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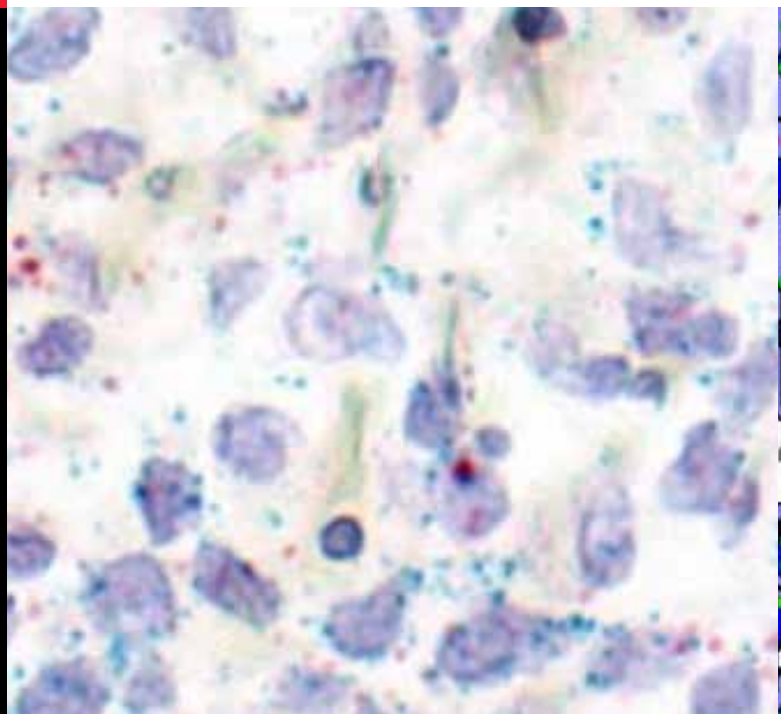

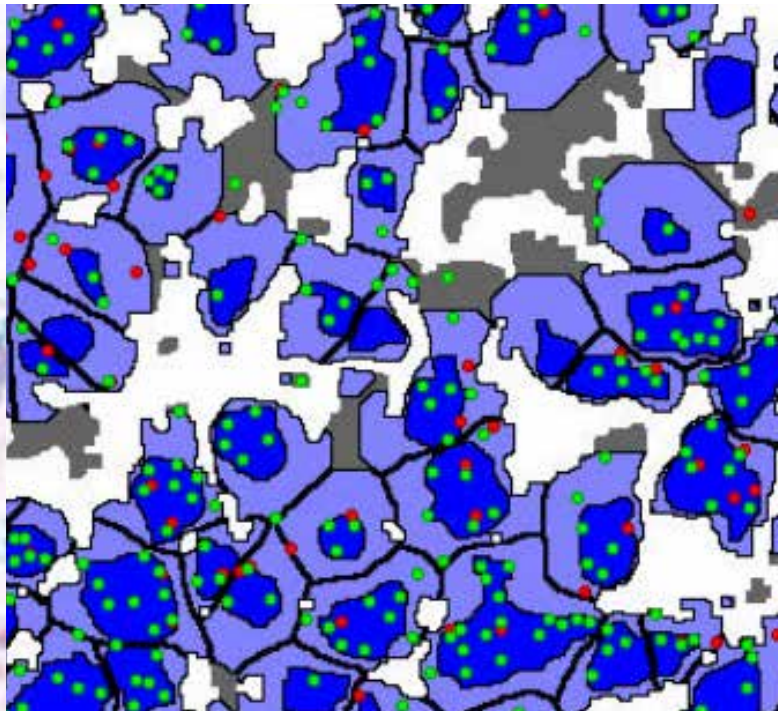

Comparison of Digital Image Analysis with Manual Reads for Chromogenic RNA ISH Slides

Catherine Conway, BSc, PhD
Sr. Product Manager, Pathology Image Analysis

Abstract

RNA ISH (Ribonucleic acid *in situ* hybridization) assays are an ever-expanding application due to the ability to evaluate molecular targets, while retaining tissue morphology. The rate limiting step in RNA ISH assays is the time consuming and error prone method of manually counting signal under a microscope. The Aperio RNA ISH Algorithm offers a reproducible, fast, and quantitative method of evaluating tissue samples that have been stained to detect RNA ISH signal. This single algorithm can be used on numerous tissue types for both single and dual-plex assays.

