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Imaging Innate Immune Responses using Dual Color Stochastic Reconstruction Optical Microscopy (STORM)

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Outline

- Motivation: Innate Immunity and Toll-Like Receptor (TLR) signaling
- Methods: Stochastic Optical Reconstruction Microscopy (STORM)
- Results: Detection of receptor clustering and co-localization of receptors with antigen
- Conclusions

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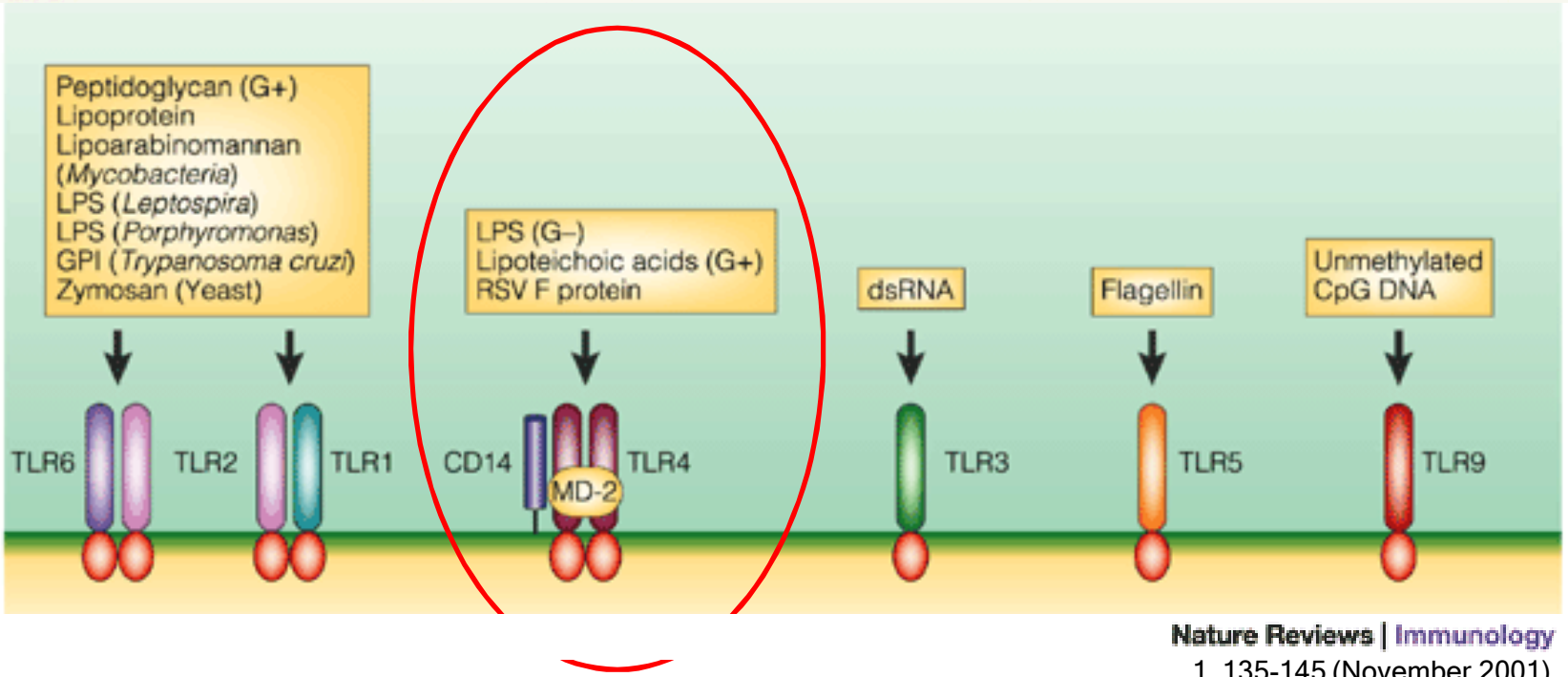
The TLR Family



Nature Reviews | Immunology
1, 135-145 (November 2001)

- LPS recognition by TLR4 is aided by accessory proteins
- Different chemotypes of LPS generate distinct immune responses
- Important implications for pathogenesis, biodefense

