Supplementary Information

Raman spectroscopy and machine learning reveals early tumor microenvironmental changes induced by immunotherapy

Santosh Kumar Paidi[1,#,&], Joel Rodriguez Troncoso[2,#], Piyush Raj[1], Paola Monterroso Diaz[2], Jesse D. Ivers[2], David E. Lee[3$], Nathan L. Avaritt[4,5,7], Allen J. Gies[4,5], Charles M. Quick[6], Stephanie D. Byrum[4,5,7], Alan J. Tackett[4,5,7], Narasimhan Rajaram[2,5,*], Ishan Barman[1,8,9,*]

[1]Department of Mechanical Engineering, Johns Hopkins University, Baltimore, MD, 21218

[2]Department of Biomedical Engineering, University of Arkansas, Fayetteville, AR, 72701

[3]Department of Health, Human Performance, and Recreation, University of Arkansas, Fayetteville, AR, 72701

[4]Department of Biochemistry and Molecular Biology, University of Arkansas for Medical Sciences, Little Rock, AR, 72205

[5]Winthrop P. Rockefeller Cancer Institute, University of Arkansas for Medical Sciences, Little Rock, AR, 72205

[6]Division of Pathology, University of Arkansas for Medical Sciences, Little Rock, AR, 72205

[7]Arkansas Children's Research Institute, Little Rock, AR, 72205

[8]The Russell H. Morgan Department of Radiology and Radiological Science, The Johns Hopkins University School of Medicine, Baltimore, MD, 21205

[9]Department of Oncology, Johns Hopkins University, Baltimore, MD, 21287

[#]Both authors contributed equally to this work.

[&] Current affiliation – School of Optometry, University of California, Berkeley, CA, 94720

[$] Current affiliation – Duke Molecular Physiological Institute, Duke University, 300 N. Duke St., Durham, NC, 27701
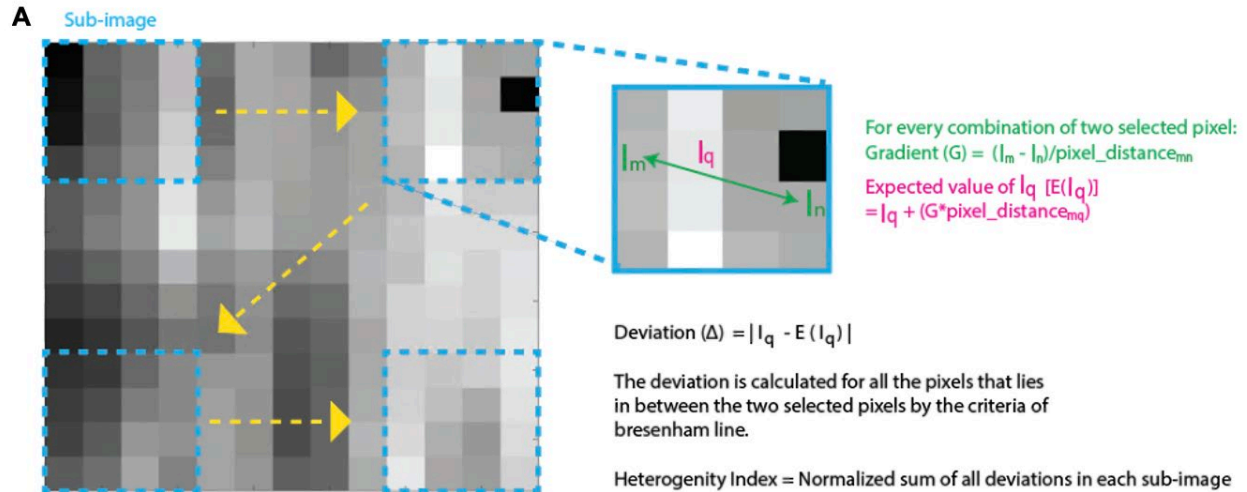
Running title: Raman spectroscopy of response to immunotherapy

*Corresponding authors:

Narasimhan Rajaram, University of Arkansas, Department of Biomedical Engineering, 700 W. Research Center Blvd., Fayetteville, AR 72701, USA, Email: nrajaram@uark.edu, Phone: 479-575-7282

Ishan Barman, Johns Hopkins University, Department of Mechanical Engineering, 3400 N Charles St, Latrobe 103, Baltimore, MD 21218, USA, Email: ibarman@jhu.edu, Phone: 410-516-0656

The authors disclose no potential conflicts of interest.

A Sub-image

For every combination of two selected pixel:
Gradient $(G) = (I_m - I_n)/\text{pixel\_distance}_{mn}$

Expected value of I_q $[E(I_q)]$
$= I_q + (G \cdot \text{pixel\_distance}_{mq})$

Deviation $(\Delta) = |I_q - E(I_q)|$

The deviation is calculated for all the pixels that lies in between the two selected pixels by the criteria of bresenham line.

Heterogenity Index = Normalized sum of all deviations in each sub-image

We find heterogenity index for each 4 pixel x 4 pixel sub-image obtained by raster scanning a 4x4 moving window across the MCR scoremaps.

