Video Article

Fabrication of Refractive-index-matched Devices for Biomedical Microfluidics

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Abstract

The use of microfluidic devices has emerged as a defining tool for biomedical applications. When combined with modern microscopy techniques, these devices can be implemented as part of a robust platform capable of making simultaneous complementary measurements. The primary challenge created by the combination of these two techniques is the mismatch in refractive index between the materials traditionally used to make microfluidic devices and the aqueous solutions typically used in biomedicine. This mismatch can create optical artifacts near the channel or device edges. One solution is to reduce the refractive index of the material used to fabricate the device by using a fluorinated polymer such as MY133-V2000 whose refractive index is similar to that of water (n = 1.33). Here, the construction of a microfluidic device made out of MY133-V2000 using soft lithography techniques is demonstrated, using O_2 plasma in conjunction with an acrylic holder to increase the adhesion between the MY133-V2000 fabricated device and the polydimethylsiloxane (PDMS) substrate. The device is then tested by incubating it filled with cell culture media for 24 h to demonstrate the ability of the device to maintain cell culture conditions during the course of a typical imaging experiment. Finally, quantitative phase microscopy (QPM) is used to measure the distribution of mass within the live adherent cells in the microchannel. This way, the increased precision, enabled by fabricating the device from a low index of refraction polymer such as MY133-V2000 in lieu of traditional soft lithography materials such as PDMS, is demonstrated. Overall, this approach for fabricating microfluidic devices can be readily integrated into existing soft lithography workflows in order to reduce optical artifacts and increase measurement precision.

Video Link

The video component of this article can be found at https://www.jove.com/video/58296/

Introduction

The development of microfluidic technology has enabled a wide range of new biomedical techniques that leverage the unique physics of microscopic-scale flows[1,2]. This includes the diagnostic techniques built on microfluidic platforms that quantify clinically relevant biomarkers, including cell stiffness[3], surface markers[4], and growth[5]. By manipulating single cells, microfluidic devices can also be used to measure biomarker heterogeneity, for example as an indicator of malignancy[6]. The ability to combine microfluidic applications with microscopy has further increased the utility of these platforms by allowing for devices that measure multiple biomarkers simultaneously[7].

QPM is a microscopy technique that measures the phase shift as light passes through and interacts with the matter inside transparent samples. The mass of individual cells can be calculated from QPM measurements, by using the known relationship between the refractive index and the biomass density[8,9]. Previous work has shown that QPM is capable of measuring clinically relevant parameters such as cell growth[10,11] and cell mechanical properties *via* disorder strength[12]. When combined with microfluidics, QPM can potentially be used to measure cell behavior in a highly controlled environment *in vitro*. One of the primary challenges facing combining QPM with microfluidics is the high refractive index of most polymers used to construct microfluidic channels *via* soft lithography[13].

An important challenge facing the combination of microfluidics with various microscopy techniques is the mismatch between the refractive index of the device material relative to the refractive index of water[14,15]. One method to address this is through the use of a low refractive index polymer such as CYTOP[16] or MY133-V2000[13]. The latter is a fluorinated ultraviolet (UV)-curable acrylate polymer that has a refractive index similar to water (n = 1.33) and that is compatible with soft lithography techniques, allowing for a smooth integration into many established microfluidic device fabrication workflows. This makes MY133-V2000 not only suitable for microfluidic device fabrication, but also allows it to be readily combined with QPM and other microscopy approaches, to measure cell behavior both at the colony and on a single-cell scale. MY133-V2000 eliminates artifacts due to phase unwrapping by producing little, if any, phase shift as light passes through the water-MY133 interface.

Although eliminating the mismatch in refractive index, one major challenge associated with the devices fabricated from fluorinated polymers, such as MY133-V2000, is the low adherence to other materials such as glass or PDMS. The present work demonstrates the fabrication of an MY133-V2000 microfluidic device using soft lithography. Using O_2 plasma to treat the surface of both the channel and the PDMS substrate combined with a custom-fabricated acrylic holder ensures that the device adheres to the substrate, creating a sealed channel. This device is

suitable for cell culture and QPM to measure the mass of cells in the channel, which has important applications for measuring the growth of live cells and the intracellular transport of cell biomass, both of which have clinical relevance in diagnostic medicine and drug discovery.

