Fluorescence imaging and image analysis of actin dynamics in living cell

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Dynamic assembly of actin cytoskeleton drives multiple intracellular biological processes in eukaryotic cells. Different types of actin networks are generated upon cellular requirement in a temporal- and spatial-specific manner. Living cell imaging of dynamic behaviors of actin filaments provides the mechanistic insights for the actin polymerization, which enhances our understanding of actin assembly per se and the actin powered biological processes. High quality living cell images enable quantitative measurement of actin dynamics that make great contributions to our knowledge of intracellular regulation of actin assembly. In this chapter, we will demonstrate the approaches for fluorescent living cell imaging and image analysis for studying actin filament assembly in budding yeast. Advanced wild-field microscope, imaging acquisition and deconvolution approaches will be used to quantitatively determine the lifetime of actin patches and the dynamics of actin cables, which support clathrin-mediated endocytosis and membrane transport, respectively.

1. Introduction

Actin cytoskeleton assembly in eukaryotic cells powers various intracellular activities, including endocytosis, polarized cell growth, vesicular trafficking, and cytokinesis [1-5]. The underlying molecular mechanisms of these biological processes and actin filament assembly are usually highly conserved among all eukaryotic species, from unicellular organism like budding yeast to multicellular organisms such as human and plants. In yeast, two major types of actin networks, comprised of branched and unbranched actin filaments, are responsible for driving multiple intracellular activities. Actin cable, which is a bundle of unbranched F-actin (filamentous actin), mediates the vesicular trafficking in cooperation with type V myosin [6, 7]. Actin cables assemble at the cell cortex by formin protein and align along the mother-bud axis [8]. Actin cables provide the tracks for the transportation of various cargoes, including the membrane bound organelles and the cytoplasmic protein aggregates [9-11]. Actin patches comprise of branched actin filaments that are nucleated by Arp2/3 complex near the plasma membrane. Actin patches involve in the force generation for the bending and internalization of plasma membrane during endocytosis process. Both above branched and unbranched actin filament networks undergo dynamic turnover via a tread milling type of mechanism, which is precisely regulated by various actin binding proteins (ABPs) in a cooperative and competitive manner [12].

Due to the nature of dynamic behavior during actin filament assembly, living cell fluorescence imaging approach showed greater advantages over the methods using fixed samples in studying actin filament assembly and disassembly, including the nucleation, elongation and de-polymerization. In addition, simultaneous dual-color live cell imaging results in rapid advances in studying the dynamics of multiple proteins at a same time for a particular actin assembly driven process, such as endocytosis, which is a highly coordinated activity involved by a serial of protein components [13, 14]. Imaging of fluorescent labeled proteins in living cells for studying actin assembly not only provides the spatial and temporal information of protein localization, but also the dynamic turnover of actin filament assembly and the progression of actin supported biological activities. For example, clathrin-mediated endocytosis in budding yeast is a membrane invagination process that requires a ordered recruitment of more than 60 functional proteins, including coat module proteins before actin polymerization, actin binding proteins and then the scission module proteins [15]. Lifetime imaging of the appearance and disappearance of each individual protein provides an unbiased map for the protein lifetime to evaluate the whole dynamic endocytosis efficiency, which is achieved by tagging fluorescent fusion protein to targeted protein at the genomic locus under the control of the endogenous promoter [13, 14]. Due to the varied dynamics and abundance of each individual protein at the endocytosis sites, quantitative measurement of the dynamic behaviors for each individual protein in a great precision is necessary for dissecting detailed mechanisms. However, it is often challenging to image and analyze the fluorescent proteins that have low abundance in vivo, which usually requires an optimized strategy that integrates a combination of considerations, including the most suitable fluorescent protein, appropriate microscope setting, and careful imaging analysis. Here in this chapter, we will have a focused introduction and discussion of a wide-field florescence microscope based lifetime imaging approach that is coupled with image analysis using deconvolution to improve the quality and feasibility of efficient data analysis. The imaging of actin-mediated endocytosis in budding yeast, using early coat module proteins Edel and Syp1 that have relatively weak signal intensity in actin patches, is demonstrated here (see result).

