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Super-resolution Analysis of TCR-Dependent Signaling: Single-Molecule Localization Microscopy

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Abstract

Single-molecule localization microscopy (SMLM) comprises methods that produce super-resolution images from molecular locations of single molecules. These techniques mathematically determine the center of a diffraction-limited spot produced by a fluorescent molecule, which represents the most likely location of the molecule. Only a small cohort of well-separated molecules is visualized in a single image, and then many images are obtained from a single sample. The localizations from all the images are combined to produce a super-resolution picture of the sample. Here we describe the application of two methods, photoactivation localization microscopy (PALM) and direct stochastic optical reconstruction microscopy (dSTORM), to the study of signaling microclusters in T cells.

Keywords

Single-molecule localization microscopy; Super-resolution microscopy; Photoactivation localization microscopy; Direct stochastic optical resolution microscopy; T cell; Microclusters

1 Introduction

In many biological systems, ligand binding to specific cell surface receptors initiates the complex process of signal transduction. Often signal propagation within cells is characterized by the formation of complexes composed of many interacting proteins that promote and regulate the distal signaling events that then lead to specific cellular responses and functional outcomes. In T cells, engagement of the T cell receptor (TCR) by a cognate antigen displayed by an antigen-presenting cell (APC) causes the rapid formation of cell surface microclusters, which contain the macromolecular signaling complexes consisting of enzymes and adapter molecules required to initiate T cell activation [1–4]. Once activation begins, the T cell undergoes a number of dramatic changes. Actin polymerization leads to large-scale morphological changes as the T cell spreads and forms contacts with the APC [5]. The micro-clusters themselves move and rearrange, eventually leading in some cases to the formation of a larger complex structure, the immunological synapse (IS), at the contact surface between the T cell and APC [6]. The IS contains a central region containing concentrated TCR and other signaling proteins surrounded by an integrin-rich ring, which in turn can be surrounded by very large glycoproteins. The exact function of the IS is complex and includes both enhancement and downregulation of signaling [7].

The microclusters involved in T cell activation have been examined using light microscopy [8, 9]. However, the resolution of conventional optical microscopes is limited by diffraction of the light coming through the lens that recombines to form a magnified image. Light from a point source appears as a large blurred spot in an image so it is impossible to see molecular details using standard optical instruments. However, in the last few decades, super-resolution techniques that allow visualization of much smaller detail have been developed, many of which are now available in commercial systems [10–13]. A number of methods, collectively referred to as single-molecule localization microscopy (SMLM), allow visualization of single molecules and hold the promise of defining the structure and heterogeneity of signaling complexes [14, 15]. These techniques share a common strategy of imaging a limited number of isolated fluorescent molecules and then using mathematical techniques to determine the location of the fluorophore (Fig. 1). The key is to visualize a small number of well-separated fluorescent molecules so that only one molecule is present in a diffraction-limited spot. An image of these well-separated spots is captured, and then the imaged molecules are photoswitched or photobleached. The process can be repeated many times to visualize thousands of single molecules in a sample. Mathematical methods are then used to determine the center of each diffraction-limited spot corresponding to the likely position of a single molecule, thus building up an image composed of molecular peaks or localizations. The first of these techniques, named photoactivation localization microscopy (PALM) [16], uses illumination with activating light to induce a photoactivatable protein to become fluorescent or to change the emission properties of a photoswitchable protein. Modulating the strength of the activating beam controls the number of protein molecules that are capable of fluorescing. Another method, stochastic optical reconstruction microscopy (STORM) [17], takes advantage of energy transfer between cyanine dye molecules to produce an activated state. A variation of this technique, direct stochastic optical resolution microscopy (dSTORM) [18–20], uses a single dye molecule that can cycle between a dark and fluorescent state. There are now many methods that use different strategies to produce a limited number of fluorophores [21], including ground-state depletion with single-molecule return (GSDIM) [22], PALM with independently running acquisition (PALMIRA) [23], and point accumulation imaging in nanoscale topography (PAINT) [24, 25], but the underlying principle is the same. The final image contains a number of points representing the calculated position of each resolved molecule, generally presented in a manner in which the size of a point in the rendered image contains information on the precision of each localization. The localization precision then limits the resolution of the image.

SMLM has been applied to a large number of biological samples, including cells involved in immune responses [26]. IgE-FcεRI complexes have been examined in mast cells [27] as well as receptors in B cells [28], but most immune cell studies have focused on the organization of signaling molecules and complexes in T cells [29–33]. Because of topological constraints and the difficulty of imaging the surface between two cells, SMLM imaging has been performed using various model systems in which the activating surface can be visualized in a single plane. These investigations have revealed nanoscale organization of the TCR and other important components of the signal transduction pathway. This chapter will focus mainly on using PALM and dSTORM with Jurkat T cells, a cell line derived from human T lymphocytes. We will also touch briefly on applying PALM to human peripheral blood

lymphocytes. In these experiments, the T cells were activated by contact with an antibody-coated cover glass, thus producing signaling complexes in a single plane near the coverslip. Our studies with these techniques have focused on investigating complexes containing two essential adaptor proteins, linker for activation of T cells (LAT) and the SH2 domain-containing protein of 76 kDa (SLP-76), which will be presented as examples in this chapter. A number of factors must be considered before embarking on any SMLM experiment. In addition to describing our experimental methods, we have tried to incorporate information on the decisions that must be made when applying SMLM to a biological problem.

