Bioluminescence dynamics in single germinating bacterial spores reveal metabolic heterogeneity

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Abstract

Spore-forming bacteria modulate their metabolic rate by over 5 orders of magnitude as they transition between dormant spores and vegetative cells, and thus represent an extreme case of phenotypic variation. During environmental changes in nutrient availability, clonal populations of spore-forming bacteria exhibit individual differences in cell fate, timing of phenotypic transitions, and gene expression. One potential source of this variability is metabolic heterogeneity, but this has not yet been measured, as existing single-cell methods are not easily applicable to spores due to their small size and strong autofluorescence. Here, we use the bacterial bioluminescence system and a highly sensitive microscope to measure metabolic dynamics in thousands of *B. subtilis* spores as they germinate. We observe and quantitate large variations in the bioluminescence dynamics across individual spores that can be decomposed into contributions from variability in germination timing, the amount of endogenously produced luminescence substrate, and the intracellular reducing power. This work shows that quantitative measurement of spore metabolism is possible and thus it opens venues for future study of the thermodynamic nature of dormant states.

Introduction

Some bacteria respond to nutrient depletion by differentiating from growing, vegetative cells into spores that can survive extreme temperatures, radiation, and long periods without nutrients, while retaining the ability to germinate and reinitiate growth in the presence of sufficient nutrients [1-3]. One of the most remarkable aspects of spore-forming bacteria is the extreme reduction of their metabolic rate, by at least 5 orders of magnitude, during sporulation [4]. Metabolic dormancy and resistance allow spores to survive for long times in harsh environments, which typically fluctuate. Dynamically sensing the environment requires energy expenditure [5], and thus is likely difficult for spores given their low or nonexistent metabolism. Therefore, sporulating populations must adapt to fluctuating environments through other strategies, such as phenotypic diversification, or "bet-hedging" [6, 7].

Significant heterogeneity is observed in populations during both the sporulation and germination transitions. For example, after nutrient limitation, only a portion of vegetative cells commit to sporulation, while other cells either lyse or survive in the vegetative state by using secondary metabolites [8]. The time at which a vegetative cell sporulates varies significantly within a population, and this time correlates with the time at which the ensuing spore germinates and in its ability to resume growth in various nutrients [9]. Spores germinate in reponse to low concentrations of nutrients at variable times [7], and this variability is partially explained by the number of germinant receptors in each spore [10]. Even in the absence of germinants, a small fraction of spores germinates spontaneously [11, 12]. These phenomena of phenotypic heterogeneity have been linked to variations in gene expression [8, 9, 12, 13]. However, the remarkable dynamics of metabolism during sporulation and germination have not been measured in individual cells, due to lack of a suitable technique.

The dynamics of energy metabolism can be measured in single vegetative bacteria using several *in vivo* methods, most of which are based on fluorescence [14]. These are difficult to apply to spores due to their strong autofluorescence, primarily from calcium dipicolinate, NADH, and

flavins [15, 16]. The intracellular redox potential drives all pathways of energy metabolism other than fermentation [17, 18], and has been measured *in vivo* in single bacterial cells using a custom-fabricated microelectrode coupled to an optical trap [19]. However, this technique requires a specialized device, operates in anaerobic conditions, and only measures a small number of cells.

An alternative approach uses the bacterial luminescence system [20]. An enzyme, luciferase, encoded by the *luxAB* genes, catalyzes the light-emitting oxidation of a substrate, luciferin, which can be supplied exogenously or synthesized endogenously by expression of the *luxCDE* genes. The oxidation reaction's rate depends on the reduced form of the electron transporter flavin mononucleotide ($FMNH_2$), whose concentration is determined by the intracellular reducing power [21]. Thus, the rate of light production directly relates to the redox potential. In addition, since the emission of light occurs without external illumination, bioluminescent techniques can be considered "zero background", and therefore offer improved sensitivity as compared to fluorescent techniques. Here we describe a microscope designed to capture the dynamics of bioluminescence emitted by single cells, which we used to non-invasively assay metabolic activity of single germinating spores of *Bacillus subtilis*.

Results

Bioluminescence microscopy of single spores

To date, the bioluminescence dynamics of sporulating and germinating cells have been measured only in populations, typically using a photomultiplier tube for photon counting [22, 23]. A *B. subtilis* strain that expresses the *lux* genes under a sporulation-specific promoter (P_{sspB}) produces spores that contain a sufficient number of luciferase enzymes to emit detectable light only upon germination [23]. Prior to germination, spores do not emit detectable light, presumably due to their dormant metabolic state.

The feasibility of measuring bioluminescence dynamics in single germinating spores is determined by the rate of photon emission, which must be sufficiently high to be resolved over

77 measurement noise. This rate increases with higher expression of the *luxABCDE* genes, as has
78 recently been achieved in *B. subtilis* by modifying the ribosomal binding sites upstream of each
79 gene [24]. The rate of bioluminescent photon emission at the population level has been reported
80 in relative light units, or RLUs [24, 25], which are defined differently for various devices. RLUs can
81 be converted to units of photons per time per cell by calibration with a luminescent standard [26],
82 although this calibration has not been included in the published results. As a rough guide,
83 microscopy of individual metabolically active *Escherichia coli*, *Synechoccocus elongatus*, *Vibrio*
84 *fischeri*, and *Vibrio harveyi* cells expressing the lux system typically measures ~60 photons min^{-1}
85 $cell^{-1}$ [27-29]. Our preliminary analysis suggested that a microscope utilizing recent improvements
86 in CCD technology could resolve bioluminescence signals as dim as 1 photons min^{-1} $spore^{-1}$ (SI
87 Appendix).

88 We therefore constructed a custom microscope for measuring the dynamics of
89 bioluminescence in single spores (Fig. 1A, Materials and Methods). The microscope includes an
90 optical path for bright field imaging, which is used to localize spores and measure their refractility,
91 as described below. The setup consists of an inverted microscope with a high-NA 63X objective
92 and a cooled, back-illuminated CCD camera. We used 10 min exposure times, sufficient to
93 resolve germination dynamics while achieving sensitivity to permit measurements from single
94 germinating spores. Over a period of 10 min, Brownian motion displaces a spore by a root-mean-
95 square displacement of approximately 50 μm. To prevent such displacements, we trapped the
96 spores by using hydrophobic glass coverslips. With this method, the displacement of spores over
97 10 min periods was below the optical resolution of the bright field images (<160 nm). The sample
98 was held in a perfusion chamber, which dynamically controlled the chemical environment (Fig.
99 1B) , and the temperature at the sample was controlled to ±0.05 ℃ by an air-to-air heat exchanger.

100 We initially investigated the dynamics of bioluminescence emitted by single *B. subtilis*
101 spores as they germinated in response to L-alanine. These spores (JDB3780) expressed the
102 *luxABCDE* genes under the control of a sporulation-specific promoter. A field of approximately

103 900 spores was imaged in bright field and luminescence every 10 min for 1.5 h (Fig. 1C). Prior to
104 the induction of germination, the dormant spores appeared dark, with high contrast, and produced
105 no resolvable luminescence signal (Figs. 1D and E). L-alanine was added to the perfusion media
106 at a concentration of 100 μM, and the ensuing germination response was recorded for 15 h.

107 After an initial lag following the addition of L-alanine, most spores showed a significant
108 decrease of contrast in bright field, and many produced luminescence well above the noise
109 background (Figs. 1F and G). The predominant source of measurement noise was sensor read
110 noise; we found that its contribution could be reduced significantly by applying a temporal low-
111 pass filter with a cutoff period of 36 min (SI Appendix). Sensor read noise has a Gaussian
112 distribution, and can therefore produce estimated photon counts that are negative [30]. We
113 determined that the technique had a sensitivity limit of 0.4×10^{-2} photons min^{-1} $spore^{-1}$ (SI
114 Appendix).

115 The dynamics of individual spores in the bright field and luminescence images were
116 analyzed, resulting in time series of bright field contrast and estimated luminescent photon counts
117 for individual spores (Figs. 1H and I). Rapid loss of contrast after the addition of L-alanine was
118 evident in most spores, indicating an early event in germination, namely, the release of calcium
119 dipicolinate from the spore [31]. Spores differed significantly in their time of germination (Fig. 1H)
120 and in their bioluminescence signals (Fig. 1I).

