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A Hitchhiker's guide to high-dimensional tissue imaging with multiplexed ion beam imaging

Yao Yu Yeo[a,b,†], **Precious Cramer**[a,†], **Addison Deisher**[a], **Yunhao Bai**[c], **Bokai Zhu**[c], **Wan-Jin Yeo**[d], **Margaret A. Shipp**[e], **Scott J. Rodig**[f], **Sizun Jiang**[a,b,g,h,*]

[a]Center for Virology and Vaccine Research, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA, United States

[b]Program in Virology, Division of Medical Sciences, Harvard Medical School, Boston, MA, United States

[c]Department of Microbiology & Immunology, Stanford University School of Medicine, Stanford, CA, United States

[d]Department of Physics, Institute of Learning and Brain Sciences, University of Washington, Seattle, WA, United States

[e]Department of Medical Oncology, Dana Farber Cancer Institute, Harvard Medical School, Boston, MA, United States

[f]Department of Pathology, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, United States

[g]Department of Pathology, Dana Farber Cancer Institute, Harvard Medical School, Boston, MA, United States

[h]Broad Institute of MIT and Harvard, Cambridge, MA, United States

Abstract

Advancements in multiplexed tissue imaging technologies are vital in shaping our understanding of tissue microenvironmental influences in disease contexts. These technologies now allow us to relate the phenotype of individual cells to their higher-order roles in tissue organization and function. Multiplexed Ion Beam Imaging (MIBI) is one of such technologies, which uses metal isotope-labeled antibodies and secondary ion mass spectrometry (SIMS) to image more than 40 protein markers simultaneously within a single tissue section. Here, we describe an optimized MIBI workflow for high-plex analysis of Formalin-Fixed Paraffin-Embedded (FFPE) tissues following antigen retrieval, metal isotope-conjugated antibody staining, imaging using the MIBI instrument, and subsequent data processing and analysis. While this workflow is focused on imaging human FFPE samples using the MIBI, this workflow can be easily extended to model systems, biological questions, and multiplexed imaging modalities.

*Corresponding author: sjiang3@bidmc.harvard.edu.
†Authors contributed equally.

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1. Introduction

The tissue microenvironment (TME) consists of many cell types, which orchestrate key roles in biological processes, including oncogenesis, microbial pathogenesis, and differential clinical responses to diseases and therapy (Hanahan & Weinberg, 2011; Jiang et al., 2022; Keren et al., 2018, 2019; Meacham & Morrison, 2013). Our appreciation of the diverse composition of the TME has traditionally been limited by the low-plex nature of tissue imaging techniques. The advent of high-dimensional imaging technologies, including MIBI, now enables the visualization of TMEs in tissue samples at unprecedented detail and depth. This allows the delineation of cellular identities, tissue architectures, and host-disease immune interactions in situ.

Prior to advancements in high-dimensional imaging, conventional methods for understanding cellular compositions within tissues have been generally limited to brightfield immunohistochemistry (IHC) or immunofluorescence (IF) (Fig. 1, top). IHC and IF are generally capable of measuring the expression of two to five proteins, respectively, within tissue sections. Orthogonally, developments in flow cytometry are routinely capable of measuring over 10 parameters, albeit within cell suspensions from dissociated tissues without their spatial context (Bandura et al., 2009; Baumgarth & Roederer, 2000; Coons, Creech, & Jones, 1941; de Matos, Trufelli, de Matos, & da Silva Pinhal, 2010; Fulwyler, 1965; Hall & Lane, 1994; Spitzer & Nolan, 2016). MIBI is a multidimensional imaging technology that circumvents the limitations of IHC, IF, and flow cytometry by retaining the native architecture of tissues, while enabling deep profiling and phenotyping capabilities across individual cells in their native tissue context (Angelo et al., 2014).

The fundamental principle of MIBI leverages upon tissue staining with antibodies conjugated to monoisotopic lanthanide reporters, followed by detection via SIMS (Fig. 1, bottom) to quantify these antigens with high resolution and sensitivity (Angelo et al., 2014). Tissue sections are first incubated with a customized panel of primary antibodies, each labeled with distinct lanthanide metal reporters. A primary ion beam is then directed at the tissue section to rasterize ions located therein in a flyback manner. This process liberates the metal reporters into secondary ions, which are then extracted and quantified using a time-of-flight mass spectrometer. Extraction of the masses present within each pixel space results in an image that simultaneously delineates the expression and spatial distribution of multiple proteins within the tissue of interest.

