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Touch-free optical technologies to streamline the production of T cell therapies

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Abstract

Currently approved adoptive T cell therapy relies on autologous (obtained from the same patient) T cells, which often suffer from poor quality that diminishes treatment efficacy. Due to the heterogeneous nature of T cell quality between and within patients, significant efforts are aimed at optimizing cell manipulation and growth conditions for potent T cell products. We believe that touch-free imaging and sensing technologies are critical to monitor single-cell features during T cell manufacturing to ensure consistent and optimally timed methods for cell manipulation and growth. Here, we discuss emerging label-free optical imaging and sensing methods, along with machine learning techniques that could enable in-line feedback to optimize T cell quality at multiple stages during manufacturing. These methods have the potential to streamline the current workflow, accelerate the manufacture of safe high-quality T cell therapies, and improve our understanding of the dynamic, heterogeneous processes of T cell manufacturing.

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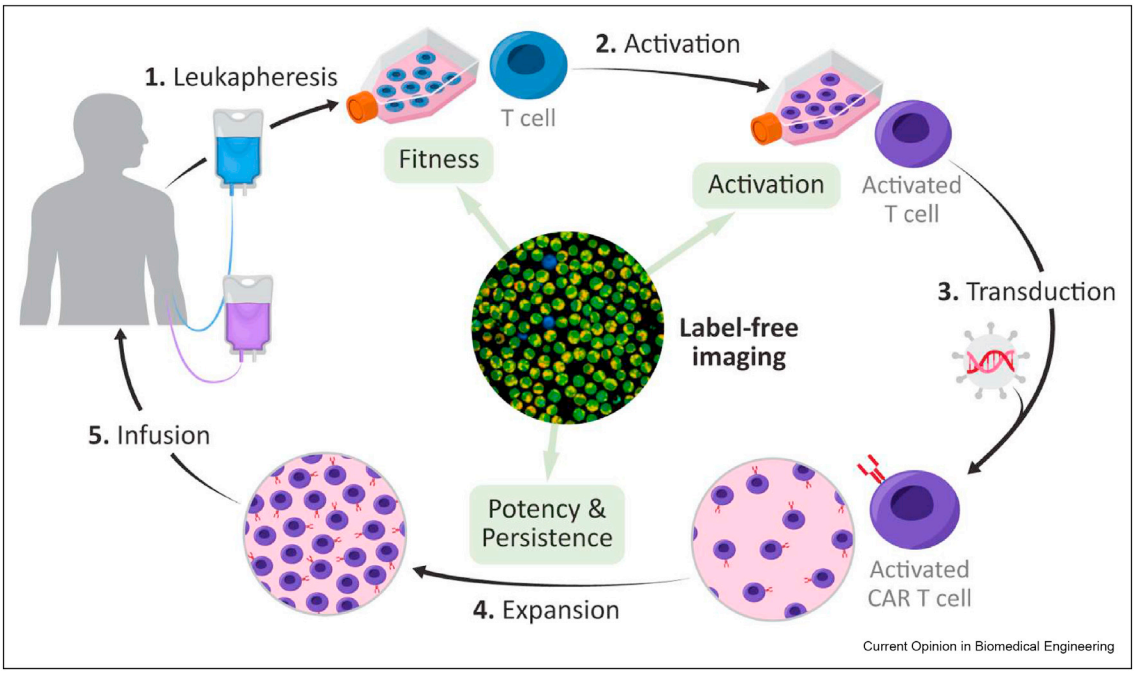
Introduction

With the ability to induce antigen-specific cytotoxicity and to generate memory to prevent tumor relapse, T cells have become a central target of cancer immunotherapies. T cell manufacturing is an exciting alternative to traditional molecular therapies with six products approved for clinical use and hundreds in clinical trials for a wide range of diseases, with decades-long remission in cancer patients having been reported [1,2]. T cell manufacturing encompasses several classes of therapy, including chimeric antigen receptor (CAR), synthetic T cell receptor (TCR), and tumor infiltrating T lymphocytes (TILs) [3]. Engagement of T cell receptor or CAR receptor with tumor antigen leads to T cell activation, which initiates a signaling cascade for T cell proliferation, effector differentiation and cytokine secretion. Activated T cells mediate specific tumor killing and determine treatment efficacy. Currently, T cell manufacturing requires a lengthy and labor-intensive *in vitro* manipulation and expansion process that typically requires weeks [4,5]. Unfortunately, due to the autologous (cells obtained from the same patient) nature of currently approved manufactured T cell products and lack of technologies for high-throughput real-time single-cell monitoring, this manufacturing process is plagued by poor cell quality and heterogeneous cell function even within a patient [6].

