Individual cells can resolve variations in stimulus intensity along the IGF-PI3K-AKT signaling axis

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SUMMARY

Cells sense and respond to signals in their local environment by activating signaling cascades that lead to phenotypic changes. Differences in these signals can be discriminated at the population level, however single cells have been thought to be limited in their capacity to distinguish ligand doses due to signaling noise. We describe the rational development of a genetically-encoded FoxO1 sensor, which serves as a down-stream read-out of IGF-PI3K-AKT signaling pathway activity. With this reporter, we tracked individual cell responses to multiple IGF-I doses, pathway inhibitors, and repeated treatments and observed that individual cells can discriminate multiple IGF-I doses and these responses are sustained over time, reproducible at the single cell level, and show cell-to-cell heterogeneity. These studies imply that cell-to-cell variation in signaling responses is biologically meaningful and support the endeavor to elucidate mechanisms of cell signaling at the level of the individual cell.

eTOC Blurb

Here we describe the rational development of a new FoxO1 fluorescent reporter, and use it to demonstrate that individual cells can accurately discriminate multiple IGF-I doses and that these responses are durable across time.

Graphical Abstract
INTRODUCTION

Cells sense and respond to their local environment by activating distinct intracellular signaling pathways. These pathways are composed of multi-step relays that use second messengers, protein translocations, and kinase/phosphatase cycles to transduce extracellular signals into an intracellular response (Lemmon et al., 2016). Given the multi-step nature of cell signaling in both space and time, much remains unknown about exactly how individual cells process extracellular signals into intracellular responses (Cheong et al., 2011, Selimkhanov et al., 2014, Suderman et al., 2017, Uda et al., 2013). Previous studies have concluded that cell signaling is inherently noisy and therefore individual cells are limited to simple detection of the presence or absence of extracellular stimuli (Cheong et al., 2011, Selimkhanov et al., 2014, Suderman et al., 2017). In contrast, we hypothesize that individual cells can finely discriminate variations in stimulus intensity and this observation can be revealed with experimental techniques that are optimized for the study of single cell responses.

The IGF-PI3K-AKT signaling pathway provides an excellent model to study cell signaling: it uses multiple modalities to encode, relay, and decode extracellular signals and also produces sustained responses (Gross and Rotwein, 2016, Sampattavanich et al., 2018, Yoneyama et al., 2018). In brief, IGF-I binds to the receptor tyrosine kinase IGF1R to stimulate intracellular kinase activity (Manning and Toker, 2017), which leads to recruitment and activation of PI3-Kinase followed by phosphorylation and activation of AKT. The protein kinase AKT can then phosphorylate a range of proteins including the transcription factor FoxO1 (Brunet et al., 1999, Manning and Toker, 2017, Rena et al., 1999, Zhang et al., 2002). Previously, we developed a fluorescent fusion protein with FoxO1 (Gross and Rotwein, 2015). Using this as a readout for IGF-I signaling activity, we found that responses to IGF-I were sustained across time and heterogeneous across the population. We surmised that this heterogeneity could arise from various sources, including: noise in the signaling pathway, variability in the reporter and its quantification, non-specificity in the reporter, or cell-to-cell variation in the encoding of the signal. Because the source of this heterogeneity impacts the interpretation of underlying biological mechanisms of signal processing, we set out here to systematically assess variation in single cell IGF-I responses.

Establishing the extent of signaling fidelity in individual cells is critical for motivating experimental approaches and molecular mechanisms at the single cell level. For instance, if individual cell responses are corrupted by noise within a signaling pathway, then population

Keywords
signaling pathways; signaling dynamics; AKT; live-cell imaging; FoxO1
mechanisms such as ensemble averaging would be necessary to accurately sense environmental signals. In contrast, single cell responses that are accurately processed but vary between individual cells are likely mediated by molecular differences at the single-cell level and therefore would benefit from single-cell studies such as cyclic immunofluorescence (cycIF) (Lin et al., 2015), mass cytometry, and single cell RNA-Seq. We describe here the development of a FoxO1 reporter optimized to assess the signal-to-noise ratio of IGF-I induced pathway activity and find that individual cells can faithfully decode multiple growth factor concentrations and that these responses are sustained over time, reproducible at the single cell level, and show cell-to-cell heterogeneity.

RESULTS

Single cells responses to IGF-I are heterogeneous and sustained over time

To robustly assess signaling noise in the IGF-PI3K-ATK pathway, we developed an improved version of our existing fluorescently-tagged FoxO1 reporter (Gross and Rotwein, 2015) (see STAR Methods, Figures S1 and S2, and Movies 1 and 2). We tested responses to a range of IGF-I concentrations and found that small changes in ligand concentration (5 pM IGF-I) could be robustly separated when single cell responses were averaged (Figure 1A). This suggests that differences in stimulus intensity can be finely discriminated at the population level. In contrast, single cell responses varied substantially within a dose and overlapped across doses, such that it would be difficult to predict treatment dose based on a cell’s response (Figure 1B–C bottom graph). This overlap in single cell responses between doses is consistent with dose-response measurements reported in other signaling pathways and has previously been interpreted as an inability of individual cells to discriminate different ligand doses (Cheong et al., 2011).

