# MICRO-INCUBATION SYSTEMS FOR MICROFLUIDIC CELL CULTURE

## CROSS REFERENCE TO RELATED APPLICATIONS

**[0001]** This application is a divisional application of Singapore Patent Application No. 11201402558Q "MICRO-INCUBATION SYSTEMS FOR MICROFLUIDIC CELL CULTURE" filed on 03.12.2012

**[0002]** This application incorporates each of the following provisional patent applications by reference:

61/367,371 filed Jul. 23, 2010

61/297,278 filed Jan. 21, 2010

61/471,103 filed Apr. 4, 2011

**[0003]** This application is related to material discussed in one or more of the following applications, each of which are incorporated herein by reference for all purposes: provisional patent application 61/037,297 filed Mar. 17, 2008, provisional patent application 61/018,882 filed Jan. 3, 2008, U.S. application Ser. No. 11/994,997, filed Aug. 11, 2008, which is a National Stage Entry of PCT/US06/26364, filed Jul. 6, 2006 and which claims priority from provisional patent application 60/773,467 filed Feb. 14, 2006 and from provisional patent application 60/697,449 filed 7 Jul. 2005, U.S. application Ser. No. 12/019,857, filed Jan. 25, 2008, which claims priority to U.S. Provisional Patent Application No. 60/900,651 filed on Feb. 8, 2007, U.S. application Ser. No. 11/648207, filed Dec. 29, 2006, which claims priority to U.S. Provisional Patent Application U.S. provisional patent application No. 60/756,399 filed on Jan. 4, 2006, U.S. application Ser. No. 12/348907, filed Jan. 5, 2009.

## COPYRIGHT NOTICE

**[0004]** Pursuant to 37 C.F.R. 1.71(e), applicants note that a portion of this disclosure contains material that is subject to copyright protection (such as, but not limited to, diagrams, device photographs, or any other aspects of this submission for which copyright protection is or may be available in any jurisdiction.). The copyright owner has no objection to the facsimile reproduction by anyone of the patent document or patent disclosure, as it appears in the Patent and Trademark Office patent file or records, but otherwise reserves all copyright rights whatsoever.

## FIELD OF THE INVENTION

**[0005]** The invention in various embodiments relates to assays, systems, and/or devices for culturing cells or other biologic material in controlled environments and that are applicable to related fields generally using microfluidic systems. Particular embodiments involve configurations that can be used with various standard automated handling systems, with active or passive loading and perfusion of medium and to provide high-throughput multi-assay automated systems for culturing, viewing, and analyzing cell growth, invasion, movement, chemotaxis or other properties. More specifically, the invention relates in specific embodiments to heat control systems for microfluidic culture plates and other automated systems for culture plates.

## BACKGROUND OF THE INVENTION

**[0006]** The discussion of any work, publications, sales, or activity anywhere in this submission, including in any documents submitted with this application, shall not be taken as an admission that any such work constitutes prior art. The discussion of any activity, work, or publication

herein is not an admission that such activity, work, or publication existed or was known in any particular jurisdiction.

**[0007]** Microfluidic cell culture is an important technology for applications in drug screening, tissue culturing, toxicity screening, and biologic research and can provide improved biological function, higher-quality cell-based data, reduced reagent consumption, and lower cost. High quality molecular and cellular sample preparations are important for various clinical, research, and other applications. In vitro samples that closely represent their in vivo characteristics can potentially benefit a wide range of molecular and cellular applications. Handling, characterization, culturing, and visualization of cells or other biologically or chemically active materials (such as beads coated with various biological molecules) has become increasingly valued in the fields of drug discovery, disease diagnoses and analysis, and a variety of other therapeutic and experimental work.

**[0008]** Numerous aspects related to microfluidic systems, devices, methods and manufacturing are discussed in the above-referenced and related patent applications. While no particular limitations should be read form those applications into any claims presented herein, these incorporated documents provide useful background material related to specific embodiments.

**[0009]** Other publications and/or patent documents that discuss various strategies related to cell culture using microfluidic systems and related activities include the following U.S. patent applications and non-patent literature, which, along with all citations therein, are incorporated herein by reference for all purposes. A listing of these

references here does not indicate the references constitute prior art.

**[0010]** Cytoplex, Inc. U.S. Pat. No. 6,653,124 "Array-based microenvironment for cell culturing, cell monitoring and drug-target validation."

**[0011]** Cellomics, Inc. U.S. Pat. No. 6,548,263 "Miniaturized cell array methods and apparatus for cell-based screening."

**[0012]** Fluidigm, Inc. Published Application 20040229349 (Nov 18, 2004) "Microfluidic particle-analysis systems."

**[0013]** Earlier work and patent applications as cited above, involving at least one of the present inventors, discuss various configurations, methods, and systems related to microfluidic cell culture and that work and those publications are is incorporated herein by reference.

## SUMMARY

**[0014]** The present invention involves various components, systems, and methods related to improved microfluidic cell culture devices and systems, in particular systems for the culturing and/or analysis and/or viewing of cells under controlled temperature and gas parameters. In one aspect, the invention involves novel microfluidic cell culture devices, systems and methods that have advantages over previously proposed culture systems providing in some embodiments, more convenient and unobtrusive heating and gas control and also providing automatic handling. In another aspect, the invention involves novel structures and methods for integrating heating and gas control with multiple microfluidic cell culture units in various multi cell culture unit systems, such as to a microtiter well plate structure including various standard well plate formats (e.g., a 96-well SBS culture plate, or other plate formats, including plates having 6, 12, 24, 96, 384 or 1536 sample

wells, as well as open bottom standard well plates, as well as plates with specific areas for cell culture and/or viewing.