2 Materials

We assume that readers are familiar with general cell culture techniques and provide only a very brief description of the reagents used to culture Jurkat T cells.

2.1 Reagents for Culturing, Plating, and Fixing Cells

1. Culturing of Jurkat T cells and imaging buffer: Cells are grown in culture flasks in RPMI medium 1640 with 10% fetal calf serum and penicillin/streptomycin. Stably transfected cell lines are maintained in the same medium with the addition of 1.3 mg/ml geneticin sulfate G418 (KSE Scientific). Jurkat T cells are available through ATCC.
2. Plating cells for microscopy: Lab-Tek II Chambered Cover Glasses, with four or eight wells (Nalge Nunc International), are cleaned with acidic ethanol made by adding 5 ml of 10 M HCl to 45 ml 70% ethanol, made from 100% ethanol. Cleaned chambers are coated with 0.01% polylysine made by diluting 0.1% poly-L-lysine with purified distilled water. The chambers are then coated with either murine IgG1 antibody against CD3e, clone UCHT1, the equivalent IgG2a clone Hit3a, or, a non-activating control reagent, murine IgG1 antibody against CD45 (clone HI30) in PBS (pH 7.4). Human PBLs are activated with a combination of anti-CD3e and murine IgG1 anti-CD28 antibodies (clone CD28.2). PBS is used to rinse the chambers. Cells are resuspended in RPMI without phenol red containing 10% FCS and 20 mM HEPES pH 7.0 before plating.
3. Fixation of samples: 4% paraformaldehyde is made by dissolving paraformaldehyde powder in warm (70 °C) PBS (pH 7.4).

2.2 Equipment and Reagents Needed for All SMLM Techniques

1. SMLM can be performed with commercial imaging systems that are equipped to detect single molecules (*see* Note 1 and Fig. 2). Most systems use an electron-multiplying charged-coupled device (EMCCD) camera with high-speed image capture for detection. The system should be equipped with high numerical aperture (NA) objectives and high-quality filters and dichroic mirrors. High-power lasers are needed, particularly for dSTORM imaging. If possible, 70–125 mW are recommended for laser lines to be used in dSTORM imaging. The computer must be capable of acquiring and storing a large number of frames.

Most SMLM is performed with a total internal reflection (TIRF) microscope to improve z resolution and to reduce out-of-focus light. Additional hardware can be added to gain resolution in three dimensions; most commonly an extra lens is added to deform the point spread function in z, thus gaining information on z positioning. Interferometer-based systems can also be used to determine z position [34]; however, this feature is not yet commercially available. Of particular note, Zeiss has licensed the use of PALM, while Nikon holds the patents for STORM imaging. We use a Nikon Eclipse Ti inverted microscope, AOTF modulated LUNB solid-state lasers (70 mW at 488 nm, 70 mW at 561 nm), and a 60× SR Apochromat TIRF lens with an Andor iXon DU888 EMCCD camera (1024 × 1024 pixels, 8 μm pixel) for PALM imaging. For dSTORM imaging, we use the 647 nm line from the same system (125 mW at 647 nm), a 100× SR Apochromat TIRF objective lens (1.49 NA) with an Andor iXon Ultra 897 EMCCD camera (512 × 512 pixels, 16 μm pixel), and the 1.5 magnifier lens in place to increase the pixel resolution which leads to better fitting of the localization peaks.

2. Several kinds of fiducial markers can be used in SMLM. 50–100 nm gold beads (microspheres-nanospheres) are popular; however, some researchers use small multiwavelength fluorescent beads such as 100 nm TetraSpeck beads (Invitrogen). We are exploring the use of 100 nm negatively charged, nitrogen-vacancy-center nanodiamonds (Adamas Nanotechnologies) in SMLM.
3. Software is needed to calculate the super-resolved molecular positions from the raw diffraction-limited images. Both Zeiss and Nikon provide complete software packages as part of their specialized SMLM systems. We use PeakSelector version 5 to identify individual molecules in PALM images and the Fiji-/ImageJ-based program ThunderSTORM to identify individual molecules in dSTORM images. However, there are a large number of commercial and free programs available. A Google search for SMLM software yielded over two million entries (also see a list of programs at <http://bigwww.epfl.ch/smlm/software/>). A comparison of these methods is beyond the scope of this chapter, but there are many publications explaining the various algorithms [35–37], and a recent study compared over 30 packages using metrics such as detection rate, accuracy, and resolution to help users choose which is the best for their purposes [38].
4. Statistical techniques can be used to further study the distribution of the localized molecules. Often, the images are analyzed using spatial statistical tools such as second-order statistics, point pattern sorting, or nearest neighbor clustering algorithms. We use a published algorithm by Wiegand and Moloney for second-order statistics [39] and customized MATLAB code for the nearest neighbor analysis of clustering [30]. Minkowski functionals can also be used to sort the point patterns generated by LAT PALM imaging [40]. However, there are many other approaches to these problems with a wide range of analysis options including open-source code, stand-alone freeware, and many commercial variations [26] (*see Note 2*).

