nature protocols

SUPPLEMENTARY INFORMATION

https://doi.org/10.1038/s41596-019-0206-y

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Qualifying antibodies for image-based immune profiling and multiplexed tissue imaging

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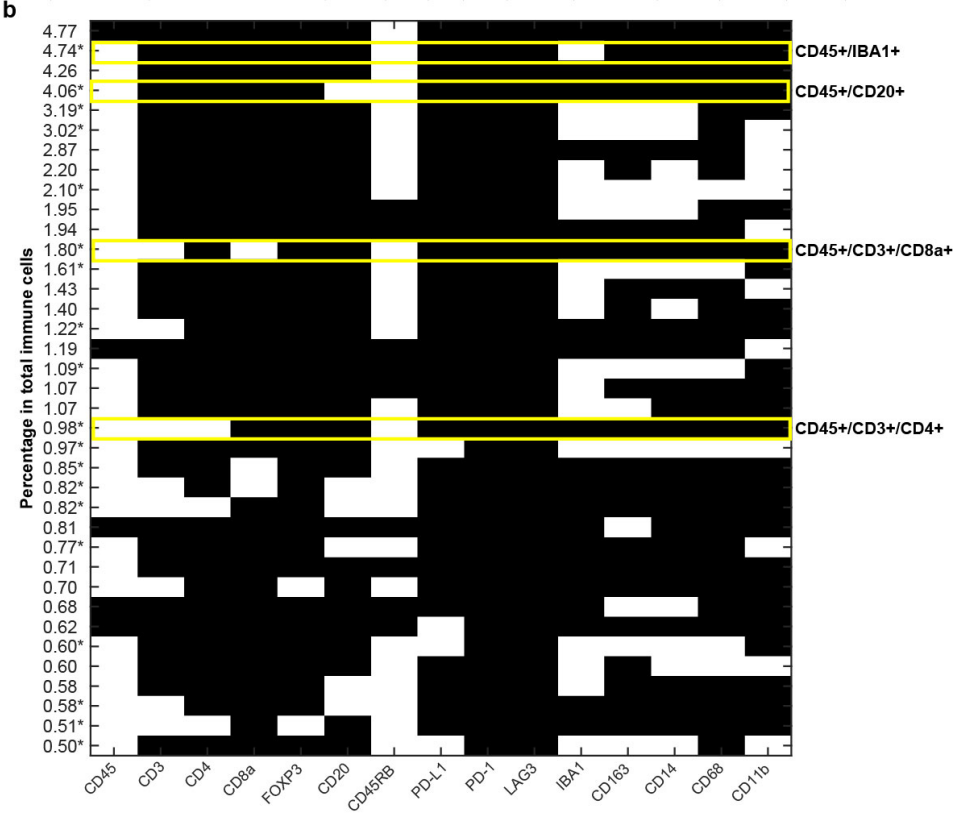
a

Markers	Main cell types	B cells	T Lymphocytes				Myeloid cells		Stromal	Tumor
			CD4+		CD8+					
			Help T	Treg	Cyto T	NKT cells	Macrophage	Dendritic		
CD19	B cells	++								
CD20	B cells	++			+					
LAG3	Checkpoint			++	++	++				
PD1	Checkpoint		+	+	+					
PD-L1	Checkpoint	+	+	+	+	+	++	++	++	++
CD45	Lymphocyte	++	++	++	++	++	++	++		
CD45RB	Lymphocyte	++	+	+	+		++	++		
CD11b	Macrophage	+	+	+	+	++	++	++		
CD14	Macrophage						++	++		
CD163	Macrophage						++	+		
CD68	Macrophage						++	++	+	
IBA1	Macrophage		+	+	+		++	++		+
CD8a	T cell (Cytotoxic)			+	++				++	
CD4	T cell (T helper)		++	++		+	++			
FOXP3	T cell (Treg)			++						
CD3	T cell marker		++	++	++	++				
Ki67	Other(Proliferation)	+	+	+	+	+	++	+	++	++
alpha-SMA	Other(Stromal)								++	
Pan-Keratin	Other(Tumor marker)									++

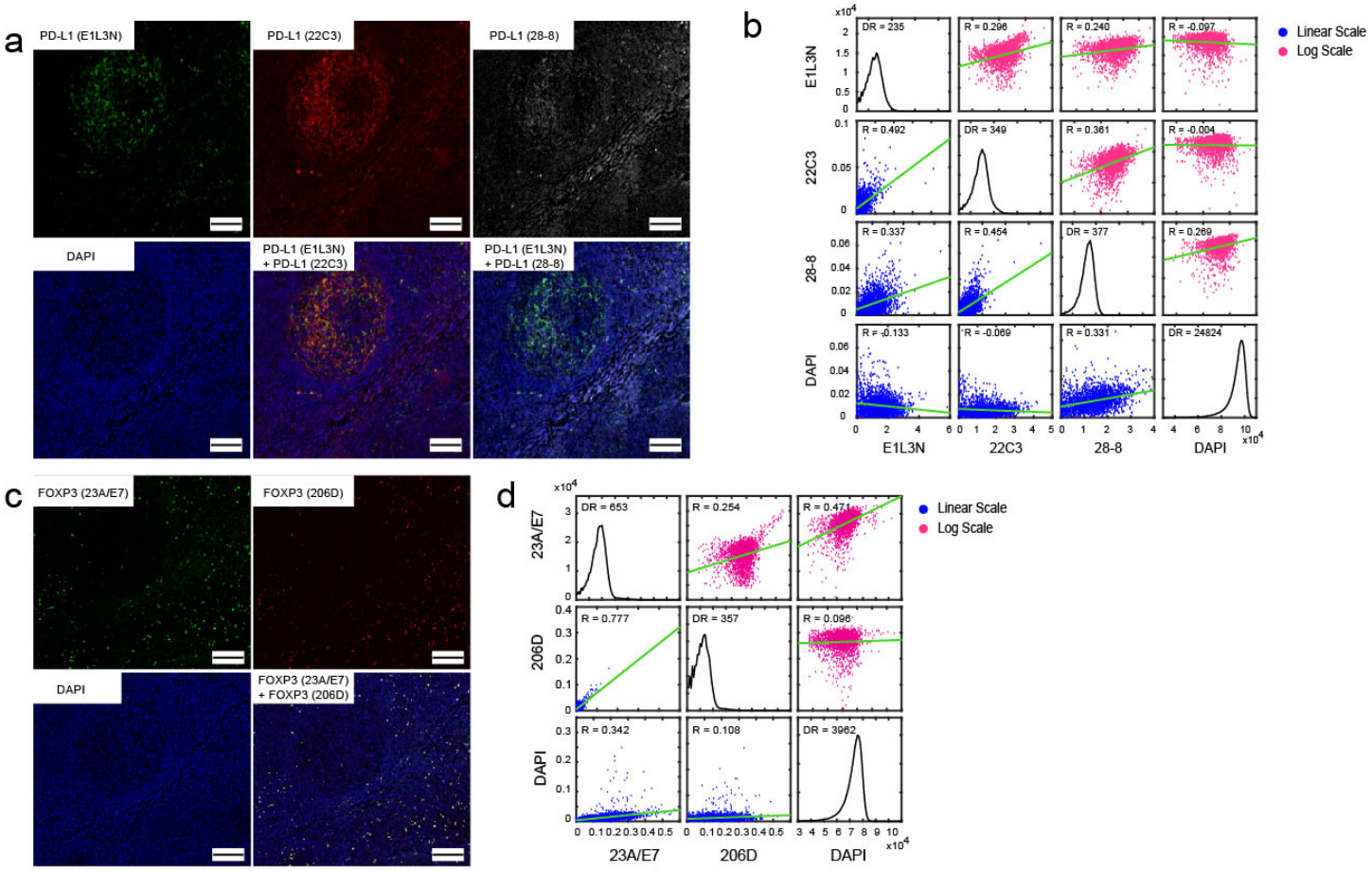
b



Supplementary Figure 1

Markers and major cell types identified by the t-CyCIF immune panel.

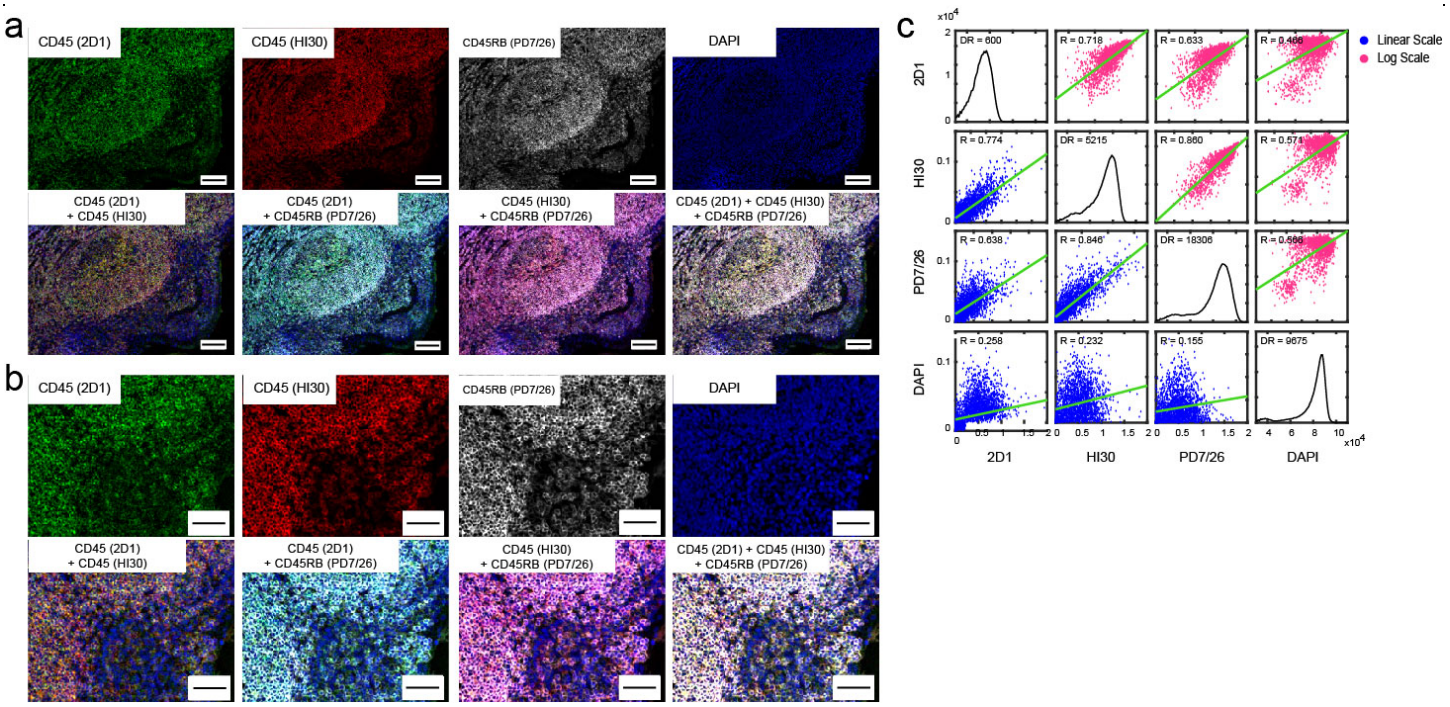
a) Canonical immune cell types and their markers. Seven major immune cell subtypes were assayed using a panel of 16 markers. Three additional markers (Ki-67, α-SMA and pan-keratin) were used to identify cell states (Ki-67 for proliferative cells) or separate immune cells from tumour cells (keratin-positive) or stromal cells (α-SMA-positive). b) Actual immune subpopulation identified from t-CyCIF immune profiling of LUNG-3-PR. 23,079 immune cells (keratin/α-SMA-negative cells) from the sample were used for binary gating of 15 different markers. A total of 1,356 different subpopulations were identified, of which 37 subpopulations represented >0.5% of total immune cells. The four subpopulations highlighted here are CD45+/IBA1+ (macrophage or dendritic cells), CD45+/CD20+ (B cells), CD45+/CD3+/CD8a+ (cytotoxic T cells) and CD45+/CD3+/CD4+ (helper T cells). Asterisks label 19 common immune cell subtypes.



Supplementary Figure 2

Multi-antibody qualification of PD-L1 and FOXP3 antibodies by t-CyCIF in human FFPE tonsil tissue.

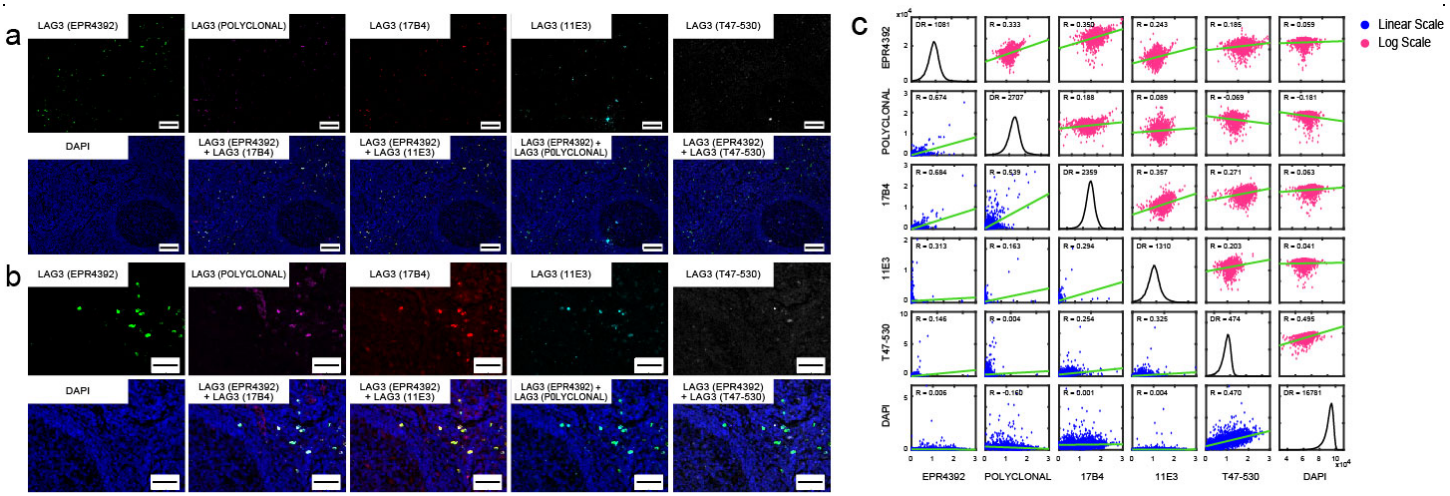
Representative images of immunofluorescence staining from human FFPE tonsil sections using a) three different antibodies for PD-L1: E1L3N, CST; 22C3, DAKO; and 28-8, Abcam (Scale bars: 100 μm). b) Plots of the pixel-by-pixel correlation of the signal intensity generated by the various PD-L1 antibodies. c) Immunofluorescence staining with two different antibodies for FOXP3: 23A/E7, eBioscience; and 206D, BioLegend (Scale bars: 100 μm). d) Plots of the pixel-by-pixel correlation of the signal intensity generated by the various FOXP3 antibodies. The plots in b and d display the correlation of a random sampling of 2,000 pixels. The plots on the lower left (with blue dots) show the original fluorescence intensity at each pixel, and the plots on the upper right (with cyan dots) show the log-transformed fluorescence intensity at each pixel. The Pearson correlation coefficients (R) are shown. DR, dynamic range. The dynamic range and correlation values from 5 different regions are presented in Supplementary Table 1.



Supplementary Figure 3

Multi-antibody qualification of CD45 antibodies by t-CyCIF in human FFPE tonsil tissue.

