Immuno-Oncology Technology 1 (2019) 11–18

Contents lists available at ScienceDirect

Immuno-Oncology Technology

journal homepage: www.esmoiotech.org

Technology Explained

A user's perspective on GeoMx™ digital spatial profiling

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ARTICLE INFO

Keywords:
NanoString
DSP
Region of interest
Multiplex
IHC
Translational research

ABSTRACT

Characterization of spatial protein expression for multiple targets from a single tissue is difficult to perform, especially due to the limitations of multiplex immunohistochemistry and tissue heterogeneity. Therefore, a new technology is required that permits detailed and simultaneous expression profiling of proteins within a defined region of interest (ROI). To address this unmet need, NanoString Technologies developed a new technology, GeoMx™ digital spatial profiling (DSP), which currently enables simultaneous and guided detection of up to 40 antibodies (probes) from a single formalin-fixed paraffin-embedded (FFPE) tissue. DSP probes are tagged with unique photocleavable DNA oligos that are released after guided ultraviolet exposure in specific ROIs. Digital quantification of the released oligos by NanoString's nCounter® system provides a detailed expression profile of proteins within these discrete ROIs. In this article, we will describe our experience with the GeoMx DSP platform using cancer FFPE tissues. These expression profiles will provide better characterization and understanding of tumor heterogeneity and the tumor micro-environment, enabling the improvement of patient therapy and the identification of potential biomarker signatures. The purpose of this article is to offer potential future users an independent insight into the DSP platform and a comprehensive idea of usability, including advantages and current limitations of the technology based on our current experience with the beta version of NanoString's DSP platform as part of the DSP beta-testing program. The GeoMx™ Digital Spatial Profiling (DSP) platform is a non-destructive technique for regional in-depth protein expression profiling. Using oligonucleotide detection technologies, the GeoMx™ DSP enables simultaneous high-level multiplexing on a single FFPE tissue. Here, we focus on our current experience derived from our biomarker research using the beta version of the DSP instrument.

Background

Regional and quantitative protein signature analysis of clinical formalin-fixed paraffin-embedded (FFPE) tissues has proved to be difficult to perform in many research areas. In particular, in the field of immuno-oncology (IO), detailed characterization of immune cell subsets within a tumor area would improve our understanding of antitumor immunity and resistance to immune checkpoint inhibition. Tumors are highly heterogeneous in their architecture, (immune) cell composition, abundance and distribution. Understanding tumor and immune cell co-localization may also be important for biomarker identification and precision immunotherapy [1–5]. At present, the CD8+ T cell:regulatory T cell ratio is an improved biomarker compared with CD8 expression alone [6–11]. Furthermore, characterizing co-expression of co-stimulatory and inhibitory receptors would improve therapeutic strategies. For example, characterization of inhibitory markers expressed on T cells (e.g. LAG-3 and TIM-3) in the tumor micro-environment may impact the decision for new (combination) therapies [12,13]. Detailed tissue analysis is highly necessary but involves a laborious staining procedure and is hampered by limited patient tissue samples. Therefore, multiplex immunohistochemistry (IHC) will provide a more comprehensive insight into the interaction and crosstalk between tumor and immune cells within the tumor micro-environment.

Multiplexing more than five antibodies has largely been restricted by the spectral overlap of available fluorophores or chromogens. Moreover, current multiplex IHC tools provide objective information about the presence and histological location of immune cells, but neglect detailed phenotypical description. Due to this limitation, additional flow cytometry analysis is required. To advance standard IHC-based multiplex tissue analyses, NanoString Technologies (Seattle, WA) developed a novel imaging and tissue-sampling platform: GeoMx™ digital spatial profiling (DSP). This is a high-level multiplexing technique that provides information about the presence and histological location of immune cell subsets, and detailed characterization of their activation, differentiation

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https://doi.org/10.1016/j.iotech.2019.05.001