We next explored whether the overlap between doses (Figure 1C bottom panel) could be explained by technical or biological factors. Single cell ligand responses were not correlated with reporter expression or basal activation level, which ruled out technical factors related to the reporter itself (Figure S3A–D). Integration across multiple time points has been used as an analytical approach to reduce measurement noise (Selimkhanov et al., 2014, Lee et al., 2014, Zhang et al., 2017). Following this, we varied the number of measurements used to calculate each cell’s response (one, two, or 15 time points). This did not change the EC50 value (Figure S3E), but distribution widths were reduced by increasing the number of time points used for quantification. Despite this reduction in variance, the responses to different doses continued to overlap, supporting the hypothesis that signaling responses to IGF-I are heterogeneous across the population (Figure S3F).

Cell responses can be defined in terms of intracellular or extracellular variation. Extracellular variation defines cell-to-cell heterogeneity across the population whereas intracellular variation assesses variance in individual cells across time. The single cell traces from IGF-I treatment suggest that individual signaling responses are consistent over time, which can be interpreted as low intracellular variation (Figure 1B). To compare the level of extra- to intra-cellular variation, we plotted the early response distributions to 25 pM IGF-I (0–90 min window, “extracellular variation”) against the late steady-state response distributions (90–180 min window, “intracellular variation”), and found that the standard
deviation of the early 0–90 min window was 12.8% compared to 6.2% for the late 90–180 min window. (Figure 1D). As a further assessment, we visualized all IGF-I doses tested at 90 and 180 min (Figure 1E–F) and found that cell responses were broadly distributed along the diagonal with relatively low variance in the off-diagonal. These comparisons show that variation between cells is considerably larger than the variation within a cell across time and suggests that extracellular variation can obscure the true fidelity of single cell responses.

Each cell has its own communication channel

Information theory has previously been applied to measure the signaling fidelity of biological systems (Cheong et al., 2011, Selimkhanov et al., 2014, Suderman et al., 2017). One assumption of these applications is that the input-output relationship is identical for all cells in the population and that observed variation is due to noise in signal interpretation, transmission, and encoding (i.e., intracellular variation). Based on this assumption, a noisy pathway would lead to responses that fluctuate in single cells over time to the same extent that responses vary over the entire population at a single time point (Figure 1G, upper). An alternative explanation for heterogeneity in signaling responses is that the input-output relationship varies between individual cells (extracellular variation), but there is minimal variation in signal transmission over time (low intracellular variation) (Figure 1G, lower). In this scenario, each cell can be viewed as having its own unique communication channel, which implies that an accurate assessment of the fidelity of cell signaling requires separation of extra- from intra-cellular variation.

To separate extra- from intra-cellular variation, we quantified variance using the standard deviation of responses from each dose of IGF-I in the early 90-minute extracellular variation window compared to the late 90-minute intracellular variation window (Figure 1H). We found that the standard deviation during the first 90-minute window increased as the IGF-I dose increased (Figure 1H, red line). This suggests different input-output relations across the population as indicated by an increase in the number of responsive cells with increasing IGF-I dose.

In contrast to the early window, the standard deviation for the late window remained constant across IGF-I doses and was similar to the 0 pM dose for the first 90-minute window (Figure 1H, blue line). These variances were equivalent across IGF-I doses and were normally distributed (Figure S3 G–H). Furthermore, we found that variation in fluorescence intensity using only a single time point (90 min) was similar across doses, in contrast to that observed for the 0–90 min window, T0-normalized, and summed responses, which all incorporate multiple time points (Figure S3I). These observations indicate that single time point measures significantly obscure the extent of intracellular variation and that response heterogeneity likely results from cells decoding the same IGF-I input signal into different FoxO1 signaling outputs.

Based on this interpretation, we reasoned that variance across time quantified from individual cells provides a more representative measure of noise for channel capacity measurements because it considers each cell to have its own unique communication channel. To test this, we used the Shannon-Hartley channel capacity equation, which allows comparison of different signal and noise values (Selimkhanov et al., 2014). Analysis of
channel capacity from a single time point yielded a value of 1 bit (Figure 1I, purple dots), which is similar to prior measures of the maximal mutual information of other signaling pathways (Cheong et al., 2011). This measure was increased to 1.2 bits when we computed channel capacity from the T0-normalized responses (green dots), and was further increased to 1.4 bits by using the summed responses drawn from 15 images (red dots). Finally, we quantified channel capacity with noise values drawn from the stable 90–180 min (intracellular variation) window and observed a channel capacity of 1.99 bits using the T0-normalized data (yellow dots) and 2.38 bits using the summed response data (blue dots). A value of 2.38 bits indicates that individual HeLa cells can discriminate at least 5 IGF-I doses.

