Science Advances

Supplementary Materials for

Highly multiplexed fluorescence microscopy with spectrally tunable semiconducting polymer dots

Ziyu Guo *et al.*

Corresponding author: Daniel T. Chiu, chiu@uw.edu; Joshua C. Vaughan, jcv2@uw.edu

This PDF file includes:

Supplementary Text on linear unmixing:

Mixed signals in spectral multiplexing experiments arise when two or more signals are detected within a single channel as a result of spectral crosstalk. In linear algebra terms, this can be written as equation (S1) where the mixed signals are a matrix multiplication between the crosstalk / calibration matrix and the "unmixed" or true signal.
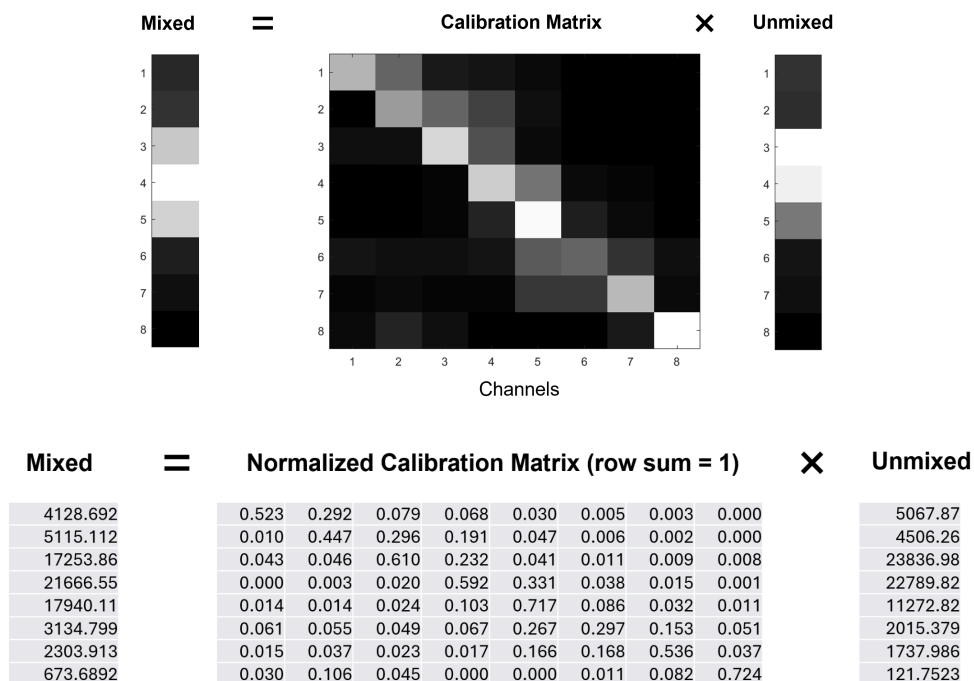
$$Mixed = CM \times Unmixed \qquad (S1)$$

Using linear unmixing, the "unmixed" images can be calculated using equation (S2) when the "mixed" images and the "calibration matrix" are known. It is a simple matrix multiplication between the inverse calibration matrix and the mixed images.

$$Unmixed = CM^{-1} \times Mixed \qquad (S2)$$

As an example, let us take the context of emission multiplexing as shown in Figure 2 or Figure S3. According to equation (S2), an 8-channel unmixed image [8×1 dimensions] can be generated by the matrix multiplication of the inverse calibration matrix [CM^{-1}, 8×8 dimensions] with the 8-channel mixed image [8×1 dimensions].

For the average signal in the images in Figure S3, equation (S1) looks like the following in visual and in tabular form:

Mixed	=	Normalized Calibration Matrix (row sum = 1)								×	Unmixed
4128.692		0.523	0.292	0.079	0.068	0.030	0.005	0.003	0.000		5067.87
5115.112		0.010	0.447	0.296	0.191	0.047	0.006	0.002	0.000		4506.26
17253.86		0.043	0.046	0.610	0.232	0.041	0.011	0.009	0.008		23836.98
21666.55		0.000	0.003	0.020	0.592	0.331	0.038	0.015	0.001		22789.82
17940.11		0.014	0.014	0.024	0.103	0.717	0.086	0.032	0.011		11272.82
3134.799		0.061	0.055	0.049	0.067	0.267	0.297	0.153	0.051		2015.379
2303.913		0.015	0.037	0.023	0.017	0.166	0.168	0.536	0.037		1737.986
673.6892		0.030	0.106	0.045	0.000	0.000	0.011	0.082	0.724		121.7523

According to the calibration matrix, 52.3% of the total signal of the first Pdot lands in its own channel, 29.2% of the signal lands in the next channel, and so on. Note that the unmixed signal intensities have changed in subtle ways in comparison to the mixed signals. These changes are much more obvious when unmixing one or two pure stains in solution. We tested our method on solutions of pure stains and simple combinations of pure stains and we were able to unmix the

