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TITLE:

Real-time Imaging and Quantification of Fungal Biofilm Development Using a Two-Phase Recirculating Flow System

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KEYWORDS:
Flow, *Candida albicans*, fungal pathogen, oropharyngeal candidiasis, biofilm, live cell microscopy

SUMMARY:
We describe the assembly, operation, and cleaning of a flow apparatus designed to image fungal biofilm formation in real time while under flow. We also provide and discuss quantitative algorithms to be used on the acquired images.

ABSTRACT:
In oropharyngeal candidiasis, members of the genus *Candida* must adhere to and grow on the oral mucosal surface while under the effects of salivary flow. While models for the growth under flow have been developed, many of these systems are expensive, or do not allow imaging while the cells are under flow. We have developed a novel apparatus that allows us to image the growth and development of *Candida albicans* cells under flow and in real-time. Here, we detail the protocol for the assembly and use of this flow apparatus, as well as the quantification of data that are generated. We are able to quantify the rates that the cells attach to and detach from the slide, as well as to determine a measure of the biomass on the slide over time. This system is both economical and versatile, working with many types of light microscopes, including inexpensive benchtop microscopes, and is capable of extended imaging times compared to other flow systems. Overall, this is a low-throughput system that can provide highly detailed real-time information on the biofilm growth of fungal species under flow.

INTRODUCTION:
Candida albicans (*C. albicans*) is an opportunistic fungal pathogen of humans that can infect many tissue types, including oral mucosal surfaces, causing oropharyngeal candidiasis and resulting in a lower quality of life for affected individuals[1]. Biofilm formation is an important characteristic for the pathogenesis of *C. albicans*, and numerous studies have been done on the

formation and function of *C. albicans* biofilms[2–5], many of which have been conducted using static (no flow) *in vitro* models. However, *C. albicans* must adhere and grow in the presence of salivary flow in the oral cavity. Numerous flow systems have been developed to allow for live-cell imaging[6–10]. These different flow systems have been designed for different purposes, and therefore each system has different strengths and weaknesses. We found that many of the flow systems appropriate for *C. albicans* were costly, required complex fabricated parts, or could not be imaged during flow and had to be stopped prior to imaging. Therefore, we developed a novel flow apparatus to study *C. albicans* biofilm formation under flow[11]. During the design of our flow apparatus, we followed these major considerations. First, we wanted to be able to quantify multiple aspects of the biofilm growth and development in real-time without requiring the use of fluorescent cells (allowing us to study mutant strains and unmodified clinical isolates easily). Second, we wanted all parts to be commercially available with little to no modifications (*i.e.*, no custom fabrication), allowing others to more easily recreate our system, and allowing for easy repairs. Third, we also wanted to allow for extended imaging times at reasonably high flow rates. Lastly, we wanted, following a period of cells attaching to the substrate under flow, to be able to monitor the biofilm growth over an extended time without introducing new cells.

These considerations led us to develop the two-flask recirculating flow system illustrated in **Figure 1**. The two flasks allow us to split the experiment into two phases, an attachment phase that draws from the cell-seeded attachment flask, and a growth phase that uses cell-free media to continue the biofilm growth without the addition of new cells. This system is designed to work with an incubation chamber for the microscope, with the slide and the tubing preceding it (2 to 5 - **Figure 1**) being placed inside the incubator, and all other components placed in a large secondary container outside the microscope. Additionally, a hotplate stirrer with an attached temperature probe is used to maintain fungal cells in the attachment flask at 37 °C. As it is recirculating, this system is capable of continuous imaging during flow (can be over 36 h depending on conditions), and can be used on most standard microscopes, including upright or inverted benchtop microscopes. Here, we discuss the assembly, operation, and cleaning of the flow apparatus, as well as provide some basic ImageJ quantitative algorithms to analyze the videos after an experiment.

PROTOCOL:

1. Assemble the Flow Apparatus.

1.1. Configure the parts listed in the **Table of Materials** according to the schematic in **Figure 1** with the considerations discussed below.

Note: For convenience, the flow apparatus is divided into two sides, the green side (everything upstream of the slide to the media flasks), and the orange side (everything downstream of the slide to the media flasks).

1.1.1. Ensure that all of the flow apparatus is air tight to prevent leaks, with the sole exception of the media flasks (1 – **Figure 1**). To accomplish this, apply plumbers tape to any male

threading before assembling except for the pulsation dampener (PD) and 2 µm filter bottle (FB), which do not require plumbers tape as the rubber gaskets keep them airtight.

1.1.2. Apply ear clamps at every barbed fitting that is under positive pressure during normal operation (*i.e.*, downstream of the pump).

1.1.3. Use color coded lab tapes to label the valve locations with an A or G (for attachment and growth, respectively), the pump location, the slide connection locations, and the 0.2 µm filter connection.

1.1.4. Determine the length of tubing to be used based on the distance between the flow system and microscope, keeping in mind that all the flow apparatus downstream of the pump to the flasks (majority of the orange side) should be in the secondary containment. Add approximately 1 m of extra tubing upstream of the slide (and preferably the bubble trap) to place within the microscope incubation chamber, as this ensures that all media reaching the slide will be at the correct temperature.

1.1.5. Place the bubble trap as close as reasonably possible to the slide, preferably inside the incubation chamber during an experiment (bubbles often form along the tubing wall); however, keep in mind that it must be connected to a vacuum to operate.

1.1.6. Ensure that the tubing between the FB and the 0.2-µm disposable filter is about 0.5 m long.

1.1.7. Add an approximately 2 cm magnetic stir bar to each media flask.

1.1.8. Obtain some form of tubing clamps to act as shut-off valves (hemostats can be used).

1.1.9. For ease of use, keep the flow apparatus in an autoclavable basket. It can be helpful to have a second smaller basket in a larger one to allow easy separation of the green side and the orange side.

1.1.10. For the attachment flask, using a 4-mm drill bit, drill an extra hole in the rubber stopper to accommodate the thermal probe (take care not to go through another hole). To get the tubing through the ports, push the tubing through with tweezers; once through, clamp the tubing to hold it in place, and then pull the tweezers back out.

