than 24 hr RT samples and 1 hour cold ischemia samples typically had suppressed signal levels even compared to standard 24 hr RT fixation.

Discussion and Conclusion

We have previously developed novel fixation protocols to address standardization of tissue collection and increased preservation of biomolecules [35]. In the previous work, we were able to show greater efficacy in preservation of highly labile protein phosphorylation events. In this study, we have extended the results for RNA species and shown a greater ability to detect both long and short RNAs using the cold +warm protocol vs standard fixation techniques.

It has been long known that mRNAs are unstable and degrade rapidly during the course of cellular metabolism, making them ideal as regulatory molecules. As such, RNAs have also become an important predictor of levels of expression of key proteins and are the target of a growing list of diagnostic molecular assays. Since RNAs are unstable, it is necessary to develop preservation techniques that are rapid and standardized to ensure these assays are measuring biologically relevant levels. This is especially true for RNA ISH techniques, using FFPE tissue that was generated for pathology, where tissues are often subjected to a variety of pre-analytical conditions that could affect the amount of signal observed. We and other researchers have found the amount of cold ischemia and the specific fixation protocol to be the biggest factors in preserving biological molecules. Again, these two factors seem to be the key in preserving both long and short RNA species.

miRNAs, especially low expressing ones, have been difficult to detect in FFPE. At least one report suggests these short RNAs are lost during target retrieval and staining [36]. Increased signals have been demonstrated when a bi-functional crosslinking agent is used to trap miRNA during fixation [36]. The use of a bi-functional linker is problematic on clinical tissues since these molecules tend to be of much higher molecular weight than formaldehyde and therefore have slower diffusion rates. Also, bi-functional linkers tend to be extremely active, making it hard to control their reactivity. Here we have shown much higher levels of miRNA preservation by using the cold+warm fixation protocol with standard off-the-shelf formalin. In theory, RNases are much less active during the cold diffusion step, thus protecting biomolecules from changes due to metabolic enzymes. When the temperature is abruptly raised, the already diffused formalin is able to act rapidly to crosslink, prevent these metabolic activities and preserve the tissue in a state much closer to the *in vivo* status.

The medical relevance of both long and short RNA species is gaining traction for precision medicine. Many clinical studies have measured RNA expression as a correlation of disease state [1,3,6,11,37-43]. While many long and short RNAs have been identified and correlated to various types of cancers, the medical value of measuring the expression of these molecules is a bit unclear. Hampering the effort is an inability to reliably detect these short RNAs due to degradation or loss. Even with the careful collection effort for this study, it is evident that samples will have an inherent variability in expression of key biomarkers. Making further meaningful progress will require more efficient and standardized collection efforts.