121

122 **Bioluminescence dynamics are synchronized with germination time**

123 In order to compare the measurements in the microscope to an established method, we used a
124 96-well plate reader to measure the population-averaged bioluminescence dynamics of spores
125 germinating in response to the addition of L-alanine at 5 different concentrations (1 μM, 10 μM,
126 100 μM, 1 mM, and 10 mM) (Fig. 2A). Time-lapse experiments were performed in the microscope
127 setup at the same L-alanine concentrations, and the population-averaged bioluminescence
128 dynamics were calculated (Fig. 2B). The dynamics as measured by both methods shared some

129 features: Signal intensity generally increased with increasing L-alanine concentration, from total
130 darkness at 1 μM, to a peak level at 1 mM, then decreased slightly at 10 mM (Fig. 2C). The L-
131 alanine concentration also affected the temporal patterns of the dynamics: In the measurements
132 performed with the microscope, a peak in the bioluminescence signal was evident approximately
133 1 h after germination with 10 mM, 1 mM, and 100 μM L-alanine, but this peak was not observed
134 following germination with 10 μM L-alanine (Fig. 2B). Although much less pronounced, this early
135 peak was also apparent in the plate reader measurements after germination with 10 mM and 1
136 mM L-alanine; with 100 μM and 10 μM L-alanine, no peak was resolved, but a "shoulder" at 40
137 min was apparent in both conditions (Fig. 2A). The primary difference in the dynamics measured
138 by the two methods was the much higher intensity of the early peak measured in the microscope.
139 We observed similar differences caused by varying the spore density in the plate reader
140 experiments (Fig. S1), which are likely due to chemical interactions between germinating spores.
141 This effect can explain the differences observed between the dynamics measured in the plate
142 reader, at a density of 3×10^7 spores ml^{-1}, and in the microscope, at a density of less than 10^5
143 spores ml^{-1}.

144 The observed population-averaged bioluminescence dynamics arise from temporal
145 fluctuations in individual spores as well as variability in the population, and these two sources can
146 be separated using the single-spore technique. In particular, individual spores germinate at
147 different times, with large variability observed at low L-alanine concentrations [32]. With
148 automated image analysis, we identified the time τ_g of rapid contrast loss in each germinating
149 spore (SI Appendix), and interpreted τ_g as the time at which each spore started germination. We
150 considered the statistics of germination dynamics by calculating the fraction of the population that
151 had not yet germinated by time t: This is the tail distribution function of germination time, $P(\tau_g>t)$
152 (Fig. 3A). At L-alanine concentrations of 100 μM and higher, most spores germinated within 1 h
153 of induction, and over 99% of spores germinated within 13 h. In contrast, spores germinated much
154 more slowly in response to the addition of 10 μM L-alanine, with only half of all spores germinating

155 within 13 h. For the 1 μM L-alanine concentration, we did not observe any germination for spores
156 up to 82 h after induction.

157 The distributions of germination time τ_g reveal significant variability in the population of
158 spores, especially evident in the 10 μM L-alanine condition. To investigate the relationship
159 between this variability and the bioluminescence dynamics, we considered the bioluminescence
160 of each cell as a function of time elapsed since germination: $\phi_g(t) = \phi(t\text{-}\tau_g)$, defined for all spores
161 that germinated during the observation interval. Note that the germination time τ_g was determined
162 exclusively by the bright field images, through a procedure that was independent of the
163 bioluminescence dynamics (SI Appendix). The population averages of $\phi_g(t)$ in each germination
164 condition indicate significant synchronization of the bioluminescence dynamics with the time of
165 germination, as evidenced by the qualitatively similar temporal patterns in the 4 conditions for
166 which germination was observed (Fig. 3B). In particular, we observe remarkable alignment in the
167 timing of a primary peak at $t \approx 40$ min, the appearance of a secondary peak between $t = 2$ h and
168 $t = 3$ h, and similar profiles of signal attenuation at later times, possibly due to degradation of the
169 lux enzymes (Fig. S2).

170

171 **Bioluminescence varies significantly across spores**

172 Aligning the dynamics to the time of germination τ_g accounted for the occurrence and timing of
173 germination as a source of the variability observed in the bioluminescence dynamics. Significant
174 variability remained in the aligned dynamics $\phi_g(t)$ (Fig. 4A). We analyzed the variability of $\phi_g(t)$
175 over spores to answer two questions: First, what are the temporal correlations of the
176 bioluminescence dynamics in individual spores? Second, how much of the variability in
177 bioluminescence is due to variations in the factors that affect the reaction chemistry, namely, the
178 redox potential, luciferin concentration, and luciferase copy number?

179 To analyze temporal correlations in the bioluminescence dynamics, we calculated the
180 temporal covariance matrix $C(s,t)$ from the photon counts ϕ_g at times s and t (SI Appendix). This

181 calculation was performed over all germinating spores from the 4 concentrations of L-alanine for
182 which germination was observed. The covariance matrix was analyzed using principal component
183 analysis (PCA): PCA applied to time series in this manner is often referred to as empirical
184 orthogonal function analysis [33]. The first principal component explained 30% of the variance
185 over individuals, while additional components each explained only a few percent of the total
186 variance (Fig. S3). Therefore, we focused on the pattern of correlations described by the first
187 component, which had temporal structure similar to the population-averaged dynamics (Fig. 4B).
188 This similarity indicates that the dynamics in most spores were well-described by the mean
189 dynamics multiplied by a coefficient specific to each spore, which we call its amplitude. This simple
190 mode of variation was useful for quantifying the contributions from different sources, as described
191 below.
192

193 **Bioluminescence variability arises from variations in redox potential and luciferin**
194 **concentration**

195 Besides the timing of germination, several factors could contribute to variability in the
196 bioluminescence dynamics. The redox potential and concentration of luciferin in each cell are two
197 obvious candidates. To investigate these, we performed an experiment with fumarate added to
198 the germination buffer, in order to drive the citric acid cycle and thus increase the intracellular
199 redox potential. In a second experiment, decanal was added as an exogenous luciferin. In a third
200 experiment, both fumarate and decanal were added to the germination buffer. Spores germinated
201 in response to the addition of 1 mM L-alanine. We calculated the population-averaged
202 bioluminescence dynamics and performed PCA for each data set independently (Figs. 4C-E).

203 In the presence of fumarate, the population-averaged bioluminescence dynamics $\langle \phi_g(t) \rangle$
204 exhibited 2 distinct peaks, approximately 40 min and 3 h after germination (Fig. 4C). These
205 dynamics appear to be similar to those observed in germination buffer without fumarate (Fig. 4B),
206 with two major differences: The addition of fumarate increased the intensity of the second peak,

207 and strongly attenuated the spore-to-spore variability during the first peak, as indicated by the
208 disappearance of the first peak from the first principal component. The addition of exogenous
209 luciferin (decanal), both with and without fumarate, resulted in population-averaged
210 bioluminescence dynamics with a single peak, occurring approximately 40 min after germination,
211 with intensity approximately 10-fold higher than the initial peak observed in the absence of
212 exogenous luciferin (Figs. 4D and 4E).

213 In all conditions, PCA revealed a single significant component of variation (Fig. S3), and
214 in each condition, this mode's eigenvector was strongly correlated with the average dynamics
215 (Figs. 4B-E). Thus the primary mode of variation in all conditions was variability in amplitude. This
216 simple pattern facilitates the quantitation of variability, since the contribution of each spore's signal
217 to the total variability can be summarized by a single variable: the signal amplitude. Thus we
218 calculated for each germinating spore the total number of photons counted during the 12 h
219 following germination, n_g.

220 The mean value of n_g varied significantly in different conditions. Therefore, to compare
221 variability in n_g across conditions, we considered the ratio of the standard deviation to the mean,
222 $\eta=\sigma_{n_g}\langle n_g\rangle^{-1}$, also known as the coefficient of variation. We also considered the asymmetry in the
223 distribution of n_g by calculating its skew, γ. The estimated statistics are summarized in Table 1.
224 Variability was high for spores germinating in the absence of exogenous factors (η=160%), and
225 the distribution of n_g had strong positive skew (γ=3.3). The addition of fumarate significantly
226 decreased the coefficient of variation and the skew (η=125%, γ=1.4). The addition of decanal
227 reduced the variability and skew more strongly (η=55%, γ=0.6). The addition of both fumarate
228 and decanal had the largest effect (η=39%, γ=0.32). We conclude that the large amount of
229 variability in bioluminescence signal observed in spores germinating in the absence of exogenous
230 factors was predominantly due to variability in both intracellular luciferin concentration and redox
231 potential.

232 The bioluminescence dynamics following germination are influenced by metabolic
233 changes that occur early in germination, including the initiation of electron transport [23, 34]. In
234 addition to inducing germination, L-alanine may also fuel energy metabolism [9]. To investigate
235 this possibility, we considered the photon counts n_g from spores germinating in response to 4
236 concentrations of L-alanine, in germination buffer (Fig. S4). The population-average $\langle n_g \rangle$
237 increased 2-fold as the L-alanine concentration increased from 10 μM to 1 mM, then decreased
238 at 10 mM. The variation in amplitude with L-alanine concentration arose primarily from a
239 subpopulation of the brightest spores (Fig. S5). The weak, non-monotonic dependence of the
240 bioluminescence signal on L-alanine concentration indicates that exogenous L-alanine does not
241 significantly drive energy metabolism in germinating spores.
242
243 **Discussion**
244 The spore is an extreme phenotypic state that allows an organism to survive extended periods of
245 starvation, drought, and radiation. Metabolism decreases to extremely low levels during
246 sporulation, then rapidly increases during germination. Laboratory studies of populations
247 undergoing the sporulation and germination transitions have also revealed large amounts of
248 variability over individuals, suggesting that phenotypic diversification in natural spore-forming
249 populations is a bet-hedging strategy for surviving in fluctuating environments. It is therefore
250 important to study metabolic dynamics in individual spores.
251 No previously described methods for quantifying metabolic dynamics in single cells are
252 applicable to *Bacillus* spores. Therefore, we developed a microscopic technique for this purpose,
253 using a bioluminescent reporter for the intracellular redox potential. In this paper, we measured
254 bioluminescence dynamics from individual spores germinating in response to L-alanine, which is
255 both a germinant and a potential nutrient. Comparing the bioluminescence dynamics measured
256 in individuals to the population average, we exposed significant variability in the population. A
257 major source of variability was shown to be associated with differences in the timing of

258 germination. We could remove this contribution from the bioluminescence dynamics by
259 simultaneously using bright field microscopy to precisely determine the time of germination of
260 each spore. The bioluminescence dynamics could then be analyzed for its temporal correlations.
261 The analysis indicated that the primary mode of variation was associated with the amplitude of
262 the bioluminescence signal produced by each spore.

