

Development of a Visual Scoring Method to Determine Inflamed, Excluded or Desert Immune Phenotype in Non-Small Cell Lung Cancer, Colorectal Cancer, and Urothelial Cancer

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INTRODUCTION

The induction of an effective immune response to a tumor is an essential element of cancer immunotherapy. Functional immune cell populations recruited to the tumor microenvironment must infiltrate and interact directly with tumor cell populations. The spatial distribution of inflammatory cells relative to tumor cells can be used as a biomarker to identify patients likely to respond immunotherapy or to track these responses¹. A reproducible pathologist scoring method is required to interpret the heterogeneity of an inflamed tumor as well as provide the overall inflammatory status of that tumor. We describe a visual scoring approach to identify the desert, excluded and inflamed immune phenotypes in non-small cell lung cancer (NSCLC), colorectal cancer (CRC) and urothelial cancer (UC) samples with the aim to create a robust, reproducible method for pathologists to identify these significant patterns of tumor inflammation.

Each case was analysed for the presence of CD8 infiltration of tumor associated stroma (TAS) and tumor cell nests (TCN) using image analysis and pathologist visual scoring methods. This dataset gave us the opportunity to assess the accuracy of pathologist CD8 cell density estimates by comparing raw density bin scores to CD8 cell number and density results of the image analysis. Similarly, we were able to use the pathologist case phenotype to investigate the relationship between immune phenotype and CD8 cell density as determined by image analysis in the TAS and TCN. The reproducibility of immune phenotype scoring was assessed by comparing the raw density bin scores and case immune phenotypes from two pathologists using method I. Based upon these results, the method was simplified and refined and the same two pathologists scored cases using immune phenotype scoring method II. (Figure 1).

