High-Throughput Imaging and High Content Analysis of Disease Relevant lncRNAs Examined by RNA Fluorescence *in situ* hybridization (RNA FISH)

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ABSTRACT

The fate of any given mRNA has remained difficult to experimentally follow and has left mechanisms of unexpected gene expression phenomena in the dark. Examples are differential stability of mRNA expression from different promoters, and inhibition of expression in trans from co-transfected plasmids encoding N1-spermidine/spermine, acetyltransferase and a second unrelated gene. Recent advances in single molecule fluorescence in situ hybridization (smRNA FISH) now bring such central aspects of cellular mRNA metabolism into view. Streamlining both the design and synthesis of probe sets of multiple tiled singly labeled 20-mer oligonucleotides with balanced GC content have made most mRNAs targetable. In contrast to traditional cell-disruptive RNA analyses - northern blotting, qPCR, and microarrays - that yield average and relative information on the content of the probed mRNA, smRNA FISH affords discrete and cell-specific copy. The genome is extensively transcribed, yet more than 50% of RNA transcripts lack protein coding potential. In recent years, long noncoding RNAs (lncRNAs) have been shown to play extensive roles in all the central aspects of gene function including imprinting, epigenetic regulation, transcription, splicing and nuclear/cytoplasmic trafficking, translation and RNA stability, with wide ranging effects on cell cycle and differentiation. Due to the increasingly central role of lncRNAs in gene transcription and disease states, it is important to develop tools to allow direct visualization and quantification of lncRNAs that could be useful both in the basic science arena and in the clinical setting with possible uses both in diagnostics and prognostics.

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 fluorescence *in situ* hybridization (RNA FISH). In contrast to traditional cell-disruptive RNA analyses (e.g. northern blotting and qPCR) that report relative population averages of RNA content, single molecule RNA FISH affords specific and sensitive detection of RNAs and quantitatively establishes the intracellular localization of the target lncRNAs.

We have validated an imaging based platform to perform RNA FISH in both manual (coverslip) and automated (384 well) formats that allows tremendous multiplexing and throughput using high throughput microscopy. Moreover, we developed high content analysis routines that permit quantification of RNA particles at the single cell level and determine their localization and pattern (texture). As specific lncRNAs (e.g. MALAT1) are known to target specific intranuclear domains (e.g. splicing speckles) we developed classification methods based upon sets of intensity and texture features to compare intracellular patterns of novel/less characterized ncRNAs. Moreover, this framework will also permit to address changes in lncRNA expression and patterns during the cell cycle since large number of cells can be analyzed simultaneously. We are now expanding our analysis to primary tumor tissues where lncRNAs patterns will be correlated with known FISH-compatible biomarkers, thus providing a picture simultaneously for intra- and inter-cell variation.

In summary, the results and tools presented here will enable the study of disease related lncRNAs in a high content, high throughput mode. These efforts will enable gaining better understanding of how expression and localization of lncRNAs may contribute to the diseased phenotype, and add a large selection of novel biomarkers that can be interrogated for improved diagnosis, prognosis, and therapeutic choice.

lncRNA FISH

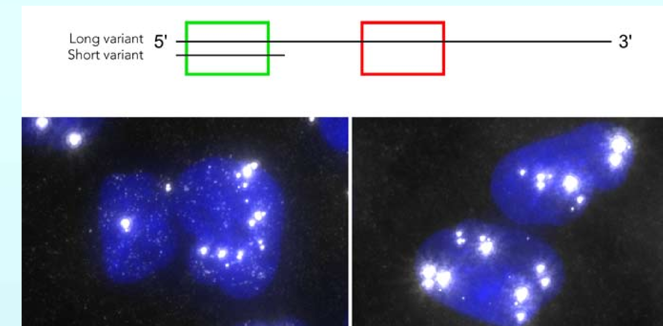

Figure 2. NEAT1 variant-specific RNA FISH. Probe sets detecting the short and the long form (left panel) or the long form only (right panel) were used for RNA FISH in human A549 cells.

