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Label-free optical imaging and sensing for quality control of stem cell manufacturing

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Abstract

Human stem cells provide emerging methods for drug screening, disease modeling, and personalized patient therapies. To meet this growing demand for scale-up, stem cell manufacturing methods must be streamlined with continuous monitoring technologies and automated feedback to optimize growth conditions for high production and consistency. Label-free optical imaging and sensing, including multiphoton microscopy, Raman spectroscopy, and low-cost methods such as phase and transmitted light microscopy, can provide rapid, repeatable, and non-invasive monitoring of stem cells throughout cell differentiation and maturation. Machine learning algorithms trained on label-free optical imaging and sensing features could identify viable cells and predict optimal manufacturing conditions. These techniques have the potential to streamline stem cell manufacturing and accelerate their use in regenerative medicine.

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Keywords

Stem cell, Cell manufacturing, Label-free imaging, Optical microscopy, Raman spectroscopy, Machine learning.

Abbreviations

CNN, convolutional neural network; DIC, differential interference contrast; FAD, flavin adenine dinucleotide; FLIM, fluorescence lifetime imaging microscopy; hPSC, human pluripotent stem cell; HSC, hematopoietic stem cell; HSpec, hyperspectral imaging; MSC, mesenchymal stem cell; NAD(P)H, nicotinamide adenine dinucleotide (phosphate); NSC, neural stem cell; OCT, optical coherence tomography; ORR, optical redox ratio; QPM, quantitative phase microscopy; SVM, support-vector machine; TPEF, two photon excited fluorescence; UMAP, uniform manifold approximation and projection.

Introduction

Human stem cells hold great promise for drug screening, disease modeling, and regenerative medicine, with the ability to self-proliferate and differentiate into various cell types [1]. Key areas of research interest include generating more accurate and higher throughput disease models and more realistic platforms for drug discovery and toxicology. These studies have focused on both pluripotent (hPSCs) and adult stem cells, which include a variety of multipotent and self-renewing types found in specific niches, such as mesenchymal (MSC), neural (NSC), and hematopoietic stem cells (HSC) [2]. The goal of stem cell manufacturing is personalized medicine using patient-matched samples that better capture heterogeneity in human disease and can eventually replace lost or damaged tissue in non-regenerative organs such as the brain, heart, and eyes [3,4].

Currently, stem cell manufacturing is relatively small scale for use in basic laboratory and pre-clinical settings [2]. However, future applications in large-scale drug discovery and regenerative medicine will require robust cell manufacturing technologies to ensure large, standardized cell batches. Efficient differentiation of stem cells and maturation of differentiated cell lineages are typically bottlenecks in the cell manufacturing process [3,4]. Batch-to-batch and line-to-line variability in stem cell differentiation hinders basic science and clinical applications [4]. While considerable improvements in large-scale culture systems have been made [2], stem cell differentiation outcome is frequently verified using techniques such as immunofluorescence, quantitative polymerase chain reaction (qPCR), and flow cytometry, which are disruptive, low throughput, labor intensive, and not conducted in real time (Table 1) [5,6]. To maximize the potential of efficient differentiation and maturation, and therefore use in further laboratory and clinical settings, label-free monitoring of stem cells is required.

Here, we discuss how label-free optical imaging and sensing, including multiphoton metabolic imaging [7–10], hyperspectral imaging [11–13], and Raman spectroscopy [14–19] can be coupled with machine learning [9,20–23] to streamline quality control of stem cell manufacturing. Label-free optical technologies could provide rapid, repeated, and non-invasive screening during the stem cell manufacturing process. Machine

Table 1

Advantages and disadvantages of current techniques and emerging optical technologies for monitoring stem cell manufacturing.

