SUPPLEMENTARY MATERIALS

MANUSCRIPT TITLE:

A spatially anchored transcriptomic atlas of the human kidney papilla identifies significant immune injury in patients with stone disease

AUTHORS

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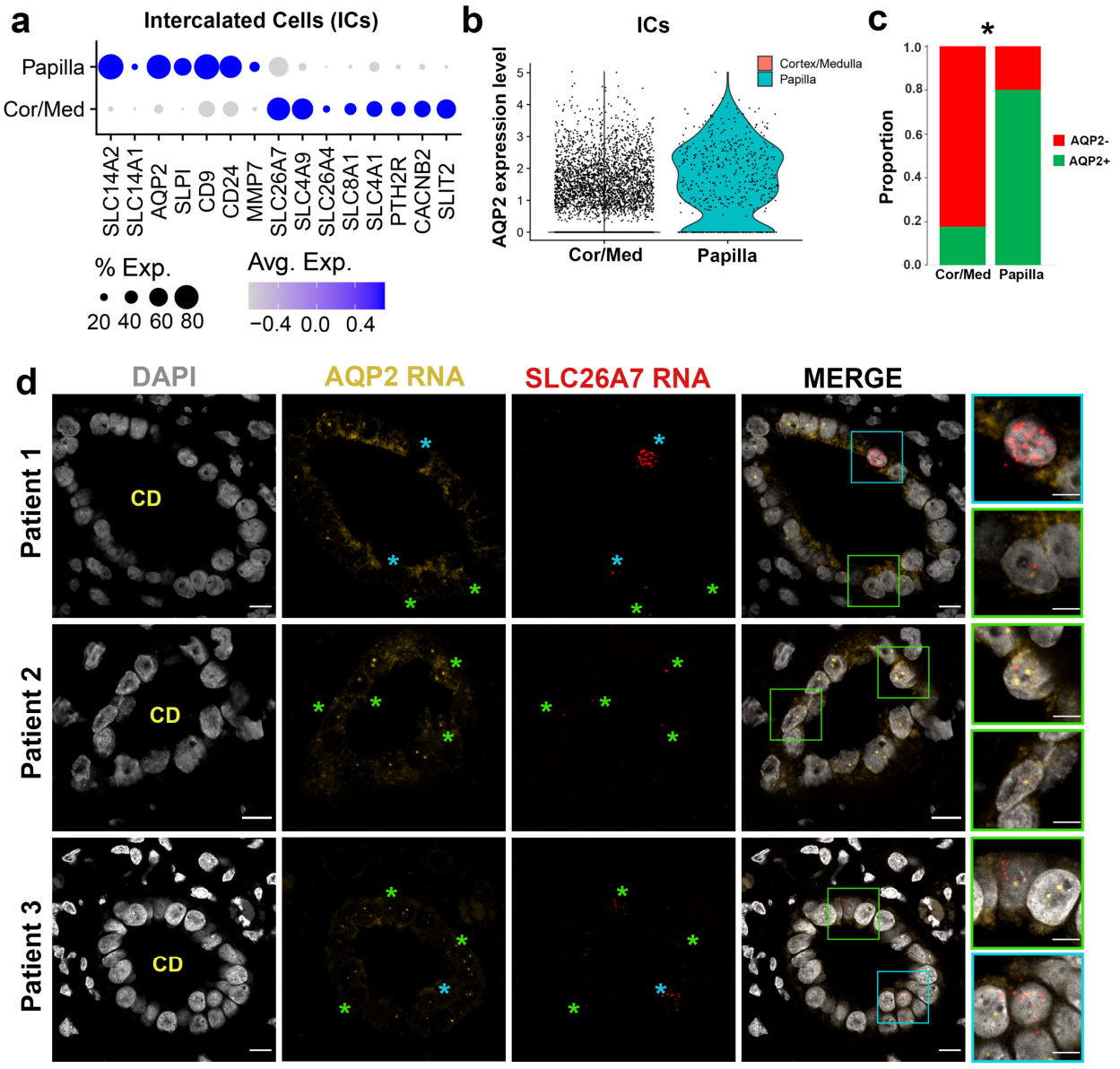
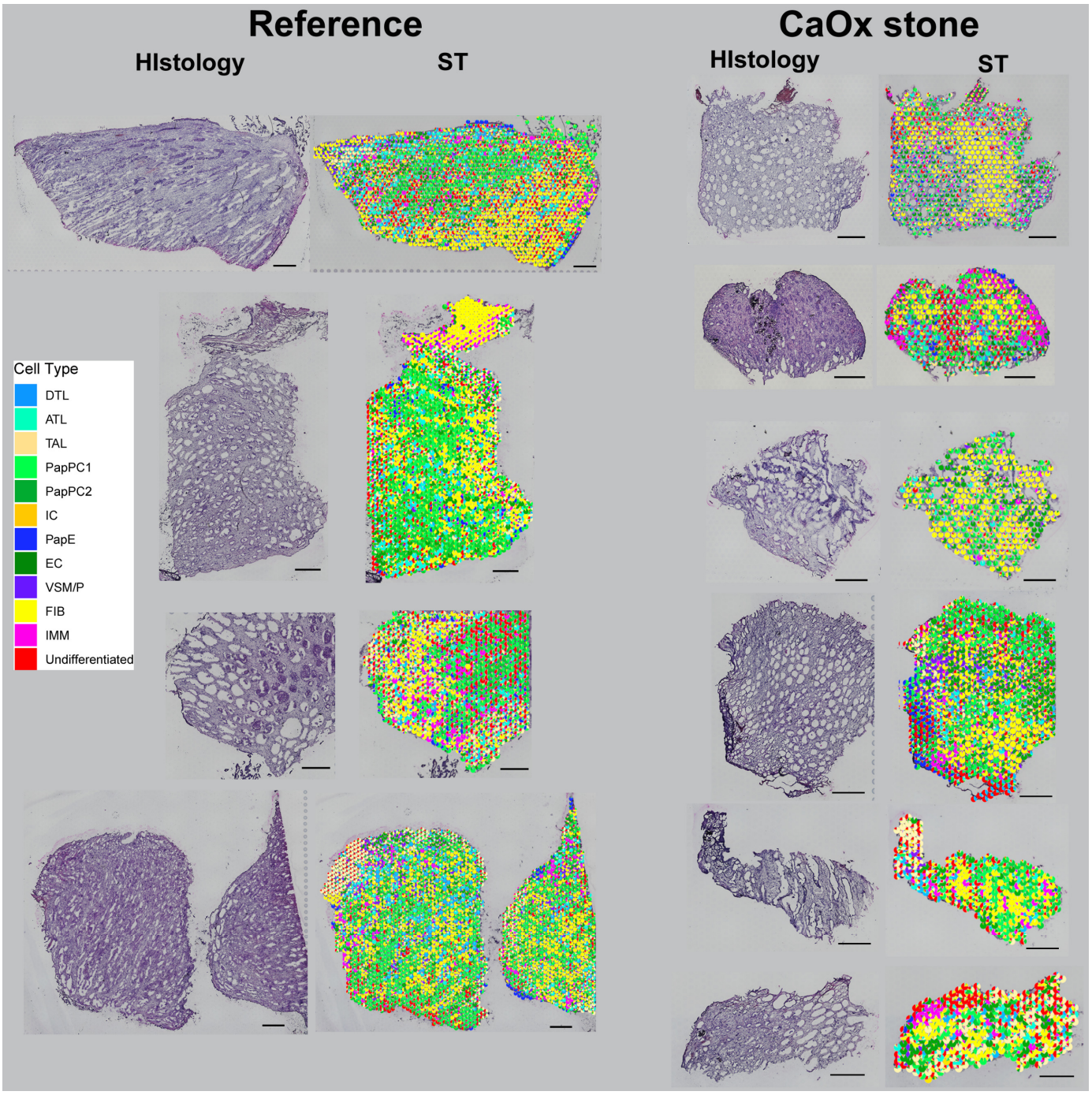
Supplemental Figures

