Multiplexed Imaging For Brain Tissue

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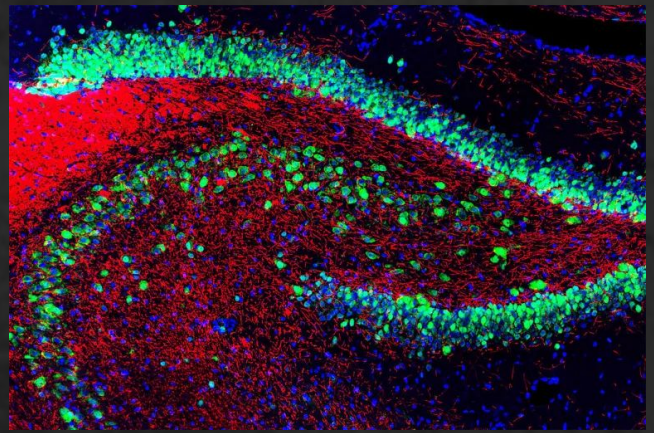
TReNDS Reading-Group Oct 2022

Motivation

Staining of brain cell types is critical not only to identify brain cell structure but to identify specific cells in the brain.



Why Multiplex?

Multiplex techniques enable neuroscientists to measure multiple molecular targets, simultaneously, in a single experiment.

This technique facilitates molecular profiling of individual cells in the context of a tissue, thereby providing a snapshot of cellular function.

Antibody-based detection of proteins can reveal the localization of a particular protein, suggesting a particular function. For example, in neurons, NeuN is localized to the nucleus.

Using multiplexing techniques, researchers can examine the protein's co-localization of their favorite protein. For example, colocalization of their protein of interest with a postsynaptic protein like PSD95 can suggest a postsynaptic function.

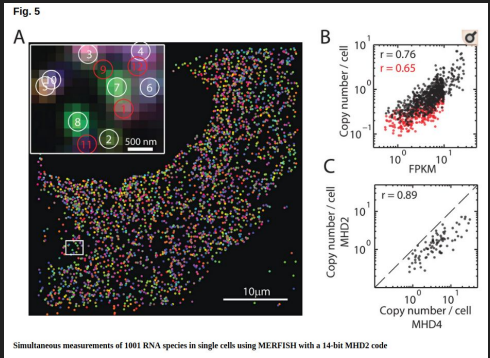
Multispectral Image Support

Assay Name	Biological Ent	Assay Type	Bulk or Single	File Format(s)	# of Samples planned	Median File Size
H&E	histology	imaging	bulk	.svs (aperio). .ir	230	50 MB/svs
CODEX	protein	imaging	single cell	.tiff	30	3Gb/tiff 10-300 files per sample
Ex-SEQ	RNA	imaging	single cell	.tiff	30	5 gb/tif, 20 tiffs per sample
MERFISH	RNA	imaging	single cell	tiff, txt	30	Should be similar to CODEX
MIBI	protein	imaging	single cell	.tiff, msdf (raw)	30	< 50mb/tiff. <500mb/msdf
Spatial transcri	RNA	NGS+imaging	near-single cell	.tif, .fastq, .tsv	30	14 Gb (raw imaging), 200 Mb (processed imaging)

Deep Profiling of Mouse Splenic Architecture with CODEX Multiplexed Imaging

Goltsev Y, Samusik N, Kennedy-Darling J, Bhate S, Hale M, Vazquez G, Black S, Nolan GP

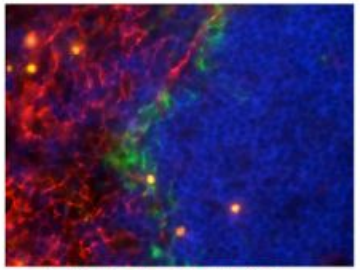
Cell, 2018 – Journal Article



RNA imaging. Spatially resolved, highly multiplexed RNA profiling in single cells

Chen KH, Boettiger AN, Moffitt JR, Wang S, Zhuang X

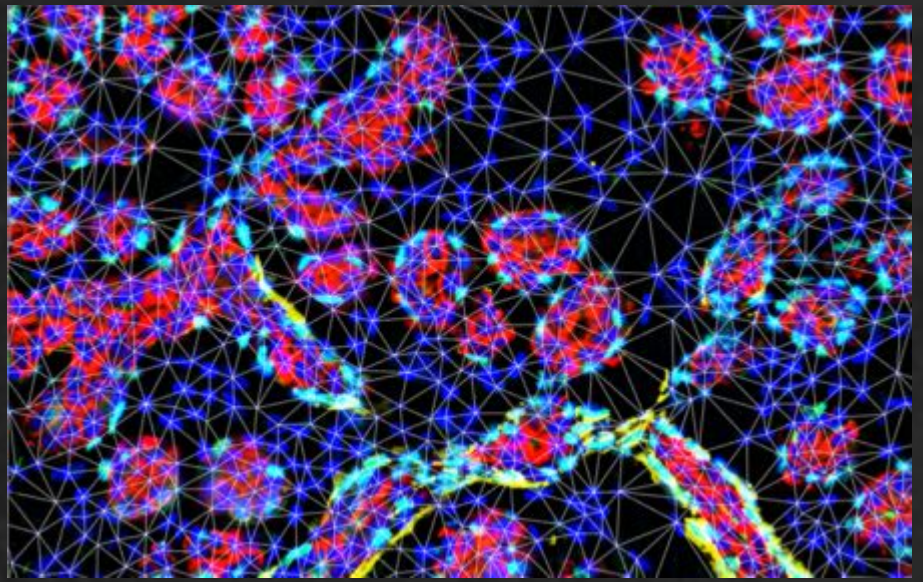
Science, 2015 – Journal Article



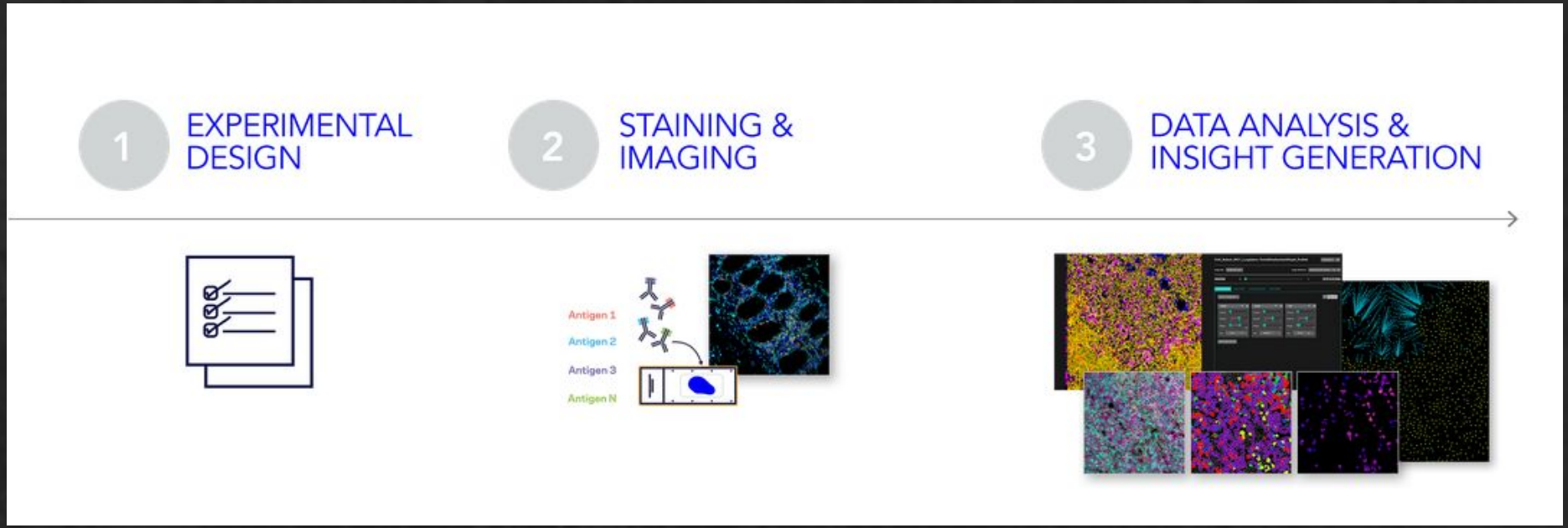
Source : Gutman

Spatial Proteomics

Imaging-based spatial proteomics methods enable quantitative and spatial analysis of over 40 protein markers across a whole tissue section at single-cell resolution.



