An Optimized Fluorescent 9-plex Spatial Proteomics Approach with Spectral Unmixing to Identify Cell Types in the Brain

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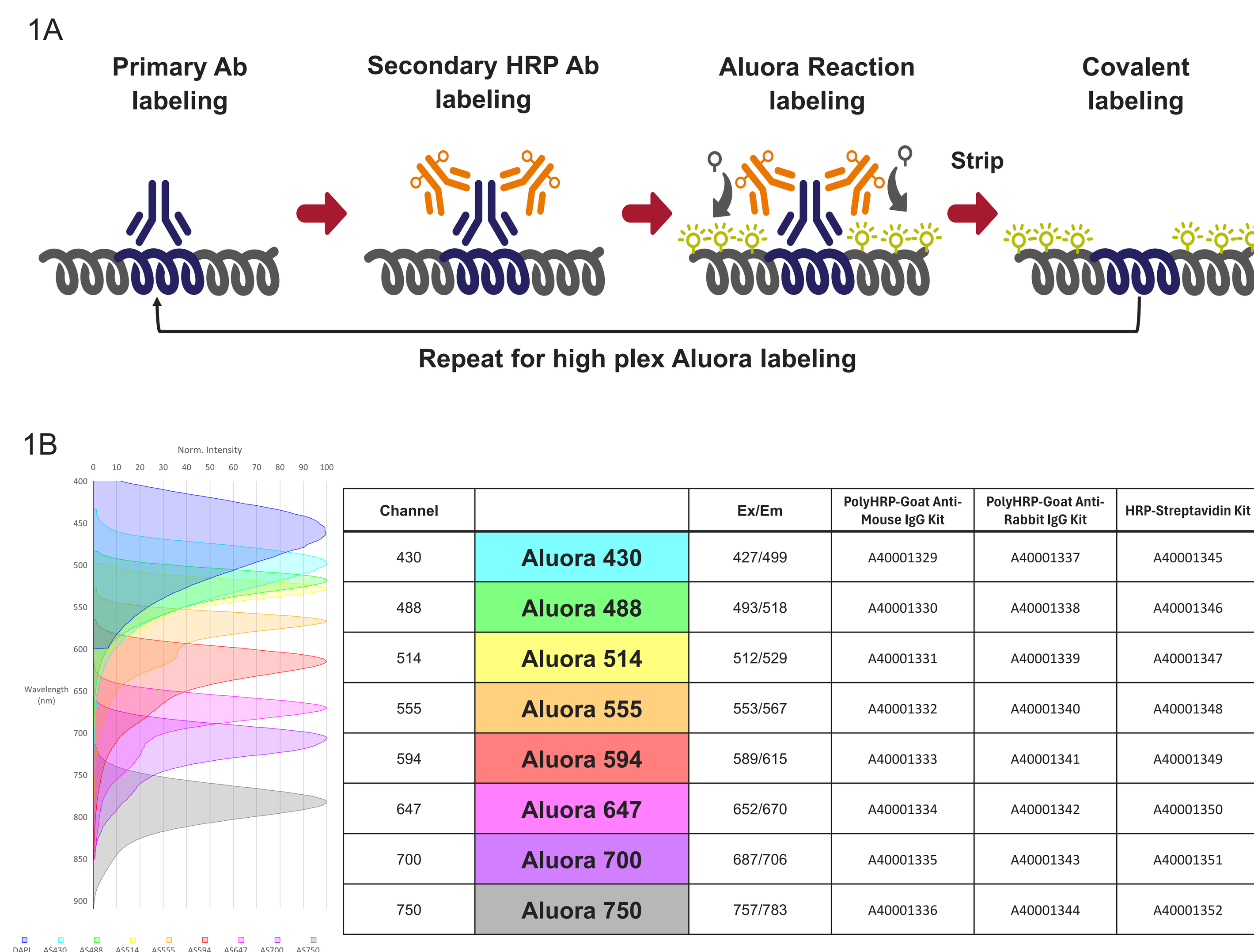
Abstract

