Method: In vitro analysis of pericentriolar material assembly

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CHAPTER OUTLINE

Abstract

Centrosomes are major microtubule-organizing centers in eukaryotic cells and play a critical role in embryonic development and asymmetric cell division. Centrosomes comprise a pair of centrioles surrounded by an amorphous proteinaceous meshwork called the pericentriolar material (PCM). Robust deposition of PCM around the centrioles is essential for a centrosome to achieve full microtubule nucleating potential. Despite the wealth of information on PCM composition and function, the mechanism and regulation of PCM assembly have been difficult to ascertain, due in part to the lack of an in vitro system. Here, we describe methods to establish an in vitro system to study PCM assembly

in *Caenorhabditis elegans*. Specifically, we describe (1) how to express and purify the *C. elegans* PCM proteins SPD-5, SPD-2, and PLK-1 from baculovirus-infected insect cells, (2) how to assemble these proteins into PCM-like structures in vitro, and (3) how to quantify this assembly process in a semiautomated fashion.

INTRODUCTION

Centrosomes are composed of two barrel-shaped centrioles that organize a matrix of proteins called the pericentriolar material (PCM). The PCM expands dramatically during mitosis and recruits tubulin complexes and effector proteins that regulate microtubule nucleation, anchoring, and dynamics (for a review on PCM assembly, see Woodruff, Wueseke, and Hyman (2014)). The PCM is built through the deposition of large coiled-coil domain-containing proteins around the centrioles (Dix & Raff, 2007; Hamill, Severson, Carter, & Bowerman, 2002; Kemp, Kopish, Zipperlen, Ahringer, & O'Connell, 2004; Pelletier et al., 2004; Zhu et al., 2008). When assembled, these proteins serve as scaffolds for the downstream effector proteins. The conserved Polo and Aurora A kinases regulate PCM assembly by directly phosphorylating these core coiled-coil domain-containing proteins (Barr & Gergely, 2007; Conduit et al., 2014; Haren, Stearns, & Lüders, 2009; Lee & Rhee, 2011). Despite our understanding of PCM composition and function from these in vivo studies, mechanistic questions regarding how the nanometer-scale PCM proteins organize into a micron-scale, functional organelle remain largely unanswered due in part to the lack of an in vitro *system* for PCM reconstitution.

The main obstacle to reconstituting PCM has been the expression and purification of the large coiled-coil domain-containing proteins that constitute the PCM matrix. Often, proteins that contain coiled-coil domains are insoluble, unstable, and/or express very poorly. Researchers can overcome this problem by making truncated constructs. This strategy has been useful for mapping the protein interaction domains of several centrosome proteins and, more specifically, in identifying a multimerization domain in centrosomin (Boxem et al., 2008; Conduit et al., 2014; Dzhindzhev et al., 2010). However, to reconstitute functional PCM-like assemblies in vitro, full activity of the PCM scaffold proteins is required; thus it is imperative to purify full-length proteins.

We recently developed a method for reconstituting *Caenorhabditis elegans* PCM in vitro using purified full-length SPD-5, PLK-1 (Polo-like kinase 1), and SPD-2 (Cep192) proteins (Woodruff et al., 2015). The results from this study indicate that the coiled-coil domain-containing protein SPD-5 can polymerize into supramolecular scaffolds that recruit downstream PCM proteins. Furthermore, SPD-2 and PLK-1 enhance the formation of SPD-5 assemblies in vitro. Here, we give a detailed description of the methods we used to study PCM assembly in vitro. Our protein expression protocol uses baculovirus vectors and has been optimized for enhanced expression, stability, and solubility of these three proteins inside *Spodoptera frugiperda* (SF9) cells cultured in suspension. SPD-5, PLK-1, and SPD-2 contain

protease-cleavable affinity tags and are purified using tandem affinity steps. We also describe how to assemble and quantify PCM-like structures using fluorescently labeled SPD-5 and unlabeled PLK-1 and SPD-2.

