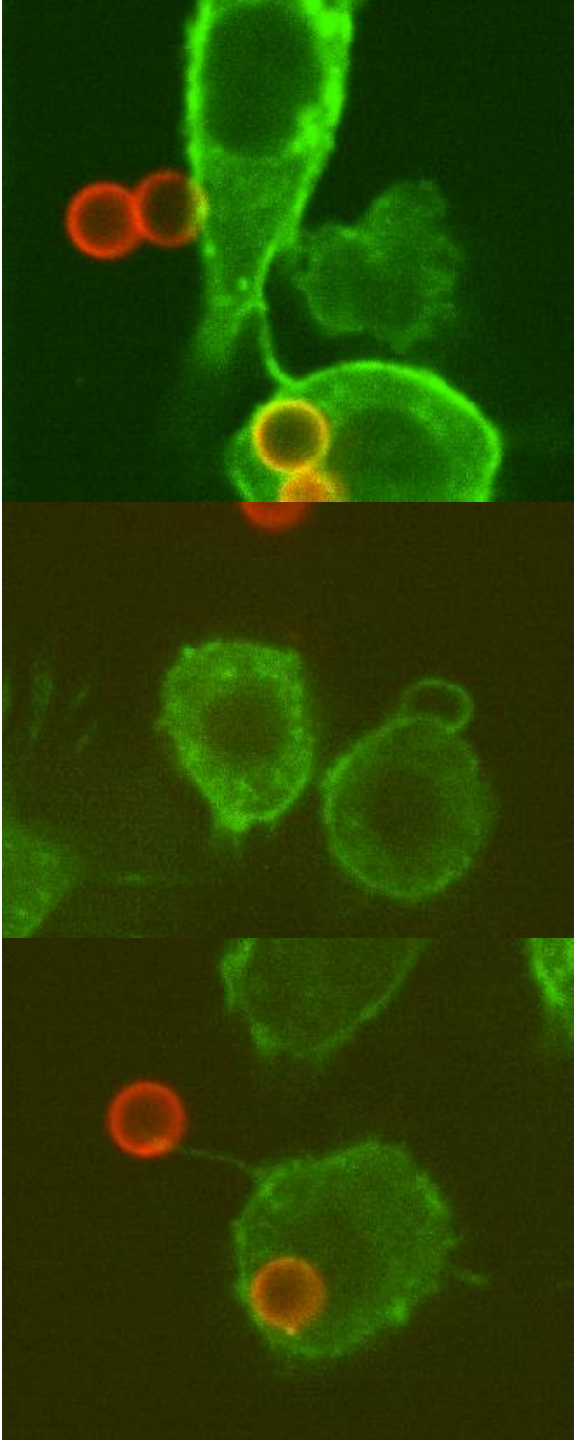

To eat or not to eat:

Decision-making in macrophage phagocytosis

Emily Suter

Fletcher Lab, UCB Bioengineering

2021-12-03 CCC Quarterly Meeting

Overview - macrophage phagocytosis in the Fletcher Lab

- Current projects:
  - How are activating and inhibitory signals integrated in phagocytosis?
  - Can we spatially map complex phosphorylation states within the phagocytic cup?

- Key project characteristics:
  - Link physical properties to biological behavior
  - Explore biological process at many length scales
  - Utilize experimental systems of varying complexity

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Macrophage phagocytosis decisions have clinical implications.

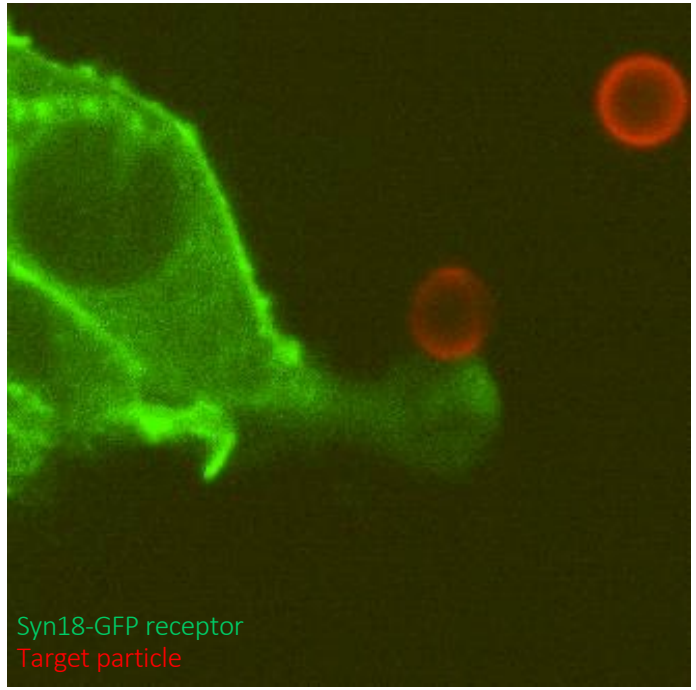
Macrophages:

- Detect and phagocytose foreign, diseased, and apoptotic cells
- Constantly evaluating target's threat
- Decisions affect more than just single target cell

Over-activation:
rheumatoid arthritis, EAE, lupus

Under-activation:
pathogens, cancer, apoptotic cells

Macrophage engulfing target



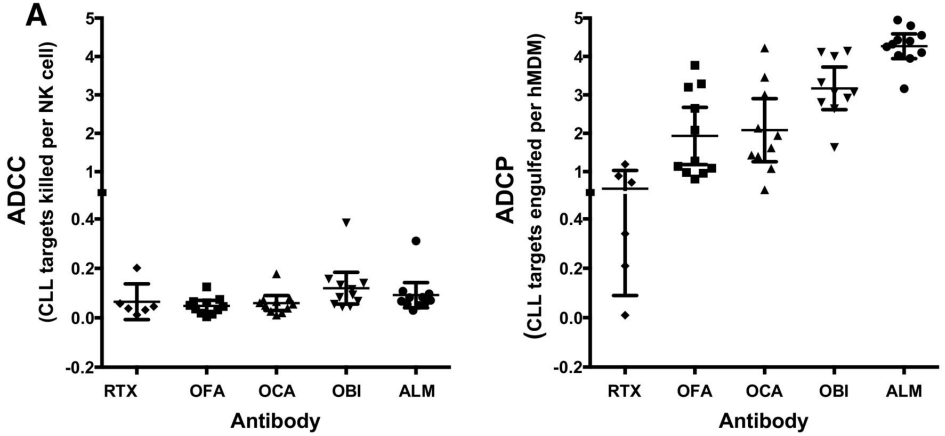
Macrophage ADCP is critical mechanism for therapeutic antibodies.