In this paper, we describe a validation study that was performed to verify the correlation between the Aperio RNA ISH Algorithm and the current gold standard method of manual interpretation. A total of 30 digital slides ranging in tissue source and assays types were scored manually by a scientist and the resulting data were correlated with scores obtained from the Aperio RNA ISH Algorithm. In both modalities, the number of cells, count of signal within the cell, and signal in all tissue were recorded. The high level of correlation between the two methods ($R^2 > 0.99$) confirms that automated image analysis can be used as a fast and reproducible alternative to the traditional methods of manual interpretation.

Introduction

RNA ISH is a rapidly growing method for the analysis of molecular targets within tissue samples. It enables identification of individual copies of targets, while maintaining tissue morphology, a feature that is lost in other methods such as PCR[1]. RNA ISH technology is being used in many areas of cancer research today[2-5]. In addition, there is the potential benefit of being able to combine RNA ISH assays with traditional IHC assays, thereby enabling users to visualize both RNA and protein status on a single slide[6]. Manual interpretation of RNA ISH signal is time-consuming and typically uses the semi-quantitative approach of ordinal scores (0, 1+, 2+, 3+), which often mask discrete cohorts that are readily identifiable when fully quantitative analysis is performed. Moreover, manual reads are subject to inter- and intra-observer variability, resulting in a lack of reproducibility and standardization in staining interpretation[7–10].

The Aperio RNA ISH Algorithm identifies and counts single or dual-plex chromogenic signals and distinguishes whether the expression is within the nuclear or cytoplasmic cellular compartments of FFPE (formalin-fixed paraffin-embedded) tissue. It has been optimized for use on brightfield digital slides from Aperio scanners or the Ariol system, at both 20x and 40x magnification. Individual signals or clusters of signals can be identified within the subcellular compartments, in uncategorized tissue (i.e. tissue not identified as a nucleus or cytoplasm) or across all tissue within the sample. The resulting data provides quantitative numerical counts of cells, signals and clusters. In addition, a semi-quantitative score is also generated by the algorithm, RNA ISH Score (0, 1+, 2+, 3+, 4+). Herein, we describe the validation study that was performed to investigate if the Aperio RNA ISH Algorithm correlates with the current gold standard of manual interpretation.

Methods & Materials

A total of 30 FFPE whole tissue sections were gathered from human and animal sources (kidney, liver, prostate, breast, small intestine, head and neck, ovarian cell lines, colon, placenta, skin, and rat kidney). Tissue sections were stained with single-plex RNA ISH probes (Red, Brown or Green chromogens) or dual-plex (Red/Green, Brown/Green chromogens) either manually or on the BOND RX IHC/ISH stainer. Glass slides were digitized at 20x or 40x on an Aperio AT2, Aperio CS2, Aperio VERSA scanner, or the Ariol system (Fig 1).

Digital slides were viewed in Aperio ImageScope v12.3, and 66 areas were annotated as regions of interest for analysis. All annotations were selected by an experienced scientist familiar with Aperio ImageScope and RNA ISH staining. First, the number of cells, signal count within cells and signal count present in all tissue areas were evaluated during a manual review of the digital slides. Second, the Aperio RNA ISH Algorithm was used to automatically quantify RNA ISH signal. The algorithm was optimized by a second similarly-experienced scientist, to reduce chance of bias, and the results for each annotation were correlated with the manual interpretation data.

Figure 2 shows the user interface within Aperio ImageScope when tuning the algorithm. The user is able to optimize input parameters to determine which cell types and signals are included in the analysis.

Figure 1: Research instruments from Leica Biosystems used during the RNA ISH validation study. (A) BOND RX, (B) Aperio AT2, (C) Aperio CS2, (D) Aperio VERSA

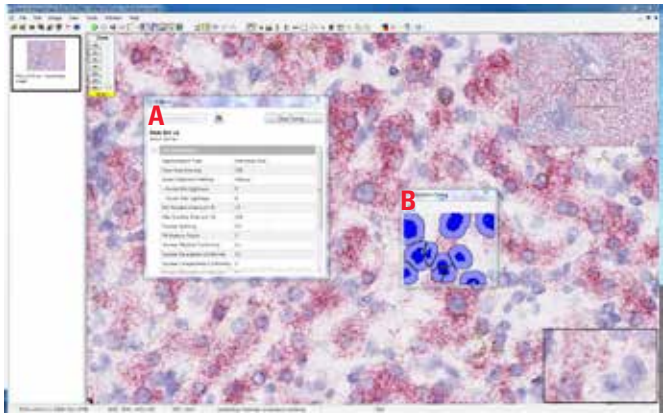

Figure 2: *User's view within Aperio ImageScope when tuning Aperio RNA ISH Algorithm to detect positive staining, where (A) shows the adjustable input parameters and (B) shows the live view tuning window, providing real-time indication of algorithm performance when input parameters are changed.*

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Results

The Aperio RNA ISH Algorithm generates both numerical and visual mark-up results to help the user understand what the algorithm is quantifying. Figure 3 illustrates the original image (A) and a mark-up (B) generated by the algorithm.

