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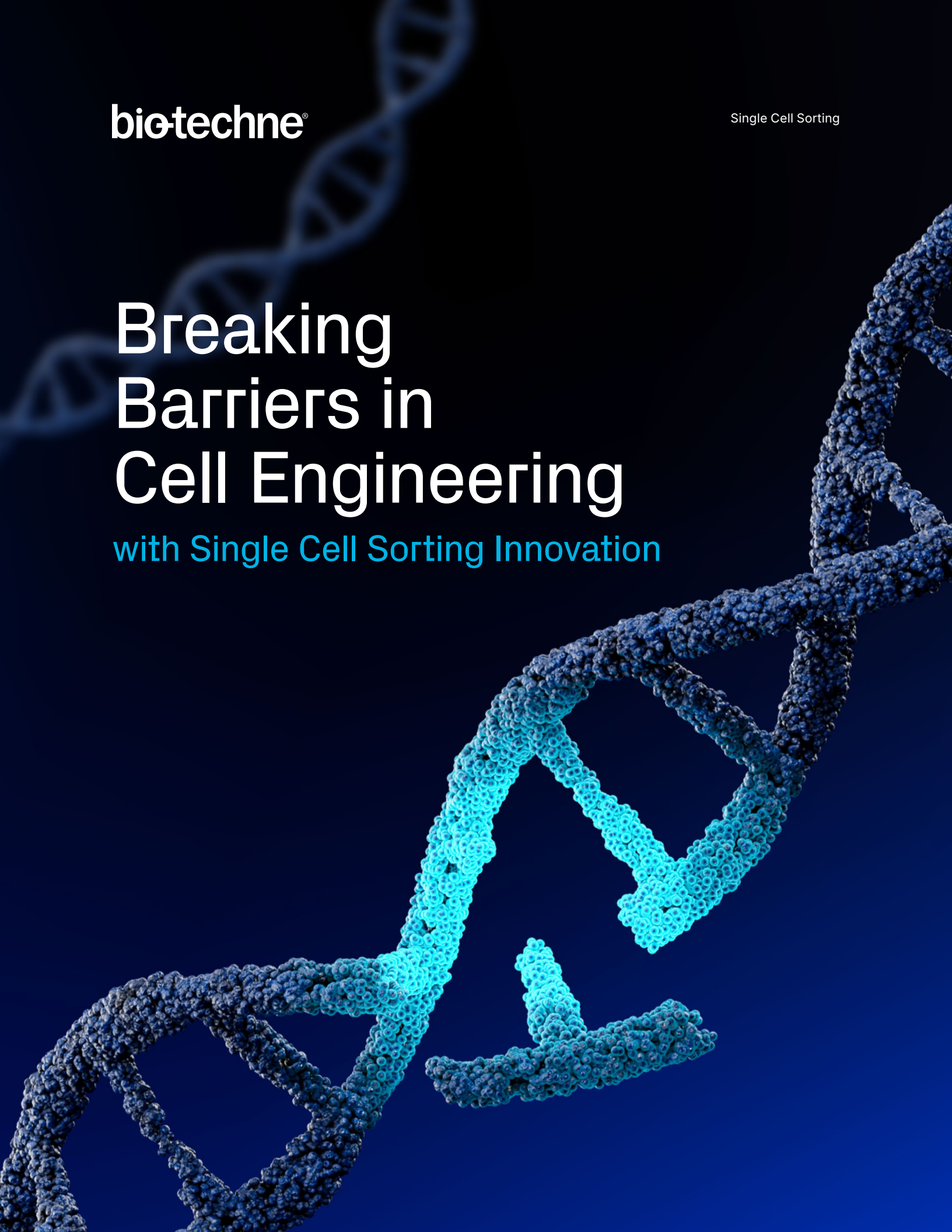
Single Cell Sorting

Breaking Barriers in Cell Engineering

with Single Cell Sorting Innovation



Contents

Introduction

Unlock the secrets hidden within cells and embark on a journey to revolutionize your understanding of cellular behavior. Welcome to the world of single-cell analysis a groundbreaking tool that is reshaping the landscape of cell engineering. In this captivating ebook, presented in collaboration with Bio-Techne, we invite you to discover the boundless potential of single-cell analysis.

The intricate variations among individual cells can hold the key to unlocking unprecedented insights into therapeutic efficacy and safety. Single-cell analysis offers a transformative solution, allowing researchers to unravel the mysteries of each cell in isolation, resulting in a profound and accurate comprehension of cellular behavior.

Within the pages of this resource, we focus on the remarkable benefits of single cell sorting technology in the realm of cell engineering workflows. Its impact spans far and wide, transcending boundaries in biotechnology, pharmaceuticals, and regenerative medicine. Prepare to witness a paradigm shift as we illuminate the path toward a new era in the development and production of cellular therapies.

And that is not all. This ebook is a treasure trove of knowledge, featuring exclusive content that will captivate your scientific curiosity. Turn to page 6 and discover an insightful article on cell line development for therapeutic proteins – a must-read for those seeking to master this vital aspect of cellular therapies. On page 20, immerse yourself in a captivating interview with the esteemed Dr. Oscar Parez-Leal, as he delves into the world of CRISPR and high-content imaging – a conversation that will leave you inspired.

From unraveling the complexities of cellular heterogeneity to harnessing the immense power of single cell sorting technology, this ebook is the ultimate guide for researchers and professionals seeking to elevate their understanding of cellular behavior and revolutionize their cellular therapy workflows. Embrace the future of cell engineering and embark on a transformative journey – one cell at a time.

Join us in the realm of single-cell analysis and discover the immense power it holds in unlocking the secrets within each individual cell.

We hope you enjoy!

Taylor Mixides
Deputy Editor, *Drug Target Review*

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Single-Cell Analysis

Transforming cell biology and therapy development

Single-cell analysis is a rapidly growing field that is transforming the way we study cells and their functions. It Involves the isolation, characterization and manipulation of individual cells to gain insight into their properties and behaviors. Although to date it has made its greatest impact in the fields of neuroscience, immunology and oncology, it now promises to enhance our understanding of individual cells in numerous other contexts.[1]

With a wide range of therapy areas, as well as enhancing basic research, the development of single-cell technologies has enabled systematic investigation of cellular heterogeneity in numerous tissues and cell populations, yielding fresh insights into the composition, dynamics and regulatory mechanisms of cell states in drug development and disease.[2]

The characterization of individual cells produces a vast amount of data that can be difficult to interpret and analyze. However, new computational tools and algorithms are being developed to help researchers make sense of this data and identify meaningful insights. However, there are several challenges that researchers face when analyzing single-cell data. One of the biggest challenges is the high level of technical noise and biological variation inherent in single-cell measurements, which can make it difficult to distinguish true signals from noise. Another issue is the sheer volume of data generated by single-cell experiments, making efforts to identify meaningful patterns and relationships among different cells problematic. There is also a lack of standardized methods for analyzing single-cell data, which can lead to variability in results and hinder the reproducibility of findings.

Addressing these challenges will require continued development of computational methods and tools, as well as increased collaboration and standardization within the scientific community.