Available online 30 May 2019

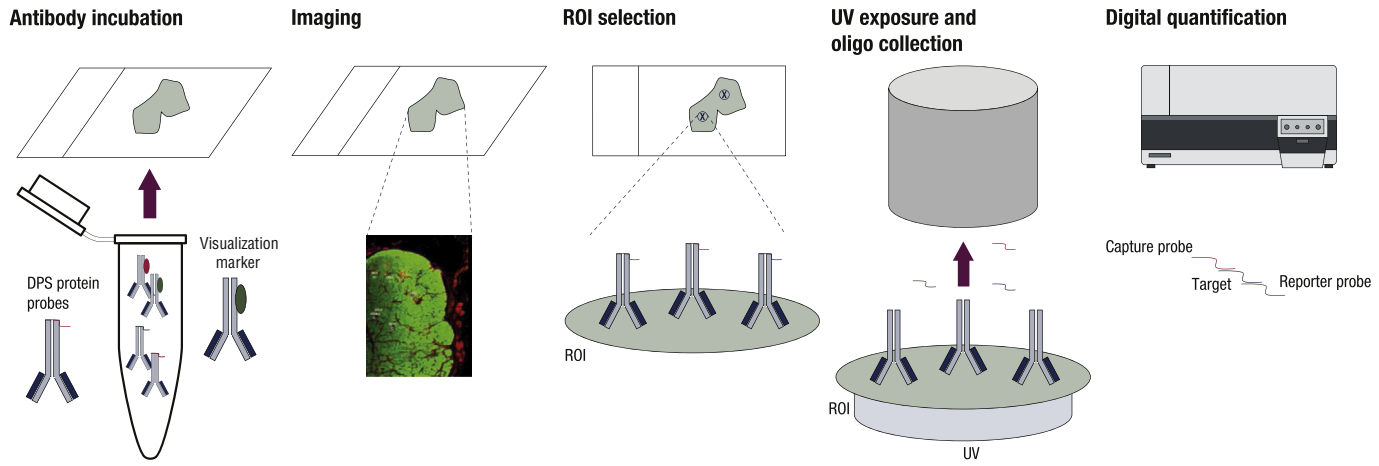

Figure 1. Protein digital spatial profiling (DSP) working procedure. Formalin-fixed paraffin-embedded tissue slide preparation involves incubation with an antibody mixture which contains up to four visualization markers and 40 DSP probes. Following imaging, regions of interest (ROIs) are selected based on visualization of the tissue. Sequential ultraviolet (UV) light exposure of each ROI results in the release of indexing oligos from the DSP probes, allowing their quantification on NanoString's nCounter® system.

and immune checkpoint expression [14].

The capability of DSP technology in the field of IO holds promise in advancing the current standard analysis of tumor biopsies. Thus, this article aims to give an insight into the DSP technology, the current recommended experimental set-up and an independent view on the advantages and limitations of the system based on our own experience and research. It should be noted that all observations are derived from our experience with the beta version of the DSP instrument, which we are currently testing, and may not apply fully to the commercially available instrument.

The working procedure of protein DSP

DSP technology simultaneously characterizes regional and quantitative protein expression of up to 40 markers related to immune cell activation and tumor cell classification on a single FFPE tissue section [14]. The DSP procedure implements five non-destructive steps: a standard FFPE tissue preparation step, a tissue incubation step with a mixture of visualization markers (VMs) and DSP probes, an imaging and region of interest (ROI) selection step, an ultraviolet (UV) exposure and oligo collection step, and a quantification step on NanoString's nCounter® system (Figure 1) [14]. In total, this procedure takes 3 days from tissue slide preparation until data analysis, with a throughput of four slides per day. Below, we will provide a more detailed review of our experiences with the DSP platform and its advantages and limitations.

Tissue preparation

Tissue preparation for DSP analysis is comparable to standard IHC, and is therefore an established procedure that does not involve additional steps or reagents. A major advantage of DSP is the non-destructive staining procedure. Tissue sections can be preserved after staining/acquisition for further haematoxylin and eosin or chromogenic IHC staining. Moreover, processed slides can be stored long term for additional DSP analysis. Further analysis will require additional antigen retrieval and re-incubation with a cocktail of VMs and DSP probes.

Tissue incubation with VMs and DSP probes

For DSP analysis, the tissue sample requires an incubation step with a master mix containing the VMs and DSP probes (Table 1). The VMs are tissue-compartment-specific fluorescent-labeled antibodies and a DNA marker used to visualize the tissue morphology. ROI selection from VMs has the advantage of allowing researchers to select areas based on the immune cell type of interest and the surrounding environment. The DSP platform enables multispectral imaging but also minimizes emission-spectral overlap by including four distinct light-emitting diodes. This limits the morphological characterization of the tissue to three different markers. Currently, there are no options within the DSP platform to extend the number of VMs, and thus thorough selection of markers or the use of serial tissue sections is required. Based on our experience, combining more than four VMs by applying an intermediate bleaching step is not recommended due to remaining tissue stain and the consequent possibility of false-positive signals.

The tissue is also incubated with the DSP probe panel, which consists of a fixed core panel that can be extended with two additional modules (Table 1B). These DSP probes are not multiplex-limited by spectral resolution, as they are not fluorescently labelled but are labelled with barcode indexing oligos. These oligos are linked to each antibody via a UV-cleavable linker modified from a previous concept [15]. During the beta test phase, the DSP probe panel consists of 40 antibodies. Theoretically, the DSP probe panel could be extended to a maximum of 96 antibodies

Table 1
Overview of (A) visualization markers used currently to distinguish between the tumor and immune cell compartment, and (B) digital spatial profiling protein probes included in the core panel and the additional modules

A

Visualization markers	Compartment
Syto13	DNA
S100B/Pmel17 or PanCK	Tumor
CD45	Immune cell
CD3	Immune cell

B

Core panel		Module 1	Module 2
Beta-2-microglobulin	CD56	CD137	CD127
CD11c	HLA-DR	LAG3	CD25
CD20	SMA	OX40L	CD80
CD3	Fibronectin	TIM-3	CD86
CD4	TGFB1	VISTA	ICOS
CD45	PD-L1	ARG1	PD-L2
CD68	GZMB	B7-H3	CD40
CD8	Ki-67	IDO1	CD40L
CTLA4	PD1	STING	CD27
PanCK	IgGs	GITR	CD44

due to the technical capability of the quantification process in the nCounter system. For our projects, we extended the standard DSP probe panel with 11 additional antibodies which were conjugated to unique oligos by NanoString's barcoding service [16]. Using standard IHC, we confirmed the efficient staining performance of the antibodies after oligo conjugation (Figure 2A) and sufficient DSP counts above the background control (Figure 2B). In total, antibody selection, conjugation and validation took 4 months; hence, thorough advance planning of projects is required.

