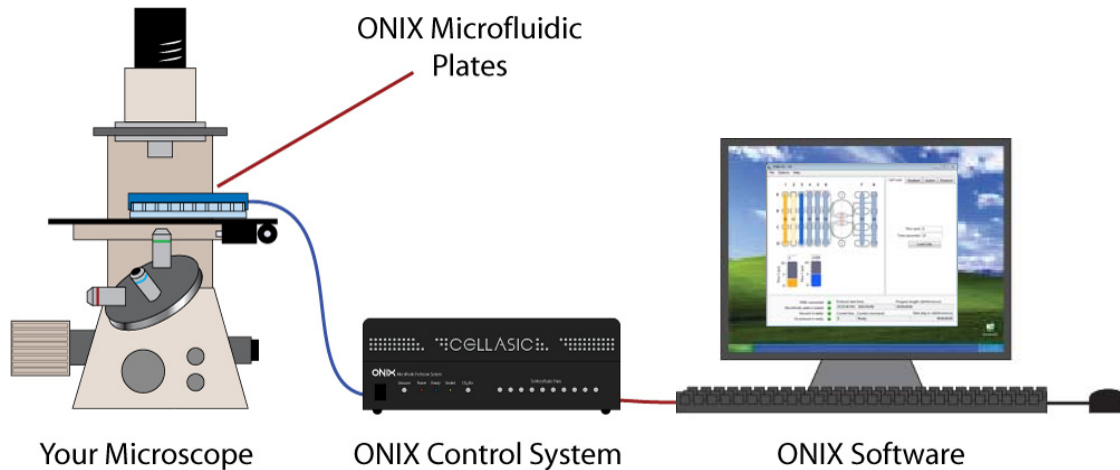




Microfluidic Perfusion System

Live cell imaging made simple. The ONIX enhances cell culture quality with advanced microfluidic perfusion technology. The system complements your microscope to provide a total solution for capturing the highest quality data with minimal effort.



The ONIX Advantage

- Automated perfusion for multi-day timelapse imaging of cellular responses
- Application specific microfluidic plates address many experiment types
- Easy setup and intuitive user interface saves time without sacrificing flexibility



ONIX System

The system integrates all the components necessary for high quality cell culture on your microscope stage. Our innovative media and CO₂ delivery method optimizes cell health during long term experiments. Intuitive software automates all operation to make data collection simple and painless.

ONIX Microfluidic Plates

Our microfluidic cell culture plates provide a highly stable cell environment for unprecedented cell culture quality. ONIX plates are available in a wide range of application specific designs to match your research needs. All solutions are directly pipette into the wells of the custom plate. Cells are cultured in microchambers with a glass coverslide floor for optimal high magnification



Technical Specs

Microfluidic Perfusion System

Pressure Output: $0-10 \pm 0.02$
8 Pressure Channels
Power Input: 110-240V
USB Data Connection
Built-in Pressure/Vacuum Pumps
Operated via FG Software

CO₂: Preset at 5%, Adjustable $0-15\% \pm 0.2$
CO₂ Input: 15 PSI
CO₂ Consumption: 1 mL/min

Temperature Control

Use with Microscope Enclosure Heaters
Compatible with Objective Heater for Immersion Imaging
No Humidity Control Necessary

Microfluidic Plates

Standard SBS 96-well Plate footprint
Imaging Substrate: #1.5 glass coverslip
Uninterrupted Culture Time: 5+ Days
Perfusion Flowrate: 1-100 $\mu\text{L/hr}$
Typical Chamber Volume: $<1 \mu\text{L}$
Input Volume: 300 μL per well
For Inverted Microscopes Only

FG Software

Computer Requirements:
Windows 7, Vista, XP, 2000 Vista
USB 1.0 Connection or Higher
Pentium III-Class PC (500 MHz or higher)

Features and Benefits

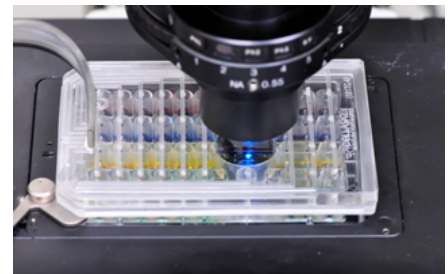
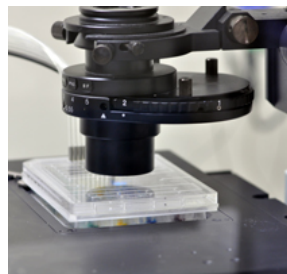
Integrates with your microscope: Fit to any inverted microscope to create a live cell imaging station.

Microfluidic Cell Culture: Proprietary microfluidic chamber and precision laminar flow creates a highly stable, optimal cell environment

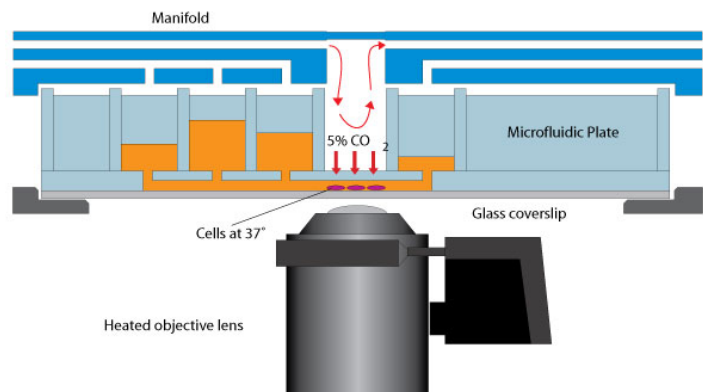
Multiplexed Culture Chambers: Independent flow chambers allow up to 16 simultaneous experiments per run.