The ability to uniquely identify cell types increases greatly with the number of biomarkers that can be used concurrently. Traditional immunohistochemistry (IHC) approaches have limitations in terms of resolving spectrally distinct signals for multiple targets. We have developed a system for flexible labeling and amplification of protein targets for fluorescent IHC with spectral unmixing, which enables the separation of overlapping spectral signatures and the accurate identification of eight biomarkers within a single tissue section. Using the EVOS S1000 Spatial Imaging System to generate high-resolution images of whole tissue sections combined with quantitative image analysis software, we can measure the spatial distribution of distinct cell populations based on identified biomarkers. This allows for the specific localization of neuronal and glial subpopulations within a single tissue section. These results highlight the advantages of using a spectrally unmixed 9-plex sample analysis (8 biomarkers and a nuclear counterstain) to investigate the spatial relationships between cell types in the brain.

Multiplex Labeling

Aluora™ Spatial Amplification Kits

Aluora kits use tyramide-like signal amplification labeling technique for enhanced sensitivity and intensity. Combining the brightness of Invitrogen™ fluorescent dyes with poly-HRP-mediated signal amplification labeling permits high-fidelity multiplexing of a variety of validated antibody clones.



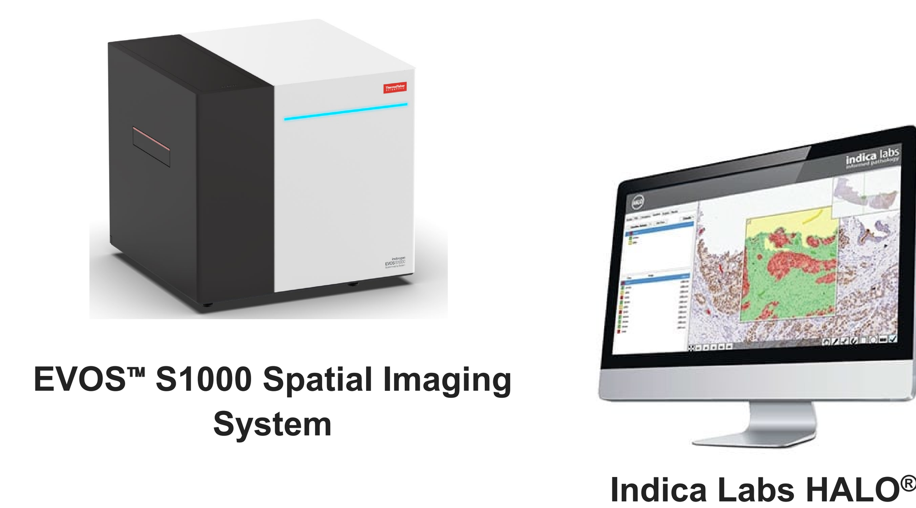
Channel		Ex/Em	PolyHRP-Goat Anti-Mouse IgG Kit	PolyHRP-Goat Anti-Rabbit IgG Kit	HRP-Streptavidin Kit
430	Aluora 430	427/499	A40001329	A40001337	A40001345
488	Aluora 488	493/518	A40001330	A40001338	A40001346
514	Aluora 514	512/529	A40001331	A40001339	A40001347
555	Aluora 555	553/567	A40001332	A40001340	A40001348
594	Aluora 594	589/615	A40001333	A40001341	A40001349
647	Aluora 647	652/670	A40001334	A40001342	A40001350
700	Aluora 700	687/706	A40001335	A40001343	A40001351
750	Aluora 750	757/783	A40001336	A40001344	A40001352

Figure 1. Multiplex labeling workflow with the Aluora kits uses horseradish peroxidase (HRP) for enzymatic conversion of the Aluora dyes to covalently bind tissue on and surrounding the protein epitope targeted primary antibody. A) Diagram of workflow. B) Emission spectra and table of Aluora products.

Materials and methods

Sample Preparation

Formalin-fixed, paraffin-embedded (FFPE) mouse coronal brain tissue sections (5µm) were obtained from Zyagen Inc. These slides were then processed using a Leica Bond RXm and stained with primary antibodies (table right) and the Aluora Spatial Amplification kits, as described in Figure 1. Images were acquired and spectrally unmixed on the EVOS S1000.



