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Visualizing Early Immune Response to Bacterial Infection: Reorganization at the Nanoscale

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NNSA National Nuclear Security Administration

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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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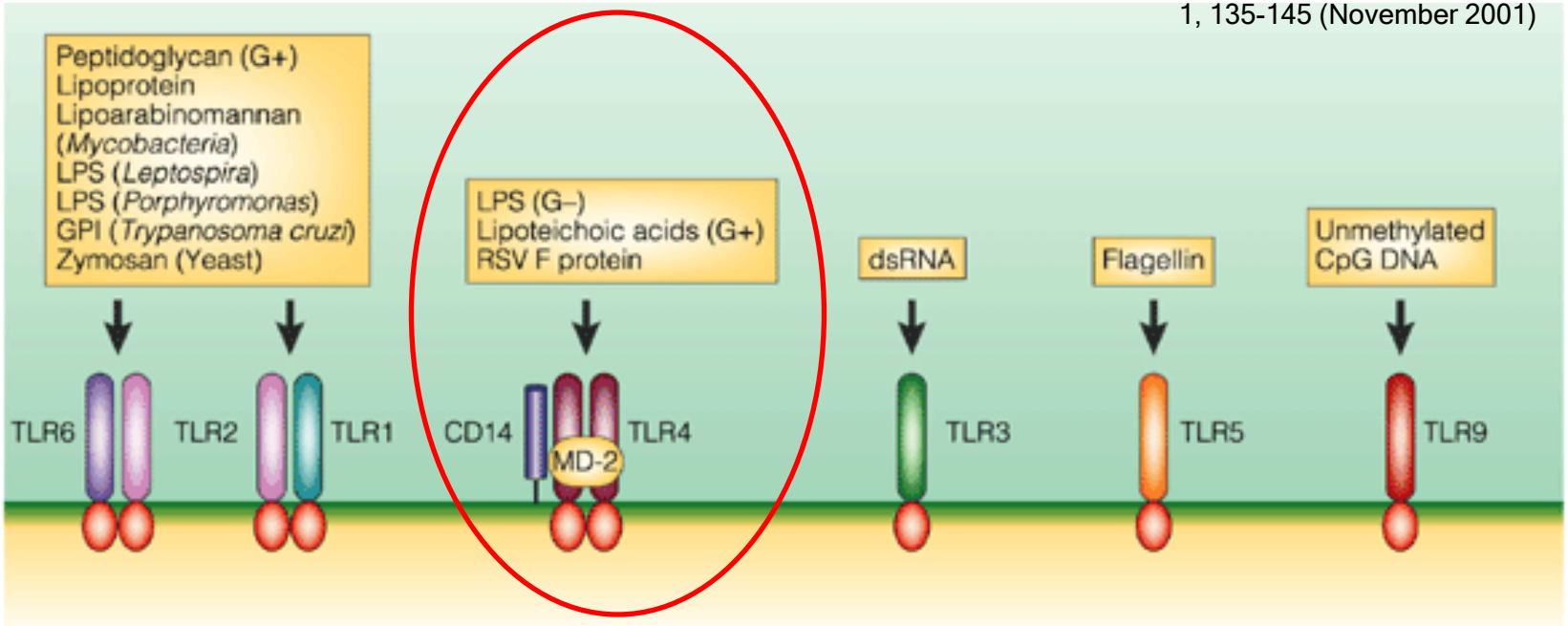
TLRs: Important in Pathogenesis, Biodefense



- Important element in mammalian innate immunity
- TLR4 recognizes LPS, starts signaling cascade
- LPS aided by accessory proteins
- Different chemotypes of LPS generate distinct immune responses

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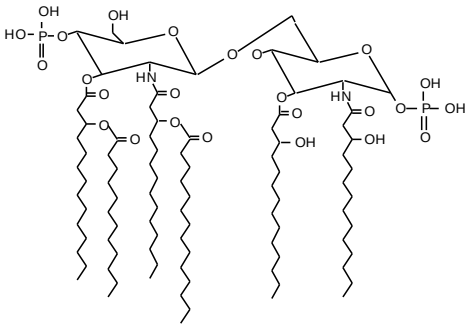
Chemotypes of LPS Exhibit Differential Immune Response

