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Imaging protein dynamics in live mitotic cells

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Abstract

To ensure that genetic material is accurately segregated during mitosis, eukaryotic cells assemble a mitotic spindle, a dynamic structure composed of microtubules and associated regulatory, structural and motor proteins. Although much has been learned in the past decades from direct observations of live cells expressing fluorescently tagged spindle proteins, a complete understanding of spindle assembly requires a detailed analysis of the dynamic behavior of component parts. Proteins tagged with conventional fluorophores, however, make such an analysis difficult because all of the molecules are uniformly fluorescent. To alleviate this problem, we have tagged proteins with a photoactivatable variant of GFP (PA-GFP), thereby allowing one to follow the behavior of a subset of tagged molecules in the cell. Here, we describe methods to tag and express proteins with PA-GFP, locally photoactivate the recombinant protein and record the dynamic behavior of the photoactivated molecules in live cells. We provide examples of photoactivatable proteins in mammalian and yeast cells to illustrate the power of this approach to examine the dynamics of spindle formation and function in diverse cells.

Keywords

photoactivation; mitosis; yeast; mammalian cells

1. Introduction

The assembly of a mitotic spindle ensures that dividing cells are properly equipped to congress and segregate their duplicated genomes. Errors in this assembly process can ultimately lead to aneuploidy, a potentially devastating fate. While it is known that several hundred proteins associate with mammalian spindles [1], functional roles have yet to be assigned to many of these proteins, and consequently, the overall process of spindle assembly is incompletely understood. To appreciate more completely how cells build mitotic spindles, one would ideally know the location, dynamic behavior and activity of each requisite protein.

Because position and function are often entwined (i.e., proteins located near kinetochores and centrosomes are more likely to be involved in checkpoint signaling and pole focusing, respectively, than vice versa), a practical step toward understanding a mitotic protein's role in spindle assembly is to first determine where it localizes. The use of indirect immunofluorescence and genetically tagged fluorophores has provided a great deal of this

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spatial information; however, such tools do not convey a tagged protein's dynamic behavior because many copies of the tagged protein are fluorescent, thus obscuring dynamic turnover. A photoactivatable variant of GFP (PA-GFP) was engineered to address this problem [2].

PA-GFP, which contains a threonine to histidine mutation at position 203 of the wild-type EGFP protein, only absorbs 488nm light (and therefore only fluoresces) after an initial exposure to ultraviolet light [2]. Since UV illumination can be spatially controlled (see Methods), a user-selected subset of PA-GFP tagged proteins can be made fluorescent while simultaneously keeping the remaining population in an inactivated state. Such techniques have been utilized to report the poleward movement of both tubulin and the plus end-directed kinesin, Eg5 [3, 4].

2. Methods

2.1. Constructing and Expressing PA-GFP fusion proteins in mammalian cells

PA-GFP is currently available as both N- and C-terminal fusion plasmids. Linearizing either of these plasmids, and subsequently ligating an appropriately digested cDNA of interest, is the simplest method for creating tagged proteins. We subsequently transfer the tagged cDNA of interest into mammalian expression vectors with a gene for antibiotic resistance, so that we can positively select cells expressing the transgene (see below). We find that using IRES vectors, which express the tagged gene and the selection marker from a single promoter, improves the selection process.

For transient expression in mammalian cells, lipid-based transfection protocols can be used to introduce a recombinant PA-GFP vector into the cells of interest. The particular protocol and reagent chosen depends on the cell type. For LLC-Pk1 cells, however, we have found that nucleofection offers a comparatively higher level of transfection efficiency and requires a smaller amount of purified plasmid.

