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Signal transduction at the single-cell level: Approaches to study the dynamic nature of signaling networks

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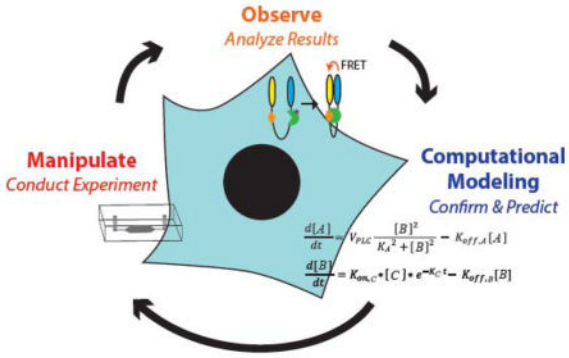
Abstract

Signal transduction, or how cells interpret and react to external events, is a fundamental aspect of cellular function. Traditional study of signal transduction pathways involves mapping cellular signaling pathways at the population level. However, population averaged readouts do not adequately illuminate the complex dynamics and heterogeneous responses found at the single-cell level. Recent technological advances to observe cellular response, computationally model signaling pathways, and experimentally manipulate cells now enables studying signal transduction at the single-cell level. These studies will enable deeper insights into the dynamic nature of signaling networks.

Graphical abstract



Introduction

Defining cellular signaling pathways is important to understand many biological processes including tissue development, immune response, cancer development, cellular growth and migration, and more. Traditional biological approaches to study cellular signal transduction

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include bulk assays to measure cellular response at the population level. Although these studies have been useful in mapping signaling pathways and making qualitative predictions, population averaging can often mask the spatial and temporal dynamics important in physiological processes. Furthermore, population averaging results in information loss regarding biological variability that often has important physiological implications.

Over the last few years, technological advancements in measuring, manipulating, and modeling signaling activities at single-cell resolution has enabled researchers to go beyond the limitations of population-averaged bulk assays. These new studies often reveal that previous knowledge about signaling dynamics at the population level may not be the complete picture. For example, earlier studies of the p53 signaling pathway provided support for damped oscillations to stimulus [1]. However, single-cell studies revealed that there are no damped oscillations in the individual cell. Instead, differences in pulse cycle between cells created a population average behavior showing damped oscillations [2]. More recent work on this pathway elucidates the complex dynamic patterns of p53 activities that cells use to encode and transmit information [3]. Analysis based solely on population level readouts completely masked these insights. Similarly, single-cell studies of the NF-κB pathway show complex digital activation patterns in individual cells that are hidden by population level studies [4,5]. Single-cell studies have uncovered the importance of temporal dynamics in information transmission through cellular signaling networks [6]. Furthermore, single-cell information exposes the complexity of cellular response distribution. The implications of cellular heterogeneity is a key area of research important to understanding fundamental issues such as variability of drug response at the cellular and organism level [7].

The ability to fully comprehend signal transduction at the single-cell level requires advancements in how we observe cells, model cellular behavior, and manipulate biological systems. However, single-cell studies continue to utilize the same overarching approach as traditional population level studies (Figure 1). Observing cells at the single-cell level is now possible using better fluorescent biosensors and single-cell analysis techniques. Additionally, the development of complex computational algorithms can dissect the dynamics and distribution of single-cell behavior found in the complicated and rich datasets produced by single-cell measurements. Computational models confirm intricacies in cellular network behavior that are difficult to elucidate through observation alone. Additionally, predictions made using computational models facilitate directed experiments. Although the specific techniques to manipulate biological systems is different, the overarching theme of changing the internal and external environment of cells remains the same between single-cell and bulk-level assays. In the following review we will discuss the specific methods and developments used to observe, model, and manipulate biological systems to study dynamic signal transduction at the single-cell level.

Observe: Dynamical measurements of signaling activities at single-cell resolution

Fluorescent biosensors and computational image analysis have enabled evaluating the distribution of the cellular response across a population in real-time. Traditional biological techniques such as western blots only provide a population average level readout (Figure 2). As a result, this type of analysis lacks both temporal and spatial resolution. In contrast, fluorescent biosensors can capture dynamic cellular events in living cells at subcellular resolution. Increased cellular, spatial, and temporal resolution has contributed to rich datasets that require image analysis algorithms to fully capture the complexity of the data. Furthermore, meaningful interpretations of single-cell datasets require advanced statistical methods such as dimensionality reduction and information theory to quantify and properly interpret the distribution of cellular behavior.

Sampling Cellular Signaling Networks-Expanding the Palette of Fluorescent Biosensors

Reliable dynamic biosensors are crucial for live single-cell analysis of signal transduction. Fluorescent biosensors function by coupling one or more fluorescent proteins to an activity sensing domain. Biosensors have been developed for a number of different signaling molecules by designing the sensing domain for a specific signaling molecule [8]. The intricate process of designing sensing domains compatible with fluorescent proteins to properly report signaling molecule activity has been reviewed elsewhere and will not be addressed here [8–11]. Rather, here we will discuss advancements in biosensor development that have led to an enriched variety of fluorescent proteins with optimized molecular properties and improved modular design of fluorescent reporters.

