IMAGING SEPTUM FORMATION BY FLUORESCENCE MICROSCOPY

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Running head: Fluorescence imaging of the septum

Abbreviations: B-BG, branched $\beta(1,3)$ -glucan; L-BG, linear $\beta(1,3)$ -glucan; CW, Calcofluor white.

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SUMMARY

Fungal cleavage furrow formation during cytokinesis relies in the coordinated contraction of an actomyosin-based ring and the centripetal synthesis of both new plasma membrane and a special wall structure named division septum. Through transmission electron microscopy the septum exhibits a three-layered structure with a central primary septum, flanked at both sides by the secondary septum. In contrast to the chitinous primary septum present in most of fungi, the fission yeast *Schizosaccharomyces pombe* does not contain chitin, instead it divides through the formation of a linear $\beta(1,3)$ glucan-rich primary septum, which has been shown to be specifically stained by the fluorochrome Calcofluor white. Recent findings in *S. pombe* have revealed the importance of septum synthesis for the steady contraction of the ring during cytokinesis. Therefore, to study the molecular mechanisms that connect the extracellular septum wall with the other components of the cytokinetic machinery located in the plasma membrane and cytoplasm, new experimental approaches are needed. Here we describe the methods developed to image the septum structure by fluorescence microscopy, with a special focus in the analysis of septum progression by the use of time-lapse microscopy.

KEY WORDS

cell wall; septum; polysaccharide; $\beta(1,3)$ -glucan; Calcofluor white; glucan synthase; cytokinesis.

1. INTRODUCTION

The *S. pombe* septum is a special structure of wall material formed during the cytokinesis. Its synthesis is intimately coupled to the actomyosin ring contraction and the addition of new plasma membrane. Once completed, the septum physically separates the original cell in two identical new cells. Next, the controlled dissolution of the glucans in the septum edging and the primary septum in the complete septum structure, allows cell separation and the end of cytokinesis [1-4]. When observed by transmission electron microscopy, the septum displays a three-layered structure (Figure 1A), with a central disk of primary septum flanked at both sides by the secondary septum [5]. The septum is mainly composed of interlinked glucose polysaccharides [6]. Immunoelectron microscopy using colloidal gold-labeled antibodies specific against different β -glucans has helped to define the organization of these polysaccharides in the *S. pombe* septum [7]:

1) Linear $\beta(1,3)$ -glucan (L-BG) is mostly found in the primary septum structure. This polysaccharide consists of $\beta(1,3)$ -linked glucose units forming linear chains in a conformation of single-helix with a small proportion of triple-helix structures. The L-BG has been shown to be responsible for the primary septum structure observed by electron microscopy [8]. The primary septum organization is similar to those found in plants, which is also formed by a L-BG named callose [9], or in *Saccharomyces cerevisiae*, which is formed by chitin. The single-helix conformation of L-BG makes the primary septum more susceptible than the secondary septum to degradation by $\beta(1,3)$ -glucanases [10]. The L-BG is recognized with higher affinity than the rest of *S. pombe* wall polysaccharides by the fluorochrome Calcofluor White (CW), and is responsible for the strong labeling of the septum wall with this fluorescent dye [8] (Figure 1A and B).

2) Branched $\beta(1,3)$ -glucan (B-BG) is located along all the septum structures, including the primary septum. It is made of $\beta(1,3)$ -linked glucose units forming linear chains with $\beta(1,6)$ -linked branches of $\beta(1,3)$ -linked chains. Unlike L-BG, the B-BG chains form closed triple-helix structures that are more resistant than those of L-BG to degradation by $\beta(1,3)$ -glucanases [11-13]. The B-BG is essential for cell integrity and for secondary septum formation. In addition, the B-BG plays a crucial role in cytokinesis, linking the cell wall to the plasma membrane and contractile actomyosin ring, which is needed to couple the septum synthesis with the plasma membrane and ring progression [4].

3) $\beta(1,6)$ -glucan is only located in the secondary septum. It is a highly branched polysaccharide formed by a main chain of $\beta(1,6)$ -linked glucose units and 75% of $\beta(1,3)$ -linked branches of $\beta(1,6)$ -linked chains [14-15].

