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Measurement and Modeling of Signaling at the Single-Cell Level

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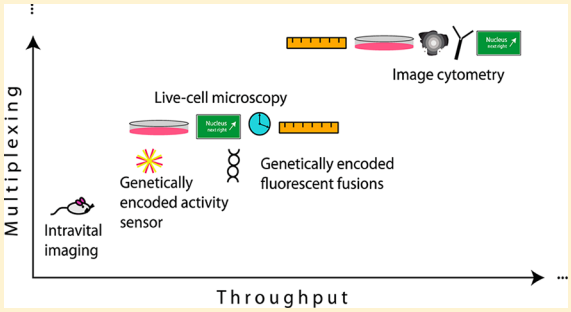
ABSTRACT: It has long been recognized that a deeper understanding of cell function, with respect to execution of phenotypic behaviors and their regulation by the extracellular environment, is likely to be achieved by analyzing the underlying molecular processes for individual cells selected from across a population, rather than averages of many cells comprising that population. In recent years, experimental and computational methods for undertaking these analyses have advanced rapidly. In this review, we provide a perspective on both measurement and modeling facets of biochemistry at a single-cell level. Our central focus is on receptor-mediated signaling networks that regulate cell phenotypic functions.



Improved understanding of how cell signaling events are affected by extracellular cues and lead to cellular outcomes like survival, death, and proliferation will be crucial for the development of therapeutics for addressing pathologies such as cancers and inflammatory disease. It is understood that complex networks of signaling interactions are at work in transduction and that, rather than individual pathways working in isolation, crosstalk and network-wide effects determine behavior; thus, systems biology approaches, in particular mathematical modeling of signaling data, have proven vital to this endeavor. It is also known that measurements made on bulk cell populations may miss key information, as even genetically identical cells respond variably to the same cues, and that heterogeneity is a key feature of many processes of great interest, such as cancer metastasis[1,2] and tumor cell responses to drugs.[3−5]

Cell-to-cell heterogeneity arises in many physiological contexts. Cells involved in a process of interest may differ in genetic makeup (as is often the case in tumors), type (as when multiple cell types interact to produce a functional tissue), and interaction partners (including other cells and/or extracellular matrix). Asymmetric interactions between cells that lead to divergent cell outcomes are crucial in development as well as tissue homeostasis, for example, in asymmetric cell fate determination through Notch signaling.[6] Tissues may be comprised of cells of multiple types in various stages of differentiation (e.g., stem, progenitor, and mature cells), which must be either separated accordingly in groups for analysis or analyzed at the single-cell level.

The cell cycle presents another source of heterogeneity between cells at a given point in time, with nonsynchronized cells occupying different points in the cell cycle. Even if such cells are "running the same program", it may be hard to determine the nature of this program by monitoring the average of all the cells over time. Via measurements on single cells within a cell population, it becomes possible to access information about time-dynamic programs happening at the individual cell level. For example, Son et al. used a microfluidic platform to observe how growth rates of mammalian cells changed across the cell cycle, allowing them to propose a potential mechanism for cell size homeostasis.[7]

Single-cell approaches are therefore likely to be valuable in a variety of contexts. To this end, new techniques are being developed for measuring signaling at the single-cell level, and mathematical models are being used to interpret and learn from these data. Here we discuss these technological, methodological, and conceptual advances, describing current approaches for measuring and modeling signaling at a single-cell level, with a focus on kinase signaling.

Value of Data at the Single-Cell Level. Measurements at the single-cell level require extremely sensitive assays and careful assessment and minimization of technical error and may require highly specialized equipment or large data storage and handling resources (e.g., in the case of live-cell imaging). In cases where an average model generated using population-level measurements represents signaling events taking place in individual cells, data at the single-cell level are not necessary. This may be more likely in situations where interactions between cells are symmetric, the processes of interest are not cell cycle-dependent, and variable time delays are minimal. However, when this is not the case, single- or few-cell measurements are needed to understand the system under study. It would be valuable to identify such cases to optimize resource allocation (using traditional assays where more convenient, cost-effective, and/or feasible) while minimizing information loss, to avoid missing key features of a system. Though there is no simple formula for determining in advance whether single-cell measurements will be needed in a particular setting, we can identify contexts that may make it more likely. As we discuss below, these include situations involving binary

