Deep Spatial Phenotyping of Microglia in Human Alzheimer's Disease Brain Tissue

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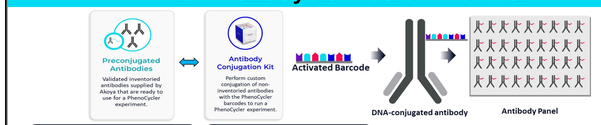
1. Introduction

Neuroinflammation is increasingly linked to the etiology and progression of Alzheimer's disease (AD). Microglia, the critical stewards of the brain's immune microenvironment, display phenotypic and state diversity in AD as shown in single-cell transcriptomic and proteomic analyses on tissue homogenates[1]. These approaches lack spatial information, which is crucial to interpreting microglial involvement in neuroinflammation. We thus set out to develop a workflow that provides both, deep biomarker readouts and excellent spatial resolution. To this end, we developed a **PhenoCycler®–Fusion** (**PCF**) workflow that is compatible with formalin-fixed human brain tissue. The PCF is a novel spatial biology platform that generates ultrahigh-plex spatial data for millions of cells on a single tissue section within a matter of hours. Using this technology, we characterized the immune microenvironment – with emphasis on microglia – in normal and AD brain tissues. Our findings suggest an enrichment of certain microglial subtypes in AD brain tissues and in proximity to Aβ plaques. In its sum, this study provides (1) a method for ultrahigh-plex immunofluorescent analyses of AD pathology and new insights into the inflammatory microenvironment of AD.

2. PhenoCycler-Fusion Workflow

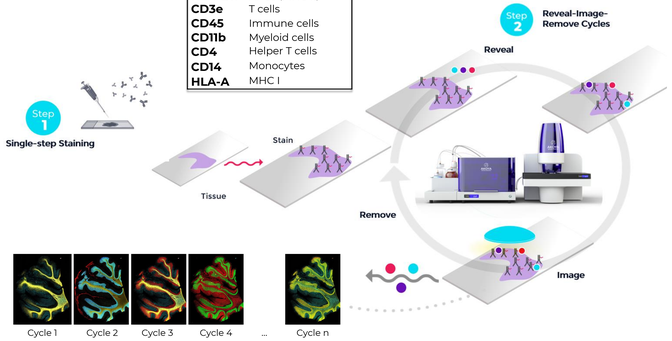


Neuroscience ABs	
NeuN	Neurons
MAP-2	Neurons
H2A.X	Neurons
N-filament	Neurons
GFAP	Astrocytes
Iba-1	Microglia
Olig-2	Oligodendroglia
Vimentin	Astrocytes, NSCs
CD31	Vascular
Collagen IV	Vascular
Claudin-5	Vascular
CD34	Vascular
PSD-95	Synaptic
Syp-physin	Synaptic
SMA	Vascular

Neuroinflammation ABs	
TMEM119	Microglia
AQP4	Astrocytes & Vessels
CD68	Microglia & Macrophages
CD11c	Microglia
CD163	Microglia & Macrophages
PCNA	Proliferating cells
ApoE	Plaques
β-Amyloid	Plaques
iNOS	Macrophages

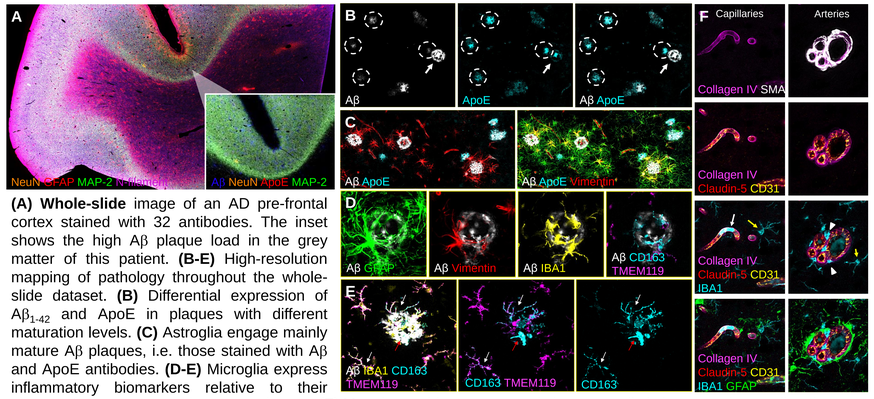
Immune ABs	
CD8	Cytotoxic T cells
HLA-DR	APCs (MHC II)
CD3e	T cells
CD45	Immune cells
CD11b	Myeloid cells
CD4	Helper T cells
CD14	Monocytes
HLA-A	MHC I

The **PhenoCycler-Fusion** workflow is compatible with a wide range of commercially available antibodies. Antibodies can also be customized via conjugation to activated oligonucleotide barcodes that are complementary to existing antibody panels (e.g. Neuro Panel to the left). Following conjugation, antibodies are titrated and tested for appropriate target recognition, and then added to a panel. We routinely deploy panels with >100 antibodies.



