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ORIGINAL ARTICLE

Quantitative RNA assessment and long-term stability in the FFPE tumor samples using Digital Spatial Profiler

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Background: Long-term storage of tissue slides has been reported to induce reduced biomarker (e.g. proteins and messenger RNA) detection. This study aimed to evaluate the impact of long-term storage time (0, 16, 24 and 36 weeks) and treatment conditions (non-paraffin and paraffin dipping) at 4°C on RNA quality in formalin-fixed and paraffin-embedded (FFPE) tissue sections.

Materials and methods: NanoString GeoMx Digital Spatial Profiling (DSP), a novel platform that allows spatial profiling, was used to profile RNA in human bladder cancer FFPE tissue sections.

Results: We observed excellent consistency of quantitative DSP RNA counts of all targets between two different treatment conditions ($R > 0.97$, Pearson correlation) at each time point and among all four different storage time points ($R > 0.96$, Pearson correlation) within each treatment condition. No significant difference was observed in the percentage of target genes with sufficient signal across two different treatment conditions at any time point (0 week, $P = 0.96$; 16 weeks, $P = 0.76$; 24 weeks, $P = 0.96$; 36 weeks, $P = 0.76$, Kolmogorov—Smirnov test) and across all four different storage time points ($P > 0.05$, Kolmogorov—Smirnov test) in either treatment condition.

Conclusion: Although both treatment conditions provided similar results in terms of count reproducibility and signal preservation, we recommend paraffin dipping to generate reproducible RNA results and optimize sample storage. Technology behind the NanoString GeoMx DSP platform shows a robust and reproducible RNA signal from multiple targets in the FFPE tissue sections stored at 4°C for at least up to 36 weeks.

Key words: GeoMx Digital Spatial Profiler, formalin-fixed paraffin-embedded slides, long-term stability, RNA signal preservation

INTRODUCTION

Formalin-fixed and paraffin-embedded (FFPE) tissue samples stored after initial diagnostic testing in large medical archives or unique biobanks are widely used for both research and clinical purposes. Formaldehyde fixation of tissues is the standard choice for tissue preservation since late 19th century. Besides excellent tissue morphology preservation, formaldehyde fixation results in the preservation of cellular content within proteins, and between proteins and nucleic acids by crosslinking and chemical modification.[1] This approach allows long-term storage of tissue samples within a paraffin block at ambient room temperature.[2,3]

Many retrospective studies including prognostic and/or predictive biomarker evaluation in oncology research studies rely on using archival patient tissue blocks of various ages with long-term follow-up data. Although stability for protein antigens and DNA in intact tissue blocks has been reported, it is known that storage of tissue sections on slides experiences a gradual loss of these biomarkers.[2,4-7] A number of studies with inconsistent results have been reported for antigen stability on stored slides, where some show that antigenicity is lost after 1 week of storage, and others report that this happens only after storage for several months or even years.[7,8] This loss of antigenicity is likely the result of multiple factors including storage environment, temperature and time.

In the past, RNA preservation has been questioned in both aged tissue blocks and stored tissue sections for a defined time, which leads to biased results and inappropriate interpretations of RNA expression profiles in FFPE samples. This has been explored for multiple techniques including RT-PCR,[9] capillary electrophoresis, microarrays,[10] RNAseq[11] and RNA *in situ* hybridization (ISH).[12]

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Table 1. Clinical–pathological characteristics of the cohort

Patient ID	Age, years	FFPE block age at sectioning	Sex	Surgery	Cancer	Diagnosis	Specimen
Blca1	53	8 years	Male	Resection	Bladder	Papillary urothelial carcinoma, high grade	FFPE
Blca2	62	5 years	Male	Resection	Bladder	Urothelial carcinoma, high grade	FFPE

FFPE, formalin-fixed and paraffin-embedded.