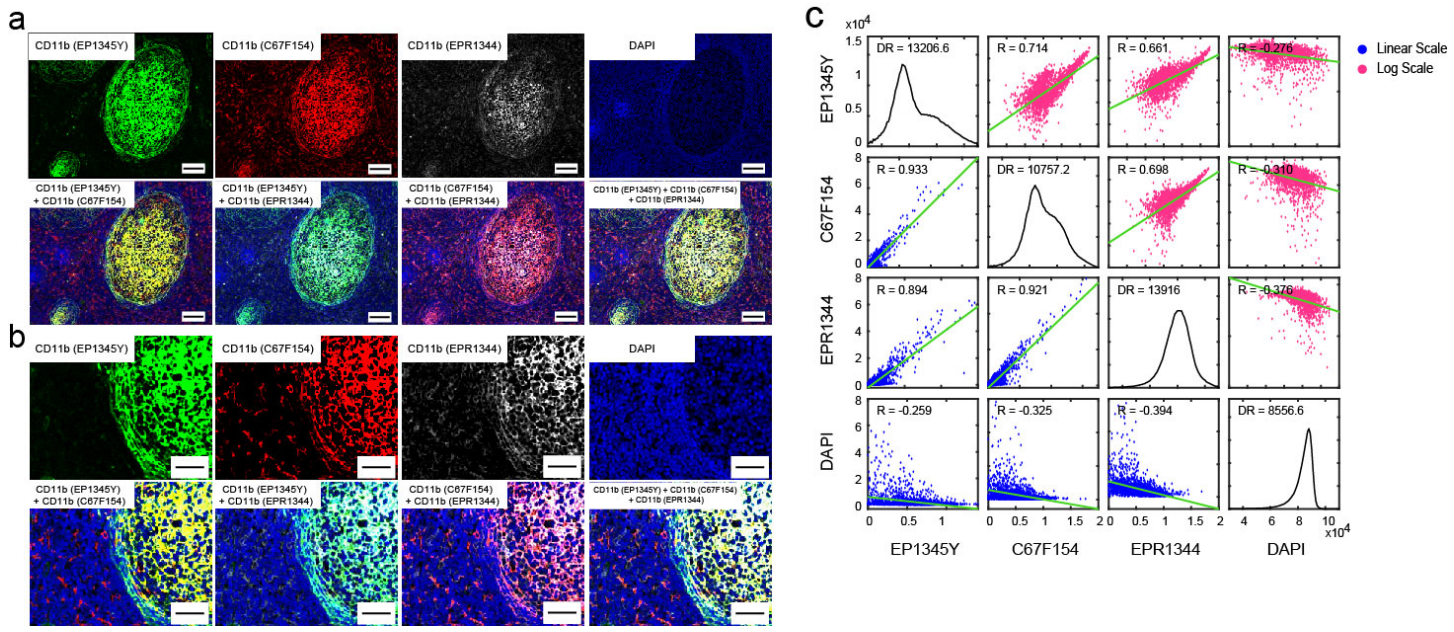
Individual and merged a) low (Scale bars: 100 µm) and b) high (Scale bars: 50 µm) magnification images of immunofluorescence from three CD45 antibodies: 2D1, R&D; HI30, BioLegend; and PD7/26, eBioscience. c) Plots of the pixel-by-pixel correlation of the signal intensity generated by these CD45 antibodies. The plots display the correlation of a random sampling of 2,000 pixels. The plots on the lower left (with blue dots) show the original fluorescence intensity at each pixel, and the plots on the upper right (with cyan dots) show the log-transformed fluorescence intensity at each pixel. The Pearson correlation coefficients (R) are shown. DR, dynamic range. The dynamic range and correlation values from 5 different regions are presented in Supplementary Table 1.



Supplementary Figure 4

Multi-antibody qualification of LAG3 antibodies by t-CyCIF in human FFPE tonsil tissue.

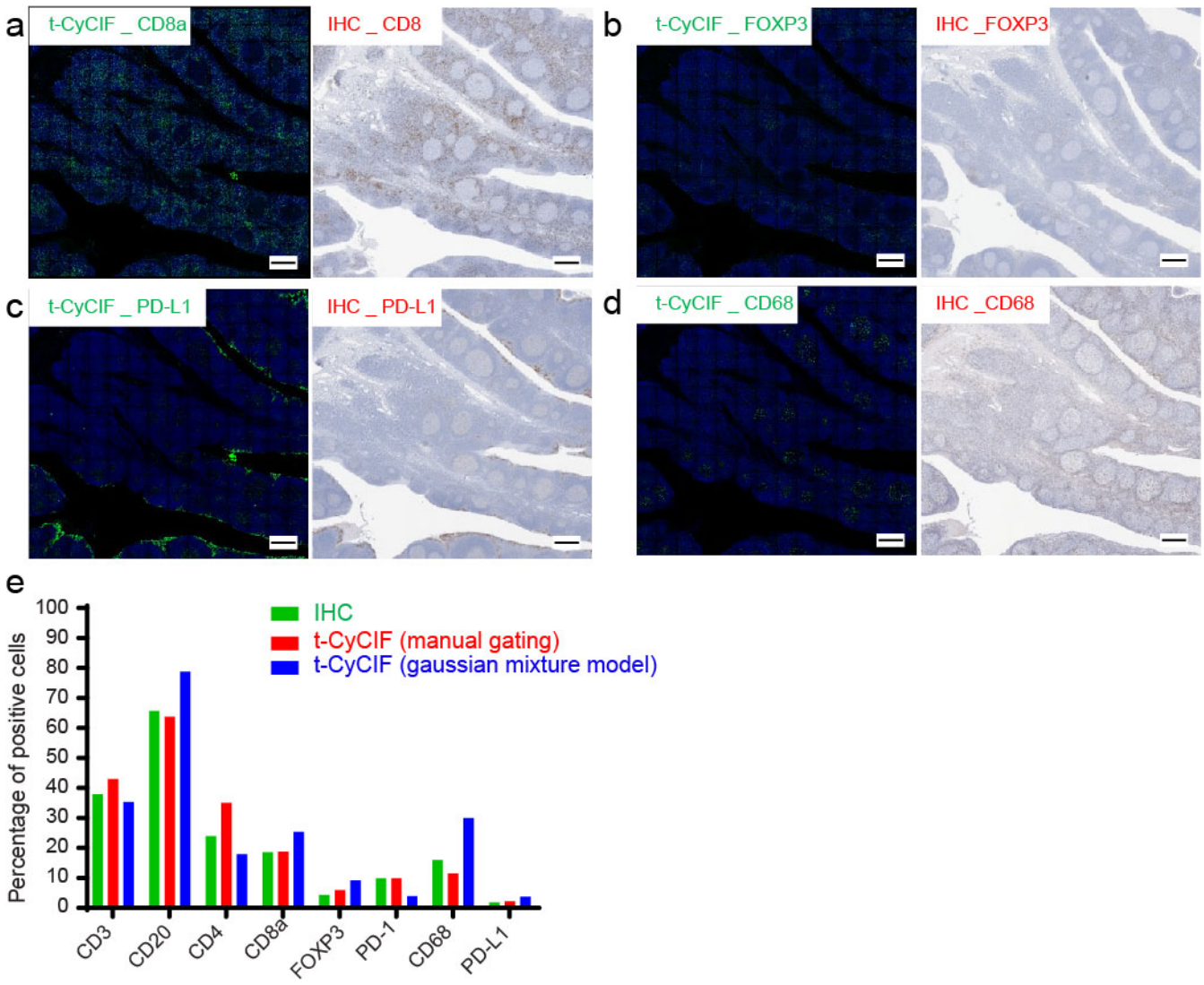
Individual and merged a) low (Scale bars: 100 µm) and b) high (Scale bars: 50 µm) magnification images of immunofluorescence from five LAG3 antibodies: EPR4392, Abcam; Polyclonal, R&D; 17B4, Lifespan; 11E3, Abcam; and T47-530, BD Bioscience. c) Plots of the pixel-by-pixel correlation of the signal intensity generated by the various LAG3 antibodies. The plots display the correlation of a random sampling of 2,000 pixels. The plots on the lower left (with blue dots) are of the original fluorescence intensity at each pixel, and the plots on the upper right (with cyan dots) are of the log-transformed fluorescence intensity at each pixel. The Pearson correlation coefficients (R) are shown. DR, dynamic range. The dynamic range and correlation values from 5 different regions are presented in Supplementary Table 1.



Supplementary Figure 5

Multi-antibody qualification of CD11b antibodies by t-CyCIF in human FFPE tonsil tissue.

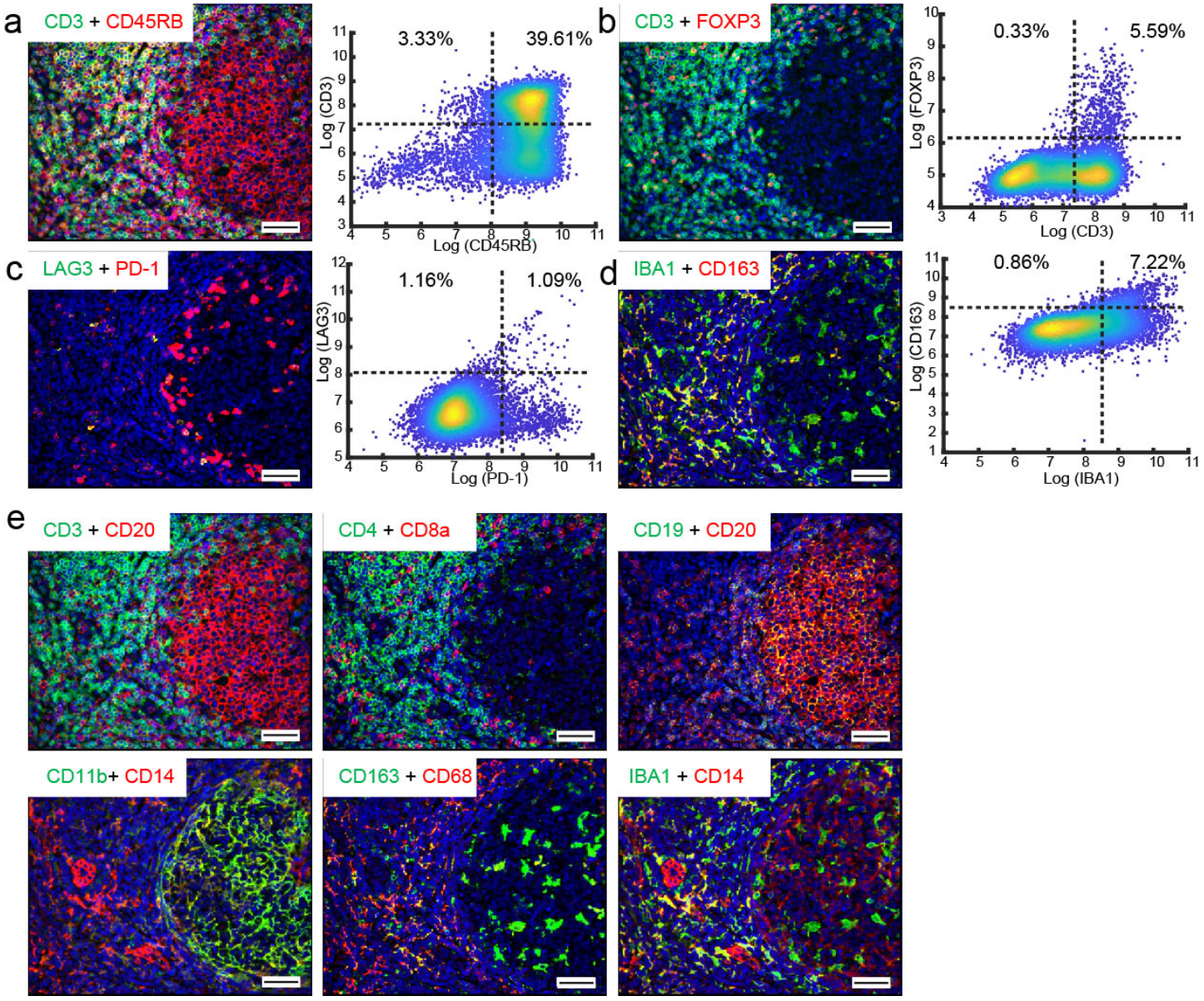
Individual and merged a) low (Scale bars: 100 µm) and b) high (Scale bars: 50 µm) magnification images of immunofluorescence from three CD11b antibodies: EP1345Y, Abcam; C67F154, eBioscience; and EPR1344, Abcam. c) Plots of the pixel-by-pixel correlation of the signal intensity generated by the CD11b antibodies. The plots display the correlation of a random sampling of 2,000 pixels. The plots on the lower left (with blue dots) show the original fluorescence intensity at each pixel, and the plots on the upper right (with cyan dots) show the log-transformed fluorescence intensity at each pixel. The Pearson correlation coefficients (R) are shown. DR, dynamic range. The dynamic range and correlation values from 5 different regions are presented in Supplementary Table 1.



Supplementary Figure 6

Multi-antibody qualification by t-CyCIF and IHC in human FFPE tonsil tissue.

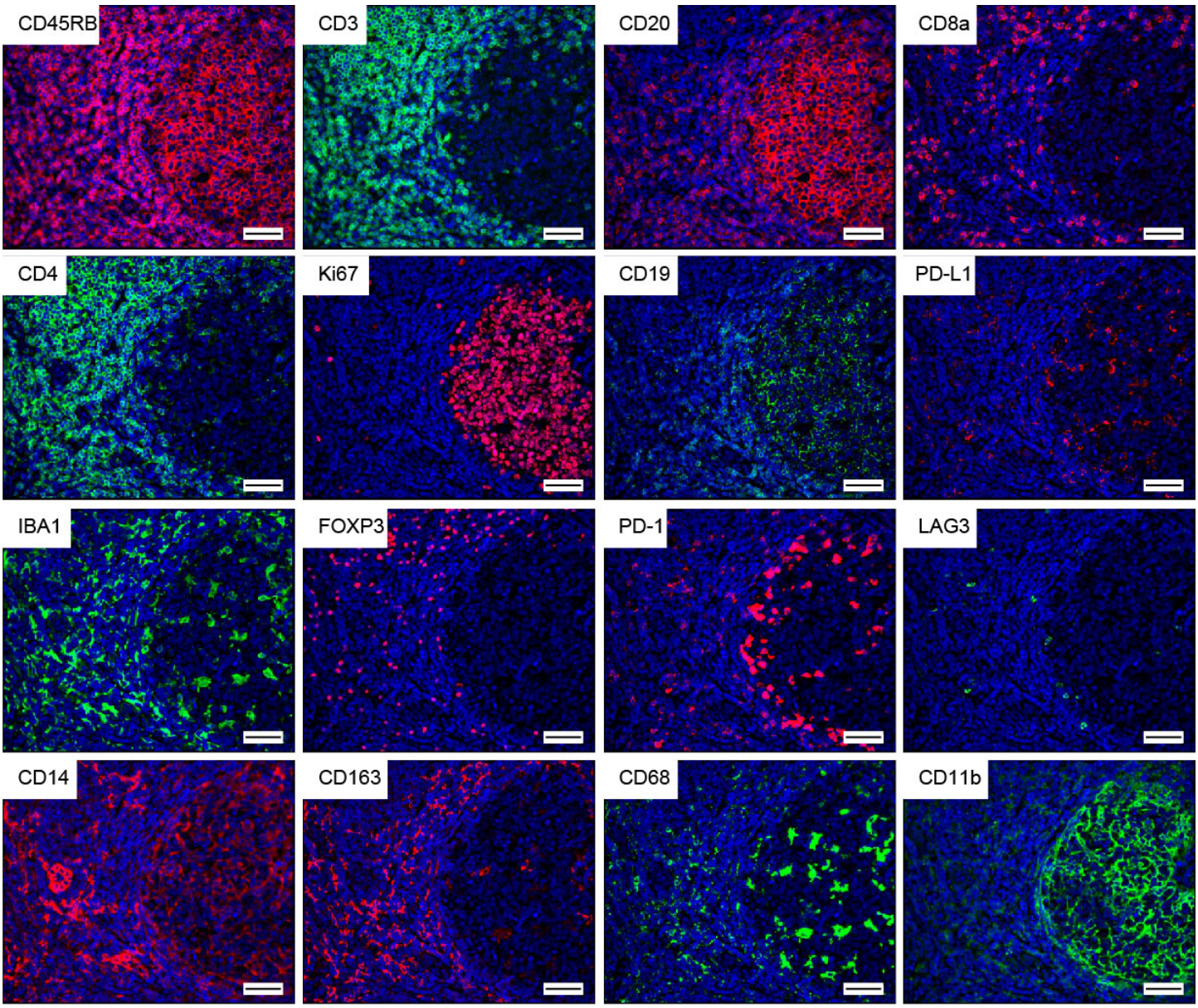
Representative images of t-CyCIF (left) and IHC staining (right) using antibodies to a) CD8a, b) FOXP3, c) PD-L1, and d) CD68 (Scale bars: 500 μm). e) Bar plot of the percentage of total immune cells in the tonsil section that were positive for the specified antibodies. The estimated number of positive cells was determined by both manual gating and by GMM analysis. The plot includes the percentage of positive cells for each immune antibody by IHC, as counted using Aperio ImageScope software.



