A protocol to quantify protein copy number in super-resolution using DNA Origami as a calibration standard

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Method Article

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Abstract

The development of methods able to access a precise molecular counting of protein copy numbers is essential, clearing the way to address several biological questions using super-resolution techniques based on single molecule localization. Here we provide a detailed protocol for the use of DNA origami in combination with GFP antibodies as a versatile platform for quantifying protein copy number in immunofluorescence based super-resolution microscopy. This calibration method is suitable to quantify the average protein copy number in a cell and to determine the abundance of various oligomeric states.

Introduction

DNA origami are as a versatile tool for quantifying protein copy number in immunofluorescence based super-resolution microscopy such as stochastic optical reconstruction microscopy \(STORM). Here we present a detailed protocol, describing all the required steps: sample preparation, imaging and image analysis to quantify proteins at the cellular level.

Procedure

1) DNA Origami Assembly: • Fold the 12-helix bundle DNA origami chassis structures in DNA origami folding buffer \(5 mM Tris \[pH 8.0], 1 mM EDTA and 16 mM MgCl2) by mixing 100 nM p8064 scaffold with 600 nM core staples, 3.6 µM handle staples, 3.6 µM biotin staples, and 9 µM fluorophore anti-handles \[1,2]. • Run the thermal folding cycle \(rapid heating to 80°C and cooling in single degree increments to 65°C for 75 min, followed by cooling in single degree increments to 30°C for 17.5 hr). • Folded chassis can be stored at either 4C or -20C. 2) Preparation of BG-oligonucleotides • Equilibrate Microbiospin6 columns in buffer \(10mM TRIS ph8, 150mM KCl, 10%v/v Glycerol). • Prepare BG-NHS and oligos: Dilute A* antihandle \(NH2) in water to 2mM, Dilute BG-NHS to 20mM in non-aqueous DMSO. • Mix at RT for 30 min: o 16 ul 2 mM A* NH2, o 32 ul 200 mM HEPES ph8.5 o 48 ul 2 mM BG-NHS • Filter in 0.1 ultrafree MC durapore membrane centrifuge 12000g, 1-4min. • Purify BG-oligos using MicroBioSpin6 columns applying 24 ul BG-oligos and centrifuging 4X times for 1 min at 1000g at 4o \[1-3]. • Store at -20C. 3) Dynein-GFP Purification: • Grind yeast cells diluted in Dynein lysis buffer \(30 mM HEPES \(pH 7.2), 50 mM KAcetate, 2 mM MgAcetate, 1mM EGTA, 10% glycerol, 1 mM DTT, 0.5 mM Mg-ATP, 1 mM Pefabloc) and spin for 1 hour at 60K rpm at 4o. • Incubate the supernatant with equilibrated IgG Sepharose beads and nutate for 1-2 hours at 4o • Wash the beads twice with 1X wash buffer \(30 mM HEPES \(pH 7.2), 250 mM KCl, 50 mM KAcetate, 2 mM MgAcetate, 1mM EGTA, 10% glycerol, 1 mM DTT, 0.5 mM Mg-ATP, 1 mM Pefabloc, and 0.1% TritonX-100). • Wash the beads twice with 1X TEV buffer \(10 mM Tris \(pH 8.0), 150 mM KCl, 0.5 mM ATP, 1 mM DTT, 1 mM Pefabloc) and incubate them \(20 minutes at RT) with BG-oligonucleotides \(20 µM). • Wash three times in TEV buffer and then incubate with TEV protease \(1:100 in TEV buffer) for 1 hour at 16oC with rotation. • Remove the beads with centrifugal filters and concentrate the protein with Amikon 100K and freeze in LiqN2 \[1-4]. • Store at -80C. 4) Preparation of DNA Origami samples for super-resolution imaging: • Prepare coverslip washed with KOH \(1M) 1M for 5’. Rinse 3 times and incubate with 100 µL of BSA-Biotin \(0.5mg/ml) for 20 min

