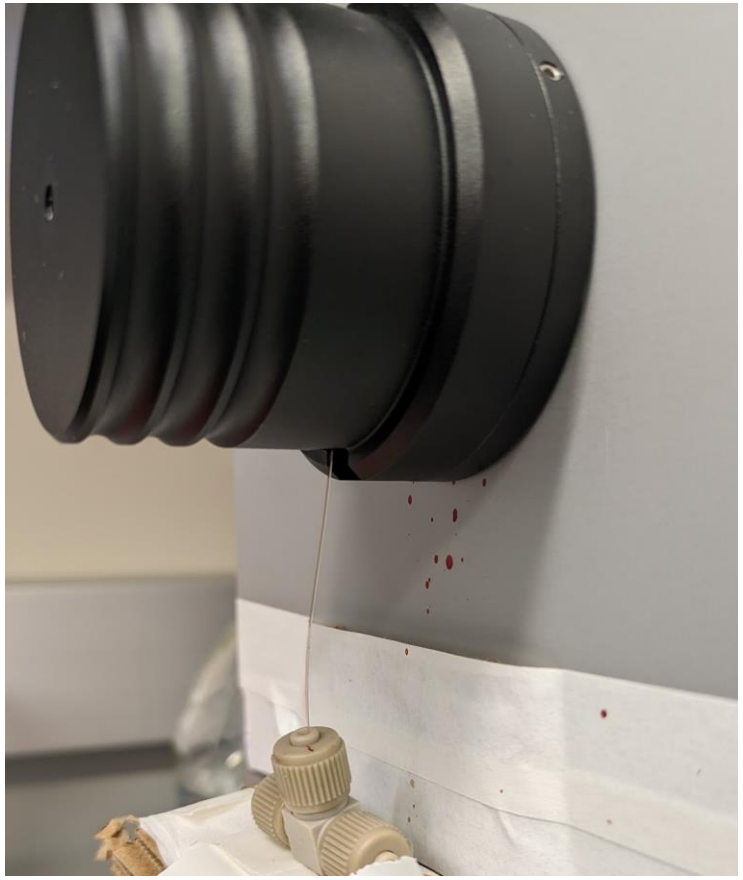

Radical Protein Footprinting in Stabilized Whole Blood

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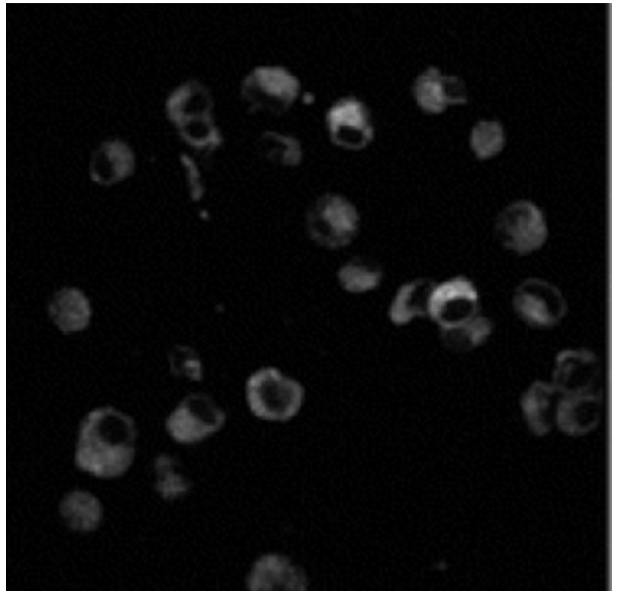
FCOI Statement: J.S.S. and L.M.J. disclose a significant interest in GenNext Technologies, Inc., a growth-stage company seeking to commercialize benchtop HRPF to support the pharmaceutical industry

Structural Proteomics by FPOP: From Test Tubes to Nematodes

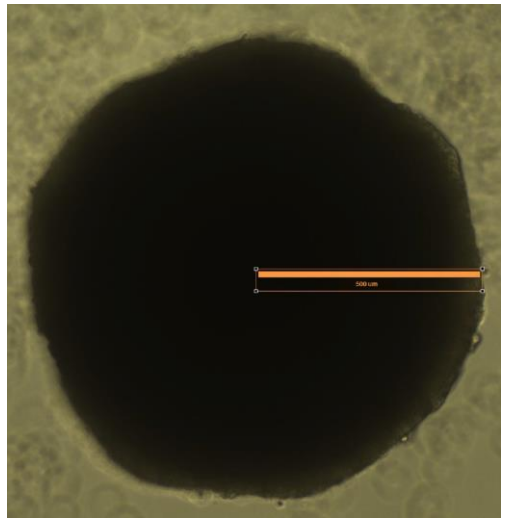
- Hydroxyl radical protein footprinting (HRPF) has long history *in vitro*
- In 2015, Espino Mali and Jones published the first example of FPOP in live cells
- Since been expanded to live nematodes and 3D cell cultures
- Mammalian tissues still out of reach due to strong tissue UV absorbance issues

Cultured Cells



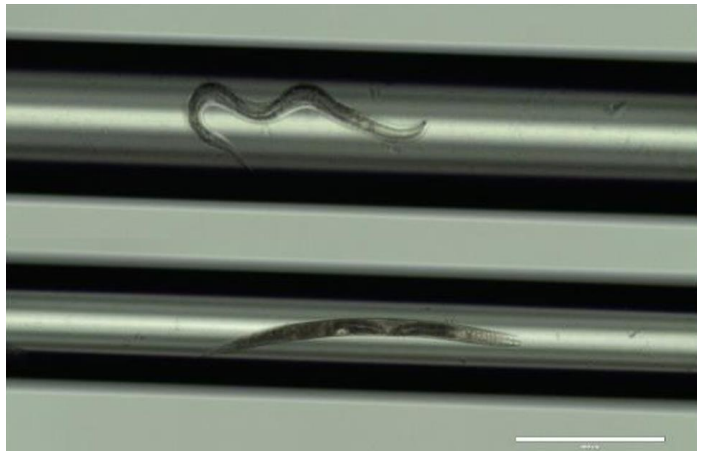
Anal. Chem. 2015, 87, 7971−7978

Solid Tumor Spheroids



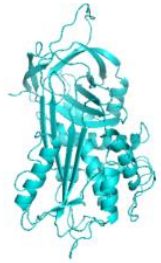
J. Am. Soc. Mass Spectrom. 2023, 34, 3, 417−425