***Escherichia coli* (control)**

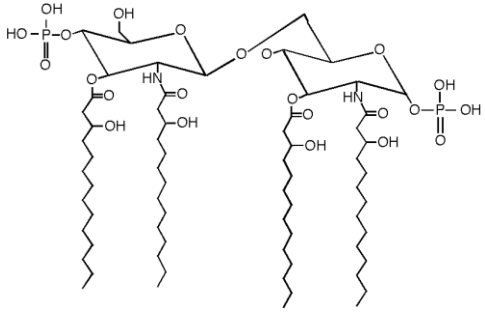
Smooth
O-polysaccharide



Bind Surface
+
↑Stimulatory

***Yersinia pestis* (37°)**

Rough
O-polysaccharide



Bind Surface
+
↓Stimulatory

Differential immune response observed 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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Receptor Clustering can be Necessary Component of Immune Response

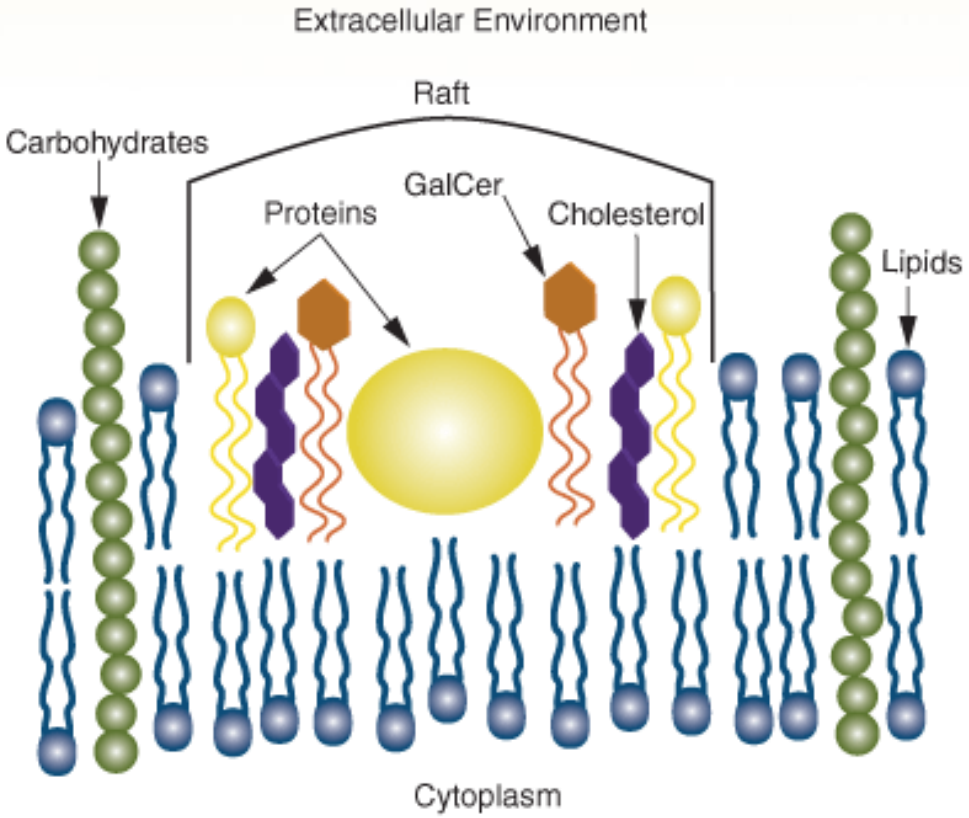

Image Courtesy of Tim Ratto, Lawrence Livermore National Labs

- Domains act as assembly areas
- Aggregation of receptors often follows activation/ligand binding
- Bulk assays have suggested that TLR4 molecules aggregate in lipids rafts within the cell membrane after LPS binding*
- Visualization at the single cell level has been limited by optical diffraction

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

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Hypothesis

TLR4 distribution in the membrane changes upon ligand binding. The spatial distribution

1) *Depends on the chemical properties of the LPS, and*
2) *Affects downstream signaling events and ultimately cellular response*

Optical super-resolution gives us a way to differentiate TLR4 clustering at a much finer scale than conventional imaging.