263 By performing experiments at different concentrations of L-alanine, we were able to show
264 that it was not a major source of variations in bioluminescence amplitude, suggesting that
265 metabolism in germinating spores was driven not by L-alanine, but rather by endogenous energy
266 sources stored during sporulation. Through the addition of exogenous nutrients (fumarate) and
267 bioluminescence substrate (luciferin), we were able to decompose the variability in
268 bioluminescence amplitude into two main sources: the intracellular redox potential and luciferin
269 concentration. Both of these sources relate to energy metabolism, as the redox potential drives
270 ATP synthesis, and the biosynthesis of luciferin depends on both ATP and NADPH [35]. Our
271 results show that we can begin to understand the metabolic transitions between the growing and
272 dormant states at the level of single cells.

273 We foresee two important extensions of the current work. First, one can study the
274 variability of metabolic dynamics in fluctuating environments. Vegetative cells can be tracked as
275 they sporulate in response to experimentally imposed starvation, and later germinate and outgrow
276 in response to nutrients [9]. By simultaneously measuring the bioluminescence dynamics in this
277 type of experiment, we can study the correlations between spores' timing of sporulation, timing of
278 germination, ability to outgrow, and metabolic state. Environments also fluctuate due to biotic
279 interactions. Bacterial spores contain high concentrations of calcium dipicolinate, a compound
280 that affects resistance and dormancy, but also induces germination in dormant spores [36].
281 Because spores release large amounts of calcium dipicolinate during germination [37], the local
282 concentration of spores can affect their germination behavior. Such interactions may explain the
283 differences we observed in the bioluminescence dynamics with respect to spore density (Figs. 2A

284 and B, Fig. S1). Interactions between spores and their effects on metabolism could be studied
285 systematically by analyzing the spatial statistics of germination timing and bioluminescence.

286 A second extension is to study the thermodynamic nature of dormant states. The
287 maintenance of order in living matter seems to require energy expenditure, in order to counteract
288 spontaneously occurring degradation [38, 39]. Therefore, a central question arises: What are the
289 energetic limits for survival? This question has primarily been studied in microbial communities
290 from low-energy environments, including deep sediments, permafrost, and subglacial lakes [40-
291 42]. Metabolic activity in such environments, averaged over the community, has been found to be
292 reduced by 5 to 6 orders of magnitude compared to the state of growth when nutrients are
293 supplied [40, 41]. These low metabolic rates are approximately equal to the energetic costs of
294 repairing molecular damage to proteins and DNA at the corresponding temperatures [41, 42].
295 However, these analyses included contributions from cells with metabolic activity that was
296 potentially significantly lower than the population average, including dormant spores [43].
297 Therefore, spores likely represent the most extreme case of metabolic dormancy in nature.

298 Most studies of metabolism in dormant spores have not detected activity [44-47].
299 Extraodinarily sensitive measurements of metabolism in populations of dormant spores using
300 radiochemical labelling of metabolites did detect activity, reduced by over 5 orders of magnitude
301 compared to the vegetative state [4]. However, the detected metabolism could have been due to
302 a rare subpopulation of germinating spores [4, 11, 12], and therefore can serve only as an upper
303 bound on the rate of metabolic activity in dormant spores. By resolving individual spontaneous
304 germination events, bioluminescence microscopy could achieve unprecedented sensitivity. The
305 intensity of bioluminescence is linear in the concentration of $FMNH_2$ at low concentrations [48],
306 so its utility as a metabolic reporter should extend to cells with very low metabolic rates, such as
307 dormant spores. A measurement on a single spore at a single time point with signal-to-noise ratio
308 ξ can be averaged over N individuals and T time points to produce a bulk measurement with
309 signal-to-noise ratio $\xi(NT)^{1/2}$. For our method applied to germinating spores, ξ is ~20 (SI

Appendix). This suggests that bioluminescence measurements could achieve sensitivity concomitant with a 10^5-fold reduction in signal compared to germinating spores by measuring, for example, a population of 10^4 dormant spores over an interval of 18 d, which is feasible using the setup described here.

A possible mechanism for long-term survival without any metabolic activity is the formation of a thermodynamically metastable state. The glassy state is an example of metastability that has been invoked to explain spore dormancy [49, 50], as macromolecules in a glass change conformation extremely slowly [51]. Therefore, a glassy environment could in principle reduce the rate of molecular damage to proteins and DNA, while also reducing the activity of metabolic enzymes and thus causing dormancy. Direct characterization of glassiness in spores is difficult, as the internal structure of the spore is highly heterogeneous [52]. Instead, one can study a feature of spores from the perspective of metastability, namely, the statistics of spontaneous germination and its dependence on environmental conditions such as temperature. This approach should be possible with our technique, which would also quantify possible differences in metabolism between spores germinating spontaneously and those germinating in response to nutrients.

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332 **Materials and Methods**

333 **Strains**

334 JDB3780 (168 *trpC2 sacA::*P_{sspB}*-luxABCDE::cm*) was constructed by transforming genomic DNA

335 from RL4921 (PY79 *sacA::*P_{sspB}*-luxABCDE::cm*) into JDB1772 (168 *trpC2).*

336

337 **Sporulation protocol**

338 Spores were prepared in Difco sporulation media (DSM). Single colonies grown on LB agar were

339 used to inoculate 3 ml cultures in test tubes. Cultures were grown with shaking at 30 ℃ for 3 h.

340 30 µl aliquots were used to inoculate 30 ml cultures in baffled flasks. After growing with shaking

341 at 30 ℃ for 36 h, spores were purified by spinning at 5,400 g for 10 min at 4 ℃ and resuspending

342 in sterile water. Cold water washes were repeated 4 times, yielding samples that were

343 predominantly refractile spores (>90%). Samples were stored at 4 ℃.

344

345 **Plate reader assay**

346 To assay bioluminescence dynamics in response to various concentrations of L-alanine, dormant

347 spores (JDB3780) were suspended in 25 mM Tris-HCl buffer at pH 8.2, at a density of 3×10^{7}

348 spores ml^{-1}. 200 µl of spore suspension was pipetted into wells of a 96-microwell plate (Nunc

349 236108). In order to avoid both signal contamination from neighboring wells as well as the

350 influence of thermal gradients, wells were filled only in the plate interior, in a checkerboard pattern.

351 2 µl of L-alanine solutions at concentrations between 1 M and 1 mM were added to the appropriate

352 wells. Each germinant condition was prepared in 3 replicate wells. Three wells with 200 µl of

353 buffer were included as blanks. The plate was sealed with a gas permeable, partially adhesive

354 film (Uniscience C20613201), and positioned within the plate reader (Perkin Elmer Wallac Victor^{2}

355 1420-011). Luminescence measurements were made without an optical filter, normal aperture

356 setting, and 10 s integration time. The temperature was maintained at 30 ± 1 ℃. Between

357 luminescence readings, the plate was shaken orbitally for 10 s with a 2 mm diameter at the slow
358 speed setting. The plate reader assay of bioluminescence in the presence of chloramphenicol
359 was performed using a similar procedure, with spores of JDB3785 suspended at a density of
360 1.2×10^{8} spores ml^{-1} and germinated with 1 mM L-alanine.
361

362 **Reagents and Media**

363 All germination experiments were conducted in germination buffer (25 mM Tris-HCl, pH 8.2).
364 Decanal was dissolved at a concentration of 15 mM in a 1:1 solution of methanol and water.
365

366 **Microscopy**

367 Sample preparation

368 All sample preparation was conducted with sterile technique at the bench.

369 Adhesion of dormant and germinating spores to glass coverslips was improved by
370 rendering the glass surface hydrophobic. Coverslips were treated with vapor deposition of
371 hexamethyldisilazane (Alfa Aesar L16519AE) in a sealed chamber for 1 h, followed by baking at
372 80 ℃ for 1 h.

373 Agarose pads were prepared by pipetting 700 µl of molten 4% low-melt agarose (Fisher
374 BP165-25) between two 22 mm diameter glass coverslips. After 1 h at room temperature, the
375 agarose pad was exposed by removing the top coverslip, and dried for 5 min.

