Technology Advancement for Integrative Stem Cell Analyses

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Scientists have endeavored to use stem cells for a variety of applications ranging from basic science research to translational medicine. Population-based characterization of such stem cells, while providing an important foundation to further development, often disregard the heterogeneity inherent among individual constituents within a given population. The population-based analysis and characterization of stem cells and the problems associated with such a blanket approach only underscore the need for the development of new analytical technology. In this article, we review current stem cell analytical technologies, along with the advantages and disadvantages of each, followed by applications of these technologies in the field of stem cells. Furthermore, while recent advances in micro/nano technology have led to a growth in the stem cell analytical field, underlying architectural concepts allow only for a vertical analytical approach, in which different desirable parameters are obtained from multiple individual experiments and there are many technical challenges that limit vertically integrated analytical tools. Therefore, we propose—by introducing a concept of vertical and horizontal approach—that there is the need of adequate methods to the integration of information, such that multiple descriptive parameters from a stem cell can be obtained from a single experiment.

Introduction

IT IS A GENERAL TRUTH that cells are the fundamental units of life and cells are interconnected to other cells and the extracellular microenvironment. The relationship between cells and complex factors has been extensively researched, but more clarification is needed in order to further explore the biology of stem cells. Since the early 1960s, research on stem cells has been ongoing and there have been many scientific milestones (e.g., embryonic stem [ES] cells, hematopoietic stem cells, mesenchymal stem cells, etc.).^{1–3} After mouse ES cells were successfully cultured in medium condition in 1981,⁴ biological studies have focused on the inherent pluripotency of stem cells at a laboratory scale.^{5–8}

Recently, ES cells and tissue-specific adult stem cells were characterized by their functional properties (self-renewal, proliferation, and differentiation)^{9–13} and categorized into one or more cell lineages according to their tissue of origin. While ES cells are derived from the inner cell mass of the blastocysts that can be propagated indefinitely in an undifferentiated state, adult stem cells exist in different tissues, including hematopoietic, neural, gastrointestinal, epidermal, hepatic, and mesenchymal stem cells. Compared with ES cells, tissue-specific adult stem cells have less self-renewal ability in a multipotent state, preserving their ability to differentiate into multiple lineages on physiological signals.¹⁴ Such pluripotent characteristics enable stem cells to be an optimal source for

the development of regenerative tissues and organs. The pluripotent properties of stem cells make them integral to the blueprint for new clinical medicine in the future (e.g., regenerative medicine, disease research, drug screening, and toxicology).^{15–18}

ES cell applications in clinical research have been limited due to the ethics; so, induced pluripotent stem (iPS) cells, and studies of nonembryo-derived counterparts, have highlighted in recent years how cell fates can be manipulated by the ectopic co-expression of transcription factors.¹⁹ Recently, it has been reported that iPS cells can newly reprogram their cell properties, in contrast to somatic cells, and adopt similar characteristics of ES cells (e.g., morphology, differentiation, and proliferation).^{20,21}

Due to their properties, stem cells have been explored for their potential application in tissue repair and renewal. Considerable efforts, including clinical trials (e.g., hematopoietic stem cells used for various hematological disorders), have been performed to develop stem cell technology, but still many problems are present and need to be resolved.^{22–24} The concept of stem cell transplantation is based on the capacity of stem cells to self-renew and regenerate tissues and organ systems. Thus, stem cell technology can be used to model and treat human ailments such as type 1 diabetes,²⁵ Parkinson's,²⁶ and cardiovascular disease.²⁷ Furthermore, stem cells can provide a source of human cells, which can be used in combination with high-throughput technologies for

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drug screening and toxicology, providing insights that traditional cell lines cannot.^{16,28,29}

Despite the enthusiasm over stem cells, there are several limitations with stem cell research that have clouded our understanding of the basic science of stem cells and delayed progress to immediate clinical application of stem cells (Table 1).^{5,30,31} For example, we have limited understanding of how to characterize and purify a homogeneous population of stem cells that is well suited for therapeutic use in terms of active cell growth and directed differentiation.³² The isolation and purification of a homogeneous population of stem cells is desired to understand the nature of stem cells for *in vitro* applications: Effective expansion and separation of pure stem cells are important in basic and applied stem cell research to probe the cellular heterogeneity in cell population. There have been a number of effective tools in the purification and isolation of stem cells; however, we are still need to have the interlaboratory and international standard techniques.^{33–35} In the case of reprogramming of iPS cells, the process is inefficient and often incomplete. In addition, recent controversy surrounding immunogenicity with regard to iPS cells^{36–38} and T-cell-mediated immune responses demonstrate the challenges that need to be overcome before harnessing the use of autologous cells for any clinical application.^{22,39,40} More fundamental and comprehensive studies of stem cells are needed to identify concrete origins or behaviors beyond the therapeutic trials.