Pressure Driven Flow: Eliminates setup time while delivering more accurate flow control compared to typical syringe pumps.

Microscale Flow Control: Software interface allows you to program solution exposures and change media solution in real time during an experiment.



The low profile manifold seals to the SBS standard footprint microfluidic plate for imaging on any inverted microscope. Cell chambers are located under the imaging window for best optics.



The innovative “microincubation” method maintains temperature and gas control to the cells during long term perfusion imaging.

M04B Microfluidic Cell Culture Plate

M04B-01

Description: The M04B plate contains 4 independent chambers, each with 6 upstream inlet channels. Each chamber is 160 micron in height and designed for long term, high magnification, live cell perfusion imaging.

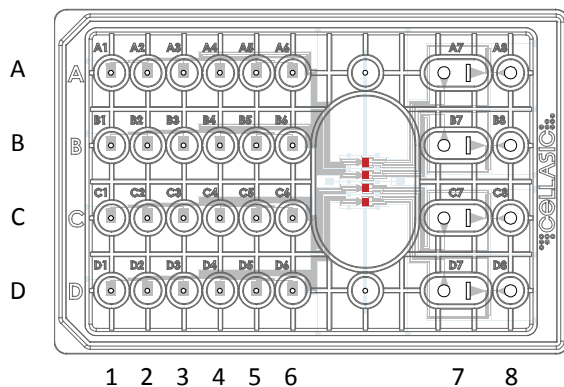
Applications:

- Time-lapsed, high magnification imaging of adherent cells (up to 160 um diameter)
- Long term continuous perfusion experiments (3 days typical, up to 14+ days)
- Solution exchange experiments (induction, inhibition, drug dosing, etc.)
- Automated immunostaining in the micro-chamber and “on-demand” fixing of live cells
- Comparing up to 4 different cell types or exposure conditions in parallel
- Temperature and gas atmosphere control (temperature shift, anoxic, etc.)

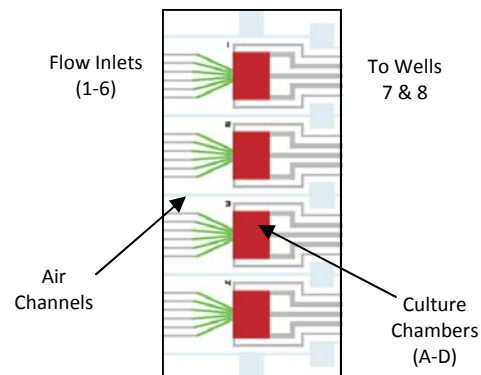
Product Specifications:

- Use with ONIX-262 system and F08 manifold
- SBS standard footprint frame (fits to typical 96 well stage holders)
- #1.5 thickness (170 um) glass slide bottom (in contact with cells)
- Capillary driven cell loading using only a pipette (10 uL sample per chamber)
- Laminar flow rates of 1-100 ul/hr (typical chamber refresh times of 10-60 seconds)
- Microfluidic gas exchange channels

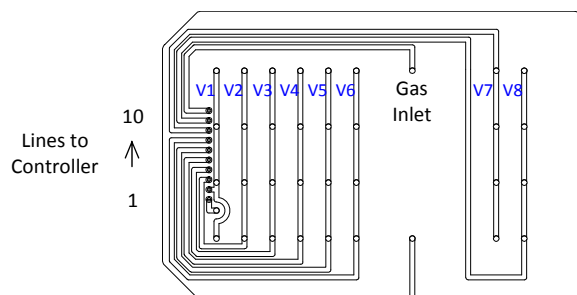
Plate Design:



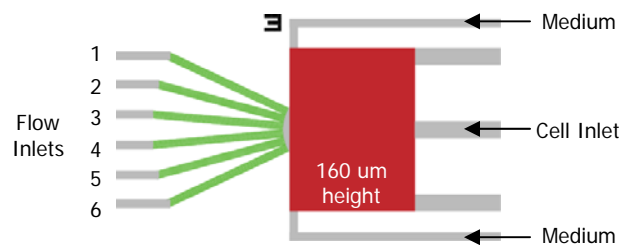
The M04B has 4 independent units (A-D), each with 6 inlet wells (1-6), a cell inlet/flow outlet (7), and a medium well (8). All four culture chambers are located under a single imaging window.



The 4 culture chambers are located under a large imaging window for high magnification phase objectives and to minimize travel distance. All channels are resistance matched for uniformity.



The F08 manifold has 8 pressure channels (V1-V8) to control flow rates through the microfluidics. A vacuum line is used to seal the plate to the manifold, and a gas line enables atmosphere control.

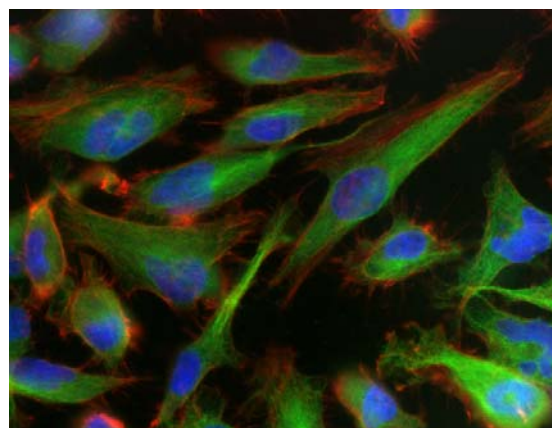


The culture chamber is 1.5 x 2.0 mm in area with a height of 160 microns. Six switching inlets enter at a central point to ensure uniform flow profiles. The medium channels feed the cells during pre-culture.

