High Throughput Spatial-Omics Sample Processing Using the GeoMx® Digital Spatial Profiler

Katrina van Raay, Christine Kang, Margaret Hoang

FOR RESEARCH USE ONLY. Not for use in diagnostic procedures.
NanoString Technologies, Inc., Seattle, WA 98109
MK5851 | July 2023

nanoString®

High Throughput Sample Processing on DSP

Introduction

Spatial omics offers promising new insights into the study of human disease from archival formalin-fixed, paraffin-embedded (FFPE) tissue, such as the ability to determine drug mechanism of action, discover novel biomarkers, and profile the immune response. Yet an emerging unmet need in spatial omics is the standardized processing of patient samples from large cross-sectional or longitudinal cohort studies. To enable large-scale spatial omic studies with the GeoMx® Digital Spatial Profiler (DSP), we have simplified the workflow by enabling batch processing of slides to maximize the instrument run rate.

We tested two steps in the GeoMx slide preparation workflow that serve as practical stopping points for large-scale cohort studies. Following tissue sectioning or *in situ* hybridization with GeoMx assay probes and morphology markers, we stored the resulting slides for different amounts of time prior to runs on the DSP instrument and have shown high concordance for gene expression counts using the Human Whole Transcriptome Atlas (WTA) between samples that were treated with and without storage. Our findings impact the experimental design of high throughput GeoMx workflows, supporting the generation of large spatial omic datasets from FFPE archival tissue that can be used to better understand mechanisms of health and disease.

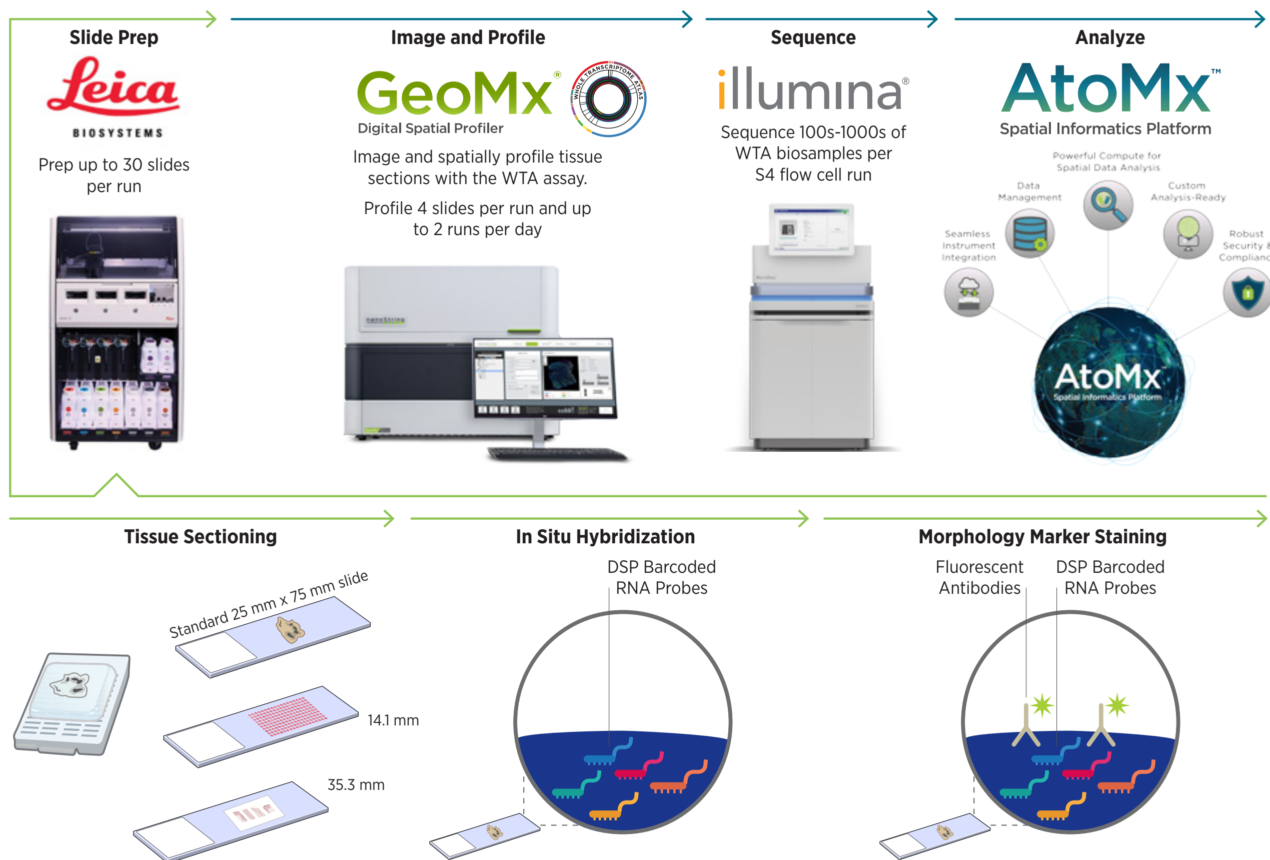


Figure 1. Automated high-throughput, end-to-end solution for the NanoString GeoMx DSP platform. Top: Automation includes slide prep on the BOND RX Fully Automated Research Stainer from Leica Biosystems, spatial profiling on the GeoMx DSP instrument, high-throughput readout on production-scale sequencers like the Illumina NovaSeq 6000, and data analysis and collaboration on NanoString's cloud computing solution for spatial omics analysis, the AtoMx™ Spaital Informatics Platform. Bottom: Slide preparation steps include FFPE sectioning and mounting to ready the biospecimen slides, *in situ* hybridization to spatially profile whole transcriptome expression, and morphology marker staining to visualize the tissue architecture.

