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Perspective

# Integrated Process Design for Single-Cell Analytical Technologies

**J. Christopher Love**
Dept. of Chemical Engineering, Massachusetts Institute of Technology, 77 Massachusetts Ave., Cambridge, MA 02139

*DOI 10.1002/aic.12413*
*Published online August 20, 2010 in Wiley Online Library (wileyonlinelibrary.com).*

*Keywords: systems engineering, unit operations, single-cell analysis, lab-on-a-chip*

## Introduction

Analytical technologies that discern different genetic, phenotypic and functional attributes of living cells are critical throughout biomedical research and biotechnology. Cells are an essential component of many biological systems, and involved in problems ranging from diagnosing and treating human diseases to manufacturing a therapeutic biological molecule in large quantities. A cell comprises a multitude of biomolecular networks of genes, proteins, and metabolites. Each cell senses changes in its local environment, integrates that information through its molecular networks, and responds by secreting proteins, moving, proliferating, or dying, among other possible functional outcomes. Understanding the relationships among the inputs or cues in the environment that trigger a response and the expression/regulation of genes for a specific cell is essential for both traditional cell biology and synthetic biology.[1] Determining the diversity in functional responses of cells and how it influences the state and evolution of other cells in a network, such as a tissue or bioreactor, are also important to assess the mechanisms of disease,[2] the efficacy of a therapy,[3] or the productivity of a biomanufacturing process.[4]

The complexity of cellular systems has promoted the maturation of bioanalytical tools that measure qualities or attributes of isolated components (Figure 1). DNA sequencing technologies provide knowledge about the genetic composition of a cell. Immunoassays or mass spectrometry yield insight into the types of proteins inside a cell or secreted by a cell. Correlating results from independent assays is necessary to determine the relationships between these biological components. These correlations are made, however, in a gross manner since different cells are used in each assay. The connections are rarely resolved directly at the level of individual cells. For example, mapping how the expression of mRNA transcripts relates to the rates of secretion for a clonal cell line used in biomanufacturing requires one assay to measure the bulk quantities of mRNA present in a population of cells (RT-qPCR, DNA microarray), and a separate one to measure the total protein released by the cells (ELISA). These results assume uniformity among the states of individual cells in the culture; they yield average measures of the system, and, thus, provide limited insight to how a network of cells interact.

There is increasing realization, however, that significant heterogeneity in operational states exists among populations of cells, even in ones where all cells are genetically identical.[5] Multicellular organisms obviously have additional heterogeneity arising from multiple types of differentiated cells in tissues and organs. The current methods for analyzing cellular samples have hindered the ability to resolve these heterogeneities precisely, and limit the breadth and depth of knowledge that is obtained from a given sample. This perspective considers how the approach to the design and implementation of bioanalytical processes could change to improve the efficiency of extracting information from any given sample, especially those related to the study of human biology and disease. It presents the challenges of studying chronic human diseases with existing analytical technologies, describes how applying fundamental concepts from chemical engineering could provide an alternative approach, and outlines how lab-on-a-chip technologies could evolve to enable new, efficient bioanalytical processes for cellular systems.

## The challenges of understanding and monitoring chronic human diseases

Chronic human diseases are one area of global significance where the difficulty of characterizing heterogeneities among cellular systems is readily apparent. They also provide a useful example to frame the challenges in developing bioanalytical technologies. Treatments to manage persistent infections, cancers, autoimmune and inflammatory diseases, including cardiovascular disease, have advanced significantly over the last 30 years, but our ability to detect them during the early stages of development and to monitor the efficacy of therapies have not progressed at the same rate.[6]

Most, if not all, chronic diseases involve sustained interactions between the immune system and some pathogenic species (e.g., virus, tumors, self). The immune system is a multicellular network of cells (dendritic cells, macrophages,

J. C. Love is also affiliated with The Ragon Institute of MGH, MIT, and Harvard, Eli and Edythe L. Broad Institute, and David H. Koch Institute for Integrative ?Cancer Research.
J.C. Love's e-mail address is clove@mit.edu.

