Fractional proliferation: a method to deconvolve cell population dynamics from single-cell data

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We present an integrated method that uses extended time-lapse automated imaging to quantify the dynamics of cell proliferation. Cell counts are fit with a quiescence-growth model that estimates rates of cell division, entry into quiescence and death. The model is constrained with rates extracted experimentally from the behavior of tracked single cells over time. We visualize the output of the analysis in fractional proliferation graphs, which deconvolve dynamic proliferative responses to perturbations into the relative contributions of dividing, quiescent (nondividing) and dead cells. The method reveals that the response of ‘oncogene-addicted’ human cancer cells to tyrosine kinase inhibitors is a composite of altered rates of division, death and entry into quiescence, a finding that challenges the notion that such cells simply die in response to oncogene-targeted therapy.

Quiescence-growth model of cell population dynamics

We used our approach to monitor the proliferation dynamics of drug-treated cells. Cell counts extracted from the image data show that vehicle- and cycloheximide (CHX)-treated PC9 cells exhibited linear proliferation (in log scale). In contrast, treatment with erlotinib, lapatinib, PLX-4720 or doxorubicin resulted in nonlinear effects on proliferation of PC9 cells (Fig. 2a). We observed similar effects in other cell lines (Supplementary Fig. 2b). We analyzed the expression of a marker of S or G2 phases (mAG-geminin) in drug-treated cells: erlotinib, lapatinib, PLX-4720 induced arrest in G1 (or G0), and doxorubicin induced arrest in S or G2 (Fig. 2b). These results suggest a correlation between cell cycle arrest and nonlinear proliferation. We also observed this correlation in erlotinib-treated PC9 cells analyzed using flow cytometry (Supplementary Fig. 2a, b) and in MCF10A cells deprived of serum (Supplementary Fig. 2c). Nonlinear population dynamics are traditionally modeled using logistic or Gompertz equations, which have been applied to cultured cells as well as tumors. A major assumption of these models is the idea of a ‘carrying capacity’ representing the maximum size a population can reach in a given environment. There is no clear relationship between carrying capacity and the biological processes that affect cell population size, such as cell division, quiescence and death. To explain the nonlinear proliferation dynamics using biologically relevant parameters, we constructed a quiescence-growth...
Figure 1 | The fractional proliferation methodology. The principal inputs are cell population counts and metrics of single-cell fate obtained by automated time-lapse imaging. The quiescence-growth model is fit to cell count data to estimate rates of cell division, death and entry into quiescence. These estimated rates are statistically bound and can be evaluated with the quiescence-growth model to provide initial insights into the underlying biology without requiring experimental determination of the rates. The EMG model of IMT distribution and the likelihood-of-quiescence model extract experimental rates of division and entry into quiescence from single-cell tracking data. Incorporating experimentally derived rates constrains the quiescence-growth model and produces fractional proliferation graphs that dynamically resolve the change in cell counts into fractions of dividing and quiescent cells.

Data-derived quiescence-growth model parameters

Good fits of the cell count data with the quiescence-growth model may be achieved through different combinations of parameter values (Supplementary Fig. 3, Supplementary Table 1 and Supplementary Software 1), particularly death and division rates, which oppose each other. We therefore constrained the model with experimentally measured rates from single-cell tracking of time-lapse images.

We first tracked single cells across time-lapse image stacks to quantify observed cell lifespans, which are defined as the time between an initial mitotic event and (i) a death event, (ii) another mitotic event (defining an intermitotic time, IMT), or (iii) the end of the experiment (EoE) (Fig. 3a, Online Methods and Supplementary Video 1). Cell lifespans demarcated by an initial mitotic event and the EoE may belong to either dividing or nondividing (quiescent) fractions.

For death rates, we identified death events by shrinkage and disintegration of nuclei (Supplementary Fig. 4a), tallied these events and converted them directly to rates (Online Methods).

For division rates, we first determined the variability of IMT in the population by examining IMT distributions, which are non-Gaussian by visual inspection (Fig. 3b,c and Supplementary Figs. 5 and 6), fail several statistical tests of normality (Supplementary Tables 2 and 3) and cause bias in the estimation of division rate if a simple mean value is used. We searched for model distributions that adequately capture the variability (Fig. 3b). Among several possible models we examined (Supplementary Table 2), an exponentially modified Gaussian (EMG) model fits the observed IMT distributions from a wide range of cells and conditions (Fig. 3 and Supplementary Figs. 5 and 6) while minimizing the number of parameters to three. An additional benefit is that EMG model parameters are mathematically and biologically separable: that is, their values are differentially affected by drugs (Supplementary Fig. 6).