Besides the β -glucans, the *S. pombe* septum also contains α -glucan. This polysaccharide has not been detected yet by immunoelectron microscopy, although it has been shown that the $\alpha(1,3)$ -glucan is localized with the B-BG in both primary and secondary septum [3-4]. It is absent in the budding yeast and in *C. albicans*, but is present in the cell wall of filamentous and dimorphic fungi [16,3,17,4]. *S. pombe* α -glucan is a linear polysaccharide formed by chains of D-glucose bound by $\alpha(1,3)$ linkages and with a small proportion of $\alpha(1,4)$ -linked residues located at the reducing end [18]. Like B-BG, this polysaccharide is essential for cell integrity and for secondary septum formation. It also plays an essential role in the primary septum adhesion strength needed to support the physical force of the internal turgor pressure during cell abscission [3].

Recent studies have revealed the importance of septum synthesis for the proper actomyosin ring contraction and cleavage furrow formation in *S. pombe* [4,19-20]. Direct visualization of living cells is an important method for cytokinesis studies and fluorochromes can be used for cell wall and septum fluorescence microscopy analysis. The most commonly used is CW, also called Blankophor (Blankophor GmbH & Co.) or Fluorescent Brightener 28 (Sigma-Aldrich). Taking advantage of the higher affinity of CW for the L-BG of the primary septum, here we describe a method to follow the synthesis of the septum by time-lapse fluorescence microscopy (Figure 2A). Besides we describe additional methods developed to image the septum structure either in live or fixed cells.

CW (Figure 2D) is a water-soluble dye that interacts with various polysaccharides, but displays a higher affinity for chitin and cellulose. It intercalates into nascent chains of chitin, and at high concentrations this prevents chitin microfibrils assembly [21]. However, at very low concentrations it can be used as a live-cell stain. Concentrations of CW ranging between 10 to 100 $\mu\text{g ml}^{-1}$ are sublethal and higher concentrations are lethal for *S. cerevisiae* cells [22], while *S. pombe* cells, which do not contain detectable chitin in their cell wall, are highly resistant to CW [21], growing in the presence of concentrations of up to 1.5 mg ml^{-1} of the dye [23-25]. Compared to these concentrations, our method to follow septum formation and progression by time-lapse

fluorescence microscopy uses a highly reduced concentration of CW (1.25 to 5 $\mu\text{g ml}^{-1}$) which does not affect the physiology of *S. pombe*.

CW is excited with UV light and fluoresces in a spectrum near-UV light (optimum excitation wavelength of 347 nm and peak emission wavelength of 450 nm), close to the range of DAPI and Hoechst dyes used for nuclear staining. The low excitation / emission peaks allow the use of CW for multiple fluorescence labels (Figure 2B) in combination with the different versions of the GFP and/or RFP fluorescence proteins, or with other vital dyes which have major excitation peaks to higher spectral wavelengths [26-27].

Although here we mainly describe a time-lapse method based on the CW staining fluorescence, this method can be adapted to other dyes used to stain the cell wall polymers after the previous testing of the doses that do not alter cytokinesis and permit to image the derived-septum fluorescence along the time. Aniline blue and its derivative Sirofluor (also named Water blue, Cotton blue, Poirriers blue, Methyl blue) (Sigma-Aldrich; Polysciences, Inc.; Merck Millipore; Kingston Chemistry; etc) are also used to stain the primary septum, but the concentration to be used varies considerably depending on the supplier. This dye binds to the single-helix and open triple-helix conformations of the L-BG but not to the closed triple-helix conformation of the B-BG [28]. In budding yeast it has been assumed that this dye stains specifically the closed triple-helix B-BG of the secondary septum and not other polysaccharide of the septum [29]. Since in this yeast it has not been described the existence of a single-helix L-BG, the specific affinity of Aniline blue in the cell wall of budding yeast still must be determined. Congo red (Sigma-Aldrich) binds to the chitin fibrils in the fungal cell walls and it is thought to interfere with cell wall assembly by binding to chitin as CW does [21-22]. Solophenyl Flavine 7GFE 500 (Ciba Specialty Chemicals) and Pontamine Fast Scarlet 4B (Bayer Corp.) have also been described as useful dyes of fungal cell walls, septa, and bud scars [26]. These two dyes fluoresce in the green and red spectrum respectively, so theoretically they could be used in combination with CW for a putative differential cell wall staining.