Nematodes

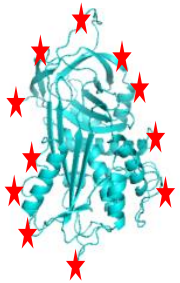


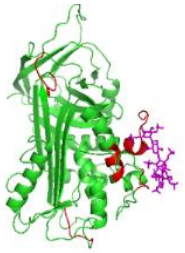
Anal. Chem. 2019, 91, 10, 6577−6584

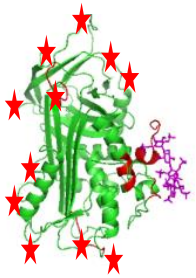
General HRPF Workflow



·OH

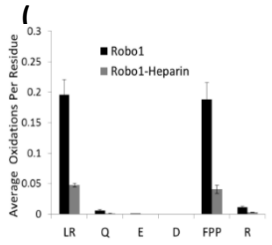


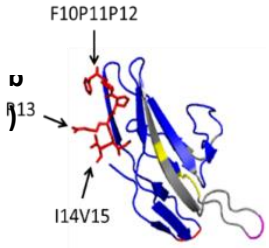


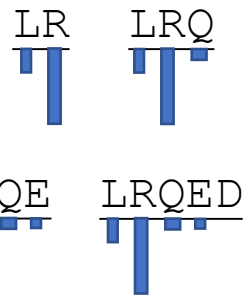


Denature
& Digest

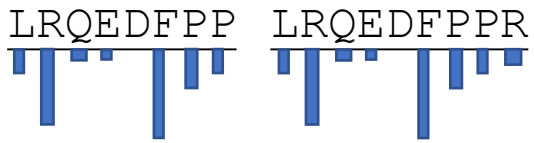
LC-MS

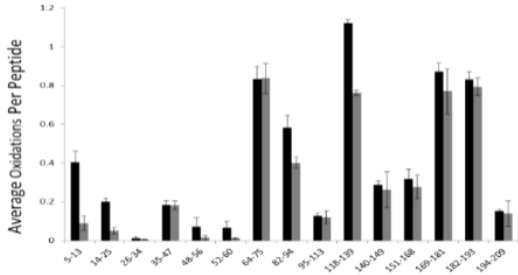






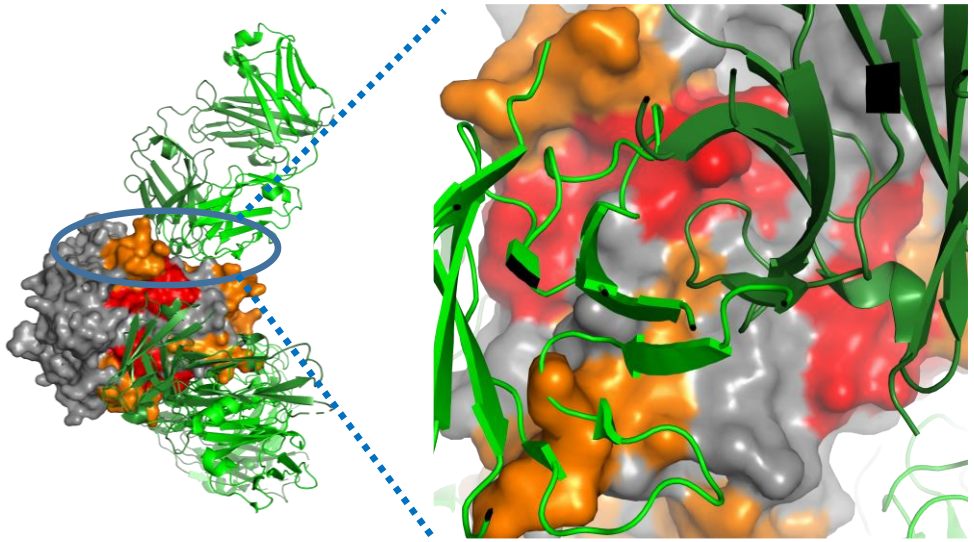
Amino Acid-Level Changes
Structural Models



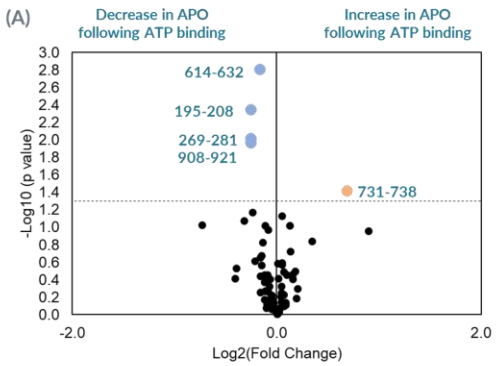


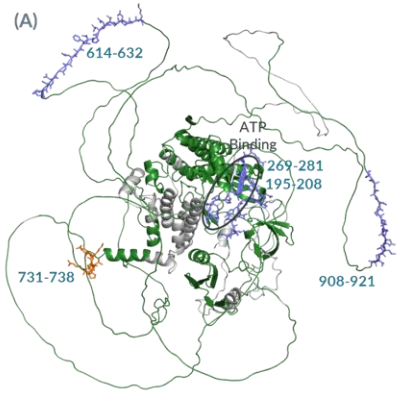
Peptide-Level Changes

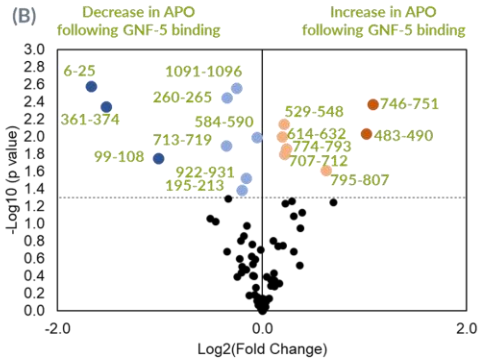
High Resolution Epitope Mapping

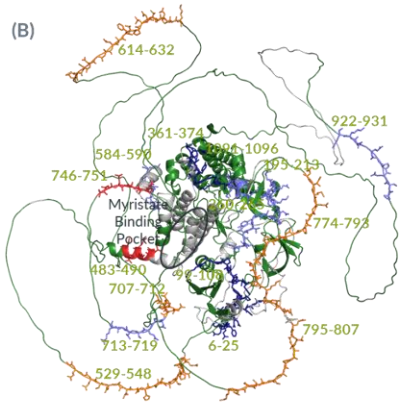


Allosteric Modulators

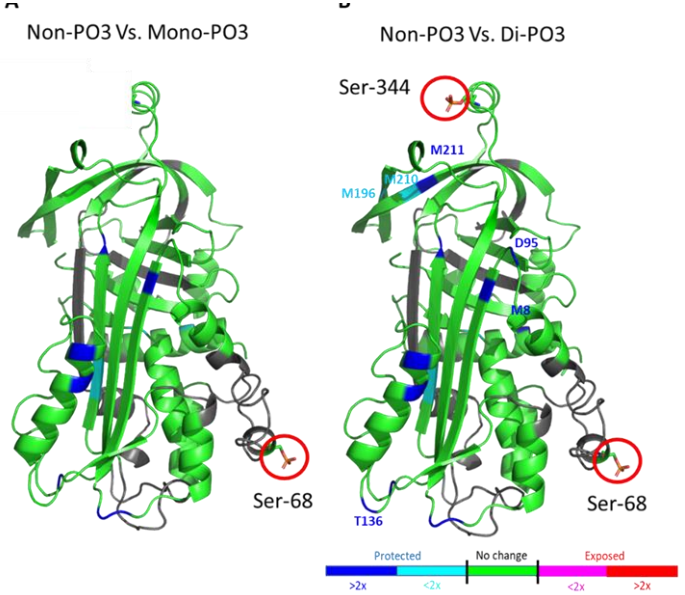








Structural Impacts of PTMs



In-Blood FPOP

- Major potential impact in structural pharmacology
  - Anti-drug antibody interactions
  - Drug:target interactions
  - Drug:off-target interactions
  - Post-administration aggregation
- Major potential for structural proteomics in human fluids
  - Liquid tumor analysis
  - Biology and diseases of blood, lymph, CSF, urine, etc.

Anti-drug antibody prevalence in oncology checkpoint inhibitor therapy

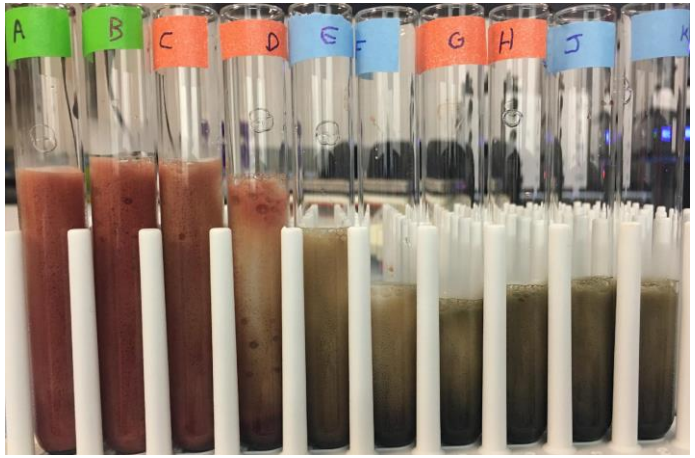
mAb	EMA first approval	Structure	Isotype	Target	ADAs %
Atezolizumab	2017	Humanized	IgG1, kappa	PD-L1	30–54.1
Avelumab	2017	Fully human	IgG1, lambda	PD-L1	4.1–5.9
Cemiplimab	2019	Fully human	IgG4, kappa	PD-1	1.3
Durvalumab	2018	Engineered human	IgG1, kappa	PD-L1	1.7–6.6
Nivolumab	2015	Fully human	IgG4, kappa	PD-1	4.1–37.8
Pembrolizumab	2015	Humanized	IgG4, kappa	PD-1	0.7–2.5
Ipilimumab	2011	Fully human	IgG1, kappa	CTLA-4	1.1–26
Nivolumab + ipilimumab	–	–	–	–	23.8–37.8

