Detection of lncRNAs implicated in disease by Stellaris® RNA Fluorescence *in situ* hybridization (RNA FISH)

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ABSTRACT

Most RNAs produced by the extensive transcription of the genome lack protein coding potential. Long non-coding RNAs (lncRNAs) have been shown to play important roles in all the central aspects of gene function and expression including imprinting, epigenetic regulation, transcription, splicing and nucleo-cytoplasmic trafficking, translation and RNA stability. These processes all have wide ranging effects on cell cycle and differentiation. LncRNAs cannot be detected by immunohistochemistry, as no protein product is produced. While fluorescence *in situ* hybridization (FISH) with labeled DNA probes against chromosomal targets can locate lncRNA genes, their transcription is not detected or quantified. The role of lncRNAs in both basic science and clinical research now requires direct visualization and quantification of lncRNAs at the single cell level.

We recently streamlined both the design and synthesis of fluorescently labeled probe sets containing multiple, tiled 20-mer GC-balanced oligonucleotides for use in RNA FISH. In contrast to traditional collective and cell-disruptive RNA analyses (*e.g.* northern blotting and qPCR) that report relative population averages of RNA content, single molecule (sm)RNA FISH affords specific and sensitive detection and quantification of RNAs, while establishing the intracellular localization of the target lncRNAs.

In this study, we examine various established lncRNAs as well as lncRNAs that have never been visualized at the single cell level by FISH. Some of these include MALAT1, NEAT1, XIST, H19 and PCA3. The results and tools presented here will further enable the study of disease-related lncRNAs. This will in turn yield a greater understanding of how expression and localization of lncRNAs may contribute to the disease phenotype, and add a large selection of novel biomarkers that can be interrogated for improved diagnosis, prognosis, and therapeutic choice.

METHODOLOGY

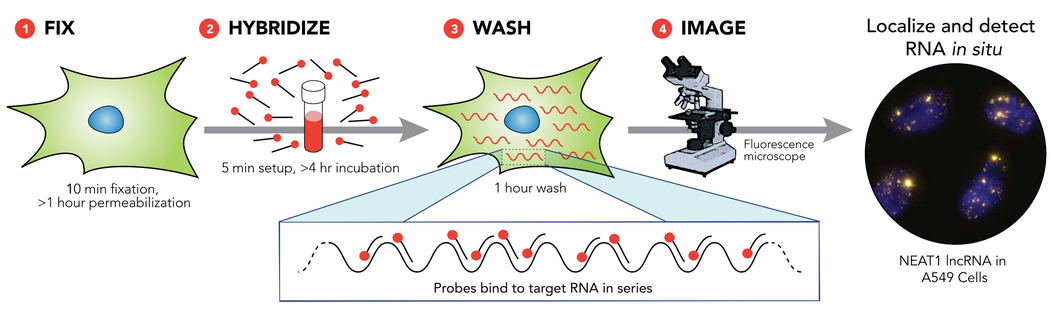
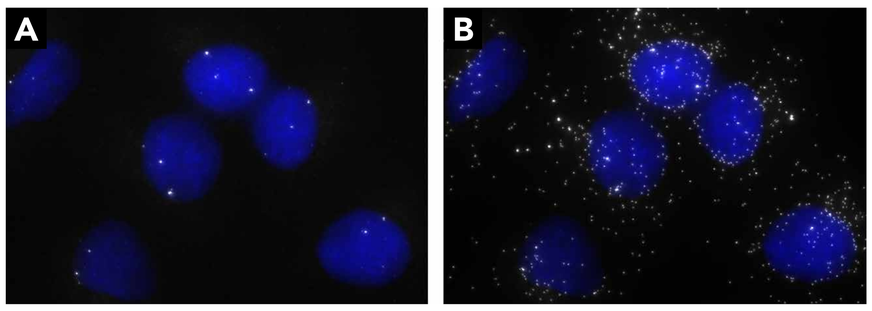


Figure 1. Schematic of Stellaris RNA FISH assay
For each target, a mix of multiple 20-mer oligonucleotides, each labeled with a single Quasar® 570, Quasar 670, or CAL Fluor® Red 610 fluorophore was designed* and synthesized. All probe sets contained at least 32 oligos. Adherent cells were grown on #1 cover glass and subsequently fixed and permeabilized. Hybridizations were carried out for 4 to 16 hours at 37 °C in 50 µl hybridization solution (10% dextran sulfate, 10% formamide in 2X SSC). Samples were then washed, DAPI stained, and imaged.

