Published in final edited form as:

Methods Mol Biol. 2016 ; 1369: 45–57. doi:10.1007/978-1-4939-3145-3_4.

High-speed super resolution imaging of live fission yeast cells

Caroline Laplante¹, Fang Huang², Joerg Bewersdorf^{2,4}, Thomas D. Pollard^{1,2,3,*}

¹Department of Molecular Cellular and Developmental Biology, Yale University, PO Box 208103, New Haven, CT 06520-8103 USA

²Department of Molecular Biophysics and Biochemistry, Yale University, PO Box 208103, New Haven, CT 06520-8103 USA

³Department of Cell Biology, Yale University, PO Box 208103, New Haven, CT 06520-8103 USA

⁴Department of Biomedical Engineering, Yale University, PO Box 208103, New Haven, CT 06520-8103 USA

Summary

We describe a step-by-step method for high-speed fluorescence photoactivation localization microscopy (FPALM) of live fission yeast cells. The resolution with this method is tenfold better than spinning disk confocal microscopy.

Keywords

Super resolution; fluorescence microscopy; FPALM; live cells; fission yeast

1. Introduction

Imaging cellular structures with a tenfold improvement over classical light microscopy techniques has been achieved using single-molecule switching nanoscopy (SMSN) techniques called PALM, FPALM or STORM [1–3]. These methods localize single fluorescent molecules in a field with nanometer precision as they are stochastically switched on and off. Precise localization of thousands of separately blinking emitters over thousands of consecutive camera frames achieves 25- to 40-nm resolution.

Until recently, data acquisition was too slow for live cell imaging. The readout speed of EMCCD cameras limited the rate of localizing molecules. Furthermore, the photoactivatable or photoswitchable fluorescent proteins available to tag proteins of interest had some limitations, such as oligomerization and/or fluorescence properties. Two recent developments opened the possibility for fast SMSN of live cells: ultra-fast acquisition with the more sensitive scientific complementary metal–oxide–semiconductor (sCMOS) cameras [4,5] combined with brighter monomeric photoconvertible fluorescent proteins such as mEos3.2 [6].

*Correspondence: thomas.pollard@yale.edu.

We show how to combine recent advances in high-speed FPALM [4] with endogenous gene targeting of the fission yeast *Schizosaccharomyces pombe* to achieve super resolution images of endogenously mEos3.2-tagged proteins in live cells on a second time scale.

2. Materials

Prepare all solutions using ultra pure (deionized) water and store them at room temperature (unless indicated otherwise by the manufacturer). Filter sterilize (0.22 μ m pore size filter) all solutions to be used for FPALM imaging.

2.1 Gene targeting components

We followed the general protocols described by Bähler et al. [7].

1. High fidelity polymerase such as Phusion (New England BioLabs) and accompanying buffers and solutions. Store and handle the enzyme according to the manufacturer's instructions.
2. Long primers resuspended to 50 μ M in ultra pure water [7] (Table 1).
3. Fission yeast transformation vectors containing the mEos3.2 DNA sequence to use for generating insertion DNA fragments.
4. dNTPs resuspended in ultra pure water at 10 mM.
5. PCR product purification kit such as spin column DNA purification kit by Qiagen.
6. 10X TE: 100 mM Tris-HCl, pH 7.5, 10 mM EDTA. Adjust pH to 7.5 with HCl and filter sterilize [7].
7. 10X LiAc: 1 M lithium acetate (102 g/mole). Adjust the pH to 7.5 using acetic acid. Filter sterilize [7].
8. PEG/LiAc/TE: 40% (w/v) PEG 3350 (Sigma P4338), 1X LiAc, 1X TE. Filter sterilize [7].
9. 1X TE: Dilute 10X TE with ultra pure water.
10. 1X LiAc: Dilute 10X LiAc with ultra pure water.

2.2 Yeast growth media

For both growth media, we added the 5 supplements (adenine, L-histidine, L-leucine, uracil and L-lysine) for the auxotrophic markers expressed by our strains. Forsburg gives recipes for each [8].