Cancer Chemother. Pharmacol. (2022) 89:577–584

Catalase Activity and Inhibition

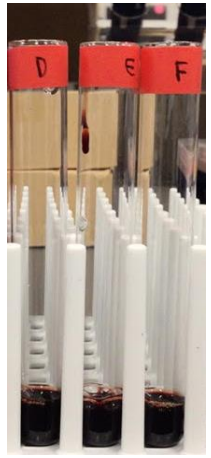
500 µL blood
80 µL 30% H_2O_2

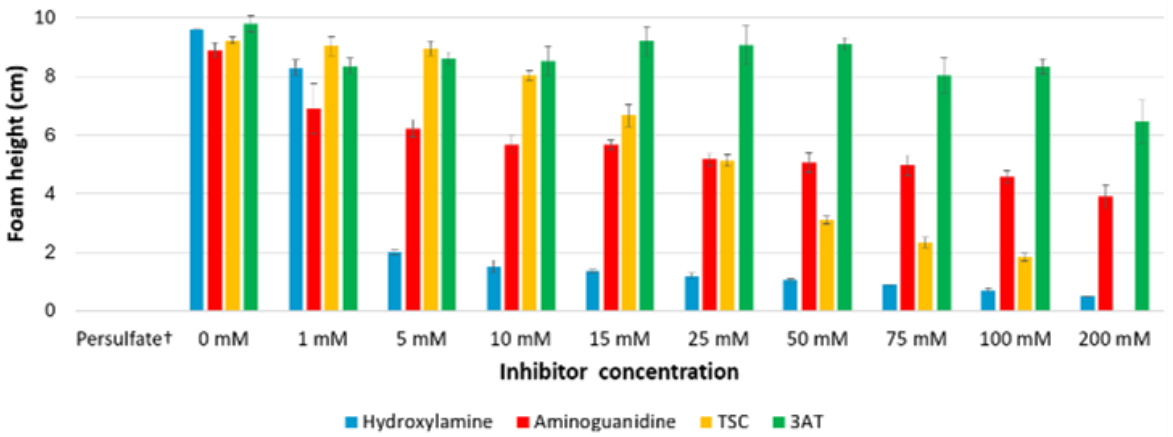
5 mM HA 100 mM HA
No inh. 25 mM HA 142 mM HA



500 µL blood
80 µL 2.5M $Na_2S_2O_8$

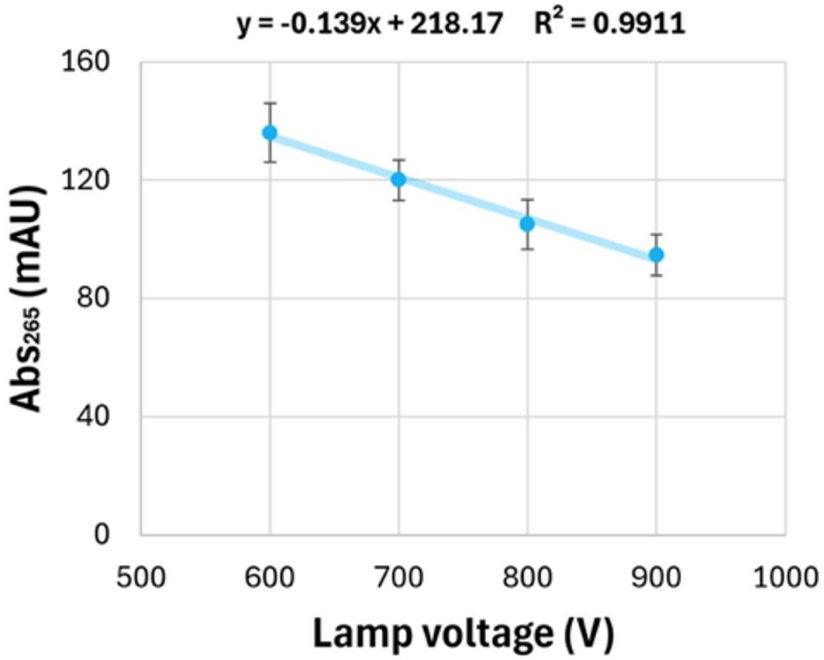
No inh.

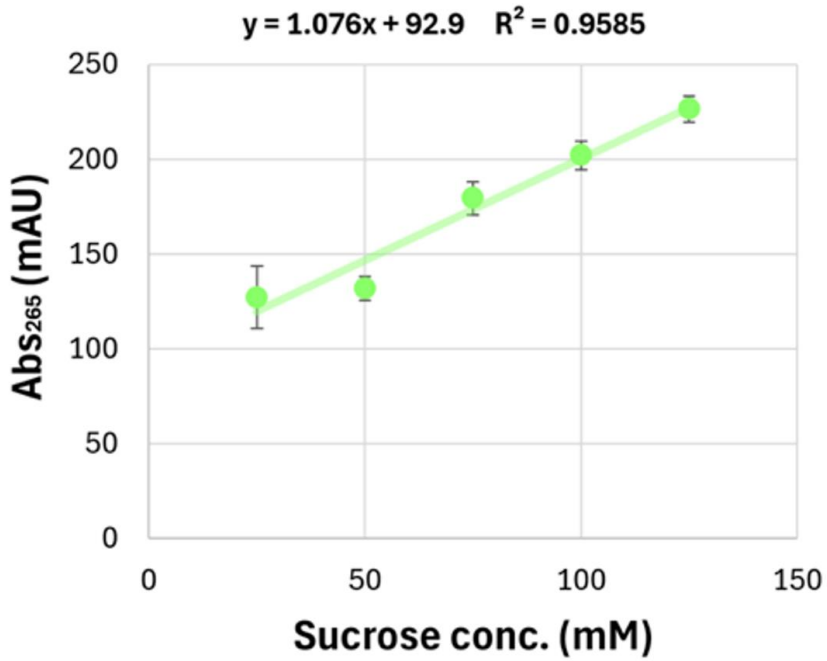




- Small amount of mild detergent captures bubbles made by catalase
- No amount of quencher tested completely inhibited blood catalase
- High concentrations of inhibitor changed blood characteristics markedly
- No measurable gas production with persulfate

