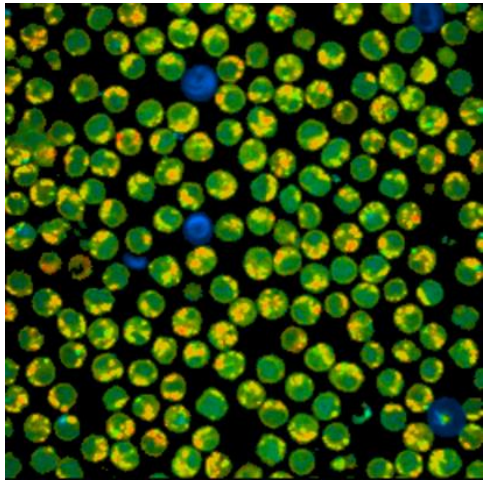

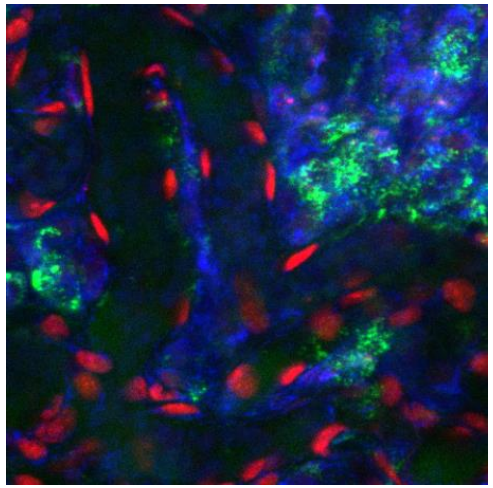

Autofluorescence imaging of immune cell metabolism

Alexa Heaton, Dan Pham, Alex Walsh, Katie Mueller, Anna Hoefges, Peter Rehani, Nathaniel Burkard, Katherine Budde, Alexander Rakhmilevich, Amy Erbe, Kelsey Tweed, Isabel Jones, Kris Saha, Paul Sondel, **Melissa C. Skala**

Investigator, Morgridge Institute for Research
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Biomedical Engineering
UNIVERSITY OF WISCONSIN–MADISON

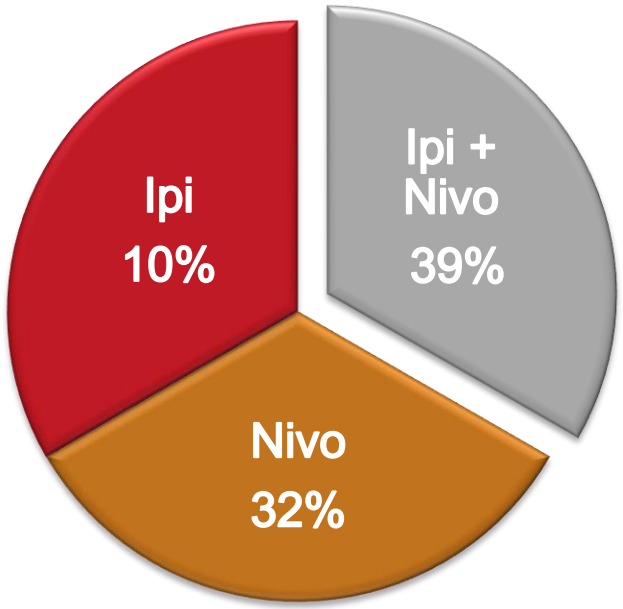
MORGRIDGE
INSTITUTE FOR RESEARCH

Disclosures

I have no disclosures

Melanoma immunotherapies

3-Year Survival[3]



Ipilimumab (Yervoy)
Nivolumab (Opdivo)

61% of patients have progressive melanoma after immune checkpoint inhibitors alone

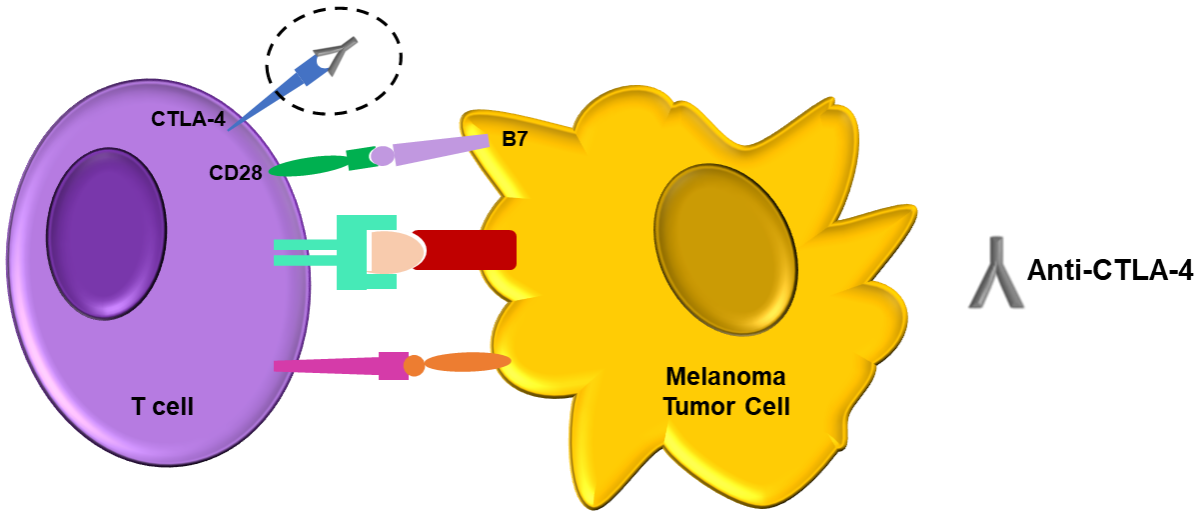
Improvements in patient outcome with checkpoint inhibitors require new tools to understand tumor immune response.

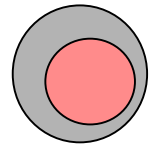
[1] Nogrady *Nature* **2020** [2] Zaidi and Jaffee *J. Clin. Invest.* **2019** [3] Wolchok *et al. N. Engl. Med.* **2017**

Metabolic changes with T cell activation

T cells activate when they recognize their antigen on the surface of antigen presenting cells

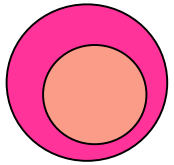
Checkpoint inhibitors (anti-CTLA-4) blocks tumor cells from turning off T cell killing





Quiescent CD8 T cell

Activation



Activated CD8 T cell (CTL)

↑↑ Glycolysis

Can autofluorescence provide label-free measure of T cell activation?

Autofluorescence of NAD(P)H and FAD: Optical Redox Ratio

Autofluorescent
NAD(P)H
FAD

Not fluorescent
$NAD(P)^+$
$FADH_2$

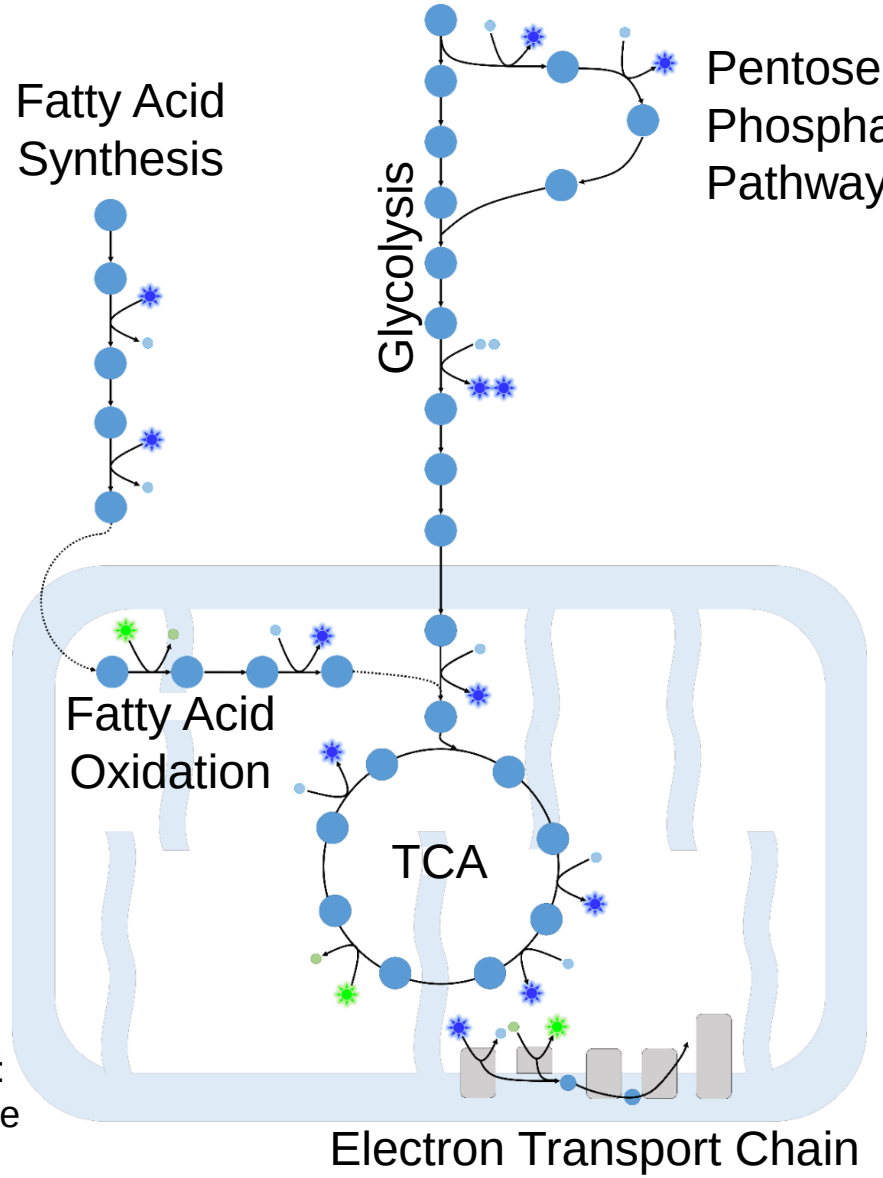


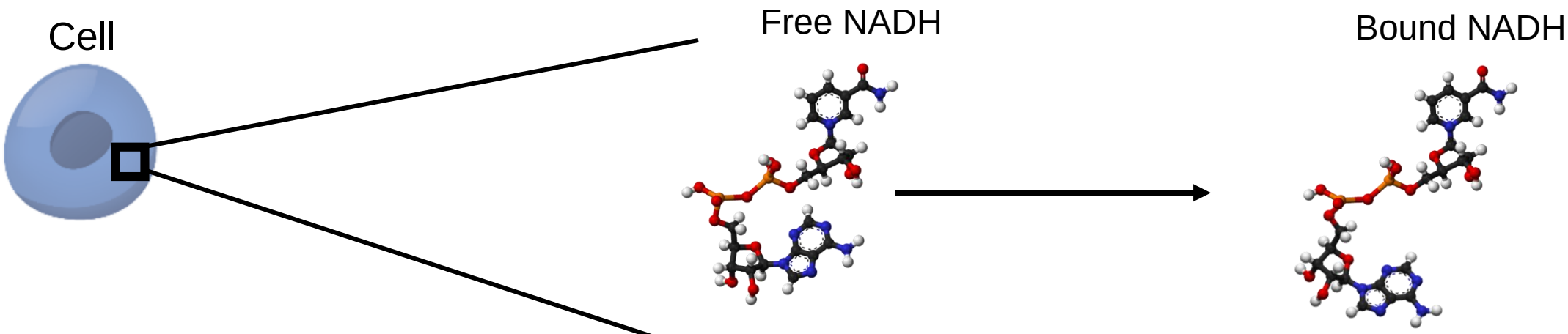
Figure credit: Amani Gillette

$$\text{Optical Redox Ratio} = \frac{I_{NAD(P)H}}{I_{FAD}}$$

Redox ratio = relative amounts of electron donor & acceptor in a cell

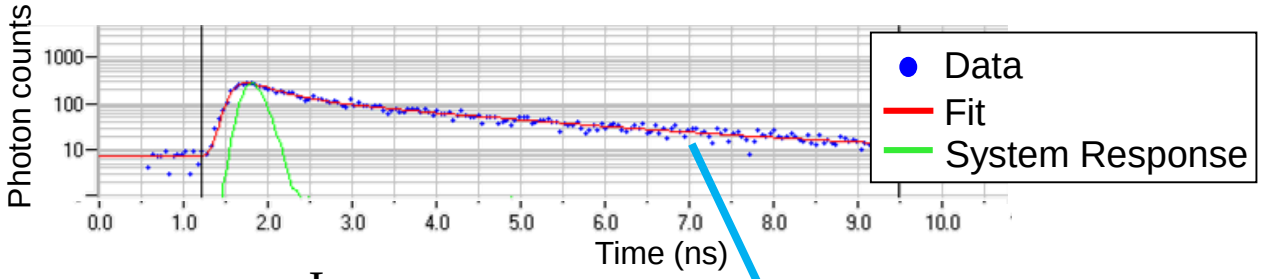
1. Chance et al., J Biol Chem 1979 **2.** Georgakoudi et al Annu. Rev. BME (2012) **3.** Quinn et al. Sci Rep (2013) **4.** Heaster et al, J. Biophotonics (2017)

