 doi:10.20944/preprints201806.0096.v1

Article

Z Probe, an Efficient Tool for Characterizing Long Non-Coding RNA in FFPE Tissues

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Abstract: Formalin-Fixed Paraffin Embedded (FFPE) tissues are a valuable resource in studying different markers and mechanistic molecules (protein, DNA and RNA) in order to understand the etiology of different cancers as well as many other diseases. Degradation and modification of RNA is the major challenge in utilizing FFPE tissue samples in medical research. Recently, non-protein coding transcripts long non-coding RNAs (lncRNAs), have gained significant attention due to their important biological actions and potential involvement in cancer. There is no validated method except qRTPCR or RNAseq to evaluate and study lncRNA expression. We have standardized and are reporting a sensitive Z probe based *in situ* hybridization method to identify, localize and quantitate lncRNA in FFPE tissues. This assay is sensitive to single transcript and localizes lncRNA in individual cells within tumor. We have characterized a tumor suppressor lncRNA-NRON (non coding repressor of NFAT), which is scarcely expressed, a moderately expressed oncogeneic lncRNA UCA1 (urothelial cancer associated 1), and a highly studied and expressed lncRNA MALAT1 (metastasis associated lung adenocarcinoma transcript1) in different cancers. High MALAT1 staining was found in colorectal, breast and pancreatic cancer. MALAT1 expression increased with the progression of the stage in colorectal cancer and invasiveness in breast cancer.

Keywords: long noncoding RNA; MALAT1; UCA1; NRON; Z probe; colorectal cancer; pancreatic cancer; breast cancer

1. Introduction

LncRNA a class of noncoding RNA has gained significant traction with exponential publications and interests from the scientific community. The role of miRNA in regulation of gene expression has been extensively studied in the last decade since its discovery. LncRNA provides a novel way of regulating gene expression and function at all levels of DNA, RNA or proteins. In 2005 when 512 known lncRNA were systematically studied [1], It was not anticipated that the number will grow exponentially to 19,175 potentially functional lncRNAs in human genome. Many of these potential lncRNA identified by FANTOM5 analysis of CAGE (cap analysis of gene expression) data and overlapping expression qualitative trait loci (eQTL), have not been functionally described, it is anticipated that this number of lncRNA will increase significantly [2]. Many of the lncRNA are been characterized by revisiting the array datasets on publicly available resources [3-7]. LncRNA, which

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are now at the center of various physiological and pathological processes have been linked to various cellular pathways including progression of different cancers and various diseases. It is imperative to invest efforts in understanding the roles of these new class of regulators and further elucidate the mechanism.

Immunohistochemistry(IHC) for decades has been the primary diagnostic method of choice for identifying important biomarkers at the protein level in cancer and other diseases. The ability of IHC to detect important protein receptors and membrane bound proteins makes it an attractive diagnostic method. The information gained by IHC helps in the designing of a better therapeutic regimen for the treatment of cancer and other diseases [8]. RNA *in situ* hybridization has been used widely to analyze mRNA in different studies [9-11]. Quantitative RT-PCR, RNA sequencing and microarrays are the current gold standard for detecting lncRNA in cells and tissues. The problem with this method is its inability to differentiate different cell populations within the tissue and the compartmental location of biomarker, which are important information for better targeted therapy [12]. The limitation also lies in low copy numbers and lack of localization information while using these techniques. Most lncRNA studies are based on bioinformatics analysis and correlation studies from the data available from public resources [2,6,7]. A major problem in understanding lncRNA's role in cancer progression and other diseases is a lack of an efficient tool to characterize them. A new Z probe based technology was reported first time to investigate into the levels of program death ligand-1 and its receptor PD1 mRNA expression in tissues cohorts of non-small cell lung cancer. [13]. If used as a corroborative diagnostic tool, this method may provide more confidence as the validation step to identify the same cancer biomarkers observed with IHC. In the interest of having a specific, sensitive and reproducible lncRNA assay, we analyzed and standardized Z probe based chromogenic method for further diagnostic detection of lncRNAs as cancer biomarkers. This method with rigorous steps, sensitivity and specificity, can improve patient lives through identification of localized gene expression within individual cancer cells as well as expression changes in different cell populations of cancer tissues.

