An easy and economical in vitro method for the formation of *Candida albicans* biofilms under continuous conditions of flow

Priya Uppuluri and Jose L. Lopez-Ribot*
Department of Biology and South Texas Center for Emerging Infectious Diseases; The University of Texas at San Antonio; San Antonio, TX USA

*Candida albicans* can develop biofilms on medical devices and these biofilms are most often nourished by a continuous flow of body fluids and subjected to shear stress forces. While many *C. albicans* biofilm studies have been carried out using in vitro static models, more limited information is available for biofilms developed under conditions of flow. We have previously described a simple flow biofilm (SFB) model for the development of *C. albicans* biofilms under conditions of continuous media flow. Here, we recount in detail from a methodological perspective, this model that can be assembled easily using materials commonly available in most microbiological laboratories. The entire procedure takes approximately two days to complete. Biofilms developed using this system are robust, and particularly suitable for studies requiring large amounts of biofilm cells for downstream analyses. This methodology simplifies biofilm formation under continuous replenishment of nutrients. Moreover, this technique mimics in vivo flow conditions, thereby making it physiologically more relevant than the currently dominant static models.

**Introduction**

Candidiasis is usually associated with in-dwelling medical devices (i.e., dental implants, different types of catheters, heart valves, artificial joints, central nervous system shunts and others), which can act as substrates for biofilm growth.1,2 These material surfaces absorb proteins or other organic materials when exposed to surrounding body fluids such as urine, blood, saliva and synovial fluid, thereby initially promoting *C. albicans* adhesion.3 Additionally, the exposure of *C. albicans* cells to body fluids may provide a continuous source of nutrients and a means for the removal of waste products, thereby promoting subsequent robust biofilm formation. The majority of *C. albicans* biofilm studies in vitro have utilized static models for biofilm formation.4 Biofilm formation under static conditions has been very popular due to ease of operation, low costs, and the ability for rapid processing of large number of samples. These models entail proliferation of cells adherent on different types of surfaces such as silicon elastomer, plastic, glass slides or acrylic strips/disk under non-shaking conditions.5 Our group has previously described a simple and robust 96-well microtiter plate model of *C. albicans* biofilms ideal for high throughput processing of multiple samples.4-8 A major drawback for these static models is that the biofilms are developed for 24–48 h without replenishing nutrients and most phenotypic and molecular investigations are undertaken following partial or total depletion of these resources. Thus, the static models fail to mimic the conditions of flow, shear stress and nutrient availability that fungal cells normally encounter within the host in a majority of settings. More recent studies have tried to overcome some of these problems by developing *C. albicans* biofilm models under conditions of flow. In a flow model, *C. albicans* biofilms can be formed either by rocking in a single compartment with limited amounts of medium, or through perfusion in more complicated

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*Correspondence to: Jose L. Lopez-Ribot; Email: jose.lopezribot@utsa.edu
systems such as CDC reactors, flow chambers, modified Robbins device or microfermentors.10–14 We have recently described a simple flow biofilm (SFB) model that can be easily assembled with materials commonly available in most microbiological laboratories.15 This model involves a controlled flow of fresh medium via Tygon tubing into a 15 ml polypropylene conical tube holding a silicon elastomer strip to which C. albicans cells have been previously attached. We reported that under flow conditions C. albicans biofilms grew up to three times faster and mature biofilms were more robust compared to those formed under static conditions.

**Materials**

**Reagents.**

(a) Sabouraud-dextrose agar (Becton Dickinson, cat. no. 211584), to prepare plates for maintenance and short term storage of C. albicans isolates.

(b) YPD: Yeast Peptone Dextrose (1% w/v yeast extract, 2% w/v peptone, 2% w/v dextrose) liquid medium for propagation of C. albicans cultures.

(c) RPMI-1640 without sodium bicarbonate supplemented with L-glutamine (Cellgro, cat. no. 50-020-PB) and buffered with 165 mM morpholinepropanesulfonic acid (Fisher, cat. no. BP308) to pH 7 for biofilm development. From now on this medium will be referred to simply as RPMI 1640.