ROI selection procedure, UV exposure and oligo collection

The DSP platform provides additional benefit to standard IHC or full-section multiplex platforms by enabling regional/spatial analysis. Based on the tissue morphology, ROIs can be selected which vary in size (10–600 μm in diameter) and form (Figure 3). The shape of the ROI can vary from geometric to a rare cell population level. Additional segmentation within a geometric ROI allows distinction to be made between the tumor and the tumor micro-environment, or between multiple immune cell types (Figure 3). However, the selection of single cells is not recommended for the beta instrument at present due to the low signal:noise ratio of the DSP probes, requiring at least 10 cells/ROI for sufficient counts. Selection of ROIs results in guided UV light exposure using two digital micromirror devices (DMDs) in the instrument. These DMDs are small mechanical systems that contain an array of steerable reflective micromirrors (Texas Instruments, Dallas, TX). DMD-directed UV light illuminates all selected ROIs sequentially, resulting in the release of indexing oligos solely within the boundaries of the set ROI. To confirm precise guidance of UV light by the DMDs, we used CAGE-532, a dye that is initially colorless and non-fluorescent but which releases a highly fluorescent signal when illuminated with UV light (Figure 4A). Further validation revealed low counts in a control 'glass' ROI compared with a

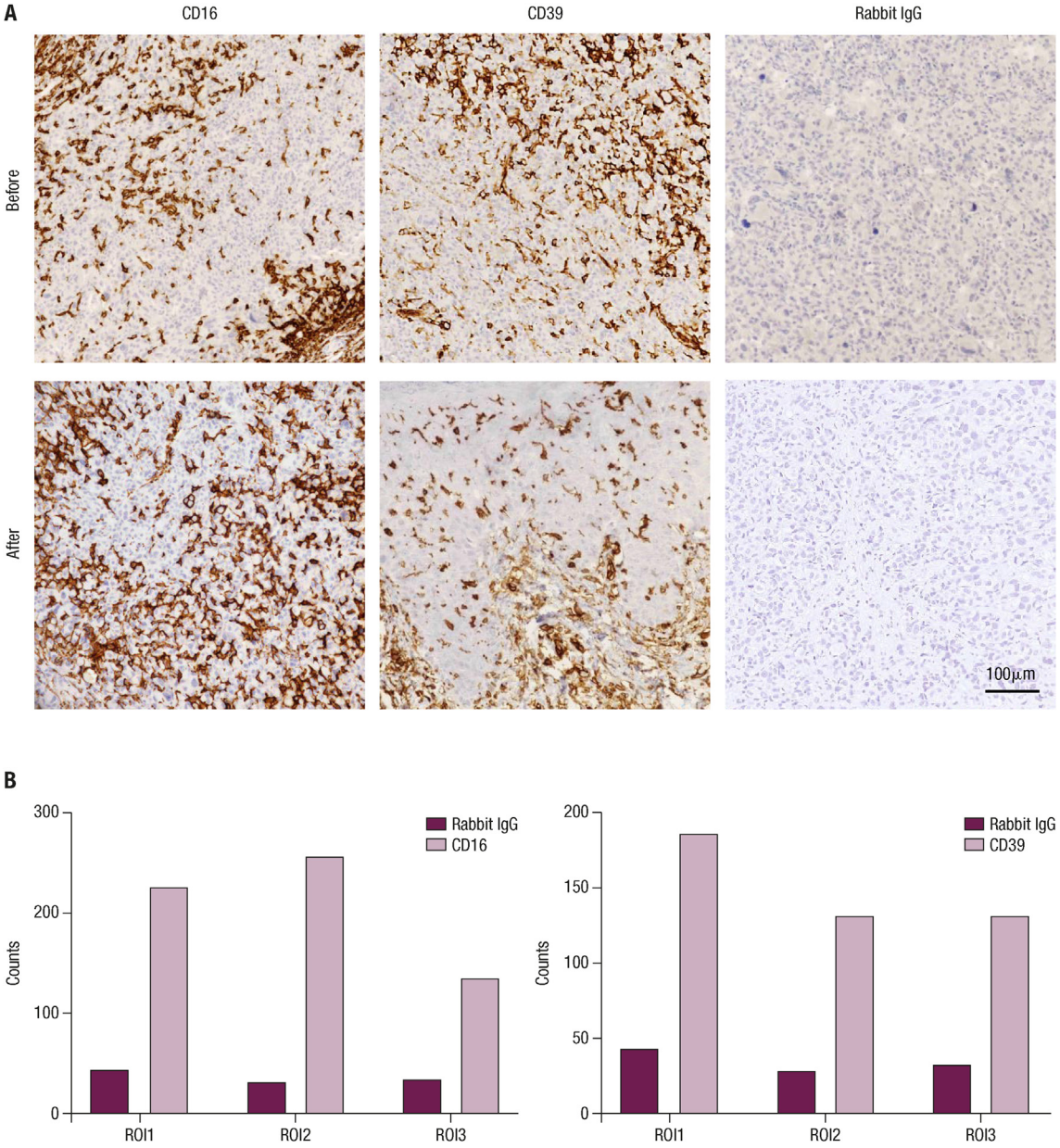

Figure 2. Comparison of staining efficiency before and after oligo conjugation of antibodies. (A) Standard immunohistochemical staining for CD16 and CD39 before and after oligo conjugation of the antibodies. Rabbit immunoglogulin G (IgG) was used as the background control. Scale bar 100 μm. (B) Housekeeping (HK)-normalized digital spatial profiling counts after oligo conjugation of antibodies from three discrete regions of interest (ROI1–3). Violet bars, rabbit IgG; pink bars (upper figure), CD16; pink bars (lower figure), CD39.

