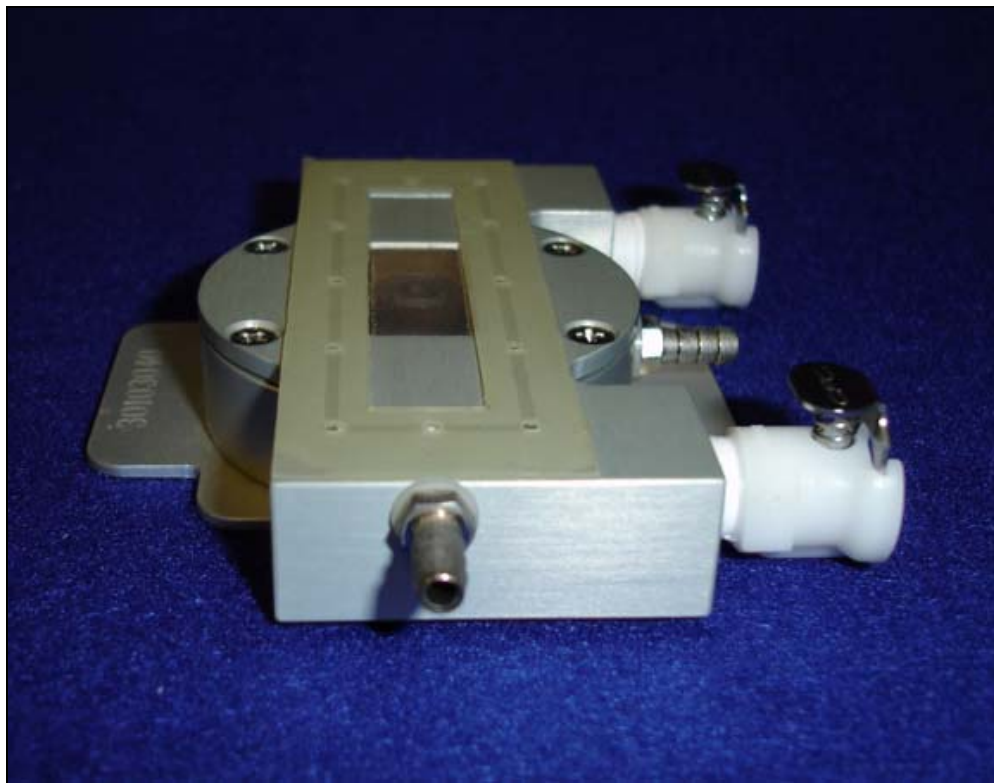




FlexFlow™ Manual

(for use with the FX-5000™ Tension System)



07-15-10

Rev: 1.1

Culturing Cells in a Mechanically Active Environment™

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1. FLEXFLOW™ SYSTEM COMPONENTS

Your FlexFlow™ system should have the following items:

1. MasterFlex® L/S™ model 7550-10 pump with Easy-Load® II pump head and power cord
2. MasterFlex® pump RS-232 interface cable
3. 25' of silicone MasterFlex® L/S™ 16 tubing
4. FlexFlow™ device with quick disconnects
5. Silicone lubricant
6. Microscope-adaptable FlexFlow™ base
7. Two pulse dampeners with quick disconnects
8. FlexFlow™ bypass connector
9. FX-5000™ Tension System adapter
10. Miscellaneous fittings packet
11. Male quick disconnects packet
12. Large tubing clamps packet
13. Small tubing clamps packet
14. Front and side FlexFlow™ port quick disconnects packet
15. Fluid collection tray
16. Vacuum tubing packet (10' clear vinyl, 10' blue polyethylene)
17. White PVC inline volume additive
18. Six collagen-coated thin Culture Slips® (sterilized)
19. Six collagen-coated StageFlexer® membranes (sterilized)
20. Three untreated thin Culture Slips® (not sterilized)
21. Three untreated StageFlexer® membranes (not sterilized)
22. One cell culture medium bottle with quick disconnects
23. One vacuum bottle with quick disconnects
24. FlexFlow™ manual
25. StreamSoft™ software and manual
26. Culture Slip® Placement and Gasket Grease Application Video CD

Check your system to be sure that everything above is included. If not, please contact Flexcell International.