Protocol

1. Fabrication of the Polydimethylsiloxane Negative

1. **Preparation of polydimethylsiloxane**
   1. Measure 18 g of PDMS silicone elastomer and 1.8 g of the curing reagent. Pour the curing reagent into a measuring boat containing the elastomer.
   2. Mix the elastomer and the curing reagent vigorously for 1 min and put the mixture into a vacuum chamber for 30 min.
   3. Remove the PDMS from the vacuum, pour 15 g onto the negative using a cookie cutter (radius = 3.8 cm) to keep the PDMS from running off the side, and cover the remaining PDMS mixture.
   4. Put the mold containing the PDMS into the vacuum chamber for 10 min.
   5. Remove the mold containing the PDMS from the vacuum chamber, place it on a hot plate set to 150 °C for 60 min to cure, and cover it with aluminum foil.
   6. Pour the remaining PDMS onto an inverted glass Petri dish (10 cm in diameter), using another cookie cutter as a mold, to create a pad of PDMS. Put this into the vacuum chamber until the PDMS negative is completely cured.
   7. Transfer the Petri dish with the PDMS from the vacuum to the preheated hot plate to cure at 150 °C for 60 min.

2. **Plasma surface treatment of polydimethylsiloxane**
   1. Insert a sharp razor blade underneath the PDMS and the cookie cutter to carefully remove the PDMS from the negative.
   2. Use a sharp hobby knife to cut out the negative from the larger piece of PDMS; it should be cut with enough buffer area to attach another piece of PDMS on top of it without obstructing the negative.
   3. Insert a sharp razor blade underneath the cured PDMS with the negative to carefully remove the PDMS from the Petri dish.
   4. Use a hobby knife to cut out a piece of PDMS from the pad that is the same size as the piece cut for the negative. Then, cut a rectangle out of the new piece of PDMS with enough room to fit it around the microchannel when it is placed on top of the negative.
   5. Put both pieces of PDMS (the negative and the rectangle) onto a flat substrate (such as a slide made from glass, quartz, polystyrene, or other material) and insert them into a radio frequency (RF) plasma cleaner. Close the door and seal the chamber by evacuating the air using a vacuum pump. Inject air up to a pressure of 400 mTorr using a digital vacuum pressure controller.
      NOTE: If the substrate used for the plasma cleaning has not been used in the plasma cleaner, put it into the plasma cleaner by itself for 60 s prior to treating the negative, in order to prevent the acrylic base layer from sticking to the substrate.
   6. Turn the RF setting to high, and a pink air plasma should appear in the viewing window. Plasma-treat the two pieces of PDMS for 30 s, then turn the RF setting off. Allow air to slowly reenter the chamber in order to prevent a turbulent air flow from disturbing the contents of the chamber.
   7. Remove the PDMS from the plasma cleaner and place it on a benchtop. Then, carefully invert the PDMS rectangle over the negative and press it down with a pair of forceps. Let it rest for 10 min to allow the two pieces of PDMS to adhere to each other.

3. **Fluorosilane surface treatment of the polydimethylsiloxane negative**
   1. Use a dropper to put 2 drops of trichloro(1H,1H,2H,2H-perfluoro-octyl)silane (PFOTS) into a small weigh boat and insert it into a glass vacuum chamber.
   2. Invert the PDMS onto a piece of aluminum foil (so that the microchannel negative faces upward) and put it into the glass vacuum chamber. Then, evacuate the chamber continuously for 24 h.

2. Fabrication of the MY133 Microchannel

1. **Forming the MY133-V2000 microchannel structure**
   1. Fill the PDMS negative with 400 µL of MY133-V2000.
      NOTE: The negative must be slightly overfilled.
   2. Place the MY133-V2000-filled PDMS negative into the vacuum chamber for 2 h to remove any bubbles.
   3. Remove MY133-V2000 from the vacuum and slowly press a glass slide against the top of the slightly overfilled negative to create a flat surface of MY133-V2000 and to prevent oxygen from inhibiting the polymerization.
      NOTE: PDMS, at the channel surface, will partially inhibit the polymerization, enabling bonding in later stages. The glass slide has a slightly reduced UV transparency (35%) compared to quartz, which helps to prevent any yellowing of the cured device.
   4. Insert MY133-V2000 into a 400 W UV oven and set the UV radiation to 50% of the maximum intensity for 300 s to cure the MY133-V2000 microchannel.
      NOTE: The peak wavelength used to cure the device is approximately 375 nm with a bandwidth of about 25 nm. Approximately 4,500 mJ/cm^2 of power was used to cure the microchannel, which is slightly more than double the minimum curing power recommended by the manufacturer.