Main Pipeline



Source : IONPATH

nature COMMUNICATIONS

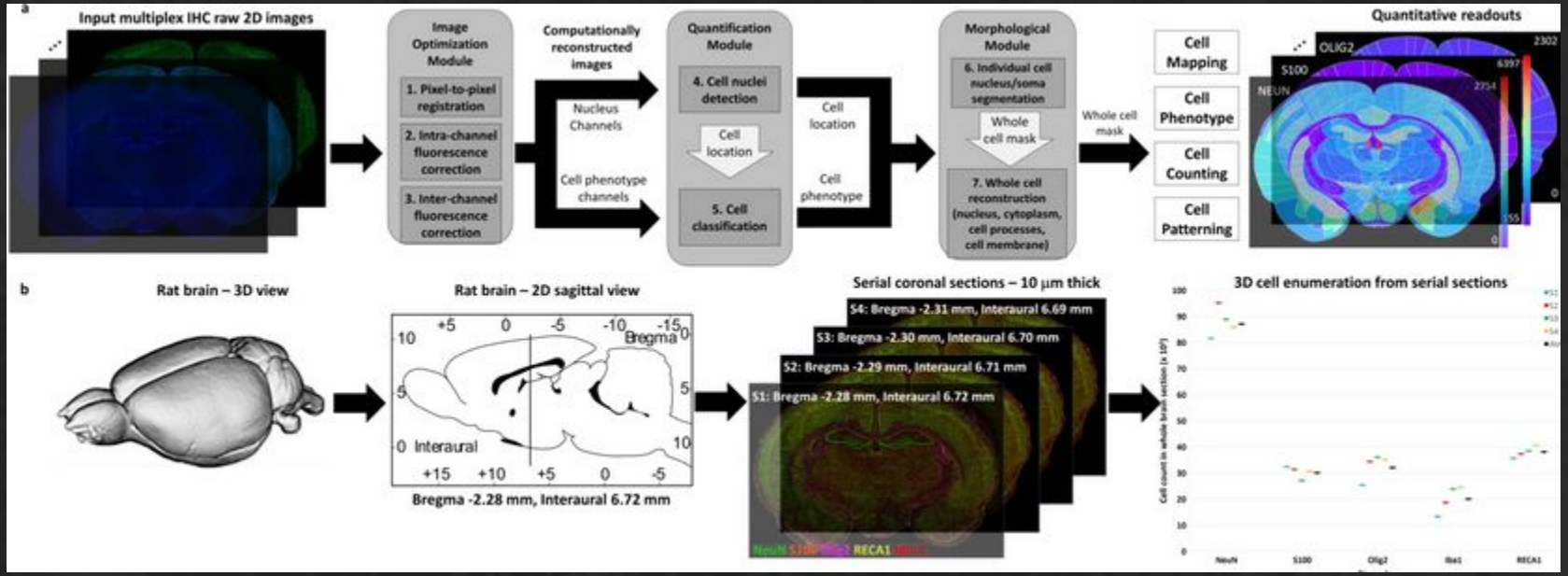
ARTICLE

https://doi.org/10.1038/s41467-021-21735-x OPEN

Whole-brain tissue mapping toolkit using large-scale highly multiplexed immunofluorescence imaging and deep neural networks

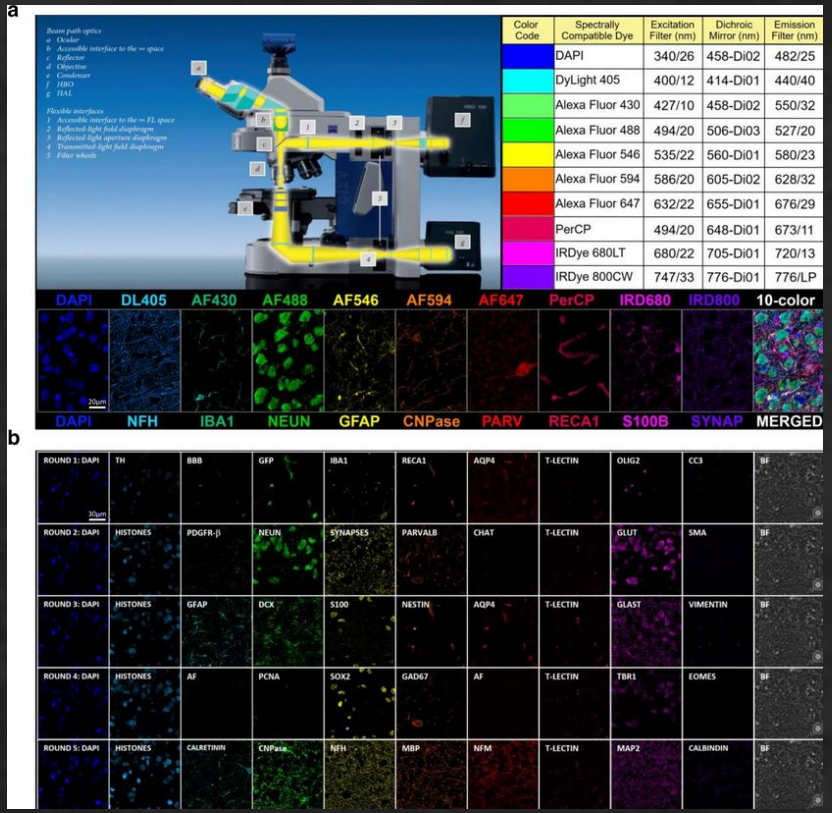
Dragan Maric[1,3,4✉], Jahandar Jahanipour[1,2,3], Xiaoyang Rebecca Li[2], Aditi Singh[2], Aryan Mobiny[2], Hien Van Nguyen[2], Andrea Sedlock[1], Kedar Grama[2] & Badrinath Roysam[2,4✉]

Overview of the whole brain tissue phenotyping pipeline



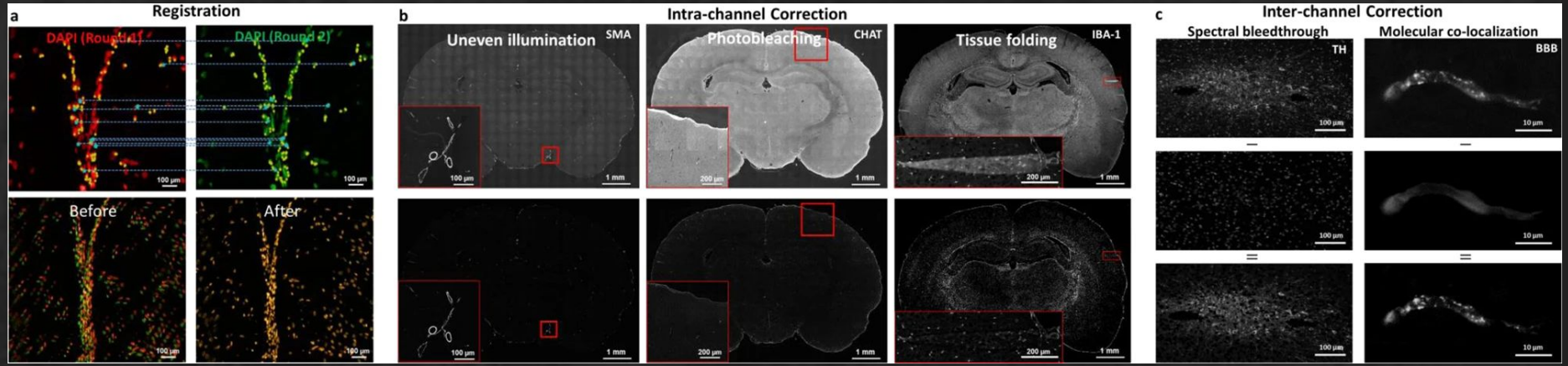
Source : Maric et al.

Overview of the multiplex IHC staining and multispectral epifluorescence imaging platform.



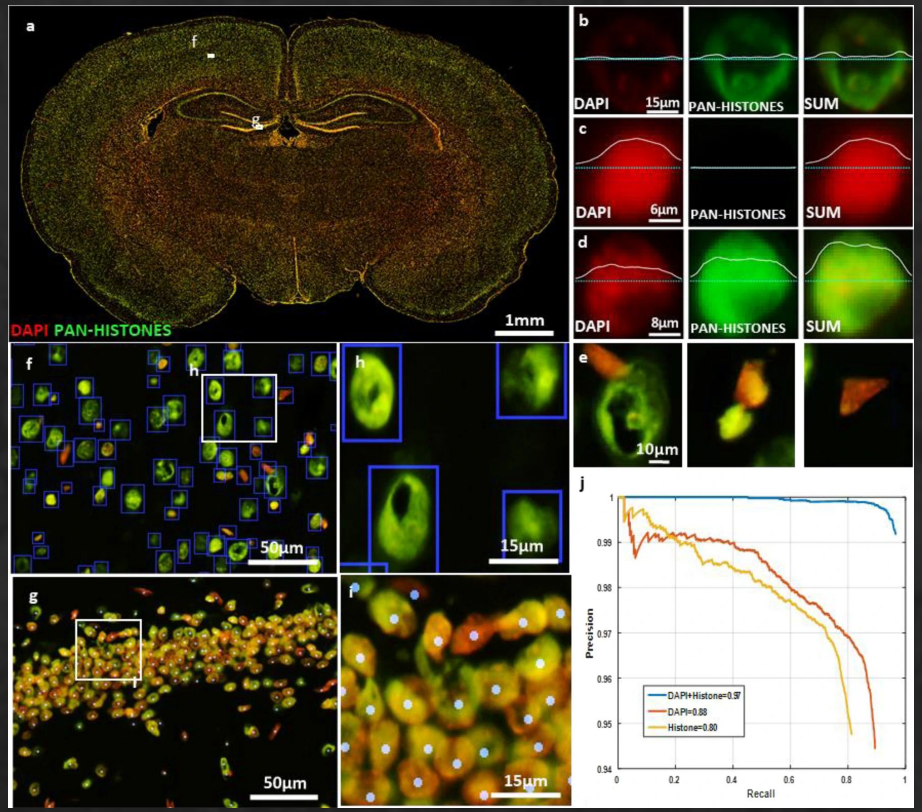
Source : Maric et al.

