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Characterization of Differential Toll-Like Receptor Responses below the Optical Diffraction Limit**

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Abstract

Many membrane receptors are recruited to specific cell surface domains to form nanoscale clusters upon ligand activation. This step appears to be necessary to initiate signaling, including pathways in innate immune system activation. However, virulent pathogens such as *Yersinia pestis* (the causative agent of plague) are known to evade innate immune detection, in contrast to similar microbes (such as *E. coli*) that elicit a robust response. This disparity has been partly attributed to the structure of lipopolysaccharides (LPS) on the bacterial cell wall, which are recognized by the innate immune receptor TLR4. As such, we hypothesized that nanoscale differences would exist between the spatial clustering of TLR4 upon binding of LPS derived from *Y. pestis* and *E. coli*. Although optical imaging can provide exquisite details of the spatial organization of biomolecules, there is a mismatch between the scale at which receptor clustering occurs (<300 nm) and the optical diffraction limit (>400 nm). The last decade has seen the emergence of super-resolution imaging methods that effectively break the optical diffraction barrier to yield truly nanoscale information in intact biological samples. This study reports the first visualizations of TLR4 distributions on intact cells at image resolutions of <30 nm using a novel, dual-color stochastic optical reconstruction microscopy (STORM) technique. This methodology permits distinction between receptors containing bound LPS from those without at the nanoscale. Importantly, we also show that LPS derived from immuno-stimulatory bacteria resulted in significantly higher LPS-TLR4 cluster sizes and a nearly two-fold greater ligand/receptor colocalization as compared to immuno-evading LPS.

Keywords

super-resolution imaging; toll-like receptor; TLR4; STORM; receptor clustering

1. Introduction

Spatial recruitment and clustering of cellular receptors into lipid rafts or other membrane domains appears to be required for a wide variety of signaling pathways to proceed, including a number of vital immune responses [1-3]. Among these, toll-like receptors (TLRs) are thought to exhibit such behavior after exposure to certain pathogen-associated molecular patterns (PAMPs) [4]. TLRs play a vital role as pattern recognition receptors (PRRs) in mammalian innate immunity by initiating a number of rapid, cell-mediated responses including microbicide and cytokine release in response to infection. The pathway incorporating the TLR4 member of this receptor family has been particularly well characterized, and is triggered by binding of lipopolysaccharides (LPS) present in the outer membrane of Gram-negative bacteria [5]. Triantafilou et al. have shown that LPS-induced

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dimerization and subsequent higher-order clustering of TLR4 into lipid rafts correlate with overall inflammatory response [4, 6-7]. Further, it was shown that hexaacylated LPS from *E. coli* resulted in an apparent increase in clustering of receptors within membrane lipid raft domains; however, a non-stimulatory lipid A analog produced no such increase in raft domain associations, as well as a corresponding lack of increased cytokine production [7]. Nonetheless, receptor clustering events arise at length scales within the 10-500 nm regime, complicating efforts to quantitatively characterize these phenomena at the single cell level.

Difficulties in measuring receptor recruitment exist in part due to the mismatch between the scale at which protein clustering occurs, and the imaging tools historically available to reliably measure their spatial characteristics in intact cells. Optical molecular imaging can reveal exquisite details of biomolecular spatial distributions in intact cells [8-9]; however, the fundamental limit of diffraction prevents conventional light microscopy from achieving truly nano-scale imaging. Although techniques such as total internal reflection fluorescence (TIRF) [10], Förster resonance energy transfer (FRET) [11], image correlation spectroscopies (ICS) [12], as well as nanoparticle-based methods [13-14] can provide indirect information below the diffraction limit, none of these approaches can directly visualize protein distributions at resolutions below *ca.* 200nm. To address this limitation, the past decade has seen the emergence of a number of “super-resolution” optical microscopy methods that are capable of resolutions in the range of 10-50nm, while retaining the high molecular contrast and minimal sample manipulations necessary in conventional fluorescence imaging [15]. Among these include photo-activation localization microscopy (PALM) [16], and stochastic optical reconstruction microscopy (STORM) [17], often collectively grouped into a “stochastic photoswitching” or “localization based” superresolution category [18]. The principle behind these techniques involves optimizing fluorescent probes and imaging conditions such that only a small, random subset of the total fluorophore-tagged biomolecules in a sample is visible at a given time. As such, the relatively few molecules emitting photons at each time point are in general well-separated from each other by at least a diffraction-limited distance. Because of this relatively large spatial separation, an isolated fluorophore’s position can be computationally estimated with sub-diffraction precision, typically less than 50nm using commercial instrumentation [19]. By repeatedly imaging random, isolated biomolecules over many cycles, it permits the computational localization of nearly every biomolecule of interest in the sample, but at a much higher precision than would be possible using conventional optical methods. This approach opens the door to examining receptor clustering behavior at near electron microscopy resolutions, yet retains the high molecular contrast and *in situ* capability of optical imaging.