signals with >99% accuracy: less than 1% of the signal was misassigned. Our actual unmixing software routine, which is provided on GitHub and Zenodo together with sample data (see main text, Data and materials availability), uses a small variation on the above procedure that ensures the returned unmixed signals lack negative components which would be unphysical.
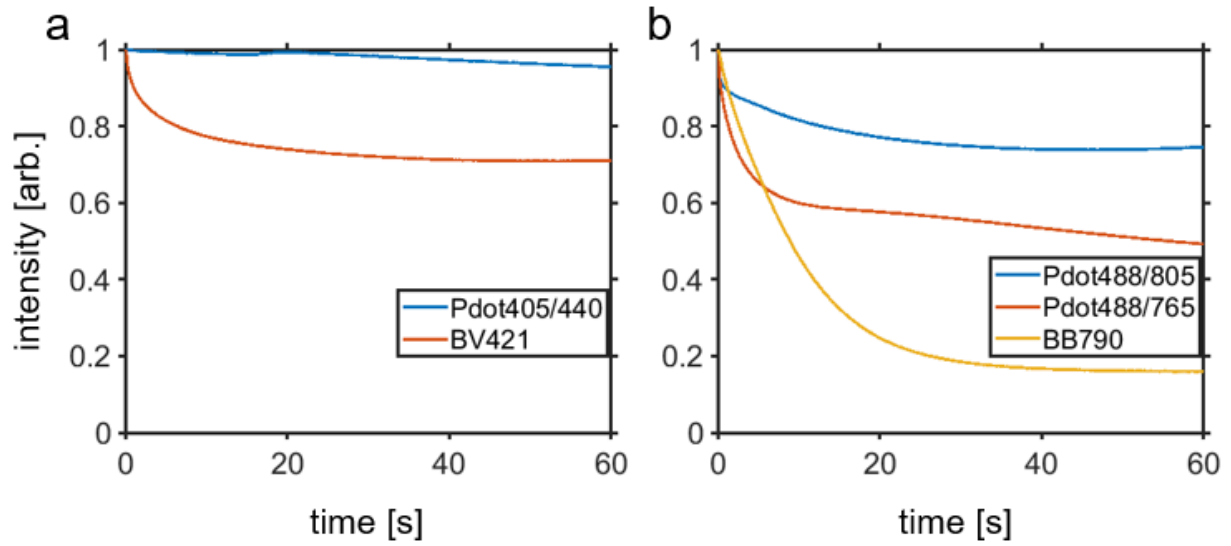
Fig. S1. Comparing the photostability of Pdots against other dyes commonly used in flow cytometry. (**a**) Comparison of the photostability of Pdot405/440 and Brilliant Violet 421, both excited at 405 nm (N=1). (**b**) Comparison of the photostability of the long Stokes-shift probes Pdot488/765, Pdot488/805, and BB790, all excited at 488 nm (N=1). All probes were illuminated at intensities comparable to that used for imaging.
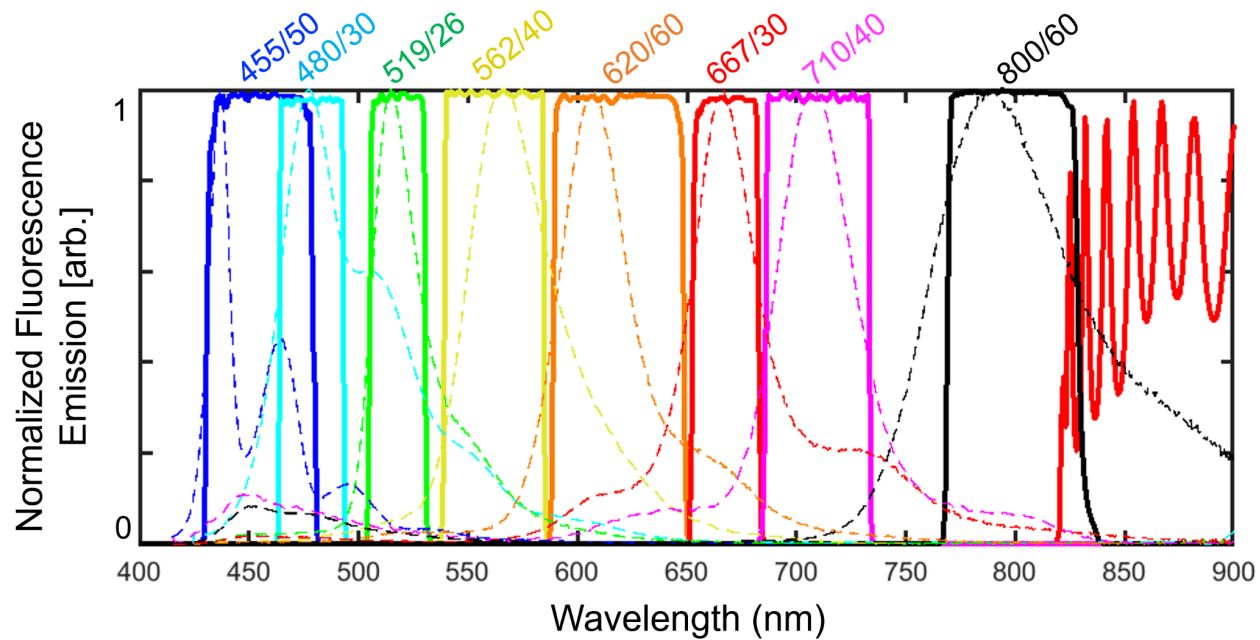
Fig. S2. Emission spectra and associated filters overlay. Emission spectra of eight 405-excited Pdots (dashed lines) along with the associated dichroic filter reflectance (red oscillating line above 825 nm) and emission filter transmission (solid colored lines).
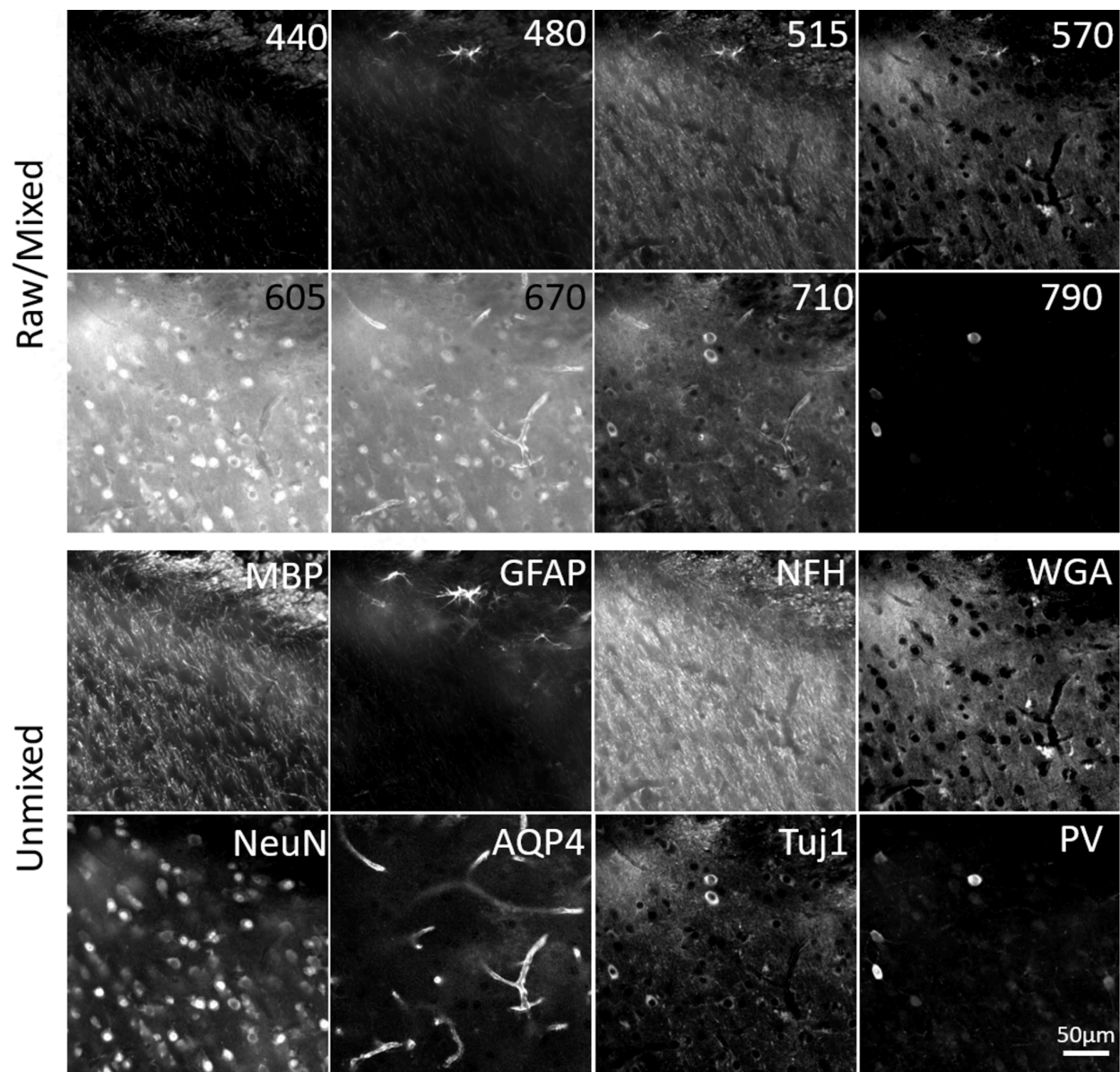
Fig. S3. Raw and processed image comparison for the data set shown in Fig. 2. Comparison of raw (mixed) images acquired at different emission wavelengths and the processed (unmixed) images that correspond to the primary antibody targets in mouse brain tissue, imaged at the amygdalar and piriform area.
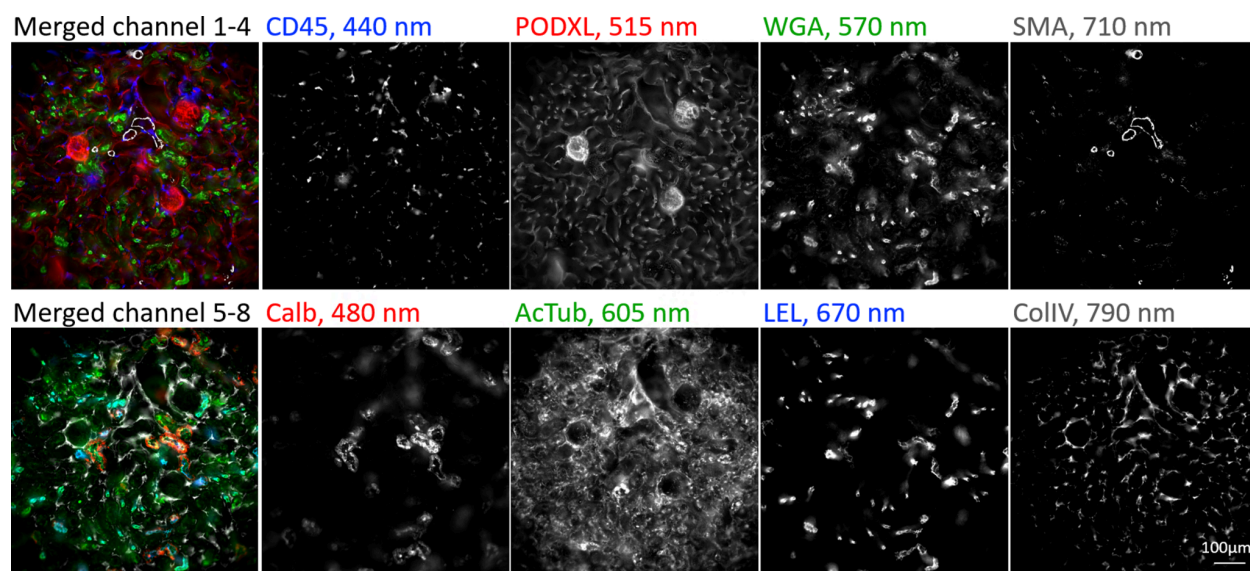
Fig. S4. Eight-channel emission multiplexing in the mouse kidney. Unmixed images from indirect immunostaining of a 50 μm kidney slice using eight 405 nm-excited Pdot conjugates and imaged at the cortex region of the kidney.
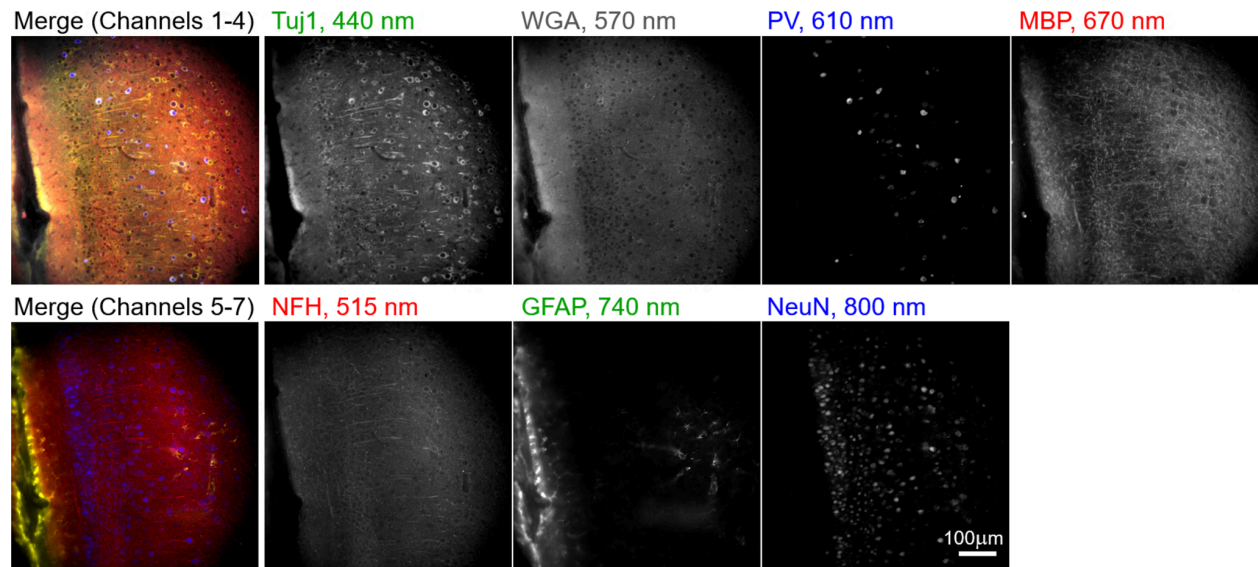
Fig. S5. Seven-channel emission multiplexing in the mouse brain. Unmixed images from indirect immunofluorescence staining of a 100 μm thick mouse brain slice using seven 355 nm-excited Pdot conjugates and imaged at the anterior cingulate area of the brain.