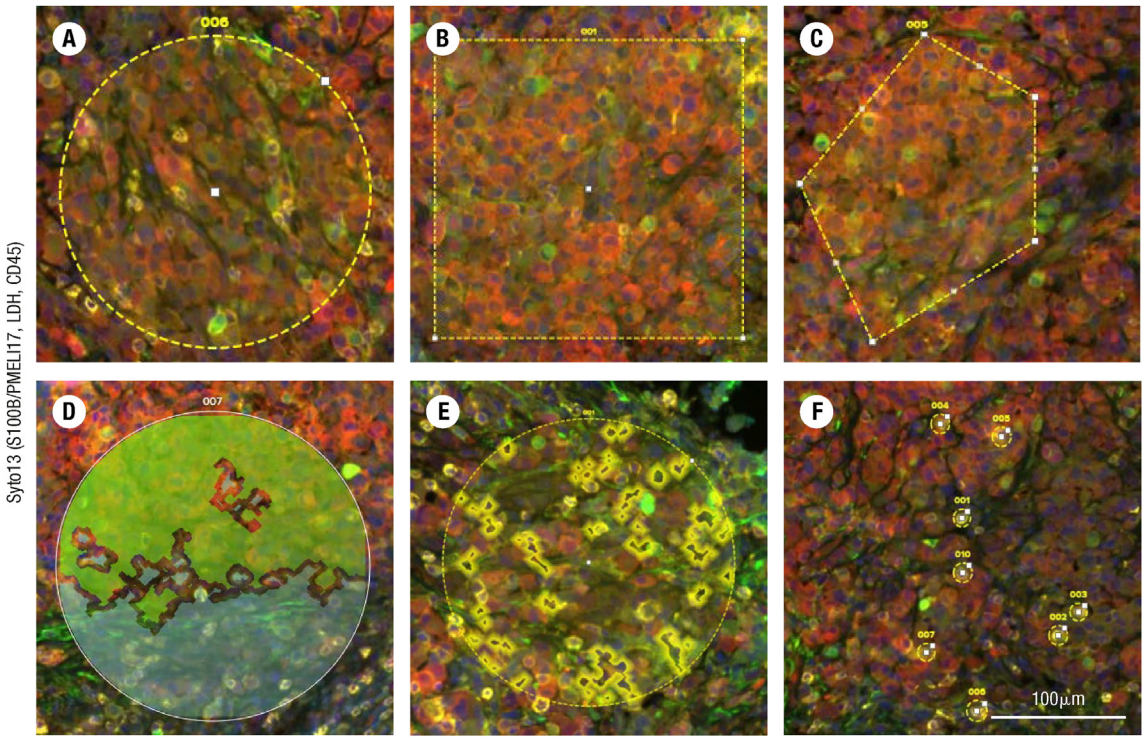

Figure 3. Selection of type of region of interest (ROI). Three types of regions can be selected within the digital spatial profiling platform. Tissue biopsy was stained with S100B/PMEL17 (green), LDH (red) and CD45 (yellow) visualization markers. (A–C) Geometric ROIs can be selected, ranging from circles (A) to rectangles (B) to user-defined polygons (C). (D,E) Segmentation within a geometric ROI is generated based on visualization markers, and can currently distinguish between tumor and stroma (D) or specific cell type populations (E). (F) Single-cell ROIs are generated based on visualization markers which allow the analysis of either one or multiple single cells within a field of view. Scale bar 100 µm.

neighboring tissue ROI (Figure 4B), and precise protein expression according to the segment type (Figure 4C).

In our experience, defining representative ROIs requires strong guidance from an experienced pathologist to avoid biased analysis, especially with heterogeneous tissues. A detailed description of the manner of ROI selection will also be a prerequisite in DSP publications. Our experiments show high reproducibility between comparable sections (Figure 5), but strong variations occur in comparisons of tumor sections with different immune cell abundance ('hot' vs 'cold' tumors) (data not shown). Ideally, spatially structurally delimited but impure tumor regions should be selected. We therefore prefer the selection of ROIs according to the tumor structure (e.g. intratumor, peritumor and pure stromal ROIs). To focus on immune cell populations, segmentation within these impure tumor ROIs can be performed. In the current state, the DSP analysis tools could be improved to incorporate the needs of users analysing complex tissue samples, especially in high-density regions (Figure 4D). By selecting distinct ROIs, this platform enables users to obtain spatial data for protein targets relative to each other, as well as in relation to tissue architecture and immune infiltration. However, spatial information between certain ROIs can only be assumed and cannot be measured, and a tool which incorporates a distance of spatial information would be valuable.