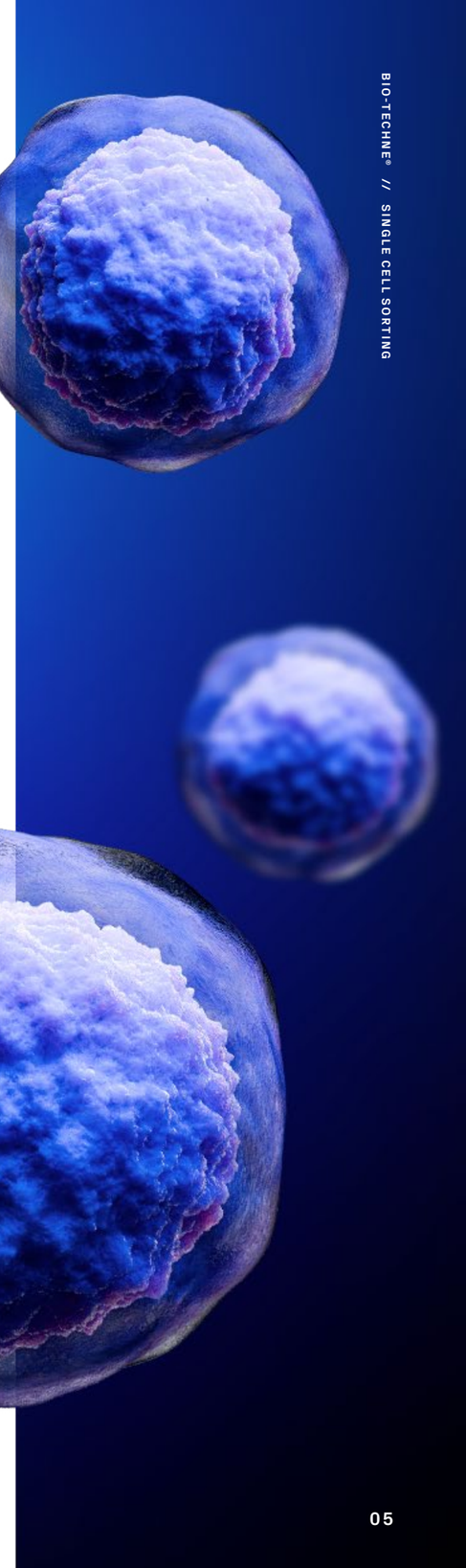
Many methods have been successfully used for the analysis of genomic data from bulk samples. However, the relatively small number of sequencing reads, sparsity of data, and cell population heterogeneity present significant analytical challenges in effective data analysis.[2]

Single-cell analysis is a critical tool in cell engineering workflows, allowing researchers to isolate and characterize individual cells. By identifying rare cell types, researchers can develop more targeted and effective therapies. Single-cell isolation methods, such as microfluidics and fluorescence-activated cell sorting (FACS), have their own advantages and limitations. FACS is commonly used in immunology and oncology research, while microfluidics is particularly useful for studying rare cell populations.

Single-cell sorting technology has been used in cell engineering workflows to improve outcomes or accelerate progress. For example, in CAR T-cell therapy for cancer, single-cell sorting technology has been used to identify T cells that are most effective at killing cancer cells and isolate them for use in therapy. However, there are common challenges or limitations that scientists face when performing single-cell sorting, such as cell damage or loss during the sorting process.

When choosing a single-cell sorting technology, scientists should consider factors such as cell type, sample size and downstream analysis. Looking to the future, advancements in single-cell sorting technology are expected to have a major impact on the development of new treatments for a wide range of diseases. These advancements may include the development of new sorting techniques or the integration of single-cell analysis with other omics technologies.

Overall, single-cell analysis and sorting technology are rapidly evolving fields that are critical in advancing cell engineering workflows and developing more targeted and effective therapies.



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Cell Line Development

Cell line development for therapeutic proteins – current perspectives and future opportunities

Development of cell line expression systems to produce biologic medicines is complex, multi-stage and time consuming, requiring specific expertise and access to suitable technologies. This can limit the development of novel medicines to existing users and may restrict companies developing new medicines. In this article, major methods and technologies used in cell line development (CLD) are reviewed, key limitations identified and solutions assessed. A suggested roadmap for the development and optimisation of an expression platform is ultimately presented.

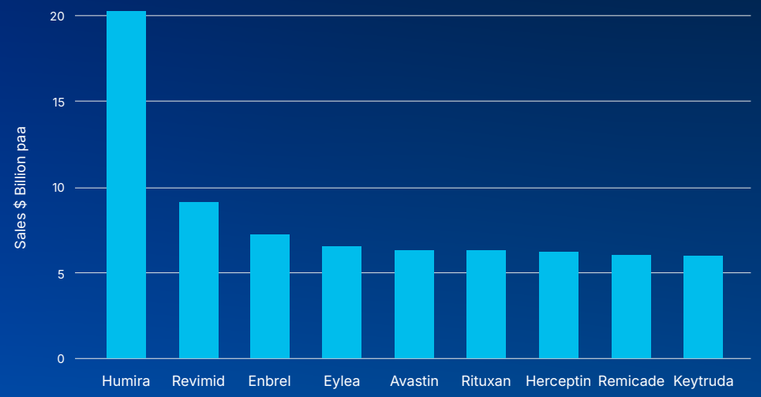

Figure 1: Graph showing that in 2018, most of the world's best selling medicines were biologics.

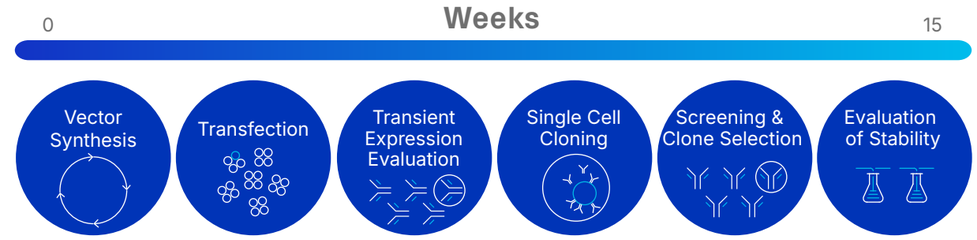

Figure 2: A flow chart showing the traditional cell line development workflow.

Biologics are one of the most successful classes of medicines today.[1] In 2018, most of the world's best-selling medicines were biologics (Figure 1). Most biopharmaceuticals are manufactured using genetically modified cell lines. Cells are constructed from immortal host cells transfected with a genetic construct or vector. Recombinant cells are then grown in large industrial bioreactors and proteins purified from the cell culture medium. Creation of productive cell lines relies on three pillars: the cell line, the vector and the screening process. Careful development and optimisation of each pillar is required to develop high-producing, commercial processes. A typical CLD workflow is shown in Figure 2.

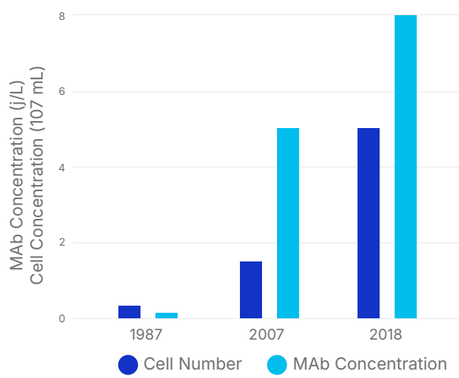

Figure 3: A bar chart showing that the concentration of protein produced has risen more than 30-fold over a 30-year period due to iterative optimization of each pillar of the cell line development process.

Over a 30-year period the concentration of protein produced has risen more than 30-fold (Figure 3). Improvements in titre have been achieved by iterative optimisation of each pillar of the CLD process, as well as improvements in cell culture medium, feeds and the manufacturing process used. Despite these successes, the process used to produce cell lines remains poorly understood, difficult to access commercially and over reliant on extensive screening to isolate highly productive clones.

