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Toxicology: Present-Day Difficulties for Investigating Safety Science and Target Safety Evaluation of Major Human Hepatocytes

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Abstract

Preclinical toxicology has developed into a more thorough understanding of toxicity pathways from its descriptive beginnings. Technological developments have made it possible to forecast safety occurrences, mitigate adverse effects, and create safety biomarkers. Because they are able to self-renew and specialize into different types of human body cells, stem cells are currently being employed for in vitro toxicological applications. However, as compared to primary human hepatocytes, stem cell-derived HLCs perform worse in the liver. More research on the concentration and/or time-response of rifampin, phenytoin, clotrimazole, and phenobarbital in six formulations was carried out. By using Western blotting, bupropion hydroxylation, and testosterone 6β-hydroxylation, respectively, CYP2B6 and CYP3A4 protein and activity were evaluated. Huh7 cells cotransfected with CYP2B6 (NR1)5-LUC reporter plasmid and hPXR were used in reporter gene experiments to assess hPXR activation by the 14 drugs. The LUC reporter Transcripts of nuclear receptors (aryl hydrocarbon receptor, pregnane X receptor, constitutive androstane receptor, peroxisome proliferator-activated receptor α), P450s (CYP1A2, 2C9, 2D6, 2E1, 3A4), phase 2 enzymes (UGT1A1, GSTA1, GSTA4, GSTM1), and other liver-specific functions were found to be expressed at comparable levels in both confluent differentiated and The levels of numerous transcripts were significantly raised in the presence of 2% dimethyl sulfoxide. Measurement of baseline activity of many P450s and their response to prototypical inducers, as well as study of metabolic profiles and cytotoxicity of several drugs, verified the functional similarity of HepaRG cells to primary cultured human hepatocytes.

Keywords: Drug safety, risk assessment, signal transduction, HepaRG, peroxisome proliferator-activated receptor α, pregnane X receptor

INTRODUCTION

Integrative pharmacology helps to deepen the understanding of how a drug's biological action (pharmacodynamics) and its behavior in the body over time (pharmacokinetics) interact. In drug development, this approach, often referred to as lead optimization, allows medicinal chemists to modify the structure of a drug to increase the likelihood of its success as a medication. Three-dimensional (3D) cell cultures offer a more realistic environment for biological cells, enabling them to interact in ways that better resemble in vivo conditions compared to traditional two-dimensional cultures [1–3].

One of the major challenges in drug development is Drug Induced Liver Injury (DILI), along with cardiotoxicity. Many cases of DILI are linked to unusual metabolic processes or unexpected drug reactions. To evaluate the safety

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of drugs, a composite safety score is calculated by analyzing systemic toleration, clinical chemistry, and multiorgan pathology. Studies have shown that potent cytotoxicants, which are toxic at low concentrations, tend to cause more severe effects in vivo compared to less potent substances [4, 5].

A commonly used tool in these studies is the human hepatocellular carcinoma cell line HepG2, which has many characteristics of normal liver cells. This cell line is useful in screening new chemical compounds for potential toxicity. Alternative approaches are becoming more important as the use of animal models declines. For example, advanced methods like the HepG2-BAC-GFP system have been employed to study the stress response pathways triggered by pharmaceuticals. Additionally, techniques using lentiviral vectors targeting key proteins and transcription factors help researchers to identify toxic mechanisms in liver cells [6].

To improve the detection of potential hepatotoxicity risks, especially during lead optimization, there is a growing need for more predictive in vitro tests. In vitro systems have long played a key role in toxicology studies, including the use of primary cell cultures, immortalized cell lines, and more complex systems like liver slices or perfused livers. These systems continue to be critical tools for advancing drug safety evaluations [7, 8].