Fluorescence lifetime

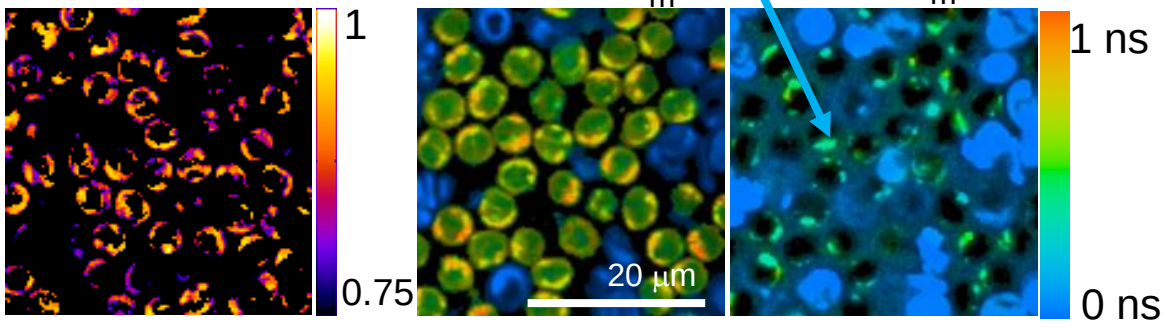


Optical metabolic imaging (OMI) of NAD(P)H and FAD

Two-photon fluorescence lifetime imaging microscopy (FLIM)



$$\text{Redox Ratio} = \frac{I_{NADH}}{I_{FAD}}$$



$$I(t) = \alpha_1 e^{-t/\tau_1} + \alpha_2 e^{-t/\tau_2} + C$$

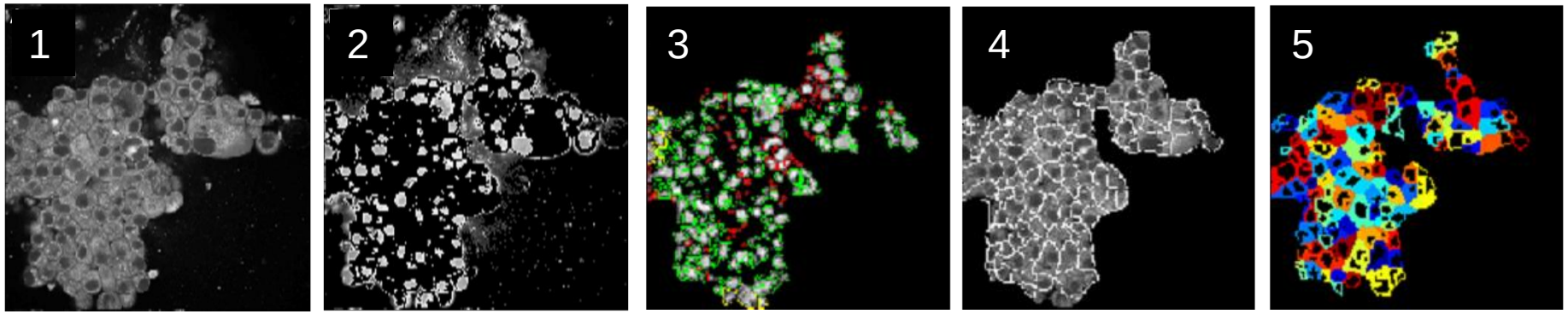
$$\tau_m = \alpha_1 \tau_1 + \alpha_2 \tau_2$$

NAD(P)H τ_1	Free NAD(P)H
NAD(P)H τ_2	Bound NAD(P)H
FAD τ_1	Bound FAD
FAD τ_2	Free FAD
α	Fractional component

2-Photon Fluorescence Microscope

- 750 nm excitation (400-480 nm emission) for NAD(P)H
- 890 nm excitation (500-600 nm emission) for FAD
- 1040nm excitation (690/45 nm emission) for CD69-PerCP
- 100X/1.45 NA objective
- B&H SPC150 board; GaAsP PMT
- 4.8 µs pixel dwell time, 60 second integration

Single cell segmentation & population modeling



1. NAD(P)H intensity image

2. Threshold to identify nuclei

3. Set nuclei as primary objects

4. Propagate out from nuclei to identify cells

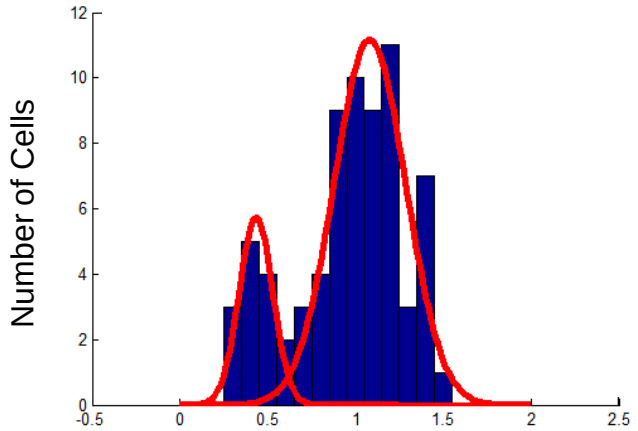
5. Cytoplasm = cell - nuclei

Use mask to extract for each cell:

1) NAD(P)H τ_1
2) NAD(P)H τ_2
3) NAD(P)H α_1
4) FAD τ_1
5) FAD τ_2
6) FAD α_1
7) NAD(P)H photon count
8) FAD photon count

Calculate combination variables:
Redox ratio, NAD(P)H τ_m , FAD τ_m

Identify cell sub-populations with AIC criteria[3]

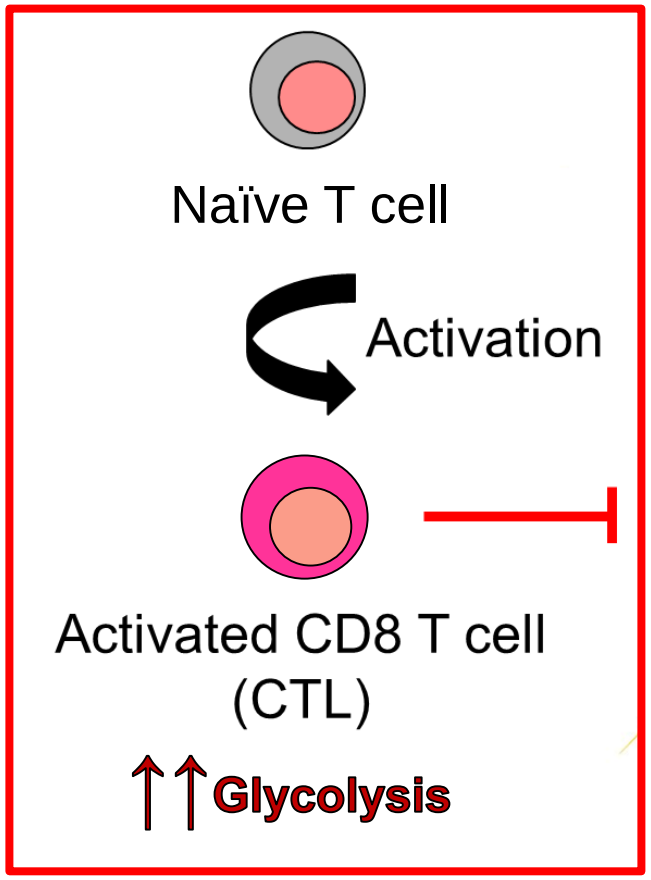


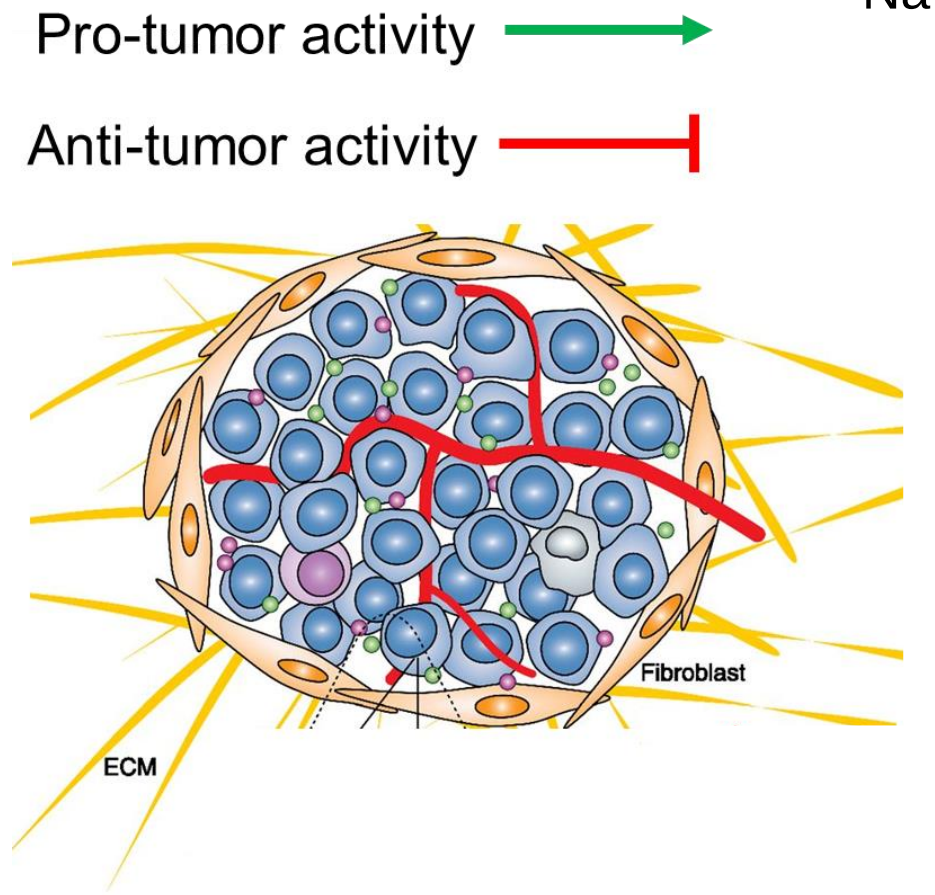
- Validated *in vitro* to accuracy <1% [4]

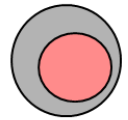
1. www.cellprofiler.org 2. https://www.cellpose.org/ 3**.** Walsh et al. Proc SPIE 2014. 4**.** Akaike H. IEEE Transactions 1974. 5**.** Walsh et al., Biomed. Opt. Exp. 2015

T cells in cancer

Both CD4+ and CD8+ T cells are required for effective treatment with checkpoint inhibitors[1]





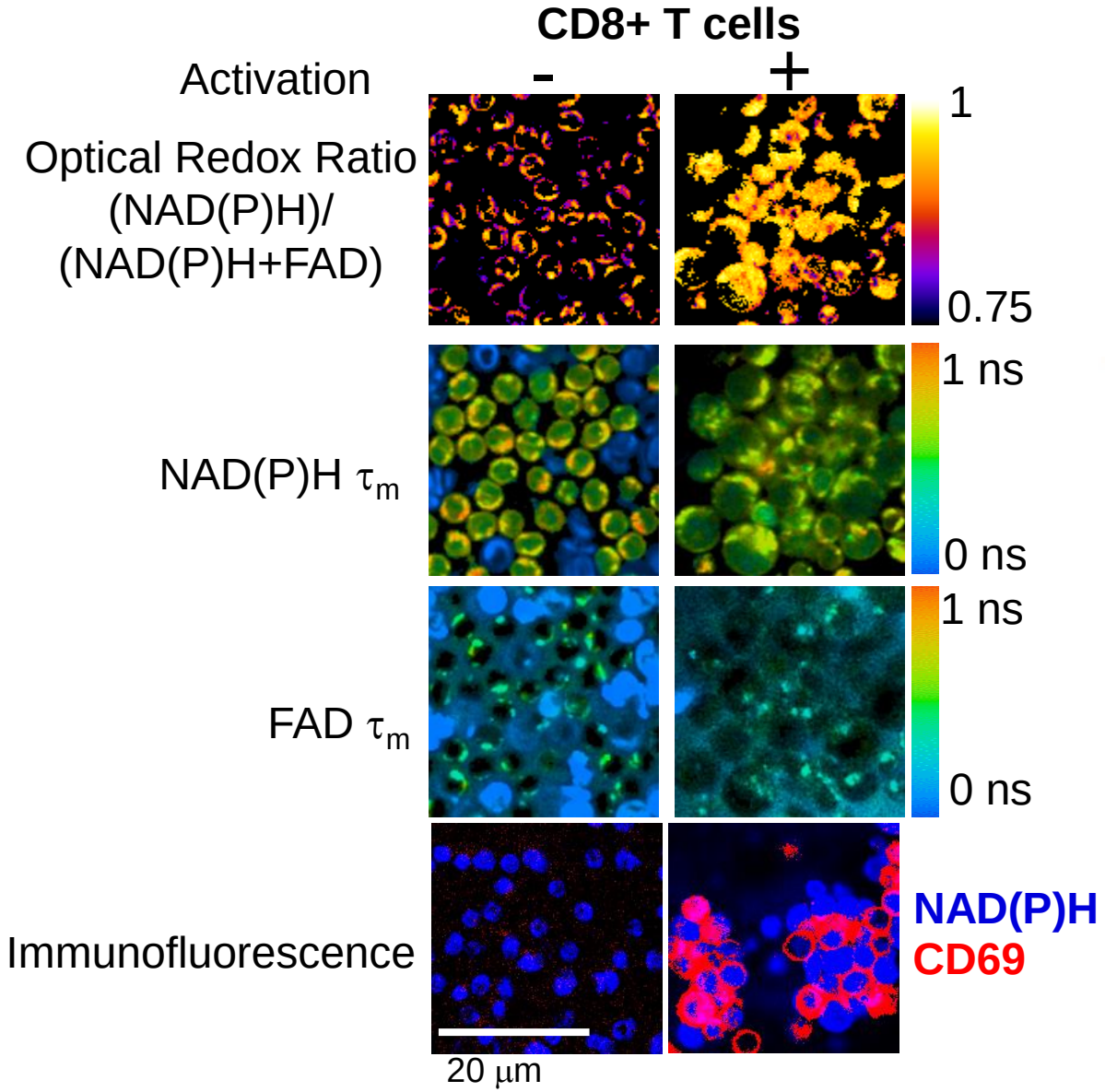


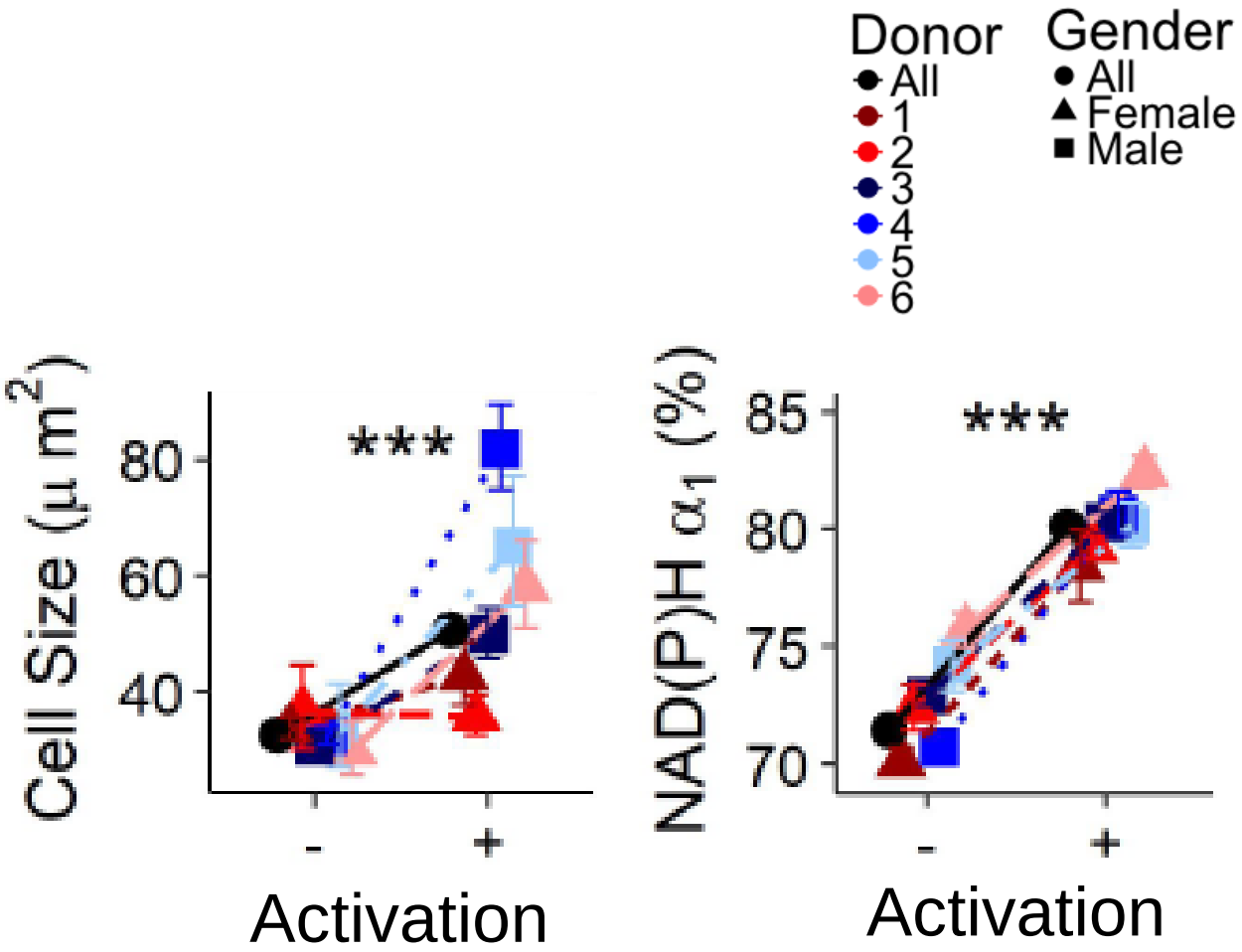
Helper (CD4+) T cells

Modified from: Dumitru, et al. Front Immunol, 2018.

