

Immuno-Oncology Research with the Hyperion Imaging System

A high-parameter imaging solution at subcellular resolution to characterize the immune repertoire in the tumor microenvironment

Introduction

The Hyperion™ Imaging System is the world's first and only commercially available metal-tagged-antibody-directed Imaging Mass Cytometry™ (IMC™) platform. This platform is capable of simultaneous detection of 4 to 37 protein markers at subcellular resolution in biological samples including fixed tissue sections or cells deposited onto glass slides (liquid biopsies). The system directs a laser beam focused at 1 μm^2 to sample proteins stained with metal-tagged antibodies and directs these metal tags to analysis by inductively coupled plasma time-of-flight (TOF) technology, the basis of CyTOF® mass cytometers. A discrete signal from each ionized metal tag is detected based on differences in mass instead of wavelength, and at 1 Da resolution with minimal background. The Hyperion Imaging System revolutionizes imaging with its ability to detect 4 to 37 proteins markers simultaneously from a single scan, without signal overlap, thus surpassing current capabilities of fluorescence imaging.

Unprecedented biological insights with high-parameter imaging mass cytometry empower scientists in translational and clinical research across a breadth of applications including immuno-oncology (Villarreal-Espindola, 2017). The sheer diversity inherent in the immune repertoire combined with a range of tissue-specific cellular phenotypes requires an imaging solution that offers the right balance between subcellular resolution and spatial visualization to measure a relatively large number of both cell surface and intracellular protein markers.

This application note presents a systematic Imaging Mass Cytometry workflow from sample preparation to image acquisition and data analysis for a typical immuno-oncology study to identify the immune cell population in human lung carcinoma samples on a tissue microarray (TMA).

The Imaging Mass Cytometry workflow

A four-step workflow is illustrated in Figure 1 to represent high-parameter imaging of metal-tagged Maxpar® antibodies on the Hyperion Imaging System and image analysis using the MCD™ Viewer.

The Imaging Mass Cytometry workflow

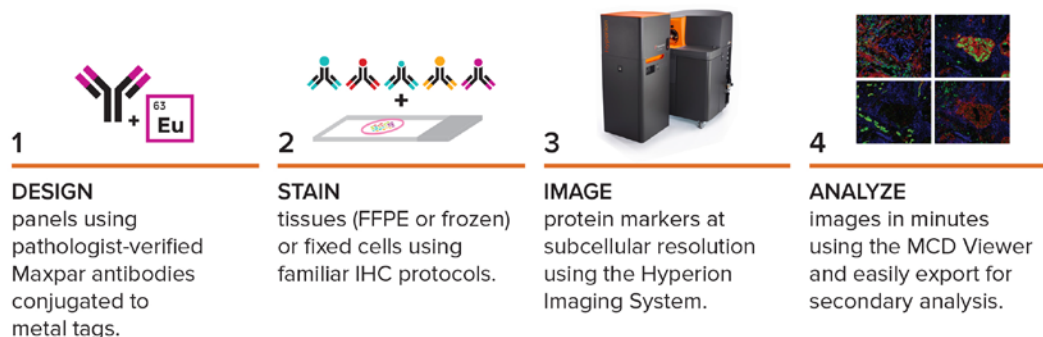


Figure 1. The Imaging Mass Cytometry workflow. A simple four-step workflow to deliver highly multiplexed imaging.

Multiplex 4 to 37 protein markers on one slide

Imaging Mass Cytometry using metal-tagged antibodies empowers scientists to characterize one or multiple cell populations of interest and provides a visual context of the heterogenous tissue microenvironment. This characterization is possible on the Hyperion Imaging System because of the 135 channels and 37 metal tags used to simultaneously detect multiple proteins from one scan and on one slide. For the human lung carcinoma study, the formalin-fixed, paraffin-embedded (FFPE) tumor microenvironment is characterized by three histological entities: 1) structural and transmembrane proteins, to visualize histological landmarks, 2) immune population with CD markers, and 3) to identify the tumor population as shown by proliferative activity and p53 expression (see Table 1).