2.3 Reagents for PALM Imaging

1. PALM imaging requires the use of proteins that either change from dark to fluorescent state (photoactivatable) or change emission (photoswitchable) in response to illumination by activating light. These genetically encoded tags must then be conjugated to a protein of interest, and their corresponding cDNAs are usually expressed by a strong, constitutive promoter. We use three photoactivatable proteins in our PALM studies: Dronpa (ex 488 nm/em 505 nm) (MBL International Corporation), PA-mCherry (ex 561 nm/em 565 nm) [41], and PA-GFP (ex 488 nm/em 505 nm) [42]. The constructs used in published studies [30] were generated in EGFP-N1 or EGFP-C1 vectors (Clontech) that contain a CMV promoter.
2. Transfection and expression of proteins. Any standard transfection system can be used that allows reasonable expression in the cell line being studied. We generally transfect E6.1 Jurkat T cells using a Nucleofector shuttle system and the Amaxa T kit (Lonza). The same system is used to transfect human PBLs.
3. Production of stable cell lines. We make stable cell lines expressing photoactivatable constructs by single-cell cloning with antibiotic selection. The EGFP-N1 and C1 vectors we use contain a neomycin-resistance cassette allowing the use of geneticin (G418) as the selection agent.
4. A system capable of sterile cell sorting is usually needed to enrich samples for PALM imaging of transiently transfected cells and to sort cells for two-color imaging. We use a MoFlo Astrios EQ (Beckman Coulter Life Sciences). It is necessary to activate PA-mCherry and PA-GFP prior to sorting. We use 400 nm illumination light, in our case using a light-emitting diode (CoolLED) for photoactivation. We find it useful to have stable cell lines available as standards that can be used to set the fluorescence levels in the gates used to collect samples.

2.4 Reagents for dSTORM Imaging

Reagent grade chemicals may be obtained from Sigma-Aldrich unless otherwise noted.

1. Labeling of primary antibodies. Alexa647 dye (or other dyes like Cy5 may be used with individually optimized conditions) is conjugated to antibodies through lysine residues using succinimidyl esters of the dyes.
2. Staining of dSTORM samples. Samples are permeabilized with 0.1% Triton-X diluted in water. 1% fish-scale gelatin in PBS is used as a blocking buffer. Samples are rinsed and stored in PBS.
3. dSTORM buffers. The dSTORM imaging buffer requires a GLOX solution stock of 14 mg glucose oxide and 50 μ l 17 mg/ml catalase in 200 μ l 10 mM Tris-HCl (pH 8.0)/50 mM NaCl. 10 μ l of GLOX solution is added to 960 μ l 50 mM Tris-HCl (pH 8.0)/10 mM NaCl/10% glucose (method B from the Nikon N-STORM protocol) with the addition of 100 mM 2-mercaptoethanol, 20 mM cysteamine, and 2 mM cyclooctatetraene (see Note 3).

3 Methods

3.1 Cleaning and Coating Chambers

1. Chambers are incubated with 0.5 ml (4-well chambers) or 0.25 ml (8-well chambers) acidic ethanol for 15 min followed by the removal of the solution by aspiration and drying at 45 °C for at least 30 min.
2. The chambers are then incubated with the same volume of 0.01% polylysine for 15 min followed by the removal of the solution by aspiration and drying at 45 °C for at least 30 min. Gold beads are added to cleaned chambers *before* polylysine (*see* Note 4). 100 nm gold beads are sonicated and diluted in methanol and then plated into chambers. The chambers are dried and then coated with polylysine as above. Nanodiamond fiducial markers are added to the clean chambers *after* polylysine coating, but before antibody coating. Fiducials should be added at a high enough concentration to insure that at least five fiducial markers are present in each image.
3. The polylysine-coated chambers are then coated with either stimulatory anti-CD3 ϵ antibodies or non-stimulatory anti-CD45 antibodies at 10 μ g/ml using 0.4 ml for 4-well chambers and 0.2 ml for 8-well chamber with an overnight incubation at 10 °C. Chambers for activating human PBLs are coated with a combination of 10 μ g/ml anti-CD3 ϵ antibodies and 10 μ g/ml anti-CD28 antibodies. After the antibody solution is removed, the chambers are rinsed three times in PBS and stored in PBS until used (*see* Note 5).

