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Super-Resolution Microscopy and Tracking of DNA-Binding Proteins in Bacterial Cells

Stephan Uphoff

Department of Biochemistry, University of Oxford, Parks Road, OX1 3QU, Oxford, United Kingdom

Summary

The ability to detect individual fluorescent molecules inside living cells has enabled a range of powerful microscopy techniques that resolve biological processes on the molecular scale. These methods have also transformed the study of bacterial cell biology, which was previously obstructed by the limited spatial resolution of conventional microscopy. In the case of DNA-binding proteins, super-resolution microscopy can visualize the detailed spatial organization of DNA replication, transcription, and repair processes by reconstructing a map of single-molecule localizations. Furthermore, DNA binding activities can be observed directly by tracking protein movement in real time. This allows identifying subpopulations of DNA-bound and diffusing proteins, and can be used to measure DNA-binding times *in vivo*. This chapter provides a detailed protocol for super-resolution microscopy and tracking of DNA-binding proteins in *Escherichia coli* cells. The protocol covers the construction of cell strains and describes data acquisition and analysis procedures, such as super-resolution image reconstruction, mapping single-molecule tracks, computing diffusion coefficients to identify molecular subpopulations with different mobility, and analysis of DNA-binding kinetics. While the focus is on the study of bacterial chromosome biology, these approaches are generally applicable to other molecular processes and cell types.

Keywords

Super-resolution fluorescence microscopy; single-molecule imaging; single-particle tracking; DNA-binding proteins; DNA repair; lambda Red Recombination; *Escherichia coli*

1 Introduction

Super-resolution fluorescence microscopy (1) has matured as a technique and is now widely applied in fundamental research. Photoactivated Localization Microscopy (PALM) (2) and Stochastic Optical Reconstruction Microscopy (STORM) (3) reach image resolution below the diffraction limit of light by localizing individual spatially isolated fluorophores. This is achieved by optically switching fluorophores from a non-fluorescent state to a fluorescent state such that only a sparse subset of fluorophores is visible at any time. Automated computer analysis detects fluorescent spots and determines their centroid positions. A super-

resolution image can then be reconstructed from the list of molecule localizations that have been recorded sequentially over a series of images.

The study of microorganisms particularly benefits from the ~10 fold increase in image resolution, allowing the visual examination of subcellular molecular structures, such as cell wall components, cell division machinery, and chromosomes (4–15). Here, we focus on the application of PALM and photoactivated single-molecule tracking to study DNA-binding proteins in *Escherichia coli*. Conventional fluorescence microscopy obscures the measurement of most of these proteins because they bind DNA transiently and are distributed throughout the bacterial nucleoid. By imaging single molecules, unsynchronized reaction events and small molecular subpopulations can be observed without population averaging.

Beyond recording static structures, the ability to determine precise localizations of single fluorescent molecules has enabled tracking proteins in live cells (16). With the super-resolution microscopy concept, this approach can now be applied for arbitrary densities of labelled molecules (17): each photoactivation event gives a glimpse into the function of a single protein. Reaction events, such as the binding of a DNA repair enzyme to a DNA damage site are marked by a change in the diffusion characteristics (9). Progress has also been made in the application of live-cell super-resolution microscopy to study DNA-binding proteins in eukaryotic cells (18, 19).

This chapter provides a detailed protocol covering the sample preparation for PALM imaging and data analysis procedures. Similar general principles also apply to other super-resolution microscopy modalities such as STORM. First, the protocol shows the construction of *E. coli* strains carrying an endogenous photoactivatable fluorescent fusion protein using lambda Red recombination (20). As opposed to exogenous plasmid expression systems, this approach maintains native expression levels and ensures complete replacement of the native gene with the fluorescent version. The following steps in the protocol include the preparation of cell cultures for microscopy, PALM data acquisition, and data processing to obtain single-molecule localizations and tracks. Once localizations and tracks have been recorded, there are many options for further analysis. Here, the most common and general approaches are presented, such as reconstruction of super-resolution images, mapping single-molecule tracks, computing diffusion coefficients to identify molecular subpopulations with different mobility, and analysis of DNA-binding kinetics.