376 Prior to spotting the pad, dormant spores were washed twice with water (5400 g, 10 min,
377 4 ℃). 2 µl of the spore suspension was pipetted onto the center of the dried pad, and the spot
378 was allowed to completely evaporate (~10 min). The pad was then separated from the bottom
379 coverslip, and inverted onto a hydrophobic glass coverslip (30 mm diameter, #1.5 thickness,
380 Bioptechs). The coverslip-bonded pad was left to evaporate for 30 min, then assembled into the
381 bottom of the perfusion chamber (Bioptechs, ICD with heated lid, gas and media perfusion).

382 Molten 4% low-melt agarose was added to the exposed portion of the coverslip, and allowed to
383 gel for 30 min. Creating a contiguous layer of agarose in this manner was found to attenuate
384 coverslip bounce resulting from the perfusion flow.
385
386 Perfusion
387 A peristaltic pump (Cole-Parmer, Masterflex L/S digital miniflex, dual-channel) was used to draw
388 media from a reservoir bottle, through sterile tubing (Cole-Parmer, C-Flex #13, i.d. 0.8 mm), into
389 the perfusion chamber, and finally into a waste reservoir bottle. Tubing was connected with
390 standard Luer locks, and the bottle openings were wrapped tightly with Parafilm and covered in
391 aluminum foil. At the beginning of each experiment and whenever the medium was changed (for
392 example, when germinant was added), the flow was primed at 1 ml min^{-1}. Otherwise, the flow rate
393 was 50 µl min^{-1}.
394 Prior to conducting each imaging experiment, the perfusion tubing was sterilized by
395 flowing 100 ml 20% bleach, followed by 100 ml isopropanol, followed by 5 subsequent bottles
396 containing 250 ml sterile, purified water (Milli-Q). The perfusion chamber was cleaned by rinsing
397 with 20% bleach, isopropanol, and distilled water, then air dried.
398
399 Imaging
400 The imaging setup consisted of an inverted optical microscope (Zeiss Axiovert 135), a 63X 1.4
401 NA oil immersion DIC objective (Zeiss 1113-108) operated without the Wollaston prism, and a
402 sensitive, back-illuminated, cooled CCD camera (Princeton Instruments, PIXIS 1024B).
403 A collimated, 445 nm LED (Thorlabs, Solis-445C) was used as external illumination for
404 bright field imaging. For acquisition of bright field images, the LED was pulsed for 50 ms at low
405 current (40 mA), and was otherwise fully off.
406 Autofocus was maintained via a pupil obscuration method applied to an infrared (780 nm)
407 beam reflected from the glass-media interface (Applied Scientific Instrumentation, CRISP). The

408 defocus detector controlled the focal position through a DC servo motor connected to the
409 mechanical focusing shaft of the microscope (Applied Scientific Instrumentation, MFC-2000).
410 Luminescence images were acquired with 10 min exposure time. The readout was
411 performed at 100 kHz (read noise 3.56 e^-), high gain (1.00 e^- ADU^{-1}), and with 2x2 binning. The
412 CCD was cooled to -65 ℃ with a combination of thermoelectric and low-vibration liquid cooling
413 (Princeton Instruments, CoolCUBE II). At this temperature, the dark current was measured at 10^{-3}
414 e^- $pixel^{-1}$ s^{-1}.
415 The microscope and camera were enclosed inside a box made of styrofoam-lined black
416 plastic. Exterior edges and corners of the box were covered with opaque black fabric and taped.
417 A removable styrofoam-lined plastic panel provided access to the microscope and sample holder,
418 and was held in place during data acquisition with Velcro straps and metal L-brackets. The
419 temperature within the box was controlled by a thermoelectric device (TE Technology, AC-220),
420 coupled to a PID feedback device (TE Technology, TC-720) monitoring a thermistor placed as
421 close as possible to the perfusion chamber. The interior temperature of the box was maintained
422 at 30±0.05 ℃. The entire imaging apparatus was supported by a vibration-damped optical table,
423 situated in a temperature-controlled room (28±0.1 ℃).
424
425 **Image analysis and statistics**
426 See SI Appendix.
427

428 **Table**

media	#spores	mean: $\langle n_g \rangle$ [photons]	coefficient of variation: η	skew: γ
germination buffer only	11,882	1,300±19	161% [154%, 169%]	3.326±0.022
germ. buffer +0.4% fumarate	1,976	2,862±81	125% [117%, 137%]	1.449±0.055
germ. buffer +30 μM decanal	928	18,600±340	55% [52%, 59%]	0.56±0.080
germ. buffer +30 μM decanal +0.4% fumarate	284	14,000±320	39% [35%, 43%]	0.32±0.14

429

430 **Table 1. Bioluminescence signal amplitude statistics.** Columns denote media, the number of
431 spores analyzed, and the statistics of n_g, the photon counts per spore integrated over the 12 h
432 after germination. The ± signs indicate 1 standard error. The bracketed interval in the coefficient
433 of variation column is the 95% confidence interval.

434

435 **Figure Legends**

436 **Fig. 1. Schematic of bioluminescence microscope and perfusion chamber with example**
437 **images and time series. (A)** Microscope schematic showing 3 light paths: bright field (blue),
438 luminescence (green), and autofocus infrared (red). For bright field imaging (blue path), the
439 external illumination is focused near the bottom of the agarose pad (yellow slab), where sample
440 cells are sandwiched against a glass coverslip. The sample emits luminescence (green path) in
441 all directions. A temperature-controlled, light-tight box (thick black lines) encloses the microscope.
442 **(B)** The perfusion chamber flows medium around the agarose pad (yellow) that traps sample cells
443 against a glass coverslip on the chamber bottom, sealed by an O-ring (orange). **(C)** Bright field
444 image of P_{sspB}-*luxABCDE* spores, before the addition of germinant (time 0 h). Highly refractile
445 dormant spores appear dark, *i.e.*, high contrast. The field of view contains approximately 900

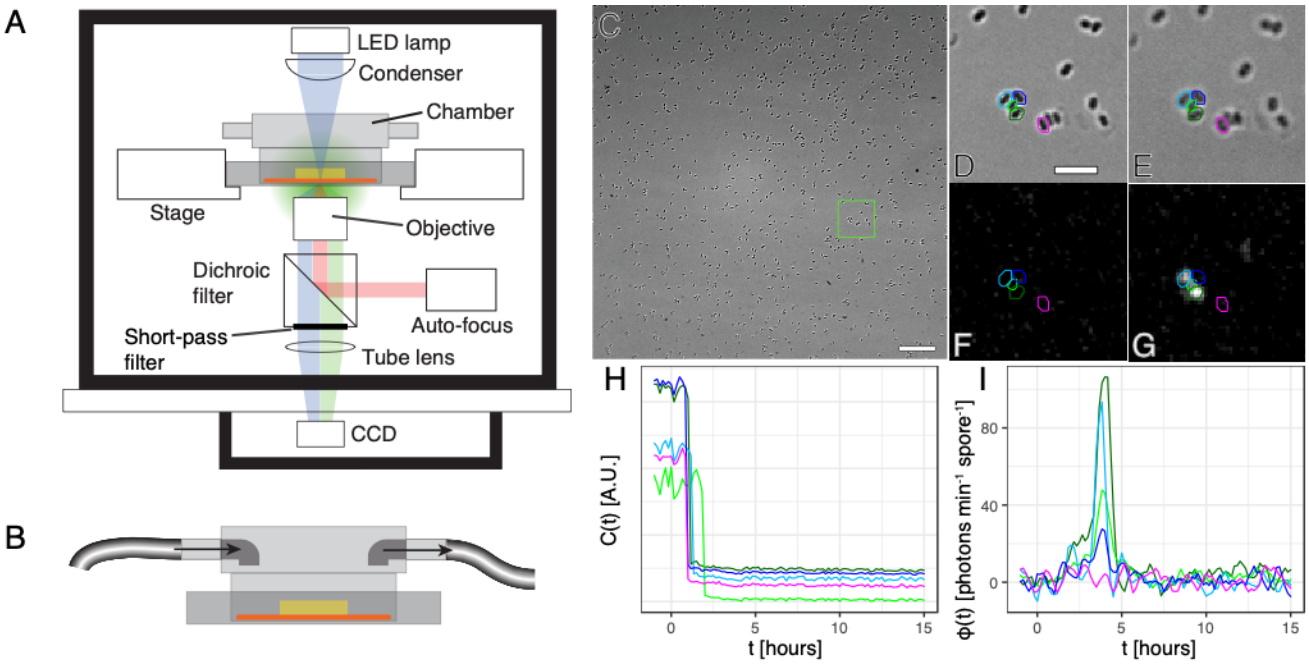
446 spores. Scale bar: 20 μm. **(D,E)** The subregion bounded by the green box is shown in panels (D)
447 (bright field) and (E) (luminescence), showing the lack of luminescence signal from dormant
448 spores. **(F,G)** The same region is depicted 3.5 h after the addition of 100 μM L-alanine, showing
449 spores' typical loss of bright field contrast upon germination (F), and the corresponding increase
450 in luminescence signal (G) that occurs in some cells after germination. **(H,I)** Time series of bright
451 field contrast (H) and luminescent photon counts (I) of the 5 representative spores outlined in (D)-
452 (G). The rapid loss of contrast shown in panel (H) defines the time of germination, τ_g, for each
453 spore. Colored cell outlines are dilated by 1 pixel for visualization. Panel (D) scale bar: 5 μm.
454
455 **Fig. 2. Bioluminescence dynamics of spore populations germinating in response to L-**
456 **alanine. (A)** The bioluminescence emitted by P_{sspB}-*luxABCDE* spores germinating in a range of
457 L-alanine concentrations was assayed in a 96-well plate reader. Each well contained
458 approximately 6×10^6 spores. Lines depict the mean signal over 3 replicates; the standard
459 deviation was negligible on this scale. **(B)** The bioluminescence signal $\phi(t)$ was measured in
460 individual spores germinating in response to various concentrations of L-alanine added at time 0
461 h. The mean signal over all spores (>600 individuals per concentration) is plotted at each time
462 point. **(C)** The bioluminescence signals in (A) and (B) were integrated over the period between 0
463 and 12 h after the addition of alanine to calculate the population-averaged photon counts n,
464 plotted as a function of alanine concentration.
465
466 **Fig. 3. Time of germination varies in populations and synchronizes with bioluminescence**
467 **dynamics.** Dormant spores were exposed to 5 concentrations of L-alanine at time 0 h. **(A)** The
468 fraction of spores that had not yet germinated by time t, $P(\tau_g>t)$, is plotted on a linear scale (left)
469 and a log scale (right). An offset is added to avoid divergence of zero values on the log scale. **(B)**
470 The luminescence signal of each spore as a function of time elapsed since the time of germination
471 is $\phi_g(t) = \phi(t-\tau_g)$, defined for spores that germinated during the acquisition interval. The population