The imaging of dynamic internalization of actin patch proteins in budding yeast has been well reported using ABPs, such as Abp1 and Sac6 [15-17]. However, the living cell imaging of actin cables has been limited due to a short of specific actin cable binding proteins that express at endogenous level without affecting actin filament assembly in vivo. In many cases, the functionality of actin binding proteins or actin per se are sensitive to the tagged fluorescent proteins with a protein size of around 27kDa. In budding yeast, a green fluorescent protein (GFP) fused actin-binding protein
140 (Abp140) was reported as an actin cable marker, which specifically bind to actin cable without affecting the dynamics of actin filament assembly in vivo when expressed at its endogenous level [18]. Subsequently, LifeAct, a 17 aa peptide derived from the Abp140, was found to be one of the best in vivo living cell actin cable reporters that was widely used to localize actin filaments in different eukaryotic species [19-21]. However the fluorescent proteins fused Lifeact reporters need to be used carefully with appropriate expression level in vivo. Recently, the Pollard Group showed clear evidences that fluorescent protein tagged LifeAct could cause potential defect in actin filament dynamics in a concentration-dependent manner, which suggested the caution in using highly overexpressed LifeAct-mCherry and emphasized the advantage of low concentration of such actin marker to label living cell actin filaments [22]. Therefore, expressing a fluorescent labeled actin binding protein at a low expression level or under the control of endogenous promoter is critical in providing physiological-relevant results in studying actin dynamics in living cells. However, it is always challenging to quantitatively measure the images with low intensity if the fluorescent signal is relatively low, such as the Abp140-GFP labelled actin cables in budding yeast. In addition, the tracking and analysis of dynamic filamentous structures, such as actin cables, is not as straightforward as the analysis of spots like signals, such as actin patches or endosomal compartments. Because the relatively weaker reporter for actin cable always requires an optimized balance between the imaging speed and the quality of images. Here, we will use budding yeast as an example to describe the approaches of imaging and data analysis to achieve high quality actin cable images by following Abp140-3GFP, which is fluorescently tagged at its genomic locus and expressed under the endogenous promoter. The approaches that we describe here also have broad implications in the studies of actin dynamics in mammalian systems when low level of fluorescent LifeAct reporter is expressed.

Taken together, this chapter will use the imaging of actin-patch and actin cable in living yeast cells as examples to demonstrate wide-field microscope imaging and image analysis, which would be generally applicable in studying dynamic and fine structures in living cells.

2. Materials

Strains, culture medium and chemicals

2.1 Yeast strains

GFP or monomeric RFP tags were integrated at the genomic locus to generate C-terminal fusions of each protein as described by standard PCR-based methods [14]. A list of strains used in this chapter is provided in Table 1.

<table>
<thead>
<tr>
<th>Strain</th>
<th>genotype</th>
</tr>
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<tbody>
<tr>
<td>Ede1-RFP, Syp1-GFP</td>
<td>MATα his3Δ200 ura3-52 leu2-3,112 EDE1-mRFP::HIS3 SYP1-GFP::KanMX</td>
</tr>
<tr>
<td>ABP140-3GFP</td>
<td>MATα his3Δ200 ura3-52 leu2-3,112 ABP140-3GFP::HIS3</td>
</tr>
<tr>
<td>bni1Δ, ABP140-3GFP</td>
<td>MATα his3Δ200 ura3-52 leu2-3,112 bni1Δ::CgLEU2 ABP140-3GFP::HIS3</td>
</tr>
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2.2 Culture medium

Yeast extract peptone dextrose (YPD) plates: 2 % bacto-agar, 1 % bacto-yeast extract, 2 % bacto-peptone, 2 % dextrose. For fluorescent imaging, cells were all cultured in Synthetic Drop-out (SD) Tryptophan to minimize the autofluorescence. Synthetic Drop-out medium: 0.067 % yeast nitrogen base (YNB), 20 μg/mL of each adenine, uracil, L-histidine and L-leucine, and 2 % dextrose.

2.3 Chemicals

Concanavalin A (ConA): from Canavalia ensiformis (jack bean) Type IV, lyophilized powder (Sigma, USA). A stock solution (10 mg/ml) was prepared by dissolving the lyophilized powder in distilled water, which was kept in −20 °C for 6-12 months without obvious loss of activity. The working concentration of ConA is prepared fresh at 1 mg/ml in water.