The protocol is illustrated using data of DNA polymerase I (PolI), a typical DNA-binding protein with key functions in DNA replication and DNA repair in *E. coli*. Photoactivated single-molecule tracking has been applied to directly visualize binding events of single PolI enzymes at DNA repair sites following DNA alkylation damage (9).

2 Materials

2.1 Lambda Red recombination

1. *E. coli* AB1157 background strain (other *E. coli* K12 strains such as MC4100 or MG1655 can also be used).

2. Plasmid pKD46 (20), encoding the lambda Red integration proteins under an arabinose-inducible promoter. pKD46 carries an ampicillin resistance marker and the temperature sensitive origin of replication repA101ts (to be grown at 30°C).
3. Plasmid encoding the photoactivatable fluorescent protein PAmCherry (21) with an N-terminal flexible linker and an antibiotic resistance marker (e.g. see (9, 22)).
4. PCR primers to amplify the PAmCherry insertion fragment from the template plasmid. The protocol provides guidance on primer design.
5. PCR kit with a high fidelity polymerase.
6. Dpn1 enzyme.
7. LB medium and LB agarose plates.
8. Antibiotics as required for pKD46 plasmid and selection of the lambda Red insertion (e.g. ampicillin, kanamycin).
9. 10% arabinose solution: freshly dissolved in dH₂O and sterilized using a 0.2 µm filter.
10. PCR primers to verify the PAmCherry insertion.

2.2 Cell culture and slide preparation

1. LB medium and LB agarose plates.
2. Supplemented M9 medium. Recipe for 500 ml medium: 100 ml 5x M9 salts, 1 ml 1 M MgSO₄, 500 µl 100 mM CaCl₂, 10 ml 50x MEM amino acids, 5 ml 100 µg/ml L-proline, 50 µl 0.5% thiamine, 5 ml 20% glucose, dH₂O up to 500 ml. Sterilize with 0.2 µm filter.
3. Microscope coverslips (no 1.5 thickness) burnt in a furnace at 500°C for 1 h to remove fluorescent background contamination.
4. Low fluorescence molecular biology grade agarose (e.g. BioRad).

2.3 Microscope

Detailed descriptions of how to design and assemble a custom Total Internal Reflection Fluorescence (TIRF) microscope for single-molecule imaging can be found in (2, 13, 23, 24). Suitable commercial instruments are also available from different manufacturers. The essential components are:

1. 100x NA1.4 oil immersion objective.
2. Electron multiplying CCD camera.
3. 405 nm laser with at least 20 mW output power.
4. 561 nm laser with at least 50 mW output power.
5. TIRF excitation module.

6. Transmitted light illumination.

2.4 Data analysis

1. Automated data processing can be performed in Matlab (Mathworks). Optional toolboxes with useful functions include:
2. Image Processing Toolbox, containing functions to read and write image files, as well as filtering, registration and segmentation tools.
3. Statistics Toolbox, containing tools for plotting data histograms and performing statistical tests.
4. Optimization Toolbox, containing curve fitting functions (e.g. lsqcurvefit).

3 Methods

3.1 Lambda Red integration to generate an endogenous PAmCherry fusion

This protocol follows the method developed by Datsenko and Wanner (20) that employs the phage lambda Red recombinase to generate an endogenous C-terminal PAmCherry fusion with a protein of interest in *E. coli*. The gene encoding PAmCherry with a flexible N-terminal linker is PCR amplified from a template plasmid together with an antibiotic resistance gene that allows for selection of the chromosomal integration.