1. METHODS

1.1 EXPRESSION OF SPD-5::GFP, SPD-2, AND PLK-1 IN SF9 INSECT CELLS

We engineered viruses that express (1) MBP PreScission::SPD-5::GFP-PreScission-6xHis, (2) MBP-TEV::SPD-2::TEV-6xHis, and (3) PLK-1::PreScission-6xHis (see Figure 1(A)). We found that adding an MBP tag to the N-termini of SPD-5 and SPD-2 dramatically increased their expression and stability. The N-terminal tag does not affect function. PreScission or TEV protease sites were inserted to allow cleavage and removal of all purification tags. PreScission protease is preferable, as it is more active at 4 °C. However, we found experimentally that PreScission protease cleaves SPD-2 internally at a cryptic site. Thus, TEV protease must be used to remove purification tags from SPD-2.

Our protocol is adjusted to ensure low multiplicity of infection (MOI) during transfection and amplification (MOI = 0.1−1). We also limit the number of virus amplification steps; thus, we use P1 virus stocks to initiate protein expression. These steps help prevent the formation of defective virus particles that show reduced target protein expression and/or express truncated target proteins.

1.1.1 Materials

ESF 921 Insect Cell Culture Medium, Protein-Free (expression systems)
Fetal Bovine Serum (PAA)
Protease Inhibitor Cocktail III (Calbiochem)
PMSF (Sigma)
SF9-ESF *S. frugiperda* insect cell line
SF9 cell resuspension buffer
    50 mM Tris HCl, pH 7.4
    150 mM NaCl
    1% glycerol
    0.5 mM DTT
Protease inhibitors (final concentration)
    1 mM PMSF
    100 μM AEBSF
    0.08 μM Aprotinin
    5 μM Bestatin
    1.5 μM E-64
    2 μM Leupeptin
    1 μM PepstatinA
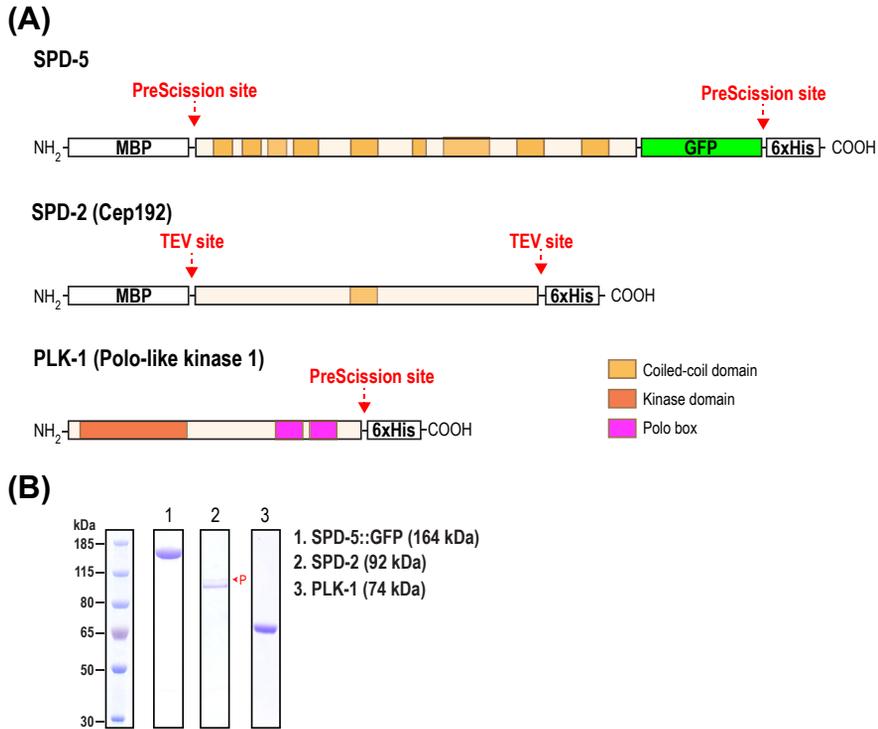
FIGURE 1 Purification of *Caenorhabditis elegans* PCM proteins.

(A) Schematic of the SPD-5, SPD-2, and PLK-1 proteins expressed using baculovirus-mediated infection of insect cells. During purification, all affinity tags can be removed by site-specific proteolysis with either PreScission or TEV protease. (B) Coomassie-stained gels showing purified SPD-5, SPD-2, and PLK-1 after removal of affinity tags. The expected molecular weights of the full-length proteins are listed on the right. The red (gray in print versions) P indicates a phosphorylated species of the full-length SPD-2. This band disappears after phosphatase treatment (data not shown).

