Published in final edited form as:

Trends Biotechnol. 2016 June ; 34(6): 458–469. doi:10.1016/j.tibtech.2016.02.009.

Redefining signaling pathways with an expanding single-cell toolbox

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Abstract

Genetically identical cells respond heterogeneously to uniform environmental stimuli. Consequently, investigating the signaling networks that control these cell responses using “average” bulk cell measurements can obscure underlying mechanisms and misses information emerging from cell-to-cell variability. Here we review recent technological advances including live-cell fluorescence imaging-based approaches and microfluidic devices that enable measurements of signaling networks, dynamics, and responses in single cells. We discuss how these single-cell tools have uncovered novel mechanistic insights for canonical signaling pathways that control cell proliferation (ERK), DNA damage responses (p53), and innate immune responses (NF- κ B). Future improvements in throughput and multiplexing, analytical pipelines, and *in vivo* applicability will all significantly expand the biological information gained from single-cell measurements of signaling pathways.

Embracing the instructive power of cell-to-cell variability

Signal transduction networks precisely regulate cellular processes in response to environmental stimuli. Paradoxically, the level and states of signaling proteins vary significantly between genetically identical cells. Recently, attempts to measure and explore the consequences of this cell-to-cell variability have surged. This trend is closely coupled to technical advances in single-cell approaches. The natural perturbations provided by cell-to-cell variability directly enable the discovery of novel signal–response relationships [1]. Indeed, measuring signals and biological responses in the same single cell can reveal mechanisms of regulation that would otherwise be obscured in a cell population.

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In this review, we highlight how technological advances in single-cell measurements have been used to gain fundamentally new insights into canonical signaling pathways. While single-cell approaches have been applied to many pathways, including those regulating wound healing, cell migration and chemotaxis (e.g. PKC, PKA and calcium signaling), we focus here on three pathways with key functions in oncogenesis and immunity: the **extracellular signal-regulated kinase (ERK)** pathway (see Glossary), which regulates cell proliferation; the **p53** pathway, driving the DNA-damage response; and the **nuclear factor-kappaB (NF-κB)** pathway, a key transcription factor in inflammatory and stress responses (Box 1).

From immunoblotting to fluorescent reporters and optogenetics in the ERK pathway

In a pioneering example of single-cell analysis of signaling pathways, Ferrell and Machleder used immunoblotting to measure ERK phosphorylation in individual progesterone-treated *Xenopus leavis* oocytes [2]. While bulk measurements showed a graded ERK response – with more progesterone, more of ERK was phosphorylated – single-oocyte responses were “all-or-none” – ERK was always either completely phosphorylated or completely dephosphorylated. This all-or-none ERK phosphorylation was shown to arise from coupling an ultrasensitive cascade with a positive feedback loop within each cell [2].

Fluorescent reporter proteins and ERK oscillations

Most mammalian cells are much smaller than *Xenopus* oocytes and much too small for single-cell conventional immunoblotting. Microfluidics-based single-cell immunoblots were recently developed [3], but have not been widely applied. However, the development of **fluorescent protein (FP)-based reporters** has enabled visualization of signaling dynamics in single cells. While immunoblots provide single endpoint measurements, live-cell imaging can track signaling *dynamics* within single cells. Cohen-Saidon and colleagues fused a fluorescent protein to ERK (FP-ERK) at its endogenous locus in human non-small cell lung cancer cells and tracked nuclear translocation of FP-ERK following epidermal growth factor (EGF) stimulation [4]. They observed that while the absolute amount of ERK translocating to the nucleus varied considerably between cells, the maximum fold-change over the basal nuclear ERK abundance was much less variable. They further observed that some cells exhibited multiple peaks in ERK nuclear localization. Using a similar strategy, Shankaran and colleagues expressed FP-ERK in human mammary epithelial cells and observed sustained asynchronous oscillations in nuclear FP-ERK across cells stimulated with EGF [5]. These single-cell measurements confirmed the existence of ERK oscillations predicted much earlier by Kholodenko’s theoretical models [6]. Quantitative characterization of these oscillations revealed they were dependent on cell density and required continuous EGF stimulation; it would take additional single-cell tools to derive mechanistic insights.

