Next-Generation Sample Preparation for Next-Generation Applications

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Next-Generation Sample Preparation for Next-Generation Applications

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Introduction

"Next-generation Sample Preparation for Next-generation Applications" explores the novel and automated Laminar Wash™ system, the only suspension-cell sample preparation workstation designed to eliminate the upstream in-process variabilities caused by the traditional sample preparation method, including labor intensive and hands-on workflows, inconsistencies across users and locations, difficult and time-consuming technology operations, and centrifuge-based mechanical stress on the samples.

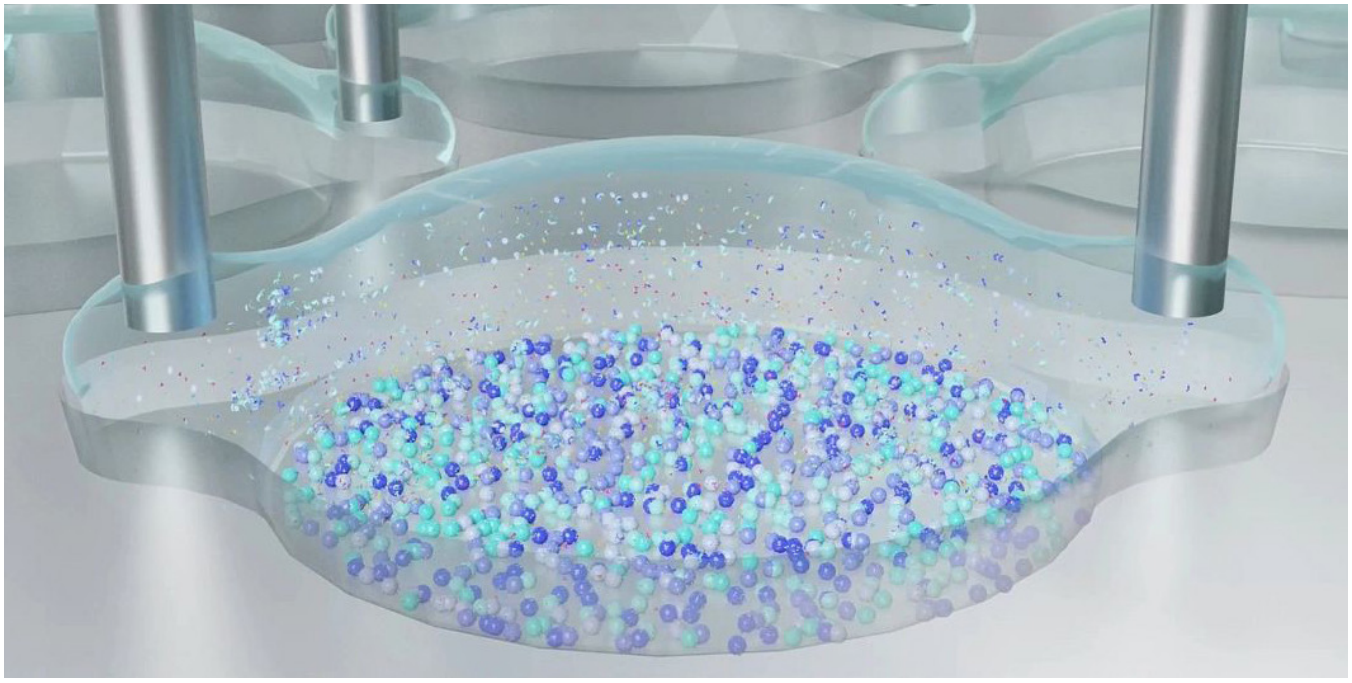
Readers will discover the benefits of the Laminar Wash™ system, including increased efficiency, reproducibility, and workflow optimization. The eBook explores the gentle Laminar Wash™ technology and the advanced user-friendly software of the system that automate tedious and error-prone manual sample preparation steps. In addition, the eBook discusses the broader implications of this technology for scientific research and analysis in a variety of fields (cell and gene therapy, single-cell multiomics, biomarker discovery, and tumor microenvironment, to name a few). It showcases real-world case studies and success stories, highlighting how automating sample preparation workflows with the Laminar Wash™ system revolutionizes data quality, throughput, and experimental precision.

Overall, this eBook aims to empower scientists and researchers with a comprehensive understanding of the Laminar Wash™ system and its potential impact on their scientific endeavors. By embracing this innovative method, researchers can enhance the accuracy, efficiency, and reliability of their sample processing, leading to accelerated discoveries, improved analytical capabilities, and advancements in various fields of science.

About Curiox Biosystems, Inc:

Next-generation therapies deserve next-generation sample preparation solutions. Curiox has brought together surface chemistry and instrumentation expertise to overcome critical challenges slowing the pace of life-science research. By focusing on common assay steps and workflows where miniaturization and automation are currently underutilized, the company has developed innovative technologies that simultaneously improve both productivity and data quality, accelerating the pace of therapeutic development.

Curiox–Technology that Enhances Single-Cell Workflow and Addresses Unique Problems in Cellular Heterogeneity



Authors: Melvin Lye, MBA, BCMAS and Mahrukh Banday, DVM, PhD

Abstract

Single-cell and single-nuclei transcriptomics present countless potential avenues of research. Sequencing individual cells and nuclei enables us to look at the remarkable diversity of biomarkers and cellular events in heterogeneous populations, which are more reflective of true biology. Unfortunately, such merits are overshadowed by limitations in the technique's workflow. Curiox's state-of-the-art Laminar Wash™ system, HT2000, pioneers a novel method of washing suspension cells that introduces enhanced scalability, quality, and flexibility into the workflow of single-cell and single-nuclei transcriptomics, addressing its existing constraints, and expanding downstream applications. In doing so, the Laminar Wash™ technology opens up a world of research possibilities for single-cell and single-nuclei transcriptomics, revolutionizing our definitions of true biology.

Introduction

Currently, single-cell and single-nuclei transcriptomics possesses a lot of potential to open doors

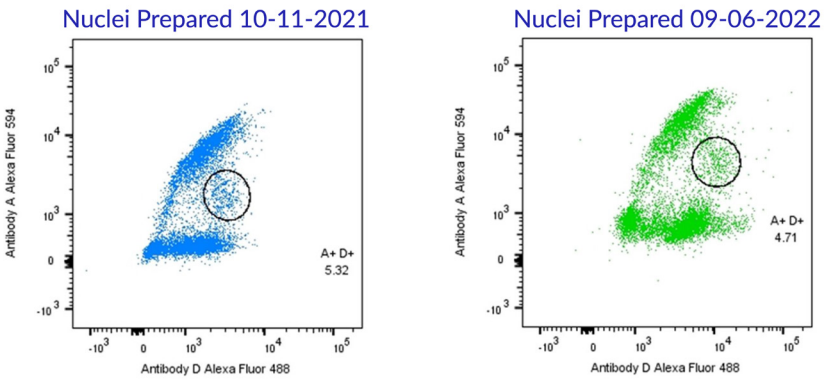
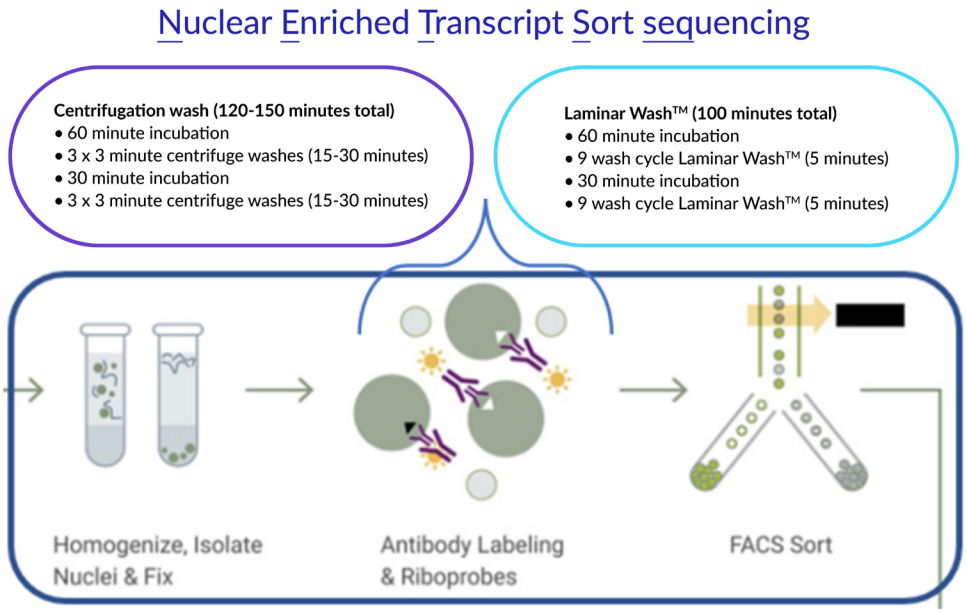


Fig 1. Consistency between nuclei stain experiments across different timepoints. Combining fully optimized Laminar Wash™ staining protocol to label nuclei prepared from matching tissue dissections (same donor and same brain region) on different days produces consistent staining to help identify highly selectively novel targets using Cerevance's proprietary Nuclear Enriched Transcript Sort sequencing (NETSseq) platform.

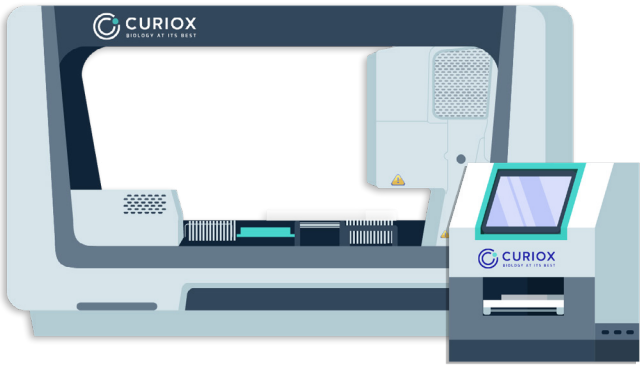
to new avenues of research. Through sequencing the individual cells and nuclei themselves, we are now able to look at a remarkable diversity of biomarkers and cellular events. Such phenomena are typically masked by bulk sampling methods and were previously overlooked. This is thus a noteworthy breakthrough for the industry as materials of true biological nature often display considerable cellular heterogeneity. By delving into the uniqueness of individual cells and nuclei, the field is poised to achieve an advanced understanding of true biology and hence its potential.

Unfortunately, the merits of single-cell and single-nuclei transcriptomics are currently overshadowed by limitations in their existing workflow. As part of the transcriptomics workflow, cell and nuclei samples are washed to remove cell debris and unbound signals before data analyses are carried out. However, centrifuge-based technologies spell out long wash times between antibody incubations and impose a constraint in the number of concurrent washes that can be executed. This exponentially increases the time required for processing large batches of samples,

Fig 2. Significant time savings and scalability through reduced washing time and increasing parallel sample processing.



limiting the technique's scalability. Furthermore, due to its high minimum nuclei input threshold, centrifuge-based washing is unable to handle samples with small nuclei amounts without noticeable yield reductions. Having such drawbacks within the workflow greatly restricts the downstream applications, and thus potential, of single-cell and single-nuclei transcriptomics in the industry.



Laminar Wash™ AUTO 1000 and Laminar Wash™ HT2000

Setting out to address the evolving needs of the industry, Curiox developed a technology that improves the workflow of single-cell and single-nuclei transcriptomics for greater efficiency and support for cellular heterogeneity. HT2000 is a Laminar Wash™ system that introduces an innovative method of washing suspension cells using surface tension, a departure from the conventional centrifuge-based process. This approach introduces enhanced scalability, quality, and flexibility, thereby expanding downstream applications while bringing us one step closer to understanding the diverse nature of true biology.

Curiox Laminar Wash™ Technology

The Curiox HT2000 consists of a proprietary Laminar Wash™ system and 96-well Laminar Wash™ plate. The uniquely designed Laminar Wash™ plate is spotted with an array of hydrophilic dimple-shaped spots for suspension cell samples to be loaded onto. Once the plate is presented into the Laminar Wash™ system, a manifold of nozzles is lowered into the samples on the plate, which creates a gentle laminar flow that washes the samples without the mechanical stress and cell losses associated with centrifugation-based washing.

The technology is tailored to address many of the existing limitations of the conventional centrifugation method. While the Laminar Wash™ system reduces the entire washing process, the 96-well Laminar Wash™ plate allows for more sample washes to run in tandem. Both features help to achieve time savings and thus improve scalability of the workflow, allowing bigger batches of samples to be processed with ease. The Laminar Wash™ system can also accommodate smaller inputs as low as 0.1×10^6 nuclei without compromising the quality of data. Previously impractical research avenues involving samples that are scarce or challenging to acquire such as clinical samples and antibodies are now within reach. In addition, using laminar flow instead of centrifugation results in stress-free and cleaner samples, minimizing effects on RNA integrity or sequence quality due to the turbulence associated with centrifugation.

