Cancer is a disease of aberrant gene expression, and delineating the critical gene expression events during cancer development and progression is vital for cancer research. Here we summarize the role of novel RNA in situ hybridization technology in expanding the tool set available to researchers, thereby overcoming the challenges of cancer research in four key areas:

1. Understanding tumor heterogeneity in gene expression
2. Studying non-coding transcripts
3. Developing biomarkers
4. Driving and refining the diagnostic strategies of the future

Cancer is becoming an increasingly critical medical challenge, with the number of new cases expected to rise by about 70% globally over the next two decades.¹ Since this disease essentially arises on the genomic level, investigating the tumor’s genomic and transcriptomic landscape is invaluable for both basic and translational cancer research.

Cutting-edge research employs a range of approaches to unravel the complexities of cancer, looking at the disease from all levels – the genome, the transcriptome, and the proteome. Over recent years, cancer transcriptomic profiling programs have proven that, like protein, RNA is a rich source of biomarkers for diagnosis, prognosis and predicting therapeutic response (recently coined as theranosis).²,³ Indeed, the significance of RNA within the cell has become increasingly apparent, with many discoveries indicating structural and regulatory functions.

Alongside our growing understanding of RNA, methods for the analysis of this molecule are also evolving. The evolution of Next Generation Sequencing (NGS) technology over the past 10 years has offered cancer researchers the ability to look at the cancer genome and transcriptome, while PCR has offered researchers the ability to determine gene expression levels averaged across heterogeneous cell populations. However, up until now, a lack of robust methods for in situ analysis of RNA transcripts has held back the potential of RNA studies in cancer research. With the latest developments in RNA in situ hybridization (RNA ISH), however, this situation is changing.

Why RNA?

Cellular physiology involves many components besides RNA, and it is common to study cancer at the level of DNA and protein. So why focus on RNA?

The cancer genome: Gross changes (gene amplifications, deletions and gene rearrangements) in the genome can be detected in situ using fluorescence in situ hybridization (FISH), but looking at DNA only provides limited information.

The cancer transcriptome: Not merely a messenger, RNA is a versatile and functionally significant molecule, reflecting the dynamic nature of a cancer cell. Novel RNA ISH techniques are quickly realizing this potential.

The cancer proteome: Proteins are the final products of protein-coding genes in the genome, and their analysis can provide a wealth of insights. However, antibody-based detection is limited. Only 33% of 60,000 human genes code for proteins,⁴ and as antibodies have variable levels of sensitivity and specificity, high quality antibodies are not available for the majority of proteins.
RNA in situ hybridization

Enabling the analysis of RNA in its morphological context is vital for cancer research. The spatial resolution provided by ISH presents a new data dimension, for example providing precise localization of target RNA in single cells and allowing localization and quantitation of RNA expression in specific cell types e.g. stromal versus tumor expression. Only with the ongoing technological advances (Figure 1) has the sensitivity, specificity and ease of use improved sufficiently to make the method both established and accessible.

Non-isotopic ISH is based on fluorescently labeled (known as fluorescence in situ hybridization – FISH) or biotin or hapten-labeled nucleic acid probes (chromogenic in situ hybridization – CISH). It has presented the most pragmatic RNA ISH approach for a number of years, improving turnaround time, sensitivity and safety in comparison with isotopic ISH. However, due to the short nature of RNA targets allowing only a limited amount of labels to be incorporated into the probes, FISH and CISH lack sufficient sensitivity for the majority of expressed genes. The signal-to-noise ratio is also limited due to a high probability of non-specific binding and cross hybridization in the highly complex tumor sections.

Addressing these issues and expanding the RNA analysis tools available for cancer researchers, the next generation of RNA ISH has now been developed. For example, RNAscope® ISH technology based on ACD’s (Advanced Cell Diagnostics Inc., Hayward, CA) unique probe design and signal amplification system (Figure 2). In order to substantially improve the signal-to-noise ratio, RNAscope technology employs a proprietary probe design strategy in which two independent probes (double Z probe pairs) must hybridize to the target sequence in tandem for signal amplification. Since it is highly unlikely that two independent probes will hybridize to neighboring non-specific targets, this design concept ensures selective amplification of target-specific signals while preventing amplification from non-specific binding.

This approach dramatically improves the sensitivity and signal-to-noise ratio of RNA ISH to enable single-cell gene expression analysis in situ with single-molecule sensitivity. Although this has been demonstrated previously through alternative techniques, the RNAscope method allows robust single RNA molecule detection in routine formalin-fixed, paraffin-embedded (FFPE) clinical specimens, unlocking the full potential of RNA.