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To make final resuspension buffer + protease inhibitor solution, add 10 μL Calbiochem Protease Cocktail III and 50 μL PMSF (200 mM) to 10 mL SF9 cell resuspension buffer.

1.1.2 Protocol

1. Generate baculoviruses expressing the SPD-5, PLK-1, and SPD-2 constructs through general viral transfection protocols. Harvest virus 4–5 days after transfection. Pass cell supernatant through a 0.45 μm filter. This is the P0 stock.

2. Infect 50 mL SF9 cells in suspension (log phase, 0.5 million cells/mL) with 12.5 μL of P0 virus. Add fetal bovine serum to a final concentration of 2%.
3. Incubate cells at 27 °C with moderate shaking. Harvest cells after 72 h by centrifugation. Save the supernatant and discard the cell pellet.
4. Pass the supernatant through a 0.45 μm filter. This is the P1 stock.
5. Infect 500 mL SF9 cells (log phase, 1 million cells/mL) with 5 mL of P1 virus.
6. Incubate cells at 27 °C with moderate shaking.
7. Harvest cells after 72 h by gentle centrifugation (700× g for 5 min; slow deceleration). Discard supernatant by decanting.
8. Add protease inhibitors to resuspension buffer.
9. Resuspend cells in 5 mL resuspension buffer + protease inhibitor solution.
10. Split resuspended cells into three 15 mL Falcon tubes (there should be approximately 3 mL of cell suspension per tube).
11. Flash freeze cells in liquid N_2. Store at −80 °C.

1.2 PROTEIN PURIFICATION

1.2.1 Purification of SPD-5::GFP

SPD-5 sticks nonspecifically to a wide variety of resins commonly used for protein purification. Traditional protein purification techniques that require large volumes of resin, such as size exclusion chromatography, are not advisable. One exception to this rule is Ni-NTA. Also, we found that using batch binding and gravity elution columns produces higher protein yields than using packed columns with high pressure solvent flow (e.g., fast protein liquid chromatography (FPLC) systems).

1.2.1.1 Materials

Ni-NTA affinity resin (Qiagen)
Amylose affinity resin (New England Biolabs)
Glutathione sepharose 4B (GE Healthcare)
15 mL disposable chromatography columns (Bio-rad)
30 K Amicon Ultra centrifugal filter units (Millipore)
0.22 μm cellulose acetate spin filters (Agilent Technologies)
Superdex 200 HR 10/30 column (GE Healthcare)
PreScission protease (1 mg/mL; GST-tagged)
TEV protease (1 mg/mL; GST-tagged)
Buffer A
    50 mM Tris HCl, pH 7.4
    500 mM NaCl
    30 mM imidazole
    1% glycerol
    0.1% CHAPS

Buffer B
>50 mM Tris HCl, pH 7.4
>500 mM NaCl
>250 mM imidazole
>1% glycerol
>0.1% CHAPS

Buffer C
>50 mM Tris HCl, pH 7.4
>500 mM NaCl
>1% glycerol
>0.1% CHAPS
>0.5 mM DTT

Protease inhibitors
>1 mM PMSF
>100 μM AEBSF
>0.08 μM Aprotinin
>5 μM Bestatin
>1.5 μM E-64
>2 μM Leupeptin
>1 μM PepstatinA

1.2.1.2 Protocol

1. Assemble all reagents and cool down centrifuge, centrifuge tubes, and the dounce homogenizer.

   a. Put the dounce homogenizer on ice and wash once with cold water, then once with cold Buffer A.

   b. Label the ultracentrifuge tubes with tape, then put them on ice.

   c. Close the door of the Beckman Ultracentrifuge, turn on the vacuum, and cool down to 4 °C.

   d. Prepare Ni-NTA columns. Pour 5 mL of Ni-NTA beads into a plastic column. Wash twice with water (50 mL), then once with Buffer A (25 mL).

   e. Prepare amylose columns. Pour 2.5 mL of amylose beads into a plastic column. Wash twice with water, then once with Buffer C.

   Note: Steps 2–3 must be performed quickly!!!

2. Add protease inhibitors to lysis buffer.

3. Add 12 mL lysis Buffer A (with protease inhibitors) to 3 mL of frozen cell suspension (fill Falcon tube to the top). Quickly thaw the cells by incubating in a beaker with warm water.