Enhancing Downstream Applications

As the industry shifts its attention towards personalized medicine, more studies are now investigating whole tissues and diverse cell populations. Hence, unique problems associated with utilizing single-cell and single-nuclei transcriptomics for diverse samples arise. Samples that are more heterogenous require more thorough washing to produce cleaner samples for better data analysis, which are currently out of reach for centrifuge-based washing techniques. As a result, the heterogeneity of the samples of interest often introduces a substantial amount of noise in the data, making data analyses challenging for samples of such nature.

The lack of sensitivity for smaller subpopulations within a sample is another bane of existing wash technologies. Centrifugation is often not equipped to retain rarer subsets of cells within a sample, resulting in a preferentialism of subpopulation ratios detected, and a misrepresentation of such samples in the data.

The Laminar Wash™ system presents a solution to these challenges. By avoiding extra processing stress that may affect cell size and cycle state, the technology improves barcode impurities and biological consistency. Curiox's scientists have carried out studies on the system's capabilities in this aspect. The team found that the Laminar Wash™ system had superior retention of rare subpopulations compared to centrifugation, allowing for better analysis of heterogeneous samples. They also established that in comparison with centrifugation, the novel system was more efficient at debris removal. This results in cleaner samples which reduce noise in downstream data analysis, allowing weaker signals to be detected with greater ease. The Laminar Wash™ system is a viable platform that supports recovery rate, which meant that less starting material was required for the workflow. Savings on production costs can be achieved by a switch from centrifuge-based washing to the Laminar Wash™ system.

The Laminar Wash™ system has since been integrated into a wide range of studies on whole tissues and diverse cell populations, enhancing their workflows and helping to overcome unique challenges in each investigation.

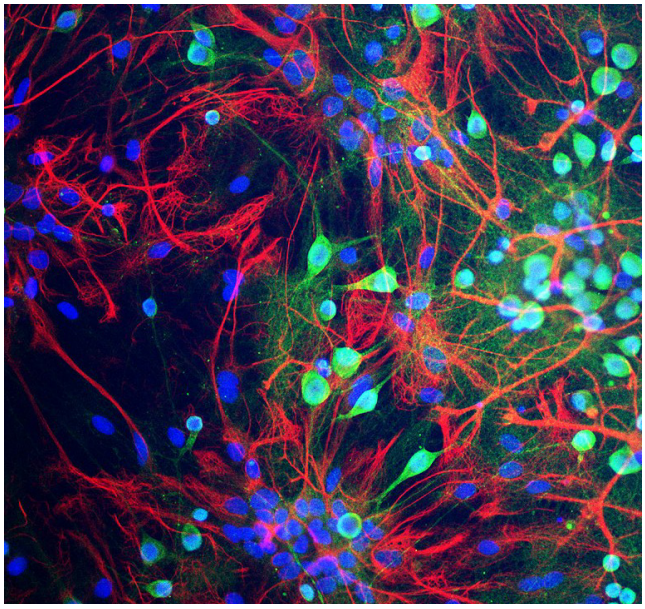
Profiling Brain Cell Types in Healthy and Diseased Brain Tissues

To develop new therapies for neurological and psychiatric diseases, Cerevance, a therapeutics company, identifies novel targets using its proprietary Nuclear Enriched Transcript Sort Sequencing (NETSseq) platform. The team has since investigated profiling specific brain cell subsets in healthy and diseased human postmortem central nervous system (CNS) tissues, using NETSseq to identify and sort the cell type–specific nuclei in the samples. The extensive procedure consisted of tissue

homogenization with nuclei extraction, fixation, and antibody staining followed by fluorescence-activated sorting of nuclei into their separate types. These cell type–specific nuclei populations will then be used for downstream transcriptomic and epigenomic comparisons between disease and non-disease control tissue to identify potential targets for treatment of neurological diseases.

To investigate the subtle differences in cell type ratios between diseased and healthy brain tissues, it was crucial for their workflow to maintain the frequencies of the different cell types in their samples. This ensured that representative data could be obtained for comparative analyses.

By integrating Curiox's HT2000 with their NETSseq platform, Cerevance's workflow was improved in the form of significant time savings and greater flexibility in the minimum nuclei sample size that could be processed. A higher nuclei retention was also achieved compared to when centrifugation was utilized. This not only produced quality representative data, but also allowed for smaller starting materials. Moreover, similar, or superior staining of nuclei was achieved with 50% less antibodies as a result of the effective washing, spelling favorable reagent and cost savings for Cerevance. Curiox's technology has thus worked synergistically with the NETSseq platform to produce superior quality data for target discovery, whilst achieving time and resource savings.



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Neurons from rat brain tissue stained green with antibody to ubiquitin C-terminal hydrolase L1 (UCH-L1), which highlights the cell body strongly and the cell processes more weakly. Astrocytes are stained in red with antibody to the GFAP protein found in cytoplasmic filaments. Nuclei of all cell types are stained blue with a DNA binding dye.

Characterizing of B Cells and Their Functions in Different Immune Scenarios

B cells are important immune cells vital in the adaptive immune response due to their ability to secrete antibodies. Within the B cell population, B cells can be sorted by using CD19, CD27 and IgD markers to the respective subpopulations. Despite the ability to classify B cells using these markers, it is reported that the double negative population (DN, CD19+ CD27- IgD-) have differing functionality, indicating a heterogeneous population. Furthermore, the DN population is also observed to be associated with autoimmunity conditions such as systemic lupus erythematosus (SLE). To maximize immunological insight, researchers at the University of Surrey and

King's College London aimed to further characterize the DN population into different subsets. After the DN population is classified to various subsets, the functionality of these B cell subsets could then be further elucidated.

With the advancement in single-cell transcriptomics analyses, B cells can be classified into different subsets within a subpopulation using gene expression data instead of just three surface markers. However, a pressing challenge for these studies is that only 5 to 10% of patients' peripheral blood mononucleated cells (PBMC) samples are B cells. Furthermore, classifying them into different subsets within a subpopulation meant that there was a lower number of B cells to work with. If B cell recovery during sample processing is low, B cell subsets present in the minority risk being undetected.

To minimize cell loss during sample processing, the HT2000 was used to process the samples. With high cell recovery, the researchers gathered sufficient data and identified a novel subset of B cells in the DN population. The novel B cell subset is IgE-rich, suggesting that it may have roles to play in autoimmunity or allergic reactions. Without Curiox's technology, the novel B cell subset is unlikely to have been identified. This is because only 5 to 10% of the PBMCs are B cells, and approximately 0.5% of the total B cell population is classified into this novel subset. Hence, the low cell recovery of the traditional centrifugation method lacks sensitivity, while the Curiox provides a sensitive platform allowing for high cell recovery.

Challenging the conventional wisdom of immunology

With the proliferation of highly sophisticated technology in the field of scientific research, conventional immunological concepts are bound to be challenged. This phenomenon is observed in the study on B cell clone evolution post SARS-CoV-2 infection.

Investigating the Evolution of B Cell Clones in the context of COVID-19

Since the emergence of SARS-CoV-2 in December 2019, there has been an increased interest in studying the evolution of antigen-specific B cell clones post-infection. The traditional understanding of antibody response towards a novel pathogen is that IgM would be the dominant antibody class before class switching to other antibody isotypes. To the surprise of several research groups, IgA and IgG isotypes emerge early in SARS-CoV-2 infection, even before IgM. Various research groups also suggested that the early rise in IgA and IgG isotypes was due to the reactivation of memory B cells (MBCs) specific to seasonal beta coronaviruses. These results suggest that the pool of MBCs present in our bodies has a greater breadth of antigenic binding than previously thought. Therefore, this led to the hypothesis that

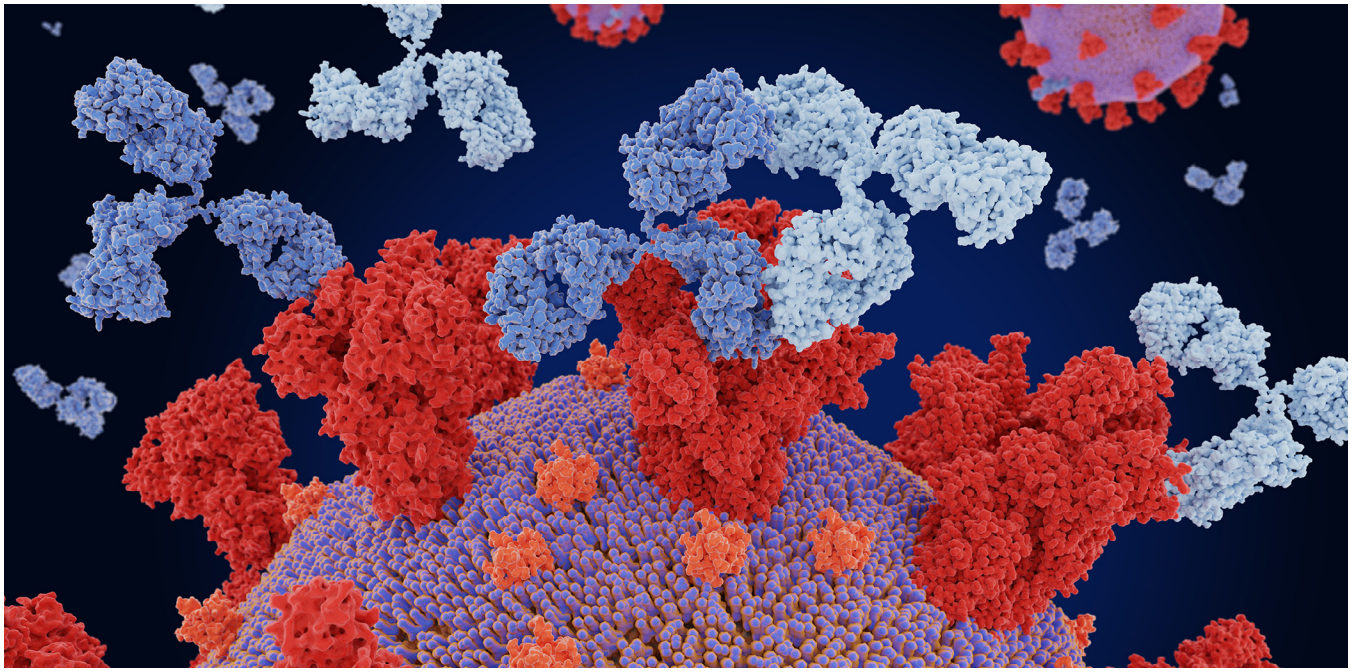

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Illustration of two different therapeutic monoclonal antibodies (y-shaped) binding to different antigenic sites on a SARS-CoV-2 virus spike protein (red).

the evolution of MBCs continues post-infection and can protect against possible emerging variants of the virus.

To better understand the evolutionary dynamics of MBCs post-SARS-CoV-2 infection, a longitudinal study is conducted by a group at the University of Gothenburg, Sweden. The study requires the collection of PBMCs from six patients before conducting single-cell RNA-seq, CITE-seq, and BCR-seq. The small sample size coupled with sequencing techniques requiring a minimum number of cellular counts meant that cell recovery is crucial. The conventional centrifuge-based washing method may result in low cell recovery; hence, the downstream analysis will be negatively impacted. By using the HT2000 system, the team managed to process PBMC and sufficiently recover cells needed for analysis.

The HT2000's ability to process PBMC samples allowed researchers to uncover B cell evolutionary dynamics post-infection. Indeed, this research group discovered that MBCs increase their binding and neutralization breadth post-infection. Interestingly, monoclonal antibodies derived from SARS-CoV-2-specific MBCs started recognizing viral variants within three months despite only being exposed to the original Wuhan strain.

Expanding Horizons Beyond True Biology

Aside from inquiring into true biology, the industry is also simultaneously shifting gears towards

confronting the conventions of biology. The field of cell engineering challenges scientists to think out of the box and come up with creative and unconventional solutions to tackle problems in biology. The Laminar Wash™ system improves continual analytics between in vivo and in vitro conditions and mimics the biological continuum by avoiding extra processing stress that may affect functionality. Such capabilities better equip single-cell and single-nuclei transcriptomics for cell engineering, expanding the technique's applications beyond what we know as true biology.

Conclusion

Curiox's breakthrough Laminar Wash™ technology offers greater efficiency, flexibility, and quality in the washing of samples that is part of the single-cell and single-nuclei transcriptomics workflow. This presents superior scalability and savings in resources compared to existing centrifugation-based technology. Besides providing enhanced support for existing applications in single-cell and single-nuclei transcriptomics, the Laminar Wash™ also opens doors to a myriad of new applications and research avenues for the technique. The technology has since seen applications in a wide range of fields, including immunology and neuropharmacology. With such breakthrough technology, researchers may now rise to the challenge of uncovering the sheer diversity within heterogeneous cellular populations and exploring their own unique definitions of true biology.

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Harnessing Cell Therapy's Cancer-Killing and Tissue-Reviving Potential

Researchers are deploying a range of genetic engineering techniques to realize the true potential of cellular therapies

BrainStorm Cell Therapeutics, a developer of adult stem cell therapeutics for neurodegenerative diseases, produces MSC-NTF cells, that is, autologous cultured mesenchymal bone marrow stromal cells that secrete neurotrophic factors. MSC-NTF cells may be used to deliver multiple NTFs and immunomodulatory cytokines directly to damaged neurons. To expand and differentiate MSC-NTF cells ex vivo, the company uses its NurOwn platform. At present, the company is evaluating how well the cells slow or stabilize the progression of amyotrophic lateral sclerosis.

