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Bioreactor Technologies to Support Liver Function In Vitro

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Abstract

Liver is a central nexus integrating metabolic and immunologic homeostasis in the human body, and the direct or indirect target of most molecular therapeutics. A wide spectrum of therapeutic and technological needs drive efforts to capture liver physiology and pathophysiology in vitro, ranging from prediction of metabolism and toxicity of small molecule drugs, to understanding off-target effects of proteins, nucleic acid therapies, and targeted therapeutics, to serving as disease models for drug development. Here we provide perspective on the evolving landscape of bioreactor-based models to meet old and new challenges in drug discovery and development, emphasizing design challenges in maintaining long-term liver-specific function and how emerging technologies in biomaterials and microdevices are providing new experimental models.

Keywords

Tissue engineering; bioreactor; microfluidic; organ on chip; hepatocytes; liver non-parenchymal cells; drug development; drug toxicity

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1. Introduction

The human liver is the largest solid organ in the body and carries out a complex array of functions, ranging from metabolic homeostasis of nutrients and many hormones in the systemic circulation, to synthesis of virtually all plasma proteins, and serving as a nexus for myriad immune functions that balance innate and adaptive immunity and thus control tolerance and inflammation. The liver also metabolizes drugs, xenobiotics, and endogenous hormones and waste products, and produces bile to aid digestion and as route of excretion of liver waste. Acute and chronic ailments of the liver arising from infection, pharmacological drug consumption, poor diet and lifestyle, alcoholism, cancer metastasis, and other factors remain significant sources of morbidity and death around the world. Over 360 million people have chronic liver infections, which can result in end-stage liver disease and death. While some of these ailments can be prevented or treated with known approaches, many lack suitable therapeutic options due to incomplete mechanistic understanding. Hence, a wide spectrum of therapeutic and technological needs drive efforts to capture liver physiology and pathophysiology in vitro.

Given the diversity of liver functions, especially its role connecting metabolism and immunologic regulation, it is not surprising that a multitude of different in vitro models have arisen to capture both individual facets and more integrated functions of the liver. It is arguably unlikely that a single in vitro model of liver will ever serve all needs in the drug development or basic research communities, as any model is a balance between what information is needed and the cost and complexity of setting up, validating, and maintaining the model. Here we provide perspective on the evolving landscape of models to meet old and new challenges in drug discovery and development. An analysis of liver models directed toward commercial, pre-clinical drug development appears elsewhere in this issue (A. Roth et al, this issue) and in other recent reviews [1–3]. Hence, we focus here on design and application of bioreactor formats designed to support complex liver-specific functions in culture for a range of applications, noting that only a few of these formats have been commercialized and that much of the potential for bioreactor technologies in drug development is yet to be realized.

We consider “bioreactors” to include approaches that involves a designed or programmed fluid flow as an integral part of the culture format, where flow is used to enhance molecular transport, provide mechanical stimulation, control addition of drugs or biological regulators on a diurnal or other temporal basis, or to otherwise influence cell function and/or assay performance in ways that are not readily accessible in static culture formats. The evolution of bioreactor formats over the past few decades has taken place in parallel with tremendous advances in synthesis and fabrication of functional biomaterials scaffolds for driving 2D and 3D organotypic tissue organization and function, as well as mechanistic understanding of how cells integrate biophysical and chemical cues in their environments [4]. After providing a brief overview of liver physiology, we examine features of various bioreactor formats in the context of application needs, describe engineering parameters and their influence on in vitro hepatic function, and identify emerging technologies aimed at modeling more complex tissue functions and integrating systems biology approaches for analysis of complex system behavior. In light of the growing interest in models based on human cells, we especially

highlight the recent advances in modeling human liver behaviors with bioreactor systems. We note that many organotypic systems are operated in static rather than bioreactor format, and refer the reader to other reviews for additional perspective on these culture formats [1,2,5].

1.1.The liver feeds and protects us

The basic energy needs of organisms must be fulfilled continuously despite dramatic fluctuations in nutrient availability during normal diurnal feeding cycles and in times of starvation or excess. These demanding metabolic forces resulted in evolution of the liver, a metabolic integrator that, at 2–4% of body weight, is the largest solid organ in the body. The liver is located at a unique hemodynamic confluence where, unlike other organs, it receives blood from arterial and venous supplies. The hepatic portal vein brings in nutrient-rich blood rich from the intestine while the hepatic artery brings oxygen-rich blood from the heart. Of the total hepatic blood flow (100–130 mL/min per 100 g of liver, 25% of cardiac output), one-fifth to one-third is supplied by the hepatic artery [6]. With the exception of some fats that are adsorbed into intestinal lymph, compounds absorbed by the intestine first pass through the liver, which senses and regulates the concentrations of glucose, lipids, amino acids that circulate systemically to brain, muscle, and other organs. Metabolic functions extend beyond homeostasis of nutrient levels to a wide spectrum of metabolic transformations of xenobiotics encountered in the portal (or systemic) blood. In parallel with these metabolic transformations, the liver regulates diverse body functions through the production of most serum proteins. The liver controls the availability of lipophilic hormones (through production of binding proteins ranging from albumin to transcortin), guides clotting processes (through production of fibrinogen and many enzymes involved in clotting), and regulates inflammation (through production of proteases and protease inhibitors).

In addition to the vascular network, the liver is permeated by a biliary network that originates as small canaliculi between adjacent hepatocytes, draining hepatocyte-produced bile into biliary ductules that coalesce and empty into a common duct leading to the gall bladder, which stores bile for secretion into the intestine during digestion. Bile salts, along with many other small molecules secreted into bile, are in large part re-absorbed by the ileum, and returned to the liver by way of the portal vein, thus completing a portal enterohepatic circulation [7]. Maintenance of the enterohepatic circulation is vital for several liver and gastroeintestinal functions including bile flow, solubilization and excretion of cholesterol, clearance of toxic molecules, intestinal absorption of lipophilic nutrients, and metabolic and antimicrobial processes [8].

The liver is also the first line of defense against microbes that breach the gut barrier, and against a number of antigens arising from food and from gut microflora. Microbial products such as flagellin, lipopolysaccharide (LPS; endotoxin), and peptidoglycans permeate into portal blood in healthy individuals (e.g. LPS is present at ∼1.0 ng/mL in portal venous blood [9]) where they provide constant stimulation to the extensive immune system in the liver [10] and influence immune tolerance in both the liver and the entire body [11].

1.2. Liver structure and function

Hepatocytes are relatively large, mitochondrial and Golgi-rich cells organized into epithelial plates and are the primary cell type responsible for metabolic functions in the liver such as synthesis of serum proteins, intermediary metabolism of amino acids, lipids, and carbohydrates, and detoxification of xenobiotic compounds. Hepatocytes comprise over 80% of liver volume but 50–60% of total liver cell number. Hepatocytes are traditionally described as “parenchymal” cells and together with cholangiocytes, the cells that form the biliary tree, they comprise the epithelial cells within the liver (Figure 1). The primary functional unit of the liver is the hepatic lobule. It consists of a roughly hexagonal arrangement of rows of hepatocytes (epithelial plates) radiating outward from a central vein. Located along the lobule perimeter, the portal triad consists of a small portal vein, hepatic artery, and bile duct. Blood enters the lobules through branches of the portal vein and hepatic artery, and it flows through liver sinusoids toward the central vein. On their basolateral side, hepatocytes are surrounded by the sub-endothelial space of Disse where lymph is collected for delivery to lymphatic capillaries [12] (Figure 1). Quiescent hepatic stellate cells comprise 5–8% of total liver cells, are present within the space of Disse at a ratio of approximately 1 stellate cell for 6 hepatocytes, store 80% of body vitamin A, and participate in hepatic wound healing, regulation of sinusoidal blood flow, angiogenesis and hepatocyte growth [13]. Sinusoidal endothelial cells comprise 15–20% total liver cells and form a fenestrated endothelium that is unique among capillary beds. Most fenestrations (<200 nm) are clustered together in groups of 10–100 called liver sieve plates, which occupy 2–20% of the liver endothelial surface [14,15]. These fenestrations allow direct cell-to-cell contact and free diffusion of molecular and macromolecular complexes like LDL (25–27 nm in diameter) and HDL particles (8–11 nm in diameter).

Kupffer cells are resident liver macrophages that comprise 8–12% of total liver cells and reside within the hepatic sinusoids where they act as one of the first lines of defense against antigens passing through the gastrointestinal barrier. Although the liver sinusoids lack a true basement membrane, the space of Disse also contains basement-membrane-like matrix, which is essential for the differentiated and normal functions of all the hepatic cellular compartments [16]. Hepatic stellate cells, sinusoidal endothelial cells, and Kupffer cells, together with additional cells from the immune system (e.g. multiple subsets of intrahepatic lymphocytes such as Natural Killer (NK) cells, Natural Killer T (NKT) cells or hepatic dendritic cells) comprise the “non-parenchymal” hepatic cells (NPCs), which account for approximately 40% of total liver cells [11,17,18].

The apical faces of adjacent hepatocytes form bile canaliculi. Bile is secreted by hepatocytes into the bile canaliculus and then drains toward bile ducts, which are lined by duct epithelial cells named cholangiocytes. The link between hepatocyte canaliculi and the biliary tree is named the Canal of Hering, which is also believed to be a niche for liver progenitor cells [19]. While hepatocytes initially secrete bile into the bile canaliculus, cholangiocytes modify bile of canalicular origin by a series of coordinated spontaneous and hormone or peptide regulated secretion/reabsorption of water and electrolytes before it reaches the small intestine [20]. Although parenchymal liver cells are critical in delivering key functional attributes of liver such as metabolic function, the non-parenchymal cells are equally critical

to define liver microstructure and function, can sense and decode many environmental cues such as the presence of LPS, viral particles, dead cells or a toxin, and can communicate with hepatocytes to generate appropriate phenotypic response. This response includes, but is not limited to, cell death, cell proliferation, and in some cases altering metabolic pathways.

Recreation of liver physiology in culture requires isolation and reassembly of at least some subset of these heterogeneous cell types, preferably from human sources. Although elutriation and other methods have been used by specialized laboratories to isolate relatively pure populations of individual cell types, widespread access to culture the non-parenchymal cells requires methods for preserving and shipping them from the point of isolation. Currently, cryopreserved hepatocytes and cryopreserved Kupffer cells are available commercially, enabling at least one important part of the immune system (liver macrophages) to be included in routine cultures. Whether iPS-derived cells will emerge to fill the gaps in availability of liver NPCs is an open question, as the utility of iPS-derived hepatocytes is only slowly being defined on an application-by-application basis.

1.3.The liver as an immunological organ

The liver is a central integrator of immunological modulators. It contains the single largest population of macrophages in the body [21], but also the highest density of NK cells and NKT cells, and the largest reticulo-endothelial cell network [11,22]. Adding further to the complexity of the immunological networks in liver, hepatic parenchymal cells exhibit some immunological properties such as expression of Toll-like receptors (TLRs) and secretion of IL-6 and other cytokines [23,24]. Remarkably, the healthy liver routinely takes up immune activators from the portal blood, and LPS can drop 100-fold in concentration between portal and peripheral venous blood without exhibiting an overt innate immune response [9]. At the same time, however, failure to clear pathogens can result in systemic infections, often leading to death. These facts suggest that there are unique hepatic immunoregulatory mechanisms that maintain a balance between tolerance and immunity. This tolerogenic attribute of liver creates a window of vulnerability for well-adapted pathogens such as hepatitis C virus, and is illustrated by the persistence of microbial infections and tumor metastases in the liver.

This tolerogenic state inside the liver comes about through cooperation between both innate and adoptive immune systems. Unusual cell types that act as antigen-presenting cells (APCs) exist in the liver and include resident hepatic cells such as sinusoidal endothelial cells, Kupffer cells, and stellate cells [25]. Such antigen presentation usually results in tolerance characterized by CD8+ T cells with low effector functions, such as IFN-gamma production [25,26]. Additionally, the threshold of innate immune response is higher within the hepatic microenvironment. Thus, low-level constitutive exposure to portal LPS has been shown to induce regulatory mediators such as IL-10, causing cell-autonomous hypo-reactivity to subsequent LPS stimuli [27–29]. It can be stated that liver’s immune system is maintained in a steady state of active tolerance that can be reversed by sufficiently strong pathogen-specific signal.

Due to the high threshold for the initiation of an adaptive T cell response in the liver, innate immune mechanisms assume greater significance. To be able to decode environmental

stimuli, various cell types in the body express an array of different receptors known mainly as pattern recognition receptors (e.g. TLRs). These receptors can sense the presence of various pathogen-associated molecular patterns (such as LPS) as well as damage-associated molecular patterns that are linked to cellular injury such as cellular high-mobility group protein B1, which is released from necrotic tissue [24]. So far, 13 mammalian TLRs have been identified. These receptors can be expressed both on cell surface (e.g.TLR2,4,5) or endosomes (TLR-3,7,9). They recognize a wide range of exogenous and endogenous molecules such as LPS and HMGB1 (TLR-4), flagella (TLR-5), bacterial associated unmethylated CpG DNA and mitochondrial DNA (TLR-9) [24]. The presence of various TLR receptors in different types of liver cells establish a complex cross-talk between these cells which can shape innate and adoptive immunity and regulate unique tissue behavior.

There are currently few in vitro models that properly mimic the complex immunologic nature of liver tissue. As more and more drugs and drug formulations are being developed to target the immune system for chronic inflammatory diseases and cancer, pre-clinical assessment of off-target effects in the liver is of growing importance and an area that remains underserved. A major challenge to developing models is the accessibility to sources of immune cells that complement the hepatocytes and other NPCs, hence the use of bioreactors for these challenging applications awaits parallel development of better ways to isolate and incorporate immune cells into these systems.

1.4 Drug metabolism in the liver

Briefly, drug metabolism in liver generally involves a sequence of Phase I reactions, resulting in structural alterations including oxidation, reduction and hydrolysis, followed by Phase II conjugation reactions that increase the solubility of the drug via modification with glucuronate, sulfate, or other polar molecules. Although Phase I reactions may be carried out by any combination of a diverse group of over 50 cytochrome P450 (CYP450) monooxygenases, a subset (the CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP3A4, and CYP3A5 enzymes) metabolize 90%of drugs [30][31]. Metabolites may be readily excreted if they are rendered sufficiently polar after Phase I, but otherwise undergo Phase II reactions involving conjugation with highly polar endogenous molecules including glucuronic acid, sulfates, certain amino acids (glycine, glutamine, ornithine), endogenous acids (acetylation) or with glutathione [30,32]. Some compounds undergo a further Phase III transformations such as glutathione conjugation via a peptidase-mediated intermediate [33]. The transport of drugs and their metabolites into hepatocytes from blood and out into bile is regulated by families of transporters located on the basolateral and canalicular surfaces [34,35].

2.The needs for liver tissue engineering in drug development

The liver is a complex and multi-faceted organ, which plays a central role in drug metabolism, toxicity, and a number of diseases that impose significant health burdens worldwide. Animal models are widely used in drug development and are a regulatory requirement for assessing the toxicity of new chemical entities despite being imperfect predictors of outcomes in humans [36]. This underscores the need for relevant and reliable in vitro tissue models as a compliment to animal models for pharmacological evaluation. Liver tissue engineering and bioreactor technologies offer the opportunity to produce multi-

cell type, multi-organ in vitro models which step towards capturing the physiological complexities of whole animal models, while avoiding some of the scientific and ethical issues. Liver tissue engineering will have applications in basic research exploring fundamental liver biology. Here, we will concentrate on potential applications in pharmaceutical drug development, noting that we expect bioreactor technologies will add to (and not replace) the compendium of existing in vitro assays based on liver cell culture. We outline a few of the key application areas, and consider individual application areas in more depth in Section 4 in the context of reactor technologies.

2.1.ADME and Toxicity

Hepatocyte models are critical in drug development to test the toxicity of small molecule drugs and to perform preclinical evaluations of drug metabolism in humans. Many of these needs are well-served by relatively simple in vitro cultures that focus on a particular facet of drug disposition or toxicity, as reviewed elsewhere [2]. For example, overall disappearance rates of a parent compound, induction of common CYP450s, drug-drug interactions, and inhibition of bile acid transport can be assessed with hepatocyte suspension or short-term cultures of plated hepatocytes [37–39]. However, the desirable pharmacokinetics (PK) features of small molecule drugs have evolved over time, and an increasing number of slowly cleared, highly lipophilic compounds now fill drug discovery pipelines. Accurate PK prediction of these compounds demands in vitro hepatocellular models, which combine high response fidelity and long-term retention of function, with controlled plasma-protein drug binding and platform drug binding.

The prediction of hepatotoxicity is an inherently complex undertaking and myriad assays featuring combinations of covalent binding, carcinoma cell lines, primary hepatocytes and high content imaging have been developed to screen for hepatotoxins [40–42]. When combined with animal testing, these assays are generally successful in preventing overt hepatotoxins reaching the clinic, save for a few notable examples such as fialuridine. The greater challenge is to identify idiosyncratically toxic drugs that appear safe in healthy patients in early-phase clinical trials but exhibit toxicity in larger patient populations, via mechanisms that appear to require integrated responses of parenchymal and non-parenchymal cells, particularly interactions with the immune system. These drugs result in significant harm to patients through drug induced liver injury (DILI), incur costly late stage clinical trial failures, and withdrawal from the market of approved drugs.

The mechanisms underpinning DILI with drugs showing issues at the 1 in 10,000 patient level are not completely understood, inflammation is often implicated. For example, it is known that an increase in gut permeability, due either to disease or due to the actions of a drug on the gut barrier, can induce a mild inflammatory state in the liver. The subsequent activation of inflammatory signaling pathways in hepatocytes can then synergize with metabolic stresses, often associated with drug metabolism, and ultimately cause liver failure [43,44]. However, these types of effects often cannot be discovered with single-cell type, two-dimensional (2D) models. In a screen of almost 100 drugs from the DILI list, only a fraction of the known toxins were correctly identified as toxic using an assay involving mixtures of drugs and cytokines applied to 2D monocultures of hepatocytes [43]. This

finding underscores that while relatively high-throughput 2D cultures remain valuable for early stage screens, and can be well described with perspectives from systems biology, in many instances there is a need for more advanced in vitro tissue models capable of mirroring complex in vivo physiology.

2.2.Disease Models

Liver disease as a result of infection, e.g. Hepatitis B (HBV) and Hepatitis C (HCV) or lifestyle choices e.g. alcoholic fatty liver disease, is a growing problem leading to increasing health burdens in both the developed and developing world. If untreated, patients with liver diseases typically progress through fibrosis to cirrhosis and onto liver cancer and liver failure. As an example of the scale of the problem, the World Health Organization figures shows 240 million people are chronically infected with HBV, 600,000 of whom will die from the consequences of infection each year. Viral hepatitis well exemplifies how liver tissue engineering and bioreactors might address an unmet need in the drug development process. New therapies for viral hepatitis are under active development, as evidenced by the December 2013 approval of the HCV treatment Sofosbuvir (Sovaldi, Gilead Biosciences Inc, Forest City, CA), however the in vitro and animal models for both HBV and HCV fail to capture many salient aspects of the human response to these infections.