Monoclonal Abs for cancer immunotherapy

Generic name	Trade name	Type	Target	FDA approval
Rituxumab	Rituxan	Chimeric	CD20	1997
Transtuzumab	Herceptin	Humanized	HER2	1998
Gemtuzumab ozogamicin	Mylotarg	Humanized conjugated with calicheamicin	CD33	2000
Alemtuzumab	Campath-1H	Humanized	CD52	2001
Ibritumomab tiuxetan	Zevalin	Murine conjugated with radioactive ^{90}Y	CD20	2002
Bevacizumab	Avastin	Humanized	VEGF	2004
Cetuximab	Erbitux	Chimeric	EGFR	2004
Panitumumab	Vectibix	Human	EGFR	2006
Ofatumumab	Arzerra	Human	CD20	2009
Denosumab	Xgeva	Human	RANKL	2010
Brentuximab vedotin	Adcetris	Chimeric conjugated with MMAE	CD30	2011
Ipilimumab	Yervoy	Human	CTLA-4	2011
Pertuzumab	Perjeta	Humanized	HER2	2012
Ado-transtuzumab emtansine	Kadcyla	Humanized conjugated with DM-1	HER2	2013
Obinutuzumab	Gazyva	Humanized	CD20	2013
Nivolumab	Opdivo	Human	PD-1	2014
Pembrolizumab	Keytruda	Humanized	PD-1	2014
Ramucirumab	Cyramza	Humanized	VEGF	2014
Dinutuximab	Unituxin	Chimeric	GD2	2015
Elotuzumab	Empliciti	Humanized	SLAMF7	2015
Daratumumab	Darzalex	Human	CD38	2015
Necitumumab	Portrazza	Human	EGFR	2015
Atezolizumab	Tecentriq	Humanized	PD-L1	2016
Avelumab	Bavencio	Human	PD-L1	2017
Durvalumab	Imfinzi	Human	PD-L1	2017
Inotuzumab ozogamicin	Besponsa	Humanized conjugated with calicheamicin	CD22	2017
Rituximab and hyluronidase human	Rituxan Hycela	Chimeric with hyluronidase human	CD20	2017
Bevacizumab-awwb	Mvasi	Humanized	VEGF	2017
Transtuzumab-dkst	Ogivri	Humanized	HER2	2017

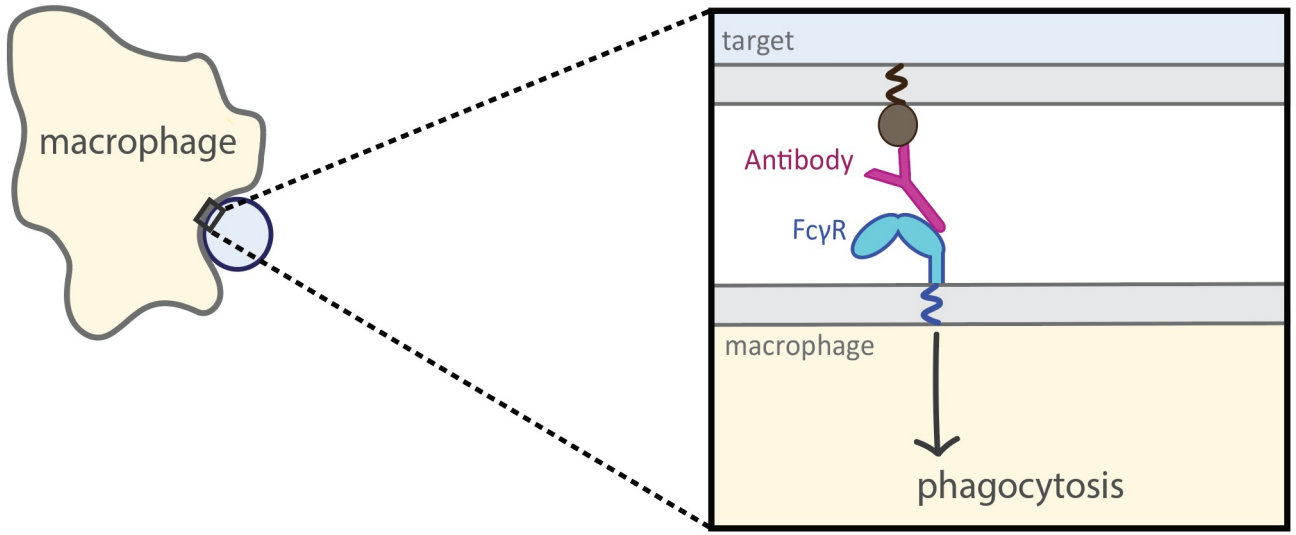
Kimiz-Gebologlu I, et al. *Mol Bio Rep* (2018)

NK Cell ADCC vs. Macrophage ADCP



VanDerMeid KR, et al. *Cancer Immun Res* (2018)

Inhibitory checkpoints dampen pro-phagocytic effects of therapeutic antibodies.



Fcγ Receptors

- Bind Fc portion of IgG antibodies
- Most drive activation/phagocytosis

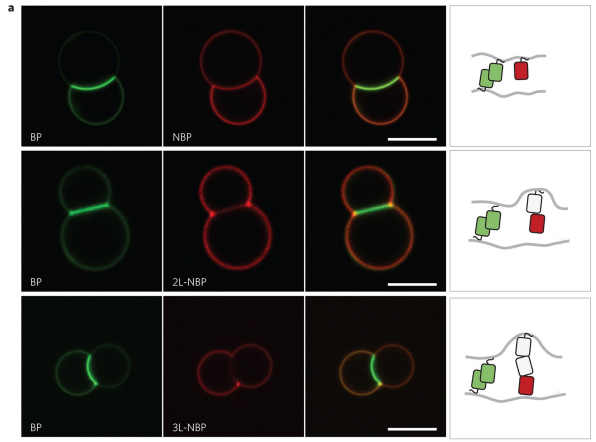
SIRPα

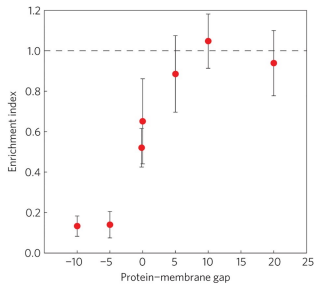
- Bind CD47, a "marker of self"
- Inhibits activation/phagocytosis

Cancer presents confusing decision of disease vs. self.

Cell-cell interfaces have complex and important spatial dynamics.

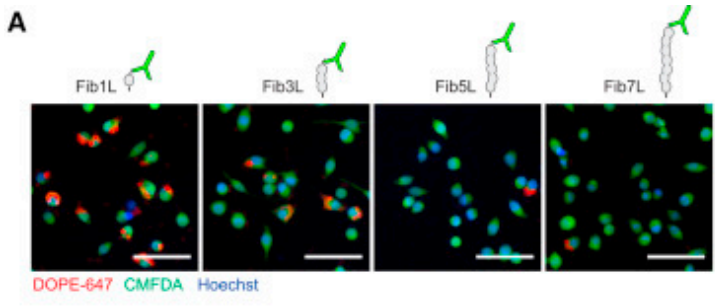
Short binding proteins exclude tall non-binding proteins

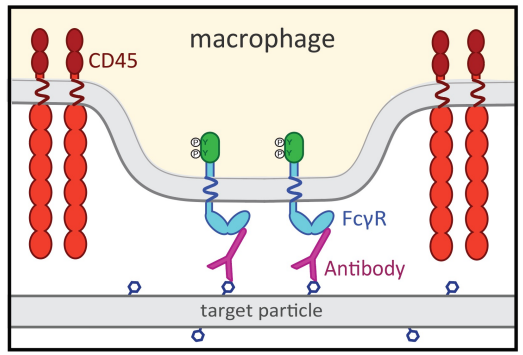




Schmid E, et al. *Nat Phys* (2016)

