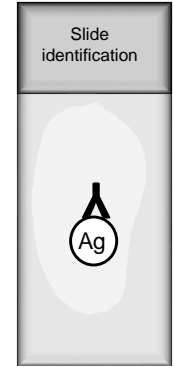
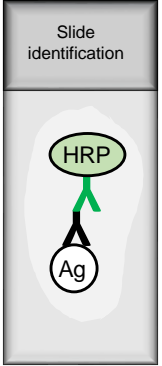


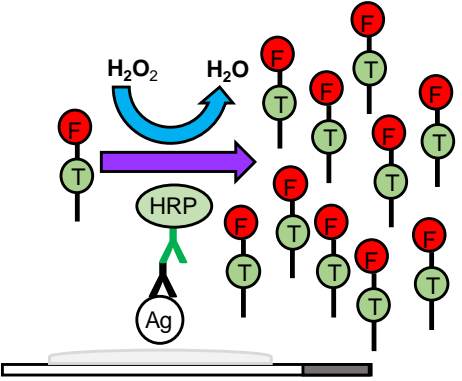
Supplementary Figure 1



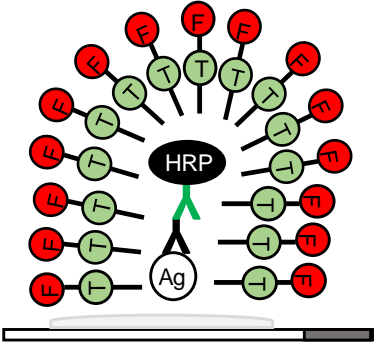
First primary antibody (Ab1) incubation



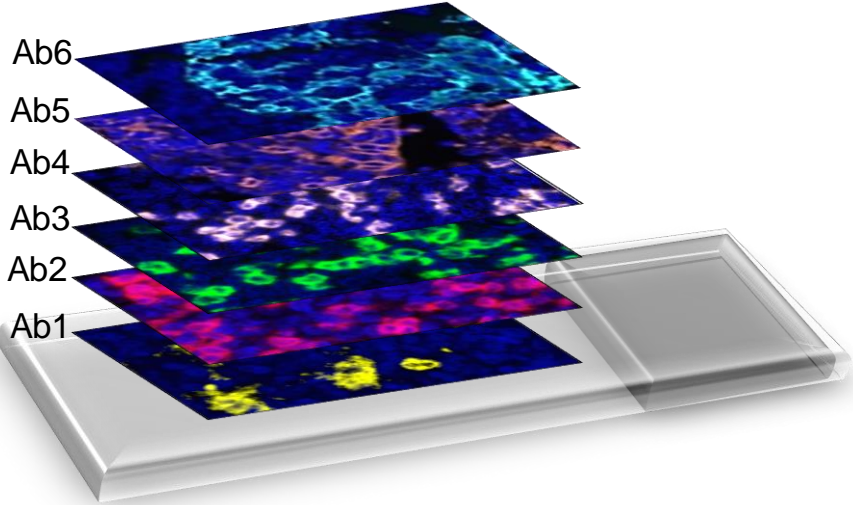
Introduce HRP



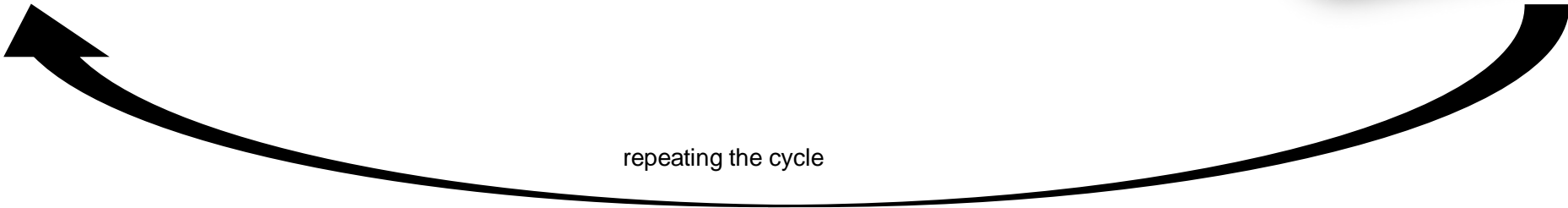
TSA incubation and HRP catalyzes with TSA to free radicals formation