This review focuses on advanced stem cell analytic technologies that are well suited for integrated, multi-scalar measurements of stem cells, and it highlights recent examples which illustrate this concept in bioanalytical technologies. There are several excellent recent reviews that detail the many specific technologies available for stem cell analysis.^{41,42} The objectives of our review are (1) to introduce the concept of horizontal integration—collecting information

from cells dynamically in any developmental process—of current stem cell analytical technologies; (2) to provide an overview of the areas in stem biology and biotechnology where integrated nanotechnology processes are needed; (3) to offer comparative classes of technologies demonstrated to date which appear best positioned for integrative measurements of stem cells; (4) to review recent examples of analytical measurements on stem cell tracking that suggest the type of modularity required for integration with other assays; and (5) to outline some of the technical challenges and opportunities facing integrated stem cell analysis (iSCA) with the aim of improving advanced technologies in stem cell research.

Current Stem Cell Analysis Methods and Their Limitations

Due to the heterogeneous nature of the cellular microenvironment, it is still not clear how stem cells can maintain (or alter) the dynamic equilibrium with distinct functional states. The question of whether there are intrinsic or extrinsic regulators (temporal or spatial) such as cell contact, cell-to-cell communication, and extracellular matrix that are influenced by the microenvironment has not been clearly answered.^{43–45} Therefore, understanding the cellular heterogeneity is a challenge that can be addressed with systems biology, and researchers are taking the approach of assessing general features of target cells (e.g., phenotype, genotype, protein secretion etc.). The current endeavor for stem cell research applying micro fabrication-assisted technology is summarized in four categories (Fig. 1). These research categories do not include all of stem cell biology, but each part of the research areas is closely interconnected and provides the series of snapshots on the characteristics of stem cells: binary linkages between genotypes, phenotypes, or functions of stem cells. In addition, they highlight several challenges in biomedical research and biotechnology where proper tools for comprehensive analyses of stem cells are expected.

The integration of multiple measurements on stem cells would increase the number or types of information that can be extracted in a given experiment, and would enable multiple defined operations to obtain maximum multiparametric information from cells in bioanalytical processes.⁴⁶ One of the essential features of any technologies for stem cell assays, therefore, would be the capability to isolate and track target stem cells or stem cell colonies in defined compartments for multiparametric analyses.⁴⁷

In addition, it is essential not only to decipher the true nature of stem cell behavior, but also to understand the plasticity that is influenced by a heterogeneous environment along with cell-fate decisions and progeny (mechanotransduction,⁴⁸ asymmetric cell division,⁴⁹ gene regulatory networks,⁵⁰ and so on). However, conventional biological/biotechnological approaches demonstrate little about the various aspects of an individual cell's fate that may determine the profound transformation of stem cells. Evaluating all aspects of cellular diversity at a particular point in time in the same cell is complicated owing to technical difficulties and the limitations of the current technology. Although these strategies represent essential technical advances, more improvement for analyses is desired for both temporal and spatial cellular responses.

TABLE 1. CURRENT STEM CELL RESEARCH TOPICS AND TECHNICAL LIMITATIONS THAT HINDER THE INVESTIGATION OF EACH TOPIC

Study area of interest	Cell isolation and purification
	Profiling and selecting the well-identified population of stem cells
	Cell fate-differentiation
	Pluripotency of stem cells
	Controllable stem cell fate
	Cell proliferation and mobility
	Suitable microenvironment for stem cell proliferation
	Stem cell homing and migration
	Cell function
	Immunosuppressive function
Technical limitations	Wound healing
	Low efficiency in cell purification
	Nonstandardized method of stem cell culture
	Not-fully determined phenotype markers
	Limited methods of cell tracking
	Inadequate <i>in vitro</i> microenvironments
	Not-fully understood mechanism of cell function

FIG. 1. Summarized categories and classes of micro-fabricated system for stem cell research. **(a)** Microfluidic assays with active valved traps contain sequential steps, including different valves and traps for optimal mixing and custom manipulations. **(b)** Microfluidic assays with passive traps consist of trapping arrays constructed on a chip that can immobilize cells passing through them. **(c)** Droplet encapsulation of cells in microfluidic channel can be used to enumerate and differentiate specific single cells in well-defined space. **(d)** Microarray stem cell assay systems accommodate homogeneous microwells that can trap stem cells with a high occupancy rate. Schematics adapted from Lecault *et al.*,⁵⁸ Hong *et al.*,⁵⁹ Brouzes *et al.*,⁸³ and Moeller *et al.*⁹⁰ Color images available online at www.liebertpub.com/teb

Micro-Fabricated Devices: “Lab-on-a-Chip”