*Stellaris probe designer and protocols: www.biosearchtech.com/stellaris

Microscope specifications used for image acquisition:
Nikon Eclipse Ti
Andor Clara CCD camera
Prior Lumen 200 Illumination System
CFI Plan Apo VC 60X Oil

RNA FISH IN ADHERENT CELL LINES



Cell#	MKI67 Exonic RNA Spots	MKI67 Intronic RNA Spots
1	192	3
2	92	3
3	114	3
4	128	3
5	201	3

Figure 2. Identification of MKI67 mature and nascent RNAs (transcription bursts).
A Stellaris probe set labeled with a Quasar 670 dye targets the intronic RNAs of MKI67 (a cellular marker for proliferation) in the human lung adenocarcinoma cell line, A549 **(A)**. A second probe set labeled with a CAL Fluor Red 610 dye targets the exonic RNAs of MKI67 **(B)**. The overlaid images reveal colocalization with the intronic and exonic sets that target nascent RNAs **(C)**. Quantification of RNA spots detected was performed **(D)**.

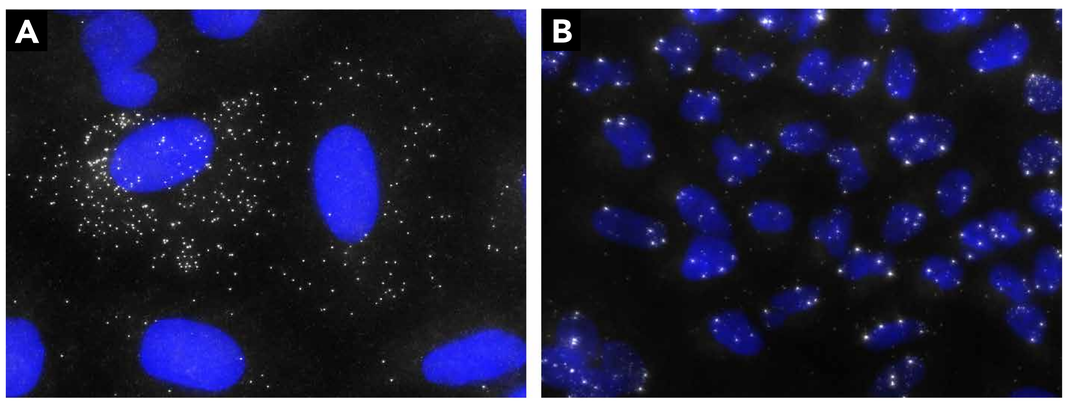


Figure 3. LncRNA expression in adherent cells.
(A) Cell-to-cell gene expression variation of H19 RNA in the human lung adenocarcinoma cell line, A549.
(B) PVT1 RNA distribution in the human lung adenocarcinoma cell line, A549.

RNA FISH AND IF IN ADHERENT CELL LINES

MALAT1 is a predictive RNA biomarker for metastasis development in lung cancer.

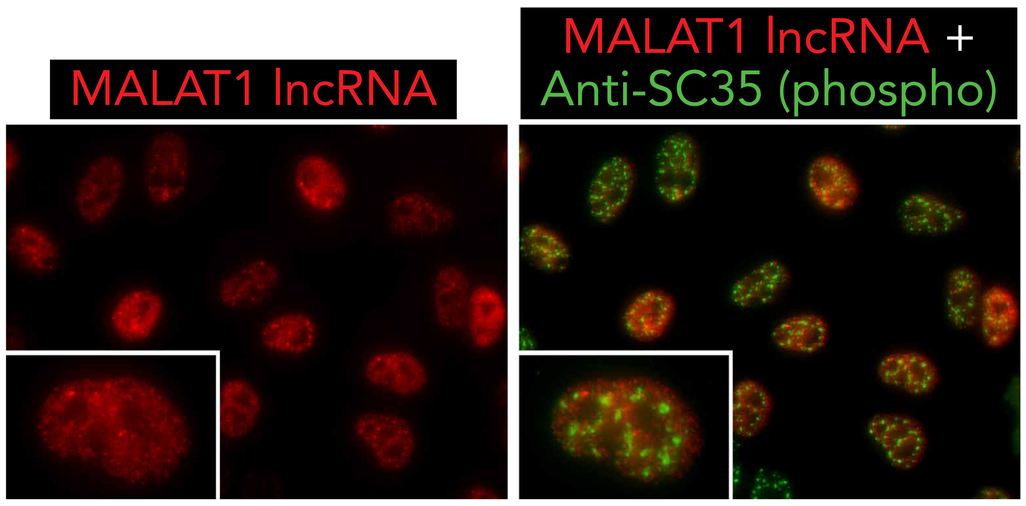


Figure 4. Colocalization of MALAT1 lncRNA and SC35 protein in nuclear speckles.
RNA FISH with a Quasar 570 labeled MALAT1 probe set (red) combined with immunofluorescence with an anti-p-SC35 antibody (green) reveals co-localization in the human lung adenocarcinoma cell line, A549.