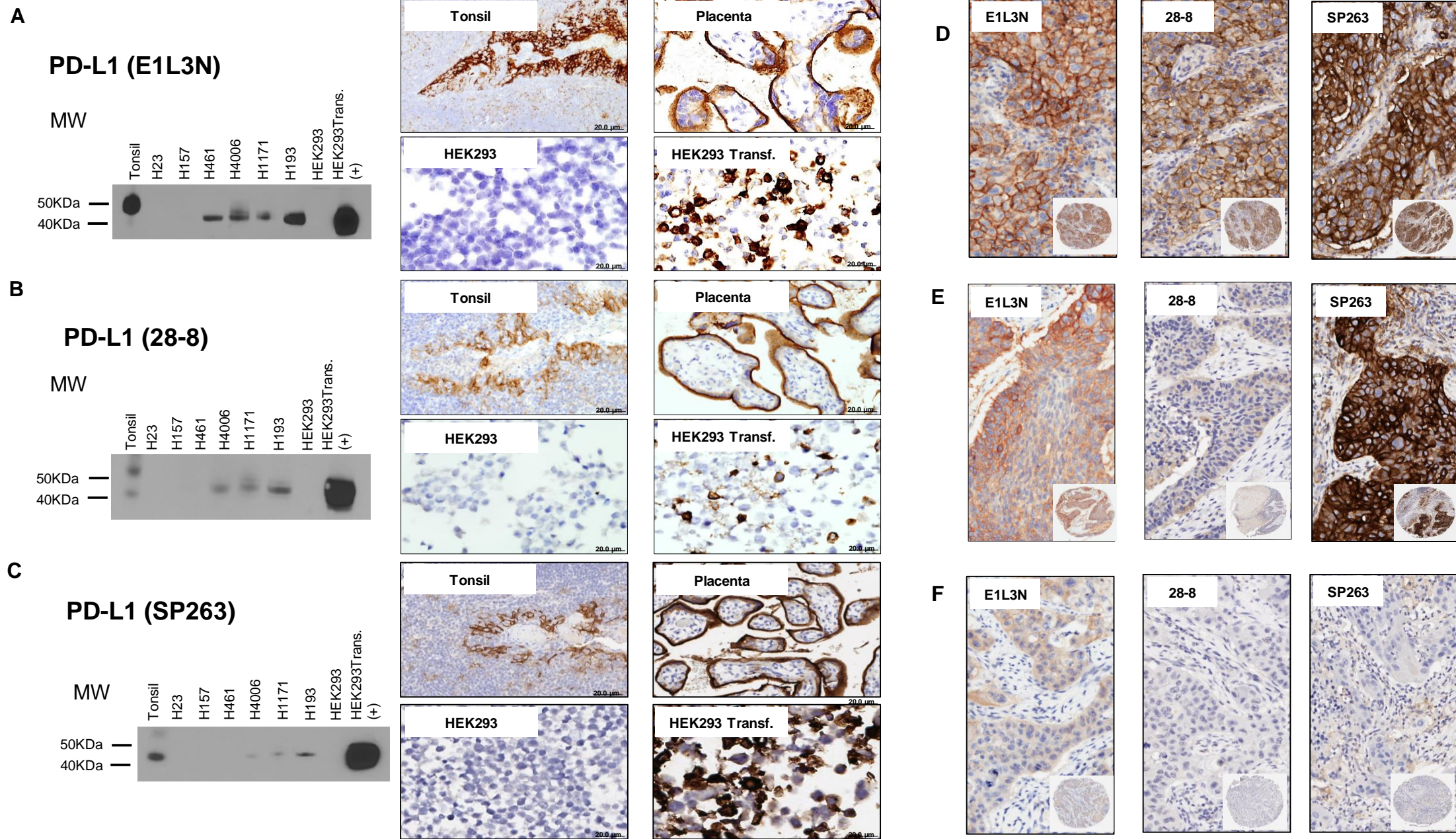
Covalent bonds formation with TSA residues next to HRP