The ability to observe cells in a high-throughput facility, or so-called “lab-on-a-chip” (e.g., microfluidic devices, microwells, and so on), provides critical and quantitative information about fundamental biological systems.^{51–54} Microsystems including microwells or microhurdle-based docking with adequately controlled spatiotemporal resolution have been used for cell trapping and analysis and have also been categorized as cell analytic technologies. “Lab-on-a-chip” devices and systems have become a conspicuous feature of cell analyses due to small reagent volumes, dynamic control of reagents, high throughput, biocompatibility, and sensitivity.^{55,56} Microfluidic analysis methods have long been a focus of technology development; it is only recently that these tools have impacted stem cell research. The maturation of microfluidics from its infancy into adolescence over the past 10 years has yielded many approaches to trap cells and manipulate their microenvironments in defined ways.^{55–59} The simplest strategy for isolating cells involves the placement of weirs or traps that lie in line with the fluid flow.^{60–62} The pressure exerted by the flowing fluids holds the cells in place for imaging cytometry and the measurement of some dynamic properties, including calcium flux on stimulation,⁶³ cell motility,⁶⁴ or proliferation.⁶⁵ Some in-line traps also enable the juxtaposition of two or more cells together to enable cellular fusions,⁶⁶ or assessments of cell-mediated activation.⁶⁷ However, only a few examples have been reported regarding the use of microfluidic approaches to analyze stem cells. There are currently

two prevalent technological approaches for the analysis of stem cells: microfluidics and microwell arrays.^{47,68,69}

There are three common microfluidic methods for positioning and trapping cells: in-line, actively-valved traps; passive traps; and reverse emulsions or “droplets.”⁷⁰ Arrays of microwells provide a second technology class that may stand alone or be combined with microfluidics. We highlight here the primary advantages and disadvantages of these four approaches for positioning and manipulating cells and their environments, and then provide a comparative analysis of how certain attributes critical for single stem cells assays could be compared across these classes.

Microfluidic-Based Cell Trap Technologies

One maturing approach in microfluidics is the use of actively actuated traps (Fig. 1a).^{66,71–73} These systems use computer-controlled, pneumatically driven systems to manipulate small quantities of fluids precisely through multiple modules. The utility of these systems for the detection and amplification of DNA or RNA from individual cells has been described.^{74,75} In-line passive traps, where a cell enters the junctions to control the mobility, exert an attractive force and a repulsive force toward the stagnation point where the flow axis was piled through another perpendicular to isolate cells (Fig. 1b). The integration of microfluidic features, combining cell analyses with a cross-slot channel geometry, creates the flow convergence to halt a single cell. The advantage of this approach is that a cell flowing in the fluidic channel is trapped. To obtain sophisticated controlling of the

cell arrays, hydrodynamic effects are applied with an external field gradient such as optical, electromagnetic, and acoustic for feasible confinement of cells.^{76–78}

However, the narrow dimensions of the channels near the valves (usually <10 µm) have limited their use in these systems for the extended analyses of stem cells. A pragmatic challenge for these systems is that the minimal areal density needed for each trap is defined by a significant number of control elements and the number of external connections required for each element. Thus, the total number of cells that can be screened in these types of devices is less than that in other fluidic systems with comparable dimensions. One advantage of in-line traps is that the yield for the number of traps containing a defined number of cells is often high (>95%).^{79,80} It is worth noting, however, that examples to date with this efficiency typically have not been conservative with regard to the number of cells used to fill the traps. Other disadvantage of an in-line trap microfluidic assay for iSCA is the shared, dynamic microenvironment. A common network of capillaries facilitates the application of specific stimuli to all cells in parallel, but it is difficult to define the exact environment around a given cell. Molecular factors secreted or released by the cells themselves may influence other cells positioned downstream. In addition, it is difficult to assign specific secretions to particular cells in the system, and it is challenging to retrieve individual stem cells of interest with precision.⁵⁷

Droplet-Based Microfluidic Technologies

A rapidly developing approach in microfluidics involves the generation of reverse emulsions of water in oil in capillaries (Fig. 1c).^{81–83} Small aqueous “droplets” encapsulate cells and provide independent reactors for conducting assays in a confined and predefined environment to measure proliferation,⁸⁴ gene expression,⁸⁵ and enzymatic activity.⁸⁴ Droplet-based assays are the other type of microfluidic system typically used for analysis of single cells.^{56,83} Trapping single cells in aqueous droplets is advantageous for controlled separation of cells, simultaneous tracking of cell lineages, increasing cell survival, and other critical processes.⁸⁶ A recent report of a microfluidic fluorescence-activated droplet sorter describes a proof-of-concept experiment that integrated a common cell-sorting method with the advantages of microfluidic cell culture and cell manipulation in microenvironments.⁸⁷ Two advantages of this approach are its continuous operation and ease of integration with electrostatic and optical methods for manipulating and sorting cells. For example, a device analogous to a flow cytometer has been used to sort droplets containing cells that express particular enzymes at rates of approximately 300 droplets per second.⁸⁷

