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3D tissue-like assemblies: A novel approach to investigate virus–cell interactions

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ABSTRACT

Virus–host cell interactions are most commonly analyzed in cells maintained *in vitro* as two-dimensional tissue cultures. However, these *in vitro* conditions vary quite drastically from the tissues that are commonly infected *in vivo*. Over the years, a number of systems have been developed that allow the establishment of three-dimensional (3D) tissue structures that have properties similar to their *in vivo* 3D counterparts. These 3D systems have numerous applications including drug testing, maintenance of large tissue explants, monitoring migration of human lymphocytes in tissues, analysis of human organ tissue development and investigation of virus–host interactions including viral latency. Here, we describe the establishment of tissue-like assemblies for human lung and neuronal tissue that we infected with a variety of viruses including the respiratory pathogens human parainfluenza virus type 3 (PIV3), respiratory syncytial virus (RSV) and SARS corona virus (SARS-CoV) as well as the human neurotropic herpesvirus, varicella-zoster virus (VZV).

1. Introduction

Three-dimensional (3D) tissue culture systems are used in cell biology and physiology, immunology, cancer research, microbiology (bacteriology and virology) and tissue engineering to facilitate investigations in a more physiological setting than is obtained with 2D-tissue culture systems. Many bioreactor systems and other experimental devices have been used to establish 3D tissues that emulate properties similar to human and mammalian tissues *in vivo* [1–3]. Here we describe our approach to establish an optimized 3D tissue culture system using a rotating wall vessel (RWV) reactor to study viral infections and virus/host interactions.

1.1. Early attempts at 3D tissue culture systems

Tissue culture explant models were established in the 1920s, which allowed maintenance and expansion of organ tissues in culture or on filters [4,5]. For certain tissues (e.g. skin), it was critical to keep the cultures at the air–liquid interface, thereby providing conditions similar to the *in vivo* environment [6]. To maintain native tissue architecture, 1–5 mm^2 tissue explants were grown on collagen-coated cellulose sponges surrounded by plasma clots in roller tubes [2,7]. A number of these systems are well established and widely used in contemporary research. However, the disadvantages of explant systems are that *ex vivo* tissues from humans are limited and often difficult to obtain. Explant cultures are also short lived and prone to necrosis due to insufficient nutrient and oxygen transfer within the tissue. Furthermore, it remains almost impossible to genetically modify primary tissues, while cultured cells can be readily modified using various targeted genome-editing techniques such as transcription activator-like effector nucleases (TALENS) and clustered regularly interspaced short palindromic repeats (CRISPR/Cas9) [8–10]. To circumvent these limitations, culture systems have been established that allow *de novo* assembly of analog 3D tissues for a variety of applications.

The establishment of high-density 3D tissues *in vitro* using mammalian cells is challenging due to shear forces, turbulence, inadequate oxygenation and restricted nutrient transfer that can disrupt or damage the cultures. Peripheral cells commonly grow readily while cells in the center of the tissue undergo necrosis due to nutrient and oxygen deprivation. To provide a constant source of nutrients, chambers were developed that allowed

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metabolic exchange between a pool of medium and the culture chamber by diffusion across a membrane. This histo-physiological gradient mimics to some degree, the diffusion of essential nutrients in tissues [7,11]. To enhance the establishment of 3D tissues *in vitro*, Nandi and colleagues embedded the cultures in collagenous gels providing a scaffold for tissue growth [12]. The collagen gels allow establishment of mammary-like tissue with ductile structures from mammary cells. These cultures show sustained growth for several weeks and closely resemble the corresponding tissue *in vivo* [12]. Taken together, the early advances allowed the maintenance of tissue explants and *de novo* assembly of 3D tissues, while retaining their normal shape and special associations [2].

1.2. Establishment of 3D tissues in bioreactors

To improve the 3D tissue culture systems, bioreactors were introduced. Bioreactors are commonly used to produce pharmaceutical proteins such as interferon, growth hormones, and insulin molecules and to allow the maintenance of stable growth conditions over a long time period. In addition, bioreactors can provide defined culture conditions at exact time points, which is often required for the assembly and differentiation of 3D structures. Basic steps involved in construction of 3D tissue structures include tissue assembly, growth, formation of extracellular matrix (ECM) and basement membranes. Cell differentiation is associated with cellular specialization and vascular (or pseudo-vasculature) formation and is required for the establishment of an *ex vivo* tissue comparable to their *in vivo* counterparts (Fig. 1). To achieve this, each 3D tissue structure must be optimized for the following parameters (i) co-location of particles (tissues) of different sedimentation rates, (ii) three-dimensional spacial freedom that maximizes cell-to-cell and cell-to-microcarrier adherence required for assembly of the complex tissues, (iii) extremely low fluid shear stress and turbulence and (iv) oxygenation by active or passive diffusion with the exclusion of all air bubbles and permitting only dissolved gasses to enter/exit the bioreactor chamber thereby yielding a vessel devoid gas/fluid interface (zero headspace) [13,14]. Standard vertically oriented bioreactors allow minimal assembly of complex functional mammalian tissues; however, the normal fluid mechanical effects in these systems often leads to excessive shear forces, turbulence and in many cases, inadequate oxygenation and nutrient transfer that cause cell death and pose critical barriers to the establishment of the functional 3D culture systems (Fig. 1).

