Spatially variant immune infiltration scoring in human cancer tissues

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Supplementary Figures

B7 – Lung Cancer

CD68/HLA-DR/CD206/CD163

CD103/CD8α/CD4/TCF1

PanKeratin/ E-cadherin/ SMA/Collagen I

CD45RO/ CD3/ Granzyme B/CD20

F7 – Matched Metastatic Lymph Node

CD68/HLA-DR/CD206/CD163

CD103/CD8α/CD4/TCF1

PanKeratin/ E-cadherin/ SMA/Collagen I

CD45RO/ CD3/ Granzyme B/CD20
Supplementary Figure 1. Representative IMC images of primary lung cancer sample B7 and its matched metastatic lymph node sample F7. Scale bars represent 500-µm.
### C3 – Lung Cancer

| CD68/HLA-DR/CD206/CD163 | CD103/CD8α/CD4/TCF1 |
|--------------------------|----------------------|
| ![Image](C3_Lung_Cancer_CD68_HLA-DR_CD206_CD163.png) | ![Image](C3_Lung_Cancer_CD103_CD8alpha_CD4_TCF1.png) |
| PanKeratin/E-cadherin/SMA/Collagen I | CD45RO/CD3/Granzyme B/CD20 |
| ![Image](C3_Lung_Cancer_PanKeratin_E-cadherin_SMA_Collagen_I.png) | ![Image](C3_Lung_Cancer_CD45RO_CD3_Granzyme_B_CD20.png) |

### G3 – Matched Metastatic Lymph Node

| CD68/HLA-DR/CD206/CD163 | CD103/CD8α/CD4/TCF1 |
|--------------------------|----------------------|
| ![Image](G3_Matched_Metastatic_Lymph_Node_CD68_HLA-DR_CD206_CD163.png) | ![Image](G3_Matched_Metastatic_Lymph_Node_CD103_CD8alpha_CD4_TCF1.png) |
| PanKeratin/E-cadherin/SMA/Collagen I | CD45RO/CD3/Granzyme B/CD20 |
| ![Image](G3_Matched_Metastatic_Lymph_Node_PanKeratin_E-cadherin_SMA_Collagen_I.png) | ![Image](G3_Matched_Metastatic_Lymph_Node_CD45RO_CD3_Granzyme_B_CD20.png) |
**Supplementary Figure 2.** Representative IMC images of primary lung cancer sample C3 and its matched metastatic lymph node sample G3. Scale bars represent 500-µm.
### D1 – Lung Cancer

| CD68/HLA-DR/CD206/CD163 | CD103/CD8α/CD4/TCF1 |
|--------------------------|---------------------|
| ![Image](image1)         | ![Image](image2)    |
| ![Image](image3)         | ![Image](image4)    |
| ![Image](image5)         | ![Image](image6)    |

| PanKeratin/ E-cadherin/ SMA/Collagen I | CD45RO/ CD3/ Granzyme B/CD20 |
|---------------------------------------|-------------------------------|
| ![Image](image7)                      | ![Image](image8)              |
| ![Image](image9)                      | ![Image](image10)             |
| ![Image](image11)                     | ![Image](image12)             |

### H1 – Matched Metastatic Lymph Node

| CD68/HLA-DR/CD206/CD163 | CD103/CD8α/CD4/TCF1 |
|--------------------------|---------------------|
| ![Image](image13)        | ![Image](image14)   |
| ![Image](image15)        | ![Image](image16)   |
| ![Image](image17)        | ![Image](image18)   |

| PanKeratin/ E-cadherin/ SMA/Collagen I | CD45RO/ CD3/ Granzyme B/CD20 |
|---------------------------------------|-------------------------------|
| ![Image](image19)                    | ![Image](image20)            |
| ![Image](image21)                    | ![Image](image22)            |
| ![Image](image23)                    | ![Image](image24)            |


**Supplementary Figure 3.** Representative IMC images of primary lung cancer sample D1 and its matched metastatic lymph node sample H1. Scale bars represent 500-µm.
Supplementary Figure 4. Representative IMC images of primary lung cancer sample D2 and its matched metastatic lymph node sample H2. Scale bars represent 500-µm.
D5 – Lung Cancer

CD68/HLA-DR/CD206/CD163

CD103/CD8α/CD4/TCF1

PanKeratin/ E-cadherin/ SMA/ Collagen I

CD45RO/ CD3/ Granzyme B/ CD20

H5 – Matched Metastatic Lymph Node

CD68/HLA-DR/CD206/CD163

CD103/CD8α/CD4/TCF1

PanKeratin/ E-cadherin/ SMA/ Collagen I

CD45RO/ CD3/ Granzyme B/ CD20
Supplementary Figure 5. Representative IMC images of primary lung cancer sample D5 and its matched metastatic lymph node sample H5. Scale bars represent 500-μm.
### D9 – Lung Cancer

| CD68/HLA-DR/CD206/CD163 | CD103/CD8α/CD4/TCF1 |
|-------------------------|----------------------|

| PanKeratin/ E-cadherin/ SMA/Collagen I | CD45RO/ CD3/ Granzyme B/CD20 |

### H9 – Matched Metastatic Lymph Node

| CD68/HLA-DR/CD206/CD163 | CD103/CD8α/CD4/TCF1 |
|-------------------------|----------------------|

| PanKeratin/ E-cadherin/ SMA/Collagen I | CD45RO/ CD3/ Granzyme B/CD20 |
Supplementary Figure 6. Representative IMC images of primary lung cancer sample D9 and its matched metastatic lymph node sample H9. Scale bars represent 500-µm.
Lung Cancer Tissues

B7

Matched Metastatic Lymph Node

F7

Age: 42
Sex: Male
Pathology diagnosis: SCC
TNM: T3N1M0
Grade: 3
Stage: IIIA
Type: Malignant

C3

G3

Age: 63
Sex: F
Pathology diagnosis: SCC
TNM: T3N1M0
Grade: 3
Stage: IIIA
Type: Malignant

D1

H1

Age: 65
Sex: M
Pathology diagnosis: ADENOCA
TNM: T2N1M0
Grade: 3
Stage: IIIA
Type: Malignant
Supplementary Figure 7. Representative H&E images of primary lung cancer samples and their matched metastatic lymph node samples in patients’ cohort 1.

