Multiplexed immunofluorescent imaging and analysis of Alzheimer's brain tissue

Spatial neurobiology with the MACSima™ Imaging System

Background

Alzheimer's disease progression can be highly complex, consisting of multiple interactions of many different cell types, and each cell type may also possess a unique marker-expression signature. In this disease, cognitive impairment is preceded by alterations in neurons, microglia, and astrocytes. This, in turn, leads to neuroinflammation, alteration in vessels, necroptosis, and the accumulation of amyloid beta plaques and tau tangles.[1] Accumulation of intracellular hyperphosphorylated tau protein (pTau) is a key pathological feature of Alzheimer's disease and shows promise as a novel biomarker.[2]

Standard immunofluorescence analysis is limited to a maximum of five markers, which only allows for the analysis of a small subsection of disease pathophysiology. The recent development of highly multiplexed tissue imaging methods aims to enhance research studies and clinical practice with precise, spatially resolved single-cell data.[3] The MACSima™ Imaging Platform, utilizing fully automated cyclic immunofluorescence, is designed to overcome previous limitations, by providing a novel and automated system to analyze up to hundreds of markers on a single-tissue sample.[4]

In the study described here, MACSima Imaging Cyclic Staining (MICS) and analysis was used to compare Alzheimer's hippocampus tissue with healthy hippocampus tissue sections. A quantitative analysis was performed on the two tissue samples using MACS® iQ View image analysis software.

Methods

Sample preparation
Frozen tissue sections of the hippocampus region of human adult normal brain and human adult Alzheimer's brain (8 µm thick) were purchased from a commercial source (BioChain®) and prepared according to standard immunohistochemistry procedures. The tissue sections were placed on the slides, fixed with 4% PFA, stained with DAPI, and inserted in the MACSima Instrument for imaging.

Panel design, tissue staining, and image acquisition
A total of 31 primary antibodies were selected to analyze the tissue samples. The panel was designed to identify neurons, microglia, astrocytes, blood vessels, white matter, synapse-dense regions, and markers indicative of potential immune responses. Each antibody was pre-conjugated with fluorescein isothiocyanate (FITC), phycoerythrin (PE), or allophycocyanin (APC), and the panel was designed to maximize marker-labeling specificity and signal detection, while minimizing emission spillover.

The MACSima Imaging System was used for automated tissue labeling and imaging, utilizing iterative immunofluorescent-staining cycles. These cycles include staining with DAPI and up to three fluorochrome-conjugated antibodies per cycle, multi-field imaging, and signal erasure through photo-bleaching or enzymatic release.[4] Between each cycle, additional images are acquired to ensure the complete removal of any fluorescent signal. Staining and washes are conducted with the internal liquid handling system and images are acquired with an epifluorescence microscope with a 4× objective for regions of interest (ROI) selection and a 20× objective for image collection, using violet-, blue-, green-, and red-imaging channels.

Image analysis and cell mask creation

The data acquired with the MACSima System were analyzed using MACS iQ View Software. Typically, the first step in analyzing and quantifying spatial biology data in detail is the segmentation of the ROI. Most cell types can be segmented into nuclei and cytoplasm with established algorithms in MACS iQ View Software. However, neurons have complex and elongated shapes that are often present in several layers. Therefore, an alternative superpixel segmentation was used, as described below.

Results

Images of normal and Alzheimer's hippocampus tissue sections that had been stained with the full antibody panel were analyzed and selected markers were compared. The images shown in (fig.1) display staining with classic neuronal markers synaptophysin, vimentin, myelin basic protein (MBP), and neuronal nuclear protein (NeuN). Interestingly, the staining patterns highlight regions of lower tissue density in the Alzheimer's sample (fig. 1B) that are absent in normal tissue (fig. 1A). Three small ROI were selected in each tissue section to perform further analysis.

For this purpose, a data analysis pipeline was created in the Workflow Editor of MACS iQ View and applied to all of the ROI (fig. 2). First, a cell mask was created using the superpixel tool to select the areas of the tissue sections containing neurons. The superpixel feature was used to create a grid over the ROI using 10-pixel diameter squares. Superpixels with positive staining for either DAPI (cell nuclei), neurofilament H (neuronal cytoskeleton), or synaptophysin (synaptic regions) were selected to create a cell mask from which the area with positive pTau staining could be analyzed. While this study focused on pTau, other markers from the extensive staining panel could easily be selected in a similar workflow.