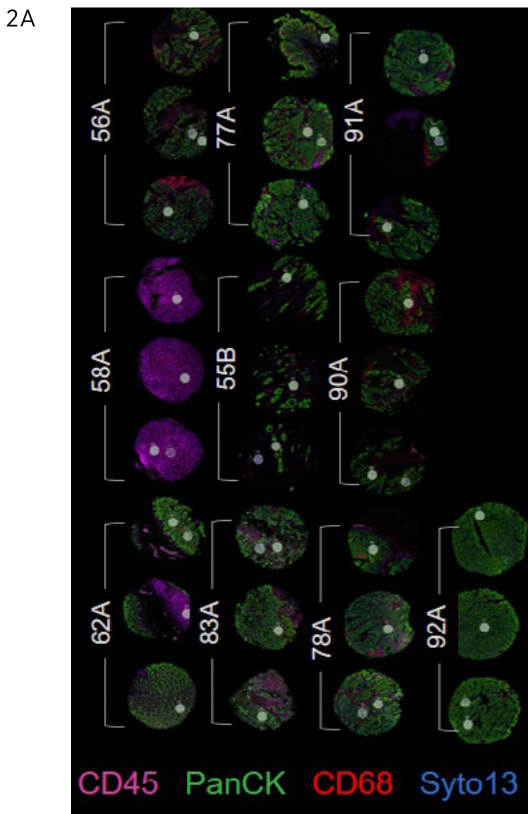
FOR RESEARCH USE ONLY. Not for use in diagnostic procedures.
nanoString®

Results

Long-term dry storage after FFPE tissue sectioning and mounting onto microscope slides

FFPE blocks help preserve and protect excised tissue, but increased oxidative exposure by tissue sectioning may enhance the rate of biomolecule degradation and loss. To test the effect of dry storage of FFPE tissue sections on GeoMx WTA assay performance, we first designed and purchased an FFPE tissue microarray (TMA) from a commercial vendor (Acepix). The TMA is composed of colorectal cancer (CRC) tissue from 10 patients with three cores represented per patient (Figure 2A). The original FFPE patient block ages ranged from 2 to 11 years and DV200 scores ranged from 69-90% (Figure 2B). The TMA block was chilled on ice for 20 mins and then sectioned on a microtome (Leica) into 5 µm thick sections. The resulting sections were then floated in a 42° C water bath to dewrinkle and mounted on a positively charged microscope slide (Superfrost Plus). Mounted slides were dried vertically at room temperature (RT) for 2 days. For the control without storage, two slide replicates were immediately processed as described in the GeoMx DSP Automated Slide Prep User Manual (MAN-10151), using the Semi-Automated Slide Preparation Protocol (FFPE) with the GeoMx Human WTA assay (GMX-RNA-NGS-HuWTA-4) and the GeoMx Solid Tumor TME Morphology Kit (GMX-RNA-MORPH-HST-12). Following slide preparation, standard protocols were followed as described in the GeoMx DSP Instrument User Manual (MAN-10152) and GeoMx DSP NGS Readout User Manual (MAN-10153).

2A



2B

Sample	Block Year	RIN	DV200
55B	2017	1.9	78
56A	2014	1.9	80
58A	2012	1.6	74
62A	2011	1.7	74
77A	2017	2.3	84
78A	2017	2.5	79
83A	2015	1.9	69
90	2020	2.0	80
91	2020	2.1	84
92	2020	2.1	90

2C

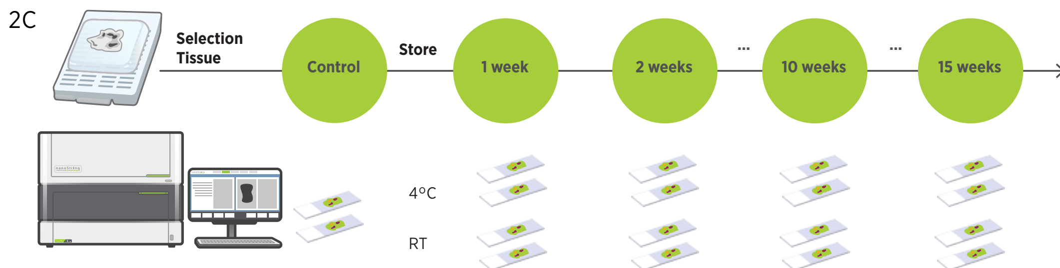


Figure 2. Experimental design to test the effect of dry storage conditions of FFPE tissue sections on the GeoMx WTA assay. A) Fluorescent image from the GeoMx DSP instrument of the FFPE tissue microarray (TMA) used in the section storage experiment. The TMA was composed of colorectal cancer (CRC) tissue from ten patients with three 2 mm diameter cores from each FFPE block. Morphology markers visualized the TMA using antibodies to CD45 (hematopoietic cells, pink), PanCK (epithelial cells, green), and CD68 (macrophages, red) along with Syto13 (DNA, blue). The 300 µm diameter regions of interest (ROIs) for GeoMx WTA profiling are shown on the tissue cores as translucent circles. B) FFPE block year with RNA Integrity (RIN) and DV200 scores (Agilent Tapestation) for each patient block used in the CRC TMA. C) Schematic of the experimental design to assess the effect of time since TMA sectioning on GeoMx WTA assay performance. Two replicate slides per temperature storage condition and time point were assessed compared to control.