Revealing Tumor Heterogeneity

Tumors from patients receiving the same histological diagnosis are often highly heterogeneous both in biology and clinical behavior. Recent NGS studies have uncovered a high degree of genetic heterogeneity in tumors at different metastatic sites in the same patient or between patients (known as inter-tumor heterogeneity), and even in the same tumor (known as intra-tumor heterogeneity). Moreover, tumor properties also change over time (temporal heterogeneity). This presents an even greater challenge for cancer therapy – as a patient may not necessarily respond to a single treatment. Assessing molecular heterogeneity in the tumor is a challenging task, and in-solution based methods such as RT-qPCR and RNA-seq for accurate quantitative RNA analysis destroy all morphological context and spatial resolution within the sample, limiting researchers to comparing RNA expression information among heterogeneous cell populations.

Employing RNA ISH, Dr. Nallasivam Palanisamy and co-authors demonstrated the existence of rare subsets of prostate cancer with heterogeneous molecular aberrations involving ETS family genes, utilizing both standard immunohistochemistry (IHC) and RNA ISH with ACD’s RNAscope technology (see Application Example 1).

Tumor microenvironment

The tumor microenvironment describes the tumor’s non-cancerous components, which include immune cells, fibroblasts, signaling molecules, the extracellular matrix and surrounding blood vessels. There is a highly complex two-way interplay between the tumor and its microenvironment, with the tumor both influencing its environment, and receiving signals from the microenvironment to affect how it grows and spreads. Understanding the localization of molecular markers within the microenvironment allows a more complete characterization of the tumor to better predict tumor progression and response to therapy.

RNA ISH is particularly well suited to these investigations for several reasons. Receptor-ligand interactions stimulate or attenuate key signaling pathways, while autocrine versus paracrine signaling may define different subtypes of cancers. However, this information can only be gleaned through in situ hybridization to visualize the localization. In particular, RNA ISH’s ability to precisely identify the source of secreted proteins is a distinct advantage over IHC because the mRNA will always localize in the cell of origin – whereas secreted proteins become diluted and diffused within the inter-cellular space.
APPLICATION EXAMPLE 1

Analyzing prostate tumor molecular heterogeneity by combined immunohistochemistry and novel RNA ISH\textsuperscript{20,21}

RESEARCHER:
Nallasivam Palanisamy, MSc., MPhil., PhD. Associate Scientist, Henry Ford Health System; Associate Research Professor (Adjunct), University of Michigan.

RESEARCH GOALS:
Refining approaches for molecular classification to replace morphological assessment of tumors. This involves the discovery of new molecular markers in cancer – particularly recurrent gene fusions, and understanding their role in cancer development.

METHODS:

**Discovery - RNA-seq**
Initial transcriptome sequencing presents an unbiased characterization of a given sample, identifying biomarkers in both protein-coding and non-coding genes.

**Validation - RNA ISH**
For subsequent biomarker profiling, RNA detection is the only option when looking at non-coding genes (Figure 3A). Even some of the markers based on protein-coding genes do not have good antibodies (Figure 3B). Dr. Palanisamy explains: “ETV1, ETV4 and ETV5 genes are overexpressed in a small subset of prostate cancer, and RNA screening is the method of choice. Even for the genes with good antibodies, if the protein level is variable or always too low for detection, supporting protein analysis with information on RNA expression forms an unequivocal assessment.”

**Combined IHC & RNA ISH**
This is performed on the same slide in a sequential manner - RNA ISH followed by IHC. Given the limited availability of tissue from a small biopsy, it is important to develop methods to detect more than one type of marker on the same slide. Dr. Palanisamy comments: “Development of combined protein and RNA detection methods may overcome many concerns for accurate detection of biomarkers, and I can see this being the standard practice in future molecular cancer profiling.”

FIGURE 3: RNA ISH in prostate tumor tissue. A) Probing for non-coding PCA3 transcript in whole tissue section using RNAscope technology. B) Tissue microarray probing for ETV1 rearrangement, for which a good antibody is unavailable for protein detection. Showing positivity only in the ETV1 rearrangement-positive case, with no nonspecific staining in the negative cases. Entire tumor area in all three cores representing the positive case is detected by RNA ISH with RNAscope technology.\textsuperscript{20,21}
Analysis of Non-coding RNA: Beyond IHC

Discovering the world of non-coding RNAs has unlocked many secrets into how life's incredible phenotypic diversity arises from a relatively small and fixed set of genes. Today, new classes of RNAs are being discovered on a regular basis that do not code for proteins, but are instead involved in genetic regulatory control and a wide range of cellular activities. Amazingly diverse and changeable, these transcripts have the potential to produce any number of splice variants, and in the latest data release, version 22, by the GENCODE project we now know that there are approximately 60,000 human genes transcribing ~200,000 RNA species. Moreover, of the 60,000 genes, only 20,000 genes code for proteins while 25,000 genes do not code for proteins but are transcribed into RNA.