In addition to area-specific characterization of the tissue, full tissue section analysis can also be performed on this platform by selecting adjacent square ROIs throughout the tissue. However, in our opinion, it is not recommended or feasible to carry out this time-consuming and costly procedure. Therefore, the DSP platform is not the preferred tool and we recommend the use of specialized sequential staining platforms, such as CODEX (Akoya Biosciences, West Coast, 1505 O'Brien Drive, Suite A-1, Menlo Park, CA, USA) or InsituPlex (Ultivue Inc., Cambridge, MA) [17,18].

Quantification using NanoString's nCounter instrument and data analysis

For digital quantification, the photocleaved oligos are hybridized to NanoString barcodes and processed on the nCounter instrument. This results in digital counts corresponding to the abundance of each targeted protein within each ROI. These digital counts can be analysed in the provided web-based software, in which data are associated with the tissue scan and ROIs. Several implemented functions allow comparison of the acquired DSP probe counts from different ROIs in (clustered) heatmaps, boxplots and bar graphs, and perform statistical analysis. Our experience to date with the software is that comparison of data from two to three slides is simple. However, experimental groups involving more than three tissues and three points of comparison (e.g. patients' response, treatment and dose) require careful annotation to enable clustering of the data, or bioinformatics support.

Due to the high-dimensional analysis of the DSP platform, technical and biological variations need to be controlled for during data comparison and interpretation. To address various sources of technical variation, NanoString implemented several internal control mechanisms to normalize for variables, including External RNA Control Consortium (ERCC) controls, housekeeping (HK) proteins and immunoglobulin G (IgG) controls (Table 2). ERCCs are included as positive and negative controls for technical variation during hybridization, while HK proteins, number of nuclei or area size are included to normalize the variation of cellularity within the ROIs (Table 2). To date, there is no established guideline for normalization; IO pathologists, researchers and bioinformaticians need to develop consensus guidelines. We found the best method for analysis was to normalize to ERCC, scale counts to nuclei counts, and then normalize to either HK proteins or IgG controls. However, we have also identified some exceptions that require careful normalization in order to avoid artificial elevation of counts. For example, when using a geometric ROI on 'glass' as a background control