1. Arakawa, Akiko, et al. Frontiers in immunology (2019).

Representative OMI of T cells





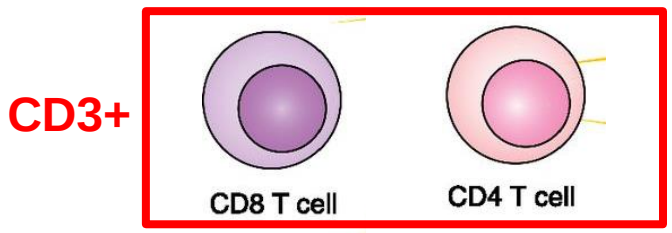
n = 89-1998 cells

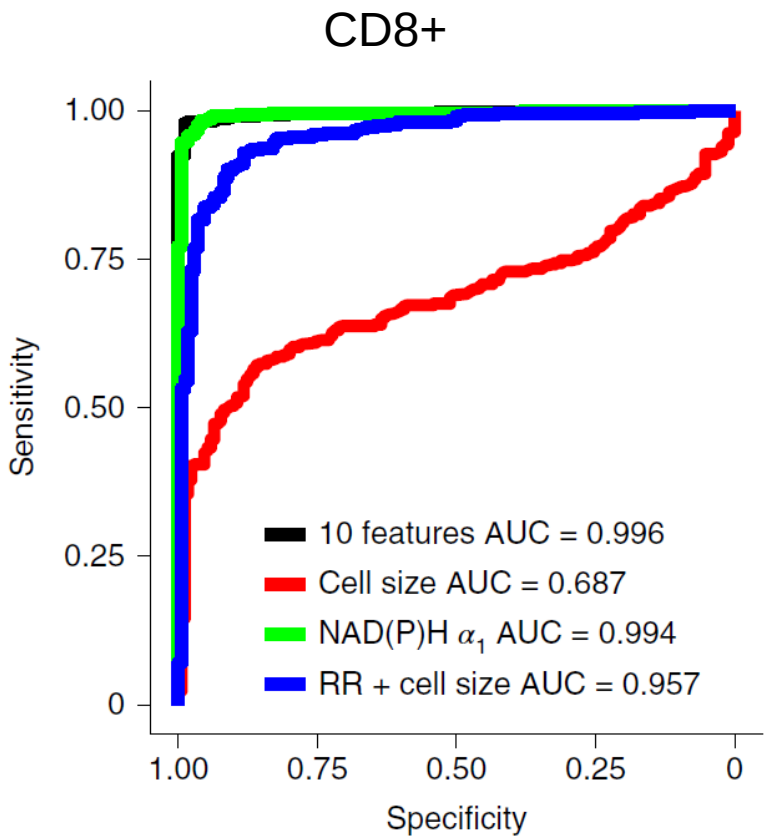
Mean +/- 99% CI, *** p<0.001, p-value from GLM adjusted for donor

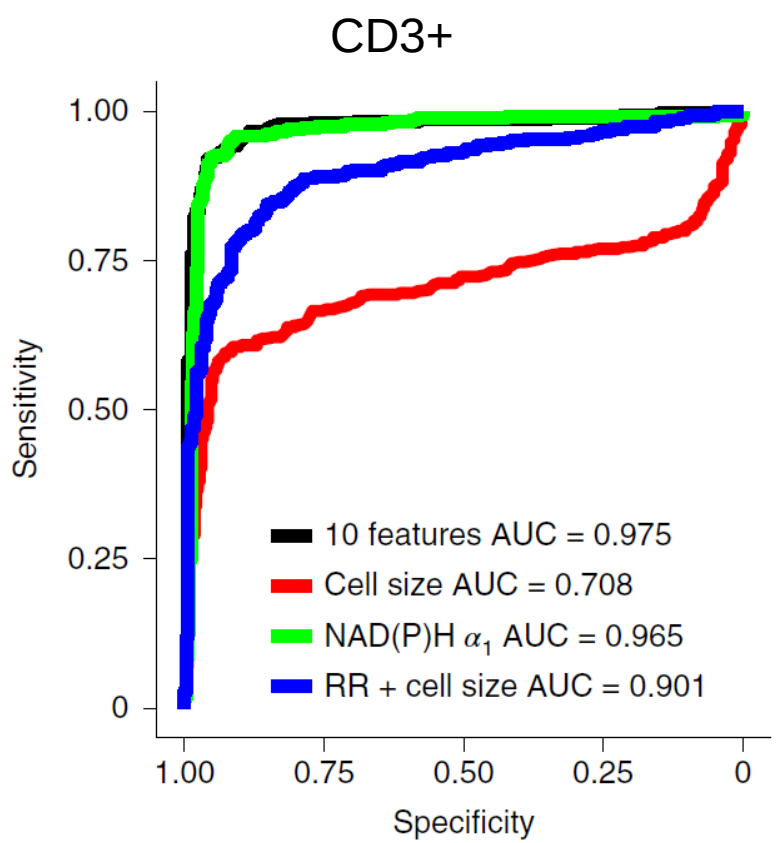
Walsh et al., Nat. Biomed. Eng. 2021

OMI classifies T cell activation

ROC curves for logistic regression models to classify activation state



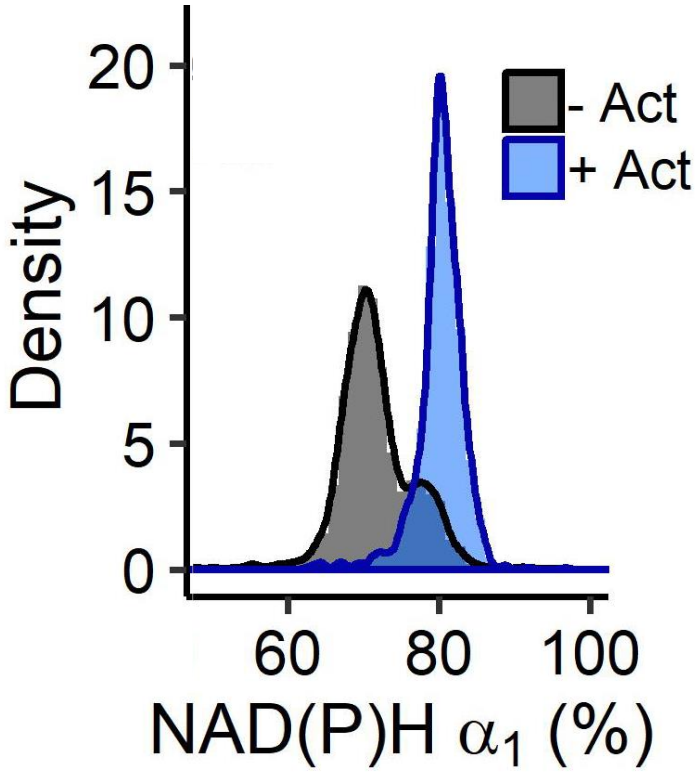


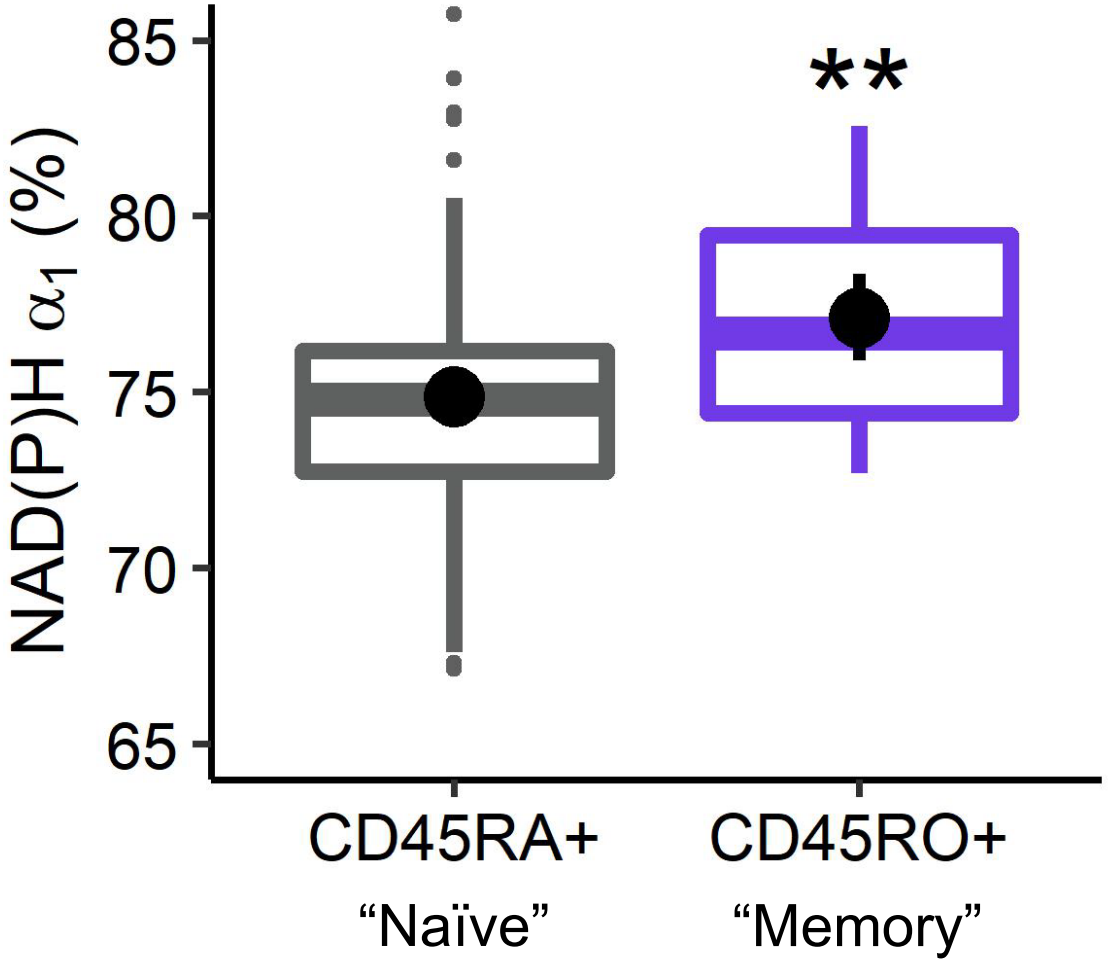


- Training Data: 4 donors
  - 2655 CD8+ cells (82%)
- Testing Data: 3 donors
  - 595 CD8+ cells (18%)

Walsh et al., Nat. Biomed. Eng. 2021

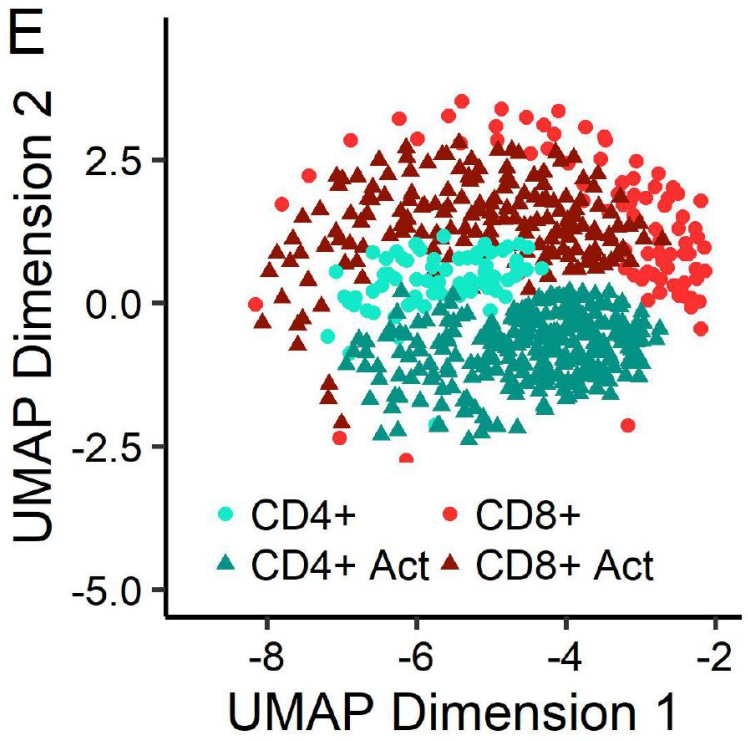
OMI discriminates quiescent CD8+ T cell sub-sets