We posited that localization based super-resolution imaging could enable an examination of TLR4 behavior with unique detail. Quantitative, *in situ* measurements of TLR4 behavior within this spatial regime have not been achieved to date, despite the wealth of potential new information such studies may provide. In particular, the role of LPS chemotype on TLR4 behavior is not fully understood. LPS structures differ across bacterial species and growth conditions, with variations occurring in the O-antigen domain as well as within the fatty acid composition of the lipid A portion, which binds to MD-2/TLR4 [20]. Interestingly, overall cellular inflammatory response also varies with LPS chemotype: in general, those forms comprised of hexaacylated lipid A promote considerably more endotoxic activity (and resultant cytokine production) than penta- or tetraacylated LPS, such as that found in *Yersinia pestis*, the causative agent of plague [21-23]. It is known that a number of bacterial pathogens, including *Y. pestis*, have evolved mechanisms that can bypass an innate immune response, thereby enhancing virulence [22, 24]. Such bacteria are thought to suppress cytokine production that is in part mediated by recognition of LPS by TLR4 [25-26]. Given the apparent correlation between cellular inflammatory response and TLR4 recruitment to lipid rafts, we hypothesized that a quantitative discrimination between TLR4 response to

immune-stimulatory and immune-evading bacterial LPS chemotypes, respectively, may exist at the nanoscale.

In this study, we show the first optical super-resolution characterization of TLR4/LPS distributions in the cell membranes of intact macrophages. We utilize a direct stochastic optical reconstruction microscopy (dSTORM) [27] technique, incorporating a novel simultaneous dual-color detection scheme that permits localizing both TLR4 and LPS (as well as their co-localization) in a single step. We show that this technique allows characterization of receptor/ligand distributions *in situ* with spatial resolutions of 40-50 nm, nearly an order of magnitude beyond the optical diffraction limit. Due to the increase in spatial detail, we are able to quantify nano-scale differences in TLR4 distributions due to LPS stimulation, as well as discern significant variations in receptor clustering and TLR4/LPS associations in response to hexaacylated (*E. coli*) vs. tetraacylated (*Y. pestis*) LPS exposure – thus giving further insight into mechanisms of innate immune evasion.

2. Results

2.1 Single Color Imaging of TLR4 Clustering

We investigated whether P388D1 murine macrophage-like cells displayed nano-scale differences in the spatial distribution of natively expressed TLR4, in response to hexaacylated and tetraacylated LPS exposure. To mimic typical pathogen exposure, we utilized a hexaacylated, “smooth” form of LPS, along with a “rough”, tetraacylated LPS chemotype, the forms of LPS produced by *E. coli* and *Y. pestis* cultured at 37 °C, respectively. LPS-induced TLR4 clustering has been hypothesized to occur in lipid rafts whose dimensions are <200 nm, while potentially being present within a background of isolated receptors [4, 6]. Because of this, conventional optical imaging, such as confocal microscopy, is limited in its utility as a quantitative measure of receptor distributions within the nano-scale regime. Electron microscopy (EM) can achieve sub-nanometer resolution; however, the inefficiency of immuno-gold labeling, as well as the extensive sample preparation needed, and its potential artifacts, remain as impediments to quantitative EM molecular imaging [28].