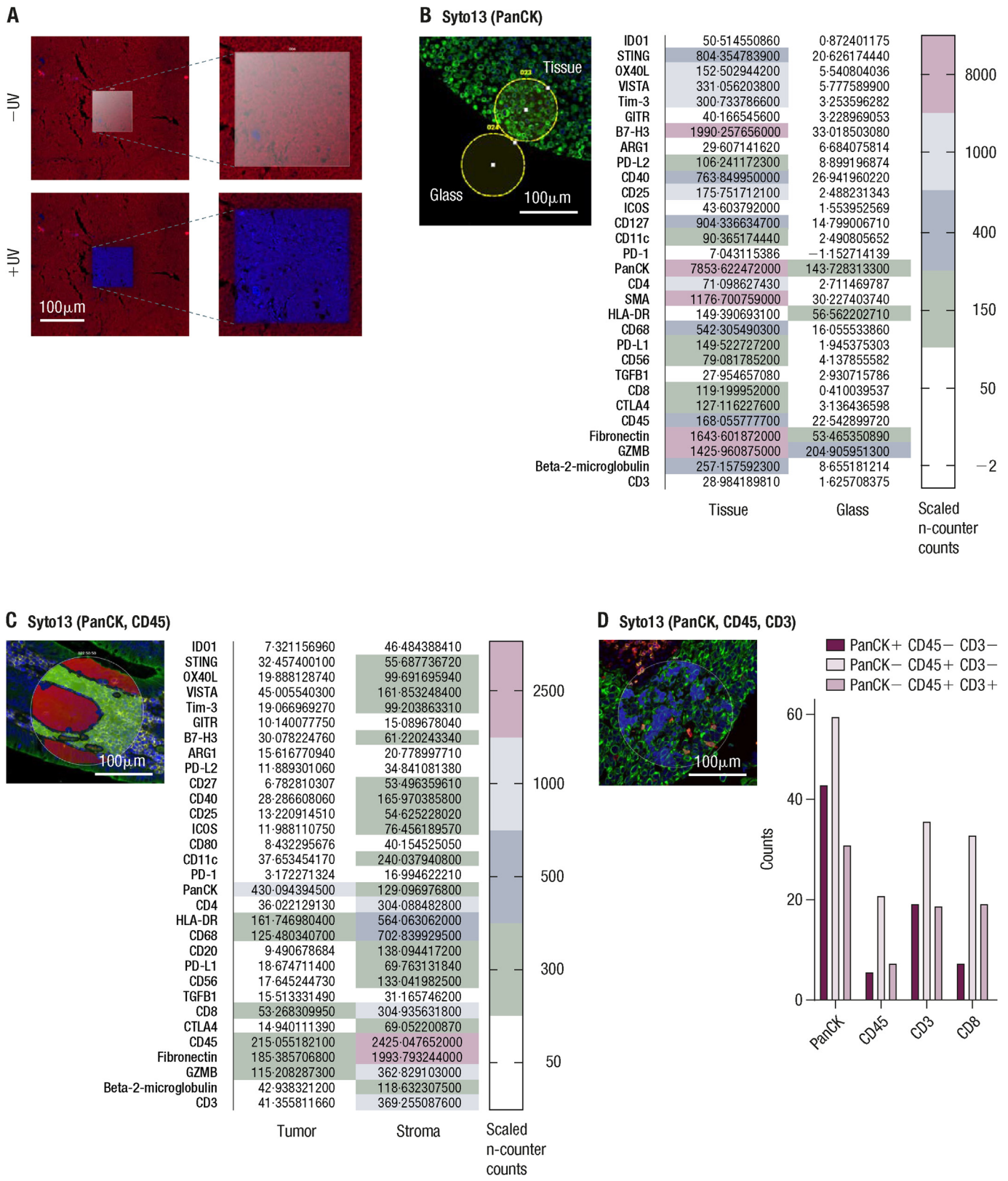

Figure 4. Precise ultraviolet (UV) guidance by digital mirror devices. (A) CAGE-dye-stained tonsil tissue with geometric region of interest (ROI) before and after UV exposure. Scale bar 100 μm. (B) Circular ROIs (200-μm diameter) on cell pellet array and 'glass' and respective protein expression levels after digital spatial profiling (DSP) analysis. DSP counts are normalized to immunoglobulin G (IgG) controls to correct for noise. Scale bar 100 μm. (C) Geometric ROI on colorectal cancer tissue with internal segmentation for 'tumor' (red) and 'stroma' (green). Scale bar 100 μm. Heatmap of region-specific nCounter counts normalized to nuclei. (D) Circular ROI (200-μm diameter) with segmentation on 'PanCK+ CD45- CD3-' (blue), 'PanCK- CD45+ CD3-' (yellow) and 'PanCK- CD45+ CD3+' (red) cells and respective DSP counts after normalization to IgG controls. Scale bar 100 μm.