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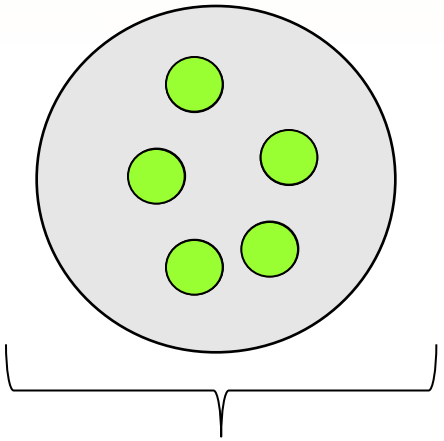
STORM = Subdiffraction Spatial Resolution

Stochastic Optical Reconstruction Microscopy



diffraction-limited spot size

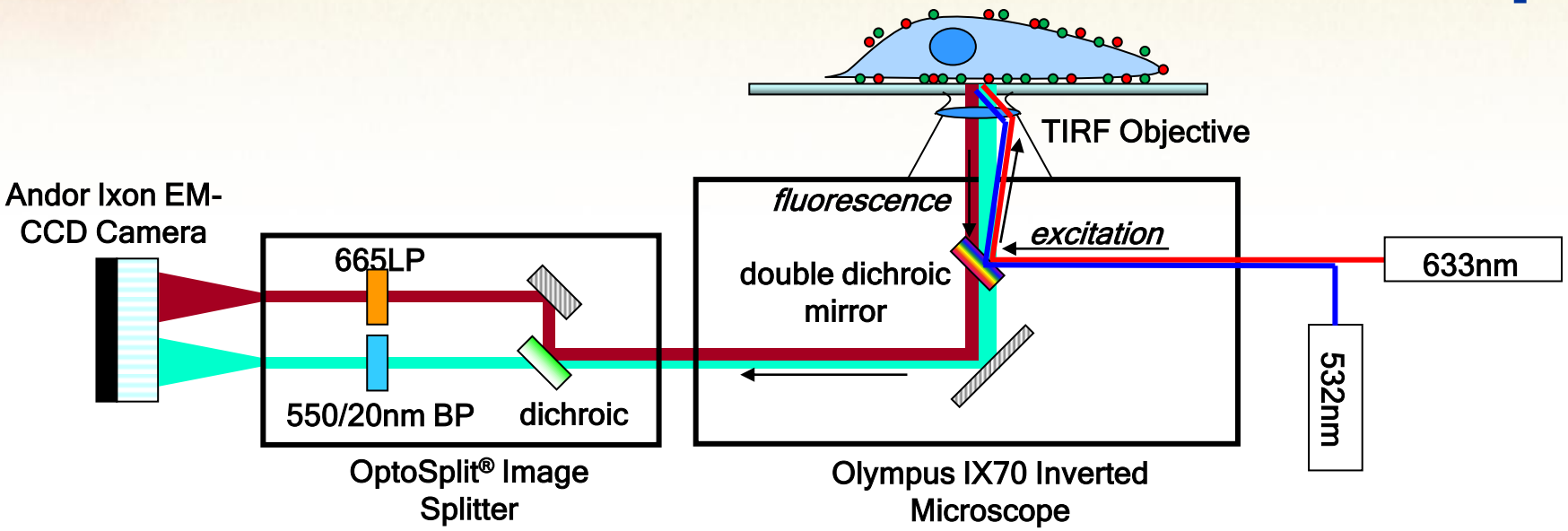
- Assuming <1 fluorophor per diffraction-limited area, it's position can be determined with nanometer precision.

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 stochastic "photoswitching"
  - Only a small subset of fluorophors is visible at any given time.
- Photoswitching for organic dyes can occur in buffer containing small thiol (i.e. BME) and oxygen scavenging system. (dSTORM)

Heilemann, et. al, Angew Chem Int Ed Engl. 47: 6172:6176 (2008)

Multicolor STORM Setup



Unique capabilities:

- Four excitation λ's (405, 488, 532, 633nm), variable angle
- Simultaneous dual-color emission
- Capable of >50fps over 30μm x 30μm FOV

Advantageous in:

- Receptor reorganization
- Nanoparticle-membrane interactions, uptake
  - Engineered NPs
  - Natural NPs - Viral trafficking