Highest Quality Live Cell Imaging

CELLASIC's microfluidic cell culture technology delivers unmatched quality for live cell imaging.

- Stress-free cell loading using a pipette inside your sterile hood via our capillary flow method.
- Gravity driven perfusion lets you "pre-culture" cells for many days in a standard incubator.
- The #1.5 thickness (170 μm) glass coverslide floor is matched to immersion and high NA objectives and amenable to surface coating.
- A large imaging window allows light to pass freely for optimal phase contrast quality.
- The four microchambers are located within a 1.5 cm

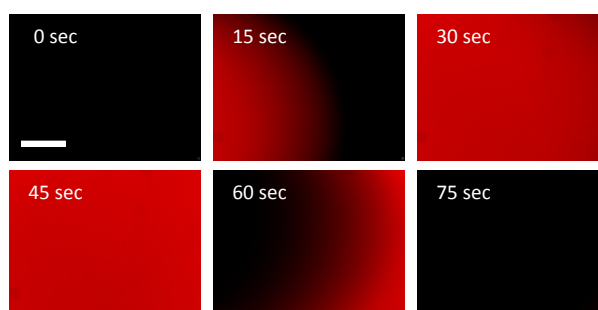


HeLa cells immunostained in the microchamber after perfusion culture.

Solution Switching

Our advanced perfusion control enables experiments not possible with existing instrumentation.

- Six switchable inlet wells for each chamber.
- Highly laminar flow provides uniform exposure profiles and sharp transition boundaries.
- Low shear ensures minimal stress on cells.
- Rapid solution exchange rates and <10 nL dead volume for fine time resolution experiments.
- Perfusion rates of $\mu\text{l/hr}$ allow continuous multi-day experiments on your microscope.

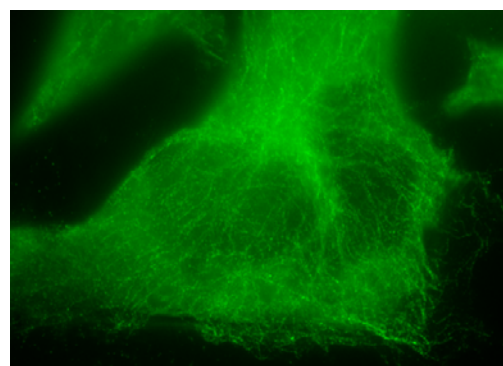


Solution exchange profile between a fluorescent dye and buffer solution in a 10X field of view. Scale bar = 200 μm .

On-Demand Immunostaining

The M04B plate allows *in situ* immunostaining of cells monitored in the microfluidic chambers.

- Automate staining and washing protocol using up to 6 inlet solutions.
- Laminar flow mechanics provide excellent washing and staining kinetics with minimal reagent usage.
- Fix cells "on-demand" by flowing fixative into chamber during cell imaging. Fix cells when they reach a desired state (mitosis, apoptosis, response to drug, etc.) to obtain precise detail on single cell biology.

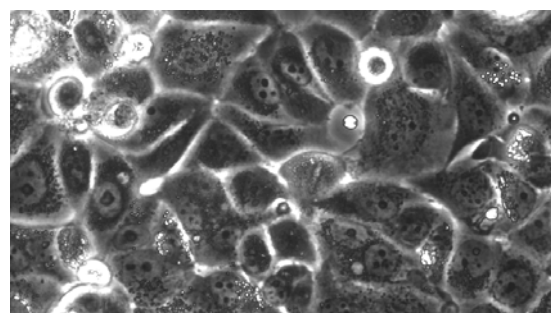


α -tubulin staining in the microfluidic chamber. HeLa cell, 100X

Long Term Cell Culture

The innovative microfluidic plate is designed for long term cell happiness.

- Continuous perfusion ensures fresh medium and waste removal, even at confluent cell densities.
- Small chamber areas allow uniform temperature control using only an objective heater.
- The sealed manifold delivers 5% CO_2 through specialized microfluidic gas diffusion channels.
- Gravity driven medium channel allows pump-free culture in any incubator.



MCF-7 breast cancer cells growing at high density after 6 days continuous perfusion.

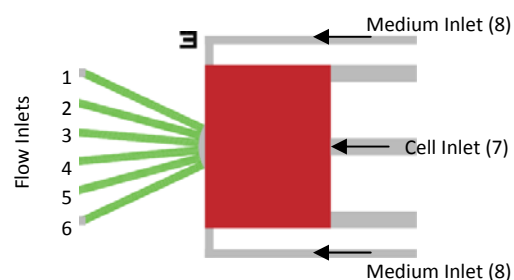
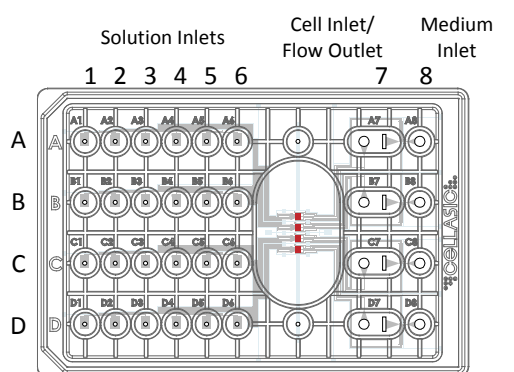