Given the rare observation of lanthanides in biological samples, their use in biological imaging results in a low intrinsic background. The mass spectrometry imaging modality of MIBI also circumvents the autofluorescence generally associated with fluorescence imaging. The uniqueness of the antibody-isotopic combinations and SIMS approach also allows for their simultaneous quantification, different from the cyclical approaches required for multiplexed immunofluorescence imaging. Tissues are thus not subject to multiple iterations of staining and imaging, which may introduce potential artifacts or limit the multiplexing capabilities (Hickey et al., 2022). MIBI also enables robust quantitative analyses, as primary antibodies are labeled using maleimide-labeled polymers containing multiple coordination sites capable of retaining up to 20 metal ions per polymer, with 4–6 polymers labeling each

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antibody (Han, Spitzer, Bendall, Fantl, & Nolan, 2018). This allows for high sensitivity and dynamic range for the quantification of protein targets and beyond (Keren et al., 2019).

Currently, the MIBI platform can detect approximately 40 markers, with the number of possible markers expected to continue expanding with new developments in chemical conjugation reagents. The MIBI has since been commercialized by Ionpath Inc. and is now available for mainstream usage. While the MIBI is capable of imaging any form of biological samples compatible with slides, we detail here the workflow for successful MIBI staining and imaging of archival FFPE tissue samples. Principles from this workflow can be easily adapted for fresh-frozen tissue imaging and other high-dimensional imaging modalities.

2. Antibody selection, validation, and conjugation

It is pertinent to select antibodies that recognize their targets with high specificity and minimal cross reactivity. A well-designed high-plex antibody panel should also provide information on cellular phenotypes and function within the tissue context. For instance, CD45RO, CD45RA and CD8 can be combined to identify memory and naive subpopulations of CD8+ T cells, Ki-67 to identify proliferative cells, and PD-1, CTLA-4, LAG-3, TIM-3 and TIGIT to evaluate T cell immunoregulatory pathways. Importantly, many of these markers are not limited to T cells and can be expressed on other cell types within the tissue, showing the importance to include markers delineating major cell types expected within the tissue of interest. The addition of a ubiquitous cell surface marker (e.g., Na/K-ATPase or HLA-ABC) may also greatly facilitate downstream cell segmentation.

Antibodies are manually validated by performing traditional IHC on FFPE tissue or cell pellet sections. While FFPE tissue sections are ideal, cell pellet sections can also be useful for testing distinct targets, including specific viral antigens or proteins known to be differentially expressed between cell types. Although many commercially available antibodies have been pre-evaluated via IHC on FFPE tissue sections, we highly recommend to re-validate antibodies manually as the antigen retrieval conditions, type of tissue, and tissue fixation conditions may vary broadly. In general, monoclonal antibodies are preferred as they allow batch-to-batch consistency, particularly for antibody conjugation, but polyclonal antibodies have performed well at times. For more details on target selection and validation, we refer readers to the following resources (Hickey et al., 2022; Liu et al., 2022).

After the antibody panel is assembled, each validated antibody is assigned to a different metal isotope for conjugation. The choice of metal isotope to be conjugated is dependent on the expression level of the antigen to be detected, the binding efficiency of the antibody, and the potential mass bleed-through (usually +1, +16 and +17 m/z). Readers are referred to the following resource that may help guide these choices (Keren et al., 2019). Prior to conjugation, antibodies must be in carrier-free formulations to ensure accurate antibody quantification and maximize labeling efficiencies. Most additives, such as small molecule preservatives (e.g., sodium azide, glycerol, trehalose), and standard buffer salts are generally acceptable (Hartmann et al., 2019).

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3. Methods

3.1 Pre-treatment of gold-coated slides with Vectabond®

MIBI requires sample conductivity for efficient ionization of the metal isotope-conjugated antibodies. This requires tissue sections to be mounted onto gold-coated slides for sample conductivity, and to minimize background interference or sample charging. To promote the adherence of tissue sections, gold-coated slides are pre-treated with Vectabond®, a reagent that chemically modifies the surface of the gold slides with positive charges to generate a highly adherent surface.

1. In a fume hood, fill a glass staining dish with 125mL of 100% acetone and 2.5mL of Vectabond®.
2. Place gold-coated slides onto a staining rack and incubate in the diluted Vectabond® for 5min.
3. Rinse the Vectabond®-treated gold-coated slides in distilled water, taking care to not create any bubbles. This should stop quench any excess reagents. Gently tap the slide rack to remove excess water.
4. Air dry the slides and store in a cool, dry place for future use.

3.2 Tissue preservation and sectioning

FFPE is a standard procedure for tissue preservation due to its cost-effectiveness, ease of handling, and benefit of long-term storage at ambient temperatures. Tissues harvested post necropsy or biopsy are fixed with formalin, embedded into paraffin blocks, and then sectioned onto gold-coated slides pre-treated with Vectabond®. These steps can be performed at most histology core facilities accessible to the reader. FFPE tissue blocks are best stored in a cool, dry place for future use. We recommend the storage of sectioned tissue slides within a sealed chamber, such as a vacuum desiccator, to preserve protein epitope integrity.