repeating the cycle



Supplementary Figure 2



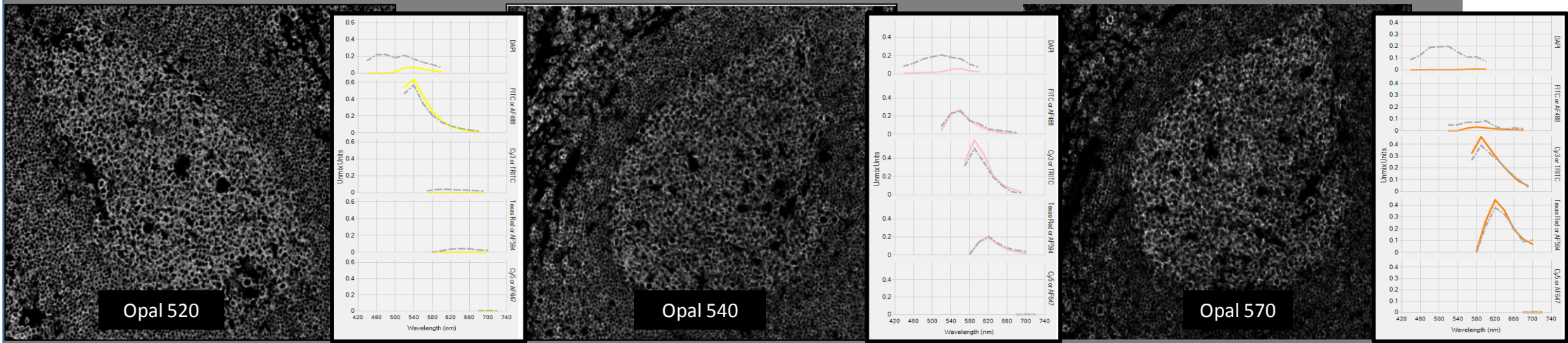
Supplementary Figure 3

Build Library Settings
Load Image:
Sample Format: Fluorescence
Fluor: Opal 520



View: Color Component

2



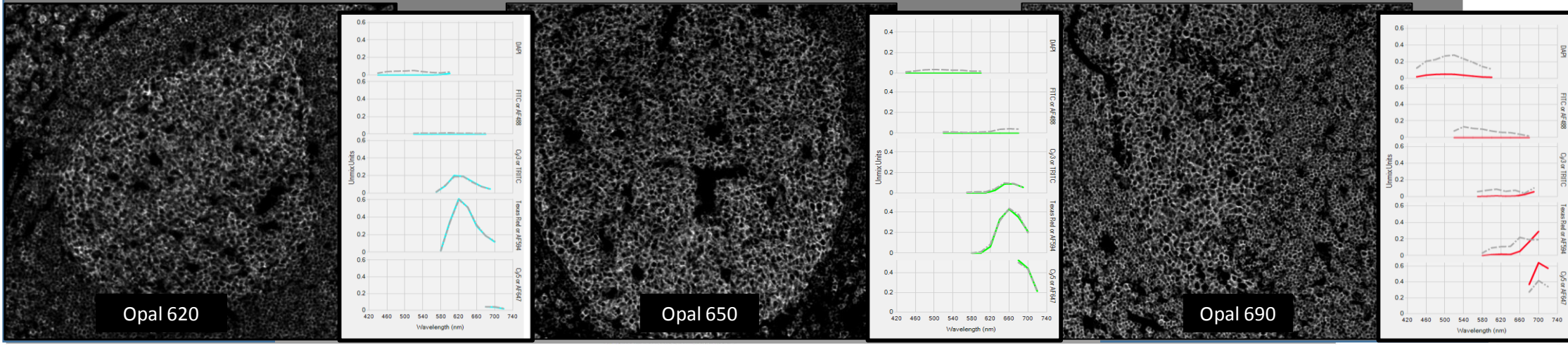
Opal 520

Opal 540

Opal 570

3

Extract



Opal 620

Opal 650

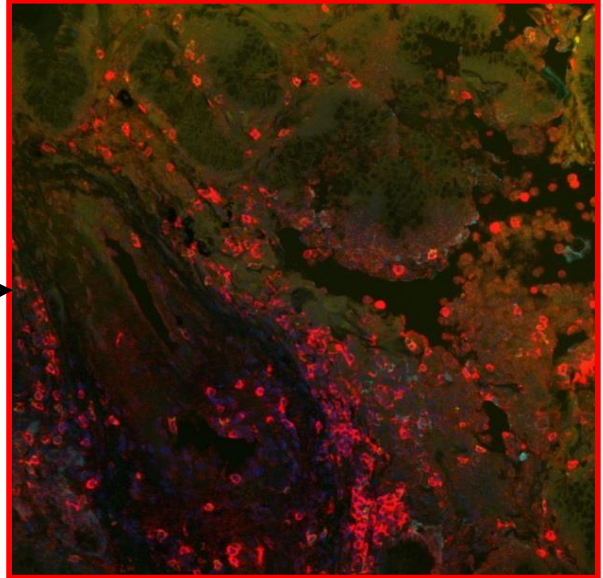
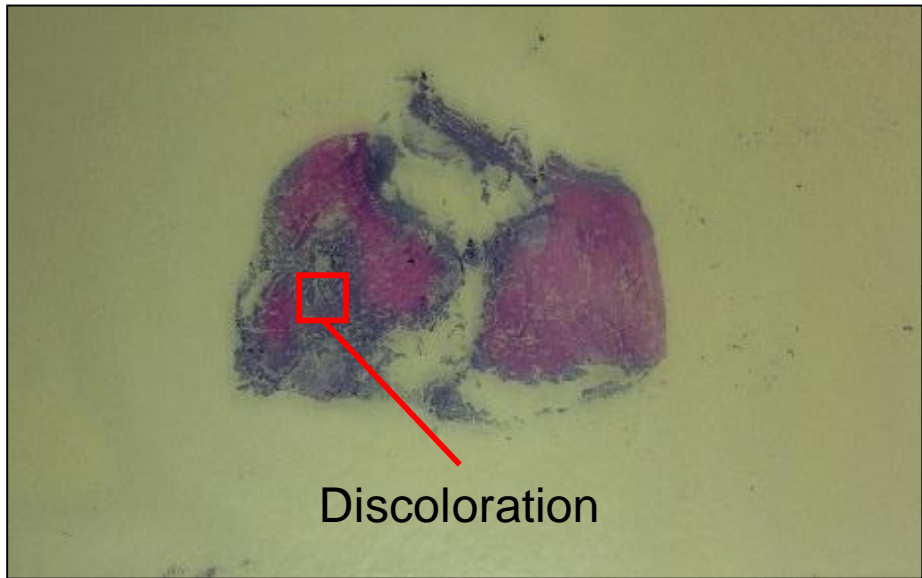
Opal 690

