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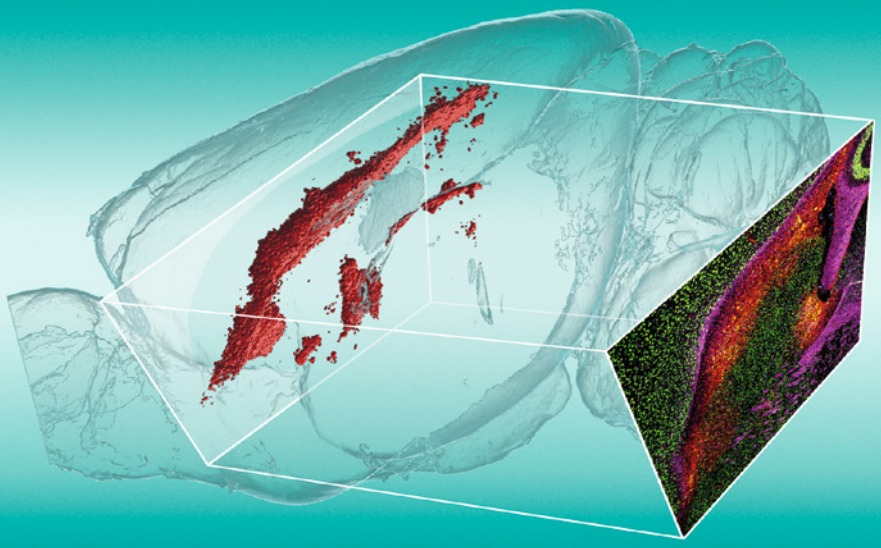

A 3D-2D spatial biology workflow for precision tissue profiling

Abstract

Spatial biology has revolutionized our understanding of tissue architecture by enabling the contextual analysis of cellular and molecular constituents within their native microenvironments. However, conventional methodologies are confined to two-dimensional thin sections, limiting the capacity to capture large-scale tissue organization and potentially missing critical cellular relationships. To overcome these limitations, we present an integrated workflow that combines volumetric 3D imaging with high-plex spatial analysis, enabling multiscale investigation of complex biological systems within intact specimens.

In this study, using a glioblastoma mouse model, we implemented whole-tissue 3D immunofluorescence (3D-IF) labeling and optical tissue clearing to visualize intact mouse brain hemispheres with the UltraMicroscope Blaze™ Light Sheet System. This approach provided a comprehensive 3D context of tumor-bearing tissue, and enabled anatomically precise targeting of regions of interest (ROIs) through a novel process termed light sheet–guided histology. Selected ROIs were then subjected to MACSima® Imaging Cyclic Staining (MICS), which enabled high-plex proteomic profiling at single-cell resolution within the original tissue. Importantly, the 3D-IF fluorochromes remained stably detectable post-sectioning, allowing for retrospective localization of ROIs and validation of marker distribution. Moreover, epitope integrity was preserved throughout the entire workflow, from tissue clearing to multiplex imaging, showing the compatibility of MICS with previously cleared tissues.

This integrated 3D-to-multiplex workflow represents a transformative advancement in spatial biology, bridging macro- and micro-scale insights of the same specimen[1]. It significantly enhances the ability to investigate tissue heterogeneity, cellular microenvironments, and pathophysiological alterations with high precision. As such, this workflow holds substantial promise for accelerating discoveries in fundamental and preclinical research, particularly in fields such as oncology, immunopathology, and neurobiology, where spatial context is essential.

