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Super-Resolution Microscopy Reveals Protein Spatial Reorganization in Early Innate Immune Responses

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Motivation

- Traditional optical imaging is limited to 200-400nm resolution
- Many biological processes operate at an order of magnitude below this
- Currently, optical super-resolution techniques are approaching EM resolutions and can image intact/live cells
- **We have applied STORM imaging to monitor TLR4 receptor organization and co-localization with lipopolysaccharides (LPS) on the cell membrane with better than 40nm resolution**

Outline

Background & Methods

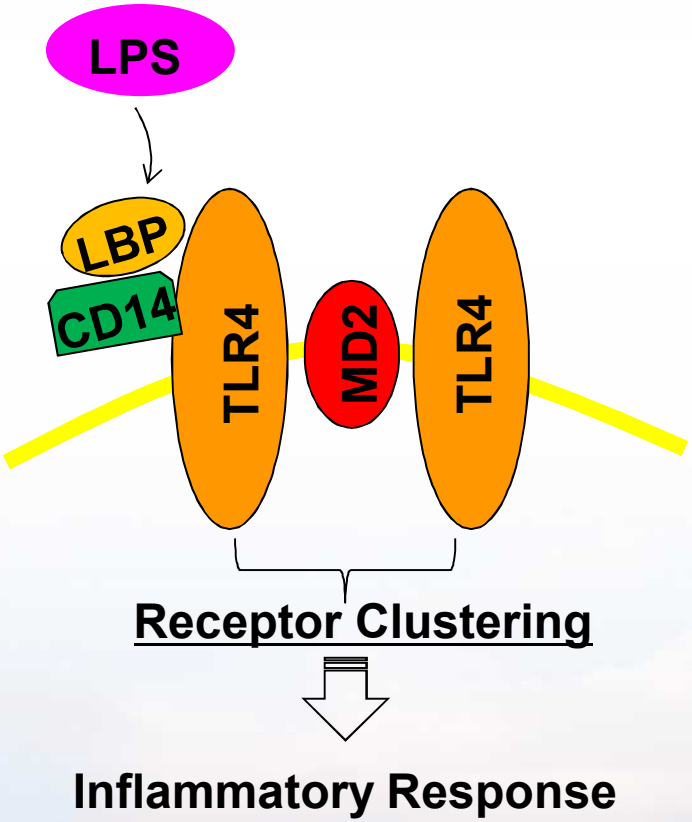
- TLR4 signaling and the innate immune system
- STORM imaging, concepts and methodology

Results

- Initial validation using quantum dots
- TLR4 receptor clustering in macrophage cells
- Dual-Color STORM of TLR4 and LPS

Conclusions/Future Work

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).

Receptor Clustering

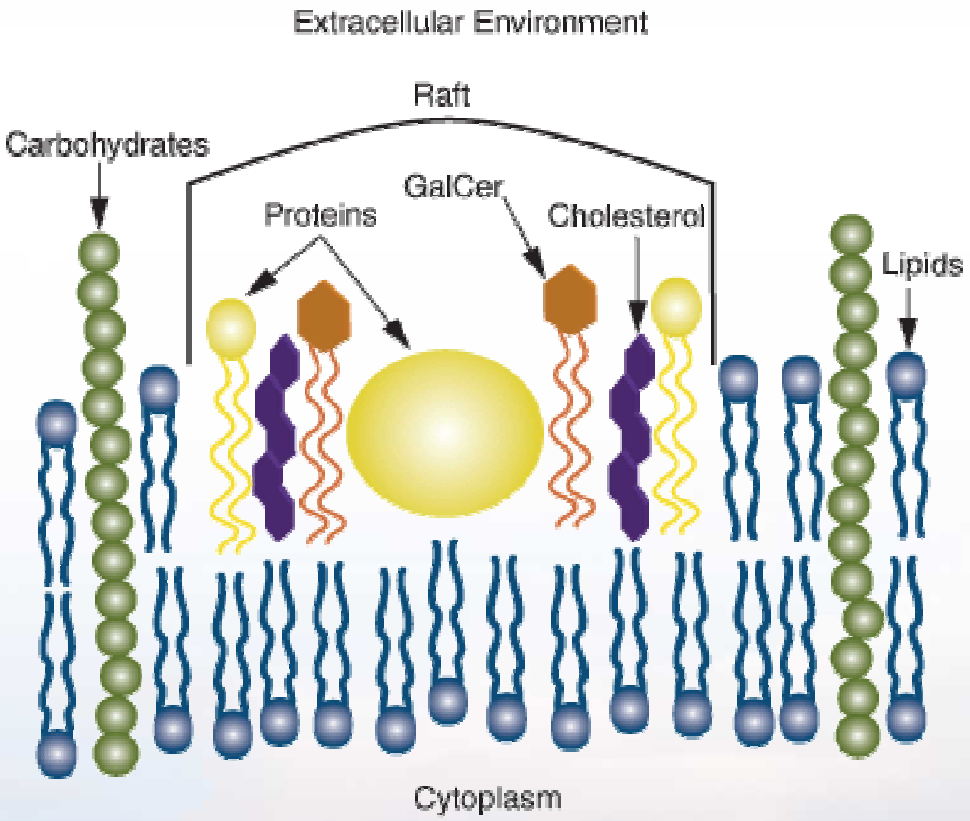
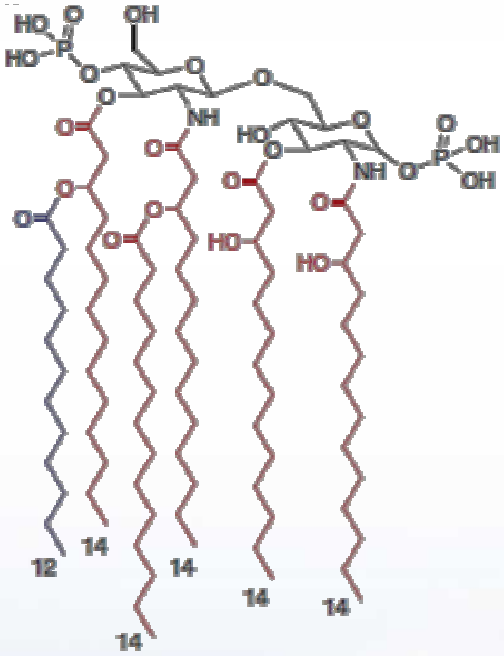