2. APPLYING FLUID SHEAR STRESS TO CELLS: BACKGROUND

It is well accepted that cells in the vascular system, particularly endothelial and smooth muscle cells are subjected to fluid shear as blood courses through an artery or vein. In atherosclerosis or post-angioplasty, the endothelial cell lining can be compromised, exposing the underlying smooth muscle cells to fluid flow. However, it is now appreciated that all cells are subjected to shear stress when tensile or compressive forces are placed on tissues causing fluid movement and associated fluid-induced shear stress. Osteoblasts, chondrocytes, ligament and tendon cells, bladder epithelial cells and every other cell are exposed to fluid shear to varying degrees. Cells respond

to fluid-induced shear stress by opening or closing ion channels, signaling through second messenger pathways, activating transcription factors, altering gene expression, polymerizing actin stress cables, redistributing focal adhesions and altering cell shape.

Investigators in the field have utilized roller bottle apparatus, rocking culture plates, pumps and flow loops with in-line samples, cone and plate viscometers, parallel plate flow devices and microinjectors that pass fluid over cells to create fluid-induced shear stresses on cells. However, a commercial system that can provide reproducible fluid-



induced shear stress has not been available until now. Flexcell® International Corp. offers a fluid-induced shear stress system

with the capabilities to test your cells for shear stress responses.

3. THE FLEXCELL® FLEXFLOW™

The FlexFlow™ is a parallel plate laminar flow device designed to apply fluid shear stress and/or cyclic strain to cells in culture while providing a means for viewing cell activity under a microscope in real time. The FlexFlow™ adapts to the StageFlexer®, a device used to apply cyclic strain to cells while viewing under a microscope.

the area of the membrane covering the flex post. This area is 0.25 cm². If you are doing flow only and wish to grow your cells on a Culture Slip®, the usable flow area increases to 7.0 cm². This flow area assumes that no cells are cultured within 0.10" of the fluid entrance port on the left or right side of the device (depending on the flow direction).

Cells can be grown either on the surface of a silicone StageFlexer® membrane or a thin or standard Culture Slip®. When culturing cells on the StageFlexer® membrane, the available culture area for flow alone is 2.85 cm² (total exposed area of the membrane). When subjecting the cells to cyclic strain on this membrane, cell growth area is limited to

Cells can be viewed on a standard microscope with a light source illuminating the cells from above or below. The FlexFlow™ operates with a peristaltic pump and/or the Flexcell® FX-5000™ Tension System to flow and/or stretch cells under specific regimens as they are viewed under the microscope.

4. FLEXFLOW™ ASSEMBLY

Initial assembly of the FlexFlow™ involves eight parts (Fig. 1):

1. StageFlexer® body
2. Yellow gasket
3. Stretch/flow or flow-only post
4. Silicone membrane
5. O-ring
6. FlexFlow™ body
7. Four screws
8. Culture Slip®

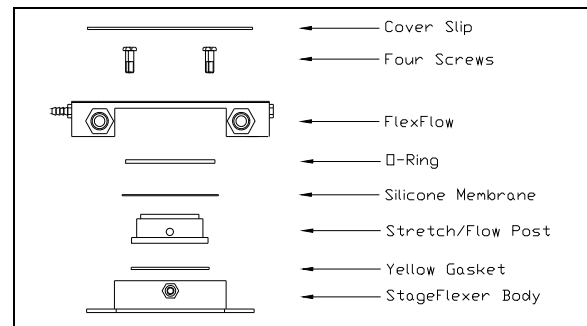


Figure 1. FlexFlow™ assembly order.

To assemble the unit, first apply silicone grease to the bottom inside surface of the StageFlexer® body. Place the yellow gasket into the StageFlexer® body. Apply another layer of silicone grease to the top surface of the yellow gasket. Place the stretch/flow or flow-only post on top of the gasket and rotate the post so that one of its long sides is



perpendicular to the fitting on the front of the StageFlexer®. If you are planning to stretch your cells, use the stretch/flow post and apply a thin layer of silicone grease (provided with the system) to the entire top of the post, especially the circular center.

NOTE: *The stretch/flow post has an annulus at its center. This post should be used only when you are stretching with or without flow. If you are doing flow only, always use the flow-only post, the post with a flat surface and four miniature vacuum holes.*

The next step in assembly is dependent on whether you are culturing cells on the silicone membrane or a Culture Slip®. You should not attempt the following steps until you have fully assembled the flow system (see section 5) and are prepared to use it according to the instructions. Therefore, it is best to read the following two sections first, then proceed with them once you have completed reading the remainder of the manual.

