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Single-cell technologies to dissect heterogenous immune cell therapy products

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Abstract

Single-cell tools have dramatically transformed the life sciences; concurrently, autologous and allogeneic immune cell therapies have recently entered the clinic. Here, we discuss methods, applications, and considerations for single-cell technologies in the context of immune cell manufacturing. Molecular heterogeneity can be profiled at the level of the genome, epigenome, transcriptome, proteome, metabolome, and antigen receptor repertoire in isolation or tandem through multiomic approaches. Such data provides a detailed characterization of heterogeneity within cell products and can be linked to potency readouts and clinical data, with the ultimate goal of identifying critical quality attributes to predict patient outcomes. Non-destructive approaches hold promise for monitoring cell state and analyzing the impacts of genetic modifications within engineered products. Destructive omics approaches could be combined with non-destructive technologies to predict therapeutic potency. These technologies are poised to redefine cell manufacturing toward rapid, cost-effective, and high-throughput methods to detect and respond to dynamic cell states.

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Introduction

Engineered immune cell products are a promising frontier in cancer immunotherapy, with an explosion of candidates recently entering clinical trials [1]. Adoptive T cell therapies, including chimeric antigen receptor (CAR) T cell therapies, have enjoyed particular success; there are over 800 active CAR T trials worldwide [2], and 5 products are now approved in the US [3]. More nascent immune cell products, including CAR-NK [3] and CAR-Macrophage [4] cells, as well as fourth-generation T cell products (e.g., TRUCKS [5]), are also achieving increased interest and momentum in clinical trials. However, engineered immune cell products remain heterogeneous in quality and phenotype, making patient outcomes difficult to predict. Immune cell heterogeneity refers to diversity in characteristics, including activation, memory acquisition, and exhaustion, which have historically been defined by protein biomarkers [6,7]. However, so-called 'canonical' subsets don't capture all clinically relevant cell states, and individual subsets may have functional variation. Therefore, new approaches are needed to comprehensively define the cellular and molecular heterogeneity of engineered immune cell products and determine which critical quality attributes (CQAs) best predict potency [8].

In recent years, a parallel explosion of single-cell technologies has emerged to assay cellular and molecular characteristics. Given the diversity and plasticity of cellular phenotypes, single-cell assays are a valuable tool to understand heterogeneous cell populations at high resolution. While a comprehensive summary of single-cell technologies [7,9] is beyond the scope of this article, we highlight tools developed within the last five years to assay six facets of molecular heterogeneity within immune cells: (1) the genome, (2) the epigenome, (3) the transcriptome, (4) the proteome, (5) the metabolome, and (6) the antigen receptor repertoire (Figure 1, Table 1). We follow this technology summary with a discussion of specific considerations and applications in a cell manufacturing context. For the purposes of this review, we will focus on therapeutics such as (CAR) T and Natural Killer (NK) cell therapies.

Acronyms

CAR	Chimeric antigen receptor
CQA	Critical quality attribute
NK	Natural killer
CRS	Cytokine release syndrome
SNP	Single nucleotide polymorphism
scDNA-seq	Single-cell DNA sequencing
LiRA	Linked-read analysis
SCI-seq	Single-cell combinatorial indexed sequencing
scATAC-seq	Single-cell assay for transposase-accessible chromatin sequencing
scBS-seq	Single-cell bisulfite sequencing
sci-MET	Single-cell combinatorial indexing for methylation analysis
scRNA-seq	Single-cell RNA sequencing
TCR	T cell receptor
Th2	T Helper 2
CyTOF	Cytometry by time-of-flight
MS	Mass spectrometry
NMR	Nuclear magnetic resonance
MALDI-MSI	Matrix-assisted laser desorption/ionization-mass spectrometry imaging
T-ATAC-seq	Transcript-indexed assay for transposase-accessible chromatin sequencing
CITE-seq	Cellular indexing of transcriptomes and epitopes by sequencing
REAP-seq	RNA expression and protein sequencing assay
PLAYR	Proximity ligation assay for RNA
scM&T-seq	Single-cell methylome and transcriptome sequencing
sci-CAR	Single-cell combinatorial indexing of chromatin accessibility and mRNA
CRISPR	Clustered regularly-interspaced short palindromic repeats
GVHD	Graft-versus-host disease
CRISP-Seq	Clustered regularly interspaced short palindromic (repeats)/(RNA)-sequencing
CROP-Seq	CRISPR droplet sequencing
dscATAC-seq	Droplet single-cell assay for transposase-accessible chromatin using sequencing
dsciATAC-seq	Droplet single-cell assay with combinatorial indexing for transposase-accessible chromatin using sequencing; indrops, indexing droplets
HDST	High-definition spatial transcriptomics
scNT-seq	New RNA tagging sequencing
SERS	Surface-enhanced Raman scattering
EHR	Electronic health record