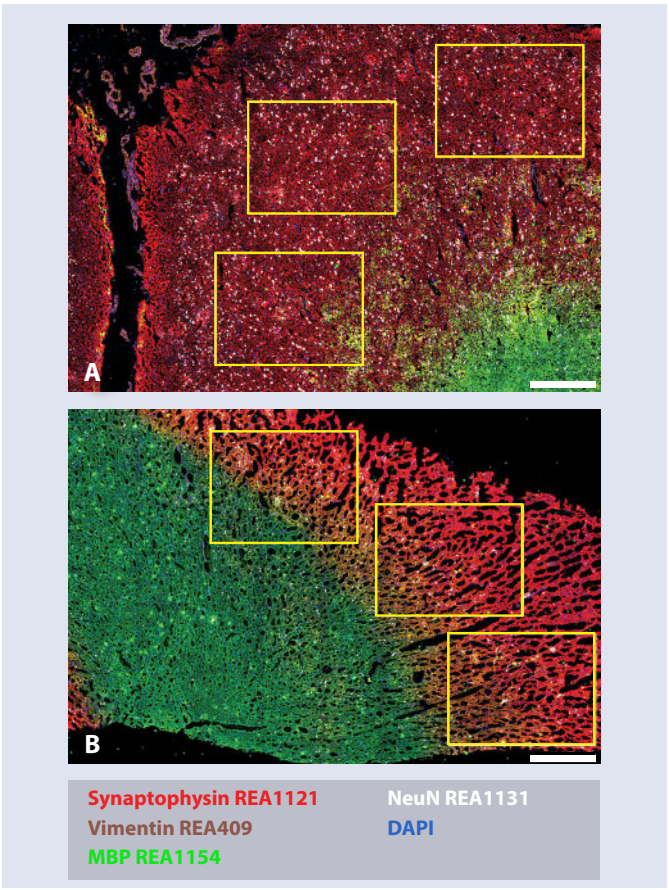

Figure 1. Multiplexed imaging highlights obvious differences in tissue density between normal (**A**) and Alzheimer's (**B**) human hippocampus tissue sections. The following markers are shown: synaptophysin in red, vimentin in brown, MBP in green, NeuN in white, and DAPI in blue. Both images display ROI selected (yellow boxes) for further data analysis. The scale bar corresponds to 500 µm.