Author: Katarina Zimmer

Scientists have long conceived of using living cells as treatments for human ailments. In the 19th century, various kinds of animal cells were injected into people in attempts to treat age-related diseases or cancer. Fortunately, the field took on a more serious face in the mid-20th century, when researchers' understanding of the human immune system greatly improved.

In the late 1950s, the American physician Edward Donnall Thomas and other scientists began treating leukemia patients by irradiating their bodies to eradicate the cancer and giving them infusions of bone marrow cells from healthy

donors to restore their blood-forming systems. The donors' tissues rarely engrafted into the recipients, however, and many patients died from serious immune reactions or infections.

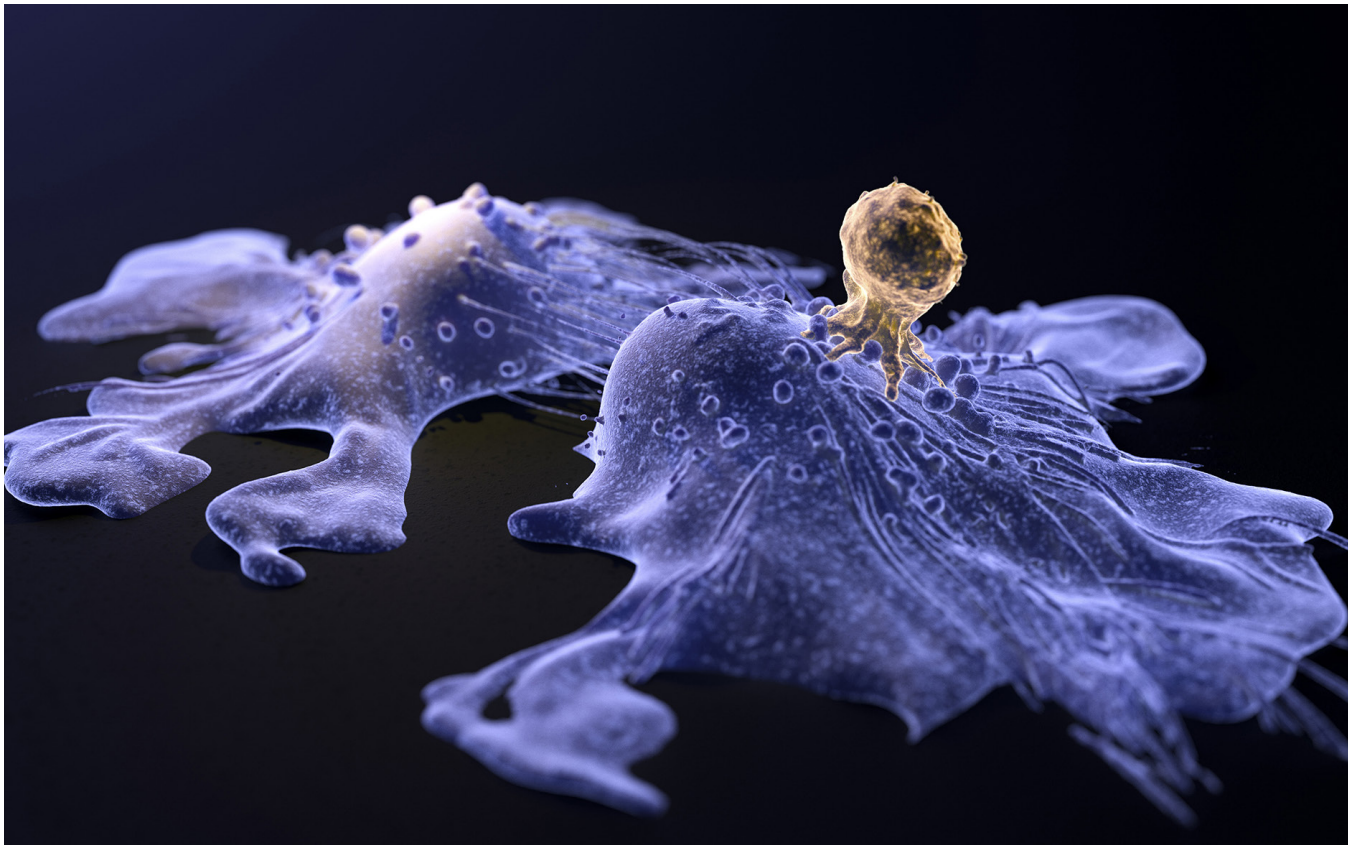
Thomas found more success when he sourced the bone marrow cells from an identical twin of the recipient, which led to a remission of the leukemia until it eventually recurred. The key, he and others learned, was genetic similarity of cell surface receptors called human leukocyte antigens (HLAs) to prevent recipients' immune systems from rejecting the foreign donor tissue. Over the years, he and others developed ways of HLA-matching donors with suitable recipients, as well as immunosuppressive treatments that helped prevent the transplanted cells attacking the recipients' tissues.[1, 2] Eventually, bone marrow transplantation became an important therapy for certain blood cancers and other diseases.

These developments also sparked another line of research. Thomas and others noticed that donor immune cells would sometimes help eradicate rogue tumor cells that remained in recipients' bodies. Researchers surmised that infusing immune cells alone into patients' bodies could help combat blood cancers—a vision that came to fruition years later when advances in genetic engineering facilitated the development of chimeric antigen receptor (CAR) T-cell therapies. These involve extracting a patients' T cells, genetically engineering their receptors to target specific cancer cells, and infusing them back into patients' bloodstreams. In what the U.S. Food and Drug Administration (FDA) called a "historic action," the first CAR T-cell therapy, tisagenlecleucel, was approved in 2017 to treat certain patients with acute lymphoblastic leukemia for whom standard therapy had been unsuccessful.[3] Since then, a number of CAR T-cell therapies have been approved for a range of blood cancers.

Now that cell therapies—and genetically engineered cell therapies—are a mainstay of modern medicine, scientists are working to find more applications for them. Many are tinkering with immune cells to make them work for broader groups of patients and to direct them against solid tumors, which has so far proven challenging with CAR T cells. Others, meanwhile, are tinkering with stem cells to revive damaged tissues in degenerative diseases, in the hope of tackling progressive neurodegenerative diseases such as multiple sclerosis (MS) and amyotrophic lateral sclerosis (ALS). *GEN* spoke with a number of scientists and company leaders to learn more about the challenges and successes at these two therapeutic frontiers.

Making CAR T cells better killers

The California-based company ImmPACT Bio is working on developing more effective CAR T-cell therapies. One limitation of some popular CAR T-cell therapies is that cancer cells often lose the B-cell receptor they're designed to target—the



Thom Leach / Science Photo Library/Getty Images

Illustration of a T cell (brown) attacking cancer cells (purple). CAR-T therapy is a type of immunotherapy that genetically modifies a patient's own T cells to recognize and destroy cancer cells.

CD19 protein, for instance—which can allow the malignancy to recur, says Sumant Ramachandra, MD, PhD, Imm-PACT Bio's president and CEO. He explains, "There's usually an antigen loss by malignant B cells when they're under selection pressure by CD19-CAR therapy."

ImmPACT Bio specializes in developing logic-gated CAR T-cell therapies. In these therapies, the CAR T cells are engineered to follow logical rules, such as targeting either one of two antigens. The company's lead product is a CAR T-cell construct designed to target either the CD19 or CD20 B-cell receptor.

The construct was developed by one of the company's scientific founders—Yvonne Chen, PhD, of the University of California, Los Angeles (UCLA). UCLA researchers recently concluded a safety study of 10 patients with relapsed or refractory non-Hodgkin lymphoma who received an infusion of their own T cells after the T cells had been engineered to express the construct. In Phase I data reported in a preprint, nine patients responded to the therapy and seven achieved complete remission after two months.[4]

Importantly, no patients experienced neuro-toxicity or severe levels of cytokine release

syndrome—common side effects of conventional CAR T-cell therapies and a reason why the therapies are given in the inpatient setting. Fewer side effects mean that outpatient administration is possible. "If your therapy is very tolerable," Ramachandra elaborates, "you can now increase access."

ImmPACT Bio researchers are now planning another Phase I study to test the CAR T-cell therapy with the company's own industrialized manufacturing process, alongside efforts to develop other logic-gated CAR T-cell therapies for solid tumors.

Reengineering T cells for solid tumors

A2 Biotherapeutics, a firm based in California, is also pursuing a CAR T-cell therapy for solid tumors. One of the challenges involved is that many of the CAR T-cell therapies tested so far target tumor-associated antigens that are also expressed in healthy tissue, which the T cells end up attacking. "With regular cell therapies … there is this on-target, off-tumor toxicity that can happen," explains Talar Tokatlian, PhD, a senior scientist at A2 Biotherapeutics. "Depending on the tissue, it can be very problematic."

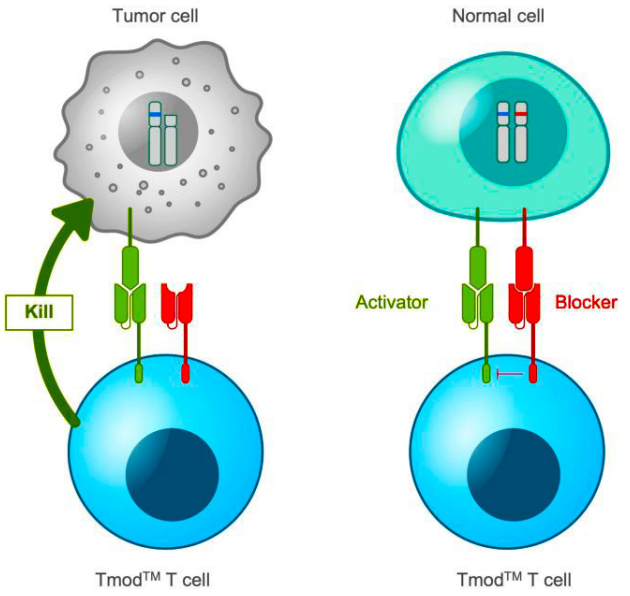
The company's solution consists of logic-gated CAR T-cell constructs that target tumor-associated antigens but are directed not to target cells with antigens that are expressed only on healthy cells. One of the company's leading candidates targets the carcinoembryonic antigen (CEA) present in colorectal and certain other cancers, but is blocked by HLA-A*02, a commonly expressed antigen in healthy tissues. Tokatlian says that for a subset of patients whose tumors have lost expression of HLA-A*02, the treatment would selectively eliminate tumor cells while protecting healthy tissues.

One 2022 study reported that these CEA-targeting "Tmod" cells selectively killed CEA-expressing tumor cells and avoided HLA-A*02-expressing cells in mouse cancer models.[5] The team observed similar results with Tmod cells that target the tumor-associated antigen mesothelin and are blocked by HLA-A*02.[6]

The limitation, Tokatlian points out, is that these therapies apply only to patients who are heterozygous for HLA-A*02 and have complete loss of the antigen in tumor cells. The goal is to find more combinations of activating and blocking antigens to identify other subgroups that could benefit. "Once we show that this works," she says, "then the next step is to make sure we can apply this therapy to a lot of different patients."

Mobilizing natural killer cells

Boston-based Catamaran Bio is using another immune cell type entirely to tackle solid tumors. Natural killer (NK) cells, part of the innate immune system, have several advantages over



A2 Biotherapeutics has invented the Tmod platform to develop cell therapies that possess, to a very high degree, the ability to distinguish between tumor cells and normal cells. Tmod exploits gene losses that occur in tumors, but not in normal cells, by utilizing receptor pairs. In each pair, one receptor is an "activator," and one is a "blocker." When both receptors are engaged, or when only the blocker is engaged, the T cell is not activated. Essentially, the blocker functions as a safety switch. It does not protect tumor cells that lack blocker antigens.

T cells in that they're capable of killing tumor cells even if those cells don't express the target antigen. In addition, the way that NK cells identify foreign cells makes them less likely to attack a recipient's tissues if they're derived from a different person—facilitating the development of off-the-shelf therapies that don't need to be manufactured individually for each patient. And NK-cell therapies are expected to be much safer than the CAR T-cell therapies, suggests Alvin Shih, MD, CEO of Catamaran Bio.

The challenge with developing any cellular therapy for solid tumors, however, is that the tumor microenvironment is inherently immunosuppressive, often harboring cytokines like transforming growth factor-b (TGFb) that have inhibitory effects on immune cells. That's why Catamaran Bio researchers have engineered NK cells, derived from peripheral blood, to carry a TGFb dominant negative receptor that enables the cells to capture the cytokines without activating any intracellular pathways and shuttle them out of the tumor microenvironment. Rather than using a virus to introduce the receptor-encoding genes into the NK cells, the researchers use a transposon-based system. According to Shih, this system is more efficient and allows for the insertion of larger genes.