1. Rich yeast medium (YE): Forsburg gives the recipes for YE liquid medium and agar plates [8].
2. Edinburgh minimal medium (EMM) used for imaging: (see Note 1) Forsburg gives the recipe for EMM [8]. Follow the protocol for the ingredients. (see Note 2)

3. 50 mL Pyrex Erlenmeyer flasks with deep baffles and loose caps such as Corning Life Sciences No. 4446–50.
4. Wood applicators (Puritan, REF 807). Autoclave in stainless steel canisters. (*see* Note 3)

2.3 Imaging materials

1. n-propyl gallate to prevent photobleaching: Make a 100X stock (10 mM) solution by adding 0.22 g n-propyl gallate (Sigma P3031) to 100 mL EMM5S. Dilute to 10X (1 mM) with EMM5S, filter sterilize, make 1 mL aliquots and freeze at -20°C . Use at 0.1 mM final concentration in EMM5S imaging medium.
2. Coverslips number 1.5. We use Fisherbrand 22×22 mm No. 12–541-B.
3. Glass microscope slides 25×75 mm $\times$ 1 mm thick. We use Thermo Scientific plain, pre-cleaned slides No. 420–004T.
4. Gelatin pads: Add 0.25 g porcine gelatin (Sigma G2500, gel strength 300, Type A) to 1 mL EMM5S + 0.1 mM n-propyl gallate to obtain a 25% (w/v) gelatin mix. (*see* Notes 4 and 5) Heat to 65°C for 10–15 min. Pipette 30 μL of gelatin onto a clean microscope glass slide and immediately place another glass slide on top to flatten the gelatin into a thin disk about 10 mm in diameter [9] (*see* Note 6, Fig. 1). Store gelatin pads in a sealed, humid container lined with wet paper towels at 4°C for up to 2 days to prevent drying of the pad. Wrap the slides loosely in aluminum foil to avoid contact with the paper towels.
5. VALAP: Combine equal parts by weight of Vaseline, Lanolin and Paraffin wax (flakes work best) in a disposable heat resistant container. (*see* Note 7) Heat the mixture to $\sim 70^{\circ}\text{C}$ in a fume hood until the ingredients mix uniformly. Aliquot VALAP into 1 mL microcentrifuge tubes, cool to room temperature, cap and store at room temperature.

3. Methods

Among the currently available photoactivatable or photoswitchable fluorescent proteins for tagging fission yeast proteins we have experience with mEos2 and mEos3.2, a version of mEos2 mutated to eliminate oligomerization [6]. (*see* Note 8)

3.1 Synthesize long PCR product

1. Design long primers specific to the gene to be tagged. Long primers for insertions are made of two parts; the first section anneals to the vector used as template (Table 1 for sequences to use, Fig. 2 for organization of vector template) and the second section anneals to the genomic sequence flanking the gene of interest (*see* Note 9). Order oligonucleotides and have them purified by polyacrylamide gel electrophoreses (PAGE).

2. Design and order checking primers. Checking primers are used to confirm the insertion of the mEos3.2 tag in the genome. Use Table 2 and the Bähler website to design your primers.
3. Use 0.25–0.5 ng of vector as amplification template DNA per 50 µL PCR reaction. (*see* Note 10)
4. Synthesize the PCR products following the polymerase instructions and using buffers provided by the manufacturers (Fig. 2). (*see* Notes 11 and 12) Keep 1–2 µL of the unpurified PCR product to run by agarose gel electrophoresis.
5. Purify the PCR products using a purification kit with spin column (Qiagen) to remove contaminants from our PCR products. Elute the purified DNA in ultra pure water. (*see* Note 13) Keep 1–2 µL of purified PCR product for DNA gel electrophoresis.
6. Run the samples of unpurified and purified PCR product by DNA gel electrophoresis to ensure the recovery of an amplified PCR product of the expected size.

3.2 Gene targeting in fission yeast

1. Follow Bähler's protocol for fission yeast transformation [7]. We use 50 µL of PCR amplified DNA for each transformation.
2. Plate the transformed cells onto YE5S agar plates, incubate at 25°C for 24 h.
3. Replica plate the transformed cells onto selective media YE5S plates, incubate at 25°C for 3–4 days or until colonies are visible.
4. Select colonies and re-streak onto selective YE5S agar plate. (*see* Note 14)