Relative to other microfluidic approaches, one disadvantage for these systems is the inefficient loading of droplets with cells. The distributions of cells into droplets follow Poisson statistics. Most droplets analyzed, therefore, are empty, and the capacity of the system is underutilized. The use of spatial constraints to control the rate of cells moving into nascent droplets⁸⁸ and inertial flow focusing⁸⁹ is currently being used to address this challenge. A second disadvantage for droplet-based systems is that the materials required to measure a particular biological activity (e.g., antibody secretion, enzymatic activity) should be present on

formation or added to the droplets afterward. While sequential joining of droplets is feasible,⁸³ it is less apparent how to enable complex assays requiring washes or dilutions of reagents. Extended cultures (>4–6 h) on primary mammalian cells may also be difficult to achieve in these systems due to the limited volumes of media available and the need to track the locations of many individual droplets during this period. The segregation of droplets into defined arrays is one means to address this particular challenge.⁸⁴ Finally, the recovery of specific drops containing single cells of interest, rather than the enrichment of certain populations, remains a challenge.

Microwell-Based Analysis System

Successful development of optimized cell culture conditions and a controllable microenvironment in microarray devices has facilitated various studies examining cell responses, signaling, cell death, and other cell functions (Fig. 1d).^{90–93} For example, T-cell and B-cell interactions and the mechanisms of signaling between the two types of cells have been studied by locating cells in a microarray.⁹⁴ Rapid, sensitive detection of cell–cell signaling, peptide fragments, and pathogens has been performed on the antibody-functionalized surface of microwells.⁹⁵ In particular, localizing a number of single mouse ES cells in specific locations by patterning in the microarray has been shown to modulate cell–cell signaling.^{92,96} Development of microwell surface functionalization methods and controlled localization of cells has helped decipher the complex cell signaling pathways involved in the defense against pathogens.

One critical step for microarray analysis of stem cells is the development of the ability to specifically load cells in wells. Much research has focused on the development of a method to provide researchers with optimized loading conditions. For example, different cell-loading parameters in microwells have been optimized for adherent and nonadherent cells.⁹⁷ Microwell dimensions and seeding number of cells can be adjusted to promote optimal cell culture. In addition, functionalization of the microwell surface has been suggested as an efficient way to home cells inside the wells. Surface modification of microwells was demonstrated by coating wells with cationic polymers, which helped specifically adsorb the protein of interest.⁹⁸ The streptavidin-functionalized surface in microwells can then be used to bind nonadherent B cells, thereby increasing their homing efficiency. Since culturing cells in microwells is different than culturing them in a two-dimensional culture plate, and may be more similar to the *in vivo* environment, there have been several efforts to probe cell behavior in three-dimensional (3D) culture and to optimize the culture conditions.⁹⁹ Therefore, stem cell culture in a 3D array may simulate the cellular microenvironment. A report on engineering a 3D polydimethylsiloxane microwell array for culturing cells showed the potential of studying various cell behaviors and screening them for specific functions.¹⁰⁰ Multiplexed detection methods for a microarray device have also been developed, enabling multiplexed measurements and high-throughput analysis of cells.¹⁰¹

One primary advantage of microwell-based systems for stem cell analysis is their inherent simplicity; there are no external connections and it can be integrated into standard research practices for *in vitro* assays. A second advantage is that minimal manipulation is required to isolate cells.

Typically, the cells are dispersed onto an array by pipette and allowed to settle by gravity. It is also straightforward to recover specific cells by micromanipulation to rescue particular genes or generate clonal lines of interest.^{102–104} The integration of microfluidic systems with these types of arrays can further expand their utility by enabling the modulation of microenvironments or application of stimuli.^{105,106}

Similar to most examples of microfluidic assays, one of the main disadvantages in these systems is the efficiency of distributing stem cells into individual wells. The number of wells satisfying the cell-occupying criteria relies on the cell-seeding density and the dimensions of each microwell. In addition, a usual image analysis process for the obtain data from microwell array is time consuming and labor intensive, as one should be able to characterize each snapshot of wells (usually more than 1000 snapshots per one array). It is desirable to prepare computer-based automated image analysis tools to process images and identify target stem cells reliably, which may not be easily available for many labs.¹⁰⁷ To overcome such obstacles for effective assays, methods for reducing the size of the wells, or recycling cells during deposition, can improve loading of the arrays with single cells to achieve loading efficiencies of approximately 70%.^{108,109}

Development of *In Vivo* Assays for Stem Cell Monitoring

It is noteworthy that an *in vitro* culture system for stem cell tracing such as a microfluidic microwell has a main advantage, as noted earlier, on any artificial cellular system and could have an ease of observation through using a ubiquitous microscope. As summarized in Table 2, current *in vitro* techniques bear ability to analyze the most topics for stem cell research. However, the main disadvantage of *in vitro* assays would be the lack of dynamic information owing to the difficulties in tracing cells for a long time. *In vivo* assays of stem cells after grafting would be important, therefore, for dynamic analyses of stem cells that may complement the limitations of *in vitro* assays (Table 2).

