Supplementary Material

Supplementary Table 1. **Patient demographics.** ** Unknown pathology score. DO = Disease onset. PM = Post-mortem. LOAD = Late onset Alzheimer's disease. EOAD = Early onset Alzheimer's disease

	Diagnosis	**Sex**	**DO**	**Age**	**Braak**	**Amyloid load**	**PM delay**	**APOE genotype**	**Familial genetic variant**
C1	Control	F		91	2	2/3	03:47	3/3	
C2	Control	M		73	2	1	08:00	3/3	
C3	Control	M		82	**	**	05:30		
C4	Control	F		87	1	0	08:30	3/3	
C5	Control	F		72	0	0	07:15	3/3	
C6	Control	M		93	**	**	08:30		
C7	Control	M		82	1/2	1/2	07:30	3/4	
C8	Control	F		89	3	1/2	06:30	3/4	
C9	Control	F		72	1	1	06:50	3/3	
AD1	LOAD	F	80	88	6	5	04:40	3/4	
AD2	LOAD	M	69	73	5	5	04:45	4/4	
AD3	LOAD	F	>65	82	4	4	04:35	3/4	
AD4	EOAD	F	60	73	6	5	07:17	3/3	
AD5	EOAD	M	61	72	6	5	05:15	3/4	
AD6	EOAD	F	40	67	6	4	04:30	3/4	
AD7	EOAD	F	64	91	6	4/5	04:20	3/3	
AD8	EOAD	M	47	59	5	4/5	05:25	3/3	APP duplication
AD9	LOAD	F	85	90	6	4/5	03:55	2/3	
AD10	EOAD	F	51	70	6	5	04:20	4/4	
AD11	LOAD	F	87	89	4	3-4	04:30	3/3	
AD12	EOAD	F	34	43	6	5	04:15	3/3	PSEN1

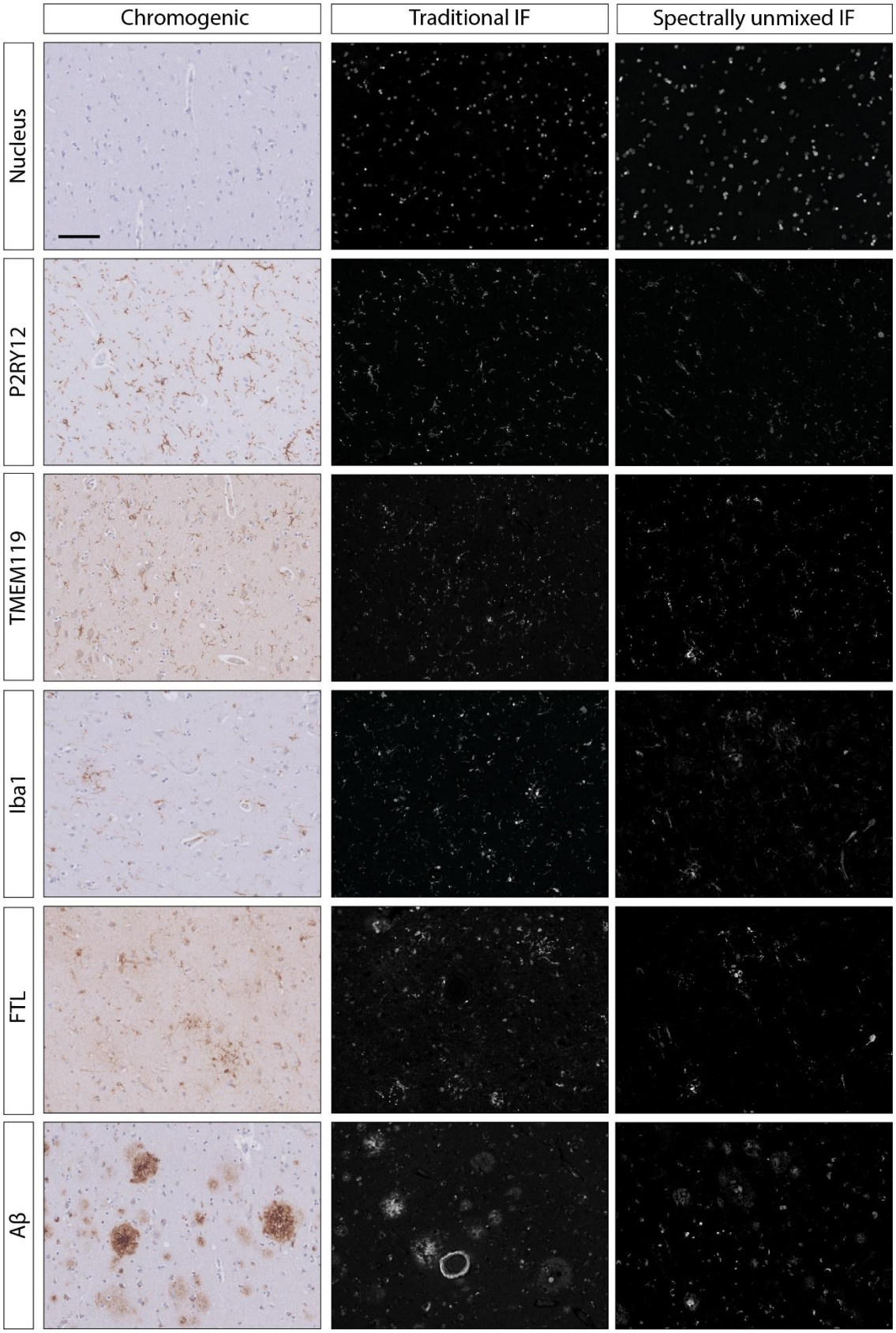
Supplementary Table 2. Details microglia multispectral immunofluorescence (mic-mIF) panel

