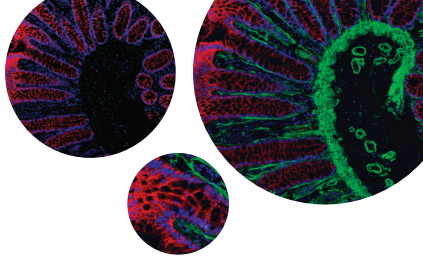
MYTH vs FACT



Uncovering the Truth about Imaging Mass Cytometry

Myths about high-multiplex imaging carry just enough plausibility to be perpetuated but are not based in fact. As experts in the field of Imaging Mass Cytometry™ (IMC™) and designers of the Hyperion™ Imaging System, Fluidigm takes great pride in sharing their transforming capabilities.

By uncovering the truth behind such myths, we present a clearer picture of the growing opportunities that high-multiplex imaging, and particularly IMC, offers.

Myth 1

Several new high-multiplex technologies are proven and published.

Fact 1

Imaging Mass Cytometry™ (IMC™) is the most proven and published high-multiplex imaging technology.

Myth 2

All imaging technologies must manage autofluorescence and signal overlap, and software can compensate for this regardless of approach.

Fact 2

Avoid problematic autofluorescence and background issues with Imaging Mass Cytometry.

Myth 3

High-multiplex imaging is slow.

Fact 3

Significant bias can be introduced when comparing various high-multiplex imaging approaches.

Myth 4

All workflows used to acquire and analyze data from different high-multiplex imaging approaches are similar.

Fact 4

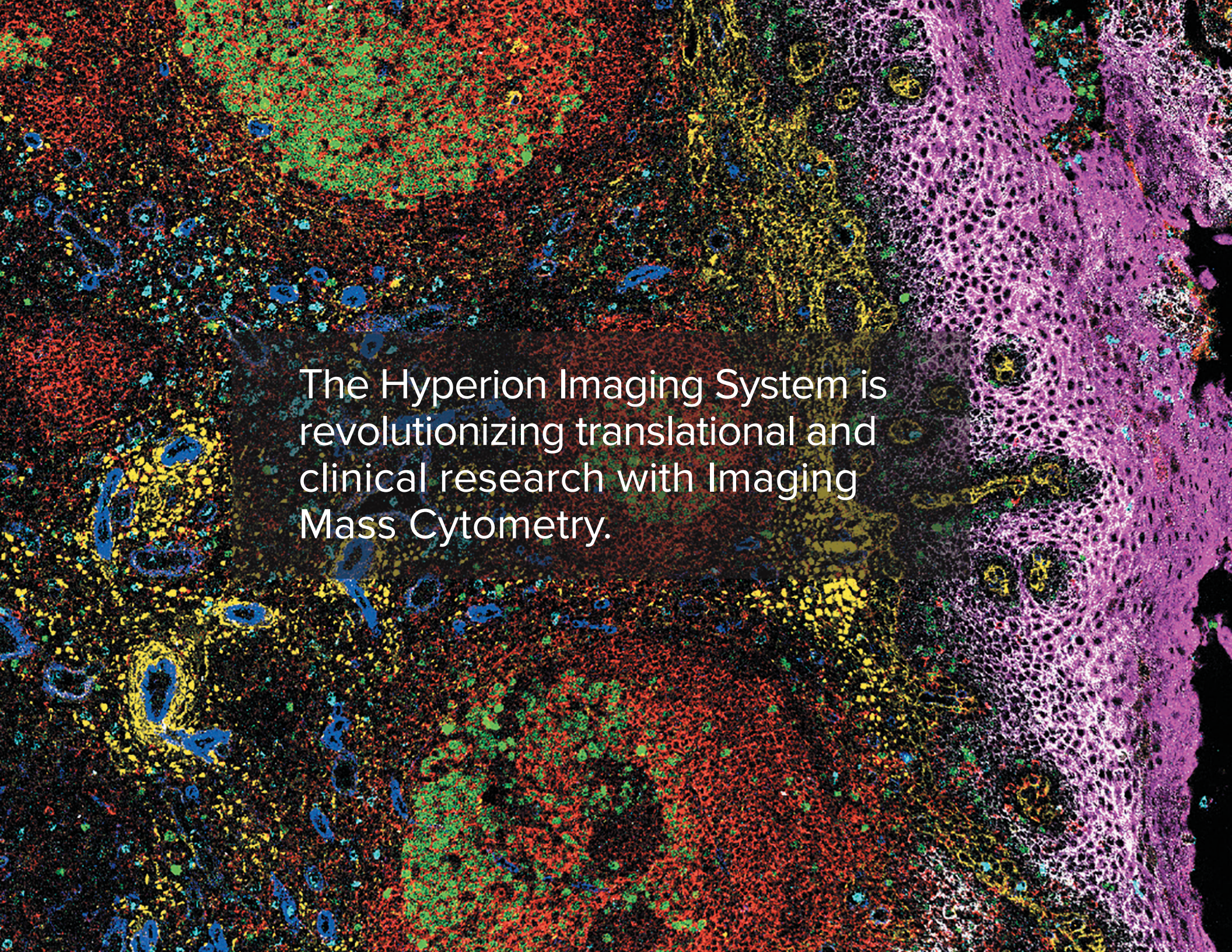
High-multiplex imaging workflows are not all the same.