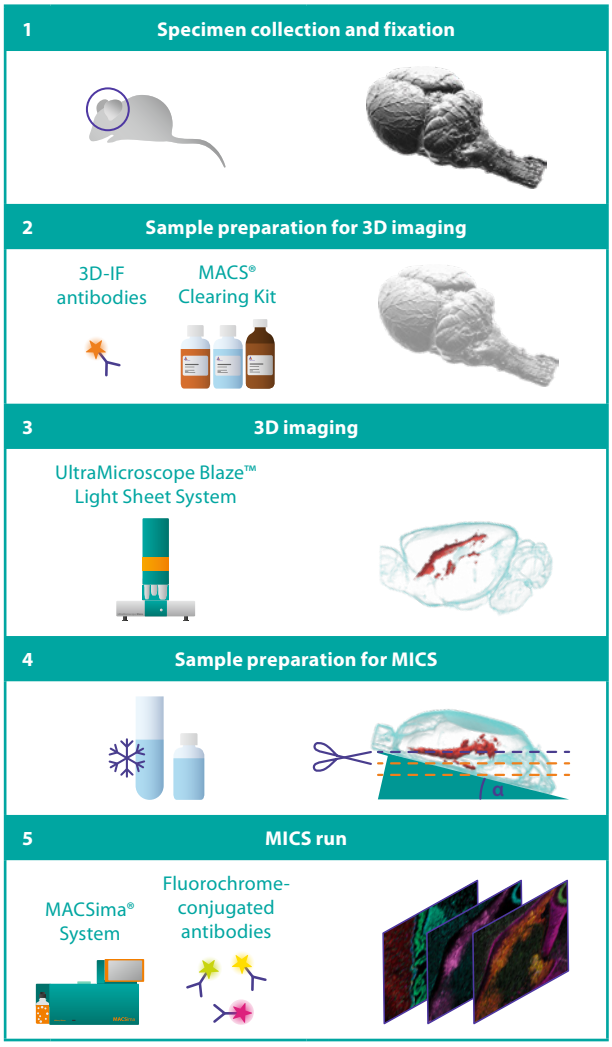

Figure 1: Illustration of a spatial biology workflow that combines 3D imaging on the UltraMicroscope Blaze and MICS technology using the MACSima Platform.

Introduction

The spatial organization of cells and molecular constituents within intact tissues influences physiological function, disease progression, and therapeutic outcomes. Conventional molecular assays often require tissue dissociation or thin sectioning, which disrupts native tissue architecture and limits spatial context.[2] While recent multiplexing spatial proteomic technologies provide rich molecular data at single-cell resolution, these approaches are generally confined to two-dimensional (2D) tissue sections with thicknesses ranging from a few µm to 50 µm, potentially missing rare or spatially restricted cell populations and the broader three-dimensional (3D) tissue microenvironment.[3,4]

To overcome these limitations, integration of volumetric 3D imaging with multiplex molecular profiling has emerged as a novel transformative strategy in spatial biology[8]. Tissue clearing combined with light sheet fluorescence microscopy (LSFM) enables unbiased, high-resolution imaging of entire organs or large tissue volumes, preserving anatomical context at cellular resolution.[5] However, LSFM has limited multiplexing molecular characterization capabilities. Conversely, cyclic immunofluorescence-based platforms, like MICS technology, enable highly multiplexed proteomic profiling of tissue sections without comprehensive 3D context.[2]

Here we present a workflow that synergistically combines 3D immunofluorescence light sheet imaging with subsequent light sheet–guided sectioning and MICS-based spatial analysis on the same specimen. This integrated approach preserves fluorescent labeling throughout clearing, imaging, and sectioning processes, enabling precise spatial alignment between 3D structural context and deep proteomic phenotyping. Such spatially resolved, multi-scale analysis is particularly powerful for preclinical research applications, including mapping tumor heterogeneity and immune infiltration in cancer[6], or dissecting region-specific molecular signatures in neurodegeneration and neuroinflammation[7]. By providing both comprehensive 3D anatomical context and high-dimensional cellular profiling from a single sample, this workflow enhances our ability to characterize complex tissue microenvironments with high spatial fidelity and molecular detail.

This integrative methodology represents a significant advancement in spatial biology, enabling targeted biomarker discovery, mechanistic disease studies, and spatially guided therapeutic development in preclinical and translational research.

Methods

The methods used are illustrated in figure 1 and can be accessed at STAR Protocols.[8]

1. Specimen collection and fixation

After transcardial perfusion using PBS/EDTA and PFA buffer, mouse brain hemispheres were extracted and fixed in a 4% PFA/PBS solution for 2.5 hours at 2–8 °C, and washed in PBS afterwards.