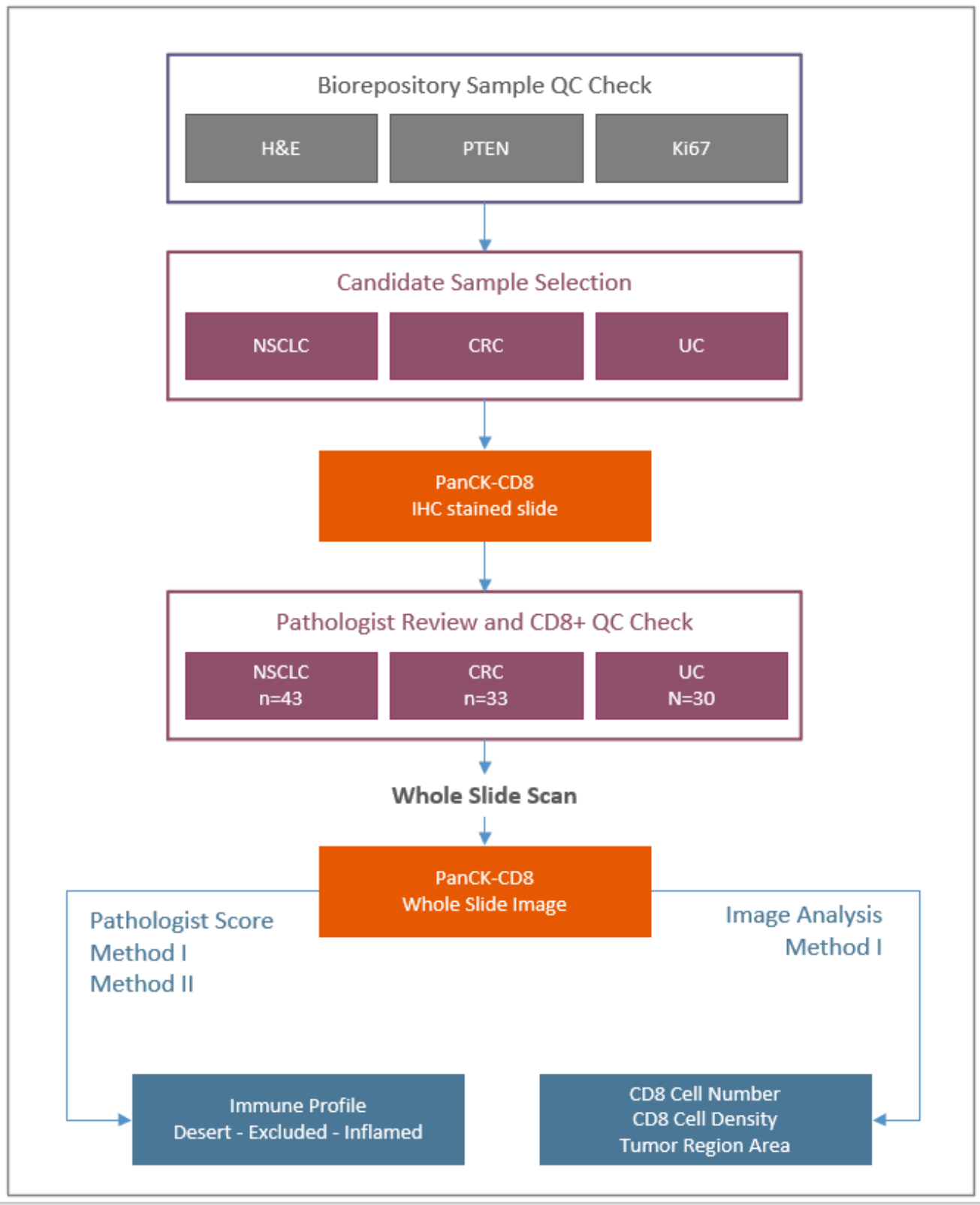


Figure 1. Workflow. Candidate NSCLC, CRC and UC FFPE sample (n=273) were selected from the CellCarta biorepository and tissue sections were stained for H&E, PTEN and Ki67. Samples which passed QC were stained with the PanCK-CD8 duplex IHC assay and a subset of samples were selected with low/moderate to high levels of CD8+ cell infiltration. Visiopharm image analysis and pathologist immune phenotype scoring was performed on NSCLC (n=43), CRC (n=33) and UC (n=30) samples.

METHODS

CD8 Image Analysis

Commercial FFPE NSCLC, CRC and UC resection samples were stained by H&E and with the PanCK-CD8 duplex chromogenic IHC assay on Ventana Discovery Ultra. Whole-slide images were with a Panoramic SCAN digital slide scanner (3DHISTECH, Budapest, Hungary) using a Zeiss plan-apochromatic objective (magnification: 20x, numerical aperture: 0.8) and a Hitachi (HV-F22CL) 3CCD progressive scan color camera (resolution: 0.23 µm/pixel). Scanned images were examined in Panoramic Viewer (3DHISTECH) to check for image quality and to confirm that the whole tissue section was captured. Images were imported in Visiopharm (version 2019.07) and a tumor region of interest (ROI) was delineated on the images in Visiopharm based on the pathologist H&E annotation. An analysis protocol package (APP) was developed in Visiopharm using the Author module. Based on the PanCK IHC staining (Ventana DISCOVERY Pure kit), an epithelial compartment ROI is automatically created within the total tumor ROI. The APP output variables contain the number and density of CD8+ IHC stained (3,3'-diaminobenzidine (DAB)) cells inside and outside the PanCK stained epithelial compartment within the total tumor ROI (Figure 1).