	Staining target	**Antibody**	**Isotype**	**Antigen retrieval**	**dilution**	**Incubation time**	**Secondary Conjugate**
1.	P2RY12	HPA014518, Sigma Aldrich	rIgG	10mM Citrate buffer (pH=6.0)	1:2500	2h	Poly HRP + Opal 520
2.	TMEM119	HPA051870, Sigma Aldrich	rIgG		1:250	ON	Poly HRP + Opal 570
3.	Light Chain Ferritin (FTL)	AB69090, Abcam	rIgG		1:100	ON	G-α-Rab Alexa 594
4.	Aβ (17–24)	SIG-39220, Biolegend	mIgG2b		1:250	ON	Goat-α-mIgG2b Alexa 647
5.	Iba1	MABN92, Millipore	mIgG1		1:20	ON	G-α-mIgG1 CF680
6.	Nucleus, DAPI	D9542-1mg, Sigma Aldrich	n.a.		0.1 µg/ml	5 min	n.a.

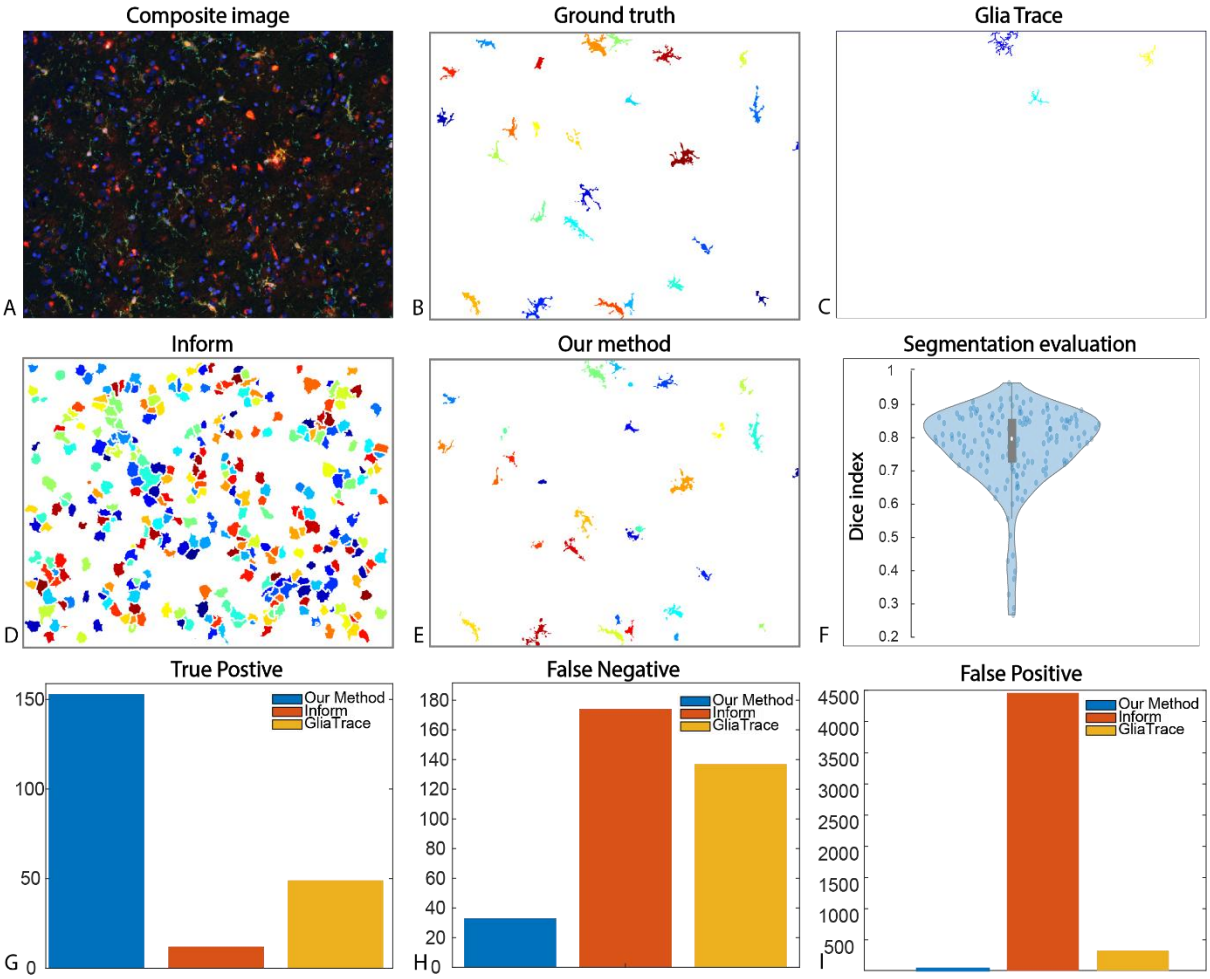
Supplementary Table 3. **Key resources**

Product	Catalogue nr.	Supplier	Dilution
Primary antibodies			
Purified anti-β-Amyloid	SIG-39220	Bio legend	1:250
Anti-Iba1/AIF1	MABN92	Millipore	1:20
Anti-Ferritin Light Chain (rab)	ab69090	Abcam	1:100
Anti-Ferritin Light Chain (mIgG2a)	SC-74513	Santa Cruz	1:100
Anti-P2RY12	HPA014518	Sigma Aldrich	1:2500