*SCC: squamous cell carcinoma, * ADENOA: adenocarcinoma. Scale bars represent 500-µm.
Lung Cancer Tissues

H2

- Age: 59
- Sex: M
- Pathology diagnosis: ADENOCARA
- TNM: T3N1M0
- Grade: 3
- Stage: IIA
- Type: Malignant

Matched Metastatic Lymph Node

H5

- Age: 60
- Sex: M
- Pathology diagnosis: ADENOCARA
- TNM: T2N1M0
- Grade: 3
- Stage: IIA
- Type: Malignant

H9

- Age: 52
- Sex: F
- Pathology diagnosis: ADENOCARA
- TNM: T2N1M0
- Grade: 3
- Stage: IIA
- Type: Malignant
**Supplementary Figure 8.** Representative H&E images of primary lung cancer samples and their matched metastatic lymph node samples in patients’ cohort 1.

*SCC: squamous cell carcinoma, * ADENOCA: adenocarcinoma. Scale bars represent 500-µm.
Supplementary Figure 9. Virtual H&E images of the patients' cohort 2 using imaging mass cytometry data. Scale bars represent 200-µm.

a. Multiple markers were used to identify the cytosol (Vimentin, CD68, MHC-II, Granzyme B, CD20, E-cadherin, Pancerin) and the nucleus (Histone 3, H3K9me3, Ki67, FoxP3, Intercalator 191Ir/193Ir). The cytosol was presented in a purple shade whereas the nuclei were presented in a blue shade.

b. Virtual H&E images of the patients' (n=14) lung cancer samples were illustrated.
Marker Intensity Variation with the Cell-based Segmentation on UMAP
Supplementary Figure 10. Variation in the mean intensity of single cell markers across all samples in cohort 1 was displayed on t-distributed stochastic neighbor embedding (t-SNE) embedding. Red indicated a high expression level and blue demonstrated a low expression level at the single-cell level (Number of patient samples = 12 and n = 264,191 cells).
Combined Markers
Tumor/ Stroma/ CD8α/ M1/ M2
Supplementary Figure 11. Representative tissue images of cohort 1 patients’ primary lung cancer samples and their matched metastatic lymph nodes (B7-F7, C3-G3, D1-H1, D2-H2, D5-H5, D9-H9) were shown by combining tumor region (purple), stroma region (yellow), CD8α (green), M1 (red), and M2 (cyan). Scale bars represent 200-µm.
Supplementary Figure 12. Representative tissue images of cohort 1 patients’ primary lung cancer samples and their matched metastatic lymph nodes including B7/F7, C3/G3, and D1/H1 were presented. Tissue regions were marked as tumor region (purple), stroma region (yellow), CD8α (green), M1 (red), and M2 (cyan). Scale bars represent 200-µm.
**Supplementary Figure 13.** Representative tissue images of cohort 1 patients’ primary lung cancer samples and their matched metastatic lymph nodes including D2/H2, D5/H5, and D9/H9 were provided. Individual anatomical regions were marked as tumor region (purple), stroma region (yellow), CD8α (green), M1 (red), and M2 (cyan). Scale bars represent 200-µm.
**Supplementary Figure 14.** Comparisons of spatial cell neighborhood graphs constructed at different pixel distances radius between centroids were illustrated. A radius of 10-µm was used for the far left column to connect the cell centroids, 20-µm for the center-left panel, 30-µm for the center-right panel, and 40-µm for the right panel. The optimal radius was chosen empirically with the radius such that the constructed graph captured cell neighboring information at the best. Scale bars represent 50-µm.
Marker Intensity Variation with the Pixel-based Segmentation on UMAP
Supplementary Figure 15. The intensity variation of pixel-level markers across all samples in cohort 1 using the Uniform Manifold Approximation and Projection (UMAP) embedding (n=5,093,784 pixels).
**Supplementary Figure 16.** Pixel-level immune microenvironment classification outperforms the cell-level segmentation in identifying stromal and dense tumor regions. Comparisons of cell-level and pixel-level immune microenvironment classification were shown with zoomed-in examples per region (right). White Scale bars represent 500-µm and yellow scale bars represent 50-µm.
A) PD-L1 Expression on M2-polarized tumor-associated macrophages

B) Infiltration of proliferating antigen-experienced T-cells

C) Infiltration of tissue-resident memory T-cells with M1-polarized macrophages

D) Infiltration of regulatory T-cells
**Supplementary Figure 17.** Visual representation of immune cellular phenotypes included PD-L1+ M2 tumor-associated macrophages, antigen-experienced T-cells, tissue-resident memory T-cells, and regulatory T-cells using imaging mass cytometry raw data. Scale bars represent 50-µm.

**a** PD-L1+ M2 macrophages were shown with the expression of CD68 (blue), CD206 (red), CD163 (yellow), and PD-L1 (white).

**b** Proliferating antigen-experienced T-cells were demonstrated with the expression of CD8α (blue), CD45RO (red), and Ki67 (yellow).