   Note: All remaining steps must occur at 4 °C.

4. Once the cell suspension is loose (partially unfrozen), place the falcon tube on the rotator and rotate for 15 min in the cold room.

5. Check if the cells are fully thawed and resuspended. If not, then pipet up and down a few times.
6. Pour the cells into the clean, prechilled dounce homogenizer. Add 10 mL of Buffer A (+protease inhibitors) to the falcon tube and then pour into the dounce. Gently plunge up and down five times.
7. Use a pipet to remove the homogenized lysate and dispense it into a prechilled ultracentrifuge tube.
8. Balance the tubes by weighing them and adding buffer.
9. Place tubes in a 50.2 Ti rotor, then place the rotor in the Beckman Ultracentrifuge. Spin at 40,000 rpm for 25 min.
10. Remove tubes from the rotor and pour off the supernatant into a prechilled 50 mL falcon tube. This is the high-speed supernatant. Save 20 μL for later analysis on an SDS-PAGE gel.
11. Pour the high-speed supernatant over the prepared Ni-NTA column. Allow the supernatant to drip through once (about 10 min). Collect this flow through in your 50 mL falcon tube. Then, pour the flow through back into the column. Close the column and put in on the rotator and rotate for 60 min or longer.
12. Open the column and let the unbound protein flow through. Save the flow through for later analysis.
13. Wash off non-specifically bound proteins from the Ni-NTA. Fill the column to the top with Buffer A. Repeat 5 times. Check for unwanted proteins in the flow through using a Bradford assay.
14. Elute bound protein with Buffer B. Close the column and rotate it on the rotator for 30 min or longer. Open the column and collect the eluate in a 50 mL falcon tube. Save 20 μL for later analysis on an SDS-PAGE gel.
15. Load the eluate onto the prepared amylose column. Close the column and rotate on the rotator for 60 min or longer.
16. Open the column and collect the flow through. Save 20 μL for later analysis on an SDS-PAGE gel.
17. Wash the column with Buffer C. Repeat 5 times. Check for unwanted proteins in the flow through using a Bradford assay.
18. Elute your protein from the column by adding 25 mL Buffer C plus 100 μL PreScission protease (1 mg/mL). Close the column and rotate on the rotator overnight in the cold room.
19. Open the column and collect the eluate. Once the column is empty, add 20 mL of Buffer C and collect the leftover protein in the same tube used for collecting the eluate.
20. Add 500 μL of prewashed glutathione beads. Incubate for 1 h. This will remove the PreScission protease.
21. Pass eluate + glutathione beads over an empty chromatography column to remove the beads. Collect the flow through in a new 50 mL Falcon tube.
22. Concentrate the flow through using a 15 mL 30 K Amicon concentrator. Just use one. Spin at 4000 rpm for 10 min in a tabletop centrifuge with a swinging

bucket rotor. Dump the flow through. Load again. Repeat until all of the eluate has been concentrated down to about 1 mL (the time needs to be determined experimentally, but spin for at least 10 min).

23. Add 14 mL of Buffer C to the concentrator. Spin until the protein is concentrated to about 1 mL. This step will ensure that any remaining cleaved His tags are removed (the concentrator has a 30 kDa cutoff, so the His tag should easily flow through).

24. Measure the absorbance of the protein at 280 nm using a NanoDrop 1000 and determine protein concentration from the absorbance by entering its extinction coefficient (E(1%)).

25. Distribute protein in 15 μL aliquots to prechilled PCR tubes. Label them and snap-freeze them in liquid nitrogen. Plunge freezing is the best way to ensure uniform and quick freezing.

26. Store the PCR tubes in a small cardboard box and keep at $-80\,^{\circ}\mathrm{C}$.

27. Analyze high-speed supernatant, Ni-NTA flow through, Ni-NTA eluate, amylose flow through, amylose eluate, and final product on an SDS-PAGE gel.

1.2.2 Purification of SPD-2

Follow the same protocol used for SPD-5::GFP described above. Important: use the appropriate protease for cutting (TEV instead of PreScission protease). At the very end, spin protein at 80,000 rpm for 10 min to remove any aggregates that may have formed.