2. **Building the acrylic holder**
   1. Draw the acrylic base layer using a vector drawing software. Make sure the base layer is a rectangle that is 1.5 mm thick, 75 mm long, and 25 mm wide, with a centered rectangle that measures 25 mm x 11 mm.
   2. Draw the acrylic mid-layer using a vector drawing software. Make sure the mid-layer is a rectangle (1.5 mm thick, 75 mm long, and 25 mm wide) with a centered square (5 mm x 5 mm) and two circles (both with a diameter of 3 mm) separated by 15 mm.
   3. Draw the acrylic top layer using a vector drawing software. Make sure the top layer is a rectangle (3 mm thick, 30 mm long, and 25 mm wide) with a centered square (5 mm x 5 mm) and two circles (both with a diameter of 3 mm) separated by 15 mm.

4. Cut out the device designs created in the vector drawing software with a laser cutter. Use the sample settings of 100% power and 30% speed. Run the program twice to ensure that the acrylic is cut out.
5. Tap the holes in the acrylic top layer using a size M5 0.80-mm tapping tool.
6. Wipe the acrylic pieces with acetone to remove any residual marks or burns.

3. **Bonding of MY133-V2000 in the acrylic holder**
   1. Attach the base layer of the acrylic to the glass substrate using an adhesive, such as cyanoacrylate super glue. Place two small drops on the edges of the acrylic and then use a disposable tool to evenly spread the glue. Place the glass substrate onto the acrylic and allow the glue to dry using the weight of the glass to hold it in place.
   2. Coat the exposed glass with 100 µL of PDMS using a positive displacement pipette; then, insert the base layer into a vacuum spin coater and set it to 1,500 rpm for 2 min to evenly coat the glass with a PDMS film approximately 10-µm thick.
   3. Remove the base layer from the spin coater and carefully wipe away any excess PDMS that coated the acrylic with acetone and paper towel. Be careful not to disturb the uncured PDMS.
   4. Bake the base layer with the PDMS in an oven at 65 °C for 2 h to cure the PDMS.
   5. Pipette 1 mL of PDMS onto a glass slide and, then, use another glass slide to evenly spread the PDMS until it starts to ooze out the sides. Cure this on a hot plate at 150 °C for 10 min.
   6. Cut the cured PDMS into a rectangle with the same dimensions as the MY133-V2000 device and, then, using the mid-layer of the acrylic as a mold, punch holes for the reservoir and cut a square viewing window in the PDMS to make the PDMS gasket.
   7. Place the MY133-V2000, channel side up, on the same or a similar flat substrate as used for plasma-treating the PDMS in step 1.2.5. Place the acrylic base layer containing the cured PDMS substrate alongside the MY133-V2000 channel in the O_2 plasma cleaner. Set the vacuum pressure to 200 mTorr and the RF level to high and surface-treat the channel and glass substrate for 30 s.
   8. Using forceps, immediately place the MY133-V2000 channel side-down in the rectangular cutout of the base layer acrylic such that the MY133-V2000 contacts the PDMS.
   9. Place the PDMS gasket on top of the MY133-V2000 device, lining up the holes in the gasket with the reservoirs in the device.
   10. Place the unfinished device onto a raised platform above the bench to provide a clamping surface for device assembly.
   11. Extract 3 mL of acrylic cement using a syringe. Distribute enough acrylic cement on top of the base layer to provide a thin coating. Make the coat as even as possible and do not let the material seep into the channel.
   12. Place the mid-layer acrylic piece on top of the base layer acrylic. Make sure that the holes line up with the reservoirs of the MY133-V2000 channel.
   13. Use 3 small clamps to hold the base layer and mid layer together as tightly as possible for 2 min while the acrylic cement hardens, to bond the pieces of acrylic together. Ensure that the reservoirs are not obstructed during this step to prevent air from being trapped underneath the MY133-V2000 device.
   14. Remove the pressure from the device and place the acrylic cement on the mid-layer in the places of anticipated contact with the top layer of the acrylic.
   15. Place the top layer of the acrylic on the mid-layer piece of the acrylic, ensuring that the holes are aligned with the reservoirs, and allow it rest on the bench for 2 min while the acrylic cement dries.