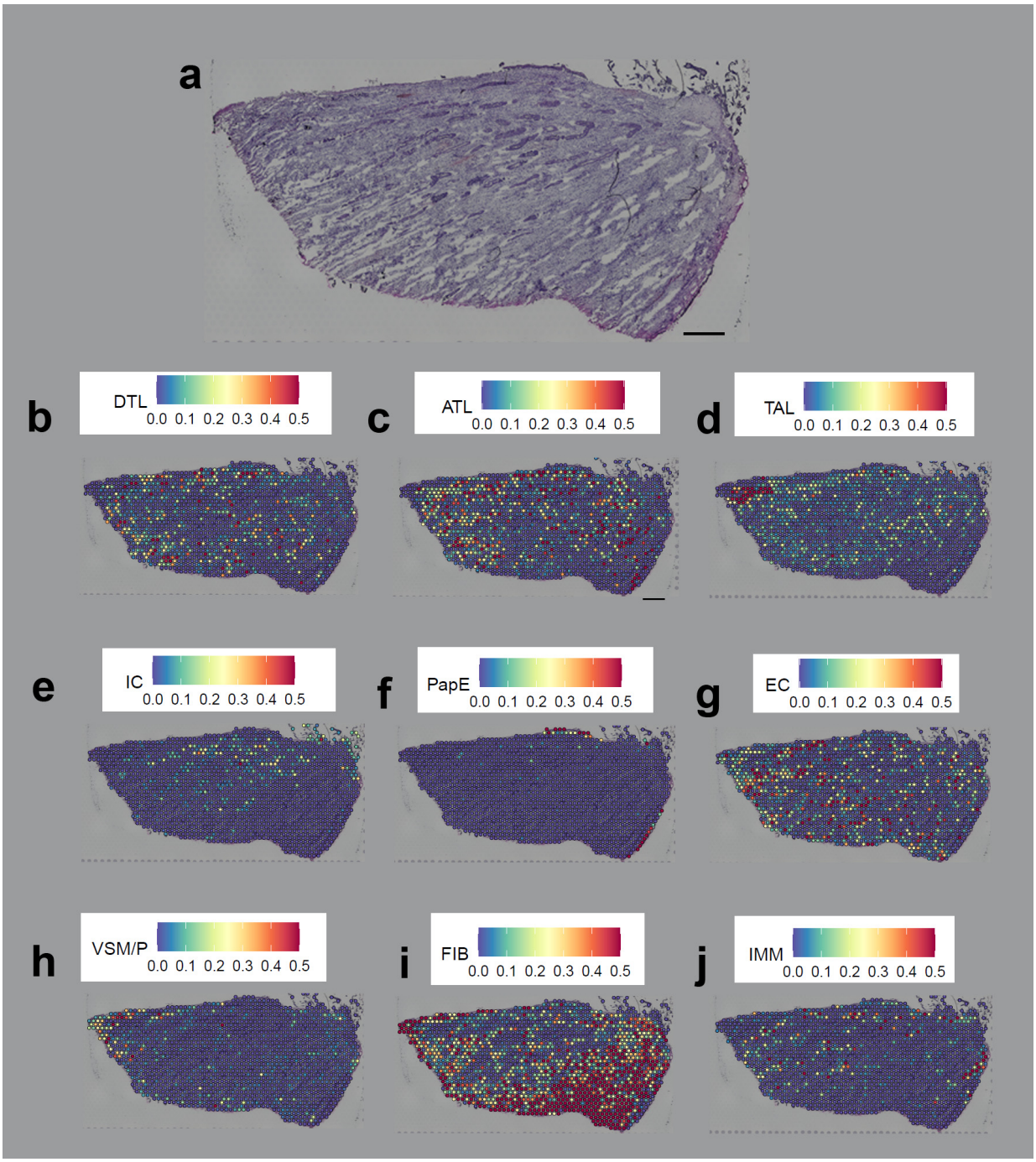


Supplementary Fig. 1: Intercalated cells in the papilla. (a) Differential gene expression between cortico-medullary and papillary intercalated cells based on snRNAseq (full dataset in Figure 1). Papillary intercalated cells have higher expression of AQP2 (b) than cortico-medullary ICs, and most IC cells in the papilla express AQP2 (c). Asterisk in (c) denotes statistical significance ($p < 0.0001$) using two tailed Fisher's exact test comparing proportions of AQP2 negative cells in the corticomedullary vs. papillary ICs. Panels in (d) show single molecular fluorescence in situ hybridization (smFISH) for AQP2 and SLC26A7 from 3 different stone papillary biopsy patients (scale bar = 10 μm). SLC26A7 is a chloride/bicarbonate exchanger specific to IC[1] and a marker for human IC cells from the atlas by Lake et al.[2] In the papilla, few IC collecting ducts cells express high levels of SLC26A7 with no AQP2 expression (cells

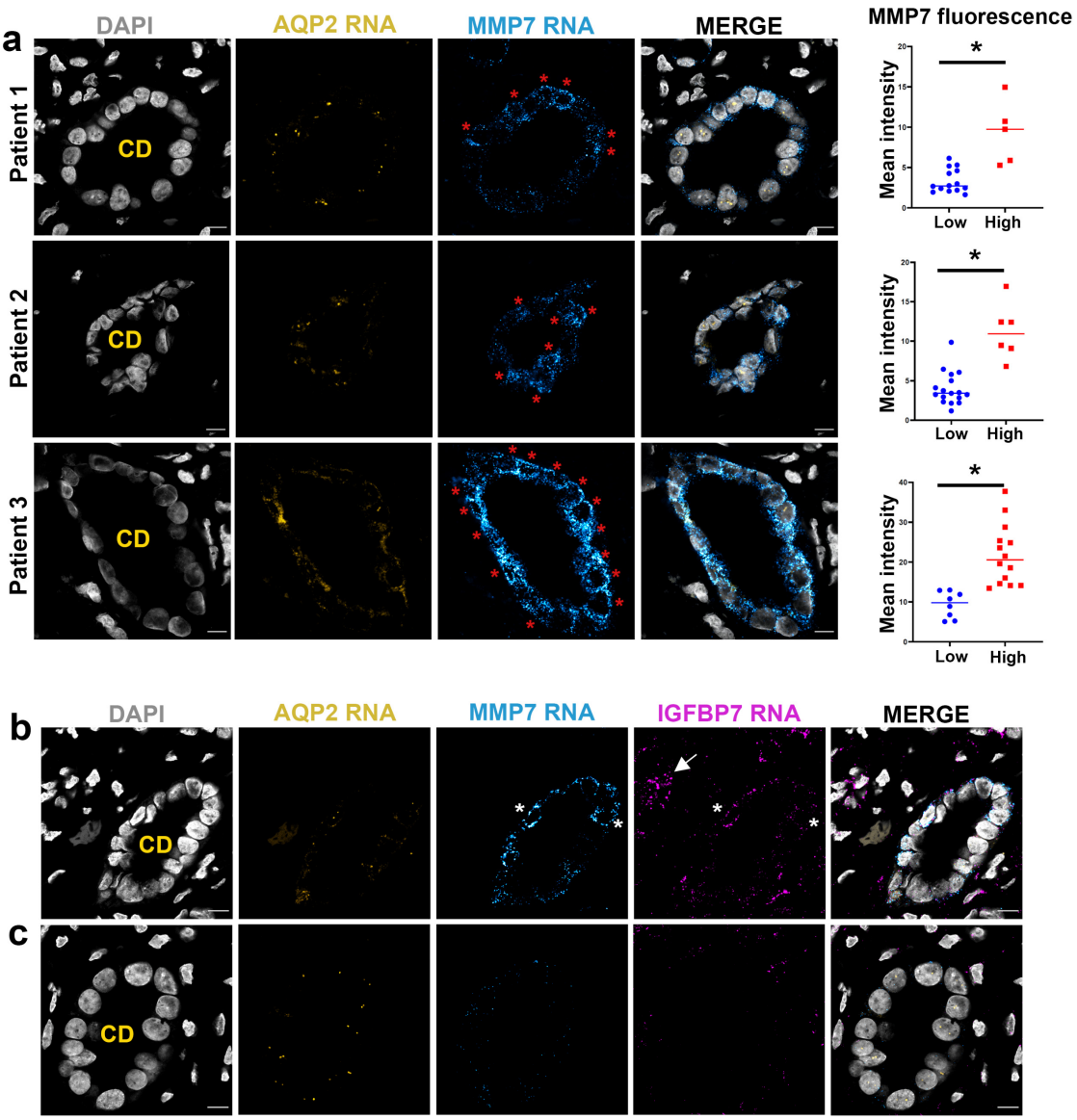
marked by blue asterisks). Many scattered collected duct cells express moderate SLC26A7 along with AQP2 (cells marked by green asterisks). The smFISH data supports that papillary IC cells frequently express AQP2 and is consistent with the snRNAseq profile of papillary ICs for the expression of AQP2 and SLC26A7 in panel (a). The boxed cells in the corresponding merge panels are enlarged in the far right (scale bars = 5 μm in the enlarged boxes).