A

H&E with oxidation artifact

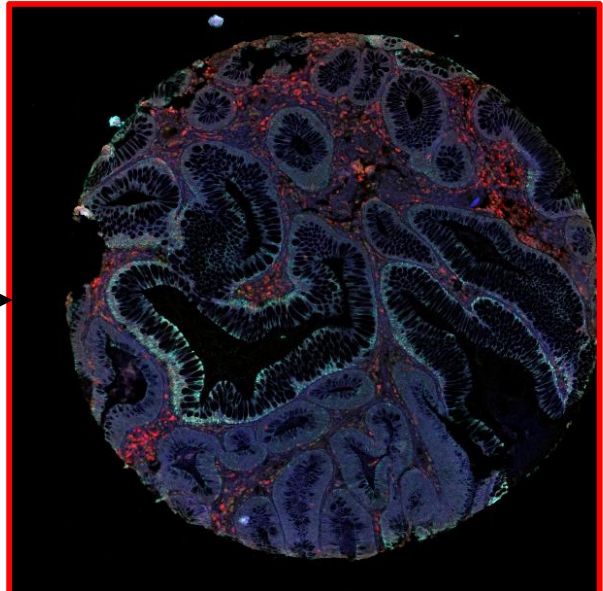
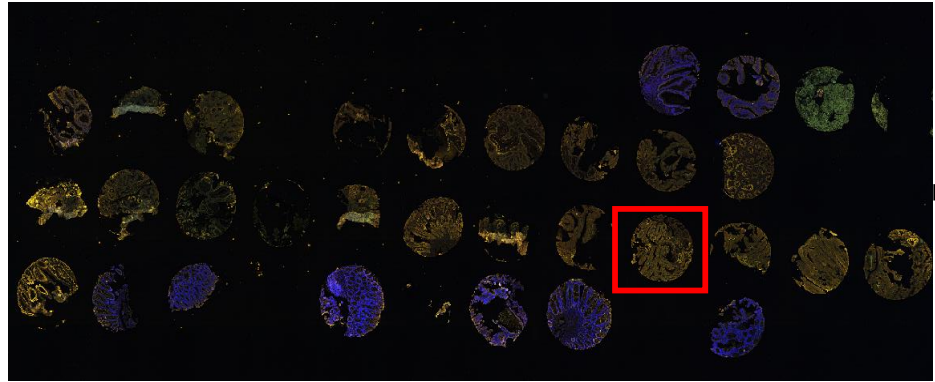
Multiplex staining artifacts

10 years
(block storage)

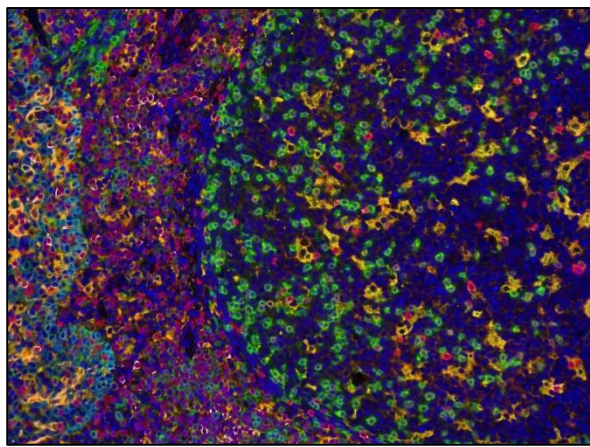


B

3 years
(TMA slide storage)



C. Control (Human tonsil)



CK/PD-L1/CD3/CD8/PD-1/CD68/DAPI

CK/PD-L1/CD3/CD8/PD-1/CD68/DAPI

Supplementary Figure 5

The screenshot displays the 'Tissue Segmentation Training' software interface. At the top, a workflow bar includes 'Prepare Images', 'Segment Tissue' (highlighted with a red box and labeled '1'), 'Segment Cells', 'Phenotype Cells', and 'Export'. Below this, the 'Tissue Segmentation Training' panel is divided into several sections:

- Tissue Categories:** A table with columns for category name and color, and a 'Draw' column with a radio button. The categories are TUMOR (Red), STROMA (Green), and GLASS (Blue). This section is highlighted with an orange box and labeled '2'.
- Components for Training:** A list of markers with checkboxes: CD68 (Opal 520), PD-L1 (Opal 570), PD-1 (Opal 650), CD3 (Opal 690), and DAPI (DAPI). A 'Pattern Scale' dropdown is set to 'Large'.
- Train Tissue Segmenter:** A section with 'Recent Trainings' and 'Segmentation Options'. The 'Segmentation Resolution' is set to 'Fine'. Other options include 'Trim Edges By (pixels)' (5), 'Minimum Segment Size (pixels)' (500), and 'Discard if touching image border'. This section is highlighted with a green box and labeled '3'.
- Bottom Panel:** A 'Please train a segmenter.' message and buttons for 'Segment Image', 'Segment All', and 'Advance'. This section is highlighted with a blue box and labeled '4'.

