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Measuring Time Courses of Endocytic Actin Patch Assembly in Fission Yeast

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Yeast actin patches assemble at the sites of clathrin-mediated endocytosis, where actin assembly by the Arp2/3 complex helps promote endocytic internalization using a mechanism conserved from yeast to man. Work in budding yeast has identified over 40 proteins that assemble into endocytic patches and function at different stages of endocytosis. In order to understand how these proteins assemble into patches to drive endocytosis, we need to establish their order of assembly into endocytic sites, measure their intracellular concentrations, and determine their concentrations in patches.

To measure the time courses of patch assembly, we adapted the method of Wu and Pollard [1] to measure the numbers of molecules in patches over time rather than at a single time point. In yeast, genes are easily tagged with fluorescent protein sequences directly in native chromosomal loci so that a tagged protein is expressed from its endogenous promoter as the sole source of that protein. The method is based on the fact that the total cell fluorescence intensity from imaging is directly proportional to the number of molecules measured by immunoblotting [1].

Yeast actin patches are dynamic structures that assemble and disassemble in 20 seconds. Therefore, imaging patches requires fast acquisition with good temporal resolution and high sensitivity afforded by the spinning disk confocal microscopy. To track the time courses of assembly and disassembly of individual patches, we developed improved methods for photobleaching correction, cytoplasmic background subtraction, and aligning data in time. Finally, we developed rigorous tests for the effects of fluorescent protein tags on the functions of tagged proteins.

For initial analysis, we measured time courses for 16 patch proteins, including key proteins involved in actin assembly [2]. We tagged these proteins with YFP or GFP and collected a series of time lapse images along with seven standard proteins, for which total cell concentration is known [1]. We measured total cell fluorescence intensities of standard proteins in order to construct a linear calibration curve (Fig. 1A), which converts fluorescence intensities into numbers of molecules.

We manually tracked intensities and positions of individual patches over time. We subtracted cytoplasmic background measured in areas away from the patches (Fig. 1B), since we had found that the original concentric circle method [1] underestimated patch fluorescence due to the inflated cytoplasmic background intensities. We converted fluorescence intensities into the numbers of molecules using a standard curve and aligned data in time to the start point of patch movement, corresponding to the initiation of endocytic internalization. GFP is sufficiently photostable to allow collection of a full Z-stack at each time point. We imaged YFP-tagged proteins in a single confocal section and corrected for the fraction of patch fluorescence present in a single section. With this correction, measurements in 3D with GFP and measurements in 2D with YFP gave identical results (Fig. 1C).

Aligning data in time revealed that not only the order of assembly but also the numbers of molecules in patches were highly reproducible. Thus, we averaged the numbers of molecules in individual patches at

each time point to generate average time courses of the numbers of molecules for key patch components (Fig. 1D). We verified the order of patch assembly by imaging pairs of proteins labeled with contrasting fluorescent proteins YFP and CFP. This analysis revealed that tagging of Arp2/3 complex with fluorescent proteins results in slower actin patch assembly. Therefore, we corrected measured time courses for the Arp2/3 complex (Fig. 1C) to match the time course of actin binding protein fimbrin.

These measurements provided the basis for the mathematical modeling of patch assembly [3]. The model yielded insights into the mechanisms controlling actin assembly driven by a wave of Arp2/3 complex activator protein WASp. Yet little is known about the mechanisms of WASp localization and regulation at endocytic sites. To begin dissecting these mechanisms, we determined the timing of localization to patches for 24 additional proteins. We tagged these proteins with GFP and combined them with mCherry-tagged fimbrin Fim1 by genetic crosses. We imaged the strains by spinning disk confocal microscopy and carried out detailed image analysis to classify proteins into three classes: early proteins that may recruit WASp to endocytic sites, late proteins that may regulate WASp, and proteins that did not localize to patches. This classification has allowed us to focus on proteins that may recruit and regulate Wsp1 during endocytosis.

References:

[1] J.Q.Wu and T.D. Pollard, Science 310 (2005), p. 310-314.
[2] V. Sirotkin et al., Mol Biol Cell 21 (2010), p. 2894-2904.
[3] J. Berro, V. Sirotkin, and T.D. Pollard, Mol Biol Cell 21 (2010), p. 2905-2915.

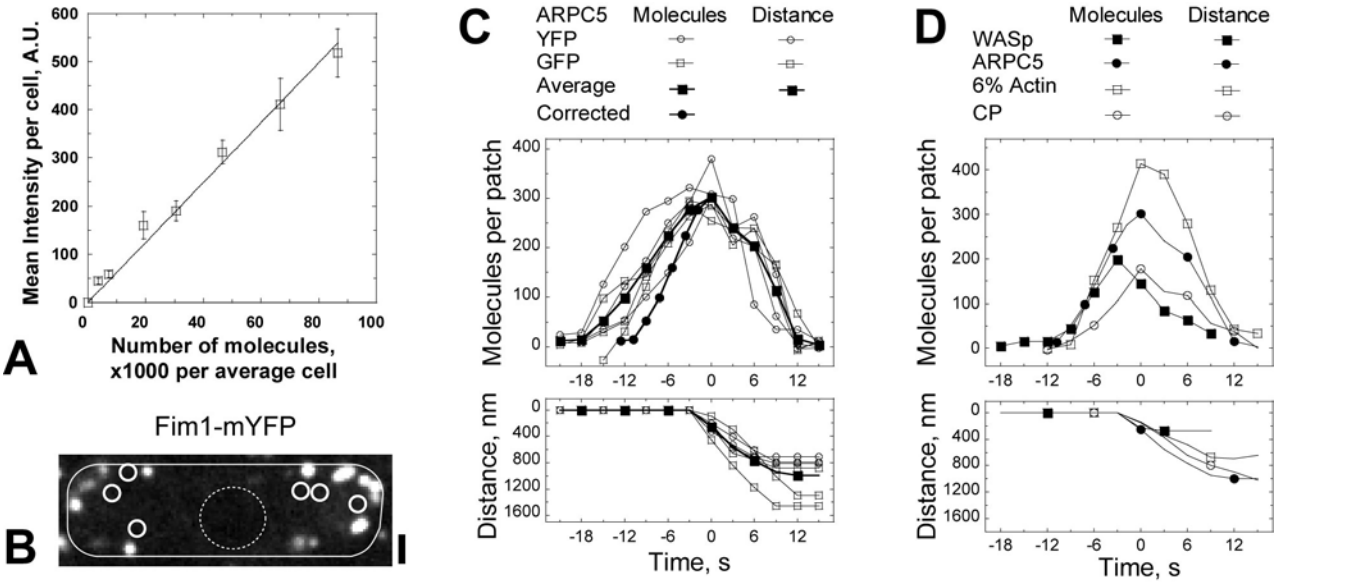

Figure 1. Measuring time courses of endocytic actin patch assembly in fission yeast. (A) Calibration curve. (B) Cytoplasmic background areas (circles) located away from patches and the nucleus (dashed circle) in a confocal section through the middle of a cell (outlined). Bar, 1 μm. (C) Raw, average and corrected average time courses of the number of molecules and distance for the ARPC5 subunit of Arp2/3 complex measured with GFP in 3D and YFP in 2D. (D) Summary time courses of the number of molecules and distance for WASp, ARPC5, actin, and actin Capping Protein (CP). The data are aligned to the time of initiation of patch movement (time zero).

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