Effect of Different Fluorescence Iabelling Methods of the Actin Cytoskeleton for Storm Application

Yijun Tao
King's College London, London, UK
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Abstract: The Abbe limit was a major challenge in optical microscopy, making it difficult to observe microscopic biological structures. However, the development of STORM, a new fluorescence localization microscopy technique, has enabled much higher resolution imaging by causing dye molecules to blink under laser light. Advances in computational analysis have further improved image processing and resolution, leading to clearer observations and a better understanding of biological structures. Actin, a common protein in eukaryotic cells, forms F-actin, a filament involved in essential processes like cell division, migration, and muscle contraction. Fluorescence labeling is crucial for visualizing F-actin and other cellular structures, allowing scientists to study these important processes. This project explored two methods to visualize actin structures in Hela cells: phalloidin labeling with Alexa-647 dye and DNA-PAINT using GFP. ThunderSTORM analysis produced normalized images for both methods, and resolution values were calculated using FRC. The results indicated that phalloidin labeling provided higher resolution but lower-quality images, while DNA-PAINT with GFP offered better-quality images but with lower resolution.
***Keywords*:** cell, optical microscopy, flourscence labelling, DNA-PAINT

1. Introduction and Motivation

The actin cytoskeleton plays an essential role as a dynamic network in many biological processes, including cell motility, tumor cell transformation and metastasis. Actin filaments (F-actin) bind to at least 60 different actin-binding proteins (ABPs) in the cell which can regulate their organization and rheological properties assembly and disassembly.

Electron microscopy enables the observation that demonstrates the F-actin directs its fast growing barbed ends towards the cell edges[2]. The organization and dynamics of F-actins are differed based on their location in the cell, however the mechanisms that regulate these properties are not fully understood yet. Quantitative techniques with high spatial and temporal resolution are crucial for the investigation of the properties of actin cytoskeleton in order to understanding the role of actin in cell migration, cytokinesis and transformation better[1].

To achieve this goal, electron microscopy is a useful tool. However, electron microscopy does not enable the observation of living cells. Optical microscopy is still the main tool to observe biological structures, but traditional optical microscopy still contains some limitations, diffraction limitation(Abbe limit) is one of the main limitations to optical microscopy.

1.1 Introduction of the STORM

The traditional optical microscopy has significant limitations to observe cellular structures at micro-scales and nano-scales because of the diffraction limitation. Once the sample structure is smaller than 200nm, the image formed will be blurred and impossible to resolve.[3]. Stochastic optical reconstruction microscopy (STORM) is a super-resolution technique which is based on individual fluorophores with high accuracy localization which can switch on and off through using different colours of lights(lights with different wavelength).[4][3] The entire imaging process of the STORM is combined with cycles of each imaging process. Only a proportion of the fluorophores will be switched on in each cycle, the rest are kept optically inactive to avoid overlap in the images[4]. Repeating this process until the entire image of the object has been reconstructed properly.

STORM microscopy produces images with resolution to around 20nm[12]. The manipulation of STORM does not require hardwares but softwares like Fiji(ImageJ) [13], instead, Gaussian fitting is supposed to be applied to determine the centroids of PSF(point spread functions)which achieves a minimal uncertainty on any microscope in 3D.[14]. A super-resolution image is reconstructed and shown in computer by determining centroids of PSFs from each fluorophores. Each fluorophore is considered as a point that can blink through switching on and off to photo-switchable dyes such as cyanine Cy5(double conjucted structure dyes). Phalloidin-Alexa Fluor 647 is also a type of photo-switchable dye used in this project. Giving an example, red laser (633 nm) enables to switch Cy5 off: from the fluorescent state to the dark state while green laser

enables to switch Cy5 on: from the dark state to the fluorescent state.(Figure 1 shows how photo-switchable fluorophores was switched on and off through absorbing lasers with different wavelengths in STORM)

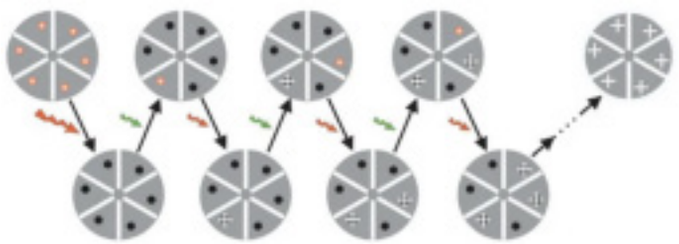

Figure 1. An example of how STORM reconstruct image through the manipulation of different colours of lights illuminating cyanine dyes[4]