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Receptor Clustering

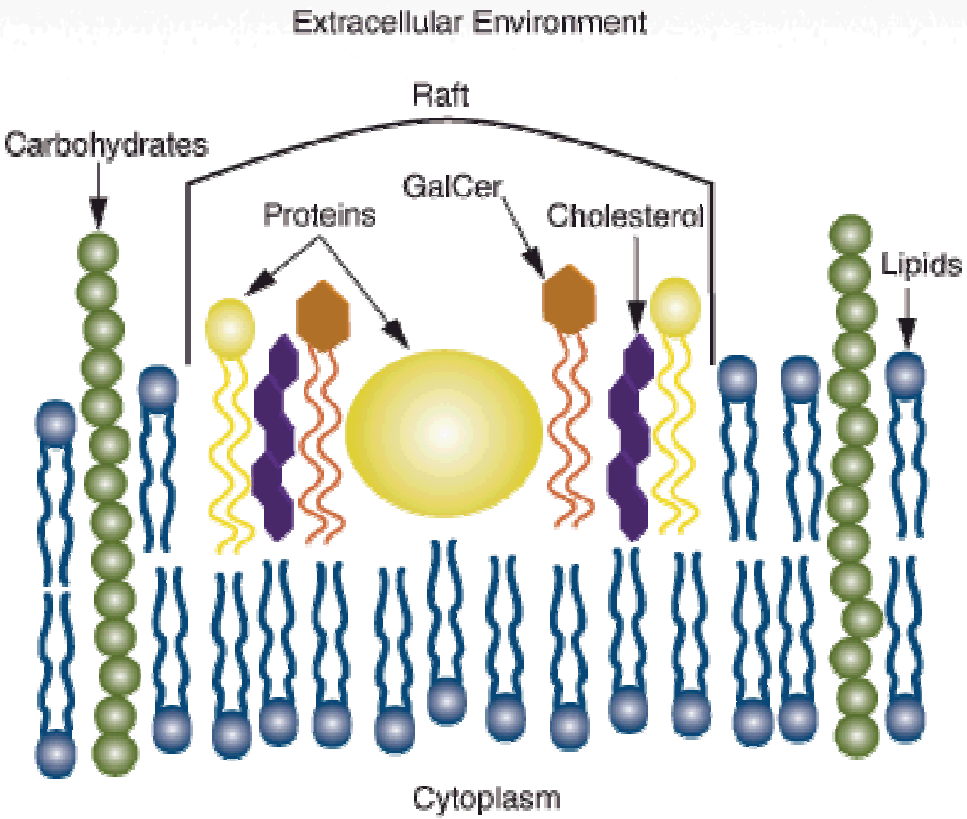

Image Courtesy of Tim Ratto, Lawrence Livermore National Labs

- Domains act as assembly areas
- Aggregation of receptors often follows activation/ligand binding
- Receptor reorganization can be a necessary component of immune response
- Bulk assays have suggested that TLR4 molecules aggregate in lipids rafts within the cell membrane after LPS binding*

*Triantafilou, et. al, *Biochem. J.* 381(Pt 2): 527-536

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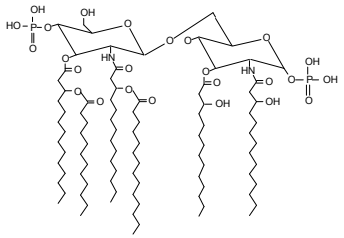
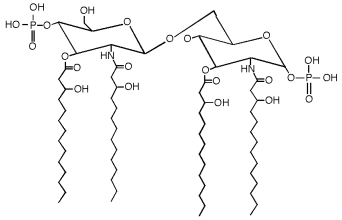
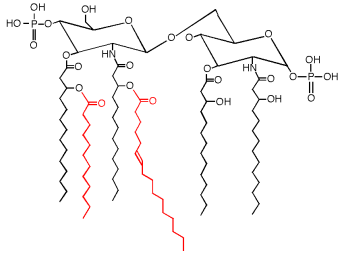
Unanswered Questions

Escherichia coli **(control)**	***Yersinia pestis*** **(37°)**	***Yersinia pestis*** **(21°)**
Smooth O-polysaccharide	Rough O-polysaccharide	Rough O-polysaccharide
		
Bind Surface + ↑Stimulatory	Bind Surface + ↓Stimulatory	Bind Surface + ↑Stimulatory

Differential immune response observed with chemotypes of LPS is not fully understood.

- LPS from *E. coli* binds & produces an immune response
- LPS from *Y.* pestis (plague @ 37 °) binds, but does not

Are there clues in the nano-scale arrangement of the early immune response at the membrane interface?

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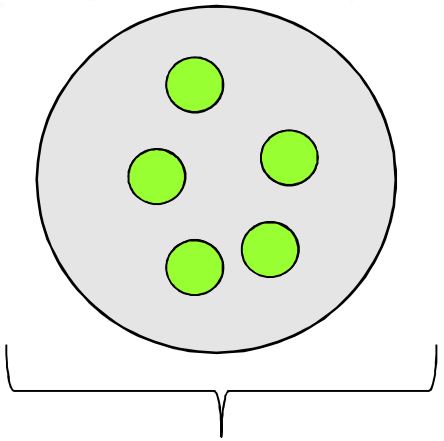
Can optical super-resolution give us a way to differentiate receptor clustering on a much finer scale than conventional imaging?

STORM Imaging

Stochastic Optical Reconstruction Microscopy



~400nm diffraction-limited spot size

- Assuming <1 fluorophor per diffraction-limited area, it's position can be determined with nanometer precision.

Rust, et. al, Nat. Meth. 3: 793 - 796 (2006)

- The Abbe resolution limit can effectively be broken if the fluorophors in a sample can be imaged *independently* from each other.
- In STORM, this means incorporating "photoswitching", whereby only a small subset of fluorophors is visible at any given time.
- Photoswitching for organic dyes can occur under 10-100mW excitation in buffer containing small thiol (i.e. BME) and oxygen scavenging system.

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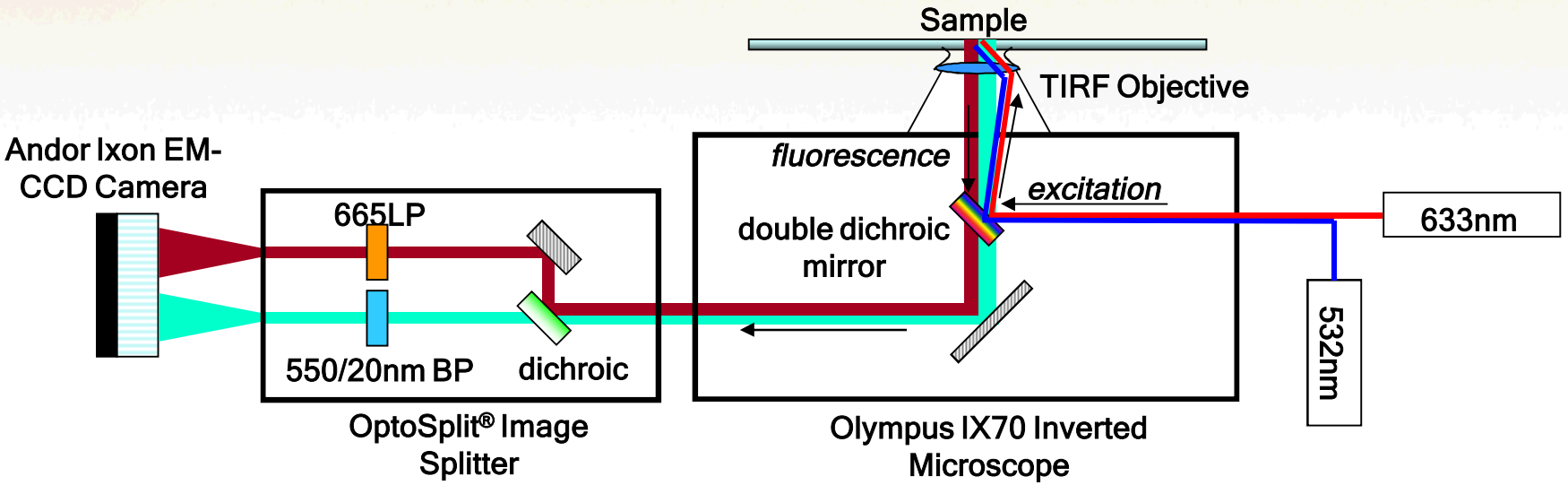
Imaging Setup