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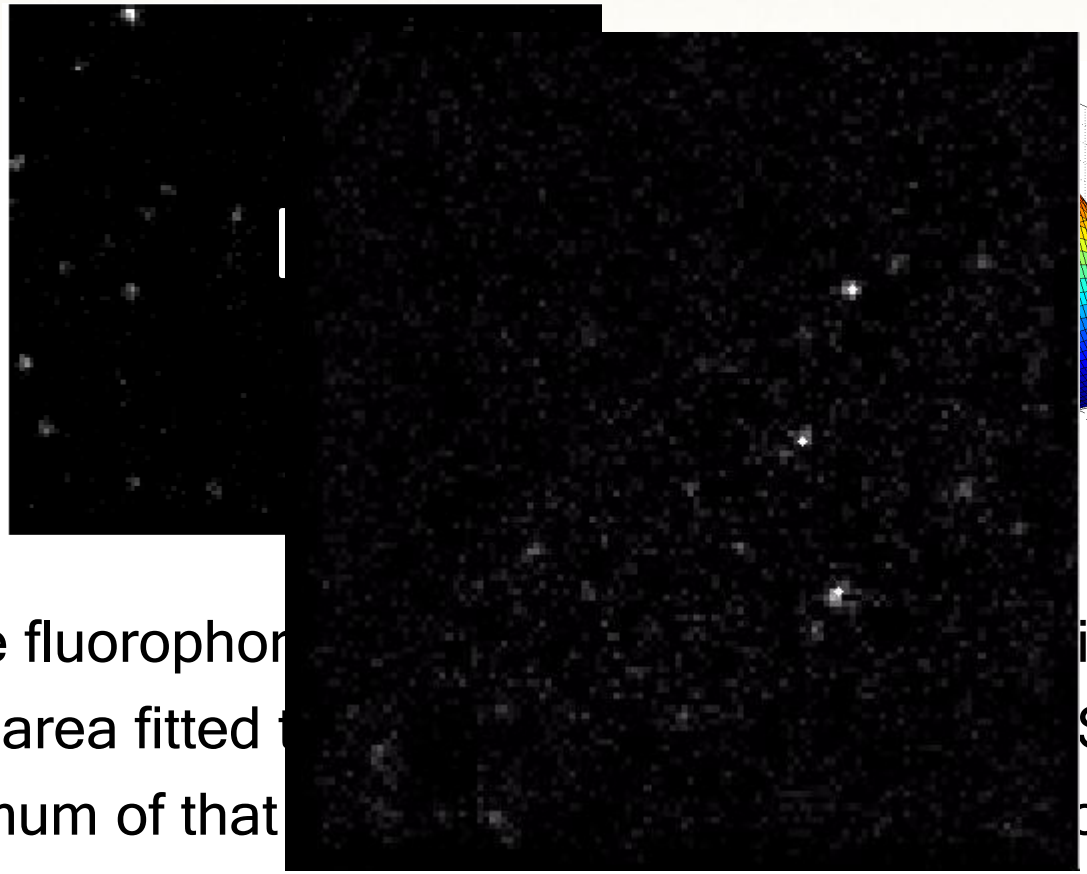
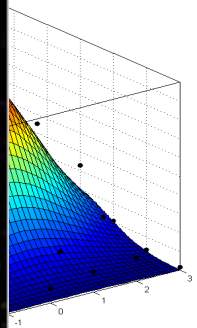
Fluorophor Localization



nm , 44±23nm)



Uncertainty ~ 1/√N
(SNR)

- Single fluorophor ified in each frame
- Local area fitted t SF
- Maximum of that of the fluorophor
- Typically, location fit uncertainty 40-60nm
- Process repeated over 1k-10k frames to build STORM image