To overcome these problems, NASA engineers and scientists developed the integrated rotating-wall vessel (RWV; US patent 5,026,650; Fig. 2A and B) that allows establishment of 3D tissues on microcarriers or other matrices under near neutral buoyancy, controlled oxygenation, low shear stress and minimal turbulence [13,15,16]. The microcarriers serve as structural support for assembly and growth of 3D tissues [17]. Cells efficiently form bead-to-bead or matrix-to-matrix bridges in the RWV bioreactor at low agitation rates [18], typically resulting in aggregates of approximately 12–15 microcarrier beads. The RWV system eliminates excessive agitation from stirring (vertical bioreactors) and minimizes other shear forces that damage the microcarrier 3D tissues [19,20]. RVW systems are available in various commercially available volumes and can be scaled up to 1 liter.

One advantage of the RVW system is the high reproducibility and efficiency of establishing 3D tissue-like assemblies (TLAs) with characteristics of human tissues. Numerous publications confirm the similarity of structural organization and biomarker expression on the surface of the TLAs with their *in vivo* counterparts [21–25]. For example, we selected both DNA (VZV) and RNA (RSV, PIV3 and SARS-CoV) viruses to infect neuronal or respiratory TLAs, respectively, that were shown by transcript and protein analysis to reflect their physiological counterparts [26–29]. The unique feature of the RWV system maintains cell viability of the virus-infected TLA tissues for longer time periods than their 2D-tissue culture counterparts. Importantly, high-titer, cell free virus (1×10^5–10^7/ml; Hepatitis C, 1×10^5–10^7; RSV; and 1×10^7/ml, PIV3 5×10^6) [25,28,30,31] can be obtained from the supernatants of infected TLAs. Cytopathic effects (CPE) were detected in TLAs infected with SARS and RSV; however, this CPE did not impair the ability of the RWV cultures to replicate. In the case of VZV we succeeded in our objective to construct and infect neuronal TLAs with the virus that remained viable for months during which time the virus/host interaction was monitored [27]. Here we describe the establishment of neuronal and lung (TLAs) in the RWV system that can be infected with various viruses.

2. Materials and equipment

2.1. Rotating-wall vessel (RWV) bioreactor assembly

RWV bioreactors are available as batch fed or continuous flow, and allow tissue growth under 3D conditions in an optimized fluid suspension environment. The system is based on a rotating cylinder completely filled with fluid. Shortly after the cylinder begins to rotate, the fluid in the cylinder becomes coupled to the wall and rotates at the same speed as the vessel and TLAs rotate at the same speed (angular velocity) as their supporting fluid. Since TLAs have a slightly higher density than the fluid, they become suspended without stirring and with extremely low shear (0.15 dynes/cm^2). Oxygenation is achieved by passive diffusion to avoid gas bubbles and associated shear forces [14]. 3D cultures in the RWV system (Fig. 2) require the following equipment, materials, media and supplies.

2.2. Equipment

2.2.1. Required

Inverted microscope, hemocytometer Reichert, tissue culture incubator with sufficient air flow to dissipate heat (e.g. ThermoForma Model 3950),[1] laminar flow hood, fume hood; autoclave; bench top 4° refrigerated centrifuge, iStat handheld blood gas analyzer,[2] RWV bioreactor and power supply, model number RCCS1, Synthecon,[3] Milli-Q water system,[4] 50-micron polyester mesh, No. 7-51/36[5] BEEM capsules, No. 2310,[6] Optional equipment: Scanning Electron Microscope Jeol 330T and JEOL-JEM 1010 transmission electron microscope[7] or Environmental Scanning electron microscope, FEI Quanta 250 Environmental Scanning Electron Microscope[8] (SEM, ESEM).

2.3. Materials (*, materials found to provide optimal results)

T-75 (Corning 430641U)*, T-150 (Corning, 430824)* and T-225 (Nunc-159933)* culture flasks[1]; 50-ml (14-375-150)* and 15-ml (S50712)*, polypropylene, sterile, Fisherbrand conical tubes[1], Fisherbrand 1, 2, 5, 10, 25 and 50 ml, sterile, single pack serological pipettes[1]; Corning[9] 1L disposable Filter units, (431096)[1]*; Fisherbrand disposable borosilicate Glass 9-in. disposable pipettes,

[1] ThermoFisher Scientific, Waltham, MA.
[2] iStat Abbott Laboratories, Abbott Park, IL.
[3] Synthecon, Friendswood, TX.
[4] Millipore Corp., Milford, MA.
[5] Tetko, Inc., Briarcliff, NY.
[6] SPI, West Chester, NY.
[7] Jeol, Peabody. MA.
[8] FEI Quanta, Hillsboro, Oregon.
[9] Corning, Corning, NY.

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