4.1 CULTURING CELLS ON THE SILICONE MEMBRANE

After cells have been cultured on the silicone membrane, carefully place the membrane over the top of the stretch/flow or flow-only post, as centered as possible with respect to the post and StageFlexer® body. Be sure that your cells are facing upward and that the culture area is centered over the flow area on the post. If you are stretching, be sure that the cells are centered over the cylindrical flex post at the center of the stretch/flow post. Place the O-ring over the outer edge of the silicone membrane such that it will seat in the groove around the top surface of the StageFlexer® body once compressed.

Carefully place the FlexFlow™ body onto the silicone membrane, post and

StageFlexer® body. Line the rectangular shape of the post up with the rectangular hole in the FlexFlow™ and press the FlexFlow™ onto the post to clamp the membrane in place. The O-ring should press into the groove around the top surface of the StageFlexer® body, thereby clamping and sealing the membrane. Place the screws into the four holes in the FlexFlow™ and tighten until the membrane is fully clamped and flush with the top surface of the FlexFlow™. Be sure that the O-ring in the StageFlexer® is compressed between the StageFlexer® top and the FlexFlow™ bottom surface. When compressed correctly, there should be a very small gap between the FlexFlow™ and StageFlexer®.

4.2 CULTURING CELLS ON A CULTURE SLIP®

If you choose to culture your cells on a Culture Slip® instead of the membrane, use a blank membrane (a membrane without cells) in the device. Be sure to remove the plastic film backing from the membrane before using it with the device. This membrane can be reused as you do experiments with different Culture Slips®. Read section 7.3 for instructions on how to place the Culture Slip® onto the device.

When culturing cells on the Culture Slip®, there are some important things to consider. First, the area in which cells should be grown on the Culture Slip® is limited because part of the Culture Slip® will sit on the gasket while the remainder is exposed to flow. When plating, the available width for plating cells on the Culture Slip® is 1.4 cm. That is, 0.7 cm from each side of the center point in the width direction. It is helpful to lay the Culture Slip® centered on top of the FlexFlow™ before plating and mark with a felt pen or sharp point marker where the gasket boundaries are on the width and fluid



entrance boundaries (or Lexan post boundaries if you are illuminating from the bottom) of the device. Given these boundaries, you can better plate your cells and more precisely place the Culture Slip® onto the device once you begin the experiment. Once cells are plated as desired,

it is also helpful to mark off the boundaries within which the cells are growing, especially if you are doing spot cultures. These markings will help you find the cells more easily when viewing through the microscope.

5. SETTING UP THE FLOW SYSTEM

The flow system consists of eight major components:

1. Peristaltic pump (software controllable)
2. Tubing and quick disconnects
3. Two fluid pulse dampeners
4. FlexFlow™
5. Bypass connector
6. Cell culture medium bottle
7. Vacuum bottle

The setup is shown in Figure 2. The lengths of tubing between each component should be kept as short as possible and cut according to the most convenient setup for your lab.

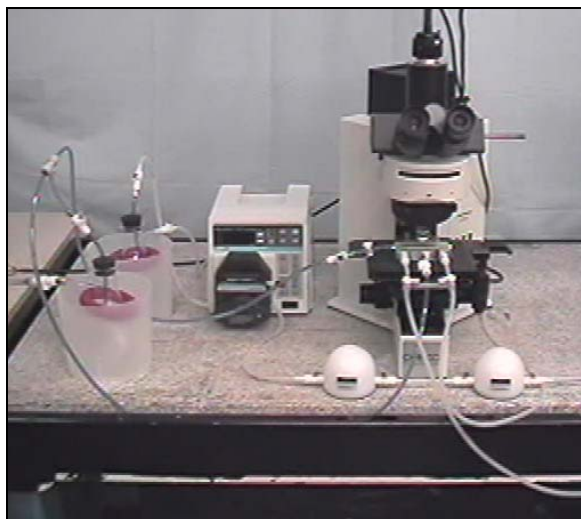


Figure 2. Flow system setup (from left to right): vacuum bottle in plastic container, cell culture medium bottle in plastic container, peristaltic pump, fluid pulse dampener 1, FlexFlow™ on microscope stage, and fluid pulse dampener 2.