- Olympus IX-71, 60x, NA 1.45 TIRF objective
- Capable of up to four excitation wavelengths (choose among 405, 488, 532, and 633nm), variable angle
- Optosplit® image splitter projects multiple emission wavelengths simultaneously onto EMCCD (Andor iXon)
- Capable of >50fps over 30µm x 30µm FOV

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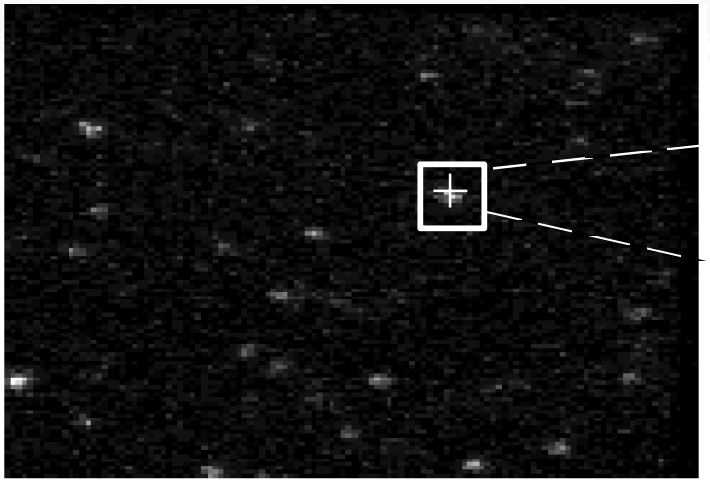
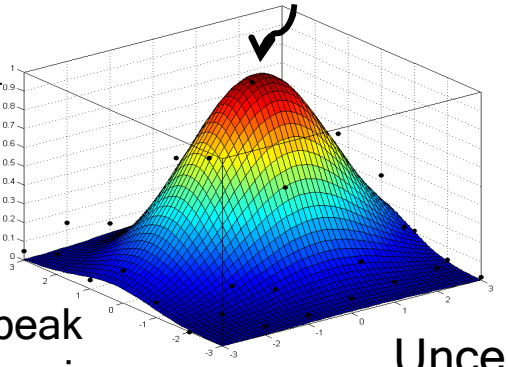
Localization Algorithm



$(x_0 , y_0) = (-61\pm31\text{nm} , 44\pm23\text{nm})$



Fit peak
to Gaussian

Uncertainty ~ $1/\sqrt{N}$
(SNR)

- Single fluorophors with minimum SNR are identified in each frame
- Local area fitted to 2-D Gaussian surface as ~PSF
- Maximum of that surface is most likely position of the fluorophor
- Typically, location fit has 95% confidence intervals of 50-60nm for
- Process repeated over 1k-10k frames to build STORM image

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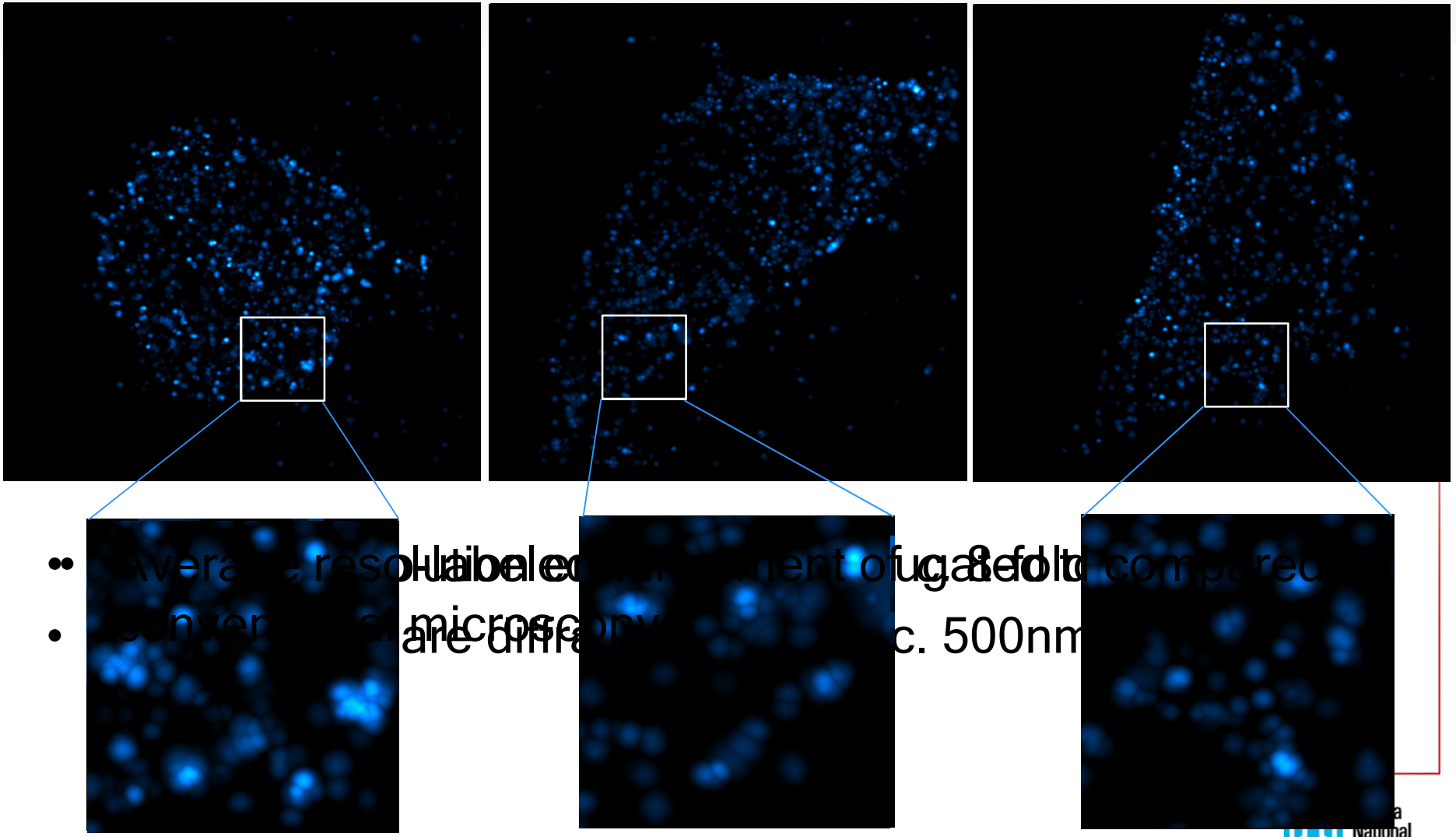
Imaging TLR4 using STORM