Figure 1. Plate Layout

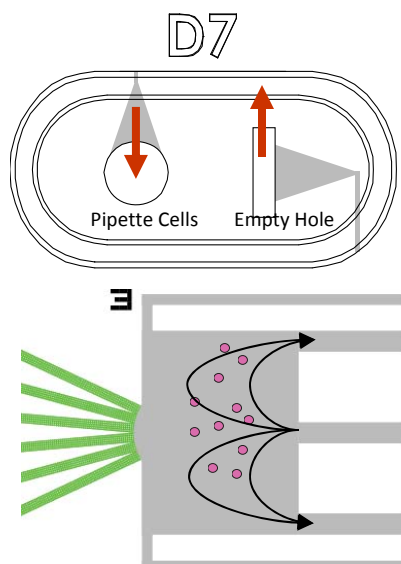


Figure 2. Capillary Cell Loading Method and Flow Profile



Figure 3. Manifold Sealed to Plate on Microscope Stage

Operation Instructions

1. The M04B microfluidic plate contains 4 parallel chambers, each with 6 solution inlets (Figure 1). Each row of wells (A-D) addresses the corresponding chamber. The plate is shipped pre-primed with sterile PBS containing penicillin/streptomycin/amphotericin B, which can be replaced with a buffer of choice prior to experiment. Plate performance guaranteed up to the 6 month expiration date.

Cell Loading

2. Prepare a cell suspension of $0.5\text{-}5 \times 10^6$ cells/ml. Aspirate the cell loading well (7). Remove the liquid from the left (circle) hole (Figure 2). Pipette 10 μl cell suspension into the hole. Without delay, remove the liquid in the right (rectangle) hole. This will cause capillary action to pull the cells into the chamber for ~ 2 min, as depicted in figure 2. Check loading density on a microscope. If more cells are desired, repeat this step.
3. Optional Pre-Culture. Pipette 300 μl of culture medium into well 8 and 50 μl of PBS into well 7 to initiate gravity perfusion of the medium at ~ 40 $\mu\text{l}/\text{day}$ from well 8 to 7. Place the plate in an incubator for cell attachment and growth. Replace the medium in well 8 (and empty 7) as necessary for long term culture.

Live Cell Imaging

4. Fill the six sets of flow inlet wells (col 1-6) with up to 300 μl of solution. If less than 4 units will be used, fill the unused inlet wells with buffer.
5. Seal the microfluidic plate to the F08 manifold: Clean the manifold gasket with 70% ethanol and blot dry. Place the microfluidic plate on a flat surface. Align and set the manifold over the wells of the plate. Turn on the vacuum switch on the ONIX box and push down on the manifold with slight force for ~ 5 seconds to ensure uniform contact during sealing. Alternately, gently squeeze the plate and manifold together by placing your fingers underneath the plate with thumbs on top of the manifold. When a proper seal is formed, the green “sealed” light will be lit. Make sure a proper seal is formed before proceeding. Leave the vacuum on during the course of the experiment. (If the seal is unsuccessful, turn off the vacuum switch and wait for the blue “ready” light to turn back on. Repeat step 5 above. If a seal cannot be formed, please contact CellASIC.)
6. Place assembly on an inverted microscope (Figure 3). Focus on the center of the imaging area. The culture region is 1.5×2 mm in size with a 160 micron ceiling height.
7. Turn on the “CO₂/Air” switch on the ONIX control box. The CO₂ will fill the air channels in the microfluidic plate and diffuse through the gas permeable chamber walls. Use the ONIX CO₂ Sensor to set the desired gas mixture.
8. Open the ONIX FG software on a computer attached to the USB line from the control box. Select the “M4” tab from the front page. If you do not see the tab, make sure you have an updated version of the software (www.cellasic.com/Products-downloads) or contact CellASIC.

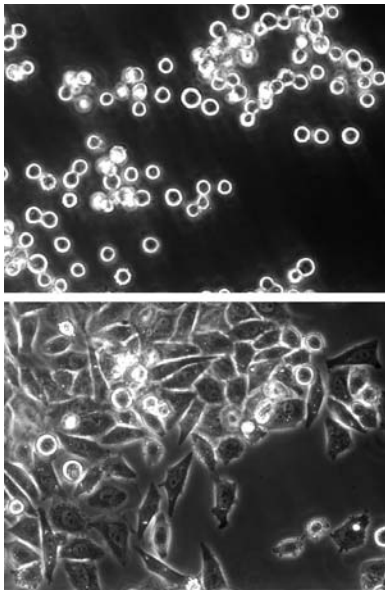


Figure 4. Cells Attaching to Glass After Overnight Perfusion

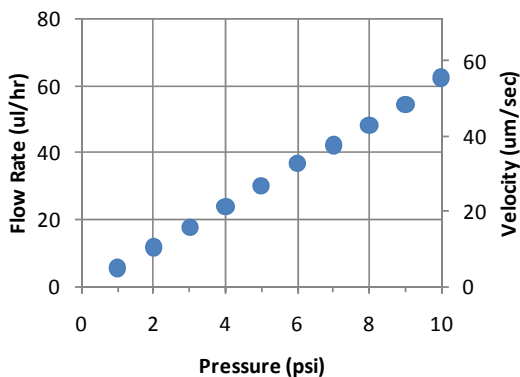


Figure 5. Flow Rate and Chamber Flow Velocity

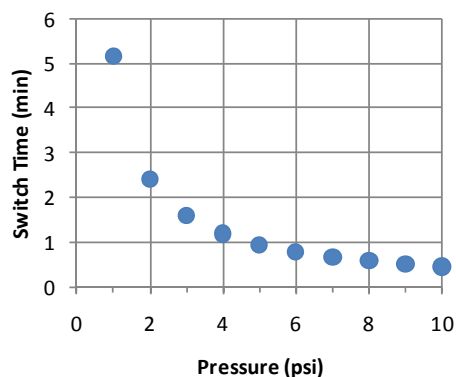


Figure 6. Total Chamber Switch Time

Operation Instructions (cont.)

