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MICROGRAVITY STUDIES ON CELLS AND TISSUES: FROM MIR TO THE ISS

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Abstract. *In vitro* studies of cells and tissues in microgravity, either simulated by cultivation conditions on earth or reduced by spaceflight, are essential for the identification of mechanisms underlying gravity sensing and transduction in biological organisms. In this paper, we review rotating bioreactor studies of engineered skeletal and cardiovascular tissues carried out in unit gravity, a Shuttle-Mir study of cartilage tissue engineering, and the ongoing development and testing of a Cell Culture Unit for cell and tissue cultivation aboard the ISS.

INTRODUCTION

Exposure to microgravity can affect cells, tissues and the whole organisms at a variety of levels (Churchill 1997, Nicogossian 1994). Significant loss of bone mass, muscle strength, and cardiovascular fitness occur even when astronauts exercise regularly (Hughes-Fulford 1996, Churchill 1997), but the mechanisms underlying these physiological changes are not yet understood. Microgravity also changes growth patterns and carbohydrate processing in plants (Tripathy 1996, Bonting 1992), and the behaviors of aquatic organisms (de Jong 1996, Wiederhold 1997). Biological experiments carried out in microgravity could potentially improve our understanding of the basic mechanisms underlying the above effects. In particular, controlled *in vitro* studies of cells and tissues can be designed to distinguish the specific effect of reduced gravity from the complex milieu of factors involved in spaceflight (i.e. launch, landing, cosmic radiation). Microbes (e.g. yeast) could provide genetic models for studies of molecular responses to microgravity, while unicellular aquatic plants (e.g. *Euglena*) could serve as simple model systems that could be extended to higher level plants. Mammalian tissue culture studies could improve our understanding of the human responses to weightlessness (e.g. during embryogenesis, prolonged immobilization, or spaceflight) and expedite the development of countermeasures for long duration space flight (e.g. a Mars mission).

Flight studies offer a unique opportunity to study the effects of the gravitational force on cell growth and tissue development. Microgravity studies of cells and tissues refer to two different experimental situations: ground-based studies, in which the biological specimens are cultured in a state of continuous free fall (simulated microgravity), and space studies (actual microgravity). The combination of ground and space studies can potentially answer basic questions related to the biological mechanisms of gravity sensing, transduction, and response (e.g. cell migration, tissue development), as well as secondary effects of gravity on mass transfer in the cellular microenvironment. In this paper, we review (1) tissue engineering studies carried out in a rotating bioreactor (the Slow Turning Lateral Vessel, STLV) on earth, (2) a space study of tissue engineered cartilage carried out in a flight-qualified rotating bioreactor (the Biotechnology System, BTS) aboard the Mir Space Station, and (3) the ongoing design and testing of a new Cell Culture Unit (CCU) for use aboard the International Space Station (ISS).

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TISSUE ENGINEERING STUDIES IN ROTATING BIOREACTORS ON EARTH

The rotating bioreactor is a tissue culture vessel developed at NASA's Johnson Space Center (JSC) (Schwarz, 1992) and is commercially available from Synthecon (Houston, TX). **Fig. 1a** shows one design, the STLV, which is configured as the 110 mL annular space between two cylinders, the inner of which is covered with a silicone membrane for gas exchange. Vessel rotation speed can be adjusted such that the contents (i.e. inoculated cells or cell-biomaterial constructs) either rotate synchronously with the device, or are maintained suspended by a dynamic equilibrium between the acting gravitational, centrifugal, and drag forces (F_g, F_c, and F_d, respectively) (**Fig. 1b**, Freed, 1995). In the case that the tissue is cultured on earth in a state of continual free-fall, the rotating bioreactor simulates some aspects of actual microgravity. Fluid-dynamic experiments were carried out using rotating bioreactors containing 3-dimensional (3D) constructs. Efficient mixing in the bulk phase was induced by construct settling, as demonstrated by residence time distribution studies (Freed, 1997a). Flow conditions were laminar and measured shear stresses were below 1 dyn/cm^2, as assessed by particle image velocimetry (i.e. cross-correlation of particle displacement fields in a rotating vessel containing a construct and microparticulate tracers) (Neitzel, 1998). Rotating vessels can thus provide efficient, low-shear mass transfer to growing tissues during cultivation.

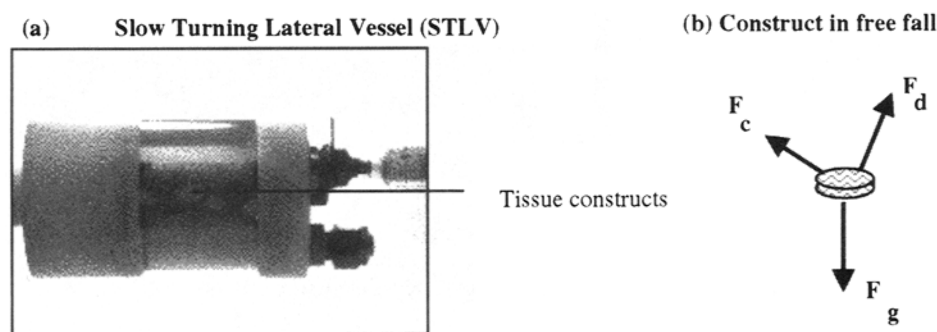

FIGURE 1. Working principles of the Slow turning Lateral Vessel (STLV). (a) The STLV is 110 mL vessel that can house ~ 12 tissue constructs, each 5-10 mm dia x 2-6 mm thick. **(b)** By adjusting the vessel rotation speed (e.g. 12 – 40 rpm), the constructs can be maintained in a state of continual free-fall due to a dynamic equilibrium between the acting forces (gravitational F_g, centrifugal F_c, and drag F_d).