The dSTORM method can be implemented on a conventional optical microscope platform [27], in contrast to other super-resolution techniques that require complex and costly instrumentation [29-31]. Further, it does not require the use of photoactivatable fluorescent protein transfection [32] which likely alters cell physiology and responsiveness. dSTORM involves the photon-dependent conversion of organic fluorophor tags to and from “dark” or “light” states – generally termed photoswitching. Zhuang and colleagues initially showed that covalently linked pairs of Cyanine dyes can be photoswitched using an alternating sequence of activation and excitation laser pulses [17]. Further studies determined that single fluorophors could exhibit similar behavior [27] and protocols were refined such that photoswitching was observed using simultaneous activation/excitation illumination [33]. While a physical understanding of this photoswitching process is still being developed, mass spectroscopy studies suggest that the presence of small thiol-containing molecules within the sample engage in a reversible photon-dependent reaction with the organic fluorophors, rendering it alternately fluorescent or non-fluorescent [34]. This permits super-resolution imaging on samples prepared essentially identically to that needed in conventional immunofluorescence microscopy.

P388D1 cells were initially exposed to 100 nM LPS purified from either *E. coli* (hexaacylated form) or *Y. pestis* (tetraacylated form), respectively, for 30 minutes at 37 °C. As a negative control, a third set of samples were exposed to 10 µg/mL flagellin, which although induces similar cytokine production, operates through a TLR5-dependent pathway

and is not expected to result in TLR4 activation^[35]. Following exposure to these respective PAMPs, cells were immediately fixed and immunostained with anti-mouse TLR4 IgG antibodies labeled with Atto532 (see Experimental Section and Supplementary Figure S1 for further details). Prior to imaging, samples were immersed in 1x PBS containing both an oxygen scavenging system (see Experimental section for details), as well as 140 mM β -mercaptoethanol. We have found that Atto532 displays bright, stable photoswitching behavior under these conditions.

Samples were imaged through an objective-based, inverted TIRF microscope in order to selectively visualize cell membrane-associated components. To produce the photoswitching necessary for dSTORM imaging, samples were illuminated at moderately high powers, typically between 15-20 mW, using a solid-state 532 nm laser. 3,000-7,000 images were acquired at 10-20 fps across a 25 μ m field of view. Image stacks were then analyzed in Matlab, and individual emitting dyes were identified in each frame. In order to ensure that we were sufficiently sampling the microscope's point-spread function (PSF) onto the detector, we repeatedly imaged 0.2 μ m fluorescent beads to measure the PSF width, as estimated by a Gaussian standard deviation. Analysis of *c.* 85,000 PSFs yielded a standard deviation of 1.05 ± 0.07 pixels, thus rendering an entire PSF within a 25 pixel² area. To ensure that the repeatability of our imaging scheme was sufficient, we again imaged sub-diffraction sized fluorescent beads using the same acquisition and detection parameters that were used for the cell samples. 3500 localizations were made of 25 beads, respectively, and subjected to the Gaussian NLLS fit procedure. A histogram of the localization repeatability yielded a FWHM of 13 nm. This indicates that, for our studies, the repeatability due to random fluctuations and thermal stage drift was similar or better than the resolution computed in the cell images, thus not necessitating fiducial markers for localization correction. Finally, to compute the number of photons detected per molecule, per image, we used the following relation^[36]:

$$N_p = \frac{LG_{AD}}{G_{EM}\eta} \quad (1)$$

Where *L* is the pixel value in analog to digital units (ADU), *G_{AD}* is the number of electrons per ADU, *G_{EM}* is the electron multiplication factor, and η is the quantum efficiency at the wavelengths in question (see S. Quirin, et al. PNAS, 109, 675-679). For our system, *G_{AD}* = 62.27 e-/ADU, *G_{EM}* = 108, and η = 0.95 and 0.90 at 550 and 650 nm, respectively. Using these parameters, we find that the Atto 532 dye produces an average of *c.* 3500 photons per 0.05 s acquisition, while the Alexa Fluor 647 yielded an average of *c.* 750 photons over the same exposure time, due to its relatively low quantum yield (0.33).