Received: June 24, 2012
Revised: August 31, 2012
Published: September 6, 2012

dx.doi.org/10.1021/bi300846p | *Biochemistry* 2012, 51, 7433−7443

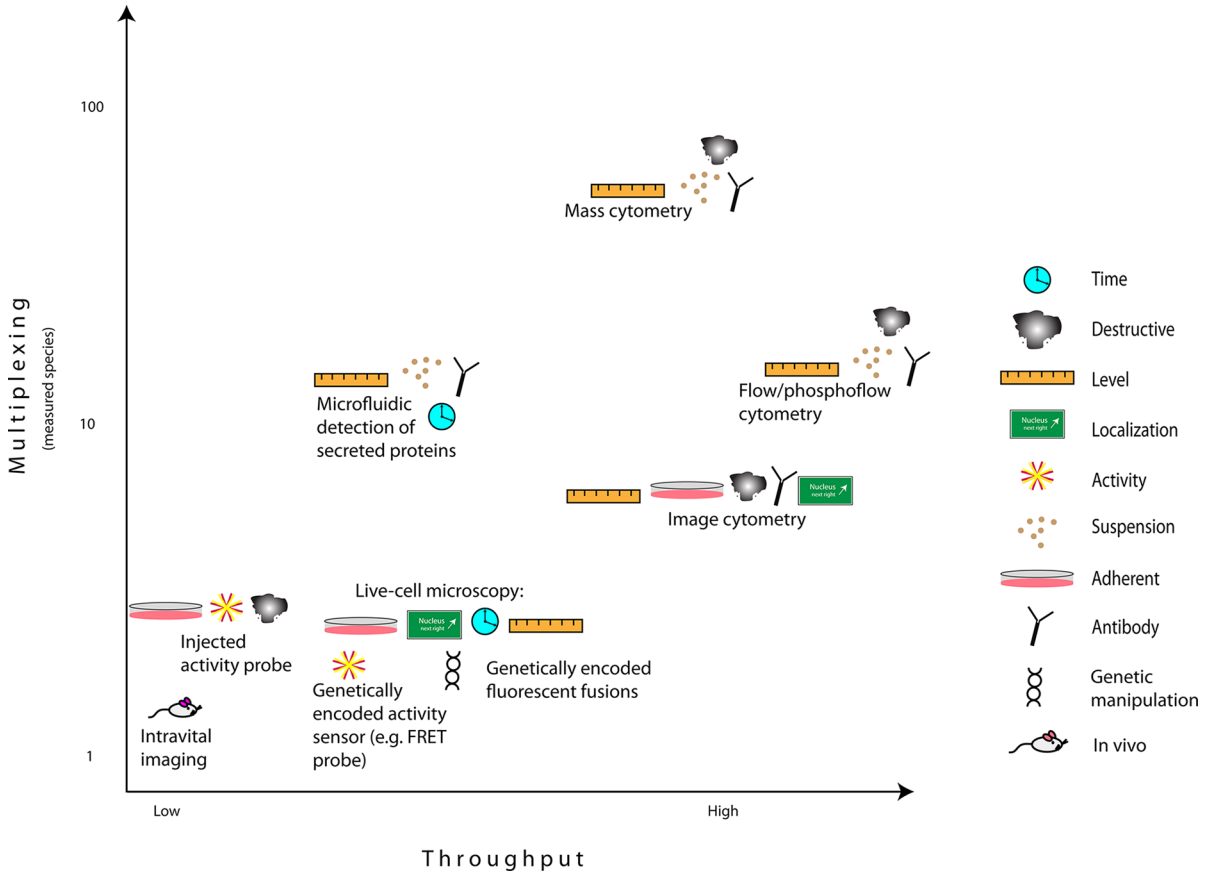


Figure 1. Methods for measurement of signaling proteins from single cells. Methods are placed according to the levels of multiplexing and throughput that they provide. Symbols are given for each technique to indicate advantages, disadvantages, or requirements of that technique, to allow for an easier visual comparison of approaches.

cellular outcomes, multiple subpopulations of cells, or behaviors exhibited by only a small subset of cells.

Some degree of heterogeneity between cells is inevitable as a result of intrinsic noise, an inherent contribution of chance underlying biochemical events.[8] A key issue, however, is to identify contexts in which heterogeneity is important for cell or tissue function. Such a situation could be indicated, for example, by instances of cellular regulation of heterogeneity.[9,10] Such examples are appearing more frequently in the literature. Here we mention two such studies, in which single-cell measurements revealed that population-averaged measurements missed crucial information.

Paszek et al. observed one example of cell-to-cell variability that appears to be regulated by the cell.[11] By altering the time delay between the transcription of two inhibitors of NF-κB (IκBε and IκBα) in mammalian cells, the authors observed that this time is tuned in normal cells to maximize the heterogeneity of NF-κB activity between cells. On the basis of simulation using a hybrid stochastic differential equation model, the authors proposed that this behavior could provide for a more uniform paracrine signal at the tissue level, preventing a potential overload of inflammatory response in any one location.

Another instance of cell-to-cell heterogeneity potentially serving a function for a population was identified by Yuan et al.[12] This study employed multicolor flow cytometry to reveal a bimodal activation of the PI3K pathway in MCF10A mammary epithelial cells upon EGF stimulation. The authors observed that this response was robustly maintained in the cell population, and that cells with activated Akt corresponded to cells with high levels of PI3K. They proposed that maintenance of this bimodality might play a protective role against oncogenicity in these cells.

This study also demonstrated that a subpopulation of cells experienced dramatic dynamic changes in PI3K levels that were not visible by bulk level Western blot, because of the confinement of these changes to a relatively small subpopulation.[12] Such an example represents one general situation in which single-cell measurements are useful, a case in which each of multiple subpopulations of cells exhibits a different behavior. Several other general cases necessitate single-cell resolution. Where absolute levels of a protein are important for a threshold-based binary decision, a measurement at the bulk level will smear out this thresholding, making it appear as though an intermediate level of protein results in an intermediate response, when an intermediate response might never in actuality occur.[13] Similarly, in cases where the timing of an all-or-none decision differs between cells, a bulk measurement might misleadingly make it appear that an intermediate time corresponds to an intermediate level of response. For example, commitment to apoptosis upon treatment with the cytokine TRAIL occurs in a switchlike fashion for each individual HeLa cell, yet the time to commitment varies widely, such that the death response examined at the population level would appear graded.[3]

In addition, single-cell techniques are crucial for understanding processes in which only a few outlier cells exhibit a behavior of interest. For example, cancer cell invasion and

dx.doi.org/10.1021/bi300846p | *Biochemistry* 2012, 51, 7433−7443

metastasis are marked by heterogeneity.[1] Individual cells have been observed undergoing chemotactic migration away from the primary tumor in vivo, and differences in gene expression were observed between these invading cells and cells remaining in the tumor.[14,15] Live-cell tracking data obtained by the Quaranta group using high-throughput automated microscopy showed that invasive cancer cell lines were marked by a greater spread in observed motility, with a few cells showing much higher motility than the majority of the population. It is not yet clear whether these particular cells are the ones responsible for metastasis; further single-cell investigation will be needed to confirm or refute this idea.[16]

MEASUREMENT

Overview of Signal Measurement. Many aspects of cell signaling are accessible at the single-cell level. A number of methods allow measurement of gene expression, levels of secreted and intracellular proteins and phosphorylated proteins, protein localization, and protein activities, in some cases over time. Electrophysiological measurements and monitoring of ion-sensitive dyes are also performed on single cells; our focus, however, will be on protein-level measurements.