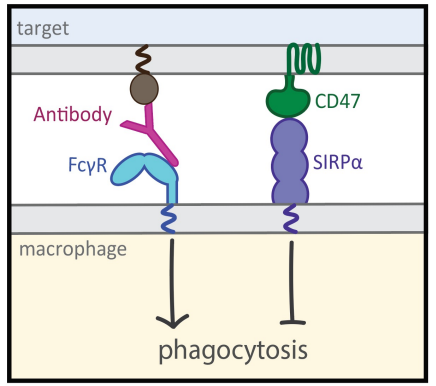
Short antigens yield more efficient phagocytosis due to CD45 segregation





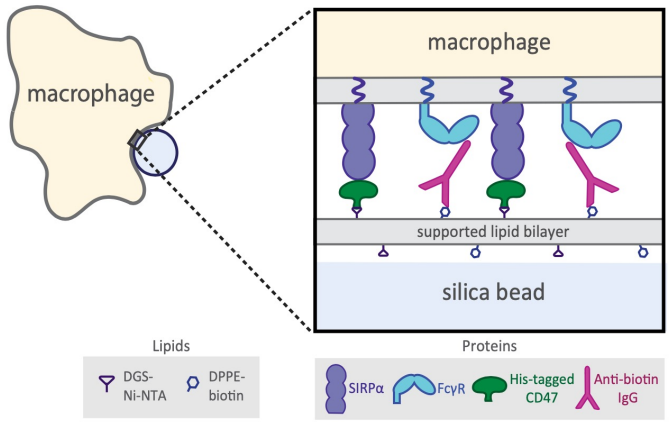
Bakalar M, et al. *Cell* (2018)

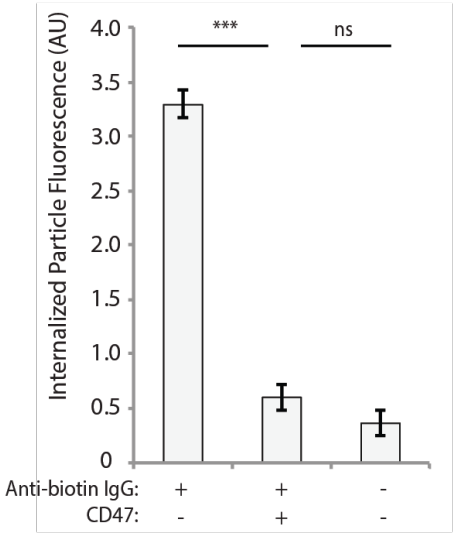
Can we use reconstitution to explore more complex macrophage signaling?



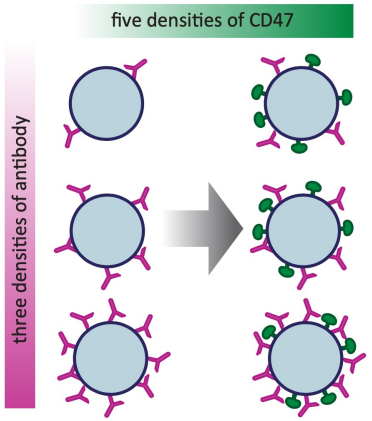
Antibody:CD47 ratio dictates phagocytosis in reconstituted system.

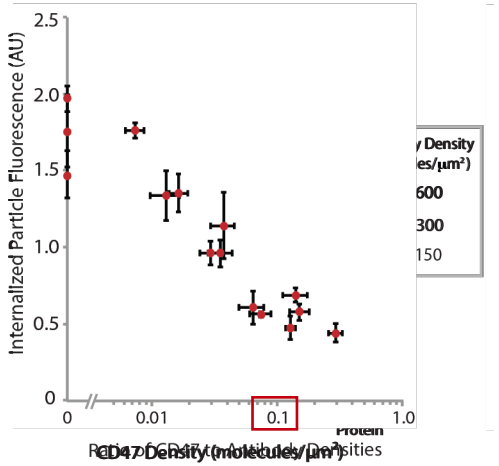
Reconstitution of CD47 inhibition





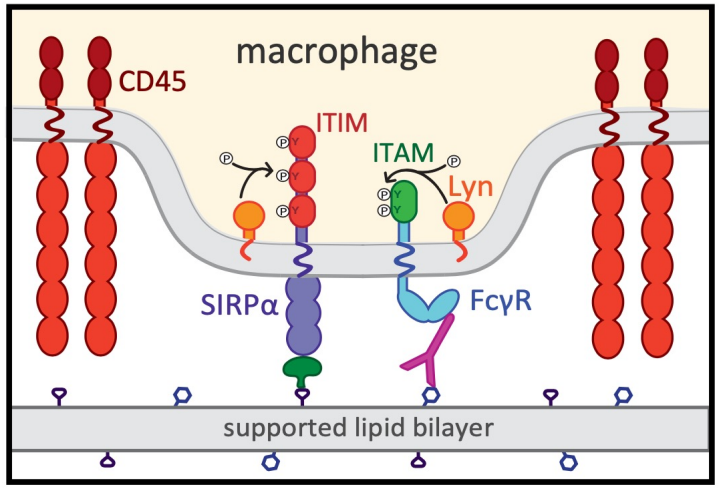
Varying surface ratio of CD47:antibody



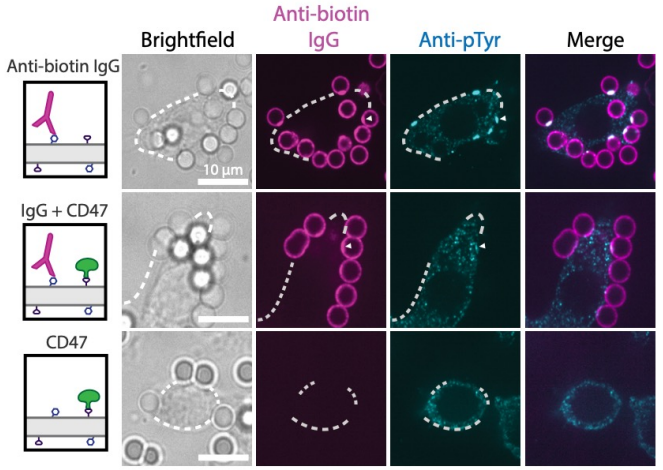


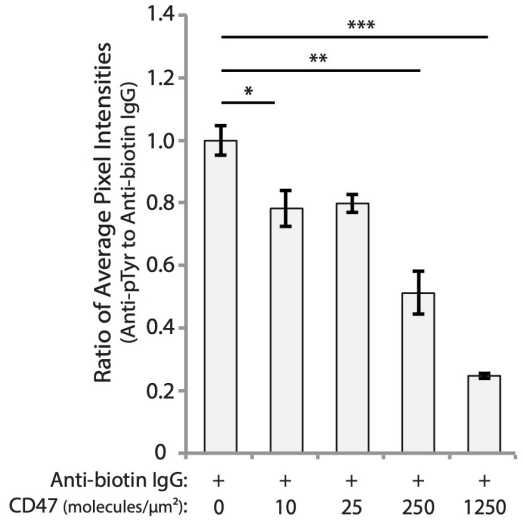
SIRPα enrichment decreases phosphorylation at macrophage-target interface.

