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Supplementary Materials for

Feature-rich covalent stains for super-resolution and cleared tissue fluorescence microscopy

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Supplementary Materials

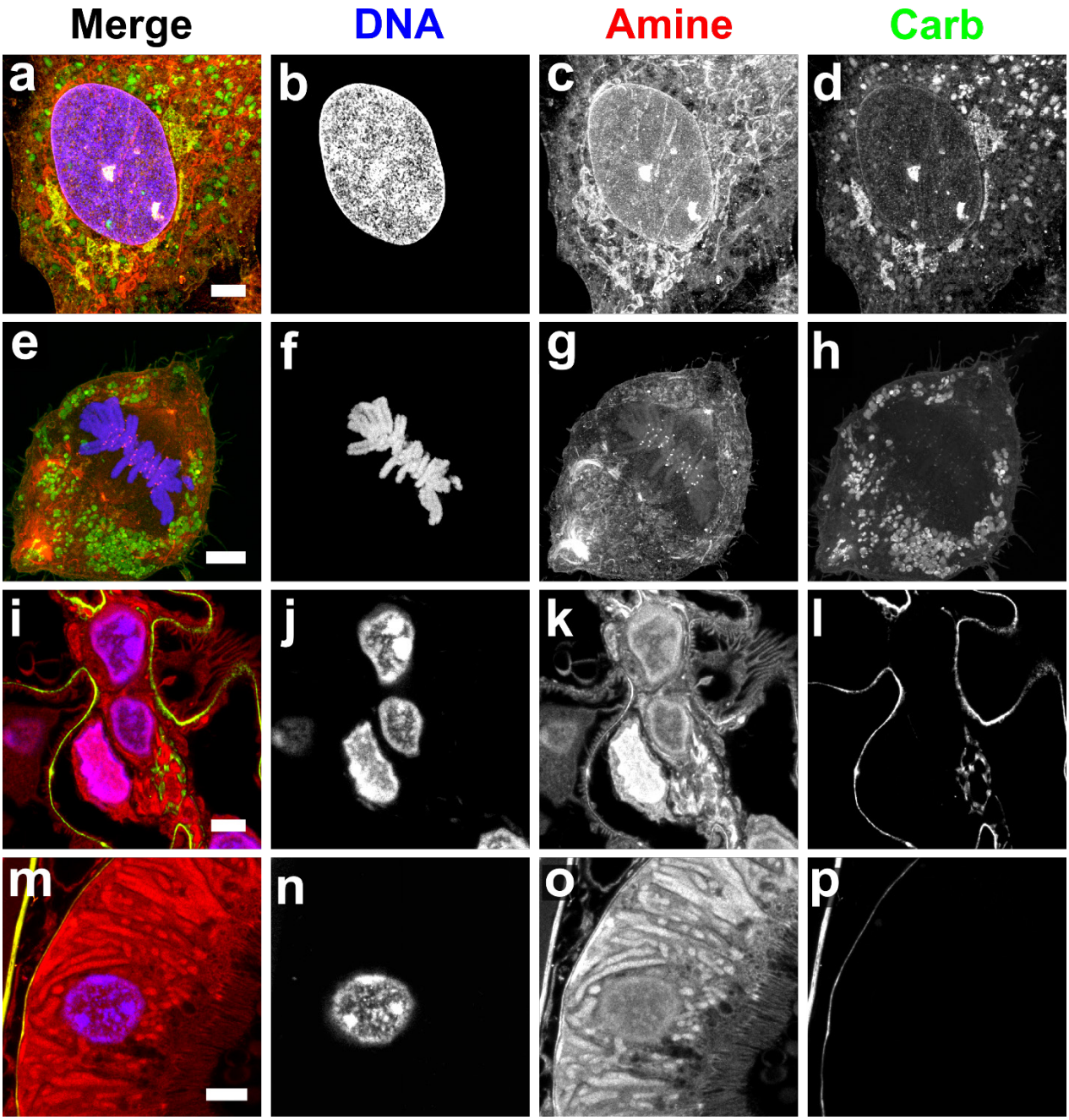


Fig. S1. **Single-channel images from the multichannel data in Fig. 2 and 3.** Confocal microscopy images of expanded specimens stained for carbohydrates, amines, and DNA (see also **Fig. 2** and **Fig. 3**), together with their respective individual channels. These include (**a-d**) a single interphase RPE cell, (**e-h**) a single dividing RPE cell, (**i-l**) a region of a mouse kidney glomerulus, and (**m-p**) a region of a mouse kidney proximal convoluted tubule. Scale bars are 3 μm and are in pre-expansion units.