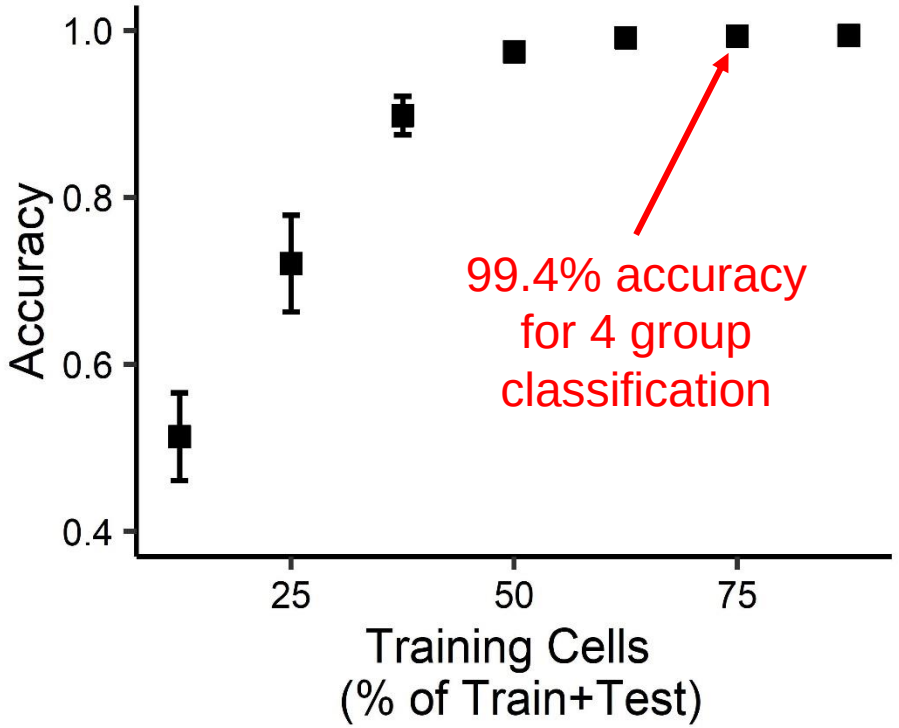




Walsh et al., Nat. Biomed. Eng. 2021

OMI classifies helper (CD4+) vs. cytotoxic (CD8+) T cells in peripheral blood



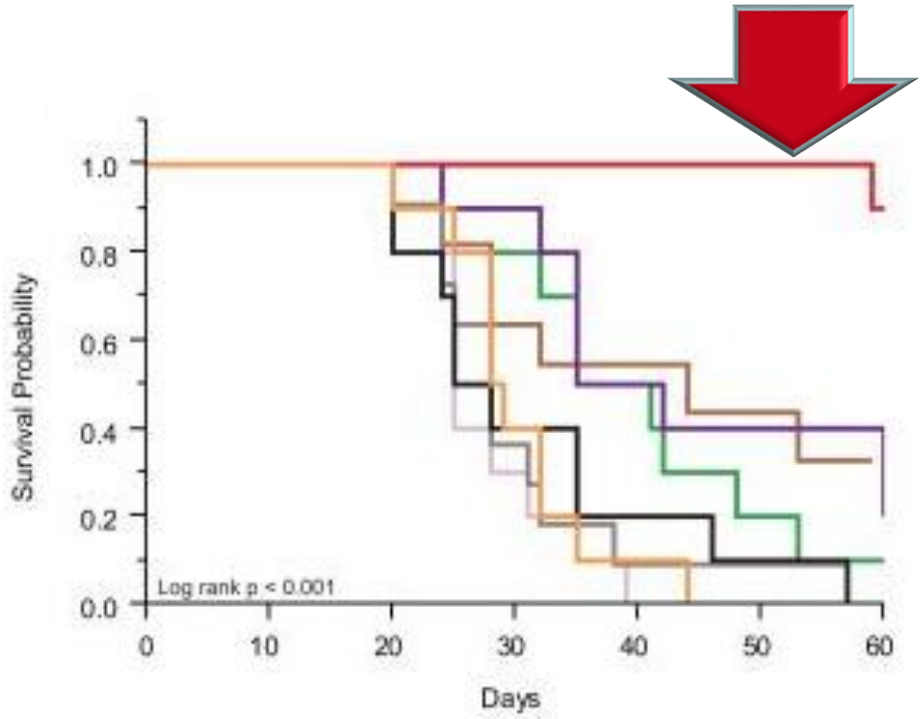


Walsh et al., Nat. Biomed. Eng. 2021

Curative melanoma immunotherapy in mice

- Curative immunotherapy for melanoma demonstrated in mice[1-2]
  - Radiation + immuno-cytokine + checkpoint inhibitor
  - Cures ~90% GD2+ tumors
  - T cells critical
  - Currently in Phase II clinical trials

- **How is this therapy working? How can we translate to other cancer types?**



OMI of tumor and CD8 T cell metabolic changes *in vivo* during curative immunotherapy

[1] Morris *et al. Cancer Res.* **2016** [2] Morris *et al. Cancer Immunol. Res.* **2018**

Mouse tumor model

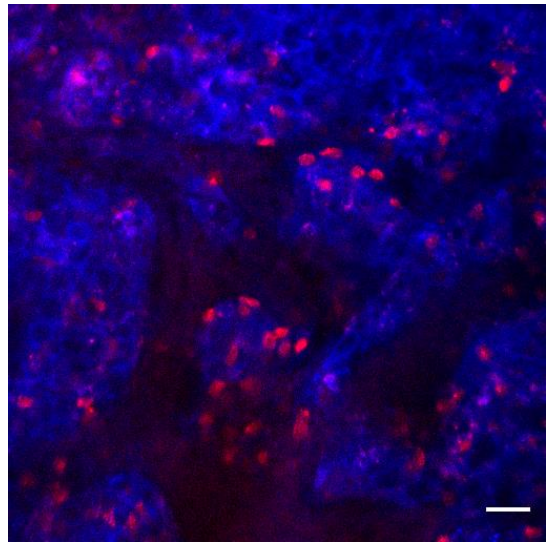
Mouse

- C57BL/6 mice with CRISPR/Cas9 knock-in of mCherry to CD8+ T cells only

Tumor

- B78 murine melanoma: derived from B16, transfected to upregulate GD2 and lack melanin[1]
- Disialoganglioside (GD2) overexpressed on cell surface[2-3]
  - Melanomas, neuroblastomas, gliomas, lung cancers, soft tissue sarcomas

***In Vivo* Melanoma Tumor**

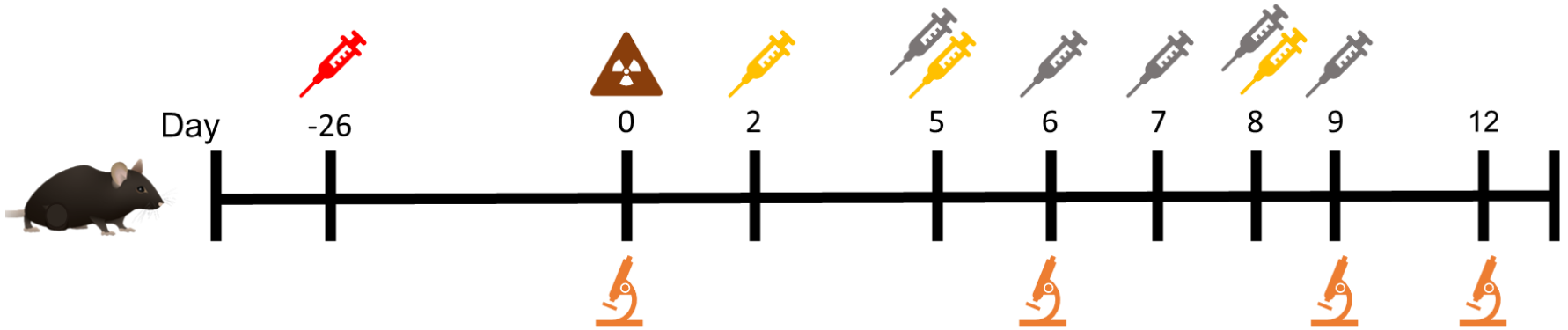


CD8-mCherry

NAD(P)H mCherry

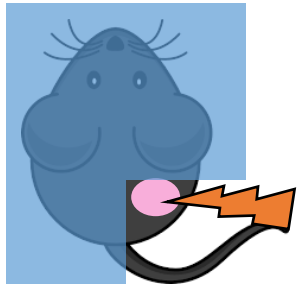
[1] Haraguchi *et al. PNAS* **1994** [2] Navid *et al. Curr. Cancer Drug Targets* **2010** [3] Nazha *et al. Front. Oncol.* **20201**

Treatment regimen



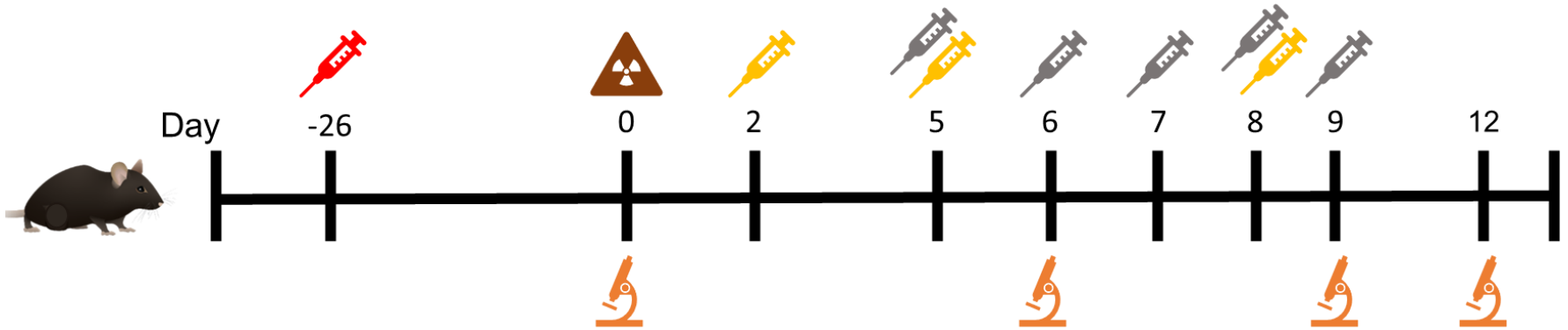
2×10^6 B78 cells injected I.D. into right flank
RT (12 Gy)
α-CTLA-4 (200 µg) I.P.
Hu14.18-IL2 (50 µg) I.T.
OMI, flow, mIF

- Low dose radiation to tumor only
  - Mouse shielded by lead
- Tumor antigens released; immune cells recruited to tumor



[1] Demaria and Formenti *Front. Oncol.* **2012**

Treatment regimen



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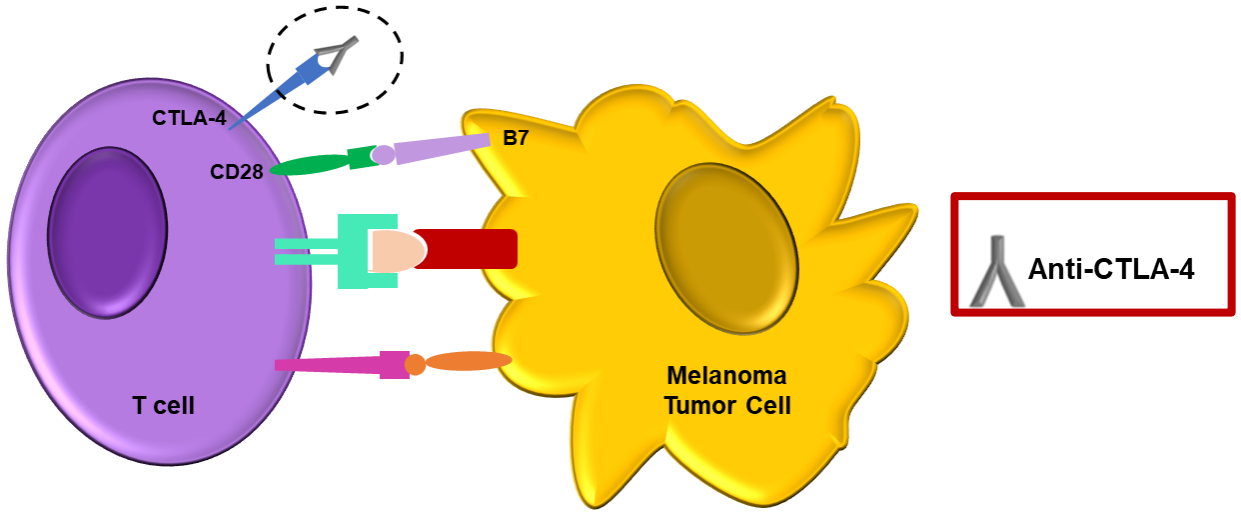
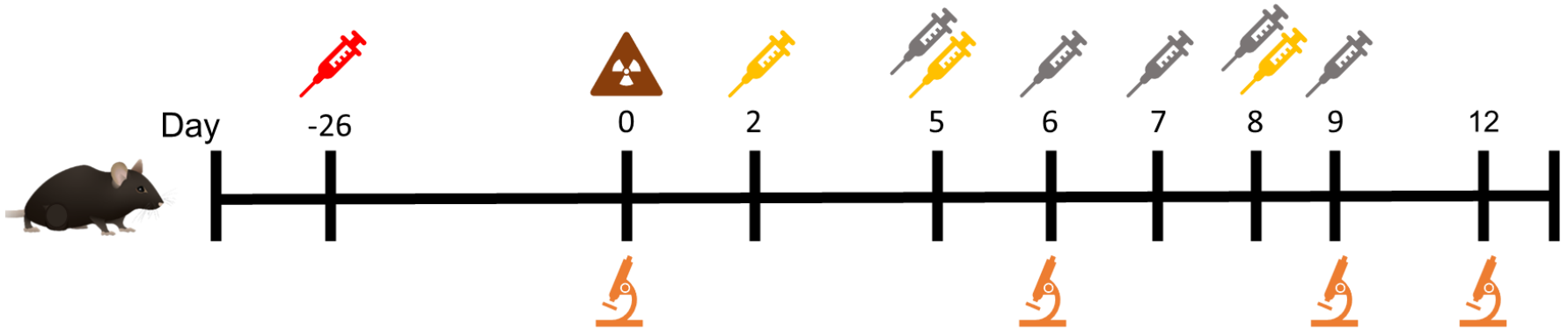
I.D. into right flank
RT (12 Gy)
α-CTLA-4 (200 μg) I.P.
Hu14.18-IL2 (50 μg) I.T.
OMI, flow, mIF

- Anti-CTLA-4 or Ipilimumab antibody
- Blocks tumor cells from turning off T cell killing
- Can eliminate suppressive T_{reg}



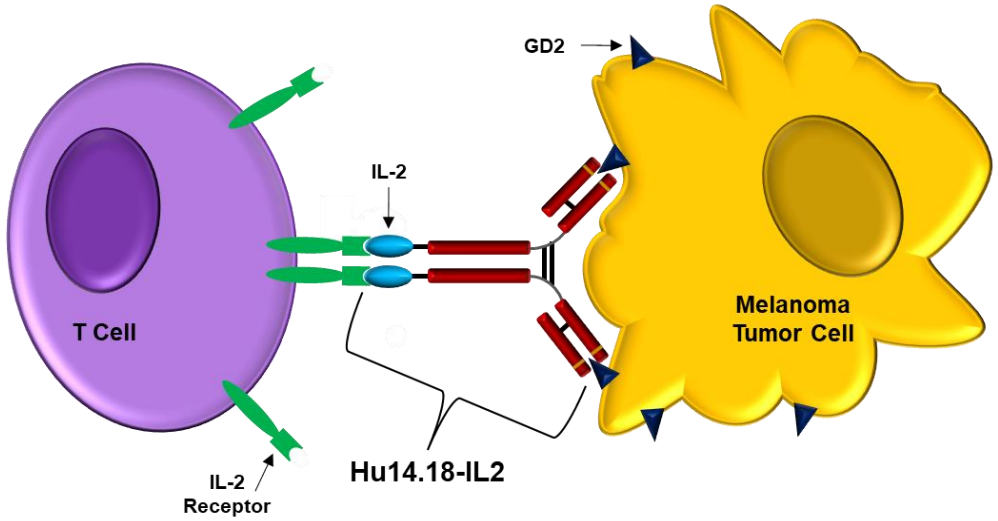
[1] Waldman *et al. Nat. Rev. Immunol.* **2020**