**c** Tissue-resident memory T-cells were indicated with the expression of CD8α (yellow) and CD103 (red).

**d** Regulatory T-cells were presented with the expression of CD4 (red) and FoxP3 (yellow).
Percentile cut off for Low (0.0, 2.255] < Medium (2.255, 6.695] < High (6.695, 10.0]
**Supplementary Figure 18.** High-dimensional patient stratification for the immune infiltration.

a Weighted CD8-T infiltration scores within the two patients’ cohorts were shown using a bar plot. CH1 refers to cohort 1 and CH2 refers to cohort 2. 75th percentile scores were considered high (Red) and 25th percentile scores were considered low (Purple). The remaining scores were considered intermediate (Green).

b 2D scatter plot of the CD68-T vs. CD8-T distribution for the two patients’ cohorts based on their immune infiltration scores (low in purple, intermediate in green, and high in red) was demonstrated.

c UMAP provided the distribution of the patients within the new patients’ cohort based on their SpatialVizScore for all possible interactions (T-CD8, T-M1, T-M2, etc).

d-e 3D scatter plot of the CD8-T, M1-T, and M2-T distribution for the patients within the second cohort of patients was presented. The arrows indicated the ascending distribution of CD8-T scores.
Supplementary Figure 19. Comparisons of single-cell segmentation techniques using a deep-learning method (CellPose) and open-source software (CellProfiler) were presented. Segmentation results were overlaid on virtual H&E for samples in cohort 2. Single-cell segmentation results from both methods were similar in terms of performance. Scale bars represent 200-µm.
Primary Lung Cancer ROIs

Cluster 1
Cluster 2
Cluster 3
Cluster 4
Cluster 5
Cluster 6
Cluster 7
Cluster 8
Cluster 9
Cluster 10
Cluster 11
Cluster 12
Cluster 13
Cluster 14
Cluster 15
Cluster 16
Cluster 17
Cluster 18
Cluster 19
Cluster 20
Cluster 21
Cluster 22
Cluster 23
Cluster 24
Cluster 25
Cluster 26
Cluster 27
Cluster 28

Cluster 1
Cluster 2
Cluster 3
Cluster 4
Cluster 5
Cluster 6
Cluster 7
Cluster 8
Cluster 9
Cluster 10
Cluster 11
Cluster 12
Cluster 13
Cluster 14
Cluster 15
Cluster 16
Cluster 17
Cluster 18
Cluster 19
Cluster 20
Cluster 21
Cluster 22
Cluster 23
Cluster 24
Cluster 25
Cluster 26
Cluster 27
Cluster 28
**Supplementary Figure 20.** Benchmarking SpatialVizScore against HistoCAT was presented. The top row demonstrated the marker expression profile for regions of interest (ROIs) in cohort 1 patients and their distribution on a t-SNE plot. The bottom row provided the results from the HistoCAT’s unsupervised clustering with the cluster marker expression and their distribution on a t-SNE plot.
Supplementary Figure 21. Benchmarking SpatialVizScore against HistoCAT was shown. The top row indicated expression profiles of the marker for ROIs in lymph nodes of cohort 1 patients and their distribution on a t-SNE plot. The bottom row demonstrates the results from the HistoCAT’s unsupervised clustering with the cluster marker expression and their distribution on a t-SNE plot.
Supplementary Figure 22. Benchmarking SpatialVizScore (a) against Giotto (b) was shown. Our method results in a similar clustering result compared to Giotto. Giotto shows the centroid of the segmented cells whereas our method shows the cell mask with different morphological variations.
Spectrum of Macrophages Polarization

Pro-inflammation  Anti-inflammation

Tumor with **low** M2/M1 Ratio
- Favorable prognosis
- Longer overall survival
- Better drug response

Tumor with **high** M2/M1 Ratio
- Aggressive tumors
- Poor drug response
- Poor prognosis

Cell-Based M2/M1 Analysis

Pixel-Based M2/M1 Analysis
Supplementary Figure 23. The spectrum of macrophage polarization was implemented within SpatialVizScore.

a Macrophages exhibit a spectrum of phenotypes that are classified as M1, M2a, M2b, M2c, and M2d. They exhibit a range of pro-inflammation to anti-inflammation functions.

b M2/M1 ratios showed a more consistent correlation with lung cancer disease progression and outcomes than the discrete count of M1 and M2 macrophages.

c-d M2/M1 ratio was quantified by two approaches including the cell-based (c) and the pixel-based (d) analysis. Created with BioRender.com. White Scale bars represent 500-μm, and yellow scale bars represent 50-μm.
**Supplementary Figure 24.** M2/M1 ratio analysis using both the cell-based analysis and the pixel-based analysis was presented. The first column indicated cell-based M2/M1 spectrum analysis. The second column demonstrated pixel-based M2/M1 spectrum analysis. The respective zoomed-in region was shown in the last two columns. White Scale bars represent 500-µm and yellow scale bars represent 50-µm.
**Supplementary Figure 25.** Validation of the SpatialVizScore on breast cancer H&E images with drug response data was shown.

**a** The workflow of the analysis included annotating tumor regions on patients’ H&E images, classifying cell phenotypes based on their morphologies, generating their network, and quantifying their neighborhood score.

**b** Examples of spatial variant immunoscore for non-responder (left-top) compared to responder (left-bottom). Responder patients yielded higher immunoscores than non-responder patients (right). P-value calculated using Wilcoxon Rank Sum Test. Box plot demonstrated the distribution of the data with a minimum, first quartile (Q1), median, third quartile (Q3), and maximum. Original Data was overlaid on the boxplot.
Supplementary Figure 26. Validation of the SpatialVizScore rationale on lung cancer H&E whole slide images was demonstrated.

a The workflow of the analysis included annotating tumor regions on patients’ H&E images, classifying cell phenotypes based on their morphologies, generating their network, and quantifying their neighborhood score.

b Benchmarking SpatialVizScore in generating the spatially variant immune scores that agreed well with the previously reported tumor-infiltrating lymphocytes map approach using deep learning (Saltz et al, Cell Reports 2018).
**Supplementary Figure 27.** Cell-level segmentation and clustering showed the heterogeneity of markers’ expressions within the patient population was presented.

**a** Representative schematic indicated the process of cell-level segmentation. Cell segmentation was performed using the deep-learning CellPose algorithm. The nuclei were segmented using signals from intercalators conjugated to 191Ir and 193Ir along with other markers associated with the nucleus including Histone 3, Ki67, and FoxP3. The cytosol was segmented by expanding the nuclei by 2 pixels. Number of cells n = 44,379. Created with BioRender.com

**b** The resulting data was clustered using all markers in the antibody panel excluding widely expressed markers including signals from intercalator 191Ir and 193Ir, histone 3, and MHC-II. The resulting clusters are visualized on the tissue samples by attributing each cell mask to the corresponding cluster color, yielding distinct separation of different anatomical regions. Scale bars represent 200-µm.