2. MATERIALS

2.1. Reagents

- CW fluorescent brightener 28 (Sigma-Aldrich). A stock of CW to 10 mg ml⁻¹ is made by adding 100 mg of the fluorescent brightener 28 in 10 ml of distilled water and filter sterilized. The final stock can be stored in the dark at 4°C for at least 2-3 months. Discard when the solution displays signs of precipitated material (see **Notes 1 and 2**).
- 10% potassium hydroxide.
- Lectin from Glycine max, soybean (Sigma-Aldrich). Prepare 1 mg ml⁻¹ by adding the corresponding volume of distilled water to the original vial with the powder. Next the solution is divided into 100 to 200-µl aliquots and stored at -20°C.
- Fresh standard fission yeast culture media: yeast extract plus supplements (YE4S), or Edinburgh minimal medium + supplements (EMM4S). Recipes can be found at: <http://www-bcf.usc.edu/~forsburg/media.html>.

2.2. Disposables and Equipment

- Uncoated plastic or glass 15 µ-Slide 8 well for Live Cell Analysis (Ibidi GmbH). These slides (Figure 2C) allows standard immunofluorescence protocols and live imaging of cells growing in liquid medium over extended periods of time on an inverted microscope (see **Note 3**).
- Inverted fluorescence microscope.

3. METHODS

3.1. Time-lapse fluorescence microscopy of the septum formation in *S. pombe*

This method is basically as described in [3-4], with some modifications and tips to improve its understanding and make it simpler. We prefer to image the cells in liquid growth medium instead of using agarose embedding medium (see **Note 4**). Using liquid medium allows to maintain the cells in the growth conditions more efficiently and without any stress. In addition, this method is more conservative because it does not apply pressure on the cells, contrarily to the pressure caused by the coverslips used in agarose embedding medium

3.1.1. Preparing the cells.

Use early log-phase cells growing in the same liquid medium and temperature that are going to be used for imaging. Maintain the cells in early log-phase with the appropriate dilutions.

Incubate 4-ml of early log-phase cells in 25-ml flasks using an incubator shaker set to 200 rpm shaking speed, which allows a stable incubation temperature and the adequate aeration of the cells (see **Note 5**).

3.1.2. Coating the slide.

The uncoated slide must be coated with lectin to promote the cell adhesion to the bottom surface.

1. Add the lectin (1 mg ml^{-1}) 20-30 min before image acquisition onto the bottom surface of a well of the μ -Slide (5- μl for plastic or 2.5- μl for glass bottom slides).
2. Gently spread the lectin to cover uniformly the well bottom surface. The side of a clean pipette tip can be used for spreading. Let the lectin dry at room temperature and wash the well twice with 300- μl of growth medium each, before addition of cells (see **Notes 6 and 7**).

3.1.3. Seeding cells.

1. Prepare a fresh stock of growth medium with CW. Dilute the CW to the desired final concentration ($1.25\text{-}5 \text{ }\mu\text{g ml}^{-1}$) in 10-ml of the same growth medium where the cells are growing.
2. Centrifuge 750- μl of early log-phase growing cells at 2348 g during 1 min. and resuspend them in 300 to 500- μl of liquid medium containing CW.
3. Apply 300- μl of the cell suspension into a well of the μ -Slide and leave it at room temperature for 1.5 min. Avoid shaking or moving the μ -Slide as this will result in a non homogenous distribution of the cells (see **Note 8**).
4. After seeding the cells, carefully remove the medium and wash the well twice with 300- μl of liquid medium containing CW. The washes permit to remove the cells that are not attached to the bottom surface of the well.

5. Add 300- μ l of liquid medium containing CW and cover the μ -slide with the supplied lid. Then the cells are ready to be observed and imaged on the inverted fluorescence microscope.