The main workspace shows two panels, A and B. Panel A is a raw fluorescence microscopy image with segmented regions in red, green, and blue. Panel B shows the same image with a segmentation map overlaid, where the red, green, and blue regions correspond to the Tumor, Stroma, and Glass categories respectively. A toolbar with various icons is visible between the two panels.

Tissue segmentation visualization tools



Show/Hide the tissue segmentation map



Show/Hide the training regions



Tumor



Stroma



Glass

Supplementary Figure 6

File Edit Views Tools License Help

Prepare Images Segment Tissue **1** Segment Cells Phenotype Cells Export

Cell Segmentation Settings

Segment

Nuclei Cytoplasm Membrane

Components

DAPI (DAPI) Nucleus, Relative Intensity: 0.22

CD3 (Opal 690) Membrane, assists nuclear splitting.

CK (Opal 620) Cytoplasm

Add...

2

Show Preview 3

Nuclear Component Splitting

Nuclear Staining: A mixture of quality

Splitting Sensitivity: 0.42

Minimum Nuclear Size: 62

Assisting Component Splitting

Assisting Staining: A mixture of quality

Splitting Sensitivity: 0.42

Minimum Nuclear Size: 46

Other Settings

Fill Nuclear Holes Smaller Than: 19

Refine cells after segmentation

Membrane and Cytoplasm Settings

Cytoplasm Thickness: 3.0

Membrane Search Distance: 10.0

Membrane Staining: Continuous with sham edges

Segment Image Segment All Advance 4

A **B** **C**

Cell segmentation visualization tools



Show/Hide the nuclear segmentation map



Show/Hide the cytoplasm segmentation map



Show/Hide the membrane segmentation map

Supplementary Figure 7

5

1

2

3

4

A

B

View Editor

Data Displayed: Composite

Rendering Options: Brightfield Fluorescence

Scaling: Scale Views for Each Image Individually Scale Views Equally for All Images in the Project Scale Views Based on Selected Images

Component Display

Display Intensity: True Adjustable

Display Color: True Color False Color

- CD68 (Opal 520) Yellow
- PD-L1 (Opal 570) Orange
- PD-1 (Opal 650) Green
- CD3 (Opal 690) Red
- DAPI (DAPI) Blue
- CD8 (Opal 540) Pink
- CK (Opal 620) Cyan
- Autofluorescence Black

Reset to Default

Image Options: Tissue Segmentation Map Training Regions Processing Regions Equalize Display Histogram