For example, the current workflow for CAR T cell manufacturing (Figure 1) begins with T cell collection from patients (leukapheresis), followed by T cell activation and gene transfer, then expansion to reach $\sim 10^8$ T cells for infusion [7]. These engineered T cells then undergo release testing, which checks for the presence of CAR T cells ($\geq$5%) and lack of contamination [8]. During the manufacturing process, T cells are subject to environmental cues and extended *in vitro* culture, which often leads to exhaustion or terminal differentiation that compromises treatment efficacy and persistence [9]. Specialized manufacturing systems for adoptive T cell therapies have recently emerged, such as Miltenyi CliniMACs Prodigy, but current manufacturing of FDA approved products still heavily rely on manual labor and bioreactors adopted from the biologics industry. However, the requirements for manufacturing integrity are inherently different between T cell and biologics production.

Since T cell manufacturing relies on living T cells as the therapeutic agent, it requires a complex and rigorous manufacturing process with significant heterogeneity within and between the end products. Currently, approved T cell products require autologous cell sources so input materials vary for each manufacturing batch, resulting in unique manufacturing conditions for each patient. This causes highly variable end products and unpredictable therapeutic efficacy [10]. Without appropriate technology to monitor the dynamics and

Figure 1



Opportunities for label-free optical imaging and sensing of the chimeric antigen receptor (CAR) T cell manufacturing process. Label-free optical imaging can assess patient T cells for fitness before entering the manufacturing process, identify T cell activation efficiency, and assess T cell function to predict potency and persistence in the patient prior to release.

heterogeneity within and among batches, the current T cell manufacturing process faces an increased rate of batch failure, while also requiring specialized facilities and highly skilled labor. Additionally, the lengthy manufacturing process expose patients to high risks of disease progression while waiting for their T cell products, which, up to 15% of the time, do not pass release criteria [11,12]. Together, these manufacturing challenges significantly increase treatment costs, limit hospital treatment capacity, and compromise patient outcomes, all of which limit the full potential of manufactured T cell products.

T cell manufacturing would greatly benefit from touch-free technologies that provide real-time measurements to inform process interventions that improve batch quality and consistency (Figure 1). Non-invasive and rapid analytical measurements could reduce labor, decrease the risk of contamination, decrease batch failure rates, and reduce manufacturing cost. Here, we discuss label-free optical imaging and sensing methods including two-photon endogenous fluorescence microscopy, Raman spectroscopy, and quantitative phase microscopy along with machine learning methods to streamline T cell manufacturing. Label-free optical imaging and sensing methods are attractive for monitoring T cell manufacturing because they enable rapid, frequent, and non-invasive assessments of cell function and potential culture contamination. Machine learning decisions based on label-free assessments could be used to adapt manufacturing processes in real-time for improved end products and provide go-no-go decisions. Overall, this adaptive workflow could streamline the manufacturing process and ultimately improve patient outcomes for an array of manufactured T cells under development for numerous diseases.

Current methods to monitor T cell manufacturing

In-line bulk measurements

Bioreactor systems that were originally developed for biologics manufacturing have been adopted for clinical-scale T cell expansion. Sensors to monitor environmental cues such as pH, dissolved oxygen, and temperature have been integrated into these bioreactors (in-line, or real-time measurements within the bioreactor) because these cues are known to affect T cell growth, viability, and differentiation into distinct phenotypes [9,13]. Additionally, in-line optical probes measure media turbidity as an indirect measure of total cell density (TCD) while permittivity-based sensors assess viable cell density (VCD) via intracellular biocapacitance mass [14,15]. While TCD and VCD reflect culture growth throughout the manufacturing process, these readouts are subjected to confounding factors. For example, the presence of viral vectors or contamination increases media turbidity and cell size increases during T cell activation raise total bio-