3.1.4. Time-lapse microscopy.

Consider the time scale of the dynamic processes being imaged to specifically optimize the image acquisition intervals and the total time of cell imaging. In a wild-type *S. pombe* strain septum formation takes approximately 20-30 min depending on the temperature and growth medium selected for the specific experiment. However, in diverse mutant strains septum formation may take longer. Although the septum forms a disk structure, 3D acquisition over the time is not required. As an example, a typical capture uses: 100 $\times$ /1.40 IX70 objective lens; the camera set up to 2 $\times$ 2 binning; 30 to 120 sec interval between successive images; 0.250 sec exposure time with 10% of light transmission intensity; and 30 to 60 min of total cell imaging time (see **Notes 9** and **10**).

3.2. Fluorescence microscopy of the septum structure in *S. pombe*

3.2.1. CW-staining of live cells

To image directly the primary septum of live cells, early log-phase cells grown in liquid medium can be visualized directly by adding a solution of CW to the sample (1.25 to 50 μ g ml⁻¹ final concentration):

1. Use precleaned/ready to use microscope slides. Always clean the surface of the slide with a coverslip to remove the remaining dust particles. This will help to image all the cells in focus, in a single focal plane. The single layer of cells between slide and coverslip permits to keep the cells stopped, without the cell movement that would be caused by convection of the liquid medium in a broader space.

2. Centrifuge 500 to 1000- μ l of growing early log-phase cells at 2348 g during 1 min. Discard the supernatant leaving 10 to 25- μ l of the liquid medium, depending on the amount of cells to be imaged. Resuspend the cells and add CW to make 1.25 to 50 μ g ml⁻¹ final concentration. Alternatively, remove the entire liquid medium and resuspend

the pellet of cells in 10 to 25- μ l of water, PBS or liquid medium containing CW (1.25 to 50 μ g ml⁻¹ final concentration).

The standard concentration of CW used for septum staining is 50 μ g ml⁻¹ final concentration; but lower concentration can be useful for differential staining in non-saturated conditions (see **Notes 11** and **12**).

3. Deposit 1.75- μ l of the cell suspension in the slide and place a clean coverslip over the sample. To obtain a monolayer of cells distributed uniformly, be sure that the cell suspension is covering all the surface of the coverslip. Depending on the time used for imaging cells in a single slide, the coverslip can be sealed with melted VALAP (1:1:1 vol mixture of vaseline, lanolin, and paraffin) to prevent the sample from drying.

4. Image the cells under a microscope equipped with a UV lamp and the appropriate filter (see **Note 13**).

3.2.2. CW-staining of fixed cells

When acquisition of multiple label fluorescences is required, the cells may be occasionally fixed to preserve the fluorescence of a specific dye or tagged protein or its localization to a specific cellular structure. Several methods for *S. pombe* cells fixation have been described [30]. For CW-staining cold 70% ethanol is the most generally used:

1. Centrifuge 500 to 1000- μ l of growing early log-phase cells at 2348 g during 1 min.
2. Add 1-ml of cold 70% ethanol and resuspend by vortexing the pellet of cells. In this step the samples can be stored at 4°C.
3. Before microscope acquisition rehydrate the cells by removing the ethanol by centrifugation as above and adding 1-ml of water. Centrifuge the cells and resuspend the pellet in 10 to 25- μ l of a solution containing CW and proceed as described for CW-staining of live cells (see section 3.2.1).

Ethanol fixation can be used to image the nucleus (DAPI or Hoechst staining) and septum (CW-staining) simultaneously. CW fluorescence interferes with the signal from the DAPI or Hoechst staining. Thus proper CW concentration should be adjusted

before imaging. Here we briefly describe a protocol that works well for live and fixed cells. Centrifuge early-logarithmic phase cells, live or fixed with cold 70% ethanol, and resuspend them in $20 \mu\text{g ml}^{-1}$ Hoechst or DAPI. Keep the cells at room temperature in the dark for several minutes, wash them to remove the Hoechst or DAPI, and add 10 to 25- μl of a $10 \mu\text{g ml}^{-1}$ CW solution. All these fluorophores are visualized with the same UV filter (see **Note 14**).

An alternative method using mounting media, CW and DAPI can be found in [30].

