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Effect of Labeling Density and Time Post Labeling on Quality of Antibody-Based Super Resolution Microscopy Images

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Abstract

Super resolution microscopy (SRM) has overcome the historic spatial resolution limit of light microscopy, enabling fluorescence visualization of intracellular structures and multi-protein complexes at the nanometer scale. Using single-molecule localization microscopy, the precise location of a stochastically activated population of photoswitchable fluorophores is determined during the collection of many images to form a single image with resolution of ~10–20 nm, an order of magnitude improvement over conventional microscopy. One of the key factors in achieving such resolution with single-molecule SRM is the ability to accurately locate each fluorophore while it emits photons. Image quality is also related to appropriate labeling density of the entity of interest within the sample. While ease of detection improves as entities are labeled with more fluorophores and have increased fluorescence signal, there is potential to reduce localization precision, and hence resolution, with an increased number of fluorophores that are on at the same time in the same relative vicinity. In the current work, fixed microtubules were antibody labeled using secondary antibodies prepared with a range of Alexa Fluor 647 conjugation ratios to compare image quality of microtubules to the fluorophore labeling density. It was found that image quality changed with both the fluorophore labeling density and time between completion of labeling and performance of imaging study, with certain fluorophore to protein ratios giving optimal imaging results.

Keywords

super resolution microscopy; single molecule localization microscopy; photoswitching; small molecule fluorophore

1. INTRODUCTION

Super resolution microscopy (SRM) has overcome the historic spatial resolution limit of light microscopy, enabling fluorescence visualization of intracellular structures and multi-protein complexes at the nanometer scale.^{1,2} Using single-molecule localization microscopy, the precise location of a stochastically activated population of photoswitchable fluorophores is determined during the collection of many images to form a single image with resolution of ~10–20 nm, an order of magnitude improvement over conventional microscopy.³ One of the key factors in achieving such resolution with single-molecule SRM is the ability to accurately locate each fluorophore while it emits photons.^{4,5} Localization precision is positively correlated with photon output and inversely correlated to the amount of time the fluorophore spends in the fluorescence “on” state because accuracy of localization precision of individual molecules improves with higher photon number and emitted photons for multiple fluorophores within the sample are less likely to overlap, respectively.^{6,7} Image quality however, is not only related to localization precision of individual molecules, but also to the key aspect of appropriate labeling density of the entity of interest within the sample.^{8,9} While ease of detection improves as molecules of interest are labeled with more fluorophores and have increased fluorescence signal, there is potential to reduce resolution with an increased number of fluorophores that are on at the same time in the same relative vicinity for SRM imaging.

As an example of this, the current work demonstrates the quality of super resolution images of fixed microtubules that were labeled via indirect immunofluorescence staining with a range of fluorophore densities while maintaining the primary and secondary antibody concentration across samples. The range was selected based on fluorophore to protein ratios used for various single molecule localization and other imaging applications. Fluorophore to protein ratios of less than 1:1 are used in the evaluation of single molecule photoswitching properties⁶ while ratios of multiple fluorophores per protein are common in conventional fluorescence microscopy to enhance detected fluorescence signal.¹⁰ Images were evaluated qualitatively based on the density of the fluorescence signal detected along the microtubule structures and by the contrast of the microtubules to the background. Imaging was performed at two time points after labeling, the same day and the following day, to evaluate labeling density and stability over time. It was found that image quality changed with both the fluorophore labeling density and the time interval between labeling and imaging, with certain fluorophore to protein ratios giving optimal results at both time points.