The aim of this long-term study was to systematically evaluate the impact of storage time and sample processing conditions on FFPE tissue sections with the GeoMx Digital Spatial Profiling (DSP) system (NanoString, Seattle, WA), a novel platform that allows spatial RNA profiling. This platform allows simultaneous probe-based detection of multiple RNA transcripts from single FFPE tissue section in a quantitative and spatially resolved manner.[13] For this purpose, FFPE tissue blocks (human bladder cancer) were acquired commercially and processed to obtain consecutive sections. Unstained sections were divided into two batches, one for storage without any coating and one for storage with paraffin wax coating. All unstained slides were then stored at 4°C for 0, 16, 24 and 36 weeks and further examined for RNA transcript stability.

MATERIALS AND METHODS

Samples

Two bladder cancer FFPE blocks <10 years of age, from two different patients, were commercially acquired (Table 1).

Digital Spatial Profiling

Briefly, sections were deparaffinized at 60°C for 60 min and then subjected to antigen retrieval with Tris-EDTA, pH9 buffer (BOND™ Epitope Retrieval 2, AR9640, Leica Biosystems, Wetzlar, Germany) at heat-induced epitope retrieval for 15 min, followed by enzyme retrieval with 1 μg/ml proteinase K (AM2546, Ambion, Austin, TX) at 37°C for 15 min on a Leica Bond Rx system (Leica Biosystems). Next, tissues were hybridized at 37°C overnight with the GeoMx™ Immune pathway panel human RNA core of 84 unique photocleavable oligonucleotide-labeled DNA probes targeting immuno-oncology RNA targets (NanoString). Subsequently, tissues were incubated with blocking solution (Buffer W, NanoString) for 30 min, followed by staining with a visualization cocktail of nuclear stain (SYTO 13, NanoString) and two fluorescent-labeled antibodies to detect tumor cells [pan-cytokeratin (CK)] and all immune cells (CD45, NanoString) for 1 h at room temperature in a humidity chamber. Once the staining was completed, slides were loaded on to GeoMx DSP instrument, where they were scanned to produce a digital fluorescent image of the section. Individual regions of interest (ROIs) were then created with a maximum of 400 μm diameter and two molecularly defined tissue compartments were generated by fluorescent visual co-localization: tumor (pan-CK+) and tumor microenvironment (TME) compartment (CD45+). Once each ROI was compartmentalized, UV light was directed on to the specific compartments (tumor and TME), releasing photocleaved oligonucleotides which are collected via microcapillary aspiration and transferred into a 96-well plate. Oligonucleotides were then hybridized to four-color, six-spot optical barcodes and digitally counted in the nCounter system (NanoString) according to the manufacturer's instructions. Using the GeoMx interface software (NanoString), digitally counted barcodes corresponding to RNA probes were normalized with internal spike-in controls (ERCCs) to account for system hybridization variation. The ERCC normalized counts were then further normalized to the geomean of the four housekeeping genes (*OAZ1*, *POLR2A*, *RAB7A* and *SDHA*).

Statistical analysis

All datasets were analyzed and plotted using R software (version 4.0.2) and relevant R libraries. Normalized $\log_2$ counts were used for the statistical analyses. For analyzing the expression correlation, the Pearson correlation coefficient (*R* coefficient) was reported for paired groups; $R > 0$ indicates a positive correlation, while $R < 0$ suggests a negative correlation. The expression difference between two groups was determined using the two-sided Kolmogorov–Smirnov test, while the one-way analysis of variance test was utilized for comparison across multiple groups. Normalized $\log_2$ counts were further zero-centralized for generating the heatmap. The Euclidian method was used to calculate the individual distance, and the average hierarchical clustering method was used to determine the expression clusters.

RESULTS

The two commercially acquired human FFPE tissue blocks (bladder cancer) were processed to obtain 11 consecutive tissue sections each. The first section was hematoxylin–eosin stained and reviewed by trained pathologists to confirm tumor tissue present in the consecutive unstained sections (Supplementary Figure S1, available at https://doi.org/10.1016/j.iotech.2021.100069). Sections 8 and 9 were folded for one sample and excluded from this study for both FFPE blocks. The remaining eight unstained sections were divided into two batches, one for storage without paraffin dipping (sections 2, 4, 6 and 10) and one for storage with paraffin dipping (sections 3, 5, 7 and 11). All unstained slides were then stored in a low humidity environment with a desiccator at 4°C for 0, 16, 24 and 36 weeks, thus rendering two experimental settings. At each time point, four FFPE sections, paired consecutive non-paraffin- and paraffin-dipped slides from both patient specimens, were evaluated for RNA stability using the GeoMx DSP system