**Removable Multi-Chamber Plate Manifold with Gas and/or Temperature Control**

**[0015]** In particular embodiments and examples, design features include providing a cell culture device in a convenient format that allows for the elimination of tubing and connectors to the plates themselves and provides temperature control and monitoring mostly or entirely contained in a removable manifold that fits onto the plates with a gas seal, thereby providing the ability to maintain long-term cell culture with temperature and/or gas control in a culture platform that maintains the ability to easily observe cells and that is easily removable from the culture plate. A system of the invention can be used with a variety of cell culture units on culture plates, such as those described in the above referenced patent applications, including cell culture units for determining cellular invasion, culture units with gel culture media, and a variety of other culture units as described herein or in incorporated related applications.

**[0016]** Living cells generally require careful control of their physical environment, including nutrient and gas exchange, temperature regulation, and protection from stress. Advanced micro-scale cell culture methods such as described in above referenced patent applications enable structural control (microfabrication), and perfusion control (microfluidics).

**Micro-Incubation, And Micro-Incubator Chamber/Well in Contact Microfluidic Cell Culture Chambers**

**[0017]** According to specific embodiments, the present invention is directed to an additional area of this field, referred to at times herein as micro-incubation, to provide control of temperature and gas atmosphere for use in micro-scale cell culture systems in a way that is unobtrusive to observational equipment and that further is easily attached and removed from a culture plate in specific embodiments.

**[0018]** According to specific embodiments, the invention provides a miniaturization of the traditional cell culture incubator concept to perform dynamic, continuous temperature and gas regulation directly to a microfluidic chamber. In example implementations, this is possible through the creation of a micro-incubator chamber of about 5 mL volume in contact with the microfluidic cell culture chambers (1 ul). The temperature and gas content of the micro-incubator quickly transfers to the cell culture chamber (by conduction and diffusion). In specific embodiments, the micro-incubator is maintained by a novel manifold design as described herein. Manifolds according to specific embodiments of the invention can be controlled by various systems and software.

**System for Time-Lapse Microscopy of Living Cells**

**[0019]** Many products and methods enable time-lapse microscopy of living cells. Three approaches that are commonly known can generally be understood as: (1) full microscope enclosures, (2) stage-top incubators, and (3) perfusion chambers.

**[0020]** Full microscope enclosures surround the entire microscope except for some heat generating or heat sensitive components such as the camera and illumination sources. The air within the enclosure is circulated and maintained at the

desired temperature and gas environment of the sample. An advantage of this method is that temperature control of the whole microscope greatly reduces focus drifts due to room temperature fluctuations, but numerous drawbacks include expensive and customized construction, obstruction of access to microscope, and high consumption of energy and gases. Also, exposure to humidity and repeated temperature changes may damage microscopes.

**[0021]** Stagetop incubators surround only a small volume intended to house one or more Petri dishes, slides or other culture platform. These provide local temperature regulation and enable limited gas environment control. They are convenient, but do not provide the same level of control as a microscope enclosure, as the stagetop incubator must mimic the mechanics of an enclosure, but in a smaller size and adapt to the microscope. For example, uniform temperature control in a stagetop incubator is limited by the heat sink of the stage itself, and cutouts to provide for the light path for optical clarity further reduce uniformity. In addition, the complexity of the design of stagetop incubators make them expensive.

**[0022]** Perfusion chambers generally consist of an assembly that encloses a flowing liquid volume, with heating elements either directly through the walls of the chamber or immediately upstream of the inlet flow path. The design of the control elements need to be carefully considered, as issues such as heat/mass transfer may make proper maintenance of a steady state condition difficult. At present, flow chambers are infrequently used in live cell imaging due to the myriad difficulties of adapting them for typical uses.

**[0023]** The present invention, by contrast, integrates temperature, flow, and gas control directly to a

microfluidic culture plate via the use of a manifold that seals to the microfluidic plate. While manifolds similar in some aspects are discussed in some of the above referenced applications to control perfusion on microfluidic plates, the previous designs did not incorporate all of the features described herein as such incorporation was difficult due to the compact nature of the manifold and culture plate. The present manifold design includes novel temperature or gas "microincubator" compartments created by operation of the manifold. These compartments have been demonstrated to provide novel and critical advantages for the proper integration for live cell imaging needs.

**[0024]** The present invention enables direct cell culture on a microscope stage without the use of external environment chambers such as enclosures or stage-top heaters. The "microincubator" concept according to specific embodiments of the present invention provides precise control, long-term cell culture, and ease of dynamic changes of conditions that has not been available in this context in other designs.

**[0025]** While many of the examples discussed in detail herein are designed to be used in conjunction with a standard or custom well plate, according to specific embodiments the microfluidic structures and culture units and systems and methods of various configurations as described herein can also be deployed independently of any well-plate, such as in various integrated lab-on-a-chip systems that are not configured to be used in conjunction with well plates or various other microfluidic devices or systems.