3.2 Plating and Fixing Cells

1. Stable Jurkat lines expressing photoactivatable proteins for PALM or untransfected cells for dSTORM are typically passaged the day before use so that the cells are in mid-log phase on the day of the experiment.
2. Sorted cells transiently transfected with photoactivatable proteins are usually used 24 h after sorting. Imaging buffer is equilibrated at 37 °C in a humidified 5% CO₂ tissue culture incubator, and the antibody-coated chambers are also warmed.
3. The chambers are rinsed once with imaging buffer and 0.3 ml (4-well chambers) or 0.15 ml (8-well chambers) of imaging buffer is placed in each well. The cells are resuspended in imaging buffer at these concentrations: 2.5×10^6 cells/ml for cells to be fixed after 2.5 min of incubation or 4×10^6 cells/ml for cells to be fixed within 2–2.5 min (*see* Note 6). If possible, no fewer than 1×10^5 cells should be plated in each chamber. 100 μ l (4-well chambers) of the resuspended cells is plated by placing the pipette tip on the bottom of the chamber and gently pipetting onto the bottom of the chamber (Fig. 3). For 8-well chambers, 50 μ l of resuspended cells is plated into each chamber.
4. The chamber is placed in a 37 °C humidified 5% CO₂ tissue culture incubator for the desired time (*see* Note 7). At the appropriate time, cells are fixed by gently

adding 600 μ l (4-well chambers) or 300 μ l (8-well chambers) of 4% paraformaldehyde to the chamber.

5. The cells should be incubated in fixative for 30 min at 37 °C. The fixative is removed and the chamber is rinsed three times with PBS. The fixed cells should be imaged as soon as possible (*see* Note 8).
6. If multiwavelength fluorescent beads are used as fiducial markers, they should be added just before imaging, allowing sufficient time for the beads to settle in the chamber before imaging.

3.3 PALM Imaging

1. Because we already have plasmids containing many of the signaling proteins found in microclusters conjugated to fluorescent proteins, we usually produce PALM reagents by replacing the fluorescent protein tag with a photoactivatable version using standard methods. Generally, the Age1 and BsrG1 sites are used to exchange the fluorescent proteins [30]. This strategy was used to produce LAT-Dronpa and SLP-76-PA-mCherry from versions expressing yellow fluorescent protein. We have always used probes conjugated to full-length proteins, but shortened versions containing the domains of interest could also be used (*see* Note 9).
2. Cells can be transfected with any method that will allow imaging of the cells. For Jurkat T cells and human PBLs, we transfect cells with the LONZA Nucleofector shuttle system, program H-10, and the Amaxa T kit. Either transiently transfected cells or stable cell lines can be used for imaging (*see* Note 10). It is important to have a very high percentage of transfected cells in the sample.
3. Generally, the transfection efficiency in lymphocytes is low, so we routinely sort transiently transfected cells to generate samples containing mainly transfected cells. Dronpa has basal fluorescence so it can be sorted using conditions for GFP fluorescence (488 nm excitation, 500–520 emission). PA-mCherry and PA-GFP must be activated before sorting. We illuminate the cells with a 400 nm light-emitting diode (LED) source (CoolLED) for 10 min before sorting. Windows can be set using Dronpa or GFP stable cell lines for the green window and PA-mCherry or Cherry for the red window to select an appropriate expression level. Transiently transfected cells are sorted at 24 h, allowed to recover overnight and then plated and fixed 48 h after transfection.
4. Alternatively, single-cell cloning can be used to produce stable cell lines that do not require sorting. We usually produce cell lines by single-cell cloning in the presence of 1.3 mg/ml G418. This method was used to produce a stable cell line expressing LAT-Dronpa as well as a TCR-PA-mCherry cell line that was used to set the sorting gates for all other cells expressing PA-mCherry.
5. The expression level of the protein of interest should be determined in sorted cells or cell lines using Western blotting or a comparable method. We generally

image cells expressing about two times endogenous levels of the protein of interest.

6. Multiplexed PALM imaging can also be performed in cells expressing two photoactivatable proteins either by transfecting cells with two plasmids or super-transfecting a stable cell line with a second protein. In both cases, it is usually necessary to sort the cells to obtain a sufficient number of cells expressing both photoactivatable proteins (*see Note 11*).
7. The activation protocol depends on the photoactivatable protein. Dronpa can be easily activated by low-power 405 nm light or 360 nm light. We use simultaneous illumination with a DAPI cube (excitation 340–380 nm) and arc lamp for Dronpa. PA-mCherry and PA-GFP require stronger illumination to photoactivate. We use 10–20 s of 405 light or maximal intensity of the arc lamp with a CFP cube (excitation 426–446 nm) for PA-mCherry and 60 s to activate PA-GFP.
8. All imaging is performed in TIRF mode. Since Dronpa can be activated and imaged at the same time, a large number of frames can be collected in each sequence. We generally collect 2500 frames/sequence and at least two sequences per sample using continuous activation illumination and simultaneous imaging with an optical configuration suitable for visualizing GFP (excitation 488 nm, emission 505–525 nm) (Fig. 4a). PA-mCherry must be activated and then imaged separately with an optical configuration suitable for mCherry visualization (excitation 561 nm, emission 580–630 nm). Often, no fluorescence will be visible after 500–700 frames. We typically activate and image 500 frames/sequence and collect 5 sequences/sample although often the pool of excitable molecules will be exhausted before all the sequences are captured. PA-GFP is imaged after activation using the same optical configuration as Dronpa. In our hands, PA-GFP fluoresces for 1000–1500 frames after activation. We generally collect 1250 frames/sequence and 4 sequences/sample (*see Note 12*).
9. For two-color PALM imaging, we use a Dronpa-conjugated molecule such as LAT-Dronpa paired with a PA-mCherry-conjugated molecule such as SLP-76-PA-mCherry (Fig. 4b). The Dronpa construct is always imaged first as described above. Once all the Dronpa images are collected, the sample is illuminated with a stronger activating light, and then the PA-mCherry images are captured. The photoactivation and imaging of PA-mCherry can be repeated several times.