1.2.3 Purification of PLK-1

1. Follow steps 1–13 in the SPD-5::GFP purification protocol.

2. Elute PLK-1 from the Ni-NTA resin by adding 25 mL Buffer B plus 100 μL PreScission protease (1 mg/mL). Incubate overnight.

3. Save the eluate and pass through a 0.22 μm cellulose acetate spin filter to remove large particles.

   Note: Subsequent steps can occur at room temperature.

4. Connect a 24 mL Superdex 200 size exclusion column to a FPLC system. Wash column with two column volumes of filtered and degassed water, followed by two column volumes of filtered and degassed Buffer C.

5. Inject the filtered protein solution into the FPLC, run, then collect 0.5 mL fractions in a multiwell plate.

6. Analyze the fractions using SDS-PAGE gel electrophoresis followed by Coomassie staining. PLK-1 should elute as a single peak in the 14–15 mL fractions.

7. Collect the fractions containing full-length PLK-1 and concentrate using an Amicon 30 K concentrator.

8. Measure protein concentration, aliquot protein into PCR tubes, flash freeze, and store as per steps 24–28 in the SPD-5::GFP purification protocol.

1.3 SPD-5::GFP NETWORK PREPARATION

SPD-5::GFP will assemble into micron-scale networks over time in a concentration-dependent manner. This means that SPD-5::GFP will form networks during the final concentration step during purification. However, performing a proper network assembly assay requires SPD-5::GFP to be in the unassembled state. Diluting SPD-5::GFP into a high salt, high detergent buffer and incubating overnight at 4 °C will ensure that all preexisting networks are dissolved. Network assembly can then be initiated by diluting SPD-5::GFP into a low salt, no detergent buffer and raising the temperature to 23 °C. Inert red fluorescent beads are added to the sample to aid in maintaining proper focus during imaging. If blocked properly, these beads should not interfere with SPD-5::GFP network assembly or morphology.

1.3.1 Materials

HEPES-KCl Buffer

    25 mM HEPES, pH 7.4
    150 mM KCl
    0.5 mM DTT
Network Dilution Buffer
    50 mM Tris HCl, pH 7.4
    500 mM NaCl
    1% Glycerol
    0.1% CHAPS
    0.5 mM DTT
0.2 μm FluoroSpheres, red fluorescent beads (Molecular Probes)
    Before use, wash the beads in water, then block the beads in a solution of
    5 mg/mL BSA or 5 mg/mL ovalbumin for 16 h at 4 °C. Wash the beads 3×
    to remove unbound blocking agent, then resuspend in HEPES-KCl buffer
    and store at 4 °C. You will need to adjust the final bead concentration so that
    you have >10 beads per field of view during microscopy. Fewer beads will
    result in improper autofocusing.
20× ATP·MgCl$_2$ mix
    4 mM ATP
    200 mM MgCl$_2$
    Dilute into HEPES-KCl buffer. Aliquot and store at −20 °C.
0.5 mg/mL ovalbumin
    Dissolve ovalbumin (Sigma) in HEPES-KCl buffer. Aliquot and store at
    −20 °C.

1.3.2 Protocol

1. Day 1: Dilute SPD-5::GFP to 250 nM using Network Dilution Buffer. Incubate overnight at 4 °C. Dilution and overnight storage will promote disassembly of preexisting SPD-5 networks.

2. Day 2: Set up a master network reaction in 1.5 mL microcentrifuge tubes.
Example 1. 12.5 nM SPD-5 assembly reaction

1 μL	250 nM SPD-5::GFP
1 μL	Preblocked red fluorescent beads
1 μL	0.5 mg/mL ovalbumin
17 μL	HEPES-KCl buffer
20 μL	

Example 2. 12.5 nM SPD-5 + 12.5 nM PLK-1 + 12.5 nM SPD-2
assembly reaction

1 μL	250 nM SPD-5::GFP
1 μL	250 nM PLK-1
1 μL	250 nM SPD-2
1 μL	Preblocked red fluorescent beads
1 μL	0.5 mg/mL ovalbumin
1 μL	20X ATP·MgCl$_2$ mix
14 μL	HEPES-KCl buffer
20 μL	

