Author's response to reviews

Title: An exfoliation and enrichment strategy results in improved transcriptional profiles when compared to matched formalin fixed samples.

Authors:

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Author's response to reviews: see over
Dear Ms. Makri,

The reviewer’s comments were examined and the manuscript changed accordingly. The manuscript was also formatted to be in agreement with the journal’s checklist. In addition, two “native English speaking colleagues” have reviewed and proofread the manuscript. The reviewer’s points are addressed below:

Reviewer 1:
Point 1. Additional sentences were incorporated on page 7 describing the “typical” automated processing performed in this hospital institution. The sentences were: 

**The tissue section was allowed to fix for 8 more hours to simulate typical fixation conditions for tissue anatomical surgical pathology specimens. Following fixation, the tissue was processed on a Miles Scientific Tissue Tek VIP automated processor.** The program entailed an additional 2.5 hours in 10% NBF, followed by dehydration and then three separate cycles of infiltration by paraffin wax lasting 30, 60 and 60 minutes. The type of fixative (10% Neutral Buffered Formalin) was annotated 3 sentences prior to these changes.

Point 2. The typographical error on page 8 was changed from “hemocytometer change” to “**hemocytometer chamber**”.

Point 3. As per the suggestion of reviewer 1, conditions on which the cap were maintained after laser capture microdissection were corrected. The word frozen was deleted from page 8.

Point 4. A statement was included in the “RESULTS - RNA recovery and Degradometer results” section addressing the absence of 18S and 28S peaks in Group 3. The following sentence was incorporated into page 13: **The absence of 18S and 28S peaks in the latter two groups may be reflective of the enrichment process, as others have reported encountering absent ribosomal peaks but retention of intact mRNA levels for specific genes (16).** Additionally, a reference was provided. The authors of this reference found that 21% of their samples exhibited degraded rRNA electropherograms profiles (as is the case in this manuscript - Fig 5B and C) but retained mRNA levels of specific genes similar to samples that were considered by capillary electrophoresis as high quality. These changes are on page 13.

Point 5. A sentence was added in the discussion clarifying the quality of frozen tissue: **Group 3 represents the current gold standard for expression analysis because RNA molecules are suspended by freezing for subsequent examination.** These changes are located on page 17.
Point 6. Normal colonic tissue was specifically chosen for this experiment because the enrichment techniques works with these cells. The technique works equally well in procuring cells from adenocarcinomas of the colon. However, we did not want to get any variability in gene expression that may be the result of regional location in the tumor. That is why we chose normal colon tissue to profile by these 3 techniques. The exfoliation technique works well with most tissue types except bone and fat. There has been no problem in getting cells from tumors either. The limitations of this technique lie in the enrichment step, and in the identification of which antibodies to conjugate to the magnetic beads. These statements have been incorporated as the last sentences in the Discussion on page 18 as: **In our practice, exfoliation of cells works well for the recovery of intact cells from all normal and tumor tissue types excepting bone and fat. The current limiting factor in the technique lays in the enrichment approach, specifically, the identification of antibodies specific to preferentially expressed plasma membrane proteins on different cell types. We envision that in the future, when the plasma membrane proteome becomes published, this technique can be expanded to include other tumor types and experiments designed to collect only certain cell types.**

Point 7. The title was changed by deleting the word “clinical”. The new title is: **An exfoliation and enrichment strategy results in improved transcriptional profiles when compared to matched formalin fixed samples.**

Reviewer 2:

We acknowledge that the use of magnetic beads may slightly alter the transcriptome in the exfoliated/enrichment approach relative to the frozen specimen. A number of alterations are accordingly made. Since there were no specific points to address, the changes relevant to the magnetic beads are addressed in the following sections:

Abstract (Background):