Anti-TMEM119	HPA051870	Sigma Aldrich	1:250
Secondary antibodies			
Opal 520 Reagent Pack	FP1487001KT	Perkin Elmer	1:100
Opal 570 Reagent Pack	FP1488001KT	Perkin Elmer	1:100
Goat anti-Rabbit IgG (H+L), Alexa Fluor 594	A-11037	ThermoFisher	1:200
Goat anti-Mouse IgG2b, Alexa Fluor 647	A-21242	ThermoFisher	1:200
Goat anti-mouse IgG1, CF680	20253	Biotium	1:200
Goat anti-Mouse IgG (H+L), Alexa Fluor 546	A-11003	ThermoFisher	1:200
Goat anti-Rabbit IgG (H+L), Alexa Fluor 546	A-11010	ThermoFisher	1:200
Swine Anti-Rabbit Immunoglobulins/Biotin	E0353	DAKO	1:400
Rabbit anti-Mouse Immunoglobulins/Biotin	E0354	DAKO	1:200
VECTASTAIN Elite ABC-HRP Kit, Peroxidase	PK-6100	Vector Laboraties	n.a.
Extra resources			
BrightVision+ Poly- HRP-Anti Mouse/Rabbit IgG Biotin-free	VWRKDPVO1 10HRP	Immunologic	n.a.
1X Plus Amplificiation Diluent	FP1498A	Perkin Elmer	n.a.
ProLong Diamond Antifade mountant	P36961	ThermoFisher	n.a.
DAPI	D9542-1mg	Sigma Aldrich	0.1ug/mL
DAB sigma	D5637	Sigma Aldrich	0.5ug/mL