To set up the system, first place all components in the position that you desire them to be when the system is complete. This prior positioning will help you determine how long each piece of tubing should be. Place both bottles in a safe place such that they will not be prone to tip over. ***The bottle with the two smaller female quick disconnects and an open hole in one of the ports is used for the fluid medium. The bottle with the two larger quick disconnects and no open ports holes in the top is the vacuum bottle.***

NOTE: *In the following sections, for all silicone tubing connections to barbed fittings, use the small white tubing clamps provided with the system.*

Determine how much tubing will be needed to go from the outlet quick disconnect fitting at the top of the fluid medium bottle (this is the fitting which is attached to the long, straight piece of tubing in the bottle), through the pump head, and to the fitting on the first fluid pulse dampener (can be either of the two). Cut this amount of tubing. Connect one end of this tubing to the barbed end of one of the quick disconnects in the packet labeled *Male Quick Disconnects (used throughout system)*. Connect this male quick disconnect to the outlet quick disconnect on the top of the fluid medium container. Place the tubing into the pump head so that it is in position to be clamped. Connect the other free end of the tubing to



the barbed end of another male quick disconnect as before, and connect this quick disconnect to the first pulse dampener. Cut a very short length of tubing, about 3-4", put a male quick disconnect on each end, and connect the two pulse dampeners together with this tubing.

Cut another length of tubing long enough to reach from the free end on the second fluid pulse dampener to the FlexFlow™. Cut this tubing. Add a male quick disconnect to one end and connect this end to the second fluid pulse dampener. Leave the remaining end of the tubing free.

Cut another length of tubing that will reach from the inlet quick disconnect (this is the fitting which is attached to the short, bent piece of tubing in the bottle) of the cell culture medium bottle to the FlexFlow™. Add a male quick disconnect to one end and leave the other end free.

Locate the bypass connector included with the system (Fig. 3). Connect the two ends of the silicone tubing coming from the stopcocks to the two ports on the FlexFlow™ such that the stopcock valve levers are facing upward. Connect the open ends of the stopcocks to the ends of the tubing coming from the cell culture medium container and the tubing coming from the second pulse dampener. The tubing coming from the second pulse dampener should be connected to the port that you desire to be the flow inlet and the tubing from the cell culture medium container should be connected to the port that you desire to be the flow outlet.

Connect one end of the blue 1/4" O.D. tubing to the barbed fitting at the vacuum port on the side of the FlexFlow™. Connect the other end to the smaller 1/4" quick disconnect on top of the vacuum bottle. Cut this length of tubing about 3-4" away from the FlexFlow™

device and put a pair of quick disconnects in line. Cut a length of the clear 3/8" O.D. vinyl tubing to reach from your vacuum source to the larger 3/8" quick disconnect on top of the vacuum bottle. Connect one end to your vacuum source and the other end to the quick disconnect on the vacuum bottle.

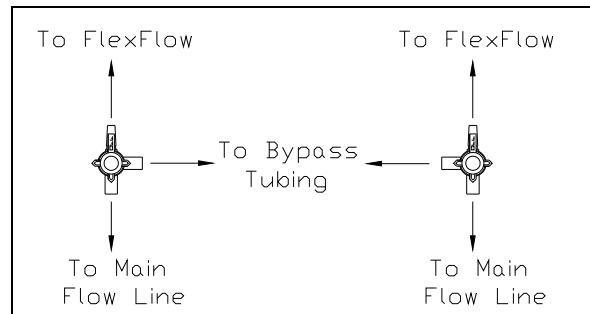


Figure 3. Stopcock setup on the bypass connector.

NOTE: To assure a proper seal on the FlexFlow™, your vacuum source should be capable of pulling a minimum of -75 kPa static vacuum.

5.1 FLOW ONLY

If you plan to flow only (i.e., no stretching), you will need to apply vacuum to the front port of the FlexFlow™ as well as the side. Cut another piece of the blue 1/4" O.D. tubing long enough to reach from the front of the FlexFlow™ to a point near the vacuum bottle on the blue vacuum line that you just connected. Cut the blue vacuum line that you just connected near the flask. Using the grey plastic "T" fitting provided with the system, connect the two cut pieces of the vacuum line that you just cut. Connect another piece of tubing from the remaining end of the "T" fitting to the barbed fitting on the quick disconnect at the front of the FlexFlow™. This connection will provide a static vacuum to both the flow post and Culture Slip®. A grey two-way fitting is supplied with the system to use when you no longer need to supply static vacuum to the front port of the FlexFlow™.