Table 1. Designing a panel of antibodies to characterize the immune population in the human lung carcinoma microenvironment

| | Antibody | Metal Tag | |
|-----------------|---------------------------------|-----------|-------|
| Structural | Alpha-smooth muscle actin (SMA) | 141Pr | |
| | Vimentin | 143Nd | |
| | Collagen type I | 169Tm | |
| | Beta-catenin | 165Ho | |
| | E-cadherin | 158Gd | |
| Cancer | HMWK | 144Nd | |
| | Ki-67 | 168Er | |
| | p53 | 150Nd | |
| Immuno-oncology | CD45 | 152Sm | |
| | CD3 | 170Er | |
| | CD8a | 162Dy | |
| | CD20 | 161Dy | |
| | CD68 | 159Tb | |
| | PD1 | 164Dy | |
| | CTLA4 | 161Dy | |
| | CD31 | 145Nd | |
| | Myeloperoxidase | 146Nd | |
| | p40 | 167Er | |
| | CD44 | 171Yb | |
| | Nuclear | | 191Ir |
| | | | 193Ir |

Panel design addresses these histological entities and drives the selection of antibodies. The Maxpar catalog for the Hyperion Imaging System features over 80 pathologist-verified antibodies for Imaging Mass Cytometry on formalin-fixed human tissues. A selection of tags is available to achieve multiplexing of 4 to 37 protein markers on one or more regions of interest. Human lung carcinoma microarray sections are stained with two cocktails of at least 20 antibodies on two sequential sections. A short list of these antibodies is identified in Table 1. Assignment of metal tags to each antibody is listed in this table. The staining was performed using a standard immunostaining protocol that is illustrated in Figure 2.

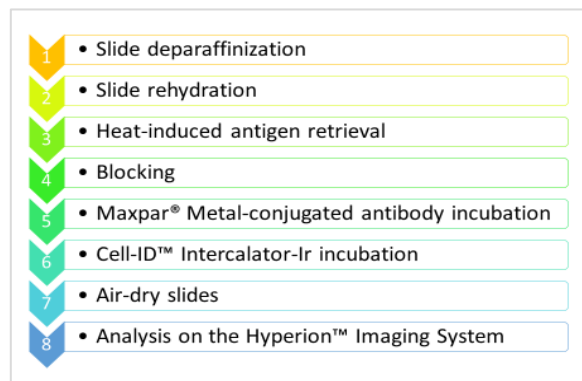


Figure 2: Summary of the Hyperion Imaging System staining protocol for FFPE tissue sections. An Imaging Mass Cytometry staining experiment follows the same protocol as for standard immunofluorescence (IF).

Automate multiplexed imaging at subcellular resolution

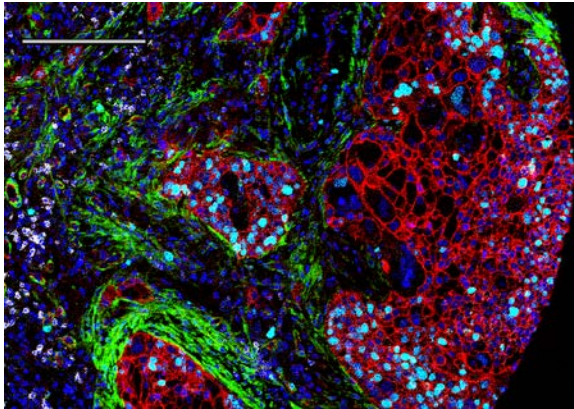


Figure 3. Subcellular imaging on the Hyperion Imaging System; Ki-67 staining in cyan overlaid on nuclei staining in blue. The structural protein beta-catenin is stained in red, CD8a in white and alpha-SMA in green. Scale bar is equal to 100 μm .

The Hyperion Imaging System engages a plug-and-play imaging workflow that includes the selection of one or more regions of interest (ROIs) and automation of precise laser-directed sampling and guidance of resulting data to the CyTOF image acquisition software. This accommodates studies that require a balance between high throughput and high-parameter imaging of multiple biological samples on a single slide such as a tissue microarray. Multiple ROIs in the human lung carcinoma array (see Figure 3), once stained and air-dried, were sampled by a UV laser pulsed at 200 Hz frequency and at $1 \mu\text{m}^2$ spot size. The resulting plume traveled to the plasma ion source, where the metal tags were ionized. Isotopes associated with individual $1 \mu\text{m}^2$ spots were detected based on time-of-flight and then indexed against the source location. This indexed signal map is the image data acquired at the end of a scan. It is stored in MCD file format, and as an option, in TXT format.

Visualize spatial context of high-parameter data in minutes

The Hyperion Imaging System generates image acquisition data to visualize proteins and their sub-cellular expression within minutes. For example, the MCD Viewer software that accompanies the Hyperion Imaging System imports ion current images of all metal tags from a panel from one lung carcinoma sample to produce 16-bit single TIFF or multiple OME-TIFF images of publication quality and with a scale bar. Figure 4 shows the selected 20 tags as a composite rendering of eight multiple OME-TIFF images. Each image can be spectrally unmixed to display the expression of multiple proteins, and three proteins are rendered in each image from A to H. Basic statistical data including the number of ion counts detected per pixel can be saved for each ROI and exported as a tab-delimited text file for third-party analysis.