All in all, the core operating principle of the STORM is overlapping diffraction images of fluorescent samples had been stained before imaging, a smooth, blurred picture will be created and capatured by the camera in every frame, the fluorescent probes utilized by the STORM could be switched on and off so that only small and optically resolvable fluorophores can be observed and detected. This is known as the stochastic switching of single-molecule fluorescence signal, which also enables their positions with high localization accuracy to be determined from the central of the fluorescent spots.

1.2 DNA-PAINT

DNA points accumulation for imaging in nanoscale topography (DNA-PAINT) offers an easy way to image molecular structures under the super-resolution microscopy. The DNA is known as the genetic material as well as the RNA and mRNA, which have been showing their programmability and specificity properties in various fields.[6] The mRNA's programmability has been recently applied in this Covid Pandemic. The mRNA vaccine consists of series of genetic materials which were coded to defend virus, bacteria, and other antigens. A package of molecules will into cells through the lipids on the membrane, where it will be translated into simulation that provoke an immune response within in the human body to make antibodies. [7][8]

In DNA-PAINT, the association of the fluorophore transient is to target a molecule which is mediated by the pairing of a DNA sequence that is combined with less than 10 nucleotides. [9]

The target sequence (docking strand) attached to a molecule of interest, it also can attach with antibodies, nanobodies or other high affinity probes. The dye-labelled DNA strands (imagers) carry the fluorophores, which diffuse freely in the imaging buffer.

During the DNA hybridization process, the fluorophores are immobilized temporarily near the target molecules, and will be excited by the illuminate sources(laser). Then, the light emitted will be tracked and captured by camera as a diffraction limited flash. Only a fraction of fluorophores will be imaged through adjusting the sequence, concentration, the DNA strands' ratio , and the imaging buffer's composition, enabling stochastic super resolution microscopy imaging.

1.3 FRC Calculation

STORM enables a theoretically infinite resolution, meanwhile the localisation of fluorophores was limited by various of factors[18] such as camera noise. Those factors lead to numerical values of resolution in each imaging process, those numerical resolution values in this project was measured through FRC.

FRC enables measurements to the effective image resolution in a straightforward and objective way[16]. FRC analysis also enables the restoration of blind images. Fluorophore localisation issues made by Gaussian fitting or machine issues can also be detected and measured by FRC. FRC can be applied to denoise images by filtering frequency domain. In 2019, fluorescence microscopists proposed novel image deconvolution methods used FRC to estimate effective PSFs(point spread functions) from images directly[16].

Generally, resolutions of each microscopy image is estimated in two methods: based on the traditional Abbe limit, measuring the minimum distance between two adjacent objects that is resolvable in each image; estimating the resolution based on intensity distribution analysis, such as the estimation of FWHM(full width half maximum). However,both of these two methods require mutual measurements come from clearly subjective decisions to find objects or structures that are suitable to measure and resolve. None of these methods can be achieved automatically, making statistical data samples through mutually repeated measurements lead to a tired and tedious work which could creat errors more easily.

FRC [17][18]has been essentially used to estimate resolution of images from electro-microscopys for decades[16]. FRC

has recently been adapted to estimate resolution of images from optical microscopys such as STORM as well as addressing issues caused in traditional methods to estimate resolution of images. The application of FRC is based on a normalized cross correlation histogram measure calculated in the frequency domain between two images of the same region of interest with independent noise sources. Therefore, when applying the FRC calculation, it is essential to divide one image into two of its sub-images through filtering different parameters on Fiji(ImageJ) software after doing "Plugins-ThunderSTORM-run analysis" on Fiji, parameters can be used to filter the image can be found on the page that appears automatically after running ThunderSTROM. Noting the parameter used to divide images in this project is their "id" parameter, filtering parameter was done through selecting all odd order ids by setting "id%2=1" and selecting all even order ids by setting "id%2=0".

In FRC calculation, spatial frequency of the two sub-images are divided into bins that produces a series of concentric rings in the polar-form frequency domain images[16].