Treatment regimen



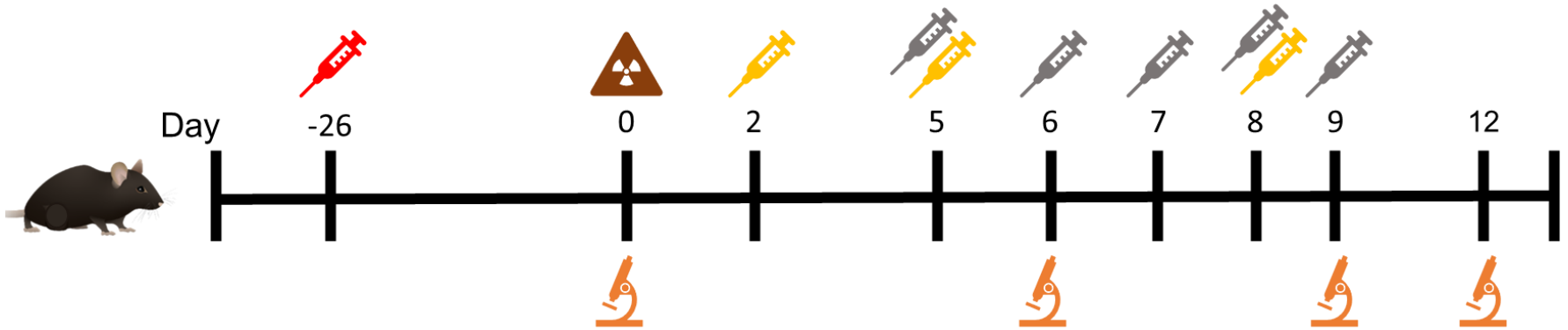
2×10^6 B78 cells injected
I.D. into right flank
RT (12 Gy)
α-CTLA-4 (200 µg) I.P.
Hu14.18-IL2 (50 µg) I.T.
OMI, flow, mIF

- Hu14.18-IL2 immunocytokine
- IL2 activates T and NK cells
- Anti-GD2 targets tumor cells



[1] Gillies *et al. Proc. Nati. Acad. Sci.* **1992** [2] Osenga *et al. Clin. Cancer Res.* **2006** [3] Yang *et al. J Immunol.* **2012**

Treatment regimen



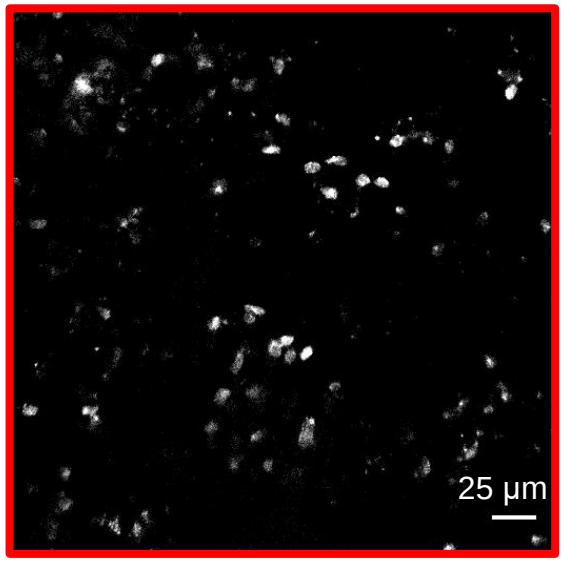
2×10^6 B78 cells injected
I.D. into right flank
RT (12 Gy)
α-CTLA-4 (200 µg) I.P.
Hu14.18-IL2 (50 µg) I.T.
OMI, flow, mIF

- Intravital OMI on treated and untreated mice
- After imaging, tissues harvested for flow cytometry and multiplex immunofluorescence

[1] Haraguchi *et al. PNAS* **1994** [2] Navid *et al. Curr. Cancer Drug Targets* **2010**

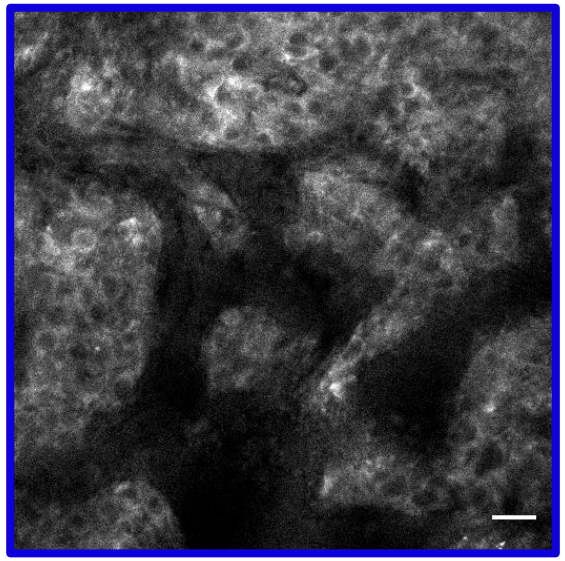
Single cell segmentation of *in vivo* images

CD8 T Cells

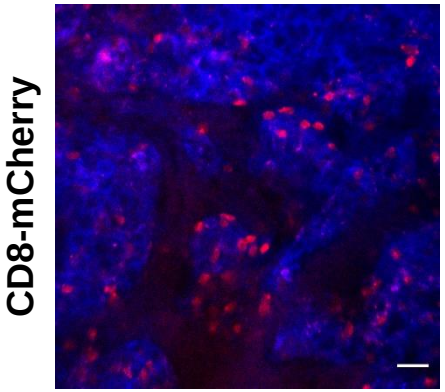


- mCherry intensity images
- Whole cell
- Manually segmented via custom CellProfiler pipeline

B78 Tumor Cells

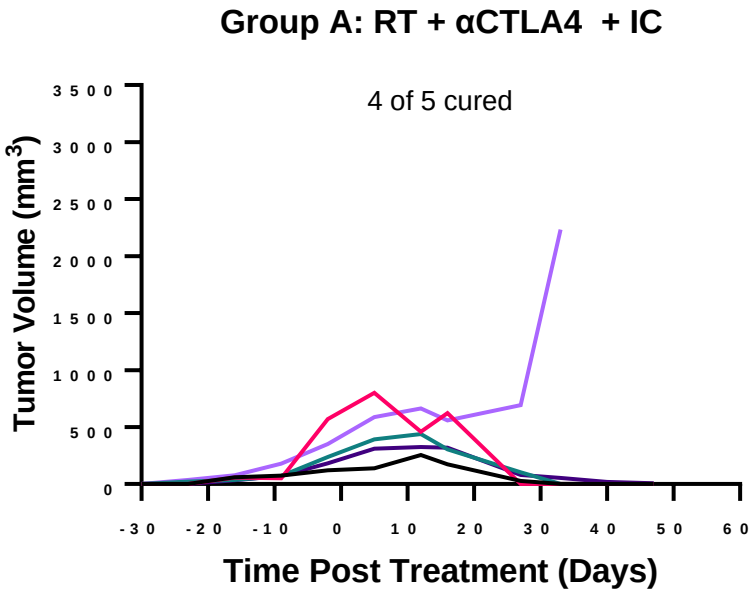


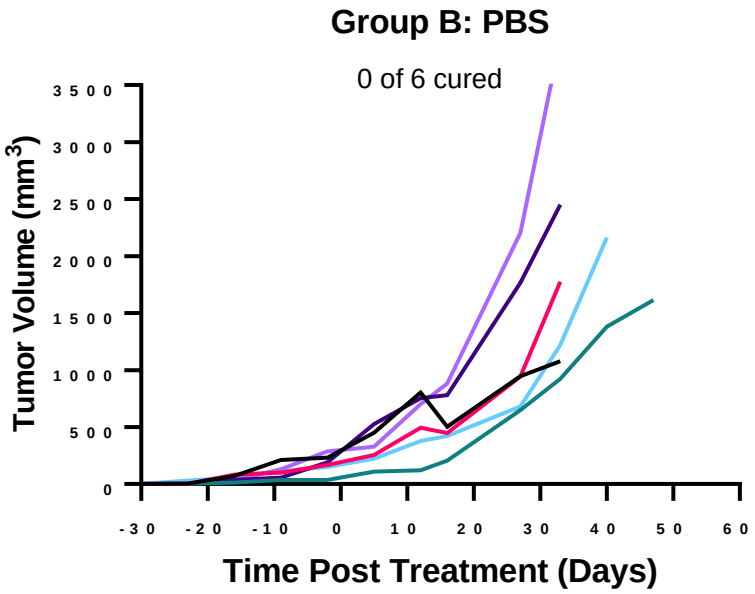
- NAD(P)H intensity images
- Whole cell
- Automatically segmented via Cellpose through a Napari viewer

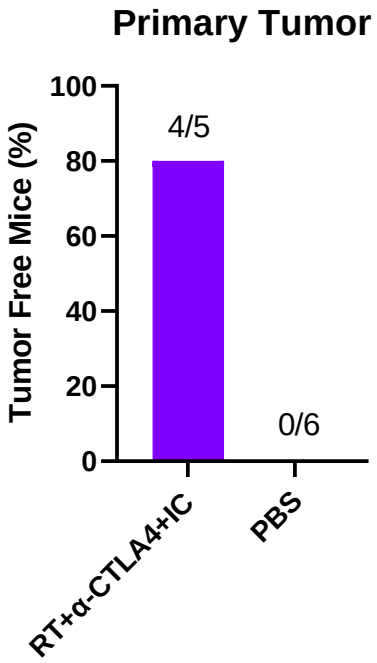


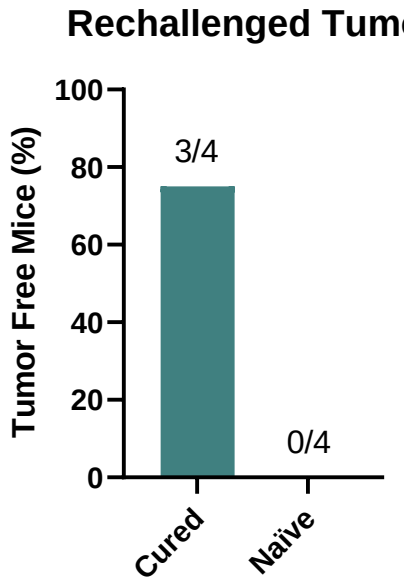
[1] Cellprofiler www.cellprofiler.org [2] Cellpose www.cellpose.org

Melanoma tumor growth and cure rate





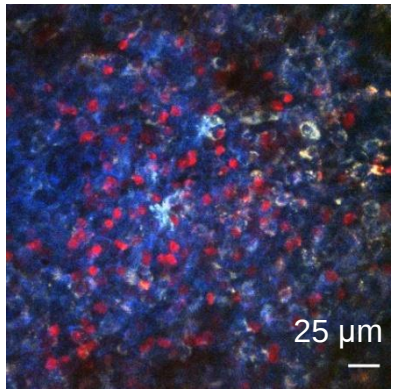




Visualization of tumor microenvironment

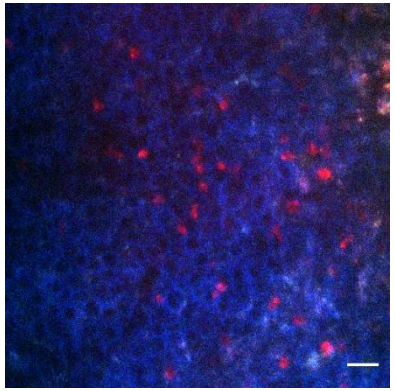
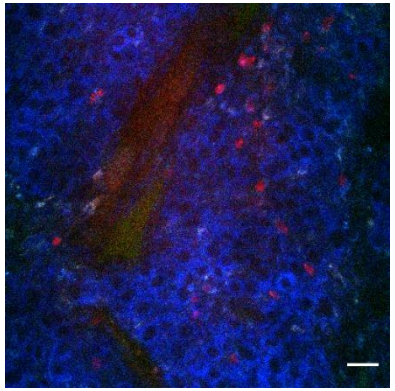
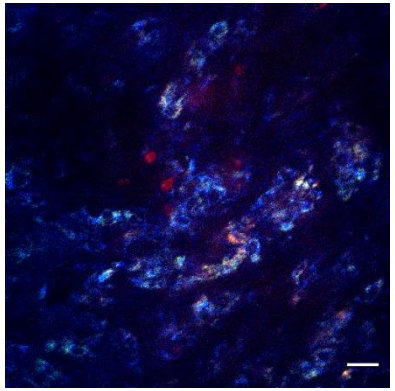
In Vivo
Fluorescence Intensity Images
mCherry
NAD(P)H FAD

Day 0 Pretreatment

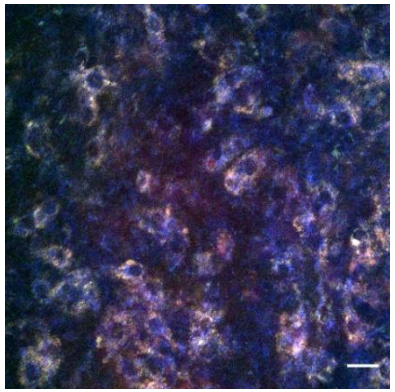
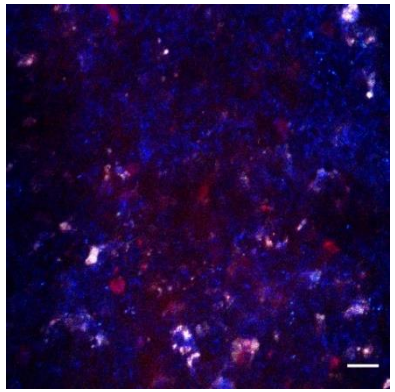
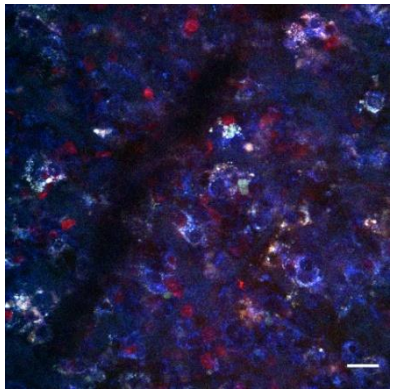