1. Transform the target *E. coli* strain with plasmid pKD46. This plasmid encodes the Lambda Red integration factors and an ampicillin resistance marker. The transformed strain needs to be grown with ampicillin at 30°C to maintain the temperature-sensitive plasmid.
2. Find the gene sequence of the protein you wish to tag with PAmCherry using the online *E. coli* Genome Browser: http://microbes.ucsc.edu/cgi-bin/hgGateway?db=eschColi_K12
3. Design lambda Red PCR primers flanking the PAmCherry and antibiotic resistance genes on the template plasmid and add overhangs with homology to the chromosomal insertion site. For the forward lambda Red PCR primer, use 40 – 50 nt of homology at the 3' terminal end of the gene just upstream of the stop codon and add a primer sequence at the 5' terminus of the fusion linker on the template plasmid. For the reverse PCR primer, use 40 – 50 nt of homology immediately downstream of the stop codon of the gene and add a primer sequence downstream of the antibiotic resistance gene on the template plasmid.
4. Run a 50 µl PCR to amplify the lambda Red insertion fragment from the template plasmid using a high fidelity DNA polymerase.
5. Add 1 µl of DpnI enzyme to the PCR product and incubate for at least 2 h at 37°C. This digests the template plasmid.
6. Run the PCR product on a 1% agarose gel (e.g. 100 V for 1 h). Cut out the 2.7 kb DNA band and extract the DNA using the Qiagen gel extraction kit.

Elute the DNA in a small volume of dH₂O (e.g. 30 µl) to obtain a concentrated solution.

7. Grow a 5 ml culture of the target strain carrying plasmid pKD46 in LB medium with 50 µg/ml ampicillin at 30°C overnight.
8. Dilute 500 µl of the culture in 50 ml LB medium containing 50 µg/ml ampicillin and 0.2% arabinose. Grow the culture at 30°C until it reaches an OD of 0.6.
9. Place the culture on ice for 10 min and swirl to chill it abruptly.
10. Spin down the culture in a chilled 50 ml tube for 10 min in a centrifuge at 2900 x g, cooled to 4°C.
11. Repeat several cycles of centrifugation and resuspension of the culture in decreasing volumes of ice-cold dH₂O: 50 ml, 35 ml, 2 ml, 500 µl. Keep the culture on ice between steps and centrifuge at 4°C.
12. Add 1 – 6 µl of DNA (typically 30 – 200 ng/µl concentration) to 60 µl of cell suspension and incubate on ice for 15 min.
13. Electroporate the cells with DNA in a pre-chilled cuvette. Following electroporation, immediately add 1 ml of LB medium (at room temperature) and mix gently.
14. Recover the cells at 37°C for 1 h and plate on LB agarose containing the appropriate antibiotic to select for insertions. Incubate plates at 37°C overnight to promote the loss of the temperature sensitive plasmid pKD46.
15. Plates typically show a few up to several tens of cell colonies. Pick several colonies and streak again to isolate single colonies. Replica plate on LB agar with 50 µg/ml ampicillin to confirm the loss of plasmid pKD46.
16. Verify correct lambda Red insertion by colony PCR and sequencing using primers flanking the insertion site.
17. Use P1 transduction (25) to move the PAmCherry fusion allele to a strain which had not been transformed with plasmid pKD46.
18. Functionality of the fusion protein should be assessed by comparing the growth rate of the generated strain to the wild-type strain and using other appropriate assays (e.g. sensitivity to DNA damage for a strain carrying a fusion of a DNA repair protein). See Note 1 for more information.

3.2 Cell culture

The following protocol is for the culture of *E. coli* AB1157 strains exhibiting wild-type phenotypes. Mutant strains may require different growth conditions.

1. Two days before a microscopy session: Streak cells from a frozen glycerol stock onto an LB agar plate containing the appropriate antibiotic to select for the lambda Red insertion strain.