Our calculation of the average maximal channel capacity likely still underestimates the true value due to limitations in the measurement of individual cell responses. To test the amount of measurement noise included in our calculation of the intracellular variation, we eliminated signaling noise by treating cells with Leptomycin B (LMB), which results in complete and sustained nuclear localization of the reporter (Figure S3J). After LMB treatment, variation in nuclear intensity across time can be attributed to measurement noise. Signal variation 90–180 min after treatment with LMB was nearly equivalent to the variation observed after a submaximal dose of 17.5 pM IGF-I (Figure S3K–L). This indicates that measurement noise is the primary source of the Shannon-Hartley noise value, rather than signaling noise (intracellular variation). This suggests that the true channel capacity may actually be greater than our calculations suggest.

**Individual cells can recapitulate population-derived dose response curves to reveal high signaling fidelity**

To test the prediction that single cells can discriminate multiple signal intensities, we treated cells with four IGF-I doses distributed across the dose-response range. Treatment with single doses of IGF-I served as reference controls (Figure 2A). The average population response to a single dose matched the response from cells treated with the same dose as part of a multiple-dose treatment (Figure 2A), with some modest signal decrement when cells were maintained in IGF-I over relatively long time periods (e.g., see late phase of 125 pM response). Likewise, population frequencies for multiple-dose treated cells were nearly equivalent to time- and dose-matched controls maintained with a single dose (Figure 2B). These multiple-dose data can recapitulate a dose response curve from individual cells that are similar to traditional population-level dose response curves in which different cell populations were treated with escalating doses of IGF-I (Figure S4A).

We next sought to assess whether individual cells could discriminate multiple IGF-I doses and whether all cells have equivalent input-output relationships. We tracked 400 single cell responses after multi-dose treatment (0, 17.5, 37.5, and 125 pM IGF-I) and found that individual cells show distinct responses to each IGF-I dose (Figure 2C, Movies 3–4). Moreover, we observed substantial variation across the population: some cells showed modest responses to all doses while others showed measurable responses to even the lowest dose tested. Comparison of traces from 20 cells centered at the 25th and 75th percentiles revealed similar responses within each subpopulation but distinct differences between them (Figure 2D). Likewise, the average dose-response relationships from these cell
subpopulations and a subpopulation of cells centered at the 50th percentile revealed consistent responses within each subpopulation (Figure 2E). Moreover, we found that an individual cell’s response to one treatment dose was significantly correlated with its response to a subsequent treatment (Figure S4B) and that responses of multi-dose treated cells were significantly different from time- and dose-matched cells maintained in the same dose (Figure S4C). Together, these studies indicate that individual cells can discriminate at least four distinct IGF-I concentrations and that the input-output relationship is consistent for individual cells but variable across the population.

Our multi-dose single cell data indicates that cells can discriminate multiple concentrations of IGF-I when the concentration of IGF-I is sequentially increased. This raised the question of whether reductions in IGF-I concentrations could be similarly resolved. To test this, we treated cells with 17.5 pM IGF-I for 90 min and then either increased the IGF-I concentration to 37.5 or maintained cells in 17.5 pM IGF-I. We also treated cells with 37.5 pM IGF-I for 90 min and then either decreased the IGF-I concentration to 17.5 pM or maintained the same concentration for an additional 90 min. At the population (Figure 3A) and single cell levels (Figure 3 B–C) both increases and decreases in IGF-I concentration can be accurately discriminated in individual cells.

To elucidate the relationship between pathway activation and inhibition, we treated cells with 25 pM IGF-I, a submaximal dose that leads to a wide variation in single cell responses (see Figure 1C), followed by dose escalation of the PI3Kα inhibitor alpelisib (0–500 nM). At the population level, we observed the expected dose-response relationship to alpelisib treatment (Figure 3D). We compared cells from the upper (blue traces) and lower (red traces) quantiles after treatment with 25 pM IGF-I and found that these two populations showed distinct responses after inhibitor treatment (Figure 3E). Comparison of all drug doses revealed similar separations between quantiles, up to 500 nM alpelisib, the dose at which signaling from IGF-I is nearly completely blocked (Figure 3F). These observations indicate that a cell’s response to PI3K inhibition is determined by its response to IGF-I and are consistent with a model in which there is variation in how individual cells encode a signaling input.

We further explored the durability of individual cell responses by testing how individual cells respond to identical IGF-I doses that are separated by a 2 hr washout period. We found that a cell’s first response correlated with its second response ($R^2 = 0.55$) (Figure 4 A–B). This value was similar to the correlation values from the multi-dose treatments (Figure S4B) and indicates that within a cell, signaling responses to the same dose of IGF-I are relatively durable across a 5-hour time frame.