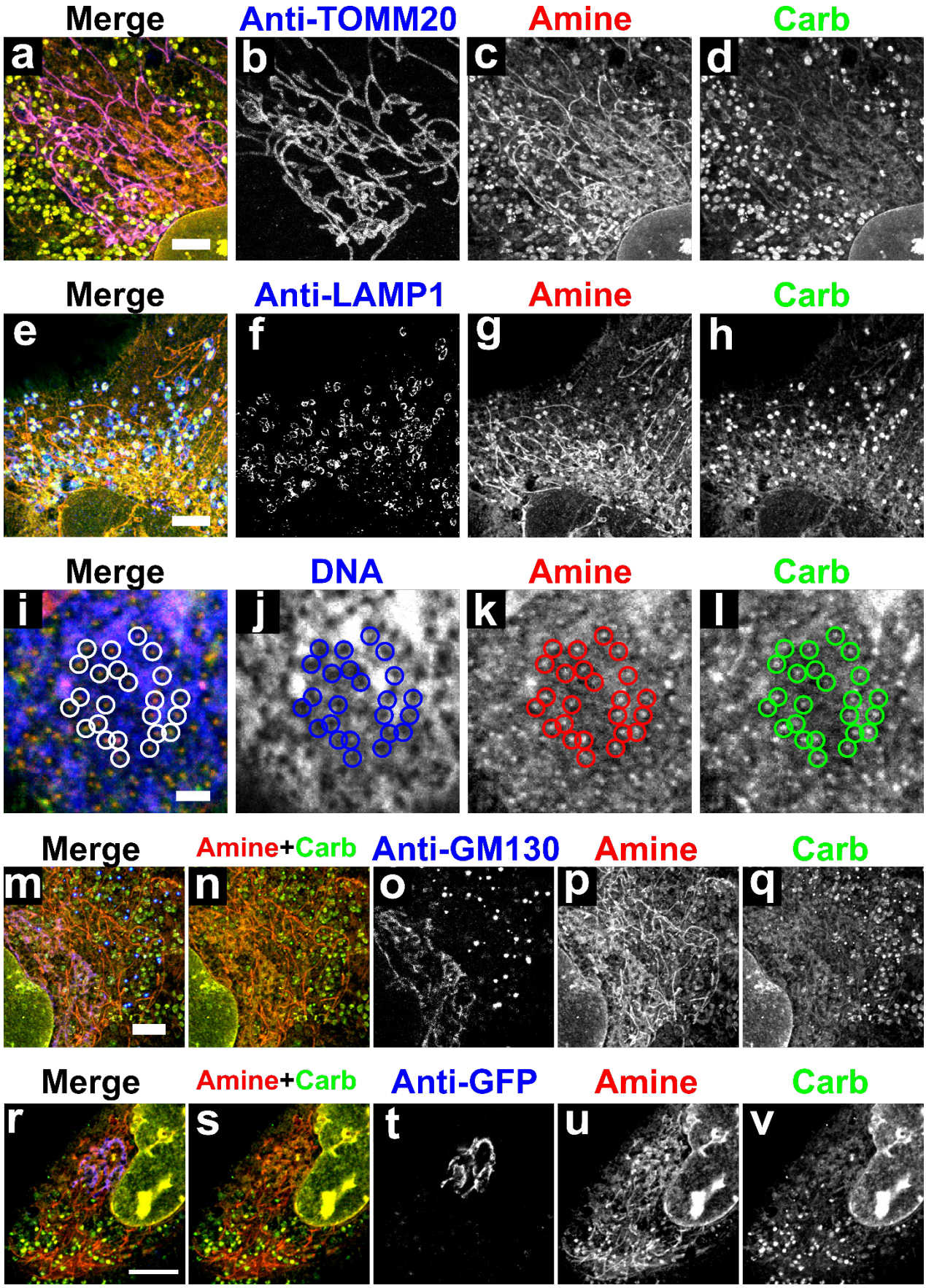


Fig. S2. Validation of sub-cellular features identified by FLARE staining in expanded RPE cells. Confocal microscopy images of expanded RPE cells showing that mitochondria, lysosomes, nuclear pores, and parts of the Golgi apparatus are readily identifiable from covalent stains of

amines and carbohydrates. (**a-d**) An immunostain against the mitochondrial protein TOMM20 colocalizes extensively with the filamentous structures in the amine channel. (**e-h**) An immunostain against the lysosomal protein LAMP1 colocalizes extensively with bright punctae in the carbohydrate channel. (**i-l**) In this zoom-in view of a region of a nucleus, heterochromatin voids in the DNA channel colocalize with punctae in the amine and carbohydrate channels and are indicative of nuclear pores (same cell as in **Fig. 2a**, but here a projection of a thin volume near the bottom of the nucleus). (**m-q**) An immunostain against cis-Golgi matrix protein GM-130 partially colocalizes with a distinct perinuclear signal coming from amine and carbohydrate channels. **(r)** Multichannel, maximum intensity projection of an expanded transfected RPE cell that was labeled with anti-GFP, (**s**) corresponding combined amine and carbohydrate channels, and (**t-v**) corresponding single-channel images. All scale bars are in pre-expansion units. Scale bars, 3 μm (**a**, **e**, **m, r**), 500 nm (**i**).

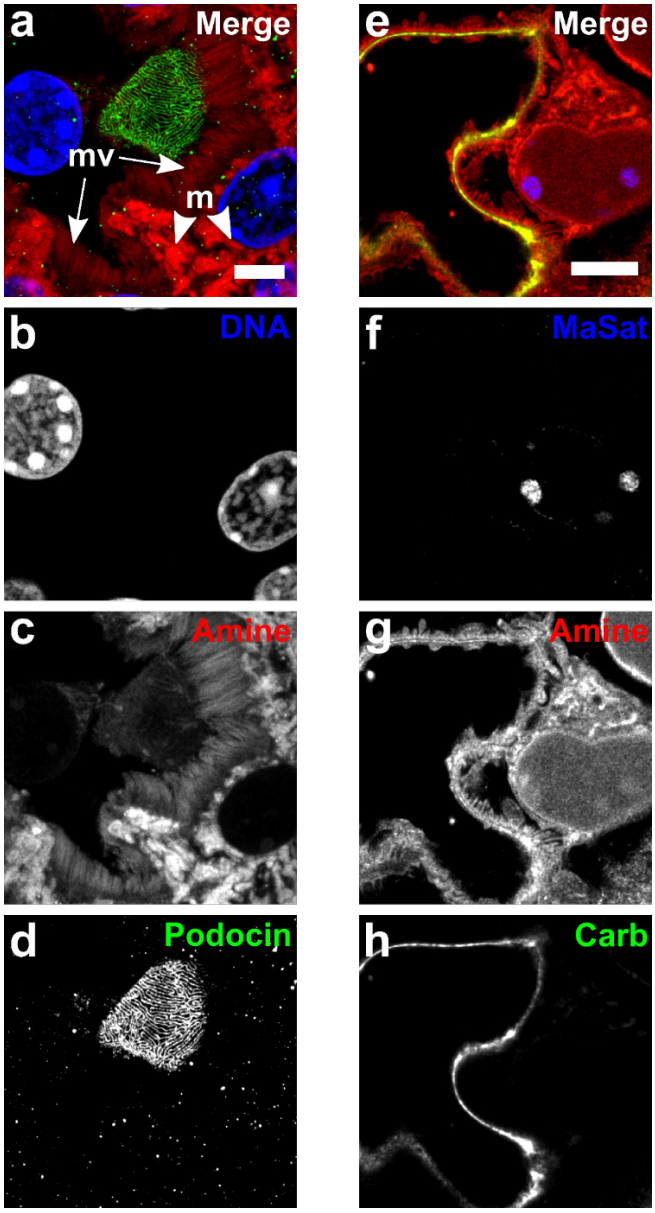


Fig. S3. **Use of FLARE staining on mouse kidney tissue with enzymatic digestion expansion protocol or DNA FISH protocol. (a-d)** Confocal microscopy images of expanded mouse kidney. Prior to covalent labeling, tissue was processed with an expansion microscopy (21) (ExM) procedure that uses enzymatic digestion, rather than a magnified analysis of the proteome (20) (MAP) procedure that uses heat and detergent to dissociate tissues as was done for all other expansion data in this paper (see **Materials and Methods** and **Supplementary Table 1**). Prior to gelation, the sample was immunolabeled against podocin and covalently stained for amines. After gelation, the sample was homogenized using enzymatic digestion, expanded, and stained with the DNA-binding dye Hoechst (see **Materials and Methods** and **Supplementary Table 1**). (**a**) Maximum intensity projection of a region showing interdigitated podocytes (green, podocin) on a glomerulus as well as a proximal convoluted tubule lined with microvilli ('mv') and containing abundant mitochondria ('m'). (**b-d**) corresponding single-channel images. (**e-h**) Confocal microscopy images of an expanded mouse kidney tissue that was stained for carbohydrates, amine, and then DNA FISH against pericentromeric major satellite (MaSat) DNA. (**e**) Multichannel, maximum intensity projection and (**f-h**) corresponding single channel images. Scale bars are 3 μm and are in pre-expansion units.