Note: If it is not possible to add the extra hole to the rubber stopper, a wide-mouth screw bottle with a four-port screw cap may work in place of the flask and rubber stopper.

1.2. Once the flow system has been fully assembled, close the valves of both green and orange side growth flasks. Use water with the attachment flask tubing and a graduated cylinder to calibrate the peristaltic pump according to the manufacturer's instructions.

2. Perform an Experiment.

2.1. The day before the experiment, begin pre-heating the microscope incubation chamber to 37 °C, and prepare an overnight culture of a fungal strain (fluorescence is not required).

2.2. Gather single use components and pump, and place in a sterile biosafety cabinet.

2.3. Remove the bubble trap and the temperature probe from the flow apparatus and place these in the biosafety cabinet.

2.4. Detangle and organize the tubing, if necessary.

2.5. Autoclave the flow apparatus, including the stir bars, for 30 min to ensure sterility; when finished, transfer to the biosafety cabinet.

2.6. Attach the bubble trap, temperature probe, and all single use components (except the slide) as depicted in **Figure 1**.

2.6.1. For the 0.2 µm filter (11 – **Figure 1**), remove the plunger from the 1 mL syringe to make it as an "adapter". Force the tubing from the FB into this end, and attach the 0.2 µm filter to the tubing leading to the growth flask.

2.6.2. Apply silicone vacuum grease around the barb of the slide adapter (take care not to get any grease on the inside) prior to connecting it, as this helps prevent air leaks into the system.

2.7. Fill the attachment flask with 100 mL of 1% (w/v) yeast extract, 2% (w/v) peptone, and 2% (w/v) glucose (YPD), and fill the growth flask with 200 mL of YPD. Ensure that the green side tubing reaches the media in each flask.

2.8. Ensure that all valves are open. Attach the bubble trap to a vacuum, and connect the pump to the green side tubing downstream of the bubble trap.

2.9. Pump the fluid at a flow rate of 3.3 mL/min to completely fill the green side, then dispense and discard approximately 1-2 mL of the media because the first couple of milliliters often contain dead cells or random debris. Ensure that the green side of the tubing is filled with media, and has no bubbles downstream of the bubble trap before proceeding.

2.10. Fill the channel slide and the reservoir with YPD, taking care not to introduce bubbles.

2.11. Connect the slide to the flow apparatus, and pump more fluid to create a buffer of about 0.5 m on the orange side. This is to prevent accidentally trapping air in the slide in the event of backflow.

2.12. Prepare the flow apparatus for the transport to the microscope: Clamp closed the inlet

and outlet of the bubble trap, and clamp the green and orange side attachment flask valves closed. Ensure that the screw caps for the PD and FB are tight as they can loosen during autoclaving.

2.13. Disconnect the pump from the tubing to make transport easier. Then move all components, including the hotplate stirrer, into a secondary container near the microscope.

2.14. Prepare the flow apparatus for imaging.

2.14.1. Attach the temperature probe to the hotplate stirrer and begin heating the attachment flask to 37 °C. Stir the media at 300 rpm and maintain this for the whole experiment.

2.14.2. Mount the slide on the microscope, and use tape if necessary to tightly secure it.

2.14.3. Attach the bubble trap to a vacuum (do not undo the clamp yet).

2.14.4. Connect the pump to the flow apparatus at the location indicated on **Figure 1**.

2.14.5. Start the pump at a flow rate of 3.3 mL/min, allow it to run for approximately 5-10 s, and then remove the bubble trap inlet/outlet clamp.

2.14.6. Allow the pump to continue running while the attachment flask heats up. Once media has circulated throughout the flow system, check for normal operation.

2.14.6.1. Check fittings for air leaking in upstream of the pump (some bubble formation is normal), or fluid leaking out downstream.

2.14.6.2. Check that the growth media flask, PD, and FB are all dripping media from the inlet tube (if not, this could indicate a clogged filter, or an overtightened ear clamp).

2.14.6.3. Using the microscope, check for attached or rolling cells on the channel slide. An excessive number of cells may indicate contamination during set-up, or that the polytetrafluoroethylene (PTFE) membrane of the bubble trap needs replacing.

2.15. Once the attachment flask and incubation chamber are both at 37 °C, add enough overnight culture of the fungal cells to the attachment flask to reach 1×10^6 cells/mL.

Note: The volume to add in µL can be calculated using this formula:

$$\frac{1 * 10^9}{Concentration\ of\ Cell\ stock\ (cells/mL)} * 100\ mL$$

2.16. Wait 15 min to allow the cells to acclimate.

2.17. Open both green and orange side attachment flask valves while closing both growth flask valves to start the flow of cells.

2.18. Wait for approximately 5 min to allow cells to reach the slide, and allow for initial focusing of the microscope (this time may need to be adjusted depending on the length of the green side tubing). During this time, adjust the microscope to the same imaging parameters used in previous experiments. If this is the first run, follow these steps:

2.18.1. Switch to a low magnification air objective.

2.18.2. Find and focus on an attached cell or small budding cell.

2.18.3. Configure condenser for Köhler illumination, then switch to darkfield.

2.18.4. Set the exposure time to 300 ms.

2.18.5. Adjust the illuminating intensity until a small cell is dim yet clearly visible against the background (a signal to background ratio of approximately 7-8 for a budding daughter cell is a reasonable value). Note/mark the illuminating intensity for future experiments.

2.18.6. Configure the software to acquire an image every 2 min over 2 h.

2.19. Begin image acquisition for the attachment phase. Check back after approximately 5 and 10 min to ensure that focus has been maintained. If not, attempt to adjust the focusing immediately after the next image is acquired.

2.20. Immediately after the attachment phase has finished, save the file, and then open both green and orange side growth flask valves while closing both attachment flask valves. Take care not to bump the stage if any valves are inside the incubation chamber.