We have carried out a variety of studies using an *in vitro* system with three components: isolated cells, polymeric templates and tissue culture bioreactors (**Fig. 2**). In particular, cells (i.e. chondrocytes, bone marrow stromal cells, BMSC, and cardiac myocytes respectively isolated from cartilage, bone marrow, and cardiac tissue) were cultured on 3D synthetic, biodegradable polymer scaffolds (i.e. meshes of polyglycolic acid, PGA, or sponges of polylactic co-glycolic acid, PLGA) in rotating vessels. The chondrocytes formed engineered cartilage containing glycosaminoglycan (GAG) and collagen type II (Freed, 1998) that responded to compressive loads in a characteristic manner (Vunjak-Novakovic, 1998). The BMSC formed engineered bone contained mineralized extracellular matrix (ECM), collagen type X, osteopontin and bone sialoprotein (Martin, 1998 a&b). The cardiac myocytes formed cardiac muscle that expressed specific proteins (cardiac myosin and troponin-T, sarcomeric tropomyosin), contained sarcomeres and intercalated discs, and contracted both spontaneously and in response to electrical stimulation (Freed, 1997b, Bursac, 1998, Carrier, 1998). Rotating bioreactor cultivations of a wide variety of normal and transformed cell types were recently reviewed (Unsworth, 1998).

As compared to conventional tissue culture methods, rotating bioreactors provided a signficantly better environment for cartilage tissue engineering (Freed, 1997b; Vunjak-Novakovic, 1998). Constructs based on bovine calf chondrocytes and PGA scaffolds cultured for 6 weeks in STLVs had a significantly higher fraction of cartilaginous ECM (i.e. GAG) and superior mechanical properties (i.e. equilibrium modulus) compared to otherwise identical constructs grown statically (Vunjak-Novakovic 1998, **Fig. 3**), and remained inferior to native calf cartilage. It is possible that dynamic changes in hydrodynamic forces acting

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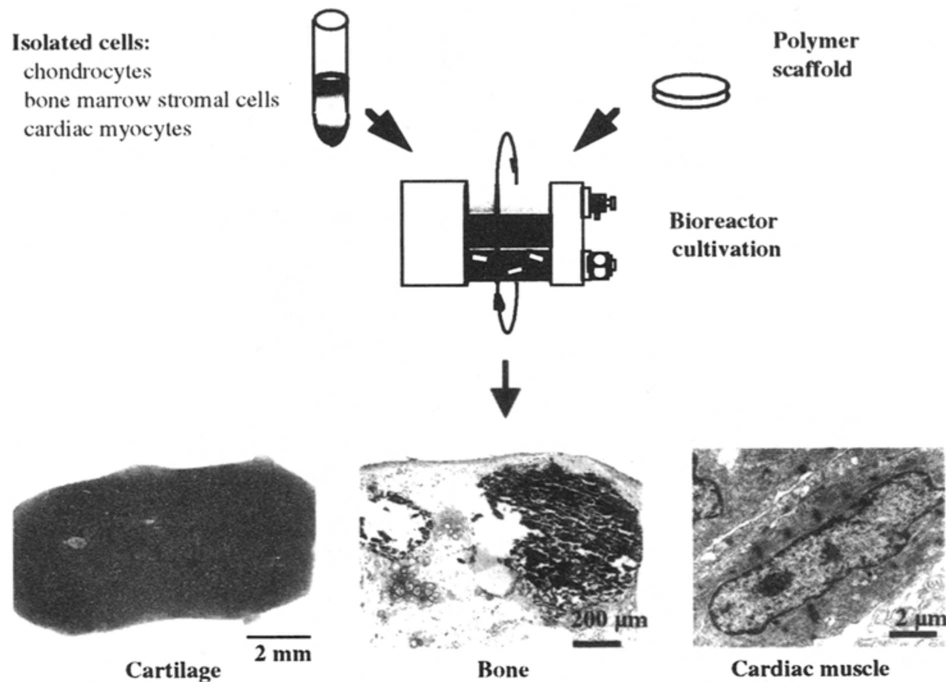

FIGURE 2. Model System. Isolated cells (e.g. chondrocytes, bone marrow stromal cells, or cardiac myocytes) are seeded onto polymer scaffolds and cultured in rotating bioreactors. The photographs show representative examples of the resulting engineered cartilage, bone and cardiac muscle (light and transmission electron micrographs).