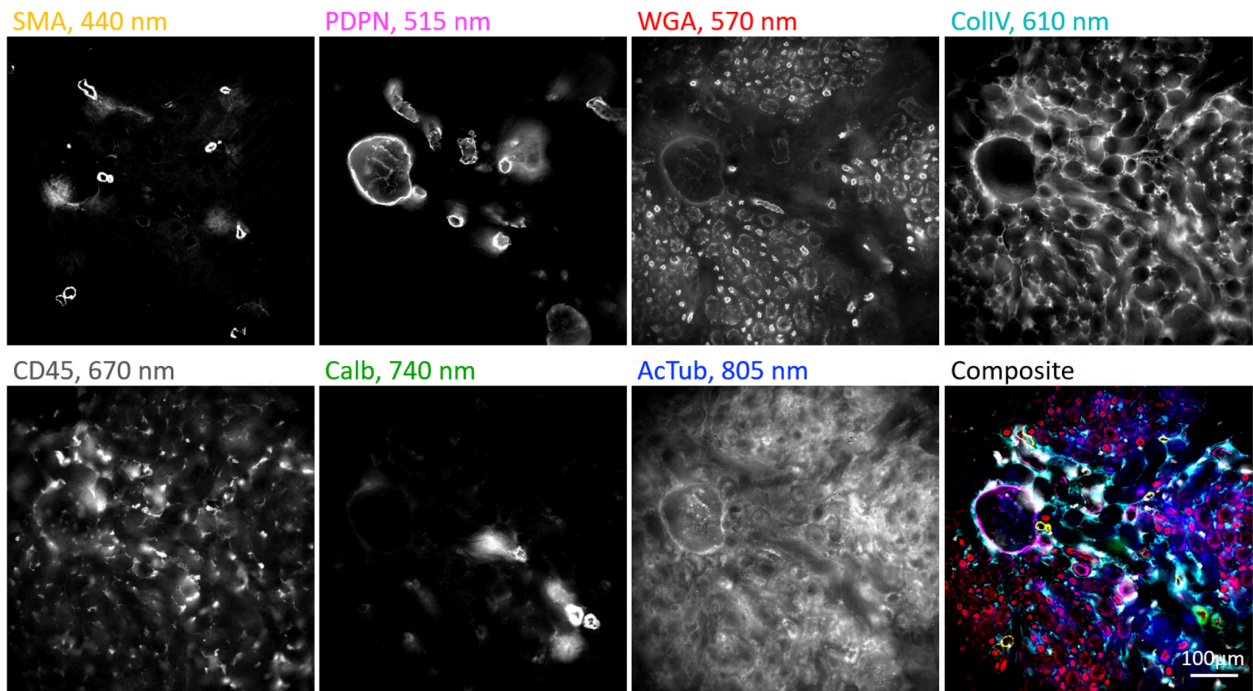
Fig S6. Seven-channel emission multiplexing in the mouse kidney. Unmixed images from indirect immunostaining of a 50 μm kidney slice using seven 355 nm-excited Pdot conjugates and imaged at the cortex region of the kidney.
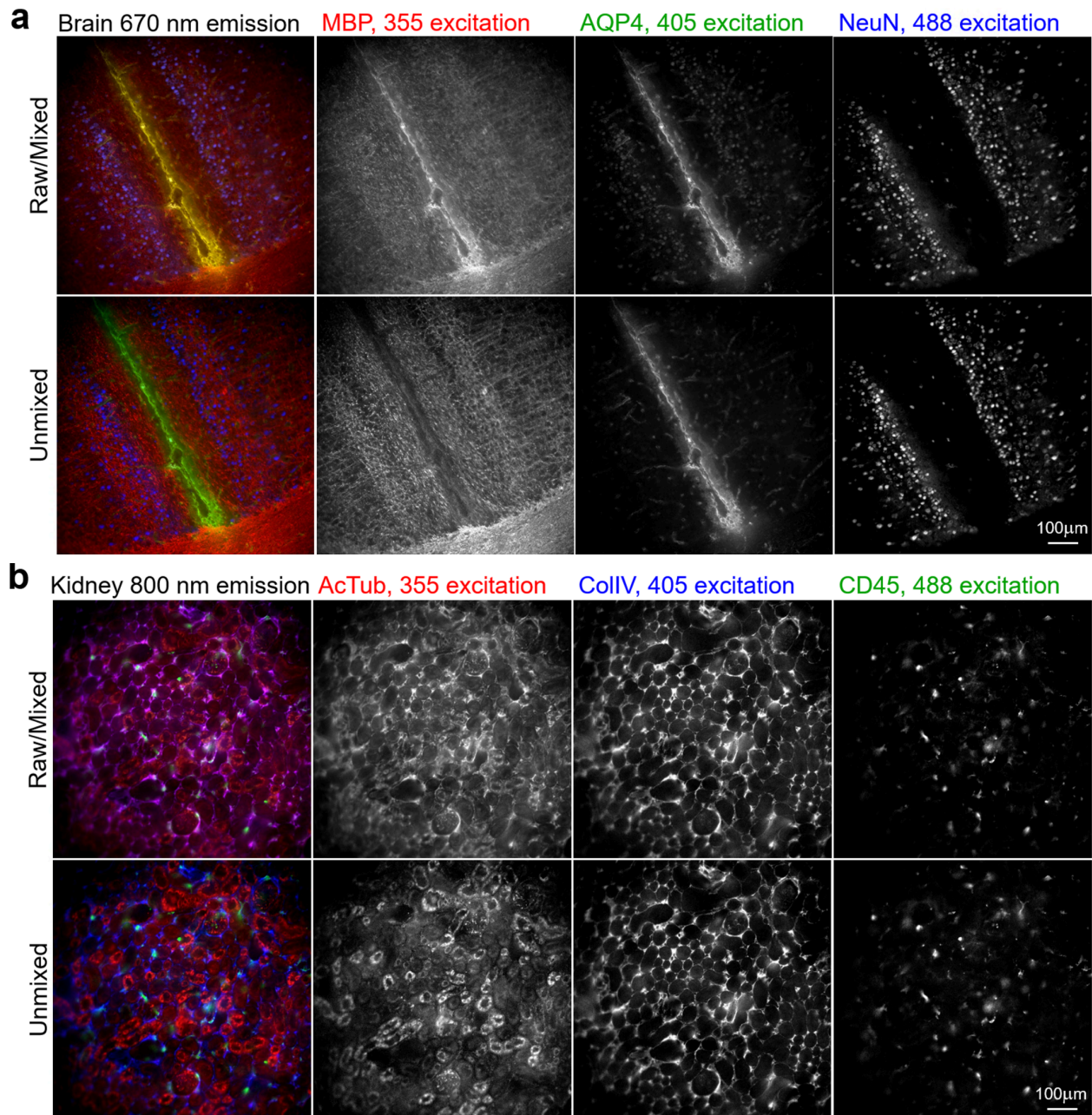
Fig. S7. Three-channel excitation multiplexing in the mouse brain and mouse kidney. A comparison of mixed (rows 1,3) and linearly unmixed (row 2,4) images for the data set shown in Fig. 3. (**a**): A 100 μm thick mouse brain slice was stained using three Pdots with different excitation maxima. The brain section was imaged above the medial corpus callosum region and emission was collected with the same emission filter for all three channels. (b): A 100 μm thick mouse kidney slice was stained using three Pdots with different excitation maxima. The kidney section was imaged at the cortex region and emission was collected with the same emission filter for all three channels.
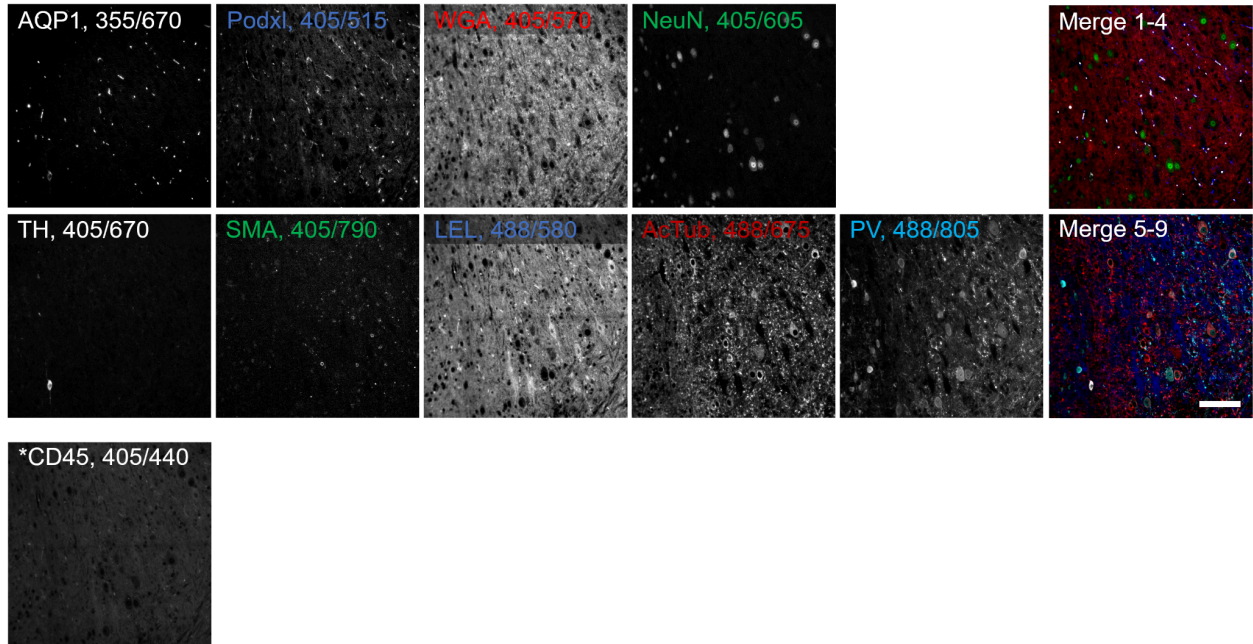
Fig. S8. 10-channel Pdot staining of mouse cerebellum FFPE tissue. The images in the five columns to the left show grayscale images for individual molecular targets in the mouse cerebellum. The rightmost column shows 4-channel composite images of the same tissue region. CD45, shown with an asterisk (*) in the bottom row, is not expressed in this region of the brain. The scale bar is 100 μm. Labels indicate the protein targets or lectins together with Pdot excitation/emission wavelengths. (AQP1 = aquaporin 1, PODXL = podocalyxin, WGA = wheat germ agglutinin lectin, NeuN = neuronal nuclei, TH = tyrosine hydroxylase, SMA = alpha-smooth muscle actin, LEL = lycopersicon esculentum tomato lectin, AcTub = acetylated tubulin, PV = parvalbumin, CD45 = leukocyte common antigen).