**c** UMAP visualized the distribution and the separation of the resulting 15 clusters from the single-cell phenotypes showing the heterogeneity in markers’ expressions within the patient population.

**d** Correlative heatmap demonstrated the co-expression of markers across the 15 clusters that make up the data set.

**e** Marker abundance heatmap presented the cluster distributions for all patients’ tissues.
**Supplementary Figure 28.** Tissue neighborhood analysis was demonstrated using single-cell data to identify the tumor/epithelial, stromal, and immune composition of patients’ tissues.

a Representative schematic provided the process of neighborhood analysis. Each cell is assigned to a distinct type (tumor/epithelial, stroma, CD8+, or CD68+) based on the highest intensity values of markers. Tumor cells (T) were marked by the expression of pankeratin and e-cadherin, and stromal cells (S) were marked by the expression of collagen Type 1 and SMA. Additional cells were marked by the expression of CD8α and CD68. T-marked cells may also be major epithelial cells in paracancerous/adjacent tissues. A Cell network was generated by connecting every cell centroid to its neighboring centroids within a 30-μm distance. Created with BioRender.com

b Cell network graphs on patients' samples were shown. Magenta indicated tumor/epithelial regions, yellow demonstrated stromal regions, green corresponded to the CD68+ regions, and blue provided the CD8+ tissue regions. Scale bars represent 200-μm.

C Box plot demonstrated the tissue composition of patients’ samples. Tumor/epithelial regions were the most predominant followed by stromal regions, CD68+, and lastly CD8+ regions. N = 14. Box plot demonstrated the distribution of the data with a minimum, first quartile (Q1), median, third quartile (Q3), and maximum. Original Data was overlaid on the boxplot.

d Heatmap yielded the distribution of neighborhood scores across different tissue regions.
**Supplementary Figure 29.** Spatial interaction maps and immunoscopying of the tumor microenvironment in lung cancer were presented using single-cell segmentation masks to reveal the immune continuum of patients’ cancerous and paracancerous tissues.

**a** Cell network and spatial proximity maps on lung cancer samples demonstrated the interaction of tumor/T (in magenta), stroma/S (in yellow), tumor-CD68 (in green), and tumor-CD8 (in blue). T-marked cells may also be major epithelial cells in paracancerous/adjacent tissues. Scale bars represent 200-µm.

**b** Spatial interaction map provided the distribution of tumor/epithelial and CD68+ immune cells. It follows a continuum where it’s highest for immune inflamed and immune suppressed tumors but damps down for immune cold tumors.

**c** Spatial interaction map yielded the distribution of tumor/epithelial and CD8+ immune cells. It follows a continuum across immune inflamed, immune suppressed, and immune cold tumors.

**d** Spatial density map demonstrated the distribution of tumor/epithelial density across the patients’ samples.
**Supplementary Figure 30.** Spatial immune phenotyping, interaction maps, and immunoscoring of the tumor microenvironment in lung cancer were presented using single-cell segmentation masks to the unique distribution of immune infiltration patterns.

**a** Cell mask on lung cancer samples represented the immune phenotypes in cancerous and paracancerous tissue samples, highlighting the cluster distribution on the patients’ tissues. Scale bars represent 200-µm.

**b** Spatial interaction map provided the distribution of tumor/epithelial and immune cell markers across the three different immune states.

**c** Correlative heatmap demonstrated the co-expression of markers across the 10 clusters from the multiplexed dataset.

**d** Heatmap yielded the distribution of scores from the marker neighborhoods across different tissue regions.
Supplementary Figure 31. The immune continuum for lung cancer patients, containing cancerous and paracancerous tissues, ranges can be classified into immune inflamed, immune cold, and immune desert.

a Bar graphs with stacked columns demonstrated the composition of patients’ tissues from tumor/epithelial in red, stroma in magenta, CD68+ in blue, and CD8+ in green. The top bar graph corresponded to the composition of patients’ tissues from the lowest to highest CD68+ scores whereas the bottom bar graph displayed the composition of patients’ tissues from the lowest to highest CD8+ scores.

b The distribution of immune markers across patients' samples (top) after arranging them from the lowest CD8+ to the highest (bottom).

c Individual marker distribution plots across patients samples were arranged from the lowest immune CD8+ scores (immune cold tumors) to the highest immune CD8+ cores (immune inflamed tumors). The markers included SMA, Vimentin, Pankeratin, H3K9me3, PD-L1, CD44, FoxP3, CD4, E-cadherin, CD68, CD20, CD8α, PD1, Granzyme B, Ki67, collagen 1, CD3, and CD45RO.
**Supplementary Figure 32.** Pixel-level classification and clustering reveal the distinct distributions within patients’ samples.

**a** Representative schematic demonstrates the process of pixel classification. The intensity information for all markers at all pixel locations (pixel size = 1-µm) was extracted and normalized. The background signal was filtered by eliminating pixel locations with an intensity value lower than 0.3. The resulting marker intensity data considered as non-background was clustered. Number of pixels \( n = 1,589,784 \).

**b** The resulting pixel intensity data was clustered using all markers in the antibody panel excluding widely expressed markers including signals from intercalator 191Ir and 193Ir, histone 3, and MHC-II. The resulting clusters are visualized on the tissue samples by attributing each pixel to the corresponding cluster color, indicating distinct separation of different anatomical regions. Scale bars represent 200-µm.