2.2. Generating Cell Lines Expressing PA-GFP fusion proteins in mammalian cells

Although the distribution and dynamics of PA-GFP-tagged proteins can be examined in transiently transfected cells, generating a stable cell line is highly recommended. This is because following transient transfection, the expression level of the recombinant protein can vary greatly among cells in the population and, at high levels of expression, the tagged protein can alter cellular processes. In addition, because PA-GFP tagged proteins are not fluorescent until photoactivated, choosing cells expressing the appropriate level of the PA-GFP protein is difficult. For these reasons, we generate and characterize one or more cell lines expressing the PA-GFP tagged protein. To do this, transfected (or nucleofected) cells are grown for ~48 hours to allow expression of the antibiotic resistance gene, and then subjected to 2 weeks of positive selection in the presence of the appropriate antibiotic. Following this period of selection, drug-resistant cells are transferred to 100mm dishes at densities suitable for cloning rings [5]. Within 1–2 weeks of growth, single colonies will be visible by eye; each colony is a potential cell line. Isolated colonies can then be transferred into single wells of a 24-well plate, then (after sufficient growth) transferred to 2 wells of a 6-well plate. For screening purposes, one of these wells should contain a coverslip. Cells growing on coverslips can subsequently be analyzed for expression of the PA-GFP tagged protein by photoactivating full fields of view. Multi-well plates with a coverslip bottom are also convenient for screening large numbers of clones.

For LLC-Pk1 epithelial cells, which grow as colonies, cloning discs can also be used to remove individual colonies from the surface of the culture dish. For cells that do not grow as colonies, the selected cells can be trypsinized, diluted, and plated in a 96-well format at a density of ~1cell/well. These potential cell lines can be expanded and examined.

Once a cell line is obtained, we perform several assays to verify that the expressed protein has no detectable effects on cell division. The mitotic index and cell cycle time are measured, and the percentage of cells with abnormal mitotic spindles is determined. Western blotting is used to quantify the amount of the tagged protein relative to the endogenous protein. Depending on the particular cellular process that is under investigation, other assays can be used to demonstrate that the tagged protein has no (or minimal) effects on cellular processes.

2.3. Constructing and Expressing PA-GFP fusion proteins in budding yeast

At present, yeast expression vectors for PA-GFP tagging are not commercially available. Therefore, it is first necessary to ligate a cDNA of interest with a promoter appropriate for expression in yeast. The promoter-cDNA fragment can then be fused to PA-GFP (as N- or C-terminal fusions) in a pRS plasmid carrying a selectable marker and a CEN or 2- μ sequence, which will enable episomal expression of the PA-GFP fusion protein in the presence of the endogenous untagged protein. CEN and 2- μ plasmids are low and high copy plasmids, respectively, thus allowing differing levels of expression. Alternatively, inducible promoters (e.g., *GAL1p*, *MET3p* and *ADHIp*) can be used (during the construction of the promoter-cDNA fragment) to control the strength and timing of expression.

A second, and more powerful approach to tag a gene of interest is to introduce PA-GFP at the chromosomal locus via homologous recombination. This approach is rapid, since integration of PA-GFP only involves a PCR amplification step followed by yeast transformation. The PCR product should contain PA-GFP and a selectable marker, flanked by homologous sequence corresponding to the targeted locus. PCR tagging cassettes were recently constructed [6] and were made available with a variety of selectable markers, including the commonly used *HIS3* and *TRP1* auxotrophic markers, as well as the *kan^r* antibiotic resistance marker.

2.4. Photoactivation

PA-GFP tagged proteins were photoactivated using a Nikon Eclipse TE300 inverted microscope equipped with a X-Cite 120 (EXFO America, Plano, TX) epi-illuminator. A 100W mercury arc lamp or a 405nm laser can also be used for photoactivation [11]. We use a D405/20 filter cube (Chroma Technology, Rockingham, VT) to select light of the appropriate wavelength for activation (413nm). The duration of exposure to UV light required for full activation was determined for each PA-GFP tagged protein. To do this, cells were exposed to 413nm light for various intervals and the resulting fluorescence was quantified from images acquired using identical acquisition settings. The minimal exposure that resulted in full activation was used for subsequent experiments.

