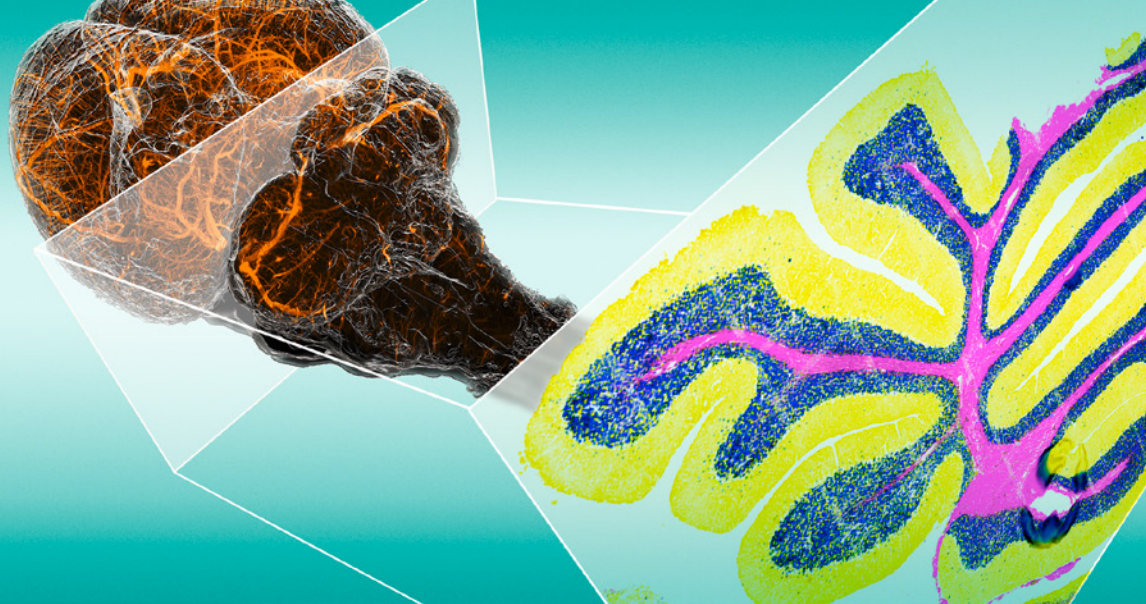**Miltenyi Biotec**

Maximizing spatial biology

## A workflow combining 3D imaging with multiplex analysis

Abstract

Spatial biology is an emerging field that studies the spatial organization of cells and molecules within tissue to better understand complex biological processes. Currently available methods may only provide information from thin tissue sections, limiting analysis to rather roughly selected areas. Our workflow combines 3D imaging with multiplex spatial analysis to get an overview of complex large samples, identify target structures within them, and to further analyze a carefully selected region in depth with hundreds of markers – all on the same valuable specimen.

In this study, we utilized 3D-immunofluorescence (3D-IF) staining and tissue clearing to prepare mouse brain hemispheres for 3D light sheet imaging with the UltraMicroscope Blaze™. With the full specimen's 3D view, we were able to identify target regions in order to prepare tissue sections that precisely cover these specific parts of the specimen, a process termed light sheet guided histology. The tissue sections were further analyzed with MACSima™ Imaging Cyclic Staining (MICS), providing expression levels of up to hundreds of protein markers from individual cells on a single sample. Remarkably, the fluorochrome conjugates used for 3D-IF staining remained detectable after sectioning, enabling us to verify the location of our target region and markers. Furthermore, the epitopes remained stable throughout the entire process of sample preparation for 3D imaging, 3D imaging itself, and sample preparation for MICS. Thus, we have demonstrated that it is possible to apply our MICS technology to a previously cleared tissue.