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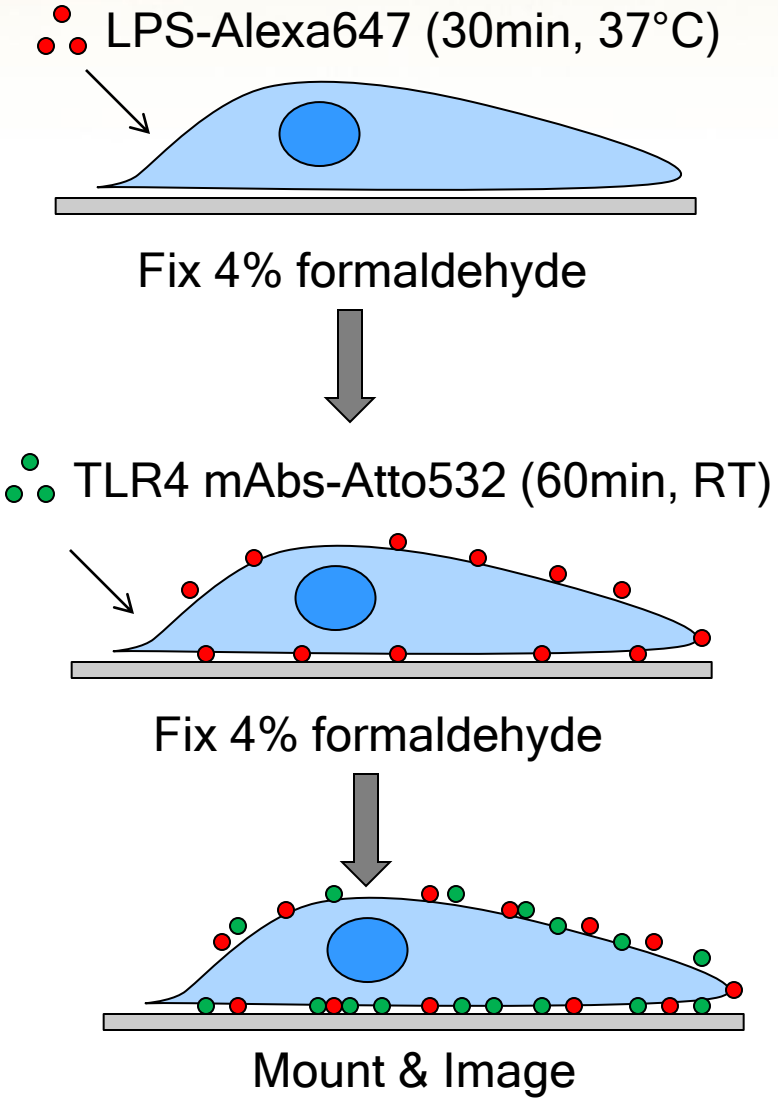
Experimental Design



- 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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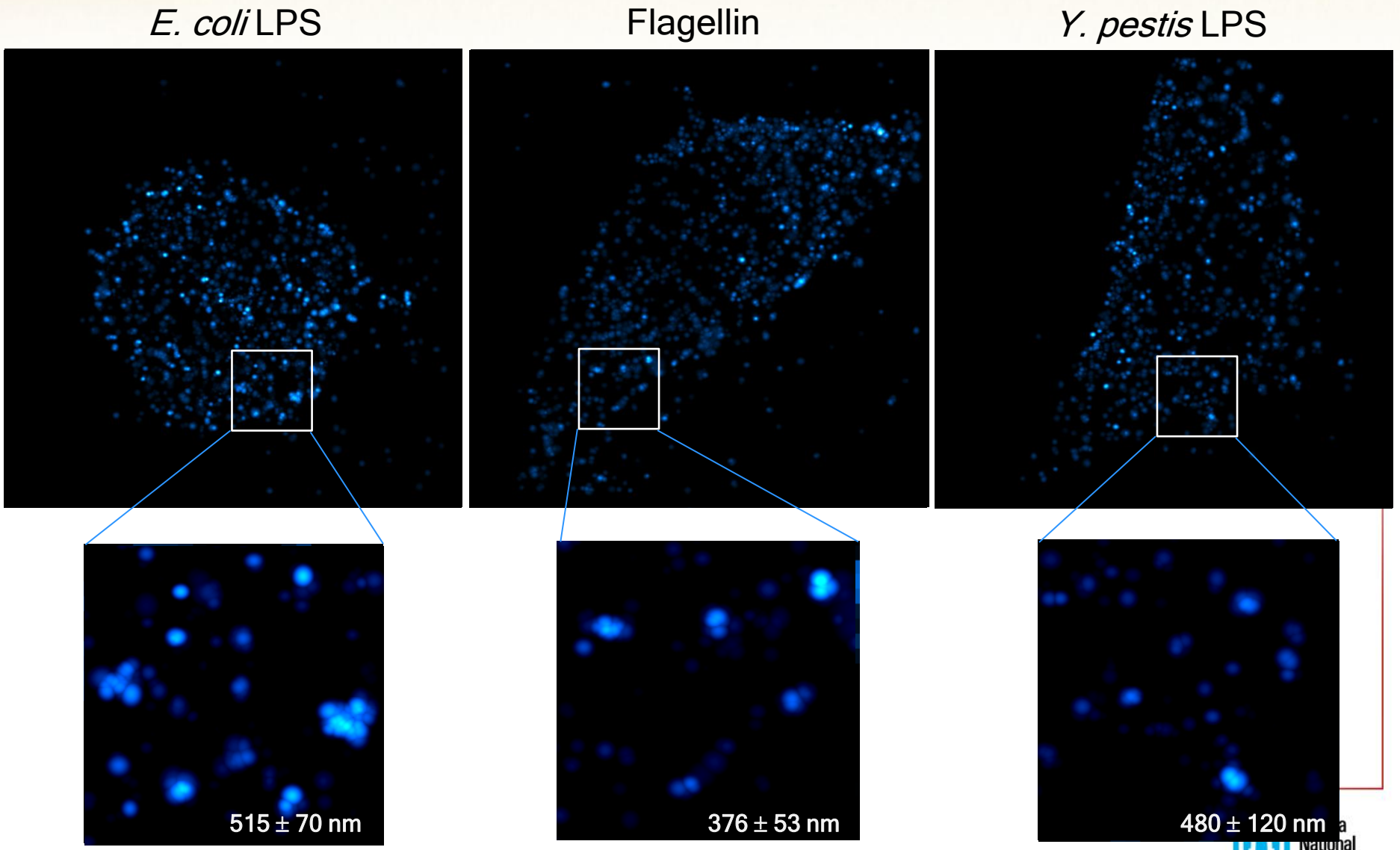
TLR4 Clustering is Specific to LPS