For region of interest (ROI) selection strategy, one 300 µm diameter circle was placed over each core to capture > 100 nuclei of epithelial cells (PanCK+) and/or immune cells (CD45+, CD68+), avoiding blood vessels, necrotic tissue, and tissue processing artifacts. A total of 50 areas-of-illumination (AOIs) were collected, five from each patient that included three geometric circles and one circle segmented into two compartments, PanCK+ and PanCK- (see Figure 2A). The remaining slides were stored dry in slide holders with desiccator packets at either 4°C or RT. After 1, 2, 10, and 15 weeks of storage, two slides from each storage condition (4°C or RT) were prepared following the same protocol as the control (Figure 2C) including overlaying the same ROIs onto each core.

Despite the oxidative exposure of cut tissue sections through dry storage, WTA expression results show high concordance of Q3 normalized counts of matched AOIs between the no storage control and each storage timepoint at 4°C (Figure 3A, R = 0.90 ± 0.02, AVG ± SD) or RT (Figure 3A, R = 0.90± 0.02, AVG ± SD). Counts were highly concordant between slides stored at 4°C and RT (Figure 3B, R = 0.94 ± 0.01, AVG ± SD), suggesting that section storage temperature for at least up to 15 weeks was not a significant parameter. As reference, concordance between the two replicate slides of the control (R = 0.95 AVG) and within each timepoint (4°C at R = 0.94 ± 0.01, AVG ± SD; RT at R =0.95 ± 0.005, AVG ± SD) was also high as expected (data not shown). Median number of genes detected in the no storage control (median = 6214 and range 6135 - 6403) was similar over time in each storage condition (4°C with median = 9024 and range 7999 – 10374; RT with median = 8776 and range 7989 – 9294) (Figure 3C). Taken together, our results demostrate that 5 µm thick cut and mounted FFPE tissue sections can be stored dry with a dessicator pack at either 4°C or RT for at least 15 weeks with comparable WTA assay performance.