We adapted a method of calculating division rates in bacterial cultures12 to utilize all EMG parameters fit to the observed IMT distribution (Supplementary Notes 1 and 2). This method accounts for the dispersion of individual IMT, especially the...
slowly dividing cells in rightward-skewed tails. In addition, this calculation of the division rate takes into account the age structure of an asynchronously dividing cell population under the assumption of exponential growth.

For quiescence rates, a crucial question is whether cells with lifespans demarcated by an initial mitotic event and the EoE are quiescent or whether they would have divided if the experiment had continued (Fig. 3a). We estimated the probability that a cell is quiescent by implementing a likelihood-of-quiescence model based on a statistical survival formula 13 (Online Methods and Supplementary Notes 1 and 2). The older the undivided cell relative to the distribution, the lower the likelihood that it would have divided after the EoE and the higher the likelihood that it would have been quiescent. The rate of entry into quiescence was then calculated using the fraction of quiescent cells in the population and is relative to the calculated rate of division. Notably, without properly accounting for the slowly dividing cells in the population with a well-fit model (such as an EMG), the rate of entry into quiescence could be significantly overestimated.

We experimentally derived the three quiescence-growth model parameters from single-cell tracking data of CA1d cells (Fig. 3c) treated with erlotinib (1 and 8 μM). Compared to the control (dimethylsulfoxide (DMSO)-treated) cells, more erlotinib-treated cells reached the EoE without dividing and were estimated to be quiescent by the likelihood-of-quiescence model (Table 1). The IMT distribution of cells treated with 8 μM erlotinib was flattened and elongated rightward (Fig. 3c). Rates calculated from the single-cell tracking data, EMG parameters and estimated quiescence fraction confirmed a decreased rate of division, a slight increase in death rate and a tenfold increase in the rate of entry into quiescence at either erlotinib concentration. These experimentally measured rates were in agreement with the rates estimated from fitting the quiescence-growth model to the cell count data (Supplementary Fig. 3 and Supplementary Table 1), thus indicating that the quiescence-growth model provides an accurate description of cell proliferation and validating its prediction that the nonlinear proliferation response to erlotinib (Fig. 2a) is explained by altering the rate of entry into quiescence (Fig. 3c). We observed that few cells died during

Table 1 | Quiescence-growth model parameters obtained from single-cell tracking data

|            | DMSO  | 1 µM erlotinib | 8 µM erlotinib |
|------------|-------|----------------|---------------|
| \( f_{\text{EoE}} \)  | 0.04  | 0.26           | 0.47          |
| \( f_d \)    | 0.04  | 0.26           | 0.41          |
| \( d \)       | 0.0379| 0.035          | 0.0241        |
| DT\(_d\)    | 18.3 h| 19.8 h         | 28.8 h        |
| q           | 0.0015| 0.0013         | 0.0018        |

The following parameter values were calculated from the data shown in Figure 3c: fraction of live cells reaching the end of experiment, \( f_{\text{EoE}} \); fraction of quiescent cells, \( f_d \); division rate, \( d \); doubling time of dividing cells only, \( DT_d \); and rate of entry into quiescence, \( q \). The death rate \( (d) \) was obtained directly from analyzed image stacks. Values for \( d \) and \( q \) are in h\(^{-1}\).
the experiment, and cells considered quiescent did not exhibit preapoptotic nuclear condensation (data not shown).

**Fractional proliferation graphs**

We then used the parameters obtained from single-cell tracking data (Supplementary Video 2) in the quiescence-growth model to produce graphs of the total population deconvolved into fractions of dividing and quiescent cells (Fig. 3d and Supplementary Software 2). At ~24 h the fractions of cells in the two compartments were equal (Fig. 3d); in contrast, at 80 h the quiescent fraction was 82% and the dividing fraction was 18%.