Supplementary Figure 7

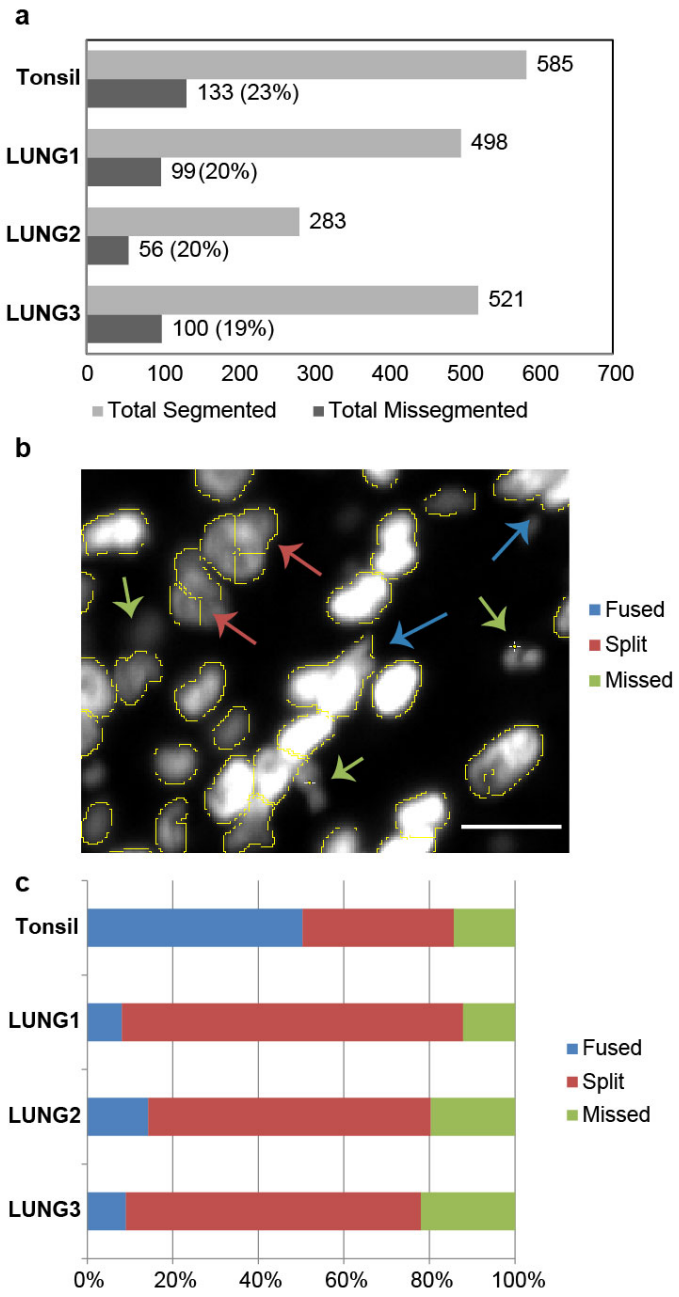
Immune profiling in human FFPE tonsil tissue by t-CyCIF.

Merged images of t-CyCIF data (left) and scatter plots (right) from a random sampling of 10,000 cells for a) CD3 and CD45RB, b) CD3 and FOXP3, c) LAG3 and PD-1, and d) IBA1 and CD163. e) Additional merged images for CD3 and CD20, CD4 and CD8a, CD19 and CD20, CD11b and CD14, CD163 and CD68, and IBA1 and CD14 (Scale bars: 50 μm).



Supplementary Figure 8

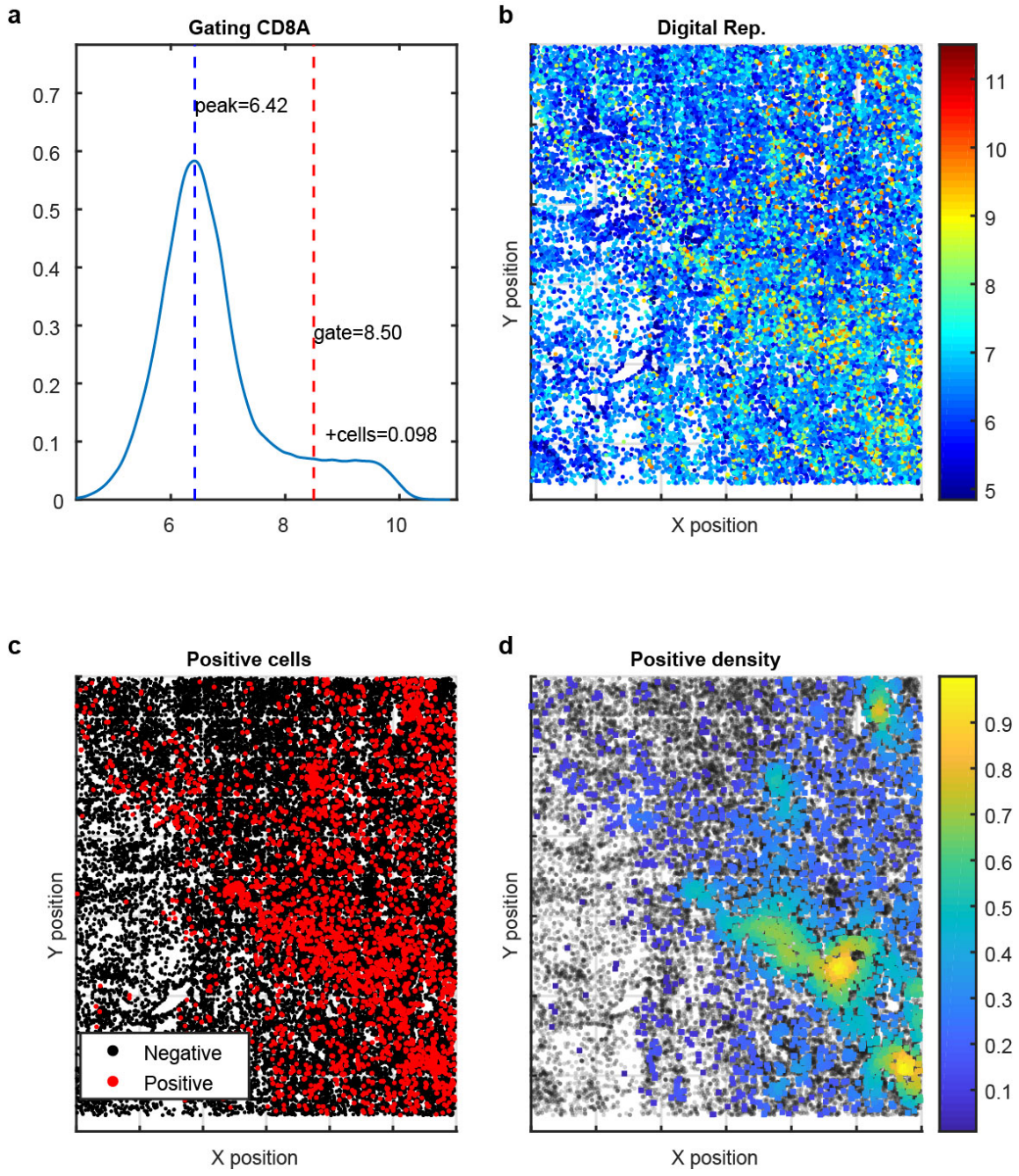
Individual t-CyCIF images for 16 different immune markers in human FFPE tonsil tissue. Scale bars: 50 μm.



Supplementary Figure 9

Evaluation of segmentation accuracy and error composition.

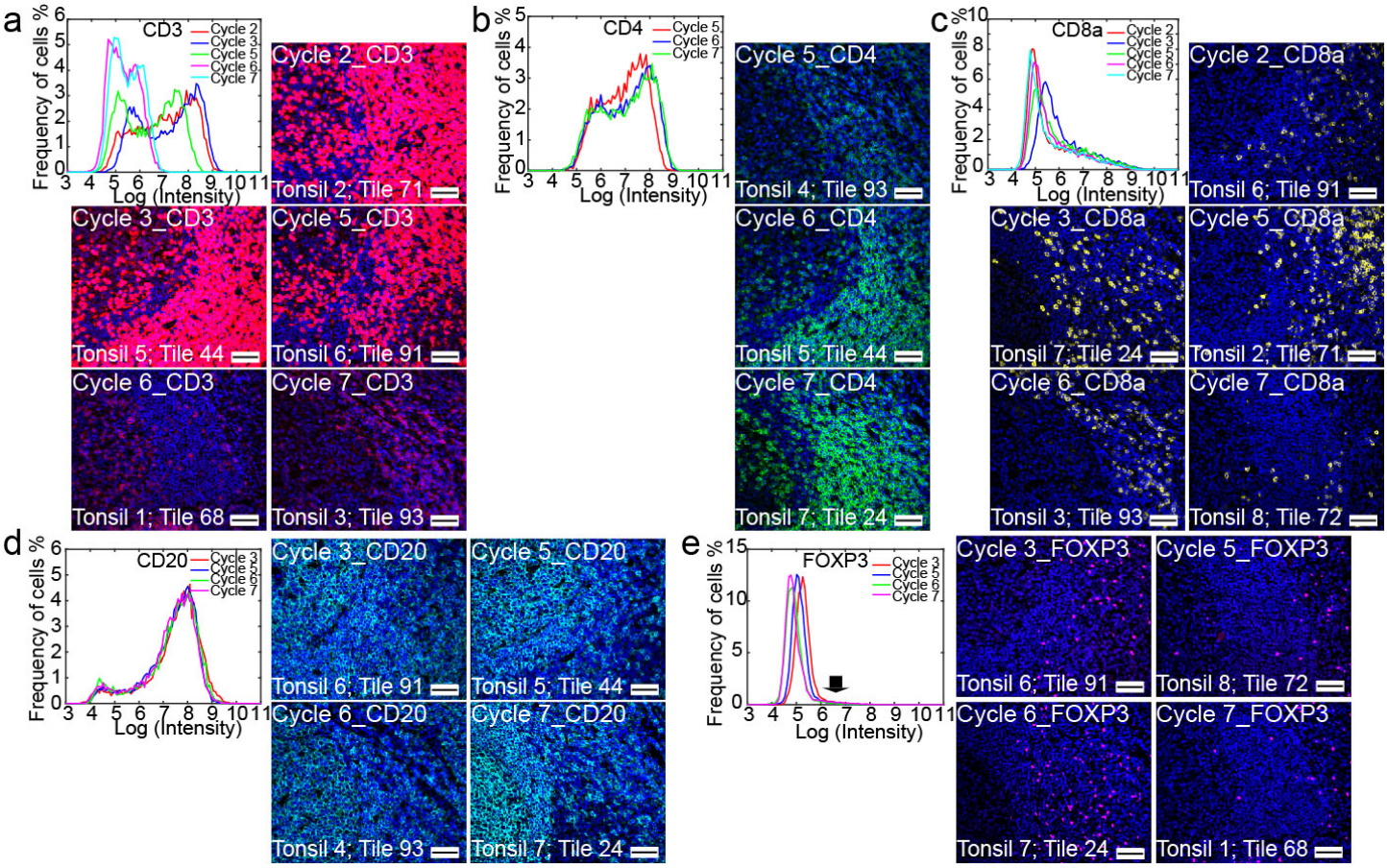
a) Overall error rates of segmentation. Several hundred segmented masks were validated by human review, and the errors versus total counts of segmented cells from four different samples (tonsil, LUNG-1-LN, LUNG-2-BR and LUNG-3-PR) are plotted here. The average error rate was ~20% (S.E. 2%) for each sample. b) Illustration of the types of errors encountered. An example single-channel grey-scale Hoechst image is shown, with segmented masks highlighted in yellow. Three major types of segmentation errors were found: fused (blue arrows), split (red arrows) and missed (green arrows) cells (Scale bar: 20 μm). c) The composition of the different types of segmentation errors from the four samples.



Supplementary Figure 10

Illustration of manual inspection and gating of intensity data.

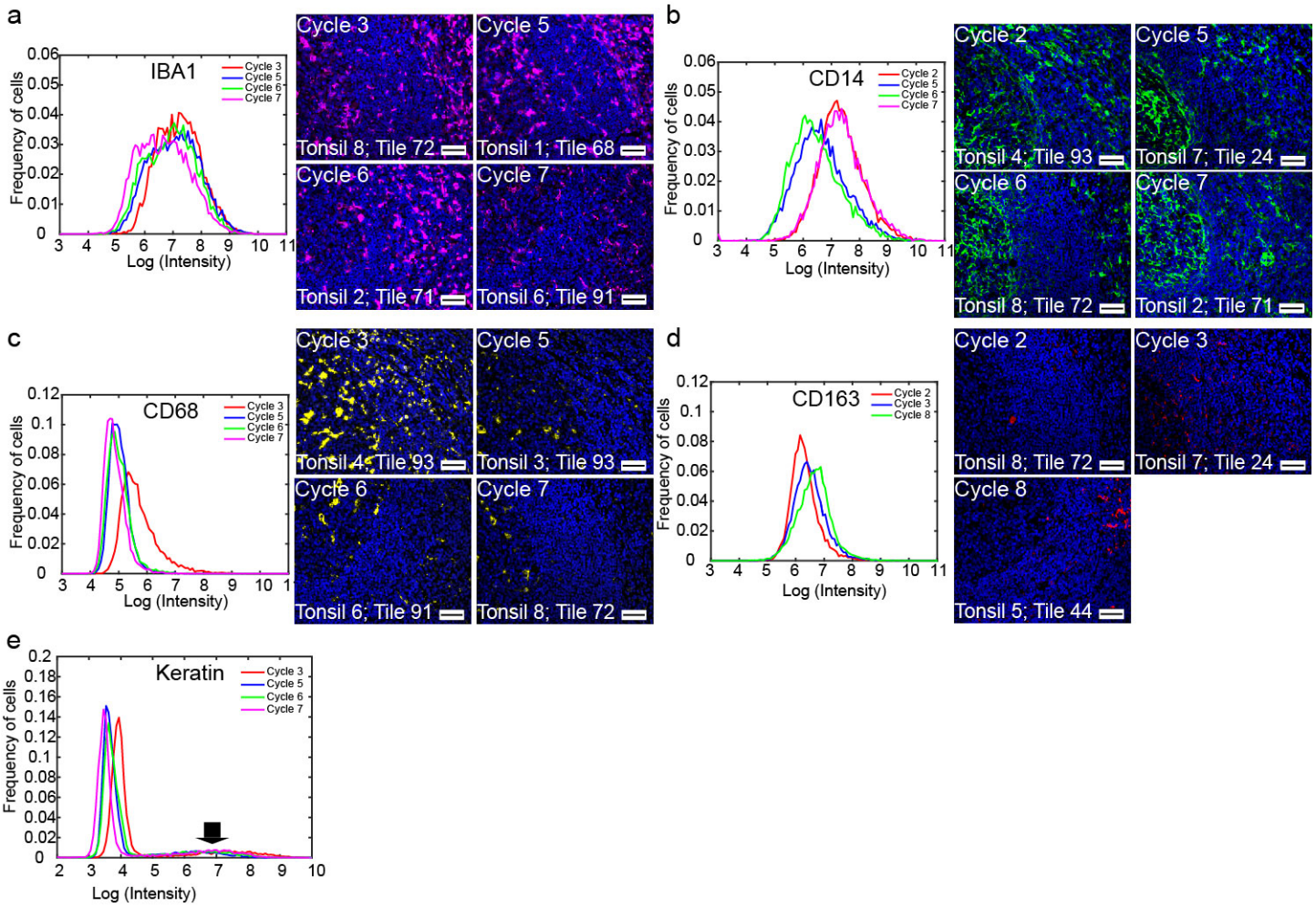
a) Histogram of single-cell intensity from CD8a staining in LUNG-3-PR. Human inspection of this signal profile was used to set the gate/threshold at 8.5 log. The digital representation of the same intensity data was projected into physical maps of b) the original intensity data in log scale, c) data with all positive cells coloured in red, and d) the relative density of positive cells.



Supplementary Figure 11

Analysis of effect of t-CyCIF cycle number on antigenicity in human FFPE tonsil tissue.