More recently, in an elegant example of multiplexing single-cell measurements within one pathway, Albeck and colleagues combined several live-cell reporters to study EGF-induced ERK activity [7]. A **Förster resonance energy transfer (FRET)**-based ERK activity reporter tracked near real-time ERK activity (Fig. 1A; [8]); a Fra-1-based integrative

reporter of ERK (FIRE) mimicked downstream effector activity as FIRE stability, and thus fluorescence, increased upon phosphorylation by ERK (Fig. 1B); and finally, accumulation of fluorescent protein-tagged geminin reported S-phase entry (proliferation). They observed short, asynchronous pulses of all-or-none ERK activity, with pulse frequency and duration increasing with EGF concentration (Fig. 2, Key Figure, A). Monitoring FIRE, they showed that effectors integrate ERK activity over time, and thereby tune the S-phase entry probability. Interestingly, ERK pulse frequency modulation was found to be EGF-specific; stimulation by neuronal growth factor (NGF) via the TrkA receptor modulates ERK activity amplitude instead [9]. Therefore, the feedback mechanisms regulating pulse duration are not contained within the ERK pathway, which is shared between both receptors, but rather reside at the receptor level.

Alas, FRET sensors limit measurement multiplexing in individual living cells because they require two fluorescent proteins. To circumvent this, Regot and colleagues developed kinase activity biosensors that convert phosphorylation to nuclear translocation [10] (Fig. 1C). This **kinase translocation reporter (KTR)** technology is generalizable to multiple kinases, enabling them to multiplex dynamic measurements of ERK and two other mitogen-activated protein kinases (MAPKs), p38 and c-Jun N-terminal kinase (JNK), in the same cells. JNK and p38 are generally activated by stress stimuli while ERK is not, yet crosstalk has been observed between these pathways. Regot and colleagues observed frequent fluctuations in basal ERK activity (similar to those observed using FRET reporters), and less prominent fluctuations in basal p38 and JNK activity. The stress stimulus anisomycin transiently increased p38 and JNK activity in every cell, while a few cells showed a brief ERK activity pulse that quickly dissipated. However, if a p38 inhibitor was added after anisomycin, both ERK and JNK activity increased in cells with decreased p38 activity, demonstrating that p38 negatively regulates the ERK and JNK pathways.

While we have focused on live-cell reporters, fixed-cell imaging is also informative. For neuronal differentiation within a PC-12 cell population, sustained ERK activity must follow NGF treatment [11]. However, Chen and colleagues combined phospho-ERK and phospho-Akt immunofluorescence to show that instead of phospho-ERK alone, each cell's phospho-ERK to phospho-Akt ratio determines proliferation vs. differentiation [12]. The ratio boundary is regulated by a negative feedback loop from the Akt to ERK pathway, elucidating how complex signaling dynamics can result in relatively simple, single-cell behavior control mechanisms.

Optogenetics to control signaling dynamics

Perturbations are some of biologists' best tools: overexpression, knockouts, drugs, etc. Many signaling studies rely on varying stimulus duration and/or amplitude, but **optogenetics** permit very precise temporal control of activation of *intracellular* signaling proteins and inherently integrate with imaging. To investigate if and how signaling information can be dynamically encoded, Toettcher and colleagues linked ERK pathway activation to light-induced membrane localization of **opto-SOS**. SOS is an upstream regulator of the Ras-ERK pathway which can only interact with and activate Ras when membrane-bound [13] (Fig. 1D). Leveraging their approach for single-cell input-output relationship measurements, they

used light to vary membrane-bound catalytically active SOS (**SOS_{cat}**) abundance and thus Ras activity, and then measured nuclear ERK. Repeatedly returning to the same cell, they generated single-cell dose-response curves. These varied between cells yet were reproducible within the same cell, elegantly demonstrating that single cells discriminate input information more precisely than population-level data suggests. Furthermore, population-level proteomics measurements after sustained vs. transient light exposure revealed that some proteins respond only to sustained ERK. Of these, phospho-STAT3 responded to a 2-hr sustained signal but not two 1-hr pulses of the same amplitude, demonstrating that distinct dynamics trigger distinct downstream responses.

Combining single-cell measurements for mechanistic insight

Using their ERK FRET-biosensor [8], Aoki and colleagues observed, as did Wiley and colleagues [5], basal ERK activity pulses of cell density-dependent frequency [14]. Intermediate cell densities yielded the highest ERK activity frequency and were linked to increased cell proliferation (like EGF-induced high-frequency ERK pulses [7]). Intriguingly, they noted that a cell's ERK activity pulses were reproducibly propagated to neighboring cells. To investigate the propagation mechanism, they combined three experimental methods: they optogenetically activated Raf (upstream of ERK), and then tracked ERK activity via FP-ERK localization in optogenetically perturbed cells, and via an ERK FRET-biosensor in neighboring cells. They showed that locally restricted light-induced ERK activity pulses were sufficient to transfer ERK activation to neighboring cells. Inhibitor experiments strongly suggested that this lateral activation occurred via an EGF-mediated autocrine mechanism.