E. coli LPS

Flagellin

Y. pestis LPS



- Average resolution of ~8nm
- Conventional microscopy is diffraction limited to c. 500nm

Ripley's K-function Analysis

- K-function is a normalized measure of point clustering

$$K(r) = \frac{A}{N^2} \sum_{j \neq i}^{N} \sum_{i=1}^{N} \frac{I(d_{ij} < r)}{w_{ij}}$$

- Complete spatial randomness (CSR)

$$K(r) = \pi r^2$$

- Transform to H-function to gauge deviation from CSR at each test radius
- Peaks (or inflection points) in H(r) indicate characteristic cluster sizes

$$H(r) = \sqrt{\frac{K(r)}{\pi}} - r$$

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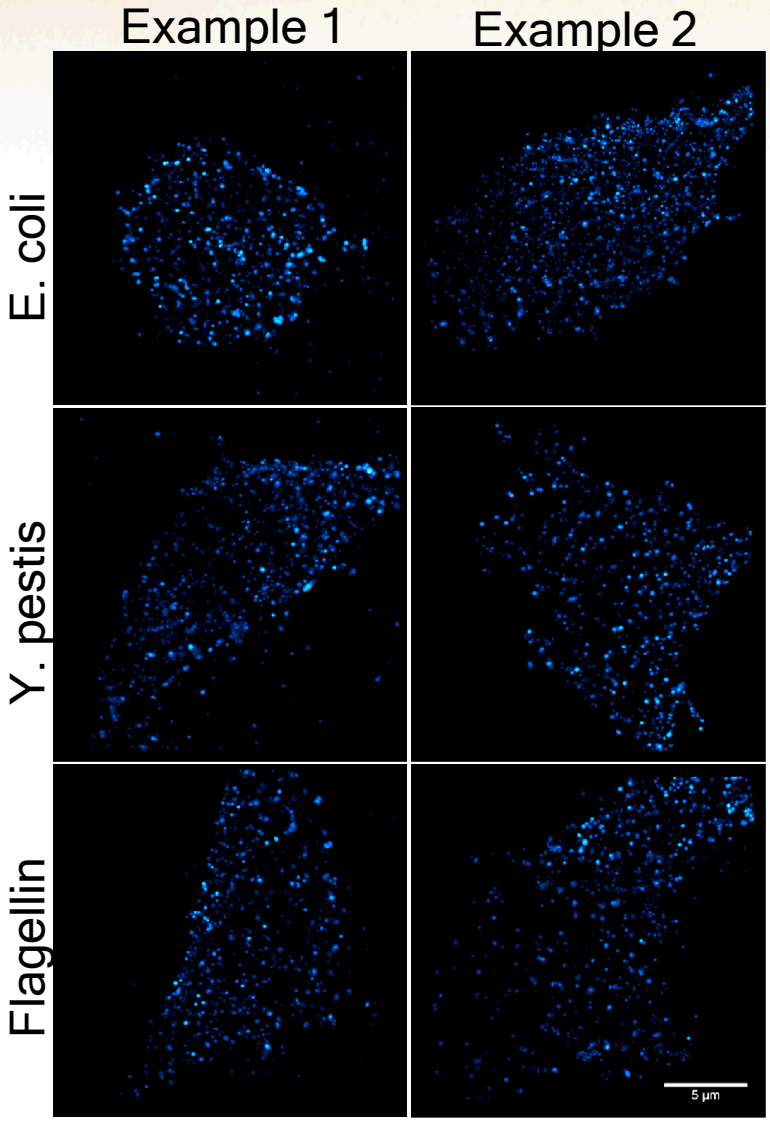
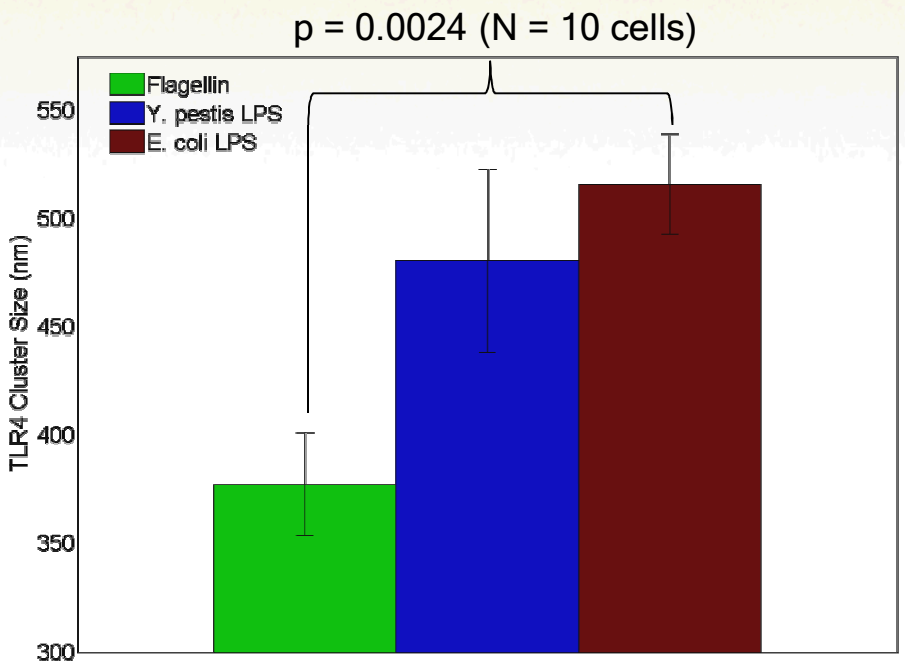
TLR4 Cluster Analysis





- Ripley's K-analysis indicates that *E. coli* LPS induces significant clustering over negative control (flagellin)
- Suggests that *pestis* induces less clustering, but not significant
- TLR4-LPS complex?