472 average of $\phi_g(t)$ is plotted for each concentration of L-alanine. Note that no spores germinated in
473 1 μM L-alanine and so no curve for that condition appears in (B).
474
475 **Fig. 4. Variability in bioluminescence dynamics over spores. (A)** Bioluminescence as a
476 function of time since germination is analyzed in terms of variability over individuals. Data include
477 all spores that germinated in response to L-alanine at 4 concentrations (Table 1). The time series
478 of $\phi_g(t)$ for a randomly chosen subset of 500 cells are plotted as thin black lines. The solid red line
479 indicates the median over the 11,000 cells comprising the data set, and the dashed red lines
480 indicate the 16% and 84% quartiles. (**B-E**) Principal component analysis was performed on time
481 series $\phi_g(t)$ from 4 experimental conditions: germination buffer only (B), 0.4% sodium fumarate
482 (C), 30 μM decanal (D), and 30 μM decanal plus 0.4% sodium fumarate (E). The dark gray line
483 shows the population average $\langle\phi_g(t)\rangle$ for each condition. The first principal component for each
484 condition explained 30%, 55%, 68%, and 58% of the total variance, respectively. The red line
485 shows the loadings of the first principle component, *i.e.*, $\lambda_1^{1/2}w_1(t)$, where λ_1 is the largest
486 eigenvalue of the covariance matrix, and w_1 is the first principle component, scaled to unit norm.
487 The average and loadings are plotted on separate axes. The first principal component and the
488 population average were highly correlated in all conditions: defining $r=\mathrm{cor}(w_1(t),\langle\phi_g(t)\rangle)$, $r_{\text{buffer-}}$
489 $_{\text{only}}$=84%, r_{fumarate}=97%, r_{decanal}=89%, $r_{\text{decanal+fumarate}}$=99% (all p-values $<2.2\times10^{-16}$).
490