2.21. Unplug the thermometer probe from the hotplate stirrer.

2.22. Remove the attachment flask from the hotplate stirrer and place the growth flask in its place.

2.23. Configure the software to acquire an image every 15 min over 22 h and begin image acquisition for the growth phase. Re-focusing should not be necessary, but it is highly recommended to check the flow apparatus after a few hours.

2.23.1. Check fittings for air leaking in upstream of the pump (again some bubble formation is normal), or fluid leaking out downstream

2.23.2. Check that the growth media flask, PD, and FB are all dripping media (if not, this could

indicate a clogged filter, an overtightened ear clamp, or a clog at a barbed fitting if the cells being used flocculate).

2.23.3. Check the fluid level in the FB. If the media is approaching the top of the bottle (over 1.5 cm above the top of the filter), tighten both screwcaps (do not loosen them, as this flask is under pressure). If they will not tighten further, continue the experiment (though this may result in a leak), and replace the rubber gaskets on the PD and FB after the next cleaning.

2.24. When the growth phase acquisition has finished, save the file, and then open the green and orange side attachment flask valves which will make a noise as the pressure releases on the orange side. Pull up on the green side tubing coming from both media flasks until they are at least several centimeters above the media. Run the pump at a high speed (approximately 100 mL/min or hold the fast forward button on the pump) to remove all the media from the tubing, which makes cleaning much easier. When emptied, disconnect the flow apparatus from the pump, and remove it from the microscope.

3. Clean the Flow Apparatus.

3.1. Remove all non-autoclavable components (single use components, bubble trap, and temperature probe), and autoclave the flow apparatus for 30 min. Discard used single-use components, clean probe with 70% ethanol, and set aside bubble trap.

3.2. After autoclaving is finished, discard media, and rinse and set aside media flasks. Then re-connect the bubble trap, connect an ibidi channel slide (reusable) to be used for cleaning, and connect the flow system to the pump at the location shown in **Figure 1**.

3.3. Clamp closed the orange side growth flask valve.

3.4. Place approximately 200 mL of undiluted bleach into a beaker. Place the rubber stoppers into the bleach, and then start the pump at a high speed to circulate bleach throughout the flow apparatus (except all the filters). Once filled with bleach, stop the pump because leave the pump on at a high speed can wear and break the tubing.

3.5. After bleaching for 15 min, hold the rubber stoppers above the beaker and start the pump again to remove the bleach from the flow apparatus.

3.6. Repeat steps 3.4 and 3.5 twice with excess water instead of bleach to rinse the flow system. During this time, clean the filters only with water because other cleaning agents will corrode or clog the filters.

3.6.1. Place the tubing that would normally connect to the 0.2 μm media filter (coming from the 2 μm FB) into the beaker water with the rubber stoppers from step 3.6.

3.6.2. Disconnect the tubing attached to the inlet of the 20 μm inline filter, which can usually

be pulled apart with ease despite the ear clamp.

3.6.3. Use a vacuum filter flask and a long section of tubing through a spare 3-hole stopper to create a vacuum system that can connect to the flow apparatus.

3.6.4. Connect this vacuum system to the inlet of the 20 µm filter inlet, and start the vacuum; this will pull water through the filters in the reverse direction, removing dead cells.

3.6.5. Pull at least 200 mL of water through the filters, then remove the tubing from the water to empty the filter lines of water.

3.6.6. Disconnect the vacuum system from the 20 µm filter, and reconnect the filter to its normal tubing.

4. Quantifying the Videos

Note: All files need to be converted to the tag image file (TIF) format to work. Additionally, to compare between experiments, it is critical that all images are taken with the same microscope and imaging parameters, as discussed above.

4.1. Download and install ImageJ if not already installed.

4.2. Download the supplemental macro file, and place it in the ImageJ\macros folder.

4.3. Adjust the provided macro:

4.3.1. Open an image stack from a previous experiment in ImageJ, and select a time point with cells present.

4.3.2. Select from the menu via **Image | Type | 8-bit**.

4.3.3. Select from the menu via **Image | Adjust | Threshold**. Check the **Dark Background** box. Set the right side dropdown menu to Red.

4.3.4. Adjust the lower value until all cells are covered in red with minimal excess noise (some non-cell speckling is okay and will be processed out by the macro). Make note of this lower value.

4.3.5. Close both the Threshold window and the open image.

4.3.6. Select from the menu via **Plugins | Macros | Edit**. When prompted to open a file: **move up one folder level**, then select the macros folder and open the flow biofilm quantification macro file.

4.3.7. Change the 15 value in all instances of "setThreshold(15, 255);" to the value determined in step 4.3.4. Save the file and close this window.

4.4. Select from the menu via **Plugins | Macros | Install** and select the flow biofilm quantification file.

4.5. Now, under the **Plugins | Macros** menu, six new options for various video quantifications appear. Run the *Complete analysis* and select the attachment and growth video files when prompted to perform all available analyses on the acquired data and automatically generate output files.

REPRESENTATIVE RESULTS:

Representative images of a normal overnight time-lapse experiment using wild-type *C. albicans* cells at 37 °C can be seen in **Figure 2A** and **Supplemental Video 1**. The images have been contrast enhanced to improve visibility. Quantification of the original data was performed, and representative graphs can be seen in **Figure 2B**. To generate these graphs, the data were first normalized to the imaging area (*i.e.*, divided by the total imaging area), and the detachment was further normalized to the biomass, as described above. Additionally, the attachment and detachment show the cumulative values over time, rather than the individual frame values generated by the flow biofilm quantification macro. Once the graphs have reached this stage, statistical comparisons can be performed through regression analyses.