2. Results

2.1. Schematic and Controls

Schematic work flow of RNAScope assay using the Z probes is represented in Figure 1. Each Z probe has a18-25 base complimentary sequence to the target lncRNA and a 14 base sequence complementary to the pre-amplifier. Three double ZZ probes are sufficient for a signal, but this assay utilizes 20 double ZZ probes, which covers around 1kb of the target transcript.

FFPE sectioned HeLa cells from ACD were used first to validate the assay as shown in Figure 2(c). PPIB (Peptidylprolyl isomerase B), a human gene and DapB (dihydrodipicolinate reductase), a bacterial (*E.coli*) gene, were used as positive control and negative control probe respectively. DapB did not show any signal in pancreatic and colorectal cancer tissues, but PPIB stained nicely on the tissues (pancreatic and colorectal cancer) as well as on the FFPE sectioned HeLa cells. This result demonstrated the sensitivity and specificity of the assay and that FFPE tissues can be tried with the experimental probe staining with similar results as with the embedded cells.

 doi:10.20944/preprints201806.0096.v1

2.2. Validation of assay

In order to validate the assay method in identifying lncRNAs on FFPE tissues we selected a low, moderately and highly expressed lncRNA. LncRNA NRON, is scarcely expressed and is very hard to quantitate even by qRTPCR. Figure 3(a)(i) shows NRON signal in xenograft FFPE tissue, similarly moderately expressed oncogenic lncRNA UCA1 was very specifically located in colorectal cancer tissue in the epithelial population (Figure 3 (a)(ii)), highlighting the sensitivity of the assay method. Further, we chose highly expressed lncRNA MALAT1 for staining in colorectal, breast and pancreatic cancer. MALAT1 showed a very prominent staining in these three cancer tissues, mostly in epithelial cell population.

2.3. Quantitative measure of Progression and Invasiveness

Prominent lncRNA MALAT1 staining encouraged us to utilize this assay to analyze MALAT1 as a cancer progression and invasiveness marker. Interestingly lncRNA MALAT1 stained very well rwith respect to the the progression of colorectal cancer. Figure 4(a) represents MALAT1 staining in different stages of CRC with mean area intensity represented below in different stages. Figure 4(b) represents the quantitative stain intensity in different stages of CRC tissues. The MALAT1 stain intensity is significantly correlated among different stage (except stage III and stage IV) progression. Additionally, we observe higher MALAT1 expression in epithelial cells than in stroma cells. This might indicate a preferential expression within cell types and perhaps different regulatory mechanism.

Finally, to assess whether the assay method can quantitatively measure the invasiveness, we used a tumor microarray (TMA) with normal to adjacent tumor (NAT) and matched invasive breast cancer tissues and reassuringly, invasive breast cancer tissues have a higher MALAT1 staining (as shown in Figure 5(a), mean area intensity is higher in both invasive tissues as compared to NAT. Figure 5(b) shows a significant difference in the stain intensity between NAT and invasive tissues indicating lncRNA MALAT1 as a marker of invasive breast cancer.

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2.2. Figures, Tables and Schemes