Next, we tested the durability of signaling responses between sibling pairs by first imaging cells for 22 hr in growth media to establish sibling relationships, serum starving cells for 2 hr, and then treating with 37.5 pM IGF-I for 90 min. This revealed that responses were correlated between sibling pairs, although to a lesser extent then sequential treatments in the same cell ($R^2 = 0.31$) (Figure 4C–D). Further analysis of the sibling pairs indicated that the time since division had no relation to the extent of response, indicating there was no cell cycle dependency (Figure 4E). Additionally, the signaling difference between siblings did
not increase based on the time since division (Figure 4F). These observations suggest that a stable molecular mechanism underlies signaling variation between cells and aligns with the concept of high signal resolution in individual cells. Moreover, it suggests a heritable component to signaling potential.

**DISCUSSION**

Understanding how individual cells respond to diverse stimuli is a fundamental issue in cell biology (Altschuler and Wu, 2010, Janes, 2016, Pelkmans, 2012). To that end, we describe here the development of a FoxO1 reporter with a higher specificity and increased dynamic range compared to our previous FoxO1 reporter (Gross and Rotwein, 2015). With this reporter, we observed that individual cells could faithfully and repeatedly discriminate multiple IGF-I doses, and this ability was maintained across time and cell divisions. These experiments provide insights into intracellular and extracellular variation and how molecular features can be linked to the regulation of various biological processes.

Much effort has been put toward modeling cell signaling as an information transmission problem, with cells acting as “channels” that transmit extracellular signals into intracellular molecular responses. Using the paradigm of Information Theory, previous studies of the Erk, NFkB, and Ca²⁺ signaling pathways found channel capacities from 1–1.7 bits (Cheong et al., 2011, Selimkhanov et al., 2014, Uda et al., 2013, Zhang et al., 2017). An underlying assumption of these earlier studies was that all cells process signals over an identical channel. Here we focused our channel capacity studies on IGF-I induced activation of the PI3K-AKT pathway which produces sustained AKT signaling responses (Gross and Rotwein, 2016, Sampattavanich et al., 2018), thereby allowing separation of intracellular from extracellular variation. Our analysis revealed that individual cells stably and durably encode the same IGF-I signal into different signaling outputs. This indicates that each cell should be viewed as having its own unique communication channel when modeling cell responses, which is in contrast to assumptions of previous modeling efforts (Cheong et al., 2011, Selimkhanov et al., 2014, Uda et al., 2013, Zhang et al., 2017). With this updated assumption, we calculated a channel capacity of 2.38 bits (discrimination of 5 doses), which is nearly double previous reports for other pathways and likely still an underestimate of the true channel capacity. Our estimate is similar to a recent analysis of GPCR signaling (Keshelava et al., 2018). Taken together, we hypothesize that robust signal discrimination likely generalizes to other signaling pathways when intracellular--rather than extracellular--variation is considered. Considering that tissues can display substantial spatial variation, this capability provides a molecular mechanism by which cells can have fine-grained control over their ability to sense and respond to signals in their local environments.

Use of our improved FoxO1 reporter to track signaling responses uncovered minimal variation in IGF-PI3K-AKT pathway activity over the time frame studied. When combined with the knowledge that cells can robustly separate multiple ligand doses, we expect that variation in signaling responses can be directly linked to variation in cellular factors. This suggests that combining live cell measurements with data-rich single cell techniques including cyclic immunofluorescence (Lin et al., 2015), mass cytometry (Maeda et al., 1988), and single cell RNAseq (Ziegenhain et al., 2017) will provide powerful approaches to...
dissect the regulatory underpinnings of cell-to-cell variation in cell signaling. In the IGF-
PI3K-AKT pathway, we speculate that extracellular variation in the input-output relationship
could arise because cells differ in their expression of the IGF-I receptor or another pathway
component (Meyer et al., 2012). However, variation could also arise from differences in
basal signaling (Voliotis et al., 2014), cell shape (Swanson et al., 1991), nuclear import/
export machinery (Regot et al., 2014), or phosphatase activity. Future experiments that
combine rich single cell measurements with live cell imaging will be critical for identifying
which biological mechanisms underlie and also create extracellular signaling variation.
While technically demanding, such experimental paradigms have proven informative (Lane
et al., 2017).

Our data indicate minimal intracellular variation in the IGF-PI3K-AKT signaling pathway
and instead imply that individual cells can faithfully and reliably encode extracellular
signals. We postulate that low signaling variation could be mediated by redundancy in signal
processing and the stability of pathway components across time. For example, the average
HeLa cell expresses approximately 5,000 copies of IGFIR and also highly expresses
downstream signaling proteins (Nagaraj et al., 2011). Additionally, the different pathway
components are relatively long-lived, with half-lives of ~24 hrs (Boisvert et al., 2012,
Cambridge et al., 2011). This model of averaging across molecules in a cell is similar to
averaging across cells in the population, which has been proposed as a mechanism to
achieve accurate responses to variations in the extracellular milieu (Cheong et al., 2011).
Future experiments could test this model by lowering expression of a pathway component to
create a bottleneck or rendering a pathway component unstable such that its concentration
fluctuates across time. Of particular interest is exploration of the regulation of signaling
pathways that rely on protein synthesis and degradation to encode signals.