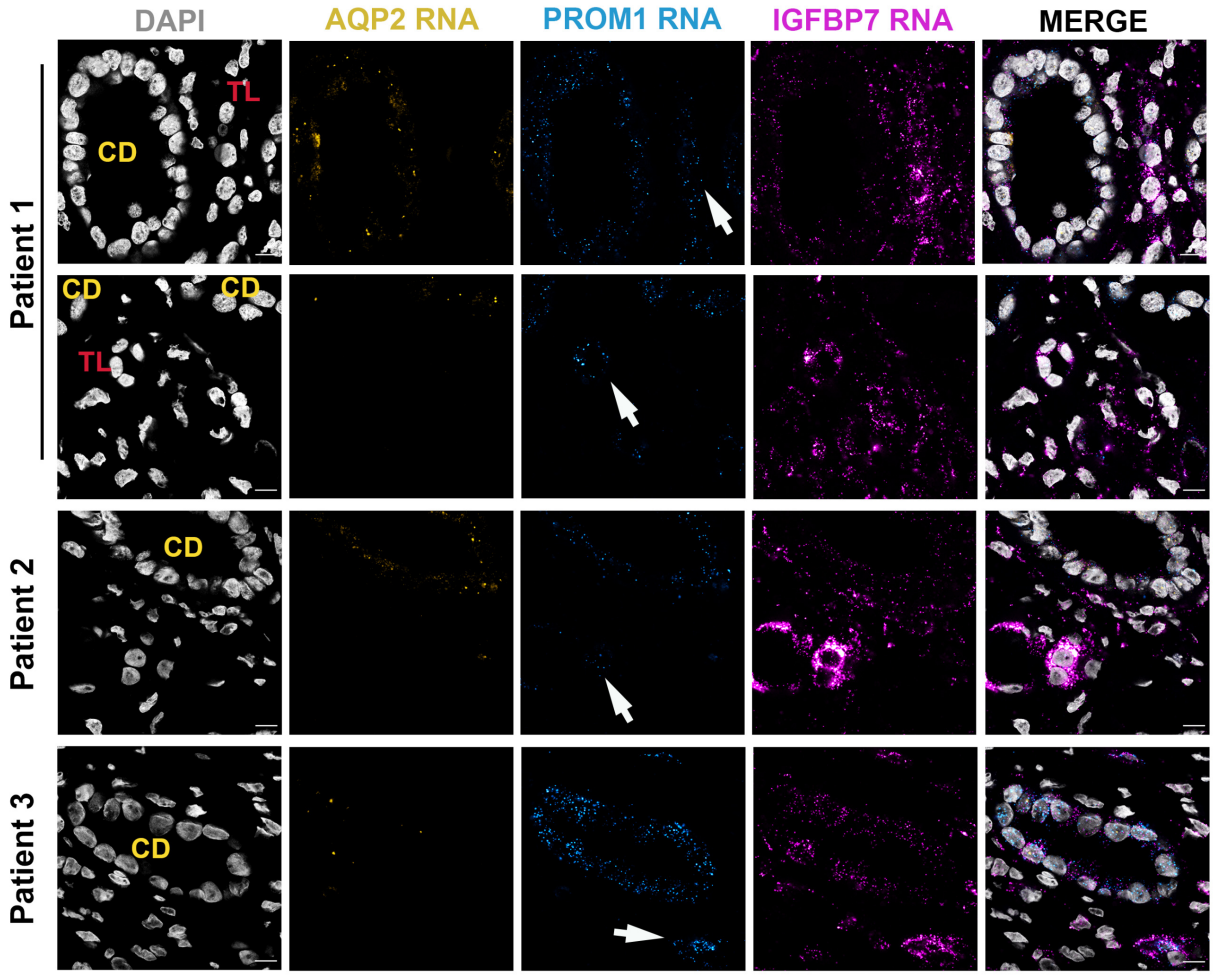
Supplementary Fig. 2: Spatial transcriptomics atlas of the papilla from reference and stone patients- Label transfer and mapping of the snRNAseq cell classes discussed in Figure 1 onto spatial transcriptomic spots in all papilla samples used, with underlying histology shown for each specimen (descending thin limbs- DTL; ascending thin limbs- ATL; thick ascending limbs- TAL; Papillary principal cells- Pap-PC1 and Pap-PC2; intercalated cells-IC; papillary epithelium- PapE; endothelial cells- EC; vascular smooth muscle / pericyte-VSMP; fibroblast-FIB; immune cells-IMM, undifferentiated cells). CaOx= Calcium oxalate. Scale bars = 500 µm.



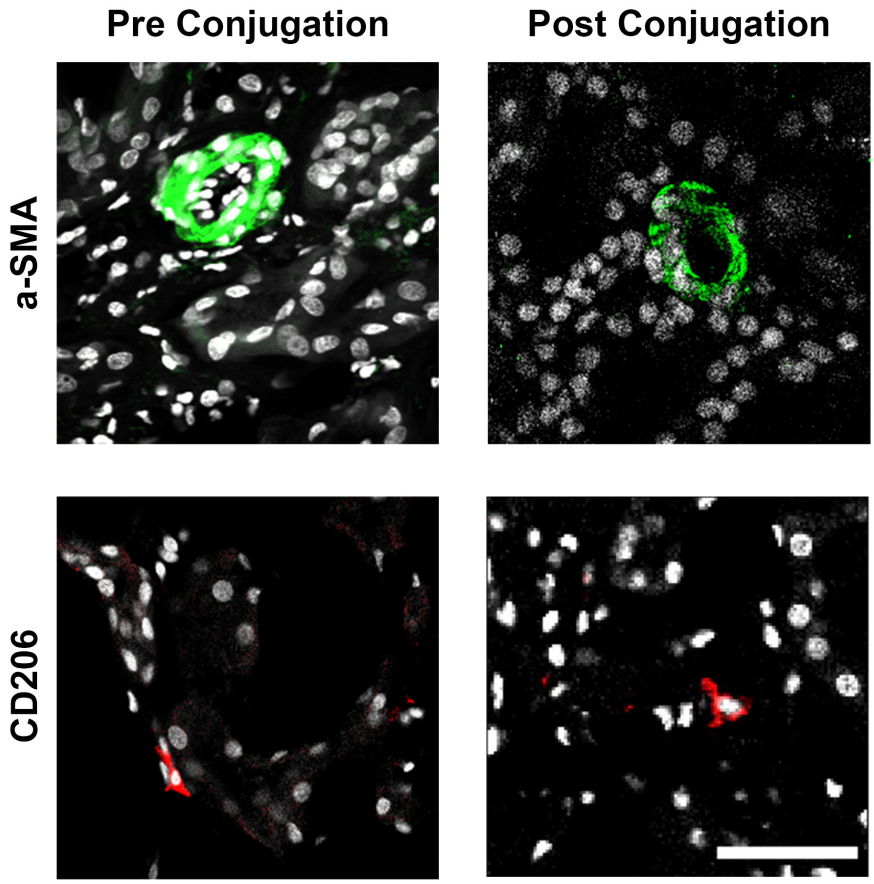
Supplementary Fig. 3: Distribution of various cell signatures for the reference specimen shown in Figure 1. Feature plots showing the mapping of the cell signatures in a representative reference tissue used in Figure 1. (a) shows underlying histology. (b-j) show the distribution of cell signatures indicated in the trop left of each panel. See Supplementary Table 1 for list of abbreviations. Scale bars = 500 µm.