3.4 dSTORM Imaging

1. Primary antibodies should be directly conjugated to the dye of interest (*see Note 13*). We conjugate antibodies with Alexa647 with a standard kit using the manufacturer's recommended protocol (Molecular Probes/Invitrogen).
2. Cell staining. Fixed cells are permeabilized for 5 min with 0.1% Triton-X, incubated in blocking buffer for 30 min and then with the Alexa647-conjugated primary antibody (50 ng/ml) at room temperature for 1 h (*see Note 14*). Stained samples are washed five times with PBS and stored in PBS at 4 °C in the dark.

The dilution of the primary antibody depends on the avidity of the particular antibody; we usually use 50 ng/ml. Induced blinking of Alexa647 requires a reaction with a thiol-containing compound so after five washes with PBS, dSTORM imaging buffer is added in excess to the samples, that is, at 2 ml/4-well chamber and 1 ml/8-well chamber. Samples are sealed with a glass coverslip to protect them from air.

3. Imaging sequence. The sample is illuminated with a high-power laser to induce dye blinking. The cell sample is imaged for 20,000–30,000 frames in dSTORM imaging buffer (Fig. 4c).

3.5 Analysis of SMLM Data

1. Determining the molecular localizations from the raw diffraction-limited images is a complicated process. Most SMLM software packages process the raw images, correct for stage drift, identify intensity peaks representing candidate molecules, determine the position of the molecules producing the peaks, and finally create a rendered image of the localizations. Each package has its own algorithms and required inputs for performing these steps, so rather than explain how to use a particular one, we will highlight some of the considerations for these steps. However, all choices may not be available for every step in every package.
2. Preprocessing steps. Background subtraction is strongly advised, followed by noise reduction to enhance the detection of local maxima corresponding to emissions from candidate molecules while reducing false positives. SMLM software packages generally include a number of standard techniques, such as average, median, erosion, and Gaussian filter masks, which can be used to reduce the noise in the image (*see Note 15*). SMLM imaging sequences generally last for many minutes and stage drift will affect the images. Drift correction can be performed either by tracking fiducial markers present on each frame of the imaging sequence [16, 17] or with cross-correlation algorithms that use the sample structure to correct for movement during the image sequence [43] (*see Note 16*). We prefer fiducial markers for correcting drift. If more than one color is used in SMLM, chromatic aberration will be a problem [44]. In this case, fiducial markers can be used to align channels and to monitor the accuracy of the correction.
3. The user has to set a number of parameters, including thresholds for peaks (local or global). The user may also need to set the criteria for rejecting peaks using measures such as the amplitude of the fitted function, symmetry, or skewness. Some thought should be given to these parameters as they will directly influence the quality of the SMLM data.
4. Most programs report an estimated localization precision or σ that shows the error in the calculated position of each molecule. This information can be used to judge the quality of both the original images and the SMLM localizations. The most important factors are the number of photons and noise (*see Note 17*).

5. In both PALM and dSTORM, some fluorophores move to a long-lived dark state instead of photobleaching. These molecules can then blink or return to fluorescent state. Thus, a single molecule could be counted multiple times in the final SMLM image [45, 46]. This is easier to correct in PALM imaging as the amount of blinking is lower. We collect localizations with a spatial radius 50% larger than the sigma if they occur within 15 frames and group them into one localization. The number of localizations found as the time gap (number of frames) increases fits a negative exponential function; thus, for larger time intervals, the improvement becomes asymptotically lower. Our grouping conditions lower the number of detected molecules to 115% of the asymptotic plateau. Other methods such as scanning for temporal clusters can also be used to account for blinking [45]; however, grouping errors are common in SMLM and bedevil all of the analyses (*see* Note 18).
6. Most SMLM software also contains algorithms to translate the list of positions of localized molecules into an image. The final image is a point pattern formed by placing filled spots at the location of each molecule. Most simply, each localization can be drawn as a single dot of a set size, but generally the spot size, intensity, or color is used to convey information on error in the calculated location of that particular molecule. In the most common representation, each spot in the final image is formed by Gaussian functions centered at the molecular position with an intensity that represents the number of photons and a width that depends on the localization precision. Smaller, brighter spots represent better localizations. In this representation, the edges of molecules can overlap. Alternatively, images can be made where the intensity corresponds to the probability density of the fitted Gaussian distribution. Finally, spots can be produced by binning localizations into an appropriate 2D grid. Binned representations can be improved by using jittered 2D histograms where the jitter is proportional to the localization accuracy [47]. The density of the label also influences the accuracy of the image (*see* Note 19). In our PALM images that are produced by the PeakSelector software, the spot intensity reflects the probability density (Fig. 4a, b). In our dSTORM images that are produced by ThunderSTORM, the spots are produced by Gaussian functions (Fig. 4c).
7. The distribution of points in SMLM images can be analyzed to determine if there is a pattern. Because these images are not pixel-based intensity maps, traditional image processing algorithms are not appropriate in most cases. Many SMLM studies have used second-order statistics that compare the points in an image to a null or random model to determine nonrandom distributions [29–31]. Many of these methods have been borrowed from other fields that have developed methods to examine distribution of objects. Care is needed to pick the correct method and null model [39]. We use a heterogeneous null model to account for variations in the membrane contact surface in our analyses of single-color PALM images (Fig. 5). The details of this analysis have been published [30]. Very different results can be obtained from the same data depending on how the analysis is performed [48]. Second-order statistics provide an aggregate answer