In preclinical studies, Catamaran Bio scientists engineered these TGFb-capturing NK cells to target the HER2 receptor present in breast tumors.[7] Mice with HER2-expressing tumors that received the NK cells survived longer than controls. "There's a significant difference," Shih remarks, "between the placebo-treated and the CAR-NK-treated animals, both in terms of tumor volume as well as survival."

Harnessing mesenchymal stem cells

Meanwhile, the New York–based Brainstorm Cell Therapeutics is deploying mesenchymal stem cells (MSCs), which play various supportive roles in the body, to stall tissue loss. "Some cells within our bodies … have these amazing properties

in and of themselves," says Stacy Lindborg, PhD, executive vice president and chief development officer at Brainstorm.

Lindborg says Brainstorm's therapy, NurOwn, has a multipotent mechanism of action that can tackle progressive diseases, including the autoimmune disease MS as well as ALS, which leads to paralysis and death. The treatment consists of MSC cells that are isolated from individual patients' bone marrow, induced to express neurotrophic factors, and delivered into patients' cerebrospinal fluid, from where the MSC cells migrate to areas of inflammation. In some animal models of neurodegenerative diseases, implanting MSC cells that secrete neurotrophic factors had a neuroprotective effect.[8, 9]

Brainstorm recently published results for a Phase II study of 18 patients with nonrelapsing, progressive MS who received multiple treatments of NurOwn.[10] The therapy appeared to be safe, and 19% of patients saw improvements in a walk speed test after 28 weeks. Interestingly, researchers also observed an increase of neuroprotective proteins and a decrease in inflammatory biomarkers in treatment recipients' cerebrospinal fluid—an observation also made in a Phase III study of NurOwn in 263 ALS patients.

However, that study didn't meet its primary endpoint, with 33% of NurOwn recipients seeing an at least 1.25-point change in disease progression on an ALS functional rating scale after 28 weeks, compared with 28% of placebo-receiving patients—a difference the FDA didn't consider sufficient to grant NurOwn a Biologics License Application.[11] Buoyed by revised results, Brainstorm is now resubmitting its request. (The company found that the original results had been distorted by a statistical error in the analyses.[12] Revised results for the study show that NurOwn significantly slowed disease progression on a secondary endpoint for a patient subgroup with less severe disease.)

Repairing motor neurons in ALS

Clive Svendsen, PhD, executive director of the Board of Governors Regenerative Medicine Institute at Cedars-Sinai Medical Center in Los Angeles, and his colleagues are also using stem cells to tackle neurodegenerative diseases, including retinitis pigmentosa, Parkinson's disease, and ALS.[13]

Their approach, however, is based on a neural progenitor cell line derived from a human fetal cortical tissue sample isolated many years ago. The cells are genetically engineered to express glial cell line–derived neurotrophic factor (GDNF), which promotes neuron survival. The strategy is to transplant the cells directly into the spinal cord; they then migrate to sites of degeneration and differentiate into GDNF-secreting astrocytes, a cell type that supports motor neurons.

Svendsen and his colleagues recently tested these cells in a Phase I/IIa study, transplanting

them into one side of the lumbar spinal cord of 18 ALS patients, who received immunosuppressive drugs to prevent transplant rejection. The treatment appeared safe.[14] Notably, tissue analysis of 13 individuals who had died from ALS showed that the implanted cells survived and secreted GDNF. "That's remarkable given we didn't even know if they'd survive in an adult spinal cord, let alone in the environment of ALS," Svendsen says.

The team is planning another study that involves implanting the stem cells into ALS patients' motor cortexes to tackle degeneration at the upper motor neurons. Svendsen notes that slowing disease progression requires treating both sites. He acknowledges that the road ahead is long. "This is a marathon," he remarks. "It's a series of safety steps, leading to a therapy which could protect both sides of the system."

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Current State and Future State of Sample Preparation in Cell and Gene Therapy

Targeting the Tumor Microenvironment and Cytokines

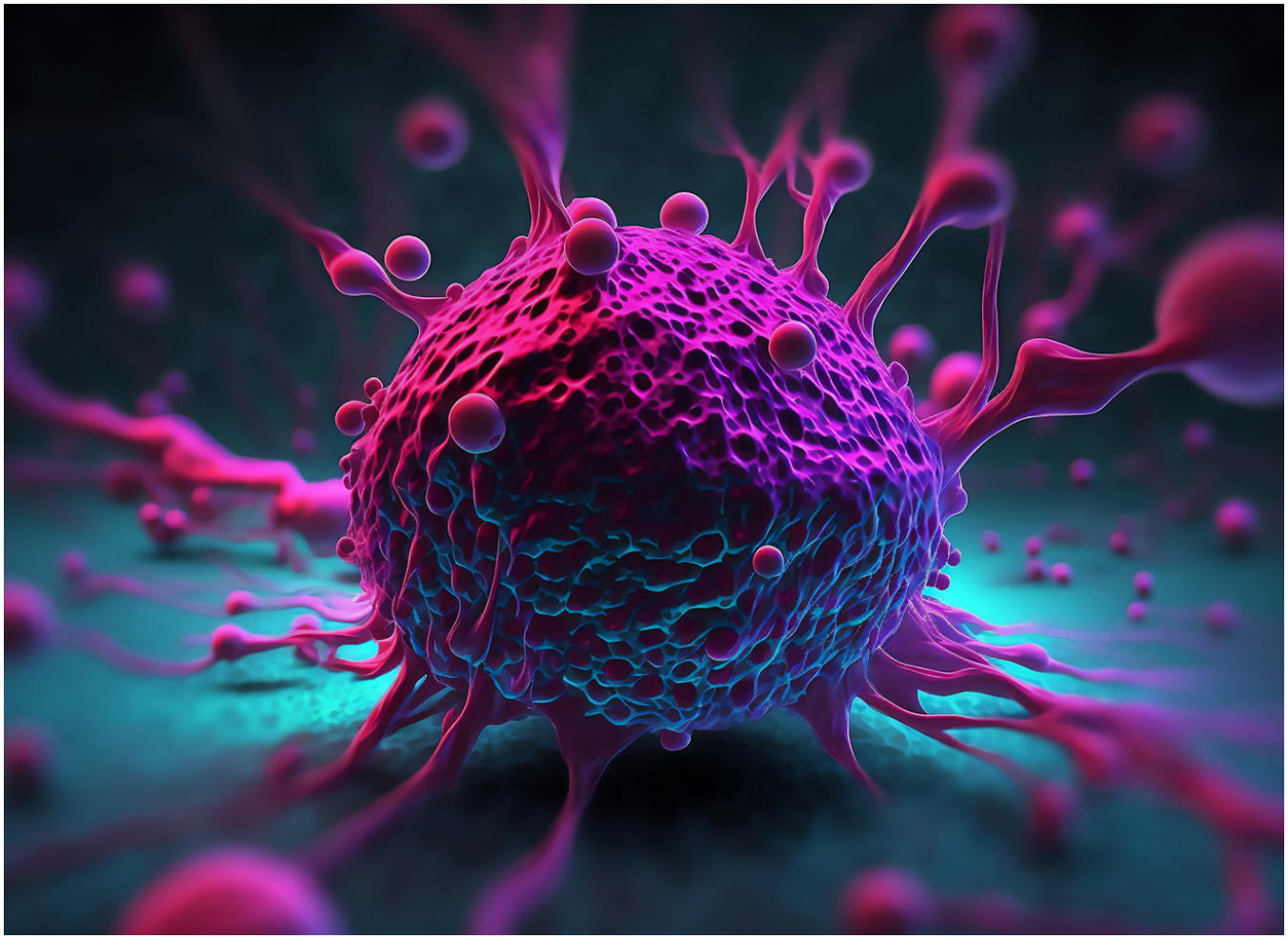
Authors: Sam Virolainen, Christoph Eberle, PhD, MICR, FRSPH, and Melvin Lye, MBA, BCMAS

Recent discoveries in basic research have fundamentally transformed our understanding of cancer. Advancements in techniques like single-cell genomics in tandem with the ability to study patient samples ex vivo have enabled researchers to understand both clinical and molecular aspects of tumors that were unknown just years ago. However, even with these tremendous advances, challenges remain. Cancer studies rely on the isolation of often rare and heterogeneous tissue samples, and there is an urgent need to translate fundamental discoveries made in basic research labs into clinical trials and the next generation of therapeutics for patients. A practical starting point to addressing these challenges and moving the field forward involves approaching cell preparation in a way that emphasizes functionality, has been tested in a variety of experimental contexts, and will be vital in ushering in the next generation of scientific discoveries beyond the field of cancer research.

The answer to this question is already in the research community's toolbox, and it involves employing basic principles of debris removal and quality control to enable high-quality and -resolution research. Laminar Wash™ technology, pioneered by Curiox Biosystems, has already met current needs of the research community in a variety of contexts, from isolation of rare immune cell types in tumors from primary samples to discovery of a novel biomarker in heart tissue. These applications show the potential of this technology to open the door to discoveries we currently see as unattainable, and the basic principles underlying this technology have the potential to grow and evolve in concert with new discoveries and the needs of the research community.

Accurate Characterization of Tumor-Infiltrating Lymphocytes

A major challenge in tumor microenvironment (TME) research is understanding how some immune cells can infiltrate and kill cancerous cells. Tumor Infiltrating Lymphocytes (TIL) are immune cells with the ability to successfully mount an anti-tumor attack despite the complex milieu of the TME. Understanding what enables the anti-tumor immunity of these infiltrating lymphocytes at the cellular level could inform novel therapies. Yet isolation of TILs remains challenging. Since



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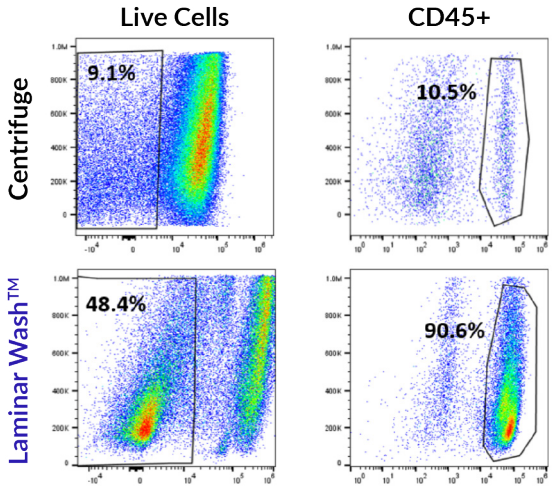
these are co-located with an array of other cell types in addition to the tumor cells themselves, the tissue environment is highly heterogeneous. Therefore, obtaining a pure TIL population is experimentally cumbersome, and their phenotypic characterization by flow cytometry or single-cell sequencing can likewise be difficult, especially since conventional isolation methods tend to result in less viable cell recovery. This hampers the development of reproducible findings that could inform our understanding of TILs, their biology, and how these could be harnessed in the context of anti-tumor therapies.

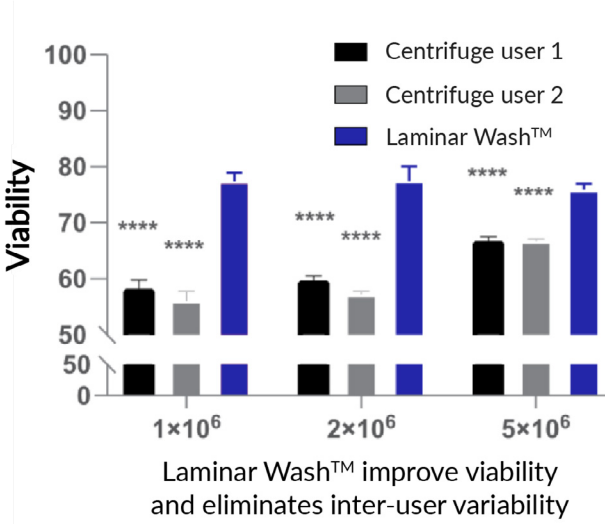
The Laminar Wash™ system answers this call by improving efficiency and accuracy of TIL isolation. A recent study employing the Laminar Wash™ system shows the promise of this technology to address current gaps in TME research. Instead of relying on multiple centrifugation steps that result in cell loss and are labor intensive, Laminar Wash™ employs a gentle, gravity-dependent method that allows for higher cell viability, cell purity, and reproducibility. A recent study employed Laminar Wash™ for isolation of TILs from primary murine tissue and showed that samples prepared with Laminar Wash™ have higher viability



Laminar Wash™ increases the fraction of live and target TILs, revealed by flow cytometry

and cell recovery, with the Laminar Wash™ method improving cell viability of tumor dissociated cells by nearly 20%. This allows researchers to have access to a cell population enriched with TILs for downstream applications. In addition to a more viable pool of cells, the Laminar Wash™ system also improved the resolution of TIL populations, with the gentle washing of this system leading to better defined populations with distinct subgroups of the samples. This is important because targeted therapy relies on accurate prediction of cell subpopulations, making recovery and resolution critical. In addition to the experimental benefits, the Laminar Wash™ approach also has the potential to save time and resources. In the same study, the authors showed that the Laminar Wash™ method is also less time-intensive than centrifuge-based methods.