STAR METHODS

LEAD CONTACT AND MATERIALS AVAILABILITY

Further information and requests for resources and reagents should be directed to the Lead
Contact, Laura Heiser (heiserl@ohsu.edu). The following plasmids generated in this study
have been deposited to Addgene: pSBbi-FoxO1_1R_10A_3D (plasmid number: 106278) and
pSBbi-FoxO1_1R_13A_3D (plasmid number: 106279).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

HeLa cells were validated by STR profiling and tested for mycoplasma contamination. The
female cells were grown in DMEM supplemented with 10% FBS in a tissue culture
incubator maintained at 37°C and 5% CO₂.

METHOD DETAILS

Molecular structure informs rational optimization of a novel FoxO1 reporter—
In previous work, we developed a fluorescently-tagged FoxO1 protein (Gross and Rotwein,
2015) as a live-cell reporter to study IGF-I responses at the single cell level. We observed
variability in nuclear localization of the reporter across a population of HeLa cells cultured
in low signaling, serum free conditions (Gross and Rotwein, 2017). To robustly assess
signaling noise in the IGF-PI3K-AKT pathway, we first sought to reduce the basal variation in our readout of pathway activity through modification of non-AKT phosphorylation sites.

To construct a reporter with increased specificity, we used the template of our previously developed reporter, termed Fox Tracer 1 (FT1), and deleted a 234 amino acid segment from the C-terminus. Next, we made alanine substitutions to 10 serine and threonine sites previously identified as phosphorylation sites of other kinases (Asada et al., 2007, Huang et al., 2006, Ozcan et al., 2012, Woods et al., 2001, Yang et al., 2008) (Figure S1 A–C). Preliminary testing revealed that the peptide sequence around S326 impacted nuclear export of this reporter, so we created a series of reporters to identify a variant that maximized the rate of nuclear export. A comparison of these reporters revealed that response rates increased with peptide charge, with a maximum export rate for the FT2-DDD variant (Figure S1 D–F, Movies 1–2). As a negative control for the FT2-DDD reporter, we created a second reporter with the three canonical AKT phosphorylation sites—Thr24, Ser253, and Ser316—mutated to alanine. As expected, this reporter (FT2-DDD 3A) did not translocate in response to IGF-I (Figure S1E). We also performed several experiments that confirmed that localization of the FT2-DDD reporter corresponded to canonical measurements of AKT pathway activity (Figure S2). In summary, a comparison of the FT1 reporter to the FT2-DDD reporter in HeLa cells revealed that the FT2-DDD construct is optimized for tracking the fidelity of signaling responses based on its: increased specificity (Figure S1 A–C), reduced population variance in both serum free conditions and following IGF-I treatment (Figure S1G), and increased measurement range (Figure S1H).

Molecular cloning—The FoxO1-Clover reporter was constructed from a modified pSBbi-RP plasmid (Kowarz et al., 2015) (plasmid number: 60513 Addgene, Cambridge, MA) that had dTomato replaced with mCherry appended with N- and C-terminal nuclear localization signal (NLS) tags. To recreate the FoxO1-Clover reporter using the new transposase vector, pLenti-FoxO1-Clover was digested using NaeI and XbaI restriction sites and ligated into the modified pSBbi plasmid. To create the FT2-LAP reporter, DNA for FT2-LAP was synthesized (IDTDNA) with the following amino acid substitutions: S209A, H212R, S215A, S246A, S284A, S295A, S298A, S300A, S326A, S380A, S391A, T399A. The synthesized DNA contained 5’ BglII and 3’ Hind3 restriction sites that enabled ligation into the pSBbi-FT1 plasmid. Mutations to FoxO1 amino acids 325–327 (LSP peptide) were introduced via PCR using primers containing the desired mutation/s and ligated in frame using Scal and Hind3 restriction enzyme sites. DNA for the FT2-DDD-3A construct was synthesized (IDTDNA) and contained six additional substitutions compared to FT2-LAP: T24A, S253A, S316A, L325D, S325D, and P327D. The FT2-DDD (plasmid number: 106278) and the FT2-DDD-3A (plasmid number: 106279) are available from Addgene.

Transfection and selection of stable cell lines—To create stable lines expressing each reporter variant, cells were transfected using Lipofectamine 3000 (ThermoFisher) with the respective FoxO1-clover expression plasmid and the transposase expression plasmid pSB100X (plasmid number: 34879, Addgene, Cambridge, MA) at a ratio of 4:1. Two days following transfection cells were selected by incubation with puromycin (2 μg/ml) for 7-
days. Single cell clones expressing FT2-DDD were created through limiting dilution in 96-well plates.

**Microscopy**—Live cell imaging was performed using an EVOS FL Auto microscope (ThermoFisher) with a stage top incubator maintained at 37°C, and in 95% air, 5% CO2. Cells were imaged every 3 minutes using a 10X fluorite objective (N.A. 0.3), and a GFP LED light cube (excitation peak, 472/22 nm; emission peak, 510/42 nm).