References

1. Benjamin H, Lebanony D, Rosenwald S, Cohen L, Gibori H, et al. (2010) A diagnostic assay based on microRNA expression accurately identifies malignant pleural mesothelioma. J Mol Diagn 12: 771-779.
2. Hall JS, Taylor J, Valentine HR, Irlam JJ, Eustace A, et al. (2012) Enhanced stability of microRNA expression facilitates classification of FFPE tumour samples exhibiting near total mRNA degradation. Br J Cancer 107: 684-694.
3. Kalmar A, Wichmann B, Galamb O, Spisak S, Toth K, et al. (2013) Gene expression analysis of normal and colorectal cancer tissue samples from fresh frozen and matched formalin-fixed, paraffin-embedded (FFPE) specimens after manual and automated RNA isolation. Methods 59: S16-19.
4. Kebebew E, Peng M, Reiff E, McMillan A (2006) Diagnostic and extent of disease multigene assay for malignant thyroid neoplasms. Cancer 106: 2592-2597.
5. Kim S, Lee H, Kim H, Kim Y, Cho JE, et al. (2015) Diagnostic performance of a cytokine and IFN-gamma-induced chemokine mRNA assay after Mycobacterium tuberculosis-specific antigen stimulation in whole blood from infected individuals. J Mol Diagn 17: 90-99.
6. Lebanony, D, Benjamin H, Gilad S, Ezagouri, M, Dov A, et al. (2009) Diagnostic assay based on hsa-miR-205 expression distinguishes squamous from nonsquamous non-small-cell lung carcinoma. J Clin Oncol 27: 2030-2037.
7. McCoy MH, Post K, Sen JD, Chang HY, Zhao Z, et al. (2014) qPCR is a sensitive and rapid method for detection of cytomegaloviral DNA in formalin-fixed, paraffin-embedded biopsy tissue. J Vis Exp.
8. Parsons SD, Menezes AM, Cooper D, Walzl G, Warren RM, et al. (2012) Development of a diagnostic gene expression assay for tuberculosis and its use under field conditions in African buffaloes (Syncerus caffer). Vet Immunol Immunopathol 148: 337-342.
9. Schiavon BN, Jasani B, de Brot L, Vassallo J, Damascena A, et al. (2012) Evaluation of reliability of FISH versus brightfield dual-probe in situ hybridization (BDISH) for frontline assessment of HER2 status in breast cancer samples in a community setting: influence of poor tissue preservation. Am J Surg Pathol 36: 1489-1496.
10. Scolnick JA, Dimon M, Wang IC, Huelga SC, Amorese DA (2015) An efficient method for identifying gene fusions by targeted RNA sequencing from fresh frozen and FFPE samples. PLoS One 10: e0128916.
11. Spector Y, Fridman E, Rosenwald S, Zilber S, Huang Y, et al. (2013) Development and validation of a microRNA-based diagnostic assay for classification of renal cell carcinomas. Mol Oncol 7: 732-738.
12. Tafe LJ, Schwab MC, Lefferts JA, Wells WA, Tsongalis GJ (2010) A validation study of a new molecular diagnostic assay: The Dartmouth-Hitchcock Medical Center experience with the GeneSearch BLN assay in breast sentinel lymph nodes. Exp Mol Pathol 88: 1-6.
13. Zheng Z, Andersson AF, Ye W, Nyren O, Normark S, Engstrand L (2011) A method for metagenomics of Helicobacter pylori from archived formalin-fixed gastric biopsies permitting longitudinal studies of carcinogenic risk. PLoS One 6: e26442.
14. Duchaine TF, Slac FJ (2009) RNA interference and micro RNA-oriented therapy in cancer: rationales, promises, and challenges. Curr Oncol 16: 61-66.
15. Grosshans H, Slack FJ (2002) Micro-RNAs: Small is plentiful. J Cell Biol 156: 17-21.
16. Botling J, Micke P (2011) Fresh frozen tissue: RNA extraction and quality control. Methods Mol Biol 675: 405-413.
17. Culpin RE, Sieniawski M, Proctor SJ, Menon, G, Tryfonia Mainou F (2013) MicroRNAs are suitable for assessment as biomarkers from formalin-fixed paraffin-embedded tissue, and miR-24 represents an

appropriate reference microRNA for diffuse large B-cell lymphoma studies. J Clin Pathol 66: 249-252.