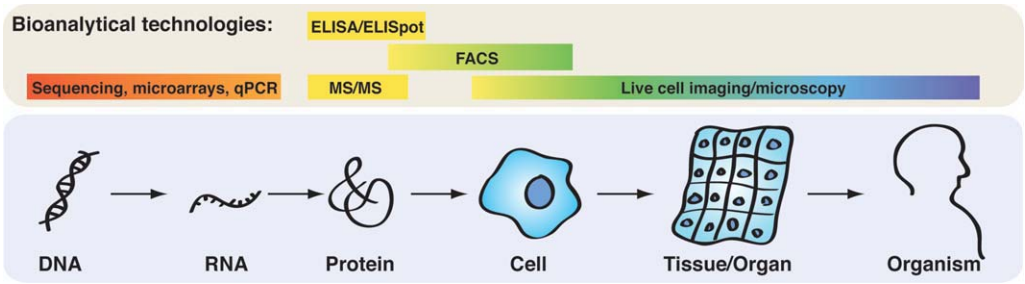


**Figure 1. Schematic summary of the breadth of biological scales for which existing bioanalytical technologies are best-suited.** qPCR, quantitative polymerase chain reaction; ELISA, enzyme-linked immunosorbant assay; ELISpot, enzyme-linked immunospot; MS, mass spectrometry; FACS, fluorescence-activated cell sorting. The bars indicate the breadth of biological scales typically covered by each technology.

natural killer (NK) cells, T cells, B cells) that interacts through ligand-receptor complexes mediated by manifold secreted proteins (cytokines, chemokines, antibodies), and glycoproteins expressed on the cells' surfaces. Analyses of the reported connections among the cells of the immune system suggest that the number of connections among members of the immune network is comparable to that for the world-wide web or social networks.[7] The nature of any response by the immune system, whether to a virus or a tumor, is complex and multifaceted. Analyzing these responses, therefore, requires measuring many aspects, including the types of cells that respond, the magnitude and nature of their response (secretion of cytokines, proliferation, cytolytic activities), and the dynamics of the response.

Presently, evaluating multiple attributes of an immune response related to a particular disease requires a collection of independent assays like flow cytometry and immunoassays (ELISA, ELISpot),[8,9] as well as other functional assays to characterize proliferation or cytotoxicity. Comprehensive analysis by a panel of such assays requires a minimum of ~1,000,000–10,000,000 cells. For most clinical research on human diseases, these cells originate from a blood sample. A sample from an adult (~4 mL per vacutainer) may yield ~1,000,000 or more immune cells, and larger volumes can be obtained safely in most cases. These samples are convenient and inexpensive with low risk to the patient compared to those collected from affected tissues. A significant drawback, however, is that the cells of interest — disease-specific ones — are not common in circulation for many diseases (~1 in 10,000 or less). In addition, the amount of blood collected from pediatrics and infants is usually much less, hindering the study of diseases in this group of individuals whose immune systems are not yet fully developed.

Another disadvantage of sampling blood is that the nature and responsiveness of the cells in circulation may differ significantly from those located in the actual site of infection or inflammation. Studying the biology of human diseases in affected tissues is feasible, but obviously, the amount of material recovered from small "pinch" or needle biopsies is limited in volume and expensive to collect. While the number of disease-specific cells in these samples is usually enriched relative to blood, the total number of cells available per biopsy for study is small (~10,000–100,000). The inefficiencies of existing bioanalytical assays, therefore, prohibit comprehensive analysis of the samples that contain the most useful knowledge about the pathology of a disease.[10]