The results of this study show that there already is a simple and effective answer to the challenges associated with TME research and primary tissue analysis, and this answer begins at the sample preparation level. However, primary tissue analysis in the context of the TME is one of many facets in which Laminar Wash™ has already been tested for accuracy.



Laminar Wash™ improve viability and eliminates inter-user variability

Tumor microenvironment characterization

Co-Cultures as Potency Assays for Potential Tumor Targets

Co-culture systems are commonly used to identify potential therapeutic targets and to study cell-to-cell crosstalk in a controlled cellular environment, and Laminar Wash™ has already empowered researchers to use these systems in the context of tumor biology. The Marion Curtis, PhD, group from the Mayo Clinic recently showed that inhibiting protein phosphatase 4 (PP4), a key regulator of the DNA damage response (DDR), enhanced the anti-tumor functions of immune cells such as natural killer (NK) cells, natural killer T cells, and CD4+ T cells. This study used in vitro

co-culture assays to show that inhibition or deficiency of PP4 results in increased sensitivity of ovarian cancer (OC) cells to carboplatin, a chemotherapeutic. Moreover, the team also showed that inhibition of PP4 increased inflammatory signaling and promoted the anti-tumor properties of NK cells. These co-cultures provided a strong rationale for PP4 as a therapeutic target in OC that was able to be further validated in vivo, with the group showing that knockdown of PP4C in a mouse model of OC resulted in decreased tumor growth and enhanced immune cell infiltration of the tumors in the mice. In other words, the co-culture assays served as a measure of potency that was able to be replicated in an animal model system. This data highlights the significance of both the co-culture system as well as the potential for Laminar Wash™ technology to empower studies like this that identify therapeutic targets through use of primary tumors and mice as well as potency co-culture assays.

Novel Biomarker Identification in Primary Tissue Samples

While Laminar Wash™ technology has been used in studies directly relevant to the TME, it has also been applied in other contexts that show its ability to adapt to new environments and scientific hypotheses, particularly those involving primary tissues. The Rebekah Gundry, PhD, group from the University of Nebraska Medical Center recently published a study that identified a surface protein that is more abundant in healthy heart tissue compared to failing heart tissue. While expression of cell type–specific proteins can serve as valuable biomarkers for disease, not all proteins are displayed on the cell surface, making their detection by flow cytometry or similar methods challenging. Moreover, proper sample preparation and debris removal methods ensure that specific cell populations, particularly rare populations in primary tissues, are accurately quantified and characterized. This is particularly important as surface proteins are expressed and displayed in a context- and cell type–specific manner, meaning that proper cell preparation ensures that a key marker is not "missed" in the experimental process. A solution to this challenge is the development of various protein databases that catalog various proteins in specific cell types. However, relying on these databases alone is not sufficient for discovering novel biomarkers. To overcome this, Gundry's team developed an innovative tool called "CellSurfer" that 1) generates mass spectrometry-based quantification and identification of surface proteins, 2) curates and annotates these data using known databases, and finally 3) prioritizes markers that may be potential biomarkers. This tool's strength lies in its combination of both experimental evidence and computational approaches, while methods solely relying on computational approaches have failed to identify all potential disease markers. One such marker is leucine-rich single-pass membrane protein 2 (LSMEM2)—a protein identified using CellSurfer that previously had conflicting

predictions for its function and structure when using other tools. The team employed the Cell-Surfer workflow to primary cardiomyocytes from failing and non-failing preserved heart tissue and were able to show that the primary cardiomyocytes had LSMEM2 while failing heart tissue did not, identifying a novel biomarker for heart disease. The major strength in this study involves its effective application of multiple methods to ensure accuracy, and the novel biological conclusions in the study are possible because of this approach. A key component of this approach is the increased recovery and resolution of primary tissue possible through Laminar Wash™, showing its ability to evolve and adapt to a variety of research needs.

The path forward in studies of TME is through the proper and accurate implementation of technologies that we already have and that can adapt to an evolving analytics environment. Laminar Wash's™ benefits extend well beyond cell recovery and reducibility, particularly as the field of oncology targets specific cell types. Marion Curtis's group found that inhibition of PP4 increased the anti-tumor properties of NK cells, a timely discovery as the field is deeply interested in dissecting the tumor-killing capacity of NK cells. The ability to characterize tumor-killing immune cells would enable the development of novel therapeutics, and the studies described above show how this data and understanding are in our reach. We simply need to apply the correct technology so we do not miss them.

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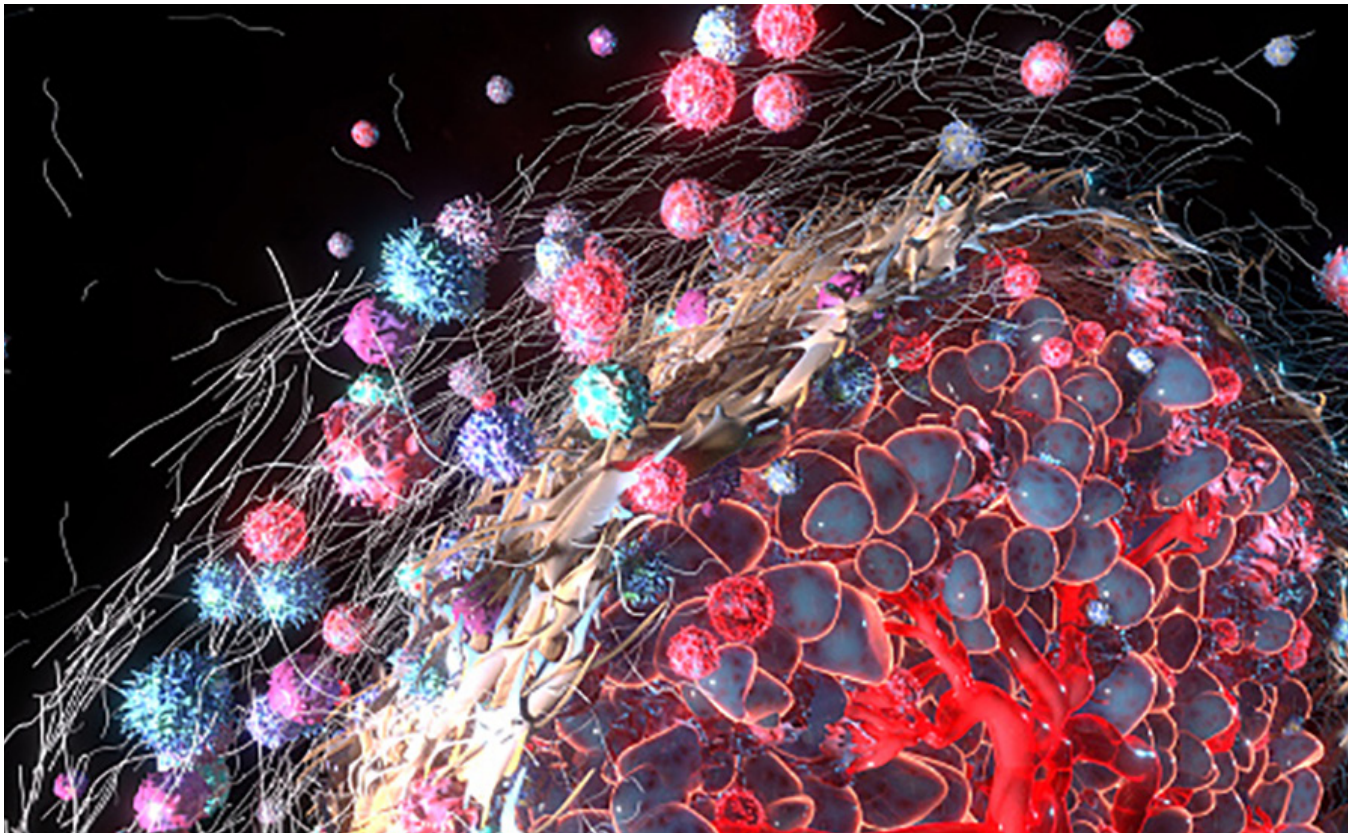
Measuring Cancer Cell Death to Optimize Treatment Selection



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Author: Anjali A. Sarkar, PhD

Treatments for cancer patients generally follow standard clinical protocols that have the highest response rates or survival rates on average, but each patient presents a unique etiology. A new paradigm is emerging that offers oncologists the opportunity to predict how cancers in the body might respond to specific drugs or their combinations before patients receive chemotherapy, opening new avenues for personalized therapy with optimal likelihood of success.

Over the past two decades, Robert Nagourney, MD, a practicing oncologist in Los Angeles, and his team at the Nagourney Cancer Institute have developed a laboratory technique for generating functional profiles that measure how cancer cells respond to a variety of drugs. Nagourney and his team claim this approach is more powerful than the genomic testing offered at most centers.

"At the individual level, responders are 100% responsive and non-responders are 0% responsive. What patients are looking for is to know, to

Robert Nagourney

Robert Nagourney, MD, a practicing oncologist in Los Angeles, and his team at the Nagourney Cancer Institute have developed a functional profiling protocol that measures how cancer cells respond to a variety of drugs before patients are treated with them.

the best of their knowledge, where they fit into the response expectations," says Nagourney.

The technique, called EVA/PCD (Ex Vivo Analysis of Programmed Cell Death) and developed by Nagourney's team, assesses which drugs cause cancer cells to die and is gaining popularity in the treatment of a variety of cancers, particularly advanced-stage pancreatic cancers.

In addition, the team has compiled a large database of over 10,000 human cancer studies that use the EVA/PCD protocol in breast, ovarian, lung, pancreatic, and other types of cancers. Using the database in clinical decision-making, they report a two-fold increase in clinical response and improved one-year survival in over 2,500 published patient outcomes.

The database allows categorizing average patients into those above and below average, with performance characteristics of about 80% sensitivity and 80% specificity. "Using this approach, our objective response rates improve by a factor of 2.04, $p<0.001$, and our one-year survival is higher by 1.44-fold, $p=0.02$," says Nagourney.

Key methods in cancer research

According to Nagourney, phosphoproteomics, metabolomics, and primary cultures of human tumors are the three top methodological advances in cancer research, diagnostics, and therapeutics. Commenting on the importance of focusing on protocols and detailed methodologies, Nagourney says, "We have spent an enormous amount of time on developing resources, diagnostics, and drugs, but I think we have given short shrift to technologies that connect these developments to clinical therapy. Methodology that turns gee-whiz science into practical utility has been lacking."

Genetic abnormalities that lead to cancer result in molecular anomalies that could serve as diagnostic, prognostic, and predictive biomarkers for the disease. Identifying these biomarkers in different tumorigenic pathways makes it possible for clinicians to select the most appropriate therapy for each patient. "When talking of

[method-based] breakthroughs—phosphoproteomics, epigenomics (Peter Jones' or Steven Baylin's work), or other developments critical in advancing [cancer] therapeutics—my bent is to say those technologies that more closely approximate phenotypes are closest to the biological behavior of a cell."

Phosphoproteomics is a promising method that helps identify biomarkers to diagnose disease progression and assess therapeutic efficacy, and new targets that can be drugged to treat cancer. Functional, physical, and chemical interactions among proteins are often orchestrated in time and space through post-translational phosphorylation. Therefore, understanding the interconnected gamut of phosphorylated proteins provides key signatures of cancer that can be used to develop a more granular understanding of specific disease phenotypes and determine optimal and personalized treatment paradigms.

"I am a great believer in metabolomics. Our laboratory includes mass spectrometry capabilities because we believe [the metabolome] is a very good approximation of phenotype," says Nagourney. "Human tumor primary cultures offer the ultimate phenotypic expression in real time."

Penetrating problems

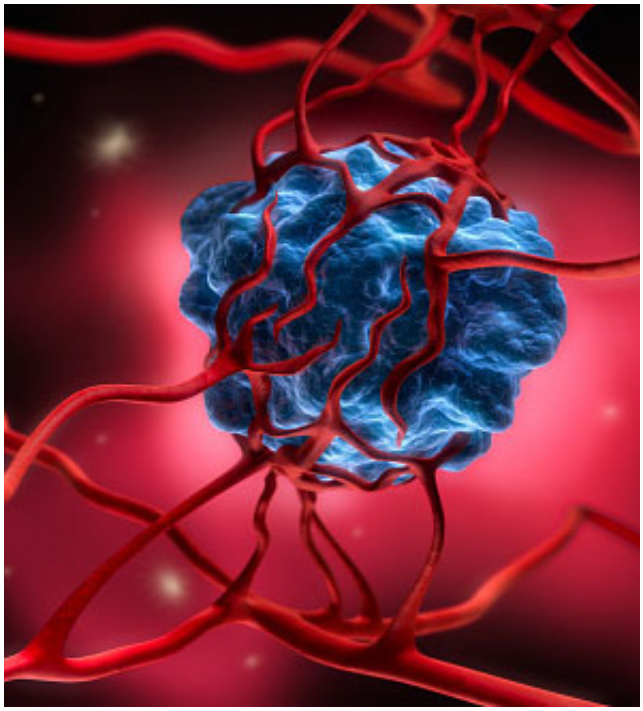
Nagourney began his career studying hematologic malignancies. His early papers focused on the ability to induce programmed cell death in primary suspension cultures and predict the outcomes of childhood leukemia.