2.4 Imaging and data analysis

Live-cell images were taken by fluorescence microscopy Leica DMI8 (Leica, Germany) that is equipped with a 100X objective lens (NA=1.4), SPECTRA X light engine (Lumencor, USA), and a sCMOS camera ORCA-Flash4.0 LT Digital (Hamamatsu, Japan). The acquired Z stack images were subjected to Huygens Deconvolution software package.
3. Result

3.1 Quantitative measurement of actin patch’s lifetime in budding yeast by wide-field microscopic imaging system

In budding yeast, actin patch-mediated endocytosis is comprised of several protein modules that follow a pattern of recruitment and detachment in a temporal manner. The arrival and disappearance of each individual protein at endocytic site have a highly regular timeline and defined dynamics, which are precisely coordinated with each other for driving effective internalization of endocytic pits at plasma membrane. The staying time of each protein at plasma membrane for endocytosis is termed as life time, which indicates their functional period in driving the endocytosis process. The quick growth and easily manipulated genome of budding yeast have allowed us to fuse fluorescent protein to a serial of endocytic proteins for studying and dissecting the endocytosis in a great detail. Using advanced fluorescense microscopy approaches in both WT and mutant yeast strains, researchers are able to understand the underlying molecular mechanisms by which a protein of interest interferes endocytosis events, via a rapid characterization of the dynamic behaviors of fluorescent fusion-tagged endocytic proteins in any mutant strain. Here, we use two early module proteins, Ede1 and Syp1, as examples to illustrate their lifetime at endocytic sites and demonstrate the imaging process. Both Syp1 and Ede1 arrive early at the endocytic sites and play important roles in controlling the rate of endocytic site turnover, where Syp1 functions in the polarized distribution of endocytic sites and Ede1p modulates endocytic site formation [23]. Both proteins have been tagged by fluorescence proteins at C-terminal of endogenous locus, resulted in Ede1-RFP and Syp1-GFP [23, 24]. Previous studies have shown that Ede1 and Syp1 have a wide range of life time, from 30 s to 4 min [23], with relatively dim fluorescent signal at endocytic sites.

To improve the image quality for fast quantitative analysis of Ede1-RFP and Syp1-GFP, live-cell images were taken by wide-field fluorescence microscopy equipped with scientific CMOS (sCMOS) camera. Making general recommendation of camera is difficult because the camera field has been developed rapidly in the past few years and usually a main consideration could be finding a best compromise between price and performance. sCMOS has gained its popularity due to its advantages of lower noise and higher frame rates to produce better quality images comparing to other cameras. Here, cells expressing Ede1-RFP and Syp1-GFP were first inoculated from YPD plate and grew in SD medium without tryptophan at 25°C overnight. Cells were attached to Concanavalin A (1 mg/ml)-coated coverslips by 10 minutes of incubation at room temperature of 23°C. Image acquisition was done by streaming the movie at a rate of 3 s/frame and 60 frames in total. In order to improve the contrast and resolution of the images of Ede1 and Syp1, which do not present at endocytic sites with very high concentration, image deconvolution by Huygens deconvolution software was applied before quantitative image analysis. We found such combination of image acquisition and deconvolution provides a great advantage to improve the image quality for targeted protein that encounters low expression levels, which is evident in our images of Ede1-RFP and Syp1-GFP using wide field microscope [Fig. 1A]. Huygens deconvolution software has largely removed the images noise and improved the image quality. Deconvoluted images were subjected to quantitative tracking of the protein life-time by kymograph generated in the ImageJ software. Then the length of protein trajectories of Ede1 and Syp1 that retained on the plasma membrane was measured [Fig. 1B]. Both original and deconvoluted movies were analyzed and quantified. We found deconvolution did not show statistic difference for lifetime measurement but improved the image quality with better contrast and lower background noise, which contributed to an easier image analysis and quantification [Fig. 1C]. We also examined the lifetime distributions of both early endocytic proteins Ede1-RFP and Syp1-GFP [Fig. 1D], both of which showed a wide range of lifetime during their recruitment of binding partner proteins, in consistent with previous report [23].
3.2 Study of actin cable dynamics in budding yeast by wide-field microscope fluorescence imaging