9. Flow properties are given in Figures 5 and 6. Figure 5 shows the total flow rate out of each inlet well. The 2nd y-axis, flow velocity, is of the laminar front moving through the chamber, or the local switch rate for cells in the chamber. Figure 6 gives the time for the laminar front to move through the entire chamber. If more than one channel is open at a time, multiply the flow rate/velocity by the number of open channels. Note: flow of liquid from wells 7 or 8 is roughly 100x faster than wells 1-6.
10. Use the tabs on the right side of the software control window (Figure 8) to set up your experiment.
11. For custom flow scheduling, use the "Protocol" tab to enter text based commands. The commands are given in Figure 8.
12. Click "Run" to run the perfusion program.
13. Acquire images using your standard microscope methods.

Pressure Driven Cell Loading

14. Alternately, cells can be loaded using the ONIX flow controller by filling 100 ul of cell suspension into well 7 (both holes), and flowing well 7 at 0.2 psi for ~10 seconds. Cells will exit to well 8. In general, this requires a higher density of cells compared with the capillary method.

Rapid Solution Exchange

15. For experiments requiring rapid solution exchange, the following techniques can be used.
16. Flow at high pressure for the initial transition, then reduce flow for long term exposure. Multiple inlets can be used containing the same solution to speed flow. For example, wells 3, 4, 5, 6 can be filled with the switch solution. Schedule flow for 3, 4, 5, 6 simultaneously at 5 psi, getting a switch time of ~15 seconds. Reduce the flow rate to 0.5 psi (from all 4) for long term exposure. For very high pressure flow (8-10 psi) it is recommended that columns 1 or 2 be avoided to prevent manifold unsealing.
17. For extremely fast switching, utilize the medium inlet channel (col 8). Replace the liquid in col 8 with the desired switching solution. Flow 8 at the desired pressure. The switch time will be ~100x faster than listed in Figure 6. For example, at 2 psi, the chamber will be replaced in approximately 2 seconds (~20 ul/min). Make sure not to flow well 8 for extended periods of time in order to avoid emptying the well and introducing air bubbles.

Surface Coating

18. To coat the chamber surface, add 200 ul of coating solution to well 8. Leave 50 ul of solution in well 7. Allow gravity perfusion to coat the surfaces for 1-24 hours for full coverage. Flush out coating solution with buffer or medium for 1 hour via gravity perfusion (add 300 ul to well 8) prior to loading cells. Note: parameter optimization (solution concentration and exposure time) will result in best performance.
19. For additional protocols, contact CellASIC.

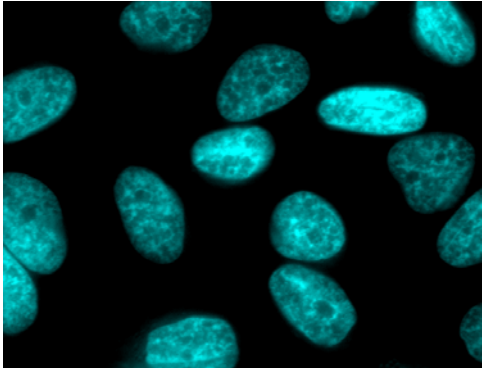


Figure 7. Nuclear staining in live cells (63X)

Operation Tips

20. During capillary cell loading, empty the bottom holes using a standard pipette to avoid introducing air bubbles. A vacuum aspirator can be used, but be careful to quickly and gently empty the liquid.
21. Cells can be “redistributed” in the chamber after loading by tilting the plate in the desired direction for a few minutes.
22. For long term cell perfusion, a pressure of 0.5-1 psi generally provides adequate nourishment with minimal stress.
23. For longer term experiments requiring less than 6 solutions, use multiple wells for each solution.
24. An objective heater is adequate (with immersion lenses) to maintain cell health; for air lenses, an enclosure is best.

Software Operation

Note: Flow X controls V1 and V2, Flow Y controls V3-V8

Column:	1	2	3	4	5	6	Duration (min):	Flow (psi):
1	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	2	2
2	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	10	4
3	<input type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	10	5
4	<input type="checkbox"/>	<input type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	25	5
5	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	30	4
6	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	10	2
7	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	0.1	1.5
8	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	0.5	1.5
9	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	0	0.25
10	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	0	0.25

Flow X (psi): 3
Flow Y (psi): 6.253

Protocol start time: 01:00:00 PM 2000/01/01
Program length: 00:00:00:00
Current line: 0
Current command: Ready
Next step in: 00:00:00:00

Figure 8. ONIX FG M4 Interface

Text Commands:

- setflow X n** ; where $n = 0.25-10$ (psi) ; sets flow rate on X (orange)
- setflow Y n** ; where $n = 0.25-10$ (psi) ; sets flow rate on Y (blue)
- open V1** ; V1, V2, ..., V8, all ; opens pneumatic valve
- close V1** ; V1, V2, ..., V8, all ; closes pneumatic valve
- wait n** ; n is minutes ; holds current condition until next step
- end** ; ends the program ; shuts off all valves and resets regulators
- %** ; Put at the beginning of line for comments

```

1 % Sample Protocol
2
3 setflow Y 2
4 open V3
5 open V5
6 wait 10
7 setflow X 4.5
8 open V1
9 wait .5
10 close all
11 end
12
  
```

Y04C Microfluidic Yeast Plate: 4-5 um

Y04C-01

Description: The Y04C plate contains 4 independent chambers, each with 6 upstream inlet channels. Each chamber has microfluidic trap regions of 4.0, 4.5, and 5.0 micron in height to hold cells in a single focal plane during long term, high magnification, perfusion imaging.