FcR and SIRPα phosphorylation by Lyn



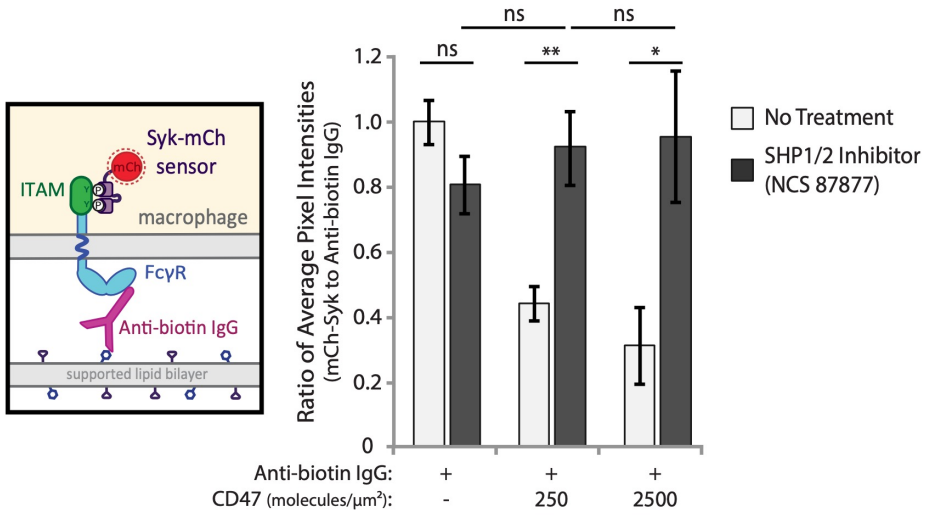
CD47 engagement reduces phosphorylation



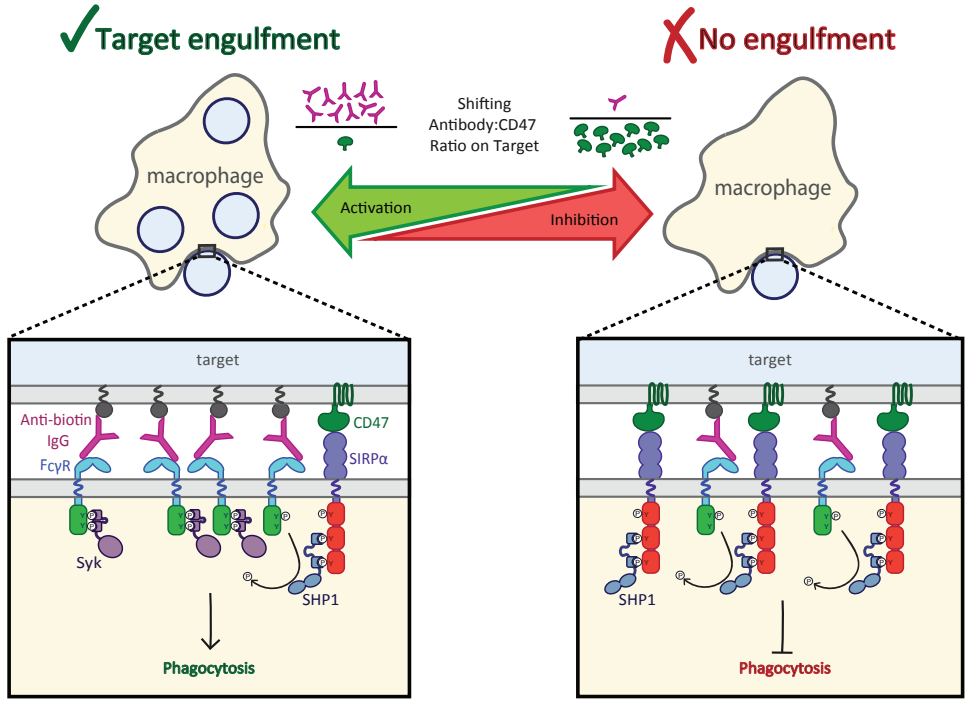


ITIM-associated phosphatases decreases FcγR phosphorylation.

CD47 reduces Syk recruitment to FcRs

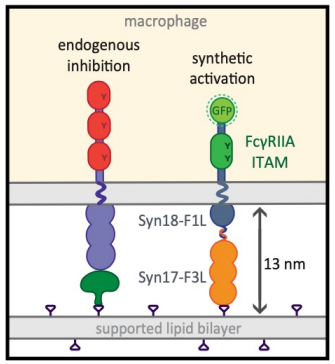


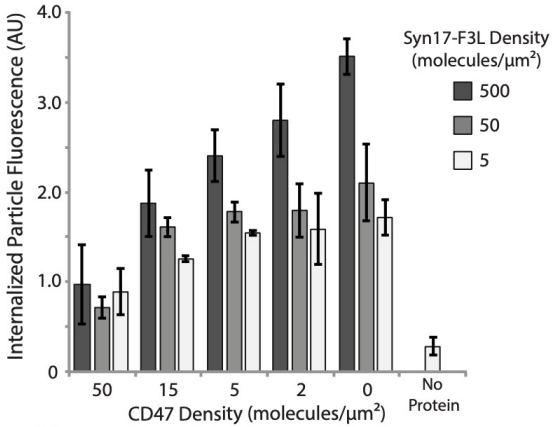
Proposed model:

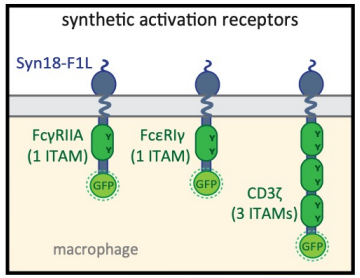


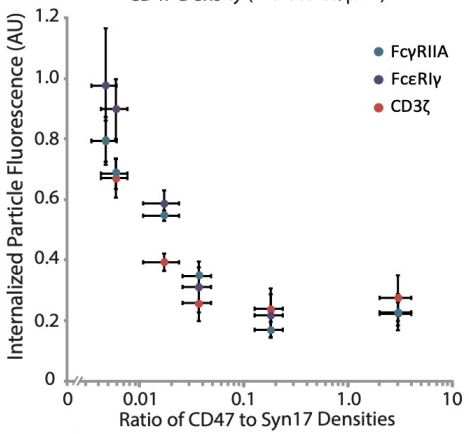
ITIM:ITAM ratio dictates phagocytosis levels with synthetic and tumor cell targets.