To demonstrate *in vivo* relevance, the same group visualized ERK activity in the skin of transgenic mice expressing their ERK FRET-biosensor [15]. Tracking skin cells over hours using two-photon microscopy, they observed occasional ERK activity bursts that propagated radially to neighboring cells, confirming the occurrence of both ERK activity pulses and their propagation *in vivo* [16].

A different biosensor combination, an ERK FRET-biosensor with ERK-KTR, was shown to improve time-resolution of peak detection in individual cells [9]. Indeed, differences in reporter activation and deactivation kinetics enable detection of a rapid deactivation event by the ordered loss of FRET then KTR signal in an individual cell [9]. By taking advantage of this resolution, the ERK pulses were shown to depend on sustained EGFR activity; upon EGFR activity disruption, ERK activity pulses immediately stopped before peak amplitude was reached.

Finally, Handly and colleagues combined an ERK FRET-biosensor with a live-cell calcium (Ca^{2+}) signaling reporter and a **microfluidic device** to study paracrine signaling in wound healing [17]. Wounded cells are known to release signals to surrounding cells that increase Ca^{2+} , resulting in paracrine EGF secretion that activates ERK and coordinates wound healing in a gradient centered on the wound. By tracking spatiotemporal signaling in individual cells following wounding, the researchers demonstrated that paracrine EGF yielded an ERK signal that was less noisy than the Ca^{2+} signal. This improved the signal-to-noise ratio (SNR) for cells to compute distance from the wound. Importantly, the distance

traveled by paracrine EGF appeared restricted, maximizing SNR without significantly decreasing the EGF gradient magnitude.

Imaging DNA-damage response and the p53 tumor suppressor protein pathway

Combining signaling dynamics and response measurements

Single-cell observations of FP-tagged p53 dynamics have enabled quantitative studies of basal dynamics [18] and cellular responses to different DNA-damaging agents. After γ -irradiation, FP-p53 is pulsatile, with pulse number varying between cells as pulse probability correlates with amount of damage [19-21]. Gamma-irradiated cells typically arrest and then proliferate again when their DNA is repaired. In contrast, UV-irradiated cells exhibit a sustained FP-p53 increase, with amplitude and duration proportional to UV dose; large UV doses induce cell senescence or death [22, 23]. Recently, Purvis and colleagues combined two technological innovations to explore how single-cell p53 dynamics control cell fate. First, they modulated p53 dynamics using timed stepwise drug addition to change the p53 profile of γ -irradiated cells to that of UV-treated cells, successfully switching the cellular outcome to senescence. Second, they combined same-cell measures of FP-p53 dynamics with single-molecule RNA fluorescence in situ hybridization (smFISH)-measured transcript abundance for two p53 target genes. With these same-cell measurements, they clearly demonstrated that qualitatively different p53 *dynamics*, not just integrated p53 activity, regulate gene transcription and cell fate [23].

Imaging protein complex formation

One commonality for the pathways highlighted in this review is that application of widefield or confocal fluorescence microscopy to characterize signaling dynamics has been informative. Nevertheless much could be gained by leveraging other microscopy approaches. This is exemplified by Gaglia and colleagues, who used **fluorescence correlation spectroscopy (FCS)** to study the dynamics of p53 tetramerization [24]. By characterizing tetramerization in many single cells, they inferred an order of events: tetramerization of existing p53 proteins precedes new synthesis of p53 induced by DNA damage. Furthermore, they found that while p53 oligomerization state varied between cells prior to damage, all cells converged to a predominance of tetrameric p53 after damage. Single-cell FCS also allowed measurements of p53 stability in different oligomerization states. Combining these data with mathematical modeling led to the surprising insight that DNA damage induces both overall stabilization of p53 and tetramerization, but tetramerization alone is sufficient for activation of transcription.

A **protein-fragment complementation assay (PCA)** also allows studying protein oligomerization with live-cell imaging. Applying this approach to p53, the same group showed that even as p53 accumulates under increasing DNA damage, the p53 tetramerization rate remains unchanged, dampening the overall damage response (Fig. 2B; [25]). Importantly, because p53 tetramerization and accumulation are asynchronous across a cell population, characterization of assembly rate absolutely required single-cell approaches. Several other signal transduction systems are thought to rely on signaling proteins

oligomerization, and application of the approaches developed for p53 will undoubtedly lead to new insights into how oligomerization regulates different cellular decision processes.