"I did the first work on a drug called 2-chlorodeoxyadenosine. We discovered it was curative for hairy-cell leukemia. The beauty of it was that you had a primary culture—a human tissue that gave you an immediate handle on how a cell responds to stress and the appropriate drug or drug-combination inducing stress that led to cell death," says Nagourney.

However, when Nagourney moved from Scripps to UC Irvine and attempted to apply the same method to predicting outcomes in solid tumors, he ran into obstacles.

"When you make the jump from leukemias, which are single-cell suspensions, to solid tumors, all that lovely data is pulled apart. Disaggregated cellular systems were not predictive of solid tumor biology. We had to go back and explore what constituted a good solid tumor model. We changed our disaggregation, enzymatic, and density gradient technologies, and began to turn our pure little cultures into dirty cultures—clusters and aggregates. When we began to study solid tumors in aggregates, the predictive validity began to approach that of leukemia data."

Over the years, Nagourney and his team have moved from studying "clean" single-cell suspensions to messier cultures that closely approximate



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"It would be interesting to examine, for example, how might the proximity to the blood vessels influence drug sensitivity," notes Nagourney.

the native state of cancer tissue and contain all elements of the in vivo tumor microenvironment, including stroma, intercellular messengers, inflammatory cells, vascular elements, and cytokines. "We began to work in micro-aggregates or spheroids. Now we might call them explants."

Heterogenous primary tumor cultures, although more representative of reality, were accompanied by new challenges. "For example, many drugs do not permeate multiple levels of cells. You have to adjust the aggregate size so that you get diffusion throughout the tissue aggregate. If the cell clusters are too large, larger drugs like doxoubicin cannot permeate. There is no vasculature, but you have aggregates that still have their vascular communications intact."

In the absence of blood vessels infiltrating primary tumor explants cultured in vitro, ensuring proper diffusion is essential.

"Once we optimized the right size and dimensions of the tumor [explants] we achieved appropriate permeation of the drugs into the microenvironment and began to get nice dose-response curves. That's the technology we now use," explains Nagourney.

Heterogeneity of cellular architecture in tumors in vivo suggests that the location of specific cancer cells in primary spheroid cultures and, in particular, the degree to which their location ex vivo mimics in vivo geographic orientations, determines how predictive the assay results might be. Nagourney says, "We seek to injure [cancer] cells and induce cell death under conditions that mimic or recreate the in vivo condition. It's not a perfect reproduction. It would be interesting to examine, for example, how might the proximity to the blood vessels influence drug sensitivity. We have not gone down to that level of granularity."

In his ex vivo assays to gauge the response to smaller drugs, Nagourney often finds cell death across the entire spheroidal explant population. Monoclonal antibody studies using spheroids are a different matter, however, since these are

large molecules and do not permeate into the interior of explants.

"We developed a technique that we used to study cetuximab in its original evaluations before it was approved. We were examining cetuximab alone and in combination with small molecule TKIs (tyrosine kinase inhibitors) for EGFR (epidermal growth factor receptor) and made some interesting discoveries based on our onion peeling approach. We could see layers of cells that were exposed to the monoclonals peeling off as they died, leaving a central viable core. That's about as far as we've extended the granularity of assessing cell distributions," says Nagourney.

The platform is developed to test small molecules that permeate into explants. Nagourney's team is currently working to apply the technology to test the efficacy of larger drugs and cell therapies.

"We are getting very close to having a methodology that uses this platform to examine checkpoint inhibitors," says Nagourney. This method assesses cytolytic effects of activated T cells. The challenge in maintaining patient-derived tumor explant cultures in a state as close as possible to their native state is that they cannot be propagated, amplified, or sub-cultured.

"One of my concerns for groups who are doing propagated 3D spheroids is that they will not be as predictive," says Nagourney. "What we have to convince people is that these are primary culture explants in their native state." The team is currently using the approach to study the cytolytic activity of tumor-infiltrating lymphocytes (TILs).

Nagourney is hesitant to use commercially available 3D organoid culture systems to study cancer, although he recognizes the burgeoning interest in using such models in testing drug toxicity. "My problem with tumor organoids that are propagated from cellular materials that then grow into 3D cultures is that they are for the most part monocultures, which are not representative of the actual state of affairs in human tissue. We are concerned that as these technologies grow in the cancer field, they may not be predictive."

Explant studies in tumor biology have gone through a lot of fits and starts since their inception in the 1950s. "It went through a period when people were propagating cells in 2D, doing clonogenic assays and thymidine incorporation. That didn't work and people ran away from the field in the 80s and early 90s."

Two fundamental advances renewed success in the face of prior failures. The first was the recognition of programmed cell death as a therapeutic goal, where cancer cells aren't just prevented from growing and dividing but are induced to die. The second is the development of 3D heterogenous culture systems.

Nagourney's team does not limit its histological and metabolomic analyses to studying apoptotic markers of cell death. "Apoptosis is only one form of cell death—there is necroptosis, ferroptosis, and autophagic changes. These are not captured if you simply look at caspase activation," explains Nagourney. "In hematologic tumors, the caspase 3/7 activity was a good correlate. In solid tumors, it is not."

Nagourney prefers global assessments of cell viability to techniques such as BH3 loading that forces cell death through apoptosis. "A cell dying in culture by apoptosis that in the body would not die by apoptosis will give false results," says Nagourney. His team captures both apoptotic and non-apoptotic cell death by assaying mitochondrial function, ATP content, and histochemical staining to assess morphological attributes.

"Our original work was largely morphologic," notes Nagourney. "The delayed loss of membrane integrity, visible under the microscope with histological stains and counterstains, remains the team's most reliable measure for assaying cell death, particularly in the context of drug development."

In addition, Nagourney's team uses luminometer-based assessments of ATP content and MTT and XTT endpoints to assay mitochondrial succinate dehydrogenase activity as a metabolic measure of cell death.

Orthogonal validation

"We tend to do a number of different measures as we run these dose-response curves so that we get different angles on what is causing cell death. Morphologically you can often distinguish an apoptotic cell, which has characteristic features, from a more autophagic cell and a necroptotic cell."

Scoring how cells appear to be dying from morphologic features gives the team a good handle on how a drug affects a cancer cell and the molecular signaling pathway that induces it to die.

Cancer cells change constantly to metastasize, evade therapy, and adapt to immune pressure. The U.K.'s TRACERx (TRAcking Cancer Evolution through therapy) project, led by Charles Swanton, FRCP, PhD, at the Francis Institute, is tracking how cancer cells vary as they migrate from primary to metastatic lesions. The multi-million-pound initiative aims to improve diagnosis, better tailor therapies, and forestall recurrence. During the early stages of the disease, cancer cells express what Swanton described as "truncal neoantigens" that involve a select set of tumor suppressor genes such as p53 or KRAS.

"Over time, under conditions of stress, tumor cells will develop new tricks that allow these stressed cells to survive new conditions. You might see variations of mutations that change the course of the therapy," says Nagourney. In vitro assays reflect these changes in cancer

progression observed in vivo and are equally predictive in testing metabolic and morphologic markers in primary and metastasized solid tumors.

"Most of our patients come to us at fairly advanced stages. Very often they have liver metastasis or extensive lymphadenopathy. We get a biopsy of that. At this stage, they do become somewhat more resistant. We often feel that since they share the truncal mutation and may have acquired new mutations, if we can find activity in those distant sites, it often characterizes the population as a whole—both primary and metastatic."

Cancer cell death

Nagourney is a great believer in the work of John Reed. "His group suggests cancer is a disease of cell survival. Ultimately cancer is a cell that wants to stay alive," says Nagourney. Although a cancer cell may proliferate before it is targeted by a drug, Nagourney is not interested in proliferation. He says, "The endpoint of cell death is the most important measure in a cancer laboratory because only a living cell can proliferate, and a dead cell cannot kill you."

In a 2018 paper in *Oncotarget* and a 2021 paper in *Gynecological Oncology*, Nagourney's team explored the metabolic features that enable cancer cells to stay alive and outsmart the immune system. "These can often be measured metabolically in the blood or in tissue culture media. The signatures correlate with drug resistance. There is a continuum from the state of a cell gaining a survival advantage, utilizing that survival advantage to remain alive, to ultimately propagating and metastasizing. They use those same survival advantages to resist chemotherapy and other targeted therapies."

These subtle abnormalities in cell biology that ultimately result in cancer are similar to inborn errors of metabolism, believes Nagourney. "Metabolic studies may for the first time give us a handle on the phenotype from a drop of blood. We're extremely excited by the possibility of applying that in the clinical setting."

Database

Nagourney's team has compiled laboratory analyses of over 10,000 patients into a comprehensive proprietary database. The database enables the prediction of whether a patient is likely to respond to a specific treatment with a high degree of accuracy. Through contracts with various nations, the institute offers the database as a service to patients around the world.

"For almost every type of cancer, if you ask me, is this degree of, say, taxane or platinum likely to confer response? Based on the distributional analyses of the continuum of sensitivity and resistance, we can place every patient against their cohort," points out Nagourney.

The database also enables in vitro Phase II trials. "It's an excellent way for the pharmaceutical industry to have a bridge between, as I mentioned previously, gee-whiz science and practical utility," he says.

Nagourney's team has used the database to test the efficacy of a drug called gemcitabine for Eli Lily. Gemcitabine, a difluorodeoxycytidine originally developed as an antiviral, was repurposed for the treatment of sarcoma, ovarian, lung, lymphoma, and bladder cancers. "The laboratory results predicted virtually all of the ultimate FDA approvals."

Toward precision

Despite their robust predictive advantage, Nagourney laments that these technologies have been underestimated in clinical practice and decried in editorials.

"They continue to suggest these technologies need to prove they save lives. I have never seen a clinical trial where a genomic platform has been forced to show it saves lives," he says. "What we prove is the performance characteristics of the test within the standards."

Nagourney believes initial errors of reasoning that led to failed strategies of blocking cell proliferation, and disaggregating tumors into single cells instead of focusing on cell death, has created a bias against the technology. "Once you use this technology, it's very hard to go back to clinical oncology as it is practiced."

Nagourney believes guideline-driven medicine is increasingly on a collision course with precision medicine. "We better get on the right side of that," says Nagourney, "because what we're seeing is increasing standardization of therapies while patients are clamoring for individualized care."

Nagourney celebrates the recent advances in genomics, transcriptomics, and gene editing using CRISPR, epigenomics, and proteomics.

"But the ultimate measure of the human phenotype is cell biology studies," he explains. "That's going to be so important to grasp the complexity of cell behavior. We need to be humble in our scientific pursuits so that we allow this extraordinary biological complexity, redundancy, and promiscuity of events to teach us. In my work, I get the answer—other people have to provide the question."

Nagourney hopes the in vitro tumor explant testing platform in combination with the database could be the key to achieving personalized care so that cancer patients are treated based on the latest research optimized for their specific condition and not just what has been approved for a narrow, generalized indication.

Using Flow Cytometry to Identify Metrics That Are Biologically Meaningful and Sensitive to Variations in a Cell Therapy Manufacturing Process

Authors: Melvin Lye, MBA, BCMAS, Christoph Eberle, PhD, MICR, FRSPH, Mahwish Natalia, PhD, and Virginia Litwin, PhD

Introduction

Cell therapies are proving to be highly effective therapeutic modalities for cancers and other indications such as diabetes, autoimmune diseases, spinal cord injuries, and neurological disorders. The types of cells being used as the basis of cellular therapies include stem cells (hematopoietic, skeletal muscle, mesenchymal stem) pancreatic islet cells, and leukocytes (lymphocytes, dendritic cells). Owing to the success in the treatment of blood cancers, cellular immunotherapies (tumor-infiltrating lymphocyte (TIL) therapy, engineered T cell receptor (TCR) therapy, chimeric antigen receptor (CAR) T cell therapy, natural killer (NK) cell therapy) have garnered broad attention.

Successful commercialization of any novel therapy is a long, complex, costly, and arduous process, which is fully dependent upon generating robust data suitable for decision making at every stage of the drug development pathway from pre-clinical evaluation to manufacturing. When the therapy is actually a living cell, each and every process, including manufacturing, regulatory, distribution, testing, and delivery, is considerably more demanding compared to small molecule drugs or even protein-based therapies. Reproducible manufacturing processes are necessary but not sufficient to achieve consistent product from dose to dose. Robust, well-characterized methods that can sufficiently monitor the manufacturing and in-process evaluation of cellular therapies are also required. Several assays, each measuring unique physical and biological characteristics of the cell product, need to be developed for each stage of the manufacturing process. The goal of such assays is to identify metrics that are both biologically meaningful and sensitive to variations in the manufacturing process.