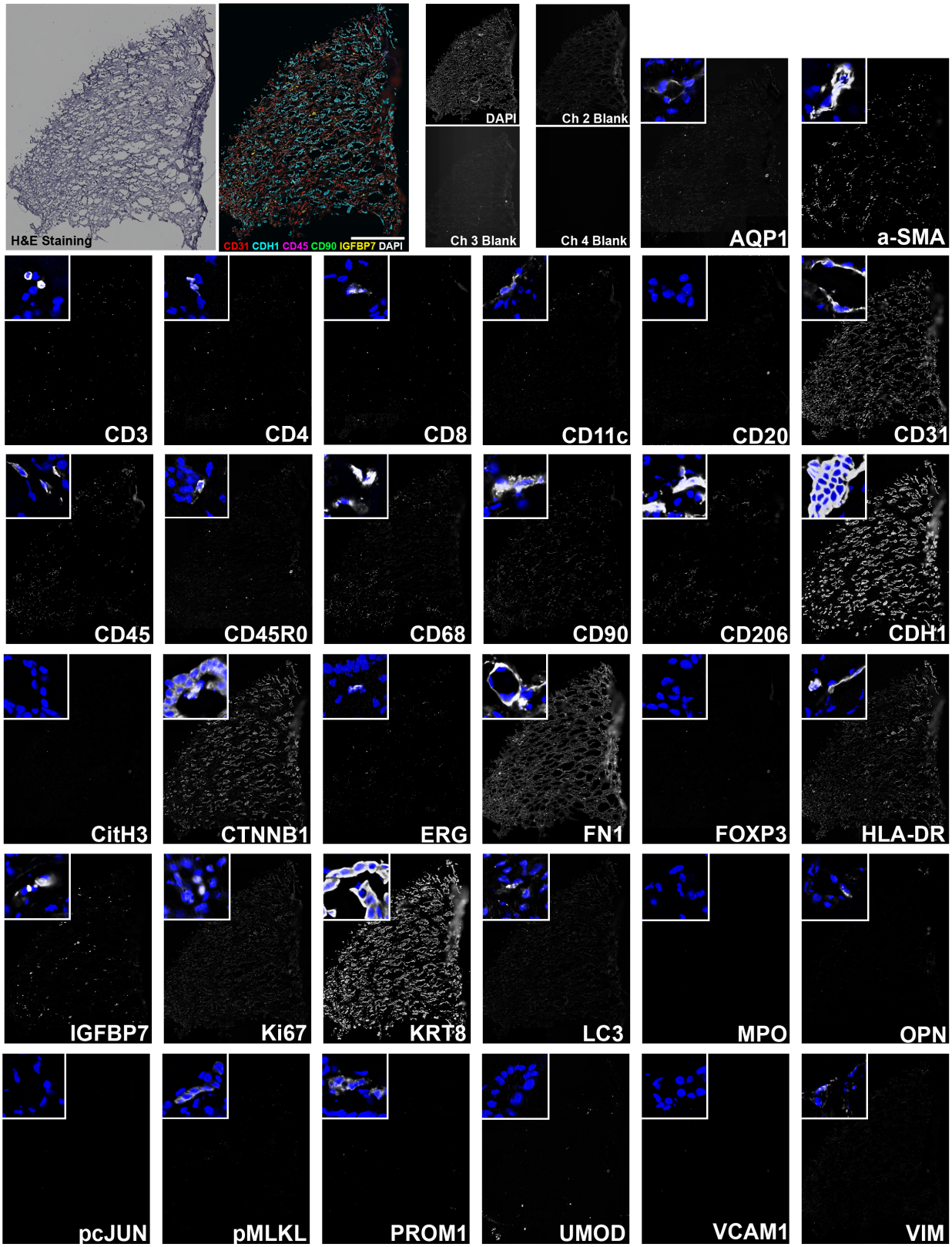
Supplementary Fig. 4: validation of Pap-PC1 and Pap-PC2 cells using single molecular fluorescence in situ hybridization (smFISH) (extended data to Figure 1). (a) smFISH for Aquaporin 2 (*AQP2*) and *MMP7* to distinguish PapPC1 and PapPC2 in papillary specimens from 3 different patients with Calcium oxalate stone disease. Collecting ducts (CD) are morphologically distinct and express *AQP2*. Red asterisks denote cells with high *MMP7* expression which correspond to PapPC2. The *MMP7* fluorescence associated with these cells (n=5 for patient 1; n=6 for patient 2; n= 14 for patient 3) was significantly higher than the rest of the cells (n=14 for patient 1; n=15 for patient 2; n=8 for patient 3) in each corresponding CD (cells manually segmented and fluorescence mean intensity measured using ImageJ. (* statistical significance by two tailed unpaired t-test performed independently for each patient (p= 0.0001 patient1, p< 0.0001 patient 2, p= 0.0003 patient 3)). (b) Few PapPC2 cells (white asterisks) also express *IGFBP7*, which is also consistent with data in Figure 1F. The arrow shows undifferentiated cells outside of CD with high IGFBP7. (c) Collecting duct from the same patient in (b) but different section, not expressing *MMP7* or *IGFBP7*, consisting mostly of PapPC1 cells. Scale bars= 10 μm.



Supplementary Fig. 5: validation of undifferentiated cells using single molecular fluorescence in situ hybridization (smFISH) (extended data to Figure 1). smFISH for *IGFBP7* and *PROM1* was performed on papillary specimens from 3 different patients with Calcium oxalate stone disease. The images highlight undifferentiated cells with high expression of *IGFPB7* and frequently positive for *PROM1* (arrows). These cells localize to the interstitium or in cells with morphology of thin limbs (TL). Scale bars = 10 µm.



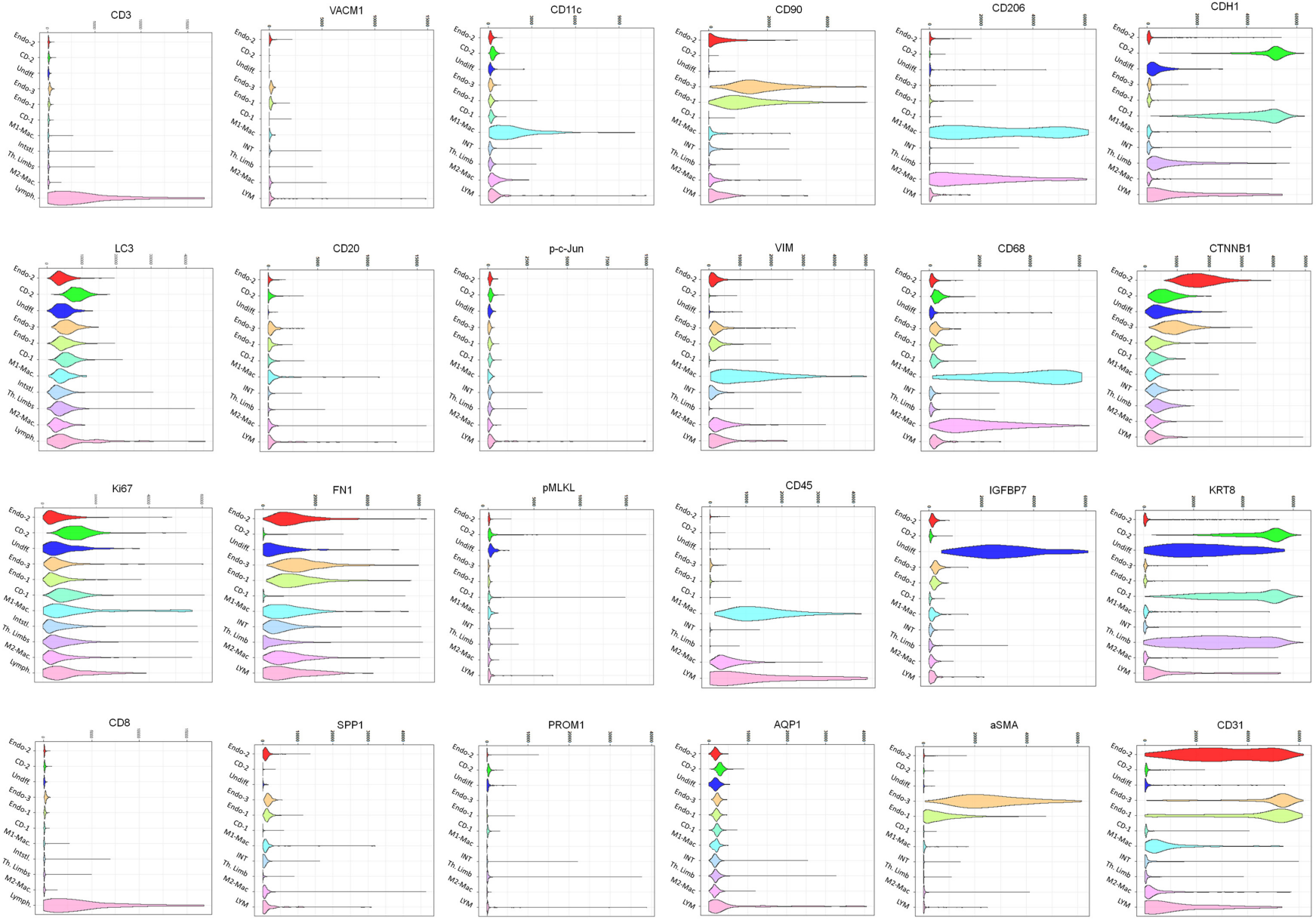
Supplementary Fig. 6: Example of antibody validation before and after conjugation for CODEX imaging. Examples shown here for immunofluorescence staining using antibodies against alpha smooth muscle actin (a-SMA; green), a marker of large vessels and CD206 (in red), a marker of M2 macrophages. Panels on the left represent antibody staining prior to the conjugation of these antibodies to oligonucleotides, while panels on the right represent staining with these antibodies after completed conjugation process. DAPI-stained nuclei are visualized in grey. Scale bar = 50um.