**Drug response of oncogene-addicted cells**

We applied this approach to investigate the dynamic response of PC9 cells to erlotinib (Fig. 4 and Supplementary Fig. 1c). PC9 cells harbor activating mutations of the epidermal growth factor receptor (EGFR) and are ‘oncogene-addicted’ and hypersensitive (that is, concentration of drug required to cause a 50% decrease in cell number (G1S0 < 20 nM) to EGFR tyrosine kinase inhibitors (TKIs). These cells are representative of EGFR-mutated tumor cells in lung cancer patients that respond favorably to treatment (or other EGFR-specific TKIs). The prevalent view is that PC9 cells have a strong apoptotic response to EGFR TKIs, but, to our knowledge, the relative contributions of cell death versus quiescence in the PC9 response to erlotinib remain uncharacterized.

From cell count data derived from image stacks, PC9 cells exhibited exponential proliferation with a rate = 0.0183 h−1 (doubling time = 38 h) in medium containing the vehicle (DMSO); however, single-cell tracking data show that this value underestimates the rate of division and does not consider the contribution of quiescence and death (Fig. 4d). Proliferation decreased in a nonlinear fashion upon erlotinib treatment (Fig. 4a), and cells accumulated at the EoE (Figs. 3a and 4b). From single-cell tracking data, 53% and 73% of cells with initial mitotic events within the first 25 h entered quiescence in response to 15.6 nM and 62.5 nM erlotinib, respectively, compared to 4% in the control cells. The IMT distributions showed a rightward skew in response to erlotinib characterized by an increased k parameter value, whereas all other parameter values remain essentially unchanged (Fig. 4c). We calculated the rates of division, death and entry into quiescence from the single-cell tracking data and produced fractional proliferation graphs (Fig. 4d). As for CA1d cells, using these rates in the quiescence-growth model correctly predicted the experimentally determined cell counts (Fig. 4d). The rate of cell death was 0.0015 h−1 in DMSO and increased to 0.0048 h−1 in 62.5 nM erlotinib, but this higher death rate cannot by itself explain the decreased proliferation in response to erlotinib. The q parameter for 62.5 nM erlotinib-treated cells increased ~40-fold over that of DMSO-treated cells (Fig. 4d). These results indicate that in oncogene-addicted PC9 cells, the antiproliferative response to erlotinib is primarily due to increased entry into a nondividing or quiescent state, and not to apoptosis as is commonly assumed.

We verified that erlotinib treatment increased the fraction of quiescent PC9 cells by flow cytometry on Ki-67– (Supplementary Fig. 7) and p27–immunostained cells (not shown). We confirmed these observations in another oncogene-addicted model cell line (A375, representing BRAF V600E–mutated melanoma) (Supplementary Note 3 and Supplementary Figs. 7 and 8). The nondividing quiescent state we observed was not equivalent to a preapoptotic state; after 96 h in 1 µM erlotinib, a drug washout experiment showed that proliferation resumed to pretreatment levels (Supplementary Video 1 and Supplementary Fig. 9). Also, a large fraction of cells remained viable (Ki-67 positive) after more than 90 h of erlotinib treatment (Supplementary Fig. 7). Determining the eventual fate of these cells (division, death or extended quiescence) would require studies of longer duration.
Application to primary cells
We assessed the performance of our approach on primary cells using baculovirus-based transduction of a genetically encoded fluorescent nuclear probe that is easy to use and commercially available (CellLight Nucleus, Invitrogen). Labeled nuclei were detectable within 12 h, persisted for 5–6 d, and were amenable to automated counting and manual tracking. As an example, the proliferation of primary human squamous cell carcinoma cells is shown in Supplementary Figure 10 and Supplementary Video 3.

It is worth noting that an EMG distribution also describes the distribution of IMT in primary cultured cells. Additional experimentation will be required to determine whether this distribution is applicable to all cell types.

DISCUSSION
The fractional proliferation method provides quantitative insight into cell proliferation in response to perturbations and permits deconvolution of the relative contribution of multiple cell fates to cell population dynamics. Using this approach, it is possible to capture the behavior of minor subpopulations of cells (for example, stem or progenitor cells, clonal variants and drug-resistant phenotypes) within perturbed populations. The importance of these subpopulations is increasingly appreciated, and methods to study them should be broadly applicable.