This analysis is repeated for all the MCR Scores and for all the tumors.
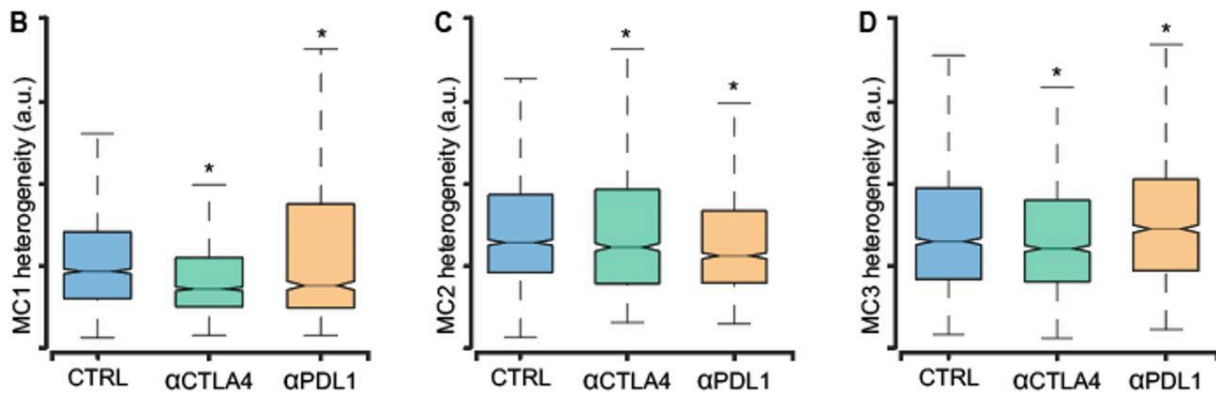
Figure S1. Raman assessment of changes in spatial heterogeneity in response to immunotherapy. (**A**) A schematic of the method used for the measurement of heterogeneity index is shown. Box and whisker plots showing the median and interquartile range for the spatial heterogeneity indices derived from the MCR score maps of the tumors in the treatment groups are presented for (**B**) MC1, (**C**) MC2 and (**D**) MC3. The statistical significance of the differences in the heterogeneity of the immune checkpoint inhibitor-treated tumors compared to the controls are assessed by Wilcoxon rank sum test. * indicates p-value < 0.05.
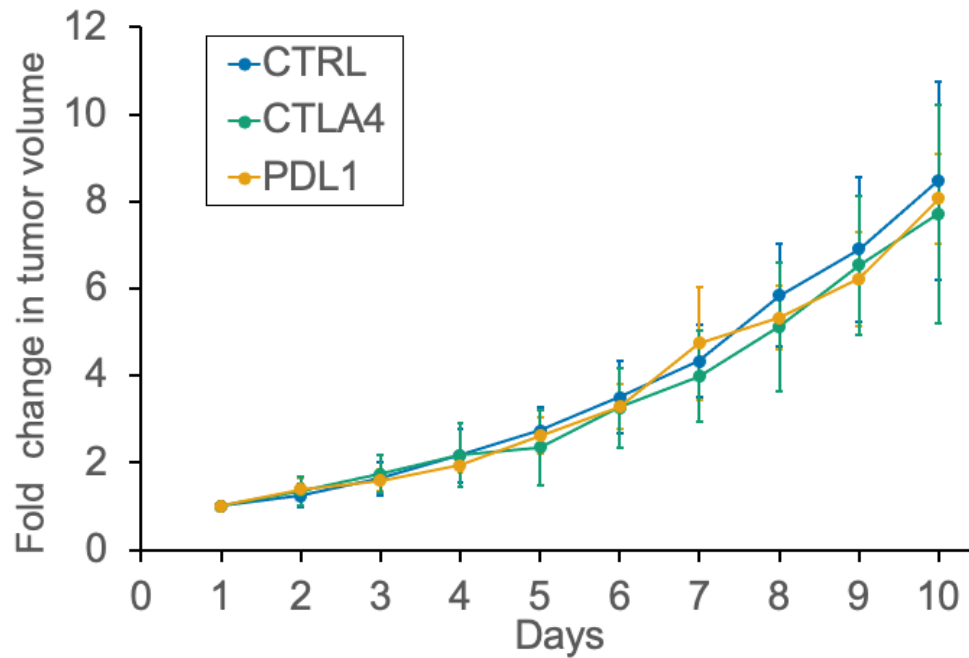
Figure S2. Evolution of tumor volume with immunotherapy. The curves show the mean (marker) and standard deviation (error bars) of the volume (fold change compared to day 1) for tumors in the CTRL, αCTLA4, and αPDL1 treatment groups show mean and standard deviation of the volumes (fold change compared to day 1).

Table ST1. Table of MCR-ALS component spectral peak assignments

MC1	MC2	MC3	MC4	MC5	Raman band assignment from literature
					Observed Raman peaks in the MCR loadings (cm⁻¹) / **Raman band assignment from literature**
	793				O-P-O stretching in DNA
	813				O-P-O stretching in DNA and RNA
		851	849		C-C stretch of proline in collagen
				908	Formalin contamination during tissue fixation
		930	930		C-C vibration in collagen backbone
		1042			Proline in collagen
				1042	Formalin contamination during tissue fixation
1078					C-C stretch
	1073				PO_2^- symmetric stretching in DNA
	1235				PO_2^- asymmetric stretching in DNA
				1247	Formalin contamination during tissue fixation
		1256			Amide III in collagen
			1262		Amide III in collagen
1266					CH_2 in-plane deformation (Triglyceride)
1302			1301		CH vibration (Triglyceride)
		1315			CH_3CH_2 twisting modes of collagen
					CH_3CH_2 wagging modes of collagen and nucleic acids
1442			1442		CH_2 bending mode (Triglyceride)
		1448			CH_2 bending mode in collagen
				1489	Formalin contamination during tissue fixation
1654					C=C lipid stretch
		1657	1657		α-helical structure of amide I in collagen

Table ST2. Assignment for the top spectral predictors derived from random forest analysis

Observed Raman peaks	Raman band assignment from literature
981	C-C stretching (proteins)
1065	=CH bending (lipids)
1323	C-C stretch (lipids)
1386	δCH3 symmetric band (lipid)
1574	Nucleic acid modes
1596	C=C in-plane bending mode (phenylalanine)
1652	Amide I (collagen) / C=C stretch (lipids)
1763	C=0 stretch (lipids)