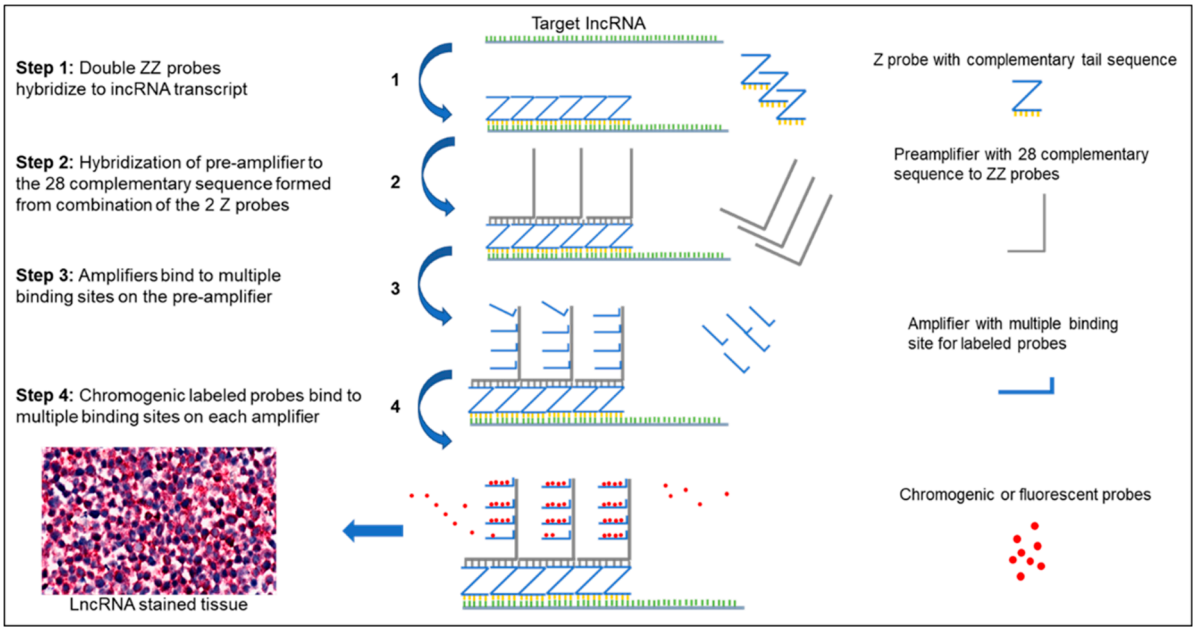

Figure 1: A schematic representation of Z probe based RNAScope assay for lncRNA analysis. Starting with Z probes hybridizing with target sequence creating a double ZZs up to 20 groups side by side. Preamplifier then binds to the complementary sequence on the 28 base tail (top of the ZZ). Pre-amplifiers contain multiple binding sites for amplifiers to bind to and the amplifiers also have multiple binding sites for labeled probes to bind. Upon chromogenic stain, the label probes fluoresce red color.

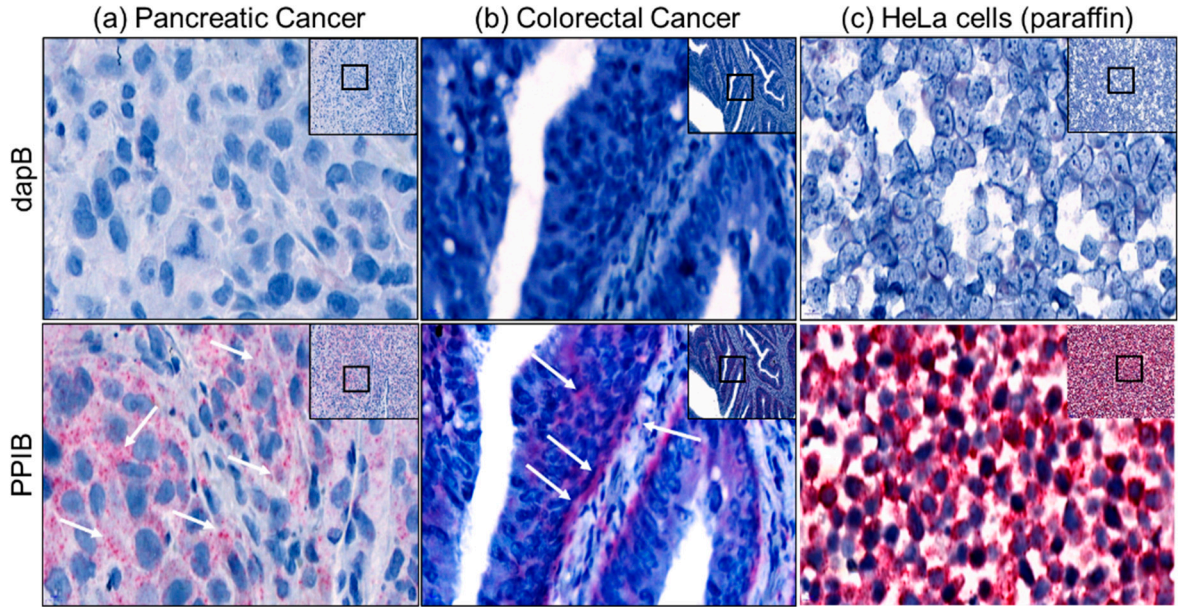

Figure 2: Validation and optimization of the Z-probe staining. Paraffin embedded and sectioned, pancreatic cancer tissue, colorectal tissue and HeLa cell pellet were stained with a negative control probe DapB (full form) and positive control probe PPIB (full form). The PPIB stained well with pancreatic, colorectal and HeLa cells (bottom panel **(a)**, **(b)** & **(c)**. 20x (inset) and 80x magnification using caseview software, scanned and analyzed on Pannoramic 250 Flash III. Arrows point at specific staining.