Imaging and fluidics to measure NF- κ B pathway signaling and outputs

Transcription factors of the NF- κ B family are found in almost all cell types and are critical regulators of immunity, stress responses and development. Studies of the regulatory network controlling NF- κ B activity first leveraged single-cell approaches over ten years ago when Nelson and colleagues reported the dynamics of nuclear translocation of FP-tagged RelA, the transcriptionally active subunit of canonical the NF- κ B pathway. This work exemplified how asynchrony within a cell population obscures the true dynamics if assayed using bulk measurements (Fig. 2C; [26]).

Indeed while TNF-induced NF- κ B dynamics were originally characterized as strongly damped oscillations [27], single-cell studies tracking FP-RelA have shown that some cells exhibit persistent, albeit asynchronous and noisy, oscillations [26, 28]. By tracking NF- κ B oscillations for 20 hours in mouse 3T3 fibroblasts, Hughey and colleagues observed significant cell-to-cell variability in the period of oscillations, although the average period was constant for different stimuli [29]. Intriguingly, the intracellular variability in period was smaller than its intercellular variability (Fig. 2C), suggesting that preexisting cell-to-cell differences ('extrinsic noise') explain the width of distributions in single-cell oscillatory period. These differences may be regulated to produce a population-invariant dynamical feature. To explore extrinsic noise sources influencing NF- κ B translocation dynamics, Cheng and colleagues measured and modeled FP-RelA nuclear translocation in RAW 264.7 mouse macrophages following lipopolysaccharide (LPS) stimulation. They found that intracellular protein abundance regulated two distinct signaling paths downstream of LPS respectively contributing to variations in initiation timing and duration of NF- κ B nuclear localization [30].

Microfluidic-enabled single-cell measurements

Microfluidic devices allow efficient and reliable stimulation and monitoring of cell cultures. Tay and colleagues used a multiplexed microfluidic device [31] for a detailed dose-response investigation of FP-RelA nuclear translocation dynamics in TNF-treated mouse 3T3 fibroblasts [32]. After careful measurements in hundreds of cells they concluded that although the percentage of responding cells in a population exhibits a clear TNF dose-dependence, many characteristics of single-cell response dynamics are dose-independent. Importantly, White and colleagues reached similar conclusions for human cells treated with TNF in conventional culture system [28, 33].

Microfluidic systems also provide exquisite control of stimulus delivery, enabling the observation of single-cell responses to fluctuating inputs that can inform on the structure of underlying signaling networks. For instance, Kellogg and colleagues used the microfluidic system mentioned above to investigate how mouse fibroblasts respond to TNF saw-tooth oscillations [34]. They showed that, for their cells, oscillatory TNF synchronized NF- κ B nuclear translocation dynamics. Surveying a range of TNF oscillation periods, synchronization was optimal when the input period was near the natural NF- κ B oscillation

period of cells under constant TNF. With data on hundreds of individual cells for each stimulation profile, the authors dissected how different noise sources contribute to synchronization quality. They found that intrinsic noise - noise within the NF- κ B network itself - likely helps individual cells synchronize to the input. By contrast, noise arising outside the NF- κ B network gives flexibility to the cell population as a whole: greater cell-to-cell variability in natural period ensures that for a wide range of input periods, at least some cells are synchronized. By coupling these single-cell NF- κ B dynamics measurements to population-based measurements of RNA abundance, they showed that synchronization increased transcriptional output, implying that the NF- κ B-driven transcriptional network may have evolved to be particularly responsive to a certain stimulus frequency range.

More recently, Zambrano and colleagues used microfluidics to impose square-profile TNF pulses (rather than saw-tooth oscillations studied above) on FP-RelA expressing mouse embryonic fibroblasts. Transcriptome profiling of cells synchronized with this pulsatile TNF input, demonstrated that while genes encoding unstable mRNAs exhibited pulsatile expression dynamics, genes with long-lived mRNAs showed instead a steady concentration or slow increase. Intriguingly, these two gene sets are functionally distinct; pulsatile genes principally encode chemokines and their receptors and the others encode innate immune response and extracellular matrix remodeling genes [35].

