Supplementary Material & Methods

In vitro pmel-1 T cell activation assay

Pmel-1 TCRtg mice were sacrificed, and the spleens were harvested. Single cell suspensions were prepared with complete RPMI medium supplemented with 5µg/ml human gp100 (25 - 33) (AnaSpec, #AS62589), treated with DMSO (Carl Roth, #4720.1), CBX (Sigma-Aldrich, #C4790) or 10j (Merck KGaA, #385581) for 2 hours before DMSO, 11-Dehydrocorticosterone (United States Biological, #D3224-99), Corticosterone (Cayman Chemical, #16063) or Dexamethasone (Cayman Chemical, #11015) were added and cultured for 72 hours. Phorbol-12-myristate 13-acetate (PMA) (50ng ml-1, Sigma-Aldrich, #P1585), ionomycin (1µg ml-1, Sigma-Aldrich, #I3909), Golgi Plug (1:1000, BD Biosciences, #555029) and Golgi Stop (1:1000, BD Biosciences, #554724) were added four hours prior cell surface and intracellular staining. IFN- expression by gp100 peptide activated Pmel-1 CD8+ T cells was analyzed with the indicated antibodies by flow cytometry. Single cell suspensions were stained with LIVE/DEAD™ Fixable Red Dead Cell Stain Kit (1:2000, ThermoFisher Scientific, #L34971) to exclude dead cells, stained with the antibodies anti-mouse CD16/32 (1:200, BioLegend, #101301), anti-mouse Vβ 13 TCR (1:500, BD Biosciences, #746769), anti-mouse CD8a (1:1000, BioLegend, #100740), anti-mouse CD45 (1:1000,Thermo Fisher Scientific, #58-0451-82) and anti-mouse CD90.1 (1:1000, BioLegend, #202528), fixed and permeabilized and stained with the antibody anti-mouse IFN-γ (1:250, Thermo Fisher Scientific, #17-7311-82). For intracellular staining the BD Cytofix/Cytoperm Fixation/Permeabilization Kit (BD Biosciences, #554722) was used according to manufacturer's protocols. All data were acquired using a Cytek Aurora flow cytometer (Cytek Biosciences) and analyzed using FlowJoTM v10 software for Windows (BD Biosciences).

Co-detection by indexing tissue imaging

Co-detection by indexing experiments were performed following the protocol published by others (Black et al., 2021).

Tissue specimen preparation
FFPE tissues were heated at 55°C, for 30 minutes, on a slide warmer heating plate and were then let cool down at room temperature (RT). Tissues were rehydrated in the following solutions: 100% xylene (5 minutes, two times), 100% ethanol (5 minutes, two times), 90% ethanol (5 minutes), 70% ethanol (5 minutes), ddH2O (5 minutes, two times).

Antigen retrieval
Heat-induced antigen retrieval was performed at high-pressure, at 97°C, for 20 minutes, using a pressure cooker, in which tissues were immersed into a beaker containing the antigen retrieval solution (Target Retrieval Solution, pH 9, Agilent, S2367) Tissues were then let cool down at RT.

Autofluorescence quenching and staining with DNA-conjugated antibodies
In order to reduce autofluorescence of tissues, samples were immersed into a quenching solution (20 mM NaOH, 4.5% (v/v) H_2O_2, in PBS), then placed between two LED lamps for 90 minutes. The solution was changed with fresh one after the first 45 minutes.

After the autofluorescence quenching process, tissues were washed in 1X TBS IHC buffer containing Tween 20 (table 2) for 10 minutes at RT. Then tissues were covered with 100 µL of blocking solution (table 2) and incubated for 1 h, at RT, in a humidity chamber, as described by Black and colleagues (Black et al., 2021). At the end of incubation, the blocking solution was removed and 100 µL of the