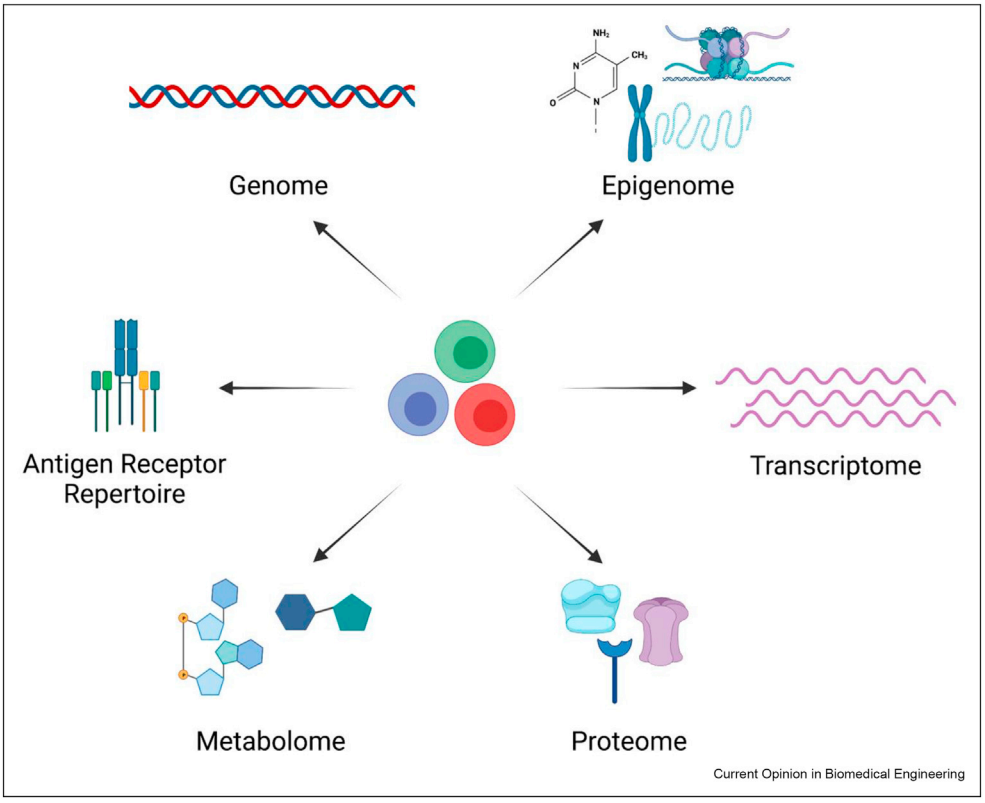
Cellular and molecular heterogeneity

Genome

To consider heterogeneity, we begin with the genome. Genomic diversity is a major factor in predictive medicine, particularly for identifying patient-specific genomic biomarkers to predict outcomes [10]. This problem is magnified in the context of immune cell therapies, as a patient's genetic background informs both their response to the therapeutic and how the product itself will behave after manipulation *ex vivo*. Genomic predispositions may influence the likelihood of severe cytokine release syndrome after CAR T cell therapy, as they can affect a patient's cytokine secretion levels [11]. Furthermore, specific mutations occurring during gene transfer can drive unique *in vivo* behaviors for particular CAR T cell clones. One notable case involved complete remission for a patient in which a single CAR T cell clone harbored a gene disruption during viral gene transfer, which combined with the patient's background genotype, disrupted both alleles of the *TET2* locus [12]. Loss of *TET2* expression caused the clone to expand to dominate the patient's CAR T fraction, demonstrating the impact of a single mutation on a product's success. While lucky for the patient in question, this case highlights the need to monitor engineered cell products for acquired deleterious mutations, which could cause insertional oncogenesis and other severe consequences. Tracking genomic modifications at the single-cell level could therefore be essential to understand the efficacy and safety of therapeutic products.

Recent advances in single-cell genomic analyses [13,14] have achieved high coverage, uniform amplification, and successful identification of single nucleotide polymorphisms while decreasing monetary cost. Single-cell DNA sequencing (scDNA-seq) approaches, such as linked-read analysis (LiRA) have allowed true genomic heterogeneity to be defined at the single-cell level while avoiding the propagation of single nucleotide errors during analysis [15]. While whole-genome sequencing remains costly for routine use, these technologies are expected to become increasingly cost-effective and integrate into standard biomanufacturing workflows [16]. Methods to simultaneously profile thousands of cells in parallel (e.g., single-cell combinatorial indexed sequencing (SCI-seq) [17]) offer the potential to assess known loci of interest in engineered cell products while capturing diversity from a large enough portion of the cell product to identify expansion events indicative of specific clones with enhanced proliferation. Such tools could capture known predictive biomarkers for clinical outcomes and analyze parameters such as copy number variation in products featuring integrated transgenes (e.g., CAR T cells). In addition, they could be leveraged to monitor the effects of genome editors on engineered immune cell products. For CRISPR-based technologies, in particular, off-target edits or chromosomal translocations after multiple edits are a critical parameter to monitor to ensure patient safety, as well as unintended on-target modifications (e.g., large on-target deletions) [18]. Many off-targets can be predicted *in silico* or through non-biased whole-genome assays to profile guide RNA targeting fidelity. scDNA-seq approaches could therefore be used to more deeply probe the frequency of off-target edits at