Microfluidic systems also enable variation of stimulus duration. Using a single short LPS pulse to drive NF- κ B activation in mouse fibroblasts, it was shown that varying either concentration and duration changed the response probability for individual cells, yet minimally influenced peak nuclear NF- κ B [36]. Only concentration significantly affected dynamics in each responsive cell, with increasing concentration resulting in earlier responses, thus hinting at an “analog” cellular response component. Both data and model point to cells using an “area rule” whereby their probability of translocating NF- κ B is proportional to the area under the curve of LPS concentration vs. LPS duration. Therefore although individual cells may respond predominantly “digitally” (“on” or “off”), the response of the full population is modulated by stimulus amplitude and duration. Microfluidics-enabled precisely timed growth factor inputs have also refined knowledge of the signaling network structure controlling ERK dynamics and cell fate [37].

Linking NF- κ B signaling to responses

Early single-cell studies of transcription regulation by NF- κ B dynamics combined single-cell nuclear FP-RelA dynamics with population-based RNA measurements [32, 38, 39]. Therefore, although the true dynamics of RelA localization and its cell-to-cell variability were characterized, these dynamics were not directly linked to RelA activity. Newer approaches have coupled live-cell FP-RelA tracking with single-cell gene activity assays. Sung and colleagues used live-cell imaging of RAW 264.7 mouse macrophages co-expressing FP-RelA with mCherry driven from a RelA-dependent promoter sequence of the TNF gene [40]. Same-cell tracking of FP-RelA dynamics with mCherry gene activity showed that while most cells translocated FP-RelA and transcribed mCherry only transiently, a small fraction of cells treated with high-concentration LPS had a prolonged nuclear FP-RelA peak and long-lasting mCherry expression. These striking single-cell

dynamics revealed a previously unknown positive feedback controlling RelA expression whereby RelA drives expression of the transcription factor Ikaros, which in turn binds to the RelA promoter and increases its expression. The RelA-Ikaros-RelA positive feedback switches on only in high-LPS-treated cells, overcoming the well-characterized NF- κ B network negative feedbacks via I κ B and A20 [39, 41]. This network topology effectively discriminates between low and high LPS, allowing macrophages to mount a commensurate innate immune response.

One of our groups applied another approach to same-cell readouts of NF- κ B dynamics and transcriptional output. By combining nuclear FP-RelA tracking with endpoint smFISH-based transcript counting, we demonstrated that the NF- κ B pathway exhibits fold-change detection (Fig. 2D) [42]. Quantifying the relationship between transcript number and fold-change in nuclear RelA (ratio of maximal over initial nuclear intensity) was impossible with population-level transcription assays. Because fold-change detection is a property of very few network topologies, this result allowed us to identify a type I incoherent feed-forward in the TNF-induced NF- κ B regulatory network.

Single-cell measurements to track regulation by cell-to-cell communication

A counterintuitive concept emerging from single-cell NF- κ B studies is that the variability in the timing of I κ B and A20 negative feedback appears optimized to *increase* cell-to-cell heterogeneity in NF- κ B oscillations [28]. It was hypothesized that this heterogeneity could exist to “smooth out”, over time, paracrine signals from NF- κ B-regulated cytokines (e.g. TNF) producing a steady population response. By combining fluorescent reporters or by using microdevices, we can now directly test the consequences of heterogeneous paracrine signaling via single-cell measurements.

Indeed, signal transduction studies often emphasize intracellular networks yet extracellular, paracrine signals also influence cell behaviors. Single-cell analyses are improving our understanding of how paracrine signals produced in response to an initial input, feedback to alter intracellular signaling dynamics. For example, TNF induces only transient nuclear RelA increases due to negative feedback by I κ B α and A20, yet LPS induces more sustained NF- κ B responses. The RelA-Ikaros positive feedback discussed above is one explanation [40]; another is positive feedback by LPS-induced TNF secretion [43]. Measurements of LPS-induced NF- κ B signaling in single murine fibroblasts showed that only some cells had a sustained NF- κ B response, suggesting that not all cells received the secondary TNF signal [44]. By mixing wild-type cells with TNF-responsive, LPS-receptor knockout cells, the same study showed that the secondary TNF signal was a low-concentration, paracrine signal from the LPS-responsive cells, not an exclusively autocrine (same-cell) signal.

This finding suggests that LPS-induced NF- κ B signaling would be substantially altered if cells were isolated from the population, therefore modifying downstream responses. The development of microwell assays for isolated single-cell cultures makes it possible to investigate the behavior of cells in the absence of signals from neighboring cells. In a study by one of our groups, we took advantage of a microwell-based miniaturized sandwich immunoassay developed to measure secretion from individual cells [45]. We stimulated isolated individual human U937 macrophage cells with LPS and multiplexed measurements

of their secreted TNF, interleukin-6 (IL-6), IL-10 and other cytokines. We found that the total secretion of IL-6 and IL-10 was lower compared to conventional cultures, demonstrating that loss of paracrine signals attenuates inflammatory signaling (Fig. 2E; [46]). Heterogeneity of TNF secretion was especially high, with a few cells accounting for a majority of TNF secretion in the population. Follow-up experiments were consistent with a role for paracrine TNF in increasing IL-6 and IL-10 secretion; however, direct measurements of NF- κ B signaling will be required to determine if paracrine TNF acts by sustaining NF- κ B signaling in surrounding cells.