**Reporter Characterization**—To characterize each HeLa reporter cell line, cells were serum starved for 90 minutes in Fluorobrite media (ThermoFisher) supplemented with 2 mM L-glutamine, 0.1% bovine serum albumin, penicillin and streptomycin. Cells were then treated with 250 pM R3-IGF-I for 90 minutes followed by the combined addition of the dual PI3K and mTOR inhibitor PI-103 (1 μM) and LMB (100 nM) for 90 minutes. From these experiments, we quantified: the average rate of reporter nuclear exclusion following IGF-I treatment, the average rate of nuclear localization following PI-103 and LMB, and the average measurement range, which was defined as the difference between the percent nuclear localization after serum starvation and the percent nuclear localization 90 min after IGF-I treatment. Export and import rates were calculated using the population average response and fit via non-linear regression (Graph Pad Prism 7). All cell lines were imaged using the same 75 millisecond imaging duration.

**IGF-I single and multi-dose experiments**—For IGF-I dose response experiments, HeLa cells were plated in 12-well plates (Falcon) allowed to grow for 48 h and then serum starved for 90 min in imaging media. Cells were then treated with: 0, 5, 10, 15, 20, 25, 30, 40 50, 75, 125, or 250 pM R3-IGF-I. For multi-dose experiments, cells were imaged 30 minutes prior to IGF-I addition and then treated with 0, 17.5, 37.5, or 125 pM R3-IGF-I for 270 min. In separate wells, cells were imaged in SFM for 30 minutes and then treated with three progressively higher doses of IGF-I for 90 min each (17.5, 37.5, or 125 pM IGF-I). For IGF-I reduction experiments, cells were treated with 37.5 pM R3-IGF-I. After 90 min 54% of the IGF-I containing media was removed and replaced with an equal volume of warmed SFM media such that the new predicted IGF-I was 17.5 pM.

To track repeated IGF-I treatments in individual cells, HeLa cells were serum starved for 90 min and then treated with 37.5 pM R3-IGF-I. After 90 min the cells were then washed three times with SFM and imaged for 2 hr. The cells were then treated a second time with 37.5 pM R3-IGF-I and followed for 90 min. To track sibling responses, HeLa cells were imaged every 30 min for 20 hr in DMEM containing 10% FBS. Cells were then removed from the microscope washed three times in SFM and placed back in the microscope incubator. After 2hrs in SFM cells were treated with 37.5 pM R3-IGF-I and imaged every 3 min for 90 minutes.

**Drug Treatments**—To quantify the relationship between reporter expression and sub-maximal IGF-I responses, cells were serum starved for 90 minutes and then treated with 17.5 pM R3-IGF-I. After 90 minutes cells were treated with 1 μM PI-103 and 100 nM LMB for 90 minutes. To track variation in individual responses across time, cells were treated with either 100 nM LMB, or 17.5 pM R3-IGF-I for 180 minutes.
For tracking cell responses to PI3Kα inhibition, cells were serum starved for 90 min and then treated with either 0, 25, or 125 pM R3-IGF-I. After treatment for 90 min cells were exposed to 0, 12.5, 25, 37.5, 250, or 500 nM alpelisib for 90 min.

**Immunoblots**—Protein lysates were collected on ice in RIPA buffer containing protease and phosphatase inhibitors following a PBS wash. Lysates were spun at 16,000 RCF and the supernatants were collected and used for further analysis. Protein lysates (12.5 μg/lane) were separated using precast NuPAGE™ 4–12% Bis-Tris Protein gels followed by transfer to Immobilon-FL membranes, and blocked with 10% SeaBlock solution. Primary antibodies were used at dilutions of 1:1,000 with 16 h incubations at 4°C, and secondary antibodies were used at dilutions of 1:2,500 with 90 min incubations at 20°C. Images were captured using the LiCoR Odyssey. Primary antibodies were purchased from Cell Signaling: anti-phospho-FoxO1Ser256 (# 9461), anti-FoxO1 (# 14952), anti-phospho-PRAS40Thr246 (# 2997), anti-PRAS40 (# 2691), anti-phospho-AktSer473 (# 4060), anti-Akt (# 4691). Secondary antibodies included IR800- conjugated goat anti-mouse IgG (Rockland, Gilbertsville, PA) and goat anti-rabbit IgG conjugated to Alexa Fluor 680 (Invitrogen, Carlsbad, CA).

**QUANTIFICATION AND STATISTICAL ANALYSIS**—Images were pre-processed using the ImageJ plug-ins (NIH, Bethesda, MD) StackReg (image translation) and Gaussian Blur (2 pixels). Background fluorescence was calculated per plate for each time point by taking the average fluorescence of three regions of interest that did not contain cells. To quantify signaling responses, individual cells were manually tracked by selecting a location in the center of each cell nuclei using the ROI plug-in. The fluorescence intensity of that location was then assessed across time. Cells that migrated over the course of the experiment such that the T0 location no longer aligned with later time points were excluded from analysis. Additionally, cells that divided, died, were multi-nucleated, or overlapped with another cell were excluded from analysis. Image acquisitions were transiently stopped while cell treatments were added and the plate was mixed. Images acquired immediately following cell treatments were removed as they were recurrently out of focus due to the manual mixing.