H&E Staining
CD31 CDH1 CD45 CD90 IGFBP7 DAPI
DAPI
Ch 2 Blank
Ch 3 Blank
Ch 4 Blank
AQP1
a-SMA
CD3
CD4
CD8
CD11c
CD20
CD31
CD45
CD45R0
CD68
CD90
CD206
CDH1
CitH3
CTNNB1
ERG
FN1
FOXP3
HLA-DR
IGFBP7
Ki67
KRT8
LC3
MPO
OPN
pcJUN
pMLKL
PROM1
UMOD
VCAM1
VIM

Supplementary Fig. 7: Quality control and cyclical staining of CODEX

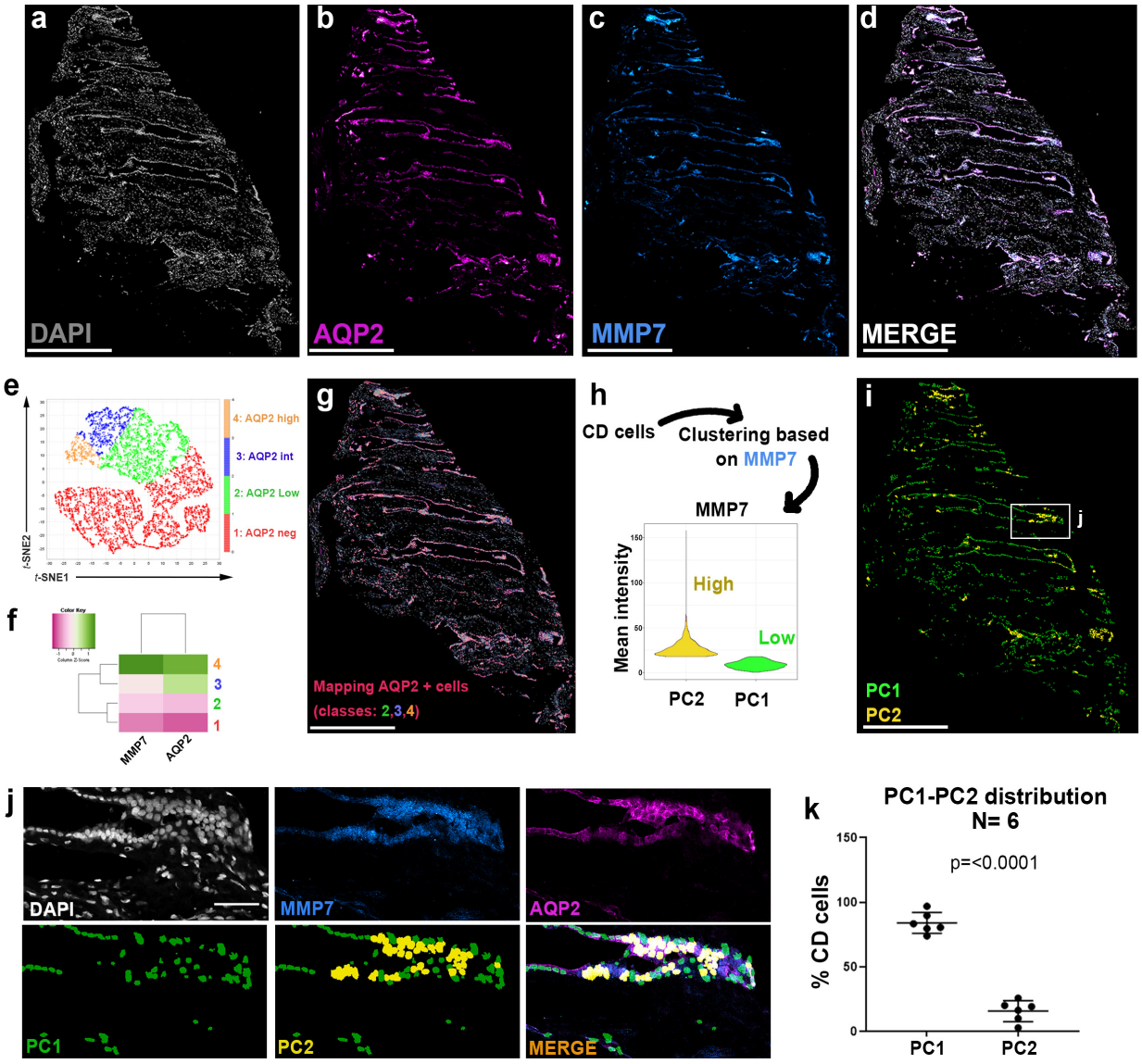
Following antibody binding, CODEX imaging goes through cycles of fluorescent probe labeling-imaging and stripping in 4 channels: DAPI- Green (channel2) red (channel 3) and far red (channel 4).

From upper left to Right: Top left panel shows H&E staining of the section that underwent CODEX staining and imaging. A representative image after CODEX is shown to its right (showing merged stains for endothelial marker CD31, distal nephron and CD marker E-cadherin (CDH1), pan leukocyte marker CD45, followed by the mesenchymal/endothelial cell marker CD90 or THY1,injurt marker IGFBP7 and DAPI for nuclei). This is followed by representative images of DAPI alone, and the blanks from channels 2, 3, and 4 that were used for background subtraction during image processing. The panels shown subsequently represent images of each of the antibodies included in the staining panel across the whole reference tissue. Insets show DAPI in blue and antibody staining in white to show staining patterns at the cellular level. Certain markers (CD20, Citrulline H3, FOXP3, MPO, pcJUN, UMOD, VCAM1) were negative in the reference tissue. Cellular level examples are still shown for these antibodies. Scale bar = 1 mm


CD3
VACM1
CD11c
CD90
CD206
CDH1
LC3
CD20
p-c-Jun
VIM
CD68
CTNNB1
Ki67
FN1
pMLKL
CD45
IGFBP7
KRT8
CD8
SPP1
PROM1
AQP1
aSMA
CD31
Endo-2
CD-2
Undiff.
Endo-3
Endo-1
CD-1
M1-Mac.
Intstl.
Th. Limbs
M2-Mac.
Lymph.
INT
Th. Limb
LYM

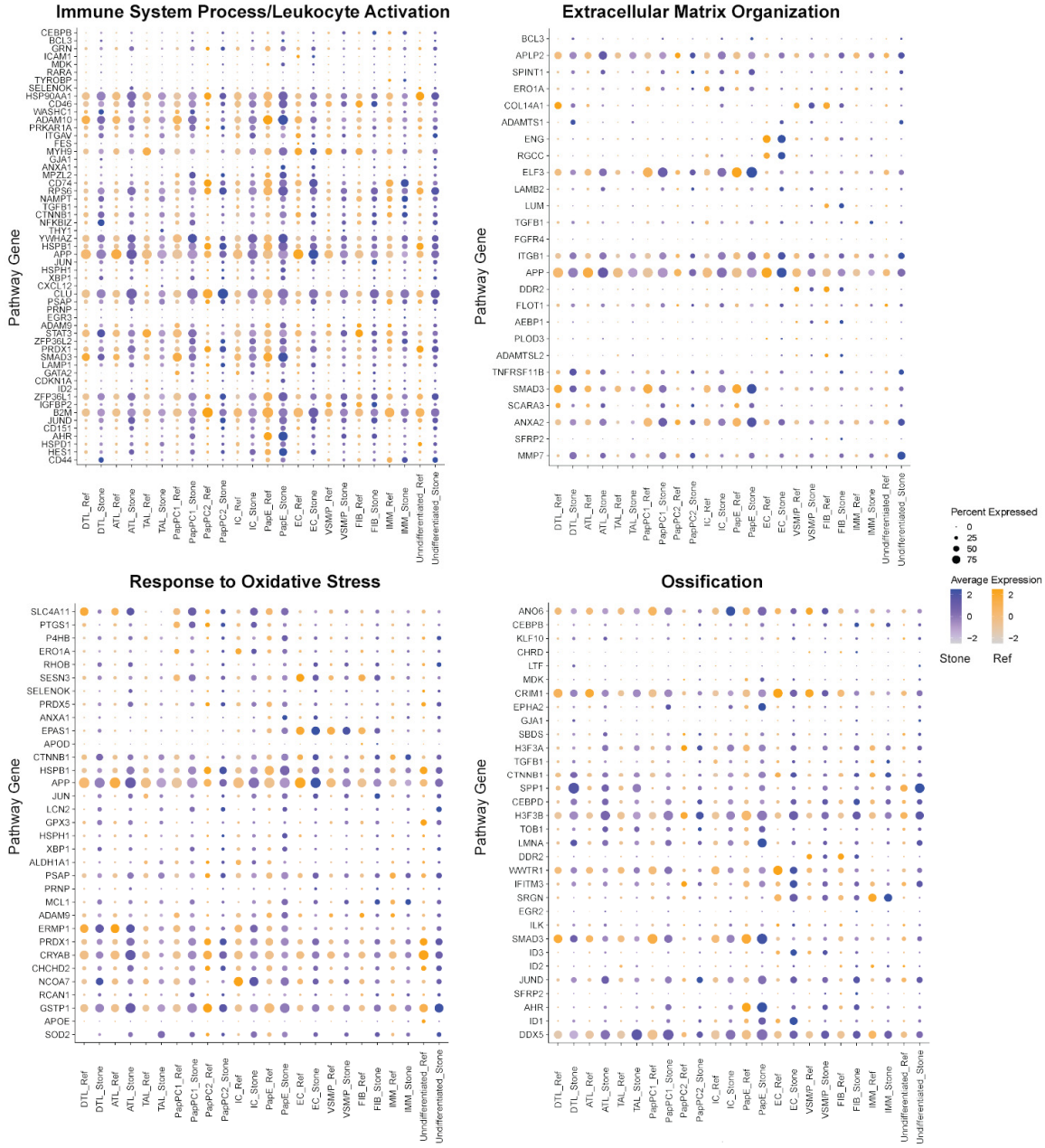
Supplementary Fig. 8: Marker profile for the cell types identified in CODEX analysis in Figure 2

Immunofluorescence intensity profile of the cell classes identified using unsupervised analysis in Figure 2 sorted by cell markers used in CODEX (few markers with low or no signal in the reference tissue were not included). In addition to validating the cell cluster designation based on expression profile, this visualization will also detect any potential cross talk between various markers.