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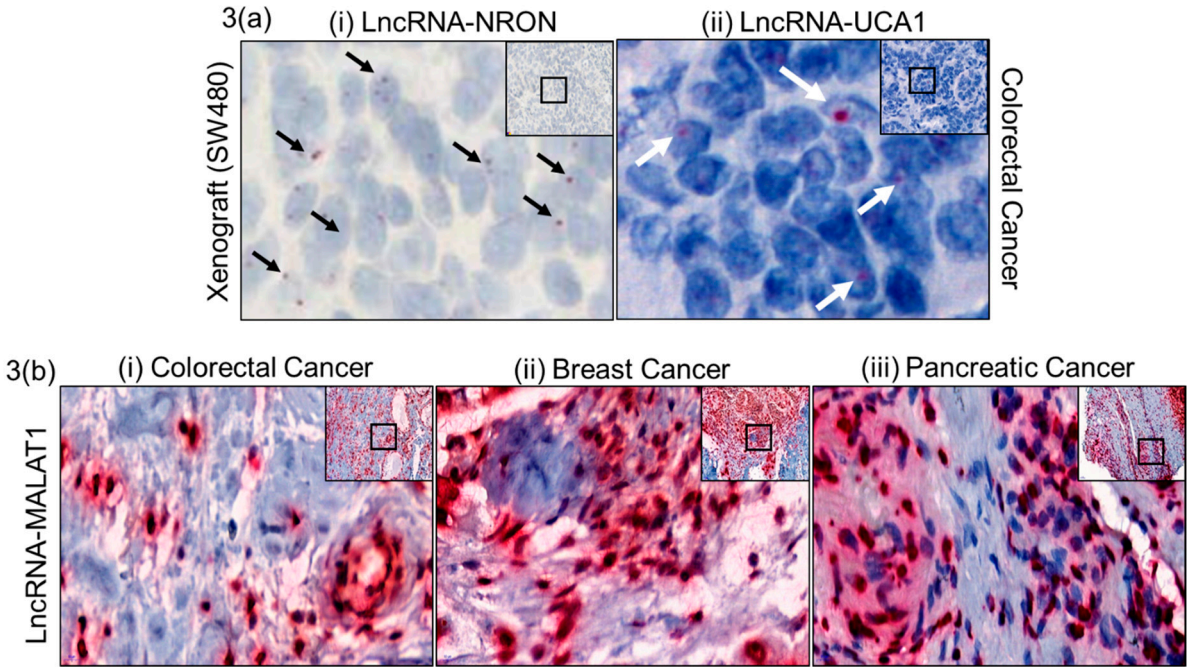
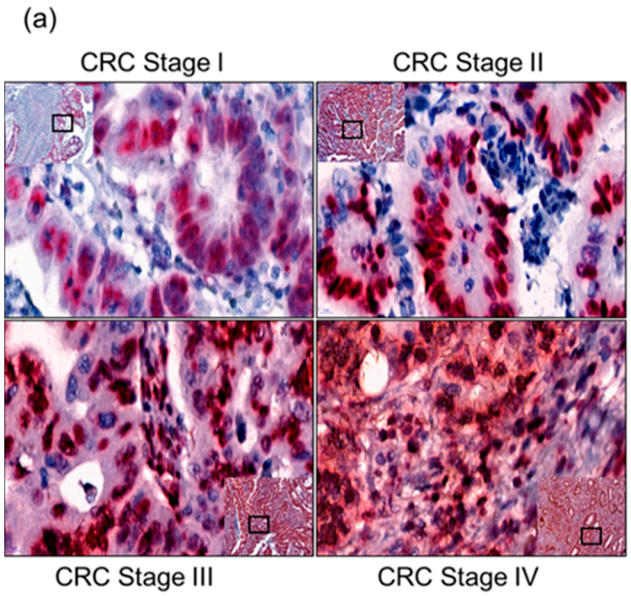
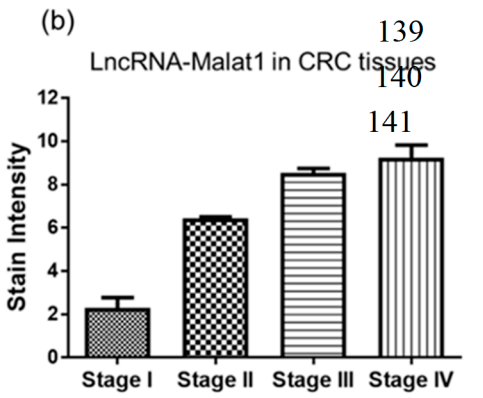