Inquiry Toxicology Objectives and Resources

Toxicology focuses on studying the harmful effects of chemicals on living organisms, aiming to understand and manage the risks associated with exposure to dangerous substances. This includes identifying harmful compounds, such as chemicals, drugs, or pollutants, that can negatively impact humans, animals, or the environment. A key aspect of toxicology is determining the relationship between a substance's dose and its effects, helping to identify the threshold at which toxicity occurs. The field also delves into how toxic substances affect biological systems at the molecular, cellular, and organ levels. In real-world scenarios, toxicology plays a crucial role in assessing the risks of exposure and offering recommendations to reduce potential harm [9, 10].

Toxicological Developments with Significant Impact

Our research on pharmaceutical companies, conducted in both 2015 and 2020, revealed noticeable shifts in the importance of various technologies. We also ranked these technologies based on their current and projected value over the next two to five years, highlighting those that could significantly impact drug discovery and development. Over the five-year period, there was a marked increase in the perceived potential of mRNA profiling, organs-on-chips, high-content imaging (HCI), modeling and simulation, and induced pluripotent stem cells (iPSCs) to transform the field [11, 12]. In contrast, technologies like gene editing, microRNA, in vivo imaging, and mass spectrometry imaging (MSI) were seen as having a declining influence (Figure 1).

Cytotoxicity as a Predictor of Clinical Acute Hepatotoxicity

Cytotoxicity is often used as an early indicator of potential acute liver toxicity in the drug discovery process. A set of five cell lines, including CYP450 enzyme-transfected cells and a non-metabolizing parental cell line, is routinely used to predict whether a compound might cause liver failure before in vivo testing. This screening helps determine if toxicity is linked to the parent compound or an active metabolite and whether CYP450 enzymes can detoxify a toxic compound [13, 14].

While certain receptors, like CAR, can induce CYP3A4, their role is generally minor. These screens also check for enzyme level increases due to the activation of nuclear receptors. It has been observed that hepatic enzyme induction in rats can lower ALT levels, although extra-hepatic factors, such as muscle damage, can also raise serum ALT levels. Often, elevated AST levels exceed those of ALT when both are elevated [15, 16].

Some drugs, like perhexiline maleate, cause toxicity through their chemical structure rather than CYP450 metabolism. In contrast, the metabolism of other drugs, such as danazol, involves CYP3A4,

which creates an active metabolite that may contribute to liver toxicity. The exact mechanism behind danazol's hepatotoxicity remains unclear, but the selective inhibition of CYP3A4 by its metabolite is thought to play a role. Similarly, the metabolism of felbamate through CYP450 enzymes leads to the production of a toxic metabolite, which has been linked to its liver toxicity [17, 18].

Primary hepatocyte cultures, while useful for studying drug metabolism, face challenges, such as high costs, limited availability, and variability between donor samples. Moreover, human hepatocytes undergo rapid changes in response to compounds, making them less reliable for long-term toxicity studies. However, recent advances have shown that co-culturing fresh human hepatocytes with fibroblasts in organized micropatterns can extend their functional lifespan, allowing for better long-term assessments of hepatotoxicity and enzyme induction (Figure 2 & Table 1) [19].