(d) Phosphate-buffered saline, PBS (10 mM phosphate buffer, 2.7 mM potassium chloride, 137 mM sodium chloride, pH 7.4) (Sigma, cat. no. P4417).

(e) Sterile water: Autoclaved Distilled water.

(f) Tygon® tubing, 2 mm internal diameter (Cole-Parmer®)

(g) Standard forceps (Roboz Surgical Instrument Co.)

(h) Sterile surgical blade scalpel (Dynarex Co.)

(i) Erlenmeyer flasks (Corning)

(j) Large plastic container

(k) Biological safety cabinet (Nuaire)

(l) Vortex mixer (Fisher)

(m) Orbital shaker (New Brunswick)

(n) Centrifuge (Eppendorf)

(o) Peristaltic pump (Masterflex L/S® Easy-Load® II, Cole-Parmer®)

(p) Hemacytometer (Hausser Scientific)

(q) Microscope (Fisher).

(r) Silicon elastomer sheet (Cardiovascular Instruments Corp.)

(s) Bovine serum (B-9433; Sigma)

**Reagent set up.**

- After preparing the corresponding solutions in distilled water following manufacturer’s instructions, microbiological media need to be autoclaved (YPD) or filter-sterilized (RPMI 1640).

- Bovine serum is normally kept at -20°C and should be thawed to room temperature.

**Equipment set up.**

- The incubator should be set up at a constant temperature of 37°C.
- The peristaltic pump should be introduced into the 37°C incubator.
- Please refer to Figure 1 for a picture of the entire set up and the fully assembled apparatus inside the 37°C incubator.

**Procedure.**

**Preparation of equipment and inocula— timing ~24 h.**

1. Cut silicon elastomer sheet to yield several strips of 1 cm x 9 cm dimension. Sterilize by autoclaving.

2. Thaw Bovine serum at room temperature or 37°C and treat a 1 cm x 9 cm silicon strip with the serum overnight at 37°C (inside a sterile 15 or 50 ml conical tube).

3. At the same time, inoculate flasks containing YPD liquid medium (typically 25 ml of medium in a 150 ml flask) with a loopful of cells from the stock culture of C. albicans (from a fresh subculture on Sabouraud plates) and incubate overnight in an orbital shaker (180 rpm) at 30°C.

Figure 1. (A) Picture of the fully assembled apparatus inside the incubator. (B) A close-up picture of the tube with the cut-off bottom and the inserted tubing.
Note: C. albicans is a Risk Group 1/ BSL1 microorganism. Always remember to use good aseptic/sterile techniques for all microbiological manipulations and follow federal and institutional guidelines for proper use of disposal of biohazard materials.

(4) Harvest cells from the overnight grown liquid culture by centrifugation (approximately 3,000 g for 5 min at 4°C), remove supernatant and wash twice in sterile PBS (by resuspending the pellet in approximately 20 ml of ice-cold buffer, vortexing vigorously, followed by centrifugation as above).

(5) Resuspend cell pellet in RPMI 1640 medium. Prepare 1:100 and/or 1:1,000 fold dilutions in RPMI 1640 medium and count using a hemocytometer and a bright field microscope with a 40x objective.

(6) After counting, calculate the volumes needed to prepare a 13 ml suspension of cells at a final density of 1.0 x 10^7 cells/ml in RPMI 1640, in a 15 ml conical tube.

**Adhesion step—timing -90–120 min at 150 rpm agitation.**

(7) Wash the silicon strip twice in sterile water or PBS and introduce into the 15 ml conical tube containing the C. albicans culture. Incubate 2 h at 37°C statically in an horizontal position.

(8) After 2 h, remove the silicon strip from the 15 ml conical tube and wash it with sterile water twice. Use this silicon strip containing adhered C. albicans cells to develop biofilm as mentioned in the subsequent steps.