Many assays require the destruction of the cell, by lysis or fixation. In such cases, measurements obtained at multiple time points necessarily are taken from different cells, and thus, this approach may increase the difficulty of separating cell-to-cell variation from variation over time. On the other hand, several techniques allow monitoring of live cells over time. Live-cell imaging such as phase-contrast imaging for overall morphological characteristics can be performed nondisruptively. Genetically encoded reporters can also be introduced to monitor expression, localization, or activities of proteins.[17] For a review of approaches for obtaining dynamic signaling measurements, see Spiller et al.[18] Alongside the advantage of time-resolved information, however, each of these live-cell assays carries disadvantages. Making genetic changes risks perturbing the system under study. The processes of microinjection or electroporation used to introduce some nongenetic probes are likely to perturb the cell, and the probe concentration required for monitoring might disrupt the processes of interest. Time-lapse cell imaging requires immense data storage and processing capabilities.[19] Indeed, each signaling assay approach carries associated advantages and disadvantages. Figure 1 provides an illustration of such tradeoffs. For example, while lacking time resolution for a given cell, some destructive assays may be more easily multiplexed or offer higher throughput than live-cell measurements.

Multiplexing, or the ability to measure several characteristics or species from a given sample, is an important aspect of measurement that adds power to the ability to interpret the data. The relationship between different species' variations may be essential for understanding a system, and measurements of species separately from distinct cells may miss this type of information. For example, in a population of cells, species A may be observed at high levels in some cells and low levels in others and measuring marker B separately may reveal the same pattern, yet it may not be possible to determine whether a correlation between the two exists.[9] Modeling techniques for extracting this type of information without performing the multiplexed experiment may be possible in some cases[20] but represent an active area of research, as discussed below. Because of the limited amount of cellular material, the challenge of multiplexing increases when working with single cells. Measurement techniques that utilize some form of signal amplification are therefore helpful, and thus, gene expression measurements employing nucleic acid amplification have held an advantage over measurements at the protein level. However, given the ability to observe multiple turnover from the same enzyme, protein activity can provide a readout that amplifies its own signal, presenting an opportunity in this arena.

Gene Expression. In this review, we focus on protein-level measurements in signaling rather than on genome- and gene expression-level information. However, we point here to several methods that have made it possible to measure gene expression from individual cells.

Several recent reviews discuss single-cell genome and transcriptome analysis methods.[21,22] Methods for single-cell transcriptome analysis include quantitative polymerase chain reaction and reverse transcription polymerase chain reaction via microfluidic device[23,24] and single-cell RNA sequencing (RNA-seq).[25] A recent interesting approach is whole exome sequencing from single tumor cells.[26,27] In addition, microfluidic Sanger sequencing has been used to sequence the genome of single cells.[28] Navin et al. were able to study tumor evolution through the use of "single-nucleus sequencing" (SNS), using whole genome amplification (WGA) and Illumina sequencing to quantify copy number from flow-sorted tumor cell nuclei.[29]

An exciting new approach called stochastic profiling identifies sets of genes that are regulated heterogeneously between cells. This technique accesses single-cell-level information without the need to take measurements of individual cells, but rather of small numbers of cells. Tens of cells are obtained from tissue by laser-capture dissection and interrogated for expression of many genes; this procedure is performed repeatedly, and the fluctuations in gene levels are statistically analyzed for patterns to reveal genes that may be coregulated.[30] This technique has already revealed interesting connections between FOXO and RUNX1 transcriptional programs.[31]

Much work is underway examining noise in gene expression and how it may be mitigated or exploited by cells. For a detailed treatment of this subject, we refer the reader to several reviews,[32−34] as well as an interesting recent study that shows how statistical approaches to analyzing fluctuations in expression can yield useful information about signaling pathways.[35]

Protein Levels and Localization. While gene-level information is useful, information at the protein level better illuminates relevant cell signaling events. A range of methods exists for measuring levels and localization of proteins and phosphorylated proteins from single cells.

Flow cytometry has long been used for measuring protein levels in single cells in a high-throughput manner. This technique requires cell fixation when used to measure levels of intracellular proteins but can also be used for live-cell measurements, in the case of flow-activated cell sorting for surface markers (FACS). However, cell culture conditions are disrupted by this process, particularly for adherent cells, which must be placed in suspension for use in this assay. Flow cytometry-based methods have the advantage of high cell throughput but rely on the existence of reliable antibodies for targets of interest.

Information about post-translational modifications provides yet another level of utility, and in the past few years, phospho-flow cytometry has made it possible to measure the phosphorylation state of intracellular proteins, using phospho-

specific antibodies.[36,37] Multicolor flow cytometry has provided multiplexing for up to 17 simultaneously measured species from a single cell, although for technical reasons typical usage often employs fewer species.[37−39] Because of overlapping spectra, the use of fluorescent tags places practical limitations on the number of species that can be resolved.

The recent technique of mass cytometry evades this limitation through the use of mass tags rather than fluorescent tags and combines the flow cytometry approach with mass spectrometry, allowing resolution on the order of 100 parameters per cell. For a review of this technique and comparison to phospho-flow cytometry, see Bendall et al.[40] Bendall et al.[41] used mass cytometry to measure more than 30 parameters, including both surface markers and internal signaling proteins, from individual primary cells from human bone marrow. The authors were then able to map related cell types using surface markers and to superimpose on that map cell signaling responses under various stimulation conditions, bringing into view a wide picture of signaling in hematopoiesis.