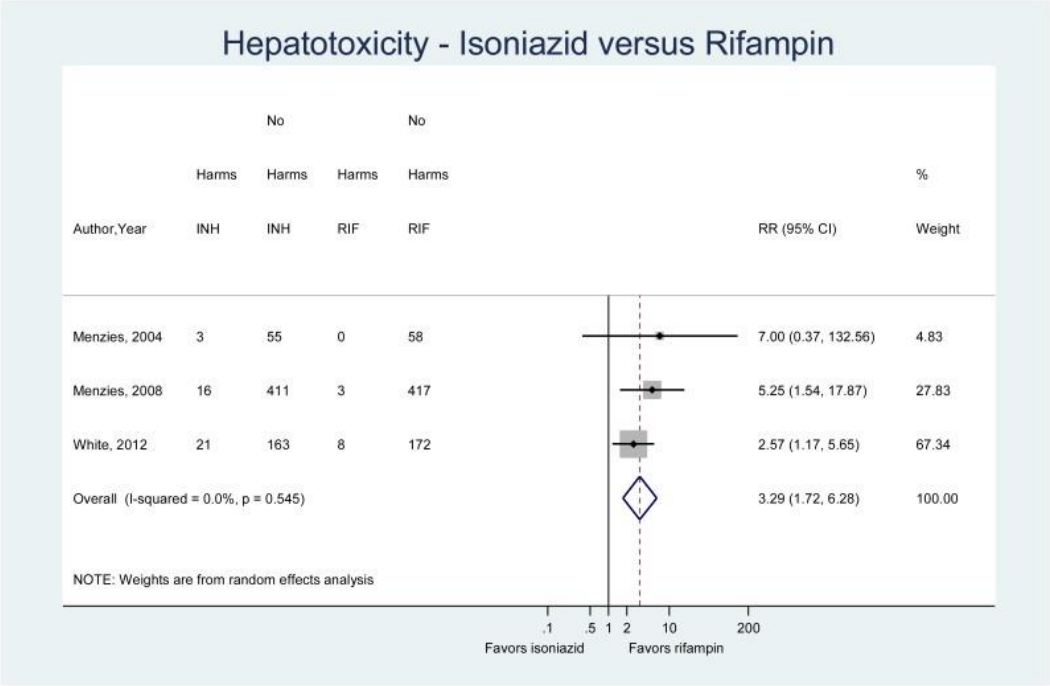

Figure 1. Hepatotoxicity – isoniazid versus rifampin.

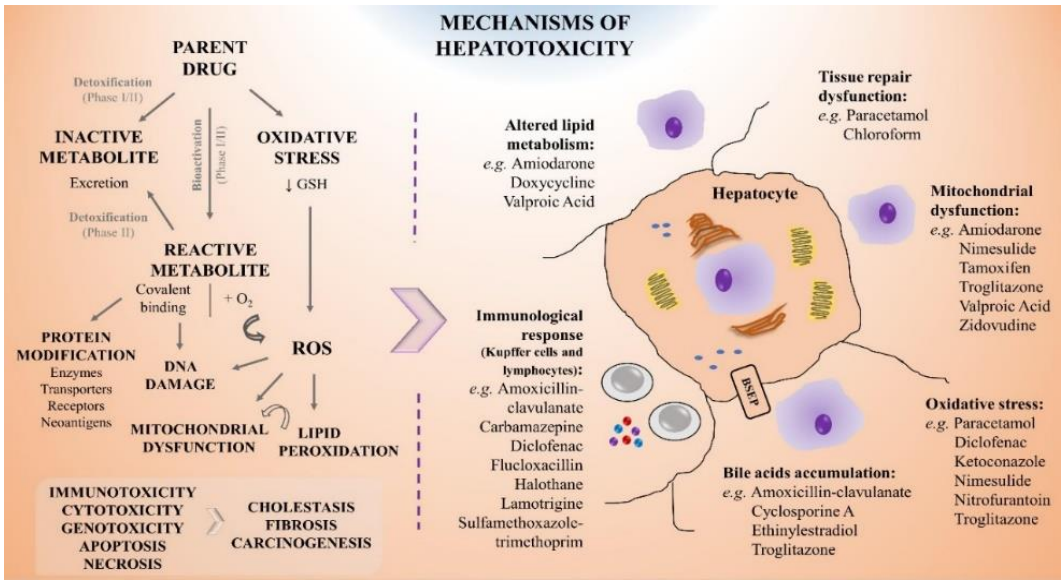

Figure 2. Mechanisms of hepatotoxicity.

Table 1. An overview of 3D hepatic cell-based models previously used for studying DILI.