EVOS™ S1000 Spatial Imaging System

Indica Labs HALO®

Primary antibody	Catalog no.	Staining concentration
Calbindin D28K Recombinant Polyclonal Antibody (15HCLC)	711443	1:500
GFAP Antibody	ab7260	1:1,000
TLE1 Recombinant Rabbit Monoclonal Antibody (ARC0793)	MA5-35377	1:5,000
MAP2 Monoclonal Antibody (AP18)	MA5-12826	1:1,000
Olig2 Antibody (211F1.1)	MABN50	1:1,000
NECAB1 Polyclonal Antibody	PA5-54734	1:500
FOXP2 Monoclonal Antibody (CL5312)	MA5-31419	1:5,000
GAD67 Antibody (1G10.2)	MAB5406	1:500

Data Analysis

Analysis of the multiplex immunofluorescence stitched image was performed on the Indica Labs HALO® (version 4.0.5107.318) software. 81,926 cells were identified across a coronal section using the Indica Labs-HighPlex FL version 4.2.14, and the Halo AI Nuclei Seg V2-FL classifier.

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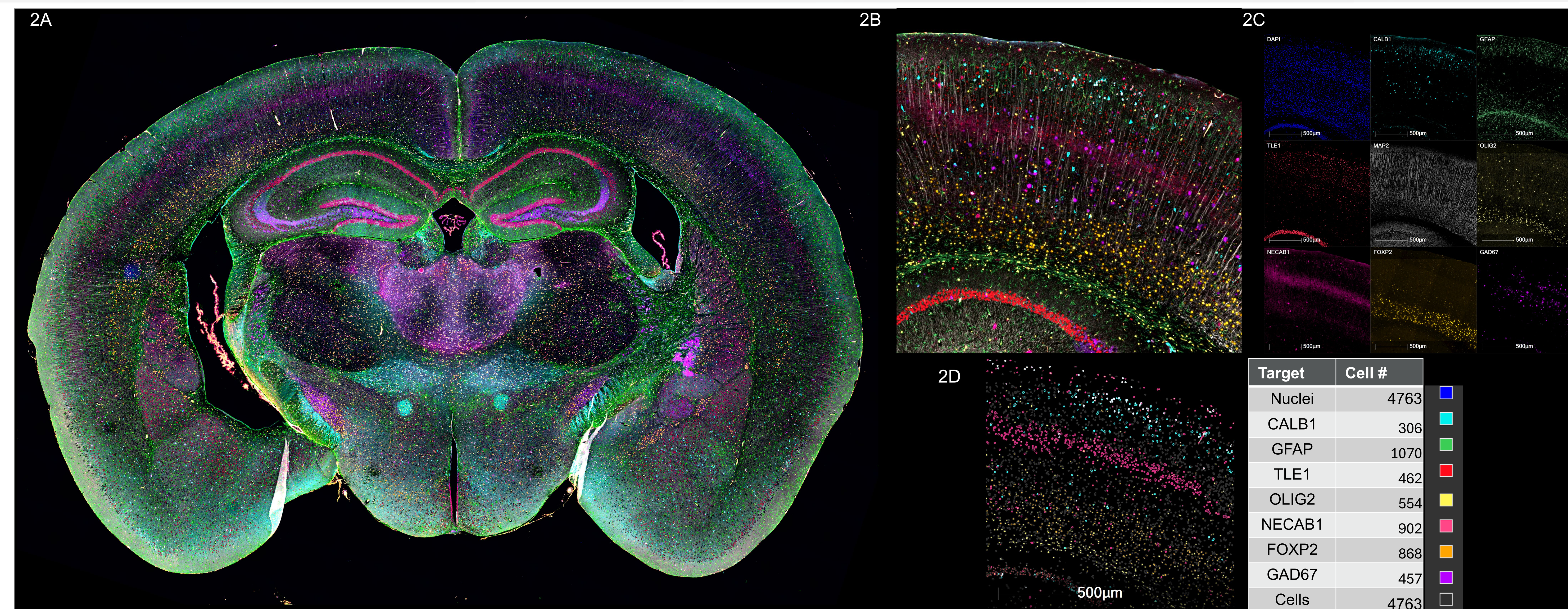
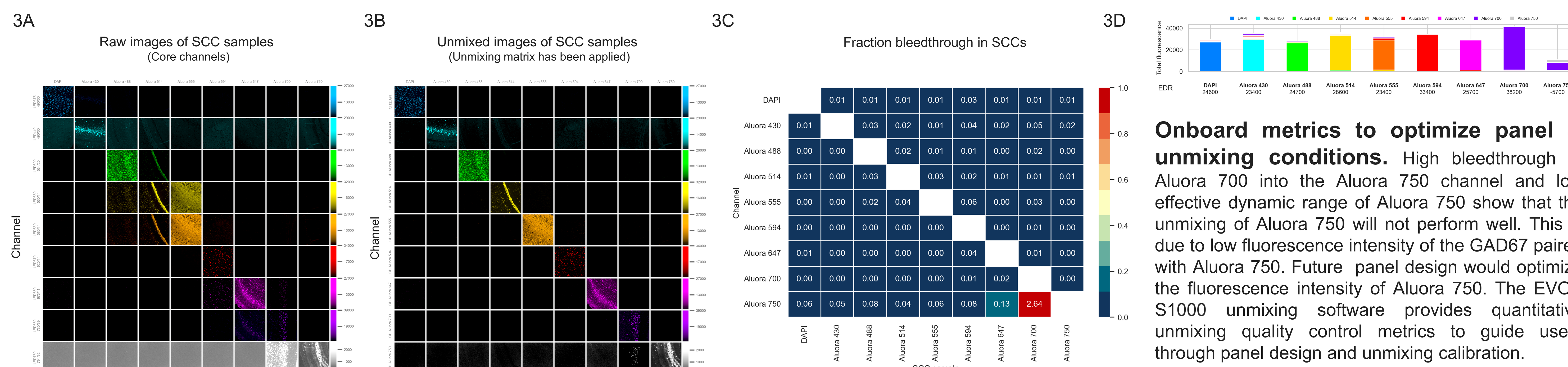