Image Courtesy of Tim Ratto, Lawrence Livermore National Labs

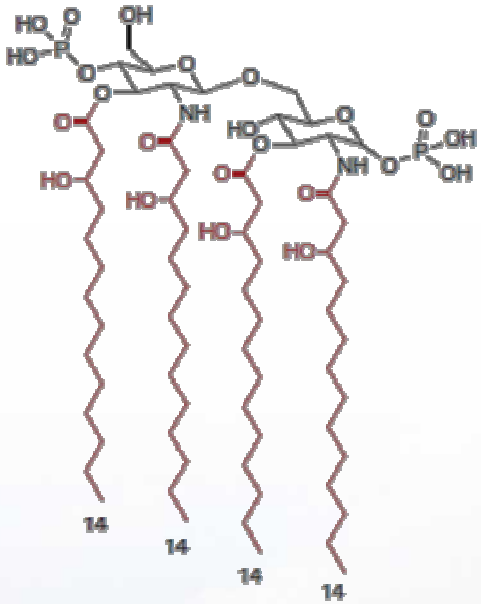
- Domains within cell membrane act as concentration points and assembly areas for many receptors
- Aggregation of receptors often follows activation/ligand binding
- Bulk assays have suggested that TLR4 molecules aggregate in lipids rafts within the cell membrane after LPS binding

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

Unanswered questions



E. coli



Y. Pestis

Miller, et. al, *Nat. Rev. Micro.*, **3**:36-46

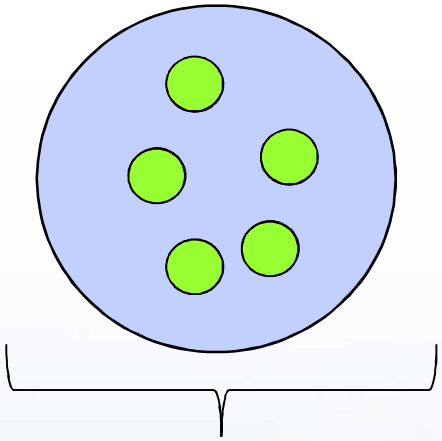
- LPS from *E. coli* produces an immune response
- LPS from *Y.* pestis (plague) does not

- Are there clues in the nano-scale arrangement of this signaling system that will give further insight?

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

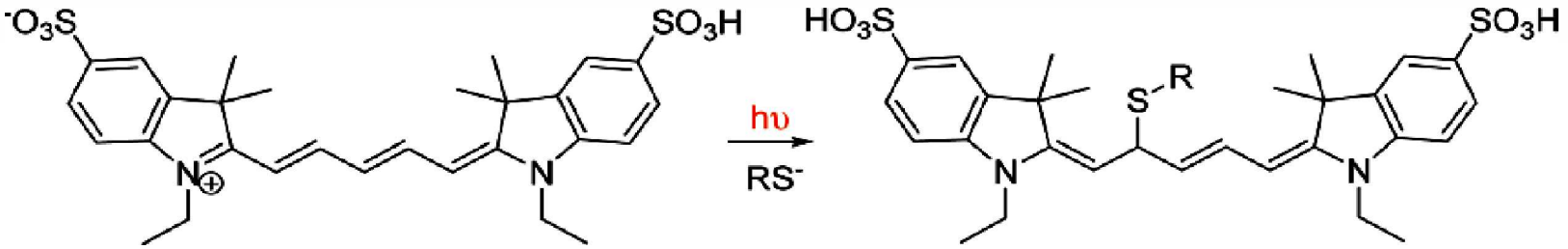


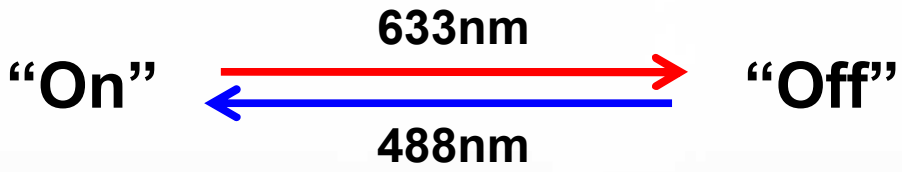
~300nm diffraction-limited spot size

- **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 subset of fluorophors is visible at any given time**
- **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)