capacitance mass, affecting TCD and VCD measurements, respectively. Additionally, cell viability is not a sufficient quality attribute as recent clinical data for an approved CAR T therapy (Tisagenlecleucel) showed no significant correlation between batch viability and clinical outcome [16]. Overall, these in-line bulk sensors offer a non-invasive, real-time readout of the current manufacturing state and have been integrated into feedback control loops, enabling automated interventions to attempt to maintain consistent culture conditions [17]. However, they do not offer direct measurements of cell function and product performance (Table 1). T cell manufacturing is an intricate process that relies on a heterogeneous cell population to achieve therapeutic safety, quality, and efficacy. This emphasizes the importance of measuring cell characteristics beyond environmental cues and cell viability.

At-line and off-line sub-sampling measurements

Multiple methods exist to evaluate the secretome and nutrients present in the media that can be used to provide insight into the functional profile of T cells in culture at-line (sample is removed for testing near the bioreactor) or off-line (sample is removed for testing in a separate laboratory) [18]. Cytokine profiles have been associated with disease manifestation and T cell phenotypes while metabolite assessment can monitor active metabolic pathways that regulate cell function.

Table 1

Advantages and disadvantages of current and emerging optical methods to monitor T cell manufacturing.

Technique	Advantages	Disadvantages
Current methods		
Environmental factors	+ Nondestructive/label-free + Metabolic information + Real-time	- Non-direct measure - Lacks single-cell resolution - Doesn't capture heterogeneity - Optimized per desired cell type
Total cell density Viable cell density	+ Nondestructive/label-free + Real-time	- Lacks single-cell resolution - Doesn't capture heterogeneity - Confounding factors - Non-direct measure
Secretome analysis	+ Nondestructive/label-free + Metabolic information	- Lacks single-cell resolution - Doesn't capture heterogeneity - Non-direct measure - Off-line measurement
Flow cytometry	+ High throughput + Rapid + Single cell resolution	- Disruptive - Off-line measurement
Label-free optical technologies in development		
TPEF	+ High sensitivity + Nondestructive/label-free + Single-cell resolution	- Expensive - Requires skilled user
FLIM	+ High sensitivity + Nondestructive/label-free + Metabolic information + Single-cell resolution	- Expensive - Requires skilled user - Slow/low throughput
THG	+ High sensitivity + Nondestructive/label-free + Single-cell resolution	- Expensive - Requires skilled user
Raman spectroscopy (bulk analysis)	+ Biochemical information + High throughput	- Specialized equipment - Complicated analysis - Lacks single-cell resolution
Raman spectroscopy (single-cell analysis)	+ Biochemical information + Single-cell resolution	- Specialized equipment - Complicated analysis - Slow/low throughput
ATR-FTIR	+ Biochemical information + High throughput	- Specialized equipment - Complicated analysis - Lacks single-cell resolution
Low-cost methods (quantitative phase microscopy)	+ Inexpensive + Nondestructive/label-free + Readily available	- Lacks metabolic/biochemical information

The most common method to measure secreted cytokine is enzyme-linked immunosorbent assay (ELISA), which provides information on cytokine levels within the media. Alternatively, mass spectrometry measures key metabolites and nutrients secreted by T cells [19]. While several at-line media analyzers with automatic sampling have been commercialized (*e.g.*, Roche Cedex Bio), these media evaluation methods do not assess the heterogeneity of different cell populations and their relative frequency within the product. Finally, the most common off-line single-cell analysis technology in T cell manufacturing is flow cytometry, which measures the expression of surface proteins that have been well characterized for cell health and phenotypes [10,20,21]. Currently, flow cytometry and other single-cell assays require sampling cells from bioreactors for off-line analysis, which introduces contamination risk and limits the frequency of measurements. These assays also involve destructive sample preparation and are highly complexed with time-consuming data acquisition and analysis. Therefore, despite providing important information on T cell function for predictions of therapeutic potency and persistence, they cannot be readily integrated into the manufacturing workflow (Table 1).

Label-free optical technologies in development to monitor T cell manufacturing

Endogenous nonlinear microscopy

Effective T cell manufacturing requires T cell activation and propagation of distinct functional T cell subsets [22,23]. Current methods to quantify T cell function and activation require antibody labeling or cytokine secretion measurements from the media as described above. These methods do not allow for label-free single-cell monitoring of T cell function so that the tested product can remain intact. Current methods also prevent repeated measurements of single-cell T cell function over time within intact, unlabeled samples.