3. Testing and Use of the MY133-V2000 Device

1. **Leak testing**
   1. Test the MY133-V2000 device by adding 10 µL of food dye or deionized water into one of the reservoir holes to test for the adhesion and flow.
   2. Insert a tube (with a 1/8-in outer diameter) into the reservoir and connect the other end to a vacuum trap. Turn the vacuum on to pull the dye or water through the channel to ensure that a successful device is created.
   3. Check under a microscope to verify that there are no leaks in the channel or reservoir, fill the reservoir with ethanol, and allow the ethanol to sit at room temperature for 10 min. Then, pull the ethanol through using the vacuum, to clean the dye or water out of the channel.
   4. Spray the channel with ethanol and put it into a sterile polystyrene dish. Wrap it tightly with parafilm and store it in a sterile environment until needed.
      NOTE: At this point, the device must be handled aseptically in order to prevent contamination. When ready to use the device, the outside of the container should be sterilized using ethanol and brought into a biosafety cabinet prior to opening the container.

2. **Plating MCF7 cells in the MY133-V2000 device**
   1. Grow MCF7 cells on 10-cm Petri dishes in a complete media consisting of Dulbecco's minimum essential medium (DMEM), supplemented with 10% fetal bovine serum, penicillin-streptomycin, glutamine, and non-essential amino acids, in a cell culture incubator that maintains a humid atmosphere containing 5% CO_2 and 20% O_2 at a temperature of 37 °C. Grow the cells to approximately 80% confluence before seeding them in the microchannel.
   2. First, spray the polystyrene dish containing the microchannel with 70% ethanol. Then, bring it into the biosafety cabinet before removing the parafilm protecting the channel from the ambient laboratory environment.
   3. Immobilize human-plasma-derived fibronectin on the microchannel surface by diluting it in Dulbecco's phosphate-buffered saline (DPBS) to 10 µg/mL. Then, inject 20 µL of this solution into the channel and incubate it at 20 °C for 45 min.
   4. Wash the fibronectin solution out of the channel by injecting 20 µL of cell culture media into the channel, and allow it to incubate at 20 °C for 10 min.
   5. Wash the cells with 10 mL of DPBS, and trypsinize the cells by adding 1 mL of 1x trypsin. Incubate the cells at 37 °C for 7 min. Neutralize the trypsin with 9 mL of complete media.
   6. Inject 20 µL of the cells into the channel and incubate them at 37 °C for 45 - 120 min to allow the attachment to the substrate prior to imaging.

Representative Results

This protocol describes the fabrication of MY133-V2000, a fluorinated polymer with a low refractive index matching that of water. A key feature of this protocol is how to overcome the lack of adhesion that is characteristic of fluorinated polymers by using oxygen plasma and by fabricating the device within an acrylic holder to provide the extra mechanical force required to seal the channel against the PDMS substrate (**Figure 1**). The low refractive index of the final device is clearly shown on a macroscopic scale (**Figure 2**). The edges of a channel made from this material are clearly seen in air (**Figure 2A**) but become difficult to distinguish when immersed in water (**Figure 2B**) due to the close match in refractive index. This provides a quick check of the optical properties of the material after fabrication. A successfully completed device is shown in **Figure 2C**, where the MY133-V2000 microchannel can be seen encapsulated by the acrylic that provides the extra mechanical force required to seal the microchannel. A successful device should show good adhesion (free of air bubbles) between the device and the substrate. The presence of an air bubble at the center of the device channel indicates that air was not allowed to escape during bonding, most likely due to an obstruction of the reservoirs when the acrylic cement sets. This device also shows well-adhered acrylic/glass layers with minimal bowing of the glass substrate. Problems in this area can be addressed by either spreading the adhesive between the acrylic and glass layers more evenly or by reducing the thickness of the microfluidic device to prevent stress on the holder. Finally, the reservoirs in all three panels show clear edges, with no air gaps at the edges. This is another potential problem area for device-substrate adhesion that is eliminated by the use of a device mold with perfusion ports built in.

On a microscopic scale, few, if any, artifacts are introduced due to phase unwrapping by matching the refractive indices of the microchannel and the cell culture media. This demonstrates that the mass of cells can be precisely quantified even in the vicinity of the channel walls (**Figure 3**). Collectively, these results show, on both a macroscopic scale and on a microscopic scale, the advantages of using a fluorinated polymer to match the indices of refraction between the fabrication material of the microchannel to aqueous cell culture media.