The area of photoactivation was controlled either by the field diaphragm or by pinholes and slits (Lenox Laser, Glen Arm, MD) mounted in a filter wheel (Ludl Electronic Products, Hawthorne, NY) placed in a conjugate image plane in the light path. Such pinholes and slits permit greater control of the photoactivated area. For experiments on mitotic spindles, we find that a slit of dimensions 25 μ m $\times$ 3mm, which generates an area of activation of $\sim$ 2 μ m width in the image plane, is ideal. With this set-up, the slit is well defined and sharp, facilitating tracking of the photoactivated area in time-lapse image sequences. To align the spindle (or other cellular feature) relative to the slit, which is in a fixed position, we use a rotating stage. Once a spindle is located, the stage is rotated until the spindle long axis is perpendicular to the slit; for reference the location of the slit can be noted on an eyepiece reticle or within the image acquisition software.

Images were acquired prior to and following photoactivation with a spinning disk confocal scan head (Perkin Elmer, Waltham, MA), an Orca ER cooled charge-coupled device camera

(Hamamatsu, Bridgewater, NJ) and a 100X 1.40 NA objective. Images were collected using either a single wavelength filter cube (488) or a dual filter cube (488/568).

We have also performed photoactivation using a Nikon laser scanning confocal microscope (Nikon C1) equipped with a 100X 1.45NA objective lens. Photoactivation was performed using a 405nm laser; the area of activation was restricted using the Nikon C1 confocal software. The duration of exposure to photoactivating light was similar using either system.

In summary, photoactivation experiments can easily be performed using a standard research microscope equipped with an epi-illuminator, field diaphragm, and appropriate filter cube, equipment that is readily available in most laboratories. Following photoactivation, images can be collected using wide field or confocal microscopy, and quantified using readily available software (e.g., Image J). Thus, this method is easily utilized.

2.5. Analysis of PA-GFP tagged proteins in mitotic mammalian cells

Microtubules in the mitotic spindle move poleward, a process called flux [7]. The first clear demonstration of flux was obtained using photoactivation of caged-fluorescein labeled tubulin [3]. Prior work, using fluorescence recovery after photobleaching (FRAP) had failed to detect microtubule motion, presumably because of difficulty resolving the bleached region against the non-bleached fluorescent background [8]. Using photoactivation, however, the activated region is detected against a dark background, providing a superior signal-to-noise ratio. PA-GFP has the further important advantages that preparation of chemically modified tubulin and microinjection of the modified protein are not required. In addition, by preparing a cell line, all the cells express the genetically encoded probe, facilitating selection of appropriate cells for experimentation.

We have tagged α -tubulin, a major structural component of the mitotic spindle, and TPX2, a Ran-regulated spindle assembly factor, with PA-GFP and prepared cell lines expressing each protein [9,10]. To analyze the dynamics of these tagged proteins, mitotic spindles were photoactivated using a rectangular slit positioned perpendicular to the long axis of the spindle and time-lapse images were acquired. Following photoactivation, appropriate software (e.g., Metamorph and Image J) can be used to select the photoactivated area and examine its behavior. In the example shown (Fig. 1), α -tubulin was photoactivated and rectangular boxes, typically 1–2 μ m in height and positioned parallel to the spindle's long axis, were placed around a photoactivated mark of interest in order to create montages. Rates of motion could be obtained from the slope within the montage. Furthermore, the dissipation of photoactivated fluorescence could be used to measure the turnover of each tagged protein [11].

Using an LLC-Pk1 cell line expressing PA-GFP-tubulin, Ferenz and Wadsworth [10] recently showed that spindle microtubule dynamics change as cells progress from prophase to metaphase. In prophase cells, microtubules move both toward and away from the spindle poles at a wide range of rates. In the metaphase spindle, however, only motion directed toward the spindle poles is detected and fast movements are eliminated. Because prophase cells are a small percentage of all the mitotic cells, these results clearly demonstrate the importance of generating cell lines expressing genetically encoded probes for analysis of microtubule dynamics at particular stages of the mitotic cycle.