3.2.3. Immunofluorescence of the linear $\beta(1,3)$ -glucan of the primary septum.

Since the primary septum contains L-BG, its structure can be studied through immunofluorescence by using a specific monoclonal antibody against the L-BG [7],[31]. *S. pombe* immunofluorescence method is essentially described in [32] with some modifications [8]. Briefly, cells are fixed with 100% ethanol for 30 min at -20°C , washed and incubated with 0.15 to $5.0 \mu\text{g ml}^{-1}$ of Novozyme-234, also named Glucanex (Sigma-Aldrich) for 45 min at 30°C in PEMS buffer (100 mM Pipes pH 6.9, 1 mM EGTA, 1 mM MgSO_4 , 1.2 M sorbitol) to allow a controlled and progressive partial digestion of the cell wall. Then labeling of the primary septum is performed using primary anti-L-BG-specific monoclonal antibody 400-2 (1:100 dilution; Biosupplies), and secondary Alexa Fluor 594 labeled anti-mouse antibody (1:400 dilution) (see **Note 15**).

4. NOTES

1. CW is described as hazardous and potentially carcinogenic. Wear protective gloves, eye shields, and a dusk mask to prevent inhalation during stock preparation.
2. Depending on the supplier the CW cannot be directly dissolved in water so its dissolution may be aided by the addition of 10 N KOH dropwise, with mixing until the solution becomes clear.
3. In addition to the 15 μ -Slide 8 well, this method can be used with glass bottom dishes (P35G, MatTek), although we favour the use of the μ -Slides because they can be inserted in most of microscope stages and can be used for 8 individual experiments.

4. As an alternative method to the live fluorescence imaging with lectin-coated μ -Slides or bottom-glass dishes the cells can be mounted in agarose pads, where the agar embedding growth medium keeps the cell in a immobilized position and provides an acceptable environment for sustained cell growth. Briefly, these agarose pads contain the appropriate yeast growth medium plus 2% w/vol of agarose. Place approximately 100 μ l of hot melted agarose onto a clean glass slide and immediately place a second glass slide on top of the agar drop. After a few minutes to let the agar to cool and solidify, remove the top glass slide from the agar pad by gently but quickly sliding apart both glass slides. Next add quickly 1-2- μ l of cell suspension onto the solid agar pad, place a coverslip on top of the cells and seal it with melted VALAP (a 1:1:1 vol mixture of vaseline, lanolin, and paraffin). Then the cells are ready to be imaged for a long-term period [33].

5. To improve the cell wall and septum labeling, CW can be added to the growth medium and the cells can be incubated in this medium for 12-24 h or longer times before image acquisition. Use the same concentration of CW (1.25 to 5 μ g ml⁻¹) before and during image acquisition.

6. Coating can be prepared a day in advance, but it is preferable to make it the same day of use. Keep the coated μ -Slide at room temperature until its use. Be sure to keep track of which well is coated.

7. Poly-L-lysine or Concanavalin A coated μ -Slides do not completely immobilize fission yeast cells; however they can be used for budding yeast cells microscopy.

8. The cells must be handled and seeded carefully. The absence of adequate care can lead to non-flattened cells, resulting in regions of the cells out of focus and hence, with multiple focal layers. In our case 1.5 min of seeding works well in most of the cell backgrounds displaying wild type morphology. However the seeding time may vary depending on the concentration of lectin used to coat the well, the growth medium or the cell shape. For example, liquid medium containing sorbitol is denser and therefore the cells need longer times (2.5 to 3 min) to seed and attach to the well surface. On the contrary, longer mutant cells need less time to seed (0.5 to 1 min).

9. Phototoxicity and photobleaching are two major problems in live cell analysis. Although it has been described that CW fades rapidly when exposed to excitation

wavelengths [26] in our experimental conditions we never detected any bleaching of the CW fluorescence over the time.

10. CW is excited with UV or near-UV light, phototoxicity can be also an issue. Phototoxicity describes a general class of damaging effects on live cells based on either long term or short but extreme exposures to a light source. Phototoxicity can be a result of DNA damage (UV light) or protein damage (infrared light). Besides, excessive excitation of a fluorophore can lead to oxide radical formation and may also negatively impact cell growth [34]. To avoid unexpected effects during acquisition, 3D microscopy of the septum over the time is not recommended unless necessary for a specific experiment. On the contrary, reducing the percentage of light transmission to 10% and using 0.250 s of exposure time, permit to extend the movie acquisition time up to at least 90 time points during 90 min using the CW fluorescent dye to follow septum formation.