3.3 Selection of the transformed strains

1. Verify insert by PCR. Using a sterile wood stick, collect yeast cells (a tip full of yeast cells from a healthy culture growing on a YE5S agar plate) and resuspend into 50 µL of ultra pure water, vortex well to mix. Boil for 15 min and spin down at max speed in a tabletop centrifuge for 2 min. Take 10 µL of the supernatant genomic DNA for a 50 µL diagnostic PCR reaction using the mEos3.2 “checking” primers (Table 1 and Bähler website). Run the PCR products by agarose gel electrophoresis. Strains with an insert should have amplified a PCR product of the desired size.
2. Verify expression of the fusion protein by fluorescence microscopy. An abundant protein tagged at its endogenous locus with mEos3.2 can be detected by fluorescence microscopy to confirm its expression. Image the green, non-photoconverted, fluorescent species of mEos3.2 using excitation at 488 nm and the emission filter used to detect GFP. This works well for abundant proteins, especially if they concentrate in a specific organelle or structure (such as the cytokinetic contractile ring), but the green fluorescence of mEos3.2 bleaches

rapidly, making detection of low abundance and/or diffusely distributed proteins difficult.

3.4 Cell preparation for microscopy

Use standard sterile techniques to prevent contamination. Grow cells in liquid media in the dark to prevent photobleaching of the fluorescent protein. Solutions used for imaging are filter sterilized (size of pore 0.22 μm) to remove particles that cause fluorescent background.

1. Grow cells in loosely capped Pyrex Erlenmeyer flasks (volume 50 mL) in a shaking water bath set to 25°C in the dark for 36 to 48 h in 10 mL of YE5S medium prior to imaging. Dilute the cultures twice daily to maintain the cells at OD_{595nm} 0.05 to 0.5. (*see* Note 15)
2. Cell cycle synchronization (Optional): Cross the strain expressing the tagged protein with a *cdc25-22* strain, a temperature sensitive mutation that arrests the cell cycle at the G2-M transition at the non-permissive temperature of 36°C. Select the resulting progeny to express your tagged protein and the *cdc25-22* mutation. Cells with the *cdc25-22* mutation grow very long when incubated at 36°C and this phenotype can be used for selection. The day of imaging, dilute your *cdc25-22* mutant cells to OD₅₉₅ 0.2 and incubate in a shaking water bath at 36°C for 4 h to arrest cells at the G2-M transition. Prior to imaging, return the arrested cell cultures to room temperature (22–25 °C) and proceed expeditiously with the following steps to prepare the cells for imaging as they enter mitosis synchronously.
3. Collect 1 mL of cells (OD₅₉₅ 0.2 to 0.4) by centrifugation at 5000 rpm for 30 s with a tabletop centrifuge and discard the supernatant. (*see* Note 16)
4. Wash the cells by resuspending the pellet in 1 mL EMM5S. Collect the cells as above. Discard the supernatant.
5. Wash the cells by resuspending the pellet in 1 mL EMM5S with 0.1 mM n-propyl-gallate. Collect the cells as above. Discard the supernatant.
6. Resuspend the pellet in 10–50 μL EMM5S with 0.1 mM n-propyl-gallate depending on the desired density of the cell suspension.
7. Pipette 5 μL of cell suspension onto the gelatin pad of a prepared microscope slide. (*see* Note 17) Cover the cells with a coverslip and seal with VALAP.
8. Image the cells immediately.

3.5 FPALM setup and cell imaging

Huang et al. describe the FPALM microscope illustrated in Figs. 3 and 4 [4]. The cost of the two lasers, acousto-optical tunable filter (AOTF), sCMOS camera, optical components, an optical bench and a computer is currently about 100,000 USD. With help from someone knowledgeable about optics and programming, a cell biology laboratory should be able to modify its own fluorescence microscope for FPALM imaging.

1. Focus on cells of interest using bright field illumination, preferably DIC or phase contrast optics. Once the cells are in focus, turn off the bright field illumination.
2. Set the power of the 561 nm imaging laser at the sample plane to 4 kW/cm². (*see Note 18*)
3. Use the AOTF to set the 405 nm photoactivation laser intensity to 0 W/cm² and then increase the power at the imaging plane by 9 W/cm² every 5 s, increasing the photoconversion rate to compensate for the progressive, irreversible photobleaching. Ramping up the photoconversion power is more reproducible with an automated software program (for example, LabView) than by manual adjustments.
4. Acquire images until all of the mEos3.2 in the field has been photoconverted, imaged and irreversibly bleached. Then move on to a new field. Active mEos3.2 “blinks” on and off a few times by temporarily entering a dark state before being photobleached. (*see Note 19*)
5. Set frame rate. We acquire data with a Hamamatsu sCMOS camera at 200–400 Hz. Other frame rates may be more suitable depending on the properties of the sample. If using our data analysis method and a Hamamatsu ORCA-Flash4.0 sCMOS camera, disable the automatic pixel correction in the acquisition software (check for similar settings on cameras from other manufacturers). (*see Note 20*)