Quantifying the influence of cell morphology and microenvironment

Fluorescence-based assays are widely used, yet imaging offers much more information about cells than intensity of fluorescent reporters or immunofluorescence signals. Sero and colleagues leveraged this information, computing dozens of morphological features for thousands of individual breast epithelial and tumor cells [47]. Using these data, they defined influence networks between cellular morphological features and RelA nuclear abundance pre- and post-TNF treatment (Fig. 2F). The mechanisms by which cell shape influences intracellular signals are still ill-defined although some foundations have been laid. Several years ago, the influence of imposed cell shape on cellular behaviors were observed using micropatterned surfaces [48]. More recently, Pelkmans and colleagues used imaging to identify signaling pathways via which cell shape and cellular context influence plasma membrane composition, susceptibility to viral entry and multicellular patterning [49, 50].

Concluding Remarks and Future Directions

Where does the future lie for single-cell analyses (see Outstanding Questions)? Many methods described above are limited by being relatively low throughput (tens to hundreds of cells) and have low multiplexing capabilities (a few measurements per cell). This is particularly limiting when statistical and computational analysis methods require thousands of single-cell observations and measurements for multiple signaling nodes in each cell. Creating efficient data collection and analysis pipelines will open new areas of investigations, as Selimkhanov and colleagues recently showed. They collected time courses of signal activation for nearly a million cells by live-cell imaging and automated image processing [51]. With these data, they applied information theoretic methods and demonstrated that despite substantial observed cell-to-cell variability, signaling networks transmit quantitative information with high fidelity within single cells. The information content in signaling pathways is an open question. In contrast to the work of Selimkhanov and colleagues, Cheong and colleagues concluded that the information content in the NF- κ B pathway is limited to little more than one bit – when considering only single post-TNF treatment time points [52]. Similarly, Uda and colleagues used fixed-cell measurements of phospho-ERK and concluded that the ERK pathway capacity is also limited to one bit [53]. What is becoming clear is that we will first need to leverage single-cell dynamic data to better understand noise propagation within and between pathways [54] as well as uncover the mechanisms by which dynamic features can encode quantitative information [55]. Then we can assess the true information capacity of different signaling networks.

As we have seen, imaging is at the core of many single-cell approaches, but can be limited by a low multiplexing capability. Single-cell RNA sequencing (RNA-seq) has broken that barrier for the measurement of transcriptional responses [56]. Although the issue of multiplexing has not yet been solved for dynamic signaling protein measurements, two technologies have recently been reported that enable imaging of many more antibody-based measurements from fixed-cell samples. **Mass cytometry** is an exciting new method with the potential to measure 30-50 species in fixed single cells. Current experimental designs typically combine signaling with phenotypic cell-surface markers yet still yield more than ten signaling measurements per cell, leading to new insights about signal regulation [57, 58]. When combined with laser-based, image-guided cell capture, mass cytometry can even be used to make single-cell measurements in tissues [59]. The second method is elegantly simple: high-multiplexing is achieved via cycles of low-multiplexed conventional immunofluorescence with gentle signal elimination methods that enable multiple rounds of immunofluorescence staining to measure at least 15 signaling proteins per cell [60].

Finally, it will be important to validate new mechanisms of signaling regulation discovered from single-cell measurements *in vivo*. With the availability of mice with genetically encoded live-cell signaling reporters [15, 61] combined with intravital imaging [62, 63] and the rapid adaptation of tools such as RNA-seq, smFISH and mass cytometry to intact tissues [59, 64, 65], these answers may be available very soon.

Glossary

Extracellular signal-regulated kinases (ERK)

Intracellular protein kinase conserved in eukaryotic cells that play central roles in regulating cell proliferation and differentiation in response to extracellular ligands.

Fluorescence correlation spectroscopy (FCS)

FCS allows direct coupled measurements of fluorescence intensity (a measure of total fluorescent protein concentration) and of brightness of individual fluorescent particles (inferred by the average fluorescent intensity of individual spots). A higher brightness-to-intensity ratio indicates a higher degree of protein oligomerization, enabling quantification and live-cell tracking of this common regulatory mechanism for signaling proteins.