Day 6 **Day 9** **Day 12**

Control Mice

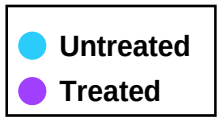
Treated Mice

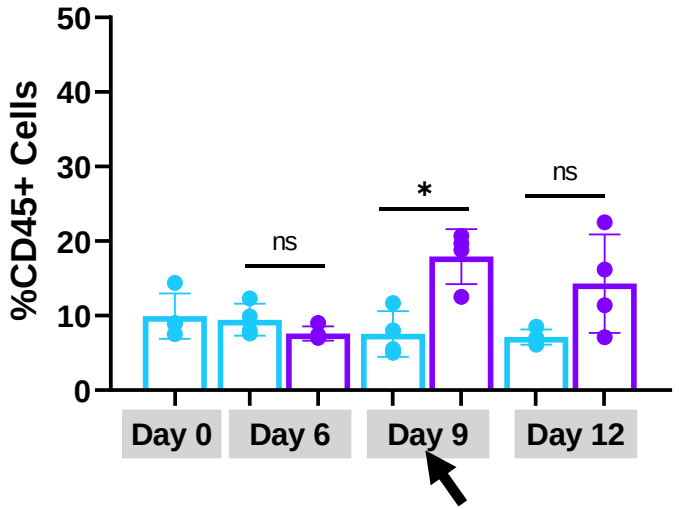
OMI: n = 2 mice per day

Functional changes during immunotherapy

Flow Cytometry

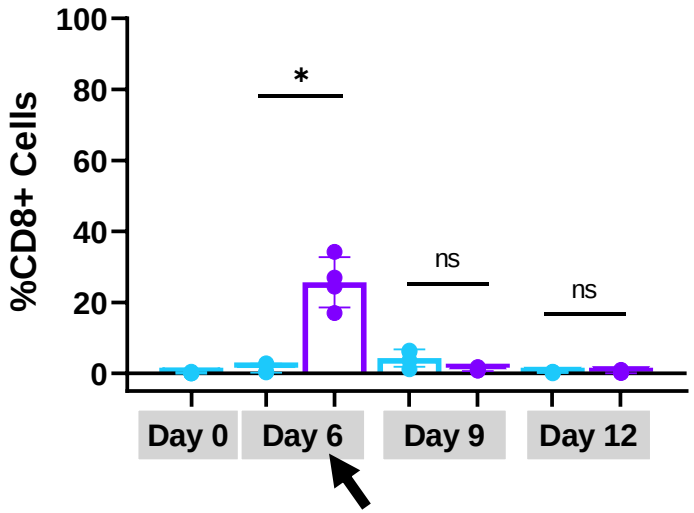


Infiltrating CD8 T Cells



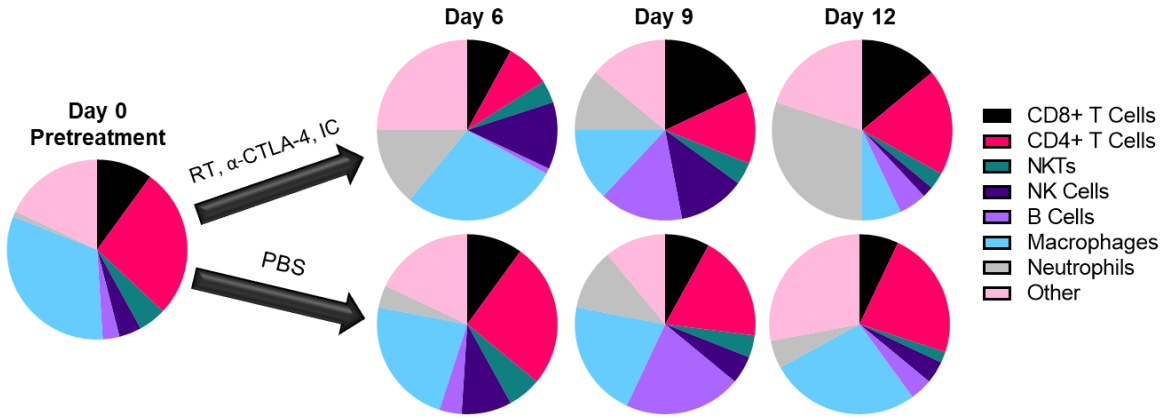
Peak CD8 T cell infiltration day 9

CD69+ CD8 T Cells

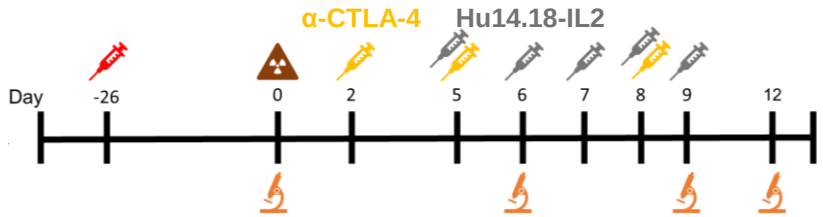


Peak CD8 T cell activation day 6

CD69: early activation marker

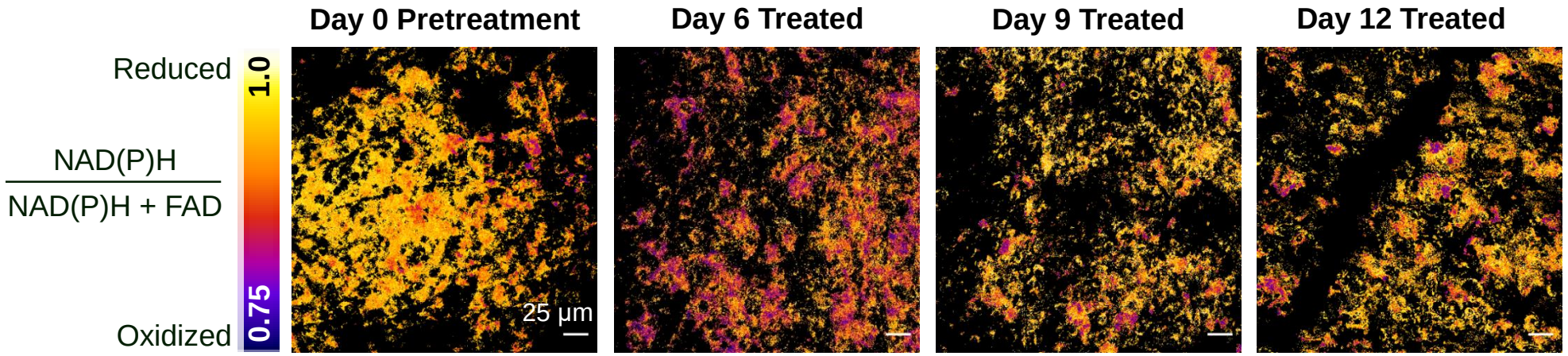


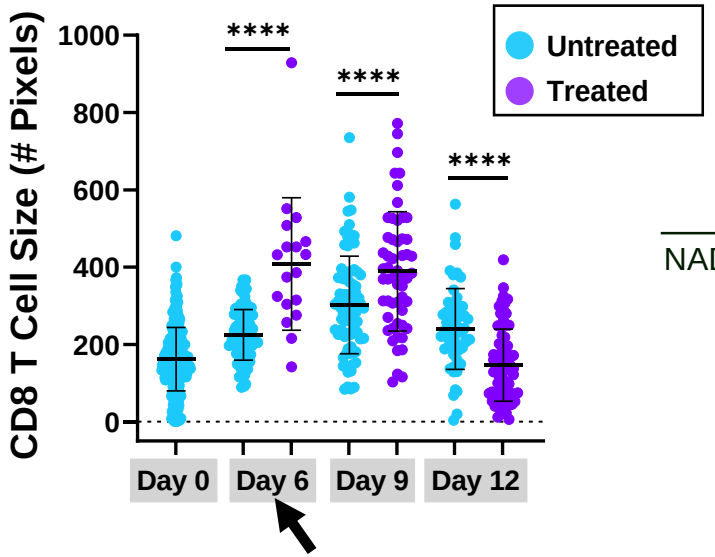
Increased neutrophils, decreased macrophages with treatment



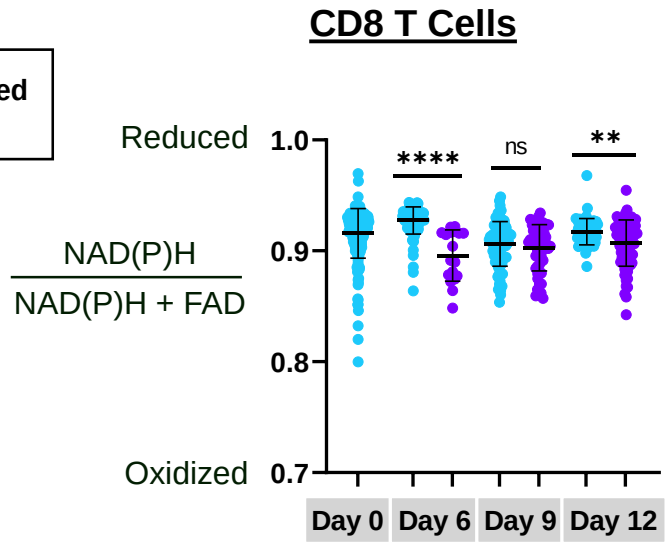
Mean ± SD; n = 4 mice per group
Mann Whitney U Test

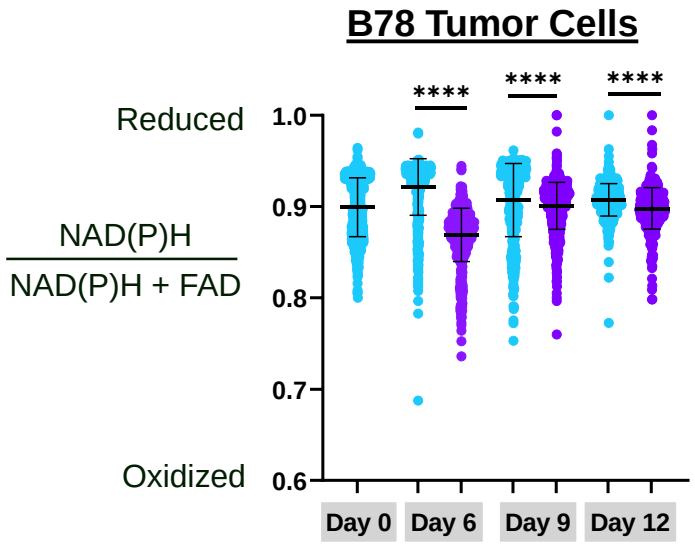
Redox changes *in vivo* during immunotherapy





Peak CD8 T cell activation day 6

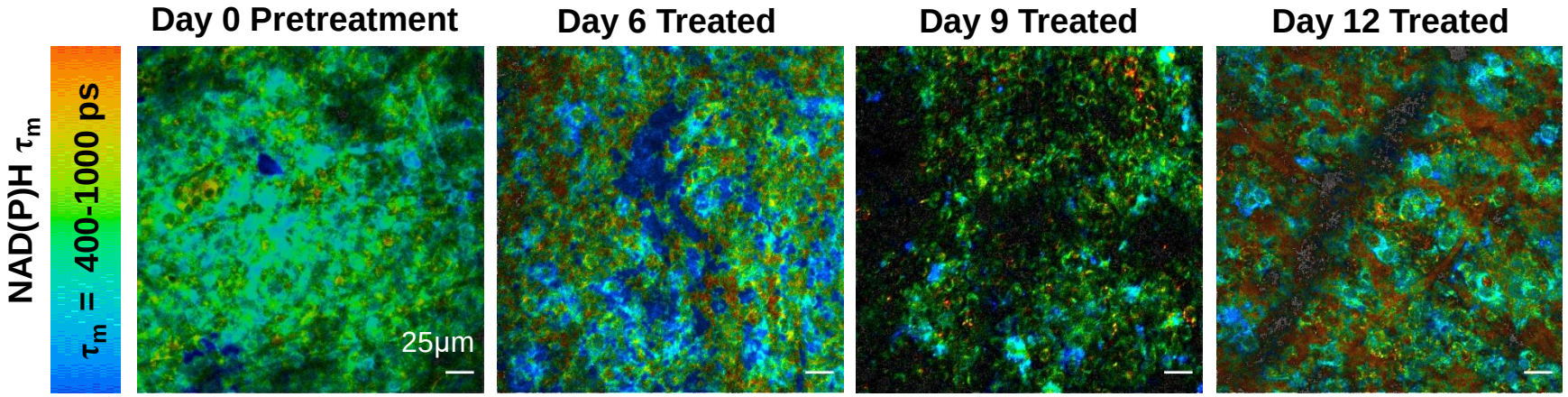


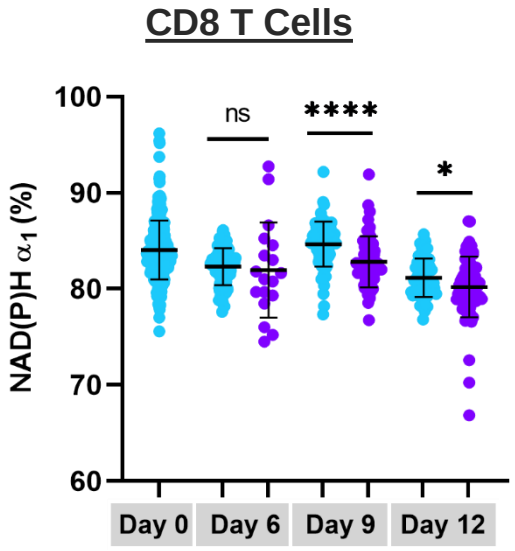


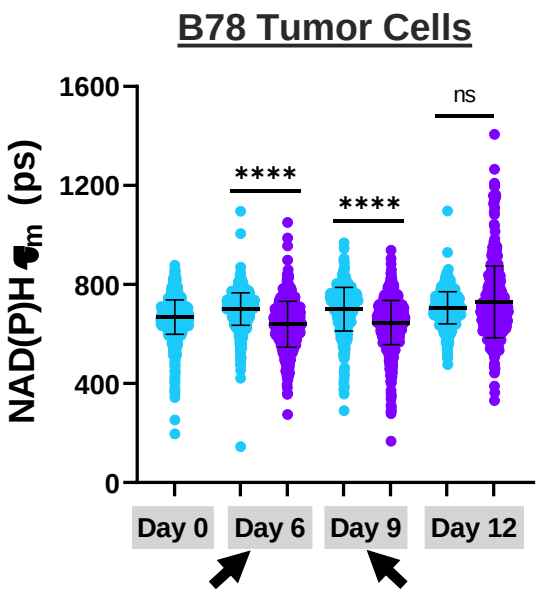
Both tumor and CD8+ T cells become more oxidized with treatment

Mean ± SD; n = 2 mice per day; Mann Whitney U Test;
CD8 T Cells: Control 244 = cells, Treated = 148 cells;
Tumor Cells: Control 2,161 = cells, Treated 2,009 = cells

Fluorescence lifetime changes *in vivo* during immunotherapy







Metabolic Changes During Immunotherapy			
		B78 Tumor Cells	CD8 T Cells
NAD(P)H τ_1	Lifetime Free	↓	↑
NAD(P)H α_1	% Free	↑	↓
NAD(P)H τ_2	Lifetime Bound	↓	↑
NAD(P)H α_2	% Bound	↓	↑
NAD(P)H τ_m	= $\alpha_1\tau_1+\alpha_2\tau_2$	↓	↑

Exhaustion? Metabolic constraints?