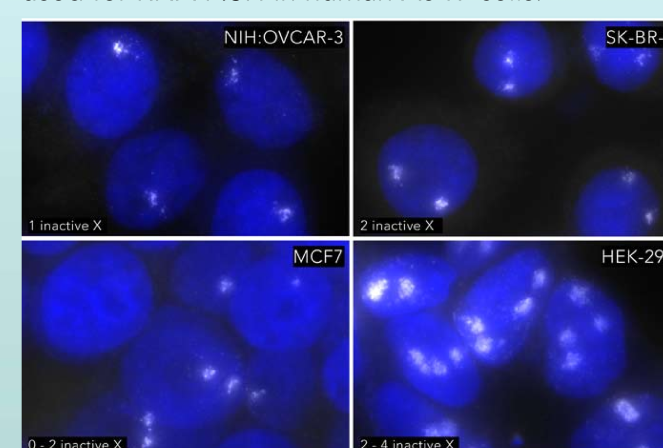

Figure 3. XIST clouds in four female cancer cell lines with variable X-chromosome karyotype.

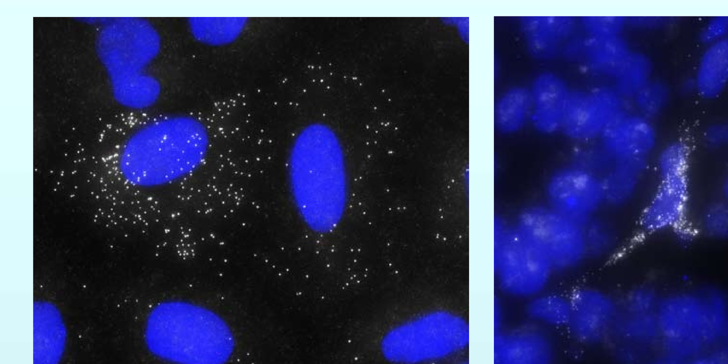

Figure 4. Cytoplasmic localization of H19 lncRNA in human A549 cells (left panel) and frozen ovarian tissue (right panel). Note pronounced cell-to-cell variability.

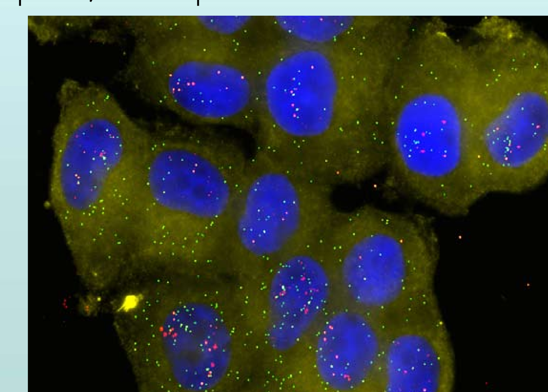

Figure 5. Multiplex analysis of ERBB2 (HER-2/Neu) mRNA and protein and NEAT1 long lncRNA by immunofluorescence and RNA FISH in T47D human ductal breast epithelial tumor cells.