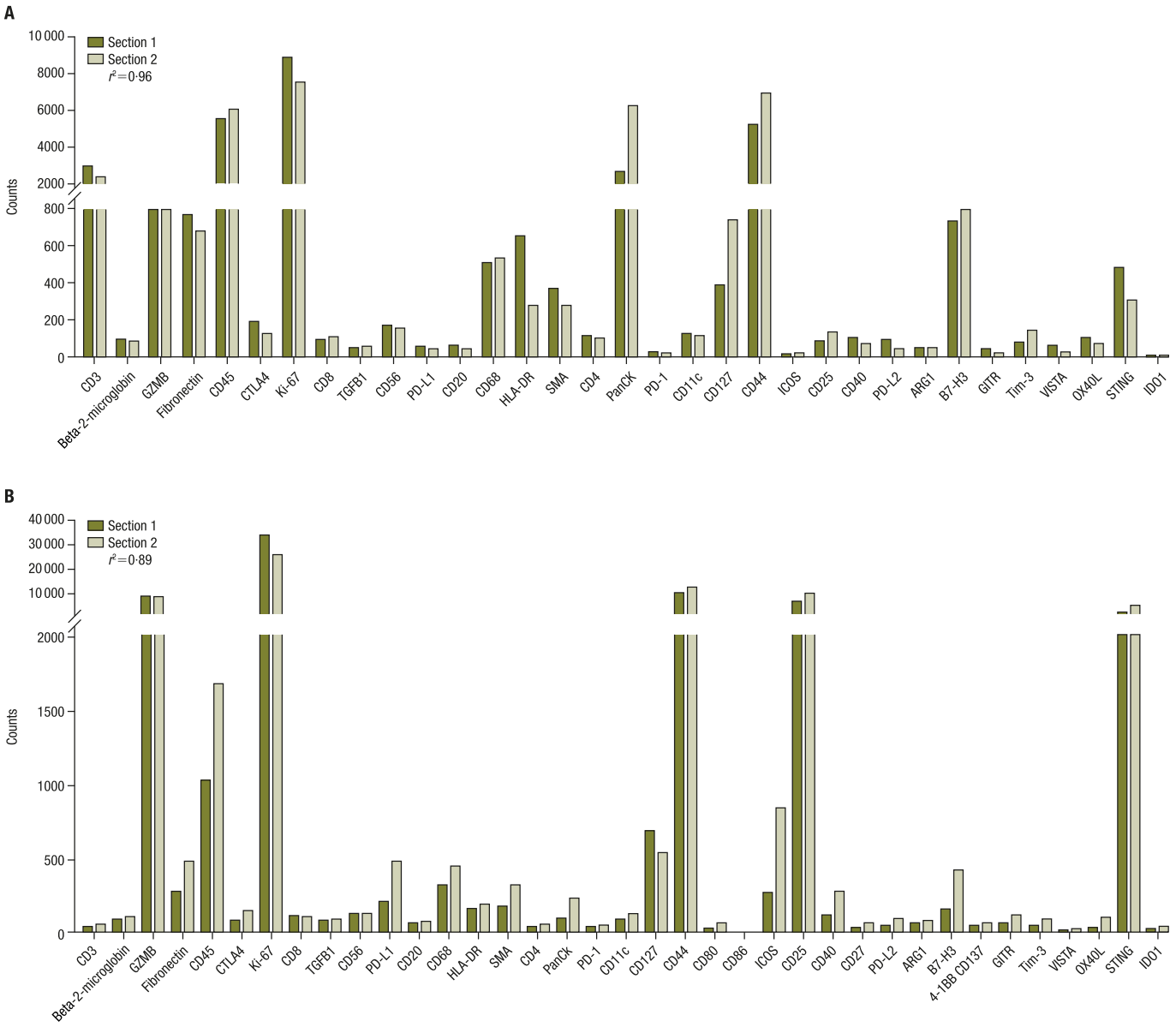

Figure 5. Reproducibility of digital spatial profiling analysis. Independent expression analyses of depicted proteins were performed on two serial sections of (A) cell pellet array and (B) colorectal cancer tissue (B). Expression levels are shown for one 200-μm-diameter circular region of interest (ROI)/section and counts were normalized to nuclei and housekeeping proteins. ROIs of serial sections were chosen in the same tissue area to allow close comparison. r^2 value indicates correlation between expression profiles from sections 1 and 2. Section 1, dark grey bars; section 2, light grey bars.

or when comparing tumor and rare cell segmentation with a significant difference in cell number, data cannot be scaled to nuclei or normalized to HK proteins. This would result in false-positive counts; instead, normalization to IgG controls is recommended.

Of note, analysis and interpretation of counted indexing oligos have to be carried out carefully as a single count does not necessarily reflect protein expression on a single cell. This is due to potential variation in antigen density between cells and the fact that cells may bind simultaneously to multiple oligo-conjugated antibodies. Heterogeneous oligo-antibody conjugation is regulated and controlled by NanoString to a certain extent by analysis of the 'degree of labeling'. Using the nCounter system, they estimate the oligo numbers for each clone, and remaining unconjugated antibodies are purified via high-performance liquid chromatography [16]. Normalization of the counts to cell nuclei will give an indication of the density of positive cells or protein expression. However, these variabilities hinder estimation of the percentage of cells co-expressing certain markers, unlike other tools such as flow cytometry.

Table 2
Data analysis and normalization: (A) Technical and biological sources of variation during the digital spatial profiling (DSP) working process. To subtract the variation, NanoString Technologies offers 'different types' of normalization for protein DSP; (B) Isotype controls and housekeeping proteins included in the DSP probe panel

A

	Source of variation	Type of normalization
Technical variation	nCounter quantification	• ERCC correction
Biological variation	ROI size	• Area normalization
	ROI cellularity	• Reference protein/gene normalization
		• Nuclei count normalization
	ROI background	• Three isotype controls

B

Isotype controls	Mouse IgG1, IgG2a and rabbit IgG
Housekeeping proteins	Histone H3, GAPDH and ribosomal protein S6

ROI, region of interest; ERCC, External RNA Control Consortium.