Historical perspective – host cells used for protein production

CLD requires a suitable host line, which is genetically modified by transfection with a genetic vector. Most biotherapeutic proteins are produced using Chinese hamster ovary cells (CHOs).[2] CHOs are an ideal host for recombinant protein production as they generate proteins that are biocompatible with humans, are amenable to serum/suspension adaptation and can be used with several selection systems. CHOs were first isolated in 1958[3] with the subclone CHO-K1 distributed and lodged in culture collections by the late 1960s (CHO-K1 -ATCC® CCL-61TM). A distinct lineage, CHO-S, generated simultaneously with CHO-K1 was received at Gibco (now Thermo) during the 1980s.[4] Many CHO subtypes have been generated. CHO-DXB11, lacking one allele for dihydrofolate reductase (DHFR), was generated in 1980.[5] CHO-DXB11 allows selection of transfected clones using methotrexate (MTX), an inhibitor of DHFR.[6] A CHO line deficient in both alleles of DHFR, CHO-DG44, was isolated in 1983.[7]

Selective markers exploit the cell phenotype (eg, DHFR- or GS-), allowing selection of expressing

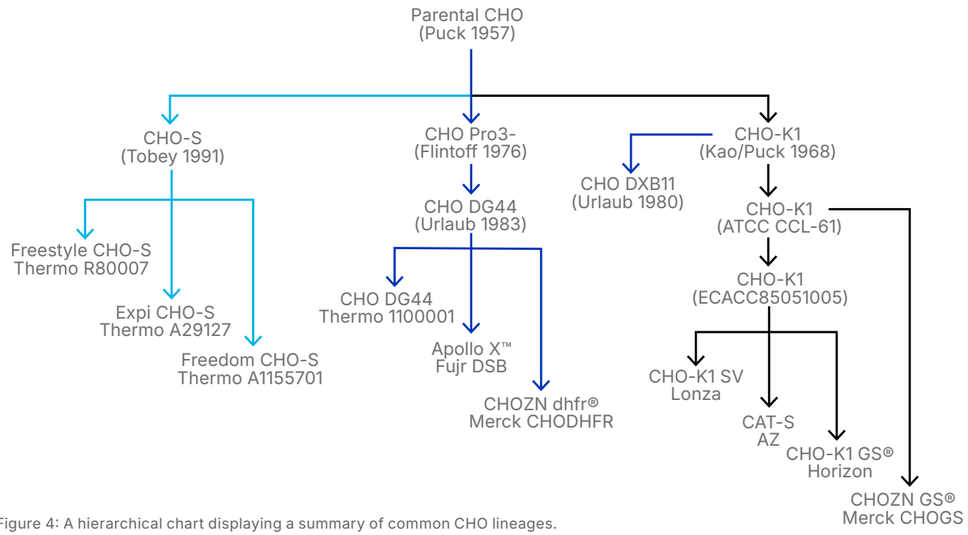

Figure 4: A hierarchical chart displaying a summary of common CHO lineages.

clones using inhibitors such as methotrexate (MTX) or methionine sulphoxamine and/or nutrient-deficient medium, allowing non-transfected cells to be selected out in culture. CHO-DG44 with DHFR/MTX selection was used for production of the first therapeutic protein, Activase, and remains the leading expression system for producing monoclonal antibodies (mAbs). CHOs are anchorage dependent, exhibiting a fibroblast-like phenotype in culture and requiring adaptation to suspension growth for large-scale commercial bioreactor culture and fetal bovine serum (FBS), an excellent physiological fluid routinely used to culture animal cells *in vitro*, was used to isolate early CHO lineages. Due to concerns of introducing infectious agents into the cell line, the use of serum is no longer acceptable for producing therapeutics.

Commercial CHO expression systems require serum and suspension adaptation (SSA) of the host. Many SSA CHO-K1 and DG44 have been generated: CHO-K1 SV by Lonza for use with the glutamine synthase (GS) expression system, CAT-S by AZ8 and ApolloX CHO-DG44 developed by Fuji. More recently, gene editing has been used to generate SSA CHO-K1 cells with single gene deletions. Horizon produced a GS- CHO-K1 knockout with Merck, creating CHO-K1 DHFR- and GS-. These lines are designed for common selection systems, namely GS and DHFR. SSA CHOs are often subject to commercial restrictions. A summary of common CHO lineages is shown in Figure 4.

CHOs are known to undergo genetic and phenotypic changes over time in culture.[9,10] Adaptation of CHOs to serum and suspension growth results in major changes to cell growth and physiology.[11] Therefore, each SSA-adapted CHO will have a unique and distinct physiology.

The 'plasticity' of the CHO genome can be exploited by directed evolution. As CLD steps can be physiologically challenging to cell lines, pre-adaptation may improve performance during cell line construction. By exposing CHOs to process workflows, such as cloning or nutrient limitation, cells will gradually adapt, potentially improving processability. Directed evolution can yield improvements in cell growth rates, capacity for protein synthesis/secretion and process 'fit'.[12] Apollox (described above) was isolated using this method.

Finally, it is also a regulatory requirement that the "origin, source and history of cells" is documented, detailing all steps in the generation of the parental cell line from the progenitor – normally an ampoule from the deposited culture collection.

Expression vectors and selection

The second element required for construction of recombinant CHOs is a vector. Vectors are complex, multi-component DNA strands that facilitate the expression of foreign protein within the cell and allow for selection of positively transfected cell lines.

A vector contains the gene of interest (GOI), a promoter allowing transcription of the GOI, a selective marker to select expressing cells, and other genetic elements (signal peptides, un-transcribed regions [UTR] and poly A sequences) required by cells to transcribe, translate and export the GOI (Figure 5).

Linear depiction of an expression vector

Vector elements were first derived from natural sources such as viruses. Native genetic elements can be sub-optimal for expression and prone to undesired events such as gene silencing and deletion. In order to alleviate detrimental events, improved or modified promoters have been developed. Probiogen™ has developed promoter variants, used in the Freedom CHO-S CLD system (Thermo™ A1369601). Optimization of signal peptides can also improve mAb secretion.[13] An exciting development is the design of synthetic genetic elements specific to the host that can be more effective in interacting with CHO transcriptionfactor receptor elements (TRFE). Synpromics™ was the first company to develop and market synthetic promoters. While early results are promising, outcomes can be unpredictable.[14] Heterogeneity of CHO cells following SSA can require bespoke development for each host. Evidence for improved expression in industrial processes remains limited.

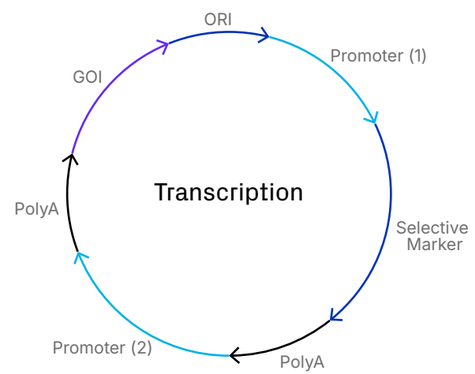

Figure 5: A circle chart illustrating transcription of the gene of interest, a selective marker to select expressing cells, and other genetic elements required to transcribe, translate and export the GOI.

Monoclonality

It is a regulatory requirement that cells producing therapeutic proteins are derived from a single clone (ICH Q5D). Monoclonality is routinely determined statistically following limiting dilution cloning (LDC) in multi-well plates.[15] Deposition of less than one cell per well allows statistical assessment of monoclonality. Realistically, achieving an acceptable probability of monoclonality requires two consecutive rounds of cloning. The cloning and expansion of cell lines in multi-well plates is extremely labor intensive and time consuming. Therefore, several procedures have been implemented to eliminate one round of cloning, automate transfer and screening, or reduce the number of clones handled and screened. Initially, methods using either LDC or capillary-aided single-cell deposition, with manual or automated plate imaging, enabled elimination of one round of LDC.[16] Automated plate handling, cloning and imaging systems are now extensively used with instruments, providing visual demonstration of monoclonality (Solentim™). In a further advance, automated combination of cell deposition with integrated imaging is performed using instruments such as the ClonePix™, CytoMine™ or Berkeley Lights™. These instruments can also accelerate CLD. By eliminating one round of LDC, use of the CytoMine™ can reduce the time to produce a cell line by more than two months.