3.3 Preparation of tissue sections for staining

The highly cross-linked nature of FFPE tissues often masks protein epitopes and prevents efficient antibody staining (Bogen, Vani, & Sompuram, 2009; Leong & Leong, 2007; Shi, Key, & Kalra, 1991; Stumptner, Pabst, Loibner, Viertler, & Zatloukal, 2019). Deparaffinization, rehydration, and Heat-Induced Epitope Retrieval (HIER) are steps aimed at unmasking protein epitopes through the partial reversal of chemical crosslinks between epitopes (Bogen et al., 2009; Leong & Leong, 2007; Shi et al., 1991; Stumptner et al., 2019) (Fig. 2). Below is an optimized HIER protocol from our lab that has worked robustly across multiple species and tissue types.

5. *Deparaffinization*: Gold-coated slides containing FFPE tissue sections are baked at 70°C for 1h in a conventional laboratory oven. The paraffin should be visibly melted at the end of the hour. Should the paraffin still appear as a white solid, we recommend baking for longer and using a thermometer to ensure that the oven is at a consistent temperature.

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6. *Rehydration*: Post baking, tissue sections are immediately soaked in xylenes for 10min twice before being subjected to further washes below. Xylenes is a hydrophobic reagent used to dissolve and remove any remaining paraffin on the slide, and should be handled in a fume hood with extreme caution due to its toxicity.

   The following steps can be performed manually or using an automated staining device such as the Leica ST4020 Small Linear Stainer in this protocol. The Linear Stainer is set to perform 3 dips per interval and 180s per container of reagent. Prepare the reagents in the following order:

   i. Xylenes (×3)

   ii. 100% EtOH (×2)

   iii. 95% EtOH (×2)

   iv. 80% EtOH

   v. 70% EtOH

   vi. ddH_2O (×3)

   vii. Holding tank with ddH_2O

   Once the slides have completed their 2 × 10min xylenes washes, they can be placed onto the Linear Stainer to begin the rehydration process. The graded concentrations of ethanol to water are optimized to facilitate the removal of xylenes while gently rehydrating tissue sections.

7. *HIER*: While tissue rehydration is proceeding, prepare the PT module accordingly:

   a. Program the PT module with the following parameters: prewarm to 75°C → heat to 97°C for 20min → cool to 65°C.

   b. Fill the PT module chamber with 1 × PBS.

   c. Fill a slide holder with 1 × Antigen Retrieval Buffer (pH 9.0) and place it in the PT module.

   d. Ensure that the PBS level in the PT module is below the slide holder, without triggering the “low water level” sensor of the PT module.

   e. Click “Start” on the PT Module, which will trigger it to prewarm the Antigen Retrieval Buffer to 75°C. The PT Module will maintain the temperature until stopped.

   After rehydration (Step 6 above), transfer the slides from the ddH_2O holding tank into the prewarmed slide chamber in the PT Module containing the warm 1 × Antigen Retrieval Buffer. Close the lid and click “Start” once more to start the HIER process. You should hear a clicking sound to indicate that the PT Module has auto-locked the chamber and is proceeding to the preset temperature.

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Note that HIER can also be performed by completely submerging the slides in a beaker containing 1 × Antigen Retrieval Buffer (pH 9.0) and boiling on a hot plate. A pressure cooker has also been used commonly by histology labs with success. We find the PT module to give the most consistent results.

8. After HIER, we cool down the slides before circling each tissue section with a hydrophobic PAP pen. This will facilitate downstream blocking and staining steps by helping to localize reagents.

    a. Once the PT module cools to 65°C and starts to beep, remove the slide chamber containing the slides and allow it to cool further for ~30min to reach ambient temperature.

    b. Rinse the slides twice in ddH_2O for 2min each using Coplin jars.

    c. Flick off excess ddH_2O and circle each tissue section with a hydrophobic PAP pen, taking care to avoid contact between the pen and the tissue.

    d. Briefly air dry the slide by gently flicking the slide a few times.

    e. Return the slide back into ddH_2O to ensure the sections remain wet. The user is encouraged to visually inspect the slides at this point to confirm that the hydrophobic barrier drawn using the hydrophobic PAP pen is intact around the tissue of interest.

    f. The slides are now ready for the blocking and antibody staining. Ensure slides are always well hydrated to avoid potential non-specific staining. This is also a good pause point to check and ensure that the hydrophobic barrier from the pap pen is intact around the tissue.