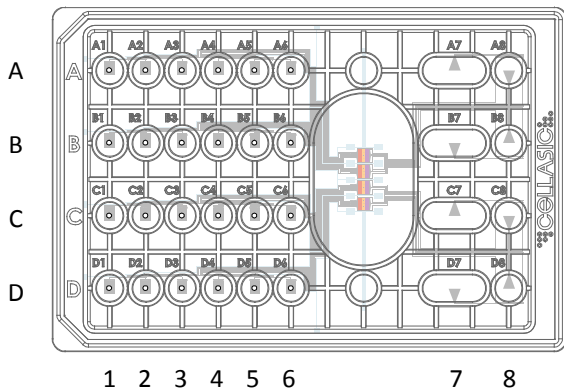
Applications:

- Time-lapsed, high magnification imaging of yeast cells (4-6 um diameter)
- Long term continuous perfusion experiments (3 days typical, up to 14+ days)
- Solution exchange experiments (induction, inhibition, drug dosing, etc.)
- Comparing up to 4 different cell types or exposure conditions in parallel
- Cell division tracking (follow mother/daughter cells over generations)
- Temperature and gas atmosphere control (temperature shift, anoxic, etc.)

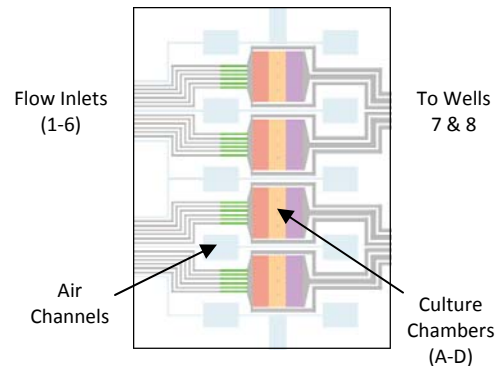
Product Specifications:

- Use with ONIX-262 system and F08 manifold
- SBS standard footprint frame (fits to typical 96 well stage holders)
- #1.5 thickness (170 um) glass slide bottom (in contact with cells)
- Three trap heights per chamber (4.0, 4.5, 5.0 micron)
- Laminar flow rates of 1-100 ul/hr (typical chamber refresh times of 10-60 seconds)
- Microfluidic gas exchange channels

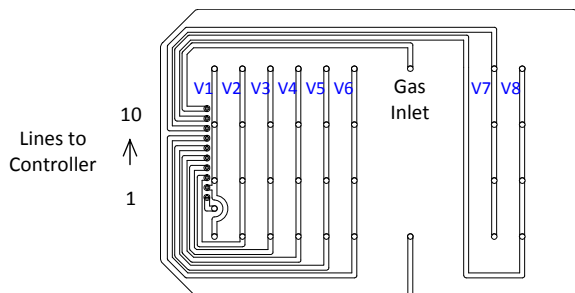
Plate Design:



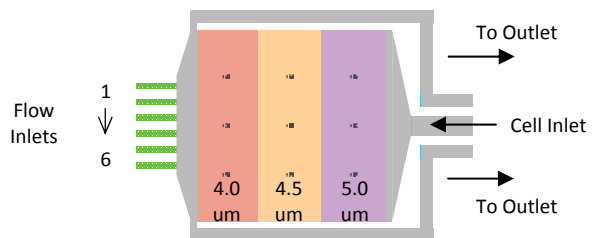
The Y04C has 4 independent units (A-D), each with 6 inlet wells (1-6), a cell inlet (8), and a large outlet well (7). All four culture chambers are located under a single imaging window.



The 4 culture chambers are located under a large imaging window for high magnification phase objectives and to minimize travel distance. All channels are resistance matched for uniformity.

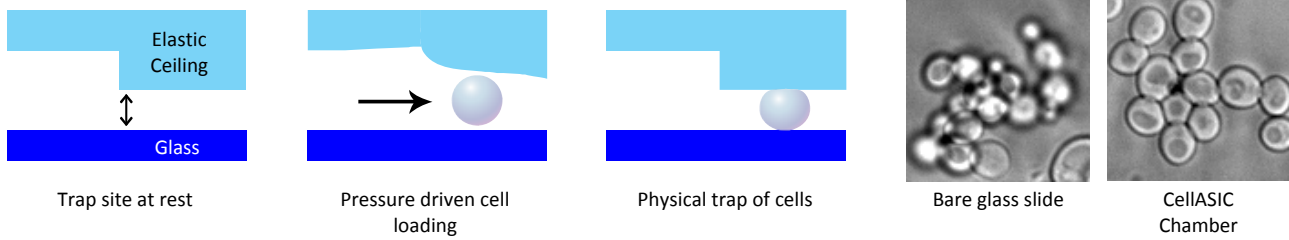


The F08 manifold has 8 pressure channels (V1-V8) to control flow rates through the microfluidics. A vacuum line is used to seal the plate to the manifold, and a gas line enables atmosphere control.