Myth 5

Only specialists can successfully prepare and perform a high-multiplex imaging experiment.

Fact 5

It's easy to get an IMC project underway with minimal training and panel design support from our expert FAS team.


The Hyperion Imaging System is revolutionizing translational and clinical research with Imaging Mass Cytometry.

1 MYTH Several new high-multiplex technologies are proven and published.

FACT **Imaging Mass Cytometry™ (IMC™) is the most proven and published high-multiplex imaging technology.**

In 2017, Fluidigm pioneered development of and made commercially available the Hyperion Imaging System using IMC. This system was the first high-multiplex subcellular spatial imaging platform, offering significant progress over single-to-low-plex methods like H&E staining, immunohistochemistry (IHC) and immunofluorescence (IF), for in-depth phenotyping with a simple workflow.

The transformative Hyperion Imaging System earned the top spot in the Analytical Scientist Innovations Awards in 2017, leading as a vital translational and clinical research tool.

In 2019, other technologies were introduced, including co-detection by indexing (CODEX®), MIBI™-TOF and digital spatial profiling. In contrast to IMC (Figure 1), the limited hands-on experience and peer-reviewed publications with these other technologies currently restricts the community's understanding of detailed specifications and applications of each.

Figure 1. Unrivaled high-multiplex imaging with The Hyperion Imaging System.

2 MYTH

All imaging technologies must manage autofluorescence and signal overlap, and software can compensate for this regardless of approach.

FACT

Avoid problematic autofluorescence and background issues regardless of tissue type with the metal-tagged antibodies used in Imaging Mass Cytometry.

Autofluorescence can be problematic when using fluorescent labeling. In the shorter wavelengths, decreased signal-to-noise ratio can lead to loss of signal resolution, reduced sensitivity and the potential for false positives[1,2]. Certain sample preparations (particularly with FFPE) or tissue types, including liver, brain, lung and any tissues containing NADH, fatty acids or collagen, further confound imaging with increased autofluorescence[1]. Fluorescence detection also suffers from spherical and chromatic aberrations that can reduce signal and resolution even more. So, with longer wavelengths the fluorophore needs to be carefully paired to avoid functional markers.

Signal overlap, or spillover, a significant issue with fluorescence, can mask low expression levels and reduce specificity[3]. Special care must be taken in choosing appropriate fluorophores to avoid spectral overlap when using multiple tags simultaneously (Figure 2).

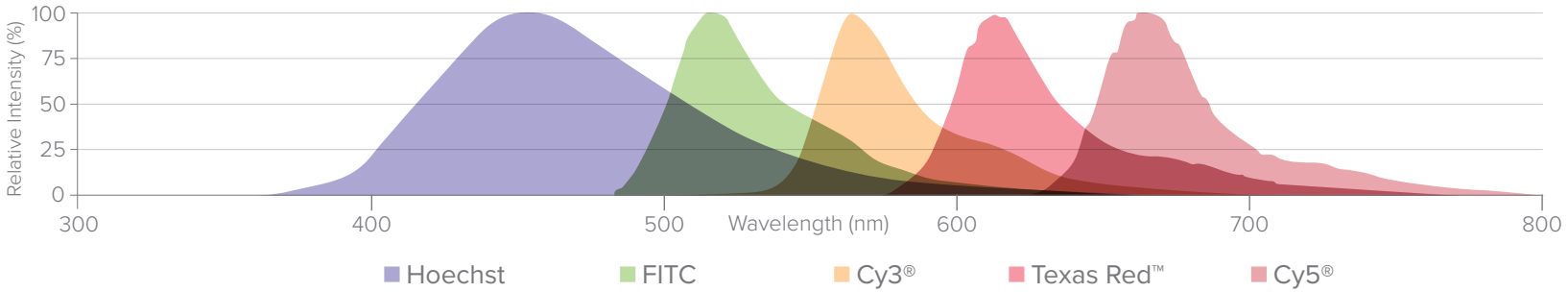


Figure 2. Emission spectra for a selection of commonly used fluorophores.