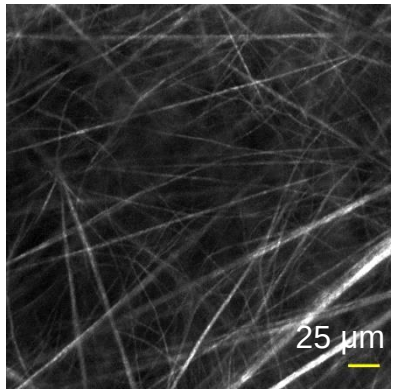
Early ↓ NAD(P)H τ_m → treatment response

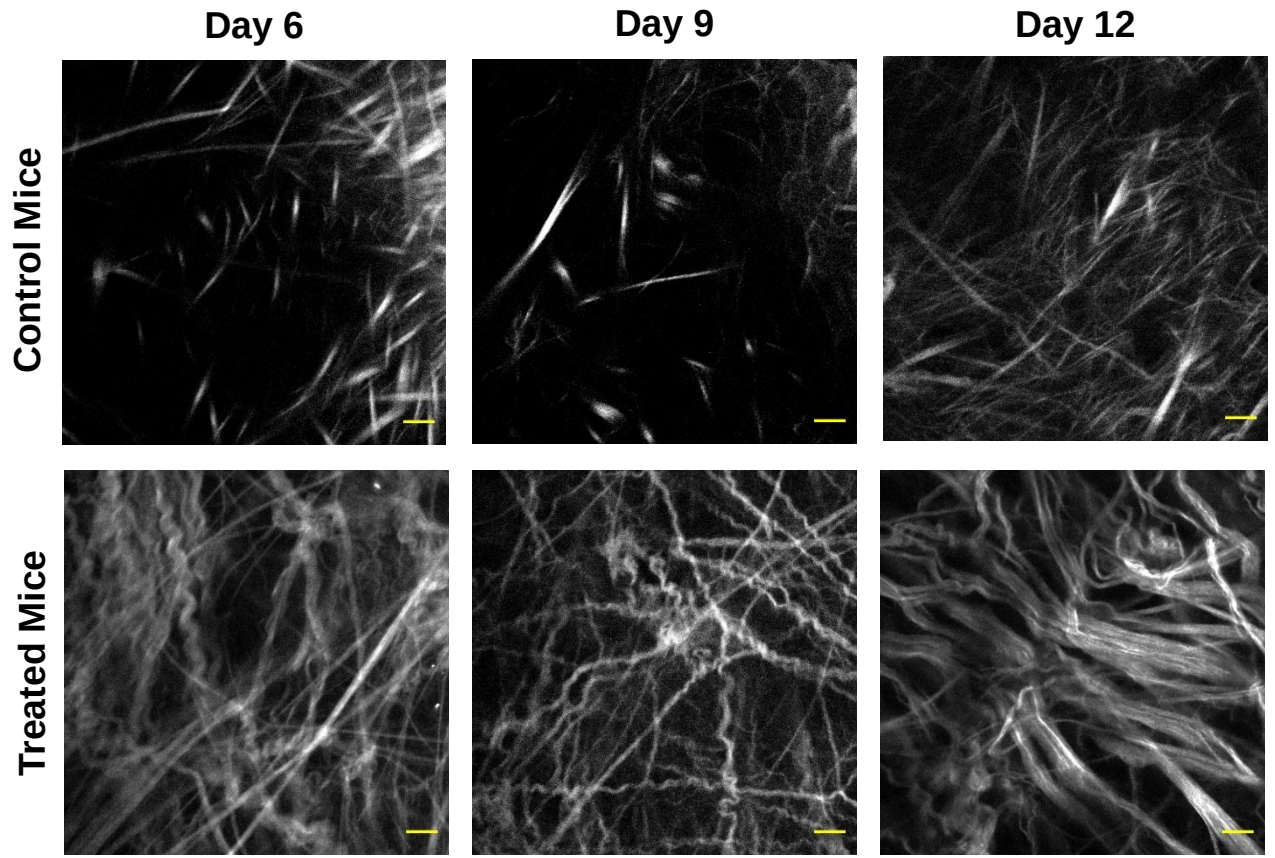
Mean ± SD; n = 2 mice per day; Mann Whitney U Test;
CD8 T Cells: Control 244 = cells, Treated = 148 cells;
Tumor Cells: Control 2,161 = cells, Treated 2,009 = cells

Collagen remodeling during immunotherapy

In Vivo
Second Harmonic Generation Images
Collagen

Day 0 Pretreatment





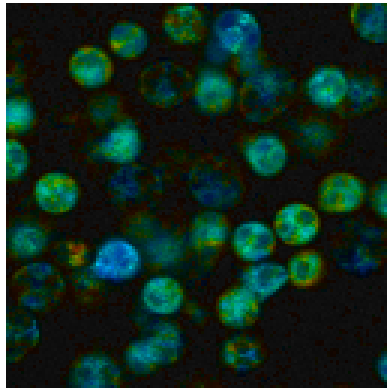
SHG excitation: 1041 nm laser; SHG: n = 2 mice per day,
Flow: n = 4 mice per group

[1] Bredfelt *J Biomed. Opt.* **2014** [2] Hanley *Oncotarget* **2016** [3] Ouellette *Bioengineering* **2021** [4] Jolly *Cancer Res.* **2016** [5] Schnoor *J Immunol.* **2018** [6] Chen *Acta Pharm. Sin. B.* **2020**

Other immune cell types

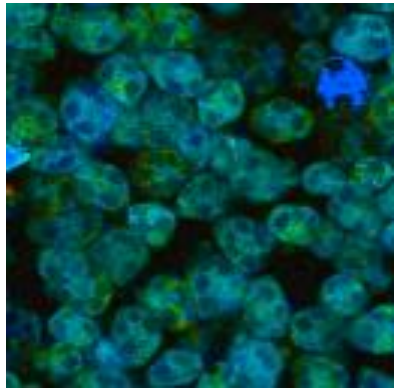
Neutrophils

Quiescent



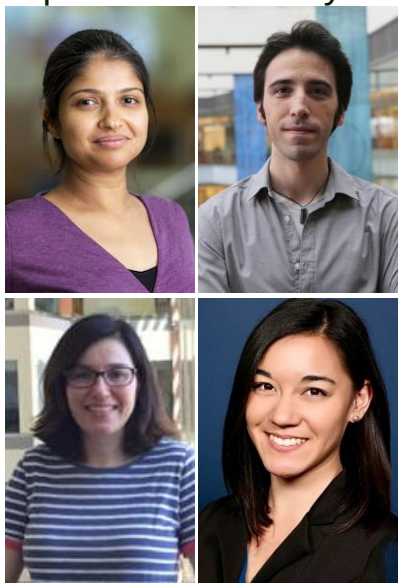
↓ optical redox ratio
↑ NAD(P)H τ_m

Activated



↑ optical redox ratio
↓ NAD(P)H τ_m

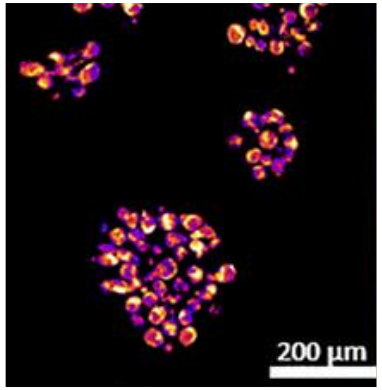
Rupsa Datta Jose Ayuso



Veronika Miskolci Tiffany Heaster

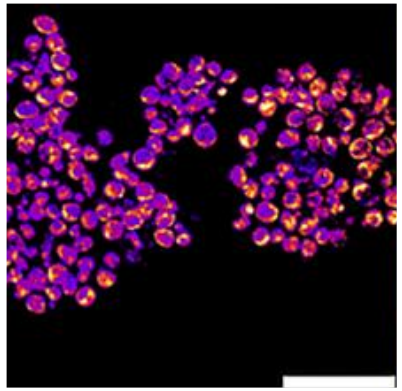
NK cells

Naïve



↑ optical redox ratio
↑ NAD(P)H τ_m

Tumor-exposed

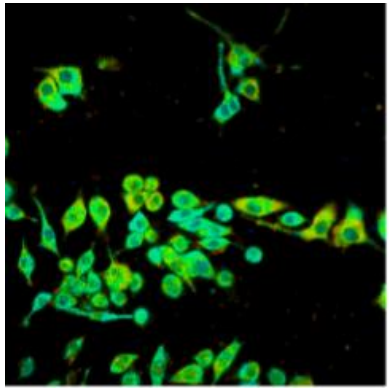


↓ optical redox ratio
↓ NAD(P)H τ_m

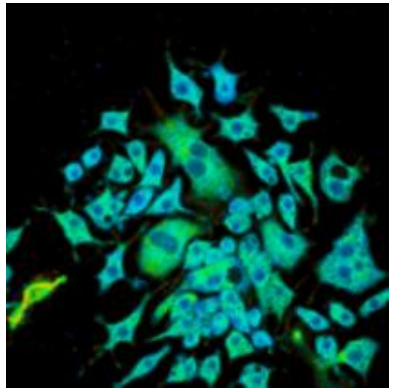
Macrophages

M2

↑ optical redox ratio
↑ NAD(P)H τ_m



M1

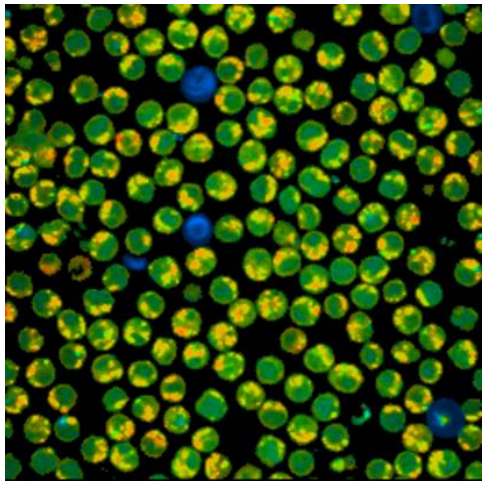


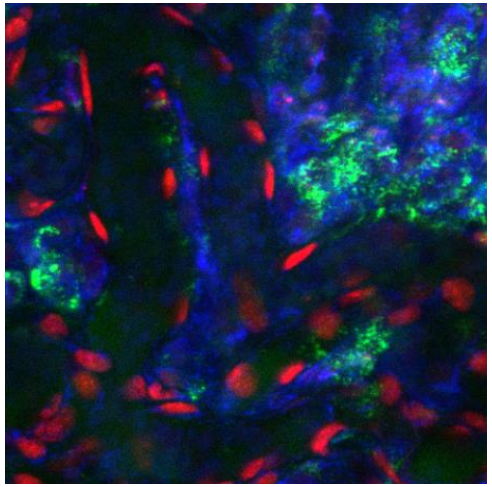
↓ optical redox ratio
↓ NAD(P)H τ_m

Heaster T, Cancer Res 2020
Ayuso JM, Sci Adv. 2021
Heaster T, Front. Bioeng. Biotech. 2021
Miskolci V, Elife 2022

Summary: Autofluorescence imaging of immune cell metabolism

- Label-free autofluorescence can monitor T cell activation and function
  - Optimize cancer therapies that modulate T cell function
- Autofluorescence *in vivo* can monitor dynamic tumor and T cell metabolism
- Autofluorescence is sensitive to immune cell function in neutrophils, NK cells, and macrophages.





Datta R. et al. J Biomed Opt 2020
Heaster TM et al., Cancer Res. 2020
Walsh AJ et al., Nat. Biomed. Eng. 2021
Samimi K et al., Opt. Lett. 2021
Heaster TM et al., Front. Bioeng. Biotechnol. 2021
Ayuso JM, Sci Adv. 2021
Miskolci V et al, ELife 2022

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Skala Lab Undergraduates and High Schoolers, **Former lab members**

Collaborators: **Kris Saha**, Christian Capitini, Dustin Deming, **Paul Sondel**, Cheri Pasch, Mark Burkard, David Beebe, Anna Huttenlocher, Sean Palecek

We are recruiting graduate students and postdocs; please email mcskala@wisc.edu if interested. http://morgridge.org/skala

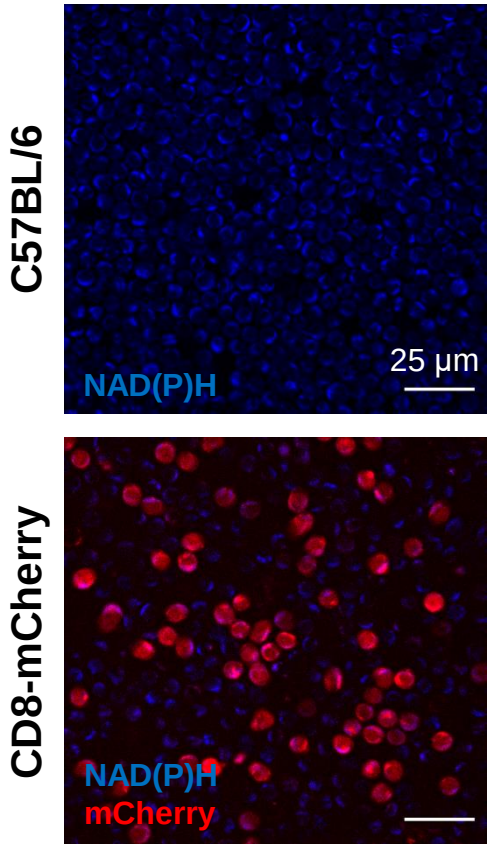
NCI R01 CA205101 NCI P01 CA250972
NCI R01 CA211082 NCI R37 CA226526
NEI U01 EY03233 NIAID U24 AI52177
NEI R21 EY033558
NCATS U01 TR002383

MORGRIDGE INSTITUTE FOR RESEARCH

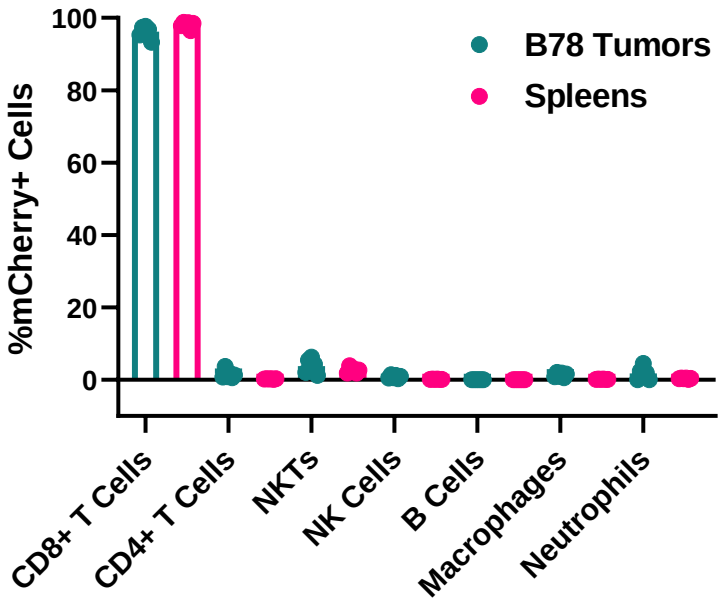
Carbone Cancer Center
UNIVERSITY OF WISCONSIN
SCHOOL OF MEDICINE AND PUBLIC HEALTH