Clone screening – isolation of a highly productive host

Following LDC, multiple rounds of screening are performed to exclude poorly expressing lines and identify the highest producers. Since the cost of a therapeutic protein will depend in part on the cell line productivity, it is vital to identify the highest producer possible. Surrogate screening methods, such as multi-well plates and shake flasks, are used; however, these are rarely predictive of the bioreactor systems used for manufacturing.[17] In order to better characterize and rank clones faster while reducing labor, several novel instruments have been developed. Both the CytoMine™ and Berkeley Lights™ instruments allow pre-enrichment of the transfected selected cell population, meaning fewer clones need to be handled and screened. Augmenting deposition and imaging with early measures of expression can also greatly reduce the number of clones handled and screened. Incorporation of representative scale-down bioreactors (eg, ambr™) further improves clone screening, allowing selection of process-ready, highly productive cell lines. Implementing automated single-cell deposition, imaging, population enrichment and miniaturized bioreactors can result in labor savings of over 50 percent, reduction in timelines by up to three months and may lead to higher producing clones.

Restrictions for commercial access

Although the development pathway for mAbs is well defined, access to technology can be a limiting factor for new market entrants. Much of the technology, such as SSA-adapted cell lines, optimized vector elements and improved workflows are proprietary or subject to commercial restrictions. Attempts to provide high-quality kits for CLD, such as Freedom CHO-S marketed by Thermo, have only been partly successful due to the poor expression levels obtained. Restrictions on access to technology for development can provide a roadblock for the development of new medicines. Ultimately, in the author's opinion, open source, technically advanced cell line systems are needed if continued growth in the industry is to continue.

About

Dr. Jonathan H Dempsey

Jonathan is the Managing Director of Dempsey Consulting and a founding Partner of Pathway Biopharma Consulting. Jon has a degree in biotechnology and a PhD from the University of Edinburgh. With almost 30 years' experience in the development and manufacture of biologics, with companies such as Lonza Biologics, Cambridge Antibody Technology and Invitrogen, Jon has deep knowledge in cell line development and in innovative technologies impacting this field.

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Gene-Edited Cell Lines

Unlocking the potential of gene-edited cell lines: A promising tool for clinical research and compound screening

Recent advances in gene editing technologies have made it possible to precisely modify the genome of a wide range of cell types, including immortalized cell lines. The recent advent of genome-editing technologies has enabled a new paradigm in which the sequence of the human genome can be precisely manipulated to achieve a therapeutic effect.[1] These gene-edited cell lines are a valuable tool for investigating the effects of genetic alterations on cellular physiology and can be used to model disease and identify potential therapeutic targets. By introducing specific genetic modifications into these cell lines, researchers can gain insights into the underlying mechanisms of various diseases.

One of the most widely used gene editing tools is the CRISPR/Cas9 system, which enables precise and efficient editing of specific genes within the genome. This technique has revolutionized the field of gene editing, allowing researchers to create gene-edited cell lines with high precision and ease. Most clinical use of CRISPR to date has focused on ex vivo gene editing of cells. However, *in vivo* use of CRISPR technologies can be confounded by problems such as off-target editing, inefficient or off-target delivery, and stimulation of counterproductive immune responses.[2] Additionally, other gene editing tools such as TALENs and zinc finger nucleases have also been used to generate gene-edited cell lines.

The potential applications of gene-edited cell lines in clinical research are vast. These cell lines can be used to model a variety of genetic diseases and study the effects of specific genetic mutations on cellular physiology. For example, gene-edited cell lines have been used to model genetic disorders such as cystic fibrosis, sickle cell anemia and Huntington's disease, among others. By studying the effects of specific genetic mutations on cellular physiology, researchers can gain insights into the underlying mechanisms of these diseases and identify potential therapeutic targets.

In addition to modeling genetic diseases, gene-edited cell lines also have significant potential for use in compound screening and drug development. By introducing genetic alterations that mimic disease-associated mutations, these cell lines can be used to identify potential drugs that may be effective against these diseases. These cell lines can also be used to test the efficacy of potential drugs and identify potential side effects, thus accelerating the drug development process.

Moreover, the use of gene-edited cell lines in drug development has already shown promising results. The field of gene editing has emerged to make precise, targeted modifications to genome sequences.[2] For example, researchers used gene-edited cell lines to identify a potential drug for cystic fibrosis, which is currently undergoing clinical trials. Another study used gene-edited cell lines to identify a potential drug for sickle cell anemia, which also showed promising results in preclinical trials.

In conclusion, the use of gene-edited cell lines in clinical research and drug development represents a promising avenue for accelerating the development of new therapies and improving our understanding of disease mechanisms. With the continued advancements in gene editing technologies, gene-edited cell lines will likely become an increasingly important tool for biomedical research in the years to come.

Taylor Mixides
Deputy Editor
Drug Target Review

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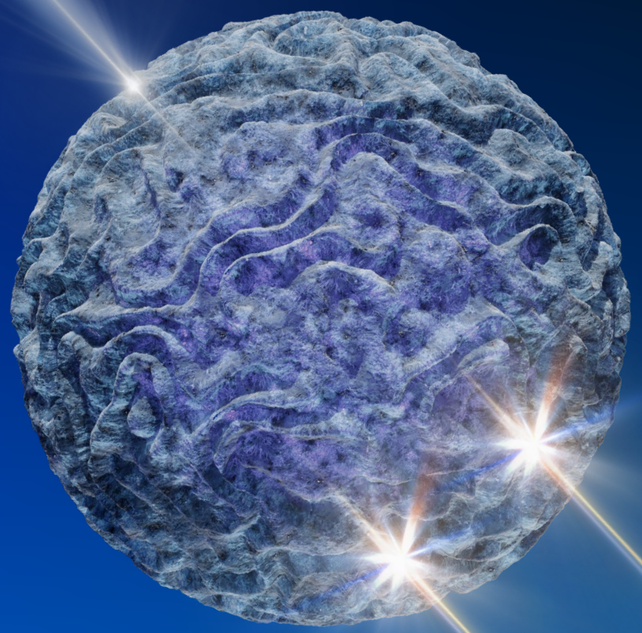
Cell Analysis Breakthroughs

From discovery to innovation: A deep dive into single-cell analysis and sorting technology



In this interview, Dr. Michelle Duong highlights the challenges and benefits of single cell analysis and sorting technology in producing genetically modified cell lines. The need for reliable single cell dispensing devices and the advantages of identifying clonal hits in protein production and drug discovery. The choice of a sorting technology should consider compatibility with imaging and integration into automated workflows. The interviewee provides an example of how single cell sorting technology improved efficiency in delivering clonal edited cell populations. Future developments are expected to enhance cell viability, expansion, and clonal recovery. Overall, single cell analysis and sorting technology play a vital role in cell engineering workflows, offering insights into cellular behavior and accelerating progress in genetically modified cell line production.

Single-cell analysis and sorting technology play a vital role in cell engineering workflows, offering insights into cellular behavior and accelerating progress in genetically modified cell line production. This Q&A reflects on the importance of carefully selecting reliable single-cell dispensing devices and compatible sorting technology. Michelle provides an example of how single-cell sorting technology improved efficiency in delivering clonal edited cell populations. Future developments are expected to enhance cell viability, expansion and clonal recovery.