The most common approach to interrogate the transcriptome for non-coding RNA species involved in cancer is to begin with a fully comprehensive transcriptomic discovery program, utilizing technologies such as the microarray or RNA-seq. Once a set of transcripts exhibiting differential expression between healthy and tumor tissue has been defined, subsequent studies focus on validating their functional significance and clinical relevance. For non-coding genes, however, antibody detection techniques such as the well-established IHC, cannot be applied.

RNA ISH therefore presents the ideal alternative to IHC, greatly facilitating cancer researchers in determining the expression and localization of non-coding RNA species, which is vital for understanding the underlying biology of neoplasia. RNAscope technology is ideally suited to validate such long non-coding RNA genes, with a rapid two-week turnaround time to generate target-specific probe reagents. One recent study is explored in Example 2.

RNA as a Biomarker*

Biomarkers have long been used for diagnostic testing and the identification of potential therapeutic targets, growing exponentially in their importance within the era of personalized cancer medicine. A valuable biomarker is capable of uncovering a specific biological trait or measurable change directly associated with a physiological condition or disease status, and they therefore tend to be one of the three main functional components of the cell: DNA, RNA or protein.

As discussed, the routine detection of non-coding RNA presents a particular set of challenges, since they do not have protein counterparts for antibody-based detection. This is one of the reasons why the ability to detect RNA in cancer biopsy samples is so valuable, opening up the use of IncRNA as a biomarker. With several IncRNA candidates showing promising diagnostic and prognostic utility, RNA ISH allows for effective and reproducible in situ detection of these IncRNA biomarkers.

APPLICATION EXAMPLE 2

Realizing the potential of long non-coding RNA as a cancer biomarker

RESEARCHER:
Rohit Mehra, M.D. Clinical Assistant Professor of Pathology at Michigan Center for Translational Pathology

RESEARCH GOALS:
Long non-coding RNA (lncRNA) plays an important role in the pathogenesis of genitourinary cancers, especially prostate cancer.

The biology of lncRNAs is a relatively new field of study, and we do not yet have a full understanding of their roles in normal and disease states. Of the several lncRNAs that play an important role in prostate cancer, one in particular - SChLAP1 - may have clinical utility as a prognostic or diagnostic biomarker. For this, accessible methods for routine in situ lncRNA detection are vital.

METHODS:
Discovery – RNA-seq Comprehensively profiling the transcriptome of >100 prostate cancer tissues and cell lines found that ~20% of RNA transcripts in prostate cancer represent novel, uncharacterized lncRNA genes.

From this set, 121 candidate lncRNAs were nominated for further investigation.

Validation – RNA ISH One of these was re-named SChLAP1, and in the cohort studied, RNAscope technology effectively stratified patient outcomes by predicting more rapid biochemical recurrence, clinical progression to metastatic disease (defined by a positive bone scan) and prostate cancer-specific mortality.

Functional studies Our early studies, which involved the use of RNAscope technology, found that SChLAP1 antagonizes the genome-wide localization and regulatory functions of the SWI/SNF chromatin modifying complex. Furthermore, through antagonizing the tumor-suppressive functions of the SWI/SNF complex, it contributes at least in part to the development of lethal cancer. Further studies to uncover the molecular mechanisms by which SChLAP1 functions are ongoing.

Dr. Mehra comments: "This technology allows us to directly visualize gene expression in the target tissue of interest – for example, within the same sample we can tell whether gene overexpression occurs in benign prostate glands, high grade prostatic intraepithelial neoplasia (HGPIN – a pre-cancerous state) or prostate cancer."
Cancer immunotherapy

Immune checkpoints are often suppressed in many cancers, and reinstating the anti-tumor actions of the patient’s own immune system has demonstrated tremendous potential.