3.6 FPALM data analysis

Huang et al. [4] describe the steps to localize the central position of each blinking molecule in each image (Fig. 5). Each pixel of a sCMOS camera has its own noise, so the key to using a sCMOS camera for FPALM is to characterize the camera noise. Use either GPU or CPU processors for the analyses depending on the complexity of the computation. We implemented all algorithms in MatLab, using CUDA-C-MatLab code for the GPU algorithms [4].

1. Characterization of sCMOS camera noise: Take a series of dark images with zero expected incident photon (camera aperture capped and lights turned off in the room) to characterize the offset and variance of each pixel on the camera. Following this step, take a series of images over a range of light levels ranging from 20 to 200 photons per pixel to characterize the gain of each pixel. Huang et al. describe a more detailed protocol [4]. Incorporate these statistics in the single molecule detection, position estimation, uncertainty estimation and rejection processes described below [4].
2. Image Segmentation (IS): Filter the raw, unprocessed sCMOS images, using a series of uniform filters to reduce the peak of the background fluorescence noise. Use a maximum filter to identify local maxima in the filtered image and isolate regions of potential single molecule emitters in square sub-regions of the raw images [10] (Fig. 5).

3. Maximum Likelihood Estimation (MLE): Fit each sub-region with a two-dimensional (2D) Gaussian using a maximum likelihood estimator that incorporates the statistics of sCMOS camera noise [4].
4. Cramér-Rao Lower Bound (CRLB): Estimate the uncertainties of the single molecule position estimates using Cramér-Rao lower bound taking into account the sCMOS camera noise model [4] (Fig. 5).
5. Log-Likelihood Ratio (LLR): Reject overlapping emitters and non-converging fits using a goodness of fitting metric called the LLR. The LLR metric follows a χ^2 distribution with degrees of freedom N-K (N: number of pixels in the subregion; K: number of parameters in the fitting) [4,11] (Fig. 5).

3.7 Reconstruction of super resolution images

These processes generate a list of single molecule coordinates and their corresponding uncertainties for use in quantitation or to reconstruct super resolution images (Fig. 6).

1. Generate 2D histogram images on a fine pixel map (typical pixel size 5 nm) using MatLab. The count for each pixel represents the number of localization within that region.
2. To aid with visualization, convolve the 2D histogram images with a 2D Gaussian [$\sigma = 7.5$ nm] with each localization and display using a color map such as 'Gray' or 'Hot' in MatLab (Fig. 6B).
3. We use the 'Jet' color map in MatLab to color code each localization position according to its time of appearance during acquisition (Fig. 6C). The same information can be used to reconstruct images as time-lapse movies by combining frames acquired over 1–2 s and playing them in succession.

3.8 Quantitation and simultaneous observation of two tagged proteins

Single-molecule switching nanoscopy can be used to count fluorescent emitters [12] [13] and should be valuable in the future for counting absolute numbers of proteins tagged with photoconvertable fluorescent proteins in live cells. In our experience, the numbers of detected single molecules of proteins tagged with mEos3.2 in nodes (precursors of the cytokinetic contractile ring) is proportional to their numbers measured by quantitative fluorescence microscopy [14].

The best photoactivatable proteins have similar emission wavelengths, so they are not useful for two-color live cell imaging. However, we have localized pairs of proteins tagged with mEos3.2 in cytokinesis nodes by comparing the distributions of single molecule detections in cells expressing each protein separately and the two proteins together.

Notes

1. Do not use rich growth medium YE5S for imaging. It exhibits autofluorescence in the range of emission wavelengths used for imaging and will cause an undesired increase in the fluorescence background of your datasets.