Although the behavior of spindle microtubules has been extensively examined, less is known about the dynamic behavior of other spindle components. TPX2 is a microtubule binding protein that is required for microtubule formation at kinetochores in mammalian cells [12]. To understand how TPX2 contributes to spindle assembly, we have generated a cell line expressing PA-GFP-TPX2. Photoactivation experiments show that, like tubulin, TPX2 moves poleward in mitotic cells (Fig. 2). One possible explanation for this movement is that TPX2 binds

microtubules, which flux poleward. Alternatively, TPX2 could be the cargo of molecular motor proteins that move poleward. Measurements of the dynamic behavior of both microtubules and spindle associated proteins will provide new information that will guide our understanding of spindle assembly and mitosis.

2.6. Analysis of PA-GFP tagged proteins in mitotic budding yeast cells

We used PA-GFP to track the mobility of Num1p, a protein that has been proposed to function as a cortical anchor for the dynein complex during mitosis in yeast. Using the PCR-mediated integration technique (section 2.3), we inserted PA-GFP at the 3' end of the chromosomal *NUM1* gene. Num1p is a 313 kDa protein containing a coiled-coil domain, a putative Ca^{2+} -binding domain, thirteen 64-residue tandem repeats, and a C-terminal pleckstrin homology (PH) domain. Although it is known that the PH domain is required for Num1p's cortical localization, the spatiotemporal regulation of Num1p's cortical foci assembly is not known. Previous studies have provided indirect evidence, using conventional GFP-labeled protein, that the assembly of Num1 at the bud tip occurs coincident with the time that new Num1p protein is translated during S/G2 phase, suggesting that newly synthesized Num1p may be targeted to the bud cortex and is responsible for anchoring cortical dynein [13,14]. Using a PA-GFP tag to track Num1p protein dynamics, we discovered that some Num1 foci located at the daughter bud cortex assemble from Num1 molecules previously located at the mother cortex (Fig. 3). Our data support the hypothesis that Num1p proteins that are located at the mother cortex early during mitosis are spatially and temporally targeted to the bud cortex. This targeting from mother to bud may be required to mediate dynein activation at the bud cortex.

3. Conclusions

In conclusion, we find that photoactivation of PA-GFP tagged proteins is a powerful method to examine protein dynamics in live mitotic cells. Our work shows that the approach is suitable for diverse cell types (i.e., mammalian cells and budding yeast), can be applied to a wide range of proteins and is easy to perform using standard laboratory equipment. Data analysis is easily performed using image analysis software. Finally, many proteins have already been shown to retain their native function after tagging with GFP, so substitution of a PA-GFP tag, and performing photoactivation, is experimentally uncomplicated, and is likely to provide new information about protein dynamics.

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Figure 1.

Poleward flux of microtubules in mitosis. (A) Photoactivation of PA-GFP-tubulin in an LLC-Pk1 cell. A metaphase cell was aligned with its long axis perpendicular to the slit used to control the area of photoactivation ($-0:05$). Fluorescent images prior to ($0:00$) and immediately following a 5sec photoactivation ($0:06$). (B) Quantification of poleward flux from kymographs. A rectangular box, $1.2\mu\text{m}$ in height and parallel to the spindle long axis, was positioned around a photoactivated mark of interest. The resulting montage allows the rate of motion to be calculated. Times are in min:sec. Bar = $10\mu\text{m}$ (A) and $5\mu\text{m}$ (B).

Figure 2.

Poleward transport of TPX2 in mitosis. (A) Photoactivation of PA-GFP-TPX2 in an LLC-Pk1 cell. (B) Quantification of poleward transport from kymographs. Photoactivation, quantification and figure layout are as described in Figure 1, except that PA-GFP-TPX2 was photoactivated for 15sec and a 1.7 μ m box was used to create the montage. Times are in min:sec. Bar = 10 μ m (A) and 5 μ m (B).

Figure 3. Targeting of Num1p to the bud cortex. Photoactivation of Num1p-PA-GFP in budding yeast. The boxed region in the mother cell defines the area of photoactivation. Photo-marked molecules appeared in the bud cortex at 70min (arrows). Bar = 2 μ m.