to whether there is clustering and what is the length scale of the clustering. These statistics are not meant to determine cluster size. An alternative approach uses algorithms that examine the proximity of any given molecule to its nearest neighbors to study cluster distribution and size [26]. There are many variations of this approach, and the choices of thresholds and other variables can influence the outcome. Obviously, accurate cluster analysis also requires an accurate correction for multiple localizations from a single molecule. Finally, SLML techniques, as currently implemented, rarely give accurate counts of the number of molecules, although methods are being developed to improve counting [49, 50]. Both overcounting and undercounting errors are common [48].

8. Analysis of SMLM images of two or more components is even more complicated. As mentioned earlier, if different optical configurations are used for the different species, chromatic aberration can introduce additional errors in the accuracy in assigning each position. For two-component images, second-order statistics such as the bivariate pair correlation function (PCF) or Ripley's K-functions can be used to compare the distributions in an image to random patterns or random mixing. We use the bivariate pair correlation with a random sampling null model to analyze two-color images as described earlier (Fig. 6) [30]. Colocalization measures for pixel-based images including Pearson's or Mander's are generally not suited for point patterns. Furthermore, these techniques can only analyze pairs of molecules, so additional methods are needed for analysis of patterns and relationships between three or more components.

4 Notes

1. While a SMLM can be performed on commercial microscopes, the system must be sensitive, contain high-quality optics, employ lasers of sufficient power, and be protected from thermal instability and vibration to reduce drift. References are available that describe how to assemble a SMLM system [18, 51, 52]. If TIRF illumination is used, nonuniform illumination may cause systematic variation in the localization accuracy as areas with fainter illumination may produce fewer photons per molecule. In extreme cases, molecules in some parts of the sample may not be detected. We use only a small area in the center of the field where the illumination is most uniform for imaging and collects no more than three T cells per field for PALM images and only one T cell per field for dSTORM imaging.
2. These statistical methods are applicable to one- and two-color point patterns but would require sequential pair-wise analysis of SMLM images containing more than two components. New methods are needed to examine the relationships between three or more proteins.
3. There are a wide variety of dSTORM buffers in use ranging from simple buffers and commercial mounting [53] to mixtures optimized for maximum photon count [54]. It is worth performing some preliminary experiments to determine the best buffer for a particular application before beginning a dSTORM project.

4. The fiducial marker should be chosen before preparing the chambers, as different fiducials are added at different times in the protocol. Many researchers have used gold beads as stable fiducials. For optimal adherence, they should be added to the chamber before the polylysine coating, although gold rods will adhere reasonably well to an antibody-coated coverslip. However, we have found that the signal diminishes at shorter wavelengths, so gold fiducials sometimes fail as green or multicolor markers. Fluorescent beads make good fiducials, and because they can be added after the sample is in place, they are less likely to overlap with localizations from the sample. However, they are not strongly bound to the surface and can be slightly mobile in the chamber. Beads that fluoresce at multiple wavelengths are readily available. Unfortunately, they can be bleached when performing STORM imaging. We have found that negatively charged nitrogen-vacancy-center nano-diamonds are very good fiducial markers. They produce stable fluorescence in multiple channels, and because the fluorescence is an integral part of the crystal structure, they do not photobleach. Nanodiamonds must be added before the antibody coating as they will not adhere to the coated surface.
5. Dry polylysine-coated chambers may be stored at room temperature for many weeks. Antibody-coated chambers should be coated shortly before use although the plated antibodies are generally active for at least 1 week.
6. The number of cells securely attached to the coverslip is lower at early timepoints, so more of the plated cells are removed in the washes. Increasing the number of cells helps compensate for this effect.
7. Once the cells have been plated, the chambers should be moved carefully to avoid dislodging the activated cells.
8. PALM samples are light sensitive. Once the cells are plated into the chamber, the sample should be protected from light. Storing the samples below 10 °C diminishes the activation of photoactivatable proteins. PALM samples should be stored at room temperature.
9. There are many choices for photoactivatable and photoswitchable proteins [55, 56]. For single-molecule imaging, photon yield is one of the most significant considerations. In addition, photostability and the spontaneous photoactivation rate should also be considered [10]. It is important to choose fluorescent proteins that do not form aggregates either by themselves or as conjugated molecules [57]. The photoswitchable protein tdEos is often used because it is very bright, but because it is a tandem dimer, care should be taken to insure that it does not affect the localization and function of the protein being studied. For multicolor PALM, it is important to choose suitable pairs [48].
10. The relative merits of transient transfections and stable cell lines are generally the same for PALM imaging as other experiments. Stable cell lines offer a more uniform expression but require a substantial time investment. In several cases, we found additional mutations in Jurkat cell lines selected for stable expression of

signaling proteins, so all cell lines should be carefully evaluated to be sure they behave normally. If a single construct will be used for many PALM experiments, the advantages of a stable cell line may outweigh the disadvantages.