Increasing the variety of optimized fluorescent proteins enables multiplex imaging to expand the number of potential biological readouts. Significant efforts have been made to optimize fluorescent proteins to enhance brightness and contrast, increase photostability, and expand the range of available colors, where color is defined as the unique excitation and emission spectra of a protein. For example, cyan, green, and red fluorescent proteins have improved in all three areas with the development of mTurquoise2, mClover3, and mRuby3, respectively [12,13]. Improving fluorescent proteins also enables expanding the range of available colors for a specific molecule. Increasing the variety of colors for a specific molecule allows researchers to select a fluorescent protein color that does not interfere with the spectra of other fluorescent proteins in a given experiment. Researchers now have a much wider range of fluorescent proteins and can choose them based on the needs of the experiment rather than resorting to what is available [10,14].

The development of fluorescent biosensors to report dynamic cellular activity has evolved rapidly over the past 15 years. Kinase activity is of particular interest due to the large number of biological processes regulated by protein phosphorylation such as cell growth, cell cycle, and immune response [15]. The ability to monitor kinase activity using genetically encoded FRET biosensors presented a modular design adaptable to different proteins with increased dynamic range [16]. Zhang et al. first developed a genetically encoded protein kinase A (PKA) activity reporter (AKAR) in 2001 using a generalizable

FRET backbone [17]. The development of AKAR2 enabled measurement of reversible PKA activity by increasing cellular phosphatase sensitivity [18]. By taking advantage of better fluorescent proteins, such as those discussed above, further improvements were made in AKAR dynamic range with AKAR3 and AKAR4, with each iteration brighter than the last [19,20]. AKAR also expanded beyond the commonly used CFP/YFP FRET pair with GFP/RFP AKAR2 and CFP/RFP AKAR3 variants [21,22]. Using improved fluorescent proteins not only increased the dynamic range of the sensors, but also enabled co-imaging with other FRET pairs to increase the number of biological readouts per experiment [22]. In addition to improvements in design and color range, AKARs were also modified to localize to different areas of the cell to measure PKA dynamics in specific locations such as the plasma membrane (AKAR4-Kras) and sarcoplasmic reticulum (SR-AKAR3) [23,24].

The advent and improvements made to AKARs also triggered the development of a multitude of FRET kinase reporters. Perhaps the most intuitive adaptation was to other protein kinases with activity reporters for protein kinase B, protein kinase C, and protein kinase D [25–27]. Other kinase reporters also followed the AKAR design such as the c-Jun N-terminal kinase (JNK) activity reporter JNKAR1 and the extracellular signal-regulated kinase (ERK) activity reporter EKAR [28,29]. To make the AKAR design more generalizable and improve the dynamic range, Komatsu et al developed an intramolecular FRET biosensor with an optimized backbone using a longer linker to make the fluorescent protein pair completely “distance-dependent” as opposed to “orientation-dependent” [30]. The increased length of the backbone, termed the Eevee (EV) backbone, makes the FRET backbone adaptable to kinases and GTPases. Removing the need to optimize each sensor per biological readout makes biosensor development simpler and faster. This backbone was adapted to make FRET biosensors for PKA (AKAREV), ERK (EKAREV), JNK (JNKEV), among many others [30]. FRET kinase biosensors continue to be used to measure single-cell dynamics to solve complex problems while still improving in design. For example, the EKAREV sensor was used to quantitatively measure ERK dynamics during proliferation and was again improved in design to increase the signal-to-noise ratio [31,32].

A deeper understanding of signaling networks requires measuring multiple dynamic biological outputs simultaneously. Although FRET biosensors present advantages over single-protein reporters such as an increased dynamic range, the use of two fluorescent proteins makes measuring multiple biological outputs during a single assay challenging. Regot et al developed a kinase activity reporter that measures the phosphorylation of kinases using reporter translocation rather than fluorescent strength as an activation indicator [33]. Here, rather than comparing the ratio between two fluorescent proteins, the fluorescent ratio between the nucleus and cytoplasm measures the level of kinase activation in a cell. Additionally, using a few simple design principles, KTRs can be adapted for a variety of kinases.

Live single-cell fluorescent imaging provides quantifiable dynamic spatio-temporal data not available with population level analysis techniques (Figure 2). The use of fluorescent biosensors now enables better subcellular resolution in addition to spatial and temporal dynamics of a specific protein, gene, or second messenger of interest. Despite extensive advancements made in fluorescent proteins and biosensors, experiments remain limited in

the number of biological readouts during a single experiment in comparison to assays such as western blots that have a plentiful selection of detection antibodies (Figure 2). This is in part due to limitations set by the number of fluorescent proteins able to be used in a single read-out. Although each fluorescent protein color has a unique excitation and emission spectra, overlap between spectra makes using multiple proteins in a single experiment challenging. Practically, experiments remain limited to a maximum of 3–4 fluorescent proteins depending on the specific microscope configuration. Furthermore, biosensor development requires specific expertise in protein kinetics and structure, constraining the variety of available sensors for specific biological readouts. Continuing developments to improve fluorescent protein properties as well as improved modular designs will open new avenues for improved live-cell multiplex imaging.