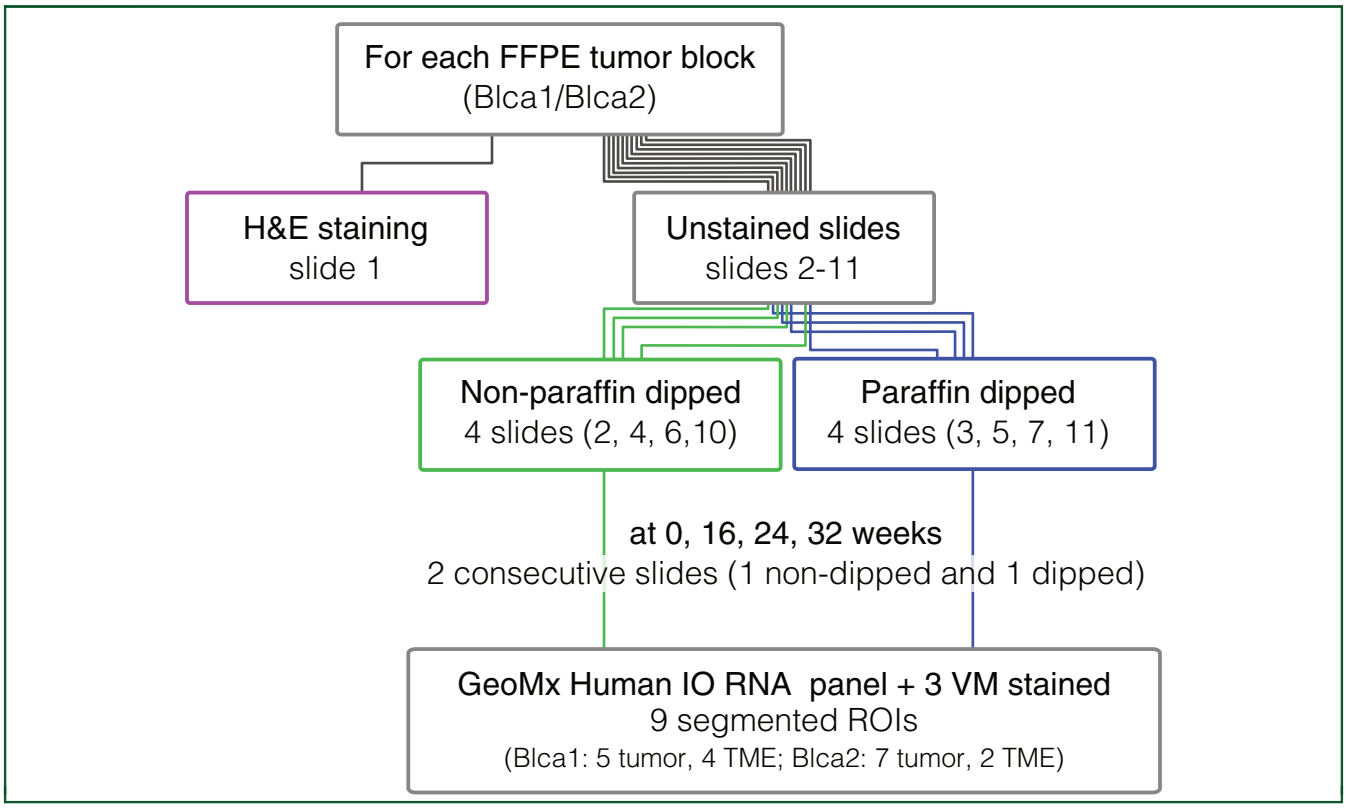

Figure 1. Study plan to evaluate GeoMx RNA count stability over time.
FFPE, formalin-fixed and paraffin-embedded; H&E, hematoxylin—eosin; IO, immuno-oncology; ROIs, regions of interest; TME, tumor microenvironment; VM, visualization marker.