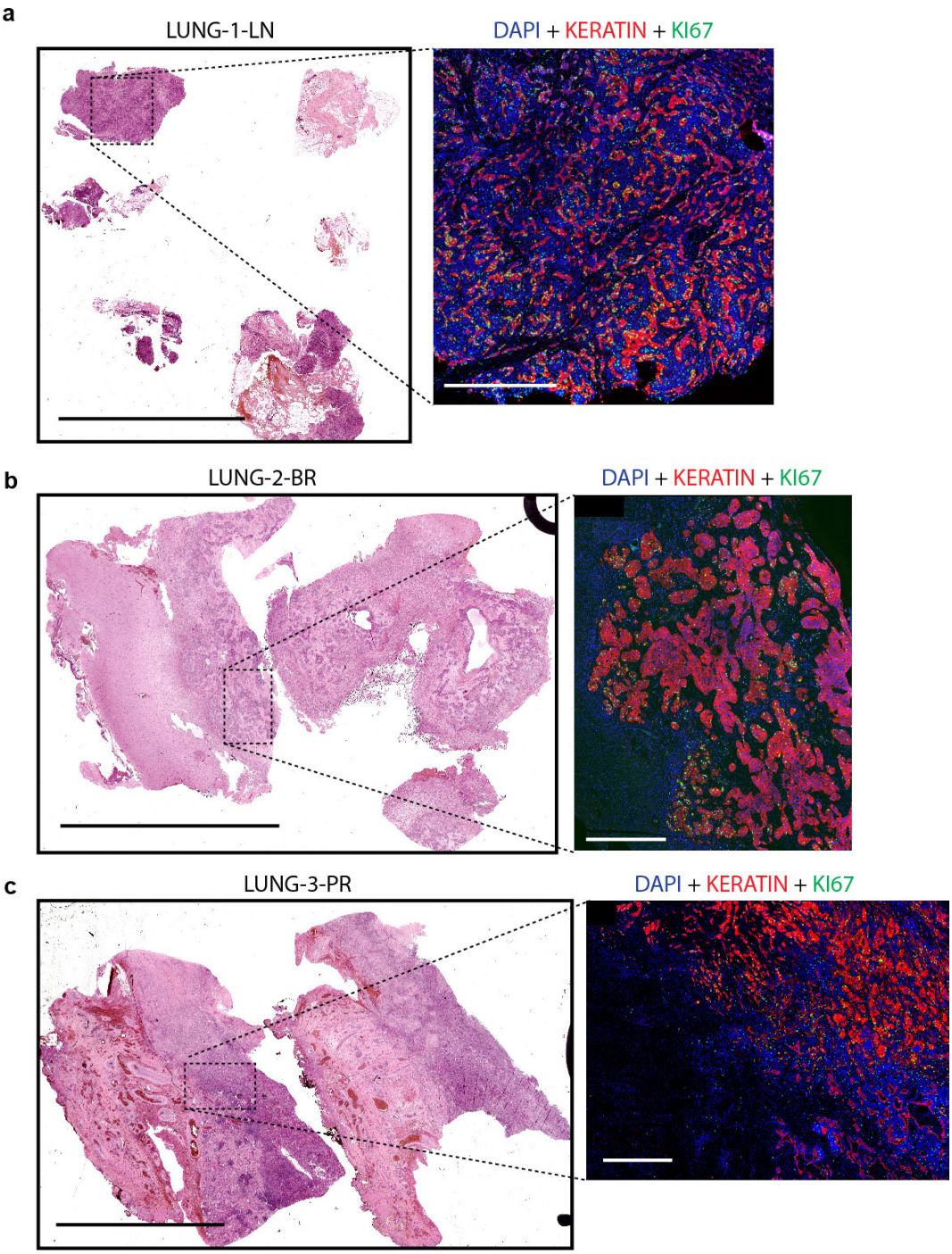
Histograms of signal intensity (upper left) and representative images of t-CyCIF from eight sequential sections of human FFPE tonsil tissue across different staining cycles for a) CD3, b) CD4, c) CD8a, d) CD20, and e) FOXP3. The same antibody concentration and exposure time for imaging were used for each cycle (see Supplementary Table 5, 6 for additional details). The histogram plots were made from single cell data to show the intensity distribution from the different populations from different cycles. For the images shown, the same threshold was used for each marker for visualization and comparison. In e, the black arrow indicates the FOXP3+ population (Scale bars: 50 μm).



Supplementary Figure 12

Analysis of effect of t-CyCIF cycle number on antigenicity in human FFPE tonsil tissue.

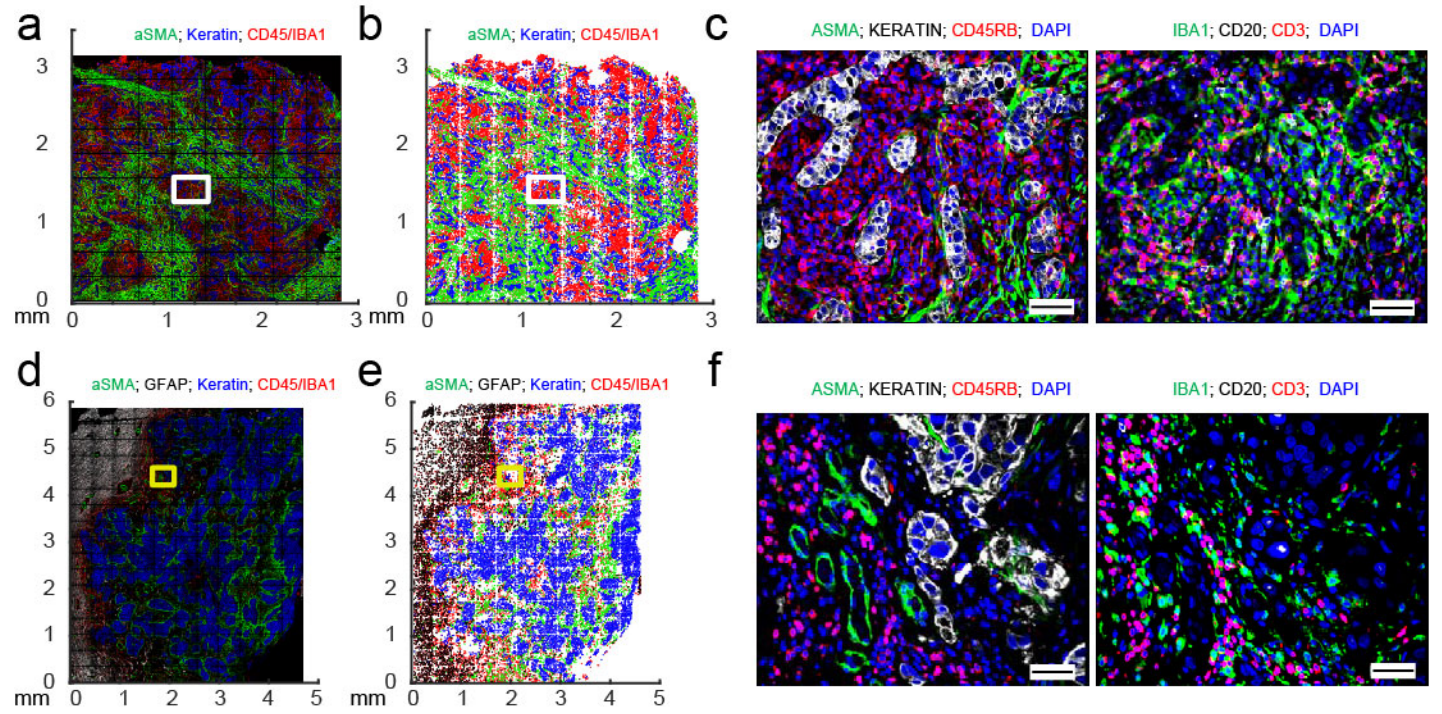
Histograms of signal intensity (upper left) and representative images of t-CyCIF from eight sequential sections of human FFPE tonsil tissue across different staining cycles for a) IBA1, b) CD14, c) CD68, d) CD163, and e) keratin. The same antibody concentration and exposure time for imaging were used in each cycle (see Supplementary Table 5, 6 for details). The histogram plots were made from single cell data to show the intensity distribution from the different populations from different cycles. For the images shown, the same threshold was used for each marker for visualization and comparison. In e, the black arrow indicates the keratin+ population (Scale bars: 50 μm).



Supplementary Figure 13

H&E images of lung cancer specimens.

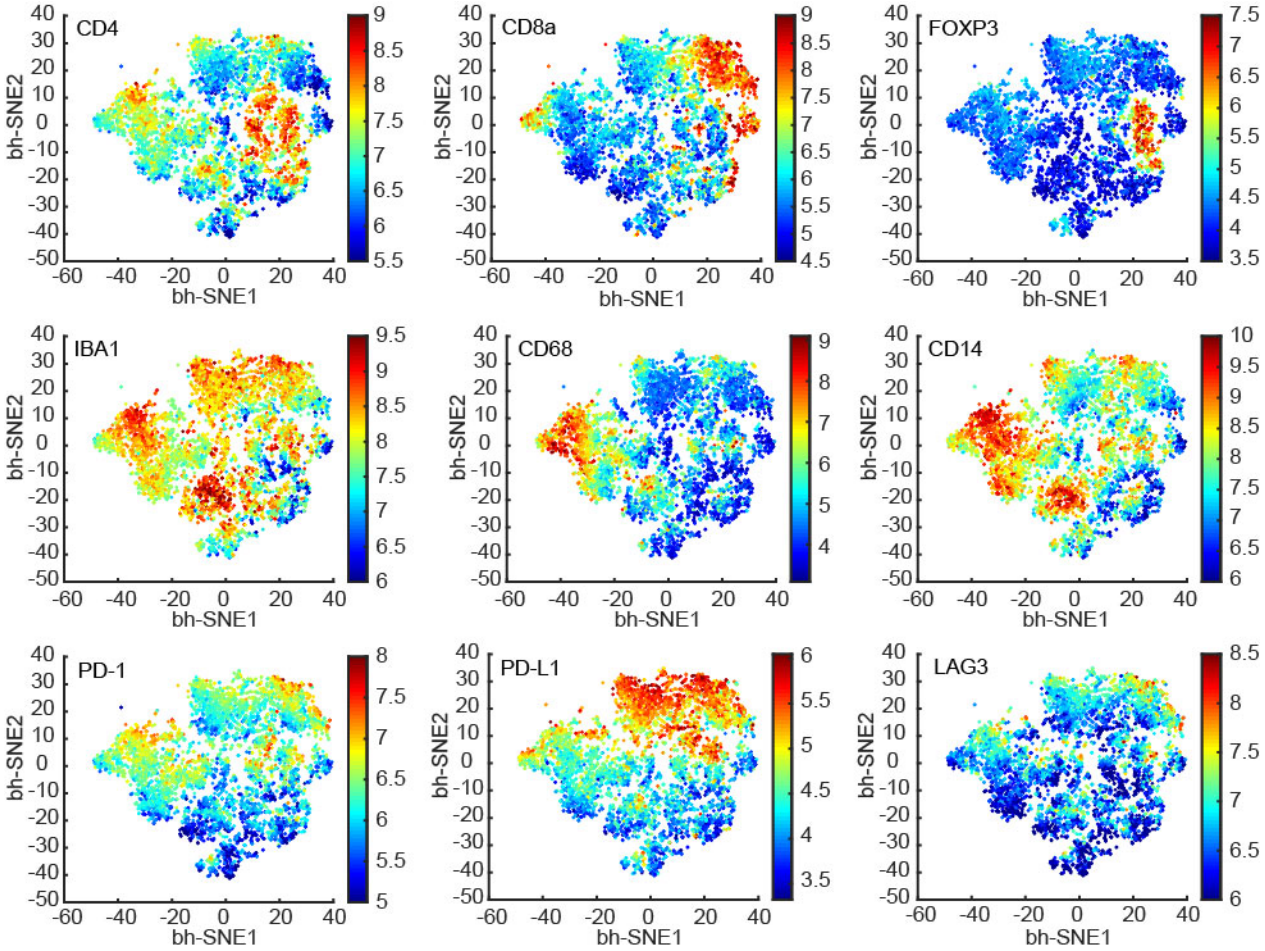
Whole slide scans of H&E-stained slides (left) and t-CyCIF images (right) from a) lung adenocarcinoma metastasis to the lymph node (LUNG-1-LN), b) lung squamous cell carcinoma metastasis to the brain (LUNG-2-BR), and c) primary lung squamous cell carcinoma (LUNG-3-PR) (Scale bars: 15 mm in H&E images; 1 mm in t-CyCIF images).



Supplementary Figure 14

Geographic visualization of t-CyCIF data in LUNG-1-LN and LUNG-2-BR.

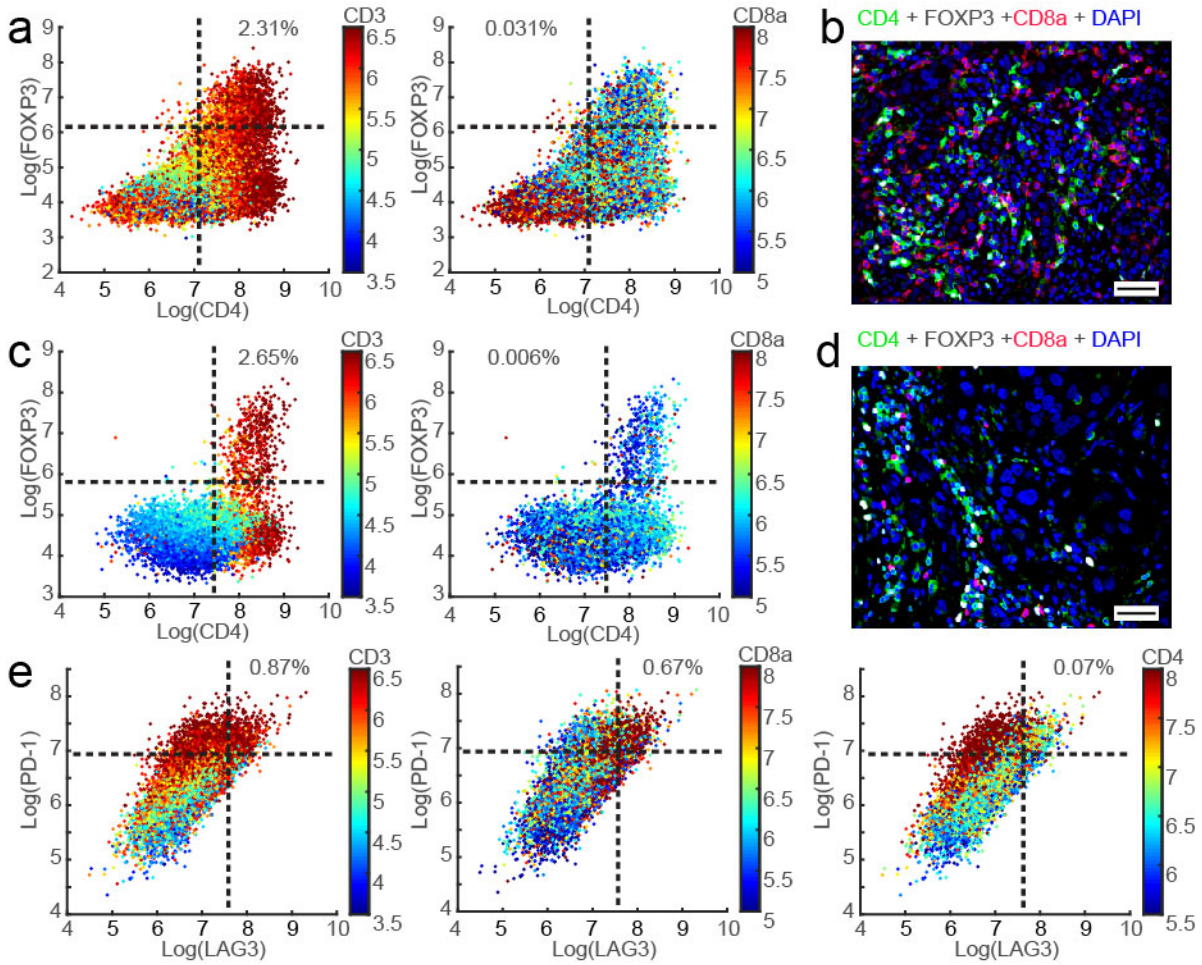
a) Montage of t-CyCIF images and b) corresponding dot plot for tumour cells (keratin+, blue), fibroblasts (α-SMA+, green) and immune cells (CD45+ or IBA1+, red) in LUNG-1-LN. c) Representative merged images of t-CyCIF data for α-SMA, keratin, CD45RB, IBA1, CD20, and CD3 from LUNG-1-LN (Scale bars: 50 μm). d) Montage of t-CyCIF images and e) corresponding dot plot for tumour cells (keratin+, blue), fibroblasts (α-SMA+, green) and immune cells (CD45+ or IBA1+, red) in LUNG-2-BR. f) Representative merged images of t-CyCIF data for α-SMA, keratin, CD45RB, IBA1, CD20, and CD3 from LUNG-2-BR (Scale bars: 50 μm).



Supplementary Figure 15

t-SNE analysis of immune cells from lung cancer samples.