Synthetic receptors respond to activation-inhibition ratio

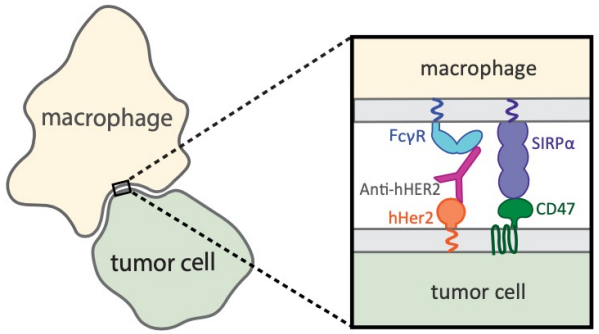


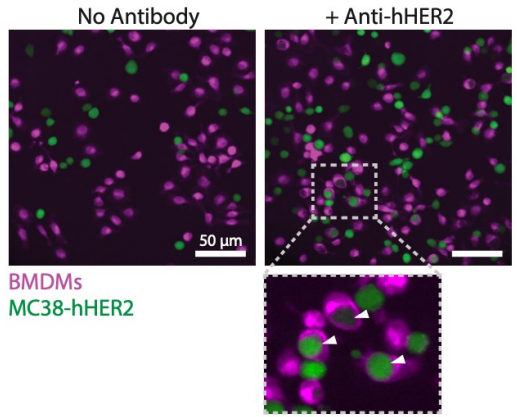


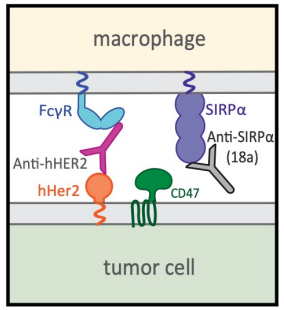


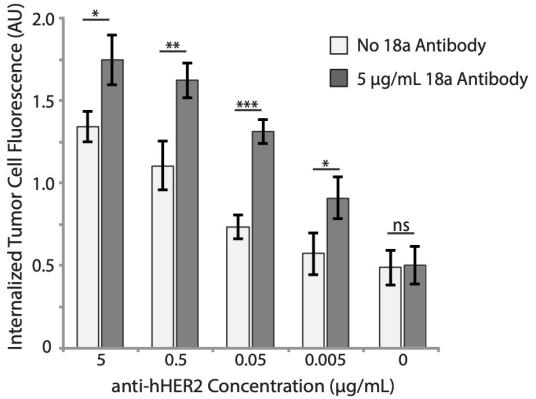


Therapeutic antibodies shift activation-inhibition ratio









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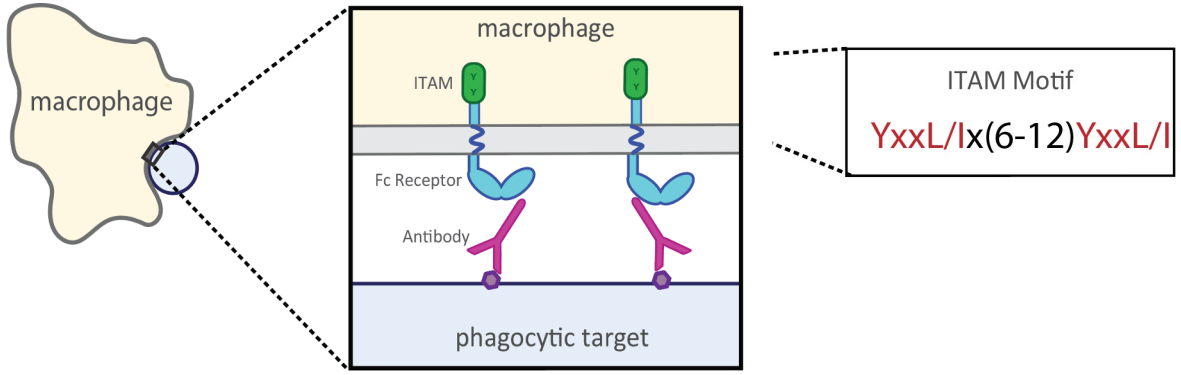
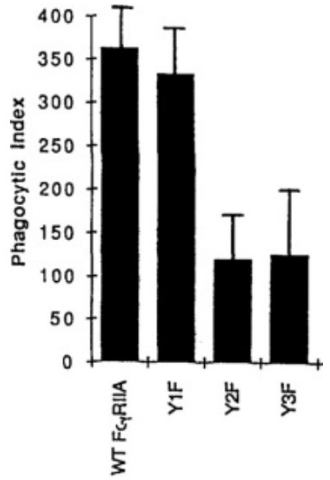
Both ITAM phosphorylation sites are important for phagocytic signaling.

- Immunoreceptor tyrosine-based activation motif (ITAM):
  - Two tyrosine phosphorylation sites become activated upon antibody engagement
- Mutation of either tyrosine reduces phagocytosis
  - Other studies have probed kinetic and functional changes of different phosphorylation states
- However, importance of N- vs. C-terminal phosphorylation is largely unstudied in a cellular context
  - Could C-terminal tyrosine serve as a sort of “kinetic proofreading”?



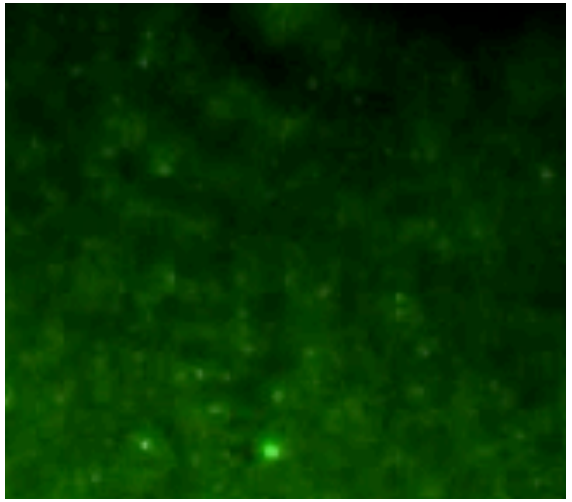


Mitchell et al. *Blood* (1994)

Can we create a cellular map of ITAM phosphorylation state?

A more detailed map allows us to ask…

- How distinct are is phosphorylation in different FcR clusters?
- Where are singly vs. doubly phosphorylated receptors located?
- Are there patterns to N- vs. C-terminal phosphorylation?
- What are phosphorylation patterns at activation-inhibition interfaces?

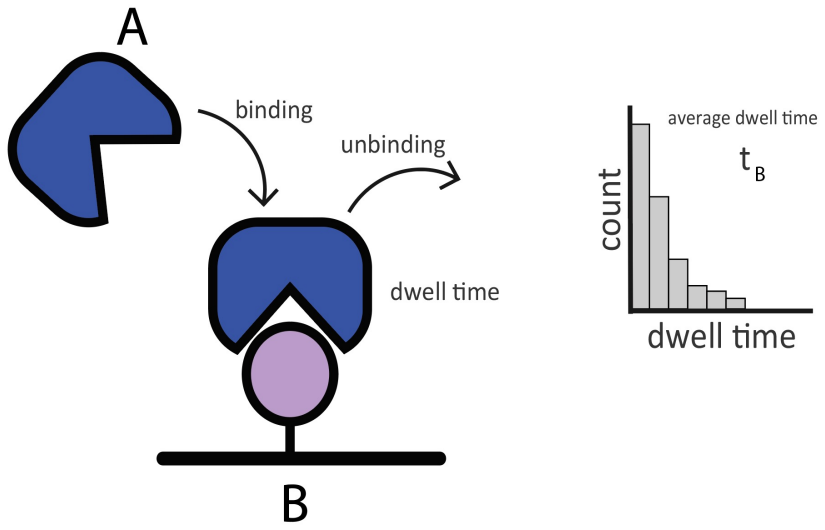


Macrophage spreading on anti-biotin IgG SLB

GOAL:
Combine **single molecule localization precision** and **kinetic information** to generate images of hyperlocal Syk binding affinity to map ITAM phosphorylation.