Overview of the post-acquisition image optimization module

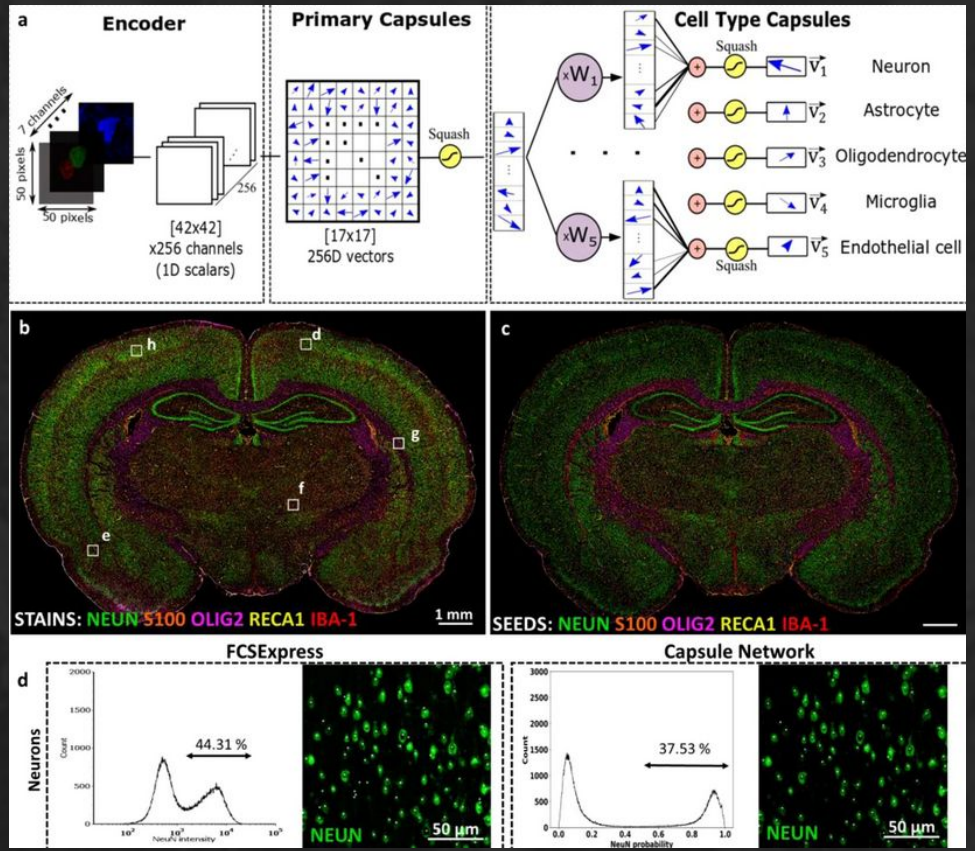


Source : Maric et al.

Improved nuclei detection in whole brain images using a multiplexed approach

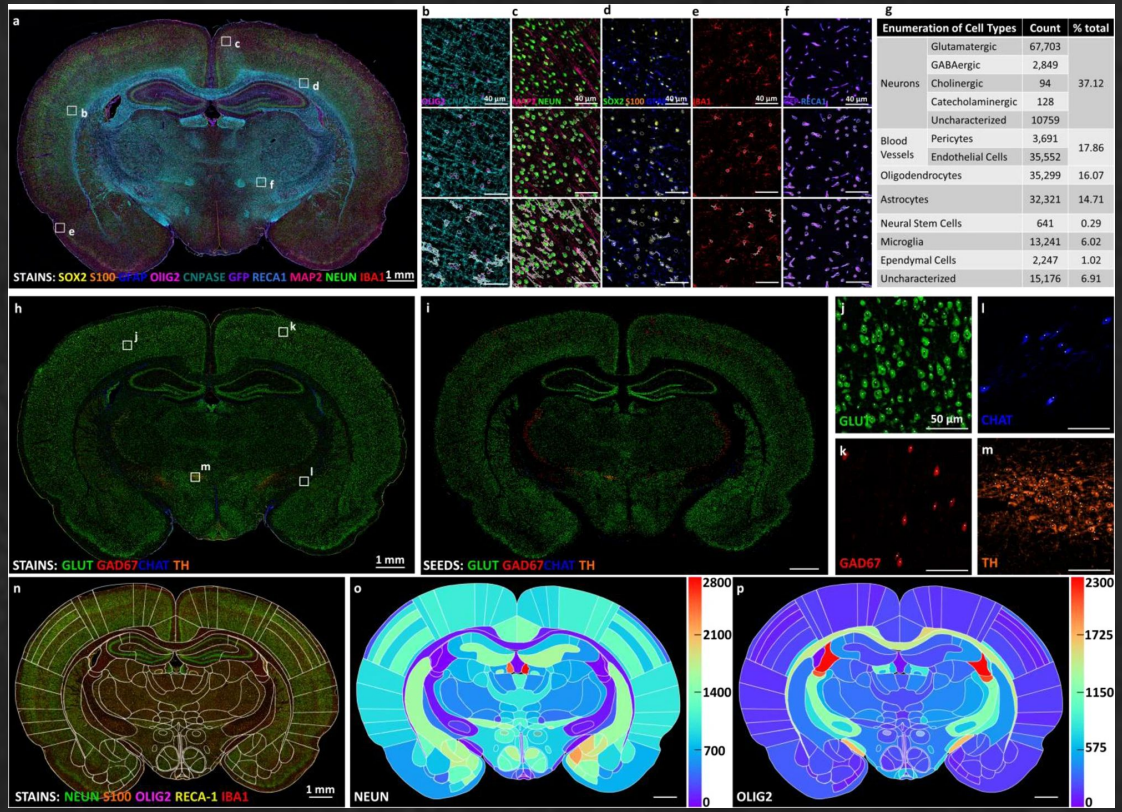


Source : Maric et al.



Source : Maric et al.

Summary of quantification and morphological modules



Enumeration of Cell Types		Count	% total
Neurons	Glutamatergic	67,703	37.12
	GABAergic	2,849	
	Cholinergic	94	
	Catecholaminergic	128	
	Uncharacterized	10759	
Blood Vessels	Pericytes	3,691	17.86
	Endothelial Cells	35,552	
Oligodendrocytes		35,299	16.07
Astrocytes		32,321	14.71
Neural Stem Cells		641	0.29
Microglia		13,241	6.02
Ependymal Cells		2,247	1.02
Uncharacterized		15,176	6.91

Source : Maric et al.

Thank you

Table–1

Supplementary Table 1. Library of validated primary antibodies for multiplex IHC staining of rat brain tissue sections

Panel 1 - Neuroinflammatory Cell Phenotyping:

Antibody	Target/Function	Vendor	Product #	RRID #	Host Ig class	Clone	Conjugate	Reactivity	Application
CD3	All T cells	Thermo Fisher Sci	MA1-7630	AB_2073347	Mouse IgG3	RIV9	Unconjugated	Hu, Ms, Rt	IHC-FoFr
CD45R/B220	All B cells	Thermo Fisher Sci	14-0460-82	AB_467275	Mouse IgG2b	HIS24	Unconjugated	Rt	IHC-FoFr
MHC Class II	Dendritic Cells	Thermo Fisher Sci	14-0920-82	AB_467384	Mouse IgG1	HIS19	Unconjugated	Rt	IHC-FoFr
IBA1	All Microglia, Macrophages	Wako Chemicals	019-19741	AB_839504	Rabbit IgG	Polyclonal	Unconjugated	Hu, Ms, Rt	IHC-P, IHC-FoFr
IBA1	All Microglia, Macrophages	Synaptic Systems	234006	AB_2619949	Chicken IgY	Polyclonal	Unconjugated	Hu, Ms, Rt	IHC-P, IHC-FoFr
IBA1	All Microglia, Macrophages	Synaptic Systems	234004	AB_2493179	Guinea Pig IgG	Polyclonal	Unconjugated	Hu, Ms, Rt	IHC-P, IHC-FoFr
CD68	Activated Macrophages	Abcam	ab125212	AB_10975465	Rabbit IgG	Polyclonal	Unconjugated	Hu, Ms, Rt	IHC-FoFr
TMEM 119	Resident Brain Microglia	Synaptic Systems	400211	AB_2814935	Mouse IgG2a	97G1C1	Unconjugated	Rt	IHC-P, IHC-FoFr

Panel 2 - Neural Stem Cells and Astroglial Cell Lineage Phenotyping:

Antibody	Target/Function	Vendor	Product #	RRID #	Host Ig class	Clone	Conjugate	Reactivity	Application
Human/Mouse/Rat Sox2	Neural Stem Cells, Neural Progenitors	BD Biosciences	561469	AB_10694256	Mouse IgG1	O30-678	Unconjugated	Hu, Ms, Rt	IHC-P, IHC-FoFr
Human/Mouse/Rat Sox2	Neural Stem Cells, Neural Progenitors	Thermo Fisher Sci	14-9811-82	AB_11219471	Rat IgG2a	Btjce	Unconjugated	Hu, Ms, Rt	IHC-P, IHC-FoFr
Mouse/Rat Nestin	Neural Stem Cells, Neural Progenitors	Millipore Sigma	MAB353	AB_94911	Mouse IgG1	Rat 401	Unconjugated	Ms, Rt	IHC-FoFr
Vimentin	Astrocytes-Differentiating Stage	Synaptic Systems	172004	AB_2832221	Guinea Pig IgG	Polyclonal	Unconjugated	Ms, Rt	IHC-FoFr
Vimentin	Astrocytes-Differentiating Stage	Synaptic Systems	172006	AB_2800525	Chicken IgY	Polyclonal	Unconjugated	Hu, Ms, Rt	IHC-P, IHC-FoFr
Vimentin	Astrocytes-Differentiating Stage	Millipore Sigma	AB5773	AB_11212377	Chicken IgY	Polyclonal	Unconjugated	Hu, Mk, Ms, Rt	IHC-P, IHC-FoFr
S100β	Astrocytes-Fully Mature Stage	Millipore Sigma	MAB079-1	AB_571112	Mouse IgG2a	15E2E2	Unconjugated	Hu, Mk, Ms, Rt	IHC-P, IHC-FoFr
GFAP	Astrocytes-Reactive Stage	Agilent/Dako	556330	AB_396368	Mouse IgG2b	Cocktail	Unconjugated	Hu, Mk, Ms, Rt	IHC-P, IHC-FoFr
GFAP	Astrocytes-Reactive Stage	Thermo Fisher Sci	13-0300	AB_2532994	Rat IgG2a	2.2B10	Unconjugated	Hu, Mk, Ms, Rt	IHC-P, IHC-FoFr
GFAP	Astrocytes-Reactive Stage	Thermo Fisher Sci	PA1-10004	AB_1074620	Chicken IgY	Polyclonal	Unconjugated	Hu, Mk, Ms, Rt	IHC-P, IHC-FoFr
GFAP	Astrocytes-Reactive Stage	Agilent/Dako	Z0334	AB_10013382	Rabbit IgG	Polyclonal	Unconjugated	Hu, Mk, Ms, Rt	IHC-P, IHC-FoFr
GLAST	Astrocytes-Glutamate Transport	Synaptic Systems	250114	AB_2619957	Guinea Pig IgG	Polyclonal	Unconjugated	Hu, Ms, Rt	IHC-P, IHC-FoFr
GLAST	Astrocytes-Glutamate Transport	Miltenyi Biotec	130-095-822	AB_10829302	Mouse IgG2a	ACSA-1	Unconjugated	Hu, Ms, Rt	IHC-P, IHC-FoFr
AQP4	Astrocytes-BBB Integrity	Abcam	ab9512	AB_307299	Mouse IgG3	[4/18]	Unconjugated	Hu, Ms, Rt	IHC-P, IHC-FoFr

Panel 3 - Oligodendroglial Cell Phenotyping:

Antibody	Target/Function	Vendor	Product #	RRID #	Host Ig class	Clone	Conjugate	Reactivity	Application
Olig2	Oligodendrocytes-Gene Transcription	Millipore Sigma	MABN50	AB_10807410	Mouse IgG2a	211F1.1	Unconjugated	Hu, Ms, Rt	IHC-P, IHC-FoFr
CNPase	Oligodendrocytes-Soma, Processes	Millipore Sigma	MAB326	AB_2082608	Mouse IgG1	11-5B	Unconjugated	Hu, Ms, Rt	IHC-P, IHC-FoFr
CNPase	Oligodendrocytes-Soma, Processes	Novus Biologicals	MA5-35002	AB_2848907	Mouse IgG2b	CL2887	Unconjugated	Hu, Ms, Rt	IHC-P, IHC-FoFr
Myelin-MAG	Oligodendrocytes-Myelinating	BioLegend	851702	AB_2749880	Mouse IgG2b	B11F7	Unconjugated	Hu, Ms, Rt	IHC-P, IHC-FoFr
Myelin-PLP	Oligodendrocytes-Myelinating	Millipore Sigma	MAB388	AB_177623	Mouse IgG2a	PLPC1	Unconjugated	Hu, Mk, Ms, Rt	IHC-P, IHC-FoFr
Myelin-MBP	Oligodendrocytes-Myelinating	BioLegend	808402	AB_2564742	Mouse IgG2b	SMI99	Unconjugated	Hu, Ms, Rt	IHC-P, IHC-FoFr
Myelin-MBP	Oligodendrocytes-Myelinating	Millipore Sigma	MAB386	AB_94975	Rat IgG2a	aa 82-87	Unconjugated	Hu, Ms, Rt	IHC-P, IHC-FoFr

Panel 4 - Neuronal Cytoarchitecture:

Antibody	Target/Function	Vendor	Product #	RRID #	Host Ig class	Clone	Conjugate	Reactivity	Application
NeuN	Neurons-Fully Mature	Millipore Sigma	MAB377	AB_2298772	Mouse IgG1	A60	Unconjugated	Hu, Ms, Rt	IHC-P, IHC-FoFr
NeuN	Neurons-Fully Mature	Millipore Sigma	ABN78	AB_10807945	Rabbit IgG	Polyclonal	Unconjugated	Hu, Ms, Rt	IHC-P, IHC-FoFr
NeuN	Neurons-Fully Mature	Millipore Sigma	ABN91	AB_11205760	Chicken IgY	Polyclonal	Unconjugated	Ms, Rt	IHC-P, IHC-FoFr
NeuN	Neurons-Fully Mature	Millipore Sigma	ABN90P	AB_2341095	Guinea Pig IgG	Polyclonal	Unconjugated	Hu, Mk, Ms, Rt	IHC-P, IHC-FoFr
Tubulin β3	Neurons-Cytoskeletal	Millipore Sigma	T8660	AB_477590	Mouse IgG2b	SDL.3D10	Unconjugated	Hu, Ms, Rt	IHC-P, IHC-FoFr

Table–2

Supplementary Table 2. Library of spectrally compatible secondary antibodies for 10-plex fluorescence IHC staining

Secondary Antibody	DL405[a]	DY395XL[c]	AF430[a]	AF488[a]	AF546[a]	AF594[a]	AF647[a]	PerCP[e]	IRD680LT[d]	IRD800CW[d]
Goat Anti-Mouse IgG1	115-475-205[b]	Custom[d]	Custom[b]	A21121[a]	A21123[a]	A21125[a]	A21240[a]	115-125-205[b]	926-68050[d]	926-32350[d]
Goat Anti-Mouse IgG2a	115-475-206[b]	Custom[d]	N/A	A21131[a]	A21133[a]	A21135[a]	A21241[a]	115-125-206[b]	926-68051[d]	926-32351[d]
Goat Anti-Mouse IgG2b	115-475-207[b]	Custom[d]	N/A	A21141[a]	A21143[a]	A21145[a]	A21242[a]	115-125-207[b]	926-68052[d]	926-32352[d]
Goat Anti-Mouse IgG3	115-475-209[b]	Custom[d]	N/A	A21151[a]	N/A	A21155[a]	115-605-209[b]	115-125-209[b]	Custom[d]	Custom[d]
Goat Anti-Mouse IgM	115-475-075[b]	Custom[d]	N/A	A21042[a]	A21045[a]	A21044[a]	A21238[a]	115-126-075[b]	926-68080[d]	926-32280[d]
Goat Anti-Rat IgG (min x Ms)*	112-475-167[b]	Custom[d]	N/A	A11006[a]	A11081[a]	A11007[a]	A21247[a]	112-126-071[b]	926-68029[d]	926-32219[d]
Goat/Donkey Anti-Rabbit IgG	711-475-152[b]	Custom[d]	A11064[a]	A11034[a]	A11035a	A11037[a]	A21245[a]	711-126-152[b]	926-68023[d]	926-32213[d]
Goat/Donkey Anti-Chicken IgG	703-475-155[b]	Custom[d]	N/A	A11039[a]	A11040a	A11042[a]	A21449[a]	703-126-155[b]	926-68028[d]	926-32218[d]
Goat/Donkey Anti-Guinea Pig IgG	706-475-148[b]	Custom[d]	N/A	A11073[a]	A11074a	A11076[a]	A21450[a]	706-126-148[b]	926-68030[d]	926-32411[d]
Donkey $F(ab')_2$ Anti-Sheep IgG	713-475-147[b]	N/A	N/A	A11015[a]	A21098a	A11016[a]	A21448[a]	713-126-147[b]	N/A	N/A

AF: Alexa Fluor; DL: DyLight; DY: Dyomics; IRD: Infra Red Dye; PerCP: peridinin-chlorophyll-protein; XL:Extra Long.

[a]Thermo Fisher Scientific; [b]Jackson ImmunoResearch; [c]Dyomics; [d]LI-COR Biosciences; [e]BD Biosciences.

*Min x Ms: Highly adsorbed for minimal crossreactivity with mouse IgG/IgM antibodies.

Tabulated entries represent vendor product/catologue numbers.

Custom: Custom conjugated by vendor.