“Direct” Photoswitching





- Cyanine-based dyes have been shown to switch between “on” and “off” states in the presence of small thiol-containing molecules and an oxygen scavenging system
- Process is photon-dependent, and the rate can be adjusted via relative intensities of a probe (i.e. 633nm) and activation (i.e. UV-488nm) beam

Dempsey, et. al, *J. Am. Chem. Soc.*, 131(51): 18192–18193 (2009)

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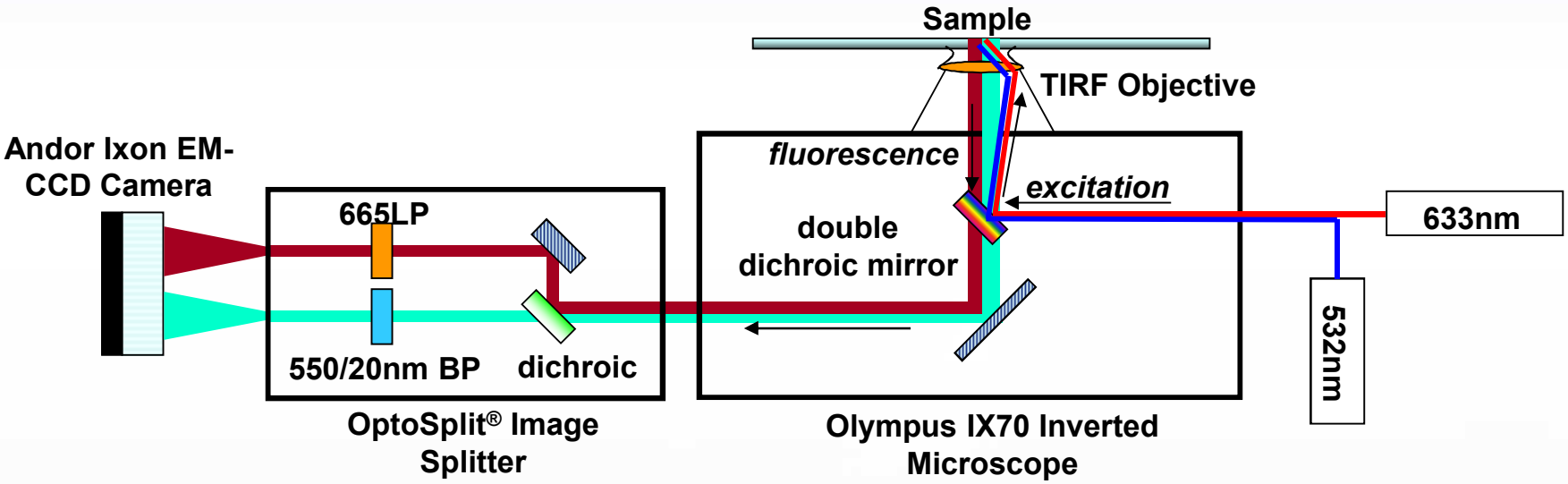
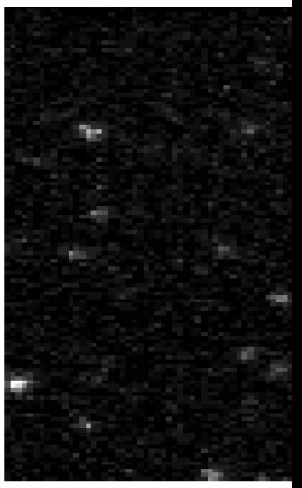
Imaging Setup