Phenotypes: CD68+ CK+ CD3+ others

6

C

D

E

F

G

H

Phenotyping visualization tools

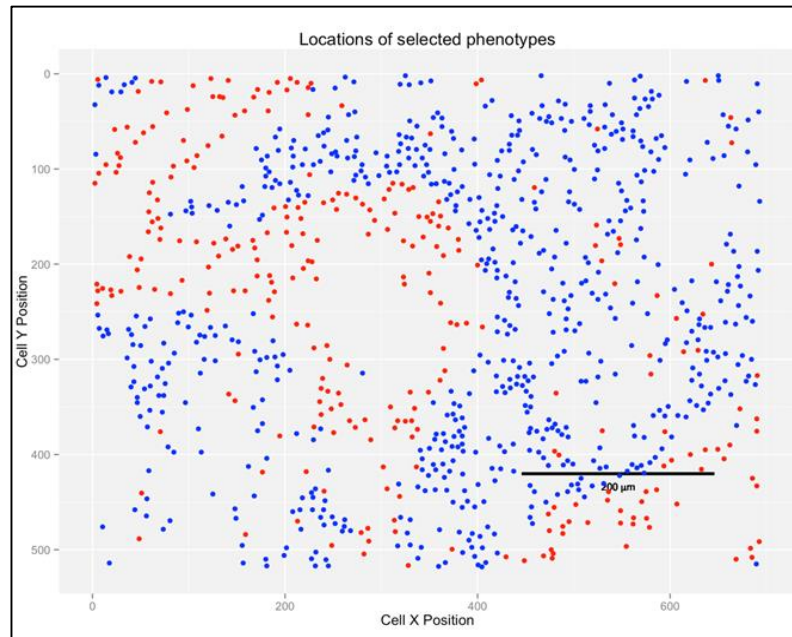
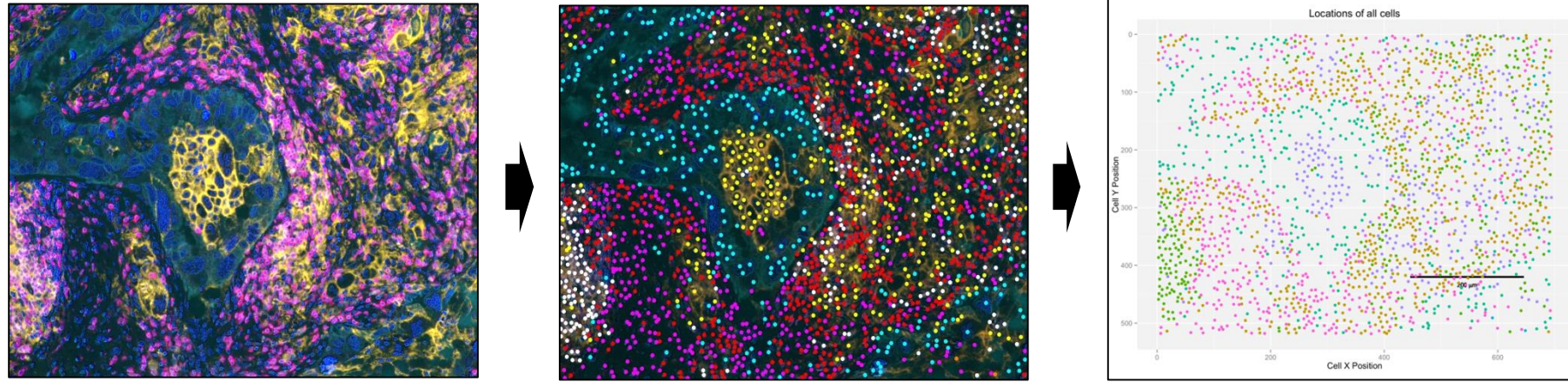
Edit a cell's phenotype