Figure 1 illustrates the principle of sub-diffraction localization of individual molecules that is central to the dSTORM approach. Typical densities of visible fluorophors ranged from 5-50 per 25 μ m field of view. Instances where fluorophor signal was of poor signal-to-noise (SNR) of less than 2, or insufficiently separated (less than 6 pixels) from other labeled receptors were identified and not considered for subsequent analysis. A non-linear least squares (NLLS) fit was performed for each suitable point in the data set to approximate the point-spread function as a 2D Gaussian surface. A novel adaptive algorithm was developed to choose the pixel neighborhood over which to apply the NLLS fit such that the detector noise was evenly distributed about the estimated centroid of the 2D Gaussian surface. By repeatedly measuring the localization of sub-diffraction fluorescent beads, we have found that an apparent "splitting" of the NLLS-fitted centroid occurs when the localization is near the boundary between two pixels. Because of random signal fluctuations (primarily due to Poisson emission statistics and detector noise), the pixel position at which the apparent peak

intensity occurs can alternate positions over several switching cycles, creating a bimodal distribution of localizations. We have found, however, that by (1) computing the intensity weighted PSF center-of-mass (COM), and then (2) positioning the nearest pixel boundary to the COM at the center of the NLLS fitting neighborhood, repeated localizations resulted in a unimodal distribution, thereby increasing overall accuracy. Refer to Supplementary **Figure S2** for further details.

After the sub-diffraction position of each fluorophor was calculated, as well as the localization uncertainty (which is inversely proportional to the square-root of the number of detected photons), dSTORM images were reconstructed to reveal the overall TLR4 distribution. Figure 2 compares dSTORM and conventional TIRF images of TLR4 distributions. Figure 2(A-C) indicate representative conventional resolution images of P388D1 cells that have been treated with hexaacylated (A) or tetraacylated (B) LPS, as well as a flagellin (C) control, respectively. Corresponding enlarged areas (indicated by white boxes) are shown in Figure 2(g, i, and k). Without applying the dSTORM reconstruction, resolution in these images is determined by optical diffraction with a theoretical point-spread function diameter of approximately 470nm. However, Figure 2(D-F), with corresponding enlarged sub-images in Figure 2(h, j, and l), illustrate the increase in available detail after application of the dSTORM reconstruction algorithm. The superresolution point-spread function in this case is SNR dependant, but the average localization uncertainty, across all imaged samples, was found to be ± 11 -30 nm, at least an order of magnitude increase in effective resolution.

2.2 Cluster Size Analysis using Ripley's K-function

Due to the increase in resolution afforded by dSTORM, we were able to quantitatively analyze image data with greatly enhanced precision, particularly with regard to evaluating TLR4 clustering within the plasma membrane. Although image segmentation algorithms are often used to gauge image feature sizes, the presence of both clustered and isolated receptors in the reconstructed images can bias segmentation algorithms towards smaller cluster diameters. As an alternative, we chose to employ Ripley's K-function analysis [37]. The K-function (see Experimental Section, equation 1) is a common spatial statistics approach, historically used for large-scale geological/ecological imaging and mapping applications and more recently for characterizing protein distributions in cells [38-39], and acts as an estimate of spatial randomness or aggregation within a large positional data set. It is often transformed to the so-called H-function (see Experimental Section, equation 2), whereby local maxima in H correspond to areas of least randomness, and can be considered a characteristic cluster size. However, when determining point clustering within a complex background of isolated points, Kenworthy and colleagues found that values of r corresponding to inflection points in H also served as accurate measures of cluster size [40]. For our analysis, when a clear local maxima in H was not present for a given cell image (seen in approximately half of cell samples), the secondary inflection point method was used to determine a characteristic TLR4 cluster size. If multiple peaks/inflection points were present, the smallest cluster size within the range of 10 nm to 1 μ m was selected. Supplementary **Figure S3** illustrates representative H-functions taken from images of cells exposed to hexaacylated and tetraacylated LPS, as well as flagellin, respectively.

Figure 3 shows results of Ripley's K-function cluster size analysis on TLR4 distributions after various stimuli, averaged over $N = 10$ cells per stimulus type. Cellular innate immune response was targeted either through a TLR4-independent pathway via flagellin (green) or hexaacylated LPS (from *E. coli*, shown in red). Consistent with previous reports [4, 35], stimulatory LPS produced significantly ($p < 0.01$, $N = 10$) larger characteristic TLR4 cluster sizes (515 ± 70 nm) than cells stimulated with flagellin, a TLR5-specific ligand, (376 ± 53

nm). Cells stimulated with tetraacylated LPS (from *Y. pestis*, shown in blue), displayed a somewhat intermediate cluster size of 480 ± 120 nm, but were not statistically distinguishable from either flagellin or hexaacylated LPS. We hypothesized that contributions from isolated and/or non-ligand bearing receptors could potentially mask differences in TLR4 distribution patterns between *E. coli* and *Y. pestis* LPS-treated cells. This was in part supported by the observation that the calculated characteristic cluster sizes were approximately 2-3 fold larger than the typical diameter of lipid rafts ^[3], suggesting the presence TLR4 molecules near, yet unincorporated into these domains.