Model	Cell Line	Endpoint Assay	Contribution/Main Findings	Reference
Spheroid	3D HepaRG 3D HepG2.	• Cell viability assay – (Promega GmbH). • CYP3A4 activity. • Confocal microscopy. • HPLC glucose and lactate quantification.	The study concluded that 3D cell culture models better mimic in-vivo tissue and improve cellular functionality and that the 3D HepaRG organotypic cultures represent a high throughput system for drug toxicity screening.	Mueller et al., 2014 [20].
			The study observed that CYP3A4 activity is higher in the 3D HepaRG cultures.	
			Repeated dose toxicity of chlorpromazine assessing its effects on glucose and lactate metabolism concluding that sub-toxic concentrations of chlorpromazine induced significant metabolic changes in both 2D and 3D HepaRG cultures upon acute and repeated dose exposure.	
Bioreactor	3D HepaRG.		HepaRG cells were cultured up to 68 days in a 3D multicompartment capillary membrane bioreactor, which enables high-density cell culture under dynamic conditions. 3D HepaRG cells maintained CYP450 activities that could be induced and inhibited similar to in-vivo.	Darnell et al., 2011 [21].
Bioreactor spheroids	HepG2/C3A.	• Protein content – photomicrographs. • Cell viability – ATP assay, Adenylate kinase assay. • Glycogen synthesis. • Urea assay. • Cholesterol assay. • Gene expression.	Evaluated physiological capabilities of the spheroids (cell survival, growth rate, glycogenesis, ATP, cholesterol and urea synthesis, and drug metabolism) and the expression of key genes related to the main liver pathways in spheroids cultured for an additional 24 days after full recovery (day 18) and discovered that the HepG2/C3A, can recover their main functions after trypsinization within 18 days.	Wrzesinski et al., 2013 [22].
Spheroids	HepG2	• Cell viability – MTS assay. • Genotoxicity – Comet assay. • Gene expression – qPCR. • Protein expression – western blot.	Developed an approach for genotoxicity testing with 21-day-old HepG2/C3A spheroids using a bioreactor system that showed higher basal expression of genes encoding metabolic enzymes compared to monolayer culture.	Štampar et al., 2019 [23].
Spheroids	HepG2	• Confocal microscopy. • Light sheet microscopy. • Cell viability – live/dead staining. • Luminescence-based ROS measurements.	Provided new information on the effect and penetration of SiO_2 NPs in HepG2 spheroids, the role of nanoparticle exposure in different phases of spheroid formation, and limitations of nanoparticle penetration in cellular spheroids.	Fleddermann et al., 2019 [24].
Spheroids	HepG2 HepaRG	• Cell viability – TB assay. • Urea assay. • Albumin assay. • Mononuclear micronucleus assay. • Cytokinesis block micronucleus assay.	Developed a 3D HepG2 hepatocyte model that demonstrated increased efficacy for genotoxicity over 2D monoculture. The model maintained stability and liver functionality over extended culture periods making it suitable for assessing the genotoxicity of direct and indirect-acting mutagens.	Conway et al., 2020 [25].

Spheroids	HepG2	• Cell viability assay – colorimetric assay, Almar blue assay. • Albumin assay. • Urea assay.	Provided evidence that the cell environment has a significant influence on cell sensitivity and that liver spheroid is a useful and novel tool to examine nanoparticle dosing effect even at the level of in-vitro studies.	Dubiak-Szepietowska et al., 2016 [26].
Bioreactor	Primary human liver cells	• Intracellular enzyme activity – lactate dehydrogenase (LDH), aspartate aminotransferase (AST), alanine transaminase (ALT), and glutamate dehydrogenase (GLDH), as well as urea and ammonia concentrations. • Hematoxylin-eosin (HE) and immunofluorescence staining. • qRT-PCR.	Results validate the suitability of the microscale 3D liver bioreactor to detect hepatotoxic effects of drugs in-vitro under perfusion conditions.	Freyer et al., 2018 [27].