Show/Hide the phenotypes

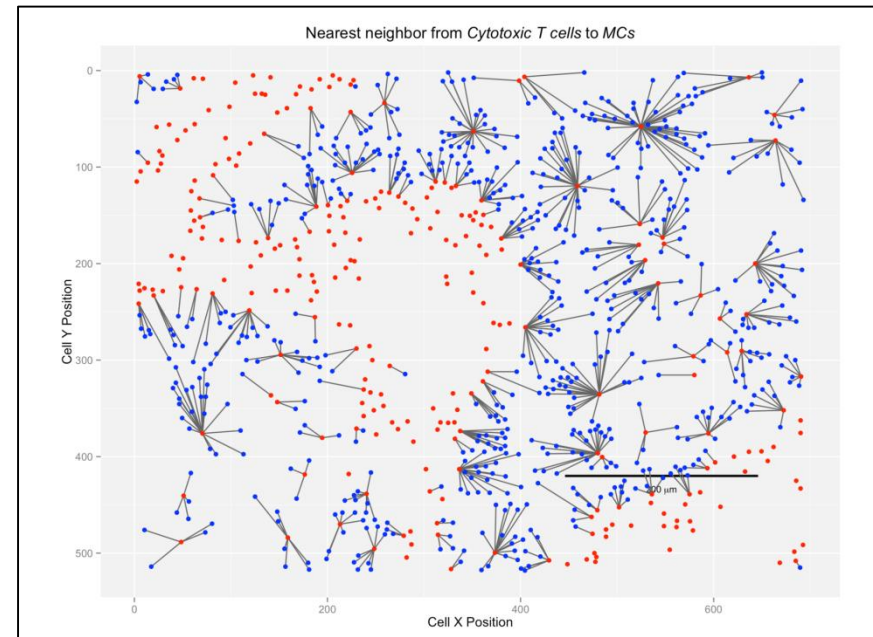
Marker's trainer

A **C** **E** **G**

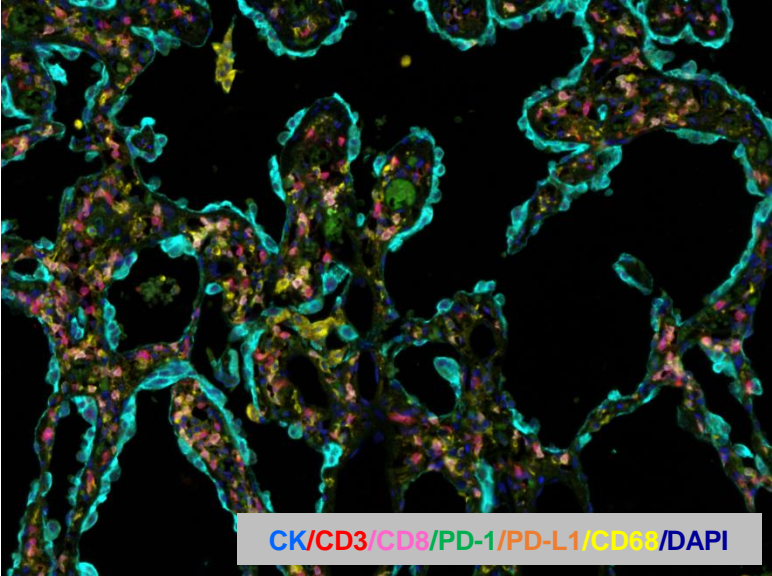
Supplementary Figure 8



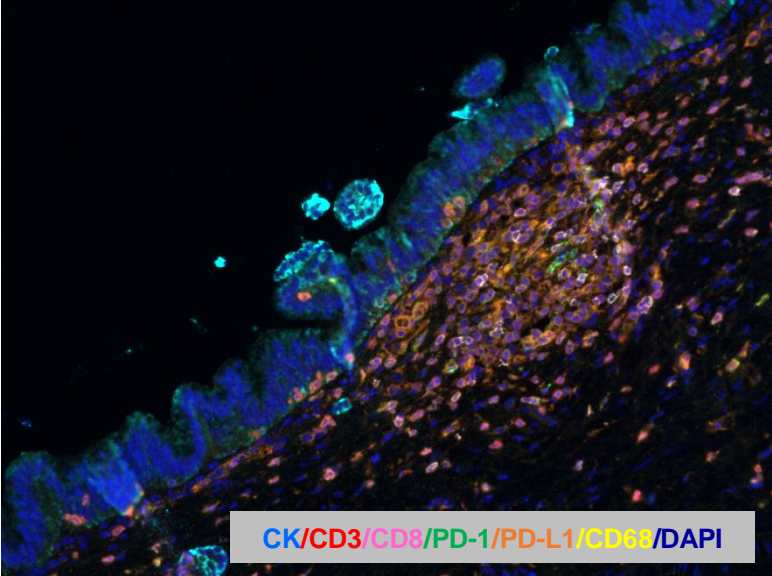
● CD3+/CD8+ Cytotoxic T Cells **● Malignant Cells**



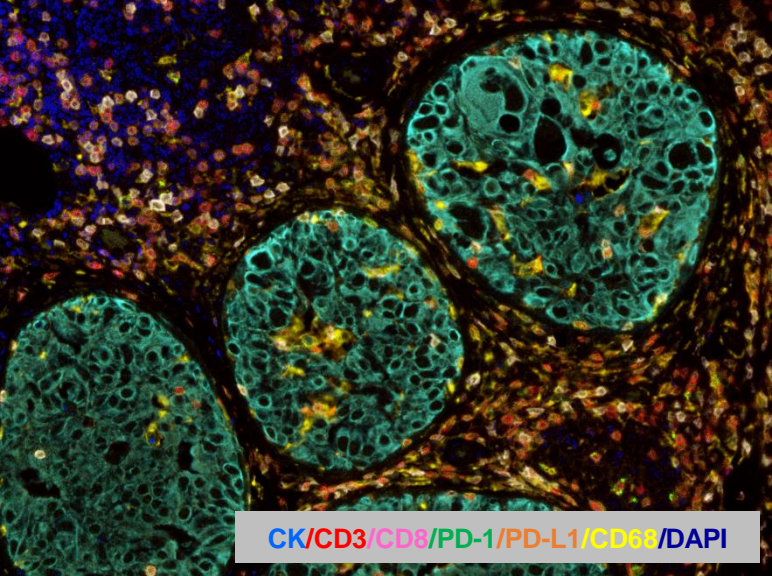
Mean Distance Between Cytotoxic T cells to MCs = 285.9 microns