The specific example of the difficulty in developing a vaccine for the human immunodeficiency virus type 1 (HIV-1) illustrates many of these challenges. HIV-1 infects CD4+ T-cells and integrates its genome directly into the cells'. Relative to other viruses such as influenza, the replicating machinery of HIV-1 is extremely error-prone, which leads to many variants of the virus both within an individual and globally.[11] The shifting composition of the virus makes it difficult to mount an immune response that effects durable, lasting control, and if untreated, leads to AIDS for most individuals. A small percentage of individuals, however, are able to control the virus naturally.[12] Many develop effective cytolytic CD8+ T-cell (CTL) responses that limit the propagation of the virus from infected cells. Several recent efforts to develop a vaccine against HIV-1 have sought, therefore, to elicit similar HIV-specific CTLs in the vaccinee, but an open question remains: What is the best means to evaluate the efficiency of the vaccine intended to raise an appropriate population of HIV-specific CTLs by a candidate vaccine? The standard metric employed in clinical trials of vaccines has been the number (or frequency) of HIV-specific CD8+ T-cells that release interferon-gamma (IFN$\gamma$), a proinflammatory cytokine associated with successful responses to viral pathogens. A recent trial (Merck/STEP) demonstrated, however, that there was very poor correlation between the frequency of HIV-specific T-cells releasing IFN$\gamma$ and the ability of the vaccine to protect against infection or reduce viral loads after infection.[13] Analysis of subjects who have naturally generated CTLs that effectively inhibit replication of the virus *in vitro* have shown that there is a vast range of functional variations within these populations of HIV-specific CD8+ T-cells.[3] These results suggest that the signature for a protective response to a vaccine is likely more complex than a single functional measure scored by one assay (IFN$\gamma$)—one that existing bioanalytical systems have been insufficient for defining to date.

A further constraint on monitoring immune responses in large-scale clinical trials such as the Merck/STEP trial is economics. The number of samples collected is large when many persons are enrolled (1,000–10,000). The size of the samples and the frequency of collection are, therefore, limited. As discussed earlier, current analytical approaches are relatively poor at extracting information from small samples. Large numbers of cells are required to perform comprehensive

analyses of cellular immune responses using a collection of independent assays. In this situation, incomplete analysis produces imperfect knowledge about how a vaccine or treatment affects an individual, and hinders decisions about modifying subsequent designs.[13,14]

## Principles from chemical engineering should guide the design of bioanalytical processes

A fundamental idea in chemical engineering is that processes are designed and developed to transform a material (or set of materials) into products or defined outcomes. Usually, these products are more useful or valuable than what was available to start. Examples of processes include refining crude oil, remediating soil to remove toxic compounds, fabricating electronic goods, or making specific products from raw materials. In the context of analyzing biological samples, the transformation of interest is one that converts living cellular systems into comprehensive knowledge about that system and how it is influenced by external cues, such as drugs or pathogens. The ideal product for a bioanalytical process, therefore, should be perfect knowledge about the biological system (whether a clinical sample as described above or others such as a population of clonal cells used in manufacturing). It is, perhaps, unreasonable to expect that complete understanding of a living system could be extracted from a single sample, but applying rational engineering practices to the design and implementation of bioanalytical processes should improve the efficiency with which information, and subsequently knowledge, is extracted relative to current practices (Figure 2).

Biological systems comprise large and complex networks of variables: intracellular molecules (genes, proteins, metabolites, glycans), extracellular features (surface-expressed proteins that delineate a type of cell or its differentiated state, morphology, size), dynamic functional qualities (secreted proteins, proliferation, cytolytic ability, motility), and the numbers and types of cells, among other traits (Figure 2a). Studying how these systems respond to external cues (e.g., drugs, vaccines, pathogens, and reactor conditions) requires gathering information about these variables that uncovers the direct and indirect relationships among them. The integration of those data can then provide new understanding or models for how the biological system operates. For example, experimental data on one or more variables may provide insights on how cells sense changes in their local environments, and respond by moving, secreting proteins, proliferating, or dying, or how they influence the state and evolution of other cells in a network, such as a tissue or bioreactor, to impact a macroscopic outcome (progression of a disease, titers of manufactured biomolecules). Identifying the structure of the models that best describe these complicated behaviors, therefore, depends on designing a set of appropriate experiments to reveal underlying relationships among the variables.

To probe the relationships among variables in a biological system, a researcher must currently define a set of self-standing, independent experiments that probe specific relationships, ignoring others. Biology's complexity has promoted the maturation of analytical tools that—*by design*—constrain the data extracted to a single variable (or a set of related variables) from one or more populations of cells. For example, flow cytometers extract the numbers of cells that express certain biological molecules, while ELISA or ELISpots extract the types of proteins released by cells from a given sample. Each independent assay also typically uses a different set of the cells from a sample—an inefficiency that hinders studies where the amount of material is scarce (e.g., biopsies). Thus, the standard design of experiments limits the extent to which many relationships among the variables of a biological system can be determined, and is uneconomical when extracting knowledge from a given sample.

