

# Event timing at the single-cell level

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## Abstract

The timing of a cellular event often hides critical information on the process leading to the event. Our ability to measure event times in single cells along with other quantities allow us to learn about the drivers of the timed process and its downstream effects. In this review, we cover different types of events that have been timed in single cells, methods to time such events and types of analysis that have been applied to event timings. We show how different timing distributions suggest different natures for the process. The statistical relations between the timing of different events may reveal how their respective processes are related biologically: Do they occur in sequence or in parallel? Are they independent or inter-dependent? Finally, quantifying morphological and molecular variables may help assess their contribution to the timing of an event and its related process.

**Keywords:** *live cell imaging; event timing; single cells; quantitative biology; systems biology*

## INTRODUCTION

Phenotypic variability within a population of cells has been extensively studied in microbial [1, 2], viral [3, 4] and multicellular organisms [2, 5–8]. Many of the studied examples focus on variability in cell states at a given time. A different form of variability is that of timing of cellular events. Often this form of variability leads to cell state variability: in the trivial sense, at a certain point in time some cells will be before a given event while others have already passed it (Figure 1A). At a deeper level, differential timing of molecular or cellular events can lead to different decisions at the cell level. Differential timing within a population of cells may lead to spatial organization such as symmetry breaking and pattern creation phenomena. At the other end, timing variability is itself caused by other forms of cellular variation, and therefore we can learn about the variation of its sources through studying timing. While cell-to-cell variability in gene expression and in cell states received much attention over the past decade [5, 8, 9], variability in timing has been less studied. In this review, we cover recent examples of quantitative studies in event timing, methods to time events and types of analysis that

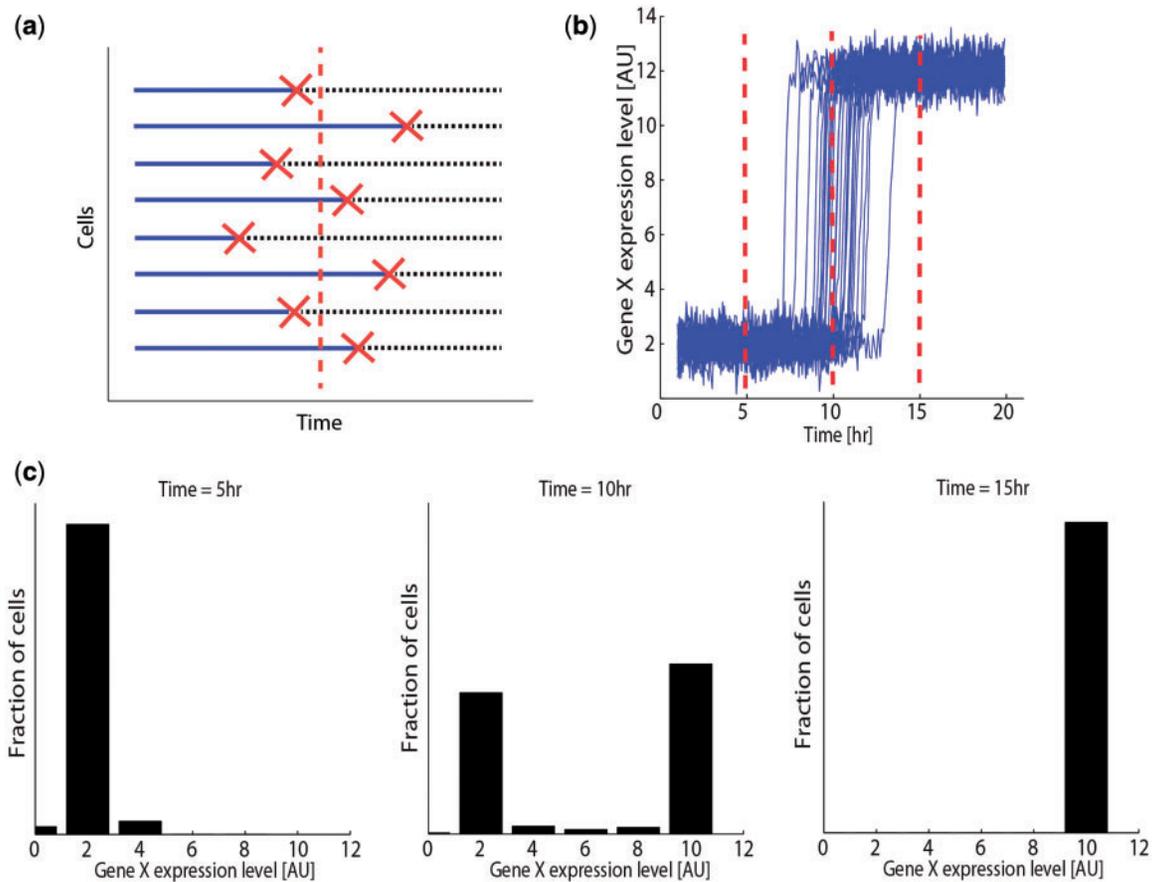
can be performed on event times in order to gain new biological insights.