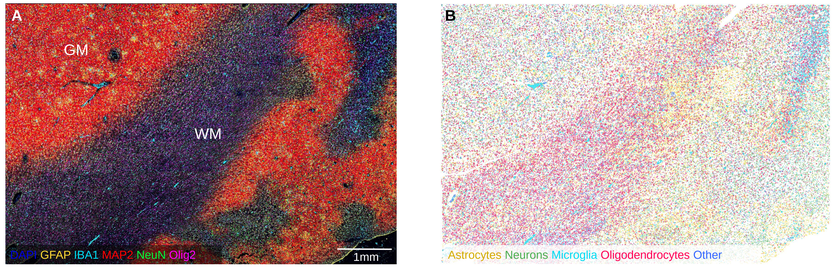
The PhenoCycler-Fusion workflow consists of iterative cycles of labelling, imaging and removing fluorescent reporters. In each imaging cycle, three fluorescent reporters are assayed to their corresponding barcode-conjugated antibodies and imaged via standard fluorescent optics. Thereafter, the three reporters are removed, and a new cycle images additional reporters. The process is fully automated, and data are acquired **across whole slides at single-cell resolution**.

3. Comprehensive Mapping of Alzheimer's Pathology in Human Brain Tissue

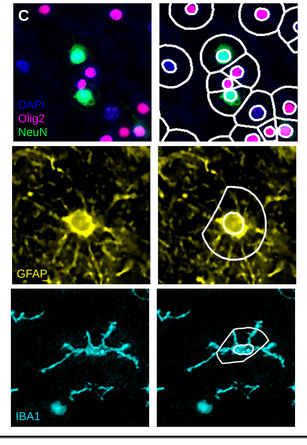


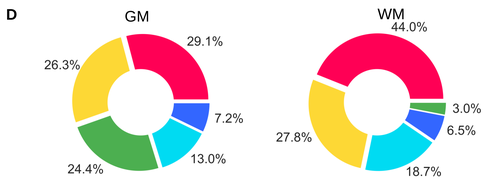
(A) Whole-slide image of an AD pre-frontal cortex stained with 32 antibodies. The inset shows the high Aβ plaque load in the grey matter of this patient. **(B-E)** High-resolution mapping of pathology throughout the whole-slide dataset. **(B)** Differential expression of $A\beta_{1\text{-}42}$ and ApoE in plaques with different maturation levels. **(C)** Astroglia engage mainly mature Aβ plaques, i.e. those stained with Aβ and ApoE antibodies. **(D-E)** Microglia express inflammatory biomarkers relative to their distance from mature and immature plaques. **(F)** Mapping resident vs. systemic immune cells is key to understanding the AD inflammation. We have combined markers for the vasculature with immune cell antibodies to distinguish these systems.

4. Stardist Nuclear Segmentation for Neuronal Cell Classification



(A) Fluorescent image showing major cell-type defining markers. **(B)** Spatial map showing cell classification results for 110,088 DAPI-based segmentation masks, obtained using Stardist. GM: Grey Matter, WM: White Matter (WM).

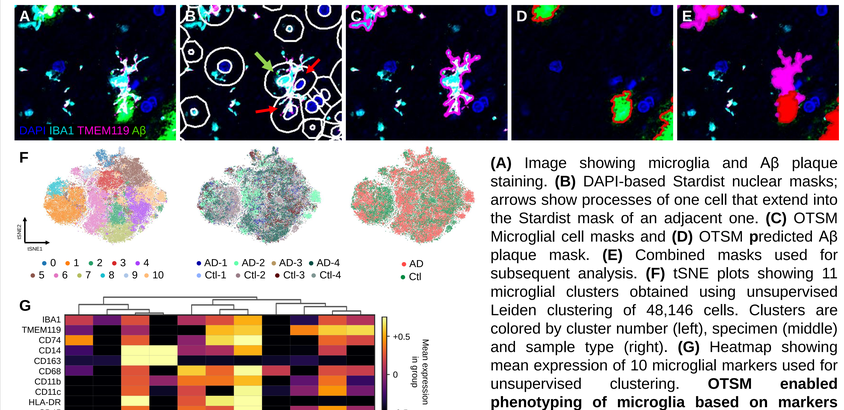




(C) Stardist-based masks showing localized nuclear signals for Neurons (NeuN) and Oligodendrocytes (Olig2). **(D)** Overall abundance of Stardist-identified cells is similar in GM (n= 59,783) and WM (n=50,305), with more oligodendrocytes in WM vs. more neurons in GM.