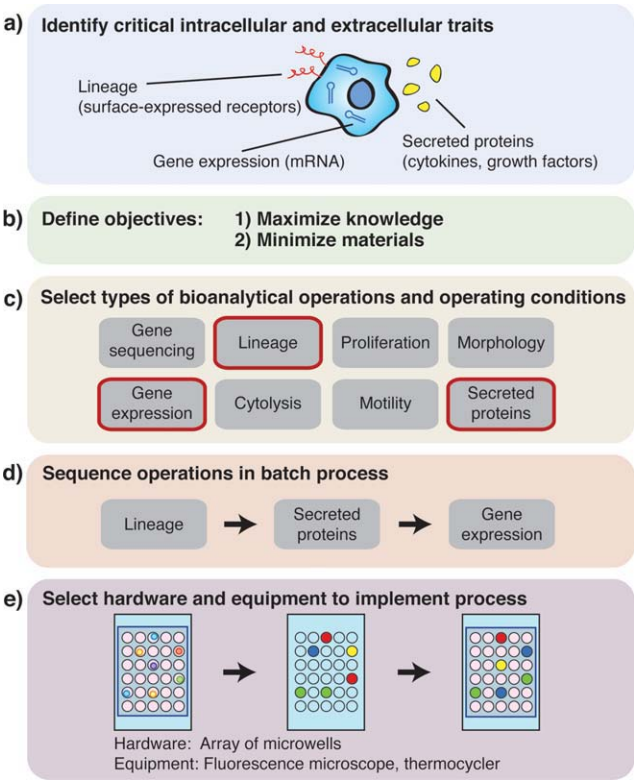


**Figure 2. Schematic overview of a design process (a–e) for an integrated bioanalytical process using a collection of unit operations to extract correlated data about multiple biological attributes for many single cells in parallel.**

In designing an economical process for manufacturing chemicals or other products, two objectives usually are (1) to maximize the yield of the product, and (2) to minimize inputs or raw materials. By analogy, a process for analyzing a biological system should aim, therefore, to maximize the knowledge gained, but without requiring additional material or samples for each incremental analysis (Figure 2b). An integrated network of operations that uses a given biological sample as a common substrate would improve the efficiency and extent to which information and knowledge is collected.

## Unit operations as knowledge generators

To design flexible, integrated bioanalytical processes, it would be useful to have set of modular operations from which to construct any specific experiment. Unit operations in chemical engineering traditionally rely on fundamental physical, chemical, or mechanical principles that supersede any specific

process or embodiment. Some unit operations transform a chemical or material from one state to another; others separate one compound from a mixture. Here, in the context of bioanalytical processes, it is useful to define unit operations as individual processes that act on a certain biological substrate to generate specific knowledge or information about a variable of interest (Figure 2c).

A logical substrate to consider as an input to a bioanalytical unit operation is the cell. As outlined perviously, cells are the focal point of biological systems. They contain the biomolecular networks of genes, proteins and metabolites, and mediate interactions within multicellular networks. Understanding the heterogeneities among individual cells provides a direct view of the range of states present, and the relative importance of each.[15] Unit operations, therefore, should return information about different attributes of a given cell. Examples include determining a cell's genetic composition, its expression of genes and proteins, its functional capacities, and its relationship or role in the network of cells to which it belongs. Consistent with classically defined unit operations in chemical engineering, how a specific operation is implemented may vary. For phenotyping cells based on surface-expressed glycoproteins, laser-based cytometry or optical microscopy are convenient. For measuring secreted proteins, mass spectrometry or immunoassays would be suitable.