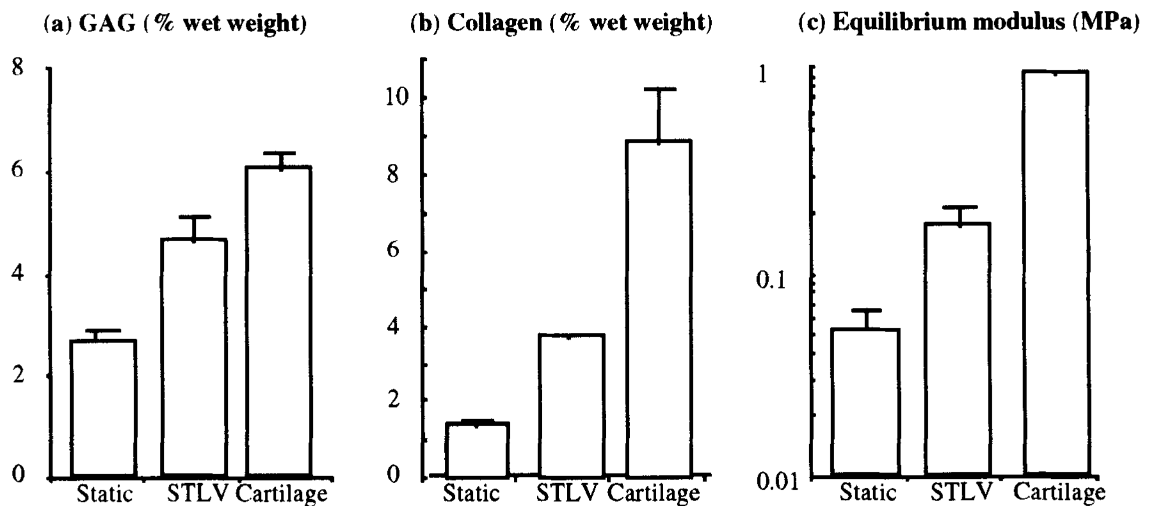

FIGURE 3. Rotating bioreactors improve the composition and function of engineered cartilage. Chondrocyte-PGA constructs cultured for 6 weeks in STLVs were compared to otherwise identical constructs cultured statically for 6 weeks and to freshly explanted native calf cartilage with respect to (**a, b**) wet weight fractions of GAG and collagen, and (**c**) equilibrium modulus (i.e. mechanical stiffness). Data are the Ave$\pm$SD of 3-6 independent measurements.

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on free-falling constructs in rotating bioreactors enhanced ECM deposition and assembly in constructs, in a manner similar to that induced by dynamic compression of cartilage explants (Sah, 1989) and intermittent hydrostatic loading of chondrocyte-PGA constructs (Carver & Heath, 1999).

TISSUE ENGINEERING OF CARTILAGE IN SPACE

The Biotechnology System (BTS) is one of the components of the cell culture flight hardware being developed by NASA-JSC for the ISS (NASA website). The BTS consists of a bioreactor connected to a recirculation loop consisting of a silicone membrane gas exchanger (Avecor model 0400-2A, Plymouth, MN) and a peristaltic pump (Randolph model 250, Manchaca, TX). The unit, which is maintained at 37°C, has access ports for infusing fresh media and gas (10% CO_2 in air), sampling bioreactor media, and removing waste media (**Fig. 4a**). The bioreactor within the BTS is configured as the 125 mL annular space between two cylinders, the inner of which is covered with a nylon mesh and has a disc attached at one end which serves as a viscous pump (**Fig. 4b**). The BTS has approximate dimensions of 17 x 20 x 25 inches (2 1/2 middeck Shuttle lockers) and weighs 137 lbs.