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

Determining Localization

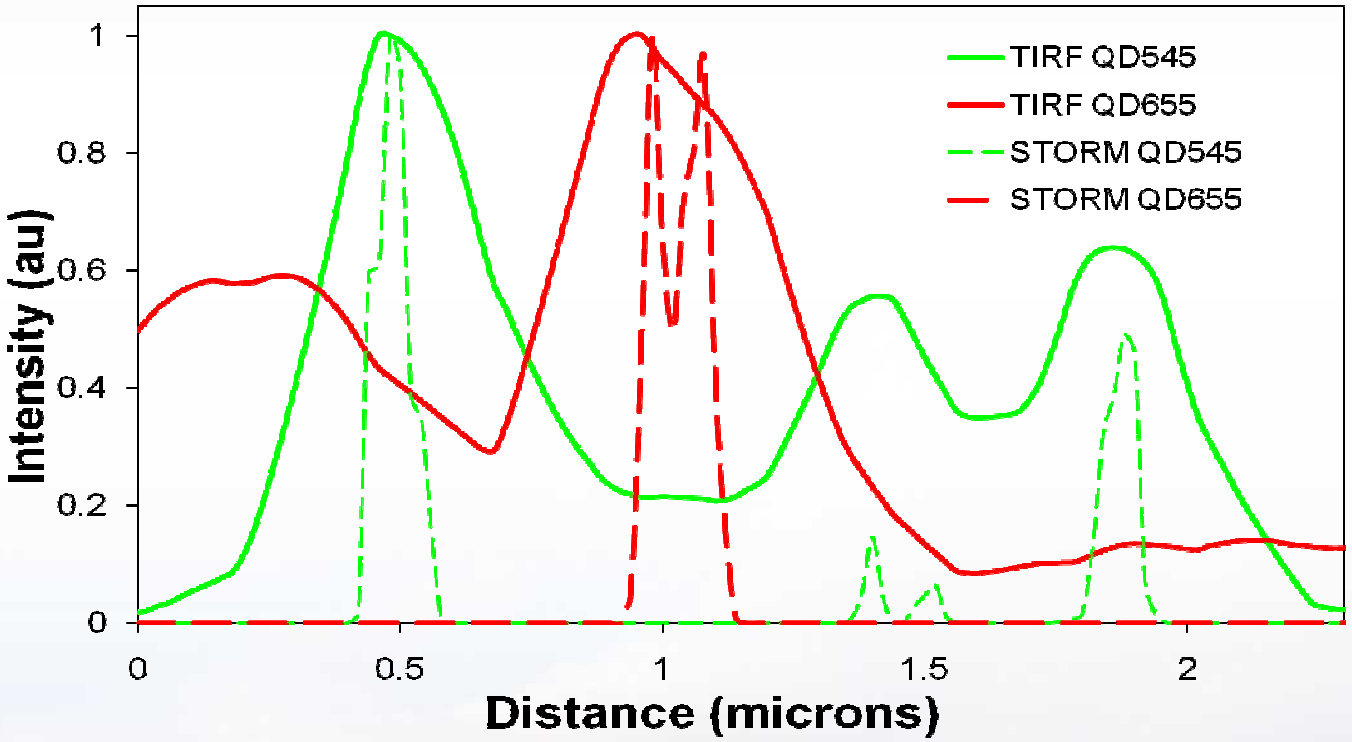


4±23nm)

ncertainty ~ 1/√N
(SNR)

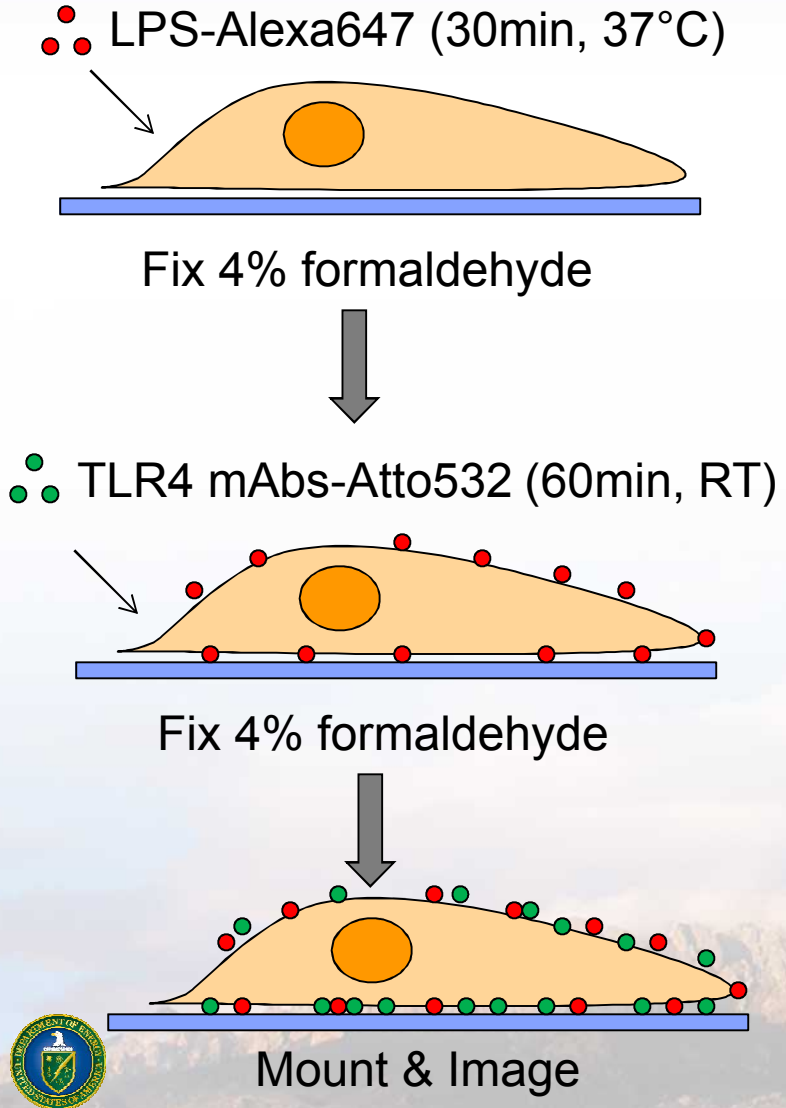
- Single Fluo in each frame
- Local area f mation of PSF
- Maximum o e fluorophor
- Typically, fit for location
- Process rep M image