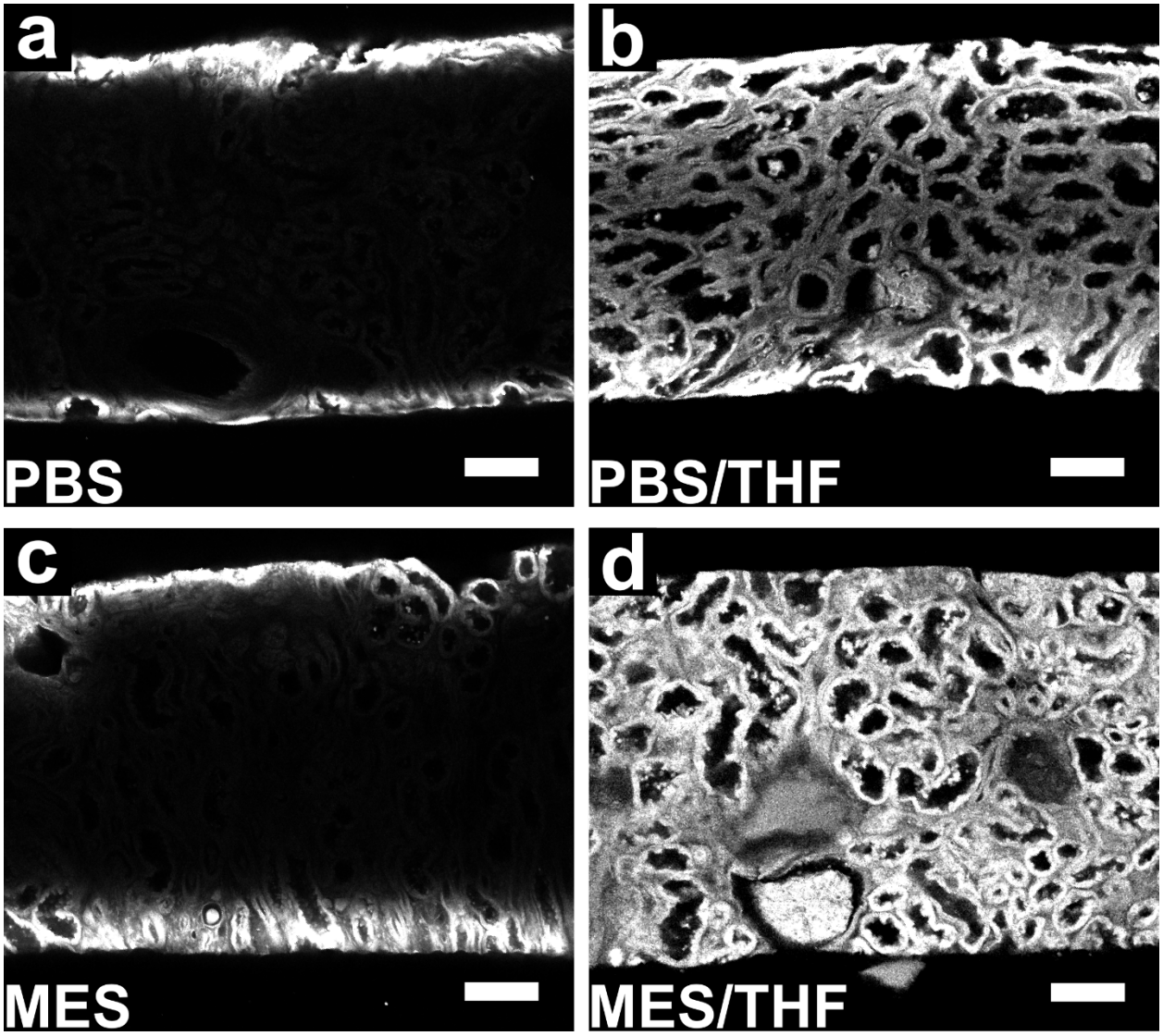


Fig. S4. Enhancement of FLARE stain uniformity in unexpanded thick tissue. Comparison of stain uniformity for amine labeling of ~500 μm thick human kidney sections stained for 15 hours with 1 μg/mL ATTO 647N-NHS (see also **Fig. 4** for panel c and d). Sections were stained, cut perpendicular to the section face, and imaged on a confocal microscope to measure the stain intensity at different depths for (**a**) a pH 7.4 1×PBS solution, (**b**) a pH 7.4 1×PBS:THF (1:1) mixture, (**c**) a pH 6.0 MES buffer solution, and (**d**) a pH 6.0 MES:THF (1:1) mixture. Scale bars are 100 μm.

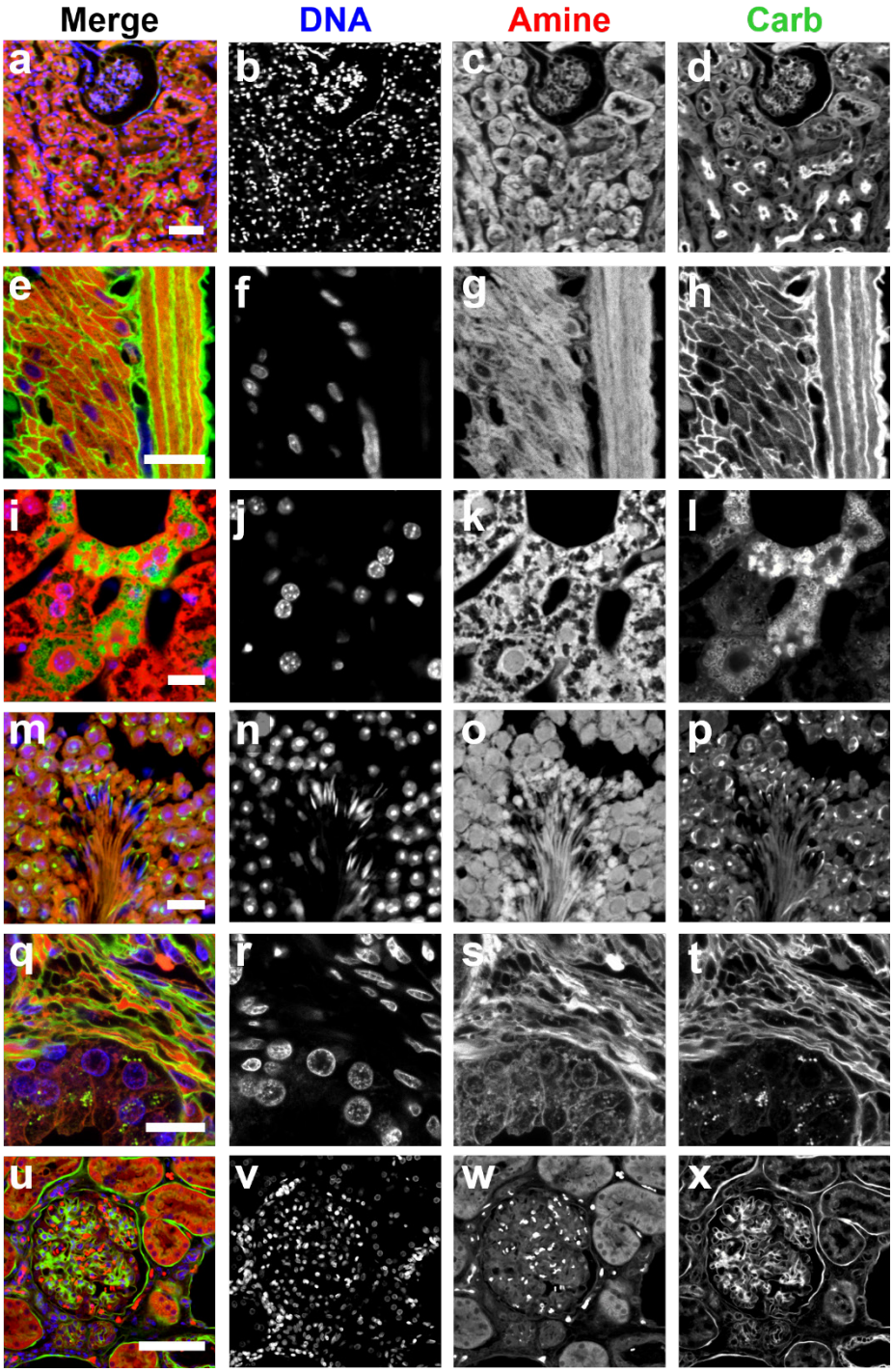


Fig. S5. Single-channel images from the multichannel data in Fig. 4 and 5. Confocal microscopy images of unexpanded, optically cleared tissue specimens that were FLARE-stained for carbohydrates, amines, and DNA (see also **Fig. 4** and **Fig. 5**), together with their respective individual channels, including (**a-d**) a region of mouse half kidney tissue, (**e-h**) circular and longitudinal muscle from mouse intestine, (**i-l**) mouse liver, (**m-p**) mouse testis, (**q-t**) human prostate, and (**u-x**) a formalin-fixed, paraffin-embedded (FFPE) human kidney. All images are maximum intensity projections as described in Supplementary Table 1. Scale bars are 50 μm (**a**), 10 μm (**e**, **i**, **m**, **q**), and 100 μm (**u**).

