Aperio RNA ISH Algorithm Validation*

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ABSTRACT
RNA ISH (Ribonucleic acid in situ hybridization) assays are an ever-expanding application due to the ability to evaluate molecular targets with the added benefit of retaining tissue morphology. The rate limiting step in RNA ISH assays is the time consuming and error prone method of manually counting signal when reviewing under a microscope. The Aperio RNA ISH algorithm offers a reproducible, fast, and quantitative method of evaluating tissue samples which 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.

Within this paper, we describe a validation study, which was performed in order 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 (> R² 0.99) confirm 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, whilst maintaining tissue morphology, a feature which is lost in other methods such as PCR. RNA ISH technology is being used in many areas of cancer research today. In addition, there is the potential benefit of being able to combine RNA ISH assays with traditional IHC assays, thereby enabling users to be able to visualize both RNA and protein status on a single slide.

Manual interpretation of RNA ISH signal is time-consuming and typically reverts to the semi-qualitative approach of ordinal scores (0, 1+, 2+, 3+), which often mask discrete cohorts that are readily identifiable when quantitative analysis is performed. Moreover, manual reads are subject to inter- and intra-observer variability, resulting in a lack of reproducibility and standardization in formalin fixed paraffin embedded tissue staining interpretation.

The Aperio RNA ISH algorithm identifies and counts single or dual-plex chromogenic signals and will distinguish whether the expression is within the nuclei or cytoplasmic cellular compartments of FFPE tissue. It has been optimized for usage on brightfield digital slides from Aperio scanners or the Ariol System, at both 20x and 40x resolution. Individual signals or clusters of signals can be identified within the subcellular compartments, in uncategorized tissue (i.e. tissue not identified as a nuclei 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 which was performed in order to ensure 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/Brown, 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.

Digital slides were then viewed within 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. 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. The Aperio RNA ISH algorithm was used to automatically quantify RNA ISH signal. The algorithm was optimized by a second scientist and the results for each annotation were correlated with the manual interpretation data. Figure 1 shows the 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 within the analysis.

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 2 illustrates the original image (A) and a mark-up (B) generated by the algorithm. Figure 2 represents a dual-plex assay with fast red and green chromogens. The mark-ups represent nuclear counterstain (blue), cytoplasm (light blue), background tissue (grey) and Fast Red signal (red) and green signal (green).
The Aperio RNA ISH algorithm was compared with the expert manual review, by correlating the level of agreement between counts when evaluating the total cell number, the total signal number in cells and in all tissue. Figure 3 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 a considerable amount of agreement between the automated image analysis algorithm and the expert manual review across 66 annotations over 30 digital slides.

Figure 4 illustrates the application of the algorithm within a strongly stained section of tissue. Such tissue is not easily quantifiable when performing a manual review.

**DISCUSSION**

The human eye is 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. When manually scoring RNA ISH staining there are a number of factors which make the review particularly challenging, even more so than when evaluating more traditional assays such as IHC or FISH. With RNA ISH, reviewers must count the number of “dots” present, thus enabling this assay to be truly quantitative. However, within 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+. Therefore, multiple scoring systems can exist for a single assay, which is often confusing and hard to correlate across different cohorts or reviewers. Signal which 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, this is due to both the chromogen used but more likely due to the focal plane of digitization resulting 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 such a number of variables there are numerous opportunity for human error and inter- and intra-observer variability.
During this validation, extreme caution was applied when performing the manual review. As a result, the review was very time consuming and took a single reviewer approximately one week to perform manual analysis across the 66 annotations. Once trained, the same areas could be assessed within 1 hour when performing the automated image analysis using the Aperio RNA ISH algorithm. In addition, this algorithm can be deployed within Aperio eSlide Manager software, thereby enabling users to remotely access images and perform high-throughput server-side batch analysis, which greatly improves turnaround time when evaluating tissue sections. However, due to the volume of slides, the analysis was performed locally on a user’s computer using Aperio Image Analysis Workstation.

**CONCLUSION**

The level of correlation between the algorithm and the human review, demonstrates that once trained correctly, the algorithm could be used as a viable method of interpreting RNA ISH signal. When the algorithm is used as part of a digital pathology workflow, ideally the scientist or pathologist would identify the regions of interest for analysis. Automated signal counting and classification would be performed by the algorithm and all results could 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 simply not possible when performing traditional manual review utilizing a microscope.

**REFERENCES**

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