3. Distribute master mix in 20 μL aliquots to other 1.5 mL microcentrifuge tubes.
Incubate for desired amount of time at 23 °C.
4. Place an 18 × 18 mm coverslip on the Pressing Device (illustrated in Figure 2(A)).
5. Create centering marks on a nonfrosted microscope slide using the slide guide.
To do this, place the slide on top of the Slide Guide (illustrated in Figure 2(A))
and trace the black lines using a pen.
6. Pipet 2.3 μL of a reaction into the center mark area of the marked microscope
slide. Important: Do not turn the slide after pipetting, instead pipette the reaction
on the bottom side of the slide directly. Turning the slide will cause the drop to
spread and dry out, thus affecting the morphology of the networks.
7. Place the slide with the hanging drop sample on the foam supports of the Pressing
Device (see Figure 2(A)). Align the slide with the cover slip using the centering
marks.

Note: The Pressing Device is needed to prevent bubbles from forming under the
coverslip. Bubbles will cause denaturation and unwanted aggregation of protein that
will significantly alter your results. The centering marks and cover slip holder are
necessary to maintain reproducibility in later analysis steps.

8. Press down on the slide gently. Use both hands and apply pressure evenly
above the foam supports. Press until the hanging drop just contacts the coverslip.
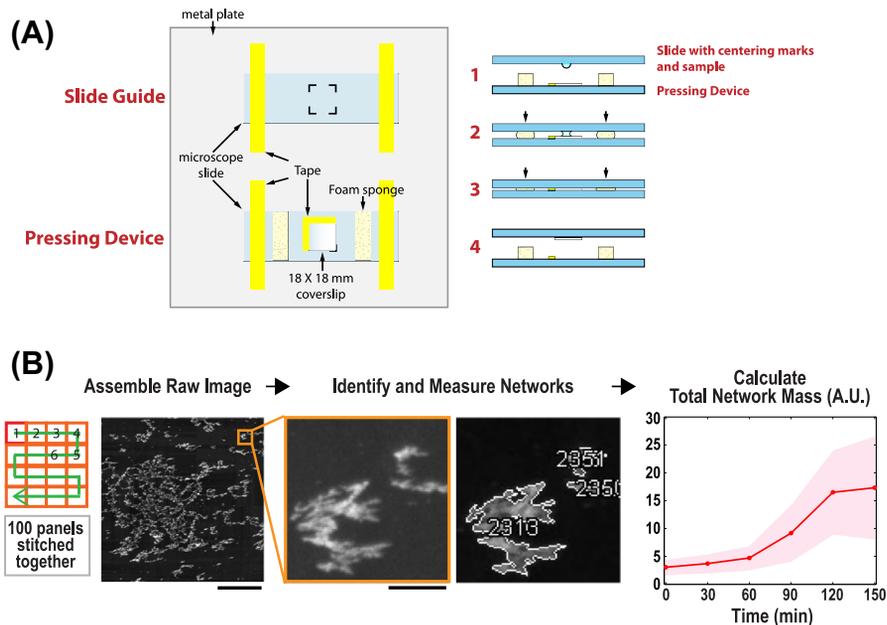
FIGURE 2 Analysis of SPD-5::GFP assemblies.

(A) Schematic of the devices used to prepare the samples for imaging. These devices ensure reproducible positioning of the cover slip and prevent the formation of bubbles in the sample. A new nonfrosted microscope slide is placed over the slide guide and the centering marks are traced with a pen. The sample is applied in the center of the centering marks on the bottom side of the marked slide (Step 1). An 18 × 18 mm coverslip is loaded into the Pressing Device. Second, the sample slide is placed on the Pressing Device and gentle pressure is evenly applied above the two sponges, bringing the sample droplet in contact with the coverslip (Step 2). The slide is then pressed down farther until the cover slip adheres to the free slide (Step 3). Finally, the free slide is lifted up and the newly adhered cover slip is sealed with nail polish (Step 4). The sample is now ready for imaging. (B) Overview of the imaging and analysis protocol. The objective scans across the sample and collects 100 adjacent panels in a square pattern. Every two panels, the focal plane is readjusted using an autofocusing routine that identifies fluorescent red beads in the sample (not shown). The image panels are stitched together to assemble the final raw image. This image is analyzed with an image processing script that identifies and calculates the integrated fluorescence intensity for each SPD-5::GFP network. The integrated fluorescence intensities for all of the networks in a sample are summed up, giving the Total Network Mass. Finally, after performing several experimental replicates, the mean Total Network Mass is determined and plotted. The graph on the right depicts the formation of SPD-5::GFP networks over time for an initial concentration of 25 nM SPD-5::GFP. The red (gray in print versions) line represents the mean Total Network Mass per time point and the pink shaded area represents the 95% confidence interval (n = 10). Scale bars, 25 μm (first panel), 2.5 μm (inset).