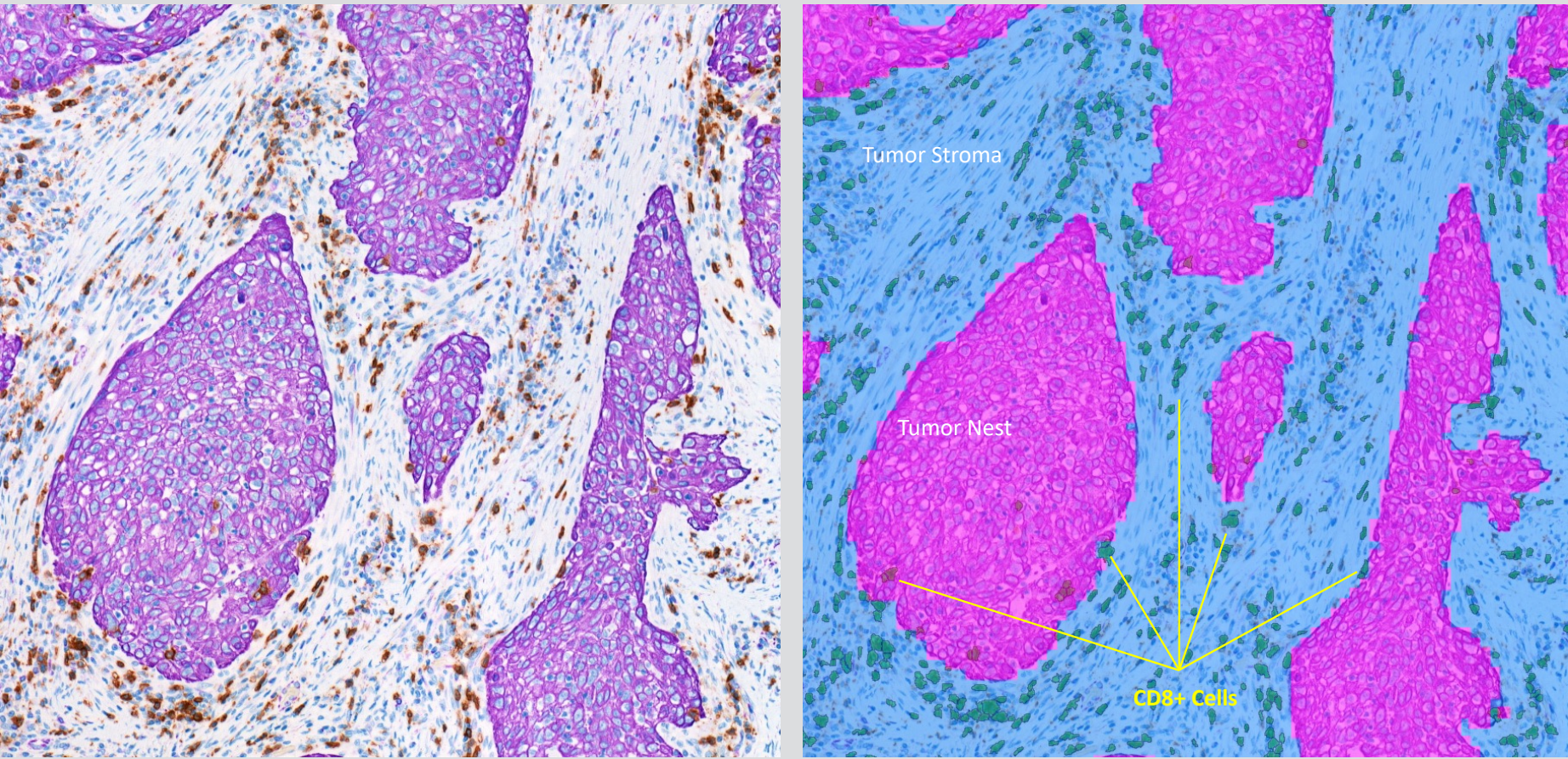


Figure 2. PanCK/CD8 Visiopharm Image Analysis. Tumor area containing intraepithelial and peri-tumoral stroma was analyzed by Visiopharm image analysis. The PanCK/CD8 IHC stained slide is shown on the left with the PanCK tumor cells stained purple and the CD8+ cells stained brown. The Visiopharm mask of the same area is shown on the right. In this image the areas identified as stained with PanCK are covered in a pink mask, the tumor associated stroma is covered in a blue mask and the CD8 stained cells are masked green.

CD8 Immune Phenotypes

A pathologist visual scoring method for the assessment of inflammatory cell populations in tumor associated stroma (TAS) and tumor cell nests (TCN) was developed by CellCarta. The density of inflammatory cells is estimated as a percent relative to the area being evaluated. Tumor stroma and tumor cell nests are scored separately in four cell density bins for no, low, moderate and high density of CD8+ cells. In the TAS, areas of high density infiltrates are clearly visible at low power (5x) and often present in cell clusters or aggregates. In areas of moderate density, loose clusters or groups of CD8+ cells are visible at 10x magnification. Areas of low density are sparsely populated with individual CD8+ cells which require confirmation at 20x. This general magnification rule can also be applied to CD8 infiltration in the TCN, but with the PanCK IHC, it is necessary to confirm the presence of CD8 stained cells at 20X. Examples of low, moderate and high density infiltration of the TAS and TCN are shown in the wsi below. Density values in the TAS and TCN are used to determine the desert, excluded and inflamed immune phenotype using an arbitrary cut-off of 20%.

% TAS area CD8+ve	+ % TAS area Low Density CD8	+ % TAS area Moderate Density CD8	+ % TAS area High Density CD8	= 100% TAS area
% TCN area CD8+ve	+ % TCN area Low Density CD8	+ % TCN area Moderate Density CD8	+ % TCN area High Density CD8	= 100% TCN area
Phenotype	Description			Density
Desert	No or very few CD8+ cells in tumor cell nests and tumor associated stroma.			Moderate + High Density TCN <20% No + Low Density TAS <50%
Excluded	CD8+ cells present in the tumor associated stroma but absent or in low numbers in the tumor cell nests. A predominant cuffing pattern on the tumor periphery.			Moderate + High Density TCN <20% Moderate + High Density TAS <20%
Inflamed	Extensive, generalized CD8+ cell infiltration of tumor cell nests. CD8+ cells may also be present in tumor associated stroma but only TCN density scores are used to identify this phenotype.			Moderate + High Density TCN >20%