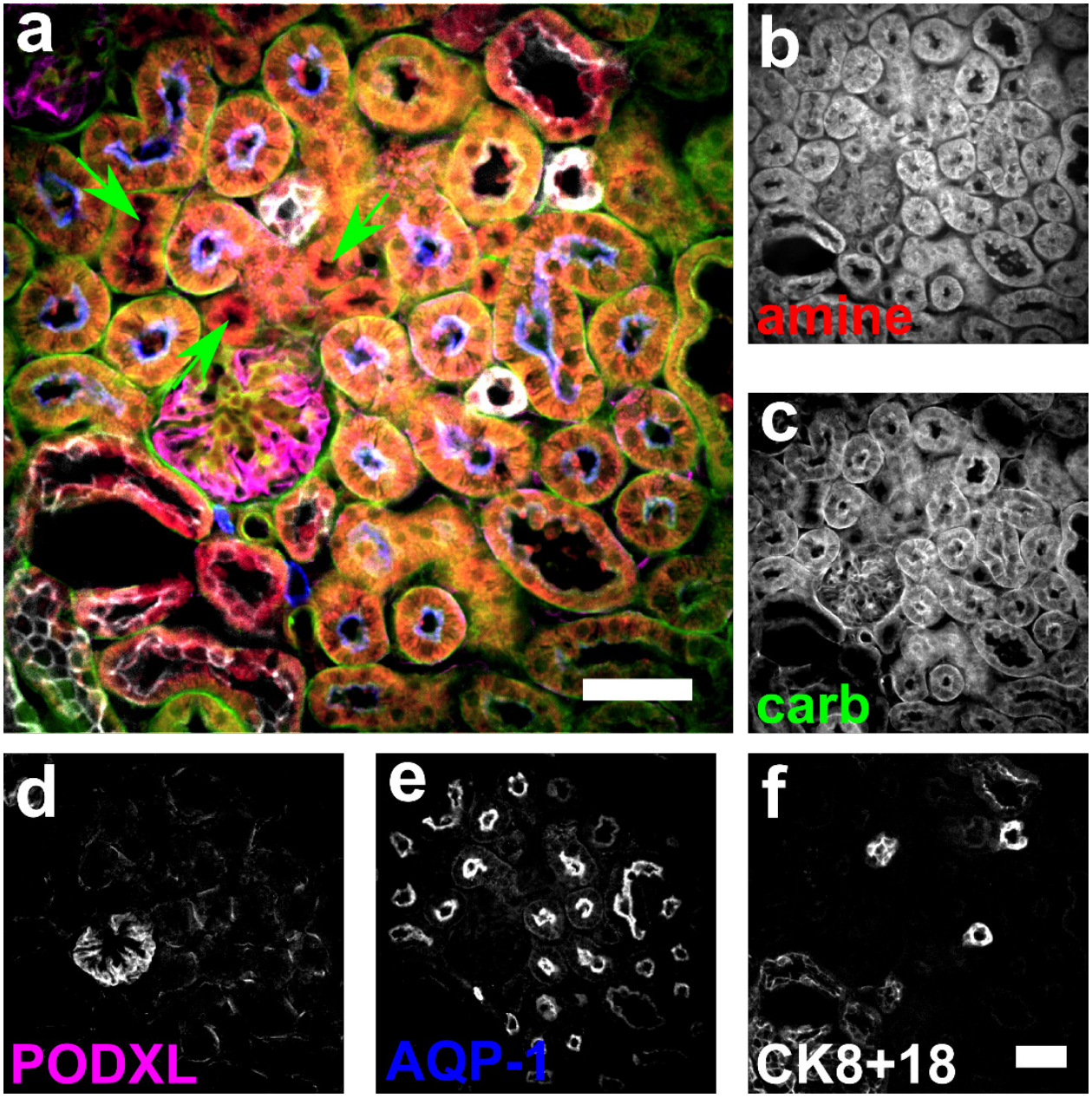


Fig. S6. Concurrent use of FLARE staining with immunostaining to identify general features in mouse kidney. (**a**) Confocal microscopy image of a mouse kidney tissue section that was stained for carbohydrates and amines, and then immunostained for podocalyxin (PODXL, labels glomeruli), aquaporin-1 (AQP-1, labels proximal tubules), and cytokeratin 8+18 (CK8+18, labels collecting ducts). Renal tubules that lack antibody signals here are most likely distal convoluted tubules and are indicated by green arrows. (**b-f**) Corresponding single-channel images of the specific channels indicated. Scale bars are 50 μm.