What events can be timed in single cells? One type is landmark structural/developmental events. Cellular processes that include structural changes, such as the mitotic cell cycle, meiosis, sporulation or apoptosis, all include several landmark events that can be visually identified and timed. The timing of cell division in bacteria was studied quantitatively by Powell >50 years ago [10]. In yeast, timing of S phase in the cell cycle was analyzed [11, 12], as well as different phases of meiosis and sporulation [13]. An easy event to spot and time is cell death in its different forms, including bacterial lysis following Lambda-phage activation [14, 15], and cell death in *TRAIL*- or *TNF*-induced apoptosis in mammalian cells [16]. Landmark cell-state switches have been timed in bacteria, including exit from dormancy [17], sporulation [18, 19] competence [1, 20] and filamentation [21]. Another type of event is functional events: those that correspond to the function of the cells and are often repetitive. These include action potentials in neuronal cells or a contraction of cardiac muscle cells.

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**Figure 1:** Relations between timing variability and phenotypic variability. **(A)** Time tracks for individual cells. The ‘x’ sign marks a landmark event, separating between two cell states (marked by continuous and dashed line). Cell-to-cell variability in the timing of the event results in phenotypic variability in the population at a given time (dashed vertical line). **(B)** Simulated expression level time tracks in individual cells, showing variable onset times. **(C)** Expression level histograms for three different time points in (B). The histogram at  $t=10$  hr shows high expression variability resulting from the onset time variability.

Molecular events can be timed for specific molecular species in the cell. One can define discrete events in the context of localization or expression of the mRNA or the protein of a given gene. Localization events have been quantified, for example in response to external signals. Hersen *et al.* [22] showed a direct correlation between the magnitude of osmotic stress and the time it takes the master regulator of osmo-stress response in yeast (*Hog1*) to localize into the nucleus. In other cases, the temporal measurements of such localization events exposed previously unknown oscillatory behavior, where a steady localization was expected. Cai *et al.* [23] reported short stochastic bursts of nuclear localization of *Crz1* in single cells in response to change in extracellular calcium. They showed that ‘calcium’ concentration controls the frequency, but not the duration, of those bursts.

Nuclear-cytoplasmic localization oscillations were also reported for *Msn2* in response to stress [24] and for *NF- $\kappa$ B* in response to stimulation [25].

Expression events can be defined for the expression of a specific gene: for example, its onset time or shut-off time. Such events have been used as indicators of larger cellular responses, as well as the activity of upstream signaling pathways. Onset of protein production in the context of different processes was timed in bacteria [14, 17] and yeast [13, 26]. Onset times of early meiosis genes served as a molecular event (marking transcriptional response) that preceded structural changes (replication, nuclear division). Activation of early and late lytic genes marked different stages in ‘lambda-phage’ activation [14]. Turning on and off of a synthetic *GAL*-inducible promoter was analyzed in yeast lineages [27]. Lahav *et al.* [6] studied protein

expression dynamics of *p53* and its negative regulator *Mdm2* in single cells, showing that this circuit generated pulses in *p53* levels in response to DNA

damage. Expression oscillations were also measured in the bacterial ‘SOS’ [28] and iron homeostasis [29] systems.