During actin cable assembly, monomeric actin is incorporated into the existing barbed end of the actin filament by nucleation factor formin proteins, Bni1 and Bnr1, in the budding yeast. Individual actin filaments are cross-linked into actin cables bundles by cross-linking factors, such as Sac6 and Scp1. Actin cables are polymerized with a wide range of elongation rate at around 1-3 µm/s. These dynamic processes of actin filament nucleation, bundling and elongation are highly regulated by several key ABPs that function in a cooperative and competitive manner [12]. Disruption of actin cable regulatory ABPs could eventually lead to severe defects in actin cable dynamics, including the density, intensity, and the length. Quantification of the phenotypic parameters of actin cable dynamics are usually more challenging compared to the actin patch analysis due to the relative weak fluorescent signal of actin cable reporter ABP140. Here, we demonstrate the imaging and data analysis of actin cables in wild type yeast cells and a formin mutant (bni1Δ). The Huygens deconvolution software was subsequently applied to the acquired original images to improve the contrast and resolution. Deconvoluted movies of elongating cables were then analyzed by ImageJ and FilamentTracer function of Imaris to quantify the velocities, number, length and average intensity of actin cables. In order to directly visualize the dynamic actin cable phenotype, Abp140 was tagged by three GFP. Fluorescent Abp140 labels actin cable by binding the sides of actin filaments, with a higher preference in binding to actin cable when both types of branched and unbranched actin filaments are presented. Actin cables are nucleated and elongated in a long distance in the cell, which is generally more sensitive to environmental stimulation or small molecule inhibitor perturbation than the compact actin filament network inside actin patch [17]. For example, low concentration of Latrunculin A at 1-5 μM can rapidly depolymerize actin cable before actin patch signals disappeared [16, 17]. But when the cells experience a loss of actin cables, actin patches labeling by Abp140-3GFP could also be observed. To capture a 3D whole-cell image to visualize the actin cable phenotype, images were acquired continuously at 0.25 µm intervals through the entire cell with an exposure time of 0.2s per slice to create a stack in the Z axis. The microscopy parameters used were based on the Nyquist's sampling theorem, which could be calculated at https://svi.nl/NyquistCalculator. In order to calculate the actin
cable polymerization rate, movies were streamed at a rate of 0.25 s/frame with 160 frames in total at the cortical focal plane.

To quantify the actin cable phenotype, the 3D scanning images achieved from wide-field fluorescent microscope were first deconvoluted to improve the image quality and were then subjected to Imaris software for filament tracking and quantification. The tracking of individual filament in Imaris was based on the software detection of cable signals by the auto-path function, as an example shown in Figure 2B. The number, length and total intensity of actin cables were measured in more than 25 cells [Fig. 2C-E]. In the bni1Δ mutant, the number and length of actin cables showed a slightly decrease, which is likely due to the reduced ability in actin filament nucleation by deleting one of the two major formin proteins. The total signal intensity of individual filament gives an overview of the actin cable phenotype. Changes of actin cable intensity reflect potential differences in the level of the cross-linking in the bundle, the total number, and the length of individual single actin filament in actin cables. Therefore, by sequential image process and analysis using Huygens and Imaris software, detailed phenotypic parameters of actin cables could be identified. Such stepwise imaging and analysis approach in yeast WT and mutant strains reveal deep insights of the well-orchestrated process of actin filament assembly. In addition, dynamic polymerization of actin cables could also be investigated by quantifying the actin cable velocity using ImageJ. The actin cables for measuring their elongation speed were randomly selected from more than 20 cells, in which one representative example is shown in Figure 2F. To obtain the velocity of the polymerized actin cable, the distance of cable movement was divided by elapsed time. Actin cable velocity of both WT and bni1Δ were measured, in which bni1Δ showed a slightly decreased speed in actin filament elongation, compared to the WT cells [Fig. 2G].

Figure 2. Actin cable dynamics in wild type and bni1Δ cells. A. Representative actin cable image by maximum Z projection from Abp140-3GFP signals in wild type and bni1Δ cells. B. An example of tracking actin cables in a wild type cell by Imaris. C. Quantification of actin cable number in WT and mutant (n = 30). D. Quantification of the actin cable length in WT and mutant (n = 30). E. Quantification of the total intensity of each actin cable in WT and mutant (n = 30). F. A representative image of an elongating actin cable showing the movement at different time points. G. Quantification of actin cable elongation speed in WT and mutant (n = 30).
cable indicated by Abp140–3GFP. Red arrows point to the position of the point end of the elongating cable. Yellow arrows indicate the elongating end along the movie at a time interval of 0.25 s. G. Actin cable velocity indicated by Abp140-3GFP in wild type and bni1Δ cells (n = 80). Bars, 2 μm.