Fig. S9. 10-channel Pdot staining of mouse kidney FFPE tissue. The images in the four columns to the left show grayscale images for individual molecular targets in the mouse kidney. The rightmost column shows a 7-channel composite image of the same tissue region. NeuN, TH, and AcTub, shown with a asterisk (*) in the bottom row, were either not expressed in the kidney or showed very low levels of staining in this specimen. All scale bars are 100 μm. Labels indicate the protein targets or lectins together with Pdot excitation/emission wavelengths. (AQP1 = aquaporin 1, PODXL = podocalyxin, WGA = wheat germ agglutinin lectin, NeuN = neuronal nuclei, TH = tyrosine hydroxylase, SMA = alpha-smooth muscle actin, LEL = lycopersicon esculentum tomato lectin, AcTub = acetylated tubulin, PV = parvalbumin, CD45 = leukocyte common antigen).
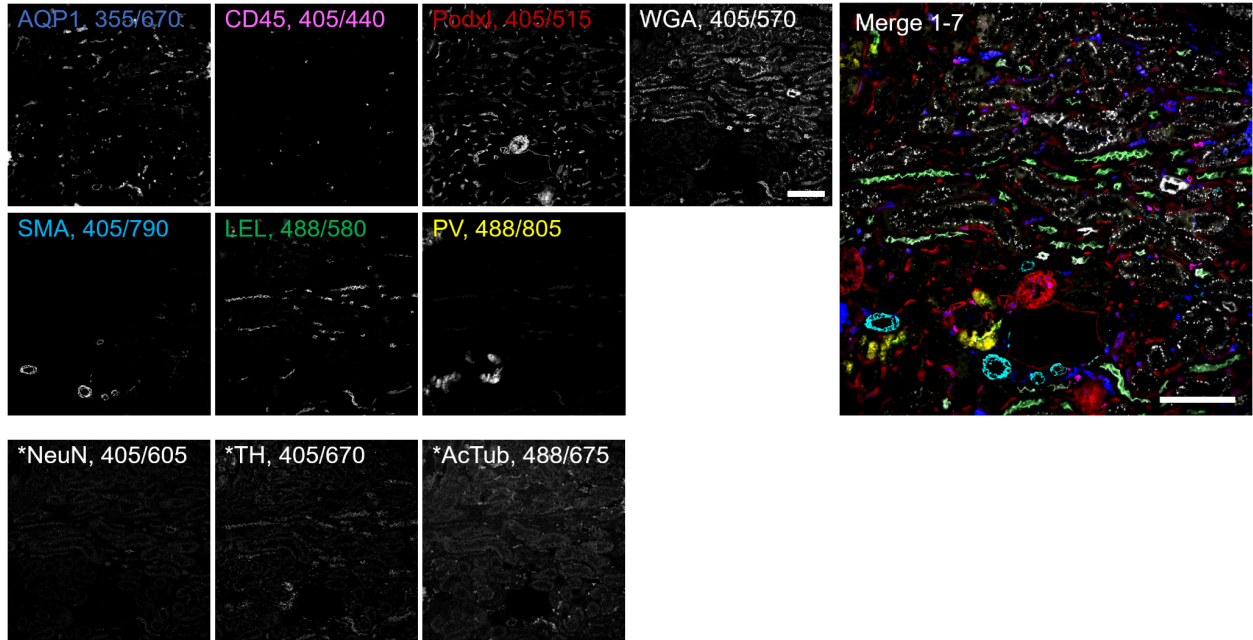
Fig. S10. 10-channel Pdot staining of mouse thymus FFPE tissue. The images in the four columns to the left show grayscale images for individual molecular targets in the mouse thymus. The rightmost column shows 4-channel composite images of the same tissue region. NeuN and AcTub, shown with asterisks (*) in the bottom row, were either not expressed in the thymus or showed very low levels of staining in this specimen. The scale bar is 100 μm. Labels indicate the protein targets or lectins together with Pdot excitation/emission wavelengths. (AQP1 = aquaporin 1, PODXL = podocalyxin, WGA = wheat germ agglutinin lectin, NeuN = neuronal nuclei, TH = tyrosine hydroxylase, SMA = alpha-smooth muscle actin, LEL = lycopersicon esculentum tomato lectin, AcTub = acetylated tubulin, PV = parvalbumin, CD45 = leukocyte common antigen).
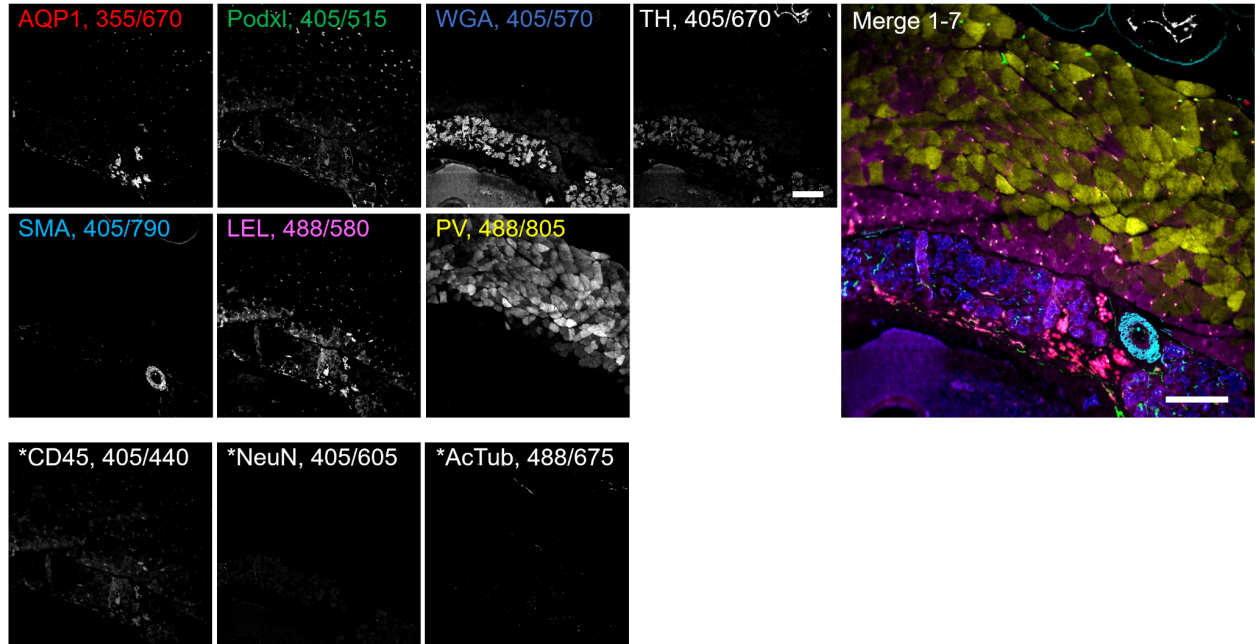
Fig. S11. 