PALM Microscope User Manual

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Microscope components:

The laser excitation is delivered from a Toptica Multi Laser Engine with 405 nm, 488 nm, 561 nm, 640 nm lasers via a single-mode optical fibre. The fibre cable is very sensitive and should not ever be bent. Translation of the fibre output and lens assembly perpendicular to the beam allows continuous adjustment of the excitation angle for TIRF, near-TIRF, or brightfield imaging. The fluorescence emission is recorded on an Andor iXon 897 ultra electron multiplying CCD (EMCCD) camera at a magnification resulting in a pixel length of 96 nm/pixel. The ASI controller is used for sample positioning and focusing. The button on top of the joystick switches between coarse and fine x-y positioning. The switch on the controller front switches between coarse (z-motor) and fine (z-piezo) focusing. The controller is also used to set the perfect focus; instructions can be found in the ASI manual.

Laser safety:

The lasers are class IIIB and can cause serious damage if the eye is exposed to a focused beam. The instrument is built such that all beams are contained within the enclosure. The lasers must be switched off when the enclosure is opened to insert or remove a sample. Adjustment of the excitation angle must be performed with the rubber cover shielding the beam.

Switching on the microscope:

1. Switch on the electrical sockets with plugs for the EMCCD camera, Toptica laser box, ASI controller, and the computer + screen.
2. Switch on the computer.
3. Switch on the EMCCD camera via the on/off button at the top of the camera. Start the camera software “Andor Solis”.
4. Switch on the laser box using the key switch. An automatic startup program runs. Start the laser control software “Toptica MLE”. Select connection settings “USB to Serial converter” and connect. If the software is in “Read only” mode, select connections settings “User” with password “palm” and re-connect. Operating instructions can be found in the Toptica Manual.
5. The microscope is ready for use.

Switching off the microscope:

1. Transfer all collected data. Data must not be stored on the local hard drive after the experiments are finished
2. Close the data acquisition programs and shut down the computer.
3. Switch off the EMCCD camera, Toptica laser box, ASI controller.
4. Remove all samples.
Example experiment: Photoactivated single-molecule tracking in live *E.coli* cells.

This protocol describes photoactivated single-molecule tracking using PAmCherry fusion proteins expressed in *E.coli*. The following figures show the concept and representative results.

*Figure 1* Photoactivated single-molecule tracking in live *E.coli* cells. (A) The fluorescent protein PAmCherry can be photoactivated from an initial nonfluorescent state upon irradiation with 405 nm light. The bright state is excited at 561 nm and emits fluorescence around 600 nm until the fluorophore bleaches irreversibly. (B) Controlling the photoactivation rate allows imaging only a single stochastically activated PAmCherry fusion protein per cell at any time while the arbitrarily large pool of molecules that have not yet been activated or have already been bleached remains in a dark state. (C) The position of the fluorescent molecule is determined from the centroid of the isolated PSF and tracked for several frames until photobleaching. (D) Tracks of many molecules are recorded in a sequential manner.

*Figure 2* Photoactivation of DNA polymerase I – PAmCherry fusion proteins in live *E.coli* cells. Scale bars: 1 µm. (A) Transmitted light microscopy image of cells immobilized on an agarose pad. (B) Phototactivating a single PAmCherry fluorophore in one cell. (C) A higher photoactivation rate increases the number of fluorescent molecules. (D) Integrated PAmCherry fluorescence from a PALM movie.
1) **Cell culture:**

1.1) Streak an *E.coli* strain with a PAmCherry fusion gene from a frozen glycerol stock on a Luria Broth (LB) agarose plate with selective antibiotics (here, 25 µg/ml kanamycin) and incubate at 37 °C overnight.

1.2) Inoculate a 5 ml LB culture from a single cell colony and grow at 37 °C shaking at 220 rpm for 3 hours.

1.3) Dilute the culture 1:10,000 into 5 ml minimal medium (M9 medium, 0.2% glycerol) and incubate at 37 °C shaking at 220 rpm overnight.

1.4) The following morning, measure the optical density (OD) using a spectrophotometer and dilute the culture in 5 ml fresh minimal medium to OD 0.025. Grow for 2 hours at 37 °C shaking at 220 rpm to early exponential phase (OD 0.1).