2. The next day, inoculate an LB culture from a single cell colony and grow for 3 – 5 h at 37°C.
3. Add 1 µl of LB culture to 5 ml of supplemented M9 medium and grow overnight at 37°C.
4. The next morning, dilute 25 µl of the culture in 5 ml of supplemented M9 medium and grow for 2 h at 37°C so that cells are imaged during early exponential growth phase (~OD 0.05 – 0.1).
5. Pellet cells in a benchtop centrifuge at 3300 x g for 3 min.
6. Thoroughly resuspend the cell pellet in 10 µl of supernatant medium.
7. Microscopy should be performed immediately after preparation of the concentrated cell suspension.

3.3 Microscopy

1. Prepare 5 ml of a 1% low fluorescence agarose solution in supplemented M9 medium.
2. Spread 1 ml of melted agarose solution on a microscope coverslip and place a burnt coverslip onto the solidifying gel to create a flat pad.
3. Remove the burnt coverslip from the gel pad and drop 1 µl of concentrated cell suspension onto it. Place a new burnt coverslip on top to sandwich the cells.
4. Place the sample on the microscope and bring cells into focus using transmitted light illumination.
5. Activate the electron multiplying gain of the EMCCD camera and display the live camera data. Only background noise should be visible. Typical frame acquisition rates for single-molecule imaging in live cells are between 10 – 100 frames/s.
6. Switch on the 561 nm laser for excitation of PAmCherry and the 405 nm laser for photoactivation. Wait for individual fluorescent spots to appear inside cells and adjust the TIRF illumination angle to maximize the brightness of the spots.
7. Record a short movie, examine the saved images, and optimize the acquisition settings (frame rates & laser intensities) as explained in Note 2.
8. For data acquisition, first expose cells to 561 nm excitation for a few seconds to bleach the autofluorescence background, then start recording a movie. Switch on the 405 nm laser for PAmCherry photoactivation. For tracking experiments, activated molecules should be visible for a few frames until photobleaching. Gradually increase the 405 nm intensity over the course of the movie while the pool of pre-activated molecules depletes. Keep the density of activated molecules sparse at less than one spot per

cell in each frame. Record a movie of several thousand frames until most molecules have been activated (Fig. 1).

9. Several movies of different cells can be recorded for approximately 45 min before the agarose gel starts to dry out.

3.4 Reconstructing a super-resolution map of localizations

1. Use a localization algorithm to identify fluorescent spots and determine their centroid with high precision. Several algorithms have been developed for this purpose, e.g. (26–28). See Note 3 for guidance.
2. Display the resulting localizations as a scatter plot (Fig. 2a-b).
3. Generate a super-resolution image by binning localizations into a two-dimensional colour-coded histogram (Fig. 2c). Alternatively, each localization can be rendered as a Gaussian spot with a width corresponding to the estimated localization error. The super-resolution image is then reconstructed as the super-position of all Gaussian spots (Fig. 2d).
4. The super-resolution image can be used to analyse protein structures (7, 12, 13), foci (8, 10), or partitioning of molecules between different cellular compartments (15, 29).

3.5 Short exposure times to quantify diffusion

In live cells, protein movement can be measured by recording a PALM movie at high frame rates and short exposure times (e.g. 15 ms/frame) and using an automated tracking algorithm.

1. Determine molecule tracks by linking localizations that appear nearby in consecutive frames. Simple algorithms use a fixed tracking window within which localizations get connected (30), while other methods apply global tracking analysis (31). See Note 4 for more information on the following analysis procedure.
2. Compute the mean-squared displacement (MSD) from the (x,y) localizations for each track with at least $N = 5$ localizations: $MSD = 1/(N - 1) \sum_{i=1}^{N-1} (x_{i+1} - x_i)^2 + (y_{i+1} - y_i)^2$.
3. Compute the apparent diffusion coefficient (D^*) per track using the mean-squared displacement: $D^* = MSD/(4 \cdot \Delta t)$. Here, Δt is the time interval between frames.
4. Plot a histogram to examine the distribution of diffusion coefficients. Molecule subpopulations with different mobility (e.g. mobile and bound molecules) can be identified as distinct species in the histogram (Fig. 3).
5. In the case of a bound and a mobile population of molecules, the fraction of molecules in the bound state can be quantified using a threshold on the apparent diffusion coefficient (Fig. 3b-c).