4. Discussion

The understanding of dynamic cellular events and subcellular networks has been greatly advanced by high-resolution living-cell imaging techniques, which provide spatial and temporal information of individual biological activity. The study of actin filament networks is one of the classical examples that uses living cell imaging approach to dissect the detailed mechanisms of the dynamic actin treadmilling. Due to the advantages of experimentally tractable features and fewer protein homologs, budding yeast has been a great model organism to study the fundamental basis of actin assembly. Here, we demonstrated the examples of using wide-field microscopic imaging to study the dynamics of actin networks in living budding yeast. Because quantitative biology has much more advantages over descriptive investigations in understanding the underlying mechanisms of the specific phenotypes. We demonstrated several quantitative approaches to analyze the dynamic actin filament structures, which provide mechanistic insights on the function of targeted protein candidates in a particular biological process.

In this chapter, we demonstrated the imaging and image analysis approaches that showed an improved image quality for easy quantitative measurement in studying the dynamics of actin cable and endocytosis. In general, a series of considerations should be taken into account during living cell imaging to improve the overall image quality, which is critical to understand the physiological or pathological process that is investigated. However, due to the varied dynamic behaviors of different proteins, it may not be practical to perform a cell biology imaging using fixed microscope parameters for different proteins. A few factors that should be considered in the design of a particular live cell imaging experiment, such as the selection of fluorescent protein, the detecting camera, the image acquisition parameters, and the use of auto-focus system. A broad range of fluorescent protein variants have been developed over the past several years with increased quantum yield, photostability, and protein maturation times, which should all be taken into consideration in choosing fluorescence protein reporters. In addition, the improvement of the images quality does not only rely on the suitable fluorescence protein tagging, but also depend on the high quantum efficiency provided by the detection unit, such as scientific CMOS (sCOMS) or EMCCD cameras.

Yeast has been proved to be a premiere model system for studies of the actin cytoskeleton due to the advantage of the high genetic tractability and the availability of large-scale libraries, including null mutants of every yeast gene and GFP-tagged yeast protein under the control of endogenous promoter. However, living cell imaging could be challenging in studying a highly dynamic process by imaging fluorescent fusion of a weakly expressed protein. To visualize the yeast subcellular structures, such as actin filament networks, a high-magnification objective lens (100×) with numerical aperture (NA) at least 1.4 is necessary. And to record the dynamic changes of actin filament, a camera with capabilities for taking images with fast speed, high sensitivity, and low background noise is important as well. In addition, a number of factors should also be paid extra attention during the sample preparation and imaging acquisition. In order to reduce the background fluorescence signal, a culture medium of synthetic drop-out tryptophan could be used if it is compatible with the yeast genotype. Last but not least, a standardized imaging protocol could be helpful and time saving for the followed imaging processing steps. Optimization of imaging acquisition conditions to minimize light exposure as much as possible is extremely critical to achieve high quality results. The exposure time, illumination intensity and Z-slice number always need to be optimized for every single fluorescent proteins depends on the signal intensity and the dynamics of the targeted protein. The excessive illumination and long exposure times that often cause bleaching of fluorophores and phototoxic stress should be avoided if it is possible. Due to less condensed actin network and therefore lower labeling density of reporter protein, imaging of actin cable is in general more difficult than the imaging of actin patch. To enhance fluorescent signal, triple GFPS were tagged to Abp140 [16, 17, 25] for monitoring actin cable dynamics. Because of the fast elongation speed of actin cable in a range from 1-3 μm/s, movie acquisition by live streaming model was used with exposure time with around 200-250 ms, without compromised in both time resolution and actin cable signals.

The imaging and data analysis approaches we discussed here to study actin filament dynamics using inexpensive wild-field microscope coupled with image deconvolution could also be broadly applied to study living cell processes in multiple eukaryotic systems and meet the increased demands in researches using rapid advanced technologies, such as powerful genome-editing system. Recently, the revolutionary genome-editing technologies, including zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALEN), and CRISPR/Cas, made it possible to efficiently tag fluorescent fusion to targeted protein at genomic locus under the control of native promoter [26-30]. However, the weak expression and dim signal will still be one of the main challenges for quantitative cell biology to study key acting binding proteins with low abundance, such as formin protein. Appropriate choose of imaging and analysis methods as we described here will greatly facilitate these cell biology studies.
References