2 MYTH

All imaging technologies must manage autofluorescence and signal overlap, and software can compensate for this regardless of approach.

FACT

Avoid problematic autofluorescence and background issues regardless of tissue type with the metal-tagged antibodies used in Imaging Mass Cytometry.

Metal tag spectra are based on mass and are not endogenous to biological systems, so no background issues occur. Due to the high sensitivity of mass cytometry, neighboring isotopes can be readily resolved without spectral unmixing algorithms. This separation enables routine use of metal tags in high-multiplex experiments with minimal signal overlap (Figure 3). Any spillover can easily be accounted for by optimizing and tuning instruments, using only ultrapure isotopes, properly titrating reagents and adjusting panel design to account for metal impurities and expected intensity of each channel[3,4]. Further developments in IMC workflows are enabling generation of high-quality data devoid of spillover regardless of these standard recommendations[3].

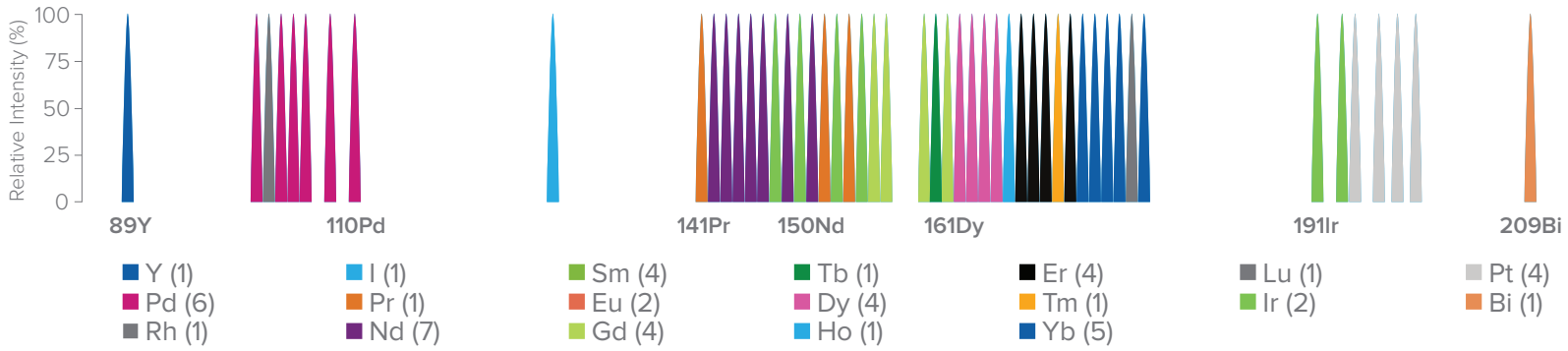


Figure 3. Metal-tagged antibodies used in IMC have separate and distinct signals

3 MYTH High-multiplex imaging is slow.

FACT

When what matters is a deep understanding of cell phenotype and function within a tissue microenvironment, there is no better solution than IMC.

Differences in time to results and instrument speed can depend on resolution, sample size or region of interest (ROI), the number of markers simultaneously imaged and scans needed to produce an informative image and for data analysis. The key here is to focus on what information is desired from an experiment, whether investigating targeted ROIs to effectively obtain depth of information or whole-slide imaging for a broader overview.

Various technologies can be made to appear faster than others, depending on the application:

- Sample throughput and time to results using CODEX are impacted by numerous clear and scan cycles necessary to capture multiple parameters in a single section[5]. These cycles can also create issues with tissue and antigen loss from repetitive clearing potentially leading to variability in measurements.