ALTERED lncRNA NUCLEAR PATTERNS

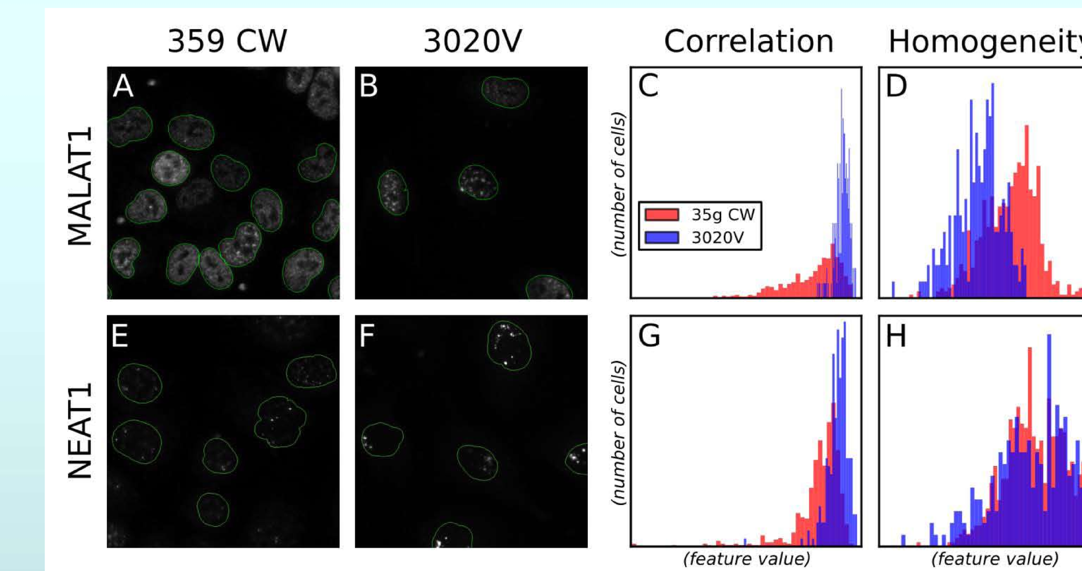

Figure 7. Quantitative analysis of subnuclear lncRNA patterns in primary stromal endometrial cell lines.

Stellaris FISH probes were used to label the lncRNA MALAT1 in cell lines derived from two patients with different endometriomas (A and B) obtained from Dr. Shannon M. Hawkins (Dept. Ob/Gyn, BCM, Houston, TX). DAPI staining was used to identify nuclear boundaries (green outlines). While MALAT1 is punctate in both cell lines, it exhibits a more consistent nucleoplasmic staining in the 359CW cells. This difference is reflected in two texture features, including one that captures the linearity of structure (correlation, C) and one that captures the homogeneity of a pattern (homogeneity, D). Similar analysis for NEAT1 (E, F, G, and H) shows that its correlation is statistically different between the two cell lines while its homogeneity is not, highlighting that different measures can define different types of pattern changes.