The abstract is changed to reflect the role of the exfoliation/enrichment technique relative to FFPE and frozen specimens: Identifying the influence formalin fixation has on RNA integrity and recovery from clinical tissue specimens is **integral to determining the utility of using archival tissue blocks in future molecular studies.** For clinical material, the current gold standard is unfixed tissue that has been snap frozen. Fixed and frozen tissue however, both require laser capture microdissection to select for a specific cell population to study. The recent development of a sampling method capable of obtaining a viable, enriched cell population represents an alternative option in procuring cells from clinical material for molecular research purposes. **The expression profiles of cells obtained by using this procurement approach, in conjunction with the profiles from cells laser capture microdissected from frozen tissue sections, were compared to the expression profiles from formalin fixed cells to determine the influence fixation has on expression profiles in clinical material.**

Abstract (Conclusions):
“to traditional methods” is deleted and replaced by “relative to formalin fixed tissue”. The last sentence is deleted and replaced by “The exfoliation/enrichment technique also represents an economical alternative that will yield comparable results to cells enriched by laser capture microdissection from frozen tissue sections.”

Page 4:
As per the reviewer’s suggestion, bottom line changed to read “there has been no other procurement method for comparison”

Page 5:
“closest” is deleted and replaced by “a close”
“serve” is deleted and replaced by “theoretically”
“help” is incorporated before the word “define” in the same sentence
“Only after using this procurement technique” is deleted and replaced by “Using this procurement technique in conjunction with analysis of frozen LCM cells”

Page 11:
“of either an alteration in transcript levels or” was incorporated into the sentence starting with “For the remainder of genes in the transcriptome…..” (second to last sentence in second paragraph).

Page 13:
Under the section headed as “Microarray results”, two sentences were added after the sentence “This represent 1.9% of the gene probe sets in the chip.” The added sentences were “In order to gain further insight into these changes, we examined the Gene Ontology Biologic Process ascribed to these altered gene transcripts. The largest contingents belonged to genes involved in transcription, signal transduction, protein metabolism, cell adhesion and cytoskeletal organization”

Page 15-16:
“This exfoliation and enrichment approach represent the closest approximation to control cells currently possible from heterogeneous clinical material” sentence was changed to “This exfoliation and enrichment approach represents a superior alternative in the procurement of specific cells relative to FFPE clinical material”

Page 16:
Last sentence of first (continuing) paragraph was deleted and replaced with “Additionally, based on the amounts of RNA recovered, this procurement technique represents a more economical and expeditious method for obtaining specific cell populations from clinical material for molecular studies. A 5 ml vial of antibody bound magnetic beads costs less than $1,000. We utilized only 40µL for each sample in Group 1. Thus, the potential to acquire 125 samples for under $1,000 exists.”

Page 16-17:
Second, third and fourth sentences of last paragraph on page 16 (and continuing to page 17) are deleted and changed with: “Group 3 represents the current gold standard for
expression analysis because RNA molecules are suspended by freezing for subsequent examination. Since Groups 1 and 3 were not exposed to fixative and demonstrated highly reproducible replicates, they served as a baseline from which to compare expression profiles. The expression profiles between Groups 1 and 3 demonstrated a 2 fold log 2 difference in only 1.5% of their transcripts. The differences between Groups 1 and 3 although small, may be attributable to some degree in the procurement approach of the cells in Group 1. The manipulation of these cells, exposure to buffer and binding of these cells to antibodies conjugated to magnetic beads can lead to changes in the transcript levels for certain genes. Examination of the Gene Ontology Biological Process for these gene probe sets confirmed that the majority of these changes could be attributable and the result of the manipulation the cells in Group 1 experienced.”

Conclusions
The last statement in the last paragraph “and to a lesser degree to similar cells that have been frozen and laser capture microdissected” was deleted and replaced with the sentence: “Taking into consideration the small number of genes that have been noted to have an altered gene expression level, this technique can also be utilized as an alternative to frozen tissue.”

A number of small grammatical corrections were performed. Thank you for your time and consideration.

Sincerely,

Wilfrido D. Mojica, M.D.