Fluorescent fusion proteins

A cDNA fusing the sequence for a fluorescent protein (FP; e.g. green fluorescent protein (GFP) or mCherry) to that of the signaling protein of interest is transfected into cells.

Förster resonance energy transfer (FRET)

FRET occurs when a donor fluorophore in an excited electronic state transfers its excitation energy to a nearby (~10 nm) acceptor fluorophore such that when the sample is excited at the donor wavelength, acceptor-emitted fluorescence is detected. Because FRET is strongly distance-dependent, donor-emitted fluorescence becomes visible as the donor-to-acceptor distance increases. A successful FRET biosensor design strategy is to create a linker between donor and acceptor fluorescent proteins that contains a kinase substrate peptide sequence and a phospho-binding (PB) domain [8, 66]. Upon phosphorylation by the kinase

of interest, the peptide binds to the PB domain, causing a conformational change that brings together donor and acceptor and produces a detectable FRET signal.

Kinase translocation reporter (KTR)

KTRs are genetically encoded biosensors that combine a negatively phosphoregulated nuclear localization sequence (NLS) with a positively phosphoregulated nuclear export sequence (NES). This enables visualization of kinase activity by translocation of a fluorescent protein-based KTR from the nucleus (non-phosphorylated state) to the cytoplasm (phosphorylated state) in single cells [10].

Mass cytometry

In this approach, cellular targets are labeled with rare earth metal-tagged antibodies. Cells are nebulized and analyzed by time-of-flight mass spectrometry to quantify abundance of the metal-tagged proteins of interest. Currently, measurements of over 50 targets can be made from each cell.

Microfluidic devices

Microfluidic devices are typically channel-based devices for cellular assays where at least one channel dimension is in the sub-millimeter range. They take advantage of laminar flow, whereby parallel streams of fluids mix only by diffusion, not convection, a property that water-based fluids characteristically take in such small-dimensional systems.

Nuclear factor- κ B (NF- κ B)

A family of transcription factors that regulate immunity and stress responses.

Optogenetics

Phytochromes (Phy), photoreceptive signaling proteins in plants, respond a particular wavelength of light with a conformational change that increases affinity for Phy interaction factors (PIF). By linking the Phy-PIF module subunits to signaling proteins whose activity is controlled by recruitment or induced proximity, light can be used to gain precisely spatiotemporal control of signaling activity ([67, 68] and recent review [69]).

OptoSOS

Opto-SOS is a light-controlled genetically encoded protein tool generated by fusing the catalytic domain of SOS (**SOS_{cat}**) to a Phy interaction factor (PIF) and YFP. SOS (Son of Sevenless) is a guanine exchange factor that binds to Ras and stimulates its exchange of GDP in favor of the more abundant GTP, thereby activating Ras. In cells expressing membrane-bound PhyB, YFP-PIF-SOS_{cat} translocates to the membrane upon exposure to red light (650 nm) thereby bringing SOS_{cat} in close proximity to Ras, inducing Ras activation.

Protein-fragment complementation assay (PCA)

A fluorescent protein is split into two complementary fragments, and each is used to tag two different proteins that are potential binding partners. When split, the fragments are not fluorescent and have low affinity for each other, but if the tagged proteins stably bind to each other, the fluorescent fragments can interact and fold, leading to fluorescent signal recovery [70].

Single molecule RNA fluorescence in situ hybridization (smFISH)

smFISH enables visualization of single mRNAs by labeling fixed cells with 30-50 short singly labeled oligonucleotide probes whose sequences tile along a target mRNA [71]. Consolidated binding of the probes on individual mRNAs produces bright fluorescent spots detected by fluorescence microscopy and counted with computational image analysis tools.

Tumor suppressor protein p53

A transcription factor that regulates the DNA-damage response.

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Box 1**Overview of the ERK, p53, and NF- κ B pathways****Extracellular signal-regulated kinase 1/2 (ERK)**

Growth factors, including epidermal growth factor (EGF) and nerve growth factor (NGF), regulate proliferation and differentiation in many cells types via the activation of a mitogen-activated protein kinase (MAPK) cascade that ends in phosphorylation of the ERK family kinases, of which ERK2 is the most studied. ERK is active when phosphorylated and can then localize predominantly in the nucleus and phosphorylates transcription factors. Thus, many technologies discussed herein are designed to measure ERK localization or, more directly, its activity. Improper ERK activation is implicated in cancer, in many cases due to mutations in growth factor receptors.