**c** UMAP visualized the distribution and the separation of the resulting 16 clusters from the pixel-level phenotypes.

**d** Correlative heatmap demonstrated the co-expression of markers across the 16 clusters that make up the data set.

**e** Marker abundance heatmap provided the cluster compositions for all patients’ tissues.
**Supplementary Figure 33.** Spatial interaction maps and immunoscoring of the tumor microenvironment in lung cancer using pixel-level classification outperform in segmenting stromal regions.

a Pre-identified marker images were used to mark distinct tissue regions (Tumor/epithelial: pankeratin and e-cadherin, Stroma: SMA and collagen Type 1, CD8α, and CD68) resulting in 4 final images. The maximum projection image is generated by dividing each ROI image into patches with pixel size = 5-μm where each patch is assigned a tissue region based on the maximum intensity value of the pre-identified markers list. Pixel neighborhood analysis was then performed within the unique tissue regions.

b Maximum projection images of ROIs were shown using magenta for tumor/epithelial regions, yellow for stromal regions, green for CD68+ regions, and blue for CD8+ regions. Scale bars represent 200-μm.

c Box plot indicated the tissue composition of patients’ samples. Tumor/epithelial regions are the most predominant followed by stromal regions, CD68+, and lastly CD8+ regions. N = 14. Box plot demonstrated the distribution of the data with a minimum, first quartile (Q1), median, third quartile (Q3), and maximum. Original Data was overlaid on the boxplot.

d Heatmap demonstrated the density across different tissue regions.
**Supplementary Figure 34.** Pixel-level immune microenvironment classification outperforms the cell-level segmentation in identifying stromal and dense tumor regions.

a Tissue regions in an 800-µmx800-µm section can be visualized by assigning false colors to isotope signals and their conjugated antibodies and delimiter showing the region of comparison in each ROI. Scale bars represent 200-µm.

b Zoomed-in views of tissue regions in each ROI were shown. Scale bars represent 50-µm.

c Single cell level reconstruction of tissue was presented. The view was created with the cell mask assigned to the corresponding cell type color.

d Pixel level reconstruction of tissue was demonstrated. The view was created with pixel patches region assigned to corresponding marker type color with patches size of 5.

e Pixel-level reconstruction of tissue was presented. The view was created with pixel patches region assigned to corresponding marker type color with patches size of 2.

f Comparison of area density for tumor, stroma, CD8+, and CD68+ between single cell and pixel-level tissue reconstruction method. N = 14. Asterisk indicates the statistical significance for pairwise comparison. P-value calculated using Wilcoxon Rank Sum Test (ns: 0.05 < p, *: p <= 0.01). Box plot demonstrated the distribution of the data with a minimum, first quartile (Q1), median, third quartile (Q3), and maximum. Original Data was overlaid on the boxplot.

g Line plot demonstrated the variation of area density across ROI for single cell level reconstruction of tissue.

h Line plot provided the variation of area density across ROI for the pixel-level reconstruction of tissue.
**Supplementary Figure 35.** Cell-level segmentation and pixel-level classification are in agreement in immunoscopying lung cancer patients’ samples.

**a** Cell-level segmentation and pixel-level classification analysis were used to quantify spatially variant immunoscores. Created with [BioRender.com](http://BioRender.com)

**b** Heatmaps demonstrated the cell-level and the pixel-level immunoscores for all cancer subtypes that were included within the patient population.

**c** Representative schematic provided the difference between cell-based and pixel-based immunoscopying. The cell-based immunoscore relies on cell segmentation masks to identify individual cells. The cell network is generated by identifying the distance between cell centroids within a diameter of 30 µm distance. The pixel-level immunoscore relies on the pixel-based classification where the 800 µm x 800 µm patients’ tissues are divided into 160x160 square patches with patch size = 5 µm. Each patch is assigned a phenotype based on the highest intensity of marker expression. Similar to the cell network, the pixel network is generated to assess the neighborhood of the anatomical tissue region.

**d-f** Correlation heatmaps between all scores for all ROIs were generated by **d** cell-level segmentation and pixel-level classification, **e** cell-level segmentation only, and **f** pixel-level classification only.
## Supplementary Tables

| Marker         | Clone       | Metal Tag | Dilution |
|----------------|-------------|-----------|----------|
| CD20           | H1          | 161Dy     | 1:400    |
| CD3            | Polyclonal  | 170Er     | 1:100    |
| CD4            | ERP6855     | 156Gd     | 1:200    |
| CD45RO         | UCHL1       | 173Yb     | 1:50     |
| CD68           | KP1         | 159Tb     | 1:50     |
| CD8a           | C8/144B     | 162Dy     | 1:100    |
| FoxP3          | 236A/E7     | 155Gd     | 1:30     |
| Pan-Keratin    | C11         | 148Nd     | 1:100    |
| Granzyme B     | EPR20129-17 | 167Er     | 1:100    |
| Ki-67          | B56         | 168Er     | 1:50     |
| PD-1           | ERP4877(2)  | 165Ho     | 1:50     |
| PD-L1          | SP142       | 150Nd     | 1:50     |
| SMA            | 1A4         | 141Pr     | 1:200    |
| Collagen Ty1   | Polyclonal  | 169Tm     | 1:300    |
| E-cadherin     | 24E10       | 158Gd     | 1:50     |
| Histone 3      | D1H2        | 171Yb     | 1:50     |
| Vimentin       | D21H3       | 143Nd     | 1:100    |
| CD44           | IM7         | 153Eu     | 1:100    |
| MHC-II         | 6C6         | 175Lu     | 1:200    |
| H3K9me3        | EPR16601    | 149Sm     | 1:400    |
| HLA-DR         | LN3         | 174Yb     | 1:200    |
| CD11c          | Polyclonal  | 154Sm     | 1:100    |
### Supplementary Table 1. Antibody panel used for imaging mass cytometry.