491 **Figures**

492

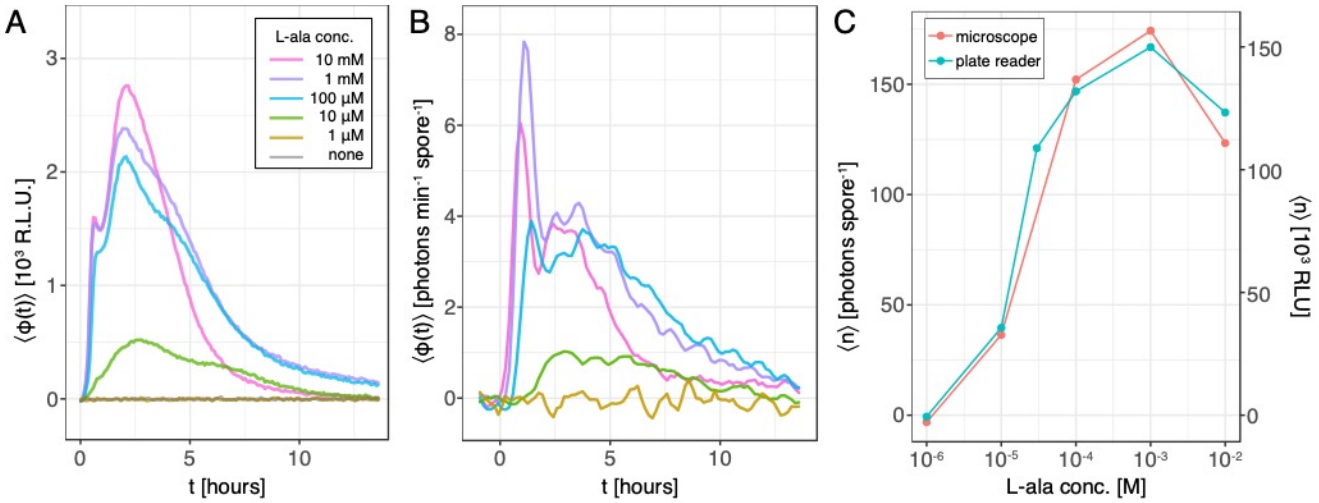
493 **Figure 1**



494

495

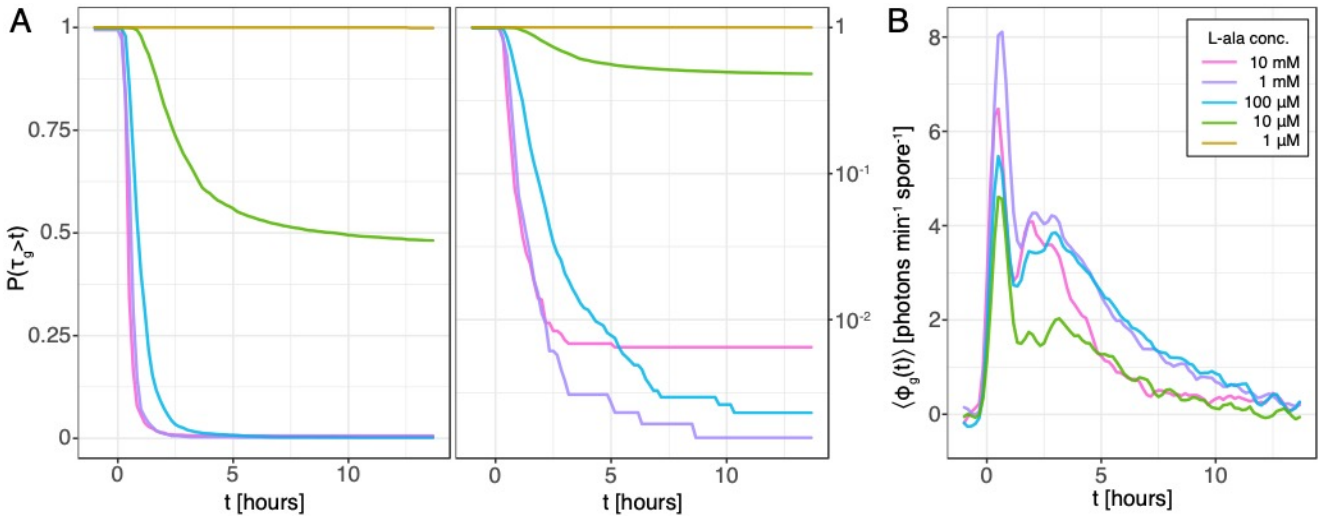
496 **Figure 2**



497

498

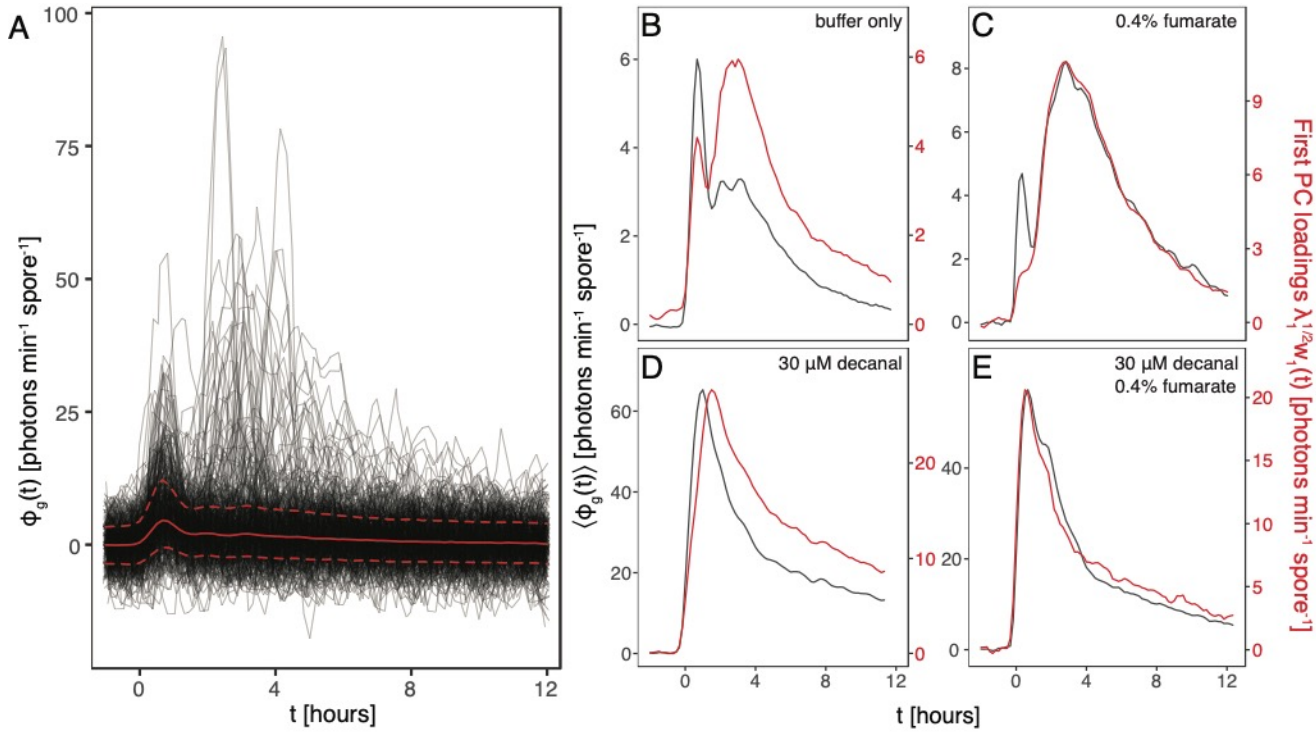
499 **Figure 3**



500

501

502 **Figure 4**



503
504

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644

Supporting Materials and Methods

Sensitivity limits of bioluminescence microscopy

Sensor read noise, dark current, and high energy particles such as cosmic rays generate a noise background for imaging. The signal measured from a steadily emitting source accumulates with increasing exposure time t. Noise due to dark current also accumulates with t, but modern cooled CCDs have very low dark current, so that sensor read noise dominates for t up to about 1 h. The number of high energy particle artifacts per image also increases with t. These artifacts can be identified automatically [1], so that pixels affected by these artifacts can be removed from subsequent analysis. From a practical perspective, t must be short enough to ensure that the majority of pixels are not affected by these artifacts. The flux of high energy particles from cosmic rays is about 1 cm^{-2} min^{-1} [1]. In our preliminary experiments, artifacts from high energy particles limited t to 10 min.

After removing artifacts due to high energy particles, imaging noise is dominated by sensor readout noise. We consider the following parameters: the average rate of photon emission per spore, ψ; the average number of pixels in the luminescence image of a spore, N; the RMS readout noise per pixel σ_r; the exposure time t, and the collection efficiency of the imaging setup s. Note that the collection efficiency s is the ratio of the number of emitted photons to the number of electrons measured by the sensor. It includes geometric effects, *i.e.*, losses due to the collection geometry, reflections from optical surfaces, and the quantum yield of the sensor.

The signal to noise ratio for each pixel in the image of the spore, χ_p, is the number of electrons per pixel per exposure arising from emitted luminescence, divided by the magnitude of the readout noise: $\chi_p = s\psi t N^{-1}\sigma_r^{-1}$. The signal from each spore is the sum over the signals from the pixels comprising its image. The readout noise is independent for each pixel, so the signal to noise ratio per spore, χ, equals $\chi_p N^{1/2} = s\psi t N^{-1/2}\sigma_r^{-1}$. The sensitivity limit of the technique is taken to be the rate of photon emission per spore for which the signal to noise ratio χ is equal to 1: $\psi_{min} = s^{-1}t^{-1}N^{1/2}\sigma_r$.

Optimizing the sensitivity of the technique amounts to maximizing collection efficiency s, minimizing the number of pixels per spore n, and minimizing the sensor read noise σ_r. Practically, s is less than 1/2 electrons per photon, since at least half of photons are emitted away from the collection objective. The numerical aperture of the objective should be as high as possible, to maximize collection of the remaining photons. The quantum efficiency of back-illuminated CCDs in the spectral range of bacterial bioluminescence (~490 nm) typically exceeds 0.9 electrons per photon. Accounting for these effects, we estimated that we could achieve a value of s as high as 0.3 electrons per photon. For the camera we used in the microscope, the parameters were as follows: s = 0.3 e^- photon^{-1} (estimated), t = 10 min, N = 10 pixels, σ_r = 3.6 e^-, which determines the sensitivity limit $\psi_{min} \approx$ 3.8 photons min^{-1}. Published results typically report the number of measured photons per time ($\gamma^{-1}s\psi$, where γ is the gain, expressed in electrons per photon), rather than the number emitted per time (ψ), and so we introduce a new symbol for the former rate: $\Phi = \gamma^{-1}s\psi$, and estimate the sensitivity limit $\Phi_{min} = \gamma^{-1}s\psi_{min} \approx$ 1 photons min^{-1} (γ = 1 e^- photon^{-1} for our setup).

We analyzed the signal to noise ratio of our measurements on a per-spore basis. The noise background was quantified by considering the statistics of luminescence signal from the reported experiments in regions of the image that did not contain spores. These regions were defined to each include the same number of pixels as a real spore. Twelve thousand such regions were analyzed, and the standard deviation of the estimated photon counts from each region was calculated: σ = 3.5 photons min^{-1}. The bioluminescence dynamics per spore $\Phi(t)$ varied over time and across spores. Detection of the signal from a particular spore is primarily determined by that spore's maximal signal. For the purposes of determining a single signal to noise ratio that would describe the typical sensitivity of the technique, we calculated the population-average of the temporal maximum bioluminescence signal:

$$\phi^* = \langle \max_t \phi(t) \rangle$$

The signal to noise ratio is given by $\chi = \Phi^{*}\sigma^{-1}$. Its values were χ = 4.2 (germination buffer only), 5.1 (0.4% fumarate), 22 (30 μM decanal), and 18 (30 μM decanal + 0.4% fumarate).

Image analysis

Background subtraction

Spatial inhomogeneities were observed in long exposure images, even those acquired in complete darkness without a luminescent sample. These inhomogeneities were found to be nearly constant over time and were treated as a form of background. To remove this background, a sequence of at least 20 images was acquired in darkness prior to each experiment, and the temporal median of this sequence, *i.e.*, the estimated background, was subtracted from each luminescence image subsequently acquired during the experiment.

Removal of artifacts due to high energy particles

Each luminescence image included several small regions of extremely bright pixels, due to high energy particles, such as cosmic rays, ionizing the CCD. In order to prevent these artifacts from corrupting the luminescence signal, we developed a routine to automatically detect pixels that appeared to be affected by such events. Pixels affected by artifacts had a particularly identifying feature: extreme brightness, that appeared rapidly in time and with very high spatial contrast. Temporal and spatial high-pass filters were applied to the images, and the resulting images were segmented, using a threshold chosen to separate the vast majority (>99%) of affected pixels from unaffected pixels. The affected regions were grown by 2 pixels to avoid any remaining signal contamination. The mean number of artifacts was 2.0 min^{-1}, and the mean number of pixels affected by each artifact was 28.

Spore identification and segmentation

Spores were identified by determining the maximally stable extremal regions of the bright field image, using the VLFeat software library [2, 3]. The parameters of the method were tuned manually to identify >99% of spores, with a negligible false identification rate (<0.1%).

Determination of Germination Time τ_g

The bright field contrast of each spore was determined by calculating the spatial gradient of each bright field image, ∇I_{BF}. The magnitude of the gradient was averaged over the pixels p corresponding to each spore to calculate its contrast:

$$C(t) = \sum_{p} \|\nabla I_{\mathrm{BF}}(t)\|^2$$

The dynamics of contrast of germinating spores typically appeared as noisy step function, declining from a high value prior to germination to a low value after. The time of germination τ_g was determined for each spore by fitting the measured contrast with a regression tree having a single split, using the rpart package for R, v. 4.1-15. The automatically determined τ_g was found to exactly match the value determined by manually considering the time-lapse images in 100 spores selected randomly from 4 data sets.

Statistical analysis

Temporal filtering

Camera read noise and photon counting noise (shot noise) corrupt the counts of bioluminescence photons. Each of the noise sources is expected to have zero temporal autocorrelation, while the bioluminescence dynamics are expected to have significant autocorrelation. Defining the photon counts within each pixel p as $X_p(t)$, we expect the power spectral density of X_p to be different for pixels located within the image of a spore vs. pixels located outside (background). To text this, we estimated the power spectral density of $X_p(t)$ over 110,000 pixels within the image of spores ("signal": $P_S(\omega)$) and over 200,000 pixels not within the image of spores (background: $P_{BG}(\omega)$).