Target	Cell #
Nuclei	4763
CALB1	306
GFAP	1070
TLE1	462
OLIG2	554
NECAB1	902
FOXP2	868
GAD67	457
Cells	4763

Figure 2. Mouse brain tissue stained with the 8-plex Aluora spatial amplification assay and DAPI. Mouse coronal brain tissue section was processed and stained with the Aluora spatial amplification 8-plex assay, 20x Imaging and spectral unmixing performed on the EVOS S1000 Spatial Imaging System. A) Composite image, B) zoomed-in inset, C) individual unmixed channels, D) Quantification of target positive cells identified using HALO®.



Onboard metrics to optimize panel & unmixing conditions. High bleedthrough of Aluora 700 into the Aluora 750 channel and low effective dynamic range of Aluora 750 show that the unmixing of Aluora 750 will not perform well. This is due to low fluorescence intensity of the GAD67 paired with Aluora 750. Future panel design would optimize the fluorescence intensity of Aluora 750. The EVOS S1000 unmixing software provides quantitative unmixing quality control metrics to guide users through panel design and unmixing calibration.

Figure 3. Spectral unmixing enables high-plex spatial biology imaging. A) Single color control (SCC) samples showing spectral overlap in raw images where fluorophore is detected by neighboring core channels. B) Application of the unmixing matrix separates signals into their respective channels. C) Quantification of fluorescence in "Unmixed images". Fraction bleed-through is the ratio of the signal observed in off target channels to the signal of the target fluorophore after unmixing has occurred. D) Effective dynamic range (EDR) = Target fluorescence – bleedthrough.

Conclusions

We developed a highly flexible and sensitive Aluora labeling method capable of 8-rounds of protein labeling. Combined with the EVOS S1000, which enables spectral unmixing of overlapping dyes, we demonstrate the ability to distinguish the unique staining patterns of 8 different biomarkers and DAPI. Onboard metrics assist with the optimization of complex panel design. These high-resolution images allow for precise quantification of 8 targets and identification of cell types in an entire mouse brain section at 20x magnification.

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