3.4 Antibody staining and crosslinking

Prior to MIBI staining, antibodies should be validated via IHC to ensure their specificity against target antigens (Uhlen et al., 2016) (see Section 2). Although a validated antibody may be specific for its target antigen, non-specific binding to other molecules or regions in the tissue can still occur. Blocking reagents, such as serum from an unrelated species and bovine serum albumin (BSA), are therefore incubated with the tissue slide to mask non-specific binding sites prior to antibody staining. Post antibody staining and washing, tissues are crosslinked to retain the antibody-bound complexes for downstream MIBI imaging. This crosslinking step is also thought to help retain tissue morphology when the dehydrated tissue slides are introduced to the high vacuum conditions of the MIBI imaging chamber, akin to high resolution electron-microscopy imaging (Angelo et al., 2014).

9. Block the slides in MIBI Blocking Buffer for 1h at room temperature to reduce non-specific binding of the Antibody Cocktail. The slides should be kept in a humidity chamber to minimize evaporation.

    a. The humidity chamber can be created by filling an empty pipette tip box with water and damp paper towels.

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10. During the blocking step, prepare the Antibody Cocktail by diluting antibodies in MIBI Antibody Diluent Buffer. Take note to maintain all antibodies and the Antibody Cocktail mixture on ice to minimize antibody denaturation.

    a. The antibody concentration titrated during IHC validation is an excellent starting point to determine the optimal antibody concentration for MIBI. However, the optimal concentration for each antibody often requires further optimization, due to variables including antibody labeling efficiency, metal isotope conjugated, and tissue type. Therefore, further titrations on control tissues are highly recommended prior to the full MIBI imaging session.

    b. We recommend using a brand-new box of pipette tips during this step, both to reduce potential cross-contamination, and to better keep track of the pipetting order of the antibodies.

11. After 1h, gently tip the slide on its side and tap on a Kimwipe to remove excess MIBI Blocking Buffer. Add the Antibody Cocktail (from Step 10), ensuring that the entire tissue is covered while minimizing bubbles. Typically, ~100μL is enough to cover the entire slide, and this volume can be further reduced for smaller tissue sections.

12. Incubate the tissue slides with the Antibody Cocktail at 4 °C overnight in a humidity chamber. This can be made using an empty pipette box containing damp paper towels in the bottom well.

13. The next day, wash the slides in MIBI Wash Buffer for 5min with gentle agitation. Repeat this twice more.

14. Flick off excess MIBI Wash Buffer and arrange the slides on paper towels, facing up in a fume hood.

15. In the fume hood, crosslink the antibodies and tissue by pipetting MIBI Post-Fixation buffer onto the tissue sections, and leaving the slides for 15min. We recommend ~250μL of MIBI Post-Fixation buffer for each slide. Pipette gently to avoid spilling the MIBI Post-Fixation buffer.

    a. CAUTION: The MIBI Post-Fixation Buffer must be handled with care and disposed of in appropriate waste containers as it contains PFA and glutaraldehyde that are toxic reagents.

16. Handling the slides containing MIBI Post-Fixation Buffer one-at-a-time, dunk the slides into a Coplin jar containing 1 × PBS, before moving the slide into a separate Coplin jar containing Tris Quenching Buffer. Slides should be handled individually and with care. Slides should be washed in Tris Quenching Buffer for three times, at least 1min each.

    a. CAUTION: All buffers from this step should be disposed of in well-labeled chemical waste bottles. Coplin jars can be rinsed with more Tris Quenching buffer to remove any residual crosslinkers.

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17. Dehydrate the slides in increasing concentrations of ethanol on a robotic linear staining (3 dips per interval, 60s intervals), or manually as follows:
    i. ddH_2O (×3)
    ii. 70% EtOH
    iii. 80% EtOH
    iv. 95% EtOH (×2)
    v. 100% EtOH (×2)
    vi. Empty holding tank
18. Store the dehydrated slides in a vacuum desiccator prior to imaging on the MIBI-TOF. Slides are very stable at this point if stored appropriately. We have been successful in imaging slides up to 2years post-staining with no noticeable degradation of signal.

3.5 MIBI imaging

MIBI images should be acquired with consideration of the manufacturer's guidelines. For the data presented in this article, images were acquired using a custom alpha-iteration of the MIBI-TOF mass spectrometer equipped with a duoplasmatron ion source (Jiang et al., 2022; Keren et al., 2019). The machine's parameters are set as previously described (Bai et al., 2021). Images were extracted from the binary mass spectrum files and processed with MIBIAnalysis tools (Keren et al., 2018).

3.6 Data processing

For each acquired field of view (FOV), the time- and space-resolved TOF data are extracted into individual TIFF files for each mass channel. Next, FOVs are subjected to background removal, which is generally done using Au (gold) as it constitutes the blank regions of the MIBI slides not covered by tissues. Subsequently, the data is "denoised" via Gaussian smoothing and manual thresholding using the K-nearest neighbor algorithm before a final aggregate removal (Fig. 3A). We refer readers to the following resource for a detailed explanation of the algorithms behind this process (Baranski et al., 2021).