In Vitro Persulfate Oxidation in Fox® System



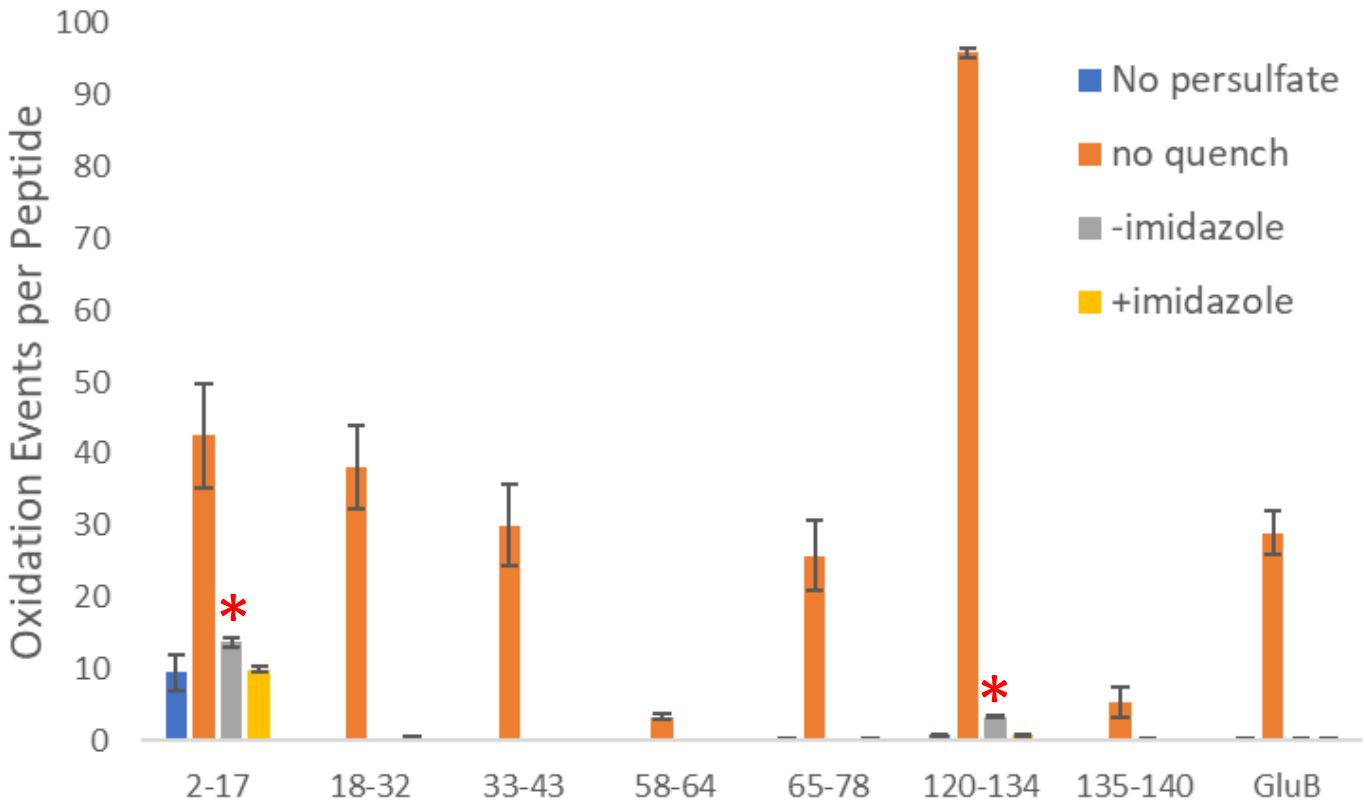


- Fox Photolysis System can efficiently photoactivate persulfate to oxidize proteins, peptides and small molecules
- Real-time adenine dosimetry works *in vitro*; absorbance decreases as more radical is created, decreases as more radical is scavenged

New Persulfate Quench System

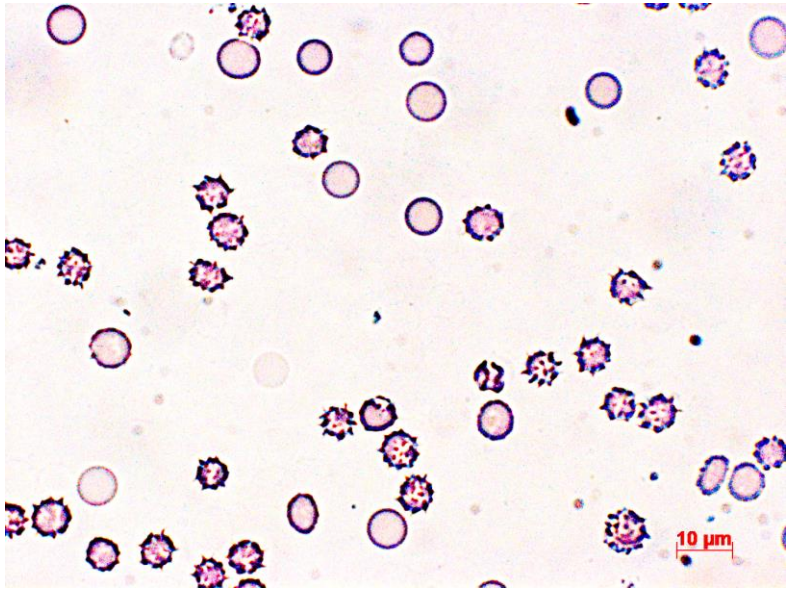
- Peroxide-based FPOP quenching with DMTU and methionine still had some peptides with elevated background oxidation
- Addition of 200 mM imidazole to quench eliminated background oxidation in these peptides
- Hydroquinone and GSH also seem to work, but have other issues

Protein in quench, 100 mM persulfate, 900V lamp
Quench: 200 mM DMTU, 70 mM methionine, ± 200 mM imidazole

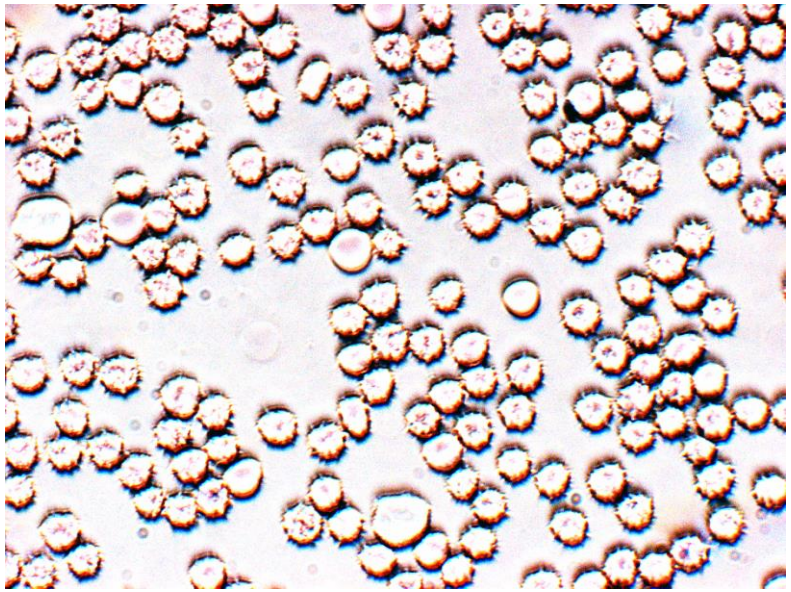


* = significant increase from no persulfate to –imidazole quench

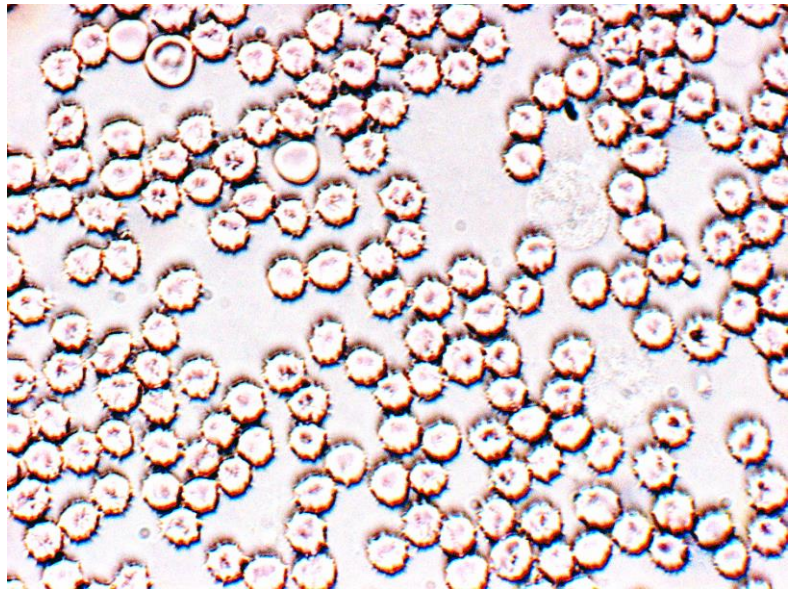
Blood Cell Morphology in 200 mM Sodium Persulfate