CYP450 Induction of Enzymes

A significant concern with drug–drug interactions is their potential to cause toxicity and alter effective drug exposure levels. There are two common ways these interactions can lead to toxicity. The first is through competitive or noncompetitive inhibition of CYP450 enzymes, which are critical for metabolizing many drugs. When these enzymes are inhibited, a toxic drug may accumulate in the body. For instance, taking terfenadine alongside other CYP3A4 substrates can lead to prolonged QT intervals and dangerous ventricular arrhythmias [4].

The challenge in studying these interactions often lies in the variability of enzyme activity among human donors, which means experiments typically require cells from multiple donors to ensure accurate results. Additionally, a positive control drug that induces CYP450 isoforms is used for comparison [6].

To improve cell culture technologies, scaffolds are used to provide structural support, simulating the extracellular matrix (ECM) to create a more natural cellular environment. These scaffolds help cells adhere, interact with biomaterials, and ensure proper nutrient and gas exchange for cell growth. The goal is to replicate the biocompatible properties of native cells within the ECM. Scaffolds can be made from natural materials like fibers, collagen, or hyaluronic acid, as well as synthetic materials, such as peptides or titanium.

The Effects of Hepatic DME Induction on Clinical Implications and Toxicity

Understanding the complexities of hepatic drug-metabolizing enzyme (DME) induction is essential for assessing potential risks associated with various substances. When evaluating these risks, it's important to consider several key questions: which enzymes show increased levels after induction? What mechanisms are responsible for this induction? Are there genetic variations in humans that affect the metabolic processes involved? Additionally, what other external factors, such as exposure to different substances, influence microsomal enzyme activity?

In preclinical evaluations, researchers assess various factors related to a molecule's safety, including enzyme specificity and the potential to generate reactive metabolites, alongside induction potential. Reactive metabolites are scrutinized for their role in preclinical toxicity, as any change in the balance between toxic metabolite production and detoxification due to hepatic DME induction can have significant toxicological implications. This is particularly relevant for substances that induce phase I or phase II metabolic processes [10].

There are several reasons why certain drugs may pose risks, including increased activation of harmful metabolites, decreased detoxification, reduced inactivation, and shifts in the balance between activation and inactivation. Numerous known cases illustrate how enzyme induction can lead to hepatotoxicity, highlighting the importance of monitoring these processes. Other consequences of induction, such as alterations in thyroid and reproductive hormones, are also noteworthy.

Various xenobiotics, including alcohol, cigarette smoke, and certain medications, can significantly induce human DMEs, leading to clinically relevant effects. Understanding these interactions is crucial for developing safer therapeutic strategies and mitigating potential adverse effects [11].

Higher Metabolites Exclusive to Humans Compared to Preclinical Animal Species Resulting from Hepatic DME Induction

Preclinical safety assessment studies play a crucial role in identifying potential human toxicity and ensuring the safety of a test medication at proposed clinical dosages. Typically, these studies reveal that test substances have more pronounced effects in animal models compared to humans for most phase I and phase II metabolic reactions. Consequently, the predictions regarding drug safety derived from preclinical studies are generally adequate for evaluating safety in most cases.

However, challenges can arise when metabolites unique to humans are identified, complicating the risk assessment of xenobiotics. In instances where major and minor metabolic pathways differ significantly between species, human-specific metabolites – sometimes referred to as "unique" or "dominant" metabolites – may not be adequately tested in nonclinical safety evaluations. This can result in an overestimation of risk if a metabolite that only occurs in humans is absent from the animal models used for testing [8].

Recent regulatory guidance has emphasized the importance of assessing these human-specific metabolites, particularly when they are found at levels exceeding 10% of total drug-related exposure and are significantly higher in humans than the maximum levels observed during toxicity studies. In such cases, it is essential to conduct a thorough toxicity evaluation of these distinct human metabolites to ensure comprehensive safety assessments.