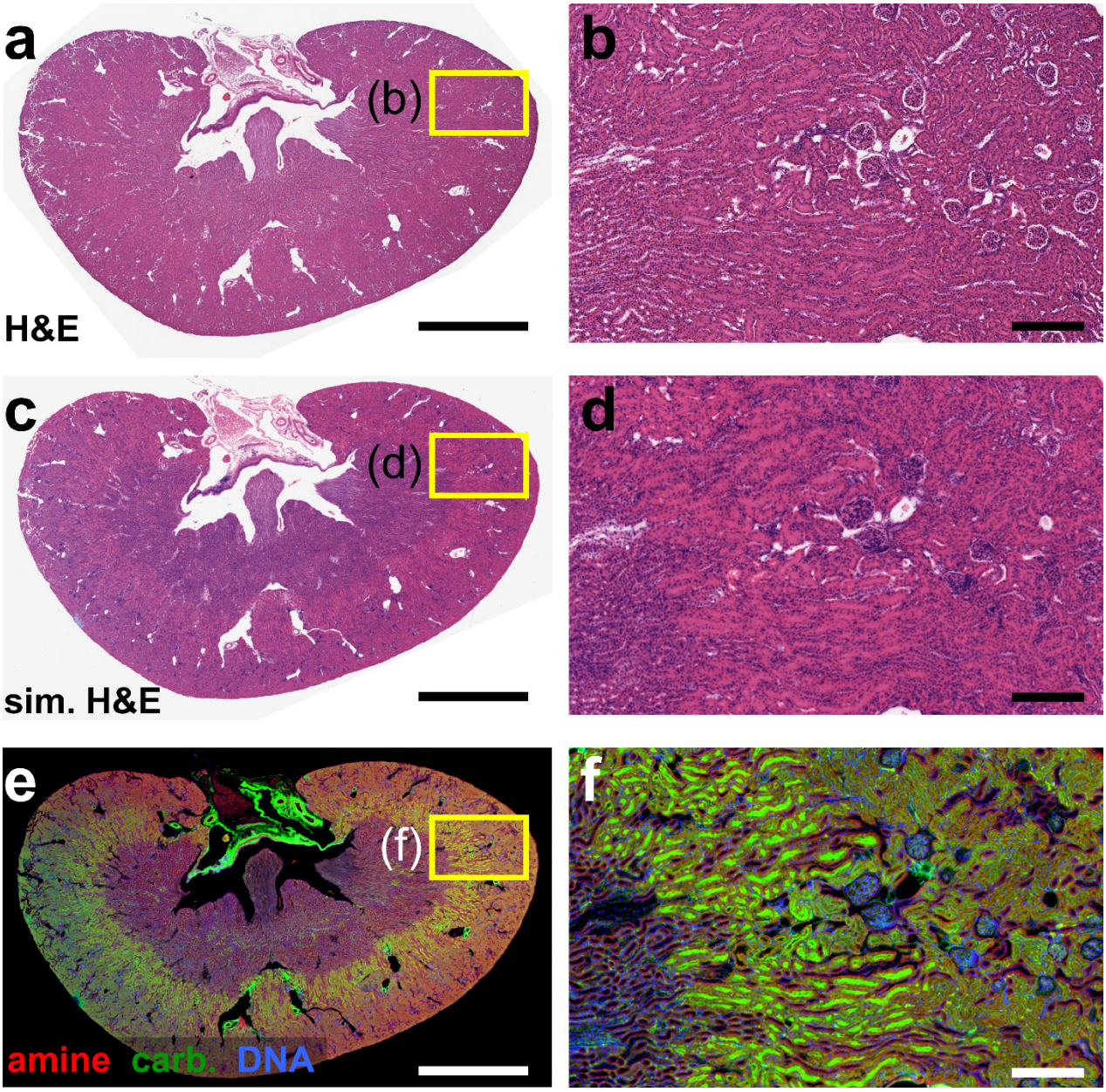


Fig. S7. Side-by-side comparison of H&E and FLARE stains on FFPE mouse kidney sections. Two consecutive 10 µm thick mouse kidney FFPE sections were used for (**a-b**) H&E and (**c-f**) FLARE stains, respectively. The FLARE stain DNA and amine channels were converted to a simulated (sim.) H&E image **(c-d)**, showing a similar pattern as for H&E. However, the molecular distribution and general features are substantially more informative in (**e-f**) the standard fluorescence display. Scale bars are 2 mm (**a, c, e**), 200 µm (**b, d, f**).

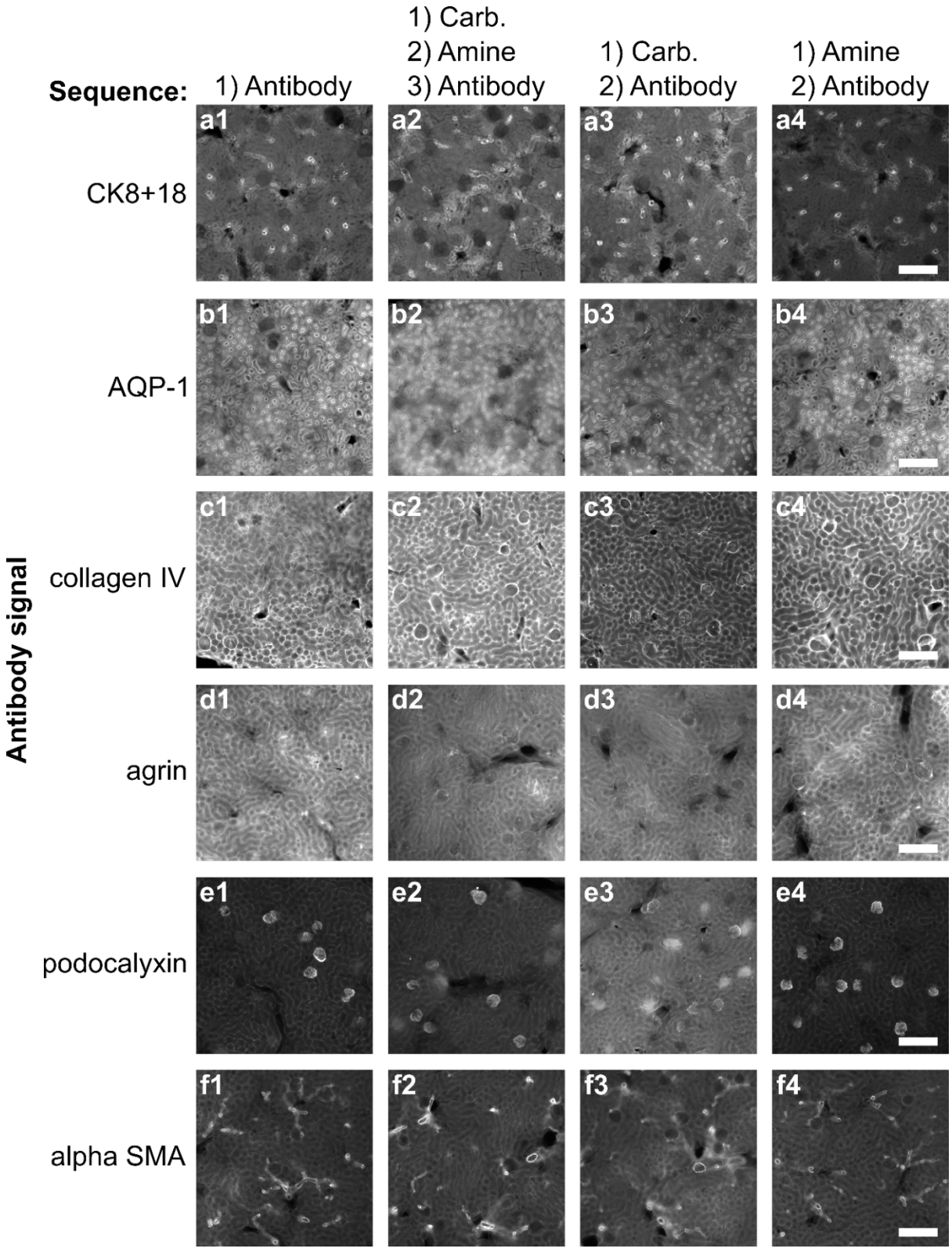


Fig. S8. Effect of FLARE staining on antibody binding for unexpanded mouse kidney tissue. Unexpanded mouse kidney tissue was labeled with various covalent reactions (or none) as indicated in the column headings, immunostained against the protein indicated in each row, and then imaged by widefield fluorescence microscopy. None of the six immunostains were perturbed by the covalent labeling of amines (compare columns 1 and 4) but agrin immunostaining was partially perturbed by covalent labeling of carbohydrates (compare columns 1 and 3). Scale bars are 200 μm.