In your experience, what are some common challenges or limitations that scientists face when performing single-cell analysis or sorting, and how can they be overcome when trying to produce genetically modified cell lines?

Assumption: Small biotech companies or isolated groups within larger companies.

Scenario 1: Protein production > groups are moving away from generating proteins such as antibodies via transient expression and are moving towards stable cell line expression. This requires a device that is capable of reliably performing single-cell dispense (SCD) in order to help identify clonal hits. When working for a medium size company, I noticed that while my team in operation had a single cell dispenser, other teams in research and development (R&D) and product development (P&D) did not; often having to perform manual dilution and estimation for single-cell dispense events.

Scenario 2: Library screening for drug discovery from pool to clone. SCD is needed to isolate a clonal population to effectively generate assay-ready cells for use downstream for either small molecule screening or metabolic pathway analyses.

Can you explain why single-cell analysis is important in cell engineering workflows, particularly when trying to produce genetically modified cell lines, and what kind of information it can provide that traditional bulk analysis cannot?

In addition to my previous answer, in the case of protein production, having a best performing producer aka hits will facilitate the establishment of a reliable source titer and shorten the project timeline. And for cell and gene therapy, the clonal population allows the researchers to specifically pair up phenotype and genotype association.

There are several different types of single-cell sorting technologies available. Can you provide an overview of the most common methods and the advantages and limitations of each when it comes to producing genetically modified cell lines?

I did not personally operate the SCD (Single Cell Despensing) instrument in the course of my work but the model that my team used was Scienion.

What are some key factors that scientists should consider when choosing a single-cell sorting technology for their specific cell engineering workflow that involves the production of genetically modified cell lines?

Compatibility with imaging technology or, at least for my previous team, how well the SCD can fit into the automated workflow for a large-scale genome editing facility.

Can you provide some examples of how single-cell sorting technology has been used in cell engineering workflows to improve outcomes or accelerate progress in producing genetically modified cell lines?

Without the Scienion SCD instrument my last company would not be able to deliver clonal edited cell populations to their customers with an average promise date of eight weeks. I believe their timeline is now even less.

Looking to the future, what new developments or advancements do you anticipate in the field of single-cell analysis and sorting technology, and how might they impact cell engineering workflows that involve the production of genetically modified cell lines?

There are various critical parameters to evaluate the quality of cells post SCD including viability of single cell per well and clonal expansion. Overall low clonal recovery is a primary concern for SCD users.

How do you see single-cell analysis and sorting technology evolving over time, and what kind of impact might it have on our understanding of cellular behavior and function, particularly in the context of producing genetically modified cell lines?

a. Clonal population panel enabled by SCD coupled with genotype and phenotype analyses can illuminate new learnings on how to develop treatments for pervasive diseases

b. SCD is a great setup for any high throughput arrayed screening for example small molecule screening to facilitate drug discovery

c. Adding onto the high throughput screeningcampaign, this equipment enables faster information generation thus feeding to a broader machine learning model.Identification of clonal hits “top producers” therefore reducing time and cost for, say, antibody manufacturers at large scale.

About

Michelle Duong

Michelle is currently a field application scientist at MaxCyte supporting cell and gene therapy and protein production solutions for Bay Area customers from R&D to GMP settings. She previously worked as a field application scientist at Inscripta supporting a fully automated CRISPR platform that provided an end-to-end solution for gene engineering. She also worked as a bench scientist at Synthego using CRISPR gene editing with their ECLIPSE platform. She conducted her doctoral studies at Cornell University, where she received a MS in Toxicology and a PhD in Bioengineering and focused her research on CRISPR-Cas-9 editing of bacteriophages for biomarker detection purposes. The bacteriophages in her doctoral work were field tested in Kenya by the Bill & Melinda Gates Foundation for water safety trials.

CRISPR Cell Line Development

Streamlining CRISPR cell line development for high-efficiency drug screening

In a recent study published in *Biomolecules*[1] researchers from Temple University in Philadelphia, USA discovered a novel approach to identifying tubulin polymerization inhibitors, which could aid in the development of more effective cancer treatments. The team used a CRISPR-edited cell line that expresses fluorescently tagged β-tubulin and a nuclear protein to visualize tubulin polymerization dynamics via high-content imaging (HCI) analysis.

Inhibiting tubulin polymerization has shown to be an effective strategy for inhibiting the proliferation of cancer cells. Previously, identifying compounds that could inhibit tubulin polymerization has required the use of *in vitro* assays utilizing purified tubulin or immunofluorescence of fixed cells. However, a recent study by researchers utilized live cell tracking to monitor the changes in Haralick homogeneity values over a period of three hours after treatment with three compounds (ON-01910, HMN-214 and KX2-391) that inhibit tubulin polymerization.

The team screened a library of 429 kinase inhibitors, resulting in the identification of the three compounds that inhibit tubulin polymerization. The results showed that all three compounds inhibited tubulin polymerization rapidly, with changes detectable in the first 20 minutes of treatment. Colchicine yielded the highest change in Haralick texture homogeneity, followed by KX2-391 and then ON-01910. Molecular docking was performed to evaluate the potential interaction of the hit compounds with β-tubulin to confirm that the effect of tubulin polymerization inhibition was achieved by directly blocking tubulin function.

The analysis suggested that these compounds all interacted with the colchicine binding site. The docking analysis revealed several key differences between colchicine and the new compounds, with KX2-391 being more effective in inhibiting tubulin polymerization than the other two compounds. ON-01910 had an aromatic ring with three methoxy-substituted groups that interacted with most of the amino acids that are key for the binding of colchicine, while HMN-214 contained two polar groups within the non-polar binding cavity of tubulin, which may have made the binding less stable and could explain why it was the least potent.

The group used CRISPR gene editing to insert fluorescent tags into the genome of two proteins involved in cell division, allowing them to visualize the behavior of these proteins in live cells. Microfluidic single-cell sorting was then used to isolate cells to derive a clonal line; this enabled researchers to perform whole-well imaging for selection of an ideal clone that could expand a cell line to use in the study.

Traditionally, methods for isolating single cells involved a two-step process of first enriching cells into a pool using a conventional cell sorter (FACS) and then isolating single clones using a limiting dilution method. However, this approach often results in a relatively small number of viable clones due to the high sorting pressure of FACS machines and potential damage to the cells. To address this issue, the researchers employed a microfluidics-based single-cell sorter, which enabled selection and deposition of target cells as singlets into 96- or 384-well plate in one step using low sorting pressure that's 10-30 times lower than that of conventional FACS sorter.

The Bio-Techne Cell Sorter and Single Cell Dispenser improved the ease-of-use and outcome of the cloning workflow by gently isolating target cells as single cells to generate clonal cell populations for use in the study. This approach ensured a high rate of outgrowth among isolated clones and allowed the researchers to visualize the behavior of fluorescently tagged endogenous proteins in live cells, providing valuable insights into the effect of compounds on cell division.

The use of innovative technologies such as microfluidic single-cell sorting holds great promise for improving our approach for cell biology research and discovery of new treatments for diseases such as cancer.

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CRISPR Tagging and High-Content Imaging

Dr. Oscar Perez-Leal delves into the promising fusion of **CRISPR and high-content imaging** in drug development

Dr. Oscar Perez-Leal discusses his ground-breaking work using CRISPR genome editing for drug discovery. By adding labels to endogenous target proteins, he creates accurate physiological models to track drug effects.