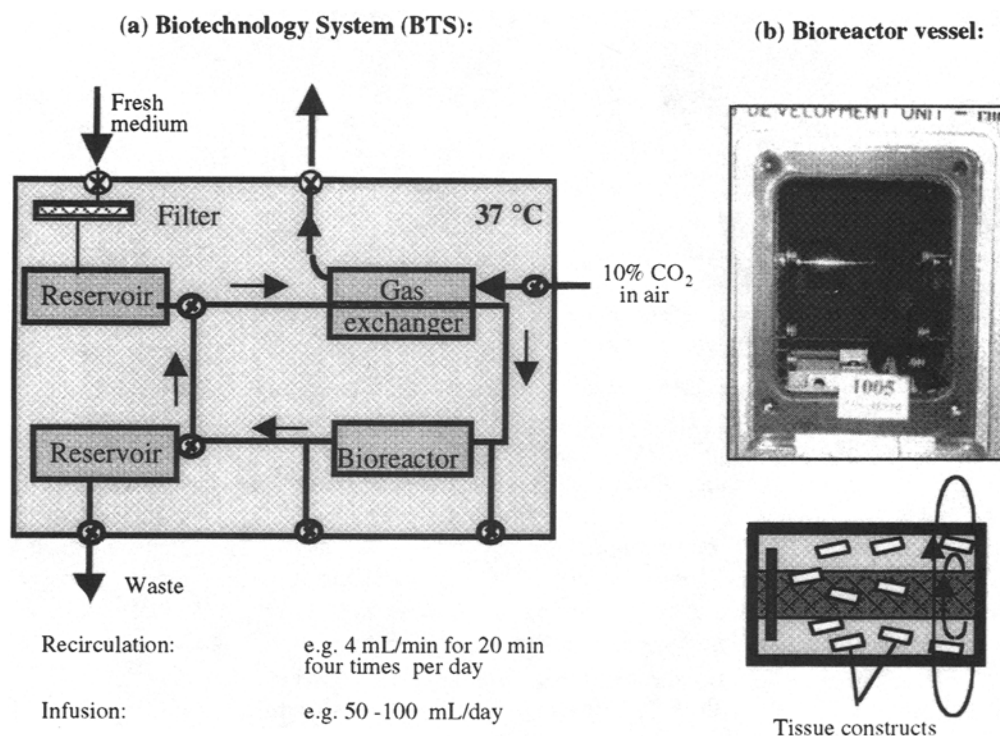

FIGURE 4. Working principles of the Biotechnology System (BTS). The BTS is flight hardware that contains a 125 mL volume rotating, perfused vessel that can house approximately 10 tissue constructs each measuring 5-9 mm diameter x 4-8 mm thick. Medium recirculation (i.e. between the bioreactor and gas exchanger) and infusion (i.e. from a reservoir), and rotation speeds of the inner and outer cylinders are preset and computer-controlled in-flight.

Constructs based on bovine calf chondrocytes and PGA scaffolds were cultured for 3 months in STLVs on earth and then transferred into the BTS for an additional 4 months of cultivation either on Mir or on earth. Specifically, one BTS containing ten constructs was transferred to Mir's Priroda module via the US space shuttle STS-79 (9/16/96 launch) and brought back to earth via STS-81 (1/22/97 landing). A second BTS with ten constructs served as an otherwise identical study conducted on earth, at NASA-JSC. Concentration gradients within the bioreactors were minimized by differential rotation of the inner and outer cylinders at 10

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and 1 rpm, respectively, in microgravity, and by the convection associated with gravitational construct settling during solid body rotation of the bioreactor at 28 rpm in unit gravity (Freed, 1997c). Medium was recirculated between the bioreactor and the gas exchanger at 4 mL/min for 20 min four times per day, and 50 to 100 mL of fresh medium was infused into the system approximately once per day.

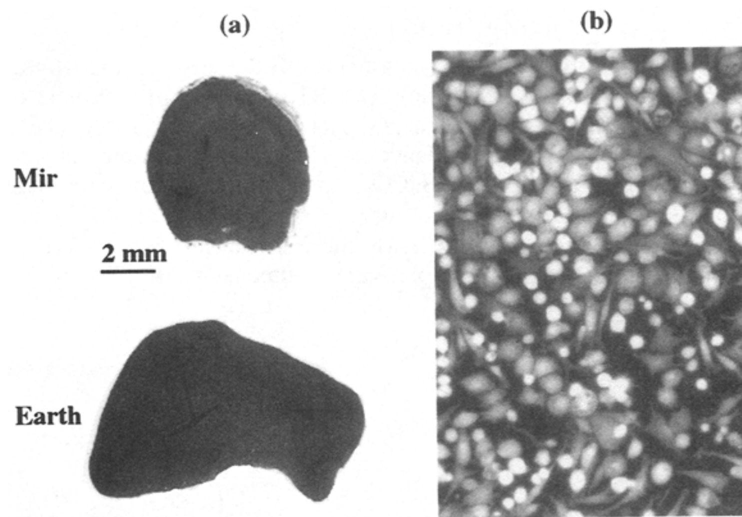

FIGURE 5. Cell and tissue growth in space. (**a**) full histological cross-sections of constructs grown on Mir and on earth, stained with safranin-O, (**b**) cells isolated from a Mir-grown construct after 2 days of monolayer culture; stained regions correspond to cells expressing intracellular esterase.

Under these conditions, metabolic parameters were maintained within previously established target ranges (e.g. pH between 6.9 and 7.4; oxygen tension between 71 and 127 mmHg, as assessed in space using a portable cartridge (G3+, I-Stat, Princeton, NJ). Constructs were assessed at the time of launch and after 4 additional months of cultivation on either Mir or earth, and compared to natural calf articular cartilage.

Constructs grown on Mir tended to become more spherical while those grown on earth maintained their initial discoid shape, as assessed histologically (**Fig. 5a**) and from the respective aspect ratios. These findings might be related to differences in cultivation conditions, as follows. Videotapes showed that constructs floated freely in microgravity but settled and collided with the rotating vessel wall at 1 g. On Mir the constructs were exposed to uniform shear and mass transfer at all surfaces such that the tissue grew equally in all directions, while on earth the settling of discoid constructs tended to align their flat circular areas perpendicular to the direction of motion, increasing shear and mass transfer circumferentially such that the tissue grew preferentially in the radial direction. Constructs from both groups appeared continuously cartilaginous over their entire cross-sections (4-8 mm), with the exception of fibrous outer capsules (0.15 - 0.45 mm thick). Cells isolated from post-flight tissues were alive and metabolically active, as demonstrated by their ability to attach to Petri dishes, proliferate, and enzymatically convert an unlabeled substrate into a fluorescent dye (**Fig. 5b**). Mir-grown constructs consisted of 95-99% viable cells as assessed by trypan blue exclusion, and incorporated radiolabeled tracers into macromolecules 30 h post-flight at rates comparable to those measured for earth-grown constructs.