The important technique that enabled the most of current *in vivo* stem assays was molecular imaging. Molecular imaging technologies have long been developed in biological and medical science, as within decades the constant development of numerous new techniques (e.g., direct particle labeling, reporter gene labeling) and imaging machinery comprehensively is able to observe stem cells.^{110–112} When

analyzing cellular behavior, in particular, one naturally identifies the best candidate for monitoring. Images of cells might not be perfect due to heterogeneity, possibly resulting in misinterpretation of data. The ideal experimental design for tracking stem cells should monitor cells at the single cell level over a prolonged period of times.^{113,114} With continuous improvements in labeling technologies for molecular imaging and approaches to track specific cellular components, the long-term monitoring of signals, instead of being restricted to discrete time points, will enable tracing of cellular behaviors for both *in vitro* and *in vivo* processes.^{115–117} *In vitro* experimental design has the disadvantage of short-term observation (<48 h) due to the limited space. This spatial problem fundamentally cannot be avoided when analyzing events that occur over long-term periods from hours to a few weeks, such as differentiation of stem cells.¹¹⁴ These studies showed that the limited passage numbers of the same cell or population expansion in nonhomeostatic conditions.^{68,118,119} There are only a few examples using a high-throughput method to access differentiation of stem cells where *in vitro* conditions are inadequate.^{51,58,117} In the absence of an *in vitro* approach that considers all aspects of stem cell-driven regeneration, *in vivo* monitoring approaches are the main alternative to visualize stem cells in a precise spatiotemporal manner, where a 3D environmental milieu is already available, including chemical components and physical cues.^{45,120,121} Furthermore, adult stem cells within tissues continuously self-renew to maintain the undifferentiated stem cell population. In order to identify lineages of stem cells, traditional tools to measure them have been adopting the invasive analysis of tissue sections in combination with appropriate tissue/cell staining techniques. Recently, there have been reports of applying the intravital microscopy techniques that are able to track stem cells *in vivo* over a long period of time.¹¹² With the help of molecular imaging techniques that enable the longitudinal, noninvasive assessment of the *in vivo* behavior of stem cells, the studies of cell survival, distribution, immunogenicity, and tumorigenicity have been actively performed after stem cell transplantation. With advances of labeling techniques, many groups have demonstrated “Direct labeling techniques” such as iron particle label,^{122,123} radionuclide label^{124,125} for noninvasive imaging of stem cell migrations, engraftment, and morphological differentiation. In addition, the reporter gene labeling has been shown, coupling a reporter gene with a complimentary reporter probe where it is

TABLE 2. COMPARING *IN VIVO* VERSUS *IN VITRO* STEM CELL ASSAYS FOR THEIR CAPABILITY IN STUDYING EACH TOPIC OF INTEREST AND THEIR ADVANTAGES AND DISADVANTAGES

	In vitro	In vivo
Cell isolation and purification	++	--
Cell fate	+	+
Cell proliferation and mobility	+	+
Cell function	++	–
Advantage	Ease of observation Controllable parameters of microenvironment High-throughput	Long-term survival Considering all aspects in heterogenous microenvironment
Disadvantage	Relatively short-term survival Nonhomeostatic condition	Biomolecular imaging required Relatively expensive

++ , excellent; + , good; – , not desirable; -- , not applicable.

capable of generating long-term signal contrast exclusively in live cells.¹²⁶ The technical advantages and disadvantages of imaging machinery such as fluorescence imaging, magnetic resonance imaging, positron emission tomography, single photon emission computed tomography, and bioluminescence imaging are well described elsewhere.¹²⁷

Recent Examples of Integrated Techniques for Stem Cell Analysis

We suggest here certain critical attributes that are needed to implement processes for iSCA to enable multiple linked measurements on individual stem cells across more than one biological scale or activity (DNA, mRNA, lineages, secretion, and motility). We then expand on how recent examples of stem cell tracking technologies can be applied to stem cell biology according to these criteria.

Despite the numerous advantages of microsystem-based assays (speed, number of events scored, and multi-parametric analysis), they are not well suited for evaluating all aspects of cellular diversity at different times in the same cell.¹²⁸ Such assays typically yield less information about cellular systems, such as clinical samples or production-ready hosts used for manufacturing therapeutic proteins, where modifying the cells of interest is neither practical nor desirable. Here, we describe several areas where there are significant opportunities for integrating stem cell assays to enhance our knowledge of complex cellular systems.