Unit operations that act on a common substrate are useful, because they allow a particular process to be constructed by selecting a set of operations, defining their specific operating conditions, and ordering them to define a process. The substrate is carried from one operation to the next to yield a product or outcome. For manufacturing chemicals, independent operations usually are connected physically by pipes that carry the substrate and products through the process. Here, the substrate—many single cells—must be carried from one analytical operation to another in a manner that retains registration with the data collected for each one in each operation. This arrangement requires both a pipeline for information flowing out of the operation and mechanisms to transition cells to the next operation. The development of new micro- and nanotechnologies for handling and interfacing with many single cells and their molecular components in parallel are well-suited for these tasks, and should lead then to standard modular components with which to design integrated bioanalytical processes.

## Microtechnologies for implementing single-cell unit operations

The maturation of technologies based on principles from chemistry, materials science, physics, and electrical engineering have generated an interesting and practical set of tools for interacting with and analyzing single cells. Collectively, these tools are often referred to as Lab-on-a-Chip (LOC) or micro-total analytical systems ($\mu$TAS).[16,17] Many of these tools use devices in which the smallest length scales approximately match the scale of the biological elements of interest. For example, to manipulate and position cells precisely, critical features are typically 1–100 $\mu$m. Smaller features ($\sim$10 $\mu$m to nanometers) are advantageous for applying localized stimuli to cells or separating DNA, proteins, organelles, and other biological components from individual cells.

There are three dominant classes of microsystems currently used for single-cell analysis. First, microfluidic devices use either actively actuated systems or passive, inline systems to trap individual cells.[18–20] Second, reverse emulsions of water-in-oil—so-called "droplet" microfluidics—enable the entrapment of single cells in (sub)nanoliter volumes for cytometry and heterogeneous immunoassays.[21–24] Third, planar arrays of microwells allow the spatial localization of single cells for subsequent interrogation and manipulation.[25–28] Each technology provides certain advantages and disadvantages. Conventional microfluidic systems offer the most control over the position and microenvironment of the cells. The areal density required per trapped cell, however, typically limits the number of cells analyzed per device. Droplets containing cells provide defined objects that can be manipulated and imaged in a continuous and precise manner, but the range of assays available for evaluating multiple aspects of primary cells is still limited. Arrays of microwells are the simplest platform for isolating and interrogating cells that can be widely used in biology labs without specialized equipment. The ability to tune the microenvironment of each cell in the array in real time is more challenging compared to microfluidic-based systems, though.

The majority of examples to date for single-cell analysis that use these technologies have focused on miniaturizing existing macroscale analytical tools to allow resolution of individual cells. That is, most examples still assess only one of many possible variables of the cell or its constituent parts—for example, the type or amount of protein secreted, the types of glycoproteins expressed on its surface, or its propensity to proliferate upon activation. While useful for conserving reagents, and resolution of heterogeneities, these approaches, like their macroscopic counterparts, still do not aim to maximize the extraction of information about the system analyzed. Current examples of single-cell analytical technologies are also essentially unique designs. That is, they are implemented to conduct a single operation. Nevertheless, these technologies should allow the definition of standardized, modular operations with which to design reconfigurable batch processes for testing a specific hypothesis or pursuing discovery-oriented research.

## Designing batch processes for integrated single-cell analysis

The ability to manipulate and measure biological attributes of single cells is necessary, but not sufficient, for designing optimized, sample-conserving bioanalytical processes. To collect comprehensive data about each cell in a given sample that defines its genetic and phenotypic variations, one must consider how to implement a batch process wherein many cells are subjected to a series of operations, each designed to recover certain information about individual cells (Figure 2d). Some operations could occur in parallel, while others would be serial. Because the operations would act on each cell, the data generated should allow straightforward correlations of many biological variables directly.