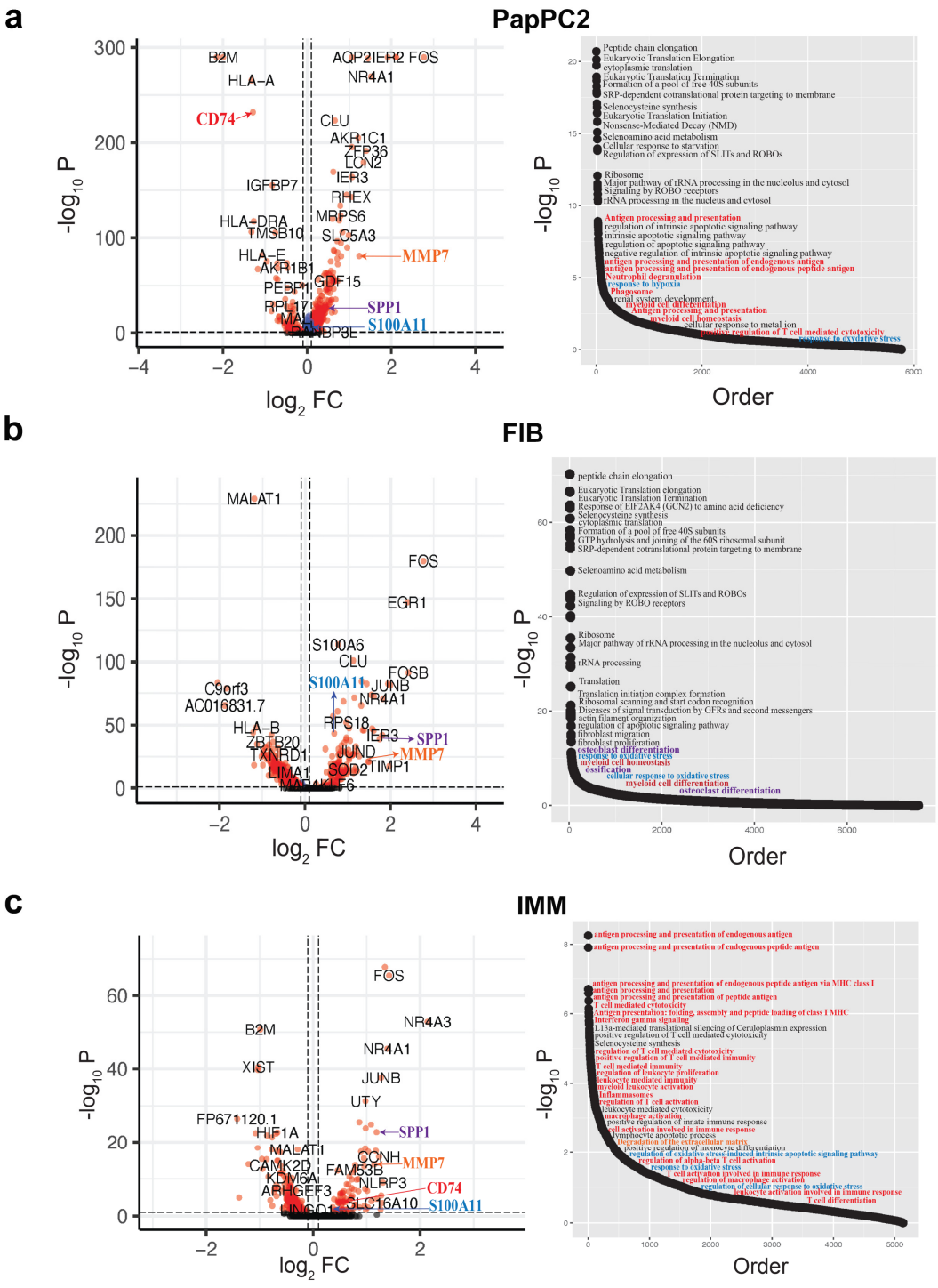


Supplementary Fig. 9: Validation of PapPC1 and PapPC2 cells using large scale 3D imaging and tissue cytometry. (a-d) Representative slices from a confoncal immunofluorescence 3D image volume of a papillary tissue section from a patient with calcium oxalate stone disease stained with AQP2, MPP7 and DAPI. After segmentation with VTEA software, unsupervised analysis was performed based on AQP2 and MMP7 fluorescence (e-f). Collecting duct classes (AQP2+) were distributed in 3 classes based on the fluorescence intensity, and these were mapped for validation in the image volume (g), where nuclear overlays (red) of AQP2+ cells map over AQP2+ collecting ducts. CD cells with high AQP2 expression (class 4) had also high MMP7 expression (f), which is consistent with a PapC2 transcriptomic profile from Figure 1. CD cells were then re-clustered (h) using an unsupervised analysis based on MMP7 fluorescence (MMP7 high and MMP7 low classes), and these were mapped back using nuclear overlays to CDs in the image volume in (i). The boxed area in (i) is enlarged in (j),

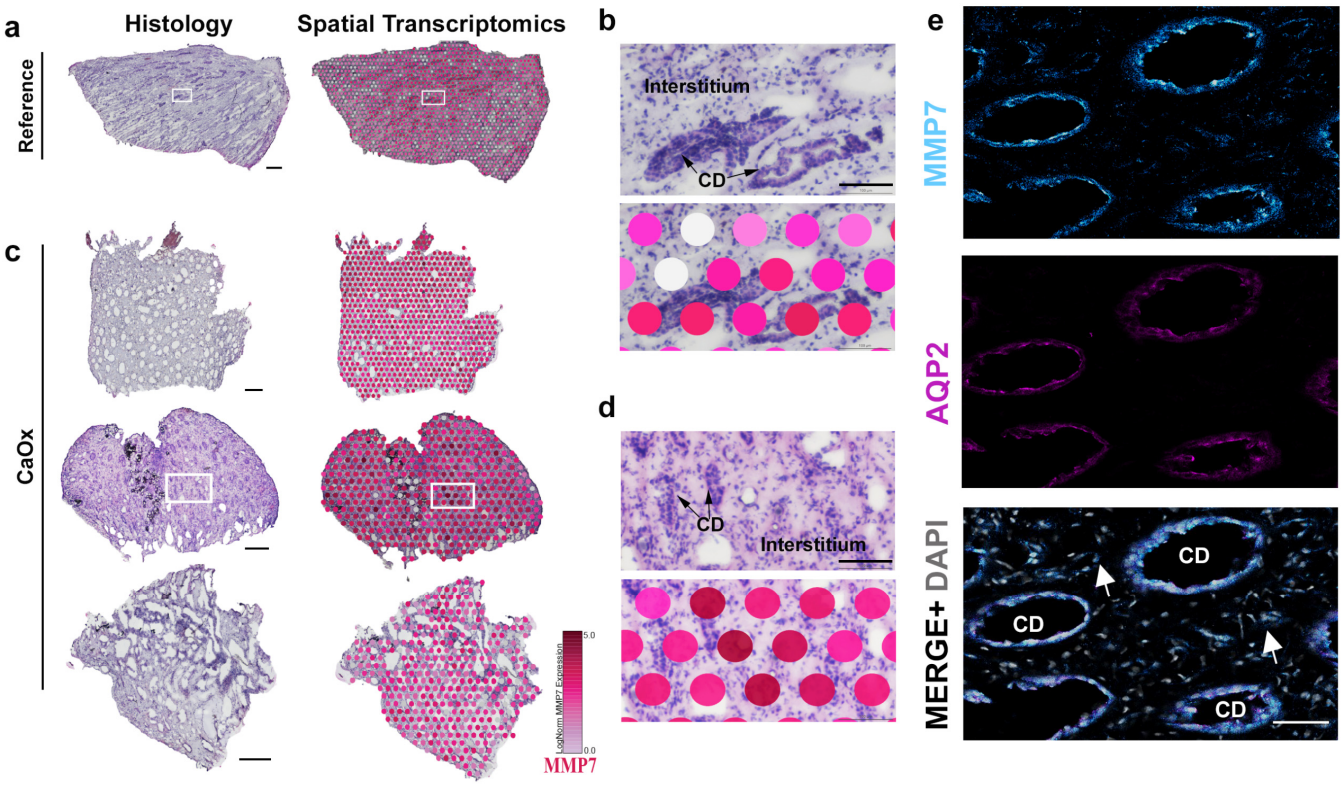
showing the staining of AQP2 and MMP7 and the distribution of PapPC1 and PapPC2 (using nuclear overlays) within on CD. (k) Similar analysis was done on 5 other samples (n=3 reference and n=3 stone independent specimens in total), showing consistent distribution and a higher proportion of PapPC1 across samples ($p < 0.0001$ for a difference in proportions of PapPC1 vs PapPC2 using a two tailed paired test). Scale bars in a-d, g, i = 500 μm. Scale bar in j = 50 um. Mean (large bar) and standard deviation (flanking small bars) are shown for each group in (k).