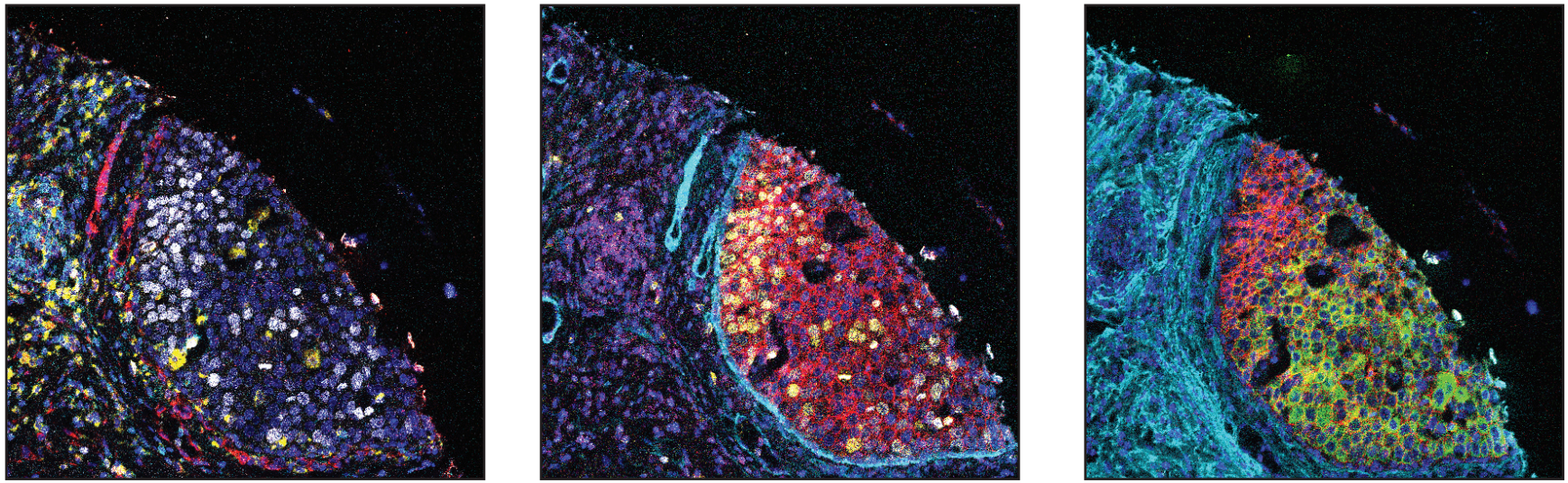
- Digital spatial profiling can combine steps to process multiple samples in parallel but cannot profile at single-cell resolution, and thus does not result in enough data points to sufficiently phenotype and decipher cellular composition and function[6].

- MIBI-TOF can achieve greater resolution under special operating circumstances, though increasing resolution increases the time required to generate an image[7].

3 MYTH High-multiplex imaging is slow.

FACT

When what matters is a deep understanding of cell phenotype and function within a tissue microenvironment, there is no better solution than IMC.



Typical study requirements that target selected ROIs to discover specific cell subsets, cell behavior and potential interactions are better enabled with high-resolution technologies offering true spatial context as shown here with IMC. Selected markers show immune populations and tissue structure in human breast cancer tissue.

4 MYTH

All workflows used to acquire and analyze data from different high-multiplex imaging approaches are similar.

FACT

The IMC workflow is familiar and straightforward. There are several, sometimes unexpected, differences between high-multiplex imaging technology workflows.

With high-multiplex imaging, there are several workflow-associated differences to consider, including the use of standardized protocols, single-scan acquisition, panel design and file manipulation (Table 1).

IMC provides a quantitative dataset from a standard IHC workflow. Apart from requiring more costly gold-plated slides, MIBI-TOF, like IMC, uses the same standard IHC workflows to stain tissues. With both IMC and MIBI-TOF, multiple metal-labeled antibodies are stained simultaneously and detected in a single scan. IMC data contains cellular context and is collected in a small, single file without additional manipulations. However, MIBI-TOF requires additional denoising to generate an image[7]. IMC also allows for real-time visual assessment of antibody staining at single-cell resolution so that cellular relationships can be discerned.

The CODEX platform requires tissue to be prepared on a small coverslip[5], which is incompatible with existing prepared samples on standard slides. Since this workflow generates a set of images based on several scans of a smaller marker set per scan, additional file handling via image stacking and proper alignment is necessary to view a comprehensive image of all markers[5].

Digital spatial profiling quantifies oligo counts and performs predictive counting[6], where oligo number per antibody can be highly heterogenous between clones and impact data interpretation. Analysis does not thoroughly relay information about cell phenotype and which cells express which markers, or accurately pinpoint cell location and cell interactions[9].