Canine Blood



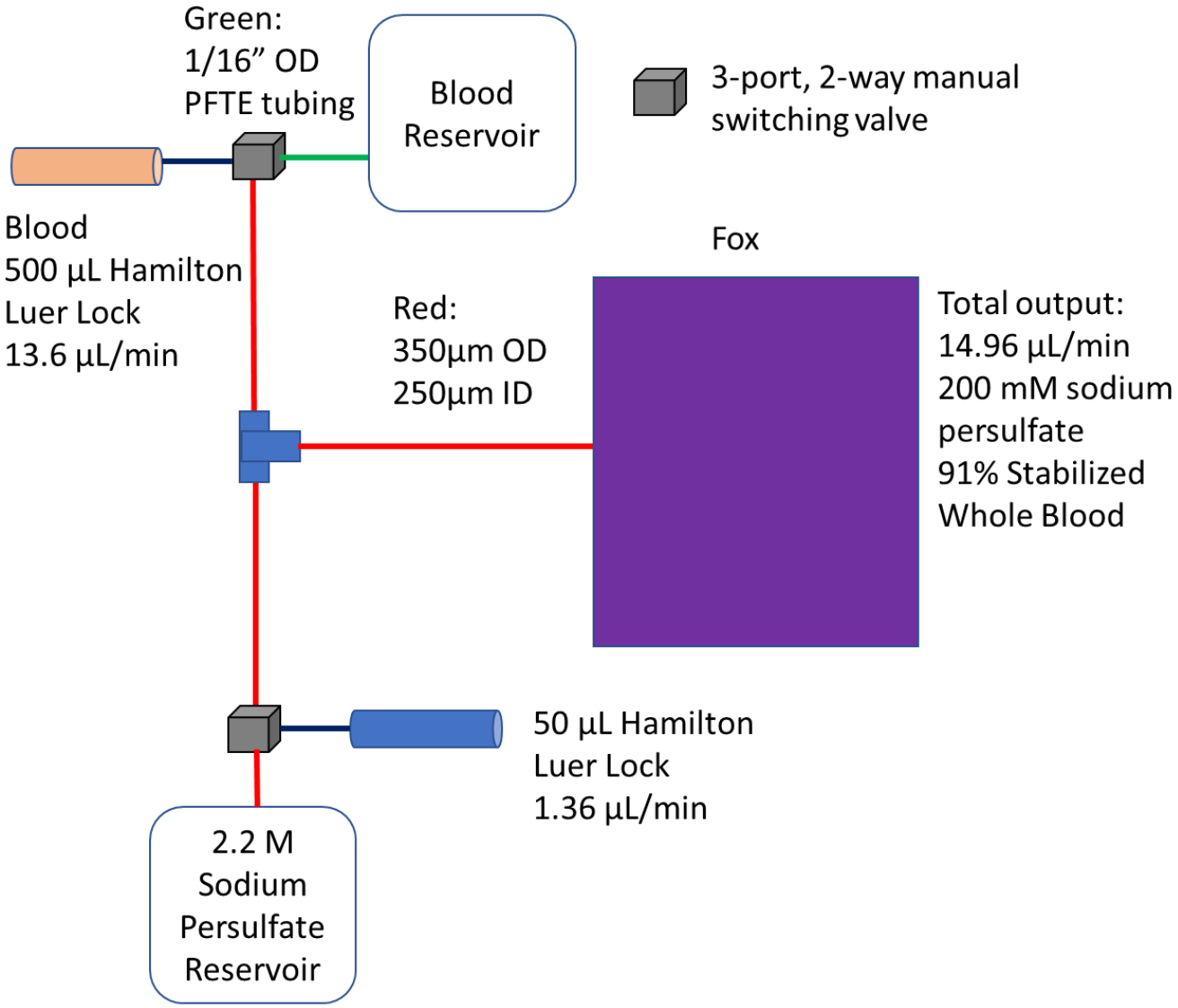
+200 mM sodium persulfate

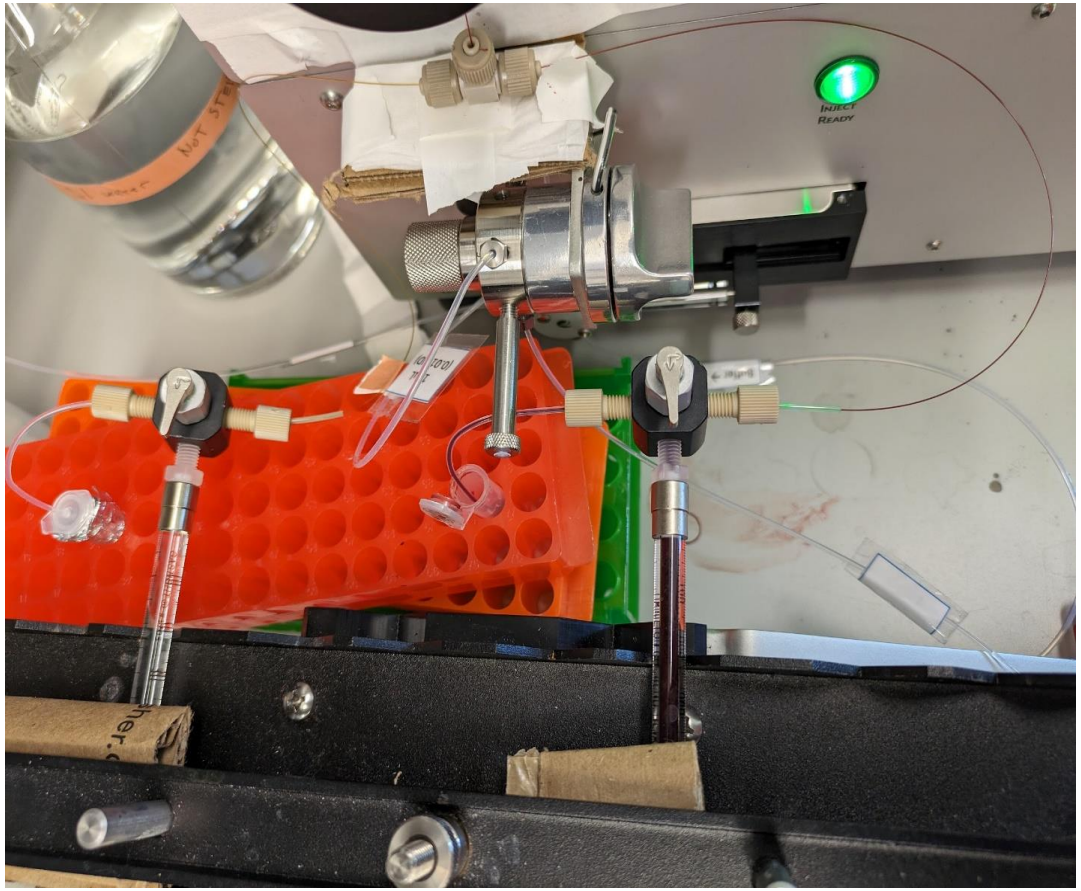


+200 mM sodium chloride

- No increase in ghost cells observed
- Moderate increase in hypertonicity observed in In-Blood RPF conditions
- Gross morphological changes indistinguishable from addition of equivalent concentration of sodium chloride on footprinting timescale (<1 minute)