The Technology of Omics

Excessive hype surrounded toxicogenomic (TGx) in the early 2000s, but the field's initial results were underwhelming. TGx is the molecular evaluation of toxicological effects using transcriptomics or the cellular output of gene expression (i.e., proteomics and metabolomics). A multi-sector study conducted in 2010 revealed that, despite the use of TGx data for over a decade, the widespread use of TGx to enhance decision-making remained unfulfilled. On the other hand, it is now believed that applying these technologies can significantly improve preclinical toxicity research. Techniques like the L1000 technique, which is employed in the NIH Library of Integrated Network-based Cellular Signatures (LINCSs) initiative, provide high-throughput analysis of signatures and transcriptional profiling. In early drug research, the pharmaceutical industry frequently uses transcriptional profiling to rank compounds in order of priority. The fact that 42% of respondents to our poll in 2015 and 65% in 2020 thought this would alter the game for investigative toxicology shows how mature the technology is becoming (Figure 2). A lead series' medicinal chemistry optimization may be tracked by looking at the gene expression profiles of the compounds, which can be utilized to capture a variety of poly-pharmacological effects. Advances in NMR and mass spectroscopy workflows as well as analytical techniques now mean that metabolomic changes in cells, supernatants, and also bio-fluids from in vivo animal studies or clinical samples can now be readily quantified and used either for hypothesis-free profiling (often consisting of thousands of cellular metabolites) or for biased approaches that measure more focused metabolic subsets. The monitoring of metabolic changes following drug treatment, in conjunction with bioinformatics processing analysis to identify biological processes perturbed or to perform statistical comparisons with metabolic signatures of chemical/drug libraries, can facilitate the identification of toxicological mechanisms or hazards [14].

Considerations for Cell Sourcing

With approximately 500 activities, the liver is frequently referred to as the body's chemical factory. Among them is the synthesis of proteins (albumin, clotting factors, etc.), the metabolism of cholesterol, the formation of bile, the metabolism of glucose and fatty acids, the detoxification and metabolism of endogenous (bilirubin, ammonia) and exogenous (drugs, environmental toxins) chemicals. Hepatocytes process xenobiotics through three stages of metabolism and transport. The first-pass metabolism of lipophilic substances into water-soluble metabolites for the body's excretion is known as phase I, and the cytochrome P450 family (CYP450) of enzymes that are experts in oxidation-reduction processes primarily catalyze these reactions. Highly polar substances like glucose, glucuronic acid, sulfate, or glutathione are conjugated to xenobiotics and/or their metabolites by phase II enzymes. Despite the term "metabolic detoxification" being used to describe phase I and II metabolism in the liver, many xenobiotics are transformed into hazardous or pharmacologically active molecules. NPCs support and regulate the development, functioning, and in certain situations, the pathological pheWhile primary human hepatocytes (PHHs) account for nearly 80% of the liver's volume (60% of all cells) and carry out the majority of liver activities, nonparenchymal cells (NPCs), which make up around 6.5% of the liver's volume (40% of all cells), surround PPHs. Networks of arteries and ducts make up the remaining liver volume. Liver sinusoidal endothelial cells (LSECs), hepatic stellate cells (HSCs), Kupffer macrophages (KMs), cholangiocytes (biliary epithelial cells), and pit cells (intrahepatic lymphocytes or natural killer cells) are notable examples of liver NPCs. Through the synthesis of paracrine factors, these NPCs support and regulate the development, functioning, and in certain situations, the pathological phenotype of the liver (Table 2).

Table 2. Comparison of various cardiomyocyte cell sources for research applications.

Cell Source (Type)	Advantages	Disadvantages
Adult rat ventricular myocyte (ARVM)	Easy to obtain, mature.	Xenogenic.
Neonatal rat ventricular myocyte (NRVM)	Easy to obtain.	Xenogenic, immature.
Adult porcine ventricular myocyte	Genetically closer to human cells than rodents.	Xenogenic.
Human embryonic stem cell-derived cardiomyocyte (hESC-CM).	Same species, unlimited cell source.	An ethical issue, allogenic, immature.