2. Sample preparation for 3D imaging

After fixation, cleared mouse brain hemispheres were prepared for 3D imaging as follows:

2.1 Permeabilization

Fixed samples were incubated in the Permeabilization Solution of the MACS® Deep Clearing Kit for 24 hours on a MACSmix™ Tube Rotator.

2.2 Antibody staining

Following this step, an anti-GFP-Alexa Fluor® 647 Nanobody from Chromotek was used for immunostaining. The 3D-IF antibody was diluted in the Antibody Staining Solution of the MACS® Deep Clearing Kit, and the samples were incubated for 7 days at 37 °C with gentle shaking. Note that prolonging the incubation time may lead to improved staining results.

2.3 Dehydration

After antibody incubation, the mouse brain hemispheres were washed with Antibody Staining Solution and dehydrated through a series of ascending ethanol (EtOH) dilutions in PBS with 2% Tween® 20 (v/v). This dehydration process was performed on continuous agitation at 28 °C, starting from 30% and up to 100% EtOH.

2.4 Tissue clearing

Finally, the mouse brain hemispheres were optically cleared by incubation in the Clearing Solution of the MACS Deep Clearing Kit for at least 24 hours at room temperature, under slow rotation on a MACSmix Tube Rotator.

Sample preparation for 3D imaging was performed in accordance with Miltenyi Biotec's protocol for immunostaining and clearing of mouse brain hemispheres. To access the detailed protocol and a complete list of available 3D-IF antibodies with data sheets and recommended dilutions, refer to the QR codes below.

LEARN MORE

Check out the protocol

▸ **miltenyibiotec.com/Mouse-brain-staining-clearing**

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3. 3D imaging and identification of the ROI

3D imaging of optically cleared mouse brain hemispheres was performed using the UltraMicroscope Blaze in combination with MACS Imaging Solution. A 4 µm thin light sheet was used to ensure optimal x-, y-, and z-resolution. An initial overview was captured with the 1.1×/0.1 MI Plan objective to identify regions of interest. For detailed imaging, the 12×/0.53 MI Plan objective was used. Fast tiling scan mode was utilized to acquire mosaics. Denoising and contrast compression algorithms were used to further enhance the detailed images.[2]

4. Sample preparation for MICS

After 3D imaging, cleared mouse brain hemispheres were prepared for MICS imaging as follows:

4.1 Rehydration

To rehydrate the sample, a series of descending EtOH dilutions were employed in PBS with 2% Tween 20 (v/v), starting from 100% EtOH (8 hours), 90% EtOH (overnight), 70% EtOH (4 hours), 50% EtOH (4 hours), 30% EtOH (overnight), PBS only (8 hours), and finally PBS only (overnight). All steps were performed at 28 °C at 400 rpm on a ThermoShaker.

4.2 Cryoprotection

The specimens were subjected to cryoprotection in 30% sucrose (w/v) in PBS at 2–4 °C until they sank to the bottom of a 25 mL Falcon tube and tissue was kept in solution until it sedimented completely (24–72 hours).

4.3 Angle determination and correction for cryosectioning

Surfaces were generated using the semi-automated surface detection tool in Imaris (v9.9.1) by selecting the appropriate source channels and applying smoothing. Thresholds were adjusted to create solid, representative surfaces. The cutting plane tool was then used to intersect the ROI and define the embedding orientation. Angled planes were created to simulate tissue alignment during sectioning and guide cryosectioning. Angle measurements were performed in ImageJ using the angle tool to guide cryoembedding preparation.

4.4 Embedding and freezing

Tissues were acclimated in embedding matrix for at least 30 minutes at room temperature in the dark to minimize fluorescence loss. For correct angular orientation during cryosectioning, an agarose block was trimmed to the previously defined angle using a printed angle guide and confirming orientation with a 2D protractor. Samples were then transferred to embedding molds prefilled with a thin layer of matrix and positioned atop the angled agarose blocks for correct tissue orientation. Molds were filled until the tissue was fully covered, ensuring correct alignment and snap-frozen in precooled isopentane (chilled in liquid nitrogen) for 30–60 seconds until fully solidified.