5.2 FLEX AND FLOW

If you plan to flex and flow your cells, the barbed quick disconnect fitting at the front of the FlexFlow™ will need to be connected to the FX-5000™ Tension System "Flex In" and "Flex Out" ports. To make this connection, use the StageFlexer®→FX-5000™ adapter. This adapter has a large "T" connector with a piece of blue and clear tubing, both with quick disconnects on the ends, and a longer piece of blue tubing with

a quick disconnect on the end. Connect the quick disconnect on the longer piece of blue tubing to the quick disconnect on the front of the FlexFlow™. Connect the shorter pieces of blue and clear tubing to quick disconnects connecting to the "Flex In" and "Flex Out" tubing on the FX-5000™. Cut the larger clear tubing on the StageFlexer®→FX-5000™ adapter at its midpoint, and add the white PVC pipe volume (included with the system) inline to help stabilize the FX-5000™ with sufficient air volume.

6. ASSEMBLING THE FLEXFLOW™ PRIOR TO EXPERIMENTS

Assemble the FlexFlow™ as described in section 4, "FlexFlow™ Assembly." If you plan to do flow only (no stretching) at this point, use the flow post with a flat top. If you plan to flex and flow, use the post with the circular annulus at the center (stretch/flow post). This open area is used to apply vacuum that will stretch the membrane. *Before placing the membrane onto this post, apply a thin layer of silicone grease (provided with the system) to the entire top of the post, especially the circular center.*

If you are culturing cells on the silicone membrane, carefully use this membrane in the assembly of the FlexFlow™. Otherwise use a blank membrane without cells. If this is your first time using the system, use the blank membrane included with the system to practice before actually starting your experiment.

Once the membrane is intact and the FlexFlow™ is screwed into clamping position, check all fluid and vacuum connections to be sure that they are secure. The entire system should now be in place except for the coverslip on the FlexFlow™.

7. STARTING YOUR EXPERIMENT

7.1 FIRST TIME USERS

On your first trial, you will want to use water to determine exactly how much fluid will be required to fill the entire system. Once this volume is determined you can use the exact amount of cell culture medium. To run this initial test, fill the cell culture medium bottle with 400 mL of water. Run the fluid through the system (described in

the following section). Once the fluid has fully circulated through the system, check the remaining fluid in the bottle and subtract this amount from 400 mL to determine your needed volume. Add some additional fluid to this number, about 75 to 100 mL. This excess media in the bottle will prevent air bubbles from entering the system.



7.2 ALL USERS

If you have used your system before and know the exact amount of cell culture medium needed, add this amount to the cell culture medium flask.

Before placing cells in the device, cell culture medium should be pumped through the system to remove all air bubbles. Air bubbles may damage cells by shearing them off the membrane or Culture Slip®. The following procedure must be followed to insure that all air bubbles are removed from the system.

Turn the “off” lever arm of each stopcock on the bypass connector in the direction of the FlexFlow™ so that pumped medium will bypass the FlexFlow™ and go through the tubing between the stopcocks. Set the direction of the motor on the peristaltic pump to provide flow immediately toward the pulse dampeners. Start the peristaltic pump at about 100 mL/min. Allow fluid to pump through the entire system (except for the FlexFlow™). Once the system is full, tilt the pulse dampeners, one at a time, at an angle of approximately 20 degrees, such that the direction of the flow is going from the vertex of the angle to the open end of the angle. Leave the pulse dampener in this position until the fluid comes through the outlet fitting again, then lay the pulse dampener down horizontally. This process will allow the pulse dampener to fill to a level slightly higher than the fittings, thereby creating a bubble trap for any air bubbles that may accidentally enter the system. Do the same with the second pulse dampener. Once this process is complete and the system is full of fluid, shake the tubing throughout the system as necessary to remove all air bubbles (leave the pump running). You may notice air bubbles trapped in the tubing at various high points. Air bubbles are usually trapped in two ways:

1. In corners or on side surfaces
2. On upper surfaces (always float upward)

Two ways to move air bubbles through the system:

1. Shake the tubing where the bubbles are trapped
2. Lift the tubing or system component upward such that the flow direction is upward, and the bubbles float to the top.