2. METHODOLOGY

2.1 Fluorophore-labeled antibodies

The photoswitchable fluorophore Alexa Fluor 647, succinimidyl ester form (Life Technologies) was conjugated to donkey anti-mouse secondary antibody (Jackson ImmunoResearch) in a range of fluorophore to antibody ratios. The conjugation reactions were set up with 6.5×10^{-10} moles of antibody in phosphate buffered saline adjusted to pH 8 with disodium phosphate with a variety of molar ratios of Alexa Fluor 647 (Table 1). The reactions were mixed for three hours at room temperature while protected from light before concentrating with 10 kDa molecular weight cut off spin filters (EMD Millipore) and further

purifying with desalting columns (Zeba Spin, Thermo Scientific). The final fluorophore to antibody ratios (Table 1) were calculated using absorbance maxima measurements at 280 nm to quantify protein and 650 nm to quantify Alexa Fluor 647. Alexa Fluor 647 had been conjugated to donkey anti-mouse several times at the 1:1 ratio setup resulting in slightly different final ratios.

2.2 Immunofluorescence Labeling of Microtubules

SKBR3 cells were grown in 8-well chambered coverglass slides (In Vitro Scientific) to ~75% confluency. After washing with PBS, the cells were extracted for 20 seconds with 0.5% Triton X-100 (Sigma Aldrich) in PEM (100 mM PIPES (pH 7.0), 1 mM EGTA and 1 mM MgCl_2), fixed for 10 min with 3.2% paraformaldehyde and 0.1% glutaraldehyde (Electron Microscopy Science) in PEM, and washed with PBS. Cells were then reduced for 10 min with 10 mM sodium borohydride, washed with PBS, and blocked/permeabilized for 30 min with 3% bovine serum albumin (EMD Millipore) and 0.5% Triton X-100 in PBS (BSA/Triton X-100). Cells were incubated with Bovine Alpha-Tubulin Mouse mAb (Life Technologies) at 2 $\mu\text{g}/\text{ml}$ in BSA/Triton X-100 for 30 min, washed with PBS, incubated with fluorophore-labeled donkey anti-mouse secondary antibody at 1×10^{-7} M antibody concentration in BSA/Triton X-100 for 30 min, fixed with 0.25% glutaraldehyde in PBS for 10 min and washed with PBS.

2.3 Super Resolution Microscopy Imaging

Imaging was performed on a Nikon ECLIPSE Ti-U inverted microscope equipped with a Nikon 60x oil immersion objective (NA = 1.49) using total internal reflection fluorescence (TIRF) configuration of the light path. Samples were excited with a 647 nm laser line (Coherent OBIS) at a fluence rate of 1.11 kWcm^{-2} . Videos were collected through a 708/75 nm single-bandpass filter (Semrock). An electron-multiplying charge coupled device camera (Photometrics Evolve) recorded videos in a 256×256 pixel area with 107 nm/pixel using a 100 ms exposure time and a fixed gain setting. All videos were collected for 10,000 frames.

Cells were imaged in imaging buffer consisting of Tris-buffered saline (TN buffer, 50 mM Tris pH 8.0 and 10 mM NaCl) with oxygen scavenger components including 0.5 mgml^{-1} glucose oxidase, $40 \text{ }\mu\text{gml}^{-1}$ catalase, and 10% w/v glucose, as well as the reducing component 10 mM β -mercaptoethylamine. Gold fiducial markers (BBi International) were added to correct for image drift during imaging and utilized during image rendering.

Cell imaging videos were collected on the same day as labeling (day 1) and one day after labeling (day 2). The samples were stored in PBS protected from light at 4°C between day 1 and day 2 imaging sessions.

2.4 Image Rendering

Videos were rendered to produce images with custom-written scripts in Matlab (Mathworks, MA). During rendering, molecules were identified as fluorescent signals detected above a threshold 4 times the root mean square of the average detected signal. Fluorescent signals were clustered together over the 10,000 frame video based on their location following correction for drift during imaging using the gold fiducial markers. Each single molecule

signal was rendered at 20 nm in size. The contrast was kept constant across all images to enable quantitative signal intensity comparison.