**[0026]** For purposes of clarity, this discussion refers to devices, methods, and concepts in terms of specific examples. However, the invention and aspects thereof may have applications to a variety of types of devices and systems.

It is therefore intended that the invention not be limited except as provided in the attached claims and equivalents.

**[0027]** Furthermore, it is well known in the art that systems and methods such as described herein can include a variety of different components and different functions in a modular fashion. Different embodiments of the invention can include different mixtures of elements and functions and may group various functions as parts of various elements. For purposes of clarity, the invention is described in terms of systems that include many different innovative components and innovative combinations of innovative components and known components. No inference should be taken to limit the invention to combinations containing all of the innovative components listed in any illustrative embodiment in this specification. Unless specifically stated otherwise herein, any combination of elements described herein should be understood to include every sub-combination of any subset of those elements and also any sub-combination of any subset of those elements combined with any other element described herein or as would be understood to a practitioner of skill in the art.

**[0028]** In some of the drawings and detailed descriptions below, the present invention is described in terms of the important independent embodiments of multi-component devices or systems. This should not be taken to limit various novel aspects of the invention, which, using the teachings provided herein, can be applied to a number of other situations. In some of the drawings and descriptions below, the present invention is described in terms of a number of specific example embodiments including specific parameters related to dimensions of structures, pressures or volumes of liquids, temperatures, electrical values, durations of time, and the like. Except where so provided in the attached

claims, these parameters are provided as examples and do not limit the invention, which encompasses other devices or systems with different dimensions. For purposes of providing a more illuminating description, particular known fabrication steps, cell handling steps, reagents, chemical or mechanical process, and other known components that may be included to make a system or manufacture a device according to specific embodiments of the invention are given as examples. It will be understood to those of skill in the art that except were specifically noted herein otherwise, various known substitutions can be made in the processes described herein.

**[0029]** All references, publications, patents, and patent applications cited in this submission are hereby incorporated by reference in their entirety for all purposes.

## BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows a side view of an example micro-incubator manifold according to specific embodiments of the invention shown in place in a micro-incubator system with a well plate and microscope viewer.

FIG. 2 illustrates one example of a top plane view of a manifold with a heat controller according to specific embodiments of the invention.

FIG. 3 illustrates one example of a topside view of a manifold with a heat controller sealed to a well-plate and mounted in a microscope viewer according to specific embodiments of the invention.

FIG. 4 illustrates one example of a culture plate with four culture units placed onto a 96-well standard SBS plate. This example shows four culture units (corresponding to rows labeled A-D) with six inlet wells (labeled A1-D6), four microfluidic culture areas placed under the large viewing

oval, and two outlet wells (7-8). This is an example only and placement and designation of the various wells and components will vary with different implementations.

FIGS. 5A-D are schematic drawings of an example implementation of a manifold with a heat controller from various views according to specific embodiments of the invention.

FIG. 6A illustrates the bottom portion of a heat exchange module according to specific embodiments of the invention. The bottom portion attaches to the pneumatic portion of the manifold. FIG. 6B illustrates the top portion of a heat exchange module according to specific embodiments of the invention.

FIG. 7 is a schematic side view of the pneumatic portions of a manifold sealed to a culture plate and showing the gas in lines connecting to an environment control volume according to specific embodiments of the invention.

FIG. 8 is a schematic showing how a manifold interfaces to a microfluidic plate according to specific embodiments wherein a positive seal is created by applying a vacuum to the cavity surrounding all the wells. The heating unit is not shown in this figure.

FIG. 9 illustrates a manifold with additional gas line and a heated objective lens according to an earlier manifold design.

FIGS. 10A-C show a top view, side view, and plan view of a schematic of an example pneumatic manifold according to earlier designs. In this example, the eight tubing lines to the right are for compressed air, and each is configured to provide pressure to a column of cell inlet wells in a microfluidic array. The left-most line in the figure is for vacuum and connects to an outer vacuum ring around the

manifold. This basic manifold design is modified using the teachings herein to produce the heated manifold.

FIG. 11 illustrates an example microfluidic perfusion system (ONIX™), microincubator controller and manifold (MIC) according to specific embodiments of the invention.

FIG. 12 illustrates an example microfluidic perfusion system (ONIX™), microincubator controller and manifold (MIC) and computer control system according to specific embodiments of the invention.

FIG. 13 shows NIH-3T3 mouse fibroblasts cultured using the microincubator system according to specific embodiments of the invention at t=0 (left) and after 15 hours (right) showing cell growth and viability. When no temperature or $CO_2$ was controlled, the cells rapidly died within 2 hours.

FIGS. 14A-B illustrate one alternative of plate and culture unit design with an example culture unit filled with blue dye with the image taken from top according to specific embodiments of the invention.

FIG. 15 is a screenshot showing integration of the ONIX microfluidic perfusion system with an open-source microscopy application.

FIG. 16 illustrates a microincubation system integrated with a microscope system for cell analysis. The dimensions of the manifold in specific embodiments allow it to sit on a standard well plate stage, with a transparent optical path that does not interfere with light microscopy. This allows time-lapsed imaging of cells cultured in the micro-incubator.

FIG. 17 is a block diagram showing a representative example logic device in which various aspects of the present invention may be embodied.