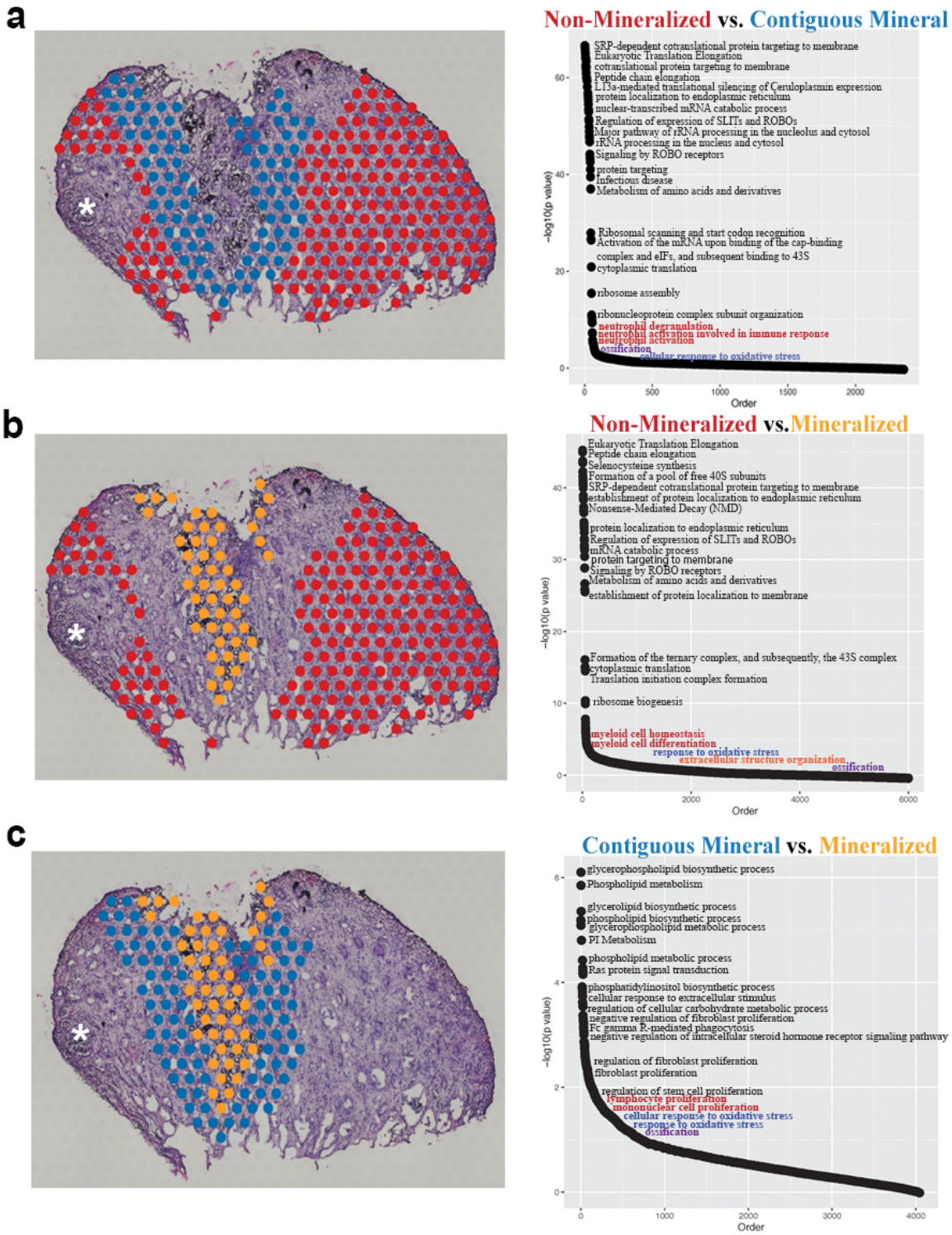
Supplementary Fig. 10: Cell type expression of select genes from pathways enriched in stone versus reference patient papilla biopsies. Genes in relevant pathways (Extracellular Matrix Organization-GO:0030198, Leukoctye Activation-GO:0045321, Ossification-GO:0001503 and Response to Oxidative Stress: GO:0006979) that were significantly increased in all stone biopsies as detected by snRNAseq analysis were compared by dot plot across cell types identified based on their transcriptomic signatures relative to the snRNAseq atlas. Undiff. = undifferentiated; DTL = descending thin limb; ATL = ascending thin limb; TAL = thick ascending limb; PC = principal cell; IC = intercalated cell; PapE = papillary epithelium; EC = epithelial cell; VSM/P = vascular smooth muscle cell; FIB = fibroblast; IMM = immune cell.



Supplementary Fig. 11: Differentially expressed genes (DEGs) and pathways for various cell types in stone vs. reference samples (extended data for Figure 3). (a), (b) and (c) correspond to PapPC2, fibroblasts and immune cells, respectively. Two tailed Wilcoxon rank sum testing was used to define DEGs. Differentially enriched pathways were determined using overrepresentation testing, as described in methods.[3, 4]



Supplemental Figure 12: MMP7 expression in the papilla. Spatial transcriptomics analysis comparing control (Reference) and three different CaOx stone patient biopsies. In reference tissue (a), MMP7 expression is localized predominantly to collecting ducts (CD) (b). In stone disease, MMP7 expression is diffusely increased and encompasses various papillary cells and structures, which is consistent with the snRNAseq expression and the expression signature mapping on ST (Figure 3). Scales bars in (a) and (c): 0.5 mm for reference and top stone sample, 0.25 mm for other 2 specimen; for (b) and (d): 0.1 mm. (e) Immunofluorescence confocal imaging for AQP2 and MMP7 in a papilla sample from a stone patient confirms the predominant localization of MMP7 to CDs. A less intense signal could also be detected in the surrounding interstitium (arrows). Scale bar in (e) = 10 µm



Supplementary Fig. 13: Differentially enriched pathways based on regional analysis and spatial association with mineralization. (a-c) correspond to various comparisons indicated in the top right. Pathways linked to myeloid activation, oxidative stress, matrix remodeling and ossification are colored in red, blue, orange and purple, respectively. Differentially enriched pathways were determined using overrepresentation testing, as described in methods.[3, 4]

Supplementary Table 1- List of abbreviations

TERM	MEANING
CaOX	Calcium oxalate
CD	Collecting duct
IC	Intercalated cell
PC	Principal cell
CODEX	Co-detection by indexing
POD	Podocyte
EC	Endothelial cells
VSM/P	Vascular smooth muscle/pericyte
IMM	Immune
PT	Proximal tubule
PEC	Parietal epithelial cell
DTL	Descending thin limb
ATL	Ascending thin limb
DCT	Distal convoluted tubule
CNT	Connecting tubule
PC	Principal cell
IC	Intercalated cell
PapE	Papillary epithelium
EC	Endothelial cell
FIB	Fibroblast
NEU	Neural cell
PapPC1	Papillary principal cell 1
PapPC2	Papillary principal cell 2
smFISH	Small molecular in situ hybridization
RP	Randall's plaque
AQP	Aquaporin
MMP	Matrix metalloproteinase

Supplementary Table 2: Papilla biopsy usage in spatial assays and post-sequencing quality control