One major immune checkpoint of interest is the Programmed cell death-1 (PD-1) pathway (Figure 4). In cancer, interactions between the PD-1 cell surface receptor and its ligand PD-L1 function to suppress lymphocyte function within the tumor microenvironment. In fact, blocking of PD-L1 has demonstrated promising anti-cancer activity in several malignancies.33

PD-L1 also presents great potential as a biomarker, since several studies have found its expression is related to T-cell infiltration within the tumor microenvironment, and even disease outcome. In order to effectively utilize PD-L1 as a biomarker, it is critical to develop standardized assays. However, conventional approaches have proven inadequate in this case. Measurement of PD-L1 protein was found to be limited by the lack of standardized immunohistochemical methods and variable performance of antibodies, and mRNA analysis has instead presented a more viable alternative.25,34,35

A study by Du et al. (2014) looked at the relationship of tumor infiltrating lymphocytes and PD-L1 expression in meningioma, comparing IHC with RNA ISH using RNAscope technology.25 Applying the RNAscope assay for PD-L1 mRNA detection, the researchers confirmed assay specificity, observing signal only in tissues known to harbor PD-L1-expressing cells (e.g. tonsil and Hodgkin’s lymphoma) and not in several other normal human tissues. Notably, even though they observed a significant correlation between PD-L1 mRNA expression as detected by RNAscope in situ hybridization and PD-L1 protein expression as detected by IHC using two different anti-PD-L1 antibodies, the signal-to-noise ratio of the RNAscope probe was far better than that observed with IHC detection. Their data highlights the importance of testing PD-L1 mRNA alongside antibodies in clinical trial settings, and RNascope technology has proven to be instrumental in this, capturing correlations between clinical response and PD-L1 expression levels.

RNA from Bench to Bedside

Through a number of innovative research studies such as those discussed, it has become apparent that RNAscope ISH technology is a true driving force behind clinical applications of the future. The key to any technology is not only its capability, but its accessibility, and enabling researchers to integrate these tools into their existing workflows opens doors to a host of unique applications and novel discoveries. Easily slotting into any lab, the colorimetric or fluorescent readout from the RNascope assay is read under a standard microscope.

FIGURE 4: Detection of PD-L1 mRNA in various tissues. A) PD-L1 mRNA detection in human colon FFPE tissue and B) in human lung FFPE tissue using the RNascope Leica (BROWN) kit.

FIGURE 5: Accurate and reliable detection of HER2. HER2 expression in human breast cancer FFPE tissue using RNascope 2.0 HD Reagent Kit-Brown
Rapid development of companion diagnostics

Companion diagnostics are vital in guiding cancer therapeutics, and are central to the personalized medicine revolution.

A prime example of application of RNA ISH in this area is using HER2 status (Figure 5) in the management of breast carcinoma – guiding the use of the anti-HER2 agent Herceptin. This has in the past been limited by laboratory-to-laboratory variability, equivocal testing and intra-tumoral heterogeneity, and one comprehensive study in particular has found that quantitative in situ RNA measurement at the single-cell level may be able to address these issues, for broad application in companion diagnostics.

The retrospective study applied RNAscope ISH technology to quantify single-cell HER2 mRNA levels in 132 invasive breast carcinomas, and compared the results with those obtained by qPCR, FDA-approved FISH and IHC. Both RNAscope ISH and qPCR results were 97.3% concordant with FISH in cases where FISH results were unequivocal. However, RNAscope ISH results were superior to qPCR results in cases with intra-tumoral heterogeneity or equivocal FISH results. This novel assay may in the future enable ultimate HER2 status resolution as a reflex test for current testing algorithms.

Viral causes are also prominent among the variety of contributing factors to tumor development, and in these cases the routine detection of viral genes demands a specific, sensitive and accessible technique. Human Papillomavirus (HPV) is a causal agent in head and neck squamous cell carcinoma, and while evidence for transcriptionally active HPV oncogenes E6/E7 is regarded as the gold standard for presence of clinically relevant HPV infections, detection of E6/E7 mRNA can be challenging using conventional techniques. PCR amplification of HPV DNA is more sensitive, but it is less specific than DNA ISH, and RNA ISH with RNAscope has been found to provide both sensitive and specific detection (Figure 6), facilitating the move towards companion diagnostics in the future.

Further reinforcing this shift in clinical research strategies, in 2014 the FDA updated its guidelines for industry in developing companion diagnostics. It now recommends that the need for companion diagnostics is identified at an earlier stage in the drug development process, planning for co-development of the drug and companion diagnostic test. The ultimate goal of such guidance is to stimulate early collaborations that will result in faster access to promising new treatments for patients living with serious and life-threatening diseases.

Summary

The RNAscope in situ assay joins IHC and DNA FISH to complete the in situ tool set for cancer researchers to discover, develop and implement a new generation of tissue-based and cell-based techniques, integral to the promise of personalized medicine.

* For a more detailed review on RNA’s potential as a biomarker, see our previous whitepaper “The RNA Revolution: A Guide to RNA as a Biomarker and its Detection”
5. GENGODCE version 22 http://www.gencodegenes.org/stats/current.html
1. WHO Cancer http://www.who.int/mediacentre/factsheets/fs297/en/

References
Experience unprecedented molecular specificity and morphological context in one sensitive assay at acdbio.com/cancer