2. For EMM5S, we autoclave the dextrose separately from the rest of the ingredients. We make a 20% dextrose solution by dissolving 20 g of dextrose into 100 mL, final volume, of ultra pure water. In a separate flask, we mix the remainder of the ingredients in 900 mL ultra pure water. We autoclave the two solutions separately, mix them together and then add the vitamin stock solutions.
3. We autoclave wood sticks in stainless steel metal canisters made for sterilizing glass pipets.
4. Some sources of gelatin are autofluorescent. Sigma gelatin G2500 has minimal to no autofluorescence at the emission wavelengths used for FPALM.
5. Immediately after adding the gelatin to the liquid medium, cap the tube and vigorously flick the tube to mix the gelatin and liquid together. The gelatin creates a “plug” on top of the liquid if not mixed immediately.
6. When making the pad, the gelatin will spread out too thinly between the glass slides if it is too hot and not enough if it is too cool. This takes a bit of practice.
7. These items can all be purchased at a pharmacy.
8. Some fission yeast strains with genes tagged with mEos2 were sick or inviable, but were healthy when tagged with monomeric mEos3.2.
9. Use the Bähler website (<http://www.bahlerlab.info/resources/>) to obtain the sequences of the primers that anneal to the genomic sequence.
10. We made mEos3.2 vectors by replacing the coding sequence of GFP [6] with the mEos3.2 sequence in the vectors described by Bähler et al. [7]. Our vectors express *Kan* marker for selection in yeast and *Amp* marker for selection in bacteria.
11. We use 0.5 kb/min to calculate the duration of the polymerization step.
12. We use 4 PCR reactions of 50 µL each for one transformation. We combine the products of 4 PCR reactions together, purify them following the manufacturer’s instructions and resuspend the purified DNA in a final volume of 100 µL of ultra pure water.
13. We elute the DNA twice from the spin column by reapplying the first flow through to the column a second time and centrifuging. Some water volume is lost in the spin column, so the recovery is <100 µL. Each transformation reaction takes 50 µL of DNA and diagnostic gel electrophoresis requires 1–2 µL of DNA.
14. This second round of selection eliminates false positive colonies.
15. Allow the cells to adapt to the liquid media by growing them for 36 to 48 h in a shaking water bath set to 25°C in YE5S liquid medium prior to imaging. Failing to do so may result in phenotypic artifacts.
16. Centrifuging fission yeast cells at higher speeds or for longer times causes the nucleus to sediment to one pole of the cell.

17. Insert a clean razor blade between the two glass slides and in one sharp motion separate the slides, exposing the gelatin pad on one surface. Use the exposed gelatin pad immediately to prevent drying of the pad.
18. We set the power to 4 kW/cm² at the sample plane to achieve an average of 200 photons per emitter per frame.
19. The intensity of the 561 nm imaging laser photoconverts mEos3.2 at a high enough rate (without 405 nm illumination) to be useful for focusing on the region of interest of the cells (surface or cross-section through the cell).
20. Huang et al. describe the method to correct for the pixel-dependent noise [4].

Acknowledgements

Research reported in this publication was supported by National Institute of General Medical Sciences of the National Institutes of Health under award number R01GM026132 to TDP, a grant 095927/A/11/Z from the Wellcome Trust to JB, a James Hudson Brown—Alexander Brown Coxe Postdoctoral Fellowship to FH and a HFSP Long-Term Fellowship to CL. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. JB discloses significant financial interest in Bruker Corp. JB and FH disclose significant financial interest in Hamamatsu Photonics K.K.