FIG. 18A is a block diagram showing an automated piston driven system according to specific embodiments of the invention. FIG. 18B illustrates perspective views of the

automated piston driven system of FIG. 18A in the ejected and sealed states.

FIG. 19 (Table 1) illustrates an example of diseases, conditions, or states that can be evaluated or for which drugs or other therapies can be tested according to specific embodiments of the present invention.

FIGS. 20A-D are schematics of an example implementation of electronic control circuits for a manifold according to specific embodiments of the invention.

## DETAILED DESCRIPTION OF SPECIFIC EMBODIMENTS

### 1. OVERVIEW

*Definitions*

**[0030]** A "particle" refers to biological cells, such as mammalian or bacterial cells, viral particles, or liposomal or other particles that may be subject to assay in accordance with the invention. Such particles have minimum dimensions between about 50-100 nm, and may be as large as 20 microns or more. When used to describe a cell assay in accordance with the invention, the terms "particles" and "cells" may be used interchangeably.

**[0031]** A "microchannel" or "channel" or "flow channel" generally refers to a micron-scale channel used for fluidically connecting various components of systems and devices according to specific embodiments of the invention. A microchannel typically has a rectangular, e.g., square, or rounded cross-section, with side and depth dimensions in a preferred embodiment of between 10 and 500 microns, and 10 and 500 microns, respectively. Fluids flowing in the microchannels may exhibit microfluidic behavior. When used to refer to a microchannel within the microwell array device of the invention, the term "microchannel" and "channel" are used interchangeably. "Flow channel" generally denotes

channels designed for passage of media, reagents, or other fluids or gels and in some embodiments cells. "Culture channel" or "cell culture channel" generally denotes a portion of a cell culture structure that cells are designed to flow through and also remain during cell culture (though the cells may be localized into a particular culture area of the culture channel in some embodiments). "Air channel" generally denotes a roughly micron-scale channel used for allowing gases, such as air, oxygen enriched mixtures, etc., to pass in proximity to flow channels or culture areas. "Perfusion channel" is sometimes used to indicate a flow channel and any perfusion passages or structures that allow media to perfuse to the culture area.

**[0032]** A "perfusion barrier" refers to a combination of solid structures and perfusion passages that generally separate a flow channel from a cell culture area or chamber. The perfusion passages are generally smaller than the microchannel height and/or width (for example, on the order of 5-50% or on the order of about 10%) and are designed to keep cells, other culture items, and in some embodiments gels, from migrating into the flow channels, while allowing some fluidic flow that is generally of a much higher fluidic resistance than the fluid flow in the flow channels. In one example embodiment, the perfusion barrier has a perfusion passage that is 4 microns high and that otherwise runs most of the length of the microchannel. In other embodiments, a perfusion barrier has many perfusion passages that are about as high as the microfluidic channel, but about 4 microns wide.

**[0033]** A "microfluidics device" refers to a device having various station or wells connected by micron-scale microchannels in which fluids will exhibit microfluidic behavior in their flow through the channels.

**[0034]** A "microwell array" refers to an array of two or more microwells formed on a substrate.

**[0035]** A "device" is a term widely used in the art and encompasses a broad range of meaning. For example, at its most basic and least elaborated level, "device" may signify simply a substrate with features such as channels, chambers and ports. At increasing levels of elaboration, the "device" may further comprise a substrate enclosing said features, or other layers having microfluidic features that operate in concert or independently. At its most elaborated level, the "device" may comprise a fully functional substrate mated with an object that facilitates interaction between the external world and the microfluidic features of the substrate. Such an object may variously be termed a holder, enclosure, housing, or similar term, as discussed below. As used herein, the term "device" refers to any of these embodiments or levels of elaboration that the context may indicate.

**[0036]** Microfluidic systems provide a powerful tool to conduct biological experiments. Recently, elastomer-based microfluidics has especially gained popularity because of its optical transparency, gas permeability and simple fabrication methods. The present invention involves integrated microfluidics used for various culture and assay applications and systems for providing heating control and automating various handling of culture plates. Advantages of specific embodiments include use of a standard-sized microtiter plate format, tubing free plates, and easy and effective mating of plates with a manifold to provide gas recirculation and heating control.

**[0037]** According to further embodiments of the invention, as has been previously described, a novel cell loading system uses a pneumatic manifold and pneumatic pressure to place

cells in the micro culture area. With the addition of this cell loading system, microfluidic cell culture and analysis can be fully automated using other automated equipment that exists for handling standard titer plates. In the present invention, heating and gas circulation elements are incorporated into the manifold to provide a micro-incubator system.

**[0038]** In further embodiments, a gravity driven flow culture configuration utilizes the medium level difference between the inlet and outlet well as well as engineering the fluidic resistances to achieve the desirable flow rate in nL/min regime can be used to "passively" flow culture medium for long periods of time (e.g., up to 4 days) without the use of bulky external pumps or tubes in an environment such as an incubator to control temperature and then the heat controlled manifold, as provided herein, can be used for control of the cell culture during observation.

**[0039]** In some embodiments, a custom pneumatic flow controller can be attached to the gas and electric connectors in the manifold and thereby used to load the cells into the culture regions, to switch between different exposure solutions, and to control the temperature of the culture region. A digital software interface can be used to allow a user to program specific inputs (pulses, ramps, etc.) over time to expose the cells to complex functions during time-lapse imaging while maintaining or varying temperature and gas exposure as desired.