2.3 Dual Color Imaging of TLR4 and LPS

To test this hypothesis, we performed dual-color dSTORM on cells prepared as described above, using LPS conjugated to AlexaFluor 647, in addition to Atto532-labeled TLR4. Alexafluor 647 has been shown to be well suited for localization-based superresolution imaging ^[19], and its emission profile is spectrally distinct from the Atto532 used to tag TLR4 receptors. Alexafluor 647 dyes were attached to the LPS O-oligosaccharide portion, so as to not interfere with binding to TLR4, via hydrazide-based linkages. Cells were exposed to fluorescently-tagged LPS, fixed, and immunostained for TLR4 as described above. Cells were then exposed to simultaneous 532 nm and 638 nm laser illumination in the TIRF microscope, and images were acquired using the same settings as before. A filter-based optical splitter was used to project the images of TLR4 and LPS distributions onto separate areas of the EMCCD camera simultaneously, thereby avoiding any movement artifacts that can be brought about by sequential dual-color imaging. Images from each channel were co-registered via the use of sub-diffraction-sized polystyrene beads, which contain broadly excitable fluorescent dyes that are visible in both image channels. A linear transformation was used to map the bead centroids from one channel to the other, with an average co-localization error of less than 50 nm across the field of view, consistent with the localization uncertainty demonstrated in the single color experiments. Figure 4 shows two-color overlay dSTORM images of TLR4 and LPS distributions, shown in blue and orange, respectively. Figure 4A indicates cells treated with hexaacylated LPS, while Figure 4B indicates tetraacylated LPS. To quantify relative TLR4-LPS co-localization among the two chemotypes, we initially ignored the dimmest 10% of the observed signals to ensure that any spurious signal was not considered. Areas of co-localization were then determined by the presence of signal from TLR4 and LPS within a radial distance of 50 nm (corresponding to the average localization error). Due to the near-molecular level of detail, co-localization analysis of superresolution images provides greatly enhanced probability that two species are bound to each other, as compared to conventional imaging. Figures 4C and D illustrate areas of receptor-ligand overlap, indicated in white. Quantification of the fraction of total LPS that was co-localized to TLR4 indicated a significant ($p < 0.01$, $N = 10$ cells) difference of more than 1.8-fold ($29 \pm 12\%$ vs. $53 \pm 21\%$) between the *Y. pestis* and *E. coli* derived chemotypes, respectively, as shown in Figure 4E. In Figures 4C and D, there appears to be limited amounts of TLR4-LPS complex that are not clustered (as evidenced by a lack of single, isolated white pixels). However, the overall aggregation order of these complexes is noticeably greater in cells exposed to hexaacylated LPS. To quantify this observation, Ripley's K-function analysis was repeated as before, but only on areas of TLR4/LPS co-localization. In contrast to the TLR4-only results shown in Figure 3, which implicitly considers clustering of both ligand-bound and unoccupied receptors, the behavior of the ligand-bound complex indicates a significant reduction in receptor clustering produced by *Y. pestis*-derived LPS as compared to the *E. coli* derived chemotype ($p = 0.031$, $N = 10$ cells), as illustrated in Figure 4F. Taken together, these results suggest that differential innate immune responses may be correlated to events occurring at the cell membrane at relatively early time points in the infection cycle.