FRC calculation also gives a numerical number of resolution to each image (as shown in the results section). Sample and microscope characterize the calculation of FRC as well as the resolution calculated by FRC for each image.

2. Aims of this project

The aims of this project contains two main goals: the first goal is observing actin cytoskeleton structure of Hela cells bind with phalloidin-dye and DNA-PAINT respectively under STORM and gives Normalized Gaussian images;the second goal is calculating resolution values through FRC calculation and compare the advantages and disadvantages for two labelling methods. Fiji(ImageJ) software was applied in this project to process raw images produced from STORM and gave a clearer performance to actin cytoskeleton structure.

3. Analysis and Discussion

ThunderSTORM analysis enables a much better visualisation of raw images captured by camera on STORM by localising each blink of fluorophore through PSF method. However, some uncontrollable factors lead to a numerical value of resolution in each image while the numerical value of resolution are supposed to be unlimited in theory. Entire resolution values in Hela cell image achieved by Phalloidin labelling are smaller than the values achieved by DNA-PAINT labelling. Phalloidin labelling achieved a better resolution as shown in the results, it means that this is a better labelling method. The small clusters can lead to artifically higher resolution as small dots tend to give better resolution vales. Use of MEA in imaging buffer can not be ignored in phalloidin labelling, it helps the formation of fine actin structures in STORM imaging. If only talking about Normalized Gaussian ThunderSTORM images, existence of big, discrete, bright white dots appeared on images means bad labelling. However, phalloidin is toxic which limits its application to label living cells[19][20] for imaging especially in the application to investigate F-actins, because F-actin analysis in living cells dramatically relies on modifying GFP derivatives which tag actin directly or other actin-binding domains[21]. GFP can be used to label actins directly for deeper investigation while the chimeras of actin and GFP can interfere the normal functions of actin cytoskeleton, leading to artifacts during imaging experiment[22][23] and influencing the final resolution.This explains that in this experiment, Normalized Gaussian images under phalloidin labelling give a worse performance to actin structures based on subjective judgement but a higher resolution was also achieved by phalloidin labelling

FRC calculation gives numerical resolution values to each ThunderSTORM image by defining a cut-off frequency in the frequency domain through FRC measurement[16], errors might happen during imaging experiment were taken into consideration, such as machine noise. Any frequency higher than the cut-off frequency will not be detected. Therefore, resolution value is given based on cut-off frequency. PSF method used in ThunderSTORM is the integrated Gaussian. There is a possibility to estimate PSF as the resolutions in each image are known through FRC calculation.

FRC analysis was done through finding statistically independent sub-images, sub-images mean that same details were shared with different noise realizations. Each FRC analysis contains two sub-images in this project, one image was divided into two sub-images by selecting pixels in odd/even index[16]. Both two sub-images have the identical dimensions. Doing FRC calculation for each sub-image and averaging values to process asymmetries in frequency domain. Identical field of view and focal plane in each image also enable FRC measurement under an almost fixed resolution value with 100nm pixel size[16]. Calibrated value σ corrects pixel pitch.

In DNA-PAINT, different protocol does not make significant difference on resolution. SNR (signal-to-noise ratio) in ThunderSTORM Normailzed Gaussian images that protocal involves cTBS is lower, which means the quality of cTBS protocol images are worse. PBS is more encouraged to be used in DNA-PAINT cells staining.

GFP was applied as dyes in this project to process DNA-PAINT labelling, it did give a better quality to DNA-PAINT images than phalloidin labelling images, but did not achieve a better resolution. To deal with this issue and make the

improvement, Lifeact-GFP, small fluorophore labeled peptides that can be applied as actin probe to label cytoskeleton and visualize its structures. Lifeact-GFP is generated by fusing a short 17-amino-acid peptide (Lifeact) from yeast Abp140 with GFP[24] Lifeact enables specific and selective binding with F-actin in the cytoskeleton as well as permitting the investigation of actin structures in natural environment without interfering actin dynamics in vitro or in vivo therefore causing errors in microscopy images.

Fluorescence dyes were used to label samples during experiment, their properties define that those fluorescence dyes will bleach under the illumination of lasers and influence imaging. To reduce the effect comes from bleaching, it is helpful to cover samples with lab tissues when samples are not being imaged.

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