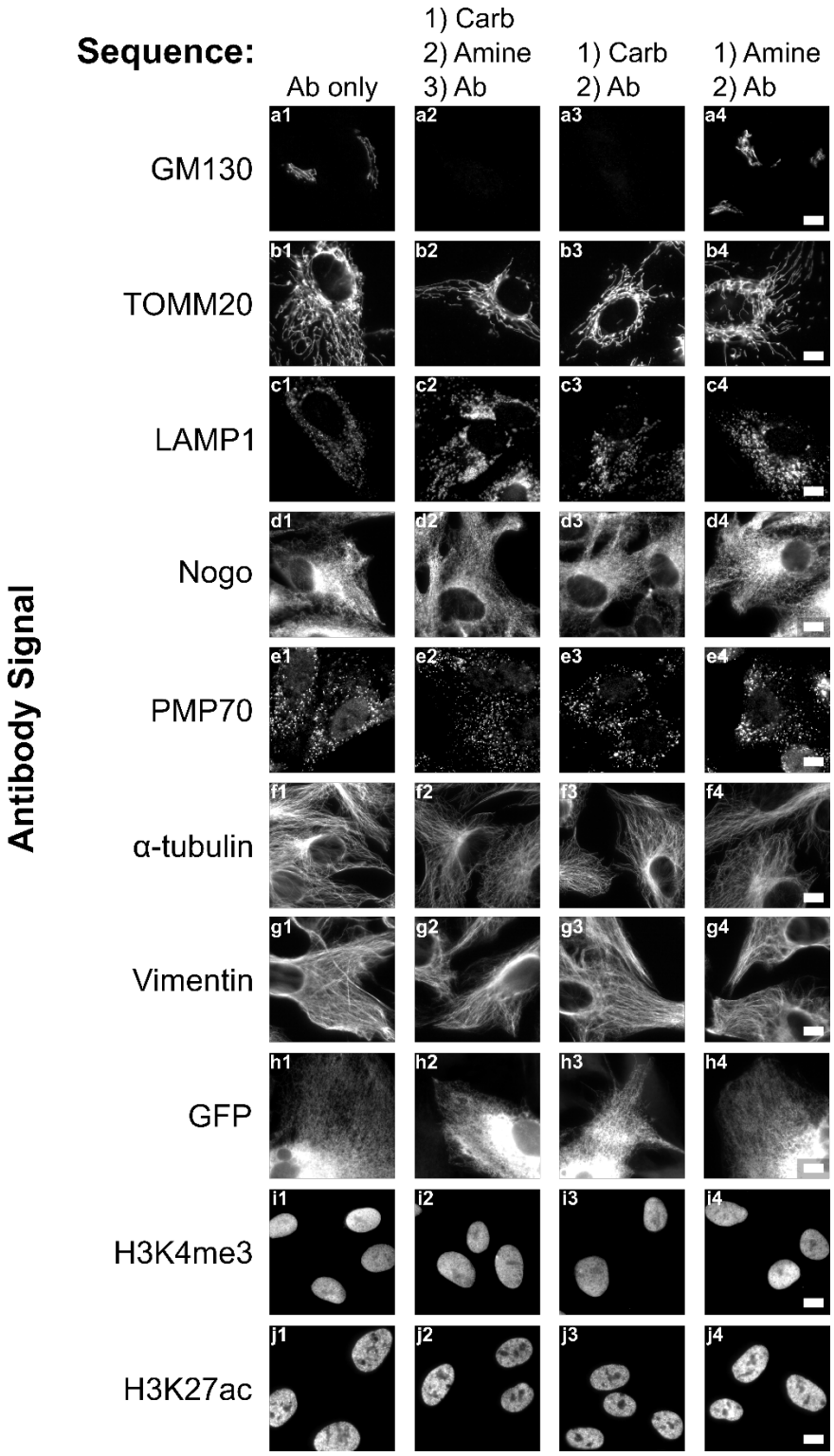


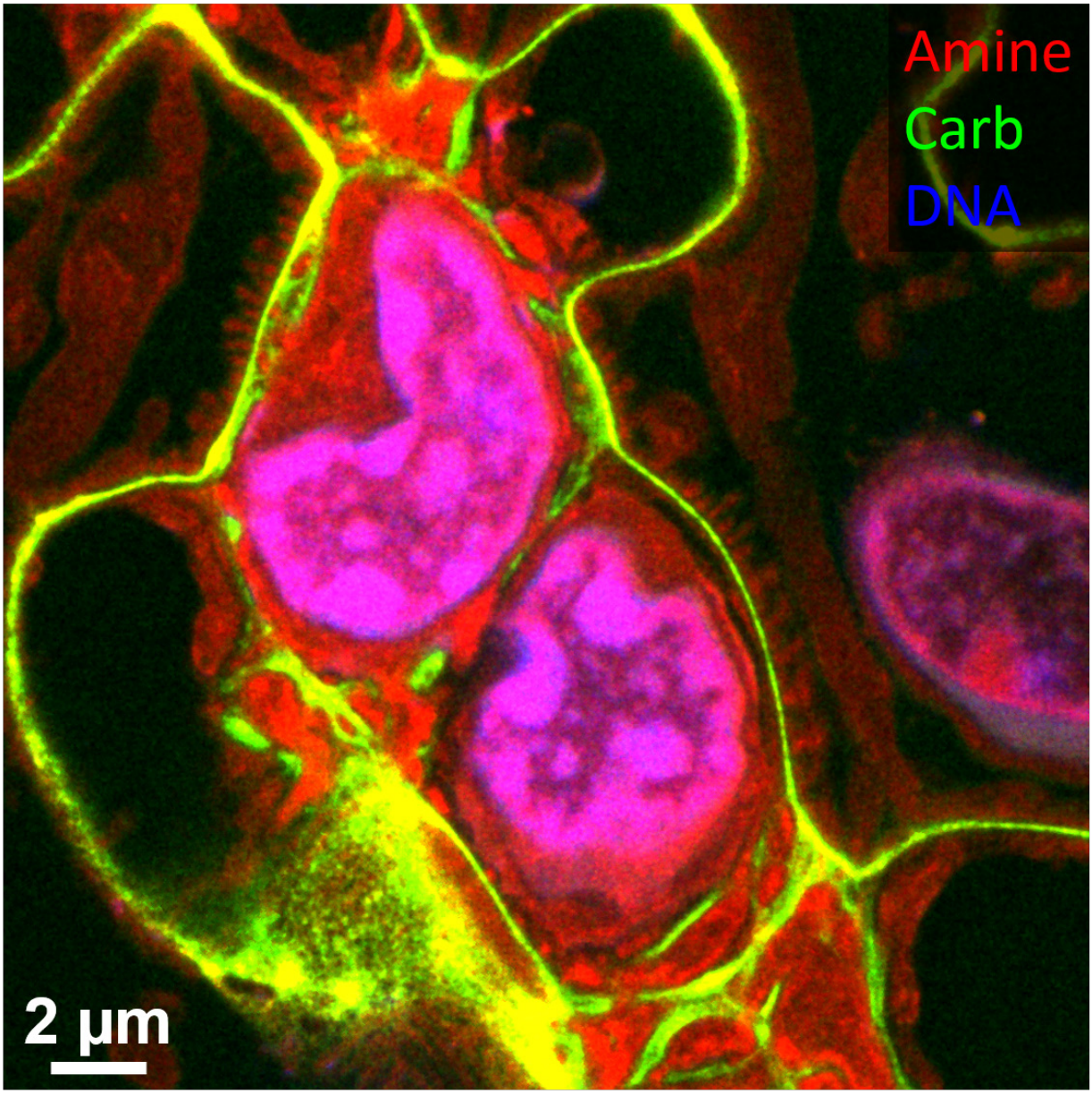
Fig. S9. Effect of FLARE staining on antibody binding for unexpanded RPE cells. Unexpanded RPE cells were labeled with various covalent reactions (or none) as indicated in the column headings, immunostained against the protein indicated in each row, and then imaged by widefield fluorescence microscopy. None of the ten immunostains were perturbed by the covalent labeling of amines (compare columns 1 and 4) but GM130 immunostaining was disrupted by covalent labeling of carbohydrates (compare columns 1 and 3). Scale bars are 10 μm. Ab = antibody; Carb = carbohydrate covalent stain; Amine = amine covalent stain.