To delineate single cells within the tissue section, cell segmentation is next performed using Mesmer from the DeepCell library (Greenwald et al., 2022; Van Valen et al., 2016). We recommend using a strong nuclear marker (e.g., Histone H3) and membrane markers that robustly stain the cell membranes of all cells (e.g., Na/K-ATPase or HLA-ABC) to ensure optimal performance (Fig. 3B). We manually adjust the model_mpp, maxima_threshold, and interior_threshold parameters to fine-tune segmentation results depending on the tissue of interest and MIBI imaging parameters. Single-cell features (scaled by cell size) are then extracted from the high-dimensional MIBI images into single-cell FCS files (Bai et al., 2021). These files can be imported into most flow-cytometry specific software, or easily read by most programming languages.

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For further processing, we generally normalize channel counts for each individual cell using arcsine transformation, before performing a floor and ceiling thresholding (generally within the range of 0.1–99.9%) to account for noise or oversaturated signals. The final count for each cell is then normalized to a global 0–1 scale for each channel. Selected cellular features, usually ones delineating cell phenotypes, are clustered using FlowSOM (Van Gassen et al., 2015) and visualized using Marker Enrichment Modeling (MEM) (Diggins, Greenplate, Leelatian, Wogsland, & Irish, 2017) (Fig. 3C, left). We recommend over clustering followed by merging of similar clusters to facilitate more accurate and granular cell type identification. Finally, phenotype maps of each cell can be visualized by plotting the annotated cellular composition within each FOV (Fig. 3C, right). All computational scripts related to data and analysis here can be found at https://github.com/SizunJiangLab/MCB-MIBI.

3.7 Materials

3.7.1 Reagents and equipment

Products	Source	Identifier
Gold-coated slides	Ionpath	567001
Staining dish/rack and glass cover	EMS	71426
Vectabond® Reagent	Vector Labs	SP-1800
Acetone	Fisher	A949-4
Xylenes (histological grade)	Sigma-Aldrich	534056-500
200 Proof Ethanol	Pharmco	111000200CSPP
Ultrapure H_2O	Fisher	10977-023
ImmEdge® Hydrophobic Barrier PAP Pen	Vector Labs	H-4000
TBS IHC wash buffer with Tween 20	Cell Marque	935B-09
Targeted retrieval solution pH 9	Agilent (Dako)	S2375
Slide chamber	EMS	62705-01
Donkey serum	Sigma-Aldrich	D9663-10ML
Sodium azide	Sigma	S8032
Triton X-100	Sigma	T8787
Bovine serum albumin (BSA)	Fischer	BP1600-100
16% PFA (Aqueous, EM grade)	EMS	15710
8% Glutaraldehyde (Aqueous, EM grade)	EMS	16020
10× PBS	Fisher	70011044
1 M Tris-HCl buffer, pH 7.5	Fisher	15567027

3.7.2 Equipment

Heratherm OGS60 Lab Oven	Fisher	51028112
Thermo Scientific™ Lab Vision™ PT Module	Fisher	A80400012
Leica ST4020 Small Linear Stainer	Leica	14050946425

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Cabinet Style Vacuum Desiccator	SP Bel-Art	F42400-4031
Clear orbital shaker	Boekel	270200

3.7.3 Recipes

3.7.3.1 95% Ethanol

Reagent	Final concentration	Amount (mL)
100% Ethanol	95% v/v	950
ddH_2O	n/a	50

Store at RT for up to 6 months.

3.7.3.2 80% Ethanol

Reagent	Final concentration	Amount (mL)
100% Ethanol	80% v/v	800
ddH_2O	n/a	200

Store at RT for up to 6 months.

3.7.3.3 70% Ethanol

Reagent	Final concentration	Amount (mL)
100% Ethanol	70% v/v	700
ddH_2O	n/a	300

Store at RT for up to 6 months.

3.7.3.4 1 × Antigen retrieval buffer (pH 9.0)

Reagent	Final concentration	Amount (mL)
10× pH 9.0 Dako	1×	8
ddH_2O	n/a	72
Total	**n/a**	**80**

Make fresh for each use.

3.7.3.5 10% Bovine serum albumin (BSA) w/v

Reagent	Final concentration	Amount
BSA	10% w/v	5g
ddH_2O	n/a	50 mL
Total	**n/a**	**50 mL**

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Aliquot in 10mL aliquots and store at −20°C for up to a year.

3.7.3.6 MIBI wash buffer

Reagent	Final concentration	Amount (mL)
10% w/v BSA	0.1% w/v	10
20× TBS-T	1×	50
ddH_2O	n/a	940
Total	**n/a**	**1000**

Store at 4°C for up to a year.