### TECHNICAL BOX: HOW TO TIME EVENTS?

Timing of events in single cells requires two components: an experimental setup for imaging live cells during the relevant process and a characterization of the event in terms of a visual phenotype, which can be tagged either manually or (preferably) by means of an automated image analysis procedure.

The zero time point can often be naturally defined as the switch of experimental conditions (e.g. shift to starvation or differentiation medium). Similarly, we can induce later reference points by changing conditions at defined times along the experiment [30]. The cellular events can then be timed with respect to these external reference time points. The use of microfluidic devices allows us to precisely control external signals while imaging the cells and is therefore very powerful in these kinds of studies.

Events such as apoptosis are followed by gross changes to the cell or nucleus morphology. Often there are preceding phenotypes as well (e.g. blebbing in apoptosis). Different stages in differentiation or reprogramming are characterized by shrinking or expansion of the cell or by a change in aspect ratio (e.g. from round to elongated). Mitosis can be easily identified in fluorescently tagged nuclei by the fission of a single nucleus into two parts. In yeast meiosis, the two meiotic divisions (MI and MII) are characterized by a switch from one to two, back to one and then four fluorescent foci [13]. Moreover, during this process there is a typical pattern of changing distances between the foci.

An additional way to time the transition into a new cell state, or phase in a process, is through the expression of a molecular marker. Entry into competence in bacteria, different phases of lysis by phage, cell cycle phases, loss or gain of pluripotency in stem cells have all been timed using the turning on or off of a molecular fluorescent marker. Observed molecular levels are usually of a continuous nature, and so often a threshold is set in order to define a discrete event from these markers. Such ‘threshold events’ can also be defined for continuous morphological quantities. For example, a threshold on cell length in growing bacteria was set to define a ‘filamentation’ event [21].

Some events, such as lineage restriction points or ‘point of no return’ in a developmental process, can often be measured and timed indirectly in a ‘destructive’ fashion by changing the external conditions. For example, to time a commitment point to meiosis in yeast (which requires a starvation signal), one has to return the yeast to rich conditions and observe which of the cells reverted back from the meiotic plan and which of them carried on with the process. Similarly, the response to addition of a pheromone in yeast can distinguish between cells that are before (pre-start) or after (post-start) the commitment point to mitosis [30]. This technique does not allow the continuous timing of the event in all cells, but instead only measures which cells already passed the event at a particular point in time.

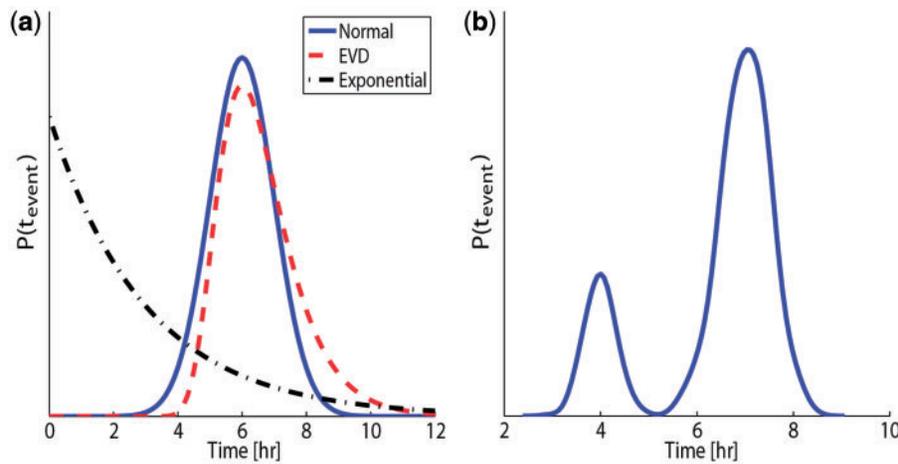
### ANALYZING THE TIMING OF A SINGLE EVENT

Once we have timed an event in a large population of cells, the first thing we can analyze is its distribution.