The integrated mathematical models we developed describe the emergence of population behavior from experimental data measuring single-cell fates. We note that these models are not definitive—for example, they will need to be adjusted to more accurately accommodate age structure in asynchronously dividing populations; to apply them to stem cells, a rate of entry into one or more differentiated states would have to be included. However, image data sets such as those described here can be analyzed by future models. Furthermore, the approach could be enhanced by the incorporation of other readouts, such as immunofluorescence of molecular markers or live-cell reporters of molecular activity.

Time-lapse video microscopy has been used for decades, but its high-throughput implementation has only recently been enabled by technological advances in automated microscopy instrumentation coupled to computation. Methods to automate the extraction of mitotic phases and duration from time-lapse movies were recently developed17–19. These approaches focus will be required to determine whether this distribution is applicable to all cell types.

Note: Supplementary information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS
D.R.T. conceived of the approach, D.R.T. and P.L.F. cultured, treated and imaged cells, D.R.T. analyzed images, S.P.G. and D.R.T. developed the mathematical models, D.R.T. and S.P.G. fit model parameters to data, and D.R.T. and V.Q. cowrote the paper.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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ONLINE METHODS

Software. We provide (i) an interactive Mathematica CDF Player file to explore the effects of altering quiescence-growth model parameter values on cell proliferation plots (Supplementary Software 1), (ii) an ImageJ macro for automated cell counting (enumeration of nuclei) (Supplementary Note 2), and (iii) fracprolif, an extension of the freely available statistical software package R\textsuperscript{31} (http://www.r-project.org/) that incorporates all of the code (Supplementary Software 2) used to analyze single-cell tracking and cell count proliferation data and generate fractional proliferation graphs (described in Supplementary Note 2).

Cell culture and labeling. The following cell lines were used: MCF10A, MCF10A-CA1d (abbreviated as CA1d), SQ20B and PC9. MCF10A and CA1d cells were cultured in DMEM/F12 medium containing 10% equine serum, 5% FBS, 20 ng/ml epidermal growth factor, 10 μg/ml insulin, 500 ng/ml hydrocortisone and 100 ng/ml cholera toxin. SQ20B cells were cultured in DMEM containing 20% FBS and 400 ng/ml hydrocortisone, and PC9 cells were cultured in RPMI 1640 medium containing 10% FBS. Primary human tumor-derived cells were obtained from a patient with squamous cell carcinoma of the tongue and were grown continuously in culture in keratinocyte (serum-free) growth medium (Invitrogen). Cell lines were engineered to express the histone H2B–monomeric red fluorescent protein (H2B-mRFP) fusion protein using lentivirus-mediated transduction as previously described\textsuperscript{32}. Brightly fluorescent single-cell clones were expanded and compared to parental populations using traditional proliferation measurements to ensure they were representative of the initial population. Primary cells were induced to express H2B-mRFP using recombinant baculoviral particles (CellLight Nucleus, Invitrogen) at 20 particles per cell according to the manufacturer’s instructions.

Imaging with extended temporally resolved automated microscopy (ETRAM). Imaging was performed on 96-well plates (BD cat. no. 353219) using a BD Pathway 855 with a 20× (0.75 NA) objective in a CO\textsubscript{2}- and temperature-controlled environment. Images were acquired every 6–30 min using BD Attovision 1.6.2 software with the instrument in confocal mode (spinning disk). Nine adjacent images were captured at 0.4-s exposure and 2 × 2 binning to comprise a single 3 × 3 montage (approximately 800 μm\textsuperscript{2}) from each of approximately 40–60 wells per experiment, and images were acquired for at least 72 h. Example montaged images are shown in Supplementary Figure 1a. Assays minimally included duplicate or triplicate wells, and complete experiments were performed at least twice. Cells were seeded at 2,500–5,000 cells per well and were allowed to grow overnight, which yielded approximately 200–600 cells at the onset of imaging. Cells were imaged until confluence (approximately 3,000 cells in an image) was achieved in control wells. Erlotinib concentration-response curves on PC9 cells were performed five or more times.