| CD163 | EDHu-1 | 147Sm | 1:100 |
|-------|--------|-------|-------|
| CD206 | 5C11   | 152Sm | 1:200 |
| CD95  | EPR5700| 160Gd | 1:250 |
| CD103 | EPR4166(2) | 151Eu | 1:400 |
| TCF1  | C63D9  | 144Nd | 1:35  |
| Intercalator | — | 191Ir/193Ir | 1:400 |

| Patient | Tissue | Age | Sex | Organ/Anatomic Site | Pathology Diagnosis | TNM | Grade | Stage | Type  |
|---------|--------|-----|-----|---------------------|---------------------|-----|-------|-------|-------|
| TMA: LC814a (Cohort 1) |
| B7      | 42     | M   | Lung | Squamous cell carcinoma | T3N1M0 | 3   | IIIA  | Malignant |
| C3      | 63     | F   | Lung | Squamous cell carcinoma | T3N1M0 | 3   | IIIA  | Malignant |
| D1      | 65     | M   | Lung | Adenocarcinoma         | T2N1M0 | 3   | IIIA  | Malignant |
| D2      | 59     | M   | Lung | Adenocarcinoma         | T3N1M0 | 3   | IIIA  | Malignant |
| D5      | 60     | M   | Lung | Adenocarcinoma         | T2N1M0 | 3   | IIA   | Malignant |
| D9      | 52     | F   | Lung | Adenocarcinoma         | T2N1M0 | 3   | IIA   | Malignant |
| F7      | 42     | M   | Lymph Node | Metastatic Squamous cell carcinoma of B7 | – | – | – | Malignant |
| G3      | 63     | F   | Lymph Node | Metastatic Squamous cell carcinoma of C3 | – | – | – | Malignant |
| H1      | 65     | M   | Lymph Node | Metastatic Adenocarcinoma of D1 | – | – | – | Malignant |
| H2      | 59     | M   | Lymph Node | Metastatic Adenocarcinoma of D2 | – | – | – | Malignant |
| H5      | 60     | M   | Lymph Node | Metastatic Adenocarcinoma of D5 | – | – | – | Malignant |
| H9      | 52     | F   | Lymph Node | Metastatic Adenocarcinoma of D9 | – | – | – | Malignant |

TMA: LC1002a (Cohort 2)
|   |   |   | Tumor | Tumor | Tumor- | Tumor- | Tumor- | Tumor- | M1-M1 | M1-M2 | M2- | M2- | CD8+ | CD8+ | CD8+ | CD8+ |   |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| B1 | 63 | F | Lung | Squamous cell carcinoma | T1N0M0 | 2 | I | Malignant |
| B3 | 55 | M | Lung | Squamous cell carcinoma | T2N0M0 | 2 | I | Malignant |
| B5 | 39 | F | Lung | Squamous cell carcinoma | T2N1M0 | 2 | II | Malignant |
| C6 | 40 | M | Lung | Cancer adjacent lung tissue | - | - | - | - |
| D7 | 66 | M | Lung | Squamous cell carcinoma | T2N2M0 | 3 | IIIA | Malignant |
| D9 | 65 | M | Lung | Squamous cell carcinoma | T2N0M0 | 3 | I | Malignant |
| E1 | 56 | M | Lung | Squamous cell carcinoma | T2N1M0 | 2 | II | Malignant |
| B6 | 39 | F | Lung | Cancer adjacent lung tissue | - | - | - | - |
| F3 | 54 | M | Lung | Adenosquamous carcinoma | T2N1M0 | 3* | II | Malignant |
| F2 | 58 | M | Lung | Cancer adjacent lung tissue | - | - | - | - |
| G1 | 73 | M | Lung | Adenocarcinoma | T2N0M0 | 2 | I | Malignant |
| G5 | 54 | F | Lung | Mucinous adenocarcinoma | T3N0M0 | 1 | IIIA | Malignant |
| E5 | 52 | M | Lung | Squamous cell carcinoma | T4N1M0 | 2* | IIIB | Malignant |
| E7 | 65 | M | Lung | Squamous cell carcinoma | T2N1M0 | 3 | II | Malignant |

**Supplementary Table 2. Patient specifications for cancer type, stage, and grade.**
| Patient Sample | Cell count density | Cell neighbor interaction density |
|----------------|-------------------|----------------------------------|
|                | Tumor             | Stroma                          | IP_T | IP_S | IN_T | IN_S |
| B1             | 0.7808            | 0.1222                          | 0.0068 | 0.0008 | 0.0393 | 0.0132 |
| B3             | 0.3335            | 0.3743                          | 0.0512 | 0.0331 | 0.0877 | 0.1076 |
| B5             | 0.6776            | 0.1491                          | 0.059  | 0.0275 | 0.0957 | 0.03  |
| C6             | 0.251             | 0.1695                          | 0.0987 | 0.0695 | 0.1551 | 0.081 |
| D7             | 0.2997            | 0.2393                          | 0.0872 | 0.099  | 0.11   | 0.1439 |
| D9             | 0.4331            | 0.2247                          | 0.0406 | 0.0256 | 0.0494 | 0.0267 |
| E1             | 0.2044            | 0.2297                          | 0.1289 | 0.1464 | 0.0723 | 0.0972 |
| B6             | 0.2703            | 0.2873                          | 0.0268 | 0.0272 | 0.0683 | 0.0462 |
| F3             | 0.217             | 0.1978                          | 0.099  | 0.1726 | 0.0462 | 0.1306 |
| F2             | 0.2098            | 0.1641                          | 0.0621 | 0.0653 | 0.1004 | 0.0732 |
| G1             | 0.0955            | 0.3996                          | 0.004  | 0.0587 | 0.035  | 0.0759 |
| G5             | 0.5114            | 0.213                           | 0.0663 | 0.0619 | 0.0571 | 0.0445 |
| E5             | 0.6521            | 0.0871                          | 0.0108 | 0.0004 | 0.0399 | 0.0095 |
| E7             | 0.8312            | 0.0833                          | 0.0216 | 0.0117 | 0.0141 | 0.0135 |
**Supplementary Table 3. Spatially variant Immunocore using the cell-based segmentation**