STORM images = diffraction limited (~400 nm)

TIRF images = 10 increased (40-50 nm)

Ripley's K-function Analysis

- K-function is a normalized measure of point clustering
- Complete spatial randomness (CSR)
- Transform to H-function, deviation from CSR at each test radius
- Peaks (or inflection points) in H(r) indicate characteristic cluster sizes

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

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

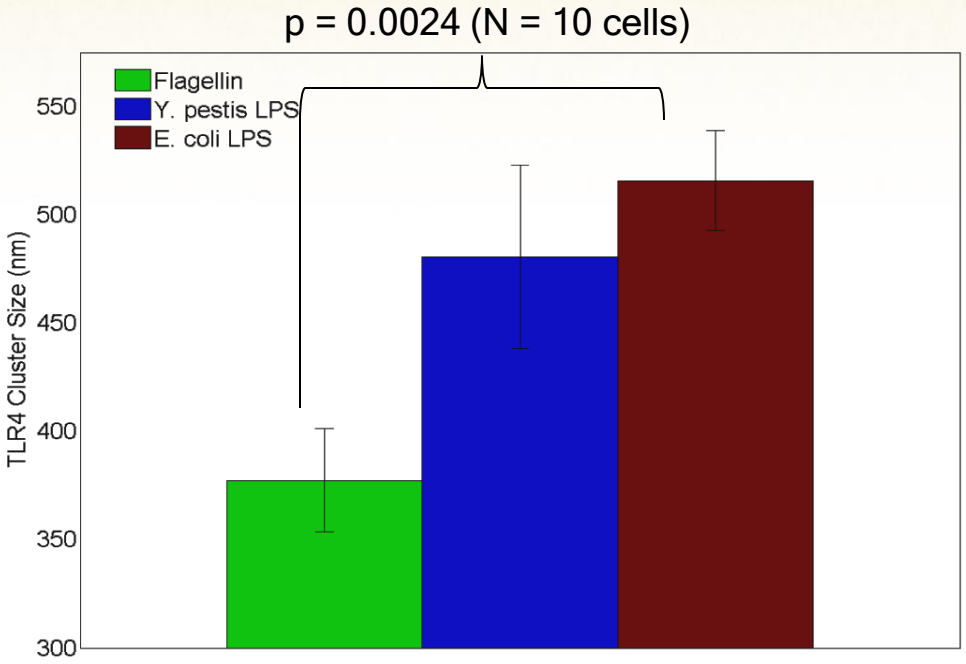
$$H(r) = \sqrt{\frac{K(r)}{\pi}} - r$$

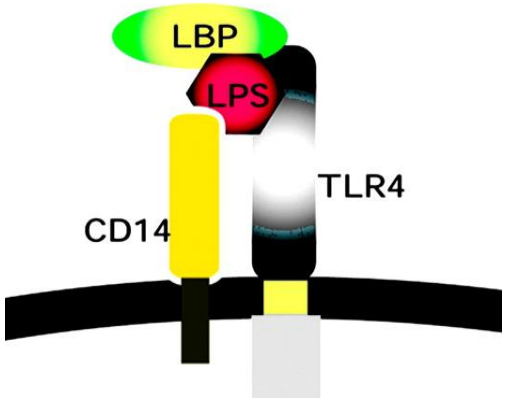
Ripley, B.D. *J. R. Statist. Soc.* B41:368-374 (1979)

Kriskowski, M.A., et al, Biophys. J. **97**(4), 1095-1103, (2009)

Differential TLR4 Clustering is Significant

- 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?

