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Review

Redefining Signaling Pathways with an Expanding Single-Cell Toolbox

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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 and stress responses (NF-κB). 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.

Trends

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.

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.

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 laevis* oocytes [2]. While bulk measurements showed a graded ERK response – with more progesterone, more of ERK was phosphorylated – single-oocyte responses were

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Box 1. Overview of the ERK, p53, and NF-κB 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 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-κB)

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

'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

Glossary

Extracellular signal-regulated kinases (ERKs): intracellular protein kinases conserved in eukaryotic cells that play central roles in regulating cell proliferation and differentiation in response to extracellular ligands.

Fluorescence correlation spectroscopy (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): 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 reporters (KTRs): 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