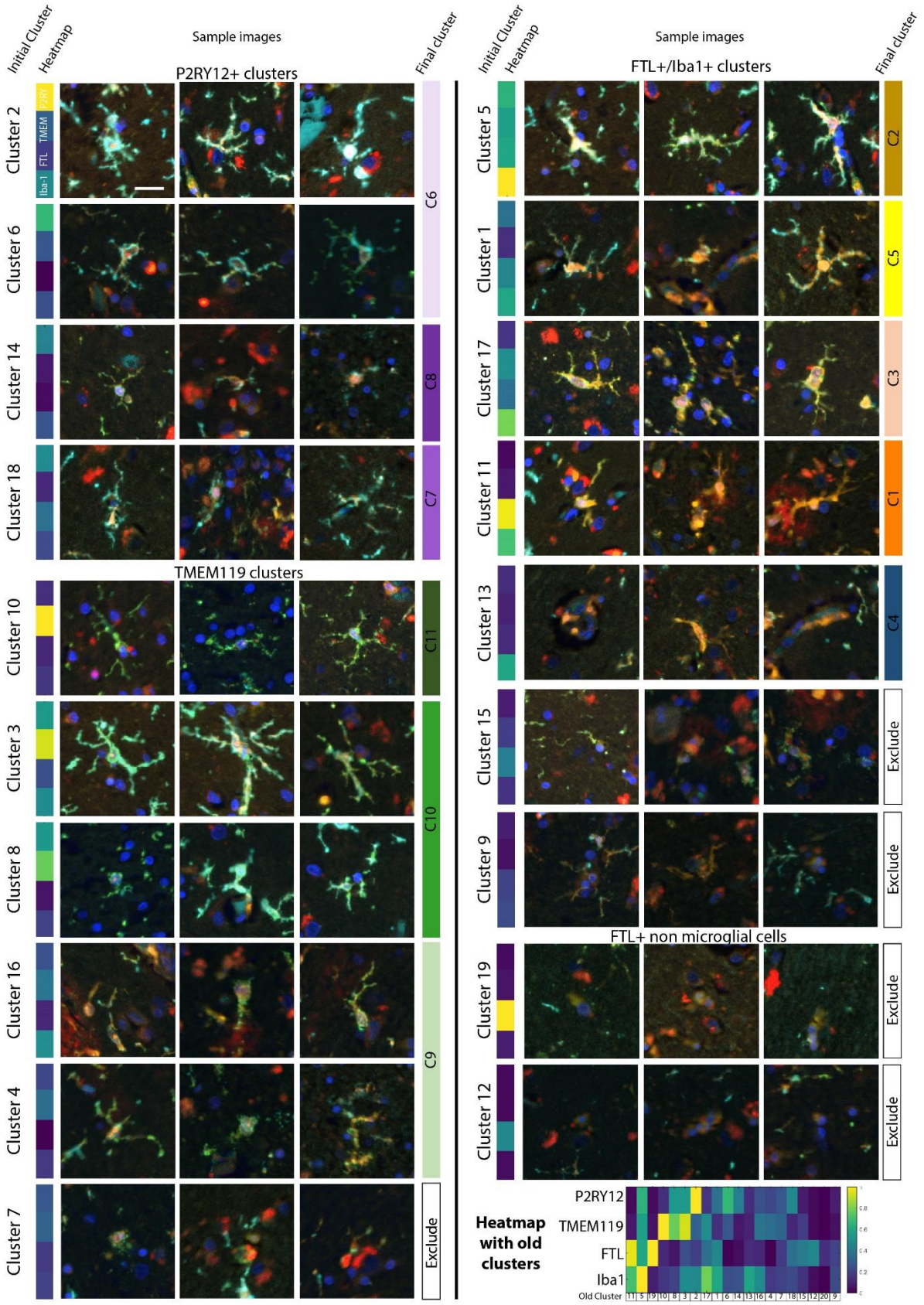
Supplementary Fig. 1. Sample images of DAB-enhanced, single immunofluorescence and multiplexed immunofluorescence stainings of all antibodies used in our multispectral immunofluorescence panel. Scale bar, 100 μm.