Table S1. Summary of sample preparation and imaging conditions.

Fig.	Sample	Fixation	Tissue Sectioning	Carbohydrate Stain	Amine Stain	Other Stain(s) and Stain Order	Expansion or Clearing	Imaging
2	RPE cell	PFA/GA 10min	---	1hr, 20mM $NaIO_4$ in 100mM NaOAc with 1M NaCl, pH5; then 3hr, 6.65μM hydrazide-AT565 in 100mM NaOAc, pH5; then 30 min, 50mM $NaBH_3CN$ in 100mM NaOAc, pH5.	1hr, 3.0μM NHS-AT647N in PBS.	DNA: 30min, 1.96μM SYBR Green in DI water Order: gel, covalent stains, expand, DNA.	Expansion	Confocal, 63× 1.2NA water lens. Image thickness: a) 738nm, b) 462nm, c) 738nm, d) 1.85μm, e) 1.57μm, f) 462nm. Filter: none.
3	Mouse kidney	PFA 1hr	100μm vibratome sections	Same as **Fig. 2**.	6hr, 5.9μM NHS-AT647N in 100mM MES, pH6.	DNA: 30min, 1.87μM Hoechst 33258 in PBS, pH7.4. Order: gel, covalent stains, DNA, expand.	Expansion	Confocal, 63× 1.2NA water lens. Image thickness: 92.3nm. Filter: 2.
4a-b	Human kidney	PFA 1hr	500μm vibratome sections (resliced after labeling)	---	1.2μM NHS-AT647N, 15hr in a) MES, b) MES:THF (1:1).	---	Clearing	Confocal, 10× 0.4NA air lens. Image thickness: 1.52μm. Filter: none.
4c-d	Mouse kidney	PFA 1hr	500μm vibratome sections (resliced after labeling)	4hr, 100mM $NaIO_4$ in 100mM NaOAc, pH5; then 15hr, 1.2μM hydrazide-AT565 in c) NaOAc and d) THF:NaOAc (1:1); then 30min, 50 mM $NaBH_3CN$ in THF:NaOAc (1:1).	---	---	Clearing	Confocal, 10× 0.4NA air lens. Image thickness: 1.52μm. Filter: none.
4e	Mouse kidney	PFA 1hr	500μm vibratome sections	---	2 hr, 17.7μM NHS-AT647N in THF: MES (1:1).	Immuno: Rb×Collagen 5 μg/mL 72hr; D×Rb AT488 10μg/mL, 72hr; at 4°C. Order: immuno, covalent stain, clear.	Clearing	Confocal, 10× 0.4NA air lens. Image thickness: 1.52μm. Filter: none.
4f-h	Mouse kidney block	PFA 1hr	1mm vibratome section	5hr, 100mM $NaIO_4$ in 100mM NaOAc, pH5; then 6hr, 3.33μM hydrazide-AT565 in THF:NaOAc (1:1); then 1hr, 100mM $NaBH_3CN$ in THF:NaOAc (1:1).	8hr, 5.9μM NHS-AT647N in THF: MES (1:1).	DNA: 15hr, 20μM SYBR green in H_2O: PBS (1:1). Order: covalent stains, DNA, clear.	Clearing	Light sheet microscope, 20× 0.43NA oil lens. Image thickness: f-h) 446nm. Filter: none.
5a-f	Mouse intestine, liver, testis, human prostate	PFA 1hr, intestine, testis; PFA 6hr, liver; formalin 20hr, prostate	100μm vibratome sections	30min, 100mM $NaIO_4$ in 100mM NaOAc, pH 5; then 2hr, 3.33μM hydrazide-AT565 in THF:NaOAc (1:1); then 30min, 100mM $NaBH_3CN$ in THF:NaOAc (1:1).	2hr, 5.9μM NHS-AT647N in THF: MES (1:1).	DNA: 30min, 1.87μM Hoechst 33258 in THF: PBS (1:1). Order: covalent stains, DNA, clear.	Clearing	Confocal, a) 20× 0.7NA air lens, b-f) 63× 1.4NA oil lens. Image thickness: a) 8.06μm, b) 2.26μm, c) 377nm, d) 377nm, e) 1.89μm, f) 377nm. Filter: a-e) none, f) 2.
5g	Human FFPE kidney	Formalin 24hr	60μm microtome sections	Same as **Fig. 5a-f.**	Same as **Fig. 5a-f.**	Same as **Fig. 5a-f.**	Clearing	Confocal, g) 20× 0.7NA air lens. Image thickness: 1 μm. Filter: 2.
S1a-h	RPE cell	Same as **Fig. 2**	---	Same as **Fig. 2.**	Same as **Fig. 2.**	Same as **Fig. 2.**	Expansion	Same as **Fig. 2.**
S1i-p	Ms. kidney	Same as **Fig. 3**	---	Same as **Fig. 3.**	Same as **Fig. 3.**	Same as **Fig. 3.**	Expansion	Same as **Fig. 3.**
S2a-d	RPE cell	Same as **Fig. 2**	---	Same as **Fig. 2.**	Same as **Fig. 2.**	Immuno: Rb×TOMM20 2μg/mL 90min; D×Rb biotin 3μg/mL 45min; streptavidin AF488 5μg/mL in PBS 15hr; at ~20°C. Order: immuno, gel, covalent stains, streptavidin, expand.	Expansion	Confocal, 63× 1.2NA water lens. Image thickness: a-d) 646nm. Filter: none.
S2e-h	RPE cell	Same as **Fig. 2**	---	30min, 20mM $NaIO_4$ in 100mM NaOAc with 1M NaCl, pH5; then 1.5hr, 2.66μM hydrazide-AT565 in 100mM NaOAc, pH5; then 15min, 50mM $NaBH_3CN$ in 100mM NaOAc, pH5.	1hr, 1.2μM NHS-AT647N in PBS.	Immuno: Rb×LAMP1 4μg/mL 90min; D×Rb AF488 5μg/mL 45min; at ~20°C. Order: covalent stains, immuno, gel, expand.	Expansion	Confocal, 63× 1.2NA water lens. Image thickness: e-h) 923nm. Filter: none.
S2i-l	RPE cell	Same as **Fig. 2**	---	Same as **Fig. 2.**	Same as **Fig. 2.**	DNA: Same as **Fig. 2.** Order: Same as **Fig. 2.**	Expansion	Confocal, 63× 1.2NA water lens. Image thickness: i-l) 277nm. Filter: none
S2m-q	RPE cell	Same as **Fig. 2**	---	Same as **Fig. 2.**	Same as **Fig. 2.**	Immuno: Ms×GM130 2μg/mL 90min; D×Ms biotin 3 μg/mL 45min; streptavidin AF488 5μg/mL in PBS 15hr; at ~20°C. Order: immuno, gel, covalent stains, streptavidin, expand.	Expansion	Confocal, 63× 1.2NA water lens. Image thickness: m-q) 1.15μm. Filter: none.
S2r-v	RPE cell transfected	Same as **Fig. 2**	---	Same as **Fig. 2.**	Same as **Fig. 2.**	Immuno: Rb×GFP-AF488 4μg/mL 15hr; at ~20°C. Order: gel, covalent stains, immuno, expand.	Expansion	Confocal, 63× 1.2NA water lens. Image thickness: r-j) 185nm. Filter: none.