Mass spectrometry technology to allow proteomics on individual cells is still developing. Such techniques tend to require multiple preprocessing steps, making scaling down to the single-cell level more difficult. However, metabolites and peptides present in the cell in larger quantities have been assayed in single cells. For example, the Zenobi group has recently performed metabolomics profiling on single yeast cells using high-density microarrays for mass spectrometry (MAMS).[42] For a recent review of single-cell peptide and metabolite profiling techniques, see Rubakhin et al.[43]

The subcellular localization of proteins provides additional information not accessible by flow cytometry. A number of imaging techniques allow observation of protein translocation within the cell over time. Detailed rates of protein movement can be accessed using techniques that employ photobleaching of fluorescently labeled protein, such as fluorescence recovery after photobleaching (FRAP) or fluorescence loss in photobleaching (FLIP). Fluorescent probes that undergo photoactivation and photoconversion can also be used for tracking protein movement. Fluorescence correlation spectroscopy (FCS) can yield concentrations and diffusion rates by tracking the flux of fluorescent molecules through the confocal volume.[18] For an excellent recent review of the use of genetically encodable fluorescent probes in the study of signaling dynamics, see ref 17.

Genetically encoded probes for monitoring protein localization include fusions of proteins of interest with fluorescent proteins. For example, oscillations in NF-κB translocation have been observed in high throughput at the single-cell level. Tay et al. used a microfluidic platform combined with live-cell imaging to monitor responses of NF-κB to TNFα in thousands of cells over time.[44] Nuclear translocation of a p65 fluorescent fusion was taken as a representation of NF-κB activity. The authors observed that TNFα sensitivity varied by cell and presented a model involving a combination of graded and all-or-none responses of NF-κB to TNFα in individual cells. Assay platforms such as this one are allowing an increasingly detailed look at a topic that has been heavily pursued in the literature.[45−50] For a review focusing on the interplay of experimental and modeling work in this field, see ref 51.

In another study monitoring a fluorescent fusion protein, Batchelor et al. used time-lapse microscopy to investigate the time dynamics of p53 in the response to UV stress of MCF7 cells bearing a p53−Venus fusion.[52] This study observed a graded response of p53 to UV stress, in contrast to earlier work showing p53 pulses with other kinds of DNA damage [double-stranded breaks (DSBs)],[53−55] revealing that p53 exhibits different temporal responses to different types of stress. It will be interesting to see whether these temporally patterned responses encode part of the signal that is then taken in by downstream components. The authors showed that when their mathematical model[53] was adjusted to account for a particular topological difference (lack of a single negative feedback interaction) in the UV response pathway relative to the response to DSBs, the model was able to explain the strikingly different temporal responses.

Fluorescently labeled proteins can be used to obtain other types of temporal information, as well. Eden et al. introduce a "bleach-chase" technique for monitoring protein half-lives in individual cells.[56] This technique allowed the intriguing observation that several drugs that affected the cell growth rate had differential effects on the half-lives of longer- and shorter-lived proteins, such that the half-lives of proteins with longer half-lives under normal conditions were affected more strongly by the drug conditions. Because these differential effects represent drug-induced shifts in the proteome, this finding could potentially have interesting implications for the effects of drugs on signaling networks.

Protein Activity. Information about protein activities is extremely valuable, providing more direct access to actions taking place in the cell. Activity assays performed on cell lysates provide end-point measurements, while genetically encoded reporters allow monitoring of protein activity over time.

Traditional radioactive kinase activity assays are based on an initial step of immunocapture of the kinase of interest, and thus, direct single-cell analogues of this method are difficult to realize. In steps toward this goal, however, Fang et al. have been able to reduce the required sample size to 3000 cells, measuring ABL kinase activity from AML patient samples using a [^{32}P]ATP radioassay on a microfluidic device.[57]

Fluorescence-based measurement of kinase activity from cellular lysates using peptide probes[58] has been demonstrated at a single-cell level of sensitivity through the use of microfluidic devices.[59] Work is in progress to adapt this to use directly with single adherent cells.[60]

Fluorescently labeled peptide probes for kinase activity have also been introduced into cells by microinjection; the cells were subsequently lysed, and capillary electrophoresis was used to separate the substrates and gauge the kinase activities that were present in the cell, allowing the measurement of three kinase activities from a single mammalian cell.[61] This approach, however, is limited by the fact that injection of substrates disrupts the cell, as well as by challenges in achieving specificity of these peptides, their reaction parameters relative to those of the native substrate, and their susceptibility to cleavage within the cell. Moreover, this technique does not allow time course measurements from a single cell, providing only an end-point measurement, although it could be seen as a way of accessing information within the cell that could not be accessed with the kinase out of its native environment.

A technique called activity-based protein profiling has been used with bulk-level cell lysates, making use of "mechanism-based" probes to observe the activity of many enzymes that share a common mechanism but are not necessarily related in sequence.[62,63] This technique observes phosphorylation rates of a panel of peptide substrates using mass spectrometry. While this technique has not been performed at the single-cell level,

Kubota et al. have demonstrated sensitivity down to nanogram amounts of bulk lysate.[64] This is consistent with the sensitivity required for single-cell amounts of lysate, as the amount of total protein in some mammalian cells is approximately one nanogram [in this instance, back-calculated from results of a total protein assay on bulk samples of known approximate cell number obtained from the hepatocellular carcinoma HepG2 cell line (authors' unpublished observation)].