At the time of launch and after additional cultivation on Mir and on earth, constructs contained 13, 14, and 19 million cells, respectively (Freed, 1997c). On earth, construct wet weights increased 1.7-fold between 3 and 7 months, which could be attributed to increasing amounts of cartilage-specific tissue components (i.e. collagen type II and GAG) (**Fig. 6 a&b**). In contrast, on Mir construct wet weights increased 1.3-fold over the same time interval, due to deposition of collagen and unspecified components (**Fig. 6 a&b**). The

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fraction of the total collagen that was type II decreased, but not significantly between the time of lauch and landing, demonstrating relatively good maintenance of the chondrocyte phenotype (Freed, 1997c). The equilibrium modulus of earth-grown constructs was comparable to that of native calf cartilage and was 3-fold higher than for Mir-grown constructs, as assessed in radially confined compression (**Fig. 6c**).

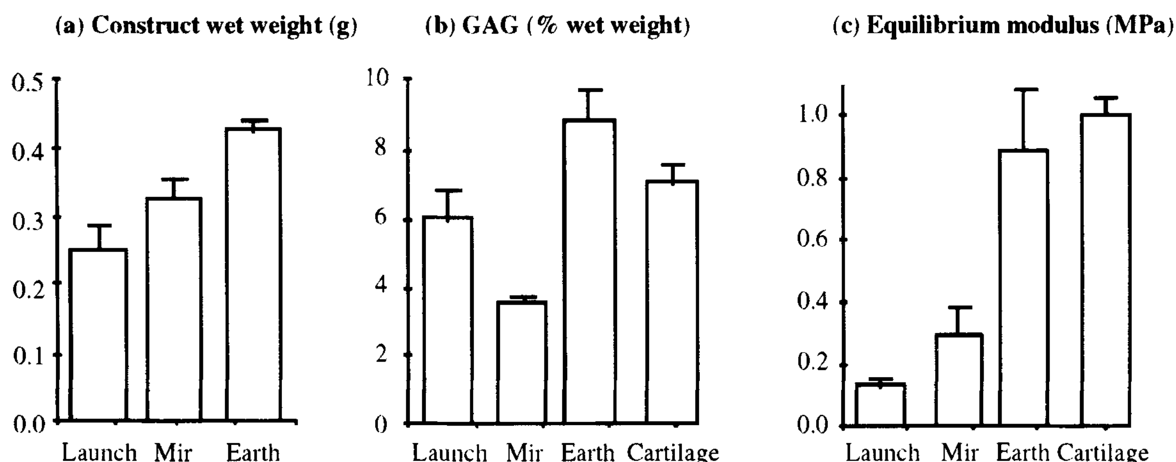

FIGURE 6. Spaceflight affected tissue structure and function. Constructs based on calf chondrocytes and PGA scaffolds and cultured for 3 months in STLVs on earth prior to launch are compared to constructs cultured for an additional 4 months on Mir or on Earth, and to freshly explanted native calf cartilage with respect to (a) wet weight, (**b**) GAG as a fraction of wet weight, and (**c**) equilibrium modulus. Data are the Ave$\pm$SD of 2-7 independent measurements.

The finding that constructs in the earth group had markedly higher wet weights, GAG fractions, and compressive moduli than constructs in the Mir group might be attributed to differences in construct cultivation conditions (i.e. free floating vs. gravity settling). In particular, spherical shape, relatively low GAG fraction and inferior mechanical properties of Mir-grown constructs might be attributed to reduced physical forces in the microgravity environment of space. However, we cannot distinguish between the relative contributions of microgravity, launch/landing, and local environmental factors (e.g. cosmic radiation), emphasizing the need for control studies carried out at unit gravity in space.

DEVELOPMENT OF A NEW CELL CULTURE UNIT

The Cell Cultur Unit (CCU) is a flight hardware currently being developed by Payload Systems Inc. in conjunction with M.I.T. and NASA's Ames Research Center (ARC) for use aboard the ISS (Searby 1998). The unit is designed to accommodate diverse specimens (animal, microbial, and plant cells; suspension and attachment cultures; tissues ≤ 4mm in diameter; non-feeding aquatic specimens), and to meet the requirements of long duration and sequential experiments, in both reduced and unit gravity. The CCU contains up to 24 Cell Specimen Chambers (CSCs) each of which is connected to a silicone tubing gas exchanger by a recirculation loop consisting of a peristaltic pump (model P625/66/NC Instech, Plymouth Meeting, PA) and reservoirs for fresh media, additives (e.g. growth factors, fixatives), and waste (**Fig. 7a**). The bottom surface of the CSC is made of optical quality glass coated with magnesium fluoride for on-line video observation. The CSC contains a porous membrane that restrains biological specimens within its central compartment. Medium enters the central compartment and exits from the annular cell-free space. Each CSC loop can be isolated from the remainder of the system if and when needed (e.g. in the event of contamination or after fixation of the CSC contents). CSCs will also be removable and will be compatible with the ISS compound microscope in the glovebox of the Life Sciences module. Individual CSCs (8, 12 or 24 in number with respective volumes of 30, 10 or 3 mL) are grouped in quadrants in order to allow four independent experimental groups, and mounted on a circular CSC tray that can be rotated (**Fig. 7b**). Light

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(wavelengths of 400-730 nm, intensities of 10-30 μmol/m^{2}s) can be provided for studies of photosynthetic cells. An automated sampling system allows collection and storage of up to 60 samples of cells or cell-free media per experiment. The CCU has dimensions of 18.5 x 21 x 21 inches (2 Shuttle middeck lockers), and weighs 147 lbs. One CCU will be operated within the Habitat Holding Rack; a second CCU will be operated within the ISS centrifuge to serve as an on-board unit-gravity control.