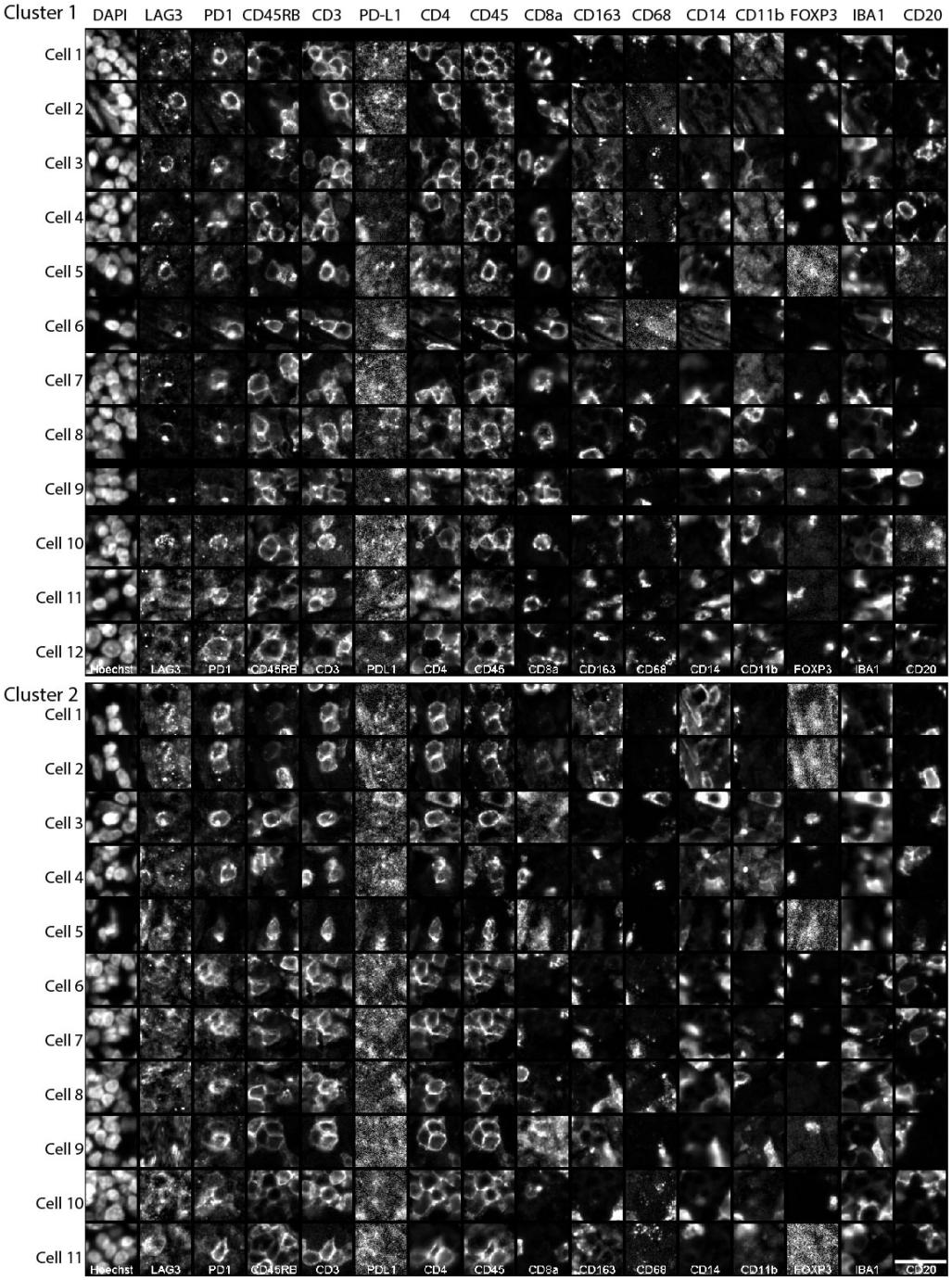
t-SNE plots of immune cell markers CD4, CD8a, FOXP3, IBA1, CD68, CD14, PD-1, PD-L1 and LAG3 from a random sampling of 2,000 immune cells for LUNG-1-LN, LUNG-2-BR and LUNG-3-PR. The staining for each of the indicated markers is mapped by colour (red = high, blue = low).



Supplementary Figure 16

Low-frequency immune cell types detected and confirmed by t-CyCIF in LUNG-1-LN and LUNG-2-BR.

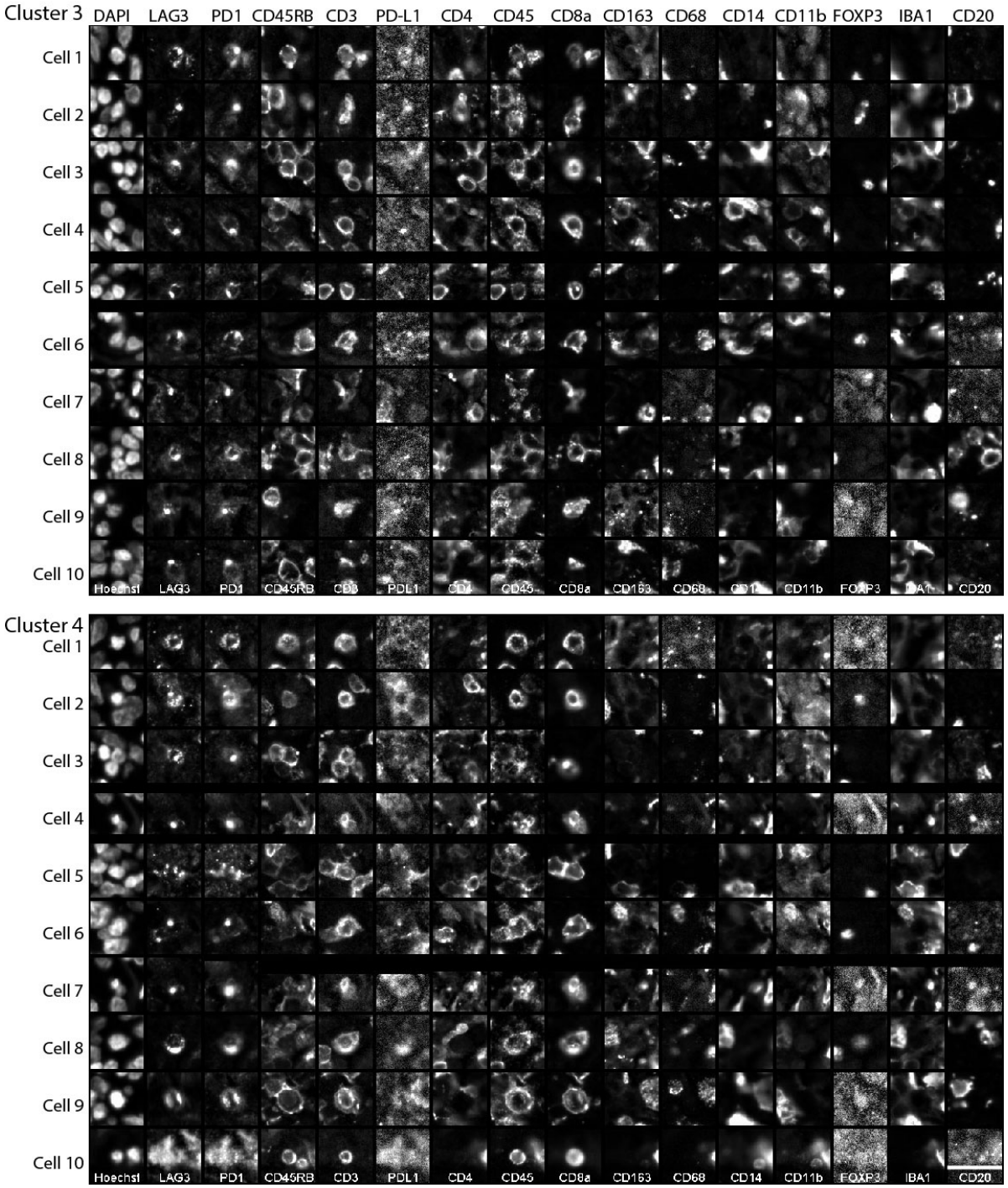
a) Scatter plots of CD4 and FOXP3 expression in LUNG-1-LN, with CD3 and CD8a expression mapped by colour (red = high, blue = low). 2.31% of the immune cells were CD45+/CD3+/FOXP3+/CD4+/CD8a-, while 0.031% of the immune cells were CD45+/CD3+/FOXP3+/CD4-/CD8a+. b, Representative image of t-CyCIF data for CD4, CD8a and FOXP3 in LUNG-1-LN (Scale bar: 50 μm). c. Scatter plots for CD4 and FOXP3, with CD3 and CD8a mapped as in panel a in LUNG-2-BR. 2.65% of the immune cells were CD45+/CD3+/FOXP3+/CD4+/CD8a- while 0.006% of the immune cells were CD45+/CD3+/FOXP3+/CD4-/CD8a+. d) Merged image of t-CyCIF data for CD4, CD8a and FOXP3 in LUNG-2-BR (Scale bar: 50 μm). e) Scatter plots for LAG3 and PD-1 expression in LUNG-1-LN, with CD3 and CD8a expression mapped as in panel a. 3.6% of immune cells were CD45+/CD3+/PD-1+, 0.87% were CD45+/CD3+/PD-1+/LAG3+ cells, 0.67% were CD45+/CD3+/ PD-1+/LAG3+/ CD4-/CD8a+, and 0.07% were CD45+/CD3+/PD-1+/LAG3+/ CD4+/CD8a-. See Supplementary Table 8 for additional details on these data.



Supplementary Figure 17

Galleries of images of immune markers expressed in rare cell clusters 1 and 2.

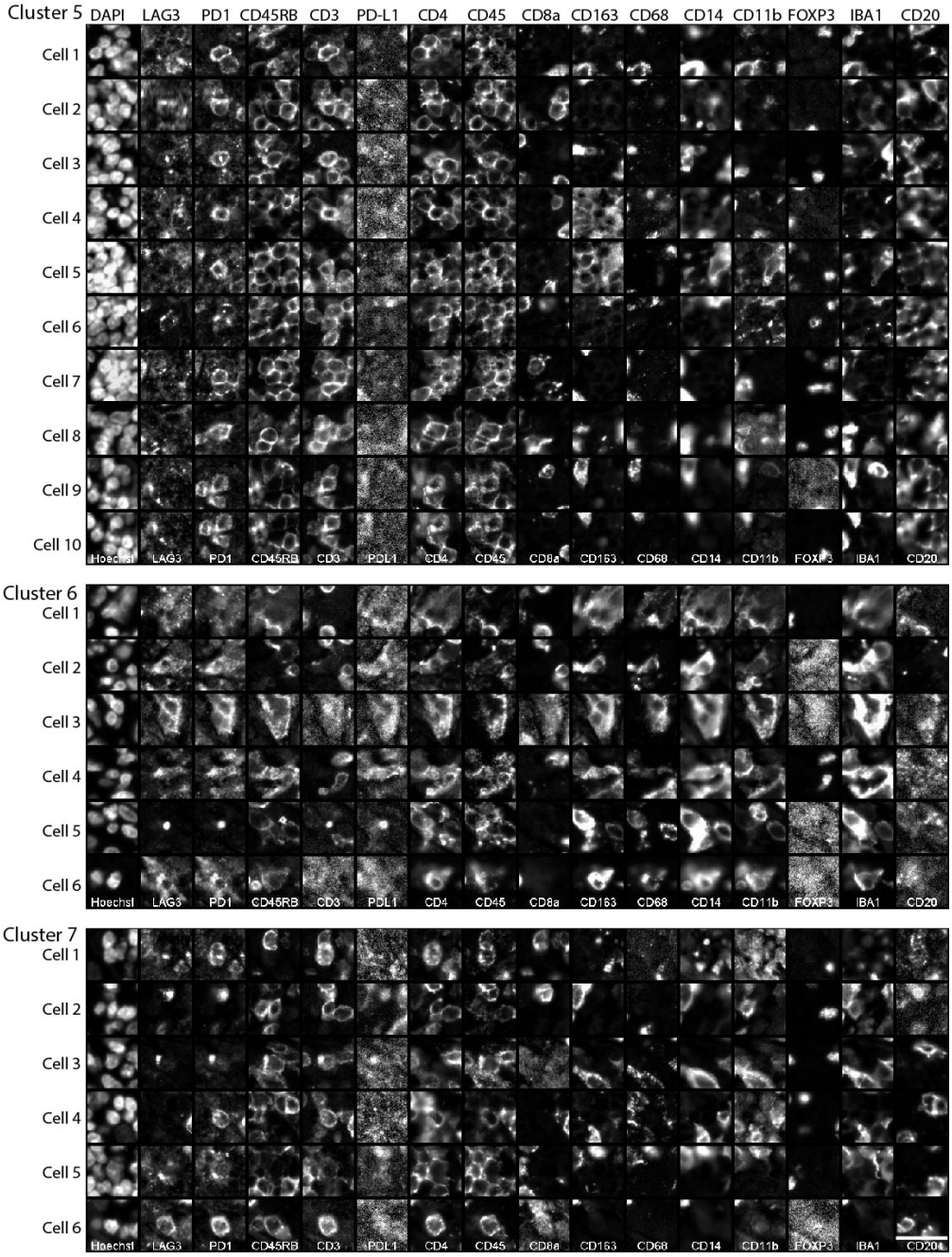
Galleries of images of immune markers (LAG3, PD-1, CD45RB, CD3, PD-L1, CD4, CD45, CD8a, CD163, CD68, CD14, CD11b, FOXP3, IBA1, CD20) and DNA stain for 12 individual rare cells identified in cluster 1 using automated methods that putatively express CD45+/CD45RB+/CD3+/CD8a+/PD-1+ (top) or 11 individual rare cells in cluster 2 that putatively express CD45+/CD45RB+/CD3+/CD4+/PD-1+ (bottom) (Scale bar: 25 μm). Supplementary Table 10 indicates whether visual review of these images by a trained pathologist was deemed to be consistent with the automated calls.



Supplementary Figure 18

Galleries of images of immune markers expressed in rare cell clusters 3 and 4.

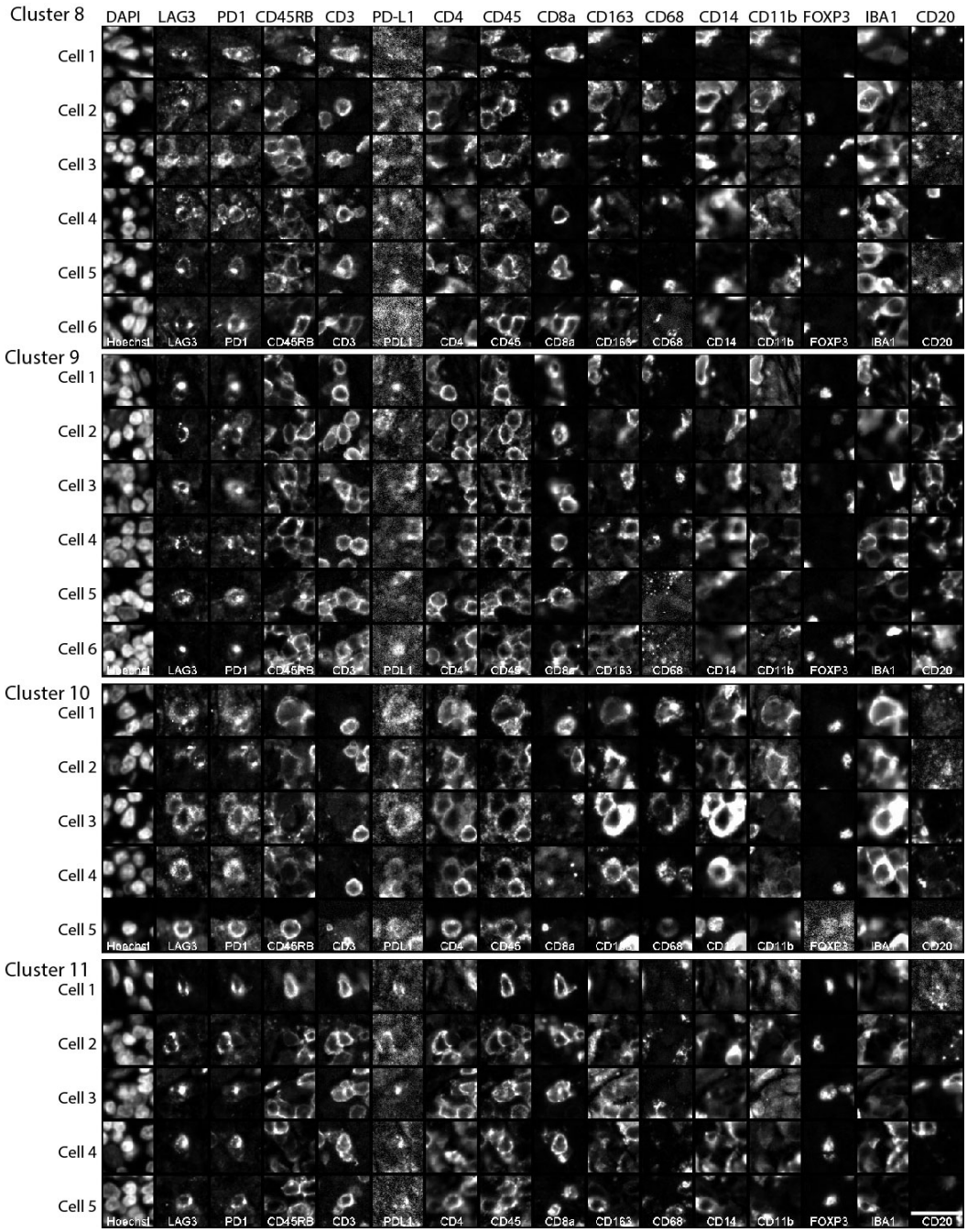
Galleries of images of immune markers (LAG3, PD-1, CD45RB, CD3, PD-L1, CD4, CD45, CD8a, CD163, CD68, CD14, CD11b, FOXP3, IBA1, CD20) and DNA stain for 10 individual rare cells in cluster 3 identified using automated methods that putatively express CD45+/CD45RB+/CD3+/CD8a+/PD-1+/LAG3+ (top), or 10 individual rare cells in cluster 4 that putatively express CD45+/CD45RB+/CD3+/CD8a+/PD-1+/LAG3+/PD-L1+ (bottom) (Scale bar: 25 µm). Supplementary Table 10 indicates whether visual review of these images by a trained pathologist was deemed to be consistent with the automated calls.