11. CW signal depends on the concentration of cells in the sample. To more cells concentration, less CW signal will be detected.

12. We have observed that much lower concentrations of CW, such as $1.25 \mu\text{g ml}^{-1}$, are still able to stain all the septa of a wild type strain. Concentrations below $1.25 \mu\text{g ml}^{-1}$ can also be used by increasing the exposure time. The analysis with low CW concentration can be useful for strains affected in the formation of the primary septum, where $50 \mu\text{g ml}^{-1}$ of CW can saturate and eventually mask a defective staining of the septum. This is the case of Bgs1-depleted cells, whose defects in the primary septum structure are clearly observed using CW at $1.25 \mu\text{g ml}^{-1}$, but are not detected at the standard concentration of $50 \mu\text{g ml}^{-1}$. Under conditions of low CW concentration many Bgs1-depleted septa did not stain or displayed a very weak and irregular CW-staining [8].

13. CW only stains the L-BG and therefore, the primary septum structure. No fluorochrome has been described for B-BG, α -glucan or secondary septum labelling. Lectins that bind the outer layer of cell wall galactomannoproteins have been used to image the cell outline, but they do not label the septum, probably because the outer layer of galactomannoproteins is absent of this structure. Therefore, to study the complete septum morphology by fluorescence microscopy, the only option available nowadays is the analysis of the septum thickness by using fluorescent tagged-proteins

or probes that localize to the septum plasma membrane (glucan synthases, acylated GFP, syntaxin Psy1, etc) and therefore, that are surrounding the septum structure.

14. When imaging fixed cells notice that while some cellular structures are well preserved after rehydration, others can result affected. The last is the case for the septum structure. When S. pombe cells are fixed with ethanol (or methanol, formaldehyde, etc.) and imaged, the septa appear as thicker and curved structures with a very weak CW-stain when compared with the septa of live cells. This is probably due to alterations in the wall structure and/or linkage between polysaccharides, causing fluffy and expanded wall structures.

15. For optimal primary septum L-BG labeling, we observed that $1\text{ }\mu\text{g ml}^{-1}$ of Novozyme preserves the cell shape and allows labeling of all the septa in wild-type cells. However this concentration could not be adequate for cells of mutants affected in the cell wall structure, and therefore in this case a range of Novozyme 234 or Glucanex concentrations should be previously tested.

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FIGURE LEGENDS

Figure 1. In *S. pombe* cells the fluorochrome Calcofluor white (CW) binds specifically and with high affinity to the L-BG of the primary septum. Cell morphology and septum structure of wild-type cells (A) and cells from germinated *bgs1* Δ spores (B) observed by phase contrast, CW-stain fluorescence and transmission electron microscopy (TEM). Differently from the wild-type septa, the *bgs1* Δ septa do not contain primary septum and L-BG, and are not stained with Calcofluor. Images adapted from [8].

Figure 2. Progression of septum formation followed by time-lapse microscopy of CW-staining fluorescence. (A) Time-lapse showing the progression of the primary septum formation in a wild-type cell by CW-staining fluorescence. Early log-phase wild-type cells growing in YES liquid medium at 28°C were collected and processed for time-lapse CW-staining visualization. Images were captured every 1 minute at 28°C. (B) A defect in B-BG synthesis causes misdirected actomyosin ring contraction and septum progression (arrow). Fluorescence time-lapse of the progression of the primary septum (CW), the septum plasma membrane (GFP-Psy1) and the contractile actomyosin ring (Rlc1-RFP) of a cell affected in the synthesis of the B-BG. Early log-phase cells of the Bgs4 mutant allele *cwg1-1* growing in YES liquid medium at 37°C were collected and processed as in A. Images were captured every 1 minute at 37°C. Images adapted from [4]. (C) Scheme of the μ -Slide 8 well (Ibidi GmbH, <http://ibidi.com>) used for the time-lapses presented in A and B. The dimensions of the μ -Slide 8 well are those of a standard slide and it can be mounted and the cells observed in any microscope with slide holder. Images adapted from <http://ibidi.com>. (D) Chemical structure of CW fluorescent brightener 28 (Sigma). Image adapted from <http://www.sigmaaldrich.com>.

Figure 1

Figure 2