Cho J , Tsichlis P N PNAS 2005;102:2350-2355

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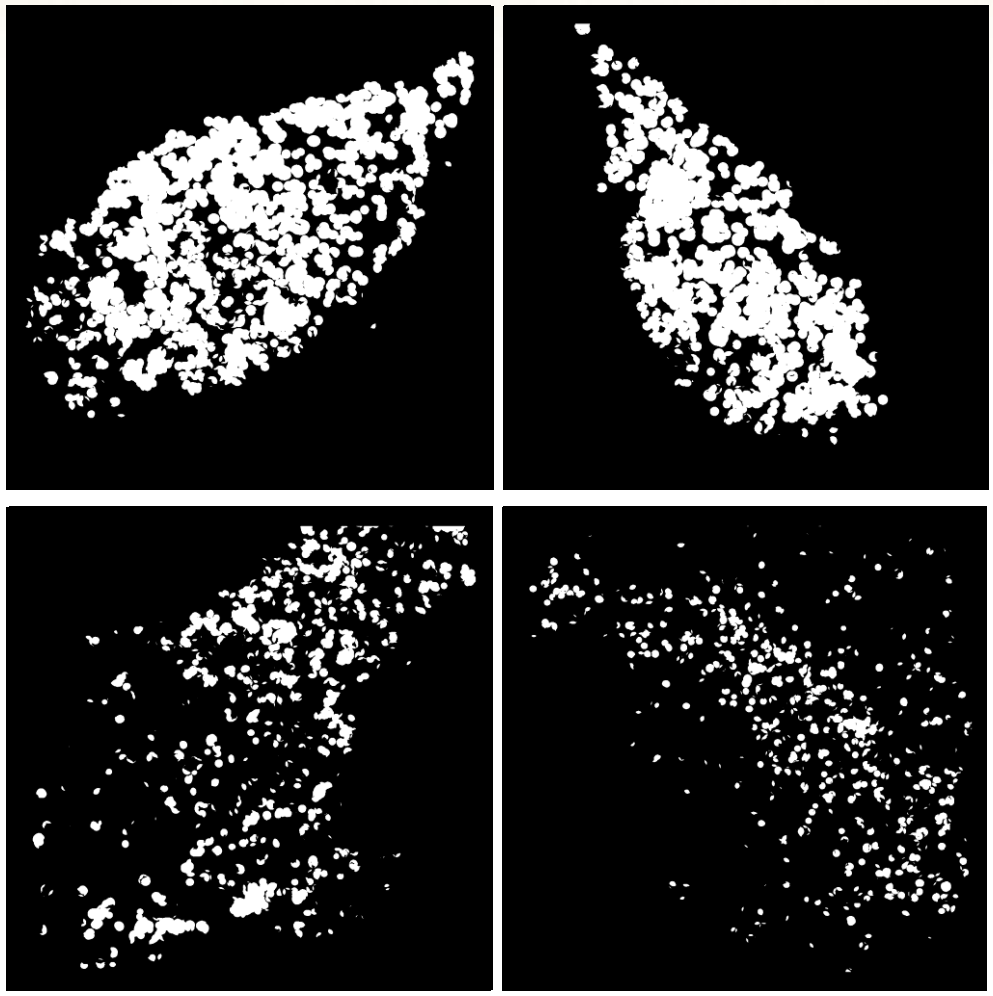
Colocalization of TLR4 & LPS



- 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
  - Custom implementation of Ripley's K-Function