Kinase activities can also be monitored over time in individual cells. A number of genetically encoded FRET sensors make this possible (e.g., for JNK[65]), with the caveat that few signaling activities can be measured simultaneously from a cell: even with dramatic advances in biosensor technology, three simultaneous measurements is the present-day expected limit.[66] To deal with this limitation, Machacek et al. proposed an approach termed "computational multiplexing", a framework for integrating information from independent experiments into an overall model of a signaling network.[67] The authors made use of fluctuations of simultaneously measured activities to infer relationships between these activities and demonstrated an ability to predict relationships between activities measured pairwise across different cells, given common conditions and a subset of activities in common between cells.

Microfluidics-Based Approaches to Single-Cell Measurement. Microfluidic devices are increasingly utilized as a means to allow and automate handling of miniscule samples, to subject cells to carefully controlled cues (e.g., chemotactic gradients), to provide sensitive readouts of biochemical assays using minimal sample amounts, and to observe single-cell behavior over time. Such measurements could include mRNA levels,[68] secreted[69,70] or intracellular protein or phosphoprotein levels,[41] or enzyme activities.[60] Assays performed using microfluidic devices could involve microscopic imaging (or other monitoring) over time of living cells, or end-point assays involving cell fixation or lysis. There are several major considerations in using these devices. If cells are cultured within the device, their growth characteristics in the device must be checked to be comparable to standard culture methods. As with any new assay, technical error components must be carefully characterized and accounted for in these new platforms to ensure the ability to discriminate biologically relevant differences in signal.[70,71] Challenges remain in making new devices accessible for general usage and compatible with existing techniques and platforms, which will allow their utility to be maximized.

One major application of microfluidic devices is in maximizing the amount of information that can be obtained from precious clinical specimens. For example, Sun et al.[71] present a microfluidic image cytometry platform allowing concurrent measurement of levels of four signaling proteins from the PI3K pathway from individual mammalian cells. The authors applied this technology to make measurements on solid tumors (dissociated into cell suspensions), using brain tumor biopsies. Heterogeneity was observed in protein levels between cells within tumors as well as between tumors. Measurement results were demonstrated to be consistent with traditional (but less precise) immunohistochemistry scoring. The authors made use of self-organizing maps[72] to show that patients could be stratified on the basis of these measurements into clusters that correlated with patient outcomes in terms of survival and tumor progression.

The work of Shin et al.[70] incorporates innovative approaches to both measurement and modeling. This study used a microfluidic device to measure levels of secreted protein from single human macrophages stimulated with LPS, to characterize interactions between secreted proteins with and without perturbations. Single stimulated cells isolated into micro-chambers were assayed for a panel of 12 secreted proteins via antibody arrays ("barcodes"). Barcodes were then developed using detection antibodies and fluorescent labels, and calibration curves used to convert these readouts to the number of molecules detected. Notably, the authors did a careful analysis utilizing both experiment and simulation to evaluate the experimental error of this assay and determine contributions from biological differences versus technical error. The authors used the observed biological fluctuations to compute a covariance matrix relating the measured proteins to reconstruct their relationships in a network. Applying principles of maximal entropy, the authors showed that the effects of small perturbations on the system could be predicted on the basis of the fluctuations in protein levels measured in unperturbed cells.

The same group[73] used a similar assay platform to measure levels of phosphorylated membrane and cytoplasmic proteins from cells captured and lysed in nanochambers containing antibody arrays, focusing on proteins in the PI3K pathway in cancer cell lines under several perturbation conditions (e.g., EGF stimulation). Comparing the single-cell measurements to bulk measurements showed that protein−protein interactions could be recapitulated in this system, and that additional information was gained about fluctuations of these proteins from cell to cell that could reveal information about regulation and allow predictions of protein-level responses to perturbations as in the earlier work. A disadvantage of this approach for use with adherent cells is that following stimulation the cells must be trypsinized to be loaded into the device, which could affect the signaling responses under study.

Connecting Signals to Responses. A major goal in investigating signaling is to understand how signaling events lead to phenotypic outcomes. Cell phenotypic behavior can be quantified in a number of ways. Depending on the context, some features already discussed as "signals" could also be considered aspects of phenotype (for example, protein secretion, ligand shedding, or cell surface markers). In addition to characteristics that can be measured as previously discussed, live-cell microscopic imaging allows the observation of individual cell phenotypic behaviors over time such as migration, proliferation, and morphological changes.

To elucidate the connections between signaling and phenotype, it will be useful to have directly comparable signaling and phenotypic data. Practical experimental limitations mean that often phenotypic measurements are made separately from measurements of signaling, and thus, it is necessary to connect signals to phenotype from separate experiments and make optimal use of data on signal and phenotype that come from different cells. Single-cell approaches may provide the ability to assess signaling and phenotype in more closely related conditions. Practical considerations of assays in bulk sometimes necessitate taking these two types of measurements under differing conditions (for example, certain phenotypic assays for migration require sparsely plated cells, whereas signaling measurements made from bulk lysates are typically made on confluent cultures for the technical reason of obtaining sufficient yield). Single-cell methods that allow similar conditions for both signaling and

phenotypic measurements, or ideally measurements of both from the same cell, present a great advantage in clarifying the connection between signals and responses.

New techniques are increasingly making it possible to measure signaling and phenotype concurrently from the same cell. One exciting example is in the investigation of signaling in cancer metastasis. Giampieri et al.[74,75] made use of intravital imaging using fluorescent reporters of TGFβ activity to observe TGFβ signaling in individual breast cancer cells simultaneously with their motility behavior in tumors grown in the mammary fat pad of mice. This approach revealed that TGFβ signaling was necessary though not sufficient for increased single-cell motility and greatly affected the mode of metastasis.

Integrating such data into an understanding of the connections between signaling events and phenotypic outcome poses a significant challenge. Because this mapping is typically governed by multiple inputs and complicated network connections, approaching such a question often demands the aid of modeling techniques.