Animal models of HBV are limited to the duck, woodchuck, human chimeric mouse and chimpanzee [45], while the available in vitro models are based on carcinoma cell lines, such as HepG2 and HepaRG [46], cultured in monolayers. The in vitro tools for HCV is broader, both from the viral molecular biology perspective (HCV replicon) and the in vitro models perspective (infectable carcinoma cells lines and a growing compendium of protocols for infection in primary human cells) [47]. The available models for HBV and HCV have limitations such as the inability to readily infect with patient isolates to facilitate the study of different viral genotypes and resistant viruses. In common with ADME and toxicity applications, it is likely the inability of standard 2D culture to maintain well differentiated primary hepatocytes for extended periods restricts current in vitro viral hepatitis models, and the addition of non-parenchymal cells as modulators of the immune environment.

2.3.Engineered Liver Platforms: Meeting the needs of both the cells and the user

Several reviews have addressed the general challenges of capturing human tissue complexity in vitro [48][4], and we highlight some of them here in the context of requirements of reactor design (Table 1). Any bioreactor useful for drug evaluation must be robust enough to sustain the cultured tissue for long time-scales, allow regular sampling or gathering of metrics without compromising sterility, and sustain the needs of the cultured tissue without demanding excessive researcher involvement. The reactor must be designed to deliver the proper levels of nutrients, cytokines, and gases, and to encourage or limit, as appropriate, various cell-cell or cell-matrix interactions. It is also advantageous if bioreactors can be designed that are readily scaled, to allow moderate- or high-throughput screening without proportional scale-up of operational effort.

From the performance perspective, the most overwhelming biological design constraint for *in vitro* liver bioreactors is meeting the basic metabolic demands of the cells or tissue.

Oxygen tension is a controlling factor in many aspects of liver phenotype varying along the sinusoid from 85 μM periportal to approximately 45 μM pericentral. Hepatocytes consume oxygen at 10- to 100-fold the rates of most cells, and because hepatocellular function is so intimately linked to oxygen tension there exists an enormous need to balance oxygen consumption with oxygen delivery. In cell culture, oxygen is depleted very quickly compared to other key nutrients like glucose and amino acids, which are present in culture medium at roughly comparable concentrations to those in blood. Each hepatocyte contains over 1500 mitochondria which consume oxygen at a rate of 0.3 – 0.9 nmol/sec/10^6 cells [49], while the average rate of oxygen utilization by many other cells is about 2 – 40 picomol/sec/10^6 cells [50]. This is an especially substantial challenge in 3D cultures, as the oxygen gradient across a layer of 5 cell diameters, which represents a distance of approximately 120 μm, ranges for liver from normoxic to hypoxic.

In common with many other tissues, the appropriate cellular polarization and paracrine cellular communication found in 3D liver cultures has been associated with improved longevity of function in culture. Hence, a number of interesting strategies have been explored and are under development to meet the general challenge of creating relatively uniform perfusion through 3D cultures in a manner that delivers an adequate supply of oxygen while preventing deleterious effects of supraphysiological flow rates through the tissue. Supraphysiological flows can cause mechanical stress in the form of shear or stretch that impairs function directly through mechanical stimulation, but can also dramatically disrupt the appropriate autocrine and paracrine signals sent between cells. The movement of growth factors, peptide hormones, and cytokines in the extracellular space in most tissues is greatly influenced by even the slow interstitial convective flows from blood capillaries to lymph that prevail in vivo [51]. The balance of these important autocrine and paracrine signals can be greatly disrupted by high perfusion rates in vitro. Further, the need for a scaffold to organize cells in 3D is often challenging to couple with methods for generating convective flow. Arguably the greatest challenge in comparing data from different bioreactor designs and operating conditions is the disparate combinations of effective local fluid velocities, oxygen concentrations, and matrix microenvironments, which all together govern the phenotypic responses of the in vitro cellular system.

To put this spectrum of considerations into perspective, we analyze existing liver bioreactors according to increasing conceptual complexity. We start by assessing relatively straightforward implementations of flow over monolayer configurations, and move on to review various 3D bioreactors in classic and microfluidic/microfabricated formats that attempt to create complex microenvironments for hepatocyte and non-parenchymal cell cultures. All of these bioreactors are assessed in terms of not only how well they support liver function, but also how usable they are in the drug development process for particular applications. We note that relatively few of the bioreactors that have been described in the literature are accessible to the average user; i.e., few systems have yet made the transition from the academic laboratory to user-friendly formats or are commercial availability. By reviewing the entire spectrum of systems described in the literature, we hope to stimulate greater awareness of the challenges and opportunities, and thus foster further development of these approaches in applications where they add value.

3.Bioreactors

A wide variety of bioreactor formats have been developed or adapted for liver culture over the past four decades. A summary of the major features and commercial availability is provided in Table 2, and a detailed description of the historical development and current features is provided in this section, followed by a focus on applications in Section 4.

3.1.Bioreactors to manipulate phenotype in monolayer culture

The most conceptually simple bioreactor involves inducing flow over or through a monolayer of hepatocytes or co-cultures of hepatocytes with other cell types, which may be plated on or within matrix such as collagen gels. Such systems have undergone an evolution from early work aimed toward very large scale culture for extracorporeal liver support, to intermediate “macroscale” reactors directed toward physiology and drug development, to microfluidic systems directed at increasing throughput, improving control over microenvironment conditions, and integrating with other organ mimics. Though conceptually simple, reactors based on variations of monolayer culture remain an important practical use and a foundation to more complex configurations.

3.1.1.Macroscale monolayer-based reactors—The benefits of medium flow, often termed “perifusion” when slow flow medium replenishment across the cell surface is employed, have been investigated almost since the beginning of cell culture. Studies have shown improved survival and metabolic function in the form of ureagenesis of primary rat hepatocytes over 6 days when cultures were perfused compared to maintained static with daily medium changes [52]. In a continuous-flow system designed to feed petri dishes in shaker, the kinetics of insulin uptake and stimulation of glucokinase and pyruvate kinase were shown to be more physiological in the perifused system [53].

Early work directed toward extracorporeal liver support applications showed primary dog hepatocytes cultured in a monolayer on collagen-coated plates with perfusion flow across the top of the monolayer retained acceptable urea and ammonia metabolic function for at least 2 weeks, allowing support of anhepatic dogs when very large surface areas (200 plates each 200 cm^2) were used [54]. Hepatocellular metabolic function relevant for liver support improved in this culture system by adapting the collagen gel sandwich format, which provides both matrix stimulatory cues and modulates mechanical shear experienced by cells [55]. A similar flat plate perfusion arrangement used to assess the effects of NPC co-cultured with hepatocytes on the metabolic functions of primary rat hepatocytes confirmed the positive benefit of co-culture and linked function to shear stress (range 1–5 dyn/cm^2) as the rates of albumin secretion declined with application of flow compared to static, while rates of urea and ammonia metabolism were enhanced [56][57].

These early reactors stimulated a more systematic mathematical design-oriented approach to controlling oxygen concentration and fluid shear in low dead-volume reactors by shrinking the channel height to a 0.1–0.5 mm range, incorporating an optional oxygen-permeable upper membrane to de-couple fluid shear and oxygen gradients, and incorporating optional shallow microgrooves (0.1 mm) into the culture substrate to physically protect cells from shear [58,59]. A notable result was that for primary rat hepatocytes co-cultured with J2

fibroblasts under well-oxygenated conditions in a flat plate flow-through configuration, hepatocellular function (albumin secretion and urea synthesis) was well-maintained and comparable to the static control during 3-day perfusion for shear rates <0.33 dyne/cm^2, but showed decline for shear rates in the 5–21 dyne/cm^2 regime [58].

As an alternative to an oxygenation membrane, microgrooves incorporated into the flat substrate allow a high flow rate of medium to deliver oxygen while protecting cells from shear. As expected, very low flow and low shear conditions (<0.5 dyne/cm^2) were associated with oxygen depletion and loss of viability for both configurations but the grooved substrates allowed viability and function to approach that of static control cultures at high flow rates, presumably by maintaining shear <1 dyne/cm^2 compared to 15 dyne/cm^2 for the flat configuration. A very similar flat plate configuration for rat hepatocyte cultures was implemented to create very well-defined, systematically varied oxygen gradients that allowed recapitulation of zonal expression of metabolic enzymes and toxicology of acetaminophen [60][61], with no noticeably deleterious effects on viability or gene expression using flow conditions that resulted in shear stresses of 1.25-7 dyne/cm^2, and hypoxia-related death under very low flow was well-documented to occur zonally. An additional series of studies on shear stress effects on co-cultures of primary rat hepatocytes with liver non-parenchymal cells focused on the 1–5 dyne/cm^2 regime using urea secretion and alanine transaminase (ALT) release as metrics of response, and found enhanced function with perfusion relative to static culture even at the high end of the range though the magnitude of ALT release, a marker of cell damage, was elevated in perfusion compared to static culture even at the low end of the range [56,57]. These studies also showed that increasing shear induced cells to undergo morphogenesis into spheroidal structures (Figure 2).

Taken together, these studies have somewhat ambiguous implications for the effects of the magnitude of shear stress on hepatocellular function in the mid-range ($\sim$ 1 dyne/cm^2), and highlight how challenging it is to uncouple the multiple external cues that cells integrate (adhesion signaling, growth factor signaling from autocrine and paracrine sources, oxygen and other nutrients) to study any one factor in a univariate manner, especially when the phenotypic metrics are not identical. For example, the dramatic improvement in function of primary rat hepatocyte co-cultures with fibroblasts on collagen-modified polydimethylsiloxane (PDMS, silicon rubber) substrates as a means to enhance oxygenation [62] may also arise in part due to more physiological presentation of the adhesion matrix or to partitioning of lipid-soluble hormones and nutrients into PDMS [63,64] providing a local depot regulating gene expression in a favorable manner. Similarly, culture in recessed wells may allow enhanced accumulation of autocrine factors that favorably regulate cell function [65].

Indeed, underscoring this complexity, when primary rat hepatocytes were cultured in collagen gel-covered monolayer configuration using deep cylindrical PDMS wells continuously perfused via inlet and outlet ports placed 1 cm above the culture surface, a configuration termed “MultiChamber Modular Bioreactor (McMB)” by the developers (Figure 3a,b), analysis of cell viability and albumin secretion as a function of flow rate showed a biphasic response to flow. The loss of viability at low flow compared to static

control conditions was attributed to oxygen limitations, while the loss of viability at high flow attributed to shear effects, even though the shear rates were estimated to be well below 0.01 dyne/cm^2 at the highest flow rate [66]. In this configuration, the effective cell concentration was a relatively low 0.02×10^6 cells/mL (in a total volume of 10 mL) and a possible alternative explanation for lower function at higher shear is rapid washing out of autocrine factors for the high (1 mL/min) flow rate. Another approach to protecting cells from shear stress is to shield the monolayer from flow with a flat microporous membrane of a transwell sort, which has the possible additional advantage of increasing polarization via having fluid access on both sides of the monolayer. A commercial perfusion system (Minucell) provides a membrane cassette structure that can be placed in flow, and using this arrangement, Yu and coworkers used a mathematical modeling framework of nutrient and drug transport and fluid shear to guide an investigation of operational parameters (perfusion rates, in situ oxygenation method) that would optimize the viability of primary rat hepatocytes, showing greatly improved metabolic function over 14 days of culture compared to static culture as assessed by albumin secretion, biliary structure, and maintenance of ethoxyresorufin-O-deethylase (EROD) metabolizing capacity, a measurement of cytochrome P4501A induction [67]. Additionally, culture of primary rat hepatocytes in a perfused collagen gel-overlay configuration at an estimated shear stress of 0.6 dyne/cm^2 showed dramatic improvement of multiple hepatocellular functions (albumin secretion, metabolic enzymes, bile acid transport). However in this report, the fluid shear was provided by modification of the transwell culture configuration and the cell concentration was approximately an order of magnitude greater for comparable medium exchange rates in order to employ confluent cell monolayers on a large (75 mm diameter) transwell [68]. This is an intriguing approach for providing access to both sides of the monolayer while providing a “liver sinusoidal” value of shear stress (Figure 3c).

Perfused monolayer culture configurations, such as those described above for primary rat cells, have also been applied to pig cells [69], and were shown to be beneficial for human primary cells relative to static conditions [70][71]. Interestingly, fresh primary human cells cultured in the McMB described above were very robust in long-term (2–3 weeks) culture, when cultured at flow rates of 0.25–0.5 mL/min with a 3-fold greater cell concentration (0.06×10^6 cells/mL). These cells showed greatly improved hepatocellular function compared to static cultures as assessed by gene expression of a large panel of CYP450, Phase II enzymes, and transporter genes with confirmatory drug metabolism activity assays [71]. Metabolism activities for almost all genes except CYP2D6 were found to be comparable to freshly-isolated cells. Although the authors at least partly attribute the enhanced function to shear stress, the values they estimate are several orders of magnitude lower than those in previous studies ($\sim 5 \times 10^{-7}$ dyne/cm^2), hence it is interesting to speculate that factors such as altered autocrine accumulation of extracellular signaling molecules might play a role as well. Greatly enhanced function of cryopreserved primary human cells cultured under perfusion monolayer conditions was also observed in a reactor comprising a sandwich of two $\sim$20 cm^2 circular gas-permeable membranes spaced $\sim$0.5 mm apart, with hepatocytes cultured on the surface of a collagen-coated membrane and compared to collagen gel-cultured static conditions over a period of over 4 weeks [70]. Albumin and total protein secretion rates were significantly higher in the perfused

conditions over the first 3 weeks, though interestingly total albumin secretion in the static condition, while lower than in perfused, was approximately constant over the time period. The differences in albumin may be related in part to the 10% oxygen saturation used in the gas phase as albumin secretion is positively regulated by oxygen up to highly supraphysiological levels [72]. The cells were surprisingly robust over more than 3 weeks, as the cultures were used to analyze protective effects of IL-6 on exposure to 80 μM diclofenac over a period of 3 days; presumably due to the large number of cells used for each reactor ($\sim 10^6$) Untreated controls were not shown, but the results indicated that cells recovered their full function after diclofenac treatment [67].

3.1.2.Microfluidic "flat plate" configurations—The experience described above with various "macro" flat plate configurations yielded several important insights about rat and human hepatocyte and heptatocyte/NPC co-cultures. At the least, phenotypic outcomes comparable to static culture could be achieved, thus providing a way to measure metabolism and toxicity under controlled exposure. At best, reactors improved the phenotypic responses compared to control static culture systems. Cells cultured under select flow conditions can maintain certain metabolic functions at nearly the levels of freshly-isolated cells, which motivates further adaptation of the "flat plate" configuration to more accessible, user-friendly, higher throughput, and scalable technologies. Thus, we review the literature broadly, including analysis of systems that are not commercialized.

Several advances in microfabrication technologies have together yielded tremendous opportunities to create highly tailored microenvironments and flow patterns. These include (i) the application of high-resolution silicon microfabrication technologies to create fine-scale structural features in silicon, the so-called "micro-electro-mechanical systems (MEMS)" devices, coupled with the innovation of using microfabricated silicone structures as molds to cast PDMS parts that can be easily bonded to glass to create fluidic structures and (ii) photopolymerization and masking approaches.

Application of microfluidic approaches to liver cell culture began to emerge about ten years ago in several labs, both as semi-empirical feasibility studies aimed at demonstration of long-term cell survival in PDMS devices [73][74][75][76] and as design-principle based visions of how to transform physiologically based pharmacokinetic (PBPK) measurements and toxicity analysis by creating appropriately-scaled cell to medium ratios, organ cross-talk and in situ cellular activity sensors [77,78]. Leclerc and co-workers exploited the ease of fabricating intricate features in PDMS surfaces to create a device wherein the culture medium flows along the top of a PDMS slab containing square microwells (0.5 mm) and interconnecting angled channels, all recessed 100 μm beneath the top of the flow surface to provide a low shear, relatively uniform flow environment with about 2 cm^2 total of culture surface area [74][73] (Figure 4). They first demonstrated basic operating parameters (flow rates, medium exchange rates, oxygen transfer) for culturing HepG2 cells for 2 weeks in an initial device [73,79]. Common challenges encountered in this microfluidic system included medium evaporation and debris bubble clogging of channels resulting in non-uniform flow, in addition to the complication not encountered with primary cells of the tendency for cell proliferation during culture. With shear stresses estimated in the range of 0.03–0.25 dyne/cm^2 for flow rates in the range of 20–30 μL/min for up to 20×10^7 cells, these studies

showed that for HepG2 frequency of medium change and availability of oxygen for large cell numbers limited the growth and viability of cells, especially when several devices were concatenated in a stack to increase cell number as might be desirable for extracorporeal devices [79].

A series of subsequent papers characterizing HepG2/C3a behavior in this system, mainly in short term culture (4 days), further refined the design and operating conditions. The feasibility of carrying out transcriptomic, proteomic, metabolic and toxicologic characterization with this arrangement, was illustrated and showed that HepG2/C3a phenotypic responses including albumin secretion, sensitivity to acetaminophen toxicity, and relative expression of CYP450 and transport genes appeared more physiologically close to primary hepatocytes in the microfluidic compared to static petri dish culture, a result that may in part due to the quasi-3D aggregate structures that form in regions of the recessed structures [74,79,80][81–84]. Initial studies analyzing primary rat [85,86] and human [74,87] cells in short term culture (4 days) indicate that these cells were less likely to form aggregates, and optimization of operating conditions for these cells is evolving. The results assessing drug metabolism with cryopreserved human cells are instructive in two key ways, first showing that these cells attach and survive in both the recessed features as well as the top of the PDMS exposed directly to flow. Secondly, experimental confirmation that after 4 h of perfusion in cell-free devices, hydrophobic drugs such as midazolam, dextromethorphan, and desethylamodiaquine are very significantly (60–95%) depleted due to partitioning into PDMS, a phenomena reported by others for hydrophobic compounds in PDMS devices [63,64], a topic we discuss in more detail in Section 4.