Air bubbles will generally flow out of the system, but to be safe you will want to work through the system from beginning to end to be sure that all are removed.

7.3 ADDING THE CULTURE SLIP®

The following instructions can be seen on video. This video can be found on the CD that is included with your system. It is recommended that this file be viewed before and after reading these instructions for clarification.

At this point your pump should be moving fluid through the system with no air bubbles. The bypass connector should be closed off to the FlexFlow™. The FlexFlow™ itself should be fully assembled apart from the Culture Slip®. All tubing connections to the FlexFlow™ should be intact. Before proceeding, place the device onto the fluid tray provided with the system. This tray is used to collect any fluid spills.

First, apply a thin layer of silicone grease to the gasket on the top of your FlexFlow™. Be careful not to fill any of the vacuum holes with grease. This layer will help to seal between the Culture Slip® and gasket during flow.

NOTE: The following series of steps should be performed several times before the actual



experiment. The technique may take some practice to perfect and should not be tried with cells for the first time.

Turn on your vacuum source to the vacuum bottle. Then, open the stopcock attached to the fitting on the side of the FlexFlow™. Be sure that the stopcock is only open for airflow from the vacuum source to the FlexFlow™ (i.e., none leaking to the atmosphere). There should be an air sound coming from the holes in the gasket on top of the device. Next, turn the peristaltic pump speed down to its lowest setting. Be sure that the FlexFlow™ is sitting flat on the surface of fluid collection tray. Simultaneously turn the two stopcocks on the bypass connector such that the "off" position of each is facing the other. That is, each should be aligned with the bypass line on the bypass connector.

At this point, fluid will begin to flow into the FlexFlow™ chamber(s) and eventually come out of the top and fill the gasket area. Some fluid may be pulled through the gasket holes on top of the device, so be sure that your vacuum bottle is intact. Once the volume contained by the inner gasket width, length, and height is full of fluid, stop the pump. You may have to use a Culture Slip® to move the fluid so that the pool inside of the gasket is completely full with no air contained. The fluid pool should be like a bubble over the edges of the gasket due to surface tension.

Next, take the Culture Slip® that you are culturing/practicing with, and place one of its lengthwise sharp edges across one side of the gasket such that if you dropped the slip from that point, it would land on the gasket centered over the flow area and covering all vacuum holes (you may want to practice placing the Culture Slip® over the gasket in this way before actually pumping the fluid into the gasket pool area).

Once the Culture Slip® is angled properly, carefully lay it down onto the gasket. This process will force the excess media out of the pool and into the vacuum lines while leaving nothing but media in the flow area. At this point, there should be no air bubbles present in the flow area or anywhere in the system previous to this point. The only remaining air in the system will be left at the outlet port of the FlexFlow™. This air will be pushed out as you start your experiment.

You are now ready to start shearing and/or flexing your cells. *If you are using the StreamSoft™ software, you do not need to refer to the remaining two paragraphs in this section. Simply refer to the StreamSoft™ manual for instructions on running the pump at your desired shear stresses.*

If you are not using the software but are controlling the pump directly, you will need to determine the flowrate needed to achieve the shear stress that you wish to apply to your cells. For this value, refer to the chart at the end of this manual entitled "FlexFlow™ Shear Stress Table." The values on this chart have been calculated **according to your particular FlexFlow™**. If you lose them, please contact Flexcell, and we will provide you with replacement values.

Be sure that your pump is set to the proper tubing size (16). Set the pump to the flowrate that you desire. Start the pump. After 10-15 seconds the system should be stabilized.

7.4 USING THE FLEXFLOW™ WITH THE FX-5000™ TENSION SYSTEM

To connect the FlexFlow™ to the FX-5000™ Tension System, use the StageFlexer®→FX-5000™ adapter supplied with the unit. This



adapter has a large "T" connector with a piece of blue and clear tubing, both with quick disconnects on the ends, and a longer piece of blue tubing on the other end, also with a quick disconnect. Connect the quick disconnect on the long end of the blue tubing to the quick disconnect on the front of the FlexFlow™. Connect the two quick disconnects on the other end of the adaptor to the "Flex In" and "Flex Out" tubing on the FX-5000™. Add the white PVC pipe volume inline with the larger clear tubing on the StageFlexer®→FX-5000™ adapter.