(Figure 1). For each specimen, nine similar spatially segmented ROIs (either tumor or TME) were selected across both sample processing types and time points and profiled for 84 RNA targets (Figure 2). Unsupervised hierarchical clustering of all these data points revealed that tumor and TME signals were mostly separated, which was to be expected based on prior internal work (unpublished data). The two samples also showed certain differences but no obvious

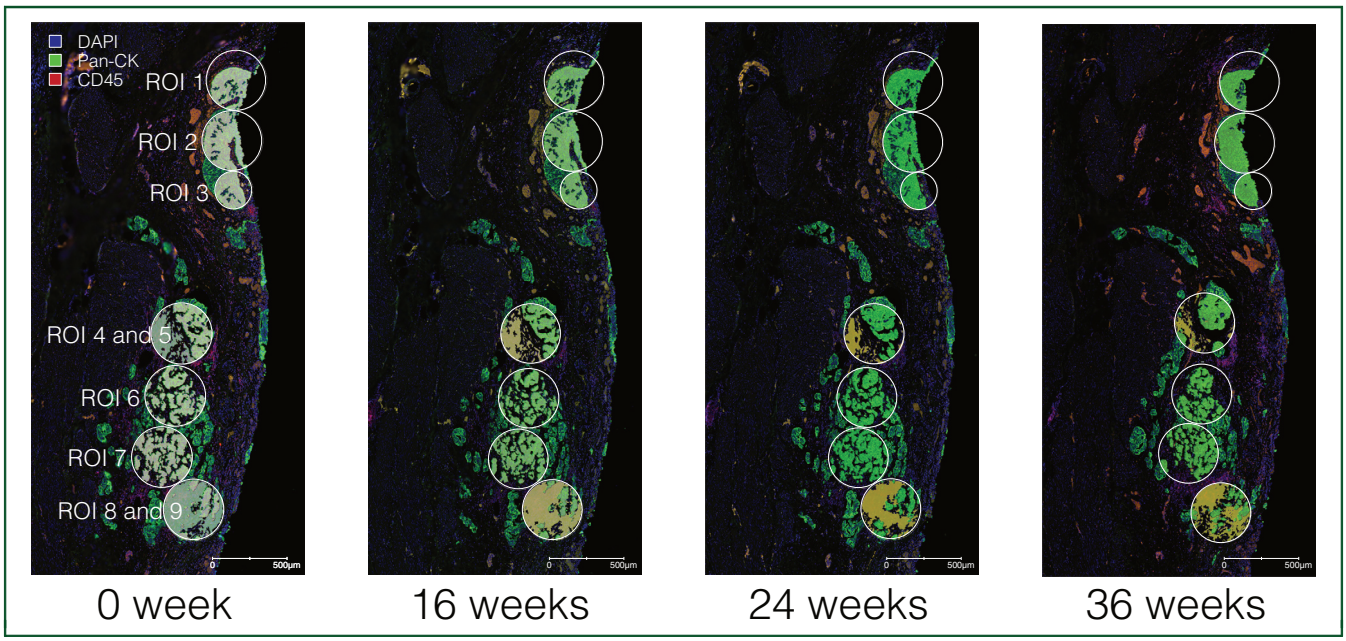

Figure 2. ROI selection strategy.
Representative DSP images with segmented ROIs (ROIs 5 and 9 are TME and the rest are tumor ROIs) from Blca2 paraffin-dipped slide for all the time points. Spatially identical ROIs were selected irrespective of treatment and time point for each patient.
CK, cytokeratin; DSP, Digital Spatial Profiling; ROI, region of interest; TME, tumor microenvironment.