Fujii and coworkers developed the concept of protecting cells from shear by perfusing the opposite side of porous flat membrane with the cultured cells using microfluidics. A bilayer PDMS device incorporated either a polyester or microfabricated PDMS membrane between two PDMS slabs containing a well above the membrane for cell monolayer culture and flow channels below the membrane [76]. They showed that in the perfused microdevice, albumin secretion rates of primary rat hepatocytes over two weeks of culture were stable, comparable to those of cells maintained in transwell-type membrane inserts in static culture, and were much higher than those in standard 2D petri dish culture. Cells in the perfusion culture demonstrated slightly superior conversion of ammonia compared to cells in static transwell culture, so this demonstration shows how a device might be used in a continuous mode. Borenstein and coworkers described a similar approach to protecting hepatocytes from shear due to high-velocity flow, while also providing a suitable culture substrate to induce cell polarization [75]. They created a bilayer PDMS device incorporating a 0.4 μm pore-size polycarbonate membrane separating the medium flow from the hepatocyte layer cultured on the membrane above the flow (Figure 5a,b). An intricate microchannel flow path inspired by vascular network geometry, essentially created a microscale version of transwells perfused on the bottom of the membrane by a “vascular network” etched into the adjacent PDMS layer, which exposed the hepatocytes to negligible levels of shear stress. Each region of the hepatocyte monolayer on the membrane was in contact with a “vascular channel” for about 25% of the monolayer surface (Figure 5b,c). Although this sparse network might seem limiting, both HepG2 and primary rat hepatocytes remained viable in the system for at least a week, provided the polycarbonate membrane culture surface was coated with collagen and

the flow rate was adjusted to accommodate the metabolic needs. Primary hepatocytes increased their per-cell rate of albumin secretion over a week of culture in the device, and secreted albumin at higher rates than in control static cultures [75]. Subsequent diffusion-reaction modeling of oxygen transport and consumption in this device suggests that oxygen may become limited at high cell densities [88], however experimental verification of oxygen gradients would shed more light on the apparent high viability of dense hepatocyte monolayers. To our knowledge, these devices have not been commercialized.

The potential of microfluidic systems as "micro cell culture analogs (μCCA)" of humans to represent PBPK was analyzed and demonstrated in the seminal work by Shuler in co-workers [77,78], and the term μCCA is now commonly used in the in vitro tissue bioreactor community to designate the organ-cross talk system pioneered by Shuler. A detailed analysis of scaling of in vivo organ residence times and how to translate them into interlinked multi-organ crosstalk systems within constraints of low fluid shear stress and oxygen delivery was presented. The multi-organ crosstalk systems were represented by monolayer cultures of lung, liver, and fat cell lines, and the fluid shear stress was constrained to less than 5 dyne/cm^2 for the liver compartment (Figure 6). Further, they provided proof of principle for naphthalene toxicity in a cross talk of human HepG2/C3a cells with lung cells[78]. While interacting micro-organ systems are discussed in more detail below, these papers made interesting observations about how the morphology and function of liver cells was exquisitely governed by cell density, flow rates, and matrix coatings in the liver compartment where collagen or fibronectin was required to prevent cell detachment. These studies also underscored the difficulties in obtaining defect-free devices given the tendencies of bubbles and debris to clog the systems. A straightforward PDMS microfluidic system for culturing HepG2 on collagen-coated glass under shear stresses ranging from 1.4–60 dyne/cm^2 confirmed observations from macroscale systems that sensitivity to shear appears to have onset around 5 dynes/cm^2. In this system, shear stress was systematically varied not only by flow microchannel dimensions but addition of high molecular weight dextran to the medium to increase medium viscosity, and thus shear for a given flow rate. In 3-day culture experiments, cells at higher shear rates became progressively more rounded, detached, and reduced secretion of albumin[89] (Figure 7). A variation on this original design is being further developed in collaboration with a commercial partner under the auspices of the NIH-funded "Microphysiological Systems Program."

The challenges in translating the benefits of perfusion culture for more sensitive primary cells from macroscopic to a microfluidic format are illustrated in a study employing the well-characterized rat hepatocyte-fibroblast micropatterned co-culture system described above to a PDMS device comprising a microperfused 8 × 8 array of interconnected (i.e., not separately addressable) 3 mm diameter micropatterned wells. Channels were fed by a flow rate constrained to keep shear well below 0.01 dyne/cm^2 while providing 1.4 mL/day of medium in a one-pass configuration, and providing oxygen by a secondary network of gas flow channels above the medium flow channels [90]. In cultures maintained for 32 days, albumin and urea secretion rates, interestingly, stabilized after 5 days in both perfused and control cultures of comparably-seeded static cultures open to a medium volume of 4 mL, but the rates in the perfused system were only ∼50% of the controls. The significant decrease in absolute level of metabolic performance in the perfused microfluidic system was speculated

to arise from possible differences in local concentrations of soluble signaling factors, oxygen limitations, or nutrient limitations, as the shear stress appeared to be within an acceptable physiological range. Another implementation of the co-culture arrangement involves co-culture in the “liver” compartment of the HμREL® chip [91], which was designed based on the principles described by Shuler and coworkers, where the authors demonstrated clearance of several compounds by cryopreserved human hepatocytes was more physiological compared to the chip containing hepatocytes alone. Commercial applications of the HμREL® chip are still evolving.

As outlined by Shuler, these issues, along with smallness of the volumes and cell numbers in the microfluidic systems, drives incorporation of on-board instrumentation to monitor cell activity and function noninvasively. His group illustrated this by measuring CYP450 activity non-invasively during culture of HepG2/C3a cells embedded in Matrigel in the μCCA device described earlier [92]. HepG2/C3a cells in the μCCA showed appreciable basal levels of CYP450 activity in a manner not previously observed in standard culture, although comparison to static cultures in Matrigel may have revealed contributions of Matrigel alone. This work stands out for the very detailed analysis of the reaction/diffusion effects on the conversion of CYP450 substrates in the μCCA, including a breadth of investigation into effects of cell density and substrate concentrations.

Whether various flat plate microfluidic reactor configurations focused on liver will gain significant traction in drug discovery pipelines or in more general liver physiology research is still an open question, as various implementations of traditional macroscale flat plate reactors described earlier remain accessible and can provide high levels of function. Macroscale systems in principle require more cells and reagents, which make microscale systems appear attractive for saving costs of primary cells and experimental compounds that may be expensive to synthesize early in the drug development process. Tradeoffs in microscale systems include drug adsorption and complex operation, and other approaches are continuously being developed to address specific assays such as low clearance compounds and reactive metabolites [93]. Thus, the advantages of microfluidic systems may be greater for more complex applications that require microscale 3D architectures to retain function, or applications where interconnection with other microscale organ mimics provides greatly enhanced information [94–96].

3.2. Evolution of hollow fiber bioreactors as prototypical 3D liver culture environments

The phenomenological advantages of 3D culture of liver cells arguably dates back to experiments culturing hepatoma cells in some of the earliest incarnations of hollow fiber reactors [97]. Since hollow fiber technologies are still being actively pursued today for 3D liver physiological culture [98], a review of the evolution of hollow fiber bioreactor technology for liver cell culture establishes a contextual foundation for other 3D bioreactor technologies.

Hollow fiber cell culture reactors emerged over 30 years ago as a means to culture mammalian cells on a large scale for biotechnology applications, such as protein and vaccine production, complementing approaches based on stirred-tank reactors and roller bottle cultures [99]. Commercial user-friendly versions implemented with pH, oxygen, and

medium exchange controls are in use today for production of proteins, vaccines, and other therapeutics [100]. Rooted in technology developed for kidney dialysis and protein purification, the classic hollow fiber mammalian cell culture reactors comprise a bundle of semipermeable hollow fiber ultrafiltration or dialysis-type membranes encased in a housing such that liquid pumped down the fiber lumens exchanges molecular components with cells cultured in the extracapillary space outside the fibers; i.e., exchange occurs in a manner that mimics facets of capillary blood-tissue molecular exchange in vivo. Because the membrane physically separates cells from fluid flow, high fluid flow rates can be maintained inside the capillary to minimize gradients along the capillary length, while protecting fragile cells from shear forces of flow. This same arrangement protects cells from the stresses of the air-liquid interface that occurs in roller bottle culture. Other major design features include small fiber dimensions on the order of 0.2–0.4 mm diameter and close packing of fibers. This affords a high surface-to volume ratio at the same time that the diffusion distances from the central fiber lumen, the source of oxygen and fresh nutrients, to and through the tissue mass outside the fibers are kept within a range of a few hundred microns to support in theory adequate oxygen and nutrient delivery to the entire cell mass.

An interesting feature and challenge of the hollow fiber configuration is the control of molecular exchange properties between the nutrient feed and tissue compartment by selection of membrane permeability properties. As a practical matter in protein production applications, appropriate choice of permeability allows the desired protein product to either be concentrated in extracapillary space which has relatively low contamination by serum proteins in medium, or to diffuse into the lumen and be automatically separated from cells on a continuous basis, thus facilitating subsequent purification in either case. One of the earliest reports of hollow fiber epithelial culture, of colon cancer cells to produce carcinoembryonic antigen (CEA), illustrated that low-permeability (10 to 50 kDa cut-off) membranes enhanced differentiated function of cells as the tumor cells formed 3D organoid-like structures, secreted mucus, and produced higher rates of the CEA relative to 2D cultures [101]. While enhanced function is likely partially attributed to the adhesion environment driving a 3D structure, the serendipitous concentration of autocrine survival, growth and differentiation factors in the pericellular environment can be greatly enhanced in the hollow fiber arrangement compared to 2D culture.

The application to liver cell culture was almost immediate, first with a report that a rat hepatoma line could carry out bilirubin conjugation in hollow fiber culture with either 10 kDa or 50 kDa cut-off membranes [97], with speculation that the rates of conjugation may be limited by the transport of the bilirubin carrier protein albumin across the membrane. Next it was found that primary rat cells cultured up to 45 days in a 7-cm long hollow fiber reactor with 520-0.3 mm diameter fibers maintained almost half the diazepam-metabolizing capacity after 10 days provided the perfusion medium was equilibrated with 30% oxygen atmosphere [102]. Not surprisingly, given the similar design features between hollow fiber cell culture reactors and blood dialysis units, liver cell culture in hollow fiber reactors soon emerged as a potentially promising approach for extracorporeal liver support to provide metabolic capacity in synthesis of urea and metabolism of toxic metabolites like bilirubin [103][102,104][105][106][107]. Detailed reviews of early work of the approximately dozen groups using various bioreactor formats for clinical extracorporeal liver support, including

analysis of clinical constraints, can be found in a review by Rozga and co-workers [108], while later developments and clinical applications can be found in more recent reviews [109][110][111][112][113][114][115]. Here, we summarize findings and observations as context for in vitro culture models and focus on reactor configurations that have been scaled down for use in drug development.

While no precise consensus exists on the minimum number of liver cells needed in an extracorporeal device to support a liver failure patient, in part due to differences in types of cells and use of adjuvant charcoal-stripping steps to remove toxins from patient plasma, estimated needs range from 10–20% of the patient's liver mass. The scale is unequivocally enormous – tens to hundreds of grams of cells – a scale that presents an almost insurmountable barrier to using primary human hepatocytes on a routine basis, especially when the use of reactors is on an emergency and not pre-scheduled basis. Hence, of five reactor systems that progressed to clinical trials in the US and Europe, four were developed for use with primary porcine cells, one with the human HepG/C3a cell line [115] and nascent incorporation of HepaRG cells [116]. Translation to the clinic has been arduously slow, hampered by the high cost, difficulty in obtaining clear-cut results in clinical trials, and competition for subsets of patients by a cellular reactors that remove toxins [115].

Based on encouraging results for long-term liver cell survival in clinical-scale devices, at least two groups have developed scaled down versions of their clinical reactor for application in drug development and study of liver physiology. The system developed by Gerlach and co-workers employs an appealing design that to a certain extent uncouples oxygen delivery from transport of molecular components in the medium [98,117– 121]. The design comprises three separate sets of hollow fiber interwoven so that culture medium flows between two sets of fibers in an almost interstitial-like manner. The third set provides a flow of gas containing a pre-set oxygen concentration, so that all regions of the reactor can be fed by oxygen locally (Figure 8). Cells are seeded into the extracapillary space and maintained under perfused conditions. Early studies aimed at clinical application using a reactor volume of close to 1 L showed, promisingly, that primary porcine cells formed tissue-like structures incorporating non-parenchymal cells, and that biochemical activity was relatively stable over 30 days [118]. The principles for scaling the reactor from the clinical scale (800 mL, 1.4×10^{10} cells) to an intermediate scale (8 mL, 2.6×10^{8} cells) and a small scale (2 mL, 1.2×10^{8} cells) were illustrated using 3 week cultures of fresh primary human cells from 5 different donors, where the medium refreshment rates and perfusion flow rates were scaled for each reactor size based on computational models previously developed by the group. Immunohistochemistry revealed formation of tissue-like structures with expression of markers of hepatocytes as well as biliary cells (CK19), Kupffer cells (CD68) and endothelial cells (VWF), and polarization of key transporters. Interestingly, the specific per cell rate of secretion of albumin was greatest for the 2 mL reactor. Drug metabolizing capacity (phenacetin, Cyp 1A; midazolam, Cyp3A; diclofenac, Cyp2C9) declined 50–80% from the day 3–5 period to the day 10–23 period [122]. A subsequent study with this design further miniaturized to a 0.5 mL volume (25×10^{6} cells) showed that primary human hepatocytes maintained for 10 days in culture performed comparably well in terms of protein secretion and drug metabolizing capacity across 4 drugs in serum-free medium compared to serum-containing medium, but that serum-free culture enhanced the expression

of several transporter genes [119]. This same system enhanced the differentiation of human fetal liver cells compared to static petri dish culture, especially when combined with a hyaluron-based extracellular matrix for embedding the cells [123,124], and has been extensively characterized for differentiation of the human hepatic cell line HepaRG, illustrating stable differentiation [120].

An alternate hollow fiber design combined fiber matrix sheets interspersed with hollow fibers to similarly address the high oxygen demands of hepatocytes by providing a set of hollow fibers for gas exchange in the context of adhesion to the fibers was developed by Chamuleau and coworkers for clinical application and has also been scaled down from a clinical version to a version suitable for pharmacological studies [125– 127][128,129] [116,130,131]. The large clinical-scale version of this reactor system was showed comparable to the Gerlach 3-fiber system in maintaining liver-specific function of primary porcine cells over 7 days in culture, and both maintained >90% function of the 10 billion cells seeded [132]. The hepatocytes in this system occupy several different niches and experience a complex combination of cues where some of the cells attach within the walls of the fiber mat and are distant from the source of oxygen, and others form aggregates in spaces between the hollow fibers [127]. In the liver, glutamine synthetase (GS) is normally expressed in the pericentral zone, a region that experiences the lowest oxygen concentration, and carbamoyl phosphate synthetase I is normally expressed in the periportal and central zones. An interesting analysis of localization of expression within the bioreactor of GS and carbamoyl phosphate synthetase I showed that cells near the gas capillaries (i.e., cells experiencing the highest oxygen concentration) expressed the highest level of GS, in contrast to what would be expected in vivo. The authors suggest that combinations of substrate and autocrine factors may contribute to this zonation pattern [127]. This reactor system has also been optimized for stable differentiation and maintenance of HepaRG cells in long-term culture in the absence of DMSO for use in drug metabolism studies [116,130,131].

Although hollow fiber reactors incorporate many elegant design principles for uncoupling fluid flow, matrix, support, and medium perfusion, others have observed that “despite these advantages, the high cost and difficult set-up of hepatocyte (hollow fiber reactors) probably limit their use in drug metabolism and transport studies” [113]. Adsorption of hydrophobic drugs to the components of hollow fiber systems may also be a limitation in some applications, and together with challenges in in situ imaging of tissue structures and relative difficulty in accessing the tissue space, these reactor formats may need additional evolution to enter routine use. Some of the publications for the scaled-down versions of hollow-fiber reactors were co-authored by investigators in pharmaceutical companies, and these investigators are now a resource in the community for providing perspective via personal communication on the strengths and weaknesses of these systems.

3.3.Stirred suspension bioreactors for liver cell culture

The propensity of hepatocytes to spontaneously aggregate when seeded on poorly-adhesive surfaces, and for aggregates to maintain liver-specific functions to a greater degree than cells in monolayer culture, has been observed and reported for decades [133][134]. Formation

and maintenance of spheroids in static culture is almost an art, as cell seeding density, culture medium depth, and other factors all conspire to create local microenvironments that are difficult to control (particularly the concentration of oxygen) and reproduce across labs. Stirred-tank bioreactors have long been used in large-scale culture of mammalian cells to provide an environment allowing control of oxygen, pH, and continuous addition and removal of medium. With appropriate design of impellers, the fluid mechanic microenvironment in such systems can provide relatively uniform low-shear mixing and the commercial availability of reactor systems makes them accessible for general use. The utility of such a reactor configuration for efficient (80% of cells incorporated) formation of relatively uniform, functional spheroidal aggregates of primary rat hepatocytes that exhibit evidence of appropriate polarization of bile canaliculi was illustrated over 15 years ago [135].

Recently, Alves and co-workers applied this approach to primary human hepatocytes from 3 different donors showing that appropriate choice of operating conditions such as stirring rate, maintenance of culture oxygen level at the physiologically-relevant level of 60 μM led to formation of aggregates with average dimensions of 80 μm diameter by the end of the second week of culture [136]. This length scale was expected to be well below diffusion limitations for oxygen, though steep gradients in proteins from the medium as well as autocrine factors would be expected. Interestingly, cells were maintained in serum-free medium after the initial 3-day aggregation period, and the per-cell rate of albumin secretion increased over two weeks of culture for all donors. Immunohistochemistry of the spheroids after two weeks of bioreactor culture revealed an interesting distribution of albumin concentrated around the periphery and somewhat depleted in the spheroid core and Cyp3A was strongly expressed in about half the cells with no particular localization within the spheroid (Figure 9). Similar to the previous observations with rat cells, the human spheroids exhibited strong evidence of a bile canilicular network penetrating the spheroids, as assessed by proper apical polarization of atypical protein kinase C (aPKC) and excretion of fluorescent 5-(and-6)-carboxy-2’,7’-dichlorofluorescein diacetate (CFCDA) into the biliary structures. In general, the spheroids maintained high, stable levels of CYP gene expression and ability to be induced by rifampicin to metabolize ethoxycoumarin. This study also highlighted the high degree of variability between donors: a 10-fold difference in absolute levels of albumin secretion despite similar trends of increasing per cell rates of secretion over 2 weeks, variability in the formation of actin stress fibers and polarization, and differences in trends of gene expression of CYP enzymes as a function of time. Presumably, different donors had different initial states, and all adjusted to the environment in the bioreactor. These observations underscore the need to characterize systems with multiple different donors in order to generalize results.

The strength of using the stirred-tank bioreactor is that the conditions are well controlled potentially improving overall success given large interdonor variability, and a large number of spheroids can be maintained for extended periods, thus allowing efficient use of large numbers of cells from a single donor. Screening a large number of conditions against the spheroids would require a step of removing the spheroids and dispensing them to individual wells or flow-through systems for applying the test conditions, a process that should be entirely feasible. In previous studies, spheroidal cell aggregates seeded into a support

scaffold attached to the walls of the scaffold and formed tissue-like structures through which medium could be perfused [137–139]. Hence, this culture method may provide a means to maintain aggregates on a large scale in a rather inexpensive bioreactor for later seeding into a more complex bioreactor format where perfusion directly through aggregates enables additional functionalities such as seeding with tumor cells to study metastasis.