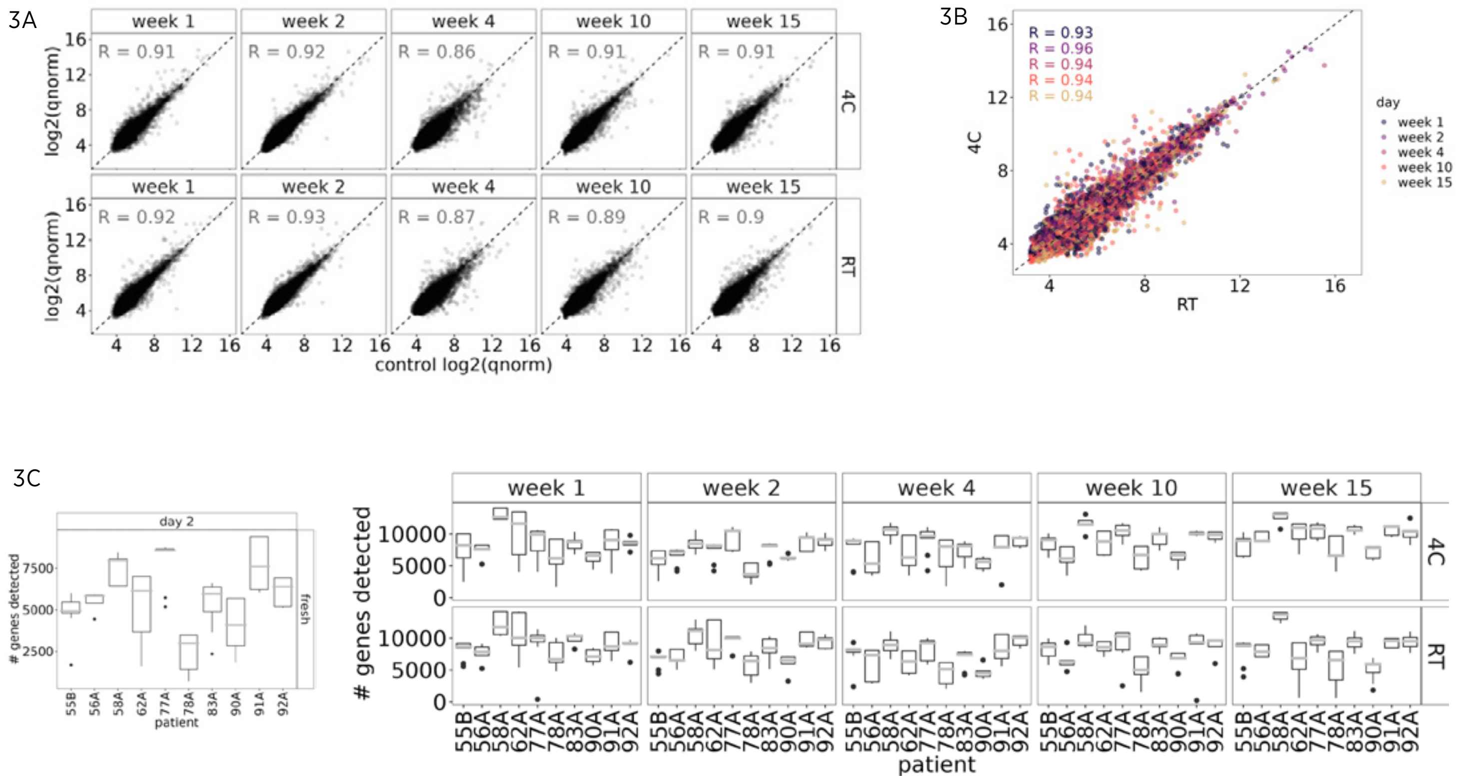


Figure 3. Long-term dry storage of FFPE tissue sections does not impact GeoMx WTA performance. A) Scatter plots of WTA normalized counts comparing no section storage control counts (x-axis, Q3 normalized, log2 scale) versus with section storage counts (y-axis, Q3 normalized, log2 scale) at indicated timepoints stored at 4°C (top row) or room temperature (RT, bottom row). B) Scatter plot of WTA normalized counts comparing temperature of section storage within each timepoint with RT (x-axis, Q3 normalized, log2 scale) versus 4°C (y-axis, Q3 normalized, log2 scale). C) Box plots indicating number of WTA genes detected (out of 18K+ total genes) in each AOI (n=5) per CRC patient (x-axis). No section storage control (left) and with section storage with indicated storage time for 4°C and RT (right). WTA genes detection based on target gene counts that are confidently greater than non-specific background and measured as limit of quantitation (LOQ) = GeoMean(NegProbes)*GeoSD(NegProbes)^2.0.

FOR RESEARCH USE ONLY. Not for use in diagnostic procedures.

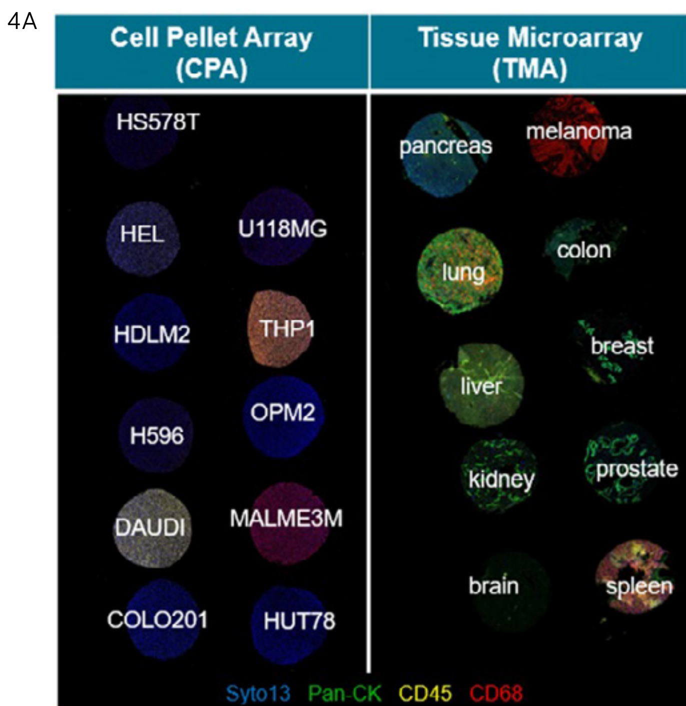
nanoString

Long-term wet storage after *in situ* hybridization of WTA probes in FFPE tissue sections

To test the effect of batch slide processing with storage, 28 slides were processed as described in the GeoMx DSP Automated Slide Prep User Manual (MAN-10151) using the Semi-Automated Slide Preparation Protocol (FFPE) with the GeoMx Human WTA assay (GMX-RNA-NGS-HuWTA-4) and GeoMx Solid Tumor TME Morphology Kit (GMX-RNA-MORPH-HST-12). For biosamples, we used 14 FFPE near-serial sections of a tissue microarray (TMA) custom designed with 10 different human tissue types (Acepix) and 14 FFPE serial sections of a cell pellet array (CPA) with 11 different human cell lines (Acepix). Slides were hybridized with human WTA probes overnight and stained the following day with three morphology markers (PanCK, CD45, and CD68) and the nuclei stain Syto13. After batch preparation of the 28 slides, four of these slides (two CPA and two TMA) were immediately loaded onto the GeoMx instrument for ROI selection and collection to serve as the control workflow. The remaining 24 slides were stored in 2X SSC (Saline Sodium Citrate) at 4°C in light-sealed containers for 1 day, 2 days, 3 days, 1 week, 2 weeks, and 3 weeks before the GeoMx DSP instrument run (Figure 4B). For CPAs, two different sizes of geometric circles (50 µm and 200 µm diameter circles) were collected per cell line for a total of 22 AOIs per CPA. For TMAs, 1-4 AOIs of geometric circles or segmented compartments were collected per tissue type with a total of 22 AOIs per TMA, including PanCK+ and PanCK- segmented compartments in pancreas, lung, spleen, and breast tissues. Standard downstream protocols were followed as described in the GeoMx DSP Instrument User Manual (MAN-10152) and GeoMx DSP NGS Readout User Manual (MAN-10153).