## 2. Microfluidic Culture System and Array

**[0040]** The applications referenced above discussed a variety of different cell culture configurations and fabrication techniques. Portions of the operation of the cell culture areas and materials are useful as background to the present

discussion. In some examples therein, one or more micro culture areas are connected to a medium or reagent channel via a grid of fluidic passages (or diffusion inlets or conduits), wherein the grid comprises a plurality of intersecting high fluidic resistance perfusion passages. In one discussed example, passages in the grid are about 1 to 4 μm in height, 25 to 50 μm in length and 5 to 10 μm in width, the grid allowing for more even diffusion between medium or reagent channels and the culture area and allowing for easier manufacturing and more even diffusion. The earlier application further discussed that the high fluidic resistance ratio between the microchamber and the perfusion/diffusion passages or grid (e.g., ratios in the range of about 10:1, 20:1 to 30:1) offers many advantages for cell culture such as: (1) size exclusion of cells; (2) localization of cells inside a microchamber; (3) promoting a uniform fluidic environment for cell growth; (4) ability to configure arrays of microchambers or culture areas; (4) ease of fabrication, and (5) manipulation of reagents without an extensive valve network. Examples were illustrated wherein a grid-like perfusion barrier can be much shorter than the culture area or can be near to or at the same height, according to specific embodiments of the invention and further wherein various configurations for culture devices were illustrated.

## 3 Pneumatic Manifold with Heat Control

**[0041]** As discussed above, one difficulty in a number of culture systems is how to control the heating and temperature of the culture area while allowing for observation of the cellular processes. Previous solutions have relied on heating sources applied to the well-plate,

for example, from the microscope viewer. (E.g., see FIG. 11), or containing the entire system in a controlled environment.

**[0042]** The present invention provides an improved culture system by placing all or nearly all heat and gas controls in or attached to a typically removable manifold that in preferred embodiments is configured to be operational will not interfering with observational equipment. The invention will be more easily understood with reference to the specific examples illustrated, though these examples are intended to be illustrative and not limiting. FIG. 1 shows a side view of an example micro-incubator manifold according to specific embodiments of the invention shown in place in a micro-incubator system with a well plate and microscope viewer. FIG. 2 illustrates one example of a top plane view of a manifold with a heat controller according to specific embodiments of the invention. FIG. 3 illustrates one example of a topside view of a manifold with a heat controller sealed to a well-plate and mounted in a microscope viewer according to specific embodiments of the invention. FIG. 4 illustrates one example of a culture plate with four culture units placed onto a 96-well standard SBS plate. This example shows four culture units (corresponding to rows labeled A-D) with six inlet wells (labeled A1-D6), four microfluidic culture areas placed under the large viewing oval, and two outlet wells (7-8).

**[0043]** Thus, according to specific embodiments, a MicroIncubator Manifold can interface to a variety of microfluidic plates and provides one or more of cell-loading, perfusion, temperature and gas environment control. A convective heat exchanger adds or removes heat to the gas mixture using a Peltier thermoelectric device. The cells are kept warm through conducted heat from the warm gas mixture and the desired gas concentration diffuses in the media

surrounding the cells through a gas permeable membrane on the microfluidic plate.

**[0044]** As can be seen from FIG. 1, when the manifold according to specific embodiments is in place over a culture plate, a sealed gas chamber is formed from the heat exchanger, under the manifold and into the area above the culture microfluidics. Gas introduced into the area above the microfluidics is circulated by a fan or other gas circulatory means in the heat exchanger, thereby providing control of both the gas environment and the temperature above the culture area using the manifold.

**[0045]** This design, according to specific embodiments of the invention, solves the problem of providing effective heating in a small space to deliver the controlled temperature to the cells themselves while also controlling the gas composition. According to specific embodiments of the invention, the microincubator manifold includes both a gas input and a recirculating fan to control the gas composition.

**[0046]** In the example implementation shown in FIG. 1, controlled and pressurized gases enter the manifold through the gas lines 5. The pneumatic portion of the manifold is shown for convenience in this example in two pieces, top piece 10a and bottom piece 10b, which comprises a gasket for tightly fitting to plate 20, the plate containing a number of wells 22. The pneumatic operation of the manifold and fitting to the well plate is as described in herein referenced patent applications. The manifold also includes heat exchange module 40, with internal heat exchange fins 42 and a transparent cover plate (e.g., glass or acrylic) 44. When the manifold is placed over plate 10, the open region above the culture areas is connected with the heating element to create a gas circulation region 30. To show the device in context, microscope and lighting elements 102 and

104 are illustrated as they would generally be used in operation. The well plate can be any standard or custom well plate as described herein. It will be understood from the teachings provided herein that different configurations of manifold 40 are constructed to accommodate different well plate designs.

**Recirculating on the Manifold.**

**[0047]** In this example design, the gas and heating controls and elements are entirely contained in the manifold and the manifold can mate with any number of differently configured micro-well plates, including microwell plates that have no specific modifications to allow them to receive a heat source.

**[0048]** According to specific embodiments, two fans are used in the heat exchange element one to circulate the sealed gas in the gas area and one to interact with the heat exchanger.