References

1. Betzig E, Patterson GH, Sougrat R, Lindwasser OW, Olenych S, Bonifacino JS, Davidson MW, Lippincott-Schwartz J, Hess HF (2006) Imaging intracellular fluorescent proteins at nanometer resolution. *Science* 313 (5793):1642–1645. doi:10.1126/science.1127344 [PubMed: 16902090]
2. Hess ST, Girirajan TP, Mason MD (2006) Ultra-high resolution imaging by fluorescence photoactivation localization microscopy. *Biophysical journal* 91 (11):4258–4272. doi:10.1529/biophysj.106.091116 [PubMed: 16980368]
3. Rust MJ, Bates M, Zhuang X (2006) Sub-diffraction-limit imaging by stochastic optical reconstruction microscopy (STORM). *Nature methods* 3 (10):793–795. doi:10.1038/nmeth929 [PubMed: 16896339]
4. Huang F, Hartwich TM, Rivera-Molina FE, Lin Y, Duim WC, Long JJ, Uchil PD, Myers JR, Baird MA, Mothes W, Davidson MW, Toomre D, Bewersdorf J (2013) Video-rate nanoscopy using sCMOS camera-specific single-molecule localization algorithms. *Nature methods* 10 (7):653–658. doi:10.1038/nmeth.2488 [PubMed: 23708387]
5. Ma X, Kovacs M, Conti MA, Wang A, Zhang Y, Sellers JR, Adelstein RS (2012) Nonmuscle myosin II exerts tension but does not translocate actin in vertebrate cytokinesis. *Proceedings of the National Academy of Sciences of the United States of America* 109 (12):4509–4514. doi:10.1073/pnas.1116268109 [PubMed: 22393000]
6. Zhang M, Chang H, Zhang Y, Yu J, Wu L, Ji W, Chen J, Liu B, Lu J, Liu Y, Zhang J, Xu P, Xu T (2012) Rational design of true monomeric and bright photoactivatable fluorescent proteins. *Nature methods* 9 (7):727–729. doi:10.1038/nmeth.2021 [PubMed: 22581370]
7. Bahler J, Wu JQ, Longtine MS, Shah NG, McKenzie A 3rd, Steever AB, Wach A, Philippsen P, Pringle JR (1998) Heterologous modules for efficient and versatile PCR-based gene targeting in *Schizosaccharomyces pombe*. *Yeast* 14 (10):943–951. doi:10.1002/(SICI)1097-0061(199807)14:10<943::AID-YEA292>3.0.CO;2-Y [PubMed: 9717240]
8. Forsburg SL, Rhind N (2006) Basic methods for fission yeast. *Yeast* 23 (3):173–183. doi:10.1002/yea.1347 [PubMed: 16498704]
9. Maddox PS, Bloom KS, Salmon ED (2000) The polarity and dynamics of microtubule assembly in the budding yeast *Saccharomyces cerevisiae*. *Nature cell biology* 2 (1):36–41. doi:10.1038/71357 [PubMed: 10620805]

10. Huang F, Schwartz SL, Byars JM, Lidke KA (2011) Simultaneous multiple-emitter fitting for single molecule super-resolution imaging. *Biomedical optics express* 2 (5):1377–1393. doi:10.1364/BOE.2.001377 [PubMed: 21559149]
11. Wilks SS (1938) The Large-Sample Distribution of the Likelihood Ratio for Testing Composite Hypotheses. *The Annals of Mathematical Statistics* 9 (1):60–62
12. Lee SH, Shin JY, Lee A, Bustamante C (2012) Counting single photoactivatable fluorescent molecules by photoactivated localization microscopy (PALM). *Proceedings of the National Academy of Sciences of the United States of America* 109 (43):17436–17441. doi:10.1073/pnas.1215175109 [PubMed: 23045631]
13. Rollins GC, Shin JY, Bustamante C, Presse S (2015) Stochastic approach to the molecular counting problem in superresolution microscopy. *Proceedings of the National Academy of Sciences of the United States of America* 112 (2):E110–118. doi:10.1073/pnas.1408071112 [PubMed: 25535361]
14. Wu JQ, Pollard TD (2005) Counting cytokinesis proteins globally and locally in fission yeast. *Science* 310 (5746):310–314. doi:10.1126/science.1113230 [PubMed: 16224022]
15. Forsburg SL (1994) Codon usage table for *Schizosaccharomyces pombe*. *Yeast* 10 (8):1045–1047. doi:10.1002/yea.320100806 [PubMed: 7992504]

Fig. 1. Diagram of gelatin pad. A 30 μL aliquot of hot gelatin sandwiched between glass microscope slides flattens into a pad about 10 mm in diameter [9].

Fig. 2.

Schematic of cassettes used as PCR templates to generate the fragments to be used to tag a protein coding sequence in the genome on the C-terminus (top, pFA6a-mEos3.2-kanMX6) or N-terminus (bottom, pFA6a-kanMX6- P_{GOI} -mEos3.2). Thin arrows within the boxes show directions of transcription. Large arrows outside the boxes indicate PCR primers for amplifying the insertion cassette (bottom) and checking primers to verify insertion (top). The primers are not to scale. See Table 1 for primer sequences annealing to the vector and Table 2 for checking primer sequences. For the N-terminal tagging cassette, insert the promoter for the gene of interest into the Bgl II-Pac I site. The breaks in the box for P_{GOI} (Promoter of Gene Of Interest) indicate that promoters vary in length.