Figure 1



Cellular and molecular heterogeneity within engineered immune cells. Single-cell omics technologies can be used to characterize the cellular and molecular heterogeneity of engineered immune cell products by profiling the genome, epigenome, transcriptome, proteome, metabolome, antigen receptor repertoire, or a combination thereof.

defined loci, an important step toward ensuring patient safety.

Certain limitations must be considered when applying any scDNA-seq approach. In addition to the overall high cost of whole genome amplification, the amplification and library preparation process introduces errors and biases that can be difficult to distinguish from the true signal. For instance, read counts may be distorted by preferential amplification of one allele over another. Entire alleles or sites may also fail to amplify, further biasing results [14]. Approaches to address these challenges may be limited to certain genomic features. For example, LiRA relies on the proximity of heterozygous germline single nucleotide polymorphisms (SNPs) to identify acquired single nucleotide variations, while SCI-seq is designed specifically to identify copy number variants. Different amplification methods (e.g., PCR-based vs multiple displacement amplification) can also be applied in the context of certain genomic features to balance the need for uniform coverage with high fidelity amplification [14]. Finally, additional methods are needed to track other forms of genomic variation at the single-cell level, including insertions and deletions (indels) and large deletions or translocations [19,20] produced during gene editing.

Epigenome

After considering underlying genomic diversity, we move to a level of molecular organization at larger length scales: the epigenome. The epigenome describes the collective chemical modifications and proteins that interact with the human genome to regulate gene expression, including chromatin state, histone or nucleotide modifications, and histone variant exchanges [21]. Epigenetic modifications provide the information to establish a cell's functional identity and, therefore, directly mediate cell plasticity; these modifications have implications for the potency of heterogeneous engineered immune cell products at varied differentiation states. Epigenomic footprints have been associated with immune cell states, including T cell memory [22] and exhaustion [23,24], which correlate with clinical outcomes [25,26].

Table 1

Technologies to assay single-cell molecular heterogeneity. We include selected methods developed within the last five years to profile the genome, epigenome, transcriptome, proteome, metabolome, and antigen receptor repertoire within single cells. While many omics-based approaches are destructive, there is an increasing trend toward developing non-destructive methods to profile cellular heterogeneity.

Cellular/Molecular Characteristic	Technology	Destructive?	Reference
Genome	scDNA-seq	Yes	Reviewed in (Gawad et al. 2016, Lähnemann et al. 2020)
	LiRA	Yes	Bohrson et al. 2019
	SCI-seq	Yes	Vitak et al. 2017
Epigenome	scATAC-seq	Yes	Mezger et al. 2018
	dscATAC-seq	Yes	Lareau et al. 2019
	dsciATAC-seq	Yes	Lareau et al. 2019
	scBS-seq	Yes	Clark et al. 2017
	sci-MET	Yes	Mulqueen et al. 2018
Transcriptome	Drop-seq	Yes	Macosko et al. 2015
	inDrops	Yes	Zilionis et al. 2017
	Chromium next GEM (3′ and 5′)	Yes	Zheng et al. 2017 (10X Genomics)
	Spatial transcriptomics	Yes	Stahl et al. 2016
	Slide-Seq	Yes	Rodriques et al. 2019
	HDST	Yes	Vickovic et al. 2019
	scNT-seq	Yes	Qiu et al. 2019
Proteome	CyTOF	Yes	Gadalla et al. 2019
	Spectral flow cytometry	Yes	Park et al. 2020
	Abseq [47]	Yes	Shahi et al. 2017
Metabolome	MALDI-mass spectrometry	Yes	Dueñas et al. 2017
	SERS	No	Sun et al. 2019
	In-cell NMR	No	Luchinat et al. 2020
Antigen Receptor Repertoire	T-ATAC-seq	Yes	Satpathy et al. 2018
	Chromium single-cell immune profiling [92]	Yes	Fritz et al. 2017
Multiomic Approaches	CITE-seq (RNA/protein)	Yes	Stoeckius et al. 2017
	REAP-seq (RNA/protein)	Yes	Peterson et al. 2017
	PLAYR (RNA/protein)	Yes	Frei et al. 2016
	scM&T-seq	Yes	Angermueller et al. 2016
	sciCAR	Yes	Cao et al. 2018
	T-ATAC-seq	Yes	Satpathy et al. 2018
Other non-destructive approaches	Optical metabolic imaging	No	Walsh et al. 2020
	Holographic microscopy/optical d iffraction tomography	No	Hejna et al. 2017 Nygate et al. 2020 Lee et al. 2020
	Nanostraw intracellular sampling	No	Cao et al. 2017