3. Conclusions

Using novel optical superresolution microscopy, we have quantitatively characterized toll-like receptor (TLR) distributions within the plasma membrane in response to LPS binding in intact cells at an unprecedented scale (<30 nm). Reports have indicated that ligand-induced TLR clustering into lipid rafts correlates with cytokine production [4, 6, 41]. However, this result has mainly been shown in bulk assays that preclude a quantitative, single cell assessment of receptor sequestration in the membrane via measurement of TLR4 cluster size. Through single-cell imaging studies, we have shown directly that exposure to *E. coli*-derived LPS results in an increase in characteristic TLR4 cluster size by nearly 40% over flagellin, which operates in a TLR4-independent fashion. Furthermore, through the addition of a multicolor detection capability, we have resolved differences in TLR4:LPS complex distribution in response to stimulatory and less-stimulatory lipopolysaccharides (LPS). We found that both the extent of LPS colocalization with TLR4, as well as the accompanying clustering of receptor-ligand complexes was significantly greater in the case of hexaacylated LPS vs. the tetraacylated form. Importantly, these differences occur at spatial scales well below the optical diffraction limit, and thus impossible to resolve using conventional methods. Further investigations, such as correlating TLR4 clustering with LPS concentration will likely shed further light on the role of this endotoxin in innate immunity. Based on previous dose-response assays (not shown), the studies reported here utilize a relatively high LPS dose to maximize image quality, while at the same time preserving differences in overall inflammatory responses between LPS chemotypes. Improvements in optical detection technology should allow future imaging studies to probe behavior at lower endotoxin exposure levels.

A molecular understanding of the role LPS chemotype plays in determining TLR4 spatial behavior is still developing. It is known that other putative LPS recognition receptors may be present in monocyte/macrophage cells, including the macrophage scavenger receptor [42], as well as heat shock proteins (HSPs) [43], among others; notably, both have been suggested to modulate cytokine production [42, 44]. Relative specificities of the various LPS forms to these receptors may, in part, explain the differential cytokine response. Our observation that a stimulatory LPS co-localizes with TLR4 to a greater extent than a non-stimulatory chemotype is consistent with this possibility. Furthermore, structural studies have indicated that the number of acyl chains contained within the lipid A portion of the LPS molecule determined the overall shape and binding angle of LPS with respect to the membrane. This, in turn, has implications for downstream signaling [23, 45]. Similarly, Park and colleagues showed that 6 lipid A acyl chains were necessary for LPS to bridge both MD2 and TLR4 during complex formation [46]. Overall, it is becoming increasingly apparent that nanoscale protein rearrangements can sensitively affect cell and tissue-wide responses. As such, the role of optical superresolution imaging including the strategies developed for this work will likely play an increasing role in elucidating these behaviors.

4. Experimental Section

Cell culture

PD388D1 murine macrophage cells were cultured at 37 °C in a CO₂ environment (5%) using RPMI media (ATCC) supplemented with FBS (5%, Gibco). For all experiments, cells were seeded on #1 glass coverslips, previously cleaned with piranha solution (12 M H₂SO₄ and 30% H₂O₂, mixed 1:3), at 2×10^5 cells/mL.

Labeling Reagents

Anti-mouse TLR4 antibodies (eBioscience, clone UT41) were conjugated to Atto532 fluorescent dye (Attotec), following the manufacturer's instructions via succinimidyl ester linkages. Briefly, antibodies were concentrated in 1x PBS using a 10 kD MWCO centrifugal filter (Millipore) to a final concentration of 2 mg/mL. A NaHCO₃ solution (1M) was added at a 1:10 dilution factor, followed by addition of Atto532 (10 µg dye per mg antibody). After incubation for 1 hr, labeled antibodies were separated from un-reacted dye via a centrifugal size exclusion gel column.

LPS from *E. coli* was purchased from Sigma, catalog number L-3129. LPS from *Y. pestis* strain KIM6+ was prepared by magnesium-ethanol precipitation after the method of Darveau and Hancock [47]. All preparations were examined for purity by polyacrylamide gel electrophoresis and silver staining [48]. Stock solutions were quantified by KDO content assay [49]. In some experiments as indicated, LPS was labeled with Alexa 647-hydrazide (Invitrogen) by the method of Triantafyllou et al. [50].