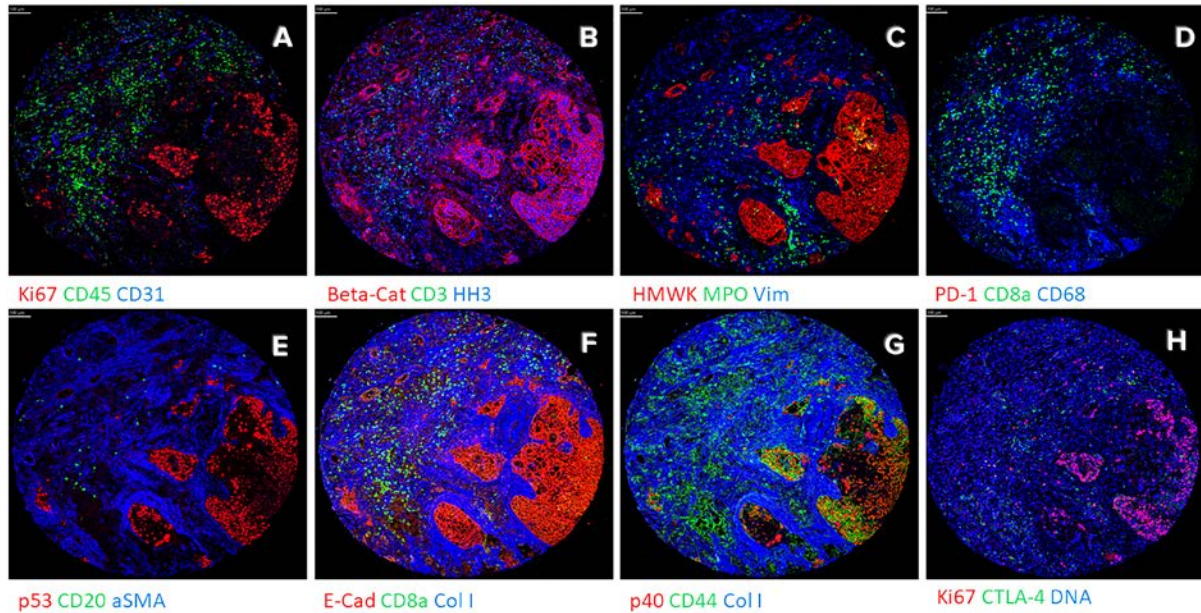


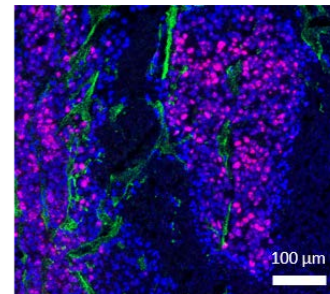
Figure 4: Hyperion images of 20 immune, tumor and structural markers identified on a 1 mm x 1 mm lung carcinoma core in a formalin-fixed, paraffin-embedded tissue microarray slide. Scale bars are equal to 100 μ m.

A Hyperion analysis workflow

The three-step analysis workflow illustrates a standardized analysis pipeline for multiplexed data with the histoCAT™ and aforementioned MCD Viewer software that accompany the Hyperion Imaging System.

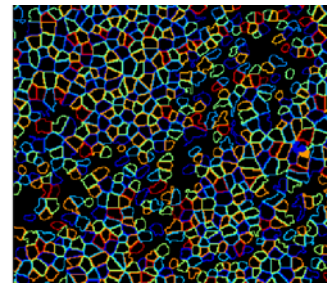
Step 1

View and create images using the MCD Viewer. Detect presence of proteins of interest, produce high-quality images with selected combinations of markers and confirm experimental success using the MCD Viewer. Export files in various formats for subsequent secondary analysis.



Step 2

Define image segmentation. Often, the follow-up step in image analysis is to segment cell populations, which can be done in a variety of third-party software types including CellProfiler. After that, a cell mask is imported into histoCAT for subsequent high-order analysis.