MODELING

Introduction. Because of the complex nature of signaling and the quantity of available data, intuitive interpretation of signaling networks is increasingly difficult, creating a need for models to interpret signaling data and characterize the networks underlying these observations. Building such models requires quantitative measurements, as well as appropriate computational analysis and modeling methods for synthesizing and interpreting signaling data to gain insight and make predictions.

One initial task in modeling signaling is to map the connectivity between species in a network, first defining which nodes are relevant and then how they interact with one another. Participants in the network can be defined using literature and checked against experiments in the relevant specific context of cell type and conditions. Once a framework is in place to define the species that interact, experiments may be needed to further determine the nature of these interactions. Observations of the system over time, or network perturbations such as drugs that inhibit activation of particular signaling nodes, provide information that can be used to better define these interactions. A model created in this way might then be used to predict, for a given network structure, the effects of certain stimulation conditions over time or at steady state, with or without inhibition of a particular node (e.g., to predict the effects of drugs, or combinations of drugs, in various environmental contexts). Such a model could also reveal previously unseen interactions in the network. For example, Morris et al. used a logic-based modeling approach at the bulk level to evaluate a literature-derived network in a specific experimental context (a hepatocellular carcinoma cell line under treatment with inflammatory cytokines), allowing prediction of unexpected network crosstalk effects (from TGFα to JNK) that were then experimentally verified.[76]

Models of cell signaling contend with a number of challenges, including the fact that relevant events operate over a large range of time scales (from seconds for signaling events to hours or days for phenotypic outcomes such as cell division), as well as a large range of length scales (from nanometers for protein interactions to millimeters for events at the tissue scale). The contributions of physical organization within the cell also have key effects that may be difficult to model. Other potential issues include missing data in data sets, conflicts in the literature, and the difficulty of integrating data from experiments that may not be directly comparable. The use of single-cell data introduces additional complexities, which may include the interpretation of time dynamic data across cells versus within a given cell, stochasticity arising from small numbers of proteins and inherent noise in the system, and a potentially greater likelihood of missing data.

Overview of Modeling Techniques. Kholodenko et al. give an excellent review of methods for modeling signaling networks.[77] Many of these methods have generally been used with bulk-level data but are also applicable for single-cell data. We give a brief overview of methods in use for modeling signaling and provide examples of their use with single-cell data.

When mechanistic information is available for the biochemical interactions of components of a signaling pathway, ordinary differential equation (ODE)-based models are often used to describe the mass-action kinetics of the system.[78] Translating these models to a single-cell level raises several concerns. Stochasticity can play a significant role in single-cell signaling events, so a deterministic model may not faithfully represent events at a single-cell level. Stochastic effects can come into play in differences in the levels of signaling proteins from cell to cell (termed "extrinsic noise") as well as the effects of chance on events governing gene and protein expression and other biochemical events ("intrinsic noise").[8] Spatial inhomogeneity within the cell may also affect modeling strategy, given that many key signaling events occur based on localization, for example, in signaling complexes at the cell membrane; such effects can be incorporated with the use of partial differential equation (PDE) models. For a review of stochastic and spatial modeling approaches for single-cell data, see ref 79.

As an example of an ODE model used with single-cell data, Spencer et al. used live-cell microscopy and flow cytometric measurements to investigate cell-to-cell variability observed in times to death for HeLa cells after stimulation with TRAIL. They were able to closely simulate observed variation using experimentally measured means and deviations of five apoptosis-regulating proteins in a mass-action ODE model for TRAIL-induced apoptosis, suggesting that the variability in this timing resulted from differences in protein concentration between cells.[3] Applications of single-cell measurement and modeling to apoptosis are discussed in detail in a recent review.[80]

Where less mechanistic information is at hand, other modeling approaches can be used to take advantage of available data. At the other end of the spectrum are fully data-driven methods such as clustering, PCA, or PLSR, each of which extracts combinations of variables that describe the most variation in the data.[81] Such an approach can help to identify measured species that correlate with particular aspects of the cellular response. For example, Rivet et al.[82] used a microfluidic chip to lyse and fix cells for imaging for multiple biomarkers and developed a multivariate regression model capable of predicting T cell age.

In the middle of the spectrum of mechanistic detail lie Bayesian networks identifying probabilistic relationships among variables, decision trees that provide rules connecting signals to responses, and logic-based modeling capable of incorporating a degree of mechanistic information in terms of parameters for interactions between species (e.g., in the case of fuzzy logic modeling).[83] These methods are focused on describing how signaling species, and potentially responses, are connected in a network.

Network inference methods such as decision trees and Bayesian networks yield statistical relationships between species. However, data obtained from applying network interventions may allow causal interpretation of these relationships. Such methods require a great deal of data. Thus, high-throughput single-cell level data such as flow cytometric data can be appropriate for these methods. Sachs et al.[84] applied a Bayesian network approach to infer causal interactions between MAPK pathway proteins in a multicolor flow cytometry data set. The authors demonstrated by averaging the single-cell data (and comparing averaged data points to the same number of single-cell data points) that the presence of single-cell resolution was crucial to the accuracy of the network constructed. In a subsequent work, the authors[20] describe a technique for performing network construction without the need for all species to be simultaneously measured, to extend the size of networks that can be modeled beyond the limits of experimentally feasible multiplexing. Luo and Zhao[85] describe additional developments using Bayesian network modeling applied to single-cell flow cytometry data, focusing on pooling information from interventional experiments to obtain relationships between network components and investigate ways of incorporating intrinsic noise and technical error.