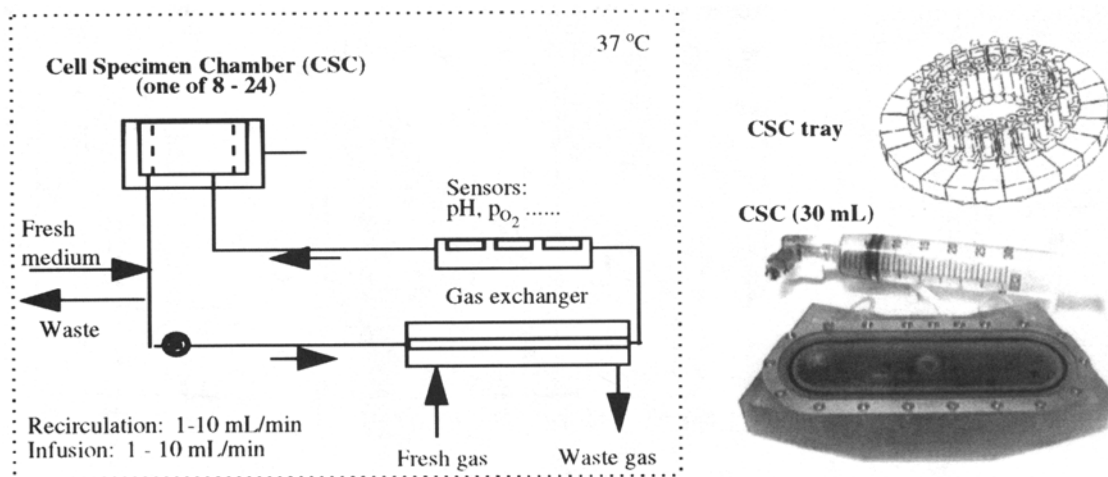

FIGURE 7. Working principles of the Cell Culture Unit (CCU). The CCU consists of up to 24 Cell Specimen Chambers (CSCs) that can house a variety of biological specimens. Each CSC is connected to a gas exchanger by an individual recirculation loop and to reservoirs of fresh and waste media. Medium parameters (e.g. oxygen tension, pH) are monitored on-line using sensors.

The CCU should provide for on-line monitoring and control of culture medium parameters. However, the selection of appropriate biosensors is limited by the small size of system components and problems associated with sensor drift and recalibration in-flight. In the current prototype, temperature is monitored using small thermocouples. Recent developments in fluorophores, optics and microelectronics have made it possible to monitor oxygen concentration and pH using fluorescence lifetime frequency modulation (FLFM). The working principle behind FLFM is that variations in medium analyte concentration induce variations in the fluorescence lifetime of a chemical immobilized in a sensor patch (Szmacinski 1995).

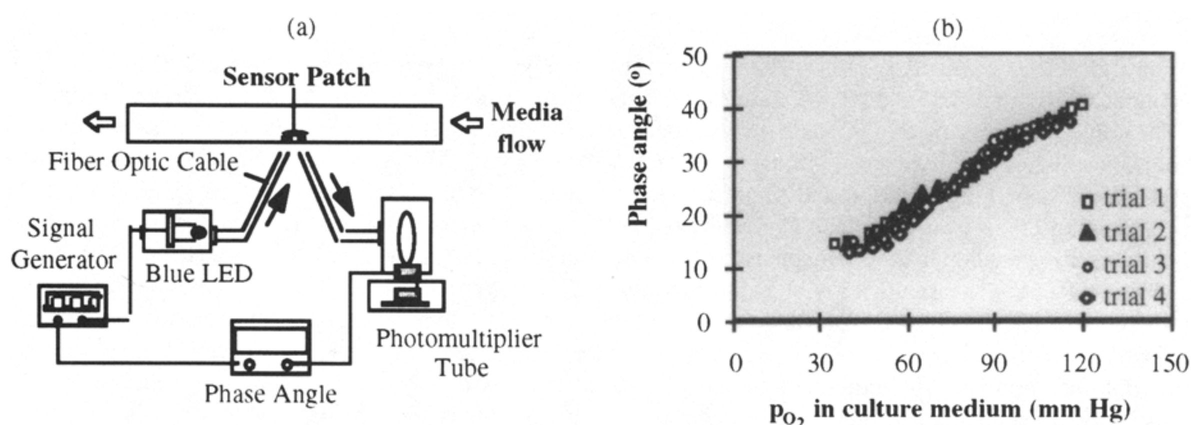

FIGURE 8. Oxygen sensor: (**a**) schematic of the fluorescence lifetime frequency modulation system, (**b**) correlation between the partial pressure of oxygen (PO_2) and the phase angle, in medium with 10% serum as observed in 4 sequential trials carried out over a period of 1 month without recalibration.