Technique	Advantages	Disadvantages
Traditional (gold standard) methods [5,6]		
Flow cytometry	+High throughput +Single cell	-Invasive and/or destructive -Labor intensive
Immunofluorescence	+Highly specific	-Slow/low throughput -Labor intensive -Requires skilled user
Quantitative polymerase chain reaction (qPCR)	+Highly specific +Rapid	-Destructive
Label-free optical imaging & Sensing		
Two photon excited fluorescence [7,10]	+High sensitivity +Nondestructive/label-free +Single cell resolution	-Expensive -Requires skilled user
Fluorescence lifetime imaging [7,9,11,38,39]	+High sensitivity +Nondestructive/label-free +Single cell resolution +Metabolic information	-Expensive -Requires skilled user -Slow/low throughput
Hyperspectral imaging [11–13]	+High sensitivity +Non-destructive/label-free +Single cell resolution	-Specialized equipment -Large datasets
Raman spectroscopy [14–19]	+Biochemical information +Rapid	-Specialized equipment -Lacks single cell resolution
Low-cost methods (**phase** [21,22], **transmitted light** [41], **differential contrast** [42])	+Inexpensive +Nondestructive/label-free +Easily integrated into widely used microscopes	-Lacks metabolic/biochemical information

learning and classification algorithms trained on label-free optical features of cells could predict optimal manufacturing conditions and identify which cells are functionally viable for benchtop or clinical needs. We describe the advantages of these label-free optical imaging and sensing methods for streamlining the stem cell manufacturing workflow (Figure 1).

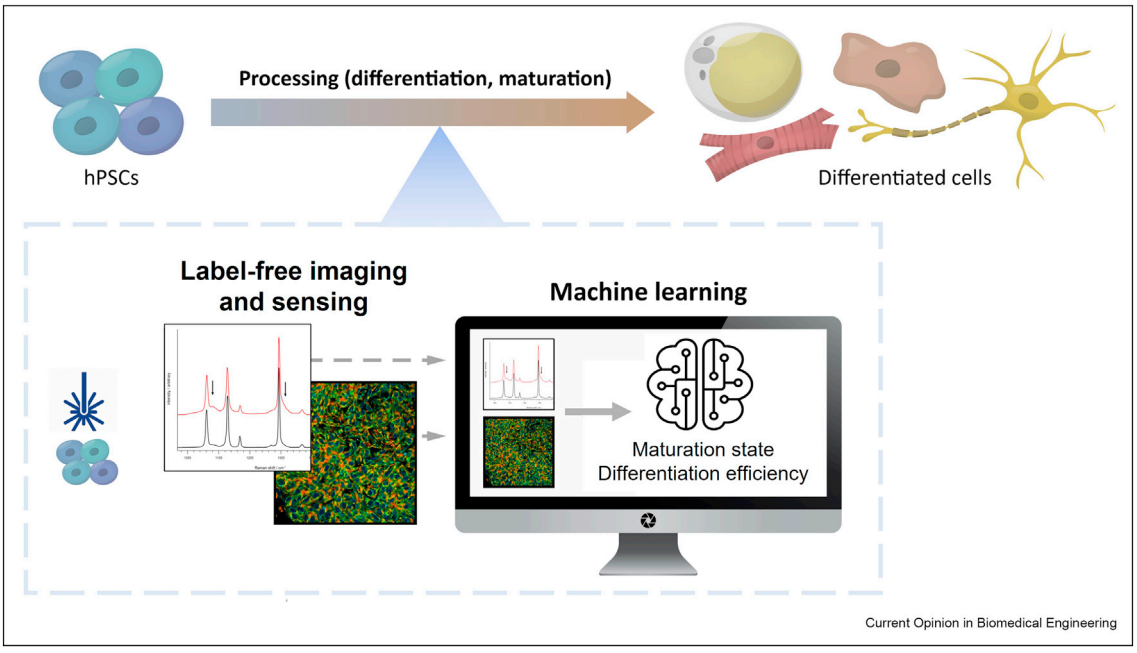
Label-free optical imaging and sensing to monitor stem cell differentiation and maturation of differentiated cells