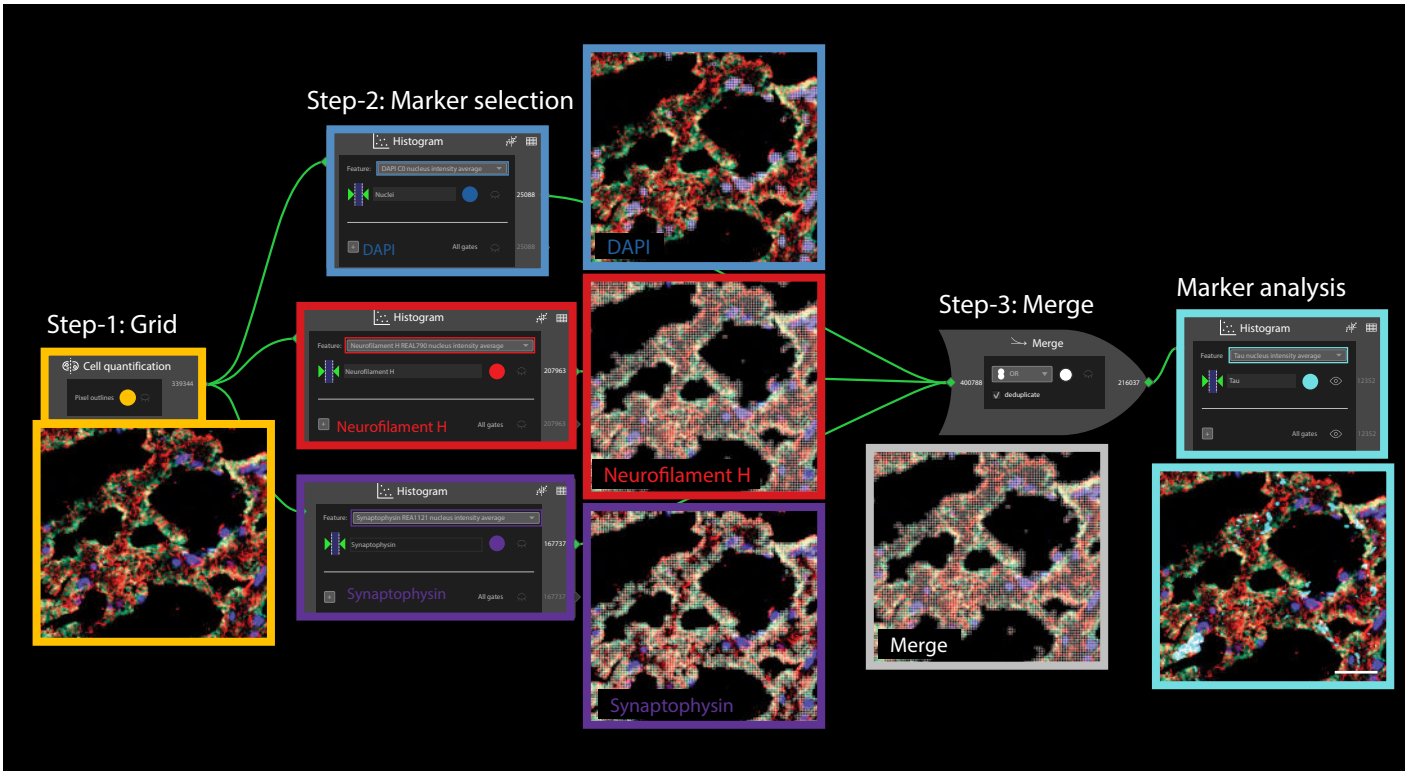

Figure 2. Data analysis pipeline as defined in MACS iQ View Workflow Editor. Step 1-Grid application of superpixel grid over selected ROI, **Step 2-Marker selection** reported superpixels based on average pixel intensity for each marker. DAPI (blue), neurofilament H (red), synaptophysin (purple). **Step 3-Merge** the combined pixels selected in Step 2. **Marker analysis** of pTau marker expression within the cell mask. The scale bar corresponds to 100 µm.

Data analysis and cell mask application

MACS iQ View Software was then used to quantify the percentage of neuronal cells and the synapse density in the Alzheimer's tissue section in comparison with the normal tissue section. Figures 3A and 3B display the cell mask applied to an ROI from normal and Alzheimer's hippocampus tissue sections, respectively. As with the overview images shown in figure 1, the tissue density in the Alzheimer's sample is visibly lower than the normal tissue. Using MACS iQ View it is possible to quantify this difference by calculating the ratio between the number of superpixels containing either neurofilament H (NF-H), synaptophysin, or DAPI stain, and the total superpixels in the ROI. The average quantity of tissue density on all three ROI of both normal and Alzheimer's tissue sections was determined. As illustrated in figure 3C, the Alzheimer's tissue presents a reduction of tissue density equal by about 20% compared to the normal tissue.

Similarly, the cell mask was used to calculate the averaged percentage of the pTau marker in the ROI of each tissue section, which was absent in normal tissue (fig. 3D), but visible as white puncta in the Alzheimer's tissue (fig. 3E). The pTau marker detection, identified as pixels in the software, was equal to 0.01% in the normal tissue section and 4.49% in the Alzheimer's tissue section (fig. 3F).