Sample Preparation

Prior to all endotoxin exposures, P388D1 cells were washed in 1x PBS. Samples were exposed to lipopolysaccharide (100 nM) derived from either *E. coli* or *Y. pestis*, or flagellin in RPMI (10 µg/mL) + FBS (5%) media, respectively, which have been shown to be sufficient to stimulate murine macrophage cytokine production [51]. Cells were allowed to interact with the labeled LPS chemotypes or flagellin for 30 min at 37 °C. Cells were then fixed in freshly prepared buffered paraformaldehyde/sucrose (4%) at 4 °C. Immediately following, cells were washed 3 times in 1x PBS, and incubated with a 1:200 dilution of fluorescently tagged anti-TLR antibodies, prepared as described above, for 1 hr at room temperature. Optimum antibody dilution factor (1:200) was determined by comparison against identically labeled non-specific mouse IgG, with typical specific/non-specific labeling ratios in excess of 10-fold (See supplemental Figure 1). Cells were again fixed as before and stored in 1x PBS. Prior to imaging, cell samples were immersed in a STORM imaging buffer, containing glucose (10% w/v), β-mercaptoethanol (BME, 140 mM), glucose oxidase (500 µg/mL), and catalase (40 ug/mL) (all obtained from Sigma Aldrich) in 1x PBS [19], and mounted on standard microscope slides.

Image Acquisition

Samples were imaged using a custom objective-based TIRF microscope (Olympus, IX-71). Illumination was provided by fiber-coupled solid state lasers emitting at 532 and 638 nm, respectively (Crystal Laser, GCL-025-S and RCL-025-640). Illumination intensity was 15-20 mW total power, corresponding to a total irradiance of approximately 10 W/cm² at the sample. Samples were illuminated at both wavelengths for 1-2 minutes prior to image acquisition in order to render a majority of dye molecules in the "dark" state. In both single and multi-color experiments, signal from the sample was projected through a dual-channel image-splitter (Cairns Research, OptoSplit) containing a 570/25 nm BP, and 665 nm LP emission filters to isolate signal from the Atto532 and Alexafluor647 dyes, respectively. Images corresponding to each dye were projected simultaneously on an electron multiplied charge coupled device (EMCCD) (Andor Technologies, iXon). 5,000-10,000 images were captured for each cell, at 0.05-0.1 seconds per frame in order to achieve sufficient SNR. In single color imaging experiments, only the channel corresponding to Atto532 signal was considered. In multi-color experiments, the two OptoSplit image channels were registered with respect to each other using 200 nm diameter polystyrene beads containing multiple fluorophors (Invitrogen, Tetraspek). Using the corresponding bead images in both channels as fiduciary markers, a simple linear transformation was then calculated to map the

coordinates of one image channel onto the other, with average error of less than 50 nm (data not shown).

STORM Image Reconstruction and Analysis

All image processing and analysis was conducted using custom-written algorithms in Matlab incorporating the Image Processing, Curve Fitting, and Statistics Toolboxes. Image TIFF stacks were first pre-processed via background subtraction using a sliding window method. Particles corresponding to single photo-emission events in each frame were identified with a minimum signal-to-noise ratio of 2-4. Subsequently, a 6×6 pixel area surrounding each particle peak was subjected to a constrained non-linear least squares (NLLS) fitting routine to approximate the image data as a 2D Gaussian surface. We implemented a novel adaptive neighborhood selection algorithm by initially computing the point spread function (PSF) centroid, and selecting neighboring pixels at an optimum symmetry surrounding this point. We have observed that neglecting to optimize neighborhood symmetry around the PSF peak can lead to inaccurate NLLS estimates due to noise bias. Please refer to Supplementary Figure S2 for further details. Individual NLLS estimates were rejected based on several criteria: poor fit to the data (measured by adjusted R^2 value), proximity to the image edge, as well as large Gaussian standard deviations, caused by asymmetric PSFs. We have found through repeated imaging of sub-diffraction fluorescent beads that the microscope PSF displays an equivalent standard deviation of 1.05 ± 0.07 pixels. STORM images were constructed using the location data obtained from the NLLS fits and the localization uncertainty (which is proportional to the inverse square-root of the number of photons collected from each photoswitching event ^[19]).