In static cell analysis techniques, despite the promising biological assays based on various nanotechnology, there is no validated system for dynamic stem cell tracking.^{129,130} These techniques should include unmodified samples and safe and efficient analysis in a noisy and dynamic environment. Therefore, there is a need for integrated system tools for a platform that contains adequate administration of a stem cell culturing system.

One challenge in the stem cell measurement system is how to collect dynamic cellular behavior from the environmental stimuli where cellular responses consistently interact with the microenvironment and any cells closely happen to be in the static culture system, which is beyond the conventional dish-based methods.^{43,59} In addition, recovery of the stem cells of interest for further analysis and correlation with the captured real-time secretion products is absolutely critical, but much less attention is paid to the biology and dynamic behaviors of the cultured stem cells themselves.¹³¹ Although

static cell analyses are distinctly valuable for interpretation of biological behavior, the same approach performed multiple times will give rise to stochastic events, resulting in misinterpretation as there are gradual changes in stem cells across a distribution range.^{42,132}

One of the main hurdles that current nanotechnology should overcome for iSCA is how one can integrate all the information obtained from stem cell analysis to interpret the overall attributes. The connection between the different states of the same stem cell (e.g., ground state and pluripotent state) is crucial for understanding the links to functional attributes.^{9,133–135} For example, can the transcriptome or secretory profile of the same stem cell be used to pinpoint the differences between the different states for that cell?¹³⁶ A fluctuation response in gene expression levels due to intrinsic factors and/or environmental milieu prevents us from interpreting information on cell fate kinetics and fate choice. Since stochastic events contribute to high noise in existing gene expression data and there is possible heterogeneity in the laboratory conditions, the available data are restricted, especially in terms of functional attributes that lack markers for analysis.¹³²

Specific markers are necessary to interpret stem cell behavior through the analysis of phenotypic responses, which can distinguish stems cells in different states. Owing to the lack of defined stem-cell specific markers, the physiological assessment of stem cells is rarely linked to function. The process of defining new surface markers based on molecular mechanisms or cellular morphological analysis yields only one parameter-labeled individual stem cells. Furthermore, obvious changes in phenotype do not guarantee more intrinsic properties of the dynamic variations of stem cells, which are prone to karyotypic abnormalities.^{137–139}

Horizontal Integration of Information for iSCA

Dynamic variation between stem cells and somatic cells might help explain how stem cells can give rise to diverse stages of cells, ranging from a ground state to a pluripotent state. Considering the cells are derived from different sources, how meaningful is it to define qualities of diverse cell types or gather large quantities of information on two different cell populations? With vertical collection, are the comparisons between conditions meaningful if the cells have a different origin, even if they have undergone the same procedure? (Fig. 2a) Based on these questions, we

FIG. 2. Horizontal integration of information obtained for stem cell. Vertical integration (a) makes it difficult to compare data sets obtained at the different time points, as its result does not deliver information about dynamics and different cell states. Horizontal integration (b), however, enables such data interpretation possible, as the assay contains the multiplexed information with regard to time factor. Color images available online at www.liebertpub.com/teb

anticipate that the advantages of horizontal integration of information would be helpful to understand the nature, behavior, and destiny of stem cells by combining the analysis technique with vertical approaches as well. Dynamic analysis of characteristics of stem cells, which enable keeping track of specific stem cells over the period of time, may be beneficial in answering earlier questions that the current analysis approach only includes vertical integration of data (Fig. 2b).

The majority of reported assays or technologies for assessing phenotypic traits of cells focus on only one parameter of interest, or a related class of parameters. For example, cytometers typically report on one or more surface-expressed markers (for live cells) or intracellular components (for fixed cells). Immunoassays, such as ELI-Spot or ELISA, detect the quantities of one or more secreted proteins, but do not include information about the phenotypic identity or other functional attributes of the cells analyzed. A common goal in the advancement of these conventional assays (and miniaturized variants) has been to enhance the degree of “multiplexing” and “integrate” the information collected from each experiment. Multiplexing or integrating in biology usually implies a set of parallel measurements on a related set of parameters (several expressed receptors or secreted products).^{102,103,140–143} The related term “high-content” generally describes extracting multiple, different parameters from a set of data, usually images (e.g., protein localization, morphologies, and size).^{66,83,87,108,144,145} Neither of these terms, however, describes a comprehensive view of the phenotypic and functional diversity within a population of cells or within the surrounding microenvironment.