Flow cytometry, a highly specific and sensitive platform for single-cell analysis, is ideally suited to provide in-depth analysis throughout the life cycle of cell therapy products. The data can be used to assess the impact of changes in the manufacturing process. Assays for cell product identification can be used to evaluate efficacy of the cell isolation procedure and, when developed

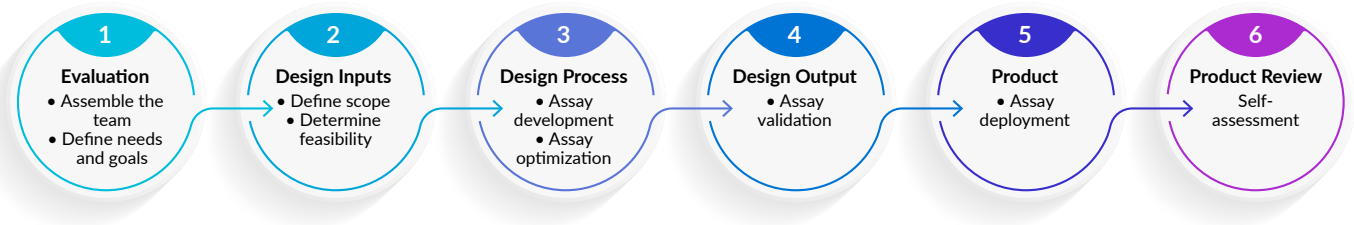

Fig 1. Critical Stages in the Design Control Process

to be high-sensitivity assays, can be used to monitor even low levels of cellular impurities. Other flow cytometric readouts can be used to support optimization of cell culture media and scale-up. Extensive multiparametric immunophenotyping methods can be used to characterize the cell products and monitor phenotypic drift along the pathway from manufacturing to delivery and post-infusion. To be able to monitor how the phenotypic profile changes over time, and the impact those changes have on the infused cell product, assays must be sensitive, specific, and highly reproducible.

The quality attributes (CQA) for most cell therapies remain poorly defined and will be different for each type of cellular product and each manufacturing process, and standardized measurements of CQA have yet to be established. In this regard, additional challenges arise from the lack of reference materials for monitoring the analytical measurement and the absence of regulatory guidance for the validation of such processes. The general consensus in the field is that standardized measurements would accelerate cell therapy development and regulatory approval of these novel modalities. Unfortunately, standardized measurement processes are likely to be several years in the making, while the manufacturing and clinical development activities are occurring today. Fortunately, there are existing, generic strategies and tools such as Design Control[1] and Measurement Assurance[2] that can be applied to a wide variety of complex processes, including the production and in-process evaluation of living therapies, to ensure better outcomes. Additionally, the consensus validation strategies for single-cell measurement technologies, such as flow cytometry, can also be applied.

Strategies

Design Control

The Design Control management practices can serve as a valuable tool when creating and mapping new processes and conducting risk assessments.[1] In the area of cell characterization, Design Control provides a framework for the team members to assemble and discuss, at a high level, the requirements for the product testing process (Figure 1). The first step of this approach is the Evaluation, where the team is assembled to discuss and define the goals and needs. For cell manufacturing,

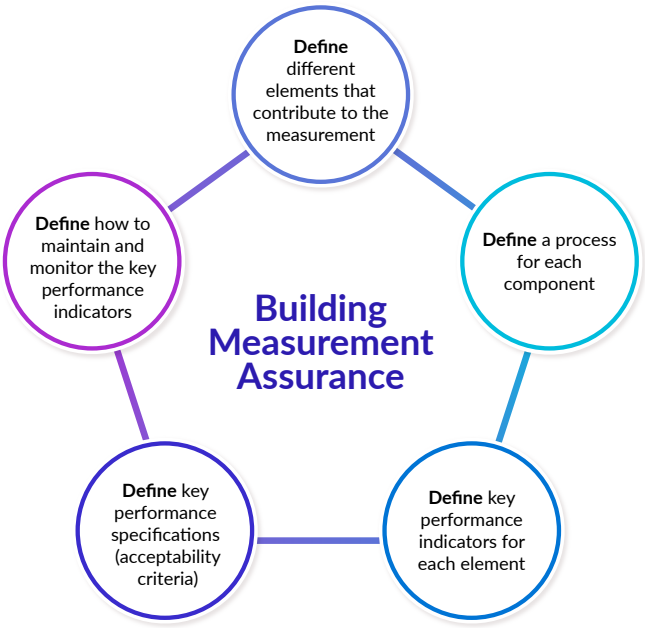

Fig 2. Building Measurement Assurance

this team would likely include assay-analytics-focus representatives from Quality Assurance, Quality Control, and Analytical Lead (mainly, the Subject Matter Expert who is developing the assay and defining in-process questions for different stakeholders). Often, this group includes a Regulatory Team representative. The process often begins by identifying the metrics of identity, phenotype, impurity, potency, and viability that will sufficiently characterize the cellular therapy, ushering the definition of properties that confirm acceptable production or identify variations in the manufacturing process. This is accompanied by identifying analytical methods for the evaluation of each metric. With the priority of site-readiness, this team summarizes the ease of use, assay throughput, hands-on time, feasibility, and cost of the assays. In essence, a successful Control Strategy needs to be married to assay transfer logistics.

Measurement Assurance

Applying the concept of Measurement Assurance can help the Design Control Team define and evaluate the processes in a more granular manner. Measurement assurance is a systematic approach that informs on the comparability and confidences in the result to enable data-driven decision making. For each assay used to monitor cell therapy production, the Design Control Team would first define the different elements that contribute to the measurement and then define a process for each element. Next, the key performance indicators and acceptability criteria for each element would be defined, and finally a process would be identified for maintaining and monitoring the key performance indicators.

Flow Cytometry

The elements of a measurement made by a flow cytometer include the instrument; the method itself, including the assay development and the validation; the data reporting processes; the assay and instrument monitoring; and the documentation (Figure 2). These elements are well described in the international guidance document addressing the validation of methods performed by flow cytometry, published by the Clinical and Laboratory Standards Institute (CLSI) in 2021.[6]

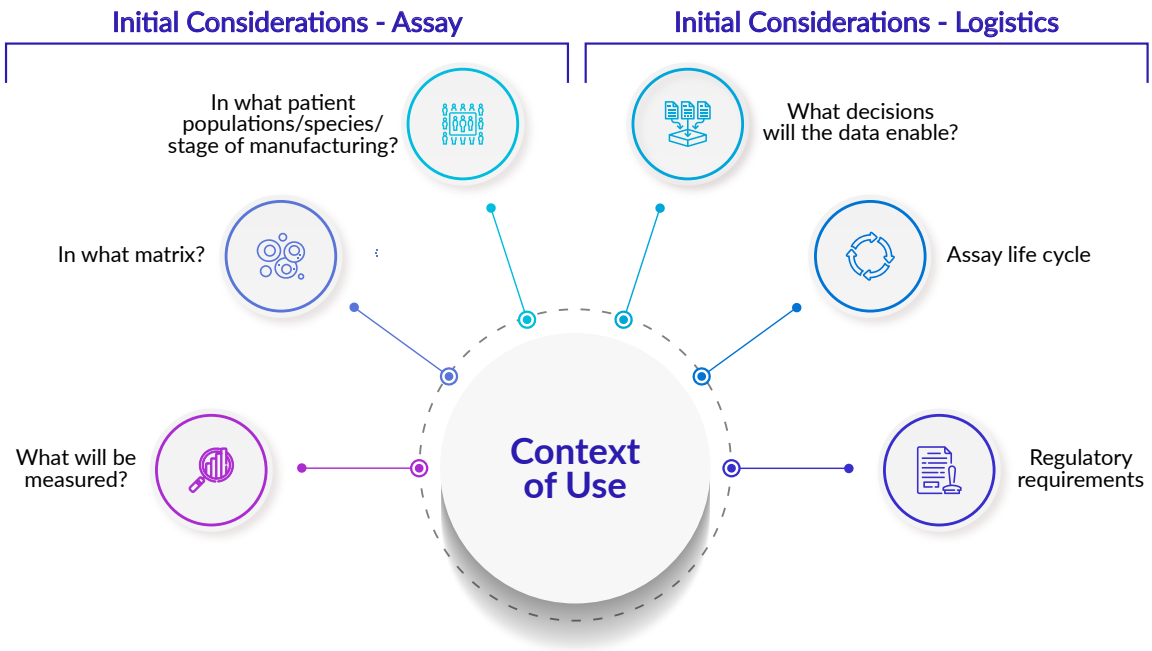

Fig 3. Assay Context of Use. Considerations when developing a new assay and determining assay performance criteria for a given context-of-use.

Assay Development

When designing a new flow cytometric method, the first step is to establish the Context-of-Use (COU) of the assay by determining what results the assay will report, what decisions the data will enable, and the regulatory requirements (Figure 3).[6] In addition, the overall life cycle of the assay should be considered. For example, the product characterization and release assays will likely be transferred from the discovery or manufacturing groups to the quality assurance (QA) laboratories. The most efficient practice that will lead to the best continuity of the data is to ensure that all the testing labs use the same instruments, methods, and QC samples (ref assay transfer paper). When this is not feasible, robust assay transfer validation, including sample exchange, should be conducted following the approach described by Cabanski et al.[3]

After establishing the COU, the next steps in designing the panel are to select the antibodies to be used for both positive and negative cell subset selection, screen monoclonal antibody (mAb) clones, and select the optimal fluorophore for each mAb as described in depth in CLIS H62[6]. The appropriate viability and/or apoptosis assessment based on the panel design should then be selected. Finally, the gating strategy and templates would be designed.

Measurement Assurance Assay Optimization – Pre-analytical Variables

After the panel has been designed, pre-analytical variables should be optimized. Considerations

during the optimization phase include evaluating the number of cells to acquire, which will impact the final sensitivity of the method, as described in depth in Sommer et al.[4] In addition, the washing, lysis, and fixation steps should be evaluated. The goal is to select processes that will not damage the cells, result in a loss of representative populations in the test samples, or introduce debris.

Sample centrifugation is an integral part of the cell isolation and staining procedures. Centripetal force and the centrifugation times must be optimized so that impurities are removed, recovery is maximized, and cell damage is minimized. Unfortunately, even after extensive optimization, centrifugation can result in cell damage and/or incomplete debris removal. More unfortunate still, there is a risk that even in a highly optimized flow cytometric method, damaged cells and debris may be mistaken for actual cellular subsets. Newer technologies such as the Laminar Wash™ System from Curiox Biosystems can replace centrifugation and mitigate these risks. In a side-by-side comparison of the Laminar Wash™ System to traditional centrifugation, the viability of tumor infiltrating lymphocytes isolated from solid tumors in a murine model increased by about 10%, but the recovery increased by nearly two-fold (Figure 4).

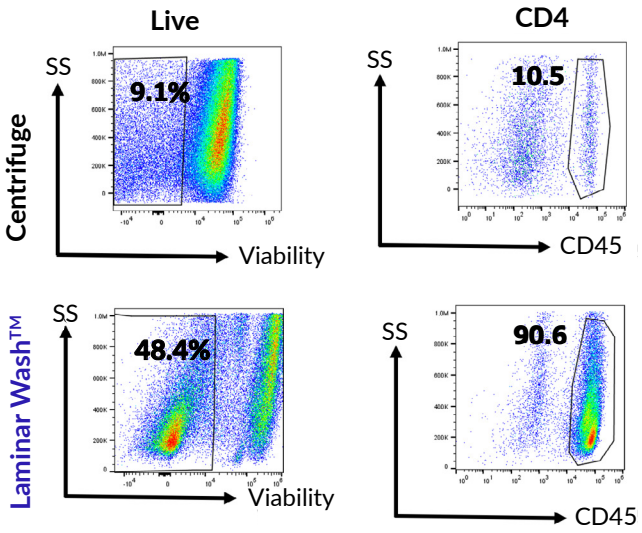

Fig 4. Laminar Wash™ compared to centrifugation. Solid mouse tumors (B16-F10) from untreated animals were processed into single cell suspensions. For immunostaining tumor infiltrating lymphocytes (TILs) were washed simultaneously by traditional centrifugation and Laminar Wash™ technology. Viability and CD45+ frequencies with representative dot plots were compared in technical triplicates.

Measurement Assurance Validation

After an analytical method has been developed and optimized it should be validated, no matter the regulatory requirements or regulatory setting. The CLSI document H62 embraces the fit-for-purpose validation approach, where each method would be validated based on the context-of-use.[6] For example, the stability of a cellular product is measured through cellular viability and functionality assays. In contrast to the viability measurements, which are rapid and precise, the functional assays are lengthy, difficult to control, and complex. Thus, validation strategies for these two assay types will be quite different.

The challenge when validating methods supporting cell therapies is not how to validate each parameter, but what the regulatory requirements are in each setting where the assay is used.