Previous thought assumed that no stopping point existed after in situ hybridization of 18K+ WTA probes due to probe-target instability over time with the progressive loss of signal. However, the average melting temperature (Tm) of the human WTA probes are ~80°C and with storage at low temperatures (e.g., 4°C) and high salt conditions (e.g., 300 mM NaCl, 30 mM sodium citrate for 2X SSC buffer), probe-target complexes are predicted to remain stable. Indeed, our results show high concordance between WTA normalized counts in the control compared to all storage timepoints in the TMA (R =0.91 ± 0.01, AVG ± SD; see Figure 5A) and CPA (R = 0.95 ± 0, AVG ± SD; data not shown). For reference, the correlation between the two replicate slides of the control TMA at R = 0.94, AVG; CPA at R = 0.93, AVG) and replicates within each timpoint (TMA at R = 0.94 ± 0.01, AVG ± SD; CPA at R = 0.92 ± 0.01, AVG ± SD) was also high as expected (data not shown). We further assessed if the proposed trend existed with the number of genes detected decreasing through time of dry storage. Contrary to this hypothesis, we did not observe a decreasing trend of the median number of genes detected per AOI (n = 39-43 AOIs per timepoint, including the two replicate slides, with the exception of day 14 which only had

4A



4B

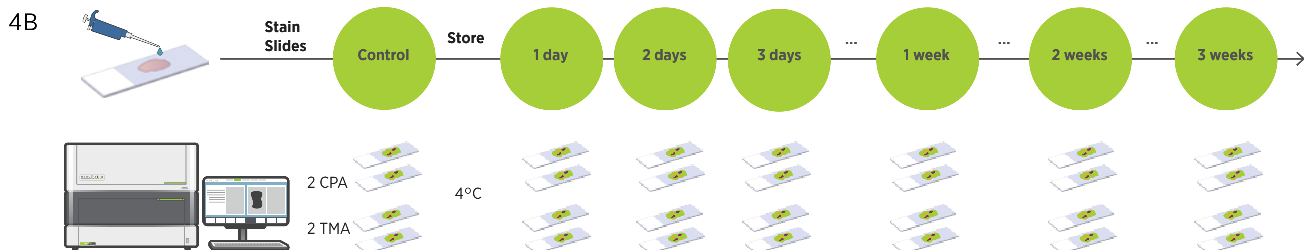


Figure 4. Experimental design to test the effect of wet storage conditions after in situ hybridization and morphology marker staining of FFPE samples on the GeoMx WTA assay. A) Fluorescent image from the GeoMx DSP instrument of the FFPE cell pellet array (CPA) and FFPE tissue microarray (TMA) used in the stained wet storage experiment. Morphology markers used to visualize the array were PanCK (epithelial cells, green), CD45 (hematopoietic cells, yellow), and CD68 (macrophages, red) along with Syto13 (DNA, blue). B) Schematic of study plan to assess batch slide processing and storage on GeoMx WTA assay performance. Control is the standard workflow with no storage. Timepoints indicate slide storage duration at 4°C in 2X SSC buffer after batch slide processing of WTA in situ hybridization and morphology marker staining. Four slides were assessed at each timepoint with two replicates of each sample type, CPA and TMA.

FOR RESEARCH USE ONLY. Not for use in diagnostic procedures.

one slide with 22 AOIs) through storage times of 1 day to 3 weeks within nine different human tissues on the TMA (Figure 5B) Note that the colon tissue on the TMA was excluded from this analysis due to tissue loss during slide processing. There were no significant differences in the number of genes detected in most comparisons between the no storage control and each storage timepoint for each tissue ($p > 0.05$ in 28 of 30 comparisons via unpaired t-tests, see Figure 5B). Consistent with the TMA, the CPA also showed a consistent number of genes detected in each of the 11 cell lines over the same storage time course [data not shown]. Our results demonstrate that FFPE tissue sections after in situ hybridization of human WTA probes can be stored in 2X SSC buffer at 4°C for up to three weeks without significant impact to WTA performance.

Long-term wet storage after morphology marker staining of FFPE tissue sections

Prior to loading the WTA hybridized FFPE slides onto the DSP instrument, there is a short incubation step with fluorescently labeled antibodies and a nuclei stain. Morphology markers allow for visualization of the tissue and are used to guide ROI selection, segmentation, and nuclei counting. Therefore, from the same aforementioned experiment, we asked how long-term wet storage affects morphology marker intensity.