Multiphoton microscopy

As stem cells differentiate and the resulting cell types mature, they undergo dramatic shifts in structural organization, morphology, and metabolism [6,24]. Therefore, metabolite sensing of culture medium (*e.g.*, mass spectrometry, bioluminescent assays) is currently under development to monitor stem cell manufacturing [25,26]. These and other methods (*e.g.*, immunofluorescence, flow cytometry) are biochemically specific but time-consuming and invasive [27,28]. Alternatively, label-free optical imaging and sensing are advantageous for stem cell manufacturing because these technologies provide rapid, touch-free monitoring of cell structure and function using endogenous sources of light and contrast. Various label-free, touch-free, and long-term optical monitoring methods can be used to capture structural and functional changes throughout stem cell differentiation and subsequent cell maturation, providing a comprehensive picture of development and favored growth conditions (Figure 1).

Cell stemness is regulated in part by metabolism, with energy dependence shifting from glycolysis to oxidative phosphorylation as differentiation progresses [24,29,30]. Structural and metabolic fluctuations vary temporally, by cell type, and heterogeneously between cells within the same culture, so there is a need for nondestructive, long-term observational methods that provide functional readouts of these changes on a single cell level. The general shift from glycolysis to oxidative phosphorylation 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 intrinsically fluorescent [31–33]. Two-photon endogenous fluorescence (TPEF) imaging of these cofactors can be used to quantify the optical redox ratio (ORR), frequently defined as the ratio of NAD(P)H to FAD fluorescence intensity. ORR has been used to identify metabolic changes in various stem cell lineages, including mesenchymal stem cells (MSC) differentiating into adipocytes [8,13,34], chondrocytes [7], osteocytes [7,10], and (epi)dermal cells [35]. Additionally, NAD(P)H and FAD lifetimes are distinct in their free and protein-bound conformations, and can provide

Figure 1



Label-free optical imaging and sensing can monitor stem cell differentiation and maturation. Machine learning algorithms trained on optical imaging and sensing data can identify viable cells to predict optimal culture conditions and streamline biomanufacturing.

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 [33,36,37]. Fluorescence lifetime imaging microscopy (FLIM) has been used to separate hPSC-derived cardiomyocytes into high- and low-differentiation efficiency groups within the first 24 h of differentiation [9], distinguish undifferentiated MSCs from osteogenic cells [38], separate neurons from neural progenitor cells [39], and monitor differentiation and long-term maturation in hPSC-derived retinal organoids [11,12]. In addition to cellular morphological features such as cell alignment, short/long axis ratio, and surface area, high-resolution multiphoton imaging also allows for more detailed assessment of subcellular structures. For example, NAD(P)H autofluorescence can be used to analyze mitochondrial organization and elongation during adipocyte differentiation [34] while lysosomal (lipofuscin) autofluorescence changes with oxygen concentration in MSCs, indicating oxidative stress [10].

Hyperspectral imaging (HSpec) can be used with TPEF to capture both spatial and broad spectral information from cellular autofluorescence. The spectral signature can provide specific chemical information resulting from light scattering and absorption that shift with molecular and structural changes during stem cell differentiation. HSpec involves excitation at a single wavelength and detection over multiple channels in the emission range; spectral information can then be extracted from each pixel in the image [40]. Phasor-based FLIM and HSpec have been used together to detect retinol in self-organizing stem cell-derived retinal organoids [11,12]. Retinol is produced in the visual cycle of mature photoreceptors, and HSpec imaging of this fluorophore provides a functional readout of differentiation within these organoids. Depending on the molecule of interest, HSpec may be used to distinguish early functional markers such as small lipid droplets present in adipocytes by day 3 of differentiation [13]. Multiphoton microscopy, including FLIM, HSpec, and TPEF, presents several advantages over traditional fluorescence microscopy including the use of infrared excitation for deeper tissue penetration and reduced photodamage as well as improved optical sectioning and high resolution. The specificity and environmental sensitivity of these techniques as well as their potential for single-cell resolution presents a unique ability to quantify dynamic cell-level heterogeneities within intact, unperturbed cultures compared to existing standards that cannot resolve individual cells or use destructive techniques that cannot monitor this heterogeneous response within the same population over time, making them ideal for extended

monitoring of metabolic and structural changes during cell maturation [11,12]. However, multiphoton systems are large, expensive, and complex, limiting their use in routine biomanufacturing (Table 1).