Sampling the Cellular Signaling State: Acquiring Cellular Signaling State Distributions

Measuring the signaling state, or the level of activation of a specific molecule in a signal transduction pathway, at the single-cell level based on fluorescent biosensors as described above requires quantifying the fluorescent levels at single-cell resolution. Typically, flow cytometry or fluorescence microscopy approaches are utilized to measure cellular signaling states. Although fluorescent microscopy approaches enable live-cell imaging to fully capture signaling state dynamics, measuring multiple readouts simultaneously remains limited, as discussed above. On the other hand, flow cytometry methods are only able to measure cellular signaling states at a single time point, but are capable of measuring multiple readouts simultaneously. Specifically, maturation of fluorescent flow cytometry methodology has made previously highly challenging and demanding experiments, such as the simultaneous measurement of >10 color channels, more commonplace. In addition, developments in readout technology substantially increases the multiplexing capacity. For example, mass cytometry combines time-of-flight mass spectroscopy with the readout of a flow cytometer. Specifically, single-cells are captured and the concentration of isotopically pure rare metals conjugated to antibodies [34] and nucleic acid probes [35] are measured. Mass cytometry pushes the boundaries of multiplex measurements and can now concurrently measure >40 channels in a single-cell. The wealth of information produced by mass cytometry methods has already made important contributions to understanding the distribution of single-cell responses [36–38].

Unlike cytometry, advances in fluorescent microscopy are not focused towards measuring throughput or increasing multiplexing capacity. Rather, recent developments focus on increasing resolution by utilizing super-resolution approaches. Therefore, hardware used for high-content imaging systems has not significantly changed in recent years. Instead, improvements in software allow better measurements. Developing better analysis software is important since interpreting and quantifying microscopy images is a non-trivial process. Existing microscopes effectively allow image acquisition at rates of a few images per second. Therefore, a standard multi-well overnight acquisition can generate >100,000 images. Manual analysis of these large datasets is practically impossible. Moving from manual to automated image analysis to properly and efficiently identify cells, track cells, and measure changes in biosensor state over time requires computer vision and sophisticated algorithms (Figure 3). The majority of computational image analysis approaches for high-

content screening (HCS) follow a similar workflow [39]. In the first step, called segmentation, cells are identified in the image and distinguished from each other and from regions without cells (Figure 3). Subcellular structures and organelles can also be segmented and associated with their parent cell. In live-cell time-lapse microscopy, tracking cells over the entire sequence of acquisitions is a critical step to obtain a cellular response time-series at single-cell resolution. Additionally, quantifying the phenotypic states of cells, i.e. cellular shape, size, microenvironment, etc, is necessary to better understand heterogeneity between single-cells [40–45]. A large number of cellular segmentation and tracking algorithms have been developed and are available in commercial and open source software packages [46–49].

Sampling Cellular State Space: Challenges in Quantifying the Distribution of Cellular Behavior

While catchy, the phrase “single-cell analysis” is misleading to a degree. Single-cell analysis does not aim to understand the behavior of a particular individual cell. Rather, single-cell analysis aims to understand population behavior by analyzing single-cell distributions. New statistical tools enable the analysis of complex cellular state distributions which enable deeper insights into underlying biology.

Single-cell statistical analysis methods are needed to interpret increasingly complex biological data. Many single-cell datasets not only characterize biological responses at a single-cell resolution, each cellular response is measured at a multivariate level resulting in highly complex datasets. Increasing data complexity makes gaining even an initial intuition of raw data prior to analysis difficult. For example, a typical mass cytometry dataset generates a data matrix of ten thousand rows and thirty-eight columns. Understanding and properly interpreting such a large dataset is non-trivial. To address this complexity a large array of statistical analysis methods have been developed to simplify complex data in a manner that attempts to capture the relationships between cells. This technique, often called dimensionality reduction, is used in the initial stages of data analysis to identify natural groupings between data types(e.g. types of genes). Groupings with similar relationships provide a simplified representation of complex high-dimensional data. A challenge with dimensionality reduction is that the interpretation of the simplified representation is often not obvious. Proper interpretation of the simplified data requires understanding what assumptions were made to simplify the dataset. Techniques like principal component analysis identify a less complex and lower dimensional representation of the data that preserves most of the variance. On the other hand, techniques like isomap, t-SNE and its variants such as viSNE preserve local relationships between neighboring cells [50–52]. In the case of the mass cytometry dataset mentioned above, t-SNE transforms the data matrix from ten thousand by thirty-eight into a simplified matrix of ten thousand by two. This simplification maintains single-cell information while combining information from several distinct readouts (e.g. columns) into two quantities that can be plotted against each other in a standard scatter plot.

The suitability of dimensionality reduction methods continues to expand as single-cell datasets become richer and more complex. However, it is important to note that such methods are a visual aid to initially interpret intricate data and does not provide quantitative

information. Quantitative analysis of single-cell data that does not depend on an initial simplified interpretation requires computational tools that provide meaningful statistics summarizing single-cell response distributions. Fortunately, a large array of existing data analysis methods combined with freshly developed quantification methods is able to accomplish these tasks. One example of the adoption of such methods is the growing usage of information theory in the analysis of variability in signal transduction. Information theory has powerful techniques to measure relationships between random variables [53]. New developments and tools in information theory enable better insights into complex and highly variable cellular responses [6,37,54].

Computational Modeling: Confirm & Predict

The American psychologist Kurt Lewin famously said “There is nothing as practical as a good theory.” [55]. Traditionally, biological sciences have not been as receptive to mathematical modeling as other disciplines such as engineering, physics, and chemistry. A major contributing factor is the difficulty of integrating biological data with mathematical models. Even with a physiologically sound mathematical model, it is difficult to measure or estimate kinetic parameters that produce useful predictions. Despite these challenges, there have been significant advancements in modeling several major signal transduction pathways.