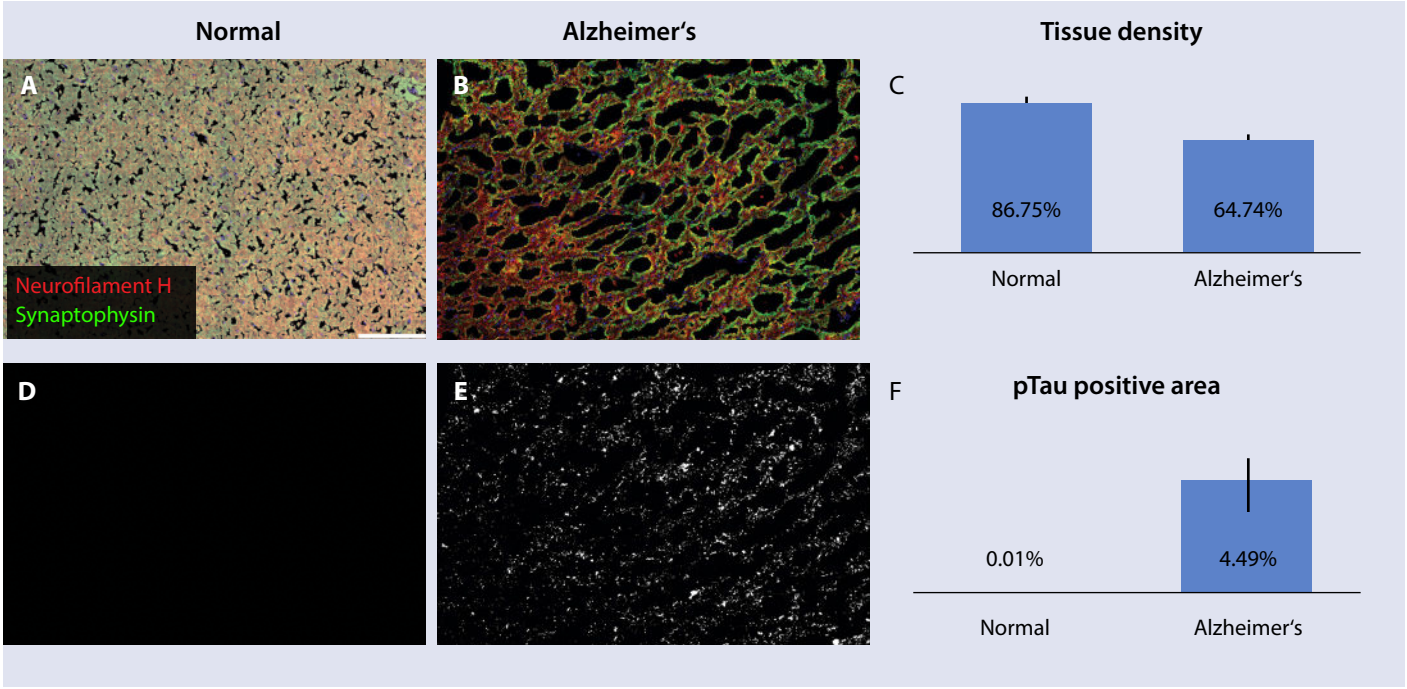

Figure 3. Images represent the superpixel cell mask applied to one ROI from normal tissue sections and Alzheimer's hippocampus tissue sections. (**A**) and (**B**) show the staining with NF-H (red), synaptophysin (green). The average quantity of tissue density and standard deviation (SD) was calculated based on pixels of three ROI and compared between tissues (**C**). Expression of the pTau in normal (**D**) and Alzheimer's (**E**) tissue within cell mask. Average and SD of the pTau positive area of three ROI are calculated and compared (**F**). The scale bar corresponds to 100 µm.

Conclusion

The MACSima Imaging Platform has the ability to analyze a large number of key neural markers simultaneously and effectively quantify differences in tissue samples. As demonstrated here, a comparison could be made between hippocampus tissue sections of healthy donors and those affected by Alzheimer's disease.

MACS iQ View Image Analysis Software provides a superpixel analysis tool that allows the segmentation and analysis of irregularly shaped cells such as neurons. With the creation of a simple workflow, it was possible to quantify both the loss of synaptic density and a greater amount of phosphorylated tau protein in the Alzheimer's tissue, compared to the normal tissue.

This study demonstrates that combining MICS and comprehensive data analysis with MACS iQ View Image Analysis Software provides a revolutionary approach to the study of neurological diseases such as Alzheimer's utilizing multiplexed spatial biology.

References

1. Scheltens, P. *et al.* (2021) Alzheimer's disease. Lancet. 397(10284):1577-1590. doi: 10.1016/S0140-6736(20)32205-4

2. Ossenkoppele, R. *et al.* (2022) Tau biomarkers in Alzheimer's disease: towards implementation in clinical practice and trials. Lancet Neurol. 21(8):726-734. doi: 10.1016/S1474-4422(22)00168-5

3. Lin, J. *et al.* (2023) High-plex immunofluorescence imaging and traditional histology of the same tissue section for discovering image-based biomarkers. Nat. Cancer. 4:1036-1052. doi.org/10.1038/s43018-023-00576-1