T cells undergo dramatic metabolic changes with activation and exhaustion, switching from primarily oxidative phosphorylation in naïve cells to glycolysis in activated cells to fatty acid oxidation with exhaustion [24,25]. This metabolic shift requires the regulation of key metabolic coenzymes including the reduced form of nicotinamide adenine dinucleotide (phosphate) (NAD(P)H) and oxidized flavin adenine dinucleotide (FAD), which are autofluorescent [26–28]. Two-photon endogenous fluorescence (TPEF) imaging of these co-factors can quantify the optical redox ratio (ORR), frequently defined as the ratio of NAD(P)H to FAD fluorescence intensity. Additionally, NAD(P)H and FAD fluorescence lifetimes are distinct in their free and protein-bound conformations, and can provide information on protein-binding activities, preferred protein-binding partners, and other environmental factors like pH and oxygen, which cannot be quantified using fluorescence intensity alone [28–31]. Previously, fluorescence lifetime imaging microscopy (FLIM) has been used to separate activated and quiescent T cells [32–34].

Beyond endogenous fluorescence, nonlinear microscopy can also be used for third harmonic generation (THG) microscopy, which is a label-free method to detect lipid bodies within cells. Prior studies have used THG microscopy to detect increases in lipid bodies with T cell activation at the single cell level [35]. While these label-free endogenous fluorescence methods provide high sensitivity at the single cell level to enable characterization of heterogeneous populations, they are expensive and are not currently high throughput (Table 1). Further development of these technologies is needed to create sensitive, high-throughput systems for automated in-line analysis of single cells during T cell manufacturing.

Optical spectroscopy

As T cells become activated, they produce biochemical products or signatures. Raman spectroscopy is a label-free vibrational spectroscopy-based technique that assesses naturally generated inelastically scattered light with wavelength shifts that are specific to chemical bonds. This Raman signature can provide single cell information based on the molecular species present in the sample, including lipids and nucleic acids, while minimizing phototoxicity [36,37]. Vibrational spectroscopy methods such as Raman have been used to identify dynamic chemical signatures, such as cytochrome C levels and lipid levels, in individual T cells that change with activation [38–40]. Beyond single-cell spectra, bulk in-line Raman spectroscopy sensors, together with near infrared, dielectric, and fluorescence spectroscopies, have been developed to characterize media composition and cytokines during T cell expansion [38,41,42]. Bulk in-line fluorescence spectroscopy sensors can measure metabolites in cell media such as tryptophan, tyrosine, riboflavin, and NAD(P)H via their autofluorescence properties. Several of these systems have been validated against known samples, which showed moderate to strong correlation with standard off-line assays such as high-performance liquid chromatography (HPLC) [43–45]. Another spectroscopic method, attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR), has also detected differences in absorbance spectra with T cell activation [46]. The spectroscopic techniques discussed here provide specific biochemical information that can be used to discriminate T cell function, however when used at the single cell level they require highly specialized equipment and complicated analysis, while the bulk sensors lack single-cell resolution (Table 1). Additionally, further development of simplified analysis strategies is needed for future adoption of these technologies in T cell manufacturing (Table 1).

Low-cost label-free optical imaging methods

Several other label-free optical imaging methods can monitor T cell manufacturing, primarily morphological changes with T cell activation. For example, transmitted light and quantitative phase microscopy (QPM) capture cell morphology using inexpensive and widely accessible hardware [47–51]. These methods can be used to monitor cellular organization and density, which change throughout the T cell manufacturing process [48,52]. Although these technologies are low-cost and widely available, they do not provide the specific biochemical information available from two-photon endogenous fluorescence microscopy and Raman spectroscopy, limiting their sensitivity for complex and subtle changes in T cell function (Table 1).