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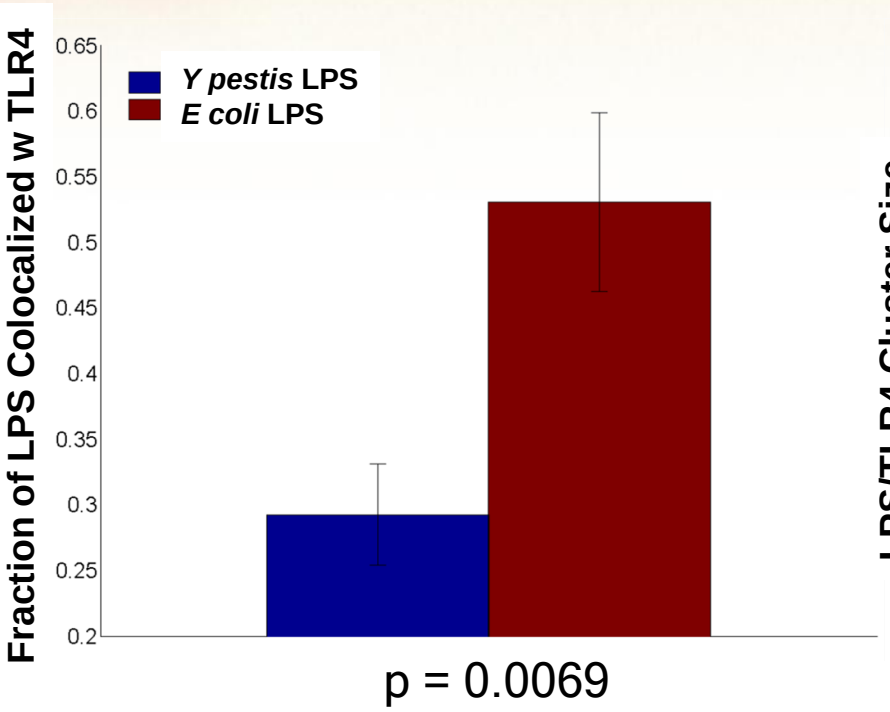
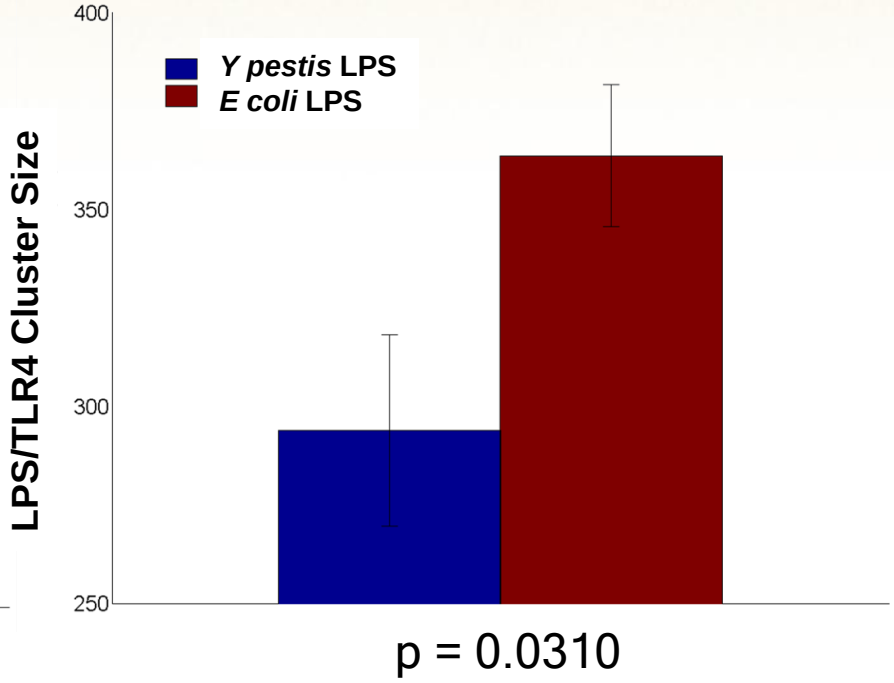
Y. pestis LPS is less Efficient at Recruiting TLR4 into Clustered Domains





- Significantly less co-localization of *Y. pestis* LPS with TLR4 compared to *E. coli LPS*
- Significantly smaller *Y. pestis* LPS-TLR4 clusters than E. coli LPS-TLR4 clusters

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Conclusions

- Visualization of TLR4 and TLR4-LPS distributions in individual, intact macrophage cells
  - *E. coli* LPS produces a significant increase in TLR4 cluster size within 30 minutes, as compared to a non-specific ligand and non-stimulatory control
  - *Y. pestis LPS* exhibits less co-localization with TLR4 and is less able to recruit TLR4 into clusters as compared to *E. coli* LPS → correlated with down-stream signaling response
  - Role of co-factors ?
- Super-Resolution imaging allows for measuring subtle changes that aren't apparent in conventional microscopy
  - Spatial-temporal behavior of multiple components
  - Broad impact in cell signaling research

Acknowledgements

- Dr. Roberto Rebeil (NBACC) for isolation of *Y. pestis* LPS

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