11. When a stable cell line is super-transfected, the levels of expression of the two proteins tend to be inversely related, so it can be difficult to find cells with acceptable levels of both photoactivatable proteins. Cells that are dually transfected with both plasmids tend to give proportional labeling of both photoactivatable proteins, making it easier to locate cells that can be imaged.
12. The exact conditions needed for imaging will depend on expression level as well as the photoactivatable protein that is being used. As mentioned in Note 9, there is a wide range in the number of photons produced by different photoactivatable proteins. Also, as the expression level increases, the amount of activating light may need to be decreased to avoid activating too many molecules. Generally, the strategy in PALM imaging is to vary the activation energy to control the number of activated molecules so that well-spaced single molecules are imaged. To a large extent, the optimal length of the collection series following activation is controlled by the characteristic production of photons by the fluorescent protein. Increasing the excitation laser power may speed up the imaging and bleaching of the activated molecules; however, as the excitation power increases, there is increased risk of bleaching without capturing fluorescence. When we image PA-mCherry or PA-GFP, by the end of our imaging series, we have visualized almost all of the molecules that we can activate in that particular sample, so increasing the number of cycles would not produce many more localizations.
13. Using indirect immunostaining puts two immunoglobulin molecules between the protein being studied and the fluorophore being imaged by dSTORM. This increases the distance between the detected molecule and the actual target, significantly increasing the uncertainty in the actual location of the protein being studied. Therefore, all dSTORM probes should be directly conjugated to as small an entity as possible. If Fab fragments or single-domain antibodies can be found, these are the best choices for primary antibodies for use in dSTORM.
14. Many fluorophores can be used for dSTORM imaging. Alexa647 and Cy5 have the optimal combination of photon count and duty cycles [56]. Other dyes are usable, but with decreased performance [58].
15. Noise reduction filters have been evaluated for use in SMLM. One report preferred median smoothing [59], while a second study recommended convolution with a Gaussian kernel [47].
16. Drift correction is implemented differently in different software packages. We prefer methods based on fiducial markers. Fiducial markers are required for channel correction when performing multicolor SMLM.
17. There are several different measures that are important in assessing the quality of SMLM data [36, 60]. The most commonly reported measure is σ or localization precision which gives the error in the calculated position. Most studies use a

standard formula to estimate the error that takes into account the number of photons, the pixel size, and the noise in the image [61]. Increasing the number of photons/localization greatly increases the localization precision. dSTORM has a great advantage in photon production as the best dyes, Alexa647 and Cy5, produce 6000 photons/burst, while photoactivatable proteins are generally in the range of 200–600 photons/burst [15, 16, 62]. The image resolution or the ability to distinguish two different points in the image can be affected by experimental errors so a given image may not actually achieve a resolution equal to σ . Many papers on SMLM techniques show images to demonstrate that they can resolve a known structure such as microtubules [22, 58, 63]. In addition to these intrinsic standards, DNA origami has been used to produce molecular rulers that could be used to verify resolution [64]. A technique used in electron microscopy, Fourier ring correlation, has been applied to SMLM to determine the true resolution [65, 66]. However, to analyze microclusters, most researchers are interested in accurately determining the position of each protein in a multi-protein complex. Unfortunately, the localization error is not an adequate measure of the error in the actual position of the molecule. If the error is distributed normally, the probability of finding a single molecule in a circle with a radius of σ is only 33%. That is, if the σ is 20 nm, 66% of the molecules will be outside a circular area with a diameter of 40 nm. A circle of probable location with a radius of 3σ or 60 nm will contain the molecule of interest 99% of the time. However, a probable location somewhere within an area of 11,000 nm² is not sufficient to define the molecular structure of a signaling complex. Moreover, when a single molecule blinks, the calculated positions of the different blinks may not overlap [46]. The true location of the molecule could be anywhere within the territory defined by the set of blinks.

18. The difficulty in properly assigning multiple localizations to the correct molecule or the grouping of localizations remains one of the most stubborn problems in SMLM [67]. Without this crucial correction, it is impossible to perform a detailed molecular analysis of microclusters and the immune synapse.
19. As the spatial resolution improves, it becomes harder to produce a labeling density that meets the Nyquist density requirement of two measurements per resolution unit. If too many molecules are missing because of low labeling efficiency or low label detection, the image will be incomplete and inaccurate [68]. Labeling density in dSTORM is limited by antibody binding affinities and steric hindrance of antibodies when the epitopes are close together. In the crowded milieu of a signaling complex, it may be quite difficult to label every protein of interest. PALM offers the theoretical possibility of labeling every protein in the absence of unlabeled endogenous proteins. However, because PALM reagents produce fewer photons, it may be difficult to visualize every photoactivated protein, which will reduce the density of detected molecules and limit the accuracy of the image.