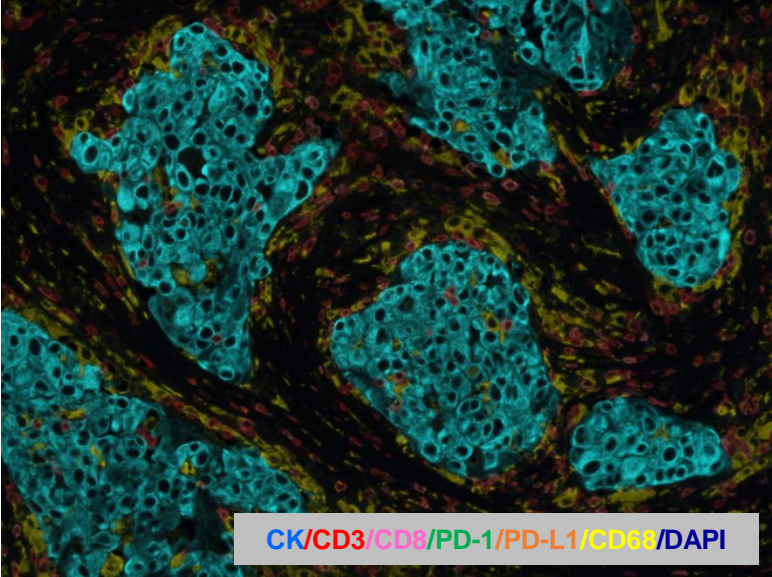
Lung Adenocarcinoma In Situ



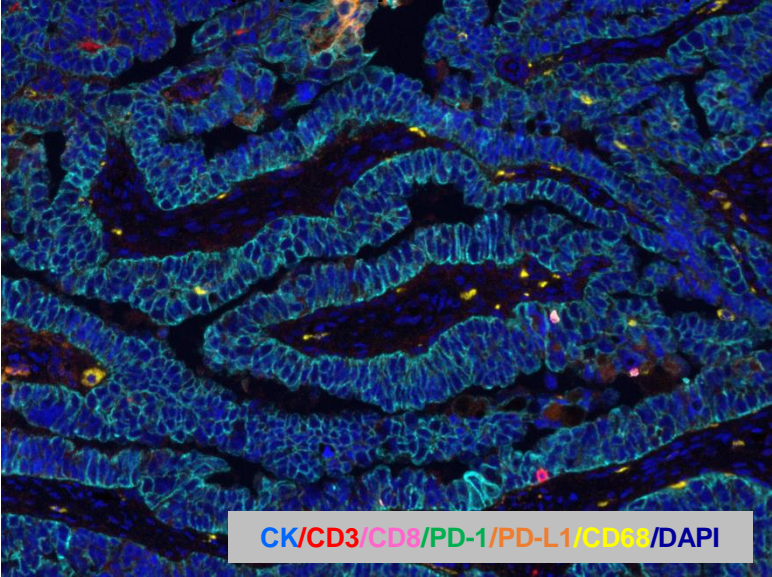
Intraductal papillary mucinous neoplasm



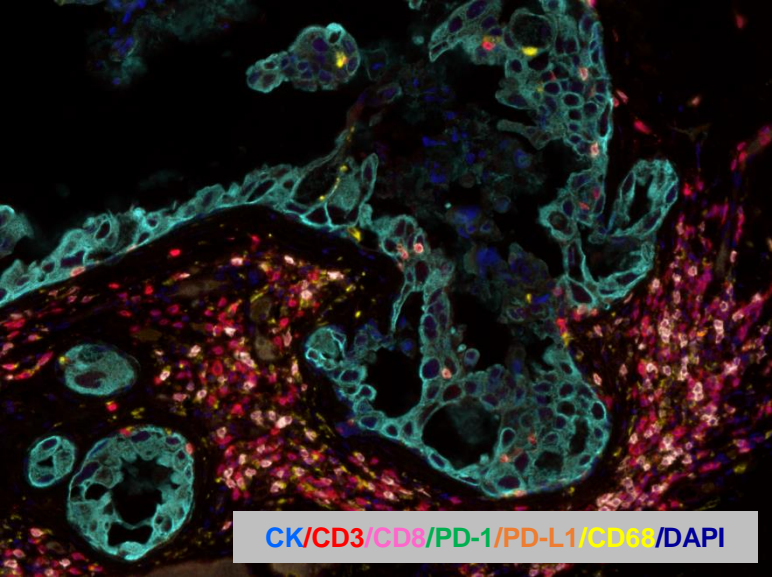
Ductal Carcinoma In Situ



Lung adenocarcinoma



Pancreatic adenocarcinoma



Breast cancer

Supplementary Table 1. Immune profiling cases using multiplex immunofluorescence from 2015 to 2018 in our Immunoprofiling Laboratory.

Organ	Histology type	Cases (N)	Slides (N)	Tissue category	Panel (N)	ROIs (N)
Lung	ADC/SCC	829	1503	WS/CNB/TMA	5	7774
Mesothelium	Mesothelioma	52	104	CNB	2	520
Head and neck	SCC	269	332	WS	4	1660
Esophagus	SCC	22	44	WS	2	220
Liver	HCC	15	30	WS	2	150
Colon/rectum	CRC	172	313	CNB/TMA	3	1565
Ovary	ADC	240	10	TMA	2	240
Breast	ADC/DCIS	144	249	WS	2	1245
Soft tissue	Sarcoma	278	556	CNB	2	2780
Brain	GBM	43	82	WS	2	410
Skin	Merkel cell carcinoma	82	82	WS	1	410
Pancreas	IPMN	130	260	WS	2	1300
Other	Miscellaneous	238	577	WS/CNB	2	2885
Total	11	2514	4142	3	5	21159

ADC, adenocarcinoma; SCC, squamous cell carcinoma; HCC, hepatocellular carcinoma; CRC, colorectal carcinoma; DCIS, ductal carcinoma in situ; GBM, glioblastoma; IPMN, intraductal papillary mucinous neoplasm of the pancreas; WS, whole section; CNB, core-needle biopsy; TMA, tissue microarray; ROI, region of interest.