Bayesian network modeling affords several useful features. With the use of perturbations, not all nodes need to be measured to define network interactions. Other advantages of Bayesian approaches are their ability to handle missing data, which may be particularly applicable when dealing with single-cell data, as well as providing an estimate of the uncertainty in the model's predictions. The ability to quantify model uncertainty is crucial. It is important to be able to assess how well the assembled network is constrained by the data, because there could be many models (or sets of parameters for a given model) consistent with the data. Useful insight may in fact be gained from interpreting families of models rather than any single model.[76]

Information theoretic approaches have increasingly been employed for understanding the flow of signaling information in networks. In an interesting example of application of this type of technique at a single-cell level, Cheong et al.[86] consider a cell's ability to take in information from its environment in the presence of noise in signal transduction and present a framework using mutual information for how information is transmitted. If transduction is noisy, then it is possible that the same input could result in different outputs and thus the cell could lose information about the input. The authors use the metric of mutual information to provide a quantitative assessment of the number of input values the cell can distinguish and in this way evaluate the fidelity of information flow in NF-κB responses of single cells to TNFα stimulation. While single pathways were seen to transmit few bits of information (e.g., NF-κB could respond to two input concentrations of TNFα, present or absent), it was observed that considering pathways signaling together as part of networks could make up for information lost to noise.[86]

Mathematical Approaches for Preprocessing Data. Modeling methods may be needed to handle and process data even before it can be approached in attempts at modeling for biological insight. Initial mathematical preprocessing of raw data allows for judicious employment of modeling techniques aimed at providing insight into aspects of a cell signaling system or allowing prediction of behavior. For example, the normalization method used may significantly affect the outcome of PLSR; it is often wise to try multiple preprocessing approaches to determine their effects on the resulting model.

Preprocessing approaches are often required when using measurement techniques such as live-cell imaging that involve massive amounts of data. As an example, consider the case of extracting relevant features from images of cells. Loo et al.[87] used a support vector machine-based method to obtain phenotypic features and markers (e.g., actin) from fluorescence microscopy images of drug-treated cells. With this technique in hand, the authors were then able to develop methods for investigating heterogeneity in the population by separating it into subpopulations, as will be discussed below.

As another example, Bendall et al.[41] used a minimal-spanning-tree algorithm (termed SPADE) (a way to map high-dimensional data to a two-dimensional structure that visually represents relationships in the data) to obtain a mapping of cell types by surface markers, to investigate differences in signaling responses between cell types, as discussed above. The authors used PCA to project 13-parameter surface marker measurements down to a single "progression axis" that provided a means for observing how signaling changed along the trajectory of B cell maturation. As the field moves toward gathering increasingly multidimensional data, techniques will be needed for visualization and dimensionality reduction of these data, and such modeling techniques will go hand in hand with this work.[88]

Modeling Heterogeneity. An important means of interpreting single-cell data involves characterizing heterogeneity between cells. A recent review by Altschuler and Wu[9] focuses on ways to characterize and interpret observed heterogeneity and therefore allow its consideration as a meaningful and measurable feature of cell populations. As Altschuler and Wu mention, given a distribution of a characteristic of interest across a population of cells, one question is whether differences in function are implied by the location in the distribution of the measured value for a particular cell. A cell at the edge of the distribution might exhibit behavior similar to that of a cell at the middle, or the response of interest might differ greatly between these two cells. The former situation might occur, for example, if the cell responds in a graded manner to a level of a signaling protein (where increasing levels lead continuously to a corresponding increase in response), while the latter could be the case if the response occurs instead in an all-or-nothing fashion (such that a level above a threshold results in a switch of cell behavior to another state, whereas gradual increases one on side of the threshold or the other do not). A combination of these types of responses could also be the case, as with the combination of graded and all-or-nothing responses of NF-κB to TNFa stimulation observed by Tay et al. in mouse fibroblasts monitored by live-cell imaging.[44]

One way to model cell-to-cell differences is by incorporating methods for determining cell- or subpopulation-specific model parameters. For example, a recent methodological study by Hasenauer et al.[89] combined differential equations modeling with probability distributions on the parameters as a way to model heterogeneity. The authors developed a method based on Bayesian inference for deriving such distributions with simulated flow cytometric measurements. In an example of this type of approach applied to imaging data, Kalita et al.[50] used time-lapse microscopy to observe synchronous oscillations of NF-κB nuclear translocation using a RelA fluorescent protein fusion. The authors used an ODE model to describe the

kinetics of NF-κB translocation, along with Bayesian inference to estimate model parameters. After observing that a single model with fixed rate constants was unable to describe the data and examining cases in which cells were not well fit by this model, the authors were able to distinguish two subpopulations of cells with differing kinetic parameters, such that performing parameter inference for these two subsets of cells separately produced a better fit to the data. Identifying subpopulations of cells with respect to translocation dynamics allowed the authors to then propose factors most relevant to these cell-to-cell differences.

Indeed, to characterize heterogeneity in a cell population, it may be useful to divide the population into subpopulations having differing distributions of the characteristic of interest (for example, different mean levels of a particular signaling protein or several proteins) and either model the behavior of each subpopulation independently or parametrize the same model structure separately for each. As discussed above, if one subpopulation is very limited in number, a bulk measurement might describe the vast majority of cells, but those few cells that differ in behavior might be very important (e.g., a few cells capable of metastasizing away from a tumor). If subpopulations are large and very different from one another, then the bulk measurement may not reflect events in any individual cell (as when a bulk measurement camouflages an all-or-none response as a graded response).