FIGURE AND TABLE LEGENDS:

Figure 1: Schematic of the two-phase recirculating flow apparatus. Connecting black lines indicate tubing, and arrowheads indicate the direction of flow during normal operation. **A.** A general schematic overview of the flow system is illustrated. For convenience, the flow system is divided into a green side (upstream of slide) and an orange side (downstream of slide). Bold numbers correspond to parts listed in the Table of Materials. Labels for valves simply mark the location for tubing clamps or hemostats to be placed during experiments. Filter order is as follows: 8 – 20 µm inline filter, 9 – 10 µm inline filter, 10 – 2 µm filter bottle (FB), and 11 – 0.22 µm single use disposable filter. Schematic is not to scale. **B.** A close-up view of the rubber stopper for the attachment flask, illustrating the four components that pass through the ports: the media outlet, the 0.2 µm air filter that allows gas exchange, the temperature probe (requires drilling an extra hole), and the media return. **C.** A close-up view of the pulsation dampener (PD) and the FB, as well as the screw cap assembly used for each port. These bottles need to be air-tight to function. The outlet tubing for the PD should reach deeper into the bottle than the inlet tubing for proper functioning. The gray rectangle in the FB represents the steel filter.

Figure 2: *Candida albicans* wild-type cells grown under flow at 37 °C. A. Representative darkfield microscopy images of the microcolonies that form under flow at 37 °C at the indicated time points. Scale bar indicates 100 µm. **B.** Representative image quantification data. The total biomass within the imaging region (determined by densitometry analysis), the cumulative rate of cell attachment, and the percent biomass detachment (detachment rate normalized to the

biomass) over time are shown for each strain. Data are means of n ≥ 3 experiments.

Supplemental Video 1. *Candida albicans* wild-type cells grown under flow at 37 °C. This time-lapse darkfield microscopy video shows the attachment of WT cells to the substrate during the attachment phase (time indicated in the upper left-hand corner; images acquired every 2 min), followed by the subsequent growth and development during the growth phase (starts at 2 h; images acquired every 15 min). Cell-seeded media (1×10^6) were used during the attachment phase, while cell-free media were used during the growth phase. Flow is from the right to left. Scale bar indicates 50 µm.

DISCUSSION:

Using the flow system as outlined above allows for the generation of quantitative time-lapse videos of fungal biofilm growth and development. To allow for comparisons between experiments it is of critical importance to ensure that the imaging parameters are kept the same. This includes ensuring that the microscope is set up for Köhler illumination for each experiment (many guides are available online for this process). Aside from imaging parameters, there are some important steps to keep in mind when working with the flow apparatus. First, it is important to ensure that the bubble trap is maintained under vacuum during fluid flow, as failure to do so will lead to air being pulled in through the bubble trap. Similarly, when the bubble trap is not under vacuum (*i.e.*, when transporting the flow apparatus) both the inlet and outlet must remain clamped shut; otherwise air will leak in through the PTFE membrane. This clamp does not need to be removed until the bubble trap is once again placed under vacuum. Lastly, it is very important to monitor your flow apparatus for potential clogs or leaks. The most efficient way to check for clogs in your system is to check that there are media dripping from the inlets of the attachment or growth flask, the PD, and the FB. The media from these should be dripping at relatively similar rates if everything is operating smoothly. If a clog is present, you can generally determine the location as the tubing just upstream of the clog will be more rigid.

Once the data have been obtained, we provide numerous ImageJ macros to quantitate the videos. These macros determine multiple parameters of biofilm growth and development, including a measure of the biomass, and the rate that cells attach to and detach from the surface or biofilm. Descriptions of the provided macros are provided below.

Complete analysis performs all the analyses listed below, and automatically outputs the data. This macro can be executed without an open image file while all others require an open video. When executed, it will prompt the user to open an attachment stack file, then a growth stack file. Following this, it will automatically analyze the images and output a data folder containing all the data tables, as described below, to the same folder as the attachment image file. The *attachment counter* macro is performed only on the attachment file; all other analyses are performed on a concatenated stack of the attachment and growth files. The output data files generated are text files, but should be imported into excel for ease of use.

The *sum intensity* analysis will analyze each frame of the active window. It adds up all gray values for each pixel that is above the lower threshold designated in step 4.3.7, and outputs

one cumulative value per frame. The values generated are proportional to the biomass present within the frame, up until any camera saturation that occurs. The data should then be normalized to the area of the imaging region; this is not performed by the macro.

The *coverage area* analysis will analyze each frame of the active window for the area of the frame that is covered by cells (above the lower threshold value) as a percentage.

The *attachment counter* will use frame subtraction to determine the sum intensity of all the cells that attach between each frame. Thus, the first data point is the biomass of the cells that attach between frames 1 and 2; the second data point is the biomass of the attaching cells between frames 2 and 3, etc. These data should be normalized to the area of the imaging region. For easier readability, it is also helpful to integrate this value prior to graphing.

The *detachment counter* works the same as the *attachment counter*, but reverses the frame subtraction, such that it determines the sum intensity of the cells that detach between each frame. These data should also be normalized to the area of the imaging region, and integrated prior to graphing. Prior to integration, these data can be further normalized to the total sum intensity of the preceding frame calculated in the *sum intensity* analysis. This new value represents the proportion of cells that detach from the biofilm at that time point, which is often more valuable data, since the biomass of the detaching cells will increase with increasing biofilm biomass.

While the flow system presented here is more complicated to build and operate than other flow systems, it does offer several advantages. Many of these advantages result from our use of a commercially available channel slide. The tissue culture treatment available for these slides is sufficient to allow *Candida* cells to adhere to the surface. Additionally, the profile of this channel slide being similar to a traditional slide allows it to easily be used on a wide variety of microscope systems, including upright microscopes using transmitted light at low magnification. Using this type of microscope allowed us to use darkfield microscopy, which made the quantification of data much easier, especially compared to fluorescent microscopy (as there was no photobleaching and low phototoxicity). Traditional microscopy (without optical sectioning), is power conservative, meaning out of focus cells contribute similar numbers of photons to an image as in focus cells of similar size[12]. This means that, despite our single plane of imaging, the full 3D growing biofilm is still being quantified throughout the experiment, even though the higher regions are out of focus. This single-plane imaging has the advantage of dramatically lowering the phototoxicity damage to cells, but does not provide any information on the 3D architecture of the biofilm. However, this flow system can also be used with fluorescent cells and confocal microscopes to obtain this information[13].