The ability to obtain both, a comprehensive 3D context, and detailed information about cellular diversity in a spatial context from a single sample makes our workflow game-changing in spatial biology.
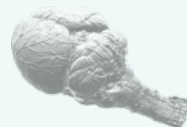
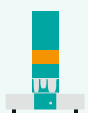**1. Specimen collection and fixation**
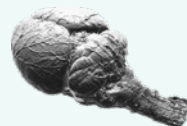
2. Sample preparation for 3D imaging

3D-IF antibodies

MACS® Clearing Kit

3. 3D imaging

UltraMicroscope Blaze™ Light Sheet Microscope

Definition of target region

4. Sample preparation for MICS

Cryosectioning of target region

5. MICS run

MACSima™ System

Fluorochrome-conjugated antibodies

Figure 1: Illustration of a spatial biology workflow that combines MICS technology with prior 3D imaging.

Introduction

Cells within tissues interact closely and can mutually influence their function and states, emphasizing the need to consider the spatial context of a particular sample for a comprehensive understanding. The MACSima Platform and its MICS technology are specifically designed to address this point by providing information on the expression levels of hundreds of protein markers on individual cells within the spatial context of tissues sections (Kinkhabwala *et al.*, 2022).

However, biological systems exist in a 3D space, and a comprehensive view of the spatial context is crucial to unleash the full power of spatial biology. To fully comprehend cellular and molecular processes and facilitate the development of drug-based treatment methods (Pfeiffer *et al.*, 2022) or analyze distribution of tumor cells in a diagnostic context (Merz, Jansen *et al.*, 2021), it is essential to obtain a complete overview of a sample, such as a brain or a solid tumor, in which a specific region of interest (ROI) can then be defined.

For this reason, we have developed a workflow that combines MICS technology with prior 3D imaging, enabling light sheet guided multiplex analysis. In detail, we fixed, stained, and cleared entire mouse brain hemispheres for 3D imaging using the UltraMicroscope Blaze Light Sheet Microscope. The hemispheres were later rehydrated, frozen, and sectioned for multiplexing with the MACSima Platform.
This workflow adds another dimension to the field of spatial biology, allowing scientists to capture a more comprehensive view on biological structures without sacrificing finer details.

Methods

The methods used are illustrated in the workflow in figure 1.

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 h at 2–8 °C.

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

2.1. Permeabilization
After fixation, the samples were washed in PBS and incubated in the Permeabilization Solution of the MACS® Clearing Kit for 24 h on a MACSmix™ Tube Rotator.

2.2. Antibody staining
Following this step, 3D-IF antibodies, (REAfinity™ recombinant primary antibodies conjugated to bright and photostable Vio® Dyes, validated for 3D imaging) were used for immunostaining. The 3D-IF antibodies were diluted in the Antibody Staining Solution of the MACS Clearing Kit, and the samples were incubated for 14 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 incubating them in the Clearing Solution of the MACS® Clearing Kit for at least 6 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/
immunostaining-and-clearing-
of-mouse-brain-hemispheres**

LEARN MORE
Check out our 3D-IF antibody portfolio
▶ **miltenyibiotec.com/
3D-IF-antibodies**

To get the most out of your tissue clearing, scan the QR code below for tips and tricks.

LEARN MORE
Get all the tips and tricks you need to optimize your tissue clearing
▶ **miltenyibiotec.com/
tips-and-tricks-for-tissue-clearing**

3. 3D imaging
The UltraMicroscope Blaze Light Sheet Microscope was used to obtain 3D images of optically cleared mouse brain hemispheres. For excitation, a 4 μm thin light sheet and Dynamic Horizontal Focusing were used to ensure optimal x-, y-, and z-resolution. For detection, a 12×/0.53 MI Plan immersion objective lens equipped with a dipping cap for organic solvents was utilized along with a 1.66× post magnifier lens, resulting in a total magnification of approximately 20×. A 7 × 7 tiles mosaic was acquired to cover the entire mouse cerebellum, with a z-step size of 4 μm and 20% overlap between tiles to allow stitching of the data into a single 3D image. Denoising and contrast compression algorithms were used to further enhance the images (Merz, Jansen *et al.*, 2021).