10-channel Pdot staining of mouse prostate FFPE tissue. The images in the four columns to the left show grayscale images for individual molecular targets in the mouse prostate. The rightmost column shows a 7-channel composite image of the same tissue region. CD45, NeuN, and AcTub, shown with asterisks (*) in the bottom row, were either not expressed in the prostate or showed very low levels of staining in this specimen. All scale bars are 100 μm. Labels indicate the protein targets or lectins together with Pdot excitation/emission wavelengths. (AQP1 = aquaporin 1, PODXL = podocalyxin, WGA = wheat germ agglutinin lectin, NeuN = neuronal nuclei, TH = tyrosine hydroxylase, SMA = alpha-smooth muscle actin, LEL = lycopersicon esculentum tomato lectin, AcTub = acetylated tubulin, PV = parvalbumin, CD45 = leukocyte common antigen).
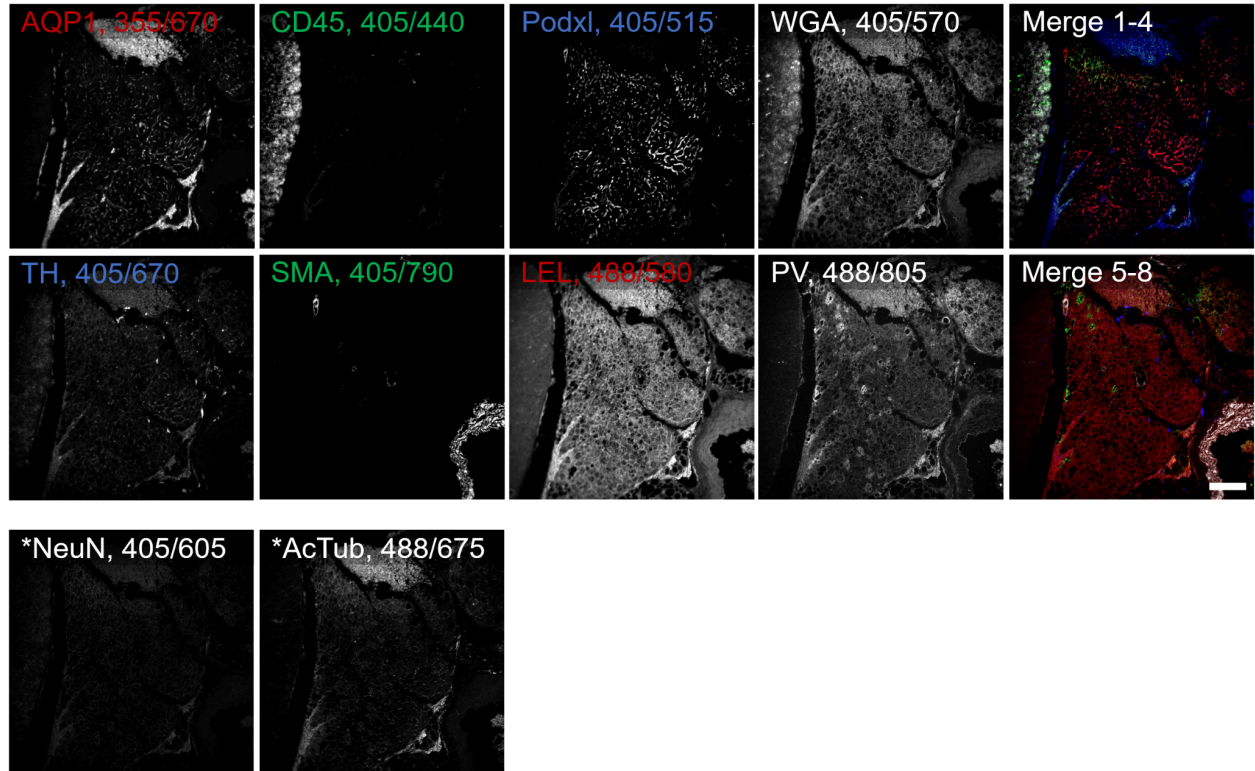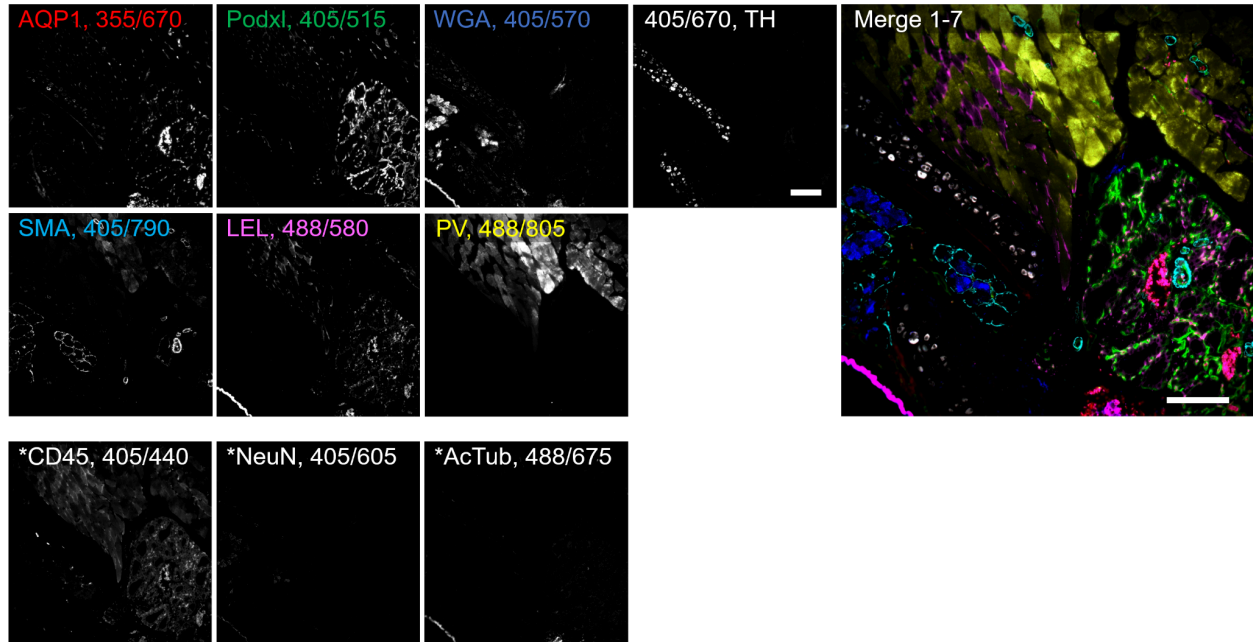
Fig. S12. 10-channel Pdot staining of mouse larynx FFPE tissue. The images in the four columns to the left show grayscale images for individual molecular targets in the mouse larynx. The rightmost column shows a 7-channel composite image of the same tissue region. CD45, NeuN, and AcTub, shown with asterisks (*) in the bottom row, were either not expressed in the larynx or showed very low levels of staining in this specimen. All scale bars are 100 μm. Labels indicate the protein targets or lectins together with Pdot excitation/emission wavelengths. (AQP1 = aquaporin 1, PODXL = podocalyxin, WGA = wheat germ agglutinin lectin, NeuN = neuronal nuclei, TH = tyrosine hydroxylase, SMA = alpha-smooth muscle actin, LEL = lycopersicon esculentum tomato lectin, AcTub = acetylated tubulin, PV = parvalbumin, CD45 = leukocyte common antigen).
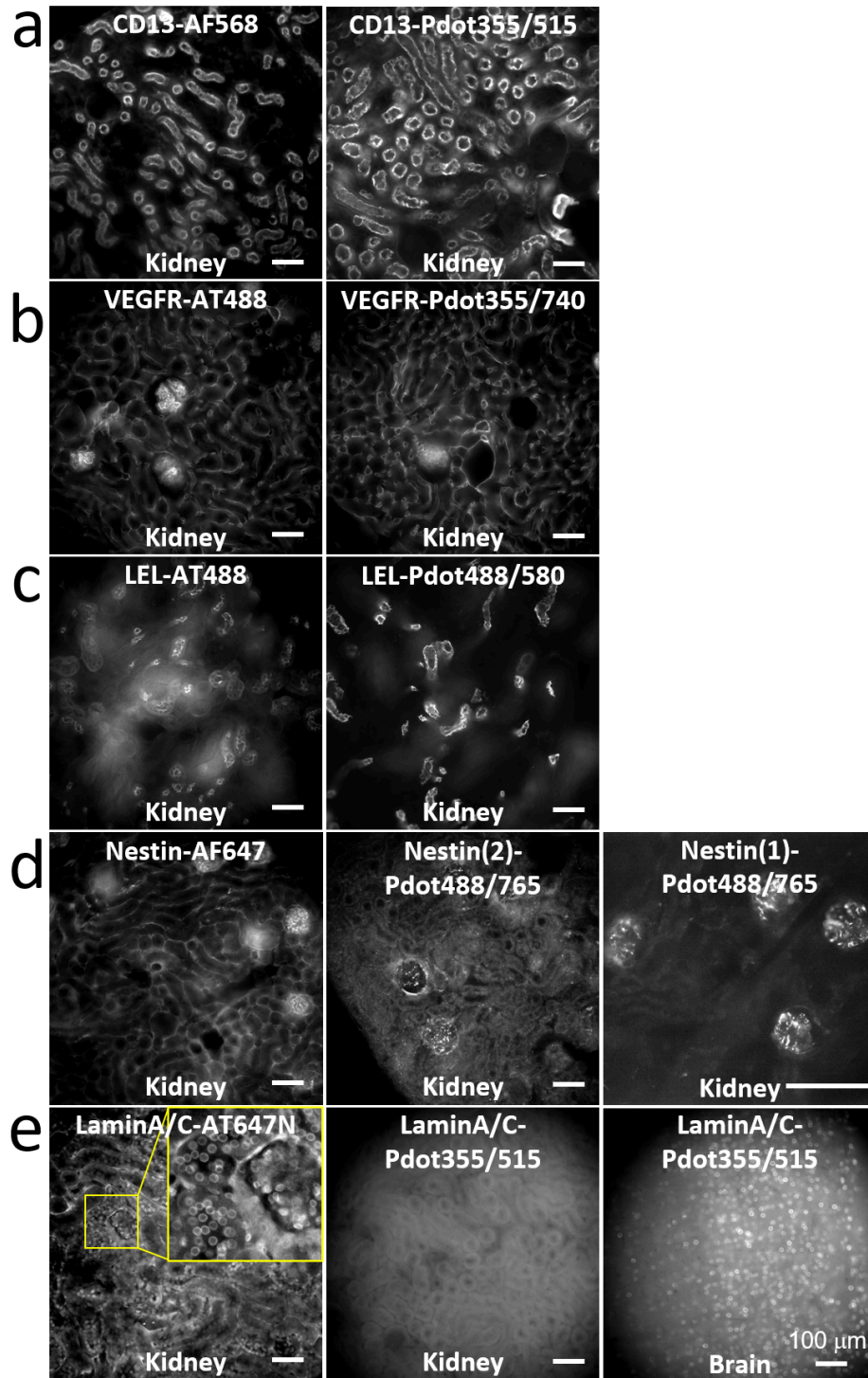
Fig. S13. Methodology for validation of Pdot-antibody conjugates. Kidney and brain tissue sections were stained for a single molecular target using direct or indirect immunostaining. The left column shows the results of staining using antibodies conjugated with conventional small molecule dyes. The right column shows the results of staining using antibodies conjugated with Pdots. Rows (**a-c**) showed the correct staining pattern using antibody-Pdot conjugates or lectin-Pdot conjugates. In row (**d**), Nestin(2) antibody, when conjugated to Pdot488/765, showed