Pioneering works in several important canonical signaling pathways such as calcium, NF-kB and MAPK utilized mathematical modeling to gain biological insights not available using experiments alone. Calcium signaling pathways regulate a multitude of biological process such as transcription, cell motility, and muscle contraction through diverse cellular response patterns [56]. Various mathematical models have been proposed to explain the oscillatory behavior of cellular calcium response. For example, models proposed by De Young & Keizer and Dupont & Erneux rely on the biochemical properties of IP3 channels to explain calcium induced calcium release [57,58]. Other models consider ryanodine receptor and voltage-gated channels to explain this phenomenon [59,60]. In addition to models describing cellular calcium oscillations, there are numerous models describing calcium spikes in non-excitable cells by incorporating surface receptor signaling and receptor desensitization parameters [61,62]. The NF-κB pathway provides an additional important example of the utility of mathematical modeling. Initial work by Hoffmann et al. provided a comprehensive mathematical model of the IκB-NF-κB signaling module [63]. Hoffmann et al successfully accounted for the population level temporal behavior of NF-κB in the EMSA data using their model. A classic study by Huang & Ferrell on the MAPK pathway predicted that the MAPK signaling cascade would produce a steep dose response curve, allowing cells to switch from one state to another [64]. This prediction was subsequently supported by experiments.

Cellular heterogeneity measurements, as indicated by single-cell datasets, motivate the use of mathematical models to uncover the underlying causes of cellular heterogeneity based on the mechanistic details of the signaling pathway. Albeck et al. and Spencer et al. utilized computational modeling to explain the heterogeneous apoptosis behavior in the TRAIL pathway [65–67]. By changing the rate parameters downstream of the TRAIL receptor or the protein concentration levels in the mathematical model, heterogeneity in timing delays to

apoptosis can be accounted for in the simulated data. In another study concerning the TRAIL pathway, Eissing et al. used a properly reduced model to perform a bistability analysis to deduce the diverging behaviors in the TRAIL pathway [68]. Nelson et al. used computational modeling in conjunction with single-cell NF-κB measurements to demonstrate how varying transcriptional activity of IκBα can alter the NF-κB oscillation frequency [69]. Lee et al. parameterized a dynamic model of NF-κB induced transcription using a combination of single-cell nuclear NF-κB measurements and transcript numbers in the same single-cells [70]. From their model they were able to determine how cells can detect a fold-change in NF-κB levels as opposed to absolute concentrations to induce transcription. Nonlinear systems analysis techniques, such as bifurcation analysis, were employed by Koenigsberger et al. to model calcium oscillations to study the mechanisms for smooth muscle cells to synchronize their oscillations [71].

Mathematical modeling is a useful tool to gain useful biological insights at the single-cell level. Feedback properties of signaling pathways can also be exploited to study the underlying causes of cell-to-cell variability. Birtwistle et al. observed bimodal behavior in the MAPK/ERK cascade which produces two populations of ppERK output in response to EGF stimulation [72]. By inspecting the negative feedback loop structure in the mathematical model of the pathway and performing computational simulations, they deduced that the heterogeneity in RasGTP levels coupled with varying ERK activation threshold levels eventually produced bimodal behavior in the cell population. Ferrell et al. studied the progesterone stimulation of the MAPK pathway leading to oocyte maturation [73]. While the population average data conveyed a graded response, single-cell data revealed an all-or-none response. Analysis of the mathematical model of the pathway showed that bistability and positive feedback within the pathway provided the switch for cell fate decision making in the cell. Using single-cell microfluidic perturbations in combination with data-driven clustering of dynamic ERK profiles, Ryu et al were able to construct an updated MAPK model to determine differences in cellular fate decision making [74]. Ultimately they were able to use their mathematical model to determine how cellular fate decisions can be rewired with different growth factors. Feinerman et al. studied T-cell receptor signaling using both single-cell data and mathematical modeling [75]. They investigated how variation in signaling proteins changes cellular responsiveness. Interestingly, the co-receptor and negative feedback loop of SHP-1 together regulate the activation threshold and the switch behavior of the cell's responsiveness to regulate the diversity of cellular phenotypes. A number of other models based on single-cell datasets have been developed for a variety of signaling systems [76,77].

In addition to moving from population level to single-cell level analysis, there has also been an increasing focus on adapting modeling approaches to account for biological noise and model parameter uncertainty. Elowitz et al. succinctly described intrinsic biological noise, the stochastic thermal fluctuation internal to the system, and extrinsic biological noise, the fluctuation external to the system of interest and is deterministic within the same cell but different among cells [78]. Others in the biological modeling community such as Janes & Lauffenburger and Gutenkunst et al. have indicated that the structure of a model is more important than the individual kinetic parameters when making predictions because multiple sets of kinetic parameters can fit a model equally well [79,80]. This implies that assigning

distributions of parameter values to the model will increase single-cell model predictability (Figure 4). Tay et al. described the level of NF-κB and TNFR-1 using lognormal parameter distributions in order to account for extrinsic noise between cells [4]. Cheng et al. applied probabilistic based mathematical modeling to the TRIF pathway [81]. They modeled four key parameters in the areas of TLR4 synthesis, MyD88 activation, TRIF activation, and endosome maturation as probability distributions rather than as fixed values. The simulated signaling pathways generated similar heterogeneous behaviors as found in the experimental data. Eydgahi et al. used Bayesian and Monte Carlo methods and calibrated a mathematical model of apoptosis single-cell data to obtain probability distributions for all kinetic parameters in the model [82]. This approach allowed for discrimination between competing mathematical models of apoptosis.