Pathologist Analysis Method

Tumor Associated Stroma

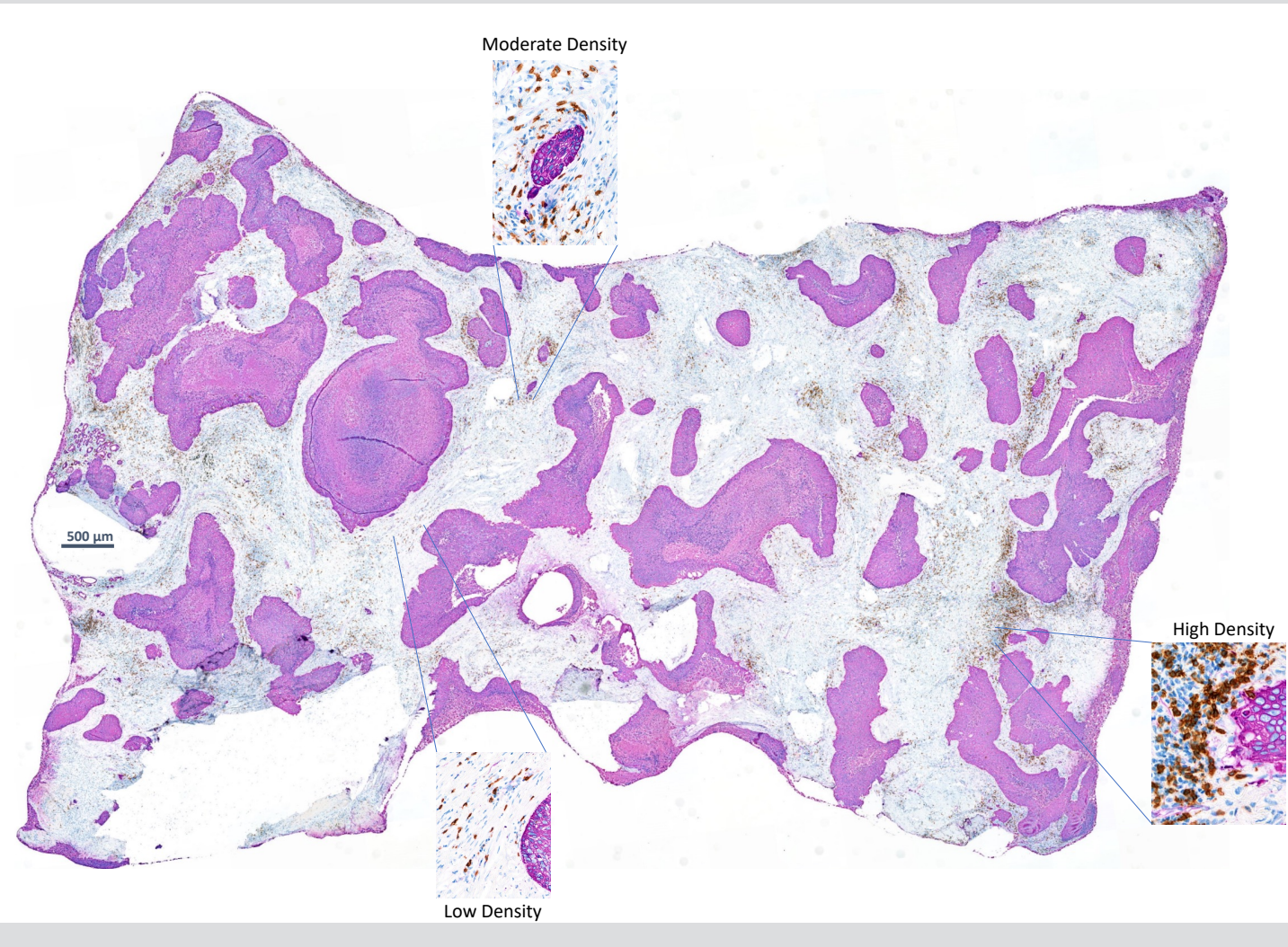


Figure 3. Pathologist CD8 Density Levels in Tumor Associated Stroma. Representative images of the three density levels scored by pathologists. Pathologists survey the PanCK-CD8 stained tissue at low, moderate and high power to estimate the percent of the TAS area covered in no (0), low (1), moderate (2) and high density CD8 cells.