Although much of the effort in developing lab-on-a-chip solutions has focused on vertical integration from sample collection to purification to analysis, horizontal integration of analytical processes is also essential for comprehensiveness. We have started to explore several ways in which a common array

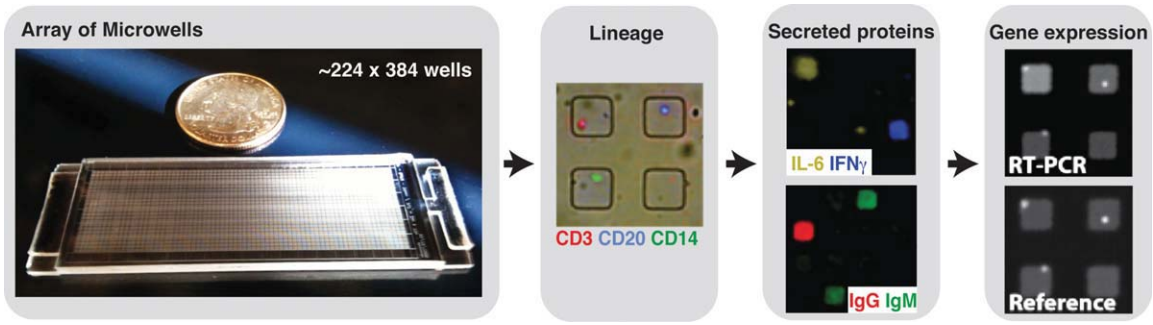


**Figure 3. Example of one type of process for integrated single-cell analysis using modular single-cell bioanalytical operations.** The elastomeric array of microwells (left) contains more than 85,000 individual wells to isolate cells deposited from a suspension. This platform allows in-well imaging to enumerate the number and types of cells (Lineage), functional assays to detect secreted proteins by microengraving (Secreted proteins), and gene expression by gene-specific reverse transcription and amplification (Gene expression). These operations can be reconfigured to monitor dynamics or increase multiplexing of analysis. Each square shown in the representative single-cell images is 50 $\mu$m wide. CD, cluster of differentiation; IL, interleukin; IFN, interferon; Ig, immunoglobulin; RT-PCR, reverse transcription-polymerase chain reaction.

of microwells can allow multiple analytical processes for a cell(s) isolated in each well (Figure 2e). The array of microwells provides a common platform for implementing different assays, and information for each well can be linked to data from multiple assays both spatially and temporally.

In one configuration for an exemplary process, the cells distributed into an array could be interrogated sequentially to determine their lineages, secretory profiles, and gene expression (Figure 3). Cells distributed in the array can be imaged to determine surface-expressed glycoproteins that identify the type of cell and to assess their viability. Because the array allows us to isolate living cells, we can then use it to perform a number of functional assays. One process, called microengraving, makes it possible to capture the proteins secreted from the same cells.[29,30] Using the array as an engraving plate, the proteins released by the cells are captured on a glass slide placed on top of the array. With fluorescent reagents with affinities for specific proteins, we can assess the number of cells secreting specific proteins, as well as the rates of secretion for four or five analytes per engraving.[31,32] It is also possible to co-load multiple cells in the wells to monitor dynamic processes like cell-mediated activation or cytolytic activity. These functional assays can be performed either in series or in parallel, making it possible to design multistage assays to monitor the dynamic evolution of responses or to increase the number of analytes characterized per cell. Finally, these processes are nondestructive, which allows the recovery of viable cells for expansion *in vitro*,[32,33] or massively parallel detection of expressed mRNA transcripts.[34]

The evaluation of the diversity in the humoral, or antibody, response raised in a model vaccination provides a representative problem to illustrate how these interchangeable operations can enable a comprehensive bioanalytical process.[35] We immunized mice with a prime-boost regime using ovalbumin—a model protein designed to mimic a recombinant immunogen in a vaccine—and then collected a series of data for a set of B cells using a combination of image-based cytometry and serial microengraving. For each B cell among thousands evaluated, we collected data on the surface-expressed proteins on the cells themselves, as well as biophysical properties of their antibodies, including the isotype of the antibody produced, and the specificity and affinity of each one for the immunogen. This dataset was equivalent to at least four independent conventional assays, and provided quantitative insights to the nature of the humoral response generated with single-cell resolution. Such an approach applied in clinical trials would allow detailed analysis of the diversity of antibody responses raised by a candidate vaccine, and facilitate understanding about how the immunogen used shapes the maturation and repertoire of antibodies produced.