Characterization of CD8-mCherry reporter mouse

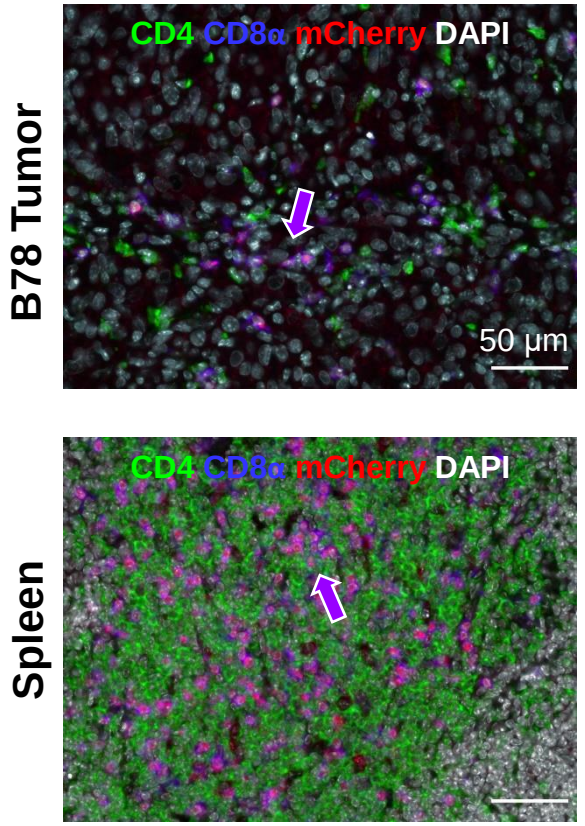
A - Optical Metabolic Imaging



B - Flow Cytometry



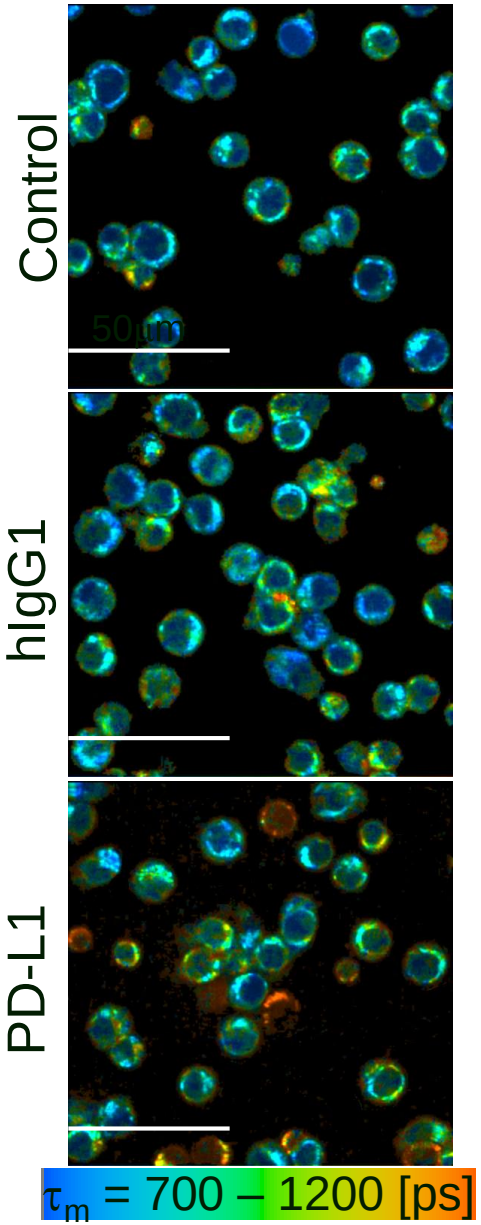
C - Multiplex Immunofluorescence



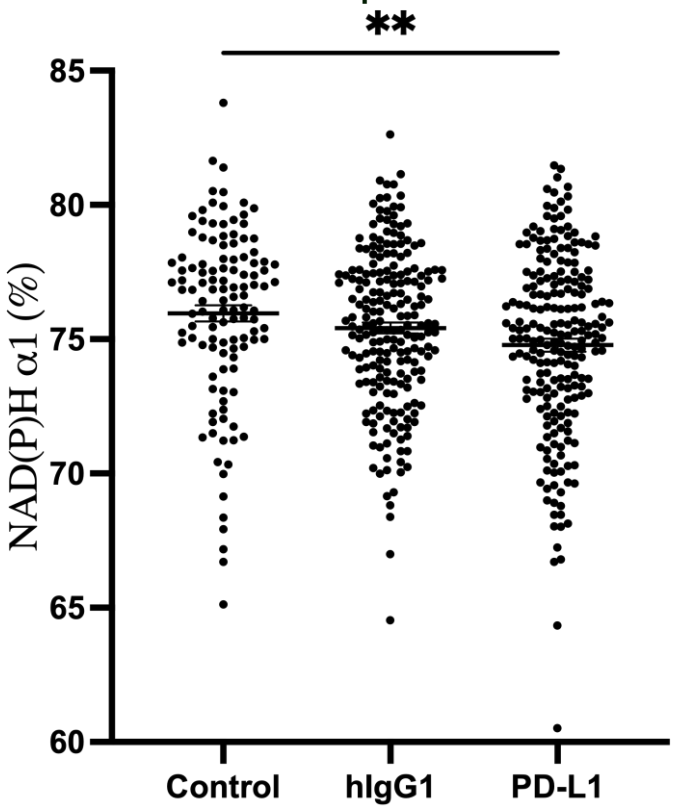
Flow: n = 6 mice, mean ± SD

OMI is sensitive to T cell exhaustion

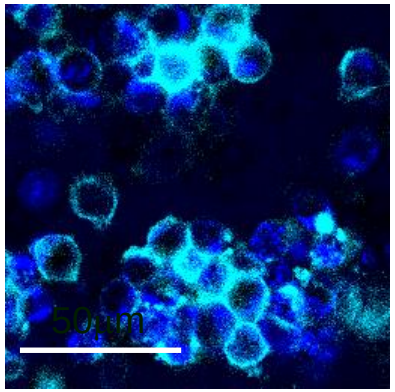
Dan Pham



15-30 mins post treatment

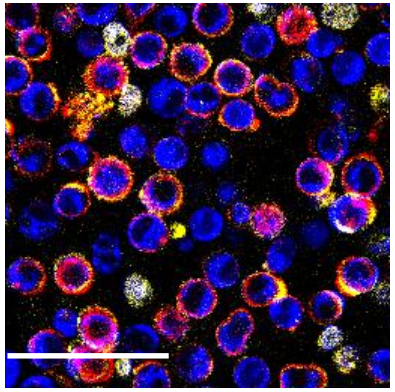


Control



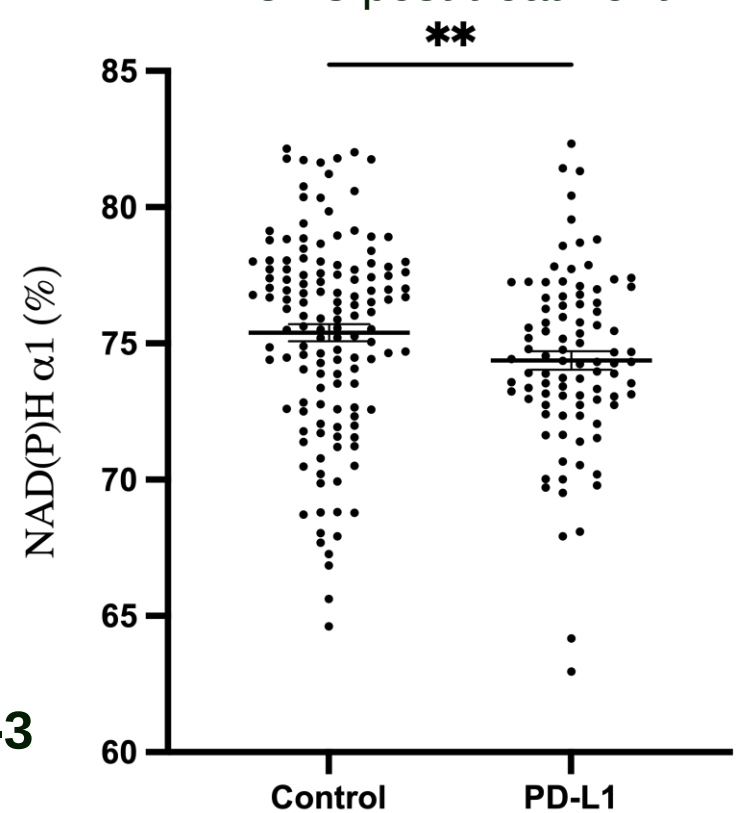
NAD(P)H CD69

PD-L1



NAD(P)H LAG-3 TIM-3

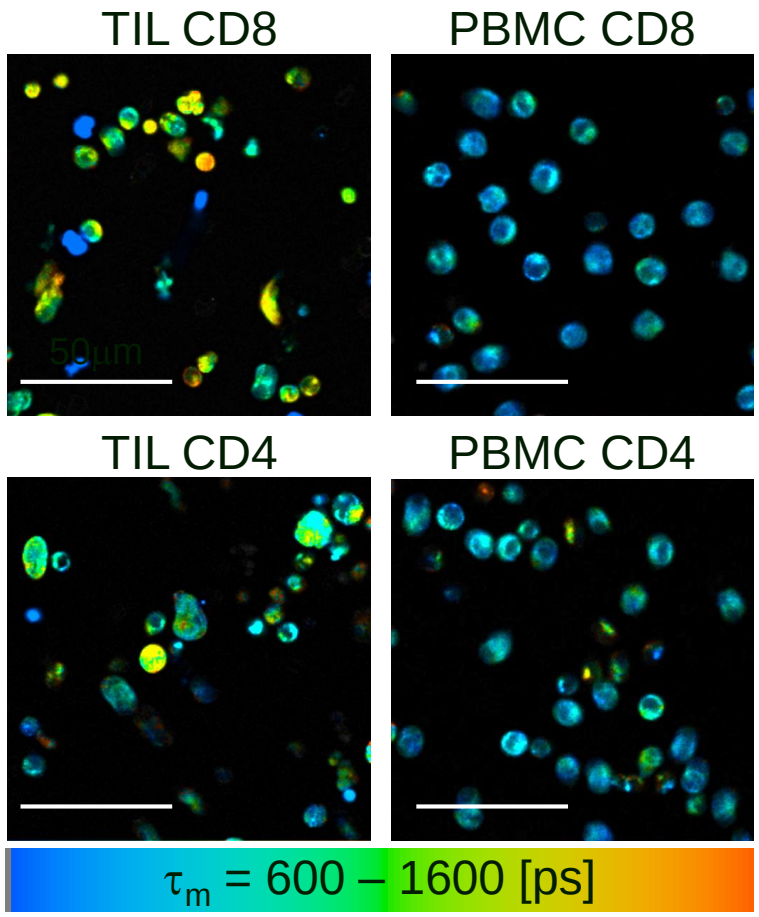
48hrs post treatment



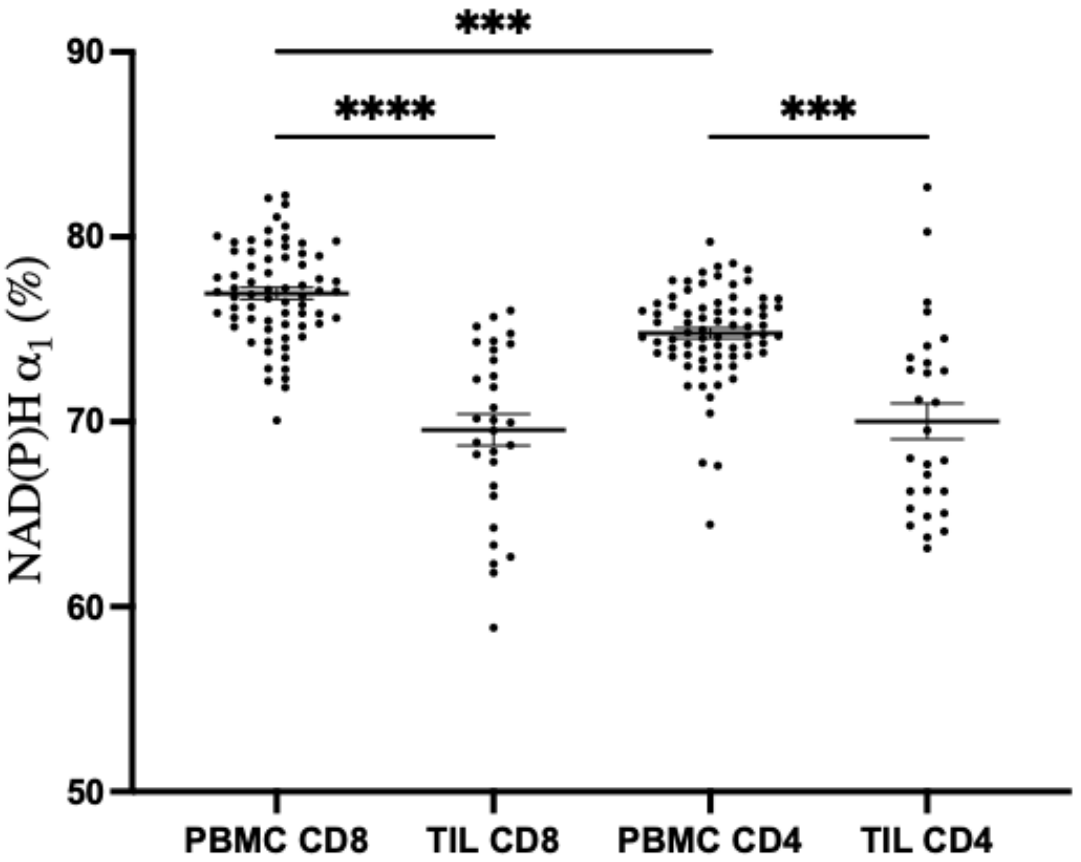
1 donor shown, same statistics for 2nd donor

* p< 0.05, ** p<0.01, *** p<0.001, **** p<0.0001

OMI of tumor infiltrating T lymphocytes (TILs) vs PBMC



TILs isolated (negative selection) from **glioblastoma patient** fresh surgical sample (**n=1**) and imaged immediately after isolation



* p< 0.05, ** p<0.01, *** p<0.001, **** p<0.0001