Supplementary Figure 19

Galleries of images of immune markers expressed in rare cell clusters 5, 6, and 7.

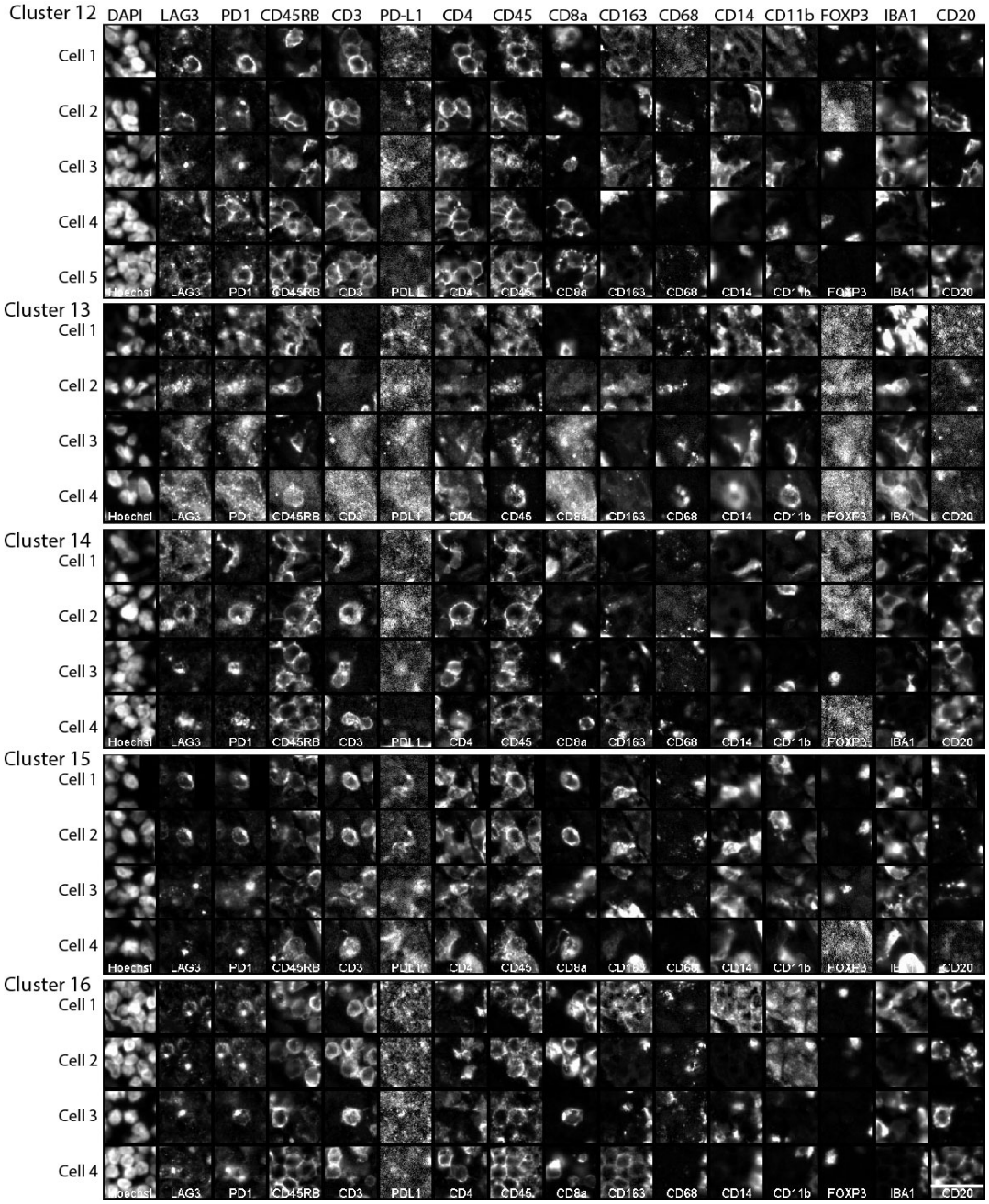
Galleries of images of immune markers (LAG3, PD-1, CD45RB, CD3, PD-L1, CD4, CD45, CD8a, CD163, CD68, CD14, CD11b, FOXP3, IBA1, CD20) and DNA stain for 10 individual rare cells in cluster 5 identified using automated methods that putatively express CD45+/CD45RB+/CD3+/CD4+/CD20+/PD-1+ (top), 6 individual rare cells in cluster 6 that putatively express CD45+/CD45RB+/IBA1+/CD163+/CD14+/CD68+/CD11b+/PD-1+/LAG3+/PD-L1+ (middle), or 6 individual rare cells in cluster 7 that putatively express CD45+/CD45RB+/CD3+/PD-1+ (Scale bar: 25 μm). Supplementary Table 10 indicates whether visual review of these images by a trained pathologist was deemed to be consistent with the automated calls.



Supplementary Figure 20

Galleries of images of immune markers expressed in rare cell clusters 8, 9, 10, and 11.

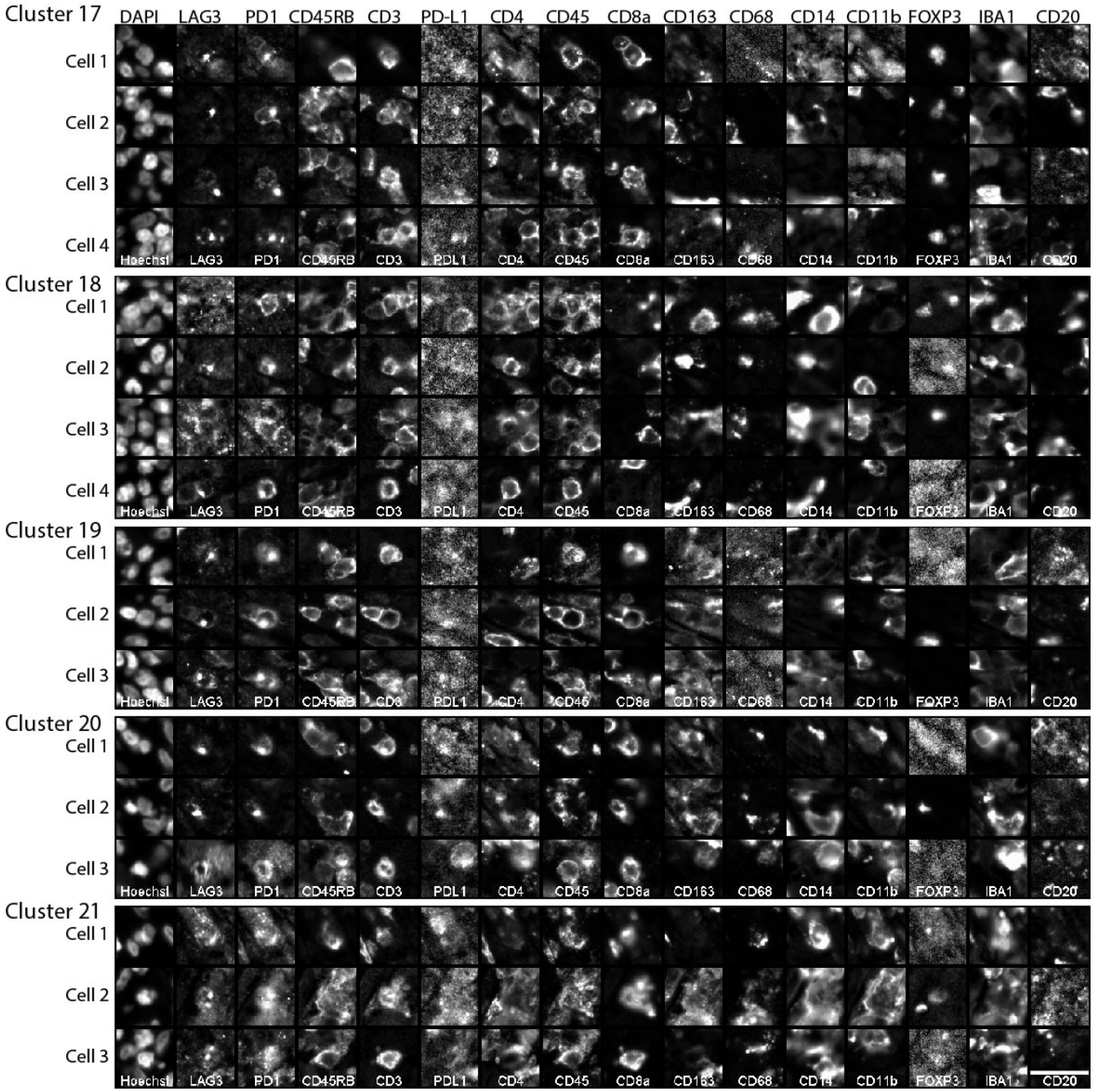
Galleries of images of immune markers (LAG3, PD-1, CD45RB, CD3, PD-L1, CD4, CD45, CD8a, CD163, CD68, CD14, CD11b, FOXP3, IBA1, CD20) and DNA stain for 6 individual rare cells in cluster 8 identified using automated methods that putatively express CD45+/CD45RB+/CD3+/CD8a+/IBA1+/PD-1+, 6 individual rare cells in cluster 9 that putatively express CD45+/CD45RB+/CD3+/CD8a+/CD20+/PD-1+/LAG3+, 5 individual rare cells in cluster 10 that putatively express CD45+/CD45RB+/IBA1+/CD163+/CD14+/CD68+/PD-1+/LAG3+/PD-L1+, or 5 individual rare cells in cluster 11 that putatively express CD45+/CD45RB+/CD3+/CD8a+/FOXP3+/PD-1+/LAG3+ (Scale bar: 25 µm). Supplementary Table 10 indicates whether visual review of these images by a trained pathologist was deemed to be consistent with the automated calls.



Supplementary Figure 21

Galleries of images of immune markers expressed in rare cell clusters 12, 13, 14, 15, and 16.

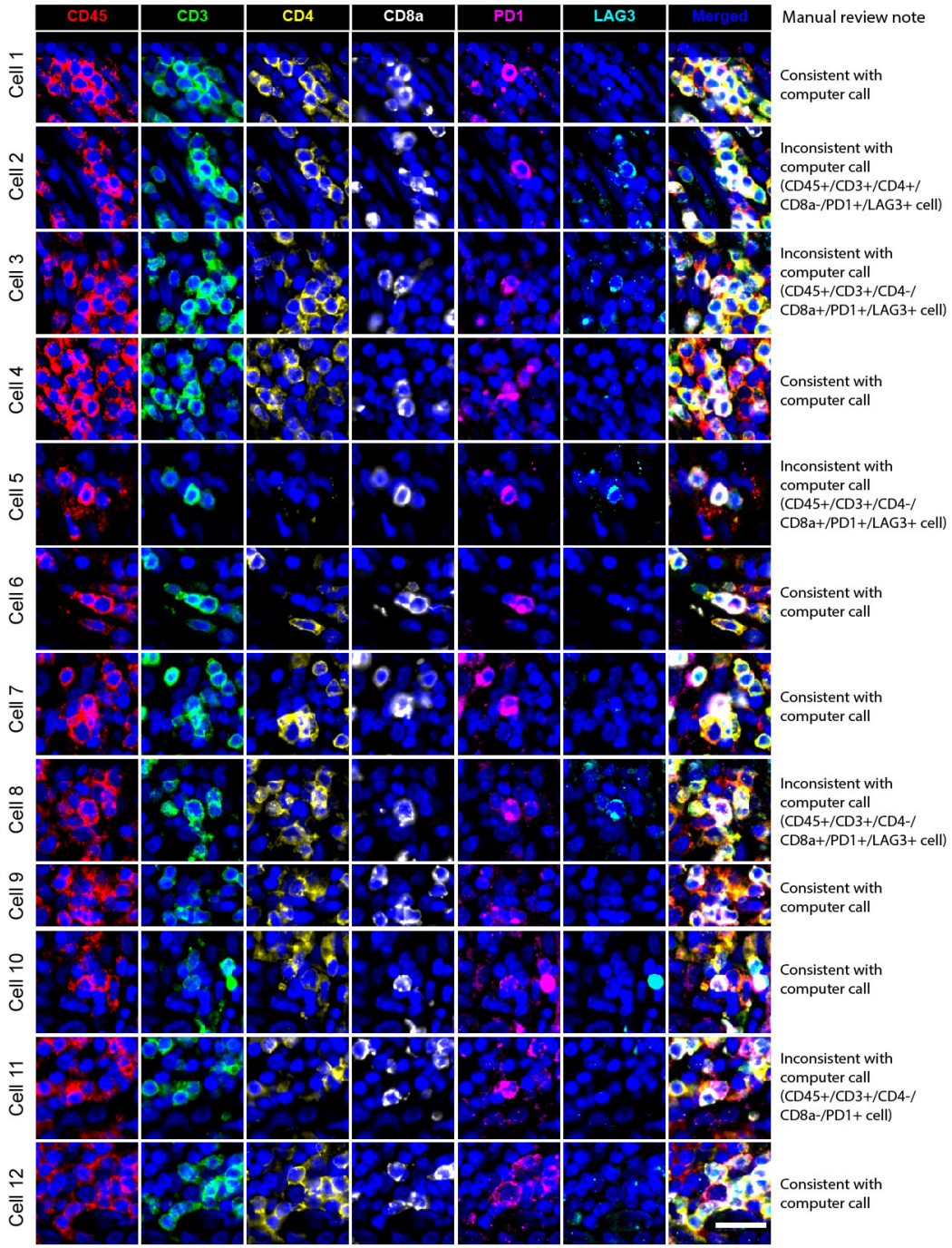
Galleries of images of immune markers (LAG3, PD-1, CD45RB, CD3, PD-L1, CD4, CD45, CD8a, CD163, CD68, CD14, CD11b, FOXP3, IBA1, CD20) and DNA stain for 5 individual rare cells in cluster 12 identified using automated methods that putatively express CD45+/CD45RB+/CD3+/CD4+/CD8a+/PD-1+, 4 individual rare cells in cluster 13 that putatively express CD45+/CD45RB+/IBA1+/CD14+/CD11b+/PD-1+/LAG3+/PD-L1+, 4 individual rare cells in cluster 14 that putatively express CD45+/CD45RB+/CD3+/CD20+/PD-1+, 4 individual rare cells in cluster 15 that putatively express CD45+/CD45RB+/CD3+/IBA1+/CD14+/CD11b+/PD-1+/LAG3+/PD-L1+, or 4 individual rare cells in cluster 16 that putatively express CD45+/CD45RB+/CD3+/CD8a+/CD20+/PD-1+ (Scale bar: 25 μm). Supplementary Table 10 indicates whether visual review of these images by a trained pathologist was deemed to be consistent with the automated calls.



Supplementary Figure 22

Galleries of images of immune markers expressed in rare cell clusters 17, 18, 19, 20, and 21.

Galleries of images of immune markers (LAG3, PD-1, CD45RB, CD3, PD-L1, CD4, CD45, CD8a, CD163, CD68, CD14, CD11b, FOXP3, IBA1, CD20) and DNA stain for 4 individual rare cells in cluster 17 identified using automated methods that putatively express CD45+/CD45RB+/CD3+/CD8a+/FOXP3+/PD-1+, 4 individual rare cells in cluster 18 that putatively express CD45+/CD45RB+/CD3+/CD4+/IBA1+/CD163+/CD14+/PD-1+, 3 individual rare cells in cluster 19 that putatively express CD45+/CD45RB+/CD3+/CD8a+/CD11b+/PD-1+, 3 individual rare cells in cluster 20 that putatively express CD45+/CD45RB+/CD3+/CD8a+/IBA1+/PD1+/PD-L1+/LAG3+, or 3 individual rare cells in cluster 21 that putatively express CD45+/CD45RB+/CD3+/CD8a+/IBA1+/CD14+/CD68+/CD11b+/PD-1+/PD-L1+/LAG3+ (Scale bar: 25 µm). Supplementary Table 10 indicates whether visual review of these images by a trained pathologist was deemed to be consistent with the automated calls.