3.4. Reactors for liver slice culture

Liver slice culture occupies an important niche in the spectrum of in vitro liver models. Liver slice culture typically involves coring out a 0.5–1 cm diameter cylinder of liver tissue from donor material, then slicing the cylinder into sections 100–200 μm in thickness. The resulting slices are maintained in some sort of agitated culture system such as on a shaker or mini-roller bottles to enhance transport of nutrients, oxygen, and drugs to the surface of the slice. This approach has been applied to livers of many species and has been especially well-developed by Groothuis and coworkers [1,140–144], and is attractive because it allows efficient use of donor material and creates cultures with a physiological balance of both hepatocytes and NPC. Hence, it is attractive as an approach to study inflammatory responses including early stages of stellate cell activation in fibrosis and drug-induced liver injury; although it is somewhat limited by the relatively short life span of slices in culture (on the order of days), even when they are maintained in equilibrium with supraphysiological concentrations of oxygen in the gas phase [1,140–144] (Figure 10).

A comparison of several different methods of convective mass transfer including rocking, perfusion across the top of the slice, and perifusion around both sides of the slice showed that the viability and performance of rat liver slices is improved with constant perifusion, i.e. with controlled continuous feed and removal of medium that also provides convective mass transfer to both the surface of slices [145]. An innovative microfluidic system comprising a 25 μL chamber to hold devices in a flow path was designed by Groothuis and Verpoort to provide perifusion and simultaneously improve detection of short-lived metabolic intermediates [3,64,142–144], exploiting the single-pass perfusion capability and high cell to medium ratio in microfluidic devices as described above in seminal work applied to hepatocyte cultures [77]. By connecting the microscale perifused culture system to a drug infusion system and to an outlet valving system that enabled online sample injection into an HPLC, they were able to demonstrate quantitative Phase II metabolism in rat tissue of ethoxycoumarin to its glucoronidated, sulfated, and hydroxylated metabolites, and were able to capture unstable Phase II metabolites of diclofenac [146]. In these initial studies, slice viability was limited to about 24 h, but longer-term survival could be improved to over two days by embedding the slices in Matrigel within the device [144]. Matrigel likely contributed in many ways to improved function as it prevented the slices from sticking to one of the microdevice surfaces enabling the maintenance of perifusion, but may also have provided supportive matrix and growth factor signaling to the cells on the surface of the slice. It is possible that longer-term survival might be achieved by further improvements in oxygen delivery. Although the medium in these studies was equilibrated with a gas phase comprising 95% oxygen, the medium flow rate is 10 μL/min for $\sim 0.5 \times 10^6$ cells, which would be expected to deliver only about 20% of the oxygen needs of the tissue based on our finding that a flow rate of 250 μL/min of medium equilibrated with 20% oxygen was

required to maintain a comparable number of cells in a 3D reactor where oxygen concentrations post-perfusion were measured [147]. Excellent perspective on how these types of microfluidic systems for slice culture and other forms of culture, including limitations of the PDMS materials and possible mitigations of these limitations [64], is provided in recent reviews by Groothuis and co-workers [1,3,143].

3.5. Microscale and microfabricated reactor systems for 3D liver culture

As described in sections above, microfluidic reactor systems have substantial attraction for increasing throughput via multiplexing, decreasing cell and reagent requirements, enabling automated in situ metabolic monitoring via sensors or imaging, allowing on-line analytical capabilities, and enabling PK scaling, especially when concatenating multiple organ systems [95][3]. Arguably their greatest potential is in facilitating formation and very local microperfusion of capillary bed-sized tissue structures that mimic features of the in vivo tissue organization and function, and many methods are emerging to integrate the diverse expertise required in biomaterials, microfabrication, microfluidics, and liver cell biology to meet these needs [139][48][148] .

The ability to culture cells at high density to drive tissue formation without exogenous matrix has driven the design of several “gel-free” microdevices. A seminal example of exploiting the high-resolution, arrayable features of microfabrication to create 3D microenvironments in this regard for hepatocyte culture is the sinusoid mimic developed by Lee and co-workers [149][150] (Figure 11), based on prior work demonstrating feasibility with other cell types. The design comprises a 3-layer sandwich consisting of a glass support slide, a PDMS layer containing the complex flow/membrane barrier features created by microfabrication, and an upper acrylic plate bonded to the PDMS layer. Cells are seeded at high density into a “hepatic cord” region 150 μm wide, 30 μm high, and 440 μm long that is separated from a 50 μm wide flow path by a PDMS wall containing 2 μm sieve holes along the top. The flow path circumvents the entire cell-containing region such that a slow convective flow moves through the sieve region across the cells in the cord due to the pressure drop between the inlet and outlet of the flow path. Cells are loaded as a suspension in controlled fashion into the cord-region to achieve close-packing and high density; each cord holds about 500 hepatocytes. When seeded with HepG2/C3a at low density (∼100 cells) and maintained until confluence, cells secreted more albumin than controls in dishes [150] and primary human cells seeded at high density showed a toxic response to diclofenac at high concentrations (IC50 or 334 μM). With primary rat hepatocytes, a feature of the culture system is that tight packing promotes cell-cell contacts similar to those in spheroid culture such that matrix is not needed to maintain function [149]. Interestingly, seeding at low density results in rapid loss of viability of primary rat hepatocytes. In this system, the cell mass likely acquires a quasi-3D structure, as the height of the culture region (30 μm) is comparable to the size of a hepatocyte. Increasing the depth of this region may be difficult, as the aspect ratio of the “sinusoid” wall features is high. Although this device design minimizes the use of PDMS through using glass and acrylic as the top and bottom layers, the presence of PDMS may still impair widespread use in drug development with hydrophobic drugs. This reactor system was commercialized by CellASIC (Hayward, California) and is

now marketed through EMD Millipore, though publications describing its use by pharmaceutical companies for liver applications are not yet available.

Another approach to creating a gel-free microfluidic tissue culture PDMS microdevice has been developed over several iterations by Yu and co-workers (Figure 12a,b)[48,151–154] [155][156]. Their configuration includes a synthetic matrix added post-seeding to stabilize the cultures, and appears to be designed for moderate and not high-density cell culture. In this arrangement, a central region for cell culture is separated from adjacent 200 μm wide flow channels on each side by parallel rows of “micropillars”, creating a porous boundary that delineates a culture chamber 200 μm wide by 600 μm long by 100 μm height. In the original description of the device, following seeding of primary rat hepatocytes, the culture was not strictly matrix-free, as support for the cells was added by post-seeding coacervation of a collagen-synthetic polyelectrolyte to stabilize the 3D arrangement of cells, and this arrangement led to viability over 72 h along with evidence of metabolic function [151]. Similar manipulation of cells to promote aggregation was subsequently shown for HepG2/C3a culture in the same device, where chemical modification of the cell surface was used to drive cell aggregation [155]. The role of the coacervated collagen matrix is not entirely clear, and may possibly be a means to keep cells stable in a 3D configuration without packing them to a high density. The dimensions of the culture chamber suggest that dense packing of tissue density may create an oxygen-limited situation, as oxygen diffuses into the 200 μm wide culture region only from the sides with micropillars, and the pillars obstruct about 50% of the available surface area for diffusion. With the added matrix and the cell densities employed, the function of rat hepatocytes appears to be improved in terms of absolute albumin secretion per cell and glucuronidation in three days of culture. Though in a multiplexed format, the rate of albumin secretion declined to a greater extent in the microfluidic arrangement [156]. Inclusion of microbeads releasing TGF-β1 along with the cells when seeding improved the functional behavior of primary rat hepatocytes and increased their sensitivity to the toxicity of acetaminophen (APAP), though it is not clear whether empty beads may also have played a role in improving cellular function [157]. In a recent study with the same device, an improved method of seeding that involves no added matrix and instead uses a mechanical compression to aggregate cells appears to improve the functional behavior, hastening the appearance of bile canaliculi compared to standard seeding. Interestingly, although the per cell albumin secretion rate is higher in the microfluidic device than in standard monolayer culture initially, the rates are comparable after 5 days of culture [153]. To our knowledge, this system has not been commercialized.

An interesting design with similar aims to create a cord-like structure using matrix-free high-density seeding was described by the group at Corning Incorporated [158] (Figure 12c). In this design, the central cord-like cell culture region is also bounded on two sides by micropillars that provide exchange with the adjacent flow path. However, this design features tiny micropillar supports on the bottom of the culture region to promote 3D cell-cell interaction, and a means of perfusion of the region below the cells with the aim of capturing secreted biliary products although this was not specifically demonstrated. The dimensions of the culture region are not specified in the text, but scale bars in the figure suggest the width of the culture region is about 100 μm and the authors suggest based on ATP measurements that the region holds about 10,000 hepatocytes. There is clear evidence of 3D tissue-like

structure formation after two weeks of culture of primary human hepatocytes with a perfusion rate of 5 μL/h, while non-perfused cultures did not survive. The notable result was retention of polarized MRP2 expression and biliary transport function in the 3D culture for two weeks, compared to 2D culture. This is a promising step toward creating an independently accessible biliary network, though the PDMS might prove highly absorptive to many compounds solubilized in bile. Other tissue engineering approaches that have not yet been adapted to microfluidic culture but that enhance biliary function as assessed by production of biliary components may provide additional ways to manipulate cells in microfluidic culture [159]. Further applications or commercial fate of this Corning Incorporated device are as yet unpublished.

Our lab has combined microfabrication approaches and microfluidics to recapitulate tissue-like features on the scale of the hepatic capillary bed, using hepatocytes or mixtures of hepatocytes, NPCs, and tumor cells, taking into account the constraints on oxygen supply and demand, shear stress, ease of set-up and use, and constraints of materials of construction that are compatible with a range of drug types [137–139,160–162][147,163][164]. The move to silicon microfabrication approaches arose from combined observations that (i) control of biophysical forces in the microenvironment could drive morphogenesis of hepatocyte spheroids and mixed cultures of endothelial cells and hepatocytes [133,165] and (ii) and that though such biophysical cues could to some extent be captured by scaffolds generated through three dimensional printing approaches, allowing tissue-like structures to evolve in morphogenic fashion under perfusion flow in a bioreactor format [160]. The resolution of the 3DP™ printing process was inherently limited to length scales of 200–300 μm for the materials of interest.

We thus designed a simple reactor system for 3D morphogenesis and continuous perfusion, employing as a scaffold a 200 μm thick silicon wafer “chip” fabricated via deep reactive ion etching with an array of 100 −1000 through-holes (depending on the application) with a 300 × 300 μm rounded square cross-sectional geometry. This geometry was determined semi-empirically to drive morphogenesis of 3D structures when coated with a concentration of collagen I that fostered a moderate level of adhesion of rat hepatocytes [138,139]. This scaffold was placed atop a microporous membrane, backed by an identical support scaffold, and placed in a perfusion reactor arrangement that provided independent flow streams across the top and bottom of the “chip”, and for slow flow through the tissue in the channels of the chip, where flow through the tissue could be reversed in direction (Figure 13a). The importance of the filter was 2-fold: (i) cells or spheroids seeded into the channels were initially kept in place by the filter until they could adhere to the walls of the channels under constant perfusion flow directed toward the filter (flow was reversed after adhesion), and (ii) the filter was instrumental in distributing flow relatively uniformly through the array of 100–1000 different tissue units during operation even if the tissue structures were different in different channels of the array, as the filter provides the overwhelming hydraulic resistance to flow across the filter-tissue combination. Initial studies with this configuration demonstrated morphogenesis including presence of cells with features of fenestrated endothelium, accompanied by sustained retention of albumin secretion for two weeks of culture at much higher levels than monolayers or spheroids, and evidence of local tissue

microperfusion as evidenced by 2-photon observance of expression of EGFP following adenoviral gene delivery [139][138].

This arrangement led to enhanced retention in primary rat hepatocytes of liver-specific gene expression (transcription factors, nuclear receptors, transporters, and Phase I and Phase II enzymes) and drug-metabolizing capacity (testosterone metabolism), over two weeks of culture [137]. This system also fostered morphogenesis of heterotypic structures of rat hepatocytes and non-parenchymal cells such that sinusoidal endothelial cells could be maintained in the absence of VEGF for two weeks in culture [164]. The findings that Kupffer cells and endothelial cells exhibited robust survival for two weeks led to application of heterotypic cultures, scaled to get the approximate ratios of hepatocytes to stellate cells, sinusoidal endothelial cells, and Kupffer cells, to the problem of inflammation-stimulated idiosyncratic toxicity [37]. This latter study included analysis of clearances of several drugs, including hydrophobic compounds, and demonstrated that heterotypic cultures including immune cells provided a robust inflammatory response that could potentiate drug toxicity. While the initial versions of the system used standard peristaltic pumps to circulate medium, a microfluidic pumping system was developed to improve materials compatibility with drugs, and allow multiplexing in a multiwell plate format while preserving the capacity to maintain sinusoidal endothelial cell survival [147,162] (Figure 13b). The microfluidic pumping system multiwell plate design operating parameters are guided by creating a physiological oxygen gradient across the tissue, as measured by oxygen microprobes [147]. We recently developed an improved microprobe system to provide multiplexing across multiple plates for in situ monitoring of function [72]. Although the scaffold design is relatively simple, it allows facile culture initiation, the ability to set up functional heterotypic cultures, and provides a format to grow tumor cells in the context of a liver microenvironment [163]. This system has been commercialized by CN Bio Innovations, Ltd (formerly Zyoxel, Ltd; Oxford, UK).

In addition to this system, microfluidic and microfabrication systems are increasingly being combined to create 3D heterotypic interactions between hepatocytes and non-parenchymal cells, often drawing from approaches used for other organ systems. A microfluidic system comprising two flow channels separated by a central gel-containing region, used for analyzing angiogenesis across geometrically well-defined gels and chemical gradients with high-resolution imaging [166], was applied to analyze the process of angiogenesis into 3D hepatocyte tissue structures [167]. Although this study was limited by the use of collagen gels and endothelial cells derived from non-liver sources, the approach demonstrated robust formation, survival and function of 3D hepatic structures from primary rat hepatocytes in a format amenable to high content imaging. This system has recently been applied to analysis of extravasation of tumor cells from microvascular networks [168], and accordingly may find application in more complex liver angiogenesis applications in the future. This system is being commercialized by AIM Biotech in Singapore.

Recently, a clever and provocative use of microfluidics was described that allows the organization of hepatic tissue “cords” of primary rat hepatocytes surrounded by mouse Swiss 3T3 albino fibroblast cell line, leading to very long term function (60 day) when cells were maintained in static culture [169]. The dimensions of the hepatic cord were on the

order of approximately two cell diameters, appealing for many applications. Further work is needed to determine whether these tissue cords can be transferred to perfusion systems in a manner that allows controlled flow of nutrients and drugs.

3.6.New technologies for capturing liver complexity

A host of ways of combining microfluidic technologies with advances in synthetic biomaterials are emerging, and will likely greatly enhance the ability to create multi-scale tissue models that can be maintained in continuous perfusion [48,170]. In addition to sophisticated experimental tools for constructing controlled cell and tissue microenvironments, there is a growing appreciation for the quantitative rules that govern how cells and tissues integrate chemical and mechanical cues. Design-based tissue engineering is becoming more common as we gain a greater understanding of how cells respond to microenvironmental perturbations and how environmental inputs can come together to generate a basal phenotype [4,171]. Already, a few straightforward experimental tools used for in vitro models have had high impact because they are both easy to adopt and capture a complex in vivo situation in quantitative fashion. For example, an accessible and popular “tissue engineering tool” employs 2D polyacrylamide gel substrates that can be tuned to specific bulk stiffness and modified with various extracellular matrix molecules. This system has been used to illuminate the interplay between extracellular matrix stiffness and soluble signaling cues in driving fibrotic phenotype of stellate cells [172]. Matrix stiffness, together with biophysical presentation mode of epidermal growth factor (EGF) also influences morphogenesis of primary hepatocytes in quasi 2D culture [173] and 3D matrix stiffness influences differentiation of hepatic progenitors in hyaluronan gels [174].

Further, a range of “solid free form fabrication” (SFF) methods are starting to be deployed in conjunction with microfluidics. Most methods make scaffolds or sacrificial molds for cells, though they can also be used to manipulate the cells themselves, with varying degrees of success in maintaining cell viability. All such methods fabricate complex 3D objects as a series of thin layers, guided by a computer programs that direct creation of solid features within each layer [48,161]. Three-dimensional printing (3DP) illustrates key features that define any SFF process: feature size resolution (i.e., the smallest size line or building-block that can be created), flexibility to create features over a hierarchy of scales within the same object (e.g., the ability to create branching vascular-type architectures from artery-size down to capillary-size), speed of fabrication (usually inversely related to resolution), and ability to handle multiple different kinds of materials especially in the same build process [175]. The 3DP printing process creates scaffolds by depositing drops of a binder or glue from a print head or nozzle onto a layer of fine powder of a (bio)polymer or ceramic, such that wherever drops of the solvent are printed, particles are glued together. The 3DP process initially seemed appealing for fabricating scaffolds for seeding with liver cells, because it can create branching structures across several hierarchies of scale, can handle multiple materials in a build, and is relatively fast, but as described above [160], features size resolution is practically limited to ∼200 μm by the physics of droplet generation and particle binding. Direct printing of cells embedded in matrices that gel on deposition (e.g. collagen, Matrigel, or alginate) or of dense cell suspensions supported by inert agarose co-printed as a physical support to guide formation of 3D tissues has also been demonstrated with feature sizes of

about 300 μm [176]. The same kind of forces that allow hepatocytes or mixtures of hepatocytes with other liver cell types to form highly functional tissue-like spheroids on relatively non-adherent surfaces drive formation of dense, viable, tissue-like structures within the agarose support, although the fabricated features are larger than those that typically survive without local microperfusion in bioreactors [177].

Stereolithography, a layer-by-layer process in which UV light is used to polymerize macromers by illuminating only selected regions of a thin layer of precursor solution, is becoming more widely used in tissue engineering and is also sometimes referred to as 3D printing [178]. Stereolithography can be used to make solid scaffolds using a wide range of materials, but it has been a particularly attractive means for microfabricating hydrogel scaffolds from synthetic, semi-synthetic, and natural polymers modified to contain photosensitive reactive groups. Polyethylene oxide (PEO)-based macromers, modified with adhesion ligands and protease-cleavable sequences, or mixed with matrix proteins, are the most common hydrogels employed for microfabrication used for creating scaffolds for post-seeding, as well as for encapsulating cells during fabrication. They have successfully been used to encapsulate viable liver cells as well as many other types of cells [48][178,179]. Standard lithography approaches are not a magic bullet for creating the kinds of complex features needed for creating a vascularized liver in vitro, as practical feature sizes are ∼100 μm, and putting multiple materials within a single build remains challenging. Also, time is of the essence when lithography is applied to cell encapsulation, as precursor solutions contain photo-initiators that can be cytotoxic. Thus, complex geometries with a small number of layers are generally feasible but multi-layer structures are more challenging.