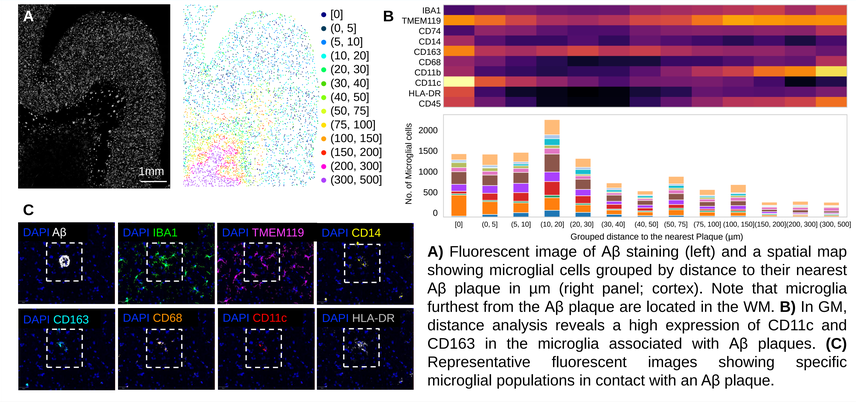
The Stardist cell masks failed to capture cellular processes in astrocytes and microglia (see GFP & IBA1 on left). To correctly segment individual microglia, we thus developed **OTSM** (pronounced 'Awesome'), which stands for Otsu's Thresholding based Segmentation and Merge. This method obtains a binary mask around microglial cells using Otsu's thresholding (2), followed by a merging step to merge disconnected processes using a predetermined distance cutoff. **Box 5** summarizes our results.

5. OTSM ('Awesome') Segmentation Accurately Registers Microglia



(A) Image showing microglia and Aβ plaque staining. **(B)** DAPI-based Stardist nuclear masks; arrows show processes of one cell that extend into the Stardist mask of an adjacent one. **(C)** OTSM Microglial cell masks and **(D)** OTSM **p**redicted Aβ plaque mask. **(E)** Combined masks used for subsequent analysis. **(F)** tSNE plots showing 11 microglial clusters obtained using unsupervised Leiden clustering of 48,146 cells. Clusters are colored by cluster number (left), specimen (middle) and sample type (right). **(G)** Heatmap showing mean expression of 10 microglial markers used for unsupervised clustering. **OTSM enabled phenotyping of microglia based on markers expressed in their ramifications.**

6. Inflammatory State of Microglia Depends on Proximity to Aβ Plaques



A) Fluorescent image of Aβ staining (left) and a spatial map showing microglial cells grouped by distance to their nearest Aβ plaque in µm (right panel; cortex). Note that microglia furthest from the Aβ plaque are located in the WM. **B)** In GM, distance analysis reveals a high expression of CD11c and CD163 in the microglia associated with Aβ plaques. **(C)** Representative fluorescent images showing specific microglial populations in contact with an Aβ plaque.

7. Conclusions and Outlook

- More than five million Americans are living with Alzheimer's Disease, but our understanding of the etiology and progression of this disease remains limited.
- We developed a comprehensive Spatial Biology workflow aimed at uncovering the inflammatory biology of AD in human patient samples *in situ*.
- Our work encompasses the development of a custom antibody panel, an imaging workflow, as well as a novel bioinformatic analysis method.
- Deployment of this workflow on post-mortem AD tissues allowed us to study different microglial cell populations, according to biomarker profiles and spatial distribution; in doing so, we identified different area and disease-associated microglial subpopulations in the cerebral cortex.
- Our workflow will allow large-scale unsupervised analyses of the AD inflammatory microenvironment that is needed to better characterize neurodegenerative disease.

1. Wang H (2021). Microglia Heterogeneity in Alzheimer's Disease: Insights From Single-Cell Technologies. *Front. Synaptic Neurosci.*
2. N. Otsu (1979). A Threshold Selection Method from Gray-Level Histograms, *IEEE Trans. on Sys, Man, & Cybernetics*

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