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 EtOH dilutions were employed in PBS with 2% Tween® 20 (v/v), starting from 100% EtOH (4 h), 90% EtOH (4 h), 70% EtOH (overnight), 50% EtOH (4 h), 30% EtOH (4 h), and finally, PBS only (overnight). All steps were performed at 28 °C at 400 rpm.

4.2 Cryoprotection

The specimens were subjected to cryoprotection in 30% sucrose (w/v) in PBS at 2–8 °C until they sank to the bottom of a 50 mL Falcon tube.

4.3 Embedding and freezing

Any excess sucrose on the sample surface was removed with a tissue wipe. The specimens were embedded and frozen by covering them with optimal cutting temperature (OCT) tissue freezing medium. After an acclimation period of 30 min at room temperature, mouse brain hemispheres were transferred to a plastic embedding mold of the appropriate size, covered with fresh OCT tissue clearing medium, and frozen at –80 °C for at least 1 h prior to cryosectioning.

4.4 Cryosectioning

The mouse brain hemispheres were cut into 10 μm thick sections at –20 °C and collected on adhesion slides. The sections were initially stored at room temperature for at least 30 min 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 much as needed, allowing for the immunostaining of hundreds of markers on a single sample. For this feasibility study, sequential staining of 12 fluorochrome-labeled antibodies was performed, together with 4',6-Diamidino-2-phenylindol (DAPI). The raw data obtained from the selected ROI was processed for registration and background correction. The resulting dataset was then imported into the MACS iQ View Analysis Software, which enabled the visualization of tissue composition with the use of multiple markers.

Results

A 3D volume of the entire hemisphere was captured (fig. 2A). The hemisphere was co-stained with Neurofilament-Vio R667 and Parvalbumin-Vio R667 (fig. 2B, C). Such staining provided a structural overview of the brain in 3D at single cell resolution (fig. 2C), allowing for more accurate selection of the section for subsequent MICS, and also enabled the identification of the same biological structures within light sheet microscopy and MICS datasets.
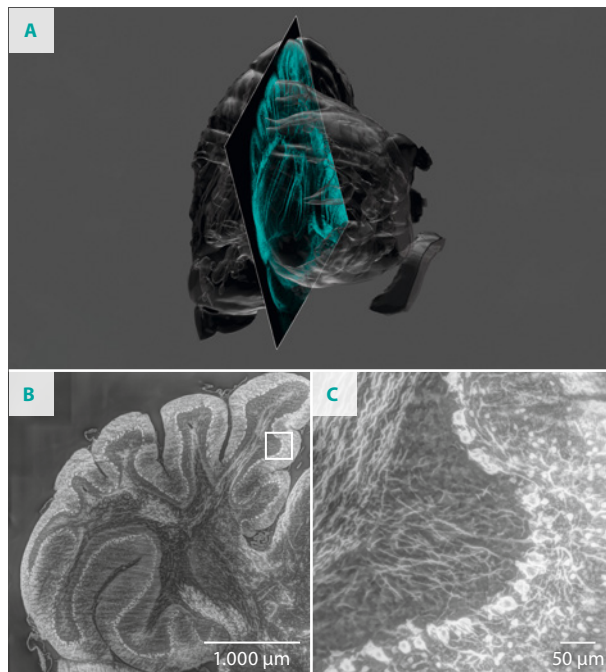

Figure 2: 3D rendering of a mouse brain cerebellum. The target region used for subsequent MICS analysis is depicted in cyan (A). Single optical section extracted from the 3D image stack corresponding to the region chosen for the following MICS run, co-stained with Neurofilament-Vio R667 and Parvalbumin-Vio R667 (B), and a detail view corresponding to the framed area (C).