Connecting data that describe the relationship between the different attributes of cells (e.g., genotypes and phenotypes) is usually straightforward for simple binary linkages. Measuring multiple, complex functional or operational responses for the same individual stem cell remains a challenge. However, some functional attributes that are useful to measure concurrently for a cell include the secreted factors (cytokines, chemokines, growth factors, and antibodies),^{146,147} their ability to proliferate,¹⁴⁸ their interactions with other cells,¹⁴⁹ and their migratory behaviors. The direct relationships between molecular states of a stem cell (transcript or protein abundances) and their functional activities are also difficult to define in many cases. Individual stem cell assays, therefore, can provide one view on the biological attributes of a cell, or multiple cells, but a set of independent experiments are required to test the relationships among multiple attributes (Fig. 2). Although the results of these experiments can be related to one another, the correlations of data to individual stem cells are lost, and the resolution of the variations among populations of stem cells remains coarse.

There is a need, therefore, to engineer technologies that can integrate and link dynamic, multiplexed data from stem cells across a range of biological scales and functions (Fig. 2). Many miniaturized analytical platforms, or “Lab-on-a-Chip” technologies, have focused on a vertical integration of processes; that is, micro-total analysis systems integrate processes from sample acquisition to analysis, but typically focus on measuring a single class of data (e.g., number or type of cells). This approach is realizing important technological

advances for well-defined diagnostic tools, but for discovery-oriented biomedical research, there remains a need to improve the horizontal integration of stem cell analysis to provide a more complete view of the diversity within populations of cells. An integrated approach to iSCA should offer the ability to connect data about multiple aspects of the genetic, phenotypic, and operational variability and different states of stem cells.^{53,129} Such integrated data with stem cells would offer new insights into the true diversity among populations of cells that is difficult to define with stand-alone analytical tools and bulk correlations.

Strategies which integrate multiple bioanalytical operations of stem cells horizontally should also incorporate certain principles that facilitate the interpretation of data from individual stem cells. To understand the extent of variation in a population, it is critical to observe large numbers of cells, 10^4 – 10^6 per assay, especially when considering rare populations. To make multiple measurements on individual stem cells, it is also important to identify approaches that can connect multiple modules for measuring different classes of data together, either serially or in parallel. This requirement for iSCA necessitates systems that can isolate and track specific stem cells through a complete process—a requirement well suited for microfabricated systems as discussed next. One requirement for designing processes which comprise multiple modular operations is that such approaches would also enable dynamic measurements of one or more classes of data for large numbers of individual cells.⁷⁰ This dimension of time is common for monitoring cellular behaviors such as motility, morphology, and growth by microscopy, but is less commonly applied to the evolution of functions such as secretion.¹⁰⁴ The other implication of knowing the location of individual stem cells throughout an analytical process is that it should be possible to design strategies that conserve the available sample.

Various intrinsic and extrinsic factors can modulate activation, demonstrating cellular plasticity that depends on the dynamic aspect of nuclear factors as well as changes in terms of relative protein expression levels. These various determinants such as growth factors and epigenetic modifiers are constantly connected to stem cells, thus regulating their ability for self-renewal, proliferation, and differentiation.^{45,47,150} However, to assess characteristics of a stem cell, we need technical access to different time points of the same stem cell or its lineages.

During other stages of development, in the case where *in vitro* tracing is impossible (or only partially possible), live-cell tracking between proliferation and differentiation is not well suited for examining secretory functions of cells, and recovering specific cells of interest can be difficult. To maximize the utility of extracting information from the same cell, new integrated technologies have emerged with single cell tracking for defining and interacting with individual stem cells within extensive large numbers of cells of interest.^{114,115,151}

Many investigators have exploited different combinations of determinants that influence specific decisions of stem cell fate *in vitro* to elucidate the complex crosstalks between stem cells and nonstem cells.^{45,152,153} Only a few researchers have focused on how to integrate and observe two cell types, however, for studies of dynamic cellular interactions at the single cell level due to technical problems that could not

practically access spatiotemporal resolution which may complement conventional approaches using standard plastic culture dishes.^{31,51}

Recently, a new method called microengraving has enabled multiplexed analysis of secretions from single cells localized in a microwell.^{142,154} A large number of single cells were simultaneously assessed (10,000–80,000 cells per plate) and analyzed independently for their response to stimulation. This technique has also been suggested as a way to retrieve cells of interest after the measurement for assessment of postassay responses and cloning. Application of this technology for deciphering and profiling the underlying mechanism for stem cell function, for example, the well-known immunosuppressive effect of mesenchymal stem cells to T cells, has been demonstrated (Fig. 3a).¹⁵⁵

In addition, the artificial niche microarray platform, comprising soft hydrogel tethering EMC proteins, has been suggested in which cell-culture structure topographically contains multiple niche effectors to instruct stem fate (Fig. 3b).^{156,157} In order to prove the role of combining cell to artificial niches, they have demonstrated not only the effect of cell–cell interactions on the differentiation of mesenchymal stem cells but also the self-renewal of single stem cells by combining robotic protein spotting with a microwell patterning method. The effect of stem cell–nonstem cell interaction and their niches, key determinants of stem cell fate, or differentiation¹⁵⁸ (or de-differentiation, trans-differentiation) has yet remained not fully investigated at the single cell level.