4.5 Cryosectioning

The angle-corrected and embedded sample was cut into 10 µm thick sections at –20 °C and collected on adhesion slides. To determine the desired tissue section used for the analysis on the MACSima Platform, sections were investigated using a fluorescence microscope and cross-compared to the simulated sections obtained from the 3D-IF dataset. The sections were initially stored at room temperature for at least 30 minutes and eventually transferred to –80 °C for long-term storage.

5. MICS run

Multiplex analysis was performed using MICS technology, which employs a three-step iterative cyclic staining process performed automatically by the MACSima System. In this process, samples are stained with multiple fluorochrome-conjugated antibodies. Next, an image is acquired and processed using a widefield microscope. Finally, the fluorescence signal is removed by photobleaching or fluorochrome release mechanisms. This cycle can be repeated as often as needed, allowing for the immunostaining of hundreds of markers on a single sample.

For this feasibility study, sequential staining of 108 fluorochrome-labeled antibodies was performed, together with 4',6-Diamidino-2-phenylindole (DAPI). The raw data obtained from the selected ROI was processed for registration and background correction. The 3D-IF antibody signal was detected at the beginning of the MICS run prior to the autofluorescence bleaching cycle. The resulting dataset was then imported into MACS iQ View Spatial Biology Analysis Software, which enabled the visualization and analysis of tissue composition with the use of multiple markers.

Results

High-resolution 3D imaging of optically cleared mouse brain was performed using the UltraMicroscope Blaze to visualize tumor cell distribution in the intact tissue context (fig. 2A). The volumetric dataset enabled precise identification of the tumor-containing ROI and its spatial orientation relative to the brain surface. To accurately section along the tumor plane, the angle between the ROI and surface plane was measured (fig. 2B and Methods 4.3). Based on this, a custom agarose approach was developed to correct for angular deviation, ensuring that the ROI plane was aligned parallel to the cryostat cutting plane (fig. 2C and Methods 4.4). This allowed for precise cryosectioning through the tumor area, preserving structural integrity and enabling downstream molecular analysis using MICS (fig. 2D).

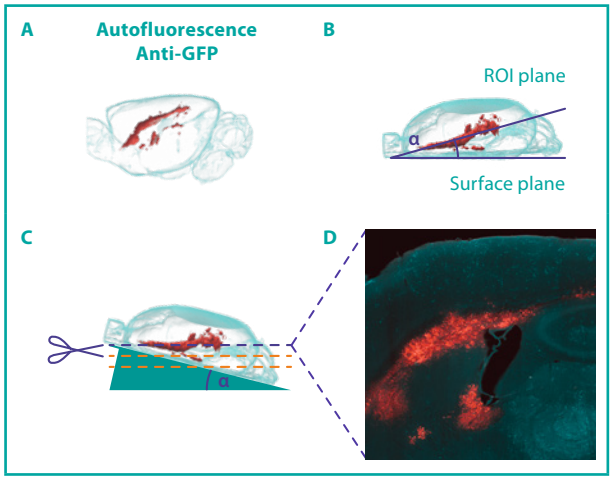

Figure 2: 3D imaging and identification of the plane of interest for MICS. 3D render of the mouse brain imaged with the UltraMicroscope Blaze and 12×/0.53 MI Plan objective (A). An optical cutting plane that includes the ROI is identified (ROI plane), and the angle between ROI plane and surface plane is measured (α) (B). After rehydration, an agarose triangular block is generated to compensate for α and bring the ROI plane parallel to the cryostat cutting plane (purple) (C). Optical slice of the ROI plane that was further processed for MICS, imaged with the 12×/0.53 MI Plan objective and 1.66× post-magnification (D).

