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Nanometer resolved single-molecule colocalization of nuclear factors by two-color super resolution microscopy imaging

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ABSTRACT

In order to study the detailed assembly and regulation mechanisms of complex structures and machineries in the cell, simultaneous *in situ* observation of all the individual interacting components should be achieved. Multi-color Single-Molecule Localization Microscopy (SMLM) is ideally suited for these quantifications. Here, we build on previous developments and thoroughly discuss a protocol for two-color SMLM combining PALM and STORM, including sample preparation details, image acquisition and data postprocessing analysis. We implement and evaluate a recently proposed colocalization analysis method (aCBC) that allows single-molecule colocalization quantification with the potential of revealing fine, nanometer-scaled, structural details of multicomponent complexes. Finally, using a doubly-labeled nuclear factor (Beaf-32) in *Drosophila* S2 cells we experimentally validate the colocalization quantification algorithm, highlight its advantages and discuss how using high molecular weight fluorescently labeled tags compromises colocalization precision in two-color SMLM experiments.
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1. Introduction

DNA–protein interactions mediate a vast amount of cellular processes. At the nuclear level they control essential DNA processes (e.g. DNA transcription, repair and segregation) and at the whole cell they can regulate complex interaction networks controlling, among others, cell cycle, metabolism and homeostasis. In particular, chromatin remodelers and the transcription machinery are essential players in the regulation of gene expression. A large set of approaches have been developed to study the mechanisms of protein-protein and protein-DNA interactions in the nucleus. Two main limitations arise when employing some of the most widely extended approaches. On the one hand, *in vivo* high-throughput technologies (e.g. bacterial one-hybrid, DNase footprint, chromatin immunoprecipitation, luminescence technologies, chromosome conformation capture [1,2]) average population information and hinder access to the single-cell dynamics. On the other hand, *in vitro* bulk, single-molecule and structural studies (e.g. electrophoretic mobility shift assay, fluorescence anisotropy, stop-flow, atomic force microscopy, optical and magnetic tweezers, X-ray crystallography, Nuclear Magnetic Resonance and Electron Tomography [3,4]) study the interacting partners outside the cellular context in which additional elements can play specific and perhaps essential roles (e.g. molecular crowding, presence of known or unknown partners).

In vivo fluorescence microscopy overcomes the above mentioned limitations and enables the non-invasive observation of protein organization and localization in live cells with high specificity. Multicolor fluorescence imaging of cellular components has the potential to reveal spatial proximity (colocalization) of the labeled species. To study molecular interactions on the <10 nm length scale Förster Resonance Energy Transfer (FRET) techniques are most commonly applied [5]. At larger distances, the colocalization or the degree of spatial coincidence between the molecular species of interest are estimated in a qualitative or quantitative manner using diffraction-limited microscopies [6]. However, the sensitivity of this colocalization strategies is limited by the maximum resolution attainable in standard fluorescence microscopy (~250 nm) and by the fact that the fluorescence signal collected will arise from a population of molecules emitting simultaneously within the diffraction-limited volume, averaging the heterogeneity and dynamics of protein-protein and protein-DNA interactions.

Over the past decade, several subdiffraction resolution imaging techniques have undergone remarkable developments in instru-

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mentation and analytical tools, which allow the investigation of nanometer-sized cellular components. The three main approaches are known as Structured Illumination Microscopy (SIM), Stimulated Emission Depletion Microscopy (STED) and Single-Molecule Localization Microscopy (SMLM) [7–9]. SMLM methods combine the stochastic photoactivation of a single fluorophore per diffraction-limited volume at any given time and its spatial localization with nanometer precision. From the coordinates of all localized emitters, a reconstructed image at super-resolution (~20–30 nm) can be obtained. Several SMLM strategies have been designed differing mostly in the fluorescent probe employed and the mechanism for achieving stochastic photoactivation, with Photo-activated localization microscopy (PALM/fPALM) and Stochastic Optical Reconstruction Microscopy (STORM/dSTORM) being the most widely applied methods [10–14]. So far, these technologies have revealed novel properties of various subcellular structures and machineries. Hence, combining multi-color labeling with SMLM imaging should allow the simultaneous observation, at the single-molecule level, of two or more components in molecular assemblies, revealing mechanistic and regulatory aspects that so far remain unattainable. The number of probes to perform efficient multi-color labeling in SMLM experiments is limiting mostly due to spectral overlap, particularly when it comes to genetically encoded probes [15,16]. Successful strategies have proven the potential of two-color imaging using both photoactivatable proteins [17–20] and single [21,22] or multiple [23–25] synthetic fluorophores. However, despite its great potential, multi-color SMLM methodologies are yet to spread amongst the biological community, possibly due to limitations in the implementation of these complex technologies. In particular, the existing bibliography is limited on practical aspects of SMLM, such as stringent protocols for sample preservation and highest labeling density, detailed imaging conditions and thorough data interpretation.