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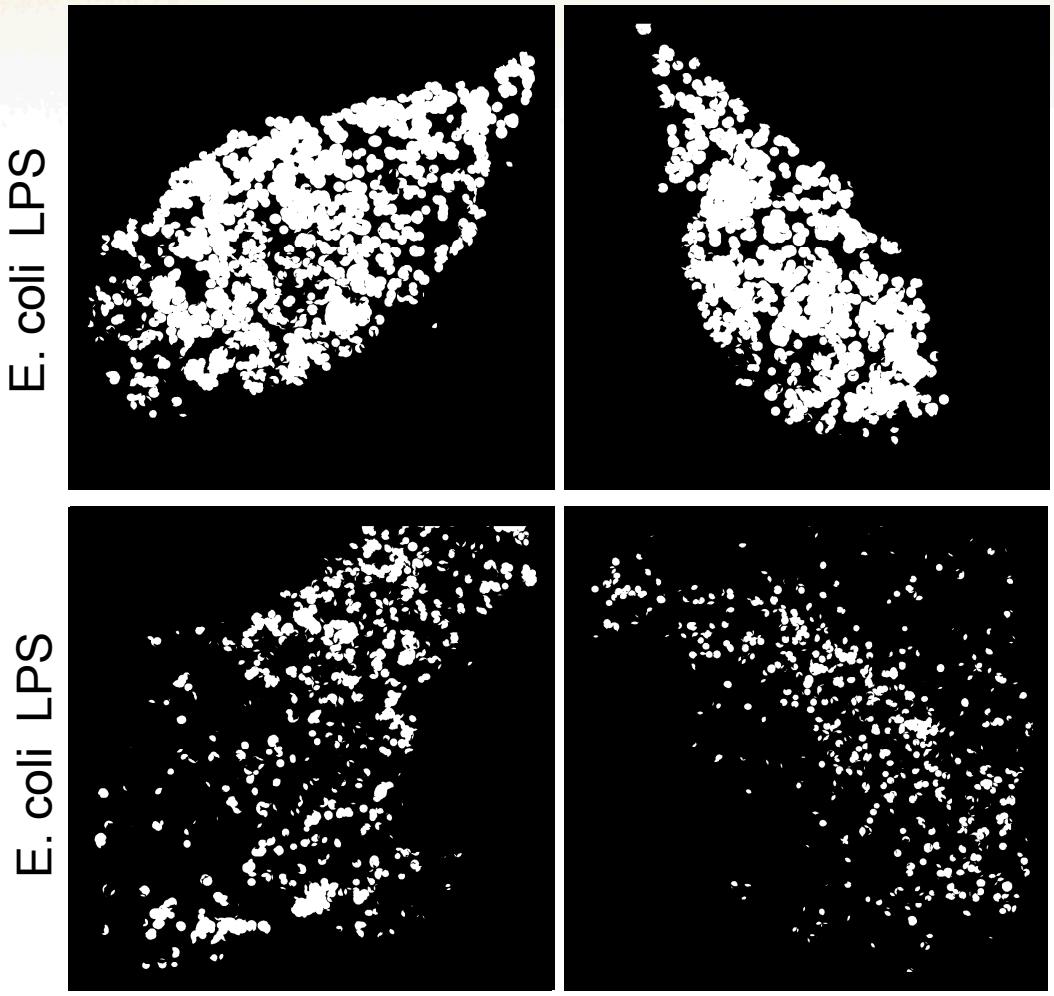
Colocalization of TLR4 & LPS



- Dual-color STORM imaging
- TLR4 – Atto532
- LPS – AlexaFluor647
- Image registration via multi-dye PS beads (average error ~50nm)
- Perform cluster analysis on co-localized points

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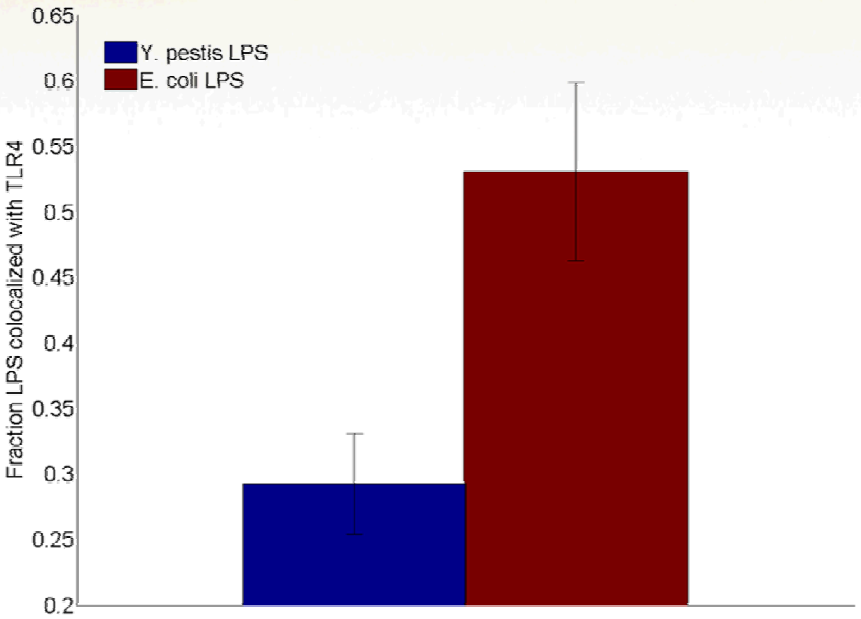
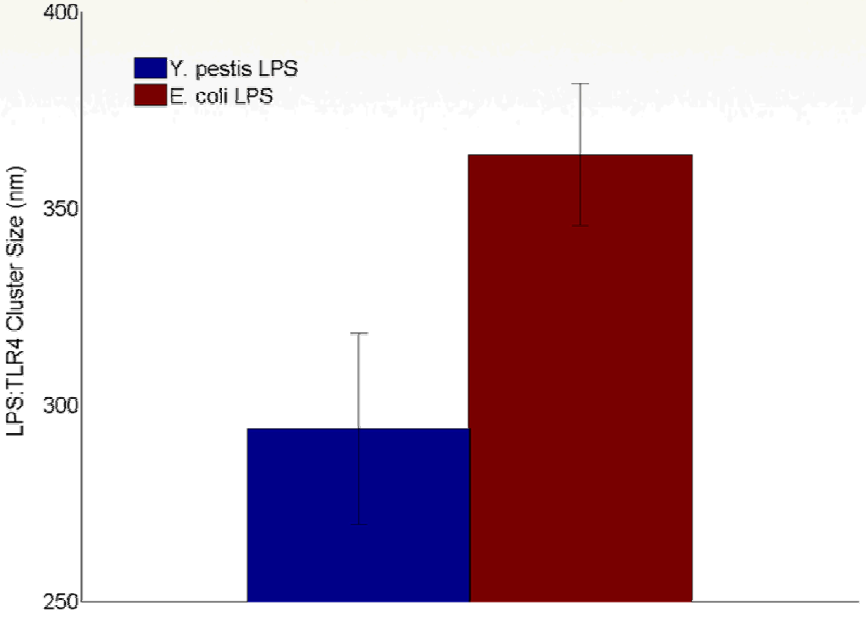
TLR4-LPS Complex Analysis