3. RESULTS

As expected, the image quality of the microtubule images changed with different Alexa Fluor 647:antibody ratios imaged on the same day as labeling (Figure 1A and 1B). At the lowest Alexa Fluor 647:antibody ratio of 0.2:1, the labeling was the sparsest and has the least amount of contrast between the microtubules and the background. At the next higher fluorophore to protein ratios of 0.4:1 and 0.8:1, the labeling was denser with the best contrast between the microtubules and the background. With the highest fluorophore to protein ratios of 1.7:1 and 3:1 the microtubules were still densely labeled, but the localization precision and image quality were decreased as the microtubules appeared wider than at lower fluorophore to protein labeling ratios. One explanation for the optimal imaging on day 1 with the 0.4:1 and 0.8:1 fluorophore to protein ratios could be the increased distance between fluorophore molecules compared to ratios of 1.7:1 and 3:1, allowing for better localization during rendering since molecules were less likely to be in the fluorescent “on” state within the same image and thus overlap.

Microtubule images of the same samples taken in different locations were also collected one day after microtubule labeling (day 2) (Figure 1C and 1D) and were compared to day 1 images. The same trend was seen on day 2 as on day 1 with the higher Alexa Fluor 647:antibody ratios resulting in higher labeling density. However, the contrast between the microtubules and the background decreased at all labeling ratios as the labeling density appeared lower on day 2. The cause for intensity decrease was not precisely determined. Fresh imaging buffer was mixed and applied on day 2 imaging as per protocol, and different locations of the samples were imaged on day 2 as compared to day 1 imaging to rule out photobleaching as the cause. It could be possible that there was a slow dissociation between the labeled antibodies and the microtubules as they were stored in PBS overnight, however this was likely minimal due to strong fixation following staining.

4. CONCLUSION

Fixed microtubules were labeled with secondary antibodies prepared with a range of Alexa Fluor 647 to secondary antibody ratios. In comparing image quality of microtubules to the ratio of Alexa Fluor 647:antibody directly after labeling, the best images were obtained using labeling with 0.4:1 and 0.8:1 ratios, demonstrating that there is an optimal fluorophore to protein ratio for antibody based SRM imaging. Image quality decreased as time between microtubule labeling and imaging increased to 24 hours post labeling as the labeling density visualized decreased. The fluorophore to protein labeling ratios of 0.8:1 and 1.7:1 resulted in the highest quality images on day 2 with the fluorophore to protein labeling ratio of 3:1 still proving to be over-labeled. Therefore, to obtain consistent imaging, a ratio of 0.8:1 was found to be the most optimal fluorophore to protein ratio under our labeling and imaging conditions since it gave the highest quality imaging results on days 1 and 2. The work presented herein demonstrates that image quality differences seen with varied fluorophore to antibody labeling ratios as well as a delay between labeling and imaging are important

parameters that effect overall image quality even when optimal photoswitchable fluorophores such as Alexa Fluor 647 are used for single molecule labeling.

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Figure 1.

Rendered images of microtubules labeled with a range of Alexa Fluor 647:antibody ratios from 0.2:1 to 3:1, keeping the primary and secondary antibody concentrations constant. **A.** Images collected on the same day as labeling (day 1) (scale bar = 5 μm). **B.** Magnified rendering of images collected on the same day as labeling (day 1) within the box in **A** (scale bar = 0.5 μm). **C.** Images collected one day after labeling (day 2) (scale bar = 5 μm). **D.** Images collected one day after labeling (day 2) within the box in **C** (scale bar = 0.5 μm).

Table 1.

Conjugation reaction conditions of Alexa Fluor 647 to donkey anti-mouse secondary antibody and final calculated molar ratio of Alexa Fluor 647 to antibody post purification.

Conjugation Reaction Conditions			Final Alexa Fluor 647: antibody (molar ratio)
Alexa Fluor 647 (moles)	Antibody (moles)	Alexa Fluor 647: antibody (molar ratio)	
6.5×10^{-10}	6.5×10^{-10}	1:1	0.2:1
6.5×10^{-10}	6.5×10^{-10}	1:1	0.4:1
6.5×10^{-10}	2.6×10^{-9}	4:1	0.8:1
6.5×10^{-10}	5.2×10^{-9}	8:1	1.7:1
6.5×10^{-10}	7.8×10^{-9}	12:1	3:1