Table–3

Supplementary Table 3. Standard filter selection for 10-color epifluorescence imaging with matrix crosstalk analysis performed by AVR Optics using Semrock SearchLight Spectra Viewer

Standard 10-color Filter Setup 1		Filter Set Specifications[a]			Signal from Primary Set[b]	Filter Signal Crosstalk (% Transmission)[c]									
Fluorophore	Standard Filter Set	Exciter	Dichroic	Emitter	Fluorescence Signal (mW)	Fluorophore	DAPI	DL405	AF430	AF488	AF546	AF594	AF647	PerCP	IRD680LT	IRD800CW
DAPI	DAPI-5060C	FF01-377/50	FF409-Di03	FF02-447/60	7.03E-07	DAPI	100.00%	201.99%	0.47%	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
DyLight 405	LED-DAPI-B	FF01-378/52	FF409-Di03	FF02-447/60	1.42E-06	DL405	47.68%	100.00%	0.24%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Alexa Fluor 430	LuciferYellow-C	FF01-438/24	FF482-Di01	FF01-538/40	2.81E-07	AF430	0.21%	0.00%	100.00%	31.10%	2.23%	0.00%	0.00%	0.38%	0.05%	0.00%
Alexa Fluor 488	FITC-3540C	FF01-482/35	FF506-Di03	FF01-536/40	6.08E-07	AF488	0.00%	0.00%	15.07%	100.00%	1.63%	0.00%	0.00%	0.17%	0.00%	0.00%
Alexa Fluor 546	TRITC-B	FF01-543/22	FF562-Di01	FF01-593/40	8.61E-07	AF546	0.00%	0.00%	0.17%	0.36%	100.00%	53.54%	0.00%	0.07%	0.00%	0.00%
Alexa Fluor 594	SpRed-B	FF01-586/20	FF605-Di02	FF01-628/32	1.65E-06	AF594	0.00%	0.00%	0.01%	0.01%	5.94%	100.00%	1.84%	0.03%	0.00%	0.00%
Alexa Fluor 647	Cy5-4040-C	FF02-628/40	FF660-Di02	FF01-692/40	6.38E-07	AF647	0.00%	0.00%	0.00%	0.00%	0.07%	9.84%	100.00%	4.67%	29.94%	0.00%
PerCP	N/A*	FF01-488/50	FF640-FDi02	FF01-676/29	7.61E-07	PerCP	0.00%	0.00%	1.41%	0.39%	0.89%	4.30%	0.68%	100.00%	0.55%	0.00%
IRDye 680LT	Cy5.5-C	FF02-655/40	FF685-Di02	FF01-716/40	5.37E-07	IRDye 680LT	0.00%	0.00%	0.00%	0.00%	0.01%	0.45%	44.88%	2.50%	100.00%	0.00%
IRDye 800CW	Cy7-B	FF01-708/75	FF757-Di01	FF02-809/81	8.68E-07	IRDye 800CW	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.09%	0.10%	14.06%	100.00%

Standard 10-color Filter Setup 2		Filter Set Specifications[a]			Signal from Primary Set[b]	Filter Signal Crosstalk (% Transmission)[c]									
Fluorophore	Standard Filter Set	Exciter	Dichroic	Emitter	Fluorescence Signal (mW)	Fluorophore	DAPI	DL405	DY395XL	AF488	AF546	AF594	AF647	PerCP	IRD680LT	IRD800CW
DAPI	DAPI-5060C	FF01-377/50	FF409-Di03	FF02-447/60	7.03E-07	DAPI	100.00%	201.99%	1.37%	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
DyLight 405	LED-DAPI-B	FF01-378/52	FF409-Di03	FF02-447/60	1.42E-06	DL405	47.68%	100.00%	0.68%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Dyomics 395XL	LF405/LP-B	FF01-390/40	Di02-R405	BLP01-405R	6.70E-06	DY395XL	10.78%	17.46%	100.00%	1.75%	3.19%	9.13%	0.52%	7.51%	3.04%	0.00%
Alexa Fluor 488	FITC-3540C	FF01-482/35	FF506-Di03	FF01-536/40	6.08E-07	AF488	0.00%	0.00%	7.80%	100.00%	1.63%	0.00%	0.00%	0.17%	0.00%	0.00%
Alexa Fluor 546	TRITC-B	FF01-543/22	FF562-Di01	FF01-593/40	8.61E-07	AF546	0.00%	0.00%	0.76%	0.36%	100.00%	53.54%	0.00%	0.07%	0.00%	0.00%
Alexa Fluor 594	SpRed-B	FF01-586/20	FF605-Di02	FF01-628/32	1.65E-06	AF594	0.00%	0.00%	0.15%	0.01%	5.94%	100.00%	1.84%	0.03%	0.00%	0.00%
Alexa Fluor 647	Cy5-4040-C	FF02-628/40	FF660-Di02	FF01-692/40	6.38E-07	AF647	0.00%	0.00%	0.03%	0.00%	0.07%	9.84%	100.00%	4.67%	29.94%	0.00%
PerCP	N/A*	FF01-488/50	FF640-FDi02	FF01-676/29	7.61E-07	PerCP	0.00%	0.00%	1.59%	0.39%	0.89%	4.30%	0.68%	100.00%	0.55%	0.00%
IRDye 680LT	Cy5.5-C	FF02-655/40	FF685-Di02	FF01-716/40	5.37E-07	IRDye 680LT	0.00%	0.00%	0.00%	0.00%	0.01%	0.45%	44.88%	2.50%	100.00%	0.00%
IRDye 800CW	Cy7-B	FF01-708/75	FF757-Di01	FF02-809/81	8.68E-07	IRDye 800CW	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.09%	0.10%	14.06%	100.00%

[a]Filter set specifications are based on the selection of standard off-the-shelf filter sets that most closely matched the spectral properties of the selected fluorophores, as determined using Semrock SearchLight Spectra Viewer. Standard filter setup 1 was selected for a panel of 10 commonly used spectrally compatible non-tandem flurophores. Standard filter setup 2 was selected to include a custom DY-395XL, a mega-Stokes shift dye, in place of AF430, to improve spectral compatibility with AF488.

[b]Estimated fluorescence signal in mW of the primary filter set. This parameter was used as a reference in Semrock SearchLight Spectral Viewer to make sure the filter set would produce sufficient signal in comparison with single fluorophore optimized sets.

[c]Filter crosstalk was computed by AVR Optics using Semrock SearchLight Spectra Viewer for the fluorophores and filter specifications listed above using the following microscope setup: Zeiss AxioImager.Z2 epifluorescence microscope, Excelitas X-Cite 200DC mercury vapor short arc broad spectrum lamp, and Hamamatsu Orca-Flash 4.0 V2 sCMOS camera. Cross-talk values exceeding 10% signal transmission required for optimal 10-color fluorescence imaging are highlighted in yellow.

Details of each standard filter set specifications are available in SearchLight here: https://searchlight.semrock.com/?sid=f46f2125-6930-4622-86c0-a9d02c5a6f84.

*N/A: Not Available; Standard PerCP filter set for epifluorescence imaging is not currently available from Smrock. Instead, filters were selected based on a good signal and signal-to-noise without regard to other fluorophores in the 10-color panel.

Abbreviations: AF: Alexa Fluor; DAPI: 4',6-diamidino-2-phenylindole; DL: DyLight; DY: Dyomics; FITC: Fluorescein isothiocyanate; IRD: Infra Red Dye; PerCP: Peridinin-chlorophyll-protein; TRITC: Tetramethylrhodamine-isothiocyanate;

Table–4

Supplementary Table 4. Custom filter selection for 10-color epifluorescence imaging with matrix crosstalk analysis performed by AVR Optics using Semrock SearchLight Spectra Viewer

Custom 10-color Filter Setup 1	Filter Set Specifications[a]			Signal from Primary Set[b]	Filter Signal Crosstalk (% Transmission)[c]									
Fluorophore	Exciter	Dichroic	Emitter	Fluorescence Signal (mW)	Fluorophore	DAPI	DL405	AF430	AF488	AF546	AF594	AF647	PerCP	IRD680LT	IRD800CW
DAPI	FF01-340/26	FF458-Di02	FF01-482/25	6.58E-08	DAPI	100.00%	0.00%	1.69%	2.28%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
DyLight 405	FF01-400/12	FF414-Di01	FF01-440/40	3.53E-07	DL405	5.95%	100.00%	0.14%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Alexa Fluor 430	FF01-427/10	FF458-Di02	FF01-550/32	9.61E-08	AF430	0.33%	0.00%	100.00%	12.28%	7.39%	0.00%	0.00%	0.29%	0.00%	0.00%
Alexa Fluor 488	FF01-494/20	FF506-Di03	FF01-527/20	3.38E-07	AF488	0.00%	0.00%	4.53%	100.00%	0.10%	0.00%	0.00%	0.10%	0.00%	0.00%
Alexa Fluor 546	FF01-535/22	FF560-Di01	FF01-580/23	4.28E-07	AF546	0.00%	0.00%	0.22%	1.12%	100.00%	9.00%	0.00%	0.07%	0.00%	0.00%
Alexa Fluor 594	FF01-586/20	FF605-Di02	FF01-628/32	1.59E-06	AF594	0.00%	0.00%	0.01%	0.01%	5.90%	100.00%	1.89%	0.03%	0.00%	0.00%
Alexa Fluor 647	FF02-632/22	FF655-Di01	FF01-676/29	4.48E-07	AF647	0.00%	0.00%	0.00%	0.00%	0.08%	3.93%	100.00%	4.08%	1.90%	0.00%
PerCP	FF01-494/20	FF648-Di01	FF01-673/11	2.02E-07	PerCP	0.00%	0.00%	0.50%	0.41%	0.62%	3.18%	0.61%	100.00%	0.00%	0.00%
IRDye 680LT	FF01-680/22	FF705-Di01	FF01-720/13	1.87E-07	IRDye 680LT	0.00%	0.00%	0.00%	0.00%	0.00%	0.04%	7.70%	1.79%	100.00%	0.00%
IRDye 800CW	FF01-747/33	FF776-Di01	FF01-776/LP	5.96E-07	IRDye 800CW	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.08%	100.00%