Morphology markers for CD45 (immune cells), PanCK (epithelial cells), CD68 (macrophages), and Syto13 (nuclei) were used to visualize the CPA and TMA slides. Fluorescent signal remained visible for up to three weeks in storage (Figure 6A). Intensity of signal was evaluated using a custom ImageJ script to measure

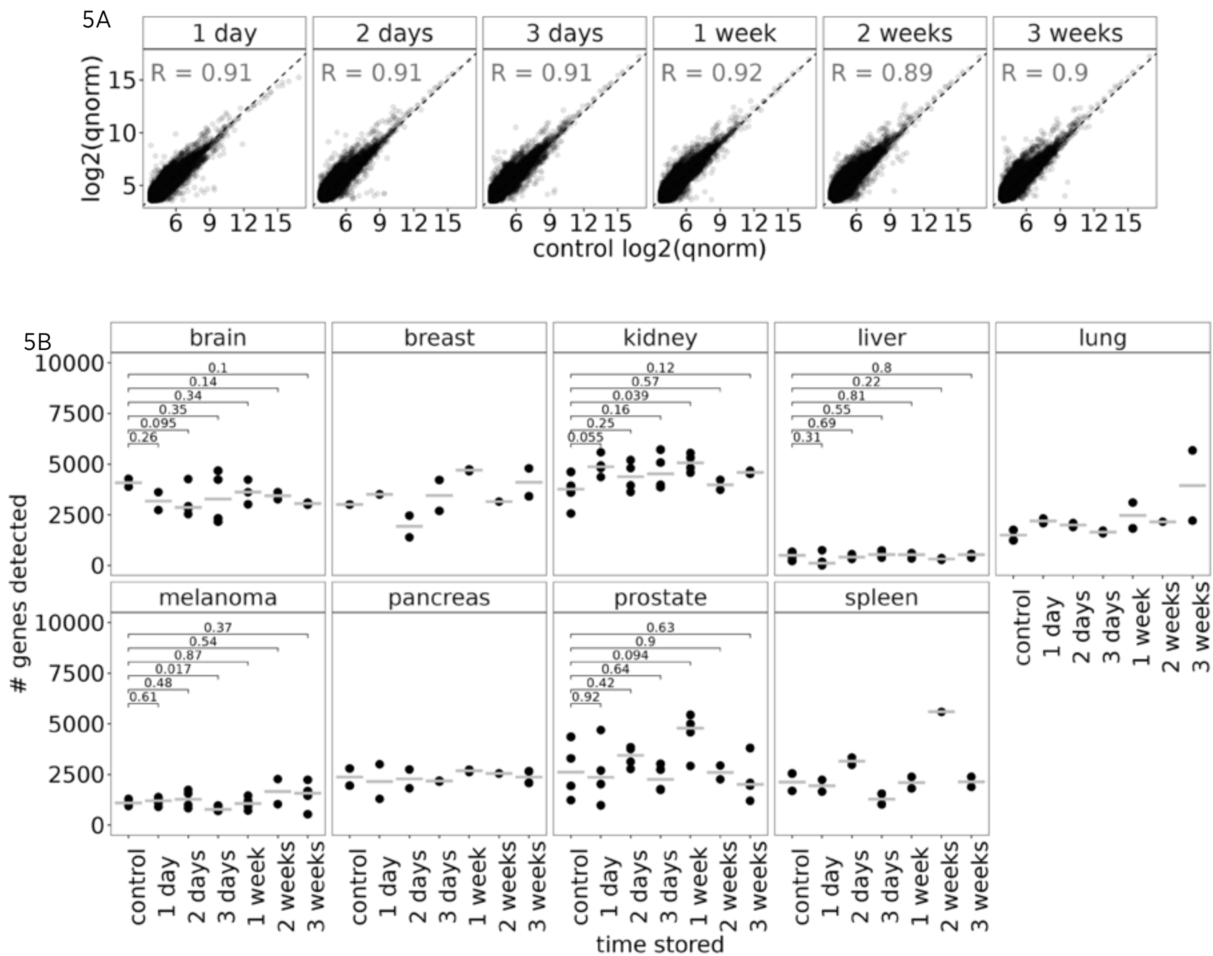


Figure 5. Long-term wet storage of in situ hybridized FFPE tissue sections does not impact GeoMx WTA performance. A) Scatter plots of WTA normalized counts between the no storage control (x-axis, Q3 normalized, log2 scale) and each storage timepoint (y-axis, Q3 normalized, log2 scale) in TMA slides. B) Dot plot of number of genes detected (y-axis) in each AOI (each dot) in nine human tissue types on the TMA over the wet storage timepoint (x-axis). Grey bar indicates median. P-values from unpaired t-tests between the control and each timepoint are shown for timepoints with at least 3 ROIs.

FOR RESEARCH USE ONLY. Not for use in diagnostic procedures.
nanoString

fluorescent signal in each ROI/AOI compared to matched background region (signal-to-noise ratio, SNR). Over the storage time course, fluorescence SNR remained stable for CD45, PanCK, and CD68 with an average SNR greater than three, our threshold for adequate segmentation (Figure 6B). PanCK segmentation was functional even after three weeks post-staining (Figure 6C). For Syto13, we observed a reduction after 2 days (see Figure 6B). Despite the Syto13 SNR decrease, nuclei counting was successfully performed on stained slides stored up to 3 weeks (Figure 6D). Results demonstrate that morphology markers remain functional when stored at 4°C in 2X SSC for at least three weeks. Because the morphology marker incubation step is short, researchers may opt to apply, or reapply, visualization reagents on the day of the DSP run without major impact to the workflow.