Highlighting the critical role of the Bio-Techne Cell Sorter and Single Cell Dispenser in efficient screening, Dr. Perez-Leal credits its capacity to isolate genetically modified cells, facilitating the generation of clonal cell lines. The device's simplicity and reliability make it an ideal tool for producing viable clones for experimentation. He also explores the merits of fluorescently tagged proteins and the transformative potential of CRISPR-edited cells in the development of cancer treatments. Finally, he emphasizes the revolutionizing impact of integrating CRISPR editing with high-content imaging analysis on drug discovery, as it allows real-time cellular observations without the need for staining or fixing cells.

Can you tell us about your work and its importance?

My current work focuses on utilizing CRISPR genome editing to modify cells lines for developing cellular models for drug discovery. The idea is to use CRISPR to introduce labels to potential drug targets or genes encoding proteins that define specific subcellular structures. Once we have these modified, labeled cells, we can employ various techniques to track changes to our targets or the cellular structures we aim to modify with the drugs.

This approach enables us to create more physiologically accurate models, avoiding the need for artificially inducing a cell to produce a recombinant protein, or fixing and staining the cell. We can monitor the real, endogenous targets that we aim to modify with drugs and gain insight into the physiological processes. In essence, my work's primary objective revolves around harnessing these advanced technologies to improve drug discovery models.

How does the emphasis on the endogenous nature of proteins in your model cell lines differentiate the current approach from conventional methods? Can you provide further insights on whether this represents a new trend in the field?

Ever since the advent of CRISPR, there has been a growing interest within the scientific community to use this technology to subtly modify cells, thereby maintaining the majority of physiological processes intact for improved modeling. The goal is to effect minimal alterations while preserving the cell's native state as much as possible.

In the past, we used to insert complete artificial cassettes into cells to develop models that express specific genes for drug discovery purposes. However, a significant issue with this approach is that it often led to model-specific activations. These activations, while observable in the model, do not necessarily occur in other cells, which can be misleading.

By focusing on endogenous targets and limiting the extent of modifications, we strive to preserve the cell's native state. This approach, which represents a growing trend in the field, enhances the physiological relevance of our cell models and reduces the risk of observing artificial responses.

What do you see as the key challenges in the development of CRISPR-edited cell lines?

The key challenges I've identified in the development of CRISPR-edited cell lines include inefficiency, unintended modifications, and difficulties in introducing CRISPR components into cells.

Firstly, CRISPR genome editing remains inefficient for certain types of modifications, especially when inserting large DNA segments into the genome. This inefficiency stems from the sporadic presence of DNA repair enzymes that execute these modifications throughout the cell cycle. Only cells at a specific stage in the cell cycle can receive these genetic alterations, making it challenging to obtain a pure population of cells with the desired modification.

Secondly, off-target effects pose a significant problem. CRISPR, while powerful, is not perfect. When modifying a specific DNA sequence, unintended alterations can occur elsewhere in the genome. This necessitates the analysis of multiple clones to ensure your cell clone has only the intended modification.

Finally, the successful introduction of CRISPR proteins or plasmids into cells varies significantly between cell types. Some cells may present more challenges for CRISPR modification due to difficulties in delivering these components into the cells.

Considering the challenges mentioned, the importance of facilitating clone screening becomes even more significant. With the decreasing likelihood of finding cells that meet the desired criteria, an efficient method for screening a larger number of clones becomes crucial. Could you elaborate on how addressing these challenges necessitates the development of an effective and efficient screening approach?

Absolutely. The ability to eliminate undesirable cells that have failed to undergo the desired modification is crucial. Alternatively, if there's a method to enrich the population of cells that have successfully undergone the modification, it can significantly aid the sorting process. Being able to selectively sort these enriched cells streamlines the workflow and enhances the efficiency of the overall process. Such strategies provide valuable tools for ensuring the success of experimental procedures and ultimately contribute to achieving the desired outcomes.

How is the Bio-Techne Cell Sorter and Single Cell Dispenser used in your workflow currently, and what are the key benefits you see in our product that make a difference in the work you do?

I specifically use the Bio-Techne platform in the final stage of establishing the cell line. Within my process, there is a critical step where I eliminate cells that lack the desired genetic modification introduced by the FAST-HDR system – a system I developed to facilitate CRISPR-based gene tagging. One significant advantage of this system is that only cells receiving the intended genetic modification have the ability to produce an antibiotic selection protein. By selectively eliminating cells that lack this modification, I am left with a pool of cells that are all genetically modified, albeit with potential variations in the number of modified alleles.

To achieve a genuinely pure and homogeneous cell line, it becomes necessary to isolate individual cells and generate separate clones. During my search for suitable equipment to accomplish this, I initially experimented with conventional flow cytometry cell sorting devices. However, I encountered significant challenges in growing cells from the sorted populations.

The process itself is harmful to the cells. The survival rate was extremely low, and the size and unavailability of the conventional flow cytometry machines posed additional challenges. The inconvenience of transporting my cells to a distant location equipped with such machines added further complexity to the process. I started exploring other options and found devices that were able to perform single-cell dispensing. One standout feature of the Bio-Techne cell sorting and single-cell dispensing device that greatly appeals to me is its utilization of a simple and unique microfluidic chip dedicated to each experiment, ensuring sterility and minimizing the risk of contamination. This capability significantly reduces the chance of unintentional cross-contamination between different cell lines. When I initially tested the device in our laboratory, I was immediately impressed by its user-friendly nature. Encouraged by the positive experience, I arranged a demo in the lab and conducted four separate experiments to isolate four distinct cell lines, including two cancer cell lines and two stem cell lines.

All four experiments yielded successful results on the first attempt. I was able to obtain single clones for each cell line, which served as strong evidence of the device's remarkable power and efficacy. This positive outcome left me thoroughly convinced of the Bio-Techne platform's tremendous capabilities.

One aspect that particularly resonated with me was the device's ability to remain functional even during periods of infrequent use. Unlike many other devices that require routine maintenance, the Bio-Techne Cell Sorter and Single Cell Dispenser does not deteriorate if left unused for weeks or months. I appreciate this feature as it allows me to store the device when not in use, ensuring its reliability and preventing major blockage issues or malfunctions.

Utilizing this machine has proven instrumental in streamlining the development process of single-cell clones for the cell lines I am working on. It has greatly facilitated the generation of valuable research tools, contributing to the overall progress and efficiency of my work.

Could you please provide some comments on the improvements in clonal outgrowth that you mentioned earlier? Specifically, I recall you mentioning the difficulty in growing cells derived from sorted populations. It would be interesting to hear your perspective on any advancements or strategies that have addressed this challenge.

Indeed, that's an excellent point. When using traditional flow cytometry equipment, the number of clones obtained in a 96-well plate is typically quite limited, often less than 10. Manual cell dilution may yield around 10 to 12 single clones. However, a significant disadvantage of this method is the difficulty in verifying that the clones truly originate from a single cell, which introduces a level of uncertainty into the results.

The Bio-Techne platform has shown remarkable performance in addressing this challenge. For conventional cancer cell lines, an average of 40 to 50 clones can be obtained in a 96-well plate, offering a significant increase in available options for further analysis. Even with more delicate iPSC stem cells, which tend to struggle when cultured individually, the device demonstrates its efficacy by producing around 14-20 well-growing clones per plate. This is particularly noteworthy considering the added challenge of starting from a single iPSC stem cell.

Overall, the device's ability to generate a substantial number of viable clones not only gives researchers a substantial pool of options to work with, but also provides greater assurance of each clone's origin, which is highly advantageous for subsequent experiments and investigations.

Prior to having the Bio-Techne cell sorting platform for your single stem-cell sorting, what was the clonal outgrowth percentage that you typically obtained?