**[0049]** FIGS. 5A-D are schematic drawings of an example implementation of a manifold with a heat controller from various views according to specific embodiments of the invention. The pneumatic portions of the manifold operate similarly to previously disclosed designs. The heat exchange module is described in more detail below.

**Manifold Heat Exchange Module**

**[0050]** A heat exchange module in one example embodiment controls the temperature within the chamber by the cycling of air at desired temperature. In specific embodiments, temperature control is provided by a thermoelectric Peltier module, which are well known devices that convert an electric current into a temperature gradient and can also be used as a temperature controller that either heats or cools. While other heating sources can be used in a manifold of the

invention, a Peltier module is a presently preferred mechanism as it can be fully incorporated into the heat exchange module and the manifold.

**[0051]** A heat exchange module in an example implementation has three main outer pieces: (1) a metal top enclosure, (2) a plastic bottom enclosure and (3) a manifold with an oval cutout that allows air to flow into the cell culturing chamber of the plate and cycle back out.

**Plastic Bottom Enclosure**

**[0052]** FIG. 6A illustrates the bottom portion of a heat exchange module according to specific embodiments of the invention. The bottom portion attaches to the pneumatic portion of the manifold. FIG. 6B illustrates the top portion of a heat exchange module according to specific embodiments of the invention. In a specific embodiment, the plastic enclosure of the bottom portion is attached to the top of the manifold (or example by screws or glue or other means). The bottom of this enclosure has a cut out of two 2 mm deep flow paths. These paths merge into one over the imaging chamber. When they merge, the depth of the path rises to 3 mm. Around the outskirt of the flow path is an o-ring that prevents the exchange of air between the flow path and the environment. The size of the flow paths must be wide enough to allow a desirable amount flow to circulate into the cell culture chamber. This decreases the difference in temperature in the plate cell culturing region and the Heat Exchange Module (at the location of the temperature probe).

**[0053]** A vertical extrusion sits above the paths. It contains 3 chambers. Above one flow path is a heat sink and above the other sits a fan. The third chamber connects to another chamber in the metal top enclosure to make room for a PCB board that connects the wires of the Peltier module, a

small temperature probe, a relay, connections to the microincubator controller and 2 thermal cutoffs (one for each heat sink). The temperature probe is routed from the electronics chamber through a screw hole on the fan and into the top of its flow path to measure the temperature of air cycling through.

**[0054]** The plastic enclosure also has a cut out from the top that forms a frame for a piece of 2 mm glass. This piece of glass sits right above the cell culturing chamber in order to create optimal condition for microscope imaging without disrupting heating.

**Metal Top Enclosure**

**[0055]** The metal top enclosure (FIG. 6B) has fin extrusion features that allow it to act as a heat sink for the other side of the Peltier module. In addition, it optionally contains a chamber for a second fan to speed up the cooling process as well as room for a thermal cutoff that connects to the electronics chamber of the plastic bottom enclosure.

**[0056]** After the fan is placed inside the enclosure and the thermal cutoff attached to the smaller chamber, thermal grease is applied to the top of the Peltier module in order to attach it to the metal top enclosure. In a specific example, the bottom of the plastic enclosure is securely fastened to the top enclosure, for example using screws or glue.

**[0057]** When temperature in the cell culturing region has to be raised, the Peltier module heats up the bottom heat sink by cooling the top heat sink. The bottom fan blows hot air across the bottom heat sink into the flow path beneath it. The heated air enters the cell culturing chamber as cooler air is circulated back to the fan. When temperature in the culturing region has to be lowered, the Peltier module cools

the bottom heat sink by raising temperature on the top (up to ambient temperature).

**[0058]** FIG. 7 is a schematic side view of the pneumatic portions of a manifold sealed to a culture plate and showing the gas in lines connecting to an environment control volume according to specific embodiments of the invention.

**[0059]** FIG. 8 is a schematic showing how a manifold interfaces to a microfluidic plate according to specific embodiments wherein a positive seal is created by applying a vacuum to the cavity surrounding all the wells. The heating unit is not shown in this figure.

## 4. Earlier Pneumatic Manifold

**[0060]** While gravity or passive loading is effective for some microfluidic cell culture devices and desirable in some embodiments, a proprietary pneumatic manifold was previously described in the above referenced applications. This may be mated to the plate and pneumatic pressure is applied to the cell inlet area for cell loading and for culturing during invasion assays. FIGS. 10A-C show a top view, side view, and plan view of a schematic of an example pneumatic manifold according to earlier designs. In this example, the eight tubing lines to the right are for compressed air, and each is configured to provide pressure to a column of cell inlet wells in a microfluidic array. The left-most line in the figure is for vacuum and connects to an outer vacuum ring around the manifold. The manifold is placed on top of a standard well plate. A rubber gasket lies between the plate and manifold, with holes matching the manifold (not shown). The vacuum line creates a vacuum in the cavities between the wells, holding the plate and manifold together. Pressure is applied to the wells to drive liquid into the microfluidic channels (not shown). A typical pressure of 1 psi is used;

therefore the vacuum strength is sufficient to maintain an air-tight seal. In one example there are 9 tubing lines to the pressure controller: 8 lines are for compressed air and 1 line is for vacuum (leftmost). In specific example embodiments, each column is connected to a single pressure line. Columns above the cell imaging regions are skipped.