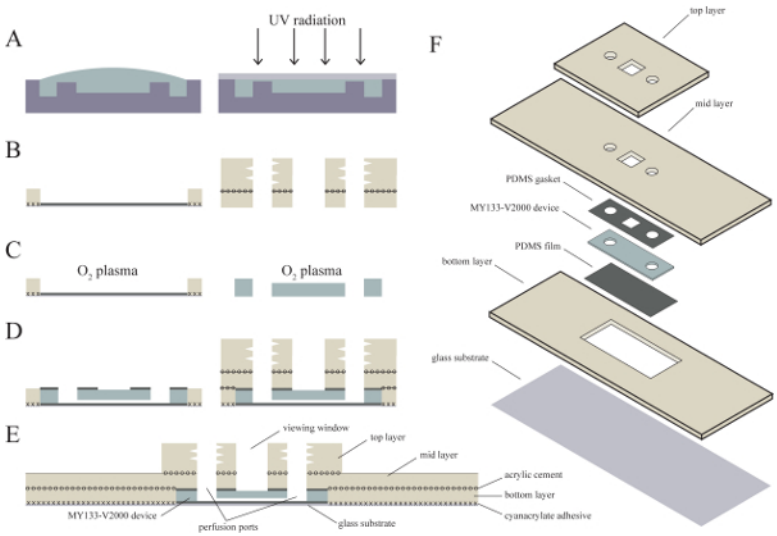

Figure 1: MY133-V2000 soft lithography workflow. (**A**) After filling the mold with MY133-V2000, the meniscus is compressed using a glass slide to create a flat surface and cured in a UV oven. Note that the height of the negative of the reservoirs is comparable to the height of the mold to create deep reservoirs 3 mm in diameter. (**B**) After cutting the acrylic, the bottom acrylic layer is glued to a glass coverslip prior to spin-coating it with PDMS. The top two acrylic layers are laminated together using acrylic cement. (**C**) The cured MY133-V2000 device (channel side up) and the coverslip are surface-treated using O_2 plasma. (**D**) Finally, the device is assembled by adhering the surface-treated sides of the device and coverslip together. The PDMS gasket is then placed on top of the MY133-V2000 device and the acrylic is attached using acrylic cement, with the holes of both the PDMS gasket and the mid-layer of the acrylic lining up with the reservoirs. The assembled device is then clamped by hand for 2 min until the acrylic cement dries. (**E**) An exploded view of the device shows how the device is assembled. (**F**) A drawing shows the cross-section through the microchannel of the finished device. Please click here to view a larger version of this figure.

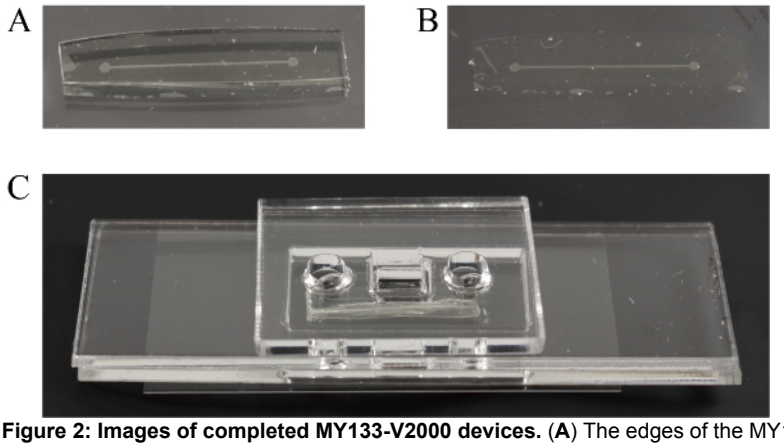

Figure 2: Images of completed MY133-V2000 devices. (**A**) The edges of the MY133-V2000 device are distinctly visible in air. (**B**) When submerged in water, the edges of the same MY133-V2000 device disappear because of the close match in refractive indices. (**C**) This photograph shows a finished device with an acrylic holder. Please click here to view a larger version of this figure.

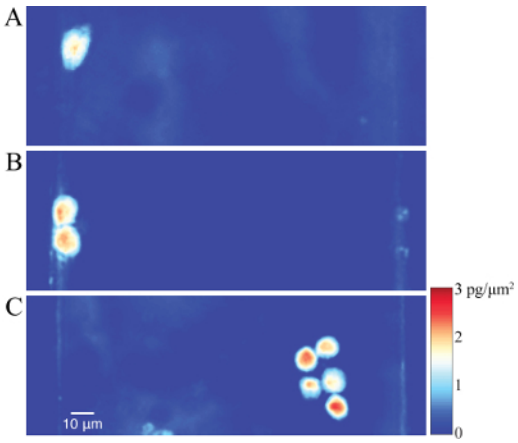

Figure 3: Cell mass can be precisely quantified near microchannel structures. Measurements of the cell mass of freshly seeded MCF7 cells in the MY133-V2000 microchannel show no artifacts due to phase wrapping near (**A** and **B**) the wall or (**C**) the center of the channel. These artifacts are avoided in the MY133-V2000 channel because of the closely matched refractive indices of the channel material and the cell culture media. Please click here to view a larger version of this figure.