The unique two flask recirculating setup of our flow apparatus also has many advantages. First, many flow systems require that the slides be pre-seeded with cells, however our use of a separate cell-seeded attachment flask allows us to image and quantify cells as they adhere to the slide while under flow, and we feel that this is more similar to what occurs *in vivo*. Additionally, we have previously been able to adjust our microscope for high-speed imaging

and image adhesion events as they occurred in real-time, as opposed to quantifying them after the fact[11]. Second, having a cell-free growth flask that recirculates and can be maintained cell-free over an extended duration allowed us to understand how biofilms grow under flow for over 24 h, a duration that cannot typically be accomplished with non-recirculating systems. We have not yet determined the upper time limit of what can be achieved with our flow system, but we have successfully completed 36 h experiments; however, the longer the experiment, the greater the chance of a leak or clogged filter. Numerous factors can affect the potential duration of an experiment, including the growth rate of the cells, how adhesive they are, and the degree of hyphae formation, making it difficult to define an upper limit on the duration of an experiment. However, if much longer durations are desired than can be achieved with the flow apparatus as presented, the filters can be replaced with an in-line ultraviolet (UV) sterilization box as has been previously described[8]. This sterilization box may also allow this flow apparatus to be used to image bacteria; our previous attempts to image bacterial strains resulted in rapid clogging of the 0.2 µm filter. Ultimately, we opted not to adopt UV sterilization, as the box is custom fabricated, and as this would result in recirculating dead cells.

Another advantage of this flow system is that it is reasonably inexpensive relative to commercial systems, especially if you need to purchase a microscope with it. In our lab, we were able to purchase a basic transmitted light benchtop microscope and place the entire microscope inside a large standard convection incubator. The only major requirement is that the microscope should have a shutter function (either mechanical or electrical) in order to perform time-lapse microscopy.

While this system is versatile and offers many advantages, it is a low throughput method. Our flow apparatus is unable to grow multiple strains in parallel, unlike other available flow systems. Due to the extensive preparation and cleaning time, we are only able to perform two experiments a week. However, many other flow systems are rather costly, and may clog when *Candida* cells are grown under hyphae forming conditions.

Additionally, this flow system is quite complex compared to others, and can be difficult to keep in operation. After many experiments, filters begin to clog, tubing begins to wear thin, and parts start to rust or become loose; thus requiring these components to be replaced. The use of filters makes this system incompatible with growth conditions of some fungal strains; in particular, anything that induces flocculation will rapidly clog the 20 µm in-line filter. However, with sufficient experience using the flow system, it becomes easier to detect potential issues before they result in a failed experiment. One thing that can be done to make the everyday operation of the flow apparatus a little simpler is to have a machinist make a replica of the bubble trap housing out of an autoclavable material (such as aluminum or stainless steel), allowing you to autoclave the bubble trap with the rest of the flow apparatus such as the PTFE membrane and adapters that are autoclavable.

In conclusion, the two-phase recirculating flow apparatus presented here represents a unique model to image and quantify *in vitro* biofilm formation of fungi under flow and in real-time. While the system has its limitations, it is highly adaptable and works well with most

microscopes.

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DISCLOSURES:
The authors have nothing to disclose.

REFERENCES:
1. Pankhurst, C.L. Candidiasis (oropharyngeal). *BMJ clinical evidence*. **2012**, 1304 (2012).

2. Ramage, G., Vandewalle, K., Wickes, B.L., López-Ribot, J.L. Characteristics of biofilm formation by Candida albicans. *Revista iberoamericana de micología*. **18** (4), 163-170 (2001).

3. Nobile, C.J., Mitchell, A.P. Regulation of cell-surface genes and biofilm formation by the C. albicans transcription factor Bcr1p. *Current biology: CB*. **15** (12), 1150-1155 (2005).

4. Blankenship, J.R., Mitchell, A.P. How to build a biofilm: a fungal perspective. *Current opinion in microbiology*. **9** (6), 588-594 (2006).

5. Araújo, D., Henriques, M., Silva, S. Portrait of Candida Species Biofilm Regulatory Network Genes. *Trends in microbiology*. **25** (1), 62-75 (2017).

6. Lane, W.O. *et al.* Parallel-plate flow chamber and continuous flow circuit to evaluate endothelial progenitor cells under laminar flow shear stress. *Journal of visualized experiments*. (59), e3349 (2012).

7. Bakker, D.P., van der Plaats, A., Verkerke, G.J., Busscher, H.J., van der Mei, H.C. Comparison of velocity profiles for different flow chamber designs used in studies of microbial adhesion to surfaces. *Applied and environmental microbiology*. **69** (10), 6280-6287 (2003).

8. Zhang, W., Sileika, T.S., Chen, C., Liu, Y., Lee, J., Packman, A.I. A novel planar flow cell for studies of biofilm heterogeneity and flow-biofilm interactions. *Biotechnology and bioengineering*. **108** (11), 2571-2582 (2011).

9. Uppuluri, P., Lopez-Ribot, J.L. An easy and economical in vitro method for the formation of Candida albicans biofilms under continuous conditions of flow. *Virulence*. **1** (6), 483-487 (2010).

10. Diaz, P.I. *et al.* Synergistic interaction between Candida albicans and commensal oral streptococci in a novel in vitro mucosal model. *Infection and immunity*. **80** (2), 620-632

(2012).

11. McCall, A., Edgerton, M. Real-Time Approach to Flow Cell Imaging of Candida albicans Biofilm Development. *Journal of fungi (Basel, Switzerland)*. **3** (1), 13 (2017).

12. Zhang, B., Zerubia, J., Olivo-Marin, J.-C. Gaussian approximations of fluorescence microscope point-spread function models. *Applied optics*. **46** (10), 1819-1829 (2007).

13. Tati, S. *et al.* Candida glabrata Binding to Candida albicans Hyphae Enables Its Development in Oropharyngeal Candidiasis. *PLoS pathogens*. **12** (3), e1005522 (2016).