Recently, single-cell epigenomic technologies have developed unprecedented throughput and resolution. Commercial systems for assay for transposase-accessible chromatin using sequencing (scATAC-seq), notably from 10X Genomics, have recently been widely available. Lareau et al. [27] described a droplet microfluidics-based method to perform scATAC-seq on a massive scale by superloading beads into a conventional droplet-based microfluidic system, enabling simultaneous profiling of hundreds of thousands of cells. As with advances in scDNA-seq [17], this dramatic increase in throughput improves the resolution at which distinct epigenetic states can be detected within a cell product. Chromatin state data from scATAC-seq can be corroborated with other assays, such as single-cell bisulfite sequencing (scBS-seq) or single-cell combinatorial indexing (sci-MET) for DNA methylation [28,29]. Single-cell epigenomic analysis has notably been applied at scale to assess regulatory elements during immune cell development and tumor penetrance [30]; such data could inform as-yet—undefined cell differentiation profiles encountered after genetic manipulation during the manufacturing process and provide key insights into epigenetic profiles that correlate with patient outcomes.

As with single-cell DNA sequencing approaches, single-cell epigenomic technologies are limited by costs, data sparsity, and noise [31]. scATAC-seq, which relies on DNA accessibility, relies on only two copies of a given locus per cell. Only 1—10% of expected accessible peaks per cell can generally be expressed in scATAC-seq datasets, a capture rate far lower than scRNA-seq approaches that may recover 10—45% of expressed genes in a given cell [32]. This sparsity can make it difficult to assess variability between cells and can complicate the biological interpretation of significant features. Open questions include differential chromatin accessibility between two alleles at a given

locus within a single cell and the total number of open chromatin regions that can be selected from within a cell [33]. New tools to increase coverage could address these questions and may better inform the interpretation of single-cell epigenomic datasets from therapeutic cell products.

Transcriptome

Single-cell transcriptomics has emerged as a landmark technology throughout the biotech field. scRNA-seq workflows typically involve droplet-based isolation of single cells, cDNA amplification of the transcriptome, cDNA barcoding, and sequencing. Common methods include Drop-seq [34] and InDrops [35], as well as the commercial 3′ and 5' Chromium Next GEM Solutions from 10X Genomics [36]. As with scDNA-seq and scATAC-seq, the scRNA-seq technology landscape is moving toward high-throughput solutions, including new modalities featuring spatial [37–39] and temporal [40] resolution to establish the *in situ* position or rate of RNA biogenesis.

scRNA-seq is increasingly being applied in the context of engineered immune cell therapies in the clinic. Recently, single-cell transcriptomes for 101,326 single cells were generated for 12 pediatric patients with acute lymphoblastic leukemia after *in vitro* stimulation with either a CAR-specific antigen or a TCR-mediated activation control [41]. Notably, activation states after CAR stimulation were highly heterogeneous, and relapse events correlated with patients that demonstrated deficiencies in the Th2 compartment of the cell product. This result highlights the potential for scRNA-seq to predict clinical outcomes through omics-level assessment of active gene expression within the therapeutic product. The high cost of scRNA-seq remains a barrier to use as a routine assay for cell products; however, the breadth and depth of information about cell state offers significant advantages in defining the heterogeneity of the product.

As with other single-cell omics datasets, sparsity remains a limitation in scRNA-seq approaches, although this can be addressed, to some extent, by increasing sequencing depth. Computational methods to 'impute' missing values resulting from data sparsity include model-based methods to distinguish technical and biological zeros and data-smoothing to define and adjust 'similar' cells to one another among other approaches [14]. Imputation can cause inherent biases within a dataset resulting in false positives, and quantification of error remains a key challenge within the field [14]. Other open problems include the development of standardized methods to identify and annotate cell types, as manual cluster annotation can be subjective and inconsistent. Cell atlases [42] are being developed to meet this need, although they are currently limited by low granularity for some tissue samples. Furthermore, *ex vivo* engineered cell products may not always match *in vivo*-derived reference maps.