Tumor Epithelial Nests

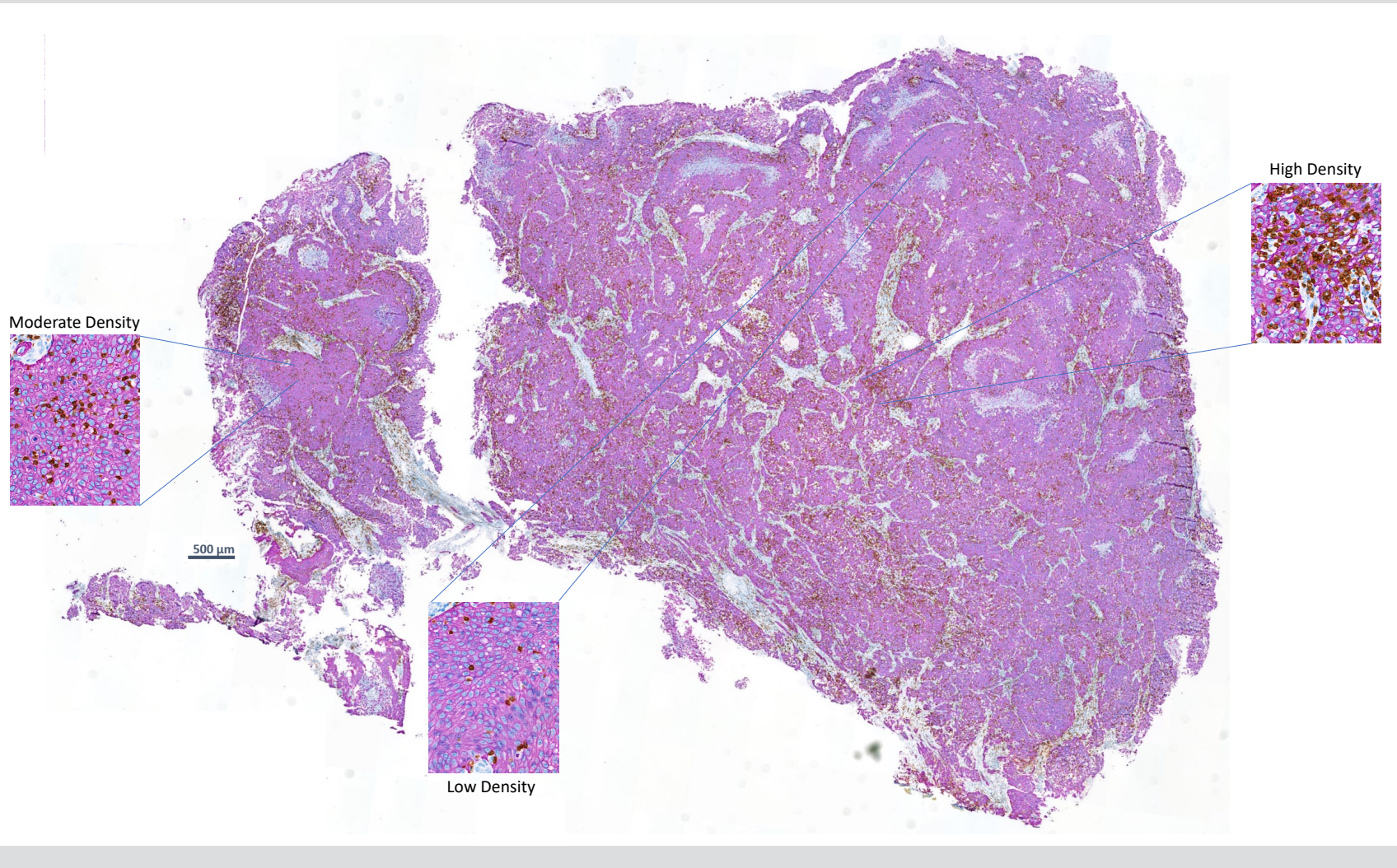


Figure 4. Pathologist CD8 Density Levels in Tumor Cell Nests. Representative images of the three density levels scored by pathologists. Pathologists survey the PanCK-CD8 stained tissue at low, moderate and high power to estimate the percent of the TCN area covered in no (0), low (1), moderate (2) and high density CD8 cells.

RESULTS

Samples – Most biorepository samples selected for further analysis were resection samples containing tumor cells nests and tumor associated stroma. Following analysis with the Visiopharm algorithm, the mean area in mm², mean ratio of tumor cell nests to tumor associated stroma and the average number of CD8+ cells is reported in Table 1 below. NSCLC, CRC and UC samples were of comparable size, ratio of TCN:TAS and samples covered a dynamic range of CD8 cell infiltration from 644 to 210318 CD8+ cells. The heterogeneity of this sample set allowed for the analysis and comparison of different levels and patterns of tumor CD8+ cell infiltration.

NSCLC, CRC and UC Analysed Case Collections					
Tumor Indication	# Cases Analysed	Sample Type	Mean Tumor Area mm ² (range low - high)	Mean TCN:TAS Ratio (range low - high)	Mean CD8+ Cells (range low - high)
Non Small Cell Lung Cancer	43	All Surgical Resection	80.1 (18.4 - 376.9)	0.87 (0.22 - 2.75)	27708 (1640 - 210318)
Colorectal Cancer	33	All Surgical Resection	95.0 (31.9 - 172.2)	0.91 (0.15 - 2.35)	19752 (1580 - 81844)
Urothelial Cancer	30	27 Surgical Resection 2 TURBT 1 Biopsy	98.5 (17.1 - 235.7)	1.33 (0.13 - 6.02)	18150 (644 - 92858)

Table 1. NSCLC, CRC and UC Selected CD8+ Case Collection. A random sample of NSCLC, CRC and UC FFPE samples were obtained from the CellCarta biobank stained with H&E, PTEN and Ki67 to check sample quality. A candidate case set was stained with PanCK/CD8 brightfield IHC assay and a subset of samples containing CD8+ cells were selected. Tumor area containing intraepithelial and peri-tumoral stroma was annotated on the H&E and IHC stained slides. The whole tumor area and PanCK+ and CD8+ sub-regions were analysed by Visiopharm image analysis. The absolute number of CD8+ cells, total tumor area (mm²) and ratio of the area of tumor cell nest (TCN) to area of tumor associated stroma (TAS) are reported.