RNA FISH IN FROZEN TISSUE

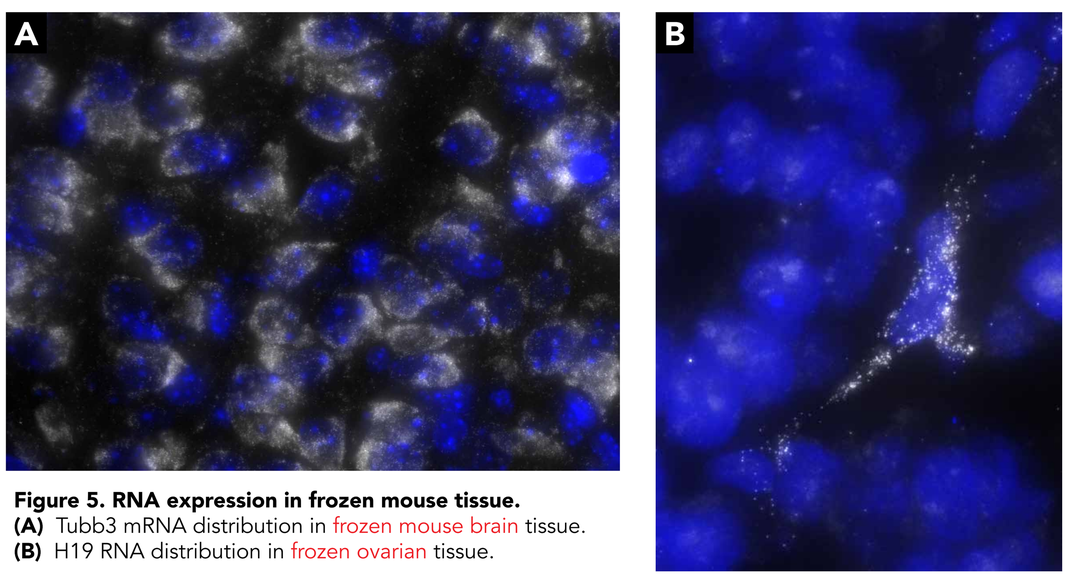


Figure 5. RNA expression in frozen mouse tissue.
(A) Tubb3 mRNA distribution in frozen mouse brain tissue.
(B) H19 RNA distribution in frozen ovarian tissue.

RNA FISH IN FFPE TISSUE

Overexpression of PCA3 lncRNA and ERG are specific prostate biomarkers.

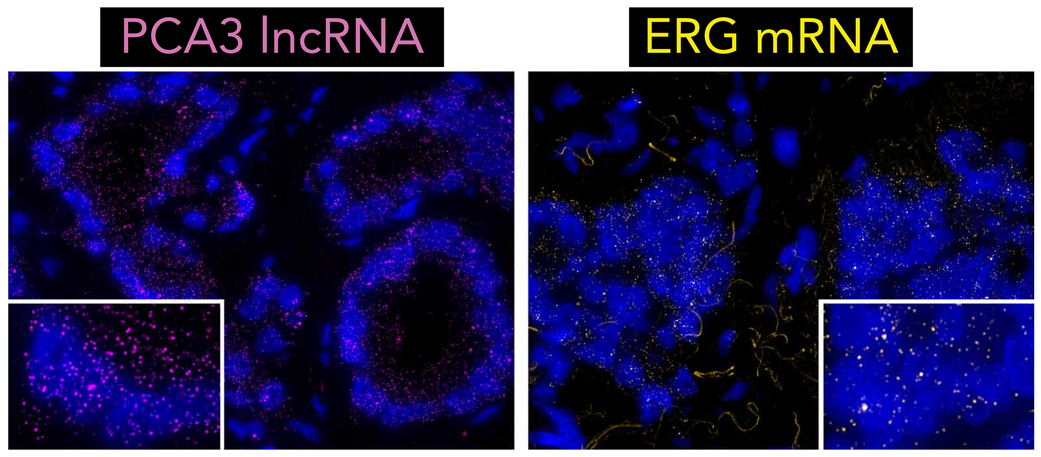


Figure 6. Prostate cancer biomarkers.
The status of PCA3 and ERG gene expression in these FFPE prostate cancer tissues was assessed by Stellaris RNA FISH.

CONCLUSIONS AND REFERENCES

Stellaris FISH is an RNA detection method that enables detection, localization, and quantification of RNA at the single cell level. Single molecule RNA FISH (smFISH) provides an accurate method to capture the stochastic behavior of genes, providing further insight into cell-to-cell gene expression variation. Utilizing spectrally distinct fluorescent labels, Stellaris RNA FISH can distinguish different RNA variants from one or multiple genes allowing for additional insight into correlated gene expression at the single cell level. The amplification and overexpression of genes in cell lines and tissue can be robustly assessed with this technology, allowing the Stellaris method to serve as a proxy for immunofluorescence and DNA FISH.

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