Overall, the methods discussed above made specific assumptions regarding the shapes of the distributions of certain kinetic parameters. A possible alternative approach is to fit the individual cell trajectories to the mathematical model using Bayesian sampling methods such that each cellular trajectory will have a set of parameter distributions. A potential advantage of using direct Bayesian sampling is that it assumes little concerning the form of parametric distributions that could potentially have complex structure not initially assumed by researchers.

Manipulate: Biological Insights through Internal and External Manipulation

Experimentally investigating a cellular signaling system involves either changing the outside or the inside of a cell. The outside is perturbed by changing the cellular environment by either adding or removing a specific factor. Similarly, the inside of a cell can be changed by over expressing or removing specific genes of interest. Although the concept behind experimental manipulation does not change with single-cell techniques, the specific techniques used to manipulate cells has evolved.

Microfluidics: Environmental Changes at a Micro Scale

The application of microfluidics to biological research has powerful implications for single-cell signal transduction measurements. Microfluidics enables the researcher to manipulate fluids with a high level of control at the submilliliter scale. This high level of control permits spatial and temporal manipulation of the cellular environment. The ability to customize a microfluidic chip to the specific needs of an experiment removes previous limitations set by currently available tissue culture technology. The complexity of a microfluidic chip depends on the specific biological question. Complexity ranges from very simple to extremely complicated depending on the biological phenomena in question (Figure 5). The increased usage of microfluidic devices in biological research over the past two decades has been extensively reviewed elsewhere [83–86]. Here we discuss the spatial and temporal benefits of microfluidics devices that range from very simple to highly complex.

Temporal modulation allows researchers to control the duration and frequency of changes to the cellular environment. Environmental changes include modifications to growth media conditions or stimulating cells via a perturbation of interest. No longer are researchers limited to population level bolus additions of stimulus at a single time point. In the Suel lab,

media conditions are manipulated to determine the specific growth mechanisms of biofilms [87,88]. Here, Prindle et al. use an unconventionally large microfluidic design to observe the growth of a biofilm [87]. Although there are only two ports allowing media to flow in and out of the cell chamber, they were still able to add and remove specific components of the media to determine factors necessary to biofilm growth. Another simple design by Herson et al measures the signaling pathway response over varying input frequencies [89]. In order to ensure rapid media changes with distinct period times, Herson et al employ a "Y" design where each arm of the Y is connected to a different media solution. The simplicity of these designs enables easier manufacture and implementation of the design. However, they are limited in throughput and can only change between two different inputs. On the other end of the spectrum, the Tay lab uses a complex microfluidic design that is able to precisely vary the duration and dose of the stimulus at a high throughput level to determine how cellular outputs are influenced by dynamic inputs [90–92]. However, the complicated design requires higher technical expertise.

In addition to temporal stimulation, microfluidic device designs also allow for spatial modulation. Spatial modulation is important in cases where gradient information is important such as in wound healing or chemotaxis. Spatial perturbations are possible using microfluidic devices due to the low Reynold's number characteristic of microfluidics devices. This property ensures that any gradient formation and mixing is due to diffusion rather than convection within the device. Handly et al. take advantage of this property to study paracrine communication of the initial wound response using a simple two-layer microfluidic design [93]. On the bottom is a cell chamber and the top an air layer. The ceiling of the cell chamber contains a pillar such that when air pressure is increased in the air layer the pillar lowers down onto the cells to mechanically wound the cells. The lack of convection within the device ensures that any molecules released into the extracellular environment move between cells according to diffusive principles rather than flow through the device. Chen et al. employ a more complicated design to study the migration of heterogeneous tumor cells at the single-cell level [94]. Their device design involves capturing single cells in narrow capillaries and applying a gradient across the capillary to mimic concentration gradients of chemokines that induce tumor cell migration. The formation of this gradient directly takes advantage of even-mixing facilitated by diffusion in microfluidic devices. The geometry of these capillaries also imitates the shape of blood and lymphatic capillaries to provide a more physiologically relevant study of tumor cell migration. Again, although each device design probes a spatially relevant biological question, the degree of device complexity is determined by the specific needs of the experiment.

Optogenetics- Intracellular Manipulation through Extracellular Stimulus

Advancements in optogenetics have opened up the possibility to expand spatio-temporal stimuli to intracellular signaling states. Traditional cellular manipulation involves perturbing the entire population of cells through genetic or pharmacological manipulations. However, these types of irreversible population level changes do not allow for selective spatial activation and dynamic temporal inputs which are important in testing hypotheses related to cellular communication. Optogenetic approaches use light to perturb genetically

manipulated cells and can do so in a spatial and temporal manner to investigate cellular signal transduction (Figure 6).

Although initial applications of optogenetics focused on neurobiology, the ability to regulate intracellular signaling pathways in a spatio-temporal manner using photoactivatable proteins has made optogenetics a functional tool to study signal transduction. The application of light-activated proteins varies from conformational changes to uncaging. However, the basic ability to perturb an intracellular signaling pathway with high spatio-temporal resolution remains the same. Toettcher et al. developed optogenetic tools able to activate isolated signaling nodes within the cell to determine how different temporal inputs regulate downstream responses [95]. Using a photoactivatable Ras protein, they measure how the dose and frequency of Ras activation determines downstream ERK response. The kinetics of the Raf/MEK/ERK pathway were also investigated using optogenetics by Zhang et al [96]. Using temporal activation patterns they were able to induce PC12 cell differentiation similarly to NGF stimulation.