S3a-d	Mouse kidney	PFA 1hr	100μm vibratome sections	---	1hr, 3.0μM NHS-AF546 in PBS.	DNA: Same as **Fig. 3a-d**. Immuno: Rb×Podocin 5μg/mL 18hr; D×Rb AT488 5μg/mL 18hr; at 4°C. Order: immuno, covalent stain, gel, expand, then DNA.	Expansion	Confocal, 63× 1.2NA water lens. Image thickness: 1.04μm. Filter: none.
S3e-h	Mouse kidney	PFA 1hr	100μm vibratome sections	Same as **Fig. 3.**	Same as **Fig. 3.**	DNA FISH: denature 10min at 92.5°C; hybridize 18hr at 37°C with 100nM MaSat oligo + 100nM AT647N oligo. Order: gel, covalent stains, DNA FISH, then expand	Expansion	Confocal, 63× 1.2NA water lens. Image thickness: 92nm. Filter: 2.
S4	Human kidney	PFA 1hr	500μm vibratome sections	---	1.2μM NHS-AT647N, 15hr in: a) PBS, b) PBS:THF (1:1), c) MES, d) MES:THF (1:1).	---	Clearing	Confocal, 10× 0.4NA air lens. Image thickness: 1.52μm. Filter: none.
S5a-d	Mouse kidney	PFA 1hr	1mm vibratome section	Same as **Fig. 4g**	Same as **Fig. 4g**	Same as **Fig. 4g**	Clearing	Same as **Fig. 4g**
S5e-x	Same as for **Fig. 5a-g**	Same as **Fig. 5a-g**	Same as **Fig. 5a-g**	Same as **Fig. 5a-g**	Same as **Fig. 5a-g**	Same as **Fig. 5a-g**	Clearing	Confocal, e-t) 63× 1.4NA oil lens, u-x) 20× 0.7NA air lens. Image thickness: Same as **Fig. 5a-g** Filter: e-l) none, m-t) 2, u-x) none.
S6	Mouse kidney	PFA 1hr	100μm vibratome sections	30min, 100mM $NaIO_4$ in 100mM NaOAc, pH 5; then 1hr, 3.33μM hydrazide-Al568 in NaOAc; then 30min, 100mM $NaBH_3CN$ in NaOAc.	1hr, 3.0μM NHS-AT647N in 1× PBS	Immuno: 2μg/mL G×PODXL, Rb×AQP-1, Gp×CK8+18 18h; 2μg/mL D×G-AFdye405, D×Rb-AF488, D×Gp-AF750 18h; at ~4°C. Order: covalent stains, immuno	Neither	Homebuilt spinning-disk confocal, 20× 0.45NA air lens; imaging cocktail was tris 200mM pH8.0, 10% glucose, 1mM Trolox, 0.4mg/mL glucose oxidase, 0.2% catalase. Image thickness: single plane Filter: none
S7a-b	Mouse kidney	Formalin 24hr	10μm microtome sections	---	---	---	Neither	Aperio ScanScope AT2 digital whole slide scanner for H&E.
S7c-f	Mouse kidney	Formalin 24hr	10μm microtome sections	Same as **Fig. S6**	Same as **Fig. S6**	Immuno: 2μg/mL primary ab. 18h; 2μg/mL secondary ab. (AF 488 conjugates) 18h; at ~4°C. Order: covalent stains, immuno	Neither	Widefield, 20× 0.45NA air lens. Image thickness: single plane. Filter: none.
S8	Mouse kidney	Same as **Fig. 3**	100μm vibratome sections	Same as **Fig. S6**	Same as **Fig. S6**	Immuno: 2μg/mL primary ab. 18h; 2μg/mL secondary ab. (AF488 conjugates) 18h; at ~4°C. Order: covalent stains, immuno	Neither	Widefield, 4× 0.2NA air lens. Image thickness: single plane. Filter: none.
S9 a-e	RPE cell	PFA/GA 10min	---	30min, 20mM $NaIO_4$ in 100mM NaOAc with 1M NaCl, pH5; then 1.5hr, 2.66μM hydrazide-AT565 in 100mM NaOAc, pH 5; then 15min, 50mM $NaBH_3CN$ in 100mM NaOAc, pH5.	1hr, 1.2μM NHS-AT647N in PBS.	Immuno: 2μg/mL primary ab. 90min; 2μg/mL secondary ab. (AF488 conjugates) 45min; at ~20°C. Order: covalent stains, immuno	Neither	Widefield, 60× 1.2NA water lens. Image thickness: Single plane. Filter: none.
S9 h	RPE cell	PFA/GA 10min	---	Same as **Fig. S9 a-e**	1hr, 1.2μM NHS-AT565 in PBS.	Immuno: 2μg/mL of Rb×GFP-AF647 90min; at ~20°C. Order: covalent stains, immuno	Neither	Same as **Fig. S9 a-e**
S9 f-g	RPE cell	0.5% Triton-x100 extraction 30sec, PFA/GA 10min	---	Same as **Fig. S9 a-e**	Same as **Fig. S9 a-e**	Same as **Fig. S9 a-e**	Neither	Same as **Fig. S9 a-e**
S9 i-j	RPE cell	4% PFA 10min	---	Same as **Fig. S9 a-e**	Same as **Fig. S9 a-e**	Immuno: permeabilize 10min with 0.1% Triton-x100 in PBS; 2μg/mL primary ab. 18h at ~4°C; 2μg/mL secondary ab. (AF488 conjugtaes) 2hr at ~20°C. Ab. incubations in 10% (w/v) BSA in PBS. Order: covalent stains, immuno	Neither	Same as **Fig. S9 a-e**

Acronyms: Ab=antibody; AF=Alexa Fluor; AT=ATTO-TEC; DI=deionized; FFPE=formalin-fixed, paraffin-embedded; NA=numerical aperture; RPE=retinal pigment epithelium cell line.

Additional notes: median filter indicates number of pixels used for application of 3D median filter if used on confocal data sets. Image thickness refers to the thickness of the data displayed in terms of the pixel sizes of the data set, where distances are in pre-expansion units for expanded tissues.



Movie S1. Animation of FLARE-stained expanded mouse kidney. Shown is the first frame from an animation of an expanded mouse kidney section that focuses on a small volume of a glomerulus. The animation shows successive z-slices of the confocal stacks, and highlights the basement membrane of capillary loops and mesangial matrix (green) as well as the fine details of the interdigitated podocyte epithelial cells (red). The same data set was used to create Fig. 3a-c. The animation covers a depth of 12.1 µm. All distances are in pre-expansion units.