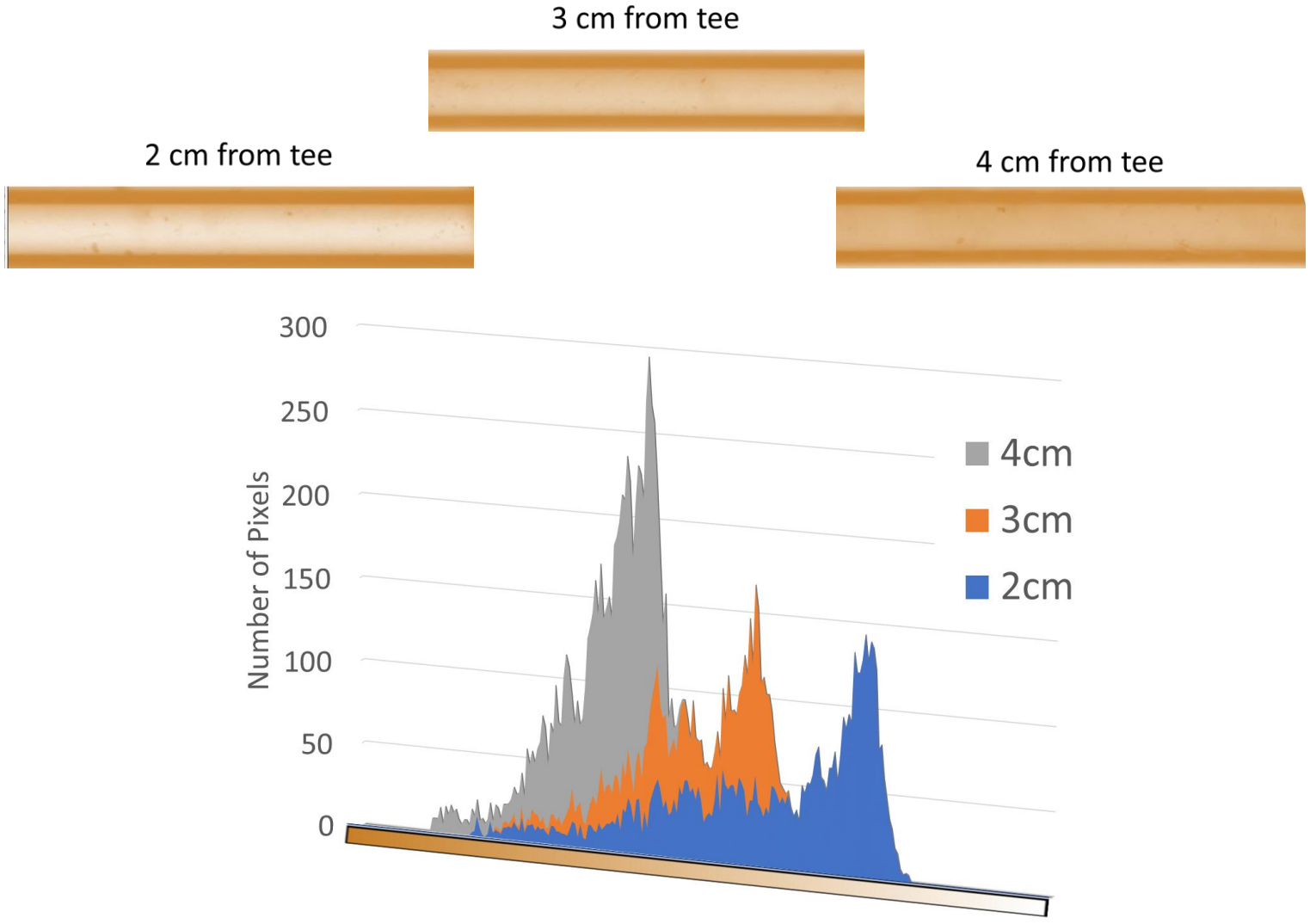
In-Blood FPOP System Design



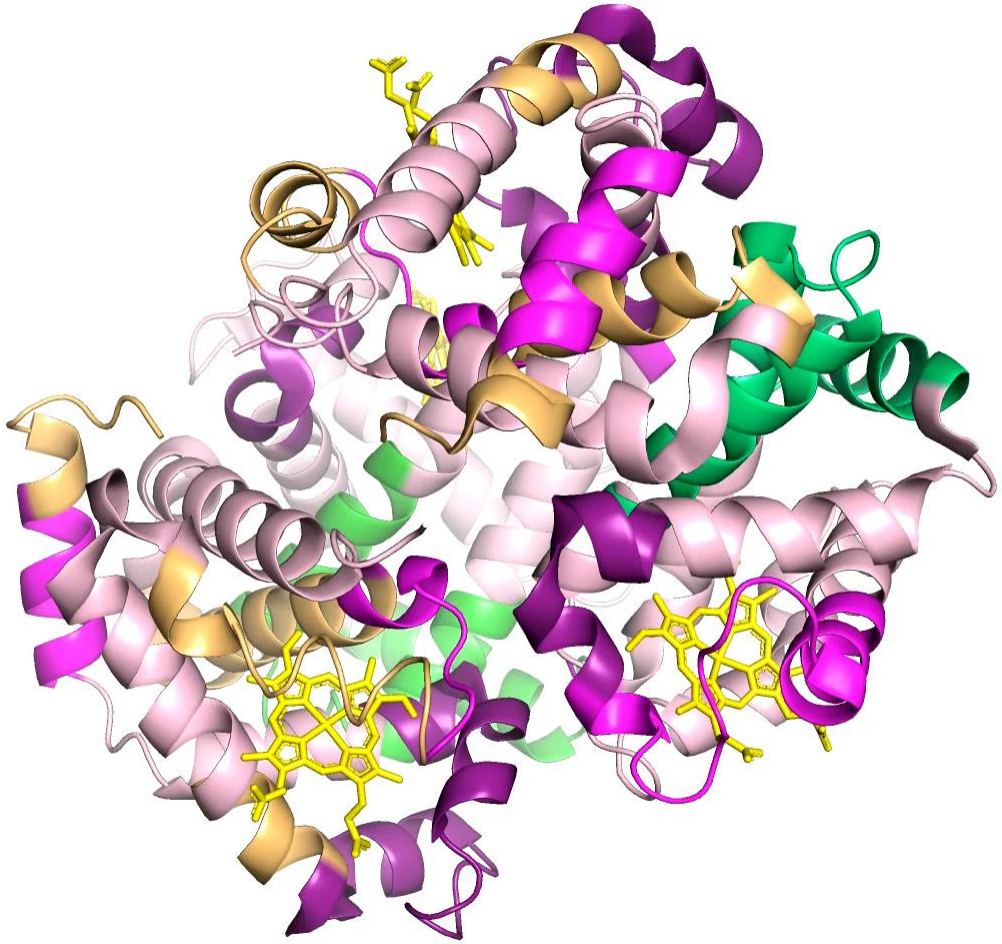


Persulfate Mixing: Dye Imaging Test

- Two dyes: Red (1.3 µL/min) and Blue (13 µL/min)
- Ran each dye by itself and measured in capillary to set color RGB values
- Set RGB values for two dyes in ImageJ Colour Deconvolution2 plugin
- Measured distribution of red dye across cross-section of capillary at different disatances from mixing tee



Hemoglobin
900V Flash Voltage



Green: Alpha-globin regions detected not oxidized
Lt. Orange: Beta-globin regions detected not oxidized
Yellow: Heme