METHODS

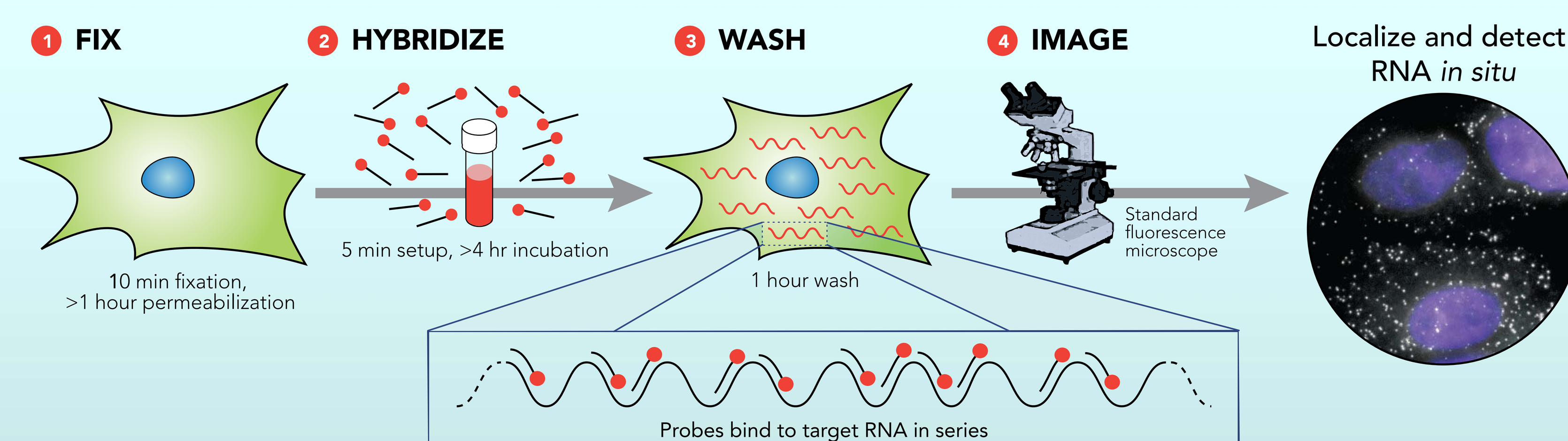

Figure 1. Stellaris® RNA FISH.

For each target, a mix of multiple 20-mer oligonucleotides, each labeled with a single Quasar® 570 or 670 fluorophore was designed* and synthesized. All probe sets contained ≥ 36 oligonucleotides each. Hybridizations were carried out on coverslips or slides for 4 to 16 hours at 37 °C in 100 µl hybridization solution (10% dextran sulfate, 10% formamide in 2X SSC). Samples were then washed, nuclei stained with DAPI, and imaged.

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

NUCLEAR RNA LANDMARKS

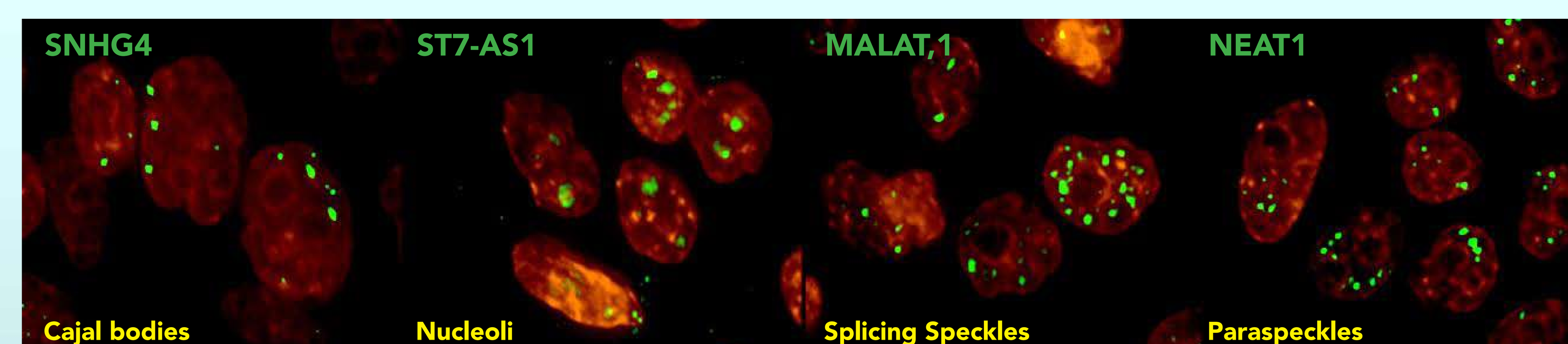

Figure 6. Detection of lncRNAs with Stellaris FISH probes.

Four prototypical lncRNA FISH probe sets were used to demonstrate unique subnuclear localization patterns known to be associated with nuclear domains. The pro-snoRNA containing SNHG4 localizes to Cajal bodies (where its snoRNAs are processed), ST7AS1 localizes to nucleoli (ribosome synthesis), MALAT1 concentrates to RNA splicing speckles (mRNA splicing), and NEAT1 accumulates in paraspeckle bodies (transcription factor regulation). Probe sets directly labeled with Quasar 570 dyes were used in HeLa cells, and manually carried out in a 384 well, glass bottom plate (Greiner). Nuclei are labeled with the DNA specific dye DAPI (blue). Imaging was performed with a multiwell plate-capable DeltaVision deconvolution microscope using a 40x/0.95NA air lens.

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ACKNOWLEDGMENTS AND DISCLAIMERS

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