Supplementary Figure 23

Galleries of images of immune markers expressed in rare cell cluster 1.

Image galleries of cells identified by automated systematic cell calling that express CD45, CD45RB, CD3, CD8a, and PD-1 (rare cell cluster 1, Figure 8) (Scale bar: 25 µm) were reviewed by a trained pathologist who then annotated whether the automated calls were consistent with visual review (notes are shown in the right-most column; visual review confirmation for all rare cell populations is presented in Supplementary Table 10). Pseudo-colour images and merged images of the markers are shown here; monochromatic images for this rare cell cluster are shown in Supplementary Figure 17.

Supplementary Table 1. Dynamic range and Pearson's r of the dynamic range between antibodies.

PD1 antibody							
DR value (5%)	Region 1	Region 2	Region 3	Region 4	Region 5	Mean	std
EPR4877(2) (AB1)	20330	14263	16145	4802	16161	14340.2	5775.6718
NAT105 (AB2)	1314	1062	1137	654	1135	1060.4	245.41251
EH33 (AB3)	19184	12397	12587	4117	12569	12170.8	5348.6358
D4W2J (AB4)	42568	29480	34088	9898	34073	30021.4	12202.604
DAP1	9863	12595	11132	12305	11152	11409.4	1088.8417
r value	Region 1	Region 2	Region 3	Region 4	Region 5	Mean	std
AB1 vs AB2	0.91	0.77	0.82	0.64	0.86	0.8	0.1031988
AB1 vs AB3	0.99	0.99	0.99	0.99	0.99	0.99	0
AB1 vs AB4	0.96	0.98	0.97	0.97	0.97	0.97	0.0070711
AB2 vs AB3	0.92	0.79	0.83	0.64	0.87	0.81	0.1065364
AB2 vs AB4	0.87	0.75	0.79	0.64	0.84	0.778	0.0898332
AB3 vs AB4	0.96	0.97	0.96	0.97	0.96	0.964	0.0054772
AB1 vs DAPI	-0.1	-0.01	-0.06	-0.02	-0.07	-0.052	0.0370135
AB2 vs DAPI	0.07	0.24	0.15	0.15	0.13	0.148	0.0609918
AB3 vs DAPI	-0.1	-0.02	-0.06	-0.05	-0.08	-0.062	0.0303315
AB4 vs DAPI	-0.12	-0.03	-0.08	-0.07	-0.1	-0.08	0.0339116
PDL1 antibody							
DR value (1%)	Region 1	Region 2	Region 3	Region 4	Region 5	Mean	std
AB1	387	403	458	507	510	453	57.109544
AB2	631	579	578	723	601	622.4	60.222919
AB3	563	439	799	793	676	654	154.38264
DAP1	32558	28201	29925	31639	33205	31105.6	2039.1162
r value	Region 1	Region 2	Region 3	Region 4	Region 5	Mean	std
AB1 vs AB2	0.5	0.39	0.49	0.63	0.52	0.506	0.0856154
AB1 vs AB3	0.35	0.32	0.47	0.59	0.45	0.436	0.1071448
AB2 vs AB3	0.48	0.45	0.7	0.74	0.64	0.602	0.1304607
AB1 vs DAPI	-0.11	-0.18	-0.01	-0.09	-0.15	-0.108	0.0649615
AB2 vs DAPI	-0.02	-0.04	0.09	-0.01	0	0.004	0.0502991
AB3 vs DAPI	0.36	0.27	0.28	0.17	0.25	0.266	0.0680441
FOXP3 antibody							
DR value (1%)	Region 1	Region 2	Region 3	Region 4	Region 5	Mean	std
AB1	614	1169.1	1286	1163	1612	1168.82	359.86068
AB2	337	677.14	845	668	982	701.828	241.7891
DAP1	3993	3380	3107.1	3637	3537.1	3530.84	326.80625
r value	Region 1	Region 2	Region 3	Region 4	Region 5	Mean	std
AB1 vs AB2	0.77	0.9	0.87	0.88	0.93	0.87	0.0604152
AB1 vs DAPI	0.35	0.3	0.25	0.23	0.22	0.27	0.0543139
AB2 vs DAPI	0.12	0.14	0.12	0.11	0.12	0.122	0.0109545
CD45/CD45RB antibody							
DR value (5%)	Region 1	Region 2	Region 3	Region 4	Region 5	Mean	std
AB1	603	626	978	836	619	732.4	167.31497
AB2	5187.7	6141	8169	8635.7	5705	6767.68	1538.7942
AB3	18104	25262	23220	25934	25728	23649.6	3281.6269
DAP1	9646	8759	10044	10966	9718	9826.6	795.24763
r value	Region 1	Region 2	Region 3	Region 4	Region 5	Mean	std
AB1 vs AB2	0.78	0.77	0.77	0.8	0.68	0.76	0.0463681
AB1 vs AB3	0.65	0.71	0.65	0.73	0.53	0.654	0.0779744
AB2 vs AB3	0.85	0.91	0.85	0.9	0.79	0.86	0.0479583
AB1 vs DAPI	0.28	0.03	0.03	0.11	0.13	0.116	0.1023719
AB2 vs DAPI	0.26	0.01	0.14	0.05	0.11	0.114	0.0960729
AB3 vs DAPI	0.18	-0.04	0.05	0	0.01	0.04	0.0845577
LAG3 antibody							
DR value (1%)	Region 1	Region 2	Region 3	Region 4	Region 5	Mean	std
AB1	3446.3	2910.1	2317	2574	1885.1	2626.5	591.76039

AB2	8014.1	6985.1	5816	6880	8280	7195.04	986.54369
AB3	4219	4720	5285	4473	4971	4733.6	416.32655
AB4	3752	2941	4006.3	2565.1	3758.1	3404.5	617.93549
AB5	757	567	655	572	664	643	78.099296
DAP1	22329	15912	15626	14576	15270	16742.6	3162.5648
r value	Region 1	Region 2	Region 3	Region 4	Region 5	Mean	std
AB1 vs AB2	0.67	0.7	0.53	0.61	0.42	0.586	0.1132696
AB1 vs AB3	0.71	0.62	0.35	0.54	0.41	0.526	0.1477498
AB1 vs AB4	0.29	0.46	0.19	0.3	0.11	0.27	0.1317194
AB1 vs AB5	0.14	0.1	0.09	0.13	0.09	0.11	0.0234521
AB2 vs AB3	0.54	0.58	0.32	0.48	0.28	0.44	0.1334166
AB2 vs AB4	0.17	0.36	0.11	0.22	0.06	0.184	0.1154556
AB2 vs AB5	0.01	0.05	0.06	0.04	0.04	0.04	0.0187083
AB3 vs AB4	0.32	0.43	0.19	0.31	0.18	0.286	0.1035857
AB3 vs AB5	0.26	0.17	0.22	0.21	0.27	0.226	0.0403733
AB4 vs AB5	0.32	0.15	0.24	0.19	0.24	0.228	0.0637966
AB1 vs DAPI	0.01	0	0.04	0.02	0.05	0.024	0.0207364
AB2 vs DAPI	-0.15	-0.13	-0.1	-0.17	-0.14	-0.138	0.0258844
AB3 vs DAPI	0.03	0.07	0.08	0.01	0.19	0.076	0.069857
AB4 vs DAPI	0.01	0.04	0.03	0.02	0.03	0.026	0.0114018
AB5 vs DAPI	0.48	0.31	0.26	0.47	0.34	0.372	0.0983362
CD11b antibody							
DR value (5%)	Region 1	Region 2	Region 3	Region 4	Region 5	Mean	std
AB1	12852	9843.7	7853	27859	4263	12534.14	9117.506
AB2	10564	10345	7603	23983	5501	11599.2	7231.1966
AB3	13803	13227	14560	27334	10172	15819.2	6649.2788
DAP1	8703	9409	10083	10521	10152	9773.6	720.52745
r value	Region 1	Region 2	Region 3	Region 4	Region 5	Mean	std
AB1 vs AB2	0.92	0.93	0.87	0.97	0.8	0.898	0.0653452
AB1 vs AB3	0.89	0.78	0.62	0.87	0.5	0.732	0.1678392
AB2 vs AB3	0.92	0.8	0.67	0.88	0.62	0.778	0.1300769
AB1 vs DAPI	-0.25	-0.19	-0.24	-0.27	-0.18	-0.226	0.0391152
AB2 vs DAPI	-0.31	-0.22	-0.28	-0.3	-0.17	-0.256	0.0594138
AB3 vs DAPI	-0.38	-0.22	-0.35	-0.34	-0.17	-0.292	0.0914877

DR: Dynamic Range; r: Pearson correlation coefficient

Supplementary Table 2: t-CyCIF immune profile antibody panel.

Antibody name	Target gene	Clone	Company	Cat#	Conjugation	Cycle No	Tonsil No	RRID (RESEARCH RESOURCE IDENTIFIERS)	Dilution
PD1 AB1	PD1	EPR4877(2)	Abcam	ab201825	Alexa Fluor® 647	2	1		100
PD1 AB2	PD1	NAT105	Abcam	ab195885	Alexa Fluor® 488	2	1		100
PD1 AB3	PD1	EH33	Cell Signaling Technology	43248	Primary mouse	1	1		100
PD1 AB4	PD1	D4W2J	Cell Signaling Technology	86163	Primary rabbit	1	1		100
PD-L1 AB1	PD-L1	E1L3N	Cell Signaling Technology	15005S	Alexa Fluor® 647	2	2		100
PD-L1 AB2	PD-L1	22C3	DAKO	M365329-1	Primary mouse	1	2		100
PD-L1 AB3	PD-L1	28-8	Abcam	ab213358	Alexa Fluor® 555	2	2		100
FOXP3 AB1	FOXP3	236A/E7	eBioscience	41-4777-80	eFluor 570	7	3	AB_2573608	100
FOXP3 AB2	FOXP3	206D	BioLegend	320113	Alexa Fluor® 647	7	3	AB_439753	100
LAG3 AB1	LAG3	EPR4392(2)	Abcam	ab180187	Primary rabbit	1	4		100
LAG3 AB2	LAG3	Polyclonal	R&D systems	FAB2319P	PE	2	4	AB_2133351	100
LAG3 AB3	LAG3	17B4	Lifespan	LS-C344749	Atto 647N	2	4		100
LAG3 AB4	LAG3	11 E3	Abcam	ab40465	Primary mouse	1	4	AB_776102	100
LAG3 AB5	LAG3	T47-530	BD Bioscience	565617	PE	3	4		100
CD11b AB1	CD11b	EP1345Y	Abcam	ab52478	Primary rabbit	1	5	AB_868788	100
CD11b AB2	CD11b	C67F154	eBioscience	53-0196-80	Alexa Fluor® 488	2	5	AB_2637195	100
CD11b AB3	CD11b	EPR1344	Abcam	ab204271	Alexa Fluor® 488	3	5		100
CD45 AB1	CD45	2D1	R&D Systems	FAB1430P-025	PE	3	5	AB_2237898	100
CD45 AB2	CD45	HI30	BioLegend	304056	Alexa Fluor® 647	3	5	AB_2564155	100
CD45RB AB3	CD45RB	PD7/26	eBioscience	53-9458-80	Alexa Fluor® 488	4	5	AB_2574448	100

Supplementary Table 3: Antibody information for the immune t-CyCIF panel.

no	Antibody name	Function	Clone	Company	Cat#	Conjugation	RRID (RESEARCH RESOURCE IDENTIFIERS)	Dilution	Final Concentration (ug/ml)	Validation approaches
1	**CD19**	B cells	EPR5906	Abcam	ab196468	Alexa Fluor® 488		100	5	Cell level, Tissue level
2	**CD20**	B cells	L26	eBioscience	50-0202-80	eFluor® 660	AB_11151691	250	0.8	Cell level, Tissue level
3	**LAG3**	Checkpoint	EPR4392(2)	Abcam	ab180187	Primary rabbit		100	4.5	Pixel level, Cell level, Tissue level
4	**PD1**	Checkpoint	EPR4877(2)	Abcam	ab201825	Alexa Fluor® 647		100	5	Pixel level, Cell level, Tissue level
5	**PD-L1**	Checkpoint	E1L3N	Cell Signaling Technology	15005S	Alexa Fluor® 647		100	2	Pixel level, Cell level, Tissue level
6	**CD45**	Lymphocyte	2D1	R&D Systems	FAB1430P-025	PE	AB_2237898	100	0.25	Pixel level, Cell level, Tissue level
7	**CD45RB**	Lymphocyte	PD7/26	eBioscience	53-9458-80	Alexa Fluor® 488	AB_2574448	100	5	Pixel level, Cell level, Tissue level
8	**CD11b**	Macrophage	C67F154	eBioscience	53-0196-80	Alexa Fluor® 488	AB_2637195	100	5	Pixel level, Cell level, Tissue level
9	**CD14**	Macrophage	EPR3653	Abcam	ab196169	Alexa Fluor® 647		100	5	Cell level, Tissue level
10	**CD163**	Macrophage	EPR14643	Abcam	ab218293	Alexa Fluor® 488		100	5	Cell level, Tissue level
11	**CD68**	Macrophage	D4B9C	Cell Signaling Technology	79594	PE		100	3.1	Cell level, Tissue level
12	**IBA1**	Macrophage	EPR6136	Abcam	ab195031	Alexa Fluor® 488		250	2	Cell level, Tissue level
13	**CD8a**	T cell (Cytotoxic)	AMC908	eBioscience	50-0008-80	eFluor® 660	AB_2574148	100	2	Cell level, Tissue level
14	**CD4**	T cell (T helper)	Polyclonal	R&D Systems	FAB8165G	Alexa Fluor® 488		100	0.5	Cell level, Tissue level
15	**FOXP3**	T cell (Treg)	236A/E7	eBioscience	41-4777-80	eFluor 570	AB_2573608	100	2	Pixel level, Cell level, Tissue level
16	**CD3**	T cell marker	EP4426	Abcam	ab208514	Alexa Fluor® 555		100	5	Cell level, Tissue level
17	**Ki67**	Other(Proliferation)	D3B5	Cell Signaling Technology	11882s	Alexa Fluor® 488	AB_2687824	100	1	Cell level, Tissue level
18	**alpha-SMA**	Other(Stromal)	EPR5368	Abcam	ab202509	Alexa Fluor® 555		250	2	Cell level, Tissue level
19	**Pan Keratin**	Other(Tumor marker)	AE1/AE3	eBioscience	41-9003-80	eFluor® 570	AB_11217482	100	2	Cell level, Tissue level
20	**GFAP**	Other (Glial)	GA5	eBioscience	41-9892-80	eFluor 570		100	2	Cell level, Tissue level

Supplementary Table 4: Clinical antibodies used for immunohistochemistry validation.

Antibody name	Clone	Company	Cat#	Resource	RRID (RESEARCH RESOURCE IDENTIFIERS)	Dilution
CD3	polyclonal	DakoCytomation	A0452	rabbit polyclonal	AB_2335677	300
CD4	SP35	Cell Marque	104R-14	rabbit monoclonal	AB_1516770	150
CD8	C8/144B	Cell Marque	108M94	mouse monoclonal	AB_1158205	100
CD20	L26	DakoCytomation	M0755	mouse monoclonal	AB_2282030	600
CD68	KP1	DakoCytomation	M0814	mouse monoclonal	AB_2314148	1000
FoxP3	259D	BioLegend	320201	mouse monoclonal	AB_430884	100
PD-1	EH33	Cell Signaling	43248	mouse monoclonal		200
PD-L1	E1L3N	Cell Signaling	13684	rabbit monoclonal		100

Supplementary Table 5: Antigenicity with cycle number in t-CyCIF.