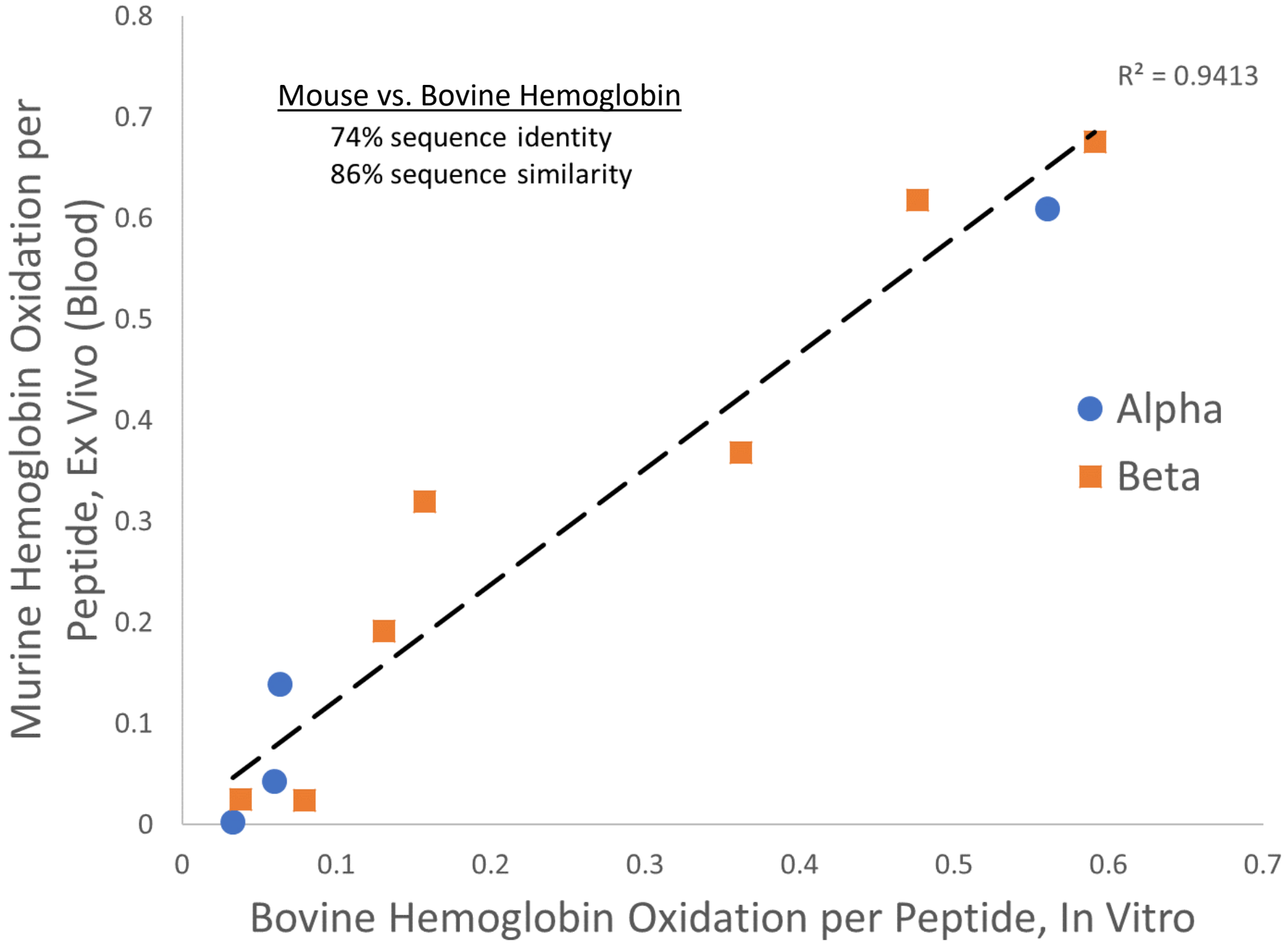
Avg. Oxidations per Peptide

In Blood

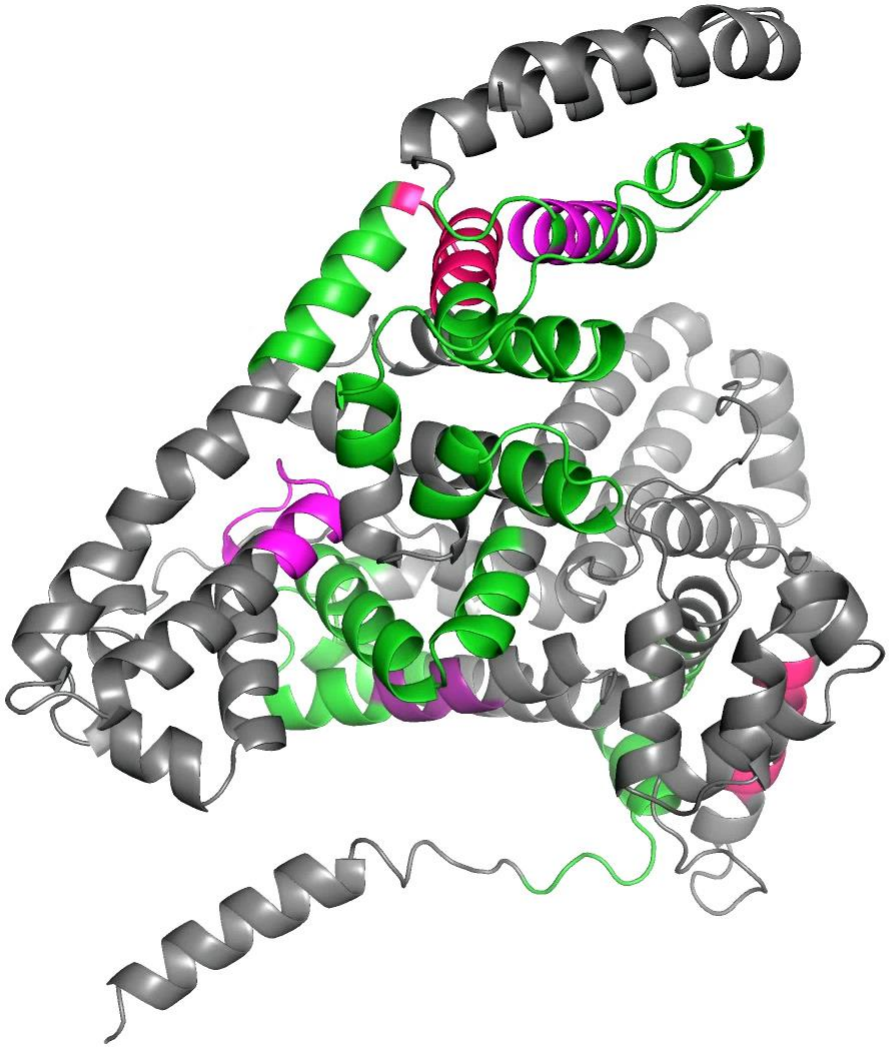
- 200 mM persulfate
- 900V lamp
- Murine blood