Raman spectroscopy

As stem cells differentiate and the resulting lineages mature, specific cell types will produce biochemical products or signatures. Raman spectroscopy assesses light interactions with these chemical bonds and can produce detailed characteristic signatures based on the molecular species present in the sample including lipids [13,19], bone minerals [14,15,17], and nucleic acids [18,19]. For example, analysis of Raman spectra has been used to identify HSCs, which exhibit more distinct nucleic acid signatures and less distinct protein and lipid-related signatures relative to their differentiated linages including B cells and granulocytes [18]. As osteoblasts differentiate from MSCs, they develop mineralized matrices containing hydroxyapatite, also reflected by Raman spectral changes [14,15,17]. Similarly, differentiated adipocytes exhibit dramatically larger lipid peaks compared to adipose-derived stem cells [16] while developed neurons exhibit reduced nucleic acid and lipid with increased protein signatures relative to NSCs [19]. Raman spectroscopy can identify complementary chemical signatures that change during stem cell differentiation and subsequent cell maturation while minimizing phototoxicity and eliminating the potential for fluorescence spectral crosstalk; however, Raman spectroscopy does not provide single cell resolution and is best suited for characterizing populations (Table 1).

Low-cost label-free optical imaging methods

Several other label-free imaging methods can identify cellular structure and morphology information, which change during stem cell differentiation. Cell structure is readily captured using complementary modalities that are included or easily added to microscopes present in most biological laboratories; these include transmitted light [41], quantitative phase [21,22], and differential interference contrast (DIC) [42] microscopies. These methods can be used to study differentiating (sub)cellular structures and organization, including cell morphology [22,41], organelle distribution, and cell density [42]. Phase distribution images can be produced using DIC microscopy to localize and quantify mitochondrial content and distinguish partially and fully reprogrammed iPSCs at varying metabolic stages [42]. Though optical coherence tomography (OCT) has not been widely demonstrated for use in stem cell applications, this label-free technology can also detect morphological changes in overall shape and organelle distribution of hPSC-derived retinal organoids [43]. Although these technologies are low-cost and widely available, they do not provide the specific biochemical information available from multiphoton microscopy and Raman spectroscopy (Table 1).

Machine learning methods to classify label-free optical images and spectra

Label-free imaging produces high dimensional datasets, which requires rapid and powerful computational methods for image segmentation, analysis, and classification. Scale-up of stem cell manufacturing will require considerable effort to optimize culture methods and determine batch standards. To reduce manufacturing and cell characterization time, machine learning methods can be incorporated into image processing and then used to find associations between multi-dimensional features of cell function and media conditions. These algorithms are broadly classified into supervised learning (*e.g.*, regression, decision trees, neural networks), which determine a correct output based on a pre-defined input, and unsupervised learning (*e.g.*, clustering, component analysis), which identify underlying relationships within the data independent of user-defined input [20,44]. Both categories of machine learning tools can be used to extract information from images or chemical spectra to make associations or predictions, which is highly complementary to label-free monitoring.

Machine learning techniques have been used to predict hPSC differentiation with the goal of reducing the time and resources needed to generate differentiated cells [23]. These algorithms are applied at various stages of the image processing pipeline including pre-processing, segmentation, and isolation of features prior to classification [20]. Morphological and structural variables that shift throughout stem cell differentiation, including cytoplasmic-nuclear ratio, total area, and sphericity, can be extracted from label-free images, and used to build classifiers [20,23]. Methods such as convolutional neural networks (CNN), support-vector machine (SVM), random forests, and regression models are well suited to incorporate imaging data and successfully sort populations or predict cell fate [20].