To determine the degree of TLR4 and TLR4-LPS clustering, we implemented a custom Ripley's K-function analysis on the localization data obtained through the NLLS procedure described above. The K-function is an estimate of spatial randomness in a positional data set, and is expressed as a function of, test radii, r , such that:

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

Where A and N denote the image area and total number of localized points, respectively. I is the indicator function, with a value of unity if the distance between the j -th and i -th points is less than r , and zero otherwise. w_{ij} refers to a weighting factor that accounts for the bias incurred due to edge effects ^[52]. In the case of complete spatial randomness (CSR), whereby points are distributed by a spatial Poisson process, $K(r) = \pi r^2$. Thus, the K-function can be transformed to the so-called H-function, given by:

$$H(r) = \sqrt{\frac{K(r)}{\pi}} - r \quad (3)$$

$H(r)$ can be defined as a measure of the K-function's departure from complete spatial randomness (CSR) at each test radius r . Local maxima, or inflection points (in the case of a highly heterogeneous mixture of isolated and clustered points) in $H(r)$ have been shown to be indicative of a characteristic clustering size, both theoretically and experimentally ^[40].

Co-localization of TLR4 and LPS chemotypes was determined via analysis in Matlab. First, the dimmest 10% of pixels were ignored in order to suppress consideration of spurious PSF fits. Then STORM images were undersampled by 3-fold, producing a pixel resolution of approximately 50 nm – corresponding to the average localization accuracy achieved using

the image registration procedure. The presence of signal from both image channels in the same pixel was then considered a point of co-localization.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Schematic illustration of the localization principle behind dSTORM imaging. In each acquired image frame (left), single molecules are identified via a minimum signal-to-noise ratio (SNR). An adaptive algorithm is then used to choose an appropriate neighborhood surrounding the selected point-spread function (right). The sub-diffraction localization of the corresponding fluorophor can then be determined by employing a non-linear least squares (NLLS) regression in order to find the centroid (denoted by x_0 and y_0) of the fitting function, typically a 2D Gaussian surface.

Figure 2.

Comparison between conventional TIRF (A-C) and dSTORM (D-F) images of TLR4 distributions in P388D1 macrophage cells. Cells were exposed to 100nM of either hexaacylated LPS (A and D) or tetraacylated LPS (B and E) for 30min at 37°C. Control samples were exposed to 10 μ g/mL flagellin (C and F) under identical conditions before fixation and immunostaining for TLR4 using a primary antibody (eBioscience) conjugated to Atto532 dye (Attotec), which displays excellent photoswitching properties. Application of the dSTORM imaging approach increases the effective resolution nearly an order of magnitude as compared to conventional imaging (11-30nm vs. 235nm, respectively). To illustrate the apparent increase in image detail, sub-images with 2.5 μ m fields of view are shown in (g-l), corresponding to white boxes indicated in (A-F).

Figure 3.

Ripley's K-function analysis was used to quantitatively assess the relative level of TLR4 clustering in response to stimulatory LPS (from *E. coli*, shown in red), non-stimulatory (from *Y. pestis*, shown in blue), and TLR4 non-specific flagellin (shown in green). The analysis reveals that hexaacylated LPS binding to TLR4 results in a significant ($p < 0.003$, $N = 10$ cells for each treatment) increase in receptor clustering within the membrane, as compared to the flagellin control.

Figure 4.

Incorporation of dual-color detection dSTORM imaging to visualize TLR4 and LPS distribution and colocalization. P388D1 macrophage cells were exposed to 100nM hexaacylated (A) and tetraacylated (B) LPS conjugated with Alexafluor 647 dye (Invitrogen), for 30min at 37°C. After fixation and immunolabeling for TLR4 expression, samples were imaged as described in the Experimental section. Following dSTORM analysis, the localization of TLR4 and each LPS type was compared, and relative colocalization between receptor and ligand was computed. Colocalization was assessed by the presence of both LPS and TLR signal within a radius of 50nm, corresponding to the image registration accuracy. (C-D) indicate areas of colocalization between TLR4 and either hexaacylated or tetraacylated LPS, respectively. (E) Quantification of the fraction of total LPS signal co-localized to TLR4, as described in Figure 4. Hexaacylated LPS (red), derived from *E. coli* was found to be significantly ($p < 0.007$, $N = 10$ cells) more associated with TLR4 than tetraacylated LPS from *Y. pestis* (blue), suggesting nearly two-fold higher specificity of stimulatory LPS to TLR4. (F) Ripley's K-function analysis was applied to areas of LPS/TLR4 colocalization (shown in white in (C-D)), indicating significantly ($p < 0.04$) higher order clustering of TLR4 in response to stimulatory LPS over the non-stimulatory chemotype.