p = 0.0069



p = 0.0310

- Analysis of TLR4-LPS complex reveals significantly less co-localization of *Y. pestis* LPS compared to *E. coli*
- *Y. pestis* LPS appears less efficient at recruiting TLR4 into clustered domains

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Conclusions

- Superresolution imaging allows for measuring subtle changes on cell membrane that aren't apparent in conventional microscopy
- Challenge with *E. coli* LPS produces a significant increase in TLR4 cluster size within 30 minutes, as compared to a non-specific ligand (Flagellin)
- Dual-color STORM imaging allows us to perform multiplexed measurements of receptor/ligand organization at the nanoscale
- TLR4 co-localization with *E. coli* LPS is significantly higher than with *Y. pestis LPS*
- Higher-order LPS clustering in *E. coli* case vs. *Y. pestis*

Acknowledgements

- Roberto Rebeil for isolation of the *Y. pestis* LPS

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- Laboratory Directed Research and Development program at Sandia National Laboratories.
- The National Institutes of Health NIH Director's New Innovator Award Program, 1-DP2-OD006673-01

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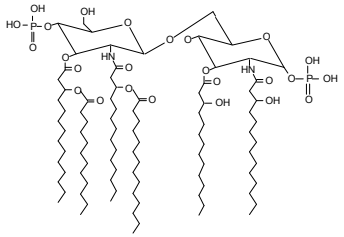
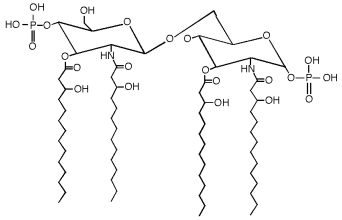
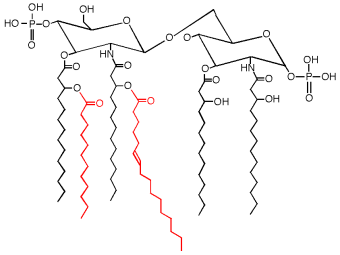
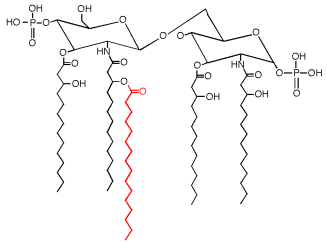
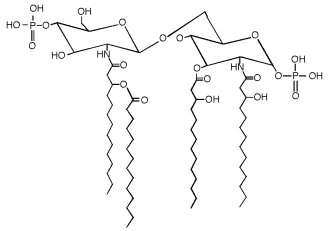
Forms of LPS

Escherichia coli **(control)**	***Yersinia pestis*** **(37°)**	***Yersinia pestis*** **(21°)**	***Yersinia pseudo-tuberculosis*** **(37°)**	***Yersinia enterocolitica*** **(37°)**
Smooth O-polysaccharide	Rough O-polysaccharide	Rough O-polysaccharide	Rough O-polysaccharide	Smooth O-polysaccharide
				
Bind Surface + ↑Stimulatory	Bind Surface + ↓Stimulatory	Bind Surface + ↑Stimulatory	Bind Surface + ↑Stimulatory	No binding + ↓Stimulatory

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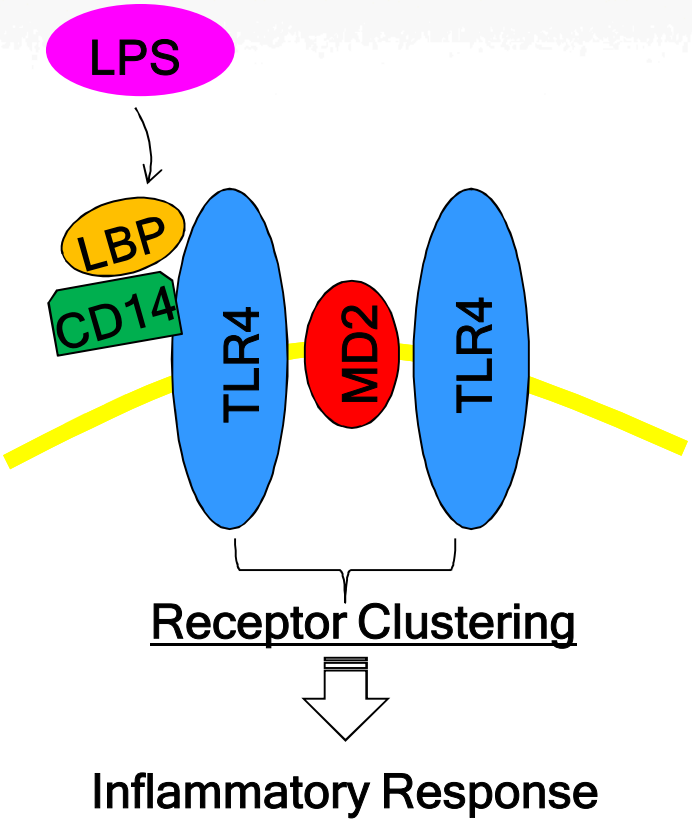
TLR-4 Receptor Signaling