Optogenetics also enables precise spatial manipulation of cells, either at the subcellular or multicellular level. This type of stimulation is important when examining specific proteins required for cellular behavior or determining how gradient formation determines cellular response. Wu et al. utilize a photoactivatable Rac to produce cell protrusions and ruffling at specific subcellular locations to control the direction of cellular motility [97]. The Gautam group developed an optogenetic method to spatially manipulate GPCRs at a subcellular level to create gradients of GPCR activation within the cell [98,99]. By forming gradients of GPCR activation within cells they were able to control the movement of the immune cell. At the multicellular level, optogenetics enables experiments where only a specific portion of cells are stimulated to determine how the surrounding cells respond to the stimulated cells. Wang et al. demonstrate this principle by activating Rac using light in a single cell within a cluster of border cells in Drosophila [100]. Although only a single cell was activated, communication between cells caused the other cells within the cluster to move according to the activation level of the initial cell. The ability to precisely localize activation in a group of cells has exciting potential in the realm of cellular signaling. These types of experiments are useful when considering gradient formation across a tissue during embryonic development, wound healing, in addition to cellular migration.

Combining the spatiotemporal control of optogenetics with the genome editing abilities of CRISPR-Cas-9 has exciting potential in the study of signal transduction at the single-cell level. At the fundamental level, genome editing using CRISPR-Cas9 is effective in population level studies. However, advancements in light-inducible CRISPR-Cas9 systems [101–103] enables researchers to make specific edits to the genome with high spatiotemporal control. Combining optogenetics with CRISPR-Cas9 enables temporal activation of specific genes to study biological outputs as well as activation of specific genes in localized regions. The development of more optogenetic systems that utilize CRISPR-Cas9 will provide an exciting set of tools in the study of single-cell signal transduction.

Combining Single-Cell Manipulation Methods for Effective Study of Signal Transduction

Using microfluidics, optogenetics, and CRISPR-Cas9 allows for controlled manipulation of both the intra- and extracellular environment. Although advances are being made to combine these technologies, such as with the photo-inducible CRISPR-Cas9 systems mentioned above [101–103], studies that fully integrate all three technologies is difficult. A combination of these three approaches has powerful abilities in controlling the spatial and temporal manipulation of cellular environment and function. As these technologies mature and become commercially available it will increase the adaptation of systems biology approaches to study signal transduction. For example, user friendly microfluidic designs or commercially available microfluidic-like devices expand single-cell study beyond traditional cell culture tools. Additionally, increasing usage of CRISPR-Cas9 and optogenetic technologies will expand the available selection of target genes and signaling systems.

Outlooks

Approaches to study signal transduction networks at the single-cell level are in a renaissance period. The ability to observe, manipulate, and model biological systems using constantly advancing single-cell techniques drives new discoveries and enables deeper insights into the inner working of cells. As cellular dynamics and heterogeneity are key aspects to understanding signaling pathways, the adoption of single-cell approaches is critical for future progress. One of the key challenges that limits the adoption of these approaches is technical. Single-cell studies require tools from engineering, biology, and computer science. Unfortunately, these three disciplines are not well integrated in traditional curriculum. However, this is changing with increasing numbers of undergraduate and graduate programs emphasizing the importance of quantitative training.

Here we outlined recent advances pivotal towards progress in understanding the dynamic nature of signaling networks at a single-cell resolution. In parallel, other single-cell technologies that can probe the internal state of the cells have made tremendous progress. Omics technologies, including both “molecular profiling” and “molecular perturbations” [104] provide rich datasets useful with many benefits over currently available microscopy methods. Omics technologies have the ability to examine thousands of genes, proteins, and post-translational modifications at one time whereas microscope technologies are limited in the number of nodes within a network they are able to monitor concurrently. We anticipate that integrating single-cell approaches within signal transduction, such as measuring dynamic single-cell signaling responses, with OMICs single-cell approaches, such as RNAseq, will play a major role in future work concerning signaling networks.

Signal transduction studies at the single-cell level provide information about the dynamic nature of biological signaling networks. Although these approaches follow the same scientific methodology of hypothesis, experiment, analysis, and conclusion, the specific approaches to decipher the intricacies of single-cell variability differ. Advancements in these technologies have come a long way to make answers to biological questions at the single-cell level possible. Future advancements of single-cell approaches and integration with other technologies shows promise for exciting developments in understanding biological network dynamics.