The mean event time may point to interesting biological phenomena, particularly when it deviates a lot from what was expected. For example, mitochondrial outer-membrane permeabilization (MOMP) [16], yeast meiosis [13] and bacterial sporulation [18] all displayed very large times on average, compared with natural time scales of their respective systems, pointing toward specific delay mechanisms. The suggested mechanisms include

both specific regulatory circuits (such as positive feedback) as well as utilization of internal or external resources (through autophagy or cannibalism, respectively) [5].

The shape of the distribution may teach us something on the factors that dominate the timing of the event. An event that hinges on a single memory-less process will show an exponential distribution [31] (Figure 2A). An extreme value distribution (EVD), such as the Gumbel distribution [32], Figure 2A) can suggest that the event waits for the last (or maximal) of a group of processes that occur in parallel. For example, the pachytene checkpoint before the first meiotic division (MI) verifies that all



**Figure 2:** Timing distributions. **(A)** Exponential, normal and extreme value distributions of event times. The EVD corresponds to a maximum over events. **(B)** A bimodal distribution.

recombination events have been completed properly [33]. The timing of MI will therefore be determined by the last recombination process, and if these events were independent the corresponding timing distribution will resemble an EVD.

A more typical case is a Gaussian distribution (Figure 2A). This timing distribution may be the result of a single process with a normal distribution, or more likely, a series of independent processes that occur in sequence, and therefore the timing of the end point (being the sum of the individual step times) tends by the central limit theorem toward a Gaussian distribution.

Distinguishing between an EVD and a normal distribution, both unimodal, may require a very large sample size. An easier case to discern is a multi-modal distribution (Figure 2B). Such a distribution may point to a hidden division of the data points to several groups. For example, a multi-modal distribution of commitment times to meiosis turned out to be the result of size dependence: large cells had a different distribution than small cells (they tended to commit to the process earlier), and so the bimodality of the timings actually reflected a bimodality in size distribution [13]. The differentiation process of embryonic stem cells (ESCs) is accompanied by an elongation of the G1 phase [34] due to an additional checkpoint. Measuring the length of G1 in a population of differentiating ESCs may therefore result in a bi-modal distribution, stemming from the sub-populations that are either before or after the gain of the new checkpoint.

Once the wild-type distribution is established, it can be used as a phenotype against which the effect

of different mutants can be evaluated. One can check the influence of specific mutations on either the mean timing of an event or its dispersion. Naively, we expect mutations that are deleterious to a process to increase its duration and its variance. Siegal-Gaskins and Crosson compared division time and elongation rate between wild-type *Caulobacter crescentus* cells and mutants in a regulator of polar cell development [35]. The mutation caused an increase in the mean and dispersion of both the division time and the elongation rate. Mutants that lead to reduction in mean time or variance may point to proteins that serve to regulate the rate of the process. Bean *et al.* [11] compared G1 length distributions between different cell-cycle-related mutants. While some mutants (such as *swi4*) increased mean time and variance, others (*cln3*, *mbp1*, *rme1*) increased the mean time while decreasing the variance (thus lowering the relative variability). At the same time, another timing marker exposed that the same mutation extended the variability in a previous phase of the cell cycle (Start). The variability of G1 duration decreased as ploidy increased, or when the gene dosage of G1 cyclins was increased [12]. When timing bacterial lysis by bacteriophage, both the mean lysis times and their variability showed a dependence on the allelic variants of a key gene [15]. Interestingly, the allele effects on the mean and the variance were independent. Cagatay *et al.* [36] showed that a synthetic variant of the circuit controlling entry to competence leads to less variable duration times of the competent state than the native circuit. This example shows how synthetic biology

approaches can be useful in the study of process timing and its variability.

## RELATIONS BETWEEN EVENT TIMES

When a process can be broken into several sub-intervals, one can analyze the relations between their durations and the contribution of each sub-interval to the whole process. The addition of a molecular marker allowed breaking the G1 cell cycle phase and entry into meiosis into two sub-intervals [12, 13]. Both in MOMP and yeast meiosis it has been shown that the initial ‘wait state’ is the longest and most variable in the process, while the subsequent events are more regularly and tightly timed [13, 16]. Such findings can focus the study of delay mechanisms to the relevant stage of the process [5].