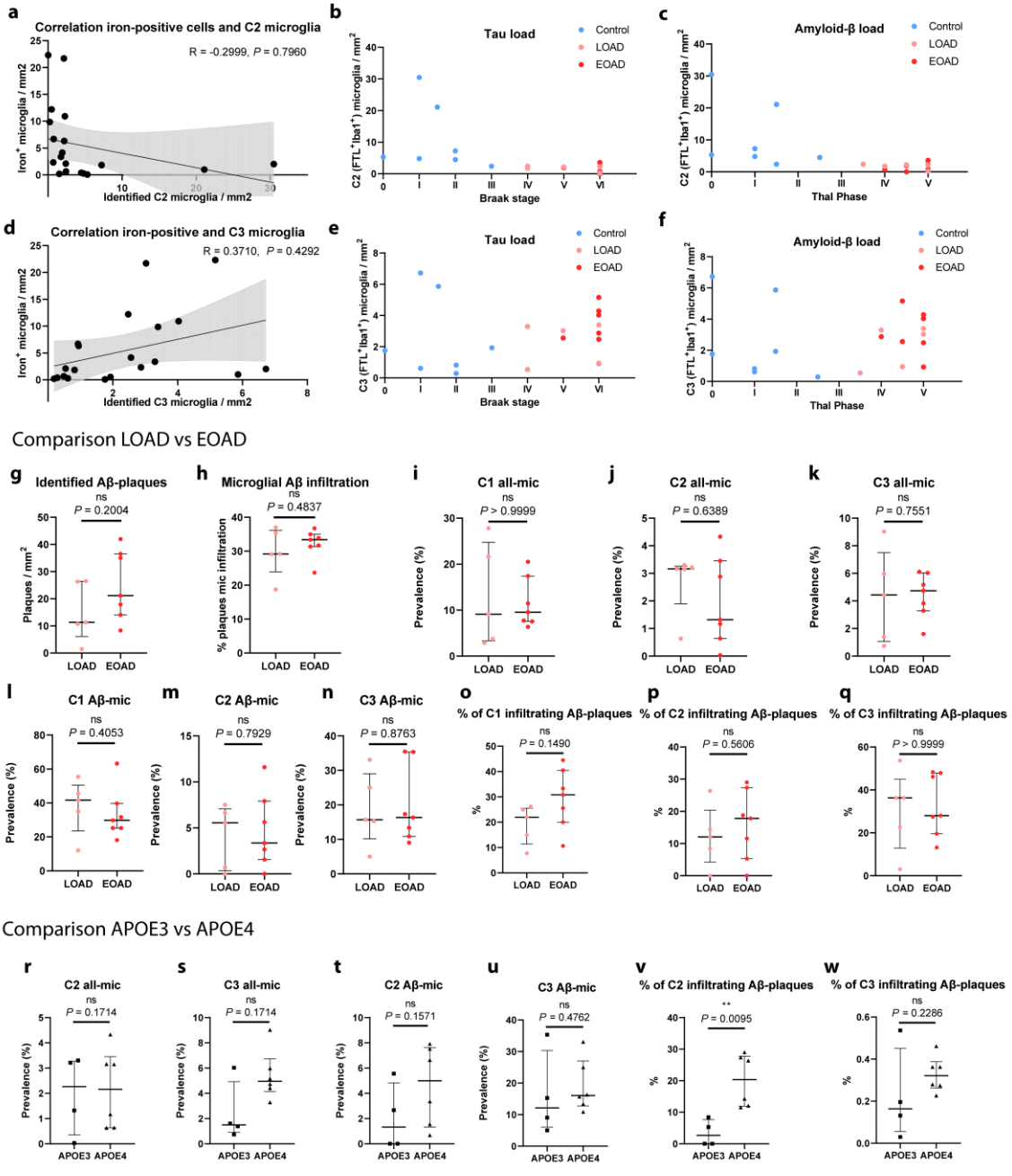
Supplementary Fig. 2. Example of multispectral immunofluorescence data extracted from the grey matter of an AD-patient**.** The segmentation mask derived: manually by the specialist (**b**), using the GliaTrace toolbox (**c**), automatically from the "Inform" image acquisition software (**d**) and from our proposed segmentation pipeline (**e**). **f** Dice's coefficient for each of the 156 cells segmented by our method. Number of correctly identified (**g**), missing (**h**) and falsely identified cells (**i**).