6. An alternative approach to quantify subpopulations with different mobility is to fit the distributions using an analytical equation (5, 11, 15).

3.6 Long exposures to quantify binding kinetics

The time a single photoactivated molecule can be observed for is limited by irreversible photobleaching. This duration is typically only a few frames, which complicates quantification of binding kinetics from tracking data recorded at high temporal resolution. A simple solution to this problem is to reduce laser intensities – which extends the photobleaching lifetime – while equally increasing exposure times to circumvent the reduced emission intensities. At long exposure times, mobile molecules will appear as blurred spots, while bound molecules result in diffraction-limited spots. A spot finding algorithm can be used to extract diffraction-limited spots only, and the average binding time corresponds to the average number of frames a diffraction-limited spot is visible for. It is important to correct for the residual effect of photobleaching on the apparent binding time by measuring photobleaching rates under identical illumination conditions using fixed cells or a sample for which binding times are very long compared to the average bleaching time. Crucially, photobleaching should not be faster than the average binding time because small errors in the estimation of the photobleaching rate will cause very large errors in the estimation of the binding time constant. More information on this type of analysis can be found in reference (9).

3.7 Clustering analysis

Clustering algorithms identify foci of localizations that might represent a spatial organisation of proteins performing their function in cells. Methods to quantify clustering include k-means, nearest-neighbour clustering, and point-correlation analysis. These algorithms report the size and number of localizations per cluster. K-means assigns all observed localizations to a fixed number of clusters. This number has to be defined prior to the analysis. Nearest-neighbour clustering groups localizations that are within a user-defined distance threshold (8). Point-correlation analysis examines the spatial correlation between localizations, and compares the observed distribution to a random homogenous distribution of points (32, 33).

4 Notes

1. Protein functionality can be affected by the fusion to a fluorescent protein. Therefore, care should be taken to closely compare growth characteristics and specific phenotypes of the strain carrying the fluorescent fusion protein to the wild-type strain. For non-essential proteins, functionality of the fusion can also be evaluated by comparison with a strain in which the gene is deleted (34). Additional control experiments can be performed that abolish specific activities of the protein (e.g. by introducing point mutations, drug treatments, or deletion of protein interaction partners).

Furthermore, many fluorescent proteins are prone to dimerize or multimerize which can cause aggregation artifacts of fusion proteins (35, 36). Localization clusters may therefore represent protein aggregates

instead of a genuine spatial organization of the native protein. Moreover, photoactivatable fluorescent proteins typically blink, i.e. they randomly convert between bright and dark states once they have been photoactivated by 405 nm light (37, 38). Each fusion protein may therefore be observed several times, which can result in apparent clustering of tracks and complicates counting molecule numbers. Conventional fluorescence imaging with a monomeric fluorescent protein fusion can be performed to evaluate the genuine presence of clusters (35).

2. Before data acquisition it is important to identify appropriate imaging conditions. To this end, record a short movie, examine the images, and change the microscope settings as required. For example: (A) Increase the 561 nm laser power or use a longer camera exposure time if the fluorescent spots are too dim against the background noise. (B) Decrease the 561 nm laser power or use faster camera exposures if the spots are very clear but appear to bleach very quickly. Estimation of diffusion coefficients requires observing the same molecule for multiple frames. (C) Shorten the camera exposure time if the fluorescent spots appear blurry due to fast movement of the fusion protein during each frame. Appropriate laser intensities and exposure times will vary between instruments due to differences in the alignment and quality of optical components.
3. Microscopy images are subject to several factors that complicate the precise localization of single fluorophores. These factors include image pixelation, camera read noise, electron multiplying noise, background noise, and photon shot noise. Background noise can be reduced by TIRF excitation, while photon shot noise is dictated by the fluorophore brightness. Once all experimental factors have been optimized, the localization algorithm is key to maximize the localization precision (39). Most localization algorithms either use least-squares (26, 40) or maximum likelihood parameter estimation (27, 28).