Interestingly, under ideal conditions, the physical limits of lithography as governed by the competing kinetics of free radical generation, reaction, and diffusion are in the ∼1 μm range [180]. This has enabled “projection microstereolithography”, which offers much more fine-tuned control of light along with additional materials handling capabilities, and has recently been shown to push that limit, offering an ability to create scaffold features across a range of hierarchies from a few microns up to about a cm in the same object with speeds that could realistically be used for manufacturing. This technology has just recently been applied to tissue engineering and may offer a breakthrough for creating the branching vessel architectures on hierarchies from sinusoid to larger vessels [181,182] (Figure 14). Finally, an interesting twist on the fused deposition approach based on the “lost wax” process has recently been applied to creating microscale perfused vascular liver structures by extrusion of arrays of molten sugar cylinders, which can subsequently be infused with gel suspensions containing cells, and dissolving the sugar to yield perfusable channels that can be lined with endothelial cells to create hierarchical structures on the length scale of the sinusoid [183]. Although the combination of steps needed to achieve channels at the capillary scale may limit the accessible hierarchies, this approach is compatible with microfluidic perfusion and has shown promise for creating vascularized structures in vitro that can be transplanted in vivo [184], though further work is needed to translate this into widespread application.

4. Opportunities and Challenges for Use of Bioreactors in Drug Development

Liver bioreactor cultures for drug development are arguably at an inflection point. Many technologies have been developed, and though a few bioreactor formats have been commercialized, there is yet no “standard format” as the user experience is still evolving. Many forces in the landscape will determine their roles in the drug development process as the capabilities of various systems are matched against the needs. The evolution of liver bioreactors for extended culture is occurring in tandem with evolution of new approaches to extract information from more straightforward hepatocyte cultures and even from post-market surveillance data, as well as evolution in drug properties and FDA interest in better predictive models. Standard hepatocyte cultures have the inherent advantage of being widely accessible to almost any laboratory in the pharmaceutical industry, and can be set up for relatively high throughput screens adaptable to imaging or biochemical assays using defined protocols that can be replicated in multiple different laboratories. Many ADME/Tox assays based on such cultures are widely used because they provide valuable information throughout the drug development process from lead optimization through to pre-clinical testing.

Bioreactor cultures, in contrast, suffer from at least three barriers to widespread application. First, the technologies themselves are typically relatively complex and inaccessible to the average academic or industry laboratory. Although many technologies are developed and produced initially in academic laboratories that publish the reactor design and fabrication procedures, specialized expertise and equipment is needed to replicate the fabrication process, and at least some training is needed to operate the systems. Hence, access to reactor technology is through either collaboration with the academic lab, which typically will have very limited throughput and capacity for multiple users, or through commercialized versions of the reactors. Commercial reactor systems include those produced by CellASIC/EMD Millipore (microfluidic), HμREL® (microfluidic), CN Bio Innovations (formerly, Zyoxel; open platform with microfabricated scaffold), HemoShear (macroscopic, controlled shear) and Minucell (general macroscopic perfused reactor for 3D culture). Even commercially produced reactors require an investment of both capital and time to adopt on-site, so a variety of business models involving contract projects at the commercial vendor as well as placement of bioreactors are seen in the landscape.

A second barrier to use of bioreactors is the fragmented nature of the application landscape, with many different needs. Each application is complex, as otherwise standard cultures would suffice, and no one dominant application is driving the development of a defined configuration and protocol. A bioreactor that might be used to study metabolic disorders, for example, may have a very different configuration and operation mode than a bioreactor used to study fibrosis or viral infection, and each of these specialized applications has relatively fewer users than traditional ADME/Tox assays. Thus, the resources needed to develop an assay or application may be greater than the early commercial returns; this situation arguably drives a model wherein commercial vendors maintain in-house research and development teams that work with users to develop appropriate assays with a team of in-

house experts that can design quantitative experiments by judicious modest alterations in the fundamental bioreactor technology.

A third barrier to use of bioreactors is that many of the applications that cannot be easily addressed in standard culture and are attractive to study in bioreactor culture involve multiple cell types, ranging from stellate cells and liver sinusoidal endothelial cells to Kupffer cells and other immune cells, and biliary cells. Although cryopreserved hepatocytes and Kupffer cells can be purchased commercially, sources of the NPCs – even if there were means to organize them into appropriate tissue structures – are not easy for the average user to access. A few core facilities, such as the NIH-supported liver core at the University of Pittsburgh, provide cells to the community, but protocols for purifying and preserving NPCs are not robust, and NPCs are not yet popular targets of stem cell differentiation protocols. Hence, again, the full range of functionalities that bioreactors have to offer may remain in-house at commercial entities that can build relationships with suppliers of primary liver parenchymal and non-parenchymal cells. New technologies for rapid cell purification, such as inertial microfluidic separators [185] may improve the ability to isolate and incorporate multiple different liver cell populations in defined fashion, but such technologies themselves must move into mainstream application. Thus, convergence of multiple different technologies to create complex in vitro systems may be necessary to have bioreactor culture become truly widespread.

Despite these barriers, the tremendous costs of drug failures in clinical trials, as well as the need for better models of efficacy in disease, will likely drive bioreactor technologies into practical use in incremental fashion over the coming few years. We consider here a few application areas where bioreactor technologies may provide benefit. We note that since almost all applications of liver bioreactors in drug development are in the exploratory phase, it is premature to make firm recommendations about particular systems or applications.

4.1 Low-clearance compounds

The number of low-intrinsic-clearance drugs has increased significantly over the past few years due to a compendium of high throughput screens early in the drug discovery process that identify high clearance as a liability and select against it [186]. Measuring the clearance of such compounds by traditional substrate depletion methods using suspension or plated hepatocyte assays can be difficult, as the time scale for significant metabolism to occur exceeds the time scale the cells for which cells maintain metabolic capacity.. Bioreactor cultures that offer stable metabolic function for a week or more can potentially be used to detect clearance of such compounds, although protocols must be developed carefully to ensure that drug loss due to adsorption onto or partitioning into the bioreactor components is either minimized or accounted for, and that the medium exchange rates are low enough to allow significant depletion to occur.

The problem of non-specific drug loss in the bioreactor system is not trivial, as most bioreactor systems described in the literature employ some type of drug-absorbing elastomers in the pumps, tubing, or the reactor housing and cell scaffold support. Virtually all microfluidic systems create fine-scale structures using the versatile polymer polydimethylsiloxane (PDMS). PDMS is the same material used to deliver the steroid

hormones in the implantable drug delivery system Norplant, in which the steroid hormones diffuse through a PDMS barrier in which they are highly soluble compared to their solubility in saline. The drawbacks of working with PDMS when quantitative control of highly lipophilic compounds is needed have been described by several labs, and though the problem can be partially and temporarily mitigated by various surface treatments, the PDMS molecules are highly mobile and the surface rearranges over time [63,187–189]. Other elastomers suitable for microfluidics are now being explored, but no prominent solution has emerged for widespread use, in part as the properties that allow elastomers to work well in microfabrication overlap with those that foster drug partitioning to some degree. This limitation is driving development of reactor systems that eliminate common microfluidic-type elastomers and use different methods of microfabrication and assembly of cellular microenvironments to achieve tissue function [37].

4.2. Influence of Polymorphisms on Drug ADME/Tox

Many CYP450s, Phase II enzymes, and transporters exhibit known polymorphisms that can affect drug clearance and toxicity, and the common polymorphic forms in a population can vary with geographic location and ethnicity. In some cases, such as common variations in CYP2D6 and 2C19, predictive pharmacogenomics tests have emerged as a means to stratify patients for proper administration and dosing of drugs [189]. In principle, the effects of these known polymorphisms can be captured adequately using existing pre-clinical assays based on hepatocytes or subcellular fractions. However, some polymorphisms operate in a complex landscape of other factors that dominate the outcome of metabolism and toxicity assays. For example, although the uridine diphosphate glucuronosil transferase isoform 1A1 (UGT1A1) is correlated in some patients with irinotecan toxicities, irinotecan metabolism involves several enzymes other than UGT (i.e., carboxyl estherases, CYP450 isoforms), and transmembrane transporters (ABCB1, ABCC1, ABCG2, SLCO1B1) making difficult the identification of patients with an optimal sensitivity and specificity, and a large part of variability among patients still remains unexplained [190]. Similarly, known polymorphisms in CYP3A4, CYP2C9 and CYP2C19 do not capture the susceptibility to drug-induced liver injury in patients taking an anti-TB drug [191]. For effects that involve changes in relative expression or activities of enzymes with repeated dosing, or that involve complex interplay between multiple enzymes, bioreactor cultures that allow long-term repeated dosing may help uncover mechanisms that are not related directly to know polymorphisms. An open question is whether availability of primary hepatocytes capturing an interesting spectrum of polymorphisms could be deployed in such systems, or whether banks of iPS-derived hepatocytes (plus supporting non-parenchymal cells) will be available for these purposes. Bioreactor culture may be particularly well suited to this problem as bioreactors may be necessary for long-term culture of iPS to ensure hepatic differentiation.

Finally, many drug toxicities have been linked to polymorphisms in HLA haplotypes [192]. Whether such complex mechanisms of drug toxicity can be captured by any assay that does not include an adaptive immune response is not clear, hence additional technologies beyond bioreactors are needed for this complex mode of toxicity.

4.3. Influence of Metabolic State, Sex Hormones, and Inflammation on ADME/Tox

Up to 30% of adults in Western countries have excess fat in the liver, with 2–3% meeting the criteria for non-alcoholic steatohepatitis and up to 1% suffering from fibrosis [193]. Although excess body fat is well-appreciated to affect clearance of many drugs due to distribution effects, increasing evidence for altered functioning of liver drug transporters and metabolizing enzymes is emerging [193]. Liver metabolism of nutrients, and the relative state of stored glycogen and fat, is exquisitely governed by pancreatic hormones that are secreted in a highly complex temporal fashion. For example, insulin, somatastatin, and glucagon, are secreted in a highly dynamic reciprocal pulsatile fashion by human pancreas in response to nutrient stimulation [194], and steroid hormones that influence liver function, such as cortisol, also vary both diurnally and postprandially [195]. Further, these hormones exert differential effects on the parenchymal and non-parenchymal cells in liver.

Capturing the effects of these metabolic states in standard hepatocyte or non-flow-based cultures is challenging, as the control of hormone and nutrient concentrations requires a complex temporal schedule of dosing and medium replenishment, and driving cells to a metabolically-”fatty” state requires culture over days or even weeks, i.e., beyond the time scale that most standard hepatocyte cultures are able to remain functional. For this application, it may be sufficient to maintain hepatocyte mono-culture or co-cultures with Kupffer cells in long term culture since both these cell types can be purchased as cryo-preserved stocks – the accessibility of such models could be adequate.

Similar arguments can be made for analyzing the effects of sexual dimorphism in liver drug metabolism, a phenomenon observed in both rodents and humans [196,197], but relatively difficult to study in standard culture models [197–199]. Bioreactors may be especially useful in investigations of sexual dimorphism, as extended exposure to sex-specific drugs, along with complex regimens of pulsed growth hormone delivery, may be required to achieve sex-specific phenotypes [197–199]. Further, the effects of hormones may require complex interactions between heterotypic cell types (e.g., hepatocytes and stellate cells); hence sophisticated tissue-engineered co-culture systems may be required. This application is on the horizon pending the development of robust systems that can be deployed to meet the numerous constraints required. Sex steroids are highly lipophilic and can partition into the common microfluidic device materials as discussed elsewhere in this review, hence, bioreactors that are relatively inert toward lipophilic compounds are needed for this application.

Finally, the role of inflammation in both clearance and idiosyncratic toxicity are both areas where in vitro models may improve prediction of human responses. Some evidence suggests that inflammation influences activity of transporters and CYP450s [200], and that synergies between inflammation and drug metabolism contributed to clinically-observed idiosyncratic liver toxicity [43][201]. A modest fraction of known human idiosyncratic toxic drugs can be captured using short-term 2D hepatocyte cultures combined with cytokine treatments [43,201], and in theory, models that are not based on flow but that may allow long-term hepatic function could potentially be used to analyze the effects of long-term repeated dosing and inflammation with cytokine stimulation as a cue. However, it is challenging to co-culture hepatic immune cells with hepatocytes for long term in standard conditions, and

such non-flow models fail to capture complexity of reactive oxygen species, tissue-resident immune cell reactions, and local hypoxia that can contribute to hepatocellular death; thus motivating development of flow-based bioreactor approaches for both maintenance of function and development of gradients similar to those in the sinusoid [37].

One crucial additional element that favors bioreactors is the choice of culture medium for such studies. Most culture media used for primary hepatocytes, and for induction of differentiation from stem cells, includes the synthetic corticosteroid dexamethasone to promote and/or maintain hepatic differentiation. Dexamethasone is used clinically as an anti-inflammatory, and inhibits inflammatory responses in vitro as well [37]. Thus, a crucial element of creating appropriate in vitro models of liver inflammation includes tailoring the corticosteroid concentrations to provide physiologically relevant support of liver function while not impeding the effects of inflammation. Cortisol (hydrocortisone), the natural hormone, is greater than 90% bound to plasma proteins, and it is the concentration of free cortisol that cells sense and respond to [202]. Bioreactor cultures, if instrumented with drug dosing and constant media exchange, can potentially control steroid hormones to within a physiologically relevant diurnally varying “free hormone” range, thus enabling accurate physiological studies of inflammation. Development of such systems is currently underway in the laboratory of the authors.

Moreover, bioreactors may offer the potential for additional sensitive readouts of sublethal toxicity such as changes in oxygen consumption rate [72,147]. New relatively low-cost technologies for microscale sensing of oxygen in real time in bioreactors are moving into commercial use [72], providing additional incentive to move more assays to bioreactor culture.

4.4. Biologics and Nanomedicine ADME/Tox

Tremendous challenges exist in predicting clearance, toxicity, and off-target effects of biologics and emerging nanomedicines. For example, the anti-IL6-receptor tocilizumab, an approved treatment for rheumatoid arthritis, influences the clearance of simvastatin in humans [203]. Efforts to understand the mechanism for this observed behavior using cultured human hepatocytes gave insights, but the authors concluded that the complexities of inflammation in liver require more sophisticated in vitro models [204]. Maintaining complex co-cultures of hepatocytes and NPCs, including appropriate immune cells, will likely require a combination of both bioreactor technologies and more sophisticated tissue engineering methods to capture more facets of liver architecture, as described above. Particularly important in these applications are the liver sinusoidal endothelial cells, which gate the passage of nanoparticles into liver and which play a substantial role in the clearance of molecules via the reticulo-endothelial system (RES). Liver sinusoidal endothelial cells are fragile [205], but can be maintained in bioreactor culture for weeks without exogenous VEGF [164] though patency of vessels has not been demonstrated. An impediment to developing robust models of the liver RES is the fragility of human liver sinusoidal endothelial cells during isolation; whether other sources of endothelial cells (e.g. iPS-derived, or cross-differentiation) can substitute remains to be determined. Greater availability of bioreactors may help foster advances in liver sinusoidal endothelial cell

culture, especially if robust bioreactor systems can be deployed at the point the primary cells are isolated from donor tissue.

4.5. Models for Testing Chemotherapy Agents

Mortality from solid tumors arises primarily from metastatic sites, and an enduring challenge in predicting efficacy of chemotherapy drugs is understanding and modeling the biology of metastatic cells in distant organs [206]. Liver is a common deadly site of metastasis for many cancers, including triple negative breast cancer (TNBC) [206,207]. Patients who have no evidence of metastatic spread of TNBC at the time of diagnosis are still given an aggressive chemotherapy regimen as TNBC has a high probability of aggressive recurrence in liver, lung, and brain within the first three years after diagnosis. The limitations of xenografts are becoming apparent, and standard non-flow cultures are not well-suited to host a complex 3D micrometastases, thus driving interest in use of bioreactor cultures to study steps in metastasis including extravasation [168] and tumor growth in liver [163]. Tumor metabolism has (re)emerged as a powerful driver of malignant behavior and target of chemotherapy [208], hence, in vitro models that provide control over the hormones, metabolites, and oxygen levels regulating metabolism in the context of a 3D liver microenviroment could potentially revolutionize the pre-clinical testing of therapeutic agents against liver metastasis. This application requires a substantial coordination of tissue engineering, tumor biology, and therapeutic agent dosing, and temporal control of hormones and nutrients, and thus can be considered as on the frontier of bioreactor culture applications. This application may be driven faster by the needs of personalized medicine, as the limitations of genomic studies to predict how patients will respond to therapies are becoming more apparent [209], motivating experimental approaches that integrate complexity.

4.6 Other Disease Models

The role of bioreactor culture in modeling liver diseases such as hepatitis and fibrosis is emerging in concert with other types of in vitro culture models as well as new ways of parsing clinical data to gain insights into therapies [210,211]. Liver fibrosis is induced by chronic inflammation, hence bioreactor design for inflammation in terms of complexity of cellular constituents and the need for tissue organization are arguably enhanced for this application; the regulation of the inflammatory hormone environment by continuous flow may be a particular benefit here.

The evolving landscape of hepatitis models includes advances in understanding how to better deploy existing standard cultures of primary cells and cell lines such HuH-7 [212,213]. For example, it was recently reported that human serum greatly enhances the function of human hepatoma cells in cultures [212], enabling more diverse aspects of hepatitis infection to be investigated in an accessible cell system. The likely benefit of 3D cultures may be to retain longer-term infection/reinfection, and to better analyze the interplay of immune and parenchymal cells in controlling infection.

An underexplored area is use of bioreactors to analyzing trafficking of immune cells from blood into liver tissue. In an effort to develop an in vitro model of a hepatic sinusoid,

transwells seeded with HepG2 cells overlaid with primary human sinusoidal endothelial cells were used to study lymphocyte trafficking in liver during short-term perfusion culture [214]. The study showed that the interactions between hepatocytes and endothelial cells amplify leukocyte recruitment by regulating the expression and function of endothelial adhesion molecules. Whilst leukocytes will bind well to unstimulated endothelial monolayers in “static” adhesion assays, the presence of shear stress conferred the necessity for adequate expression of capture receptors in order to permit adhesion [215]. In humans, metabolites resulting from drug or toxin metabolism by hepatocytes may activate the overlying endothelium, thereby explaining how a toxic insult can lead to an inflammatory response. Thus, models such as this provide a system to determine the molecules involved in leukocyte recruitment into the hepatic sinusoids, and to test potential inhibitors that might control subsets of cells at the site of inflammation. More studies are required to explore the involvement of each component of the hepatic sinusoid in lymphocyte homing within the liver microenvironment.