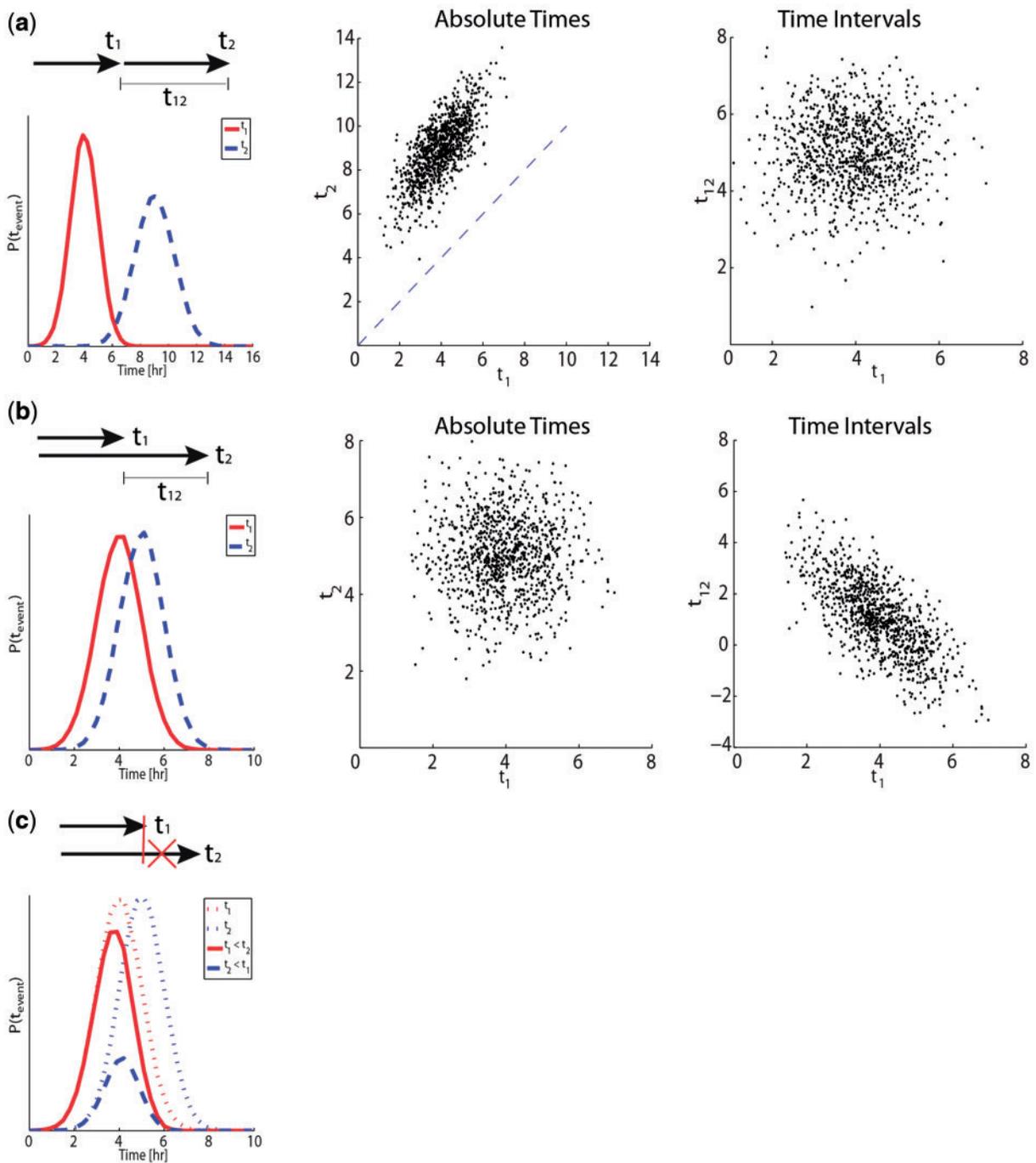
Analyzing the correlation between sequential sub-intervals over many cells provides information on interval-specific variability versus global factors that affect the timing of all the intervals. This is similar to the ‘intrinsic’ versus ‘extrinsic’ noise distinction in gene expression [37]. Both in yeast meiosis and lambda phage induction [14], consecutive intervals in the process showed no temporal correlation (Figure 3A). A similar result was seen in two sub-intervals of the yeast G1 phase [12]. This independence suggests that the dominant factor(s) controlling the timing of the events is different in each interval (‘interval-intrinsic’), and no global (‘extrinsic’) and stable factor (such as cell size) dominates the pace of all the stages in the process [31].