The culture chamber is 3.0 x 3.0 mm in area with heights of 4.0, 4.5, and 5.0 micron. Nine position markers indicate unit number and relative position.

Cell Trapping Mechanism

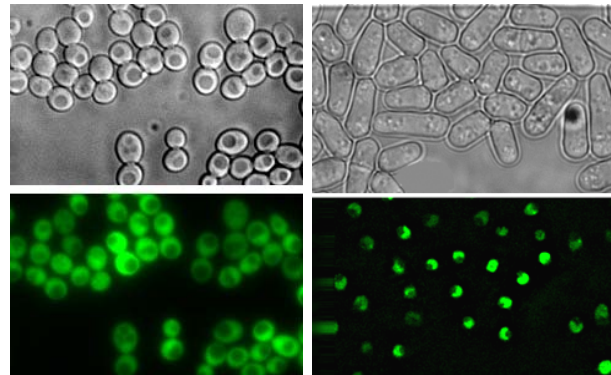


The microfabricated chamber gently holds cells against the glass imaging surface to maintain a single focal plane during perfusion imaging experiments. The Y04C has 3 trap heights of 4.0, 4.5, and 5.0 micron.

Imaging Quality

The Y04C plate is designed for optimal image quality for high end microscopes.

- The cells are held against a #1.5 thickness (170 um) glass coverslide floor, making the plate compatible with immersion and high NA objectives
- The large imaging window allows light to pass freely for optimal phase contrast quality
- The four microchambers are located within a 1.5 cm area, minimizing stage travel and focus drift



Images courtesy the Lim Lab, UCSF, and the Forsburg Lab, USC

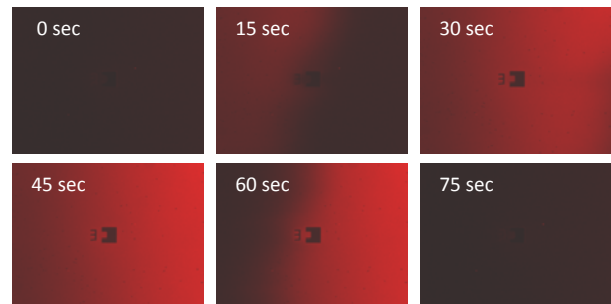
S. cerevisiae

S. pombe

Solution Switching

Our advanced perfusion control enables experiments not possible with existing instrumentation.

- Six switchable inlet wells for each chamber
- Highly laminar flow provides uniform exposure profiles and sharp transition boundaries
- Low shear ensures minimal stress on cells
- Rapid solution exchange rates and <10 nL dead volume for fine time resolution experiments
- Perfusion rates of ul/hr allow continuous multi-day experiments on your microscope

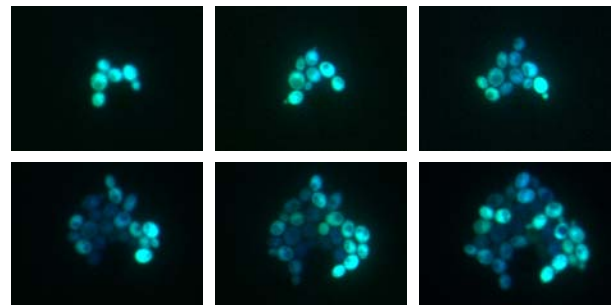


Solution exchange profile between a fluorescent dye and buffer solution in a 0.6x0.8 mm field of view (10X objective).

Long Term Culture

The innovative microfluidic plate is designed for long term cell happiness.

- Continuous perfusion ensures fresh medium and waste removal, even at confluent cell densities
- Small chamber areas allow uniform temperature control using only an objective heater
- The sealed manifold and microfabricated air diffusion channels allow user control of the gas atmosphere in equilibrium with imaged cells



Fluorescently tagged yeast cells tracked over a few cell division cycles in the CellASIC chamber.