Before using a Bio-Techne instrument for single stem-cell sorting, I encountered significant challenges with traditional flow cytometry cell sorters. I was unable to obtain viable clones or satisfactory results.

Could CRISPR-edited cells with a fluorescently tagged endogenous protein provide an advantage over traditional *in vitro* assays or fixed immunofluorescent assays for studying active compounds or drug targets?

Indeed, CRISPR-edited cells with a fluorescently tagged endogenous protein do provide a significant advantage over traditional *in vitro* assays or fixed immunofluorescent assays for studying active compounds or drug targets. The primary advantage of this methodology lies in its ability to pre-label target proteins. This feature enables a seamless transition from treating live cells with compounds to directly analysing them using a high-content imaging device, eliminating the need for any additional procedures. With the right high-content imaging device, real-time analysis becomes feasible simply by placing the plate in the machine and programming it to capture images at regular intervals.

Using CRISPR to tag proteins with fluorescent labels presents a major advantage when it comes to cell models, as it facilitates evaluations without additional cell preparation or processing. While this may seem straightforward when analysing a few items, the efficiency truly becomes apparent when handling dozens of plates, such as 384-well plates. Performing steps like fixing and staining, not to mention other associated procedures, would not only consume hours of work but could also prove exhausting for the researcher, unless they have access to advanced instrumentation or robotic liquid handlers capable of managing the multiple incubations and steps involved in the analysis process.

One of the standout benefits of our multiplex cell models is their ability to transition seamlessly from the incubator to the microscope without any additional preparation or procedures. This feature enables immediate observation and analysis of the cells, thereby saving valuable time and effort. With the labels already added to the targets, researchers can directly examine the cells, facilitating prompt insights and observations without unnecessary delays.

How might the identification of new tubulin polymerization inhibitors using CRISPR-edited cells with fluorescently tagged β-tubulin impact the development of new cancer treatments or therapies?

The exploration of novel tubulin polymerization inhibitors remains a vital pursuit in the realm of cancer therapeutics. Tubulins, given their crucial role in cellular division, have long been established as effective therapeutic targets across various cancer types. Nonetheless, the field continues to grapple with specific challenges, such as developing inhibitors that selectively target cancer cells, sparing normal cells, or designing inhibitors that demonstrate limited permeation into certain tissues (e.g., the brain), thereby concentrating their effects on the tumor.

One strategic approach to these challenges involves leveraging CRISPR-edited cells tagged with a fluorescent protein at the tubulin gene. This approach provides a real-time, visual framework to observe the dynamics of tubulin polymerization. Notably, our research indicates that the process of inhibiting tubulin polymerization is remarkably rapid, initiating within the first 10 to 20 minutes of treatment. This finding permits the development of streamlined assays, whereby one can observe the influence of various compounds on polymerization across different dosage levels.

The central advantage of employing such cell lines resides in the ability to conduct intricate experiments within a relatively uncomplicated setup. Historically, similar experiments would have necessitated the use of purified tubulin for *in vitro* assays, thereby bypassing the complexities of cellular context. The current approach, however, utilizes cells, thereby facilitating the evaluation of potential therapeutic compounds within a more physiologically relevant environment. This necessitates the compound to traverse the cell membrane, infiltrate the cell, and subsequently interact directly with tubulin at its native location. Consequently, this method offers a more authentic appraisal of a potential drug's efficacy within a biologically accurate context.

Can you describe how single-cell analysis techniques such as high-content imaging analysis and live-cell tracking in combination with CRISPR editing can be used for drug discovery?

The convergence of high-content imaging analysis and the CRISPR revolution has significantly transformed the landscape of cell biology and drug discovery. Particularly around 2015, these two fields began to intersect, leading to novel and impactful methods or drug discovery.

High-content imaging analysis of CRISPR-edited cells provides a wealth of data essential for drug discovery. By using CRISPR to fluorescently tag specific proteins or organelles, researchers can monitor the cellular response to drug compounds in real-time.

For instance, observing changes in protein location, expression, or interaction can offer critical insights into the cellular effects of potential therapeutic agents. This eliminates the need for labor-intensive cell staining and fixing procedures that were traditional bottlenecks in the process.

Further, live-cell tracking allows researchers to observe these changes over time, enabling the detection of immediate and long-term effects of a drug. This real-time observation could elucidate the cellular pathways involved in the drug's action and potentially identify off-target effects, which may not be evident from end-point assays.

This approach fosters a comprehensive understanding of a drug's mechanism of action, its efficacy, and safety profile. This rich data can guide the selection and optimization of drug candidates, thus accelerating the drug discovery process.

Moreover, the combination of CRISPR-edited cells and high-content imaging paves the way for high-throughput screening, enabling rapid testing of a vast number of potential drug compounds. Consequently, researchers can more efficiently identify promising drug candidates and discard ineffective or toxic ones.

What are some of the potential future applications of CRISPR-edited cells with fluorescently tagged endogenous proteins in drug discovery or screening efforts? And how might they impact the pharmaceutical industry?

One possible future direction for this field is integrating these technologies into stem cells and organoids to develop better models. Coupled with the emerging trend of developing 'organ-on-a-chip' systems, it is conceivable to create models that will allow drug studies without the need for animal models. Currently, our dependency on animal models for drug discovery is substantial. However, numerous studies have indicated that using animals is not always the optimal approach for all drugs. Looking ahead, it might be feasible to forego animal testing for a significant number of drug programs, proceeding directly from drug discovery to testing on organ-on-a-chip platforms that utilize human cells. These cells could be obtained from a diverse and extensive population, thereby accurately reflecting human physiology.

The possibility of conducting a mini clinical trial on a chip would provide a higher degree of certainty about a drug's efficacy in humans before proceeding to full-scale clinical trials. The field appears to be moving towards developing lab-grown organs, or even multiple organs, that can better simulate the human body. Decreasing reliance on animal models and improving physiological models offer significant advantages. There are instances where drugs work perfectly in animal models, but prove ineffective or toxic in humans. There is currently no reliable method to predict this.

How might single-cell analysis techniques such as flow cytometry or microfluidic-based cell sorting be used to select specific cells for CRISPR editing, to create genetically modified cell lines that can be used for drug discovery or screening?

Depending on the tools available in your workplace, it's highly recommended to use either flow cytometry or microfluidic-based cell sorting devices to develop cell lines. This increases the likelihood that you are indeed starting from a single cell and that your cell models originate from a single source. This approach is more precise than performing traditional serial dilution of cells to try to isolate cell lines. Undoubtedly, this method is extremely useful when developing CRISPR genome-edited cell models.

Several research groups are using CRISPR to insert genes into cells in order to overexpress proteins, antibodies or growth factors. Thus, when the aim is to develop pure cell models and isolate the products that these modified cells produce, it is highly beneficial to employ cell sorting and separation technologies.

What are some challenges that scientists might face when using single-cell sorting techniques in combination with gene editing technologies and how can they be overcome to improve the accuracy and efficiency of the process?

One of the major challenges when using gene editing on cells is that not all cells will undergo the intended modification. Thus, there is a need for a method to enrich the cells that received the intended modification from those that did not. A reliable single-cell sorting device, such as a conventional flow cytometry device, can be used for this process. However, these devices often operate under very high pressures, and the pressure exerted to move the liquids through the system can potentially damage the cells.

Another limitation that researchers might encounter is that some cells prove extremely difficult to genetically modify. Therefore, careful selection of the cell line for experiments is crucial. If the chosen cell line is too difficult to transfect/electroporate or has a slow cell division cycle, the process of identifying positive clones and growing them will be more time-consuming. Hence, when performing such modifications, it is preferable to use cell lines that double in less than 36 or even 30 hours. A traditional 24-hour cycle is optimal. However, in instances where optimal cell lines for genome editing are not available for studying a particular biological process, scientists should be prepared to spend more time optimizing the process or analysing a larger number of clones. This will ultimately ensure a comprehensive and accurate study despite the prolonged timeframe.