Cycle No	Tonsil No	Channel No	Antigen	Company	Cat No	Dilution	exposure time (s)	Lot No
2	4	4	CD14	Abcam	ab196169	1000	0.5	GR219132-2
5	7	4	CD14	Abcam	ab196169	1000	0.5	GR219132-2
6	8	4	CD14	Abcam	ab196169	1000	0.5	GR219132-2
7	2	4	CD14	Abcam	ab196169	1000	0.5	GR219132-2
2	8	2	CD163	Abcam	ab218293	300	0.5	GR314696-1
3	7	2	CD163	Abcam	ab218293	300	0.5	GR314696-1
8	5	2	CD163	Abcam	ab218293	300	0.5	GR314696-1
3	6	4	CD20	eBioscience	50-0202-80	1000	0.2	1917808
5	5	4	CD20	eBioscience	50-0202-80	1000	0.2	1917808
6	4	4	CD20	eBioscience	50-0202-80	1000	0.2	1917808
7	7	4	CD20	eBioscience	50-0202-80	1000	0.2	1917808
2	2	3	CD3D	Abcam	ab208514	150	0.5	GR3184181-1
3	5	3	CD3D	Abcam	ab208514	150	0.5	GR3184181-1
5	6	3	CD3D	Abcam	ab208514	150	0.5	GR3184181-1
6	1	3	CD3D	Abcam	ab208514	150	0.5	GR3184181-1
7	3	3	CD3D	Abcam	ab208514	150	0.5	GR3184181-1
5	4	2	CD4	R&D	FAB8165G	300	0.5	adya0116061
6	5	2	CD4	R&D	FAB8165G	300	0.5	adya0116061
7	7	2	CD4	R&D	FAB8165G	300	0.5	adya0116061
3	4	3	CD68	CST	79594S	1000	0.5	lot 1 07/2017
5	3	3	CD68	CST	79594S	1000	0.5	lot 1 07/2017
6	6	3	CD68	CST	79594S	1000	0.5	lot 1 07/2017
7	8	3	CD68	CST	79594S	1000	0.5	Lot 1 07/2017
2	6	4	CD8a	eBioscience	50-0008-82	200	0.5	E24991-10
3	7	4	CD8a	eBioscience	50-0008-82	200	0.5	E24991-10
5	2	4	CD8a	eBioscience	50-0008-82	200	0.5	E24991-10
6	3	4	CD8a	eBioscience	50-0008-82	200	0.5	E24991-10
7	8	4	CD8a	eBioscience	50-0008-82	200	0.5	E24991-10
3	6	3	FOXP3	eBioscience	41-4777-82	150	0.5	1928216
5	8	3	FOXP3	eBioscience	41-4777-82	150	0.5	1928216
6	7	3	FOXP3	eBioscience	41-4777-82	150	0.5	1928216
7	1	3	FOXP3	eBioscience	41-4777-82	150	0.5	1928216
3	8	2	IBA1	Abcam	ab195031	500	0.5	GR229273-5
5	1	2	IBA1	Abcam	ab195031	500	0.5	GR229273-5
6	2	2	IBA1	Abcam	ab195031	500	0.5	GR229273-5
7	6	2	IBA1	Abcam	ab195031	500	0.5	GR229273-5
3	3	3	Keratin	eBioscience	41-9003-82	1000	0.1	1926084
5	2	3	Keratin	eBioscience	41-9003-82	1000	0.1	1926084
6	8	3	Keratin	eBioscience	41-9003-82	1000	0.1	1926084
7	4	3	Keratin	eBioscience	41-9003-82	1000	0.1	1926084

Supplementary Table 6: Correlation in staining intensity between early and late cycles.

Part 1. Overlapped histogram region for antigenicity with cycle number in t-CyCIF.

Cycle No.	CD3D	CD4	CD8a	CD20	FOXP3*	IBA1	CD14	CD68	CD163	Keratin*
2	1		1				1		1	
3	0.76		0.45	1	1	1		1	0.7	1
5	0.64	1	0.68	0.88	0.6	0.8	0.46	0.29		0.35
6	0.21	0.77	0.79	0.91	0.31	0.74	0.38	0.29		0.45
7	0.24	0.72	0.87	0.85	0.29	0.59	0.93	0.2		0.24
8									0.5	

* The signal intensity of the FOXP3- and keratin-negative population decreased at high cycle numbers in t-CyCIF, while the signal from positive populations stabilized.

Part 2. The percentage of T cells and macrophages identified by CD3 and CD68 antibodies in different cycles by applying different cut-off values

Antibody	Cycle No	Cut off value (log)	% of positive population
CD3D	2	7.49	41.60%
CD3D	3	7.49	46.85%
CD3D	5	6.91	42.07%
CD3D	6	5.44	43.09%
CD3D	7	5.52	47.05%
CD68	3	6.55	11.14%
CD68	5	5.39	12.17%
CD68	6	5.39	13.55%
CD68	7	5.3	10.58%

Supplementary Table 7: The percentage of tumor cells, fibroblasts and immune cells (percentage of all cells) in lung cancer cases.

Cell types	Sub-types	LUNG-1-LN	LUNG-2-BR	LUNG-3-PR
Keratin+ (tumor cells)		20.49	44.45	23.16
	Keratin+/PD-L1+ (PD-L1+ tumor cells)	4.21	0.4	6.08
	Keratin+/PD-L1- (PD-L1- tumor cells)	16.28	44.05	17.07
	Keratin+/Ki67+ (Cycling tumor cells)	6.48	3.1	4.53
	Keratin+/Ki67- (Non-Cycling tumor cells)	14.01	41.35	18.63
	Ratio (Ki67+/Ki67-)	46.25	7.49	24.31
CD45+ or IBA1 + (immune cells)		59.25	34.06	58.15
	CD45+ or IBA1 +/PD-L1+ (PD-L1+ immune cells)	13.34	2.88	8.18
	CD45+ or IBA1 +/PD-L1- (PD-L1- immune cells)	45.9	31.17	49.97
	CD45+ or IBA1 +/Ki67+ (Cycling immune cells)	8.98	1.49	6.18
	CD45+ or IBA1 +/Ki67- (Non-Cycling immune cells)	50.27	32.56	51.97
	Ratio (Ki67+/Ki67-)	17.86	4.57	11.89
aSMA+ (fibroblasts)		23.39	11.78	34.98
	a SMA+/Ki67+ (Cycling fibroblasts)	1.89	0.44	1.51
	a SMA+/Ki67- (Non-Cycling fibroblasts)	21.5	11.34	33.46
	Ratio (Ki67+/Ki67-)	8.79	3.88	4.51
GFAP+ (glial cells)		0	15.63	0
	GFAP+/Ki67+ (Cycling glial cells)	0	0.09	0
	GFAP+/Ki67- (Non-Cycling glial cells)	0	15.54	0
	Ratio (Ki67+/Ki67-)	N/A	0.57	N/A

LUNG-1-LN: lung adenocarcinoma metastasis to lymph node
LUNG-2-BR: lung squamous cell carcinoma metastasis to brain
LUNG-3-PR: primary lung squamous cell carcinoma

Supplementary Table 8: The immune profile (percentage of all immune cells) in lung cancer cases.

Immune cell types	Sub-types	LUNG-1-LN	LUNG-2-BR	LUNG-3-PR
Lymphocytes components				
CD45+		63.01	59.3	87.43
CD45RB+		81.48	48.93	75.1
CD45+/CD45RB+		56.22	36.39	70.36
CD45+/CD3+ (T cells)		44.92	11.66	26.71
	CD45+/CD3+/CD4+ (CD4+ T cells)	11.75	8.48	8.44
	CD45+/CD3+/CD8a+ (CD8a+ T cells)	30.34	1.2	11.36
	CD45+/CD3+/CD4-/CD8a- (CD4-/CD8a- T cells)	5.77	2.27	8.31
CD45+/CD20+ (B cells)		1.9	0.16	13.15
CD45+/CD3-/CD20-		17.22	47.5	51.92
Inhibitory T cells components				
CD45+/CD3+/PD1-		41.32	10.92	24.22
CD45+/CD3+/PD1+		3.6	0.75	2.49
	CD45+/CD3+/CD4+/CD8a-/PD1+	1.85	0.64	0.45
	CD45+/CD3+/CD4-/CD8a+/PD1+	2.14	0.071	1.89
CD45+/CD3+/LAG3+		1.31	0.045	1.34
	CD45+/CD3+/CD4+/CD8a-/LAG3+	0.16	0.032	0.14
	CD45+/CD3+/CD4-/CD8a+/LAG3+	1.1	0.019	1.09
CD45+/CD3+/PD1+/LAG3+		0.87	0.045	1.21
	CD45+/CD3+/CD4+/CD8a-/LAG3+/PD1+	0.073	0.026	0.084
	CD45+/CD3+/CD4-/CD8a+/LAG3+/PD1+	0.67	0.013	0.97
CD45+/CD3+/FOXP3+		2.98	2.78	7.89
	CD45+/CD3+/CD4+/CD8a-/FOXP3+	2.31	2.65	6.08
	CD45+/CD3+/CD4-/CD8a+/FOXP3+	0.031	0.0065	0.66
CD45+/PD-L1+		14.81	5.32	12.34
	CD45+/CD3+/PD-L1+	10.07	0.79	4.42
	CD45+/CD20+/PD-L1+	0.35	0	0.73
Macrophages components				
IBA1+/CD163+		38.34	70.14	27.92
IBA1+/CD68+		8.59	55.8	13.57
IBA1+/CD14+		38.39	69.89	33.87
IBA+/CD11b+		14.87	53.77	18.78
IBA1-/CD11b+		3.07	6.91	11.42
IBA1+/PD-L1+		18.51	7.94	10.36
CD19		0	0	0

LUNG-1-LN: lung adenocarcinoma metastasis to lymph node
LUNG-2-BR: lung squamous cell carcinoma metastasis to brain
LUNG-3-PR: primary lung squamous cell carcinoma

Supplementary Table 9: Regional enrichment analysis (percentage of all immune cells) of rare immune cells in primary lung cancer LUNG-3-PR.

Regional enrichment analysis*	Tumor		Non-Tumor			
% of total immune cells	Mean	SEM	Mean	SEM	FC	P-Value
CD45+/CD3+/FOXP3+/CD4+/CD8a-	6.60	0.02	2.91	0.02	2.27	0.00
CD45+/CD3+/FOXP3+/CD4-/CD8a+	0.74	0.00	0.12	0.00	6.02	0.00
CD45+/CD3+/PD1+/LAG3+/CD4+/CD8a-	0.10	0.00	0.00	0.00	Inf	0.00
CD45+/CD3+/PD1+/LAG3+/CD4-/CD8a+	1.10	0.01	0.08	0.00	14.36	0.00
Manual counting**	Tumor		Non-Tumor			
% of total immune cells	Mean	SEM	Mean	SEM	FC	P-Value
CD45+/CD3+/FOXP3+/CD4+/CD8a-	6.84	1.53	1.46	1.08	4.68	0.02
CD45+/CD3+/FOXP3+/CD4-/CD8a+	1.40	0.28	0.27	0.27	5.11	0.02
CD45+/CD3+/PD1+/LAG3+/CD4+/CD8a-	0.28	0.09	0.00	0.00	Inf	0.01
CD45+/CD3+/PD1+/LAG3+/CD4-/CD8a+	1.19	0.29	0.00	0.00	Inf	0.00

Inf: Infinity
SEM: standard error of the mean
FC: fold change

* To analyze the relative frequency of rare immune cell types in the tumor region versus the non-tumor region, we defined the tumor region as all cells within 80 µm of the tumor cells (defined by keratin positivity) so as to capture direct contact between tumor cells and their immediate surroundings. The rare cells were then counted in both the tumor and non-tumor regions, and normalized to all immune cell counts (CD45+ or IBA1+) present in the imaged section. To estimate the sampling error for such rare populations, we performed bootstrapping by resampling each region's cells with replacement and applying the same cutoffs.

* *Detailed information listed below: Five frames of tumor region and non-tumor region were selected to count rare immune cells in primary lung cancer LUNG-3-PR according to presence of keratin expression (labeled tumor cells) respectively, then a t-test was performed to compare the differences between groups. The 'manual' counting was performed by visual review of images by a trained pathologist.

Detailed information of manual counting of rare immune cells in primary lung cancer LUNG-3-PR

	Tumor Region					Non-Tumor Region				
Frame	1	2	58	70	130	98	99	86	87	88
Total number of all immune cells (CD45+ or IBA1+)	203	221	337	564	338	57	42	73	36	59
CD45+/CD3+/FOXP3+/CD4+/CD8a-										
Number	15	21	36	19	11	1	0	0	2	0
% of total immune cells	7.39	9.50	10.68	3.37	3.25	1.75	0.00	0.00	5.56	0.00
CD45+/CD3+/FOXP3+/CD4-/CD8a+										
Number	4	3	7	4	3	0	0	1	0	0
% of total immune cells	1.97	1.36	2.08	0.71	0.89	0.00	0.00	1.37	0.00	0.00
CD45+/CD3+/PD1+/LAG3+/CD4-/CD8a+										
Number	4	2	6	4	2	0	0	0	0	0
% of total immune cells	1.97	0.90	1.78	0.71	0.59	0.00	0.00	0.00	0.00	0.00
CD45+/CD3+/PD1+/LAG3+/CD4+/CD8a-										
Number	1	1	0	1	1	0	0	0	0	0
% of total immune cells	0.49	0.45	0.00	0.18	0.30	0.00	0.00	0.00	0.00	0.00

Supplementary Table 10: Automated systematic identification of rare PD-1 expressing immune cell populations and visual assessment of results

Cluster	Marker expression combinations identified by automated	Automated calls (number of each cell type identified for each cluster)	Visual Review Consistent with Automated Calls	Visual Review Inconsistent with Automated Calls	Confirmed rare subtype (cluster validated)
1	CD45+/CD45RB+/CD3+/CD8a+/PD1+	1	l		1
		2		1	
		3		1	
		4	1		
		5		1	
		6	1		
		7	1		
		8		1	
		9	1		
		10	1		
		11		1	
		12	1		
2	CD45+/CD45RB+/CD3+/CD4+/PD1+	1	1		1
		2		1	
		3		1	
		4	1		
		5	1		
		6	1		
		7	1		
		8	1		
		9	1		
		10	1		
		11	1		
3	CD45+/CD45RB+/CD3+/CD8a+/PD1+/LAG3+	1	1		1
		2		1	
		3		1	
		4	1		
		5	1		
		6	1		
		7		1	
		8	1		
		9		1	
		10	1		
4	CD45+/CD45RB+/CD3+/CD8a+/PD1+/LAG3+/PD-L1+	1	1		1
		2	1		
		3		1	
		4		1	
		5		1	
		6	1		
		7		1	
		8		1	
		9	1		
		10		1	
5	CD45+/CD45RB+/CD3+/CD4+/CD20+/PD1+	1		1	
		2		1	
		3		1	
		4		1	
		5		1	
		6		1	
		7		1	
		8		1	
		9		1	
		10		1	
6	CD45+/CD45RB+/IBA1+/CD163+/CD14+/CD68+/CD11b+/PD1+/LAG3+/PD-L1+	1		1	1
		2	1		
		3	1		
		4		1	
		5		1	
		6		1	
7	CD45+/CD45RB+/CD3+/PD1+	1		1	
		2		1	
		3		1	
		4		1	
		5		1	
		6		1	
8	CD45+/CD45RB+/CD3+/CD8a+/IBA1+/PD1+	1	1		1
		2		1	
		3		1	
		4		1	
		5		1	
		6		1	
9	CD45+/CD45RB+/CD3+/CD8a+/CD20+/PD1+/LAG3+	1		1	
		2		1	
		3		1	
		4		1	
		5		1	
		6		1	
10	CD45+/CD45RB+/IBA1+/CD163+/CD14+/CD68+/PD1+/LAG3+/PD-L1+	1	1		1
		2		1	
		3	1		
		4	1		
		5		1	
11	CD45+/CD45RB+/CD3+/CD8a+/FOXP3+/PD1+/LAG3+	1	1		1
		2		1	
		3		1	

		4	1		
		5		1	
12	CD45+/CD45RB+/CD3+/CD4+/CD8a+/PD1+	1		1	
		2		1	
		3		1	
		4		1	
		5		1	
13	CD45+/CD45RB+/IBA1+/CD14+/CD11b+/PD1+/LAG3+/PD-L1+	1		1	
		2		1	
		3		1	
		4		1	
14	CD45+/CD45RB+/CD3+/CD20+/PD1+	1		1	
		2		1	
		3		1	
		4		1	
15	CD45+/CD45RB+/CD3+/IBA1+/CD14+/CD11b+/PD1+/LAG3+/PD-L1+	1		1	
		2		1	
		3		1	
		4		1	
16	CD45+/CD45RB+/CD3+/CD8a+/CD20+/PD1+	1		1	
		2		1	
		3		1	
		4		1	
17	CD45+/CD45RB+/CD3+/CD8a+/FOXP3+/PD1+	1	1		1
		2	1		
		3	1		
		4	1		
18	CD45+/CD45RB+/CD3+/CD4+/IBA1+/CD163+/CD14+/PD1+	1		1	
		2		1	
		3		1	
		4		1	
19	CD45+/CD45RB+/CD3+/CD8a+/CD11b+/PD1+	1		1	
		2		1	
		3		1	
20	CD45+/CD45RB+/CD3+/CD8a+/IBA1+/PD1+/PD-L1+/LAG3+	1		1	
		2		1	
		3		1	
21	45+/CD45RB+/CD3+/CD8a+/IBA1+/CD14+/CD68+/CD11b+/PD1+/PD-L1+/LAG	1		1	
		2		1	
		3		1	