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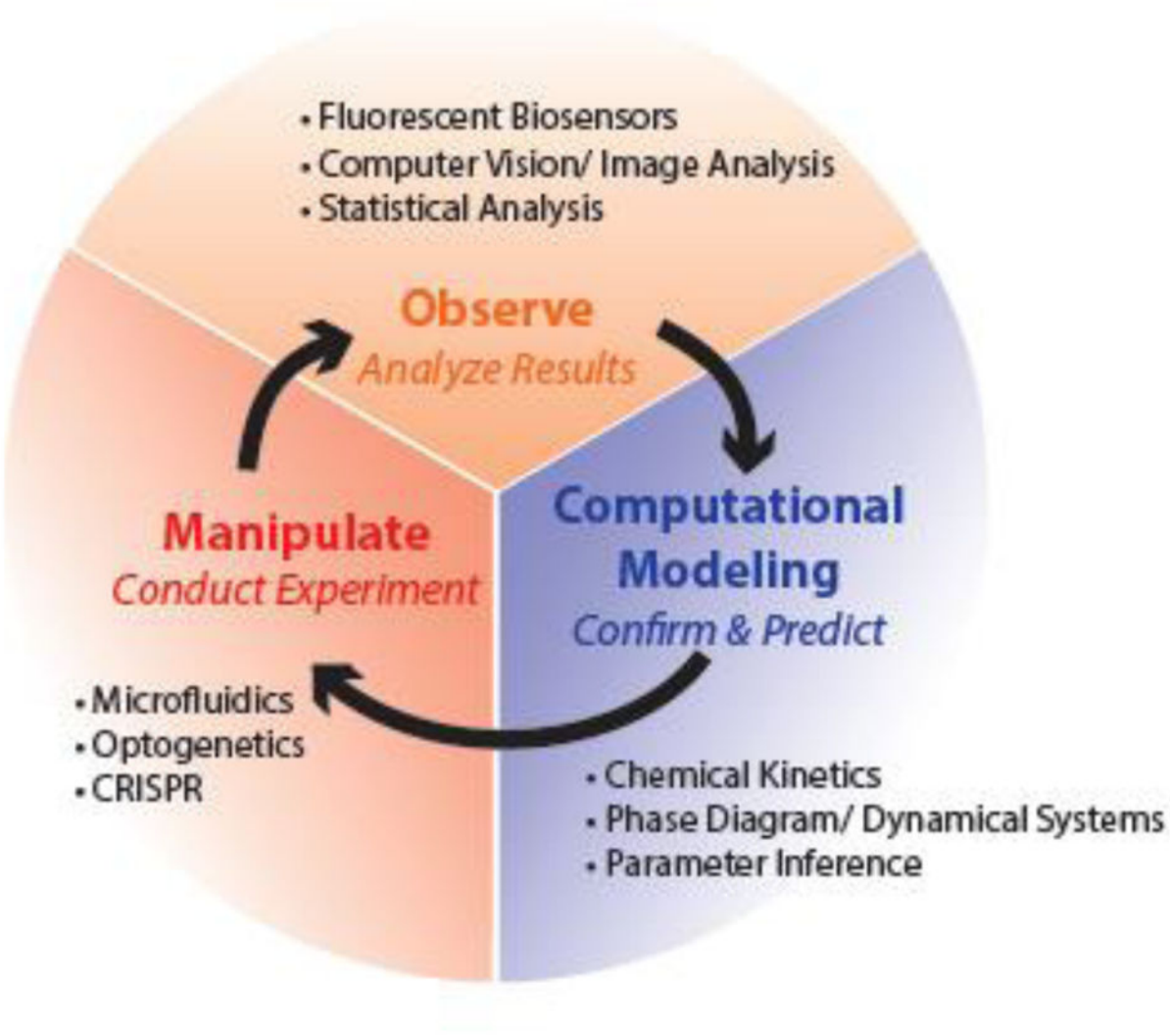

Figure 1. Studying Signal Transduction at the Single-Cell Level

Advancements in observing single-cells, computational modeling, and techniques to manipulate cells in a spatio-temporal manner enable insights into signal transduction at the single-cell level.

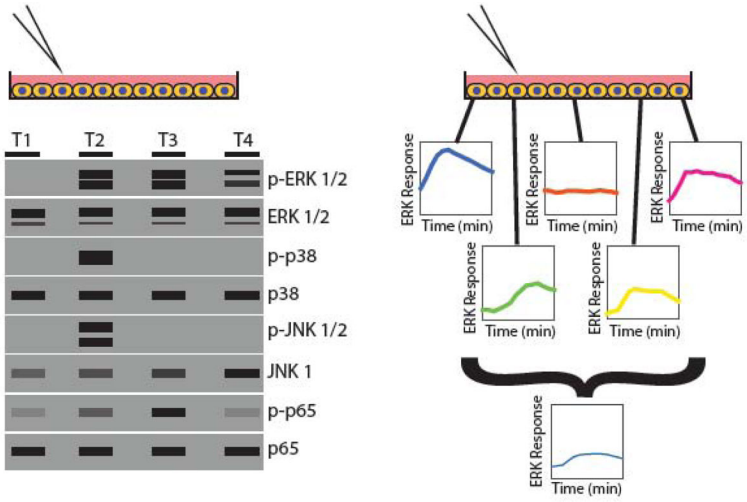

Figure 2. Bulk Assays vs Single-Cell Assays to Study Response Dynamics

Bulk-level assays, such as Western blots, provide population level response with limited temporal resolution (T1–T4, left). Fluorescent microscopy using biosensors shows the distribution of the population response at the single-cell level (right).