One of the challenges you mentioned earlier is the ability of a single cell to grow in the absence of contact with other cells. Could you elaborate on this challenge and provide further insights into how it affects the growth and viability of individual cells?

Indeed, while this characteristic is commonly associated with stem cells, it is important to acknowledge that there are other cell types, such as certain cell lines, which exhibit a comparable behavior of limited growth potential when initiated from a single cell.

This is indeed a significant challenge. Therefore, one must devise alternatives to facilitate this process. Some cells may require different culture media and a protective compound to allow them to exist as single cells with the opportunity to divide. There are various cell-specific strategies that can improve your chances of starting from a single cell.

Looking to the future, how do you see the fields of gene editing and single-cell sorting evolving, and how might their integration impact drug discovery? Is this treatment in other areas of interest, such as new models like tissue on a chip?

Improving the efficiency of CRISPR editing to enable faster and more effective simultaneous modification of multiple genes would certainly be beneficial. Moving forward, it would be advantageous to have low-pressure cell sorting systems capable of detecting multiple markers concurrently. Imagine modifying cell lines where we insert at least three different labeling proteins simultaneously. If the technology can instantly select and isolate cell populations exhibiting these three colors, it could greatly expedite the process of developing cell models for drug discovery.

Such advancements will significantly impact drug discovery, where time efficiency directly reduces operational costs. Rapid model development will not only decrease costs but also enable better disease models. This includes models for rare diseases or diseases without known targets but with observable changes in cell phenotype. By labeling these structures, we can still attempt to find drugs that may be effective against these diseases, even if we don't know the specific target, by using phenotypic drug discovery techniques.

As for the 'tissue on a chip' models, they represent a frontier that could be profoundly influenced by these advancements. We will be able to generate complex multicellular models to track biological processes in live, *in vitro* tissue for extended periods. This opens up new opportunities for understanding and treating diseases.

If the disease target remains unknown, but extensive screening is conducted, is there still a possibility of identifying effective solutions?

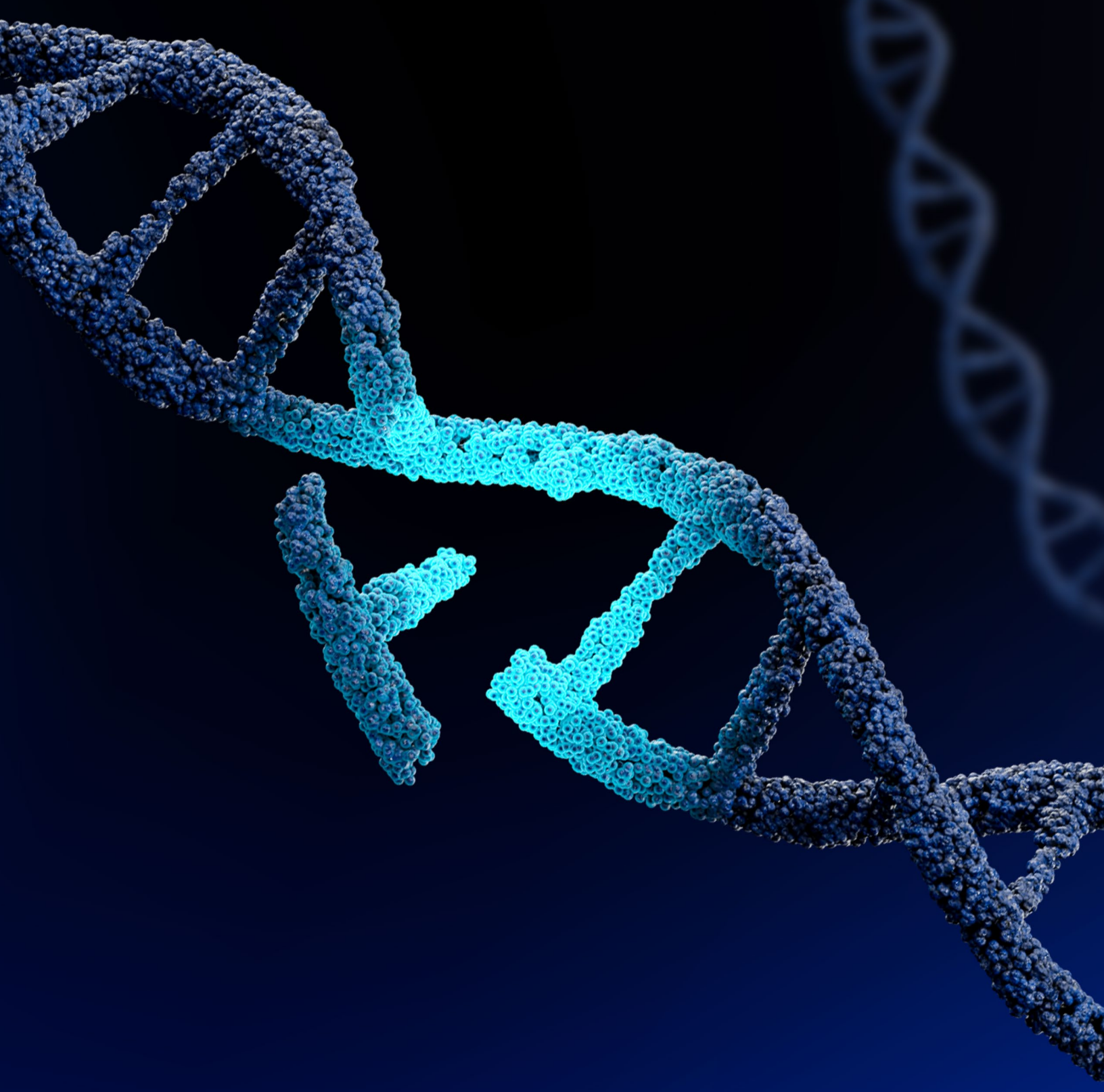
Indeed, even if a disease's target remains unknown, extensive screening can still potentially identify effective treatments. This approach operates at the phenotypic level, and subsequent methodologies can be employed to deduce the mechanism of active compounds thereafter. Crucially, having a compound that shows promise allows us to keep moving forward. It's not necessary to know the exact mechanism of action for a drug to proceed to clinical trials. What matters in practical terms is the development of drugs that are safe.

About

Dr. Oscar Perez-Leal

Oscar currently serves as an Assistant Professor at Temple University's School of Pharmacy, situated in Philadelphia, USA. His credentials include a Medical Degree from Universidad Del Norte in Barranquilla, Colombia, and postdoctoral training in Biochemistry and Molecular Biology completed at the School of Medicine, Temple University. His expertise is centered on the generation of mammalian cellular models for drug discovery applications, using CRISPR genome editing. A significant contribution of Dr. Perez-Leal's research is the creation of the FAST-HDR vector system. This innovative platform streamlines the process of endogenous gene tagging with reporter proteins, following the induction of CRISPR double-strand breaks. The functionality of the FAST-HDR system extends to multiplexing capabilities and facilitates the addition of different tags to a maximum of three genes. This enhanced capability provides an avenue for the development of intricate cellular models for drug discovery. It does so by enabling high-content imaging with live cells, thereby mitigating the need for immunofluorescence or chemical staining methods.

Learn more about the Bio-Techne Pala Cell Sorter & Single Cell Dispenser
bio-techne.com/instruments/single-cell-dispensers



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