4.7. Organ crosstalk systems

The pioneering work of Shuler and co-workers charted a path for in vitro liver micro-organ systems connected to other organs for assessment of drug disposition and toxicity, and suggested that the functions of liver may benefit from connection to these other organ systems through cross-talk of hormones and paracrine factors [95,216]. While conceptually attractive, the technical challenges to realizing this vision are formidable. In addition to the engineering challenges of designing the fluidics and hardware and scaling the sizes of interconnected organs, substantial quantitative biology infrastructure is needed to create, maintain, and characterize the phenotype of each individual organ at the requisite levels to fulfill actual needs in drug development, and most approaches in the literature use cell lines as proof of principle for design of interconnected systems. For example, Ahluwalia and co-workers have applied the liver bioreactor system described above [66] to examine interactions between mouse hepatocyte and human endothelial cultures in short-term culture, observing that the liver cells exhibited enhanced function in perfusion culture regardless of endothelial cells though endothelial function appeared to benefit from liver [217]. When this same system was used to connect hepatocytes and visceral fat cells in culture, an apparent benefit was seen over 24 h in liver function compared to static interaction or dynamic culture of hepatocytes alone [218]. Marx and coworkers described interactions between interconnected transwell-type cultures of skin biopsies and spheroids generated from the cell line HepaRG cells combined with a stellate cell line, with the interesting observation that the skin biopsies appeared to consume albumin produced by the hepatocytes, consistent with a proposed model that skin serves as a depot for albumin [219]. The spheroids used to generate the liver tissue were generated by a hanging drop method and were of dimensions greater than 300 μm diameter. The authors suggest that future work should involve examination of oxygen transfer limitations in the system.

A provocative study in capturing features of the enterohepatic interactions was reported by Groothuis and coworkers, who created a microfluidic interconnected system comprising communicating slices of liver and intestine and demonstrated that production of FGF15 by the intestine, when exposed to bile acids, subsequently travelled to the liver compartment

and influenced expression of CYP7A1 [220]. This is an intriguing physiological interaction, and this study highlights the power of microfluidics to model communication of labile compounds that are transported in small volumes and have biological effect.

A complex 4-way interaction involving compartmentalized culture of liver, lung, kidney, and fat cell lines in an interconnected microfluidic device similar to one described above [155] was described by the Yu group [154]. Interestingly, whereas this group reported that TGF-β1 enhanced function of primary rat hepatocytes in this device, it inhibited the function of the cell line HepG2/C3a in this configuration.

A very elegant microfluidic device design for interacting lung-liver-bone marrow organ systems has been used with cell lines to demonstrate the feasibility of testing the efficacy and toxicity of 5-fluorouracil according to a detailed mechanism-based PK/PD model taking into account mechanisms of mass transfer and drug metabolism in the system [95]. This device design is very appealing as it relies on gravity flow rather than pumps, and thus avoids many of the technical challenges with bubbles and other modes of pump failure and is designed to be accessible to more general users.

Development of technologies in this arena was recently stimulated by the simultaneous launch of companion NIH and DARPA-funded “Microphysiological Systems Programs” in 2012. Hence, the performance capabilities of interacting organ systems including liver-based systems will likely be advancing rapidly in the near future.

Conclusions and Future directions

Although bioreactors have been used for liver cell and tissue culture for several decades, applications in drug development are still nascent. There are diverse needs throughout the drug-development pipeline, and many remain well served by standard hepatocyte cultures. However, the liver is a highly complex organ containing many cell types, and in many instances in vitro models containing only a few cell types are not sufficient to capture this complexity and accurately predict drug-tissue interactions or model disease processes for testing efficacy. Accordingly, there is high demand for complex, three-dimensional and multi-cellular engineering tissue models that can be maintained for long time spans in the lab and produce reliable, readily obtained metrics in response to various stimuli.

As liver cells are extremely metabolic and demand high concentrations of oxygen, engineering high-density hepatic cultures can be challenging. The research community has devoted extensive effort to overcoming this obstacle, while making strides in the understanding of relevant mechanical cues, necessary chemical signaling, and fabrication of fine-scale matrix architecture. We have progressed from early reactors that employed medium flow over monolayers, spheroids, and recessed wells of cells, toward reactors capable of sustaining larger numbers of cells with greater microscale spatial organization and control of environmental parameters. It is becoming increasingly clear that by co-culturing multiple cell types found in the liver, in vitro models can be generated that are more physiologic and yield reliable and relevant information. Advances in synthetic materials and microscale fabrication techniques are proving crucial for this endeavor,

providing advanced tissue engineering tools that are being used to craft ever more sophisticated tissues.

Commercial bioreactors directed at liver are just now becoming available, with the advent of CN Bio Innovations, CellASIC, HμREL®, and Minucell systems. Such microfluidic and microfabrication technologies offer exciting promise, but in vitro models of liver tissue are only pharmacologically relevant if they yield reliable data, and technical challenges of quantitative exposure to drugs that partition into PDMS remains to be definitively solved.

Some of the most productive future directions may involve liver models interacting with other organ systems. It is known that respiratory infections such as influenza in humans are often accompanied by a hepatitis that is usually mild, implying cross-talk between organ systems. The mechanism of this kind of liver damage is not well understood, but has been reported as collateral damage subsequent to interplay between Kupffer cells and T cells [221] and might influence liver CYP450 activity [222]. Accordingly, in vitro lung-liver models that also incorporate immune cells may be useful in drug development. Without question, an exciting emerging frontier is the influence of the gut microbiome on liver function with respect to drug uptake, metabolism, and efficacy [223,224], and it is likely that models employing multiple communicating tissue types will yield fascinating results. An outstanding challenge on this “complex biology” frontier is developing quantitative models to guide what measurements to make and how to interpret them. For example, “systems biology” models are now integrating multiplex measurements of intracellular cell signaling states, extracellular cytokine and growth factor networks, and cellular phenotypic responses to external perturbations to yield non-intuitive insights into drug interventions in inflammatory diseases [225]. Designing reactor cultures that are guided by such models may enhance the ability to correlate in vitro observations with in vivo results in patients and predict clinical data more accurately. Clearly, the frontier is emerging as a highly interdisciplinary effort, requiring integration of molecular cell biology, immunology, biophysics, biomaterials and device design, and pharmacokinetic modeling, with clinical translational medicine.

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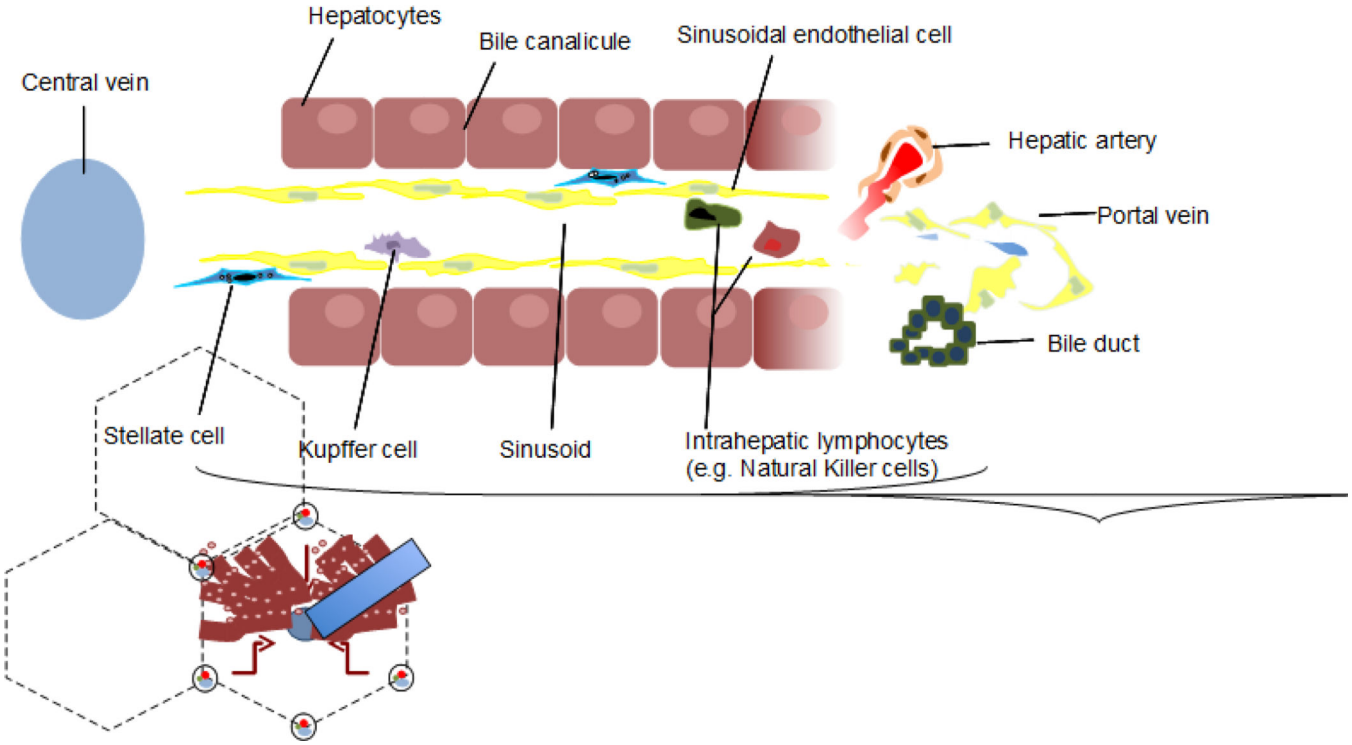

Figure 1.

Classic lobule structure and cellular compartment of hepatic sinusoid. The hepatic lobule consists of a hexagonal arrangement of hepatocytes radiate outward from the central vein. Blood originates from the portal vein and hepatic artery is delivered through the sinusoid to the central vein. Bile is secreted by hepatocytes into the bile canaliculi and drains towards the bile ducts. Endothelial cells are separated from the hepatocytes by the Space of Disse and form fenestrations along the sinusoid. Kupffer cells reside within the sinusoids and serve as resident liver macrophages. Stellate cells are present in the Space of Disse and quiescent in the healthy liver. Intrahepatic lymphocytes include a subset of immune cells that can be found in the hepatic sinusoid.

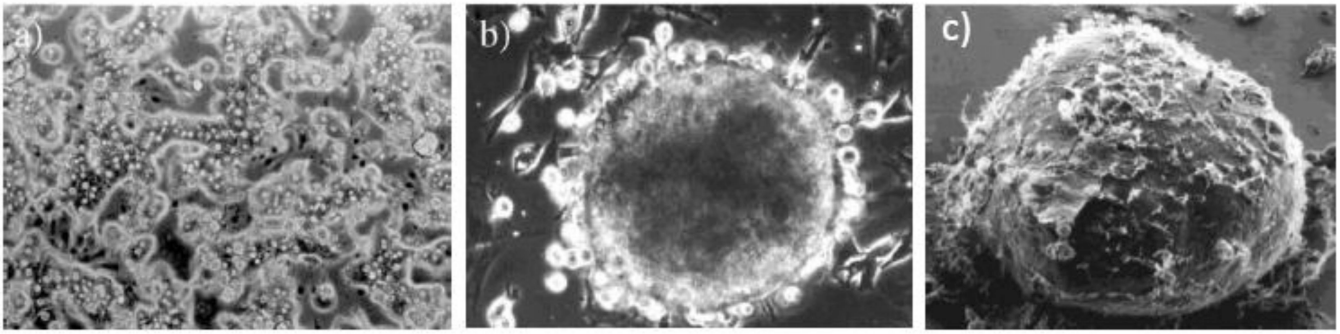

Figure 2.

Phase-contrast images of co-cultured cells in (a) static culture on day 1 or (b) perfusion culture on day 13 demonstrate the distinct change in cell morphology when cultured under shear flow. Although cell morphology was similar to static culture at early time points, perfusion induced the formation of spheroidal structures by day 5. (c) Scanning electron photomicrograph of cell aggregate in perfusion culture on day 13 [57].

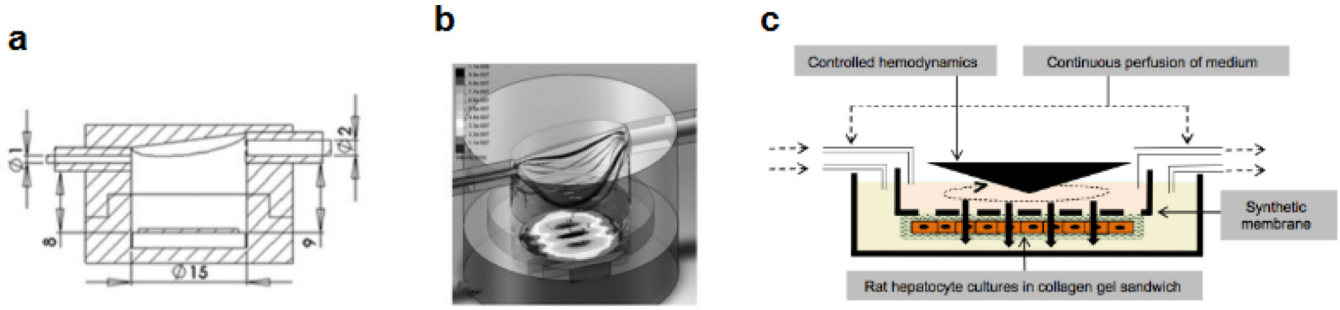

Figure 3.

(a) Multi-chamber Modular Bioreactor or McMB consists of a cell culture chamber made of PDMS and designed to accommodate the use of glass or plastic cover slips or scaffolds used for cell culture. (b) The McMB velocity profile showing stream lines. The McMB was designed with dimensions similar to a standard mutliwell plate format, and operates in a low shear regime [66]. (c) A perfused transwell device using cone and plate technology with controlled hemodynamics mimics sinusoidal circulation. Hepatocytes cultured in a collagen gel sandwich configuration within the device exhibited polarized morphology and improvement of multiple hepatocellular functions relative to nonflow culture [68].

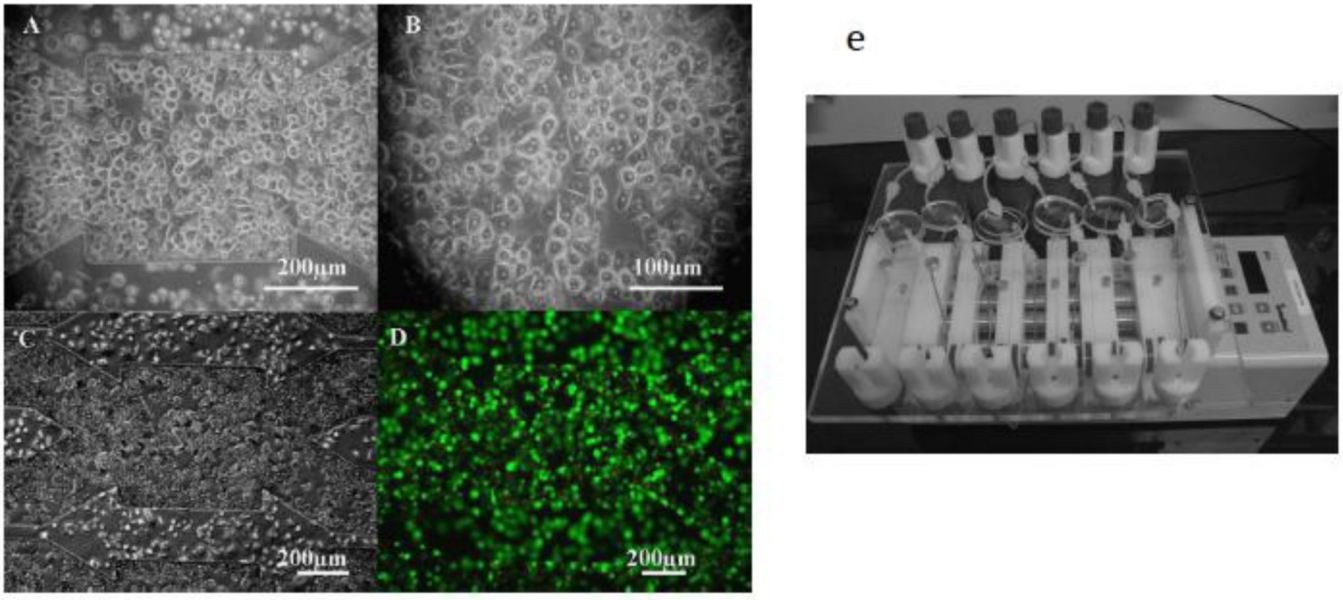

Figure 4.

Cryopreserved human hepatocytes were inoculated within the microwells of the of the PDMS and cell morphology was assessed (a, b) after 12 hours of adhesion and (c) 12 hours of adhesion and followed by 4 hours of perfusion. (d) The cell viability in the PDMS microwells was assessed with a live-dead assay after the 4 hours of perfusion (viable cells stained green by Calcein AM and necrotic cells stained red by propidium iodide (PI). No PI positive cells were observed. (e) After adhesion, cells were connected to the perfusion circuit setup operated with a peristaltic pump [87].

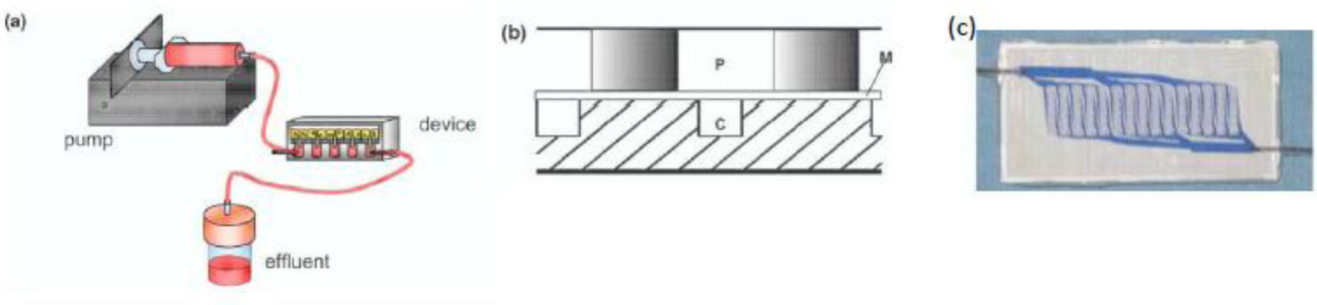

Figure 5.

(A) Bilayer PDMS device microfluidic setup with vascular and parenchymal compartments to mimic organ vasculature and support the metabolic demands of the liver cells. (B) Cross-sectional view of bilayer device showing the layers containing microfluidic channels (C), posts supporting the parenchymal chamber (P) and the intervening nanoporous polycarbonate membrane (M). (C) The assembled device was perfused with Trypan Blue through channels to demonstrate device integrity [75].