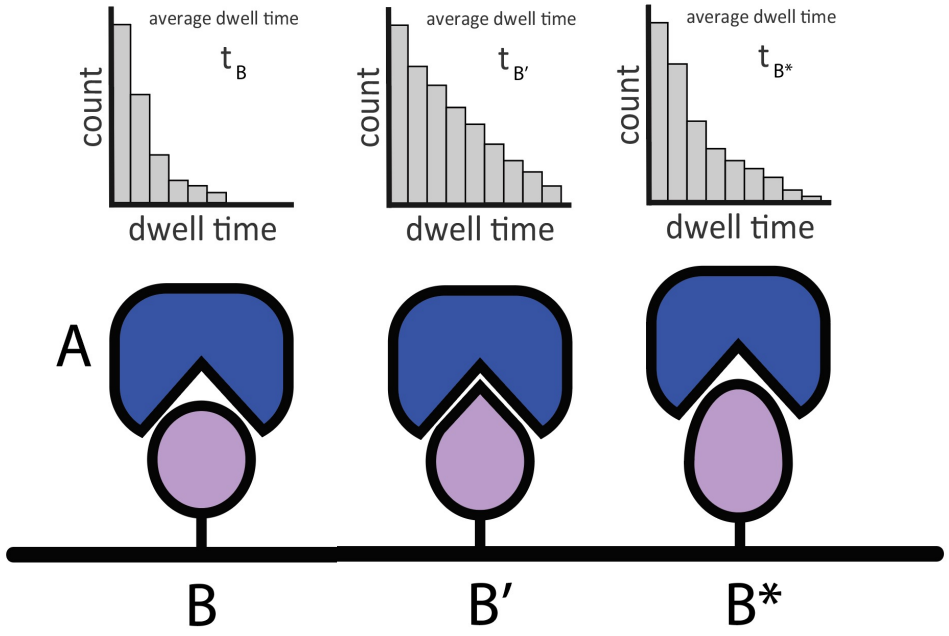
Binding kinetics are sensitive to substrate properties.

- Example system:
  - Protein A binds to protein B, then unbinds
  - Time bound = dwell time
  - A binds to B with a distribution of dwell times t_B



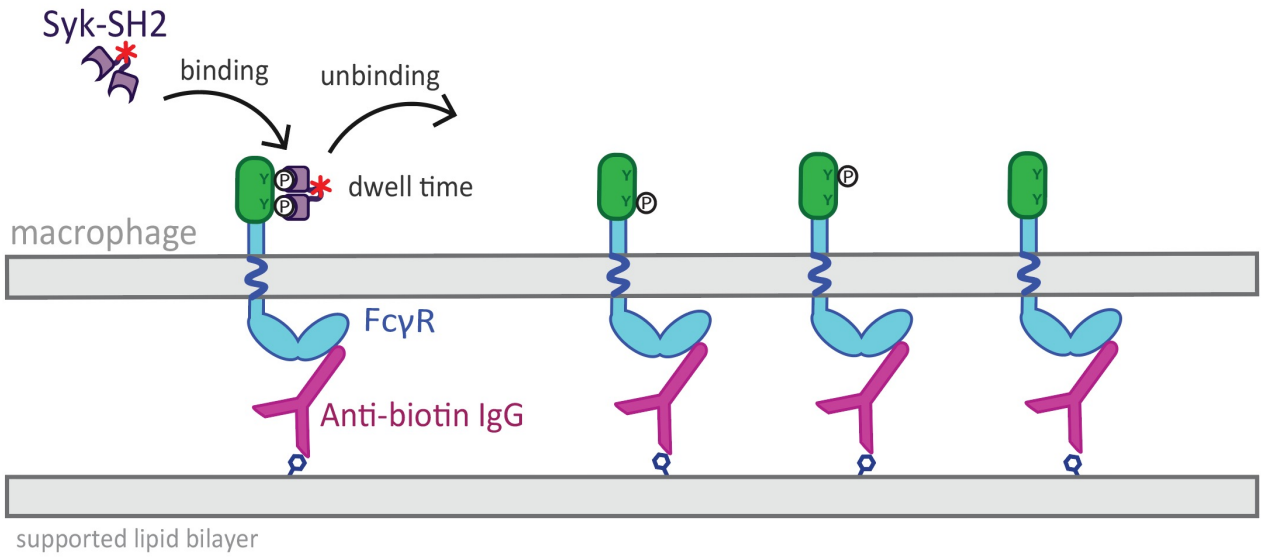
Binding kinetics are sensitive to substrate properties.

- Example system:
  - Protein A binds to protein B, then unbinds
  - Time bound = dwell time
  - A binds to B with a distribution of dwell times t_B
- Protein B can exist in multiple modified states (B’, B*, etc.)
  - Each state has distinct and characteristic distribution of dwell times
  - Importantly, measuring kinetics enables us to determine the state of B