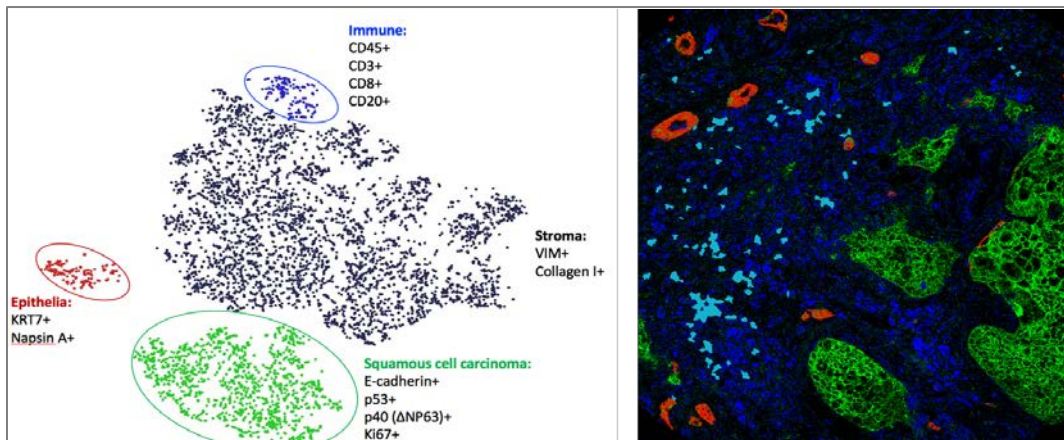


Segment specific cell populations of interest. Quantify differential expression of the epitope within cells and across cell populations.

Step 3

Perform higher-order analysis. Higher-order analysis provides a measure of differential expression of markers within a cell and between cell populations with statistical significance and in spatial context. This analysis allows for the determination of spatial relationships between cells within the tissue and can be represented with t-SNE maps (van der Maaten and Hinton, 2008) in histoCAT.

t-SNE provides the ability to sort high-dimensional data into compelling 2D maps. Following the reduction in dimensionality, the cell populations can be gated and mapped to tissue images created in histoCAT. The co-localization of markers can be determined using the neighborhood function that is guided by the user to identify subpopulations of cells that are in close proximity to the population of interest. The t-SNE in Step 3 identifies the immune cell populations CD45+, CD3+, CD20+ and CD8+ (blue), and the spatial profile can be mapped in the adjacent histology, represented as the cyan blue masks. The histoCAT analysis allows users to toggle between histology and t-SNE to a spatially resolved digital profile of the immune cell subpopulations within the tissue sample.



Conclusion

A comprehensive biology of the tumor microenvironment can be imaged for its complexity for the first time with metal-tagged antibodies given a large inventory of up to 37 available metals and up to 135 channels on the Hyperion Imaging System. Simultaneous detection of 4 to 37 metal-tagged antibodies optimizes characterization of the biology without artifacts that can be introduced with the use of serial sections. By providing an optimal balance between subcellular visualization and spatial localization of proteins, the Hyperion Imaging System revolutionizes imaging of tissue microenvironments for both FFPE and frozen tissue sections.

Related literature

- 1 Giesen, C., Wang, H.A., Schapiro, D., Zivanovic, N., Jacobs, A., Hattendorf, B., Schüffler, P.J., Grolimund, D., Buhmann, J.M., Brandt, S., Varga, Z., Wild, P.J., Günther, D., Bodenmiller, B. "Highly multiplexed imaging of tumor tissues with subcellular resolution by mass cytometry." *Nature Methods* (2014).
- 2 Chang, Q., Ornatsky, O., Siddiqui, I., Loboda, A., Baranov, V., Hedley, D.W. "Imaging mass cytometry." *Cytometry Part A* (2017).
- 3 Chang, Q., Ornatsky, O., Siddiqui, I., Straus, R., Baranov, V., Hedley, D.W. "Biodistribution of cisplatin revealed by imaging mass cytometry identifies extensive collagen binding in tumor and normal tissues." *Scientific Reports* (2016).
- 4 Mavropoulos, A., Lin, D., Lam, B., Chang, T.K.J., Bisgrove, D., Ornatsky, O. "Equivalence of Imaging Mass Cytometry and immunofluorescence on FFPE tissue sections." fluidigm.com
- 5 Ornatsky, O., Bouzekri, A., Allo, B., Watson, J. "In vitro drug effects on cancer cell morphology and functional state revealed by multiparameter Imaging Mass Cytometry." American Association for Cancer Research Annual Meeting (2017).
- 6 van der Maaten, L.J.P., Hinton, G.E. Visualizing high-dimensional data using t-SNE. *Journal of Machine Learning Research* (2008).
- 7 Villarroel-Espindola, F., et al. "Multiplexed analysis of fixed tumor tissues using imaging mass cytometry." Abstract 1635, Proceedings of the Annual Meeting of the American Association for Cancer Research. *Cancer Research* 77(13) (2017): doi:10.1158/1538-7445.AM2017-1635.
- 8 Schapiro D., Jackson H. W., Raghuraman S., Fischer, J.R., Zanotelli, V.R.T., Schulz, D., Giesen, C., Catena, R., Varga, Z., Bodenmiller, B. "histoCAT: analysis of cell phenotypes and interactions in multiplex image cytometry data." *Nature Methods* (2017).

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