Spatially-resolved whole transcriptome expression profiles cluster by cell or tissue type

Lastly, we performed analyses to validate that the duration of storage time did not significantly influence the human WTA expression profiles. We used a Uniform Manifold Approximation and Projection (UMAP) plot for dimension reduction in the cell line dataset and, as expected, found that cell line drives primary clustering (Figure 7A). Similarly, using unsupervised hierarchical clustering of the tissue data set demonstrated that clustering was driven by tissue type rather than days spent in storage (Figure 7B).

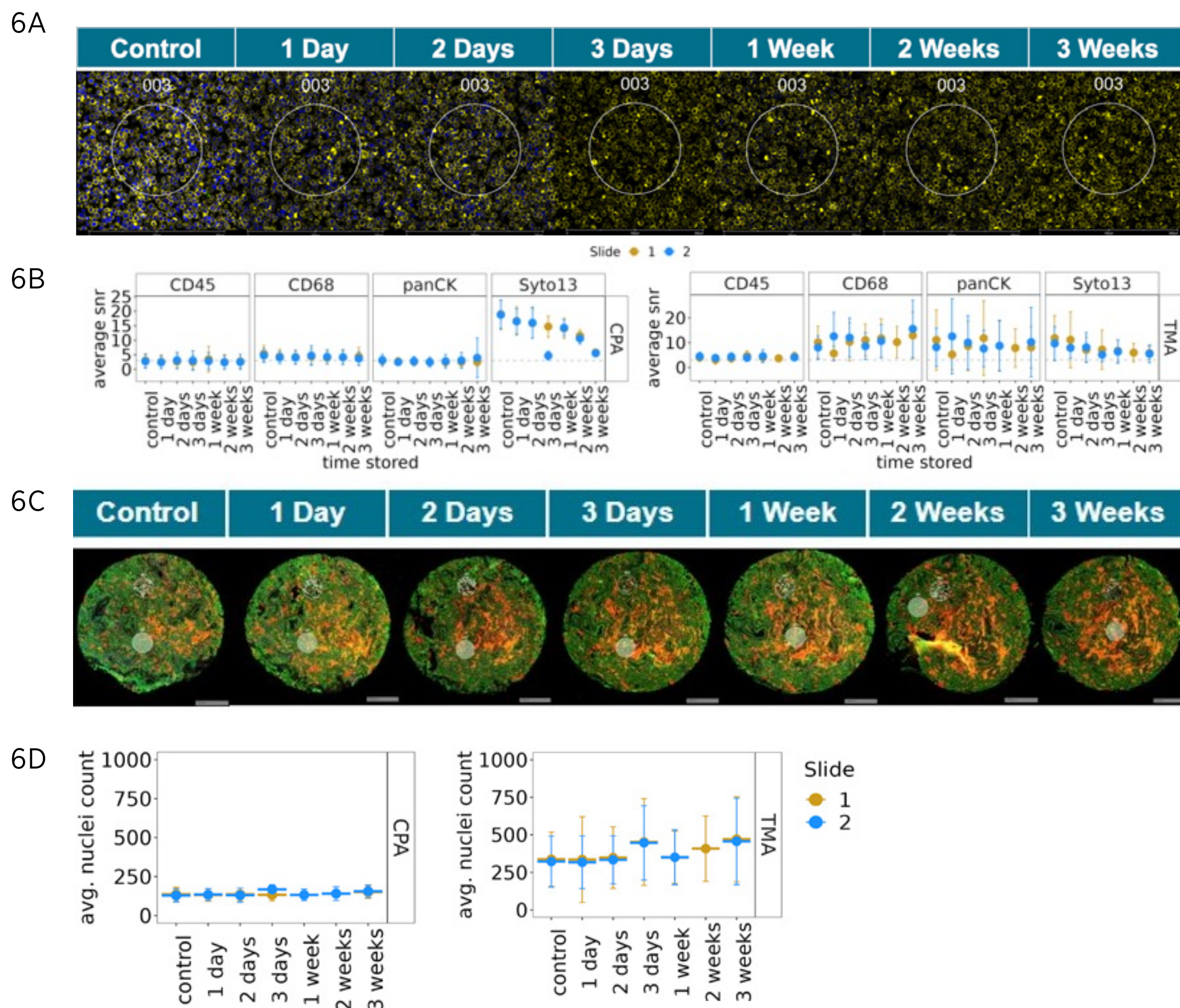
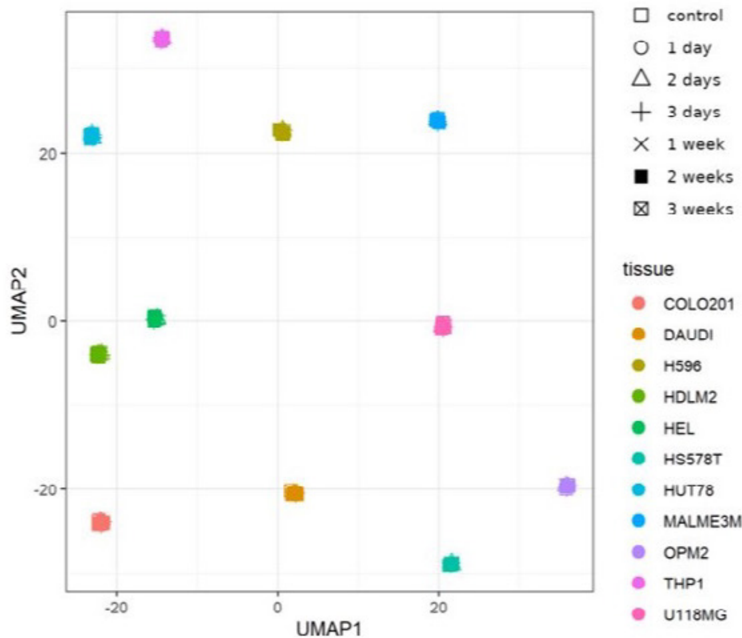