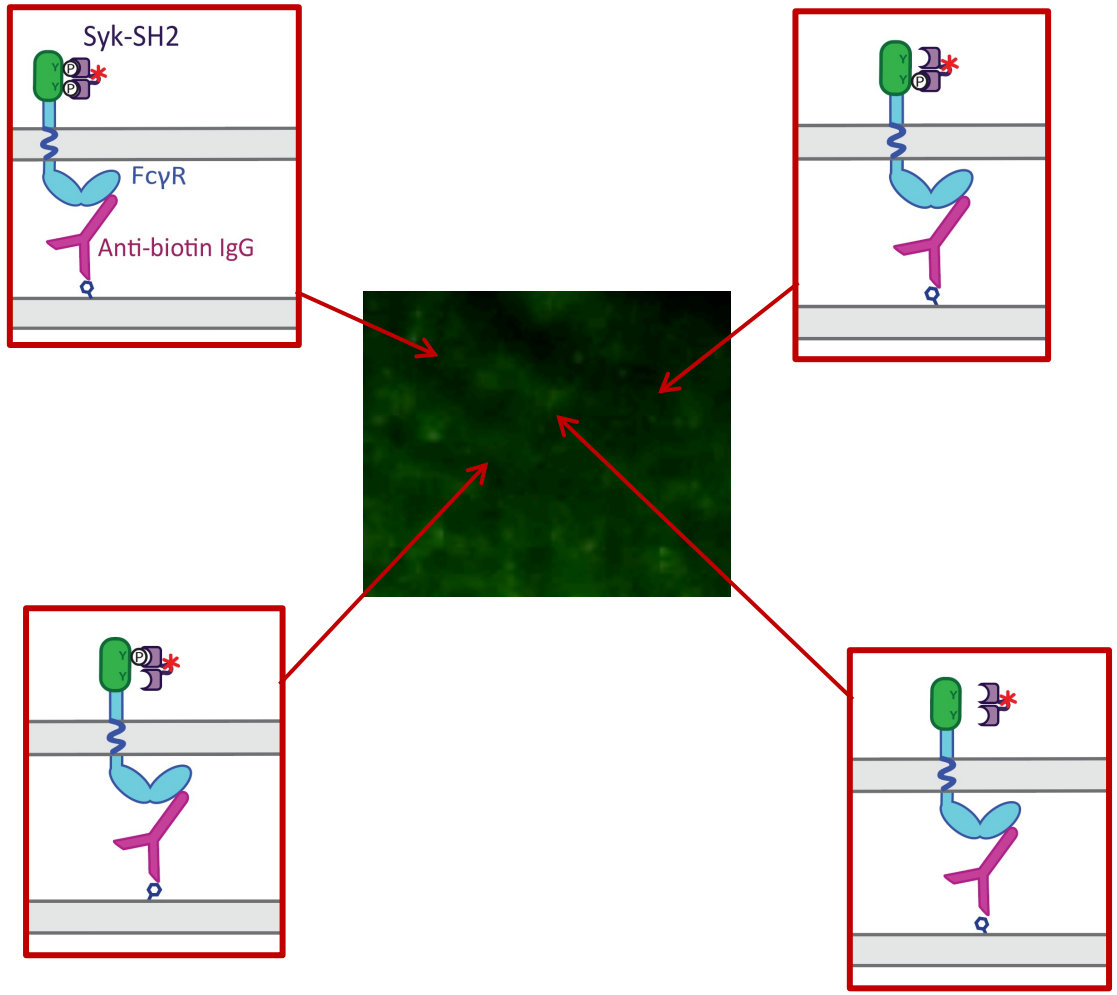


Syk-SH2 binding is sensitive to ITAM phosphorylation state.

- Dual Syk SH2 domains engage with up to two phosphorylated tyrosines
  - Four phosphorylation states bind differently with Syk
  - Previous studies show that Syk exhibits different kinetics for different ITAM phosphorylation states

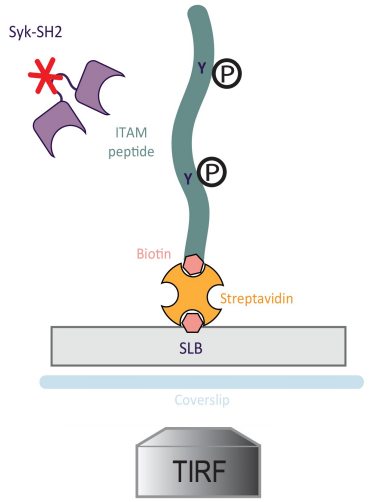


How do we map substrate state in a mixed sample?

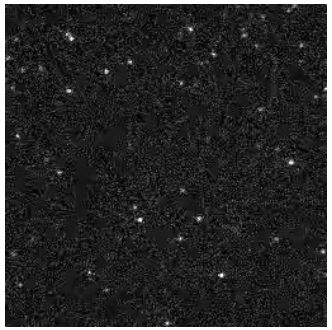


- Each receptor has distinct state we would like to capture
  - Need to spatially differentiate and kinetically differentiate each individual receptor
- Each receptor needs its own distribution!
  - Need to image for a long time to capture many binding-unbinding events for every receptor
  - Must conduct experiments in fixed samples so substrate (receptors) are not moving

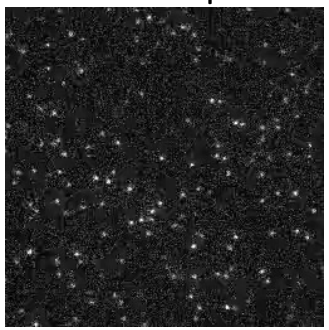
ITAM phosphorylation state changes Syk binding kinetics.



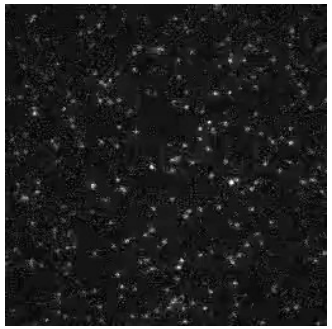
ITAM - YY



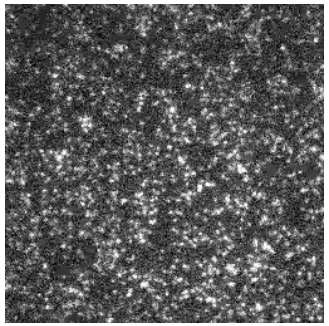
ITAM - YpY

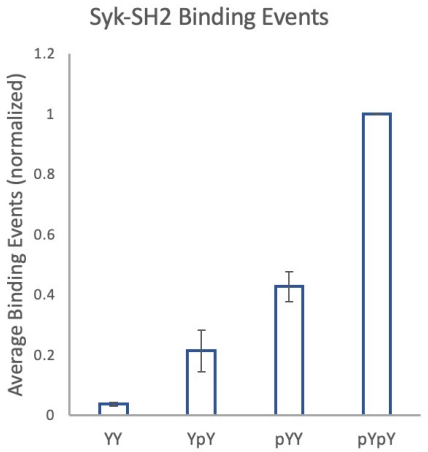


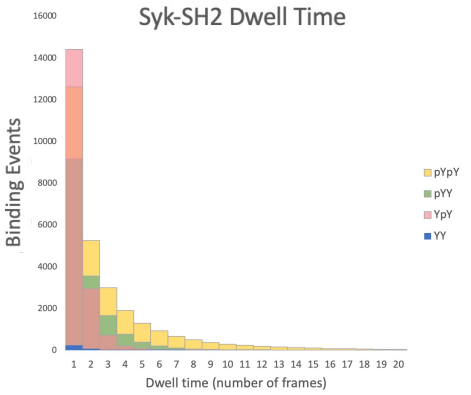
ITAM - pYY



ITAM - pYpY







ITAM phosphopeptides

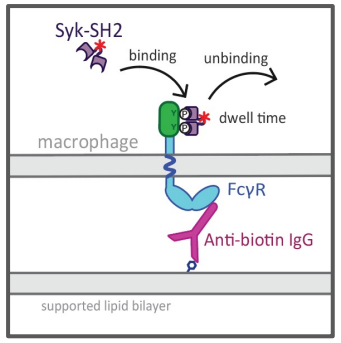
YY: *ERPPPVPNPDYEPIRKGQRDLYSGLNQR

pYY: *ERPPPVPNPD(pY)EPIRKGQRDLYSGLNQR

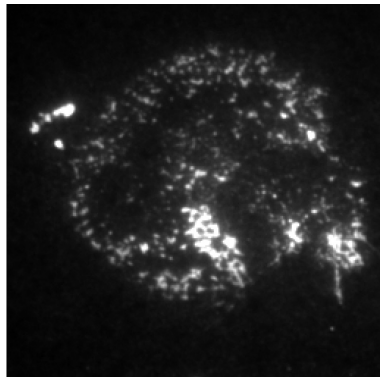
YpY: *ERPPPVPNPDYEPIRKGQRDL(pY)SGLNQR

pYpY: *ERPPPVPNPD(pY)EPIRKGQRDL(pY)SGLNQR

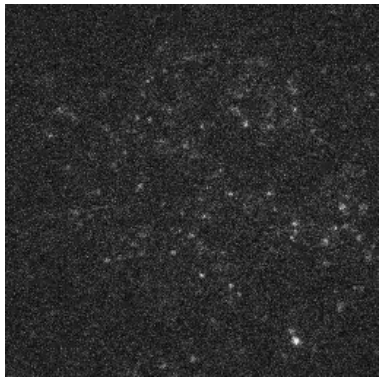
Mapping ITAM phosphorylation in WT macrophages