The FX-5000™ Tension System has the capability to regulate vacuum levels to various instruments to provide strain to elastic materials according to predetermined data. The parameters required to convert vacuum level to % elongation for the FlexFlow™ have been entered in the FX-5000™ under the platform name "FlexFlow." Simply choose "FlexFlow" as your platform when downloading a regimen, and the system will apply strain to the FlexFlow™ membrane as specified in your regimen.

8. VIEWING CELLS UNDER THE MICROSCOPE

Once fluid is flowing and the device is safely sealed, you can view your cells under shear stress. First remove the screws on your stage that hold the stage clips in place and remove the stage clips. Next, cover the stage and all lower parts with plastic wrap. Anything that could get wet due to a leak should be well covered before placing the FlexFlow™ onto the microscope stage. Once the microscope is fully covered and protected, screw the FlexFlow™ onto the stage by its base using the screw holes at the back of the large base for the FlexFlow™. The large base may need to be screwed onto the FlexFlow™ if the unit arrives with the smaller standard base attached. Tighten the stage screws until the FlexFlow™ is completely stable.

***NOTE:** If the vacuum line coming out of the left side of the FlexFlow™ is in your way, you can move it to the right side by switching the small closure bolt on the right with the barbed tubing fitting on the left. If you switch these fittings, be sure to tighten each firmly enough to compress the small gasket.*

Once your FlexFlow™ is firmly in place, you can begin viewing cells. Be careful not to apply too much pressure to the Culture Slip® with the objective. Because the Culture Slip® is a very thin piece of glass, it may easily break if too much pressure is applied. **Breaking the Culture Slip® will in turn cause medium to spill out of the device and onto your microscope.**

9. CLEANING THE FLEXFLOW™

When you finish using the FlexFlow™, the device and flow system should be cleaned by pumping fresh water through until all culture medium is removed. The salts in fluid culture medium will eventually corrode the aluminum from which the device is made, if not cleaned after each use. When

left to sit for a number of days with medium in the system, significant corrosion will begin to occur and eventually will damage the FlexFlow™ and other components in the system.



In addition to cleaning the system with the FlexFlow™ inline, the device itself should be disassembled from the StageFlexer® and cleaned internally if it is not going to be used for a period of two days or more.

If the device becomes corroded, soak it in diluted 80% phosphoric acid. This solution will begin to remove the corrosion as well as the aluminum. The device should not be left unattended while soaking. The prepared

solution should be 5% acid and 95% deionized water. Soak it for 20-30 minutes, remove and wash, and scrub with a scouring pad to remove the corrosion. Repeat the process as necessary.

Once you are finished using the system, remove the clamping pressure from the silicone tubing to increase the life of the tubing.



APPENDICES

Appendix 1: FlexFlow™ Conversion Chart



APPENDIX 1: FLEXFLOW™ CONVERSION CHART

<u>Press (-kPa)</u>	<u>% Elong</u>	<u>Strain</u>
0.00	0.0	0.000
3.59	0.1	0.001
7.05	0.2	0.002
10.41	0.3	0.003
13.64	0.4	0.004
16.77	0.5	0.005
19.80	0.6	0.006
22.72	0.7	0.007
25.54	0.8	0.008
28.27	0.9	0.009
30.91	1.0	0.010
33.46	1.1	0.011
35.92	1.2	0.012
38.31	1.3	0.013
40.62	1.4	0.014
42.86	1.5	0.015
45.02	1.6	0.016
47.12	1.7	0.017
49.16	1.8	0.018
51.14	1.9	0.019
53.06	2.0	0.020
54.93	2.1	0.021
56.76	2.2	0.022
58.54	2.3	0.023
60.27	2.4	0.024
61.97	2.5	0.025
63.64	2.6	0.026
65.28	2.7	0.027
66.89	2.8	0.028
68.48	2.9	0.029
70.04	3.0	0.030
71.60	3.1	0.031
73.14	3.2	0.032
74.67	3.3	0.033
76.19	3.4	0.034
77.72	3.5	0.035
79.25	3.6	0.036
80.78	3.7	0.037
82.32	3.8	0.038
83.87	3.9	0.039
85.45	4.0	0.040
87.04	4.1	0.041
88.65	4.2	0.042
90.29	4.3	0.043