Figure 1

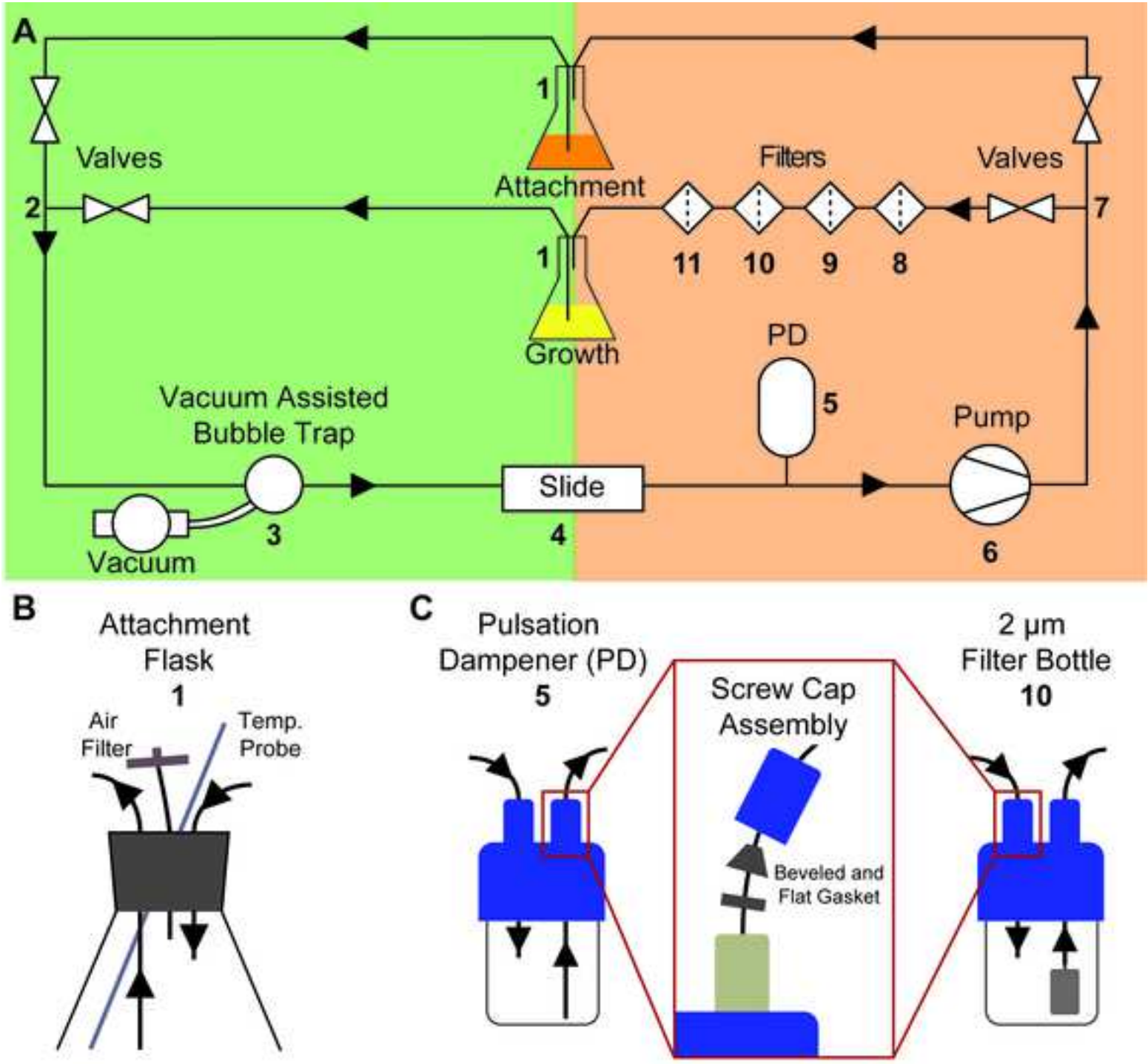
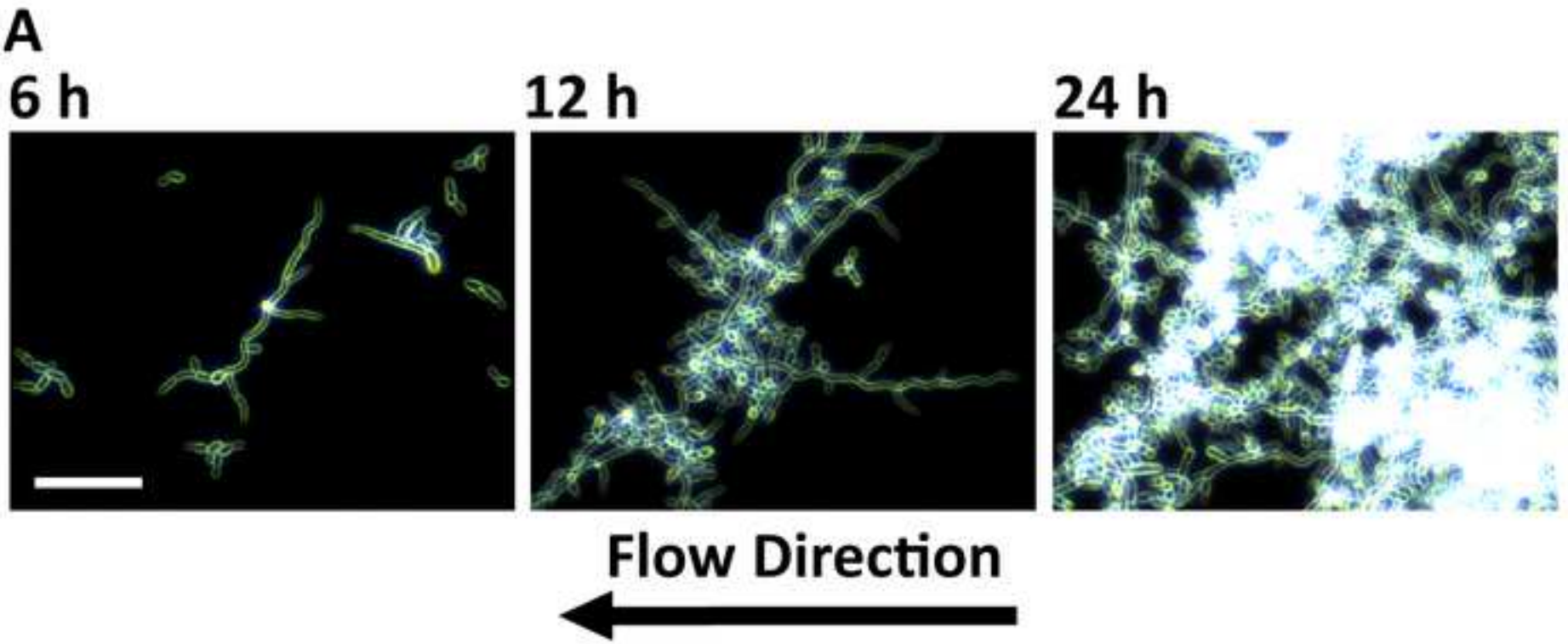