## Opportunities beyond immune monitoring

The conceptual framework outlined here for process design is ordinary for traditional chemical engineering, yet it is not inherent in how analytical processes are commonly implemented in biomedical research and biotechnology. There are significant opportunities, therefore, for chemical engineers not only to improve the specific technical systems used for single-cell analysis, but also to explore further the ways in which standard principles from the field will apply to realize more efficient operations. The context for much of this discussion has been how to study immune responses in human diseases or therapeutic interventions. The utility of integrated, comprehensive bioanalytical processes extends to many other areas of research, however.

In bioprocess development, detailed analysis of single cells within clonal populations that included rates of production, rates of division, metabolic flux, and quality of the protein produced should improve the selection of master cell lines for manufacturing. In one example, we have used integrated single-cell analysis to determine the relative ages of many single cells in parallel, and to follow their dynamics of secretion and cell division over several hours.[36] We showed that only a fraction of cells in cultures of *Pichia pastoris*—a yeast organism used for biomanufacturing—contributes significantly to the productivity of the culture at any given point in time. These cells also divided more rapidly than other clones in the popu-

lation. Analysis of the culture by bulk analytical tools did not reveal the dynamic nature of this set of cells, or others that exhibited fluctuating states of secretion, within the average behavior of the population. Understanding the fine heterogeneities among individual cells within cultures, and the dynamics with which productive subsets of cells evolve, should inform efforts to engineer and optimize new strains.

Similar processes would also allow new types of Process Analytic Technologies (PAT) for evaluating the state and evolution of the cells in a bioreactor during scale-up or manufacturing campaigns. In bioengineering, or synthetic biology, enhanced understanding of the relationships between modified genes and phenotypic diversity would also facilitate strain optimization. High-throughput, high-content drug screening and quantitative systems biology are other research areas that should benefit from redesigned bioanalytical processes that are efficient and comprehensive.

## *Conclusions and outlook*

A practical premise that has motivated the development of LOC technologies has been that miniaturizing existing bioanalytical technologies can reduce the costs associated with standard laboratory assays, improve standardization and automation of execution, and in many cases, enhance the sensitivities of the assays by relying on the unique features of small systems (e.g., high ratios of surface area-to-volume, new physics). These factors have, indeed, been important in the commercialization of some of the microtechnologies developed to date for bioanalysis, with the most noted successes being microfluidic systems for handling small volumes of reagents (e.g., Caliper, Agilent, Fluidigm). Nonetheless, the anticipated potential for these technologies has greatly exceeded their overall impact to date in diagnostics, drug discovery, and biomedical science.[37] For these types of technologies to be widely adopted in biomedical research, they must go beyond miniaturization and standardization to provide significantly greater value than is accessible with existing analytical technologies. The genomics revolution provides a useful lesson here. Miniaturization and automation were critical to realize the first drafts of the human genome,[38] but the revolutionary biology in metagenomics and personalized genomics is now occurring because there was a substantial change in the fundamental technologies used for sequencing.[39]

Another consideration for designing bioanalytical processes and their specific implementations should also be scalability. It is conceivable that many integrated single-cell analytical processes could be designed to handle small numbers of cells (~1–100). To have relevance for most applications, however, a statistically large number of cells should be sampled. For clinical samples, the frequencies of disease-specific cells can range from ~1 in 1,000 to 1 in 1,000,000.[40] Many demonstrations of single-cell technologies focus on a proof-of-concept with a few cells, but how the system could be scaled up or out should be important, even in early stages of development.

As single-cell analytical technologies mature from their infancy into adolescence, it is critical to view integration and process design as frontiers for further expansion and development. Modularity, standardization, and scalability are essential elements to include in the implementation of bioanalytical unit operations. The principles for measuring many common and many novel attributes of single cells have been established using micro- and nanosystems, yet defining efficient and economical processes with these tools to maximize the knowledge extracted from a given biological sample and inform new models of biological systems remains an open opportunity—one where chemical engineers are well-poised to contribute.

## Acknowledgments

The author thanks K. R. Love for helpful comments. This work was supported, in part, by the W.M. Keck Foundation and by the Charles A. Dana Foundation. J. C. Love is a Latham Family Career Development Professor.

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