Proteome

Single-cell proteomics is rapidly developing in concert with single-cell epigenomics and transcriptomics to characterize cell state and function. Flow cytometry has long been the dominant single-cell immune protein assay but has historically been limited by the total number of fluorophores that can be multiplexed (recently, 17–28 colors [43,44]). Two new technologies have significantly expanded the capacity to multiplex flow-based protein detection. The first, time-of-flight mass cytometry (CyTOF) uses an atomic mass cytometer to detect antibodies conjugated to heavy metal isotopes, and can simultaneously detect 40+ immunophenotyping markers [45]. A second technology, spectral flow cytometry, recently emerged as an alternative strategy. While spectral flow relies on fluorophore-conjugated antibodies for protein detection, individual fluorescent spectra can be demultiplexed from one another by capturing the entire emission spectrum rather than single peaks. This allows colors that traditionally overlap to be used together, and 40+ marker panels have been developed for immune cell subsets [46]. Other multiplexed assays to detect low abundance molecules include Abseq, a droplet microfluidics-based tool that relies on DNA barcode-conjugated antibodies to identify specific proteins [47]. While the throughput of a given Abseq experiment (10,000 cells) is lower than flow-based approaches (millions of cells), Abseq can be used to detect more distinct cell barcodes and could, in theory, be used to probe the entire proteome. In a cell manufacturing context, these techniques can be used to monitor cell ID within the engineered product before infusion, especially given recent data identifying correlations between specific protein biomarkers for T cell memory and/or exhaustion, helper T cell subsets, and clinical outcomes [25,26,41]. Such proteomic studies are all limited by the availability of different antibodies and conjugates; hence, these experiments tend to be restricted to expected phenotypes [9].

Metabolome

While protein-level data are critical for defining cell subsets, some cell behaviors can be captured most accurately by assaying for small molecules associated with various metabolic states. Mass spectrometry (MS) and nuclear magnetic resonance (NMR) are two dominant approaches to profile metabolites. Single-cell MS has gained particular popularity in recent years [48]. Analytes are sampled from individual cells via ion-beam or laser, and high levels of spatial resolution can be achieved at the subcellular level with methods, such as 3D MALDI-mass spectrometry imaging (MSI) [49]. These analytes can be profiled for lipids, amino acids,

sugars, and other compounds that inform the specific metabolic programs in use at the time of sampling, providing insights into the dynamic metabolic behavior of engineered immune cell products. For instance, many clinically relevant T cell differentiation states and behaviors have specific metabolic footprints, including activation [50], memory [51,52] and exhaustion [53]. Small molecule metabolomic data can be used to deconvolute these cell fates, particularly given that even within an individual population (e.g., effector T cells or exhausted T cells), significant metabolic and functional heterogeneity may exist that can not be easily identified by protein biomarkers alone [54]. Furthermore, metabolic shifts may actually drive changes in cell state [53]; as such, rapid metabolome feedback could anticipate changes to cell fate before they are detectable with other assays. Finally, unbiased single-cell metabolomic approaches could provide a tool for identifying the presence of rare clonal populations of elevated potency [12] and are useful for establishing the overall metabolic heterogeneity of a given product. Sample preparation can limit the accuracy of single-cell studies, as the removal of a cell from its native environment can disrupt its metabolic profile. This disruption is a particular challenge for microfluidic-based approaches. Furthermore, single-cell metabolic studies are complicated by signal sensitivity, as the input material cannot be amplified easily, in contrast to DNA- and RNA-based omic studies [55].

Antigen receptor repertoire

In the context of immune cell therapies, a deep understanding of the varied effects of the antigen receptor repertoire is critical. This is significant both for treatments that rely on the activity of specific antigen receptors (e.g., adoptive T cell therapies [56]) and for engineered products with integrated synthetic receptors (e.g., CAR T cells). For therapeutics relying on endogenous TCRs, single-cell profiling has the obvious use of defining the repertoire of antigen receptors present within a given cell population. However, even in therapeutics that do not rely on TCRs for tumor targeting, antigen receptor information can provide insights into the functionality of the product. For instance, an open question in the field asks about interactions between endogenous T cell receptors (TCRs) and CARs, particularly the dynamics of memory formation and T cell differentiation triggered by either TCR or CAR-specific antigen stimulation [57,58]. The antigen receptor repertoire also provides a unique tool to understand the fate and behaviors of specific immune cell clones. While tracking cell fate often relies on invasive tools (e.g., genomically-integrated cellular DNA barcodes [59]) to identify parent/daughter cells, the antigen receptor repertoire offers a unique way to identify cells within a population that originate from a parent clone. This can be carried out either with a receptor-specific antibody or through sequencing to identify mRNA transcripts associated with specific TCRs [7]. Satpathy et al. [60] developed T-ATAC-seq to link epigenomic signatures with the TCR repertoire by combining scATAC-seq with single-cell RNA amplification of the *TRA* and *TRB* loci to capture TCR sequences. This technique can currently profile only 96 cells per chip and is therefore limited in its ability to detect rare T cell clones. Further advances in throughput will be needed to fully leverage the potential for this technology to link TCR-based lineage tracing to the epigenome. Commercial solutions have also been developed to capture the antigen repertoire with transcriptomic data by sequencing mRNAs from the 5' end in tandem with TCR-specific primers to amplify those transcripts separately [53]. These technologies offer important advances to probe the effects of varied T cell receptors on cell phenotype and tease apart heterogeneity associated with clonal populations.