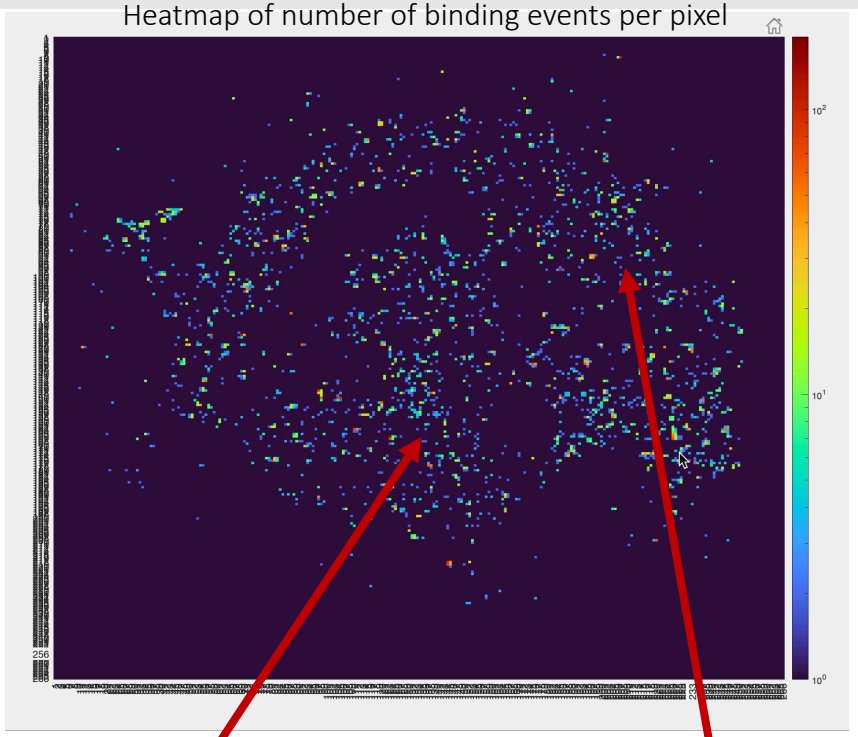
Anti-biotin IgG



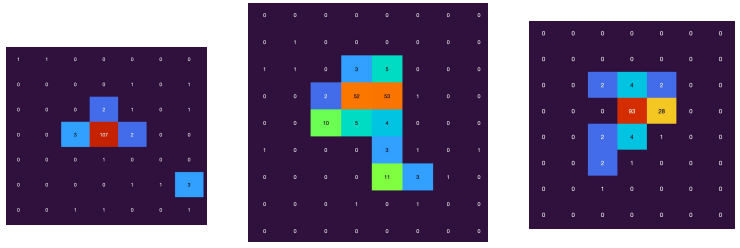
Syk-SH2

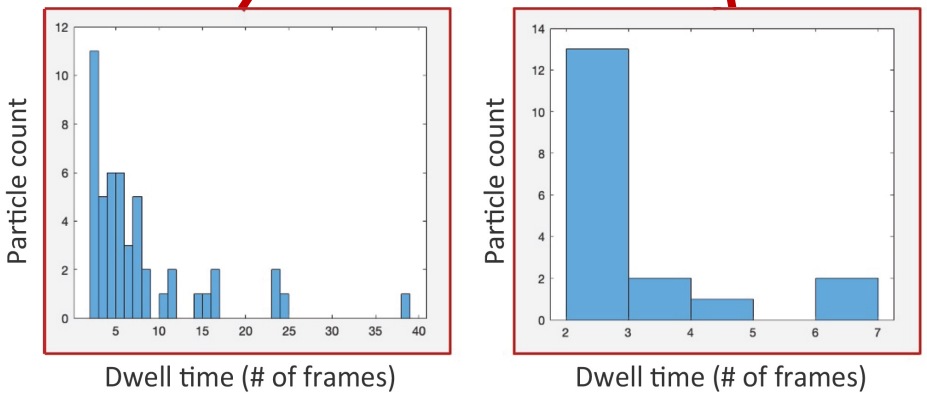


Heatmap of number of binding events per pixel



Example Syk binding clusters

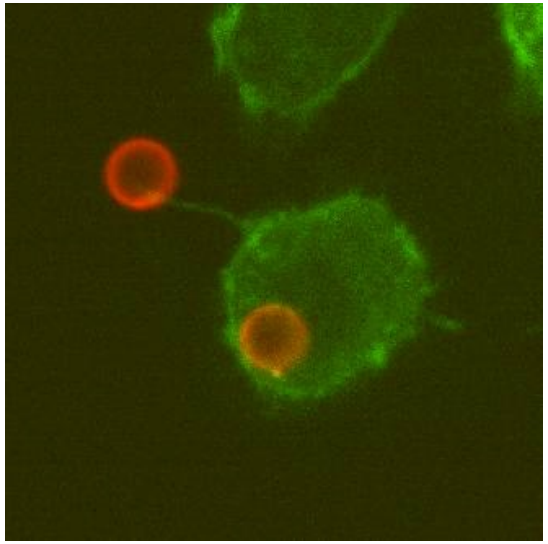


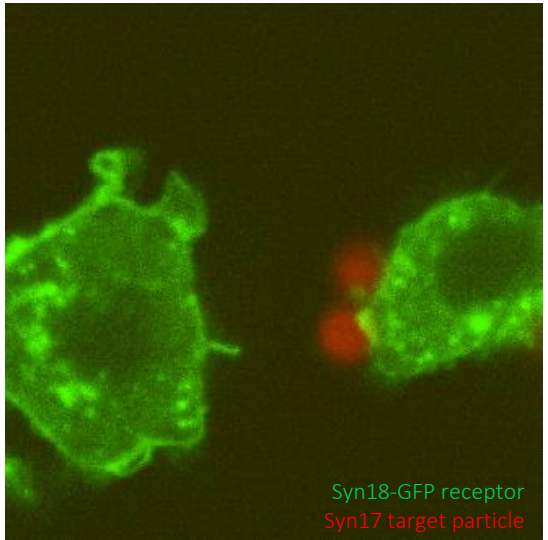


Conclusions and future directions

- **Key takeaways:**
  - Ratio of activating IgG to inhibitory CD47 dictates macrophage phagocytosis
  - ITIM-associated phosphatases halt activation by dephosphorylating adjacent FcRs
  - Combining single molecule imaging and kinetic measurement can (potentially!) map substrate variation

- **Future musings:**
  - How do macrophages explore the space around them?
  - How do macrophages coordinate signaling and cytoskeletal machinery to engulf far away targets?





Thanks!

Fletcher Lab

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Lab Alumni

Eva Schmid
Marija Pdolski
Andrew Harris
Ari Joffe
Matthew Bakalar
Kaden Southard
Carmen Chan
Tiama Hamkins-Indik
Anna Lippert

IVRI
IMMUNOTHERAPEUTICS AND VACCINE RESEARCH INITIATIVE

CHAN ZUCKERBERG BIOHUB

ADURO BIOTECH

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