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The sensor patch is placed in the path of flowing medium, excited with light using a light emitting diode (LED), and the phase shift of fluorescence emission relative to the excitation is measured using a photomultiplier tube (PMT) (**Fig. 8a**). In this system, the output is, within certain limits, independent of both the intensity of excitation light and the amount of fluorophore, minimizing the need for recalibration. We recently demonstrated that an oxygen sensing fluorophore (a complex of ruthenium) immobilized within a small silicone patch could be used to monitor medium oxygen concentrations for up to 1 month. A representative calibration curve obtained for cell culture medium at 37°C is shown in **Fig. 8b**. A stable correlation was observed between the phase angle measured using the sensor and the medium partial pressure of oxygen measured using a standard gas-blood analyzer. Extension of this same approach to monitoring medium pH, glucose and various immunomolecules is currently under investigation.

Prototype CSCs have been developed and studied with respect to biocompatibility, the presence of gas bubbles in the medium, liquid mixing, and gas exchange. The prototypes have been used in conjunction with recirculation loops (**Fig. 7a**) to test six reference biological specimens that were selected to represent the variety of samples and the ranges of experimental conditions to be studied aboard the ISS. Reference specimens included suspension cultures of yeast (*Saccharomyces cerevisiae*), tobacco, and a small aquatic plant *(Euglena gracilis)*, monolayers of myoblasts (the C2C12 cell line) and osteoblasts (derived from bone marrow stroma), and large 3D skeletal muscle "organoids" (based on embyonic chick skeletal myoblasts). System operating temperatures ranged from 23 °C (for yeast and *Euglena*) to 37 °C (for mammalian cells).

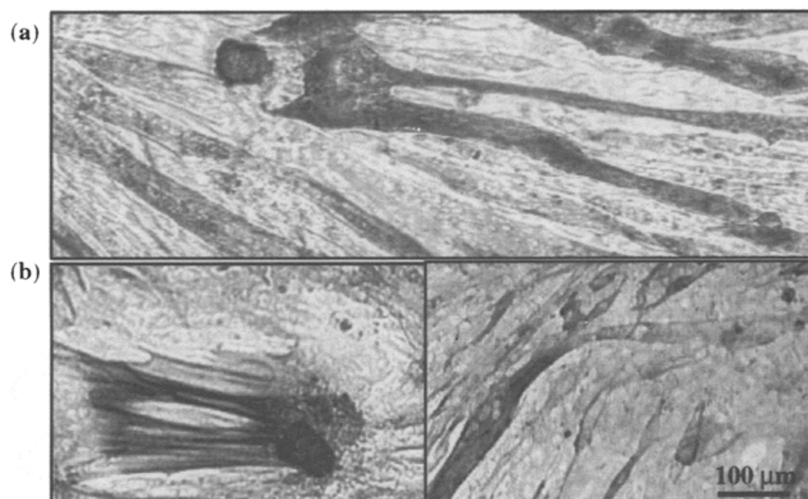

FIGURE 9. Myotube formation after 10 days of culture in C2C12 monolayers: (**a**) static dish, and (**b**) perfused CSC. Stained cells express the muscle-specific protein tropomyosin.

The growth and differentiation of C2C12 myoblasts was studied in monolayers for up to three weeks. The cells were inoculated onto 1.2 cm diameter glass coverslips that were pre-coated with laminin and collagen and placed in 10 cm diameter Petri dishes. In particular, each coverslip was inoculated with approximately 3 x 10^4 cells/cm^2 in 75 µL of medium (Dulbecco's Modified Eagle Medium, DMEM with 10% calf serum and 10% fetal bovine serum) and the dishes were placed in a 37°C humidified 5% CO_2 incubator for 2-3 h. Additional media (10 mL per dish) were added and the cells were allowed to attach for 24 h. The test system consisted of a gas-permeable reservoir bag (4R2110, Baxter Healthcare, Deerfield IL), the pump (model P625/66/NC, Instech, Plimouth Meeting PA) and a CSC operated at 37 °C and 5% CO_2. One coverslip with seeded C2C12 cells was transferred into the 3 mL CSC, the loop was filled with medium, and perfusion was established at 0.5 mL/min. Additional coverslips remained in Petri dishes to serve as

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otherwise identical static controls. After 5-6 days, the cells became confluent, and the medium composition was changed to DMEM with 2% horse serum, in order to promote differentiation. As shown in **Fig. 9**, both static dishes and perfused CSCs supported the growth of C2C12 myoblasts and their fusion into myotubes, as assessed by immunohistochemical staining for tropomyosin. In similar systems, CSC supported (1) monolayers of osteoblasts derived from bone marrow stroma, as assessed by alkaline phosphatase staining after one week in culture, and (2) 3-dimensional organoids derived from skeletal myoblasts (approximately 5 mm in diameter and 30 mm long), as evidenced by glucose consumption and lactate production after one week in culture.