partial loss of antigenicity or weak staining. When the Pdot conjugation was repeated with another Nestin antibody (Nestin(1)), it showed the correct staining pattern. In row (**e**), the LaminA/C antibody conjugated to Pdot355/515 showed little to no stain in the kidney but showed good nuclear stain in brain tissue. This difference in staining between kidney and brain may result from either a greater abundance of the protein in brain vs kidney tissue (relative to the background signal for each) or from different efficiencies of binding of the antibody to the relevant lamin a/c splice isoforms in brain and kidney.

(**a**) Rat anti-CD13 + Donkey anti-Rat AF568 (left); Rat anti-CD13, Pdot355/515 (right)
(**b**) Rat anti-VEGFR + Donkey anti-Rat AT488 (left); Rat anti-VEGFR, Pdot355/740 (right)
(**c**) LEL-AT488 (left); LEL-Pdot488/580 (right)
(**d**) Mouse IgG1 Nestin + Goat anti-mouse AF647 (left); mouse IgG1 Nestin(2), Pdot488/765 ; mouse IgG2a Nestin(1), Pdot488/765.
(**e**) Rabbit Lamin A/C + Donkey anti-Rabbit AT647N; Rabbit Lamin A/C, Pdot355/515.

Table S1. Summary of sample labeling and imaging setup. All experiments (except for Figure S13) used a Ti2-E Nikon microscope, exposure time of 100 ms for fresh-cut tissues, 1000 ms for FFPE tissues, 20x objective lens, SOLA light engine, ORCA Flash 4.0 camera, immunostaining procedure, and 20–24 hour incubation time; secondary antibodies are all from Jackson ImmunoResearch. More details are provided in the Methods section.

Fig. Tissue Thickness	Primary Antibody	Secondary Antibody	Filter Combinations		
2, S3 Brain (Mouse 1) 100 μm section	MBP GFAP NFH WGA NeuN AQP4 Tuj1 PV	Pdot405/440 Donkey anti-Rat Pdot405/480 Donkey anti-Mouse IgG1 Pdot405/515 Goat anti-Chicken Pdot405/570 WGA Pdot405/605 Goat anti-Mouse IgG2b Pdot405/670 Goat anti-Mouse IgG3 Pdot405/710 Goat anti-Mouse IgG2a Pdot405/790 Donkey anti-Rabbit	*Dichroic:* DI02-R405-25X36 Same for all	*Excitation filter:* zet405/20x Same for all	*Emission filters:* ET455/50m ET480/30m ET519/26m FF01-562/40 ET620/60m ET667/30m FF01-710/40 ET800/60
3A, S7 Brain (Mouse 1) 100 μm section	MBP AQP4 NeuN	Pdot355/670 Donkey anti-Rat Pdot405/670 Goat anti-Mouse IgG3 Pdot488/675 Goat anti-Mouse IgG2b	*Dichroics:* FF376-DI01-25X36 DI02-R405-25X36 DI02-R488-25X36	*Excitation filters:* zet365/20x zet405/20x zet488/10x	*Emission filter:* ET667/30m ET667/30m ET667/30m
3B, S7 Kidney (Mouse 1) 100 μm section	AcTub ColIV CD45	Pdot355/800 Goat anti-Mouse IgG2b Pdot405/790 Donkey anti-Rabbit Pdot488/805 Donkey anti-Rat	*Dichroics:* FF376-DI01-25X36 DI02-R405-25X36 DI02-R488-25X36	*Excitation filters:* zet365/20x zet405/20x zet488/10x	*Emission filter:* ET800/60 ET800/60 ET800/60
4 Brain (Mouse 3) 50 μm section	Pdot355/400 SOX2 (1) Pdot355/515 LMNA/C (1) Pdot355/570 MAP2 Pdot355/740 VEGFR3 (1) Pdot405/440 GFAB Pdot405/570 WGA Pdot405/605 NeuN Pdot405/670 TH Pdot405/710 PV Pdot405/790 MBP Pdot488/515 Calb (1) Pdot488/610 NFH Pdot488/675 Tuj1 Pdot488/765 Nes	All primary conjugates, no secondaries necessary	*Dichroics:* FF376-DI01-25X36 FF376-DI01-25X36 FF376-DI01-25X36 FF376-DI01-25X36 DI02-R405-25X36 DI02-R405-25X36 DI02-R405-25X36 DI02-R405-25X36 DI02-R405-25X36 DI02-R405-25X36 DI02-R488-25X36 DI02-R488-25X36 DI02-R488-25X36 DI02-R488-25X36	*Excitation filters:* zet365/20x zet365/20x zet365/20x zet365/20x zet405/20x zet405/20x zet405/20x zet405/20x zet405/20x zet405/20x zet488/10x zet488/10x zet488/10x zet488/10x	*Emission filters:* ET405/30m ET519/26m FF01-562/40 ET740/40x ET455/50m FF01-562/40 ET620/60m FF01-562/40 FF01-710/40 ET800/60 ET519/26m ET620/60m ET667/30m ET760/40
5 Kidney (Mouse 2) 50 μm section	Pdot355/440 PNA Pdot355/515 CD13 Pdot355/570 AQP2 Pdot355/610 ITGA8 Pdot355/670 AQP1 Pdot355/740 VEGFR3 (1) Pdot355/800 UMOD Pdot405/440 CD45 Pdot405/480 Megalin Pdot405/515 PODXL Pdot405/570 WGA Pdot405/605 ColIV Pdot405/670 LEL Pdot405/710 Agrin Pdot405/790 α-SMA Pdot488/515 Calb (1) Pdot488/580 PDPL Pdot488/610 REN Pdot488/675 AcTub Pdot488/765 Nes Pdot488/805 PV	All primary conjugates, no secondaries necessary	*Dichroics:* FF376-DI01-25X36 FF376-DI01-25X36 FF376-DI01-25X36 FF376-DI01-25X36 FF376-DI01-25X36 FF376-DI01-25X36 FF376-DI01-25X36 DI02-R405-25X36 DI02-R405-25X36 DI02-R405-25X36 DI02-R405-25X36 DI02-R405-25X36 DI02-R405-25X36 DI02-R405-25X36 DI02-R405-25X36 DI02-R488-25X36 DI02-R488-25X36 DI02-R488-25X36 DI02-R488-25X36 DI02-R488-25X36 DI02-R488-25X36	*Excitation filters:* zet365/20x zet365/20x zet365/20x zet365/20x zet365/20x zet365/20x zet365/20x zet405/20x zet405/20x zet405/20x zet405/20x zet405/20x zet405/20x zet405/20x zet405/20x zet488/10x zet488/10x zet488/10x zet488/10x zet488/10x zet488/10x	*Emission filters:* ET455/50m ET519/26m FF01-562/40 ET620/60m ET667/30m ET740/40x ET800/60 ET455/50m ET480/30m ET519/26m FF01-562/40 ET620/60m ET667/30m FF01-710/40 ET800/60 ET519/26m FF01-562/40 ET620/60m ET667/30m ET760/40 ET800/60
S4 Kidney (Mouse 2) 50 μm section	CD45 Calb PODXL WGA AcTub LEL α-SMA ColIV	Pdot405/440 Donkey anti-Rat Pdot405/480 Donkey anti-Mouse IgG1 Pdot405/515 PODXL Pdot405/570 WGA Pdot405/605 Goat anti-Mouse IgG2b Pdot405/670 LEL Pdot405/710 Goat anti-Mouse IgG2a Pdot405/790 Donkey anti-Rabbit	*Dichroic:* DI02-R405-25X36 Same for all	*Excitation filter:* zet405/20x Same for all	*Emission filters:* ET455/50m ET480/30m ET519/26m FF01-562/40 ET620/60m ET667/30m FF01-710/40 ET800/60