Supplementary Fig. 3. Sample images of all original clusters, and how they were merged. Scale bar, 20 μm.



Supplementary Fig. 4. Correlation of iron^{+}-microglia with C2-microglia (**a**) and C3-microglia (**d**) is nonsignificant (*n* = 20, Pearson coefficient). C2 microglia are mostly present in controls with early stage tau and amyloid-β pathology (**b-c**), whereas C3 microglia are variably present among all stages in both controls and Alzheimer patients (**e-f**). Comparison between LOAD (*n* = 5) and EOAD (*n* = 7) patients shows no differences in number of identified Aβ-plaques (g), percentage of microglia infiltration (**e**), prevalence of all-mic (**i-k**), prevalence of Aβ-mic (**l-n**), and percentage of microglia infiltrating Aβ-plaques (**o-q**) for C1, C2 and C3-microglia (Median, Mann-Whitney U test). Comparison between APOE4 (*n* = 6) vs. APOE3 (*n* = 4) shows no difference in prevalence in all-mic(**r,s**), nor Aβ-mic (**t,u**) of C2 and C3 microglia. A significantly increased proportion of C2-microglia infiltrated Aβ-plaques (v), but this was not the case for C3-microglia (**w**) (Median, Mann-Whitney U test).



Supplementary methods

1. Histological staining protocol

10-µm-thick sections were used for histochemical iron detection using an enhanced Perl's reaction previously published by Van Duijn *et al.* (2013). Sections were incubated for 80 min in 1% potassium ferrocyanide, washed three times in 0.1 M phosphate buffer, followed by 100 min incubation in methanol with 0.01 M NaN_3 and 0.3% H_2O_2. Subsequently, sections were washed again and incubated for 80 min in a solution containing 0.025% 3′3-diaminobenzidine-tetrahydrochloride (DAB, Sigma) and 0.005% H_2O_2 in 0.1 M phosphate buffer. The reaction was stopped by washing with tap water. A consecutive 10-µm-thick section was used for IHC detection of FTL. Sections were deparaffinized with xylene, washed with alcohol and blocked with 0.3% H_2O_2/methanol for 20 min. Subsequently, they were incubated with FTL (1:100, Santa Cruz) overnight at room temperature and after washing with PBS incubated with anti-rabbit alkaline phosphatase (1:50, Vector) for 1 h. Sections were washed with PBS and incubated with Vector Blue for 20 min in the dark. Finally, they were rinsed and covered with aqua mount.

2. Brightfield microscopy

Chromogenically stained slides were imaged using a Philips IntelliSite Ultra Fast Scanner (Philips, the Netherlands). Whole slide images could be viewed in 2×–40× magnification using the Philips Intellisite digital Pathology Solution system. Whole slide images were exported at 4× magnification for quantification of number of iron-positive cells. Snapshots of higher magnification were taken directly in the image-viewer.