Multi-Color Validation with Quantum Dots



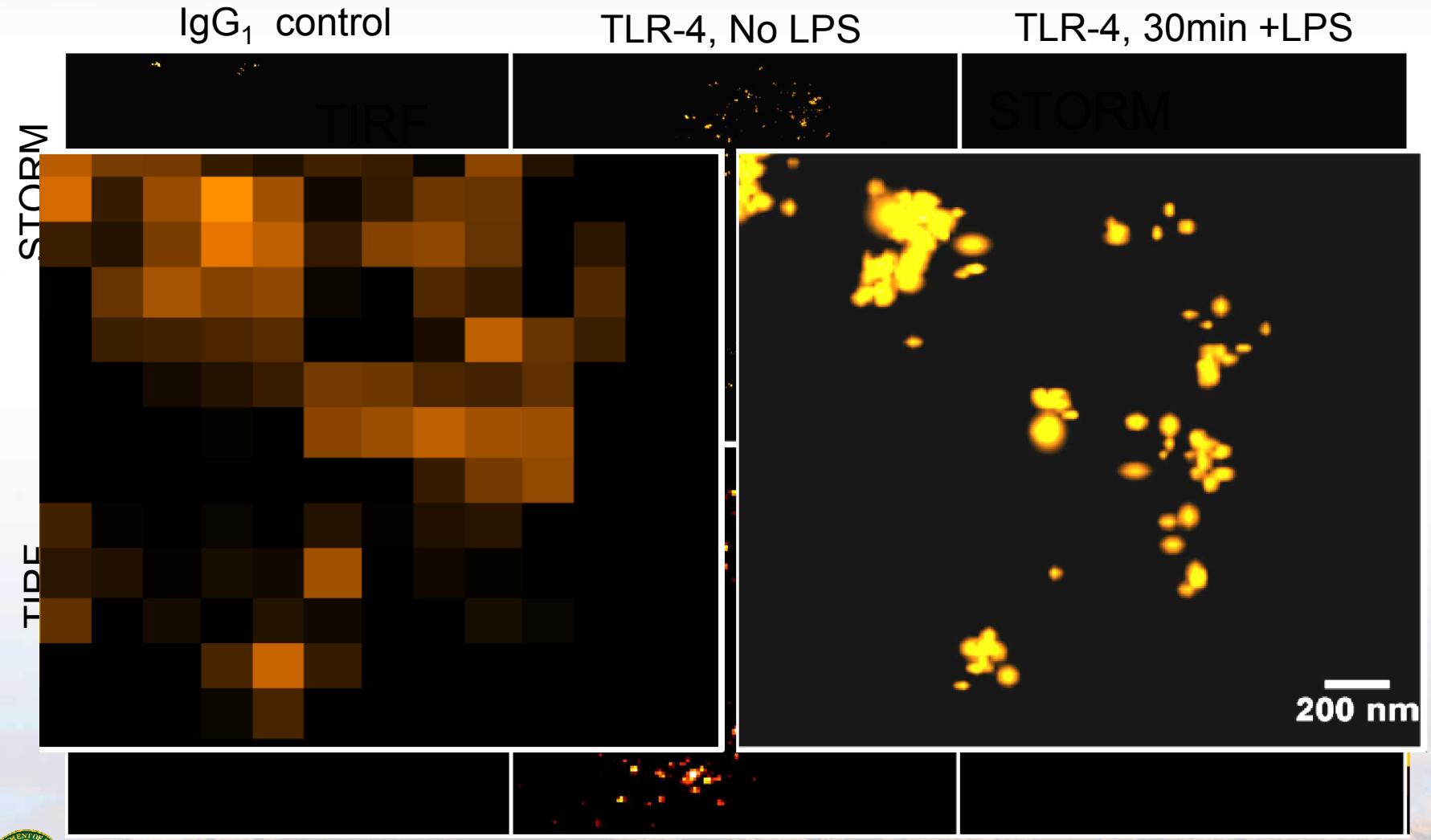
- FWHM <40nm in STORM vs. 400-500nm for conventional imaging