The power spectral density of the background was flat, while that of the signal was flat above $\omega \approx 0.84\ \mathrm{h}^{-1}$. In order to filter the background noise without significantly blurring the signal dynamics, we applied a low-pass filter (sinc filter), with a cutoff period of 36 min, to the bioluminescence signal measured from each pixel. For each spore, the sum of these counts over all pixels inside its bright field image was calculated for each time point, resulting in $\Phi(t)$.

Principal component analysis

The covariance matrix $C(s,t)$ was calculated over all germinating spores as:

$$C(s,t) = N^{-1} \sum_{i=1}^{N} \left(\phi_{\mathrm{g}}^{i}(s) - \langle \phi_{\mathrm{g}}(s) \rangle\right)\left(\phi_{\mathrm{g}}^{i}(t) - \langle \phi_{\mathrm{g}}(t) \rangle\right)$$

where $\phi^{i}_{\mathrm{g}}(t)$ is the estimated rate of photons counted from spore i at time t, and N is the number of spores in the data set. The times s and t took values corresponding to 87 time points: 12 before germination (2 h), and 75 following germination (12.3 h). Principal component analysis was performed on the covariance matrix $C(s,t)$, resulting in the eigenvalues λ_i and the eigenvectors $w_i(t)$. The eigenvalues have units (photons $\mathrm{min}^{-1})^2$ and the eigenvectors are unitless and have norm 1:

$$\sum_{j=1}^{87} \left(w_i(t_j)\right)^2 = 1$$

The temporal pattern of variation explained by mode i is quantified by $\lambda_i^{1/2} w_i(t)$, as is plotted in Figs. 4B-E for $i = 1$.

Statistics of n_{g}

The number of photons counted per spore was calculated as

$$n_{\mathrm{g}} = \sum_{t \geq 0}^{t \leq 12\ \mathrm{h}} \phi_{\mathrm{g}}(t) \Delta t$$

where $\Delta t = 10$ min is the exposure time.

For Table 1, the estimates of the skew of n_g and its standard error were calculated for each data set using method 3 of Joanes & Gill [4]. The confidence interval of the coefficient of variation was estimated using the formula of McKay [5].

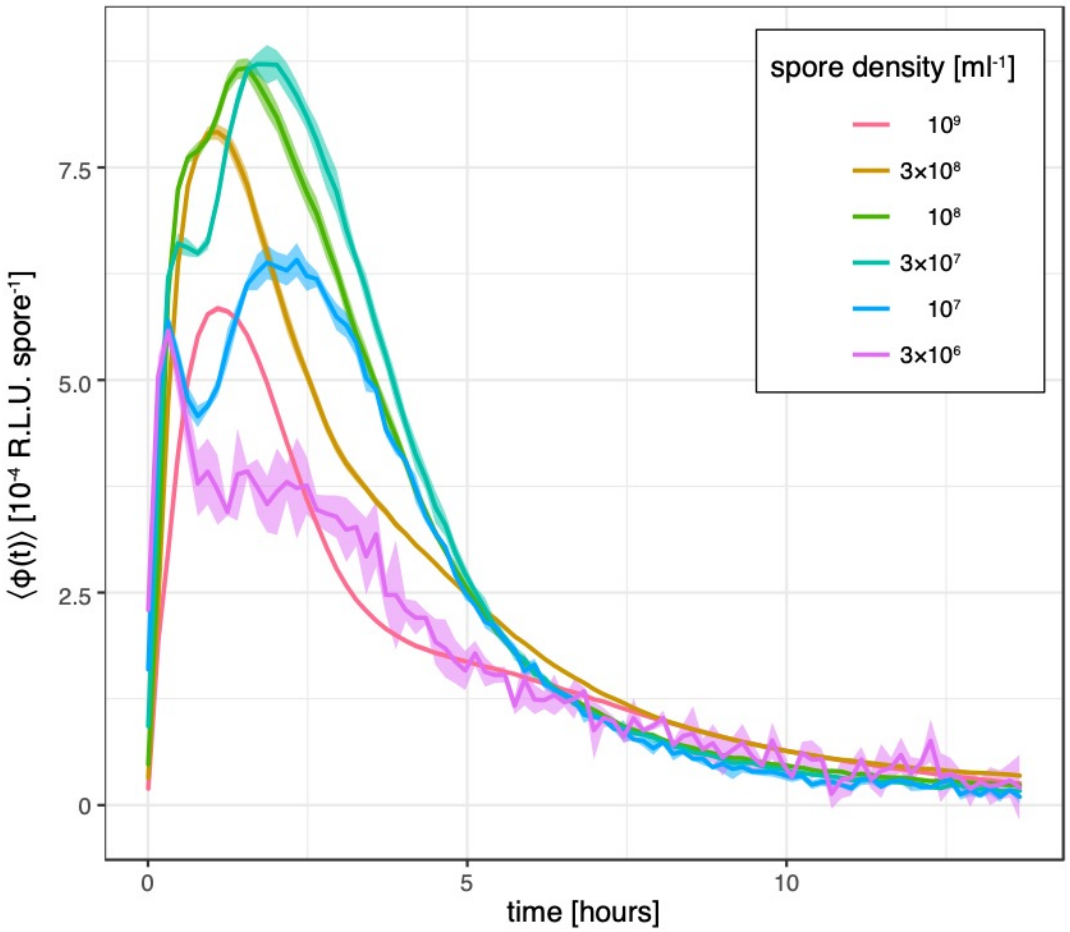

Figure S1. Bioluminescence dynamics depend on spore concentration. The population-averaged bioluminescence signal $\langle \phi(t) \rangle$ was measured in a 96-well plate reader, at a range of spore densities, and normalized by the number of spores per well to facilitate comparison between densities. Spores were induced to germinate with 1 mM L-alanine. Lines depict the mean signal over 3 replicates and the shaded region encloses ±1 standard deviation. Spore density in the experiments performed with the microscope was less than 10^5 ml^{-1}.

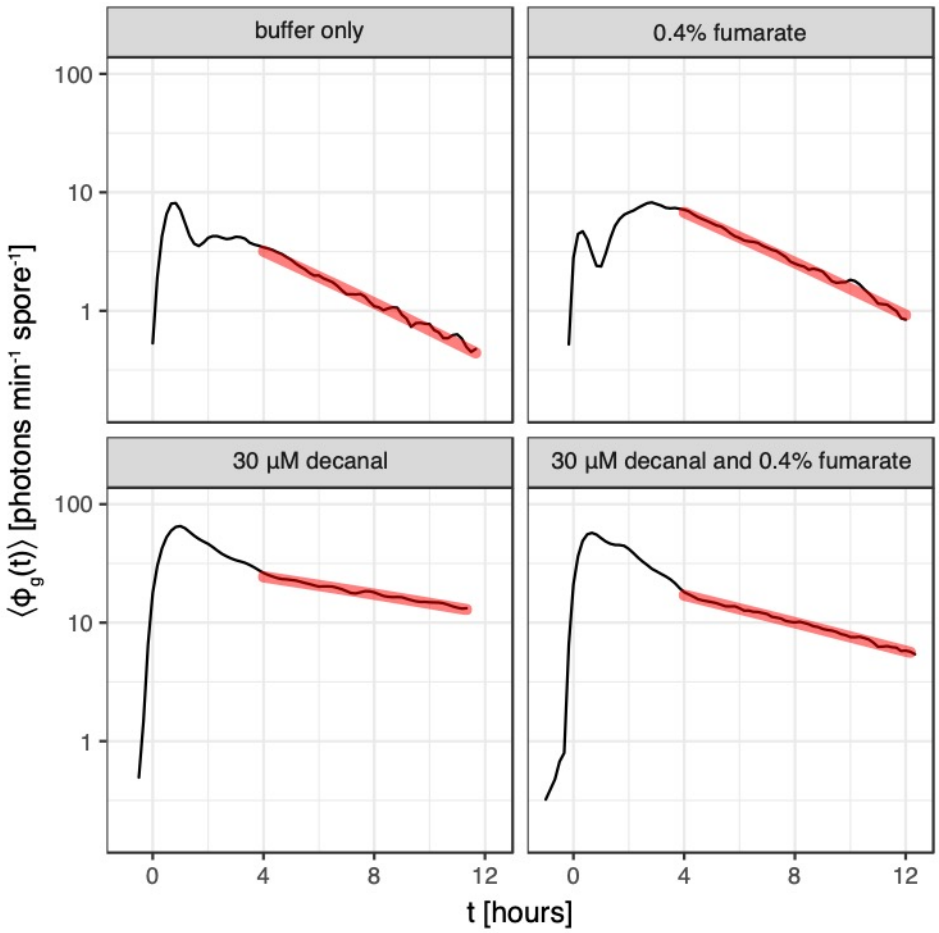

Figure S2. Bioluminescence signal decays exponentially after 4 h post-germination. The population-averaged bioluminescence dynamics of germinating spores $\langle \phi_g(t) \rangle$, depicted by the black line, exhibit exponential decline, beginning at 4 h post-germination. The rate of exponential decline r was estimated in each of 4 conditions: $r_{\text{buffer-only}}$ = 22% h^{-1}, r_{fumarate} = 22% h^{-1}, r_{decanal} = 8% h^{-1}, $r_{\text{decanal+fumarate}}$ = 12% h^{-1}. Estimated fits are depicted by red lines.