Several papers from the Altschuler group characterize heterogeneous cellular populations as mixtures of relatively few phenotypically distinct subpopulations, and responses of the overall population to perturbation as probabilistic redistributions of cells between these states. Slack et al.[90] use this idea to characterize the responses of cancer cells to drugs, using high-content imaging to read out patterns of spatial heterogeneity in immunofluorescent marker costaining within a culture, identifying subpopulations based on phenotypic features using a Gaussian mixture model. This study observed that similar patterns of heterogeneity were established in cellular responses to drugs of a given class, and that these patterns differed for drugs of different classes. Singh et al.[91] extended this work, asking whether patterns of heterogeneity reflect functional differences between cell populations. Using this technique showed that patterns of heterogeneity in basal signaling levels in untreated cancer cells could predict drug sensitivity, whereas the same was not true of noncancerous lines. Loo et al.[92] used the immunofluorescence microscopy technique to examine the process of 3T3-L1 preadipocyte differentiation. Using a Gaussian mixture model, as in the earlier work, for clustering based on levels of adipogenesis markers to identify subpopulations, heterogeneity was observed in the physical state as well as drug responses of these cells in a manner consistent with the idea that the cell population could be described by a mixture of subpopulations inhabiting phenotypically distinct states.

■ FUTURE DIRECTIONS AND OPPORTUNITIES

Single-cell techniques are needed to resolve situations in which multiple major subpopulations of cells exhibit different behavior, where only a few cells are responsible for a behavior of interest (e.g., invasion and metastasis), or where all-or-none decisions are at work (e.g., cell fate or lineage commitment). Single-cell measurements can also make a crucial contribution in clarifying the mapping between signaling state and phenotype, another component that is blurred by bulk-level measurement. Understanding the connection between signaling state and cellular outcome will be key for our understanding of disease, for example, and our ability to address questions such as which drug treatments might be effective. It will thus be extremely valuable to have data on signal and phenotype for the same individual cells. Microfluidics- and imaging-based techniques will increasingly provide access to this type of data.

Advances in both measurement and modeling can contribute greatly to the field. On the measurement side, improvements in multiplexing as well as throughput will be helpful in achieving more powerful data sets. Microfluidics and other technological advances such as mass tags and improved fluorescent probes are making this a reality. Efforts to make microfluidic platforms easy to use and compatible with more standard resources will also lead to considerable advances in the study of signaling.

On the modeling side, the field needs the ability to connect single-cell and bulk data in a meaningful way and to identify where each type of data is most useful. In the end, it will be valuable to leverage the significant amount of extant bulk data and models and integrate a range of information types into our overall understanding of signaling networks and cell decision processes. Approaches for combining different types of signaling data are broadly relevant beyond the integration of single-cell and bulk data and are being investigated.[93,94] As Albeck et al. note,[94] in some cases a small amount of single-cell data can greatly aid in the interpretation of population-level data.

Treating heterogeneity as a feature of cell populations that can be measured and modeled is a helpful conceptual advance. For example, it could lead to new approaches stemming from the idea that a drug that could reduce heterogeneity might potentially render a population more amenable to treatment. An additional conceptual advance on the modeling side is the use of statistical characterization of fluctuations to extract information such as transcriptional programs (e.g., stochastic profiling) or other network connections.

There is a natural interplay between techniques for measurement and modeling. As mentioned above, many measurement techniques require mathematical approaches to extract information from data prior to the step of extracting biological insight (e.g., Shin et al.). New measurement techniques may therefore necessitate mathematical or computational advances. For example, because of the tremendous amount of data generated by live-cell imaging, improved methods for data handling are needed in parallel with advances in this technology.[19] New and increasingly multidimensional types of data may also require new methods of visualization to aid in their interpretation. For example, as mentioned above, Bendall et al.[41] used projection and visualization methods to facilitate the interpretation of highly multidimensional data, making an overwhelming array of data accessible to visual intuition. In this way, innovations in measurement can drive innovation in modeling, and perhaps the other way around (in making it possible to deal with increasingly complicated data, in identifying the most valuable types of information to obtain, or, for example, in the sense that the stochastic profiling approach allows use of measurement techniques that can access few cells rather than single cells yet still access single-cell-level information).

It would be extremely helpful to know how we might a priori identify those situations in which single-cell techniques would be most useful. We have listed several situations in which the bulk model would be unable to distinguish very different cases

with important differences in biological interpretation and where thus single-cell data are needed. It would be helpful to elucidate defining characteristics of these situations, beyond the observed phenomenon itself, which would allow prediction of the likelihood of such a situation. This is still an open question, although there are increasingly many contexts in which the phenomenon under study is known to have relevance to cellular heterogeneity (e.g., invasion).

CONCLUSIONS

In summary, measurement techniques that can be used on single cells provide a number of compelling advantages. These include the ability to make use of very small samples, which is desirable for decreased reagent consumption but especially crucial when dealing with precious patient samples; an improved ability to resolve differences present in heterogeneous samples, which is also highly relevant for many types of patient samples (e.g., tumor tissue); the ability to access and zero in on a small fraction of the population exhibiting an interesting behavior; the ability to more fully characterize the overall distribution of a behavior in a cell population and to determine whether multiple subpopulations of cells displaying a behavior of interest are present; and the potential to provide a more direct connection between signaling state and cellular phenotype. As such, single-cell assays hold great potential for furthering our understanding of signaling processes in both normal and disease states. While challenges remain in accessing this type of information, techniques for obtaining such data and interpreting it with the aid of mathematical and computational models are advancing and will, we anticipate, lead to exciting and valuable steps forward in our understanding of signaling.

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Funding

This work was partially supported by a Ludwig Fellowship from the Koch Institute for Integrative Cancer Research to S.E.K., by National Institutes of Health Grants R01-EB010246 and P50-GM68762, and by the Institute for Collaborative Biotechnologies through Grant W911NF-09-0001 from the U.S. Army Research Office.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We are grateful to the reviewers for their very helpful critiques, Jesse Lyons and Seth Dickey for valuable comments, and Walter Holland for excellent editing assistance.

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