Multiomic integration

While the aforementioned techniques focus predominantly on the analysis of a single type of molecule, the field is rapidly moving toward approaches that integrate two or more types of molecular information, providing a more comprehensive and unbiased snapshot of cellular behavior [7]. These techniques include tools to simultaneously study the proteome/transcriptome (e.g., CITE-seq [61], REAP-seq [62], and PLAYR [63]), the transcriptome/epigenome (e.g., scM&Tseq [64] and sciCAR [65]), and the epigenome/antigen receptor repertoire (e.g., T-ATAC-seq [60]). Multiomic approaches afford the capacity to track protein biomarkers for specific immune cell compartments while simultaneously deconvoluting heterogeneity within these 'canonical' subsets. In addition, some key biomarkers include splice isoforms such as CD45RA and CD45RO, which have traditionally been used to distinguish naive, memory, and effector T cell phenotypes. These distinctions are lost in most transcriptomic assays but retained in single-cell protein/RNA workflows, which can inform gene expression heterogeneity that would be lost with protein-only studies. For instance, Corselli et al. [66] identified plasticity in exhaustion and activation phenotypes within *in vitro*-stimulated T cell populations by combining molecular cytometry (Abseq) with targeted RNA profiling, highlighting the potential for combinatorial approaches to classify cell subsets within immune cell products.

Defining and monitoring quality attributes

CQAs are needed to define and standardize characteristics of immune cell products that effectively and consistently predict clinical success [8,67]. This task is quite challenging, given the incredible diversity and plasticity of cell types within a single product, not to mention between patients. Previously, we discussed

mostly destructive methods to measure characteristics of immune cells; however, there is an increasing focus on the development of non-destructive single-cell tools to assay cell behavior. These tools are useful because they (1) are noninvasive and generally label-free and therefore less likely to perturb cell state, (2) can provide spatiotemporal resolution into dynamic processes within individual cells, and (3) leave assayed cells intact. Technologies with these capabilities are positioned to provide insights into cell functionality that can be used to monitor and provide real-time feedback to inform manufacturing.

Many non-destructive methods are well-suited to deep learning approaches to classify cells [68–70], and these data could potentially integrate with insights into CQAs from destructive methods to identify and even select for highly potent cells within the product. For instance, Walsh et al. describe the use of non-destructive optical imaging to classify T cell activation by measuring endogenous autofluorescent properties of two metabolic coenzymes, NADH and FAD, to describe their relative concentrations and binding state within the cell, thereby providing a 'metabolic fingerprint' that can be used to classify T cell activation and memory. Other tools to non-destructively capture metabolic information include single-cell Raman spectroscopy [71,72] and single-cell NMR spectroscopy, which can integrate into a bioreactor design for real-time in-cell NMR [73]. Additional methods for non-destructive single-cell analysis of cell behavior include optics-based tools, such as holographic microscopy [74,75] and optical diffraction tomography [70], which has been applied in the context of CAR T cells to study immunological synapse dynamics. Finally, non-destructive nanostraw intracellular sampling allows for longitudinal cell monitoring at the subcellular level [76]. Together, these tools offer significant promise to transform cell manufacturing paradigms toward real-time, single-cell analyses during manufacture that measure and respond to cellular heterogeneity at unprecedented levels.

To fully realize the potential of single-cell datasets to define biologically and clinically relevant CQAs, machine learning approaches have gained ground as tools to identify relevant signals for potency, toxicity, and other relevant cell behaviors from massive omics datasets. Single-cell omics datasets are ideally suited to machine learning approaches for CQA discovery, given their high dimensionality. Such tools require effective training datasets, such as tissue samples from patients who did or did not successfully respond to therapy, or batches of cells with known *in vitro* measures of potency and other relevant phenotypes. These tools can subsequently be used to identify features of significance that predict desirable cell states, with the goal of defining manufacturing CQAs that can predict therapeutic success. There is potential also to apply machine learning approaches to electronic health records to further link cell quality to single-cell molecular profiles, and ultimately, patient outcomes [77].