1.5) Concentrate 1 ml of cells in a 1.5 ml microcentrifuge tube by centrifugation at 2,300 x g for 5 minutes. Remove the supernatant and resuspend the cell pellet in 20 µl residual medium and vortex.

2) **Microscope slide preparation:**

2.1) Prepare a 1.5% low-fluorescence agarose solution in dH2O. Use a microwave to melt the agarose until the solution is clear. Mix 500 µl of the melted agarose solution with 500 µl of 2x minimal medium by gently pipetting up and down a few times.

2.2) Spread the agarose solution evenly on the center of a microscope coverslip (No 1.5 thickness). This has to be done quickly before the agarose cools, avoiding bubbles.

2.3) Flatten the pad with a second coverslip (No 1.5 thickness). To remove background fluorescent particles, coverslips were previously burned in a furnace at 500 °C for 1 hour. Burned coverslips can be stored for weeks at room temperature covered in aluminium foil.

2.4) Remove the top slide from the pad and add 1 µl of concentrated cell suspension onto the pad. Immobilize the cells by covering the pad with an unused burned coverslip (No 1.5 thickness, matching the microscope objective specification) and by pressing very gently on the slide. Cells should be imaged within 45 minutes of immobilization before the agarose pad dries. To prevent drying during longer experiments, agarose pads can be sealed using silicon gaskets.

3) **Preparing microscopy data acquisition:**

PALM relies on the detection and precise localization of single fluorescent proteins. The sensitivity and optimal alignment of the microscope is critical for the data quality. Single-molecule fluorescence microscopes typically employ Total Internal Reflection (TIR) illumination to enhance the signal-to-noise ratio by exciting only fluorophores within a thin section above the coverslip surface. Here, imaging inside *E.coli* requires highly inclined illumination or near-TIRF (Ref 6), which can be achieved on a TIRF microscope by slightly decreasing the angle of the excitation light. PALM imaging with PAmCherry requires a 405 nm photoactivation laser and a 561 nm excitation laser. Here, we describe the general protocol for acquisition of a PALM movie for single-molecule tracking. Application of the method to fusion proteins of different molecular weight or copy number per cell will require different acquisition settings (see Discussion section).

3.1) Add a small amount of immersion oil onto the microscope slide.
3.2) Place the sample on the microscope stage with the side of the agar sandwich that holds the cells facing the objective. Set the transmitted light LED illumination using the ASI console command “LED x = 50”. Important: The EMCCD camera gain has to be switched off when the brightfield LED illumination is on to prevent damage to the camera by overexposure.

3.3) Bring the cells into focus using the coarse focus adjustment (Fig. 3A). It is possible to define a cropped FOV to reduce data size and increase the camera read-out speed.

3.4) Camera settings: Define the camera acquisition settings in the Andor Solis software. Typical settings can be loaded through the “load acquisition settings” menu. Data should be saved in .fits format for analysis using the stormtracker MATLAB software. Set the frame rate (for example 15.26 ms/frame, including 0.26 ms camera readout time). See “Exposure time and excitation intensities” in the Discussion section below.

3.5) Switch off the brightfield LED illumination using the ASI console command “LED x = 0”. Switch on the EMCCD camera gain.

3.6) Display the camera data to check the dark background signal (Fig. 3B).

3.7) Switch on the 561 nm laser and check the excitation background signal (Fig. 3C).

3.8) Switch on the 405 nm laser for photoactivation of the PAmCherry fusion proteins and increase the intensity until fluorescence PSFs appear. Adjust the 561 nm excitation intensity to achieve a suitable PSF signal.

3.9) Adjust the angle of the excitation beam to illuminate only a thin section of the sample close to the coverslip surface.

3.9.1) To this end, the laser beam is focused into the back focal plane of a 100x NA 1.4 objective. Translating the focusing lens perpendicular to the beam moves the focus away from the centre of the objective causing the beam to exit the objective under an angle.

3.9.2) Aim to maximize the fluorescence intensity and minimize the background signal. Note that strict TIR excitation is optimal to image fluorophores within 100 nm of the coverslip surface, however, imaging intracellular proteins within B. coli requires deeper illumination up to 0.8 µm.