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The coverslip should then adsorb to the slide, if not, then press down a little bit more. This will spread the networks on the glass surface for easy detection.

9. Remove slide and seal the coverslip using nail polish.

Important: Once you remove sample from a tube, discard the tube. Pipetting will disrupt the networks and initiate disassembly. However, disassembly will not occur if the networks are adsorbed to glass. Thus, your squashed samples are relatively stable and can be visualized over the next 30 min.

1.4 IMAGING OF SPD-5::GFP NETWORKS

We use automated microscopy to collect a series of images and then stitch them together into one final image for analysis (see Figure 2(B), first panel). The appearance of bright, interconnected assemblies indicates that the assembly reaction was successful (Figure 2(B), middle panels). The red beads in the sample can be used for autofocusing, as the SPD-5::GFP networks (if assembled) should be in the same focal plane as the beads. This is especially useful in the earlier time points of the experiment before network formation has begun. The red beads should not be fluorescent when illuminated in the GFP channel (475 nm excitation/523 nm emission). If they are, it means that they were not properly blocked and are sticking nonspecifically to monomeric SPD-5::GFP.

Important: SPD-5::GFP networks may not be distributed homogenously under the squashed coverslip. Therefore, to ensure adequate quantification of network formation, it is essential to sample a large area. We typically collect >100 images per sample ($40\times$ objective, 1024×1024 pixels per image).

1.5 DATA ANALYSIS

SPD-5::GFP networks can be identified and analyzed using Fiji or ImageJ, which are freely available. In brief, the stitched image is thresholded to generate a mask. The mask is then used to create selections that outline the SPD-5::GFP networks. Finally, the selections are applied to the original stitched image, and the total area and mean fluorescence intensity of SPD-5::GFP networks are measured. These data can be compiled from multiple replicates and timepoints to generate a network assembly curve (Figure 2(B), last panel).

1. Open stitched image in Fiji or ImageJ.
2. Subtract background from image.
3. Under "Image," click "Duplicate."
4. Click on the duplicated image. Under "Image/Adjust," select "Threshold." Choose dark background. Adjust the lower bound until only the SPD-5::GFP networks are identified. Click "Apply." This is the masked image.
5. Invert masked image. The background should now be white.
6. Under "Edit/Selection," select "Create selection." This will outline the networks with a yellow line.

7. Under "Edit/Selection," click "Add to Manager." This will store the ROI generated in step 5.
8. Click on the original image.
9. Under "Image/Overlay," click "From ROI Manager." This will overlay the outlines generated in step 5 onto the unaltered image.
10. Under "Analyze," click "Measure." With the default settings, this will measure the total area and average fluorescence intensity of the material within the ROI. Multiplying these values will generate the integrated fluorescence intensity, which can be used as an estimate of the total amount of SPD-5::GFP network existing within the stitched image.

CONCLUSION

Here, we report how to express and purify the *C. elegans* PCM proteins SPD-5, PLK-1, and SPD-2 and how to use them to form PCM-like assemblies in vitro. We also describe imaging and analysis methods to quantify the formation of these assemblies over time. Using this system, we have recently shown that SPD-5 can polymerize into supramolecular assemblies that serve as hubs for downstream PCM proteins (Woodruff et al., 2015). In the future, this system can be used for identifying proteins that influence SPD-5 polymerization and also for identifying structural features of SPD-5 that mediate self-assembly.

Recent work from Conduit et al. (2014) suggests that the *Drosophila* protein centrosomin may perform a similar function as SPD-5 in forming the foundation of the fly PCM. However, full-length centrosomin has yet to be purified; thus, it is not known if full-length centrosomin can assemble micron-scale assemblies in vitro. We are hopeful that the methods described here can be applied to the in vitro study of PCM proteins from *Drosophila*, humans, and other centrosome-containing organisms.

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