Temporal correlation can also be analyzed for processes that occur in parallel. In fact, in the example of entry to meiosis, such an analysis suggested that arrest of mitosis and entry into meiosis, two events that were thought to be sequential, actually occur almost independently in parallel (Figure 3B). Similarly, in B-cell activation, a ‘competition and censorship’ effect was observed between two alternative fates (death and cell division) [38]. In this scenario, two or more processes occur in parallel, and the first process to reach termination (or an earlier landmark point) censors or excludes the possibility of termination of the other processes. This results in censored versions of the respective event timing distributions (Figure 3C). In such cases, one can compare the event time distributions of one event between wild-type conditions and

conditions where the second fate is inhibited. This comparison will show if there is a competition between the processes or not. This approach was used to study cell death and mitotic slippage, showing they are independent and mutually exclusive [39]. The similarity of timing distributions of these two fates provided another evidence for the ‘independent completion’ model in this system. Interestingly, a constraint on the order of two events (as in yeast cell cycle arrest and entry into meiosis [13]) do not necessarily impose a correlation between their times, if their respective time distributions are separated enough in time: two events can occur sequentially while their timing may be independent. Similarly, Amir *et al.* [29] show that resumption of cell division after a switch to low-iron conditions occurs after the onset of iron metabolism gene expression, but the timing of these two events is uncorrelated.

A special type of correlations is mother–daughter correlations, or in the case of symmetric division sibling correlations. These can point to an early state within the cell leading to a specific event and timing of an event. They can also be used to infer more information on the temporal and dependence structure of the observed process. Bean *et al.* [11] compared different interval times between mother and daughter, concluding that a tagged cell cycle regulator (*Whi5-GFP*) shows asymmetry in its nuclear residence. Kaufmann *et al.* [27] showed long-range memory in yeast through the within-lineage correlation of activation times of a synthetic positive-feedback circuit.

In *TRAIL*-induced apoptosis, analysis of sister-cell time to death showed that the more time has passed from cell division to induction of the process, the less correlated is the time to death of two sister cells, suggesting a decay in the memory state within the cells [16]. A strong dependence of this memory on protein translation further supports that it stems from changing protein levels. Similarly, oscillations in *Mdm2* in response to DNA damage showed decreasing correlation between sisters as time from cell division passed [6]. Analysis of sister-cell fates in B-cell differentiation led to a model of competition and censorship between four independent processes [38]. A similar competition and censorship was recently shown in *Bacillus subtilis* between competence and sporulation [2]. The independence structure in that system suggested these two processes progress independently with no cross-regulation before the decision between them.



**Figure 3:** Relation between two events can be distinguished by their statistics. Two events, with timing  $t_1$  and  $t_2$ , can fall into one of several classes. **(A–C)** Left: time histograms of  $t_1$  and  $t_2$ . Middle: plot of  $t_2$  against  $t_1$  for single cells. Right: plot of the interval  $t_{12} = t_2 - t_1$  against  $t_1$ . **(A)** Sequential events: the second process starts after the first one ends. This results in positive correlation between absolute times, but not necessarily between time intervals. **(B)** Parallel independent events: the two processes proceed in parallel with no interaction. This results in no correlation between absolute times, but negative correlation between the time intervals. **(C)** Parallel independent events with censorship: same as **(B)**, but the first event to occur excludes the other event. The non-censored versions of the distributions are plotted (dotted) for comparison.

By activating the competence master regulator from promoters expressed at three different phases of sporulation (before, during and after fate decision), Kuchina *et al.* [2] showed that the relative timing of *comK* with respect to the sporulation process modulates its effect on the chosen fate.

By tracking lineages of yeast cells, Kaufmann *et al.* [27] found that variability among single cells is not only due to the stochastic chemical reactions inside those cells but also due to their unique family histories. Using engineered network with two semi-stable states, they initiated the cells to the 'OFF' state. They found that cells continued to be synchronized in their switch time to the 'ON' state long after they were separated in the lineage tree. While independent individual cells have exponentially distributed switching times.