Enumerating nuclei. Nuclei were counted from ETRAM-generated image stacks sampled at approximately 1-h intervals. Images were imported into the freely available ImageJ (http://rsb.info.nih.gov/ij/) program and subjected to a macro optimized for images obtained from a BD Pathway 855. The macro (i) corrects for uneven illumination using a 50-pixel-diameter rolling ball filter, (ii) converts images to binary using a predefined threshold intensity value, (iii) segments individual nuclei using a watershed algorithm, and (iv) quantifies objects within a specified range of areas and circularity. Example images and resultant cell population plots are shown in Supplementary Figure 1a,b. The ImageJ macro is provided in Supplementary Note 2, and an example image sequence showing nuclei enumeration by ImageJ is shown in Supplementary Figure 4.

Quantifying rates of cell death. An example of a region of a manually tracked ETRAM-generated image stack is shown in Supplementary Video 1. Cell death was identified by nuclear shrinkage to less than 50% of average nuclear area and subsequent nuclear dispersion or detachment (that is, the nucleus was no longer detectable) (Supplementary Fig. 4). Rates of cell death were determined by dividing the number of events (cell deaths) detected across all frames by the total cell observation time (the number of cell nuclei in each frame multiplied by the time interval between frames); this rate does not require individual cells to be tracked over complete lifespans.

Quantifying intermitotic times and rate of division. For most cell lines, ETRAM was performed at 12-min intervals to minimize light exposure and phototoxicity, and for highly motile cells, at 6-min intervals to eliminate bias against faster-moving cells. Intermitotic times were measured by manually tracking individual nuclei through the series of ETRAM-generated images, identifying mitotic events (metaphase chromosomes) and determining the number of frames between mitotic events for each individual cell lifespan. Lifespans of cell nuclei that divided once during the experiment but reached the EoE were also determined. We tracked 100 nuclei for each condition, and more if too many cells reached the EoE without having divided, as a minimum of 50 individual IMTs represented a distribution.

Model of proliferation kinetics (quiescence-growth model). The quiescence-growth model is formulated as a pair of coupled ordinary differential equations in which \( x \) represents the dividing or birth rate, \( q \) for quiescence, \( a \) for division or death rate, \( y \) equals the total cell population. The rates are described by the three parameters: \( d \) for division or birth rate, \( q \) for quiescence rate and \( a \) for death rate.

\[
x' = (d - q - a)x
\]
\[
y' = qx - ay
\]

which has an analytical solution of the following form

\[
x(t;x_0,d,q,a) = x_0e^{(d - q - a)t}
\]
\[
y(t;x_0,y_0,d,q,a) = \left( y_0 + x_0 \frac{q}{d-q} \left(e^{(d-q)t} - 1 \right) \right)e^{-at}
\]

On a log scale the model is nonlinear because of the quiescence compartment (\( y \)). Also of note, when \( d = q \), the following
solution applies
\[
\lim_{d \to q} y(t;x_0,y_0,d,q,a) = e^{-at}(x_0 dt + y_0)
\]

When \( d \geq (q + a) \), the following asymptotic behavior is observed
\[
x + y \sim -\frac{d}{d - q} x_0 e^{(d - q - a)t}
\]

This shows that the model approaches exponential growth as long as the division rate exceeds the sum of the rates of death and entry into quiescence, and it mathematically proves that the observation of exponential proliferation of a population does not exclude the possibility of quiescence and death. Thus, only in the absence of any death or quiescence does the rate of cell division reflect the rate of proliferation of the population. On the other hand, note that the population could grow exponentially even if nearly half of the cells enter quiescence or die. Furthermore, entry into the quiescent compartment (with rate \( q \)) provides the only mechanism by which nonlinear proliferation curves can be achieved. An alternative of the model has been produced in which a different rate of death from each compartment is provided (Supplementary Note 1). However, because measuring these different rates from the single-cell data with sufficient statistical power is not yet feasible, the alternative model reverts to the form used in this method.

**Statistical methods.** To determine whether a model could describe the observed data with sufficient statistical accuracy, a one-sided Kolmogorov-Smirnoff test was performed with a two-sided test being the null hypothesis. This test determines the probability that the data and the model are sampled from the same distribution, and \( P < 0.05 \) was assumed to be statistically significant evidence that the data and the model represent different distributions.

The Shapiro-Wilk test was used to test for normality. The likelihood of one of two specific models (for example, a linear model compared to the quiescence-growth model) correctly fitting the data was determined using Akaike’s information criterion.

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