**[0061]** Pressurized cell loading has been found to be particularly effective in preparing cultures of aggregating cells (e.g., solid tumor, liver, muscle, etc.). Pressurized cell loading also allows structures with elongated culture regions to be effectively loaded. Use of a pressurized manifold for cell loading and passive flow for perfusion operations allows the invention to utilize a fairly simple two inlet design, without the need for additional inlet wells and/or valves as used in other designs.

**[0062]** While this manifold is effective for cell loading and some perfusion tasks, the manifold did not effectively provide for the recirculation of a gas over the culture area or for any heat control. As illustrated in the figure, heating was provided when necessary from the opposite side of the culture plate, for example form the vicinity of the microscope viewer.

**[0063]** The plate manifold optionally also included an additional "gas line" that is used to bathe the cells in the microfluidic device with a specified gas environment (for example, 5% $CO_2$). Other examples include oxygen and nitrogen control, but any gaseous mixture can be sent to the cells. The gas flows through the manifold into the sealed wells above the cell culture area and holes in the microfluidic device enable the gas to flow into specified microfluidic air channels, as described above. The gas permeable device layer (PDMS) allows the gas to diffuse into the culture medium prior to exposing the cells. By continuously flowing

the gas through the microfluidic plate, a stable gas environment is maintained. This provides an optional means for controlling the gas environment to placing the microfluidic plate into an incubator.

**[0064]** FIG. 12 illustrates an example microfluidic perfusion system (ONIX™), microincubator controller and manifold (MIC) and computer control system according to specific embodiments of the invention.

**[0065]** As just one example, FIGS. 14A-B illustrate one alternative of plate and culture unit design with an example culture unit filled with blue dye with the image taken from top according to specific embodiments of the invention. However, any culture units in any configuration of culture plates can be used with a correctly dimensioned manifold according to specific embodiments of the invention. This includes open top units, invasion units, liver mimetic units, gel units, etc. as described in above referenced applications.

**Cell Assay and/or Observation**

**[0066]** Cell assay can be performed directly on the microfluidic cell culture using standard optically based reagent kits (e.g. fluorescence, absorbance, luminescence, etc.). For example a cell viability assay utilizing conversion of a substrate to a fluorescent molecule by live cells has been demonstrated (CellTiter Blue reagent by Promega Corporation). The reagent is dispensed into the flow inlet reservoir and exposed to the cells via gravity perfusion over a period of time (e.g., 21 hours). For faster introduction of a reagent or other fluid, the new fluid can be added to the flow inlet reservoir followed by aspiration of the cell inlet reservoir.

**[0067]** Data can be collected directly on the cells/liquid in the microfluidic plate, such as placing the plate into a standard fluorescence plate reader (e.g., Biotek Instruments Synergy 2 model). In some reactions, the substrate may diffuse into the outlet medium, and therefore be easily detected in the cell inlet reservoir. For cell imaging assays, the plate can be placed on a scanning microscope or high content system. For example, an automated Olympus IX71 inverted microscope station can be used to capture viability of cultured liver cells with a 20× objective lens.

**[0068]** By repeatedly filling/aspirating the wells, cells can be maintained for long periods of time with minimal effort (e.g. compared to standard "bioreactors" which require extensive sterile preparation of large fluid reservoirs that cannot be easily swapped out during operation).

**Example Cell Culture**

**[0069]** Cells were cultured using the micro-incubation system to control temperature and gas atmosphere. In one example, human cancer cells (HT-1080, MCF-7, MDA-MB-231) were cultured at 37 C and 5% CO2 to monitor cell division over 24 hours. Additional cell types, including yeast, bacteria, primary cells, neurons, etc. have been successfully cultured using the microincubation system. As an example, FIG. 13 shows NIH-3T3 mouse fibroblasts cultured using the microincubator system according to specific embodiments of the invention at t=0 (left) and after 15 hours (right) showing cell growth and viability. When no temperature or CO2 was controlled, the cells rapidly died within 2 hours.

**Integrated Systems**

**[0070]** Integrated systems for the collection and analysis of cellular and other data as well as for the compilation,

storage and access of the databases of the invention, typically include a digital computer with software including an instruction set for sequence searching and/or analysis, and, optionally, one or more of high-throughput sample control software, image analysis software, collected data interpretation software, a robotic control armature for transferring solutions from a source to a destination (such as a detection device) operably linked to the digital computer, an input device (e.g., a computer keyboard) for entering subject data to the digital computer, or to control analysis operations or high throughput sample transfer by the robotic control armature. Optionally, the integrated system further comprises valves, concentration gradients, fluidic multiplexors and/or other microfluidic structures for interfacing to a microchamber as described.

**[0071]** Readily available computational hardware resources using standard operating systems can be employed and modified according to the teachings provided herein, e.g., a PC (Intel x86 or Pentium chip-compatible DOS,™ OS2,™ WINDOWS,™ WINDOWS NT,™ WINDOWS95,™ WINDOWS98,™ LINUX, or even Macintosh, Sun or PCs will suffice) for use in the integrated systems of the invention. Current art in software technology is adequate to allow implementation of the methods taught herein on a computer system. Thus, in specific embodiments, the present invention can comprise a set of logic instructions (either software, or hardware encoded instructions) for performing one or more of the methods as taught herein. For example, software for providing the data and/or statistical analysis can be constructed by one of skill using a standard programming language such as Visual Basic, Fortran, Basic, Java, or the like. Such software can also be constructed utilizing a

variety of statistical programming languages, toolkits, or libraries.