mix containing the conjugated antibodies, at the proper dilution (table 1) were added on the tissues. Samples were incubated overnight at 4°C, in a humidity chamber. The following antibodies were used SOX10 (clone SOX10/1074, abcam ab212845; working dilution: 1:50), CD45 (clone 92B11 + PS7/26, NovusBio NBP2-34528, 1:50), CD68 (clone KP-1, BioLegend 916104, 1:100); HSD11B1 (polyclonal, Cayman Cay10004303-500, 1:50).
Tissues were then washed in staining buffer 2 (S2) (table 1), fixed for 10 minutes in 1.6% paraformaldehyde solution (table 1) and then washed in PBS (2 minutes, 3 times). Samples were incubated in ice-cold 100% methanol, for 5 minutes at 4°C and washed again three times in PBS. Samples were placed in the humidity chamber and 100 µL of BS3 fixative solution (Table 1) were added on each tissue. After 20 minutes of incubation, tissues were washed again three times in PBS and stored in S4 buffer until the image acquisition.

All the antibodies used for the staining had been previously conjugated to the corresponding oligonucleotide, following the protocol published by Black and colleagues (Black et al., 2021), that we slightly adapted.

Briefly, 100 µg of each purified antibody were concentrated by centrifugation (12000g, 8 min, RT), using 50 kDa filter columns. Then, the antibodies were incubated in presence of Tris (2-carboxyethyl) phosphine (TCEP) + Ethylene diamine tetraacetic acid (EDTA) solution (table 1) for 30 minutes at RT.

The antibodies were centrifuged again, and washed three times (12000g, 8 min, RT), by adding 400 µL of Buffer C (table 1), centrifuging (12000g, 8 min, RT) and discarding the flow through each time.

Finally, 200 µg of DNA oligonucleotide solution (0.5 µg/µL, in 1X PBS) were added to the respective antibody and the mixtures were incubated 2h at RT. At the end of the incubation, tubes were centrifuged (12000g, 8 min, RT) and antibodies were washed three times by adding 450 µL of high-salt PBS to each column, and centrifuging again (12000g, 8 min, RT).

After the last centrifugation, antibodies were resuspended in 200 µL of antibody stabilizer buffer (table 1), collected in new tubes and stored at 4°C.

Buffer	Composition
1X TBS IHC buffer with Tween 20	Dilute 20x TBS IHC buffer with Tween 20 (ThermoFisher, 28360) in ddH_2O
Blocking solution	B1 reagent (1:20), B2 reagent (1:20), B3 (1:20), BC4 (1:30) in S2 buffer
Blocking reagent 1 (B1)	1 mg/mL mouse IgG (Sigma, I5381-5MG) in S2 buffer
Blocking reagent 2 (B2)	1 mg/mL rat IgG (Sigma, I4131-10MG) in S2 buffer
Blocking reagent 3 (B3)	10 mg/mL sheared salmon sperm in ddH_2O (ThermoFisher, AM9680)
BC4 solution	Mixture of the 57 nonmodified CODEX® DNA oligonucleotides, each at the final concentration of 0.5 mM, in 10 mM Tris + 5 mM EDTA pH 8.0 buffer (Life Technologies GmbH, 14041568)
Staining buffer 1 (S1)	5 mM EDTA (pH 8.0), 0.5 % (wt/vol) BSA

	(Sigma, A3059), 0.02% NaN_3, in ddH_2O
Staining buffer 2 (S2)	2.5 mM EDTA (pH 8.0), 0.25 % (wt/vol) BSA, 250 mM NaCl, 61 mM Na_2HPO_4 (Sigma, S7907), 40 mM NaH_2PO_4 (Sigma, S9638) in ddH_2O
Staining buffer 4 (S4)	500 mM NaCl, in S1
Paraformaldehyde solution	1.6% paraformaldehyde (Science Services GmnH, E15710-S) in S4 buffer
BS3 fixative	Dissolve BS3, bis(sulfosuccinimidyl) suberate (ThermoFisher, 21580) in DMSO, at a final concentration of 200 mg/mL.
Final BS3 fixative	Dilute BS3 fixative 1:50 in 1X PBS, immediately prior to use.
H2 buffer	150 mM NaCl, 10 mM Tris (pH 7.5), 0.1 % Triton X-100, 10 mM $MgCl_2$, 0.02% NaN_3, in ddH_2O.
TCEP+EDTA solution	2.5 mM TCEP+ 2.5 mM EDTA pH 8, in ddH_2O
Buffer C	1 mM Tris pH 7.0, 1 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA pH 8, 0.02% wt/vol NaN_3, in ddH_2O
High salt PBS	Add 45 mL of 5M NaCl and 25 mL of 10x DPBS to 180 mL of ddH_2O, add NaN_3 to 0.02% (wt/vol) final concentration
Stock antibody stabilizer solution	Add 0.02% NaN3 to Candor PBS antibody stabilizer solution (purchased from Thermo Fisher)
Working antibody stabilizer solution	500 mM NaCl, 500 mM EDTA (pH 8.0), in stock antibody stabilizer solution (Candor)