The high metabolic rate and rapid doubling time of yeast challenged the CCU design. In an attempt to control the metabolic activity of the cells, glycerol was used in place of dextrose as the carbon source in a complex medium of yeast extract and peptone. The test system consisted of a reservoir bag, a gas exchanger configured as a coiled loop of 1m of silicone tubing (1/16" internal diameter x 1/32 inch thick, Cole Parmer, Vernon Hills, IL), the pump, and a CSC, and was operated at 23 °C. The 30 mL CSC was inoculated with *Saccharomyces cerevisiae* at a density of 5 x10^4 cells/mL and perfused using 3 inlets, at a rate of 15 mL/min per inlet. In order to minimize membrane fouling, the direction of perfusion was periodically reversed while maintaining the same flowrate. The flow intervals employed were 10 seconds in the forward direction followed by 5 seconds in the reverse direction, resulting in a net forward flow rate of 17 mL/min. An additional culture was carried out in a 250 mL shake-flask (250 rpm) operated at 30 °C. As shown in **Fig.10a**, final cell density obtained in the CSC at 23 °C (approximately 10^7 cells/mL) was comparable to that achieved in the shake flask maintained under optimal conditions (30°C). The duration of these studies was limited to approximately 5 days by cellular production of carbon dioxide, which caused the formation of gas bubbles in medium, and membrane fouling which eventually blocked perfusion. We are currently exploring the use of a minimal synthetic medium (to maintain cell doubling times within 4-6 h) in conjunction with the use of polycarbonate membranes with larger size pores (to minimize fouling).

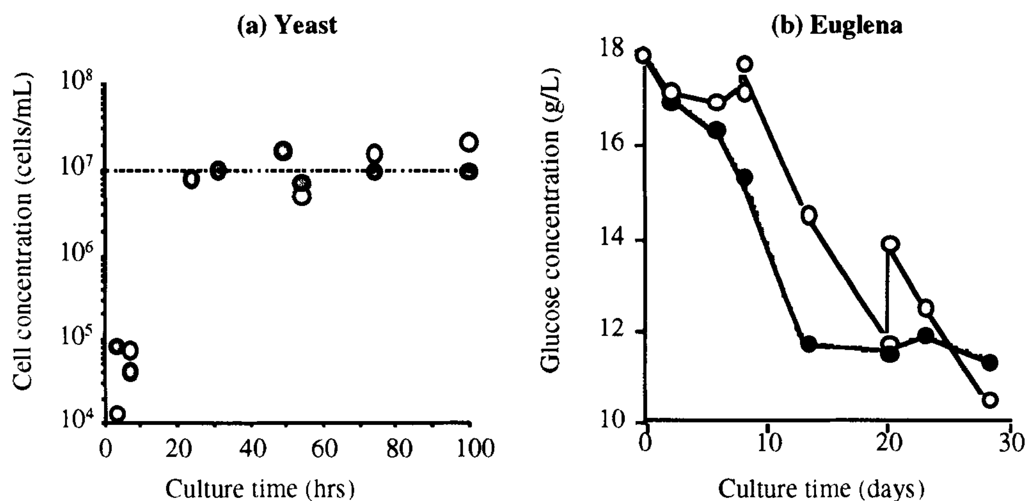

FIGURE 10. (a) Yeast (*Saccharomyces cerevisiae*) and (b) *Euglena gracilis* cultured in the CSC (open symbols) and in shake-flasks (closed symbols). Data points represent individual measurements.

Euglena gracilis is a motile unicellular plant that exhibits gravitactic, chemotactic and phototactic responses. Cells were cultured in heterotrophic medium (i.e. containing glucose) in order to eliminate their light requirement. The test system consisted of a reservoir bag, the pump, and the CSC, and was operated at 23°C. The 30 mL CSC was inoculated with *Euglena gracilis* at a density of approximately 10^4 cells/mL and continuously perfused in the forward direction through a single inlet at a rate of 3 mL/min. An otherwise identical control culture was carried out in a 125 mL shake-flask (120 rpm). **Fig.10b** shows medium glucose concentrations measured over 4 weeks of culture in the CSC and the control flask. CSC medium replacements were responsible for the increases in glucose concentration observed on culture days 8 and 20.

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Rates of glucose utilization were similar in the CSC and in the shake flask, indicating comparable overall metabolic rates. Cellular motility, aggregation and sedimentation were also comparable in the two groups. However, cells were found to have moved from the CSC into the recirculation loop. We are currently testing membranes with smaller pore sizes and cell separation filters at the CSC inlet and outlet.

SUMMARY

Microgravity studies have been carried out both on earth (simulated microgravity) and in space (actual microgravity), using a variety of cells and tissues. In ground studies, rotating bioreactors provided a favorable environment for tissue engineering (e.g. of 3-dimensional skeletal and cardiovascular tissue constructs), presumably due to the combination of efficient mass transfer and dynamic laminar flow at tissue surfaces. In prolonged cultivations of engineered cartilage in rotating bioreactors (3 months on earth followed by 4 months on earth or in space), the cells maintained viability and differentiated phenotype. Tissues had markedly higher wet weights, GAG fractions, and compressive moduli in the earth group as compared to Mir group, presumably due to the reduced physical forces in the microgravity environment of space. Further controlled studies of cells and tissues are essential for the identification of mechanisms underlying gravity sensing and transduction in biological organisms. The new cell culture unit, currently under development for use aboard ISS, is being designed to accommodate up to 24 cultures within a single experiment and to include a control unit operated at unit gravity in space, in order to extend the range of operating parameters and help distinguish specific effects of gravity from other factors in space flight (e.g. launch/landing, cosmic radiation).

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