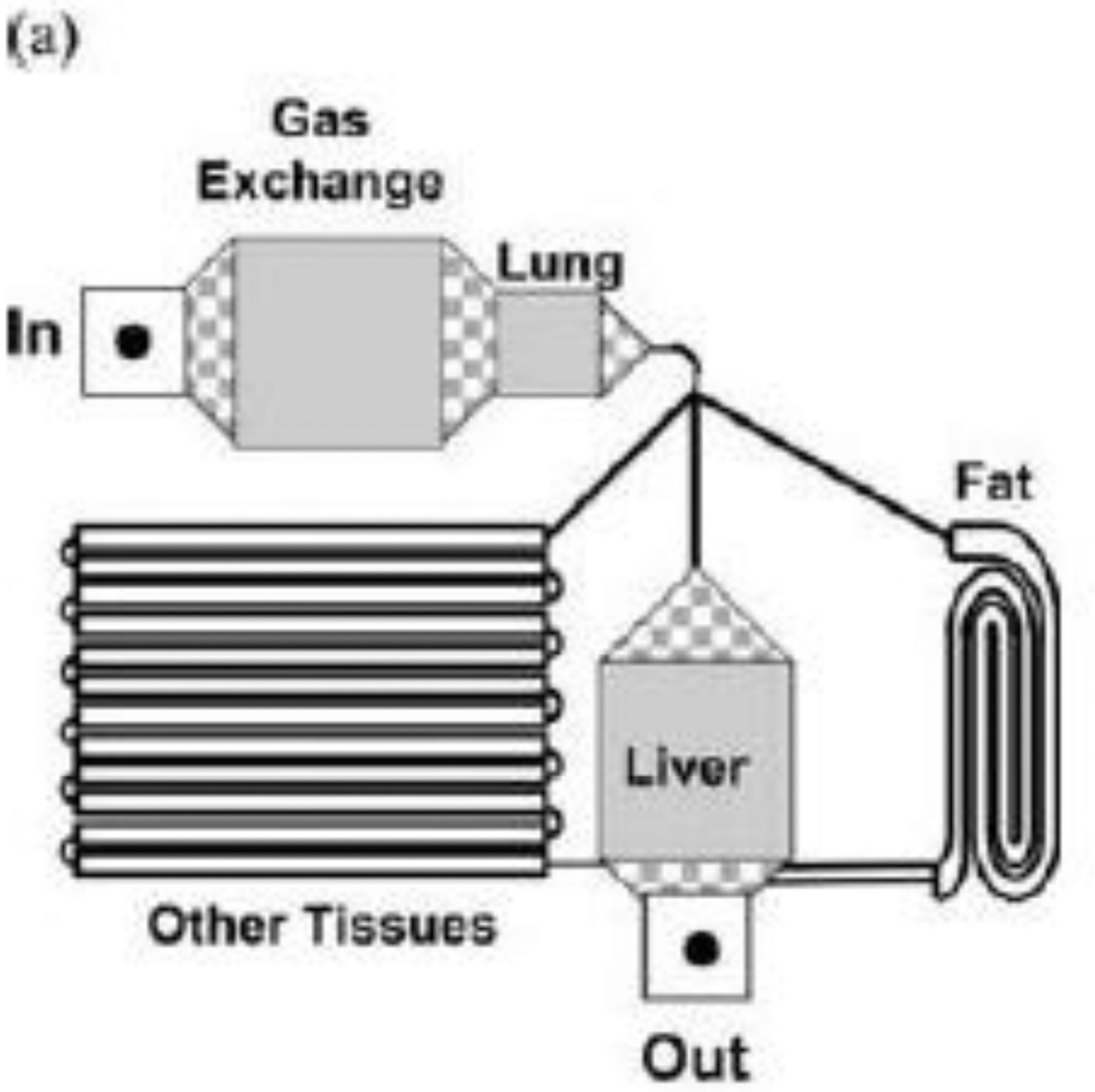

Figure 6.

A schematic diagram of two-cell system, four chamber μCCA design integrating lung, liver, fat, and other tissue to probe naphthalene toxicity. An array of channels or chambers are microfabricated with the goal of obtaining an appropriate physiological scale. Such microscale systems enable testing to be conducted on minimal amounts of scarce or expensive materials [78].

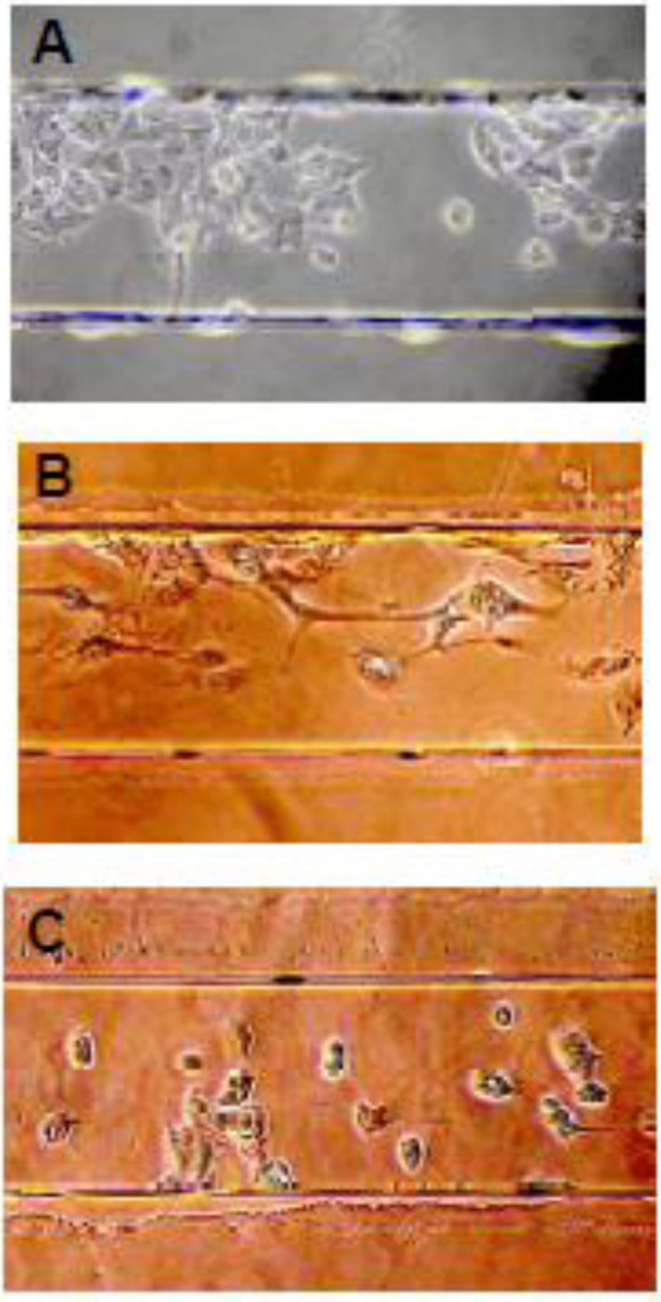

Figure 7.

The effect of shear stress on hepatocyte activity was evaluated with HepG2 cells cultured in a microfluidic system. Phase-contrast microscope images of hepatocytes under various shear stress conditions: (a) 0.14 Pa (b) 1.2 Pa and (c) 3.6 Pa demonstrate the change in hepatocyte morphology in response to shear flow [89].

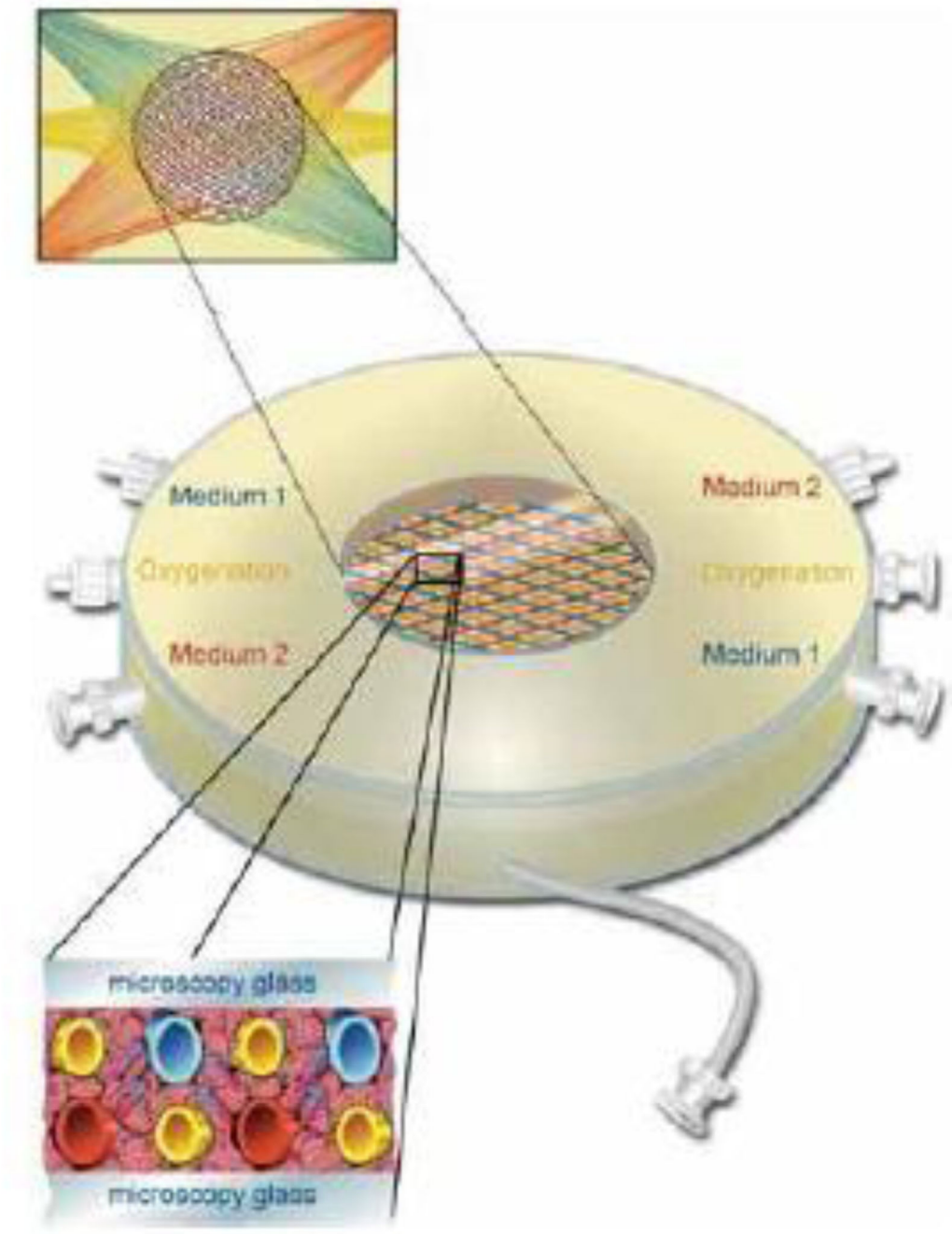

Figure 8.

Gerlach and co-workers developed a miniaturized hollow fiber perfusion design consisting of three fibers interwoven in two layers in an alternating fashion where hydrophilic membranes are employed for medium perfusion and hydrophobic membranes are employed for gas perfusion. This arrangement enables the reduction of bioreactor volume to 0.5 mL and subsequently the required number of cells [98].

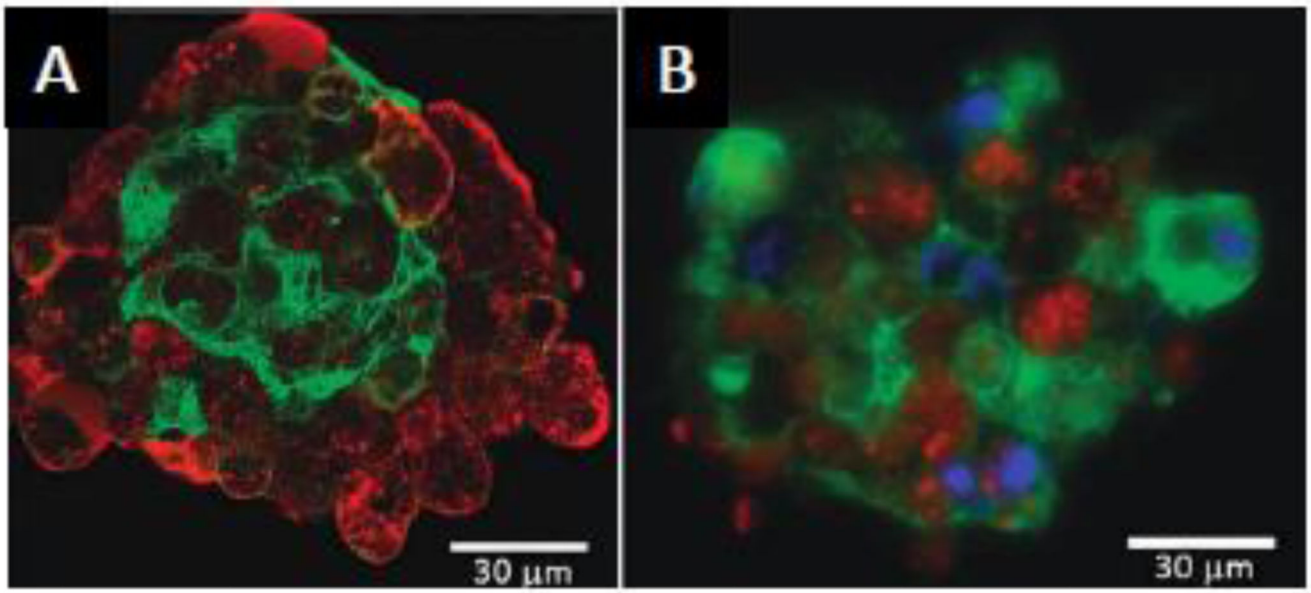

Figure 9.

A perfusion bioreactor employed to culture human hepatocyte spheroids for 3–4 weeks confirmed the presence of liver-specific markers. Immunofluorescence microscopy of liver-specific antigens after 2 weeks of bioreactor culture indicated: (a) albumin (red) was concentrated around the periphery of spheroids with lower levels expressed in the spheroid (CK18 appears green), and (b) Cyp3A (red) strongly expressed in half the cells randomly throughout the spheroid (CK18 appears green and nuclei (DAPI) appears blue) [136].

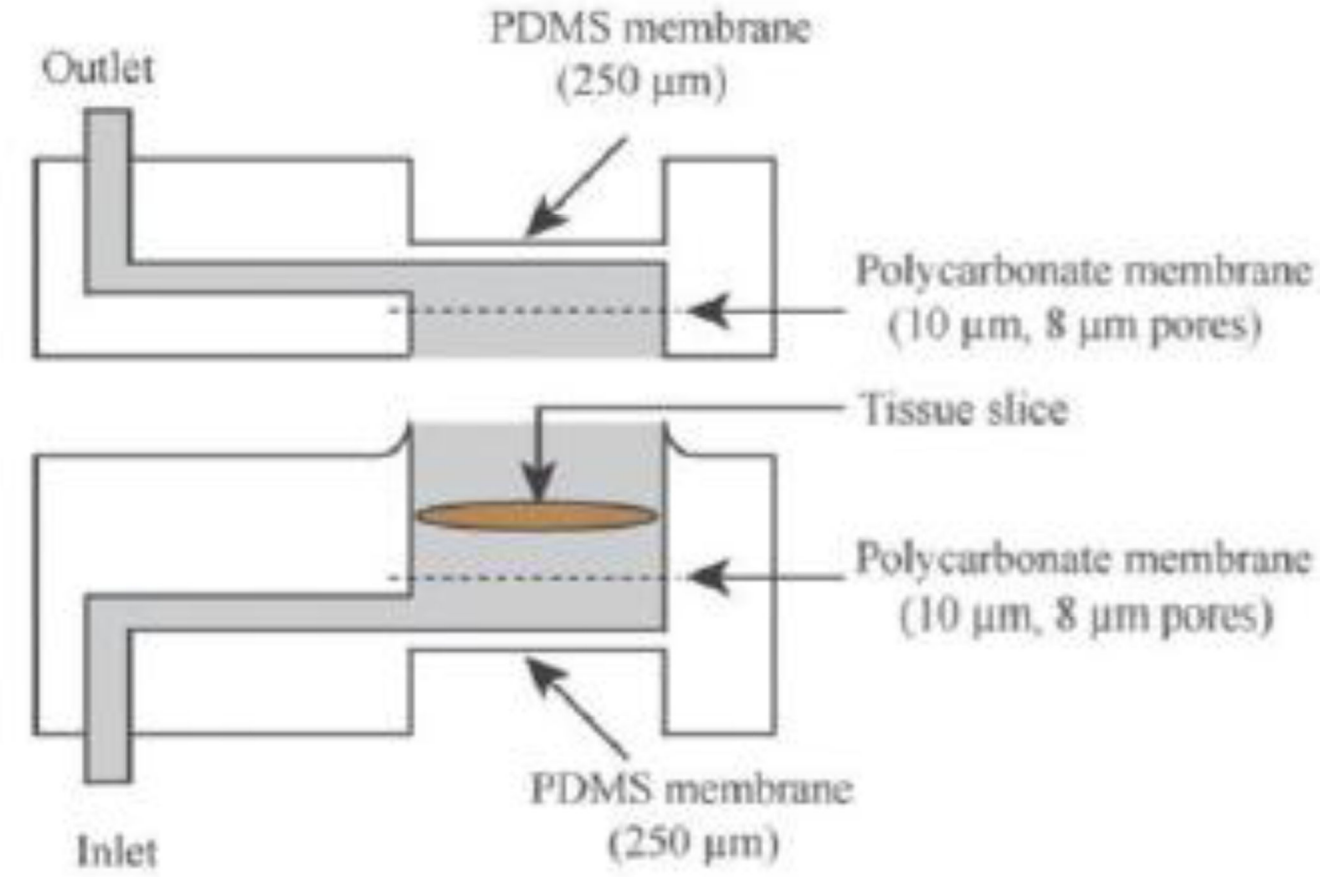

Figure 10.

A flow-through device for liver-slice incubations to enable continuous supply of nutrients and oxygen and removal of waste products. The microfluidic-based system is made of PDMS and contains a 25 μL microchamber between two polycarbonate membranes for liver slice culture. Continuous flow is supplied from the inlet via a peristaltic pump around the tissue slice and up to the outlet [143].