**Biofilm formation—timing -24 h.**

(9) Add 1.5 L of sterile RPMI 1640 medium in a 2 L Erlenmeyer flask. Place the flask within the incubator (see Fig. 1A).

Cut approximately 1 cm off from the bottom of a 15 ml clear polypropylene conical tubes using a sterile surgical blade scalp. Also bore a hole in the middle of the cap of the conical tube. For a tubing of ~4 mm external diameter, bore a hole of ~5 mm. This can be done in various ways (i.e., by introducing the sharp thin end of a scissor right into the middle of the plastic cap and turning it in a circular motion to peel off the plastic from the cap and simultaneously producing a hole; or alternatively, the hole can be made by introducing into the middle of the cap one leg of forceps that has been flame-heated, thereby melting the plastic).

(10) Cut the Tygon tubing to a length of 1.5 meters and introduce one end of the tubing into the conical tube through the hole in the cap. CAUTION: The hole in the cap should only be as wide as to tightly fit the end of the tubing. Also, only about one quarter of an inch of tubing should be introduced into the conical tube through the cap (Fig. 1B). Cover the free tip of the tubing with aluminum foil and then wrap the conical tube along with the tubing with aluminum foil. Sterilize this apparatus by autoclaving.

(11) After sterilization, tightly fix the conical tube into a tube holder at an angle (approximately 30–45°) and place inside the incubator (see Fig. 1A).

(12) Introduce the other end of the now sterilized tubing into the 2 L Erlenmeyer flask containing RPMI 1640 medium. Make sure the end of the tube touches the bottom of the flask.

(13) Connect the tubes to a peristaltic pump and adjust the settings on the pump to yield the desired flow rate. Place a large plastic container under the conical tube to collect the flow through media (this could contain a disinfectant such as diluted chlorine to kill the cells present in the eluate on contact). At this point, introduce the silicon strip containing adhered C. albicans cells into the conical tube using a pair of sterile (autoclaved) forceps. CRITICAL STEP: Make sure that the first flow of media over the silicon strip is liberal, mainly to uniformly impregnate the whole strip. If this is not done, the media will most likely trickle down from the sides of the strip, preventing even coverage of the strip for eventual biofilm formation. Once a flow (covering most of the strip surface area) is established, flow should be adjusted to the desired rate, typically 0.75 to 1 ml/ml.

(14) Close the 37°C incubator and run for 18–24 h to allow for biofilm development. The biofilms grow to macroscopic scale and are visible by the naked eye; thereby biofilm formation can be monitored by visual inspection.

(15) At the end of the incubation period stop the media flow and disassemble the apparatus. From within the 15 ml conical tube, using forceps remove the silicone strip with the biofilm on it. The resulting biofilm is now ready for a series of downstream analyses, which may include biomass determination metabolic activity, microscopic examination, antifungal susceptibility testing, recovery of biofilm cells, extraction of macromolecular components such as matrix carbohydrate, protein and RNA, among others (see “Anticipated Results” section). For recovery of biofilm cells and/or recovery of subcellular fractions, strips containing biofilms can be introduced into 15 ml or 50 ml conical tubes containing sterile PBS and vortexed vigorously. The strips can be removed and the tubes centrifuged to yield a pellet of biofilm cells for downstream use. For visualization by Confocal Scanning Laser Microscopy (CSLM), the biofilms on strips can be cut to pieces of 1 cm^2 and introduced into 6 well plates containing 25 μg/ml of fluorochrome-conjugated concanavalin A (or other suitable stain) and incubated at 37°C for 45 min, in the dark. The stained biofilms can then be visualized using a confocal microscope.

**Timing.**
Steps 1–6, growing C. albicans culture and preparation of inoculum for biofilm development -16–24 h

Steps 7–8, adherence of C. albicans cells to silicon elastomer strips -2–2.5 h

Steps 9–16, preparation of equipment and biofilm formation -18–24 h.