- Critical component of the mammalian innate immune system
- Binds lipopolysaccharides (LPS) present on the surface of gram-negative bacteria
- Understanding this system is important for understanding pathogenesis, implications for bio-defense

Triantafilou, et. al, Biochem. J. 381(Pt 2): 527-536 (2004).

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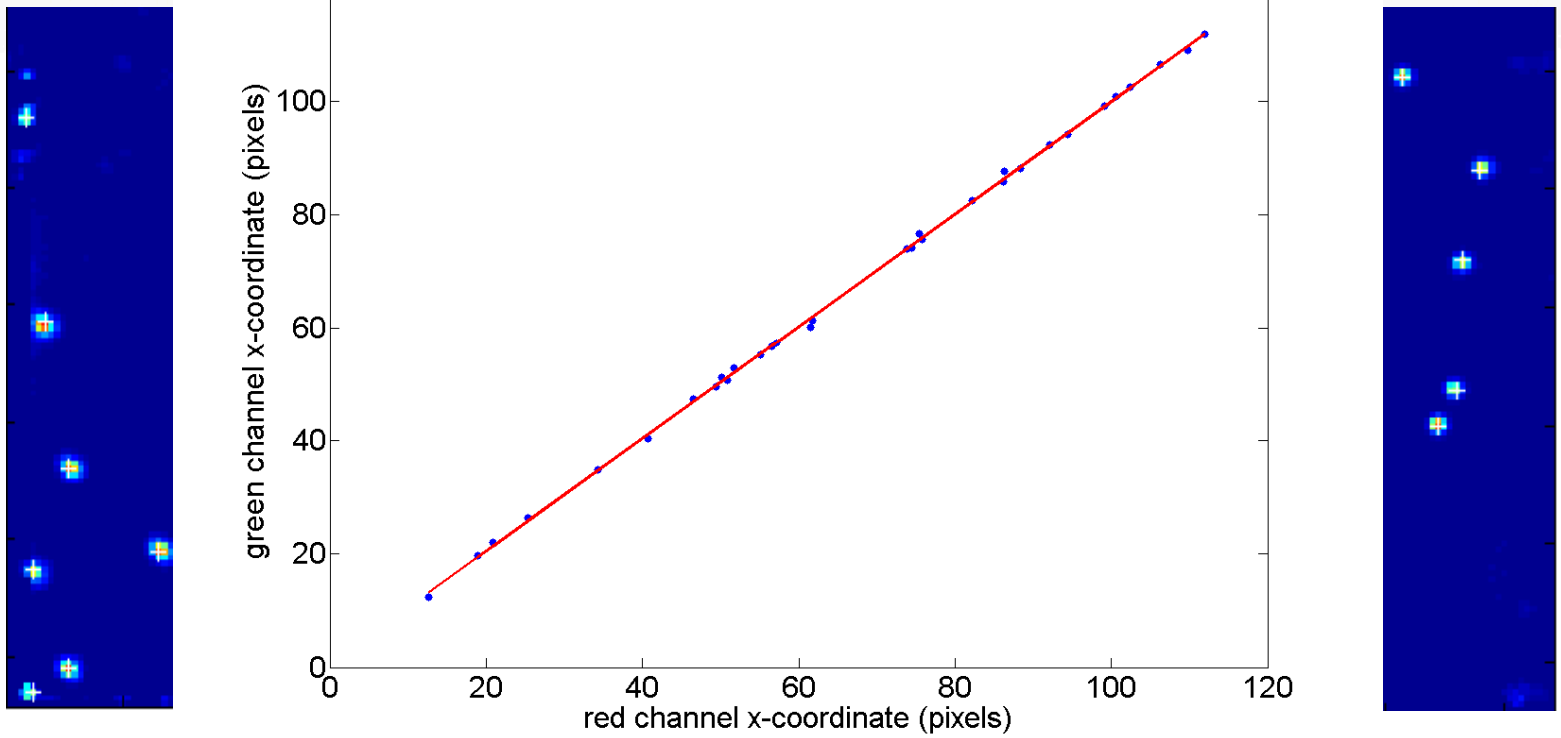
Channel Alignment



- Sub-resolution beads with multiple fluorophors used to register “green” and “red” channels
- Linear transformation results in <50nm error in position (not improved with polynomial fit).