Machine learning methods to classify label-free optical images

New label-free optical imaging techniques produce large multi-dimensional datasets, so computational image segmentation and classification methods are needed to integrate these technologies into automated T cell manufacturing workflows. Machine learning methods can automatically quantify in-line measurements of cell features (*e.g.*, cell size, autofluorescent signals, spectroscopic signatures), then use these features to generate real-time decisions for interventions (*e.g.*, media conditions, cell stimuli) in a feedback manner. The overall goal of this feedback process is to maintain consistently high-quality T cell products. Additionally, machine learning decisions could reduce the subjectivity of go-no-go decisions that are currently performed by trained technicians. Machine learning algorithms can be broadly classified as either supervised or unsupervised learning methods. Supervised learning methods require user input and training to identify a user-defined output, and are based on models including decision trees, neural networks, and regression. Unsupervised learning methods rely only on traits of the measured signal and do not require user input. These unsupervised methods, primarily clustering and component analysis, are promising for T cell manufacturing because they can become user-independent and can identify underlying patterns in the imaging data. Overall, machine learning tools can be optimized to extract information from images or other data types acquired during the T cell manufacturing process to make decisions on the quality of the sample, or interventions to optimize culture conditions. These tools are complementary to label-free imaging and sensing methods as they would allow for single-cell classification and assessment but are also broadly applicable to standard in-line measurements of culture conditions.

Recently, machine learning techniques have been used to predict T cell activation from multiple label-free imaging modalities. Morphological and structural variables that shift throughout T cell activation, including total area and sphericity, can be extracted from label-free images acquired by QPM and TPEF and used to build classifiers [49,52,53]. Additionally, several groups have trained classifiers to separate activated and quiescent T cells based on FLIM alone [32,34,53,54]. Clustering algorithms have also been used to optimize segmentation and classify T cell states with image-based textural and morphological variables acquired from image-based cytometry [53,55,56]. These machine learning techniques have great potential to streamline the T cell manufacturing workflow by monitoring cell state at the single cell level and identifying time-points for media conditioning to improve the consistency, potency, and safety of the manufactured T cell products. As with any machine learning approach, significant amounts of user-annotated data are needed to build and validate decisions, and this process must be comprehensive across manufacturing conditions (*e.g.*, cell types, manipulations, culture conditions, possible interventions) before these algorithms can be reliably implemented in the T cell manufacturing workflow.

Future applications and conclusions

Technologies that provide real-time measurements to inform timely interventions could greatly improve batch quality and consistency of manufactured T cell products. Currently approved T cell products rely on autologous cell sources that have variable quality depending on the patient, so consistently potent products are difficult to generate without an adaptive manufacturing process. In fact, recent clinical data for an approved CAR T therapy (Tisagenlecleucel) showed no significance difference in clinical outcomes for CAR T batches with viability out-of-specification when compared to batches that passed standard release criteria [16]. This emphasizes the need for an adaptive manufacturing process capable of acquiring precise measurements of cell features in real-time with in-line feedback capabilities. This approach would decrease cost, increase hospital treatment capacity, and improve patient outcomes by unlocking the full potential of manufactured T cell products. This real-time in-line monitoring and intervention approach would also provide fundamental insights to improve T cell manufacturing for the >1000 therapies currently in the pipeline [57].

Label-free optical imaging and sensing coupled with machine learning techniques could fill this need for in-line T cell monitoring and feedback to non-invasively optimize the quality of T cells at multiple stages during manufacturing. For example, initial patient T cells could be assessed for fitness before entering the manufacturing process, T cell activation efficiency and proliferation could be optimized, and final T cells products could be assessed for potency and persistence prior to release (Figure 1). Label-free optical technologies and machine learning can be used at each step in

this process, and the choice of measurement technique and predictive algorithm can be determined based on application needs. This approach has the potential to streamline current practice, accelerate the manufacture of safe, high-quality manufactured T cell products, and improve our understanding of the dynamic, heterogeneous processes of T cell manufacturing.

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Given the role as a Guest Editor, Melissa Skala had no involvement in the peer-review of this article and has no access to information regarding its peer-review. Full responsibility for the editorial process for this article was delegated to the editor Irene Georgakoudi.

Declaration of competing interest

The authors declare the following financial interests/personal relationships that may be considered as potential competing interests: Melissa Skala reports financial support was provided by National Science Foundation. Melissa Skala has patent issued to Wisconsin Alumni Research Foundation. Melissa Skala has patent pending to Wisconsin Alumni Research Foundation. Guest editor for this special issue of COBME (MCS).

Data availability

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