In addition to minimizing localization error, a key metric for the performance of localization algorithms is the recall, i.e. the fraction of successfully detected spots. The recall fraction decreases with increasing density of fluorescent spots per image. However, high densities are often desired because the time required to record a PALM movie that represents the majority of labelled molecules in the sample is limited by the number of accurate localizations per frame. Moreover, the total density of all localizations dictates the resolution of the reconstructed image according to Shannon's sampling theorem. Several algorithms have been developed that substantially improve the localization recall in images of densely overlapping fluorescent spots (41, 42).

4. Because of photobleaching, the number of localizations per track is usually small, which causes large statistical uncertainty in the estimation of the diffusion coefficient of a single molecule. For this reason, only

tracks with a certain minimum number of steps should be included in the analysis (e.g. at least 5 localizations or 4 steps). The term *apparent* diffusion coefficient is used because this measurement of the diffusion coefficient includes several biases: Confinement of molecules within the cell volume or cellular compartments leads to an underestimation of the diffusion coefficient. Furthermore, molecular movement during the exposure time reduces the apparent diffusion coefficient because the localizations represent time-averaged positions. The localization error, on the other hand, effectively adds a random step to each true position, which causes an overestimation of the diffusion coefficient. This error can be corrected by measuring the average localization error σ_{loc} using a sample with immobile molecules and applying the equation: $D^* = \text{MSD}/(4 \cdot t) - \sigma_{\text{loc}}^2/t$.

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Fig. 1. Example images of a PALM recording.

The transmitted light image shows live *E. coli* cells immobilized on an agarose pad. Cells are expressing a fusion of DNA polymerase 1 (Pol1) with PAmCherry. Example frames of a PALM movie show isolated fluorescent spots of single Pol1-PAmCherry molecules. Different molecules become photoactivated in different frames such that their precise positions can be recorded over time. Scale bars: 1 μm .

Fig. 2. Super-resolution image reconstruction.

(a) Localizations of Pol1-PAmCherry from a PALM recording of 7.500 frames (example frames in Fig. 1) are displayed as a scatter plot. (b) Localizations mapped onto the transmitted light image of cells. (c) Histogram visualization: Localizations were binned into a two-dimensional grid of subpixels ($38 \text{ nm} \times 38 \text{ nm}$, i.e. 4×4 subpixels per original pixel). The number of localizations per bin is represented according to the colours in the scale bar. (d) Gaussian kernel visualization: The image is reconstructed by summing normalized

Gaussian kernels with 40 nm standard deviation (equivalent to the localization precision) centred on the localizations. The boxed regions are shown magnified. Scale bars: 1 μm .

Fig. 3. Photoactivated single-molecule tracking analysis.

Tracks of Pol1-PAmCherry molecules were generated from the localization data in Fig. 2 and shown on a transmitted light image of cells. The histograms show the distribution of apparent diffusion coefficients. Live cells were treated with the DNA-damaging agent methyl methanesulfonate (100 mM MMS) for 20 min before imaging. Base-excision repair of MMS damage creates gapped DNA substrates to which Pol1 binds for DNA repair synthesis. Single-molecule tracking of Pol1 allows identifying such events by the low apparent diffusion coefficient. (a) Data for all tracks with at least 5 localizations. (b)

Detecting individual bound molecules by plotting only tracks with an apparent diffusion coefficient below $0.15 \mu\text{m}^2/\text{s}$. (c) All observed tracks, colour coded by their classification as bound or mobile, in red and blue, respectively. Scale bars: $1 \mu\text{m}$.