Regional Segmentation vs CD8 Infiltration

Regional Segmentation and Associated CD8+ Cell Infiltration. The relative area of tumor cell nests to tumor associated stroma within the entire tumor area is shown in Figure 5 for the NSCLC, CRC and UC sample sets. In addition, the absolute number of CD8+ cells in the tumor area is shown for each case by the yellow bell. Variation in CD8+ cell infiltration was not related directly to either the relative area of tumor associated stroma or tumor cell nests. NSCLC samples were the most highly inflamed and UC samples demonstrated the lowest overall level of CD8 infiltration, but samples were heterogeneous with regard to both the relative amount of TAS and the number of CD8 cells within the total tumor area.

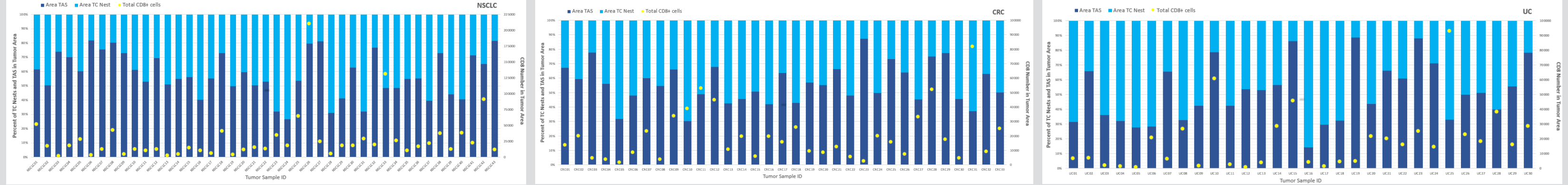


Figure 5. Tumor to Stroma ratio and CD8 cell infiltration. Each FFPE tumor sample was stained with H&E and PanCK/CD8 duplex chromogenic IHC assay. Pathologists annotated tumor area to include intraepithelial and peritumoral tumor associated stroma (TAS). The crosses of TAS in the tumor periphery did not exceed from the outer edge of the tumor nest. Whole slide images were analysed using a Visiopharm analysis algorithm developed and validated at CellCarta. The PanCK IHC purple stain was used as an epithelial compartment mask to distinguish epithelial tumor cells from the tumor associated stroma in the tumor area. Three parameters are shown for each NSCLC (top), CRC (middle) and UC case. The bars represent the percent of the whole tumor area which is tumor cell (TCN) nests versus tumor associated stroma (TAS). The yellow circle in each case represents the absolute number of CD8+ cells in the tumor area.

Correlation CD8 Density - Visual vs Image Analysis

Correlation of CD8 Densities as Determined by Pathologist and Image Analysis.

The pathologist's ability to make an accurate assessment of low, moderate and high density CD8 levels in the TAS and TCN is foundational to the determination of the desert, excluded or inflamed immune phenotype. In order to determine the accuracy of pathologist scoring, the CD8 cell density estimates were compared to the CD8+ cell densities determined by image analysis. In order to make this comparison, pathologists estimates at each density level (no CD8+ cells, low density CD8+ cells, moderate density CD8+ cells and high density CD8+ cells) were summed to obtain the total percent of the tumor area covered in CD8 cells at any density. These case density values were grouped in quartiles with Q1 for 0-25%, Q2 for 26-50%, Q3 for 51-75% and Q4 for 76-100%. The corresponding IA CD8 density for the cases in each pathologist score quartile were grouped and the mean and error bars for the IA density values were charted. The CD8 densities in TAS and TCN demonstrate the same trend in that increasing density values for pathologist estimates were associated with increasing density values by IA.

The IA CD8 density result does not account for the variation in regional densities from low to moderate to high. In order to make a comparison of pathologist estimates to IA results for each case, pathologist density scores were normalized according to 1*CD8 low density + 2*CD8 moderate density + 3*CD8 high density. These normalized CD8 densities were then charted against the IA CD8 densities for each case. In both the TAS and TCN, there was good correlation between CD8+ cell density pathologist estimates and the density results of image analysis with R² values of 0.75 and 0.61 respectively, which suggested at the case level, pathologist CD8 density estimates were aligned with image analysis results.