Additional challenges are encountered when transferring an assay from one laboratory to another, particularly when the regulatory requirements are not the same in each laboratory.[4]

Summary

Cellular therapies have already had an incredible impact for patients, but their full potential has likely not yet been realized, and many more patients' lives will be impacted by these therapies. As the compounds make their arduous journey from the manufacturing phase into the clinic, the potential life-saving benefit to a patient cannot be forgotten or underestimated. The success of this transition is dependent on the delivery of reliable data. Multiple assays should be performed that fully characterize the cell in identity, composition, and potency. The same data will not only be used for release testing and a potency assay: they are also important for identifying critical process parameters (CPPs) and the critical quality attributes (CQAs) for the cell therapy product developers and their partners. For the developer, this requires succinct steps between Analytical Development and Process Development teams in developing assays that measure the mechanism(s) of action, but also utilizing first-in-class processes and GMP-supported analytical tools, which in this case would be developing flow cytometry workflows with the COU in-mind.

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With Cell Therapy Products, Multiple Safety Issues Call for Multiple Solutions

Safety challenges posed by cell therapy products include maintenance of sterility, genetic characterization, and the minimization of unintended effects

Artisan Bio provides genome engineering tools to facilitate the development and commercialization of cell and gene therapies. These tools include molecular assays to detect and assess the consequences of off-target cuts, potentially adverse on-target cuts, and chromosomal translocations. Data packages are available that can support build specifications, and qualified assays are supplied that can facilitate late-stage development.

Author: Kathy Liszewski

Cell therapies promise to revolutionize the treatment of devastating diseases ranging from cancer to neurodegeneration. Therapeutically engineered cells can be either autologous (originating from the patient) or allogeneic (originating from donors). Although the cell therapy market is still in its infancy, it is, in the view of many forecasters, poised for exponential growth. For example, Precedence Research anticipates that the cell therapy market will

grow from its current $12.59 billion to more than $60.67 billion by 2030.

Before cell therapies reach their "prime time," many challenges must be overcome, paramount of which is appropriate safety testing. However, because cell therapies are complex and varied, safety testing methods, and the risk assessments they entail, may be complex and varied as well.

The safety testing of cell therapy products was discussed by several presenters at the Process Development for Cell Therapies Summit, an event that was held October 2022. Several of these presenters have contributed their thoughts to this article. If there is a consensus, it is this: Cell therapy, as a new field, needs novel testing techniques and personnel with the appropriate expertise.

More specific challenges include the development of compendial and noncompendial approaches to assess sterility and purity. Solutions include focusing on process design and control measures early in development. For example, staging closed cellular processes (and avoiding open steps) helps ensure sterility.

Because cell types may differ, companies may need to customize testing. For example, there are fully differentiated cells that have been altered via genome editing, and there are induced pluripotent stem cells (iPSCs) that have been altered via genome editing. (There are also iPSCs that have been generated without the use of genetic material.) To determine how cells of different types should be tested, investigators rely heavily on genetic characterization through karyotyping and plasmid loss.

Jenessa Smith, PhD, Arsenal Biosciences

Safety testing of CRISPR-based platforms must address risks such as potential off-target cuts to the genome and consequent modifications. Additionally, cryopreservation protocols must be developed that are both scalable and comparable. Finally, the terminal processing step of any process is critical for maintaining sterility and preparing samples for batch release.

Multiple safety approaches

There is no "one size fits all" when it comes to ensuring the safety of cell therapy products. Instead, a variety of safety challenges may be

encountered. "Challenges faced include having the technical expertise to run the many molecular, cell, and other tests needed to fully characterize and understand the product," says Jenessa Smith, PhD, director of process development at Arsenal Biosciences. "We are lucky to have talented scientists and leaders that can execute and define the safety of our CITE process, as an example, in a deep way with several orthogonal assays that demonstrate no significant off-target editing."

CITE is Arsenal's "CRISPR-based Integration of Transgenes by Electroporation" process. It is designed to enable precise and homogenous manufacturing and to offer greater operating leverage than viral alternatives.

Additional challenges include ensuring sterility and removal of residuals. "We face these challenges by processing all steps possible in a closed manner, moving to a fully closed system as a key goal for our work," Smith details. "Closing the process by avoiding open steps that use a biosafety cabinet is important so that sterility is maintained with the highest level of control. We also use reagents thoughtfully to mitigate the presence of any adventitious agents and choose reagents with assays available to easily test residuals."

Smith says that it's important to understand the various aspects of each product and have tests that accurately assess the functions. "We developed a matrix of potency and safety assays to understand the various functions [we can build into] our product," she explains. "[Our] novel integrated circuit T cells (ICT cells) incorporate a synthetic circuit that enhances potency, expansion, and tumor microenvironment penetration."

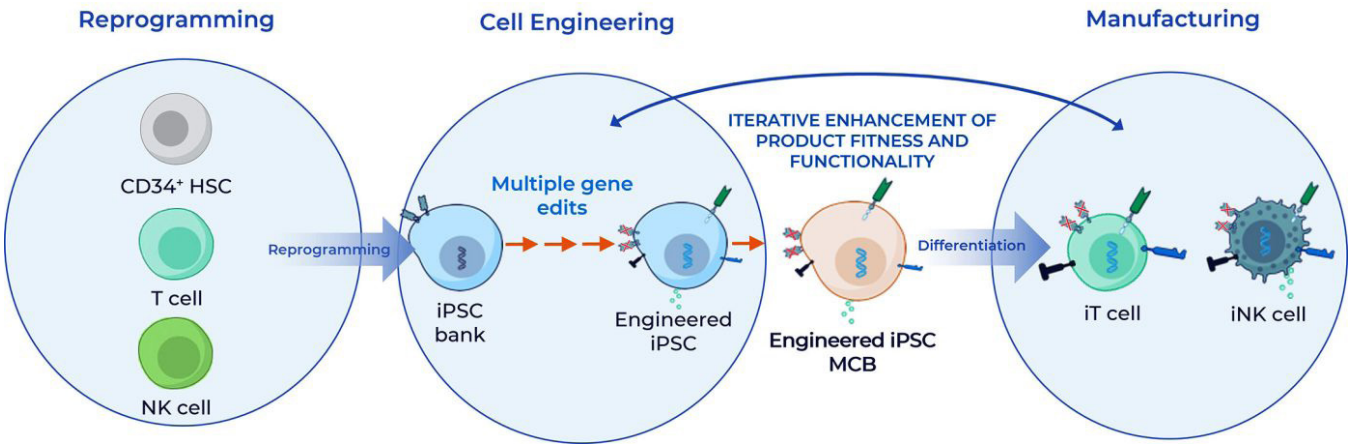
For example, Arsenal has developed an ICT cell that is capable of conditional chimeric antigen receptor (CAR) activity and other functions. This ICT cell, which is called AB-X, includes a transgene cassette with two functional modules. One is an AND logic gate designed to limit off-tumor toxicity through dual tumor antigen recognition. The other is a dual shRNA-miR to resist tumor microenvironment suppression and improve ICT cell function. (So-called AND biological gates are functional only when one input AND another are present.)

Arsenal plans to evaluate AB-X in clinical trials for treatment of platinum resistant/refractory ovarian cancer. The company also intends to test its novel ICT cells against several other solid tumor indications.

Next-generation iPSCs

Because iPSCs may differentiate into a variety of cell types, they hold many promising therapeutic applications. "Originating from a single donor, iPSC-derived therapies provide the potential for unlimited multiplex or sequential genetic engineering steps," notes Chris deBorde, PhD,

CENTURY'S END-TO-END PLATFORM HAS THE KEY COMPONENTS TO REALIZE POTENTIAL OF IPSC



Century Therapeutics integrates gene editing, protein engineering, technical development, and manufacturing capabilities to generate allogeneic, iPSC-derived NK- and T-cell therapies. The company says that by exploiting a gene editing technology called homology directed repair, and by constructing master cell banks from selected cells, it can reduce random integration events and copy number variations, achieving more predictable and consistent transgene expression.

process development engineer, Century Therapeutics. "[These steps may lead to] a defined single-cell-derived product amenable to multiple indications and improved efficacy."

Although some aspects of safety testing are similar to those for other cell-based products, there also are important differences. "Strategies for adventitious agent testing, which is heavily dependent on process raw materials and residuals, may be unique to iPSC therapies given the nascency of the field, distinct manufacturing processes, and particular raw material needs and vendors," deBorde explains. "Additionally, iPSC and other genome-edited cell therapies rely heavily on genetic characterization through karyotyping and plasmid loss. When any number of edits are made to the genome of a cell, it is critical to ensure that these edits do not cause unwanted mutations or off-target effects, particularly as iPSCs are further differentiated into immune effector cells."

According to deBorde, Century Therapeutics has developed quality control strategies for its iPSC-derived therapies that employ well-characterized compendial and noncompendial procedures, including sterility, endotoxin, and mycoplasma tests as well as a risk-based approach to adventitious testing at different points in the manufacturing process. The company also employs genetic characterization with PCR, flow cytometry, karyotyping,

Mark Lalli, PhD, Be Biopharma

and genomic sequencing to ensure a highly pure and genetically stable cell therapy product.

However, iPSC therapies offer several safety advantages. "In contrast to other allogeneic cell therapies that use multiple healthy donors for their cell source, iPSC-derived therapies can utilize a single master cell bank," deBorde notes. "This eliminates heterogeneity from the starting material and allows for a deeply characterized master cell bank to feed an entire product for its lifetime. Also, because iPSCs can have their genome edited sequentially, a single clone can be selected and then expanded that incorporates all of the specific edits targeted for that product. As a self-renewable resource, iPSCs will provide an unlimited supply of material for future products."

The company is developing genetically engineered iPSC-derived natural killer and T-cell product candidates to specifically target hematologic and solid tumor cancers.

Engineered B-cell medicines

"B cells represent a game changer for medicine," states Mark Lalli, PhD, associate director of process development at Be Biopharma. "[They promise to have a] profound patient impact across therapeutic areas." The company is creating B cells that can produce specific therapeutic proteins for disease treatment. Lalli elaborates, "Be Bio's platform is made possible through precise engineering and a convergence of gene editing, B-cell biology, and cell therapy manufacturing and development."

Safety considerations are similar to those for other cell-based therapies. Lalli explains, "With respect to safety from a manufacturing perspective, it is important to focus on process design and control to ensure sterility and identity of the final product." He also emphasizes that the final processing step of any process is critical for maintaining sterility and preparing samples for batch release. For example, cryopreservation protocols must be developed that are both scalable and comparable to ensure sterility and quality. Additionally, automating fill and finish technologies can reduce contamination as well as time and cost.

Lalli adds, "Both off-the-shelf and autologous cell therapies require standard safety tests, such

as sterility, mycoplasma, and endotoxin tests, whereas graft-versus-host disease considerations generally apply only to off-the-shelf therapies."

The company is advancing both autologous and allogeneic B-cell medicine platforms across multiple therapeutic areas with an initial focus on rare disease and oncology. The potential resulting B-cell products would be single-dose biologic protein medicines that produce an endogenous and continuous therapeutic infusion at constant levels.

Making the cut safely

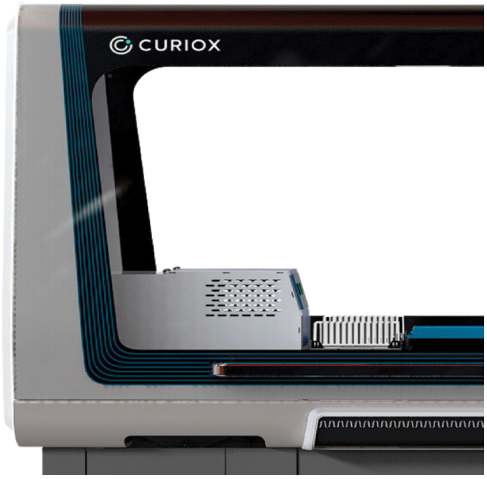
Artisan Bio is employing nonviral engineering to design scalable, cost-effective, and reliable processes for cell production, according to Nick Timmins, PhD, the company's chief development officer. He explains, "Our STAR-CRISPR platform had been developed from the outset to employ ribonucleoproteins. In simple terms: the nuclease, the gRNAs, and the DNA repair template (for knock-in) are combined in solution, and then various transfection methods can be used to deliver these components to the nucleus of cells. Our proprietary nucleases are engineered to enhance the efficiency with which ribonucleoprotein complexes transit across the nuclear membrane."

Safety concerns include potential off-target cuts to the genome and consequent modifications. Similarly, on-target cutting could potentially result in other unwanted outcomes (for example, template copy errors or structural changes). "It is important to note that these are outcomes that might occur," Timmins advises. "Careful target choice and system design can be used to minimize the probability of occurrence, and appropriate analytics can be deployed to detect any such outcomes should they occur."

The company is deploying a suite of molecular assays early in development to detect not only off-target edits, but also unintended outcomes of both off- and on-target edits. These assays are coupled to data-driven approaches for assessing the hazard potential of any such outcomes.

"We leverage the massive quantities of data obtained in databases such as COSMIC and ClinVar/GenVar to evaluate what the functional and/or clinical consequences of a potential unwanted outcome might be," Timmins elaborates. "All of this occurs very early in development during selection of optimal gRNAs before substantial costs accrue and at a time when changes are more easily made. Functional cell-based assays provide an additional opportunity to identify unintended outcomes through altered or aberrant behavior, prior to testing preclinical animal models and staging appropriately designed clinical trials."

The only time you should



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