High-multiplex imaging workflow comparison

Technology	Prep	Stain	Acquire	Image	Analysis
Imaging Mass Cytometry™ (IMC™)	Uses metal-tagged Ab on tissue sections and cells on a **standard microscope slide.**	Stain with standard immunohistochemistry (IHC) protocol.	Manually pre-select region of interest (ROI) coupled to mass cytometry.	Signal extraction of n markers—single-scan acquisition at single-cell resolution.	Real-time quantitative IMC data is collected in a **small single file** that can be used to immediately generate publication-quality images.
Digital spatial profiling (DSP)	Uses oligo-conjugated Ab/RNA probes. Tissues are sectioned on a **standard microscope slide.**	Incubate tissue with fluorescent markers, antibodies and RNA probes tagged with photocleavable barcodes.	UV-cleave oligos on manually pre-selected ROI, aspirate, deposit in 96-well plate, hybridize for counting or purify for sequencing.	Ideally suited to investigating larger 200–400 µm ROIs **as a complement** to imaging-based methods.[8,10]	**Digital counting** by nCounter®/sequencing, ROI counts layered onto previously stained reference image.
CODEX®	Uses Ab-tagged oligos hybridized to complementary oligo-linked fluorophores. Tissues are sectioned onto a **glass coverslip.**	Stain with standard immunofluorescence (IF) protocol.	Perform fluorescent probe exposure, image, cleave to remove fluorophores and prep for next cycle.	Visualize selected fluorophores in each cycle to reconstruct digital image from all cycles.	**Stitching and alignment**[5] of multiple images/tile areas collected from each cycle results in a **large file size.**
			Repeat for *n* cycles depending on number of parameter sets needed.		
MIBI™-TOF	Uses metal-tagged Ab and tissue sectioned on **specialized gold slide.**	Stain with standard IHC protocol.	Manually pre-select ROI coupled to mass spectrometry.[9]	Composite image of n markers at single-cell resolution.	Post-processing including **alignment and MIBI-specific background removal before denoising.**

The workflows for each approach vary from slide prep to data acquisition and analysis, including the need for specialized equipment and step repetition. IMC maintains a simple workflow while providing a comprehensive dataset at the single-cell level in a single scan. Published references validate the use of IHC or quantitative IF with IMC[5-8]. Green and red text highlights the differences between workflows. Red sections indicate the need to repeat steps. Note that other cyclic IF protocols on alternative platforms exist that include variations to the CODEX workflow.

Workflow impact on ease of panel design:

- Order and assignment of fluorescent-tagged antibodies used with CODEX must be well-planned to maximize signal strength with variable marker expression.
- Antibody options can also be limited, particularly with digital spatial profiling, where panels are fixed, and customization can be costly.
- Metal-tagged antibodies used for IMC and MIBI-TOF provide more flexibility to better customize panels with markers that can be easily substituted as experimental needs change.
- To simplify IMC, a selection of pre-designed panels can be used with or without additional markers.

5 MYTH

Only specialists can successfully prepare and perform a high-multiplex imaging experiment.

FACT

It's easy to get an IMC project underway with minimal training and panel design support from our expert FAS team.

"I try to convey to users who are interested in using the technology, that if you've done any amount of immunostaining in the past—whether it's IHC or immunofluorescence—from a technical standpoint much of the workflow is very similar. You're putting antibodies onto tissue, waiting a certain amount of time, washing them off. It's not that technically challenging. Even though it might feel complicated, it really is quite easy to use." —Trevor McKee, PhD, Image Analysis Manager at STTARR

The growing IMC community, unique among high-multiplex technologies, has hundreds of users from novices to early adopters who attend global and regional user meetings each year. These meetings are a fantastic opportunity to network, share successes and ideas, address challenges and learn more about IMC. Furthermore, access to panel kits, training resources and field application specialists caters to those new to the technology and those who want to expand its use.

"The user group meetings are an excellent way to network and to talk to other people, and I think in the IMC community there is a very collaborative atmosphere." —Kevin Kelly, MD, PhD, Associate Professor at Keck School of Medicine

While data analysis can be challenging for any high-multiplex experiment, workflows and data acquisition are becoming more streamlined as software advances and new approaches to analysis are developed. CyTOF® Software v7.0, released in mid-2019 for IMC, enables simple scanning procedures and enhances ROI selection, allowing users to more easily interact with pathologists to select ROIs and get all desired information from a sample in one experiment.

As the first platform capable of investigating protein expression with high-quality phenotyping resolution, IMC is supporting the expansion of high-dimensional applications to proteomics research and the study of single cell behavior. IMC has been used and integrated into more labs than any other high-parameter multiplex imaging technology. It has a growing portfolio of consumable content and is still expanding the boundaries of what is known.

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