Monitoring genome modifications

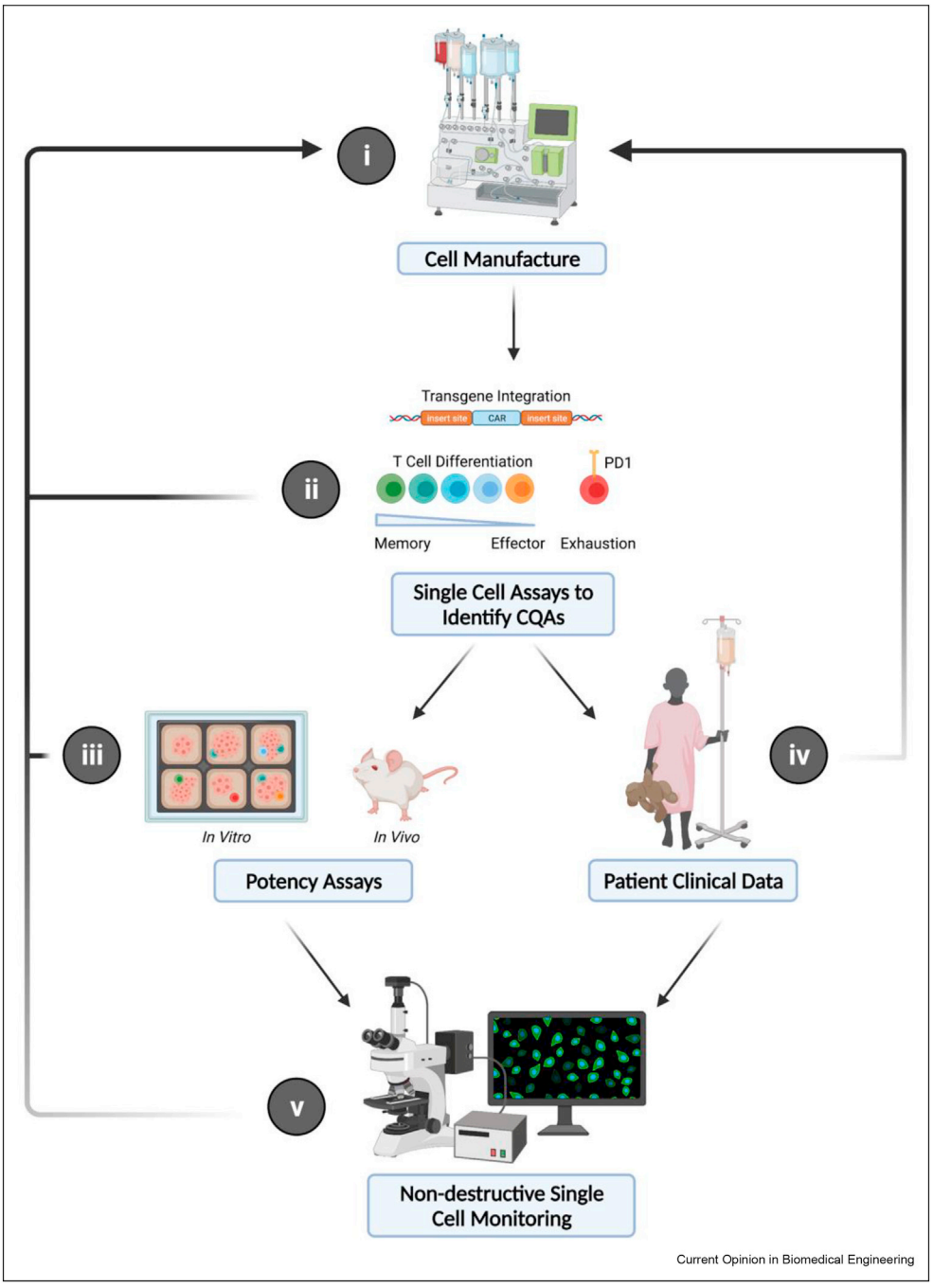
Gene transfer itself is a source of heterogeneity, as all methods are inefficient or imprecise to some extent. Genome modifications may be targeted, as in precise CRISPR/Cas9 engineering strategies [18,78], or untargeted, as is the case for most virally-transduced cells with integrated CAR transgenes [79]. A key consideration for any single-cell workflow involving a gene-edited product is the ability to identify said edits within individual cells, to (1) distinguish edited versus unedited cells and (2) classify the edit sequence and locus. Virally-manufactured CAR T cells can have significant heterogeneity in CAR copy number and CAR integration sites, two features that affect the functionality of individual cells and which should be monitored and controlled where possible. Studies have already indicated the potential for CRISPR-edited CAR T cells to achieve elevated potency [80]; as these therapeutics enter the clinic [18], we expect to see a focus on profiling and characterizing the dynamic effects of gene edits on the product. These considerations are also highly relevant in the context of allogeneic cell-based therapies, which generally require disruption of the *TRAC* and *B2M* loci to avoid graft-versus-host disease [81]. To verify the safety and purity of gene-edited allogeneic products, selection and monitoring strategies are required to confirm that all cells within the product have the desired edit. Single-cell methods could greatly augment these workflows.