Discussion

MY133-V2000 can be used as an alternative to traditional soft lithography fabrication materials such as PDMS. Previous work has shown that materials with a high index of refraction, such as PDMS, introduce significant artifacts near the channel walls due to the mismatching indices of refraction between the fabrication material and the aqueous solution inside the channel[13]. MY133-V2000 enables matching the refractive index of the microfluidic device to the aqueous solutions commonly used in biomedical applications. This reduces imaging artifacts when combining microfluidics with advanced microscopy techniques, providing a distinct advantage over traditional microfluidic fabrication materials. The artifact reduction in microfluidic channels made possible by this system enables fluorescence and quantitative phase microscopy (**Figure 3**) signals to be more precisely quantified, even in close proximity to microchannel structures[13].

As a fluorinated polymer, MY133-V2000 typically exhibits low adhesion between the channel structure and other materials, thus introducing a major limitation when compared to traditional fabrication materials. To overcome this challenge, both chemical surface treatment (O_2 plasma) in addition to mechanical compression created by squeezing the device and the PDMS gasket between the substrate and a piece of acrylic are used. The lower elastic modulus of PDMS (compared to MY133-V2000) is critical to transferring mechanical force from the acrylic to the MY133-V2000 device, because it is deformable enough to be compressed, allowing it to hold the microchannel in place while simultaneously keeping it sealed against the substrate.

Two important points of failure can be encountered when fabricating microchannels using this method and should be noted when troubleshooting. These are leakage from the reservoir due to excess slag and leakage from the center of the channel where the force from mechanical compression is at a minimum. Even a small amount of slag from punching holes for the reservoirs prevented the reservoir from adhering to the substrate properly. To prevent leaks from the reservoir, the microchannel negative contains small pillars, 3 mm in diameter, that are similar in height as the mold, so that the reservoirs can be cast as a part of the microchannel without having to use a tool to punch holes for the reservoirs. Punching holes for reservoirs after curing creates slag that must be washed away without damaging the channel (**Figure 1A**). This is difficult to achieve in practice. To prevent leaks from the center of the channel, it was critical not to cover the reservoirs during assembly in order to allow air to escape from underneath the device. If the reservoirs are obstructed when gluing the device together, air underneath the device is unable to escape, creating a space for fluid to seep out of the channel.

This protocol, therefore, presents a method to improve adhesion through both surface treatment and mounting the device in an acrylic holder, mitigating the negative effects of fluorination on the adhesive properties of the polymer. The devices were filled with fluid and incubated for up to 24 h, demonstrating that the device remains functional for the duration of a typical imaging experiment. MY133-V2000, as a fabrication material for a microfluidic device, can be combined with QPM to measure cell biomass. Previously, QPM has been shown to measure the growth of live cells with a greater precision than conventional approaches[17]. Using QPM, a live-cell drug response can be measured with single-cell resolution[11,18,19]. When combined with MY133-V2000, cells can be cultured in the channel for at least 1 d, enabling the growth rate of the cells to be precisely determined. Combining the advantages of both microfluidics and QPM allows for the measurement of growth and live drug responses of cells under controlled conditions.

Future applications of this technique involve incorporating it into more advanced microfluidic and quantitative microscopy experiments. The elasticity of MY133-V2000[13] is compatible with advanced microfluidic techniques such as pneumatic valves, further enabling the fabrication of complex geometries and experimental designs. The results presented here demonstrate the use of this material for making quantitative measurements using QPM. MY133-V2000 should also be compatible with other quantitative microscopy techniques, such as Frster resonance energy transfer or fluorescence lifetime microscopy. This approach also allows for the device to be mounted on and sealed against a PDMS substrate, enabling advanced experimental designs such as encapsulating fluorophores in the substrate. Overall, MY133-V2000 reduces artifacts when making quantitative measurements in aqueous solutions in microchannels, making it an ideal material for the fabrication of microfluidic channels for making high-precision biomedical measurements.

Disclosures

The authors have nothing to disclose.

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