Cell Imaging

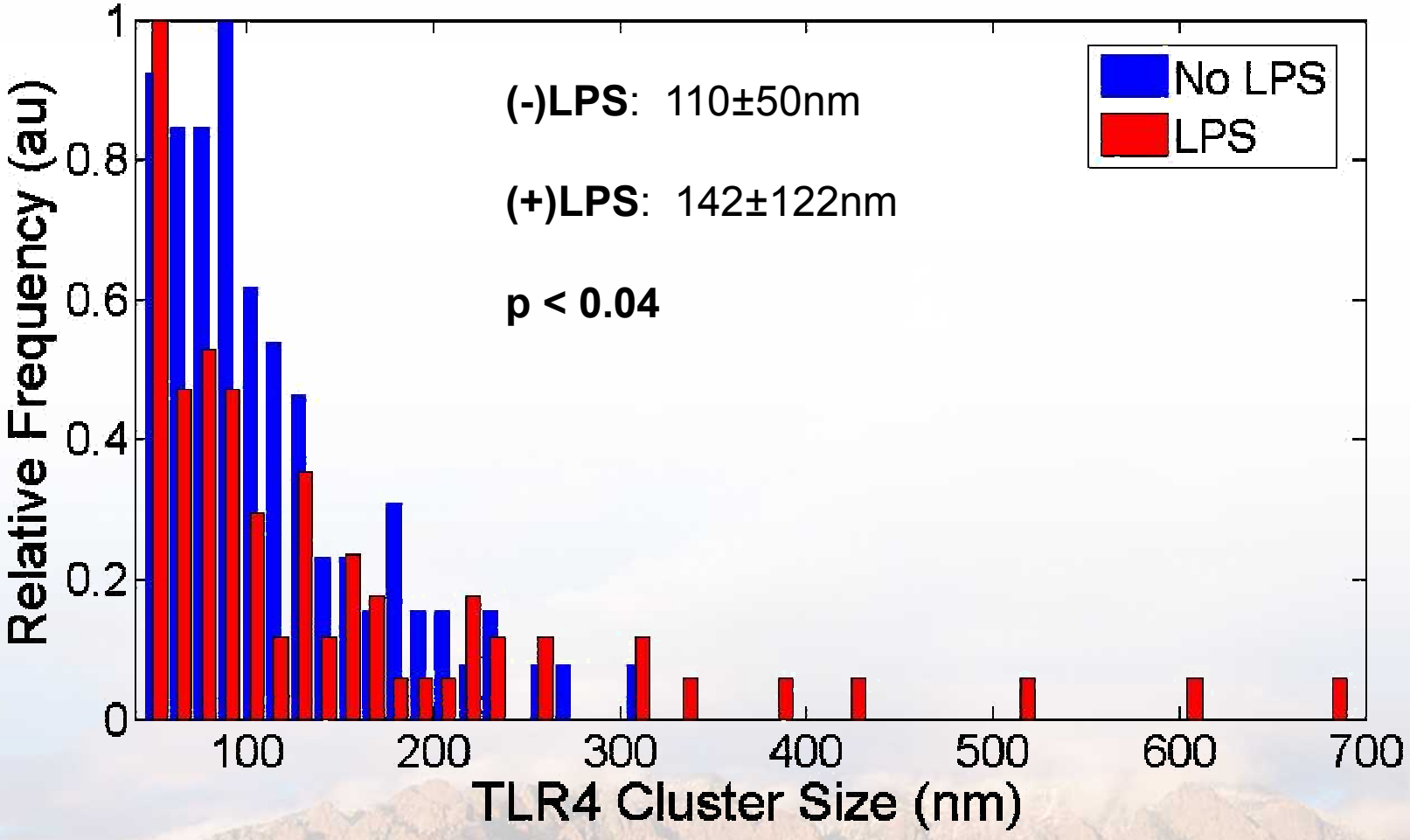


- Mouse macrophage cells (P388D1) incubated with 100nM *E. coli* or *Y. pestis*-derived LPS for 30 min at 37°C and formaldehyde fixed. LPS are labeled with Alexa Fluor 647-hydrazide via linkage with core-polysaccharide
- TLR4 receptors visualized via 1^0 antibodies labeled with Atto532
- Cells imaged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