Images courtesy the Maheshri Lab, MIT

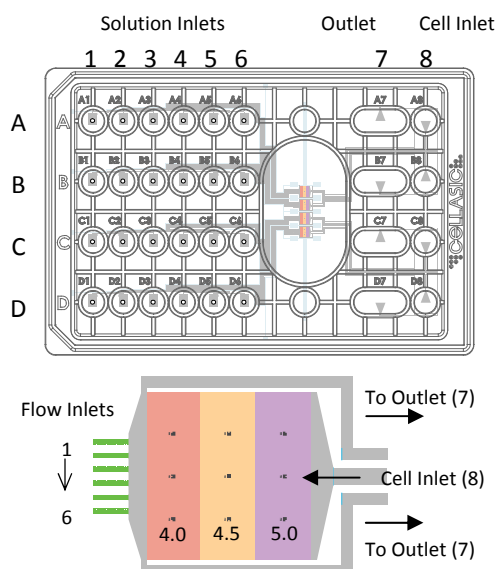


Figure 1. Plate Layout



Figure 2. Plate with Manifold on Stage

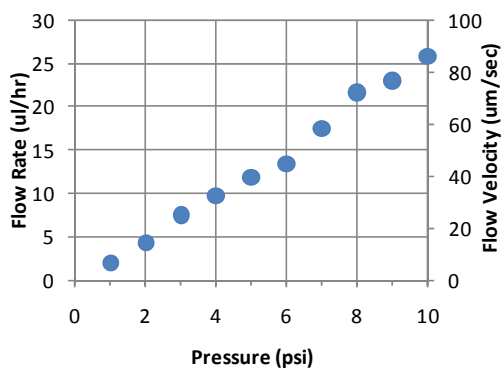


Figure 3. Flow Rate

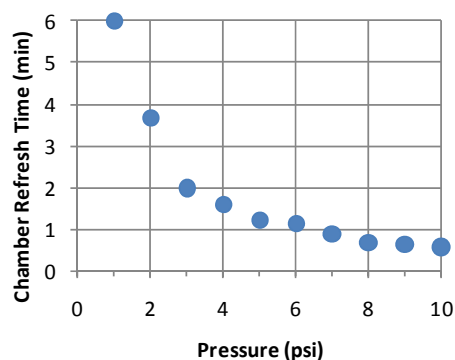


Figure 4. Chamber Switch Time

Operation Instructions

1. The Y04C microfluidic plate contains 4 parallel chambers, each with 6 solution inlets (Figure 1). Each row of wells (A-D) addresses the corresponding chamber. The plate is shipped pre-primed with sterile dl water containing penicillin/streptomycin, which can be replaced with a buffer of choice prior to experiment. Plate performance guaranteed up to the 6 month expiration date.
2. Fill the six sets of flow inlet wells (col 1-6) with up to 300 μ l of solution. If less than 4 units will be used, fill the unused inlet wells with buffer. Empty the outlet well (col 7).
3. Fill the cell inlet wells (col 8) with 50 μ l of cell suspension, making sure to cover the hole at the bottom of the well. A density of $1-20 \cdot 10^6$ cells/ml is recommended depending on desired trapping density.
4. Seal the microfluidic plate to the F08 manifold: Clean the manifold gasket with 70% ethanol and blot dry. Place the microfluidic plate on a flat surface. Align and set the manifold over the wells of the plate. Turn on the vacuum switch on the ONIX box and push down on the manifold with slight force for ~ 5 seconds to ensure uniform contact during sealing. When a proper seal is formed, the green "sealed" light will be lit. Make sure a proper seal is formed before proceeding. Leave the vacuum on during the course of the experiment. (If the seal is unsuccessful, turn off the vacuum switch and wait for the blue "ready" light to turn back on. Repeat step 4 above. If a seal cannot be formed, please contact CellASIC.)
5. Place assembly on an inverted microscope (Figure 2). Focus on the center of the imaging area. The trapping region is 3x3mm in size with 4.0, 4.5, and 5.0 micron ceiling heights in series as depicted in Figure 1. Nine location markers indicate the chamber number and location.
6. Open the ONIX FG software on a computer attached to the USB line from the control box. Select the "Y4" tab from the front page. If you do not see the tab, make sure you have an updated version of the software (www.cellasic.com/Products-downloads) or contact CellASIC.
7. On the control window (see figure 6), set "Cell Load" parameters to 8 psi and 5 seconds. Click the "Load Cells" button. Depending on your cell type/strain and desired cell density, modify and repeat the loading process as necessary.
8. Wash un-trapped cells by flowing one or more inlet solutions at 5psi for 5 minutes. This will also serve to prime the specified inlet channel by flushing out the shipping solution. Cells remaining in the trap regions are now firmly held in x,y,z for imaging.
9. Flow properties are given in Figures 3 and 4. Figure 3 shows the total flow rate out of each inlet well at the specified pressures. The flow velocity is of the laminar front moving through the chamber, or the local switch rate for cells in the chamber. Figure 4 gives the time for the laminar front to move through the entire chamber.

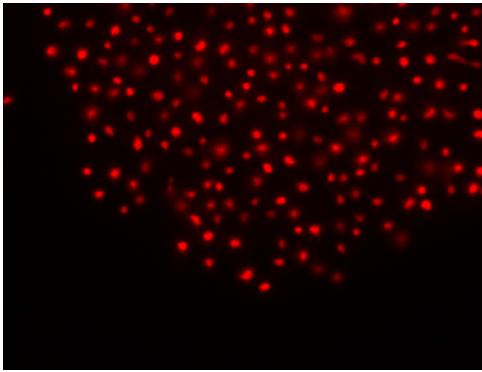


Figure 5. Single Yeast Colony after 15 Hours
Cells courtesy of the Skotheim Lab, Stanford

Operation Instructions (cont.)

10. Use the tabs on the right side of the software control window to set up your experiment. Manual operation is possible by clicking on the valve buttons and dragging or typing in values on the flow bars on the left of the software control window.
11. For custom tuning of flow scheduling, use the "Protocol" tab to enter text based commands. The commands are given in Figure 6.
12. Click "Run" to run the perfusion program.
13. Acquire images using your standard microscope methods.

Software Operation

Valve on/off Buttons

Regulator Setpoints

Note: Flow X controls V1 and V2, Flow Y controls V3-V8

Status Indicators

Function Tabs

Program Status

Figure 6. ONIX FG Y4 Interface

Text Commands:

- setflow X n** ; where $n = 0.25-10$ (psi) ; sets flow rate on X (orange)
- setflow Y n** ; where $n = 0.25-10$ (psi) ; sets flow rate on Y (blue)
- open V1** ; V1, V2, ..., V8, all ; opens pneumatic valve
- close V1** ; V1, V2, ..., V8, all ; closes pneumatic valve
- wait n** ; n is minutes ; holds current condition until next step
- end** ; ends the program ; shuts off all valves and resets regulators
- %** ; Put at the beginning of line for comments

```

Cell Load Gradient Switch Protocol
1 % Example Program:
2 setflow Y 2
3 open V3
4 open V5
5 wait 10
6 setflow X 4.5
7 open V1
8 wait .5
9 close all
10 end
11
12
  
```