**[0072]** FIG. 7 shows an information appliance (or digital device) 700 that may be understood as a logical apparatus that can read instructions from media 717 and/or network port 719, which can optionally be connected to server 720 having fixed media 722. Apparatus 700 can thereafter use those instructions to direct server or client logic, as understood in the art, to embody aspects of the invention. One type of logical apparatus that may embody the invention is a computer system as illustrated in 700, containing CPU 707, optional input devices 709 and 711, disk drives 715 and optional monitor 705. Fixed media 717, or fixed media 722 over port 719, may be used to program such a system and may represent a disk-type optical or magnetic media, magnetic tape, solid state dynamic or static memory, etc. In specific embodiments, the invention may be embodied in whole or in part as software recorded on this fixed media. Communication port 719 may also be used to initially receive instructions that are used to program such a system and may represent any type of communication connection.

**[0073]** Various programming methods and algorithms, including genetic algorithms and neural networks, can be used to perform aspects of the data collection, correlation, and storage functions, as well as other desirable functions, as described herein. In addition, digital or analog systems such as digital or analog computer systems can control a variety of other functions such as the display and/or control of input and output files. Software for performing the electrical analysis methods of the invention are also included in the computer systems of the invention.

**Auto-Sealer Automated System**

**[0074]** FIG. 18A is a block diagram showing an automated piston driven system according to specific embodiments of the invention. According to further specific embodiments, an auto-sealer somewhat similar to a plate reader commonly used in biotechnology with the main difference is the design of the system component to allow automated handling of the microfluidic plates. In this implementation, the positive seal between manifold and microfluidic plate is still accomplished by applying vacuum to the interstitial areas, but the necessary initial downward force is applied mechanically. The area above the manifold is clear to allow access by an automated liquid hander such as the Tecan EVO. Vacuum and pressure sensors as well as plate presence and carriage position sensors allow for intelligent software based error handling. FIG. 18B is an image sequence showing how the automatic sealing device accepts and seals the manifold to a microfluidic plate. A single pneumatic linear actuator (e.g., a piston) provides horizontal and vertical motion. It has been found that this single operation allows more precise control of the manifold and plate and holds the plate in place during operation of the pneumatic manifold.

**Other Embodiments**

**[0075]** Although the present invention has been described in terms of various specific embodiments, it is not intended that the invention be limited to these embodiments. Modification within the spirit of the invention will be apparent to those skilled in the art.

**[0076]** It is understood that the examples and embodiments described herein are for illustrative purposes and that various modifications or changes in light thereof will be suggested by the teachings herein to persons skilled in the

art and are to be included within the spirit and purview of this application and scope of the claims.

**[0077]** All publications, patents, and patent applications cited herein or filed with this submission, including any references filed as part of an Information Disclosure Statement, are incorporated by reference in their entirety.

**WHAT IS CLAIMED:**

1. A microincubator manifold, comprising:

a top layer carrying one or more pneumatic channels for conveying pressure or gas to a culture plate;

a bottom layer comprising a gasket for providing a removable air-tight seal to the culture plate;

and a heat exchange module, attached to the top layer; wherein manifold seals to the culture plate thereby enclosing an incubation volume of gas;

the incubation volume of gas circulating between a culture chamber or observation area of the culture plate and a heat exchange area of the heat exchange module;

thereby providing a controlled temperature and gas composition above and in communication with the culture chamber or observation area.

2. The manifold of claim 1, further comprising:

one or more pneumatic connections at the top layer for interfacing with an external pneumatic controller;

one or more electrical connections to the heat exchange module for providing power and/or sensor and/or control connections between the heat exchange module and an outside controller.

3. The manifold of claim 1, further comprising:

a transparent window that sits above the culture chamber or observation area when the manifold is sealed to the culture plate, thereby containing the incubation volume while allowing for observation of the culture chamber.

4. The manifold of claim 1, wherein the heat exchange module comprises a circulating tan, in communication with

incubation volume, for circulating gases in the incubation volume.

5. The manifold of claim 4, wherein the heat exchange module comprises a second fan for cooling a portion of a heat exchanger.

6. The manifold of claim 1, wherein the incubation volume is less than about 10 milliliters.

7. The manifold of claim 1, wherein the incubation volume is maintained at about 37 C and about 5% $CO_2$.

8. The manifold of claim 1, wherein the culture plate has the dimensions and configuration of a standard cell culture plate.

9. The manifold of claim 1, wherein the manifold can be attached or removed, such that the incubation volume can be created and removed without affecting the culture chamber integrity.

10. The manifold of claim 1, wherein incubation contents can be controlled and changed independent of microchannel flow.

11. The manifold of claim 1, wherein circulating gas is exposed only to cell culture chambers of the culture plate.

12. The manifold of claim 1, further comprising at least one vacuum connector allowing vacuum sealing between the manifold and the culture plate.

13. The manifold of claim 1, wherein temperature and gas control is independent of flow control.

14. The manifold of claim 1, wherein the manifold interfaces with a plate with the dimensions or outlook of a standard culture plate.