18. Frankel A (2012) Formalin fixation in the '-omics' era: a primer for the surgeon-scientist. ANZ J Surg 82: 395-402.
19. Hedegaard J, Thorsen K, Lund MK, Hein AM, Hamilton-Dutoit SJ, et al. (2014) Next-generation sequencing of RNA and DNA isolated from paired fresh-frozen and formalin-fixed paraffin-embedded samples of human cancer and normal tissue. PLoS One 9: e98187.
20. Liu X, Harada S (2013) RNA isolation from mammalian samples. Curr Protoc Mol Biol 4: 16.
21. Perez-Portela R, Riesgo A (2013) Optimizing preservation protocols to extract high-quality RNA from different tissues of echinoderms for next-generation sequencing. Mol Ecol Resour 13: 884-889.
22. Samadani AA, Nikbakhsh N, Fattahi S, Pourbagher R, Aghajanpour Mir SM, et al. (2015) RNA extraction from animal and human's cancerous tissues: Does tissue matter? Int J Mol Cell Med 4: 54-59.
23. Rossi ED, Schmitt F (2013) Pre-analytic steps for molecular testing on thyroid fine-needle aspirations: The goal of good results. Cytojournal 10: 24.
24. Milcheva R, Janega P, Celec P, Russev, R, Babal P (2013) Alcohol based fixatives provide excellent tissue morphology, protein immunoreactivity and RNA integrity in paraffin embedded tissue specimens. Acta Histochem 115: 279-289.
25. Hostein I, Stock N, Soubeyran I, Marty M, De Mascarel I, et al. (2011) Nucleic acid quality preservation by an alcohol-based fixative: comparison with frozen tumors in a routine pathology setting. Diagn Mol Pathol 20: 52-62.
26. Dyhdalo KS, Fitzgibbons PL, Goldsmith JD, Souers RJ, Nakhleh RE (2014) Laboratory compliance with the American Society of Clinical Oncology/College of American Pathologists human epidermal growth factor receptor 2 testing guidelines: A 3-year comparison of validation procedures. Arch Pathol Lab Med 138: 876-884.
27. Dyhdalo KS, Howanitz PJ, Wilkinson DS, Souers RJ, Jones BA (2014) Documentation of quality control and operator training at point-of-care testing: a College of American Pathologists Q-Probes study of 106 institutions. Arch Pathol Lab Med 138: 1444-1448.
28. Goldstein NS, Ferkowicz M, Odish E, Mani A, Hastah F (2003) Minimum formalin fixation time for consistent estrogen receptor immunohistochemical staining of invasive breast carcinoma. Am J Clin Pathol 120: 86-92.
29. Kalkman S, Barentsz MW, van Diest PJ (2014) The effects of under 6 hours of formalin fixation on hormone receptor and HER2 expression in invasive breast cancer: a systematic review. Am J Clin Pathol 142: 16-22.
30. Middleton LP, Price KM, Puig P, Heydon LJ, Tarco E, et al. (2009) Implementation of American Society of Clinical Oncology/College of American Pathologists HER2 Guideline Recommendations in a tertiary care facility increases HER2 immunohistochemistry and fluorescence in situ hybridization concordance and decreases the number of inconclusive cases. Arch Pathol Lab Med 133: 775-780.
31. Terrenato I, Pennacchia I, Buglioni S, Mottolese M, Arena V (2015) HER2 status determination: Analyzing the problems to find the solutions. Medicine (Baltimore) 94: e645.
32. Yamashita-Kashima Y, Shu S, Yorozu K, Hashizume K, Moriya Y, et al. (2014) Importance of formalin fixing conditions for HER2 testing in gastric cancer: immunohistochemical staining and fluorescence in situ hybridization. Gastric Cancer 17: 638-647.
33. Theiss AP, Chafin D, Bauer DR, Grogan TM, Baird GS (2014) Immunohistochemistry of colorectal cancer biomarker phosphorylation requires controlled tissue fixation. PLoS One 9: e113608.
34. Bauer DR, Stevens B, Chafin D, Theiss AP, Otter M (2016) Active monitoring of formaldehyde diffusion into histological tissues with digital acoustic interferometry. J Med Imaging (Bellingham) 3: 017002.
35. Chafin D, Theiss A, Roberts E, Borlee G, Otter M, et al. (2013) Rapid two-temperature formalin fixation. PLoS One 8: e54138.
36. Pena JT, SohnLee C, Rouhanifard SH, Ludwig J, Hafner M, et al. (2009) miRNA in situ hybridization in formaldehyde and EDC-fixed tissues. Nat Methods 6: 139-141.
37. Gilad S, LithwickYanai G, Barshack I, Benjamin S, Krivitsky I, et al. (2012) Classification of the four main types of lung cancer using a microRNA-based diagnostic assay. J Mol Diagn 14: 510-517.
38. Morton ML, Bai X, Merry CR, Linden PA, Khalil AM, et al. (2014) Identification of mRNAs and lincRNAs associated with lung cancer progression using next-generation RNA sequencing from laser micro-dissected archival FFPE tissue specimens. Lung Cancer 85: 31-39.
39. Nelson PT, Wang WX, Rajeev BW (2008) MicroRNAs (miRNAs) in neurodegenerative diseases. Brain Pathol 18: 130-138.
40. Sempere LF, Christensen M, Silahtaroglu A, Bak M, Heath CV, et al. (2007) Altered microRNA expression confined to specific epithelial cell subpopulations in breast cancer. Cancer Res 67: 11612-11620.
41. Sempere LF, Korc M (2013) A method for conducting highly sensitive microRNA in situ hybridization and immunohistochemical analysis in pancreatic cancer. Methods Mol Biol 980: 43-59.
42. Slack FJ, Weidhaas JB (2006) MicroRNAs as a potential magic bullet in cancer. Future Oncol 2: 73-82.
43. Wang WX, Rajeev BW, Stromberg AJ, Ren N, Tang G, et al. (2008) The expression of microRNA miR-107 decreases early in Alzheimer's disease and may accelerate disease progression through regulation of beta-site amyloid precursor protein-cleaving enzyme 1. J Neurosci 28: 1213-1223.