Figure 2



S Video 1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Materials Table

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Name of Material/ Equipment	Company	Catalog Number	Corresponding number in Fig. 1
Pump	Cole Parmer	07522-20	6
Pump head	Cole Parmer	77200-60	6
Tubing	Cole Parmer	96410-14	N/A
Bubble trap adapter	Cole Parmer	30704-84	3
Bubble trap vacuum adapter for 1/4” ID vacuum line	Cole Parmer	31500-55	3
In-line filter adapter (4 needed)	Cole Parmer	31209-40	8,9
Orange-side Y	Cole Parmer	31209-55	7
Green-side Y	ibidi	10827	2
* Slides	ibidi	80196	4
* Slide luers	ibidi	10802	4
Vacuum assisted Bubble trap	Elveflow/ Darwin microfluidics	KBTLarge - Microfluidic Bubble Trap Kit	3
Media flasks	Corning	4980-500	1
0.2 µm air filter	Corning	431229	1
Threaded glass bottle for PD and filter flask (2 needed)	Corning	1395-100	5,10
Ported Screw cap for PD and filter flask (2 needed)	Wheaton	1129750	5,10
Screwcap tubing connector	Wheaton	1129814	5,10
Tubing connector beveled washer	Danco	88579	5,10
Tubing connector flat washer	Danco	88569	5,10

Clamps for in-line filters and downstream Y (7 needed)	Oetiker/MSC Industrial Supply Company	15100002-100	7,8,9
Clamp tool	Oetiker/MSC Industrial Supply Company	14100386	N/A
20 micron in-line media filter	Analytical Scientific Instruments	850-1331	8
10 micron in-line media filter	Analytical Scientific Instruments	850-1333	9
2 micron inlet media filter	Supelco/Sigma-Aldrich	58267	10
* 0.22 µm media filter	Millipore	SVGV010RS	11
* 0.22 µm media filter “adapter”	BD Biosciences	329654	11
Rubber stopper	Fisher Scientific	14-131E	1
Hotplate stirrer with external probe port	ThermoFisher Scientific	88880006	N/A
Temperature probe	ThermoFisher Scientific	88880147	N/A

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Author(s):	Andrew D McCall and Mira Edgerton

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CORRESPONDING AUTHOR

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Department:	Oral Biology
Institution:	University at Buffalo
Title:	Real-time imaging and quantification of fungal biofilm development using a two phase recirculating flow system.
Signature:	[signature]
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July 7th, 2018

Dear Editors,

On behalf of the authors, we appreciate your recent invitation to resubmit our manuscript entitled “Real-time imaging and quantification of fungal biofilm development using a two phase recirculating flow system” (Manuscript #58457). We thank the reviewers for providing valuable input that we have used to improve the manuscript. Following are our specific responses (highlighted in bold) to the reviewers’ concerns. We hope that you now find our manuscript suitable for publication in the Journal of Visualized Experiments.

Sincerely,

Andrew McCall, Ph.D.

Editorial comments:
Changes to be made by the Author(s):
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.
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3. Please define all abbreviations before use.
4. Please use SI abbreviations for all units: L, mL, µL, h, min, s, etc.
5. Please include a space between all numbers and their corresponding units: 15 mL, 37 °C, 60 s; etc.
6. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets, dashes, or indentations.
7. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).
8. Lines 71-119, 137-139, 155-157, 178-180, 280-312: Please revise the protocol so that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc. The Protocol should contain only action items that direct the reader to do something. Please move the discussion about the protocol to the Discussion.

9. Line 225: Please specify the high speed used in this step.
10. Please include single-line spaces between all paragraphs, headings, steps, etc.
11. There is a 2.75 page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.
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14. Line 351: Please remove commercial language: ibidi®.
15. Discussion: Please also discuss critical steps within the protocol, any modifications and troubleshooting of the technique.

We have reviewed our manuscript and made the changes suggested above.

Reviewers' comments:

Reviewer #1:
Manuscript Summary:
This manuscript will be tremendously useful to the fungal biofilm community. The apparatus developed by the authors is slightly complex (as they acknowledge) but also quite inexpensive. A major strength is the ability to quantify several key biofilm parameters. The presentation of green and orange sides was very helpful. I also very much appreciated the authors' even handed presentation of the apparatus, though I felt that they were perhaps a little more negative about their approach than necessary. (The fact that a Bioflux sends you to the poorhouse, and that it clogs really fast when hyphae are used, are major problems with this alternative.) I also very much appreciated the section on cleaning the apparatus. Overall, bravo!

Major Concerns:
None.

Minor Concerns:
There were some minor English usage errors.
- "couple feet" or "couple ml" should be "couple of feet" or "couple of ml" I believe.
- "Ensure all valves are open" should be "Ensure that all valves are open" I believe.

We have made these changes.

Reviewer #2:
Manuscript Summary:
This manuscript describes a method for the real-time imaging of fungal attachment, growth, and detachment using reticulated flow of growth medium. The authors acknowledge that there are a number of systems available for studying the growth of fungi under flow conditions, but provide a valid justification for the visualisation of their protocol. Their arrangement has advantages in that cells do not have to be fluorescently labelled for visualisation, the reticulation of medium allows prolonged growth experiments, and the equipment should be relatively inexpensive to install. Thus there should be interest in the adoption of this technology. The authors provide detailed instructions and discuss the limitations of the system. I provide the following comments that could help improve the manuscript.