Siegal-Gaskins and Crosson found a correlation between mother and daughter cell division times in *Caulobacter pilori* cells [35].

Event timing can be used to characterize a more complex temporal structure, such as division times in cell pedigrees [11]. In this work, the effect of different mutants was evident in its effect on the structure and branch lengths of the tree of division times. While the wild-type pedigrees were more uniform in their branch lengths, the mutant pedigrees were more variable. Wang *et al.* [21] used special microfluidic devices to follow cell growth, division rate and filamentation events in the original mother bacterium along 200 generations. They found that the rate of filamentation events grew with replicative age, while it maintained constant low rate in a DNA damage response mutant.

Event timing can also point at spatial correlations between cells. For example, by using the 'Fucci' constructs that report on two phases of the cell cycle, Sakaue-Sawano *et al.* [40] demonstrated remarkable spatial correlation between the cell cycle phase of cells in specific tissue layers in the developing mouse embryo. Cell-cell interactions can coordinate timing of cellular events, as observed in circadian cycles and morphogenesis during development [41].

## DRIVERS OF EVENT TIMING

When observing the variability of event times in a population, it is interesting to ask what factors cause cells to be slower or faster in the measured process. It is easiest to first look for 'determinants of timing', or factors that correlate with the event time, and

only at the second step study the causal driver of timing behind that determinant.

Event times can be correlated with a global property of the cell. For example, cell size showed high correlation with time to enter meiosis in yeast [13] and in cell cycle time [12, 42]. The further breaking of G1 phase to two subintervals showed that the first phase is size dependent, while the second one is not [12]. Size dependence of timing can be mediated through a host of parameters, from direct mechanisms for size estimation through differences in global concentrations of expression machinery components to differences in specific proteins. It will be interesting to decipher which of these parameters are responsible for the observed size dependence.

More direct drivers of event times are molecular levels or rates, which can be measured through fluorescent reporter constructs in single cells. Time to death in *TRAIL*-induced apoptosis was shown to be most highly correlated with the rate of initiator caspase cleavage [16]. Meiosis entry time in yeast was highly correlated with the promoter activity of the master regulator *IME1* [13]. The relative timing of the master regulators controlling bacterial sporulation and competence affected which program was finally chosen [2].

Protein production in *Escherichia coli* was measured in the context of switching from dormant to reproductive state and was found to occur in a narrow time window after exit from dormancy [17]. Bacterial lysis time was found to depend on both a molecular level (that of a late lysis promoter) and a host global property (growth rate) [15].

In the previous examples, the effect of one or more factors (molecular or cellular) on event timing was quantified separately. We can deepen our understanding on those dependencies by testing the contribution of one factor in the context of the others. These tests quantify the amount of variability explained by factor X alone, compared with the variability explained by X given that factor Y is already known. How much knowledge is added? Is the information of X on the event time channeled through Y? In this way the effect of size on meiosis entry time was shown to be mediated through the master regulator's promoter activity [13].

## FUTURE DIRECTIONS

By timing events in single cells, we can learn about dependencies of these events on other molecular or

structural features in the cell through simple statistical analysis. With the progress of single-cell and single-molecule live imaging techniques, it is becoming possible to identify and time more and more events. Particularly in the case of single-molecule events, such studies are illuminating very basic facts even on well-studied processes, for example the order of arrival of different molecular components to the process site [43]. Also, all the examples discussed in this review use two-dimensional culture systems. It will be interesting to see how event detection and timing are extended into processes occurring in three-dimensional cell structures.

### Key points

- Event timing is an important phenotype that can be quantified at the single-cell level by live imaging.
- The distribution of event times can reveal the nature of the timed process.
- Statistically testing the relation between different event timings can reveal if they are sequential or parallel, and whether they are independent not.
- Timing in mother–daughter pairs, sibling pairs and whole cell pedigrees can be used to infer decision points in the underlying processes.
- Cell morphological and molecular phenotypes can be quantified for their statistical contribution to event timing.

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