Sample	Target	Conjugate	Dichroics:	Excitation filters:	Emission filters:
S5 Brain (Mouse 1) 100 μm section	Tuj1 NFH WGA PV MBP GFAB NeuN	Pdot355/440 Goat anti-mouse IgG2a Pdot355/515 Donkey anti-Chicken Pdot355/570 WGA Pdot355/610 Donkey anti-Rabbit Pdot355/670 Donkey anti-Rat Pdot355/740 Goat anti-Mouse IgG1 Pdot355/800 Goat anti-Mouse IgG2b	FF376-DI01-25X36 Same for all	zet365/20x Same for all	ET455/50m ET519/26m FF01-562/40 ET620/60m ET667/30m ET740/40x ET800/60
S6 Kidney (Mouse 2) 50 μm section	α-SMA PDPN WGA ColIV CD45 Calb AcTub	Pdot355/440 Goat anti-mouse IgG2a Pdot355/515 Donkey anti-Hamster Pdot355/570 WGA Pdot355/610 Donkey anti-Rabbit Pdot355/670 Donkey anti-Rat Pdot355/740 Goat anti-Mouse IgG1 Pdot355/800 Goat anti-Mouse IgG2b	FF376-DI01-25X36 Same for all	zet365/20x Same for all	ET455/50m ET519/26m FF01-562/40 ET620/60m ET667/30m ET740/40x ET800/60
S8–S12 Mouse Tissue Microarray 5 μm section	Pdot355/670 AQP1 Pdot405/440 CD45 Pdot405/515 PODXL Pdot405/570 WGA Pdot405/605 NeuN Pdot405/670 TH Pdot405/790 α-SMA Pdot488/580 LEL Pdot488/675 AcTub Pdot488/805 PV	All primary conjugates, no secondaries necessary	FF376-DI01-25X36 DI02-R405-25X36 DI02-R405-25X36 DI02-R405-25X36 DI02-R405-25X36 DI02-R405-25X36 DI02-R405-25X36 DI02-R488-25X36 DI02-R488-25X36 DI02-R488-25X36	zet365/20x zet405/20x zet405/20x zet405/20x zet405/20x zet405/20x zet405/20x zet488/10x zet488/10x zet488/10x	ET667/30m ET455/50m ET519/26m FF01-562/40 ET620/60m ET667/30m ET800/60 FF01-562/40 ET667/30m ET800/60
S13 Kidney and Brain (Mouse 3) 50 μm section	CD13 Pdot355/515 CD13 VEGFR Pdot355/740 VEGFR AT488 LEL Pdot488/580 LEL Nestin Pdot488/765 Nestin(2) Pdot488/765 Nestin(1) Lamin A/C Pdot355/515 Lamin A/C	AF568 Donkey anti-Rat - AT488 Donkey anti-Rat - - - AF647 Goat anti-mouse - - AT647N Donkey anti-Rabbit -	zt405/488/561/640rpc v2-UF2 FF376-DI01-25X36 DI02-R488-25X36 FF376-DI01-25X36 DI02-R488-25X36 DI02-R488-25X36 zt405/488/561/640rpc v2-UF2 DI02-R488-25X36 DI02-R488-25X36 zt405/488/561/640rpc v2-UF2 FF376-DI01-25X36	- (LED control) zet365/20x zet488/10x zet365/20x zet488/10x zet488/10x - (LED control) zet488/10x zet488/10x - (LED control) zet365/20x	ET595/44m ET519/26m ET519/26m ET740/40x ET519/26m FF01-562/40 ET700/75m ET760/40 ET760/40 ET700/75m ET519/26m

Table S2. Pdot-secondary antibody conjugates.

	Primary Antibody or Lectin	Protein Target or Lectin	Host	Vendor	Catalog #	Clonality	Pdot Conjugated Secondary Antibody or Lectin	Catalog #
Brain - 355ex	Tuj1/TUBB3	Tubulin β 3	Mouse IgG2a	BioLegend	801201	monoclonal	Pdot355/440 Goat anti-mouse IgG2a	115-005-206
	NFH	Neurofilament Heavy Chain	Chicken	Aves Labs	NFH		Pdot355/515 Donkey anti-Chicken	703-005-155
	WGA	Wheat Germ Agglutinin		Vector Labs	L-1020		Pdot355/570 WGA	-
	PV	Parvalbumin	Rabbit	Abcam	ab11427	polyclonal	Pdot355/610 Donkey anti-Rabbit	711-005-152
	MBP	Myelin Basic Protein	Rat	Abcam	ab7349	monoclonal	Pdot355/670 Donkey anti-Rat	715-005-151
	GFAP	Glial Fibrillary Acidic Protein	Mouse IgG1	Cell Signaling	3670S	monoclonal	Pdot355/740 Goat anti-mouse IgG1	115-005-205
	NeuN	Neuronal Nuclei	Mouse IgG2b	Encor	MCA-1B7	monoclonal	Pdot355/800 Goat anti-mouse IgG2b	115-005-207
Kidney - 355ex	α-SMA	alpha-Smooth Muscle Actin	Mouse IgG2a	BioLegend	904601	monoclonal (1A4)	Pdot355/440 Goat anti-mouse IgG2a	115-005-206
	PDPN	Podoplanin	Syrian Hamster IgG	DSHB	8.1.1-s	monoclonal	Pdot355/515 Donkey anti-Hamster	Unavailable
	WGA	Wheat Germ Agglutinin		Vector Labs	L-1020		Pdot355/570 WGA	-
	AQP1	Aquaporin 1	Rabbit	Abcam	ab15080	polyclonal	Pdot355/610 Donkey anti-Rabbit	711-005-152
	CD45	Leukocyte Common Antigen	Rat	BioLegend	103101	monoclonal (30-F11)	Pdot355/670 Donkey anti-Rat	715-005-151
	Calb	Calbindin	Mouse IgG1	Abcam	ab82812	monoclonal	Pdot355/740 Goat anti-mouse IgG1	115-005-205
	AcTub	Acetylated Tubulin	Mouse IgG2b	Sigma	T7451-25 UL	monoclonal (6-11B-1)	Pdot355/800 Goat anti-mouse IgG2b	115-005-207
Brain - 405ex	MBP	Myelin Basic Protein	Rat	Abcam	ab7349	monoclonal	Pdot405/440 Donkey anti-Rat	715-005-151
	GFAP	Glial fibrillary acidic protein	Mouse IgG1	Cell Signaling	3670S	monoclonal	Pdot405/480 Donkey anti-mouse IgG1	115-005-205
	NFH	Neurofilament Heavy-chain	Chicken	Aves Labs	NFH	polyclonal	Pdot405/515 Goat anti-chicken	103-005-155
	WGA	Wheat Germ Agglutinin		Vector Labs	L-1020		Pdot405/570 WGA	-
	NeuN	Neuronal Nuclei	Mouse IgG2b	Encor	MCA-1B7	monoclonal	Pdot405/605 Goat anti-mouse IgG2b	115-005-207
	AQP4	Aquaporin 4	Mouse IgG3	Santa Cruz Biotechnology	sc-32739	monoclonal	Pdot405/670 Goat anti-mouse IgG3	115-005-209
	Tuj1/TUBB3	Tubulin β 3	Mouse IgG2a	BioLegend	801201	monoclonal	Pdot405/710 Goat anti-mouse IgG2a	115-005-206
	PV	Parvalbumin	Rabbit	Abcam	ab11427	polyclonal	Pdot405/790 Donkey anti-rabbit	711-005-152
Kidney - 405ex	CD45	Leukocyte Common Antigen	Rat IgG2b	BioLegend	103101	monoclonal (30-F11)	Pdot405/440 Donkey anti-Rat	715-005-151
	Calb	Calbindin	Mouse IgG1	Abcam	ab82812	monoclonal	Pdot405/480 Donkey anti-mouse IgG1	115-005-205
	PODXL	Podocalyxin	Goat	R&D Systems	AF1556		Pdot405/515 Goat anti-PODXL	-
	WGA	Wheat Germ Agglutinin		Vector Labs	L-1020		Pdot405/570 WGA	-
	AcTub	Acetylated Tubulin	Mouse IgG2b	Sigma	T7451-25 UL	monoclonal (6-11B-1)	Pdot405/605 Goat anti-mouse IgG2b	115-005-207
	LEL	Lycopersicon Esculentum Tomato Lectin		Vector Labs	L-1170-2		Pdot405/670 LEL	-
	α-SMA	alpha-Smooth Muscle Actin	Mouse IgG2a	BioLegend	904601	monoclonal (1A4)	Pdot405/710 Goat anti-mouse IgG2a	115-005-206
	ColIV	Collagen IV	Rabbit	Abcam	ab6586	polyclonal	Pdot405/790 Donkey anti-rabbit	711-005-152
Brain - 488ex	Tuj1/TUBB3	Tubulin β 3	Mouse IgG2a	BioLegend	801201	monoclonal	Pdot488/610-Goat anti-mouse IgG2a	115-005-206
	NeuN	Neuronal Nuclei	Mouse IgG2b	Encor	MCA-1B7	monoclonal	Pdot488/675-Goat anti-mouse IgG2b	115-005-207
	MBP	Myelin Basic Protein	Rat	Abcam	ab7349	monoclonal	Pdot488/805-Donkey anti-Rat	715-005-151
Kidney - 488ex	α-SMA	alpha-Smooth Muscle Actin	Mouse IgG2a	BioLegend	904601	monoclonal (1A4)	Pdot488/610-Goat anti-mouse IgG2a	115-005-206
	AcTub	Acetylated Tubulin	Mouse IgG2b	Sigma	T7451-25 UL	monoclonal (6-11B-1)	Pdot488/675-Goat anti-mouse IgG2b	115-005-207
	CD45	Leukocyte Common Antigen	Rat	BioLegend	103101	monoclonal (30-F11)	Pdot488/805-Donkey anti-Rat	715-005-151