To date, although new methods to link genotypes to transcriptional phenotypes are gaining speed, strategies for tracking CRISPR and other gene-edits within single-cell datasets are nascent [82]. In the case of integrated transgenes with adequate sequence diversity relative to the human genome, gene-edited cells can be tracked by aligning scRNA-seq sequence reads to a custom reference genome featuring an added sequence for the transgene of interest [83]. Other notable methods to link genotype to phenotype include Perturb-seq [84,85], CRISP-seq [86], and CROP-seq [87] to identify cells featuring specific guide RNAs. These methods are limited in a clinical context as they (a) require lentiviral integration of barcoded guide RNAs to identify gene-edited cells and (b) do not provide sequence-specific information about mutations. We expect significant development in this area, as gene-editing workflows integrate with multiomic single-cell strategies to analyze gene-edited cell products.

Human clinical trial data

The end goal of many studies in this field is to inform clinical treatment. Datasets from clinical studies emerging over the last decade highlight the potential

Figure 2



Integrating multiomic and non-destructive single-cell assays to identify critical quality attributes and predict the potency of engineered immune cell therapies. (i) Engineered immune cell products are heterogeneous and reflect dynamic and plastic processes that determine cell behavior. (ii) Single-cell omics-based assays are used to identify the breadth of molecular heterogeneity within engineered cell products, which could inform clinically relevant cell states. (iii) *In vitro* and *in vivo* potency assays provide pre-clinical potency indicators that can be linked to single-cell omics datasets to predict signatures associated with potency. (iv) Clinical data can be linked with molecular information captured from the infusion product and from patient samples collected during and after treatment to establish signatures associated with positive and negative patient outcomes. (v) Single-cell signatures captured by label-free, non-destructive assays can be used to predict the potency of individual cells within a product and could integrate into biomanufacturing workflows for real-time, in-line sensing of dynamic cellular processes.

for single-cell technologies to enhance the predictive potential for individual therapeutics. For instance, Bai et al. [41] studied the infusion products of 12 pediatric patients with leukemia upon CAR antigen-specific stimulation *in vitro* and demonstrated heterogeneity in activation and T-cell memory subsets that predicted

patient outcomes. In another study, Li et al. [88] leveraged scRNA-seq to evaluate CAR T cells from a patient with primary plasma cell leukemia from both the pre-infusion product and *in vivo* samples captured at the peak and remission phases of treatment, demonstrating distinct cell states for each phase. In a third approach, Parker et al. [89] leveraged scRNA-seq datasets from the human brain to identify a population of mural cells that express CD19, the target antigen for several patients who experienced severe or fatal neurotoxicity because of cerebral edema after therapy. Mural cells surround the endothelium and protect the blood–brain barrier; thus, we see an example of how scRNA-seq can be used to pinpoint the mechanism by which treatment likely caused an adverse response. These and other examples highlight the potential for single-cell approaches to tackle challenges associated with cellular heterogeneity within both product and patient to better understand the dynamics of remission and relapse.

Outlook

In addition to the recent expansion of methods to profile various classes of biomolecules at the single-cell level, there has been a similar explosion of analysis techniques to accompany these datasets. The sheer scope of new analytical techniques requires parallel efforts to systematically benchmark these methods against one another to determine their relative use and limitations. Such methods require baseline datasets with clear, validated qualities — for instance, defined cell types and expected genomic composition, which can produce a chicken-and-egg problem. Several such datasets have been generated by mixing known and well-characterized cell lines at specific ratios with spike-in controls to simulate biological noiset [90]. Further efforts in this area could provide additional metrics to measure the quality of various analytical tools, standardize data QC pipelines, annotate cell types, and integrate data across multiple experiments, among other tasks. *In silico* simulations can also be applied to benchmark data analysis tools using defined, ground-truth model datasets [14]. Benchmarking efforts could assist with the challenge of integrating data across multiomic efforts, which are currently mostly limited to two classes of biomolecules at a time. New methods will be needed to combine three or more classes of biological information for individual cells.

In sum, various technologies have now been developed to profile the cellular and molecular heterogeneity of single cells. Important considerations for their use in cell manufacturing contexts include defining CQAs for cell potency, tracking gene edits within engineered cell populations, and informing clinical applications (Figure 2). We expect a trend toward the use of single-cell methods to identify and even select for highly potent cells within products, perhaps ultimately allowing smaller and safer dosages of cells to achieve equal or better patient outcomes. These methods may reveal new engineering targets and strategies to drive cells toward desirable states before infusion (e.g., central memory T cells with increased persistence *in vivo*). While routine use of single-cell technologies will need to balance with high costs in an already expensive treatment paradigm [91], we expect the field to move in this direction as assay costs decrease and predictive insights for specific patient outcomes improve. Many methods may ultimately integrate directly into cell manufacturing workflows through in-line sensing, as their use becomes standard.

Declaration of competing interest

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