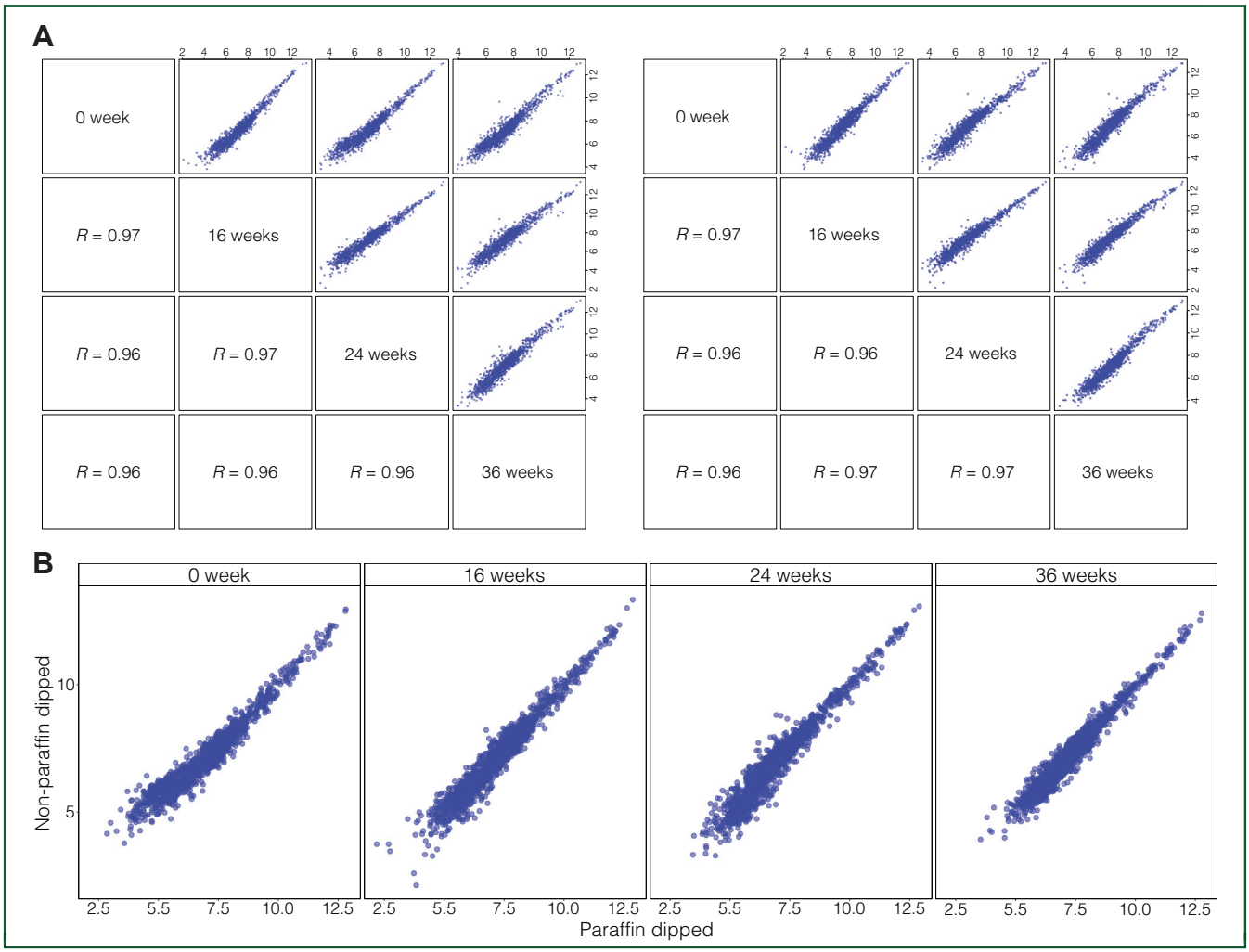

Figure 3. Reproducibility of Digital Spatial Profiling (DSP) RNA counts across time points and treatment conditions.
Correlation of all targets' $\log_2$ transformed expression data from all samples (A) across different time points for non-paraffin- and paraffin-dipped samples and (B) at each time point between non-paraffin- and paraffin-dipped samples.

clusters were found for different time points in either non-paraffin- or paraffin-dipped samples (Supplementary Figure S2, available at https://doi.org/10.1016/j.iotech.2021.100069).

To evaluate the consistency of quantitative DSP RNA counts, we first assessed RNA expression of all targets from both bladder cancer samples between different time points and observed high correlation ($R > 0.96$, Pearson correlation), in both non-paraffin-dipped and paraffin-dipped samples (Figure 3A). Then, we assessed RNA expression of all targets from both bladder cancer samples at each time point and again found excellent agreement ($R > 0.97$, Pearson correlation) between non-paraffin-dipped and paraffin-dipped samples (Figure 3B).