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Fig. 1.

Principle of single-molecule localization microscopy. A small number of fluorescent molecules are imaged in each frame. They must be well dispersed so that the diffraction-limited spots do not overlap. Then, the molecules are photobleached or moved to a permanent dark state. The center of each spot is calculated. This process is repeated thousands of times to build up an image containing the localization of thousands of molecules. Finally, the localizations are combined and displayed in a single super-resolution image

a) Light path for PALM Imaging

b) Light path for TIRF Objective

Fig. 2.

Light paths used in SMLM. **(a)** For PALM or STORM imaging, the microscope must have two lasers, one for activation and one for imaging. For dSTORM imaging, only a single high-power laser is needed. The detector must be capable of detecting single molecules; usually an EMCCD camera is used. Most SMLM is performed with TIRF excitation to limit excitation to a single z section near the coverslip. **(b)** Most TIRF systems use an objective that brings the excitation light through the objective so that it reaches the sample at the critical angle needed for TIRF

a) Plating Jurkat T cells in Chambered coverglass

b) Jurkat cells spreading on activating coverslip

Fig. 3.

Preparing activated Jurkat T cells for SLML imaging. **(a)** Cells are plated into a prepared chamber by careful pipetting. **(b)** Jurkat T cells contacting and spreading on an antibody-coated coverslip. Fiducial markers are shown in *red* under the antibody coating

Fig. 4.

Jurkat T cells imaged by PALM and dSTORM. (a) PALM imaging of LAT-Dronpa. The *left panel* shows the sum of all the diffraction-limited images (5000 frames total). The *center panel* is a rendering of the localizations found in the 5000 frame series. The color scale shows the probability density of the localizations; a larger probability density means there is a greater chance of finding a localization in a given volume. Areas with a high localization density are small and *white*. As the probability density decreases, the rendered spots become larger and darker red. The *white circle* indicates a representative fiducial marker. In this image the fiducials were 100 nm TetraSpeck beads. The *right panel* shows a magnification of the boxed area from the *middle panel*. (b) PALM imaging of LAT-Dronpa and SLP-76-PA-mCherry. 5000 frames were obtained of LAT-Dronpa followed by imaging 2500 frames of SLP-76-PA-mCherry. The *left panels* show the sum of all the diffraction-limited images of LAT-Dronpa (*top*) and SLP-76-PA-mCherry (*bottom*). The *middle panel* shows the

localizations from all of these images combined into a single two-color rendering. The *white circle* indicates a representative fiducial marker. In this image the fiducials were 100 nm TetraSpeck beads. The *right panel* shows a magnification of the boxed area from the *middle panel*. (c) dSTORM imaging of phosphorylated SLP-76 using antibodies to anti-phosphorylated SLP-76 directly conjugated to Alexa647. The *left panel* shows a diffraction-limited TIRF image of the stained sample. The *middle panel* shows the localizations from a 20,000 image series. This rendering is produced using Gaussian profiles centered at the molecular positions. The *white circle* indicates a representative fiducial marker. In this image the fiducials were 100 nm negatively charged, nitrogen-vacancy-center nanodiamonds. The *right panel* shows the TIRF image superimposed on the dSTORM rendering

Fig. 5.

Analysis of a one color PALM image. **(a)** Rendering of a central ROI chosen from the cell pictured in Fig. 4a. **(b)** Pixelation of the localizations in the ROI using a 20 nm hard-shell model. **(c)** Density map of ROI showing heterogeneity of the density of molecules in the ROI. **(d)** Univariate pair correlation function $g(r)$. The *blue line* shows the sample PCF while the *black dotted lines* show the highest and lowest PCFs from 19 Monte Carlo simulations of a random distribution using a heterogeneous Poisson process based on the density map shown in **(c)**. A sample PFC lying between these lines would be considered a random distribution with no clustering. The sample PCF lies above the highest PCF from the random simulations showing that LAT-Dronpa is more clustered than a random distribution

Fig. 6.

Analysis of a two-color PALM image. (a) Rendering of a central ROI chosen from the cell pictured in Fig. 4b. (b) Pixelation of the localizations in the ROI using a 20 nm hard-shell model. LAT-Dronpa is shown in *green* and SLP-76-PA-mCherry is shown in *red*. (c) An example of a random mixing simulation. *Red* and *green* spots were placed randomly into any position that was marked with a spot in (b). The spot locations are the same as in the sample, and the number of *green* and *red* spots corresponds to the numbers found in (b). (d) Bivariate pair correlation function $g_{12}(r)$. The *blue line* shows the sample PCF, while the *black dotted lines* show the highest and lowest PCFs from 19 Monte Carlo simulations of the random mixing model. A sample PCF lying between these lines would indicate random mixing of the two molecules in the sample. The sample PCF lies below the lowest PCF from the random simulations showing that LAT-Dronpa and SLP-76 are more segregated than would be expected in a random distribution