The resulting tissue sections were inspected using a fluorescence microscope and cross-compared to the 3D imaging dataset in order to select the desired tissue section for multiplexed analysis using MICS technology. The selected tissue section was successfully stained with 108 different Fluorescein isothiocyanate (FITC)-, Phycoerythrin (PE)-, and Vio® 780–conjugated antibodies of which 10 are shown in figure 3. The resulting MICS data were compared to a glioblastoma sample stained using MICS technology on the MACSima System without prior 3D imaging (fig. 3A and 3B). The data demonstrates that tissue morphology was comparable between the samples with and without prior 3D imaging. Moreover, the data indicates that the epitopes remained stable throughout the entire process of sample preparation for 3D imaging, 3D imaging itself, and sample preparation for MICS. Noticeably, the 3D-IF staining using the anti-GFP-Alexa Fluor 647 conjugate was preserved (fig. 3C) and staining using MICS technology demonstrated co-expression with EGFR within the glioblastoma area (fig. 3D). As depicted, various cell types, such as neuronal cells (identified by the anti-NeuN and anti-Neurofilament antibodies), astrocytes (anti-GFAP), and endothelial cells (anti-CD146) could be identified using MICS technology (fig. 3E and 3F). Importantly, tumor-associated immune cells (anti-CD45) were detected with single-cell resolution (see fig. 3F).