In Vitro

- 100 mM persulfate
- 900V lamp
- Bovine hemoglobin



Albumin
900V Flash Voltage



Green: Albumin regions detected not oxidized
Gray: Albumin regions not detected

Avg. Oxidations per Peptide

Serine protease inhibitor
900V Flash Voltage

Green: SPI regions detected not oxidized

Avg. Oxidations per Peptide

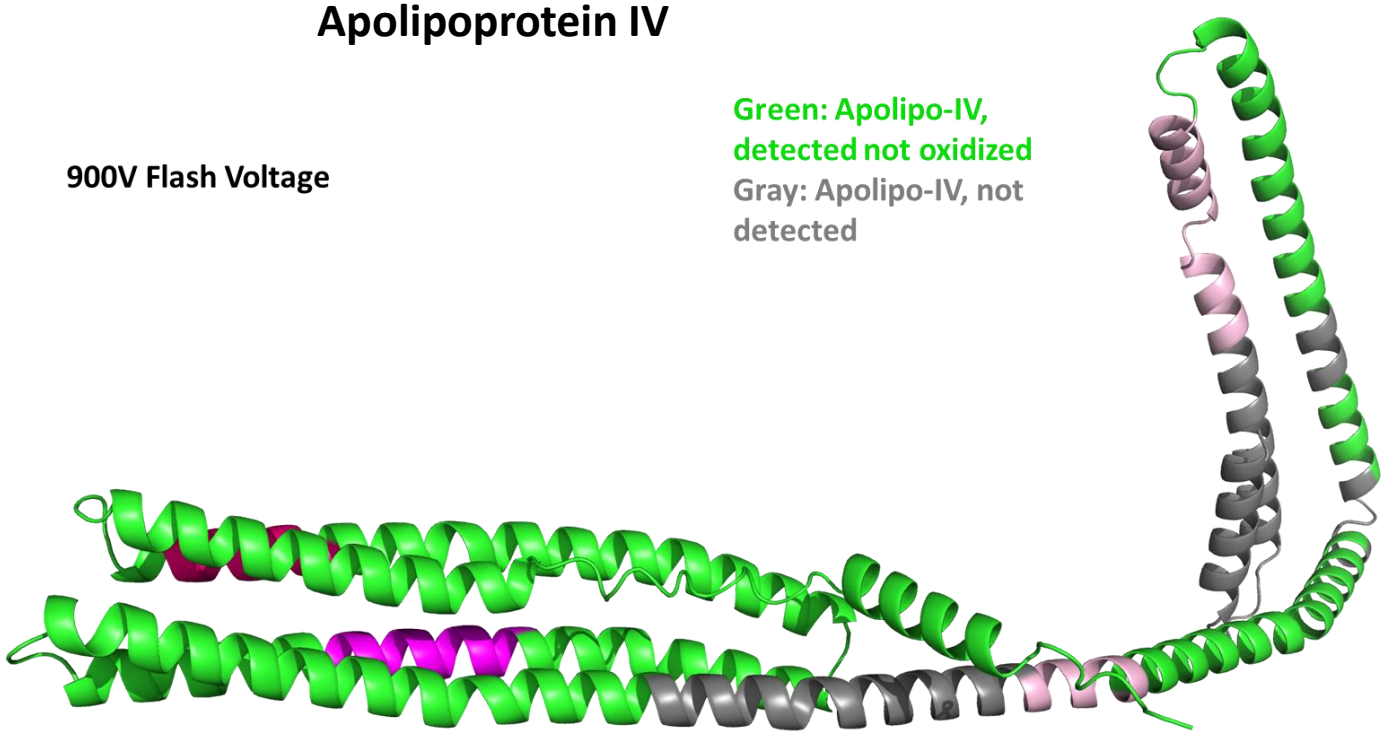
<0.2 0.2 0.4 0.6 0.8 >0.8

Apolipoprotein IV

900V Flash Voltage

Green: Apolipo-IV, detected not oxidized

Gray: Apolipo-IV, not detected

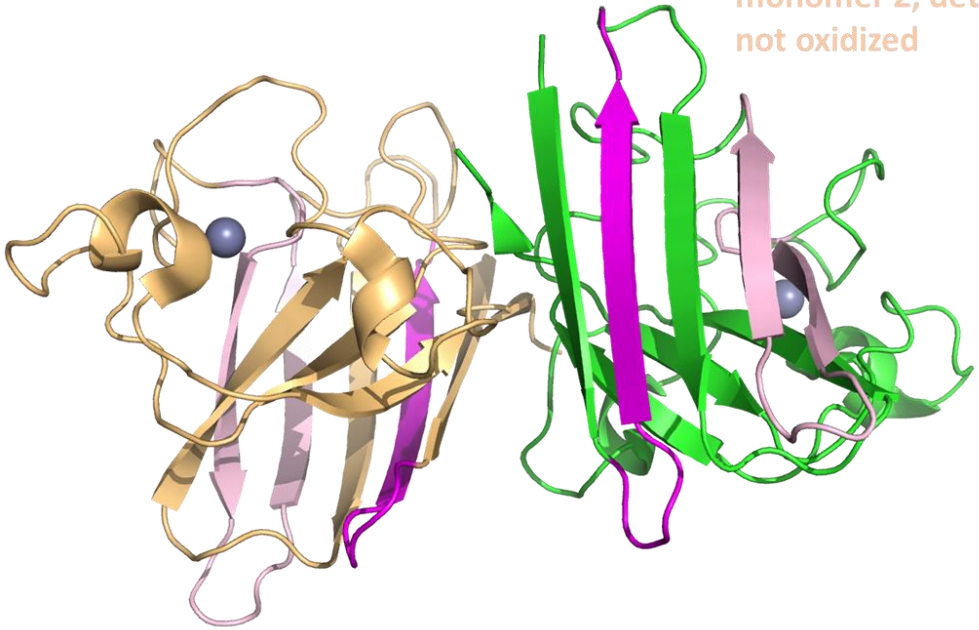


Superoxide Dismutase

900V Flash Voltage

Green: SOD monomer 1, detected not oxidized

Lt. Orange: SOD monomer 2, detected not oxidized

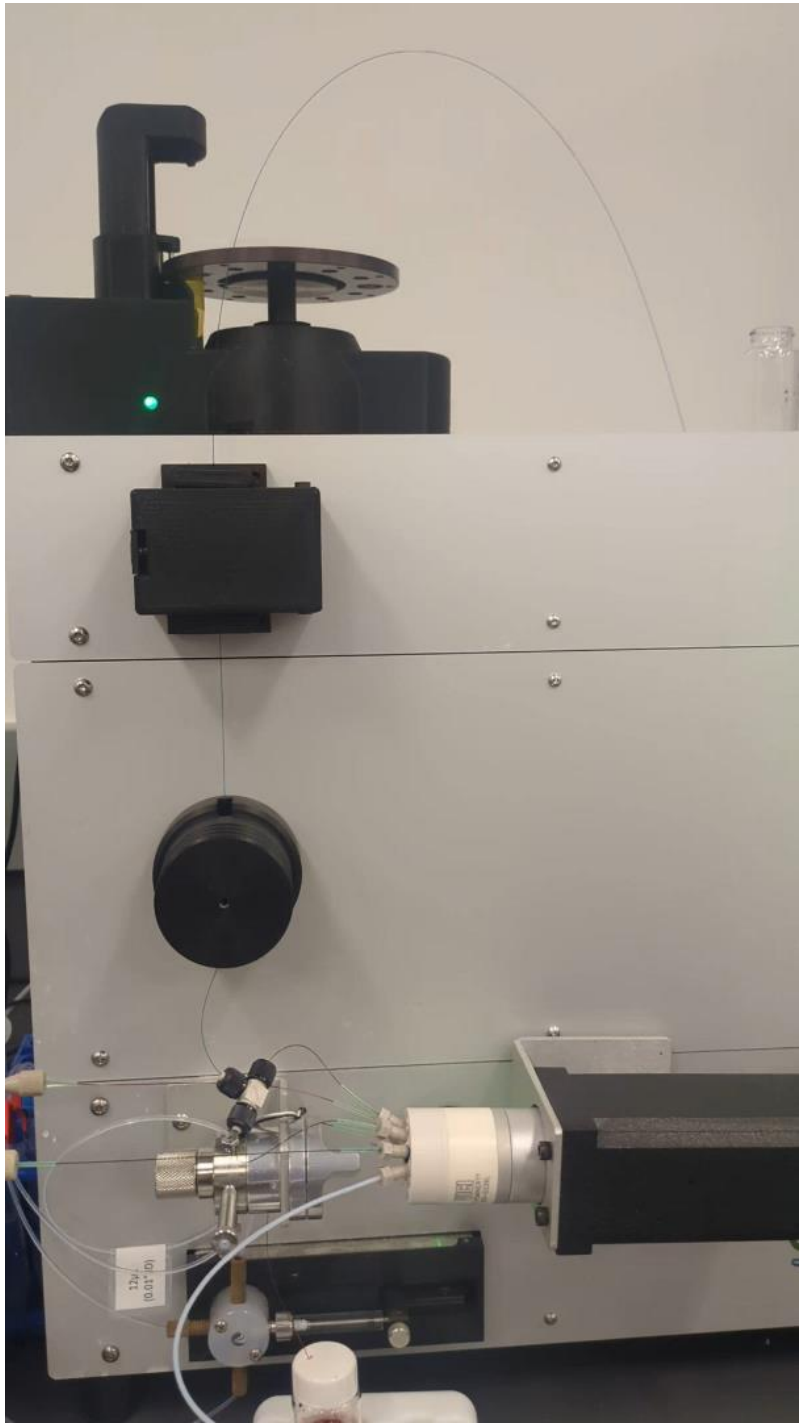


Avg. Oxidations per Peptide

<0.2 0.2 0.4 0.6 0.8 >0.8

Current Work

- Automated fluidics handling
  - Custom software fluidics control
  - Automatic low-volume switching valve
  - Hardware integration with Fox system
- Dosimetry methods
- Proteomic complexity and dynamic range
- Workflows for targeted analysis from blood



Acknowledgements

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NIGMS

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