Custom 10-color Filter Setup 2	Filter Set Specifications[a]			Signal from Primary Set[b]	Filter Signal Crosstalk (% Transmission)[c]									
Fluorophore	Exciter	Dichroic	Emitter	Fluorescence Signal (mW)	Fluorophore	DAPI	DL405	DY395XL	AF488	AF546	AF594	AF647	PerCP	IRD680LT	IRD800CW
DAPI	FF01-340/26	FF458-Di02	FF01-482/25	6.58E-08	DAPI	100.00%	0.00%	6.87%	2.28%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
DyLight 405	FF01-400/12	FF414-Di01	FF01-440/40	3.53E-07	DL405	5.95%	100.00%	0.16%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Dyomics 395XL	FF01-409/32	FF552-Di02	FF01-572/15	1.06E-06	DY395XL	0.48%	0.00%	100.00%	0.63%	5.00%	0.20%	0.00%	0.03%	0.00%	0.00%
Alexa Fluor 488	FF01-494/20	FF506-Di03	FF01-527/20	3.38E-07	AF488	0.00%	0.00%	1.35%	100.00%	0.10%	0.00%	0.00%	0.10%	0.00%	0.00%
Alexa Fluor 546	FF01-535/22	FF560-Di01	FF01-580/23	4.28E-07	AF546	0.00%	0.00%	0.80%	1.12%	100.00%	9.00%	0.00%	0.07%	0.00%	0.00%
Alexa Fluor 594	FF01-586/20	FF605-Di02	FF01-628/32	1.59E-06	AF594	0.00%	0.00%	0.15%	0.01%	5.90%	100.00%	1.89%	0.03%	0.00%	0.00%
Alexa Fluor 647	FF02-632/22	FF655-Di01	FF01-676/29	4.48E-07	AF647	0.00%	0.00%	0.03%	0.00%	0.08%	3.93%	100.00%	4.08%	1.90%	0.00%
PerCP	FF01-494/20	FF648-Di01	FF01-673/11	2.02E-07	PerCP	0.00%	0.00%	0.39%	0.41%	0.62%	3.18%	0.61%	100.00%	0.00%	0.00%
IRDye 680LT	FF01-680/22	FF705-Di01	FF01-720/13	1.87E-07	IRDye 680LT	0.00%	0.00%	0.00%	0.00%	0.00%	0.04%	7.70%	1.79%	100.00%	0.00%
IRDye 800CW	FF01-747/33	FF776-Di01	FF01-776/LP	5.96E-07	IRDye 800CW	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.08%	100.00%

[a]Filter set specifications were custom selected from off-the-shelf exciter, dichroic and emitter filters available from Semrock using the following criteria based on calculations performed within Semrock SearchLight Spectral Viewer: (1) for excitation filters, we searched for sufficient overlap between the fluorophore excitation spectrum and the excitation source, and selected filters so as to minimize cross-excitation among the 10 fluorophores of interest without detrimentally compromising the excitation of the specific fluorophore for which the excitation filter was selected. This was an iterative process as the criteria for determining sufficient excitation and minimal crosstalk were the 'primary' fluorescence signal as determined in Searchlight's optimization calculator compared to the 'secondary' signals of the other fluorophores in the panel. As a benchmark, we determined that for a given primary fluorophore, signals of ~10E-07 mW were good while signals of ~10E-08 mW were acceptable with increased integration times and verified viability experimentally; (2) the dichroic mirror was selected based on proximity to the emission filter, ideally achieving a state of transmission just before the passband of the emission filter, to give an additional level of blocking for unwanted excitation light; (3) emission filters were selected based on overlap with the fluorophore emission spectra and an average optical density (OD) blocking of OD6 across the excitation bandwidth, with as narrow bandpass properties as possible to minimize cross-talk among the 10 fluorophores of interest without detrimentally compromising specific emission signal of the specific fluorophore for which the emission filter was selected, which was also an iterative process in conjunction with the excitation filter; and (4) to improve signal to noise in cases where the excitation and emission filters are very close, where possible based on the available choices, at least a ~1% separation in wavelength of the falling edge of the excitation filter versus the rising edge of the emission filter at OD3.5 was used. Custom filter setup 1 was optimized for a panel of 10 commonly used spectrally compatible non-tandem flurophores. Standard filter setup 2 was selected to include a custom DY-395XL, a mega-Stokes shift dye, in place of AF430, to improve spectral compatibility with AF488, which exhibited a potential bleedthrough of 12.28% with custom AF430 filter set and only 0.63% potential bleedthrough with custom DY395XL filter set.

[b]Estimated fluorescence signal in mW of the primary filter set. This parameter was used as a reference in Semrock SearchLight Spectral Viewer to make sure the filter set would produce sufficient signal in comparison with single fluorophore optimized sets.

[c]Filter crosstalk was computed by AVR Optics using Semrock SearchLight Spectra Viewer for the fluorophores and filter specifications listed above using the following microscope setup: Zeiss Axiolmager.Z2 epifluorescence microscope, Excelitas X-Cite 200DC mercury vapor short arc broad spectrum lamp, and Hamamatsu Orca-Flash 4.0 V2 sCMOS camera. Crosstalk values exceeding 10% signal transmission required for optimal 10-colorfluorescence imaging are highlighted in yellow.

Details of each custom filter set specifications are available in SearchLight here: https://searchlight.semrock.com/?sid=a9cbb659-0301-4866-b249-e6a04d9033c2.

Abbreviations: AF: Alexa Fluor; DAPI: 4',6-diamidino-2-phenylindole; DL: DyLight; DY: Dyomics; IRD: Infra Red Dye; LP: Long Pass; PerCP: Peridinin-chlorophyll-protein; XL:Extra Long.

Table–5

Supplementary Table 5. List of primary and secondary antibodies for 10-plex IHC staining protocol in Fig 2b

Fluorophore	Channel#	Biomarker	Target Cell/Function	1st step reagent*	2nd step reagent*
DAPI	C1	4',6-diamidino-2-phenylindole (DAPI)	All Nucleated Cells	N/A	DAPI (TFS, D1306, AB_2629482)
DL405	C2	Neurofilament Heavy Chain (NFH)	Neurons-Axonal Processes	Chicken IgY anti-NFH (MS, AB5539, AB_11212161)	Donkey anti-Chicken IgY-DL405 (JIR, 703-475-155, AB_2340373)
AF430	C3	Ionized calcium binding adaptor molecule 1 (IBA1)	Microglia, Macrophages	Rabbit anti-IBA1 (Wako, 019-19741, AB_839504)	Goat anti-Rabbit IgG-AF430 (TFS, A11064, AB_2534111)
AF488	C4	Neuronal Nuclei (NeuN)	Neurons-Nucleus, Soma	Guinea Pig IgG anti-NeuN (MS, ABN90P, AB_2341095)	Goat anti-Guinea Pig IgG-AF488 (TFS, A11073, AB_2534117)
AF546	C5	Glial fibrillary acidic protein (GFAP)	Reactive Astrocytes	Rat IgG2a anti-GFAP (TFS, 13-0300, AB_2532994)	Goat anti-Rat IgG-AF546 (TFS, A11081, AB_2534125)
AF594	C6	2',3'-Cyclic-nucleotide 3'-phosphodiesterase (CNPase)	Oligodendrocytes	Mouse IgG2b anti-CNPase (NB, NBP2-46617, N/A)	Goat anti-Mouse IgG2b-AF594 (TFS, A21145, AB_2535781)
AF647	C7	Parvalbumin	Interneurons-Subset	Sheep IgG anti-Parvalbumin (RDS, AF5058, AB_2173907)	Donkey anti-Sheep IgG-AF647 (TFS, A21448, AB_2535865)
PerCP	C8	Rat Endothelial Cell Antigen 1 (RECA1)	Endothelial Cells	Mouse IgG1 anti-RECA1 (TFS, 14-0360-82, AB_467212)	Goat anti-Mouse IgG1-PerCP (JIR, 115-125-205, AB_2338631)
IDRye 680LT	C9	S100 calcium-binding protein β (S100β)	Pan-Astrocytes	Mouse IgG2a anti-S100β (MS, MAB079-1, AB_571112)	Goat anti-mouse IgG2a-IRDye 680LT (LCB, 926-68051, AB_2783643)
IRDye 800CW	C10	Synaptophysin	Neurons-Synapses	Mouse IgM anti-Synaptophysin (MS, MAB329, AB_94786)	Goat anti-mouse IgM-IRDye 800CW (LCB, 926-32280, AB_2814919)

*Each primary antibody/ligand/dye and secondary antibody was used at 1 μg/ml final concentration (i.e., 1:1,000 dilution of 1 mg/ml stock concentration) with sufficient volume to cover the tissue section (typically 250-500 μL/per section).

Vendor Abbreviations: ABC: Abcam; BL: BioLegend; JIR: Jackson ImmunoResearch; LCB: Li-Cor Biosciences; MS: Millipore Sigma; NB: Novus Biologicals; RDS: R&D Systems; TFS: Thermo Fisher Scientific.

Reagent information in parenthesis (by order listed): Vendor, Product#, RRID#.

RRID#: Research Resource Identifier.