Tumor suppressor protein p53

Stress stimuli that induce DNA damage, including ultraviolet light (UV), γ -irradiation and oxidative stress, lead to the activation of the transcription factor p53. p53 limits the adverse effects of DNA damage by activating DNA repair, while arresting cell cycle or inducing cell death (if DNA damage persists). Inactivation of p53 can lead to accumulation of mutations and is common in many cancers. In the absence of stimulation, p53 is bound to the ubiquitin ligase protein Mdm2, which targets p53 for rapid degradation. After stimulation, Mdm2 releases p53, leading to its stabilization and a conformational change that promotes formation of an active tetramer. The technologies discussed in this review have been developed to track nuclear abundance of p53 and its tetramerization.

Nuclear factor-kappa B (NF- κ B)

Inflammatory and stress stimuli, including the cytokine tumor necrosis factor (TNF) and the bacterial endotoxin LPS, activate NF- κ B, a family of transcription factors that regulate hundreds of genes, many of which encode cytokines or chemokines involved in immunity and stress responses. NF- κ B transcription factors are dimers and the most commonly studied family member is RelA, thought to heterodimerize predominantly with p50. In the absence of stimulation, NF- κ B is bound to the inhibitor of κ B- α (I κ B α), which leads to shuttling of NF- κ B to the cytoplasm. Upon stimulation, I κ B α is degraded and NF- κ B can then accumulate in the nucleus and bind to the promoter of target genes. Nuclear localization of NF- κ B is strongly correlated with its activity, and therefore most single-cell technologies track activity via nuclear translocation.

Outstanding Questions

- Will single-cell technologies rapidly become standardized and sufficiently flexible to enable measurements from a more diverse set of biological pathways?
- Will the development and/or adaptation of single-cell technologies for measurements in tissues and whole organisms lead to *in vivo* confirmation of signaling mechanisms discovered *in vitro*?
- If dynamical features of signaling convey information, which features are most important, and what is the mechanism by which cells interpret these dynamics?
- Will multiplexing improvements for single-cell approaches enable investigating large-scale regulatory networks?
- Can the single-cell method's throughput and multiplexing be concomitantly increased to enable the use of powerful computational methods that necessitate thousands of multiplexed single-cell observations?

Trends Box

- Measuring signaling dynamics in cell populations can obscure underlying mechanisms.
- New tools are rapidly being developed to measure signaling at single-cell resolution.
- Single-cell technologies have uncovered fresh mechanistic insights for canonical signaling pathways.
- Future innovations will increase throughput and multiplexing of single-cell measurements.

Figure 1. Overview of technologies developed to interrogate or modulate ERK activity in single cells

A) A FRET-based sensor undergoes a conformational change upon phosphorylation by ERK that is detected by a change in the ratio of donor-to-acceptor fluorescence emission intensity. B) Fluorescent KTRs translocate from the nucleus to the cytoplasm when phosphorylated by ERK. C) FIRE (Fra-1-based integrative reporter of ERK) is stabilized upon phosphorylation by ERK leading to an increase in fluorescence intensity. D) Light activates an optogenetic membrane localized ERK-activating signal (SOS_{cat}) to enable light-based control of ERK activation. FP: fluorescent protein. FHA1, Elk-1, FRA-1: substrate domains modulated by active ERK.

Figure 2. Regulatory signaling mechanisms discovered from single-cell data

A) ERK activity increases linearly with EGF concentration as measured in a cell population. Single-cell data showed that this increase is due to increased frequency of short pulses of ERK activity. B) p53 abundance increases linearly with UV intensity. Measuring both total and active tetramer abundance in single cells revealed that active tetramer abundance increases much more gradually than the total abundance, constraining the dose response. C) For NF- κ B, single-cell data showed that the strongly dampened oscillations observed at the population level are due to loss of synchrony in oscillations following the first peak. While the intercellular oscillatory period varies, the intracellular period is relatively constant, and altogether the population produces stable and largely invariant NF- κ B oscillation pattern. D) Population-averaged nuclear NF- κ B abundance and that of its target transcripts are positively correlated but vary across cells. By measuring time courses of NF- κ B translocation in single cells, it becomes apparent that the fold change in nuclear NF- κ B – not its absolute abundance – more precisely determines the transcriptional output in individual cells. E) Nano-well experiments have shown that strong inflammatory signals released in a population of LPS-treated cells are dependent on paracrine signaling. F) Single-cell studies allow the investigation of how cell shape and cell microenvironment influence signaling responses, including the nuclear localization of NF- κ B.