Table S3. Pdot-primary antibody conjugates.

Pdot	Antibody or Lectin	Protein Target or Lectin	Performance	Host	Vendor	Catalog #
355-400	CD31/PECAM-1	Cluster of Designation 31, Platelet Endothelial Cell Adhesion Molecule-1	++	Goat	R&D Systems	AF3628
355-400*	SOX2 (1)	Sex Determining Region Y-Box 2	+++	Goat	Novus Biologicals	AF2018
355-400	SOX2 (2)	Sex Determining Region Y-Box 2	++	Mouse IgG1	Thermo Fisher	66411-1-IG
355-440	Tuj1/TUBB3	Tubulin β 3	+	Mouse IgG2a	BioLegend	801201
355-440†	PNA	Peanut Agglutinin	+++	-	Vector	L-1070-25
355-440	SYNPO	Synaptopodin	+	Rabbit	Thermo Fisher	TA890150
355-515†	CD13/APN	Cluster of Designation 13, Aminopeptidase N	+++	Rat IgG2a	Thermo Fisher	MA1-33449
355-515*	LAMIN A/C (1)	Lamin A/C	+++	Rabbit	Proteintech	10298-1-AP
355-515	Podocin (1)	Podocin	+	Rabbit	Millipore Sigma	P0372-200UL
355-570†	AQP2	Aquaporin 2	+++	Rabbit	Novus Biological	NB110-74682
355-570*	MAP2	Microtubule Associated Protein 2	+++	Chicken IgGY	Novus Biologicals	NB300-213
355-610	AQP4 (1)	Aquaporin-4	+	Rabbit	Millipore Sigma	HPA014784-100UL
355-610	AQP4 (2)	Aquaporin-4	+	Rabbit	LSBio	LS-C150456
355-610	Nephrin	Nephrin	+	Goat	R&D Systems	AF3159
355-610	CD45	Cluster of Designation 45, Leukocyte Common Antigen	+++	Rat IgG2b	BioLegend	103102
355-610†	ITGA8	Integrin alpha 8	+++	Goat	Novus Biologicals	AF4076
355-610	Podocin (2)	Podocin	+	Rabbit	Thermo Fisher	PA5-37284
355-670†	AQP1	Aquaporin 1	+++	Rabbit	Proteintech	20333-1-AP
355-670	PNA	Peanut Agglutinin	++	-	Vector	L-1070-5
355-740*†	VEGFR3	Vascular Endothelial Growth Factor Receptor 3	+++	Rat	Novus Biologicals	NB110-61018, 2022
355-740	VIM	Vimentin	++	IgG2a	R&D Systems	MAB21052-100
355-805	GAD67	Glutamate Decarboxylase 1	+	Rabbit	LSBio	LS-C827246-100
355-805	CD90/Thy1	Cluster of Designation 90, Thymus Cell Antigen-1	+	Rat IgG1	Novus Biologicals	NB100-65543
355-805†	UMOD/THP	Uromodulin, Tamm-Horsfall Protein	+++	Rat IgG2a	R&D Systems	MAB5175
405-440†	CD45	Cluster of Designation 45, Leukocyte Common Antigen	+++	Rat IgG2b	BioLegend	103102
405-440*	GFAP	Glial Fibrillary Acidic Protein	+++	Chicken IgY	Thermo Fisher	PA1-10004
405-480	CD31/PECAM-1	Cluster of Designation 45, Platelet Endothelial Cell Adhesion Molecule-1	++	Goat	R&D Systems	AF3628
405-480†	Megalin/LRP2	Low Density Lipoprotein-related Protein 2	+++	Rabbit	Invitrogen	PA5-92032
405-515	NFL	Neurofilament Light Chain	+	Chicken	Aves Labs	NFL
405-515†	PODXL	Podocalyxin	+++	Goat	R&D Systems	AF1556
405-570*†	WGA	Wheat Germ Agglutinin	+++	-	Vector	L-1020-25
405-610†	ColIV	Collagen IV	+++	Rabbit	Abcam	ab6586
405-605*	NeuN	Neuronal Nuclei	+++	Mouse IgG2b	BioLegend	834501
405-670†	LEL	Lycopersicon Esculentum Tomato Lectin	+++	-	Vector	L-1170-2
405-670*	TH	Tyrosine Hydroxylase	++	Mouse IgG2a	BioLegend	818001
405-710†	Agrin	Agrin	+++	Goat	R&D Systems	AF550
405-710*	PV	Parvalbumin	+++	Rabbit	Novus Biologicals	NB120-11427
405-790†	α-SMA	alpha-Smooth Muscle Actin	+++	Mouse IgG2a	Millipore Sigma	A5228-200UL
405-790*	MBP	Myelin Basic Protein	+++	Mouse IgG2b	Invitrogen	MA1-10837
488-520*†	Calb (1)	Calbindin	+++	Rabbit	LSBio	LS-B16562-100
488-520	Calb (2)	Calbindin	++	Mouse IgG1	Abcam	ab82812
488-520	Calb (3)	Calbindin	+	Chicken	Novus Biologicals	NBP2-50028
488-580†	PDPN	Podoplanin	++	Goat	R&D Systems	AF3244

488-580	LEL	Lycopersicon Esculentum Tomato Lectin	+++	-	Vector	L-1170-2
488-610*	NFH	Neurofilament Heavy Chain	+++	Chicken IgY	Aves Labs	NFH
488-610†	REN	Renin	+++	Goat	R&D Systems	AF4277
488-675†	AcTub	Acetylated Tubulin	+++	Mouse IgG2b	Millipore Sigma	T7451-200UL
488-675*	Tuj1/TUBB3	Tubulin β 3	+++	Mouse IgG2b	BioLegend	MMS-435P
488-765*†	Nes/Nestin (1)	Neuroepithelial stem cell protein	+++	Mouse IgG2a	R&D Systems	MAB2736
488-765	Nestin (2)	Neuroepithelial stem cell protein	++	Mouse IgG1	Novus Biologicals	NB300-266
488-765	SYNPO (1)	Synaptopodin	+	Rabbit	Millipore Sigma	S9442-200UL
488-810	CK8	Cytokeratin 8	+	Rabbit	LSBio	LS-B7928-50
488-810	LMNB1 (1)	Lamin B1	++	Mouse IgG1	Proteintech	66095-1-Ig
488-810†	PV	Parvalbumin	+++	Rabbit	Novus Biologicals	NB120-11427

* labeled are used in brain 14-panel stain
† labeled are used in kidney 21-panel stain
+++ Positive and correct signals in multiple samples
++ Positive and correct signals in some tested samples
+ Images dominated by autofluorescence or exhibiting incorrect non-specific signals in most samples

Table S4. Optical filters.

	Chroma	Semrock
Dichroics (longpass)		DI02-R405-25X36
		DI02-R488-25X36
		FF376-DI01-25X36
		zt405/488/561/640rpcv2-UF2
Excitation filters	zet365/20x	
	zet405/20x	
	zet488/10x	
Emission filters	ET405/30m	FF01-562/40
	ET455/50m	FF01-710/40
	ET480/30m	
	ET519/26m	
	ET595/44m	
	ET620/60m	
	ET667/30m	
	ET700/75m	
	ET740/40x	
	ET760/40	
	ET800/60	