Author's response to reviews

Title: Preservation of Biomolecules in Breast Cancer Tissue by a Formalin-Free Histology System

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Author's response to reviews:

Dr. Deborah Saltman,
Editorial Director
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RE: MS#1945273621135923

Dear Dr Saltman,

We truly appreciated the guidance the reviewers provided in their thoughtful comments. We are submitting the revised manuscript and in the following we have addressed all of the reviewers concerns.

Reviewer #1 Dr O'Leary

1- "In the introduction the authors imply that..."

By "safe" we meant biologically safe as our method is less hazardous compared to conventional method using formalin and xylene. We have previously published our result on anti-microbial activity of our molecular fixative as detailed in reference 4. To address the concern of Dr O'Leary we have deleted the word "safe".

2- "The authors found that they were only able to amplify...."

We clarified successful PCR as: "Only copy numbers above "1" were considered reliable values". (p5 Ln.17)
There are many methods used in extraction nucleic acids from FFPE samples. So far, none has been able to provide intact high molecular weight DNA or RNA. Although FFPE tissue is being used in clinical diagnosis, it is mostly for DNA-based and qualitative studies. RNA-based quantitative test are less frequently performed clinically on FFPE samples.

3- "Also, while the approach that the authors use...."

Other investigators have shown that by amplifying short segments of RNA, similar result can be obtained from FFPE using normalization to a control gene. This approach is often based on normalization to one or more house keeping genes for sample quality or cellularity. Recent data shows that housekeeping genes are not only tissue specific but also show variability based on the method of tissue preservation (Chen 2007 ref. 9). Also, as our reviewer mentions, there is little known about low copy number genes. Therefore, instead of normalization to a housekeeping gene we chose to show the magnitude of difference in copy numbers between different methods.

We have added figure 4, which shows comparison of two methods in all samples for ER, HER2, PPIA and RN7SL1 transcript copy number. It shows that there is at least a log range difference in transcript copy number between UFPE and FFPE samples. We omitted the comparison of PCR and IHC data.

4- "More importantly, is there anything that makes the results with...."

Our aim was to validate our methodology in clinical settings. Most other non-cross linking fixatives -- beside lack of practical application -- do not show a detailed clinical validation study. Furthermore they require manual processing and embedding. Our approach, on the other hand, is based on using validated reagents, automation and standard methods to overcome the pre-analytical variability before using tissue samples for down-stream molecular studies.

We have been able to amplify RNA and DNA fragments up to 1400 bp successfully. This is not a surprising finding since it is possible to extract high molecular weight nucleic acids from these samples. We have previously shown preservation of high molecular weight RNA suitable for transcription profiling by microarray (Vincek et al 2003 ref.8) and HMW DNA for oligo-array CGH assay, with similar result to fresh tissue (Nassiri et al, manuscript submitted, USCAP 2007 abstract # 1606).

We have modified the sentence: "GAPDH could be easily detected in all UMFIX samples whereas it was rarely amplified from formalin-fixed tissue." We modified the figure 5 to represent more samples.

We have followed the advice of our reviewer and performed PCR for beta actin for fragments of 131 bp to 705 bp in 20 paired samples. Figure 6 shows that relatively large RNA segments can be amplified from UMFIX preserved samples, consistently.
We tried antigen retrieval to see if any enhancement can be achieved. We have found previously (Nadji et al 2005 ref. 7) that antigen retrieval helps in case of most nuclear and some but not all cytoplasmic proteins in UFPE samples. No one to our knowledge has studied effect of antigen retrieval on alcohol-fixed tissue before.

Reviewer #2 Dr Liotta

1- We have followed the reviewer advice in providing the scores of three pathologists who were blinded to the fixation and processing, and added in the manuscript as:

To evaluate the IHC and CISH staining, random selections of slides were evaluated simultaneously on a multi-headed microscope by three pathologists and a consensus score was reported for each slide. FISH slides were scored by one pathologist. All slides were stripped of any identifier referring to fixation or processing method (p4. Ln18).

Samples were stripped of any identifier referring to fixation or processing method during all experimental steps (p.6. Ln 1).

2- We have added Figure 2, heatmap chart for ER and HER2 IHC scores as Dr Liotta had suggested. We only stated that RNA yield was higher in UMFIX, not cDNA or DNA yield. We have modified Figure 3 to show scatter plot of all samples for RNA and DNA yield as suggested. We have added figure 4 that shows realtime PCR result of all samples for ER, HER2 and PPIA transcript copy number for both method studied.

3- We described the composition of fixative as follows: Molecular fixative is composed of methanol and polyethylene glycol at 90 to 10 percent ratios (p. 3 Ln 21).

We have added following sentence to clarify time of fixation: All samples were immersed in fixative within 30 minute of surgery. Immersion time was similar for both fixatives for each specimen (less than 24 hours for 32 cases, 24-48 hours for 18 cases, 48-72 hours for 12 cases) (p.3 Ln 22).

Size of samples in explained as: Paired tissue section (similar dimensions, 1.5x1.5 x0.2 cm) (p.3 Ln.18).

Processing is described as: Formalin-fixed samples were then processed using a conventional method (VIP, Sakura Finetek, Torrance, CA). Samples fixed in the molecular fixative were processed by a recently described automated microwave-based rapid tissue processing instrument (Tissue-Tek(R) XpressTM, Sakura Finetek, Torrance, CA) (p.3 Ln. 24).

I hope that our explanations are satisfactory.
I am looking forward to hearing from you.

Sincerely yours

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