3.7.3.7 10% Sodium azide

Reagent	Final concentration	Amount
Sodium azide	10% w/v	1g
ddH_2O	n/a	Fill to 10 mL
Total	**n/a**	**10 mL**

Aliquot in 1mL aliquots and store at −20°C for up to a year.

3.7.3.8 10% Triton X-100

Reagent	Final concentration	Amount (mL)
Triton X-100	10%	5
ddH_2O	n/a	45
Total	**n/a**	**50**

Store at RT for up to a year.

3.7.3.9 MIBI blocking buffer

Reagent	Final concentration	Amount
20× TBS IHC wash buffer with Tween 20	1×	500 µL
Donkey serum	5%	500 µL
10% Triton X-100	0.1%	100 µL
10% Sodium azide	0.05%	50 µL
Ultrapure H_2O	n/a	8.85 mL
Total	**n/a**	**10 mL**

Aliquot in 1mL aliquots and store at −20°C for up to a year.

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3.7.3.10 MIBI antibody diluent buffer

Reagent	Final concentration	Amount
20× TBS IHC wash buffer with Tween 20	1×	500 μL
Donkey serum	5%	500 μL
10% Sodium azide	0.05%	50 μL
Ultrapure H_2O	n/a	8.95 mL
Total	**n/a**	**10 mL**

Aliquot in 1mL aliquots and store at −20°C for up to a year.

3.7.3.11 MIBI post-fixation buffer

Reagent	Final concentration	Amount (mL)
16% PFA	4%	10
8% Glutaraldehyde	2%	10
10× PBS	1×	4
Ultrapure H_2O	n/a	16
Total	**n/a**	**40**

Store at 4°C for up to 3 months.

3.7.3.12 Tris quenching buffer

Reagent	Final concentration	Amount (mL)
1 M Tris-HCl buffer	50 mM	25
Ultrapure H_2O	n/a	475
Total	**n/a**	**500**

Store at RT for up to 6 months.

4. Discussion

We detail here the framework for high-dimensional tissue staining, imaging, and downstream analysis to enable novel insights into biological processes in their native tissue context. While this article focuses on the MIBI, the general protocol and framework is applicable to other tissue imaging modalities, including CODEX (Co-Detection by indDExing), CycIF (Cyclic Immunofluorescence), and IMC (Imaging Mass Cytometry) (Giesen et al., 2014; Goltsev et al., 2018; Lin et al., 2018).

Despite the broad utility of multiplexed imaging technologies, several limitations persist. Like most high-plex imaging modalities, MIBI is only applicable to fixed tissue sections and is unable to allow live imaging of biological processes and samples. Novel approaches in this regard may allow complementary approaches for a systems-level understanding of biological processes *in situ* (Ko et al., 2022). While high-plex imaging is largely limited to protein markers to date, recent developments such as PANINI (Protein And Nucleic acid

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IN situ Imaging) allows concurrent staining of proteins and nucleic acids in FFPE tissue sections, enabling the potential to address new biological questions such as the composition and microenvironment around HIV tissue reservoirs (Jiang et al., 2022). We are confident that multiplexed imaging methods, including MIBI, will allow the innovation of therapeutic intervention strategies and expand our understanding of complex disease mechanisms.

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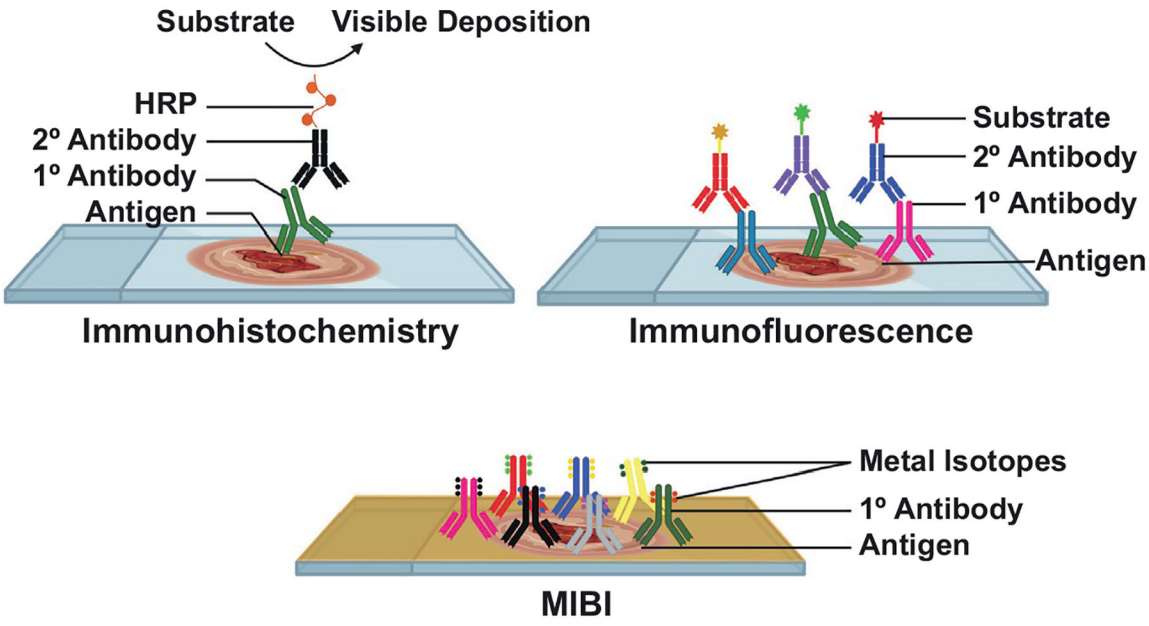