**Problem handling.** Potential problems and advice on possible solutions can be found in Table 1.

**Anticipated results.** Biofilms formed using the flow system are extremely robust and can be clearly seen by the naked eye at the end of the experiment (Fig. 2A). When examined by CSLM these biofilms are highly filamentous and typically about 1 mm thick (Fig. 2B).15

Besides the simplicity of use, the greatest advantage of growing biofilms in the SFB model is the various downstream applications that the biofilms can be utilized for, some of which cannot be achieved using a static model. For example, the SFB model has been used to study the phenomenon of biofilm dispersion, by simply monitoring the number of cells present in the flow through media thorough-out the process of biofilm development.16
Different microscopy techniques, such as scanning electron microscopy and CSLM, can be used for visualization and characterization of the resulting biofilms.\textsuperscript{15} Due to the increased biomass, number of cells and presence of ample extracellular matrix, these biofilms can be used for extraction of RNA, protein and other biochemical and phenotypic analyses, both for cells within the biofilms as well as for dispersed cells.\textsuperscript{15-17} As long as the biofilm has been successfully and uniformly formed along the entire length of the silicone strip, this can then be cut into pieces of equal length for antifungal susceptibility testing.\textsuperscript{15} See Table 2 for typical results of antifungal susceptibility testing of \textit{C. albicans} biofilms formed under flow. Antifungals can also be added at a fixed concentration to the reservoir of media so that biofilms formed using this model are subjected to a continuous flow of media containing an antifungal over a period of time, thus mimicking physiological conditions.

For simplicity this protocol describes step by step the formation of a single biofilm. However, once the technique has been mastered and depending upon the number of ports in the peristaltic pump, it is equally possible to form several “equivalent” biofilms (typically 2–4) at the same time. Depending on the experimental design, this set up with multiple biofilms formed in parallel is ideal for comparison of multiple parameters for biofilm development. It is also possible to compare the biofilm-forming ability of mutant strains versus the corresponding wild type strain, effect of different antifungals or different concentrations of the same antifungal and many other side-by-side comparisons.

To summarize, this protocol describes an easy and robust and economical in vitro model for the development of Candida biofilms under conditions of flow that uses materials and equipment widely available in most microbiological laboratories.

Table 1. Potential problems and advice on possible solutions

| Problem                        | Possible reason                                      | Solution                                                |
|-------------------------------|-----------------------------------------------------|--------------------------------------------------------|
| Lack of biofilm formation     | Problems with initial adherence of cells on the silicon elastomer strip | Make sure the silicon strip is pretreated with bovine serum overnight |
|                               | Problem with flow of media over the silicon strip | Make sure that the media flow covers the entire silicon strip. |
|                               |                                                     | Make sure that the peristaltic pump is working and pumping media continuously at a constant flow rate |
|                               |                                                     | Make sure that the media flow rate is not excessive, such that it washes away the cells |
| Microbial contamination       | Not maintaining sterility                           | Sterilize all buffers and microbiological media Decontaminate surfaces (bench and incubator) prior to use. Make sure to use good aseptic/sterile techniques to minimize chances of contamination. |