Three measures were used to quantify individual cell responses: the ‘nuclear localization’, the ‘relative nuclear intensity’, and the ‘scaled relative nuclear intensity’. The ‘nuclear localization’ for each cell was calculated by normalizing fluorescence intensities to the nuclear fluorescence intensity 90 min after leptomycin (LMB) treatment. The ‘relative nuclear intensity’ for each cell was calculated by normalizing fluorescence intensities to the nuclear fluorescence intensity at T0 following 90 min in serum free media. The ‘scaled relative nuclear intensity’ was calculated by scaling each cell’s response to the population average, where 0 pM was set to 0 and 125 pM IGF-I was set to 100.

Dose response curves were fit via non-linear regression using the Hill equation (Graph Pad Prism 7). ‘Raw’ responses were quantified using the nuclear intensity 90 min after IGF-I treatment. ‘T0 normalized responses’ were calculated by taking the 90 min value relative to the 0 min value. ‘Summed responses’ were calculating by summing the relative change of the T0 normalized data using 15 equally spaced images (every 6 min). Individual cell responses to multiple doses of IGF-I were sorted using the summed responses across the...
300-minute experiment. Heat maps were created in R (https://www.R-project.org/) using the gplots heatmap package version 3.0.1.

**Quantification of channel capacity**—To calculate the channel capacity, we used the Shannon-Hartley formula (Shannon, 1948) which was recently applied to dynamic single-cell data by Selimkhanov et al. (Selimkhanov et al., 2014) (Supplementary Materials Subsection 3.1: Signal to Noise Ratio (SNR) in single-cell experiments). Here we define the communication channel as the transmission of the extracellular IGF-I concentration into the relative change in the nuclear FT2 content of an individual cell. In the Shannon-Hartley formula: \[ CC = \frac{1}{2} \log_2\left(\frac{S^2}{N^2}\right), \]
where CC is the channel capacity in bits, S is the signal magnitude quantified by taking the variance of the average response across six relatively evenly spaced IGF-I doses representing the full dose range (0, 10, 15, 25, 40, 125 pM R3-IGF-I), and N is the noise magnitude quantified by taking the average variance within a dose and averaging across all doses (Selimkhanov et al., 2014).

In calculating the IGF-I-FoxO1 channel capacity in HeLa cells we made several assumptions:

1. Each cell in the population has its own communication channel.
2. All cells respond at a relatively uniform maximum level to saturating IGF-I doses and therefore the average maximum response for the population serves as an accurate proxy for any individual cell.
3. All cells have a similar band of additive white gaussian noise in their communication channel.
4. The ligand doses are equally distributed across the sampled response spectrum.
5. Responses as assessed by the FT2 reporter have stabilized by 90 min.
6. After individual cell responses to IGF-I have stabilized they are constant and do not further vary across time.
7. The components of the channel do not materially change across the time frame when responses are measured.
8. The IGF-I concentration is constant across time and is not degraded or locally produced.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

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Highlights

• We describe the development of an improved FoxO1 fluorescent reporter
• Variation between cells can obscure the extent of signaling noise within a cell
• Individual cells can accurately discriminate multiple IGF-I doses
• IGF-I signaling responses in individual cells are durable across time
Figure 1. Variation in signaling responses between cells can obscure the extent of signaling noise in individual cells.