RT. • Rinse 3 times in miliQ water and incubate with 100 µL Streptavidin \(0.5mg/ml) for 20’ RT. • Rinse with PBS and incubate with Carboxyl Fluorescent Particles \(Yellow, 1% w/v Spherotec SPH-CFP-0252-2, diameter 111nm 1:30.000) for 10 min. • Block the coverglass in blocking buffer containing 10% \(wt/vol) BSA \(Sigma) in DAB solution \(30mM Hepes, 50 mM KAcetate, 2 mM MgAcetate, 1mM EGTA 7.5, 10% glycerol, 1 mM DTT, 1 mM Mg-ATP, 2.5 mg/mL casein) for 10-30 minutes at room temperature. • Mix 2 µL of purified dynein \(300nM) with 2 µL of DNA origami solution \(3nM) for 30’ on ice. • Diluted motor-chassis complexes to 30 pM concentration in DAB* blocking buffer are incubated for 5’ RT on pre-cleaned and functionalized coverslip. • Rinse gently 2x in DAB* blocking buffer at 4oC. • Block with DAB* blocking solution for 15’ at 4oC. • Add primary antibody \(chicken polyclonal anti GFP, Abcam 13970) diluted 1:2000 in DAB* blocking buffer and incubate for 1h at 4oC. • Rinse 3x in DAB* blocking solution at 4oC. • Add donkey-anti chicken secondary antibody with photoactivatable dye pairs for STORM \(A405/A647) diluted 1:50 in DAB* blocking solution for 1h at 4oC • Rinse DAB* 3x in DAB blocking solution. • Add imaging buffer freshly prepared and containing GLOX solution as oxygen scavenging system \(40 mg/mL-1 Catalase \[Sigma], 0.5 mg/ml-1 glucose oxidase, 10% Glucose in PBS) and MEA 10 mM \(Cysteamine MEA \[SigmaAldrich, #30070-50G] in 360mM Tris-HCl) and supplemented with 2.5 mg/mL casein. 5) Imaging Protocol: • Set the readout power to the desired power and frame rate. • Set the activation power to ensure sparseness of the imaged molecules. • Image the DNA origami with a fixed routine based on 1 activation frame followed by 3 readout frames. • Acquire until all the molecules are completely bleached. • Change the imaging buffer every 2 hours to ensure stability of the photophysical properties of the photo-switchable dyes. For example, we used the following imaging parameters: N of frames=85000; Routine with 1 activation frame and 3 subsequent readout frames; Readout power=~1KW/cm2 for the 647 nm; Activation power<25W/cm2 using the 405 nm; Frame rate= 25Hz frame rate; 6) Image Analysis: • Analyze the DNA Origami data using the exact same parameters used for biological data: same localization method \(Least Squares Fitting, Maximum Likelihood Estimation etc...), same threshold for peak identification, same molecule linking and rejection criteria. For example, we used the following image analysis parameters: Least squares Fitting method; a threshold is set at 2000 counts for considering a molecule for further analysis, spots smaller than 200nm and larger than 500nm were excluded from further analysis; molecules localized in positions closer than 16nm are considered the same molecule and linked together • Run the deco.m code. • Load the .txt file of data by clicking the "Load data" button. A dataset \(DNAorigami_3motors.txt) is provided for testing. • Fit the data by clicking the "Fit" button. Fittings are performed by a two-step numerical minimization of the objective function, obtained as a weighted sum of the negative log-likelihood plus the entropy. At the end of the analysis the Nmax for which the objective function is the minimum will be shown. • Save the results by clicking the "Save output" button, it will generate a *.txt file containing information on the parameters used and the results of the fitting. • Simulate expected distribution of stoichiometries using the synthetic origami data to test the performance of the method as follows: Launch the deco.m code. Set the "Type of pdf" \(LogNormal or Exponential) you want to use, depending on the calibration data, and set the function parameters. Set N, the "number of pdfs" fn that compose your expected distribution. If "only even components" is checked, only the even functions \(i.e. with n=2**k, with k=1, 2,..., floor\(N/2)) will be considered. The calibration functions will be displayed in the plot on the bottom-left corner of the GUI. By

clicking the "Simulate" button, a window will pop up, asking for the "Number of samples" to simulate and to input the values of the relative weights \(wn) for the n pdfs to be simulated. By clicking OK, another window will pop up to choose the name and the path of the file to be saved. The file will be saved as **.txt, composed by a column of integers. Fit the simulated data using the deco.m code and compare the fitting results with the known distribution of monomers/dimers. The latter can be quantitate using some goodness of fit estimator. Make sure that the fit produces a reasonable estimation of your expected distribution. If the data are not satisfactorily fitted, try to increase the number of sample.

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