Papilla ID[1]	Age Range	Sex	Tissue source	Group	snRNA seq	ST	Imaging (3D-IF, CODEX, smFISH)[2]	Number of spots	% Mapping to exons	% Mapping under tissue	Data availability transcriptomics	Data Display
20-0031	60-65	M	DecDon	Ref			3D-IF					Suppl. Fig 9
20-0032	46-50	F	DecDon	Ref			3D-IF					Fig 3k Suppl. Fig 9
20-0034	36-40	F	DecDon	Ref		X	3D-IF	2438	0.92	0.83	GSE 231630	Figs 1, 3I, 6, Suppl. Figs 2, 3
20-0045	46-50	M	DecDon	Ref			3D-IF					Fig 3K Suppl. Fig 9
20-0046	46-50	M	DecDon	Ref		X	3D-IF Codex	2468	0.50	0.93	GSE 231630	Figs 2, 3I Suppl. Fig 2
20-0051	56-60	F	DecDon	Ref		X	3D-IF	1386	0.56	0.36	GSE 231630	Fig 6, Suppl. Fig 2
20-0052	61-65	F	DecDon	Ref		X	3D-IF	1376	0.56	0.45	GSE 231630	Fig 6, Suppl. Figs 2, 9
21-0053	30-35	F	DecDon	Ref			3D-IF					Fig 6
K1900387	66-70	M	DecDon	Ref	X						GSE 183279 HuBMAP[3]	Fig 1, Fig 3
K2000094	56-60	F	DecDon	Ref	X						GSE 183279 HuBMAP [4]	Fig 1, Fig 3, Fig 6
K2100041	61-65	M	Nx	Ref	X						HuBMAP [5]	Fig 1, Fig 3
K2100055	61-65	F	Nx	Ref	X						HuBMAP [6]	Fig 1, Fig 3
K2100223	56-60	F	Nx	Ref	X						HuBMAP [7]	Fig 1, Fig 3
KRP428	51-55	F	Biopsy	CaOx		X	3D-IF Codex	817	0.53	0.53	GSE 231630	Fig 3E-H, Fig 3I, Fig4, Fig 5, Suppl. Fig 2
KRP429	36-40	M	Biopsy	CaOx	X	X	3D-IF	1085	0.82	0.79	GSE 231630	Figs 1, 3A-B, 3I, 6, Suppl. Fig 2
KRP436	41-45	M	Biopsy	CaOx			3D-IF					Fig 6
KRP449	61-65	F	Biopsy	CaOx		X	smFISH	939	0.86	0.75	GSE 231630	Fig 3I, Suppl. Figs 1D, 2, 5
KRP446	26-30	M	Biopsy	CaOx	X	X	3D-IF smFISH	656	0.49	0.49	GSE 183279 HuBMAP [8] GSE 231630	Fig 1P, Fig 3I, Suppl. Figs 1D 4A, 4B-C, 5, 9
KRP460	61-65	F	Biopsy	CaOx	X						GSE 183279 HuBMAP [9]	Fig 1, Fig 3
KRP462	66-70	F	Biopsy	CaOx	X	X	3D-IF smFISH	262	0.58	0.34	GSE 183279 HuBMAP [10] GSE 231630	Fig 1, Fig 3A-B, Fig 3I, Suppl. Figs 4A, 5, 9
KRP 463	31-35	F	Biopsy	CaOx			smFISH					Fig 3I, Suppl. Figs 1D, 4A
KRP473	51-55	M	Biopsy	CaOx	X						GSE 231630	Fig 1, Fig 3
KRP475	61-65	F	Biopsy	CaOx		X	3D-IF	229	0.58	0.12	GSE 231630	Fig 3I, Suppl. Figs 2, 9
KRP478			Biopsy	CaOx			3D-IF					Fig 6
K2200020[11]	46-50	M	DecDon	AKI	X						GSE 231630	Fig 1

[1]A medullary sample (K2100202) was added to the kidney atlas in Figure 1 and uploaded to GSE206306 for data availability. The sample was not included in the table because it is not papilla.

[2]All source data for imaging is available at: doi: 10.5281/zenodo.7653239: https://zenodo.org/record/7653239#.ZGIuGc7MKUk

[3-10] Data available through HuBMAP at: [3]https://portal.hubmapconsortium.org/browse/donor/ab8258a97e0820c294d1f0ba2d261f61, [4]https://portal.hubmapconsortium.org/browse/donor/b8f375d33daa5228782abd838d851b8d, [5]https://portal.hubmapconsortium.org/browse/donor/d876de578e9d8c2ce2dcd7c1bbb00681, [6]https://portal.hubmapconsortium.org/browse/donor/9d3c93a2e2d0e81b0f18dd515739a42f, [7]https://portal.hubmapconsortium.org/browse/donor/dfd0352b60504b321899f738c860e3fc, [8]https://portal.hubmapconsortium.org/browse/donor/613776ac210f65a63462ce6f926f4557, [9]https://portal.hubmapconsortium.org/browse/donor/5c4208e2f2715c56ee8dc82b5f1b90c0, 10https://portal.hubmapconsortium.org/browse/donor/e742ac485a0614e4114a26886ed0b444.

[11]Acute Kidney Injury papillary sample included in the cell type mapping but excluded in downstream comparisons between reference and stone papilla.

ST = spatial transcriptomics; 3D-IF = 3-dimensional immunofluorescence imaging, CODEX= codetection by Indexing, smFISH= single molecule fluorescence in situ hybridization, DecDon= Deceased donor, Nx= Tumor nephrectomy, CaOX= Calcium Oxalate stone former, Ref= Reference non-stone former, AKI= Acute Kidney Injury with Blood Urea Nitrogen above 100 mg/dl.

Supplementary Table 3: antibodies used in CODEX assay

Antibody	Significance	Clone	Supplier	Catalog Number
Ki67	Proliferating cells	B56	Akoya	4250019
CD3	Pan T cells	UCHT1	Akoya	4350008
CD4	CD4+ T cells	SK3	Akoya	4350010
CD8	CD8+ t cells	SK1	Akoya	4150004
CD11c	resident dendritic cells	S-HCL-3	Akoya	4350012
CD31	endothelial cells	WM59	Akoya	4250009
CD20	B cells	L26	Akoya	4150018
CD45	pan leukocyte markers	HI30	Akoya	4150003
CD45RO	memory T cells	UCHL1	Akoya	4250023
HLA-DR	antigen presenter cells	L243	Akoya	4250006
CD90 (THY1)	PT, fibroblasts, activated endothelial cells	SE10	Akoya	4150021
E-cadherin (CDH1)	Distal nephron, CD	4A2C7	Akoya	4250021
b-catenin (CTNNB1)	Pan tubular epithelium	12F7	Akoya	4450036
Cytokeratin8 (KRT8)	Loop of Henle, distal nephron and CD	TS1	NovusBio	NBP2-34501-0.1mg
Uromodulin	TAL	Polyclonal	R&D	AF5144
a-sma	myofibroblast, arterioles	1A4	Invitrogen	14-9760-82
PROM1 (CD133)	fibrosis	AC133	Miltenyi Biotec	130-090-422
MPO	neutrophils	Polyclonal	Abcam	ab9535
CD68	activated macrophages	KP1	ThermoFisher	14-0688-82
IGFBP7	injury	Polyclonal	Acris/Origene	AP01109PU-S
p- c-Jun	stress kinase pathway	D47G9	Cell Signalling	3270BF
CD206	M2	Polyclonal	R&D	AF2534

SPP1	Osteopontin /OPN	AKm2A1	Santa Cruz	sc21742
ERG	Endothelial Nuclei	EPR3864	Abcam	ab92513
AQP1	PT, TDL	1/22	Santa Cruz	sc-32737-X
Citruline H3	netosis	7C10	Acris/Origene	AM10179PU-N
Vimentin	Fibroblasts	RV202	BD Pharmingen	550513
FOXP3	injury	236A/E7	Thermo Fisher	14-4777-82
VCAM1	non-repairing epithelial cells	EPR5047	Abcam	ab271899
Phosphor-MLKL	necroptosis	D6H3V (S358)	Cell Signaling	91689BF
Fibronectin	Injury, pre-collagen	F1	Abcam	ab271831
LC3	autophagy	Polyclonal	Sigma Aldrich	L8918-25UL

Antibodies purchased from Akoya were conjugated by vendor. Antibodies from other vendors were conjugated in-house using Akoya conjugation kits as described in methods

Supplementary Table 4: Clinical summary of urine donors for MMP7/9 studies.

	Normal N= 20	**Non-Active SF** N= 18	**Active SF** N= 18	***P***
Age	41 +/- 8.9	39 +/- 9.0	47 +/- 11.9	ns
Sex (% male)	50	67	40	ns
Race (% white, non-hispanic)	100	100	100	ns
EGFR (ml/min)	93.2 +/- 19.7	98.10 +/- 18.9	88.9 +/- 22.8	ns
Serum Creatinine (mg/dl)	0.92 +/- 0.09	0.93 +/- 0.12	1.00 +/- 0.45	ns
Urine Creatinine (mg/dl)	152.3 +/- 64.0	119.9 +/- 57.0	121.3 +/- 74.7	ns
Diabetes (%)	0	0	5.6	N/A
HTN (%)	0	0	0	N/A
Cardiac Disease (%)	0	0	0	N/A

Except for proportions, values represent mean ± standard deviation. Comparisons were done using a two-tailed ANOVA.

EGFR= Estimated glomerular filtration rate

SUPPLEMENTARY REFERENCES

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2. Lake BB*, et al.* An atlas of healthy and injured cell states and niches in the human kidney. *bioRxiv*, 2021.2007.2028.454201 (2021).

3. Yu G, He QY. ReactomePA: an R/Bioconductor package for reactome pathway analysis and visualization. *Mol Biosyst* **12**, 477-479 (2016).

4. Yu G, Wang LG, Han Y, He QY. clusterProfiler: an R package for comparing biological themes among gene clusters. *OMICS* **16**, 284-287 (2012).

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