Table–6

Supplementary Table 6. List of primary and secondary antibodies for 50-plex IHC staining protocol in Fig 2c

Stains in Round #1	Round#Channel#	Biomarker	Target/Function	1st step reagent*	2nd step reagent*
DAPI	R1C1	4',6-diamidino-2-phenylindole (DAPI)	All Nucleated Cells	N/A	DAPI (TFS, D1306, AB_2629482)
DL405	R1C2	Tyrosine Hydroxylase (TH)	Neurons-Catecholaminergic	Chicken IgY anti-TH (ABC, ab76442, AB_1524535)	Donkey anti-Chicken IgY-DL405 (JIR, 703-475-155, AB_2340373)
DY395XL	R1C3	Blood Brain Barrier (BBB)	Endothelial Cells	Mouse IgM anti-BBB (BL, 836803, AB_2687236)	Goat anti-Mouse IgM-DY395XL (LCB, custom, RRID: N/A)
N/A	R1C4	Green Fluorescent Protein (GFP)	Endothelial Cells	N/A (endogenously expressed GFP)	N/A (endogenously expressed GFP)
AF546	R1C5	Ionized calcium binding adaptor molecule 1 (IBA1)	Microglia	Guinea Pig IgG anti-IBA1 (SS, 234005, AB_2493179)	Goat anti-Gunea Pig IgG-AF546 (TFS, A11074, AB_2534118)
AF594	R1C6	Rat Endothelial Cell Antigen 1 (RECA1)	Endothelial Cells	Mouse IgG1 anti-RECA1 (TFS, 14-0360-82, AB_467212)	Goat anti-Mouse IgG1-AF594 (TFS, A21125, AB_2535767)
AF647	R1C7	Aquaporin-4 (AQP4)	Astrocyte Terminals	Mouse IgG3 anti-AQP4 (Abcam, ab9512, AB_307299)	Goat anti-mouse IgG3-AF647 (JIR, 115-605-209, AB_2338920)
PerCP	R1C8	Tomato Lectin (TL)	Endothelial Cells, Microglia	Tomato Lectin-Biotin (VL, B-1175, AB_2315475)	Streptavidin-PerCP (JIR, 016-120-084, AB_2337241)
IRDye 680LT	R1C9	Oligodendrocyte Transciption Factor 2 (Olig2)	Oligodendrocytes	Mouse IgG2a anti-Olig2 (MS, MABN50, AB_10807410)	Goat anti-Mouse IgG2a-IRDye 680LT (LCB, 926-68051)
IRDye 800CW	R1C10	Cleaved Caspase-3 (CC3)	Apoptotic Cells	Rabbit IgG anti-CC3 (MS, AB3623, AB_91556)	Donkey anti-Rabbit IgG-IRDye 800CW (LCB, 926-32213)
Bright Field	R1C11	N/A	Cell Morphology	N/A	N/A
Stains in Round #2	**Round#Channel#**	**Biomarker**	**Target/Function**	**1st step reagent**	**2nd step reagent**
DAPI	R2C1	DAPI	All Nucleated Cells	N/A	DAPI (TFS, D1306, AB_2629482)
DL405	R2C2	Pan-Histones H1, H2A, H2B, H3, H4 (HIST)	All Nucleated Cells	Mouse IgG2b anti-HIST cocktail mix (MBLI)**	Goat anti-mouse IgG2b-DL405 (JIR, 115-475-207, AB_2338801)
DY395XL	R2C3	Platelet-derived growth factor receptor beta (PDGFRβ)	Pericytes-Subset	Mouse IgG3 anti-PDGFRβ (SCB, sc-365464, AB_10847085)	Goat anti-Mouse IgG3-DY395XL (LCB, custom, RRID: N/A)
AF488	R2C4	Neuronal Nuclei (NeuN)	Neurons-Pan Specific	Chicken IgY anti-NeuN (MS, ABN91, AB_11205760)	Goat anti-Chicken IgY-AF488 (TFS, A11039, AB_2534096)
AF546	R2C5	Synaptohysin (SYN)	Neurons-Pan Specific	Mouse IgM anti-SYN (MS, MAB329, AB_94786)	Goat anti-Mouse IgM-AF546 (TFS, A21045, AB_2535714)
AF594	R2C6	Parvalbumin (PARV)	Interneurons-Subset	Guinea Pig IgG anti-PARV (SS, 195004, AB_2156476)	Goat anti-Gunea Pig IgG-AF594 (TFS, A11076, AB_2534120)
AF647	R2C7	Choline Acetyltransferase (ChAT)	Neurons-Cholinergic	Mouse IgG1 anti-ChAT (MS, MAB5270, AB_2079753)	Goat anti-mouse IgG1-AF647 (TFS, A21240, AB_2535809)
PerCP	R2C8	Tomato Lectin (TL)	Endothelial Cells, Microglia	Tomato Lectin-Biotin (VL, B-1175, AB_2315475)	Streptavidin-PerCP (JIR, 016-120-084, AB_2337241)
IRDye 680LT	R2C9	Glutaminase GLS2 (GLUT)	Neurons-Glutamatergic	Rabbit IgG anti-GLUT (Abcam, ab113509, AB_10866157)	Donkey anti-Rabbit IgG-IRDye 680LT (LCB, 926-68023, AB_10706167)
IRDye 800CW	R2C10	Smooth Muscle Actin (SMA)	Pericytes-Subset	Mouse IgG2a anti-SMA (TFS, 14-9760-82, AB_2572996)	Goat anti-Mouse IgG2a-IRDye 800CW (LCB, 926-32351, AB_2782998)
Bright Field	R2C11	N/A	Cell Morphology	N/A	N/A
Stains in Round #3	**Round#Channel#**	**Biomarker**	**Target/Function**	**1st step reagent**	**2nd step reagent**
DAPI	R3C1	DAPI	All Nucleated Cells	N/A	DAPI (TFS, D1306, AB_2629482)
DL405	R2C2	Pan-Histones H1, H2A, H2B, H3, H4 (HIST)	All Nucleated Cells	Mouse IgG2b anti-HIST cocktail mix (MBLI)**	Goat anti-mouse IgG2b-DL405 (JIR, 115-475-207, AB_2338801)
DY395XL	R3C3	Glial Fibrillary Acidic Protein (GFAP)	Astrocytes-Reactive	Rat IgG anti-GFAP (TFS, 13-0300, AB_2532994)	Goat anti-Rat IgG-DY395XL (LCB, custom, RRID: N/A)
AF488	R3C4	Doublecortin (DCX)	Neurons-Newly Born	Rabbit IgG anti-DCX (Abcam, ab18723, AB_732011)	Goat anti-Rabbit IgG-AF488 (TFS, A11034, AB_2576217)
AF546	R3C5	S100 calcium-binding protein β (S100β)	Astrocytes-Pan Specific	Mouse IgG2a anti-S100β (MS, MA8079-1, AB_571112)	Goat anti-Mouse IgG2a-AF546 (TFS, A21133, AB_2535772)
AF594	R3C6	Nestin (NST)	Immature Neural and Endothelial Cells	Mouse IgG1 anti-NST (MS, MAB353, AB_94911)	Goat anti-Mouse IgG1-AF594 (TFS, A21125, AB_2535767)
AF647	R3C7	Aquaporin-4 (AQP4)	Astrocyte Terminals	Mouse IgG3 anti-AQP4 (Abcam, ab9512, AB_307299)	Goat anti-mouse IgG3-AF647 (JIR, 115-605-209, AB_2338920)
PerCP	R1C8	Tomato Lectin (TL)	Endothelial Cells, Microglia	Tomato Lectin-Biotin (VL, B-1175, AB_2315475)	Streptavidin-PerCP (JIR, 016-120-084, AB_2337241)
IRDye 680LT	R3C9	EAAT1 (GLAST)	Astrocytes-Pan Specific	Guinea Pig IgG anti-GLAST (SS, 250114, AB_2619957)	Donkey anti-Guinea Pig IgG-IRDye 680LT (LCB, 926-68030, AB_10706310)
IRDye 800CW	R3C10	Vimentin (VIM)	Immature Neural and Endothelial	Chicken IgY anti-VIM (MS, AB5733, AB_11212377)	Donkey anti-Chicken IgY-IRDye 800CW (LCB, 926-32218, AB_1850023)
Bright Field	R3C11	N/A	Cell Morphology	N/A	N/A
Stains in Round #4	**Round#Channel#**	**Biomarker**	**Target/Function**	**1st step reagent**	**2nd step reagent**
DAPI	R4C1	DAPI	All Nucleated Cells	N/A	DAPI (TFS, D1306, AB_2629482)
DL405	R2C2	Pan-Histones H1, H2A, H2B, H3, H4 (HIST)	All Nucleated Cells	Mouse IgG2b anti-HIST cocktail mix (MBLI)**	Goat anti-mouse IgG2b-DL405 (JIR, 115-475-207, AB_2338801)
DY395XL	R4C3	Autofluorescence (AF)	N/A	N/A	N/A
AF488	R4C4	Proliferating Cell Nuclear Antigen (PCNA)	All Actively Proliferating Cells	Mouse IgG3 anti-PCNA (GTX, GTX40237, AB_423993)	Goat anti-mouse IgG3-AF488 (TFS, A21151, AB_2535784)
AF546	R4C5	SRY (sex determining region Y)-box 2 (Sox2)	Neural Precursors, Astrocytes	Mouse IgG1 anti-Sox2 (BD, 561469, AB_10694256)	Goat anti-Mouse IgG1-AF546 (TFS, A21123, AB_2535765)
AF594	R4C6	Gutamic Acid Decarboxylase 67 (GAD67)	Neurons-GABAergic	Mouse IgG2a anti-GAD67 (MS, MAB5406, AB_2278725)	Goat anti-Mouse IgG2a-AF594 (TFS, A21135,AB_2535774)
AF647	R4C7	Autofluorescence (AF)	N/A	N/A	N/A
PerCP	R1C8	Tomato Lectin (TL)	Endothelial Cells, Microglia	Tomato Lectin-Biotin (VL, B-1175, AB_2315475)	Streptavidin-PerCP (JIR, 016-120-084, AB_2337241)
IRDye 680LT	R4C9	Tbr1	Neurons-Pyramidal Mature	Rabbit IgG anti-Tbr1 (Abcam, ab31940, AB_2200219)	Donkey anti-Rabbit IgG-IRDye 680LT (LCB, 926-68023, AB_10706167)
IRDye 800CW	R4C10	Eomes (Tbr2)	Neurons-Pyramidal Immature	Rat IgG2a anti-Tbr2 (TFS, 14-4875-82, AB_11042577)	Goat anti-Rat IgG-IRDye 800CW (LCB, 926-32219, AB_1850025)
Bright Field	R4C11	N/A	Cell Morphology	N/A	N/A
Stains in Round #5	**Round#Channel#**	**Biomarker**	**Target/Function**	**1st step reagent**	**2nd step reagent**
DAPI	R5C1	DAPI	All Nucleated Cells	N/A	DAPI (TFS, D1306, AB_2629482)
DL405	R2C2	Pan-Histones H1, H2A, H2B, H3, H4 (HIST)	All Nucleated Cells	Mouse IgG2b anti-HIST cocktail mix (MBLI)**	Goat anti-mouse IgG2b-DL405 (JIR, 115-475-207, AB_2338801)
AF430	R2C3	Calretinin (CRT)	Interneurons-Subset	Rabbit IgG anti-CRT (MS, AB5054, AB_2068506)	Goat anti-Rabbit IgG-AF430 (TFS, A11064, AB_2534111)
AF488	R2C4	2',3'-Cyclic-nucleotide 3'-phosphodiesterase (CNPase)	Oligodendrocytes-Pan Specific	Mouse IgG1 anti-CNPase (MS, MAB326, AB_2082608)	Goat anti-Mouse IgG1-AF488 (TFS, A21121, AB_2535764)
AF546	R2C5	Neurofilament Heavy Chain (NFH)	Neurons-Axonal Innervation	Chicken IgY anti-NFH (MS, AB5539, AB_11212161)	Goat anti-Chicken IgY-AF546 (TFS, A11040, AB_2534097)
AF594	R2C6	Myelin Basic Protein (MBP)	Oligodendrocytes-Myelinating	Rat IgG2a-MBP (MS, MAB386, AB_94975)	Goat anti-Rat IgG-AF594 (TFS, A11007, AB_10561522)
AF647	R2C7	Neurofilament Medium Chain (NFM)	Neurons-Axonal Innervation	Mouse IgG2a anti-NFM (BT, NB500-446, AB_525746)	Goat anti-Mouse IgG2a-AF647 (TFS, A21241, AB_2535810)
PerCP	R2C8	Tomato Lectin (TL)	Endothelial Cells, Microglia	Tomato Lectin-Biotin (VL, B-1175, AB_2315475)	Streptavidin-PerCP (JIR, 016-120-084, AB_2337241)
IRDye 680LT	R2C9	Microtubule Associated Protein (MAP2)	Neurons-Dendritic Innervation	Mouse IgG3 anti-MAP2 (RDS, MAB8304, AB_2814693)	Goat anti-Mouse IgG3-IRDye 680LT (LCB, Custom, RRID: N/A)
IRDye 800CW	R2C10	Calbindin (CLB)	Interneurons-Subset	Guinea Pig IgG anti-CLB (SS, 214004, AB_10550535)	Donkey anti-Guinea Pig IgG-IRDye 800CW (LCB, 926-32411, AB_1850024)
Bright Field	R1C11	N/A	Cell Morphology	N/A	N/A