The Aperio RNA ISH Algorithm was compared with the expert manual review, to correlate the level of agreement between counts when evaluating the total cell number, the total signal number in cells, and total signal number in all tissue. Figure 4 illustrates the levels of correlation between the two methods of review. In all instances the correlation coefficient R^2 was greater than 0.99, demonstrating considerable agreement between the automated image analysis algorithm and expert manual review, across 66 annotations over 30 digital slides.

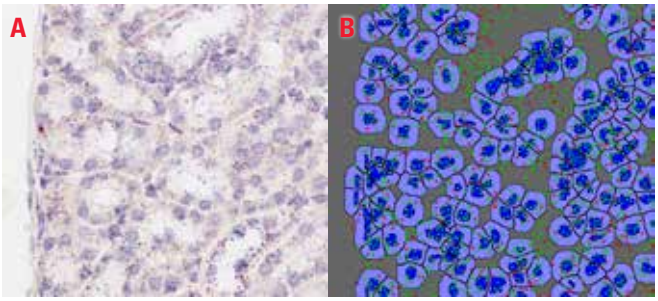

Figure 3: *Represents a dual-plex assay with Fast Red and green probes. The mark-ups (B) represents nuclear counterstain (blue), cytoplasm (light blue), background tissue (grey), Fast Red Signal (red), and green signal (green).*

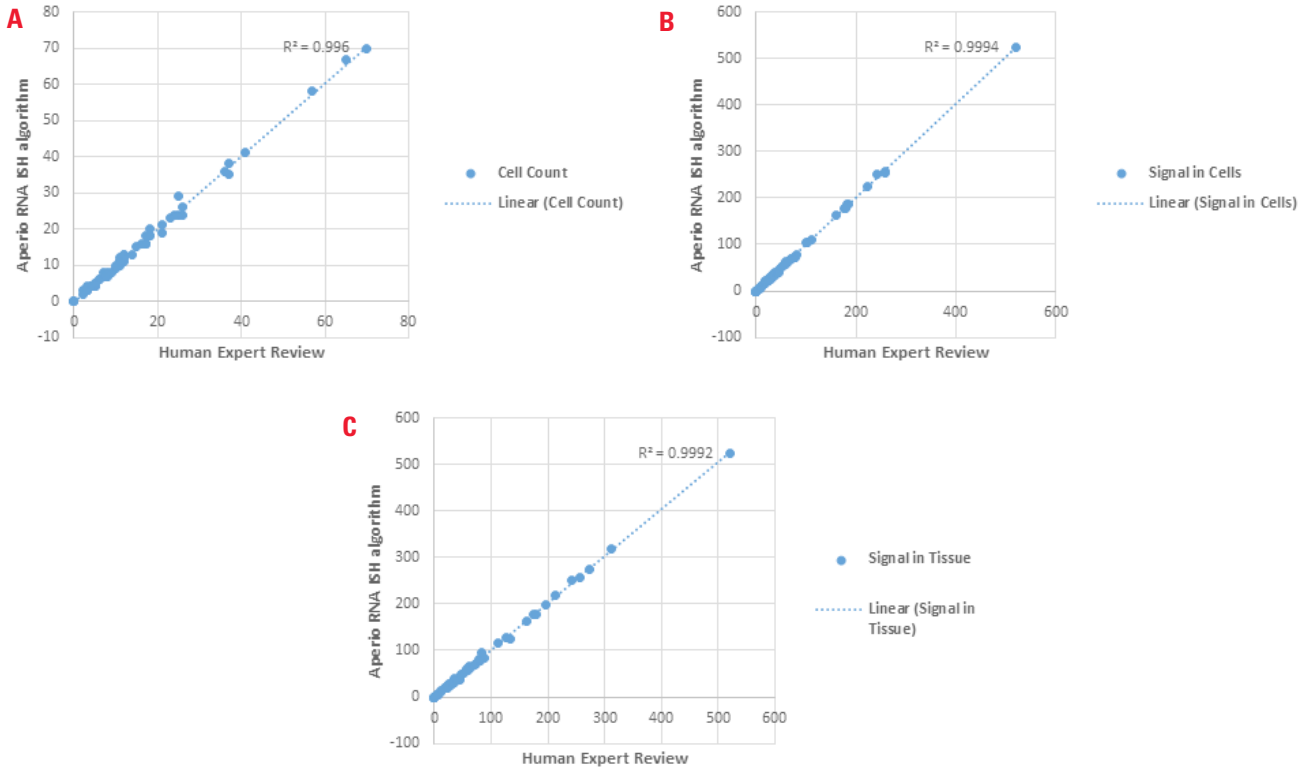

Figure 4 A,B,C: *Correlation between the human expert review and the automated image analysis algorithm when counting the number of cells present (A), signals within cells (B), signals within tissue areas (C)*