3. Antibody validation for multiplexed IHC

The six-colour mic mIF panel (Supplementary Table 2) was created and optimized following a previously described protocol (IJsselsteijn *et al.*, 2019). Individual antibody conditions were optimized using single IHC and IF. Firstly, individual antibodies were tested with chromogenic and fluorescent detection for optimal antibody concentration and antigen retrieval method with the following protocol. 5-µm-thick FFPE tissue sections were deparaffinized with xylene, washed with alcohol and endogenous peroxidase was blocked by incubating in 0.3% H202 in methanol for 20 min. Antigen retrieval was performed with either citrate buffer (10 mM, pH = 6.0) or EDTA buffer (10 mM, pH 9.0). After cooling, the sections were blocked with 0.1% BSA/PBS with 0.05% Tween for 30 min and incubated with primary antibody diluted in blocking buffer in a range of concentrations overnight at room temperature. For chromogenic detection, the slides were washed with PBS and incubated with Sw-a-Rb/biotin (1:400, Dako) or Sw-a-Mouse/biotin (1:200, DAKO) for 60 min, followed by 30 min incubation with VECTAstain elite ABC. Chromogenic substrate was developed with 0.05% DAB (Sigma) with 0.005% H_2O_2 for 10 min. The reaction was stopped with

tap water, after which the sections were counterstained with haematoxylin for 5 min and mounted with micromount. For fluorescent detection, slides were incubated with the appropriate Alexa 546 fluorophore (1:200, ThermoFisher) for 1 hour. Subsequently, the slides were incubated with 0.1 µg/mL DAPI (Sigma) for 5 min and mounted with prolong diamond (ThermoFisher). After individual antibody testing, antibodies were combined to test the viability for multiplexed immunofluorescence. The full protocol described in section 2.3 was performed for 7 slides of one control and one AD subject. One slide of each was incubated with all primary antibodies. For each of the other slides the exact same protocol was performed, but only 1 out of the 6 primary antibodies was added in the primary incubation step. These slides were used to analyse the fluorescent spectrum of the individual fluorophores on our slides, which are subsequently used to un-mix the 6 different spectra. To verify specificity, the extracted signal was compared with single immunofluorescence (Supplementary Fig. 1).

4. Multispectral microscopy and image-acquisition

Mic-mIF-stained tissue slides were scanned at 4× magnification using the Vectra 3.0 Automated Quantitative Pathology Imaging system (PerkinElmer). Following whole slide scanning, a 50% and 25% ROI grid was placed on the cortex and white matter, respectively. For each ROI, high-resolution 20× magnification images are obtained of all subjects. Spectral separation of the 6 individual dyes was performed automatically using InForm Cell Analysis software (PerkinElmer), using spectral libraries obtained with single-marker IF detection of the different fluorophores. In total, six different raw component images of the extracted spectra were exported from Inform for further analysis.

5. Confocal microscopy

20 µm sections were imaged using an Andor Dragonfly 200 spinning disk confocal system (Andor, Oxford Instruments). Sections were stained with DAPI, Alexa 488, Alexa 546 and Alexa 647, which were imaged with a 405 nm, 488 nm, 561 nm and 637 nm laser respectively. Subsequently images were exported and processed using Imaris (Bitplane, Oxford instruments). First, a Gaussian filter was applied to all channels, after which snapshots of the reconstructed 3D projection were taken.

References

van Duijn S, Nabuurs RJA, van Duinen SG, Natté R. Comparison of histological techniques to visualize iron in paraffin-embedded brain tissue of patients with Alzheimer's disease. J Histochem Cytochem 2013; 61: 785–92.

Ijsselsteijn ME, Brouwer TP, Abdulrahman Z, Reidy E, Ramalheiro A, Heeren AM, et al. Cancer immunophenotyping by seven-colour multispectral imaging without tyramide signal amplification. J Pathol Clin Res 2019; 5: 3–11.