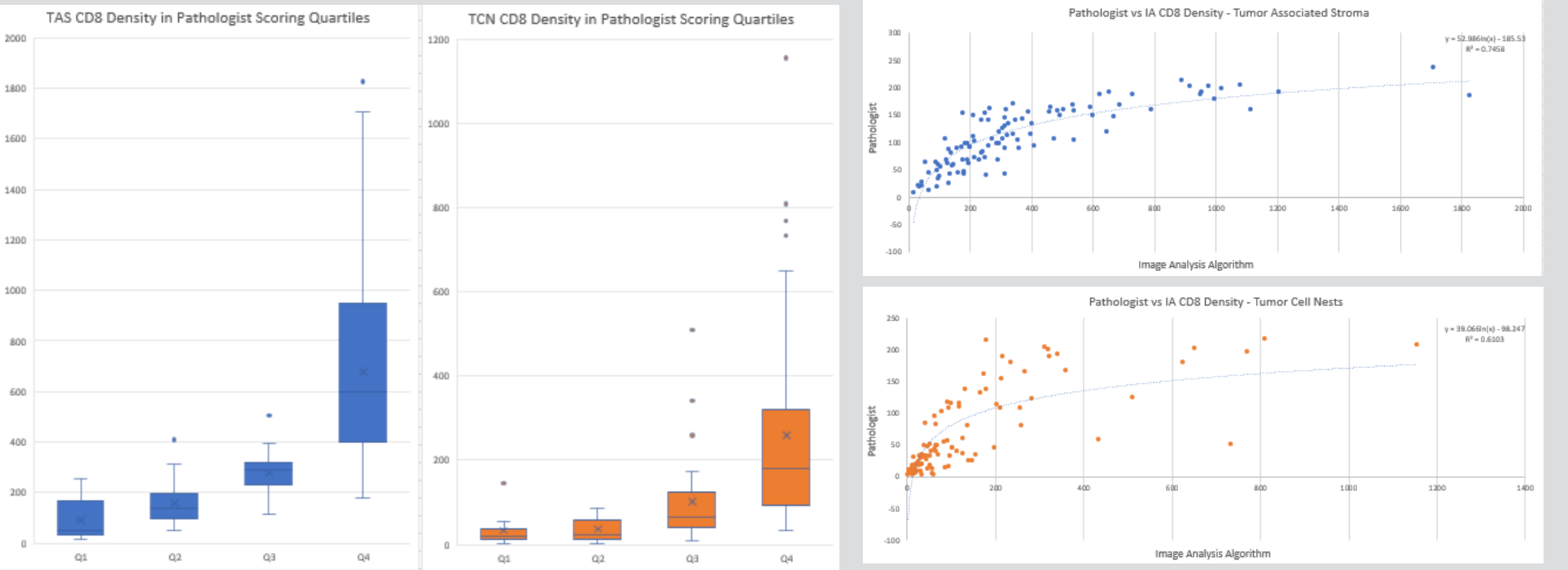


Figure 6. Correlation between pathologist and image analysis CD8 density scores. Whole slide images (wsi) of PanCK-CD8 stained tissue were analysed using a Visiopharm analysis algorithm. The PanCK IHC purple stain was used as an epithelial compartment mask to distinguish epithelial tumor cells from the tumor associated stroma in the tumor area. The density in cells per mm² of CD8+ cells was determined in the tumor associated stroma (TAS) and tumor cell nests (TCN). The mean CD8 densities for cases in each density are shown for the TAS (top left) and TCN (top right) for the low and higher density cases. The lower two panels show the normalized pathologist estimated CD8 density score against the CD8 density result from image analysis in the TAS (top blue) and TCN (bottom orange) are shown with a logarithmic scale (X).

Inter-Pathologist Concordance CD8 Density Scores

Regional Segmentation and Associated CD8+ Cell Infiltration.

The relative area of tumor cell nests to tumor associated stroma within the entire tumor area is shown in Figure 5 for the NSCLC, CRC and UC sample sets. In addition, the absolute number of CD8+ cells in the tumor area is shown for each case by the yellow bell. Variation in CD8+ cell infiltration was not related directly to either the relative area of tumor associated stroma or tumor cell nests. NSCLC samples were the most highly inflamed and UC samples demonstrated the lowest overall level of CD8 infiltration, but samples were heterogeneous with regard to both the relative amount of TAS and the number of CD8 cells within the total tumor area.

Improved Inter-pathologist Concordance for CD8 Density Estimates in TAS and TCN – Method II.

In the original 8 category scoring method for immune phenotypes, pathologists were able to make accurate, well aligned estimates of low versus high density areas of CD8 infiltration of TAS and TCN but struggled to align when more granular, subjective scoring was required. The density scoring method was adapted and streamlined to ensure that raw density scoring, which is the foundation of the immune phenotype assessment, can be performed reproducibly by different pathologists. The 8 category method was reduced to 4 categories in which pathologists determined the percent of the TAS and TCN covered in low or high density CD8+ cells. These two bins corresponded to the no/low and moderate/high bins of the 8 category method. When pathologists re-scored the NSCLC, CRC and UC cases, the raw scores for density estimates were highly concordant with low bias, although tendency for P2 to report higher density scores, particularly in the TAS, was maintained for both the 8 category and 4 category methods. This insight is helpful when reviewing cases to improve alignment.