Future Directions for iSCA

Technical advances would still be desired to integrate three or more classes of information (e.g., gene expression, secretion profiles, lineages, and growth rates) with the horizontal data integration efforts for stem cells. For iSCA, a new bioanalytical nanotechnology should be developed that may yield horizontally interpretable multidimensional datasets for stem cells. We have highlighted here several criteria for a successful iSCA.

Robustness

A critical factor for adoption of new technologies within the field of biomedical research is the ease of use and standardization of processes. Even simple assays such as ELISpots, ELISAs, and immunophenotyping by flow cytometry still require significant optimization across clinical labs to assure common practices and comparable data. The defined volumes and structures enabled in iSCA nanotechnology should reduce both inter- and intra-assay variances, but technologies should also be robust in implementation. Designing devices to interface with commonly available equipment such as microarray scanners, hybridization stations, and off-the-shelf imaging systems will facilitate the automation and standardization of processes.

Open-platform designs

Central to the widespread use of both flow cytometry and ELISA in biomedical research is the advantage of these technologies in enabling user-defined analytical operations. The flow cytometer, for example, measures the fluorescence

of stem cells in suspension. This, in turn, has enabled the development of many different kinds of assays to measure phenotypic and functional variations among cells or groups of cells, varying from enzymes on the surface of bacteria to intracellular staining of cytokines in immune cells. Many lab-on-a-chip technologies, however, are designed to provide one analytical operation—for example, to measure a specific expressed gene or to count the number of stem cells. For diagnostic purposes, this tight control on the analysis is critical, but discovery-oriented research can benefit from flexibility in design and implementation to promote broad access without the need to fabricate custom chips for every desired application. Defining interchangeable analytical operations as the basis from which unique bioanalytical processes can be constructed should be a goal in implementing iSCA nanotechnology. A related parameter is the ability of these devices to interface with existing equipment such as fluorescent microscopes and flow cytometers found in standard biomedical/clinical labs. As the need for specialized infrastructure increases, routine implementation becomes more challenging.

Multiplexing within a given analytical operation

The ability to maximize the information content from the target stem cell is the ultimate goal of iSCA nanotechnology. It is, thus, important to design strategies that multiplex data collection within a class of data (e.g., secreted or surface-expressed proteins; expressed genes) while retaining the ability to profile data across multiple classes. For example, when profiling functionally active mesenchymal stem cells, the goal should be to analyze the isotype specificity of the protein secreted from the cell, the phenotypic characteristics of the cell itself (cell surface markers, differentiation state), and the sequence of the proteins (using mRNA).

Data processing and analysis

As described earlier, if the goal is to maximize the information that can be extracted from single cells, and implementation is sought in medium- to high throughput (at least 10,000 cells), the data generated from a cell are equivalent in some regard to the large datasets generated by DNA or protein microarrays for a set of experimental conditions. If kinetic monitoring of these single cells is also desired or if a few different samples need to be analyzed as a part of a single experiment, the amount of raw data generated and the subsequent processing and analysis of the data become formidable challenges. This would necessitate robust bioinformatic approaches to collate and mine the data.

Conclusion/Perspective

Current micro/nanotechnologies have been used as effective experimental tools for the rapid and multiplexed analysis of cells. Technological advances for miniaturizing such systems and the optimization of delicate controls in profiling cells have motivated many researchers in stem cell research to employ new nanotechnology for stem cell analyses. New nanotechnology that horizontally integrates the analysis of stem cells would enable high-throughput, multiplexed analysis of cells, and the analysis would not be

FIG. 3. Examples of *in vitro* horizontal integrated stem cell analysis (iSCA). **(a)** Study of stem cell and immune cell interactions. Dynamic cell–cell physical interaction and resulted secretion of proteins at different time points from T cells can be assayed. **(b)** Schematic images of production of artificial niche microarrays. Mesenchymal stem cells (MSCs) were stimulated to differentiate into adipocytes after 11 days in each microwell. All images are from references cited in the article. Color images available online at www.liebertpub.com/teb

limited by the lack of large samples. These characteristics may significantly benefit the study of stem cells, where the number of cells available for testing is usually limited and consists of a heterogeneous population of cells. The development and application of iSCA nanotechnology may present a novel opportunity for the qualitative and quantitative characterization of stem cells, thus leading to a comprehensive understanding of fundamental aspects of stem cell-related regenerative medicine.

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Disclosure Statement

No competing financial interests exist.

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