Imaging TLR4 Receptors

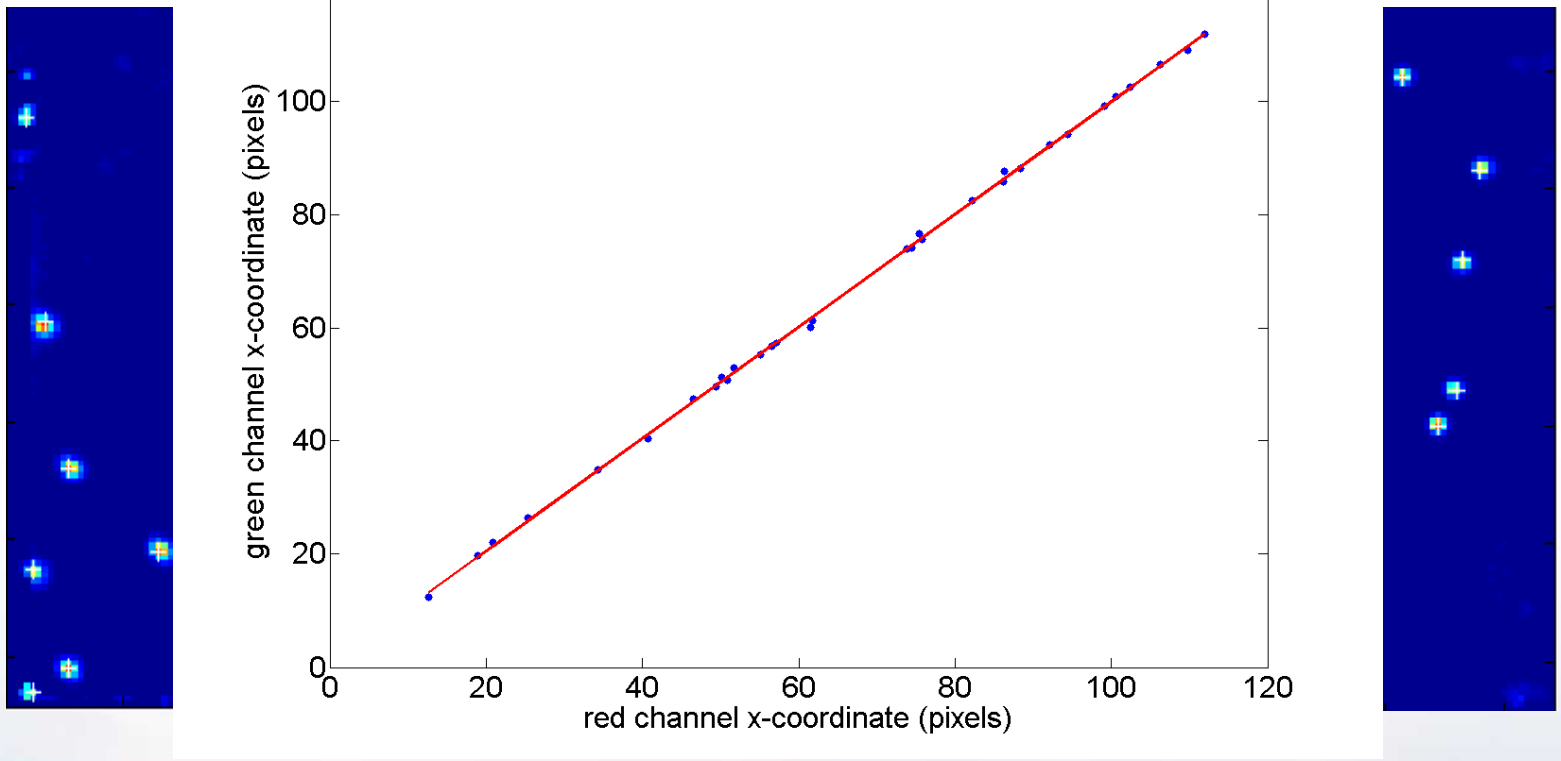


TLR4 Cluster Size



- Differences not apparent in TIRF (p = 0.7)

Dual-Color STORM

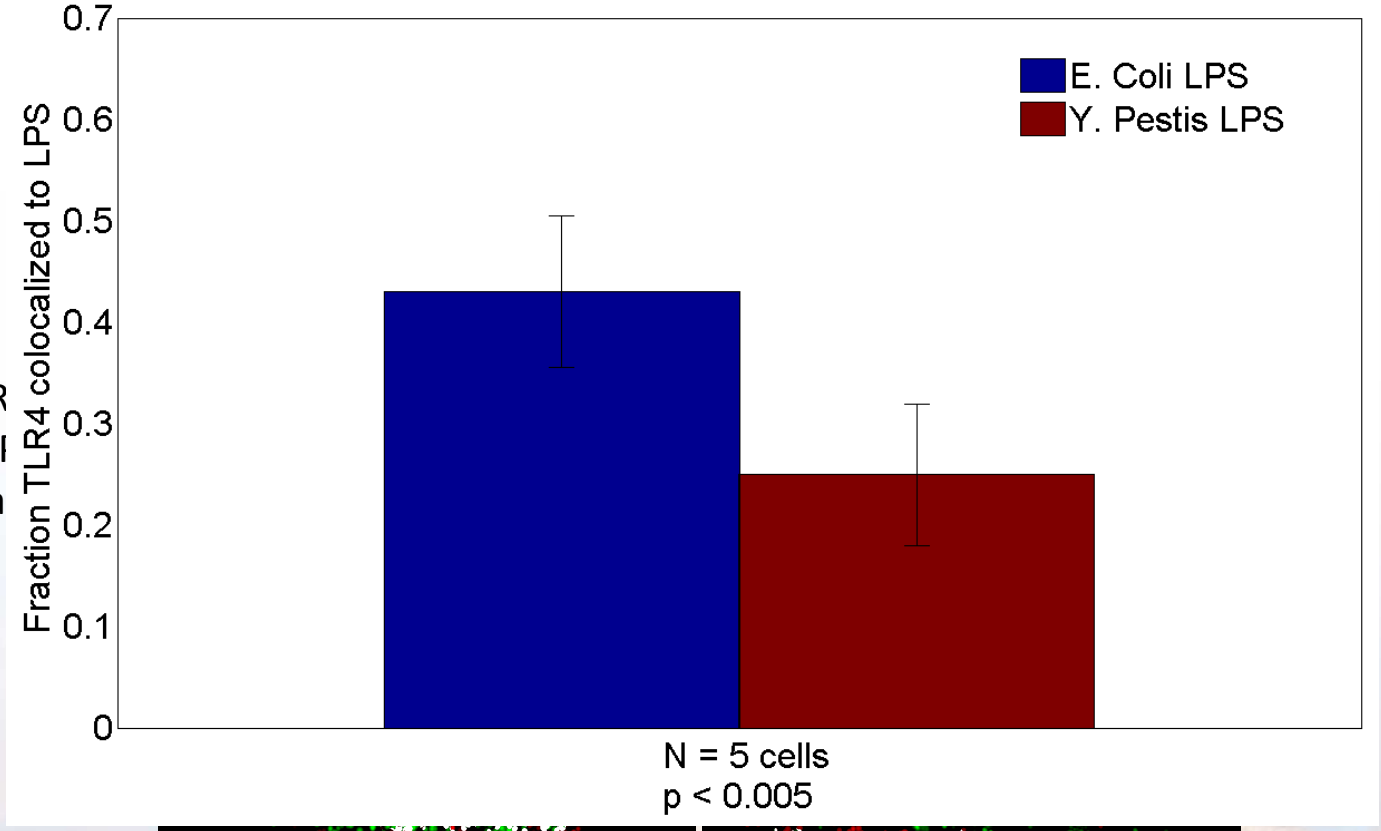


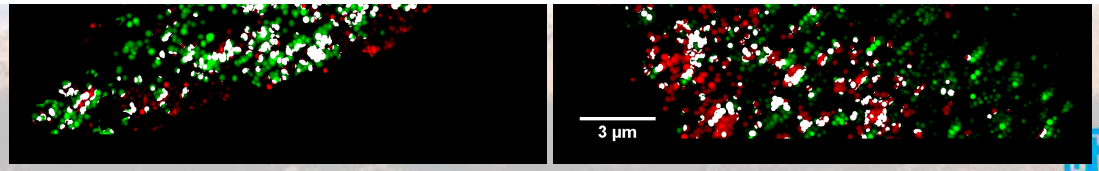
- Sub-resolution beads with multiple fluorophors used to register “green” and “red” channels
- Linear transformation results in <50nm error in position (not improved with polynomial fit).