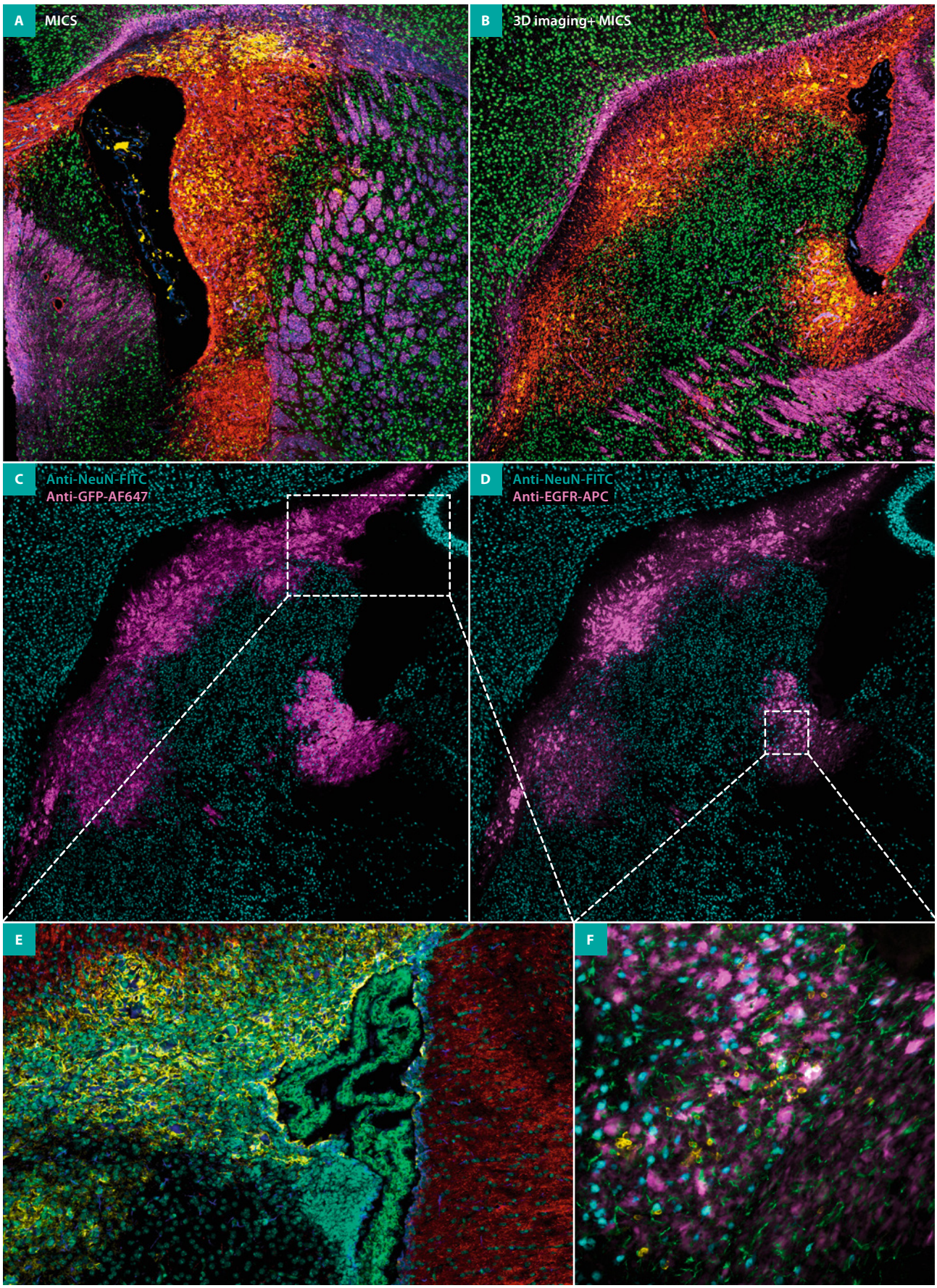

Figure 3: Mouse brain hemisphere tissue sections stained with different markers and recorded with a 20× objective lens on the MACSima System. Glioblastoma tissue section without prior 3D imaging stained with antibodies against nestin (REA575) in red, CD11b (REA592) in yellow, CD146 (REA1064) in blue, NeuN (REA1131) in green, and neurofilament (REA1127) in magenta (A). Post-3D imaging selected tissue section stained with antibodies against nestin in red, CD271 (REA648) in yellow, CD146 in blue, NeuN in green, and neurofilament in magenta (B). Selected tissue section showing NeuN in teal and GFP (anti-GFP-Alexa Fluor 647 used for 3D imaging) in magenta (C), or NeuN in teal and EGFR (REA939) in magenta (D) demonstrating glioblastoma co-expression of EGFR and GFP. Enlarged view with DAPI in cyan, neurofilament in red, CD29 (REA1074) in green, CD146 in blue, CD44 (REA664) in yellow (E). Enlarged view with GFP (3D imaging) in magenta, NeuN in blue, CD45 (REA737) in yellow, and GFAP (REA335) in green (F).

Conclusion

Spatial biology platforms often rely on thin tissue sections to study biological systems such as organs or tumors. While this method can address certain research questions, it lacks the broader spatial context that may exist beyond the selected sections. This limitation becomes especially apparent in complex tissues, such as tumor-bearing samples with diverse cellular compositions, where identifying relevant structures or key cell populations is challenging.

Here we describe a workflow that starts with 3D imaging of a large organ sample and goes on to multiplexed immunostaining and imaging of up to hundreds of markers on specific tissue sections of the same organ. The goal of our approach is to enable comprehensive 3D organ analysis, while also facilitating targeted examination of specific sections within the organ using multiplexing.

We demonstrated that throughout this workflow, combining 3D light sheet imaging with highly multiplexed tissue profiling, the tissue morphology remained intact and multiplexed detection of target proteins was retained.

This makes our workflow for light sheet–guided multiplex analysis a powerful spatial biology tool for providing a comprehensive understanding of the localization and expression of specific proteins in a dimensionally enhanced tissue context. The workflow has the potential to significantly impact applications that advance our knowledge of organ function and disease pathology, as well as for diagnostics and identifying potential therapeutic targets. For example, this workflow can be implemented in various applications, such as seeking and characterizing hidden metastasis in large tissues[2], assessing intratumor infiltration of therapeutic cells[6], and identifying the key characteristics of neurodegenerative disorders[9].

Looking ahead, we aim to further automate this workflow to strengthen the connection between 3D and multiplexing technologies, and to enhance the correlation of resulting datasets. Ongoing research will also explore the application across a wider range of tissues and use cases, expanding its impact in biomedical research and preclinical diagnostics.

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Notes

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