In this article, we build on previous developments and implement an improved protocol for two-color SMLM using PALM/dSTORM. We describe thoroughly sample preparation, cell fixation strategies, antibody labeling for dSTORM as well as setup and software adaptations for optimal imaging. We propose a detailed pipeline to perform sequential two-color SMLM imaging and discuss acquisition conditions, data post-processing including single molecule localization, chromatic and drift-related corrections, image segmentation and final image reconstruction. We discuss in detail the implementation of a new algorithm (aCBC) using a statistical estimator of the colocalization between two molecular species and perform a wide set of simulated SMLM datasets to evaluate the robustness and reproducibility of the method. We also thoroughly explore aCBC input space-parameter values to define optimal colocalization criteria and quantification according to structural properties of the observed molecular species. Finally, employing as a validation model a double labeled nuclear protein (Beaf-32) in *Drosophila melanogaster* (*D. melanogaster*) cells, we experimentally evaluate the performance of the colocalization analysis and highlight the advantages of primary over secondary antibody labeling to increase the colocalization precision results in two-color SMLM.

2. Materials and methods

2.1. Sample preparation

The protocol discussed in this section provides key steps for sample fixation and labeling for multicolor SMLM imaging of nuclear structures in *Drosophila* cells. The sample preparation conditions have been optimized to yield the highest labeling specificity and fluorescent signal. Sample particularities such as cell type, cell compartment and target molecule were taken into account and several protocols were evaluated as discussed for instance in [26,27]. Note that the handling and seeding of cells presented below can also be performed on non- or semi-adherent mammalian cell lines. All materials employed in this work were of analytical grade. All steps are to be carried out at room temperature unless otherwise indicated.

2.1.1. Cell culture and transfection with Beaf-32-mEos2

Drosophila S2 cells were grown in Schneider's Drosophila medium (Gibco) in cell culture flasks (Nunc) supplemented with 10% fetal bovine serum at 25 °C. The mEos2-Beaf-32 sequence was synthesized by Clontech before cloning into the plasmid pMT/V5-His-TOPO (DES TOPO TA Expression kit, LifeTechnologies) and used for the following transfection protocol. Transfections were performed in S2 cells that were plated at 2 million cells per well in a 6-well plate containing 2 ml of Schneider's medium per well. Cells were incubated for 2–3 h at 25 °C and transfected with Effectene reagent (Qiagen) in a mix containing 100 μl EC buffer, 0.4 μg pMT/V5-His-TOPO mEos2-Beaf-32, 3.2 μl enhancer, 20 μl Effectene. After 24 h, copper sulfate $CuSO_4$ (250 μM) was added to activate the Drosophila metallothionein (MT) promoter for metal-inducible expression of the Beaf-32 gene. The day after (i.e. 48 h after the transfection), cells were harvested.

2.1.2. Cell fixation and permeabilization

S2 cells were fixed with 4% PFA (Electron microscopy sciences) for 15 min at room temperature (RT) and next washed three times with Phosphate-buffered saline solution (PBS) for 5 min at RT. Next, plasma and nuclear membranes were permeabilized with Triton X-100 (Sigma) 0.5% for 5 min at RT and washed three times with PBS for 5 min at RT. To ensure reproducibility, all solutions are made fresh prior to each labeling experiment.

2.1.3. Affinity staining of nuclear proteins using antibodies (Immunofluorescence)

After permeabilization, nonspecific antibody binding sites were blocked with 10% bovine serum albumin (BSA) at RT for at least 1 h. Next, S2 cells were incubated with custom-raised rabbit antibodies directed against Beaf-32 (Eurogentec) at a concentration of 5 μg/ml at 4 °C for 12–16 h followed by three PBS washes. For direct immunofluorescence experiments, anti-Beaf-32 antibodies (primary antibodies) were coupled to Alexa Fluor 647 (AF647, Molecular Probes, Invitrogen) at an average density of 1–1.5 fluorophores per antibody molecule. For indirect immunofluorescence, AF647 coupling was performed on goat anti-rabbit Fab2 antibody fragments (secondary antibodies, Jackson ImmunoResearch) instead of on primary antibodies. Secondary antibody incubation was performed subsequently to unlabeled primary antibodies at a concentration of 5 μg/ml at 4 °C for 12–16 h preceded and followed by three PBS washes.

Antibodies were validated and their concentrations were determined to ensure for the best labeling density in SMLM experiments with minimal nonspecific labeling. Control experiments were performed to validate specificity of primary antibodies using RNAi and that of secondary antibodies through a negative control without primary antibody incubation, as discussed in [28,29].

Custom fluorescent labeling of affinity-purified antibodies (at a concentration >2 mg/ml) with the desired fluorophore to antibody molecular ratios was performed in PBS (pH 7.4) at RT for 1h30. The amount of AF647 succinimidyl ester added was calculated to obtain a antibody:fluorophore mass ratio of 30:1. The labeling reaction was stopped with 10 mM final concentration of Tris pH7.5. Unreacted dye molecules were discarded using dialysis.

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