Figure 3: Paraffin embedded different human cancer tissues Z probe stained for different lncRNAs. **(a)** Tumor suppressor lncRNA-NRON (very low expressed) **(i)**, and oncogeneic lncRNA-UCA (moderately expressed) **(ii)**, stained in colorectal cancer tissue. **(b)** LncRNA–MALAT1 stained using specific Z-probes in paraffin embedded **(i)** colorectal cancer, **(ii)** breast cancer and **(iii)** pancreatic cancer tissues. 20x (inset) and 80x magnification using caseview software, scanned and analyzed on Pannoramic 250 Flash III. Arrows point at specific lncRNA signal.



Stages	Mean Area intensity
Stage I	2.299
Stage II	6.437
Stage III	8.523
Stage IV	9.247



Stages	Significant	Summary	P value
Stage I vs Stage II	Yes	***	0.0003
Stage I vs Stage III	Yes	****	<0.0001
Stage I vs Stage IV	Yes	****	<0.0001
Stage II vs Stage III	Yes	*	0.232
Stage II vs Stage IV	Yes	**	0.0043
Stage III vs Stage IV	No	ns	0.5859

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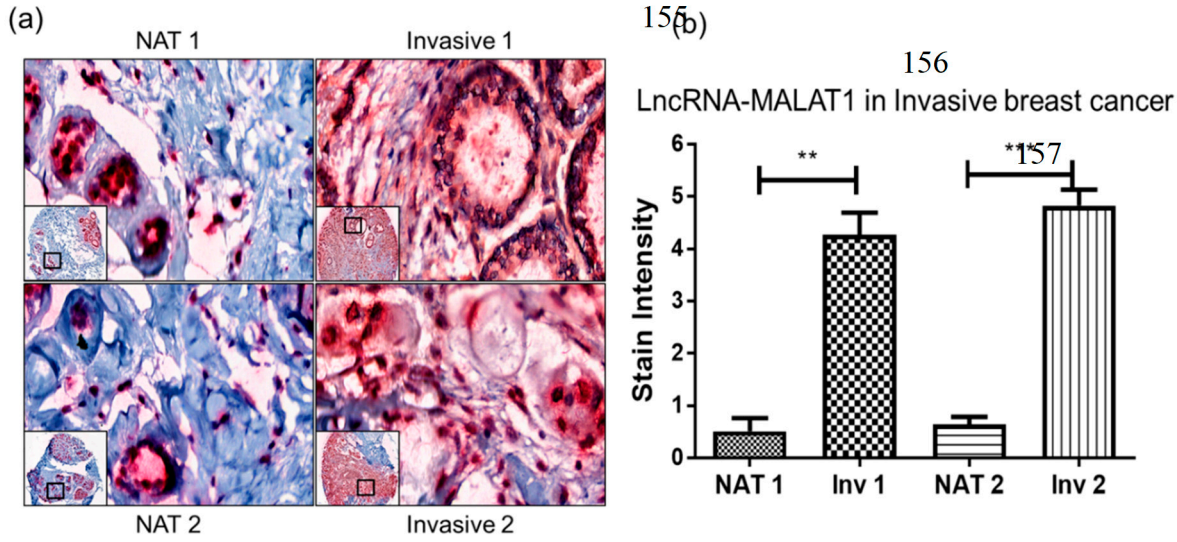
Figure 4: LncRNA-MALAT in different stages of CRC. Paraffin embedded different stages of colorectal cancer tissues were stained for lncRNA-MALAT1 and quantitated for the staining intensity. **(a)** Stages 1-IV CRC tissues show a differential stain for lncRNA-MALAT1. Stain intensity correlates to the progression. **(b)** Quantitation of the lncRNA-MALAT1 staining intensity was performed using Image J software. 10x (inset) and 80x magnification using caseview software, scanned and analyzed on Pannoramic 250 Flash III. Statistical analysis: One way ANOVA, Tukey' multiple comparison test. Compare the mean of each column with mean of other column. Arrows point at specific lncRNA signal.