Figure 5 illustrates the application of the algorithm within a strongly stained section of tissue. Such tissue is not easily quantifiable when performing a manual review due to the presence of overlapping signals and signal clusters.

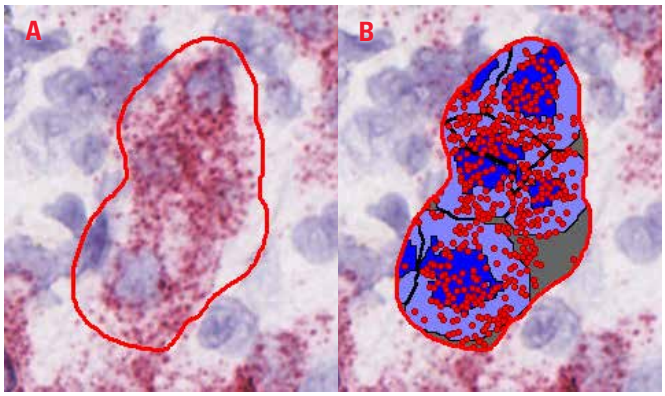

Figure 5: *Automated image analysis can easily identify cells and identify signal, which is not easy to enumerate by the human eye (B).*

Discussion

The trained human eye is typically superior to automated image analysis when identifying patterns within tissue sections. However, when enumerating dots of signal, image analysis offers equivalent scoring to human review, with the additional benefit of being truly quantitative and reproducible. The user of the Aperio RNA ISH Algorithm retains control by tuning the algorithm to suit each tissue type and RNA ISH probe(s) in use, from nuclear segmentation, to depth of cytoplasm drawn around each nucleus, to independent thresholding of each signal, to independent cluster analysis of each probe, and even the final scoring scale for reporting results.

When manually scoring RNA ISH staining there are a number of factors that make the review particularly challenging. With RNA ISH, reviewers must count the number of "dots" present, thus enabling this assay to be truly quantitative. However, in strongly positive samples it is not always possible to count each individual dot, so typically reviewers revert back to the semi-quantitative scale of 0, 1+, 2+, 3+. Multiple scoring systems can exist for a single assay, which can be confusing and hard to correlate across different cohorts or reviewers. Signal that is considered positive for RNA ISH has a very wide range of acceptance criteria, namely color, shape, size, and presence of clusters. The range of color of positive signal varies greatly, due both to the chromogen used, and the fact that the focal plane of digitization results from sectioning a 3D structure and creating a 2D glass slide. Therefore, users must try and impose a standard of not only what color represents a positive RNA ISH signal but also what intensity and size. Such variables make consistent manual scoring extremely difficult. Clusters of signal are also recorded by estimating the number of signals within a cluster, which is a very subjective method of evaluation. With a large number of variables, there are numerous opportunities for human error and inter- or intra-observer variability.

During this validation, extreme caution was applied when performing the manual review. As a result, the review was time consuming, taking a single reviewer approximately 40 hours to perform manual analysis across the 66 annotations. Once the algorithm has been tuned for the probe(s) and tissues, the same areas could be assessed in 1 hour when performing the automated image analysis using the Aperio RNA ISH Algorithm. In addition, the algorithm can be deployed within Aperio eSlide Manager software, enabling users to remotely access images and perform high-throughput server-side batch analysis, which greatly reduces turnaround time when evaluating tissue sections. In this study, due to the volume of slides, the analysis was performed locally on a workstation PC using Aperio Image Analysis Workstation software.

Conclusion

The level of correlation between results generated by the algorithm and human review demonstrates that once trained correctly, the algorithm can be used as a viable method of interpreting RNA ISH signal. When the algorithm is used as part of a digital pathology workflow, the scientist or pathologist should identify the regions of interest for analysis. Automated signal counting and classification are performed by the algorithm and results can be verified by the reviewer. Utilizing image analysis increases throughput, removes subjectivity, and provides a consistent and reproducible method of RNA ISH interpretation, which is not possible when performing traditional manual review with a microscope.

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