A. Average responses of HeLa cells that stably express the FT2-DDD reporter and have been treated with different doses of IGF-I at 0 min. Doses are indicated to the right of each trace. ($n = 300$ cells per dose). Data have been normalized relative to the fluorescence intensity measured in SFM. B. Single cell responses following treatment with 25 pM IGF-I indicate variable responses across the population (orange traces). Black trace indicates the average population response. ($n = 20$ cells). C. Frequency plots of individual cell responses to IGF-I
at 90 minutes, color coded based on the IGF-I dose as in panel A ($n = 300$ cells per dose). Lowest panel shows pooled distributions from the upper panels; gray indicates the summed response distribution over all doses. **D.** Population distributions of relative FT2- DDD signaling responses to 25 pM IGF-I from 0–90 min (orange) compared to centered distributions from 90–180 min (gray). ($n = 300$ cells per dose). **E.** Graph of single cell responses at 90 min compared to 180 min (color coding as in panel A) ($n = 300$ cells per dose). **F.** Time lapse images of HeLa cells treated with 25 pM IGF-I shows cell-to-cell variation. Scale bars: 25 pm. **G.** Schematic of two scenarios that could explain heterogeneous responses: scenario A is when individual cell responses are heterogeneous because responses have high intracellular variation; and scenario B is when cell responses are heterogeneous because cells have low intracellular variation. **H.** Graph of the standard deviation calculated from the dynamic response of cells treated with different IGF-I doses. The red trace shows the average standard deviation across 0–90 min and the blue trace shows the average standard deviation of the same cells from 90–180 min. Colored circles show the values from three independent experiments. Black bars indicate the average values. **I.** Bar plot of the average maximal channel capacity, where the signal is calculated from raw, T0 normalized (Norm), or summed cell responses (Sum) and the noise is calculated from the variation at 90 min, 0–90 min or 90–180 min. Values are drawn from three independent experiments (colored circles). See also Figures S1, S2, and S3.
Figure 2. Individual cells can accurately discriminate multiple IGF-I doses.
A. Red trace indicates cells sequentially treated with 0 pM IGF-I 0–30 min, 17.5 pM 30–120 min, 37.5 pM 120–210 min and with 125 pM 210–300 min. Control treatments show average responses of HeLa cells treated with 0 pM IGF-I 0–30 min and then with 17.5 pM (medium gray trace), 37.5 pM (dark gray trace), or 125 pM (black trace) from 30–300 min. The light gray trace shows the average response of cells maintained in 0 pM IGF-I. (n = 250 cells per condition).
B. Frequency plots showing time and dose matched responses of cells exposed to a sustained treatment (colored lines as in panel A), or from multi-treated cells (red lines). (n = 250 cells per condition).
C. Heat map showing individual HeLa cell responses to multidose treated cells shown in panel A. Each row indicates response of a single cell across time; treatment is indicated at the top and time window of response is indicated along the x-axis of the heat map. Cells are sorted based on their summed response over 300 minutes. (n = 400 cells).
D. Single cell traces of 20 cells centered at the 75th (cells 90–109 blue traces) and 20 cells centered at the 25th percentile (cells 290–309 red traces) from panel C. The black trace shows the average response from the total cell population.
E. Graph of the average relative response against IGF-I dose for subpopulations of cells that display low (blue = cells 90–109), mid (green = 190–209), and high (red = cells 290–309) responses to IGF-I treatment. Error bars indicate the standard deviation of each selected population. See also Figure S4.
Figure 3. Single cell responses to reductions in pathway activity show limited signaling noise.

A. Time course of the average nuclear fluorescence intensities from HeLa cells maintained in SFM (black trace) and treated with 17.5 pM IGF-I (light blue trace open circles) or 37.5 pM IGF-I (light red trace open circles) for 180 min. In the darker blue trace cells were treated with 17.5 pM IGF-I for 90 min and then the concentration was increased by 20 pM. Correspondingly in the darker red trace cells were treated with 37.5 pM IGF-I for 90 min and the concentration was reduced by 20 pM. (n = 250 cells per condition).

B. Single cell traces drawn from panel A where the concentration was increased by 20 pM at 90 min (blue traces), or reduced by 20 pM (red traces). (n = 25 cells per condition).

C. Relative cell responses at 90 and 180 min following a 20 pM reduction (red circles) or 20 pM increase (blue circles). (n = 250 cells per condition).

D. Time course of the average nuclear fluorescence intensities from cells treated at time 0 min with 25 pM IGF-I and then at 90 min with increasing doses of the PIK3CA inhibitor alpelisib: 0 pM (purple), 12.5 nM (blue), 25 nM (green), 37.5 nM (orange), 250 nM (red), and 500 nM alpelisib (maroon). Control traces show cells maintained in SFM (light gray) or treated with 125 pM IGF-I (dark gray). The first gray arrow indicates the time of IGF-I addition, and the second gray arrow indicates the time of alpelisib addition. (n = 250 cells per condition).

E. Representative individual cell traces drawn from the upper quartile (blue traces) and lower quartile (red traces) of cells treated at 0 min with 25 pM IGF-I and at 90 min with 25 nM alpelisib. The black trace shows the average population response.

F. Plot of the relative responses of individual cells at 180 min drawn from panel A, color-coded to indicate their relative response rate at 90 min: the top 25% (blue), middle 50% (gray), bottom 25% (red). The
black bar indicates the average population response at 180 min. (N = 250 cells per treatment).
Figure 4. IGF-I signaling responses are durable across time.
A. Dynamic responses to repeat IGF-I treatments. Blue traces indicate responses of individual cells; black trace is population average response. Cells were treated with 37.5 pM IGF-I for 90 min followed by a 120 min washout in SFM and a second 37.5 pM IGF-I treatment. B. Comparison responses to repeat treatments. Each dot represents responses of an individual cell. x-axis represents first response calculated at 90 min; y-axis represents second response calculated at 300 min. (n = 500 cells). C. Comparison of responses of sibling cells to 37.5 pM IGF-I. (n = 500 sibling pairs) D. Time-lapse images at t=0 min and at t=90 min following 37.5 pM IGF-I. Sibling pairs are labeled in white with the same letter. Scale bars: 25 μm. E. Analysis of single cell relative response against the time since division. F. Comparison of the relative difference between sibling cells and the time since division. (n = 500 sibling pairs).