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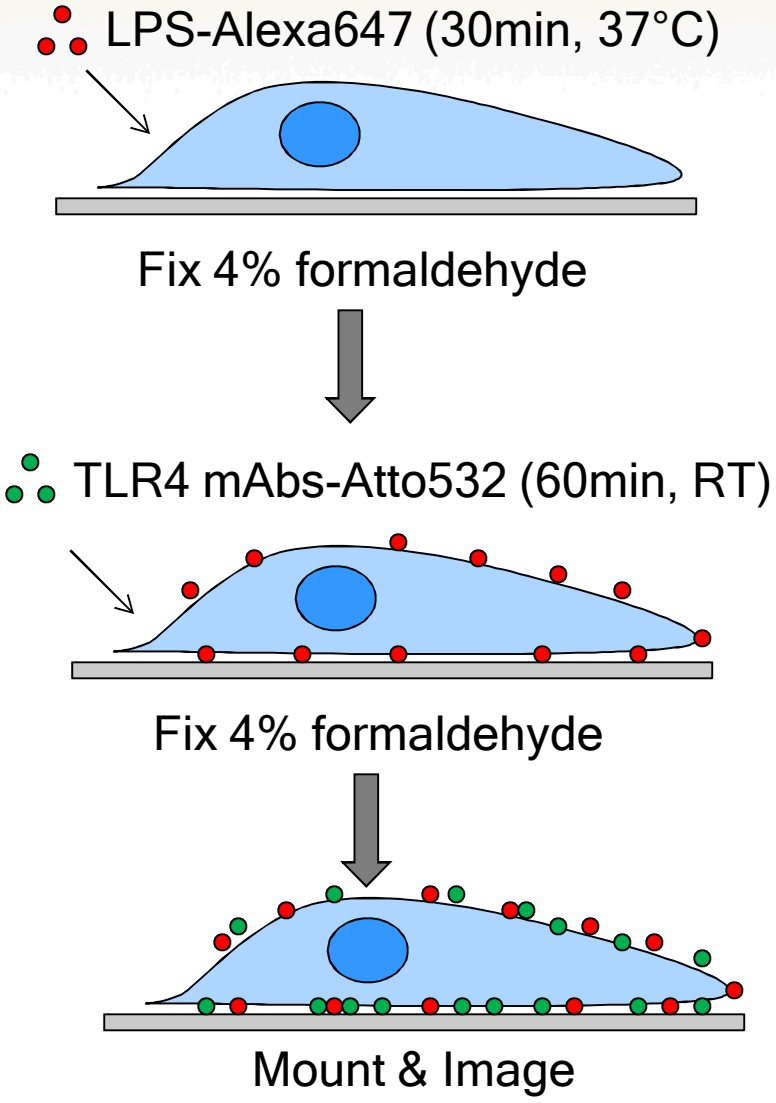
Experimental Details



- Mouse macrophage cells (P388D1) incubated with 100nM *E. coli* or *Y. pestis*-derived LPS for 30 min at 37°C and formaldehyde fixed. LPS are labeled with Alexa Fluor 647-hydrazide via linkage with core-polysaccharide
- TLR4 receptors visualized via 1^0 antibodies labeled with Atto532
- Cells imaged in O_2-scavenging buffer containing β-mercaptothiol

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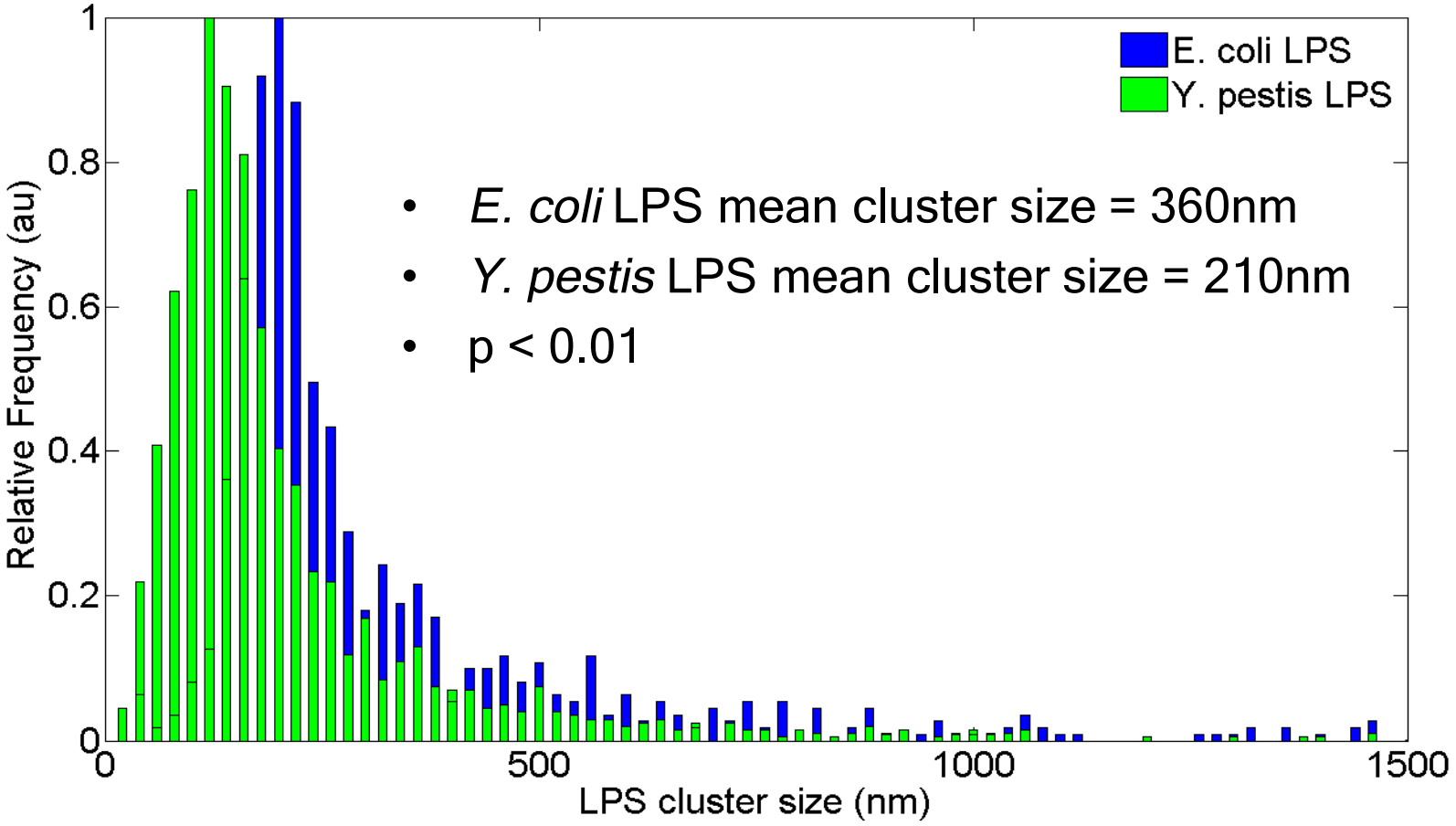
TLR4-induced Clustering of LPS



- *E. coli* LPS mean cluster size = 360nm
- *Y. pestis* LPS mean cluster size = 210nm
- $p < 0.01$

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