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References

1. Kojic EM, Darouiche RO. Candida infections of medical devices. Clin Microbiol Rev 2004; 17:255-67.
2. Ramage G, Martinez JP, Lopez-Ribot JL. Candida biofilms on implanted biomaterials: a clinically significant problem. FEMS Yeast Res 2006; 6:979-86.
3. Ramage G, Saville SP, Thomas DP, Lopez-Ribot JL. Candida biofilms: an update. Eukaryot Cell 2005; 4:633-8.
4. Ramage G, Vande Walle K, Wickes BL, Lopez-Ribot JL. Standardized method for in vitro antifungal susceptibility testing of \textit{Candida albicans} biofilms. Antimicrob Agents Chemother 2001; 45:2475-9.
5. Pierce CG, Uppdurli P, Tristan AR, Woemley FL, Jr, Mowat E, Ramage G, et al. A simple and reproducible 96-well plate-based method for the formation of fungal biofilms and its application to antifungal susceptibility testing. Nat Protoc 2008; 3:1494-500.
6. Bachmann SP, Ramage G, VandeWalle K, Patterson TF, Wickes BL, Lopez-Ribot JL. Antifungal combinations against \textit{Candida albicans} biofilms in vitro. Antimicrob Agents Chemother 2003; 47:3657-9.
7. Ramage G, Lopez-Ribot JL. Techniques for antifungal susceptibility testing of \textit{Candida albicans} biofilms. Methods Mol Med 2005; 118:71-9.
8. Ramage G, VandeWalle K, Bachmann SP, Wickes BL, Lopez-Ribot JL. In vitro pharmacodynamic properties of three antifungal agents against pre-formed \textit{Candida albicans} biofilms determined by time-kill studies. Antimicrob Agents Chemother 2002; 46:3634-6.

Figure 2. Macroscopic and microscopic observations of biofilms grown in the simple flow model. (A) Macroscopically, biofilms grown in the flow biofilm model appeared mucoid and highly wrinkled in appearance. (B) Confocal laser scanning microscopy reveals a thick biofilm with a highly filamentous top layer.
Table 2. Results of antifungal susceptibility testing of biofilms formed under conditions of flow by C. albicans type strain SC5314 against fluconazole, amphotericin B and caspofungin

| Isolate | Fluconazole | Amphotericin B | Caspofungin |
|---------|-------------|----------------|-------------|
| SC5314  | SMIC50 >1024| SMIC80 >1024   | SMIC50 4    |
|         | SMIC80 >1024| SMIC80 >32     | SMIC50 0.06 |
|         |             |               | SMIC80 0.125|

SMIC, Sessile Minimum Inhibitory Concentration. Values are in µg/ml.

9. Al-Fattani MA, Douglas IJ. Biofilm matrix of Candida albicans and Candida tropicalis: chemical composition and role in drug resistance. J Med Microbiol 2006; 55:999-1008.
10. Everaert EP, van de Bilt-Gritter B, van der Mei HC, Buuscher HJ, Verkerke GJ, Dijk F, et al. In vitro and in vivo microbial adhesion and growth on argon plasma-treated silicone rubber voice prostheses. J Mater Sci Mater Med 1998; 9:147-57.
11. Garcia-Sanchez S, Aubert S, Iraqui I, Janbon G, Ghigo JM, d’Enfert C. Candida albicans biofilms: a developmental state associated with specific and stable gene expression patterns. Eukaryot Cell 2004; 3:536-45.
12. Hawser SP, Baillie GS, Douglas IJ. Production of extracellular matrix by Candida albicans biofilms. J Med Microbiol 1998; 47:253-6.
13. Mukherjee PK, Chand DV, Chandra J, Anderson JM, Ghannoum MA. Shear stress modulates the thickness and architecture of Candida albicans biofilms in a phase-dependent manner. Mycoses 2009; 52:440-6.
14. Ramage G, Wickes BL, Lopez-Ribot JL. A seed and feed model for the formation of Candida albicans biofilms under flow conditions using an improved modified Robbins device. Rev Iberoam Micol 2008; 25:37-40.
15. Uppuluri P, Chaturvedi AK, Lopez-Ribot JL. Design of a simple model of Candida albicans biofilms formed under conditions of flow: development, architecture and drug resistance. Mycopathologia 2009; 168:101-9.
16. Uppuluri P, Chaturvedi AK, Srinivasan A, Banerjee M, Rama-subramaniam AK, Kohler JR, et al. Dispersion as an important step in the Candida albicans biofilm developmental cycle. PLoS Pathog 2010; 6:e1000828.
17. Martins M, Uppuluri P, Thomas DP, Cleary IA, Henriques M, Lopez-Ribot JL, et al. Presence of extracellular DNA in the Candida albicans biofilm matrix and its contribution to biofilms. Mycopathologia 2010; 169:323-31.