Preparation of the fluorescent oligonucleotides plate

The cyclic acquisition of the images was enabled using the Phenocycler® instrument (Akoya Biosciences). Specifically, during each cycle, the instrument enables the acquisition of the signal of up to three fluorescent oligonucleotides, corresponding to three individual antibodies, and the nuclear stain DAPI. The fluorescent oligonucleotides were diluted in a total volume of 250 µL of plate buffer (5 mg/mL sheared salmon sperm, DAPI nuclear stain 1:300, in H2 buffer, table 1), at a concentration of 400 nM, in a Corning black 96-well plate. Each well corresponds to one cycle and contains up to three spectrally distinct fluorescent oligonucleotides, ATTO550 (Absorption max. 554 nm, Emission max. 579 nm), DY647P1 (Absorption max. 653 nm, Emission max. 672 nm), DY747P1 (Absorption max. 747 nm, Emission max. 769 nm), in addition to DAPI (Absorption max. 358 nm, Emission max. 461 nm) for nuclear detection. The first and the last cycles of the cyclic run do not contain fluorescent oligonucleotides, but only DAPI (1:300).

In the following table 2, the pairs of antibody-DNA oligonucleotide and the fluorescent DNA-oligonucleotides chosen for the experiments are summarized. The DNA oligonucleotides were purchased from Biomers.

Antibody	DNA Oligonucleotide identification number	DNA Oligonucleotide Sequence (5'-3')	Fluorescent DNA oligonucleotide sequences (5'-3')
SOX 10	72	/mal/AACGCGACGGAT	/5DY647P1/ATCCGTCGCGTT
CD45	56	/mal/GGTCACATGGTCGTT	/5DY647P1/AACGACCATGTGACC
CD68	70	/mal/AACCAAACTGACCG	/5DY647P1/CGGTCAGTTTGGTT
HSD11B1	59	/mal/GCTTATTATGGACTTC	/5ATTO550N/GAAGTCCATAATAAGC

Image acquisition and data analysis

Images were acquired using a Zeiss Axio Observer 7 inverted microscope, with Colibri 7 as the LED Light source and the Plan-Apochromat 20X/0,8 M27 (a=0,55 mm) as objective. The microscope used is equipped with the Prime BSI PCIe camera.

Once acquired, the images were converted to TIF files by using Codex Manager Instrument® (Akoya Biosciences).

Then, images were processed by using CODEX® Processor 1.8.3, and the following methods were applied:

- Image stitching
- Deconvolution
- Background subtraction
- Shading correction

Images were analyzed using Halo® v3.3.2541.256 (Indica Labs). Cell segmentation was performed using the Nuclei Segmentation Classifier, while the algorithm HighPlex FL v4.1.3 was used to analyze cell phenotypes. Tumor area was marked as Region of Interest (ROI) that was used to analyze the expression of HSD11B1 in macrophages (CD45+, CD68+) and tumor cells (SOX10+).

References

Black, S., Phillips, D., Hickey, J.W. *et al.* CODEX multiplexed tissue imaging with DNA-conjugated antibodies. *Nat Protoc* **16,** 3802–3835 (2021). https://doi.org/10.1038/s41596-021-00556-8