Stages	Mean Area intensity
NAT 1	0.515
Invasive 1	4.273
NAT 2	0.641
Invasive 2	4.8

Figure 5: LncRNA-MALAT1 expression in breast cancer. **(a)** Matched breast cancer tissues (NAT vs Invasive) from two different patients, 1 & 2 were stained for lncRNA-MALAT1 using Z probe. The invasive breast cancer tissues have higher staining for lncRNA-MALAT1 as compared to normal adjacent tumor. **(b)** Quantitation of the lncRNA-MALAT1 staining intensity was performed using Image J software.10x (inset) and 80x magnification using caseview software, scanned and analyzed on Pannoramic 250 Flash III. Statistical analysis: Unpaired t test, ** $p<0.01$, *** $p<0.001$.

3. Discussion

IHC and RT-QPCR has long been the gold standard for investigating protein and RNA biomarkers respectively [8,14-16]. Lack of specificity, sensitivity and reproducibility of these methods continue to limit our interpretation of data collected. This in turn makes clinical diagnosis challenging. While IHC is beneficial in identifying protein biomarkers, the assay is ineffective in detecting lncRNA in FFPE tissues, which is the limitation of the assay design. As our understanding of long non-coding RNAs and their role in cancer development and progression augments, a more specific, sensitive and reproducible tool is warranted. The strength of Z probe assay lies on the fact that it utilizes approximately 1kb of the target transcript and it employs 20 double ZZ binding probes side by side, which is definitely much more sensitive and robust assay than conventional RNA *in situ*

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hybridization assay [9-11]. Differential expression pattern of HOTAIR, H19, KCNQ101T, MEG3, MALAT1, and ZFAS1 via RNAScope on breast cancer patient samples has been shown recently.[12]. Use of the appropriate positive and negative controls strengthen the confidence on the results. PPIB positive signal on xenograft HeLa cell and human cancer tissues simultaneously validated the procedure.

Tumor suppressor lncRNA-NRON was one of the first lncRNA identified from an unbiased screen [1]. NRON is noncoding repressor of NFAT, and it is a tumor suppressor [17] . NRON is very lowly expressed and its expression was difficult to visualize in human tumor FFPE tissue, so SW480 (colorectal cancer cell line) Xenograft FFPE tissue was used. Moderately expressed oncogenic lncRNA-UCA1 has been studied for different pathways and interactions, resulting in cancer progression [18-20]. The assay in study was able to show specific epithelial cell population expressing UCA1 as red dot. We were also able to show that MALAT1 is aberrantly expressed in TMAs of three cancer groups (Colorectal, Breast, and Pancreatic), suggesting that lncRNA MALAT1 is associated with multiple cancer and its use as a therapeutic target should be investigated further. Though MALAT1 has been studied in different cancers as a poor prognostic [21-24], aggressive and metastatic marker [21,25,26], most of the published data on MALAT1 lack visual representation of MALAT1 detection. All the studies known so far have used RT-QPCR, RNAseq or curated database analysis for their analysis. This is the first evidence of MALAT1 expression in FFPE patient tissues of breast, colorectal and pancreatic cancer by chromogenic staining, suggesting that lncRNA MALAT1 is associated with multiple cancers and its use as a therapeutic target should be investigated further. We also showed progressive expression of MALAT1 across the different stages of colorectal cancer and were able to correlate the stages with the progression of CRC. To our confidence the stain intensity of different ROI in the CRC tissues was statistically significant except for Stage III and stage IV comparison. More stage III and Stage IV CRC tissues might help to increase the statistical significance. Invasive breast tissues have been shown to have higher MALAT1 expression [21,26-28], but for the first time this study gives a visual evidence. Although we were only able to show UCA1 expression in CRC TMA, staining in TMA of other cancer group will be investigated to determine if there is differential expression of UCA1 between cancer types.

As we continue to investigate the role of long non-coding RNAs in cancer development and metastasis, this assay method will help provide visual representation of this class of RNA molecules. Collectively, this data suggest that Z probe can be an efficient tool to characterize long non-coding RNA molecules in FFPE tissues and cells. This probe provides high confidence with respect to specificity, sensitivity and reproducibility. This assay alleviates all areas of concern by simultaneously detecting lncRNA molecule of interest while suppressing background noise with more novel Z probe pair design and signal amplifiers. In both laboratory and clinical settings, this assay can be utilized to corroborate the results of both IHC and RT-QPCR strengthening our interpretation of cancer behavior especially in this new field of long non-coding RNAs.