Minor Concerns:
1. Rather than simply stating that the fungal cells do not need to be fluorescently labelled, I think an unstated advantage of the system is that it allows the study of unmodified clinical isolates increasing the relevance of growth studies.

We have added this to the introduction.

2. I think the protocol section would benefit by beginning with a general description of the system. It is not clear at the outset that some of the equipment is within a temperature-controlled cabinet and some is outside the cabinet, but uses a hot plate.

We have added a brief general description to the end of the introduction, just prior to the protocol.

3. It is unclear how or why fungal cells should adhere in the chamber. Is it treated in some way to promote adherence (such as coating with poly-L-lysine)?

The ibidi slides have a proprietary cell-adhesion coating (ibiTreat) that allows the cells to adhere. We have added this information to the manuscript.

4. It is not clear what FB ('filter bottle') refers to. There is no 'filter bottle' on Figure 1, although the 'attachment flask' has a filter as does the 'filter flask'.

We have revised figure 1, changing the filter flask to filter bottle.

5. In protocol sections 1.6 - 1.8, feet and inches are used. As this is an international journal I think that SI units should be used. Likewise, in sections 16, 18, 23 and cleaning section 5, 'm' is used as an abbreviation for minute whereas 'm' is the SI unit for metres. Usually 'minute' is abbreviated to 'min'.

We have made these changes.

6. I don't think the protocol, in section 1.12, should introduce the possibility of alternative untried configurations. Either the authors should test the alternative set-up or they should limit the protocol to the set-up they used.

In principle we agree, however I do not think everyone would be as comfortable drilling a hole in a rubber stopper, as it can be a little tricky to do (they may also not have access to a drill). This is why we mentioned the alternative configuration. We have made this more clear.

7. It is unclear why large portions of the manuscript are highlighted in yellow.

This is part of the JoVE submission process. The highlighted sections are for preparing the script for the video.

8. In protocol section 6.1 the 0.2 µm filter is referred to as 'X' does this mean '10' because I think the 0.2 µm filter in Figure 1 is filter '11'.

Our apologies, the X was meant as a place filler until Fig. 1 was finalized, this was supposed to be ‘11’.

9. In section 14.7.2, I think it should be clarified that the 'dripping media' should be coming from the input tube.

We have added this to the manuscript.

10. In section 4, line 236, do the authors mean 200 ml undiluted bleach? This is quite a lot of bleach to be pumping around the system.

Yes, undiluted bleach. We have tried using diluted bleach, but it does not clear the tubes of debris as well as undiluted bleach (based on the amount of debris in the ibidi slide at the start of the next run). We realize this is a lot of bleach, but bleach is cheap while small caliber tubing, PTFE membranes and micron pore size filters are not. Thus, we tend to err on the side of clearing the tubing as much as possible. We have clarified the use of undiluted bleach.

11. Cleaning section 6.1, what is meant by "into the water", what water?

The water being used to rinse the remainder of the flow system, from step 6. This has been clarified in 6.1.

12. Quantifying the videos section 3.7, should 'step 2.4' be 'step 3.4'?

We have fixed this typo.

13. In the analysis descriptions section 5, it would help if the analysis features, such as Complete Analysis were in italics to show that they are an analysis function.

Great idea, we have made this change.

14. Results section, lines 318-9, what does "normalized to the imaging area" mean?

It simply means dividing by the imaging area. We have clarified this in the manuscript.

15. I think the discussion section overemphasises potential difficulties with the system. In lines 390-2 I don't think it is necessary to say that it is difficult to use the system in a university - many facilities will allow long bookings of equipment. In lines 397-402 I don't think the authors should raise the possibility of forgetting steps. As scientists we should be trained to follow protocols precisely how ever many steps are involved. You can also forget steps in simple protocols with dire consequences and it is not necessary to warn people about forgetting them.

We have removed these sentences.

16. The clogging of filters by cells detached from the slide occurred to me early on in the reading of the manuscript. It is mentioned in the discussion, but it would be good to know how long growth can be followed before filters clog.

The max time varies substantially, and this is one of the reasons we didn't specify a value. Sadly, there are many variables that can influence when the filters clog.

17. I think that the bubble trap in Figure 1 should have a line to vacuum marked on the plan.

We have added a vacuum to the figure.

July 12th, 2018

Dear Editors,

On behalf of the authors, we apologize for missing some of the editorial corrections during the revision process. Following are our specific responses (highlighted in bold) to the editorial comments. We hope that you now find our manuscript suitable for publication in the Journal of Visualized Experiments.

Sincerely,

Andrew McCall, Ph.D.

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
   We have proofread the manuscript.
   2. Please revise the text in Protocol to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

   We have removed the personal pronouns; sorry for those we missed last time.
   3. JoVE cannot publish manuscripts containing commercial language. This includes company names of an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.

   We have removed all the commercial language from the manuscript.
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   6. Please number your protocol steps continuously.

   We have made this change.
   7. The highlighted protocol steps are over 2.75 page limit. Please adjust the highlighting.
   We have adjusted the highlighting.
   8. Line 55-65: Please do not use lists in Introduction.

We have made this change.

9. Line 86: Please write this step in imperative tense.

We have made this change.

10. Line 118: Please write this step in imperative tense.

We have made this change.

11. Line 125: What's the size of the hole?

We have added this information.

12. Line 272: Please write this step in imperative tense.

We have made this change.

13. Line 329: Please write this step in imperative tense.

We have removed this step.

14. Line 370: Please write this step in imperative tense.

We moved this description to the discussion.

15. Line 379: Please write this step in imperative tense.

We moved this description to the discussion.

16. Line 388: Please write this step in imperative tense.

We moved this description to the discussion.

17. Line 394: Please write this step in imperative tense.

We moved this description to the discussion.

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