Fig. 1.

Schematic of antibody staining approaches across IHC, IF, and MIBI platforms. Immunohistochemistry (IHC) and immunofluorescence (IF) often utilize secondary antibodies, conjugated to an enzyme or a fluorophore, respectively, to visualize proteins targeted by a primary antibody. MIBI directly detects antigens via primary antibodies conjugated to 4–6 polymers, each containing up to 20 metal isotopes. A maximum of 2 antigens are detectable with IHC and ~5 antigens with IF, while the current iteration of MIBI is capable of measuring 40 antigens simultaneously.

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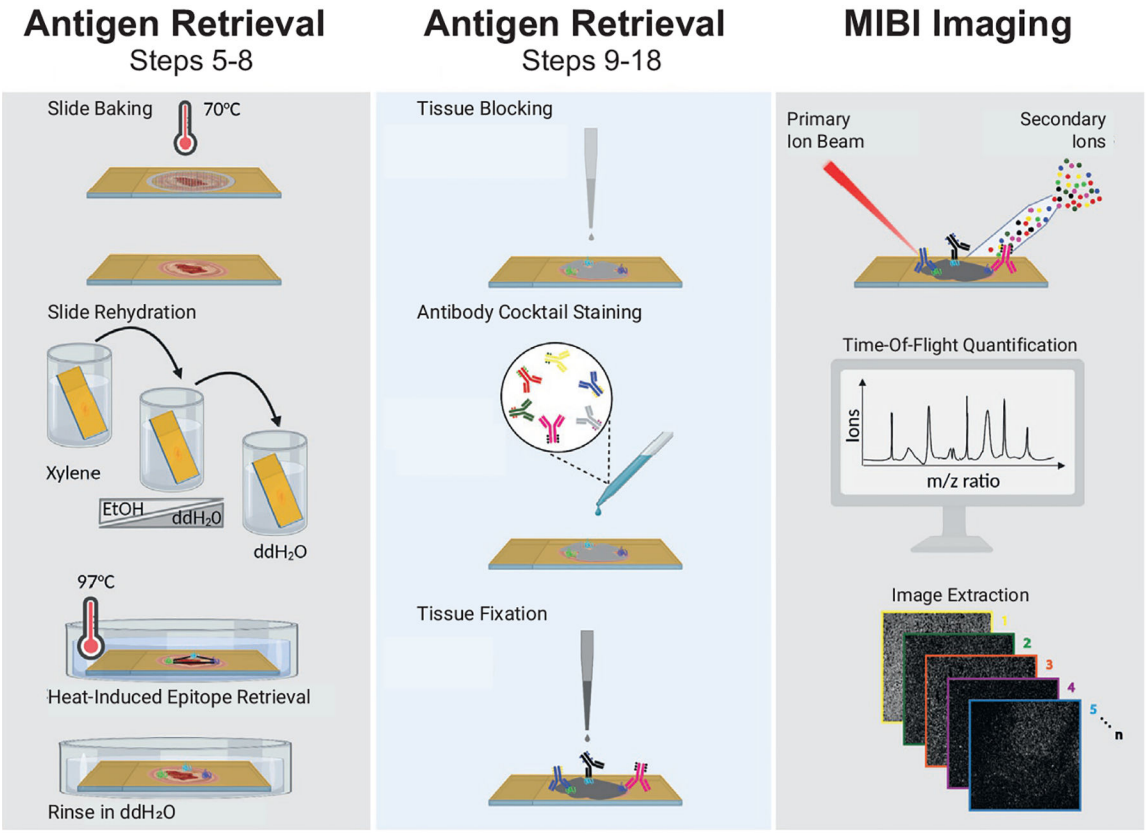


Fig. 2.

Experimental workflow for MIBI-TOF. Paraffin-embedded tissue slides are deparaffinized by baking at 70°C and soaking in xylenes. The slides are then rehydrated with decreasing concentrations of ethanol to water ratios. Post rehydration, slides are subjected to Heat-Induced Epitope Retrieval (HIER) before blocking and staining with an antibody cocktail. Antibodies are crosslinked to the tissue before MIBI imaging, where secondary ions liberated by the primary ion beam are detected, and quantified by a time-of-flight detector for downstream analysis.