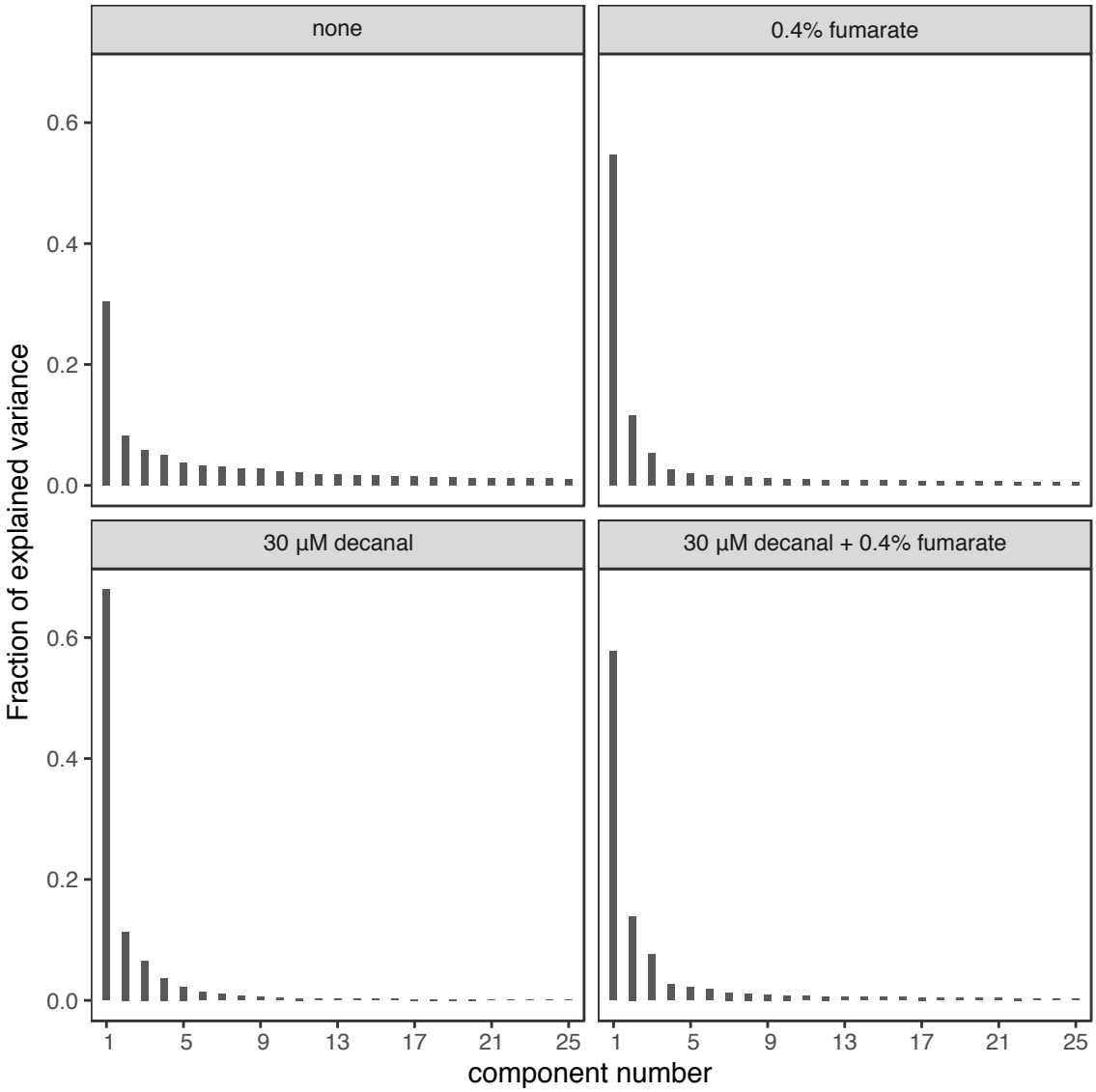

Figure S3. Variance explained by principal components. Principal component analysis was performed on the bioluminescent photon counts $\phi_g(t)$ for each of 4 experimental conditions, as described in the Results section. The variance explained by principal component number k is λ_k, and the fraction of variance explained is $\lambda_k(\Sigma_i \lambda_i)^{-1}$. The data sets included 87 time points and therefore 87 principal components; only the first 25 components are included in the plots.

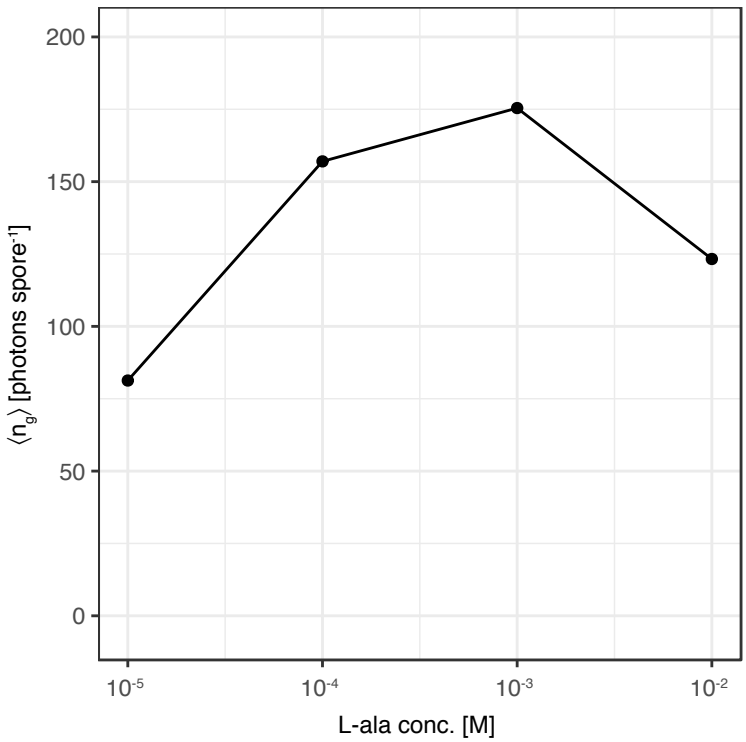

Figure S4. Bioluminescent photons counted per germinating spore. The bioluminescence signal as a function of time since germination, $\phi_g(t) = \phi(t\text{-}\tau_g)$, was integrated from 0 to 12 h post-germination to calculate the number of photons counted per germinating spore, n_g. The average of n_g over all germinating spores is plotted against L-alanine concentration.

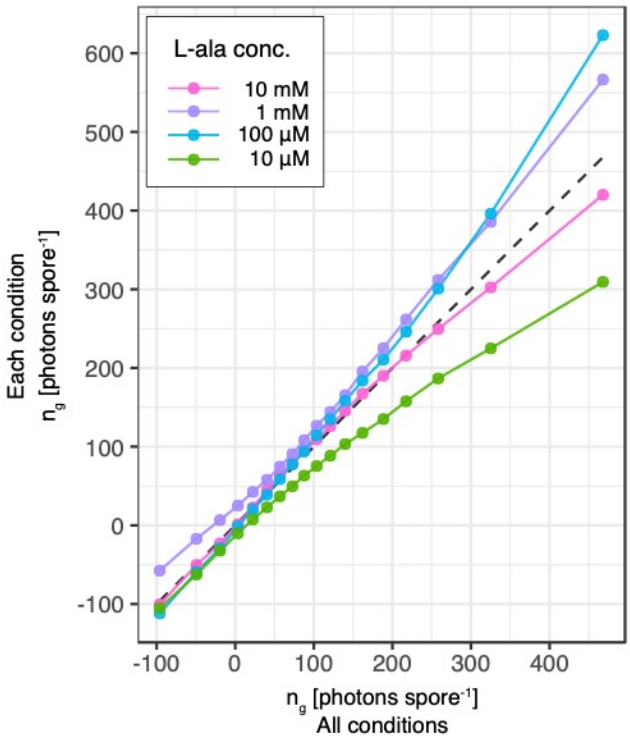

Figure S5. Quantile-quantile plot of bioluminescent photons counted per spore, germinating in response to 4 concentrations of L-alanine. Quantiles of the number of photons emitted per germinating spore during the 12 h post-germination, n_g, were determined for 4 concentrations of L-alanine. Quantiles were also calculated for the data from all 4 L-alanine concentrations, weighted for equal contribution from each condition. The quantiles in each condition are plotted on the vertical axis, as a function of the corresponding quantile in all conditions. Quantiles were determined in increments of 5%, starting at 5% and ending at 95%.

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