4) Data acquisition:

4.1) Find a new field of view (FOV) of cells in transmitted light microscopy mode (with LED on and EMCCD gain off) and focus the image. Take a camera snapshot to record the cell outlines (Fig. 3A).

4.2) Switch on the EMCCD camera gain and switch on the 561 nm laser to bleach the cellular autofluorescence and background spots on the coverslip for a few seconds before starting data acquisition. For cells grown and imaged in M9 medium and using burned coverslips there is usually very little fluorescence background; however, pre-bleaching could be useful for imaging cells in a rich growth medium such as LB. Note that intense illumination is toxic to cells so pre-bleaching should be kept to a minimum.

4.3) Start the acquisition of a PALM movie under continuous 561 nm excitation at the chosen frame rate (e.g. 15.26 ms/frame).

4.4) Switch on the 405 nm laser to stochastically activate a subset of PAmCherry fluorophores per frame. Gradually increase the 405 nm intensity over the course of the movie. Avoid high 405 nm intensities that cause cellular autofluorescence. Pay attention to the density of fluorescent
molecules - it is important to keep activation rates low such that PSFs are clearly isolated in each frame (Fig. 3D-F).

4.5) Record typically several thousand frames per movie (depending on the number of molecules to be imaged per cell); note that a single movie requires around 1 GB of hard disk space depending on the size of the FOV and the length of the movie.

4.6) Repeat the acquisition procedure for multiple FOV. Note that each FOV can only be imaged once because PAmCherry fluorophores get photoactivated and bleached irreversibly.

![Figure 3](https://example.com/figure3.png)

5) Data analysis:

Photoactivated single-molecule tracking data can be analysed using custom software written in MATLAB.

5.1) Open MATLAB and navigate to the folder containing the data to be analysed.

5.2) Type “stormtracker” into the MATLAB console to open the data analysis user interface.

5.3) Define an intensity threshold to localize PSFs.
5.4) Run localization analysis by clicking on the “localization” button. Multiple movies can be selected. The algorithm first identifies PSFs in a band-pass filtered image using a Gaussian kernel with 7 pixels diameter. The locally brightest pixel per candidate PSF serves as initial guess for fitting an elliptical Gaussian function. The free fit parameters are: x-position, y-position, x-width, y-width, rotation angle, amplitude, and background offset. The elliptical Gaussian mask accounts for molecular motion during the exposure time, which blurs and deforms the PSF. Details of the localization algorithm can be found in Ref. 8.

5.5) Plot the resulting \((x, y)\) localizations from all frames of the PALM movie onto the transmitted light microscopy image of the same FOV using the “plot localizations” button (Fig. 4A).

5.6) For automated tracking analysis, the MATLAB implementation of the algorithm described in Ref. 7 can be used. Positions that appear in subsequent frames within a user-defined tracking window are connected to form a trajectory. In the case that multiple localizations occur in the same window, tracks are uniquely assigned by minimizing the sum of step lengths. For a detailed discussion of the various considerations when calculating diffusion coefficients from single-particle tracking data, see Ref. 9.

5.6.1) The algorithm uses a memory parameter to account for transient blinking or missed localizations during a track. Here, we set the memory parameter to 1 frame; higher values can be used for tracking fluorophores with long-lived dark states.

5.6.2) Choose a suitable tracking window based on the following calibration steps.

5.6.3) Run the tracking algorithm for a range of tracking window parameters. Calculate the number of measured tracks per cell as a function of the tracking window to identify the smallest possible tracking window that does not split tracks (Fig. 4B).

5.6.4) Plot the resulting tracks on the transmitted light microscopy image of the same FOV to visualize the spatial distribution of molecule movement within cells (Fig. 4C-D).

5.6.5) If a fraction of tracks appears to cross between cells this suggests that separate molecules were erroneously linked because the tracking window was chosen too large and/or the photoactivation rate was too high (Fig. 4E).

5.6.6) Plot the cumulative distribution of the step lengths between consecutive localizations (Fig. 4F). The curve rises and saturates smoothly for sufficiently large tracking windows but shows a cut-off edge if the window was chosen too small.