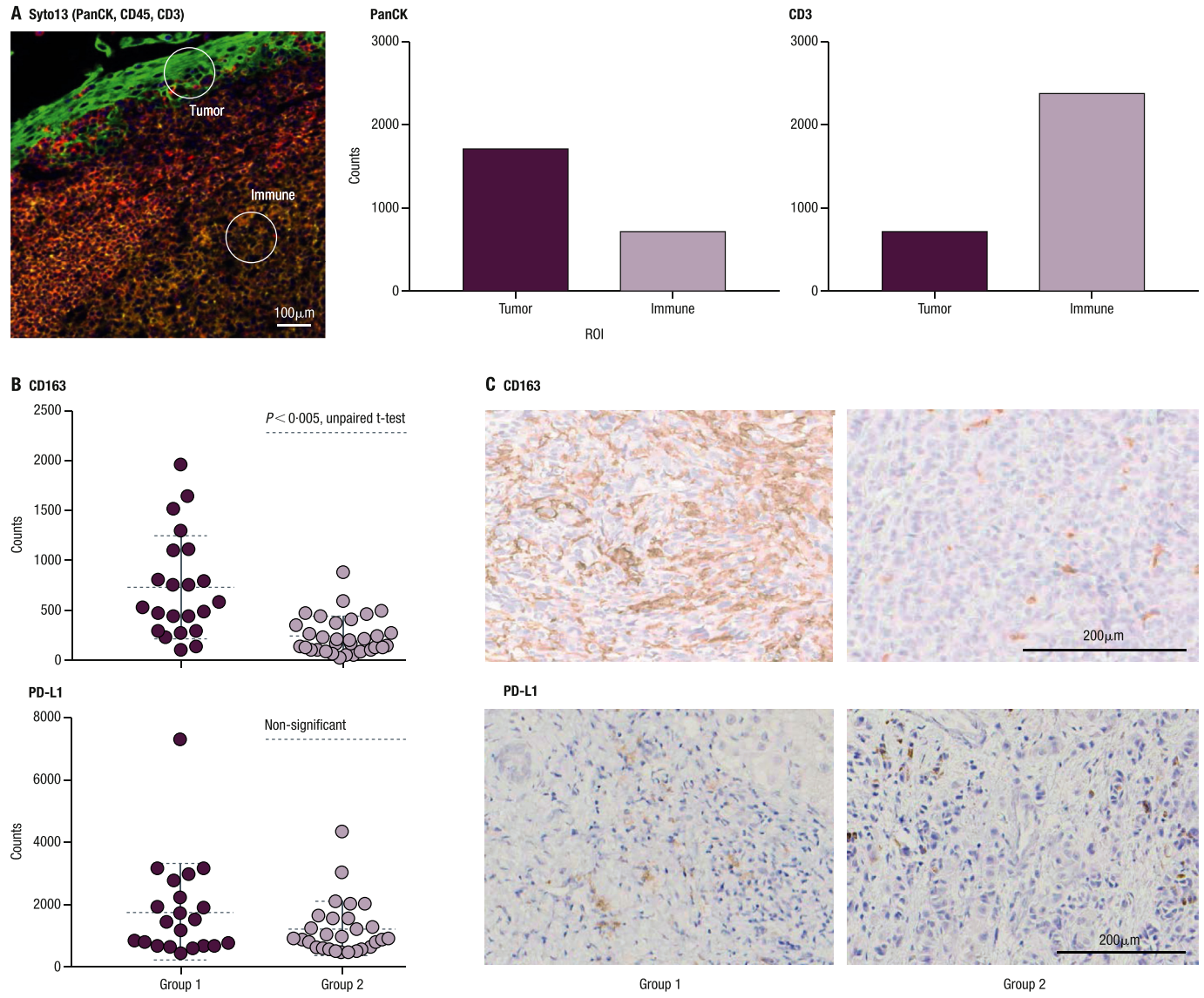

Figure 6. Correlation of digital spatial profiling (DSP) counts with immunofluorescent or standard immunohistochemical staining. (A) Tonsil tissue stained with PanCK (green), CD45 (red) and CD3 (yellow) visualization markers. Scale bar 100 μm. Graphs depict nuclei and housekeeping (HK)-normalized DSP counts of indicated proteins in 'tumor' or 'immune'-enriched regions of interest (ROIs). (B) Nuclei and HK-normalized counts for CD163 and PD-L1 obtained from melanoma patient groups 1 (n=22) and 2 (n=32). Each dot represents one ROI/patient selected by similar tissue morphology. *P<0.005, unpaired t-test. ns, non-significant. (C) Representative images for CD163 and PD-L1 were obtained from melanoma tissue samples from groups 1 and 2 using standard immunohistochemistry. Scale bar 200 μm.

At present, co-expression of proteins can be identified only for a cell population within an ROI, but not at single cell level due to the limitation of single cell analysis. Taking these variabilities into account, we currently validate our top protein candidates derived from DSP analyses by standard IHC methods. Our comparisons of DSP counts with staining obtained using VMs (Figure 6A) or standard IHC reveal good correlation, enhancing the reliability of the DSP platform (Figure 6B and C).

Conclusion and future perspectives

NanoString's DSP platform is an innovative technology that combines imaging and tissue sampling to advance the standard IHC procedures currently used by pathologists. In our view, the major advantages of the platform are its high multiplexing ability on FFPE samples, requiring low hands-on time, and the non-destructive straightforward procedure that will contribute to define biomarker signatures in discrete ROIs. Moreover, the segmentation tool enables users to focus the analysis on, for example, distinct immune cell infiltrates within a tumor area. As a beta test site, we have encountered a few correctable instabilities in the software but not in the hardware of the DSP instrument. Nonetheless, during the testing phase, we have encountered restrictions in the simultaneous combination of more than four VMs, which requires the use of serial tumor tissue sections for different research questions. During this testing period, single cell analyses were not feasible, which limited our analytical approaches.

The DSP platform is a rapidly evolving platform that, in the future, will likely include more DSP probes to cover other aspects of tissue profiling. In addition to the protein DSP, in the near future, NanoString will also provide the possibility to characterize RNA expression on FFPE tissues [14]. However, to implement the DSP platform for translational research, established guidelines are needed for data analysis, including normalization strategies, and a more comprehensive understanding of the effects of staining intensity and oligo labelling on DSP counts. This will likely be addressed in the near future with the upcoming possibility

to characterize a single cell. Similar to the recent publication by Decalf et al. [19], methodological cross-laboratory comparisons between the DSP platform and other multiplex staining methods, such as sequential chromogenic IHC multiplexing, Akoya's Codex and Vectra systems, are essential to optimize the use of each technology based on the research question and/or clinical need.

In conclusion, in our view, the DSP platform will be an important addition to current single staining IHC methods in clinical diagnostics. Recent published data, and our unpublished data, reveal sufficient characterization of the melanoma protein profile in patient cohorts that received immune checkpoint blockade therapy using protein DSP [20, 21]. We envisage that with further standardization and optimization of the working process, data analysis and extension of the visualization and DSP probe panels, DSP-based multiplex analyses can become a helpful tool towards personalized immunotherapies.

Funding

CB and TMV received a research grant from NanoString for testing the beta version of the DSP machine.

Disclosure

CB and TMV received a research grant from NanoString for testing the beta version of the DSP machine.

Acknowledgements

TMV thanks NanoString Technologies for the opportunity to join the DSP development team for a 6-month training rotation. We thank Dr AM Terry for proofreading the manuscript.

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