Table–7

Supplementary Table 7. Cell phenotyping results

Cell ID	centroid_x	centroid_y	xmin	ymin	xmax	ymax	NeuN	S100	Olig2	Iba1	RECA1	Cleaved Caspase-3	Tyrosine Hydroxylase	Blood Brain Barrier	GFP	PDGFR beta	Parvalbumin	Choline Acetyltransferas	GFAP	Smooth Muscle Actin	Glutaminase	Doublecortin	Sox2	PCNA	Vimentin	GAD67	Tbr1	Eomes	Calretinin	Nestin	Aquaporin-4	Calbindin
1	38157	29149	38142	29135	38173	29164	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
2	10900	28364	10880	28356	10921	28372	0	0	1	0	0	0	0	1	0	1	0	0	0	0	1	0	0	0	1	1	1	0	0	1	1	0
3	10939	28358	10923	28352	10955	28365	0	0	1	0	0	0	0	1	0	1	0	0	1	0	1	0	0	0	1	1	1	0	0	1	1	0
4	10833	28350	10813	28339	10853	28362	0	0	1	0	0	0	0	1	0	1	0	1	1	0	1	0	0	0	1	0	1	0	0	1	1	0
5	10790	28348	10771	28338	10809	28358	0	0	1	0	0	0	0	1	0	1	0	1	1	0	1	0	0	0	1	0	1	0	0	1	1	0
6	10903	28352	10885	28346	10921	28358	0	0	1	0	0	0	0	1	0	1	0	1	1	0	1	0	0	0	1	0	1	0	0	1	1	0
7	10968	28351	10954	28346	10982	28357	0	0	1	0	0	0	0	1	0	0	0	0	1	0	1	0	1	0	1	0	1	0	0	1	0	0
8	10899	28341	10882	28330	10917	28353	0	0	1	0	0	0	0	1	0	1	0	0	1	0	0	0	1	0	0	0	1	0	0	0	1	0
9	10962	28340	10948	28333	10976	28348	0	1	0	0	0	0	0	0	0	1	0	0	1	0	0	0	1	0	0	0	0	0	0	0	1	0
10	10869	28339	10857	28331	10882	28347	0	0	1	0	0	0	0	1	0	1	0	0	1	0	0	0	1	0	0	0	1	0	0	0	1	0
11	10993	28338	10977	28330	11009	28347	0	0	1	0	0	0	1	1	0	1	0	0	1	0	1	0	0	0	0	0	1	0	0	0	1	0
12	10674	28331	10656	28320	10692	28343	0	0	1	0	0	0	0	1	0	1	0	0	0	0	1	0	0	0	1	1	1	0	0	1	1	0
13	10720	28329	10703	28318	10737	28340	0	0	1	0	0	0	0	1	1	1	0	0	1	0	1	0	0	0	0	1	1	0	0	1	1	0
14	10698	28330	10688	28321	10708	28340	0	0	1	0	0	0	1	1	1	1	0	0	1	0	1	0	0	0	0	1	1	0	0	1	1	0
15	11032	28325	11019	28317	11046	28334	0	0	1	0	0	0	0	1	0	1	0	0	1	0	0	0	0	0	0	0	1	0	0	1	1	0
16	10674	28316	10660	28308	10688	28324	0	0	0	0	1	0	0	1	0	1	0	0	1	0	0	0	1	0	0	0	0	0	0	0	1	0
17	10779	28309	10760	28297	10799	28321	0	0	1	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0
18	11064	28310	11043	28300	11085	28321	0	0	1	0	0	0	0	1	0	1	0	0	1	0	1	0	0	0	0	0	1	0	0	0	1	0
19	10628	28308	10611	28298	10646	28319	0	0	0	0	0	0	0	1	1	1	0	0	1	0	1	0	0	0	1	0	1	0	0	1	1	0
20	11117	28300	11097	28288	11137	28313	0	0	1	0	0	0	0	1	0	1	0	0	1	0	1	0	0	0	1	0	1	0	0	1	1	0
21	10600	28304	10585	28297	10615	28312	0	0	1	0	0	0	0	1	1	1	0	0	1	0	1	0	1	0	1	0	1	0	0	1	1	0
22	10817	28302	10810	28294	10825	28310	0	0	0	0	1	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
23	11393	28293	11370	28279	11416	28307	0	0	1	0	0	0	1	1	0	1	0	1	0	1	1	0	0	0	1	1	1	0	0	1	1	0
24	10553	28295	10540	28288	10566	28303	0	0	1	0	0	0	0	1	0	1	0	0	1	0	1	0	0	0	1	1	1	0	0	1	1	0
25	10580	28287	10564	28278	10597	28296	0	0	1	0	0	0	1	1	0	1	0	0	1	0	1	0	1	0	0	0	1	0	0	0	1	0
26	10532	28288	10522	28282	10542	28295	0	0	1	0	0	0	0	1	0	1	0	0	0	0	1	0	0	0	1	1	1	0	0	1	1	0
27	11142	28283	11126	28271	11158	28295	0	0	1	0	0	0	0	1	0	1	0	0	1	0	1	0	1	0	0	0	1	0	0	1	1	0