Table 1. Pathologist Alignment CD8 Density Proportion Score Method I					
Tumor associated immune phenotype	Description	QC	Concordance	Raw P1 vs P2	95% CI Bounds
Desert	Desert	0.02	0.02	0.02	0.02
Excluded	Excluded	0.02	0.02	0.02	0.02
Inflamed	Inflamed	0.02	0.02	0.02	0.02
Desert	Desert	0.02	0.02	0.02	0.02
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Inflamed	Inflamed	0.02	0.02	0.02	0.02
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Inflamed	Inflamed	0.02	0.02	0.02	0.02
Desert	Desert	0.02	0.02	0.02	0.02
Excluded	Excluded	0.02	0.02	0.02	0.02
Inflamed	Inflamed	0.02	0.02	0.02	0.02
Desert	Desert	0.02	0.02	0.02	0.02
Excluded	Excluded	0.02	0.02	0.02	0.02
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Desert	Desert	0.02	0.02	0.02	0.02
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Inflamed	Inflamed	0.02	0.02	0.02	0.02
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Inflamed	Inflamed	0.02	0.02	0.02	0.02
Desert	Desert	0.02	0.02	0.02	0.02
Excluded	Excluded	0.02	0.02	0.02	0.02
Inflamed	Inflamed	0.02	0.02	0.02	0.02
Desert	Desert	0.02	0.02	0.02	0.02
Excluded	Excluded	0.02	0.02	0.02	0.02
Inflamed	Inflamed	0.02	0.02	0.02	0.02
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Desert	Desert	0.02	0.02	0.02	0.02
Excluded	Excluded	0.02	0.02	0.02	0.02
Inflamed	Inflamed	0.02	0.02	0.02	0.02
Desert	Desert	0.02	0.02	0.02	0.02
Excluded	Excluded	0.02	0.02	0.02	0.02
Inflamed	Inflamed	0.02	0.02	0.02	0.02
Desert	Desert	0.02	0.02	0.02	0.02
Excluded	Excluded	0.02	0.02	0.02	0.02
Inflamed	Inflamed	0.02	0.02	0.02	0.02
Desert	Desert	0.02	0.02	0.02	0.02
Excluded	Excluded	0.02	0.02	0.02	0.02
Inflamed	Inflamed	0.02	0.02	0.02	0.02
Desert	Desert	0.02	0.02	0.02	0.02
Excluded	Excluded	0.02	0.02	0.02	0.02
Inflamed	Inflamed	0.02	0.02	0.02	0.02
Desert	Desert	0.02	0.02	0.02	0.02
Excluded	Excluded	0.02	0.02	0.02	0.02
Inflamed	Inflamed	0.02	0.02	0.02	0.02
Desert	Desert	0.02	0.02	0.02	0.02
Excluded	Excluded	0.02	0.02	0.02	0.02
Inflamed	Inflamed	0.02	0.02	0.02	0.02
Desert	Desert	0.02	0.02	0.02	0.02
Excluded	Excluded	0.02	0.02	0.02	0.02
Inflamed	Inflamed	0.02	0.02	0.02	0.02
Desert	Desert	0.02	0.02	0.02	0.02
Excluded	Excluded	0.02	0.02	0.02	0.02
Inflamed	Inflamed	0.02	0.02	0.02	0.02
Desert	Desert	0.02	0.02	0.02	0.02
Excluded	Excluded	0.02	0.02	0.02	0.02
Inflamed	Inflamed	0.02	0.02	0.02	0.02
Desert	Desert	0.02	0.02	0.02	0.02
Excluded	Excluded	0.02	0.02	0.02	0.02
Inflamed	Inflamed	0.02	0.02	0.02	0.02
Desert	Desert	0.02	0.02	0.02	0.02
Excluded	Excluded	0.02	0.02	0.02	0.02
Inflamed	Inflamed	0.02	0.02	0.02	0.02
Desert	Desert	0.02	0.02	0.02	0.02
Excluded	Excluded	0.02	0.02	0.02	0.02
Inflamed	Inflamed	0.02	0.02	0.02	0.02
Desert	Desert	0.02	0.02	0.02	0.02
Excluded	Excluded	0.02	0.02	0.02	0.02
Inflamed	Inflamed	0.02	0.02	0.02	0.02
Desert	Desert	0.02	0.02	0.02	0.02
Excluded	Excluded	0.02	0.02	0.02	0.02
Inflamed	Inflamed	0.02	0.02	0.02	0.02
Desert	Desert	0.02	0.02	0.02	0.02
Excluded	Excluded	0.02	0.02	0.02	0.02
Inflamed	Inflamed	0.02	0.02	0.02	0.02
Desert	Desert	0.02	0.02	0.02	0.02
Excluded	Excluded	0.02	0.02	0.02	0.02
Inflamed	Inflamed	0.02	0.02	0.02	0.02
Desert	Desert	0.02	0.02	0.02	0.02
Excluded	Excluded	0.02	0.02	0.02	0.02
Inflamed	Inflamed	0.02	0.02	0.02	0.02
Desert	Desert	0.02	0.02	0.02	0.02
Excluded	Excluded	0.02	0.02	0.02	0.02
Inflamed	Inflamed	0.02	0.02	0.02	