Several groups have trained classifiers to separate stem cells from their differentiating counterparts based on label-free transmitted light, phase, and fluorescence microscopy images. In these studies, CNNs distinguished hPSCs from mesoderm [41] and specialized endothelial cells [21] as well as NSCs from neurons within the first day of differentiation [45]. CNNs and random forest models trained on morphological features from time-lapse microscopy images have also predicted HSC differentiation into either monocytic or erythroid

lineages within 24 h, several days prior to classical neural marker availability [46]. Uniform manifold projection and approximation (UMAP) is used to reduce high-dimensional data into low-dimensional space to lower the computational resources needed, reduce the likelihood of overfitting, and assist with data visualization [20]. UMAP has been applied to TPEF variables including NAD(P)H and FAD lifetimes and ORR to visualize the efficiency of hPSC differentiation to cardiomyocytes within the first 24 h [9]. Clustering algorithms can assist with segmentation and SVM can be used to classify stem cell colonies with image-based textural and morphological variables [22]. These techniques offer great potential to streamline the stem cell manufacturing workflow by validating cell differentiation and evaluating the multifactorial conditions that enhance cell differentiation and maturation.

Future applications and conclusions

Large-scale use of stem cells for regenerative medicine and basic science requires carefully regulated, expanded 2D and 3D culture systems [2]. To ensure safe, homogeneous products at industrial and clinical scales, extensive research is being conducted to understand the methods and environments most favorable for effective stem cell differentiation and maturation of the resulting lineages. Due to its rapid and non-invasive nature, label-free optical imaging and sensing can be used to improve and predict how environmental conditions will impact stem cell viability and functionality. These tools may be integrated along with machine learning algorithms to investigate the substantial parameter space to identify optimal culture systems, media conditions, and environmental formulations.

For example, stem cell fate is affected by properties of the surrounding substrate and growth medium. Biomaterial-inspired hydrogels offer tunable platforms to assess multiple environmental variables for stem cell culture. Hydrogels of varying composition [47,48], stiffnesses [18,48,49], and patterning [50] enhance stem cell adhesion and proliferation, in turn directing differentiation. The label-free optical monitoring methods and machine learning techniques discussed here can perform long-term screens of multiple experimental culture systems to non-invasively identify optimal material and environmental properties for stem cell differentiation.

Further, classification and monitoring are needed at multiple stages of stem cell manufacturing: patient-derived cells need to be sorted prior to inducing pluripotency, differentiated cells must be separated from their counterparts, and functionally mature cells are needed for use in animal models, clinical trials, and drug screening. Label-free optical technologies and machine learning can be used at each step in this process, and the choice of imaging or sensing modality and predictive algorithm can be determined based on application needs.

Efficient differentiation and maturation are bottlenecks for *in vitro* and *in vivo* applications of stem cells. Inconsistencies in the differentiation process have impeded the scale-up of stem cell manufacturing. To realize their research and clinical potential, new methods are needed to predict differentiation efficiency of stem cells and assess the maturation state of differentiated cells during biomanufacturing. Label-free optical imaging and sensing offer significant advantages over current standard assays including real-time, contactless, long-term, and non-destructive assessment of the structural and biochemical phenotype in single cells. Machine learning-based predictive models provide rapid identification of failed batches and may enable development of closed loop processes to correct failing batches, resulting in more streamlined manufacturing for consistent cell safety and potency. Innovative application of machine learning models to multivariate single-cell parameters of biochemistry and morphology extracted from optical signals can predict differentiation outcome early and quickly identify maturation. These models have the potential to streamline current practice, accelerate the development of improved protocols for functional differentiated cells, and improve our understanding of the dynamic, heterogeneous processes of differentiation and maturation.

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Declaration of competing interest

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

Data availability

No data was used for the research described in the article.

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* of special interest
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