Fig. 3.

Simplified schematic of custom-built FPALM setup with a sCMOS camera modified from [4]. Beams from a 405 nm laser (CrystaLaser model DL-405-50; 50 mW output power) and a 561 nm laser (MBP Communications model F-04306-102, 500 mW output power) are combined and sent through an acousto-optic tunable filter (AOTF_{NC-400.650-TN}, AA Opto-Electronic). Lenses f1 and f2 expand the beam. Lens f3 focuses the laser beams into the back focal plane of the objective (alpha PlanApochromat 100x/1.46 Oil DIC, Zeiss) on an inverted microscope stand (Axio Observer D1, Zeiss) for wide field illumination. The emitted fluorescence from a single fluorophore such as mEos3.2 is collected by the objective and separated from the excitation light by a dichroic mirror (Di01-R405/488/561/635, Semrock; for a setup limited to imaging of the mEos3.2 detection channel only, a single-edge dichroic should be used) and two bandpass filters (FF01-446/523/600/677 and BLP01-635R-25, Semrock) before being focused on the chip of the sCMOS camera (Orca Flash 4.0, Hamamatsu). Relay optics (lenses f4 and f5) magnify the image so that the camera pixel size corresponds to ~103 nm in the sample.

Fig. 4. Photograph of FPALM microscope with labeled components and the light paths from the two lasers traced in blue and green. The laser clean-up filter provides high transmission of the desired wavelengths while blocking unwanted wavelengths. Visible components from the schematic in Fig. 3 are labeled.

Fig. 5.

Analysis and reconstruction of super resolution images. Left, flow chart of image processing steps. Right, analysis of a single sCMOS camera frame with three single molecule emitters. The image analysis steps, color-coded in the flow chart, are highlighted on the picture using the same color code. The key to using a sCMOS camera is to generate the pixel-dependent noise map that includes the mean (offset), variance and the amplification gain of each pixel [4]. This map is then applied at each step required to localize single-molecule emitters and generate a cumulative coordinate map of emitter locations for the final image reconstruction.

Fig. 6.

Comparison of confocal and FPALM super resolution fluorescence micrographs of the fission yeast cytokinetic ring expressing the myosin-II regulatory light chain Rlc1p fused to fluorescent proteins. A. Spinning disk confocal micrograph of single focal plane of a cell expressing Rlc1p-tdTomato. The cytokinetic ring is pixelated and blurry with no obvious structural details. B, C. FPALM reconstruction images of a cell expressing Rlc1p-mEos3.2. B. An image reconstructed from 2000 camera frames (acquired at 200 fps for 10 s) and color coded for intensity with MatLab Hot map showing structural features of the cytokinetic ring. C. An image reconstructed from 5000 camera frames (acquired at 200 fps for 25 s) and color coded for time with the MatLab Jet map. This image shows movements of features inside the ring. Scale bar: 100 nm.

Table 1:

Sequences of long insertion primers. The sequences listed in this table anneal to the vector only. The sequences that anneal to the genomic sequences are generated by the Bähler website (<http://www.bahlerlab.info/resources/>). Use GGAGGTGGAGGT as forward 4-glycine linker sequence for forward primers (C-terminal tagging) and use ACCTCCACCTCC as reverse 4-glycine linker sequence for reverse primers (N-terminal tagging), GGA and GGT being the two most abundant glycine codons in the fission yeast genome [15].

Vector	Forward primer sequence	Reverse primer sequence
<i>pFA6a-mEos3.2-KanMX6</i>	ATGAGTGCGATTAAGCCAGAC	GAATTCGAGCTCGTTTAAAC
<i>P_{GOR}-pFA6a-mEOS3.2</i>	GAATTCGAGCTCGTTTAAAC	TCGTCTGGCATTGTCAGGCA

Table 2 :

Sequences for the checking primers of genes tagged at their endogenous genomic loci. These primers anneal to the mEos3.2 gene sequence. The primers that are not provided anneal to the sequence of the tagged gene and can be generated using the Bähler website or generated using the gene sequence.

Checking primer sequences	Forward primer	Reverse primer
C-terminal tagged genes	Design using Bähler website	GCATAACTGGACCATTGGCGG
N-terminal tagged genes	GGTGCAAGTTACCAGGCCGG	Design using Bähler website