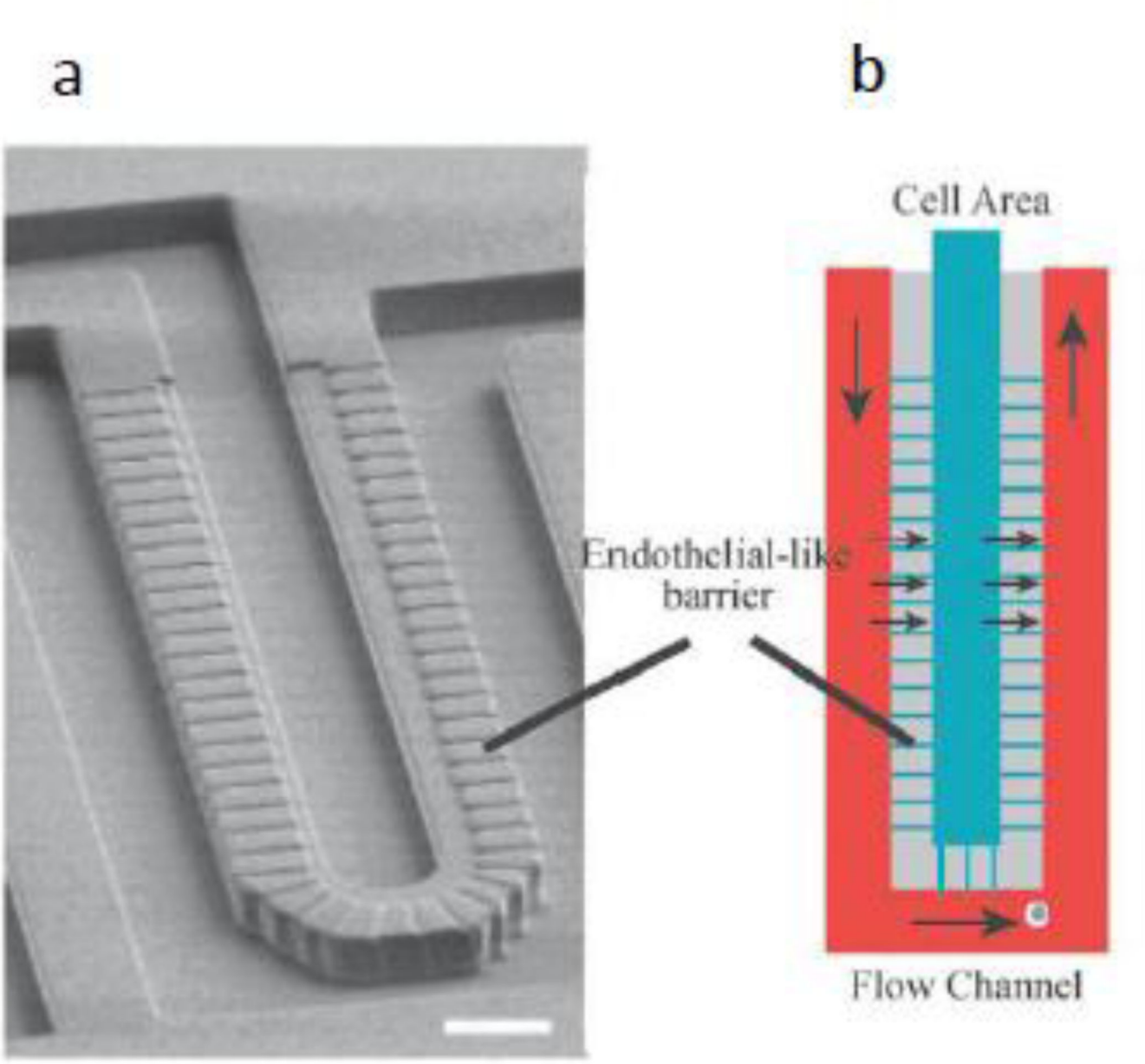

Figure 11.

A biologically inspired liver sinusoid with a microfluidic endothelial-like barrier developed by Lee and co-workers. (a) A SEM micrograph depicts the microfluidic sinusoid unit. (b) A cord of hepatocytes in the cell area region of the device is fed nutrients across the endothelial-like barrier via convective flow as media is fed through the outer flow channels. This device sustained rat and human hepatocytes for 7 days without the introduction of exogenous matrix [149].

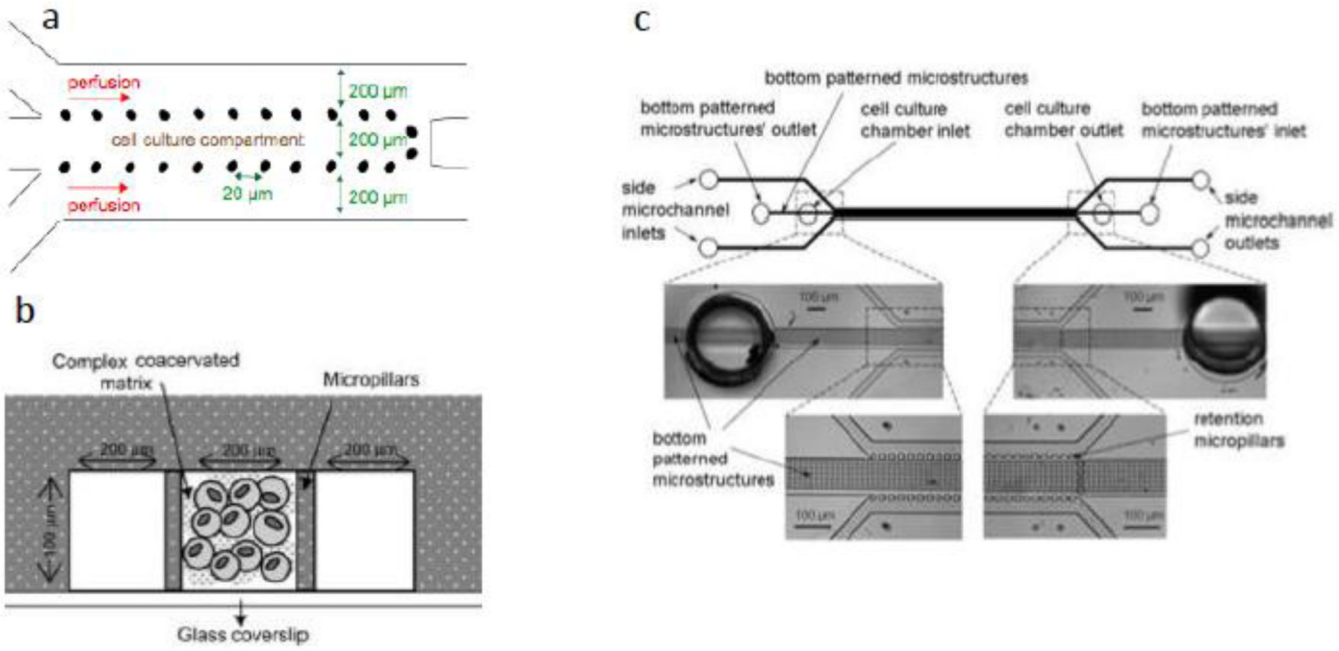

Figure 12.

(a) The gel-free 3D microfluidic channel-based cell culture system developed by Yu and coworkers. The microfluidic system has two inlets (one for culture medium infusion, one as cell reservoir) and one outlet. (b) Cross-sectional Illustration of the same system. (c) Representation of another system developed by Goral et al at Corning Incorporated to create 3D dynamic primary human hepatocyte cell culture without the addition of biological or synthetic matrices. [151,155,158]

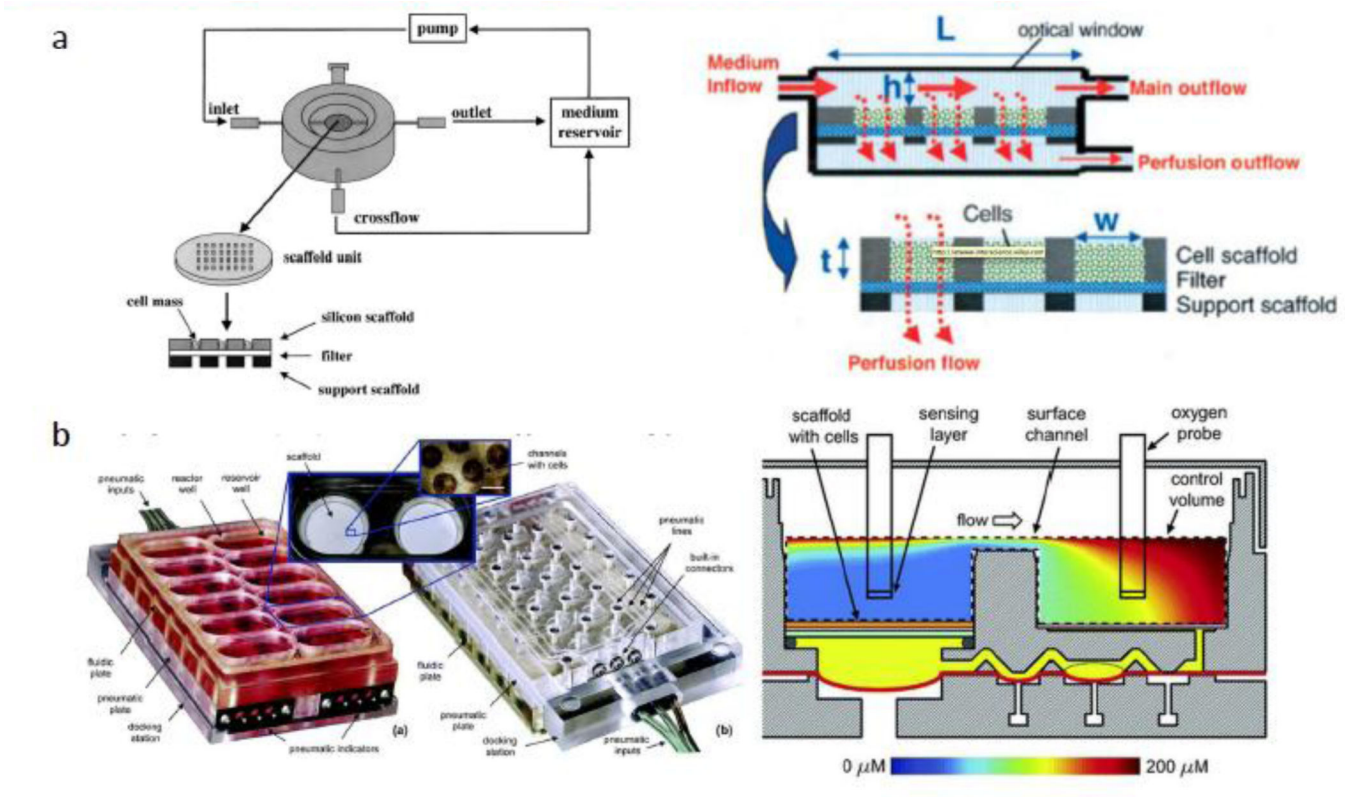

Figure 13.

Evolution of bioreactors for high-throughput 3D packed hepatocyte tissue culture. Original bioreactors (a) provided cross-flow perfusion, while also allowing through-tissue perfusion. Later reactors (b) allow the culture of up to twelve tissue constructs at a time, and are supported by a pneumatic platform that provides reliable control of flow through each tissue construct. Modeled oxygen concentrations for a perfused condition are shown in the right portion of (b)[139,138,147,156].

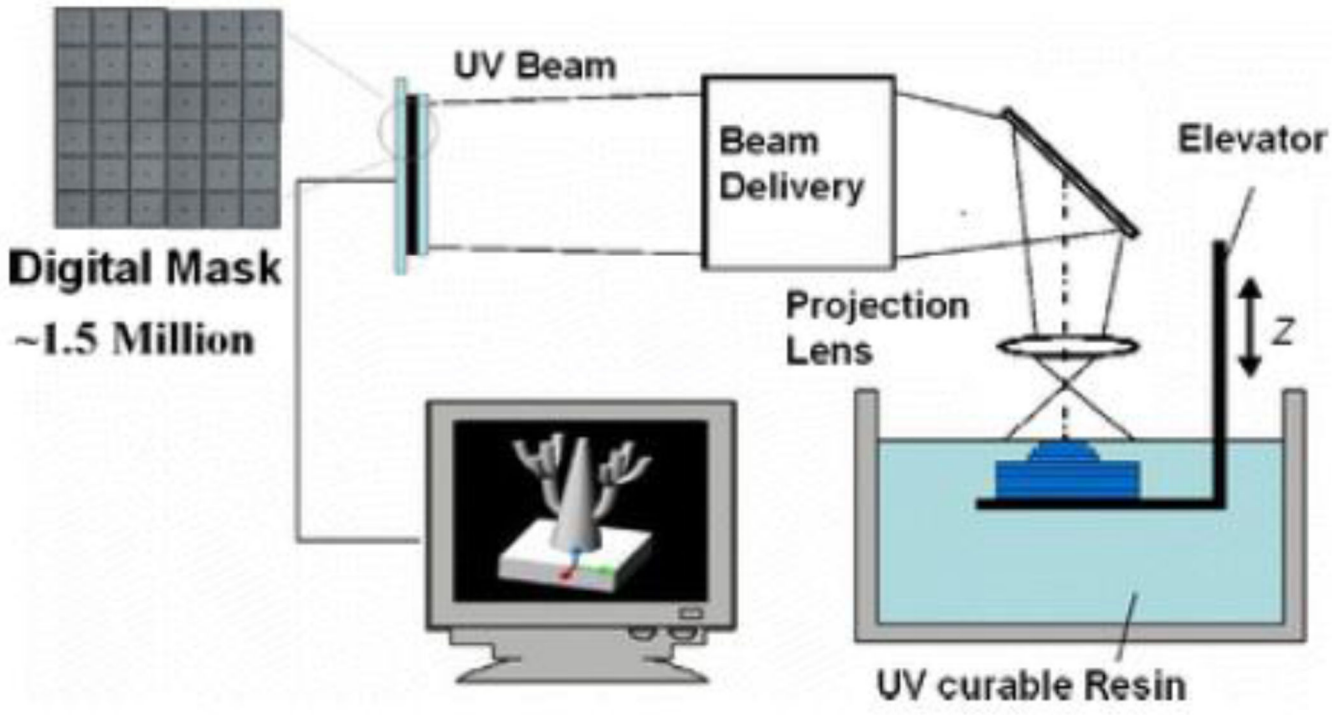

Figure 14.

A schematic showing the process of PμSLA, a recently developed technique for generating complex 3D micro-architecture tissue scaffolds with very high (~1 μm) feature resolution. Free-form fabrication techniques are likely going to prove applicable to the fabrication of scaffolds capable of guiding liver tissue morphogenesis and organization. The depicted technique is able to build structures similar to branching vasculature and biliary networks, and could prove a useful tool for future efforts to develop in vitro tissue analogues with histological architecture similar to that found in vivo [182].

Table 1

Important design considerations in liver bioreactor technologies

Design consideration	Relevance*
Drug absorption into material	Many materials commonly used in medical devices and microfluidic platforms readily absorb and sequester hydrophobic drugs. This affects accurate measurement and regulation of active drug concentration within systems, and subsequently impacts relevance for pharmacological studies [63,64].
Oxygen delivery	Oxygen is a limiting nutrient and controlling factor in many aspects of liver phenotype, and it is therefore crucial that the in vitro tissue do not become hypoxic so that all metabolic demands are met physiologically [49, 50,58, 72,126,128,134].
Fluidic shear stress	Limiting fluidic shear can be important for maintaining hepatocyte morphology and function, but this can be a difficult design constraint to satisfy while still guaranteeing sufficient oxygen delivery to cells [58,59,66,68].
Chemical cues (soluble)	Hepatocytes function is closely regulated by both autocrine and paracrine factors produced by themselves as well as non-parenchymal cells. In vitro tissue models strive to replicate this environment, providing a well-controlled system with near physiological concentration of relevant cues [37,67,173,174].
Micro- and nano-structural cues	Micro-structural cues and architecture can be very important for encouraging cell self-organization and generating macroscopic tissue morphology. Nano-structural cues, such as pore size and stiffness of adherent scaffold materials, can support a fully mature phenotype [133,174,179].
Number of cell types and ratios	There is increasing evidence that co-culture systems incorporating supporting cell types along with hepatocytes are better able to replicate physiologic metabolism and maintenance of phenotype [37,62,91,167]. It is desirable, therefore, to develop reactor platforms flexible enough to incorporate multiple cell types.
Mass tissue to media volume ratio	Cellular maturation and function rely on an intricate network of growth factors, hormones, and cytokines [37,57,77,173]. Reactors with too large of a media reservoir, or with continuous media exchange, risk diluting these factors and negatively influencing cell survival and function. Additionally, dilution of produced cellular factors can result in low concentration below the limit of many assays making quantitative measurements difficult.

* Please refer to the text for additional references.

Table 2

Summary of reactor features

Bioreactor type	Relevant findings	Commercial context	Pages for reference
Macroscale monolayer	Practical and simple to operate. Perifusion shown to improve survival and metabolic function including standard measures such as urea and ammonia production. High shear stresses associated with high flow rates negatively impact urea, ammonia, and albumin production, and can cause cellular morphogenesis into spheroidal structures. These early reactors underscore the difficulty of uncoupling cues from multiple sources, such as adhesion signaling, growth factor signaling, autocrine and paracrine interactions, and oxygen and nutrient delivery.	Minucell (Bad Abbach, Germany) provides a membrane cassette that can be placed under flow conditions for either monolayer or scaffold-based configurations. Hemoshear (Charlottesville, VA) has a perifusion monolayer system with controlled "sinusoidal" shear.	9–10
Microfluidic "flat plate"	Can create highly tailored microenvironment and flow patterns, though debris, bubble clogging, and cell proliferation can present obstacles. Oxygen delivery needs to be carefully modeled to assure adequate delivery. Promising format for organ cross-talk. Require low cell numbers and media volume, but can be complex to operate. Drug absorption can compromise assay reliability.	The HμREL chip and the CellASIC/EMD Millipore system are commercial versions, but most versions remain in prototypical form. Users with access to PDMS microfabrication facilities can potentially replicate published designs that have not yet been commercialized, although operation of devices can take training.	11–13
Hollow fiber	Longstanding technique for large-scale cell culture in biotechnology and extracorporeal liver applications; most suitable for large numbers of cells (>1 million per reactor). Protects cells from shear and provides mass transport into interstitial space, possibly concentrating beneficial autocrine factors. Except for specialized academic version, format precludes in-situ imaging, and cell recovery can be challenging.	User-friendly devices with pH, oxygen, and medium exchange controls are currently commercially available for large-scale applications (FiberCell Systems, Inc.; Bellco Glass "CellMax"). Devices in the pipeline for clinical application are now being scaled down for pharmacological studies, but still require a large number of cells for inoculation.	13–15
Stirred suspension	Can provide inexpensive, reliable control of shear and nutrient levels to generate spheroids of consistent size and morphology. Cultures can be sustained with this technique for exceptionally long periods of time. Liver spheroids have been shown to be capable of developing complex morphology, including small-scale biliary networks. Donor variability can be an obstacle to generating consistent results.	Stirred tank reactors for mammalian culture are commercially available from a number of vendors, and can be set up for stirred liver suspension culture as described in the cited literature.	15–16
Liver slice	Cultures efficiently use donor material and preserve in vitro cell and matrix ratios, but current systems cannot reliable sustain cultures for longer than a few days. Constant media perfusion with convective flow on both sides of slice improves performance; sufficient oxygen delivery is likely a limiting factor in these systems.	Liver slices are often maintained in shakers or roller-bottle formats. Their inclusion in microfluidic reactors is still experimental.	16–17
Microscale 3D	Can facilitate very local microperfusion of capillary bed-like structures and encourage formation of features that mimic in vivo tissue organization. Tight packing of cells allows matrix-less cell seeding. Systems allowing 3D cell-driven morphogenesis show higher albumin secretion than found in monolayer or spheroid cultures, and can sustain multiple cell types for long periods of time (> 2 weeks).	Some formats have been commercialized [CN Bio Innovations (Oxford, UK); CellASIC/EMD Millipore; AIM Biotech (Singapore)] and offer reactors for sale or through collaboration. Most reactors remain experimental.	17–20