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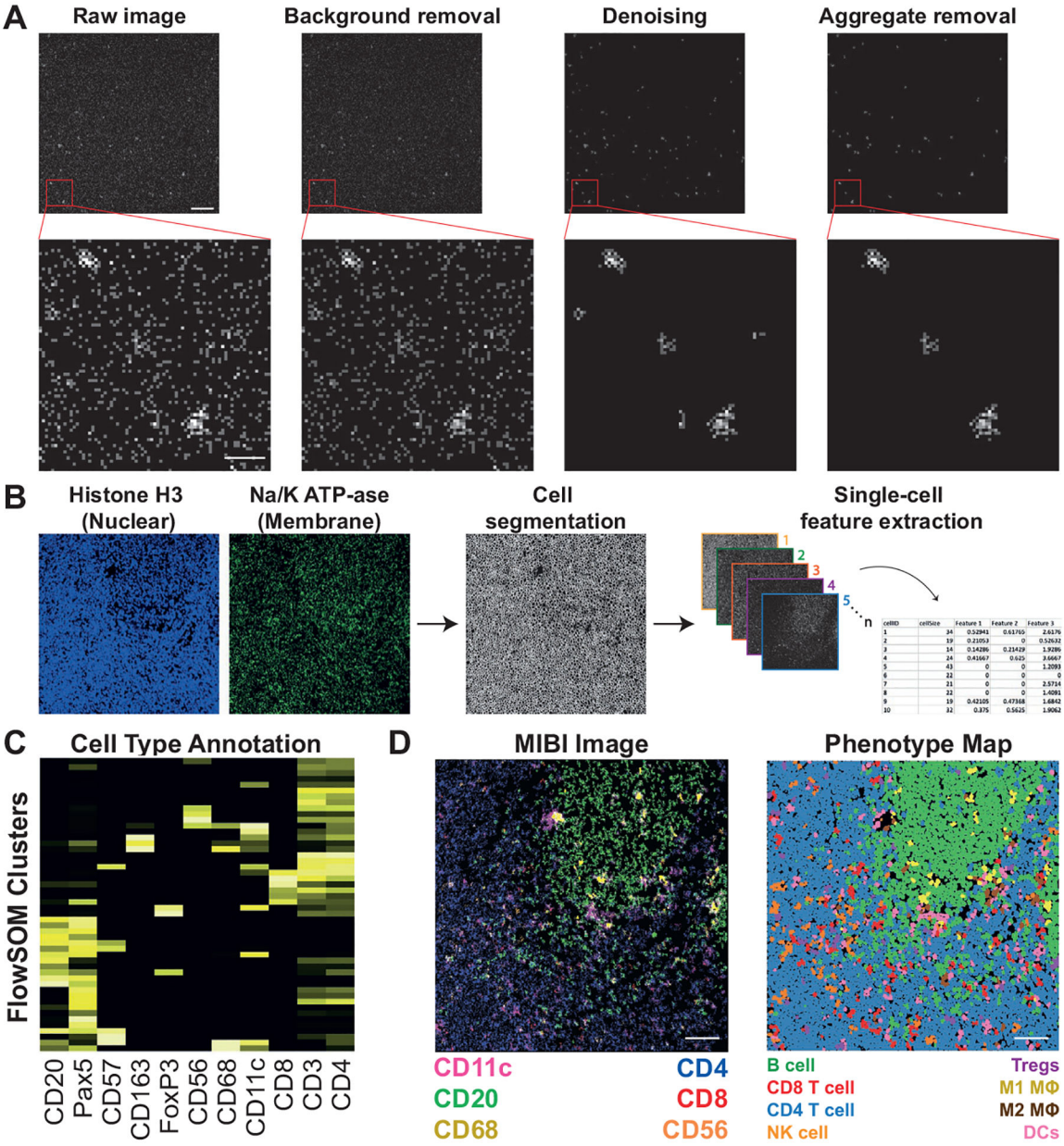


Fig. 3.

Data processing after MIBI imaging of a reactive lymph node tissue section. (A) An overview of the low-level processing steps for raw MIBI images, encompassing background removal, denoising and aggregate removal. Scale bars represents 50μm in the top row and 10μm in the bottom row. (B) Cell segmentation is performed using nuclear and membrane markers, such as Histone H3 and Na/K-ATPase in this case. Single-cell features are then extracted and scaled by cell size for downstream analysis. (C) Cells are clustered based on selected lineage-specific markers using FlowSOM. Manually annotation of cell clusters allow the visualization of cell Phenotype Maps, which represent the cell composition within their native tissue context.