5.7) To analyze the diffusion characteristics of the PAmCherry fusion protein the mean-squared displacement (MSD) between consecutive localizations for each track with a total of \(N\) steps can be computed:

\[
\text{MSD} = \frac{1}{(N-1)} \sum_{i=1}^{N-1} (x_{i+1} - x_i)^2 + (y_{i+1} - y_i)^2 .
\]

Plot a curve of MSD values over a range of lag times by calculating displacements over multiple frames (Fig. 4G) using the "plot MSD curve" button. The shape of the MSD curve can help to classify the observed molecular motion (Fig. 4H).

5.8) Calculate the apparent diffusion coefficient \(D^*\) per track from the MSD:

\[
D^* = \text{MSD}/(4 \Delta t) - \sigma^2_{\text{loc}}/\Delta t.
\]
The second term corrects for the estimated localization error. Plot a histogram of the measured $D^*$ values from all tracks in the FOV using the "plot D histogram" button.

![Image](image_url)

**Figure 4** Illustration of the tracking analysis. Scale bars: 1 µm. (A) All detected localizations of Pol1-PAmCherry in an example cell. (B) Number of tracks detected in the example cell as a function of the tracking window. Small tracking windows split molecule trajectories, which leads to artifactual high number of tracks. The dashed line indicates our choice for the tracking window parameter (0.57 µm, 5 pixels) – this gives a good compromise between detecting the full distribution of steps and keeping the trajectories of different molecules intact. (C) Example track of a single Pol1-PAmCherry molecule. (D) All measured tracks shown in random colors. (E) Tracking artifacts if the tracking window is chosen too large (here 0.8 µm, 7 pixels) or the density of PSFs per frame is too high. (F) Cumulative distributions of the step lengths for tracking windows: 0.34 µm (3 pixels, red line), 0.57 µm (5 pixels, blue line), and 0.80 µm (7 pixels, green line). Note that the 0.34 µm tracking window cuts off steps longer than 0.34 µm which clearly truncates the full distribution of steps. The 0.57 µm tracking window detects almost the same distribution of steps as does the 0.80 µm tracking window. (G) MSD curve shows confined diffusion of Pol1. (H) Schematic MSD curves for directed motion, Brownian motion, confined diffusion, and immobile particles.

**Discussion**

**Choice and expression of the fluorescent fusion protein:** There is a large palette of photoactivatable and photoswitchable fluorescent proteins (Ref. 11). The specific choice depends on the microscope characteristics, particularly the lasers and filters available. The combination of 405 nm and 561 nm is ideal for common photoactivatable fluorescent proteins. We previously chose PAmCherry (Refs. 4, 5) because it is monomeric and showed no aggregation in cells. Furthermore, irreversible photoactivation allows counting the number of activated fluorophores to measure protein copy numbers per cell. Instead of expressing the fusion protein from a plasmid, we prefer chromosomal insertion of the gene encoding for the fusion protein at the wild-type locus. This ensures complete replacement of the protein of interest with the fluorescent version and maintaining the wild-type expression level.
**Photoactivation rate:** It is important to adjust the photoactivation rate such that on average less than one molecule per cell is in the fluorescent state in any frame of the movie. This depends on the 405 nm intensity and the number of molecules left to be activated. At very low imaging densities, however, not all molecules will be imaged before the end of the movie or very long movies have to be acquired. The number of frames recorded per movie depends on the copy number of fusion proteins per cell and the mean photobleaching lifetime of PAmCherry at the excitation conditions used.

**Exposure time and excitation intensities:** Foremost, exposure times need to be sufficiently short to observe sharp PSFs with little motion blurring. However, the frame rate should be chosen to yield observable molecular motion between successive frames beyond the localization uncertainty; otherwise crucial photons are wasted by oversampling the track. The motion of unbound molecules must be sampled at sufficiently long time intervals to be clearly distinguishable from the apparent motion of bound molecules due to the localization uncertainty. When the exposure time is set, the PSF intensity should be adjusted. The localization precision of a PSF increases with the number of photons detected over the duration of a frame. Higher excitation intensities increase the photon emission rate but also the photobleaching rate and background signal. Use the lowest excitation intensity that gives the desired localization precision.

**Useful References**