| Marker | Immune Cell Phenotype | Justification for adding to the immunoscore | Ref |
|--------|-----------------------|---------------------------------------------|-----|
| CD33+  | Mast cells            | Mast cells showed an inconsistent prognostic effect on different solid tumors suggesting that they can act both as pro-tumor and antitumor cells. It could be very valuable to investigate mast cells in lung cancers. | PMID: 29651440  
  PMID: 28446910 |
| CD3+   | Regulatory T cells     | Treg cells are generally associated with poor clinical outcomes and reduced overall survival. However, Treg cells in different regions of the tumor hold different prognostic values. For example, high intratumoral Treg cells are associated with much shorter survival when compared with peritumoral Treg cells. They also hold a precious clinical value to determine patients’ eligibility to receive immune checkpoint inhibitors. Treg could be a valuable addition to the immunoscores to further increase their clinical impact. | PMID: 26462617 |
| CD80+  | Macrophages           | Macrophages can exist in several activation states including M1 and M2. M1 macrophages were associated with proinflammatory and antitumor effects whereas M2 macrophages were associated with anti-inflammatory and tolerogenic properties. CD68 alone can’t differentiate between the two phenotypes, so additional antibodies could be added to the antibody panel to further study their prognostic value and reflect the findings on the immunoscore. | PMID: 29065107 |
| CD11b+ | Myeloid-derived suppressor cells | They were shown to have immunosuppressive capabilities and were shown to support tumor invasion and metastasis. They can be added to the immunoscore to reflect their negative immune modulation nature. | PMID: 29348500 |
| CD80+  | Dendritic cells       | Given the different phenotypes that make up the dendritic cell population, they are associated with differential prognostic outcomes. CD103+ DCs and CD208+ DCs were associated with positive prognostic values. Thereby, it could be interesting to leverage multiplex panels of antibodies to decipher the differential prognostic values of dendritic cells and reflect that on the immunoscores. | PMID: 25446897 |
| CD56+  | Natural killer cells  | Prior reports showed that NK at some levels was associated with positive disease outcomes. However, if NK cells are present in high | PMID: 29018445 |
amounts, their profile changes, and they become associated with negative disease outcomes. It might be valuable to use image analysis to further investigate these thresholding effects and reflect that on the immunoscore

| CD19+ CD20+ CD21+ CD40+ | B cells | Although B cells were generally linked to good disease outcomes, some studies reported a negative prognostic value of B cells. This can be attributed to the different subtypes and phenotypes of B cells. Multiplex imaging and image analysis could be used to decipher the B cells phenotypes and their differential prognostic values. | PMID: 28626234 |
| BTLA+ CD3+ CD4+ CD40L+ CD57+ CD84+ CXCR4+ CXCR5+ ICOS+ CD10+ CD150+ | T follicular helper cells | TFH was associated with positive disease outcomes and overall survival, so it could be a valuable addition to the immunoscore to further investigate its spatial prognostic value. | PMID: 29628290 |
| CD3+ CD4+ CCR3+ CCR4+ CCR8+ CXCR4+ STAT5+ STAT6+ | T helper type 2 (Th2) cells | Th2 cells show conflicting results in solid tumors, suggesting that there are several phenotypes of Th2 cells whereas each phenotype is associated with different prognostic values. Multiplex imaging technologies along with high-dimensional image analysis can be used to decipher Th2 phenotypes and their prognostic value. | PMID: 29628290 |
| CCR4+ CCR6+ CD3+ CD4+ STAT3+ Batf+ | T helper type 17 (Th17) cells | Th17 cells have differential prognostic values based on their location within patients’ tumors. Intraepithelial Th17 cells were shown to have a positive prognostic value whereas stromal Th17 didn’t maintain the same impact. Using multiplex imaging to identify Th17 along with spatial image analysis could yield a valuable spatially variant immunoscore. | PMID: 26719303 |
| CCR1+ CCR5+ CD3+ CD4+ CXCR3+ STAT1+ STAT4+ T-bet+ | T helper type 1 (Th1) cells | It is associated with a positive prognosis for several solid tumors but was associated with reduced survival for NSCLC. | PMID: 22419253 |

**Supplementary Table 4:** The clinical significance of the spatial infiltration patterns of immune cell types

|          | CD68-T | CD68-S | CD68-CD8 | CD8-T | CD8-S | CD8-CD68 |
|----------|--------|--------|----------|-------|-------|----------|
| CH2_B7   | 0.0764 | 0.0393 | 0.0583   | 0.1844| 0.0705| 0.0361   |
| CH2_C3   | 0.0806 | 0.0439 | 0.0863   | 0.2484| 0.0954| 0.0495   |
### Supplementary Table 5. Interaction score between CD8+, CD68+, tumor, and stromal cells in both cohorts 1 and 2.

| Cell Line | CD68-T  | CD68-S  | CD68-CD8 | CD8-T   | CD8-S   | CD8-CD68 |
|-----------|---------|---------|----------|---------|---------|----------|
| CH2_B7    | 4.25    | 1.81    | 3        | 6.76    | 4.03    | 1.91     |
| CH2_C3    | 4.54    | 2.08    | 4.48     | 9.18    | 5.5     | 2.65     |
| CH2_D1    | 6.07    | 1.73    | 4.86     | 5.56    | 5.18    | 3.07     |
| CH2_D2    | 2.44    | 3.17    | 2.9      | 6.96    | 7.29    | 2.37     |
| CH2_D5    | 8.8     | 0.89    | 1.06     | 10      | 1.76    | 1.2      |
| CH2_D9    | 8.86    | 7.17    | 2.94     | 7.59    | 7.88    | 2.28     |
### Supplementary Table 6

Scaled interaction score between CD8 +, CD68 +, tumor and stroma cells in cohorts 1 and 2.

| Technology            | Multiplexing limits | Immunoscore                                                                 | Reference                                                                 |
|-----------------------|--------------------|----------------------------------------------------------------------------|--------------------------------------------------------------------------|
| Hematoxylin and Eosin (H&E) | No                 | Assessing the infiltration of tumor-infiltrating lymphocytes in solid tumors including colorectal, breast, lung, prostate, etc concerning patients’ survival. | DOI: 10.21873/anticanres.13041 DOI: 10.1007/s00262-020-02834-y DOI: 10.1016/j.celrep.2018.03.086 |
| Immunohistochemistry (IHC) | Yes, 1-3          | Assessing the infiltration of CD3+ and CD8+ within the tumor core and the invasive margin of several tumor types including colon, lung, pancreatic, etc. with survival, and disease recurrence. | DOI: 10.1007/s00262-020-02834-y DOI: 10.1186/s40425-018-0488-6 DOI: 10.1038/s41379-019-0291-z |
Immunofluorescence (IF) | Yes, cyclic experiment | Assessing the infiltration of several immune markers including CD3, CD4, CD8, CD20, FoxP3, PD-L1, etc within several tumor types including lung cancer, breast cancer, etc. | DOI: 10.1093/jnci/dju435  DOI: 10.1038/s41598-021-01116-6