4. Materials and Methods

Breast cancer TMA # BR243w was purchased from Biomax, human colorectal cancer tissues (FFPE), pancreatic cancer tissue (FFPE) sectioned were procured from department of pathology, UTHSC as per UTHSC IRB guidelines. Colorectal cancer cell line SW480 xenograft (FFPE) were sectioned using Microtome (Leica Biosystems). FFPE HeLa slides were procured from ACD (#310045) to use as positive and negative controls.

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Chromogenic staining in FFPE tissues: Formalin fixed (10% neutral buffered formalin, 16-32 hrs) and alcohol treated (70% alcohol, 24-48 hrs) paraffin embedded tissues, sectioned (5-7 M) and mounted on Superfrost slides (Fisherscientific) were used for lncRNA Z-probe staining. RNAScope 2.5 HD Detection Kit (RED) (#322360, ACD), was used as per manufacturer suggestions with modifications. After baking FFPE slides on side warmer for 1 hr at 60 °C, they were deparaffinized in fresh xylene twice for 5 min, dehydrated in 100% alcohol twice for 1 min. and treated with hydrogen peroxide (#322330, ACD) for 10 min at room temperature. Maxed epitopes were retrieved by incubating slides in boiling target retrieval buffer (#322000, ACD) for 25 min (this is a critical step as different tissues require different retrieval times) followed by protease plus (#322340, ACD) treatment for 30 min in the HybEZ (#240200, ACD) incubator preset at 40 °C. Z probes (HsNRON #508481, HsUCA1 #417521, HsMALAT1 #400811) warmed at 40 °C for 10 min, and amplifiers (#322360, ACD) were normalized to room temperature before hybridization steps. After aspirating protease plus, probes were added to respective tissues and incubated at 40 °C for 2 hrs in HybEZ incubator. Hybridized probes bound to complementary bases were amplified through sequential amplification steps from amp-1 to amp-6 followed by incubation at 40 °C in the incubator with washing (#310091, ACD) between each amplification steps. Amplifiers tagged with chromogenic labeled dye probes (mix of RED-B and RED-A at 60:1 ratio) after 10 min incubation gives a red signal. Slides were counterstained with 50% hematoxylin for 2 minutes at room temperature and washed with 0.02% ammonia water followed by distilled water. Slides finally dried at 60 °C for 15 min on a slide warmer, Xylene dipped and mounted with Ecomount (#EM897L Biocare Medical). FFPE mammalian cells sectioned on slides can also be stained using the same method.

Quantitation (ImageJ) Analysis: The stained slides were scanned on Pannoramic 250 Flash III. Images were visualized using Caseviewer software (3DHistech). Four different field (ROI: Region of Interest) of the 10x image were analyzed for the total intensity of the red stain, using Image J (nih.gov), representing the corresponding lncRNA staining. Color threshold was adjusted to identify only the red color.

Statistical analysis: Statistical analysis were performed using GraphPad Prism 7. $P<0.05$ was considered significant.

Author Contributions: Conceptualization, M.K.T, CZ, and SCC; Methodology, CZ and MKT; Software, CZ, MKT; Formal Analysis, CZ and MKT; Investigation, CZ and MKT; Resources, MKT, LG, MMY, MJ and SCC; Writing-Original Draft Preparation, CZ and MKT; Writing-Review & Editing, CZ, MKT, KD, FK, MJ, MMY and SCC; Visualization, CZ, KD, FK, and MKT; Supervision, MKT and SCC; Project Administration, MKT and SCC; Funding Acquisition, MKT, LG, MMY, MJ and SCC.

Funding: The research was partially supported by the National Institutes of Health Grant (R01CA204552, R01CA210192 and R01CA206069) to S.C.C. UTHSC-CORNET and UTHSC-Clinical CORNET award to M.K.T. This work was also partially supported by UTHSC-College of Pharmacy-Dean's Seed Grant and UTHSC New Grant Mechanism Award to S.C.C., M.M.Y. and M.J.

Acknowledgments: The authors would like to thank Dr. Marie Chisholm-Burns, Dean COP UTHSC, Dr. Lisa Youngentob, Director R&D and Dr. Steven R. Goodman, Vice Chancellor of Research for suppor through CORNET program. Department of Pathology data acquisition services are also acknowledged.

Conflicts of Interest: "The authors declare no conflict of interest."

 doi:10.20944/preprints201806.0096.v1

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