In order to assess the long-term stability of DSP RNA counts, we compared percentage of target genes with sufficient signal across different time points for non-paraffin- and paraffin-dipped samples. The number of target genes with sufficient signal did not show statistically significant difference between non-paraffin- and paraffin-dipped samples at any time point (0 week, $P = 0.96$; 16 weeks, $P = 0.76$; 24 weeks, $P = 0.96$; 36 weeks, $P = 0.76$, Kolmogorov—Smirnov test). Although no statistically significant difference was found in the number of target genes with sufficient signal between any two different time points ($P > 0.05$, Kolmogorov—Smirnov test) within non-paraffin- or paraffin-dipped samples, we observed that the number of target genes with sufficient signal was more consistent across different time points in paraffin-dipped samples (Figure 4). Furthermore, none of the genes when plotted individually showed continuous degradation between different time points in either non-paraffin- or paraffin-dipped samples (Supplementary Figure S3, available at https://doi.org/10.1016/j.iotech.2021.100069).

DISCUSSION

Genomics-based approaches are constantly evolving, such as RNAseq bulk expression profiling, providing ever-increasing insights into basic biology with the caveat that signals from rare cells can be drowned out and spatial context is missing. Single-cell RNA sequencing provides information from different cell clusters with the limitation that spatial context is still missing, and ISH of RNA gives