Figure 6. Long-term wet storage of fluorescent-antibody staining (CD45, CD68, PanCK) and nuclei-stained Syto13 of FFPE samples remains functional for visualization, segmentation, and nuclei counting. A) Representative images of one of eleven cell lines from the CPA slide (Daudi, top) and one of nine tissues (lung, bottom) from the TMA slide over the three week time course. Morphology markers are as follows: Syto13 (blue), PanCK (green), CD45 (yellow), and CD68 (red). B) Average signal-to-noise ratio (SNR) for CPA (left) and TMA (right) each day for morphology markers. The dashed line (y=3) denotes the minimum threshold for SNR for good segmentation. C) Segmentation on PanCK in one ROI in lung tissue the day of staining (control) versus after three weeks of storage. Panel shows full ROI (PanCK in green and CD68 in red), ROI with segments denoted as PanCK+ and PanCK-. D) Average nuclei count per ROI of CPA and TMA over the three week time course. All error bars are +/- standard deviation.

7A



7B

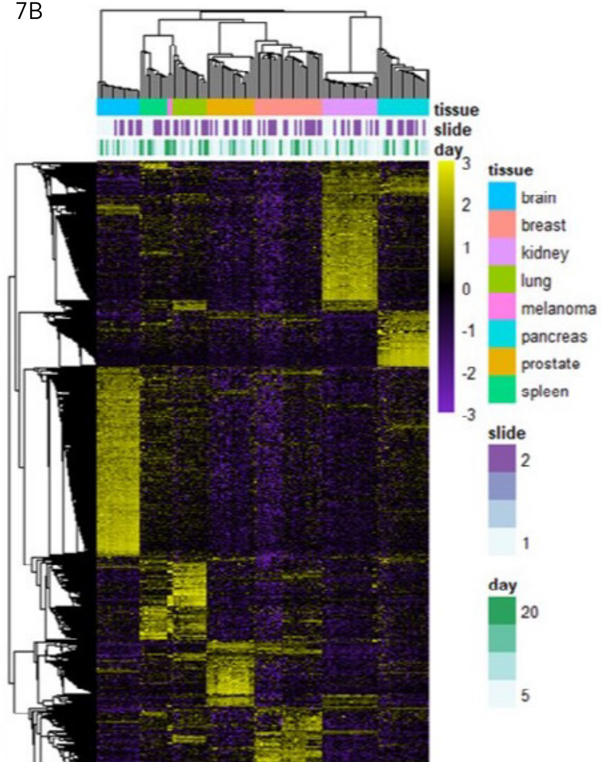


Figure 7. Human WTA expression profiles cluster based on cell or tissue type and not by storage duration. A) UMAP plot of cell line clustering. B) Unsupervised hierarchical clustering of TMA.

Conclusions

The results here demonstrate two possible modifications to the GeoMx DSP workflow that may be performed while maintaining WTA assay performance: 1) FFPE tissue blocks may be sectioned and stored at either 4°C or RT for up to 15 weeks before in situ hybridization with assay probes and staining with morphology markers and 2) FFPE slides prepared on the Leica BOND RX followed by overnight WTA hybridization and stringent washes may be stored in light-sealed containers in 2X SSC for up to 3 weeks. Furthermore, for best results, we recommend morphology markers are applied the day slides are run on GeoMx DSP. This sample processing flexibility not only enables high-throughput workflow designs for large-scale spatial omic studies but also improves user experience.

For more information, please visit nanostring.com

NanoString Technologies, Inc.
530 Fairview Avenue North
Seattle, Washington 98109

T (888) 358-6266
F (206) 378-6288

nanostring.com
info@nanostring.com

Sales Contacts
United States **us.sales@nanostring.com**
EMEA: **europe.sales@nanostring.com**

Asia Pacific & Japan **apac.sales@nanostring.com**
Other Regions **info@nanostring.com**

FOR RESEARCH USE ONLY. Not for use in diagnostic procedures.
© 2023 NanoString Technologies, Inc. All rights reserved. NanoString, NanoString Technologies, the NanoString logo, GeoMx, and AtoMx are trademarks or registered trademarks of NanoString

Technologies, Inc., in the United States and/or other countries. All other trademarks and/or service marks not owned by NanoString that appear in this document are the property of their respective owners.

July 2023 MK5851

nanoString