4. Kinkhabwala, A. *et al.* (2022) MACSima imaging cyclic staining (MICS) technology reveals combinatorial target pairs for CAR T cell treatment of solid tumors. Sci. Rep. 12:1911. doi.org/10.1038/s41598-022-05841-4

Materials

Miltenyi Biotec multiplex antibodies panel				
Product name	**Clone**	**Fluorophore**	**Order Number**	**Vendor**
Synapsin-1 Antibody, anti-human/mouse/rat, PE, REAfinity™	REA1125	PE	130-119-359	Miltenyi Biotec
Synaptophysin Antibody, anti-human, APC, REAfinity	REA1121	APC	130-119-348	Miltenyi Biotec
CD68 Antibody, anti-human, PE, REAfinity	REA886	PE	130-114-460	Miltenyi Biotec
CD11b Antibody, anti-human, PE, REAfinity	REA1321	PE	130-128-773	Miltenyi Biotec
GFAP Antibody, anti-human/mouse/rat, APC, REAfinity	REA335	APC	130-123-846	Miltenyi Biotec
CD1c (BDCA-1) Antibody, anti-human, PE	AD5-8E7	PE	130-113-302	Miltenyi Biotec
CD45 Antibody, anti-human, PE	5B1	PE	130-113-118	Miltenyi Biotec
CD56 Antibody, anti-human, APC	AF12-7H3	APC	130-113-305	Miltenyi Biotec
CD141 (BDCA-3) Antibody, anti-human, PE	AD5-14H12	PE	130-113-318	Miltenyi Biotec
CD16 Antibody, anti-human, PE, REAfinity	REA1324	PE	130-128-774	Miltenyi Biotec
CD146 Antibody, anti-human, APC, REAfinity	REA773	APC	130-111-323	Miltenyi Biotec
CD14 Antibody, anti-human, PE, REAfinity	REA599	PE	130-110-519	Miltenyi Biotec
Vimentin Antibody, anti-human, PE, REAfinity	REA409	PE	130-123-774	Miltenyi Biotec
β-Tubulin 3 Antibody, anti-human/mouse, PE, REAfinity	REA1152	PE	130-120-265	Miltenyi Biotec
HLA-DR Antibody, anti-human, PE, REAfinity	REA805	PE	130-111-789	Miltenyi Biotec
Podoplanin Antibody, anti-human, REAlease®	REAL468	PE	130-121-373	Miltenyi Biotec
MBP Antibody, anti-human/mouse, REAfinity	REA1154	PE	130-120-342	Miltenyi Biotec
CD3 Antibody, anti-human, APC, REAfinity	REA1151	APC	130-120-269	Miltenyi Biotec
Neurofilament Antibody, anti-human/mouse, PE, REAfinity	REA1127	PE	130-119-496	Miltenyi Biotec
NeuN Antibody, anti-human/mouse/rat, PE, REAfinity	REA1131	PE	130-119-493	Miltenyi Biotec
CD34 Antibody	REA1164	PE	130-120-515	Miltenyi Biotec
HLA-ABC Antibody, anti-human, PE, REAfinity	REA230	PE	130-120-055	Miltenyi Biotec
MAP2 Antibody, anti-human/mouse, PE, REAdye_lease	REAL1048	PE	130-128-142	Miltenyi Biotec
CD31 Antibody, anti-human, PE, REAfinity	REA1028	PE	130-117-225	Miltenyi Biotec
VGLUT2 Antibody, anti-human/mouse/rat, PE, REAfinity	REA1144	PE	130-119-732	Miltenyi Biotec
GLUT1 Antibody, anti-human, PE, REAdye_lease	REAL763	PE	130-127-119	Miltenyi Biotec
Neurofilament M (160 kD) Antibody, anti-human/mouse, REAdye_lease	REAL962	FITC	130-129-275	Miltenyi Biotec
CD326 (EpCAM) Antibody, anti-human, PE, REAfinity	REA764	PE	130-110-999	Miltenyi Biotec
Neurofilament H (200 kD) Antibody, anti-human/mouse, FITC, REAdye_lease	REAL790	FITC	130-129-274	Miltenyi Biotec
CD20 Cytoplasmic Antibody, anti-human, PE, REAfinity	REA1087	PE	130-118-293	Miltenyi Biotec

Other products				
Tau (pSer202/ pThr205)	AH36	APC	SMC-601D	StressMarq Biosciences

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