The selected section was successfully stained with 12 different Fluorescein (FITC) and Phycoerythrin (PE) conjugated antibodies of which 9 are shown in figure. 3, indicating that the epitopes remained stable throughout the entire process of sample preparation for 3D imaging, 3D imaging itself, and sample preparation for MICS. As depicted, various cell types, such as neuronal cells (identified by the anti-NeuN and anti-Neurofilament antibodies), astrocytes (anti-GLAST), and oligodendrocytes (anti-MBP and anti-O4) were detected with single-cell resolution. Moreover, the imaging capabilities of the MACSima Platform made it effortless to identify subcellular compartments, such as VGLUT1 (fig. 3D).

Noticeably, the 3D staining using the VioR667 conjugates was preserved.
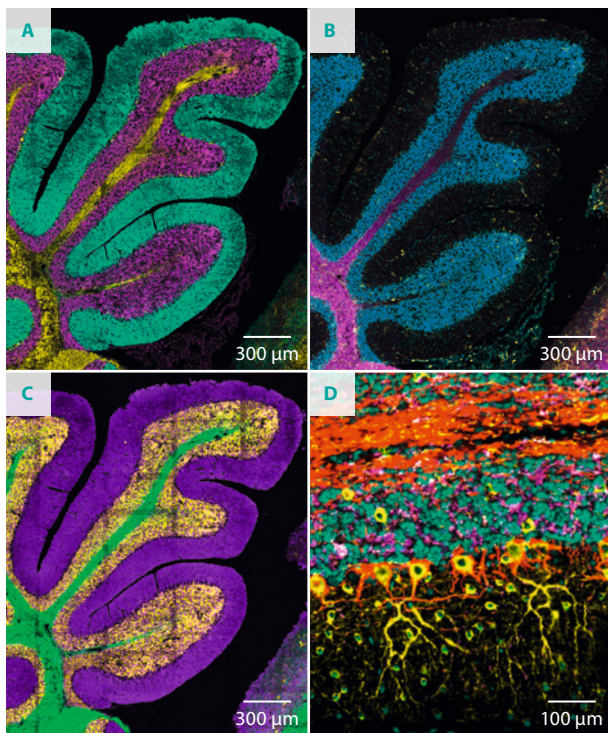
Figure 3: Mouse cerebellum tissue section, stained with different markers and recorded with a 20× objective lens. Neurofilament is shown in yellow, GLAST in cyan, DAPI in magenta (A), O4 in magenta, DAPI in blue, CD11b in yellow (B), NeuN in yellow, Synaptophysin in purple, and MBP in green (C). Enlarged view with neurofilament in red, β-tubulin III in yellow, VGLUT1 in magenta for visualization of neurons, and DAPI in cyan for visualization of nuclei (D).

Conclusion

Spatial biology platforms typically utilize few thin tissue sections to analyze biological systems, such as organs. This approach poses a risk of missing critical contextual information that may exist beyond the chosen sections. Identifying the right tissue plane to define sections of interest is one of the main challenges, particularly when relevant structures or cells are located in complex tissue samples like tumors. Such samples often have distinct cellular compositions and spatial arrangements in different regions.

Here we describe a workflow that starts with 3D imaging of a large organ sample and leads to 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 have successfully demonstrated that the fluorochromes used for 3D imaging and the tissue epitopes remained stable throughout the entire process of sample preparation and imaging.

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 tissues. 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 (Merz, Jansen *et al.*, 2021), assessing intratumor infiltration of therapeutic cells (Pfeiffer *et al.*, 2022), and identifying the key characteristics of neurodegenerative disorders (Bhatia *et al.*, 2022).

Ultimately, this workflow will become even more automated, with the goal of strengthening the connection between 3D and multiplexing technologies, along with enhancing the correlation of the resulting datasets. Furthermore, ongoing research and experimentation will focus on testing this workflow in diverse tissues and/or applications, thereby expanding the potential scope of this technology in the future.

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