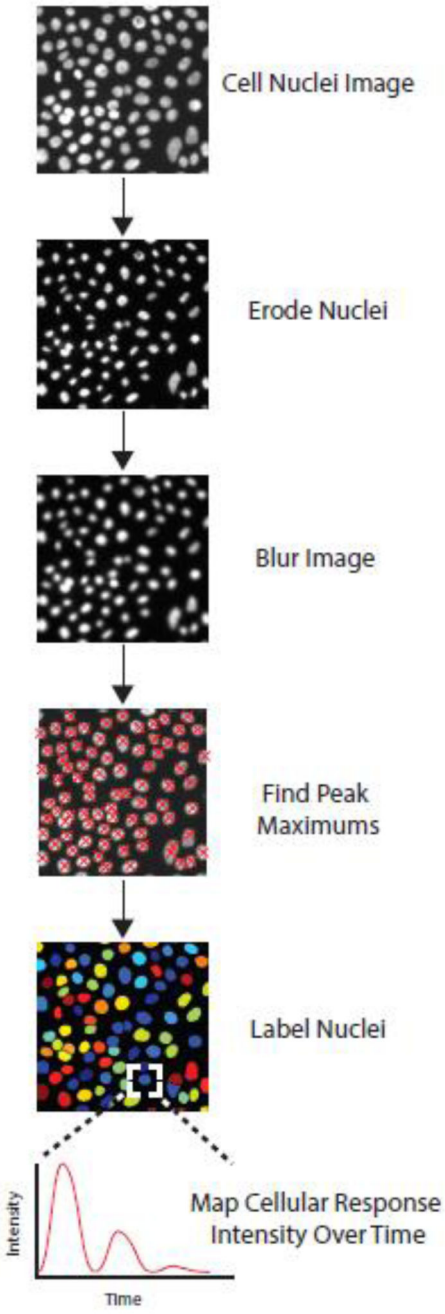

Figure 3. Cellular Segmentation Analysis

Cellular segmentation separates cells from each other and the background of the image. A raw image of the cells (here an image of nuclei stained with Hoeschst is shown) undergoes a sequential process to eventually create a label for each cell. A series of images showing the response can be mapped to this cell label to create a cell response time series.

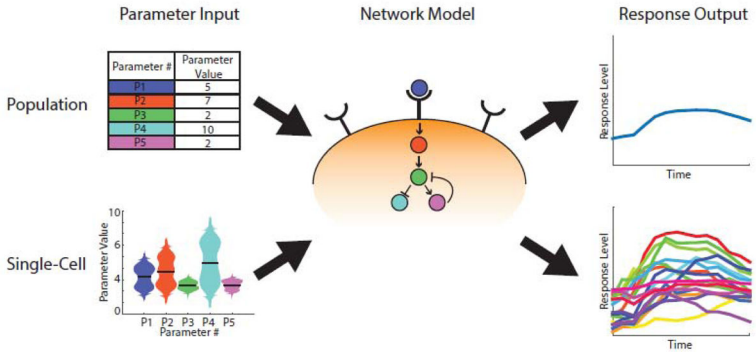

Figure 4. Population vs Single-Cell Computational Model Parameterization

Although a network model between population and single-cell level models remains the same, differences in parameter selection and distribution elicit different response outputs. Population level parameters have a single value per parameter whereas single-cell level parameters consist of a range of values. Population level parameters provide a single response output that represents that entire population of cells whereas single-cell level parameters show the distribution of responses within the population.

Figure 5. Complexity of Microfluidic Chip Design Depends on Desired Experimental Output

Simple microfluidic designs (left) do not equate to less information in comparison to complicated designs (right). Rather, the complication level of each design depends on the required information from the experiment whether it be spatio-temporal dynamics of wound response (left) or cellular response to dynamic, temporal inputs (right, image courtesy of Savas Tay at the Institute for Molecular Engineering at the University of Chicago).

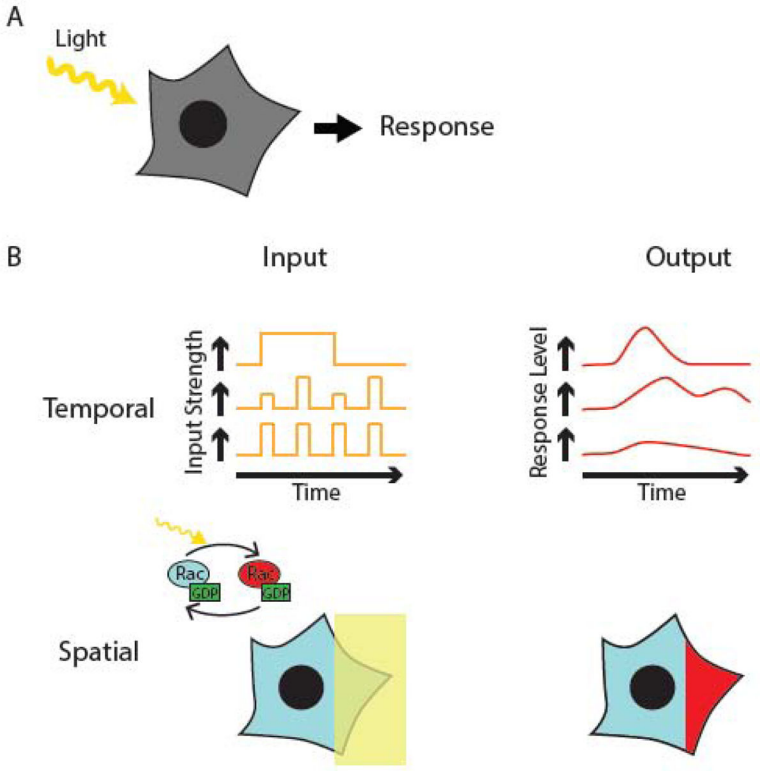

Figure 6. Optogenetics enables controlled spatial and temporal inputs

A. Genetically manipulated cells are perturbed by light to elicit a response. B. Cells can be manipulated with light both temporally and spatially using optogenetics. Dynamic light inputs can generate varying cellular response outputs (temporal). Optogenetic control of specific signaling molecules, such as the GTPase Rac, enables precise spatial control over which area of the cell is activated (spatial).