Imaging mass cytometry (IMC) | Yes, simultaneous detection ~35 markers | Dissecting the immune microenvironment in diffuse large B cell lymphoma using a panel of different markers checkpoint markers (PD-1, PD-L1, TIM-3, VISTA, LAG-3), chemokines and their receptors (CXCR3, CCR4), immune markers (CD3, CD3, CD8, CD45RO, FoxP3, Granzyme B, etc) | DOI: 10.1101/2021.02.01.21250775

**Supplementary Table 7.** Comparing other imaging technologies with IMC for developing immunoscores for cancers.

| Patient ID Number | Race | Clinical Response | Age (years) | Number of prior treatment lines | Checkpoint Inhibitor | Chemotherapy partner | Overall survival (months) |
|-------------------|------|-------------------|-------------|---------------------------------|----------------------|-----------------------|--------------------------|
| 1                 | black | non-responder     | 46          | 3                               | pembrolizumab        | Nab-paclitaxel        | 23                       |
| 2                 | black | non-responder     | 45          | 5                               | atezolizumab         | Nab-paclitaxel        | 18                       |
| 3                 | black | non-responder     | 32          | 0                               | pembrolizumab        | Gemcitabine/ Carboplatin | 5                       |
| 4                 | black | responder         | 66          | 0                               | atezolizumab         | Nab-paclitaxel        | 21                       |
| 5                 | white | responder         | 67          | 0                               | atezolizumab         | Nab-paclitaxel        | NA                       |
| 6                 | black | responder         | 39          | 2                               | pembrolizumab        | Gemcitabine/ Carboplatin | NA                     |

**Supplementary Table 8.** Breast cancer patient characteristics.

| Patient barcode | Cancer subtype | Tumor Status | Vital Status | Last contact day to | Death day to | Therapy type | Treatment best respond |
|-----------------|----------------|-------------|--------------|---------------------|-------------|--------------|-----------------------|
| TCGA-05-4402    | TUMOR FREE    | Dead        | [Not Available] | 244 | Chemotherapy Responder |
| TCGA-44-7660    | TUMOR FREE    | Alive       | 162          | [Not Applicable] | Vaccine | Non Responder |
| TCGA-44-A47F | TUMOR FREE | Alive | 246 [Not Applicable] | Chemotherapy | Responder |
|-------------|------------|-------|----------------------|--------------|-----------|
| TCGA-49-AAR2 | TUMOR FREE | Alive | 1945 [Not Applicable] | Chemotherapy | Responder |
| TCGA-50-5055 | WITH TUMOR | Alive | 785 [Not Applicable] | Chemotherapy | Non Responder |
| TCGA-50-5068 | WITH TUMOR | Dead | 1499 | 1499 | Chemotherapy | Non Responder |
| TCGA-53-A4EZ | TUMOR FREE | Alive | 280 [Not Applicable] | Chemotherapy | Responder |
| TCGA-55-1596 | TUMOR FREE | Alive | 1375 [Not Applicable] | Chemotherapy | Responder |
| TCGA-55-5899 | [Not Available] | Alive | 87 [Not Applicable] | Chemotherapy | Responder |
| TCGA-55-6712 | WITH TUMOR | Alive | 24 [Not Applicable] | Chemotherapy | Non Responder |
| TCGA-55-6979 | WITH TUMOR | Dead | [Not Available] | 237 | Chemotherapy | Non Responder |
| TCGA-55-6982 | WITH TUMOR | Dead | [Not Available] | 995 | Chemotherapy | Non Responder |
| TCGA-55-6983 | TUMOR FREE | Alive | 1826 [Not Applicable] | Chemotherapy | Responder |
| TCGA-55-7815 | TUMOR FREE | Alive | 54 [Not Applicable] | Chemotherapy | Non Responder |
| TCGA-55-7914 | WITH TUMOR | Alive | 3 [Not Applicable] | Chemotherapy | Non Responder |
| TCGA-55-8513 | TUMOR FREE | Alive | 146 [Not Applicable] | Chemotherapy | Non Responder |
| TCGA-55-8615 | TUMOR FREE | Alive | 15 [Not Applicable] | Chemotherapy | Non Responder |
| TCGA-55-A48Y | TUMOR FREE | Alive | 42 [Not Applicable] | Chemotherapy | Responder |
| TCGA-62-A471 | TUMOR FREE | Alive | 883 [Not Applicable] | Chemotherapy | Responder |
| TCGA-64-5778 | TUMOR FREE | Alive | 926 [Not Applicable] | Chemotherapy | Non Responder |
| TCGA-69-7974 | WITH TUMOR | Alive | 184 [Not Applicable] | Chemotherapy | Non Responder |
| TCGA-71-6725 | TUMOR FREE | Alive | 61 [Not Applicable] | Chemotherapy | Non Responder |
| TCGA-73-4675 | TUMOR FREE | Alive | 40 [Not Applicable] | Chemotherapy | Responder |
| TCGA-73-7498 | TUMOR FREE | Alive | 621 | [Not Applicable] | Chemotherapy | Responder |
| TCGA-86-8279 | TUMOR FREE | Alive | 21  | [Not Applicable] | Chemotherapy | Responder |
| TCGA-86-8674 | TUMOR FREE | Alive | 0   | [Not Applicable] | Chemotherapy | Non Responder |

**Supplementary Table 9.** Cohort 3 of 26 TCGA lung tumor patients with clinical information.