TLR4 & LPS Co-localization

E. coli LPS | *Y. pestis* LPS

Red = TLR
Green = LI
White = coloca





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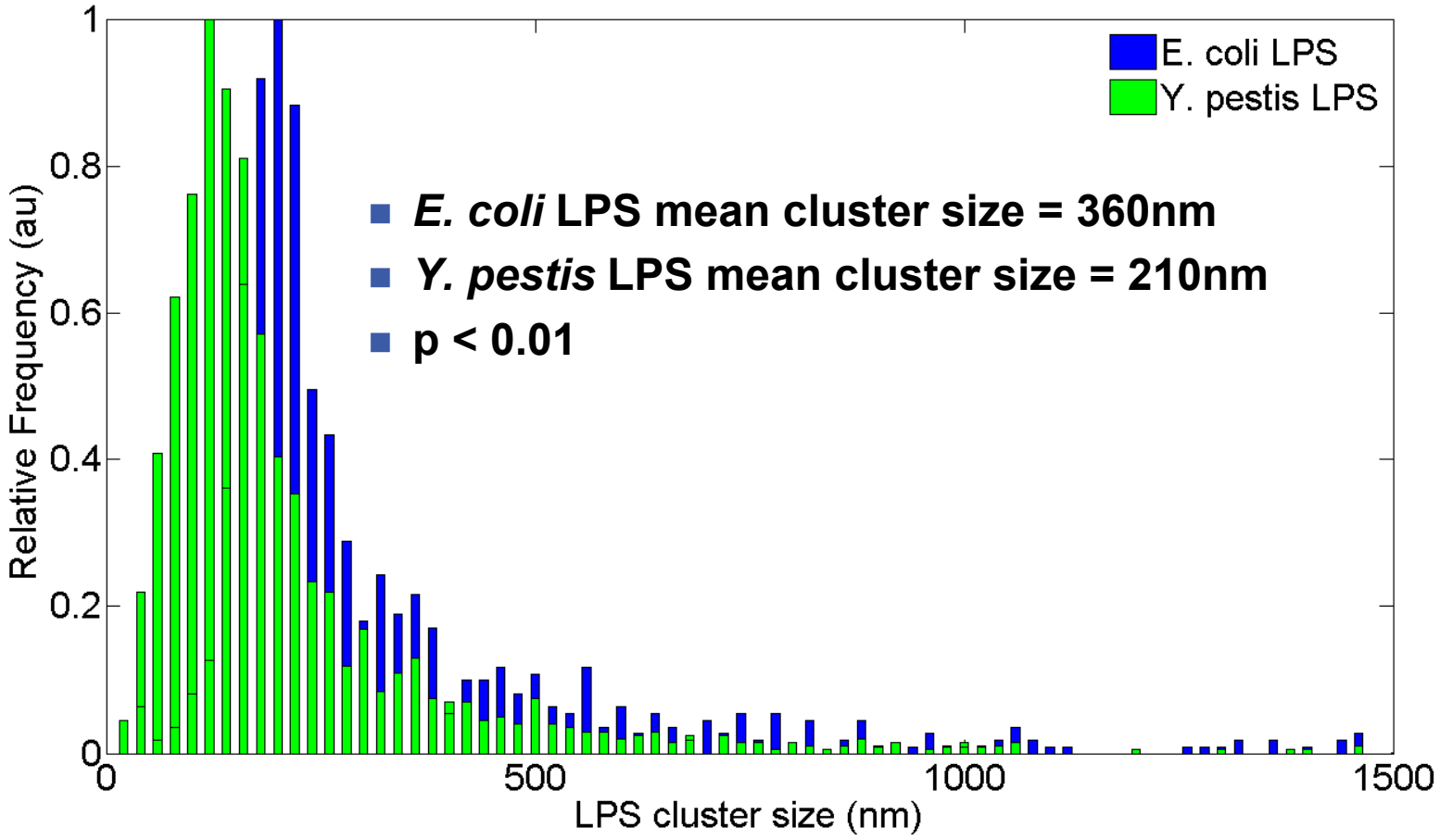
TLR4-induced clustering of LPS



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

Conclusions

- Direct photoswitching-based STORM gives more than an order of magnitude increase in effective resolution (40nm vs. 400nm)
- Challenge with LPS produces a significant increase in TLR4 cluster size within 30 minutes
- 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*
- Dual-color STORM imaging allows us to perform multiplexed measurements of receptor/ligand organization at the nanoscale

Acknowledgements

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  - The National Institutes of Health through the NIH Director's New Innovator Award Program, 1-DP2-OD006673-01
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