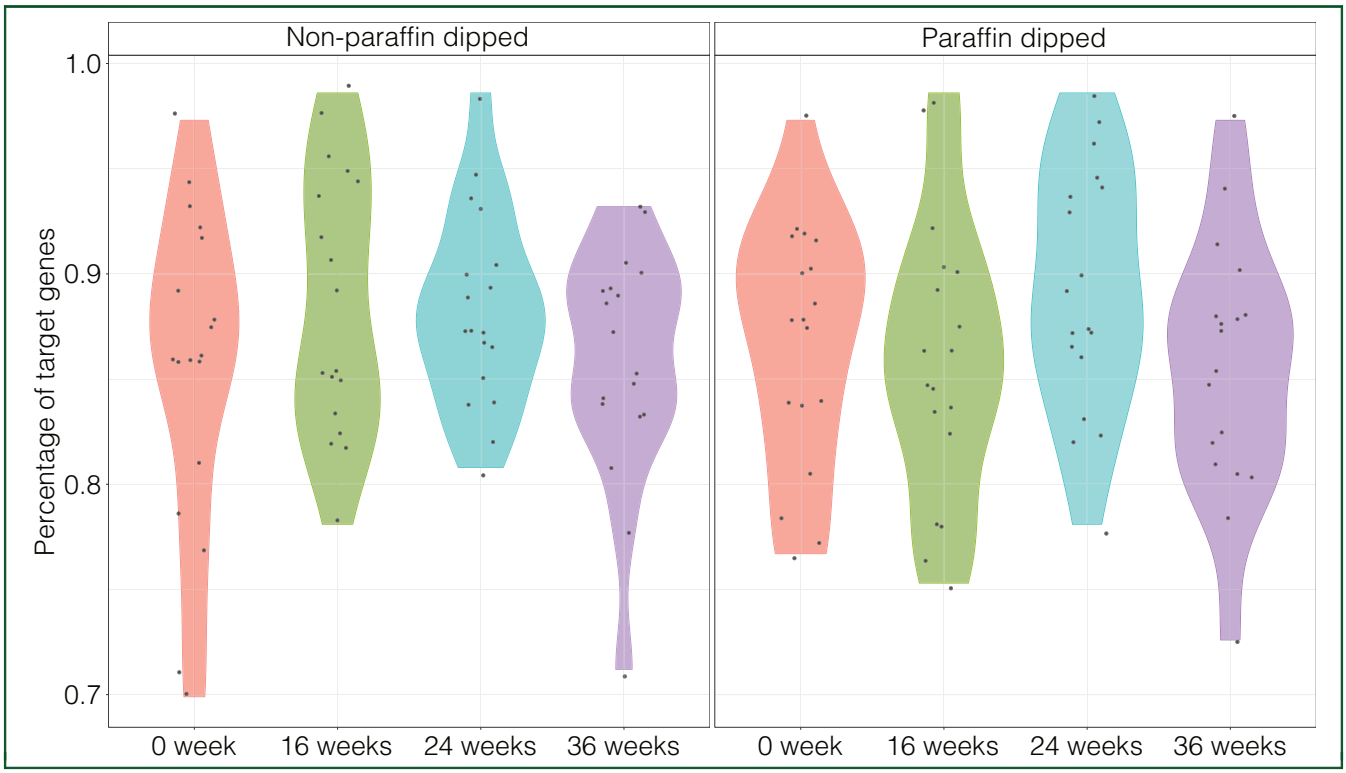

Figure 4. Consistency of genes with sufficient signal across time points and treatment conditions.
Comparison of percentage of target genes with sufficient signal across different time points for non-paraffin- and paraffin-dipped samples.

spatially resolved information but lacks quantitative information and is limited to a few markers. NanoString GeoMx DSP technology allows generation of quantitative, high-plex and spatially resolved RNA results. As this technology is very new, there are a number of unanswered technical and methodological questions. Biomarker studies rely on samples provided by patients. There are many limitations in terms of the type and quantity of samples that can be obtained in clinical studies: for example, fresh tissue is better than FFPE tissue but the latter is the standard sample type generally readily available from patients. Furthermore, a complete FFPE block is desired versus some slides, but these are precious samples and it is not always possible for the full block to be provided. Thus, we thought to understand the impact of cut-slide stability on NanoString GeoMx DSP technology RNA expression profiling.

Several studies have been conducted on RNA expression profiling from archived human FFPE blocks and on the effect of degraded or poor-quality RNA from FFPE blocks on transcript expression analyses.[14-18] It is well known that storing FFPE unstained slides at room temperature deteriorates protein immunoreactivity for many antigens when compared to storing tissues as FFPE blocks at room temperature.[4,5,7,19] Furthermore, storage of FFPE unstained slides at lower temperatures (4°C, −20°C and −80°C) has been shown to preserve antigenicity similar to tissues kept in FFPE blocks at room temperature.[20] To preserve antigenicity in such FFPE unstained slides, paraffin dipping has been recommended and their storage in nitrogen chamber prevents further tissue oxidation.[4,21] Thus, in our study, we have begun efforts to determine the impact of storage time and treatment conditions (non-paraffin and paraffin dipping) at lower temperature (4°C) on RNA stability in FFPE tissue sections using probe-based DSP technology. While NanoString GeoMx DSP is a novel platform, the technology behind the platform shows the kind of robustness and preservation of RNA signal from multiple targets up to 36 weeks irrespective of paraffin dipping.

The current study has to be interpreted in the context of its limitations. Firstly, it is a pilot study in which we conducted hypothesis testing on commercially acquired FFPE blocks from two different patients, as is sometimes done in research type work. To compensate for this, we selected multiple ROIs from two morphologically distinct compartments, tumor and TME, for each patient specimen across different treatment conditions and storage times. Furthermore, this study includes the usage of a single cancer indication for assessing the long-term stability of quantified RNA targets. As such, the data shown here must be considered as a proof of concept and will require confirmation in additional indications. However, in testing long-term RNA stability, this technology has significant advantage including tissue heterogeneity exclusion from an assay.

In conclusion, this study illustrates the potential of high-plex DSP to preserve long-term antigenicity of quantified transcripts in the FFPE tissue sections stored under lower temperature 4°C. Although non-paraffin- and paraffin-dipped FFPE slides provided similar results in terms of count reproducibility and signal preservation, when an FFPE tissue block cannot be provided, we recommend paraffin

dipping FFPE tissue slides to generate reproducible RNA results and maximize sample storage. Altogether this observation is particularly valuable in low resourced settings such as clinical testing where routine access to FFPE blocks is challenging.

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DISCLOSURE

BD and TC are employees of H3 Biomedicine. While conducting this study, SG was employed at H3 Biomedicine and at present is a Merck employee.

SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1016/j.iotech.2021.100069.

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