Challenges and Prognosis

The technologies covered in this article are representative examples of some recent approaches that our survey participants believed were most likely to close gaps in our mechanistic understanding of drug safety and help with drug candidate selection. However, the discussion is not exhaustive because the goal of this article is to provide an overview of the field's current state rather than to go into great detail about each approach. Organs-on-chips is one of the promising technologies that still needs a lot of effort to improve. However, the process of separating cells from tissues is essential, which results in the loss of important data on local cellular interactions. Understanding biological processes might be greatly enhanced by the ability to locate hundreds or even thousands of transcripts in situ using newly developed, especially for complex tissues like the brain. Investigational toxicologists have fundamental challenges when deciding which approaches to invest in because the pharmaceutical industry will continue to base expenditure decisions on the trade-off between demonstrated costs and hypothetical effects (Figure 3).

RESTRICTIONS

Even if all of the various 3D culture techniques may be "done deal," meaning they are simpler to use and more similar in vivo than 2D techniques, there are still a lot of issues and unfulfilled demands. In fact, the repeatability of biomimetic scaffolds varies greatly between batches. Additionally, it may be challenging to recover all of the larger and more tortuously shaped cells for study when employing scaffolds. 3D matrices have too many components and the building of structures is tough and laborious. There are limitations to the ability to handle post-culturing

processing and the ability to scale up or down a single 3D format. Depending on the scaffold size, material transparency, and microscope depth, imaging may become challenging [15].

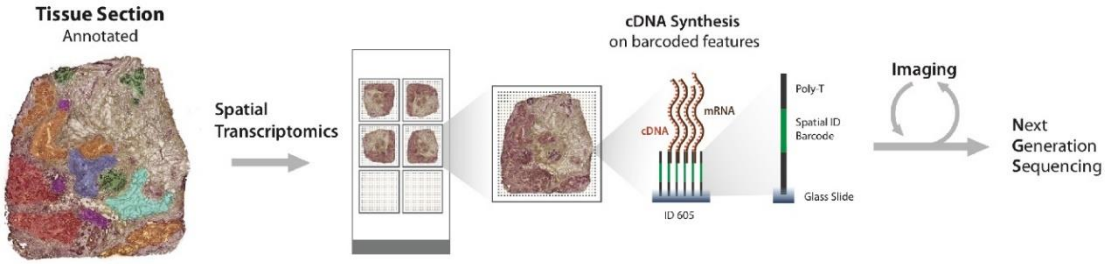

Figure 3. Workflow of spatial transcriptomics for tissue section analysis.

CONCLUSIONS

For uses in research, toxicity assessment, and safety screening, the availability of precise, illuminating in vitro tests is becoming an increasingly significant barrier. 3D cell culture significantly improves cell division, growth, and function. Only by integrating many essential fields, such as materials science, cell biology, and bioreactor architecture, can 3D culture models be successful. The development of 3D scaffolds that provide a true substitute for the in vivo environment has been made possible by technological advancements. Labeled ligands, enzyme substrates, or tracer molecules are often needed for the majority of cellular tests that depend on the detection of radio emission, absorbance, fluorescence, and luminescence. Even though RTCA is becoming more and more popular in drug development, we felt it was necessary to show that this technique is stable and dependable enough to be used in medium-throughput screening. Thus, we wanted to demonstrate the use of RTCA in quickly determining ideal culture conditions for several cellular types, such as fibroblasts, hepatocytes, cardiomyocytes, and hybrid neuroblastoma/neuronal cells, as well as to assess the assay's repeatability across many studies. Cellular consistency and quality control are major concerns due to the growing dependence on cells in drug development. RTCA profiles were compared with cell-imaging data at 24, 48, and 69 hours to gain a better understanding of the signature produced in neuroblastoma (ND7/23) cells treated with cytochalasin B, a modulator of tubulin polymerization. For all tested doses (1.1–30 μM), cytochalasin B caused a sharp drop in cell index within a few hours, with a flat signal for 10 and 30 μM up to 69 h. Cell imaging data did not show a decrease in cell viability.

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